The Effects of Genotype, Location, and the Development of the Hard-to-Cook Defect on Black Bean (*Phaseolus vulgaris*) Electrophoretic Patterns

by

Darcy R. Driedger

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

The effects of location, cultivar, storage, and susceptibility to the hard-to-cook (HTC) defect on electrophoretic patterns of black beans (Phaseolus vulgaris) were investigated. To investigate these effects, six black bean cultivars were grown in three locations in Guatemala. The time required for adequate cooking was determined for each sample immediately after harvest using a Mattson bean cooker. The cooking times for beans from two locations were determined again after approximately six months of storage at 25°C. The isoenzymes of peptidase, peroxidase, polyphenol oxidase, acid phosphatase, esterase, phosphoglucosoisomerase, and glutamate oxaloacetate transaminase from the cotyledon, root, stem, and leaf tissues were separated electrophoretically. The electrophoretic patterns of cotyledon storage proteins soluble in 5 M acetic acid and in 0.1 M acetic acid containing 1.0% mercaptoethanol were determined using acid-polyacrylamide gel electrophoresis (acid-PAGE). SDS-PAGE patterns from cotyledon proteins soluble in sodium chloride solution, acetic acid, ethanol, and from residual proteins were also examined.

To further investigate the effect of storage, two cultivars were stored for 6 months at three temperature levels (9°, 23°, and 36°C). At each temperature, two lots of beans were adjusted to 12% and 16% moisture. Cotyledon isoenzymes, 5 M acetic acid soluble cotyledon storage proteins, and phaseolins were monitored electrophoretically.

The cooking time of fresh samples was significantly affected by cultivar, location, and the interaction between cultivar and location ($p<0.001$). After six months of storage, cooking times were higher for most samples. The increase was significantly affected by cultivar

($p<0.001$). The cultivars San Martin and ICTA Line 85-12 exhibited greatest fresh hardness as well as the greatest increase in hardness during storage. High temperature and high moisture promoted the development of HTC beans during storage.

Cotyledon esterase electrophoretic patterns exhibited locational variation. No other patterns were affected by location. Stem esterase, root esterase, leaf peroxidase, root peroxidase, and leaf acid phosphatase exhibited cultivar variation but not all cultivars could be uniquely classified on the basis of isoenzyme patterns. ICTA Line 85-12 and San Martin displayed unique esterase isoenzymes (R_f = 0.41 and 0.45) while San Martin also had a unique leaf acid phosphatase isoenzyme (R_f = 0.94), indicating that these isoenzymes might be related to cooking quality.

Genotype had a greater effect on seed protein electrophoretic patterns than on enzyme patterns with variation observed using acid-PAGE (acetic acid/ mercaptoethanol extraction) and SDS-PAGE (sodium chloride, acetic acid, and residual extractions). All cultivars could be uniquely classified on the basis of storage protein patterns. No clear relationship was observed between storage protein patterns and cooking quality.

The effect of storage on cotyledon enzyme electrophoretic patterns was minimal. No changes were observed after 24 weeks under any storage conditions. Phaseolin protein patterns were likewise not affected by storage. Three 5 M acetic acid soluble protein bands (Rm = 55, 59, 63) weakened in intensity over time when beans were stored at 36°C/16% moisture. These changes were not evident when the beans were stored at 9°C/12% moisture.

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1. INTRODUCTION

1.1 Background

In terms of global production, legumes provide almost as much dietary protein as wheat, and substantially more than corn or rice (Figure 1.1). This is reflected in Latin America where beans are, after cereals, the most important source of protein, calories, and other nutrients (Paredes-Lopez et al., 1989). Because the production of beans requires less land and capital investment than the production of livestock, beans tend to be a more important protein source for the poor than for the rich. Significant gains in the availability of beans would correspondingly result in great improvements in the nutritional status of a vast segment of the world's population.

Increasing the world's production of beans has been hampered by post-harvest handling problems. The development of hard-to-cook (HTC) beans during storage is a serious problem on Central America (Stanley and Aguilera, 1985). Hard-to-cook beans are characterized by requiring dramatically more cooking time than do fresh or soft beans. The development of the HTC condition is accelerated by storage at high temperature and moisture levels (Figure 1.1). These conditions are common, if not unavoidable, in Central America.

The HTC defect imposes economic losses on a group of farmers that are already economically disadvantaged. It has been documented that Guatemalan beans that were bought fresh for US$0.66/kg had to be sold for US$0.15/kg after inadequate handling and storage (Booth, 1980). In all of Central America and Panama, the HTC condition was estimated to be responsible for losses equivalent to US$12 million in 1977 (Gonzalez de Mejia, 1982).

TABLE 1.1

World production of protein by major food legumes and cereals in 1975 (1000 tonnes).

Crop	Protein
Soybeans	25 800
Dry beans	11 500
Peanuts	2 500
Legumes, total	39 800
Wheat	42 600
Corn	25 800
Rice	24 000
Cereals, total	92 400

Source: Rockland and Radke (1981).

Economic losses occur at the consumer level as well. Garcia et al. (1988) estimated that in the Peten region of Guatemala alone, the increased cooking time required for HTC beans cost consumers the equivalent of US$2 million of electrical energy annually. This value is somewhat deceptive as most residents of the Peten heat with wood rather than electricity. Nevertheless, with deforestation of the Peten's rain forest already a serious problem, the real cost of the HTC defect may be best considered in terms of the release of CO_2 to the atmosphere and the destruction of large O_2 generating areas that accompany deforestation.

Strategies to combat the HTC defect must consider the economic situation of most Central American bean producers. The mean annual

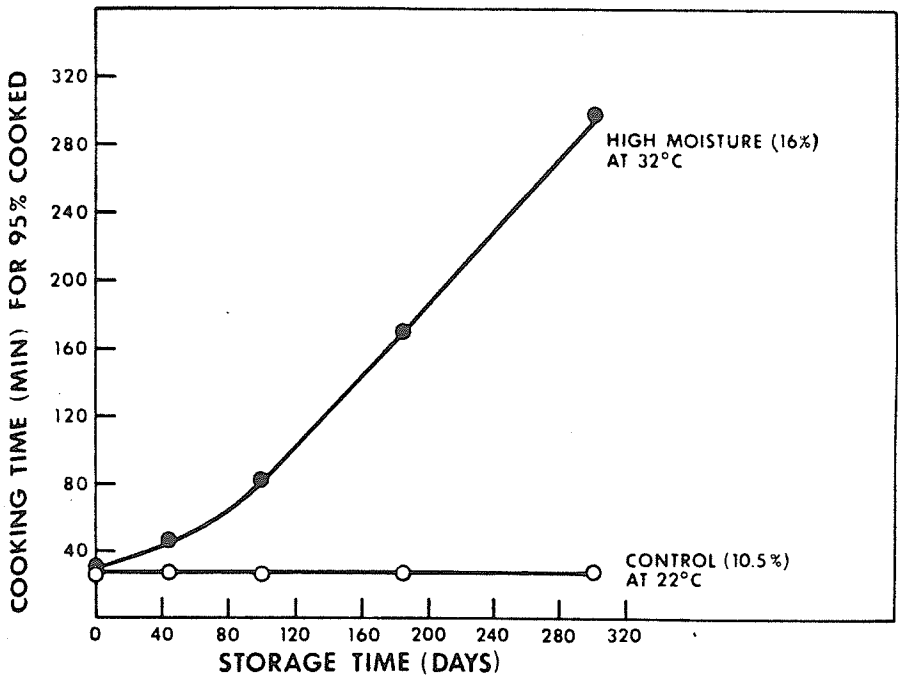

Figure 1.1 Cooking rate of test beans stored at high moisture and temperature compared to control beans stored at low moisture and temperature.

Source: Kon and Sanshuck (1981).

income for the poorest 20% of the Guatemalan population is only US$350 (Table 1.2). This realistically means that there is no money available to invest in technological improvement. Any technology that effectively helps the poorest farmers must be practically free while at the same time requiring no capital costs for maintenance. Plant breeding offers a possible solution to this problem. The development of a bean cultivar resistant to hardening under adverse conditions would provide that farmer with a cheap, maintenance free technology to avoid losses due to the HTC defect.

Conventional methods of cultivar identification of plants are based on physical and morphological differences. Because black bean cultivars are often identical in appearance, it is very difficult for plant breeders to identify different varieties if they do not have access to pedigree information. Electrophoresis is one technique that has been used successfully to identify bean cultivars on the basis of chemical differences rather than physical differences (Bassiri and Adams, 1978; Hussain et al., 1986). These studies, however, used bean cultivars that were already distinguishable by morphological characteristics. An electrophoretic technique that could rapidly distinguish morphologically identical black bean varieties would assist plant breeders in developing a cultivar that is resistant to the HTC defect. Moreover, if electrophoretic patterns could be used to predict the susceptibility of cultivars to the HTC defect, time-consuming storage studies could also be avoided.

TABLE 1.2

Average annual income of the poorest 20% of the population of several Central American countries in 1980 (adjusted to approximate 1990 US dollars).

Country	Income
Guatemala	350
El Salvador	150
Honduras	260
Nicaragua	200
Costa Rica	560

Source: CEPAL (1984).

1.2 Objectives

This thesis is divided into two parts. The main part of the study addresses the following three objectives:

1. To develop an electrophoretic technique capable of identifying morphologically identical black bean cultivars.

2. To identify electrophoretic patterns unique to cultivars resistant to the HTC defect.

3. To determine if bean electrophoretic patterns are stable among growing locations.

A smaller second study (Chapter 5) was designed to address the last objective:

4. To determine if bean electrophoretic patterns are stable during storage.

2. LITERATURE REVIEW

2.1 Bean Taxonomy

The black bean belongs to the species Phaseolus vulgaris L. The genus Phaseolus, which belongs to the family Leguminosae, represents a wide variety of beans, such as mung beans (P. radiatus L.), Lima beans (P. lunatus L.), runner beans (P. coccineus L.), and adzuki beans (P. angularis (Willd.) Wight). The species P. vulgaris is distinguished by leaflets that are entire, ovate, acuminate, and have a marked pulvinus at the base. The flowers are borne on axillary, few-flowered lax racemes and the pedicels are 5-8 cm in length (Kay, 1979).

P. vulgaris shows considerable variation in plant size and in the size, shape, and color of the seeds. There is not, however, a clear consensus as to how to classify the subspecies. From an evolutionary perspective, Singh (1989) has suggested 12 gene pools based on phaseolin electrophoretic patterns, morphological characteristics, and geographical distribution. On the other hand, the groupings used in international trade are based solely on seed characteristics: pea beans (navy), white kidney beans (Great Northern group), red kidney beans, pinto beans, cranberry beans (Romano), black beans, yellow eye beans, brown beans, and pink beans (Kay, 1979). Both systems are not categorical as there are many intermediate forms.

Regardless of which classification system is used, there exist many genotypes within each subspecies. Moreover, the genotypes often appear to be morphologically identical. This is a particular problem for germplasm banks which must determine if morphologically identical accessions are also genotypically identical. Further characterization by biochemical means, such as electrophoresis, is usually required.

2.2 Factors the Influence Electrophoretic Patterns

2.2.1 Genetics

Because proteins are the products of gene activity, they give a good indication of the genetic make up of an organism. Transcription converts the DNA genetic code into mRNA which is in turn translated into a protein (Figure 2.1). There are many different proteins in a given organism serving as enzymes, structural components, storage vehicles, and a wide variety of other functions. Separation techniques, such as chromatography and electrophoresis, can be used to separate the proteins on the basis of their size, polarity, electrical charge, or other characteristics. With respect to electrophoresis, if a change in the DNA affects the size or the charge, or even the existence of a protein, it will also affect the electrophoretic pattern. For this reason, electrophoresis is a useful tool for cultivar identification. Cultivars, by definition, are genetically different plants, and since their DNA codes differ, each cultivar should have its own protein complement, and hence, its own electrophoretic pattern.

There are, however, several other factors that affect the proteins present in a given plant tissue - they are not solely the function of the DNA. For example, it has often been shown that enzyme electrophoregrams can differ significantly between leaf and root tissues of the same plant, even though the DNA of the cells in the different tissues is identical (Ramirez et al., 1987; Bassiri and Adams, 1978). The synthesis of specific proteins in a cell can be regulated by such things as differential transcription, translational repression, mRNA stability, and post-translational modifications. Weeks (1981) has described protein biosynthesis regulation in a well written

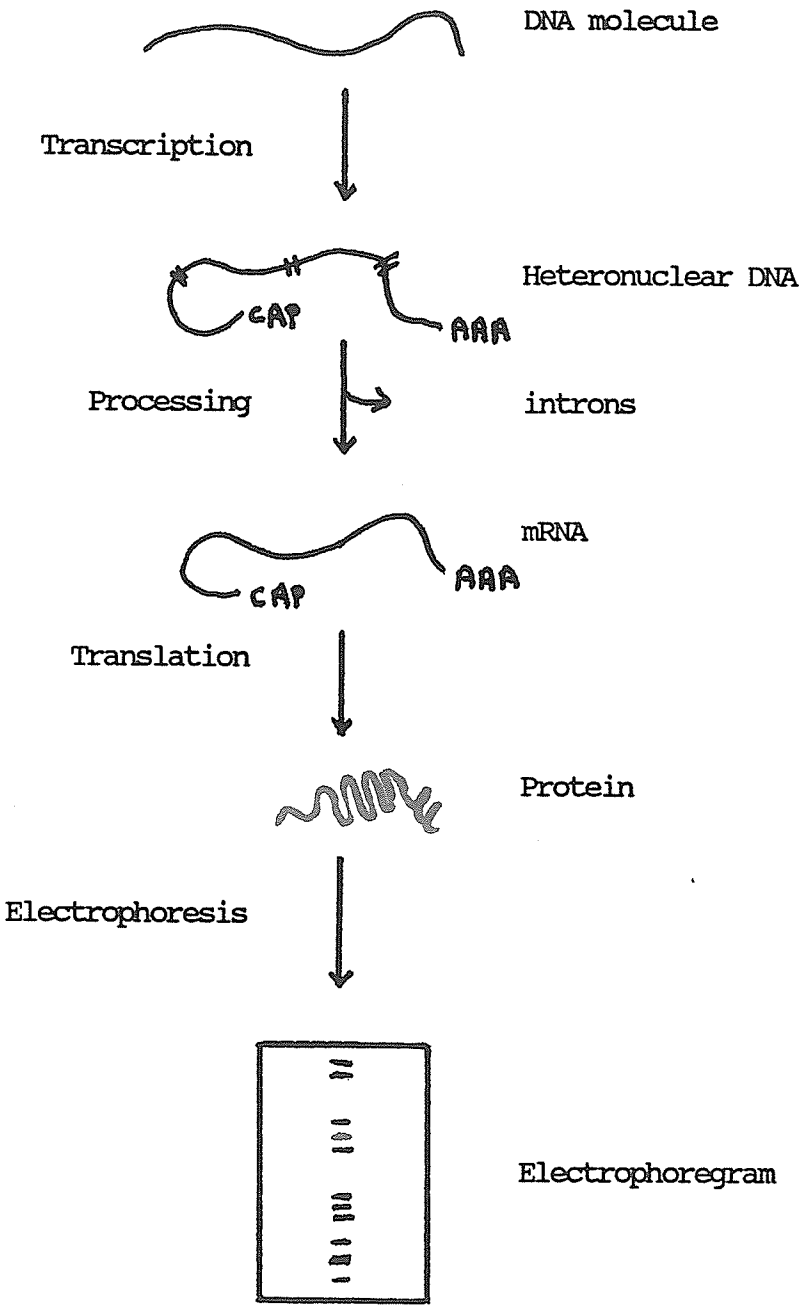

Figure 2.1 The relationship between the DNA code and protein electrophoresis.

review.

Intercellular differences in protein make up due to protein synthesis regulation would not, however, be expected within cells of the same tissue at the same physiological age. Cells within the same tissue would be subject to the same forces of regulation and would, therefore, produce the same set of proteins. The literature confirms that electrophoretic patterns are highly reproducible within the same type of tissue, but that differences in the age or type of plant tissue analyzed can have a dramatic effect on electrophoretic patterns (Chang and Harrold, 1988; Ramirez et al., 1987).

2.2.2 Location

Because nitrogen for protein synthesis must ultimately come from the soil, it follows that soil nitrogen levels may affect the protein make up of a plant. It is well known that increasing the nitrogen levels in the soil increases the yield of a crop. Leleji et al. (1972) found, however, that yield and crude protein were generally negatively correlated. Souza et al. (1973) reported that added levels of nitrogen, phosphorous, and potassium had no effect on the percent protein in the seed nor on the protein efficiency ratio (PER) of P. vulgaris. The stability of the PER would indicate that the protein characteristics of the seed are not affected by fertilizer levels.

The minimal effect of location on the seed protein complement has been confirmed by actual electrophoretic work. Lee and Ronalds (1967) reported that effect of location on wheat gliadin patterns was negligible in comparison with varietal differences. Adriaanse et al. (1969) tested beans that had been grown in Tanzania and in Holland, on clay and sandy soils, and with fertilization levels of 0-120 kg N/ha.

Although they found some minor differences, they still concluded that electrophoregrams were independent of external conditions of growth. More recently, field beans grown in Colombia and in Canada produced identical SDS-PAGE and acid-PAGE patterns (Hussain et al., 1986). Field peas grown in four Canadian locations during two growing seasons showed no location or season effect (Hussain et al., 1988a).

2.3 Isoenzyme Electrophoresis

2.3.1 Biochemical Background

It has been well established that, what are referred to as single enzymes, often exist in several molecular forms. When Markert and Moller (1959) used starch gel electrophoresis to separate protein extracts from beef heart, they found that there were several regions in the gel that exhibited lactate dehydrogenase activity. Multiple regions of activity were also discovered for citrate and malate dehydrogenase. They concluded that there must be numerous different molecules that are capable of catalyzing the same reaction. The term "isozyme" was designated to refer to the distinct molecular forms of an enzyme. The term "isoenzyme" is also commonly used.

Isoenzymes may originate from several gene loci, each coding for one isoenzyme. They may also exist as the products of allelic genes, sometimes called allozymes (Figure 2.2). Studies show that these isoenzyme patterns are inherited according to Mendelian laws. Unlike the inheritance of characteristics such as eye color, the isoenzymes produced by one allele do not mask the product of the other allele. The products of both allelic genes are expressed in heterozygous individuals (Moss, 1982).

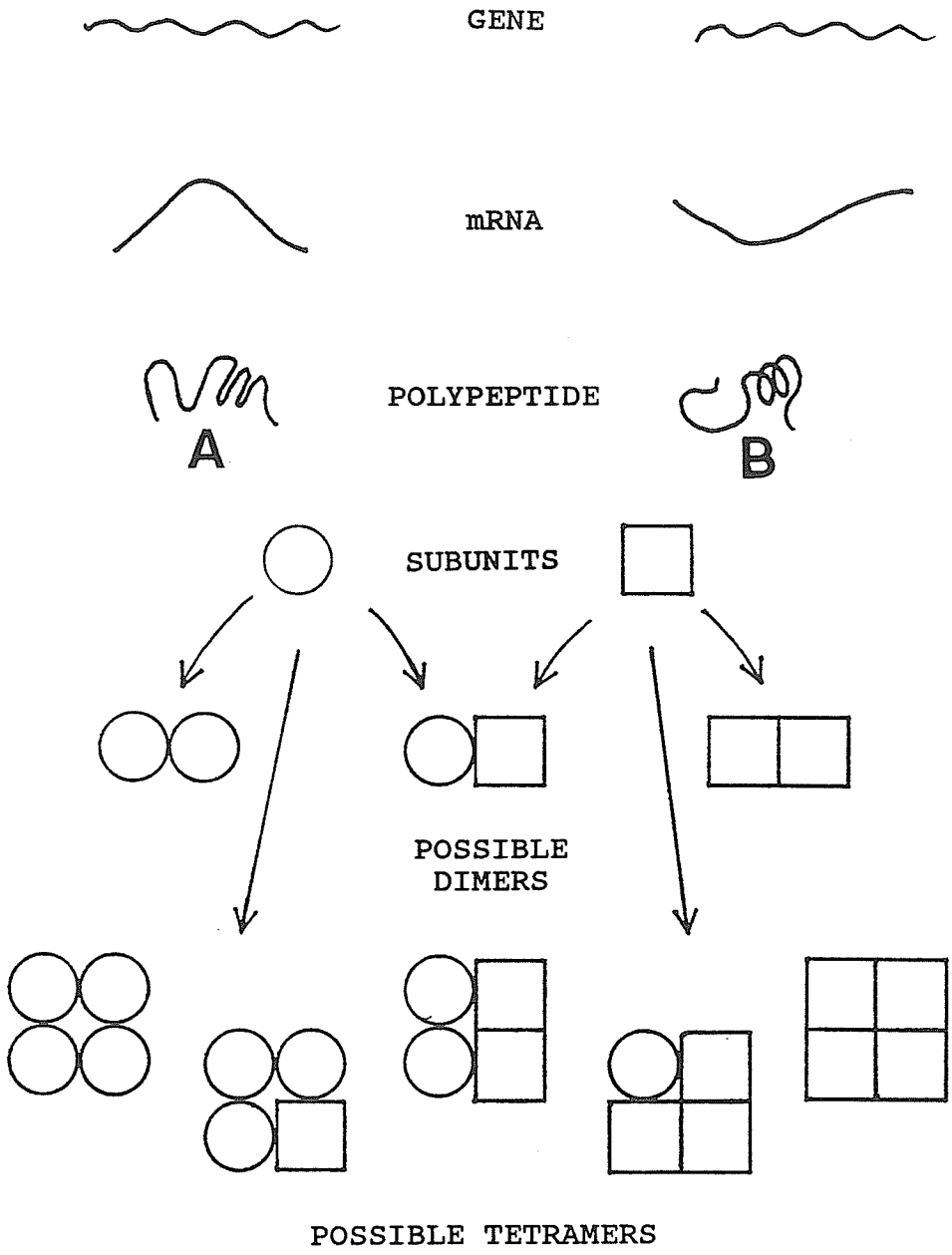

Figure 2.2. Origin of isoenzymes. Distinct genes code for polypeptides A and B which may act as monomeric isoenzymes. Polypeptides A and B may also act as the subunits for polymeric isoenzymes (allozymes).

Although members of a set of isoenzymes catalyze the same or very similar reactions, they do differ in other functional characteristics. For example, they can differ in size or charge as Markert and Moller (1959) demonstrated electrophoretically. It has also been shown that isoenzymes can differ in specific activity (Pesce et al., 1967), susceptibility to inhibitors (Plagemann et al., 1960), substrate specificity (Rosalki and Wilkinson, 1960), and in stability (Harris, 1980). Moss (1982) acknowledges that isoenzymes can have quite different characteristics and that distinguishing between isoenzymes and completely different enzymes can be difficult.

2.3.2 Cultivar Identification.

Schwartz (1960), working with maize, reported that isoenzyme electrophoretic patterns often varied among cultivars of the same species. As a result, it was recognized that electrophoresis could be a useful for plant cultivar identification. Successful isoenzyme procedures for cultivar identification have since been developed for potatoes (Desborough and Peloquin, 1968), peanuts (Cherry and Ory, 1973), barley (Fedak, 1974), and soybeans (Gorman and Kiang, 1977), as well as for many other crops.

Although others had reported the existence of isoenzymes in Phaseolus vulgaris (Robb et al., 1965; West and Garber, 1967), Bassiri and Adams (1978) were the first to investigate the possibility of using enzyme electrophoretic patterns for bean cultivar identification. Using starch gel electrophoresis, they analyzed the leaf, stem, and root tissue of 34 bean cultivars. The cultivars analyzed were widely representative of Phaseolus vulgaris and included lines from the Great Northern, kidney, navy, pinto, and black groups. On the basis of leaf

and stem esterases and leaf, stem, and root peroxidases, they were able to classify the cultivars into 28 groups. While many of the cultivars were readily identifiable by morphological differences, the value of this work was that it demonstrated that enzyme electrophoresis had potential for the more difficult identification of cultivars within a specific commercial class.

Weeden (1984) did a similar study but limited the material analyzed to only white seeded snap beans. Unlike the previous study, this material was difficult to classify visually. Starch gel electrophoresis was used to analyze ten enzymes in imbibed seed, seedling leaf, hypocotyl, and root tissue. Of the 90 cultivars investigated, 52 exhibited unique combinations of electrophoretic patterns. the remaining 38 were segregated into 14 sets, resulting in a total of 66 classes of material among the 90 cultivars. Although not able to uniquely identify each cultivar, these results indicated that electrophoresis was useful for discriminating cultivars within the same commercial class.

Although Weeden (1984) developed a relatively sensitive method of cultivar identification, it was not a rapid procedure. To do 10 enzyme assays for each cultivar would be very time- consuming. The use of polyacrylamide gel, which generally gives better resolution than starch gel, might have reduced the number of enzymes needed for the same level of discrimination. Cassava cultivars have been identified on the basis of esterase isoenzymes alone when separated on polyacrylamide (Ramirez et al., 1987).

Both researchers (Bassiri and Adams, 1978; Weeden, 1984) reported that differentiating enzymes were normally found in seedling tissue

extracts. Extracts from ungerminated seeds have not given satisfactory discrimination (Hussain et al., 1986). This is probably because enzymes are relatively dormant in seed, becoming much more active upon germination.

2.4 Protein Electrophoresis

Using a general protein stain, such as Coomassie Blue, has several advantages over enzyme stains for cultivar identification. With a general protein stain, there will be many more bands present in the gel, resulting in a greater chance to observe diversity among cultivars. Secondly, the proteins may be denatured with SDS as there is no need to conserve enzyme activity. Finally, there is no need to grow out seedlings as is needed for enzyme electrophoresis.

Graham (1963) was among the first to use protein electrophoresis for cultivar identification. She demonstrated that the starch gel electrophoretic patterns produced from the acetic acid soluble proteins of wheat flour could be used to discriminate varieties of Triticum vulgare. Cultivar identification procedures have since been developed for many crops including peanuts (Tombs, 1963), peas (Hynes, 1968), and faba beans (Barratt, 1980) among others.

With respect to beans, Adriaanse et al. (1969) performed an experiment in which water soluble seed proteins were extracted and separated on starch gel under acid conditions (pH 3.1). A wide variety of cultivars was examined, including green beans, string beans, and bacon beans. It was found that even closely related cultivars of the same type of bean could often be identified. They were not, however, able to discriminate among all varieties. The effects of nitrogen

fertilization, climatic conditions, and soil properties were negligible. There were some minor, inconsistent differences in cultivars grown under different conditions, but they did not affect cultivar identification.

A study by Hussain et al. (1986) investigated field bean cultivar identification using two systems. In the first, 0.1 M acetic acid containing 1% 2-mercaptoethanol was used to extract seed proteins which were separated on polyacrylamide gel under acid conditions (pH 3.1). In the second, residual proteins that remained after sequential extraction with sodium chloride, ethanol, and acetic acid solutions, were separated using SDS-PAGE. The seven cultivars analyzed were clearly identifiable using both systems and results were reproducible. They also reported that the patterns were not affected by location or by a period of storage of six months at ambient laboratory conditions. A later study indicated that there could be significant variation within different accessions of the same cultivar, although intra-accession variation was generally nil (Hussain et al., 1988d).

No work has yet been published indicating that protein electrophoresis is capable of discriminating among cultivars of the same commercial or color class as has been reported with enzyme electrophoresis (Weeden, 1984). A protein method would be preferable because of its convenience and speed. A single protein gel could give more information than several enzyme gel and would be no more difficult to produce.

2.5 Bean Hardening

Electrophoretic cultivar identification techniques have been of most interest to plant breeders who must be able to characterize their germplasm collections. In the past, the development of new plant varieties has been driven by agronomic considerations, such as the desire for increased yield or disease resistance. There is, however, an increasing amount of work being done using plant breeding to address nutritional considerations. The development of a low-erucic acid/low glucosinolate rapeseed, now known as canola, is an example of a successful genetic solution to a nutritional problem.

With respect to beans, one of the most pressing nutritional problems is the hardening that develops during storage under adverse conditions (Stanley and Aguilera, 1985). In addition to the considerable economic losses (Gonzalez de Mejia, 1982), hardened beans require substantially longer cooking times, increasing the susceptibility of nutrients to thermal destruction. The Protein Efficiency Ratio (PER) has also been reported to decrease with hardness (Antunes and Sgarbieri, 1979).

There are two phenomena that are associated with bean hardening. "Hardshell" refers to beans that do not imbibe sufficient water during the soaking step, that is, the seed coat acts as a barrier to water uptake. Hardshell is not a great problem in Guatemala as beans are normally cooked without a prior soaking step. The "hard-to-cook" (HTC) phenomenon refers to soaked legumes that do not soften during cooking, even though they have imbibed sufficient water. In this case, the seed coat does not act as a barrier. Because it is such a serious problem in Central America, this thesis will deal exclusively with HTC phenomenon.

2.5.1 Factors Affecting Bean Hardening

Four factors are known to affect the development of the HTC defect during storage: time, relative humidity, temperature, and the moisture content of the seed. Moisture content and temperature are considered the most important (Elias, 1982). Relative humidity is a factor in that it affects the seed moisture and time is a factor only when temperature and moisture levels are above certain levels. Pinto beans stored at 10% moisture and 8°C did not require increased cooking time even after two years of storage (Burr et al., 1968). Aguilera and Hohlberg (1988) suggests that beans having a moisture content below 8-9%, or stored at a temperature below 17°C, will not harden significantly, independent of the other variable. Data collected from the Peten region in Guatemala indicates that beans are routinely stored with moisture levels above 15% and at temperatures above 25°C, making them highly susceptible to the HTC problem (Garcia and Elias, 1987).

In addition to the above four factors, there is growing evidence that genetics play a role in the development of the HTC defect. Several workers have reported that the hardness of freshly harvested beans is significantly affected by genotype (Ghaderi et al., 1984; Hosfield et al., 1984; Proctor and Watts, 1987). There is also evidence that there are varietal differences in the rate of hardness increase during storage. Burr et al. (1968) stored pinto, Sanilac, and Lima beans at 21°C and 16% moisture for 12 months. Using a modified Mattson Cooker, Sanilac and Lima beans exhibited greater hardening rates than pinto beans. Vindiola et al. (1986) placed navy, pinto, brown, red, and black beans under storage at 45°C and 100% R.H. Hardness was tested by squeezing single cooked beans between the forefinger and thumb. They

reported that there were differences in the hardening rates among the cultivars. White beans hardened very quickly while pinto and brown beans hardened at a slower rate. Michaels and Stanley (1988) established a broad sense heritability value of 0.53 for the HTC defect, a value they considered high enough to indicate significant genetic control.

Although there is general agreement on the factors that influence bean hardening, there is no consensus as to the physical or chemical mechanisms that are responsible for the development of the HTC condition. Several theories have been put forward: (1) Protein-phytic acid complex: Phytic acid is known to react with proteins and it has been suggested that the resultant insoluble complex may be responsible for the hardening of bean texture (Lolas and Markakis, 1975; Elias, 1982). (2) Starch retrogradation: Hellendoorn (1979) proposed that hardening may be caused by a similar physical process as that of the retrogradation of starch, that is, amylose molecules coming to associate together more closely, producing a tough starch matrix, resulting in a harder bean. (3) Starch gelatinization: Using scanning electron microscopy, Aguilera and Steinsapir (1985) reported that hard beans had more ungelatinized starch granules than did soft beans after the same amount of cooking time. It is postulated that the starch of HTC beans has some sort of resistance to gelatinization. (4) Lipid deterioration: Muneta (1964) postulated that lipid oxidation and polymerization may cause changes in water permeability which in turn would affect cooking time. More recently, it has been shown that hydrolysis of phosphatidylcholine and phosphatidylethanolamine occur in soybean during storage (Priestly and Leopold, 1979). This would lead to

cell membrane degradation and increased solute leakage resulting in decreased cell wall turgidity and decreased cell wall separation on cooking. Solute leakage would also physically bring together enzymes and substrates involved in the hardening mechanism. (5) Changes in the middle lamella of cotyledon cells: There is considerable microscopic evidence indicating that hard beans show less cell separation upon cooking than do soft beans (Hincks and Stanley, 1986). It is believed that cross-linking of components in the middle lamella of HTC beans is responsible for hardness.

Of the five proposed mechanisms, changes in the middle lamella of the cotyledon cells seem to enjoy widest support. The middle lamella refers to the region where the walls of plant cells abut and are held together. Pectin is one of the principle components of this area. Upon cooking, the pectin is rapidly degraded causing the cells to separate from one another, producing the soft texture associated with cooked beans. Any changes in the middle lamella that would cause the cells to hold together more tightly would result in beans that are HTC. To support this theory, there is substantial evidence that relatively soluble pectin becomes cross-linked with divalent cations to produce an insoluble pectate complex that cements cell walls together. There is also recent evidence that lignin-like complexes form in the middle lamella where they would prevent cell separation.

2.5.2 Pectate Formation

Phytic acid (PA: myo-inositol hexaphosphoric acid) is the major reservoir of phosphorous in the seed (Lolas and Markakis, 1975). It has been implicated in pectate formation as the main supplier of the

divalent cations needed for the cross-linking of pectin. Phytic acid has strong chelating potential and binds ionically to Ca^{2+} and Mg^{2+}, as well as Fe^{3+} and Zn^{2+}, making them unavailable biologically (Reddy et al., 1988). It is proposed that the enzyme phytase hydrolyzes PA into its constituent inositol and inorganic phosphorous and releases the Ca^{2+} and Mg^{2+}. The divalent cations are then free to combine with the pectin in the middle lamella to form an insoluble Ca^{2+}-Mg^{2+} pectate complex (Figure 2.3).

Several researchers have reported correlations between PA levels in the seed and cooked legume texture. Hincks and Stanley (1986) reported that black beans stored under adverse conditions (30°C/85% relative humidity) exhibited a dramatic increase in cooked hardness corresponding to a significant decrease in PA levels. Kon and Sanshuck (1981) added PA and EDTA to soaking water and produced significantly shorter cooking times. The added PA and EDTA were thought to chelate any free divalent cations, making them unavailable for pectate formation. Bhatty and Slinkard (1989) found a significant relationship ($p<0.01$) between phytic acid content and shear force values of cooked lentils. They concluded that PA was largely, if not entirely responsible for the observed differences in the cooking quality of lentil.

Other researchers have found no correlation between PA and cooking quality. Proctor and Watts (1987) analyzed three navy bean cultivars in three locations. They observed that although cooking times varied significantly, no relationship could be established between phytate content and cooking times. Studies with lentils have likewise shown no relationship between PA and cooking quality (Wassimi et al., 1978).

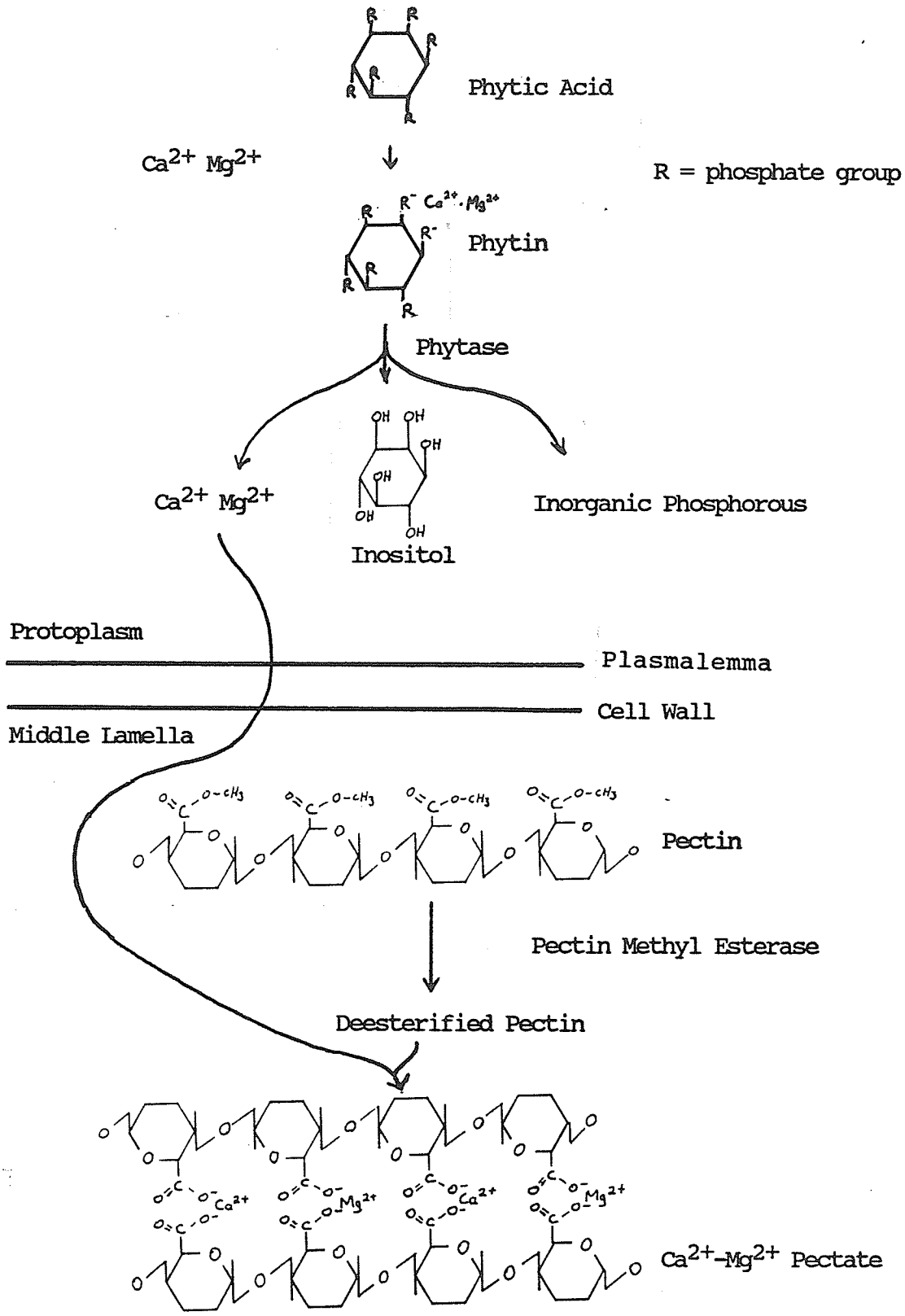

Figure 2.3 Postulated pectate formation mechanism involving phytase and pectin methyl esterase.

There is also evidence supporting the role of phytase in the hardening process. The activity of phytase has been shown to increase with temperature which would correspondingly promote the development of the HTC condition (Lolas and Markakis, 1977). Vindiola et al. (1986) soaked HTC beans in 0.05 M sodium fluoride, a strong phytase inhibitor, and observed a dramatic improvement in cookability over beans soaked without fluoride, indicating that hardness could be almost completely prevented by the inhibition of phytase.

The role of pectin methyl esterase has not been extensively investigated. It has been suggested that pectin methyl esterase activity would increase the number of sites on the pectin molecule capable of binding Ca^{2+} and Mg^{2+}. Jones and Boulter (1983b) reported that pectin of fresh black beans was 51% esterified. This value dropped to 15% after storage at 34°C and 75% relative humidity for 6 months suggesting that pectin methyl esterase was active in the stored beans.

Jones and Boulter (1983b) reported that along with the deesterification of black bean pectin, there was a corresponding increase in cell wall Mg^{2+} from 210 ug/g (dwb) to 354 ug/g and in cell wall Ca^{2+} from 120 ug/g to 173 ug/g. The correlation between Mg^{2+} and percentage insoluble pectin was $r=+0.93$ ($p<0.001$) (Jones and Boulter, 1983a). Using kidney beans, Moscoso et al. (1984) confirmed that insoluble pectin levels increased during storage at high temperature and moisture levels. Some studies have failed to detect changes in pectic substances as beans harden (Kon, 1968), but in general, the evidence supports a conversion of pectin to Ca^{2+}-Mg^{2+} pectate in HTC beans.

The mechanism for the transportation of Ca^{2+} and Mg^{2+} from the protoplasm to the middle lamella remains unexplained. Dissolved material can pass through the cell wall quite easily but the plasmalemma would be impermeable to divalent cations. It has been suggested that phospholipid hydrolysis may degrade cell membranes sufficiently to allow Ca^{2+} and Mg^{2+} to move into the middle lamella (Priestly and Leopold, 1979).

2.5.3 Lignin Formation

Lignin is an important constituent of plant cell walls and can also be found in the middle lamella (Esau, 1965). It has been suggested that an increase in cell wall adhesion may be due to an increase in the level of lignin or lignin-like compounds in the middle lamella/cell wall region (Hincks and Stanley, 1987). As the lignin complex develops and cell bonding increases, cell separation during cooking would be more difficult. The proposed mechanism suggests that the aromatic amino acids resulting from proteolysis serve as precursors for the formation of lignin by the actions of peroxidase and polyphenol oxidase (Figure 2.4).

The existence of protease activity in the bean seed has been well established (Nielsen, 1988). Ching and Schoolcraft (1968) suggested that protease activity in stored seeds is related to hydration and temperature, which means that protease activity would increase with storage temperature and moisture. Ultracentrifuge patterns have shown that high storage temperatures and humidities accelerate the reduction of high molecular weight protein fractions and the related formation of smaller fractions (Stanley and Aguilera, 1985). Correspondingly, both gel filtration and electrophoresis have shown that the degradation of

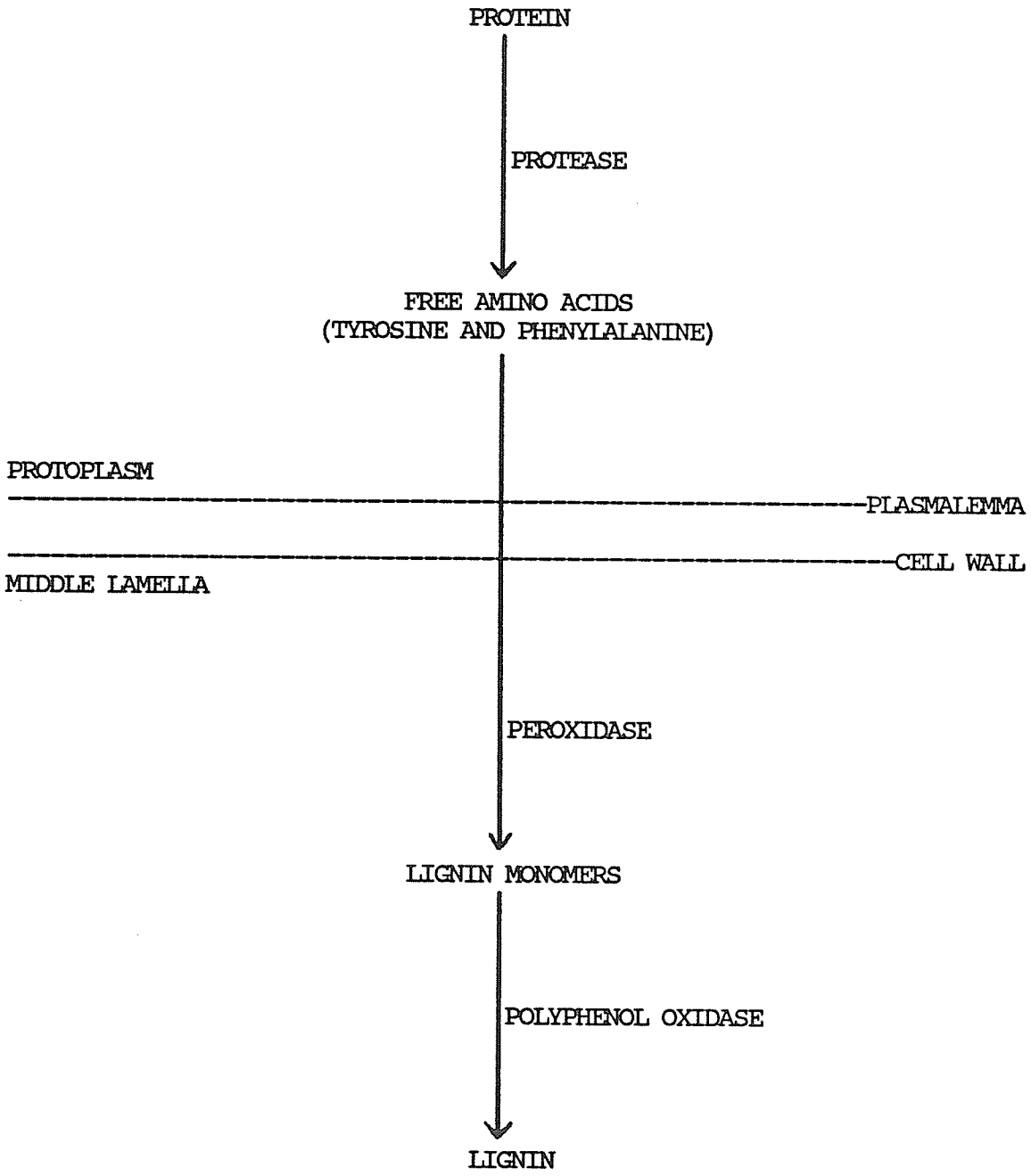

Figure 2.4 Postulated lignin formation mechanism involving protease, peroxidase, and polyphenol oxidase (Hincks, 1985).

seed proteins accelerates under adverse storage conditions, presumably due to increased protease activity (Hohlberg and Stanley, 1987; Hussain et al., 1989c). Hohlberg and Stanley (1987) reported protease activity in black bean seed but there were no significant differences among storage times or storage conditions.

Goodwin and Mercer (1972) have given evidence that free aromatic amino acids, such as tyrosine and phenylalanine, serve as precursors to lignin monomers. Hincks (1985) proposed that cotyledon peroxidase and polyphenol oxidase work in conjunction to produce a lignin-like substance in the middle lamella. Evidence from Whitmore (1978) confirms that peroxidase is involved in lignin formation. Although peroxidase activity occurs in the bean cotyledon (Hohlberg and Stanley, 1987), the data concerning polyphenol oxidase is contradictory. Elias (1982) reported significant levels of polyphenol oxidase activity while Hohlberg and Stanley (1987) observed no activity at all. Results are complicated by the inclusion of the seed coat which has a high phenolic content.

The literature is also contradictory with regard to the actual existence of lignin in hard beans. Molina et al. (1976) established a correlation coefficient of 0.91 for the relationship between lignified protein content and cooked bean texture. Hincks and Stanley (1986) used microscopy to show the build-up of a lignin-like compound in the middle lamella during storage. Other researchers, however, have reported no increase in lignin in beans that hardened significantly during storage (Srisuma et al., 1989; Rozo et al., 1990). The literature, therefore, remains ambivalent about lignin formation as an explanation for bean hardening although most recent studies tend to deemphasize its role.

2.6 Electrophoresis to Predict the HTC Defect

While the effectiveness of electrophoresis for cultivar identification has been well documented, there are also studies showing that electrophoretic patterns can be correlated to the functional properties of the cultivars. Using 71 wheat cultivars of diverse quality, Campbell et al. (1987) reported very highly significant correlations between specific protein bands produced by SDS-PAGE and both dough extension and dough resistance. Hussain et al. (1988d) suggested that electrophoretic patterns may be correlated with seed shape and seed coat luster of field bean cultivars.

Because both pectate formation and lignin formation are thought to be enzymatic processes, isoenzyme electrophoretic patterns may have the potential to identify HTC cultivars. It is postulated that HTC cultivars contain unique isoenzymes that are particularly active in the hardening process. It may be that two cultivars exhibit the same level of overall phytase activity, but that the HTC cultivar contains a phytase isoenzyme with an especially broad optimal temperature curve which allows it to hydrolyze PA under a variety of conditions while the resistant cultivar shows equivalent activity only within a narrow temperature range. On the other hand, it may be that HTC varieties contain an esterase isoenzyme that is particularly active toward pectin while resistant varieties have esterases that are more general in nature.

The identification of an electrophoretic pattern unique to HTC cultivars would not only give information as to the biochemical mechanisms involved in hardening, but it would also assist plant breeders in screening newly developed lines with regard to their

cooking quality. Rather than having to grow sufficient sample to do a four to six month storage study, cooking quality could be predicted by electrophoresis in just a few days using only two or three seeds.

All the enzymes considered to be involved in pectate and lignin formation have been shown to exist in multiple molecular forms in beans (Table 2.1). The assays that have been used to identify esterases and acid phosphatases identify broad families of enzymes which include pectin methyl esterase and phytase.

TABLE 2.1

Multiple molecular forms of enzymes involved in postulated hardening mechanisms reported in bean tissues.

Enzyme	Tissues Exhibiting Multiple Molecular Forms	Reference
Acid phosphatase	leaf, stem, root	Bassiri and Adams (1978)
	leaf, hypocotyl, root	Weeden (1984)
Esterase	leaf, stem	Bassiri and Adams (1978)
	leaf, hypocotyl	Weeden (1984)
	leaf	West and Garber (1967)
Peptidase[1]	seed	Weeden (1984)
Peroxidase	leaf, stem, root	Bassiri and Adams (1978)
	root	Weeden (1984)
Polyphenol oxidase[2]	leaf	Robb et al. (1965)
	stem/leaf, seed[3]	Zenin and Park (1978)

[1] Substrate: L-phenylalanyl proline
[2] Substrate: L-DOPA
[3] Velvet bean (Stizolobium deeringianum)

3. Materials and Methods: Effect of Genotype, Location, and the Development of the Hard-to-Cook Defect on Bean Electrophoretic Patterns

3.1 Materials

Six cultivars of black beans (Phaseolus vulgaris L.) were obtained from the Institute of Nutrition of Central America and Panama (INCAP) in Guatemala. Two of the varieties were native to Guatemala while the other four were improved cultivars (Figure 3.1). The seeds of all the cultivars were almost identical in appearance with the exception of San Martin which had slightly larger seeds. Seedlings were also identical in appearance making visual cultivar identification impossible.

It is commonly believed that improved varieties are harder than the native, or "criollo" varieties. This was confirmed by the work of Rios-Sierra (1988). Rios-Sierra (1988) also reported cooked hardness values for the improved cultivars Tamazulapa, Quetzal, and Ostua, cultivars which were analyzed in this study as well. Her results indicated that, when kept under the same storage conditions (4°C), Tamazulapa was the hardest of the three cultivars, followed by Ostua and then Quetzal.

Each cultivar was grown in three locations in Guatemala during the fall of 1988 (Table 3.1). The samples grown at Jutiapa and in the INCAP greenhouse were planted at an early and a late date so that each cultivar had two harvest dates. After harvest, the moisture content of the beans was established using a Dole 400-B Moisture Tester[1]. The samples were kept in paper bags and stored under laboratory conditions (23°C).

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[1] James Dole Corporation, 1400-T Industrial Way, Redwood CA 94063.

NATIVE LINES:

*PECHO AMARILLO - Native material developed by local farmers in region of Quesada, Jutiapa in Guatemala.

*SAN MARTIN - Native material of San Martin, Chimaltenango in Guatemala. Selected from 800 plants with certain characteristics.

IMPROVED LINES:

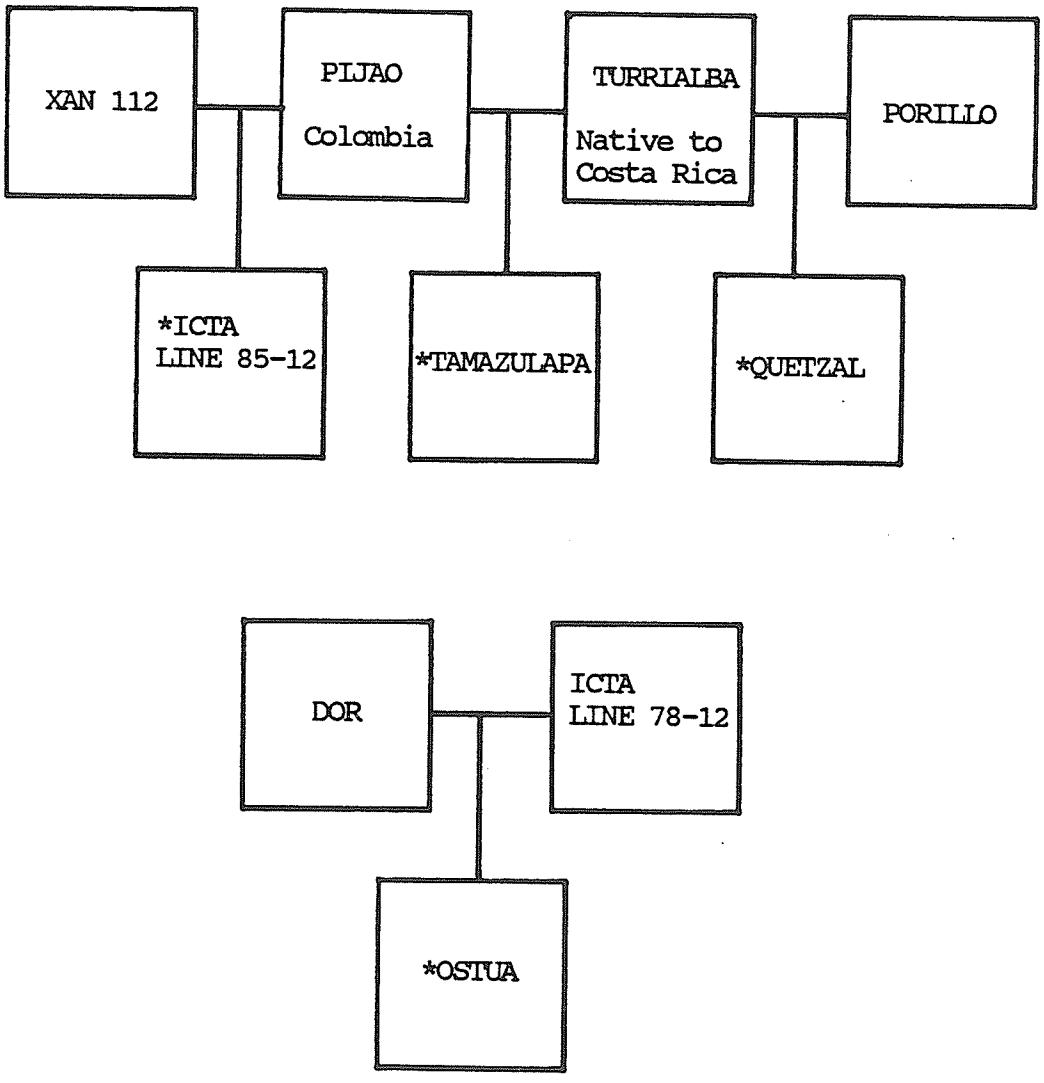

Figure 3.1 Pedigrees and descriptions of cultivars. Names with asterisks indicate cultivars used.

TABLE 3.1

Locations where the samples were grown and their harvest dates.

Jutiapa	906 meters above sea level. Hot, dry climate. First harvest: September 05, 1988. Second harvest: January 26, 1989.
INCAP Experimental Farm	1560 meters above sea level. Situated near Guatemala City Moderate, dry climate. Harvest: November 14, 1988
INCAP Greenhouse	1499 meters above sea level. Situated in Guatemala City. Moderate, controlled climate. First harvest: September 26, 1988. Second harvest: March 03, 1989.

3.2 Cooking Quality

The cooking quality of freshly harvested beans was determined for all samples. The samples from the first harvest at Jutiapa and from the second harvest in the greenhouse were analyzed again after approximately six months of storage at laboratory conditions.

Cooking quality was determined using the Mattson Bean Cooker equipped with 90 g weights. The plunger tips were 1 mm in diameter. The beans were placed in distilled water and quickly brought to a boil. Cooking continued until all the plungers dropped. Five seeds from each

cultivar were analyzed and cooking time was recorded as the mean time it took for the five plungers to drop. All cultivars were analyzed in triplicate.

3.3 Protein Determination

Nitrogen content of the seed with the coat removed was measured by the Micro-Kjeldahl Method using a Kjeltech Analyzer from Tecator[2] (Model Auto 1030 Analyzer) (AOAC, 1975). The protein conversion factor was 6.25.

All samples were then dried before being ground for protein analysis, therefore, protein values are considered to be on a dry weight basis.

3.4 Electrophoretic Procedures

3.4.1 Electrophoresis of Enzymes

3.4.1.1 Sample Preparation

The procedure for sample preparation and the electrophoresis of enzyme extracts is based on the methods of Hussain et at. (1988b) with some minor modifications.

For seedling analysis, seeds of each cultivar were germinated between moist paper towels. When the emerging hypocotyl was 2-3 cm long, the seed was planted in vermiculite and allowed to grow for 12 days under greenhouse conditions. On the twelfth day, the seedling was separated into its roots, primary leaves, and stems. The stems were

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[2] Tecator AB, P.O. Box 70, S-26301, Hoganas, Sweden.

considered to be the region between the cotyledon leaves and primary leaves. The tissue was kept frozen until analyzed. For the analysis of cotyledon enzymes, three beans from each cultivar were soaked overnight in distilled water and the seed coats removed the following morning.

Samples were crushed thoroughly in cold extraction buffer using a glass rod as a pestle. For starch gel electrophoresis, enough extraction buffer was used to produce a pasty consistency. For polyacrylamide gel electrophoresis, slightly more buffer was used so that a thin layer of supernatant would form when the sample was allowed to stand for several minutes. Esterase band quality was improved by centrifuging the crushed sample for 20 minutes at 15 600 X G at 5°C in an Eppendorf 5414 micro centrifuge[3]. The band quality of the other enzymes was not improved by centrifugation. The extracts were kept on ice until applied to the gel. The extraction buffer used was a 0.1 M tris-maleate solution, pH 7.4, containing 20% glycerol, 5% soluble PVP-40, 0.5% Triton X-100, and 14 mM dithiothreitol. The Triton X-100 and dithiothreitol were added just before use.

3.4.1.2 Starch Gel Electrophoresis

A 10.6% starch gel was prepared using a tris-citrate/ lithium borate mixture (Table 3.2). One hour prior to use, the gel was refrigerated. Whatman No. 3 filter paper was used to make 5 mm X 10 mm wicks which were dipped into the crushed samples to absorb the extract. The wicks were then blotted with paper towels to remove the excess moisture and inserted into a slit in the gel. Wicks dipped in 1.0%

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[3] Eppendorf Geratebau, Netheler and Hinz GmbH., Postfach 65 06 70, 2000 Hamburg 65, GDR.

bromophenol blue were inserted at either end of the gel to act as markers.

Moist pieces of cloth were used make contact between the electrode buffer and gel. The run was initiated with 10 mA of current until the bromophenol dye had clearly moved into the gel, normally requiring 15 minutes. The wicks were then removed, the upper cooling plate put in place, and the current increased to 20 mA for 30 minutes, then to 30 mA for another 30 minutes, and finally to 35 mA until the end of the run. The run was terminated when the bromophenol front had travelled 10 cm from the origin. Ice water was circulated through the apparatus during the entire procedure. After electrophoresis, the gels were removed and sliced into 1.5 mm thick slabs for staining. Starch gels gave best results for acid phosphatase, peptidase, peroxidase, and phosphoglucosoisomerase stains.

3.4.1.3 Polyacrylamide Gel Electrophoresis

A 10% polyacrylamide gel was used to separate peroxidase and polyphenol oxidase isoenzymes while a 12% gel was used for esterase and glutamate oxaloacetate transaminase. A 3% percent stacking gel was used in all cases. The optimal amount of sample to be applied to the gel was determined experimentally. Amounts ranged from 12 ul for leaf esterase to 35 ul for root esterase. A marker solution (0.01% bromophenol blue in extraction buffer) was applied to indicate the location of the front during the run. Electrophoresis was initiated at 50 V and gradually increased (50 V every 30 minutes) to 250 V. Total time of electrophoresis was approximately 6 hours. Ice water was circulated

TABLE 3.2

Electrophoresis buffer systems for isoenzyme separation.

Starch Gel Electrophoresis:	
Electrode Buffer:	Lithium borate pH 8.1
	0.03 M lithium hydroxide, 0.19 M boric acid
Gel Buffer:	9 parts tris citrate buffer pH 8.4 1 part lithium borate buffer pH 8.1
	Tris citrate buffer: 0.05 M tris, 0.008 M citric acid
Polyacrylamide Gel Electrophoresis:	
Electrode Buffer:	0.1 M tris borate pH 9.0
Stacking Gel Buffer:	0.125 M tris-HCl pH 6.8
Separating Gel Buffer:	0.325 M tris-HCL pH 8.8

during the entire procedure. All polyacrylamide electrophoresis was performed using the LKB 2000 electrophoresis unit.

3.4.1.4 Enzyme Stains

Acid phosphatase:

Sodium naphthyl acid phosphate (50 mg) and Fast Garnet GBC Salt (50 mg) were dissolved in 50 ml of 0.1 M sodium acetate, pH 5.0, and filtered. The gel was incubated in the stain solution for 1-2 hours at 37°C, rinsed with water and fixed overnight in 50% ethanol, 1% acetic acid (Hussain et al., 1988b).

Esterase:

A sodium phosphate buffer, pH 7.0, was prepared by mixing 60 ml of 0.15 M sodium phosphate monobasic and 40 ml of 0.15 M sodium phosphate dibasic. The gel was placed in the buffer for 10 minutes at 37°C. A small amount of acetone (5 ml) containing 50 mg of α-naphthyl acetate and 25 mg β-naphthyl acetate was then mixed with approximately 10 ml of water and added to the buffer. This was incubated at 37°C for an additional 15 minutes. Fast Blue RR (75 mg) was dissolved in 20 ml of water, filtered, and added to the buffer. After one hour of staining, the gel was washed with water and fixed overnight in 50% ethanol, 1% acetic acid (Hussain et al., 1987).

Glutamate Oxaloacetate Transaminase:

A solution was prepared by dissolving 200 mg of L-aspartic acid and 100 mg of α-ketoglutaric acid in 75 ml of 0.1 M tris-HCl, pH 8.0. Just prior to use, 6 mg of pyridoxal-5-phosphate and 75 mg of Fast Blue BB salt were added. The solution was filtered and the gel incubated one hour at room temperature in the dark. It was fixed overnight in 50% ethanol, 1% acetic acid (Hussain et al., 1988b).

Peptidase:

Solution A consisted of 10 mg L-glycyl-L-leucine, 10 mg L-phenylalanyl-L-proline, 0.75 mg snake venom (Sigma A5147), 2.45 mg peroxidase (Sigma P8125), and 40 mg $MnCl_2 \cdot 4H_2O$ in 35 ml 0.02 M disodium phosphate, pH 7.5. Solution B consisted of 2% agar in 0.02 M disodium phosphate, pH 7.5, which was boiled and then cooled to lukewarm. Solutions A and B were mixed along with 15 mg of 3-amino-9-ethylcarbazol dissolved in 1 ml of N,N-dimethyl formamide. The mixture was poured over the gel and allowed to solidify. The gel was covered

with plastic wrap and allowed to incubate overnight at 37°C. The starch gel with agar overlay was fixed in 50% ethanol, 1% acetic acid. This procedure is a modification of Harris and Hopkinson (1976).

Peroxidase:

A mixture was prepared containing 3-amino-9-ethylcarbazol (60 mg) dissolved in 5 ml of N,N-dimethyl formamide and 75 ml 0.2 M sodium acetate, pH 5.0. The gel was placed in this mixture and 3 drops of 30% H_2O_2 added. Incubation time was one hour at room temperature. The gel was fixed 50% ethanol, 1% acetic acid overnight (Hussain et al., 1988b).

Phosphoglucosoisomerase:

A solution was prepared by dissolving 10 mg fructose-6-phosphate, 6 mg NADP, 6 mg 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), 20 mg $MgCl_2 \cdot 6H_2O$, a trace of glucose-6-phosphate dehydrogenase (Sigma G7750), and a trace of phenazine methosulphate in 75 ml of 0.1 M tris-HCl, pH 8.0. The gel was incubated one hour in the dark at room temperature and fixed overnight in 50% ethanol, 1% acetic acid (Hussain et al., 1988b).

Polyphenol oxidase:

The gel was incubated for 2 hours at 37°C in 75 ml of 0.1 M potassium diphosphate monobasic, pH 6.5, containing 125 mg of L-DOPA. It was fixed overnight in 50% ethanol, 1% acetic acid (Choi et al., 1987).

3.4.2 Electrophoresis of Seed Proteins

3.4.2.1 Acid-Polyacrylamide Gel Electrophoresis

3.4.2.1.1 Sample Preparation

Seeds were soaked overnight in distilled water to facilitate the removal of the seed coats. The seed coats were removed the following day and the beans allowed to dry overnight in a convection oven at 37°C. The dried beans were milled with a Wiley Mill[4] through a 40 mesh screen. Extractions were performed by mixing 50 mg of ground sample with 200 ul of extraction solution. The two extraction solutions used were 5 M acetic acid and 0.1 M acetic acid containing 1.0% 2-mercaptoethanol. The mixtures were vortexed for 3 minutes and allowed to incubate at 40°C for one hour. After centrifugation at 15 600 X G for 20 minutes at room temperature, the supernatant was mixed (1:1) with a solution of 40% sucrose and 0.5% methyl green in aluminum lactate buffer, pH 3.1. Twenty-five ul of the resulting solution was used for electrophoresis.

3.4.2.1.2 Electrophoresis

Electrophoresis was carried out on an apparatus described by Sapirstein and Bushuk (1985). The samples were separated by a modification of the method described by Hussain et al. (1989b) (Table 3.3). Current was set at 20 mA for the first 30 minutes and increased to 30 mA for the remainder of the run. Electrophoresis was terminated when the green dye front reached the bottom of the gel.

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[4] Arthur H. Thomas Co., Philadelphia PA.

TABLE 3.3

Electrophoresis buffer systems for protein separation.

Acid-Polyacrylamide Gel Electrophoresis:

Gel mixture:

acrylamide	8 g
N,N-methylene bisacrylamide	0.4 g
ascorbic acid	0.1 g
ferrous sulphate	0.0015 g
aluminum lactate buffer, pH 3.1	80 ml
sucrose	5 g
hydrogen peroxide 3%	150 ul

Electrode buffer:

2.5 g aluminum lactate (Fluka Chemical Corp. Hauppage NY) in 1 L water, adjusted to pH 3.1 with lactic acid.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis:

Residual protein extraction buffer:

1:1 mixture of Solutions A and B.

Solution A: 0.05 M tris-HCl, pH 8.3

Solution B:

0.5 M tris-HCl, pH 6.8	12.5 ml
10% SDS	20.0 ml
Glycerol	10.0 ml
Mercaptoethanol	5.0 ml
Distilled water	2.5 ml

Dissociating buffer:

0.625 M tris-HCl, pH 6.8, containing 40% sucrose, 2% SDS, 1% mercaptoethanol, 2 mM EDTA, and 0.01% bromophenol blue (Brown et al., 1982).

Stacking gel buffer:

0.125 M tris-HCl, pH 6.8, 0.1% SDS.

Separating gel buffer:

0.625 M tris-HCl, pH 8.8, 0.1% SDS.

Electrode buffer:

0.025 M tris, 0.192 M glycine, adjusted to pH 8.3 with HCl, 1% SDS.

The gels were stained overnight in a filtered solution of 0.2 g Coomassie Blue R-250 (dissolved in 10 ml of 95% ethanol) in 240 ml of 12% trichloroacetic. The stained gels were destained with 12% trichloroacetic acid (Sapirstein and Bushuk, 1985).

The relative mobility (Rm) of protein bands was determined by assigning an arbitrary value of 100 to the fastest migrating band present in all samples. The Rm of specific bands was determined by dividing the migration distance of the band by the migration distance of the reference band and multiplying by 100.

3.4.2.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

3.4.2.2.1 Sample Preparation

A sequential extraction was performed using sodium chloride, ethanol, and acetic acid. The procedure was started by mixing 200 mg ground cotyledon with 2.0 ml of 0.4 M NaCl. The mixture was vortexed for 3 minutes and allowed to stand for one hour at room temperature. The mixture was then centrifuged at 27 000 X G for 20 minutes at 5°C. The supernatant was removed and the procedure repeated with 2.0 ml of 70% ethanol and then with 2.0 ml of 0.1 M acetic acid. Just prior to electrophoresis, the clear extracts were mixed (1:1) with the dissociating buffer, placed in a boiling water bath for 5 minutes, and then cooled quickly in ice water.

For the extraction of the residual proteins, the remaining pellet was vortexed for 3 minutes with 800 ul of residual protein extraction buffer. The mixture was placed in a boiling water bath for 5 minutes,

allowed to cool, and centrifuged as above. Best results were obtained by applying 15 ul of the dissociated NaCl extraction and 20 ul of the residual protein extraction to a 12% gel. No bands were produced from the ethanol and acetic acid extractions.

Because the ethanol and acetic acid fractions did not produce results from the sequential extraction, fresh samples were extracted directly with the two solvents. The same extraction procedure was used except that 100 mg of ground sample was mixed with 400 ul of solvent. Although the direct ethanol extraction still did not produce any bands, good results were obtained when 18 ul of the dissociated acetic acid extraction was applied to a 14% gel.

3.4.2.2.2 Electrophoresis

SDS-PAGE was carried out on an LKB 2001 electrophoresis unit according to the procedure of Laemmli (1970). Separating gel concentration was 12% for the separation of NaCl soluble and residual proteins. Because acetic acid soluble proteins were smaller, a 14% gel was used for them.

Voltage was kept at 50 until the tracking dye had moved into the separating gel. Voltage was then increased by 50 every 30 minutes up to 250 V. The current was limited to a maximum of 70 mA. When the tracking dye reached the bottom of the gel, the slabs were removed and stained overnight in 0.25% Coomassie Blue R-250 in 50% methanol, 1% acetic acid. Gels were destained with a methanol:acetic acid:water (6:1:14 v/v/v) solution until the background was clear.

4. Results and Discussion: Effect of Genotype, Location, and the Development of the Hard-to-Cook Defect on Bean Electrophoretic Patterns

4.1 Cotyledon Protein Content

For the six cultivars grown in three locations, analysis of variance indicated that there was no overall cultivar effect nor was there an overall location effect on the percentage of protein in the cotyledon (Table 4.1). The mean protein content for each cultivar ranged from 22.89% for Ostua to 24.33% for Pecho Amarillo (Table 4.2). With regard to location, the mean protein content for the beans grown at Jutiapa and at the Farm were similar at 23.46% and 22.67% respectively. Beans grown in the greenhouse were somewhat higher at 25.16%. None of the differences between means were significant.

There was however, a significant interaction between cultivar and location, indicating that cultivar differences were not consistent in ranking from location to location. For example, Tamazulapa, Quetzal, and Pecho Amarillo exhibited least protein content when grown at the Farm, while Line 85-12 showed the least protein percentage when grown at Jutiapa (Table 4.2). Tamazulapa, Line 85-12, and Quetzal yielded highest protein in the greenhouse, while Pecho Amarillo yielded highest at Jutiapa. Location had no significant effect on cotyledon protein for the cultivars San Martin and Ostua.

The results agree fairly well with the literature. Several researchers have shown significant varietal and locational differences for protein content (Tandon et al., 1957; Lantz et al., 1958). These studies however, included beans from a wide range of colour classes. When only black bean strains were studied, no significant strain effect was observed although there was a significant interaction between

TABLE 4.1

Analysis of variance for cotyledon protein measurements.

Source	df	SS	F
Cultivar	5	9.71	0.49
Location	2	38.97	4.92
Cultivar X Location	10	207.99	5.25[1]
Error	18	71.25	
TOTAL	35	327.93	

[1] $p<0.05$ (Using the Kimball inequality)

TABLE 4.2

Cotyledon protein in six cultivars of Guatemalan black beans grown in three locations.

	Location			
Cultivar	Jutiapa	Farm	Greenhouse	Mean
Tamazulapa	24.46ab[1]	17.30a	28.04b	23.26
San Martin	25.41	23.11	24.20	24.24
Quetzal	23.99b	22.17a	25.83c	24.00
Line 85-12	19.51a	27.51b	24.60b	23.87
Ostua	20.70	24.45	23.53	22.89
Pecho Amarillo	26.70c	21.49a	24.80b	24.33
Mean	23.46	22.67	25.16	23.77

[1] Means in the same row followed by different letters are significantly different ($p<0.05$).

strain and the season during which the sample was grown (Hosfield et al., 1984). A significant interaction between location and variety was also reported by Tandon et al. (1957).

4.2 Seed Moisture

Samples grown in the greenhouse were the driest when put into storage with mean moisture levels of 10.52% and 10.99% for the first and second harvests respectively (Table 4.3). The mean moisture levels for the Jutiapa samples were 14.13% and 14.04% for the first and second harvests respectively. The samples grown on the Farm were the most moist with a mean value of 18.81%.

4.3 Initial Cooking Time

Analysis of variance for the initial cooking time showed highly significant overall cultivar and location effects (Table 4.4). The cultivar-location interaction was also significant.

With regard to overall mean cooking times for cultivars, Quetzal had the lowest cooking time at 51.0 min (Table 4.5). Pecho Amarillo and Ostua were similar at 53.7 and 54.0 min respectively. Tamazulapa and Line 85-12 were similar at 55.1 and 55.5 min respectively. San Martin was substantially higher than the other cultivars at 62.8 min.

With regard to location, beans grown at the Farm cooked most quickly at 50.0 min (Table 4.6). Both harvests from the greenhouse were similar at 53.7 and 51.6 min for the first and second harvests respectively. The longest cooking times were for samples from Jutiapa, 59.5 and 61.9 min for the first and second harvests respectively.

TABLE 4.3

Initial moisture content (%) of the samples.

Cultivar	Sample Origin				
	Jut1	Jut2	Farm	Grn1	Grn2
Tamazulapa	13.89	12.38	19.24	9.96	10.39
San Martin	14.82	12.37	18.96	11.51	11.23
Quetzal	14.64	16.69	18.48	10.73	11.35
Line 85-12	13.40	13.66	18.71	10.74	11.84
Ostua	14.21	15.49	18.19	10.58	11.09
Pecho Amarillo	13.83	13.65	19.28	9.57	10.01
Mean	14.13	14.04	18.81	10.52	10.99
S.D.	0.53	1.73	0.43	0.68	0.67

The significant interaction between location and cultivar is evident in Table 4.7 which illustrates how different cultivars responded differently in the various locations. Three cultivars, San Martin, Quetzal, and Pecho Amarillo, had their shortest cooking times when grown at the Farm, while the other three cultivars produced their shortest cooking times when grown in the greenhouse. All cultivars required the most cooking time when grown at Jutiapa with the exception of San Martin which required most cooking when grown at the Farm.

This data indicates that cultivar and location affect initial cooking time about equally. The range for the mean cooking time for the cultivars was 11.8 min, while for the origin of the beans, the range was almost identical at 11.9 min. The geographic location where the beans were grown was more important than the time of the harvest. At

TABLE 4.4

Analysis of variance for initial cooking time.

Source	df	SS	F
Cultivar	5	1188.4	12.61[1]
Origin	4	1899.6	25.20[1]
Cultivar X Origin	20	1001.3	2.66[2]
Error	60	1130.7	
TOTAL	89	5220.0	

[1] $p<0.001$ using the Kimball inequality.
[2] $p<0.01$ using the Kimball inequality.

TABLE 4.5

Mean cooking time (minutes) according to cultivar immediately after harvest using the Mattson Bean Cooker.

	Cultivar					
	QUE	PEC	OST	TAM	L85	SAN
Time (min)	51.0a[1]	53.7ab	54.0ab	55.1ab	55.5ab	62.8b
S.D.	4.7	7.7	6.6	4.6	7.8	5.9

[1] Means followed by different letters are significantly different ($p<0.05$).

TABLE 4.6

Mean cooking time (minutes) according to sample origin immediately after harvest using the Mattson Bean Cooker.

	Origin				
	FARM	GRN2	GRN1	JUT1	JUT2
Time (min)	50.0a[1]	51.6a	53.7ab	59.5bc	61.9c
S.D.	4.6	7.0	7.0	3.6	3.8

[1] Means followed by different letters are significantly different ($p<0.05$).

both Jutiapa and at the greenhouse, the time of harvest did not significantly affect the cooking time.

There was no relationship between the initial cooking time and the moisture content of the beans. Beans from Jutiapa had the longest cooking time while having an intermediate moisture level of approximately 14% (Table 4.3). Beans from the Farm had the shortest cooking time but had the highest moisture content (approximately 19%).

These results confirm what has been previously reported by Ghaderi et al. (1984) and Proctor and Watts (1987) who observed that cooking quality of freshly harvested beans was significantly affected by both location and genotype. A significant interaction between location and genotype has also been reported in the literature (Hosfield et al., 1984; Ghaderi et al., 1984). Several workers have reported that location actually plays a larger role than genotype with respect to the cooking quality of legumes (Bhatty et al., 1983; Proctor and Watts, 1987). This indicates that the development of a good-cooking genotype must be specific for the location where it will be grown since not all genotypes respond in the same manner in a given location.

TABLE 4.7

Cooking time (minutes) immediately after harvest using the Mattson Bean Cooker.

Cultivar	Origin				
	Jut1	Jut2	Farm	Grn1	Grn2
Tamazulapa	60.6b[1]	58.7b	54.9ab	50.9a	50.2a
San Martin	60.7ab	66.9b	53.3a	67.5b	65.6b
Quetzal	52.3ab	58.5b	46.5a	48.5a	49.1a
Line 85-12	60.5bc	66.5c	52.4ab	50.9a	47.4a
Ostua	62.0b	60.1b	50.3a	50.5a	47.1a
Pecho Amarillo	61.2c	60.7c	42.8a	54.0bc	50.0ab

[1] Means in the same row followed by different letters are significantly different ($p<0.05$).

4.4 Final Cooking Time

After approximately six months of storage at ambient conditions, the mean cooking time for the beans from the first harvest at Jutiapa increased 13.3 min (Table 4.8). The beans form the second harvest at the greenhouse increased in mean cooking time only 2.7 min. The difference in the increase can be related to the moisture levels of the beans from the two locations. Jutiapa beans had a mean moisture level of 14.13%, while the greenhouse beans were drier at 10.99%. In general, beans will not harden appreciably during short-term storage if moisture is kept below 14% (Aguilera and Hohlberg, 1988). For this reason, greenhouse beans remained relatively stable during storage.

For the high moisture beans, the increase in cooking time for San Martin (26.3 min) was dramatically higher than for the other cultivars and almost twice that of Line 85-12 (14.0 min), the cultivar which

exhibited the next largest increase. Ostua showed the lowest increase in cooking time (8.0 min). San Martin also had the highest moisture content of the cultivars harvested at Jutiapa (14.82%), while Line 85-12 had the lowest at 13.40% (Table 4.3). Since high moisture levels promote the development of the HTC defect, this might suggest that part of the wide difference in cooking time between San Martin and Line 85-12 was due to their different moisture levels. However, it is felt that the variation in moisture among the cultivars was too small to account for the variation observed in cooking time. Therefore, the data indicate quite clearly that San Martin and Line 85-12 are particularly susceptible to the HTC defect while Ostua seems somewhat resistant.

Varietal differences in hardening rates during storage have been well documented for Phaseolus vulgaris (Burr et al., 1968; Vindiola et al., 1986). Michaels and Stanley (1988) reported that even within the black colour class, the rate can vary substantially between cultivars. This evidence suggests there is at least some degree of genetic influence on the development of the HTC defect. It follows then, that breeding may be an effective strategy to reduce losses due to the HTC defect.

TABLE 4.8

Increase in the mean cooking time (minutes) after six months of storage at ambient conditions.

Cultivar	Origin	
	Jut1 (14.13%)[1]	Grn2 (10.99%)
Tamazulapa	9.7a[2]	0.5ab
San Martin	26.3b	-6.2a
Quetzal	11.0a	4.6b
Line 85-12	14.0a	6.5b
Ostua	8.0a	4.5b
Pecho Amarillo	11.0a	3.0b
Mean	13.3	2.7

[1] Numbers in parentheses are the mean moisture contents for the samples from that location.
[2] Means in the same column followed by different letters are significantly different ($p<0.05$).

4.5 Enzyme Electrophoretic Patterns

The isoenzymes of peptidase, peroxidase, polyphenol oxidase, acid phosphatase, esterase, phosphoglucosoisomerase, and glutamate oxaloacetate transaminase were separated by electrophoresis to investigate the relationship of their electrophoretic patterns with cultivar and location. The results are discussed in the following section.

4.5.1 Peptidase

Peptidase activity was most evident in the cotyledon where three bands were observed (Figure 4.1). Faint bands were also present in the root and in the stem extracts but no activity was noted in the leaf. The band with the R_f value of 0.38 stained the darkest of the three cotyledon bands and was present in all tissues except the leaf. No cultivar variation was noted in the peptidase patterns.

Most proteolytic activity would be expected to occur in the seed relative to the other parts of the plant. Seed proteins are broken down during germination to provide free amino acids necessary for plant growth. In order for this to happen, a complement of proteases and peptidases would be needed to hydrolyze the seed proteins. This was reflected in the electrophoregrams which showed peptidase was much more active in the cotyledon than in the other tissues. Weeden (1984) also found that peptidase activity was most easily detected in seed extracts although only two bands were reported using L-phenylalanyl-L-proline as a substrate.

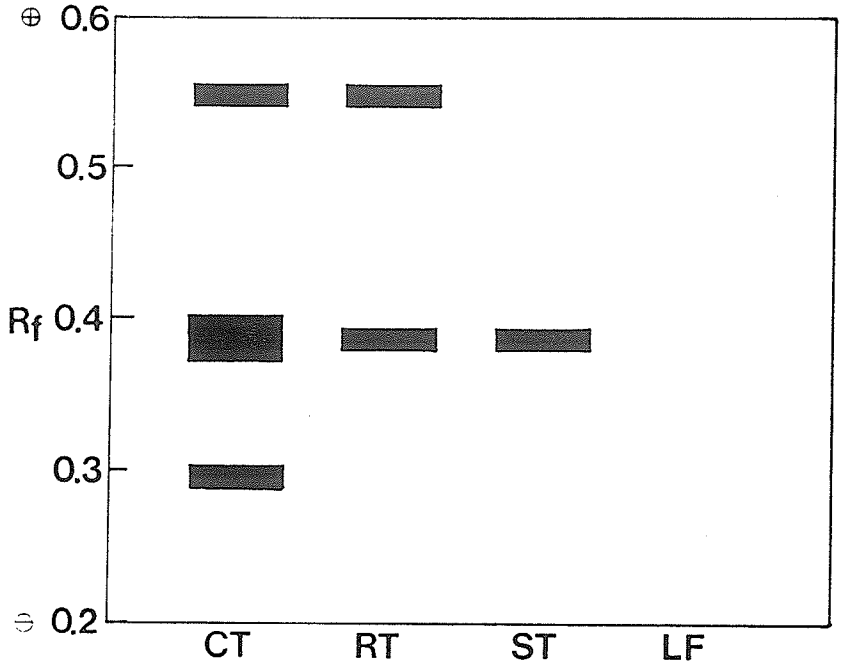


Figure 4.1 Representation of peptidase bands oserved in the cotyledon (CT), root (RT), stem (ST), and leaf (LF).

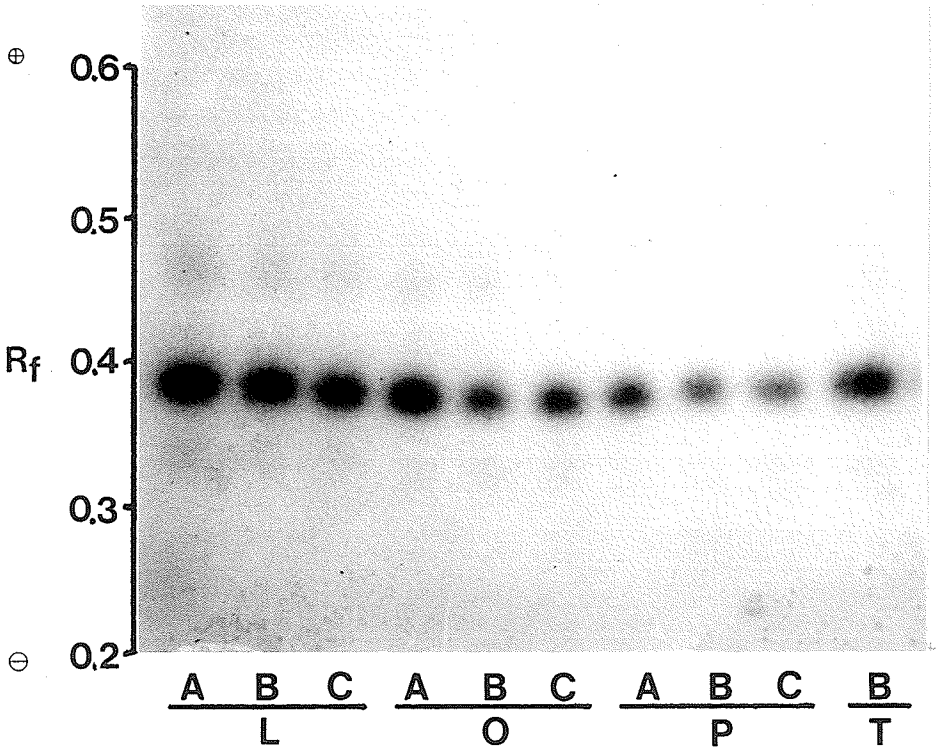


Figure 4.2 Cotyledon peptidase electrophoregram of Line 85-12 (L), Ostua (O), and Pecho Amarillo (P). Samples were harvested from Jutiapa (A), the INCAP Farm (B), and the greenhouse (C).

4.5.2 Peroxidase

Peroxidase activity was observed in all four tissues analyzed (Figure 4.3). Clearest and most intense bands were observed in the root extracts (Figure 4.4). The patterns produced from the leaf and stem extracts were also clear. Cotyledon bands were faint and inconsistent and could only be seen when separated on polyacrylamide gel.

Both leaf and root peroxidase patterns exhibited cultivar variation in the cathodal region of the gel. Line 85-12 exhibited a unique slow migrating band (R_f = -0.26) and the absence of the faster migrating band observed in the other cultivars (R_f = -0.33). Weeden (1984) reported a similar variation (R_f = -0.32, -0.35) in the cathodal region of the root peroxidase of white seeded beans.

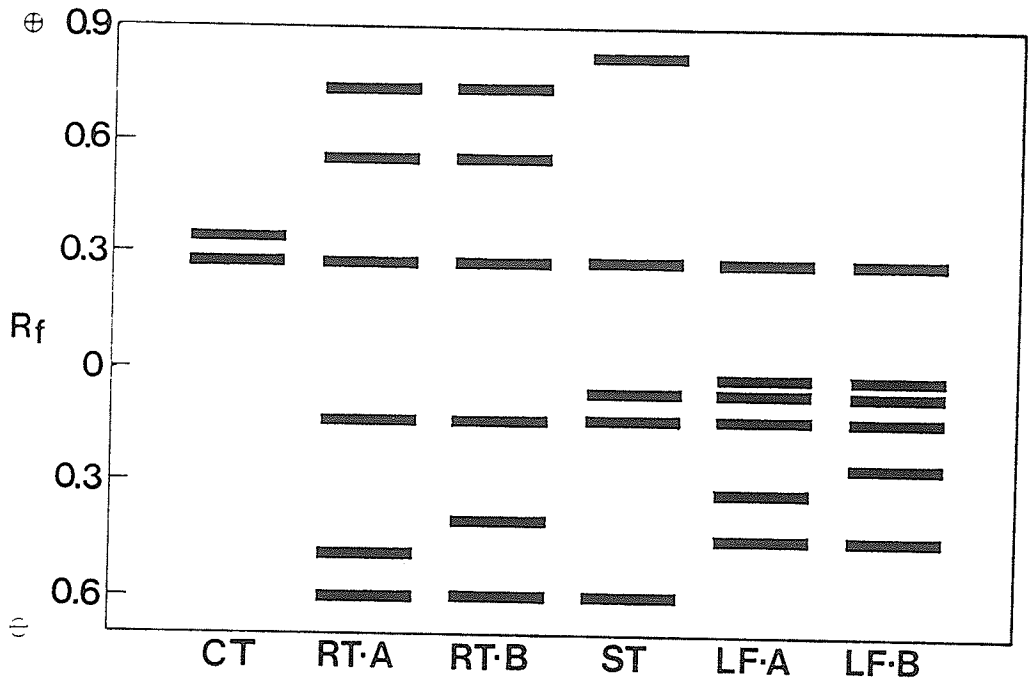


Figure 4.3 Representation of peroxidase bands observed in the cotyledon (CT), root (RT), stem (ST), and leaf (LF).

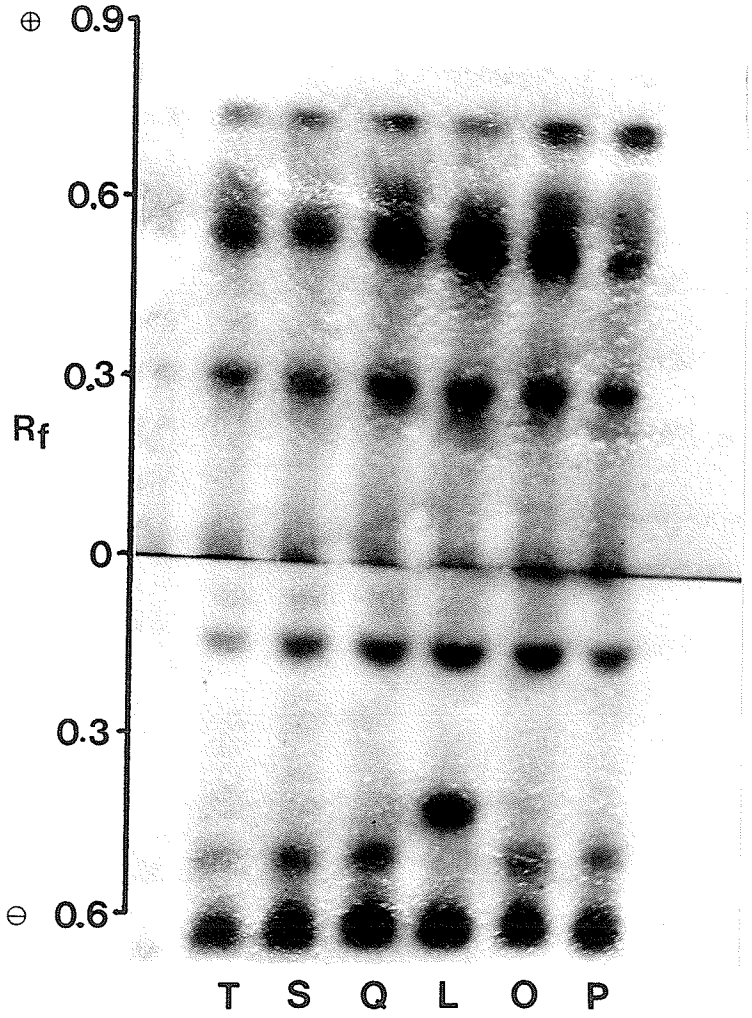


Figure 4.4 Root peroxidase electrophoregram of Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

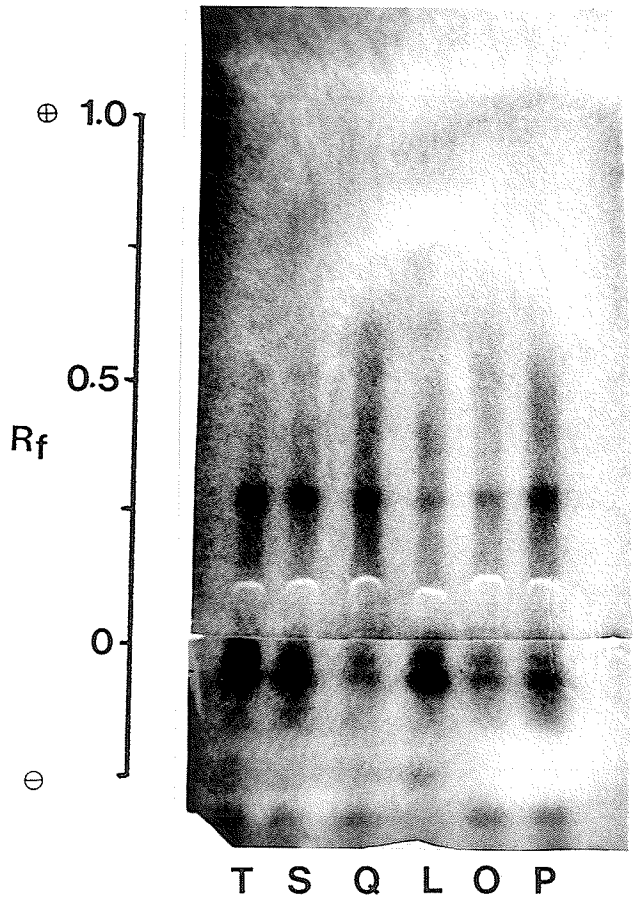


Figure 4.5 Leaf peroxidase electrophoregram of Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

4.5.3 Polyphenol Oxidase

Root extracts produced a clear polyphenol oxidase pattern (Figure 4.7). Stem and leaf extracts produced bands too light to be recorded with photography. No polyphenol oxidase activity was observed in the seed. No cultivar effect was observed.

When both root polyphenol oxidase and root peroxidase were separated on polyacrylamide, the resulting patterns were practically identical (Figures 4.7, 4.8). Although this was most obvious with the root patterns, the same phenomena was later recognized in the leaf and stem extracts. It appears as though the substrates for the peroxidase and polyphenol oxidase assays were acted upon by the same enzyme system. This was surprising because the substrates were very different in structure. L-dihydroxyphenylalanine (DOPA) used for the polyphenol oxidase assay is an aromatic amino acid while 3-amino-9-ethylcarbazol is a distinctly different three-ringed structure (Figure 4.9).

There is no clear consensus in the literature as to whether or not any appreciable amount of polyphenol oxidase activity takes place in the dormant cotyledon of Phaseolus vulgaris (Elias, 1982; Stanley and Aguilera, 1985). This study indicates that polyphenol oxidase activity takes place only in growing plant tissue. Supporting this, Udayasekhara and Deosthale (1987) reported that polyphenol oxidase activity in legume seeds increased to substantial levels only after germination.

No information specific to Phaseolus vulgaris has yet been published regarding its polyphenol oxidase isoenzymes. Using velvet beans (Stizolobium deeringianum L.), Zenin and Park (1978) reported three bands in the seed and two in the leaf and stem. Robb et al. (1965) found four isoenzymes in the leaf of Vicia faba L.

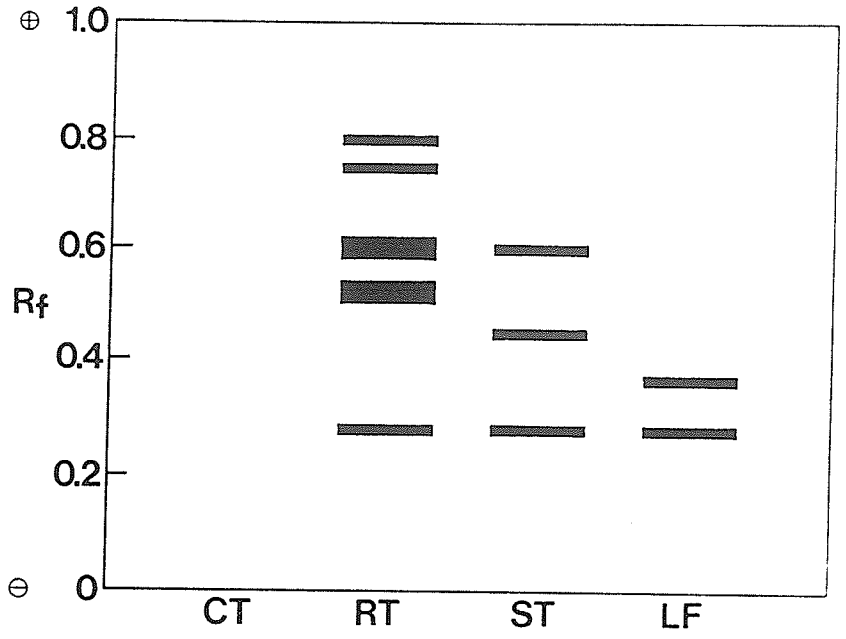


Figure 4.6 Representation of the polyphenol oxidase bands observed in the cotyledon (CT), root (RT), stem (ST), and leaf (LF).

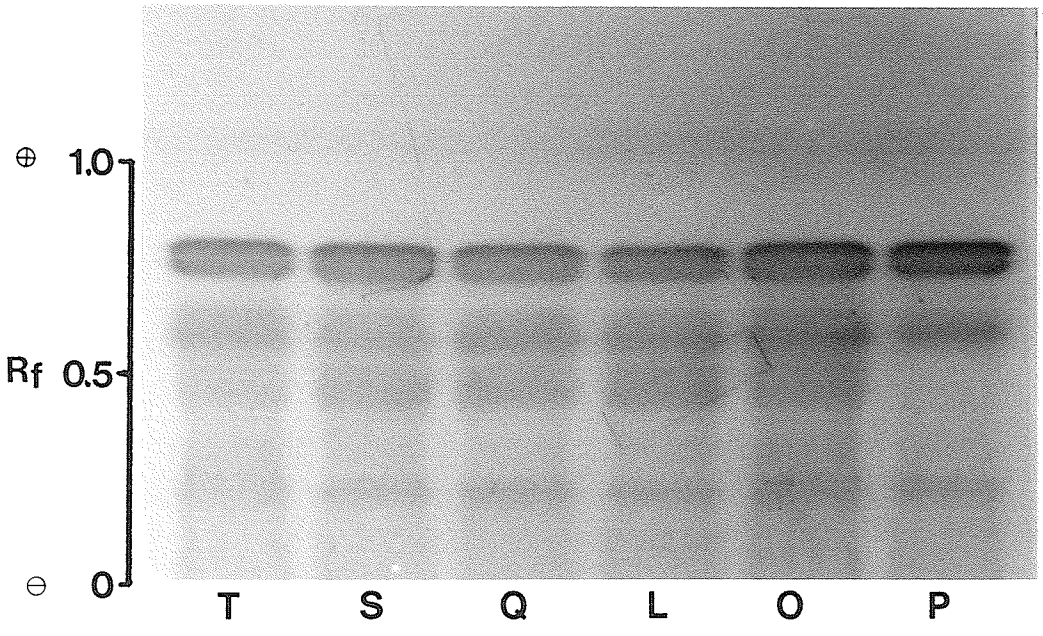


Figure 4.7 Root polyphenol oxidase electrophoregram of Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

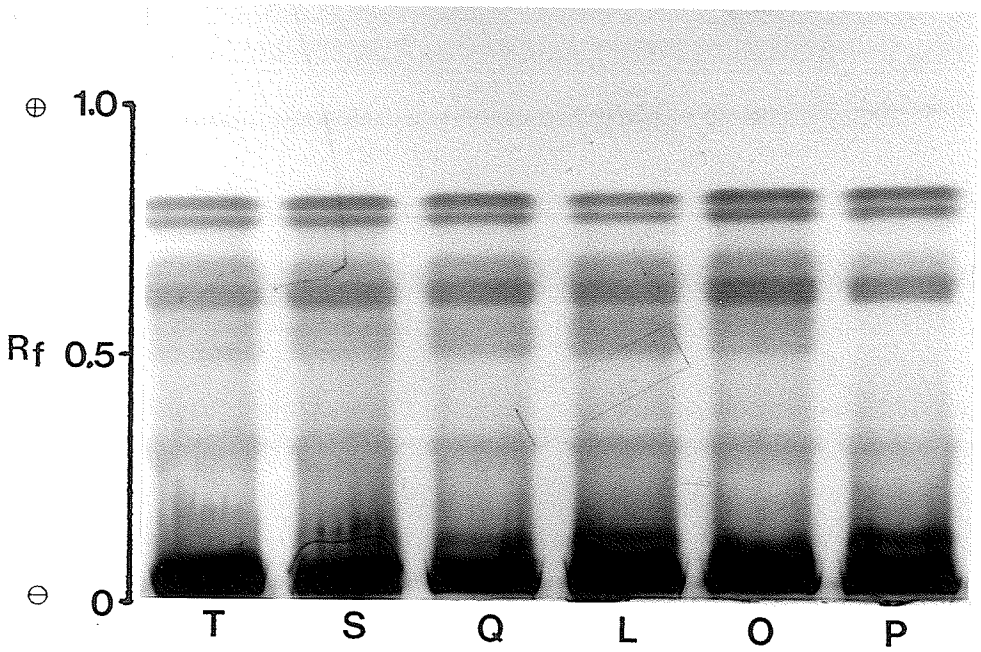
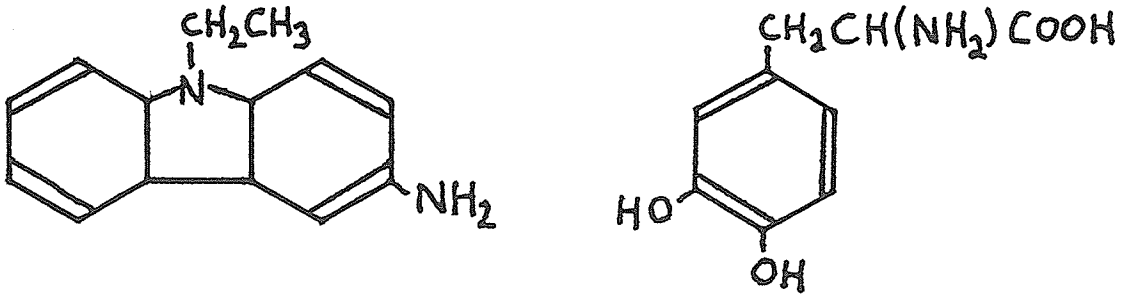


Figure 4.8 Root peroxidase electrophoregram of Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).



3-amino-9-ethylcarbazol L-dihydroxyphenylalanine

Figure 4.9 Chemical structures of 3-amino-9-ethylcarbazol and L-dihydroxyphenylalanine (DOPA).

4.5.4 Acid Phosphatase

Acid phosphatase activity was detected in all tissues of the bean (Figure 4.10). The patterns varied considerably among tissues with no one band being common to all. The cultivar San Martin produced a unique leaf pattern that showed an exceptionally fast migrating band (R_f = 0.94)(Figure 4.11). No other cultivar or location effects were observed.

Bassiri and Adams (1978) reported 10, 10, and 8 acid phosphatase bands in the primary leaves, stems, and roots respectively for Phaseolus vulgaris. This was substantially more than the 4, 4, and 3 bands observed in the same respective tissues in this study. Nevertheless, the black bean stem pattern reported in this study was essentially identical to the combined leaf, hypocotyl, and root extract acid phosphatase pattern reported by Weeden (1984) for white seeded beans.

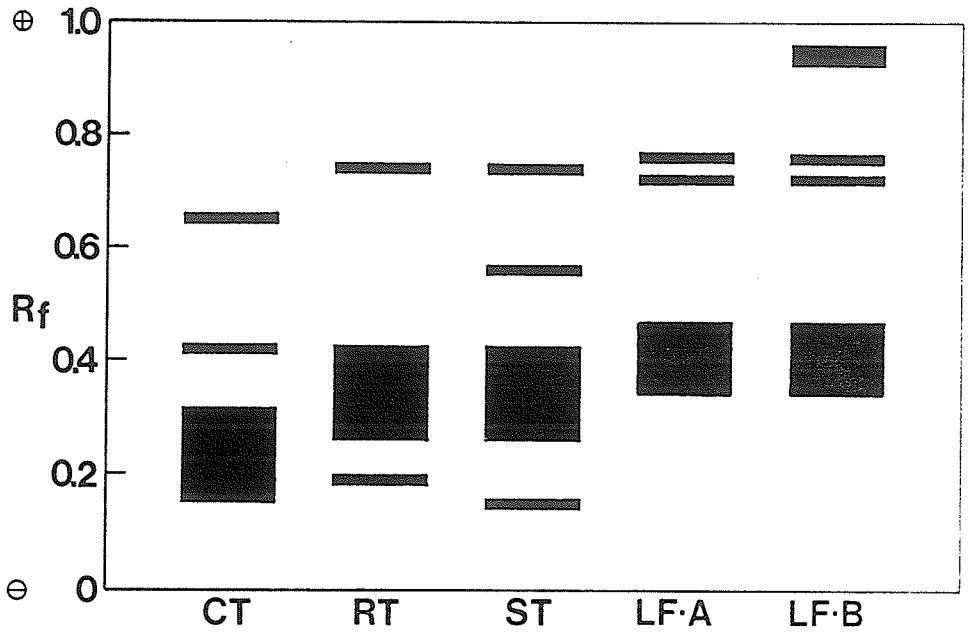


Figure 4.10 Representation of acid phosphatase bands observed in the cotyledon (CT), root (RT), stem (ST), and leaf (LF).

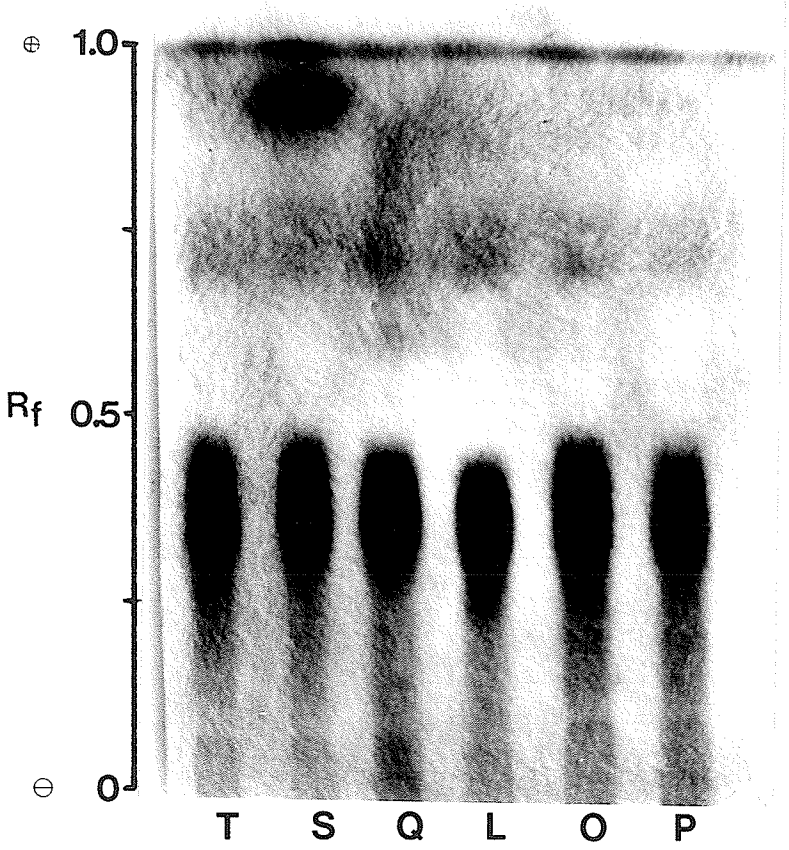


Figure 4.11 Leaf acid phosphatase electrophoregram of Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

4.5.5 Esterase

Extracts from all tissues showed prominent esterase activity (Figure 4.12). Leaf extracts gave very poor separation and it was not possible to count the bands. In the other tissues, there appeared to be three zones of activity. The most cathodal region was between the R_f values of 0.12 to 0.24, the middle region was between R_f values of 0.32 and 0.38, and the most anodal region was located between the R_f values of 0.45 and 0.54.

The band quality of the cotyledon extracts was fairly poor, possibly due to large amounts of starch in the extracts which could interfere with the mobility of the bands through the gel (Figure 4.13). No consistent cultivar differences were observed. Despite the poor band quality of the gels, it was possible to observe a band (R_f = 0.33) that was consistently present only in extracts from seeds grown at the Farm. This would suggest that there was a location effect on cotyledon esterase isoenzymes. It should be noted that samples from the Farm had a large amount of insect damage. However, this was probably not responsible for the extra band because the infected areas of the cotyledon were removed before analysis. The absence of the band in the seed samples from the greenhouse and Jutiapa might be a factor of soil conditions or climatic conditions. Minor electrophoretic variations due to environmental conditions are not common but have previously been reported for beans (Adriaanse, et al., 1969).

Both root and stem esterase patterns exhibited cultivar differences. Root extracts of San Martin had a unique band with an R_f value of 0.45 while Line 85-12 had a unique band with an R_f value of 0.41 (Figure 4.14). Stem extracts of both San Martin and Line 85-12

showed very little esterase activity in the mid-region (Figure 4.15). Line 85-12 again showed a unique band with an R_f value of 0.41. Previous studies have reported that esterase patterns are affected by bean cultivar (West and Garber, 1967; Bassiri and Adams, 1978).

In all, there were 7 bands present in the cotyledon, 11 bands in the root extracts, and 12 bands in the stem extracts. This was more than had been reported previously in beans when starch gel had been used as the separating medium. Weeden (1984) reported four distinct esterase bands in white seeded bean seedlings. Bassiri and Adams (1978), using a wide variety of Phaseolus vulgaris cultivars, reported six bands in the leaf, four in the stem, and no activity at all in the roots. It is clear that separation of bean esterases on polyacrylamide is superior to separation on starch gel. Ramirez et. al. (1987) reported similar improvements using polyacrylamide to separate cassava esterases.

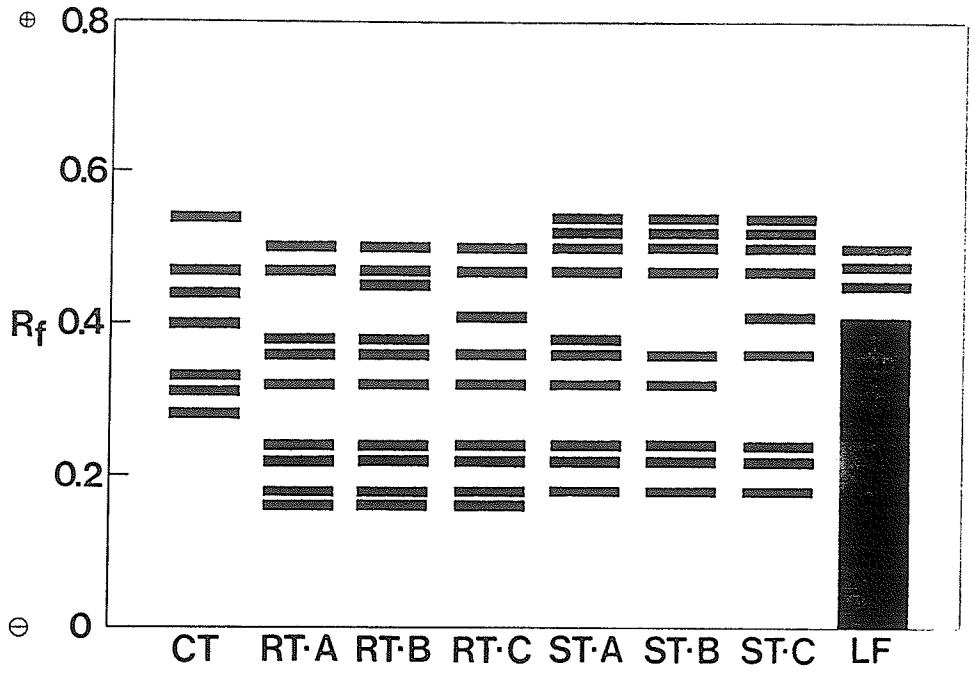


Figure 4.12 Representation of esterase bands observed in the cotyledon (CT), root (RT), stem (ST), and leaf (LF).

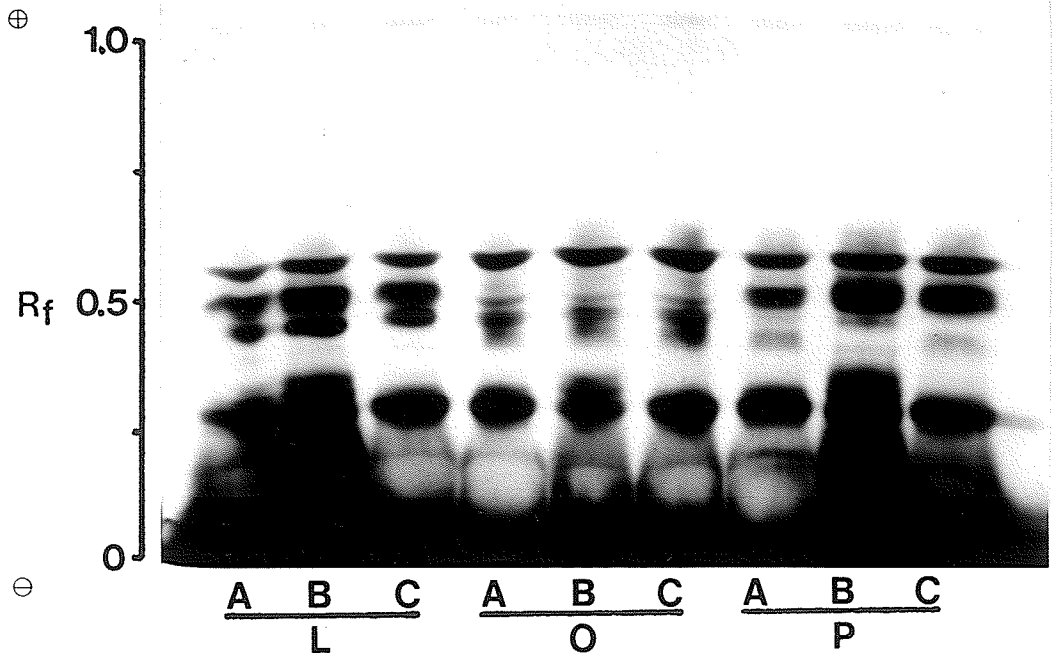


Figure 4.13 Cotyledon esterase electrophoregram of Line 85-12 (L), Ostua (O), and Pecho Amarillo (P). Samples were harvested from Jutiapa (A), the INCAP Farm (B), and the greenhouse (C).

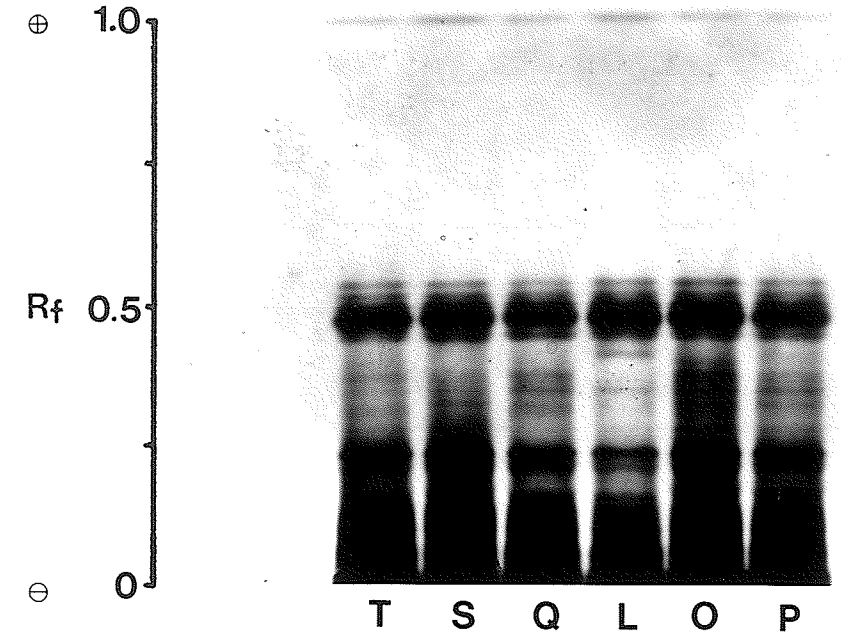


Figure 4.14 Root esterase electrophoregram of Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

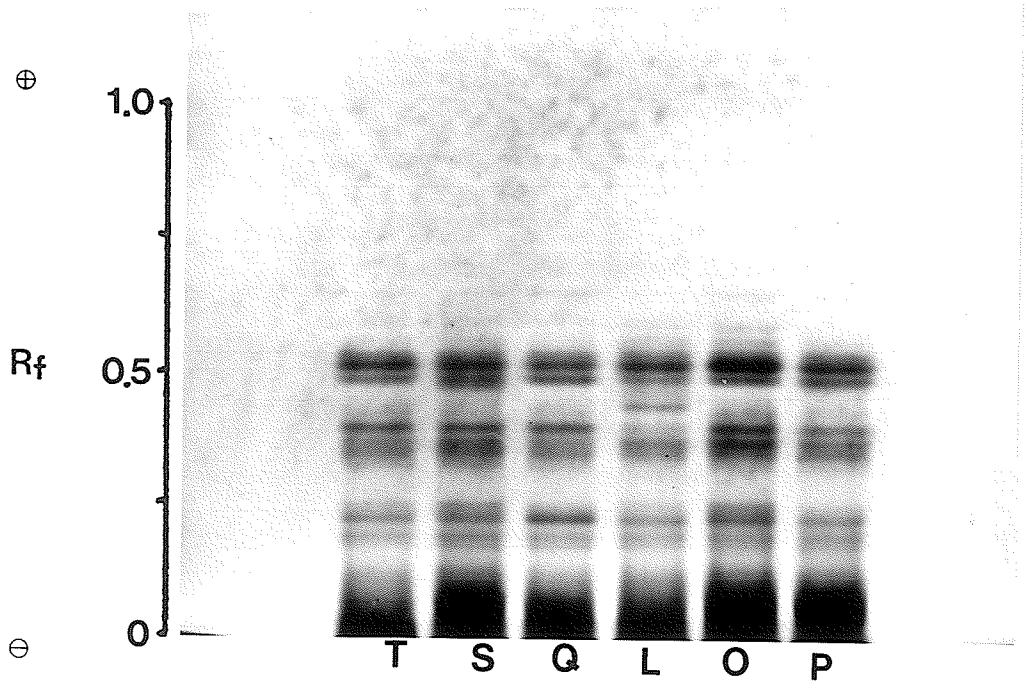


Figure 4.15 Stem esterase electrophoregram of Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

4.5.6 Phosphoglucosoisomerase

The phosphoglucosoisomerase patterns were nearly identical in all tissues (Figure 4.16). Three bands (R_f = 0.31, 0.40, 0.48) were common to all extracts while the cotyledon extracts produced an extra faint band with an R_f value of 0.35. The cotyledon extracts revealed that the fastest moving band (R_f = 0.48) was actually made up of two bands with very similar mobilities. No cultivar or location effects were observed.

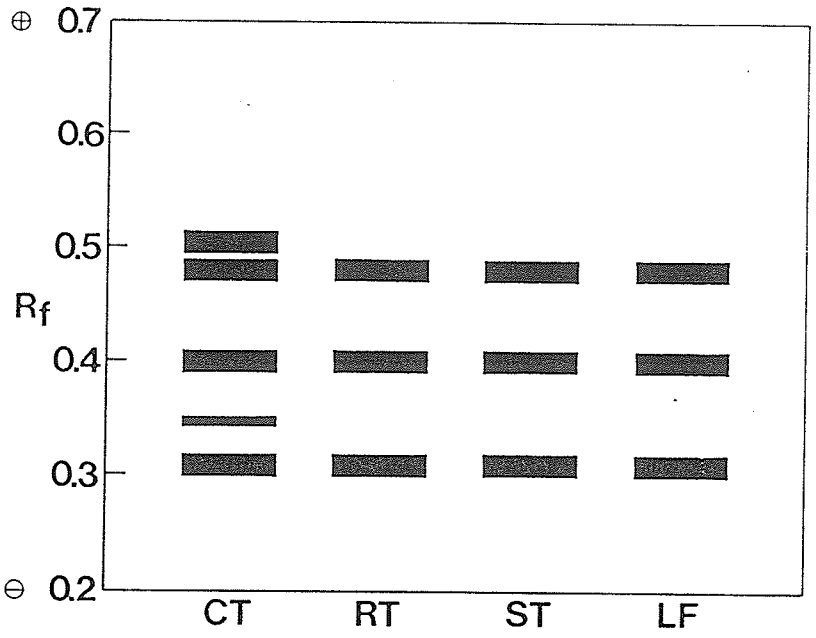


Figure 4.16 Representation of phosphoglucosoisomerase bands observed in the cotyledon (CT), root (RT), stem (ST), and leaf (LF).

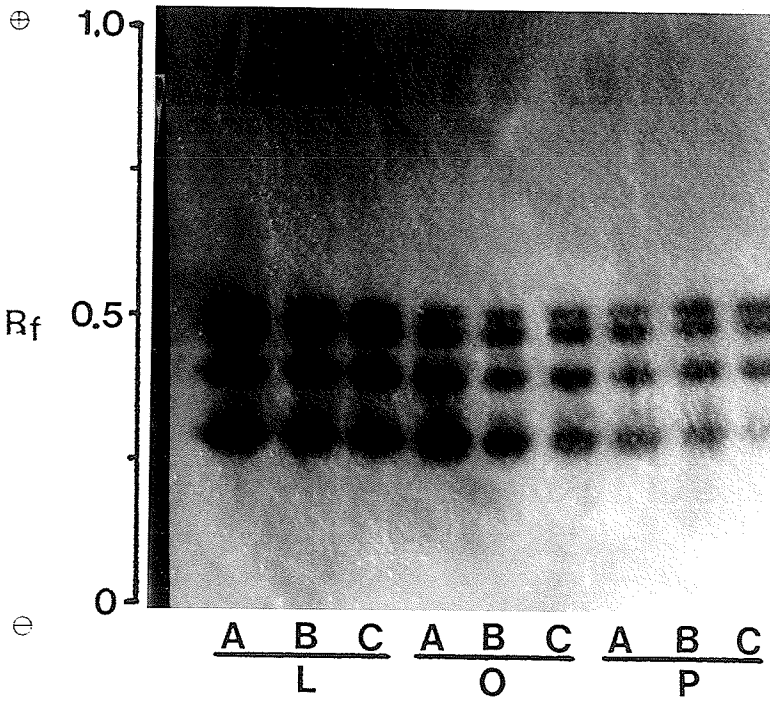


Figure 4.17 Cotyledon phosphoglucososisomerase electrophoregram of Line 85-12 (L), Ostua (O), and Pecho Amarillo (P). Samples were harvested from Jutiapa (A), the INCAP Farm (B), and the greenhouse (C).

4.5.7 Glutamate Oxaloacetate Transaminase

Glutamate oxaloacetate transaminase patterns were almost identical in all tissues (Figure 4.18). Two bands (R_f = 0.26, 0.32) were common to all tissues while the stem extracts exhibited a faint band with an R_f value of 0.29. No cultivar or location effects were observed.

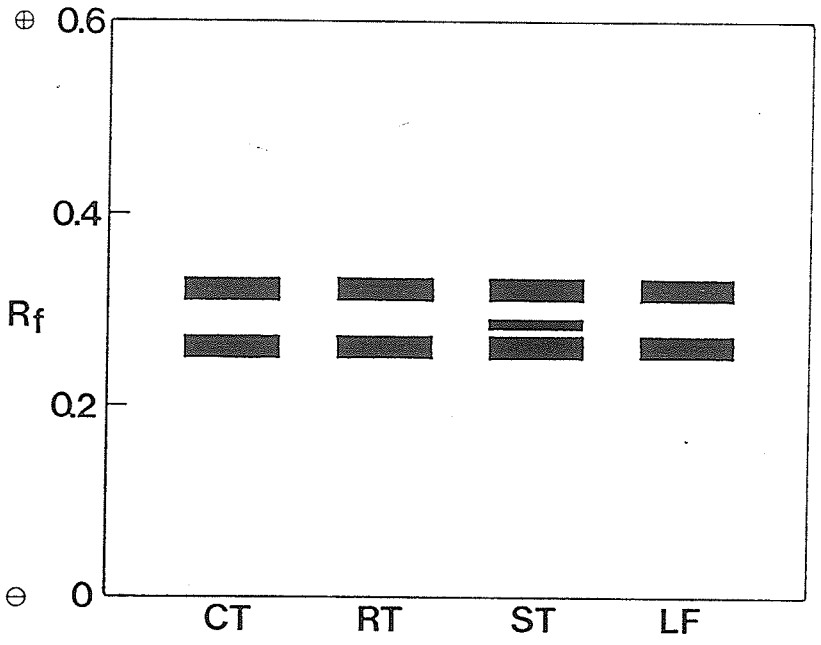


Figure 4.18 Representation of glutamate oxaloacetate transaminase bands observed in the cotyledon (CT), root (RT), stem (ST), and leaf (LF).

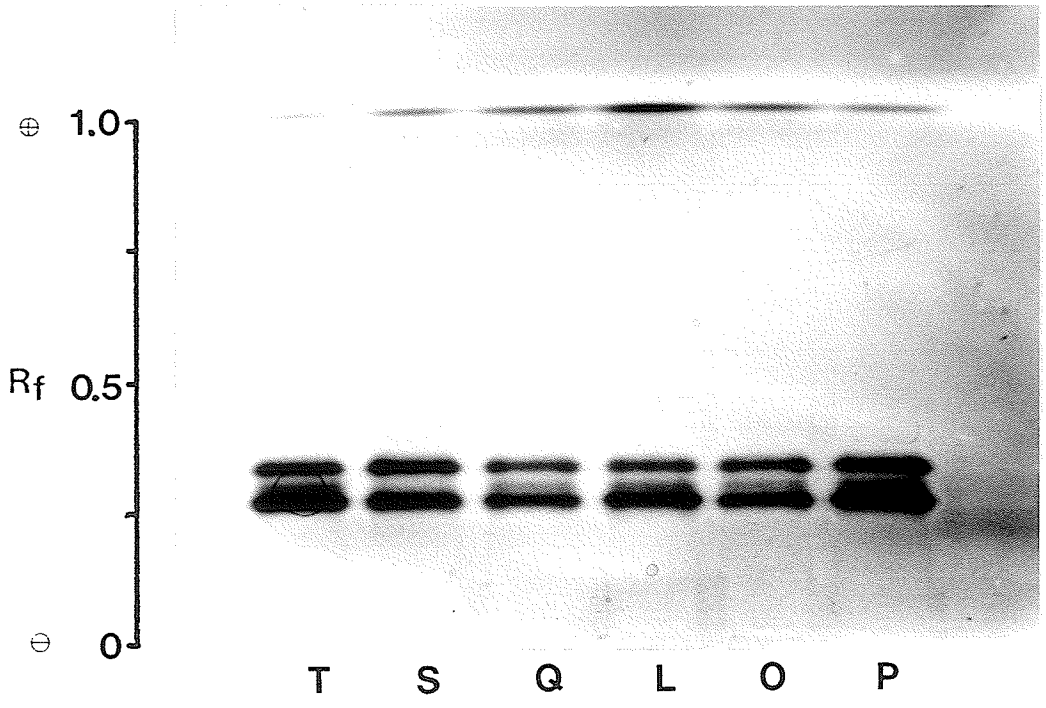


Figure 4.19 Stem glutamate oxaloacetate transaminase electrophoregram of Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

4.6 Seed Protein Electrophoretic Patterns

The effects of cultivar, location, and cooking quality on the electrophoretic patterns of various cotyledon protein extractions were investigated using acid-PAGE and SDS-PAGE.

4.6.1 Acid-Polyacrylamide Gel Electrophoresis

4.6.1.1 Extraction With 5 M Acetic Acid

A total of 13 bands were present in the 5 M acetic acid extractions (Figure 4.20). The four least mobile bands were not consistently produced in all samples. There was, however, no apparent pattern in their inconsistency. The remaining 9 bands were consistently produced and showed no cultivar or location variation.

Although a 5M acetic acid extraction has been used successfully for lentil cultivar identification (Hussain, et al., 1989a) and for Centrosema cultivar identification (Hussain, et al., 1988c), it was not able to discriminate between black bean cultivars. Further investigation indicated that a 5 M acetic acid extraction could distinguish between more distantly related Phaseolus vulgaris cultivars, such as between kidney, navy, and pinto beans (Figure 4.21).

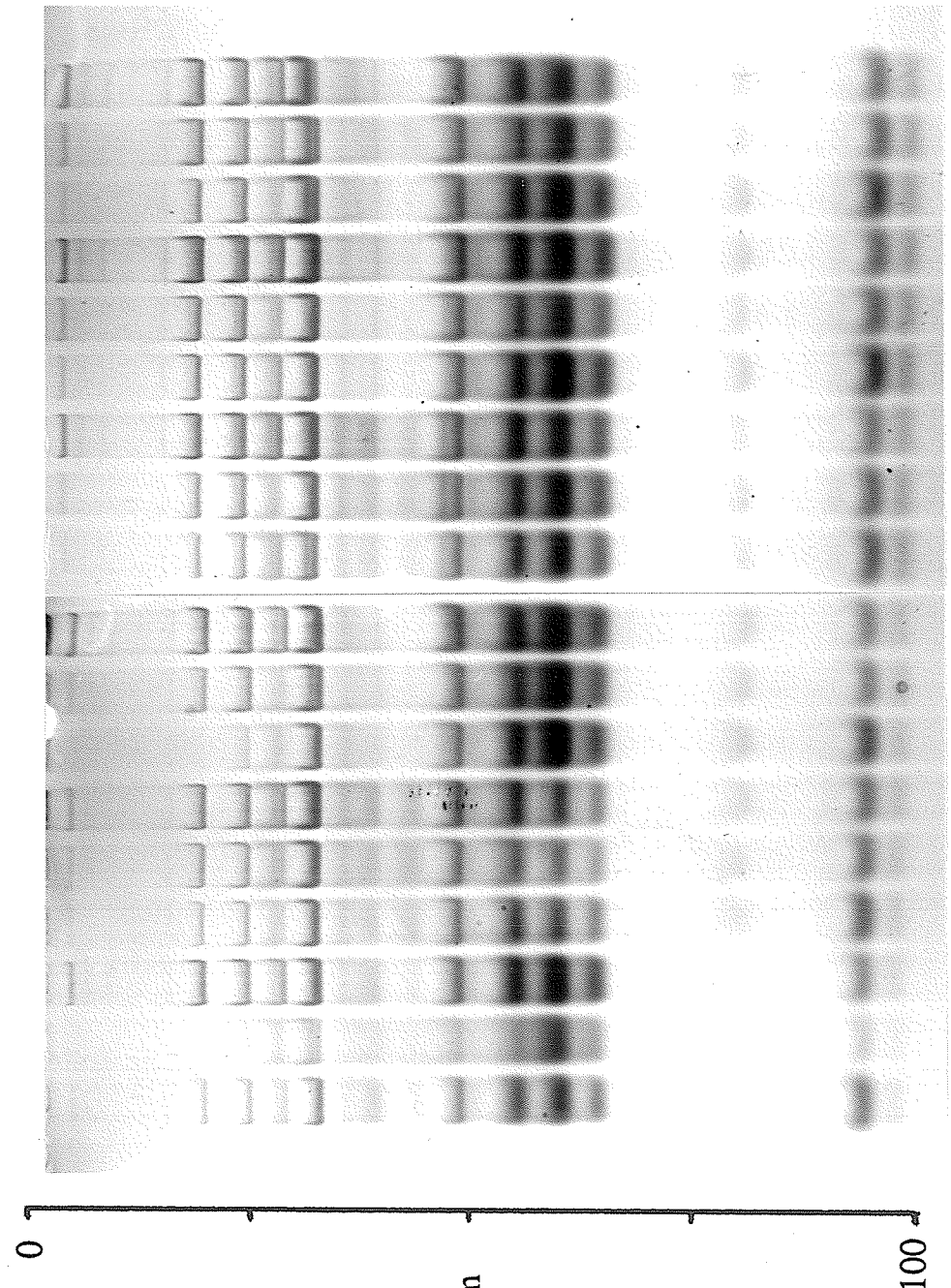


Figure 4.20 Acid PAGE electrophoregram of seed proteins soluble in 5 M acetic acid. Left to right: Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P). Patterns from left to right within each cultivar represent seeds grown in Jutiapa, the INCAP farm, and the greenhouse.

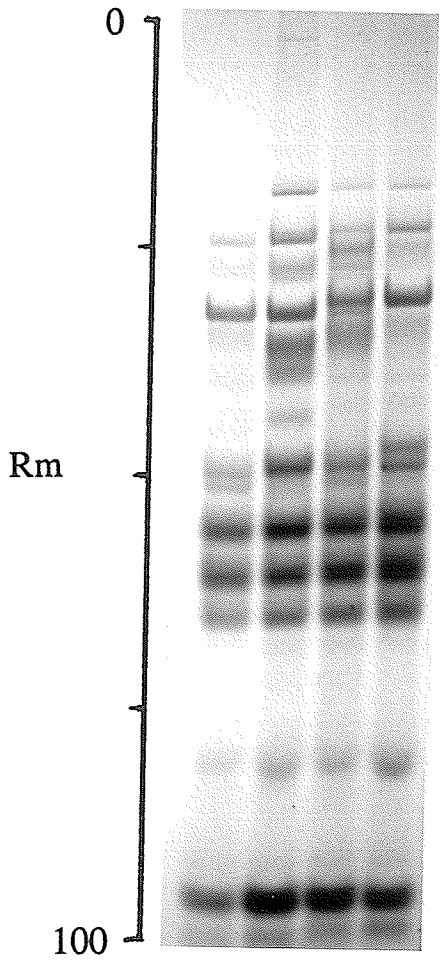


Figure 4.21 Acid PAGE electrophoregram of seed proteins soluble in 5 M acetic acid. Left to right: brown, black, white, and kidney beans.

4.6.1.2 Extraction With 0.1 M Acetic Acid and 1.0% Mercaptoethanol

Depending on the cultivar, there were 12 to 14 bands present in the electrophoregram (Figure 4.22). Cultivar variation was evident in the cathodal region of the gel (Rm less than 50). All cultivars could be identified by a unique pattern except the cultivars Quetzal and Line 85-12 which shared the same pattern (Figure 4.23). There was no location effect.

Hussain et al. (1986,1988d) had reported previously that this procedure could differentiate between Phaseolus vulgaris cultivars although the cultivars they analyzed were of several different colours and were presumably more distantly related than the black beans analyzed in this study. This study shows, however, that the procedure is useful for cultivar identification for even very closely related Phaseolus vulgaris cultivars.

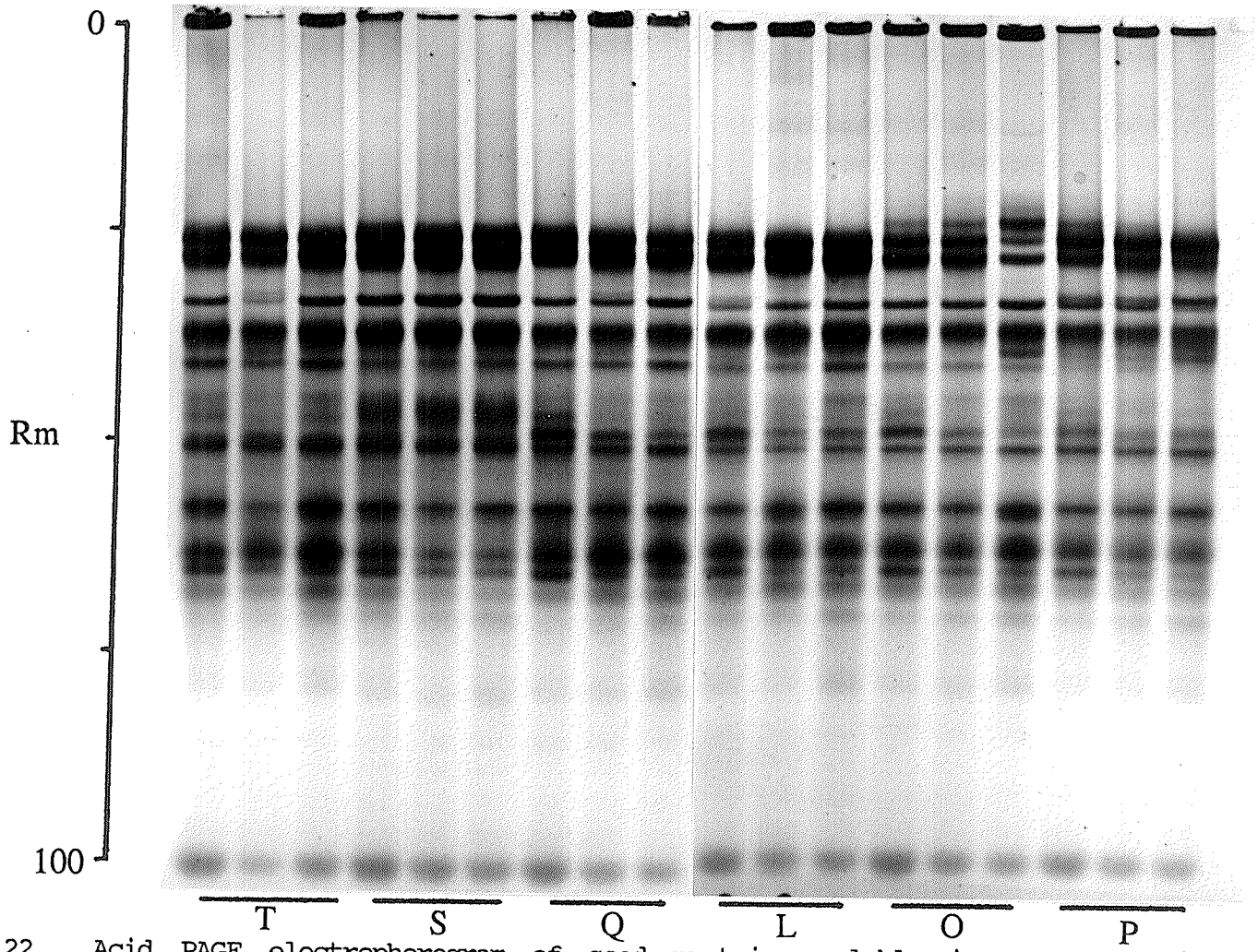


Figure 4.22 Acid PAGE electrophoregram of seed proteins soluble in 0.1 M acetic acid containing 1.0% mercaptoethanol. Left to right: Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P). Patterns from left to right within each cultivar represent seeds grown in Jutiapa, the INCAP farm, and the greenhouse.

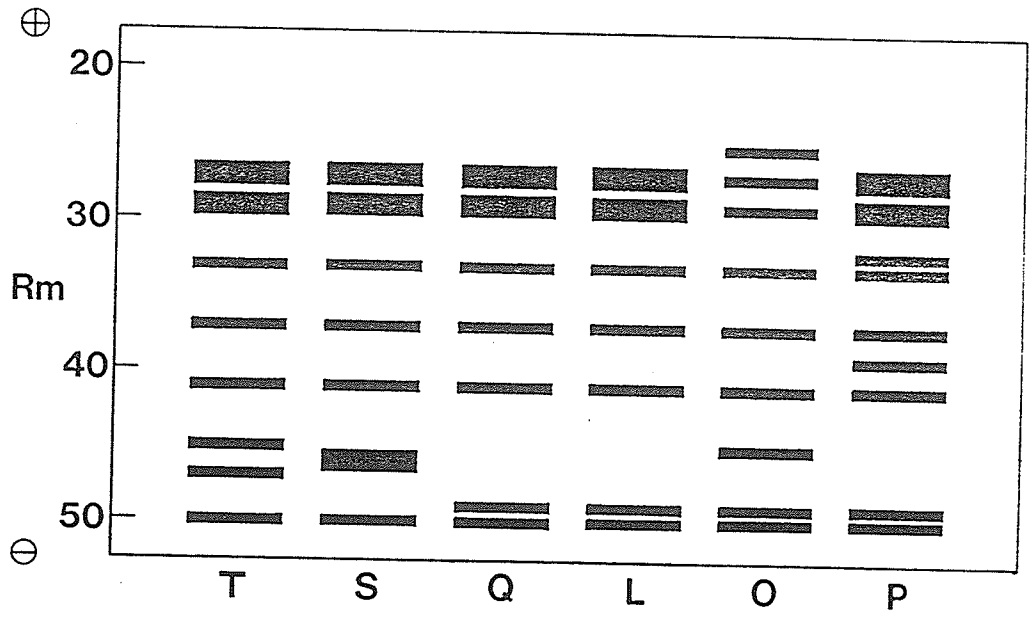


Figure 4.23 Representation of cultivar variation evident in the acid PAGE patterns of seed proteins soluble in 0.1 M acetic acid containing 1.0% mercaptoethanol. Left to right: Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

4.6.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Of the sequential extractions with 0.4 M sodium chloride, 70% ethanol, 0.1 M acetic acid, and the residual protein extraction solution, only the sodium chloride and residual protein extractions resulted in electrophoretic patterns. After this was determined, ground cotyledon was extracted directly with 70% ethanol and with 0.1 M acetic acid without doing the prior sequence of extractions. The direct acetic acid extraction produced more than 25 bands but the ethanol extraction still resulted in a blank gel.

4.6.2.1 Sodium Chloride Soluble Proteins

Depending on the cultivar, patterns with 39 or 40 bands were produced by SDS-PAGE of 0.4 M sodium chloride soluble proteins (Figure 4.24). Cultivar variation was evident only among the low molecular weight proteins (M.W. approximately 22 000 to 33 000). Four distinct patterns were observed (Figure 4.25). The cultivar Tamazulapa was not distinguishable from the cultivar Quetzal, nor was San Martin distinguishable from Ostua. Line 85-12 exhibited a similar pattern to those of Tamazulapa and Quetzal, the only difference being two bands which stained substantially darker in Line 85-12 than in the other cultivars (M.W. 22 000 and 26 000). This was not unexpected as genetically, Tamazulapa, Quetzal, and Line 85-12 are closely related; Quetzal and Tamazulapa share a parent while Line 85-12 and Tamazulapa also share a parent (Figure 3.1). There was no location effect.

Previous work done with sodium chloride soluble proteins has concentrated on using the phaseolins (G1 proteins) for determining the evolutionary origins of Phaseolus vulgaris (Brown et al., 1982; Gepts

et al., 1986). As beans from Central America characteristically have the "Sanilac" phaseolin pattern, looking only at the phaseolins would not be a useful method for the identification of Guatemalan black bean cultivars. However, as this study indicated, when the entire sodium chloride soluble protein pattern was used, reasonable discriminating power was observed with only closely related cultivars being difficult to discriminate.

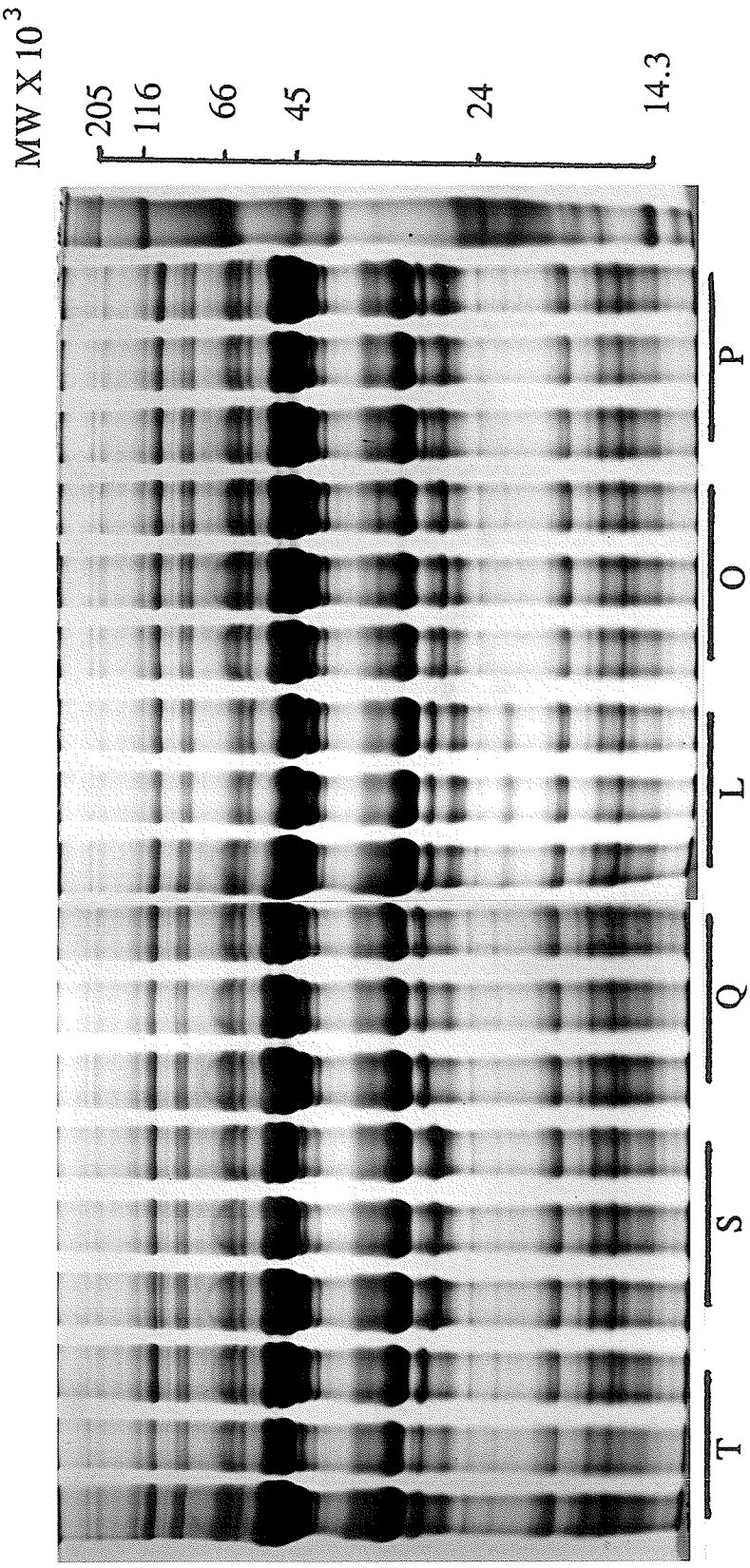


Figure 4.24 SDS-PAGE electrophoregram of sodium chloride soluble seed proteins. Left to right: Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P). Patterns from left to right within each cultivar represent seeds grown in Jutiapa, the INCAP farm, and the greenhouse.

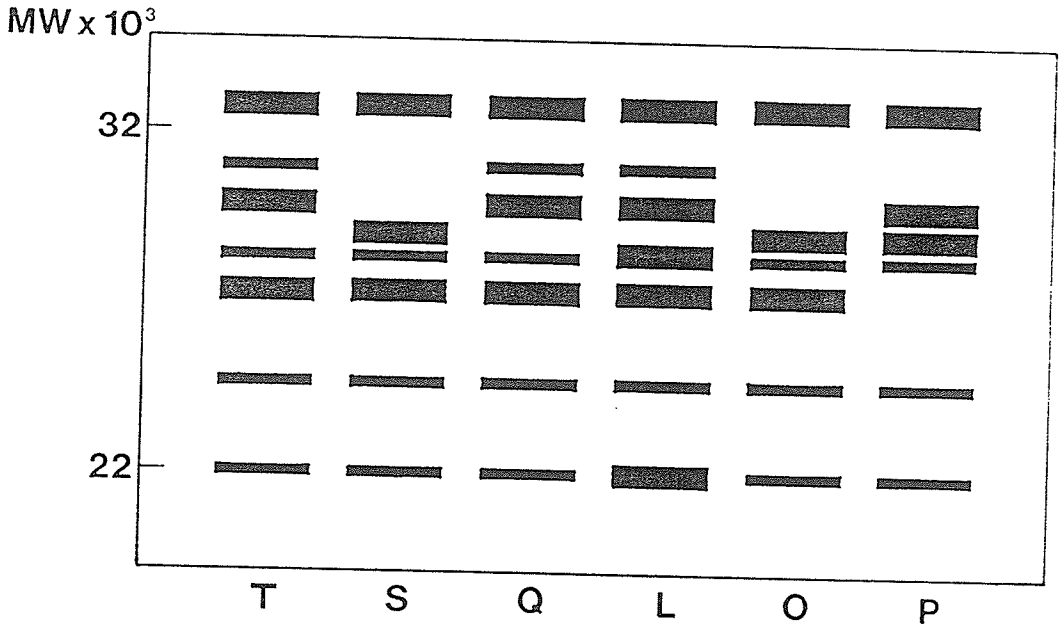


Figure 4.25 Representation of cultivar variation evident in the SDS-PAGE patterns of sodium chloride soluble seed proteins. Left to right: Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

4.6.2.2 0.1 M Acetic Acid Soluble Proteins

Acetic acid soluble proteins tended to be of lower molecular weight and were consequently separated on a 14% gel instead of a 12% polyacrylamide gel. Cultivar variation was evident among proteins from M.W. 20 000 to 30 000 (Figure 4.26). The closely related cultivars of Tamazulapa, Quetzal, and Line 85-12 had nearly identical patterns while the other more distantly related cultivars were readily distinguishable (Figure 4.27). No location effect was observed.

Some similarities were observed between reduced acetic acid soluble proteins separated in an acid medium (Figure 4.22) and the acetic acid soluble proteins separated by SDS-PAGE at basic pH. In both gels, Ostua exhibits a unique pattern near the slow migrating end of the region of variation while San Martin exhibits some unique proteins near the fast migrating end of the region.

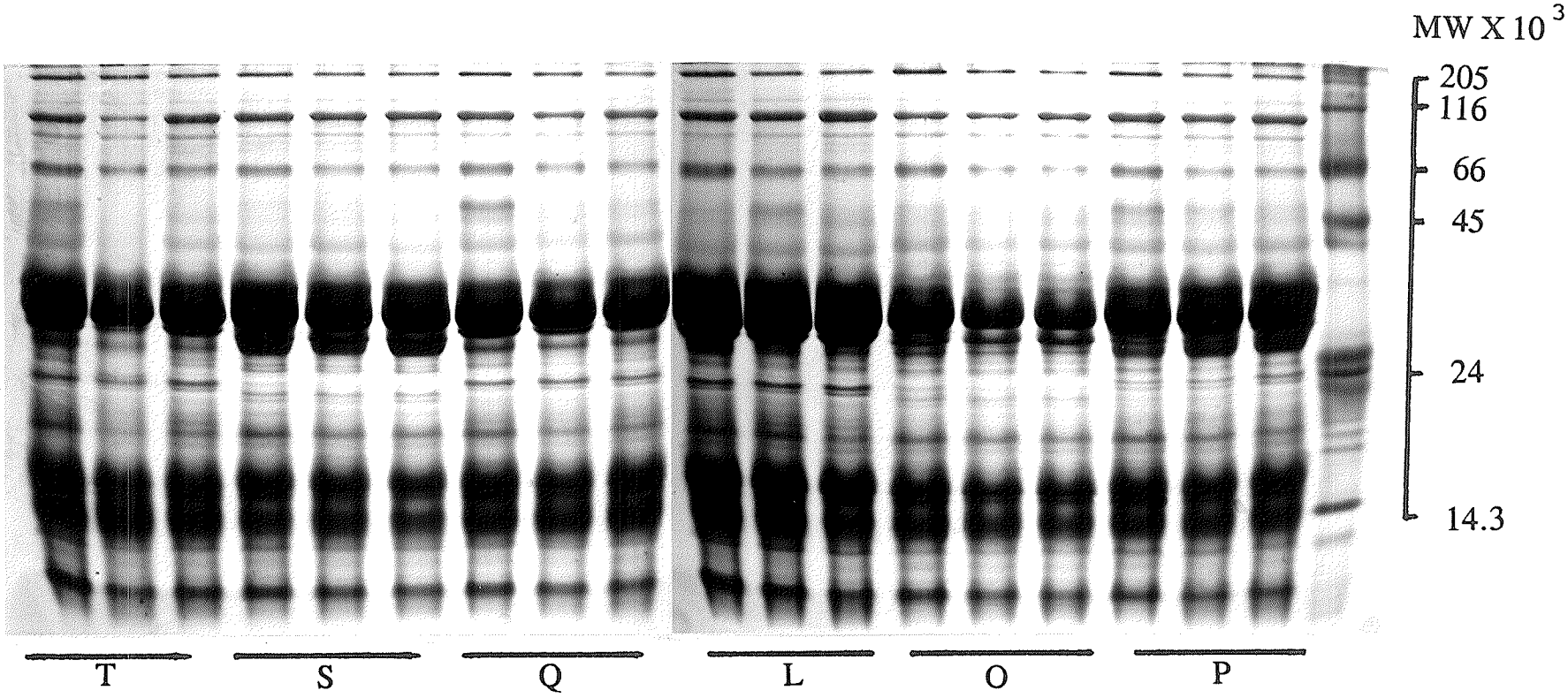


Figure 4.26 SDS-PAGE electrophoregram of acetic acid soluble seed proteins. Left to right: Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P). Patterns from left to right within each cultivar represent seeds grown in Jutiapa, the INCAP farm, and the greenhouse.

Figure 4.27 Representation of cultivar variation evident in the SDS-PAGE patterns of acetic acid soluble seed proteins. Left to right: Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

4.6.2.3 Residual Proteins

A total of 37 or 38 bands were present in the residual extracts depending on the cultivar (Figure 4.28). Cultivar variation was evident among the proteins between M.W. 19 000 and 34 000. All cultivars were uniquely identified by their electrophoretic patterns with the exception of the closely related cultivars Tamazulapa and Quetzal which shared the same pattern (Figure 4.29). No location effect was observed.

Hussain et. al. (1986) reported that this procedure could be used to distinguish between Phaseolus vulgaris cultivars. They also reported that location did not affect electrophoretic patterns. The current study confirms the earlier literature and also indicates that this procedure is sensitive enough to discriminate between all but the most closely related cultivars within the black colour class of Phaseolus vulgaris.

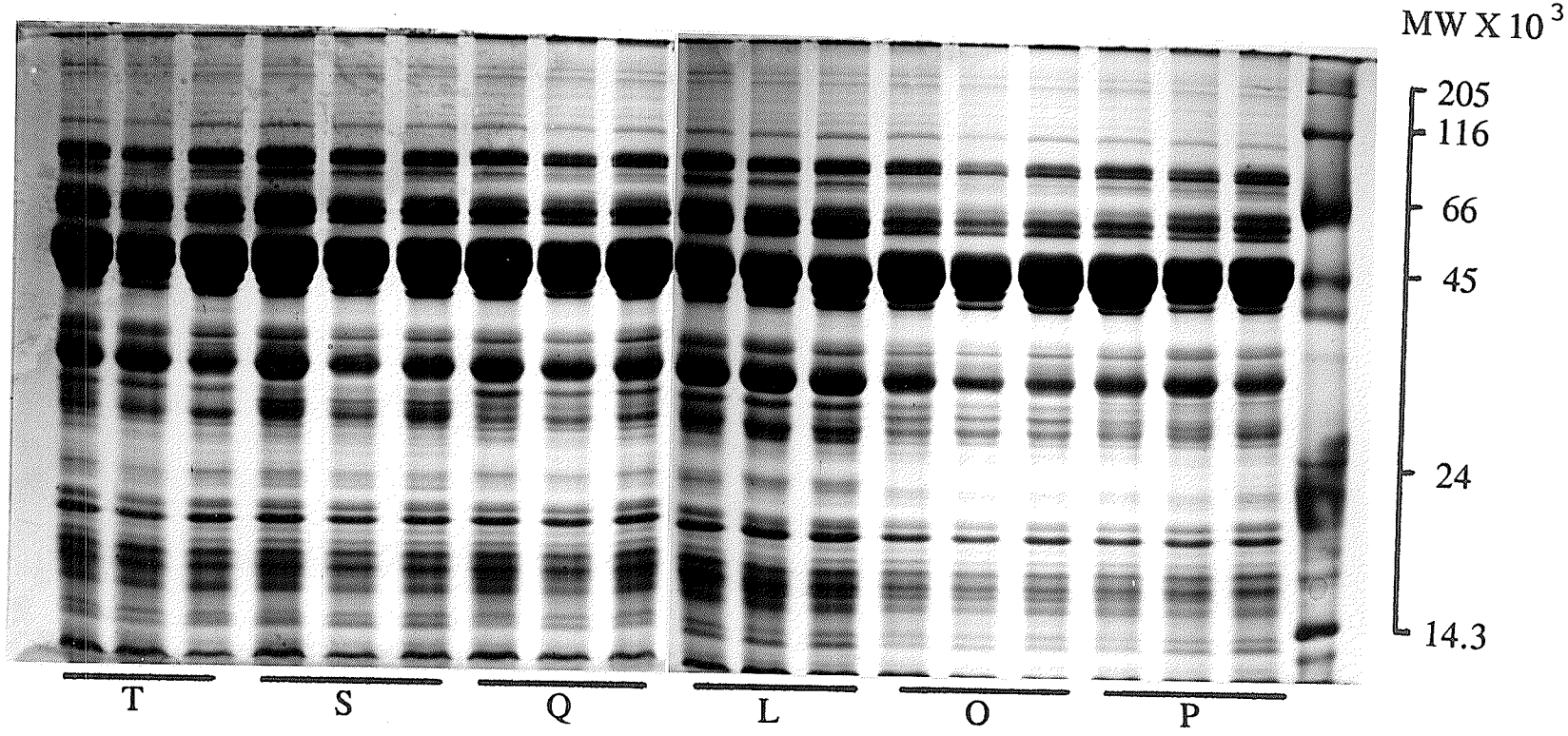


Figure 4.28 SDS-PAGE electrophoregram of residual seed proteins. Left to right: Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P). Patterns from left to right within each cultivar represent seeds grown in Jutiapa, the INCAP farm, and the greenhouse.

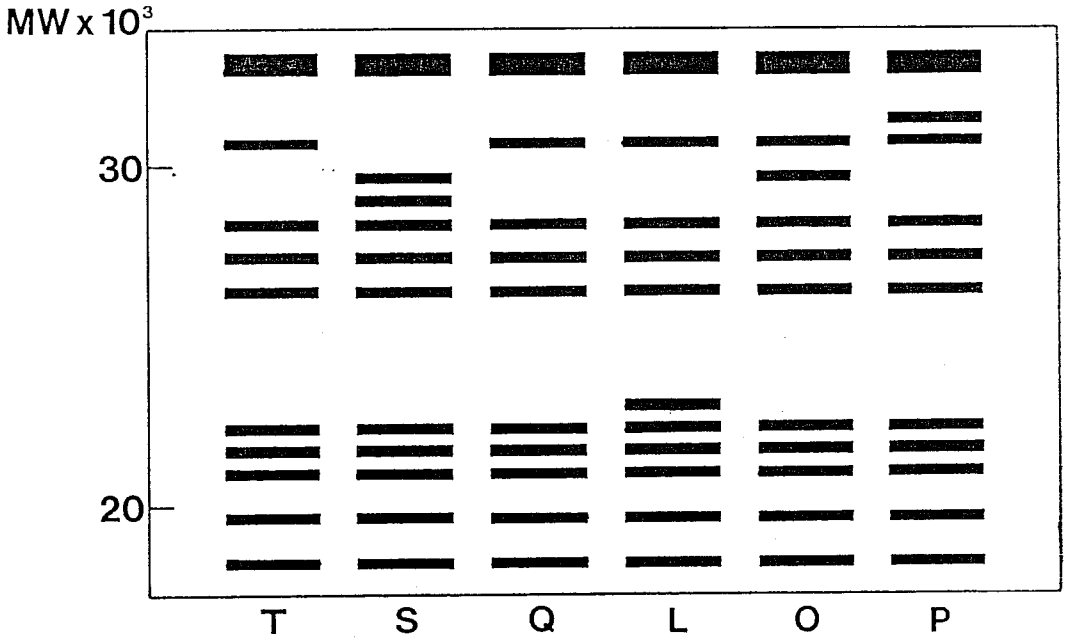


Figure 4.29 Representation of cultivar variation evident in the SDS-PAGE patterns of residual seed proteins. Left to right: Tamazulapa (T), San Martin (S), Quetzal (Q), Line 85-12 (L), Ostua (O), and Pecho Amarillo (P).

4.7 General Discussion

On the basis of isoenzyme electrophoretic patterns, the six cultivars could be separated into three groups: Line 85-12 was identifiable by its leaf and root peroxidase and by its root and stem esterase; San Martin was identifiable by its leaf acid phosphatase and by its root and stem esterase; Tamazulapa, Quetzal, Ostua, and Pecho Amarillo made up the last group as they were not distinguishable on the basis of their isoenzyme patterns. These results indicate that isoenzyme electrophoresis, using these particular enzymes, is not effective for the identification of black bean cultivars. The sensitivity, however, might have been increased by the use of other enzymes that were not analyzed in this study. For example, Weeden (1984) obtained better discrimination of white beans by using ten different enzymes in contrast to the three enzymes that exhibited cultivar variation in this study. The obvious disadvantage of using such a large number of enzymes to increase the discriminating power is that the method quickly becomes cumbersome and time-consuming.

Although enzyme electrophoresis proved disappointing with regard to black bean cultivar identification, interesting trends were noted when the patterns were compared with the cooking quality of the different cultivars. Table 4.9 shows the six cultivars ranked according to their cooking times immediately after harvest while Table 4.10 ranks the cultivars according to the rate by which they hardened during storage. In both cases, San Martin ranked higher than all the other samples, followed by Line 85-12. San Martin and Line 85-12 were also the two cultivars that produced unique isoenzyme patterns suggesting

TABLE 4.9

Enzyme electrophoretic patterns and mean cooking time (minutes) immediately after harvest.

	Cultivar					
	QUE	PEC	OST	TAM	L85	SAN
Time (min)	51.0a[1]	53.7ab	54.0ab	55.1ab	55.5ab	62.8b
Leaf POD	A	A	A	A	B	A
Root POD	A	A	A	A	B	A
Stem EST	A	A	A	A	C	B
Root EST	A	A	A	A	C	B
Leaf ACP	A	A	A	A	A	B

[1] Means in the same row followed by different letters are significantly different ($p<0.05$).

TABLE 4.10

Enzyme electrophoretic patterns and increase in mean cooking time (minutes) after six months of storage at ambient conditions.

	Cultivar					
	OST	TAM	QUE	PEC	L85	SAN
Time (min)	8.0a[1]	9.7a	11.0a	11.0a	14.0a	26.3b
Leaf POD	A	A	A	A	B	A
Root POD	A	A	A	A	B	A
Stem EST	A	A	A	A	C	B
Root EST	A	A	A	A	C	B
Leaf ACP	A	A	A	A	A	B

[1] Means in the same row followed by different letters are significantly different ($p<0.05$).

that there may be some direct relationship between certain isoenzyme patterns and cooking quality.

Clearly, not all isoenzymic variation can be related to hardening. For example, the unique peroxidase pattern produced by Line 85-12 is likely not involved in the hardening process because it was not also produced by San Martin, the hardest cultivar of the group. The stem esterase patterns present another problem; the unique pattern of San Martin is characterized by the absence of bands that were present in the softer cultivars, indicating that hard cultivars had no unique stem esterase isoenzymes which might be responsible for hardening.

The root esterase patterns, however, do appear to be linked to the hardening process. Both San Martin and Line 85-12 produced bands that were not observed in the softer cultivars. A possible explanation for the increased hardening of these two cultivars would be that the unique esterase isoenzymes that they possess are involved specifically in the demethoxylation of pectin. This would increase the number of sites on the pectin molecule capable of binding Ca^{2+} and Mg^{2+}, resulting in the formation of insoluble Ca^{2+} and Mg^{2+} pectate.

A similar explanation can be postulated for the unique, strongly staining acid phosphatase band (R_f = 0.94) found in the leaf of San Martin. If the band represents a phytase (phytase being an acid phosphatase), this would indicate that San Martin possesses a unique phytase isoenzyme capable of hydrolyzing phytic acid, releasing Mg^{2+} and Ca^{2+} for pectate formation in the middle lamella. This would explain why the cultivar San Martin was particularly susceptible to the HTC condition.

The major weakness with these hypotheses is that the isoenzyme patterns were observed in twelve day old seedling tissue and not in the seed where the hardening actually takes place. Nevertheless, it shows that San Martin and Line 85-12 do possess genes that code for unique esterase and acid phosphatase isoenzymes. The fact that they were not expressed in the seed extracts may be due to the isoenzymes being bound to other compounds in the seed and therefore not being extractable with the tris-malate extraction buffer. Another explanation may be that the production of demethoxylated pectin and the hydrolysis of phytic acid occurred during the early development of the seed and that by the time the seed was mature, the enzymatic activity responsible for hardening was more or less complete and the enzymes themselves degraded. This would explain the absence of the isoenzymes in the seed electrophoregrams. Any further hardening that occurred during storage would be attributable to the Ca^{2+} and Mg^{2+}, produced earlier by the phytase, slowly migrating from the cytoplasm into the middle lamellar region where they would help form pectate complexes.

In contrast to enzyme electrophoresis, much better cultivar discriminating power was achieved using seed proteins. Although no one protein extract was able to discriminate among all six cultivars, both the residual protein extraction and the reduced acetic acid extraction were able to distinguish five of the six cultivars (Table 4.11). All six cultivars could be uniquely identified when the reduced acetic acid extraction was used in conjunction with the sodium chloride extraction or in conjunction with the residual protein extraction. As was expected, no location effect was observed in any of the electrophoretic patterns. The literature holds only a few examples of location

affecting electrophoretic patterns, and when there has been an effect, it has not been strong enough to affect cultivar identification (Adriaanse, et al., 1969).

The cultivars Tamazulapa and Quetzal were the most difficult to distinguish with only the reduced acetic acid extraction able to identify differences. Tamazulapa also exhibited similar patterns to those of Line 85-12. The similar patterns of these cultivars can be explained by their pedigrees (Figure 3.1). Tamazulapa shares a parent with both Quetzal and Line 85-12, indicating that electrophoretic identification is most difficult with closely related black bean varieties.

This study indicates that seed protein electrophoresis is a practical and rapid method of black bean cultivar identification. In most cases, identification could be done within 36 hours using the acid-PAGE methodology. Closely related varieties however, might require additional extractions with different solvents to be correctly identified. An important advantage of seed protein electrophoresis over enzyme electrophoresis is that very little sample is required - a pattern may be produced from a single seed.

When the seed protein patterns were compared with the cooking quality of the different cultivars, some interesting trends were observed. Table 4.11 ranks the cultivars according to their cooking times at harvest and Table 4.12 according to the degree to which hardness increased during storage. In both cases, San Martin ranked significantly higher than all the other cultivars. San Martin also produced acetic acid soluble protein patterns that were markedly different than those of the other cultivars. In the reduced extract

TABLE 4.11

Protein electrophoretic patterns and mean cooking time (minutes) immediately after harvest.

Extraction	Cultivar					
	QUE	PEC	OST	TAM	L85	SAN
Time (min)	51.0a[1]	53.7ab	54.0ab	55.1ab	55.5ab	62.8b
0.1 M acetic acid, 1% ME	C	E	D	A	C	B
0.4 M NaCl	A	D	B	A	C	B
0.1 M acetic acid	A	D	C	A	A	B
Residual	A	E	D	A	C	B

[1] Means in the same row followed by different letters are significantly different ($p<0.05$).

TABLE 4.12

Protein electrophoretic patterns and increase in mean cooking time (minutes) after six months of storage at ambient conditions.

	Cultivar					
	OST	TAM	QUE	PEC	L85	SAN
Time (min)	8.0a[1]	9.7a	11.0a	11.0a	14.0a	26.3b
0.1 M acetic acid, 1% ME	D	A	C	E	C	B
0.4 M NaCl	B	A	A	D	C	B
0.1 M acetic acid	C	A	A	D	A	B
Residual	D	A	A	E	C	B

[1] Means in the same row followed by different letters are significantly different ($p<0.05$).

separated at acid pH, a very prominent band was present for San Martin at Rm = 46 (Figure 4.23). The residual pattern was not as markedly different from the other cultivars as the acetic acid pattern was, but it also showed a band (M.W. 28 500) unique to San Martin, as well as the absence of a band (M.W. 31 000) which was common to all other cultivars (Figure 4.29).

The small number of cultivars used makes it impossible to state any hard conclusions regarding a relationship between electrophoretic patterns and cooking quality. Nevertheless, this study did show that acetic acid patterns were distinctly different in the cultivar that required most cooking time. Since enzymatic processes are probably involved in the development of the HTC defect, the unique electrophoretic band observed in the hard cultivar may represent an enzyme involved in the hardening mechanism. Electrophoretic characterization of acid phosphatase showed that San Martin has a unique isoenzyme, not present in the other cultivars, that may hydrolyze phytic acid, freeing divalent cations for pectate formation in the middle lamella. One of the unique acetic acid soluble bands may represent this isoenzyme.

It is clear that electrophoresis of seed protein is a sensitive and a rapid method of cultivar identification, even within a specific colour class of Phaseolus vulgaris. There is also evidence that suggests electrophoretic patterns of fresh beans may be able to predict a cultivar's susceptibility to the HTC defect. However, acid phosphatase and esterase isoenzyme patterns appear to have more potential as predictors of HTC varieties than do seed protein electrophoretic patterns. More work, using a much greater number of

samples, needs to be done to confirm a relationship between electrophoretic results and cooking quality.

The absence of polyphenol oxidase and the minimal peroxidase activity detected in the cotyledon would suggest that the phytate pathway is more important in the hardening process than the lignin pathway. This is further supported by strongly staining cotyledon acid phosphatase and esterase patterns. strongly.

The effect of the location where the beans were grown may be ignored with regard to the electrophoresis, but it does have an effect on the initial hardness of the bean and possibly on the rate of hardening during storage. One might expect soil phosphorous to play a role in bean hardening since phytate is the major form of stored phosphorous in the plant. Since there was a significant interaction between genotype and location for initial hardness, it appears as though some cultivars are more susceptible to environmental variation than are others. Efforts to minimize the HTC defect through breeding will have to be specific to the region where the beans are grown.

5. EFFECT OF STORAGE ON BEAN ENZYME AND PROTEIN ELECTROPHOREGRAMS

5.1 Introduction

One of the most important quality characteristics of dry legumes is the cooking time required for the softening of the seed. During storage at high temperatures and relative humidities, legumes often develop the hard-to-cook (HTC) condition which results in a dramatic increase in the cooking time required to make the beans palatable (Kon and Sanshuck, 1981). Although the mechanisms involved in bean hardening are not fully understood, enzymatic processes are thought to play a considerable role. There is evidence that HTC beans exhibit less cell separation during cooking due to a build up of pectate or lignin-like compounds in the middle lamella which serve to cement the cotyledon cells together (Hincks and Stanley, 1986). The formation of both pectate salts and lignin complexes are thought to be the result of enzymatic reactions.

With respect to pectate formation, it has been proposed that the enzyme phytase hydrolyzes phytic acid into its constituent inositol and inorganic phosphorous, thereby releasing Ca^{2+} and Mg^{2+} cations. The cations are then free to combine with the pectin in the middle lamella of the bean to form an insoluble Ca^{2+}-Mg^{2+} pectate complex. To increase the number of sites available on the pectin molecule for binding the cations, the pectin may be demethoxylated by the action of pectin methyl esterase (Bhatty and Slinkard, 1989). Added phytic acid has been reported to significantly decrease cooking time in beans, presumably because it chelated the free divalent cations, preventing pectate formation (Kon and Sanshuck, 1981). Vindiola *et al.* (1986) found that hardening could be almost completely prevented by using sodium fluoride

to inhibit phytase activity. Jones and Boulter (1983b) reported that the degree of pectin esterification in black beans dropped from 51% to 15% over 6 months of storage under conditions that promoted the development of the HTC condition, giving the free divalent cations more sites to bind pectin. Other researchers, however, have found no evidence for the role of pectate formation in hardening of beans (Kon, 1968; Wassimi et al., 1978; Proctor and Watts, 1987).

Using microscopy, Hincks and Stanley (1987) reported that HTC beans had a build up of a lignin-like compound in the middle lamellar region of the cotyledon cells. An enzymatic mechanism was proposed that suggests aromatic amino acids resulting from proteolysis serve as precursors for the formation of lignin via the actions of peroxidase and polyphenol oxidase. The existence of protease activity in the bean seed has been well established (Nielsen, 1988). Correspondingly, both gel filtration and electrophoresis have shown that the degradation of seed proteins accelerates under adverse storage conditions, presumably due to increased protease activity (Hohlberg and Stanley, 1987; Hussain et al., 1989c). Goodwin and Mercer (1972) have given evidence that free aromatic amino acids, such as tyrosine and phenylalanine, serve as precursors to lignin monomers. Although peroxidase activity occurs in the bean cotyledon (Hohlberg and Stanley, 1987), the data concerning polyphenol oxidase is contradictory. Elias (1982) reported significant levels polyphenol oxidase activity while Hohlberg and Stanley (1987) observed no activity at all. There have been other recent studies that have reported no increase in lignin in beans that hardened significantly during storage (Srisuma et al., 1989; Rozo et al., 1990).

All the enzymes involved in pectate and lignin formation have been

shown to exist in multiple molecular forms in beans (Table 2.1). It may be that only one isoenzyme of any one group is involved in the hardening process. The identification of the particular isoenzymes involved in the hardening process would be a step toward identifying the possible genes responsible for making some black bean varieties more prone to the HTC defect than others. The identification of the specific proteins degraded during storage under hardening conditions would also be useful in validating the lignin formation hypothesis. The objectives of this study, therefore, were to determine the stability of isoenzyme electrophoretic patterns during the hardening process and to identify the seed proteins degraded during storage.

5.2 Materials and Methods

The samples used in this experiment were obtained from Carolina de Godinez at the Institute of Nutrition of Central America and Panama. Information regarding the hardness, phytate levels, and dietary fibre fractions of the samples has been published in her Master of Science thesis (Godinez, 1990). The hardness results presented on pages 101-105 of this thesis are the work of Godinez.

5.2.1 Beans and Storage Procedure

Two varieties of dry beans (Phaseolus vulgaris L.) were collected from the August 1988 harvest in Guatemala. Black beans, var. Tamazulapa, were bought from farmers in Jutiapa. Red beans, Line DOR-364, were obtained from the CIAT-ICTA research station, also in Jutiapa. Both varieties were divided into two lots and the moisture content of the cleaned beans was adjusted to 12% and 16% using a Dole

40-B Moisture Tester. Each moisture treatment was then divided into three lots which were packaged in sealed polyethylene bags and stored at 9°, 23°, and 36°C for 24 weeks.

5.2.2 Hardening Tests

Sixty grams of beans were soaked in 300 ml distilled water at 23°C for 18 hours. Soaking water was discarded. Beans were cooked in boiling distilled water for two hours and subsamples of 30 g drained beans were set aside. Cooked beans were allowed to cool for one to two hours, and then hardness was determined with an Ottawa Texture Measuring System (OTMS)[1] using a 10 cm^2 extrusion cell[1] and a 454.5 kg loadcell. The OTMS was attached an Apple II-E system.

The OTMS was calibrated to give an experimental maximum force of ±90 kg. The experimental time was set at 30 s. The cross head speed was set at 6.60 cm/min and the plunger was calibrated to descend to 1 mm. Required calibrations were done every time before starting the compression/extrusion test in each sample. Hardness was measured as the peak force in newtons to extrude 30 g cooked sample, using the ESRI Texture Program (1986)[2].

The soaking, cooking, and testing procedure was repeated on the following day to give replicate results. Samples were taken every three weeks during the 24 weeks of storage.

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[1] Canners Machinery Limited, P.O. Box 190, Simcoe, Ontario.

[2] Engineering and Statistical Research Institute (ESRI), Agriculture Canada, Ottawa.

5.2.3 Electrophoresis

Electrophoretic analyses were performed on fresh samples and after 3, 9, 15, and 24 weeks of storage.

5.2.3.1 Enzyme Electrophoresis

The procedure for sample preparation and the electrophoresis of enzyme extracts is based on the methods of Hussain et al. (1988b) with some minor modifications. Three beans from each treatment were soaked overnight in distilled water and the seed coats removed the following morning. Samples were crushed thoroughly in cold extraction buffer using a glass rod as a pestle. For starch gel electrophoresis, enough extraction buffer was used to produce a pasty consistency. For polyacrylamide gel electrophoresis, slightly more buffer was used so that a thin layer of supernatant would form when the sample was allowed to stand for several minutes. The extracts were kept on ice until applied to the gel. The extraction buffer used was a 0.1 M tris-maleate solution, pH 7.4, containing 20% glycerol, 5% soluble PVP-40, 0.5% Triton X-100, and 14 mM dithiothreitol. The Triton X-100 and dithiothreitol were added just before use.

Peptidase and acid phosphatase gave best results when separated on a 10.6% starch gel using a tris-citrate/lithium borate buffer (Table 3.2). Running current was initiated at 10 mA and gradually increased to 35 mA. Electrophoresis was terminated when the front had travelled approximately 10 cm. The gel was sliced into 1.5 mm slabs for staining.

Peroxidase and polyphenol oxidase were separated on a 10% polyacrylamide gel while esterase was separated on a 12% polyacrylamide gel. A 3% stacking gel was used in all cases. Optimal amounts of

extract needed for electrophoresis were determined to be 30 ul for polyphenol oxidase and peroxidase stains and 20 ul for the esterase stain. Electrophoresis was initiated at 50 V and gradually increased (50 V every 30 min) to 250 V. Total run time was approximately 6 hours.

The staining procedures used to identify the isoenzymes have been described previously: acid phosphatase and peroxidase (Hussain et al., 1988b); esterase (Hussain et al., 1987); polyphenol oxidase (Choi et al., 1987); peptidase (Harris and Hopkinson, 1976).

5.2.3.2 Seed Protein Electrophoresis

Seeds were soaked overnight in distilled water to facilitate the removal of the seed coats. The seed coat and embryo were removed the following day and the beans allowed to dry overnight in a convection oven at 37°C. The dried beans were milled with a Wiley Mill[3] through a 40 mesh screen.

Two extraction procedures were used. The first was performed by mixing 100 mg of ground sample with 400 ul of 5 M acetic acid. The mixtures were vortexed for 3 minutes and allowed to incubate at 40°C for one hour. After centrifugation at 15 600 X G for 20 minutes at room temperature, the supernatant was mixed (1:1) with a solution of 40% sucrose and 0.5% methyl green in aluminum lactate buffer, pH 3.1. Fifteen ul of the resulting solution was used for electrophoresis. Electrophoresis in 10% polyacrylamide gels at pH 3.1 was according to Hussain et al. (1989b). The gels were stained overnight in a filtered solution of 0.2 g Coomassie Blue R-250 (dissolved in 10 ml of 95% ethanol) in 240 ml of 12% trichloroacetic acid. The stained gels were

[3] Arthur H. Thomas Co., Philadelphia PA.

destained with 12% trichloroacetic acid (Sapirstein and Bushuk, 1985).

Phaseolins were extracted by vortexing 100 mg of ground sample with 3 ml 0.4 M NaCl, pH 2.4 for one min. The mixture was allowed to stand for one hour, followed by centrifugation at 20 000 X G for 20 min at 10°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on an LKB 2001 electrophoresis unit according to the procedure of Laemmli (1970) with some modifications. The dissociating buffer was 0.625 M tris-HCl, pH 6.8, containing 40% sucrose, 2% SDS, 1% mercaptoethanol, 2 mM EDTA, and 0.01% bromophenol blue (Brown et al., 1982). Six ul of the dissociated sample was applied to the gel. The separating gel concentration was 14%. Voltage was kept at 50 until the tracking dye had moved into the separating gel. Voltage was then increased by 50 every 30 min up to 250 V. The current was limited to a maximum of 70 mA. When the tracking dye reached the bottom of the gel, the slabs were removed and stained overnight in 0.25% Coomassie Blue R-250 in 50% methanol, 1% acetic acid. Gels were destained with a methanol:acetic acid:water (6:1:14 v/v/v) solution until the background was clear.

5.3 Results and Discussion

5.3.1 Bean Hardness

Figures 5.1 and 5.2 show hardening across storage time for the black beans. Beans stored at 36°C showed the greatest increase regardless of initial moisture content. Beans with an initial moisture content of 16% had higher hardening curves than those with a moisture content of 12%. Figures 5.3 and 5.4 show that the same trends were

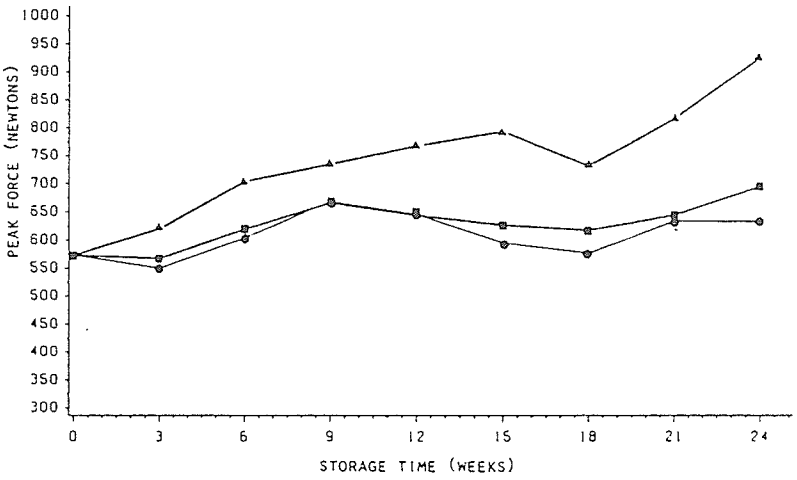

Figure 5.1 Hardness of red beans measured after two hour cooking time. Initial moisture content 12%. Samples stored at: ● 9°C; ■ 23°C; ▲ 36°C.

Source: Godinez (1990)

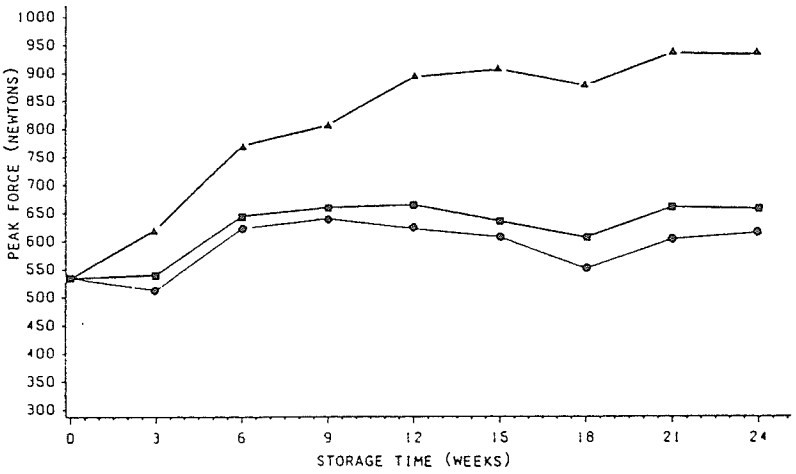

Figure 5.2 Hardness of red beans measured after two hour cooking time. Initial moisture content 16%. Samples stored at: ● 9°C; ■ 23°C; ▲ 36°C.

Source: Godinez (1990)

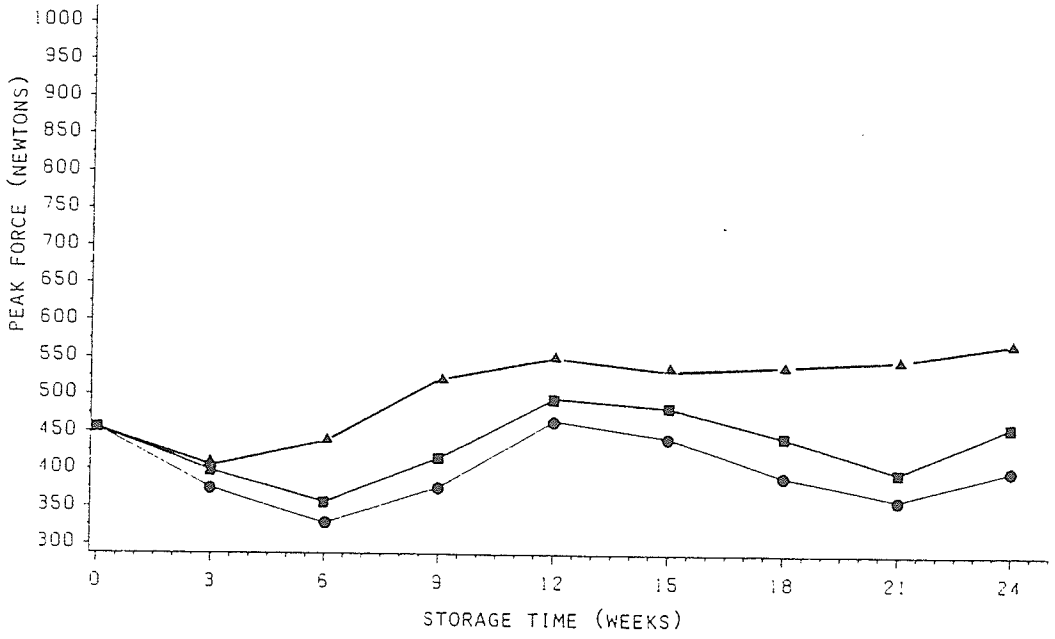

Figure 5.3 Hardness of black beans measured after two hour cooking time. Initial moisture content 12%. Samples stored at: ● 9°C; ■ 23°C; ▲ 36°C.

Source: Godinez (1990)

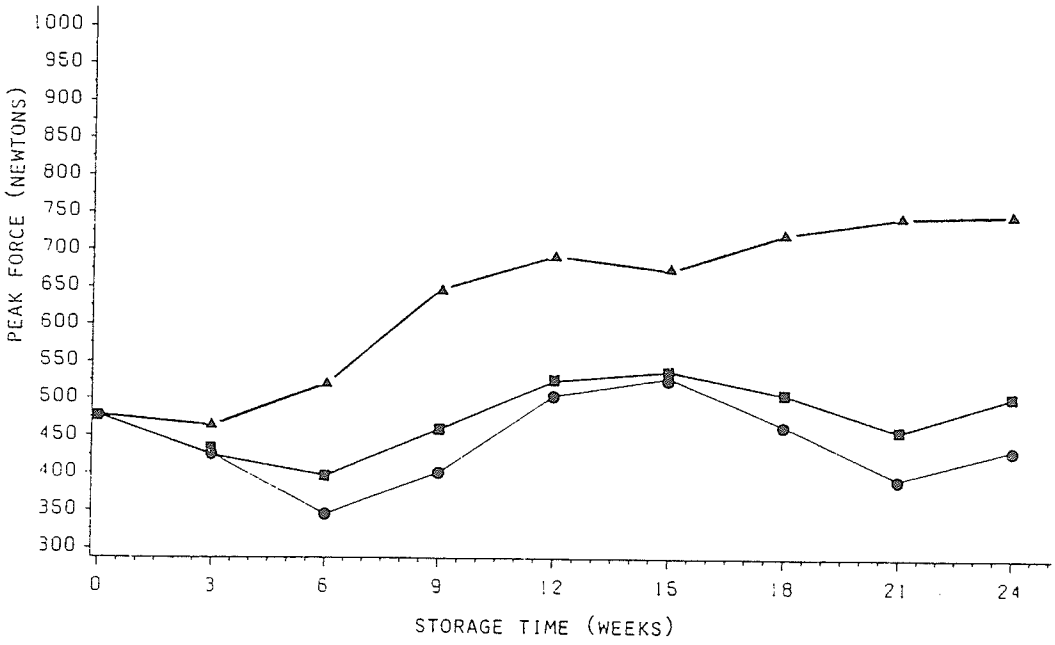

Figure 5.4 Hardness of black beans measured after two hour cooking time. Initial moisture content 16%. Samples stored at: ● 9°C; ■ 23°C; ▲ 36°C.

Source: Godinez (1990)

TABLE 5.1

Hardness main effects of pooled samples averaged across time.[1]

Main Effects	F	p
Variety	3862.96	0.0001
Moisture Content	194.05	0.0001
Temperature	1105.20	0.0001
Variety/Moisture Content	88.25	0.0001
Variety/Temperature	12.18	0.0001
Moisture Content/Temperature	86.46	0.0001
Variety/Moisture/Temperature	0.23	0.7910

[1] F = F ratio; p = probability. Based on Type III Sums of Squares.

Source: Godinez (1990)

TABLE 5.2

Hardness time effects of pooled samples.[1]

Time Effects	F	p
Week	142.17	0.0001
Variety/Week	31.63	0.0001
Temperature/Week	37.28	0.0001
Moisture Content/Week	8.19	0.0001
Variety/Temperature/Week	4.52	0.0001
Variety/Moisture Content/Week	1.80	0.0773
Moisture Content/Temperature/Week	3.65	0.0001
Variety/Moisture/Temperature/Week	1.02	0.4406

[1] F = F ratio; p = probability. Based on Type III Sums of Squares.

Source: Godinez (1990)

exhibited by the red beans although initial hardness was somewhat higher than for the blacks.

Analysis of the pooled data indicates that bean variety, moisture content, and storage temperature had significant effects on hardness (Table 5.1). The significant moisture content-variety interaction indicated that moisture effects should be interpreted separately for each variety. Table 5.2 shows a strong time effect on hardness. Bean variety, storage temperature, and moisture content had significant effects on the time effect as well, indicating that storage time should also be considered separately for each variety.

This study demonstrates a strong effect of temperature on hardness of beans with the effect of moisture content not as strong. Hardness of both varieties of beans increased when stored at 36°C, regardless of initial moisture content. Bean hardening and storage studies reported in the literature have shown similar increases in hardening resulting from higher temperature and moisture content during storage (Hincks and Stanley, 1987; Hohlberg and Stanley, 1987; Moscoso et al., 1984). This supports the hypothesis that the hardening process is enzymatic since higher temperature and moisture levels would promote enzyme activity.

5.3.2 Electrophoresis

5.3.2.1 Enzyme Electrophoresis

The enzymes of the pectate pathway were represented by acid phosphatase and esterase. Phytase is an acid phosphatase (Lolas and Markakis, 1977), but because there are other acid phosphatases, not all bands present in the gel may have phytase activity. Similarly, not all esterase bands may be specifically pectin methyl esterase isoenzymes. Both acid phosphatase and esterase were present in the cotyledon. Acid phosphatase gels showed one large region of activity in the area of R_f = 0.25 and a more distinct band with an R_f value of 0.42 (Figure 5.5). In some gels it was possible to see that the large acid phosphatase region was actually made up of 5 distinct bands. These 5 bands were evident only in samples stored at conditions of high temperature, although they were not always evident at these conditions. The reason for this is not clear. Seven esterase bands were observed. Esterase patterns were variable but no trends could be identified.

Of the three enzymes involved in the proposed lignification pathway, only peptidase showed a significant amount of activity. Two peptidase bands (R_f = 0.38, 0.54) were present in both cultivars (Figure 5.5). Peroxidase also produced two bands (R_f = 0.27, 0.33) but they were faint and not consistent. No polyphenol oxidase activity was observed. The patterns were not affected by storage.

No one isoenzyme band was notably more intense in the bean samples held at high temperature and high moisture levels although these beans hardened significantly. For this reason, it was not possible to identify specific isoenzymes involved in the enzymatic hardening

mechanisms. The weak peroxidase bands and lack of any detectable polyphenol oxidase activity would suggest that pectate formation is more apt to occur in the cotyledon than is lignin formation.

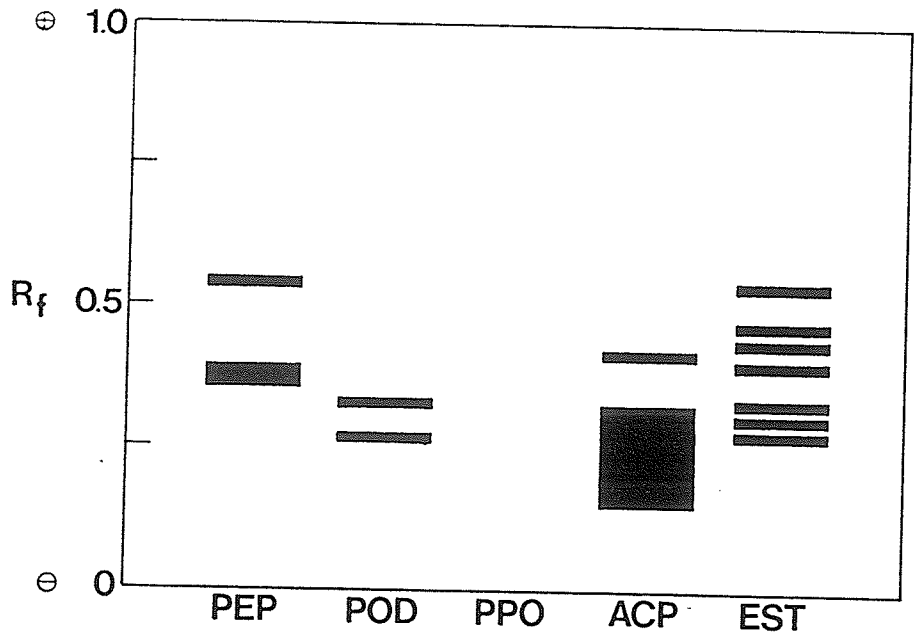

Figure 5.5 Representation of peptidase (PEP), peroxidase (POD), polyphenol oxidase (PPO), acid phosphatase (ACP), and esterase (EST) isoenzyme patterns observed in the cotyledon of red and black beans.

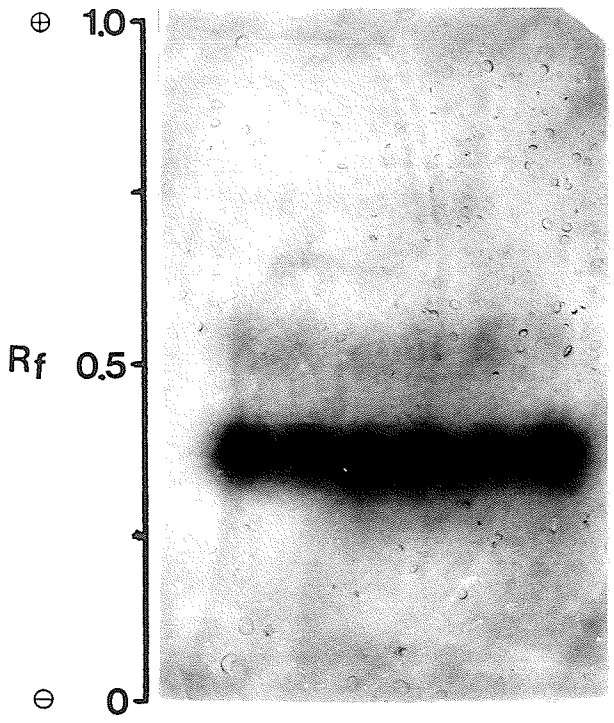

Figure 5.6 Red bean peptidase isoenzyme patterns after 24 weeks of storage. Storage conditions from left to right: 9°C/16% moisture; 36°C/16% moisture; 23°C/12% moisture; 9°C/12% moisture; 23°C/16% moisture; 36°C/12% moisture.

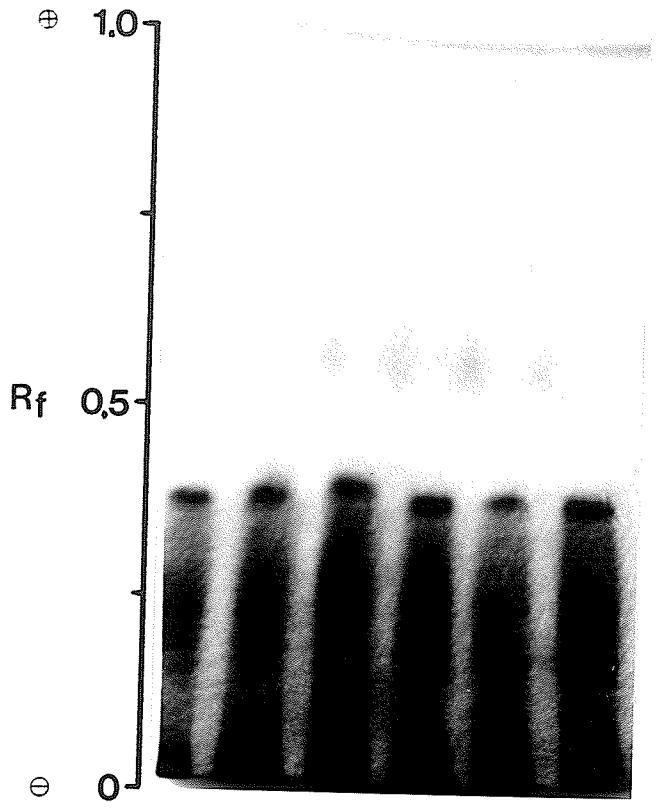

Figure 5.7 Black bean acid phosphatase isoenzyme patterns after 15 weeks of storage. Storage conditions from left to right: 23°C/16% moisture; 36°C/12% moisture; 36°C/16% moisture; 9°C/16% moisture; 23°C/12% moisture; 9°C/12% moisture.

5.3.2.2 Seed Protein Electrophoresis

A total of 19 bands were observed in the 5 M acetic acid extraction. The four least mobile bands were not consistently present in all samples (Figure 5.8). In the gels shown, the four bands in the red beans appear to be increasing in intensity during storage but this trend was not reproducible. At the low temperature and moisture levels, all other bands remained stable over storage. However, at high temperature and moisture levels, two bands (Rm = 55 and 59) clearly weakened in intensity over 24 weeks of storage (Figure 5.9). A third band (Rm = 63) weakened in intensity during storage in the black bean samples but did change appreciably in the red beans. The rest of the pattern was not affected by storage conditions or by the length of storage.

These results confirm earlier reports that 5 M acetic acid extractable proteins show changes over storage under conditions of high temperature and humidity (Hussain et al., 1989c). Using gel filtration, Hohlberg and Stanley (1987) suggested that it was the phaseolins that were being degraded during storage. Chang and Harrold (1988) used SDS-PAGE to demonstrate the breakdown of phaseolins in beans during germination. However, after 24 weeks of storage, this study found no changes were evident in the SDS-PAGE phaseolin patterns at any storage condition (Figure 5.10). This indicates that the proteins that were degraded in the 5 M acetic acid extraction were not phaseolins, but some other cotyledon protein.

Although protein degradation occurred in conjunction with the development of the HTC condition, the low levels of peroxidase and polyphenol oxidase activity would indicate that the free amino acids

resulting from proteolysis were not serving as precursors for lignification. Other researchers have also found little evidence for the role of lignification in the development of the HTC condition (Srisuma et al., 1989; Rozo et al., 1990). It may be that these proteins are being degraded preferentially as a source of nitrogen to maintain respiration or they may be involved in Maillard browning which can occur in beans during storage (Powrie, 1990). No browning was actually observed during storage. The low levels of peroxidase and polyphenol oxidase would indicate, however, that the development of the HTC condition is more the result of a build up of pectate in the middle lamella than the formation of lignin.

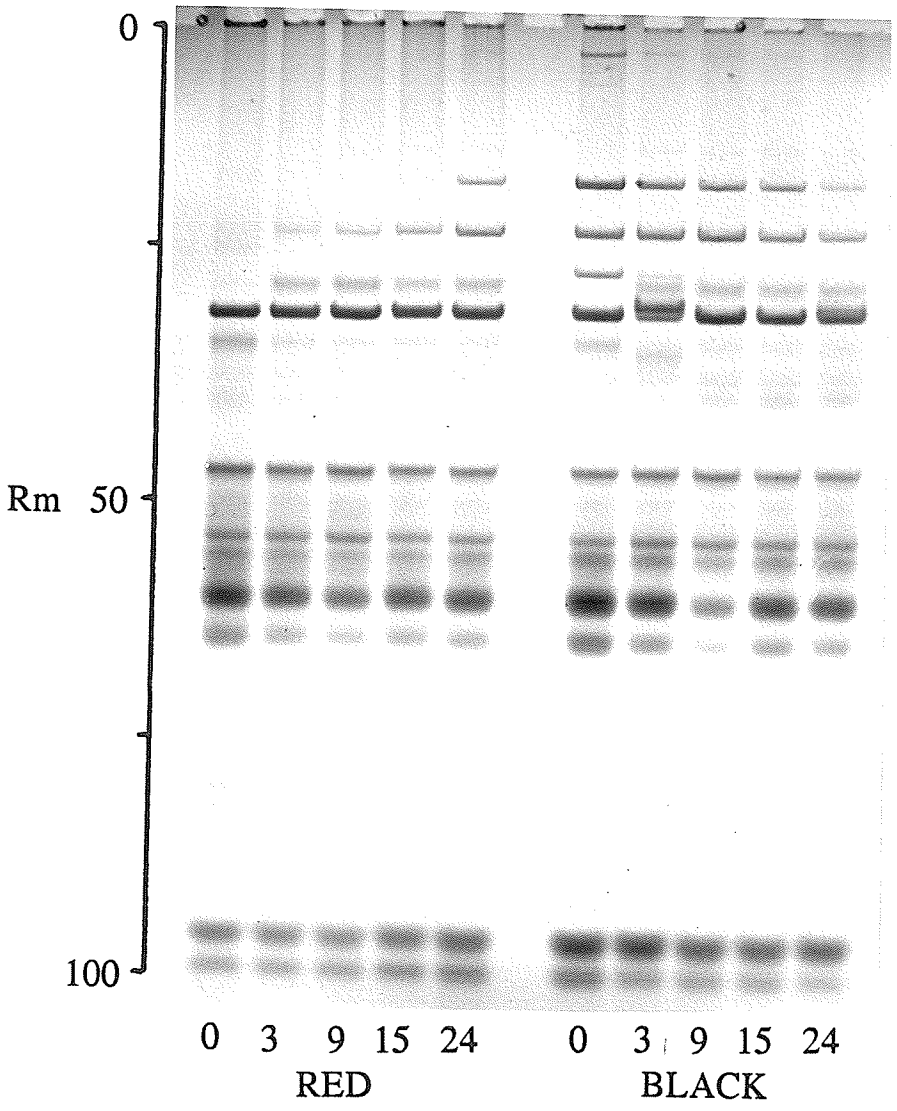

Figure 5.8 Acid-PAGE patterns of 5 M acetic acid soluble proteins from red and black beans stored at 9°C/12% moisture for 0, 3, 9, 15, and 24 weeks.

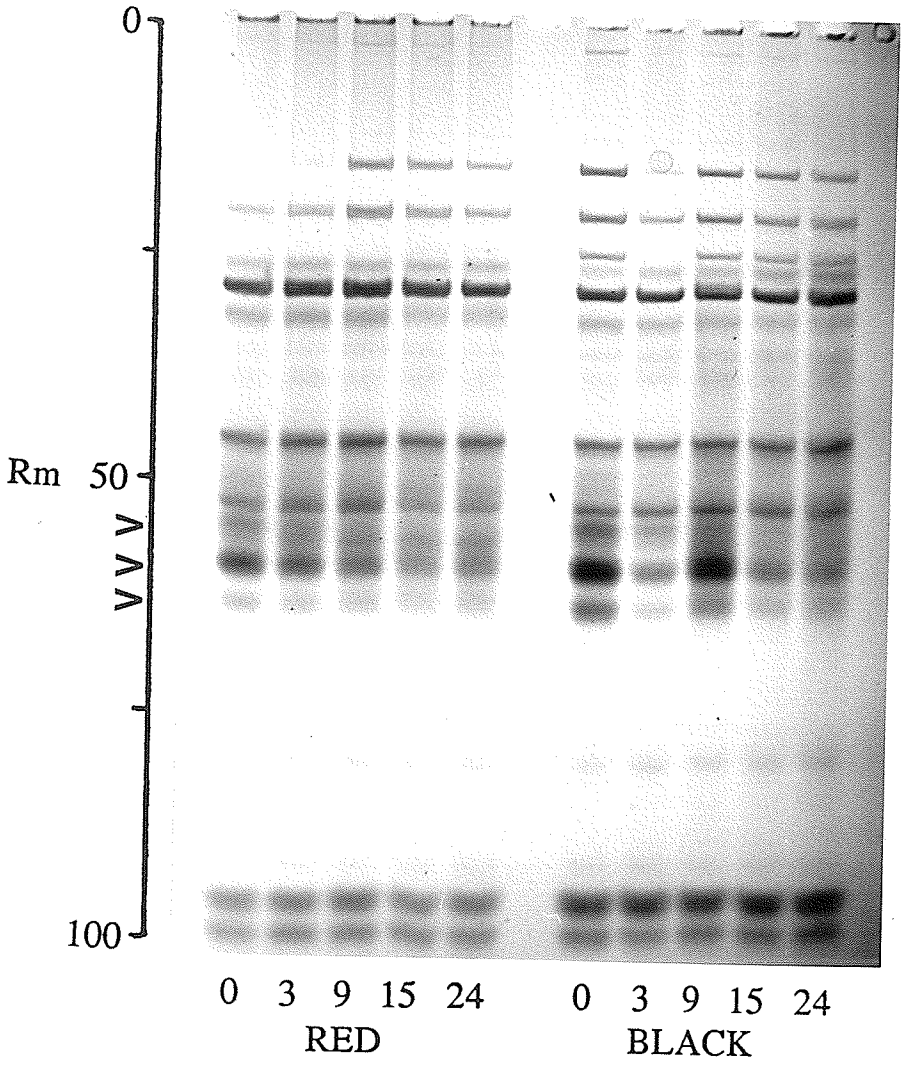

Figure 5.9 Acid-PAGE patterns of 5 M acetic acid soluble proteins from red and black beans stored at 36°C/16% moisture for 0, 3, 9, 15, and 24 weeks.

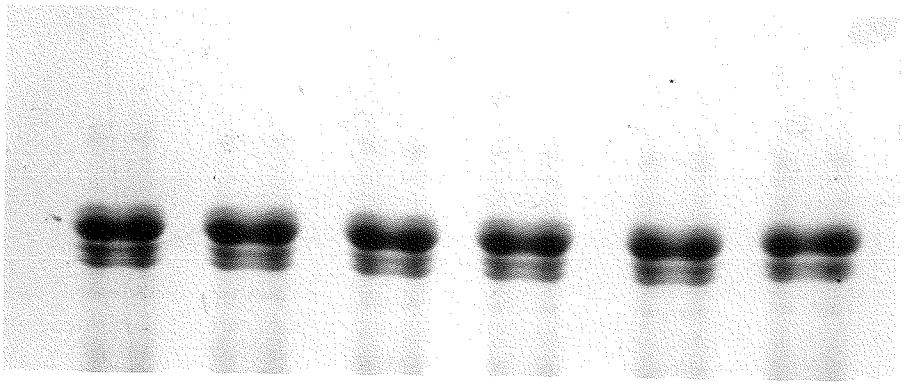

Figure 5.10 SDS-PAGE patterns of phaseolin subunits from black beans stored for 24 weeks under various conditions. Left to right: 9°C/12% moisture; 9°C/16% moisture; 23°C/12% moisture; 23°C/16% moisture; 36°C/12% moisture; 36°C/16% moisture.

6. SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 Summary

Studies were conducted to investigate the feasibility of using electrophoresis for the identification of morphologically indistinguishable black bean cultivars. Investigations were also conducted to determine the relationship between electrophoretic patterns and the development of the HTC defect in black beans.

Six black bean cultivars were grown in three distinct locations in Guatemala. Cultivar and location were found to significantly affect the time required to cook freshly harvested beans. A significant interaction between cultivar and location indicated that cookability of a cultivar is specific to the location where it is grown. When beans with a moisture content of approximately 14% were stored at ambient conditions, cooking time of all cultivars increased. The increase in cooking time was significantly affected by the cultivar.

Isoenzyme patterns were produced electrophoretically from extracts from the cotyledon and from the leaf, stem, and root tissue of 12 day old seedlings. Leaf peroxidase, leaf acid phosphatase, stem esterase, root peroxidase, and root esterase patterns exhibited reproducible cultivar variation. Only two of the six cultivars could be uniquely identified on the basis of isoenzyme patterns alone. These two cultivars (San Martin and Line 85-12) required significantly more cooking time than the other cultivars, suggesting a possible link between acid phosphatase and esterase isoenzyme complements and the susceptibility of a cultivar to the HTC defect. Isoenzyme electrophoretic patterns do not, however, appear to be useful for cultivar identification.

With respect to the electrophoresis of general seed proteins, patterns produced from residual proteins and from proteins soluble in 0.1 M acetic acid containing 1.0% mercaptoethanol were able to distinguish five of the six cultivars. All six cultivars could be uniquely identified when the reduced acetic acid extraction was used in conjunction with the sodium chloride extraction or with the residual protein extraction. San Martin, the slowest cooking cultivar, exhibited a unique acetic acid soluble protein, but no clear overall trend was observed between cooking time and seed protein electrophoretic patterns. No location effect was observed.

In a related study, the stability of electrophoretic patterns of two bean cultivars was monitored over storage under several conditions. A black variety (Tamazulapa) and a red variety (DOR-364) were stored with two different initial moisture levels (12% and 16%) and at three different temperatures (9^{o}C, 23^{o}C, and 36^{o}C) for 24 weeks.

The hardness of DOR-364 was consistently greater than that of Tamazulapa throughout the study. Temperature had the greatest effect on hardening with high temperature promoting hardening. High moisture levels also promoted hardening during storage.

Peptidase, peroxidase, polyphenol oxidase, acid phosphatase, and esterase patterns remained stable throughout storage at all conditions. With regard to seed proteins, several 5 M acetic acid soluble protein bands weakened in intensity over time in beans stored under conditions of high temperature and moisture. These change were not evident in beans stored at cooler temperatures. No changes were evident in the phaseolin patterns under any storage condition.

6.2 Conclusions

1. Cooking time of dry beans was affected by genotype as well as by the location where the beans were grown.

2. Cotyledon protein electrophoresis was effective for the identification of morphologically indistinguishable black bean varieties. Isoenzyme electrophoretic patterns, however, did not exhibit enough variation to be used for cultivar identification.

3. Acid phosphatase and esterase isoenzyme electrophoretic patterns appear to be related to cooking quality. Hard cultivars had unique isoenzymes.

4. With the exception of cotyledon esterase, none of the isoenzyme or seed protein electrophoretic patterns were affected by the location where the sample was grown.

5. Some specific 5 M acetic acid soluble seed protein bands weakened in intensity during storage at high temperature and moisture levels. These proteins were not affected at lower temperatures. All other patterns were stable over time for a period of 24 weeks.

6. The low level of cotyledon peroxidase activity and the lack of detectable polyphenol oxidase activity suggest that pectate build up in the middle lamella is a more plausible explanation for the development of the HTC defect than is lignification.

6.3 Recommendations for Future Research

1. There is fairly conclusive evidence that both genotype and location can affect the cooking time of freshly harvested dry beans (Ghaderi et al., 1984; Hosfield et al., 1984). There are implications in the literature that the same factors would affect the development of the HTC defect during storage, however, no conclusive study has yet been published. A study investigating the genotypic and locational effects on the development of the HTC condition during the post-harvest storage of dry beans would be useful in evaluating the worth of pursuing a breeding program to improve cookability. A set of 10 to 15 varieties that have been well characterized with respect to cooking quality over several seasons, locations, and storage conditions would be extremely useful for further research into the genetic effect on the development of the HTC defect.

2. This study indicated a possible relationship between esterase and acid phosphatase isoenzyme patterns and cooking time. The isoenzyme patterns of a much larger number of bean varieties need to be known in order to establish a statistically valid correlation between isoenzyme patterns and cooking times. By limiting the varieties to the black color class, genetic variation not related to cooking quality could be kept to a minimum.

3. The GBC salt acid phosphatase stain did not produce good quality results on polyacrylamide gels and resolution was poor on starch gels. Despite the poor quality of the results, acid phosphatase patterns

still showed promise as predictors for the development of HTC defect. A better staining procedure, preferably specific to phytase, may give even more dramatic results. One possibility would be to use high performance capillary electrophoresis (HPCE) to separate bean extracts and analyze each fraction for phytase activity. The small volumes required for HPCE may make the determination of phytase activity impractical however.

4. Since the peroxidase and polyphenol oxidase stains appeared to produce identical patterns, the specificity of the two assays should be investigated.

5. Because of the tedious nature of visually comparing electrophoregrams, new cultivar identification techniques should be developed using HPLC or HPCE which would allow for the easy digitization of results making computer assisted cultivar comparisons much more convenient.

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APPENDIX A

COOKING TIME OF BEANS IMMEDIATELY AFTER HARVEST - RAW DATA

Mean cooking time (minutes) of five seeds using the Mattson Cooker

I. Jutiapa, first harvest.

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	65.0	55.8	61.0	60.6
San Martin	62.0	57.8	62.2	60.7
Quetzal	55.4	44.6	52.8	52.3
Line 85-12	58.8	68.0	54.6	60.5
Ostua	66.0	61.2	58.8	62.0
Pecho Amarillo	66.2	56.8	60.6	61.2
				59.7

II. Jutiapa, second harvest.

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	62.0	53.0	61.2	58.7
San Martin	65.4	70.6	64.8	66.9
Quetzal	55.5	54.0	66.0	58.5
Line 85-12	62.0	66.0	71.4	66.5
Ostua	61.8	64.2	54.2	60.1
Pecho Amarillo	58.6	62.6	60.8	60.7
				61.9

III. INCAP Farm, first (only) harvest.

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	52.8	56.2	55.6	54.9
San Martin	52.0	50.0	58.0	53.3
Quetzal	48.2	42.8	48.4	46.5
Line 85-12	51.0	51.6	54.6	52.4
Ostua	45.4	51.2	54.4	50.3
Pecho Amarillo	47.5	46.4	34.6	42.8
				50.0

IV. Greenhouse, first harvest.

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	50.6	51.0	51.0	50.9
San Martin	72.2	70.2	60.0	67.5
Quetzal	48.2	49.0	48.2	48.5
Line 85-12	53.8	51.8	47.0	50.9
Ostua	50.6	50.0	51.0	50.5
Pecho Amarilo	57.2	54.4	50.4	54.0
				53.7

V. Greenhouse, second harvest.

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	49.0	51.4	50.2	50.2
San Martin	60.8	60.0	76.0	65.6
Quetzal	48.2	48.8	50.4	49.1
Line 85-12	44.2	53.2	44.8	47.4
Ostua	50.8	44.2	46.2	47.1
Pecho Amarillo	51.1	53.6	45.4	50.0
				51.6

APPENDIX B

COOKING TIME OF BEANS AFTER STORAGE - RAW DATA

Mean cooking time (minutes) of five seeds using the Mattson Cooker

I. Jutiapa, first harvest (storage time approximately 6 months).

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	73.2	67.4	70.4	70.3
San Martin	86.8	92.2	82.0	87.0
Quetzal	67.0	63.2	59.6	63.3
Line 85-12	74.0	71.0	79.8	74.9
Ostua	74.4	63.4	72.2	70.0
Pecho Amarillo	83.2	70.6	62.8	72.2
				73.0

II. Jutiapa, second harvest (62 day storage).

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	56.6	63.2	61.2	60.3
San Martin	59.4	70.6	69.2	66.4
Quetzal	59.0	62.2	57.8	59.7
Line 85-12	63.4	60.8	68.0	64.1
Ostua	59.0	60.0	64.6	61.2
Pecho Amarillo	60.2	58.8	60.4	59.8
				61.9

III. INCAP Farm, first harvest (62 day storage).

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	63.8	59.8	63.0	62.2
San Martin	66.8	59.2	54.4	60.1
Quetzal	51.8	54.0	61.6	55.8
Line 85-12	57.4	55.1	56.2	56.2
Ostua	56.6	53.8	57.2	55.9
Pecho Amarillo	52.6	59.8	57.2	56.5
				57.8

IV. Greenhouse, first harvest (storage time unknown).

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	50.0	50.6	45.2	48.6
San Martin	52.8	58.6	52.2	54.5
Quetzal	43.8	48.2	43.8	45.3
Line 85-12	44.4	54.2	54.0	50.9
Ostua	48.2	48.2	50.0	48.8
Pecho Amarillo	52.2	51.2	46.4	49.9
				49.7

V. Greenhouse, second harvest (storage time 157 days).

Cultivar	Trial A	Trial B	Trial C	Mean
Tamazulapa	47.4	53.4	51.4	50.7
San Martin	57.4	59.4	61.4	59.4
Quetzal	54.4	53.4	53.4	53.7
Line 85-12	52.6	55.6	53.6	53.9
Ostua	50.8	52.6	51.4	51.6
Pecho Amarillo	57.0	51.4	50.6	53.0
				53.7

APPENDIX C

COTYLEDON NITROGEN - RAW DATA

Cultivar	Location			Mean
	Jutiapa	INCAP Farm	Greenhouse	
Tamazulapa	22.10	17.99	29.86	
	26.81	16.60	26.22	
Mean	24.46	17.30	28.04	23.26
San Martin	23.24	25.70	23.96	
	27.57	20.52	24.43	
Mean	25.41	23.11	24.20	24.24
Quetzal	23.46	22.09	26.12	
	24.52	22.25	26.12	
Mean	23.99	22.17	25.83	24.00
Line 85-12	19.40	29.28	24.58	
	19.62	25.74	24.61	
Mean	19.51	27.51	24.60	23.87
Ostua	20.55	21.15	24.02	
	20.85	27.74	23.04	
Mean	20.70	24.45	23.53	22.89
Pecho Amarillo	27.00	21.51	24.47	
	26.40	21.47	25.12	
Mean	26.70	21.49	24.80	24.33
Overall Means	23.46	22.67	25.17	23.77