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**LA THÈSE A ÉTÉ
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EFFECT OF ENVIRONMENTAL CONDITIONS ON LENTIL SEEDS

by

MATILDE VIERMA DE BEZADA

A thesis submitted to the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Master of Science in Biology.

DEDICATORIA

Dedico esta tesis a mis padres Jeronimo y Conchita, con su amor y buen ejemplo me hicieron conocer los valores primordiales de la vida, y quienes me brindaron la oportunidad de alcanzar una profesion que mas tarde me permitio realizar mis estudios de Master.)

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ABSTRACT

Lentil seeds Lens culinaris Medic. under normal storage conditions discolor gradually over an 18-month period changing from an olive green to light yellow and finally to deep brown. This discoloration reduces marketability of the seeds and thus has important economic implications. In the study reported here, the rate of browning of lentil seeds exposed to a variety of environmental conditions was assessed. Browning was found to be accelerated by sources of near U.V. or visible light, an anaerobic atmosphere, high humidity and warm temperatures. Seeds placed in light and air at 30°C and 100% R.H. were discolored and overgrown with saprophytic fungi in less than a week. Germination percentage and water uptake declined as the discoloration process proceeded. The tetrazolium test showed very low or no viability in browned seeds. Electrical conductivity of the imbibition medium was highest with browned seeds indicating that membrane integrity had been lost. Exudation of soluble proanthocyanins was abundant from green seeds but limited from browned seeds. These observations suggest that browning may result from an oxidative polymerization of the low molecular weight condensed proanthocyanin precursors. Light enhances this reaction and in the process membrane proteins are presumably denatured by a tanning mechanism.

RESUME

Lors de conditions normales d'entreposage sur une période de 18 mois, la couleur des semences de lentilles (Lens culinaris medic.) passe graduellement d'un vert olive à un brun plus ou moins foncé. Cette coloration réduit la valeur marchande des semences, de ce fait, a des implications économiques importantes. Dans la présente étude, le taux de brunissement de semences de lentilles, exposées à diverses conditions d'environnement, a été déterminé. Elle a été observé que le processus était accéléré par une exposition à la lumière blanche, fluorescente ou ultraviolette, de même que par une atmosphère anaérobique, une température et humidité élevées. Des semences placées à la lumière, à 30°C et 100% H.R. avaient bruni et étaient envahies par les champignons saprophytes en moins d'une semaine. Le pourcentage de germination et l'absorption d'eau étaient abaissés chez les semences brunies. Le test du tétrazolium a montré une viabilité très faible, lorsque présente, sinon nulle chez ces dernières. La conductivité électrique du milieu d'imbibition était la plus élevée chez les semences brunes, indiquant que l'intégrité de la membrane avait été perdue. L'exudation de proanthocyanines solubles par les semences vertes était abondante alors que très rare chez les semences brunes. Ces

observations suggèrent que le brunissement pourrait résulter d'une polymérisation oxydante de précurseurs de proanthocyanines condensés de faibles poids moléculaires. La lumière favorise cette réaction et, au cours du processus, les protéines de la membrane sont probablement dénaturées par un mécanisme de tannage.

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section I

INTRODUCTION

Lentils, Lens culinaris Medic. belong to the Leguminosae, a family which includes 41 crop species such as pulses, beans, and grain legumes, and is second only to cereals as a source of human and animal food (Harlan, 1975). Nutritionally they are 2-3 times richer in proteins than cereal grains, with a protein content between 20 and 40 per cent (anonymous, 1979).

On a world scale 2 legumes are of great importance in human nutrition; the common bean P. vulgaris, in Latin America and the lentil, L. culinaris Medic. in the Indian-subcontinent. (Purseglove, 1968). It is clear from archeological evidence (Zohary, 1973a, b) that the earliest signs of lentil domestication are concentrated in the Near East. In addition to their cultivation in India, lentils are also grown in most subtropical regions of the world and at high altitudes in the tropics (Purseglove, 1968).

In Canada, lentils (L. culinaris) Medic. were first grown in the prairie provinces in 1972, using seeds obtained from the Palouse area of the U.S.A. However, the increasing area

devoted to lentil production in the Canadian prairies emphasizes the importance of the development of a cultivar adapted to the Canadian climate. After a breeding program from 1972 to 1978 using different lines of lentils, the variety Laird was granted a license in November 1978. Laird is a large-seeded Chilean type with yellow cotyledons. The seed is thicker than that of most other cultivars with a protein content of 22.5% (Slinkard and Bhatti, 1979). Saskatchewan produces most of the Laird variety grown in Canada. Most of this production is exported to Europe where it is used in soups and specialty ethnic dishes (Bhatti and Slinkard, 1979).

When supplies exceed demand the seeds are held in storage. Seed coat discoloration (a progressive change from light green to yellow and finally to dark brown) becomes evident after 10 to 18 months of storage in some if not all lentil cultivars, making them less desirable to the consumer. Therefore prices are depressed and sometimes the seeds have to be sold at no profit. Because importers strongly discriminate against lentils which have lost their light green color, browning has become an important problem to commercial producers and research workers involved in developing a desirable product for the world consumer market (Slinkard, 1978; Ali-Khan, 1980 personal communications).

The seed coat becomes darker with increasing length of storage. Sometimes the color change is relatively rapid, at other times it may occur very slowly (Hughes, 1974). These observations have led to speculations that the color change could be controlled by regulating temperature, light and relative humidity of storage rooms. Sharma and Sharma (1978) pointed out for instance that poor storage of agricultural commodities results in loss of quality of seeds, and in some cases complete destruction, due to invasion of storage fungi which bring about the degradation of seed constituents, thus rendering seeds unfit for useful purposes.

Toole et al. (1948) noted that a tan colored snap bean seed had changed to a reddish brown after storage at high temperature and high relative humidity. MacDonald and Miller (1947) pointed out that browning in fresh wheat germs was promoted by elevated temperature and moisture content. Dexter et al. (1954) found that white pea beans stored at high relative humidity and high temperatures had turned dark brown. They also noted that a rancid odor coincided with the darkening. Hughes, (1974) reported a darkening of kidney bean during storage at high temperature and high relative humidity. He also reported that cool-white light and near ultra violet appreciably darkened the bean seeds as compared to the seeds stored in the dark.

Seed coat discoloration may be related to the presence of condensed proanthocyanins which Bate-Smith and Swain (1953) recognized as responsible for a broad range of tannin reactions in plant tissues. These substances have been classed as condensed tannins but the more appropriate term "condensed proanthocyanins" will be used here (Weinges *et al.* 1969a). Weinges and his collaborators have revealed that these substances possess di-, tri- and tetra-flavan structures of a general type as shown in figure 1 in which the flavan monomer units are linked by C-C bond between the 4-position of one flavan unit and the 8-position of ring A of the next unit (Harborne, 1975).

The capacity of these compounds to tan is due to a large extent to their molecular size, and in this respect low molecular weight phenols such as flavan 3,4-diols, flavan 3-ols, chlorogenic acid, are too small to permit sufficiently effective cross linking. Highly polymerized polyphenols on the other hand are either too insoluble or too large to fit between suitably oriented polypeptide chains. Maximum tanning action is shown by tannins of intermediate size, and polyphenols with a molecular weight range of 500 to 3000 have been suggested as most satisfactorily fulfilling these requirements (White, 1956; Goldstein and Swain, 1963; Gustavson, 1954). It is desirable that the term tannin should be reserved for those polyphenols within this molecular weight

range. It is relevant to say that dimers, trimers and tetramers of the proanthocyanidins are correctly classified as tannins since they have molecular weights which fall within this range (Harborne, 1975). Roux and Evelyn (1958a) determined ebullimetrically the molecular size of different tannin fractions; they concluded that substances with a molecular weight of the order of 2000 corresponds with a polymer containing 7 flavan units. Tetra, tri or dimeric forms corresponded with a molecular weight between 1500 and 1000; monomeric forms had a molecular weight lower than 1000 (Roux and Evelyn 1958b).

To clarify the general chemical structure of the proanthocyanidin compounds, different approaches have been used. Weinges *et al.* (1968a) used proton magnetic resonance. Thompson *et al.* (1972) developed a chromatographic technique on dextran gel Sephadex LH-20 as a means of separating the individual free phenolics forms of the plant proanthocyanidins. Paper chromatography has been also utilized as an analytical method combined with spectroscopic techniques (Harborne, 1975).

In food technology it is well known that products containing seeds are susceptible to discoloration in processing. It has been recognised that condensed proanthocyanidins play a role in such a problem. Dickinson, (1957) reported

that the difference between those few varieties of broad beans (Vicia faba) that are suitable for canning and the many that are not, is the absence of condensed proanthocyanidins from the seed coats of the former. Wine is another product in which condensed proanthocyanidins have been recognised as of technical importance (Ribereau-Gayon, 1957). The principal source of these compounds has been identified as the seeds. The condensed tannins in beer are derived from both malt and hops, that from the malt originates from the pericarp in barley, being the proanthocyanidins which make the major contribution to the formation of beer hazes (Bate-Smith and Ribereau-Gayon, 1959).


In most instances, the proanthocyanidins are richest in the seed coat, and their condition changes as the fruit ripens, and after the seeds are harvested. By virtue of their tannin nature, as the cells die the proanthocyanidins become absorbed in the cell wall (Howes, 1953). Their condition then changes as compared with, for instance, that of living cells when they are mainly in solution in the vacuoles, and becomes more like that in wood. This change is accompanied by increasingly reduced solubility in methanol (Hillis and Swain, 1957) and decreasing ability to form true anthocyanidins when the tissue is digested with aqueous HCl. An excellent example of these changes is provided by the field bean (V. faba) : when still green in the pod, the seed coat

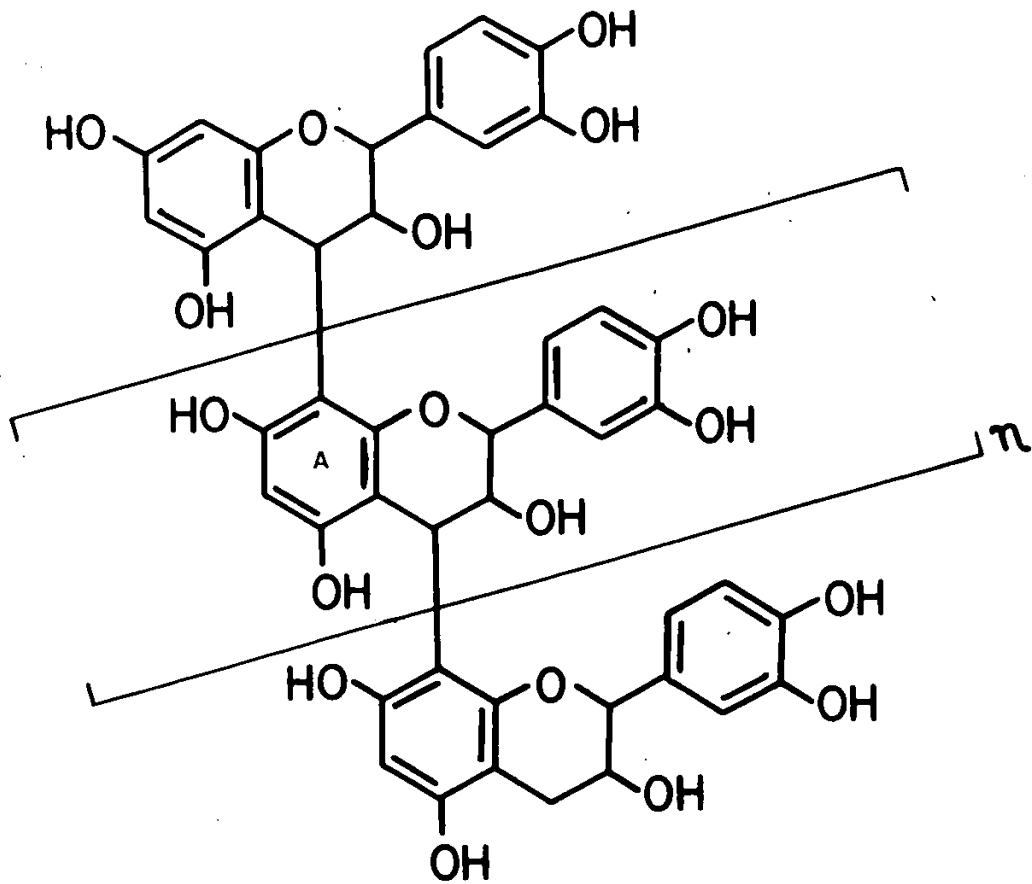
richly yields anthocyanidins when digested with aqueous HCl. When harvested ripe they still yield anthocyanidins when digested with alcoholic HCl, but during storage when the seed coats become dry, tough and discolored, the yield progressively decreases (Bate-Smith, Riberau-Gayon, 1959).

The aim of this study was to follow the discoloration phenomenon in the seed coats of lentil seeds, (*L. culinaris*) Medic. and to correlate seed coat discoloration with germination percentage, rate of imbibition, electrical conductivity, exudation of soluble condensed proanthocyanins, and different storage conditions.

These substances possess di-, tri-, and tetra flavan structures in which the flavan monomer units are linked by C-C bond between the fourth position of one flavan unit and the A ring of the next unit (Harborne, 1975).

Figure 1: Procyanidin: Precursor of Condensed Proanthocyanins.





Procyanidin

section II

MATERIALS AND METHODS

2.1 PLANT MATERIAL

Lentil seeds, (*L. culinaris* Medic) var. Laird, were obtained from J. Sheppard, Agriculture Canada. A plant introduction P.I. 345635, was provided by A. E. Slinkard, Crop Development Centre, University of Saskatchewan, Saskatoon. In order to obtain uniform samples for the different experiments performed, the seeds were sorted according to seed coat color into green (1) and light yellow (2) using an ISCC-NBS centroid color chart, standard sample No. 2106 (figure 2).

2.2 CONTROL OF STORAGE CONDITIONS

2.2.1 Light

Three different light sources were used during the storage experiments: incandescent (400-1100nm), cool white fluorescent (350-750nm) and black light blue fluorescent (300-400nm), (Smith, 1977). (See appendix).

Incandescent light was provided by lamps with Sylvania bulbs of 40 watts and a light intensity of 44 W/m² at seed level.

B.L.B. and fluorescent lights:

Fluorescent cool white and black light blue lamps were together in a wooden box. On one side were two 20 watt (black light blue) tubes (General Electric F20T12/BLB) with a light intensity of 4.2 W/m^2 at seed level and on the other side were 2 tubes of fluorescent cool white light (General Electric F20T127CN) with a light intensity of 11 W/m^2 at seed level. The B.L.B. and fluorescent cool white lights were separated by a cardboard. To keep the surroundings from over-heating, a small fan was located at one end of the box so cool air continuously circulating through the compartments. Intensity measurements were made with a Yellow Springs radiometer, model 26A.

2.2.2 Relative Humidity

Dryness (0% R.H.) was maintained using a bed of 10 grams of silica-gel under the seeds. A relative humidity of approximately 50% was obtained by saturation in a closed environment with 46% H_2SO_4 . In initial experiments 100% relative humidity was similarly achieved using 1% H_2SO_4 but in later experiments pure distilled water was used (Hand Book of Chemistry and Physics, 49th edition, 1968-69).

2.2.3 Temperature

Constant temperature rooms maintained at 5°C, 20°C or 30°C were used for temperature control.

2.3 EXPERIMENTAL DESIGN

2.3.1 Seeds Stored Under Continuous Illumination

Seeds exposed to light were stored in glass containers 15 cm high and 8.5 cm in diameter. The containers were placed in an up-side down position to allow the light to penetrate the glass and to reach the seeds sitting on the cap of the (fig 3a). The glass containers were arranged in rows, in 30x45 cm² plastic trays, covered with a cardboard box with an opening cut out at the top over which a lamp with the 40 watts bulb was fitted. The containers were rotated daily in order to get a more or less even distribution of light. For the near U.V. experiments the same type of container was used, and located inside the wooden box in which the lights were suspended directly below the tubes.

2.3.2 Seeds Stored Under Continuous Darkness

Seeds stored under darkness were kept in aluminum containers 5.2 cm high and 9.0 cm diameter. These were used in the upright position (fig 3b)

2.3.3 Control of Relative Humidity

Seeds subjected to 0% relative humidity were placed on a filter paper over silica-gel which covered the bottom of the container. The solutions of H_2SO_4 , which gave approximately 50% and 100% R.H. as well as the pure water were poured into 20 ml beakers, which were placed in the center of each container. The seeds were distributed around the beaker at the bottom of the container. All the containers were then sealed with masking tape and taken to the storage rooms.

2.4 SEED VIABILITY TESTS

2.4.1 Imbibition test

To determine the rate of seed imbibition, replicate lots of 50 seeds were weighed before soaking and this value was taken as a 100% weight. The seeds were soaked for 6 hrs in a 100 ml beaker containing 50 ml of deionised water, and then removed from the water, drained and weighed again. The second weight was expressed as percent of the initial weight, to indicate the proportional amount by which the seeds increased in weight due to water uptake. The 6 hrs imbibition time was determined by a preliminary test of rate of imbibition with green (1) seeds. A total of 16 beakers each containing 50 seeds and 50 ml of deionised water were left to imbibe for a period of 3 to 24 hrs. Replicate samples were removed every 3 hr for fresh weight determination.

2.4.2 Germination Tests

Laboratory Test:

The method followed to test germination was mainly that described in Methods and Procedure of Seed Testing, Agriculture Canada (1965). After 6hr imbibition the seeds removed from the deionised water and weighed, then placed on 200 grams of sand moistened with 15 ml of distilled water in 15 cm diameter petri plates. The seeds were kept at 20°C in continuous illumination with cool white fluorescent light. Germination was checked after 5 days; seeds were arbitrarily considered to be germinated when the radicle length was at least 1 cm.

Field trial:

This trial was performed on a plot at the Central Experimental Farm. Seeds were planted 15 cm apart and 3 cm into the soil, in rows of 50 seeds. The test was repeated at two different times during the summer of 1979. The first time (15-5-79) lots of 100 green (1) and yellow (2) seeds as well as 100 brown seeds were used. Lots of 100 green (1) and yellow (2) seeds were tested again on the 31-5-79. In the summer of 1980 only green (1) and yellow (2) seeds which were stored in the freezer for a year were tested; this time 400 seeds of each were used. Germination was checked after 2 weeks when the seedlings were between 5 and 10 cm tall.

2.4.3 Tetrazolium Test

This test is described in the Tetrazolium Testing Hand Book, Contribution No.29 to Hand Book on Seed Testing, Association of Official Seed Analysts, (Grabe, D. 1970 Ed.). Tetrazolium testing has been developed to furnish a quick estimate of seed viability.

The tetrazolium test was performed on lentil seeds after they were taken from storage and a color change on the seed coat had occurred. Lots of 100 seeds were used for each of the five different seed coat colors.

a) Imbibition of the Seeds in TTC solution:

The intact seeds were put in a 100 ml beaker containing a 1% solution of 2,3,5-triphenyl tetrazolium chloride (TTC), (1 gram of TTC in 100 ml of distilled water, pH between 6 and 8). Sufficient solution was used to cover the seeds and to allow absorption for 6 to 7 hours at 35°C and in darkness.

b) Evaluation of the test:

After staining was completed, most of the tetrazolium was siphoned off using an eye dropper. A small amount of liquid was left in the beaker to prevent the seeds from drying out.

Seed coats were removed before examination by rupturing them with a forceps. The cotyledons were then separated and the staining pattern of the internal tissues was evaluated. Viable seeds stain to a deep red color whereas non-viable seeds do not stain at all.

2.5 TESTS ON EXUDATES FROM IMBIBED SEEDS

2.5.1 Conductivity Test

The procedure followed is a modification of that of Matthews and Whitbread, 1968. The test consists of measuring the electrical conductivity of deionised water using a conductivity meter (conductivity cell, cel/VSO 1, K:0.100/cm), Beckman Instruments, INC Cedar Grove Operation, New York. The test is a measure of the amount of solutes that have leaked from the seeds during imbibition.

Two replicate lots of 50 seeds each were weighed and covered with 50 ml of deionised water. A control beaker containing water but no seeds was set with each test run. All beakers were labelled and covered with parafilm, and kept at 20°C for 6 hrs under fluorescent lights. After 6 hrs the soaking water was decanted into a second beaker, and divided into 4 equal fractions, (approximately equivalent 5 ml/seed). Fractions 1 and 2 were diluted to 125 ml with deionised water and their conductivities determined. For each replicate the conductivity reading is expressed per gram

of seed and the mean of the two readings is calculated as follows:

$$\frac{\text{Conductivity of fraction 1} \quad \text{Conductivity of Fraction 2}}{\text{Initial wt. of seed lot 1} \quad \text{Initial wt. of seed lot 2}} \div 2$$

fractions 1 and 2 were kept for further analysis. Fractions 3 and 4 were kept in 25 ml plastic bottles, and freeze-dried in a Virtis freeze-dryer. The dried exudates were redissolved in 1 ml of distilled water for later chromatography.

2.5.2 Test for Proanthocyanins

After conductivity was determined the soaking water of fractions 1 and 2 was poured separately onto a polyvinylpyrrolidone (PVP) column for adsorption of soluble condensed proanthocyanins.

Preparation of the PVP columns:

PVP powder was washed with tap water to remove the fine particles. After 10 min of settling, the water was decanted and the process repeated 19 times, in order to wash off all small particles. Burettes of 50x1.5 cm. were used. Glass-wool was placed over the drain hole of the burette to keep

it clean of any particles, so the water could run freely through the column. PVP suspended in water was poured down the column using a wide mouth 10 ml volumetric pipette. The column was drained until almost all the water had run out and to obtain a final length of 10 cm of packed PVP (fig 4). After the run the stopcock was removed, the whole column blown out from the upper end with air pressure, collected on a paper towel, and air-dried (Thakur, M. 1980).

Hydrolysis of PVP adsorbable material:

The top part of the column (discolored by tannins) was transferred to a test tube and boiled in 4 ml of 50% HCl for 15 min (Clevenger, 1971). The resultant solution was decanted, diluted to 8 ml with distilled water; and centrifuged at 2,000 g. Absorbance was determined at 540nm, using a Coleman, Junior II, spectrophotometer, Model G/20 (Marga *et al.* 1973). The red pigments were extracted using 1 ml of isoamyl alcohol. This procedure was done twice for each treatment having a total of 36 samples.

Paper Chromatography of Anthocyanidins of the Acid Hydrolyzate:

The isoamyl alcohol soluble fractions were one dimensionally chromatographed on one half of a 46x57 cm² sheet of

Whatman 1 paper; 5 samples were spotted on each paper. Each spot was made from 0.1 ml of the pigment solution, approximately 1.5 cm. in diameter, and separated by about 3 cm from its neighbours. The distance from the edge was 2.5 cm, and from the end, 7.5 cm. The papers were run descendingly for about 16 hrs using as a solvent "Forestal" (glacial acetic acid-water-conc. HCl, 30:10:3 v/v/v) (Harborne, 1967; Riberau-Gayon, 1972).

2.5.3 Paper Chromatography of the Freeze-dried Exudates

The crude exudates (fractions 3 and 4) were each chromatographed in 2 dimensions on one fourth of a 46x57 cm² sheet of paper, 0.1 ml (equivalent to 2.5 seeds) of the exudate was spotted in the right side corner, 2 cm from the short edge and 2.5 cm from the long edge. The papers were stapled along the long edge to form cylinders and chromatographed ascendingly for 2 hrs using 6% glacial acetic acid as a solvent. The papers were then air-dried, restapled along the short edge and run again ascendingly for 8 hrs in the second direction using as a solvent secondary butanol- acetic acid-water (14:1:5 v/v/v). The finished chromatograms were again air dried. The dried chromatograms were examined under long-wave U.V. and the absorbing or fluorescing spots marked with a lead pencil. The papers were then sprayed with:

1) Ferric chloride-potassium ferricyanide, 0.2% solution. After spraying, the chromatograms were washed with 2N-HCl, then in distilled water several times, and air dried. Oxidisable phenols including proanthocyanins are readily revealed as prussian blue spots on a white background.

2) Gibbs reagent (2,6 dibromobenzoquinone 4-chloroimide), 0.5% w/v in acetone, followed by sodium hydrogen carbonate (saturated aqueous solution). Reactive spots appear mauve-purple on a white background.

3) Benzidine, (diaminodiphenyl). The different phenolic acids give different colors which change with pH. (Thompson, *et al.* 1972).

2.6 DETERMINATION OF TOTAL PROANTHOCYANIN CONTENT OF THE SEED COAT

Seeds were taken directly from plants growing at the Central Experimental Farm. Pods were collected at different stages of maturity: bright green, yellow, and brown corresponding in the centroid color chart to 117, 72, and 75 respectively. The seed coat colors were the same as those of the pods. The seeds were removed from the pods, and their seed coats cut away using a razor blade. Replicate lots of 10 seed coats randomly selected from each of the three different coat colors collected from the field, were boiled separately with 4 ml of 50% HCl for 15 min, then the solution

was diluted to 8 ml using distilled water and the absorbance taken at 540 nm.

2.7 EXPERIMENTS PERFORMED

2.7.1 Long Term Experiments

2.7.1.1 Effect of Light, Temperature, and Humidity on Seed Discoloration:

18 different environmental conditions were provided as follows: using incandescent light or darkness, three different relative humidities: 0%, 50%, 100%, and three different temperatures (5°C, 20°C or 30°C). Replicate lots of 100 green (1) and yellow (2) lentil seeds variety Laird were stored under each condition for a total of 72 containers. When a color change was noted in the light-exposed seeds that lot was removed for analysis, the lots under the same storage condition of temperature and humidity but in darkness were removed from storage one week later up to the seventh week. From the ninth week to the last the samples under the same condition of temperature and humidity light or dark were taken for analysis at the same time.

The original intention was to leave the seeds until browning was observed in the light exposed containers and then to remove both light and dark samples at the same time. Seeds started to brown so rapidly at 100% R.H. in

light that the number of samples was too large to handle all at once. Thus, to overcome this darkened containers were removed one week later. By the time the seeds in the 50% R.H. containers showed some browning, the availability of an assistant (Miss Christy Nuyens) enabled handling of 8 containers at a time. By this time it became apparent that it could be several months or perhaps years before complete browning occurred under some conditions and the protocol was changed to that of removal of all samples, starting with those at the highest temperature (20°C) within as short time as possible. When the samples were removed from storage and seed lots were classified by color (see fig 2), the tests specified in sections 2.4 and 2.5 were performed on one replicate lot. The second lot was stored in the freezer for 6 months, after which they were tested for viability with TTC.

2.7.1.2 Effect of Anaerobiosis on Seed Coat Color:

100 green (1) and yellow (2) lentil seeds var. Laird were stored in an anaerobic container where the oxygen was eliminated using gas-pack disposable hydrogen + carbon dioxide generator envelope (70304 BBI division of BioQuest) at 50% R.H. using 46% H₂SO₄ and continuous illumination with incandescent light for an 11 month period. Seed coat color was checked periodically.

2.7.1.3 Effect of Freezing Temperature on Seed Coat Color:

After setting up of the long term experiment, the surplus of green (1) and yellow (2) seeds were kept in the freezer under complete darkness. Seeds were removed after one year of storage for examination of seed coat color and the TTC test performed on them.

2.7.1.4 Test to compare Humidity Controlling Agents:

In order to check the effect if any of humidity controlling agents on the discoloration of lentil seeds, replicate lots of 100 green (1) seeds each were stored at 20°C, 50% R.H. under incandescent light. The 50% R.H. was achieved using 46% H_2SO_4 in one replicate lot and a saturated solution of $CaCl_2$ for the other. The discoloration phenomenon was followed for a month period. The same experiment was repeated a second time.

2.7.2 Short term Experiments

2.7.2.1 Storage Experiment:

Since the seeds browned so quickly at 30°C, 100% R.H. in light, a second test was run as follows: Seven jars (fig 3a) containing 200 green (1) seeds variety Laird were placed in the 30°C room at 100% R.H. under continuous illumination

with an incandescent lamp. Every 24 hrs thereafter, one jar was removed for testing the rate of water uptake, germination percentage, electrical conductivity and soluble proanthocyanins.

2.7.2.2 Effect of Light Quality on Seed Discoloration:

To test which wave length range was most effective in promoting the browning phenomenon in lentil seeds, replicate lots of 100 green (1) seeds each were placed in the 30°C room at 100% R.H. under B.L.B. light, fluorescent light or incandescent light plus a U.V. filter. After 72 hrs of storage the seeds were examined for color change in the seed coat. The experiment was repeated once.

2.7.2.3 Effect of Genetic Constitution:

Using P.I. 345635 one jar containing 100 seeds was stored at 30°C, 100% R.H. under incandescent light for one week. After that the seeds were removed from the jar, examined for any color change in the seed coat, and the seed exudates further analyzed.

2.7.2.4 Effect of an antioxidant on seed exudates:

4 lots of 25 green (1) lentil seeds were removed from the freezer after storage for one year. Two lots were imbibed in 25 ml of deionised water and two lots were imbibed in 25 ml

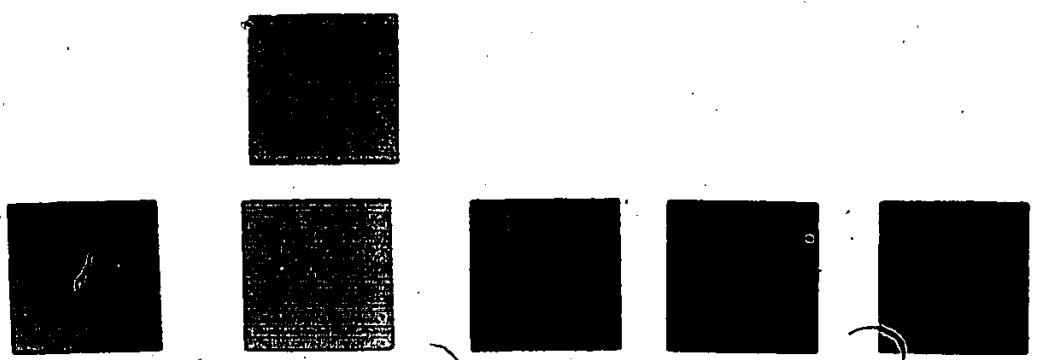
of deionised water plus 0.25 g of ascorbic acid. The seeds were kept during imbibition at 20°C and continuous illumination with fluorescent light for a period of 6 hrs, after that the water was decanted, freeze-dried, and chromatographed in two dimensions, in order to determine the effect of ascorbic acid in the oxidation of polyphenols present in the seed exudates.

A color chart was used to indicate the seed coat color changes throughout the experimental work.

Seed Coat Color	Centroid Chart No.
Green (1)	91
light Yellow (2) between	76 and 91*
Yellow-Brown (3)	77
Light-Brown (4)	58
Dark-Brown (5)	59

* Two color chips were chosen since in the color chart there was no chip which exactly matched as the seed coat for the light yellow seeds (2).

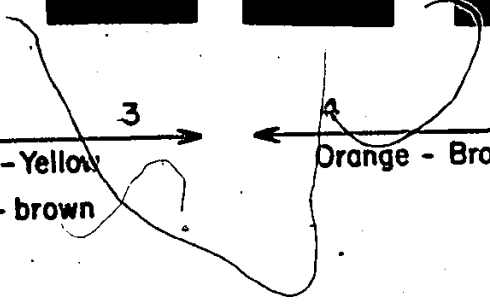
Figure 2: Centroid Color Chart.



1
Greenish
Yellow
Olive

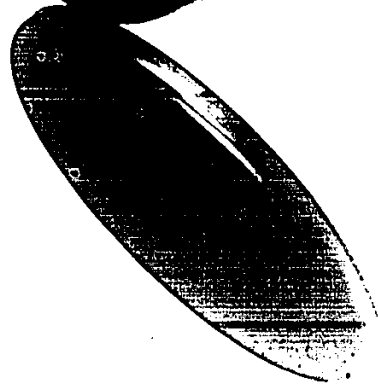
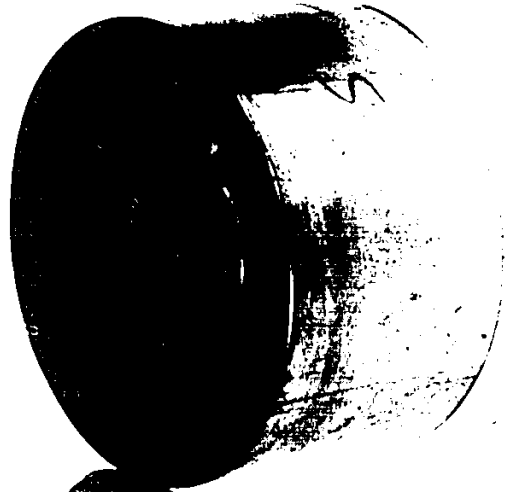
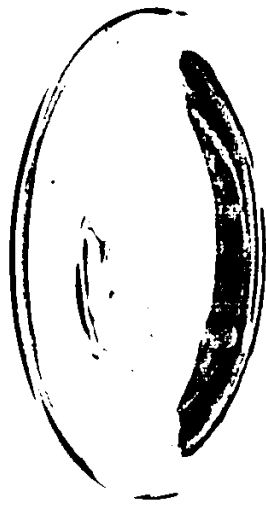
2 ← → 3
Orange - Yellow
Yellowish - brown

← 4 5 →
Orange - Brown



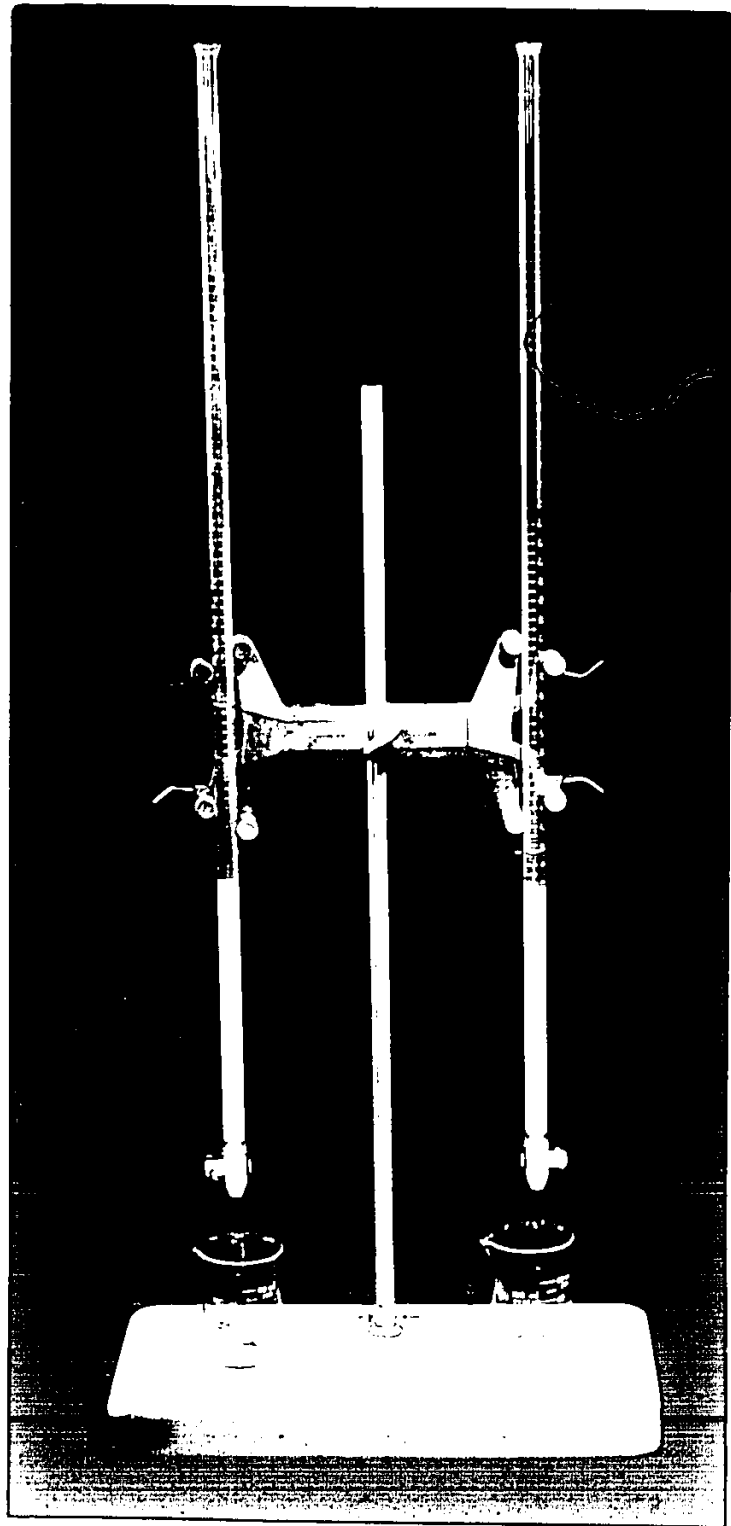
The glass containers (a) used to store light-exposed seeds were 15 cm high and 8.5 cm diameter. The aluminum cans (b) used to store dark-exposed seeds were 5.2 cm high and 9.0 cm diameter.

Figure 3: Containers for light-exposed and darkened-exposed seeds.



Burettes of 50x1.5 cm were used to set up the PVP columns. After being washed 20 times the PVP suspended in water was poured down the columns to obtain a final column length of approximately 10 cm.

Figure 4: Polyvinylpyrrolidone (PVP) columns.



section III

RESULTS

3.1 EFFECT OF LONG TERM STORAGE ON LENTIL SEEDS

3.1.1 Seed Coat Color Change

A broad range of environmental conditions (light, humidity, and temperature) was surveyed for the purpose of choosing conditions in more detailed subsequent experiments. Evidently light, high humidity, high temperature and long exposure to the conditions are important factors in the browning reaction of lentil seeds (table 1).

Storage under continuous illumination with incandescent light at 30°C and 100% relative humidity resulted in rapid discoloration of lentil seeds. (The degree of discoloration was determined at the time of sampling by reference to the color chart shown in figure 2. In the space of one week seeds changed from green (1) and yellow (2) to a dark brown (5) (Table 1, fig 5a). By contrast seeds stored in darkness under the same conditions of temperature and relative humidity had discolored only slightly if at all after two weeks (fig 5b). At 20°C and 100% R.H. all seeds stored in light were dark brown and overgrown by fungi by 3 weeks. Green (1) seeds under darkness discolored to dark brown (5) a week la-

ter, yellow seeds (2) under light turned yellow brown (3) after 3 weeks of storage. At lower humidities and temperatures the rate of browning, even in light was slower. Green (1) and yellow (2) seeds stored at 30°C, 50% R.H. discolored to a yellow brown (3) under light; in darkness green seeds changed to light yellow, and light yellow discolored to yellow brown (3). When the relative humidity was lowered to 0% all seeds stored under light changed to yellow brown (3) but seeds stored under darkness did not discolor even after 10 weeks of storage. Seeds at 20°C, 50% R.H. or 0% R.H. changed slightly under light and remained practically unchanged under darkness. Seeds at 5°C either at 50% or 0% R.H. had discolored very little even after 12 weeks of storage.

The purpose of this thesis is not to examine in detail environmental factors on browning but rather to look at some of the physiological changes occurring during browning; some of the reasons for this approach were the shortage of seed supply and time exposure with respect to economically realistic storage conditions.

3.1.2 Seed Viability Tests

3.1.2.1 Rate of Imbibition

The rate of water uptake in green (1) lentil seeds before storage was greatest within the first 3 hrs of soaking,

after this time the rate increased very slowly, until maximum imbibition had been reached by about 18 hrs, with a final increase of 260% (fig 6). In figures 7 and 8 it can be seen that when seeds are kept under different storage conditions, seeds stored at lower temperatures (5°C) for a period between 6 to 16 weeks (table 1) imbibed less water than seeds stored at higher temperatures (20°C or 30°C). An exception are the seeds stored at 30°C and 100% relative humidity in light; these samples were very badly damaged by fungal growth. In most cases seeds stored in the light imbibed more water than those kept in the dark regardless of the temperature. This pattern was found in green and yellow lentils except for the ones kept at 5°C, 0% relative humidity and 5°C, 100% relative humidity. In general seeds stored in the light at high temperature under low humidity were the ones with higher imbibition values, for instance, seeds stored at 30°C, and at 50% or 0% relative humidity had an increase of fresh weight of 295% for the former and 285% for the latter.

In figure 9 water uptake related to the seed coat color after storage is shown. Green (1) and yellow (2) and light brown (4) seeds had mean values of 236, 240, and 236 per cent increase in fresh weight respectively; whereas yellow-brown (3) showed the greatest average value of 271%, and dark brown (5) had the lowest (212%).

3.1.2.2 Germination Tests

Effect of storage conditions on germination is shown in table 1 and figure 10. Germination percentage was found to be lower in seeds stored under light (open circles) than under darkness (closed circles). An exception was green (1) and yellow (2) seeds stored at 20°C, 100% R.H. and darkness where germination percentage was zero. It was also noted that germination was lower when seeds were stored at 100% R.H. regardless of the temperature.

The relationship of germination percentage and seed coat color resulting from storage is shown in figure 11. Germination percentages are consistently lower in discolored seeds, slightly so in yellowed seeds (2) and yellow brown (3) and markedly so in browned seeds (4 and 5)

Germination was also tested in the field and it was found that green seeds showed better germination than yellow and brown seeds for the first summer (table 2). For the first planting (15-5-79) germination percentages were 86%, 76%, and 4% for green, yellow and brown seeds respectively; and for the second planting (31-5-79) germination dropped to 76% for green seeds and 68% for yellow seeds. No brown seeds were tested the second time. In the summer of 1980 germination was checked only once. As in the previous year green seeds had the greatest germination, the values being 85% for

green seeds and 79% for yellow seeds. The Student t test was applied to the set of data for the summer of 1980 and it showed that the 7% difference in germination of green (1) and yellow (2) seeds was significant at the 95% level.

3.1.2.3 Tetrazolium test

In figure 12 it can be seen that some seeds stain to a deep color whereas others do not stain. In figure 13 a close up of some of the staining patterns observed is shown. The whole embryo is stained (a), most of the cotyledon stained but the shoot-root axis not (b), or no part of the seeds stained (c). In figure 14 the staining patterns of lentil seeds of different seed coat colors are shown. Seed lots with seed coats ranging from green (1) to yellow brown (3) stained to a deep red color, but seed lots of light brown (4) and dark brown (5) stain little, if at all. The percentage of staining in relation to seed coat color is presented in table 3, green seeds (1) showed the highest percentage of staining (80%) yellowed seeds (2 and 3) showed a 74% and 62% of staining respectively. Light brown (4) and dark brown (5) seeds had the lowest staining values, 10% and 0% respectively. The tetrazolium test was repeated with green seeds from the same lot as before whose seed coats had been removed before immersion in the tetrazolium solution; 82% of the seeds were stained.

3.1.3 Effect of Anaerobiosis on Seed Coat Color

Green (1) and yellow (2) lentil seeds stored under anaerobic atmosphere did not discolor even after 11 months of storage.

3.1.4 Effect of Freezing Temperature on Seed Coat Color

Green (1) and yellow (2) seeds kept in the freezer for over a year showed a very slight change in their seed coat color. The lot of green (1) seeds had approximately 2% of yellowish seeds and yellow (2) seeds did not show any detectable seed coat color change. The lots of seeds which were kept in the freezer after the long term storage experiment remained the same color they had been when removed from the long term storage experiment.

3.1.5 Humidity Controlling Agents

Initially green (1) seeds stored under incandescent light at 20°C, 50% R.H. using H_2SO_4 or $CaCl_2$ discolored to yellow brown (3) after one month of storage, therefore no difference was found in the effect of the two different solutions on the discoloration process of lentil seeds.

3.2 TEST ON EXUDATES FROM INHIBED SEEDS

3.2.1 Electrical Conductivity

The effect of storage conditions on electrical conductivity of lentil seeds gave variable readings. In table 1 it can be seen that high temperature (30°C and 20°C) and high humidity (100%) showed the highest conductivity values. Lowering the temperature and humidity resulted in a decrease of electrical conductivity. In figure 15 a relationship between seed coat color change during storage and electrical conductivity values is shown. Conductivity was highest with darkened seeds: seeds which remained green (1) even after long term storage had conductivity values between 21 and 35, yellow (2) seeds ranged between 21 and 65, yellow brown (3) showed values between 34 and 49, and dark brown (5) had conductivity readings between 115 and 225. The vertical bars represent the standard error of the means.

3.2.2 Test for Proanthocyanin

The amount of PVP adsorbable material, as indicated by the depth of the tan color, varied from one seed lot to another. Its proanthocyanin nature was verified by the appearance of a bright red color upon boiling in acid, and by the separation of two red spots behaving as cyanidin and delphinidin on chromatograms of the isoamyl alcohol soluble fraction (fig 16). In table 1 it can be seen that absorbance of the anthocyanidin fraction was very variable ac-

ording to the storage conditions. At high temperature (30°C) and high humidity (100%) absorbance was low compared to the values of seeds after storage at low temperature (5°C) and 0% relative humidity. Figure 17 shows a quantification by absorbance measurements at 540 nm. When seeds were green (1) absorbance ranged from 0.57 to 0.90 (table 1) with a mean value of 0.67 a similar value was observed with yellow (2) and light brown (3) seeds, and with light brown (4) seeds absorbance averaged 0.50 with the seeds that had turned to a dark brown (5) absorbance was very low (0.10).

3.2.3 Paper Chromatography of the Freeze-dried Exudates

Of the 3 different colorogenic reagents used to detect the presence of soluble phenolic compounds including condensed proanthocyanins on chromatograms of crude exudates, ferric chloride-potassium ferricyanide was chosen since it showed the best results.

In figure 18 is presented representative chromatograms obtained from freeze-dried exudates of seeds of each of the 5 seed coat colors resulting from long term storage. Exudates from seeds which remained green (1) throughout the experiment had four spots mobile in both solvents, plus some spots immobile in BAW. Yellow (2) seeds showed only 3 mobile spots in both solvents and also material immobile in BAW. Yellow brown (3) seeds showed a smear in BAW with only 2

spots mobile in both solvents. Chromatograms made of exudates from light brown (4) and dark brown (5) seeds showed very little material and no mobility of spots whatsoever.

When ascorbic acid was added to green (1) seeds during imbibition, the exudate when chromatographed showed a big spot (A) on the left side of the paper, just around the area where the small spots were on exudates without the ascorbic acid.

3.2.4 Total Proanthocyanin Content

As it is shown in table 4, green seeds (1) had the highest reactivity of total proanthocyanin content (A=0.41 per 25 seeds), 25 yellow seeds (2-3) yielded a total of 0.27 absorbance followed by 25 brown seeds (4-5) with an absorbance of 0.11. These values are a result of replicate experiments.

3.3 SHORT TERM EXPERIMENTS

3.3.1 Storage experiment

3.3.1.1 Seed Coat Color Change

During the seven days of the short term storage experiment the seed coat color of lentil seeds changed gradually. After 24 hrs of storage the color remained green (1) but after 48 hrs it had turned to light yellow (2) and by the seventh day of storage, it had changed to dark brown (5).

3.3.1.2 Imbibition

Rate of water uptake decreased throughout the short term storage experiment. By the first day the value was 238% as shown in figure 19c then it gradually dropped off so that when the test was done on the seed lot with seven days of storage the value was 170%.

3.3.1.3 Germination

It was found that germination percentage remained high during the first two days of storage but by the third day it dropped to 50% and continuously decreased with time to approximately 2% during the last 3 days of storage (fig 19a).

3.3.1.4 Electrical Conductivity

Electrical conductivity of lentil seeds increased throughout the seven days of the experiment, the reading after the first day of storage was 38, it continued increasing and by the fifth day it was very high (110) and even higher (155) by the seventh day (fig 19b).

3.3.1.5 Concentration of Soluble Proanthocyanins

As is shown in figure 19d by the first day absorbance was 0.87 and by the second day had decreased to 0.70. By the third day it had gone down to 0.25 and by the seventh day absorbance was 0.10.

3.3.2 Effect of Light Quality on Seed Discoloration

Green (1) seeds stored under U.V. light at 30°C and 100% R.H. discolored from green (1) to yellow brown (3) in a period of 24 hrs, by 48 hrs they were light brown (4) and by 72 hrs dark brown (5). Seeds stored at the same conditions of relative humidity and temperature but under fluorescent light discolored to light yellow (2) after 72 hrs of storage (fig 20). Green (1) seeds stored at 30°C, 100% R.H. and under incandescent light plus a U.V. filter did not show any discoloration after a week of storage.

3.3.3 Effect of Genetic Constitution

P.I. 345635 was tested for browning as well as for presence of condensed proanthocyanins. It was found that these lentil seeds did not discolor even after a week of storage at 30°C, 100% R.H. and under continuous incandescent light. Presence of condensed proanthocyanins was not detected when seed coats were boiled in 50% HCl. Chromatographs of the crude exudate did not show any presence of phenolic compounds when run in two dimensions.

TABLE 1

Preliminary survey of a broad range of Storage Conditions on lentil seeds, (*L. culinaris*. Medic).

Storage Time*		Temp. °C.	R.H. %	S.C.C.**		Germ.*** %		Abs*** 540nm		Conductivity*** readings.	
L.	D.			Init.	Final.	L.	D.	L.	D.	L.	D.
1	2	30	100	1	5@ 2	0	66	.30	.70	225	52
				2	4 4	18	47	.60	.52	65	60
3	4	20	100	1	5@ 5@	0	1	.18	.00	116	115
				2	3 5@	61	1	.35	.00	49	158
6	7	5	100	1	4 1	39	90	.19	.80	71	23
				2	4 2	38	80	.80	.65	62	23
9	30	50	50	1	3 2	76	82	.53	--	32	21
				2	3 3	77	82	.75	.40	43	24
10	30	0	0	1	3 1	62	90	.60	.57	38	34
				2	3 2	72	81	.60	.67	42	33
10.5	20	50	50	1	2 1	84	93	.67	.60	31	23
				2	2 3	80	77	.73	.90	37	36
11	20	0	0	1	2 1	82	96	.49	.60	35	27
				2	3 2	77	86	.59	.63	37	32
11.5	5	50	50	1	2 1	79	95	.79	.57	32	21
				2	2 2	80	87	.77	.70	43	24
12	5	0	0	1	2 1	78	87	1.50	.90	65	35
				2	2 3	80	75	1.00	1.00	34	34

*Time in weeks exposed to light (L) or in darkness (D).

** S.C.C.=Seed coat color (see figure 2): Seeds were separated initially (Init.) into green (1) or yellow (2) and their color (Final) was again assessed after storage in light (L) and in darkness (D) as green (1), yellow (2), yellow brown (3), light brown (4) or dark brown (5).

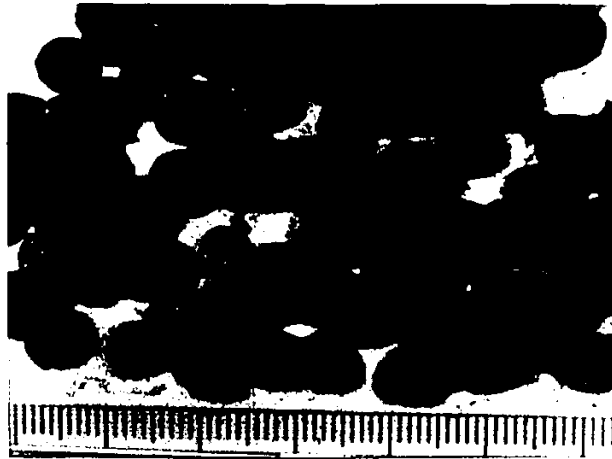
*** Germination %, absorbance of hydrolysed proanthocyanins in the exudate and conductivity of the exudate were determined in replicate. Values given in the table are the average of two readings.

@ Seed coats dark brown, fungal growth already abundant on the seeds.

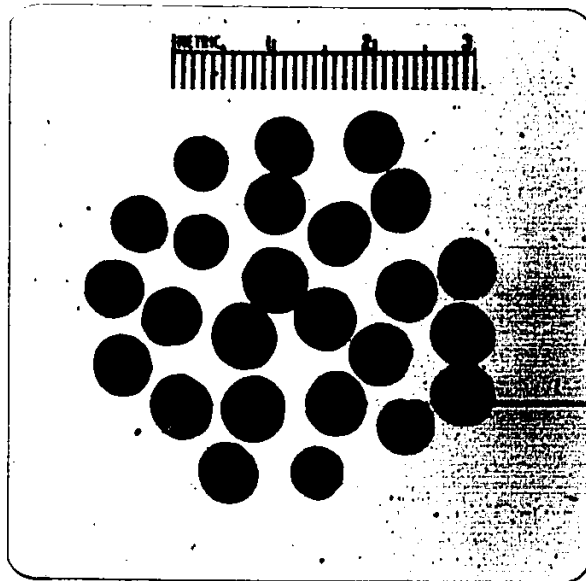
(a) green seeds (1) stored for a period of one week under incandescent light at 30°C, 100% R.H. Note a dark color of seed coats (5) dark brown. The numerical value of colors was determined by reference to color chips (fig 2). Also note a fungal growth on the seeds.

(b) green seeds (1) stored for a period of two weeks under complete darkness at 30°C, 100% R.H. Discoloration did not occur nor fungal growth.

Figure 5: Lentil seeds after storage at 30°C, 100% R.H. under light or darkness.



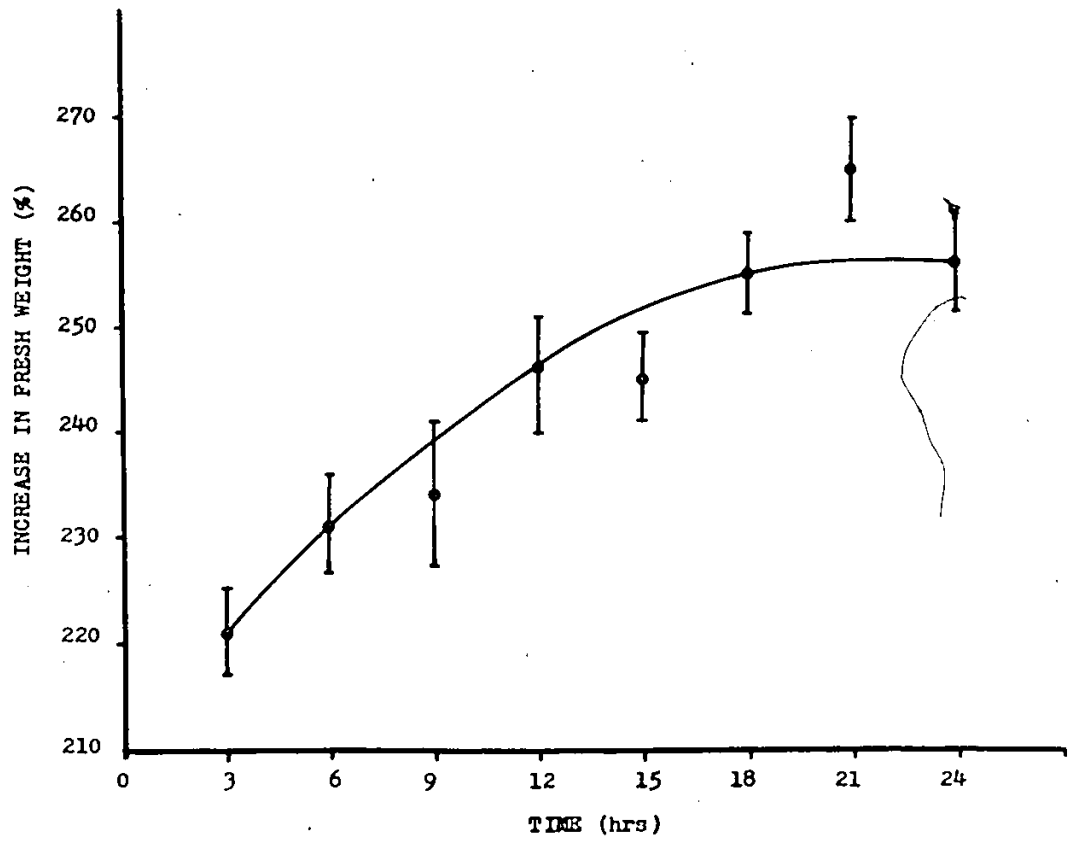
(a)



(b)

Replicate lots of 50 green (1) lentil seeds each were soaked in 50 ml of deionised water for a period ranging from 3 to 24 hrs, replicate samples were removed every 3 hrs for fresh weight determination. The vertical bars represent the standard error of the mean of two replicate samples.

Figure 6: Percentage increase in fresh weight of green (1) lentil seeds during imbibition.



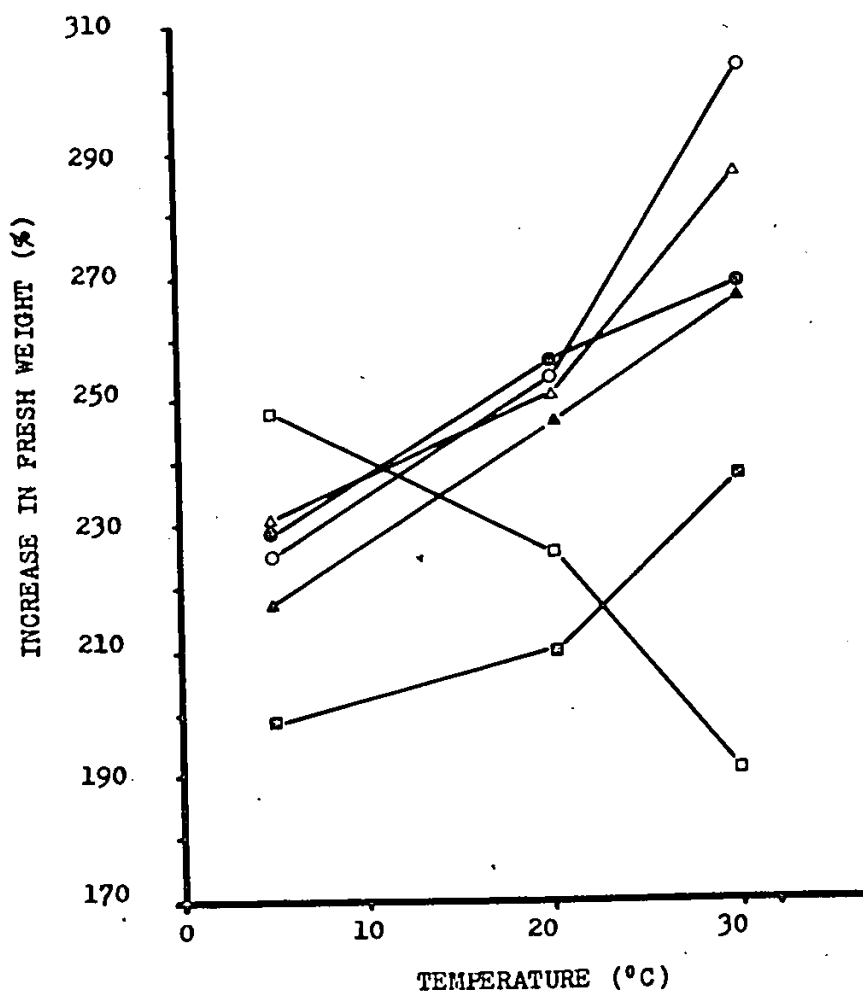
INCREASE IN FRESH WEIGHT OF GREEN LENTIL SEEDS FOR A PERIOD OF 24 hrs.

Percent increase in fresh weight of initially green (1) lentil seeds after storage at three different temperatures (5°C, 20°C or 30°C), three different humidities (0%, 50% or 100%), and in continuous incandescent light (open symbols) or complete darkness (closed symbols).

Note: Uptake of water during imbibition increases with a rise in storage temperature from 5°C to 20°C to 30°C, except for those seeds which were dark brown (5), i.e. stored at 20°C and 30°C in light and 100% R.H.

Figure 7: Imbibition percentage of initially green (1) lentil seeds after storage.

- LIGHT 0 % RH.
- DARK 0 % RH.
- △ LIGHT 50 % RH.
- ▲ DARK 50 % RH.
- LIGHT 100 % RH.
- DARK 100 % RH.

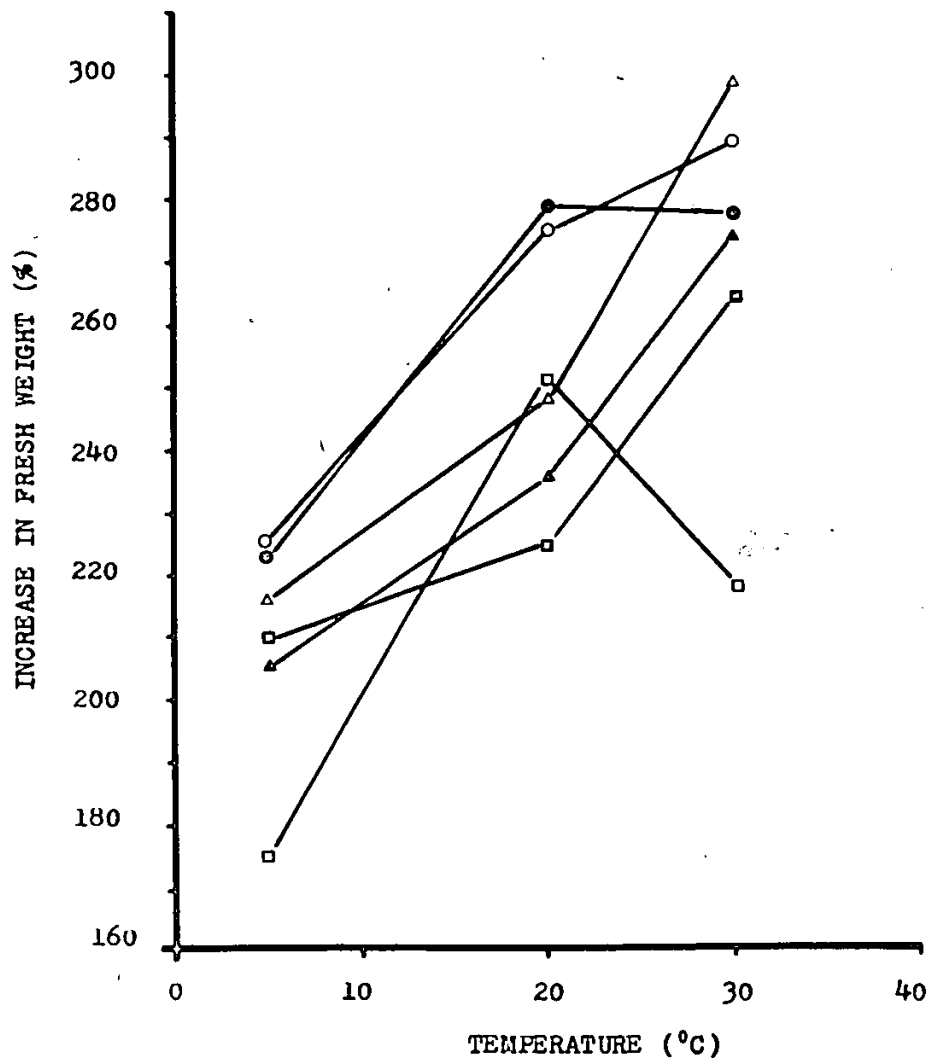


Per cent increase in fresh weight of initially yellow (2) lentil seeds after storage at three different temperatures (5°C, 20°C or 30°C), three different humidities (0%, 50% or 100%) and in continuous incandescent light (open symbols) or complete darkness (closed symbols).

Note: Uptake of water during imbibition increases with a rise in storage temperature from 5°C to 20°C to 30°C except for those seeds which were dark brown (5), i.e. stored at 30°C in light at 100% R.H.

Figure 8: Imbibition percentage of initially yellow (2) lentil seeds after storage.

- LIGHT 0 % RH.
- DARK 0 % RH.
- △ LIGHT 50 % RH.
- ▲ DARK 50 % RH.
- LIGHT 100 % RH.
- DARK 100 % RH.



Mean values of lentil seeds imbibition according to seed coat color change after storage at different environmental conditions of light, temperature and humidity. The vertical bars represent the standard error of the means.

Note: The high average of seed coat color (3) is an artefact resulting from the high proportion (5 of 9) of seed lots exposed to 30°C during storage. As indicated in fig. 7 and 8 the average imbibition of green (1), yellow (2) and yellow brown (3) is about 220%; of seeds stored at 20°C, about 250%; but of seeds stored at 30°C, about 280%.

SEED COAT COLOR	No. OF SAMPLES STORED AT		
	50°C	20°C	30°C
1	3	2	1
2	6	6	1
3	1	3	5
4	2	0	2
5	0	3	1

Figure 9: Rate of imbibition related to seed coat color change after storage.

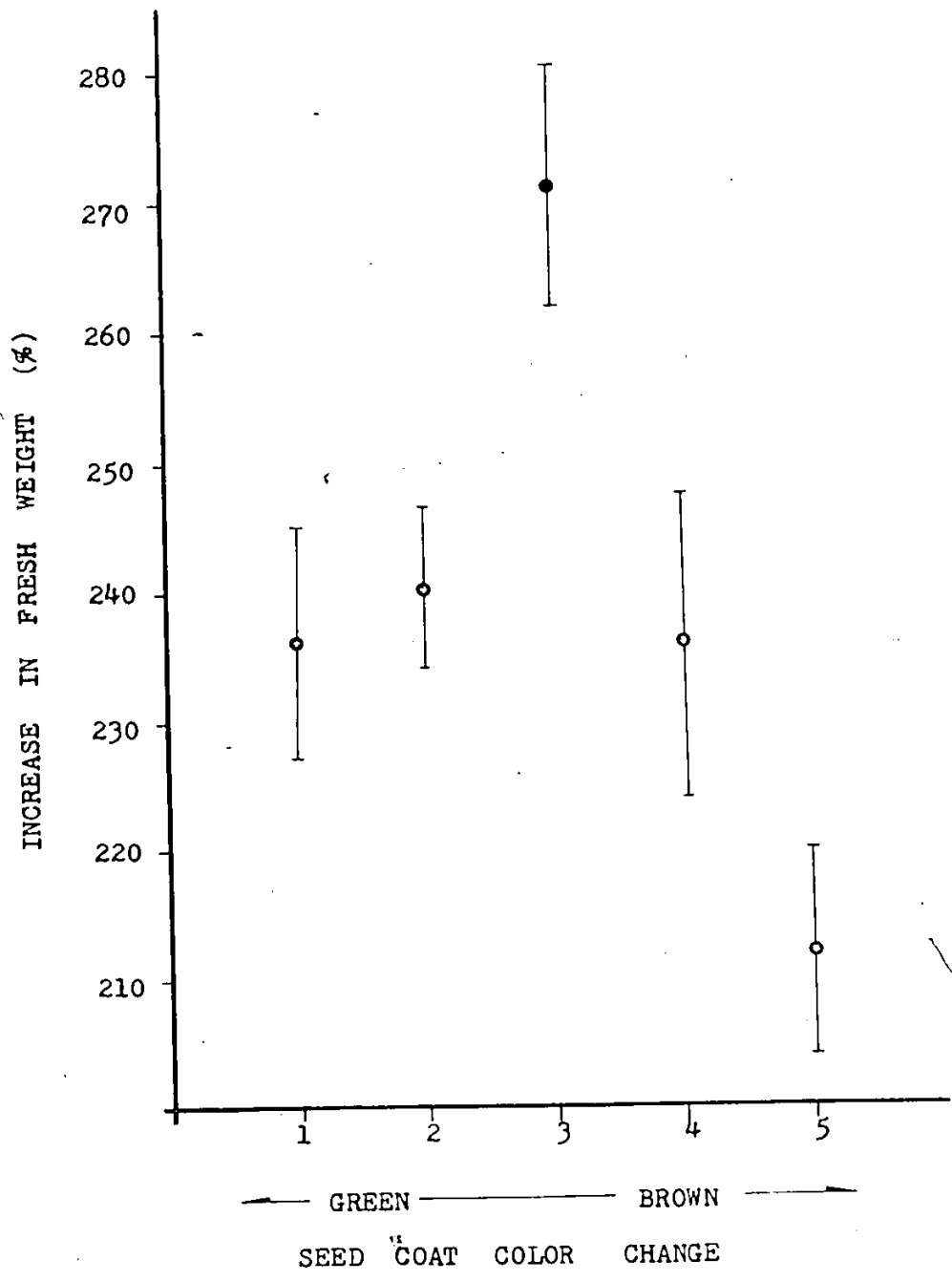


TABLE 2

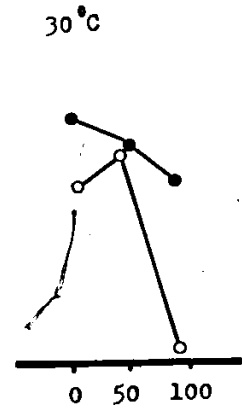
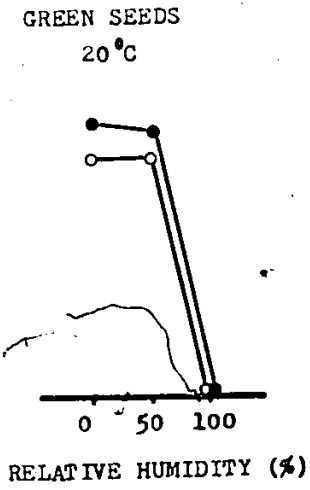
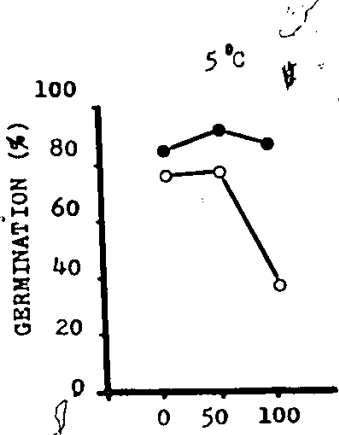
Germination % of green, yellow, and browned seeds under field conditions.

Seed Coat Color	Plantings of Summer 1979		Summer 1980
	1st of May	2nd of June	
GREEN (1)	86	76	85
YELLOW (2)	72	68	79
BROWN (4-5)	4	--	--

The data for the summer of 1980 shows a significant difference at the 95% level, by the Student t test between green (1) and yellow (2) seeds.

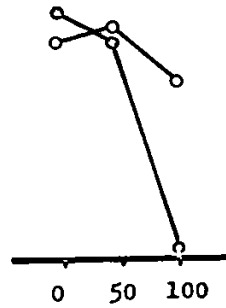
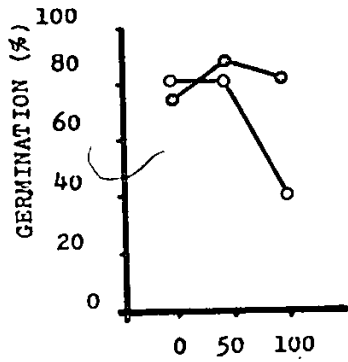
Green (1) and yellow (2) lentil seeds were stored under continuous incandescent light (open circles) or complete darkness (closed circles), at three different temperatures (5°C, 20°C or 30°C) and three different humidities (0%, 50% or 100%). Germination was determined by placing the imbibed seeds on moist sand in petri plates and at 20°C and fluorescent light for 5 days approximately.

Figure 10: Germination percentage of Lentil seeds after storage under different conditions.

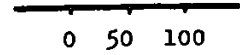


○ LIGHT.
● DARK.

YELLOW SEEDS

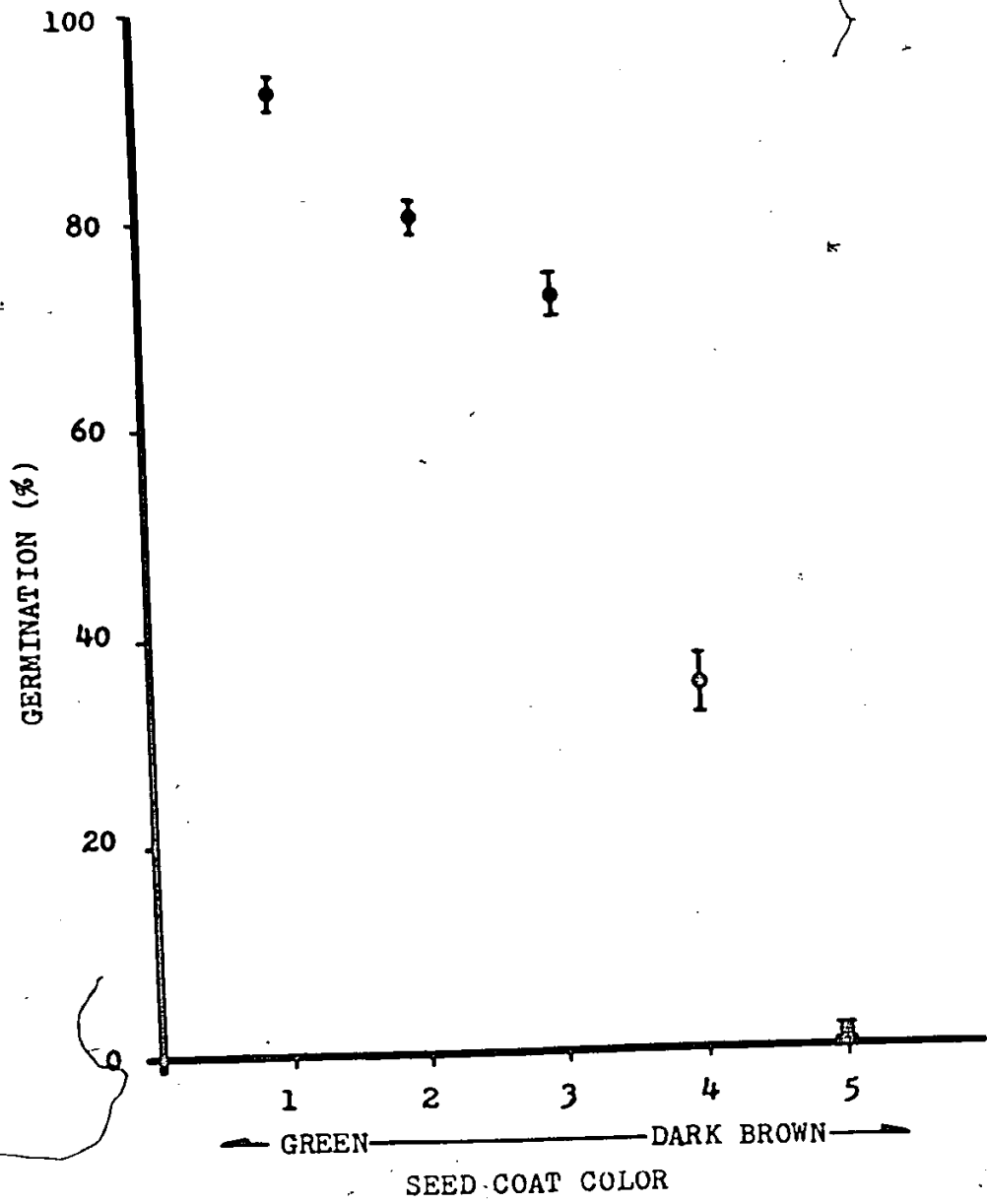


RELATIVE HUMIDITY (%)



Mean values of germination percentage of lentil seeds according to seed coat color change after storage at different environmental conditions of light, temperature and humidity (table 1). The vertical bars represent standard error of the means.

Figure 11: Relationship between seed coat color and germination percentage.



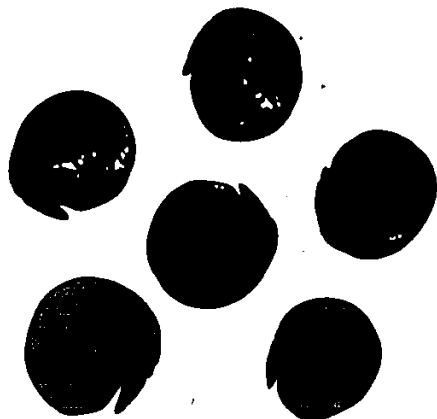
After 6 hrs imbibition in TTC at 35°C, germinable seeds stain to a deep color; whereas non germinable seeds do not stain.

Figure 12: Tetrazolium Test.

TETRAZOLIUM TEST



NON GERMINABLE

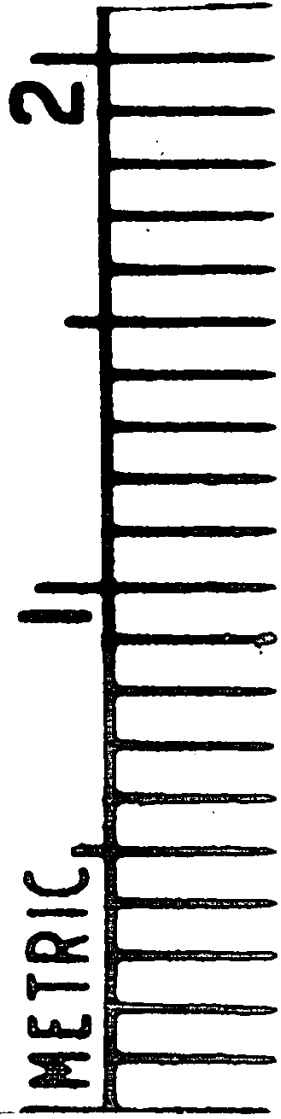
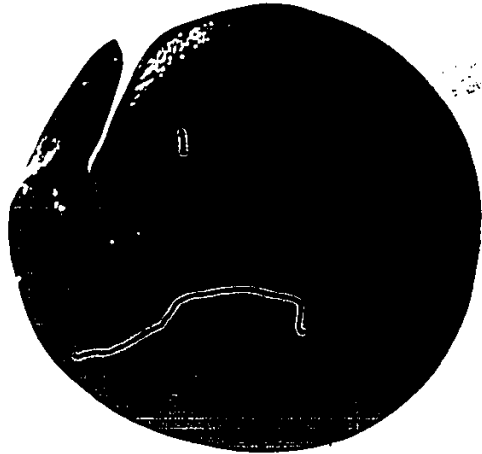


GERMINABLE

In a close up of lentil seeds after imbibition in TTC three different staining patterns can be observed:

- (a) The whole embryo stained, seeds are considered viable.
- (b) Cotyledon stained but shoot-root axis did not stain seeds are considered non viable.
- (c) When the whole embryo is not stained, the seed is dead.

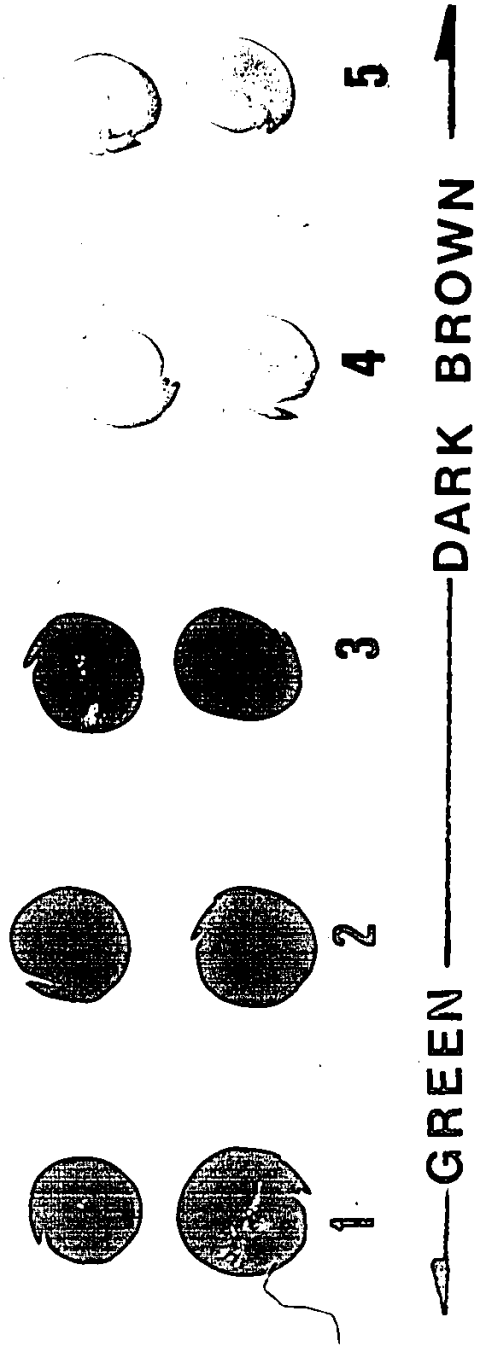
Figure 13: Close up of three different staining patterns using TTC.



Seeds with a seed coat from green (1) to yellow-brown (3) have a high number of seeds stained completely, but light brown (4) and dark brown (5) do not stain as much; so these last two lots were considered non viable.

Figure 14: TTC Test on lentil seeds which discolored during storage.

TETRAZOLIUM TEST



seed coat color

TABLE 3

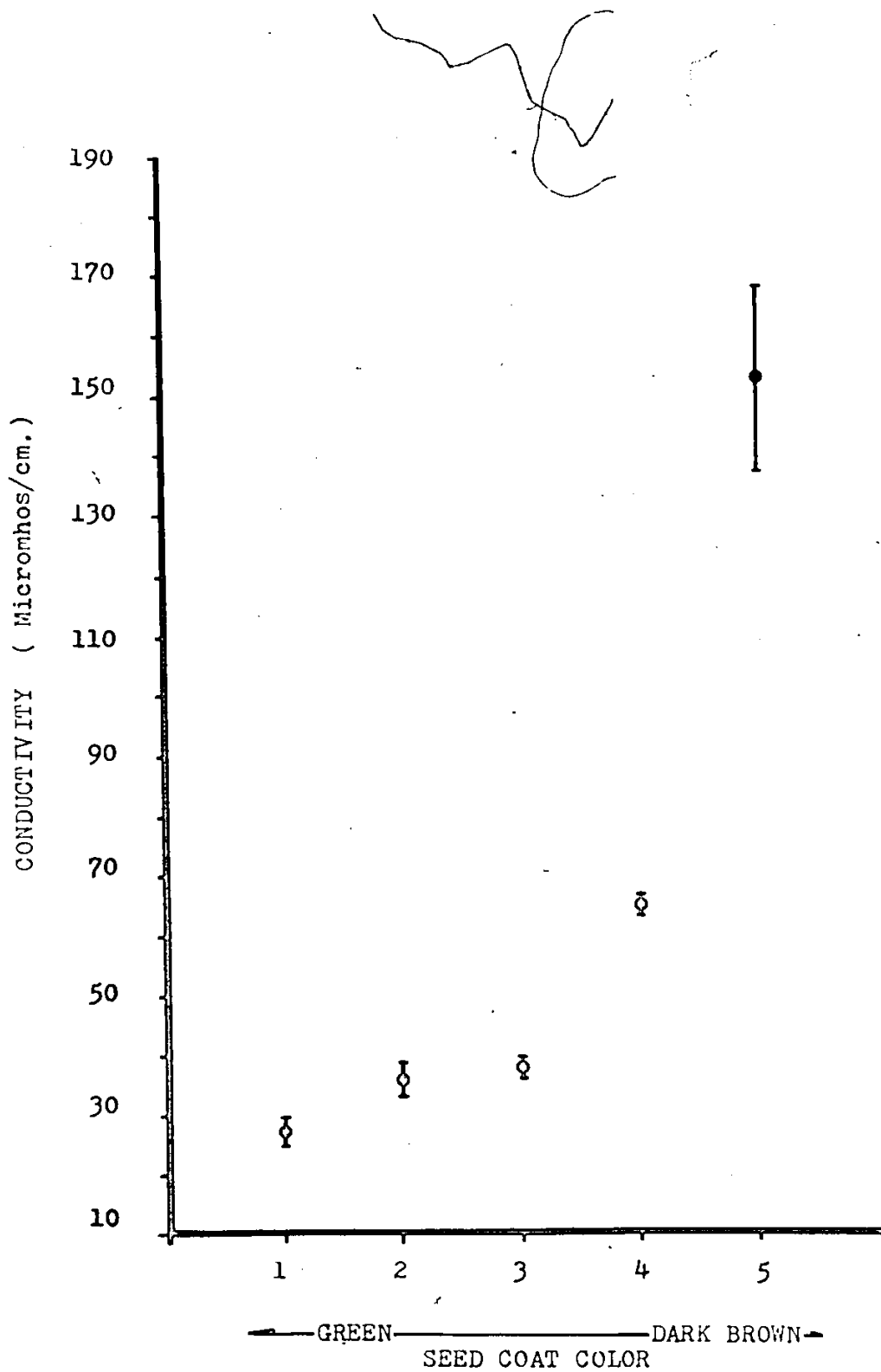
Percentage of staining of lentil seeds after storage using
TTC.

	FINAL	SEED	COAT	COLORS	
	1	2	3	4	5
stained seeds (%)	80	74	62	10	0
Not stained (%) seeds	20	26	38	90	100

The seeds were removed from storage and kept in the freezer
for 4 months before the TTC test was performed.

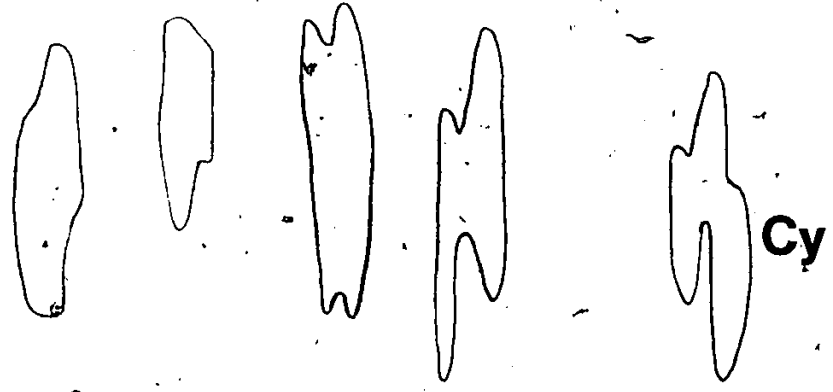
After storage at different environmental conditions lentil seeds of different seed coat colors were imbibed in deionised water for 6 hrs at 20°C and fluorescent light, and electrical conductivity determined using a conductivity meter (table 1). The vertical bars represent the standard error of the means.

Figure 15: Mean of electrical conductivity values of lentil seed exudates related to seed coat color.

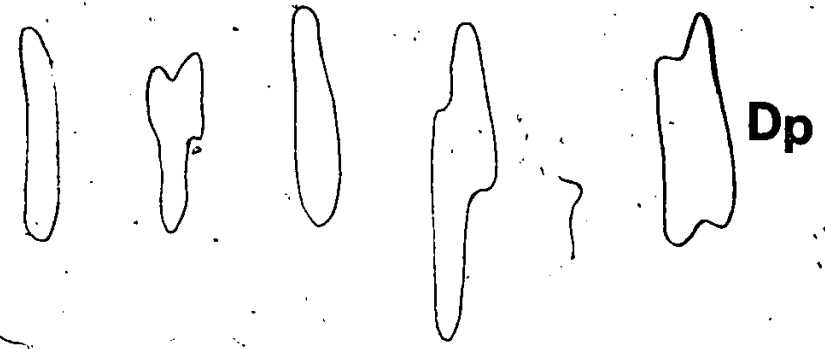


Chromatographs of anthocyanidins obtained by acid hydrolysis of the PVP adsorbable material exuded from lentil seeds of various seed coat colors during imbibition on deionised water. Spots are proportional to 2.5 seeds. Cy=cyanidin Dp=delphinidin.

Figure 16: Chromatographs of anthocyanidins from acid hydrolysis of the seed exudates.



Cy



Dp

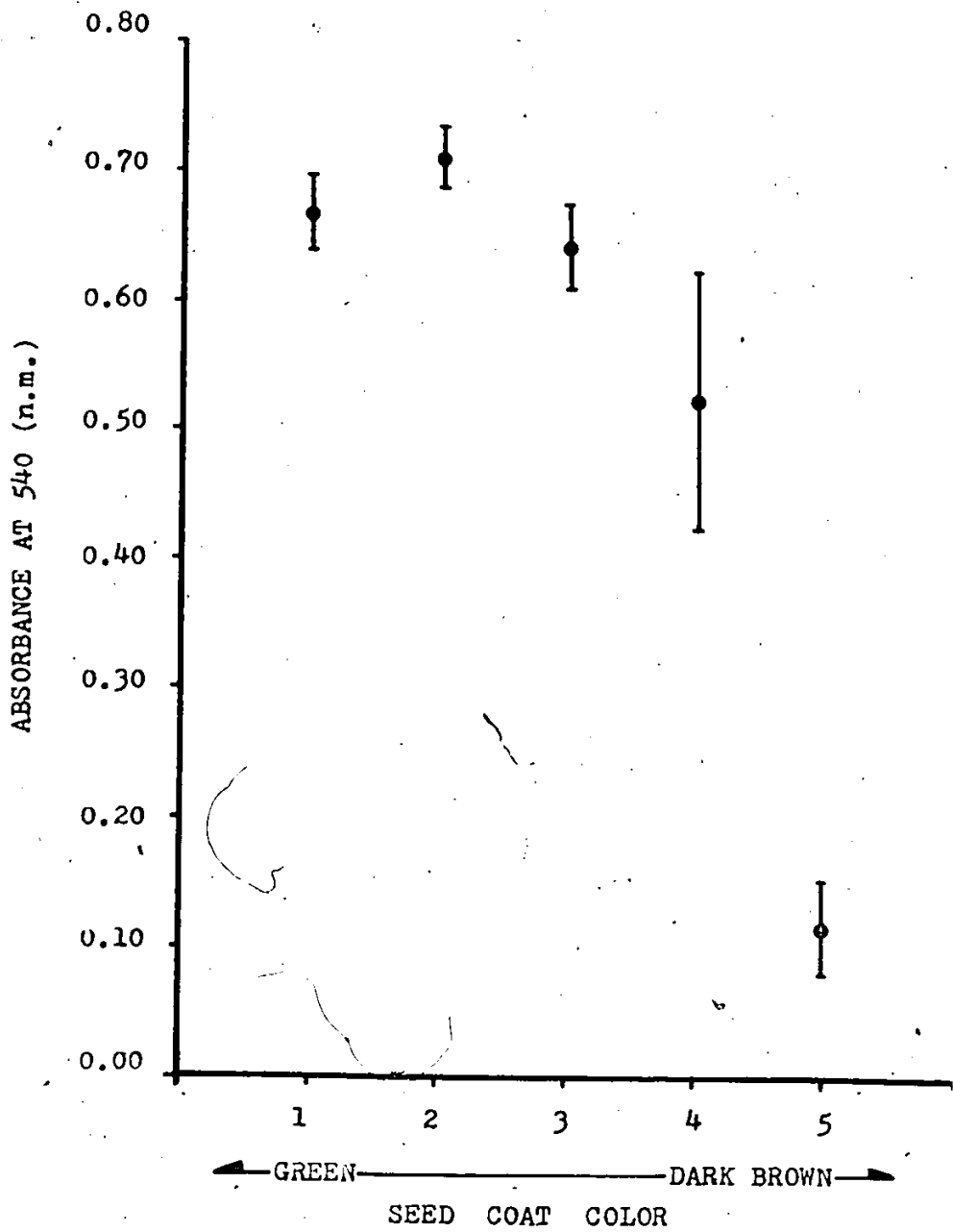
5 4 3 2 1

Forestral ↑



The hydrolyzed exudate from lentil seeds of different seed coat colors resulting from long term storage experiment was measured at 540 nm and the mean values for each seed coat color plotted (table 1). The vertical bars represent the standard error of the means.

Figure 17: Concentration of soluble proanthocyanins related to seed coat color.



Freeze-dried exudates from lentil seeds after long term storage were chromatographed in 2 dimensions. In the first direction 6% AcOH was used and in the second BuOH:AcOH:H₂O (10:1:5 v/v/v). The chromatograms were air dried followed by ferric chloride-potassium ferricyanide spray, and subsequent washing with 2N-HCl and distilled water. The lower left chromatograph was from green (1) seeds exudates which contained ascorbic acid. Spots showed as prussian blue on a white background.

Figure 18: Chromatographs of the unhydrolyzed exudates of lentil seeds after storage.

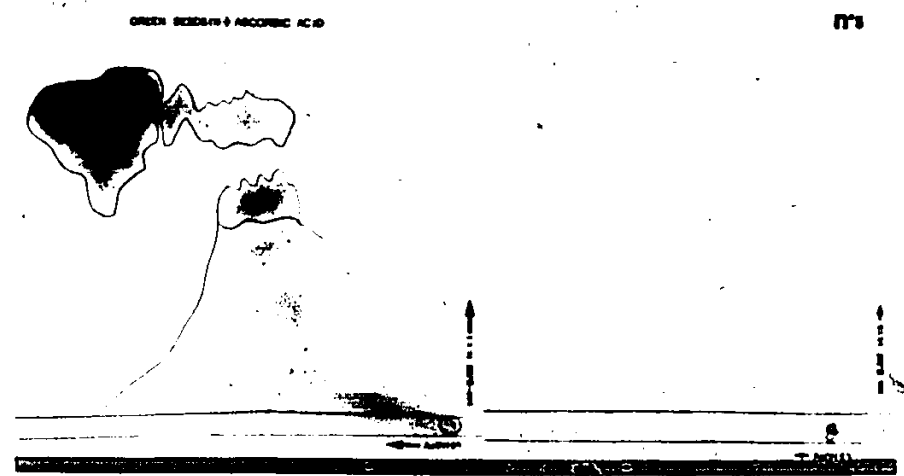
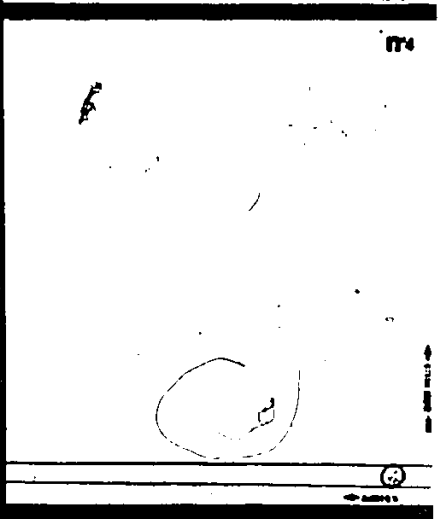
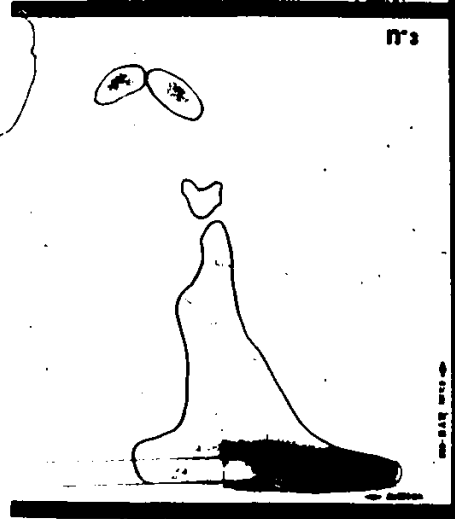
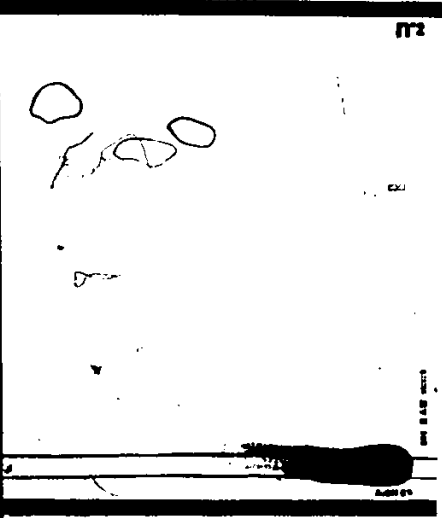
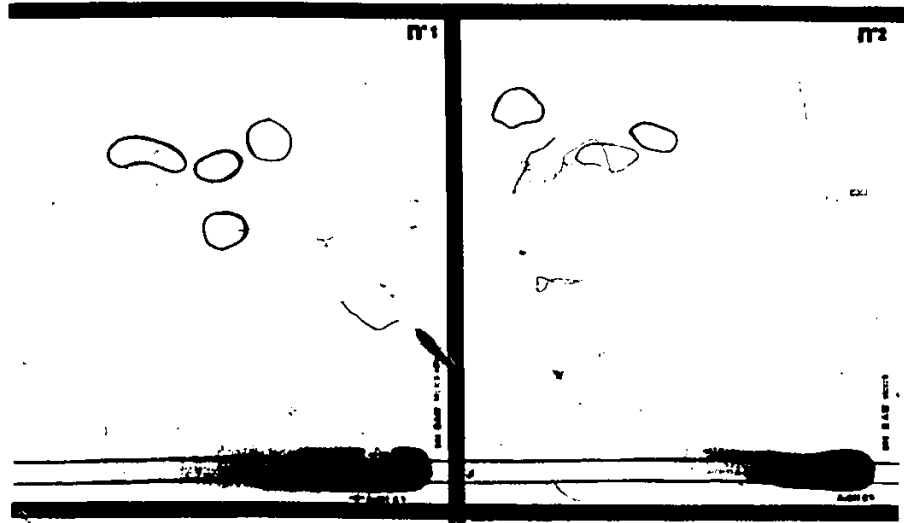


TABLE 4

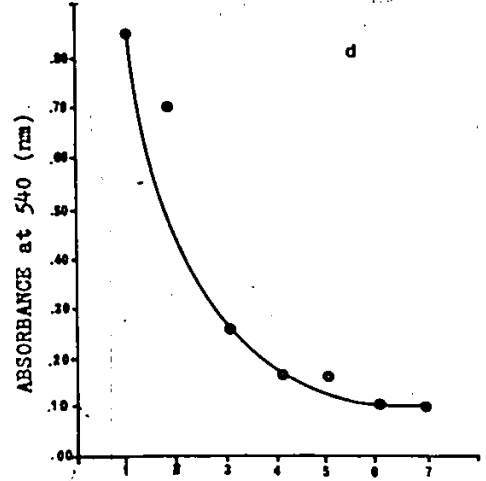
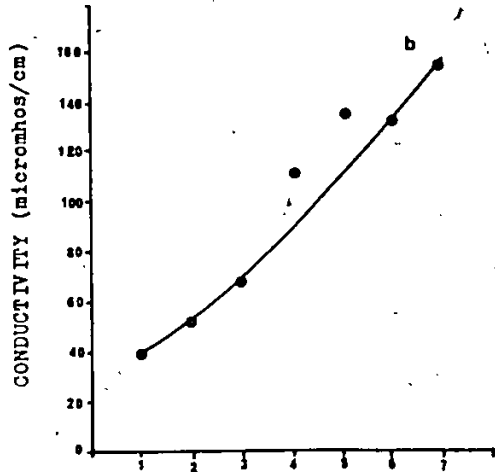
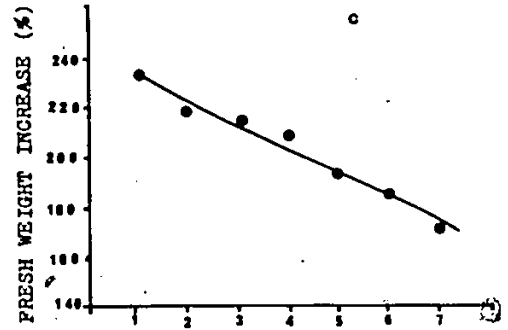
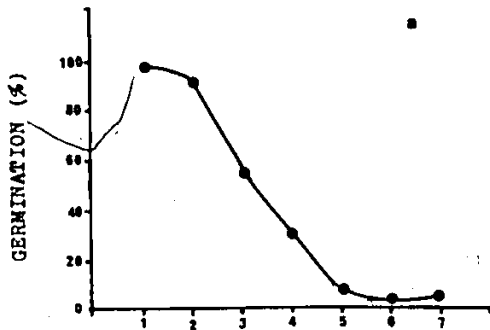
Total proanthocyanin content from lentil seeds.

Pod and Seed coat colors	No. Seed used	Absorbance Replicate 1	at 540 nm Replicate 2	Average
Green (1)	10	0.39	0.42	0.41
Yellow (2-3)	10	0.26	0.28	0.27
Brown (4-5)	10	0.10	0.12	0.11

Replicate lots of 10 seed coats from each of the three different coat colors collected from the field were boiled separately with 4 ml of 50% HCl for 15 min, then the solution was diluted to 8 ml using distilled water and the absorbance taken at 540nm.

Seeds were stored at 30°C, 100% R.H. and incandescent light for a period of one week, after which germination percentage (a) electrical conductivity (b), imbibition (c) and concentration of soluble proanthocyanins (d) were determined.

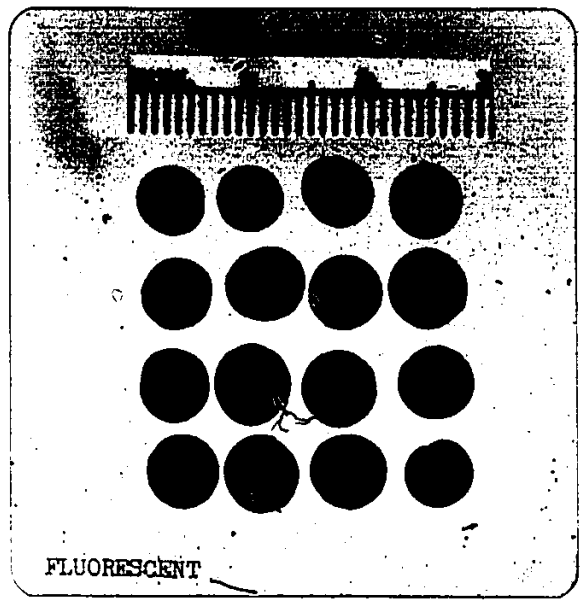
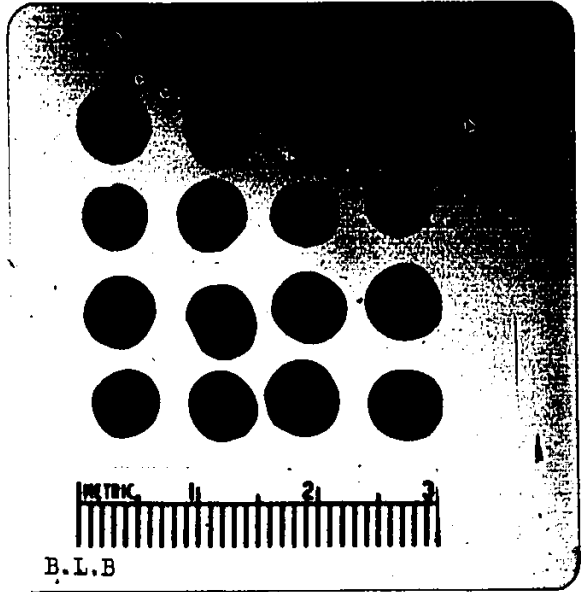
Figure 19: Effect of storage on lentil seeds in a week long experiment.



TIME (DAYS)

Green lentil seeds were stored for 72 hrs at 30°C, 100% R.H. under U.V. or fluorescent lights. Note the brown color (5) of seeds stored under B.L.B. light as compared to slight discoloration (2) of the seeds stored under fluorescent light.

Figure 20: Effect of continuous B.L.B. and fluorescent lights on green (1) lentils.



section IV

DISCUSSION AND CONCLUSIONS

It has been found that lentil seeds discolor during storage, and such discoloration is associated with the conditions existing in the storage room. As humidity and temperature increase the browning process is accelerated. Hughes (1974), found that the same discoloration pattern occurs in kidney beans. When kidney beans are stored at high temperature and high humidity the browning is faster than when seeds are kept at low temperature and low humidity. When seeds are kept in the light the darkening is also accelerated; for instance of the two lots of seeds stored under the same condition of temperature and humidity, but one under incandescent light and the other in the dark, the former discolored very rapidly whereas the later remained bright green (1) or showed only slight change. Hughes (1974) also found that light-colored red kidney bean appreciably discolored when stored with cool white light and near ultra violet compared to the dark treatment. The darkening appeared to be a continuous process during storage. Nozzolillo (1978), working with pea seeds also found seed coat discoloration during storage. Dickinson (1957) and Barker and Morris (1968), reported a discoloration phenomenon on the seed

coat of the broad bean (*Vicia faba*) during storage and canning.

Bate-Smith and Riberau-Gayon (1959), noted that the seed coat of *Phaseolus vulgaris* and the field bean (*V. faba*) discolored when left in the pod for very long or during storage. They also pointed out that such discoloration was accelerated by light.

A poor germination is associated with seed coat discoloration of lentil seeds. As lentil seeds discolor germination percentage decreases, slowly at first and then very rapidly and when seeds are dark brown germination is practically nil. Discoloration is then one factor that can be used to study seed viability. Hughes (1975), working with kidney beans and Nozzolillo (1978) with field peas noted the same correlation between discoloration and germination percentage. Steward and Carlson (1932) found that light green alfalfa seeds showed the highest germination percentage; dark brown seeds did not germinate at all. They carried out the test in the laboratory as well as in the field and germination in the field was lower compared to that in the laboratory.

Imbibition was affected both by seed coat discoloration and by the storage temperature. Little effect on imbibition was noted with green (1), yellow (2), yellow brown (3) or light brown (4) seeds as long as seeds that had been stored at the same temperature were compared. Dark brown seeds (5) however exhibited a consistently low imbibition regardless of the temperature of storage. Seeds stored at 5°C also had a low imbibition; seeds stored at 30°C had a high imbibition. The imbibition patterns can be explain as follows: In the case of green (1), yellow (2), yellow brown (3), and light brown (4) seeds imbibition was controlled by the organized membranes in the seed coat, and in the cotyledons. The high imbibition of seeds stored at 30°C suggests that some damaged may have occurred which let the water through the membranes more easily. And the low imbibition of fully browned seeds (5) may be a result of damage so severe that they have lost the capability of retaining water inside the cells. Berjak and Villiers (1972) suggested that such damage can be due to oxidation of the molecules constituting the membranes during storage. Parrish and Leopold (1978), also pointed out that low imbibition is due to deterioration in the membrane system of the seed due to accelerated aging during storage. They said that the membranes lose their ability to reform and function upon hydration. The state of cell membranes is also reflected in the solute leakage from plant material (Laties, 1964; Simon, 1974). On the basis of

this view, poor retention of solutes resulting in high leakage from imbibed embryos has been regarded as the result of either damaged (Larson, 1968) or incomplete membranes (Simon and Raja Harun, 1972). This explanation is supported by the results of the electrical conductivity. Increase of electrical conductivity of the seed exudates was inversely related to seed coat discoloration of lentil seeds. For instance, when seeds are green (1) and yellow brown (3) conductivity is low, but when seeds are light brown (4) and dark brown (5) conductivity is high. Larson (1968) noted a high leakage from dry pea embryos the seed coats had been removed during the first few hours soaking in water this did not occur from seeds imbibed intact. He attributed the high leakage to disruption of cell membranes as a result of the rapid inrush of water. Powell and Matthews (1978) associated high conductivity readings with dead or damaged tissue.

Papp (1979), related conductivity to aging in lentil seeds. She found that an older cultivar (1969) showed a higher conductivity reading than a younger (1975) lentil seed lot. In her studies the higher conductivity readings of the darker seeds was also associated with a damaging of the membranes. She pointed out that, in general, intact membranes emit few ions, whereas damaged membranes from whatever cause emit more ions. She also noted that the degree of leaching or ion loss is dependent upon the stage of seed maturity, their dormancy, age and temperature.

After conductivity was determined the tetrazolium test was performed on lentil seeds. Staining of the seed with TTC is due to the formation of red formazan on the embryos through the reduction of TTC by cell dehydrogenase (Roberts, 1951; Cothrell, 1948; Powell and Matthews, 1978). Powell and Matthews (1979), established that the presence of cracks on the surface of the seed coat was responsible for the low staining patterns of pea seeds. They also found that pea seeds without seed coats showed a very low staining pattern compared to that of seeds with seed coats, and attributed this to death of the tissue resulting from the uncontrolled entry of water. In the work reported here green seeds stained to a high percentage, but light brown (4) as well as dark brown (5), seeds did not stain at all. It can be suggested that damage to the tissue occurred during storage and not during imbibition since browned seeds imbibed the TTC solution with their seed coats.

Finally, discoloration of lentil seeds is associated with a decrease of the soluble condensed proanthocyanins exuded from the seed coat. The solubility of these compounds decreases with discoloration, being practically nil in browned seeds. Jacques (1974), suggested that there are two types of compounds that when boiled in acid give a red color; flavan 3,4-diols and flavan 3-ol dimers or higher oligomers, respectively named leucoanthocyanidins and proanthocyan-

dins. The red color is due to the transformation of both compounds to anthocyanidins, mainly cyanidin and delphinidin. The transformation of proanthocyanins into anthocyanins upon heating in acid is widely used for quantitative determination of these compounds (Quensel, 1968; Matthew, 1968; Goldainst and Swain, 1963). Some people have reported poor yield of anthocyanidins using this method (Govendarajan and Matthew, 1965; Matthew, 1969; Jewak, 1968). Considerable variability is found when exudates from lentil seeds are boiled in acid.

When total proanthocyanin content was studied in lentil seeds it was found that the development of a red color was very high in green (1) seeds whose pods were still bright green as well, but much lower when the pods were dark brown, tough and dry and the seeds were left to mature inside them. Bate-Smith and Riberau-Gayon (1959) found the same correlation in the field bean (*V. faba*). They pointed out that the browning may be due to a condensation and oxidation of primary flavanol molecules both with each other and with other constituents of the cell such as cellulose and proteins. If this is so, the formation of these oligomeric heavy molecular weight compounds may be responsible for the low degree of reactivity in the boiling acid test. Thompson (1964), suggested that the browning could be due to a polyphenol oxidase reaction. Singleton (1972), believes that the products

of browning can be shown to include very large molecules resulting from polymerization of phenolic compounds. Scott (1964), suggested that browning could be a result of oxidation of colorless phenols, which absorb only in the ultra violet into a derivative which absorbs in the visible region. Reichert et al (1979) found that discoloration of pearl millet (Pennisetum thyphoides) was also due to the presence of polyphenolic compounds predominantly located in the peripheral area of the grain. Rangaswamy et al (1974), working with a leguminous weed (Rhynchosia minima) found that during germination a brownish-black halo was formed around the seeds, they suggested that this discoloration was due to the presence of high concentrations of proanthocyanins leached from the seed coat; when ascorbic acid was used as an antioxidant no halo was formed, suggesting that such discoloration was due to the oxidation of these phenolic compounds.

A schematic chromatogram is shown in figure 21 where the different types of proanthocyanins according to their molecular weights are present; at the top of the chromatogram are found the monomeric forms (+)-catechin and (-)-epicatechin, further down the dimers then the trimers and finally the oligomers. The polymers, if any, are thought to be located at the origin in the butanol solvent. The work done on lentil seeds can be fitted within this scheme be-

cause it was found that the greener the seed coat the greater the amount of monomeric and dimeric forms. In the case of light brown (4) and dark brown (5) seeds these compounds were completely absent from the exudate. The polymerized streaks found in chromatograms from exudates of green (1) and yellow (2) seeds are due to oxidation and a certain degree of polymerization of low molecular weight dimers, trimers and oligomers during extraction and further handling of the samples since the same type of extract but containing ascorbic acid did not show the same pattern when chromatographed, instead a big spot (A fig 18) was found which may correspond to the monomeric precursors of condensed proanthocyanins.

Further support for the conclusion that soluble proanthocyanins are responsible for the browning was obtained with the following preliminary experiments: 2 replicate lots of 25 green (1) seeds were soaked in 25 ml of deionised water for one hour. They were then air-dried and stored at 30°C in light and 100% R.H. for 5 days. No color change was observed; in contrast to unwashed seeds which become dark brown (5) under the same conditions. Conductivity remained low, germination was still 88%, and absorbance of the one hour presoaking water was 0.45, the absorbance of the soaking water after storage was low, 0.1. Similar results were obtained with a 3 hr soak. The evidence clearly suggests

that removal of the soluble proanthocyanins protected the seeds from browning.

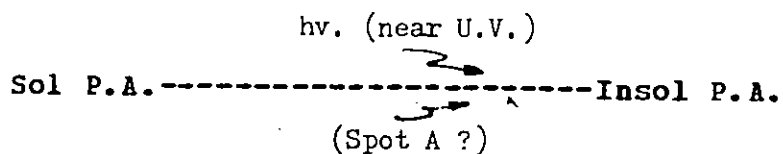
A relationship between seed coat discoloration and fungal growth was also found. Lentil seeds kept at high humidity high temperature and light had a fungal growth after only one week of storage. As discussed above as seeds discolored the exudation of soluble condensed proanthocyanins diminished. Sanders (1979), associated the concentrations of proanthocyanins with fungal growth in peanut seeds. He found that as the concentration of soluble condensed proanthocyanins decreased the percentage of colonization by Aspergillus parasiticus increased, assessing that this can be one of the several factors significantly correlated with degree of resistance of peanut to fungal invasion. Similarly, Young and Paterson (1980) found in the exudate of white clover seeds a high concentration of condensed proanthocyanins which they assumed to be toxic to Rhizobium bacteria. They hydrolysed the proanthocyanin fractions with concentrated HCl in n-BuOH and obtained a deep purple solutions which were analyzed by paper chromatography in forestal solvent. The major component of these fractions was identified as delphinidin and this finding was confirmed using spectrophotometric analysis.

In conclusion, the combination of high temperature, high humidity and light is the most damaging condition during seed storage, accelerating the browning phenomenon. Since seeds stored in the dark showed little or no discoloration regardless of the temperature and humidity in comparison to seeds stored in the light, discoloration seems to be associated with a photochemical process. This photochemical process appears to result in polymerization of soluble proanthocyanins since decrease in amount of these compounds occurs in browned seeds. An oxidation phenomenon may as well be involved since preliminary experiments have shown that lentil seeds kept at 30°C, 50% R.H. light and anaerobically did not discolor even after 11 months of storage.

The physiological changes associated with browning of lentil seeds during storage are still a matter of further studies, but it is clear that storage conditions play an important role in keeping the seeds viable and in controlling the darkening of the seed coats. More studies should be undertaken in order to find economical storage conditions that permit to keep the seeds in good physiological condition, i.e. a preliminary one hour soak in water could delay browning on a long term basis. It is clear from this work that seeds stored at 5°C, 0% R.H. under continuous darkness remain green after three months of storage, but for how much longer would they keep, and how costly is this condition?.

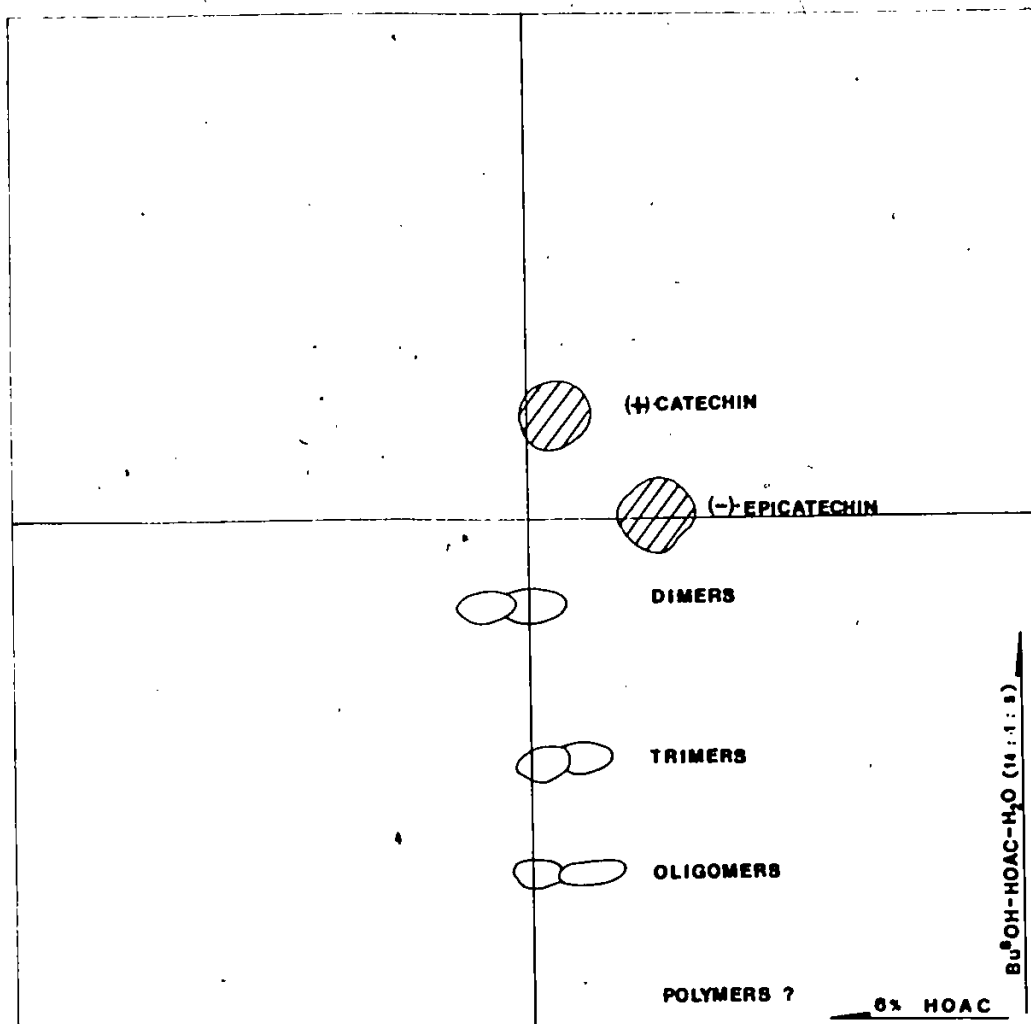
Furthermore breeding programs could be conducted using Laird variety and plant introductions such as P.I. 345635 which showed no browning after storage at high humidity and high temperature. Enzymatic studies also should be conducted to find out which enzymes are or may be involved in the browning phenomenon on lentil seeds during storage. A schematic model for the browning phenomenon is suggested below:

Colorless monomeric and oligomeric proanthocyanins in the seed coat (Sol P.A.) polymerize to brown color insoluble proanthocyanins (Insol P.A.).



Chromatogram showing the different types of condensed proanthocyanins according to their molecular weights. Taken from Thompson et al (1972).

Figure 21: Idealized chromatograph of condensed proanthocyanins.



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APPENDIX

SPECTRA OF THE LIGHT SOURCES USED IN THE EXPERIMENTS

- a incandescent light
- b blacklight blue fluorescent
- c cool white fluorescent

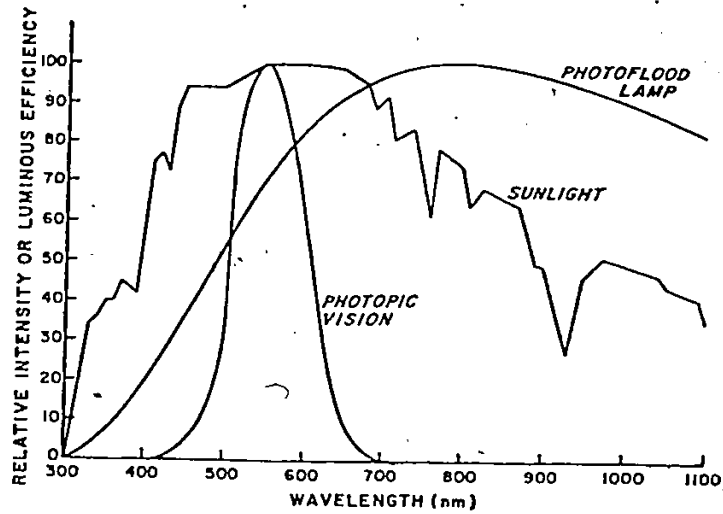


Fig. A Spectrum of (a) sunlight from the zenith at the surface of the earth. The dips in the red and infrared regions are due to absorption by water vapor [300–650 nm from R. D. Cadle and E. R. Allen, *Science* 167, 243–249 (1970); 650–1100 nm from R. B. Withrow and A. P. Withrow, Chap. 3 in *Radiation Biology*, Vol. III (A. Hollaender, ed.), McGraw-Hill, New York (1956)]. (b) 250-W incandescent photoflood lamp (color temperature, 3475 K). [From R. B. Withrow and A. P. Withrow, Chap. 3 in *Radiation Biology*, Vol. III (A. Hollaender, ed.), McGraw-Hill, New York (1956)], and (c) average human visual sensitivity.²³

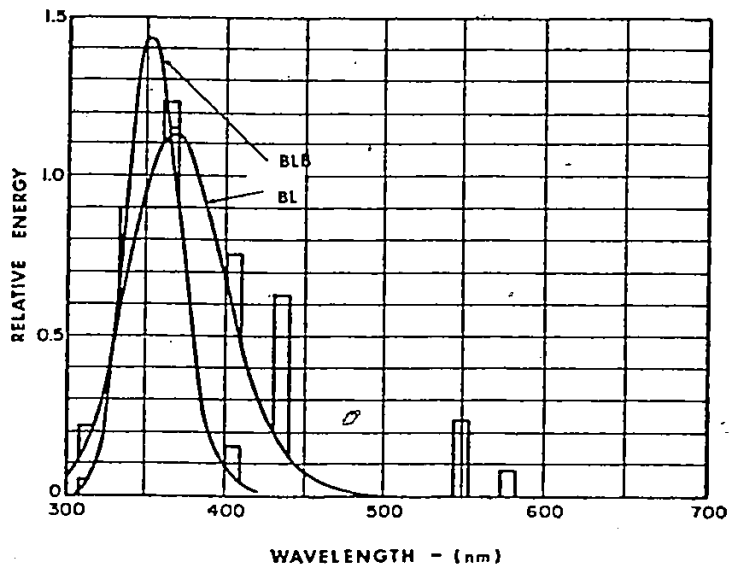


Fig. B Spectral output of 40-W T12 black-light (BL) and black-light-blue (BLB) lamps after 100 h of operation. (Courtesy of General Electric Co.)

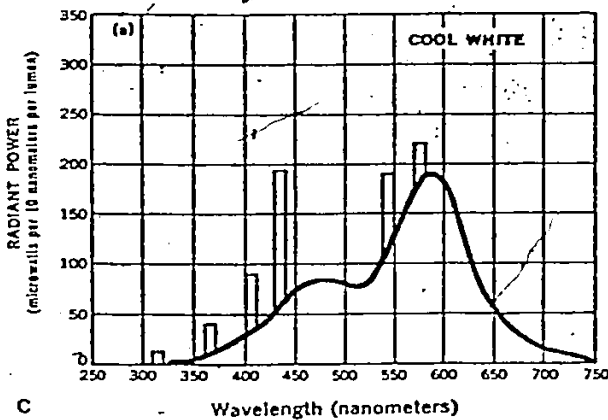


Fig. C