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CELLULAR RUPTURE AND RELEASE OF PROTOPLASM AND PROTEIN BODIES  
FROM BEAN AND PEA COTYLEDONS DURING IMBIBITION

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Abstract

Imbibition is a critical phase in germination and processing of legume seeds because cellular disruption during imbibition may influence seedling vigor and processing quality. Cellular disruption of cotyledonary surfaces of beans (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.) and the cellular contents released during imbibition were examined with scanning electron microscopy. Two types of cellular disruption were observed during imbibition: ruptures and fractures. Individual cells and small groups of cells on the surfaces of cotyledons ruptured after immersion in water. Ruptured cells had flaps of cell walls which remained attached to intact portions of cell walls. Fractured cells split in half, and remnant portions of cell walls were completely separated from each other. Disrupted cells on the interior surfaces of blister cavities were of the fractured type.

Materials released from cotyledonary tissues consisted of both dense aggregates of protein bodies and a dispersed phase of protoplasm. In some cases, protoplasm and protein bodies on cotyledonary surfaces were found adjacent to single cell ruptures and, in others, the sites of losses were not found. The presence of protoplasm and protein bodies and absence of sites for their release indicate an additional mechanism other than fracture or rupture may contribute to losses of intracellular substances during imbibition.

Introduction

Legume seeds and excised cotyledons which are soaked in water or sown in wet soil release intracellular substances into the surrounding water (Schroth and Cook, 1964; Duke and Kakefuda, 1981; Duke et al., 1983; Spaeth, 1987). Substantial quantities of nutrients can be lost from seeds during soaking, e.g., 60% of K content in 24 h (Simon, 1984). In crop production, losses of intracellular materials are associated with poor seedling vigor and stand (Larson, 1968; Powell and Matthews, 1978) because cells are ruptured and storage reserves are not available for growth of seedlings. Loss of intracellular substances is also associated with increased pathogen growth (Schroth and Cook, 1964) because nutrient sources become available to soil-borne pathogens.

The loss of intracellular substances has been attributed to large scale disruption of tissues manifested as transverse cracks (Morris et al., 1970), displacement of tissue strips (Duke and Kakefuda, 1981), and blistering of embryonic axes (Dunn et al., 1980) and cotyledonary surfaces (Spaeth, 1987). Multicellular blisters on surfaces of bean cotyledons ranged from less than 0.4 to more than 0.8 mm in diameter (Spaeth, 1987). One mechanism for release of intracellular materials from blisters is pressure-driven extrusion (Spaeth, 1987). Partially hydrated protoplasm, protein bodies and, in some cases, starch grains (granules) exhibit characteristics of a viscous fluid which is extruded as streams of material through irregular orifices in blisters.

Solute leakage from cotyledonary tissue is not always accompanied by visible tissue disruption. While cotyledons of one bean cultivar produced surface blisters, a second bean cultivar and two pea cultivars extruded small diameter streams of intracellular material but did not form visible blisters (Spaeth, 1987). Seeds with intact seed coats may leak substantial amounts of intracellular substances, yet not exhibit visible tissue disruption (Simon and Raja Harun, 1972; Duke and Kakefuda, 1981; Simon, 1984). The existence of cellular ruptures has been inferred from the appearance of intracellular substances, e.g. organelle specific enzymatic markers (Duke

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**Key words:** beans, *Phaseolus vulgaris* (L), peas, *Pisum sativum* (L), cellular rupture, protoplasm extrusion, nutrient loss, solute leakage, soaking injury, imbibitional stress, imbibitional injury, protein bodies.

and Kakefuda, 1981), which are too large for diffusion through intact membranes. Evans Blue stain has been used to demonstrate plasma membrane rupture during imbibition (Duke and Kakefuda, 1981; Schoettle and Leopold, 1984). Detailed microscopic studies of cell rupture would help to elucidate mechanisms of solute leakage and its role in reduced legume seed viability.

The first objective of this research was to examine cellular disruption on a scale smaller than transverse cracks, crescent cracks, and blisters. The second and third objectives were to identify various types of cellular disruption, and to identify some materials lost from cotyledons during imbibition. Scanning electron microscopy (SEM) was used to examine excised cotyledons and protoplasm lost during imbibition.

#### Materials and Methods

Seeds of two bean cultivars (*Phaseolus vulgaris* L. cv Apollo, a snap bean, and cv Royal Red Mexican, a dry bean) and two pea cultivars (*Pisum sativum* L. cv OSU 605, a wrinkled pea, and cv Garfield 81, a round pea) were obtained from local commercial or research sources. Seeds were equilibrated to constant water content over a saturated solution of potassium carbonate. Water content of seeds was determined by oven drying at 105°C for 48 h. Initial water contents of bean and pea cotyledons were 11 and 10% of seed dry weight, respectively.

Coats of dry seeds were carefully removed with a scalpel. Individual cotyledons were immersed in 6 ml of distilled, deionized water at 24°C. Cotyledons and extrusion streams from cotyledons were observed with a dissecting microscope during imbibition.

Cotyledons for SEM were prepared in several different ways. To prepare unfixed samples for SEM, some cotyledons were simply excised from dry seeds. Other cotyledons were excised, soaked in water for 30 min, blotted to remove excess water, and then allowed to dry at ambient temperature and humidity. In one set of Apollo bean cotyledons, a droplet of water was applied to abaxial surfaces. After the water was absorbed into the cotyledon or evaporated, the sample was reequilibrated at ambient relative humidity. Another set of bean cotyledons was fractured dry. Fracture surfaces were washed free of protoplasm and cellular inclusions as described by Wolf and Baker (1980). Dry tissues of all unfixed samples were sputter coated with gold (Hummer-Technics)<sup>1</sup>.

To prepare fixed samples for SEM, cotyledons were immersed in 6 ml distilled, deionized water until blisters or extrusion streams appeared (20 min). To preserve the structure of extruded protoplasm during fixation, a glutaraldehyde:water solution (25%) was gently added to Petri plates containing the soaking seed, and mixed with the soak water to create a 5% glutaraldehyde solution for fixing tissue and extruded protoplasm. Thirty min after addition of the glutaraldehyde solution, cotyledons were rinsed and transferred to a 2% solution of osmium tetroxide in water for 30 min. Samples were

dehydrated in a graded ethanol series (30 to 100%) (Hughes and Swanson, 1985). Fixed samples were dried in carbon dioxide with a critical point drier (Bomar SPC-1500)<sup>1</sup>, sputter coated with gold (Hummer-Technics)<sup>1</sup>. All samples were viewed and photographed at 20 kV with an ETEC U-1 Scanning Electron Microscope<sup>1</sup>.

#### Results

In addition to the large scale cracking and multicellular blisters previously reported, epidermal cells of legume cotyledons were ruptured during imbibition. Areas of Apollo bean cotyledons which were not blistered exhibited ruptured cells and the contents from the ruptured cells on adjacent unblistered tissue (Fig. 1). Only a few of the total number of cells on the surface were ruptured (Fig. 1). Holes consisted of individual or small groups of ruptured cells. Flaps of cell walls, which were formed by the rupture process, were attached to remaining walls of ruptured cells. Cell contents released from cells did not contain recognizable constituents (Fig. 1). Cell contents from ruptures did not form extrusion streams as did cell contents from blisters (Spaeth, 1987).

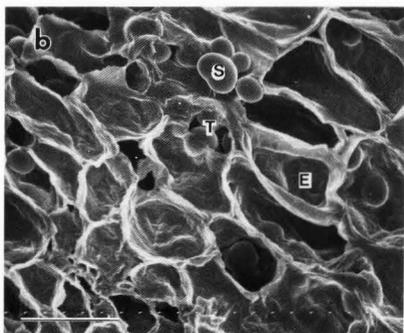
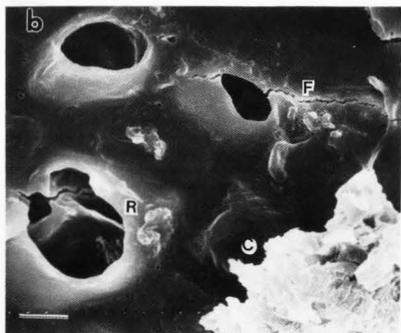
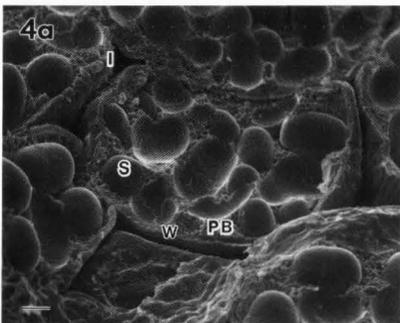
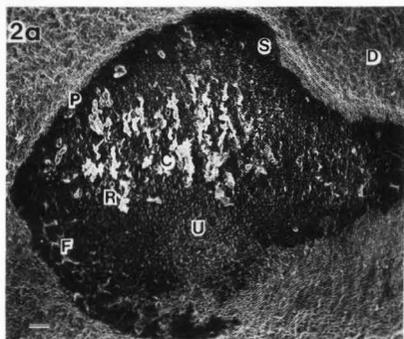
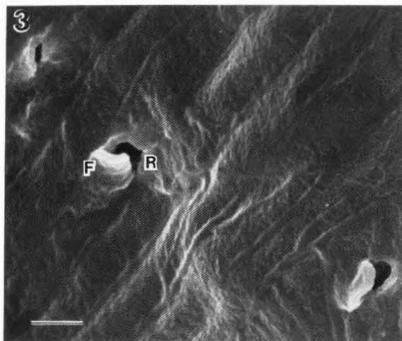
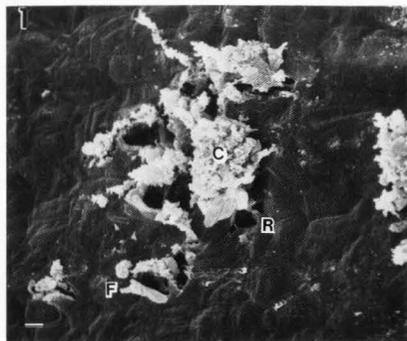
The scale of individual cell rupture was too small to be seen without SEM, therefore, it was not possible to use light microscopy to directly confirm that individual cells or small groups of cells ruptured during soaking. To

**Fig. 1.** Ruptured cells (R) with wall flaps (F) and cell contents (C) on the surface of Apollo snap-bean cotyledons soaked prior to fixation. Bar = 10  $\mu$ m.

**Fig. 2.** Surface cells of Apollo bean cotyledons which had single drops of water applied to cotyledonary surfaces. Low magnification (2a) shows dry (untreated) tissue (D), perimeter of the treated area (P), ruptured cells (R), unruptured cells (U), cell contents (C), smooth surface (S) with fissures (F). Bar = 100  $\mu$ m. Same seed at higher magnification (2b) shows ruptured cells (R), cell contents (C), and fissures (F) in the epidermal surface covered with a film of cell contents. Bar = 10  $\mu$ m.

**Fig. 3.** Ruptured cells (R) in OSU 605 pea cotyledons which were soaked but not fixed. Wall flaps (F) were attached to undisturbed portions of ruptured cell walls. Cotyledon surface was free of cellular contents. Bar = 10  $\mu$ m.

**Fig. 4.** Fracture surfaces of transverse cracks through bean cotyledons. Dry fracture without washing surface (4a) shows intercellular spaces (I), cell walls (W), starch grains (S) imbedded in protoplasm and protein bodies (PB). Bar = 50  $\mu$ m. Surface, after washing to remove protoplasm and cellular inclusions (4b), shows primarily empty cell walls (E), with few starch grains (S) on the surface, and some starch grains trapped (T) in a cell with a small hole in it. Bar = 100  $\mu$ m.



ensure that ruptures of individual cells were not caused by glutaraldehyde induced modification of cell walls, cotyledons were prepared for SEM by applying isolated drops of water to cotyledonary surfaces and allowing the drops to be absorbed or evaporate. This treatment also partially simulated the wetting and drying cycles employed by Powell and Matthews (1981).

Cells on the surfaces of Apollo cotyledons ruptured and lost intracellular materials when drops of water were applied to surfaces (Fig. 2a). Spots with irregular outlines were formed where water drops lay on cotyledon surfaces (Fig. 2a). The rough-textured areas around spots did not come into contact with water. Three zones were observed where water was applied, a rough center with ruptured cells and cellular contents, a bumpy surface without ruptures, and a perimeter which was smooth and cracked (Fig. 2a).

Cellular ruptures and intracellular materials were clearly visible at higher magnification (Fig. 2b). The intracellular materials on unfixed cotyledons were more consolidated than that on fixed cotyledons, but constituents were no more recognizable. Air-drying of soaked tissues without fixation resulted in formation of cracks and other predictable artifacts. Thin layers or films of material covered entire cotyledonary surfaces (Fig. 2a, 2b). Films were probably mixtures of soluble and insoluble components of cell contents which dried in a thin coating. Cracks in the film and cell walls probably formed during dehydration of the tissue. Dry films were similar to films found on particles of soy-flour which were moistened with buffer and dried (Wolf and Baker, 1972).

When cotyledons of OSU 605, a wrinkled pea, were soaked in water, blotted dry, and allowed to dry in air, isolated cells on the surface also ruptured (Fig. 3). Remnant flaps of cell walls hung over the empty cells. Cotyledon surfaces immersed in large quantities of water and not subsequently fixed lacked protoplasm and inclusions (Fig. 3). Protoplasm and inclusions, which in other cases were fixed to cotyledonary surfaces or restricted to small volumes of water in drops, apparently were washed away in the large quantity of soak water.

Ruptured bean and pea cells contrasted with another form of damage to cotyledonary tissues, which also contributed to losses of intracellular materials. When cotyledonary tissues of beans fracture as a result of tensile stress, e.g., crack transversely, cells in the fracture plane split completely apart (Morris et al., 1970, Spaeth, 1987) (Fig. 4a) and parts of cell walls separated completely. When protoplasm, protein bodies and starch grains were washed out of fractured cells, incomplete cell wall fragments comprised most of the surface (Fig. 4b). When holes in cell walls were small, starch grains were retained in fractured cells while protein bodies and protoplasm were washed out (Fig. 4b). Cells at fracture surfaces did not exhibit remnant flaps characteristic of ruptured cells. Blister separation surfaces (Fig. 5), were similar to tensile fracture surfaces (Morris et al., 1970, Spaeth, 1987).

The shape of OSU 605 (wrinkled pea) cotyle-

dons differed from those of bean and round pea. Before imbibition, small, isolated particles of intracellular material were observed on the wrinkled surfaces of OSU 605 cotyledons (Fig. 6a). Epidermal cells gave the surface a rough texture. During imbibition and fixation, the wrinkles changed to creases, cotyledonary surfaces became smoother, and large quantities of flecks appeared on the central portion of the cotyledon, near the creases (Fig. 6b). Blisters were not found on the surfaces of pea cotyledons as in earlier results (Spaeth, 1987).

Creases in surfaces of OSU 605 cotyledons were filled with dense particles and surfaces adjacent to the creases were covered with dense particles and patches of less dense material (Fig. 6c). Large numbers of single cell ruptures or small tissue ruptures were not found.

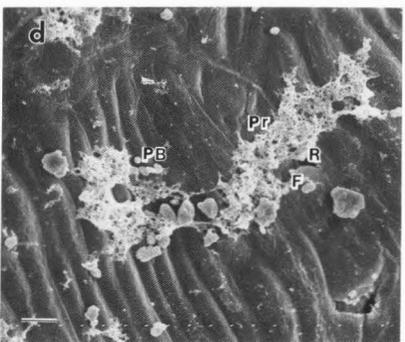
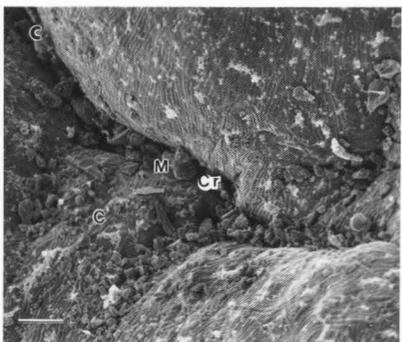
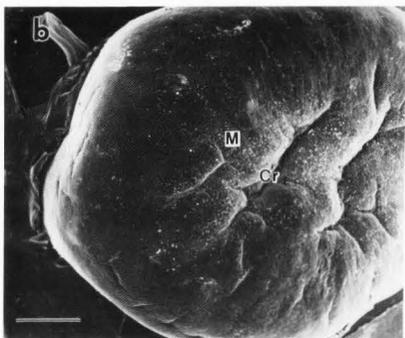
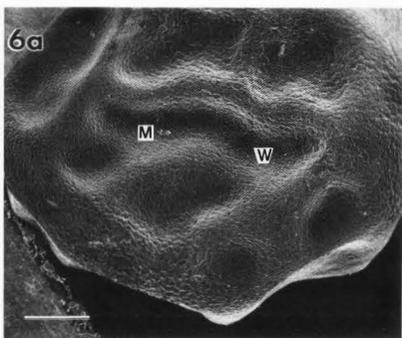
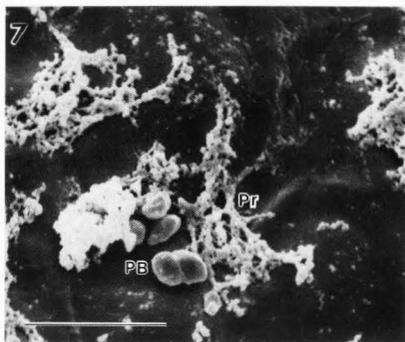
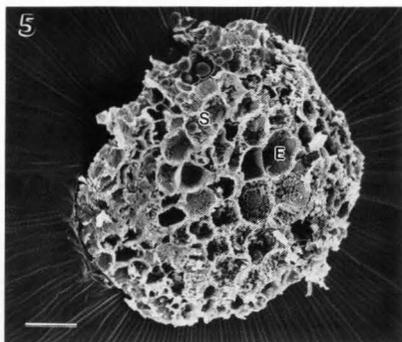
Isolated epidermal cells on cotyledonary surfaces of OSU 605 (Fig. 6d) were ruptured. Flaps of cell walls remained attached to the main portion of the wall. Protoplasm released from ruptured cells was in two forms. Dense particles were roughly spherical or irregular in shape and ranged from 1 to 10  $\mu$ m in effective diameter (Fig. 6d). The disperse phase of cellular contents often covered large areas of cotyledonary surfaces and consisted of small particles and a network of interconnecting material (Fig. 6d). Particle size distribution of the dispersed phase was similar to the granular cytoplasm found in un-aged dry-bean cells (Varriano-Marston and Jackson, 1981).

Dense particles from cotyledonary tissues were aggregates of spheroidal particles which ranged from 1 to 2  $\mu$ m in effective diameters (Fig. 6d, 7). Spheroidal particles included protein bodies which were a major component of materials from ruptured cells. Although some protoplasm and protein bodies released from cotyledonary tissues clearly came from single

**Fig. 5.** Fracture surface of blister top after removal from Apollo bean cotyledon showing empty cell walls (E) and starch grains (S). Bar = 100  $\mu$ m.

**Fig. 6.** Release of cell contents from OSU 605, a wrinkled pea. Unsoaked and unfixed cotyledon (6a) shows wrinkles (W) and material (M) on the surface. Bar = 1 mm. Soaked and fixed cotyledon (6b) shows creases (Cr) and large quantities of material (M) on the surface. Bar = 1 mm. Higher magnification of same cotyledon soaked and fixed (6c) with a crease (Cr) in surface and large quantities of cell contents (C) on the surface and large quantities of dense material (M) in the crease. Bar = 100  $\mu$ m. Highest magnification of soaked and fixed cotyledonary surface (6d) showing ruptured cells (R) with cell wall flaps (F) and cell contents including a disperse phase of protoplasm (Pr) and protein bodies (PB). Bar = 100  $\mu$ m.

**Fig. 7.** Protein bodies (PB) and disperse phase of protoplasm (Pr) on surfaces of soaked and fixed samples of Royal Red Mexican bean with adjacent cells not ruptured. Bar = 10  $\mu$ m.



cell ruptures (Fig. 6d), substantial quantities of protoplasm and protein bodies appeared on cotyledonary surfaces where cell ruptures were not observed (Fig. 6c,d,7).

A summary indicates the absence or presence, and frequency of various features observed in each of the cultivars (Table 1). Note that Royal Red Mexican and Garfield 81 had relatively few ruptures and substantial quantities of protoplasm and protein bodies on the surfaces of cotyledons after imbibition.

#### Discussion

Micrographs of cell ruptures (Figs. 1-3,6d) corroborated histochemical evidence for single cell ruptures (Duke and Kakefuda, 1981). Hypotheses about cellular rupture with complete loss of cell contents (Simon, 1984) were partially confirmed; however, cellular rupture demonstrated here was more deleterious than the membrane rupture that Simon (1984) and Powell and Matthews (1981) discuss. Cell walls as well as plasma membranes were ruptured during imbibition. Duke et al. (1983) suggested that cells might be able to repair mild cases of membrane rupture. Rupture of bean and pea cell walls during imbibition (Figs. 1-3,6d) was sufficient to preclude recovery or repair. However, it must be remembered that seed coats were not present to modify water uptake.

Cellular ruptures were observed in cotyledons which were imbibed but not fixed, and to a lesser extent in cotyledons which were fixed in aqueous solutions. Therefore, ruptures in fixed cotyledons were caused by imbibition of water from the fixation solution and were not caused by glutaraldehyde in the fixation processes. Fixation with glutaraldehyde was useful because protoplasm and protein bodies were retained on cotyledonary surfaces which otherwise would have been lost in the soak water.

Large holes were produced in walls of ruptured cells, but flaps of cell walls were attached to the undisturbed portions of cell walls (Fig. 1,3,6d). Ruptures appeared to be cases of plasmoptysis, osmotically induced rupture of cells. The presence of cell wall flaps opening to the surface was characteristic of wall rupture and explosive release of the contents.

Rupture of single cells contrasted with another process by which intracellular materials were completely lost from cotyledonary cells, cell fracture in the plane of transverse cracks. Fractures of cells in transverse cracks are caused by tensile stresses in dry tissue (Spaeth, 1986, 1987) which split cells through the middle and leave empty cell walls after protoplasm and inclusions wash out (Morris et al., 1970, Wolf and Baker, 1980). Walls of cells in the fracture plane are split in half and no longer attached to one another. Fractured cells lacked flap remnants of cell wall (Fig. 4b). Separation surfaces in blister cavities were similar to fracture surfaces associated with transverse cracks (Fig. 5). The similarity indicates that tensile stresses were probably important in the formation of blisters. Holes in epidermal cells

Table 1. Frequency of various features of injury and losses of intracellular substances from bean (Apollo and Royal Red Mexican) and pea (OSU 605 and Garfield 81) cultivars during imbibition.

Crop: Cultivar: Type:	Beans		Peas	
	Apollo Snap	RRM Dry	OSU 605 Wrinkled	Garfield Round
Feature:				
Transverse Cracks	+++ <sup>†</sup>	+	+	-
Blisters	+++	-	-	-
Cell Ruptures	+++	+	++	+
Extrusion streams				
In soak water (LM)	+++	+	+	+
On cotyledons (SEM)	+++	-	-	-
Protoplasm	+++	++	++	++
Protein bodies	+++	++	++	++

† + = presence and frequency, - = absence.

of soybean cotyledons and interior surfaces of seed coats observed by Yaklich et al. (1984) apparently were formed by a different (unidentified) process.

In contrast to the extrusion streams observed on fixed Apollo bean cotyledons and in water surrounding unfixed OSU 605 pea cotyledons (Spaeth, 1987), protoplasm and protein bodies from fixed OSU 605 cotyledons did not form extrusion streams. The disperse phase released from ruptures of OSU 605 cells was protoplasm fixed by the glutaraldehyde solution. Protoplasm quickly dispersed into soak water which did not contain glutaraldehyde. Rapid dispersion of protoplasm will make it difficult to determine to what extent fracture, rupture, extrusion, and diffusion each contribute to the aggregate quantities of material released from cotyledons.

The intracellular material observed on the surfaces of Royal Red Mexican, OSU 605, and Garfield 81 but not associated with cellular ruptures was also not in the form of extrusion streams. The dense particles from cotyledons were individual or aggregates of protein bodies (Fig. 6d,7). The disperse phases on the surfaces of Royal Red Mexican, OSU 605, and Garfield 81 differed slightly in form. Differences in protoplasm might reflect differences in composition of protoplasm from different genera and cultivars. Protoplasm and protein bodies may be one of the primary constituents of 'deposits' which have been observed on surfaces of excised cotyledons (Wolf and Baker, 1972).

The low frequency of cellular rupture in Royal Red Mexican, OSU 605, and Garfield 81 cotyledons did not appear to account for the quantities of protoplasm and protein bodies lost from cotyledons of these cultivars. Some ruptures may have been hidden by the protoplasm released from ruptured cells, or protoplasm and protein bodies may have been fixed to surfaces at some distance from ruptured cells. However, the low frequency of single cell ruptures on cotyledonary surfaces in which protoplasm was not fixed

(Fig. 3) indicated that protoplasm and protein bodies may have been released from cotyledonary cells by some mechanism other than complete rupture of cell walls.

Povell and Matthews (1981) demonstrated that substantial quantities of intracellular substances are lost from cotyledonary tissue during imbibition and that cells are able to recover from such losses. A process by which cells could lose protoplasm and protein bodies and not rupture and completely discharge cell contents has yet to be identified. The distinctions between cellular rupture and fracture, and among the forms in which intracellular substances were released, will help efforts to develop breeding, cultural, and processing strategies which minimize the problems of tissue disruption during imbibition.

#### Conclusions

Disruption of cotyledonary cells during imbibition occurs on both large and small dimensions. Large disruptions include transverse cracking and blistering. Small disruptions were cell wall ruptures. Cellular fracture is caused by tensile stresses induced during imbibition by surrounding tissue. Fractured cells split open and most of their contents were released into soak water. In contrast, individual and small groups of cells discharged cellular contents under pressure during imbibition. Cellular contents discharged from ruptured cells included protoplasm, and protein bodies in dispersed and dense phases, respectively. Efforts to measure and control leakage from seeds should take into account variation among processes by which intracellular substances are released from cotyledons during imbibition.

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<sup>1</sup>Mention of a trademark, proprietary product, or vendor does not constitute guarantee or warranty of the product by the U. S. Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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#### Discussion with Reviewers

**W. J. Wolf:** One would expect to be able to discern starch grains and protein bodies as constituents of cellular contents on the surfaces of cotyledons. Were the cell contents shown in Figs. 1 and 2b examined at higher magnification?

**Authors:** We did not find starch grains among the contents of ruptured cells in any of the seeds examined. Viewing the cell contents shown in Fig. 1 at high magnification revealed protein

bodies similar to those of OSU 605 and Royal Red Mexican (Figs. 6d and 7). However, we could not discern protein bodies in contents from ruptured cells of air-dried samples, including Fig. 2b.

E. Varriano-Marston: Structures in Fig. 3 bear some similarity to stomates. How did you distinguish between cellular ruptures and stomates on the surfaces of cotyledons?

Authors: Stomates are generally bilaterally symmetric. Cellular ruptures were often asymmetric. The large flaps of cell wall which often remained attached to ruptured cells also served to distinguish them from stomates.

K. Saio: You have shown that some cells rupture while others do not. What differences exist between ruptured and unruptured cells?

Authors: Rupture of an individual cell is probably influenced by numerous factors including the movement of water, characteristics of the cell itself and characteristics of adjacent tissue. Epidermal tissues influence water movement into subepidermal cells and may diminish cellular rupture there. Variation in strength of individual cell walls might predispose some cells to rupture. Finally, the tendency of a cell to rupture may be influenced by surrounding cells. If compressive stresses in hydrating tissue contribute significantly to pressures which cause cellular rupture, then rupture of some cells may partially relieve the stresses and, thereby, diminish the number of neighboring cells which rupture.

K. Saio: Hard beans, which cannot absorb water, can be made to absorb water if the seed coat is scarified. The rupture of cells on the cotyledonary surface may act in a similar manner to promote rapid absorption of water while also resulting in the loss of nutrients from the seeds. Please comment.

Authors: The cellular ruptures which result from imbibitional stresses should be of interest to other areas of legume seed research including the serious problems associated with hard beans. Hardness in beans is caused by different factors including the inability of water to penetrate seed coats and failure of cotyledons to soften even when seeds imbibe water. Cotyledonary cell rupture would not appear to play a role in hardness resulting from seed coat impermeability where water does not enter cotyledons, but may be involved in forms of hardness where bean seeds remain hard even after cotyledons imbibe water.

K. Saio: Losses of solid and chemical components during immersion in water clearly increase depending on time and conditions during storage (Saio et al., 1980, Cereal Chem. 57, 77-82). Are such losses related to the frequency of cellular rupture?

E. Varriano-Marston: Cultivars differ in leakage and seedling vigor. Do differences in cellular rupture cause differences in leakage and vigor?

Authors: Given the variety of processes which can contribute to losses of intracellular substances, we would not necessarily expect to find strong correlations between any one form of imbibitional injury and total losses of intracellular materials. Differences in protein leakage among cultivars were consistent with the extent of cellular fracture in excised cotyledons of susceptible and resistant cultivars (Spaeth, 1987).

R. W. Yaklich: Are the results obtained in this research applicable to seeds with intact coats?

Authors: Seed coats certainly can influence water movement into cotyledonary cells. However, fractured cells associated with transverse cracks and multicellular blisters are found in some cultivars even when seed coats are intact (Morris et al. 1970, Spaeth, 1987). Rupture of individual cells seems to be a less severe case of imbibitional injury so we expect it will be found in seeds with intact coats of some cultivars.