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SEED PHYSICAL-CHEMICAL AND MICROSTRUCTURAL DIFFERENCES BETWEEN ISOGENIC LINES OF COMMON BEAN (PHASEOLUS VULGARIS L.)

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SEED PHYSICAL-CHEMICAL AND MICROSTRUCTURAL DIFFERENCES BETWEEN ISOGENIC LINES OF COMMON BEAN (PHASEOLUS VULGARIS L.)

By

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ABSTRACT

SEED PHYSICAL-CHEMICAL AND MICROSTRUCTURAL DIFFERENCES BETWEEN ISOGENIC LINES OF COMMON BEAN (PHASEOLUS VULGARIS L.)

By

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Cookability and storability influence acceptance of bean varieties. 'Nep-2', a white seeded mutant from the tropical black bean 'San Fernando', has a different hydration rate, cooking time and processed texture. Seeds of the isolines and 'Sanilac' were held under two storage treatments and tested for differences in seed leachate conductivity and ion content of soak water, cooking time and processed texture. Fresh seed microstructure was examined by scanning electron microscopy (SEM) and relative calcium content of isoline cell walls by x-ray microanalysis.

Differences were found in imbibition rate, canned texture, cooking times and leachate contents and in response to storage for cooking time and seed leachate. 'Nep-2' had larger cotyledon cells with thicker cell walls, larger starch grains and thicker hourglass testa layers than 'San Fernando'. Calcium tended to be higher in 'San Fernando' cotyledon cell walls and in 'Nep-2' testa cell walls. Pleiotropy is offered as an explanation.

To Jesus Christ, the way, the truth, the life,
and my friend.

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INTRODUCTION

Dry beans (Phaseolus vulgaris L.) are a major source of protein, carbohydrates and fiber for people in many countries. One of the factors which influence the acceptance of a dry bean variety in a particular culture is seed coat color. Other factors of importance include cooking time and the amount of fuel required to soften the beans for chewing and digestion, the bean's texture or mouth feel after cooking and various agronomic traits such as yield, plant vigor, disease resistances and ease of harvest. In addition, the processed bean industries in various countries have standards of acceptability for beans that include freedom from cooking and canning defects such as clumping and splitting in cans. Genetic variability exists for many of these traits.

Some tropical black seeded bean lines are high yielding and have desirable disease resistances and other agronomic traits but are slow to cook and soften to a desirable texture. Black beans are not eaten to any great extent in North Temperate zone countries. People in this region who eat beans often favor white seeded market classes. Breeders have tried to develop white seeded beans from black ones via mutation breeding. In this way, the agronomic superiority of the black bean gene complex can remain intact while the seed coat is changed to the preferred color. The chemical, ethyl methane sulfonate (EMS), has been used to induce white seed coat mutations in black seeded beans (Moh, 1971).

When two genetic lines have identical genotypes except for genes controlling a single character, e.g., seed coat color, they are referred to as isolines (Fehr, 1987). Isolines are useful to conduct detailed studies of gene effects.

'San Fernando' is a tropical black seeded bean and 'Nep-2' is a white seeded mutant derived from 'San Fernando' by mutation of the P locus with EMS. The P locus controls all seed coat colors due to flavonoids (Leakey, 1988).

During the last decade (1980-1990) several workers showed that 'San Fernando' and 'Nep-2' differed markedly in physical-chemical characteristics related to culinary quality. Differences were of particular interest is as much as both bean strains should be identical genetically except for the P gene determining seed coat color.

Manifold effects associated with mutation breeding when the locus for a major gene is changed to an alternate state are generally ascribed to linkage or pleiotropy. Linkage seems unlikely in the case of 'San Fernando' and 'Nep-2' because the allele for white seed coat was not transferred to 'San Fernando' via back crossing. Gene transfer that occurs with back crossing involves a block of closely linked genes instead of a single gene (Fehr, 1987). If pleiotropy is the root of the cause of the noted differences between 'Nep-2' and 'San Fernando', then it should be of interest to breeders to know what features of the seed were changed and to what degree.

The been industry requires varieties that imbibe water readily and are free from culinary quality defects. The fine structure of cells may affect water imbibition of seeds, the degree of hydration of cotyledons, and cellular dissolution upon heating. Additional research is needed to ascertain which cotyledon and seed coat features may be associated with variable culinary quality noted for 'San Fernando' and 'Nep-2'. In order to study the isoline variability problem in detail, the present study was undertaken. The objectives of the work were to characterize fine structure to ascertain which features affect culinary quality and determine the effect of storage factors on the noted differences.

Genetics of Seed Color

In 1934, R. Praaken summarized previous research and his own test results and combined then current gene classification systems on seed and pod characters in Phaseolus vulgaris L. He showed seed coat color to follow Mendelian rules of inheritance. Seed coat color is produced by a groundfactor or P gene together with at least one complementary factor (Praaken, 1934). There are eight genes, some acting additively, which determine shade of color, or modify color such as producing mottled seed coats and determining background color in mottling. The P locus must be heterozygous or homozygous dominant for color to be expressed (complete dominance). The P factor itself produces no color (Praaken, 1934). A white seeded bean can result from all complementary color factors being homozygous recessive or from a homozygous recessive P factor (Praaken, 1934).

Praaken (1934) noted pleiotropy in that stem (hypocotyl) color and flower color were determined by the seed coat color genes. He also observed additivity in degree of color from pale white to purple for flower color and green stems or red stems for white seeded and black seeded lines, respectively.

Seed Color Mutations

In 1971, C.C. Moh reported development of seed coat color mutations from the Nuclear Energy Program (NEP) of the Inter-American Institute of Agricultural Sciences in Turrialba, Costa Rica (Moh, 1971). Seeds of black bean varieties were treated with ethyl methane sulfonate (EMS) to induce mutations which affected seed coat color in the M_3 generation. The seed coat color mutants obtained from EMS treatment varied from white and yellow to various degrees of brown (Moh, 1971). The induced white seed coat could have been from the absence of all complementary color factors or from the absence of the dominant P factor. Moh (1971) crossed the white seeded mutants with a white seeded tester line which had only the dominant enabling gene, P, but no other complementary factors. The F_1 seed coat color was black which supported Moh's supposition that the dominant P gene was missing from the white seeded mutants (Moh, 1971).

The pleiotropic effect of bean seed coat color genes on hypocotyl and flower color noted by Praaken was confirmed by Moh (1971) in his work with the EMS mutants. Moh (1971) concluded that the seed coat color mutants were true breeding for this difference. 'San Fernando' and 'Nep-2', one of the white seeded mutants from the Turrialba project, could be isogenic lines and thus differ in only one gene, that for seed coat color (Agbo et al., 1987).

White seeded dry beans (Phaseolus vulgaris L.) represent a major portion of the canned bean industry in the U.S. and

Michigan. The quality of canned and cooked beans varies among cultivars (Hosfield and Uebersax, 1980). Method and duration of bean storage and crop growing conditions also affect processed quality traits (Hosfield et al., 1984). The relationship of seed anatomical and functional qualities with seed coat color and processed quality is not clear (Agbo, 1982; Hosfield and Uebersax, 1980). White seeded navy beans (pea beans) have different processing attributes than other types of beans (Hosfield et al., 1984; Sefa-Dedeh and Stanley, 1979). Until the 1980's, little work had been performed on the genetics of cooked or canned quality and their relationship to seed coat color (Agbo, 1982). Bean seed color is an important quality and greatly affects consumer preferences (Adams and Bedford, 1973). The Hunter Lab Color Difference Meter objectively rates colors on the three scales: L--white to black, a--red to green, and b--yellow to blue (Agbo, 1982). Hosfield and Uebersax report that white beans showing a one unit difference in L value are visually distinguishable (Hosfield and Uebersax, 1980). Agbo (1982) noted that 'Sanilac' beans scored consistently higher on the 'L' scale than 'Nep-2' beans.

Water Imbibition

Most plant seeds are harvested and stored at relatively low moisture levels. Storage at low moisture levels allows seed to maintain viability for later germination. Water imbibition is an important process both for germination and

food processing. Water imbibition by seeds during soaking leads to softening of the seed coat and cotyledon because of cellular expansion (Deshpande et al., 1983). Legume seeds including dry beans are usually prepared for food by soaking in water at ambient temperatures and pressures followed by boiling (Rockland and Jones, 1974; Uebersax and Bedford, 1980).

Swanson et al. (1985) summarized several authors who noted differing entry paths for water into seeds of different legume species. He indicated that seed coat and cotyledon microstructure appear to affect rates of imbibition. Sefa-Dedeh and Stanley (1979) associated seed coat structure and dimensions with water uptake rates. Kyle and Randall (1963) reported the micropyle as the principle avenue of water entry in Great Northern beans while Adams and Bedford (1973) reported water movement through the seed coat for navy beans. Swanson (1985) and Agbo et al. (1987) showed the 'San Fernando' water uptake rate to be slower than that of 'Nep-2'.

Processed Bean Texture

Processed quality of beans can be broken down into various measurements and seed attributes called physico-chemical properties (Hosfield and Uebersax, 1980). One key measure of quality is texture (Hosfield and Uebersax, 1980). Texture affects the perceived need for chewing of a food

material. It thus influences consumer acceptance of a food (Binder and Rockland, 1964; Hosfield and Uebersax, 1980).

Cooked or canned beans may be too hard or too soft in texture and, therefore, unacceptable to the consumer (Hosfield and Uebersax, 1980). Food texture is affected by chemical and physical changes which result from storage, soaking and thermal processing (Rao and Lund, 1986; Uebersax and Bedford, 1980). Rao and Lund (1986) list techniques for objective measurement of the texture changes and softening that occur during cooking. Depending on the food, measurements such as cutting force, puncture force or back extrusion force may be appropriate although there are no hard and fast rules (Rao and Lund, 1986). The middle lamella is a layer composed of pectic substances which hold together cell walls of adjacent cells (Cutter, 1978). The "plasticizing effect" of boiling water on middle lamellae may relieve stress imposed during bean fracture (Rockland and Jones, 1974).

The Kramer Shear Press is a force measuring device adapted to a number of fresh and cooked foods (Binder and Rockland, 1964). The shear press can achieve a degree of sensitivity similar to other softness measuring devices, e.g., the tenderometer (Binder and Rockland, 1964). Read-outs of shear press forces result in printed curves or peaks depicting the amount of force required to extrude the sample through a metal grate at a constant rate (Agbo, 1982; Binder and Rockland, 1964; Hosfield and Uebersax, 1980). These

curves can be divided into two sections showing 1)force required to bring the product to a yield point prior to extrusion (shear); and, 2)force required to uniformly extrude the material (compression) (Agbo, 1982; Hosfield and Uebersax, 1980). Predominantly compression type curves resulted from lima beans extruded without seed coats, while those beans with seed coats showed greater shear components in the read-out curves (Binder and Rockland, 1964).

Bean genotypes differ for both predominant shear curve type and for force required for extrusion (kg force/100 g sample) (Hosfield and Uebersax, 1980). Hosfield and Uebersax (1980) found no clear association of seed color with shear press curve type but found black beans required more force than white beans.

Bean Seed Anatomy

Esau (1977) defines a true seed as a matured ovule containing the embryo and stored nutrients, with the integument or integuments differentiated as the protective seed coat or testa. The leguminous testa is a complex of tissues. From the outside of the seed inward, the cell layers are (Corner, 1951):

1) A palisade layer of columnar macrosclerids serving as epidermis.

2) A hypodermal layer of narrower mid-section sclerids called the "hourglass layer."

3) A mesophyl of stellate armed parenchyma cells. There are numerous air spaces among cells of this layer.

Cell walls are typical components of plant cells and impart structural strength for support of tissues and organs (Esau, 1977). Parenchyma cells typically have primary as opposed to secondary cell wall deposition, and thus are made up of cellulose cross-linked with pectins, hemi-celluloses and other polymers. Thickness of primary walls may vary among tissues of the same plant and between species for the same tissue type (Esau, 1976; Cutter, 1971). Corner (1951) lists characteristics of the palisade layer cells of seed coats (testae) as prismatic, thick walled, contiguous and more or less hexagonal in cross section. Esau (1977) defines the palisade layer cell type as macrosclerid. A macrosclerid usually has thick secondary cell walls that are highly lignified. This tissue layer develops from the outer epidermis of the outer integument of the ovule (Corner, 1951). The sub-epidermal layer of so-called hourglass cells develops from the inner epidermis of the outer ovule integuments (Corner, 1951). The hourglass layer was approximately the same thickness for 'San Fernando' and 'Nep-2' and those were thinner than 'Sanilac''s, according to Swanson et al., (1985). Agbo (1982) found the relative palisade thickness to be: 'San Fernando' > 'Nep-2' > 'Sanilac'. The relative thickness of hourglass layers was reversed, with 'Sanilac' > 'Nep-2' > 'San Fernando' (Agbo, 1982). The third cell layer making up the Phaseolus sp. seed

coat is composed of stellate armed aerenchyma and develops from the middle cell layer of the outer integument (Corner, 1951; Esau, 1977).

The hilum is a specialized area of the seed coat with a double palisade layer split by a fissure in the seed longitudinal plane (Corner, 1951). The inner central portion of the fissure contains a plug filled with tracheids which are in contact with the aerenchyma layer (Corner, 1951). This tracheid bar has cell remnants with heavily pitted secondary cell walls which provide some structural rigidity yet could allow passage of air and water. The hilum acts as a hygroscopic valve which opens during low relative humidity conditions and closes under high relative humidity to aerate the seed and regulate changes in seed moisture content (Corner, 1951; Esau, 1977). The hilum is surrounded by a raised rim or aril and plugged by dry funicular tissue in the mature testa (Corner, 1951). Corner (1951) suggests that the palisade layer's rigidity (sclerenchyma) is important for protection of the cotyledons and embryo. The aerenchyma may serve to cushion the inner seed parts (Corner, 1951) and to conduct water evenly about the cotyledons during germination and soaking (Swanson et al., 1985). The hilum may not only be important for moisture management by acting as a hygroscopic valve in the mature seed but also as a water reservoir for the immature embryo during germination (Corner, 1951).

The micropyle is the opening in the integuments of the ovule which allows entry of the pollen tube into the embryo sac for fertilization (Esau, 1977). In the testa, the micropyle remains visible as a pore located near one end of the hilum (Corner, 1951).

The main food storage of the P. vulgaris seed is in the body of the embryo, mainly in the cotyledons (Esau, 1977). The cotyledons consist of parenchyma cells and interspersed vascular bundles. The epidermal layer parenchyma cells are somewhat smaller than elsewhere in the cotyledon and do not contain much starch (Opik, 1968). During seed development, starch grains grow within plastids of cotyledonary cells (Opik, 1968). Opik (1968) reported that starch grains were about 50 μm in diameter and comprise approximately 50 per cent of the seed weight (Opik, 1968). The cotyledon parenchyma cells also synthesize storage proteins to approximately 20 per cent of dry weight at maturity (Opik, 1968). However, cultivatars of P. vulgaris vary in protein and starch amounts and in starch grain dimensions (Agbo, 1982; Esau, 1977; Swanson et al. 1985). Swanson et al. (1985) showed starch granules to be smaller in 'Sanilac' and 'San Fernando' seeds than in those of 'Nep-2'.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) has been a valuable tool in the study of seed microstructural characteristics and their role in water uptake and cooking processes (Agbo, 1982;

Rockland and Jones, 1974; Sefa-Dedeh and Stanley, 1979; Swanson et al., 1985). High resolution light microscopy is limited to observation of objects transparent to a beam of light and is therefore limited to thin specimens or sections (Postek et al., 1980). Observations of surface morphology may be made using reflected light and a dissecting or stereo microscope (Postek et al., 1980). While light microscopy continues to be a valuable tool for scientific research, the degree of resolution is limited by the nature of light waves (Postek et al., 1980). Resolution refers to the ability to separate two points as distinct entities (Postek et al., 1980). Electron beams have much smaller wavelengths than naturally visible light and consequently resolve much finer detail (Postek et al., 1980).

The SEM uses a focused beam of high energy electrons to scan the surface of a specimen. The electron beam interacts with the specimen to produce lower energy electrons called secondary electrons. Secondary electrons can be collected by a positively charged detector system and converted into an electronic signal for a cathode ray tube display system (Postek et al., 1980). The SEM has an advantage over light microscopy and transmission electron microscopy (TEM) by producing images with greater depth of field, whether at high or low magnification (Postek et al., 1980).

Seed Microstructure by SEM

Using SEM, Sefa-Dedeh and Stanley (1979) found characteristic seed coat and cotyledon structures for five legumes, two of which were P. vulgaris varieties. They related a measure of texture (Instron Universal Testing Machine) to microstructure for the five legume seeds. The middle lamella is the layer between the walls of adjacent cells which is made up of pectic substances and acts to hold adjacent cells together (Cutter, 1978). They found seed coat thickness was important in softening during soaking and that the breakdown of the cotyledonary middle lamellae led to separation of cells during cooking (Sefa-Dedeh and Stanley, 1979). Agbo (1982) used SEM observations to explain differences in functional properties such as texture and water absorption between two bean isolines, 'San Fernando' and 'Nep-2'. He related unruptured parenchyma cells of soaked cotyledons and a "rigid seed coat" to increased resistance to shear force in the black seeded variety 'San Fernando' (Agbo, 1982). He suggested that varietal differences in these characteristics could provide opportunity for variety improvement by plant breeding (Agbo, 1987).

Chapter I

Comparison of Two Isolines of Bean (Phaseolus vulgaris L.) for Water Imbibition, Cooking Time and Seed Microstructure.

Abstract

Water uptake is important for softening and cooking of dry beans. Seed microstructure has been related to imbibition, cooking and processed texture differences. 'San Fernando', a black seeded bean, has been reported as slower cooking and slower to imbibe than 'Nep-2', a white seeded mutant isoline counterpart of 'San Fernando'. Imbibition and cooking rate differences were examined with a navy bean, 'Sanilac'. A Mattson pin-drop cooking apparatus was used to test beans freshly harvested, stored at 75% RH and stored under ambient conditions. Seed coats were altered or removed for some tests. Microstructure was examined by scanning electron microscopy (SEM).

After 1.5 hours, 'Nep-2' seed imbibed at a similar rate to 'Sanilac' and more slowly than 'San Fernando'. Cooking times were highest for 'San Fernando' fresh beans but not after storage and pre-soaking. A pattern of 'San Fernando' < 'Nep-2' < 'Sanilac' was established for cell cross-sectional size, starch grain size, and cotyledon cell wall thickness.

Chapter I

Introduction

Seeds of most crop plants are harvested and stored at relatively low moisture levels which allows them to maintain viability for later germination. After they are stored, seeds need to be rehydrated to allow them to germinate and/or cook as a food for human consumption. It has long been known that when dry seeds of common bean (Phaseolus vulgaris L.) are stored for prolonged periods under unfavorable conditions they often become hard to cook and require extended cooking times to soften the cotyledons and render them palatable (Dexter et al., 1954; Muneta, 1964; Burr et al., 1964; Uebersax and Bedford, 1980; Jackson and Varriano-Marston, 1981; Jones and Boulter, 1983; Vindiola et al., 1986).

Gloyer (1921) described two conditions in which beans failed to hydrate and remained dormant. One kind of failure of water imbibition called "hardshell" was due to the impermeability of the seed coat to water (cited by Agbo et al., 1987). A second condition of dormancy in bean seeds is known as sclerema (Gloyer, 1921) and is due to the inability of the cotyledon to hydrate and expand.

Imbibition is an important physiological process for both germination and food preparation because water

imbibition by seeds during soaking leads to softening of the seed coat and cotyledon due to cellular expansion (Deshpande et al., 1983). Dry beans are generally soaked and must be cooked to render the seeds palatable, inactivate heat labile antinutrients, and permit the digestion and assimilation of protein and starch (Depshpande et al., 1983). Soaking is generally accomplished by leaving beans in cool tap water for several hours at ambient temperatures and pressure and cooking is done by boiling (Rockland and Jones, 1974; Uebersax and Bedford, 1980). Cooking the seeds causes structural changes of cells that have a bearing on consumer and food processor preferences and requirements. Cooking dry beans at high temperature (116°C) and at 10.4×10^4 Pa (15 psi) generally render them palatable but texture of genetic strains processed under the same conditions can vary significantly (Hosfield and Uebersax, 1980). In the Hosfield and Uebersax (1980) report, 'San Fernando', a tropical black seeded dry bean genotype, and 'Nep-2', a white seed coat mutant derived from 'San Fernando' (Moh, 1971), differed stikingly in textural characteristics after cooking. Later research (Agbo, 1982) showed that several physical and chemical differences related to culinary quality existed between 'San Fernando' and 'Nep-2'.

Differences between 'Nep-2' and 'San Fernando' were of particular interest in a genetic sense because isolines are identical genetically except for the single gene for which they differ. 'San Fernando' is a tropical black bean and

'Nep-2' was derived from 'San Fernando' by ethyl methane sulfonate (EMS) treatment which mutated the P or ground factor gene conditioning testa color in common bean (Moh, 1970).

Swanson et al. (1985) reviewed the literature on water imbibition by seeds of food legumes. This work indicated that water enters seeds by unique pathways depending on the species. The seed coat and microstructure of cotyledons appear to affect rates of imbibition. Sefa-Dedeh and Stanley (1979) associated legume seed coat structure and dimensions with water uptake rates. In common bean, Kyle and Randall (1963) reported that the micropyle was the principal avenue of water entry in Great Northern beans while Adams and Bedford (1973) showed water entry through the seed coat for navy beans. Agbo et al. (1987) measured imbibition over a one and a half hour soak period and found the water uptake rate greater for 'Nep-2' compared to 'San Fernando' though less than for 'Sanilac', a commercial navy bean. Agbo et al. (1987) concluded that most of the differences noted for imbibition rates were due to seed coat structure.

'San Fernando' had an occluded micropyle compared to 'Nep-2' whose micropyle was partially occluded (Agbo et al., 1987). Agbo et al. (1987) also theorized that water entered 'Nep-2' through seed coat pores. 'San Fernando' did not have seed coat pores. Agbo et al (1987) confirmed the Hosfield and Uebersax (1980) conclusion that 'San Fernando' was firmer in texture (118.1 Kg/100 g) than 'Nep-2' (87.8 kg/100 g). He

also found that cotyledon cells adhered to the seed coats of 'San Fernando' beans but that the cooking time with testae partially removed was, like the whole bean cooking time, not significantly different from that of 'Nep-2' (Agbo, 1982). Because of the difference in the two isolines regarding their cooking qualities, pleiotropy may be involved. Storage effects may also alter the expression of cooking quality in a dissimilar manner in the two isolines. The palisade and hourglass seed coat cell layers are sclerids, which thick secondary cell walls containing lignin (Esau, 1977). Hydroxycinnamic acid, a pre-cursor of lignin monomers, has been found at elevated levels in aged bean seed coats (Srisuma, et al., 1989).

Rigid walls are typical components of plant cells and impart structural strength for support of tissues and organs (Esau, 1977). Parenchyma cells typically have primary rather than secondary cell wall deposition, and thus are made up of cellulose cross-linked with pectins, hemi-cellusloses and other polymers. Thickness of primary walls may vary among tissues of the same plant and between species for the same tissue type (Esau, 1976; Cutter, 1971). In a scanning electron microscope examination of 'San Fernando' and 'Nep-2', Agbo et al. (1987) found that the palisade cell layer of 'San Fernando' was 32 µM, 30 µM and 23 µM for 'San Fernando', 'Nep-2' and 'Sanilac', respectively. Moreover, 'San Fernando' had cotyledon parenchyma cells which appeared surrounded by a densely appearing cell wall. 'Nep-2', in

contrast, had a thinner and more loosely appearing cell wall. Hourglass layer thicknesses were 13 µM, 17 µM and 21 µM for 'San Fernando', 'Nep-2' and 'Sanilac', respectively (Agbo et al., 1987). Agbo et al. (1987) also found a layer of cells resembling a "sheet of bundles" on the adaxial cotyledon surface of beans and that 'Sanilac's' "bundles" appeared thinner and more numerous.

Although Agbo et al. (1987) reported numerous microstructural differences between 'San Fernando' and 'Nep-2', actual measurements were taken only on palisade and hourglass testa layers, cotyledon cell cross sections and the longitudinal axis of starch grains. Only ranges of values were given for cotyledon cells and starch grains (Agbo et al., 1987).

Since water imbibition is a prerequisite to cellular hydration and break down upon heating and appears to be influenced by seed microstructures, the present study was undertaken. Specific objectives were to: (1) measure anatomical features of 'San Fernando' and 'Nep-2' seeds, (2) relate water imbibition and cookability of the two isolines to microstructures; and (3) determine the effects of storage on physical and chemical characteristics of 'San Fernando' and 'Nep-2' seeds; related to cookability.

Materials and Methods

Genetic Materials

'San Fernando' is a black seeded bean of tropical origin and seeds weigh between 18 and 20g/100 and are round to ovate in shape. 'Nep-2' has similar visible seed characteristics to 'San Fernando' except for a white seed coat (Moh, 1971). 'Sanilac', a white pea bean of the navy commercial class with seeds weighing between 18 and 20g/100 was used as a "control" genotype in the study.

Imbibition Study. 'San Fernando', 'Nep-2', and 'Sanilac' were grown in a nursery at the Saginaw Valley Bean and Sugarbeet Research Farm near Saginaw, Michigan in 1985. After harvesting and threshing the beans, seeds of each genotype were held in an unheated storage building in East Lansing, Michigan for two months prior to cookability tests. The moisture content of 250 gram samples was determined to be 15.3% +/- 0.5%. Beans were both cooked individually at ambient pressure in a pin-drop apparatus and processed at high pressure (10.4×10^4 Pa) in tin cans in separate experiments.

Six samples of nine seeds each were taken from each variety seed lot, weighed and placed in petri dishes with 35 ml distilled water. All samples were held at 22 degrees with lids in place except for weighing procedures. Water imbibition was determined by weight increase of each nine seed sample. Weighing procedures consisted of decanting each

petri dish in randomized order, blotting seeds on absorbant towels, and refilling dishes in the same order as decanting. Time of re-fill was noted and used to start the next soak period so that all imbibition times reflect actual soak time.

Experiment 1. Seed for this study were produced in the field in a nursery during the summer of 1985 at the Saginaw Valley Bean and Sugarbeet Research Farm near Saginaw, Michigan. After the seed of 'San Fernando', 'Nep-2', and 'Sanilac' were harvested and threshed, they were divided into two lots each compromised of individual samples of the three genotypes. The first lot was stored for 4 months and used in the canned texture equipment. The second lot was stored for 18 months at ambient conditions in an air conditioned laboratory and used in pin-drop cooking tests. The first lot beans were processed in cans and duplicate samples from each plot for each genotype were evaluated for the texture of seeds at the cooked stage of processing. Before processing, seed moisture percentage was determined on 250 g of beans using a Motomco (Model 919) moisture meter. Beans with a fresh-weight equivalent of 100 g total solids (Hosfield and Uebersax, 1980) were placed in nylon mesh bags and soaked for 60 minutes (Hosfield and Uebersax, 1980). All soaking was done in tap water containing ≈50 ppm Ca. The soaking procedure produced a product with minimum damage similar to beans cooked continuously in the high temperature systems common throughout the United States canning industry (Hosfield and Uebersax, 1980).

Number 303 tin cans (100 x 75mm) were filled with soaked beans. During the can filling, boiling brine prepared by adding 142.0 g of sucrose and 113.4 g of table salt (NaCl) to 9.1 kg of tap water containing 50 ppm Ca ion was added. Cans were sealed and cooked in a retort without agitation for 45 minutes of 116° and 10.4 x 10^4 pa (15 psi). After cooking, cans were cooled under cold running tap water and stored for 12 months at room temperature.

The texture determinations were expressed as kg force/100 g and were made by placing 100 g of rinsed and drained beans into a standard shear compression cell of a Kramer Shear Press and applying force with a dynamic hydraulic system. Total peak heights and compression component and shear component (Hosfield and Uebersax, 1980) peak heights were recorded for each sample and converted to force (kg/100 g).

The cooking time of beans of each genotype was determined with a 100-seed pin-drop apparatus designed by Mattson (1946) and modified by Burr et al. (1968). Different numbers of seed were cooked for each genotype and in each test.

Raw seed was placed in "saddles" of the apparatus and the pin terminus of weighted plungers were carefully placed on the tangential (flat) surface of seeds. Beans were blanched at 90.6°C for 30 minutes in distilled water containing 100 ppm Ca ion. To cook the beans, the pin-drop apparatus was lowered into a steam heated water bath. Beans

were cooked for 270 minutes. Actual cooking time for each seed was taken as the elapsed time from initiation of cooking until all the seed was penetrated. To quantify cooking time, an average was calculated by taking the cumulative time for all beans of a genotype to cook and dividing the sample. In some cases, several beans remained uncooked after the test. In order to quantify the cooking time in these cases, an additional and arbitrary 20 minute time increment was added to the 270 minute cooking period.

In some cooking tests, a 2.5 x 2.5 ±0.5mm aperture was cut from the seed coat on the tangential (flat) surface of seeds, thus exposing the cotyledon. However, before the aperture was cut, seeds were soaked for one hour in distilled water to facilitate removal of the seed coat "window". In some cases the entire seed coat was removed from a bean and only an individual cotyledon was cooked in each saddle of the cooking apparatus. In these instances seed were soaked for one hour to facilitate seed coat removal. The pin terminus of plungers were placed directly on the cotyledon surface of seeds with apertures.

Experiment 2. Seed for experiment 2 was produced in the greenhouse during the winter of 1987. These are referred to as "fresh" seed. Seeds of each genotype were planted in clay pots that were randomized on the greenhouse benches. After the pods from mature plants were harvested, the seed was threshed and stored for four months at ambient conditions in an air conditioned laboratory. Seed was harvested from

individual pots of each genotype and stored on a per pot per genotype basis. The cooking time of beans of each genotype was determined using the same procedures as described for experiment 1.

To prepare individual bean seed samples for scanning electron microscopy (SEM), individual seeds were wrapped in aluminum foil to improve heat conductivity and to deter any air layer from insulating the bean seeds. Each seed was then immersed in liquid nitrogen for 15 minutes, freeze dried for 60 hours using a Freeze-Mobile II freeze dryer (The Virtis Co., Gardiner, NY; Model #10-146 MR-BA), removed from the foil wrap and cross-sectioned with a razor blade. Sections were mounted on aluminum stubs and sputter coated with approximately 20 nm gold. A Model 35-C scanning electron microscope from Japan Electron Optics Laboratory (JEOL) was used at 15 keV accelerating voltage to examine specimen sections and produce micrographs for measurement and comparison of anatomical features.

The following magnification ranges were used to record feature dimensions:

Cell cross-section dimensions	400X
Starch granule dimensions	400X
Cell wall cross-sections	1000X--1500X
Cell wall thickness	3000X--5000X
Adaxial cotyledon epidermis	600X--3000X.

Cell wall thickness was measured on SEM micrographs taken after manipulation of the SEM specimen stage to provide a line of view as close to 90 degrees to the cell wall cross-section as possible. This provided consistency and comparability of measurements. In addition, the orientation of cell walls were examined to insure that cross-sections of cells were being measured rather than oblique sections and that end pieces of cells were not being measured for cell cross-sections. The same procedure was used to align and measure cotyledonary cells. Cell wall thickness was measured at 3000x to 4000x as follows: For five seeds of each genotype, each from a different plant, a total of 300 µm linear cell wall distance was measured for thickness. Average wall thickness per µm of length was determined for each measurable segment of cell wall. This required 99 readings for 'San Fernando', 94 for 'Nep-2' and 82 for 'Sanilac'. Measurements of anatomical features were obtained by comparison of feature dimensions with the measurement standard bar integral with each JEOL 35C micrograph.

Two features of starch grains were measured: (1) the long axis, and (2) the widest dimension perpendicular to the long axis of each granule. This also allowed determination of length/diameter ratios. The same procedure was used for cotyledon cell dimension measurements.

Seed coat cell layer (palisade, hourglass) thickness differences were determined by paired difference measurements on single lines covering paired cells. Length/width ratios

for starch grains and cells were obtained on a grain by grain and cell by cell basis, respectively. For adaxial cotyledon epidermal cell measurements, 71 cells were measured on four seeds of each genotype.

For cotyledon cell width and cotyledon cell wall thickness measurements, differencs were examined by Student's 'T' for significance at the 5% and 1% error levels, respectively.

Experiment 3. Seed for this experiment was produced in the greenhouse in the spring of 1986 and seed from plants harvested and threshed as described in experiment 2. However, in this case, seed was either stored in paper bags for 10 months at ambient conditions in a non-air conditioned room and then stored for five months in a dessicator in a 75% RH atmosphere at 21.0 ± 2.0°C temperature or for 15 months at ambient conditions in an air conditioned laboratory.

The cooking time of beans of each genotype was determined using the same procedures as described for experiment 1.

Results

Water Imbibition. From the weight gain of seeds during soaking it is apparent that each soaking period seeds of 'San Fernando' imbibed less water than seeds of 'Nep-2' or 'Sanilac' (Figure 1). For the first one and one half hours of soaking, seeds of 'Nep-2' imbibed less water than seeds of 'Sanilac' (Figure 1). For all three genotypes, the most

rapid water uptake based on increase in seed weight, occurred during the first one and one-half hours (Figure 1). Seeds of all three genotypes reached approximately the same hydration level in terms of percent of starting fresh weight (Figure 2). 'Nep-2' actually gained more water than 'Sanilac' during the period from 1.5 to 4.5 hours of soaking when weight gain is seem as a percent of bean weights (Figure 2).

Seed Microstructure. When measured on SEM micrographs, palisade cell layer thickness in the seed coat averaged within 1.5 micrometers for the three genotypes (Table 1). 'Nep-2' had the thickest palisade cells, followed by 'San Fernando' and 'Sanilac' (Table 1). 'Nep-2' also had the thickest hourglass cells, and 'San Fernando' the thinnest hourglass cells of the three genotypes (Table 1). 'San Fernando' had the greatest difference in thickness between the two cell layers (Table 2). 'Nep-2' was closer to 'Sanilac' than 'San Fernando' in the cell layer thickness difference (Table 2).

Cross-section of cotyledon cells showed 'Sanilac' to have the largest dimensions, with 'San Fernando' the smallest and 'Nep-2' intermediate (Table 3). From the mean ratio of long axes to wide axes (Table 3), 'San Fernando' cells appear to be more ovate than circular in cross-section shape. Cells of 'Sanilac' and 'Nep-2' appear to have a similar, more rounded cross-section shape than cells of 'San Fernando'.

'Sanilac' had the largest starch grains of the three genotypes, judging from average long and wide axes (Table 4).

'San Fernando' had the smallest starch grains, and those of 'Nep-2' were intermediate in size of the three genotypes (Table 4). The ratio of long to wide axes (Table 4) indicates 'San Fernando' had slightly more ovate shaped starch granules than 'Nep-2' and 'Sanilac' (Table 4). Epidermal cells found on the adaxial surgace of cotyledons were narrowest for 'Sanilac', widest for 'San Fernando' and 'Nep-2' cells were intermediate in width (Table 5). Cell wall thickness in cotyledons were greatest for 'Sanilac', least for 'San Fernando' and intermediate for 'Nep-2' (Table 6).

Experiment 2. Recently harvested greenhouse grown 'Nep-2' beans cooked more slowly than 'San Fernando' beans (Table 7, Figure 3). Many of the 'San Fernando' beans did not cook as indicated by a mean cooking time greater than the 270 minute base line cooking period (Figure 3). In some instances the cooking apparatus plungers were depressed into seed coats of uncooked 'San Fernando' beans but did not penetrate the seed coats or beans. Cotyledons appeared only partially softened on these uncooked seeds. Mean squares for genotypes in the analysis of variance was highly significant for the fresh beans (Table 8). When beans from the same fresh seed lot as described above (Table 1, Figure 3) were cooked with an aperture cut in the seed coats, the cooking time was greatly reduced for all three genotypes (Table 9, Figure 4). 'San Fernando' bean cooking times averaged significantly greater as seen in the "no-soak" column of

Table 9. With a pre-cooking soak of 24 hours the cooking time difference between 'San Fernando' and 'Nep-2' disappeared and 'Nep-2' beans were slower to cook than 'San Fernando', though not significantly so (Table 9, Figure 4).

The analysis of variance for this experiment (Table 10) indicates that genotypes were a significant source of variation and that the interaction effects between soaking and genotypes was highly significant.

Experiment 3. Cooking times for beans with altered seed coats, soaking treatments and two storage conditions in a 2^3 factorial arrangement of treatments, showed complex interaction effects (Tables 11, 12). The seed lots of 'San Fernando' and 'Nep-2' stored at ambient conditions showed a pattern (Table 11, Figure 5) similar to the fresh seed cooking times (Figure 3). With soaking, beans with apertures had similar cooking times (Table 11, Figure 5). Beans cooked more slowly when they were not pre-soaked for 24 hours (Table 11, Figure 5).

For beans stored at 75 percent relative humidity, cooking times were increased for both genotypes and both soaking classes in comparison to ambient storage class beans (Figures 5, 6). 'Nep-2' beans exhibited a marked increase in average cooking time for soaked beans stored at 75 percent relative humidity (Figure 6).

The storage and soak by genotype sources of variation were highly significant (Table 12).

Experiment 1. Table 13 shows mean cooking times for beans stored eighteen months in ambient conditions with three seed coat treatments: whole beans, beans with altered testae (windows cut into seed coats) and decorticated (cotyledons without seed coats). For beans with altered seed coats and bare cotyledons, 'San Fernando' beans are slowest to cook with 'Nep-2' beans intermediate and 'Sanilac' beans the fastest cooking (Table 13, Figure 7).

In the whole bean class, no 'San Fernando' seeds cooked. The arbitrary addition of 20 minutes to the 270 minute cooking period for uncooked beans produced the average value of 290 minutes for 'San Fernando' (Table 13). Likewise, 'Nep-2' and 'Sanilac' whole beans were slow to cook and several beans did not allow plunger penetration in the Mattson cooking apparatus (Table 13). The analysis of variance (Table 14) showed seed coat alteration and genotype as highly significant sources of variation with non-significant interaction effects..

Kramer Shearpress force readings indicated canned 'San Fernando' beans to be firmer than those of 'Nep-2' (Table 15, Figure 8). The compression component of the curves were greater than the shear component for both genotypes and 'San Fernando' beans showed a significantly higher average compression reading compared to 'Nep-2' beans (Table 15, Figure 8). Average shear components of the curves were similar for canned beans of both genotypes (Figure 10).

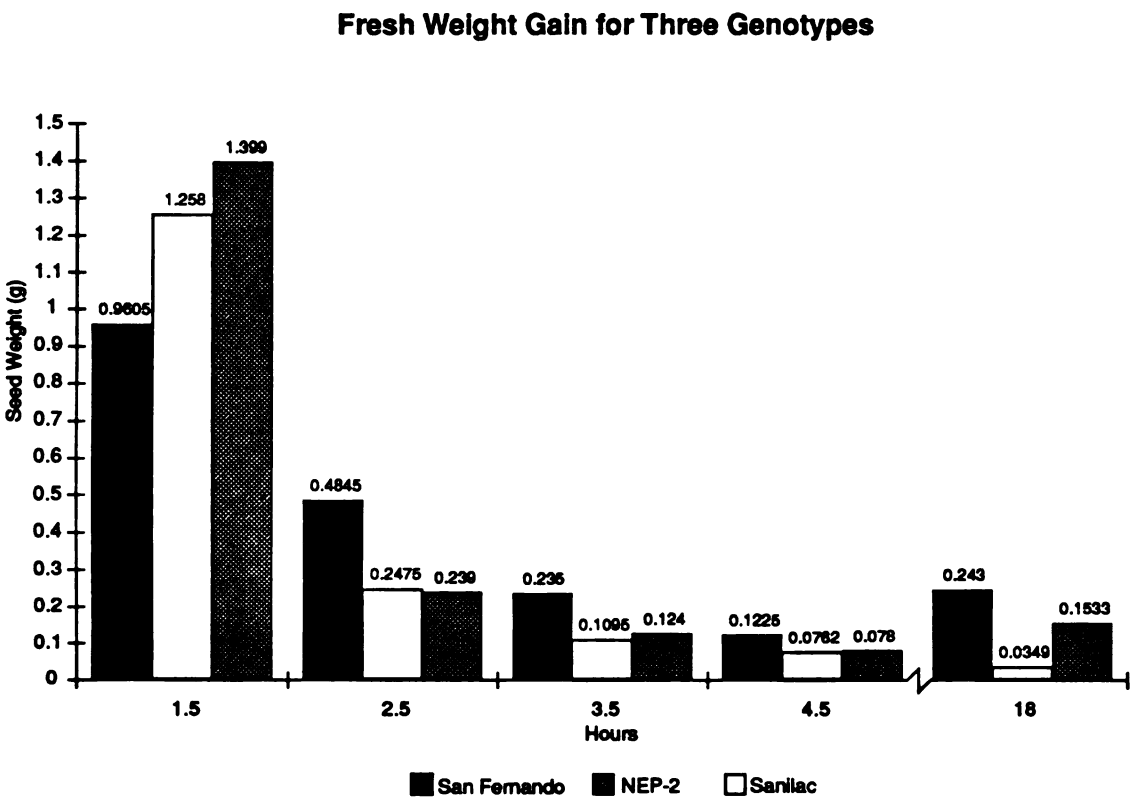

Figure 1: Weight gain of seeds of 'San Fernando', 'Nep-2', and 'Sanilac' after one hour soaking increments and a cumulative soak period for 18 hours. Data were taken after 1.5 hours of soaking and hourly thereafter for three consecutive hours. Each data point is the mean of 9 seeds and standard deviations are indicated by the numbers at graph points. The initial seed weights of the 9 seeds of each genotype were: 'San Fernando' = 1.75 g, 'Nep-2' = 1.59 g, and 'Sanilac' = 1.76 g. Beans were not stored.

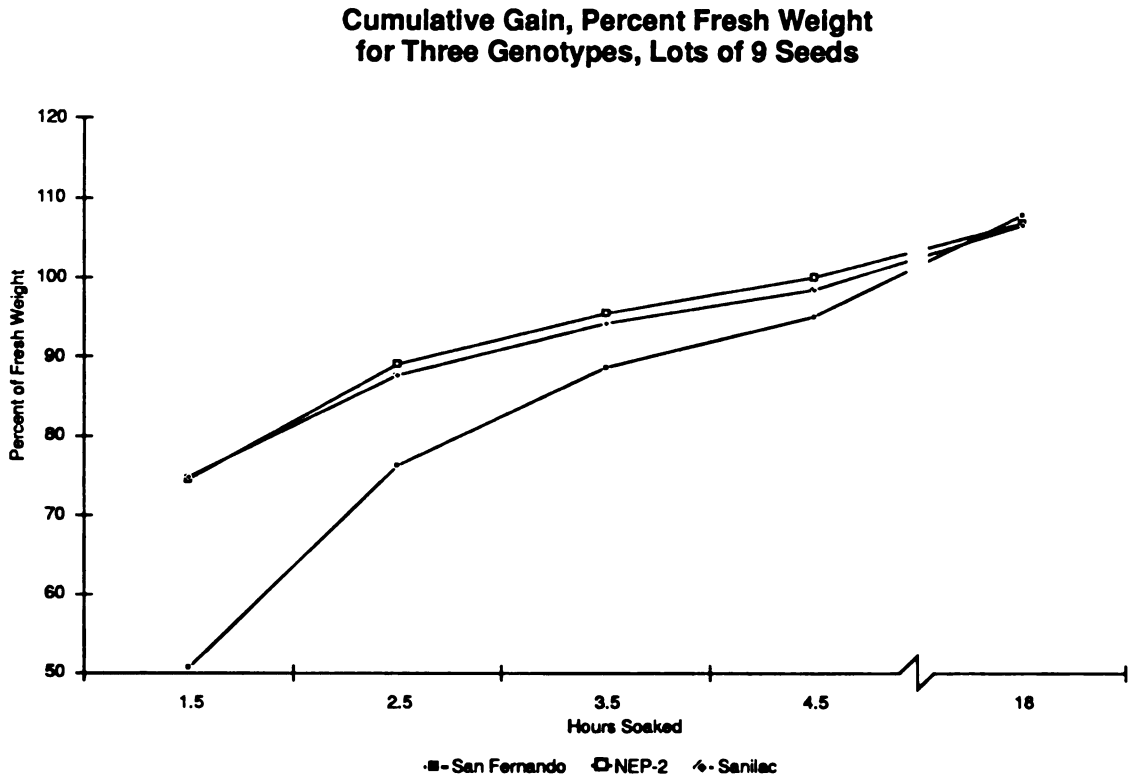

Figure 2: Average cumulative weight gain measured as a percentage of fresh weight for 9 seed samples of 'San Fernando', 'Nep-2', and 'Sanilac'. The initial seed weights of the 9 seed samples were: 'San Fernando' = 1.75 g, 'Nep-2' = 1.59 g, and 'Sanilac' = 1.76 g. Beans were not stored.

TABLE 1. MEAN THICKNESS (μM) OF SEED COAT LAYERS OF THREE DRY BEAN GENOTYPES.

	Palisade Layer	Hourglass Layer
'San Fernando' n=17	36.0	13.6
'Nep-2' n=29	36.8	19.5
'Sanilac' n=19	35.4	16.5

n=Number of observations per genotype.

TABLE 2. MEAN THICKNESS (μM) OF PALISADE LAYER VERSUS HOURGLASS CELL THICKNESS (μM) OF THREE DRY BEAN GENOTYPES.

'San Fernando' n=16	22.3
'Nep-2' n=26	16.8
'Sanilac' n=16	16.2

n=Number of observations per genotype.

TABLE 3. MEAN THICKNESS (μM) OF COTYLEDON CROSS-SECTION OF 'SAN FERNANDO', 'NEP-2', AND 'SANILAC' DRY BEAN GENOTYPES.

	Long Axis	Wide Axis	Mean Ratio L/W
'San Fernando' n=17	83.4	40.3	2.1
'Nep-2' n=24	82.6	58.3	1.4
'Sanilac' n=19	101.8	71.3	1.4

n=Number of observations per genotype.

TABLE 4. MEAN DIMENSIONS (μM) OF STARCH GRAINS OF THREE DRY BEAN GENOTYPES.

	Long Axis	Wide Axis	Mean Ratio L/W
'San Fernando' n=31	23.0	17.6	1.31
'Nep-2' n=32	24.9	19.7	1.26
'Sanilac' n=33	29.4	23.4	1.26

n=Number of observations per genotype.

TABLE 5. MEAN WIDTH (μM) OF EPIDERMAL CELLS OF 'SAN FERNANDO', 'NEP-2', AND 'SANILAC' COTYLEDONS-ADAXIAL SURFACE.

'San Fernando'	13.45a
'Nep-2'	12.83ab
'Sanilac'	11.97b

Different letters denote significant difference at 5 % level, Student's T.

TABLE 6. MEAN CELL WALL THICKNESS (μM) OF 'SAN FERNANDO', 'NEP-2', AND 'SANILAC'.

'San Fernando'	1.66a
'Nep-2'	2.01b
'Sanilac'	2.87c

Different letters denote significant difference at 1 % level, Student's T.

TABLE 7. MEAN COOKING TIME OF 'SAN FERNANDO', 'NEP-2' AND 'SANILAC' BEANS, RECENT HARVEST.

Genotypes	Minutes
'San Fernando'	276
'Nep-2'	186
'Sanilac'	184

TABLE 8. MEAN SQUARES FROM ANALYSIS OF VARIANCE FOR COOKING TIME OF FRESHLY HARVESTED BEANS OF 'SAN FERNANDO', 'NEP-2', AND 'SANILAC'.*

Source	df	M S	F
Genotypes	2	90987	22**
Error	96	4171	

*Completely random design with 33 observations per genotype.

**Significant at 1% probability level.

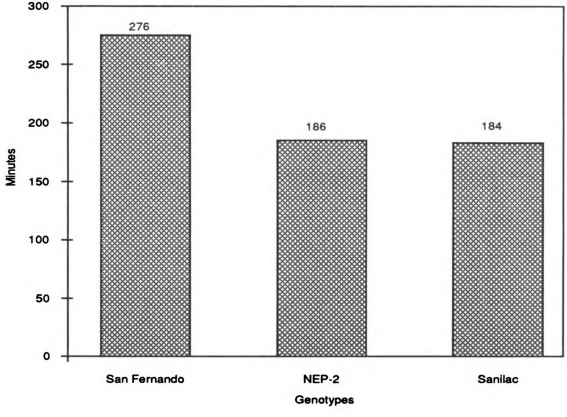

Figure 3: Mean cooking time of freshly harvested beans using a Mattson pin-drop cooking apparatus. Beans were cooked for 270 minutes preceded by a 0.5 hour blanching period. Twenty minutes were added to the time of any uncooked beans at the end of the cooking period to arrive at a cooking time.

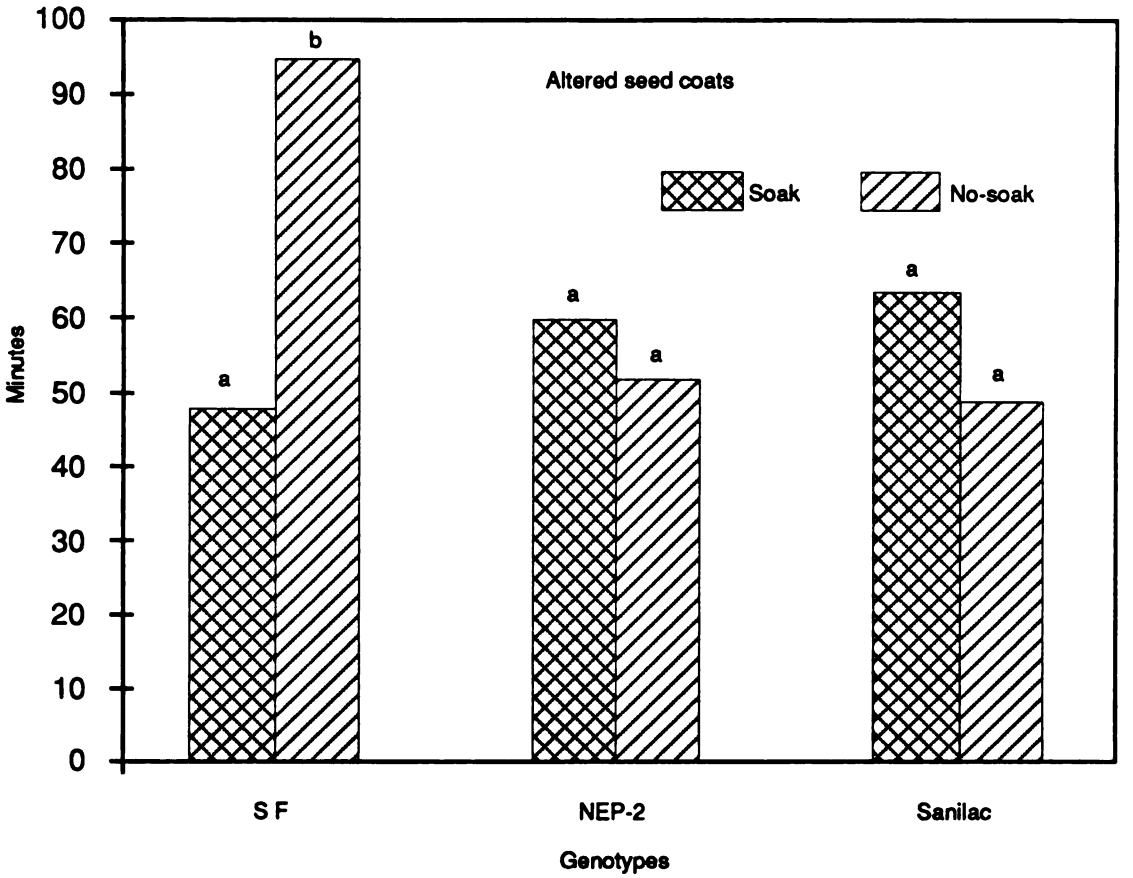

Figure 4: Mean cooking time of freshly harvested seed after removing a 2.5 x 2.5mm ± 0.5mm section of the seed coat. Beans were cooked in a Mattson pin-drop cooking apparatus and soaked in distilled water for 24 hours or unsoaked prior to cutting the seed coat aperture. Different letters on the bars indicate significant difference at the 5% probability level, lsd.

TABLE 9. COOKING TIME OF 'SAN FERNANDO', 'NEP-2', AND 'SANILAC' BEANS WITH AND WITHOUT SOAKING BEFORE REMOVING A 2.5 ± 0.5mm SECTION OF SEED COAT.

	24 hr. Soak	
Genotypes	yes	no
'San Fernando'	48 a	95 b
'Nep-2'	60 a	52 a
'Sanilac'	64 a	49 a

Same letters denote non-significant differences at 5% error level, lsd.

TABLE 10. MEAN SQUARES FROM THE ANALYSIS OF VARIANCE OF FRESHLY HARVESTED BEANS OF THREE GENOTYPES AFTER SOAKING FOR 24 HOURS AND NOT SOAKING AND REMOVING A 2.5 X 2.5 ± 0.5 SECTION OF SEED COAT (3^2 FACTORIAL ARRANGEMENT OF TREATMENTS).

Source	df	M S	F	
Soak Time	1	1258	3	ns
Genotype	2	1792	5	*
Interaction	2	6774	18	**
Error	66	368		

*, **, Significant at the 5% and 1% probability levels, respectively.

ns = non-significant.

TABLE 11. COOKING TIME OF BEANS WITH A 2.5 X 2.5 ± 0.5mm APERTURE IN THE SEED COATS STORED UNDER TWO DIFFERENT RELATIVE HUMIDITIES AND SOAKED 24 HOURS (YES) PRIOR TO COOKING OR UNSOAKED (NO).

	Storage Conditions			
	75 % RH		Ambient RH	
Genotype	yes	no	yes	no
	Minutes			
'San Fernando'	83	228	79	119
'Nep-2'	226	123	74	89

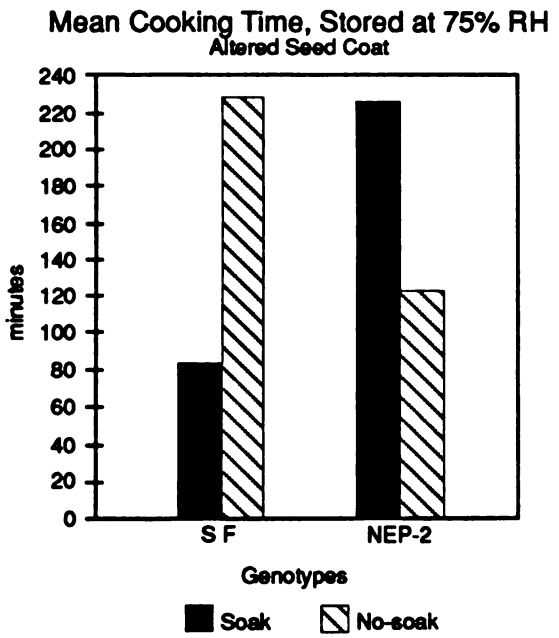

Figure 5: Mean cooking time of beans stored at 75% RH and soaked for 24 hours.

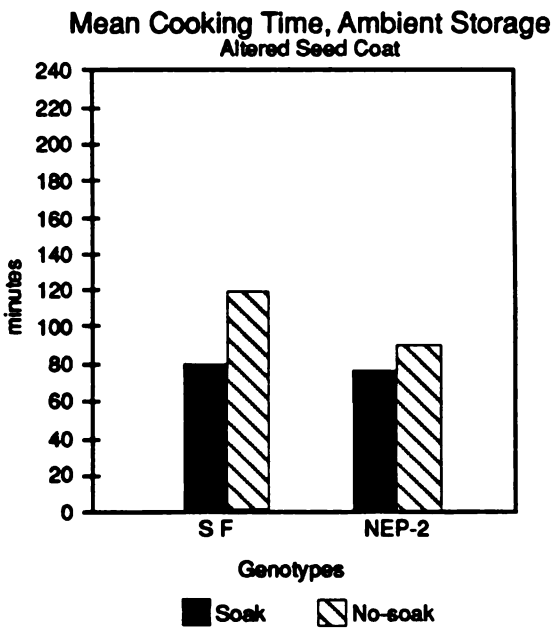

Figure 6: Mean cooking time of beans stored at ambient RH and soaked for 24 hours.

TABLE 12. MEAN SQUARES FROM ANALYSIS OF VARIANCE FOR COOKING TIME OF 'SAN FERNANDO' AND 'NEP-2' STORED AT AMBIENT CONDITIONS FOR 10 MONTHS PRIOR TO STORING AT 75% RH FOR 5 MONTHS, AND STORED AT AMBIENT CONDITIONS FOR 15 MONTHS AND SOAKED FOR 24 HOURS AT 21°C AND UNSOAKED.

Source	df	M S	F	
Soaking Time (T)	1	11737	1.3	ns
Genotype (G)	1	8	0.001	ns
T x G	1	92957	10.5	**
Storage (S)	1	112275	12.6	**
T x S	1	228	0.3	ns
G x S	1	6716	0.76	ns
T x G x S	1	62888	7.1	*
Error	72			

*, ** Significant at 5% and 1% probability levels, respectively.

ns = non significant.

TABLE 13. MEAN COOKING TIME OF BEANS OF 'SAN FERNANDO', 'NEP-2', AND 'SANILAC' WITH THREE CONDITIONS OF SEED COAT INTEGRITY AND STORED AT AMBIENT CONDITIONS FOR 18 MONTHS.

Genotype	Seed Coat Integrity		
	Whole	Aperture	Removed
'San Fernando'	290	144	49
'Nep-2'	261	122	32
'Sanilac'	271	106	30

TABLE 14. MEAN SQUARES FROM THE ANALYSIS OF VARIANCE FOR MEAN COOKING TIME OF THREE DRY BEAN GENOTYPES WITH THREE CONDITIONS OF SEED COAT INTEGRITY AND STORED AT AMBIENT CONDITIONS FOR 18 MONTHS (3^2 FACTORIAL ARRANGEMENT OF TREATMENTS).

Source	df	M S	F	
Seed Coat Integrity (I)	2	1034574	936	**
Genotype (G)	2	13963	13	**
I x G	4	1378	1	ns
Error	207	1105.302		

**significant at 1% probability level.

ns = non significant.

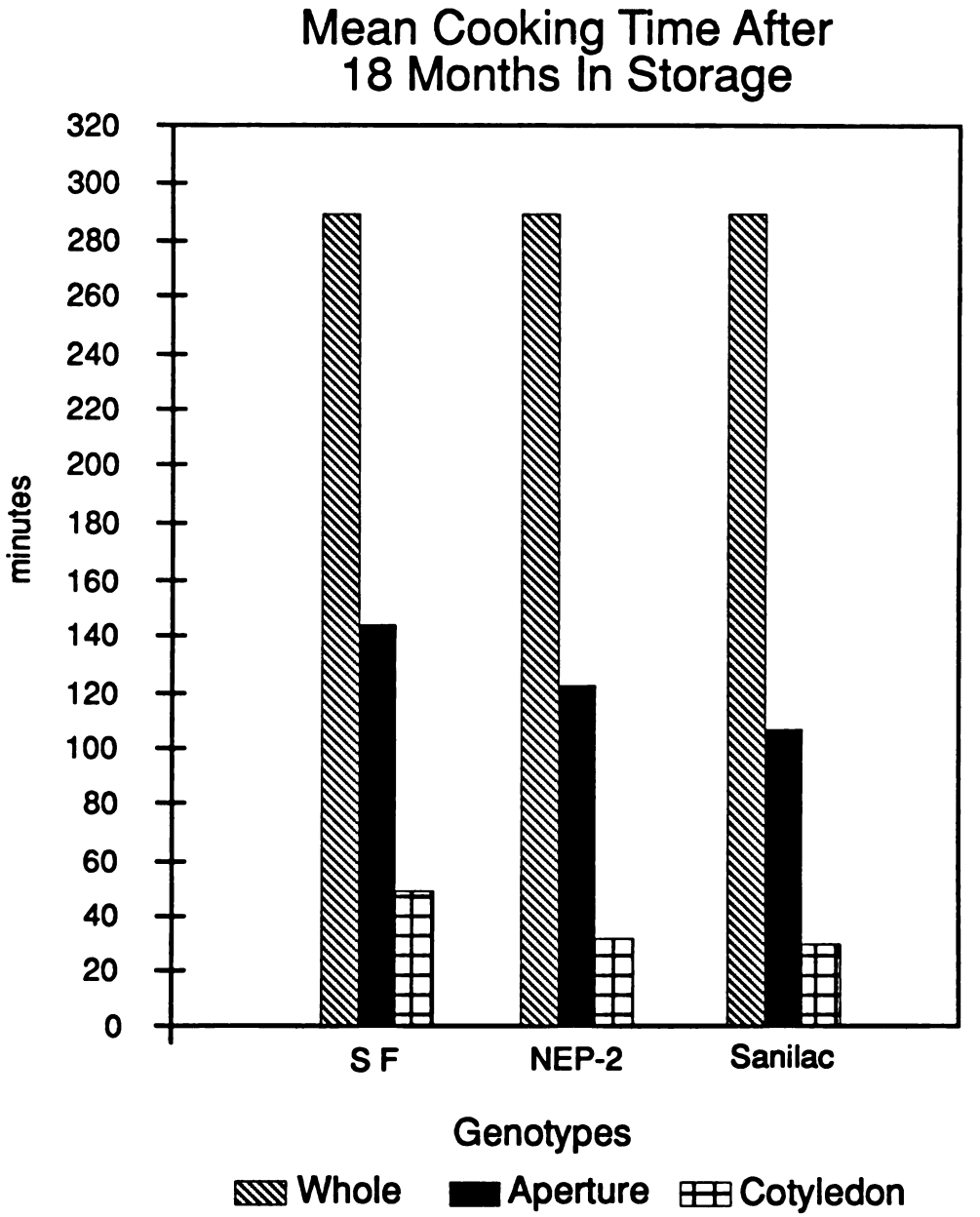

Figure 7: Mean cooking time of beans stored for 18 months under ambient conditions by level of seed coat integrity and cooked in a Mattson pin-drop cooking apparatus.

TABLE 15. TOTAL, SHEAR, AND COMPRESSION FORCES (KG/100 G) OF THERMALLY PROCESSED BEANS IN TIME CANS.

	Total**	Shear**	Compression**
'San Fernando'	170.1 g	60.0 a	110.2 d
'Nep-2'	147.7 h	61.9 a	85.8 e

** Same letters indicate non-significant differences at 5% error level, lsd, between genotypes, within shearpress elements.

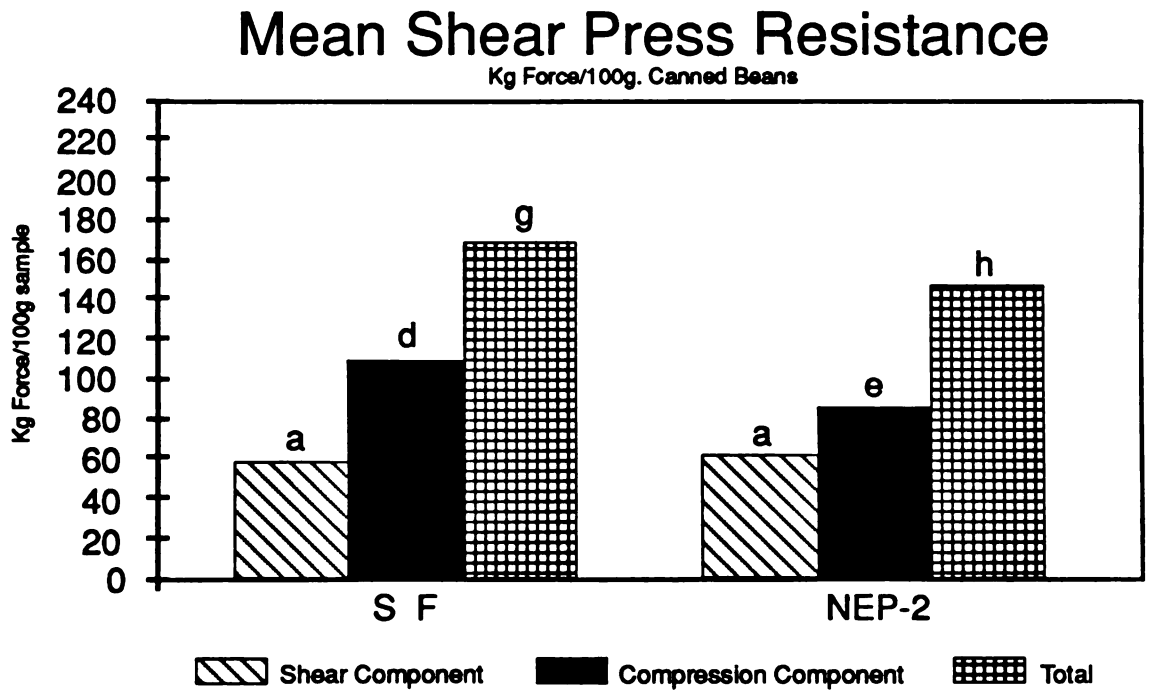

Figure 8: Mean force resistances for thermally processed beans in tin cans and determined with a Kramer Shearpress. Different letters on the bars indicate significant differences at the 5% probability level between genotypes within shearpress elements.

Discussion

The rapid rate of water uptake for the two white seeded lines compared to 'San Fernando' tends to confirm Agbo et al. (1987) in observing the importance for rapid water uptake of the open and semi-open micropyles and other seed anatomical features. Agbo et al. (1987) studied water uptake rates for a 1.5 hour soaking period. The current study extends the information to 4.5 hours of imbibition and is consistent with Kyle and Randall (1963) and Korban et al. (1981) on the relative importance of the micropyle during imbibition for the white seeded bean lines they observed. That 'Nep-2' and 'Sanilac' beans were about equal in imbibition rate after the first 1.5 hours may also point to the hilum and seed coat as entry areas for water. This would be consistent with Powrie and Adams (1973) and Varriano-Marston and Jackson (1981).

The increased cooking time for fresh whole 'San Fernando' beans over seed of the other genotypes followed the pattern noted by Agbo et al. (1987). The nearly identical cooking times of 'Nep-2' and 'Sanilac' did not confirm Agbo et al. (1987). Very high cooking times for all three genotypes, with many uncooked 'San Fernando' seeds, led to examination of cooking times with seed coat apertures. The relative position of cooking times for the three genotypes was reversed for soaked, altered beans.

Water imbibition differences for the genotypes may account for the relatively slow cooking nature of 'San

Fernando' beans and the relative position of mean cooking times for the genotypes. The 'San Fernando' seed coat may pose a barrier to pin penetration as well as water uptake. However, the significantly higher cooking time of the unsoaked 'San Fernando' beans with apertures compared to the other genotypes indicates a clear cotyledonary difference for 'San Fernando' beans. The cotyledon effect for 'San Fernando' is overcome by a 24 hour pre-soak indicating the absence of the Hard to Cook (HTC) phenomenon.

The seed stored under ambient conditions for the duration in experiment 3 confirms experiment 2. Experiment 3 also shows: 1) 'San Fernando' apparently has a cotyledon that softens more slowly during cooking than 'Nep-2' under both storage conditions; and 2) soaking will reverse or undo the slow cooking phenomenon induced by higher humidity storage in 'San Fernando' beans but not in 'Nep-2' beans.

The thicker cotyledonary cell walls of the white beans could be more heavily lignified during storage in a mechanism similar to that described by Varriano-Marston and Jackson (1981) or Hincks and Stanley (1986). The open and semi-open micropyles of 'Sanilac' and 'Nep-2', respectively, may permit restricted metabolism during high moisture storage which could lead to membrane breakdown and allow access of divalent cations from phytin to bind pectins in a mechanism similar to that described by Jones and Boulter (1983). Another enzyme which may be liberated after lamellar disintegration is pectin methyl esterase (PME) which would allow more pectin

binding sites for divalent cations according to Hincks and Stanley (1986). Varriano-Marston and Jackson (1981) speculated on cross-linking of hydroxyproline proteins in cell walls as a first step in lignification during ageing. One or more of these processes would account for the differences in 75% relative humidity stored bean cooking times of the white seeded lines compared to 'San Fernando'. Perhaps soaking for 24 hours prior to cooking allows the processes of binding middle lamellae and cell walls together to occur to an even greater extent in genotypes with open micropyles and with thicker cell walls such as 'Nep-2' and 'Sanilac'.

Experiment 1 may indicate what Agbo et al. (1987) pointed to as the influence of seed microstructure on water imbibition and extends the influence to cookability and storability. That none of the 'San Fernando' beans cooked and few of the 'Nep-2' and 'Sanilac' did so indicates a hardshell phenomenon occurring after extended storage. The increase in free hydroxycinnamic acid content of seed coats with ageing found by Srisuma et al. (1989) may also explain the toughness of tested seed coats with increased age and may be operative here.

The maintenance of the same relative position of the genotypes' cooking times over two levels of seed coat alteration suggests a clear genotype difference in cotyledons. The finding of Agbo et al. (1987) that 'San Fernando' cotyledon cells, appearing more compact and with

apparently denser cell walls, could contribute to slower water uptake are consistent with this work. 'San Fernando' cotyledon cells measured in this study were not more round in cross-section than cells of 'Nep-2', which conflicts with what Agbo et al. (1987) found. But 'San Fernando' cells were more compact in cross-section than 'Nep-2' cotyledon cells and cells of 'Nep-2' more compact than those of 'Sanilac', as Agbo et al. (1987) found. This finding could mean much greater cell surface area to be hydrated or circumference to allow movement of water during the soaking process. The relative cell wall thickness of the genotypes may help explain their relative response to ageing as seen in their relative cooking times if processes of cross-linking pectates or increased lignification are occurring in cell walls and middle lamellae.

Chapter II

Comparison of Two Isolines of Bean (*Phaseolus vulgaris* L.) for Seed Leachate Conductivity and Selected Ion Content of Soak Water and Middle Lamellae.

Abstract

Differences in bean cooking time are influenced by genetics, storage conditions and duration of storage. The linking of pectins by calcium and magnesium may cause cells to be tightly bound and not allow cotyledon softening during cooking. 'San Fernando', a black seeded bean, is slower cooking than 'Nep-2', a white seeded mutant isoline counterpart of 'San Fernando'. The soak water of seed of the isolines and 'Sanilac' from three storage treatments, fresh, 75% RH for 5 months and ambient conditions for 18 months, was tested for leachate conductivity, potassium, calcium and magnesium. Relative concentrations of calcium in cell walls and middle lamellae were determined by x-ray microanalysis for fresh seed of the isolines.

Seed soak water tested in the order 'Sanilac' > 'Nep-2' > 'San Fernando' for leachate conductivity and potassium. 'San Fernando' exceeded 'Nep-2' in calcium and magnesium levels for most treatments. Calcium in cotyledon cell walls was higher for 'San Fernando' than 'Nep-2', but the converse held for seed coat tissues.

Chapter II

Introduction

The cooking time and cooked texture of dry beans (*Phaseolus vulgaris* L.) influences their acceptability as a human food in both developing and developed countries of the world. Dry bean varieties differ in their cooking times and processed quality (Morris et al., 1950; Muneta, 1964; Hosfield and Uebersax, 1980; Hosfield et al., 1984). Differences are due to both genetic and non-genetic causes. Storage conditions of the dry seed also affects cooking time, cooked bean texture, and nutritive quality (Morris and Wood, 1956; Muneta, 1964; Molina et al., 1975; Uebersax and Bedford, 1980; Hincks and Stanley, 1986). Research conducted during the past five decades on storage factors that influence bean physical and chemical properties affecting culinary quality has shown that high relative humidity and temperature tend to act synergistically to increase cooking time of beans and may render beans unacceptable as a food (Morris and Wood, 1956; Burr et al., 1968; Molina et al., 1975; Jackson and Varriano-Marston, 1981; Vindiola et al., 1986).

Dry Beans are generally soaked and must be cooked before eating to render the seeds soft enough to be palatable,

inactivate heat labile anti-nutrients, and permit the digestion and assimilation of protein and starch (Deshpande et al., 1983). As beans cook, cotyledon cells separate along the middle lamellae. Intact cells are loosened from each other during cooking beyond that which occurs by soaking alone (Rockland and Jones, 1974; Vindiola et al., 1986). Sometimes beans exhibit a Hard-to-cook (HTC) and/or hardbean condition (Gloyer, 1921) which is manifest in the deterioration of textural quality due to the failure of the cotyledon cells to separate during cooking (Jones and Boulter, 1983). Beans subjected to poor storage conditions and which develop HTC have also been shown to have high levels of hydroxycinnamic acid in testae, which could increase the tendency of pectins to bind tightly in the cell wall (Srisuma, et al., 1989).

Some research has indicated that for beans with increased cooking time, phytate content and phytase activity of legumes may have increased (Mattson et al., 1950; Kon and Sanshuck, 1981; Hincks and Stanley, 1986). Divalent cations are theoretically liberated by phytase inside the cell and migrate to the middle lamellae where they form insoluble calcium and magnesium pectates and bind cells more strongly together than before the pectates formed (Mattson, 1946; Jones and Boulter, 1983; Vindiola et al., 1986).

Scanning electron microscopy (SEM) procedures can provide information on the relative elemental composition of a material by means of energy dispersive x-ray microanalysis

(Postek, et al., 1980). An energy dispersive system (EDS) collects and counts x-rays given off when an electron beam in a Scanning electron microscope (SEM) interacts with a specimen. Changes in electron orbitals of the specimen's elements produce x-rays with a characteristic energy for each element brought about by the accumulated forces holding the electrons in their given orbitals. X-ray counts of the characteristic energies are relative indications of elemental concentration in the area of specimen-electron beam interaction. However, x-ray counts are not considered absolute measures of concentration. Therefore, comparison of x-ray counts are also relative and approximate (Postek et al., 1980).

Electrolyte leakage from soaked seeds can be used as an indicator of decreased cookability (Jackson and Varriano-Marston, 1981) brought about by seed aging. Measurements of electrical conductivity of single seed soak water have been used in seed vigor tests but are also general indicators of membrane integrity (Powell, 1986).

Varriano-Marston and Jackson (1981) used Transmission Electron Microscopy (TEM) to note the disintegration of some cellular structures in cotyledon cells of stored beans. They hypothesized that hydrolases, including phytase, would be liberated from damaged vacuolar membranes to react with phytic acid during long term storage. Increased peroxidation of lipid membranes may be a cause of the membrane breakdown (Varriano-Marston and Jackson, 1981).

The objectives of the following studies were to:

1) Determine possible cell membrane integrity differences among the isolines;
2) Determine genotypic variability for relative amounts of divalent cation leaching during soaking and as a response to storage conditions;
3) Determine calcium content of middle lamellae in various seed tissues for the genotypes.

Materials And Methods

Design of the Storage Experiments. Seed of the three genotypes was planted in a nursery at the Saginaw Valley Bean and Sugarbeet Research Farm near Saginaw, Michigan in summer, 1985. Seeds from mature plants of each genotype were harvested, threshed and sized using 4 x 19-mm slotted metal sieves and stored in paper kraft bags at ambient humidity and temperature conditions for 18 months. After 18 months storage, the beans were refrigerated at 5.5±1.5°C until tested. These seed formed the materials for the "ambient storage" bean class.

Seed of 'San Fernando', 'Nep-2', and 'Sanilac' were planted in clay pots that were randomized on greenhouse benches from December, 1986 to March, 1987. After the plants were mature, seeds were threshed from pods and stored. Each pot was harvested separately and the respective seed was kept separate from seed of the same genotype grown in a different pot. Seed lots (seed sample of each genotype and from

individual pots) were placed in a dessicator over a saturated NaCl solution (75% RH) and held at 21.0±2.0°C for five months. The seed treated as just described formed the seed materials for the 75% RH storage class.

A third experiment seed class was composed of freshly harvested seed. These seeds came from plants of the three genotypes that were grown in the greenhouse from February to June, 1987. The planting arrangement and culture of beans was the same as described for the "75% RH" bean class. The beans grown from February to June, 1987 were not stored prior to analysis.

Seed Leachate Conductivity. The integrity of the cell membranes of seeds can be determined by measuring the electrical conductivity of leachate. Seed produced in the three experiments just described were soaked and the electroconductivity of soaked bean leachate determined with a Model ASA-610 Automatic Seed Analyzer (Neogen Food Tech Corporation, Lansing, Michigan).

To measure ion release, individual seeds were placed in a specially designed seed soaking tray that contains 100 small wells configured in a 10 x 10 grid. Wells were filled with distilled water for 24 hours at ambient conditions.

Results were measured and recorded in microamperes of current and converted to Siemens units of conductivity. Statistical analysis was conducted on microampere data.

According to Ohm's law,

$$I = \frac{V}{R}$$

where I is current in amperes, V is potential in volts, and R is resistance in ohms. Conductivity is the reciprocal of resistance and Siemens^{-1} = Ohms = mhos, the unit conductance of a body through which one ampere of current flows when the potential difference is one volt (Weast et al., 1986).

Inorganic Ion Composition of Single Bean Soak Water. Contents of individual seed soaking compartments of the Automatic Seed Analyzer tray were analyzed for calcium, magnesium and potassium. Calcium and magnesium were measured by atomic absorption spectrophotometry (AAS); potassium by atomic emission spectrophotometry. All tests were performed on a Perkin-Elmer Model 2380 Spectrometer (Perkin-Elmer Corp., Norwalk, Connecticut). The signal collector was set for a wavelength of 766.5 nm for potassium emissions. Hollow cathode lamps were used for absorption radiation sources, 422.8 nm wavelength for calcium and 285.2 nm for magnesium.

Standard absorption and emission curves were plotted for potassium using standards of 0, 10, 20, 30, 40 and 50 ppm concentration. The standard absorption curve for magnesium and calcium were based on 0, 1, 2, 3, 4, and 5 ppm solutions. Lanthanum oxide was used as a dilutant for both absorption and emission studies. The leachate solution from six soak

compartments for each variety in each storage treatment was measured for ion concentrations.

X-ray Microanalysis. Seeds from the freshly harvested and non-stored class were freeze dried, fractured with a razor blade at right angles to the longitudinal axes, mounted on aluminum stubs and carbon coated together in the same chamber for as uniform as possible depositon of carbon on all samples. A 25 keV beam was used in a JEOL-35C scanning electron microscope (Japan Electron Optics Laboratory). Each sample was examined by secondary electron image to determine that the specimen surface plane was perpendicular to the beam. Each sample was tilted 20 degrees and beam sample set at 60 picoamperes or less. X-rays were collected for 180 seconds and analyzed with a TN-2000 X-ray Analyzer using the NS-885 computer program from Tracor Northern, Inc.(Middleton, Wisconsin).

The region of interest (ROI) was chosen for the characteristic energy range of K-alpha calcium x-rays. Each sample spectrum was compared to a reference spectrum produced from a sample of calcium oxide mounted and coated in the same manner as subject specimens. A potassium oxide standard was used to determine any overlapping and interfering x-ray counts so they could be removed from calculations.

For each variety, we chose three seeds of the same apparent size from separate plants. We chose the following middle lamellae locations to collect calcium spectra: (1) between parenchyma cells in the cotyledon, (2) between

palisade cells of the seed coat, (3) between hourglass cells of the seed coat, and (4) between hourglass and palisade layers of the seed coat.

X-ray analytical spatial resolution could be up to five micrometers diameter (Echlin and Saubermann, 1977). For thick secondary cell walls in sclerified cell layers of seed coats, the volume of specimen beam interaction was anticipated to be within the cell walls of adjacent cells. For cotyledonary cell middle lamellae, the volume of interaction likely included cell walls and nearby cytoplasm.

Statistical Analysis. The leachate conductivity study for fresh seed was a randomized complete block design. Completely randomized design was the design used for leachate conductivity of the stored seed classes.

For ion content of soak water, statistical comparison consisted of Duncan's New Multiple Range Test (Steel and Torrie, 1980) to determine differences at the 5% level. Only means within storage categories within ion categories were compared for significant differences.

Results

'San Fernando' was lowest in average seed leachate conductance for all storage classes of seed—fresh, 75% relative humidity and ambient storage. 'Nep-2' was higher than 'San Fernando' and 'Sanilac' highest in leachate conductivity for all classes of seed (Table 16).

F tests were significant at the one percent error level for genotypes as the source of variation in all three seed classes (Table 17).

Of the three ions measured, potassium was in greatest concentration across all seed classes and genotypes (Table 18). The correlation between potassium concentration (ppm) and seed soak water conductivity (microamperes) was r=0.918. The soak water for 'San Fernando' bean seeds was lowest in potassium of the three genotypes in each seed storage class (Table 18, Figure 11). 'Nep-2' potassium soak water concentrations were higher than 'San Fernando' and lower than those of 'Sanilac' except for the ambient storage class (Table 18, Figure 11).

For fresh seed, 'San Fernando' seed soak water contained greater concentrations of calcium (Table 18, Figure 9) and magnesium (Table 18, Figure 10) than 'Nep-2' seed soak water. This relationship held for seed stored eighteen months, as well.

'Sanilac' seed soak water calcium and magnesium levels are intermediate for the fresh seed class and lowest of the three genotypes for the ambient conditions seed class (Figures 11, 12). 'Sanilac' soak water calcium was intermediate and magnesium highest of the genotypes for the 75% relative humidity seeds.

Some x-ray microanalysis spectra showed high variability from sample to sample (Table 19). Relative amounts of calcium in cotyledonary cell walls, middle lamellae and

nearby cytoplasm appear higher for 'San Fernando' than for 'Nep-2'. For the seed coat cell layers, the relationship appears to be the converse. Collections of calcium K-alpha x-rays were over twice the rate for palisade layer cell walls and middle lamellae of 'Nep-2' seeds compared to similar areas of 'San Fernando' seeds (Table 19). For middle lamellae of hourglass cells and thin cell walls, 'Nep-2' calcium x-ray collections were over twenty times those of 'San Fernando'. The area between palisade and hourglass cells and the surrounding cell wall material yielded over twice the number of calcium K-alpha x-rays, on average, for 'Nep-2' as for 'San Fernando' (Table 19).

TABLE 16. SEED LEACHATE CONDUCTANCE FOR SEEDS OF THREE ISOLINES, 24 HOUR SOAK IN DISTILLED WATER AT 20°C.

	'San Fernando'	'Nep-2'	'Sanilac'
	SIEMENS X 10^{-4}		
Fresh Seed	1.92	2.33	3.46
75% RH Storage	2.06	2.47	3.48
Ambient Storage	2.58	2.97	3.29

TABLE 17. MEAN SQUARES FROM ANALYSIS OF VARIANCE FOR SEED LEACHATE CONDUCTIVITY MEASURED AFTER A 24 HOUR SOAKING PERIOD ON INDIVIDUAL SEED SOAK WATER FOR FRESH SEED, SEED STORED AT 75% RH AND SEED STORED AT AMBIENT CONDITIONS.

Fresh Seed

Source	df	MS	F
Blocks	8	1032	1.4
Genotypes	2	57730	80**
Error	16	724	
Total	26		

75% Relative Humidity Storage

Source	df	MS	F
Genotypes	2	8052	48**
Error	69	168	
Total	71		

Ambient Storage

Source	df	MS	F
Genotypes	2	192	17**
Error	69	114	
Total	71		

**Significant at the 1% probability level.

TABLE 18. SELECTED CATION CONTENT OF SINGLE SEED SOAK WATER 24 HOUR SOAK IN DISTILLED WATER AT 20°C.
mean ppm

Fresh Seed			
	'San Fernando'	'Nep-2'	'Sanilac'
Calcium	3.78b	2.37a	3.02ab
Magnesium	3.38b	1.63a	3.02ab
Potassium	29.54a	39.67ab	69.76b
75% Relative Humidity Storage			
	'San Fernando'	'Nep-2'	'Sanilac'
Calcium	2.99d	5.36d	4.03d
Magnesium	5.61d	4.00d	8.09d
Potassium	92.54d	150.26de	257.12e
Ambient Storage			
	'San Fernando'	'Nep-2'	'Sanilac'
Calcium	1.68h	1.25gh	1.07g
Magnesium	4.75g	2.26h	2.19h
Potassium	118.69g	180.58g	160.68g

Means with the same letter designation within a storage X genotype treatment are not significantly different at the 5 % error level.

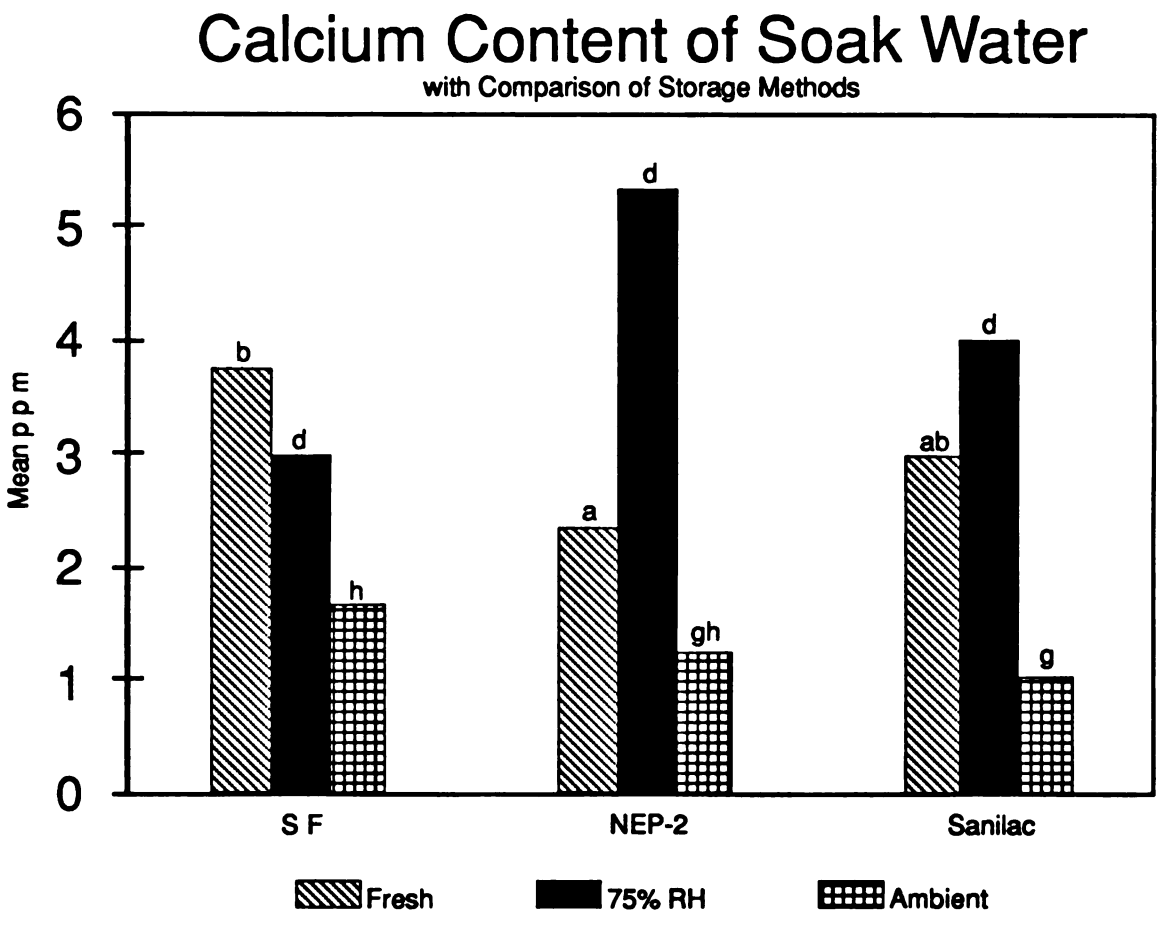

Figure 9: Calcium content of soak water in individual soak cells after 24 hour soaking period in distilled water. Measured by atomic absorption spectrometry. Bars with the same letter designation within a storage x genotype treatment are not significantly different at the 5% error level.

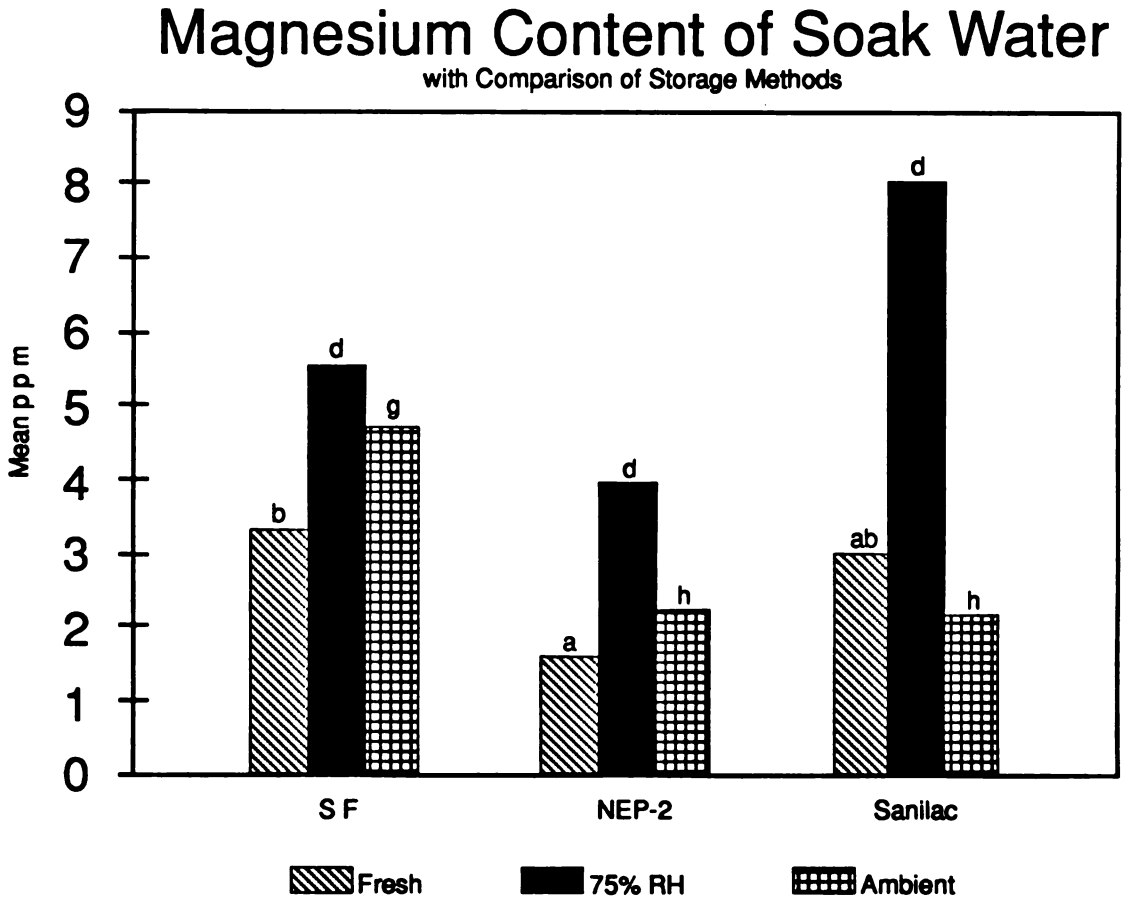

Figure 10: Magnesium content of soak water in individual soak cells after a 24 hour soaking period in distilled water. Measured by atomic absorption spectrometry. Bars with the same letter designation within a storage x genotype treatment are not significantly different at the 5% error level.

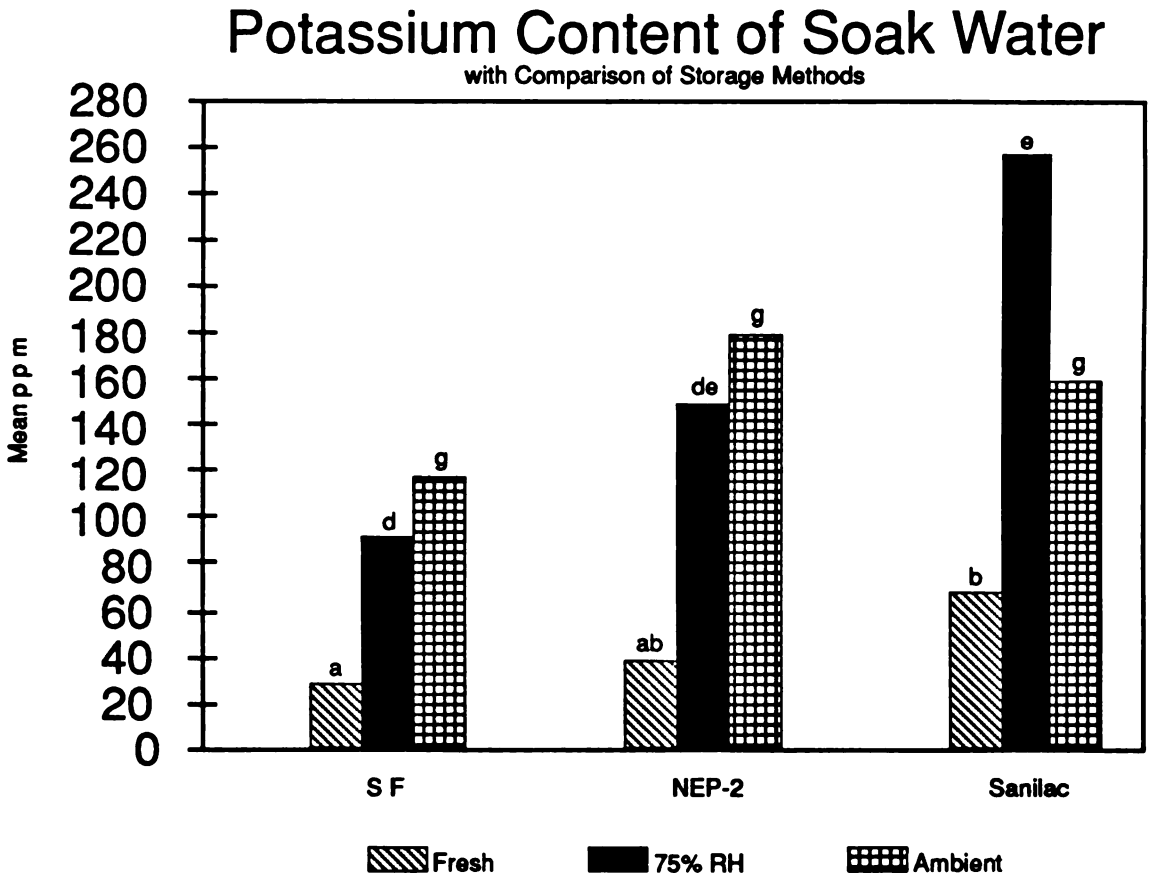

Figure 11: Potassium content of soak water in individual soak cells after a 24 hour soaking period in distilled water. Measured by atomic absorption spectrometry. Bars with the same letter designation within a storage x genotype treatment are not significantly different at the 5% error level.

TABLE 19. AVERAGE CALCIUM K-ALPHA X-RAY COUNTS, MIDDLE LAMELLAE AND CELL WALLS, FRESH SEED.

Location	'San Fernando'	'Nep-2'
Cotyledon		
Parenchyma	1514 (76%)*	768 (56%)
Testa		
Palisade	5272 (42%)	11292 (18%)
Hourglass	2955 (57%)	62772 (24%)
Palisade/ Hourglass	27037 (45%)	70209 (38%)

*coefficient of variability.

Discussion

In seed leachate conductivity, 'San Fernando' maintained the lowest levels over different storage conditions. 'Nep-2' is intermediate in leachate amount but never approaches 'Sanilac', the commercial white bean. 'San Fernando' has been shown to be slowest to imbibe (Swanson et al., 1985; Agbo et al., 1987) and the shorter duration soak this implies may account for some of the difference in leachate conductivity.

Potassium content of soak water tends to follow closely with seed leachate conductivity levels (Powell, 1986). In these tests, the relative placement of the genotypes in

conductivity holds for potassium content of soak water, except for 'Nep-2' and 'Sanilac' in the ambient storage category.

The leachate conductivity and potassium levels may vary positively with damage to plasmalemmae and intracellular membranes. The increased levels for stored beans in general coincides with the hypothesis that membranes deteriorate with seed age.

Calcium and magnesium are offered by various authors as cross linking pectins in middle lamellae during aging processes, leading to decreased cell separation during soaking and cooking. Fresh seed soak water levels for calcium and magnesium show 'San Fernando' higher than the white seeded mutant though the relationship is not as strong for stored seed, particularly for higher relative humidity storage. A mechanism explaining this would be that more of the divalent cations and phytin have traveled farther due to greater membrane leakage and perhaps due to more vacuolar liberation of phytase for the stored white beans. The reversal of the potassium/conductivity ranking between the two isolines for calcium and magnesium could be explained by this greater membrane breakdown in the white mutant coupled with insolubility of the leaked divalent cations as they bind with pectins during storage.

In fresh seed, with membranes assumed to be in good shape, we note the cotyledonary middle lamellae of 'Nep-2' tending to be lower in calcium by x-ray microanalysis. This

tends to explain the firmer processed texture of 'San Fernando' (Agbo, 1982) as greater cross linking of pectins in middle lamellae than 'Nep-2'.

In general, we observed 'Nep-2' to have a more brittle, fracturable seed coat than 'San Fernando''s tougher, more pliable testa in the dry state. 'Nep-2''s higher calcium levels in seed coat middle lamellae could also explain the higher shear acceleration for the seed coat during Kramer Shear Press evaluations of processed bean samples (Chapter I).

These studies show 'Nep-2' to be closer in observed values to 'San Fernando' than 'Sanilac' is for these derived traits. Differences are postulated between 'San Fernando' and the mutant 'Nep-2' in membrane integrity during storage and for middle lamellae differences, either in pectin amount or cross-linking degree by divalent cations. The original mutation observed by Moh (1971) at the P locus or ground factor gene appears to be pleiotropic.

SUMMARY AND CONCLUSION

Seed anatomical features noted in this study confirm much of the work by Agbo et al. (1987) to possibly explain water uptake, cooking time, and processed texture differences noted between the isolines, 'San Fernando' and 'Nep-2'. The present study has further characterized some of the anatomical differences and has extended the differences to response to aging and storage conditions. In addition, this study has identified differences in membrane integrity and points toward possible explanations for differences in some of the cooking, texture and imbibition parameters.

The studies on seed leachate conductance and ion content of soak water point to differences in the two isolines for fresh seed membrane integrity as well as for effects of storage condition on membrane integrity. The cooking rate studies show clear cotyledonary differences for both fresh seeds and stored seeds of the isolines.

Rate of imbibition could be related to gross anatomical features such as the relative opening size of the micropyle. Fine structure such as the relative thickness of palisade and hourglass layers in the seed coat, thickness of cotyledon cell walls, and width of cotyledon adaxial epidermal cells may also affect water uptake rates. Cotyledon cell wall differences may also help determine texture and cooking time differences, particularly after the seeds age.

The anatomical studies confirm Agbo et al. (1987) in the depiction of 'San Fernando' as having a smaller celled, more compactly organized seed than 'Nep-2'. 'Nep-2' differs from 'San Fernando' in many microstructural features and more closely approximates the microstructural dimensions of 'Sanilac'. This intermediate character of 'Nep-2' between 'San Fernando' and 'Sanilac' is consistent throughout these studies.

Pleiotropy for the single gene mutation found by Moh (1971) is a clear possibility. However, the differences between 'San Fernando' and 'Nep-2' appear to be of degree rather than of basic character. Many of the microstructural features examined are basic to cell and organ structure and during growth, much of the cytoplasmic machinery is given to their construction. For this reason, cells are likely to have encodings in several or many places for such structures in their genomes rather than only at a single locus such as the groundfactor locus or P gene. Thus, polygenic inheritance of the pathways leading to basic structural development is likely. The changes induced by EMS during Moh's (1971) experiments could have affected a larger region of DNA in which the P gene is located. It may also be that regulatory genes are located in close proximity to the P locus and that this study is observing the effects of pleiotropy for regulatory genes that encode for key enzymes in anabolic pathways.

The seed physical-chemical and microstructural studies lead to the following conclusions.

1) 'San Fernando' and 'Nep-2' differ for some basic seed anatomical features. These include cotyledon characteristics as well as seed coat characteristics.
2) Seed anatomical features may contribute to isoline differences in storability and plasmalemma integrity over time.
3) The pleiotropic effects of the P locus for seed coat color are numerous and far reaching in their implications for performance of a bean line.
4) Since basic structures such as cell walls have been affected by the mutation, and it is likely that there is a large amount of encoding for proteins involved in construction of basic structures cells need in large amounts, it is possible that genetic material encoding for shared pathways of cell wall materials and pigmentation was involved in a mutation event at the time of the P allele change; or that regulatory genes for such processes are closely linked with the P locus and were affected by the change in base pair sequence effected by EMS.
5) The same bean seed structural characteristics, such as a closed micropyle, that allow storage under conditions of high relative humidity with cookability may deter rapid imbibition and cooking.

6) Tests could be developed for use in breeding programs which might indicate inheritance of traits indicative of cookability and storability. These might include seed leachate conductivity, structural parameters measured by SEM and x-ray microanalysis of seed cell middle lamellae and cell walls.

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