CHAPTER III

IN VITRO APPROACHES TO REDUCE ODAP IN CELL CULTURES OF *LATHYRUS SATIVUS*

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CHAPTER-III

III.1 : INTRODUCTION

III.1.A : TISSUE CULTURE STUDIES OF LATHYRUS SATIVUS

Traditional breeding approaches to produce low neurotoxin containing Lathyrus sativus resulted in development of few low toxin lines, but unfortunately these were not stable (Narashingani, 1990). In this context tissue culture approach such as in vitro selection stands as an ideal alternative to conventional breeding. A pre-requisite for this however, is the availability of a regeneration protocol which yields plants from cultured cells and tissues.

Initial report on tissue culture studies of L. sativus was of caulogenic differentiation in shoot tip callus of L. sativus cv.LSD-1 (Mukhopadhyay and Bhojwani, 1978). It was followed by Gharyal and Maheswari's report (1980) on L. sativus plantlet formation from callus cultures which was again genotype specific since plantlet formation occured only in the cv. LSD-3, the other genotype failed to regenerate into plants. Further, it was observed (Gharyal and Maheswari, 1983) that callus initiation and subsequent organogenetic potential in cultures of shoot meristems of L. sativus was under genetic control. The genetic influence was observable not only at the broader inter-cultivar level but also between the genotypes descended from the same cultivar. However, it was possible to achieve higher levels of morphogenetic response even in recalcitrant genotypes by using physiologically altered explant. On activation of dormant lateral bud meristems

consequent to decapitation of the apical bud it was possible to induce organogenesis in the tissues of all genotypes. However, these workers did not comment on the origin of the shoot buds. Sinha et al., (1983) had claimed to have achieved true de novo differentiation in stem callus cultures of these species. They have highlighted the importance of adenine sulfate which was not used either by Mukhopadhyay and Bhojwani (1978) or by Gharyal and Maheshwari (1983). Even in this case the frequency of regeneration sharply declined after second passage and was completely lost after eighth passage, the decline being concurrent with increase in ploidy levels of culture cells.

Genetic instability of higher plant cells in vitro has now been known for some 30 years (Mitra and Steward, 1961) and its transfer to regenerants has already been reported by Hienz and Mee (1969, 1971). Today there is no doubt about the instability of cells under in vitro conditions (D'Amato, 1978; Constantin, 1981; Dathe, 1985). It was not surprising therefore that experiments were carried out to select plants with new important economic traits from cell cultures for large scale breeding. For this purpose it is irrelevant whether such mutations are already inherent in some cells of the explant, whether they occur spontaneously in vitro or induced by the application of mutagens during in vitro culture of cells (Wershun, 1989). The term somaclone was introduced by Larkin and Scowcroft (1981) as a designation for plants derived from any form of somatic cells. A somaclone is also comparable to a regenerant.

The use of plant cell and tissue cultures for the production of mutant cell lines and ultimately mutant whole plants has been well documented (Bright et al., 1985; Green, 1987; Yeoman, 1989). Various featues of microbial like plant cell offer great advantage and have contributed to the successful selection of wide range of mutants (Carlson, 1979; Widholm, 1983; and Kurchenko, 1985).

Some of the examples in which cellular selection procedures have been used for selection of cell lines with better or improved traits are : Disease resistance (Selvapandiyan, 1988), herbicide resistance (Chaleff., 1986; Breiman et al; 1989), environmental stress tolerance (Ahmed et al, 1986; Mian, 1987 and Bright et al., 1988) and antibiotic resistance (Chill et al., 1980; Subramani, 1991). Variants over producing certain end products of metabolic pathways are also obtained through selection using analogues of the end products (Widholm, 1977; Gonzales et al., 1984). By the turn of last decade (1980-90) more than 200 variant cell lines have been reported, but regrettably there were only a few variants with genetic analysis (Wershun, 1989). Tomes (1982) has reviewed the commercial status of large number of variants produced from tissue culture either with or without applying any selection pressure. It was observed that progress in this direction is quite encouraging and the coming decade will see use of cell culture technique to produce large number of plants with better agronomic traits. At present the most significant limitation for directed selection in vitro is the non-availability of traits that are understood well enough at the biochemical level to warrant extensive research in mutant selection (Vasil, 1990).

However, this limitation fortunately does not exist when one wishes to produce mutants for most of the essential amino acid biosynthesis. This because not only their biosynthetic pathways have been well understood but also most of these pathways seem to operate also in vitro, allowing one to use inhibitor or analogue of respective pathway in question, for applied and basic work (Wakasa and Widholm, 1987; Madison and Thompson, 1988; Greenberg et al., 1988; Wershun, 1989). For example in order to increase tryptophan levels, the analogue 5-methyl tryptophan is used. Level of endogenous tryptophan is controlled in the system by the feedback inhibition of the regulatory enzyme anthranilate synthetase by the end product tryptophan. The analogue 5-methyl tryptophan mimics the action of tryptophan and turns off the synthesis of tryptophan in the system thus depriving the system for this essential amino acid. The mutation in which the enzyme anthranilate synthetase is no more sensitive to the feedback inhibition by the end product, will not only survive the toxic environment of 5-methyl tryptophan but will also overproduce this important essential amino acid due to the lack of feedback inhibition. Such a strategy is quite aptly applied in number of systems to increase the level of many essential amino acids like lysine, tryptophan, methionine, etc. (Bryan, 1980; Giovanelli et al., 1980, 1985; Curtiss and Widholm, 1987). However, when we wish to apply such a strategy in case of L. sativus we are faced with number of odds. Some of them are : (a) The strategy outlined above is good to increase the level of end product. In case of L. sativus we wish to reduce the level of end product amino acid

ODAP. (b) Perhaps a strategy of auxotrophic selection used by Jacobsen and his colleagues (1985) might work in which one reduces the level of ODAP percursors or amino acid which is also primary in nature. But, success of this approach may be achieved provided there is a fundamental knowledge on the precursor of ODAP and which unfortunately so far does not exist.

Due to these limitations we are left with the option of entirely an empirical approach for our work. A laborious screening of somaclones derived in vitro will be undertaken for low neurotoxin level. Recurrent selections at each generation can be applied (Larkin and Scowcroft, 1981 ; Larkin et al ; 1984 ; Evans et al., 1984; Sree Ramulu, 1986 and Wershun, 1989).

To achieve the above defined objective, exeriments were carried out with cell suspension cultures of L. sativus to isolate, without subjecting to selection pressure, variants which differ in their ODAP content from their parental wild type cell cultures. This was achieved by exploiting the variation observed in single cell derived cell lines.

III.2 MATERIALS AND METHODS

III.2.A. Plant Material :

Seeds of cultivars Pusa-24, LSD-10, LSD-1, LSD-2, RL-15 and RL-18 were obtained from Pulse Research Station, Kanpur, India. Besides these, one local cultivar obtained from tribal region of Bharuch District of our State was used which for the sake of convenience in description is referred as 'Bharuch' (Bh). Seeds of these cultivars were germinated aseptically on filter paper in 9 cm dia meter petridishes under 16 h photoperiod. Seedling hypocotyl served as source of explants.

III.2.B.a. Glassware :

Glassware were of either Corning or Borosil brand. Erlenmayer flasks of 100 or 150 ml capacity and 25 x 150 mm test tubes served as culture vessels. Before use, all culture vessels and glassware were cleaned with solution of chromic acid prepared by mixing potassiim dichromate (100 g), sulphuric acid (500 ml) and water (1000 ml). The acid was removed by prolonged rinsing in tap water. Following this, the glassware was washed with detergent 'Teepol' and rinsed throughly with tap water. The glassware were finally rinsed with double distilled water and dried in an oven.

III. 2.B.b. Chemicals :

All chemicals were of high purity analytical grade. They were obtained from BDH, Loba Chemie, E. Merck and Fluka. Auxins, vitamins, cytokinins, antiauxins, amino acids, casein hydrolysate and authentic diaminopropionic acid were purchased from Sigma

Chemicals Company (USA).

III. 2.B.c. Sterilisation of Glassware :

Tubes, flasks, pipettes, millipore unit etc. were plugged with absorbent cotton. These were wrapped to protect them from condensed water during autoclaving. Petridishes were covered with paper. Dissection instruments such as forceps, spatula and blade holders were also autoclaved after wrapping. The sterilization was achieved at 15 psi for 20 min. at 121 °C.

III. 2.B.d. Composition and preparation of culture media :

All the media were prepared with glass distilled water. The composition of the basal media formulated by Murashige and Skoog (1962), Gamborg et al., (1968), Mante and Boll (1975) are given in table III.1. Separate stock soluions were prepared according to the composition of the nutrient medium in glass distilled water. Stocks of inorganic salt solutions were stored at 5 °C and that of vitamins and hormones at -4 °C in a deep-freezer. Supplements to the basal medium which is prepared by mixing the stock solutions, were added prior to final adjustment of the volume. The pH of the medium was adjusted to 5.5 or 5.8 depending upon the formulations using 0.1 NaOH or 0.1 HCL on pH meter (Digichem, 8201 pH meter, India). The medium was gelled with 0.8% w/v agar. After addition of agar, the medium was cooked at 100 °C and then distributed into tubes (25 x 150 mm) in 20ml aliquots. Liquid media were distributed directly in flasks. These were then autoclaved at 15 psi for 20 min. at 121 °C.

Heat labile compounds such as biotin, folic acid, etc. were sterilized by filtration through milipore membranes (0.22 µm pore size) and added to the sterile medium under aseptic conditions.

III. 2.B.e. Aseptic Techniques :

All inoculations and manipulations involving sterile cultures or media were carried out in Laminar Flow Cabinet (Klenzaids, India). Prior to use, the working surface in the Laminar Flow Cabinet was wiped either with 70% ethanol or bactericidal dettol. The UV light in the cabinet was also put on for 15 min. before beginning work to avoid contamination. The dissection instruments were kept immersed in 95% ethanol. During inoculations they were sterilized by flaming after dipping in ethanol.

III. 2.B.f. Surface sterilization and preparation of explant :

Seeds of all the varieties were thoroughly washed first with mild detergent soap solution (Teepol) and cleaned with tap water. Inside the laminar flow hood, surface disinfection of seeds was done with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 5 min. and washed thoroughly with sterile distilled water at least three to four times. Seeds were gently placed on the sterilized solution containig sucrose (2%) and agar (0.8%). About 5 to 7 seeds were inoculated into each flask. The flasks were incubated in a culture room at 25 $\pm$ 2 ^{o}C and 16h photoperiod for seed germination.

III.2.B.g. Preparation and Inoculation of explants :

Nine day old seedlings were used to prepare the explants. Since the studies were meant for induction of de novo differentiation from the nonmeristematic tissues, apical buds and axillary buds were discarded and roots (excluding root tips), hypocotyl, epicotyl, seedling leaves and cotyledons were used as explants for inoculation. Hypocotyl explants from six varieties of L. sativus, namely Pusa-24, LSD-1, LSD-2, RL-15, RL-18 and Bh were incubated on MB5 media to observe the variation in callus formation.

III. 2.C. Initiation and maintenance of callus cultures :

Callus cultures were initiated from hypocotyl explants on MB5 medium supplemented with 1 μM 2,4-D and 0.1 μM Kn as source of growth regulators, 3% (w/v) sucrose as carbon source and gelled with 0.8% (w/v) agar.

Stock cultures of callus were maintained by repeatedly subculturing the healthy fast growing callus masses on fresh medium, every four weeks. These callus tissues served as inoculum for further experiments. The static callus cultures were incubated at 25 ± 2°C with photoperiod of 16h for the duration of experiment, unless otherwise mentioned. The light intensity was 2000 lux supplied by cool daylight flourescent tubes.

III. 2.C.a. Effect of cultural parameters on growth and ODAP content in callus cultures :

Prior to the transfer of tissue on experimental media they were grown on MB5 basal medium for 8-10 days in order to minimise hormonal carryover. Size of the inoculum was 350 ± 28.5 mg fresh tissue (correspondingly dry wt. was 25.5 ± 4 mg). Tissues were incubated at 25 ± 2 °C and at the end of the culture period of 30 days they were analysed for fresh and dry weight increases and ODAP content. Growth measurements were made as a function of increase in fresh and dry weight of callus tissues. The callus was removed from culture on to a dry filter paper and any adhering agar was carefully removed. It was transferred on previously weighed aluminium foil for fresh weight and dry weight of the tissues was determined by drying the weighed mass of fresh tissue to a constant weight at 80 °C in an oven.

III.2.D. Initiation and maintenance of cell suspension cultures :

Cell suspension was obtained by transferring friable callus masses to liquid MB5 medium in 150 ml Erlenmeyer flasks and agitating on gyratory shaker at 120 rpm. Healthy fine suspension was achieved by filtering the initial suspension through a mesh of 250 µm size and only the filterate was further subcultured. At the time of 2 subculture, the suspension was again filtered through 250 µm mesh and 5 ml of the filterate was inoculated into 30 ml of fresh culture medium. The process was repeated 5 to 6 times. The growth of cell suspension was monitored in terms of gain in fresh weight, dry weight and packed cell volume.

About 5 month old cell suspension which was maintained on MB5 medium, was used for growth analysis. 5 ml of the cell suspension on a stationary phase was pipetted into 30 ml of fresh media, and allowed to grow for 18 days. The growth of cell suspension was monitored in terms of gain in fresh weight, dry weight and packed cell volume as mentioned below. For growth measurement, three replicate cultures were harvested at regular time intervals of 3 days, standard deviation was calculated to ascertain statistical significance of the growth data. In the case of cell suspension cultures, the cells were harvested over filter paper circles by suction filtration using a vacuum pump and the fresh weight and dry weights were recorded.

Packed Cell Volume (PCV) : A known volume of suspension was transferred to graduated centrifuge tubes. They were spinned in a centrifuge (Remi, India) for 10min at 5000 x g and the volume of sedimented cells recorded. This PCV is the volume of the cell as a function of the volume of cell suspension culture.

III. 2.E. Influence of various cultural parameters on growth and ODAP content :

Prior to a transfer of suspension culture on experimental media, the cells were grown in MB5 basal medium for one week in order to minimise hormonal carryover. Size of inoculum on experimental media was 5 ml of cell suspension (350 $\pm$ 28 mg fr.wt. or 12.95 $\pm$ 3 mg dr. wt.). The cultures were incubated fo 18 days on a gyratory shaker at 125 rpm at 25 $\pm$ 2 ^{o}C and 16 h photoperiod. They were analysed for fresh weight, dry weight increases and ODAP

accumulation. The effects of organic amino nitrogen compounds on growth and ODAP accumulation was studied in callus and cell suspension cultures. MB5 basal medium was supplemented with organic amino nitrogen, as mentioned below :

Component in the medium	Normal strength (mg/l)	Levels tried (mg/l)			
		X 0.25	X 0.5	X 1.5	X 2
Glycine	20	5	10	30	40
Glutamic acid	7.5	1.875	3.75	11.25	15
Glutamine	60	15	30	90	120
Aspartic acid	7.5	1.875	3.75	11.25	15
Asparagine	10	2.5	5	15	20

Three sets of experiments were carried out, keeping the strengths as of the amino acids as above, supplemented either in combination or alone, uniformly. They are (1) effect of total reduced nitrogen compounds at different levels on growth and ODAP content in callus cultures (2) effect of individual amino acid/amide as sole reduced nitrogen source on growth and ODAP accumulation in suspension cultures (3) effect of individual amino acid/amide at different concentrations on growth and ODAP accumulation, while maintaining the concentrations of other amino acid at normal strength in suspension cultures.

III. 2.F. Plant regeneration by organogenesis from callus :

Callus of variety Bharuch induced on MB5 medium supplemented with 1 μM 2,4-D and 0.1 μM Kn was transferred to MB5 medium supplemented with 0.5-2.5 μM 2,4-D in combination with 0.1-0.5 μM BAP or Kn.

In a subsequent experiment, since it was observed that out of 5 levels tried for each growth regulator, 1.5 μM 2,4-D and 0.3 μM BAP showed best response. This level of each of the growth regulator was combined with all five level of each of other. In this way 22 combinations of growth regulators were tested. The nature of morphogenetic response was evaluated in terms of root and shoot bud formation.

After 25 days when the shoots developed from the buds, they were dissected from the callus and transferred onto 1/2 strength MB5 basal medium containing 0.5 μM IBA for root initiation. Following this rooted shoots were washed free of any adhering media and planted in soil : vermiculite (1:1 v/v) mixture for acclimatization under diffused light conditions in a net-house. Plants were initially covered with a glass beaker to maintain humidity. The acclimatization was gradual by partly opening the beaker. Plants were watered with 1/2 strength Hoagland's nutrient solution every day.

III. 2.G. Immature embryo culture :

Developing pods from garden grown L. sativus cv. Bharuch plants were used as the source of immature embryos. Pods between 40-55

mm in length approximately were harvested at 16 days post-anthesis. They contained developing seeds in the size range of 5 $\pm$ 1 mm.

The pods were surface sterilized using 0.1% mercuric chloride ($HgCl_2$) for 3 minutes. They were rinsed thoroughly at least four times with sterile water and held in a flask containing sterile water till the time of excision of developing seeds. Immature embryos were isolated from seeds by making a slit in the seed coat at the broad end of the seed and applying gentle pressure at the tapering end until the entire embryo emerged out. The embryo axis was removed by making a cut through both cotyledons, distal to the shoot apex region and discarded. The deembryonated cotyledons were placed with adaxial surface down on the media containing MB5 salts (Table III.1.A) and B5 vitamins (Table III.1.B) supplemented with 3% sucrose and 0.8% agar at pH 5.8. Media were supplemented with 2,4-D or NAA (either 1 mg/l, 3mg/l and 5 mg/l of each) in combination with BAP (0.1, 0.3, or 0.5 mg/l). When the proliferating callus showed appearance of embryoids, it was subcultured on medium of similar composition but with 0.1 mg/l BAP and 1 mg/l each of NAA and 2,4-D for further differentiation. After formation of fully differeintiated embryos, they were transferred to half strength MB5 basal medium.

For histological sections, tissues at various stages of cultures were fixed by immersing them in formalin : glacial acetic acid : ethyl alcohol 5: 5 : 90 (v/v/v) for 24 h. Following dehydration in tertiary butyl alcohol, the material was infiltrated and embedded in paraffin wax (of 55°C melting point) in an oven and

paraffin blocks were prepared. Microtome sections (10 μ thickness) were cut, mounted on slides and stained sequentially with 0.1% safranin and 0.1% fast green (Jensen, 1958).

III. 2.H. Cell plating and selection :

One year old batch suspension culture of L. sativus was used for cellular selection for low neurotoxin clone. Cells in the rapidly dividing phase of the batch cultures were used. The cell suspension was filtered successfully through stainless steel mesh of 850, 600, 320, 140 and 120 μm^2 sizes under aseptic conditions respectively. The filtrate of 120 um size of mesh consisted of clumps having 2-4 cells arranged in linear filaments. These served as the plating units. This filterate was centrifuged at 300 x g for 10 min to remove the spent medium. The concentrated cell clumps were suspended in fresh medium at a density of 2×10^4 cells per ml. Two ml of this cell suspension was pipetted into 9 cm diameter petridish. Two ml of 1.2 % agar-based MB5 medium supplemented with 1 μM 2,4-D and 0.1 μM Kn which was maintained in liquid conditions at 40 ^{o}C was poured in petridish. It was mixed with cell suspension by gentle swirling of the plate and allowed to gel. Thus 5×10^4 plating units were seeded in each petridish. The petridishes were sealed with PARAFILM and incubated in the culture room under diffused light of 16h photoperiod at 25 $\pm$ 2 ^{o}C. After 10-14 days of incubation, colonies of 1-2 mm in diameter were recorded. Plating efficiency was calculated using the following formula :

$$\% \text{ Plating Efficiency} = \frac{\text{No. of colonies per petridish}}{\text{No. of plating units seeded}} \times 100$$

When these colonies grew to the size of 3 to 4 mm in diameter they were individually transferred on to 0.8% agar based MB5 medium containing 1 μM, 2,4-D and 0.1 μM Kn. Each cell colony proliferated into calli on this medium. After about 30 days of growth these calli were analysed for ODAP and tested for their morphogenetic potential.

III. 2.H.a. Selection of cell lines containing low neurotoxin, ODAP:

Cell clones were isolated using single cell plating technique of Bergman (1960) and Bhatt & Bhatt (1984), which is mentioned above. Approximately 4×10^4 cells were plated in a 9 cm diameter plate. After 30 days of incubation colonies equal or bigger than 2-3 mm in diameter were counted under a dissecting microscope and plating efficiency was calculated. The clones were numbered randomly and after they attained the size of 2-3 mm, they were subcultured onto fresh medium of similar composition in order to achieve sufficient growth. Following this, a small fraction of each cell clone was utilized for analysis of ODAP and the remaining was allowed to grow further till it was used to initiate cell suspension. The clones analysed were categorised based on their ODAP content into three groups : Low toxin (LT) lines, Medium toxin lines and High toxin lines. For the purpose of this experiment clones from only LT lines were allowed to grow further.

III. 2.H.b. Recurrent selection :

The cell suspension from low toxin (LT) lines were again plated as above and this round of selection was numbered as Round-1. LT lines were again identified from plated cell clones and they were designated as LT-1. This process was repeated three more times and each subsequent round was numbered Round-2, Round-3 and Round-4, with screening being carried out at each round for LT lines. The respective selected clones in each round were designated as LT-1, LT-2, LT-3 and LT-4.

III. 2.H.c. Evaluation of low toxin containing cell lines :

LT-1, LT-2, LT-3, LT-4 cell lines were derived from clonally derived callus on MB5 medium. Each cell line was maintained for atleast 5-6 months (10-12 subcultures) in liquid MB5 medium. Along with the LT cell suspension, a parental suspension from which the selection for variants was routinely maintained by regular subcultures. When necessary, cell plating was carried out from this parental culture and this was referred as non-selection plate. The stability and variation of cell lines were determined by comparision of growth during one single passage. At the end of this passage ODAP content was determined as mentioned in section-II.2.E. For each such comparision 3 replicates of each line were used and the experiment was repeated twice.

III. 2.H.d. Morphogenesis of callus derived from plated cells :

Callus of about 2-3 cms diameter derived from plated cell colonies were transferred on to MB5 medium gelled with 0.8% agar and

supplemented with various combinations of 2,4-D (0.5-2.5 uM) and BAP (0.1-0.5 uM) concentration in each combination. The cultures were incubated at 26 ± 2 °C. Illumination was provided with cool flourescent light (2000 lux).

The morphogenic, green nodular callus was allowed to grow on the same medium for 2-3 weeks. When the shoot buds grew into approximately one cm long shoots, the shoots along with an attached portion of the callus were transferred to MB5 1/2 basal media containing 0.5 µM IBA to initiate roots. These were incubated as above. After root formation, the resulting 2-3 cm tall plants were transferred to soil and vermiculite mixture (1:1: v/v) for acclimatization.

III. 2.H.e. Analysis of ODAP from selected clones and regenerated plants :

ODAP was estimated from leaf, stem and roots of the regenerated clones according to the standard colorimetric procedure (Rao, 1978) as described in Section II.2.E.

III.3. RESULTS AND DISCUSSION

III.3. Initiation and establishment of callus and suspension cultures :

This section describes experiments performed to initiate callus cultures from different parts viz. leaf, hypocotyl, and cotyledons on a completely defined medium. For designing standard medium, effects of various nutritional hormonal parameters on callus growth were studied. Experiments were also conducted to initiate fine suspension from the callus culture and examine their growth dynamics.

III.3.A. Studies with callus cultrues : In the preliminary studies, three different basal media were tested for callus initiation from hypocotyl, leaf and cotyledon explants. Explants were inoculated onto MS (Murashinge and Skoog, 1962), B5 (Gamborg, 1968) and MB5 (Mante and Boll, 1975) media (Table III.1). All these media contained 3% sucrose and were supplemented with 1 μM, 2,4-D and 0.1 μM Kn.

Although callus induction took place on all the three media from the cut surface of explant in the 1-2 weeks of the culture, callus tissue grown on these media markedly differed in percentage of the callus induction, growth and texture. The frequency of callusing and the growth of the tissue, measured as fresh and dry weights was maximum in MB5 medium followed by B5. The growth of callus was poor in MS medium (Table III.2) and was almost one fourth of what was obtained on MB5. The callus was

whitish green and friable on MB5 medium whereas it was yellowish green to brown on other 2 media. Among the three different explants (Table-III.2), frequency and magnitude of growth was greatest with hypocotyl explant, than with leaf and cotyledons. As a result it was preferred for the subsequent experiments. Response of hypocotyl explants on MB5 medium supplemented with varying hormone concentrations is shown in Table III.3. 2,4-D induced callus formation after more than two weeks time in only 15-18% of the explants. When 2,4-D was combined with Kn, the best growth of the callus was obtained at 1 µM 2, 4-D + 0.1 µM Kn which showed 100% frequency of callus induction in only 5 days time. Further the morphology of the callus varied on different media. Callus on 2, 4-D alone in the media led to compact type of callus whereas combination of 2, 4-D + Kn induced friable or semi-friable type of callus (Table - III.3).

Among the six varieties tested for callus formation, genotypic variations were observed in terms of extent of callus formation and its texture (Table-III.4) Hypocotyl of variety 'Bharuch' responded best by giving most profuse callus mass with greatest fresh and dry weights. Texture was also of most desirable type semi-friable and whitish green. Most of the other varieties formed a compact callus with much lower callus mass.

Patterns of Callus growth : The callus growth followed a typical sigmoid growth curve (Fig.III.1). On fresh and dry weight basis, a distinct and prolonged lag phase was observed for initial 12 days. This was followed by a phase of linear growth till day 30.

Fig.III.1. Pattern of Callus Growth.

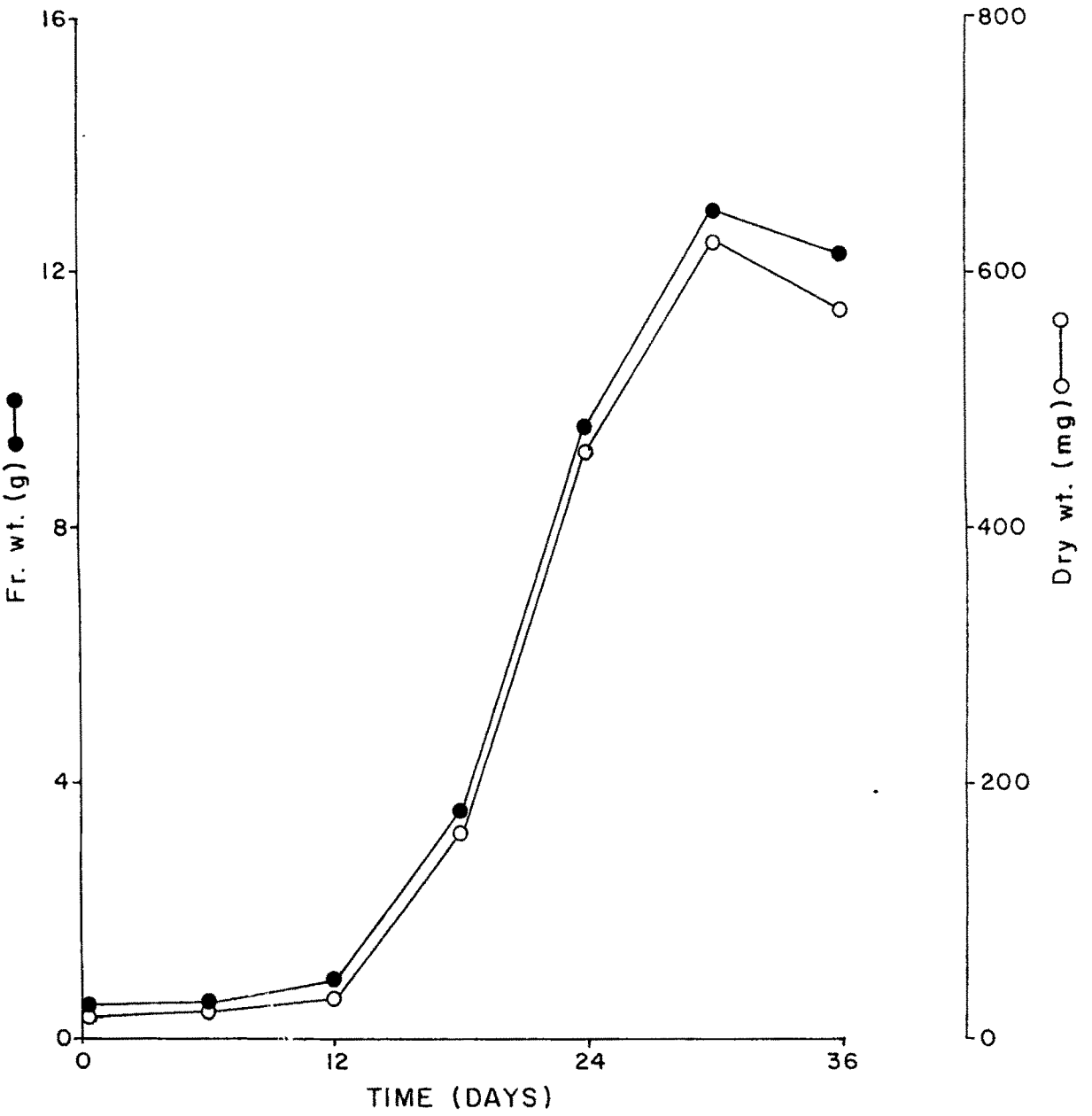
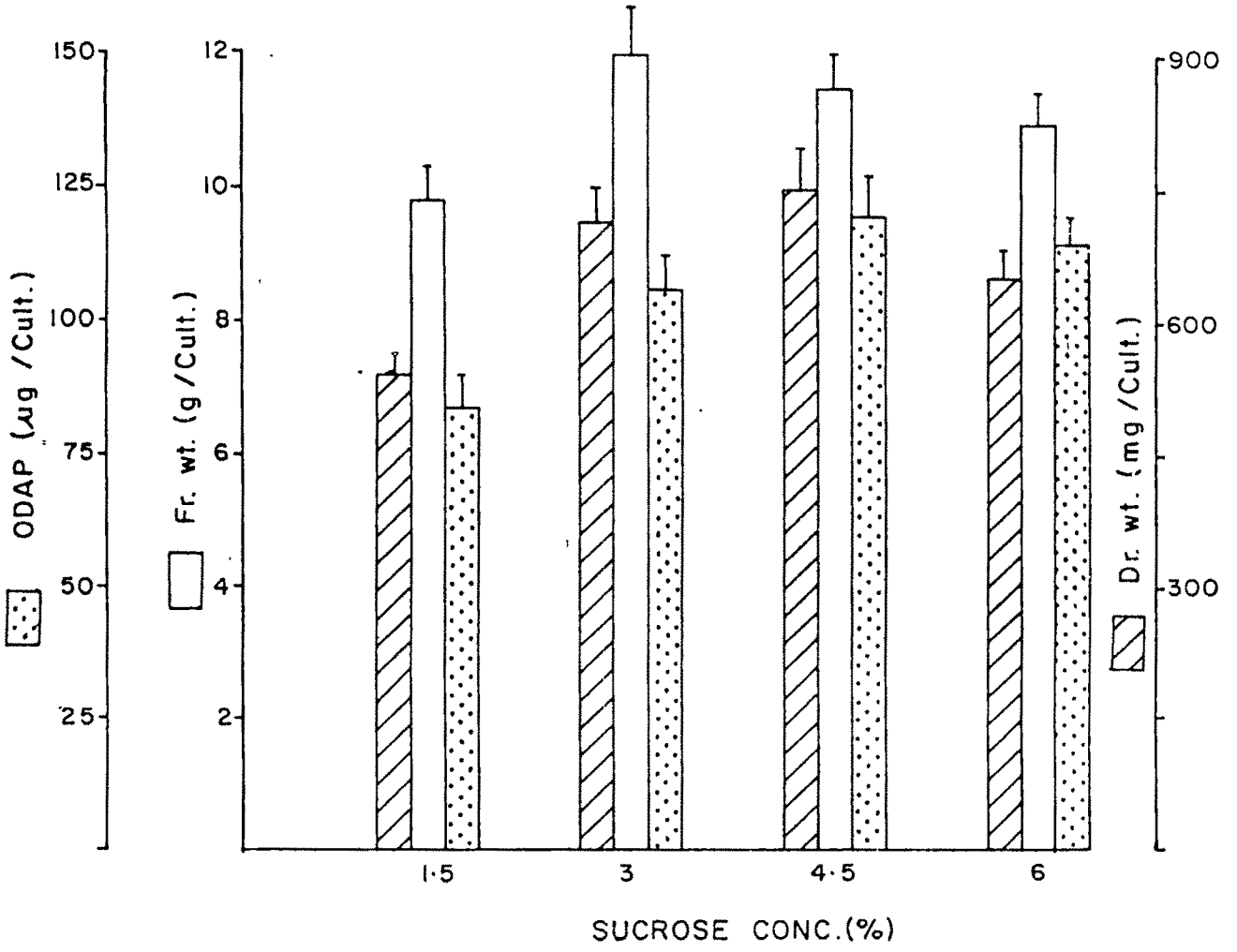


Fig.III.2. Changes in fresh weight, dry weight and ODAP content of callus as influenced by various levels of sucrose in the culture media.



During this period, there was 37 fold increase in fresh weight and 28 fold increase in dry weight respectively in the callus tissue. Thereafter the growth declined and the callus tended to become compact and dark brown.

III.3.A.a. Effect of sucrose levels on growth and ODAP production in callus cultures : To determine the influence of sucrose on growth and ODAP synthesis, the standard growth medium (MB5 +1μM 2,4-D + 0.1 μM Kinetin) was supplemented with various concentration of sucrose ranging frm 1.5 to 6%. Callus did not grow at all in the medium without sucrose. Addition of 1.5% sucrose enhnaced the growth of callus as well as ODAP production considerably but the growth was maximum (Fig.III.2) at 3% sucrose. Further increase in the sucrose level (4.5%) reduced the growth (15% reduction in fresh weight and 12.5% in dry weight compared to 3%), but increased ODAP production. Both growth and ODAP content dropped with further increase in sucrose level beyond 4.5% and callus turned hard and brown.

III.3.A.b. Effect of reduced organic nitrogen compounds on growth and ODAP content in callus cultures of five varieties of Lathyrus sativus : MB5 basal medium contains organic nitrogen in the form of glycine, glutamic acid, glutamine, aspartic acid and asparagine as described in Table III.1.C. The influence of these nitrogen compounds in combination was studied by adding these to the callus culture media at different levels as follows : 0.5, 0.75, 1 (normal) 1.5 and 2 times to the normal strength. The callus tissue did not survive without organic nitrogen source. The results presented in Table III.5 illustrated that there was a

gradual stimulation of growth as more organic nitrogen incorporates into medium upto 1.25 times the normal level. There was a 2-3 fold increase in fresh weight in the varieties Bh, P-24 and LSD-6 when nitrogenous compounds were raised from 0.5 to 1.25 times. Similarly, dry matter accumulation was enhanced by 2.25 to 2.7 folds in all the varieties.

Table III.6 shows changes in ODAP accumulation in callus tissue under the influence of various nitrogenous compounds. ODAP continued to increase parallel to the increase at all levels of reduced nitrogen tested. Although growth in terms of dry matter of the callus was slightly higher at 1.25 times as compared to normal 1 x level, the differences were not very marked. Of the five varieties examined, Bh showed the least increase in ODAP (less than 2 fold) when nitrogenous compounds increased 3 fold. Whereas in other varieties, these values were more than 2 fold, with LSD-6 responding most to ODAP accumulation to a value above 3 fold.

III.3.B. Studies with suspension cultures :

Establishment of suspension culture : The callus from which cell suspension was derived was very friable when grown on standard medium (MB5) and hence suspension culture consisting of free cells and cell aggregates was achieved with greater ease by agitation in the liquid culture medium. Thus a fine, uniform suspension was obtained which consisted of free cells and cell aggregates ranging from 2 to 20 cells/clump. Initially the suspension formed large aggregates, which were removed at the

beginning of each subculture till 5-6 subcultures. Thus eventually the fine consistency of the suspension was achieved.

III.3.B.a. Growth kinetics of cell suspension culture : For all parameters of growth (viz. fresh weight, dry weight and packed cell volume, a lag phase of 9 days was observed after fresh inoculation (Fig. III.3.A.). Thereafter a rapid growth commenced which lasted upto day 15. The growth became almost stationary by day 18 during which the suspension was ready for subculture. During the exponential phase there was a 25 fold increase in fresh weight, 22 fold increase in dry weight and 16 fold increase in PCV.

Thus the maximum growth was obtained on the 15th day which is twice as rapid as that of static cultures. All subsequent experiments on secondary metabolite ODAP, were carried out using suspension culture.

III.3.B.b. Changes in ODAP levels in the batch suspension cultures : When ODAP was analyzed during a batch cycle of suspension cultures, the changes in ODAP followed a parallel trend to that of growth parameters (Fig. III.3.B.). Growth remained stationary upto 18 days, thereafter it declined. The level of ODAP in cells stabilised at the stationary phase.

Fig.III.3.A. Growth kinetics of cell suspension culture.

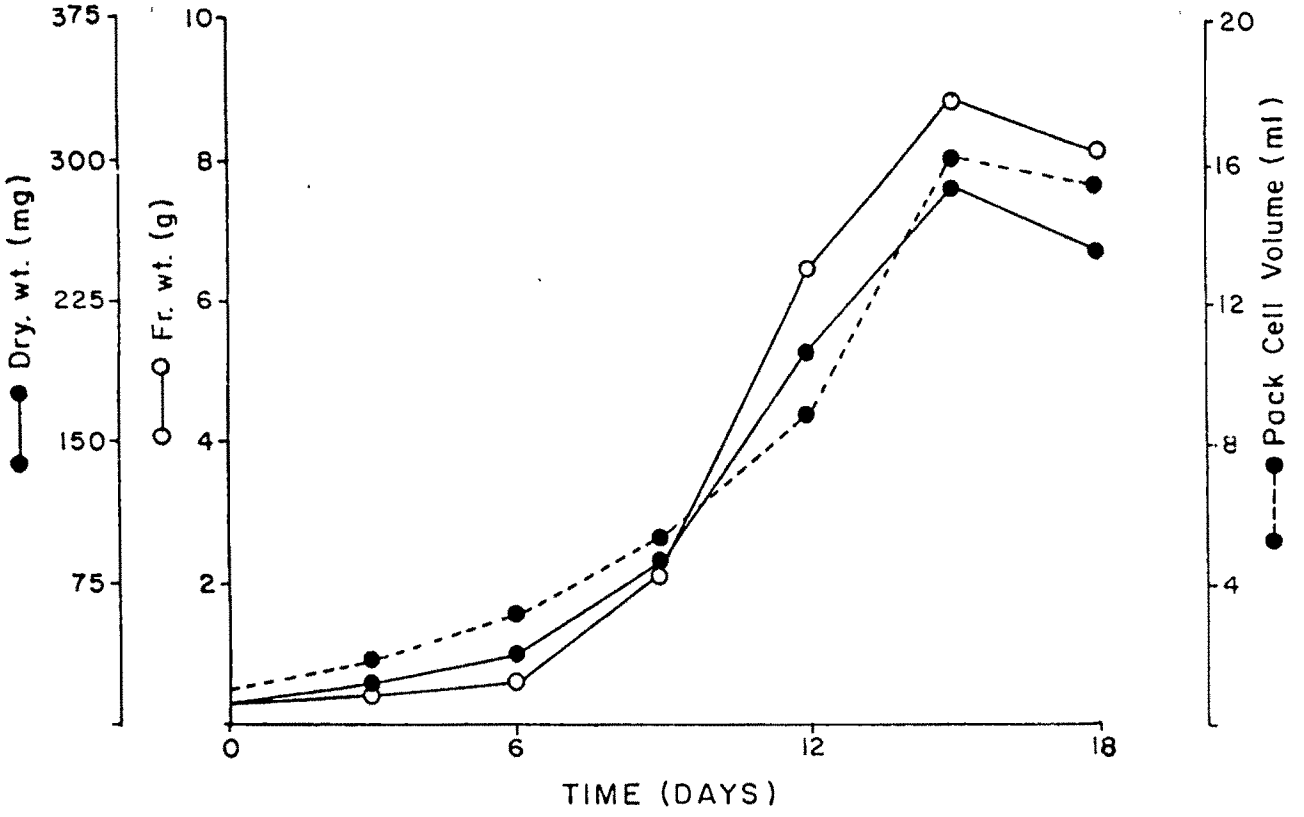
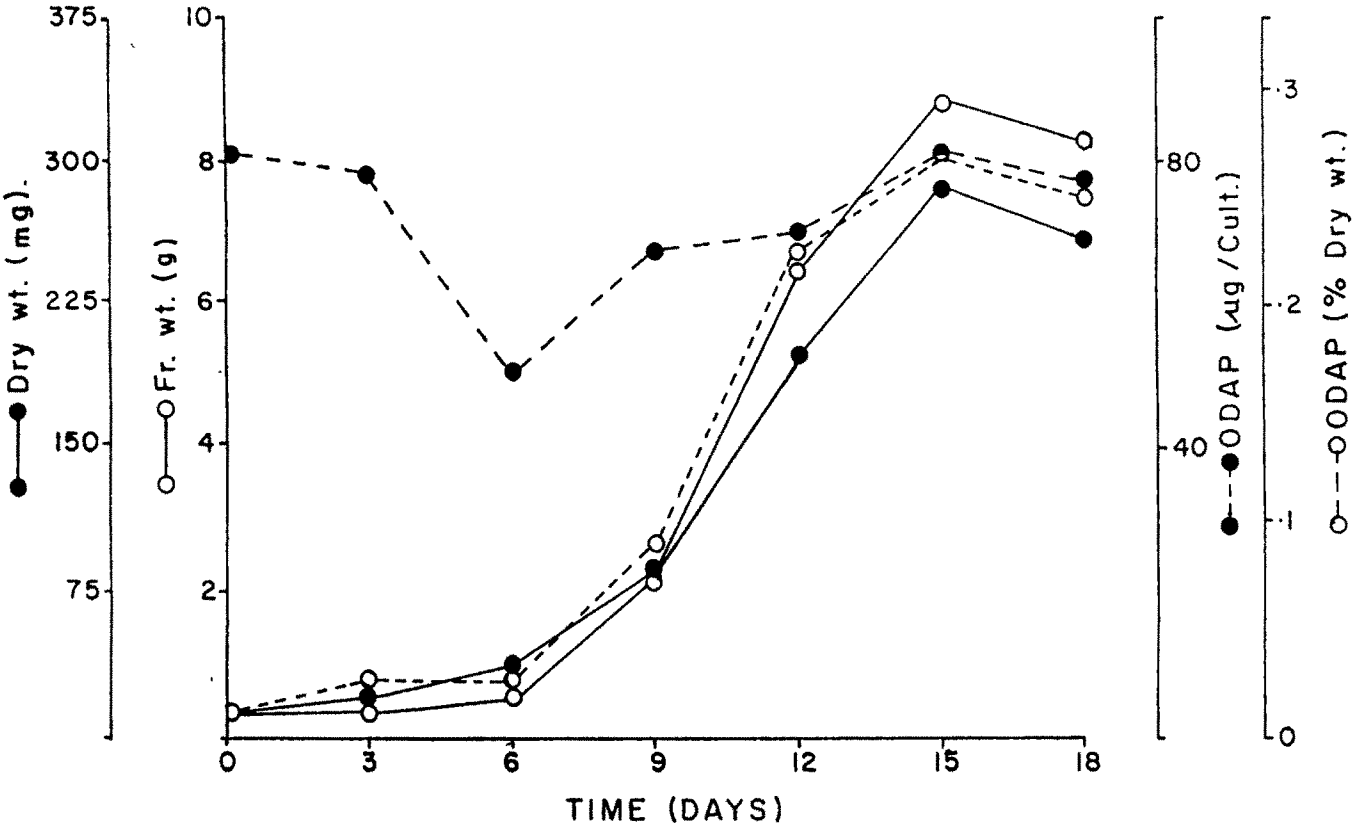


Fig.III.3.B. Growth kinetics and changes in ODAP accumulation in cell suspension culture.



III.3.B.c. Effects of Amino acids/amides on growth and ODAP accumulation in suspension cultures of L. sativus : Experiments were carried out to study the effects of exogenous nitrogen on ODAP production in the batch suspension culture. The amino acids were added individually to the culture medium at the same concentration as that in the standard media. The effect of individual amino acids as compared to combined amino acids as nitrogen source on growth and ODAP accumulation was observed (Table III.7). In general when any one of the amino acids tested were used singly as a sole amino nitrogen source, it had shown inhibition, though the degree of inhibition varied from amino acid to amino acid.

Administration of glycine or glutamic acid singly, inhibited cell growth in terms of fresh wt. by 47 and 55% and dry wt. by 38 and 46% respectively compare to normal medium. Accumulation of ODAP was inhibited over a range of 46 to 63% . Glycine caused the least inhibition, whereas aspartic acid caused maximum inhibition of ODAP. However, incorporation of asparagine in the media had shown very little inhibition of growth, both in terms of fresh weight and dry weight. With respect to ODAP content, the value in the cells grown in control medium resembled more closely to those of cells grown on media with only asparagine or arginine.

III.3.B.d. Effect of individual amino acids/amides at different concentration on growth and ODAP accumulation in suspension cultures of L. sativus : Based on the above results individual amino acids/amide were tried at different concentrations in the medium, in such a way that the level of the remaining amino acids

was maintained at their normal strength.

In general, the cell growth inhibition was reduced by amino acid in combination when compared with any one of the amino acid added singly. Thus in Figures III.4.A-4.E. values of amino acids represented are two levels lower and two levels higher than the medium normal level. In all cases, the normal level is denoted using an asterik(*).

As indicated in Fig. III.4.A. and 4.B. incorporation of glycine and asparatic acid at all the different levels tested had little effect either on growth or ODAP content. Reduction of glycine and asparatic acid at 0.25 times supressed cell growth only to 11.7 and 12.2% respectively by fresh wt. and 6.3 and 8.7% by dry wt. However, ODAP content showed a 13.5 to 14% decrease at 0.25 times concentration both in glycine and aspartic acid. Moreover at higher concentration of 2 fold there was no noteworthy deviation of growth or ODAP content with both the amino acids.

The effect of glutamic acid and glutamine both on growth of cells and ODAP levels was quite dramatic (Fig. III.4.C. and 4.D.). As compared to the control value at 7.5 mg/1 glutamic acid, both at lower and higher level reduction of growth and ODAP were registered, compared to the normal. There was a 60% growth inhibition in terms of dry weight of cells when the concentration of glutamic acid was reduced to 0.25 times. At higher concentration too, the growth inhibition was 24.6% by dry wt. The same trend was also observed with ODAP content (Fig. III.4.C.). There was 46% decrease in ODAP level when the amino

acid level was reduced to 0.25 times and 29% inhibition of toxin synthesis when glutamic acid level rose to 2.0 times. Maximum growth and ODAP content were noticed at normal concentration of glutamic acid at 7.5 mg/l.

Glutamine did not show the same degree of supression of growth like glutamic acid at lower levels of amino acid (Fig. III.4.D.). But it reached a plateau at concentrations higher than normal. When the glutamine concentration in the medium was reduced to one quarter of the normal strength, reduction in fresh weight was 43.5% and that in dry weight was 36% of that in normal. Changes in ODAP level showed a parallel trend with fresh and dry weights. On the other hand at lower concentration of glutamine, along-with reduction in growth it had a cascading effect on ODAP level, reducing it by 39%.

Of the amino acids/amides mentioned above, asparagine exhibited a distinctly different effect on the neurotoxin ODAP accumulation. Incorporation of asparagine in the medium at successively higher concentrations showed a linear increase in ODAP content of the cells in culture (Fig. III.4.E.). An increase of 24% ODAP level was observed for media with 2.5 mg/l to 5 mg/l asparagine, 26% for 5-10 mg/l and 9% for 10-15 mg/l levels. On the contrary, growth was not affected markedly (mean increase of fresh wt. by 27% and 18.5% by dry wt.) showing a slight increase upto the normal level and negligible inhibition at higher concentrations. Fig. III.5 reveals a linear relationship of ODAP with asparagine at molar concentrations showing maximum accumulation of ODAP at

Fig.III.4.A. Effect of different levels of aspartic acid on growth and ODAP accumulation in cell suspension culture.

Fig.III.4.B. Effect of different levels of glycine on growth and ODAP accumulation in cell suspension culture.

Fig.: III.4.A.

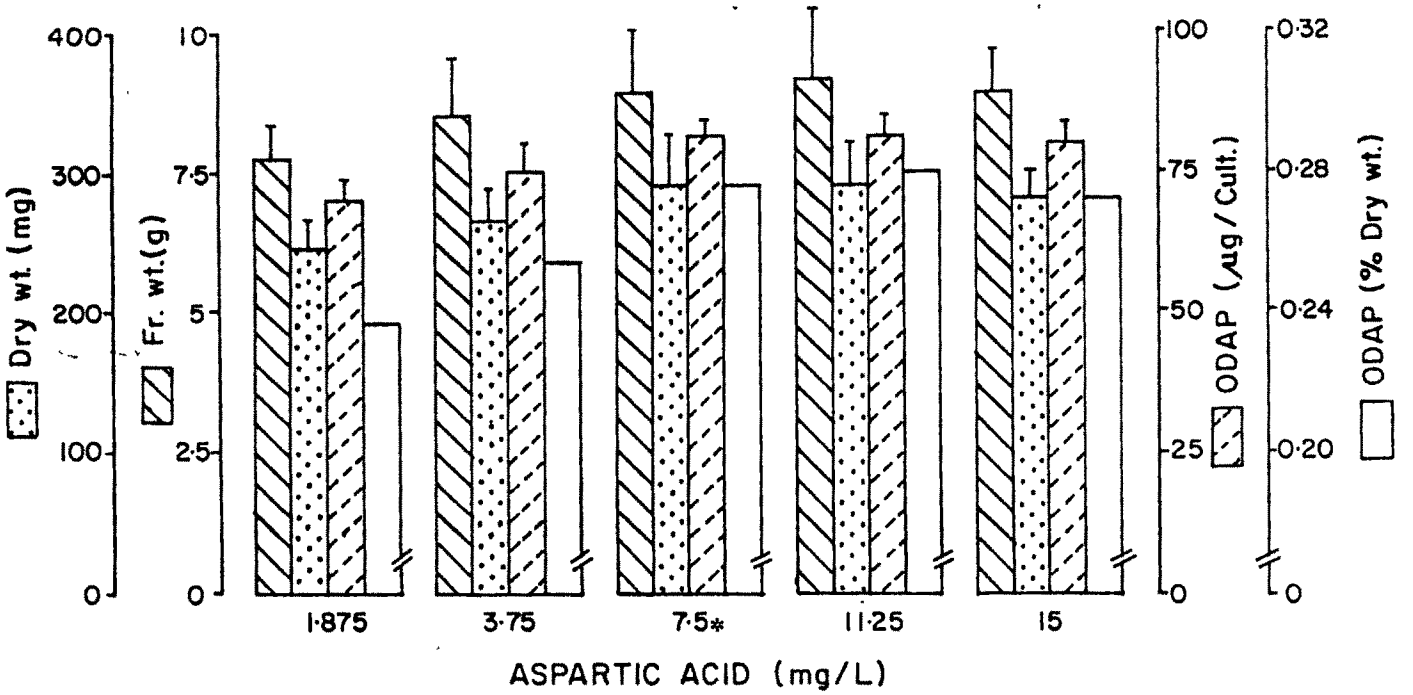


Fig.: III.4.B.

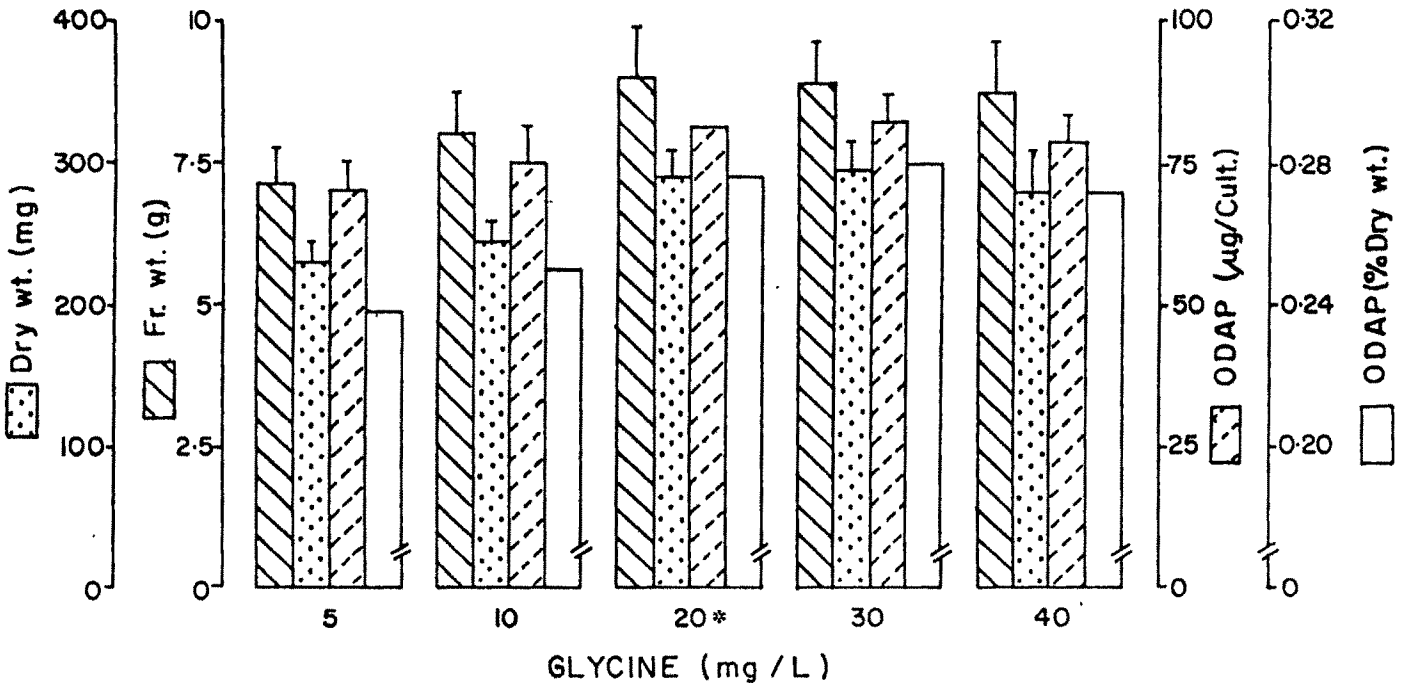


Fig.III.4.C. Effect of different levels of glutamine on growth and ODAP accumulation in cell suspension culture.

Fig.III.4.D. Effect of different levels of glutamic acid on growth and ODAP accumulation in cell suspension culture.

Fig.: III.4.C.

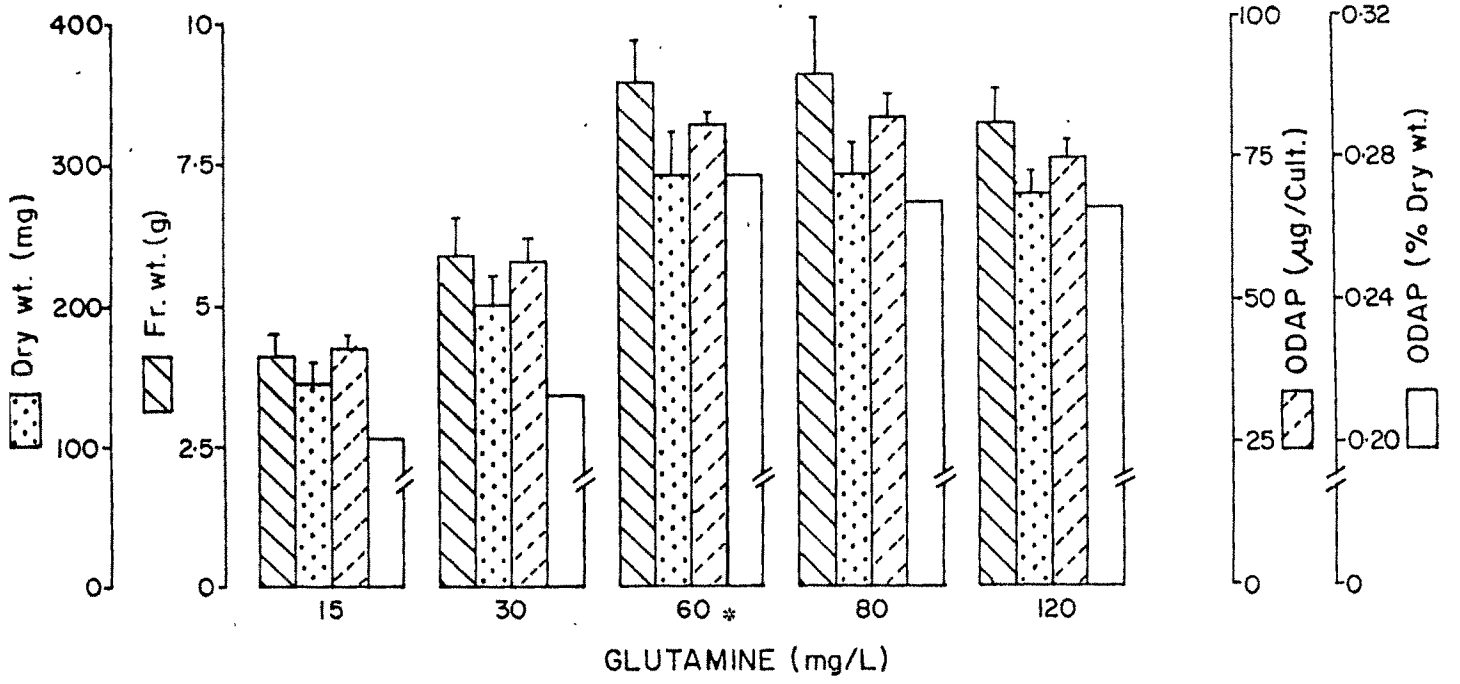


Fig.: III.4.D.

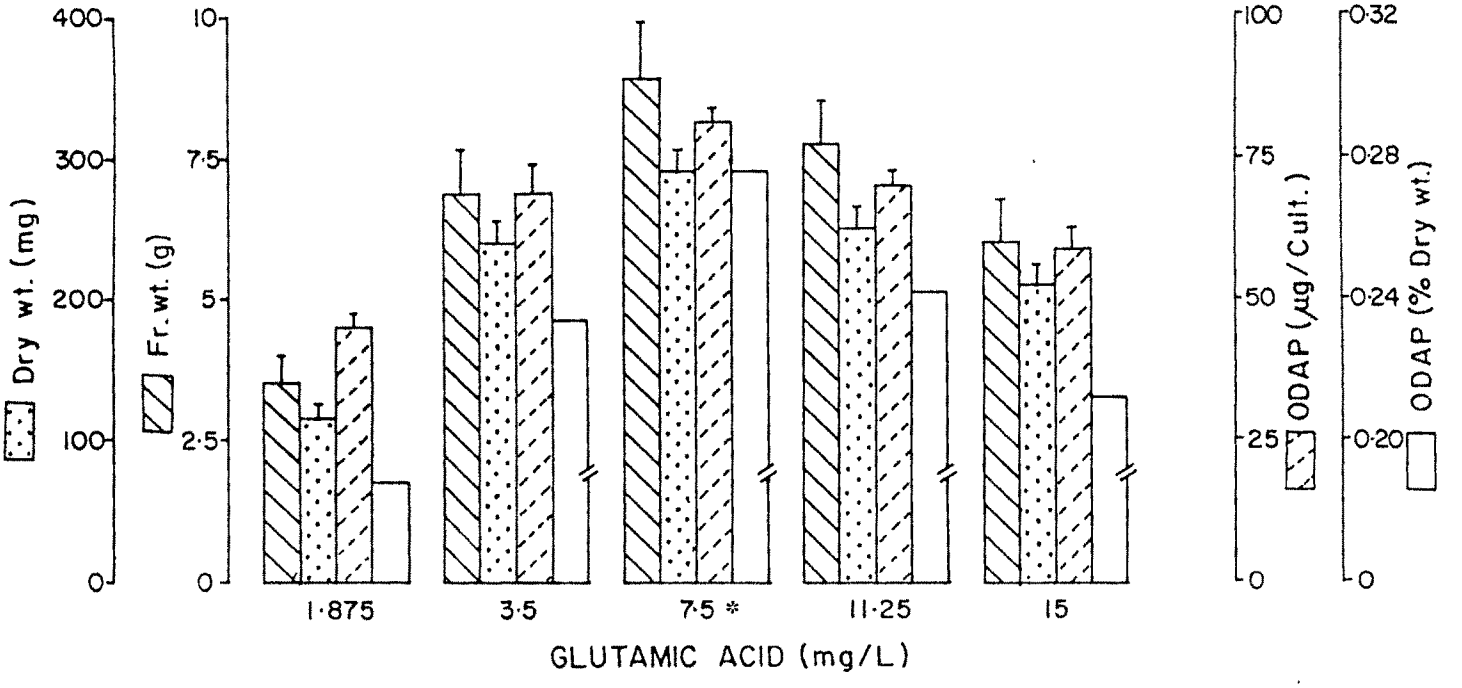


Fig.III.4.E. Effect of different levels of asparagine on growth and ODAP accumulation in cell suspension culture.

Fig.III.5. Linearity between asparagine at different molar concentrations added to culture media and ODAP levels of cultured cells.

Fig.: III.4.E.

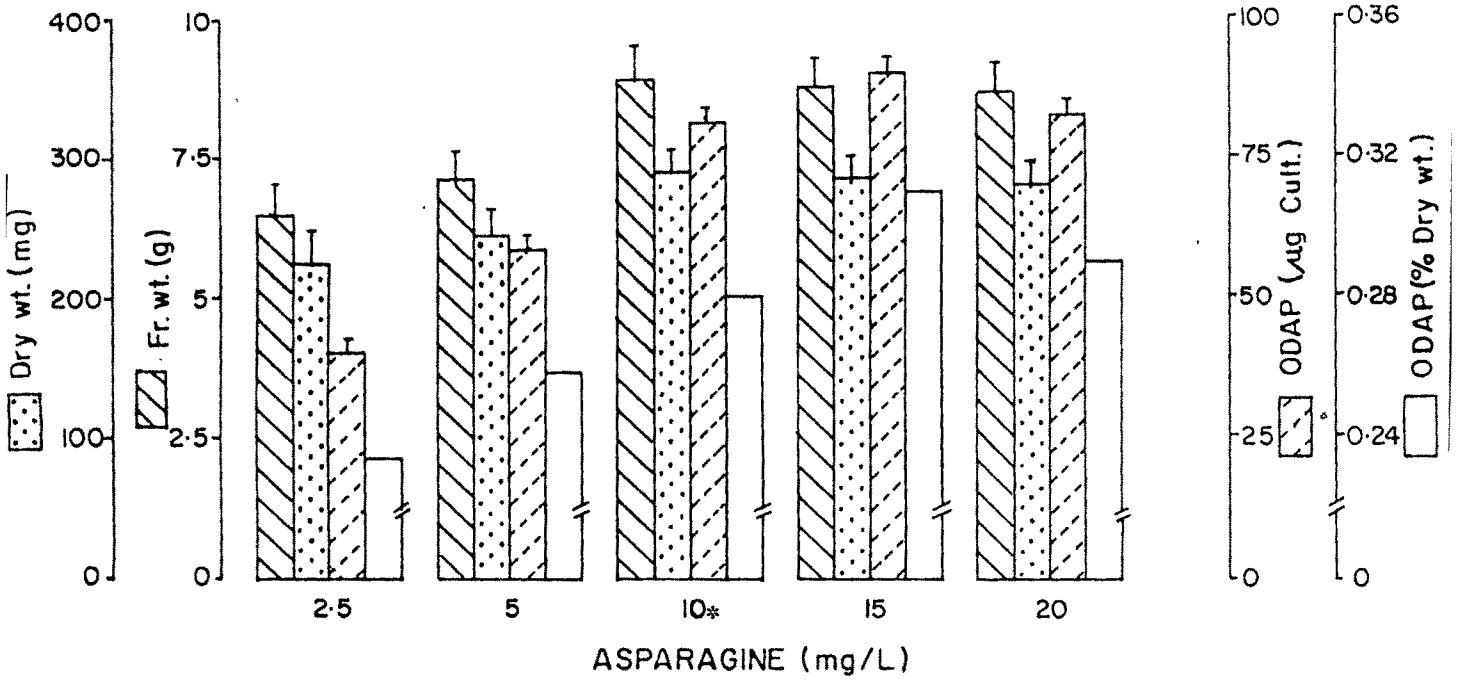
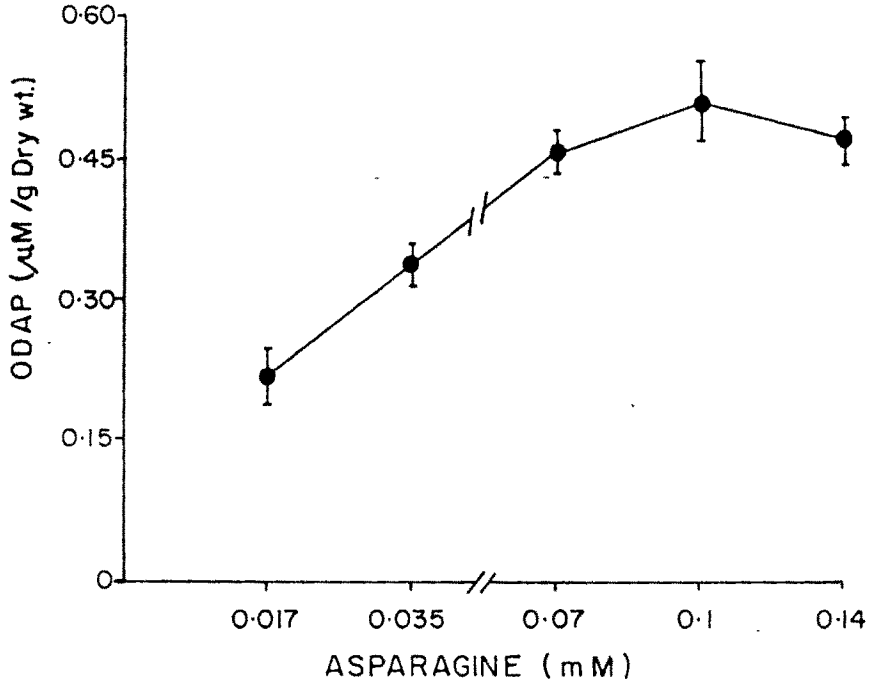


Fig.: III.5



0.10 mM of asparagine and thereafter it becomes stable.

This seemed to indicate that asparagine behaves like immediate precursor of diamino propoinic acid, since it was utilised by the cell culture more effectively for the production of ODAP than the rest of the amino acids/amides.

III.3.C. Plant regeneration by organogenesis from callus cultures : Callus derived from hypocotyl from variety Bh was transferred after about 3 months on MB5 media for regeneration. Different combinations of growth regulators tested and nature of response obtained are summarised in Table III.8.

Callus transferred on majority of media combinations showed tendency to form either roots (Fig. III.6.A.) or nodular undifferentiated callus which failed to differentiate further (Table III.8.A). Shoot buds were formed (Fig.III.6.B) in only two combinations. Subsequent shoot development is shown in Fig. III.6.C. Highest response (38% calli with formation of shoot buds) was observed in the medium with 1.5 μM 2,4-D and 0.3 μM BAP. To some extent, shoot buds were also formed on medium with 2.0 μM NAA and 0.5 μM BAP (16% calli showing shoot buds). Roots developed occasionally from shoot-bud bearing callus. It took about 18 days for shoot-buds to appear after the callus was transferred to the experimental medium.

In an effort of further fine tuning, optimal concentrations of BAP were tested in combination with 4 levels of the 2, 4-D and vice versa. However, as shown in Table III.8.B. only the combination of optimal levels of BAP and 2,4-D proved to be the

best for shoot bud formation. After 25 days, small shoots bearing leaves developed in culture were separated out from callus when transferred on 1/2 strength MB5 basal medium containing 0.5 μM IBA, rapid growth of root-shoot axis was observed. (Fig. III 6.D.) These plants were transferred to a sterilized mixture of vermiculite and sand (1:1, v/v) in pots. Total 8 plants were acclimatized (Fig.III.6.E.). Out of these, 3 plants grew well and attained a height of 25 cm. Under natural conditions in a wirehouse, they started flowering (Fig.III 6.F.). Unfortunately, due to natural calamity, plants did not survive till fruit and seed set, hence no yield data or R1 seeds could be collected for further analysis.

III.3.D. Plant regeneration by somatic embryogenesis from cultured immature embryos : For this study, cotyledons detached from immature embryos were used. The best response was obtained when cotyledons of the size 4 ± 1 mm were used. Of the three different concentrations of 2, 4-D tried in culture media,only 3 mg/l gave the fast growing callus.The appearance of embryogenic callus on this medium was rough, opaque and compact and it was light green in colour (Fig. III.7.A.).Globular shaped somatic embryos were detected on the surface of the callus (Fig. III.7.B.) after 21 days in culture.

To obtain a similar response using NAA in place of 2,4-D, a higher concentration was required(5 mg/l). The appearance of callus on this medium was smooth-shiny, translucent and it was fleshy-white in colour (Fig.III.7.C). On the surface of the

callus, white, protruding somatic embryos were observed after 18 days in culture (Fig.III.7.E and 7.E.). As seen from Table III.9., the response obtained with NAA was 23% as compared to 2,4-D which showed only 14% response in terms of frequency of embryogenic cotyledons. Moreover, the number of embryoids formed on cotyledons cultured on medium with NAA was apparently much higher than those on medium with 2,4-D as is observed, although no quantitative data was collected.

Further differentiation of embryoids did not take place on any of the above media. Hence these proliferating calli bearing embryoids were transferred on a medium containing reduced concentrations of 2,4-D/NAA (1 mg/l each) in combination with 0.1 mg/l BAP. After 16 days of culture on these media, further growth of embryoids till formation of globular or heart shaped embryoids and embryoids with fully differentiated cotyledons was observed (Figs.III.7.F). Germination of these embryoids into polarized structures of shoot and root occurred after their transfer on half strength basal MB5 medium (Fig.III.9.G.). Plantlets with first pair of leaves appeared after about 30 days in culture on basal medium (Fig. III.7.H.). Completely developed plantlets were transferred to soil : vermiculite mixture (1:1, v/v) in a glasshouse, where they grew up to 3-4 leaf stage (Fig.III.7.I).

To ascertain the embryogenic nature of the regenerants, morphological observations were substantiated by histological studies. A large number of somatic embryos at different stages of development were detected. Figs.III.8.A to 8.K. show gradual

Fig.III.6. Organogenesis from hypocotyl derived callus.

Fig.III.6.A. Callus showing roots.

Fig.III.6.B. Callus showing shoot buds on MB5 media supplemented with 2,4-D(1.5uM) + BAP(0.3uM).


6 A

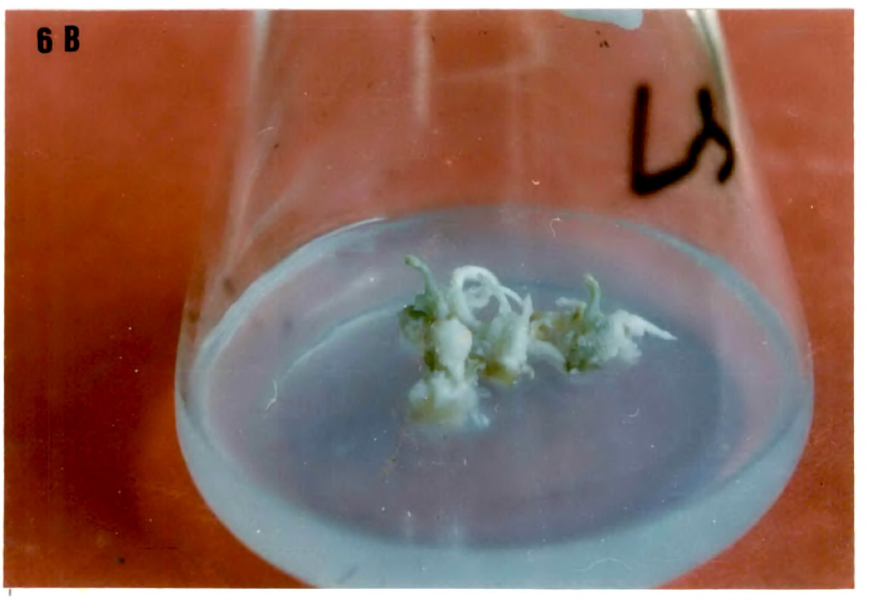

6 B

Fig.III.6.C. Subsequent development of shoot buds on MB5 + 2,4-D(1.5uM) + BAP(0.3uM). After 21 days.

A single shoot bud under higher magnication (40 X).

6 C

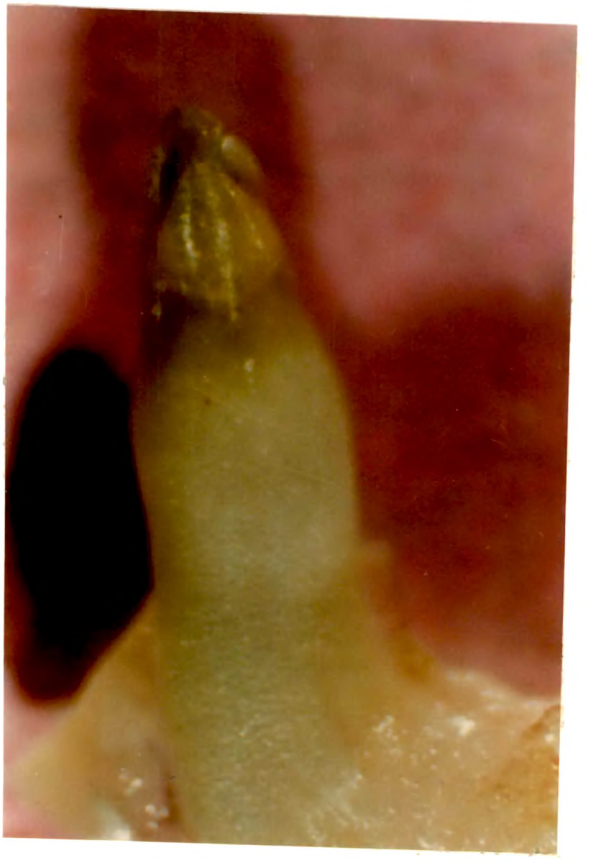


Fig.III.6.D. Fully developed plantlet with roots on 1/2 MB5 + 0.5uM IBA.

Fig.III.6.E. Acclimatized plants in pots containing soil : vermiculite (1 : 1).

Fig.III.6.F. Regenerated plant transferred to pot; at flowering stage.

6 D

E

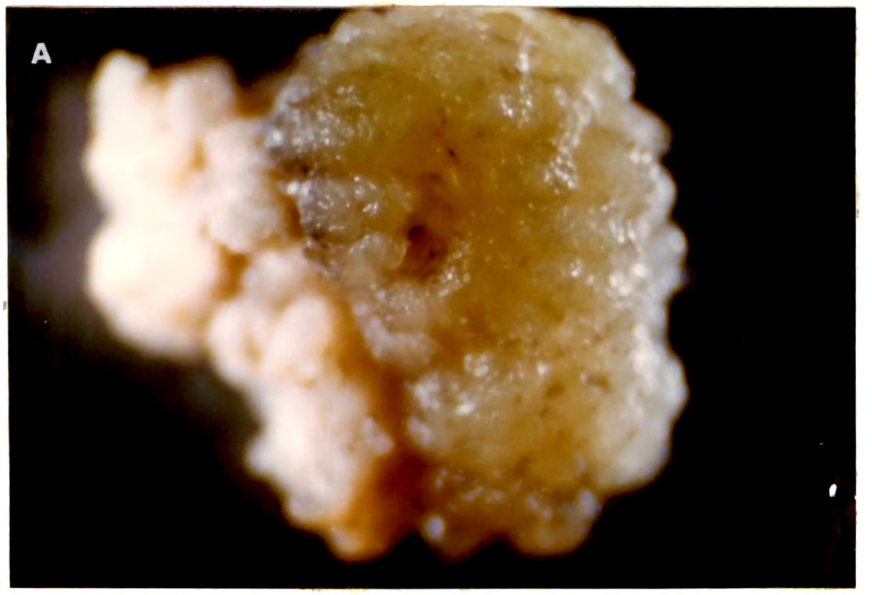
F

Fig.III.7.A. Compact, rough & light embryogenic green callus developed from cotyledons on media supplemented with 2,4 -D (3mg/l). (15 x)

Fig.III.7.B. Globular embryo on the surface of the callus. (25 x)

Fig.III.7.C. Fully differentiated embryoid into cotyledons on the surface of the calli, after 21 days. (25 x)

7



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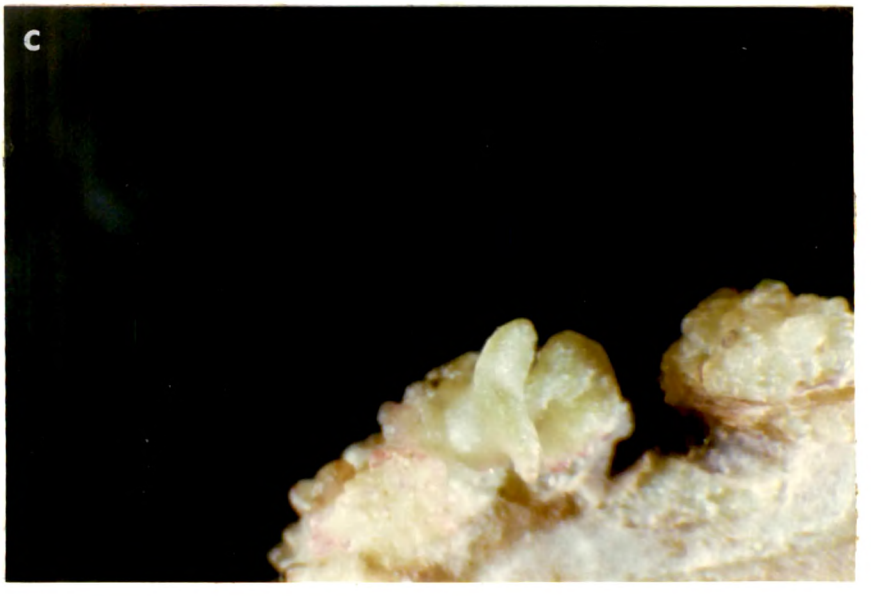
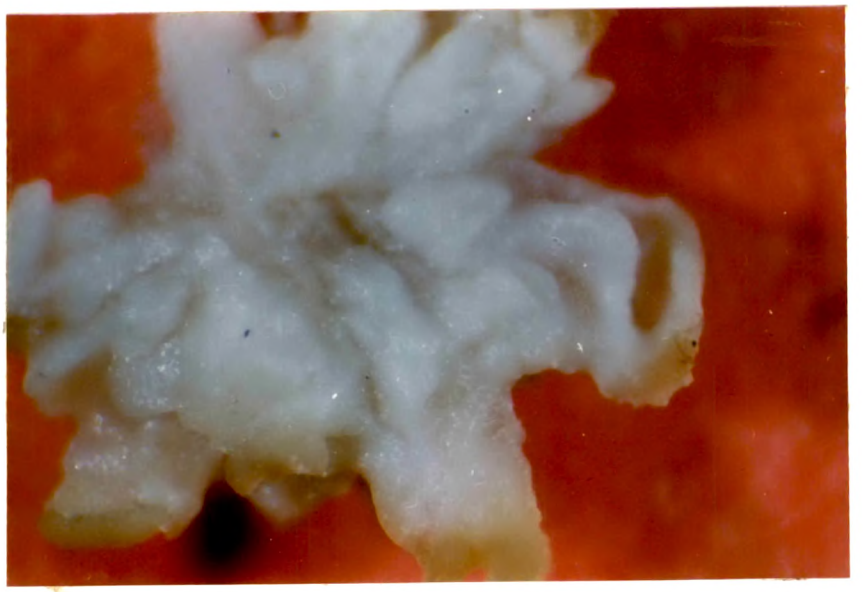


Fig.III.7.D. Smooth, shining, embryogenic callus developed from cotyledons on media supplemented with NAA (5 mg/l). (20 x)

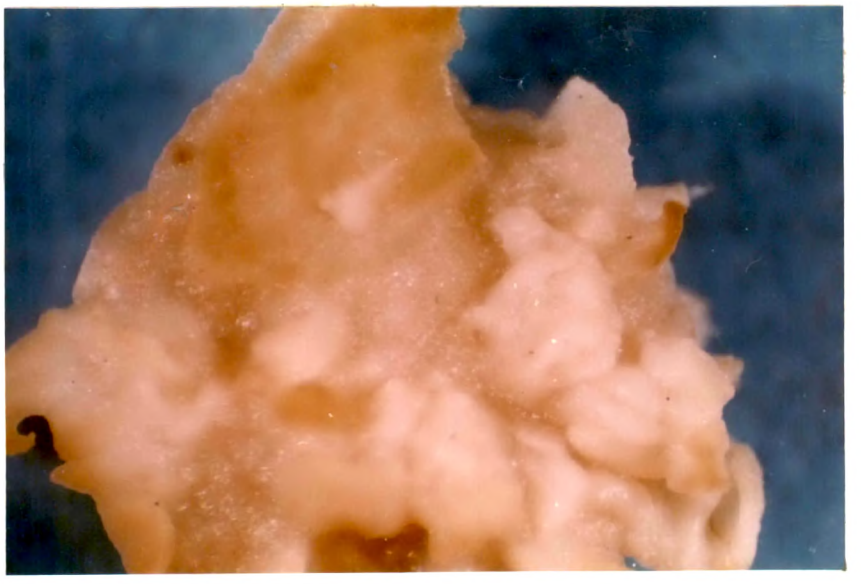
Fig.III.7.E. Subsequent development of embryoids, protruding & on the surface of calli on the above media. (20 x)

Fig.III.7.F. Embryogenic calli with fully differentiated cotyledons on media supplemented with 2, 4-D (1mg/l) + BAP (0.1mg/l). (25 x)

7D



7E



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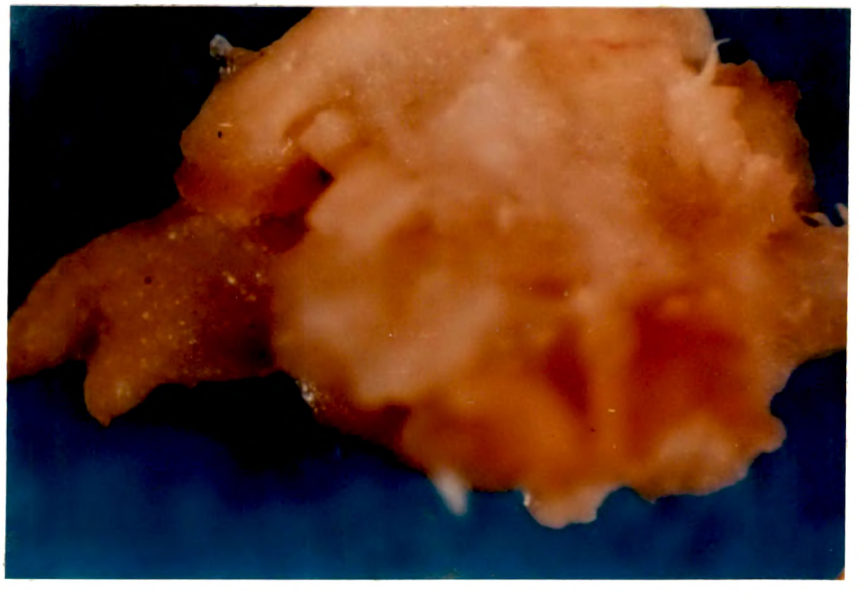


Fig.III.7.G. Germinated embryoids on 1/2 strength basal medium.

Fig.III.7.H. Further development of shoot on the above media.

Fig.III.7.I. Completely developed plants transferred to pots containing soil : vermiculite mixture.

7G

7H

7I

Fig- 8

Fig.III.8. Histological photographs(Longitudinal sections)

Fig.III.8.A. Presence of meristamatic zone on the surface of calli (15 X).

Fig.III.8.B. Section passing through series of somatic embryos (10 X).

Fig.III.8.C. Developing embryo (40 X).

Fig.III.8.D. Somatic embryo with 'suspensor' like tissue (10 X).

Fig.III.8.E. Globular embryos (10 X).

Fig.III.8.F. Spindle shaped embryos (10 X).

Fig 8

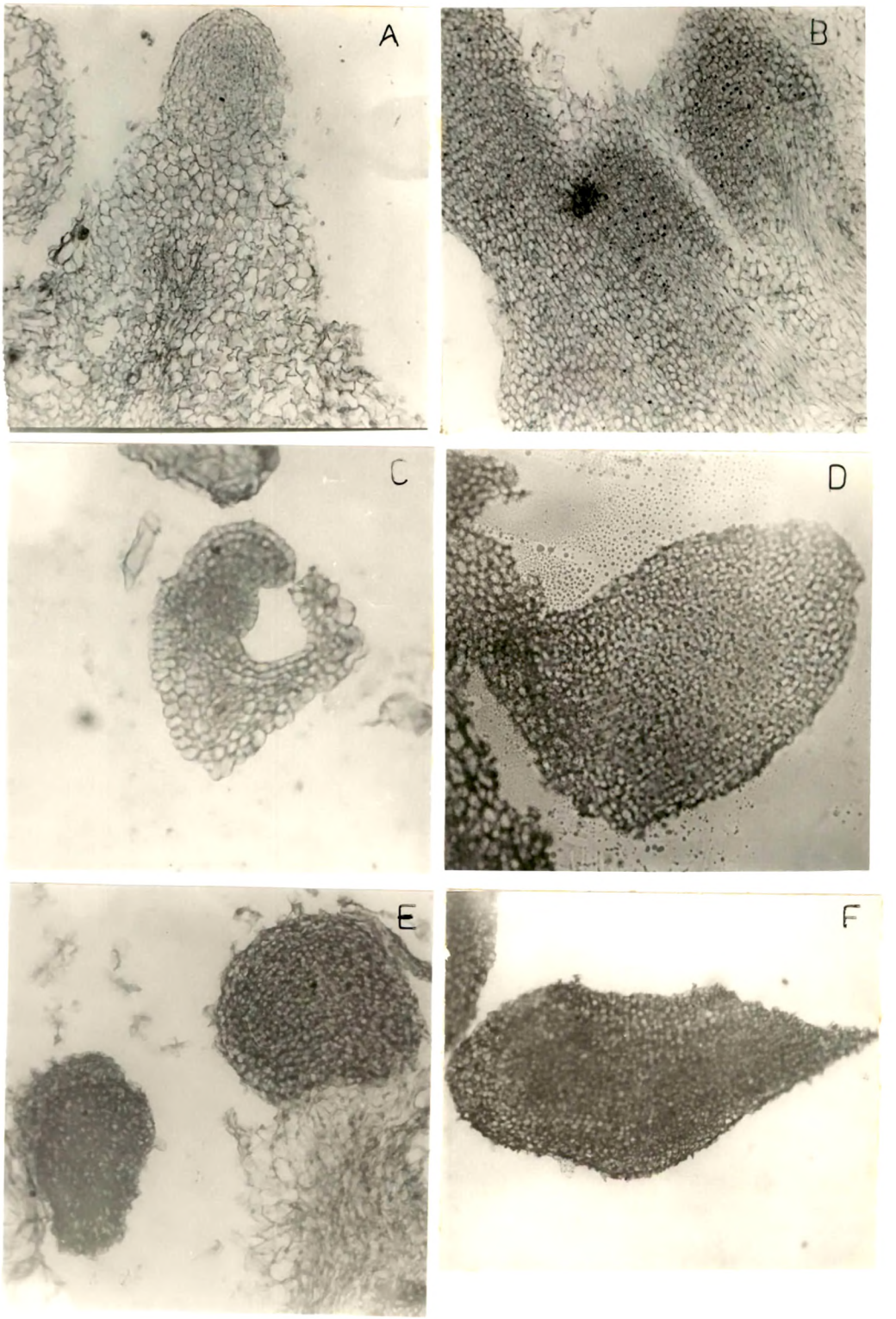


Fig-8

Fig.III.8. contd.....

Fig.III.8.G. Densely stained cells with saffranin and fast green (40 X).

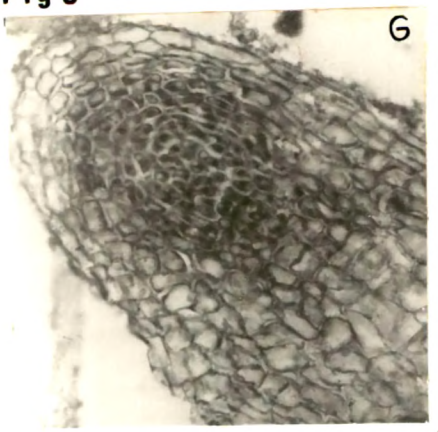
Fig.III.8.H. 't' shaped or torpedo shaped; bipolar nature (5 X).

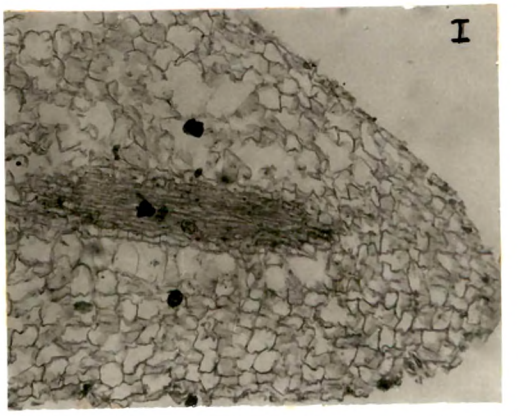
Fig.III.8.I. Developing primary vasculature at late torpedo stage (25 X).

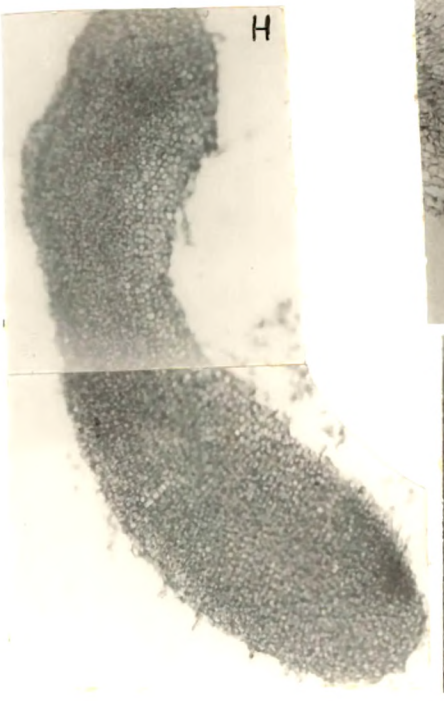
Fig.III.8.J. Primary vascular traces passing through the cotyledons (15 X).

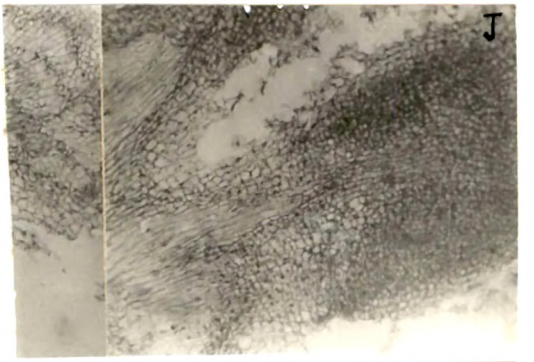
Fig.III.8.K. Tranverse section of cotyledonary part showing primary vascular bundles (25 X).

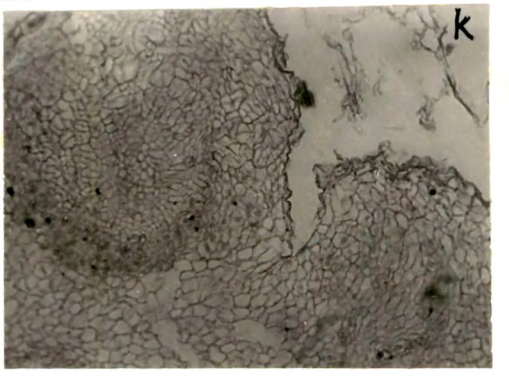
Fig 8











stages of development from small proembryoids containing large number of meristematic cells to globular, torpedo or 'T' shaped or fully differentiated cotyledonary stage embryoids.

III.3.E Recurrent selection to isolate low neurotoxin containing cell lines using single cell cloning technique :

Growth characteristics of cell suspension cultures of L. sativus : The defined medium in suspension culture denotes MB5 medium supplemented with 1μM 2,4-D,0.1μM Kn and 3% sucrose. The pattern of cell growth in suspension and ODAP accumulation were recorded from day 3 to 18. The course of cell growth in suspension displayed an initial lag phase of 9 days followed by a brisk period of growth till day 15th (Fig.III.3.B.). The growth was almost stationary till day 18th, thereafter it declined. During exponential phase the cells in suspension culture evinced 23.2 fold increase in fresh weight and 22.5 fold increase in dry weight. On percent dry weight basis ODAP level dropped during initial lag phase, increased during exponential and stationary phases and thereafter it almost remained constant.

III.E.a. Cell plating and plating efficiency (PE) of cell suspension culture of L. sativus : Plating of small cell aggregates on agar based defined MB5 medium resulted in poor plating efficiency (21%). Increasing the cell density did not improve the plating efficiency (Table III.10.). Highest plating efficiency (72%) was achieved in a medium consisting of a mixture of equal parts of conditioned medium and MB5 medium. (Fig. III.9.A). Autoclaving the conditioned medium at 15 psi for 15 minutes reduced its

growth promotary activity to 46% PE.

After 5-6 weeks when the colonies grew to 2-4 mm in diameter they were numbered randomly and they were transferred to fresh MB5 medium of similar composition. In this medium colonies grew vigorously into larger calli.

TABLE III.10. Plating efficiency (PE) in various media used for Cell plating in L. sativus :

Media used for plating	% plating efficiency
Defined MB5 medium	21
MB5 medium with 1μM 2,4-D and 0.1μM Kn + Autoclaved conditioned medium	46.26
MB5 medium containing 1 m, 2,4-D + 0.1μM Kn + Filter sterilized conditioned medium * (1:1 V/V)	72

* Conditioned medium: Spent MB5 medium of 12 day old suspension culture.

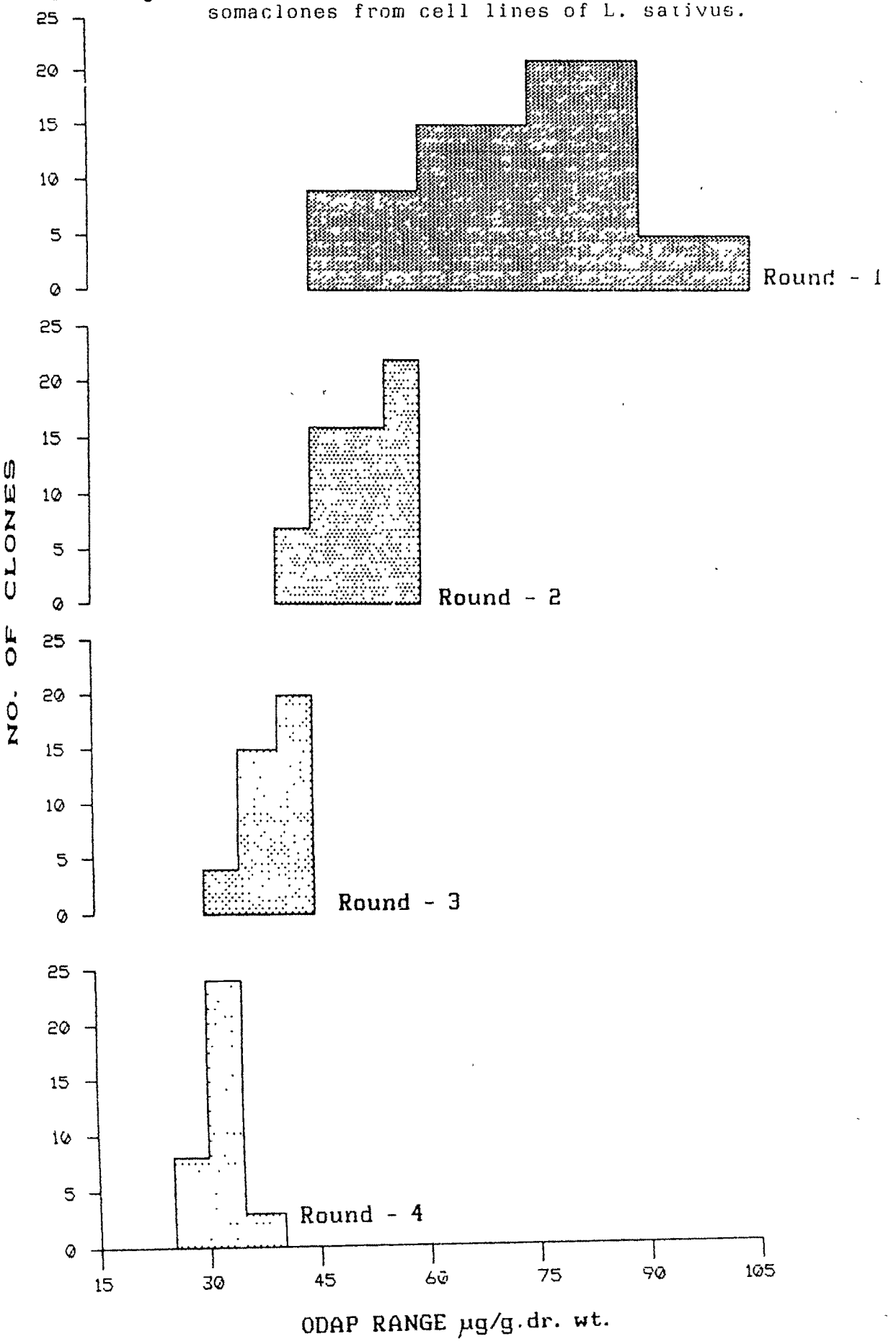
TABLE III.11. Changes in % PE and ODAP content at four rounds of selection :

Round	Plating Efficiency (%)	Mean ODAP value µg/g dr wt
I	72.23	46 ± 12
II	68.67	40 ± 14
III	68.50	30 ± 13
IV	68.32	25 ± 5

The above Table (III.11.) shows that all the four rounds of selection experiment carried out there was no significant change in the plating efficiency, although the cells had gone through 2-3 cycles of subculture in each round of selection. However, as is evident from the Fig.III.9.B there was a gradual decline in the ODAP content upon each round of selection.

III.3.E.b. Selection and characterization for variation in toxin content among single cell derived clones : In the initial round of screening the plating efficiency was 72%. Based on the ODAP content, four categories of cell clones were observed. Majority of the clones showed the same range of ODAP as that of the parental cell suspension when it was plated. This was the medium-toxin range of 76-90 ODAP µg/g dr. wt. However there were a small number of clones which had shown reduced amount of toxin

Fig.III.9.B. Recurrent selection for low ODAP containing somaclones from cell lines of L. sativus.



or LT lines which had ODAP in the range of (46-60 μg/dr. wt.). These clones were used for the subsequent recurrent selection for low toxin containing cell lines as described below. There were also some clones which were showing high toxin levels HT-lines with ODAP levels above 90 μg/g dr.wt. But the calli of these clones were rough and compact in nature and it was difficult to derive a cell suspension when they were transferred to liquid medium. Hence these clones were not used for the present purpose of the experiment.

III.3.E.c. Suspension culture : Suspension cultures were induced from low toxin containing (LT) cell lines from first round of screening (viz: LT-1). Cell lines derived from LT-1 gave friable suspension after first passage and the colour of cell suspension was pale green. The morphological nature of the cell suspension did not show any further changes in subsequent LT-2, LT-3 and LT-4 cycles.

III.3.E.d. Plant regeneration and estimation of ODAP contents in the regenerants : Plants were regenerated in calli derived from LT-4 cell lines. The plantlets were regenerated on agar-based MB5 medium containing 2,4-D and BAP of various concentrations and combinations to examine their morphogenetic potential. Shoot induction was observed only in combination of 1.5μM 2,4-D and 0.3μM BAP at frequency of 25%. In contrast the frequency of regeneration of hypocotyl derived calli was 38% under similar culture conditions. Rooting of regenerated shoots was achieved when these shoots were transferred to 1/2 strength

MB5 basal supplemented with 0.5μM IBA (Fig. III.9.C.). Regenerated plants when grown in greenhouse resembled each other as well as those derived from seeds in their morphology (Fig. III.9.D.). For further bio-chemical study regenerated plantlets were analysed for ODAP content.

III.3.E.e. Comparison of ODAP content in the vegetative parts of 30-day old plants of L. sativus : (A) seed germinated (B) Regenerants of hypocolyl derived calli (C) Regenerants of single cell derived calli of LT-4. Results are mean of 3 plants in each group.

TABLE III.12. ODAP content in the regenerants and seed derived plants

Plant	Root	Leaf ODAP (μg/g dr.wt)	Stem
A	51 ± 1.7	342 ± 16.5	378 ± 16.1
B	39.5 ± 3.9	329 ± 16.7	386 ± 14.7
C	23 ± 3.15	124 ± 10.8	135 ± 11.2

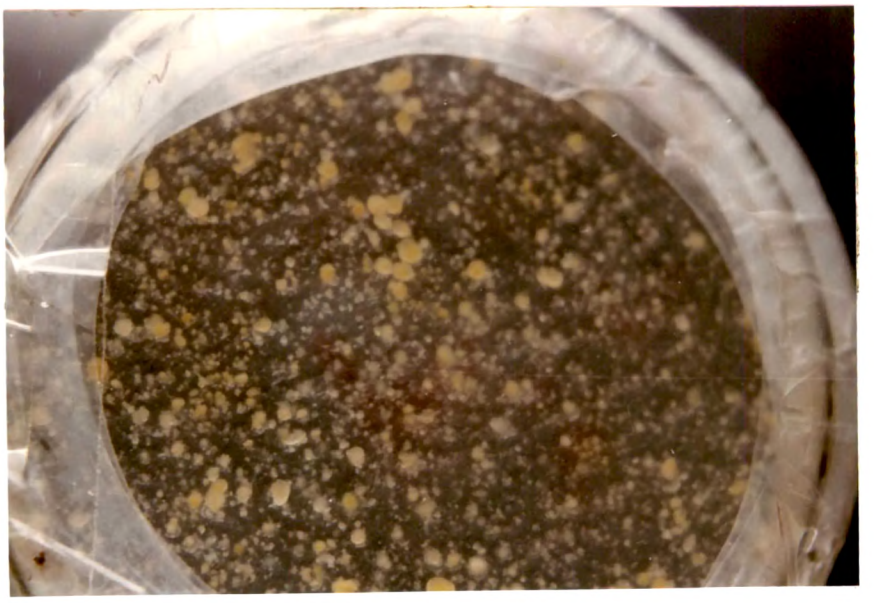
As indicated in the above Table (III.12.), the single cell derived regenerants continued to show the low level of toxin (viz. regenerated from LT-4) at the whole plant level, however the results presented are the analysis of only 3 plants. There

Fig.III. 9.A. Single cell derived cell clones after 30 days on media.

Fig.III. 9.C. Plant regenerated from LT-4 cell line.

Fig.III. 9.D. LT-4 Regenerated plant 25 days after transfer to soil : vermiculite mixture.

9 A



9 C

9 D

was almost one third of reduction in the ODAP content in the LT-4 regenerants in comparison to the seed germinated plants, both in stem and leaves. However, regenerants of hypocotyl derived calli had shown reduction in toxin content only in roots and leaves, whereas the shoots showed no such reduction.

Success in tissue culture of any plant species depends largely on a better understanding of the nutritional milieu of cultured cells and tissues (Murashige, 1974; Street, 1977). The factors which most frequently determine the success of cell cultures are explant origin and the general nutritional requirements.

It was observed in our studies that as compared to other explants, hypocotyls of L. satvius formed most desirable type of callus which was friable and light green. The tissues also exhibited very specific nutritional requirements for good callus growth.

The nutritional milieu consists of essential and optional components. The essential nutrients consists of inorganic salts, a carbon and energy source, vitamins and phytohormones/ growth regulators. Other components include organic nitrogen compounds, organic acids and complex substances which can be important but optional (Gamborg and Shyluk, 1981).

The salt composition of the modified BS medium (MB5) of Mante and Boll (1975) was most favourable for the culture of L. satvius tissues. This medium is richer than the B5 medium suggested for

legumes in general in terms of inorganic phosphate, calcium and ammonium ions and organic nitrogenous compounds (Tables III.1.A. and 1.B.).

Carbohydrate requirement of callus cultures producing secondary metabolites have been worked by a number of researchers (Subbaiah et al., 1978, Shah and Mehta, 1978., Ravishankar, 1979 and Suthar et al., 1981. Most of these studies revealed the superiority of sucrose over other carbohydrates as a carbon source for better growth and enhanced secondary product synthesis in culture.

The effect of sucrose on the yield of secondary products have been examined in a number of plant cell cultures. The optimum concentration seems to vary according to plant species. Sucrose concentration influenced the rate of synthesis and final concentration of solasodine in S. nigrum (Bhatt and Bhatt, 1984) and diosgenin in Dioscorea deltoidea cell cultures (Rokem et al., 1985). The highest diosgenin concentration was obtained with 15 g/l sucrose whereas the highest productivity was obtained with 30 g/l sucrose. The highest sucrose concentration increased anthocyanin yield in the carrot suspension culture even at high inoculum densities (Ozeki and Komamine, 1985). Increased vincristine production in Catharanthus roseus cultures was found at varying levels of carbohydrate (sucrose) source in cultures (Tabata, et al., 1990). In general, raising the initial sucrose level leads to an increase in secondary metabolite yields. In the present work, an increase (4.5%) in the sucrose concentration stimulated ODAP production in callus, but growth was adversely affected. Perhaps the effect of high initial level

of sucrose is to raise the osmotic potential of media.

Although, sucrose is generally the most suitable carbon source for plant cell cultures, many cultures can assimilate other carbon sources, albeit with lower efficiency. In Anchusa cell cultures, fructose was as effective as sucrose for growth and rosmaric acid formation whereas glucose was slightly inferior to fructose. However a 1:1 ratio of glucose to fructose also proved to be as effective as sucrose (De-Eknamkul and Ellis, 1985). Galactose was observed to be most suitable carbon source for quassin accumulation in Picrassma quassioides cell suspensions (Scragg and Allan, 1989). Sucrose was however, much superior to glucose and fructose for the formation of shikonin derivatives in Lithospermum cultures (Mizukami et al., 1977).

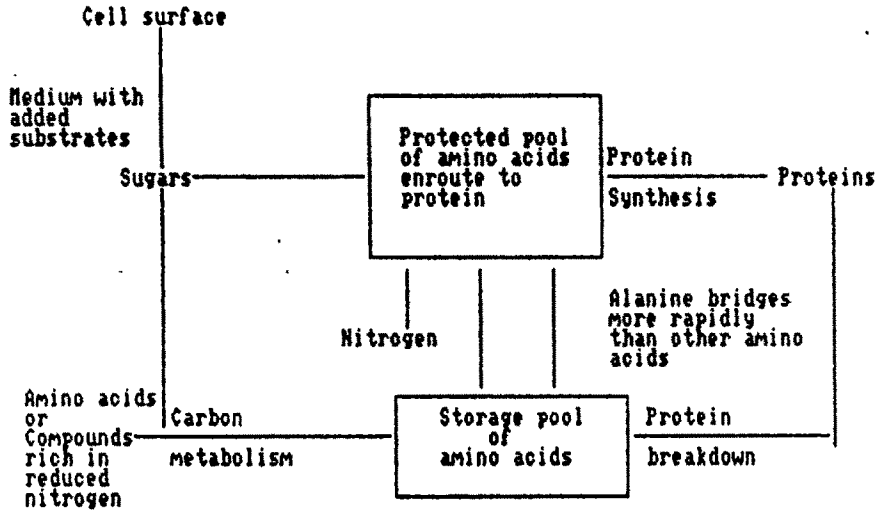
Experiments conducted to study the effect of reduced organic nitrogen compounds on ODAP levels of callus cultures revealed that the ODAP increased with increased supply of nitrogenous compounds. There was no corresponding growth stimulation in callus. Varietal differences were noticed in this regard. Amino acids administered singly to cultured cells inhibited the growth, though the degree of inhibition depended upon the type of amino acid or amide. Growth and ODAP accumulation in cells were inhibited to a greater extent by addition of glutamine and aspartic acid compared to that of other amino acids tested. Asparagine on the other hand stimulated growth and ODAP accumulation in culture.

The inhibitory effects of exogenus amino acids used singly in plant cell cultures in MS or B5 media has been shown by Filner (1966) in tobacco cell cultures and by Gamborg (1970) in soybean cell cultures. Inhibition of growth occured in cells growing on amino acids as sole nitrogen source (Street, 1966; Heimer and Filner, 1971., Behrend and Matcles, 1975., Sangwan, 1978., Pareddy and Greyson, 1989). On the contrary, combination of aminoacids like arginine with other amino acids completely reverse the inhibitory effect (Behrend and Mateles, 1975) and can stimulate growth. Such effects were also observed in maize cultures by Strauss, (1985), Green and Philips (1974) and Green et al., (1974). Heinz and Filner (1971) showed that exogenous amino acids in media inhibited nitrate reductase in cultured tobacco cells. Addition of individual amino acids may create ionic imbalance in the cells (Street, 1977).

In our study, supplementation of media with glycine and aspartic acid had no effect on growth or ODAP levels. On the other hand glutamic acid and glutamine showed inhibition in fresh weight and in ODAP accumulation.

The inhibition of ODAP accumulation in cell cultures by the primary amino acids like glutamic acid and gutamine is hard to explain. Perhaps these amino acids may be involved in ODAP accumulation and negative feedback role by them can not be ruled out. Alternatively a cross reaction could also be responsible here where a particular amino acid of one family inhibits the synthesis of amin acids belonging to another family (Miflin, 1980).

Bidwell et al., (1964) proposed a scheme to demonstrate the relationship between exogenous compounds and metabolism of proteins and carbohydrates as shown below :-



According to this scheme, protein synthesis comes either from external source or from stored amino acids. He also stated that exogenously supplied amides such as asparagine readily participated in protein synthesis unlike externally supplemented amino acids.

Our results with stimulatory effects of asparagine on cell cultures and ODAP accumulation supported the above assumption. Most strikingly asparagine behaved like a precursor of diamino propionic acid as there was a linear increase of ODAP level in cells with increase of asparagine in the medium.

Rosenthal and Bell (1979) postulated a putative metabolic relationship between primary amino acids and non-protein amino-acid ODAP of L. sativus, as shown in Fig.III.10

As per this scheme, serine is the key amino acid which leads to the formation of other amino acids such as B - cyanoalanine, DAPA etc. This was the initial attempt to elucidate the biosynthetic pathway of ODAP was put forward by Murti and Seshadri (1964). They proposed a hypothetical scheme in which ODAP can arise by oxalylation of DAPA which in turn may be formed from serine or aspuragine. These assumptions were based merely on the structural relationship of DAPA and serine. On the other hand Roy (1969) observed that, when L. sativus seeds were germinated in the presence of D,L-Serine-3-^{14}C no radioactivity was found in the isolated and purified ODAP suggesting that serine was not a precursor of ODAP.

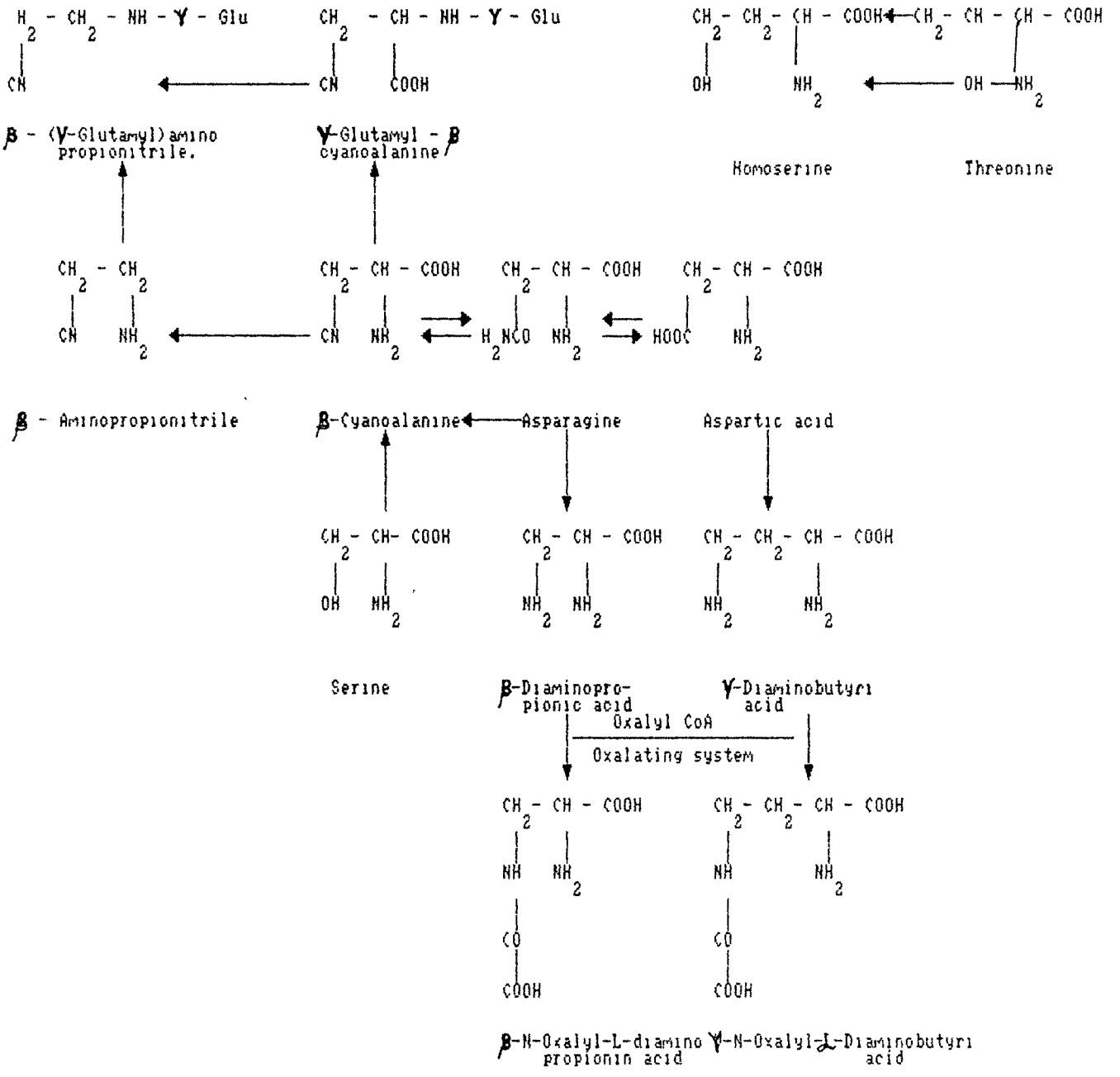
Malathi et al., (1968, 1970) showed that ODAP synthesis was a two step process. It involved oxalyl activation, followed by CoA synthetase to form oxalyl-CoA, which, in the next step condensed with L,B, Diaminopropionic acid through the mediation of DAPA oxalyl transferase to form ODAP. The schematic representation was as follows.

I. Oxalate + CoA $\xrightarrow{Mg+2}$ Oxalyl CoA + AMP + PPi

II. Oxalyl CoA + DAPA $\rightarrow$ ODAP + CoA

However, there was no mention on the origin of DAPA by these workers. Recently, Lambein et al., (1990) studied the

Fig.III.10. Rosenthal & Bell's biosynthetic pathway of ODAP (1979).

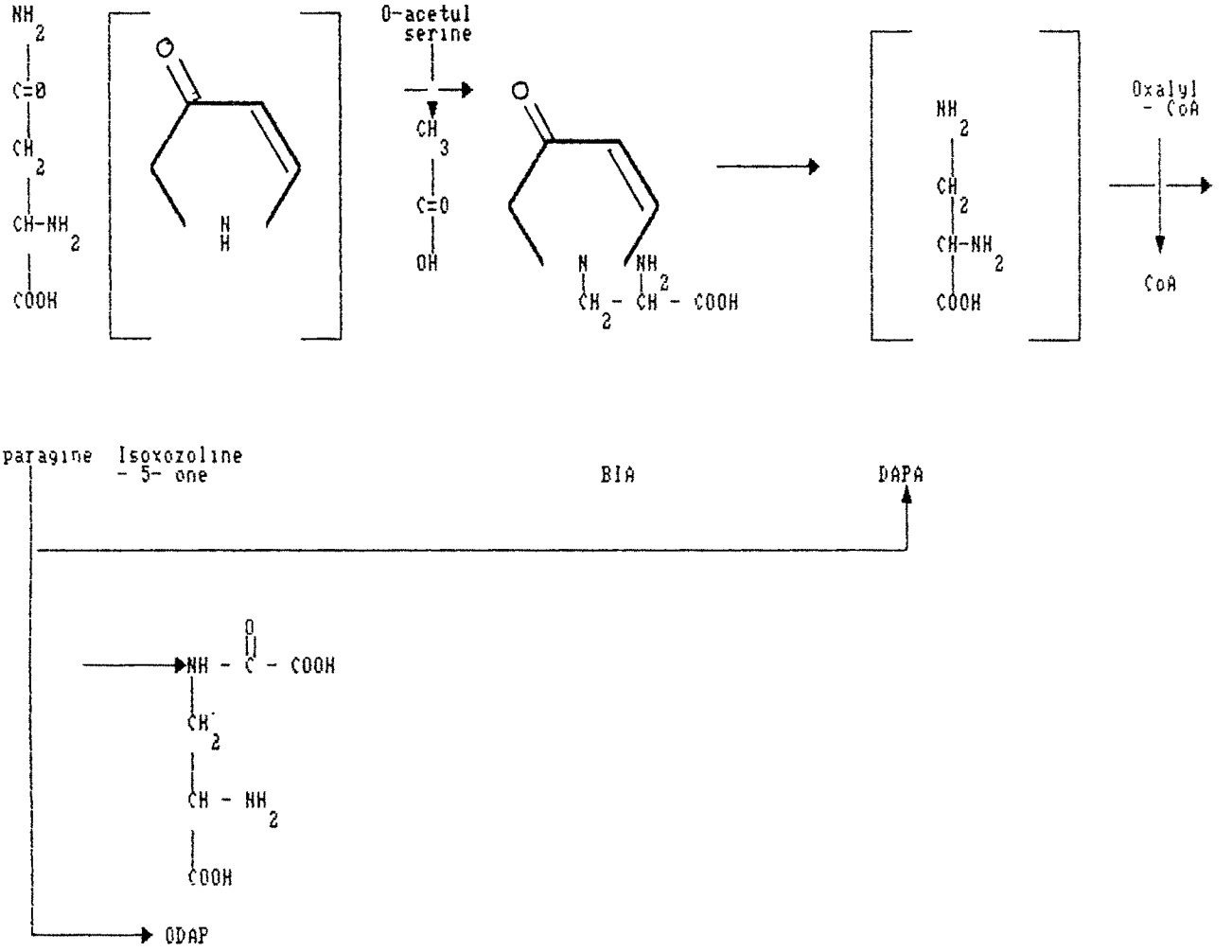


biosynthesis of ODAP in Lathyrus seedlings. They showed that a heterocyclic unstable amino acid namely β-(isoxozolin-5-on-2yl)-L-alanine (BIA) was a short-lived intermediate in the biosynthesis of ODAP via DAPA. They suggested that BIA is a prominent metabolite only during the seedling stage of L. sativus and some other closely related legumes. In seedlings, cotyledons appear to be the site of synthesis of BIA. However, since this compound is not found present in detectable amounts in young leaves and developing seeds, there is a possibility of an alternative biosynthetic pathway at this stage of plant development. Also the genera Crotolaria and Acacia produce ODAP in the seeds in the absence of detectable amounts of BIA. In these cases however BIA could still be a short-lived biosynthetic intermediate.

Lambein et al., (1984) also found that the precursor for BIA was asparagine which gives highest incorporation into the ring of BIA. These observations are supportive to our findings that supplementation of asparagine lead to the increased biosynthesis of ODAP via short lived intermediate DAPA. The schematic pathway proposed Fig. III.11 correlates our findings with Lambein et al., (1990).

Our results though preliminary, suggest that asparagine, which is structurally related to DAPA and whose incorporation in minute quantities in the media stimulated the synthesis of ODAP may be a probable precursor of ODAP. However, this assumption needs to be further substantiated by experiments involving radio-labelled asparagine and use of inhibitors of asparagine biosynthesis like

Fig. III.11: Biosynthetic pathway of ODAP in L.sativus as proposed by Lambein et al., (1990)



Figures in parenthesis have not been detected as free compounds.

those found in microbial systems such as B-aspartohydrazide or methylasparagine.

In comparison to forage lequmes, the large-seeded grain lequmes have proven to be less amenable to regeneration and in several species there are only isolated examples of plant recovery. Plants have been regenerated from shoot-tip derived callus (Glazy and Hamoui, 1981) and hypocotyl callus (Schluze et al., 1985) in Vicia faba, and from a wide variety of ex-plants in pea and peanuts (Hammatt et al.,1986). Hypocotyl and epicotyl derived callus of lima bean (Phaseolus lunatus) has also shown to produce shoots (Sreedhar & Mehta, 1984). Plantlet regeneration from hypocotyls (Shama Rao and Narayanaswamy, 1975; Xu et al., 1984), leaves (Kumar et al., 1983, 1984) and cotyledons (Mehta and Mohan Ram, 1980) in Cajanus cajan are examples of organogenesis among the grain legumes, indicating that this group of plants which was earlier considered recalcitrant, has yielded encouraging results in the recent past. As concluded rightly by Hammett et al., (1986), all these results confirm that the large-seeded grain legumes do have morphogenetic capacity.

Among the less known grain legumes such as Lathyrus, there are much lesser efforts made on regeneration of plants. In the flat pea (L. sylvestris), Coulombe et al., (1990) reported organogenesis from hypocotyl derived primary callus cultures. There is no report of organogenesis in any other species of Lathyrus.

The survey of literature on organogenesis in L. sativus reveals instances of apparent regeneration of shoots from callus cultures maintained for few or several cycles. For example, Mukhopadhyay and Bhojwani (1978) reported regeneration in primary callus cultures of shoot tip derived from plants of cv. LSD-6. Here too, only shoot formation was observed but no roots.

Gharyal and Maheshwari (1980) in cv. LSD-3 observed shoot differentiation from shoot meristem but there was no evidence of de novo shoot formation. Such observations were made for 8 other cultivars including LSD-1, P-24, etc. where cultures were maintained for short periods of time after initiation from activated lateral buds (Gharyal and Maheshwari, 1983). In long term cultures of CV LSD-3, shoot formation occured but presumably through pathway of somatic embryogenesis.

Sinha et al., (1983) reported organogenesis from callus of stem internodes in cv. LSD-1 and showed evidence of adventitious shoot-bud formation by histological studies. However, this response was genotype specific and declined sharply after 2 subcultures, diminishing completely after 8 sub-cultures.

All these reports describe regeneration in primary callus cultures and lack corroborative evidence regarding the nature of differentiation. In primary cultures, shoot buds may develop adventitiously from organized tissues of the original explant embedded in the callus (Cure and Mott, 1978) or arise from preformed buds (Widholm and Rick, 1983), either of which may be the case in all the above findings. This was verified by a

detailed study of immature leaf cultures of L. sativus later on by Bhojwani and Mukhopadhyay (1984) who showed that shoot regeneration required the presence of an organized, meristematic region in the intact explant or piece of callus. The subcultured callus continued to proliferate more shoots only when the subcultured unit contained already differentiated buds, thereby indicating either axillary or adventitious origin of the subsequent regenerants. This may provide the explanation for the culture systems of the previous workers who stated that the regeneration ability was lost after 2-3 subcultures of meristem derived callus.

The basal B5 medium used by different workers is more or less similar as is used for legumes in general and in the present study. The use of growth regulators, especially that of auxins is empirical since IAA, NAA and 2, 4-D are used in different systems. However the addition of BAP as cytokinin is common in all examples. In spite of this, the responses are very much genotype dependent. In the present study, we report organogenesis from hypocotyl derived two-year old callus which has passed through many passages of subculture. The regenerants further differentiated into fully developed plantlets which could be transferred to soil. These observations lead us to presume that organogenesis achieved in our system is by de novo differentiation in long term callus in contrast to the reports obtained by previous workers as discussed above.

The regeneration protocol thus developed is useful in experiments of selection and somaclonal variation for genetic improvement

using cell culture systems where it is necessary to maintain long term cultures before plants can be regenerated.

Immature embryos are ideal starting material for regeneration of somatic embryos and plantlets from legume species (Tilton and Russell, 1984., Grant, 1984., Lazzeri et al., 1985). The ability to regenerate plantlets from cells of immature embryos may depend on the totipotent stage of embryonic tissue (Li et al., 1985). Plant regeneration via organogenesis from callus, suspension or protoplast cultures is often associated with genetic and cytological variation (Larkin and Scowcroft, 1981). On the other hand, embryogenic cell lines maintain their competence for long period and give rise to genetically uniform and normal plant populations (Vasil, 1982). Studies have been carried out in great detail on various aspects of somatic embryogenesis in Glycine and Trifolium (Reviewed by Cui et al., 1988., Ammirato, 1989). The influence of various nutritional and physical parameters, genotypic responses and origin of the meristematic region from which the embryoids originate is studied and well standardized (Lazzeri et al.,1987).

There is no direct effort to obtain somatic embryos in Lathyrus species so far to our knowledge. In a report by Gharyal and Maheshwari (1983) on shoot formation from long term cultures of L. sativus, there is an indirect indication of somatic embryogenetic pathway of plant regeneration. They had cultured meristem apices of L. sativus for 6-8 passages on B5 media with 0.5 mg/l IAA and 1 mg/l BAP. Small plantlets were obtained when

the callus was transferred to media with higher auxin : cytokinin ratio, hence they presumed that these had originated via somatic embryogenesis. The rate of success was very low since full development of both shoot and root occurred in only a few plantlets. Moreover, no histological evidence for the origin of the plantlets was presented.

In our study on Lathyrus sativus, the optimum size of cotyledons required to generate embryogenic callus was 4 mm.Lazzeri (1985) and Barwale (1986) also reported that 4 mm size cotyledons of Glycine species were optimal for formation of embryogenic callus.In contrast, Trifolium repens cotyledons and embryo axes of groundnut form somatic embryos directly without any callus stage (Maheshwaran and Williams,1984., Hazra et al.,1989). It was observed that texture of callus of L. sativus obtained differed with nature of growth regulator used. It was smooth-shiny, creamish white, transleucent and embryogenic with NAA and rough,compact and opaque when media were supplemented with 2,4-D. These results are coherent with the observations made by Ghazi et al., (1986) on somatic embryogenesis of soybean from immature embryo culture. They found that the nature of embryogenic callus differed markedly with nature of auxin used. Lazzeri et al., (1987) also reported that NAA was a better auxin to induce normal somatic embryos as compared to IAA in soybean.In fact an entirely different route or region of origin of these embryoids was obtained from same tissue using NAA or 2,4-D (Parrott et al., 1988). Further Komatsuda and Ohyama (1988) found superiority of NAA for acceleration of normal shoot and root development over

2,4-D which inhibits the development.

The path of regeneration from cotyledon of immature embryos of soybean seems to occur via meristematic centres derived directly from organised tissue (Barwale, 1986) and Lippman and Lippman, (1984). Nevertheless embryos have been recorded from a friable primary callus although embryogenesis from subcultured callus has not been seen (Lazzeri, 1987).

Somatic embryogenesis from zygotic embryos is associated with two distinct initiation patterns. The first budding process gave rise to embryoids with suspensor like structures. The second a non-budding process produced swellings from an internal meristematic tissue that differentiated into embryos (Hu and Sussex, 1971; Pence et al., 1980). A similar situation was seen (Fig. III.8.D.) in L. sativus where somatic embryoids were attached by a narrow band of cells analogous to a suspensor.

A study by Tu and Li (1984) documented the inhibitory effects of high concentrations of 2,4-D (auxins) on differentiation of embryoids. Now it has become a routine and most frequently employed protocol of embryoid induction to utilize 2,4-D in the callus induction medium and its gradual reduction and withdrawal during subculturing. This generalized procedure has been successfully applied for induction of embryogenesis in a number of plant species (Liu and Cantliffe, 1984., Pal et al., 1985., Desai et al., 1986 and Rao and Chopra, 1989). As is generally practised, to avoid early losses of embryogenic callus reduced levels of 2,4-D and NAA are used for further regeneration.

Several authors have reported that reducing the ratio of phytohormones and gradual elimination of auxin at critical times was important for continued differentiation of embryoids. (Ammirato, 1987., Kochba et al., 1978).

The comparison of immature embryo culture system in other plants with that of the present study on L. sativus and the histological observations made to confirm their nature has led us to conclude that the nature of origin of these regenerants is by somatic embryogenetic pathway. These results would be useful for further experiments on somaclonal variation and recurrent selection in L. sativus for low ODAP. However, the frequency of embryoid formation is fairly low in the present system and optimizing the physical, nutritional and hormonal parameters as done in soybean (Lazzeri et al., 1987) would be necessary for improving the protocol so that it could be applied in breeding programs in L. sativus.

Resistant selection to antimetabolites, eg. amino acid analogues is carried out to improve nutritional quality and is the prime goal for plant breeders which is difficult to carry out at the whole plant level. Several examples of selection for resistance to tryptophan analogue 5-Methyl tryptophan (5MT) have been reported (Widholm, 1985, Ranche, 1983 and Wasaka, 1987) and the resistance is, in most cases due to the presence of a feedback altered anthranilate synthetase which allows the accumulation of free tryptophan. Anthranilate synthetase is the key feedback control enzyme in the tryptophan biosynthetic branch of the shikimate pathway (Gilechrist and Kosuge, 1980).

As mentioned earlier, since the bio-synthetic pathway of ODAP is not understood clearly yet, it is not possible to use such analogs of end products that show inhibition of the key enzymes in the biosynthetic pathway. Hence, we had to take the approach of exploiting somaclonal variation from cultured cells.

The phenomenon of novel variation or somaclonal variation observed in tissue culture derived plants is well recognised (Larkin and Scowcroft, 1981., Evans et al.,1984; Hughes, 1986 and Freytag et al., 1989) Larkin and Scowcroft (1981) were of the opinion that cell cultures are "new and exciting options for obtaining increased genetic variability relatively, rapidly and without sophisticated technology". Because of this advantage agronomists were using the genetic instability of cultivated cells for obtaining new plant breeding material. Several explanations for this variation have been documented, including gross chromosomal abnormalities, cytoplasmically controlled mutations, nuclear genic mutations and transposable elements (Orton, 1984; Peschke et al., 1987). Further, spontaneous mutations arising from tissue culture have been reported to occur with high frequency as compared to conventional mutation programmes (Rice, 1982). Recently, somaclonal variants for certain qualitative and quantitative genes have been reported (Kollipara et al., 1987., Barwale and Widholm, 1987 and Graybosch et al., 1989).

Mutant cell lines usually are obtained from protoplasts or from cell surpension cultures. The use of protoplasts have merits,

however, cell cultures are easier to handle in practice. In some instances, eg., Zea mays plant regeneration is possible from suspension cultures but not from protoplasts. Many variant cell lines (about 188) have been isolated and characterized biochemically by these methods (Flick, 1983). To a certain extent, desirable somaclones can be identified in the petriplate of plated cells better than in field trials, making somaclones variation more effecient and cost effective. This requires a correlation between the cellular and whole plant response to specific chemicals used as selective agents. Promising results have already been obtained to host - specific pathotoxins (Selvapandian et al., 1989) and for herbicide resistance (Chaleff and Parsons, 1978).

For cellular selection to be useful for plant breeding, plants must be regenerated which can pass the selected trait to their progeny. An important question in all in vitro selection experiments is whether that trait selected at the cell level will be expressed in regenerated plants. In the case of 5 MT resistance, tobacco plants regenerated from resistant cells did not express the altered form of anthranilate synthetase (Brotherton et al., 1986) while in Datura inoxia it did (Ranch et al., 1983).

In our work on L. sativus, we have been able to select some clonal variants with low neurotoxin (ODAP) content which reaches to one third the value of thant in parental, unselected cell lines. Another interesting observation was that the regererant

plants (R1) from the selected somaclones with low ODAP also retain this low values in the vegetative parts, i.e. roots, stem and leaves. For the time being those can be considered as only somatic variants till the time inheritance data is collected since proof of any mutation event occurring needs sexual transmission of the selected trait as pointed out by Maliga (1980). Coulombe et al., (1990) found there was no significant reduction of Diaminobutyric acid (DABA) in the hypocotyl derive callus cultures of flatpea Lathyrus sylvestris as compared to whole plants, although their data were also of few regenerants analysed and they also expressed need for analysing more samples before coming to any conclusion.

In contrast to well documented cases of somaclonal variations for overproduction of tryptophan and phenylalanine in rice (Wasaka et al., 1987) or Fusarium disease resistance in tobacco (Selvapandiyan, 1987) or range of fruit variations in case of tomato (Evans and Sharp, 1983), our work differs in respect that even though neither the biosynthetic pathway of ODAP is known nor the true de novo regeneration from cell cultures was known, we have been able to produce regenerants with significantly low ODAP using a novel recurrent selection procedure deviced through the course of our work. This work thus opens a door to not only improve the important drought resistant protein rich pulse crop Lathyrus sativus but gives this novel method that can be used to improve lots of other important crops of family Fabaceae and Cruciferae which are hithero not possible to improve due to lack of suitable selection procedure.

TABLE III. 1. A. : Macroelement Concentrations in Different Basal Media. All values expressed as mg/l.

Chemical	Murashige and Skoog's (MS) (1962)	Gamborgs's B5 (1968)	Mante & Boll's MB5 (1975)
NH_4NO_3	1650	-	-
KNO_3	1900	2500	2500
$CaCl_2.2H_2O$	440	150	250
$MgSO_4.7H_2O$	370	250	250
KH_2PO_4	170	-	-
$NaH_2PO_4.2H_2O$	-	150	250
$(NH_4)_2SO_4$	-	134	150

TABLE III. 1. B. : Microelement Concentrations in Different Basal Media. All values expressed as mg/l.

Chemical	Murashige and Skoog's (MS) (1962)	Gamborgs's B5 (1968)	Mante & Boll's MB5 (1975)
KI	0.83	0.75	0.75
H_3BO_3	6.2	3.0	3.0
$MnSO_4.4H_2O$	22.3	10.0	10.0
$ZnSO_4.7H_2O$	8.6	2.0	3.0
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	--
Na_2EDTA *	37.25	37.3	40.0
$FeSO_4.7H_2O$ *	27.95	27.8	27.9

* : The $FeSO_4.7H_2O$ is dissolved in approx.200.ml distilled water. The Na_2EDTA is dissolved in approx. 200. ml distilled water separately heated and mixed (under continous stirring) with $FeSO_4.7H_2O$ until it dissolves completly. The whole mixture is boiled for 5 min. After cooling the volume is adjusted to 1000 ml. Heating and stirring is needed for more stable FeEDTA complex.

TABLE III. 1. C. : Organic and Vitamin constituents in different media. All values expressed as mg/l.

Chemical	Murashige and Skoog's (MS) (1962)	Gamborgs's B5 (1968)	Mante & Boll's MB5 (1975)
Glycine	2.0	-	20.0
L-Glutami acid	-	-	7.5
L-Glumatamine	-	-	60.0
L-Asparatic acid	-	-	7.5
L-Asparagine	-	-	10.0
Urea	-	-	45.0
Adenine sulfate	-	-	2.5
L-Arginine	-	-	10.0
m-Inositol	100	100	45
Riboflavin	-	-	0.1
Thaimine HCl	0.1	10.0	1.0
Nicotinic acid	0.5	1.0	1.0
Pyridoxine HCl	0.5	1.0	1.0
Folic acid	-	-	1.0
Biotin	-	-	2.0

TABLE III. 2. : Callusing frequency, fresh weight and dry weight of 30 days old callus of different explants in 3 different media containing 2.4-D (1.0 μM) and Kinetin (0.1 μM) of cv. Bharuch.

Media	Growth parameter	Explants		
		Hypocotyl	Leaf	Cotyledon
MB5	CI*	100	73	71.5
	Fr.Wt.	9452 (± 345.2)	1201.5 (± 165)	1452 (± 225)
	Dr.Wt.	495 (± 25.6)	59.8 (± 6.9)	72 (± 8.2)
B5	CI*	62.5	65.0	63.3
	Fr.Wt.	4150.5(± 284)	634 (± 30)	545 (± 51)
	Dr.Wt.	207 (± 17.1)	31 (± 4.5)	23.8(± 4.0)
MS	CI*	48.5	50.5	61.5
	Fr.Wt.	1874 (± 109)	433.5 (± 41)	417.5(± 31)
	Dr.Wt.	85.5(± 6.3)	22.2 (± 4.4)	24.2(± 4.3)

* % Callus induction. Fr.Wt.= Fresh weight (mg/dr.wt),

Dr.Wt.= Dry weight (mg/dr.wt).

Data reprsents an average of 3 replicates.

Figures in parenthesis represents standard deviation.

TABLE III.3 : Callusing response of hypcotyl explants of L.sativus to different levels of 2.4-D and Kn, supplemented to MB5 medium + 3% sucrose.

2,4-D (μM)	Kn (μM)	Callus induction (%)	Days required for callusing	Texture of callus *
0	-	-	-	-
0.5	-	4.5	18	C + G
1.0	-	1.0	15	C + W
2.0	-	18	15	C + W
-	0.05	-	-	-
-	0.1	-	-	-
-	0.2	-	-	-
-	0.4	-	-	Browing
0.5	0.05	49.5	9	SF + YW
1.0	0.1	100	5	F + GW
2.0	0.2	65	7	SF + YW
5.0	0.5	16	15	C + G

* : C + G = Compact green; C + W = Compact white; GW = Greenish white

Y W = Yelowish white; F = Friable; S F = Semifriable; C = Compact.

The Data represents an average of 3 replicates.

TABLE III. 4. : Response of hypocotyl explants of different genotypes of L.sativus to MB5 medium with respect to callus formation.

Genotype	% Callusing	Fr.Wt. (mg)	Dr.Wt. (mg)	Texture of Callus
Bharuch	96 (± 2)	9025 (± 320.2)	481.5 (± 27.1)	Semifriable and Whitish green.
Pusa-24	61.5 (± 1.5)	4735.5 (± 312)	307.5 (± 23.2)	Compact and Yellowish white.
LSD-10	29.0 (± 0.75)	2255.0 (± 250)	148.5 (± 12.5)	Compact and green.
LSD-1	41.0 (± 1.30)	3125.0 (± 281)	205.0 (± 18.5)	Semifriable and yellowish.
RL-15	33.5 (± 1.10)	2155.5 (± 207)	145.0 (± 14.6)	Compact and Whitish green.
RL-18	31.2 (± 0.95)	2090 (± 239)	141.5 (± 14)	Compact and green.

Data reprsents an average of 3 replicates.

Figures in parenthesis represents standard deviation.

TABLE III.5 : Effect of different levels of organic reduced nitrogen compounds on growth parameters in callus cultures of five different varieties of L.sativus.

Variety	Growth parameter	Levels of reduced nitrogen compounds. 1/2	3/4	1	1.1/2	2
Bh	Fr.Wt.	3553.7 (± 221)	8665.2 (± 285)	10215 (± 347)	10168.7 (± 342)	9532.5 (± 301)
	Dr.Wt.	255.5 (± 16)	610 (± 26)	714 (± 30.5)	749.3 (± 35.1)	667.2 (± 30.5)
P-24	Fr.Wt.	3276.5 (± 211)	7830.5 (± 266)	4106.2 (± 295)	9082.5 (± 287)	8365.9 (± 280)
	Dr.Wt.	230.3 (± 14)	550.7 (± 21)	638 (± 29.1)	657.5 (± 29)	590.7 (± 29.5)
RL-18	Fr.Wt.	3116.2 (± 207)	7345.7 (± 253)	8465.5 (± 286)	8581.2 (± 276)	7269.5 (± 247)
	Dr.Wt.	218.2 (± 13)	495.1 (± 18)	566.5 (± 26.7)	571.6 (± 21)	490.2 (±26)

TABLE III.5 contd....

LSD-1	Fr.Wt.	2146.2 (± 156)	5940.5 (± 238)	6585.1 (± 257)	6816.7 (± 244)	5886.5 (± 218)
	Dr.Wt.	129.7 (± 11.2)	338.3 (± 15.1)	389 (± 18)	396.7 (± 17)	351.5 (±21)
LSD-6	Fr.Wt.	2816.2 (± 156)	6445.5 (± 251)	7265 (± 261)	7520.5 (± 247)	6589.7 (±256)
	Dr.Wt.	175.5 (± 12.8)	398.2 (± 17.5)	446.2 (± 23)	450.4 (± 25)	405 (±20)

Fr.Wt.= Fresh weight (mg/dr.wt), Dr.Wt.= Dry weight (mg/dr.wt).

Data reprsents an average of 3 replicates.

Figures in parenthesis represents standard deviation.

TABLE III.6 : Effect of different levels of organic reduced nitrogen compounds ODAP (µg/g.dr.wt) content in callus cultures of five different varieties of L.sativus.

Variety	Levels of reduced nitrogen compounds. 1/2	3/4	1	1.1/2	2
Bh	56.2 (± 6.5)	92.4 (± 8.3)	107 (± 9.5)	110.2 (± 11.1)	114.4 (± 9.1)
P-24	51.5 (± 6.1)	85.5 (± 7.9)	98.0 (± 7.8)	100.6 (± 10.9)	115.2 (± 8.7)
RL-18	58.5 (± 7.2)	110.8 (± 9.6)	135.7 (± 11.2)	141.5 (± 12.1)	145.2 (± 12.3)
LSD-1	61.7 (± 6.2)	116.8 (± 9.3)	145.2 (± 12.5)	147.5 (± 9.5)	149.5 (± 14.1)
LSD-6	55.1 (± 5.4)	103.5 (± 8.7)	126.0 (± 11.3)	132.5 (± 10.1)	188.6 (± 9.5)

Dr.Wt.= Dry weight (mg/dr.wt).

Data reprsents an average of 3 replicates.

Figures in parenthesis represents standard deviation.

TABLE III. 7 : Effect of amino acids added singly as sole amino nitrogen source on growth and ODAP accumulation in suspension cultures of L.sativus.

Amino acid/ Amide	Conc. mg/l.	Fr.Wt.	Dr.Wt.	ODAP (ug/g.dr.wt.)
Glycine	20	4730.5 (± 104)	166.5 (± 9.2)	45.8 (± 3.1)
Glutamic acid	7.5	4194.7 (± 108.5)	149 (± 6.3)	40.8 (± 2.5)
Glutamine	60	4105 (± 101.5)	143 (± 7.1)	39.8 (± 2.9)
Aspartic acid	7.5	4074.2 (± 93.5)	113.4 (± 9.5)	31.5 (± 2.9)
Asparagine	10	6668.0 (± 189)	233 (± 11.3)	74.1 (± 4.5)
Arginine	10	7064.7 (± 177.5)	253 (± 12.5)	60 (± 5.1)
Control	-	8920 (± 201)	291.5 (± 15.8)L	82 (± 1.5)

Fr.Wt.= Fresh weight (mg/dr.wt), Dr.Wt.= Dry weight (mg/dr.wt).

Data reprsents an average of 3 replicates.

Figures in parenthesis represents standard deviation.

TABLE III.8 : Morphogenetic response of calli derived from hypocotyl of L sativus. (cv. Bharuch) to various combinations of growth regulators. (μM)

Growth Regulator.	Nature of Morphogenetic response.	% Shoot regeneration.
8. A.		
2.4-D + Kin		
0.5 + 0.1	-	-
1.0 + 0.2	Nod, R+	-
1.5 + 0.3	R+	-
2.0 + 0.4	-	-
2.5 + 0.5	-	-
2.4-D + BAP		
0.5 + 0.1	R+	-
1.0 + 0.2	-	-
1.5 + 0.3	Sh++, R+	38.0 (±0.65)
2.0 + 0.4	Nod	-
2.5 + 0.5	-	-
NAA + BAP		
0.5 + 0.2	-	-
1.0 + 0.3	-	-
1.5 + 0.4	R+	-
2.0 + 0.5	Sh+	16 (± 0.21)
2.5 + 0.6	R++	-

TABLE III. 8. B. contd....

2.4-D + BAP		
1.5 + 0.1	R+	-
1.5 + 0.2	-	-
1.5 + 0.3	Sh++	39.5 (± 1.09)
1.5 + 0.4	-	-
1.5 + 0.5	-	-
BAP + 2,4-D		
0.3 + 1.0	-	-
0.3 + 1.5	Sh++	38 (± 1.05)
0.3 + 2.0	-	-
0.3 + 2.5	-	-

Sh+ = Occassional shoot bud formation.
Sh++= Profuse shoot bud formation.
R+ = Occassional rooting.
R++ = Profuse rooting.

TABLE III.9.: Response of immature embryos of L.sativus. to various concentrations of 2,4-D and NAA.

Auxin	Conc. mg/l.	Response			Embryogenesis (%)
		Embryogenesis	Callus	Swelling	
2,4-D	1	-	-	-	0
	3	+	+	+	14.8 ± 0.06
	5	-	+	-	0
NAA	1	-	-	-	0
	3	-	-	+	0
	5	+	+	+	23.1 ± 1.67

$$\% \text{ Embryogenesis} = \frac{\text{No. of cotyledons which formed somatic embryos}}{\text{Total No. of cotyledons cultured}} \times 100$$

Data represents an average of 5 replicates.