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RESEARCH REPORT

Comparative Analysis of Steady State
Protein Profiles of Drought-Hardened and
Heat-Hardened Phaseolus acutifolius (Tepary Bean)
and Phaseolus vulgaris (Common Bean)

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Comparative Analysis of Steady State Protein Profiles of Drought-Hardened and Heat-Hardened *Phaseolus acutifolius* (Tepary Bean) and *Phaseolus vulgaris* (Common Bean)

I. Introduction

The hardening process is a response of some plants to mild heat stress and limited drought stress. As a result of such induced stress, certain physiological and biochemical changes occur in these plants that enable them subsequently to tolerate a greater degree of stress caused by environmental factors, such as heat and drought (2,5). Atmospheric scientists believe that the earth is entering an era that will be hotter and drier. Different countries of the globe have experienced drought and heat cycles. Both heat and drought have caused very grave economic hardships in some countries and food shortages and famines in some other less developed countries. For example, the United States experienced one of the worst droughts in 1988, and the U.S. Congress had to appropriate more than $3.8 billion for drought related agricultural programs.

Understanding the fundamental biology of stress is of tremendous interest to plant breeders and plant biologists in determining which parent or progeny harbors genes that enable plants to adapt to heat and drought stress. Recent molecular biological approaches are expected to help biologists bioengineer plants for traits that ensure their tolerance to heat stress, drought stress, and some other types of environmental stress.

Phaseolus vulgaris is a major grain legume of Eastern Africa, Asia, and South America. The yields in Africa are relatively low with the national average in Kenya only 500 kg/ha. It is very poorly adapted to desert regions.

Its substantial degree of reproductive affinity does not exist among the species within Genus Phaseolus (10). Phaseolus acutifolius (Tepary Bean) is a narrow-leaved legume (Appendix A), which is drought tolerant, heat resistant, ozone-smog tolerant, disease resistant, salt tolerant, and has been grown in the poorest soils in Arizona, Mexico and Africa (1,5,8,11,16,17). The Tepary Bean has a potential for inter-specific hybridization with Phaseolus vulgaris (Common Bean) for drought tolerance and heat resistance (12,18). For this reason the Tepary Bean is included in this model system study.

It has been suggested that high temperature hardening of the Tepary Bean might be related to increased thermal stability of enzymes (5). Heat shock is known to induce the production of a certain set of proteins in plants (4). Little is known, however, about drought stress-induced proteins except that some of the heat shock proteins identified in soybeans are also induced by drought stress (4). It is not known if these proteins are responsible for resistance to both heat and drought stresses. Stress proteins may play a role in sensing environmental stress and triggering defense mechanisms.

Characterization and cataloguing of proteins is the first step towards identifying and isolating genes for drought and heat stress proteins. A better understanding of the physiology, biochemistry and development of stressed plants is needed if stress tolerant plants are to be bioengineered. Because the desert-adapted Tepary Bean and the heat and drought-susceptible Common Bean are closely related, they offer a prospective system with which to make such a transfer.

II. The Objectives of the Study

The objectives of this study are to:

A. Characterize and catalogue changes in the protein profiles of Tepary Bean plants upon subjection to drought and heat stresses and;

B. Compare to changes in the protein profiles of Common Bean Plants, which are less tolerant to drought and heat, when exposed to the same drought and heat stress.

III. Materials and Methods

This study was initiated during early March and completed during mid-

October, 1989 at Mississippi Valley State University, Itta Bena, MS. The major work of this investigation requiring the analysis of protein profiles and protein characterization was conducted during the summer months (May 15 - August 14, 1989) at the Plant Molecular Biology Laboratory, United States Department of Agriculture (USDA), Agriculture Research Service (ARS), Beltsville, MD. Some of the experimental plants for protein analysis were grown and treated for heat stress and drought stress at the research facilities made available by the Soybean Research Program, USDA-ARS, Delta Branch Agriculture Experiment Station, Stoneville, MS.

1. Test Plants

The Seeds of two cultivars of Phaseolus acutifolius (Tepary Bean) black tepary bean and white tepary bean, and three cultivars of Phaseolus vulgaris (common bean) light red kidney bean, dark red kidney bean, and pink common bean were supplied by Dr. Claire Federici, Department of Botany and Plant Science, Unversity of California, Riverside, California. The seeds of two cultivars of Glycine max (Soybean) PI 416.937 soybean and Davis soybean were supplied by Dr. Thomas C. Kilen, soybean Research program, USDA-ARS, Stoneville, MS. The selection of these cultivars was based upon their germplasm providing desirable traits for higher seeds yields, tolerance of adverse environmental and edaphic factors and resistance to diseases which are impediments in the developing countries of Africa, Asia and South America. The white tepary bean, USDA Plant Introduction Collection PI 319443 is one of the higher yielding, very drought tolerant cultivars, tolerant to low temperature growing conditions, resistant to bean blight (Xanthomonas Phaseoli) and is a good parent for interspecific crosses with Phaseolus vulgaris. The Black tepary bean G40035, CIAT Collection, Cali, Columbia is resistant to common bean blight and empoasca leaf hopper, but is suceptible to common mosaic virus (12).

2. Plant Growth Conditions

The seeds of all the previously mentioned seven cultivars were planted

on four different planting dates; one planting date was at Stoneville, MS and the other three planting dates were at Beltsville, MD. At Stoneville, the seeds were planted in vermiculite for germinating on April 17, 1989. For each treatment (drought stress, heat stress or nontreatment) at least ten uniformly germinated seedlings of each of the six cultivars out of seven cultivars were transplanted to soil in 8 inch pots on April 21, 1989. The soil was prepared by mixing oven dried loamy sand soil, with 73% sand, 12.5% silt, 14.5% clay and approximately 0.4% organic matter. Thus two hundred and forty planted pots were randomly placed in the green house, which was maintained at 75±5°F at day tempature and 65±5°F at night temperature and 75+15% relative humidity.

At Beltsville, for the first planting date two hundred and forty seeds of six cultivars (black tepary, white tepary, pink common bean, light red kidney bean, PI Soybean and Davis soybean) for five stress treatments and non-stress treatments were planted on May 24, 1989. For the second planting date forty seeds, ten seeds each of the four cultivars (black tepary, white tepary, pink common bean and light red common bean) were planted on June 29, 1989. And for the third planting date thirty seeds of six cultivars already mentioned for May 24 planting were planted on July 7, 1989 at Beltsville, MD. These six cultivars were grown for seed production in the green house at Beltsville (Appendix B) long after the leaf tissue was sampled for comparative analysis of steady state protein profiles.

3. Drought Stress Treatment

The seeds for drought stress treatment were planted at Stoneville, MS and Beltsville, MD locations. At Stoneville, the seeds were planted on April 17, and transplanted on April 24, 1989. After four weeks, the water for drought stress treatment for some plants was withheld from May 17-May 24. The common bean plants receiving drought stress treatment remained wilted on May 23 and did not recover from wilting on May 24. The Stoneville water-stress experiment was terminated on May 24. The weights of plants were recorded in kilograms on May 17 and May 24. The loss of water from pots was due to pot soil surface evaporation and plant transpiration. The percent-

age of kilograms of water loss of different water-stressed cultivars and actual kilogram of water loss by individual plants are listed in Table No. 1 and Appendix C respectively.

The seeds for drought-stress treatment for Beltsville location were planted on June 29. The water was withheld from July 18 - July 24 form plants receiving drought stress treatment, after they had grown for three and one half weeks. The common bean plants receiving the drought-stress treatment did not recover from wilting on July 22 or July 23. The experiment was terminated on July 24. The weights of plants were recorded in kilograms on July 18 and July 24. The percentage of kilograms of water loss of different water-stressed cultivars and averages of actual kilograms of water loss of five plants are not listed in this report which supported the observations made earlier listed and observations of Table No. 1 and the Appendix C. The experiment was repeated for comparative analysis of steady state protein profiles.

4. Heat-Stress Treatment

The cultivars of the common bean, the tepary bean and the soybean were heat stressed at the Stoneville and Beltsville locations. At Stoneville, about seven-week-old plants from the seeds planted on April 17, were exposed to growth chamber temperature maintained at 44.0°C±0.4°C for two hours on June 7 and four hours each of the following two days. The experiment was terminated on June 9, and leaves were sampled.

At Beltsville the heat stress treatment was repeated twice on less than 3 weeks old four cultivars of common bean and tepary bean plants. The seeds were planted on June 29 and plants were exposed to the growth chamber temperature maintained at 44.0°C ±2.0°C for two hours on each of the dates from July 18-July 25. The experiment was terminated on July 25 and leaves were sampled.

5. Leaf Tissue Sampling, Shipping and Preservation

Only plant tissue from recently fully expanded trifoliate leaves was

sampled for comparative analysis of steady state protein profiles. Immediately, after the termination of the water stress treatment or heat stress treatment, the sampled leaves were either freeze dried in liquid nitrogen or in dry ice. The fresh weight of the sampled tissue was recorded at the Beltsville location before freeze drying and preserving. The freeze dried leaves became brittle and were crushed to small pieces before preserving at -70°C. The leaf tissues sampled at two different dates and preserved at -80°C at Stoneville were shipped for overnight delivery to Beltsville Laboratory in dry ice for comparative analysis of steady state protein profiles.

6. Total Protein Extraction and Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (SDS-PAGE)

a) Plant Tissue Extraction

Thawed frozen leaf tissue was weighed one gram or one half gram and homogenized wih a chilled pestle and mortar by using 4 or 3 volumes (3 or 4 ml) of the extraction buffer solution which was prepared by mixing the following chemicals;

1 M Tris-Cl, PH 7.4
Mg So_4
Na HCO_3
0.25 ME DTA, PH 7.5
12.8 M B-ME
100 m M PMSF
Leupeptin
Glycerol
5 M NaCl and
GD H_20.

Extracted tissue was filtered through Miracloth previously moistened with the extracting buffer solution.

b) Plant tissue extract centrifugation

The extracted plant tissue volume was measured and transferred to prechilled plastic centrifuge tubes for centrifugation at 20,000 x g for 20 minutes. The centrifuged plant tissue supernatant was measured and stored at -20°C for analysis of protein. The pallet was washed once in extraction buffer solution and twice with extraction buffer containing no NaCl.

c) Resolution of total protein extracts by SDS- PAGE

The use of different percentages of the gradient of minigel indicated that 15% gradient for Minigel be used for best results. The buffer of Laemmli (6) was used. The chemical composition used for the buffers for lower gel and upper gel is listed as follows:

Lower Gel	Upper Gel
GDH_20	GDH_20
1.5 M Tris (LT)	0.5M Tris (UT)
30% Acrylamide	30% Acrylamide
10% APS	10% APS
TEMED	70% Glycerol
	TEMED

The gel running buffer was prepared by mixing the following:

O.25 M Tris
1.92 M Glycine
1% SDS and
GD H_20

d. Minigel Staining, and Destaining Solutions

The minigels were stained by shaking for at least one hour in a solution prepared by mixing 0.2% Coomassie blue, 50% methanol and 7% acetic acid. Stained gels were destained by shaking the stained gels at least six to fourteen hours in a solution prepared by mixing 20% methanol with 7% acetic acid.

c) Photographing and Drying the destained gels

The gels were photographed by using Polaroid film #57 or 55. The photographed gels were then dried under vacuum for at least one hour at 80°C.

7. Assay for Protein Content of samples prepared for SDS-PAGE

Three assay methods were considered to determine the amount of sample to load on the Minigel so as to get uniformity in the band size, band intensity, and width of the lanes. These methods were determined on the basis of equal membrane chlorophyll content or equivalent fresh weight of leaf tissue or quantitative and semiquantitive protein assay by Coomassie binding. The most consistant and accurate method of assaying for protein content was Esen's method modified by Dr. Mattoo (3). Five microliters of aliquots of samples with unknown amounts of protein content were compared with the standard solution of bovine serum albumin (BSA) of known concentrations as shown in the Appendix D. This method estimates the unknown protein concentration of the samples by visually matching, comparing and estimating the intensities of the stained spots against those of the BSA standards of known concentrations of protein contents.

Chlorophyll determination was made by making one percent solution of the chlorophyll from the pallets of treated and non-treated plants. It was prepared by taking ten micro liter of pallet sample and diluting it with one ml of 80% acetone. Photometric total readings of chlorophyll concentration in microgram/ml were measured at preprogrammed wavelengths of 700, 663, 645 and 600 millimicrons by Shimdzu UV-160 photometric Instrument shown in Appendix E.

8. Sample Preparation for running SDS-PAGE Mimigel

The assayed protein contents from the supernatant (soluble sample) and the pallet (membrane sample) from treated and non treated leaf tissues determined the amounts of the sample aliquots to load on gels. The supernatant sample and pallet sample were prepared by mixing with the sample buffer. For these expermients 100 micro liter soluble sample was mixed with 50 ml of 3 x sample buffer, vortexed, kept at 90° C for three minutes, before finally diluting with 1 x sample buffer and loading enough microliters to obtain 5

micrograms equivalent of protein. The pallet 50 microliter sample was mixed with 50 microliter of 3 x buffer solution, vortexed, and kept at room temperature for one hour. Before loading the pallet sample, it was diluted with 1 x sample buffer to obtain 5 microgram equivalent of protein. By following the above explained methodology, the uniformity in the protein band size, the protein band intensity and width of the lane was obtained as shown in Appendix F. Some of the equipment used in running the Bio-rad Minigels is shown in Appendix G.

IV. Results And Discussion

The common bean plants and the tepary bean plants placed under moisture stress at Stoneville, MS in May and at Beltsville, MD in July showed a marked difference in rate of wilting. During the month of May the common beans wilted 4 days after the water was withheld, whereas, tepary bean plants of the same size and age grown under the same conditions did not wilt until 7 days after water was withheld. The rate of water loss per plant per unit time due to transpiration was very similar for the common bean and that of the tepary bean, which is shown in Table No. 1. The greater heat tolerance of the tepary bean compared to that of the common bean has been adequately documented by Lin (7).

There was a great similarity between the protein bands obtained by using electrophoresis from the common beans and the tepary beans. Figure. 1. Heat stress resulted in the appearance of two novel bands on the gel indicated by arrows in Figure No.1 for both the common beans and the tepary beans. The molecular weight of these proteins was calculated to be between 18,000 and 25,000. These protein bands may represent heat stress or heat shock proteins (HSP) induced by the heat stress and are not proteins necessarily associated with heat stress tolerance. Elizabeth Vierling (14) at the University of Arizona demonstrated in her studies the dramatic and immediate changes that occur in gene expression in plants in response to high temperature stress, initiating an immediate synthesis of one or more heat shock proteins mostly of low molecular weights (20-30 k Da). She and her colleagues suggest that these proteins play an important role in stress response and recovery. R. A. Vierling

Table No. 1

Transpiration of Tepary Bean, Common Bean and Soybean as measured by water loss at permanent wilting of common beans.

Bean Type	Bean Cultivar	water loss (% of initial Pot wt.)*
I. Tepary bean		
	(a) Black	12.9
	(b) White	12.9
II. Common bean		
	(a) Light Pink	11.6
	(b) Dark Red	13.1
III. Soybean		
	(a) PI 416.937	17.1
	(b) Davis	16.7

*Means are based upon nine or more replications.

(15) at Texas A and M University showed in his studies in wheat that heat shock proteins that acquired thermal tolerance are correlated to increased levels of low molecular weight proteins. The results of the comparative analysis of steady state proteins of this study show that the similarity of the gels from the common beans and the tepary beans precludes the speculation that the greater heat tolerance of tepary beans can be related to numerous unique constitutive proteins found in tepary beans but not in common beans. Therefore, it can not be concluded that the tepary bean is more heat tolerant than the common bean, as illustrated in Fig. 1. Alternative explainations for the observed differences in heat stress tolerance could be differences in protein turn over rates and differences in the lipid portion of the membranes (9). From electrolyte leakage experiments, Lin (7) concludes that plasmalemma from the tepary bean has greater thermostability than the plasmalemma from the common bean.

As a result of water stress there were slight differences in protein band density of the gels from both the common bean and the tepary bean (Fig. 2). Vartiannian et al. studying drought induced changes in protein patterns of Brassica napus showed higher density of two dimensional gel spots of under 35 k Da proteins under drought (13). It would appear that the discussion on heat tolerance is also applicable to drought tolerance differences between the common bean and the tepary bean.

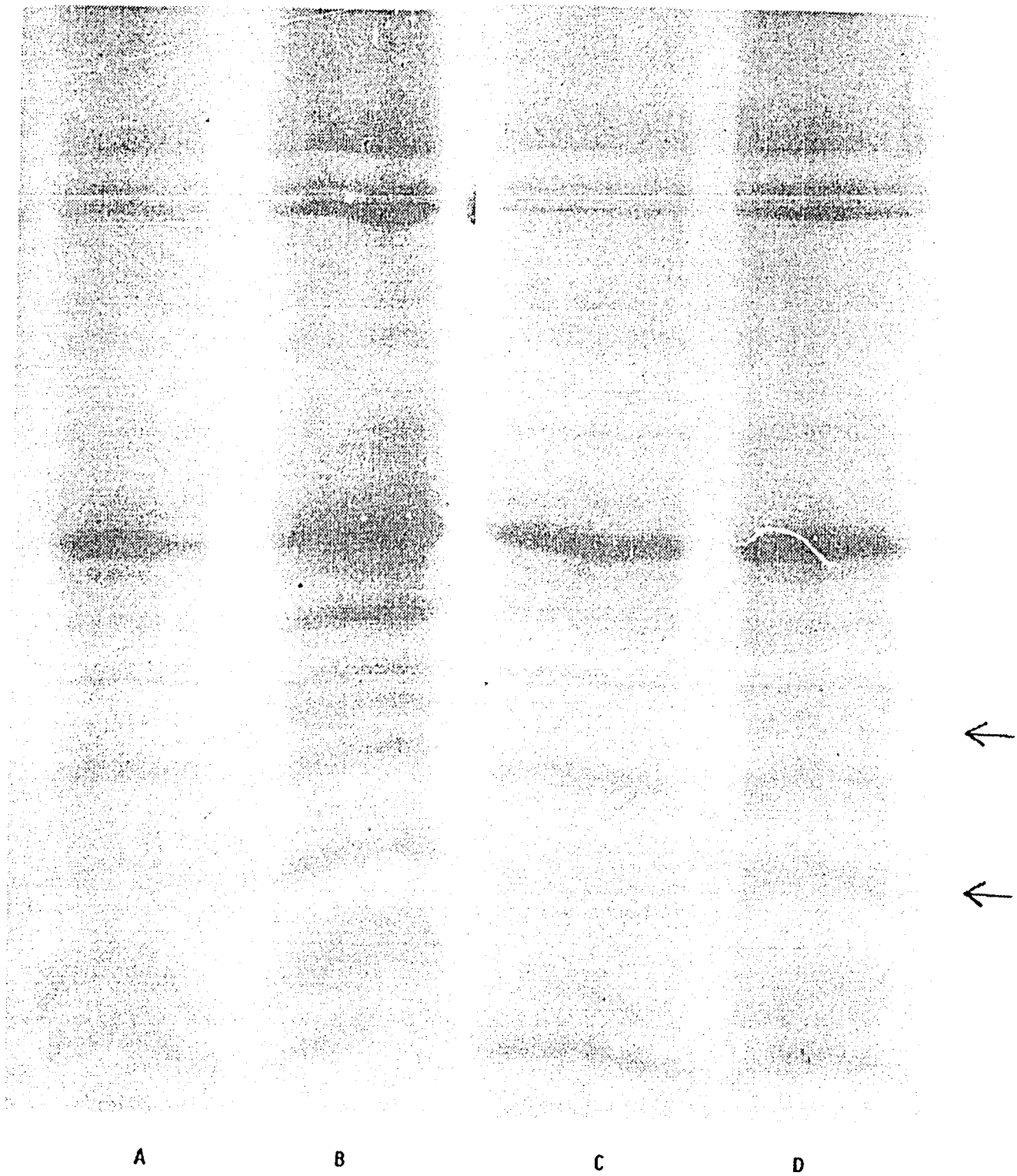
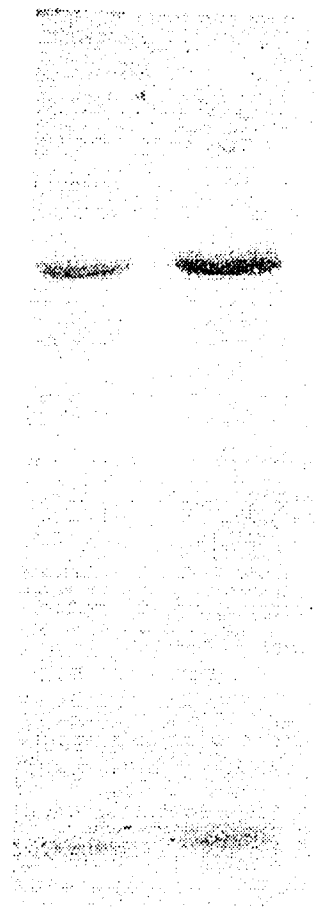


Fig. 1. Four Lanes of gels showing the differences between control and Heat-stressed plants. The arrows indicate the appearance of two novel bands in the heat-stressed plants of both Tepary bean and common bean.
A = Tepary bean control
B = Tepary bean Heat-stressed
C = Common bean control
D = Common bean Heat-stressed



A B

Fig. 2. Two lanes of gels showing slight differences in protein band density due to water stress in the Tepary bean and the common bean.

A = Black Tepary bean water-stressed
B = Pink Common bean water-stressed

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Appendix A

Phaseolus acutifolius (Tepary Bean) narrow leaved legume is on the right. *Phaseolus vulgaris* (Common Bean) wide leaved legume is on the left.

A B C D E F

Appendix B

Six cultivars grown for seed after leaf sampling.

A. PI Soybean
B. Davis Soybean
C. Light Red Kidney Bean
D. Pink Common Bean
E. White Tepary Bean
F. Black Tepary Bean

Appendix C

Plant weights in Kilograms of six cultivars on two different dates and the loss of water as the percentage of the Initial pot weight

Plant No.	Pot wt.(kg) on 5/17/89	Pot wt.(kg) on 5/24/89	Diff. in wt. between two dates	water loss % of the initial pot weight	
		1. Black Tepary Bean			
21	3.9	3.4	0.5	12.8	
26	4.2	3.6	0.6	14.3	
29	4.3	3.8	0.5	11.6	
33	4.1	3.5	0.6	14.6	
38	4.1	3.6	0.5	12.2	
41	4.6	4.0	0.6	13.0	
156	4.5	4.1	0.4		
47	4.3	3.7	0.6	14.0	
55	4.1	3.6	0.5	12.2	
59	4.4	3.9	0.5	11.4	
Total				=116.1	Average=12.9
		2. White Tepary Bean			
23	3.9	3.4	0.5	12.8	
28	4.0	3.5	0.5	12.5	
31	4.3	3.8	0.5	11.6	
37	4.1	3.5	0.6	14.6	
40	4.2	3.6	0.6	14.3	
43	4.3	3.8	0.5	11.6	
45	4.4	3.8	0.6	13.6	
50	4.2	3.7	0.5	11.9	
53	4.3	3.7	0.6	14.0	
58	4.0	3.5	0.5	12.5	
Total				=129.4	Average=12.9
		3. Light Pink Common Bean			
22	4.1	3.5	0.6	14.6	
24	3.8	3.2	0.6	15.8	
30	4.4	3.8	0.6	13.6	
32	4.0	3.6	0.4	10.0	
57	4.7	4.0	0.7	14.9	
46	4.4	3.8	0.6	13.6	
154	4.8	4.2	0.6	12.5	
52	4.4	3.9	0.5	11.4	
160	4.8	4.2	0.6	12.5	
61	4.2	3.7	0.5	11.9	
Total				=115.9	Average=11.6

4. Dark Red Common Bean

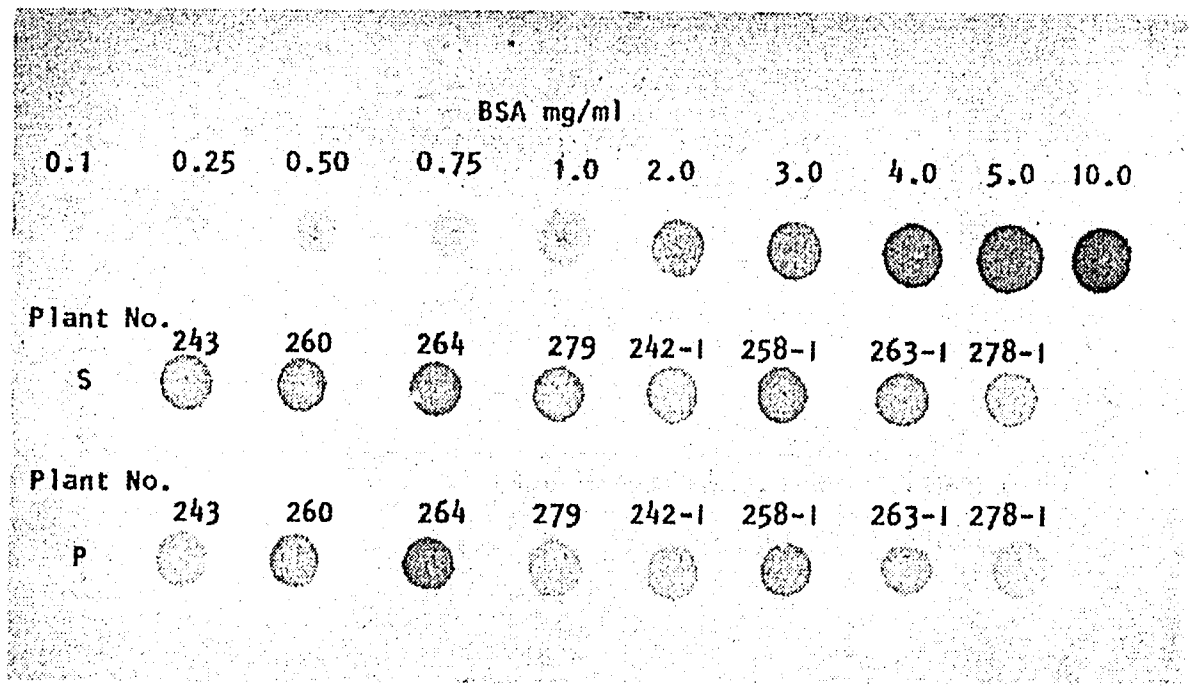
25	4.0	3.4	0.6	15.0	
27	3.9	3.3	0.6	15.4	
34	3.9	3.6	0.3		
36	4.0	3.4	0.6	15.0	
39	3.9	3.4	0.5	12.8	
42	4.0	3.6	0.4	10.0	
49	4.3	3.9	0.4	9.3	
51	4.4	3.8	0.6	13.6	
54	4.2	3.6	0.6	14.3	
57	4.1	3.6	0.5	12.2	
Total				=117.6	Average=13.1

5. PI 416.937 Soybean

171	4.2	3.5	0.7	16.7	
172	4.4	3.6	0.8	18.2	
173	3.8	3.1	0.7	18.4	
174	4.3	3.5	0.8	18.6	
175	4.0	3.4	0.6	15.0	
176	4.3	3.6	0.7	16.3	
177	4.1	3.5	0.6	14.6	
178	4.2	3.4	0.8	19.0	
179	4.0	3.3	0.7	17.5	
180	4.1	3.4	0.7	17.1	
Total				=171.4	Average=17.1

6. Davis Soybean

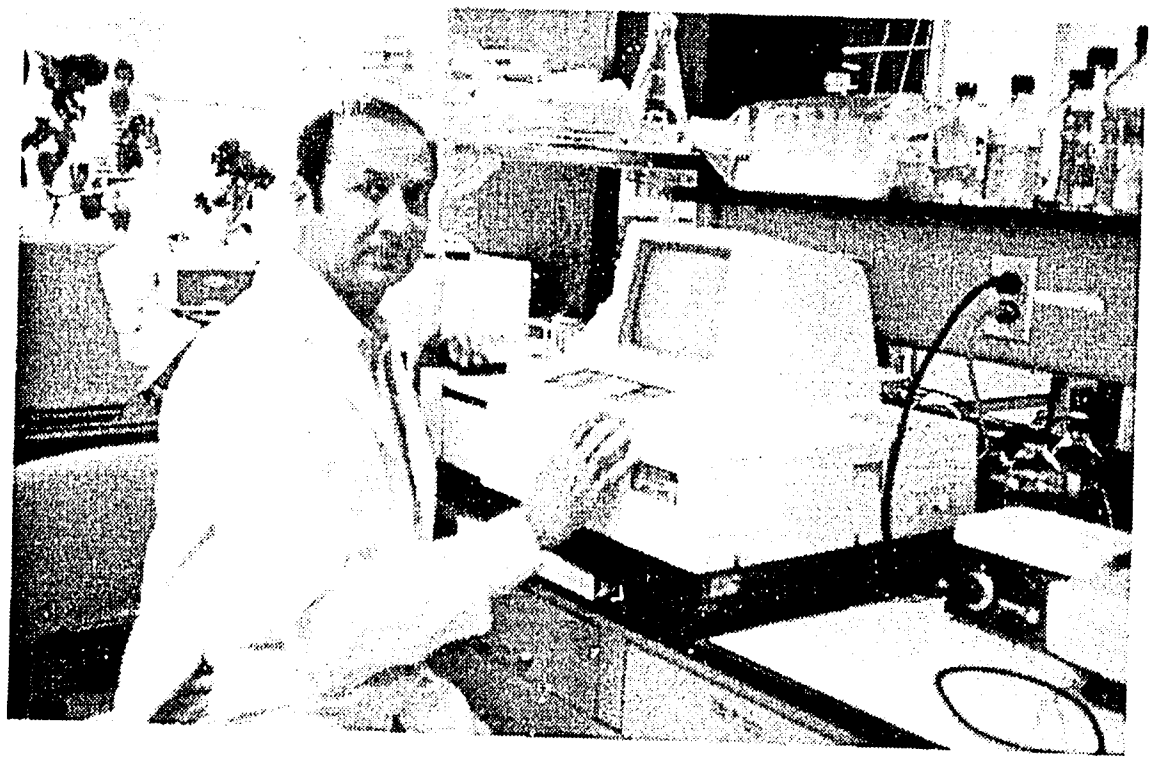
206	4.1	3.4	0.7	17.1	
207	4.2	3.5	0.7	16.7	
208	4.5	3.8	0.7	15.6	
209	4.6	3.8	0.8	17.4	
210	4.7	3.8	0.9	19.1	
211	4.1	3.4	0.7	17.1	
212	4.2	3.6	0.6	14.3	
213	4.5	3.8	0.7	15.6	
214	4.7	3.9	0.8	17.0	
215	4.2	3.5	0.7	16.7	
Total				=166.6	Average=16.7



Appendix D

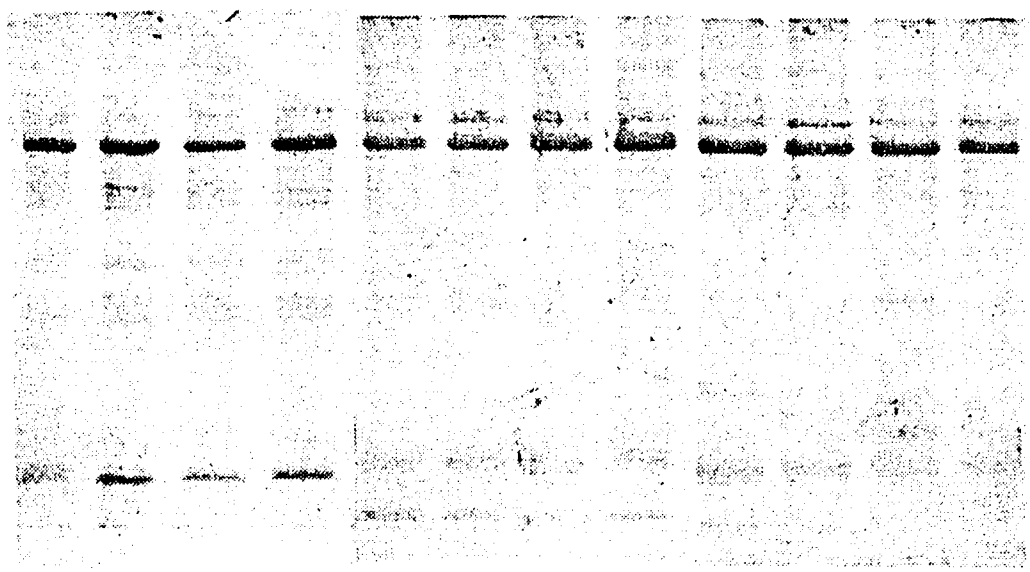
Quantitative and Semiquantative protein assay by coomassie binding.

BSA = Bovine Serum Albumin
S = Supernatant
P = Pallet



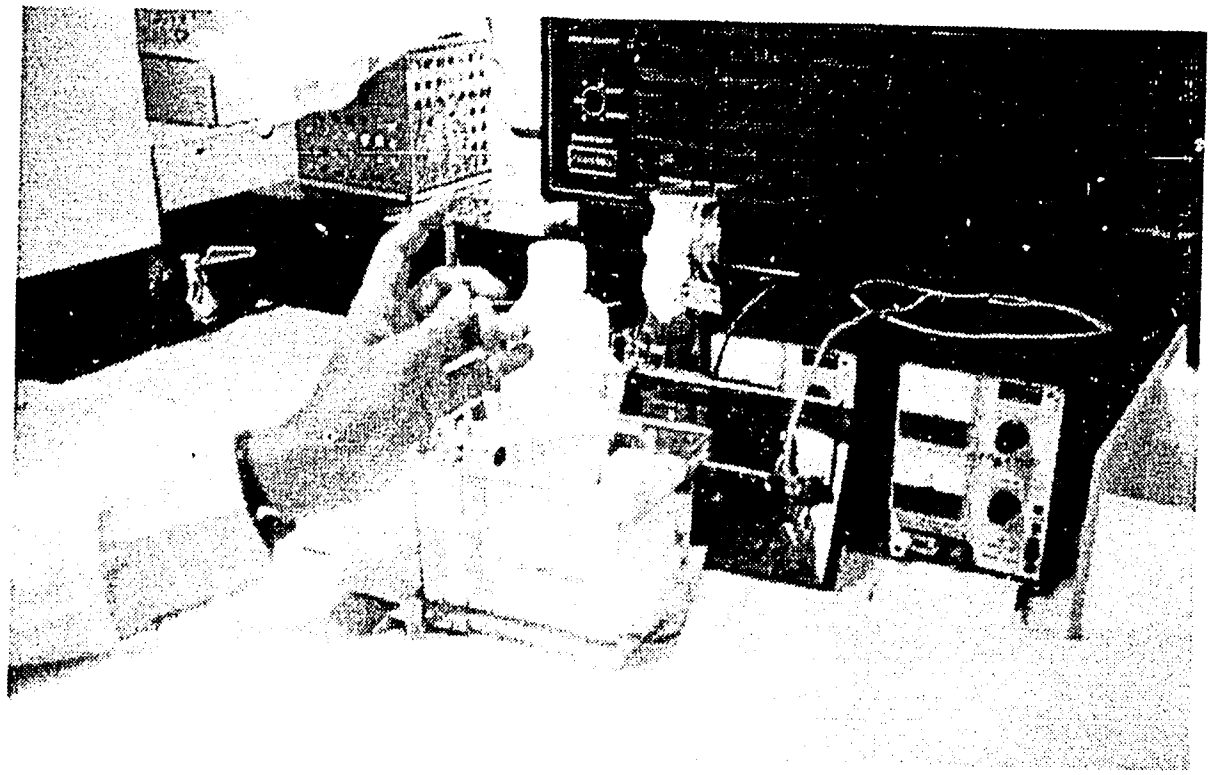
Appendix E

Shimdzu UV-160 Photometric Instrument used in measuring the concentrations of chlorophyll from samples.



Appendix F

Development of protein bands and lanes from loading of an estimated 5 microgram equivalent of protein from samples of different types of bean cultivars.



Appendix G

Some of the equipment used to run a minigel is shown.