THE EFFECTS OF THREE MICRO-NUTRIENTS, (BORON, MANGANESE, AND IODINE) INDOLE ACETIC ACID, AND ASCORBIC ACID IN THE CULTURE MEDIUM ON THE GROWTH OF TOMATO CELLS IN SUSPENSION CULTURE

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CURRICULUM VITAE
1 GENERAL INTRODUCTION

The first attempt to obtain plant tissue or cell cultures dates back to 1898, where Haberlandt tried to grow single cells from various tissues using various plants. Since Haberlandt's work very little progress was made during the next 30 years with very little progress (Kotte, 1922a, b; La Rue, 1936; Pfeiffer, 1931; Robbins, 1922a, b). The first break through was obtained by White (1934), where he reports the establishment of an actively growing clone of tomato roots. Gautheret (1934) reported that cambium pieces of certain plant species were able to proliferate into cell masses under aseptic conditions, when placed on a surface on a solidified medium containing Knop's solution (Knop, 1865) supplemented with sugar and some organic salts. The basis of all nutrient media is a mixture of mineral salts combining the essential macro- and micro-elements together with a source of carbon, usually sucrose and organic supplements such as vitamins, hormones (auxins and cytokinins) and amino acids.

Nearly all the investigators who first succeeded in cultivating plant tissues did not attempt to make an exact determination of the mineral needs of their material. For the formulation of media, either results based on plant analysis or empirically adopted solutions successfully used for the culture of entire plants were used. As an example, White (White, 1934) used the medium he had developed for his root culture experiments for all other tissue species and it happened to be satisfactory. This medium contained six macro-elements indispensable for the growth and development of higher plants (N, P, K, S, Mg and Ca), five micro-elements (Fe, B, Mn, Zn, and Cl) and two other elements (Na and I), the role of the latter two being doubtful. Gautheret (1939) used Knop's solution diluted to one-half. These media differed from that of White's, by the absence of Na and Cl and the presence of five supplementary micro-nutrients (Cu, Ni, Co, Ti, and Be) where the role of S of Ti and Be are doubtful. Beginning from 1940 a vast amount of knowledge was collected, especially to the improvement of mineral nutrient composition in media (Burkholder and Nickell, 1949; Heller, 1953; Hildebrandt et al., 1945). However, all these studies were done either pertaining to macro elements, or type of nutrient (Ex. NO₃ vs. NH₄), and or nutrient interaction in
a vast number of tissue culture media has been described for many crop species, and those described by Gamborg et al., (1968); Murashige and Skoog, (1962); Nitsch, (1967) and Wood and Braun, (1961) are used frequently. It must be emphasized that the mineral requirements of a given tissue are not fixed but depend on its physiological state. As an example the investigations of Heller (1953) have shown that vigorous colonies of carrot tissue demand more potassium than slowly proliferating ones. Calcium behaves contrawise, i.e., fast growing tissues require less calcium than slow growing ones.

Therefore in principle, the mineral composition of nutrient solutions should be adapted to each particular situation. Besides its use for the rapid vegetative propagation of plants, tissue culture now has at least four other applications of commercial potential: They are:

- to remove diseases, particularly viruses, from plants and thereby production of virus-free planting material;

- to conserve stocks of plants, particularly collections of crop plants needed for plant breeding (germplasm collections);

- to bring about genetic change and facilitate 'genetic engineering' (i.e. the deliberate transfer of genetic information between vegetative plant cells, and perhaps selection for important characters at the single cell level) in ways that will be useful to plant breeders.

- the production and extraction of valuable chemical products or secondary metabolites from cultured plant cells, rather than directly from plants grown and harvested in the field.

Since most of the environmental conditions can be precisely controlled in contrast to field conditions, tissues and cell cultures grown under aseptic conditions are better tools of testing the effects of various substances. The objectives of the present project was to
study the effects of Murashige and Skoog media-components (boron, manganese and iodine) and non media components (indole acetic acid and ascorbic acid) on the growth and development of tomato cells in suspension culture.

2 SUMMARY

The effects of boron (B), manganese (Mn), iodine (I), indole acetic acid (IAA) and ascorbic acid (AsA) in culture medium on the growth of tomato cells in suspension culture were investigated.

No increase in cell biomass was observed after 4 days without added B in medium. The highest biomasses were obtained at media B levels of 0.09 and 0.55 mM. Both low (0.005 - 0.007 mM) and high (2.30 - 4.15 mM) media B levels produced low cell biomasses. Media B levels of 1.85 mM or higher caused toxicity of cells. Depending on the B concentration in medium and the length of exposure, two observations were made. Firstly cells starved of B absorb this element from medium within a few hours. Secondly, the cells come into a almost near equilibrium with the media B levels. A very large part of the absorbed B by cells could be leached out and only very minute quantities were left in the cells. In the presence of metabolic inhibitors the B concentration in the cells and in the medium were almost at an equilibrium. These results indicated that B enters cells through passive transport and that an equilibrium exists between the cells and the medium.

In spite of a 50 fold concentration difference in medium, no differences in the biomass produced or dry weight of cells were observed with cells grown either with 0.002 or 0.1 mM Mn. Cells cultured with no added Mn (deficiency) and 0.2 mM (toxicity) Mn in the medium produced low biomasses. Deficiency as well as toxic levels of Mn in the medium had large effects on the rate of cell division as well as on cell viability. Cells grown in the presence of 0.2 mM Mn had low levels of Zn, Cu, and Fe whereas cells cultured with no added Mn in the medium had higher levels of these three micro-nutrients.

Though the biomass or dry weight produced were not significant with I levels of 0, 5, 7.5 μM in medium, this element showed a stimulating effect on growth by slightly
increasing the biomass. It is not known whether this stimulating effect was due to higher levels of nutrients found in the cells. An I level of 10 µM in medium was deleterious for the growth of cells. Studies done with labelled I showed that even after a 3h uptake period the cells contained only 0.17% of the total available iodine. The level of I determined in this study is much lower as compared with other experiments done using whole plants.

AsA in medium had no effect on biomass produced and the cell division rate. However, the dry weights, sucrose, glucose, fructose and citric acid contents were high in cells cultured with AsA in medium. Cells readily absorb AsA from medium. A rapid reduction in AsA contents in cells and in medium was observed and the reasons for such reductions are discussed.

A habituated cell suspension for auxins was obtained through a stepwise reduction in indole acetic acid concentration in medium. These cells produced almost the same biomass as cells grown either with only IAA, kinetin or both in medium. There were no significant differences in the number of embryos and plantlets produced between the control and habituated cells, suggesting that the totipotency of cells was not altered when the cells become autotrophic to IAA. The IAA analysis done using monoclonal antibodies showed, that the habituated cells produced more IAA than the control cells. Cell suspensions initiated using stem pieces from seedlings obtained from regenerated autotrophic cells, could not grow in a medium devoid of IAA. These results indicate that the process of habituation is epigenetic in nature and is not due to a mutation. Such changes are reversible and are not transmitted meiotically.

ZUSAMMENFASSUNG

Der Einfluss von Bor, Mangan, Iod und Ascorbinsäure auf das Wachstum von Tomatenzellen in Suspensionskulturen wurde untersucht.

In einem borfreien Medium ergab sich keine Zunahme der Zellbiomasse nach vier Tagen. Die höchste Biomasse wurde bei Borkonzentrationen im Medium von 0.09 und 0.55 mM gebildet. Sowohl bei niedrigen (0.005-0.007 mM) wie auch bei hohen (2.30-4.15 mM) Borkonzentrationen ergaben sich niedrige Zellbiomassen. Borkonzentrationen

Trotz eines fünfzigfachen Unterschieds im Medium ergaben sich bei Konzentrationen von 0.002 oder 0.1 mM Mangan keine Unterschiede in der Biomasse oder Trockenmasse der Zellen. Ohne Mangan (Mangel) oder bei 0.2 mM Mangan (Toxizität) wurden nur niedrige Biomassen gebildet. Manganmangel bzw. Mangantoxizität verursachten starke Veränderungen in der Zellteilung wie auch in der Vitalität der Zellen. Die Zellen wiesen bei Mangankonzentrationen von 0.2 mM niedrige Gehalte an Zink, Kupfer und Eisen auf, während diese ohne Mangan im Medium erhöht waren.

Obschon die Biomasse oder das Trockengewicht nicht signifikant durch Iodkonzentrationen von 0, 5 oder 7.5 μM im Medium erhöht wurden, ergab sich ein stimulierender Effekt auf das Wachstum. Es ist nicht bekannt, ob diese Stimulierung auf höhere in den Zellen gefundene Nährstoffkonzentrationen zurückzuführen ist. 10 μM Iod im Medium wirkten sich schädlich auf das Wachstum der Zellen aus. Nach drei Stunden nahmen die Zellen nur 0.17 % des gesamten im Medium vorhandenen Iods auf. Die in Zellen bestimmten Iodkonzentrationen sind viel niedriger als bei ganzen Pflanzen gefunden wurde.


Durch eine schrittweise Reduktion der Indolsäurekonzentration ergab sich eine Auxin autotrophe Zellsuspension. Diese Zellen produzierten beinahe dieselbe Biomasse wie
3 MATERIALS AND METHODS (GENERAL)

3.1 Cell cultures

3.1.1 Plant material

Week old tomato (Lycopersicon esculentum Mill. cv. Rodeo) seedlings were grown in plastic containers containing sand for 3 weeks in a glass house on a 16/8 day-night cycle at 24°C.

3.1.2 Induction of callus

Fully developed young leaves were harvested and surface sterilized in 60% alcohol for 30 s. Leaves were transferred to a calcium hypochlorite solution (approximately 0.02%) containing 1% Tween80 for further sterilization for 10 minutes. The leaves were washed with sterile water three times and cut into round pieces (7mm diameter) using a sterile cork borer.

The basal medium consisted of Murashige and Skoog (1962) (MS) salts (see below) with 3% sucrose and 0.8% Agar (Disco Bacto-Agar, 0140-01, Disco Laboratories Michigan, USA).

Composition of the MS medium

Macro-elements (mg L⁻¹)

Ammonium nitrate - 1650; Potassium nitrate - 1900; Magnesium sulfate - 370; Potassium-di-hydrogen phosphate - 170; Calcium chloride (dihydrate) - 440.

Micro-elements (mg L⁻¹)

Ferrous sulfate (heptahydrate) -50; Di sodium EDTA.2H₂O - 37.3; Manganeseous sulfate (monohydrate) - 16.9; Boric acid - 34.31; Zinc sulfate (heptahydrate) - 8.6; Potassium iodide - 0.83; Di-sodium molybdate (dihydrate) - 0.25; Copper sulfate (pentahydrate)- 0.025; Cobalt chloride (hexahydrate) - 0.025;
Vitamines (mg L⁻¹)
Glycine - 2; Nicotinic acid - 0.5; Pyridoxine hydrochloride - 0.5; Thiamine hydrochloride - 0.1; Myo-inositol - 100;

Hormones (mg L⁻¹)
2,4-dichlorophenoxy acetic acid - 0.4; Kinetin - 0.14;

Sugar
Sucrose - 30g L⁻¹;

The pH of the medium was adjusted to 5.8 with 1N KOH before autoclaving (1 h at a pressure of 1 bar). Leaf pieces were placed on 20-25 ml solid medium in petri-dishes (93×16mm), and incubated at 28°C under continuous fluorescent light (100 μmol m⁻² s⁻², Philips TL F 40W/33). Under these culture conditions undifferentiated friable calli appeared from the cut surfaces of leaf pieces. After 2 weeks the growing calli were again transferred to a solidified medium and incubated for further 2 weeks.

3.1.3 Cell suspensions
The leaf explantats with callus were transferred to 500 ml Erlenmeyer flasks containing 200 ml MS liquid medium. The flasks were placed on a shaker and agitated at 28°C at 100 revolutions per minute.

Cells released from explantats were 'sieved through' using sterile sieves with a pore size of 300 μm. Sieving enabled to obtain a cell suspension without leaf debris or large pieces of callus.

The cell suspensions were maintained on a MS medium, sub-cultured every 2 weeks. The conditions in the culture room were as described above.

For all experiments tomato cells in suspension culture were used.
3.2 Cell culture methods

3.2.1 Sterilization

All glass-ware used in experiments was sterilized at 180°C for 4h. Pipettes and other working material were autoclaved in a autoclave for 1h at 3 bars. All work was performed under sterile conditions on a "LF" sterile bank (Ceag Schirp Reinraumtechnik).

3.3 Measures of cell growth

3.3.1 Cell fresh weight (biomass)

The cell fresh weight (biomass) was determined by transferring a known volume of a cell suspension onto a membrane filter unit (Sartorius membrane-filter GMBH, Göttingen, Germany) containing a cellulose filter (0.8 μm) and draining under pressure. The cells on the filter paper were washed either with distilled water or MS medium depending on the type of experiment (for e.x. for the determination of macro and micro nutrients the cells were washed with distilled water). The cells were scraped out using a metal spatula and transferred to pre-weighed plastic discs and the fresh weight determined.

3.3.2 Cell dry weight

After the fresh weight determination, discs with cells were dried at a temperature of 55° for 24 hours, cooled in a desiccator and the dry weight determined.

3.3.3 Cell counting

Cell aggregates in suspension cultures were separated by a modified controlled treatment using chromium trioxide as described by Butcher and Street (1960). One volume of suspension culture was added to 2 volumes of 5% chromium trioxide and heated to 50°C on a water-bath for 5 minutes, allowed to cool and shaken vigorously for 15 minutes on a shaker (Vortex shaker, Bender and Hobein AG, Switzerland).
Depending on the thickness of the cell suspensions they were diluted, using the culture medium, and stained as described in section 3.4. The cells were counted using a cell counting slide. Care was taken so that the depth of this slide was greater than the diameter of the cells in suspension. The slide used had a depth of 1200 μ. The cells were counted at a magnification of x10. The mean of 100 counts and known volume of each field (diameter of field 105 μ, depth of field 1200 μ, volume = 0.99 μl) enables the number of cells per ml of original culture to be calculated.

3.3.4 Packed cell volume (PCV)

Packed cell volume was determined by transferring a known volume of a suspension to a 15 ml graduated conical centrifuge tube and centrifuging at 2000 xg for 10 minutes. The volume of the settled cells at the bottom of the tube is expressed as ml cell pellet per ml culture.

3.4 Cell viability studies

The viability of cells were studied using the medium of Alexander (1969) as the staining solution (contains malachite green and acid fuchsin as active substances). When stained and microscopically examined, the viable cells appear red and the non-viable cells green. This method was used to determine the viability of cells in studies done with boron.

In other studies fluorescein diacetate (FDA) staining method (Widholm, 1972) was used to determine the cell viability. This assay involves the uptake and intracellular breakdown of fluorescein diacetate by esterase activity to yield the fluorescent compound fluorescein. Application of the diluted FDA solution (0.01) of the stain is followed by examination under tungsten (total number of cells) and ultra violet (viable cells) illumination and estimated.

3.5 Analytic methods

3.5.1 Determination of pH

pH was determined using a pH electrode (Corning pH-meter 250).
3.5.2 Cell sample preparation for analysis

Dry ashing

The dried cell samples were first transferred to porcelain cups and pre-ashed over a bunsen burner. The cups were then transferred to a muffle furnace for complete ashing at a temperature of 540° C for 4h. The ashes were then dissolved in 2 ml HCl (20%) 10 minutes at room temperature, and transferred to 50 ml flasks. 1 ml solution of CsCl (5%,w/v) was added and the volume was adjusted to 50 ml with distilled water.

Extraction

Fresh cell samples were first stored at -20° C for 24h in graduated test tubes. They were then allowed to thaw and macerated using a glass rod. Internal standards were added. The cells were then homogenized with an extraction agent (see Tab. 1) and centrifuged (15 minutes at 3500 U/min). The supernatant was collected and the extraction followed twice. The extracts were mixed together and stored at -18° C until analysis.

Table 1: Extraction agents used

<table>
<thead>
<tr>
<th>Analyzed elements or compounds</th>
<th>Extraction agent</th>
<th>Extracted amount (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃⁻,SO₄²⁻,PO₄³⁻,Cl⁻</td>
<td>bi-dist. water</td>
<td>30</td>
</tr>
<tr>
<td>Sugars</td>
<td>ethanol, water and formic acid (80:19:1)</td>
<td>30</td>
</tr>
<tr>
<td>Organic acids</td>
<td>as sugar</td>
<td>20</td>
</tr>
</tbody>
</table>

3.5.3 Determination of cations

Samples prepared from dry ashing were then subjected for cation analysis using an inductively coupled plasma-Perkin Elmer photometer (Model ICP/5500).
3.5.4 Determination of N

Dry samples weighing approximately 5 mg were mixed with a small amount of V$_2$O$_5$. Analysis was done using a conductivity detector in a NCS apparatus (Carlo-Erba, Nitrogen analyzer 1500), after a complete oxidation of elements.

3.5.5 Determination of anions

10 ml cell suspension culture was vacuum filtered on an acid washed filter (Schleicher and Schüll Nr. 595) and the cells transferred to pre-weighed plastic disks and the fresh weight determined. The cells were transferred to graduated conical glass tubes and stored at -18°C for 24 h. The cells were thawed at room temperature and 1 ml water added and macerated with a glass rod. The volume was adjusted to 10 ml by adding water, mixed and centrifuged. The supernatant was collected. This extraction procedure was repeated twice and the supernatants pooled and stored at -18°C until analysis.

The anions were analyzed by high performance liquid chromatography. The system composed of a mini pump VS LDC/Milton Roy (Riviera Beach, Fl, USA), a puls attenuator, an ion analyzer ICM model 266-100 (Wescan Instruments, Inc. Santa Clara, Ca, USA) equipped with a 100 µl loop and a Wescan anion column thermostatised at 40°C. The flow rate was 2ml/min. Injection volume being 25 µl. The eluent was either 5 mmol/L phthalic acid (pH-2.8) for the analysis of phosphate (standard 5-30 ppm), chloride (standard 10-60 ppm) and nitrate (standard 15-130 ppm) or potassium phthalate (pH-4.85) for the analysis of sulfate (standard 5-30 ppm).

3.5.6 Analysis of sugars and organic acids

The internal standard for organic acids was 3-methyl-glutaric acid (5mmol/L) with 10 µl acid organic (60 mmol/L) and for sugars mannitol at a concentration of (164.68 mmol/L) was used. The extracts were then passed through two ion exchange columns, mounted in series, the first containing 6 ml Dowex 1-X2, and the second containing 6 ml Dowex 1-X8. The columns were then washed with 100 ml bi.dest water. The solutions passing through the bottom column were collected to a 250 ml round bottom
flask. These solutions containing the sugar fractions were evaporated at 50° C under vacuum in a rotation evaporator. The dry product was taken up in 2 ml of bi-dest. water and transferred to HPLC vials. The liquid was then injected to a Perkin Elmer liquid chromatograph (series 3b), equipped with a Biorad HPX-87C column and a refractions index detector (Erma Optical Works RI detector ERC-7510).

The analytical conditions were as follows.

<table>
<thead>
<tr>
<th>Flow substance</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Column temperature</td>
<td>65° C</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>45° C</td>
</tr>
</tbody>
</table>

The composition of the calibration sugar solutions were as follows.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Molecular weight</th>
<th>Concentration (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>342.30</td>
<td>1.46</td>
</tr>
<tr>
<td>Glucose</td>
<td>180.16</td>
<td>2.77</td>
</tr>
<tr>
<td>Fructose</td>
<td>180.16</td>
<td>2.77</td>
</tr>
<tr>
<td>Mannitol</td>
<td>182.17</td>
<td>164.68</td>
</tr>
</tbody>
</table>

The samples analyzed by the HPLC were -
1. A purified blank (10 ml water)
2. Standard solution of sugars purified
3. Purified samples with mannitol as the internal standard

The bottom column (Dowex 1-X8) containing the organic acids were washed with 50 ml of formic acid (8N). Elutions collected were evaporated at 50° C under vacuum and the residue dissolved in 1 ml ethanol + 1% formic acid and transferred to gas-chromatographic vials. The mixture in the vial was evaporated in a nitrogen stream and the residue was derivatized. For derivatization the following substances were added to each vial.
1. 200 µl BSFTA (Trimethylsilyl - trifluoro acetamide) containing 1% TMCS (trimethylchlorosilane)

2. 500 µl acetone

3. 10 µl non-adecanmethyl ester (60 mmol/L) - Second internal standard

The reaction took place at room temperature during 2 hours.

The solution in vials were then subjected to chromatographic analysis using a fused silica capillary column (Perkin Elmer OV-1), using nitrogen as the carrier gas (flow rate 5 ml/minute). The recovery of 3-methyl glutaric acid was calculated using non-adecan methyl ester.

The composition of the calibration solution of organic acids was as follows.

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>Molecular weight</th>
<th>Concentration (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinic acid</td>
<td>118.10</td>
<td>29.52</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>116.08</td>
<td>29.02</td>
</tr>
<tr>
<td>Malic acid</td>
<td>134.10</td>
<td>67.05</td>
</tr>
<tr>
<td>Citric acid</td>
<td>210.10</td>
<td>52.52</td>
</tr>
</tbody>
</table>
4. THE EFFECT OF VARYING MEDIA BORON (B) LEVELS ON THE GROWTH OF TOMATO CELL SUSPENSIONS

4.1 ABSTRACT

The effects of various boron (B) levels in the culture medium on the biomass production and B concentration of cells were studied using tomato cell suspensions. In study 1 no increase in cell biomass was observed after day 4 in the absence of B in the medium. These cells lost their viability by day 6. Cells grown at a B level of 0.09 or 0.55 mM in the medium had the highest biomass (doubled by day 6). Cells grown at 0.92 or 1.85 mM B had lower biomasses (doubled by day 8). Both with low (0.005-0.007 mM) and high (2.30-4.15 mM) concentration of B in the media, there was only a slight increase in biomass and the cultures failed to double their biomasses even by day 10. Cells grown with 3.70 or 4.15 mM B in the medium showed a black discoloration by day 6 and were no longer viable. Except in the high B study, the B concentration of cells were in near equilibrium with the media B. This relationship did not continue with B levels of 1.85 mM or higher. However, in the efflux study (study 2), almost near equilibrium conditions were seen even at a media B concentration of 3.7 mM since the exposure of cells was only for 4 hours. Thus cells exposed to prolonged periods for high media B concentrations are subjected to toxicity. This toxicity may have altered the membrane properties of the cells. This observation was further supported, when cells exposed to a medium B level of 3.70 mM had reduced levels of macro and micro-elements. This was due to leaky cell walls. Cells starved of B, absorb this element when transferred to a medium containing B within a few hours, suggesting that B is absorbed very quickly and attains a state of near equilibrium with the medium. In leaching studies conducted, a very large part of the absorbed B in cells, was leached out rapidly, and only minute quantities were left in cells as fixed B. Perhaps these minute quantities left in the leached cells are probably the important part of B which is essential for the growth and development of plants. The two metabolic inhibitors both 2,4-DNP and Dicoumarol did not show any effect on B uptake by cells. These results indicate that B enters the tomato cells through passive transport and that a passive
equilibrium exists between B concentration in the cells and in the media.

4.2 INTRODUCTION

Most plants require B as an essential micro-element for normal growth. The role of boron, as compared to other micro-elements, is unique for several reasons: It occurs as a non ionized molecule in the soil solution over a pH range suitable for normal plant growth (Oertli and Grgurevic, 1975). Unlike other micro-elements it has not yet been shown to be part of an enzyme system (Chapman and Jackson, 1974). Reviews by Dugger (1983), Parr and Loughmann (1983) and Pilbeam and Kirkby (1983) suggests a number of metabolic pathways which could be affected by boron deficiency, but the exact biochemical role of B in plant growth and development is still not completely understood.

Of the many aspects of the role of B in plant growth, short- and long-distance transport of boric acid and its complexes is well documented. Transport into and out of cells (short-distance transport) has been attributed largely to a passive equilibration since the B concentrations expressed on a cell sap basis were nearly equal to those of the external medium (Oertli, 1969; Seresinhe and Oertli, 1991). Moreover, although an essentially immobile element in plants, B is readily leached from leaf tissues by natural or artificially induced guttation and bleeding. This indicates that the immobility of this element cannot be explained by chemical fixation (Oertli 1960, Kohl and Oertli, 1961). A nutrient that tend towards a diffusion equilibrium between apoplast and symplast must be essentially immobile due to the unidirectional transpiration stream (Oertli and Richardson, 1970). However a small fraction of the B cannot be leached from the tissues and apparently fixed in plant tissues (Oertli and Grgurevic, 1975). It is not known if this is the fraction of B which is essential for plant growth. In contrast, Bowen (1968, 1969, 1972), Thellier and Tromeur (1968) and Bowen and Nissen (1976) concluded that B is actively accumulated in plants. However, there is a general agreement as to the long-distance transport of this element, i.e. the transport from the roots to the shoots occurs in the xylem and the net uptake of B by intact land plants is influenced by the rate of transpiration (Eaton, 1944; Kohl and Oertli, 1961; Oertli and
Kohl, 1961). When the bark and wood are in contact, the B levels in the bark tend to be higher, reflecting an accumulation against a concentration gradient (Oertli and Richardson, 1970). However, this was due to evaporative water loss from the bark surface, the result being a residual accumulation of B in the bark.

Most of the experimental work conducted to test the effects of B on growth and development has been either with intact plants or with algal tissues (Eichhorn and Augsten 1974, Smyth and Dugger 1981). Only a few studies have been made on the effects of B on cells in suspension culture. The latter is a better method of testing for a possible diffusion equilibrium for B between apoplast and symplast as compared to intact tissues.

The following studies were conducted to see the effects of B on tomato cells in suspension culture.

**STUDY 1.** The effect of varying media boron levels on cell growth (increase in biomass).

**STUDY 2.** (A) The efflux of intracellular B by leaching. (B) The effects of media B levels on the nutrient content of cells.

**STUDY 3.** The effect of two metabolic inhibitors on B absorption by cells.
4.3 STUDY 1. THE EFFECT OF VARYING MEDIA BORON LEVELS
ON THE BIOMASS PRODUCTION OF TOMATO CELLS

4.3.1 MATERIALS AND METHODS

4.3.1.1 Pre-treatment of cell suspensions

Cell suspension cultures which had been kept at a B concentration of 0.055 mM in an MS medium were separated from the culture medium using a sterile membrane filter unit (Sartorius Membrane-filter GMBH, Göttingen, Germany) and rinsed with an MS medium containing no B. Twenty-ml aliquots were transferred to 250 ml polymethyl-pentene flasks (Nalgene) containing 150 ml B-free MS medium and cultured. The cells were kept in a B-free medium to make them more responsive to the various B treatments.

4.3.1.2 Treatments

After 2 days, the cells were separated and resuspended in Nalgene flasks containing 100 ml of medium with or without B and cultured. In all studies deionized water and stock solutions of macro-nutrients, micro-nutrients, vitamins and hormones were purified by passage through columns of a borate-specific resin (IRA-743, Rohm and Haas, Philadelphia, USA). Instead using boro-silicate glass-ware, studies were conducted using plastic vessels to avoid possible B contamination.

In all studies the initial pH of the media was 5.8 ± 0.5 and 4.20 ± 0.10 at the end. In the initial experiment, B concentrations of 0, 0.09, 0.55, 0.92, and 1.85 mM were used. Based on the results of this experiment a second experiment was designed with low (0.005, 0.010, 0.023, 0.046 and 0.070 mM) and high (2.30, 2.77, 3.23, 3.70, and 4.15 mM) B levels.

4.3.1.3 Cell viability

See section 3.4 for a detailed description.
4.3.1.4 Determination of biomass and dry weight

See sections 3.3.1. and 3.3.2 for a detailed description.

4.3.1.5 Determination of B contents in cells

See sections 3.5.2 and 3.5.3 for a detailed description.

4.4 RESULTS

4.4.1 Biomass production

Cells in medium without B ceased to grow after day 4, whereas cells in all other treatments showed a continuous growth (Fig. 1-A:). A slight brown discolouration was observed after 3 days with cells in the absence of B, and this "browning effect" intensified with time. These cells lost their viability by day 6. There was no significant difference in growth between cells in media containing either 0.09 or 0.55 mM B, but their growth was significantly higher than cells in media containing 0.92 or 1.85 mM B.

Cells growing with low or high levels of B in the medium did not double their biomass even by day 10 (Fig. 1-B: and 1-C:). High levels of B in the media (2.30, 2.77 or 3.23 mM) did not hinder the growth of cells. Cells growing with 3.70 or 4.15 mM B in the media showed hardly any growth, and by day 8 these cells discolored and were no longer viable.

4.4.2 Intracellular B levels

The B contents in cells as affected by various concentrations of B in the media are presented in Figure 2. Except at lower B concentrations of 0.005 and 0.01 mM (Fig. 2-B:), all treatments showed a steady increase in cell B content, with maximum being approached by day 2. Thereafter, except in the high B treatments (Fig. 2-C:), the B levels did not vary to any great extent. Within the concentration range of 0 to 0.92 mM B in the medium, the B content in cells was almost in equilibrium with the B levels in the media.
Fig. 1. Biomass of tomato cells as affected by the level of B in the culture medium. (A: initial study, B: low levels, C: high levels) Vertical bars represent least significant differences (LSD s.e.).
Fig. 2. The B content of cells as affected by the level of B in the culture medium. (A: initial study, B: low levels, C: high levels [(V) 2.30, (C) 2.77, (o) 3.23, (Ø) 3.70 and (X) 4.15 mM). Vertical bars represent LSD (LSD 0.05).
4.5 STUDY 2. (A) THE EFFLUX OF INTRA-CELLULAR B BY LEACHING. (B) THE EFFECTS OF MEDIA B LEVELS ON THE NUTRIENT CONTENT OF CELLS.

4.5.1 MATERIALS AND METHODS

4.5.1.1 Pre-treatment of cell suspensions

Three media B concentrations namely 0.25, 0.55, and 3.70 mM were used. Cells maintained in a boron free medium was used as in study 1. 40 ml thick cell suspension samples were sterile filtered and the cells transferred to 300 ml treatment medium in Nalgene flasks.

4.5.1.2 Determination of fresh and dry weight

After an absorption period of 4h the fresh and dry weight of cells were determined using 10 ml suspension samples as described in sections 3.3.1 and 3.3.2.

4.5.1.3 Preparation of cell samples and the medium for B analysis

A separate cell suspension sample of 10 ml was transferred to a graduated plastic test tube and centrifuged (10 min). The supernatant was collected. The cell pellet was transferred to a filter unit, and washed quickly with 1 ml of a B free medium.

The B content in these cells (after a 4h absorption period) and in the supernatant was analyzed for B.

4.5.1.4 Determination of nutrients

See sections 3.5.2 and 3.5.3 for a detailed description.

4.5.1.5 Leaching of cellular boron

For leaching studies 10 ml cell suspensions were used. After the cells were
separated from the medium the cell pellets were transferred to a membrane filter unit containing a cellulose filter (0.8 μm). Draining of the medium under pressure was done as not to disperse the cells over the filter paper. 1x 2.5 cm plastic reaction vials with the bottom end cut, were then placed over the cells. These vials prevented the cells dispersing over the membrane filter during washing and helped to wash the cell pellet evenly.

The cells were washed quickly with 1 ml of a boron free solution and the leachate discarded. Thereafter, the cells were washed with increasing volumes (5, 10, 15, 20, 25, 30, and 40 ml) of a boron free MS medium and the leachates collected (for the 7 washings, seven separate 10 ml cell suspensions were used).

The leaching period was about 2-3 minutes for 5, 10, and 15 ml washings and about 5 minutes for 25, 30, and 40 ml washings. Thereafter the leached cells, and the leachates were used to determine B. The cells showed no effects of prolonged leaching.

4.5.1.6 The effect of media B levels on nutrient content of cells

The effect of these three B concentrations on the nutrient content of cells were also tested in this study. Therefore the cell suspensions in the Nalgene flasks were allowed to grow until the termination of the experiment. Cell samples for nutrient analysis were taken at day 0, 4, and 8. See sections 3.5.2 and 3.5.3 for a detailed description.

4.6 RESULTS

4.6.1 The B concentration of cells and in supernatants

The B content of cells when measured after an absorption period of 4 hours was 0.29, 0.51 and 3.80 mM for media B concentrations of 0.25, 0.55, and 3.70 mM respectively (Fig. 3-A:). The measured B contents in the supernatants were 0.22, 0.58, 3.59 mM B for the three media B levels (Fig. 3-B:).
4.6.2 The B concentration of leached cells and in leachates

With increasing volumes of wash solution the cells lost considerable amounts of intracellular B. This loss is well observed with cells cultured in the presence of 3.70 mM B (Fig. 4-B). Almost no measurable quantities of B were left in the leached cells after leached with 40 ml of wash solution (Fig. 4-A).

Fig. 3. The B concentration in cells and in supernatants as a function of three external media B concentrations. (A: cells, B: supernatants). Vertical bars indicate the standard error.
Fig. 4. The B concentration of leached cells after washing with 40 ml of wash solution and in the leachates as influenced by three media B levels. (A: leached cells, B: leachates). Vertical bars indicate the standard error.

4.6.3 The macro and micro-nutrient content of cells

The effects of the three media boron levels (0.25, 0.55 and 3.70 mM) on the macro and micro-nutrient contents of tomato cells are shown in Figures 5 and 6 respectively.

At the higher B level of 3.70 mM the cells had lower amounts of macro and micro nutrients by day 8.
Fig. 5. The effect of three B levels in medium on the macro-nutrient contents in tomato cells. Vertical bars represent least significant differences (LSD 0.05).
Fig. 6. The effect of three B levels in medium on the micro-nutrient contents in tomato cells. Vertical bars represent least significant differences (LSD$_{0.05}$).
4.7 STUDY 3. THE EFFECT OF TWO METABOLIC INHIBITORS ON B ABSORPTION BY TOMATO CELLS:

4.7.1 MATERIALS AND METHODS

4.7.1.1 Pre-treatment of cell suspensions

The effect of two metabolic inhibitors namely, 2,4-Dinitrophenol (DNP) and Dicoumarol (both at a concentration of 0.01 mM) were used to test their effects on B absorption by cells. A preliminary study showed that, the cells were not affected by these two inhibitors at above external media concentrations. Two external B concentrations, 0.25 and 0.50 mM were used. 20 ml B starved cell suspensions were sterile filtered. The cells were then transferred to 250 ml Nalgene flasks containing 100 ml of the treatment media containing either 2,4-DNP or Dicoumarol.

4.7.1.2 Determination of fresh and dry weight

5 ml cell suspension samples were pipetted and their fresh weight determined as described in sections 3.3.1 and 3.3.2.

4.7.1.3 Determination of B in cells

See sections 3.5.2 and 3.5.3 for a detailed description. B content in cell samples were determined at the beginning (time 0) and after transferring the cells to media containing inhibitors (30, 60, 120, 180, and 240 m).

4.8 RESULTS

Figure 7 shows that uptake of B by cells was not affected by both 2,4-DNP and dicoumarol at both external B concentrations. The B contents in cells were almost in near equilibrium with the media B levels at both B concentrations; at the higher B concentration (0.50 mM) the cells took 60 minutes to attain equilibrium with the medium whereas cells at the lower B concentration (0.25 mM) took as long as 2 hours.
Fig. 7. B uptake by cells in the absence and presence of two metabolic inhibitors. (A: cells grown with 0.25 mM B), B: cells grown with 0.5 mM B) Vertical bars indicate the standard error.

4.9 DISCUSSION

The growth of cells in the no B (0 mM) treatment (Fig. 1-A:) until day 4 was due to their use of endogenous intracellular B. Thereafter, whatever B left in cells was not used to produce biomass, suggesting that this B was fixed within the cell. When corrections are made for a free space of 25% determined by the india ink method of Bernstein and Nieman (1960), there is an indication that a small amount of fixed B was present. Using lily leaves (Oertli and Kohl, 1961), carrot and red beet tissues (Wildes and Neales, 1970) and sunflower roots (Tanaka, 1967), similar results were obtained where the tissues contained a small fraction of the absorbed B. Since the medium did
not contain any B, no growth was observed after day 4.

There is much evidence to show that B is intimately related to membrane permeability and cell wall integrity (Odhnoff, 1957; Dugger and Palmer, 1980; Dugger, 1983; Tanada, 1983; Goldbach, 1985). Results of Robertson and Loughman (1974) shows that a reduced rate of cell division under B deficiency is not directly attributable to the availability of B but rather to its involvement in the metabolism, transport or action of auxins. Other authors have postulated the direct involvement of B in nucleic acid metabolism in the cell (Shkolnik and Soloveva, 1962; Albert, 1965). From the above conducted studies it is not possible to say which of the above factors, if any, limit cell division and growth in the absence of B in the medium. Cells growing with very low levels of B (Fig. 1-B:), failed to double their biomasses even by day 10. This suggests that the B concentrations in the medium were suboptimal. At such low levels of B, the rate of cell division was reduced. Kirk and Loneragen (1988), observed a depressed root growth in B deficient primary and lateral roots and in leaves of soya plants. Since B is apparently important in the processes of cell growth and differentiation, and because of its very limited mobility within the plant, the observations by Kirk and Loneragen (1988) were most likely due to reduced meristematic activity under B deficiency. With an increase in B concentrations to 0.92 mM and more (Fig. 1-A: and 1-C:), the reduced biomass as compared with 0.09 and 0.55 mM B in medium, is attributed to increasing B toxicity with increasing B levels in medium. However, the B content of cells growing with 0.92 mM B in the medium (Fig. 2-A:) still appears to be in near equilibrium with medium B, but this relationship is no longer observed at B levels of 1.85 mM and higher (Fig. 2-C:). This toxicity is due to an alteration of the cell membranes. In contrast to this level of toxicity, Oertli and Kohl (1961), observed occasional symptoms of B toxicity when the concentration in the root medium was as low as 0.1 mM. In the efflux experiment (study b), the cell B content is in near equilibrium with that of the medium even at a higher B level of 3.70 mM (Fig. 3-A:). In this study the cell B contents were determined after a time elapse of only 4 hours as compared with cells in study 1 (B contents determined after 2 days). Therefore the time elapse of 4 hours was not long enough for the cells to show
Abundant evidence indicates that B is transported in the transpiration stream and that it accumulates as water is transpired from the leaf. Thus, leaf cells are exposed to higher B concentrations than roots. Oertli and Kohl (1961), found the first visual symptoms of B injury at leaf concentrations of 9.0 mmol (Kg.Fw)\(^{-1}\) and necrotic tissues above 14 mmol (Kg.Fw)\(^{-1}\). The latter level is several times higher than the 3.7 mM level (study 1), where visual symptoms of B injury were observed in cell cultures. It thus appears that the cells in culture and intact plants differ greatly in their sensitivity to B. The reduction in cell biomass beginning at a concentration of 0.92 mM (Fig. 1-A:), suggests that cells suffer from B toxicity at lower concentrations than those causing visual symptoms. When compared with cells in culture, the situation in intact plants is variable due to the uneven distribution of B. Old leaves may show symptoms of B toxicity, but the biomass production is slightly affected since the younger leaves, which are mainly responsible for photosynthesis, may contain a low and non-injurious level of B. Figure 2 shows a maximum cell B content when first measured at day 2, whereas in the leaching experiment (Fig. 3-A:) the cells took as little as four hours. In study 3 with metabolic inhibitors, depending on the external B concentration the cells took only 1-2 hours (Fig. 7-A: and 7-B:). These results indicate that B is taken up within a very short time after B starved cells are transferred to their respective media.

In these studies it is seen that the B content of cells tend to come into a near equilibrium with the external B concentration. For example the equilibrium observed between cell B concentration and medium B levels upto 0.92 mM (Fig. 2-A:) shows that a diffusion equilibrium exists within this range when measured at day 2. Similar results were observed by Oertli (1969), showing that the B concentration within and outside cells are nearly equal and that a state of passive equilibrium is reached within a short time. Bowen and Nissen (1977), could not find any accumulation against a concentration gradient, but they concluded that B uptake in barley roots satisfied many criteria for an active uptake since this transport process was compatible with the multiphasic uptake mechanisms proposed by Nissen (1971, 1974). They also concluded that uptake was temperature sensitive, being nil at 2°C and maximal at 34 to 38°C.

The two metabolic inhibitors both 2,4-DNP and Dicoumarol are known to affect the
cells in two ways. First these substances act by the uncoupling action on oxidative phosphorylation and thereby cell respiration, secondly there is indirect evidence that they also regulate membrane functions (Neumann and Jagendorf, 1965). Therefore such substances should have an effect on ion transport processes, especially those ions which move against a concentration gradient, for which energy is necessary. In this study the cells in the presence of inhibitors had almost the same B contents as the controls suggests that their presence in the medium was not inhibitory for B uptake (Fig. 7-A: and 7-B:). The near equilibrium conditions observed between control cells and cells in the presence of inhibitors therefore suggests that an active component is not involved in B absorption and that B enters the tomato cells through passive transport. Bingham et al., (1970) using 2,4-DNP and KCN as inhibitors showed that both compounds had no effect on B absorption by excised barley roots and concluded that the process of B absorption is non-metabolic. In contrast, using four metabolic inhibitors including 2,4-DNP and Dicoumarol, Bowen and Nissen (1977), showed that active B uptake by barley roots from 1 mM B solutions was inhibited. However, in their studies the unsolved question was the apparent "partial inhibition" of B uptake, where the observed inhibitions were in the order of about 29% and 58%, for 2,4-DNP and Dicoumarol respectively. These values are very much lower, where reductions in the order of 80%-90% have been observed for nearly all metabolic inhibitors. For e.x. Ordin and Jacobson (1955), observed a reduction in K⁺ absorption by excised barley roots by 90% in the presence of fluoride in culture medium. Using the algae Hydrodictyon africanum, Raven (1969b) showed, that the active influx of K⁺ was inhibited as high as by 80% as compared with the control when CN⁻ was present as the inhibitor.

For an uncharged substrate, transport against the concentration gradient is, by definition, the principal criterion for active uptake. In study 1, such a diffusion equilibrium is no longer apparent at higher external B concentrations (1.85 mM and more) (Fig. 2-A: and 2-B:), an indication that the cells were injured. Under such conditions, the cell interior was leached as rapidly as the cell wall free space, whereas in an intact cell without injury such a leaching may be slow. The fact that high media B levels affect the cell walls and perhaps also membranes was further supported when
the macro- and micro-nutrient contents of cells were determined in study b) (Figs. 5 and 6), where all these nutrients were low at an external B concentration of 3.70 mM when determined at day 8. Therefore, due to leaky cell walls, the B content of cells, in the presence of high concentrations of B in the medium, was also low due to leaching during the process of rinsing. As evident from leaching studies, a large part of the absorbed B could be leached out (Fig.4-B:). Assuming that the cells contain 90% water, on a dry weight basis the calculated B content of leached cells (external B concentration 0.25 mM) is about 0.04 ppm. (Fig.4-A:). When compared with those results of Bingham, et al., (1970), after the desorption period, barley root tissues contained about 5% of the initially absorbed B, whereas the leached tomato cells contained about 1.5% of the initially absorbed B (initially about 0.258 mM). The differences in the B concentration between leached tomato cells and barley root tissues (Bingham et al., 1970) were probably due to the differences in the desorption period.

These studies show that cells starved of B absorb this element within a very short time when they are transferred to a medium containing B. The B concentration in cells are in near equilibrium with media B levels, and the insensitivity of cells to metabolic inhibitors present in the medium suggests, that an active component is not involved and B absorption by tomato cells in suspension culture occurs through passive absorption.
5 THE EFFECT OF VARYING MEDIA MANGANESE LEVELS ON THE GROWTH OF TOMATO CELLS IN SUSPENSION CULTURE

5.1 ABSTRACT
The effects of four Mn levels in the culture medium on the biomass production, nutrient content, cell division and their viability were studied using tomato cells in suspension culture. The biomass and the dry weight was highest at Mn levels of 0.002 and 0.1 mM when compared with cells grown with 0 or 0.2 mM Mn in the medium. Cells cultured with 0.2 mM Mn in the medium turned black by day 6. Cells grown without added Mn (0 mM) turned brown by day 8. The total number of cells as well as the number of viable cells were lowest with Mn levels of 0 and 0.2 mM. Mn levels of 0.2 and 0 mM in the medium led to toxicity and deficiency respectively. By day 8 all the macro and micro-elements of cells grown either with 0.2 or 0 mM Mn were low. This was due to the change in the cell membrane permeability which led to the leaching of cell contents, at medium Mn levels of 0 and 0.2 mM. There were no significant differences in the nutrient content of cells over a Mn range of 0.1 and 0.002 mM in the medium. In spite of a concentration difference of 50 fold, tomato cells showed no deleterious effects to medium Mn concentration within the range of 0.002 and 0.1 mM.

5.2 INTRODUCTION
Manganese, an essential micro-nutrient for all higher plants, is absorbed as Mn$^{2+}$. The specific requirement for Mn, presumably is related to its tightly bound form in metalloproteins or like iron in its function in a redox system [Mn$^{2+}$/Mn$^{3+}$].

It is translocated predominantly as the free divalent cation in the xylem from the roots to the shoots (Graham, 1979). Unlike other essential trace elements such as Cu, Zn, Fe and Mo which are integral components of enzymes, Mn usually acts as an activator of enzymes and can be replaced by other metal ions. It resembles Mg in its biochemical functions and is involved in activating enzyme-catalyzed reactions such as phosphorylations, decarboxylations, reductions and hydrolysis reactions and therefore
affects processes such as respiration, amino acid synthesis, lignin biosynthesis and the level of hormones in plants. The most important role of Mn in nature is its involvement in oxygen evolution in photosynthesis in higher plants. The protein which catalyses the water splitting reaction which is located in the thylakoid membrane, consists of four Mn atoms (Amesz, 1983; Babcock, 1987; Prince, 1986). Besides this important role, it is also an important structural component of the Mn-superoxide dismutase (Mn-SOD), which is present in the chloroplast, and protects the photosynthetic apparatus from the oxygen free radical - superoxide. The inactivation of this free radical is catalyzed by Mn-SOD (Elstner, 1982).

Mn is also an important cofactor for a number of key reactions involved in the biosynthesis of plant secondary metabolites, such as caffeine, coumaric, chlorogenic acid etc., which are known as important phenolic compounds of the plants defence system (Friend, 1978, 1981).

Mn levels in the plant also affect the level of auxin in plants by influencing the level of IAA oxidase (Morgen et al., 1976) and in being a co-factor involved in the synthesis of IAA (Gross, 1980). Though not well understood it seems that Mn is also important in root nodule formation in legumes. However, the necessity for an IAA gradient within the root for effective nodulation is consistent with all available data (Hewitt, 1970; Kenten, 1955; Wagenkneckt and Burris, 1950).

Manganese differs from the other micro-nutrients in three important ways. Firstly, the insoluble oxide or oxide-Mn serves along with soil solution Mn$^{2+}$, as a source of this element to plants. Secondly, the concentration of Mn$^{2+}$ in the soil solution can vary by orders of magnitude within very short time spans. Finally, Mn may be accumulated by plants in amounts exceeding requirements by a factor of 50 or more. Generally plant needs are met at tissue levels of 20-40 mg·Kg$^{-1}$ dry matter, and toxic levels of 400-2000 mg·Kg$^{-1}$ dry matter (Foy et al., 1978; Labanauskus, 1966). Plants require a continuous supply of Mn, since reuse within the plant is limited, and particularly so at low level of supplies (Marcar and Graham, 1986; Nable and Loneragan, 1984; Robson and Loneragan, 1970). Nutritionally adequate levels are supplied by nutritional solutions containing 0.002-
0.005 mM of the element (Hoagland and Arnon, 1950; Reisenauer, 1969; Vlamis and Williams, 1962; 1967). In contrast to these levels of Mn in solution cultures, Mn levels in tissue culture media vary from 0.045 mM in B₅ medium (Gamborg et al., 1968) to 0.1 mM the highest in the MS medium (Murashige and Skoog, 1962). Therefore the Mn concentration in Gamborg's and MS medium exceeds by factors of 22 and 50 respectively when compared with the Mn concentration in the Hoagland solution.

Studies pertaining to test the effects of Mn on suspension culture systems are scarce. Most of the experimental work done to see the effects of Mn on growth and development has been conducted either with intact plants (Amberger, 1973; Graham, 1983; Nable and Loneragen, 1984; Kriedemann et al., 1985; Prince, 1986) or with tissues (Abbot, 1967; Possingham et al., 1964; Neumann and Steward, 1968). Therefore following studies were undertaken to see the effects of media Mn on the growth of tomato cells in suspension culture.

**STUDY 1.** The effects of varying media Mn levels on the biomass production and nutrient content of tomato cells.

**STUDY 2.** The effects of three Mn levels in the culture medium on the total number of cells (cell division) and their viability.
5.3 STUDY 1. THE EFFECTS OF VARYING MEDIA MANGANESE LEVELS ON THE BIOMASS PRODUCTION AND NUTRIENT CONTENTS OF TOMATO CELLS IN SUSPENSION CULTURE

5.4 MATERIALS AND METHODS

5.4.1 Cell suspensions

Tomato cell suspension cultures which had been kept at a Mn concentration of 0.1 mM in a MS medium, were used.

5.4.2 Pre-treatment of cell suspensions

Cell suspensions were first sterile-filtered and rinsed four times with an MS medium containing no Mn. The cells were resuspended in a medium without Mn, and 20 ml aliquots were transferred to 250 ml Erlenmeyer flasks containing 150 ml of Mn-free medium and cultured. In an initial test it was observed that cells treated as above could survive in a medium without added Mn for about 14 days and thereafter they lost their viability.

5.4.3 Treatments

After 10 days the cells were separated from the medium and resuspended in a medium without Mn. Twenty ml aliquots were then sterile filtered and the cells washed into 500 ml Erlenmeyer flasks using the treatment medium. The final volume of the cell suspensions were adjusted to 100 ml with treatment medium, with (0.1, 0.2, 0.002 mM) or without (0 mM) Mn.

5.4.4 Determination of biomass and dry weight

The biomass and the dry weight of cells were determined as described in sections 3.3.1 and 3.3.2 respectively. 10 ml cell suspension samples were used for the determinations.
5.4.5 Determination of nutrients in cells

See sections 3.5.2, 3.5.3 and 3.5.4 for a detailed description.

5.5 RESULTS

5.5.1 Biomass production and dry weight of cells

The effect of varying levels of media Mn on the biomass production is shown in Figure 1. The differences in biomasses under different treatments were first observed on day 4. Irrespective of the Mn\(^{2+}\) concentration in medium the biomass of cells doubled in all treatments in about three days. Cells growing in media either with 0.01 mM or 0.002 mM showed a continuous growth. The biomass of cells in the presence of 0 mM and 0.2 mM Mn in medium showed a decreasing trend beginning from day 6. Cells in the presence of 0.2 mM Mn showed a black discoloration by day 6 and those in the presence of 0 mM Mn turned brown by day 8. Cells in both latter treatments were not viable by day 10.

The dry weights of cells under varying Mn treatments are shown in Table 1. The dry weight of the cells followed the same pattern as the biomass production.

5.5.2 The nutrient content in cells

The macro and micro-nutrient contents of cells are shown in Tables 2 and 3 respectively. There were no significant differences in nutrient content of cells growing at Mn levels of 0.1 and 0.2 mM. Cells growing with 0.2 mM added Mn had lower Mg contents when measured at day 2. Cells growing either with a high (0.2 mM) or no (0 mM) Mn in medium had low amounts of all macro-nutrients at day 8, especially the reduction in K level is higher than for any other macro-nutrient. Cells growing in the presence of 0.2 mM Mn had lower Zn, Cu, and Fe whereas cells cultured without added Mn (0 mM) had higher levels of all these micro-elements. By day 8 cells growing either with 2.0 mM or 0 mM Mn in medium had lower contents of Zn, Cu and Fe as compared with cells receiving either 0.1 or 0.002 mM added Mn.
Fig. 1. The effect of four Mn levels in medium on the biomass production of tomato cells in suspension culture. Vertical bars indicate the standard error.

Table 1 The effect of varying Mn levels in media on the dry weight (mg) of cells

<table>
<thead>
<tr>
<th>Mn mM</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
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<td>4.51a</td>
<td>4.60a</td>
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<tr>
<td>0.002</td>
<td>1.45a</td>
<td>1.64a</td>
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<td>4.23a</td>
<td>4.45a</td>
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</tr>
<tr>
<td>0.2</td>
<td>1.46a</td>
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<td>3.36b</td>
<td>3.06b</td>
<td>2.85b</td>
</tr>
<tr>
<td>0</td>
<td>1.49a</td>
<td>1.71a</td>
<td>3.33a</td>
<td>3.18b</td>
<td>3.01b</td>
<td>2.83b</td>
</tr>
</tbody>
</table>

Values followed by the same letter within a column are not significantly different at the P=0.05 level as determined by the Duncan’s Multiple Range Test.
Table 2 The effect of varying Mn levels in media on the macro-nutrient contents (% Dw) in cells

<table>
<thead>
<tr>
<th></th>
<th>Mn mM</th>
<th>N</th>
<th>P</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAY 0</td>
<td>0</td>
<td>3.03 a</td>
<td>0.88 a</td>
<td>6.01 a</td>
<td>1.06 a</td>
<td>0.61 a</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>3.33 a</td>
<td>0.91 a</td>
<td>5.93 a</td>
<td>0.94 a</td>
<td>0.51 a</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3.20 a</td>
<td>0.96 a</td>
<td>5.91 a</td>
<td>1.01 a</td>
<td>0.58 a</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>3.37 a</td>
<td>0.90 a</td>
<td>6.00 a</td>
<td>1.05 a</td>
<td>0.59 a</td>
</tr>
<tr>
<td>DAY 4</td>
<td>0</td>
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<td>1.00 a</td>
<td>5.86 a</td>
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<td></td>
<td>0.1</td>
<td>3.23 a</td>
<td>1.02 a</td>
<td>6.02 a</td>
<td>0.94 a</td>
<td>0.63 a</td>
</tr>
<tr>
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<td>2.99 a</td>
<td>0.95 a</td>
<td>5.88 a</td>
<td>0.98 a</td>
<td>0.37 b</td>
</tr>
<tr>
<td>DAY 8</td>
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<td>1.99 b</td>
<td>0.59 b</td>
<td>2.00 b</td>
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</tr>
<tr>
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<td>0.91 a</td>
<td>6.05 a</td>
<td>0.96 a</td>
<td>0.54 a</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.96 b</td>
<td>0.67 b</td>
<td>1.97 b</td>
<td>0.30 b</td>
<td>0.24 b</td>
</tr>
</tbody>
</table>

Values within a column followed by the same letter are not significant at the P=0.05 significance level as determined by the Duncan’s Multiple Range Test.
Table 3 The effect of varying Mn levels in media on the micro-nutrient content (µg g⁻¹ dry weight) of cells

<table>
<thead>
<tr>
<th>Day</th>
<th>Mn mM</th>
<th>Mn</th>
<th>Zn</th>
<th>Cu</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>11.60 a</td>
<td>64.18 a</td>
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<td>140.59 a</td>
</tr>
<tr>
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<td>0.002</td>
<td>11.43 a</td>
<td>64.10 a</td>
<td>30.94 a</td>
<td>140.55 a</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>11.49 a</td>
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</tr>
<tr>
<td></td>
<td>0.2</td>
<td>11.28 a</td>
<td>63.85 a</td>
<td>30.34 a</td>
<td>140.22 a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>Mn mM</th>
<th>Mn</th>
<th>Zn</th>
<th>Cu</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0</td>
<td>9.68 c</td>
<td>68.53 c</td>
<td>32.99 b</td>
<td>150.59 c</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>79.43 a</td>
<td>56.40 a</td>
<td>26.90 a</td>
<td>138.55 a</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>81.11 a</td>
<td>57.45 a</td>
<td>27.72 a</td>
<td>139.87 a</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>120.38 b</td>
<td>51.72 b</td>
<td>22.43 a</td>
<td>126.22 b</td>
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</tbody>
</table>

<table>
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<th>Day</th>
<th>Mn mM</th>
<th>Mn</th>
<th>Zn</th>
<th>Cu</th>
<th>Fe</th>
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<tbody>
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<td></td>
<td>0.002</td>
<td>79.86 a</td>
<td>55.63 a</td>
<td>25.91 a</td>
<td>119.83 a</td>
</tr>
<tr>
<td></td>
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<td>25.22 a</td>
<td>117.61 a</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>98.24 b</td>
<td>49.21 b</td>
<td>19.73 b</td>
<td>96.20 b</td>
</tr>
</tbody>
</table>

Values followed by the same letter within a column are not significant at the P=0.05 significance level as determined by the Duncan's Multiple Range Test.
5.6 STUDY 2. THE EFFECT OF THREE MANGANESE LEVELS IN THE CULTURE MEDIUM ON THE TOTAL NUMBER OF CELLS AND THEIR VIABILITY

5.6.1 MATERIALS AND METHODS

5.6.1.1 Pre-treatment of cell suspensions

Cells growing in a MS medium with a Mn concentration of 0.1 mM were separated from the medium using a sterile membrane filter unit. The cells on the filter were then washed several times with a MS medium containing no added Mn. The cells were then transferred to a medium without added Mn and allowed to grow for 12 days. Thereafter the cells were separated from the culture medium and resuspended in 250 ml Erlenmeyer flasks containing 100 ml of medium with (0.2 mM, 0.1 mM) or without Mn.

5.6.1.2 Cell counting

The first count was taken immediately after the cells were introduced to their respective media. Cell aggregates were first separated as described in section 3.3.3. The total number of cells and the total viable cells were determined microscopically as described in section 3.4.

5.7 RESULTS

5.7.1 Total number of cells and cell viability

Varying media Mn levels showed no significant differences in the total number of cells when determined by day 4. Thereafter no significant increase in the cell number was observed in treatments receiving either 0 or 0.2 mM Mn. Whereas a continuous increase in the cell number was observed for cells growing with a Mn level of 0.1 mM. At Mn concentrations of 0 and 0.2 mM the number of non-viable cells increased dramatically with time (Fig. 3). When compared with the latter treatments, the variation in the number of viable cells at an external Mn concentration of 0.1 mM was very low.
Fig. 2. The effect of three Mn levels in the medium on the total number of cells (A:), and their viability (B:). Vertical bars indicate the standard error.

5.8 DISCUSSION

There could be two explanations for the ability of cells to grow with no added Mn (0 Mn) in the medium until day 4 (Fig. 1). Firstly, though the cells were grown in a medium without Mn and starved for some time, they would still have contained some Mn at the beginning of the experiment, and the "carry over", may have sufficed to sustain the cells for a few days. Secondly, though not in large amounts, some of the salts used for the formulation of the medium contain Mn as an impurity (e.g. both FeSO₄·7H₂O and ZnSO₄ contains about 0.05% Mn); since micro-nutrients are needed in minute quantities, even very low levels, well below optimum quantities would nevertheless enable the cells to maintain their activities for some days. Whatever Mn was left after day 4 however was not used by the cells for further biomass production,
probably indicating that it was fixed within the cells. Since Mn is an essential element for all higher plants, the growth of cells was limited after day 4. Thus it can be seen from figure 2-A that there was little variation in the number of cells following day 4, indicating that the rate of cell division was impaired when there was no added Mn in the medium. The brown color observed by day 8 indicates that the cells were dying due to a deficiency of Mn in the medium. Cells growing in a medium deficient of Mn loose their viability as seen in figure 2-B, where almost one third of the cells were non viable by day 12.

Cells growing in the presence of 0.2 mM Mn in the medium showed a black discoloration by day 8 indicating that the cells were suffering from high levels of intracellular Mn, leading to toxicity. This is indicated by the high Mn\(^{2+}\) content in the cells at day 4 (Table. 3). The Mn content of cells receiving 0.2 mM Mn was about 120 \(\mu\)g (g dry weight)\(^{-1}\)(Dw) against about 80 \(\mu\)g(g Dw)\(^{-1}\) for 0.1 and 0.002 mM Mn levels. High Mn levels in the medium arrest both cell division and cell viability (Fig. 2-A and 2-B). The common Mn toxicity symptoms observed with whole plants growing in a field or in culture solution include crinkling of the leaf in conjunction with chlorosis and brown or black spots; symptoms are often more severe on the older leaves. Later appearance of the black spots on leaves is attributed due to a greatly increased polyphenol oxidase activity and associated malfunction in phenolic and lignin metabolism (Higuchi, 1981), or the deposition of oxides of Mn (Horst and Marschner, 1978). Whether the observations made by Horst and Marschner, (1978) are also applicable to the blackening tomato cells at higher Mn concentrations is not known. Nable and Loneragan (1984), using a split-root system showed that growth of roots without an external Mn supply was 50% less as compared to roots of the same plant receiving an adequate supply of Mn from the external solution. In Mn deficient plants the formation of lateral roots ceased completely (Abbott, 1967). Reduction in root growth in the above studies is most likely due to reduced meristematic activity. The Mn status of plants has profound effects on the auxin balance of tissues and an imbalance in Mn nutrition could impair both cell division and cell elongation (Neuman and Steward, 1968). There is ample evidence to show that Mn might be a cofactor for
indole acetic acid oxidase system (Morgan et al., 1976). Morgan et al., (1976) showed that both deficient and toxic Mn levels in leaves leads to exceptionally high IAA oxidase activity which might lead to enhanced auxin degradation (IAA) in tissues. In the present experiment it is not clear whether the reduced rate of cell division in treatments receiving 0.2 and 0 mM Mn was due to an imbalance in auxins.

No significant differences were observed in the biomasses (Fig. 1) and dry weights (Table 1) of cells receiving 0.1 or 0.002 mM Mn, despite the fifty fold concentration difference. This suggests that the tomato cells in suspension culture are relatively tolerant to Mn within this concentration range.

The low levels of macro-nutrients determined by day 8 (Table. 2) in cells receiving 0.2 and 0 mM Mn is explained by the fact that toxicity (0.2 mM) as well as deficiency (0 mM) led to the death of cells. In such cells the membrane integrity is impaired. This leads to leaching of cell contents especially K, the most abundant cation in cells. That excess Mn in the medium (0.2 mM) depresses the uptake of Mg (Table. 2) has also been observed by other workers. Work done with soybeans (Heenan and Campbell, 1981), tomatoes (Le Bot et al., 1990) and with beans (Horst and Marschner, 1977) showed that toxic levels of Mn in the growing medium decrease both the Mg and the Ca content of plants. Heenan and Campbell (1981) assume that apart from depression in Mg uptake, excess Mn in the growth medium in some way blocks the binding sites for Mg. In contrast to Mg, the K contents in cells did not vary much within treatments by day 4. That toxic levels of Mn in the medium do not have direct effects on K levels has also been observed by Heenan and Campbell (1981) in their studies.

By day 4 cells cultured with no Mn in the medium had higher levels of Zn, Cu and Fe than cells supplied with either sufficient (0.1 and 0.002 mM) or toxic levels (0.2 mM) of Mn. This shows that absorption of these three elements was enhanced by a lack of Mn in the medium (Table 3). In contrast, higher levels of Mn in the medium depressed the uptake of Fe by cells. Such interactions between Mn and Fe have been observed by many workers (Sommers and Shive, 1942; Brown and Jones, 1977; Heenan and Campbell, 1983).

The critical deficiency levels of Mn are between 10-20 mg (g Dw)$^{-1}$ in mature leaves
(Ohki et al., 1970) and surprisingly this range is consistent regardless of the plant species or cultivar or the prevailing environmental conditions. In the present experiment cells receiving no Mn contained only about 10 and 6 µg (g Dw)$^{-1}$ determined by days 4 and 8 respectively. The latter values are very much lower than those obtained by Ohki et al., (1970). This was due to the loss of cell contents caused by leaching. In contrast to this narrow range of critical deficiency levels in leaves, the critical toxicity levels vary widely among plant species and environmental conditions. Even within a species, the critical toxicity levels can vary by a factor of about 3 to 5 among cultivars (Edwards and Asher, 1982). Using a large number of plant species they showed that at a level causing critical toxicity (defined as 10% reduction in dry weight), the shoots of maize and sunflower contained 200 and 5300 mg (g Dw)$^{-1}$ Mn respectively. Tomato plants grown with 50 µM Mn in the medium contained relatively high Mn concentrations of 916 µg (g Dw)$^{-1}$ and 2085 µg (g Dw)$^{-1}$ in the shoots and in the older leaves respectively (Lê Bot et al., 1990). Even at these higher Mn levels they could not observe any Mn toxicity symptoms. The values for growing tomato leaves are thus very much higher than the values obtained with tomato cells in suspension culture with 0.2 mM added Mn (day 4)( Table 3). It appears thus, that tomato cells in suspension culture, cultured with 0.2 mM Mn show far greater sensitivity to critical concentrations of Mn than whole plants.
6 THE EFFECT OF IODINE IN THE CULTURE MEDIUM ON THE GROWTH OF TOMATO CELLS IN SUSPENSION CULTURE

6.1 ABSTRACT

The effect of iodine (I₂) supplied as potassium iodide in the culture medium on the biomass production, dry weight and its effect on other mineral element composition was tested using tomato cells in suspension culture. No significant differences in biomass or dry weight of cells were observed with medium I⁻ levels of 0, 5, and 7.5 μM. However a growth stimulating effect was observed with cells grown with added I⁻ levels of 5 and 7.5 μM in the medium. Though not significant cells grown with the latter two I⁻ levels in medium had slightly higher levels of all nutrients when determined at day 6 and 12. An I⁻ level of 10 μM in the medium produced toxicity effects on cells and by day 12 these cells contained significantly less amounts of nutrients. In uptake studies done using labelled I¹²⁵ the cells contained 50 ng g⁻¹ fresh weight of this element and the cells absorbed only 0.17% from the total available iodide in medium.

6.2 INTRODUCTION

The accumulation and metabolism of certain elements which are not yet considered to be essential for higher plants are well reviewed (Bollard and Butler, 1966; Foy et al., 1978; Bollard, 1983). In these reviews the relationships of seven such elements-aluminium, iodine, selenium, chromium, cobalt, nickel and vanadium- to plant growth and metabolism are summarized. The interest shown among workers to these specific elements for plant growth and development has arisen for several reasons.

* Some are undisputably essential elements for animal nutrition and there is interest in the factors controlling their levels in plants so that animal nutrition is adequate.
* Although all of them are inhibitory or toxic to plant growth when supplied to plants at relatively low concentrations, there are numerous reports to indicate
the stimulation of growth when they are supplied at still lower concentrations.
* Plants vary considerably in their ability to tolerate potentially toxic levels of these elements and interest has developed in the mechanism of this tolerance and nature of its genetic control.
* Whether these elements are essential or not there is interest in their mobility within the plant, in the chemical forms in which they occur, and in the ways in which they may interfere with plant metabolism.

The role of I$_2$ on terrestrial plant development and their metabolism began first with the discovery of I$_2$ in sea-weed ash by Bernard Courtois towards the end of 1811. The first systematic chemical inquiry into the distribution of I$_2$ in the terrestrial mass, was begun by the French botanist and chemist Gaspard Adolpe Chatin, to whom belongs the credit of showing for the first time that deficiency of I$_2$ in man's environment is associated with the occurrence of goiter.

Presently the amount of I$_2$ in the environment is important mainly for two reasons. Firstly it is an essential nutrient element for animals, and secondly radioactive isotopes of I$_2$ (Ex. I$^{131}$: half life $1.6 \times 10^7$ years) are produced during nuclear fission. Iodine is widely distributed in nature both as organic and inorganic forms. It is the least abundant element of the halogen group, and is found in sea water, in mineral springs and in a few rare minerals such as laurite, calcium iodate and silver iodide.

One of the richest sources of iodine is sea-water, which contains about 50 $\mu$g-kg$^{-1}$ water (Shaw, 1959). In the brown sea-weed Laminaria digitata, I$_2$ forms one percent of the dry weight, thus representing a 30,000 fold concentration as compared with sea water (Shaw, 1959).

The range for Iodine in soils is 0.6 - 8 ppm with exceptional values upto 70 ppm, most of which is water soluble (Mitchell, 1955).

Studies relating the effects of Iodine on higher plants included the effects of this element in various formulations (both organic and inorganic salts) added to soils in the field, soil cultures, and nutrient solution cultures. In some cases stimulatory effects were recorded (Stoklasa, 1924; Powers, 1939; Umaly and Poel, 1970), in others Iodine was
found to be inhibitory (Fellenberg, 1925; Lewis and Powers, 1941), and some workers found no effect of added I₂ (Hagemann et al., 1942; Newton and Toth, 1962). In most of these early studies the results are difficult to evaluate since the conditions of treatment were often ill defined.

Therefore the aim of the present investigation was to examine more quantitatively the effects of iodide supplied as potassium iodide on the growth, mineral nutrient composition, and the uptake of labelled iodine by tomato cells in suspension culture.

6.3 MATERIALS AND METHODS

6.3.1 Pre-treatment of cell suspensions

Cell suspension cultures kept at a iodide (I⁻) concentration of 5 μM were sterile filtered and washed several times with a MS medium containing no I⁻. The cells were then resuspended in a I⁻ free medium for about five days to make the cells more responsive to the various treatments.

6.3.2 Treatments

The cells were then separated from the culture medium and resuspended in 250 ml erlenmeyer flasks containing 150 ml of medium with (5, 7.5 and 10 μM) or without (0 μM) I⁻.

6.3.3 Determination of biomass and dry weight of cells

See sections 3.3.1 and 3.3.2 for a detailed description.

6.3.4 Determination of mineral elements

See section 3.5.2, 3.5.3 and 3.5.4 for a detailed description.

6.3.5 Labelled I⁻ uptake by tomato cells

Labelled NaI (Iodine-125 with a half-life period of 60 days) was obtained through Paul Scherrer Institut, Würenlingen, Switzerland. 0.2 μl labelled NaI with an activity of 3.7 MBq (0.1 mCi) was dissolved in 10 ml of bi-distilled water, and 200 μl from the stock solution was added to 50 ml of cell suspension. At pre-determined intervals 5 ml cell samples were pipetted and filtered. The cells were washed with 2 ml of water and transferred to pre-weighed 20 ml scintillation polyethylene vials and the fresh weight determined. 0.5 ml of a tissue solubilizer (Soluene 100, Packard Instruments) was added
and the vials incubated at 40°C for 24 h. To each vial 10 ml of a liquid scintillation cocktail (Ultima Gold-Packard Instruments) was added and the vials subjected to liquid scintillation counting using a Liquid scintillation analyzer (Model A-500 TR, Packard Instruments). The contribution of labelled (Na-$^{125}$I) and unlabelled I (I$_2$ supplied as KI in MS medium) was 0.18 and 31750 ng/L respectively.

6.4 RESULTS

6.4.1 Biomass production and dry weight of cells

Cells growing with 10 μM I$^{-}$ in medium failed to grow after day 6 (Fig. 1). The biomass of cells growing either with 0, 5, or 7.5 μM I increased continuously reaching almost a maximum by day 12. Though there were no significant differences between the latter three treatments, cells grown with added KI in medium produced more biomass than cells grown without added KI.

![Biomass production and dry weight of cells](image)

Fig. 1. The effect of four iodide levels in the medium on the biomass production of tomato cells. Vertical bars indicate the standard error.
The dry weight of cells followed the same pattern as the biomass production (Table. 1).

**Table. 1** The effect of four iodide levels in the medium on the dry weight (mg) of tomato cells (average ± standard error). Values given are the averages of four separate experiments conducted.

<table>
<thead>
<tr>
<th>Days in culture</th>
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<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>5</td>
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<td>4.3±0.25</td>
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</tr>
<tr>
<td>7.5</td>
<td>1.9±0.07</td>
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</tbody>
</table>

6.4.2 The macro and the micro-nutrient content of cells

By day 6, the cells grown with 0 μM I level in the medium had slightly higher levels of P, K, Ca, and Mg. Low levels of both macro and micro-nutrients were determined for cells growing with 10 μM I in medium when measured on day 12 as compared with cells growing with I levels of 0, 5, and 7.5 μM in medium (Tables. 2 and 3). The nutrient contents of cells between the latter three treatments did not vary with each other significantly by day 12.

6.4.3 Uptake of labelled I by cells

The I content of cells at different sampling periods is shown in Figure 2. A saturation curve was obtained at the end of the 3 h uptake period. The amount of I absorbed by cells from the available I in medium is shown in table 4. At the end of the 3 h uptake period the cells contained only 0.17% I from the total available I in the medium.
Table 2 The effect of four I levels in the culture medium on the macro-nutrient contents (% Dw) of tomato cells (average ± standard error)

<table>
<thead>
<tr>
<th>Gamma (µM)</th>
<th>N</th>
<th>P</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAY 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.2 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>6.14 ± 0.12</td>
<td>0.98 ± 0.05</td>
<td>0.64 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>3.3 ± 0.5</td>
<td>0.8 ± 0.5</td>
<td>6.12 ± 0.10</td>
<td>1.02 ± 0.04</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>7.5</td>
<td>3.1 ± 0.8</td>
<td>0.9 ± 0.7</td>
<td>6.00 ± 0.09</td>
<td>1.01 ± 0.1</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>10</td>
<td>3.2 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>6.03 ± 0.07</td>
<td>0.97 ± 0.1</td>
<td>0.57 ± 0.05</td>
</tr>
<tr>
<td><strong>DAY 6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>6.85 ± 0.05</td>
<td>0.95 ± 0.05</td>
<td>0.59 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>3.4 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>7.05 ± 0.5</td>
<td>1.02 ± 0.06</td>
<td>0.68 ± 0.06</td>
</tr>
<tr>
<td>7.5</td>
<td>3.2 ± 0.2</td>
<td>1.2 ± 0.6</td>
<td>7.04 ± 0.16</td>
<td>1.05 ± 0.03</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>3.3 ± 0.4</td>
<td>1.1 ± 0.2</td>
<td>7.10 ± 0.12</td>
<td>0.97 ± 0.03</td>
<td>0.67 ± 0.02</td>
</tr>
<tr>
<td><strong>DAY 12</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.7 ± 0.1</td>
<td>0.8 ± 0.3</td>
<td>6.91 ± 0.07</td>
<td>0.96 ± 0.04</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>3.2 ± 0.8</td>
<td>1.2 ± 0.4</td>
<td>7.06 ± 0.4</td>
<td>1.07 ± 0.05</td>
<td>0.57 ± 0.04</td>
</tr>
<tr>
<td>7.5</td>
<td>3.3 ± 0.6</td>
<td>0.9 ± 0.1</td>
<td>7.04 ± 0.6</td>
<td>1.04 ± 0.08</td>
<td>0.56 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>1.6 ± 0.6</td>
<td>0.5 ± 0.7</td>
<td>2.82 ± 0.7</td>
<td>0.66 ± 0.04</td>
<td>0.39 ± 0.04</td>
</tr>
</tbody>
</table>
Table 3 The effect of four iodide levels in the medium on the micro-nutrient content (µg⁻¹ Dw) of tomato cells (average ± standard error)

<table>
<thead>
<tr>
<th>DAY 0</th>
<th>I μM</th>
<th>Zn</th>
<th>Cu</th>
<th>Mn</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>62.8 ± 3.4</td>
<td>29.6 ± 1.7</td>
<td>78.4 ± 1.8</td>
<td>136.3 ± 4.0</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>64.7 ± 4.2</td>
<td>30.5 ± 3.0</td>
<td>79.0 ± 0.4</td>
<td>137.0 ± 10.2</td>
</tr>
<tr>
<td>7.5</td>
<td>7.5</td>
<td>61.4 ± 2.7</td>
<td>28.6 ± 1.5</td>
<td>80.6 ± 1.8</td>
<td>140.0 ± 8.3</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>60.4 ± 5.7</td>
<td>31.0 ± 3.3</td>
<td>79.5 ± 1.0</td>
<td>141.2 ± 11.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DAY 6</th>
<th>I μM</th>
<th>Zn</th>
<th>Cu</th>
<th>Mn</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>62.3 ± 6.8</td>
<td>30.5 ± 0.9</td>
<td>77.8 ± 2.6</td>
<td>139.5 ± 10.9</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>64.3 ± 2.1</td>
<td>32.0 ± 1.5</td>
<td>81.3 ± 0.9</td>
<td>142.0 ± 12.3</td>
</tr>
<tr>
<td>7.5</td>
<td>7.5</td>
<td>62.7 ± 9.2</td>
<td>33.6 ± 2.4</td>
<td>80.4 ± 1.0</td>
<td>142.2 ± 10.4</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>63.0 ± 2.0</td>
<td>30.5 ± 3.0</td>
<td>79.6 ± 0.7</td>
<td>140.7 ± 10.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DAY 12</th>
<th>I μM</th>
<th>Zn</th>
<th>Cu</th>
<th>Mn</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>60.5 ± 2.0</td>
<td>28.9 ± 1.6</td>
<td>76.8 ± 2.6</td>
<td>139.4 ± 8.8</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>63.4 ± 9.6</td>
<td>30.1 ± 2.2</td>
<td>80.0 ± 0.2</td>
<td>142.0 ± 10.3</td>
</tr>
<tr>
<td>7.5</td>
<td>7.5</td>
<td>64.4 ± 5.8</td>
<td>31.5 ± 3.6</td>
<td>80.0 ± 1.5</td>
<td>143.3 ± 12.6</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>46.0 ± 3.2</td>
<td>20.3 ± 2.3</td>
<td>45.5 ± 3.0</td>
<td>114.3 ± 11.5</td>
</tr>
</tbody>
</table>
Fig. 2. The I⁻ content of cells after a three hour uptake period. Vertical bars indicate standard error.

Table 4 The I⁻ content of tomato cells as a function of available medium I⁻.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>I⁻ absorbed from medium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>0.07</td>
</tr>
<tr>
<td>10</td>
<td>0.11</td>
</tr>
<tr>
<td>30</td>
<td>0.12</td>
</tr>
<tr>
<td>45</td>
<td>0.13</td>
</tr>
<tr>
<td>60</td>
<td>0.14</td>
</tr>
<tr>
<td>120</td>
<td>0.17</td>
</tr>
<tr>
<td>180</td>
<td>0.17</td>
</tr>
</tbody>
</table>
6.5 DISCUSSION

There were no significant differences in biomass produced or dry weight of cells grown at a I⁻ level of 0 μM when compared with cells grown with either 5, or 7.5 μM in the medium. This suggests that this element is not an absolute necessity for the growth of tomato cells in suspension culture (Figure 1, Table 1). However, the biomass produced with 5 or 7.5 μM I⁻ levels was always higher than cells grown with no added I⁻. This shows that this element had a growth stimulating effect on cells. The response of plants to added iodine as seen from the literature depends to a large extent on the type of plant species and the environmental conditions which they are grown. Among the land plant species, some of the graminaceous plants such as oats, wheat, rye and barley are stimulated by iodine (Borst Pauwels, 1961; Lehr et al., 1958; Åberg, 1948, Ekdahl, 1948).

Whether this element performs some special function indispensable to the life of higher plants or whether it is there merely as a functionless incidental unavoidably absorbed into the plant system from the nutrient medium is yet to be proved. The exact reason or reasons, if any, for the growth stimulation action of I⁻ on tomato cell suspensions cannot be explained from this study. Cells receiving either 5 or 7.5 μM I⁻ had higher macro and micro-nutrients as compared with the 0 μM I⁻ treatment when determined at day 12 (Tables 2 and 3). Whether these differences in the nutrient levels in cells in the latter two treatments were responsible for the observed growth stimulation (Figure 1) of tomato cells is questionable, since at day 6, cells cultured with no added I⁻ in the medium too had significantly higher macro-nutrients (Table 2).

An indirect influence of this element is that it may effect the nitrogen-fixing and nitrogen converting bacteria. According to the observations of Lehr et al., (1958), I₂ stimulated the nitrification process in the soil and may thus have an indirect effect on plant growth. These authors stress that it is unlikely that the possibility that I⁻ exercises an indirect effect by influencing the uptake or action of other elements.

Cells grown with 10 μM I⁻ in medium failed to grow after day 6 and the brown color shown by cells is due to toxicity. The observations made by other workers show that if a growth stimulation effect from this element is to be expected the ideal I⁻ concentration
in the medium should be between 1 and 5 ppm. Hageman et al., (1942) working with
tomato plants observed first toxicity symptoms at a I level of 16 ppm, Umaly and Poel
(1961) observed toxicity symptoms at 10 ppm in barley and 1 ppm in peas. An I level
of 10 μM the highest concentration used in this study corresponds to about 1 ppm at
which the toxicity symptoms were observed. This suggests that tomato cells in
suspension culture show detrimental effects to this element at a much lower
concentration than whole plants.

In the uptake study using labelled NaI, the tomato cells after a three hour uptake
period contained about 50 ng g⁻¹ Fw and absorbed only 0.17 % from the total available
I in the medium (Fig. 2, Tab. 4). In contrast to this observation, many workers
(Hageman et al., 1942; Lehr et al., 1958; Borst Pauwels, 1961) have shown that the
amount of I a plant absorb is directly proportional to the amount of available I
contained in the soil or other medium which the plants are grown. Presumably plants
take this element simply with the transpiration stream. Since there are hardly any uptake
studies done with labelled I using cell suspension systems it is difficult to evaluate the
results observed in this study. After a four hour incubation period in pond water
containing only I¹³¹, Nitella tissues took up approximately 50% of the labelled I¹³¹
(Tong and Chaikoff, 1955). However these results cannot directly be compared with the
observations of the present study since the pond water in which Nitella plants grew
consisted only a small amount of radiolabelled Iodine.
7. HABITUATION OF TOMATO CELL SUSPENSION CULTURES TO INDOLE ACETIC ACID (IAA)

7.1 ABSTRACT

Heritable cellular changes, one of which known as habituation occurs in plant tissues and cell cultures. This phenomena is characterized by a newly acquired ability of plant cells to produce growth regulatory substances. Using tomato cell suspensions a habituated cell suspension was obtained through a stepwise reduction of indole acetic acid (IAA) in the medium. The habituated cells grew in a medium without an added exogenous supply of hormones. There were no significant differences between the biomass produced by habituated cells and cells grew in the presence of an exogenous supply of hormones. The habituated cells produced significantly more IAA (426.14 pmol g⁻¹ fresh weight) than the control cells (150.56 pmol g⁻¹ fresh weight). This newly acquired character of tomato cells was lost, when the cells were treated with a medium containing IAA. The process of habituation did not alter the regeneration capacity of the cells. The number of plantlets obtained from habituated cells and control cells receiving both IAA and kinetin were almost the same. These results indicate that the process of habituation does not alter the totipotency of cells and leaves the cells regenerable. However the habituated status of cells was lost during regeneration where both stem pieces from seedlings obtained from regenerated plants and stem pieces directly taken from regenerated plants could not grow in a medium lacking IAA.

7.2 INTRODUCTION

The definition of the term "habituation" or "autonomy" describes all hereditable changes in the organic nutrient requirements mainly hormones and vitamins in cultured tissues (Gautheret, 1955). The habituated state is generally very stable and cells in this condition can remain independent of an exogenous growth factor supply for many generations. The capability of callus or suspension cultures to proliferate without added auxins and cytokinins has been frequently observed and are termed as auxin habituated, cytokinin habituated or habituated for both these hormones (Meins and Binns, 1979;
Meins, 1982, 1983; Masuda et al., 1988; Jackson and Lyndon, 1990). Habituated tissues for hormones have been reported for many plant species (Einset and Skoog, 1973; Masuda et al., 1977, 1988; Meins and Lutz, 1980; Gaspar et al., 1983; Meijer, 1984; Saunders and Shin, 1986). The reason or reasons for tissues to come into a habituated state may be due to either a) increased biosynthesis of the growth substance (Cheng, 1972; Dyson and Hall, 1972; Lalou et al., 1981; Mousedale et al., 1985 Kerbauy, et al., 1986), or b) decrease in its rate of degradation (Keevers et al., 1981), or c) altered sensitivity of the cells to the growth substance or d) interaction of some or all of these. Habituated callus or cell suspension cultures have been obtained by using different techniques. Culture of tissues at 35° C, which is about 10° C above the standard culture temperatures or in the presence of trace amounts of the cytokinin, kinetin (Meins and Binns, 1978). Saunders and Danb (1984), reported that hormone autonomous callus arose at a high frequency when shoots of sugar beet were cultured on a MS medium with benzyl adenine as the sole hormone. Using Helianthus annus and Vinca rosea as explantats Sogeke and Butcher (1976), showed, that, high levels of inorganic nutrients in the culture medium can induce hormone-autonomous callus. Tobacco calluses not requiring auxin were induced by low concentrations of auxins in the medium and this habituated state could be reversed by transferring the calluses to a medium with a ten fold indoleacetic acid concentration. (Syono and Furuya, 1974). Anti-auxins supplied for a very short time to soy-bean callus provoked habituation for auxin (Christou, 1988).

The reasons or mechanisms for tissues to show a habituated state are controversial. Habituation is the result of somatic mutations are favored by White (1951), De Ropp (1951), and Kandler (1952), while Braun (1975), and Meins (1974), attributes this phenomenon to an epigenetic mechanism. Evidence for the latter view is supported by the fact that habituation is a reversible process. Others relate habituation to chromosomal abnormalities (Fox, 1963). Melchers (1979), and Butcher et al., (1975) found no direct relationship between chromosomal number and habituation.

The study of habituation or autotrophy for hormones is of interest for several reasons. Cell determination and cell differentiation can be inherited by individual cells.
reasons. Cell determination and cell differentiation can be inherited by individual cells. Since most cells are thought to be genetically equivalent, the fundamental question arises how cells with the same genotype can inherit different characters. Further the relative auxin and kinetin contents in culture media regulate the formation of shoots and roots from unorganized tissues and these substances are primary morphogens responsible for the basic body plan of plants. Habituation provides an excellent experimental system for studying stable changes in the metabolism of hormones.

There are no reports to indicate that tomato cells in suspension culture could be habituated for hormones. In this study the following experiments were conducted using tomato cell suspensions to study the process of habituation.

STUDY 1.
In this study a method is described to induce habituated cells for indole acetic acid. The growth (cell biomass) and the mineral element composition of these two habituated cell lines are compared with hormone dependant cells. Since the MS medium contains both an auxin and a cytokinin it was also tested whether the cells could grow in a medium containing either IAA or kinetin as the only hormone.

STUDY 2.
Cells habituated for IAA were then tested for reversibility. This was done by transferring the habituated cells firstly into a medium containing IAA for some time and secondly transferring them to a medium containing no IAA. The growth of these cells were then compared with hormone dependant cells.

STUDY 3.
This experiment was done to test whether the habituated status of cells at the cellular level was due to a genetic variation or was simply epigenetic by nature (by definition epigenetic changes are not transmitted meiotically).

STUDY 4.
Cells habituated to hormones are said to produce fairly high quantities of hormones. In this experiment a cell suspension habituated to IAA and the hormone dependant cells were used to determine the IAA content by the use of monoclonal antibodies.
7.3 STUDY 1. INDUCTION OF A HABITUATED CELL LINE FOR IAA

7.4 MATERIALS AND METHODS

7.4.1 Plant material and growth conditions

See section 3.1.1. for a detailed description.

7.4.2 Induction of callus and cell suspensions

Since there are many evidences to show that cell cultures established and maintained for a long time are habituated new cell suspensions were initiated.

Callus induction was done as described in sections 3.1.2 and 3.1.3. Instead of 2,4-D as the auxin in the solid medium IAA (1mg/L) was used. In all experiments IAA was added after autoclaving of the medium using a 0.22 µm sterile filter unit (Millex-GS, Millipore S.A, France).

Cell suspensions were obtained as described in section 3.1.3, and as the auxin IAA (1mg/L) in the liquid medium.

7.4.3 Induction of habituated cell for IAA

Cell suspensions were allowed to grow in a MS medium containing IAA (1mg/L) and kinetin (1mg/L) for about 2 weeks. The cells were then separated and washed with a MS medium containing only IAA (1mg/L) as the hormone for several times, and resuspended in the same medium (Fig. 1). These cells were allowed to grow for about 14 days (subcultured every week). Thereafter the cells were separated from the medium washed with a MS medium containing IAA (0.5mg/L) several times, resuspended in the same medium and grown for further 14 days. This procedure of stepwise reduction in IAA concentration in the medium was done until there was no added IAA in the medium. Cells thus obtained is denoted as "AR" (IAA independant).

7.4.5 Determination of biomass

See section for a detailed description 3.3.1.
7.4.6 Determination of nutrient content of cells

See sections 3.5.3, and 3.5.4 for a detailed description.

Fig. 1. Stepwise adaptation of tomato cells to zero IAA concentration in medium

The growth of these "AR" cells was then compared with cell suspensions which were hormone dependant. The hormone dependant cells had four different variations.
These were namely:

1. Cells growing in a medium containing both an auxin (IAA) and a cytokinin (kinetin). Designated as (+A/+K) or control treatment.

2. Cells transferred to a medium containing only auxin (IAA) as the hormone. Designated as (+A/-K).

3. Cells transferred to a medium containing only a cytokinin (kinetin) as the hormone. Designated as (-A/+K).

4. Cells transferred to a medium containing no hormones. Designated as (-A/-K)

For the latter three treatments cells growing in a medium containing both IAA and kinetin were separated, and washed several times with treatment media. Twenty-ml aliquots (PCV of 10 ml) were then transferred to 250 ml Erlenmeyer flasks containing 150 ml of treatment media. The growth conditions supplied to the cells, the determination of cell biomass, macro and micro-nutrients are as described in sections 3.3, 3.4.1 and 3.6 respectively. Cells in all treatments were tested for their viability at each biomass determination as described in section 3.4 using FDA.

7.5 RESULTS

7.5.1. Biomass production

The non-habituated or hormone dependent cells grown without added hormones (-A/-K) ceased to grow after day 4. These cells turned brown in color by day 8 and they were no longer viable. Cells in all other treatments showed a continuous growth reaching almost a maxima by day 10 (Fig. 2).
7.5.2 Nutrient contents in cells

No large differences in nutrient contents were observed between treatments when determined by day 4. However, the cells cultivated with no added hormones had lower amounts of macro and micro-nutrients by day 8 (Figures 3 and 4).

![Biomass production graph](image)

**Fig. 2.** The biomass production of habituated "AR" cells and hormone dependant cells [(○) "AR" cells: (△) +A/+K: (▽) +A/-K: (□) -A/+K: (×) -A/-K:] (Vertical bars represent least significant differences (LSD$_{0.05}$). Where: A = IAA, K = Kinetin
Fig. 3. The macro-nutrient content of habituated "AR" cells as compared with cells in other treatments. Vertical lines above bars indicate standard error.
Fig. 4. The micro-nutrient content of habituated cells as compared with cells in other treatments. Vertical lines above bars indicate standard error.
7.6 STUDY 2: REVERSIBILITY OF "AR" CELL SUSPENSIONS TO IAA DEPENDENT CELL SUSPENSIONS

7.7 MATERIALS AND METHODS

7.7.1 Treatment of "AR" cells

The reversibility of "AR" cells (i.e. from IAA autotrophic state, back to IAA dependent state) was tested as shown below by allowing the "AR" cells to grow in the presence of added IAA for two weeks and thereafter in the absence of IAA.

A 20 ml aliquot of "AR" cell suspension was sterile filtered and washed several times with a MS medium containing IAA (1mg/L) and resuspended in this medium and cultured over a period of 2 weeks. The cells were then separated from the medium and washed several times with a medium without IAA. The cells were then resuspended
in a medium without IAA and their growth was compared with hormone dependent cells over a period of 3 weeks.

The determination of biomass was done as described in section 3.3.1.

7.8 RESULTS

7.8.1 Biomass production

Table 1 shows the growth of "AR" cells resuspended in a medium containing no IAA. No large differences in growth were observed in the biomass when determined at the end of first week. At the end of the second week the control cells had almost a four-fold higher biomass production than the "AR" cells. In the third week the habituated "AR" cells turned brown in color and their growth was drastically reduced.

**Table. 1** The biomass (mg/20 ml suspension) of "AR" cells cultured in a medium with IAA for two weeks and thereafter transferred to a medium containing no IAA (average ± standard error).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control cells</td>
<td>53.6 ± 1.6</td>
</tr>
<tr>
<td>&quot;AR&quot; cells</td>
<td>48.9 ± 2.6</td>
</tr>
</tbody>
</table>

Control cells (+IAA and + Kinetin)
7.9 STUDY 3. REGENERATION OF "AR" CELLS AND HORMONE DEPENDANT CELLS

7.10 MATERIALS AND METHODS

7.10.1 Regeneration medium

The regeneration medium consisted of MS salts, agar to solidify the medium and as exogenous hormones indole butyric acid (IBA) (1 mg/L) and as cytokinins N\(^6\)(\(\Delta^2\)-isopentynyl) adenine (2ip) (0.5 mg/L) and kinetin (0.5 mg/L).

7.10.2 Transfer of cells to the regeneration medium

The cell suspensions in the flasks were allowed to settle for about one minute, and 1 ml medium from both "AR" and hormone dependent cells were pipetted from flasks to test-tubes and diluted by adding 1 ml of the respective media. The total number of cells were determined as described in section 3.3.3. From the diluted cell suspensions 0.5 ml was sterile filtered, and the cells transferred to glass petri-dishes containing 100 ml of a solidified MS medium. To ease the counting of embryos and plantlets the cells were carefully spread over the medium using a plastic spatula. These were incubated at a light intensity of 250 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (Philips TL F 40 W/33) and at 25\(^{\circ}\)C.

7.10.3 Counting of embryos and plantlets

The embryos and whole plantlets were counted using a binocular at a magnification of x100. Whole plantlets (small buds with rootlets) thus obtained were then transferred to sterile plastic containers (9.5x11.5cm), containing 200 ml of solidified regeneration medium and allowed to grow for further 2 weeks.

7.10.4 Growth conditions in the glass house

Plantlets (1-2 leaves and about 7 cm tall) in the plastic containers with intact lids were transferred to a glass-house (16 hr light and 8 hr dark; 25\(^{\circ}\)C). After 1 week the plantlets were carefully separated from the solidified medium, roots washed several times with water. They were transferred into plastic pots containing fine sand (0.5-
0.75 mm), and 1 L beakers were placed over the plantlets to prevent damages from extreme conditions. The beakers were removed after about 10 days.

During the first two weeks the plants were fertilized with a 1/10 strength Hoagland solution weekly and thereafter with a 1 strength Hoagland solution.

7.10.5 Collection of seeds and initiation of sterile seedlings from regenerated plants

Seeds were collected from both types of regenerated plants. Seedlings were obtained under sterile conditions (see section 3.1 for a detailed description).

Stem pieces obtained directly from regenerated plants, and from sterile seedlings were then tested for their growth by transferring them to a solidified MS medium containing with (1 ml/L) or without IAA.

After 4 weeks the explants were carefully removed from the medium and the adhering medium carefully removed and their weights determined.

7.11 RESULTS

7.11.1 Development of embryos and whole plantlets

Not all the cells transferred to the regeneration medium produced whole plantlets with leaflets and roots. Embryos were first visible on the third week (Tab. 2) after transferring the cells to the regeneration medium. Some cells produced only roots, and others only aerial parts while some of the cells grew into callus masses. Only a few fully developed plantlets with both leaves and roots could be obtained, and these were visible by the fifth week.

There were no differences in the number of embryos and whole plantlets obtained between the two treatments.
Table 2 The number of embryos and whole plantlets regenerated from control cells and habituated "AR" cells after transferring to the regeneration medium (average ± standard error)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (weeks)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Control cells</td>
<td>24.33 ± 8.12</td>
<td>136.37 ± 15.5</td>
<td>151.33 ± 23.23</td>
<td></td>
</tr>
<tr>
<td>&quot;AR&quot; cells</td>
<td>30.66 ± 10.0</td>
<td>143.31 ± 11.5</td>
<td>158.00 ± 20.25</td>
<td></td>
</tr>
</tbody>
</table>

Values in parenthesis indicates structures either only with shoots or roots

7.11.2 The growth of stem pieces of seedling plants and stem pieces of regenerated plants

Irrespective of the source of explants, only stem pieces transferred to a medium containing an exogenous supply of IAA showed any growth (Tab. 3). The callus growth was first observed from the cut ends of stem pieces in about 10 days.

Thereafter profuse callus formation was seen in these tissues all along the stem pieces.
Table 3 The effect of two levels of IAA in medium on callus growth (mg) in various explantats (average ± standard error mean)

<table>
<thead>
<tr>
<th>Source</th>
<th>- IAA</th>
<th>+ IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed plant stems - habituated</td>
<td>18.0 ± 1.7 (7)</td>
<td>432.2 ± 66 (5)</td>
</tr>
<tr>
<td>&quot; - N. cells</td>
<td>17.2 ± 2.0 (6)</td>
<td>459.7 ± 72 (7)</td>
</tr>
<tr>
<td>Stems regenerated from habituated &quot;AR&quot; cells</td>
<td>13.8 ± 0.9 (8)</td>
<td>398.5 ± 65 (6)</td>
</tr>
<tr>
<td>Stems regenerated from Normal cells</td>
<td>12.3 ± 1.2 (5)</td>
<td>412.9 ± 61 (7)</td>
</tr>
</tbody>
</table>

* Initial weight of explants - 12-20 mg.
(n) no of replications

7.12 STUDY 4. DETERMINATION OF IAA IN CELLS

7.13 MATERIALS AND METHODS
7.13.1 Method
The IAA contents in cells were determined using the Enzyme Linked Immunosorbent Assay.

7.13.2 The principle of an "ENZYME LINKED IMMUNOSORBENT ASSAY" (ELISA)
In an ELISA, the immuno-tracer is labelled with an enzyme such as alkaline phosphatase or peroxidase (in this study indole-3-acetic acid-alkaline phosphatase immuno-tracer). The antibodies are first bound to the wells of a polystyrene plate. Sample is added together with the tracer. This allows the competition between the tracer and the hormone present for the available antibody binding sites. The amount of immuno-tracer bound to the antibodies on the walls is then determined by adding
an appropriate substrate (in this study p-nitrophenyl phosphate).

The amount of product formed from the substrate is measured colorimetrically and is proportional to the amount of bound enzyme-labelled tracer, which is inversely proportional to the amount of hormone in the sample (see figure below).

7.13.3 Preparation of cell samples

The preparation of cells to determine the IAA content and immunoassay technique used in this study was as described by Mertens et al., (1983), Weiler and Spanier (1981) with modifications (Weiler, personal communication).

The cell suspensions were sterile filtered, and the cells washed with bidest. water. The fresh weight of the cells were determined (between 5-10 g).

To each cell sample the following were added.

1. Polyclar AT - 1% by weight of cells
2. Absolute methanol containing 0.001% butylated hydroxy toluene - 4 ml/g fresh weight of cells
3. l-14C-Indoleacetic acid (11.5 Mbq/mg) as an internal standard giving a 20,000 DPM

50 mg of acid washed sea-sand was added to each sample and the cells were macerated using a mortar and a pestle in diffused light at 4°C. The cells were extracted three times, extracts were combined and adjusted to 80% methanol and passed through a SEP-PAK C18 reversed phase cartridge (Waters Associates, Milford, Massachusetts). This cartridge contains pigments. The effluent was collected and methanol was removed by rotary evaporation. The aqueous residue was then acidified with acetic acid (pH-3), and passed over a second C18 reversed phase cartridge. The hormonal fraction bound to the column was eluted with 5 ml of absolute methanol. The methanolic fraction was concentrated to approximately 1 ml by rotary evaporation.

The IAA was measured as the methylated compound. Therefore, the hormonal fraction was methylated using ethereal diazomethane.

7.13.4 Preparation of ethereal diazomethane solution

0.3 ml of 40% KOH (w/v) and 1 ml diethylether were mixed in a test tube and placed on ice/NaCl (-15°C). Hundred mg N-nitrosomethyl urea (Sigma N-0132) was added in portions with a plastic spatula and stirred for 10 minutes on ice/NaCl. The ether phase was removed using an Eppendorf-pipet with a plastic tip. Ether was added several times (each time about 1 ml) to the aqueous phase until ether became no longer yellow. The clear yellow ether fraction was then dried over KOH (30 min, ice/NaCl).

7.13.5 Methylation of samples

Samples in methanol were first cooled on ice. Sufficient diazomethane in ether was then added until the samples became yellow. The methylation reaction was stopped after 10 minutes. This was done by adding 2-3 drops of 0.2 N acetic acid in methanol to destroy excess diazomethane. The solvent was removed in a stream of N₂ and diluted with 1 ml of the assay buffer (Tris buffered saline-TBS).
7.13.6 Reagents used

The reagents used for the immunoassay were as follows.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount or Volume/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rabbit Anti-Mouse Immunoglobulins</td>
<td>5mg</td>
</tr>
<tr>
<td>(Idetek-Immunoglobulins a/s, Denmark)</td>
<td></td>
</tr>
<tr>
<td>2. Monoclonal Antibodies for IAA</td>
<td>2mg</td>
</tr>
<tr>
<td>(Idetek, Inc., CA, USA)</td>
<td></td>
</tr>
<tr>
<td>3. Sodium bicarbonate buffer, pH 9.5</td>
<td>60 ml</td>
</tr>
<tr>
<td>(50 mM NaHCO₃)</td>
<td></td>
</tr>
<tr>
<td>4. Saline-0.5% Tween&lt;sup&gt;20&lt;/sup&gt; solution, pH 7.0</td>
<td>250 ml</td>
</tr>
<tr>
<td>5. Tris-Buffered saline, pH 7.5</td>
<td>200 ml</td>
</tr>
<tr>
<td>(25 mM Trizma base, 0.1 M NaCl, MgCl₂·6H₂O)</td>
<td></td>
</tr>
<tr>
<td>6. DEA buffer, pH 9.8 (0.9 M diethanolamine,</td>
<td>20 ml</td>
</tr>
<tr>
<td>0.3 mM MgCl₂·6H₂O)</td>
<td></td>
</tr>
<tr>
<td>7. Substrate tablets-Sigma (p-nitrophenyl phosphate-PNPP)</td>
<td>20 mg</td>
</tr>
<tr>
<td>8. Stopping reagent (1.0 M NaOH)</td>
<td>5 ml</td>
</tr>
<tr>
<td>9. Rabbit Serum Albumin-Sigma</td>
<td>200 mg</td>
</tr>
<tr>
<td>10. Gelatin</td>
<td>20 mg</td>
</tr>
<tr>
<td>11. Standards (Methyl Indole-3-Acetate)*</td>
<td>18.92 mg</td>
</tr>
<tr>
<td>12. Diluent for IAA standards (Absolute methanol)</td>
<td>10 ml</td>
</tr>
<tr>
<td>13. Alkaline phosphatase tracer (Idetek, Inc., CA, USA)</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

To assure stability and to prevent possible contamination, 0.1 % sodium azide was added to all working reagents.

7.13.7 Preparation of micro-titration plates

Step 1) 5 mg of rabbit anti-mouse antibody was dissolved in 20 ml of NaHCO₃ buffer. After 5 minutes 200 µl of this solution was added to each well of a 96 well flat bottomed micro-titration plate, covered with a sealer and incubated at 4°C for 24 h.
Step 2) The antibody solution was decanted and each well was filled with 200 µl of saline tween solution and after about 2 minutes the tween solution was decanted. This wash step was repeated twice, and excess wash solution in the wells were removed by inverting the plate and patting dry on paper towels.

Step 3) 2 mg of the monoclonal antibody was dissolved in 20 ml of NaHCO₃ buffer. 200 µl of this reagent added to each well, covered with a sealer and incubated at 4°C for further 24 h.

Step 4) The monoclonal antibody solution was decanted into a waste container, and the plate washed three more times as stated in step 2.

Step 5) 200 mg of the rabbit serum albumin was dissolved in 20 ml of TBS, and 200 µl of this solution added to each well, covered with a sealer and incubated for 1 h at room temperature.

Step 6) The rabbit serum albumin was decanted into the waste container and step 2 repeated.

7.13.8 Serial dilutions for methyl indole-3-Acetic acid

18.92 mg of methyl indole-3-acetic acid was dissolved in 10 ml of absolute methanol = 0.01 M = 10 mM stock solution.

10 mM = 10⁶ pmole/0.1 ml

Serial dilutions from the stock solution were made as follows.

1. 5 µl stock solution + 9.995 ml TBS 500 pmol/0.1 ml (A)
2. 500 µl of (A) + 2.00 ml " 100 pmol/0.1 ml (B)
3. 1.00 ml of (B) + 1.00 ml " 50 pmol/0.1 ml (C)
4. 1.00 ml of (C) + 1.00 ml " 20 pmol/0.1 ml (D)
5. 500 µl of (D) + 1.50 ml " 5 pmol/0.1 ml (E)
6. 500 µl of (E) + 2.00 ml " 1 pmol/0.1 ml (F)
7. 500 µl of (F) + 500 µl " 0.5 pmol/0.1 ml (G)
7.13.9 Immunoassay procedure

Step 1) The 2 vials of lyophilized tracer were reconstituted each with 1 ml of distilled water. After about 5 minutes the total contents of the reconstituted tracers were transferred to 8 ml of TBS containing 0.1% gelatin and mixed. To ensure the removal of all the tracer the vials were rinsed several times with the diluted tracer.

Step 2) 100 µl of the standard (methyl indole-3-acetic acid) or sample was added to each well (three replicates per standard - 8 standards - or samples).

Step 3) 100 µl of the diluted tracer was added to each well and mixed simply by tapping the plate. The plate was then covered with the sealer, and incubated at 2-6°C for 3 h.

Step 4) The solution was decanted into the waste container and the plate washed three times with saline tween.

Step 5) The substrate solution was prepared by dissolving four 5 mg PNPP tablets in 20 ml DEA buffer.

Step 6) 200 µl of the diluted substrate solution was added to each well, covered with a sealer and incubated at 37º for 1 h.

Step 7) The reaction was stopped by adding 50 µl of stopping reagent.

Step 8) After 5 minutes the optical densities were determined at 405 nm using a vertical light path photometer.

7.13.10 Presentation of data

The calculations were done as follows.

1) The optical densities of triplicate standards and samples were averaged.

2) The % binding \((B / B_0)\) of each standard point or sample was calculated as follows.

\[
\text{Standard / Sample O.D - NSB O.D} \quad \frac{\text{Standard / Sample O.D - NSB O.D}}{B_0 \text{ O.D - NSB O.D}} \times 100
\]
$B_0 = 100 \mu l$ buffer + $100 \mu l$ tracer = 100% binding (tracer binding in the absence of antigen)

$B$ = Tracer binding in the presence of antigen

NSB = Non specific binding = 1000 pmol methyl IAA + tracer = 0% binding. $B_0$ and NSB are required to determine the maximum binding capacity of IAA.

3) % binding ($B/B_0$) versus the concentration (pmole Me.IAA) was plotted and the best fit curve drawn.

4) The sample concentration was determined by extrapolation of the sample % binding from the best fit standard curve.

The rest of the sample solution was used to measure the radioactivity of $1^{-14}$C-IAA. This was done using a Liquid Scintillation Analyzer (Packard 2500 TR, Canberra, Australia).

7.14 RESULTS

7.14.1 IAA contents of cells

The standard curve and its log transformation (inserted) is shown in Figure 5. The IAA contents of the two cell types are shown in Table 4. As compared with hormone dependent cells, the habituated "AR" cells contained a higher level (almost 3 fold) of IAA.
**Fig. 5.** The standard curve for IAA-ELISA. $B=$tracer binding in the presence of antigen. $B_0=$tracer binding in the absence of antigen.

$logit \frac{B}{B_0} = \ln \frac{B}{B_0} / (100 - \frac{B}{B_0})$

**Table. 4** The indole acetic acid contents of hormone dependant cells and habituated "AR" cells as determined by the Enzyme Linked Immunosorbent Assay

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Hormone dependant cells</th>
<th>Habituated &quot;AR&quot; cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep. 1</td>
<td>140.61</td>
<td>421.96</td>
</tr>
<tr>
<td>Rep. 2</td>
<td>158.43</td>
<td>423.81</td>
</tr>
<tr>
<td>Rep. 3</td>
<td>152.64</td>
<td>432.66</td>
</tr>
<tr>
<td>Mean</td>
<td>150.56</td>
<td>426.14</td>
</tr>
<tr>
<td>Std. error</td>
<td>5.24</td>
<td>3.30</td>
</tr>
</tbody>
</table>
7.15 DISCUSSION

The growth of non-habituated cells when transferred directly to a medium without hormones (-A/-K) until day 4, was due to their use of endogenous hormones (Fig. 2). Thereafter there is a gradual decrease in biomass suggests that the cells are losing their viability and are not able to produce endogenous auxins or cytokinins. The cells also have the ability to grow either only with IAA (+A/-K) or with kinetin (-A/+K) as the sole hormone in the medium. Therefore on a medium devoid of cytokinin, the growth of hormone dependant cells occurs only if there is an exogenous supply of auxins and vice versa. Auxins such as IAA and synthetic substances such as 2,4-dichloro-phenoxy acetic acid and indole butyric acid have the ability to cause cell enlargement and in cultured systems they also have the ability to promote cell division (Krikorian et al., 1987). Witham (1968), showed that only an auxin in the medium permitted the satisfactory growth of soybean callus on a medium lacking cytokinins. Experiments done using tobacco callus could grow in a medium lacking auxins, but these cultures required a source of kinetin (Einsett, 1977). In contrast to these results callus cultures of Catharanthus roseus required both an auxin and a cytokinin for successful cell division (Staden and Mooney, 1987).

The possible explanations as to how cells growing only with IAA (+A/-K) in the medium could produce a biomass similar to the control cells (+A/+K) are: 1) an exogenous supply of IAA as the sole hormone in the medium stimulated the synthesis of cytokinins by cells 2) cytokinins are not a pre-requisite for cell division in tomato cell suspensions when IAA is present in the medium or 3) in the absence of a cytokinin in medium the IAA may directly act as an auxin and also as a cytokinin at the same time. Though higher levels of IAA were found in habituated "AR" cells (Table 4), they produced approximately the same biomass as those cells receiving an external supply of IAA and or kinetin (Fig. 2). Therefore there is a lack of correlation between the IAA concentration measured in cells and the response of habituated tomato cells in terms of biomass production. This may be due to the lack of sensitivity of tissues to the increased hormone levels as shown by Trewavas (1981) and Trewavas and Cleland (1983). Firn (1986) suggested that this lack of sensitivity of plant tissues could be due
to factors such as change in the receptor affinity or other events which might involve other hormones or other factors. From the results obtained with the habituated tomato cells it is not possible to say, which of the above factors, if any, would have caused the cells to show insensitivity to increased IAA levels. There are numerous reports to support that cells or tissues habituated either to auxins, cytokinins or to both these hormones have the capacity to produce their own requirements, and they grow and develop in media without an exogenous supply of hormones (Cheng, 1972; Meins and Binns, 1978; Keves et al., 1981; Pengelly and Meins, 1983; Kerbauy et al., 1986, 1988). The IAA content determined for hormone dependent tomato cells (Table. 4) is about 26 ng g\(^{-1}\) fresh weight (FW). Pengelly and Torrey (1982) determined values between 30-60 ng g\(^{-1}\)FW for pea roots and Tagliani et al., (1986) a value of 50 ng g\(^{-1}\)FW for barley roots. The value obtained by Epstein et al., (1991) for a hormone dependent carrot callus is about 12.7 ng g\(^{-1}\)FW. The IAA content of habituated tissues of tobacco as determined by radioimmunoassay was 80 ng g\(^{-1}\)FW (Pengelly and Meins, 1983) and the value for the habituated tomato cells is about 74 ng g\(^{-1}\)FW. In contrast to the above results Kevers et al., (1981) showed that both non-organogenetic hormone dependent and habituated sugar beet callus contained approximately the same amount of auxin, i.e., 1300 ng g\(^{-1}\)FW. Using auxin requiring organogenetic sugar beet callus, Kevers et al., (1981) showed lesser amounts of auxins in the tissues (298 ng g\(^{-1}\)FW). This variation in the IAA contents in the two types of calluses as they showed was due to higher activity of IAA peroxidases in organogenetic calluses (Kevers et al., 1981). High levels of endogenous IAA contents determined in non-organogenetic sugar beet callus by Kevers et al., (1981) are generally found in some plant species infected with Agrobacterium tumefaciens (Weiler and Spanier, 1981). The presence of higher endogenous IAA levels in habituated tomato cells as compared with control cells, therefore, indicates, that habituation to IAA is accompanied by increased levels of IAA synthesis. Since no detectable IAA was found in cultured habituated tobacco cells Nakajima et al., (1979) concluded that, auxin-autotrophic growth is not necessarily explained by the enhanced hormonal contents in tissues.

The fact that intact plants produce auxin and other cell division factors suggests that
habituation is due to the heritable expression of genes normally inactive in cultured cells. Therefore, the hypothesis predicts that habituation should be reversible. This is in fact the case observed with habituated cells when they were transferred first to a medium containing IAA and retransferred to a medium containing no IAA (Table. 1). A treatment of 2 weeks with IAA was sufficient for the cells to turn into their original status i.e., hormone dependent. This is the reason why the cells could not thrive in a medium without added IAA. The genes responsible for the production of IAA when activated, produce IAA and the cells have the ability to grow independent of an external hormonal supply. Such habituated cells when transferred to a medium with an exogenous supply of IAA, the genes are automatically turned "off", and these cells become again hormone dependent. The increase in biomass when measured at the end of the first week in habituated cells was due to the IAA "carried over" by cells (Table. 1). Thereafter the cells are dependent on an external hormonal supply. The reduction in biomass during the proceeding two weeks indicate, that the cells are not growing and are dying. The stem pieces taken directly from the regenerated plants and stem pieces from seedlings obtained from regenerated plants failed to grow in a medium lacking IAA. However when placed on a medium with IAA they could grow into a callus mass (Table. 3). Therefore these results indicate that habituation is a reversible process and is not due to spontaneous changes in genetic constitution that are essentially irreversible. The process of habituation of tomato cells thus shows an epigenetic nature and differ from most mutations in several ways. Firstly it can occur in response to a specific inducer, in the present study the inducer being the adaptation of cells in a stepwise manner to low IAA concentrations in the medium. Secondly the variant phenotype was reversible and lastly by definition epigenetic changes are not transmitted meiotically. The number of plantlets regenerated in both treatments were almost the same (Table. 2). This indicates the induction of hormone dependent cells to a habituated state did not affect the totipotency of cells. The important observation here is that fertile plants can be obtained from the progeny of IAA habituated cells, indicating that these cells retain all the necessary information to generate complete plants, i.e., they are totipotent.
8. THE EFFECT OF ASCORBIC ACID IN CULTURE MEDIUM ON THE GROWTH OF TOMATO CELLS IN SUSPENSION CULTURE

8.1 ABSTRACT

Three added ascorbic acid (AsA) levels (20, 40, and 60 mg/L) was compared with a control (0 mg/L) in medium, to observe the effects of this substance, on the growth of tomato cells. Added levels of AsA in medium had no effect on the biomass produced by cells. However, the dry weight of cells cultured with added AsA in the medium was higher than the control (0 mg/L) cells. The dry weight of cells growing with different AsA levels in the medium did not vary significantly. The four AsA levels in medium had no effect on nutrient content of cells. Cells cultured in the presence of AsA in medium had higher levels of sucrose, glucose, and fructose when compared with the control cells. Among the organic acids determined, only citric acid was higher in cells grown with added AsA. Sucrose contributed almost twice as much to the dry weight of cells than glucose, fructose, and citric acid. The total contribution from these four components to the dry weight of cells was almost two fold when compared with the control cells. The AsA content of cells receiving an exogenous source of AsA was highest at day 2 and thereafter the AsA levels in the cells decreased with time. Almost a similar pattern in the reduction of AsA content was observed in all three media supplemented with AsA.

8.1 INTRODUCTION

The human dependence on plants as a source of the "Anti-scorbutic Factor", ascorbic acid culminated in 1932 when it was identified as L-threo-2-hexone-1,4-Lactone (Staudiger,1978; Hughes, 1983). Quantitatively, the biosynthesis of L-ascorbic acid considerably exceeds that of the other vitamins and it is thus the most widely available vitamin.

Ascorbic acid is a white crystalline substance and is synthesized both biologically and chemically from D-glucose. The most significant characteristic of L-ascorbic acid is its oxidation to dehydro-L-ascorbic acid to form a redox system, and both substances
are considered as active anti-scorbutic agents in the protection and treatment of scurvy. L-ascorbic acid is stable when dry but darkens on exposure to light. The degradation of L-ascorbic acid in aqueous solution depends on several factors, such as pH (most stable between 4-6), T°, the presence or absence of oxygen and metals such as Fe, Cu, Mn etc. It is sensitive to heat and in the presence of both heat and oxygen it is oxidized at a rate proportional to the temperature rise.

It is considered as an ubiquitous constituent of green plants (Crawford and Crawford, 1980; Jaffe, 1984; Loewus, 1988). Some animals such as primates, guinea pigs, fish and also man lack the ability to synthesize L-ascorbic acid, since they lack the enzyme γ-gulonolactone oxidase. In human and in animal nutrition L-ascorbic acid plays a major role such as storing iron in bone marrow, stimulation of the immune response of man for protection against microbial infection, lipid metabolism and presumably plays a part in the oxidation of amino acids.

It is also very important in food technology, since it is used extensively in the processing of beverages and canned foods.

Plants synthesize L-ascorbic acid from carbohydrates, mainly from glucose. In higher plants, the ascorbic acid content ranges from undetectable levels in dry seeds to more than 0.1% of fresh weight in certain fruits (Leung and Loewus, 1985). Tissues undergoing active growth usually contain 0.2-2 mg of L-ascorbic acid g⁻¹ FW, with the upper range most applicable to green leaves and inflorescences (Jaffe, 1984). In contrast, seeds of higher plants have low levels of L-ascorbic acid, but at germination and with subsequent growth they begin to synthesize more. Citrus fruits, guavas, hips, berries, peppers and parsley contain fairly high levels of L-ascorbic acid. Some plants accumulate ascorbic acid to very high levels. Terminalia spp. (2850 mg 100⁻¹ g FW weight) and West Indian cherry (1300 mg 100⁻¹ g FW) are considered accumulators of L-ascorbic acid.

There is considerable evidence to show that L-ascorbic acid acts as a regulator of plant growth and development (Tonzig and Marre, 1961; Chinoy, 1962). In addition, L-ascorbic acid has been found to be important in overcoming the auxin mediated
apical dominance, in nucleic acid and protein metabolism, delaying the senescence of
leaves, branching of cotton, nodulation and nitrogen fixation in some legumes and a
whole array of other plant reactions (Chinoy, 1962; Garg, 1966; Price, 1966; Fellenberg,

Recently the interest was evoked on the action of ascorbate, ascorbate peroxidase and
dehydro-ascorbate peroxidase, when their contribution towards the detoxifying effect or
scavenging action was identified. For example it is known that during photosynthesis,
the illuminated chloroplasts reduce di-oxygen with subsequent production of hydrogen
peroxide rather than water. Hydrogen peroxide inhibits enzymes of the photosynthetic
carbon reduction cycle, is scavenged away with the help of ascorbate and its
peroxidases (Foyer and Halliwell, 1977; Robinson et al., 1980).

A great amount of work has been done to see the effects of various factors such as
light, temperature, mineral elements, photosynthetic efficiency of tissues, stress etc., on
the ascorbic acid content of plants (Somers and Kelly, 1950; Aberg, 1945; Chinoy,
1988, Jaffe, 1984; Loewus, 1988). There is almost no experimental work done to see
the effects of added ascorbic acid in medium on the growth and development of cell
culture systems. Therefore, the aim of this study was to see the effects of added
ascorbic acid in the medium on growth, mineral element composition, sugar and organic
acid contents of tomato cells in suspension culture.

8.3 MATERIALS AND METHODS

8.3.1 Treatments

The effect of ascorbic acid on growth of tomato cell cultures were tested by the
addition of 60, 40, and 20 mg ascorbic acid L\textsuperscript{-1} medium together with a control
treatment without added ascorbic acid. Twenty ml cell suspensions were sterile-filtered
and cells transferred to 500 ml Erlenmeyer flasks containing 160 ml of MS medium.
The ascorbic acid was dissolved in 20 ml sterilized MS medium and added to cell
susinations in Erlenmeyer flasks using a sterile filter unit.
8.3.2 Determination of fresh and dry weights
For a detailed description see sections 3.3.1 and 3.3.2.

8.3.3 Determination of total number of cells
For a detailed description see section 3.3.3.

8.3.4 Determination of mineral elements
For a detailed description see sections 3.5.2, 3.5.3, 3.5.4 and 3.5.5.

8.3.5 Determination of sugars and organic acids
For a detailed description see section 3.5.6.

8.3.6 Determination of total ascorbic acid
8.3.6.1 Principle
The changes in the total ascorbic acid contents in cells and in media was measured as described by Deutsch and Weeks (1965) with fluorimetry with slight modifications.

The principle is that ascorbic acid is first oxidized with iodine to dehydroascorbic acid, which is condensed with ortho-phenylenediamine to form a fluorescent quinoxaline. The blank value is obtained by inhibiting the formation of the derivative by complex formation with boric acid.

8.3.6.2 Preparation of samples and analysis
A cell suspension sample of 20 ml was sterile filtered and the fresh weight determined. 1 ml of metaphosphoric acid (10%) was added to each cell sample/filtrate, and stored at -18°C until analysis. The cells were then thawed at room temperature and macerated with a glass rod, centrifuged and the supernatant collected. The extraction followed once more with metaphosphoric acid and the supernatants were pooled.

Determination of ascorbic acid in cell extracts and in filtrates were done using a luminescence spectrometer type LS 30 (Perkin Elmer), at a excitation of 348 nm and a emission of 423 nm.
8.4 RESULTS

8.4.1 Biomass and dry weight of cells

There were no differences in the biomass production of cells growing with varying levels of ascorbic acid (AsA) in medium (Tab. 1).

Table. 1 The biomass production (mg/10 ml suspension) of cells growing with varying levels of ascorbic acid in media (average ± standard error)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>19.7 ± 1.45</td>
<td>41.2 ± 1.94</td>
<td>96.0 ± 2.10</td>
<td>102.4 ± 2.87</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>20.6 ± 2.00</td>
<td>42.0 ± 1.38</td>
<td>96.3 ± 2.72</td>
<td>101.8 ± 0.76</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>20.4 ± 1.57</td>
<td>41.4 ± 1.30</td>
<td>92.5 ± 1.51</td>
<td>100.1 ± 0.33</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>18.6 ± 1.66</td>
<td>39.8 ± 0.34</td>
<td>98.8 ± 1.08</td>
<td>100.6 ± 0.40</td>
</tr>
</tbody>
</table>

In contrast to the biomass, cells growing in the presence of AsA in medium had higher amounts of dry matter when determined at day 4, 8 and 12, than the control cells, but no significant differences in dry weights were observed with cells receiving an external source of AsA (Tab. 2).

Table. 2 The dry weight (mg) of cells as effected by varying levels of ascorbic acid in medium (average ± standard error)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>0.38 ± 0.12</td>
<td>0.91 ± 0.04</td>
<td>2.09 ± 0.09</td>
<td>2.50 ± 0.15</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>0.41 ± 0.22</td>
<td>0.90 ± 0.12</td>
<td>2.06 ± 0.11</td>
<td>2.42 ± 0.12</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>0.42 ± 0.21</td>
<td>0.94 ± 0.22</td>
<td>2.02 ± 0.14</td>
<td>2.48 ± 0.18</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>0.43 ± 0.19</td>
<td>0.81 ± 0.11</td>
<td>1.88 ± 0.12</td>
<td>2.00 ± 0.15</td>
</tr>
</tbody>
</table>
8.4.2 Total number of cells

Various levels of AsA in medium did not show any significant differences (P=0.05) on the total number of cells (Table 3).

Table. 3 The effect of four media ascorbic acid levels on the total number of cells per ml cell suspension) (average ± standard error x10^3)

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>Trt. 0</th>
<th>4</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>6.2x10^6 ± 42^A</td>
<td>15.9x10^7 ± 57^A</td>
<td>172x10^8 ± 76^A</td>
<td>187x10^8 ± 82^A</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>7.1x10^6 ± 36^A</td>
<td>16.1x10^7 ± 61^A</td>
<td>180x10^8 ± 81^A</td>
<td>183x10^8 ± 80^A</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>5.9x10^6 ± 40^A</td>
<td>16.5x10^7 ± 63^A</td>
<td>176x10^8 ± 78^A</td>
<td>188x10^8 ± 89^A</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>6.3x10^6 ± 38^A</td>
<td>16.7x10^7 ± 68^A</td>
<td>179x10^8 ± 87^A</td>
<td>191x10^8 ± 82^A</td>
</tr>
</tbody>
</table>

8.4.3 Nutrient content of cells

There were no significant differences between treatments in the cation and anion composition of cells during the experimental period (Tables 4 and 5).

8.4.4 Sugar composition of cells

Cells cultured with added AsA in medium had higher levels of sucrose, glucose and fructose by day 4, 8, and 12 as compared with the control cells (Table 6). However, the sugar contents of cells receiving added AsA did not vary significantly (P=0.05) with each other.
Table 4 The effect of four media ascorbic acid levels on the cation content (μmol g⁻¹ Dw) of tomato cells (average ± standard error).

**Day 0**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>1004.5 ± 40.0</td>
<td>26.21 ± 1.23</td>
<td>68.69 ± 7.00</td>
<td>1099.40</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>1005.5 ± 52.6</td>
<td>26.80 ± 1.32</td>
<td>67.03 ± 2.19</td>
<td>1099.38</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>1012.2 ± 23.5</td>
<td>25.99 ± 1.73</td>
<td>69.27 ± 3.24</td>
<td>1107.56</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>1029.1 ± 34.0</td>
<td>24.89 ± 1.06</td>
<td>67.36 ± 5.00</td>
<td>1121.45</td>
</tr>
</tbody>
</table>

**Day 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>1065.9 ± 43.8</td>
<td>34.79 ± 1.12</td>
<td>78.97 ± 2.47</td>
<td>1179.66</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>1073.1 ± 66.6</td>
<td>34.06 ± 2.19</td>
<td>78.76 ± 6.78</td>
<td>1186.00</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>1078.8 ± 57.9</td>
<td>35.34 ± 3.54</td>
<td>80.65 ± 4.90</td>
<td>1194.80</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>1060.3 ± 43.6</td>
<td>36.15 ± 4.63</td>
<td>80.55 ± 2.92</td>
<td>1177.04</td>
</tr>
</tbody>
</table>

**Day 8**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>1067.8 ± 42.2</td>
<td>35.18 ± 1.20</td>
<td>80.20 ± 3.63</td>
<td>1183.18</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>1076.6 ± 54.2</td>
<td>36.88 ± 3.14</td>
<td>80.12 ± 3.93</td>
<td>1193.60</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>1078.4 ± 63.5</td>
<td>37.34 ± 1.53</td>
<td>79.92 ± 1.00</td>
<td>1195.66</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>1062.6 ± 43.3</td>
<td>39.99 ± 2.71</td>
<td>78.16 ± 2.56</td>
<td>1180.75</td>
</tr>
</tbody>
</table>
Table 5 The effect of four media ascorbic acid levels on the anion content (μmol g⁻¹ D.W) in tomato cells (average ± standard error).

### DAY 0

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cl⁻</th>
<th>NO₃⁻</th>
<th>SO₄²⁻</th>
<th>PO₄³⁻</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>124.9 ± 41.6</td>
<td>207.9 ± 24.3</td>
<td>127.0 ± 21.6</td>
<td>40.06 ± 2.0</td>
<td>499.8</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>120.4 ± 43.0</td>
<td>212.9 ± 21.4</td>
<td>119.8 ± 13.4</td>
<td>39.23 ± 1.3</td>
<td>492.3</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>119.0 ± 42.3</td>
<td>208.1 ± 42.9</td>
<td>121.6 ± 12.4</td>
<td>38.63 ± 2.0</td>
<td>487.3</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>125.1 ± 23.1</td>
<td>211.7 ± 34.9</td>
<td>125.3 ± 22.7</td>
<td>37.60 ± 2.0</td>
<td>499.7</td>
</tr>
</tbody>
</table>

### DAY 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cl⁻</th>
<th>NO₃⁻</th>
<th>SO₄²⁻</th>
<th>PO₄³⁻</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>151.3 ± 14.2</td>
<td>188.5 ± 25.6</td>
<td>146.5 ± 23.1</td>
<td>48.06 ± 4.4</td>
<td>534.3</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>149.6 ± 22.3</td>
<td>180.8 ± 11.5</td>
<td>147.5 ± 31.3</td>
<td>47.26 ± 1.0</td>
<td>525.1</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>154.1 ± 13.6</td>
<td>181.8 ± 34.3</td>
<td>143.0 ± 43.9</td>
<td>49.30 ± 5.2</td>
<td>528.2</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>153.5 ± 14.4</td>
<td>183.2 ± 43.3</td>
<td>137.8 ± 33.8</td>
<td>48.56 ± 0.3</td>
<td>523.0</td>
</tr>
</tbody>
</table>

### Day 8

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cl⁻</th>
<th>NO₃⁻</th>
<th>SO₄²⁻</th>
<th>PO₄³⁻</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>144.1 ± 17.1</td>
<td>216.2 ± 23.0</td>
<td>100.0 ± 13.7</td>
<td>55.33 ± 3.0</td>
<td>515.6</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>142.3 ± 21.4</td>
<td>223.2 ± 13.5</td>
<td>105.6 ± 24.8</td>
<td>57.66 ± 2.1</td>
<td>528.7</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>138.9 ± 38.5</td>
<td>216.7 ± 23.3</td>
<td>104.4 ± 13.0</td>
<td>55.73 ± 0.1</td>
<td>515.7</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>137.2 ± 19.1</td>
<td>208.4 ± 23.3</td>
<td>102.3 ± 21.4</td>
<td>55.76 ± 1.7</td>
<td>503.6</td>
</tr>
</tbody>
</table>
Table 6 The effect of three ascorbic acid levels in culture media on sugar contents (μmol Kg⁻¹ Fw) of tomato cells in suspension culture (average ± standard error)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>10.25 ± 0.45</td>
<td>8.75 ± 0.25</td>
<td>7.20 ± 1.0</td>
<td>26.20</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>10.00 ± 0.15</td>
<td>8.55 ± 0.65</td>
<td>7.05 ± 0.15</td>
<td>25.60</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>10.14 ± 0.36</td>
<td>8.90 ± 0.30</td>
<td>8.10 ± 0.40</td>
<td>27.14</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>10.54 ± 0.13</td>
<td>8.05 ± 0.55</td>
<td>7.15 ± 0.85</td>
<td>25.74</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>23.15 ± 0.75</td>
<td>18.15 ± 0.35</td>
<td>19.35 ± 0.45</td>
<td>60.65</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>22.00 ± 1.30</td>
<td>20.40 ± 1.10</td>
<td>19.25 ± 0.85</td>
<td>61.55</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>21.20 ± 1.20</td>
<td>19.15 ± 0.55</td>
<td>20.50 ± 0.80</td>
<td>60.85</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>12.00 ± 0.60</td>
<td>11.35 ± 1.05</td>
<td>10.00 ± 0.20</td>
<td>33.35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>20.85 ± 0.55</td>
<td>19.95 ± 0.55</td>
<td>22.70 ± 0.80</td>
<td>63.50</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>20.65 ± 0.75</td>
<td>19.85 ± 0.75</td>
<td>22.50 ± 0.70</td>
<td>63.00</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>22.60 ± 1.20</td>
<td>18.85 ± 1.00</td>
<td>22.35 ± 1.35</td>
<td>63.20</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>10.00 ± 0.65</td>
<td>14.65 ± 0.53</td>
<td>11.00 ± 0.30</td>
<td>35.50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>23.15 ± 0.55</td>
<td>23.15 ± 0.35</td>
<td>20.90 ± 1.30</td>
<td>67.20</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>23.35 ± 1.45</td>
<td>23.00 ± 0.90</td>
<td>19.70 ± 2.50</td>
<td>66.05</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>22.80 ± 0.24</td>
<td>22.70 ± 1.70</td>
<td>20.25 ± 2.05</td>
<td>65.75</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>12.05 ± 0.54</td>
<td>11.90 ± 0.40</td>
<td>12.20 ± 1.30</td>
<td>36.15</td>
</tr>
</tbody>
</table>
8.4.5 Organic acid composition of cells

When determined at day 12, cells receiving an external source of AsA had significantly higher levels of citric acid (Table 7). The citric acid content of treatments receiving an external supply of AsA did not differ with each other. Cells, grown with added AsA in medium, had a tendency to produce slightly higher levels of both Malic and Fumaric acids by day 12.

Table 7 The effect of four AsA levels in culture medium on the organic acid content (µmol Kg\(^{-1}\) Fw) of cells (average ± standard error)

<table>
<thead>
<tr>
<th>Trt:</th>
<th>Succinic acid</th>
<th>Fumaric acid</th>
<th>Malic acid</th>
<th>Citric acid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>43.00 ± 4.1</td>
<td>19.35 ± 5.0</td>
<td>180.6 ± 12</td>
<td>614.0 ± 20.2</td>
<td>856.9</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>42.03 ± 2.1</td>
<td>21.50 ± 4.0</td>
<td>169.1 ± 9.2</td>
<td>609.2 ± 33.6</td>
<td>841.3</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>38.83 ± 4.0</td>
<td>22.80 ± 6.1</td>
<td>173.2 ± 6.2</td>
<td>614.6 ± 26.2</td>
<td>849.4</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>44.30 ± 3.2</td>
<td>23.00 ± 6.0</td>
<td>179.3 ± 14</td>
<td>614.2 ± 12.6</td>
<td>860.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trt:</th>
<th>Succinic acid</th>
<th>Fumaric acid</th>
<th>Malic acid</th>
<th>Citric acid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>40.30 ± 4.1</td>
<td>27.52 ± 1.0</td>
<td>212.4 ± 8.4</td>
<td>1124.6 ± 22.0</td>
<td>1404.8</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>45.58 ± 5.1</td>
<td>27.72 ± 2.0</td>
<td>209.8 ± 9.5</td>
<td>1028.9 ± 12.1</td>
<td>1312.0</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>47.20 ± 6.0</td>
<td>30.53 ± 4.0</td>
<td>201.4 ± 9.4</td>
<td>1125.2 ± 23.1</td>
<td>1401.8</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>44.68 ± 9.2</td>
<td>26.82 ± 2.1</td>
<td>191.8 ± 10</td>
<td>812.0 ± 10.9</td>
<td>1075.3</td>
</tr>
</tbody>
</table>
8.4.6 The contribution of sugars and citric acid to the dry weight of cells

The percentage contribution of the three sugars and citric acid to the dry weight of the cells are shown in Table 6. Since organic acids were measured only at the beginning (day 0) and at the termination of the experiment (day 12), the % of individual components were calculated from the average values obtained on day 12. The contribution of sucrose, glucose, fructose and citric acid to the dry weight of cells was about 9% with cells receiving added AsA, whereas for the control cells this was about 5%.

Table 8 The percentage contribution of sucrose, glucose, fructose and succinic acid to the dry weight of cells as affected by four media AsA levels.

<table>
<thead>
<tr>
<th>Ttrt</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Succinic acid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mg/L</td>
<td>3.96</td>
<td>2.08</td>
<td>1.88</td>
<td>1.07</td>
<td>9.00</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>4.0</td>
<td>2.07</td>
<td>1.77</td>
<td>1.07</td>
<td>8.91</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>3.90</td>
<td>2.04</td>
<td>1.82</td>
<td>1.07</td>
<td>8.80</td>
</tr>
<tr>
<td>0 mg/L</td>
<td>2.06</td>
<td>1.07</td>
<td>1.01</td>
<td>0.96</td>
<td>5.10</td>
</tr>
</tbody>
</table>

8.4.7 Changes in the ascorbic acid content in cells and in media

There was almost no variation in the AsA content of control cells during the experimental period (Figure 1). The highest level of AsA content was observed at day 2 and thereafter there is a continuous reduction in cellular AsA. As expected, cells cultured with a higher level of AsA (60 mg/L) in medium had the highest level of this substance. The media AsA content followed almost the same pattern as the cells (Figure 1). There was no detectable AsA in all three media by day 12.
Total AsA in cells (mg/g FW)

A:

Total AsA content in medium (mg/L)

B:

Days in culture

0 2 4 6 8 10 12

Fig. 1. The changes in the ascorbic acid content of cells (A:), and in the media (B:) as effected by four AsA levels in culture medium. [(○) 60 mg/L; (□) 40 mg/L; (▼) 20 mg/L; (+) control:]. Standard errors smaller than the symbols are not shown.

8.5 Discussion

There were no large differences in the amount of biomass produced or the total number of cells during the experimental period between treatments suggests, that this substance has no effect on the cell division rate (Tables 1 and 3).

In contrast to the biomass, cells in treatments with added AsA had higher dry weights (Table 2). This indicates that cells in the presence of AsA have accumulated or produced more cellular substances than the control cells.

Though no significant differences in the cation and anion contents were observed (Tables 4 and 5), the sucrose, glucose, and fructose contents were higher in cells grown with added AsA in medium, by a factor of almost 2 (Table 6). Among the organic acids
only citric acid content was significantly higher in cells receiving an external source of AsA (Table. 5).

Therefore the increased dry weights of cells in the presence of AsA was at least partly due to these substances accumulated by cells. For example the three sugars and the citric acid alone contributed to about 25% of the dry weight increase of cells receiving an ascorbic acid level of 60 mg/L by day 12 as compared to the control cells grown without added AsA in medium.

Further, there are no experimental evidence yet to show that ascorbic acid has an effect on plant membranes. Therefore, permeability changes in the membranes due to the presence of ascorbic acid in the medium can be ruled out, at least by the AsA concentrations used in this study.

In higher plants ascorbic acid is produced through the conversion of D-glucose for which a number of enzymes are responsible (Loewus, 1963; Burns, 1967; Loewus et al., 1975). Loewus and Stafford (1958), saw massive conversion of $^{14}$C to soluble sugars and saccharidic polymers in grape leaves applied with labelled L-[6-$^{14}$C] ascorbic acid. The results of Loewus and Stafford (1958) were again confirmed by Saito and Kasai (1978), where they observed that $^{14}$C products were found as organic acids, above all tartaric acid, and in some products found in the hexose phosphate metabolism, when labelled L-[1-$^{14}$C]- and L-[6-$^{14}$C] ascorbic acid, were applied to grape vines. Therefore the increase in sugars and citric acid in cells grown in the presence of AsA most likely originated from the absorbed AsA by cells from the medium.

The AsA content in control cells stays constant throughout the experimental period. This shows that the turnover and its utilization within the cell occurs at a similar rate, and that a state of equilibrium is maintained (Figure 1). It is expected that ascorbic acid is taken up rapidly perhaps within minutes or few hours, after the cells are transferred to a medium containing AsA. Thereafter, there is a continuous reduction in cell AsA content suggests either it is being broken down to other metabolic products or converted to other cellular products (Figure. 1). As discussed before the possible explanation for this reduction is that, it is converted to other cellular products above all to sugars. Therefore with time lower levels of AsA was detected in cells, growing with added
ascorbic acid in medium. This is already seen with cells cultivated with 20 mg/L AsA in medium where they contain almost the same level of AsA as the control cells by day 12. The reduction in ascorbic acid content in medium with time was due to two reasons. Firstly, as shown before a large part of this substance was taken up by cells. Secondly the environmental conditions in the culture room and several elements in the culture media enhanced the breakdown of this substance to other products. Continuous shaking of flasks containing cells, illuminated conditions and metal ions such as Cu and Fe are the main factors leading to the enhanced breakdown of this substance (Barron et al., 1935; Chinoy, 1984).
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Relationship between hormone content and autonomy in various autonomous 


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