EFFECTS OF IONIZING RADIATION ON NUCLEIC ACIDS CONTENT AND DISTRIBUTION IN FENUGREEK BEANS

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN RADIATION BIOLOGY IN THE GRADUATE SCHOOL OF THE TEXAS WOMAN'S UNIVERSITY

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We hereby recommend that the dissertation prepared under our supervision by Mrs. Wan-Fen Chen Fang entitled Effects of Ionizing Radiation on Nucleic Acids Content and Distribution in Fenugreek Beans.

be accepted as fulfilling this part of the requirements for the Degree of Doctor Philosophy.

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CHAPTER I

INTRODUCTION

The radiation effects on growth inhibition, morphological variations, and cytological changes in plants have been investigated thoroughly in the past half a century. Recently, the macromolecular alterations in irradiated plants have become an interesting subject because the macromolecules may be the key substances in interpreting the morpho-physiological expression of radiation.

Essentially, deoxyribonucleic acid (DNA) stores the information which regulates growth and reproduction but ribonucleic acid (RNA) participates directly in the protein-synthesizing machinery. Therefore, nucleic acids constitute the principal target for searching for molecular factors in radiation damages. Several investigators determined the content for nucleic acids in irradiated plants (Van Huystee and Cherry, 1967; Joshi and Ledoux, 1970; Shigemitsu, 1971; Chou et al., 1971), but little work has been done concerning the distribution of nucleic acids among different components of irradiated plants. Possibly, the distribution of nucleic acids might be correlated to the rate of growth of different components since they are the substances which control the metabolic processes.

Shaw (1971) found that radiation did modify the content of nucleic acids in mung bean seedlings, as did environmental effects. Looper and Aboul-Ela (1971) reported that the dry weight of cotyledons was significantly higher in irradiated (30 KR) groups than in the controls. Possibly, there is differential activity in the various seedling components as a result of irradiation and the post-irradiated environment. On the other hand, translocation factors may be involved.

Temperature has been found to influence the growth rate of fenugreek seedlings (Looper and Aboul-Ela, 1971). A correlation was always obtained between temperature and growth rates, providing other environmental factors were held constant. In addition, temperature affects the internal biochemical system in plants more than any other environmental factor. Therefore, temperature was chosen as the post-irradiation factor in the study of irradiation influence on nucleic acid distribution.

The purpose of this study was to investigate the nucleic acid distribution in fenugreek seedlings as affected by radiation and post-irradiation temperature. The methods of nucleic acid separation involve the separation of various phosphorous compounds, the findings of which were also included in this study.

CHAPTER II

LITERATURE REVIEW

Historically, the effects of ionizing radiation on living systems were largely interpreted on a biophysical basis during the early stages of radiation biology. For instance, the "hit theory" of Blau and Altenburger (1922) and the "target theory" of Crowther (1924) were developed on the basis of the statistical relationships between dose and damage of irradiation. The target theory theorized that the cell contains a critical site or "target" and a single ionizing event or "hit" with this "target" will inactivate the cell. All hits outside the critical site were unimportant with regard to the observed effect.

As against the purely biophysical explanation of radiation effect, the involvement of biochemical pathways in radiation damage became apparent. In all the probable pathways of radiation action both direct and indirect reactions were taken into consideration (Lea, 1955).

With the introduction of microwave spectroscopy came an improvement both in biophysical and biochemical techniques. Zimmer (1960) has pointed out how microwave spectroscopy has brought to light interesting new information in radiobiology. With the help of microwave spectroscopy.

many data (Ehernberg, 1961; Zimmer and Muller, 1965; Snipes and Gordy, 1963) have been collected on the production of free radicals within the irradiated cells. Thus, the role of free radicals in initiating physicochemical reactions after the absorption of radiant energy by components of an irradiated cell was discovered. Since electron spin resonance techniques and equipment have become available, many workers (Gordy and Miyagawa, 1960; Randolph and Haber, 1961; Ehrenberg et al., 1969; Bidzilya and Zezina, 1970; Ahnstrom and Sanner, 1971) have reported that free radicals are a link in the chain of reactions leading from the absorption of the radiation to the biologically manifested effects.

Various possible explanations of inhibition of growth on plants after irradiation have been suggested by many workers. Gunckel and Sparrow (1954, 1957, 1961, 1965) contributed most of the studies on morphological irregularities. Neary et al. (1959) and Van't Hof (1963) emphasized the fact that mitotic delay caused the inhibition in growth.

RADIATION EFFECTS ON PHYSIOLOGICAL PROCESSES:

Recently, the study of ionizing radiation effects has focused on biochemical and physiological changes. Plants that have been exposed to ionizing radiation will have a disruption in the efficiencies or rates of various

physiological processes. For example, the reduction in the total photosynthetic capacity of an irradiated plant has been reported. Using Chlorella, Zill and Tolbert (1958) found that the ionizing radiation did not affect the rate of oxygen evolution but decreased CO_2 fixation, suggesting an independent action of radiation on the carbon cycle and on the photolytic system. Roy and Clark (1970) found somewhat similar results and explained that the reduction of the total photosynthetic capacity was due to the irradiation effect on the CO_2 fixation and translocation of photoassimilates. Furthermore, Gailey and Tolbert (1958) reported that 50 and 150 Krad of ionizing radiation delayed chlorophyll synthesis in etiolated plants. Kohn et al. (1967) reported a greater radiosensitivity of chlorophyll a synthesis over that of chlorophyll b. Simonis and Von Fuchtbauer (1965) found the non-cyclic photophosphorylation was the least sensitive to radiation, the cyclic type with flavin mononucleotide (FMN) of intermediate sensitivity, and the cyclic system with phenazin methosulfate (PMS) the most susceptible to radiation damage.

The other metabolic processes, like glycolysis, oxidative phosphorylation, cytochrome oxidase and catalase activities were reported to be stimulated by growth-inhibiting doses of radiation (Gordon and Weber, 1955; Gordon,

1957). Respiration, which is stimulated by radiation and increases with dose, has been reported by Romani and Bowers (1963), Romani (1964), and Clarke and Lang (1965).

RADIATION EFFECTS ON PHYTOHORMONES:

Another factor which must be taken into consideration when interpreting the radiation effects is hormones. For example, the radiation sensitivity of the hormone auxin was first reported by Skoog (1935) and has been reaffirmed by King and Galston (1960). Weber and Gordon (1953) reported the extreme radiosensitivity of auxin biosynthesis, and demonstrated that the magnitude of the radiation effect implied an interference at some very initial step in auxin biosynthesis. On the other hand, Fan and Maclachlan (1967) found that synthesis of the enzyme cellulase by pea seedlings increases dramatically in the presence of auxin. Adams et al. (1970) reported that the synthesis of RNA was necessary for auxin to promote cell enlargement in peas. Thus, it is possible that auxin may selectively activate some of the genes of maturing cells, enabling them to synthesize certain kinds of RNA and, eventually, the enzymes for which these RNA's code.

In barley seeds, gibberellin has a role in stimulating the synthesis of amylase, protease, and ribonuclease in the aleurone layer surrounding the food-storage cell of

the endosperm (Van Overbeek, 1966). It also activates cellulase and pectinase, which act on the cellulose and pectin on the seed coat cell wall, enabling the embryo to break through the softened coat during germination. Bonner (1965) has discovered that in potato tubers the gibberellic acid may chemically bind to the histones and, in some manner, act to prevent the histones from supressing the activity of the gene for mRNA synthesis. Thus, the dormancy of the freshly harvested potatoes might be broken, enabling them to commence growth. Subsequently, Chrispeels and Varner (1967) concluded that gibberellic acid may exert its control at the gene level.

Several investigations have demonstrated that treatment with gibberellic acid decreases the severity of damage induced by ionizing radiation in a variety of seeds. These findings and the apparent importance of gibberellines as inducers during the germination of seeds for the synthesis of hydrolytic enzymes (Jacobsen and Varner, 1967) led to more interest in the effect of gamma radiation on gibberellic acids. Sideris et al. (1971) studied the biological activity of gibberellic acid by measuring alpha-amylase activity and found that the relationship between irradiation dose and gibberellic acid inactivation is an exponential one. They postulated that the gibberellic acid inactivation by

radiation may be attributed either to a direct effect of gamma-rays photons on the gibberellic acid molecules, or to an indirect effect through free radicals formed in the irradiated medium.

Ethylene has been hypothesized to function through interaction with auxin. Some evidence supports the idea that ethylene inhibits the movement of auxin in pea seedlings (Galston and Davies, 1970). Ethylene has also been found to induce the activity of IAA oxidase, an enzyme that catalyzes the destruction of indoleacetic acid (Van Overbeek, 1968). Increases in the activity of this enzyme would result in a decrease in the amount of auxin in the plant (Adams et al., 1970). Recently, Ogawa and Uritani (1971) found that a low concentration of ethylene significantly stimulates the increase in overall enzyme activities in sweet potato roots. On the other hand, gamma irradiation of sweet potato tubers also results in the induction of overall enzyme activities. To determine the relationship between the two treatments would be of particular interest, because gamma irradiation induces ethylene production in lemon fruits also (Maxie et al., 1965; Maxie and Abdel-Kader, 1966).

RADIATION EFFECTS ON NUCLEIC ACIDS:

The ultimate expression of radiation damage to seeds is likely to involve genetic alterations. During the

synthesis of DNA, mRNA, and enzymes template molecules are required. Therefore, the radiation damaged templates might transmit their damage to the newly synthesized molecules, and then perhaps in the form of biological activity. For example, Weiss and Wheeler (1967) showed that RNA synthesized from irradiated DNA had a slight, but significant, difference in base ratio and neighboring base sequence from that synthesized from non-irradiated DNA. Another effect of radiation was indicated by the decreased rate of DNA and RNA synthesis. If such decreased rates were attributed to unavailability (or loss) of some templates, this would have serious consequences on the cell.

The mechanism of DNA structure disruption by irradiation has been reported by Scholes and Weiss (1962), who found that the free radical attack occurs mainly in the composition of the purine and pyrimidine bases. It has been estimated that about 75% of the OH-radicals react in attacking the nitrogen bases. The pyrimidine bases were found to be more radiosensitive than those of the purines, and thymine was destroyed to the greatest extent. Hudnik-Plevnik et al. (1962) found that DNA synthesized after irradiation contains less thymine and more cytosine and guanine than the normally synthesized DNA. They concluded that the nucleic acids synthesized after irradiation were altered in structure.

Such an alteration may be reflected in the loss of some biological function characteristic of these cell constituents. When a breakage of a strand in the DNA helix occurs, it is mainly due to an attack of the radicals on the sugar moiety, which leads to a splitting of the phosphate ester linkages along the chain and thus, to the production of phosphomonoester groups (Scholes and Weiss, 1962). A main chain double break, with separation of the sections will occur only if there is a break in each of the two strands which are less than about five nucleotide units apart (Casarett, 1968). Kruglykova (1968) has studied the action of ionizing and UV-irradiation on DNA structure. He found that the obvious change in viscosity was probably due to the rupture of H-bonds. Considerably much less information is available regarding radiation effects on RNA structure. It is likely that the changes produced are similar to those in DNA. Weissberger and Okada (1961) have reported that, at the same dosage, single-stranded RNA seemed to be more sensitive to radiation than the double-stranded DNA structure.

In vitro studies have shown that appreciable amounts of DNA were synthesized when the deoxynucleotide triphosphates of adenine, thymine, guanine, and cytosine, magnesium ions, and a portion of a DNA molecule serving as a template were mixed with DNA-polymerase just before

mitosis, during interphase and early prophase. However, a decrease in the rate of synthesis of DNA following radiation has been reported. Casarette (1968) postulated that this effect may be the results of radiation decreasing the concentration or activity levels of the enzymes regulating DNA synthesis. Moreover, since it is well known that radiation exposure delays mitotic activity, decreased DNA synthesis may be the result rather than the cause of the delay. Lajtha et al. (1958) and Yoshikura (1971) reported a radiosensitive period during mitosis. Gamma-rays were more inhibitory when applied prior to the onset rather than during the process of DNA synthesis.

All types of RNA are synthesized using 4 kinds of nucleotide triphosphates (adenosine triphosphate, uridine triphosphate, cytidine triphosphate, and guanosine triphosphate), a segment of DNA template, magnesium ions, and DNA-dependent RNA polymerase. Most of the RNA synthesis occurring at these instants is of the messenger variety. The synthesis of RNA may be delayed or depressed by irradiation. Stone (referred by Casarett, 1968) reported that the incorporation of ^{14}C-uracil by irradiated lettuce seeds showed that RNA synthesis was delayed by radiation. Higher doses of irradiation depressed RNA synthesis and delayed protein synthesis.

Other investigators have contributed further information concerning the effects of radiation on nucleic acid synthesis. Van Huystee and Cherry (1967) found that nucleic acid synthesis in cotyledons of peanuts was stimulated immediately after X-irradiation, but may be inhibited after 4 weeks of storage. Studies by Tokarskaya (1969) indicated that gamma-irradiation of pea seeds altered the genome, as shown by a decreased synthesis of mRNA and increased production of rRNA precusors. Joshi and Ledoux (1970) and Shigemitsu (1971) reported that there was no significant alteration in the content of DNA and RNA in barley seeds following ionizing radiation. Chou et al. (1971) found that the RNA content in γ-rays irradiated Penicillum expansum, L. was greater than in the control up to the fourth day, and then decreased to the same level as the control by the fifth day of incubation.

According to Casarett (1968), the enzymes of living systems have shown a variety of responses to moderate doses of radiation, even though the radiation doses required are considerably higher than those which produce mutations, chromosomal damage, or which prevent cell growth or cell division. Investigation of the effects of gamma-rays on the enzyme system of maize seeds have shown that radiation caused the decrease in most arylesterases of the first

internode after 5 days of germination, the decrease in the pH 7.5 esterases of the shoot after 6 days, and the increase in some peroxidases. Endo (1967) postulated that the effects of radiation may be on the enzyme molecules themselves, on the protein synthesizing system, or on the level of gene activity.

TEMPERATURE EFFECTS ON GROWTH:

Plant growth is controlled by the interaction of genetic and environmental factors. The biophysiological activities of plants are subject to the influences of their external and internal environments. In general, an increase in temperature results in an acceleration of the metabolic activities up to a maximum temperature. Growth of higher plants occurs in the range of 0 to 50 C. Within most (but not all) of this range, raising the temperature 10 C increases the growth rate to 2 to 3 times (Levitt, 1969).

Under natural conditions, the day temperatures are higher than the night temperatures so that there is a regular diurnal temperature cycle. These diurnal changes in temperature are important in their influence on plant growth. Went (1944) pointed that the night temperature of 20 C was best for early vegetative growth, but it decreased to 15 C as the tomato reached maturity. Using a day temperature of 18 C, Bora (1970) studied the effects of 4 night

temperatures (10, 14, 18, and 22 C) on tomato plants. It was found that a greater increase in the dry weight of the stem and root occurred with the 10 C night temperature. The maximum increase in dry weight of leaf resulted at 18 C, while the maximum increase in the leaf area was obtained at 14 C. Bora (1970) concluded that stem elongation in tomato is confined to the nocturnal hours, and that the elongation process itself may be an important element of the night temperature-sensitive growth system. Decker (1944) stated that the higher the night temperature, the larger the loss of the day's photosynthetic gain. Since the night temperatures lead to a diminishing of the respiratory loss, the thermoperiodic response may be the result of a favorable balance between the photosynthetic production of plant material during the day and the respiratory loss at night.

Since the biological reactions are under the control of enzymes, the temperature range in which they may occur is quite narrow. Thus, the existance of minimum temperatures for all plant processes is related to an enzymatically controlled condition. As the temperature rises, so does the rate of reactions until a temperature inhibitory level for enzyme activity is reached. Apparently, factors leading to the denaturation of enzymes involved in photosynthesis, respiration, absorption, transpiration, and

even cell division are active at temperatures above 30 C. Wolken and Mellon (1957) showed that the photochemical aspect of photosynthesis is independent of temperature, but that the biochemical activity, which is controlled by enzymes, is strictly temperature-dependent. Decker (1944) found that in his studies of pine, the respiration rate is more than twice as rapid at 30 C as at 20 C. Similarly, Osman (1971) reported that the respiration rate during the day was about 50% higher than during the night, that a marked increase in root respiration was evident within 12 minutes of exposing the shoot to photosynthetically active radiation, and that the decrease in respiration after excluding photosynthetically active radiation was equally sharp. It was suggested that this is a direct consequence of the translocation of photosynthetic products from the leaves to the root. Furthermore, both passive and active absorption processes are affected by temperature changes. Devlin (1969) postulated that the rate of free diffusion depends upon the kinetic energy of the diffusing molecules or ions which, in turn, is dependent upon temperature. Therefore, lowering of temperature will lower the rate of any process dependent upon free diffusion. Also lower temperatures will decrease the speed of the biochemical reactions found in the active transport.

Transpiration, another temperature-dependent action, is a necessary process in the normal growth of plants. For instance, amino acids and protein metabolism in plant is related to the water stress condition. If all factors are constant, an increase in temperature will almost always increase the rate of transpiration. Devlin (1969) attributed this to the effect of temperature on stomatal movements and vapor pressure gradients between the internal atmosphere of the leaf and the surrounding atmosphere. Hofstra (1969) found that two cool climate species, pea and broad bean, had the widest apertures at 17 to 30 C. In most of the species the apertures increased with air temperatures up to 30 C.

The effect of temperature on the course of mitosis and the mitotic cycle was studied in bean root tips by Murin (1967). These studies showed that a temperature increase from 3 to 25 C shortened the duration of the individual mitotic stages. The duration of the mitotic cycle decreased from 3 to 25 C, but further temperature increases to 30 and 35 C resulted in lengthening of the mitotic cycle. Above 35 C mitotic activity was either decreased or terminated.

The interaction between temperature and ionizing radiation has been studied in fenugreek beans (Fang, 1969; Looper and Aboul-Ela, 1971). Principally, the damage

induced by radiation is greater at lower than at higher temperatures. Lachance (1961) has explained that low temperatures may increase the solubility of oxygen which enhances the radiation damage, and may decrease the molecular movement which decreases the possibility of reorientation for recovery from the damage. Thus, either pre- or post-irradiation heat may alter the radiation damage because, (a) heat may modify the membranes to become impermeable to oxygen, (b) thermal energy may enhance the rate of radiation energy to divert away from the site of primary absorption, and (c) the high temperatures may facilitate movement of broken ends of chromosomes to restore their original position (Caldecott, 1961). Caldecott and Smith (1952) found that a sublethal heat treatment significantly reduced the radiation injury in barley root-tips. Caldecott (1961) demonstrated that pre-irradiation heat protects the seedlings of barley against injury and eliminates the increased injury in stored seeds. On the other hand, studies of post-irradiation heat resulted in a progressive increase in injury as a function of time stored. Berezina and Narimanov (1969) reported that the post-irradiation heat of cotton seeds eliminated the damaging effect of the radiation (40 KR) and promoted the recovery and the viability of the seeds. The plants which developed from the heat irradiated seeds had a retarded

rate of growth in the initial stages of development with resultant deformed leaves, altered coloration, and abnormal branching. By the time budding occurred, the morphological changes were almost completely restored.

CHAPTER III

MATERIALS AND METHODS

MATERIALS:

The fenugreek bean (*Trigonella foenum-graecum*, L.) is a fast germinating and rapid growing winter herbaceous annual legume with trifoliate compound leaves, except for the first leaf which has a single blade. It is an ideal experimental material for laboratory work under the desirable setting of a controlled environment.

Previous work with fenugreek demonstrated that 21-day-old seedlings were mature enough to show the developmental differences between control and treated groups at any parameter of comparison. J. R. Freeman (1970) has reported that the maximum amount of mineral absorption, especially phosphorus, by fenugreek seedlings is at the 21-day age. Thus, the 21-day-old fenugreek seedlings were chosen for this study.

TREATMENTS:

Co-60 (U. S. Nuclear Corporation Model Gr-9) was used as the source of gamma radiation at 600 R per minute. The dormant seeds of fenugreek, with approximately 7% moisture content, were irradiated at room temperature (20 C) for

17 minutes for a 10 KR exposure dose. In previous studies Fang (1969) observed that 10 KR was a low dose for fenugreek seeds and resulted in morphological changes without great loss in weight. Uniformity of fresh weights between irradiated and control seedlings is convenient for biochemical isolations and separations. Therefore, the comparisons between the isolated components on a fresh weight basis would be sound.

Following irradiation, both treated and control seeds were immediately hydrated under aerobic conditions, and the seeds were planted in diSpo growth pouches. (DiSpo pouches, purchased from Scientific Products, were 6 inches-square plastic bags which opened along the top side containing folded paper wicks on which the seeds were placed.) Immediately after planting, the pouches were then set into the different pre-set controlled growth chambers. Light intensity was set at 20 KLux, and humidity was maintained between 50-70%. The growth conditions and the radiation were programmed as indicated in Table 1. Thus, the radiation and the day temperature were the only two variables which were applied to alter the growth of the seedlings.

TABLE 1. TREATMENT CONDITIONS OF FENUGREEK SEEDLINGS.

Radiation	Growth Condition	
	Day (16 hr)	Night (8 hr)
None (Control)	30 C	15 C
	20 C	15 C
10,000 R (Treated)	30 C	15 C
	20 C	15 C

PREPARATION OF P-32 LABELED NUTRIENT SOLUTION:

Phosphorus, an extremely important element for plant growth, exists in cell constituents as phospholipids, in energy transfer compounds as NAD, NADP, and ATP, and in core materials as nucleic acids. For this reason, the phosphorus isotope (P-32) was chosen as the radioactive tracer for determining the content of nucleic acids, as well as the other phosphorus fractions. Labeled $NaH_2{}^{32}PO_4$ was purchased from the New England Nuclear Company (10 mCi/0.5 ml carrier free). The original 0.5 ml of 99% purity of radioactive monosodium phosphate was diluted with 100 mg of non-radioactive monosodium phosphate plus water to make a 3.3 mCi/ml of stock solution. A 0.5 ml of the stock solution was transferred to 20 liters of regular nutrient (after Hewitt reported in Devlin 1969) to make a labeled nutrient with

specific activity of 0.5 mCi/mg phosphorus. The labeled nutrient solution was then used to feed the seedlings in the pouches. Equal aliquots of 20-25 ml were added to each pouch every 2 to 3 days, depending upon the age of the seedlings. The remaining nutrients in each pouch were disposed of before each replacement. Each subtreatment was replicated 5 times (5 pouches). Within two months, the incorporated radioactivity was sufficient to be detected by the Wide-Beta II.

MEASUREMENTS:

The 21-day-old seedlings were harvested and fractionated into their component leaves, cotyledons, stems, and roots. Each component was collected from each of the 10 seedlings in one pouch as a unit. The fresh weight of each component was measured right after fractionation. The dry weight was measured after each component has been dried in an oven for 24 hours at 70°C and cooled in a desiccator.

EXTRACTION:

Two gram samples of fresh leaves, cotyledons, stems, and roots from each subtreatment were collected separately and frozen instantly in dry ice to inhibit the activity of enzymes (Tsinger and Petrovsaya-Baranova, 1970). Five determinations were made on the five replicates in each subtreatment. The extraction procedure as adapted from

Broughton (1969) is presented in Figure 1.

Each two grams of frozen component were homogenized at 5 C by a Sorvall grinder for 3 minutes on the 3-4 setting. The speed of the cold centrifugation in each subsequent step was at 12,000 X g for 10 minutes, except for the acid-extraction which was centrifuged at 14,000 X g for 10 minutes.

There were six fractions in each extraction: FRACTION I was methanol-soluble phosphorus compounds, mostly inorganic; FRACTION II was acid-soluble phosphorus compounds which represented energy transfer compounds; FRACTION III was ether-soluble phosphorus compounds which included mainly phospholipids; FRACTION IV was predominantly RNA; FRACTION V was DNA; and FRACTION VI was residue which consisted of proteins, cell wall, and $KClO_4$ (Broughton, 1969). Each fraction was made into a specified volume of which certain amounts of aliquots were taken for assay.

ASSAY OF PHOSPHORUS UPTAKE:

The phosphorus uptake in each fraction was determined by radioactive counts because the P-32 count is proportional to the amount of total absorbed phosphorus in each component.

A series of P-32 standards prepared from the labeled nutrient were used as references as indicated in Table 2. One ml samples of each fraction were transferred

FIGURE 1. OUTLINE OF PROCEDURE FOR THE EXTRACTION OF DIFFERENT COMPONENTS OF 21-DAY-OLD SEEDLINGS OF FENUGREEK. (Adapted from Broughton, 1969)

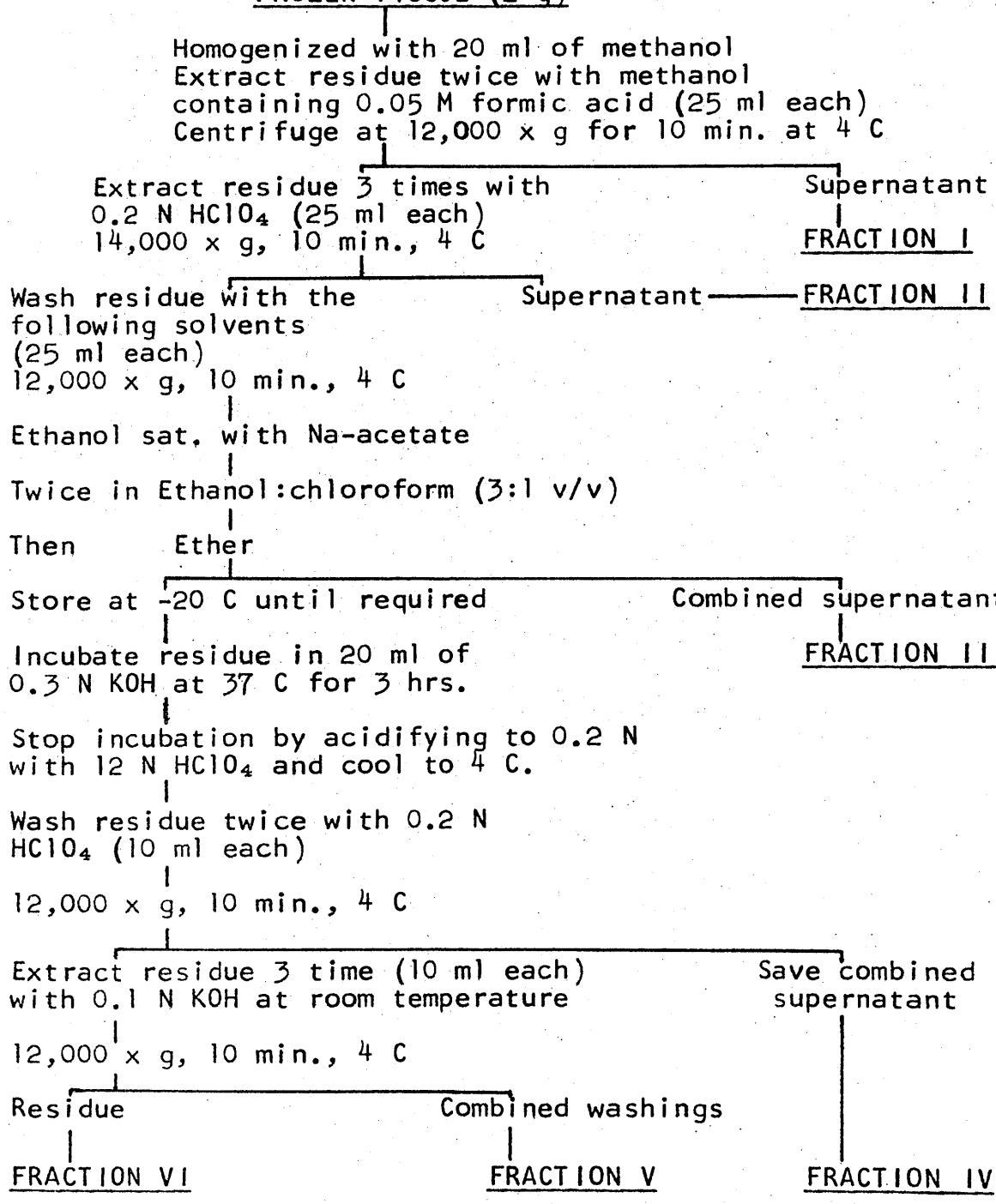

FROZEN TISSUE (2 g)
Homogenized with 20 ml of methanol
Extract residue twice with methanol
containing 0.05 M formic acid (25 ml each)
Centrifuge at 12,000 x g for 10 min. at 4 C
Extract residue 3 times with
0.2 N HClO4 (25 ml each)
14,000 x g, 10 min., 4 C
Supernatant
FRACTION I
Wash residue with the
following solvents
(25 ml each)
12,000 x g, 10 min., 4 C
Supernatant
FRACTION II
Ethanol sat. with Na-acetate
Twice in Ethanol:chloroform (3:1 v/v)
Then Ether
Store at -20 C until required
Combined supernatant
FRACTION III
Incubate residue in 20 ml of
0.3 N KOH at 37 C for 3 hrs.
Stop incubation by acidifying to 0.2 N
with 12 N HClO4 and cool to 4 C.
Wash residue twice with 0.2 N
HClO4 (10 ml each)
12,000 x g, 10 min., 4 C
Extract residue 3 time (10 ml each)
with 0.1 N KOH at room temperature
Save combined
supernatant
12,000 x g, 10 min., 4 C
Residue
Combined washings
FRACTION VI
FRACTION V
FRACTION IV

TABLE 2. A SERIES OF ^{32}P-STANDARD FOR P-UPTAKE IN FENUGREEK SEEDLINGS.

Series No.	Nutrient*/planchet	Total phosphorus**
	ml	g
1	0	0
2	0.1	4.69
3	0.2	9.38
4	0.3	14.07
5	0.4	18.76
6	0.5	23.45

*Nutrient refers to ^{32}P-labeled nutrient used for seedlings.

**Total phosphorus in the aliquot including, both the labeled and unlabeled, was calculated from the salt added to the stock nutrient as follows:

NaH_2PO_4/20 liters (l) of nutrient = 4.16 g

$NaH_2{}^{32}PO_4$/20 l of nutrient = 0.01687 g

Total monosodium phosphate/20 l = 4.17687 g

P % in NaH_2PO_4 = P/NaH_2PO_4 x 100% = 22.46%

Total P-content/20 l of nutrient = 4.17687 x 0.2246

= 0.938125 g/20 l

= 46.9 g/ml

into a 1 in. diameter planchets for counting. Both the standards and the samples were oven-dried at 40 C for 6-12 hr counted by a Beckman Wide-Beta II planchet system set at 2,000 counts, with 5% error level.

A standard curve was prepared to relate the count to the amount of total phosphorus from the standard series and to estimate the total phosphorus in aliquots of the various extracts each time they were assayed. Thus, the total P-uptake was obtained by multiplying the total volume of each fraction extraction by the total P-content of the aliquot.

ASSAY OF NUCLEIC ACIDS:

The concentrations of nucleic acids in FRACTION IV and FRACTION V were determined by three methods: (a) Radioactive counts of ^{32}P-incorporated, (b) UV absorbance at 260 m , and (c) Colorimetric reactions.

(a) Radioactive count to assay the phosphorus portion of nucleic acids was carried out as previously described. The amount of nucleic acids in the growing seedling assayed by this method represents the fraction incorporated from the absorbed phosphorus of the nutrient solution.

The phosphorus portion of nucleic acids is reported to account for about 9.22% or a 1:10.8 ratio of the DNA

(Chargaff, 1955). Magasanid (1955) indicated that the P-portion of RNA is about 9%, or a 1:11.1 ratio. Thus the nucleic acid content can be calculated from P-content as indicated by the following:

RNA-content = P-content x 11.1 x vol of FRACTION IV.

DNA-content = P-content x 10.8 x vol of FRACTION V.

(b) The ultraviolet absorbance technique is mainly an assay for the nitrogen-bases portion of nucleic acids. Its determination is based on the fact that purine and pyrimidine bases absorb light at 260 m in proportion to the concentration of nucleic acid (Bradshaw, 1966).

Yeast RNA (Nutritional Biochemicals Corporation) was used as the RNA-standard reference. Four milligrams of yeast RNA were dissolved in 24 ml of the solution which was the same extracting medium of FRACTION IV, at pH 1.1 $\pm$ 0.2 to make a 0.166 mg/ml concentration of RNA stock solution. From the stock, a series of RNA-standard dilutions were made as indicated in Table 3.

Highly polymerized DNA-disodium salt (Nutritional Biochemicals Corporation) was used as the DNA-standard reference. Ten milligrams of the DNA were dissolved in 20 ml of the same extraction medium of FRACTION V at pH 12.5 $\pm$ 0.2 to make a 0.5 mg/ml concentration of DNA stock solution. A series of DNA-standards were prepared from the stock solution as shown in Table 4.

TABLE 3. RNA-STANDARD DILUTIONS USED FOR UV-ASSAY.

Series No.	1	2	3	4	5	6
RNA in mg/ml	0.166	0.083	0.042	0.021	0.0105	0

TABLE 4. DNA-STANDARD DILUTIONS USED FOR UV-ASSAY.

Series No.	1	2	3	4	5	6
DNA in mg/ml	0.5	0.125	0.031	0.0078	0.00195	0

An ambient Hitachi Perkin-Elmer model 139 UV-Vis-spectrophotometer was used with a slit opening of 0.5 mm. The nucleic acid content of each unknown aliquot was evaluated from the standard curve which was constructed according to $A_{260\ m\mu}$ of the standard series.

(c) Colorimetric reactions are used to assay the sugar portion of nucleic acids. The series of standard RNA and DNA for color reactions were prepared as mentioned in the UV-determinations (Tables 3 and 4).

The Orcinol procedure as described by Bradshaw (1966) was used for assaying RNA content. Three milliliter aliquots of the standard, as well as the unknown RNA samples, were pipetted into test tubes and 6 ml of the Orcinol-acid reagent and 0.4 ml of the Orcinol-alcohol reagent were added to each. (Orcinol-acid reagent was prepared by slowly adding 0.5 ml of 10% $FeCl_3 \cdot H_2O$ to 100 ml of conc. HCl. The Orcinol-alcohol reagent was made by dissolving 6 mg Orcinol in 100 ml of 95% ethanol. This solution must be freshly prepared prior to use.) The prepared tubes were put in a boiling water bath for 20 min. to allow the green color to develop. At this time, all the tubes were cooled by immersion in a cold-water bath. The absorbance at 660 mμ was determined against a blank reagent which was prepared in the same way with 3 ml of the extracting

medium. Corrections were made for the light absorbancy due to the reagent and the medium. Finally, the amount of RNA in FRACTION IV was determined from a RNA-standard curve in mg/ml, and then multiplied by the total volume of FRACTION IV to obtain the actual RNA content in 2 g of fresh tissue.

The modified indole procedure described by Schmid et al. (1963) was used for assaying the DNA content from the deoxyribose portion. One ml of the standard, as well as the unknown DNA extracts, was heated with 1 ml of 10% (w/v) monochloroacetic acid (Final pH 2.5) at 90 C for 80 min. Subsequently, 1 ml of 0.06% (w/v) indole and 1 ml of 10 M HCl were added to each tube and the yellow-brown color was allowed to develop for 60 min at 80 c. All the tubes were rapidly chilled in a cold-water bath between each period of heating. Since hexoses give a pink color reacting with indole, the pink color must be completely extracted by the chloroform. These extractions were repeated on each tube 3 times with 4 ml of chloroform. The remaining aqueous solution contained the brown color compound which was formed mainly by deoxyribose. This was poured into a 3 ml capacity Pyrex cuvette to determine the light absorbancy against a modified blank reagent at 490 mμ by the same spectrophotometer as used in UV-determination.

The conversion into DNA-content was then calculated by the previously described method.

ANALYSIS OF DATA:

The experimental data were analyzed by IBM 1620 computer at Texas Woman's University using a program of simple analysis of variance (Harris, 1963, modified by Patterson, 1965).

CHAPTER IV

RESULTS

FRESH WEIGHTS:

The average weights of various components of 21-day-old fenugreek seedlings following treatments of radiation and temperature are presented in Table 5 and Figure 2. The analyses of variance are in the Appendixes no. 1, 2, 3, and 4. The least significant difference between each two means is calculated from the value of studentized range at $K_{3,16}$ (Goldstein, 1964).

Although, doses of 10 KR radiation slightly stimulated the growth at the 30 C day-temperature and inhibited the growth at the 20 C day-temperature as compared to the nonirradiated controls, such differences were not satistically significant. The temperature effect results indicated that 20 C was more favorable for growth than 30 C, by a wide significant margin, in most structures.

DRY WEIGHTS:

The average dry weights of various components of seedlings treated by radiation and temperature are shown in Table 6 and Figure 3. The analyses of variance of the four components are presented in Appendixes no. 5, 6, 7, and 8.

TABLE 5. FRESH WEIGHTS OF COMPONENTS OF 21-DAY-OLD FENUGREEK SEEDLINGS (Figures are means of 5 replicates with 10 seedlings each).

Seedling Components	Treatments*				L. S. D.**	
	30 C		20 C		5%	1%
	Control	Irrad.	Control	Irrad.		
	g	g	g	g		
Leaves	1.0557	1.1901	1.5256	1.2281	0.3363	0.4333
Cotyledons	1.0510	1.0996	1.3072	1.2334	0.2020	0.2600
Stems	0.8322	0.8774	1.2223	0.9854	0.5339	0.6880
Roots	0.9509	1.2046	2.6779	2.5094	0.4659	0.6000
Whole seedling	3.8898	4.3717	6.7330	5.9563	1.5381	1.9813

*Night temperatures were maintained at 15 C for all groups.

** L. S. D. refers to Least Significant Difference between two means.

FIGURE 2. EFFECTS OF RADIATION AND TEMPERATURE ON FRESH WEIGHTS OF 21-DAY-OLD SEEDLING COMPONENTS OF FENUGREEK BEANS. (Each column represents the mean of 5 replicates with 10 seedlings each).

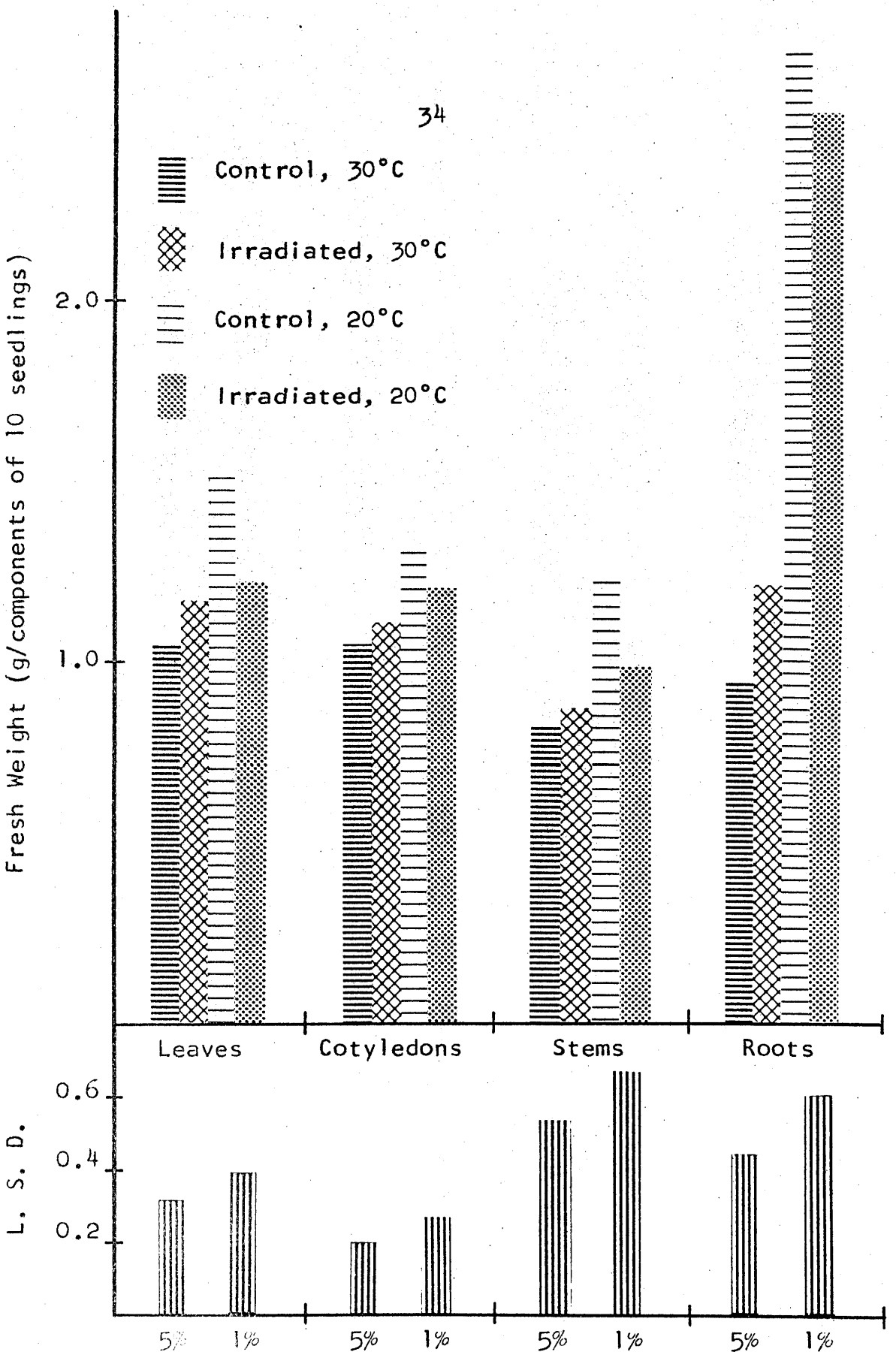

Control, 30°C
Irradiated, 30°C
Control, 20°C
Irradiated, 20°C
Fresh Weight (g/components of 10 seedlings)
2.0
1.0
Leaves
Cotyledons
Stems
Roots
L. S. D.
0.6
0.4
0.2
5%
1%
5%
1%
5%
1%
5%
1%

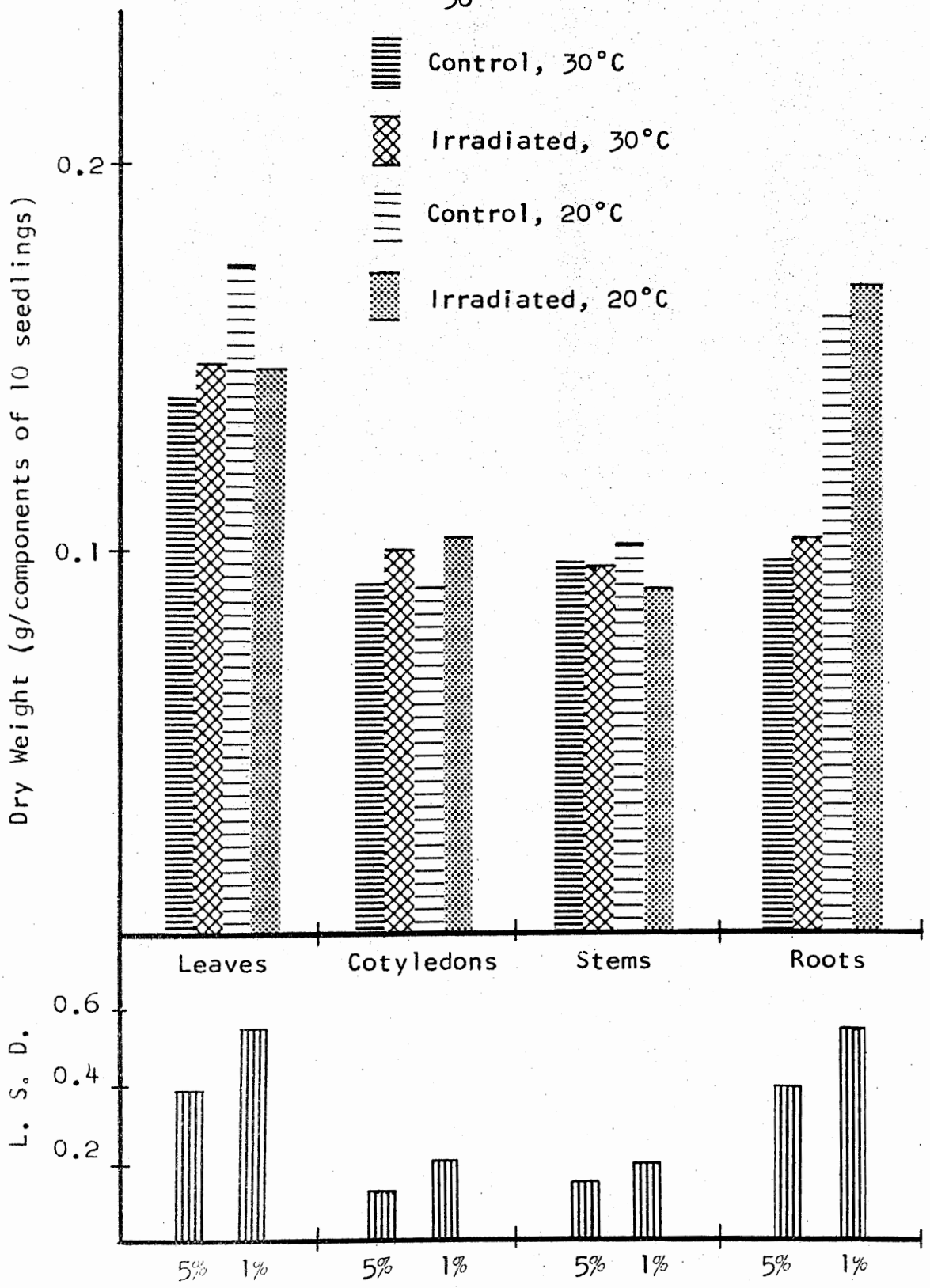
TABLE 6. DRY WEIGHT OF COMPONENTS OF 21-DAY-OLD FENUGREEK SEEDLINGS (Figures are means of 5 replicates with 10 seedlings each).

Seedling Components	Treatments*				L. S. D.**	
	30 C		20 C		5%	1%
	Control	Irrad.	Control	Irrad.		
	g	g	g	g		
Leaves	0.1403	0.1483	0.1731	0.1457	0.0400	0.0520
Cotyledons	0.0954	0.0999	0.0942	0.1099	0.0157	0.0200
Stems	0.0959	0.0941	0.1002	0.0879	0.0160	0.0200
Roots	0.1016	0.1059	0.1613	0.1686	0.0400	0.0520
Whole seedling	0.4332	0.4482	0.5288	0.5121	0.1117	0.1440

*Night temperatures were maintained at 15 C for all groups.

**L. S. D. refers to Least Significant Difference between two means.

FIGURE 3. EFFECTS OF RADIATION AND TEMPERATURE ON DRY WEIGHTS OF 21-DAY-OLD SEEDLING COMPONENTS OF FENUGREEK BEANS. (Each column represents the mean of 5 replicates with 10 seedlings each)


Control, 30°C
Irradiated, 30°C
Control, 20°C
Irradiated, 20°C
Dry Weight (g/components of 10 seedlings)
0.2
0.1
Leaves
Cotyledons
Stems
Roots
L. S. D.
0.6
0.4
0.2
5%
1%
5%
1%
5%
1%
5%
1%

The dry weights of leaves were neither affected by irradiation nor by temperature. The temperature factor did not cause a significant change in cotyledon dry weights but irradiation did. Cotyledon dry weights were significantly greater in irradiated groups as compared to the controls, especially when they were grown at 20 C.

The dry weights of stems were reduced as a result of irradiation but the reduction was not significant. In roots, apparently the 20 C day temperature provided the most suitable temperature for growth. The dry weights of roots increased significantly in the groups grown at 20 C, as compared to those at 30 C. Irradiation did not result in significant variations in root dry weights.

PHOSPHORUS UPTAKE:

The average amounts of phosphorus uptake by seedlings and their components during a three week post-irradiation period are recorded in Table 7 and Figure 4. The analyses of variance of the six fractions are presented in Appendixes no. 9, 10, 11, 12, 13, and 14.

The total phosphorus taken up by seedlings during 21 days was significantly decreased as a result of irradiation. On the other hand, the high temperature stimulated the total P-uptake significantly as seen in Table 7.

The amounts of inorganic P-compounds represented by

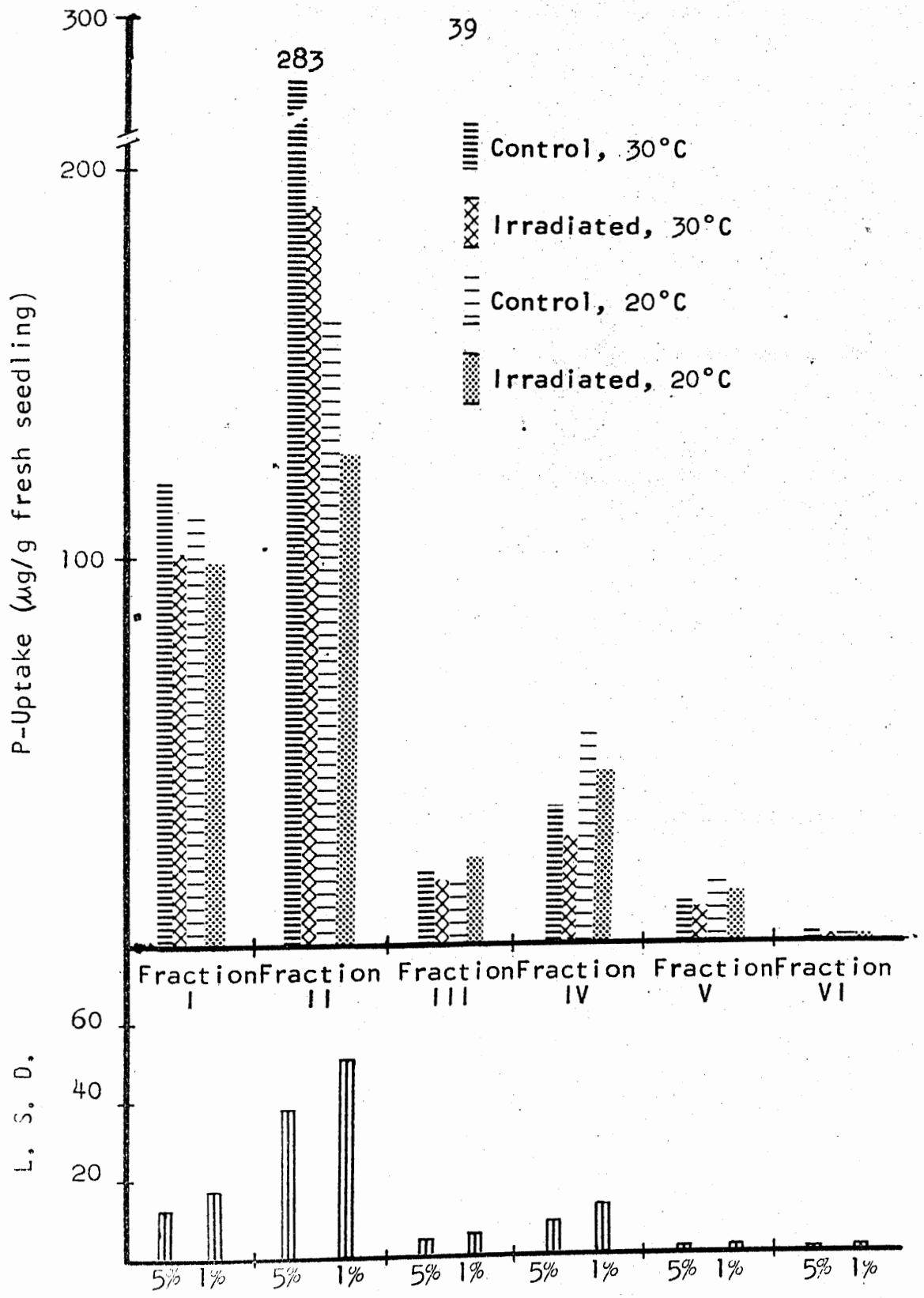
TABLE 7. THE P-UPTAKE IN µg/g FRESH WEIGHT IN 21-DAY-OLD FENUGREEK SEEDLINGS (Figures are the means of 5 replicates).

Fractions	Treatments*				L. S. D.**	
	30 C		20 C		5%	1%
	Control	Irrad.	Control	Irrad.		
	µg	µg	µg	µg		
I. Methanol-soluble	120	102	118	98	12	15
II. Acid-soluble	283	190	162	129	39	50
III. Ether-soluble	19	17	18	20	2	3
IV. RNA	37	27	55	44	8	10
V. DNA	11	9	15	12	1	2
VI. Residue	1.35	1.01	1.13	1.11	0.19	0.25
Total	471	347	370	305	62.19	80.25

*A 15 C night temperature was maintained for all groups.

**L. S. D. refers to Least Significant Difference between two means.

FIGURE 4. PHOSPHORUS UPTAKE IN μg/g OF FRESH WEIGHT IN 21-DAY-OLD FENUGREEK SEEDLINGS (Each column is the mean of 5 replicates).


39
283
300
200
100
60
40
20
P-Uptake (µg/g fresh seedling)
L. S. D.
Control, 30°C
Irradiated, 30°C
Control, 20°C
Irradiated, 20°C
Fraction I
Fraction II
Fraction III
Fraction IV
Fraction V
Fraction VI
5% 1% 5% 1% 5% 1% 5% 1% 5% 1% 5% 1%

FRACTION I were decreased in the irradiated groups at highly significant level (Table 7). Growing temperature conditions did not result in significant variations among the various treatments.

Energy transfer compounds extracted in FRACTION II were significantly reduced by radiation. The same fraction was accumulated to a significantly higher level in seedlings grown under high temperature conditions (30 C).

FRACTION III content, which included the phospholipid compounds was, almost the same in all groups except in the irradiated groups grown at 30 C. They were significantly lower than the rest of the groups.

FRACTIONS IV and V which represent RNA and DNA, respectively, were significantly reduced either as a result of irradiation, raising temperature, or both. The lower temperature provided better conditions for phosphorus incorporation into nucleic acids (Table 7).

The residue-P indicated in FRACTION VI did not show significant variability among the various treatments. It also constituted a minor part of the total uptake (less than 0.3%), indicating the efficienty of the extraction scheme.

NUCLEIC ACIDS:

RNA content:

The RNA contents of the various components of fenugreek seedlings exposed to treatments of radiation and temperature were assayed by three different methods and are presented in Table 8 and Figure 5. The last two columns in Table 8 represent the least significant difference between any two means in the same row. The column of coefficient of variation provides the relative variability among the three methods of RNA-determination. The analyses of variances are presented in Appendixes No. 15 to 26.

Data in Table 8 and Figure 5 indicated that: (a) the distribution of RNA in the various seedling components followed the same pattern among the treatments, regardless of the method of assay; (b) the varied methods of determinations yielded varied absolute amounts of RNA; (c) irradiation resulted in RNA decrease in all the seedling components except the stems of the groups grown at 20 C; (d) although radiation effects were consistent, they were not statistically significant; (e) the groups grown at 20 C contained significantly higher RNA in their leaves, cotyledons, and stems but a lower content in their roots, as compared to the groups grown at 30 C; (f) the most RNA on a per gram basis was found in leaves, followed by roots, stems, and then cotyledons, respectively.

TABLE 8. RNA CONTENT IN mg/g OF FRESH COMPONENT OF FENUGREEK SEEDLINGS. (The figures are means of 5 replicates)

Methods	Components	Treatments*				CV %**	L. S. D.***	
		30 C		20 C				
		Control	Irrad.	Control	Irrad.		5%	1%
^{32}P-counts	Leaves	0.66	0.56	1.59	1.19	14.5	0.26	0.34
	Cotyledons	0.07	0.05	0.08	0.08	20.3	0.02	0.03
	Stems	0.17	0.15	0.27	0.27	15.3	0.06	0.08
	Roots	0.72	0.46	0.51	0.43	15.6	0.15	0.19
UV-absorbance	Leaves	1.67	1.63	2.34	2.31	3.4	0.12	0.16
	Cotyledons	0.36	0.32	0.44	0.42	15.9	0.11	0.14
	Stems	0.55	0.51	0.65	0.71	13.8	0.15	0.19
	Roots	1.65	1.17	1.04	0.96	11.5	0.25	0.32
	Whole seedling	4.32	3.63	4.47	4.40		0.63	0.81
Color reaction	Leaves	3.34	3.16	4.21	3.72	11.8	0.77	0.99
	Cotyledons	1.68	1.60	1.84	1.75	10.2	0.32	0.41
	Stems	3.09	2.82	2.85	2.93	13.4	0.70	0.91
	Roots	3.32	2.79	2.18	2.13	8.8	0.41	0.53

*Night temperatures were maintained at 15 C for all groups.

**CV % refers to coefficient of variation.

***L. S. D. refers to Least Significant Difference between two means.

FIGURE 5. RNA-CONTENT IN mg/g OF FRESH COMPONENTS OF 21-DAY-OLD FENUGREEK SEEDLINGS. (Each column is the mean of 5 replicates.)

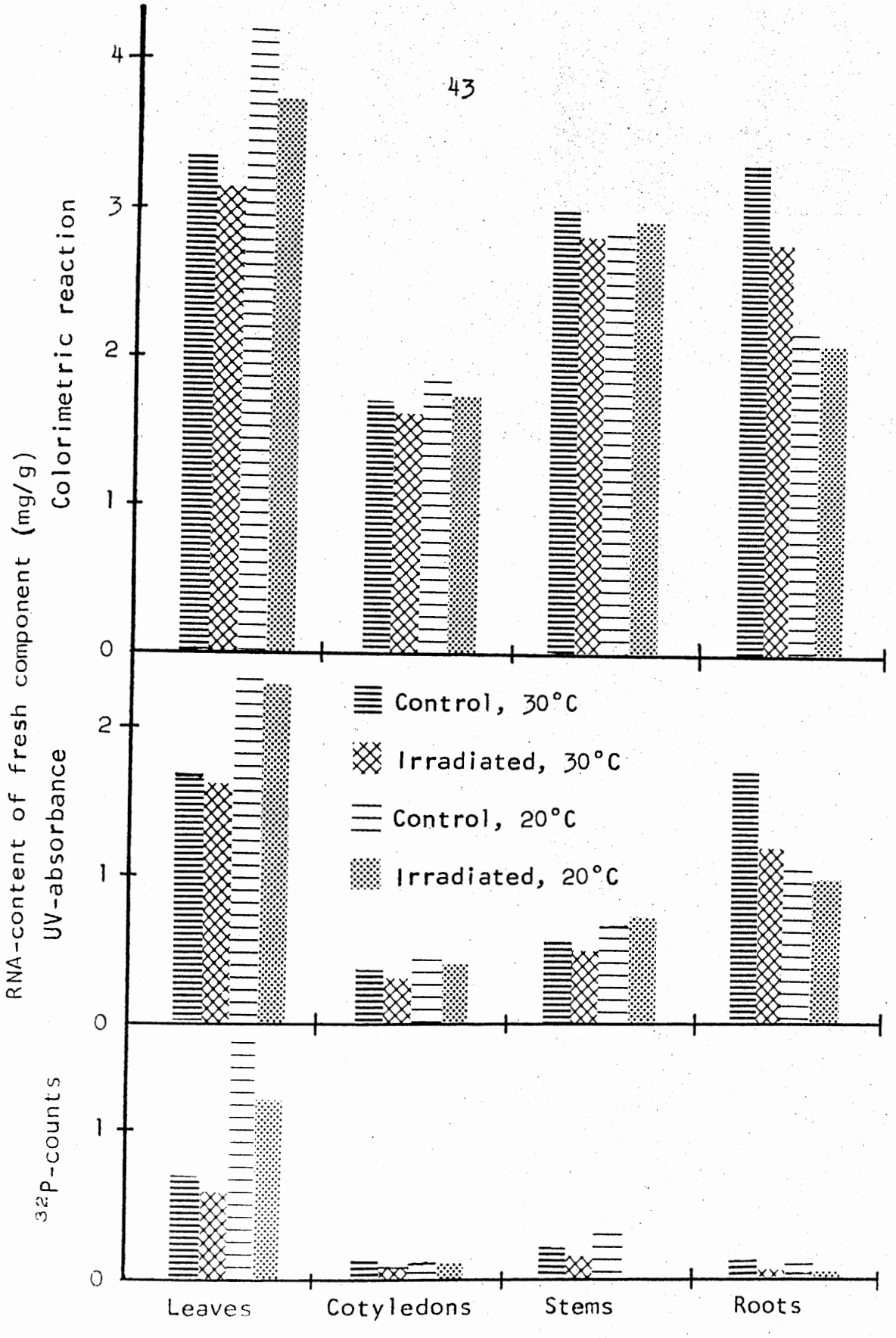
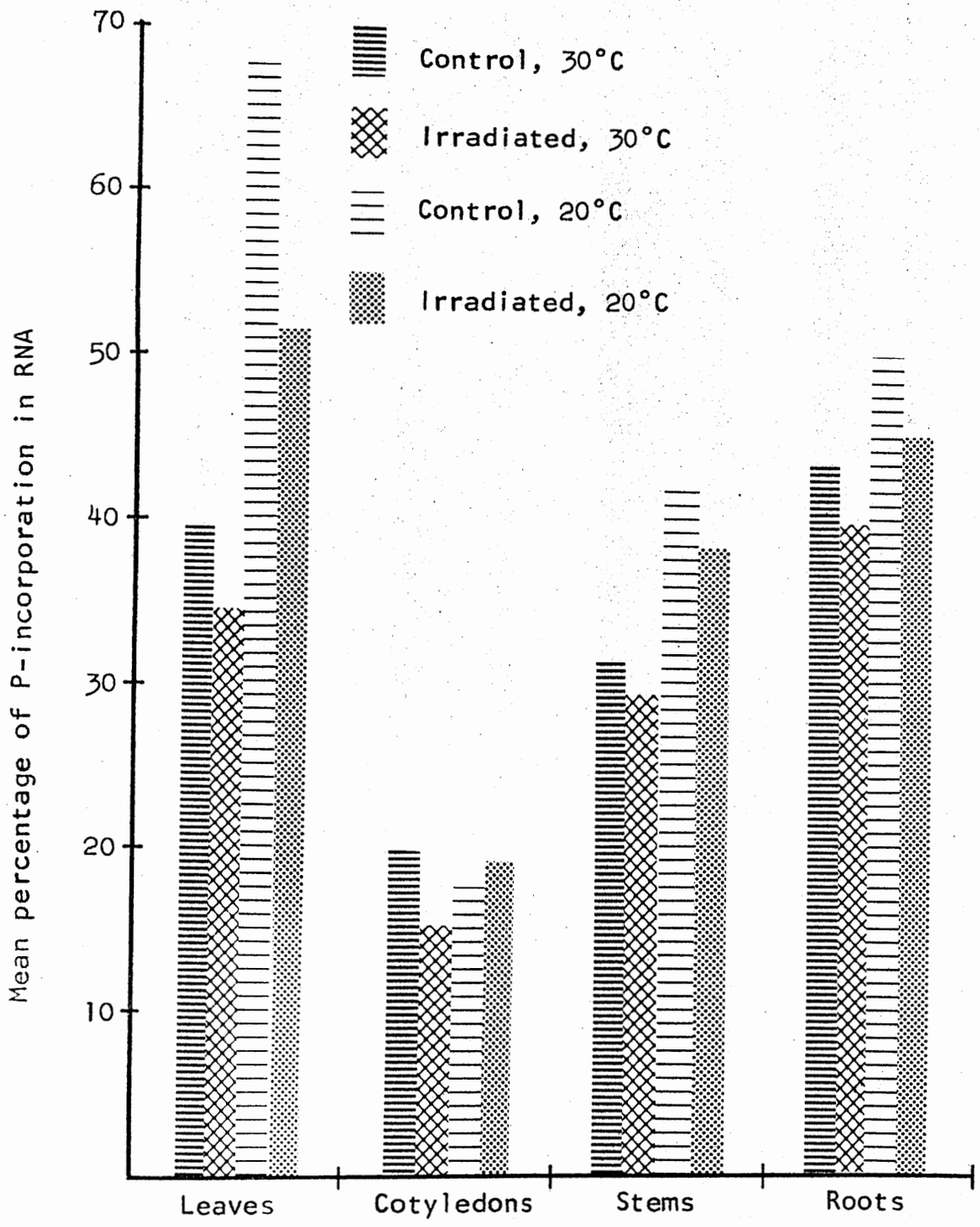

RNA-content of fresh component (mg/g)
Colorimetric reaction
UV-absorbance
^{32}P-counts
Control, 30°C
Irradiated, 30°C
Control, 20°C
Irradiated, 20°C
Leaves
Cotyledons
Stems
Roots

TABLE 9. THE MEAN PERCENTAGES OF RNA INCORPORATED BY ABSORBED-P in FENUGREEK SEEDLINGS.

Seedling Component	% RNA incorporated			
	30 C*		20 C*	
	Control	Irrad.	Control	Irrad.
	%	%	%	%
Leaves	39.5	34.3	67.9	51.6
Cotyledons	19.9	15.1	17.7	19.0
Stems	31.3	29.4	41.9	38.2
Roots	43.8	39.5	49.1	45.2

*Night temperatures were maintained at 15 C for all groups.

FIGURE 6. THE MEAN PERCENTAGES OF RNA INCORPORATED BY ABSORBED-P IN FENUGREEK SEEDLINGS.


Mean percentage of P-incorporation in RNA
70
60
50
40
30
20
10
Control, 30°C
Irradiated, 30°C
Control, 20°C
Irradiated, 20°C
Leaves
Cotyledons
Stems
Roots

The coefficients of variation in Table 8 and 10 indicated that the total nucleic acid content determined by UV-method was more precise when compared to the colorimetric method, since the coefficients were smaller. Furthermore, in the color reactions (colorimetric) presence of free sugars in the extracts usually results in higher readings (Broughton, 1970), since the reactions are specific for pentoses and deoxypentoses. The UV-determination, however, would be more realistic since it is more specific for the nitrogen bases. By this reasoning, it is appropriate to consider data on nucleic acids determined by the UV-method as representing the total content. These data were consequently used in calculating the precentage of incorporated RNA and DNA in the various seedling organs by the uptaken phosphorus (Tables 9 and 11). The data of radioactive counts is assayed to represent the incorporated part of the nucleic acids.

Results shown in Table 9 and Figure 6 indicated that the radiation treatment caused a decrease in the rate of RNA incorporation in all organs except cotyledons of the seedlings grown at 20 C. Another observation was the higher percentage incorporation under lower temperature condition. Again, the leaves showed highest incorporation followed by roots, then stems, and cotyledons last.

DNA content:

The DNA contents of the various components of bean seedlings following treatments of radiation and temperature were assayed by three methods. The results are presented in Table 10 and Figure 7. Analyses of variance of DNA contents are recorded in Appendixes no. 27 to 38. The least significant differences between any two means of DNA content are recorded in the last two columns of Table 10.

Table 10 and Figure 7 demonstrate that (a) DNA distribution, as determined by both the radioactive and UV methods, followed the same pattern, but the colorimetric technique gave incompatible results; (b) radiation resulted in insignificant variations of DNA contents; (c) the lower temperature conditions resulted in a significant increase in the DNA incorporated in leaves, and stems; (d) the total DNA showed significant increase only in the leaves but showed a decrease in the roots (Based on the UV data).

The results described in Table 11 and Figure 8 indicated that radiation did reduce the percentage of DNA incorporation in all components and treatments. The 20 C growth condition appeared to favor DNA incorporation by the bean seedlings, except for incorporation in cotyledons. Leaves, stems, and roots incorporated DNA molecules equally, but the cotyledons had less incorporation.

TABLE 10. DNA CONTENT IN mg/g OF FRESH COMPONENT OF FENUGREEK SEEDLINGS. (The figures are means of 5 replicates)

Methods	Components	Treatments* 30 C Control	30 C Irrad.	20 C Control	20 C Irrad.	CV %**	L. S. D.*** 5%	1%
^{32}P-counts	Leaves	0.19	0.18	0.39	0.28	12.0	0.06	0.07
	Cotyledons	0.03	0.02	0.02	0.02	33.7	0.01	0.2
	Stems	0.06	0.05	0.08	0.09	14.5	0.01	0.02
	Roots	0.18	0.14	0.17	0.15	10.9	0.03	0.04
UV-absorbance	Leaves	1.42	1.41	1.78	1.68	12.5	0.36	0.46
	Cotyledons	0.48	0.43	0.46	0.49	14.0	0.12	0.15
	Stems	0.44	0.41	0.39	0.45	11.7	0.09	0.12
	Roots	1.24	1.04	0.73	0.76	14.3	0.24	0.31
	Whole seedling	3.58	3.29	3.37	3.39		0.81	1.04
Color reaction	Leaves	2.03	2.13	3.11	3.28	14.7	0.70	0.90
	Cotyledons	1.13	1.04	1.44	2.17	24.4	0.63	0.82
	Stems	1.32	1.65	2.17	2.17	25.3	0.83	1.07
	Roots	0.86	0.86	1.12	1.12	27.4	0.50	0.65

*Night temperatures were maintained at 15 C for all groups.

**CV % refers to coefficient of variation.

*** L. S. D. refers to Least Significant Difference between two means.

FIGURE 7. DNA CONTENT IN mg/g OF FRESH COMPONENTS OF 21-DAY-OLD FENUGREEK SEEDLINGS. (Each column is the mean for 5 replicates.)

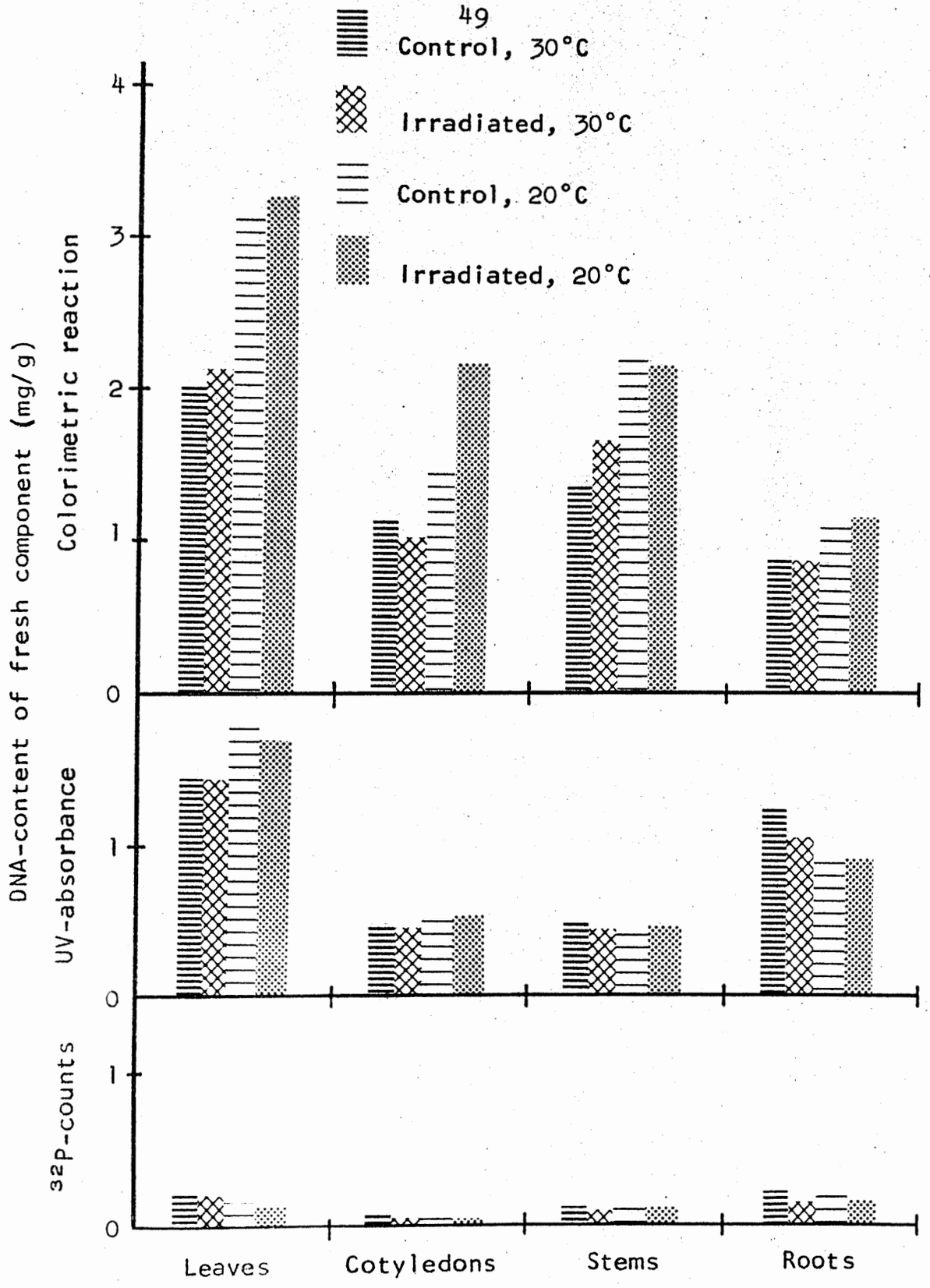
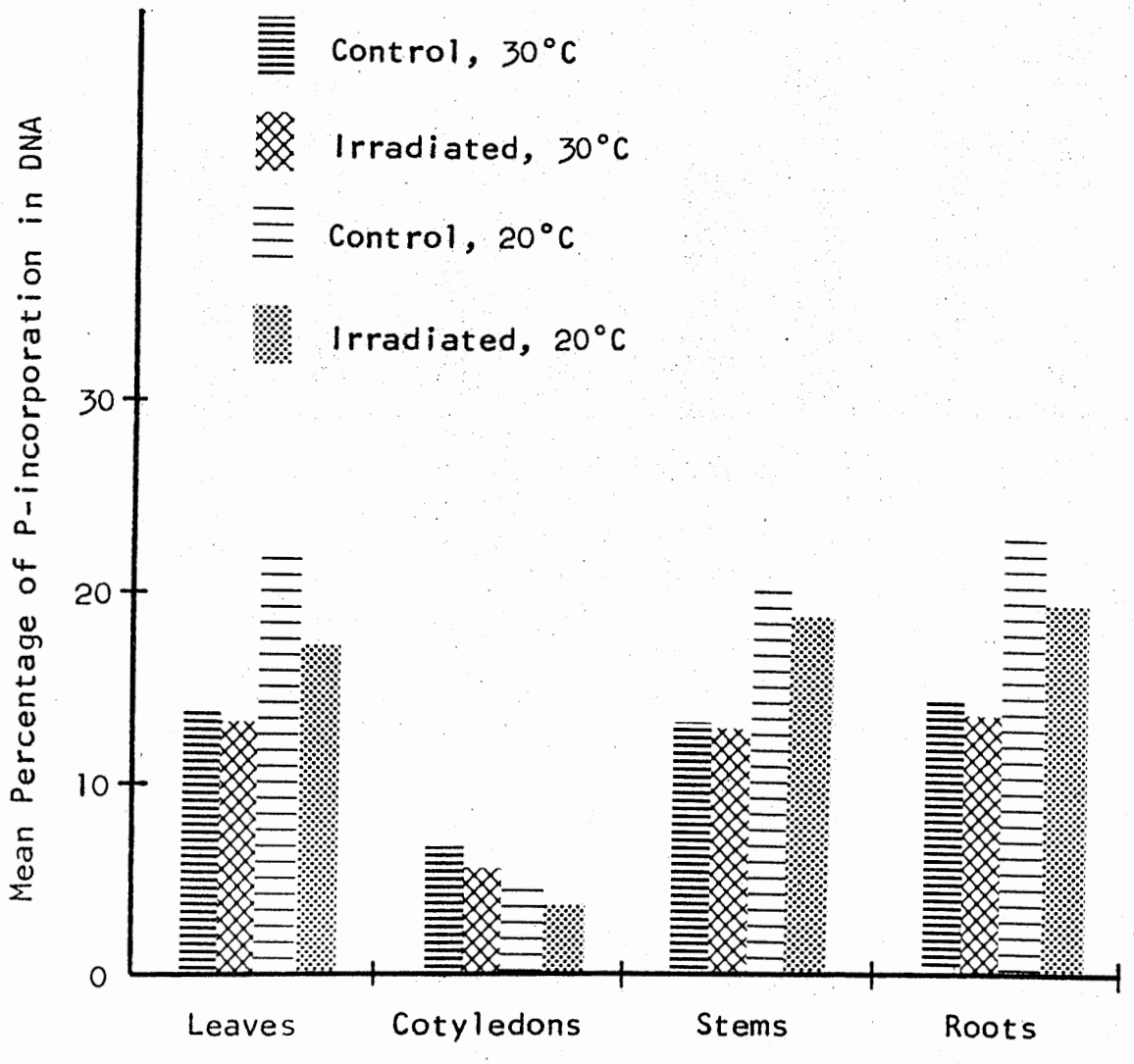

Control, 30°C
Irradiated, 30°C
Control, 20°C
Irradiated, 20°C
DNA-content of fresh component (mg/g)
Colorimetric reaction
UV-absorbance
32P-counts
0
1
2
3
4
Leaves
Cotyledons
Stems
Roots

TABLE 11. THE MEAN PERCENTAGES OF DNA INCORPORATED BY ABSORBED-P IN FENUGREEK SEEDLINGS.

Seedling components	% DNA incorporated			
	30 C*		20 C*	
	Control	Irrad.	Control	Irrad.
	%	%	%	%
Leaves	13.8	12.8	21.8	16.8
Cotyledons	6.6	5.2	4.3	3.6
Stems	13.1	12.7	19.9	18.8
Roots	14.6	13.5	22.5	19.3

*Night temperatures were maintained at 15 C for all groups.

FIGURE 8. THE MEAN PERCENTAGES OF DNA INCORPORATED BY ABSORBED-P IN FENUGREEK SEEDLINGS.


Control, 30°C
Irradiated, 30°C
Control, 20°C
Irradiated, 20°C
Mean Percentage of P-incorporation in DNA
30
20
10
0
Leaves
Cotyledons
Stems
Roots

CHAPTER V

DISCUSSION

FRESH AND DRY WEIGHTS:

The action of ionizing radiation on seeds is not confined to the surface of the seed. Therefore, this radiation may cause damage on any molecule in the seeds. Alexander and Charlesby (1954) have suggested that the occurrence of energy-transfer processes in organic macromolecules may play an important part in the biological effects of ionizing radiations, since such transfers can result in an upset of the total biological activity by the preferential decomposition of a particular component. For instance, the radiation induced radicals may disrupt the structure of DNA molecule resulting in altered information (Weissberger and Okada, 1961; Scholes and Weiss, 1962), and may also alter the configuration of latent enzymes causing inactivation of their functions (Augenstein and Grist, 1962; Augenstein and Mason, 1962). Furthermore, the de novo synthesis of nucleic acids and enzymes can be modified by the altered molecules (Cherry et al., 1962b; Van Huystee and Cherry, 1967; Weiss and Wheeler, 1967; Endo, 1967; Tokarskaya et al., 1969; Revin et al., 1970; and

Ananthaswamy et al., 1971).

Evidence gained from this study indicates that 10 KR exposure of fenugreek seeds to Co-60 gamma-rays decreases both fresh and dry seedling weights of the plants grown at 20 C but increases both fresh and dry seedling weights of the groups grown at 30 C. The result of decreasing weights of irradiated seedlings is expected, and is in agreement with previous work (Fang, 1969; Freeman, J. E., 1970; Freeman, J. R., 1970; Looper and Aboul-Ela, 1971). The reduction in seedling weight in irradiated seeds might be due to specific effects on promotive phytohormones (Weber and Gordon, 1953; Sideris et al., 1971), on inhibitory phytohormones (Maxie et al., 1965; Maxie and Abdel-Kader, 1966), on the photosynthetic efficiency (Simonis and Von Fuchtbauer, 1965; and Kohn et al., 1967) or on the respiratory enzymes (Arslanova, 1970).

Ten KR is comparatively a low dose level for dormant seeds of fenugreek. Under certain favorable environmental factors, such as 30 C, it is possible that the damage due to a low irradiation dose is repaired. Sax and Enzman (1939) pointed out that higher temperature facilitates movement of broken chromosomal ends and restoration to their original positions. Caldecott (1961) explained that the post-irradiation heat produces the thermal energy necessary to enhance the rate at which the radiation energy is diverted

away from the site of primary absorption. It is also possible that higher temperature, within active range of enzymes, may enhance all enzymatic activities, resulting in faster growth of seedlings. Subsequently, a stimulatory effect on seedling weight in irradiated groups would be possible at 30 C.

In regard to the dry weights of various components, there is an interesting finding on cotyledons. The dry weight of irradiated cotyledons, growing either at 20 C or 30 C, is heavier than the weight of non-irradiated, control cotyledons grown at the same conditions. Looper and Aboul-Ela (1971) have reported similar results with the 30 KR exposure dose. Hill et al. (1967) stated that the cotyledons of legume seedlings usually develop chlorophyll and thus carry on photosynthesis for a period of age. The food that is stored in them is gradually digested and transferred to the growing parts of the seedling. Ledoux (1962) found that irradiation rapidly inhibits the translocation of labeled macromolecules in the seed to different organs in germinating barley. He concluded that the translocation mechanism is more radiosensitive than the synthetic processes. In addition, Bostrack and Sparrow (1969) found that the number of conducting elements per bundle was reduced in the irradiated leaves of Pinus nigida. Thus, it is possible that the

reserved nutrient in the cotyledon is blocked by radiation effect resulting in heavier dry weight of irradiated cotyledons.

Phosphorus uptake:

Phosphorus is not only the constituent of certain molecules such as nucleic acids, ATP, and coenzymes, but it is also involved in the activation of basic metabolic molecules such as monosaccharides and amino acids. The overall catabolic and anabolic processes in plants depend on these activations (Devlin, 1969). Therefore, it is reasonable to conclude that phosphorus is one of the most essential elements for plants.

Obviously, the results of phosphorus uptake (Figure 4) indicated that most of the phosphorus is distributed between acid (high energy compounds) and methanol (inorganic P-compounds) fractions, followed by nucleic acid fractions, and the least in the ether fraction.

Radiation reduction effect on phosphorus uptake has been reported by Ehrenberg and Faludi-Daniel (1967) in barley seeds, Kulka and Rejowski (1970) in barley embryos, and Shaw (1971) in mung beans. Ehrenberg and Faludi-Daniel (1967) explained that the phosphate uptake probably involves a phosphorylation, since both photophosphorylation and oxidative phosphorylation are quite sensitive to radiation.

Kulka and Rejowski (1970) suggested that the ability to incorporate P-32 into nucleic acids is dependent on the growing activity of the seedling component. If the fresh and dry weights are taken as a measure of growing activity, then data presented here contradict the above mentioned hypothesis. From Tables 5, 6, and 7 it is noted that when irradiation stimulated both fresh and dry weights of seedlings grown at 30 C, phosphorus uptake was reduced. On the other hand, when radiation reduced the fresh and dry weights of those grown at 20 C, phosphorus uptake was also reduced. Thus, our data do not support the proposition of Kulka and Rejowski (1970).

If phosphorylations are actually inhibited by irradiation and phosphorylation activity is somehow correlated with phosphorus uptake, we may have an explanation why such uptake was inhibited by irradiation and promoted by high temperature. The distribution pattern of the methanol soluble and acid soluble fraction (Table 7) further substantiates this idea.

The temperature induction effects on phosphorus uptake has not been studied extensively, but such effect is probably correlated with the enzymatic activity. Devlin (1969) stated that salt absorption in roots is predominantly an active process, thus involves enzymatic acticity, and a small amount of salt is absorbed passively. Generally, the

protein nature of enzymes causes them to be particularly sensitive to temperature changes and confines their activity to a range of 0 to 45 C. Within this range the rate of biochemical reactions increases on the average 2.5 times for every 10 C increase in temperature (Conn and Stumpf, 1967). Therefore, it is not surprising that the total amount of P-uptake at 30 C groups is 22% higher than the 20 C groups, regardless of radiation treatment.

NUCLEIC ACIDS:

Randolph and Haber (1961) studied free radicals produced by X-ray irradiation of lettuce seeds. They found that the greater portion of the radicals are formed in the embryo rather than in the cotyledons. Meletti and D'Amato (1961) transplanted detached wheat and barley embryos on separated endosperms. They found that irradiated embryos did not have any advantage when they were grown on unirradiated endosperm, nor was there any effect of the irradiated endosperm on the unirradiated embryos. It appears then that the morphological damages demonstrated by the embryo or its components are results of direct hits on the embryo.

Conger and Randolph (1959) observed that exposure of wheat germ to 1 KR of Co-60 gamma-rays produced about 10^{11} radicals/mg of dry material. They noted that the decay of 65% of the radicals occurred over a 3-day period, with an

extremely slow rate of decay thereafter. Therefore, one might speculate that, in fenugreek seeds the gamma-rays produced radicals which has a slow decay rate and the major destruction in the cellular processes was still apparent at 21 days. The damage was manifested in the form of leaf variegation, fasciation, wrinkling, and general deformation (Gunckel and Sparrow, 1954).

The structure of nucleic acids (DNA and RNA) is sensitive to radiation. Scholes and Weiss (1962) reported that the effects of ionizing radiation on DNA in an aqueous system showed that radical attack occurs mainly on constituent purine and pyrimidine bases. Breakage of the strands in the DNA helix occurs mainly as a result of attack of the radicals on the hydrogen bonds. The breaking of the back bone of strands occurs mainly on the sugar moiety, which leads to a splitting of the phosphate ester linkages along the chain, and thus to the production of phosphomonoester groups.

The relative irreversibility of DNA synthesis inhibition after irradiation may be due to impaired synthesis of DNA precursors (Benes and Soska, 1962) or to the depression of DNA biosynthetic enzymes (Goutier and Bologna, 1962). Pollard (1964) has demonstrated that the DNA-RNA transcription step is particularly radiosensitive. Thus, in view of these reports, it was expected that the nucleic

acid contents of a whole seedling and its componenta at the age of 21 days be reduced as a result of irradiation, regardless of temperature. Both the DNA and RNA contents of entire seedlings were not significantly reduced by irradiation. Temperature under which seedlings were grown was apparently a major factor in altering RNA but not DNA content (Tables 8 and 10). The association of increased RNA with low temperature probably explains the increased fresh and dry weights of seedlings under that condition.

After the seeds have imbibed water, there are numerous biochemical changes in the germinating seeds. Enzymes hydrolyze the stored foods into soluble and diffusible substances which are translocated and assimilated by the embryo. Amen (1968) reported that the hydrolytic reactions catalyzed by amylases and proteases result in the formation of monosaccharides and amino acids necessary for the respiratory and biosynthetic processes of the embryo. The ribonucleases may catalyze the breakdown of nucleic acids producing cytokinins necessary for cell division. The breakdown of proteins by protease results in the formation of various amino acids, including tryptophan, a precursor of IAA to stimulate the cell elongation, as well as RNA synthesis. All these activities certainly lead to faster growth which is manifested by heavier seedling weights.

Augenstein and Grist (1962) reported that the nature of enzyme inactivation by radiation is by the disruption of specific S-S and H-bonds. Augenstein and Mason (1962) pointed out that below 250 K, radiation sensitivity is essentially temperature independent, whereas at higher temperatures the processes of charge migration associated with enzyme inactivation is the temperature-dependent step. If this interaction between temperature and radiation effect is similarly applicable to RNA disruption, then we have an interpretation for the reduction of RNA in irradiated groups at 30 C but not at 20 C. Endo (1967) found that there were stricking radiation-induced changes in enzyme systems in the maize. Cherry et al., (1962a, 1962b) calculated a 15-20% reduction in ribonuclease activity in irradiated corn endosperm upon germination. Tollier and Guilbot (1971) found that a 25 Krad dose of irradiation blocked the actions of alpha-amylase and beta-amylase in both potatoes and corn. Moreover, Anthaswamy et al., (1971) reported that the decrease in alpha-amylase, ribonuclease, and 3'-nucleotidase in irradiated wheat seeds could be regarded as a radiation induced damage. In addition, Arslanova (1970) found that irradiation impaired the dehydrogenase activity in cotton nuclei and mitochondria. The impairment of orderly biochemical processes appeared to be related to disturbance of electron transport in mitochondrial respiration and in

carbohydrate oxidation by way of the Kreb's cycle. The irradiation produced inhibition of aerobic glucose assimilation resulting in the delay of pyruvate transformations. Although we do not have data on enzyme amounts or enzyme activity, the fresh and dry weight data indirectly indicate either absence of radiation damage to the enzyme systems in fenugreek or recovery of these systems by the age of 21 days.

Dealing with the distribution of nucleic acid in plants during germination, Oota and Takata (1959) reported that large amounts of RNA are stored in the cotyledons of beans. Germination resulted in a steady decrease in the RNA content in the seedling. Cherry and Hageman (1961) found a steady decline in scutellum RNA and an increase in the synthesis of radical RNA during germination. A possible explanation for the mode of RNA utilization is that the RNA is degraded to nucleotides, translocated, and then used for growth of the embryonic plant; or that a transportable RNA is moved to the growing tissue where it is used. Cherry et al. (1965) found that at the onset of germination RNA content in the peanut cotyledons doubled in 12 days after planting. After 12 days there was a reduction in rRNA and mRNA, and an increase in sRNA. These changes in RNA appeared to be related to the final stage of senescence in the cotyledon. In the same year, Ingle and Hageman (1965) reported that the corn kernel contains little reserve of

nucleic acid; and that the increase of nucleic acid and nucleotide materials in the growing axis was due to de novo synthesis.

The experimental material and conditions which lead to the above conclusions are different and varied. However, in the light of the ideas presented, an interpretation of the distribution of nucleic acids in fenugreek seedling components may be postulated.

First, the total DNA, which was hardly affected by irradiation or temperature in the 21 day old seedlings, is probably synthesized de novo from the inherent phosphorous in the seed. This is indicated by the high content of total DNA (UV assay in Table 10) in leaves and roots as compared to the stems and cotyledons. It is hard to imagine the translocation of large DNA molecules from the store of cotyledons into the leaves and roots. These large molecules apparently are first broken down in the cotyledon then resynthesized in the other sites. The low percentages of DNA content in the seedling components representing the incorporated part (as shown by ^{32}P assay in Tables 10 and 11) point to the utilization of the stored phosphorous in the seed in DNA synthesis. It is interesting to note that the cotyledons contain relatively the lowest level of DNA, particularly the incorporated DNA, as compared to the other seedling organs. This is in spite of the observation that

at 21 days they are at a peak of growth and activity (as indicated by their healthy appearance, size and green color). Apparently they do not synthesize much DNA other than that inherently present since they attain their full size and activity early during the seedling development. The stem is mainly a transport organ, thus contains a low level of DNA. The relatively high percentage of de novo DNA synthesis in the stem probably represents the activity of its apical meristem.

Secondly, the total RNA seems to be sensitive to the radiation under high temperature growing conditions but less sensitive under low temperature. Meanwhile the RNA level in the various seedling components corresponds with their DNA level. This relationship is not surprising in light of the well known relationship between the two nucleic acids. The percentages of the incorporated RNA present (as measured by P-32 assay in Tables 8 and 9) are much higher than the corresponding DNA percentages in all seedling components, an indication of additional RNA synthesis in the 21-day old seedlings. The percentage of incorporated RNA present in cotyledons is similarly lower than in any other organ, even the stem. Thus, little synthetic activity at the RNA level in the cotyledons, as compared to the other organs, is indicated, at least at this age of seedling. Observations from Tables 9 and 11 indicate a consistent

decrease in RNA and DNA incorporation as a result of irradiation and increase in incorporation as a result of lower temperature. The variations in incorporation rates are not, however, correlated with the seedling fresh or dry weights.

Data of Tables 5, 6, and 7 show unusually favorable conditions for root growth under 20 C as compared to 30 C. At low temperature the root fresh weights increased about 100%, and the dry weight about 50%, yet the total phosphorus absorption decreased about 20%. Although the total amount of phosphorus uptake is higher under low temperature conditions, the rate of roots development is faster than the rate of their absorbing capacity.

CHAPTER VI

SUMMARY AND CONCLUSION

1. The objective of this work was to investigate the distribution of RNA, DNA, and other phosphorus compounds among the various components of fenugreek bean seedlings affected by radiation and post-irradiation-temperature. In addition, the fresh and dry weights of each component were recorded. The amounts of P-uptake and its distribution among the varied components in the different seedling organs were traced.
2. Fenugreek seeds were exposed to 10 KR of Co-60 gamma rays. Both control and irradiated seeds were sub-treated by growing them under two different day temperatures (20 C and 30 C) separately. The 21-day old seedlings were harvested for the analyses used in this study.
3. The measurements of fresh and dry weights indicated that radiation reduced weights of the groups grown at 20 C but stimulated the weights of the groups grown at 30 C. The dry weight of cotyledons were stimulated by irradiation at both temperatures. The lower temperature favored the seedling growth as compared to the higher temperature.

4. Phosphorus uptake was significantly stimulated by the higher temperature but significantly reduced in irradiated groups. The irradiation-inhibiting and temperature-stimulating effect was obvious in all seedling organs. Most of the phosphorus was in the inorganic and the acid soluble fractions. It was distributed primarly in the leaves and roots, with minimal amounts in the cotyledons.
5. The nucleic acid contents were determined by three methods. Radioactive counts represented only the amount of phosphorus incorporated into nucleic acid molecules. The color reactions gave a comparatively high readings on nucleic acids, probably because of the interference of some pentoses in the extracts. Therefore, the contents of nucleic acid assayed by UV-spectrophotometry were considered as the most accurate and were used to represent the total nucleic acids in the seedling components.
6. Radiation did decrease RNA content consistently in all seedling components. Under 20 C post-irradiation temperature, higher RNA contents were found in leaves, cotyledons, stems, but lower in roots, as compared to the groups grown at 30 C. Radiation caused the decrease in the rate of RNA incorporation but a high percentage of incorporation occurred under the lower temperature

condition.

7. Radiation resulted in insignificant variation of DNA contents. Again, the lower temperature condition resulted in a significant increase of the incorporated DNA.
8. The overall distribution of DNA and RNA was greatest in leaves, followed by roots, stems, and then cotyledons.
9. The increase in fresh and dry weights of seedling components seemed to be associated with their RNA content and RNA synthesis.
10. The rate of P-uptake appeared to be independent of the fresh and dry weights of the various components of the seedlings, especially the roots.
11. The DNA synthesis in the various seedling components consumed the stored phosphorus and by 21 days little DNA was synthesized from the absorbed phosphorus. The rate of RNA synthesis, however, from the absorbed phosphorus was much higher than DNA.
12. The amounts of nucleic acids and the percentages of their incorporation from absorbed phosphorus into the cotyledons indicates some metabolic activity in the cotyledons. Since they attain full size and activity early in the plant life, their activity is probably confined to providing the seedling proper with synthesized food material. Because radiation and temperature

resulted in slight alterations in the nucleic acid content of the cotyledons, it is reasonable to speculate that the increased dry weight in irradiated seedlings was probably due to sluggish translocation of the cotyledons photosynthetic products into the seedlings.

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APPENDIX

ANALYSES OF VARIANCE OF FRESH WEIGHT, DRY WEIGHT, AND P-UPTAKE.

No.	Measurement	Component	Mean Square Between df 3	Mean Square Within df 16	F-ratio
1	Fresh weights	Leaves	0.1962	0.0349	5.6234**
2		Cotyledons	0.0698	0.0126	5.5237**
3		Stems	0.0631	0.0880	0.7172
4		Roots	3.9079	0.0670	58.2783**
5	Dry weights	Leaves	0.0010	0.0005	2.0077
6		Cotyledons	0.0002	0.00006	3.3453*
7		Stems	0.0001	0.00007	1.6470
8		Roots	0.0063	0.00058	10.7257**
9	P-uptake by whole seedling	Fraction I	606.0945	43.9578	13.7880**
10		Fraction II	21683.8049	465.7320	46.5585**
11		Fraction III	9.2226	1.5285	6.0333**
12		Fraction IV	688.1475	19.0081	36.2027**
13		Fraction V	32.0851	0.5484	58.4962**
14		Fraction VI	0.1015	0.0118	8.5300**

@df refers to degree freedom.

*Significance at the 5% level.

**Significance at the 1% level.

ANALYSIS OF VARIANCE OF RNA CONTENT.

No.	Method	Component	Mean Square		F-ratio
			Between df@ 3	Within df@ 16	
15	^{32}P-counts	Leaves	1.1576	0.02100	55.0588**
16		Cotyledons	0.0010	0.00020	5.0477**
17		Stems	0.0210	0.00117	17.9198**
18		Roots	0.0863	0.00690	12.3981**
19	UV-absorbance	Leaves	0.7602	0.00477	159.2490**
20		Cotyledons	0.0148	0.0038	3.8988*
21		Stems	0.0434	0.0069	6.2913**
22		Roots	0.4780	0.0191	24.9867**
23	Color reaction	Leaves	1.0791	0.1835	5.8793**
24		Cotyledons	0.0497	0.0307	1.6162
25		Stems	0.0770	0.1546	0.4984
26		Roots	1.5799	0.0519	30.4092**

@df refers to degree of freedom.

*Significance at the 5% level.

**Significance at the 1% level.

ANALYSIS OF VARIANCE OF DNA CONTENT.

No.	Method	Component	Mean Square		F-ratio
			Between df@3	Within df@16	
27	^{32}P-counts	Leaves	0.0455	0.001000	45.2472**
28		Cotyledons	0.0001	0.000032	3.1368
29		Stems	0.0013	0.000190	6.8054**
30		Roots	0.0016	0.000340	4.8295*
31	UV-absorbance	Leaves	0.1819	0.0389	4.6668*
32		Cotyledons	0.0032	0.0043	0.7450
33		Stems	0.0034	0.0025	1.3773
34		Roots	0.2850	0.0183	15.5712**
35	Color reaction	Leaves	2.0712	0.1508	13.7340**
36		Cotyledons	1.3100	0.1244	10.5226**
37		Stems	0.8776	0.2138	4.1045*
38		Roots	0.0775	0.1343	0.5769

@df refers to degree of freedom.

*Significance at the 5% level.

**Significance at the 1% level.