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Changes in Phytic Acid and Phytase during Early Development of *Phaseolus vulgaris* L.

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Summary. Changes in phytic acid, phytase, and inorganic phosphate were examined in the cotyledon of *Phaseolus vulgaris* L cv. Taylor's Horticultural during embryogeny and germination. Embryogeny normally requires 36 days in this cultivar. Phytic acid is accumulated most rapidly between days 24 and 30. Coincident with this increase, relatively high values of inorganic phosphate were observed in the cotyledon. The inorganic phosphate in the developing cotyledon does not decrease until dehydration phase. This decrease cannot be entirely accounted for by the residual synthesis of phytic acid during this time. These data, along with data from the literature, suggest that close coordination exists between the biochemical systems responsible for the deposition of storage reserves in the cotyledon. Phytase activity remained undetectable during embryogeny. However, a rapid rise in phytase activity was observed commencing after day 2 of germination. The increased activity of phytase was well correlated with the disappearance of its substrate, phytic acid, from the cotyledon. Phosphate levels remained much lower than those observed during embryogeny.

Introduction

During embryo development, the cotyledon of the dicotyledonous plant passes through an initial period of rapid cell division and then through a period in which storage products are accumulated. Upon germination, the storage products are degraded by a complement of enzymes the activities of which increase rapidly during this time. Finally the cotyledon senesces relatively early in the post-embryonic development of the plant. Thus the major events in cotyledon development (cell division, cell specialization, function of the mature cell, and senescence) are well separated temporally. The anabolic nature of the storage metabolism of the cotyledon during embryonic development and the catabolic nature of the metabolism of the germinating cotyledon present two very clear phases in the developmental state of the system.

A considerable amount of information is available with regard to embryonic and early post-embryonic development of *Phaseolus vulgaris* L. (Öpik, 1968; Smith, 1973; Walbot *et al.*, 1972). Furthermore, removal of immature embryos from the ovule and culture under aseptic con-

ditions results in precocious germination, thus facilitating the study of factors involved in the shift of the cotyledon from embryonic to post-embryonic development.

A storage metabolite found in the cotyledon is phytic acid, the hexaphosphate of *myo*-inositol. Phytic acid has been found in all seeds examined for its presence, principally as the mixed salt of calcium, magnesium, and potassium (Mayer and Poljakoff-Mayber, 1963) and constitutes the major reserve of phosphate in the mature seed. The metabolism of phytic acid by seed plants has been previously examined in bean (Gibbons and Norris, 1963), lettuce (Mayer, 1958) and wheat (Sartirana and Bianchetti, 1967). However, most of this work deals either with the mature dormant[1] or the germinating seed. There have been few attempts to examine the metabolism of phytic acid in developing systems, and some of these attempts were hampered by the lack of accurate methods of staging embryo development (Makower, 1969; Jennings and Morton, 1963b; Asada *et al.*, 1969). I have investigated the metabolism of phytic acid and phytase, the enzyme responsible for the degradation of phytic acid, in the cotyledon of *Phaseolus vulgaris* during embryogeny and early post-embryonic development.

Material and Methods

Plant Material. Bush beans, *Phaseolus vulgaris* L. cv. Taylor's Horticultural (Asgrow Co., Orange, Ct., USA) were used in all experiments. For studies with embryonic cotyledons, plants were routinely grown in the Marsh Botanical Gardens, Yale University during the summer months and in controlled-temperature and light chambers during the remainder of the year. In the growth chambers the light period was 16 h per day and the temperature was maintained constantly at 23°. Light was supplied from fluorescent and incandescent lamps; the energy was 3.1 erg cm^{-2} s^{-1} at the surface of the plants.

For studies with germinating cotyledons, seeds were imbibed in tap water for 4–6 h at room temperature, then germinated in pans between sheets of moist paper toweling. The seedlings were placed in darkened cabinets and kept at 22°. No effort was made to maintain the plants in a fully etiolated condition.

Weight Measurements. Cotyledons were detached from the embryo or seedling and blotted, and their fresh weight was measured. Dry weights were obtained by drying the cotyledons to a constant weight in a 52° oven.

Determination of Phytic Acid. Phytic acid was measured according to the procedure of Makower (1970). Single cotyledons were dried and ground with a mortar and pestle. Samples of *ca.* 0.2 g were extracted 3 times with 5 ml 0.5 N HCl. After adjusting the pH to 2.0, phytic acid was precipitated from this extract by the addition of $FeCl_3$ and boiling. The precipitated ferric phytate was pelleted by centrifugation (10 min at $10000 \times g$), washed twice with 0.1 N HCl, and dispersed in 0.5 ml of H_2O and heated for about 1 h. The ferric hydroxide precipitate thus produced was collected by centrifugation (10 min at $2000 \times g$). After washing the precipitate twice with H_2O, it was dissolved in a small volume of 0.5 N HCl,

1 Dormancy is here used to describe a period of reduced activity during which growth and development do not occur.

heating when necessary to speed dissolution of the precipitate. This solution was transferred to a 25-ml volumetric flask and made to volume with H_2O. The ferric ion was then measured quantitatively by the *o*-phenanthroline method of Sandell (1959). The amount of phytic acid was calculated based on the theoretical stoichiometric binding between ferric ion and phytic acid (4 mol Fe^{3+}/1 mol phytate).

Measurement of Phytase. Phytase was extracted using the method of Gibbons and Norris (1963). Twenty cotyledons were ground with mortar and pestle in 25 ml of 0.01 M maleate buffer pH 6.5. The crude homogenate was centrifuged at $25000 \times g$ for 30 min. The pH of the resultant supernatant was lowered to 4.8–5.0 and the homogenate was re-centrifuged as before. This supernatant was then subjected to ammonium sulfate fractionation, and the 0–50% cut was retained. The protein pelleted by centrifugation was resuspended in 0.1 M maleate buffer at pH 6.5 and dialysed overnight against 0.01 M maleate, pH 6.5. The dialysed enzyme was used directly in the enzyme assay.

Phytase activity was measured by the release of *o*-phosphate from phytic acid. The reaction mixture contained 0.1 ml of enzyme and 1.9 ml of substrate buffer: 1.5 mM phytate, 1×10^{-5} M $MgCl_2$, 1×10^{-5} M $CaCl_2$ in 0.1 M acetate buffer, pH 5.2. The reaction was carried out at 37° for 2 h. Phosphate was measured according to Taussky and Schor (1953).

Orthophosphate Determinations. A single cotyledon was ground in cold 12% trichloracetic acid (TCA) in a motor-driven glass homogenizer tube. The extract was centrifuged at $10000 \times g$ for 20 min and the supernatant was transferred to a 25-ml volumetric flask. The solution was made to volume with cold TCA, and 2-ml aliquots were analysed for phosphate content by the Taussky-Schor procedure (1953).

Results

Cotyledon Development during Embryogeny and Germination

Embryo development to maturity in *Phaseolus vulgaris* requires approximately 36 days. According to the developmental timetable developed by Walbot *et al.* (1972), embryogeny in *Phaseolus* can be divided into 9 stages. The major embryonic organs are formed during the first three stages: proembryo, globular, and heart. The next two stages, known as cotyledon stages, last from about day 12 to day 30, when the cotyledon grows to fill the endosperm cavity. Stages VI through VIII encompass the maturation and dehydration phases of embryogeny. At about day 36 stage IX, dormancy, is reached. The changes in fresh weight, dry weight, and water content of *P. vulgaris* are shown in Fig. 1.

Phytic Acid

Small quantities of phytic acid are found in the cotyledon as early as day 12 of embryogeny (Fig. 2). However, phytic acid is not rapidly accumulated until after day 24 at which time a rapid rise of this metabolite in the cotyledons begins. By day 30 the rate of phytic acid accumulation has again become slow, but phytic acid continues to accumulate in the cotyledon throughout the final days of embryogeny. Approximately 90% of the total phytic acid in the cotyledon is accumulated between days 24

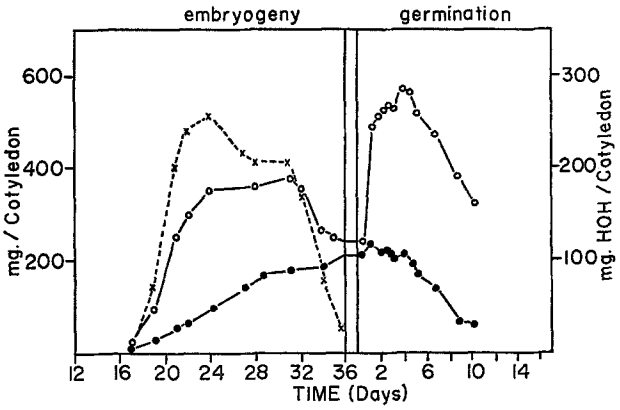

Fig. 1. Cotyledon fresh weight (o), dry weight (•), and water content (×) during embryogeny and germination

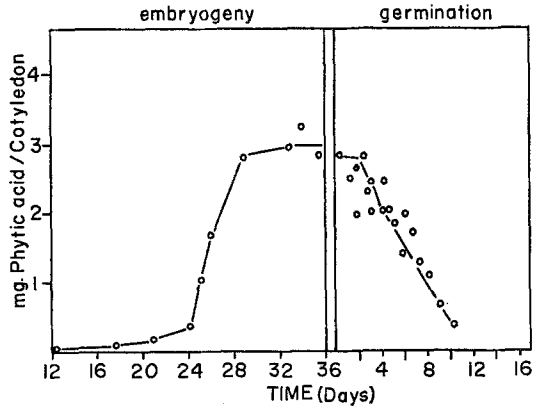

Fig. 2. Phytic acid content of the cotyledon during ontogeny

and 30 of embryogeny. The quantity of phytic acid in the cotyledon does not decrease during the first 2–3 days of germination and then declines at a constant rate for the next 8 days, resulting in a loss of 90% by day 10.

Phytase Changes

During embryogeny no phytase activity is present in the cotyledon. Phytase activity appears by day 3 of germination, reaches a maximum between days 6 through 8 and then declines as the cotyledon undergoes senescence (Fig. 3).

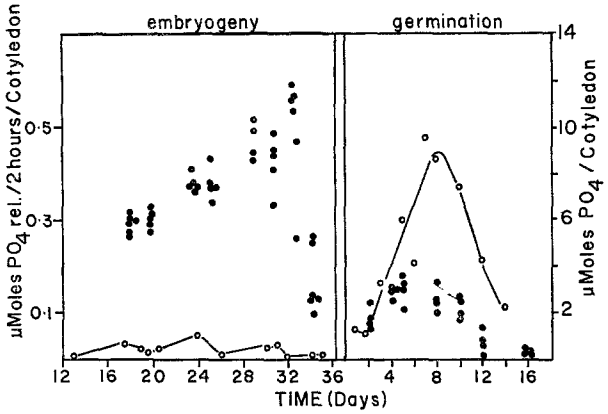

Fig. 3. Phytase activity (o) and *o*-phosphate (•) content of the cotyledon during ontogeny. The phytase activities observed during embryogeny are non-significant because they are less than the sensitivity of the test. The lower limit of the test is 0.05 µmol PO_4 released/cotyledon under these conditions of assay

Phosphate Changes

Measurements of inorganic phosphate in the cotyledon are shown in Fig. 3. Significant differences exist between the phosphate level in developing and in germinating cotyledons. During embryogeny the level of phosphate in the cotyledon progressively increases and the maximum concentration is reached between days 28 and 32 of development. The concentration of phosphate doubles between days 16 and 28, increasing from a value of 5–6 µmol to 10–12 µmol phosphate/cotyledon. As the cotyledon enters the dehydration phase and approaches full-term dormancy, the concentration of phosphate falls rapidly to a level of 2–3 µmol phosphate/cotyledon. During early seedling growth, inorganic phosphate concentration always remains low, seldom exceeding 3 µmol per cotyledon.

Discussion

The changes in fresh and dry weight of *Phaseolus vulgaris* cotyledons observed during embryogeny (Fig. 1) compare well with those observed by Walbot *et al.* (1972) in their more complete study of the growth of *P. vulgaris* embryos. The results are also consistent with the general pattern of the development of embryonic storage organs (Jennings and Morton, 1963a; Bain and Murcer, 1966). Although dry weight is accumulated at a constant rate over the entire course of late embryogeny from day 18 onwards, phytic acid is accumulated most rapidly over a more restricted period of time from day 24 to day 30 (Fig. 2).

Walbot (1971, 1973) has previously shown that RNA is accumulated in embryonic axes and cotyledons of *P. vulgaris* until day 25. Jennings and Morton (1963a, b) have observed that RNA accumulation also ceases about halfway through embryogeny in the wheat grain. In bean, the failure to accumulate RNA in the final days of embryogeny results from the cessation of RNA synthesis during the early maturation stages (Walbot, 1973). The rapid rise in phytic acid content in *P. vulgaris* cotyledons occurs just after the decline in the rate of RNA synthesis. Synthesis of small quantities of storage protein occurs very early in cotyledon development and continues quite late into embryo maturation, perhaps to day 32 or more (Kloz *et al.*, 1966). However, the largest amount of protein is produced between days 24 and 32 (Öpik, 1968; Walbot, 1973). Thus protein is accumulated in a fashion identical to the pattern established for phytic acid. Cytological evidence indicates that starch synthesis also follows this general pattern in *Phaseolus* (Öpik, 1968). Evidently, a high degree of coordination exists between the diverse systems which are responsible for the deposition of all of the cotyledonary reserve materials.

The continuous rise in inorganic phosphate concentration in the embryonic cotyledon of *P. vulgaris* from days 16 to 28 supports the conclusion that phosphate flow into the system is unrestricted over this period and that phosphate levels are unaffected by the massive synthesis of phytic acid (Fig. 3). This seems to be at variance with the report of Walbot (1973) who found a 4fold decrease in phosphate coincident with the synthesis of phytic acid. However, those values, which were obtained from axis material, were based on the fresh weight of the tissue. Replotting Walbot's data on a per-organ basis results in a curve essentially identical to the phosphate results presented here. Expressing these data on a per-organ basis is thought to reflect more accurately the nature of the changes occurring at the cellular level. Cell division in embryonic *P. vulgaris* cotyledons is most rapid during days 15 to 20. Cell number continues to increase thereafter, but at a very low rate (Öpik, 1968) so that the cell number of the cotyledon can be considered as virtually stable after day 20. No cell division occurs in the germinating cotyledon (Öpik and Simon, 1963).

After day 32 the concentration of inorganic phosphate in the embryonic cotyledon falls rapidly (Fig. 3). The most direct conclusion is that nutrient entry into the seed becomes restricted at this time. Based on the data presented in Figs. 2 and 3, there is a total of *ca.* 30–40 μmol of phosphate bound in phytic acid in the dormant cotyledon. Therefore, it is difficult to conclude that the drop in inorganic phosphate in the cotyledon after day 32 (approximately 8 μmol) results entirely from residual synthesis of phytic acid. Thus even though phosphate entry is clearly

restricted on or about day 32, additional or alternative mechanisms responsible for the observed phosphate decrease must be available to the cotyledon.

It has recently been suggested that water stress placed on the embryo by the disruption of the vascular connection to the placental tissue at the funiculus stimulates entry of the embryo into dormancy (Ihle and Dure, 1972). The water content of embryos is frequently presented as percentage by weight of the total fresh weight. Thus Walbot *et al.* (1972) have shown that the percent water content of *P. vulgaris* cotyledons decreases from 80% at day 20 at a more or less uniform rate to under 15% at dormancy. However, when presented on a per cotyledon basis, the total water content increases by more than 50% from day 20 to its maximum at day 24 and then declines by 20% over the next 3 days. This level is maintained through day 32 after which time there is a precipitous decline in the content of water.

Taken as a whole, these results present a consistent picture of early development in *Phaseolus* cotyledons. Through the early period of its embryonic development cell division in the cotyledon results in the production of a sufficient cell population to support later growth. Between days 20 and 24, just after the cotyledon has completely filled the seed cavity, critical events occur which establish a new equilibrium. RNA synthesis ceases, the rates of deposition of storage metabolites increase, and the water content stabilizes at a new, lower level. It is during this time that the system is self-directed toward dormancy. At day 32 the connection with the maternal tissue is restricted and the flow of nutrients into the cotyledon is interrupted. Dehydration occurs coincidently and full-term dormancy is reached.

Phytase activity cannot be detected in embryonic cotyledons. The rise in phytase activity during germination is coincident with a linear decrease in phytic acid content in the cotyledon. One significant difference between the events of germination and those of embryogeny is the failure of inorganic phosphate in germinating cotyledons to reach the high values observed in embryogeny even during active hydrolysis of phytic acid by phytase. It has been reported that inorganic phosphate can prevent phytase activity from appearing during germination in the wheat embryo (Sartirana and Bianchetti, 1967). Since great differences occur in phosphate concentrations between developing and germinating cotyledons in *P. vulgaris*, phosphate may play a role in the control of phytase activity during embryogeny and germination.

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