Phytic acid mobilization is an early response to chilling of the embryonic axes from dormant oilseed of hazel (Corylus avellana)

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Abstract
Dormancy of hazel (Corylus avellana L.) seeds is alleviated by a chilling treatment during which cytological, hormonal, and biochemical changes occur. Phytic acid and phosphate mobilization have been examined during this treatment. Phytic acid accounted for 0.7% and up to 3.2% of dry weight in axiferous and cotyledonary tissue, respectively. Phytic acid levels in embryonic axes were reduced by 60% within the first 3 weeks of chilling, with little subsequent change, in contrast to warm-imbibed tissue where levels did not change significantly. In cotyledons, phytic acid was mobilized to a lesser extent. Phosphate levels expressed on a fresh weight basis remained almost unaltered suggesting either the operation of a homeostatic mechanism for intracellular concentration or rapid utilization due to active metabolism. Phytase activity increased during stratification in both axiferous and cotyledonary tissue. The initial rise observed was associated with dormancy alleviation, since it occurred before the realization of full germination potential by the seeds and not in warm-imbibed tissue. Protein bodies were isolated from hazel seeds by non-aqueous density gradients. Phytase activity was closely associated with the purified organelles, where phytic acid was located by light microscopy. Overall, these findings suggest that phytic acid mobilization by phytase and previously described processes associated with protein bodies, such as considerable proteolysis, are early participants in the plethora of events leading to seed dormancy relief and germination in hazel.

Key words: Corylus avellana L., dormancy, hazel, phytase, phytic acid, phosphate, protein bodies, seed.

Introduction
Dormancy is the inability of the viable seed to commence germinative growth even when environmental conditions are physiologically favourable. In a number of seeds from temperate regions, dormancy is alleviated by a prolonged period of moist incubation at low temperatures, typically in the range 0–15 °C (referred to as chilling or stratification). Freshly harvested seeds of hazel (Corylus avellana L.) exhibit primary dormancy imposed by inhibitory substances, such as abscisic acid, present in the testa and pericarp (Ross, 1984). The seeds acquire innate, embryo dormancy upon post-harvest drying. During subsequent chilling (5 °C) of the imbibed seeds, dormancy is alleviated and germination commences upon their transfer to a higher temperature (20 °C) (Ross, 1996).

Early work on Prunus cerasus seeds indicated that dormancy relief by chilling is associated with the mobilization of phosphate (Pi) and the accumulation of organic phosphates (Olney and Pollock, 1960), suggesting considerable reserve mobilization and association with changes in the metabolic pattern. Therefore, the metabolic theory implies that dormant seeds are in some way metabolically impaired and this deficiency is removed during chilling (Ross, 1984). Previous studies in this laboratory have focused on a variety of metabolic pathways and their activities during dormancy alleviation in hazel seeds (Ross, 1996; Ross and Smith, 1992; Li and Ross, 1990a, b; Ratanakosum, 1986; Ross, 1984) and indicate that imbibed
dormant seeds are metabolically active at 5 °C, although at a rate slower than during germination. No single pathway was solely responsible for dormancy alleviation; instead it was proposed that the orchestrated and balanced changes in overall metabolism were the key to dormancy relief (Ross, 1996).

Phytic acid (IP6) is the mixed cation salt of myo-inositol-1,2,3,4,5,6-hexakisphosphate. It is ubiquitous in seeds, roots, vegetative storage organs, and pollen (Raboy, 2003). It represents the major stored phosphate resource of the seed and a pool of myo-inositol either destined to cell wall biosynthesis or rapidly utilized after conversion to pentose (Loewus and Murthy, 2000). IP6 might serve as a counter-ion for mineral storage; in hazel seeds IP6 salts contain Mg2+ and K+. (Lott and Buttrose, 1978). For further discussion on IP6 functions see recent reviews (Raboy, 2003; Loewus and Murthy, 2000).

IP6 turnover is mediated by intracellular specialized acid phosphatases (APases), the phytases (myo-inositol-1,2,3,4,5,6-hexakisphosphate phosphohydrolases, acidic pH optimum; EC 3.1.3.26) (Loewus and Murthy, 2000). Alkaline phytases have also been described (Oh et al., 2004) and Loewus and Murthy (2000) suggested that IP6 mobilization may be mediated by the action of multiple phytases. IP6 turnover has been extensively studied during seed and pollen germination (Loewus and Murthy, 2000; Gibson and Ullah, 1990; Lin et al., 1987). A profound reduction in IP6 levels is observed concomitantly to progressive germination correlating to increased phytase activity. Nevertheless, little is known about IP6 turnover during seed dormancy alleviation.

IP6 is found as globoid inclusions within the protein matrix of protein bodies (PB) in seeds (Lott et al., 1995), possibly surrounded by a membrane structure containing H+-pyrophosphatase (Jiang et al., 2001). In hazel seeds, numerous PBs were seen by electron microscopy; these exhibit internal dissolution early during the chilling treatment (Ross, 1984). As IP6 is the primary Pi store it is hypothesized that it could support wholesale metabolic reactivation during dormancy alleviation and preparation for subsequent germination. The fate of IP6 during dormancy relief is investigated by chilling in hazel. Temporal changes in phytase activity associated with IP6 and Pi mobilization are described, extending previous studies on key metabolic processes reported from this laboratory (Ross, 1996, 1984; Li and Ross 1990a, b). The subcellular localization of phytase has been investigated further and it is shown that it shares the same compartment with IP6. These results support the hypothesis that Pi mobilization is an early response of the embryonic axis to the cold temperature signal. Furthermore, combined with previous studies on the proteolytic activity in hazel seeds (Ratanakosum, 1986), it is suggested that the processes associated with PBs are important participants in the events culminating in dormancy relief and subsequent germination.

Materials and methods

Plant material

Hazel (Corylus avellana L., cv. Kent cobnut) fruits were commercially obtained, the cupules removed and the seed lot was air-dried at room (ambient) temperature until no further change in weight was observed. Dry hazelnuts were either dry-stored in airtight containers at 5 °C for no more than a year or used for stratification and warm-incubation (control) treatments as in Andriotis and Ross (2004). Dry-dormant samples (week 0 samples) were prepared from nuts removed directly from dry storage. When required, dissection of hazel seeds into cotyledonal and axiferous fractions was performed on ice and the tissue either used immediately or stored at −80 °C until required.

Germination tests

Dry, stratified, and warm-incubated seeds with the pericarp removed were surface-sterilized in 2% sodium hypochlorite for 10 min, thoroughly rinsed with sterile distilled water, and individually set to germinate in vials on sterile distilled water in temperature-controlled incubators at 20 °C, in the dark. Germination was monitored on alternate days and was said to have occurred upon radicle protrusion. All germination tests lasted for 30 d with 50 seeds per treatment.

Time-course of phytase activity during chilling

Acetone powders of cotyledonal tissue were prepared as in Andriotis and Ross (2003) and stored at −20 °C until required with no loss of enzymic activity. No acetone powders were prepared from embryonic axes; instead these were directly homogenized in extraction buffer. Crude protein extracts were prepared as in Andriotis and Ross (2003) and found to contain variable IP6 and Pi amounts. Thus, crude cotyledonal extracts were precipitated with (NH4)2SO4 (30–75%), resuspended in 100 mM acetate buffer (pH 5.5) and desalted through PD-10 columns (Amersham Biosciences, UK). Any precipitating material was removed by centrifugation and was found to contain no significant phytase activity. Extracts from embryonic axes were desalted through PD-10 columns without prior (NH4)2SO4 precipitation. No IP6 could be detected by HPLC after these operations, while endogenous levels of free Pi were reduced more than 90%.

Phytase activity assays and other analytical methods

Phytase was assayed in 300 µl reactions containing 100 mM acetate buffer (pH 5), 2.5 mM IP6 (dodecasodium salt from rice), and enzyme preparation at 37 °C for 4 h and terminated by the addition of ice-cold trichloroacetic acid (TCA) to a final concentration 10% (w/v). Following centrifugation for 10 min at maximum speed, the enzymatically liberated Pi was determined according to Fiske and Subbarow (1925). Phytase activity was expressed as in units (1 unit is the activity that liberates 1 µmol Pi mg−1 protein h−1). Protein determination and SDS-PAGE were as in Andriotis and Ross (2003).

Pi levels during chilling

To aliquots from crude protein extracts ice-cold TCA was added to a final concentration 10% (w/v). After 1 h on ice, samples were centrifuged at 13,000 g at 5 °C for 10 min and Pi determined in triplicate from three dilutions of the clear supernatants (Fiske and Subbarow, 1925).

Ion-paired C18 reverse phase HPLC determination of IP6

IP6 was determined by HPLC according to Burbano et al. (1995), with some modifications. Cotyledonal meal was defatted according
Phosphate mobilization during seed dormancy relief

Chilling alleviates hazel seed embryo dormancy imposed during post-harvest drying

During 5-weeks post-harvest drying, the seed lot developed secondary embryo dormancy (Fig. 1A) concomitant with the reduction in fresh weight. The nuts had lost c. 36% of fresh weight and the seeds exhibited 4% final germination compared to c. 80% at the freshly harvested state.

When dry-dormant fruits were stratified for 0–6 weeks a rise in germination potential was evident with progressive chilling (Fig. 1B). Seeds chilled for 2 weeks exhibited c. 25% final germination, which rose to c. 70% after 6 weeks stratification. Dormant seeds incubated at 20 °C without a chilling treatment had only a low capacity for germination (Fig. 1B). No germination was observed at 5 °C during long stratification periods (data not shown) indicating that dormancy relief and germination are distinct processes. It was concluded that chilling is an accumulating process necessary to overcome dormancy, while germination commences only upon transfer of the seeds to a higher temperature.

During stratification, the fresh weight of embryonic axes increased from 270 mg per 100 axes in the dry-dormant tissue to 420 mg per 100 axes after 6 weeks at 5 °C. Similar increase was observed in the warm-imibed seeds; however, weight of the latter was lower than the chilled axes. Little variation between weeks was observed in the dry weight of chilled axes; this reached a plateau at 130±0.2 mg per 100 axes.

IP6 mobilization is an early response of the embryonic axes to chilling

IP6 was extracted both from hazel seed axiferous and cotyledonal tissue and quantified by HPLC. In dormant axes it represented c. 0.7% dry weight and in cotyledons c. 2.5–3.2% dry weight in two successive crops with some year-to-year variation. Lower inositol phosphates (penta- to tris-phosphate) were detected in all samples (Fig. 2A, inset), however, they were not quantified. Myo-inositol mono- and di-phosphates could not be resolved by the applied HPLC method.

Axiferous IP6 levels declined rapidly after the first week at 5 °C, while after 3 weeks c. 40% of the initial IP6 levels
still remained, with little change thereafter (Fig. 2A). Such changes were not observed in warm-incubated axes; at the end of the treatment c. 85% IP6 still remained. Interestingly, an increase in extractable cotyledonary IP6 levels was observed by the fourth week of stratification, followed by a reduction thereafter (Fig. 2B). By the end of the treatment c. 65% of the initial IP6 levels still remained.

During seed and pollen germination IP6 turnover has been often accompanied by a rise in Pi levels (Silva and Trugo, 1996). In hazel axes, free Pi levels expressed on a fresh weight basis initially decreased during chilling with little change thereafter (Fig. 2C). In warm-incubated tissue, Pi levels declined throughout the experimentation period. In cotyledonary tissue, free Pi levels did not show significant change during chilling and by the end of the treatment an upward trend was seen (Fig. 2D).

**Temporal changes in phytase activity during chilling**

Substantial phytase activity was found both in embryonic axes and cotyledons of dormant hazel seeds (Fig. 3). Extractable phytase activity generally increased during chilling compared with warm-imibed tissue. After the first 3 weeks of imbibition at 5 °C a reduction was observed. However, activity increased thereafter. By the end of the treatment c. 60% (axes) and 110% (cotyledons) higher activity was determined as compared with the warm-imibed seeds.

**Phytase activity is associated with the protein bodies, the site of IP6 localization**

In order for phytase to act upon its primary substrate it should be in close association with IP6. Thus, it would be interesting to determine the subcellular localization of the hazel enzyme. The results of a crude subcellular fractionation are shown in Table 1. Almost 70% of total phytase activity was associated with the organelar fraction. Phytase solubilization was not enhanced in the presence of detergents, phospholipase C or salt suggesting that the enzyme is not an integral membrane protein or phosphatidylinositol anchored or ionically associated with membrane fractions (data not shown).

Hazel seed PBs were successfully isolated from both axiferous and cotyledonary dormant tissue by a modification of the non-aqueous methodology of Begbie (1979). Axiferous samples were loaded on the first KI/glycerol gradient and centrifuged. In fractions collected during unloading of the gradients protein levels exhibited a distinct peak at p 1.31–1.37 g ml⁻¹ (Fig. 4A). Only low contamination by cell wall fragments in the densest fractions of the gradient was evident at 400× magnification by LM, while lipid bodies and nuclei accumulated at p 1.26–1.28 g ml⁻¹ and p 1.49–1.52 g ml⁻¹ (data not shown). Phytase activity co-migrated with the protein peak, while Pi levels were higher in the top three fractions.

The fractions from the first density gradient corresponding to the protein and phytase peak were pooled and loaded onto the second, flotation gradient. All three components peaked at p 1.34–1.37 g ml⁻¹, while a second peak of Pi was observed at the denser fractions (Fig. 4B). PBs from cotyledons concentrated at p 1.31–1.40 g ml⁻¹ in the flotation gradient (Fig. 4C). Cotyledonary meal from 5-week chilled seeds was also run through the density gradients. These seeds had exited dormancy and should contain relatively few intact PBs (Ross, 1984). Protein levels were relatively evenly distributed throughout the gradient. Pi and phytase activity peaked at p 1.28–1.31 g ml⁻¹ (data not shown); therefore the distribution of all three components was different from that of the dormant tissue. When isolated
PBs were electrophoresed under denaturing conditions; storage proteins were resolved in five major subunits in the range 18–38 kDa (Fig. 5A).

IP6 globoids were visualized by LM in toluidine blue-stained sections of glutaraldehyde-fixed hazel embryonic axes. Under these conditions PBs appeared light blue, while metachromasia was observed for globoid inclusions that stained purple-blue (Fig. 5B). When observed under polarizing light these inclusions exhibited the characteristic ‘Maltese cross’ appearance (data not shown), which is not evident for starch granules in hazel seeds (Vaughan, 1970).

**Discussion**

During chilling, hazel embryonic axes are primed by an orchestrated mobilization of stored reserves (Li and Ross, 1990a; Ratanakosum, 1986) and biochemical (Li and Ross, 1990b) and ultrastructural (Ross, 1984) reorganization leading to dormancy relief. Hitherto, the various metabolic pathways studied in hazel do not appear directly linked to the fundamental control of dormancy loss; rather these are related to the process in that their activation occurs concomitant with the period of dormancy alleviation, as part of the essential preparation for germination (Ross, 1984).

In the present study, IP6 mobilization and the associated phytase activity during chilling have been investigated. IP6 levels in dormant tissue are in the range previously reported; 0.4–5% in cereals, legumes, and oilseeds (Raboy, 2003; Lott et al., 1995). Axiferous IP6 levels were significantly reduced within the first 3 weeks at 5 °C. During the same period the seeds have not exited dormancy and such changes were not detected in warm-imbibed tissue. Thus, IP6 turnover is identified as an early response of the embryonic axes (the site where the block in germination is imposed) to the low-temperature stimulus and the process is initiated before changes in other metabolic pathways have been described (Li and Ross, 1990a, b; Ross, 1984). However, neither the extent nor the rate of IP6 mobilization was as dramatic as during seed and pollen germination. Six days of germination of castor bean seeds resulted in 87% IP6 digestion (Azarkovich et al., 1999), while in lily pollen no IP6 could be determined after 3 h of pollen tube growth (Lin et al., 1987). It is suggested that the early axiferous IP6 turnover is important for metabolic activity of the resting tissue by means of supplying Pi and minerals that could reflect physiological and metabolic demands, for example, starch enzymology as has been postulated by Ogawa et al. (1979). It has also been suggested that...
expressed in units (1 unit is the activity that will release 1 mmol Pi mg\(^{-1}\) protein h\(^{-1}\)). All activity assays were performed at 37 °C, in triplicate and results are expressed as the mean ± standard errors (bars). Similar results were obtained from three independent experiments and from two successive crops.

Table 1. Crude subcellular fractionation of hazel phytase activity in cotyledons of 2-week chilled seeds

<table>
<thead>
<tr>
<th>Phytase activity (units)</th>
<th>% Total activity</th>
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<tr>
<td>Cytosolic fraction</td>
<td>0.12</td>
</tr>
<tr>
<td>Organellar fraction</td>
<td>0.23</td>
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<tr>
<td>Total</td>
<td>0.35</td>
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Phytase activity was determined at pH 5 with phytic acid (dodecasodium salt from rice) as the substrate. 1 unit is the activity that will release 1 mmol Pi mg\(^{-1}\) h\(^{-1}\). Phytase activity was expressed in units (1 unit is the activity that will release 1 mmol Pi mg\(^{-1}\) protein h\(^{-1}\)). All activity assays were performed at 37 °C, in triplicate and results are expressed as the mean ± standard errors (bars). Similar results were obtained from three independent experiments and from two successive crops.

... pyrophosphate-containing inositol phosphates (PP-IP), compounds related to IP6 and linked to the biochemical pathways involving inositol phosphate (Raboy, 2003), may serve as Pi donors for ATP synthesis. This could be potentially important during the early stages of germination prior to complete reliance on mitochondrial respiratory activity, although no direct evidence for such a process in seeds is available. Earlier studies questioned a direct link between IP6 deposition and subsequent turnover with ATP formation (Williams, 1970; and references therein).

IP6 was mobilized to a lesser extent in cotyledons, suggesting that appreciable amounts should remain to support subsequent germinative growth. The transient increase in cotyledonal IP6 could suggest net IP6 synthesis as postulated in germinating castor bean by Organ et al. (1988) and Dmitrieva and Sobolev (1984) to withstand elevated Pi. However, the barley low phytic acid (lpa) mutants store excess P as Pi instead of IP6 (Raboy, 2003). Nevertheless, seeds of lpa plants are viable and appear relatively normal, suggesting that IP6 metabolism could be not absolutely essential for seed Pi homeostasis. Furthermore, net IP6 synthesis occurs in castor bean seedlings regardless of the amount of exogenously supplied Pi (Crans et al., 1995). This could also imply that net IP6 synthesis would not significantly control endogenous Pi levels. Hazel seeds are fully imbibed after 3 weeks at 5 °C, thus the observed increase should be attributed to solubilization of the metabolite as in germinating maize seeds (Barba et al., 1997).

... germinating the rapid reduction in IP6 levels correlates with significant increase in phytase activity (Loewus and Murthy 2000; Gibson and Ullah, 1990). Typically, endogenous phytase activity is detected in ungerminated seeds; thus, some inherent activity is activated upon imbibition. This could explain the limited IP6 hydrolysis observed in warm incubated seeds. The initial increase in phytase activity during chilling occurred before the seeds had realized their full germination potential and not in those warm-imbibed for the same period. Thus, the initial rise is linked to dormancy relief, while the subsequent increase is attributed to the initiation of germinative growth and is not part of the dormancy relieving process, similar to the total APase in hazel (Andriotis and Ross, 2004) and chilled apple seeds (Rychter et al., 1972). Nevertheless, phytase was extracted from seeds held at 5 °C before activity measurements were conducted at 37 °C. Therefore, these results could indicate the potential for IP6 hydrolysis effected by the cold treatment, rather than characterize IP6 mobilization under conditions of chilling.

... Little change in extractable Pi (expressed on a fresh weight basis) was detected in hazel seeds during chilling, concomitant with IP6 mobilization, which is interpreted as evidence of active metabolism occurring in the seeds. Mukherji et al. (1971) suggested that Pi liberated from IP6 is rapidly utilized and converted to organic forms. Unaltered Pi concentration was also reported in germinating wheat (Matheson and Strother, 1969) and bean seedlings (Crans et al., 1995). Expressing Pi levels as a function of dry weight emphasizes the increased Pi content due to the hydrolysis of Pi-containing molecules (Strother, 1980).
However, when the data are expressed on a fresh weight basis, Pi levels tend to remain almost constant. A homeostatic mechanism was thus implied to operate during seed germination and increases in Pi levels may inhibit either the production or activity of phytase (Strother, 1980). Phytase is strongly and competitively inhibited by Pi (Andriotis and Ross, 2003), while the decrease in phytase activity coincided with maximal IP6 turnover. However, Gibson and Ullah (1988) questioned the in vivo inhibition of phytase during seed germination due to the sink effect of the axis.

In order for phytase to mediate IP6 turnover during chilling it should be, or brought, in association with its substrate. Intact PBs were isolated over non-aqueous gradients and found in densities of 1.31–1.40 g ml\(^{-1}\) and 1.34–1.37 g ml\(^{-1}\) in cotyledons and axes, respectively. This could reflect diversity in composition, variation in the amount of phytin and stored protein, and/or differences in the degree of tissue hydration as reported by Lott and Buttrose (1978). Phytase activity was confined in the same density range as the PBs. Protease and substantial APase activity were also associated with isolated hazel seed PBs (Ratanakosum, 1986). By LM IP6 globoids were observed embedded in the proteinaceous matrix of PBs of hazel axes. Therefore, phytase and its primary substrate co-localize to PBs in agreement with Baldi et al. (1988).

Fig. 4. Isolation of intact protein bodies from hazel seeds on non-aqueous density gradients. (A) First KI/glycerol gradient. Embryonic axes from dormant hazel seeds were homogenized in anhydrous glycerol, the sample loaded on the gradient and ultracentrifuged as described in the Materials and methods. The collected fractions were analysed for protein and Pi content and phytase activity. (B) Second shallow KI/glycerol gradient separation by the flotation of protein bodies from embryonic axes of dormant hazel seeds. The fourth fraction of the first KI/glycerol gradient in (A), corresponding to the protein peak, was overlaid with the flotation gradient. Following ultracentrifugation collected fractions were analysed as in (A), (C) As in (B) but for a sample from cotyledons of dormant hazel seeds. Similar results were obtained from at least three separate experiments. Symbols used are: closed squares, protein; open triangles, phytase activity; dashed line, Pi.

Fig. 5. (A) SDS-PAGE of protein bodies isolated with KI/glycerol density gradients from dormant hazel seeds. An aliquot of the fraction containing the protein peak from the gradients in Fig. 4 was electrophoresed under denaturing conditions and the gel stained with Coomassie Brilliant Blue. Lane 1: first KI/glycerol gradient. Lane 2: second shallow, flotation KI/glycerol gradient. The molecular weight (kDa) of standard proteins electrophoresed in the same gel is shown on the right. Letters on the left denote the major subunits observed: a, 38 kDa; b, 34 kDa; c, 23 kDa; d, 21 kDa; e, 18 kDa. (B) Localization of phytic acid globoids in protein bodies of dormant hazel seeds. Section from embryonic axes of dormant hazel seeds stained with toluidine blue and observed under bright field. Metachromatic staining (purple-blue) is observed for phytic acid globoids (arrows), while the protein matrix of protein bodies (PB) stains light blue. N: nucleus. Bar=5 μm.
In conclusion, these data suggest that IP6 mobilization is an early response of hazel embryonic axes to chilling. This could support metabolic reactivation of the resting seeds and preparation for subsequent germination. Phytase activity increased during chilling, the early rise being coupled with dormancy relief. The enzyme was associated with isolated intact PBs, the site where IP6 was also found sequestered. These results, combined with ultrastructural changes (Ross, 1984) and studies on the proteolytic activity in hazel seeds during chilling (Ratanakosum, 1986) further identify the hazel seed PBs and the processes associated with them as important and early determinants in the plethora of metabolic cues leading to seed dormancy relief and germination in this species.

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