Nutritive Role of the Seedcoats during Embryo Development in *Pisum sativum* L.¹

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ABSTRACT

Structural and metabolic features of the seedcoats of the developing pea seed indicate that events in the cells of the seedcoats are of major importance in controlling the development of the embryo. Information has been obtained on the distribution of N and P constituents of the seedcoats, embryo sac liquid, and the cotyledons of the embryo, in relation to the changes in the activities of several enzymes: aminopeptidases, β-glucosidase, and acid phosphatase. The liquid contents of the embryo sac are considered to arise as a secretion from the tegmen. High concentrations of amino acids (about 0.3 molar), NH₄⁺ (about 0.1 molar), and orthophosphate (Pi) (up to 4 millimolar) were measured in this fluid. Since Pi was the only form of P present, the data confirm the possible function of some of the seedcoat acid phosphatase activity in the provision of Pi to the embryo.

The developing legume seed has two coats, the testa and the tegmen, derived respectively from the outer and inner integuments of the ovule. The testa becomes sclerified (34, 35) and as dead tissues, the seedcoats of the mature seed serve to protect the enclosed embryo. This protective function of the seedcoats is well known. An equally important function of the living seedcoats in regulating the growth and development of the embryo has been largely ignored (8).

It is known that the seedcoats of *Pisum sativum* acquire reserves of starch and protein, which are mobilized as the seedcoats senesce and as the embryo matures (2, 6, 9, 25, 32). Mineral reserves are also mobilized from seedcoats to embryo, with varying degrees of retention (14). Clearly, metabolic and structural changes of considerable moment are taking place in the cells of the seedcoats during seed development, yet in relatively few studies have parameters relating to the seedcoats been assessed separately from those relating to the embryo.

Here, I have sought to show how changing hydrolytic enzyme activities in the seedcoats may be related to the nutritive and regulatory functions of the seedcoats during embryo development. This investigation is an extension of those in which changing enzyme activities of pea seedcoats were first reported (6, 25). Acid phosphatase (EC 3.1.3.2) was found to develop in the seedcoats from an early stage, involving the synthesis of a distinctive isoenzyme (25). However, reliable information on the distribution of P in the parts of the developing seed has been lacking. This has now been obtained in order to assess the possible function of seedcoat acid phosphatase in the provision of Pi to the embryo. The aminopeptidases previously studied (6) were examined in the seedcoats from maturing seeds only, where their declining activities were correlated with the net breakdown of protein accompanying the senescence of seedcoat tissues. In the present work, the relationships of changing aminopeptidase activities to nitrogen metabolism have been examined for both seedcoats and cotyledons at all stages of development.

MATERIALS AND METHODS

Plant Material. Pea plants, *Pisum sativum* L. cv. Telephone, were grown in the open garden from early summer. Developing seeds were obtained in the months of January and February, 1978, from pods sampled at increasing intervals from full bloom.

Preparation of Extracts. For each sample, a set of three closely matched seeds (to within 5 mg fresh weight) were taken from a single pod. Seed 1 was dissected for determination of dry matter and water content (6). Seed 2 was dissected as rapidly as possible; first the seedcoats, then the cotyledons were weighed and separately extracted with cold 0.01 M NaF as an inhibitor of acid phosphatase activity, thus providing samples for the accurate determination of Pi⁰ and Pi. Seed 3 was dissected and provided duplicate extracts prepared with cold 0.5 mM 2-ME for enzyme assays. Extracts were prepared by thorough grinding with chilled porcelain mortar and pestle, using ice-cold extracting medium at a ratio from 4:1 to 10:1 (v/w). Acid-washed sand was used only for the last (49-day) stage. The crude extracts were centrifuged immediately at 9,000g for 5 min using a Beckman Microfuge. Clear supernatants were removed and the NaF extracts were immediately sampled in duplicate for treatment with 4 volumes of 7% (w/v) trichloroacetic acid. For cotyledon extracts, acid-insoluble materials were collected by centrifugation after about 30 min and the acid-soluble fraction stored at −20 C. Seedcoat samples were first frozen to allow complete precipitation. Acid-insoluble fractions from cotyledons were washed three times with 7% trichloroacetic acid at room temperature, then extracted with trichloroacetic acid at 90 C for 15 min to yield a soluble nucleic acid fraction (10). The acid-insoluble materials were finally dissolved in 0.25 M NaOH and their protein content determined by the biuret reaction, using lipid-extracted BSA as standard (6).

Sampling of Liquid Contents of Embryo Sac. At the earliest stages, seeds were pricked with the corner of a clean razor blade and the liquid from the embryo sac was removed with a microsyringe. Measured samples were stored frozen after 50-fold dilution with distilled H₂O. pH was measured using Neutralit indicator paper (Merck, Darmstadt).

Enzyme Assays. Acid phosphatase activity (EC 3.1.3.2) was measured as before (25). β-Glucosidase (EC 3.2.1.21) was measured as the enzymic hydrolysis of p-nitrophenyl β-D-glucoside using 50 μl of extract and incubation periods up to 40 min at 30 C. This activity was assayed at pH 5.0, within the optimum from pH 4.7 to pH 5.4. The phenanthroline-insensitive (AP1) and

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3 Abbreviations: Pi⁰ ester P; 2-ME: β-mercaptoethanol.
phenanthroleine-sensitive (AP2) aminopeptidase activities were measured using leucyl β-naphthylamide as substrate according to Collier and Murray (6). This assay was performed first as color development required over 1 h. All assays were completed within 2 h of beginning extraction.

Measurement of P. Total acid-soluble P and Pi were determined according to Ames (1). Ester P was calculated as the difference between total acid-soluble P and Pi. Nucleic acid P was measured as total P content of hot trichloroacetic acid-extractable material.

Other Analyses. Acid-soluble amino nitrogen (including NH₄⁺) was measured using ninhydrin (37) with glycine as standard. NH₄⁺ was measured separately in samples of diluted embryo sac liquid using a Nessler reagent and the values for amino nitrogen were corrected accordingly (26). Because trichloroacetic acid had been used, NH₄⁺ could not be determined separately for seedcoat and cotyledon extracts by this method. The NH₄⁺ contents of the cotyledons at least are likely to be low (17) and the values given as amino nitrogen will therefore approximate amino acid content fairly closely. Total sugars (mainly hexose derived from sucrose) were measured in soluble fractions using glucose as standard (38). Reducing sugars only were measured from embryo sac liquid samples.

RESULTS

Growth of Seedcoats and Embryo. Figure 1 compares the fresh weight, dry matter content, and water content (as per cent of maximum) of the seedcoats with the same parameters of a single cotyledon of the embryo. The maximum dry matter content of the seedcoats is attained by 27 days. Total dry matter declines only slightly thereafter, as the sclereids of the outermost cell layers complete their development (34, 35) and the inner parenchymatous cells senesce and are crushed. The distinctive ontogeny of the seedcoats is reflected in their earlier and more rapid dehydration compared to the cotyledons (Fig. 1). The cotyledons show a typical biphasic pattern of dry matter accumulation (6) continuing throughout the final period of dehydration.

Changes in Major Metabolites during Seed Development. The contents of free amino acids, protein, hexose, and the various P constituents of the seedcoats and the cotyledons changed as shown in Figure 2. Dealing first with the nitrogenous compounds, it may be seen that: (a) The free amino acid content of the seedcoats is maximal early, reaching this maximum in concert with the net synthesis of proteins in the seedcoats. (b) The protein content of the seedcoats is maintained at a maximum value throughout the

![Graph](image)

Fig. 1. Changes in fresh weight (○), dry matter content (●), and water content (△, as per cent of maximum value) of seedcoats and cotyledons of pea seed during development. Data are expressed per seed (seedcoats) or per single cotyledon.
period of rapid cell expansion in the embryo. (c) The decline in the protein content of the seedcoats accompanies the slight decline in dry matter content (Fig. 1) but does not result in increased concentrations of soluble amino nitrogen in the seedcoats (Fig. 2). This situation resembles the behavior of the pod wall and subtending leaf during their senescence (41). (d) The maximum free amino acid content of the cotyledons occurs shortly after that of the seedcoats, just preceding a phase of exponential increase in net synthesis of protein. (e) The free amino acid content of the cotyledons increases slightly as the products of protein breakdown in the seedcoats (and pod wall) become available, then stabilizes at a value which is remarkably similar to those reported for whole seeds in other studies (about 50 μmol/seed; 22, 30). (f) The protein content of the mature cotyledon represents 23.3% of total dry matter, a value comparable to the 22% determined for the same cultivar in a different (cooler) growing season (6).
Although starch-sugar relations were not a major concern of the present study, the total hexose content was measured (Fig. 2). The hexose content of the seedcoats was maximal at 14 days, declining very gradually until after 41 days, then more quickly (Fig. 2). The sugar content of the cotyledons showed an initial maximum after 23 days, declined, then finally increased to a very high value (Fig. 2). Reference to an earlier study (5) on a dwarf variety of 

Telephone suggests that this final high level of sugars may be characteristic of this cultivar. Lower values (about 100 μmol/cotyledon) have been reported for cv. Victory Freezer (2) and Canners Perfection (23, 42) and very low values have been reported for field pea (e.g. μmol/cotyledon; 16), a situation correlated with a higher starch content relative to garden peas (27).

Bain and Mercer (2) found that the onset of the decline in sugar content of the cotyledons coincided with the beginning of water loss. Such a correlation was not evident in the present study (Figs. 1 and 2).

Most of the P of the seedcoats is present as Pi until after the embryo has filled the embryo sac (Fig. 2). Thereafter, P₆ constitutes more than half of the P content of the seedcoats until maturity, when the residual P of the seedcoats is entirely Pi (Fig. 2). These data are also expressed on a fresh weight basis (Table I) to aid comparison with earlier studies on whole seeds (23, 36).

In contrast to the seedcoats, the cotyledons show a Pi content which is never more than a quarter of the total measured phosphate (Table I). This proportion declines to a minimum close to 5% of total P by 41 days, but finally increases at maturity (Fig. 2 and Table I). Such an increase has also been observed for cv. Melbourne Market (unpublished data). These observations differ from those of McKee et al. (23) who reported that the Pi content of the whole seed was maintained at a maximum level of 4.5 μmol/seed throughout maturation.

The increases in acid-soluble P₆ of the cotyledons reflect the synthesis of phytate, myo-inositol hexaphosphate, the main P reserve of the pea embryo (10, 20). Since the amounts of the individual sugar phosphates are very low throughout development (36), it is clear that phytate synthesis is well under way by 31 days (Fig. 2). The synthesis of phytate proceeds faster than the accumulation of proteins (Fig. 2), yet phytate eventually shares a common location with the globulins inside the protein bodies (20). These observations suggest that the phytate is first deposited inside vacuoles as phytin and is later surrounded by acquired globulins.

Analysis and Significance of the Embryo Sac Liquid. The early maturity in Pi, amino acid, and sugar concentrations in the seedcoats (Fig. 2) coincide with a period of secretion of so-called endosperm liquid into the embryo sac. The maximum seed content of this liquid is 45 to 50 μl, with fresh weight reaching 0.070 g, which is much greater than the maximum of 0.013 g recorded for field pea (9). The pH was found to be pH 5.5, compared to pH 5.2 to 5.4 for similar secretions from bean seeds (40). Table II shows the concentrations of several key metabolites determined for embryo sac liquid obtained from the two earliest samples in this study. Compared to bean (Phaseolus vulgaris) the concentrations of Pi and NH₄⁺ are similar (40), but the concentrations of amino acids are about 6-fold greater (40). As discussed below, I consider that this liquid is not endosperm, which has disappeared from the seed before the earliest stage studied here (7), but an active secretion from the seedcoats, resulting from the metabolism of P esters and amino group donors, especially asparagine and serine, received from the phloem by the seedcoats.

Changes in Enzyme Activities. Figure 3 shows the changes which were observed in the activities of two aminopeptidases, β-glucosidase and acid phosphatase. The main features of these observations are as follows: (a) The increases in aminopeptidases in the seedcoats occurred rapidly, with both enzymes achieving maxima after 14 days. (b) The pattern of changes in AP1 activities correlated more closely with the changes in protein content of the seedcoats than did the AP2 pattern. (c) AP2 activity had practically disappeared from the seedcoats while AP1 was still measurable (cf. 6). (d) Both AP1 and AP2 activities in the cotyledons followed the pattern of accumulation of dry matter (Fig. 1) rather than that of fresh weight (Fig. 1) or protein (Fig. 2). Both activities continued to increase throughout dehydration, confirming previous observations for this cultivar (6). (e) The decline in β-glucosidase activity in the seedcoats preceded the decline in aminopeptidases and acid phosphatase. (f) The β-glucosidase activity of the seedcoats was greater than that of the cotyledons at all stages examined except the last. (g) The declines in acid phosphatase activity and AP1 activity in the seedcoats occurred at similar rates (Fig. 3), in contrast to the slower decline in acid phosphatase activity shown by seedcoats of seeds developing under cooler conditions (25). (h) Of the enzymes studied, only acid phosphatase persisted in the dehydrating seedcoats. (Figs. 1 and 3).

**DISCUSSION**

Seasonal fluctuations superimposed upon already diverse varietal potential have in the past rendered a universal description of pea seed development virtually impossible (27). It is fundamental to consider embryo development in terms of cell expansion preceded by a period of intensive cell division. This initial phase encompassing most cell divisions extends to about 10 days for garden peas (7, 23, 24) but may be considerably longer in field pea (39). It is impossible to assign the first appearances and accumulations of the various reserve materials to distinctive stages. Reserve synthesis in the embryo may begin during the initial phase of cell division (24) and continue well into the period of cytosol shrinkage accompanying desiccation (6, 24, 39, and Figs. 1–3).

The present study emphasizes the need to distinguish parameters relating to the seedcoats from those of the embryo. This has not been done in earlier work on seed development except in the studies by Raake (31–33), Bain and Mercer (2), and Flinn and Pate (9). The present work also serves to focus attention on some very large gaps in our knowledge of the pathways of nutrient transmission from the parent plant to the developing embryo.

It is clear that the phloem bears the vast majority of translocated substrates from the subtending leaves and other parts of the plant

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**Table I. Distribution of Total Phosphate and Pi as Per Cent of Total Measured Phosphate in the Seedcoats and Cotyledons of Developing Pea Seeds**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Seedcoats</th>
<th>Cotyledons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol phosphate per g fresh weight</td>
<td>μmol phosphate per g fresh weight</td>
</tr>
<tr>
<td>12</td>
<td>34.2</td>
<td>7.06</td>
</tr>
<tr>
<td>14</td>
<td>35.2</td>
<td>7.82</td>
</tr>
<tr>
<td>23</td>
<td>20.9</td>
<td>6.22</td>
</tr>
<tr>
<td>27</td>
<td>13.3</td>
<td>4.56</td>
</tr>
<tr>
<td>31</td>
<td>7.06</td>
<td>4.29</td>
</tr>
<tr>
<td>41</td>
<td>18.0</td>
<td>17.8</td>
</tr>
<tr>
<td>49</td>
<td>4.72</td>
<td>100</td>
</tr>
</tbody>
</table>

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**Table II. Concentrations of Some Metabolites in the Embryo Sac Liquid from Developing Pea Seeds**

<table>
<thead>
<tr>
<th>Component</th>
<th>Time after Flowering</th>
<th>12 days</th>
<th>14 days</th>
</tr>
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<tbody>
<tr>
<td>Pi</td>
<td>3.78</td>
<td>3.20</td>
<td></td>
</tr>
<tr>
<td>P₆</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>122</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Amino nitrogen</td>
<td>254</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>76</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

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NUTRITIVE ROLE OF SEEDCOATS IN *PISUM*

Fig. 3. Changes in activities of aminopeptidases AP1 (○) and AP2 (●), β-glucosidase (△), and acid phosphatase (□) in seedcoats and cotyledons of developing pea seeds. Activities are expressed as enzyme units per plant part (except β-glucosidase, as enzyme units × 10⁴ per plant part).

Hardham (12) observed that transfer cells did not accompany the phloem from the funiculus into the seedcoats and stated that "the functional significance of the numerous phloem transfer cells in the funicle which stop so abruptly at the junction with the ovule is puzzling ...". Since transfer cells are regarded as of paramount importance in the "loading" of translocated substrates into the phloem (11, 28), their absence from the tissues of the seedcoats should facilitate "unloading" of transferred metabolites into the cells of the seedcoats. Indeed, their absence may even be obligatory for the rapid release of such substantial quantities of solutes as are known to enter developing legume fruits (15). Since all nutrients acquired...
by the embryo must pass through the seedcoats (8, 12), the rates of transmission of most individual nutrients from the seedcoats are best gauged as their rates of accumulation in the embryo rather than as their net rates of decline within the seedcoats (Table III). It is also becoming clear that certain of the metabolites received by the seedcoats from the phloem are radically altered before their transmission to the embryo sac liquid and embryo. Sucrose, the main component of phloem sap (18, 29, 30), is transmitted with variable proportions hydrolyzed to free glucose and fructose (5, 39, 43). However, the alterations imposed by the tissues of the seedcoats on the incoming nutrient supply chiefly concern the N and P constituents.

Considerable differences in the amino acid profiles of phloem sap (18), seedcoats (9), embryo sac liquid (9), and embryo (21) have been recorded. Embryo sac fluid from field pea has an amino acid composition which varies little (9) and which shows that homoserine, alanine, and glutamine are relayed from the phloem without impediment. However, the much reduced contents of asparagine, serine, and aspartate compared to phloem sap (18) and the enhanced contents of valine and threonine (9) in this secretion bear testimony to the extensive and selective metabolism of amino acids taking place in the seedcoats prior to the release of amino acids, NH₄⁺ (Table II) and organic acids (40) to the embryo sac fluid and the embryo. Since the concentration of NH₄⁺ is generally low in plant tissues, it is likely that its generation by the seedcoats is of regulatory significance, as suggested by Smith (40). Precisely what the effects of NH₄⁺ are on the development of the pea embryo remain to be determined. It might be anticipated that the embryo would possess enzyme systems (e.g., glutamate dehydrogenase, EC 1.4.1.3) permitting it to assimilate ammonia, since intracellular concentrations of only 5 mM would be likely to uncouple phosphorylation of ADP in both mitochondria and chloroplasts (e.g., 44) and external concentrations as low as 1 mM have been shown to influence amino acid metabolism in Chlorella (13).

There is increasing evidence that much or most of the P of the phloem sap is esterified (3, 4, 15, 19), yet no P esters have been detected in the fluid secreted internally by the seedcoats (Table II). It is tempting to conclude that it is the metabolic intervention of the seedcoat tissues which requires the developing embryo to take up Pi as its sole source of P. At least some of the acid phosphatase activity of the seedcoats is likely to participate in the generation of Pi for secretion from the cells which flank the embryo sac. Obviously not all of the developing acid phosphatase activity (Fig. 3) can possibly function in this way; compartmentation is implied by the almost parallel patterns of development of P₄₀ content (Fig. 2) and of total acid phosphatase activity of the seedcoats (Fig. 3). Even the relatively low activity of acid phosphatase remaining in the seedcoats after 41 days (about 0.2 units, equal to 528 μmol of substrate converted per day) is vastly more than adequate to evacuate the P₄₀ content of the seedcoats (about 1 μmol) over the last 8-day interval (Fig. 2).

In assessing this possible role of acid phosphatase in generating Pi from P4₀ within the cells of the seedcoats, it should be pointed out that Bieseki (4) considers Pi to be the sole mobile form of P in the phloem. Hence the possibility that a substantial proportion of the Pi acquired by the embryo passes unaltered from the phloem, must be admitted. Further work will be necessary to quantify the transference of P via these alternative pathways.

Acknowledgement - The excellent technical assistance of Miss J. Wardrop is gratefully acknowledged.

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Table III. Net Rates of Accumulation of Phosphate and Amino Nitrogen by the Cotyledons Compared with the Net Losses from the Seedcoats in the Same Period (27 to 31 Days after Flowering)

<table>
<thead>
<tr>
<th></th>
<th>Phosphate</th>
<th>Amino N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Net gain by cotyledon pair (μmol)</td>
<td>21.6</td>
<td>152</td>
</tr>
<tr>
<td>Rate of net gain (μmol/day)</td>
<td>5.4</td>
<td>38</td>
</tr>
<tr>
<td>Net loss from seedcoats (μmol/day)</td>
<td>1.55</td>
<td>14</td>
</tr>
<tr>
<td>Rate of net loss (μmol/day)</td>
<td>0.39</td>
<td>3.5</td>
</tr>
</tbody>
</table>

For the purposes of calculation 1 mg protein has been treated as equivalent to 8 μmol constituent amino acids.
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