CHAPTER II

IN VIVO STUDIES IN RELATION TO ODAP IN THE SEEDS OF *LATHYRUS SATIVUS*

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CHAPTER-II

II.1. INTRODUCTION

Lathyrus sativus L.(2n = 14) also known as Grasspea, Chickling vetch and Kesaridal is a herbaceous annual legume with a winged procumbent stem (Fig. II.1).

It is a rabi (winter) crop sown in October - November and harvested in late February. It grows abundantly in dark loamy soil. Its cultivation does not need any irrigation facilities.

Moreover its seeds are protein rich (28-35% w/w on dry weight basis) and next only to soybean in its nutrional value. Due to its drought resistant character it thrives well even under adverse conditions where other drought resistant crops like millet and bajra fails. Hence during periods of famine and scarcity it is regarded as a life saving crop. The folk verses indicate the importance of this famine crop. (NIN report, 1988)

> "Matra ki roti, Matra ki dal Matra ki pati, Rakhan har"
> which means :
>
> Matra (L. sativus) as a bread and Matra as a dal,
> Matra indeed is guardian of all"

Another folk verse which shows that local people in Madhya Pradesh had already associated the pulse with the spasmatic disease. It is as follows :

"Kairat Matra piar pisan. Pisan Khai segor nasan
Hale Chandui matke kool. Ja dehko matral ki sool"
which means :

The black pea with its yellow flour from eating it comes trouble in legs.

"Flapping top not and swaying hips
Behold the ill-effects of eating matra".

(In the state of Madhya Pradesh, L. sativus is known as 'Matra').

The labourers prepare rotis (flattened bread) out of Lathyrus flour. It is sweet and tasty and is more filling than wheat or Bengalgram roti. So they prefer it over other staple foods.

Though the crop is legally banned for cultivation, Kesaridal still occupies four percent of the total pulse production in India. The state of Madhya Pradesh accounts for more the 50% of the total Lathyrus production in the country (Hartman et al., 1974; Gupta, 1980,1983).

Most of the early and rich archeobotanical finds of Lathyrus species, particularly those of the most ancient periods came from the Balkan peninsula. It has been found that the cultivation of L. sativus began there in early neolithic period, around 6000 BC as a result of the expansion of near eastern agriculture annuals into the region. This in turn encouraged development of a greater variety of legumes by the domestication of L. sativus. Hence, L. sativus may perhaps be the first crop domesticated in the Europe (Kislev, 1989).

Fig.II.1. *L. sativus* in nature, at flowering stage.

GOLDEN

Besides this, it is also cultivated in eastern Uttar Pradesh, Maharashtra, Bihar, West Bengal, Assam and parts of Gujarat. Among the other countries it is cultivated in the near east Mediterranean region.

In Mahdya Pradesh Lathyrus is extensively grown in Raipur, Bilaspur and Rewa divisions. But the cases of lathyrism invariably occurs in Rewa division only. This is due to the dietary habits of people. In Raipur, rice is always taken along with Lathyrus and both are cooked by first immersing in water for a night period (Basa preparation) and draining off the leachate containing the neurotoxin BOAA in the morning. The food is cooked subsequently. While in Rewa, chappaties are made of Lathyrus. The poor peasants unknowingly consume this toxic legume as they do not have any other means of survival.

The rapid rate of population growth in India calls for an equally rapid increase in agricultural yields. Due to imporved managerial practices and introduction of new crop varieties, productivity has increased. It appears that further increase can be brought about by expansion of the crop area which could be only upto a certain feasible limit, and to exploit undeveloped plants that are able to survive in greater extremities of the environment (Loomis et al., 1971).

The response of particular species to drought conditions varies enormously depending on the environmental conditions. Under severe drought conditions however, only those species that are capable of

changing their morphopogy and physiology to reduce water loss can take better with deficit conditions and survive. Lathyrus sativus is one such leguminous plant that has extreme tolerance to drought. Level of secondary compounds in plants modulate depending upon physiological as well as environmental condition in which the plant in question is thriving. The nonprotein amino acid ODAP is a secondary compound of L. sativus. Careful investigations of such physiological and environmental factors that effect the level of OADP in the seeds can eventually be utilized to recommend new agronomic managerial practices to the farmers in which the level of OADP in the seed is lowest. Experiments decribed in this chapter are aimed towards achieving this objectives.

To substantiate our reasoning we would like to cite few examples derived in other system by investigators. Bell (1980); Fowdan et al., (1979) have aptly reviewed the degradation of several antinutritive/secondary nutritives like trypsin inhibitor, β-cyanogenic glycosides and phytohaemaglutinins stored in seeds during seed germenation. Lipton et al., (1958) and Roy et al., (1960) have shown in two species of Lathyrus : L. odoratus and L. hirsutus that during 72 h of seed germination, β-amnopropoinitrile (BAPN) which is the osteolathyrogen compound is detoxified. It gets converted into cyanoacetic acid, (CAA) which is a non-toxic compound under the influence of the enzyme monoamine oxidase (MAO).

Mohan et al., (1966) showed substantial reduction in ODAP during sprouting of L. sativus seeds. Recently, Naveed el al., (1990) carried out a large number of tests for removal of ODAP from L.

sativus seeds. These included treatments such as soaking in water, lime water or sodium bi-carbonate. The maximum level of toxin removed was 88.9%. However, no physiological studies are carried out by any of the workers to determine the optimum level of these detoxifying agents and to understand the fate of OADP under the influence of these treatments.

Equally interesting are the effects of foliar application of nutrients like urea and micronutrients like cobalt, boron and molybdenum on yield and quality of crop plants. For example foliar application of urea on Phaseolous vulgaris increases the yield by 2.8% to 5.7% (Welmer & Wilcox, 1989) along with elevation of amino acid lysine. Foliar application of micronutrients have also been carried out previously by Misra and Barat (1981). They observed a reduction in ODAP level of seeds by foliar application of cobalt and molybdenum. However, they did not establish consistent relationship of the enzyme oxalyl Co A synthetase with ODAP level in two strains of Lathyrus. Based on these results, it was assumed that oxalyl Co A synthetase may be responsible for the degradation process of ODAP and not its biosynthesis, as is evidenced in literature.

The present chapter describes some of our experiments in an effort to reduce the lathyrogenic principle ODAP from the seeds of L. sativus using whole plant system.

They include :

1. Screening of different cultivars to show the genotypic variation with respect of ODAP content and also tissue to tissue variation within the plant.

2. Changes in ODAP levels during seed germination, as affected by osmotic stress and different nutrients.

3. Effects of nutrients like phospate (PO_4), Nitrate (NO_3) and casein hydrolysate on growth of the plant, neurotoxin content, and yield components of L. sativus under controlled environmental conditions.

4. Effects of foliar spray of micronutrients in a range of varieties with low and high ODAP levels.

II.2. MATERIALS AND METHODS

II.2 A.a Screening of seeds of different varieties of L. Sativus mature dry seeds of seven varieties were analysed for ODAP and total protein contents. These were RL 15, RL 18, LSD1, LSD2, LSD10, P-24 (Pusa-24) and Bh. (Bharuch) Average size seeds were selected from each variety and ODAP was quantitatively determined from whole seed after removal of seed coat using a spectrophotometric method as described in section II.2.E.

II.2 A.b Variation in ODAP in the vegetative parts of the plant : 12 days old seedlings of the variety Bh were used for this experiment. After excision of Roots, Hypocotyl, Stem and leaves they were allowed to dry to a constant weight at 80 ^{o}C in an oven, and then homogenized in buffer to determine ODAP as mentioned in section II.2.E. Mature dry seeds of the same variety were separated into seed coat, embryo axis and cotyledons for analysis.

II.2 B. Seed germination studies : Mature uniform size seeds of Bh variety were selected. They were placed for germination in 9 cm diam. Petriplates lined with filter papers and irrigated with 5ml of glass distilled water. Seeds were allowed to germinate and seedlings grown up to 48h. Fresh weight, dry weight and ODAP were analysed at every 24h interval.

In order to give osmotic stress on germinating seeds mannitol treatment was given. Initially, dry seeds were allowed to imbibe distilled water for 48 h. Thereafter they were placed in petriplates containing mannitol at 0, 0.1 0.3, 0.4 and 0.5 mM levels and allowed to grow further. The seedling were harvested

after 48 hours of incubation and dissected seperately into axis and cotyledons for growth and ODAP analysis.

In another set of experiments seeds preimbibed in water as mentioned above were allowed to germinate in presence of potassium dihydrogen orthophosphate ($KH2PO_4$) or potassium nitrate (KNO_3) each at 0, 0.1, 0.3, 0.4 and 0.5 mM levels or casein hydrolysate (CH) at 100, 150, 200 mgs/l levels separately. This was done by transferring presoaked seeds to 9cm diameter petriplates lined with filter paper and irrigated with 5 ml of one of the above solutions.

For all experiments on seed germination ten seeds were placed in each plate, and three such replications were maintained for each treatment.

II.2. C Sand culture experiments :

The objective of this experiment was to study the effects of nutrients such as phosphate, nitrate and casein hydrolysate on the growth, yield and ODAP content of seeds or plants grown under controlled conditions.

Sand cultures have their own advantages over liquid or soil cultures in terms of better aeration of roots, close monitoring of nutrient composition, pH and easier removal of plants without damaging root system.

Pots were devised according to the standard pot culture/sand culture method of Hewitt, (1967). For this 'T' shaped glass tubes

(9 mm diameter, medium wall) were made and one arm of the inverted 'T' tubes was inserted through a hole made on the side wall of the plastic pot near its base. The inserted end of the tube was kept parallel to the base of the pot with the help of an adhesive. The opposite arm of the inverted 'T' tube was connected to a rubber tube fitted with a pinch clip. The long arm of the 'T' tube remained in a vertical position close to the side wall of the pot. The tube served as an indicator of the level of the nutrient solution remaining at the base of the pot when the sand in the pot was irrigated through the rubber tubing. Small pebbles were arranged at the base of the pot, and the pot was filled with 2.5 Kg of sieved, acid washed sand in order to remove coarse granules and any other organic matter.

For each treatment, three pots each having 3 plants were used. Each pot received 200 ml of Hoagland's nutrient solution Table-II.1) (Hoagland and Arnon 1948). twice a week.

The pots were placed in a glasshouse where the mean maximum and minimum temperatures were maintained at $28 \pm 3^{o}C$ and $15^{o}C$ respectively with the help of desert coolers.

II.2.D. Foliar spray of micronutrients on field grown plants :
Besides a local cultivar (Bharuch) three other cultivars namely P-24, LSD-10 and LSD-2 were collected from Pulse Research Station, Kanpur, India. Sowing was done in the last week of October and the crop was raised in the fields of M.S.University Arboratum in a $15m^2$ size plot. Number of plants per plot was calculated from 50 cm x 50 cm quadrant (Plants/m^2 = 25 plants/m^2)

The soil was supplemented with 60Kg of farm yard manure. Irrigation was done five times at I, III, V, VII and IX week of the growing season.

Foliar spraying was carried out with water as (control), and in treatment with 0.5 ppm cobalt nitrate and 20 ppm ammonium molybdate when the plants were at peak of their flowering (i.e. 40th day after sowing). About 2 litres of solution was sprayed in $15m^2$ size plot. The spraying was carried out during early hours of the day. The spraying treatment was repeated after one week. The experiment was laid out in split-plot design with 3 replications and with the four varieties mentioned above.

II.2.E Estimation of ODAP :

List and procedures for preperation of various reagents are given in Table II.2.

ODAP was estimated by the method of Rao (1978). In this method, ODAP is acid hydrolysed to give diaminopropionic acid (DAPA) and oxalic acid . In a second step diaminopropionic acid reacts with orthopthalaldehyde (OPT) to give yellow colour compound stochiometric proportion, in the range of 1.5 to 15 ug. The spectrophotometric determination is done at 420 nm. Comparision of values with that of standard DAPA gives value of ODAP. For estimation of ODAP from L. sativus dhal or cultured tissue, 1.0 g of dried sample was extracted with 10ml of 0.5 mM potassium tetraborate buffer (pH 9.9), and centrifuged at 3000xg for 15 min in a clinical centrifuge. The supernatant was used for estimating

ODAP content as described above. Aliquots of the sample were taken in duplicates and volumes were made to 1.0 ml with buffer. To each tube 0.5ml of 10% trichloracetic acid (TCA) was added and centrifuged at 3000xg for 15 min. The supernatant was collected and to this 0.5 ml of 4.5 M NaOH was added. One set of tubes were kept in a water bath at 80 ^{o}C for 30 min for hydrolysing ODAP TO DAPA, whereas the others were kept at room temperature. After hydrolysis was over the tubes were cooled to room temperature. 2 ml of OPT reagent was added each tube. OPT forms an intense yellow colour complex was measured in a spectrophometer againist a blank, which contained 1 ml of buffer, 0.5ml of TCA, 0.5ml 4.5 M NaOH and 2 ml of OPT reagent. The difference in optical density before and after hydrolysis was used to determine the value of ODAP content using the standard curve. A standard curve was prepared in the following manner : Aliquots containing authentic DAPA prepared in 0.5 M potassium tetraborate buffer (pH 9.9) in the range of 4 to 20 ug were taken in test tubes in 3 replicates. The volume in each tube was adjusted to 1 ml with distilled water.

After addition of OPT reagent, the absorbance of the colour complex was recorded as described above, and a standard graph was prepared.

The concentration of DAPA in the unknown plant samples was determined using this standard graph. Since one mole of DAPA is liberated from the hydrolysis of one mole of ODAP. The value of DAPA can be considered as the value of ODAP.

II.2.G. TLC OF DAPA

For the identification of DAPA, thin layer chromatographic and UV spectrophotometic methods were used.

The alkali-hydrolysed sample in the quantitative method mentioned above was used for chromatography. The hydrolyzed extract was evaporated to dryness and the residue was dissolved in a small volume of ethanol to bring the concentration of DAPA to approximately 10 ugs/ml. A glass plate of 20 x 20 cm^2 was washed with distilled water,dried and coated with silica gel 'G' to a thickness of 0.25 mm. The plate was activated at 110 ^oC for 1 hour before use. Samples were spotted in duplicate with the help of micropipette in volumes between 0.01 ml to 0.05 ml, 1ml sample of authentic DAPA solution prepared in ethanol was also spotted in one lane.

The plate was developed with methoxy ethanol : propionic acid : water (70:15:15,v/v/v/) as the solvent system for about 2 hours.

The plate was sprayed with 0.5% ninhydrin in acetone and heated for 5 min at 80 ^oC for colour development. DAPA of the plant sample was identified by co-chromatography with authentic DAPA which has Rf - 0.81 in this condition. The UV spectrum of this plant sample as well as authentic DAPA were recorded in spectrophometer.

II.2. H. Extraction and estimation of cellular nitrogen :

The micro-kjeldhal steam distillation procedure (Umbreit et al., 1959) was employed for extraction and estimation of tissue nitrogen. About 25mg dry tissue was heated with 5ml concentrated sulphuric acid in long necked Borosil glass kjeldhal flask. A pinch of copper sulphate was added to enhance the digestion. When the mixture became colourless, the flask was allowed to cool and the contents were dilluted to 25ml with glass distilled water. 5ml of the above extract was mixed with 5.0 ml of 40% sodium hydroxide (w/v) and was subjected to micro-kjeldhal's steam distillation. Ammonia evolved was collected in a receiving flask containing indicator mixture (10 ml of 1% boric acid containing bromocresol green indicator.

From the time of the dark pink colour of the indicator mixture turned to dark green the ammonia was collected for 5 min. The solution was titrated against 0.14 N sulphuric acid till the solution turned pale pink. The volume of sulphuric acid used up during the titration procedure was multiplied by a factor 0.28 to obtain the concentration of nitrogen. Blank was prepared by heating concentrated sulphuric acid without the sample.This served as basis of correction for any ammonia present in the reagents.

II.2. I. Assay for Protein Determination :

A rapid sensitive method of Bradford (1976) was used for the estimation of protein. For each aliquot of 0.1 ml sample, 0.1 N NaOH was added. To each protein sample and standard (BSA), 5 ml of comassie blue reagent was added. The samples were throughly mixed

and allowed to develop colour at room temperature for 10 min. The itensity of the colour was read at 595 nm in a colorimeter.

TABLE 4 II.1

Composition of Hoagland's solution : (Hoagland & Arnon,1948)

A. Molar stock solutions of each of the following salts are prepared separately. Each stock solution is mixed in the following proportion to make one litre of working solution.

	ml/l of Nutrient Solution
1 M KH_2PO_4	1
1 M KNO_3	5
1 M $Ca(NO_3)_2$	5
1 M $MgSO_4.H_2O$	2

B. To each litre of nutrient solution prepared in A is added 1 ml solution of each of the following salts :

	(mM)
H_3BO_3	0.05
$MnCl_2.4H_2O$	0.01
$ZnSO_4.7H_2O$	0.001
$CuSO_4.5H_2O$	0.001
Na_2MoO_4	0.0005

C. To each litre of nutrient solution prepared in A is added 5ml of the iron stock made as follows :

$FeSo_4.7H_2O$	1 M
Na_2 EDTA	1 M

TABLE - II.2

List of reagents and standards used for experiments :

Reagents	Method of preparation	Use
Bovine Serum Albumin (BSA) (Standard stock solution)	100 mg BSA dissolved in 0.1N NaOH and volume made to 50ml to give a concentration of 2 mg/ml.	As standard in the protein estimation. -do-
Bovine Serum Albumin (working standard solution)	The BSA stock solution was diluted 1:10 to give a concentration of 0.2 mg/ml.	
Bradford's reagent for protein estimation	100 mg of coomassie brillant blue G-250 was dissolved in 50ml of 90% ethanol to which 100 ml 85% orthophosphoric acid was added. Finally volume was made upto 2000 ml with distilled water. The reagent was stored at 5 ^{o}C. The reagent was diluted by adding 4 volumes of distilled water before use.	Used for protein estimation.

TABLE - II.2 Contd....

Ninhydrin-acetone reagent (for detection of amino acids.) (for detection of amino acids).	0.4% ninhydrin was prepared by dissolving 400 mg of ninhydrin in 100 ml acetone.	For TLC of amino acids.
OPT reagent	To 100 mg of O-pthalaldehyde was added 0.2ml of meracaptoethanol and 1 ml of ethyl alcohol in a 100 ml with 0.5 M potasium tetraborate buffer, pH 9.9.	For quantitative determination of ODAP.
Potassium tetraborate buffer, 0.5M, pH 9.9	76 g of Potassium tetraborate and 15 g of boric acid were dissolved in 200 ml of distilled water. The pH was adjusted to 9.9 with 4N NaOH and then the volume made to 500 ml with distilled water.	-do-

II.3. RESULTS AND DISCUSSION

II.3. A. Screening of varieties of L. sativus for ODAP content :

Development of genotypes with zero or near zero ODAP content varieties is the major requirement in Lathyrus improvement. In order to know whether natural variation exists in ODAP and total protein content in the seeds seven varieties were screened. Fig-II.2. shows the amount of ODAP content among the seeds of seven varieties. It ranges from 3090 µg/g dr.wt. of the seed in RL-15 to P-24 is 1560 µg/g dr.wt. Total protein content shows an inverse proportion with ODAP viz., high toxic containing varieties like RL-15 shows lower amounts of protein whereas low ODAP strains like P-24 shows a high protein content. The above findings on ODAP content are at par with earlier studies of Briggs et al., (1985) who had screened 85 accessions of L. sativus. and found that ODAP levels in the seeds were in the range of 1700 to 3200 µg/g dr.wt. Attempts were made to understand the seed morphological characterstics like pattern of mottling and average seed size in comparision to that of toxin ODAP content in L. sativus. We found that colour of the seed and its size has no correlation contrary to the earlier reported (Dahiya, 1976). For example high toxin containing variety like RL-15 shows no mottling (Fig II.3) and seed size is also small (9.51 ± 0.7 mg/seed) whereas low toxin containing variety like P-24 shows slight to dark mottling.

Similar work was carried out by Roy et al., (1981) who had studied varietal differences in physico-chemical characterstics and neurotoxin content in L. sativus. They indicated that of the total

Fig.II.2.

VARIATION IN TOXIN AND TOTAL PROTEIN CONTENT AMONG 7 DIFFERENT VARIETIES OF L. SATIVUS

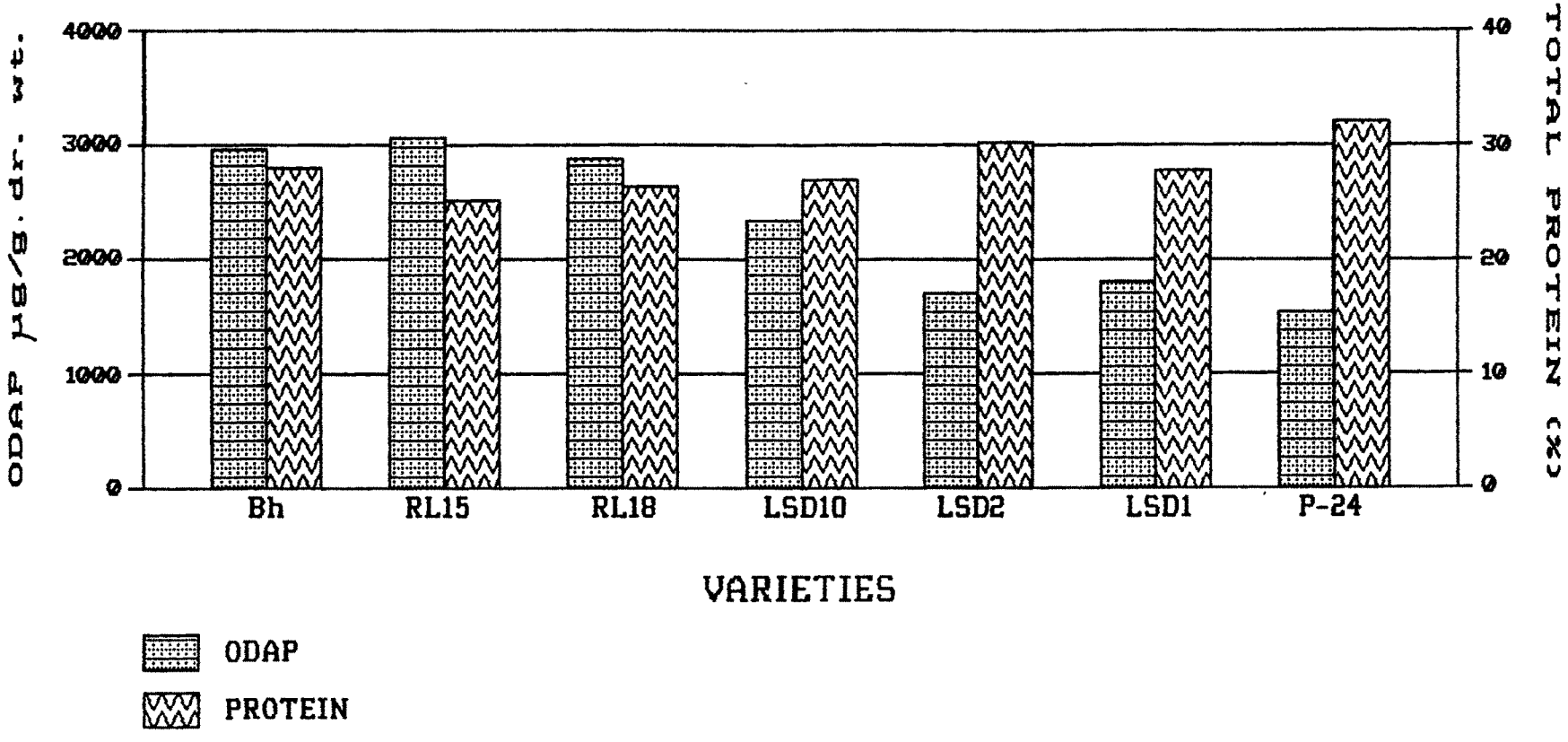
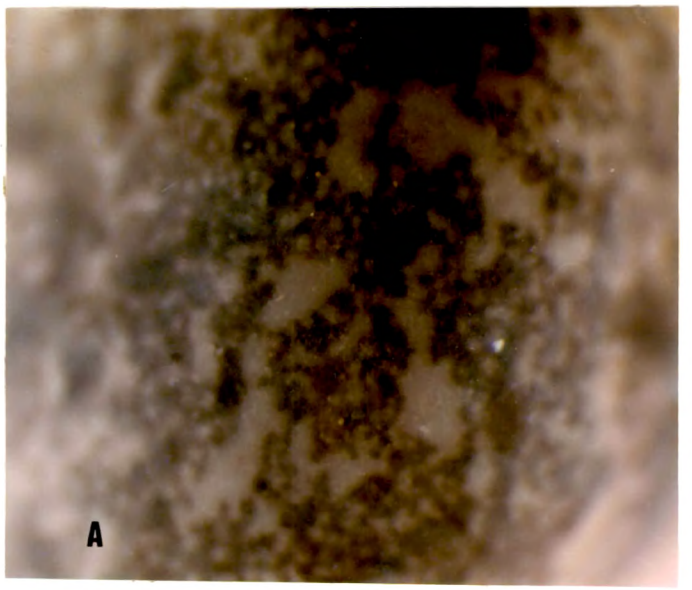
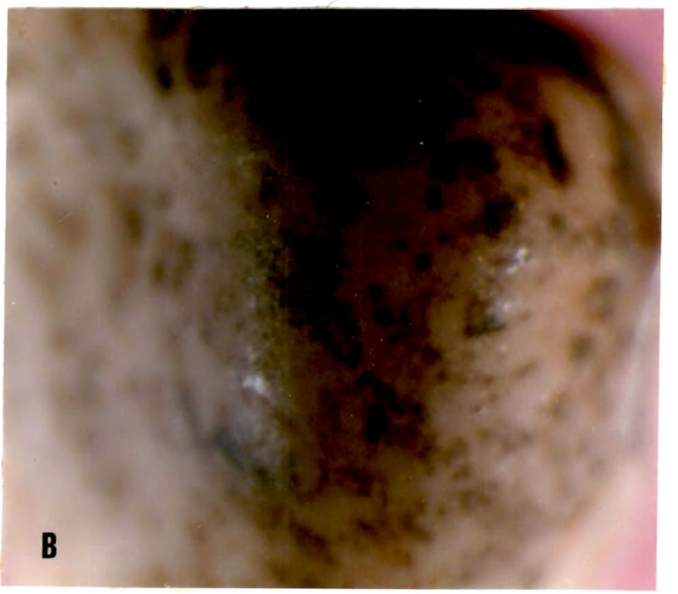
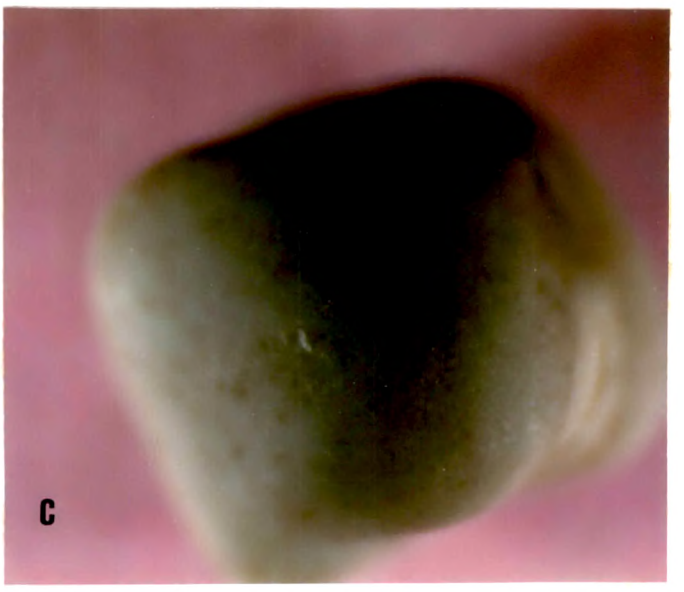


Fig.II.3. Surface texture of different varieties seeds of L.sativus.

A : Dark mottling, LSD-10, (15×)
B : Medium mottling, P-24 and (15×)
C : Nill mottling, RL-15. (15×)


A


B


C

variation existing in ODAP content, only 27% could be attributed to colour and size. Thus the physico-chemical characterstics like colour, size, moisture, fat and trypsin inhibitor activity are of little value in prediction of the seed neurotoxin level of L. sativus. They also screened 16 varieties for the varietal and locational differences in neurotoxin content. Within each variety, the ODAP content of the seed varied from one location to another. For example, the low toxin varieties showed three fold variation while in the medium toxin level varieties it was two fold. The variation within a particular environment was also not consistent. Based on these observations they concluded that a complex interaction of genotypic and environmental factors modifies the toxin content of the seeds. Within each plant itself there is a great deal of tissue to tissue variations in terms of ODAP content. Results presented in Table II.3 indicate that in the vegetative plant the leaves contain the maximum amount of toxin (375 ug) and roots have the lowest values (50 µg). The tender flattened and green stem was also rich in ODAP, however the value remained lower than that of the leaf. Within a seed the embryo axis and cotyledons possess greatest amount of ODAP and the seed coat contains the lowest (Table II.3).

Prakash et al., (1977) recorded neurotoxin variation in different tissues of the plant as a function of aging, in L.sativus. He found maximum neurotoxin in the leaf during the vegetative growth and in the embryo during reproductive stage. However, with aging of the plant the accumulation of toxin in these tissues differed respectively. Recently Kuo et al., (1987) supported the above

findings. They found extremly high concentrations of ODAP (120u mol/g fr.wt) in the pericarp of the seed followed by that in the immature seeds. The leaves contained intermediate level of ODAP as compared to the stem among vegetative parts.

II.3.B Physiological studies during seed germination :

There is a gradual increase in dry matter accumulation in the embryo axis as well as in the cotyledons. After about 48h imbibition, radicle emergence is observed. In the dry seed ODAP level is higher in the axis than that of the cotyledons (Table II.4). During germination and further growth of the seedling, ODAP level falls in the axis to 60% of the initial level whereas an increase is observed in the cotyledons till 72h of germination and than levels off during the 96h period. Earlier, Malathi et al., (1967) have also reported similar findings during seed germination in L. sativus. They reported an increase in ODAP content from 22 umol /g seed. These values are close to our values.

When the seeds are allowed to imbibe water for an initial 48h period and then germinated in presence of phosphate or nitrate ions, ODAP level significantly reduced both in cotyledons& axis a proportional decreases in ODAP (Table II.5 & 6). Nitrate treatment caused reduction only in the cotyledons, but in the embryo axis, there was a gradual accumulation of ODAP with increase in nitrate concentration.

Growth in terms of dry matter accumulation was more with nitrate as compared to that with phosphate ions. The reduced form of

nitrogen, casein hydrolysate (CH) caused more decrease of ODAP both in the axis and cotyledons than that of nitrate nitrogen (Table II.7).

The osmotic stress during germination was given by mannitol and a great degradation of ODAP was noticed along with increase in osmoticum. There was 44% reduction in ODAP in cotyledons and 26% in the embryo axis when the mannitol level was increased upto 0.5 mM. In terms of dry matter accumulation, growth was almost unaltered in the embryo axis with increase in osmoticum, and a negligible increase in dry weight of cotyledons was observed.

An understanding of the mineral nutrition and their biochemical role is of utmost importance for yield improvement of legumes since they have received much less attention as compared to cereals (Arora, 1982). The presence of non-protein amino acids in several Lathyrus and Vicia species has been taken to suggest that although they store nitrogen much more efficiently in the proteins, the non-protein amino acids may neverthless constitute a highly concentrated reserve immediately available to the embryo on germination (Bell and Tirmanna, 1965). For example in the case of Phaseolus aureus, a decrease in non-protein nitrogen was noticed 5 hours after germination with a corresponding increase in protein nitrogen (Srivastava and Kooner, 1972). Our own studies with L. sativus show that there is great degradation of ODAP upon availability of nutrients such as nitrate and phosphate which triggered germination and seedling growth (Table II. 6 & 7).

The role of essential elements during germination and seedling growth was well studied in Arachis hypogaea compared to any other legumes (Grunwald, 1975, 1981). There was a great decrease in antinutritive factors such as phenolic compounds with the availability of phosphate ions (Rao, 1979).

Bhattacharya et al., (1989) pre-treated four varieties of L. sativus seeds with the osmotic agent polyethylene glycol and potassium dihydrogen phosphate before sowing. They found that treating the seeds in this manner improved yield components and hence increased the crop yield, and no attention was paid on ODAP content of the seeds.

In the present study, application of osmotic stress of mannitol inhibited ODAP accumulation without much affecting the growth of the seedling (Table II.8). The functional role of ODAP stored in seeds may be related to the drought resistance nature of L. sativus. It is assumed that the non-protein amino acids serve as a reserve pool of nitrogen during the periods of drought but critial expermental evidences are lacking (Fowden, 1970). Perhaps ODAP would have been duly utilized for the vital metabolic processes that accompany during germination.

II.3.C. Nutritional Studies on Intact Plants in Sand Cultures :

Sand culture experiments in L. sativus var Bh were carried out with an objective to observe the effects of nutrients such as nitrate (oxidised nitrogen), phosphate and casein hydrolysate (reduce nitrogen) under strictly regulated nutrient regime. Since the plants are grown in pure sand that was made free of any organic matter, the net changes on plant growth can be attributed solely to the nutrient supply.

The results of these experiments are presented in Table II.9. After 40 days of sowing, the plants attained on an average of 34 cm height with 20-30 leaves. Upto this stage, nitrate supplied to sand does not enhance the shoot length (Table II.9.A). During later stages, at 80 days and 110 days when plant was in early flowering stage and early pod formation stage respectively, the enhancing effect of nitrate at all 3 levels tested is seen on the growth of shoot length. Analysis of variance of the data revealed that among the three levels of nitrate tested, only the lowest level was significant in which the shoot height increased to 28%, by day 110. The higher levels however did not show any significant increment of height.

Casein hydrolysate at all levels and during all three stages of plant growth, reduced the shoot height, more so during later phase of plant growth on day 110 (Table II.9 B).

In contrast to shoot length, growth in terms of day matter accumulation was stimulated by the application of nitrate and CH (Table II.10 A). With increased level of nitrate, dry matter

gradually increased. At 0.5 mM level, there was 24% increase in dry weight than that of control on day 110. In general nitrate at 0.3 mM level was most benifical in terms of incremant of dry matter. Application of CH is relatively less effective on the dry matter accumulation than nitrate (Table II.10 B). An overall growth stimulation ranging from 34-61% with all concentrations and at all three time intervals was noticed. At the highest level of CH tested at 200 mg/l, the treatment changed the morphology of stem as it became flattered with a ridge at the centre (Fig. II.4).

Pods per plant showed a significant increase by treatments of nitrate and CH highest number of pods were seen in 0.4 mM nitrate and 150 mg number of seeds/pod and hence total number of seeds/plant also showed an increase. However, the application of CH did not show any stimualtion. As a matter of fact it appeared to reduce total number of seeds per plant.

Effects of treatments was seen the most important parameter,yield component which is 100 seed weight (Table II.11 A & B). It was significantly higher in the 0.4 mM level of nitrate. In the treatement of 0.5 mM nitrate, due to no change in number of seeds per plant, yield enhancement was not seen over that of control. Similarly in case of CH,100 mg/l level had a stimulatory effect on yield, however, due to very little change in number of seeds/plant, 100 seed weight at 150 mg/l CH, was comparable to that of control.

The seeds harvested from plants grown under these treatments were analyzed for total protein and ODAP. The total protein content of seeds increased with nitrate supplementation (Table II.12). Among the three levels, 0.3 mM nitrate showed maximum increase to 35.4% protein in seeds. At 0.3 mM nitrate level, maximum reduction of 50% was observed and at 0.4 mM nitrate, the reduction in toxin contained was 45%.

Information regarding influence of nutritional factors on antinutritional compounds is meagre. The only relevant report on L. sativus. is the effect of pre-sowing of seeds with polyethylene glycon and disodium hydrogen phosphate (Bhattacharya et al., 1988). However, they have not studied the effect of osmiticum or phosphate on ODAP level of seeds.

Inode et al., (1989) who studied the effect of phosphate, nitrate and potassium in sand cultures of grain legumes found that both nitrate and potassium increased the protein synthesis on average by 2.5% in seeds, but had inhibited the accumulation of antinutritional toxic factor, phenolic compounds, almost to 50% of the control.

II.3.D. Foliar Spray of Micronutrients on Field Grown Plants :

Data of Table II.13 show the effects of foliar application of cobalt and molybdenum micronutrients on seeds. There was considerable reduction in the ODAP contents in seeds of varieties P-24 and LSD-10, both of which are low neurotoxin containing varieties. These results are in conformation with the observations made by Misra et al, (1981). Who showed that cobalt and molybdenum are effective in causing further reduction of ODAP in seeds of only low ODAP containing varieties. We also observed a significant decrease in the ODAP levels in the high neurotoxin containing varieties Bharuch and LSD-2 as well, although the effect was less pronounced. With cobalt treatment, ODAP level in all varities was between 85 to 96% of control, whereas in the molybdenum treatment, it ranged between 75 to 89%. Thus the foliar spray of the latter element caused a greater reduction in ODAP level.The protein content as well as total nitrogen levels of seeds in control plants both show a negative correlation with the ODAP levels (r=0.0013). Thus Pusa-24, the low neurotoxin variety has the highest protein levels and total nitrogen in terms of percent dry weight of seeds (Table II.14). Whereas cv. Bharuch showing highest ODAP content has the lowest protein content and total nitrogen. With foliar spray of micronutrients, there is a greater accumulation of total nitrogen in all the four varieties ranging between 109 to 116% with cobalt treatment, and 117 to 121% with molybdenum treatment with respect to control. The variety LSD-2 responded best to the cobalt treatment and Bharuch (121% over control)

responded best to the molybdenum treatment.

We also, observed that micronutrients spray induced flowering earlier by 14 days over control, even the number of flowers increased by foliar spray (Fig.II.5). The number of seeds per pod did not vary much among the treatments (Table II.15).

However, the number of pods per plant and the average weight of seed increased with both the micronutrients. This resulted in 75% more seed yield per plant with cobalt treatment and about 20% with more molybdenum.

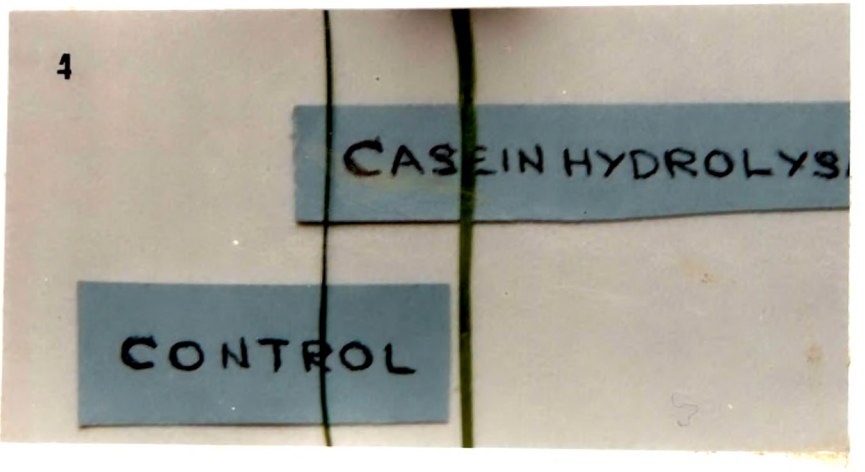
These results indicate that among the two micronutrients, molybdenum is more effective both in inhibiting ODAP accumulation as well as enhancing protein synthesis and nitrogen accumulation in the seeds. Efforts made in the past of foliar spray treatments of micronutrients in reducing the ODAP level in seeds had been partly successful in the sense that it was effective on the low ODAP containing variety P-24, but not in LSD-10, the high ODAP containing strain. Our present report demonstrates inhibitory effect of micronutrient in ODAP accumulation in low and high ODAP containing varieties as well as stimulation of protein synthesis and total nitrogen.

Schon and Blevins (1990) also reported in soybeans an increase in pod number and seeds per plant as a result of foliar application of boron and suggested that it may be due to the promotive effect of boron on pollen tube growth The role of cobalt and molybdenum in nodulation and efficient fixation of biological nitrogen in leguminous crops (Nicholas et al., 1975) and that

of boron on general growth and seed production (Willington, 1957) is suggested. It is possible that upon the availability of these essential micronutrients, diversion of the nitrogen is more towards accumulation into protein rather than non-protein amino acids. The results reported for tomoto (Possingham, 1957) lends support to this conclusion. In this work, as a result of suplementation of molybdenum, there was an increase in protein synthesis and a disappearance of non-protein amino acids such as β-alanine and γ-amino butyric acid. There is very meagre information on the role of cobalt in the nitrogen metabolism in legumes, and since effect of cobalt is similar to molybdenum though to a lower extent as observed in our work, we assume that cobalt may also be an important micro element in metabolism of non-protein amino acids.

Fig.II.4. Effect of CH (200 mg/l) on stem growth.

Fig.II.5. Dense flowering by foliar application of micro-nutrients.


4
CASEIN HYDROLYS
CONTROL

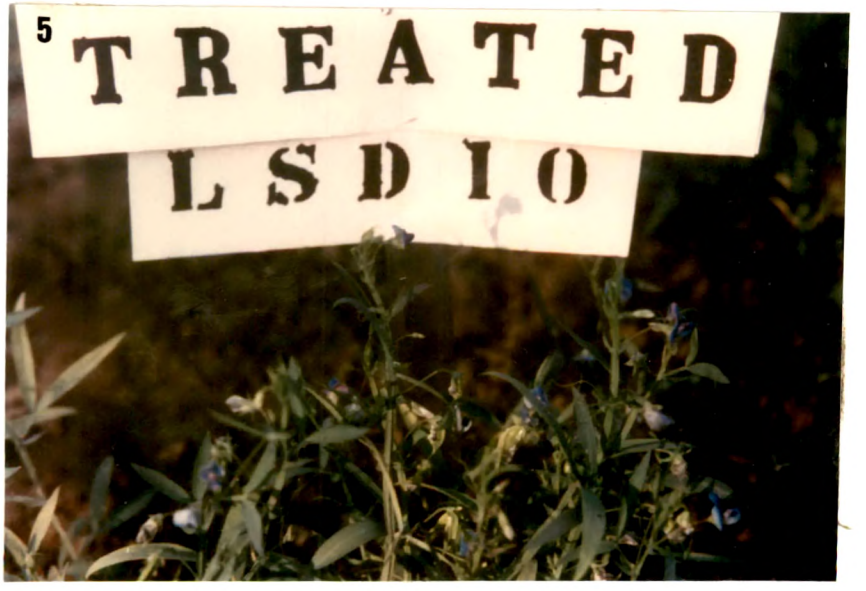

5
TREATED
LSD 10

TABLE : II.3 Distribution of ODAP in different parts of *L. sativus.* cv.Bh

	Parts	ODAP µg/g.dry weight (± S.D.)
A	Intact plant (12 day old)	
	Root	50 ± 2.5
	Hypocotyl	113 ± 6.5
	Stem	340 ± 17.5
	Leaf	375 ± 15.0
B	Dry seed	
	Seed coat	328 ± 34.5
	Embryo axis	1900 ± 125
	Cotyledons	1100 ± 78.5

TABLE II.4 : Changes in growth and ODAP contents during seed germination of *L. sativus.* cv.Bh

Time (h)	Growth parameter	Tissue	
		Cotyledon	Axis
24	FW	113 ± 15.7	18.6 ± 1.15
	DW	33 ± 5.8	4.5 ± 0.1
	ODAP	1094 ± 3.5	1930 ± 7.5
48	FW	136.3 ± 21.7	24.5 ± 4.1
	DW	42.0 ± 10.5	9.1 ± 0.7
	ODAP	1274 ± 8.0	1778.9 ± 8.5
72	FW	184 ± 17.2	71.3 ± 4.75
	DW	42.73 ± 11.2	17.3 ± 1.2
	ODAP	1793 ± 11.2	1594 ± 6.5
96	FW	215 ± 21.2	108.2 ± 9.5
	DW	48.1 ± 14.1	21.3 ± 4.2
	ODAP	1776.3 ± 15.0	1560 ± 7.1
120	FW	225 ± 22.1	138 ± 11.3
	DW	49.5 ± 13.7	26.1 ± 1.15
	ODAP	1781.9 ± 11.7	1535.6 ± 9.1

Note : FW = Fr. Wt. (mg)

DW = Dr. Wt. (mg)

ODAP = ODAP µg/g dr. wt.

TABLE II.5 : Effect of phosphate (KH_2PO_4) on fr. wt., dr. wt. and ODAP levels at seedling stage of L.sativus. Data were collected after 48 hr of incubation.

Concentration PO_4 (mM)	Growth Parameter	Tissue: Cotyledon	Tissue: Axis
Control	FW	184.5 ± 9.5	32.5 ± 0.08
	DW	32.3 ± 4.5	6.5 ± 0.75
	ODAP	1274 ± 9.0	1780 ± 8.5
0.1	FW	201.0 ± 11.2	39.5 ± 4.0
	DW	36.7 ± 6.1	9.5 ± 0.9
	ODAP	1137.0 ± 11.0	1729 ± 18.2
0.3	FW	168.5 ± 8.5	31.4 ± 2.5
	DW	29.1 ± 0.65	6.45 ± 0.7
	ODAP	991 ± 76	1335 ± 14.1
0.4	FW	153.5 ± 11.5	30.5 ± 2.1
	DW	30.1 ± 0.70	7.0 ± 0.65
	ODAP	974 ± 8.3	1301 ± 14.6
0.5	FW	155 ± 10.5	31.2 ± 2.5
	DW	26.5 ± 0.71	6.2 ± 0.4
	ODAP	872 ± 8.0	1276.2 ± 27.0

FW : Fr. wt. (mg) (± S.D)

DW : Dr. wt. (mg) (-do-)

ODAP : µg/g dr. wt. (-do-)

TABLE II.6 : Effect of different concentration of NO_3 (KNO_3) on growth and ODAP levels at seedling stage of *L. sativus*. Data were collected after 48 h of incubation.

Concentration NO_3 (mM)	Growth Parameter	Tissue: Cotyledon	Tissue: Axis
Control	FW	185 ± 6.5	32.5 ± 2.05
	DW	33.1 ± 4.9	6.5 ± 0.8
	ODAP	1275 ± 0.30	1785 ± 10.0
0.1	FW	206 ± 14.0	41.0 ± 4.9
	DW	41.1 ± 9.1	7.8 ± 0.67
	ODAP	1179 ± 16	2009.5 ± 19.6
0.3	FW	211.5 ± 16.5	43.2 ± 4.1
	DW	40.7 ± 9.6	9.1 ± 0.7
	ODAP	1026.7 ± 19.4	2125 ± 19.0
0.4	FW	237.5 ± 27.0	42.5 ± 4.01
	DW	45.7 ± 11.5	8.5 ± 0.65
	ODAP	885.7 ± 8.1	2295.0 ± 17.0
0.5	FW	231.4 ± 25.5	45.5 ± 5.06
	DW	45.5 ± 11.81	9.5 ± 0.95
	ODAP	840.5 ± 14.0	2303.5 ± 17.1

FW : Fr. wt. (mg) (± S.D)

DW : Dr. wt. (mg) -do-

ODAP : µg/g dr. wt. -do-

TABLE II.7 : Effect of different concentrations of Casein hydrolysate (mg/l) on growth and ODAP levels at seedling stage of L. sativus.

Casein hydrolysate (mg/l)	Growth Parameter	Tissue	
		Cotyledon	Axis + S.D
Control	FW	185 ± 9.5	33.1 ± 2.65
	DW	32.5 ± 4.0	7.0 ± 0.5
	ODAP	1270 ± 14.0	1780 ± 19.0
100	FW	226.5 ± 16.8	39.5 ± 3.1
	DW	44.5 ± 9.1	8.2 ± 0.9
	ODAP	1118.2 ± 16.1	1696 ± 21.0
150	FW	245 ± 18.9	38.5 ± 3.7
	DW	49.0 ± 7.1	8.25 ± 0.85
	ODAP	955.3 ± 13.8	1519.7 ± 15.0
200	FW	254.5 ± 22.7	35.7 ± 2.5
	DW	55.5 ± 9.5	7.5 ± 0.65
	ODAP	927.2 ± 9.5	1494.5 ± 16.5

FW : Fr. wt. (mg) (± S.D.)

DW : Dr. wt. (mg) -do-

ODAP : µg/g dr. wt. -do-

TABLE II.8 : Effects of osmotic stress of mannitol on growth parameters and ODAP content of germinated embryo. Seeds were incubated fo 48 h in a medium containing mannitol before alalysis.

Mannitol (mM) Concentration	Growth Parameter	Tissue	
		Cotyledon	Axis
Control	FW	183 ± 9.09	32.2 ± 2.05
	DW	32.3 ± 4.18	6.6 ± 0.9
	ODAP	1275 ± 13.6	1780 ± 11.0
0.1	FW	197 ± 11.5	31.7 ± 4.02
	DW	34.1 ± 4.1	7.3 ± 1.2
	ODAP	1257 ± 11.2	1645 ± 16.0
0.3	FW	195 ± 11.04	33.0 ± 2.8
	DW	32.3 ± 4.8	7.0 ± 2.1
	ODAP	1004.9 ± 10.6	1585 ± 11.7
0.4	FW	198 ± 13.7	31.9 ± 4.2
	DW	34.0 ± 4.1	6.7 ± 0.5
	ODAP	866.9 ± 11.3	1358.1 ± 7.1
0.5	FW	198.5 ± 11.3	32.3 ± 2.85
	DW	37.2 ± 4.8	7.5 ± 0.8
	ODAP	713 ± 6.2	1318.2 ± 7.5

FW : Fr. wt. (mg) (± S.D.)

DW : Dr. wt. (mg) -do-

ODAP : µg/g dr. wt -do-

TABLE II.9 : Effects of nitrate nitrogen and reduced organic nitrogen in form of casein hydrolysate on the growth of stem of L.Sativus. cv.Bh

9.A.

mM Nitrate	Days after sowing 40	80	110	Mean
	stem length (cm)			
0 (=control)	37	58	78	101
0.3	35	61	101	65.6
0.4	32	60	90**	60.6
0.5	35	62	92	63.0
Mean	34.75	60.25	90.2	61.7

SEm	V	T	VXT
	0.78	0.66	1.26

C.D (P=0.05)

9.B.

Casein hydrolysate (mg/l)	Days after sowing 40	80	110	Mean
	stem length (cm)			
0 (=control)	35	57	75	55.6
100	39	46	57	47.3
150	33	46	59	46.0
200	34	49	54	45.6
Mean	35.2	49.2	61.2	48.62

SEm	V	T	VXT
	0.86	0.61	1.29

C.D (P=0.05)

** significant at 1% level (CD=0.01)

TABLE II.10 : Effects of nitrate nitrogen and reduced organic nitrogen in form of casein hydrolysate on the dry weight accumulation of whole plant of L. sativus.

10.A.

mM Nitrate	Days after sowing 40	80	110	Mean Dry wt.
	Dry weight of plants (g)			
0 (=control)	4.37	12.05	15.75	10.72
0.3	4.05	12.95	17.0	11.3
0.4	5.85	15.31	18.12**	13.09
0.5	5.90**	16.44	19.55	14.09
Mean	5.04	14.18	17.7	12.3

SEm	V	T	VXT
	0.25	0.24	0.43

C.D (P-0.05)

10.B.

Casein hydrolysate (mg/l)	Days after sowing 40	80	110	Mean Dry wt.
	Dry weight of plant (g)			
0 (=control)	4.50	12.75	15.9	11.05
100	6.25	15.07	16.5	12.6
150	7.10	16.05	16.95	12.6
200	6.27	17.75**	18.72	14.57
Mean	6.27	15.40	17.01	12.89

SEm	V	T	VXT
	0.85	0.74	1.39

C.D (P=0.05)

** significant at 1% level (C_D=0.01)

TABLE II.11 : Effects of nitrogen and reduced nitrogen in form CH on yield components and yield of L.sativus grown in sand cultures.

11.A.	Yeild components		
mM NO_3	Pods/plant	seeds/pod	100 seed wt. (g)
0 (=control)	30	3	8.10
0.3	35	5	8.96
0.4	41	5.5	9.20**
0.5	36**	3.5	8.19
Mean	35.5	4.25	8.61

SEm	V	T	VXT
	1.07	1.05	1.94

C.D (P-0.05)

11.B. Casein hydrolysate (mg/l)			
0 (=control)	30	3	8.10
100	40	5	9.15
150	46**	5	8.27
Mean	38.6	4.3	8.50

SEm	V	T	VXT
	0.12	0.08	0.18

C.D (P=0.05)

** significant at 1% level (CD=0.01)

TABLE II.12. : Effect of nirtate notrogen on the total protein (%) and ODAP (%) in the seeds of L.sativus. Values expressed as % of seed dr. wt.

mM NO3 (mM)	Total Protein	ODAP
Control	28.05	0.088
0.3	35.4	0.045
0.4	34.85	0.049
0.5	32.35	0.050
Mean	32.66	0.058

SEm	V	T	VXT
	0.04	0.05	0.07

C.D. (p=0.05)

TABLE II.13. : Effect of micronutrient spray on ODAP content (% dry weight) of seeds of different varieties of *L. sativus.*

Variety (V)	% ODAP in seeds under the influence of treatment (T)			
	CONTROL	COBALT	MOLYBDENUM	MEAN
Pusa-24	0.304	0.260	0.231	0.265
LSD-10	0.412	0.398	0.361	0.390
Bharuch	0.651	0.621	0.582	0.618
LSD-2	0.602	0.551	0.522	0.558
Mean	0.492	0.457	0.424	0.457

	V	T	V x T
C.D. (P=0.05)	0.0013	0.0027	0.0046

TABLE II.14. : Effect of micronutrient spray on protein (% dry weight) of seeds and total nitrogen (% dry weight of seed in different varities of L. sativus.

TREATMENT (T)						
Variety (V)	% ODAP in seeds under the influence of treatment (T)					
Variety (V)	CONTROL		COBALT		MOLYBDENUM	
	Protein	Nitrogen	Protein	Nitrogen	Protein	Nitrogen
Pusa-24	29.10	4.64	32.1	5.13	34.0	5.44
LSD-10	26.40	4.22	29.0	4.64	31.8	5.08
Bharuch	25.20	4.03	27.8	4.44	30.7	4.91
LSD-2	26.00	4.16	30.2	4.83	30.5	4.88
Mean	26.00	4.26	29.7	4.69	31.76	5.07

		V	T	V x T
Proteins	: C.D. (P=0.05)	1.4	2.12	3.22
Nitrogen	: C.D. (P=0.05)	0.33	0.22	0.59

TABLE II.15. : Effects micronutrient spray on yield characters of L sativus.

Treatment	Yield Characters	VARIETIES Pusa 24	LSD-10	Bharuch	LSD-2
Control	Pods/plant	20	25	31	26
	Seeds/pod	3.0	4.0	2.5	3.5
	100 seed wt. (g)	7.2	7.6	7.2	7.7
Cobalt	Pods/plant	27	29	39	33
	seeds/pod	3.0	4.0	4.0	4.0
	100 seed wt. (g)	9.2	8.9	8.3	8.3
Moly-bdenum	Pods/plant	23	36	33	27
	Seeds/pod	4.0	4.0	3.0	5.0
	100 seed wt. (g)	8.5	8.4	8.4	8.9

CD (P=0.05)	V	T	V x T
pod/plant	0.92	0.69	1.47
Seed/plant	0.43	0.31	0.80
100 seed wt. (g)	0.16	0.55	0.64