Physiological and Molecular Characterization of Habituated and Non-habituated Soybean callus Lines [*Glycine max* (L.) Merr cv. Acme]

by

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PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, from January 1993 to December 1997, under the supervision of Doctor. WA Cress and Professor. J van Staden.

These studies have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

Sandra du Plessis

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ABSTRACT

A cytokinin habituated soybean callus has been isolated, utilizing the cytokinin soybean bioassay. The habituated callus line was subsequently characterized with a non-habituated callus line in relationship to levels of endogenous growth substances, ultrastructure, nitrogen metabolism and pattern of gene expression.

The cytokinin habituated soybean callus contained a higher level of endogenous cytokinin-like activity in comparison to the non-habituated callus. This higher level of cytokinin present is in part due to a lower rate of degradation. The habituated callus tissue produced very low levels of ethylene, while the non-habituated callus produced ethylene at a much higher rate (57 fold higher). than the habituated callus. In contrast to what was found in habituated sugarbeet callus, only low levels of putrescine could be detected in both callus types. The putrescine content of habituated callus tissue was lower than that of non-habituated callus tissue.

The ultrastructure of habituated callus cells exhibited several differences to what was observed in the non-habituated callus. Habituated callus cells appeared to have a thinner cell wall than that of the non-habituated callus cells. The cristae of the mitochondria in habituated cells were thicker than that of the non-habituated callus cells, indicating a lower metabolic activity. On day 14 of the growth period the nuclei of habituated callus demonstrated active RNA synthesis as indicated by the presence of several vacuolated nucleoli.

Although no significant differences between proline levels of habituated callus and proline levels of non-habituated callus were observed, it was demonstrated that there was a difference in proline metabolism between the habituated and non-habituated calli. Utilizing an inhibitor of OAT, gabaculine, it was shown that in habituated callus tissue proline originated from ornithine

during the first 14 days of growth. During the second half of the growth period, which characteristically consists of tissue with low biosynthetic activity, proline originated from glutamate. The production of proline in habituated callus from ornithine also corresponded to a period of high NH_4^+ content in both callus types, while the production of proline from glutamate corresponded to a period of low NH_4^+ content in the cells of both callus types. No such correlation was observed in proline metabolism of non-habituated callus.

A similar turning point was observed in the activity of OAT of both callus types. Although the specific activity of OAT in both callus types mirrored their changes in RNA concentration, the percentage inhibition of OAT by gabaculine was not significant from day 14 in both callus types. This may indicate a change in the catalyzing properties of OAT in both callus types. It was further demonstrated that the non-habituated callus tissue contained some inhibitor inactivating OAT activity.

With the use of gabaculine it was further shown that, in contrast to what was found in other habituated calli, there is no metabolic link between proline metabolism and putrescine synthesis.

Both the habituated callus and the non-habituated callus exhibited a high nitrogen influx during the first 14 days of the growth period. The low NH_4^+ content present in both callus types during the second half of the growth period coincided with higher levels of amino acids present in both callus types. The levels of precursor amino acids (glutamate, aspartate and alanine) did not fluctuate during the growth period, indicating a tight control on amino acid pools. Levels of amino acids further down the path of metabolism did not fluctuate drastically and there appeared to be very little difference between the levels of different amino acids measured in the habituated and non-habituated calli. Serine was the dominant amino acid in both callus types.

Total RNA concentrations of habituated callus were low in comparison to that of the non-habituated callus, except for a striking 12 fold increase on day 14 of the growth period. RNA concentrations of non-habituated callus increased gradually during the growth period and the highest concentration was recorded 21 days after subculturing. Several polypeptides were observed in the habituated callus that were not present in the non-habituated callus, utilizing IEF. Three polypeptides exhibited a change in concentration from day 6 to day 14 of the growth period in both the habituated and non-habituated callus. These polypeptides appeared to decrease in non-habituated callus, while they increased in the habituated callus.

A complete cDNA library was constructed for both of the habituated and non-habituated callus lines. Six different clones, that were over expressed in the habituated callus tissue, were isolated via subtractive techniques. One clone was characterized and showed homology to the glutamate/aspartate transport protein, the membrane component, of *E. coli*.

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ABBREVIATIONS

2,4D	2,4-dichlorophenoxyacetic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AVG	aminoethoxyvinylglycine
BSA	Bovine serum albumin
bp	base pairs
DEPC	Diethyl pyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
EGTA	[Ethylene-bis(oxyethylenenitrilo)]tetraacetic acid
FAD	Flavin adenine dinucleotide
GSA	glutamyl-γ-semialdehyde
IAA	*Indole-3-acetic acid*
IEF	Isoelectric focusing
iP	$N^6(\Delta^2$-isopentenyl)adenine
IPTG	isopropanyl-β-D-thiogalactoside
KD	Kilo dalton
LB	Luria broth
MACC	malonyl 1-aminocyclopropane-1-carboxylic acid
MOPS	3-[N-Morpholino]propanesulfonic acid
NAA	α-Naphthaleneacetic acid
NAD	Nicotinamide adenine
NADP	Nicotinamide dinucleotide phosphate
OAT	Ornithine aminotransferase (EC 2.6.1.13)
P5C	Δ^1-pyrroline-5-carboxylate
PCR	Polymerase chain reaction
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEM	Transmission electronmicroscope
TRIS	Tris(hydroxymethyl)aminomethane
x-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

CYTOKININ HABITUATION

1.1 INTRODUCTION

The suggestion that cytokinin and auxin mediated changes in the physiology of plant cells are accompanied by alterations in nucleic acid metabolism was made 40 years ago (SKOOG AND MILLER, 1957). Accordingly, elevated cytokinin levels in intact plants, excised plant organs and cultured plant cells from a variety of monocotyledonous and dicotyledonous species have been shown to induce specific changes in gene expression of a variety of gene products, such as the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco E.C. 4.1.1.39), defense related proteins, ß-1,3-glucanase (E.C. 3.2.1.6), nitrate reductase (E.C. 1.6.6.1) and several others (as reviewed by HARE AND VAN STADEN, 1997). Cytokinins have the ability to stimulate transcription and cytokinin regulated promoters have been identified (SUGIHARTO, BURNELL AND SUGIYAMA, 1992; BANOWETZ, 1992; DEHIO AND DE BRUIJN, 1992). It is assumed that the modulation of levels of these gene products is a step in the manifestation of the physiological effects of cytokinins on plant cells (BINNS, 1994; SMÜLLING, SCHÄFER AND ROMANOV, 1997). Although cytokinins were first discovered to induce cell division, they are also known to act in combination with other hormones to regulate diverse responses in plants, including seed germination, *de novo* bud formation, the release of buds from apical dominance, leaf expansion, reproductive development, senescence, chloroplast development and appropriate responses to adverse environmental stimuli (BRZOBOHATÝ, MOORE AND PLAME, 1994, HARE AND VAN STADEN, 1997). Plants expressing the *ipt* gene form *Agrobacterium tumefaciens* showed an increase in basal cytokinin levels, were reduced in structure, had released axiliary buds, smaller stem and leaf area, reduced xylem and a reduced root system (HOBBIE, TIMPTE AND ESTELLE, 1994). Most of the multiple cytokinin-regulated processes are, however, very complex. In particular developmental processes can be long term,

involving many different steps and regulatory hierarchies, making it difficult to accurately access the action of cytokinins (SMÜLLING, SCHÄFER AND ROMANOV, 1997).

Despite the wealth of literature concerning the physiological effects of cytokinins, our information concerning the mechanism by which cytokinin perception leads to a change in plant growth and development is still rudimentary. Until recently our understanding of cytokinin physiology resulted mainly from the manipulation of endogenous cytokinin levels by either application of exogenous cytokinins or the expression of cytokinin biosynthetic transgenes (HOBBIE, TIMPTE AND ESTELLE, 1994; SMÜLLING, SCHÄFER AND ROMANOV, 1997; HARE AND VAN STADEN, 1997 AND REFERENCES THEREIN). An alternative approach is to look for mutants effected in cytokinin biosynthesis or response. A few cytokinin mutants have already been described. These include the *ckr1* mutations leading to" cytokinin root syndrome" in *Arabidopsis thaliana* (SU AND HOWELL, 1992) and *Nicotiana plumbaginifolia* (BLONSTEIN, PARRY, HORGAN AND KING, 1991). The phenotype of *ckr1* mutants exhibits seedlings with longer roots and shorter roothairs than those of the wild-type plants in the absence of cytokinins. They were found to be allelic to *ein2* involved in the ethylene response pathway (CARY, LIU AND HOWELL, 1995) and to the *Aba1* locus involved in abscisic acid biosynthesis (ROUSSELIN, KRAEPIEL, MALDINEY, MIGINIAC AND CABOCHE, 1992). Another *Arabidopsis thaliana* mutant display some traits consistent with a defect in cytokinin action (DEIKMAN AND ULRICH, 1995). The recessive *Arabidopsis thaliana* mutant *amp1* displays reduced apical dominance, altered floral morphology and delayed senescence, which may result from elevated cytokinin levels (CHAUDHURY, LETHAM, CRAIG AND DENNIS, 1993).

SKOOG AND MILLER (1957) established that the relative concentrations of auxin and cytokinin in the culture medium regulate the formation of root and shoot meristems form unorganized tissue. Plant development is therefore highly regulative. Cell lineages are not, in general, fixed and even highly specialized cells remain totipotent (MEINS, 1989). There is thus no doubt that insight into plant growth and regulation will arise from determining the link between cytokinin

perception and the elicited physiological effects. The availability of strictly cytokinin-responsive elements in plants to cytokinins under certain environmental conditions represents a valuable mechanism of enhancing agricultural productivity.

Habituation of callus cultures is defined as the stable heritable loss of callus tissue for the requirement of one or more growth regulators *in vitro* (MEINS, 1989). Habituation thus provides an experimental system for studying stable changes in the metabolism of the tissue and identification of responsive elements to the hormone(s) for which the tissue is habituated, under controlled conditions in culture.

1.2 Habituation: cultural curiosity or neoplasic development in the absence of pathogens?

"Yet the results of such culture experiments (culture of isolated vegetative plant cells) should give some interesting insight into the properties and potentialities which the cell as an elementary organism processes. Moreover it will provide information about the inter-relationships and complementary influences to which cells within the multicellular whole organism are exposed."

G Haberlandt

(KRIKORIAN AND BERQUAM, 1969).

About 95 years have elapsed since Haberlandt's unsuccessful attempts to culture isolated plant cells. The great interest of Haberlandt's paper is his clear concept of the problem and his foresight as to what would be achieved (STREET, 1977). In 1939, however, three scientists, White, Gautheret and Nobecourt, successfully proceeded to the first unlimited plant cell cultures (BOXUS, 1987) satisfying both major criteria of plant tissue culture, undifferentiated and potentially unlimited growth. They introduced indole-acetic acid acid (IAA) in order to sustain an indefinite mitotic activity (BOXUS, 1987). In 1955, Skoog's research led to the production of kinetin (6-furfurylaminopurine), a synthetic cytokinin, from a DNA hydrolysate (BOXUS, 1987). SKOOG AND MILLER (1957) demonstrated that

while auxin induced cell enlargement, a simultaneous addition of kinetin is necessary to induce cell division

1.2.1 Characteristics of callus growth

Callus cultures provide a morphologically more uniform material than the whole plant (ALLAN, 1991). Callus has a high growth rate, is small and convenient to handle (ALLAN, 1991). A better understanding of the part played by growth hormones led to substantial progress in *in vitro* culturing techniques. Tissue culture pose certain advantages in the study of cellular metabolism: rapid growth, sterility and determinable and controllable environmental conditions. In contrast to suspension cultures, there is very little information available concerning callus development, with most of the research conducted by Street and co-workers during the 1970's (STREET, 1977).

Researchers have recognized two stages in callus development, namely: a so called wound response and a growth response (YEOMAN AND AITCHINSON, 1977; ALLAN, 1991). Wound responses are characterized by limited cell division and a rapid increase in metabolic activity, but this response does not necessarily lead to callus development (YEOMAN AND AITCHINSON, 1977; ALLAN, 1991). The growth response results in continued cell division and is dependent on an exogenous supply of at least auxin (YEOMAN AND AITCHINSON, 1977; ALLAN, 1991). The callus growth response can be divided into three phases, which are characterized by changes in mean cell size of the population and overall metabolism, and it culminates in the appearance of a callus (YEOMAN AND AITCHINSON, 1977; ALLAN, 1991). These stages have been termed: *induction* - when cells prepare for division, *division* - when mean cell size decrease and *differentiation* - when cells become vacuolated and the rate of cell division decrease so that there is an equilibrium between expansion and division (ALLAN, 1991). The term differentiation for the final stage is somewhat controversial since it only refers to cytoplasmic changes and no morphological differentiation associated with a specialized function is implicated.

Investigation of callus growth by evaluating the external morphology of a tobacco (*Nicotiana tabacum*) culture, showed that most rapid growth occurs where cells are in contact with the growth medium (YEOMAN AND AITCHINSON, 1977). As growth progresses the rate of growth varies in different areas within the callus (YEOMAN AND AITCHINSON, 1977). Another feature of callus growth is that the increase in fresh weight and cell number occurs at an exponential rate so that growth is balanced (YEOMAN AND AITCHINSON, 1977). It was further demonstrated that the metabolic characteristics of cells in tissue culture may be considerably modified by conditions imposed by the medium (YEOMAN AND AITCHINSON, 1977).

Plant tissues usually require exogenous sources of the growth hormones auxin and cytokinin for continuous proliferation in culture, on an otherwise complete medium (MEINS, 1989). In 1942 Gautheret reported that certain strains of carrot tissue can gradually lose their requirement of exogenously supplied auxin (MEINS, 1989 AND REFERENCES THEREIN). He called this phenomenon "à *l'auxine*" and later " *anergie* à *l'auxine*", which is translated as auxin habituation. By comparing the growth responses of the tissues on media with and without auxin Gautheret found that, during serial propagation, tissues often increase in the degree of habituation (MEINS, 1989 AND REFERENCES THEREIN). Certain similar variations can also occur for cytokinins and, more rarely, certain vitamins (MEINS, 1989).

Plant tissues and cells in culture undergo variation (MEINS, 1983). Variation is defined as phenotypic changes that are firstly stable *i.e.* it persists in the absence of the event that induced the change and secondly it is heritable *i.e.* the phenotype is transmitted to the daughter cells when they divide (MEINS, 1983). Habituation is defined as the stable heritable loss in the requirement of cultured plant cells for one or more growth factors (BUTCHER, 1977). Habituation can be inherited by single cells and could result from mutations, epigenetic changes, or a combination of both (MEINS, 1989). If a new characteristic can be transmitted sexually, it is described as genetic (STAFFORD, 1991). This infers that the variation has arisen from a structural alteration in the DNA.

Evidence suggests that it is usually only when selection pressures, such as toxin application, or stressful conditions, such as chloroplast fusion, are imposed that alterations in the mitochondrial or chloroplast genome are likely to be brought about (STAFFORD, 1991). Genetic mechanisms for observed changes in the nuclear genome include:

1. Variation in chromosome number

Polyploidy occurs naturally *in vivo* and are common amongst angiosperms (STAFFORD, 1991). The high frequency with which polyploidy occurs in callus and suspension cultures suggests that, *in vitro*, the mechanisms which normally control the occurrence of aberrant mitotic events may operate at a reduced level (STAFFORD, 1991). Events such as synchronous division of nuclei of binucleiate cells, abnormal nuclear spindle formation, chromosome lagging during anaphase, and the formation of a single nucleus after chromatid segregation due to poor functioning of the spindle, could lead to polyploidy (STAFFORD, 1991). Aneuploidy is also frequently detected in plant cell cultures as a result of aberrant mitotic events including those which follow nuclear fragmentation (STAFFORD, 1991). Non-disjunction (where both chromatids move to one pole of the spindle, instead of to opposite poles) is an additional mechanism for aneuploidy (STAFFORD, 1991).

2. Chromosomal aberrations

These aberrations include: chromatid deletions, chromatid interchanges, pseudochiasmata, and dicentric and chromosomal rings (STAFFORD, 1991). Such drastic rearrangements of the genome are likely to lead to dramatic changes in gene expression. While chromatid deletions are likely to unmask normally recessive genes, transposition of a chromosome fragment may lead to the suppression or expression of genes adjacent to the site of excision or insertion (STAFFORD, 1991).

3. Mutations

Many heritable mutations have shown a complex inheritance pattern, which suggests the contribution of a number of genes, while others clearly have a single gene basis (STAFFORD, 1991).

Heritable characteristics are not necessarily genetically determined. Whereas it is possible to suggest numerous genetic mechanisms which could provide a basis for heritable, sexually transmitted (genetic) characteristics, epigenetic changes pose more of a problem. Epigenetic alterations are heritable changes in gene expression which do not arise as a result of permanent alterations in the genome of the cell (STAFFORD, 1991). Epigenetic changes can be distinguished from mutations in several respects, namely:

- it is directed (*i.e.* it occurs at high rates $>10^{-3}$ per cell generation under inductive conditions);
- the genome is not permanently altered and the change is potentially reversible;
- the range of phenotypes generated is limited by the genetic potential of the cell and;
- epigenetic changes are not transmitted meiotically (MEINS, 1989).

There are a number of mechanisms which may underlie the epigenetic nature of variation in cultured plant cells:

1. Gene amplification

By extrapolation from animal systems, this phenomenon can be viewed as producing relatively stable/unstable changes depending upon the location of the amplified sequences (STAFFORD, 1991). It is known that gene amplification can accompany the tissue culture process, e.g. in rice certain nuclear DNA sequences became amplified during dedifferentiation of the explant tissue (STAFFORD, 1991).

2. Gene methylation

More than 25% of the cytosine residues in eukaryotic DNA in plant cells, can be methylated. In plant cell cultures, alterations in DNA methylation patterns have been observed to occur spontaneously during the process of differentiation or dedifferentiation (STAFFORD, 1991).

3 Controlling elements

These elements, also defined as genetic factors, are capable of moving around in the genome and of modifying gene expression (STAFFORD, 1991). Insertion of these controlling elements causes suppression of transcription activity at the site of insertion, or production of aberrant transcription products while excision may revert to the wild type (STAFFORD, 1991).

1.2.2 Habituation: an epigenetic variation in cell cultures

Habituation is a form of neoplasic transformation involving heritable progressive changes in cell phenotype which can result in hormone autonomous growth (CHRISTOU, 1988). Habituation has been noted in many species cultured e.g. *Crepsis capillaris* (SARCISTAN, WENDT-GALLITELLI, 1971), potato (*Solanum tuberosum*) (WIDHOLM, 1977), tobacco (BINNS AND MEINS, 1973), sugarbeet (*Beta vulgaris* L.) (DE GREEF AND JACOBS, 1979) and soybean (*Glycine max* L.) (CHRISTOU, 1988). Evidence for habituation necessarily comes from callus, because to demonstrate it one must be able to measure and compare rates of growth in the presence and absence of added growth regulators (MEINS, 1989). Habituation occurs rapidly, relative to somatic mutation, at rates of $4x10^{-3}$ per cell generation, which is 100-1000 times faster than somatic mutation rates reported for *Nicotiana spp.* (MEINS AND LUTZ, 1980). Habituation rates also depends on the physiological state of the cells. Tobacco pith tissue isolated from plants during Spring habituated 7 times faster than tissue isolated during Winter (MEINS AND LUTZ, 1980). It is a directed process that occurs due to specific inducers, although certain types of unusual constitutive genetic changes have also been observed (BINNS, 1994 AND REFERENCES THEREIN).

Tissues with different states of habituation and different degrees of competence to habituate can be derived from different types of explants (MEINS, 1989). The competence for habituation, like habituation itself has an epigenetic basis (MEINS, 1989). Studies of variation in the requirement of tobacco cells for cytokinins provided evidence that states of determination arising in ontogeny can be inherited by individual cells (MEINS AND HANSEN, 1986). These states, although stable for many generations, are not permanent and cells determined under a set of conditions can remain totipotent (MEINS AND HANSEN, 1986). The epigenetic basis of cytokinin habituation in tobacco cells was demonstrated by pith tissue from tobacco which habituated rapidly under inductive conditions in culture (MEINS, 1989). Primary explants of pith tissue habituate for cytokinins when incubated on an auxin containing medium at 35°C, 10°C above the standard culture temperature, or in the presence of trace amounts of cytokinins (MEINS, 1989).

Primary explants of tobacco pith tissue has an absolute requirement for cytokinins (MEINS AND LUTZ, 1980). When incubated at 25°C on a basal medium containing auxin, cells dramatically increased in size and the tissues became translucent but did not continue to grow in subsequent transfers (MEINS AND LUTZ, 1980). However, a large but variable portion of the explants incubated at 35°C continued to grow when subcultured at 25°C on a basal medium (MEINS AND LUTZ, 1980). The habituated state could thus be induced when incubated at 35°C.

Below a critical kinetin concentration of 1 nM explants did not habituate (MEINS AND LUTZ, 1980). Above this threshold the incidence of habituation increased with kinetin concentration, but also depended on the duration of the kinetin treatment (MEINS AND LUTZ, 1980). The concentration required for induction of 50% of the explants dropped by about 3 orders of magnitude over a 30 day period (MEINS AND LUTZ, 1980). Thus for long incubation times extremely low concentrations of kinetin will be sufficient to induce habituation.

1.2.3 Characterized changes associated with the habituated phenotype in tobacco and sugarbeet callus

Although variation in the requirement for exogenously supplied growth substances has been documented for cultured tissues of several plants, comprehensive studies have only been conducted on cultured tissues of tobacco (MEINS, 1989) and sugarbeet callus (GASPAR, HAGEGE, KEVERS, PENEL, CREVECOEUR, ENGELMANN, GREPPIN AND FOIDART, 1991).

Tobacco:

Tissues with different states of habituation and different degrees of competence to habituate can be derived from different types of explants (MEINS, 1989). In tobacco the developmental state of cells strongly influences their tendency to habituate (MEINS, 1983). There was a gradient along the stem of the tobacco plant in the incidence of cytokinin habituation of pith explants - high near the top and low near the bottom (MEINS, 1989 AND REFERENCES THEREIN). Different tissues also appeared to differ in their competence for cytokinin habituation. Pith tissue of tobacco consists of inducible cells that habituate at high rates as well as non-inducible cells, while tissues cultured from the lamina consist almost exclusively of non-inducible cells (MEINS, 1989 AND REFERENCES THEREIN). Tissues cultured from the cortex of the stem do not require cytokinins for proliferation in culture and were almost exclusively constitutively habituated (MEINS, 1989 AND REFERENCES THEREIN).

Ultimately, however, the stability of epigenetic states is under genetic regulation. In contrast to wild-type tobacco, leaves of plants regenerated from certain cytokinin habituated lines grew in culture in the absence of exogenously supplied cytokinins (BINNS, 1994 AND REFERENCES THEREIN). These studies identified two unlinked genes, Habituated leaf-1 (HI-1) and Habituated leaf-2 (HI-2), that regulated the cytokinin requirement of tobacco cultured tissues (MEINS, 1989 AND REFERENCES THEREIN). When HI-1 plants were inoculated with *Agrobacterium tumefaciens* mutants that lacked the *ipt* gene, fully autonomous

tumors developed, indicating that the aberrant locus has oncogenic functions similar to the wild-type *ipt* gene (MEINS AND HANSEN 1986).

The occurrence of the cytokinin habituated phenotype in tobacco calli seemed to be correlated to the presence nodules in the callus (MEINS AND LUTZ, 1980; KERBAUY, MONTEIRO, KRAUS AND HELL, 1987). Although, cytokinin habituated tobacco calli have the capacity to accumulate zeatin riboside (HANSEN, MEINS AND AEBI, 1987), studies demonstrated that the capacity for cytokinin autotrophic growth was, however, not correlated with zeatin riboside content (HANSEN, MEINS AND AEBI, 1987). Provided auxin was added to the culture medium zeatin riboside levels were below the limit of detection. Expression of the habituated phenotype by some tobacco calli results in cold sensitive lines and the effect of cold treatment was reversible (SYÕNO AND FURUYA, 1971; BINNS AND MEINS, 1979).

Sugarbeet:

Three sugarbeet callus lines, generated from the same plant, have been extensively investigated to note any differences that may occur on a biochemical level. The habituated non-organogenic callus is friable with a high water content and low dry weight and can proliferate in the absence of both auxin and cytokinins (GASPAR, HAGEGE, KEVERS, PENEL, CREVECOEUR, ENGELMANN, GREPPIN AND FOIDART, 1991). It is highly meristematic but unable to differentiate. Habituated sugarbeet callus has impaired cell wall development due to a lack of lignin and cellulose deposition (BISBIS, LE DILY, KEVERS, BILLARD, HUAULT AND GASPAR, 1993). At an ultrastructural level these callus cells lack well differentiated plastids and chlorophylls, and have large nuclei irregular in shape with deep invaginations and several nucleoli (CREVECOEUR, HAGEGE, CATESSON, GREPPIN AND GASPAR, 1992).

The habituated sugarbeet callus is poor in porphyrin-containing compounds which includes proteins involved mainly in H_2O_2 detoxification and desaturation of fatty acids (KEVERS, COUMANS, DE GREEF, HOFINGER AND GASPAR, 1981; BISBIS, LE DILY, KEVERS, BILLARD, HUAULT AND GASPAR, 1993). The

deficiency in these systems in habituated callus cells is exacerbated by low levels of ascorbate present (BISBIS, LE DILY, KEVERS, BILLARD, HUAULT AND GASPAR, 1993). This, and higher levels of superoxide dismutase (E.C. 1.15.1.1) and glutathione reductase (E.C. 1.8.4.7) in the habituated sugarbeet callus, suggests that the habituated callus undergoes oxidative stress (BISBIS, LE DILY, KEVERS, BILLARD, HUAULT AND GASPAR, 1993). These biochemical activities have been correlated with the loss of membrane integrity and the accumulation of proline and polyamines, which are all indicators of stress (LE DILY, HUAULT, GASPAR AND BILLARD, 1993). Habituated sugarbeet callus also appear to deviate in terms of its sugar metabolism, favouring the pentose phosphate pathway at the expense of glycolysis (BISBIS, LE DILY, KEVERS, BILLARD, HUAULT AND GASPAR, 1993). This difference in sugar metabolism includes two aspects, namely: abnormal accumulation of hexoses; and a high fructose/glucose ratio (BISBIS, LE DILY, KEVERS, BILLARD, HUAULT AND GASPAR, 1993).

1.2.4 Cytokinin habituated callus: cytokinin response altered tissue or progressive plant cancer?

Cytokinin habituated plant cells may represent an intriguing example of cytokinin response altered tissue. The physiological basis of habituation is unknown. Most physiological studies of habituation have focused on the relationship between autotrophic phenotypes and their capacity to produce the hormones for which they are habituated (MEINS, 1989). In this regard conflicting results have been obtained. Studies have indicated that auxin and cytokinin are not present in either cell type (MEINS AND HANSEN 1987; MEINS, 1989 AND REFERENCES THEREIN). Hormones were, however, also shown to be present in both cell types in similar concentrations (KEVERS, COUMANS, DE GREEF, HOFINGER AND GASPAR, 1981; KÖVES AND SZABÒ, 1987), or the hormone concentrations were elevated in habituated cells compared to non-habituated cells (DYSON AND HALL, 1972; KERAUBY, MONTEIRO, KRAUS AND HELL, 1988; MEINS, 1989 AND REFERENCES THEREIN).

This suggests that the cytokinin response system in the cytokinin-habituated tissue was constitutively switched on. However, this interpretation is not compatible with studies implicating that neither cytokinin overproduction nor a general increase in cytokinin responsiveness is responsible for the habituated phenotype. Expression of the cytokinin responsive gene β-1,3-glucanase of habituated tissue with elevated levels of cytokinins showed no down regulation unless exogenous cytokinins were also present (EICHOLTZ; HARPER, FELIX AND MEINS 1983). Similarly, analysis of bud initiation in cytokinin habituated lines of tobacco showed that stimulation of adventitious shoots required the same concentration of exogenous cytokinin than the non-habituated line (MEINS AND BINNS, 1977), implicating that neither cytokinin over production nor a general increase in cytokinin responsiveness is responsible for the cytokinin habituated phenotype.

The phenomenon of habituation also bears striking similarity to tumor transformation in crown gall diseases (GASPAR, 1995), where tumor tissues grow independent of exogenous hormones. Hormonal autonomy in crown gall as in habituated tissues leads to metabolic and physical changes in the tissue (CHRISTOU, 1988). Synthesis of a number of polypeptides is elicited as a result of the changes in physiology and/or metabolism in plants or tissues *in vitro.* (CHRISTOU, 1988).

In most cases, habituated cells keep their totipotency as do genetic tumors. Cells from habituated non-organogenic sugarbeet calli resemble in many traits those from genetic tumors, where the occurrence of neoplasia were not attributed to any external tumor inducing biotic agent, such as the presence of large nuclei, several nucleoli and micronuclei (GASPAR, HAGEGE, KEVERS, PENEL, CREVECOEUR, ENGELMANN, GREPPIN AND FOIDART, 1991). Tumor producing hybrids contain more free amino acids than normal plants and have significant differences in their levels of sugars, organic acids and alkaloids (BUTCHER, 1977). Genetic hybrids also contain higher levels of endogenous auxin and no growth factors are necessary to establish these hybrids in culture (BUTCHER, 1977).

Many characteristics of habituated sugarbeet callus cells are also common to animal cancer cells, namely: a high level of polyamines, a low level of porphyrin containing compounds, high activity of enzymes active against active oxygen species and a high level of thiobarbituric acid reactive substances (malondialdehyde and 4-hydroxynomenal) (GASPAR, 1995 AND REFERENCES THEREIN). Another trait of habituated non-organogenic callus cells, also common to true cancerous cells, is their monoclonal origin. Habituated sugarbeet callus originated as white clumps at the surface of green callus (DE GREEF AND JACOBS, 1979); while heat induced habituated tobacco callus tissue was observed as dense white hyperplastic nodules formed on a background of translucent tissue of the explant (MEINS AND LUTZ, 1980). Habituated sugarbeet callus was also responsive to allipticine an anti-cancer drug (GASPAR, 1995 AND REFERENCES THEREIN).

1.3 CONCLUSION

It would thus appear that the morphological and metabolic characteristics of habituated tissue are neither the result of hormone overproduction nor the result of a general increase of hormone responsiveness. Hence, due to the paucity of our knowledge concerning the scientific value and potential of habituated tissue for plant growth and development it is important to fully characterize the phenomenon, since it also provides an opportunity to investigate the vague but real concept of plant cancer. From various biochemical and morphological studies it is evident that the habituated phenotype bears striking similarities to cancerous cells observed in both plants and animals (GASPAR, 1995 AND REFERENCES THEREIN).

During this study a cytokinin habituated soybean callus line was isolated. The aim of this investigation was firstly, to characterize the levels of endogenous growth substances present in the habituated callus, and to compare it with what was observed in the non-habituated callus tissue. Contents of endogenous cytokinins, polyamines and ethylene production were characterized. Secondly, the ultrastructure of habituated soybean callus cells was described in comparison to

that of non-habituated callus cells. This section of the study provided valuable insight into certain biochemical properties of the habituated tissue. A change in nitrogen metabolism seems to be common to both habituated tissue and true cancer cells, hence thirdly, during this study an investigation was conducted comparing the levels of seventeen protein amino acids of habituated and non-habituated callus tissue. Proline synthesis was determined in detail including the characterization of the role of ornithine aminotransferase (OAT, E.C. 2.6.1.13) during the growth period of both callus types. Habituation has either an epigenetic or a genetic basis. It was thus of great importance to fourthly, conduct a molecular study of the habituated soybean callus tissue isolated. Total RNA levels and protein profiles were investigated. A cDNA library was constructed of each of the habituated and non-habituated callus tissue and six different clones unique to the habituated phenotype were isolated, via subtractive techniques.

CHAPTER 2

ISOLATION OF A HABITUATED SOYBEAN CALLUS LINE AND CHARACTERIZATION OF ENDOGENOUS GROWTH REGULATORS

2.1 INTRODUCTION

A key role for callus in plant tissue culture is the importance of maintaining plant material under controlled conditions (ALLAN, 1991). However, irrespective of the control imposed, heterogeneity exists both between different callus lines and within a callus itself (ALLAN, 1991). This heterogeneity is represented in established calli by differences in colour, morphology, structure, growth and metabolism (ALLAN, 1991). Callus heterogeneity can arise from:

- different genotypes within a species;
- different tissue and cell types from which the callus is initiated; and
- the methods and conditions in which calli are maintained and subcultured (ALLAN, 1991).

The morphology of the callus is dependent on its mode of growth. As a callus grows, cells are pushed upwards and outwards from the surface of the medium (YEOMAN AND AITCHINSON, 1977). This gives rise to the establishment of nutrient gradients between the cells and the substrate. These nutrients include gasses, plant growth regulators and products of metabolism which will eventually establish micro-environments (YEOMAN AND AITCHINSON, 1977). Such gradients and micro-environments can then in themselves affect growth patterns. It is therefore impossible to prevent this variation in any closed environment where nutrients are not replenished nor metabolites removed from the system. Heterogeneity implies that no two calli are identical. Calli do, however, become less variable with time and some consistency can be attained (ALLAN, 1991). The relative abundance of cell types in cultured tissues and cell suspensions is determined by three factors :

- relative rates at which cell types proliferate;
- cell-cell interactions; and

- heritable interconversions of cell types (MEINS, 1983).

A few of the main groups of phenotypic variants which have been identified in plant cell cultures are: hormone habituation, spontaneous resistance to metabolic toxins and variation in secondary metabolite yield (STAFFORD, 1991). Studies on the variation in the requirement of tobacco (*Nicotiana tabacum*) cells for cytokinins, provided direct evidence that states of determination, arising in ontogeny, can be inherited by individual cells (MEINS AND HANSEN, 1986). Heritable variation is often inferred from the persistence of a new trait upon subculturing (MEINS, 1983). These traits, although stable for many cell generations, may not be permanent. In principle, cellular variation could result from genetic mutation, epigenetic changes or a combination of both processes (MEINS, 1983). Genetic mutation involves random variations in genetic constitution such as point mutations, deletions, duplications and rearrangements of genetic material (MEINS 1983). Epigenetic changes have frequently been used to describe any culture-derived variants which exhibit some stability, but cannot be shown to be genetic (STAFFORD, 1991). Epigenesis has been defined as heritable changes in gene expression which do not arise as a result of permanent alterations in the genome of the cell (MEINS AND HANSEN, 1986). Epigenetic changes are not transmitted meiotically and are stable but potentially reversible. Alterations are directed, i.e. they occur regularly in response to specific inducers (MEINS, 1983). Changes in phenotype that persist only as long as the cells or the tissue are maintained in a new environment are referred to as physiological responses (MEINS, 1983).

Habituation is defined as the stable heritable loss in the requirement of cultured plant cells for one or more growth factors (MEINS, 1989). The heritable conversion of tobacco cells to the cytokinin habituated phenotype is a gradual, progressive process, which unlike mutation, is strongly influenced by the physiological and developmental state of the cells (MEINS, 1983 AND REFERENCES THEREIN). Once established the habituated state is extremely stable. Results obtained indicated that cytokinin habituation is the result of

epigenetic changes in the expression of a gene that is normally silent in cultured pith cells of tobacco (MEINS AND BINNS, 1977; BINNS, 1994).

Most physiological studies of habituation have focused on the relationship between autotrophic phenotypes and their capacity to produce the hormones for which they are habituated (MEINS, 1989). In this regard, conflicting results have been obtained, namely that:

- auxin and cytokinin are not present in either cell type (HANSEN AND MEINS AND AEBI, 1987; MEINS, 1989 AND REFERENCES THEREIN);
- the hormones are present in both cell types in similar concentrations (KEVERS, COUMANS, DE GREEF, HOFINGER AND GASPAR, 1981; KÖVES AND SZABÒ, 1987); or
- hormone concentrations are elevated in habituated cells compared to non-habituated cells (DYSON AND HALL, 1972; KERAUBY, MONTEIRO, KRAUS, AND HELL, 1988; MEINS, 1989 AND REFERENCES THEREIN).

According to some studies, cytokinin habituated callus of tobacco has the ability to synthesize cytokinins (DYSON AND HALL, 1972; BINNS AND MEINS, 1973; MEINS AND LUTZ, 1979). Similarly the indole-acetic acid (IAA) synthesizing capacity of auxin autotrophic tissues, via tryptophan aminotransferase (E.C. 2.6.1.27), is higher than that of heterotrophic tissues (KÖVES AND SZABÒ, 1981; SZABÒ, KÖVES AND SOMOGYI, 1994 AND REFERENCES THEREIN).

Although ethylene is not an immediate growth promoting substance, HUXTER, THORPE AND REID (1981) concluded that some of the endogenous ethylene of tobacco callus was active as a growth regulator. Low ethylene production is, however, characteristic of most habituated plant cell tissues (KÖVES AND SZABÒ, 1987; HAGÈGE, KEVERS, BOUCOUD, DUYME AND GASPAR, 1990; CAMPELL AND TOWN, 1991; HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994). Several studies have confirmed high amounts of polyamines in habituated non-organogenic callus tissue of sugarbeet (*Beta vulgaris*) (KEVERS, COUMANS, DE GREEF, HOFINGER AND GASPAR, 1981; HAGÈGE, KEVERS, BOUCOUD, DUYME AND GASPAR, 1990; HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994). The aliphatic polyamines putrescine, spermidine and spermine are

ubiquitous cellular components (PARK AND LEE, 1994) that are synthesized in part from S-adenosyl methionine (SAM), which in plant cells, can also be converted to ethylene. Polyamines are also known to reduce ethylene production by inhibition of both 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (E.C. 4.4.1.14) and ACC oxidase (HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994). Consequently it has been suggested that the dynamic equilibrium between polyamines and ethylene in cells is regulated through the availability of SAM (PARK AND LEE, 1994 AND REFERENCES THEREIN). In tobacco suspension cells, rapid growth, in terms of cell number and total protein, coincides with maximum rates of putrescine and spermidine synthesis and relatively low rates of ethylene production (PARK AND LEE, 1994). Furthermore, it was shown that treatment of rice plants with ethylene stimulated rapid growth and also enhanced activities of SAM decarboxylase (E.C. 4.1.1.50) and arginine decarboxylase (E.C. 4.1.1.19), which are both involved in polyamine biosynthesis (COHEN AND KENDE, 1986).

The problems of understanding causative associations and of explaining the complexity of epigenetic regulation in plant tissue culture have no simple solutions. Despite of several investigations, the molecular mechanism for epigenetic variation in cytokinin requirements is still unknown. During this study a cytokinin habituated soybean callus line has been isolated and this provided us with an experimental system for the investigation of stable changes under controlled conditions. In approaching this problem we firstly aimed to characterize differences between habituated and non-habituated callus with respect to the levels of endogenous plant growth regulators.

2.2 MATERIALS AND METHODS

2.2.1 Plant material and culture

Glycine max (L.) Merr. cv. Acme seeds were sterilized in 0.1% (w/v) $HgCl_2$ for 15 min and rinsed thoroughly with sterile distilled water. Sterilized seeds were subsequently germinated in 100 ml culture bottles on 0.9% (w/v) water agar.

Cotyledonary callus was initiated and grown on Miller's basal medium (MILLER, 1965) supplemented with 0.5 mg L^{-1} (2.3 µM) kinetin and 2 mg L^{-1} (11 µM) α-naphthaleneacetic acid (NAA). Calli were maintained in 100 ml culture bottles at 25°C in continuous low light conditions (0.13 µmol m^{-2} s^{-1}). Different callus lines were initiated and maintained by successive subculturing every 28 days. A habituated non-organogenic callus line which does not require added cytokinins was isolated via the soybean callus bioassay (MILLER, 1965). The habituated callus was subsequently also maintained on the same basal medium (MILLER, 1965) as the non-habituated callus. The state of callus habituation was, however, continuously monitored using the soybean callus bioassay (MILLER, 1965).

2.2.2 Cytokinin extraction and purification

Approximately 30 g of fresh callus material of habituated and non-habituated soybean lines, harvested on day 21 of the growth period, was homogenized and the cytokinins extracted in 300 ml of 80% (v/v) ethanol at 4°C for 24 hours. After filtration, samples were purified with 30 g of regenerated cation exchange resin (Dowex 50W-X8; H^+ form) by adjusting the pH to pH 2.5. Samples were passed through the resin at a flow rate of 1 ml min^{-1}. After washing with 100 ml of 80% (v/v) ethanol, the compounds were eluted from the resin using 100 ml of 5 N NH_4OH. The ethanol fraction was discarded, while the ammonium fraction was further separated by descending chromatography as described by FEATONBY-SMITH, VAN STADEN AND HOFMAN (1987). The cytokinin-like activity was determined using the soybean callus bioassay (MILLER, 1965).

Further, 30 g samples of 21 day-old callus were subjected to HPLC analysis. After extraction, the samples were purified with regenerated cation exchange resin as described above. The samples were then filtered through 0.45 µm Millipore filters, air-dried and resuspended in 500 µl of 80% (v/v) HPLC-grade methanol. These samples were filtered through 0.22 µm Millipore filters. Separation of authentic cytokinins were achieved with a linear gradient program (UPFOLD, 1992) using a Varian 5000 HPLC with a semi-preparative Hypersil 5 ODS column (250 x 10 mm) at a flow rate of 3 ml min^{-1}. Ninety fractions were collected at one minute intervals

and the cytokinin-like activity for each fraction was determined using the soybean callus bioassay (MILLER, 1965).

2.2.3 Metabolism of *trans*-zeatin and iP

Approximately 5 g of each of the habituated and non-habituated callus respectively were harvested 21 days after subculturing and incubated in a sterile flask with 1670 Bq of [8-^{14}C]-*trans*-zeatin and [U-^{3}H]-N^{6}(Δ^{2}-isopentenyl)adenine (iP) respectively. The callus material was incubated at room temperature for a period of 8 and 24 hours respectively. Callus material was homogenized in 50 ml of 80% (v/v) ethanol and kept at 4°C for 24 hours. After filtering the samples, the cytokinins were extracted and separated by HPLC as before, using an analytical Supercosil C18 reverse phase column (250 x 4.6 mm) at a flow rate of 1 ml min^{-1}. Ninety fractions were collected at one minute intervals and the radio-activity counted using a Beckman liquid scintillation counter model LS 3800.

2.2.4 Polyamine extraction and determination

Habituated and non-habituated soybean callus were harvested on days 0, 7, 14, 21 and 28 of the growth period. Polyamine levels were determined using HPLC after benzoylation (UPFOLD AND VAN STADEN, 1991). Separation of the polyamines was achieved using a gradient elution program (UPFOLD AND VAN STADEN, 1992) on a Beckman System Gold HPLC with an Ultrasphere 5 µm ODS column (250 x 10 mm) at a flow rate of 1 ml min^{-1}.

2.2.5 Ethylene production

Approximately 200 mg of each of the habituated and non-habituated callus was harvested on days 0, 7, 14, 21 and 28 of the growth period. Callus was placed on a filter disc (15 mm diameter) imbibed with 500 µl 25 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 6.1) in 15 ml glass vials and sealed with a serum cap. The vials with callus material were kept at 25 °C under culture conditions for approximately 24 h. After incubation the ethylene present was

determined in a 1 ml gas sample, withdrawn from the internal atmosphere of each vial. The gas samples were analyzed on a Varian 3400 gas chromatograph equipped with an activated alumina column and photo ionization detector at 100°C. Nitrogen was used as the carrier gas.

2.3 RESULTS

2.3.1 Isolation and growth of a habituated soybean callus line

A cytokinin habituated, non-organogenic soybean callus line, of cotyledonary origin was identified via the use of the soybean callus bioassay (MILLER, 1965). In contrast to the non-habituated callus the habituated callus was able to proliferate without the addition of cytokinins to the media (Table 2.3.1). Habituated callus was very friable with cells that separated easily in comparison to the non-habituated callus. When cultured on the same cytokinin containing medium, both the habituated and non-habituated callus exhibited a typical growth curve (Fig. 2.3.1). No significant difference ($p=0.9802$) was observed in the growth rate of the two callus types. Habituated calli, (per gram fresh weight) also had a small but significantly lower ($p=0.007$) water content than the non-habituated callus and thus exhibited higher dry mass (Fig. 2.3.2). The water content of both callus types did not vary considerably with time.

Table. 2.3.1 The total fresh weight of habituated and non-habituated soybean callus on MILLER'S (1965) media without kinetin during a 5 day incubation period. Concentrations of 1 and 10 µg L^{-1} kinetin respectively were used as controls to initiate growth of non-habituated callus. Mean values ±SE, n=5.

CALLUS GROWTH g^{-1} FW (FW_L - FW_0)	
Habituated callus	0,146 ±0.072
Non-habituated callus	0,029 ±0,020
Kinetin std. 1 µg L^{-1}	0,1022 ±0,063
Kinetin std. 10 µg L^{-1}	0,1928 ±0,088

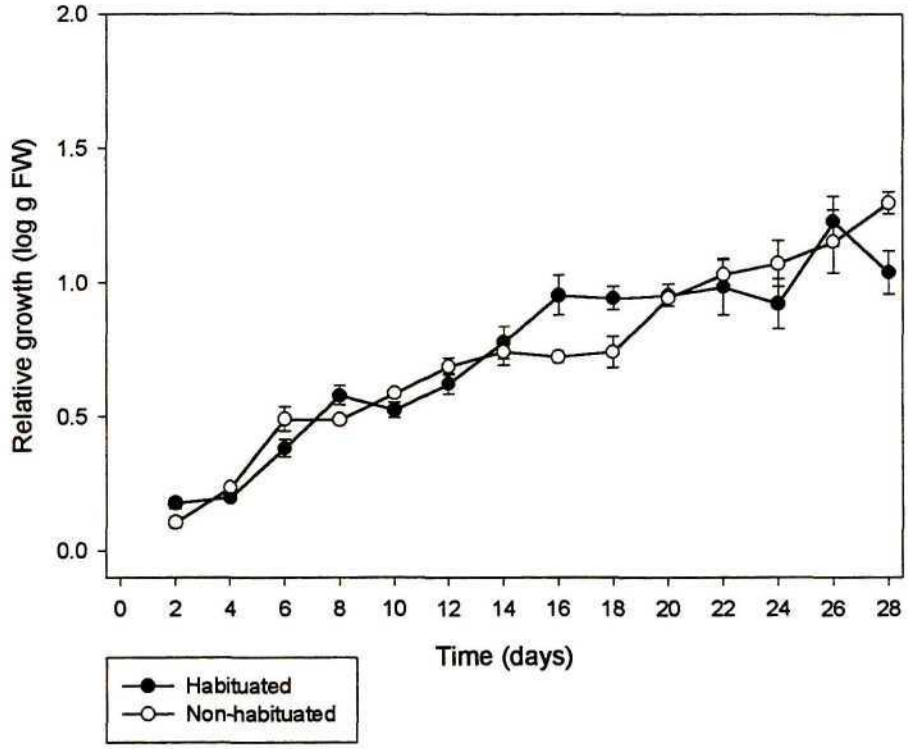


Fig.2.3.1 Relative growth of habituated (●) and non-habituated (○)soybean callus during a 28 day growth period at 25°C under low light conditions. Growth was measured as a change in fresh weight and is expressed as log (FW_L-FW_O). Mean values ±SE, n=4.

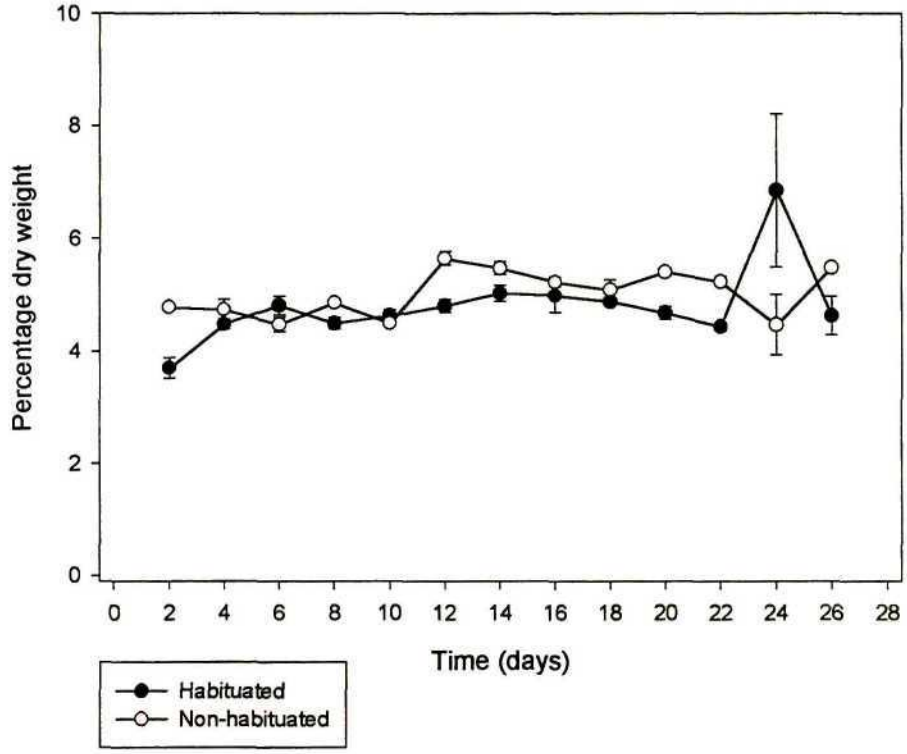


Fig.2.3.2 Change in percentage dry weight of habituated (●) and non-habituated (○) soybean callus during a growth period of 28 days at 25°C under low light conditions. Mean values ±SE, n=4.

2.3.2 Cytokinin content

Bioassay results, following paper chromatography and HPLC analysis, both revealed that cytokinin-like activity in the habituated callus was higher than in the non-habituated callus (Fig. 2.3.3). Following paper chromatography only one peak of cytokinin-like activity was detected at R_F 0.6-0.9. This peak had a similar retention time as *trans*-zeatin and zeatin riboside. Extracts of endogenous cytokinins from habituated and non-habituated callus material were subsequently subjected to HPLC analysis in order to determine the retention times of the various biologically active compounds present. Once again, peaks that co-

chromatographed with *trans*-zeatin and derivatives thereof were associated with the highest biological activity in both the habituated and non-habituated callus (Fig. 2.3.4).

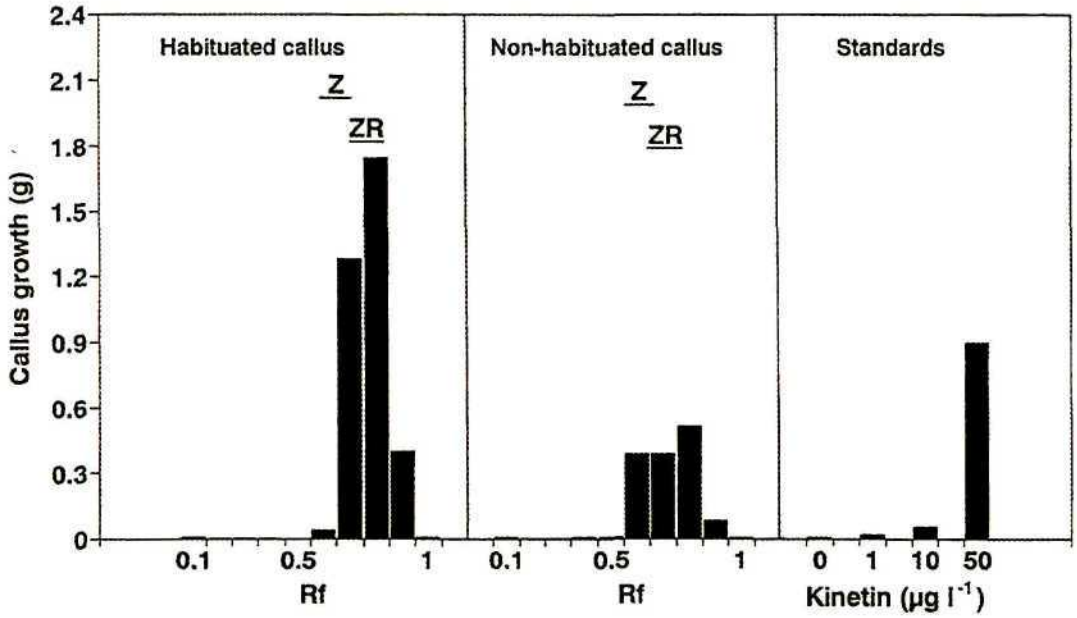


Fig.2.3.3 Cytokinin-like activity detected in extracts of habituated and non-habituated soybean callus tissue following paper chromatography. R_F values of authentic standards of Z (*trans*-zeatin) and ZR (ribosylzeatin) are indicated. The R_F value represents the ratio of the distance migrated by the component of interest divided by the distance migrated by the solvent front. Cytokinin-like activity of each sample was detected using the soybean callus bioassay (MILLER, 1965). Concentrations of 0, 1, 10 and 50 µg L^{-1} kinetin were used as controls.

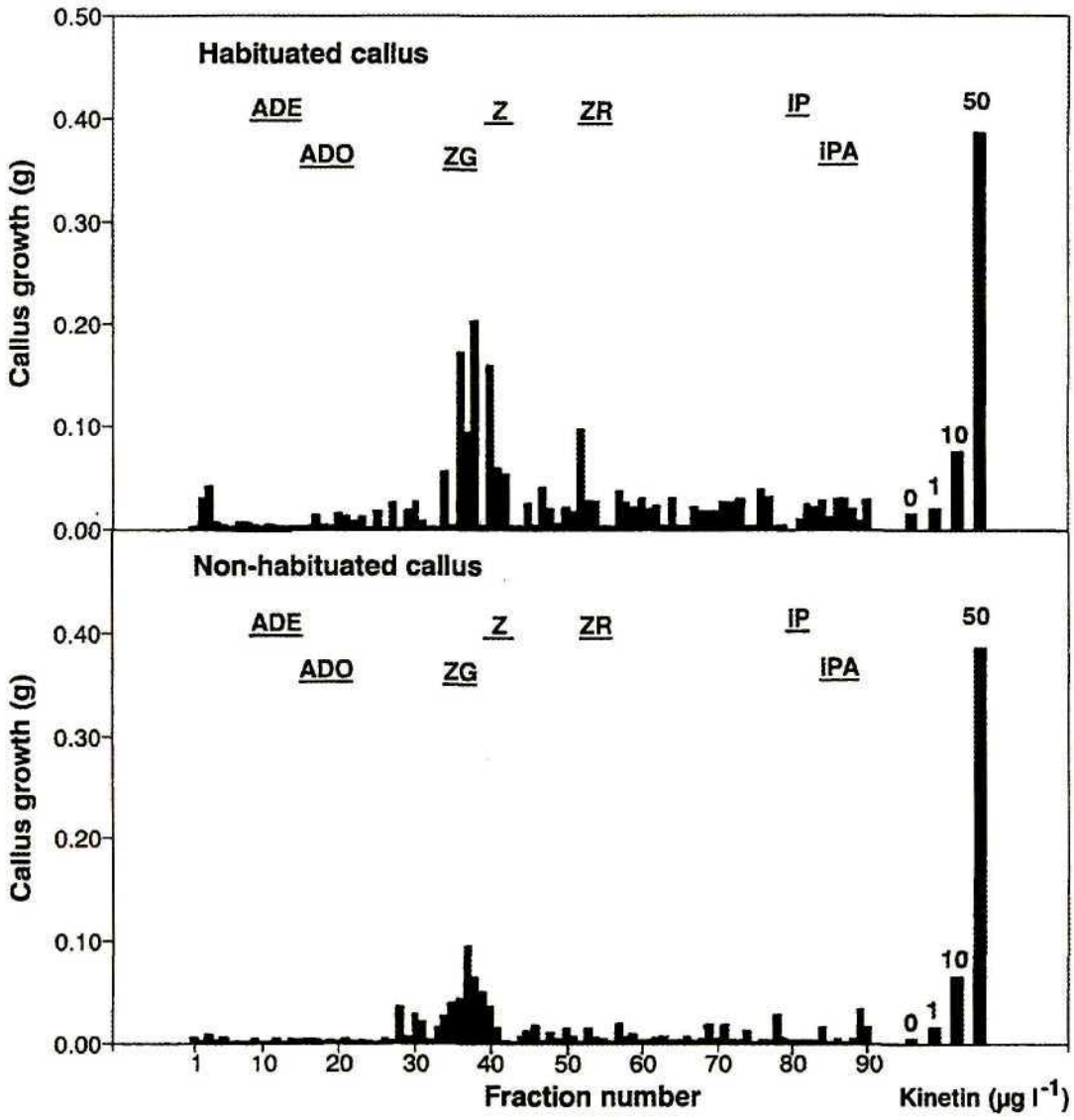


Fig.2.3.4 Cytokinin-like activity detected in extracts of habituated and non-habituated soybean callus tissue after HPLC analysis. Retention times of authentic standards of ADE (adenine), ADO (adenosine), ZG (glucosylzeatin), Z (*trans*-zeatin), ZR (ribosylzeatin) iP [N^6 (Δ^2-isopentenyl) adenine] and iPA [N^6 (Δ^2-isopentenyl) adenosine] are shown. Concentrations of 0, 1, 10 and 50 µg L^{-1} kinetin was used as controls.

2.3.3 Metabolism of radiolabelled *trans*-zeatin and iP

Non-habituated callus metabolized iP to form labelled compounds that co-eluted with the riboside and its free base adenine after a period of 24 hours (Fig. 2.3.5). The habituated callus metabolized very little of the iP, even after a period of 24 hours. There was however, no difference in the metabolism of *trans*-zeatin between habituated and non-habituated callus (Fig. 2.3.6). Very little *trans*-zeatin was metabolized and after a period of 24 hours, most of the recovered radioactivity still co-chromatographed with authentic *trans*-zeatin free base.

2.3.4 Ethylene production

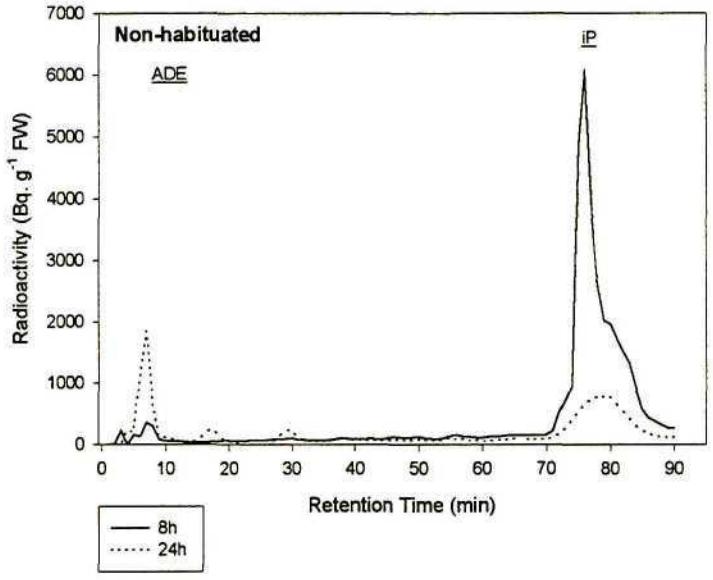
Ethylene production of habituated and non-habituated callus increased during the first 7 days of culture and then decreased drastically in both callus types (Fig. 2.3.7). No ethylene production by habituated callus could be detected from day 14 onwards. The rate of ethylene production was 37 fold lower in the habituated callus on day 7 of the growth period compared to that of the non-habituated callus of 56.03 nmol g^{-1} FW h^{-1}. Ethylene production of the non-habituated callus demonstrated a significant difference ($p=0.0001$) over time and was observed to decrease gradually during intensive growth.

2.3.5 Polyamine content

Only low levels of putrescine could be detected in both habituated and non-habituated soybean callus compared to levels of 5 ng g^{-1} FW spermine and 100 ng g^{-1} FW spermidine in habituated non-organogenic sugarbeet callus (HAGÈGE, KEVERS, BOUCAUD, DUYME AND GASPAR, 1990). In contrast to other habituated non-organogenic callus tissue (HAGÈGE, KEVERS, BOUCAUD, DUYME AND GASPAR, 1990 AND HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994) habituated soybean callus contained significantly ($p=0.0254$) lower levels of putrescine than the non-habituated callus, except on day 28 of the growth period. The habituated soybean callus contained high levels of putrescine on day 14 and 28 of the growth period (Table 2.3.2).

Table. 2.3.2 Putrescine content of habituated and non-habituated soybean callus during a 28 day growth period under low light conditions at 25°C. Fresh callus material was harvested on day 0, 7, 14, 21 and 28 of the growth period, respectively. Mean values ±SE, n=4.

Putrescine content (µmol g^{-1} FW)					
Callus	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Hab	nd	0.070± 0.004	0.474± 0.022	0.132± 0.006	1.827± 0.225
non-Hab	0.030± 0.051	0.224± 0.054	0.215± 0.011	0.512± 0.046	0.974± 0.011



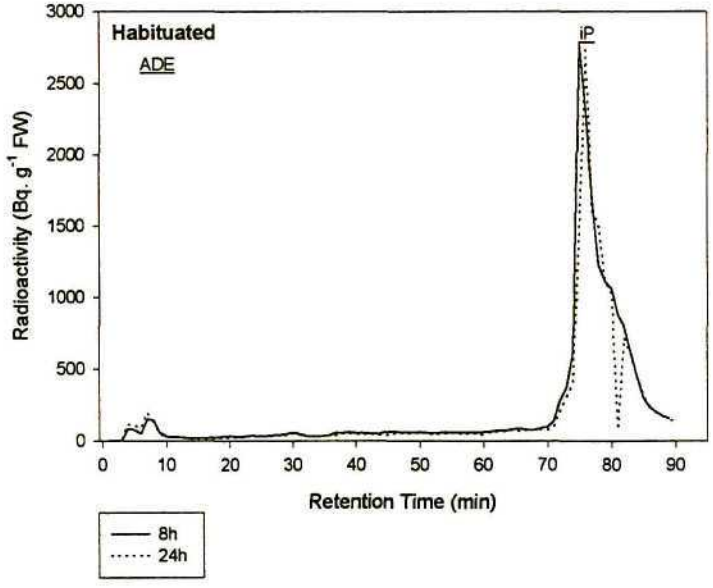


Fig.2.3.5 Radioactivity detected in extracts of habituated and non-habituated callus tissue incubated with [U^3-H]-iP (1670 Bq) for 8 and 24 hours respectively. Extracts were fractionated by HPLC and retention times of authentic standards of ADE (adenine) and iP [N^6 -(Δ^2-isopentenyl) adenine] are indicated.

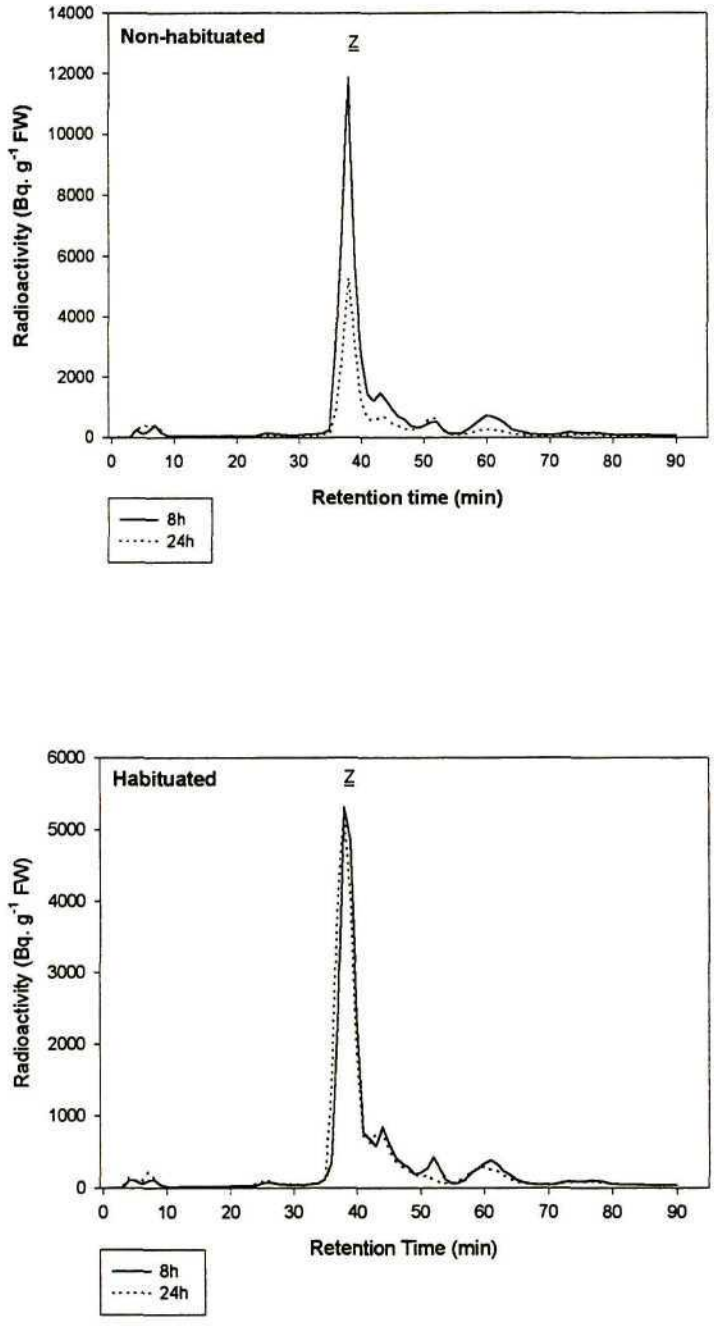


Fig.2.3.6 Radioactivity detected in extracts of habituated and non-habituated callus tissue incubated with [8-^{14}C] *trans*-zeatin (1670 Bq) for 8 and 24 hours respectively. Extracts were fractionated by HPLC and the retention time of an authentic standard of Z (*trans*-zeatin) is indicated.

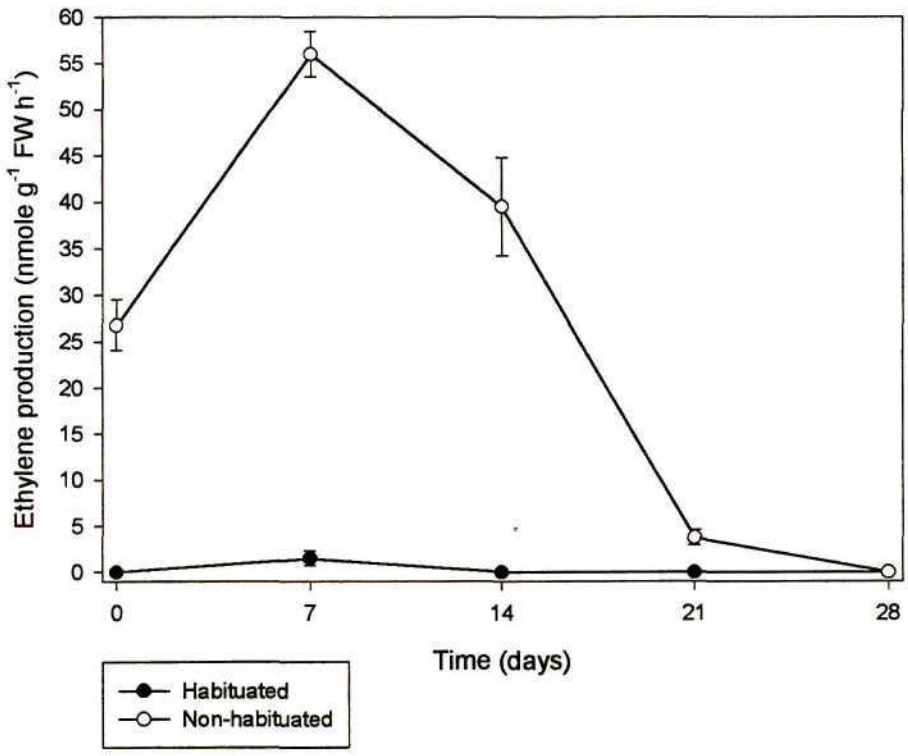


Fig. 2.3.7 Ethylene production of habituated (●) and non-habituated (○) soybean callus during a 28 day growth period at 25 °C under low light conditions. Callus was placed in a 15 ml glass vial and sealed with a serum cap. After incubation ethylene was determined in a 1 ml gas sample, withdrawn from the internal atmosphere of each vial. Mean values ±SE, n=4.

2.4 DISCUSSION

The phenomenon of plant tissue habituation discovered in carrot cells by Gautheret (1942), consists of the acquired and hereditary capacity for autonomous growth in the absence of exogenously supplied growth regulators in culture (CHRISTOU, 1988; MEINS, 1989; GASPAR, 1995). During this study, a cytokinin habituated soybean callus line was isolated through the use of the cytokinin soybean callus bioassay (MILLER, 1965). Stable cytokinin dependent tissue is normally used for the cytokinin bioassay devised by Miller (1963). It exhibit a linear response to kinetin over a concentration range of 20 nM to 50 µM kinetin (REEVE AND CROZIER, 1980). The habituated soybean callus appeared very friable with cells that separated easily. This is also characteristic of habituated *non-organogenic* sugarbeet callus (GASPAR, HAGEGE, KEVERS, PENEL, CREVECOEUR, ENGELMANN, GREPPIN AND FOIDART, 1991). Similar to other habituated callus tissue (GASPAR, HAGEGE, KEVERS, PENEL, CREVECOEUR, ENGELMANN, GREPPIN AND FOIDART, 1991) a significant difference in water content was detected between the habituated and non-habituated soybean callus tissue. Habituated tissue had a lower water content and they would thus be expected to have a higher dry mass.

During this investigation chromatographic and bioassay results indicated that cytokinins were present in both callus types. Cytokinin-like activity was however, higher in the habituated callus than non-habituated callus. Cytokinins that had similar chromatographic properties to *trans*-zeatin and its derivatives exhibited the highest activity in both callus types. Similarly it was found that habituated tobacco cells contained elevated levels of zeatin riboside (HANSEN, MEINS AND AEBI, 1987). HANSEN, MEINS AND AEBI (1987) not only produced evidence that habituated cells of tobacco can produce cytokinins but that their capacity for cytokinin autotrophic growth is correlated with the potential for zeatin riboside accumulation under inductive conditions. This zeatin riboside accumulation was further enhanced by treatment with exogenously applied cytokinins (1.4 µM kinetin) and blocked by the application of exogenous auxin (11µM NAA) (HANSEN, MEINS AND AEBI, 1987). In habituated and non-habituated tobacco

callus as well as in crown gall callus no zeatin riboside was detected in tissue grown on media containing auxin and cytokinin or only auxin (HANSEN, MEINS AND AEBI, 1987). Similarly the incorporation of IAA into the culture medium, reduced the cytokinin content of carnation ovaries (FEATONBY-SMITH, VAN STADEN AND HOFMAN, 1987). In contrast, auxin (2 mg l^{-1} or 11 µM NAA) present in the culture media during this study did not prevent accumulation of *trans*-zeatin and derivatives thereof in either habituated or non-habituated soybean callus tissue. This indicates that the cytokinin habituated phenotype and resulting accumulation of cytokinins in the callus is not affected by exogenous auxin.

Plant growth and differentiation relies on two fundamental cell activities, namely cell division and cell elongation. SKOOG AND MILLER (1957) showed that while auxin induced cell enlargement in tobacco pith cells, a simultaneous addition of the cytokinin kinetin was necessary to induce cell division (HOBBIE, TIMPTE AND ESTELLE, 1994 AND REFERENCES THEREIN). In a cytokinin-requiring strain of soybean tissue, an increase in cytokinin concentration in the media of soybean cultures gave rise to an increase in cell numbers (FOSKET AND SHORT, 1973). Closer investigation of these results revealed that cytokinin is more important as a trigger of mitosis than as a regulator of DNA synthesis and that the triggering effect of cytokinin on cell division is not mediated by a cytokinin-induced alteration of DNA synthesis (FOSKET AND SHORT 1973; EVANS, 1980). Higher levels of cytokinins in habituated soybean callus in this study did, however, not give rise to an increased rate of cell proliferation even with exogenous cytokinins present (2.3 µM kinetin). Expression of the cytokinin responsive gene β-1,3-glucanase (E.C. 3.2.1.6) of habituated tissue with elevated levels of cytokinins showed no down regulation unless exogenous cytokinins were also present (EICHOLTZ, HARPER, FELIX AND MEINS, 1983). Similarly, analysis of bud initiation in cytokinin habituated lines of tobacco, showed that stimulation of adventitious shoots required the same concentration exogenous cytokinins than the non-habituated line (MEINS AND BINNS, 1977). These studies indicate that neither cytokinin over production nor a general increase in cytokinin responsiveness is responsible for the cytokinin habituated phenotype.

The higher level of cytokinins in the habituated tissue may either be the result of an increase in cytokinin synthesis or a decrease in degradation or both. An important way to regulate the availability of active cytokinins in plant tissue is through metabolic interconversions. BINNS (1994) defined cytokinin metabolism as the conversion of [9R-5P]iP to any N^6 substituted adenine derivative cytokinin. The major conversions are the dephosphorylation and deribosylation yielding the riboside and free base respectively, the hydroxylation of the side chain to yield *trans*-zeatin derivatives and the reduction of the side chain to yield dihydrozeatin derivatives (BINNS, 1994). These molecules may be further metabolized often by O-glucosylation of *trans*-zeatin or dihydrozeatin and N-glucosylation at position 7 or 9 of the adenine base (BINNS, 1994). At present the only plant enzymes known to catalyze degradation of cytokinins to inactive compounds that lack N^6 side chain are cytokinin oxidase (E.C. 1.4.3.6) (BRZOBOHATÝ, MOORE AND PALME, 1994). For all the cytokinin oxidases studied iP is the preferred substrate but *trans*-zeatin and zeatin riboside could also act as substrates (BRZOBOHATÝ, MOORE AND PALME, 1994). Dihydrozeatin is however, resistant to cytokinin oxidase action *in vitro* and appears to be the major cytokinin in tissues with high cytokinin oxidase activity (BRZOBOHATÝ, MOORE AND PALME, 1994 AND REFERENCES THEREIN). The application of radiolabelled *trans*-zeatin and iP indicated that habituated tissue metabolized less iP than the non-habituated tissue, while both callus types metabolized very little *trans*-zeatin. The low rate of metabolism of iP in habituated tissue suggests that the higher levels of cytokinins present in the habituated tissue may in part be due to a lower rate of metabolism. Accumulation of *trans*-zeatin derivatives, which are relatively resistant to cytokinin oxidase enzyme action, could also further contribute to the higher levels present in the habituated soybean callus. Correspondingly it was demonstrated that activity of peroxidases (IAA oxidases) was also higher in non-habituated sugarbeet callus than in auxin habituated callus (KEVERS, COUMANS, DE GREEF, HOFINGER AND GASPAR, 1981; SZABÒ, KÖVES AND SOMOGYI, 1994). Habituated and non-habituated tobacco callus however, exhibited similar patterns of metabolism of exogenously supplied cytokinins (HANSEN, MEINS AND AEBI, 1987 AND REFERENCES THEREIN).

The developmental processes in plants that are controlled by cytokinins may also be affected by polyamines since the promotive effects of cytokinins on endogenous polyamines, especially on free putrescine, is pronounced (MADER AND HANKE, 1996). In contrast to habituated non-organogenic sugarbeet callus tissue (HAGÈGE, KEVERS, BOUCAUD, DUYME AND GASPAR, 1990; HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994), habituated soybean callus contained significantly lower levels of polyamines than the non-habituated callus during the growth period. Only putrescine was detected in both lines which suggests that the endogenous cytokinin level had no effect on the levels of polyamines of soybean callus tissue.

The dynamics of endogenous polyamine concentrations have been related to the progress through the cell cycle. Putrescine is the most abundant polyamine in rapidly dividing cells (MADER AND HANKE, 1996). Cells undergoing cell division contain high levels of free polyamines, whereas low levels are found in cells undergoing expansion (WALDEN, CORDEIRO AND TIBURCIO, 1997). Enhanced synthesis and accumulation of polyamines, especially putrescine, in growing tissues occurred prior to the synthesis of chromosomal DNA during the S phase (SERAFINI-FRACASSINI, BAGNI, CIONINI AND BENNICI, 1980). The S phase refers to the period of DNA synthesis prior to and necessary for mitosis. During the progression of mitosis, a decrease in polyamine accumulation and synthesis was found in both germinating seeds of *Helianthus annuus* and Chinese hamster ovary cells (SERAFINI-FRACASSINI, BAGNI, CIONINI AND BENNICI, 1980). A high level of putrescine was also observed in habituated soybean callus during the mid-log phase of the growth period (day 14) which indicates possible DNA synthesis. This corresponds with the striking increase in total RNA content on day 14 of the growth period (Section 5.3.1) and the simultaneous presence of nucleolar vacuoles (Section 3.3.1) in habituated soybean callus cells on day 14 of the growth period. Nucleolar vacuole formation is closely related to RNA synthesis in the nucleus (JOHNSON, 1969). The dynamics of the total RNA content of non-habituated callus (Section 5.3.1) also mimic the change of putrescine levels of non-habituated callus during the growth period. A decrease in putrescine content of habituated callus occurred after day 14 of the growth period. The decrease in

putrescine levels after the exponential growth phase corresponds to a phase of cell maturation as proposed by PHILLIPS, PRESS AND EASON (1987); PHILLIPS, PRESS, BINGHAM AND GRIMMER (1988). A similar decrease in putrescine levels was observed in habituated sugarbeet calli (HAGÈGE, KEVERS, BOUCAUD, DUYME AND GASPAR, 1990). Putrescine content of non-habituated callus increased gradually through the growth period. Excessive levels of polyamines, such as putrescine in the habituated callus at the end of the culture period, might also explain cell necrosis occurring after a certain time, since high amounts of putrescine induce membrane damage (HAGÈGE, KEVERS, BOUCAUD, DUYME AND GASPAR, 1990).

The low ethylene production of habituated soybean callus confirms previous data of low ethylene production by habituated non-organogenic calli of sugarbeet and tobacco (KÖVES AND SZABÒ, 1987; HAGÈGE, KEVERS, BOUCAUD, DUYME AND GASPAR, 1990; CAMPELL AND TOWN, 1991; HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994). The role of ethylene in habituation is however not clear. Ethylene production of habituated tissue was increased by treatment with 2,4-dichlorophenoxyacetic acid (2,4 D) and IAA (KÖVES AND SZABÒ, 1987). Auxins primarily act on ethylene synthesis by inducing ACC synthase in callus tissues (SZABÒ, KÖVES AND SOMOGYI, 1994). However, despite the presence of auxin in the culture media of habituated soybean callus no detectable levels of ethylene production was observed.

Polyamines are known to reduce ethylene production by inhibition of ACC synthase and ACC oxidase, enhancement of conjugation of ACC into malonyl 1-aminocyclopropane-1-carboxylic acid (mACC) and by competing for a common precursor SAM (MADER AND HANKE, 1996; HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994). The higher level of polyamines could thus be involved in the low ethylene production of habituated non-organogenic sugarbeet callus (HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994). However, since habituated soybean callus contains only low levels of putrescine as opposed to habituated non-organogenic sugarbeet callus (HAGÈGE, KEVERS, BOUCAUD, DUYME AND GASPAR, 1990; HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994) such a

hypothesis does not seem valid for this study. Although results from previous studies demonstrated ethylene and polyamines to be mutually inhibitory (PARK AND LEE, 1994 AND REFERENCES THEREIN), results form tobacco suspension cells showed that ethylene increased levels of spermine and spermidine as well as enhanced activities of arginine decarboxylase, ornithine decarboxylase (E.C. 4.1.1.17) and SAM decarboxylase (PARK AND LEE, 1994).

Ethylene on the other hand has the capacity to decrease the level of cytokinins in plant tissues (VAN STADEN, FEATONBY-SMITH, MAYAK, SPIEGELSTEIN AND HALEVY, 1987), possibly by accelerating their degradation (MADER AND HANKE, 1996). Ethylene mediated alteration in membrane permeability of suspension cells leading to a reduced uptake of kinetin from the culture medium might also result in changed contents of cellular cytokinin levels (MADER AND HANKE, 1996). Reduction of ethylene production of sunflower cell suspension cultures by both aminoethoxyvinylglycine (AVG) and BAS 111..W (a triazole growth retardant), which both inhibit ACC synthase, was accompanied by enhanced levels of cytokinins, particularly dihydrozeatin riboside and zeatin riboside (GROSSMANN, SIEFERT, KWIATKOWSKI, SCHRAUDNER, LANGEBARTELS AND SANDERMANN, 1993). Application of ACC or ethephon to the suspension cultures generated up to a three fold decrease in the contents of dihydrozeatin riboside and iP in the cells (GROSSMANN, SIEFERT, KWIATKOWSKI, SCHRAUDNER, LANGEBARTELS AND SANDERMANN, 1993). Considering these results, there may exist a casual relationship between the inhibition of ethylene biosynthesis and enhanced levels of cytokinins, in particular the accumulation of *trans*-zeatin and derivatives thereof, in habituated soybean callus.

Cytokinin habituation is an interesting and controversial topic which is complicated by our rudimentary knowledge of epigenetic changes in gene expression. The data obtained during this investigation indicated that the cytokinin habituated soybean callus line isolated, not only differs from the non-habituated callus line but also differs in several aspects form other habituated tissues. Higher levels of endogenous cytokinins in the habituated callus tissue

could in part be due to a reduced rate of metabolism. This higher level of endogenous cytokinins furthermore appears not to be effected by exogenous auxins present in the media, as was the case with cytokinin habituated tobacco cells (HANSEN, MEINS AND AEBI, 1987). Only putrescine was detected in both the habituated and non-habituated callus lines in contrast to high levels of spermine and spermidine present in habituated sugarbeet callus (HAGÈGE, KEVERS, BOUCAUD, DUYME AND GASPAR, 1990; HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994). Results indicated that the dynamics of endogenous putrescine content during the growth period of habituated soybean callus possibly relate to the progress through the cell cycle. Low ethylene production by habituated soybean callus confirms previous data of low ethylene production by habituated non-organogenic sugarbeet and tobacco calli (KÖVES AND SZABÒ, 1987; HAGÈGE, KEVERS, BOUCAUD, DUYME AND GASPAR, 1990; CAMPELL AND TOWN, 1991; HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994). Although the role of ethylene in habituation is unclear, a casual relationship may exist between inhibition of ethylene biosynthesis and enhanced levels of cytokinins in the habituated callus. Except for this possible relationship, the results discussed indicate that neither cytokinin over production, nor an increase in cytokinin responsiveness, is responsible for the cytokinin habituated phenotype.

CHAPTER 3

QUALITATIVE ULTRASTRUCTURAL COMPARISON OF HABITUATED AND NON-HABITUATED SOYBEAN CALLUS CELLS.

3.1 INTRODUCTION

The cell is the fundamental unit of most living organisms in terms of both structure and function. It represents the basic unit of biological growth and development. A plant cell has internal organization which can be divided into two main components, the cell wall and the cytoplasm with its various organelles. Compartmentation of metabolism and the sequestration of metabolic pathways into membrane bound organelles, occurs in all eukaryotic cells, but is most apparent in the cells of higher plants (DENNIS AND EMES, 1995). Compartmentation provides several advantages to the cell. Compartmentation is a means by which pathways can be regulated independently and thereby provide control of the flux through the pathways in response to the demands of cellular metabolism. The organelles also provide specialized environments more favorable to certain reactions by concentrating the metabolic intermediates of pathways (DENNIS AND EMES, 1995).

The major organelles found in plant cells are the nucleus, mitochondria, Golgi apparatus, microbodies, peroxisomes, glyoxysomes, vacuoles and plastids (DENNIS AND EMES, 1995). Although the majority of plant cells conform to this general picture, there is always a variation in size, shape and structure, which is closely related to the function of the cell type.

The most prominent cell organelle is the nucleus and it has an important role in the control of cell growth, development and reproduction. In quiescent cells the nucleus is usually flattened and positioned between the vacuole and the cell wall in a narrow band of peripheral cytoplasm (YEOMAN AND STREET, 1977). In complete contrast the nuclei of dividing cells are rounded and found distant from the cell wall, towards the center of the cell (YEOMAN AND STREET, 1977). The

nucleus consists of three distinct components: the nuclear envelope surrounding the nucleoplasm and one or more nucleoli (HALL, FLOWERS AND ROBERTS 1984). Nucleoli are most obvious in nuclei of non-dividing cells. The nucleolus's major function in cellular metabolism is the synthesis of rRNA and it thus has an important role in protein synthesis (HALL, FLOWERS AND ROBERTS, 1984). The nucleoplasm contains not only DNA but also a variety of enzymes and other compounds such as lipids. Chromatin can be visualized as dark condensed heterochromatin and lighter regions of euchromatin within the nucleoplasm (HALL, FLOWERS AND ROBERTS,1984).

Ribosomes are small subcellular particles that have been observed in tissue associated with protein synthesis. They appear as free oblate spheroids in the cytoplasm and/or attached to the outer surfaces of the membranes of the endoplasmatic reticulum, when tissue is fixated with osmium tetroxide (HALL, FLOWERS AND ROBERTS, 1984).

Mitochondria are variable and appear to be in continuous movement, they change shape rapidly and are self-replicating (HALL, FLOWERS AND ROBERTS, 1984; NEWCOMB, 1995). The surface area of the inner membrane is increased by invaginations or cristae, which may be plate-like or tubular (HALL, FLOWERS AND ROBERTS, 1984). In general, plant mitochondria are tubular compared to the plate like formation of animal mitochondria. The development of different mitochondrial forms may be related to the changing metabolic and mechanical stresses in the cells (YEOMAN AND STREET, 1977). The number of mitochondria per unit cytoplasm appears to remain similar during different developmental stages of the same cell type even though the total number of mitochondria in the cell increases. The frequency of cristae in the mitochondria however, tend to increase as mitochondrial activity and the respiratory activity of the cell rises (YEOMAN AND STREET, 1977). Major biochemical processes occurring within the mitochondria are the tricarboxylic acid cycle and oxidative phosphorylation.
Plastids are selfreplicating organelles, surrounded by an envelope comprised of two membranes (NEWCOMB, 1995). In non-photosynthetic cells, such as those of roots and cell cultures, there occur colourless plastids which have lost their

progenitor function (NEWCOMB, 1995). They are distinct from proplastids and are sites of synthesis for starch (*amyloplasts*), or lipids (*elaioplasts*), or proteins (*proteinoplasts*) (HALL, FLOWERS AND ROBERTS, 1984; NEWCOMB, 1995). Amyloplasts are characterized by the presence of one or more starch granules which distend and alter the shape of the organelle to appear elongated or oval in section (NEWCOMB, 1995).

Hormones, especially auxins and cytokinins, are essential for the success of plant tissue culture. The level of specific hormones and their ratio in the medium may influence the appearance of the culture (ZERONI AND HALL, 1980 AND REFERENCES THEREIN). Important changes in cellular structure, particularly in nuclei and nucleoli, are induced by plant growth substances (VASIL, 1973). Tomato roots grown in media containing kinetin showed a rich population of mitochondria and ribosomes (VASIL, 1973). The nucleolus appeared compact and without vacuoles. Swelling and vacuolation of nuclei as well as a marked increase in the density of nucleo-histones around nucleoli are seen in tobacco stem pith explants grown in media containing IAA, kinetin and gibberellic acid (VASIL, 1973). The greatest initial nucleolar stimulation is said to be caused by indole-acetic acid (IAA) (VASIL, 1973). Nucleolar vacuoles have been reported in several tissues and physiological situations: in callus cells of the hypocotyl from *in vitro* germinated *Lathyrus odoratus* seedlings and in cells of callus developed from immature maize embryos (CREVECOEUR, HAGEGE, CATESSON, GREPPIN AND GASPAR, 1992). In both cases calli were cultured on media containing 2,4 dichlorophenoxyacetic acid (2,4-D). However, kinetin has also been shown to increase both RNA levels within and RNA released from coconut milk nuclei (ZERONI AND HALL, 1980). The number of nucleoli in *Beta vulgaris* and *Allium sativum* calli has been shown to depend upon the growth media and hormones used for their culture (CREVECOEUR, HAGEGE, CATESSON, GREPPIN AND GASPAR, 1992). Incomplete cytokinesis and irregular nuclear morphology are also conditions described during *in vitro* culture in the presence of high exogenous auxins, namely 2,4-D (CREVECOEUR, HAGEGE, CATESSON, GREPPIN AND GASPAR, 1992 AND REFERENCES THEREIN).

Calli may be considered as teratoma-like neoformations generated at the wounded surface of plant organs in the presence of auxin and cytokinin (GASPAR, HAGEGE, KEVERS, PENEL, CREVECOEUR, ENGELMANN, GREPPIN AND FOIDART, 1991). Calli have also been compared with tumors, similar to those induced after infection of plant organs by bacterial agents like *Agrobacterium tumefaciens* (GASPAR, HAGEGE, KEVERS, PENEL, CREVECOEUR, ENGELMANN, GREPPIN AND FOIDART, 1991). The tumor disease which have been studied in the greatest detail is crown gall disease caused by *Agrobacterium tumefaciens* (BUTCHER, 1977). The process of conversion of a normal intact cell to a non-self limiting neoplasm usually involve the demonstration of continuous autonomous disorganized growth in the absence of the inciting agent (MIKI AND IYER, 1995). Transformation involves changes in properties and behavior which are perpetuated in subsequent cell generations (BUTCHER, 1977). The natural ability of this bacterium to genetically transform the plant tissue which it infects, is the basis of all gene transfer technology (MIKI AND IYER, 1995). In the occurring T-DNA, genes are present that evolved to function in plants and prominent among these are those genes that result in the production of auxins, cytokinins or both (MIKI AND IYER, 1995). This is thought to be responsible for the phytohormone independence of transformed cell growth in culture. The cells of gall tumors exhibit a marked increase in the amount of free ribosomes, extensive endoplasmatic reticulum and numerous Golgi dictyosomes with stacks of smooth cisternae (MANOCHA, 1969). The cells of sunflower (*Helianthus annuus* L.) crown gall tissue however, showed no differences in the shape of either their chloroplasts or mitochondria (MANOCHA, 1969). One to two nucleoli were found in the nucleus (MANOCHA, 1969). The nucleoli appeared uniform, without vacuoles and differentiation into *pars amorpha* (spherical body in the nucleolus of granular form) and *nucleolonema* (MANOCHA, 1969). Similar as in the case of prominent intranucleolar vacuoles, both a *pars amorpha* and a *nucleolonema* are thought to form in both plant and animal cells as a result of physiological activity of the nuclear matrix (HALL, FLOWERS AND ROBERTS, 1984).

Hormonal autonomy, a high rate of cell division and the absence of morphological differentiation are also characteristic of tumor cells (GASPAR, 1995). Cancerous cells have large nuclei, irregular in shape with deep invaginations, several nucleoli and micronuclei also occur (GASPAR, 1995). Cancer cells lose form, control, unity of design and function that favors the survival of the organism (VARMUS AND WEINBERG, 1993). Cancer cells often show a profound shift in the relationship between their nuclei and their cytoplasm, hence in many cancer cells the nucleus is almost as big as the whole cell with only a small cytoplasmic rim around it (VARMUS AND WEINBERG, 1993). Ultrastructural changes in nucleoli of rat liver cancer cells are non-specific and related to a variety of biochemical alterations in addition to changes in RNA synthesis (VARMUS AND WEINBERG, 1993).

Microscopy presents a method to study the structural organization, thanks to techniques of preparation, which allow plant structure and ultrastructure to be observed in a state as close as possible to the *in vivo* state. It is a descriptive tool to represent one aspect of a more comprehensive approach to cell or tissue function. With this study we firstly attempted to characterize the ultrastructure of habituated callus cells in comparison to that of cells of non-habituated callus. We will also observed how the structure of the different cell components corresponded with their function in the cell metabolism.

3.2 MATERIALS AND METHODS

3.2.1. Plant material

Habituated and non-habituated soybean (*Glycine max* L. Merr cv. Acme) callus lines were maintained as described in Section 2.2.1. Calli were subcultured every 28 days onto MILLER'S (1965) basal medium supplemented with 0.5 mg L^{-1} (2.3 µM) kinetin and 2 mg L^{-1} (11 µM) α-naphthaleneacetic acid (NAA). The state of callus habituation was continuously monitored using the soybean callus bioassay (MILLER, 1965).

3.2.2. Electron microscopy

Callus pieces of both callus lines were collected 14 days after subculturing onto fresh media. The calli were fixed for at least 8 hours at 4°C in 3% (v/v) glutaraldehyde prepared in 0.05 M cacodylate buffer, pH 6.86. The fixed samples were washed in two changes of buffer and then post fixed for 1 hour in 2% (w/v) osmium tetroxide prepared in the same cacodylate buffer. After being rinsed twice over a period of 1 hour, the samples were dehydrated and subsequently embedded in Epon. The tissue samples were then sectioned and the sections mounted on uncoated 200 mesh copper grids.

Sections were stained in 2% (w/v) uranyl acetate for 10 min and then lead citrate for an additional 10 min before being observed on a JOEL 100 Cx TEM electron microscope. The observations were repeated three times with different batches of calli taken from cultures at one month intervals (*i.e.* one growth period). Various pieces (10) of both callus lines were fixed for each replicate. Samples of each replicate were randomly chosen for ultrastructural examination. Micrographs were taken at 80 kV on a JOEL 100 Cx TEM.

3.3 RESULTS

3.3.1. Habituated callus cells

The ultrastructural appearance of habituated cells is illustrated in Fig. 3.3.1 A to D. The cells were large, with one or two large vacuoles and peripheral cytoplasm. However, several smaller vacuoles were often noted in the cytoplasm (Fig. 3.3.1 B). Polysomes were in abundance and some of them were bound to the endoplasmatic reticulum. Mitochondria were normal in appearance, but exhibited large cristae compared to mitochondria observed in non-habituated cells (Fig. 3.3.1 C). The plastid population consisted mainly of starch containing amyloplasts. No chloroplasts were observed. The nucleus was regular in shape and positioned close to the cell wall. Nuclear chromatin was granular in appearance. Only one nucleolus was observed within each nucleus. The nucleoli

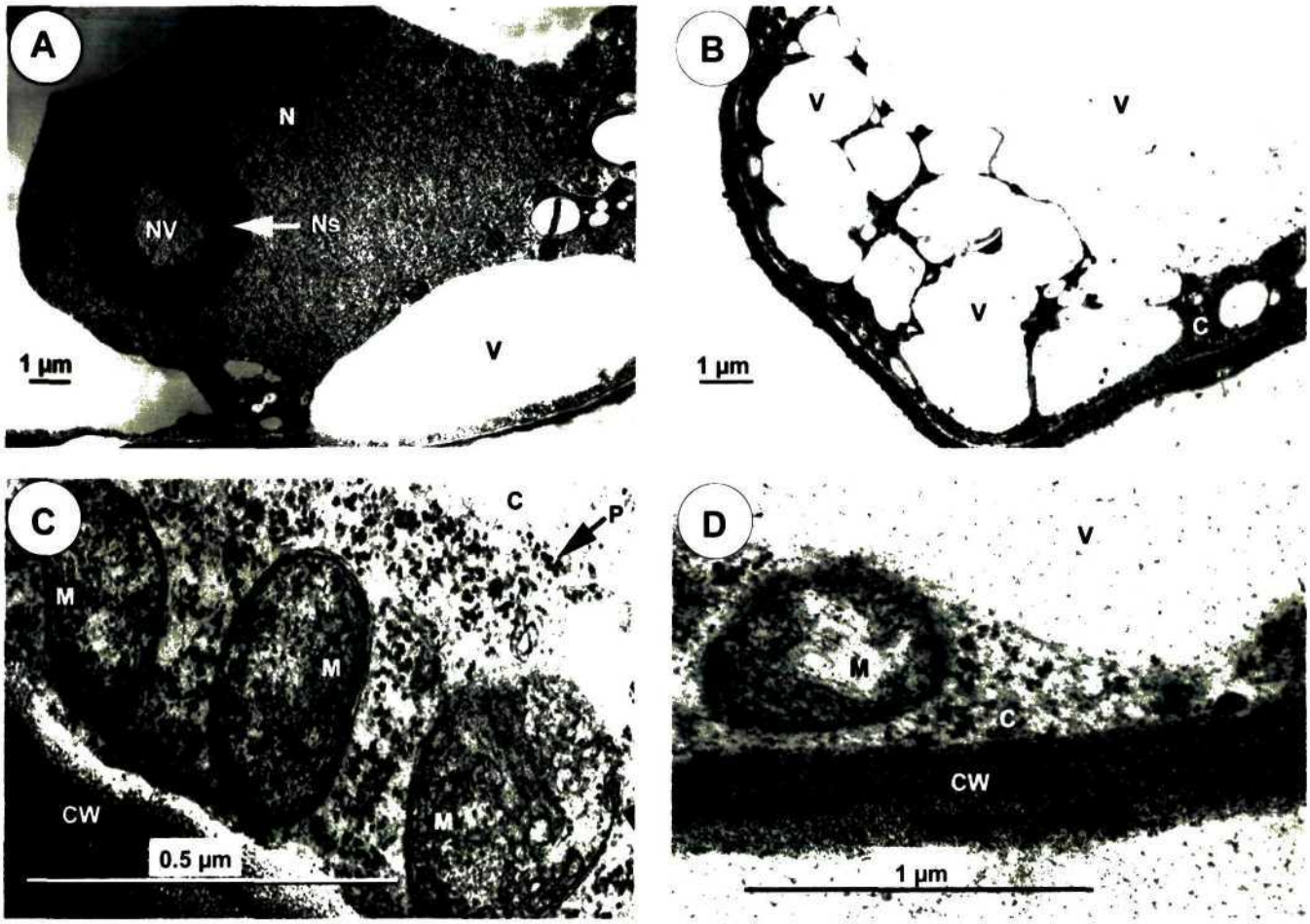
were granular in texture and frequently contained vacuoles. The nucleolar vacuoles observed varied from a large single vacuole to numerous smaller ones (Fig. 3.3.1 A). The cell walls of the habituated cells appeared thinner than those of the non-habituated cells (Fig. 3.3.2 B).

3.3.2. Non-habituated callus cells

As was noted in the habituated callus, the non-habituated callus cells also contained one or two large vacuoles and the cytoplasm is relegated to the periphery of the cell and contained several small vacuoles (Fig. 3.3.1 E). In the cytoplasm, the mitochondria appeared to have normal morphology but exhibited fine cristae (Fig. 3.3.1 F and G). Rough endoplasmatic reticulum occurred extensively through the cytoplasm (Fig. 3.3.1 H). No chloroplasts were observed. Only starch containing amyloplasts were present. The nuclei were regular in shape and granular in appearance. Each nucleus contained only one nucleolus. In contrast to the nucleoli found in the cells of habituated callus, nucleoli of non-habituated callus cells exhibited no or only a small nucleolar vacuole. The cell walls of non-habituated callus cells appeared thicker than those of habituated callus (Fig. 3.3.2 A).

Fig.3.3.1: Cross sections of habituated and non-habituated soybean callus incubated at 25°C under low light conditions. Callus material was harvested 14 days after subculture. After the callus material was prepared cross sections were made. (**A**). Nucleus (**N**) have one nucleolus (**Ns**) with a large nucleolar vacuole (**NV**) x 5000. (**B**). The cells contained one or two large vacuoles (V) with smaller vacuoles present in the cytoplasm x 6600. (**C**). Portion of the cytoplasm showing the normal shaped mitochondria (M) with large cristae and several polysomes (P) in the cytoplasm (C) x 66 000. (**D**). The cell wall (CW) of the habituated callus cells appear to be thinner than those of the non-habituated callus cells x 33 000. (**E**). Cells contain one or two large vacuoles (V) with several smaller vacuoles in the cytoplasm (C). In the cytoplasm the nucleus (**N**) contain only one nucleolus (**Ns**) with no nucleolar vacuoles x 3300. (**F**) and (**G**). The mitochondria (**M**) appear normal in shape with fine cristae. The cell wall (CW) of the non-habituated cells appear to be thicker than those of the habituated cells x 33000. (**H**) Several starch containing plastids (Pl) were observed x 16 000. Extended rough endoplasmatic reticulum (ER) appeared more frequently in the non-habituated callus cells.

Habituated callus



Non-habituated callus

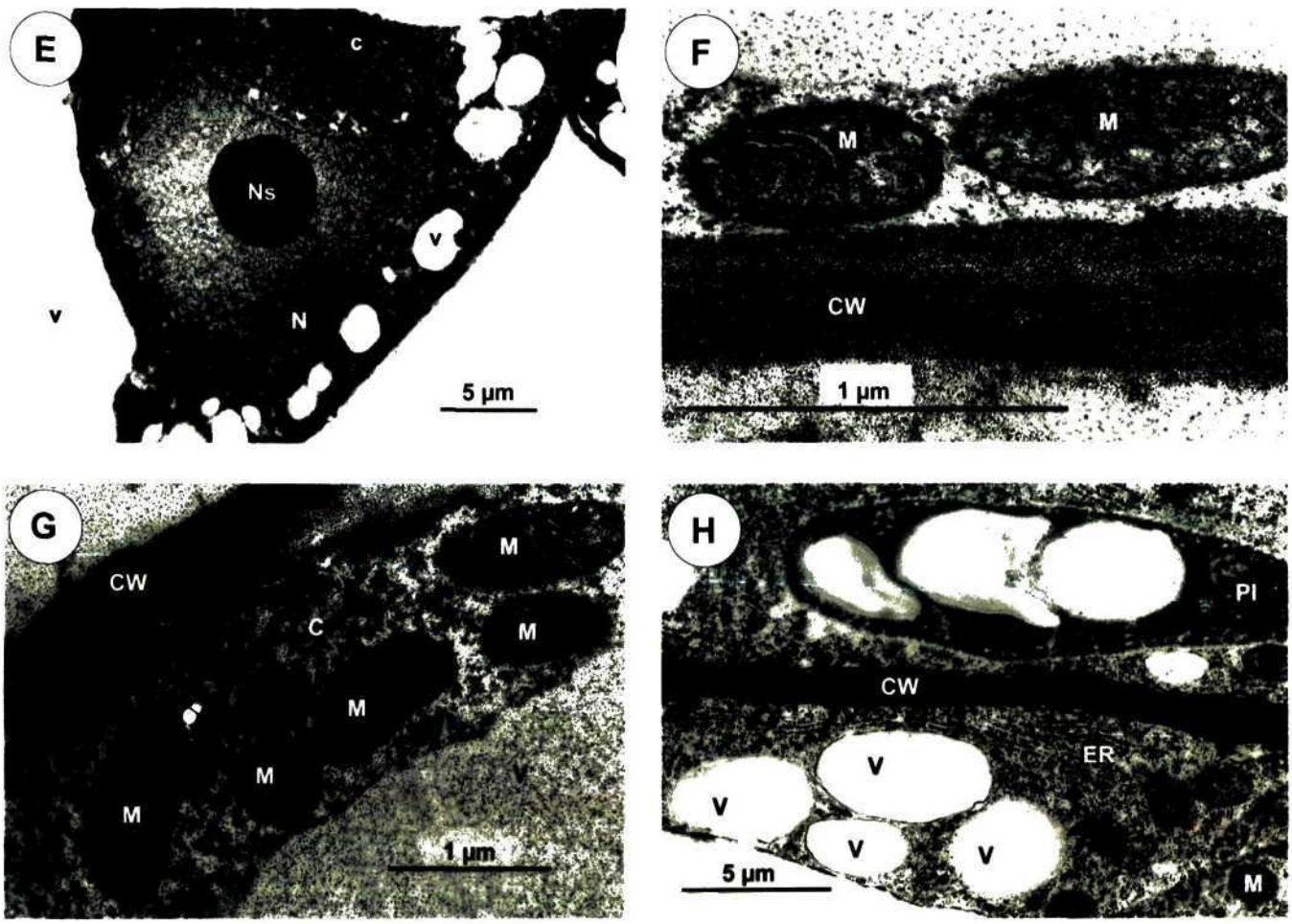
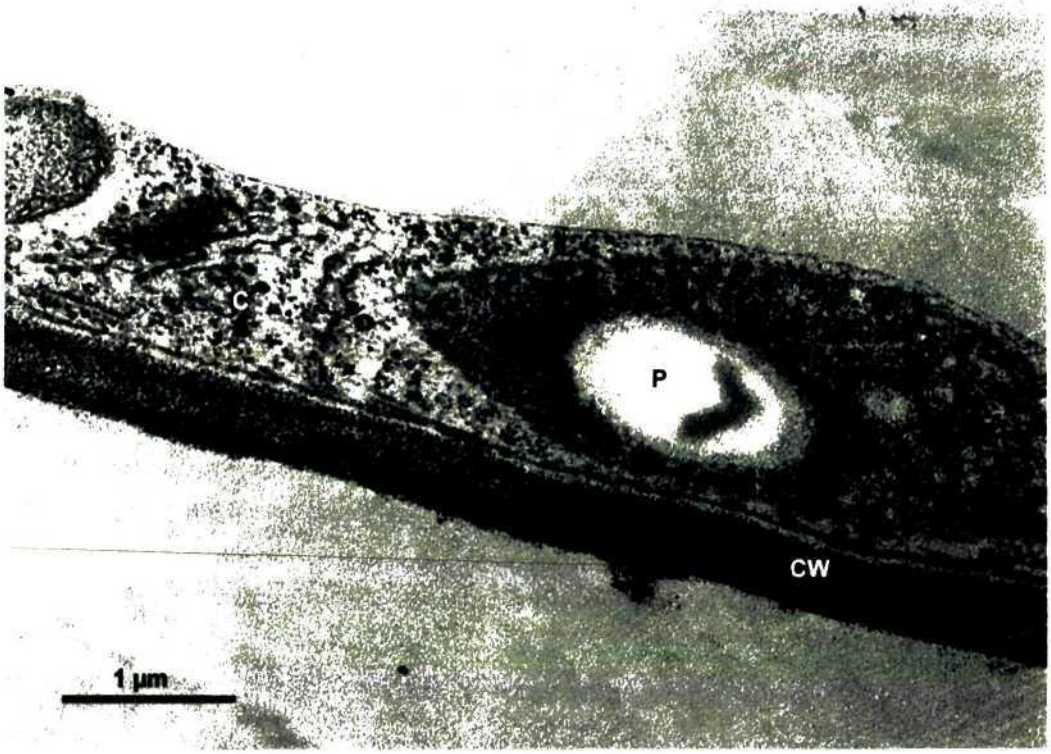
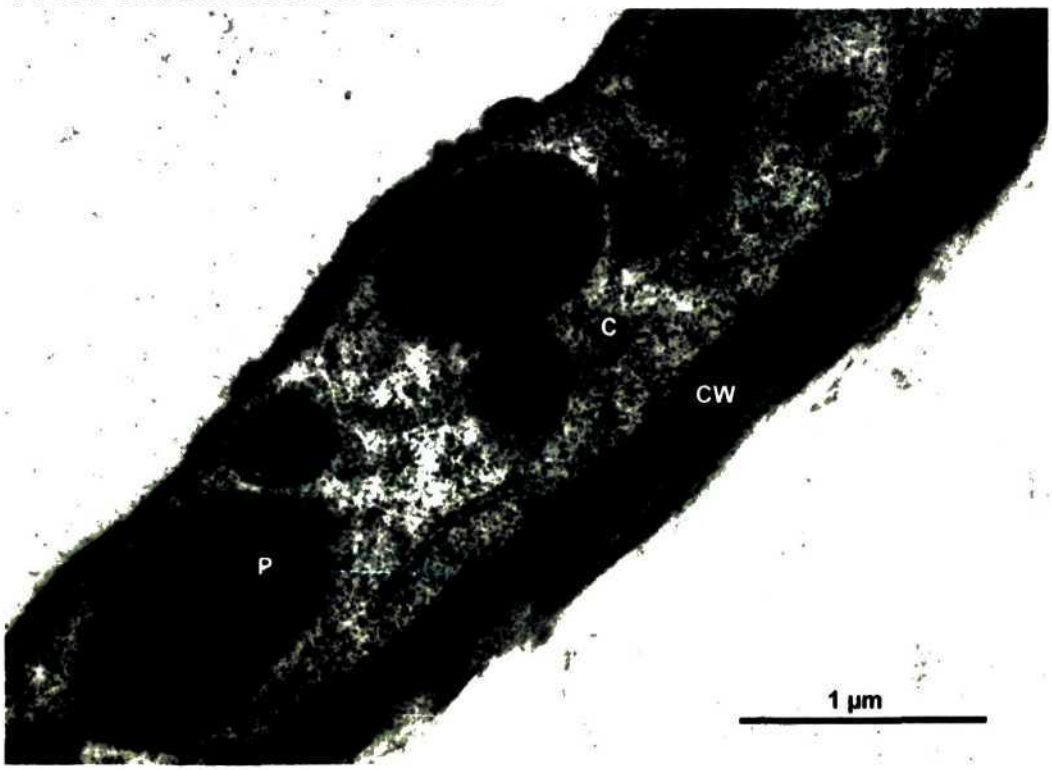


Fig. 3.3.2: Cross sections of habituated and non-habituated callus incubated at 25°C under low light conditions. Callus material was harvested 14 days after subculture, prepared and section for visualization using TEM. The cell walls (CW) of the non-habituated callus cells appear to be thicker than the cell walls of the habituated callus cells. Plastids (P) were present in both cell types.

Habituated callus



Non-habituated callus



3.4 DISCUSSION

Initiation and proliferation of plant tissue in culture require the presence of certain growth regulators, namely auxins and cytokinins. Some plant tissues, may however, lose this requirement and are able to grow in the absence of one or more of the added growth regulators. This phenomenon, which is known as habituation has never been adequately explained (ALLAN, 1991). Most studies on habituated calli were mainly concerned with the biochemical changes due to hormone autonomy. Often we can use ultrastructural characteristics to confirm at subcellular level the conclusions drawn from biochemical work and improve our understanding of cellular structure and function in relation to growth and development. The present study describes differences between the ultrastructure of a cytokinin independent and a cytokinin dependent soybean callus line.

The cell wall is a conspicuous feature of the cell and can be regarded as an organelle (HALL, FLOWERS AND ROBERTS, 1984). It is not autonomous and must be considered in its interaction and relationship with the rest of the cell (HALL, FLOWERS AND ROBERTS, 1984). The cell wall of habituated soybean callus cells, observed during this study, appeared to be thinner than that of the non-habituated cells. A deviation in the fine structure of the cell wall of habituated sugarbeet and soybean callus was also previously reported. These deviations were ascribed to the low levels of cell wall components (cellulose and lignin) present (CREVECOEUR, HAGEGE, CATESSON, GREPPIN AND GASPAR, 1992; CHRISTOU, 1988). When cell walls thicken, the average weight of each cell increases due to the deposition of α-cellulose, lignin and hemicellulose (HALL, FLOWERS AND ROBERTS, 1984). It has been suggested that hormones cause modulation of the biosynthesis and subsequent secretion of the macromolecules involved in cell wall building (ZERONI AND HALL, 1980 AND REFERENCES THEREIN). α-Naphthaleneacetic acid (NAA) (up to a concentration of 3,5 μM) was essential for cell wall regeneration in isolated protoplasts of tobacco, while kinetin at concentrations of 4,6 μM was inhibitory to cell wall biosynthesis (ZERONI AND HALL, 1980).The medium upon which the habituated and non-habituated callus were cultured, during this study, thus

contained sufficient concentrations of both auxin and cytokinin (11 M NAA and 2.3 M kinetin) in order to sustain optimal cell wall biosynthesis. The culminating effect of the higher level of endogenous cytokinins present in the habituated callus could, however, result in suboptimal cell wall biosynthesis. Ethylene is also known to control the enzyme activity involved in cell wall biosynthesis (GASPAR, 1995), such as phenylalanine ammonia-lyase (E.C. 4.3.1.5) involved in the biosynthesis of lignin (HALL, FLOWERS AND ROBERTS, 1984). Low ethylene production is characteristic of both habituated soybean callus (Section 2.3.4) and habituated sugarbeet callus (HAGÈGE, KEVERS, GEUNS AND GASPAR, 1994). One could therefore speculate that low ethylene production might have contributed to the differences observed in the fine structure of the cell wall.

In contrast to variable mitochondrial morphology exhibited by habituated sugarbeet callus (CREVECOEUR, HAGEGE, CATESSON, GREPPIN AND GASPAR, 1992), mitochondrial morphology did not differ profoundly between the habituated and non-habituated soybean callus types on day 14 of the growth period. Mitochondria of habituated soybean callus cells had larger cristae than those of the non-habituated soybean callus cells. This may represent a lower metabolic rate in the habituated callus (ENDRESS AND SJOLUND, 1976). BISBIS, LE DILY, KEVERS, BILLARD, HUAULT AND GASPAR (1993) showed that habituated sugarbeet callus possessed lower glycolytic enzyme activity. This was compensated for by higher activities of the enzymes of the pentose phosphate pathway. As does the glycolytic pathway, the pentose phosphate pathway releases CO_2 and reduces an electron carrier - $NADP^+$. $NADP^+$ is, however, not used for electron transport in the mitochondrion. Therefore, one could envisage that the variation in the mitochondrial morphology of habituated soybean callus, is indicative of metabolically inactive cells, which suggests a deviation in sugar metabolism of the habituated soybean callus compared to non-habituated callus.

Unlike habituated sugarbeet callus cells (CREVECOEUR, HAGEGE, CATESSON, GREPPIN AND GASPAR, 1992), the cells of habituated and non-habituated soybean callus cells exhibited normal nuclei, which were regular in shape and

contained only one or two nucleoli. Another noticeable difference between the cells of the habituated and non-habituated soybean callus lines concerns the nucleolus. Nucleoli of the habituated soybean callus cells contained one large or several small vacuoles, while vacuoles in the nucleoli of the non-habituated callus were less prominent or even absent. Since it is well established that nucleoli are involved in RNA metabolism (JOHNSON AND JONES, 1967), this result suggests that the nucleoli of the habituated soybean callus are more active than those of the non-habituated callus on day 14 of the growth period. JOHNSON (1969) demonstrated that vacuolated nucleoli from tobacco cells incorporated more [^{3}H] uridine as compared to nucleoli without vacuoles. It was therefore suggested that nucleolar vacuolation is closely related to RNA synthesis in the nucleus (JOHNSON, 1969). This correlates with the striking increase in total RNA levels of habituated soybean callus tissue on day 14 of the growth period (Section 5.3.1).

Cells from habituated non-organogenic soybean calli thus show only a few ultrastructural differences compared to the cells of non-habituated soybean calli, despite the higher levels of endogenous cytokinins present (Section 2.3.2). The cell wall of habituated callus cells appeared to be thinner than that of non-habituated cells. This characteristic could be associated with both the over production of endogenous cytokinins as well as the low ethylene production of the habituated phenotype. The larger cristae observed in habituated soybean callus cells are indicative of lower metabolic rates. In contrast to observations concerning structural characteristics of the nuclei of fully habituated sugarbeet callus cells (irregular shaped nuclei, occurrence of micronuclei, numerous nucleoli), the nuclei of habituated soybean callus appeared normal. The nucleoli of habituated callus cells did, however, contain several nucleolar vacuoles. This indicated a stage of active RNA synthesis.

Microscopic observations of fully habituated sugarbeet cells resemble in many traits those from genetic tumors in intra- and interspecies hybrids (GASPAR, 1995). As in the case of habituated non-organogenic sugarbeet callus cells, habituated soybean callus cells also exhibited certain traits similar to true cancerous cells: firstly, they were probably monoclonal in origin, secondly, their

reduced cell-cell adhesion - friability (resembling a loss of cell-cell adhesion in metastases) and thirdly, hormonal autonomy. Morphologic nuclear abnormalities are also intimately connected with cancerous cells, but were not observed in habituated soybean callus cells. The ultrastructural characteristics of habituated and non-habituated callus appeared to be the result of the habituated phenotype rather than only the result of a higher concentration of endogenous cytokinins present.

CHAPTER 4

NITROGEN METABOLISM: A COMPARISON OF FREE AMINO ACID CONTENT AND PROLINE BIOSYNTHESIS.

4.1 INTRODUCTION

Nitrogen is the major limiting nutrient for most plant species. Acquisition and assimilation of nitrogen is second in importance only to photosynthetic carbon assimilation for plant growth and development (VANCE AND GRIFFITH, 1995). Plants acquire nitrogen from two principal sources, namely the soil (through commercial fertilizers and/or mineralization of indigenous organic matter) and from the atmosphere (through symbiotic N_2 fixation).

Plant cells cultured on defined media normally grow on nitrate or ammonium-nitrate as nitrogen sources (GAMBORG, 1970). The concentration of nitrogen and the relative amounts of ammonium and nitrate may be critical for growth and morphogenesis of plant cells (GAMBORG, 1970). Nitrate and other nitrogen sources have a profound effect, which is species dependent, on the growth of plant suspension cultures (GAMBORG, MILLER, OJIMA, 1968). The interactions between the two inorganic nitrogen forms, ammonium and nitrate, on plant growth and uptake characteristics have been widely studied. The results of these studies have often been conflicting, with reports varying from no effect of ammonium on nitrate uptake to a strong inhibition (ASLAM, TRAVIS AND HUFFAKER, 1994 AND REFERENCES THEREIN). Soybean (*Glycine max*) cells as well as Paul's Scarlet rose (*Rosa* spp) cells growing on a defined medium, required either ammonium or glutamine in addition to the nitrate containing medium in order to proliferate (GAMBORG, MILLER AND OJIMA, 1968). The growth characteristics of *Zea mays* embryo cell cultures showed that while glutamine was an effective regulator of nitrate uptake and assimilation, the accumulation of other amino acids in the cells inhibited the uptake of nitrate without an increase in glutamine levels (PADGETT AND LEONARD, 1996).

In plants all inorganic nitrogen is, however, first reduced to NH_4^+ before it is incorporated into an organic form (WALLSGROVE, KEYS, LEA AND MIFLIN, 1983). Soil derived nitrate is reduced to ammonium by the plant enzymes nitrate reductase (E.C. 1.6.6.1) and nitrite reductase (E.C. 1.6.6.4), while the atmospheric N_2 is reduced to ammonium by the microbial enzyme nitrogenase (E.C. 1.18.6.1) (VANCE AND GRIFFITH, 1995). Ammonium taken up from the environment or produced within the tissues is rapidly assimilated into amino acids (MIFLIN AND LEA, 1980). Such a strategy helps to ensure that ammonium does not accumulate to toxic levels in tissues. The degree of ammonium assimilation in tissues varies as a function of organ development, environmental conditions, nutritional status and genotype (VANCE AND GRIFFITH, 1995). Glutamine synthase (E.C. 6.3.1.2) and glutamate synthase (E.C. 1.4.1.14) catalyze the initial steps in ammonium assimilation. Other pathways have also been implicated in ammonium assimilation, namely into alanine, aspartate and asparagine which is mediated by alanine dehydrogenase (E.C. 1.4.1.1), aspartate aminotransferase (E.C. 2.6.1.1) and asparagine synthase (E.C. 6.3.5.4) respectively, with phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) providing a portion of the carbon skeletons of these amino acids. (VANCE AND GRIFFITH, 1995; MIFLIN AND LEA, 1982).

These other pathways of ammonium assimilation represents a second tier of control for ammonium assimilation. It controls the flow of carbon between amino and organic acid biosynthesis and regulates the formation of the key amino acids aspartate and asparagine (VANCE AND GRIFFITH, 1995). Aspartate contributes to an integral part of the malate-aspartate shuttle that allows the transfer of reducing equivalents from mitochondria into the chloroplast and into the cytoplasm (LAM, COSCHIGANO, OLIVEIRA, MELO-OLIVEIRA AND CORUZZI, 1996). Asparagine is thought to be an important compound for transport and storage of nitrogen resources because of its relative stability and high nitrogen to carbon ratio (LAM, COSCHIGANO, OLIVEIRA, MELO-OLIVEIRA AND CORUZZI, 1996). Asparagine acts as the main nitrogen transporting amino acid compound in both legumes and non-legumes (LAM, COSCHIGANO, OLIVEIRA, MELO-OLIVEIRA AND CORUZZI, 1996). However, regardless of the form in which

nitrogen arrives at a tissue, be it nitrate, asparagine, glutamate or another amino acid, it is quickly redistributed within the pools of amino acids in different subcellular compartments within which aspartate, alanine and glutamate are often the major components (IRELAND, 1995).

Not only are amino acids the structural units of proteins, but they also serve as carbon and nitrogen sources for the production of most secondary products. Amino acids are present in plant tissues at higher concentrations than most other metabolites (IRELAND, 1995). The *in vitro* accumulation of amino acids, seems dependent on the culture medium (FIGUEIREDO, BARROSO AND PAIS, 1994). The analysis of 22 different species revealed that cultures grown on Murashige and Skoog medium accumulated higher amounts of amino acids than those grown on Gamborg's B5 media (GAMBORG, MILLER, OJIMA, 1968). Following the assimilation of ammonium into glutamine and glutamate these two compounds act as important nitrogen donors in many cellular reactions, including the biosynthesis of aspartate and asparagine (IRELAND, 1995). Glutamate is therefore the key compound in plant nitrogen metabolism since it is the acceptor of ammonium in glutamine synthesis, the product of glutamate synthase and the immediate precursor of the amino group of most protein amino acids (FIGUEIREDO, BARROSO AND PAIS, 1994). The concentration of amino acids that accumulates also appears to be dependent on the amount of auxin present in the media (PIÑOL, ALTABELLA, CUSIDO AND SERRANO, 1985). In media containing small amounts of auxin (1 µM α-naphthaleneacetic acid, NAA), the concentration of free amino acids, particularly proline and glutamine, was higher (PIÑOL, ALTABELLA, CUSIDO AND SERRANO, 1985).

An auxin and cytokinin habituated sugarbeet callus line has previously been associated with a disturbed nitrogen metabolism especially through accumulation of the stress related nitrogen compounds proline and polyamines, derived from the conversion of glutamate (LE DILY, BILLARD, GASPAR AND HUAULT, 1993). An elevated glutamate dehydrogenase activity together with an excess of glutamate, could be linked to the accumulation of proline and polyamines (LE DILY, BILLARD, GASPAR AND HUAULT, 1993). Proline has been reported to

accumulate in a range of plants in response to a wide range of environmental stresses (as reviewed by HARE AND CRESS, 1997). In plants, proline is synthesized from either glutamate or ornithine (DELAUNEY, HU, KAVI KISHOR AND VERMA, 1993 AND REFERENCES THEREIN). In comparison with most other amino acids, proline has the metabolic advantage of being the terminal product of relatively short and highly regulated pathways. A number of amino acids in plants can be synthesized by more than one biosynthetic pathway depending on the tissue concerned, time of day, or stage of growth (IRELAND, 1995). Cross talk between the regulation of two amino acid pathways has also been reported in plants (LAM, COSCHIGANO, OLIVEIRA, MELO-OLIVEIRA AND CORUZZI, 1996). A blockage of histidine biosynthesis for example leads to a decrease in the mRNA levels of most amino acid biosynthesis enzymes which suggests that a general control mechanism for amino acid biosynthesis occurs in plants (LAM, COSCHIGANO, OLIVEIRA, MELO-OLIVEIRA AND CORUZZI, 1996; IRELAND, 1995).

Metabolic labeling studies indicated that most of the proline accumulated in plants in response to stress, is the result of enhanced synthesis from glutamate (HARE AND CRESS, 1997 AND REFERENCES THEREIN). In the glutamate pathway, indirect evidence suggested that glutamate is converted to proline via the intermediates γ-glutamyl phosphate, glutamic-γ-semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate (P5C) (DELAUNEY, HU, KAVI KISHOR AND VERMA, 1993 AND REFERENCES THEREIN). The committing reaction in the biosynthesis from glutamate is catalyzed by a bifunctional enzyme P5C synthetase (E.C. 2.7.2.11 + E.C. 1.2.1.41) (HARE AND CRESS, 1997 AND REFERENCES THEREIN). The pathways of proline biosynthesis from glutamate and ornithine converge at the point of formation of GSA which is in tautomeric equilibrium with P5C. The latter and its reduction to proline catalyzed by the enzyme P5C reductase (E.C. 1.5.1.2), is common to both pathways (HARE AND CRESS, 1997 AND REFERENCES THEREIN).

The formation of proline from ornithine could possibly proceed via the α or δ-transamination of ornithine to Δ^1-pyrroline-2-carboxylate (P2C) or to P5C via GSA

respectively, catalyzed by ornithine δ-aminotransferase (OAT E.C. 2.6.1.13) (DELAUNEY, HU, KAVI KISHOR AND VERMA, 1993). Transamination of ornithine to form GSA specifically requires 2-oxoglutarate as the amino-group acceptor. Ornithine transamination appears to be a major catabolic pathway of ornithine and links ornithine catabolism to proline metabolism via GSA (HERVIEU, LE DILY, LE SAOS, BILLARD AND HUAULT, 1993) The equilibrium and direction of the reaction catalyzed by OAT are generally in favour of GSA formation (HERVIEU, LE DILY, LE SAOS, BILLARD AND HUAULT, 1993). The formation of GSA is followed by its spontaneous cyclisation to P5C and its reduction to proline (HARE AND CRESS, 1997 AND REFERENCES THEREIN).

Controversy surrounds the relative importance of the two biosynthetic pathways involved in P5C synthesis (HARE AND CRESS, 1997 AND REFERENCES THEREIN). Molecular studies suggest that proline is synthesized from glutamate or ornithine depending on the nitrogen status of the cell (DELAUNEY, HU, KAVI KISHOR AND VERMA, 1993). High nitrogen input induces OAT gene expression possibly via an accumulation of ornithine or arginine (the immediate precursor of ornithine) (DELAUNEY, HU, KAVI KISHOR AND VERMA, 1993). In both osmotically stressed *Helianthus tuberosus* tubers and water-stressed *Arabidopsis thaliana* seedlings, arginine was found to be quantitatively more important than glutamate in the biosynthesis of proline (WRENCH, WHRIGHT, BRADY AND HINDE, 1977; CHIANG AND DANDEKAR, 1995). Application of gabaculine, a powerful and irreversible inhibitor of OAT reduced salt-induced proline accumulation of radish cotyledons and correlated with a dose-dependent increase in OAT activity with increased salinity (HERVIEU, LE DILY, HUAULT AND BILLARD, 1995). The contribution of the ornithine pathway to salt-induced proline synthesis was, however, less at a later stage of seedling development, suggesting that the relative importance of the two pathways may be developmentally regulated (HERVIEU, LE DILY, HUAULT AND BILLARD, 1995).

Gabaculine, at very low concentrations, has been shown to irreversibly inhibit OAT extracted from various plants (HERVIEU, LE DILY, HUAULT AND BILLARD, 1995). It is therefore a convenient tool to evaluate the contribution of the ornithine

pathway to proline synthesis. Aminotransferases each have a tightly bound coenzyme pyridoxal-5-phosphate (IRELAND, 1995). This coenzyme accepts an amino group from the amino acid substrate becoming aminated to form pyridoxamine phosphate (IRELAND, 1995). The keto-acid thus produced is released and the aminated form of the coenzyme then undergoes a reversal of the process, giving up its newly acquired amino group to the keto acid substrate to produce a different amino acid product (IRELAND, 1995). When gabaculine is transaminated by the enzyme, it is converted to a cyclohexatrienyl system with one exo double bond (RANDO, 1977). Upon spontaneous aromatization this high energy intermediate is transformed into a stable compound, m-carboxyphenylpyridoxamine phosphate, which results in the covalent and irreversible modification of the cofactor. This adduct is bound tightly to the active site of the enzyme and can only be liberated under denaturing conditions (RANDO, 1977).

The use of plant tissue culture in the study of plant metabolism has several advantages, namely: a limited number of cellular phenotypes, no complicated transport systems, and a large range of nutritional manipulations can be performed. With the use of gabaculine, accumulation of proline was linked to the synthesis of ornithine, a precursor of polyamines in habituated sugarbeet callus (LE DILY, BILLARD, GASPAR AND HUAULT, 1993). Results indicated that in habituated sugarbeet callus accumulated proline is the major source of ornithine for polyamine synthesis and that aspartate and carbamyl phosphate only contribute weakly to the supply of precursors in the ornithine cycle (LE DILY, BILLARD, GASPAR AND HUAULT, 1993). It has been suggested that the disturbed nitrogen metabolism may explain the abnormal growth of the habituated sugarbeet callus (LE DILY, BILLARD, GASPAR AND HUAULT, 1993). Therefore, in our quest to shed more light upon the habituated phenotype of the isolated soybean callus line; we decided to firstly, evaluate the role of proline accumulation in putrescine formation during the growth period of habituated and non-habituated soybean callus. Secondly, we studied the free amino acid contents of habituated and non-habituated callus during the growth period to

enable us to recognize other possible differences that might occur due to the cytokinin-independent phenotype of the habituated soybean callus tissue.

4.2 MATERIALS AND METHODS

4.2.1 Plant material and culture

Habituated and non-habituated soybean callus lines were maintained as described in Section 2.2.1. Calli were maintained by successive subculturing every 28 days onto MILLER'S (1965) basal medium supplemented with 0.5 mg l^{-1} (2.3 μM) kinetin and 2 mg l^{-1} (11 μM) NAA (MILLER, 1965). The state of callus habituation was continuously monitored using the soybean callus bioassay (MILLER, 1965). In some experiments calli were subcultured onto MILLER'S (1965) basal medium supplemented with 100 μM gabaculine (3-amino-2,3-dihydroxybenzoic acid). Gabaculine was obtained from Sigma. Stock solutions of ornithine and gabaculine were made in deionized water and were filter sterilized before being added to the precooled medium.

4.2.2 Proline determination

Habituated and non-habituated soybean callus were harvested on days 0 (6 hours after subculturing), 7, 14, 21 and 28 of the growth period. Approximately 0.5 g of callus material was ground to a powder in liquid nitrogen. Proline content was then determined from the homogenate firstly, according to BATES, WALDREN AND TEARE, (1973). This method provides a rapid and simple colorometric determination of proline content for numerous samples from multiple replications. This method does not however, completely remove interfering amino acids. Therefore proline concentration was secondly determined according to BERGMAN AND LOXLEY (1970), whose method destroys interfering α-amino compounds such as ornithine with nitrous acid while the imino compounds are allowed to react with ninhydrin under acidic conditions.

Method according to BATES, WALDREN AND TEARE, (1973):

Callus material was homogenized in 5 ml of 3% (w/v) aqueous sulphosalicylic acid and centrifuged for 20 min at 15000 x *g*. Two milliliters of the supernatant was reacted with 2 ml acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) and 2 ml acetic acid in a test tube for 1h at 100°C. The reaction was terminated in an ice bath. The resultant reaction mixture was extracted with 4 ml toluene by mixing it vigorously with a test tube stirrer for 20 seconds. The chromatophore contained in the toluene was aspirated from the aqueous phase and warmed to room temperature. Absorbances were determined at 520 nm with a Beckman DU 65 spectrophotometer using toluene as blank. Proline concentration were determined from a standard curve and calculated on a fresh weight basis.

Method according to BERGMAN AND LOXLEY, (1970):

Callus material was homogenized in cold water and the proteins were precipitated with 5% (w/v) cold trichloroacetic acid (TCA). Interfering α-amino acids were destroyed via a nitrous acid treatment by adding 0.25 M $NaNO_2$ After 20 min at room temperature NH_4Cl was added to a final concentration of 0.2 M. The contents were mixed and a final concentration of 8.57 N HCl were added to the mixture. Subsequently the contents of the test tube was mixed again and heated in a boiling waterbath for 20 min. After the mixture cooled NaOH to a final concentration of 4.2 N was added to it. The contents of each tube was then diluted with distilled water in a 1:1 ratio. One milliliter of the diluted mixture was subsequently used for colour development with ninhydrin.

One milliliter of buffer (phosphate buffer solution prepared with 5.32 M phosphoric acid in 3.88 M NaH_2PO_4) and 2 ml ninhydrin solution (3% (w/v) aqueous solution) were added in a test tube to each of the nitrous acid treated aliquots. The contents of the test tubes were mixed and heated in a boiling waterbath for 100 min. After it had cooled, the contents of the test tubes were transferred to 10 ml glacial acetic acid to dissolve the reaction product. The absorbances of the resulting solutions were determined at 512 nm with a Beckman DU 65 spectrophotometer within 4 hours of the dilution procedure.

4.2.3 OAT extraction and assay

Ornithine aminotransferase (EC 2.6.1.13) was extracted from the callus tissue and assayed using the method of KANDPAL AND RAO (1982). Habituated and non-habituated callus material were harvested on days 0 (6 hours after subculturing), 7, 14, 21 and 28 of the growth period and ground to a powder in liquid nitrogen. Extraction buffer containing 100 mM potassium phosphate buffer (pH 7.9), 1 mM EDTA and 1 mM pyridoxal phosphate was added to the powder in a 5:1 volume to fresh weight ratio. The homogenate was centrifuged for 15 min at 15 000 x *g*. Solid $(NH_4)_2SO_4$ was then added to 60% saturation to the supernatant and the solution was left on ice for 30 min (HERVIEU, LE DILY, LE SAOS, BILLARD AND HUAULT, 1993). The precipitate was collected by centrifugation for 15 min at 15 000 x *g*. The supernatant was dialyzed against 6-8 changes of the same buffer containing 10 mM potassium phosphate over a period of 12-16 hours, and then used in an OAT assay.

Assays were conducted in a final volume of 0.5 ml, according to KANDPAL AND RAO (1982). The assay mixture consisting of 25 mM ornithine; 25 mM 2-oxoglutarate; 1 mM EDTA; 1 mM pyridoxal-5-phosphate; 10 mM 2-mercaptoethanol and 100 mM potassium phosphate (pH 7.4) was incubated with 50 µl of the sample at 37°C for 20 min. Addition of 2-oxoglutarate marked the beginning of the reaction. The reaction was terminated by adding 0.5 ml 10% (w/v) TCA. Colour was developed by incubating each reaction mixture with 1 ml 0.5% (w/v) o-aminobenzaldehyde in 95% (v/v) ethanol for 20 min. The amount of dihydroquinazolium derivative formed was determined by measuring the absorbance at 440 nm with a Beckman DU 65 spectrophotometer, using a molar extinction coefficient of 2.7×10^{3} M^{-1} cm^{-1}. The o-aminobenzaldehyde was obtained from Sigma and all other chemicals were of the highest quality available.

4.2.4 Amino acid analysis

Habituated and non-habituated callus material were harvested as before on days 0 (6 hours after subculturing), 7, 14, 21 and 28 of the growth period. Callus

material (0.5 g) was ground to a powder in liquid nitrogen and the homogenate was suspended in 2 ml of a 3% (w/v) aqueous solution of sulphosalicylic acid. Sulphosalicylic acid was used since it is a colourless solution and effective protein precipitant in aqueous solution which does not interfere with a ninhydrin reaction (BATES WALDREN AND TEARE, 1973). After centrifugation of the homogenate for 15 min at 15 000 x *g* the supernatant of each sample was used for subsequent analysis. Amino acid content of the samples were determined with a Beckman 6300 single column ion exchange chromatograph utilizing HPLC techniques in an instrument dedicated to amino acid analysis. Two micro flow pumps were used in the analytical flow stream, one buffer pump and one reagent (ninhydrin) pump. The standard detection system featured the classical Moore and Stein ninhydrin reagent (MOORE AND STEIN, 1948). This requires heating the combined column effluent/reagent mixture for a period, prior to survey by visible photometry at wavelengths of 440 and 520 nm.

4.2.5 Polyamine extraction and determination

Habituated and non-habituated callus, cultured onto medium with and without 100 µM gabaculine, were harvested on days 0 (6 hours after subculturing), 7, 14, 21 and 28 of the growth period. Polyamine levels were determined using HPLC after benzoylation (UPFOLD AND VAN STADEN, 1991) as described in Section 2.2.4.

4.2.6 Protein determination

The protein content of an extracted sample was measured by the method of BRADFORD, (1976), using Bovine serum albumin (BSA) as a standard. All assays were done using the Bio-Rad Protein Assay dye reagent according to the standardized procedures obtained from the manufacturer.

4.3 RESULTS

4.3.1 Optimization of free proline determination

The free proline content of habituated and non-habituated callus tissue was firstly determined according to BATES, WALDREN AND TEARE (1973). This method is a rapid assay optimized for field studies requiring numerous samples.

Table 4.3.1 Changes in the endogenous proline content of habituated and non-habituated soybean callus tissue during a 28 day growth period at 25 °C under low light conditions. Assays were conducted according to BATES, WALDREN AND TEARE, (1973). Mean values ±SE, n=3

Callus type	**Time (days)**				
	0	7	14	21	28
	Proline content (µmol. g^{-1} Fresh Weight)				
Habituated	0.286 ±0.099	0.139 ±0.02	0.091 ±0.011	0.184 ±0.05	0.136 ±0.027
Non-habituated	0.333 ±0.032	0.266 ±0.026	0.222 ±0.021	0.316 ±0.027	0.192 ±0.042

Proline levels of habituated soybean callus tissue was significantly (p=0.0226) lower than that of the non-habituated callus during the growth period (Table 4.3.1). In contrast to these results, proline levels of habituated sugarbeet callus were 3-5 times higher than that of the non-habituated sugarbeet callus during the culture period (LE DILY, BILLARD, GASPAR AND HUAULT, 1993), when proline was assayed according to BATES, WALDREN AND TEARE (1973). This method does not, however, remove other interfering α-amino acids such as ornithine and glutamine. Since there appear to be no particular increase in proline levels, colour yield of other amino acids could be of a sufficient level to interfere with the colour reaction of the ninhydrin reagent. Proline content of habituated and non-habituated soybean callus was therefore subsequently determined according to BERGMAN AND LOXLEY (1970).

Contrary to results obtained previously, there appeared to be no significant difference (p=0.3594) between the proline content of habituated and non-

habituated soybean callus tissue during the culture period (Table 4.3.2). Proline content was also significantly higher in both the habituated (p=0.0001) and non-habituated (p=0.0001) callus than was estimated previously using the method of BATES, WALDREN AND TEARE (1973). This indicates that a possible interfering substance(s) may be present in the callus tissue that caused a decrease in proline yield.

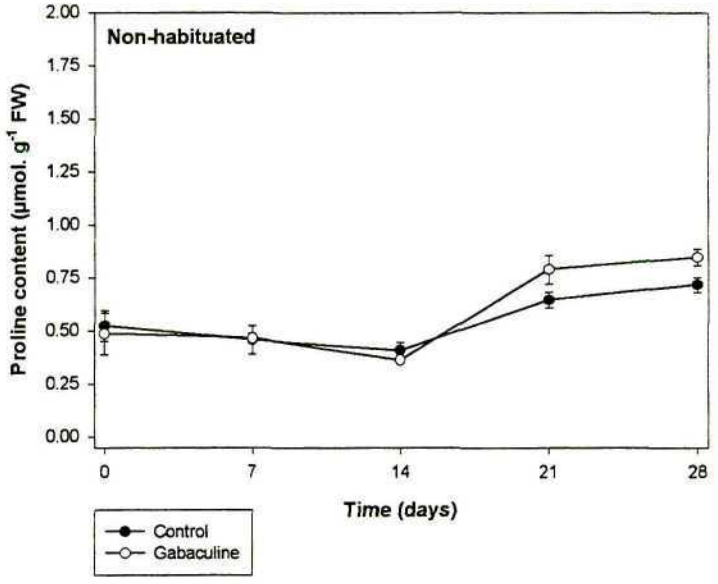
Table 4.3.2 Changes in the endogenous proline content of habituated and non-habituated callus during a 28 day growth period at 25°C under low light conditions. Assays were conducted according to BERGMAN AND LOXLEY, 1970. Mean values ±SE, n=3.

Callus type	**Time (days)**				
	0	7	14	21	28
	Proline content (µmol. g^{-1} FW)				
Habituated	0.381 ±0.036	0.526 ±0.067	0.458 ±0.055	0.386 ±0.052	0.707 ±0.048
Non-habituated	0.523 ±0.072	0.4583 ±0.065	0.620 ±0.098	0.644 ±0.036	0.716 ±0.034

4.3.2 Effect of gabaculine on proline and putrescine levels

Gabaculine, a powerful inhibitor of OAT, was used to evaluate the participation of the ornithine pathway in proline biosynthesis and to establish whether there exists a possible link between proline accumulation and putrescine production. Habituated and non-habituated soybean callus were cultured on MILLER'S (1965) basal medium with and without 100 µM gabaculine according to LE DILY, HUAULT, GASPAR AND BILLARD (1993). Gabaculine had no significant (p=0.9602) effect on the proline content of non-habituated soybean callus tissue during the growth period (Fig 4.3.1). A metabolic turning point in proline accumulation of habituated callus, cultured on gabaculine containing media was, however, evident on day 14 of the growth period (Fig. 4.3.1). During the first half of the growth period a decrease in proline content occurred when compared to the control habituated callus tissue. Proline levels of habituated callus increased,

during the second half of the growth period, to levels higher than that of the control callus.



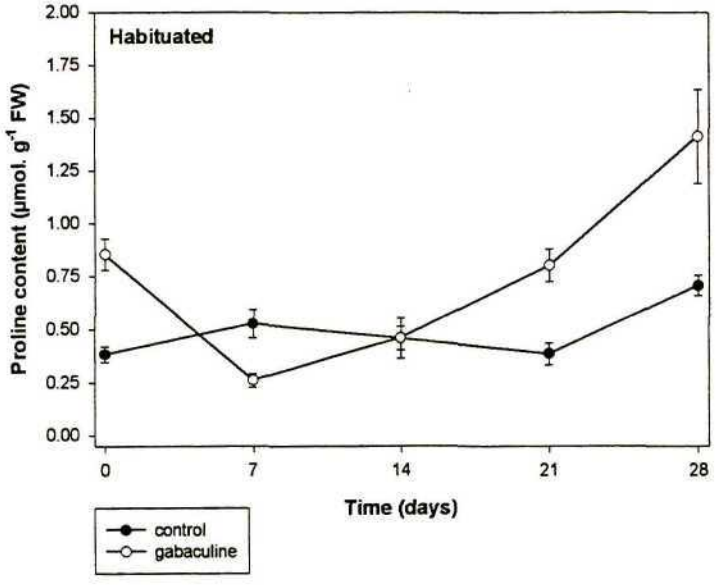


Fig. 4.3.1 The effect of gabaculine on proline content of habituated and non-habituated callus during a 28 day growth period. Calli were cultured on media containing 100 µM gabaculine. and incubated at 25°C under low light conditions. Mean values ±SE, n=4.

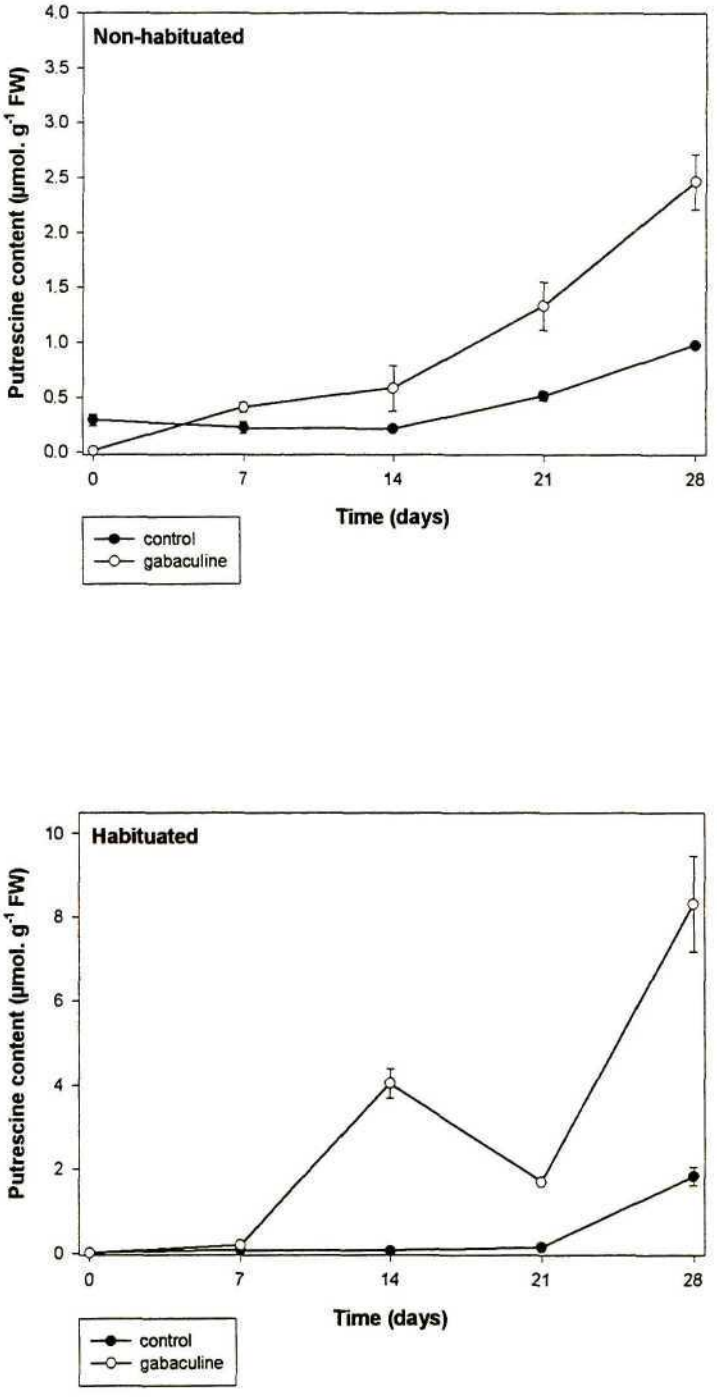


Fig 4.3.2 The effect of gabaculine on putrescine content of habituated and non-habituated callus during a 28 day growth period. Calli were cultured on media containing 100 µM gabaculine. and incubated at 25°C under low light conditions. Mean values ±SE, n=4.

Only putrescine could be detected in both habituated and non-habituated soybean callus. In both habituated and non-habituated callus tissue a similar pattern of putrescine accumulation to that of the control callus tissue, emerged during the growth period when treated with gabaculine (Fig. 4.3.2). Putrescine content of both habituated and non-habituated callus when cultured on gabaculine containing media was significantly ($p=0.016$ and $p=0.0054$, respectively) higher than the putrescine content of the control callus tissue.

4.3.3 Characterization of OAT activity during the growth period

Optimization of extraction and assay method

The numerical value of enzyme activity in a biological sample is influenced by many factors including the presence of activators or inhibitors. There are, however, several fractionation procedures available which enable the concentration of a particular enzyme in a complex, heterogeneous mixture (crude extract) to be relatively easily increased at the expense of contaminating proteins. It is furthermore important to assay at a linear rate of product formation, since product formation can decline due to: substrate depletion, equilibrium with the reverse reaction, product inhibition, instability of assay components, time dependent inhibition, assay method artifact and/or changes in the assay conditions.

Ammonium sulfate is commonly used during a procedure of salt fractionation. From the results of this study (Fig. 4.3.3) it is evident that a salt fractionation step with $(NH_4)_2SO_4$ to 60% saturation (HERVIEU, LE DILY, LE SAOS, BILLARD AND HUAULT, 1993) is necessary to produce an extract of habituated and non-habituated callus material which produce comparable enzyme kinetics. It would seem that the highest rate of product formation occurs during the first 20 minutes of the reaction. The assay does, however, contain ample substrates for the reaction to take place over a period of 120 minutes. A possible reason for the decline in product formation by the enzyme reactions of the habituated and non-

habituated extracts, treated with a salt fractionation step, is that the forward reaction has reached an equilibrium with the reverse reaction.

OAT activity during the growth period *and the effect of gabaculine*

Ornithine aminotransferase activity in non-habituated callus increased gradually during the growth period (Fig. 4.3.4). The highest rate of increase occurred during the last seven days of the growth period when OAT activity increased by 52.5% to 40.291 μmol min^{-1}. mg^{-1} protein. The specific activity of OAT from both habituated and non-habituated callus each mirrored the changes of their total RNA levels, during the growth period (Section 5.3.1). Ornithine aminotransferase activity from the habituated callus exhibited a striking 7 fold increase in specific activity from day7 to day 14 of the growth period, after which it again decreased from day 14 to day 21 of the growth period.

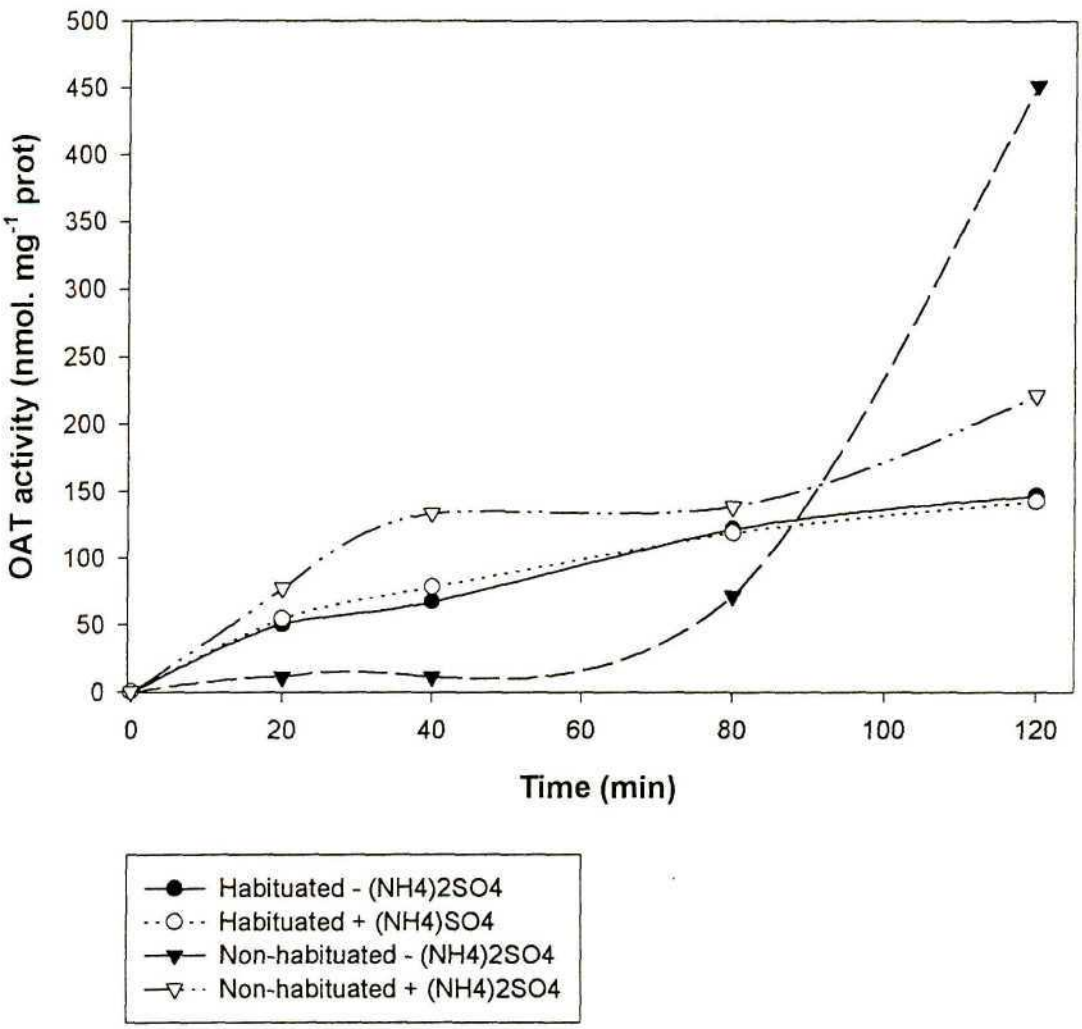


Fig. 4.3.3 The effect of a $(NH_4)_2SO_4$ fractionation purifying step during the isolation of ornithine aminotransferase (OAT) activity from habituated and non-habituated callus, respectively. Callus tissue was harvested during the exponential growth phase (day 14) and product accumulation was determined from the crude extract before and after a salt fractionation step. Mean values of two independent sets of measurements are presented.

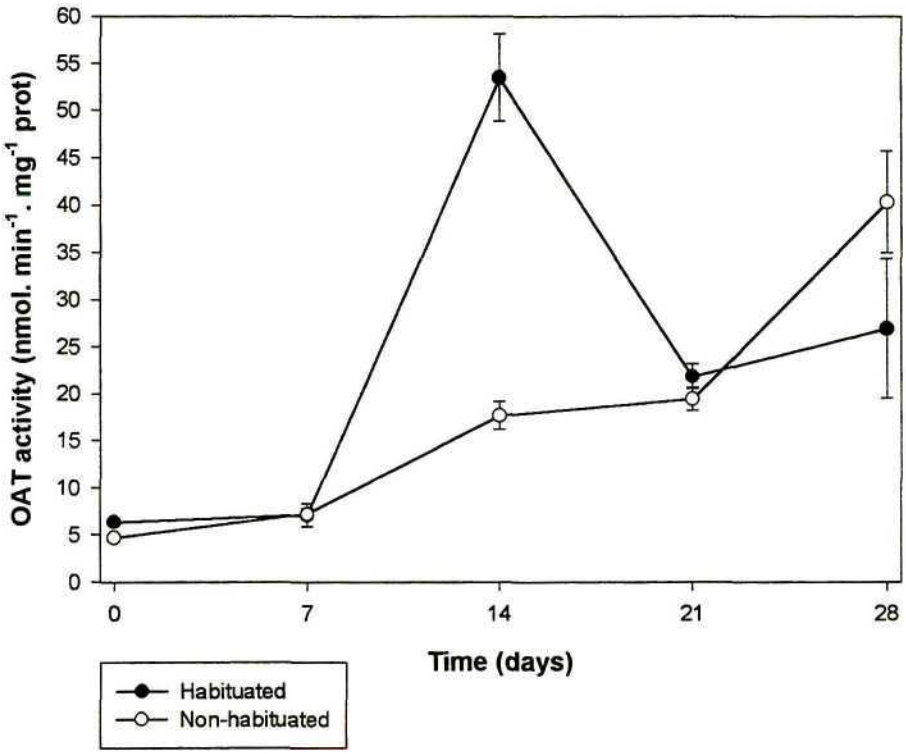
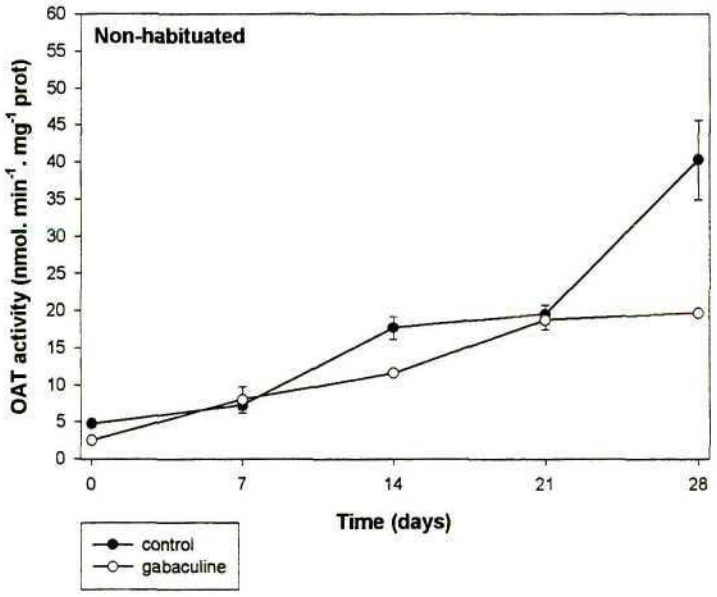


Fig. 4.3.4 The change in ornithine aminotransferase (OAT) activity of habituated and non-habituated callus during the growth period. Calli were grown on MILLER'S (1965) medium under low light conditions, at 25°C. The crude extract was purified via a salt fractionation step with $(NH_4)_2SO_4$ to 60% saturation. Mean values ±SE, n=4.

Gabaculine had a small but significant (p=0.0114) effect on OAT activity of the non-habituated callus (Fig. 4.3.5). However, OAT activity of habituated callus was inhibited by more than 50% during the first half of the growth period. Gabaculine thus had a significant effect (p=0.0046) on OAT activity of habituated callus. From day 21 of the growth period a lesser degree of inhibition occurred.



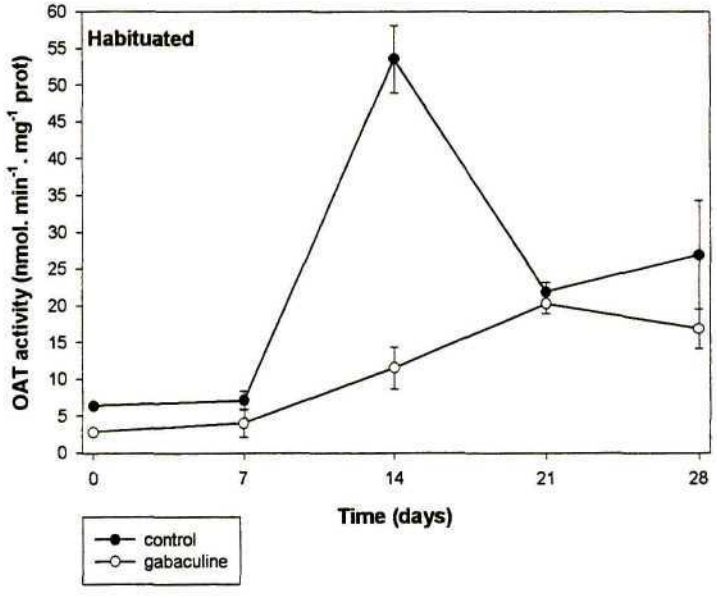


Fig. 4.3.5 The effect of gabaculine on ornithine aminotransferase (OAT) activity of habituated and non-habituated callus during the growth period. Calli were cultured on media containing 100 µM gabaculine. The crude extract was purified via a salt fractionation step with $(NH_4)_2SO_4$ to 60% saturation. Mean values ±SE, n=4.

4.3.4 Endogenous free amino acid levels

Habituated and non-habituated soybean callus growing on media containing both nitrate and ammonium-nitrate showed a striking increase in endogenous ammonium content within 6 hours after subculturing (Fig. 4.3.6). The ammonium content of both callus types increased further until day 7 of the growth period after which it gradually declined to day 21 of the growth period. The NH_4^+ content of the non-habituated callus was significantly ($p=0.002$) lower than that present in the habituated callus, during the growth period.

Seventeen free intracellular amino acids were identified during the growth period of both the habituated and non-habituated callus types. These were: aspartic acid, serine, threonine, glutamate, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine. Serine, alanine and histidine were the three main free amino acids detected in both the callus lines. The amount of free glutamate, the primary product of inorganic nitrogen assimilation, did not vary significantly (Appendix A) during the growth period of either habituated or non-habituated callus (Fig. 4.3.7). Of the other pathways implicated in nitrogen assimilation only alanine represented a major portion of the amino acid content detected in both callus types. The pattern of alanine accumulation during the growth period did however, not show any relationship with the change in ammonium content of both callus types (Fig. 4.3.10).

Both proline and arginine are biosynthetically related to glutamate. There appeared to be no relationship between proline and glutamate content during the growth period. The levels of arginine, as in the case of glutamate, did not vary significantly (Appendix A) during the growth period (Fig. 4.3.7). No significant difference between the habituated and non-habituated callus was detected in the endogenous content of glutamate, arginine and proline ($p=0.3571$, $p=0.132$ and $p=0.3872$, respectively). Proline did accumulate to much higher levels than that of glutamate and arginine, in both the habituated and non-habituated callus.

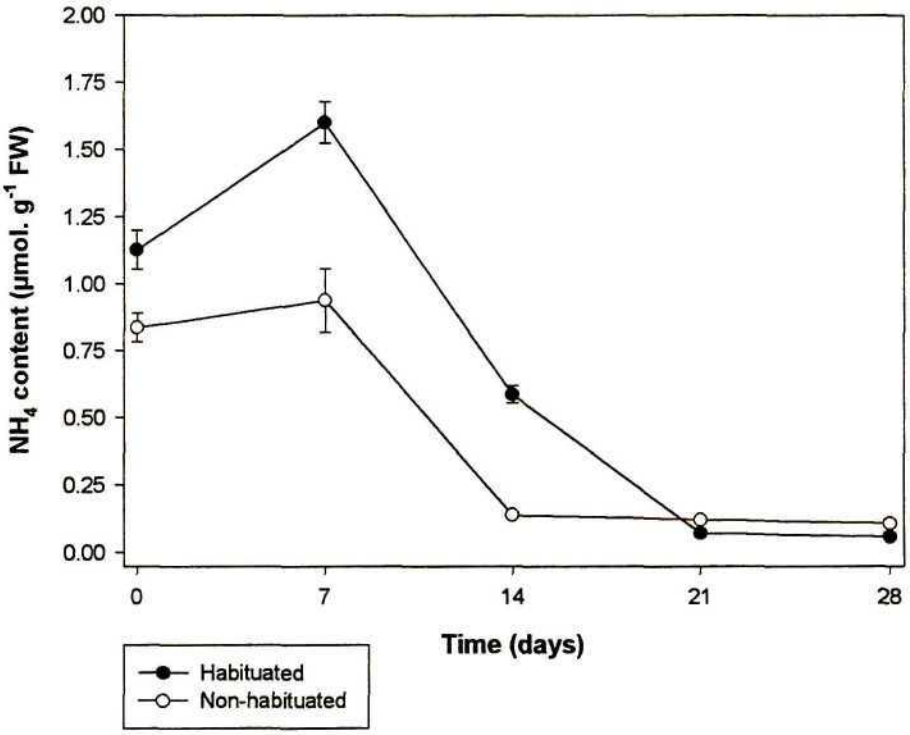


Fig. 4.3.6 The change of NH_4^+ content in habituated and non-habituated callus during a 28 day growth period. Calli were cultured on a complete (MILLER, 1965) nutrient medium containing both ammonium-nitrate and potassium nitrate. Mean values ±SE, n=3.

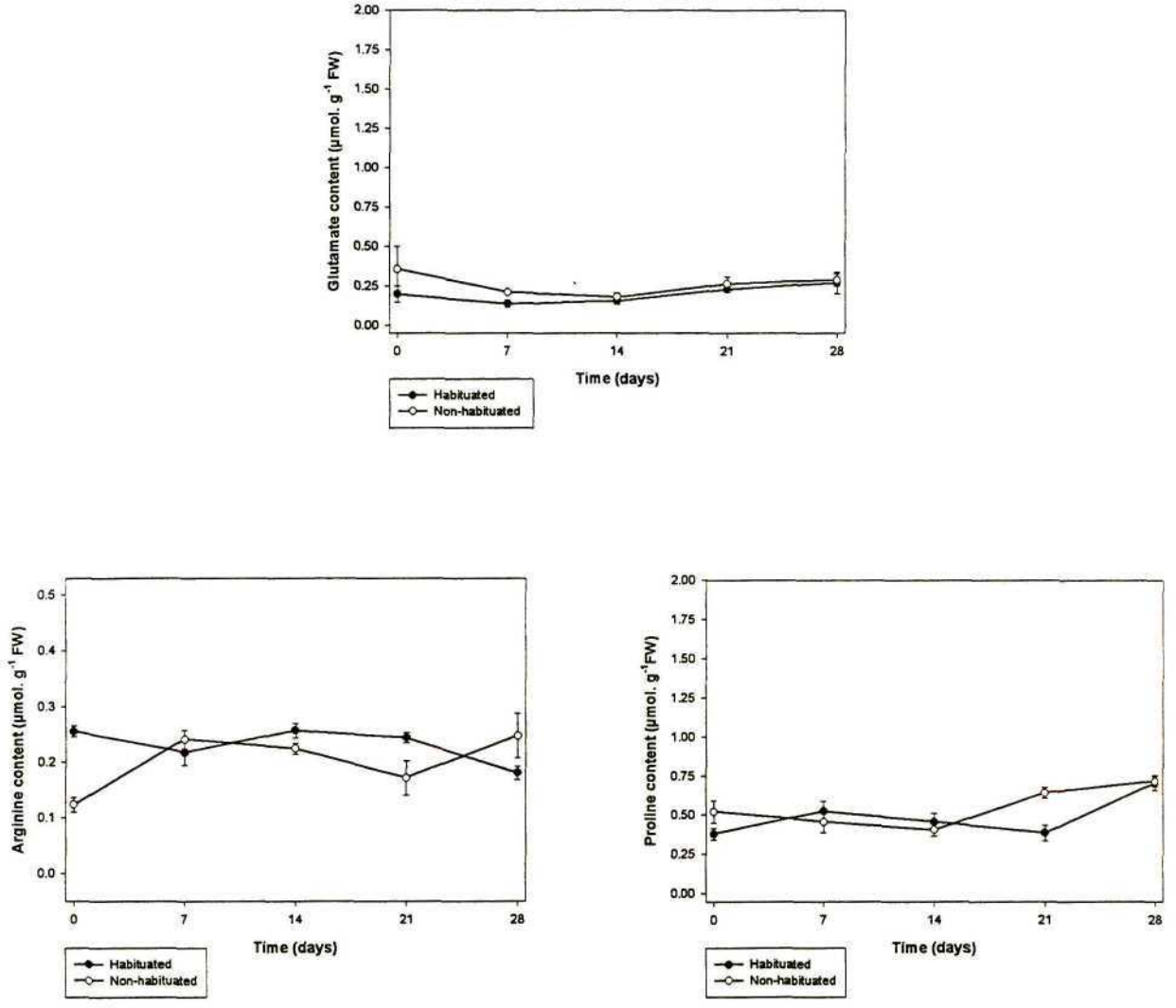
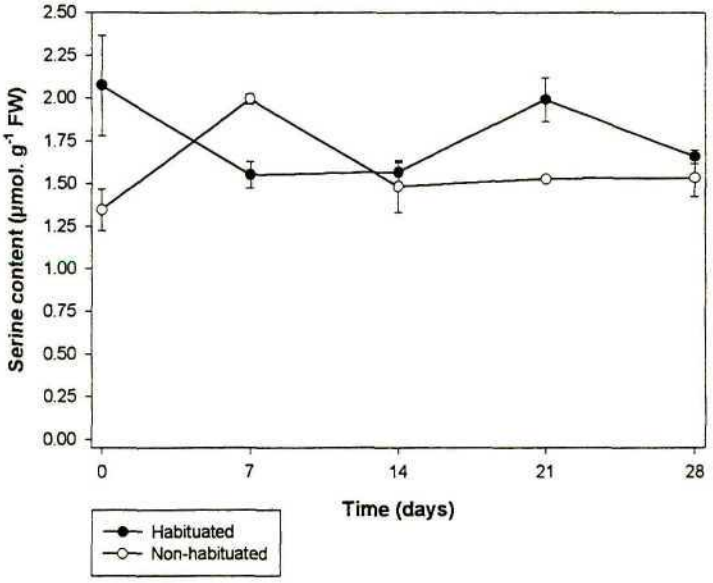


Fig. 4.3.7 The change in cellular content of amino acids from the glutamate family, glutamate arginine and proline, during a 28 day growth period of habituated and non-habituated callus. Amino acids were extracted and analysed by HPLC. Mean values ±SE, n=3.

The synthesis and interconversion of glycine and serine have been thoroughly investigated in the past (IRELAND AND HILTZ, 1995 and REFERENCES THEREIN). The changes in glycine and serine content were identical in the habituated and non-habituated soybean callus, respectively (Fig 4.3.8). Serine and glycine content of the habituated callus reached a maximum during the second half of the growth period, while the serine and glycine content of the non-habituated callus reached a maximum during the first 14 days of the growth period.

Non-habituated callus contained a significantly ($p=0.015$) higher level of aspartate than the habituated callus (Fig. 4.3.9). As with glutamate, aspartate content did not vary during the culture period of both the habituated and non-habituated callus (Appendix A). Lysine, methionine, threonine and isoleucine are all amino acids originating from aspartate. Once again the non-habituated callus contained higher levels of lysine than the habituated callus (Fig. 4.3.9). The pattern of lysine accumulation during the growth period was, however, similar in both the habituated and non-habituated callus. There was a gradual increase in lysine content after day 7 of the growth period. Free methionine was present in very low amounts in both the habituated and non-habituated callus, with no significant difference ($p=0.5247$) in the content of methionine between the two callus types. Threonine, the precursor of isoleucine, and isoleucine displayed a similar pattern to lysine during the growth period of habituated callus (Fig. 4.3.9). In contrast to aspartate, lysine and threonine there was however, no significant difference ($p=0.4366$) in the content of isoleucine between the habituated and non-habituated callus.

Alanine, valine and leucine are amino acids belonging to the pyruvate family. In the non-habituated callus the alanine content did not vary significantly (Appendix A) over time, while the alanine content of the habituated callus was variable during the growth period (Fig. 4.3.10). The highest amount of alanine appeared on day 14, that is during the exponential phase of the growth period.



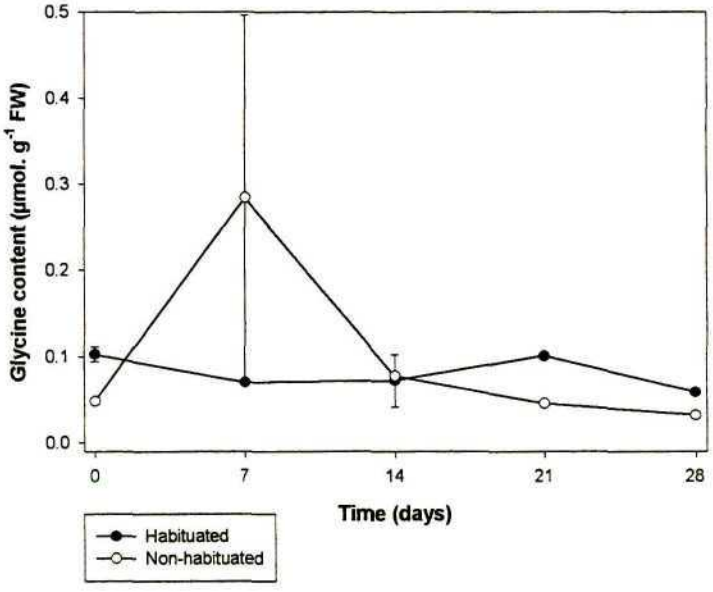


Fig. 4.3.8 The changes in cellular concentrations of serine and glycine during a 28 day growth period of habituated and non-habituated callus. Amino acids were analysed using HPLC. Mean values ±SE, n=3.

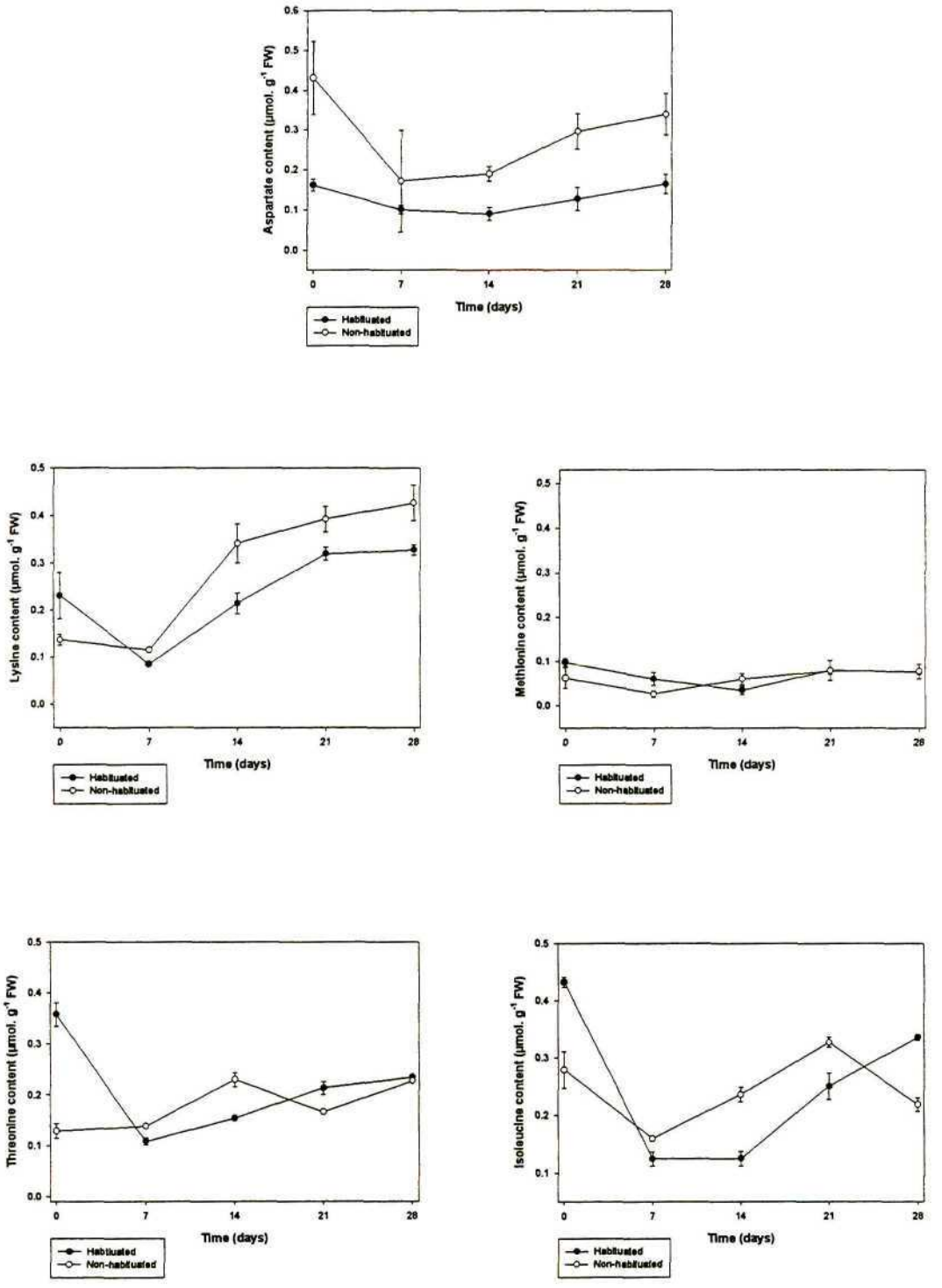
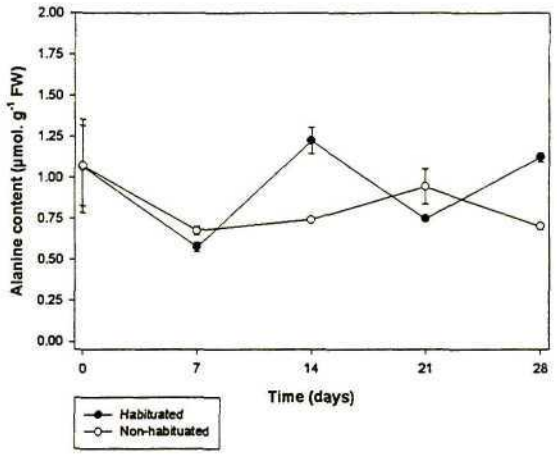
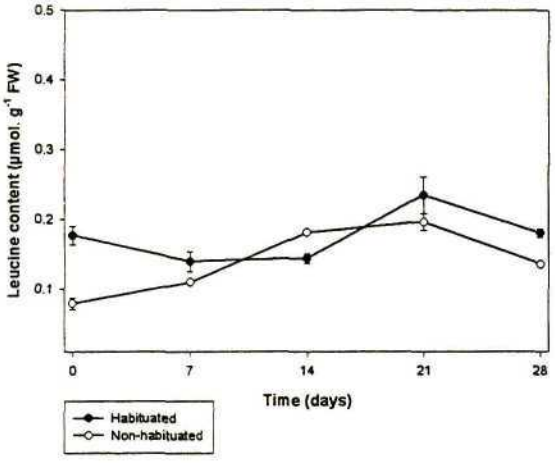


Fig. 4.3.9 The changes in cellular contents of amino acids belonging to the aspartate family (aspartate, lysine, methionine, threonine and isoleucine) during a 28 day growth period of habituated and non-habituated callus. Amino acids were analysed using HPLC. Mean values ±SE, n=3.





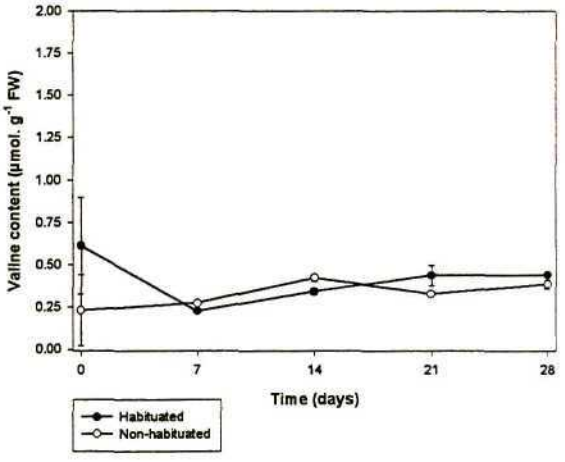


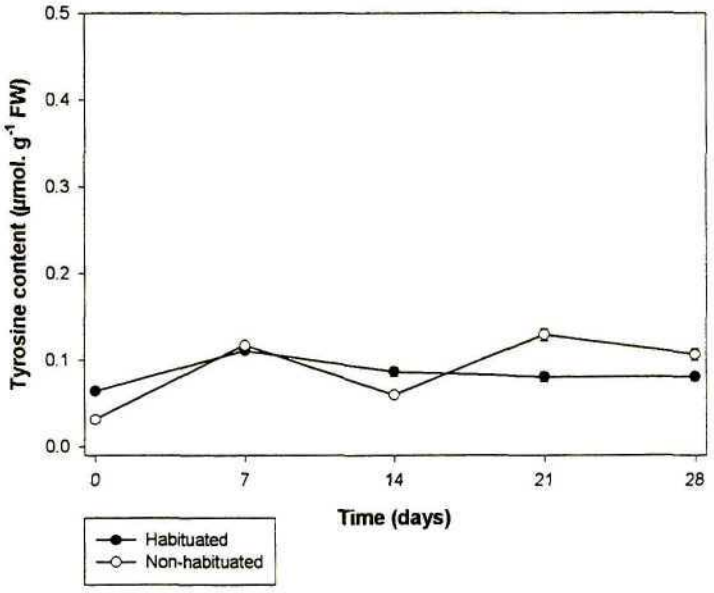
Fig. 4.3.10 The changes in amino acid contents of amino acids belonging to the pyruvate family, in habituated and non-habituated callus during a 28 day growth period. Amino acids were analysed using HPLC. Mean values ±SE, n=3.

The valine content of habituated and non-habituated callus, respectively demonstrated a similar pattern to lysine and threonine during the growth period (Fig. 4.3.10). A high amount of valine accumulated within 6 hours after the transfer to fresh media, after which it declined sharply during the first 7 days of the growth period.

Tyrosine and phenylalanine are amino acids originating from the shikimic acid pathway. Very little difference in the tyrosine levels occurred between the habituated and non-habituated callus during the culture period (Appendix A). Habituated callus exhibited a significant increase in phenylalanine content during the first 6 hours after subculturing, after which it decreased again to a level of 0.13 µmol. g^{-1} FW (Fig. 4.3.11). Phenylalanine levels of habituated callus increased again during the second half of the growth period.

The synthesis of histidine and many of the non-protein amino acids in plants remain unclear. Although there was a significant difference ($p=0.049$) in the levels of histidine between the habituated and non-habituated callus the content did not vary significantly (Appendix A) over time during the growth period in each of the callus types (Fig. 4.3.12). The high concentration (26,345 µg ml^{-1}) of histidine present in the habituated callus tissue could account for the discrepancy in proline yield when assayed with acid ninhydrin (BATES, WALDREN AND TEARE, 1973).

Cellular concentrations of individual amino acids did not change rapidly during the growth period of either the habituated or non-habituated callus tissue. No obvious correlation between the change in NH_4^+ content and any of the free amino acids detected was observed.



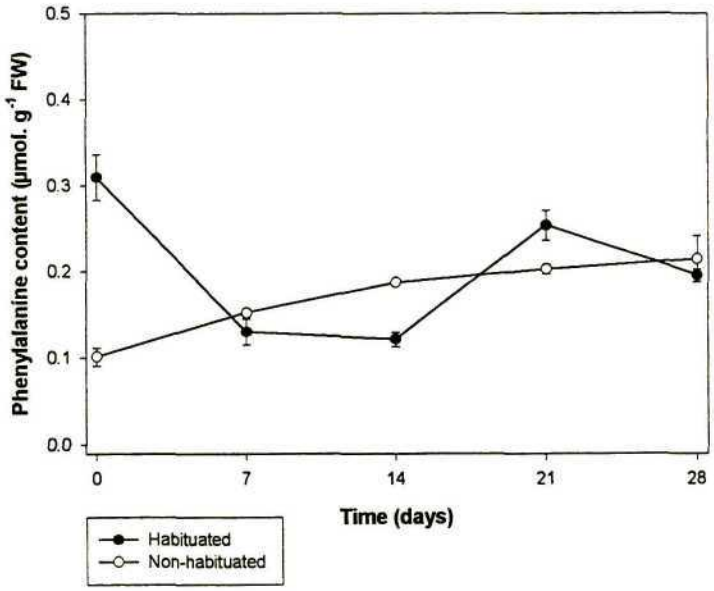


Fig. 4.3.11 The changes in cellular concentrations of tyrosine and phenylalanine in habituated and non-habituated callus during a 28 day growth period. Amino acids were analysed using HPLC. Mean values ±SE, n=3.

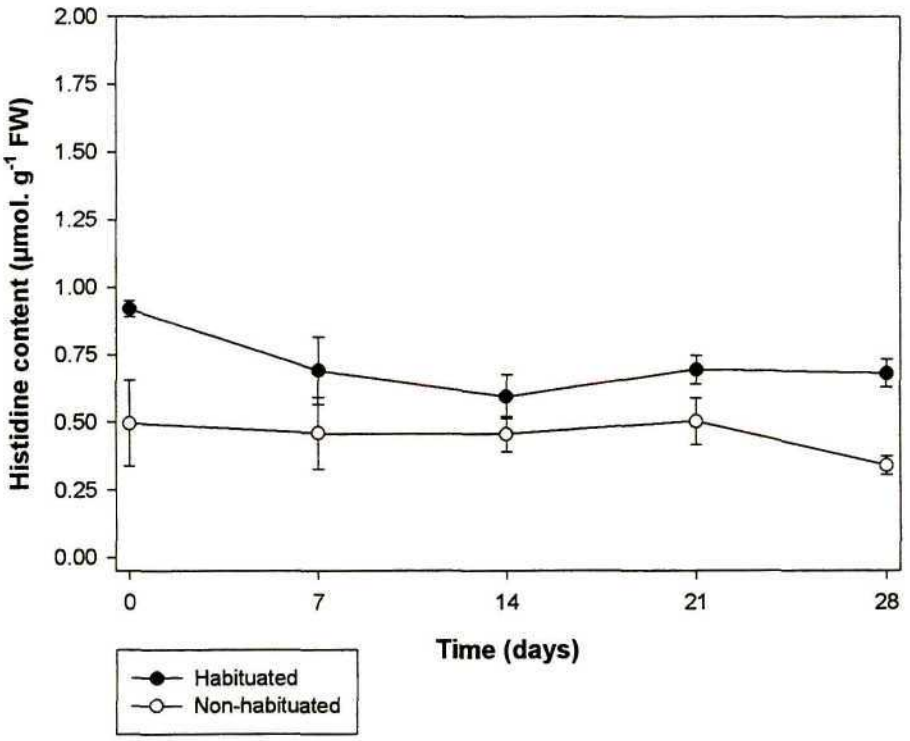


Fig. 4.3.12 The change in cellular concentration of histidine during the growth period of habituated and non-habituated callus. Histidine was determined by HPLC. Mean values ±SE, n=3.

4.4 DISCUSSION

Nitrogen is the most abundant component of the earth's atmosphere. The biosynthesis of amino acids represents the principal means of assimilation of fixed nitrogen into biologically functional molecules (ROSENTHAL, 1982). Although the central role of amino acid biosynthesis in plant metabolism and development is evident, progress in the understanding of the molecular mechanisms by which such pathways are regulated, is only recent (IRELAND, 1995). The branched and interwoven nature of these pathways for amino acid biosynthesis requires strict and precise control (IRELAND, 1995). The nitrogen and carbon flowing through for example glutamate does not all end up in a single amino acid, but is distributed to several amino acids in a defined manner in response to the needs of the cell at that particular time (IRELAND, 1995).

A common biochemical feature of fully habituated non-organogenic sugarbeet calli is a deviation in nitrogen metabolism with higher polyamine and proline levels (GASPAR, 1995). Several attempts have been made to interconnect these common disorders characterized in habituated plant tissue. The preference for the pentose-phosphate pathway to glycolysis in habituated calli (BISBIS, LE DILY, KEVERS, BILLARD, HUAULT AND GASPAR, 1993) have been associated with nitrogen metabolism to explain deficiency in tetrapyrole-containing compounds (GASPAR, 1995). The deviation in nitrogen metabolism has also been associated with polyamine accumulation through glutamate and proline (LE DILY, HUAULT, GASPAR AND BILLARD, 1993). The aim of this study was therefore to establish whether such a deviation also occurred in habituated soybean callus and whether it could be linked to other characteristics of the habituated soybean callus.

During this study, habituated and non-habituated soybean callus grown on a complete nutrient solution (MILLER, 1965) showed an increase in NH_4^+ content within 6 hours after subculturing. Although NO_3^- and NH_4^+ are both present in the nutrient medium, these results suggests that NH_4^+ was absorbed preferentially. Similar to the habituated sugarbeet callus, habituated soybean callus tissue had a 1.5-4 fold higher content of NH_4^+ than the non-habituated callus during the first 14

days of the growth period. The difference in NH_4^+ content between the two callus types could be due to a lower rate of uptake by the non-habituated callus or a higher rate of NH_4^+ assimilation.

Nearly all plant nitrogen has to pass through glutamate. Following assimilation of nitrogen into glutamine and glutamate, transaminases serve to redistribute the nitrogen to a range of other amino acids and they contribute to the maintenance of relatively stable amino acid pools (VANCE AND GRIFFITH, 1995). Amino acids can be grouped together into families, each of which are derived from a single "precursor" amino acid (IRELAND, 1995). Because more than one amino acid may be involved in the synthesis of another, a single amino acid may be assigned to more than one family and it is therefore not unusual for different authors to differ in their assignments to the families. Since the consumption of amino acids for synthetic processes requires the establishment of maintained pools (IRELAND, 1995), one can envisage tight metabolic regulation of the initial "precursor" amino acids such as glutamate and aspartate.

During this investigation, there appeared to be very little difference between the glutamate content of the habituated and non-habituated callus. In the synthesis of arginine, glutamate is first metabolized to the non-protein amino acid ornithine, via a series of acetylated intermediates (IRELAND, 1995). The arginine content of both the habituated and non-habituated callus appears to be similar to that of glutamate and it also does not vary during the growth period. The lack of variation in glutamate and arginine pools suggests fine metabolic regulation in their metabolism. Proline represents the final product of two possible metabolic pathways involving glutamate and ornithine and hence indirectly arginine (DELAUNEY, HU, KAVI KISHOR AND VERMA, 1993 AND REFERENCES THEREIN). It is an imino acid and thus has no primary amino group available for transamination. Although, there is no significant difference between the proline content of the habituated and non-habituated callus tissue it occurs at higher levels than both glutamate and arginine. This further suggests that the flux of redistributing the NH_4^+ through the "precursor" amino acids is rapid and leads to the accumulation of amino acids further down stream.

Proline was firstly determined using an assay optimized for dramatic increases in proline content (up to 100 fold) in relation to other free amino acid levels due to severe stress conditions (BATES, WALDREN AND TEARE, 1973). This method entails a modified acid-ninhydrin procedure (CHINARD, 1952). Therefore, other amino acids, such as glutamine and ornithine, may interfere with the colour yield of the proline determination, when present in high concentrations. Since there was no particular increase in proline content in either the habituated and non-habituated callus, colour yield of other amino acids could have been of sufficient levels to interfere with the colour yield of the proline determination. It was therefore decided to determine the proline content of both callus lines according to BERGMAN AND LOXLEY (1970). With this method interfering α-amino compounds are destroyed by a nitrous acid treatment, while imino compounds are allowed to react with ninhydrin under acidic conditions. Proline content, according to the second determination, appeared to be significantly higher than that determined according to BATES, WALDREN AND TEARE (1973). Contrary to what was found initially, further investigation also showed that there appeared to be no significant difference between the proline content of the habituated and non-habituated callus. These results indicated that some amino compound present in especially the habituated callus caused a decrease in colour yield when determined according to BATES, WALDREN AND TEARE (1973). An evaluation done by MESSER (1961) showed that the original procedure of proline estimation, (CHINARD, 1952) is subject to interference when the following amino acids are present in concentrations higher than 0.1-0.5 µmol ml^{-1}: glutamine, tryptophan, hydroxyproline, tyrosine and histidine. Histidine and tyrosine were shown to decrease the colour yield (MESSER, 1961). Histidine was indeed found to be present in significantly higher concentrations in the extracts of both the habituated (0.347 µmol ml^{-1}) and non-habituated (0.255 µmol. ml^{-1})callus.

Many plants distribute large amounts of nitrogen into amino acids that are not constituents of proteins (IRELAND, 1995). These non-protein amino acids comprise a very diverse and often complex group of compounds (IRELAND, 1995). Histidine comprised a significant part of the free amino acid pool in both

the habituated and non-habituated callus tissue. The habituated callus contained significantly higher levels of histidine than the non-habituated callus.

In habituated sugarbeet callus OAT, which transforms GSA and glutamate into ornithine and 2-oxoglutarate, links proline catabolism to the synthesis of ornithine, a precursor of the polyamines (LE DILY, HUAULT, GASPAR AND BILLARD, 1993). During this study gabaculine, a potent inhibitor of plant OAT (HERVIEU, LE DILY, SAOS, BILLARD AND HUAULT, 1993) was used to elucidate the relationship between ornithine and proline biosynthesis and/or catabolism in habituated and non-habituated soybean callus. Gabaculine had no significant effect on the proline content of the non-habituated callus. This indicates that, in non-habituated callus, proline is mainly synthesized via the glutamate pathway during the growth period. In habituated callus the decrease in proline content, of callus grown on gabaculine containing media, during the first 14 days of the growth period, indicates that proline is synthesized mainly from ornithine. During the second half of the growth period, however, proline content of habituated callus increased to levels higher than that of the control callus, suggesting that proline originated via the glutamate pathway. The increase in proline content further reveals that proline contributed to ornithine synthesis during the second half of the growth period, in a similar fashion to that of habituated sugarbeet callus (LE DILY, HUAULT, GASPAR AND BILLARD, 1993).

The degradation of proline is, however, also of key importance. Degradation of proline and/or P5C leads to the production of reducing power in the form of $FADH_2$ and $NAD(P)H + H^+$ during the following enzymatic reactions:

- Proline dehydrogenase (E.C.1.4.3):
  Proline + $\frac{1}{2}O_2$ + FAD $\rightarrow$ Δ^1-pyrroline-5-carboxylate + H_2O + $FADH_2$
- Δ^1-pyrroline-5-carboxylate dehydrogenase (E.C.1.5.1.12)

Δ^1-pyrroline-5-carboxylate + $NAD(P)^+$ $\rightarrow$glutamate + $NAD(P)H+H^+$

(as reviewed by HARE AND CRESS, 1997).

Two distinct P5C dehydrogenases have been detected in both mitochondria of (*Zea mays*) and cultured cells of *Nicotiana plumbaginifolia* (ELTHON AND STEWART, 1982; FORLANI, SCAINELLI AND NIELSEN, 1997). One oxidises P5C derived from proline and the other oxidises P5C derived from ornithine (ELTHON AND STEWART, 1982). FORLANI, SCAINELLI AND NIELSEN (1997) further demonstrated that one isoform of P5C dehydrogenase is expressed in actively proliferating cultured cells but decreased rapidly during the late logarithmic phase, while expression of the slightly less abundant isoform increased with the onset of the stationary phase (FORLANI, SCAINELLI AND NIELSEN, 1997). The synthesis of proline from ornithine corresponds to a period of high proliferation and biosynthetic activity during the growth period of soybean callus, while proline synthesis from glutamate corresponds to the late logarithmic phase of the growth period. One can therefore speculate that this change observed in proline metabolism during the growth period might occur in order to correspond with the pathway of proline degradation.

The synthesis of proline from ornithine in the habituated callus tissue further coincided with a high NH_4^+ content in the tissue, while synthesis from glutamate coincided with a low level of NH_4^+ in the habituated callus tissue. These results are supported by findings indicating that under conditions of high nitrogen input OAT gene expression in moth bean (*Vigna aconitifolia*) is induced, resulting in an increased conversion of ornithine to GSA and hence to proline (DELAUNEY, HU, KAVI KISHOR AND VERMA, 1993). Under conditions of nitrogen limitation in moth bean, proline was synthesized predominantly via the glutamate pathway (DELAUNEY, HU, KAVI KISHOR AND VERMA, 1993). No such correlations were recorded in proline biosynthesis in the non-habituated soybean callus.

Gabaculine treatment led to an increase in the putrescine content of both the habituated and non-habituated callus tissue. The pattern of putrescine production during the growth period was, however, similar to that of the control callus and thus revealed no relationship to proline production in either of the callus types.

The specific activity of OAT during the growth period of both the habituated and non-habituated callus mirrored the changes in the total RNA content during the growth period of both callus lines. Although the possibility still exists that the increase in OAT activity was the result of an activation of an already existing enzyme, these results strongly suggests that the increase in the specific activity of OAT, in both the callus types, was not merely the result of an increase in total protein synthesis. The necessity of a salt fractionation step during the extraction of OAT indicates that there is an inhibiting factor present in the non-habituated callus that is not present in the habituated callus. One can thus speculate that the presence of such an inhibitor could be linked to the difference in proline biosynthesis between the callus types. The highest activity occurred on day 14 of the growth period, which is also the turning point in proline metabolism. Proline synthesis via ornithine, in the habituated callus tissue, coincides with a high level of enzyme inhibition during the first 14 days. After day 14, OAT activity was inhibited to a lesser extent, while proline was synthesized from glutamate. Thus, in contrast to the pattern of enzyme inhibition, the pattern of the specific activity of OAT in the habituated callus does not reflect the change in proline metabolism during the growth period. It would thus appear that the enzyme properties of OAT in the habituated callus change during the second half of the growth period *i.e.* late exponential and stationary phases.

Other pathways implicated in ammonium assimilation includes the conversion of oxaloacetate or fumarate to aspartate, catalyzed by aspartate dehydrogenase (E.C. 1.2.1.11) and aspartase (E.C. 4.3.1.1), respectively. Similar to the glutamate content, aspartate levels did not vary significantly during the growth period of both callus lines. Non-habituated callus, however, contained significantly higher levels of aspartate than the habituated callus. This is a further indication that the lower level of NH_4^+ present in the non-habituated callus is due to a higher rate of assimilation. Lysine, threonine, isoleucine and methionine are all amino acids from the aspartate family (IRELAND, 1995). Lysine accumulated in a similar fashion in both the habituated and non-habituated callus. As for aspartate, lysine occurred at higher levels in the non-habituated callus than in the habituated callus. This suggests that the lower NH_4^+ content of the non-habituated callus

during the first 14 days after subculturing, may be due to a higher rate of NH_4^+ assimilation rather than a lower rate of uptake. Similar results were obtained for threonine. There was, however, no significant difference noted between the content of isoleucine in the habituated and non-habituated callus during the growth period. Methionine was detected only in very low levels in both the habituated and non-habituated callus. This could be due to the fact that methionine is not only withdrawn from the pool for the synthesis of proteins but also for the synthesis of other amino acids and ethylene.

Serine enters the amino acid pools where some of it is used for protein synthesis and some for the synthesis of other amino acids, but most of it is transaminated to glycine (in non-photosynthetic tissues) by the action of serine hydroxymethyltransferase (E.C. 2.1.2.1), which cleaves serine to yield glycine and methylene tetrahydropholate (IRELAND AND HILTZ, 1995). Serine was the main free amino acid detected in both the habituated and non-habituated callus. It is usually derived from photorespiratory produced glycine in photosynthetic tissues, in an effort to recover some of the carbon from glycolate produced by the oxidative reactions of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO, E.C. 4.1.1.39) (IRELAND AND HILTZ, 1995). The role of the high serine content in habituated and non-habituated callus is, however, unclear, since neither callus type contained chloroplasts. Glycine was produced in a similar pattern to serine in the habituated and non-habituated callus respectively, but at a level about 20 fold less than serine in both callus types.

Alanine is committed to ammonium assimilation by the transamination of pyruvate, catalyzed by the enzyme alanine dehydrogenase (IRELAND, 1995). Its high content in both habituated and non-habituated callus tissue is a reflection of a high pyruvate availability during the growth period (IRELAND, 1995). Similar to the other "precursor" amino acids, alanine levels did not vary significantly during the growth period of either the habituated or non-habituated callus. This emphasizes again the apparent tight metabolic control of amino acid biosynthesis. Pyruvate and acetyl-CoA also provide the carbon skeletons of the two branched amino acids valine and leucine (IRELAND, 1995). These amino acids are often

grouped together due to structural considerations but also because they share several common enzymes in their synthesis (IRELAND, 1995). Like lysine and threonine, the contents of both valine and leucine did not change significantly during the growth period in either the habituated or non-habituated callus tissue.

Tyrosine and phenylalanine appear to be synthesized exclusively from the shikimic acid pathway (IRELAND, 1995). Very low concentrations of tyrosine were present in both the habituated and non-habituated callus tissue, while phenylalanine was present at levels similar to those of leucine and isoleucine.

The phenomenon of plant tissue habituation consists of the acquired and hereditary capacity for autonomous growth, in the absence of exogenously supplied auxins and/or cytokinins in tissue culture (GASPAR, 1995). Previous studies indicated that habituated non-organogenic calli have various biochemical disorders in common (GASPAR, 1995). Several attempts have been made to interconnect these different disorders. Connecting these biochemical changes is however complicated, due to the lack of understanding of epigenetic changes in gene expression. Results obtained during this study indicated that a deviation in nitrogen metabolism also occurred in the habituated soybean callus. Since the biochemical deviation in the habituated soybean callus are different to that observed in other habituated tissues, this callus may only represent a stage during neoplasic progression as was also suggested by the morphological studies.

Both the habituated and non-habituated callus exhibited a high nitrogen influx during the first 14 days of the growth period. The low NH_4^+ content present during the second half of the growth period coincides with higher levels of the amino acids present in both callus types. NH_4^+ present in the non-habituated callus appeared to be assimilated more rapidly than in the habituated callus, as indicated by the higher levels of the "precursor" amino acids. In both callus types, "precursor" amino acid levels were significantly lower than those of amino acids down stream.

No obvious difference in proline biosynthesis was evident between the habituated and non-habituated callus. With the use of gabaculine, however, it could be established that the biosynthesis of proline in the habituated callus deviated from that in the non-habituated callus. In the habituated callus proline was synthesized via ornithine during a period of high biosynthetic activity and when high NH_4^+ levels were present. During a period of low proliferation rate, and thus low biosynthetic activity and low NH_4^+ levels, proline was synthesized via glutamate. This change in proline synthesis may correspond to a change in proline degradation during the growth period of habituated soybean callus that was not observed in non-habituated callus. Contrary to what was found in habituated sugarbeet, there exists no apparent link between proline and putrescine synthesis in either callus type.

CHAPTER 5

MOLECULAR CHARACTERIZATION OF HABITUATED AND NON-HABITUATED SOYBEAN CALLUS

5.1 INTRODUCTION

The phenomenon of plant tissue habituation consists of the acquired and hereditary capacity of cultured tissue for autonomous growth in the absence of exogenously supplied auxins and/or cytokinins (GASPAR, 1995). The heritable conversion of tobacco (*Nicotiana tabacum* (L.)) cells to the cytokinin habituated phenotype is a gradual, progressive process, which unlike mutation, is strongly influenced by the physiological and developmental state of the cells (MEINS, 1989 AND REFERENCES THEREIN). However, habituation has also been observed to occur spontaneously (GASPAR, 1995). Once established the habituated state is extremely stable.

In a series of studies on cytokinin habituation it was demonstrated that tissue cultures of tobacco (*Nicotiana* tabacum (L.) cv. Havana 425) exhibited two forms of habituation (MEINS, 1989 AND REFERENCES THEREIN). The first form involves relatively stable yet reversible changes in gene expression brought about by an unknown mechanism (BINNS, 1994). The second form of habituation in tobacco (Havana 425) involves a genetic change. Studies have identified two different habituated leaf (HI) loci as monogenic and either partially (HI-1) or fully (HI-2) dominant (BINNS, 1994). It was further suggested that the HI-1 locus had oncogenic functions (BINNS, 1994). A study done on cultured plants derived from crosses between a genotype of *Phaseolus vulgaris* which exhibited cytokinin dependence and two habituated genotypes revealed that the frequency distribution of the cytokinin requiring progeny in the F2 and backcross populations suggested the presence of a single set of alleles controlling cytokinin autonomy (MOK, MOK, DIXON, AMSTRONG AND SHAW, 1982). Ultimately, however, even the variety and stability of epigenetic states is under genetic regulation (MEINS, 1989).

Comparisons between *Arabidopsis thaliana* hormone dependent callus tissue and radiation induced hormone independent callus tissue performed at the mRNA level, indicated that several cDNA clones displayed differential expression ranging from 1.3 - 10 fold more in the autonomous callus line (CAMPELL AND TOWN, 1991). Nucleotide sequence comparisons of the cDNA fragments, however, only suggested possible functions for these gene products in the tumorous hormone independent phenotype (CAMPELL AND TOWN, 1991).

It would thus appear that controlled activation and repression of specific genes and accordingly changes in gene products must occur in the habituated callus. Early studies on the change in protein profiles during the induction of cytokinin habituation in soybean callus by phenoxyisobutyric acid, demonstrated the accumulation of higher molecular weight proteins concomitantly with the disappearance of lower molecular weight proteins (CHRISTOU, 1988). A difference in protein profiles was also evident between habituated and non-habituated sugarbeet callus (TACCHINI, XUE, GASPAR AND GREPPIN, 1995). These differences, however, were not dependent upon exogenous factors such as light or hormonal treatments, but seemed to be specific to the cell type that displayed these characteristics (TACCHINI, XUE, GASPAR AND GREPPIN, 1995).

Most physiological studies of habituation have focused on the relationship between the autotrophic phenotype and the capacity of the cells to produce hormones (MEINS, 1989). During this study it was found that the habituated phenotype callus contained elevated concentrations of cytokinins when compared to the non-habituated callus. Although, by definition, cytokinins stimulate growth of cultured cells, they also exhibit a number of other biological effects. Hence, when investigating the differences that may exist between habituated and non-habituated phenotypes it is only wise to keep in mind that if the concentration of the hormone, for which the cell type is habituated, is drastically different, that the difference in hormone concentration itself may have an effect on the characteristics of the cell type.

Our current understanding of cytokinin physiology at the cellular level results largely from the manipulation of endogenous cytokinin levels by application of exogenous cytokinins, or the expression of cytokinin biosynthetic transgenes, as well as the characterization of single gene mutants (HARE AND VAN STADEN, 1997). Cytokinins have been shown to control gene expression at transcriptional and post-transcriptional levels (SCHMÜLLING, SCHÄFER AND ROMANOV, 1997). Cytokinins modulate changes in gene expression, which are in turn assumed to affect physiological and morphological changes with which cytokinins are associated (HARE AND VAN STADEN, 1997). Plant hormones, however, often act in concert to promote growth and developmental processes. The suggestion that cytokinin and auxin mediated changes in physiology are accompanied by alterations in nucleic acid metabolism was first made by SKOOG AND MILLER (1957).

That the expression of several genes is modulated by cytokinins was shown by differential screening of cDNA libraries (CROWELL AND AMASINO, 1991; CROWELL, KALECEK, JOHN AND AMASINO, 1990), as well as by two dimensional electrophoresis of *in vitro* translation products (TERAMOTO, MAMOTANI AND TSUJI, 1993; CHEN, ERTL, YANG AND CHANG, 1987). Cytokinins stimulate greening and both protein and RNA synthesis (OHYA AND SUZUKI, 1988). Protein synthesis is often accompanied by an increase in the ratio of polyribosomes to monosomes as was noted in soybean (*Glycine max* L.) suspension cultures, excised cucumber (*Cucumis sativus* L.) cotyledons, pumpkin (*Cucurbita pepo* L.) cotyledons and apple (*Malus domestica* L.) suspension cultures (MUREN AND FOSKET, 1977; GWOZDZ AND WOZNY, 1983; CHEN, ERTL, YANG, CHANG, 1987; OHYA AND SUZUKI, 1988; ORDAS, FERNANDEZ AND RODRIGUES, 1992). Whether this increase in protein synthesis occurs concomitantly with *de novo* mRNA synthesis is unclear from the literature. Some studies indicate that cytokinins stimulate protein synthesis independently of mRNA synthesis (CHEN, ERTL, YANG, CHANG, 1987; OHYA AND SUZUKI, 1988), while other studies reveal that *de novo* mRNA synthesis occurs simultaneously with protein synthesis (GWOZDZ AND WOZNY, 1983; ORDAS, FERNANDEZ AND RODRIGUES, 1992). These stimulatory effects of cytokinins

are, however, not dependent on their growth promoting activity (GWOZDZ AND WOZNY, 1983).

Plant hormones, as well as environmental factors, are responsible for differences in the rate of synthesis of various enzymes and polypeptides in different plant cells. Cytokinins, for example also act in concert with other stimuli such as light, other phytohormones, nutrients and environmental stresses (SCHMÜLLING, SCHÄFER AND ROMANOV, 1997). An additional level of control of gene expression often omitted from studies is the cell specificity of hormone action that exists with cytokinins (SCHMÜLLING, SCHÄFER AND ROMANOV, 1997). Elevated cytokinin levels in intact plants, excised plant organs and cultured cells from a variety of monocotyledonous and dicotyledonous species have been shown to induce specific changes in the expression of several genes (HARE AND VAN STADEN, 1997). It is largely assumed that modulation of the levels of these gene products leads to manifestation of the physiological effects of cytokinins (HARE AND VAN STADEN, 1997).

Physiological and morphological results obtained during this study suggests that the elevated production of cytokinins by the habituated soybean callus tissue appears to be only a symptom of the autonomous cell phenotype, and does not appear to play an integral part in the physiological and morphological characteristics of the cell type. Despite extensive characterization of the habituated soybean callus, the phenomenon of cytokinin autonomous growth is still unexplained. The present work was, therefore, aimed at investigating the differences in protein profiles and total RNA levels of habituated and non-habituated soybean callus tissue. The identification of gene products associated with only the habituated or non-habituated tissue will further allow the molecular characterization of, and their possible role in, the phenomenon of habituation.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Habituated and non-habituated soybean callus lines were maintained as described in Section 2.2.1. Calli were subcultured every 28 days onto MILLER'S (1965) basal medium supplemented with 0.5 mg l^{-1} (2.3 µM) kinetin and 2 mg l^{-1} (11 µM) α-naphthaleneacetic acid (NAA). Calli were maintained in 100 ml culture bottles at 25°C in continuous low light conditions (0,13 µmol m^{-2} s^{-1}). The state of callus habituation was continuously monitored using the soybean callus bioassay (MILLER, 1965).

5.2.2 RNA isolation

Total cellular RNA was extracted from habituated and non-habituated callus as described by JEPSON, BRAY, JENKINS, SCHUCH AND EDWARDS, 1991. All procedures were conducted at 0-4°C. All glassware and tips used were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) and autoclaved for 60 min at 121°C. Chemicals used were of the highest quality available. Ultrapure phenol with 0.1% (w/v) 8-hydroxyquinoline was saturated with homogenization buffer and 10% (v/v) *m*-cresol was added before use.

Callus material was harvested on days 0 (6 hours after subculturing), 6, 13, 20, 24 and 30 of the growth period. Approximately 100 mg fresh material was powdered in liquid nitrogen, after which 250 µl phenol/cresol and 500 µl homogenization buffer containing: 400 mM NaCl, 50 mM Tris-HCl (pH 9), 1% (w/v) SDS, 5 mM EDTA, 4 units ml^{-1} heparin, 1 mM aurintricarboxylic acid and 10 mM dithiothreitol, were added to the plant material. The homogenized preparation was mixed carefully and centrifuged for 15 min at 10 000 x *g*. The supernatant was subsequently extracted twice with 100 µl phenol/chloroform for 5 min. Total RNA was precipitated from each sample by adding 12 M lithium chloride to a final concentration of 2 M and left overnight at 4°C. Following centrifugation of each sample for 10 min at 10 000 x *g*, the pellet was resuspended in 50 µl of 5 mM Tris-

HCl (pH 7.5). After a second lithium chloride precipitation overnight, the pellet was washed twice with 70% (v/v) ethanol and subsequently resuspended in a final volume of 10 µl sterile water. Total RNA was quantified spectrophotometrically at 260 nm with a GeneQuant RNA/DNA calculator (Pharmacia).

5.2.3 Protein isolation and analysis

Total cellular proteins were extracted from habituated and non-habituated soybean callus, on days 6 and 13 of three successive growth periods, according to MAYER, HAHNE, PALME AND SCHELL (1987). Approximately 0.5g of fresh material was ground to a powder in liquid nitrogen. Two hundred microliters extraction buffer containing: 300 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2% (v/v) Nonidet P-40, 5 mM ascorbic acid, 100 mM dithiothreitol was added to the sample, after which it was incubated with 1 mg. ml^{-1} protamine at room temperature. The sample was subsequently centrifuged for 15 min at 10 000 x *g*, after which the supernatant was removed and 9 M urea and 2% (v/v) ampholine pH 3-10 (obtained from Pharmacia), were added to the supernatant.

The concentration of proteins solubilized in sample buffer in preparation for analysis by two-dimensional polyacrylamide gel electrophoresis cannot be directly determined via commonly used methods such as the BRADFORD (1976) procedure due to interference by the combinational effect of the presence of urea, detergents, carrier ampholines and thiol compounds in the sample solubilization buffers (RAMAGLI AND RODRIGUEZ, 1985). It was however, found that when sample buffer consisting of 9 M urea, 4% (v/v) Nonidet P-40, 2% (v/v) ampholines and 2% (v/v) 2-mercaptoethanol containing solubilized samples were acidified prior to dilution, protein concentrations over the range of 0.5 - 50 µg could be reproducibly determined using a modified Bradford assay (RAMAGLI AND RODRIGUEZ, 1985).

Protein concentration of each sample was thus determined according to RAMAGLI AND RODRIGUEZ (1985), using bovine serum albumin as a standard. Prior to use the protein dye reagent, obtained from Biorad, was diluted with 3

volumes deionized distilled water and filtered through Whatman number 1 paper. To standard protein samples containing 1-50 µg in 10 µl of urea mix, 10 µl of 0.1 N HCl was added before diluting each sample to a final sample volume of 100 µl with deionized distilled water. To each sample 3.5 ml diluted dye reagent was added and the mixture was carefully mixed. After 5 min, absorbance of the sample was determined at 595 nm in a plastic cuvette against a reagent blank using a Beckman DU 65 spectrophotometer.

The total protein extract was analyzed by two dimensional gel electrophoresis (MAYER, HAHNE, PALME AND SCHELL, 1987). Isoelectric focusing (IEF) was carried out using ampholines (pH 3-10) from Pharmacia and was performed in glass tubes (100 x 1 mm) for 20 hours at 400 V. Protein (50 µg) was loaded onto the basic end of the gel using 100 mM NaOH and 11 mM phosphoric acid as electrode buffers. After the IEF, gel rods were extruded by gentle pressure into 5 ml equilibration buffer (0.5 M Tris (pH 6.8), 2.5% (w/v) SDS, 0.1% (w/v) dithiothreitol and 10% (v/v) glycerol). The second dimension electrophoresis was carried out on 1.5 mm discontinuous gels containing 12% (w/v) acrylamide with 6% (w/v) acrylamide in the stacking portion of the gel and run at room temperature at a constant current of 42 mA (LAEMMLI, 1970).

Following electrophoresis proteins were visualized by silver staining, according to MORRISSEY (1981). The gel was prefixed in 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, followed by 5% (v/v) methanol and 7% (v/v) acetic acid for another 30 min. The gel was subsequently fixed for 30 min in 10% (v/v) glutaraldehyde. After the gel was rinsed in a large volume of deionized distilled water overnight, or in several changes of water for 2 hours, the gel was soaked in 5 µg ml^{-1} dithiothreitol for 30 min. The previous solution was poured off and without rinsing a solution of 0.1% (w/v) silver nitrate was poured on and left for 30 min. After the gel was rinsed rapidly it was soaked in developer (50 µl of 37% (w/v) formaldehyde in 100 ml 3% (w/v) sodium carbonate) for 10 min. Staining was stopped by adding 10 ml 2.3 M citric acid directly to the developer and agitating for 10 min.

5.2.4 DNA modifying enzyme reactions

All restriction digestions were conducted at 37°C, utilizing enzymes obtained from Boehringer Mannheim, according to the manufacturer. Ligation reactions were accomplished using T4 ligase at 10°C, according to the manufacturer (Boehringer Mannheim).

5.2.5 cDNA synthesis

cDNA libraries were constructed according to a modified procedure of JEPSON, BRAY, JENKINNS, SCHUCH AND EDWARDS (1991). cDNA was synthesized from the total RNA isolated according to Section 5.2.1. The mRNA was translated into cDNA utilizing a 1 µg poly A^+ RNA protocol as described by the manufacturers (Amersham). Only the poly dTP primers of the cDNA synthesis kit was however, used.

UNI-Amp adapters containing an EcoR I restriction site (Clonetech) were subsequently ligated to the blunt ended cDNA, according to the manufacturer's protocols. The UNI-Amp adapters provide a template for the UNI-Amp primers (Clonetech) in order to facilitate sequence-independent PCR amplification of the cDNA synthesized. Each adapter contains a built-in restriction site, for rapid cloning of the amplified DNA. The use of a 3'-TTT overhang prevent polymerization of the adapter, so that only adapter dimers, which do not interfere with the amplification process, can form. Additionally, the restriction sites present in the adapter sequence does not form part of the UNI-Amp primer or template. This prevents primer artifacts, which frequently forms during extended PCR reactions, from being cloned. The design of the adapter allows for the use of a single primer, thus preventing primer-primer annealing.

Adapter structure:

5' CCTCTGAAGGTTCCAGAATCGATAGT**GAATTC**GTG 3'

3'-TTTGGAGACTTCCAAGGTCTTAGCTATCA**CTTAAG**CAC-P5'

Primer sequence:

5'-CCTCTGAAGGTTCCAGAATCGATAG-3'

After the adapters were ligated to the cDNA fragments, they were amplified according to the method of the UNI-Amp primer manufacturer (Clonetech). The polymerase chain reaction (PCR) reaction mix consisted of: 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl and 0.1% (w/v) gelatin); 200 µM dNTP; 0.2 µM UNI-Amp primer 2,5 units Taq Polymerase (obtained from Promega) and approximately 2 ng cDNA in a final volume of 50 µl. The cDNA was amplified via a PCR regime of 35 cycles consisting of the following steps :

- 30 sec at 94°C denaturation
- 1 min at 65°C annealing of the primer
- 2 min at 72°C polymerization of the template

The amplified cDNA was fractionated via low melting point agarose gel electrophoresis. Approximately 2 µg of cDNA was loaded onto a 1% (w/v) low melting point agarose gel and run using TAE running buffer. The agarose containing the cDNA of appropriate size (500 - 10 000 bp) were cut from the gel and stored at -70°C for subsequent use.

5.2.6 Library construction

Fractionated cDNA was amplified directly from the low melting point agarose and digested with EcoR I for the subsequent cloning steps. The cDNA fragments were cloned into the EcoR I polycloning site of a lambda bacteriophage vector λZAP II, as described by the manufacturer (Stratagene) and propagated in the *Escherichia coli* host XL1-Blue (*endA1 hsdR17(rk⁻ mk⁺) supE44 thi⁻1 cy1 λ⁻ recA1)(lac su⁻)*[F' proAB lacIqZ ΔM15 Tn10 (tet^r)]. The F' episome in the XL1-Blue strain contains the ΔM15 mutation of the lacZ gene required for alpha complementation of the amino terminus of the lacZ gene present in the λZAP II vector. The expression of both of these partial genes is required for the generation of a functional β-galactosidase protein from the λZAP II vector. Insertion into the polycloning region within the lacZ gene destroys the functioning of the lacZ gene resulting in

white plaques, while background plaques with a functional lacZ gene remain blue. This blue/white colour selection is obtained by culturing the infected bacteria on Luria broth (LB) media (10 $g.L^{-1}$ Tryptone, 5 $g.L^{-1}$ Yeast extract and 5 $g.L^{-1}$ NaCl, pH 7.5) containing 0.3 mM IPTG (isopropanyl-β-D-thiogalactoside) and 0.5 $mg.ml^{-1}$ X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

5.2.7 Subtraction library construction

Total RNA was isolated from habituated and non-habituated callus on day 14 of the growth period. cDNA was subsequently synthesized and amplified as described previously (Section 5.2.4). The subtraction library was constructed via a modified procedure of, AUSUBEL, BRENT, KINGSTON, MOORE, SIDEMAN, SMITH AND STRUHL (1987).

[+]cDNA from habituated callus was prepared with EcoR I ends while [-]cDNA from non-habituated callus was digested with Rsa I and Alu I to produce small blunt-ended fragments. The [+]cDNA was then mixed with a 50 fold excess of the [-]cDNA in a solution containing: 50% (v/v) deionized formamide, 5x SSC (15 mM Na_3 citrate, 150 mM NaCl pH 7), 10 mM $NaPO_4$ (pH 7.0), 1 mM EDTA (pH 8) and 0.1% (w/v) SDS. After the mixture was dissociated, the single stranded cDNA fragments were allowed to hybridize for 24 hours at 37°C. Following the hybridization step, annealed cDNA fragments with EcoR I ends were recovered and ligated to the cloning vector, λZAP II, packaged and transfected according to the manufacturer (Stratagene). Thus the only cDNA likely to regenerate double stranded fragments with an EcoR I site at each end are those sequences for which no complementary fragments were present in the [-]cDNA. The subsequent cloning step allowed the selection and amplification of those fragments.

The clones of interest were isolated and excised with the helper phage R408 into the phagemid pBluescript, as facilitated by the λZAP II cloning system, according to the manufacturer (Stratagene).

5.2.8 Genomic DNA isolation

Genomic DNA from habituated and non-habituated callus was isolated utilizing a mini preparation according to DELLAPORTA, WOOD AND HICKS (1983). Fresh tissue (0.5 g) was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The DNA was extracted from the homogenized tissue with 15 ml extraction buffer consisting of 100 mM Tris (pH 8), 50 mM EDTA, 500 mM NaCl and 10 mM mercaptoethanol. After the addition of 1 ml 20% (w/v) SDS the preparation was mixed thoroughly and incubated at 65°C for 10 min. Proteins and polysaccharides were removed from the sample by adding 5 ml 5 M potassium acetate and incubating it on ice for 20 min. The proteins and polysaccharides form a complex with the insoluble potassium dodecyl sulphate precipitate, which was subsequently gathered by centrifugation at 20000 *x g* for 20 min. DNA was precipitated from the supernatant by adding 10 ml isopropanol and incubating it at -20°C for 30 min. The DNA pellet was subsequently redissolved in 700 µl TE (10 mM Tris (pH 8) and 1 mM EDTA).

5.2.9 Plasmid isolation

Plasmid DNA was isolated utilizing an alkaline lysis procedure which produces plasmid DNA of a quality suitable for all routine uses such as molecular cloning, PCR and sequencing according to FELICIELLO AND CHINALI (1993). Preparation of plasmid DNA was carried out on a small scale from cells grown in 10 ml of LB medium (10 $g.L^{-1}$ Tryptone, 5 $g.L^{-1}$ Yeast extract and 5 $g.L^{-1}$ NaCl, pH 7.5). After the cells were harvested, by centrifugation at 6 000 x *g* for 2 min, the pellet was rinsed in 1 ml of cold STE buffer (0.1 M NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). Following centrifugation of each sample at 6000 x *g* for 2 min, cells were resuspended in 250 µl of solution I (50 mM glucose, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA). Cells were kept continually on ice while adding 500 µl of freshly prepared solution II (0.2 N NaOH and 1% (w/v) SDS). Samples were stored on ice for 3-5 min, after which 750 µl of 4 M potassium acetate-2 M acetic acid were added to each sample and mixed thoroughly. The samples were left on ice for a further 5-10 min after which they were centrifuged at 12000 x *g* for 5 min.

The supernatant was recovered and all nucleic acids were subsequently precipitated with isopropanol. A pellet of each sample was recovered by centrifugation at 12000 x *g* for 5 min. Each sample was then treated with 10 µg. ml^{-1} Dnase free Rnase at room temperature for 15 min. The plasmid DNA was finally precipitated with 88% (v/v) isopropanol and 0.2M potassium acetate for 10 min at room temperature and collected by centrifugation at 12000 x *g* for 5 min. The DNA was resuspended in sterile water and quantified spectrophotometrically at 260 nm with a GeneQuant RNA/DNA calculator (Pharmacia).

5.2.10 Hybridization, probe preparation and detection

Isolated DNA was analyzed using agarose gel electrophoresis utilizing TAE (40 mM Tris, 1 mM EDTA at pH 8 adjusted with glacial acetic acid) as running buffer (AUSUBEL, BRENT, KINGSTON, MOORE, SIDEMAN, SMITH AND STRUHL, 1987). In order to obtain optimal transfer, the gel was first depurinated with 250 mM HCl for 10-15 min and then denatured in a solution of 1.5 M NaCL and 0.5 M NaOH for 25 min. Before the DNA was transferred to a nylon membrane the gel was rinsed in a neutralizing solution containing 1.5 M NaCl and 0.5 M Tris-HCl pH 7.5 (AUSUBEL, BRENT, KINGSTON, MOORE, SIDEMAN, SMITH AND STRUHL, 1987). Following the processing of the gel, the DNA was transferred to the membrane via capillary blotting, overnight, facilitated by 20 x SSC. After the transfer was completed the blot was rinsed in 6 x SSC for 1 min. The DNA was fixed to the membrane by incubating the membrane for 15 min in a solution of 40 mM NaOH. The membrane was air dried and used for subsequent hybridization steps.

In all cases the ECL direct nucleic acid labeling and detection system were used for probe labeling, and detection of the target DNA, as described by the manufacturers (Amersham). The selected DNA probe was directly labeled with the enzyme horseradish peroxidase. This is achieved by completely denaturing the probe at 96°C. The peroxidase, which has been complexed with a positively charged polymer, is then chemically cross-linked with glutaraldehyde to the negatively charged single stranded DNA probe.

Hybridizations were conducted overnight at 42°C in a Hybaid hybridization oven with a specially optimized hybridization buffer included in the ECL system. After hybridization, the membrane was washed twice for 20 min respectively, in a high stringency wash buffer (6 M urea, 0.4% (w/v) SDS and 0.5 x SSC) at 42°C. The membrane was finally washed with a secondary wash buffer (2 x SSC) at room temperature for 5 min. The signal was subsequently detected with two detection solutions included in the system. The signal as generated via the reduction of hydrogen peroxide by the enzyme. The latter is coupled to a light producing reaction of luminol, which upon oxidation produces blue light. The light is detected on a blue-light sensitive film.

5.2.11 Sequencing analysis

DNA sequencing was performed by the dideoxy termination method using Sequenase version 2.0 T7 DNA Polymerase (United States Biochemicals) with T7 and T3 primers. Double stranded template was prepared as described previously (Section 5.2.7) and the procedure was performed as described by the manufacturer. The cDNA sequences obtained were compared with the GenBank + EMBL + DDBJ + PDB data bases using the BLAST search routine (ALTSCHUL, GISH, MILLER, MYERS AND LIPMAN, 1990) accessed via electronic mail server at **blast@ncbi.nlm.nih.gov**. Sequences were translated in all six reading frames and compared with the non-redundant Protein Data Base at the National Centre for Biotechnology and Information (NCBI) at Bethesda (Maryland) using blastx (ALTSCHUL, BOYUSKY, GISH AND WOOTTON, 1994).

In order to allow efficient sequence analysis of the clones isolated, a series of nested deletions from double stranded, linear plasmid DNA was generated utilizing the Erase-a-Base System (Promega) as described by the manufacturers. Exonuclease III is used to specifically digest DNA from the 5' protruding or blunt end, while leaving a 4 base 3' protruding end intact. The uniform rate of digestion of the enzyme allows deletions to be made at predetermined intervals by removing timed aliquots from the reaction.

Closed circular plasmid DNA was firstly purified, by selectively removing nicked and linear DNA by acid-phenol extraction, according to the manufacturer (Promega). Restriction digestions to generate exonuclease resistant 3' overhangs and 5' blunt or unprotected ends were done utilizing Kpn I and Cla I for deletions from the T7 primer and Sac I and Xba I for deletions from the T3 primer. Exonuclease digestion of the doubly cut DNA proceeds synchronously from the 5' end into the insert. After the DNA was treated with S1 nuclease to remove the single stranded tails remaining after exonuclease III digestion, Klenow Polymerase was added to fill in the ends. The deleted plasmid DNA was subsequently circularized and amplified in the appropriated vector. A number of subclones form each time point were then screened to select for the appropriate intervals in between the deletions.

5.3 RESULTS

5.3.1 RNA content of habituated and non-habituated callus

An investigation of the total cellular RNA levels (on a fresh weight basis), indicated a striking difference between the RNA content of habituated and non-habituated callus. During the 28 day growth period RNA levels in non-habituated callus gradually increased with time (Fig. 5.3.1) and the highest level (12.2 mg. g^{-1} fresh weight) was recorded 21 days after subculturing. Total RNA levels of the habituated callus appeared lower (Appendix A) than that of the non-habituated callus. A striking 12 fold increase in the extractable RNA content was observed 13 days after subculturing. The total RNA of the habituated callus decreased again from day 14 to day 21 of the culture period at a calculated rate of 3.02 mg. g^{-1} fresh weight. It then had a similar concentration to what was detected on day 0 of the growth period.

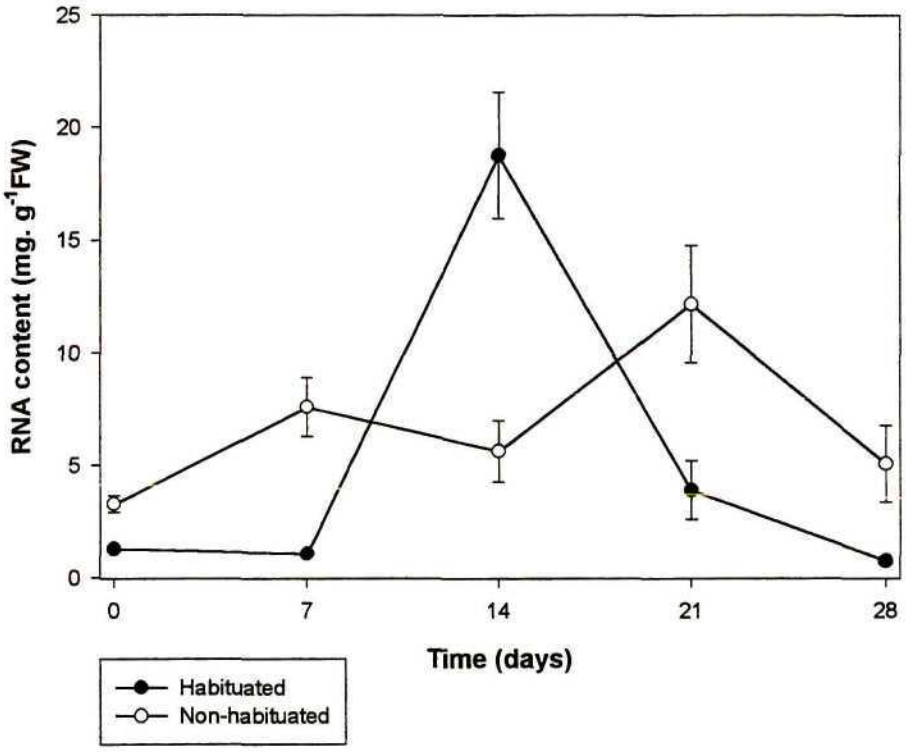


Fig. 5.3.1 The change in total RNA concentration of habituated and non-habituated soybean callus cultured on MILLER'S (1965) medium supplemented with 2 mg. L^{-1} (11μM) NAA and 0.5 mg L^{-1} (2.3 μM) kinetin, during a thirty day growth period at 25°C under low light conditions. Mean values ±SE, n=3.

5.3.2 Protein analysis

Habituated and non-habituated callus were grown in the same light conditions at 25°C. Both callus types were continually subcultured onto MILLER'S medium (1965) supplemented with 2 mg l^{-1} (11 µM) NAA and 0.5 mg l^{-1} (2.3 µM) kinetin every 28 days. Habituated and non-habituated callus tissue were harvested on days 6 and 13 of the growth period. Two dimensional gel analysis of the total protein extracted from habituated and non-habituated callus revealed both increases and decreases in the levels of several polypeptides (Fig. 5.3.2). Although several quantitative differences were observed, only qualitative differences were selected. Three polypeptides that were found to be present in both callus types, 6 days after subculturing, decreased during the exponential growth phase in the non-habituated callus. One of these polypeptides disappeared in the non-habituated callus, while the levels of these polypeptides increased in the habituated callus.

5.3.3 cDNA library construction

Separate cDNA libraries were constructed from the habituated and non-habituated callus, respectively. Each library consisted of approximately 10^6 clones of which most contained inserts between 500 - 2000 bp, although larger inserts have also been detected (Fig. 5.3.3). The library of the non-habituated soybean callus was also used to successfully isolate a putative ACC synthase gene fragment.

A subtracted cDNA library was constructed by hybridizing cDNA from habituated soybean callus with a 50 fold excess cDNA from the non-habituated soybean callus. After one round of subtraction 16 clones from the unamplified library were isolated. After cross hybridization of the different clones isolated, one of these clones, clone CH7, was chosen for further investigation (Fig 5.3.4).

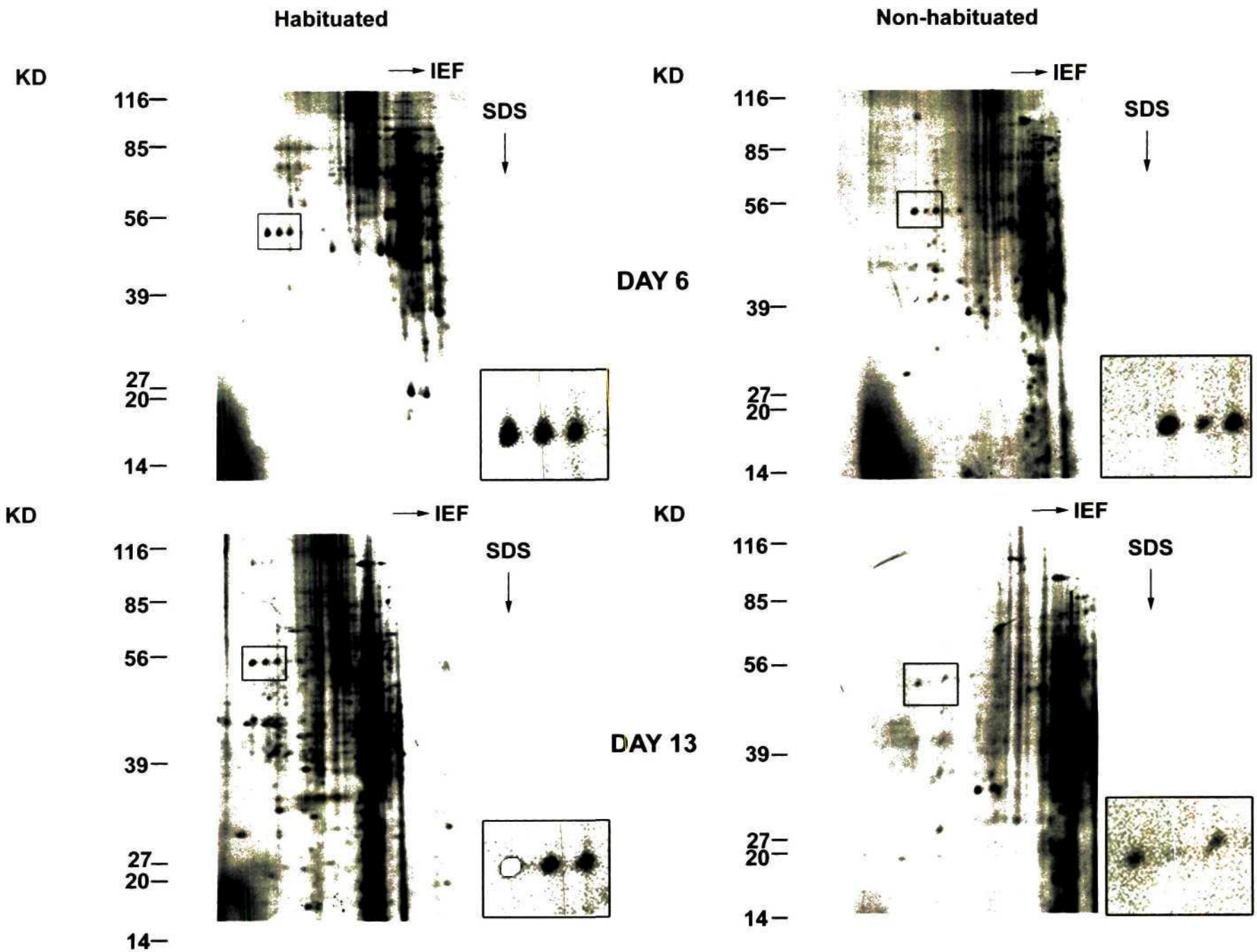

Habituated
Non-habituated
KD
IEF
SDS
116
85
56
39
27
20
14
DAY 6
DAY 13

Fig. 5.3.3 Total RNA (10 µg) was isolated from Habituated (H) and non-habituated (N) callus and used directly to synthesize the cDNA. The cDNA was subsequently amplified via sequence independent PCR after which the cDNA (2 µg) was fractionated utilizing a 1% (w/v) low melting point agarose gel electrophoresis. The fractionated cDNA was then used for the construction of cDNA libraries.

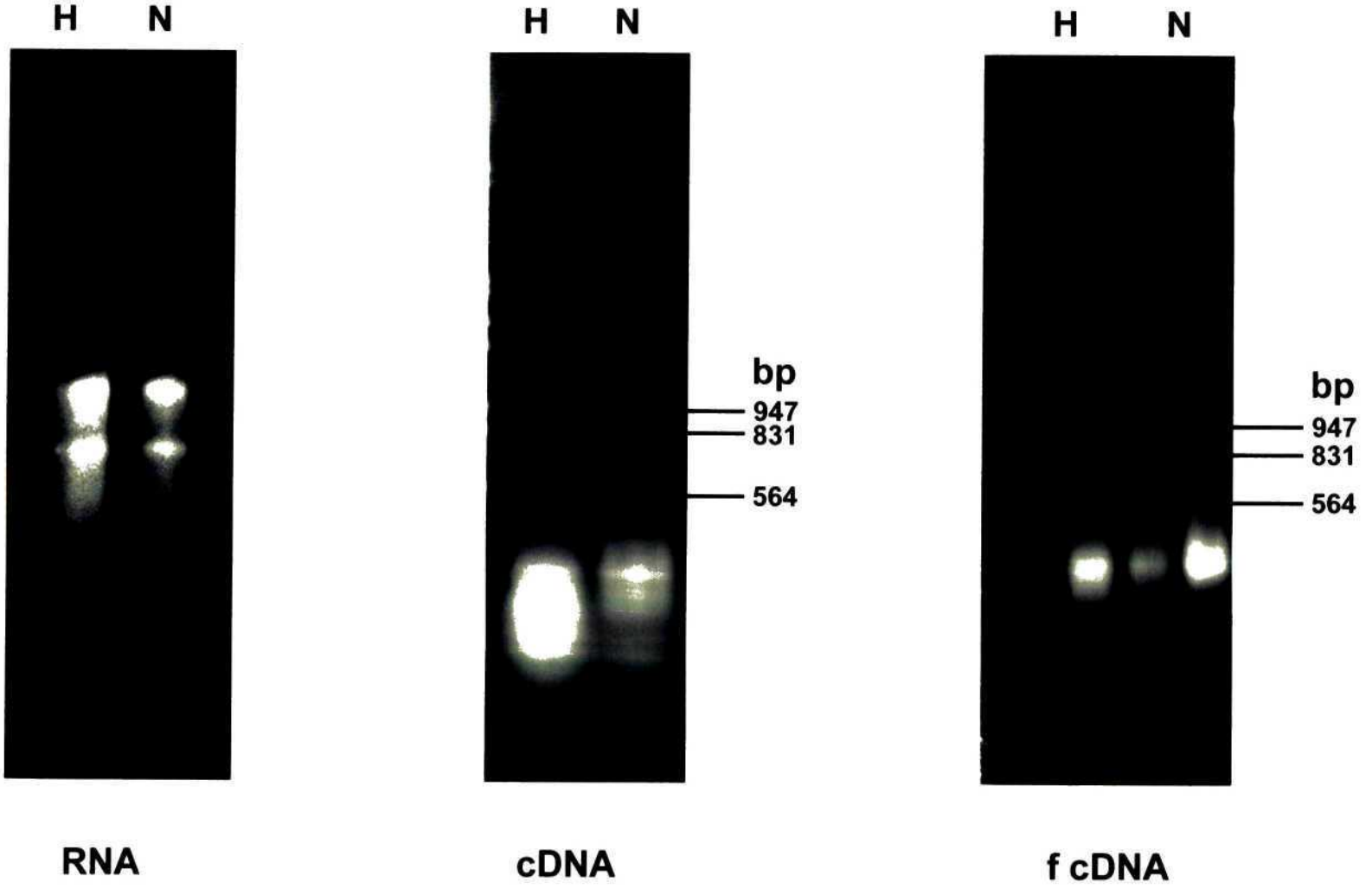

H
N
H
N
bp
947
831
564
H
N
bp
947
831
564
RNA
cDNA
f cDNA

Fig. 5.3.4 Cross hybridization of the 16 cDNA clones isolated via subtractive techniques from the habituated soybean callus (10 µg per lane). The blot was hybridized overnight with the excised insert of clone number 7 and washed at high stringency.

Fig. 5.3.5 Genomic DNA Southern blot analysis of habituated soybean callus. Total DNA (30 µg) was digested with EcoR I, Bam HI and Hind III, respectively. Hybridization was done overnight with the 900 bp fragment of the clone CH7. The blot was washed under high stringency conditions.

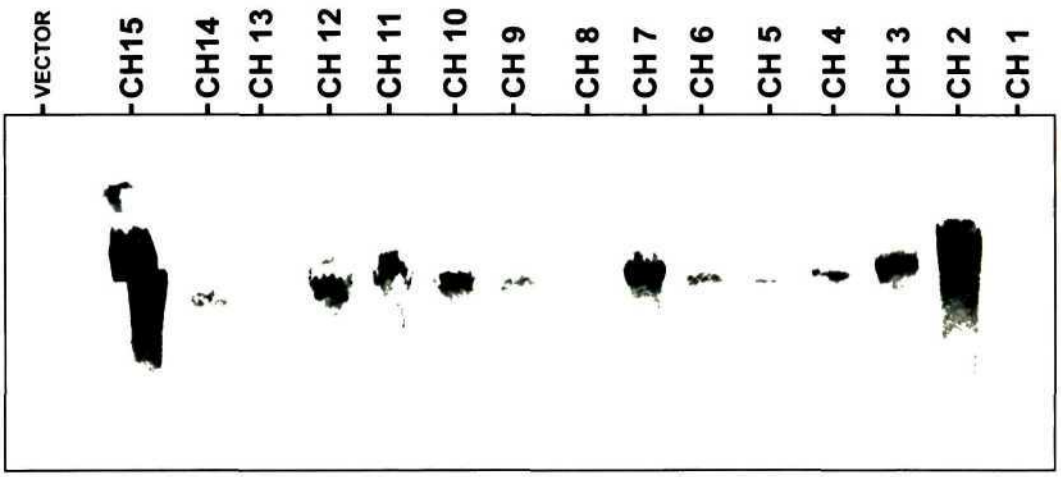


Fig. 5.3.4

Fig. 5.3.5

5.3.4 Genomic DNA analysis

To determine the sequences related to clone CH7 in the habituated soybean callus genome, total DNA (30 µg) was digested with EcoR I, Hind III and EcoR I + Hind III. The digested DNA was subjected to Southern blot analysis, using only the 900 bp fragment of CH7 as a probe. None of the enzymes used cut into the fragment. A single cross hybridizing band was detected in each of the uncut and cut genomic DNA samples. The figure Fig. 5.3.5, is a partial digestion of the genomic DNA.

5.3.5 Restriction digest and sequence analysis of CH7

The λ clone CH7, isolated from the habituated callus tissue, was excised into the phagemid pBluescript SK+ using the helper phage R408. Upon digestion with the enzymes EcoR I, Bam HI and Hind III the insert of approximately 9900 bp, produced 4 fragments of approximately 900 bp, 1500 bp, 3500 bp and 4000 bp respectively (Fig. 5.3.6). Cross hybridization results (Fig. 5.3.7) of these fragments indicated that at least three of these fragments are unique and not part of a chimeric combination of the same fragment(s).

Each of these fragments was subsequently subcloned into the phagemid pBluescript SK+ to enable sequence analysis. The nucleotide sequence and encoded amino acid sequence of the ends of two of the fragments (900 and 4000 bp fragments) revealed a high percentage of similarity to the membrane bound component of a glutamate-aspartate binding protein-dependent transport system of 2982 bp, from *E coli*. The *E. coli* sequence encompasses three components consisting of glt J (base pares 357-1097), glt K (base pares 1079-1771) and glt L (base pairs 1771-2496). The 900 bp fragment isolated from the habituated soybean callus showed 94% similarity over 225 bp on the 5' end of the insert and 89% similarity over 130 bp from the 3' end of the insert to the component glt L. The 4000 bp insert revealed a 96% similarity over 161 bp from the 5' end of the fragment to the component glt J. The other two fragments (1500 bp and 3500 bp)

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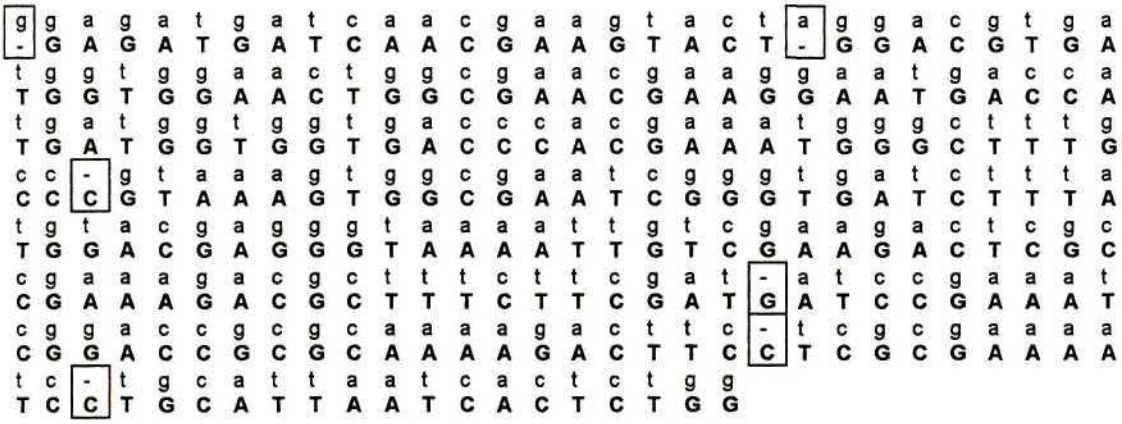
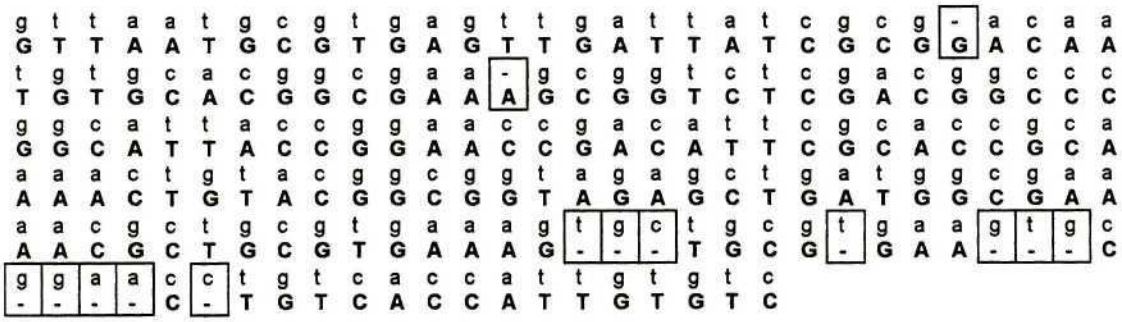
showed high similarity to λ KH100 insertion element IS5 and pBluescript cloning vectors.

The following are the results of the sequence analysis of the 5' and 3' ends of the 900 bp fragment as well as the 5' end of the 4000 bp fragment:

5' End 900 bp fragment: *E. coli* sequence is in bold



3' End of the 900 bp fragment: *E. coli* sequence in bold



5' End of the 4000 bp fragment: *E. coli* sequence in bold.

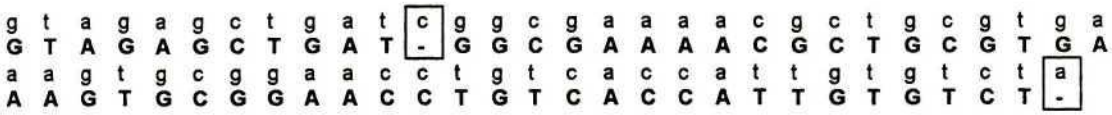


Fig.5.3.6 Restriction digest analysis of the clone CH7 isolated from the habituated soybean callus. Plasmid DNA (2µg) was digested with the enzymes EcoR I and Xho I, EcoR I, Bam HI and , Bam HI and Hind III. The digestion with Bam HI and Hind III produced 4 fragments of approximately 900 bp, 1500 bp, 3500 bp and 4000 bp respectively.

Fig. 5.3.7 Southern blot analysis of the restriction fragments of the insert of the clone CH7 isolated from the habituated soybean callus tissue. Plasmid DNA (5 µg) was digested with, Hind III and Bam HI. The blot was probed with the 900 bp and the 1500 bp fragment, respectively. After hybridization overnight the blot was washed under conditions of high stringency.

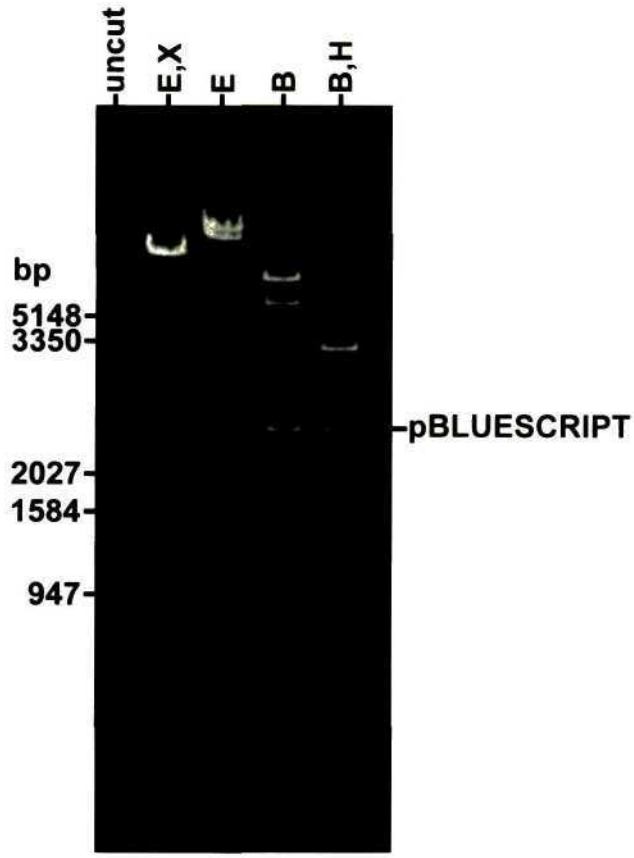


Fig. 5.3.6

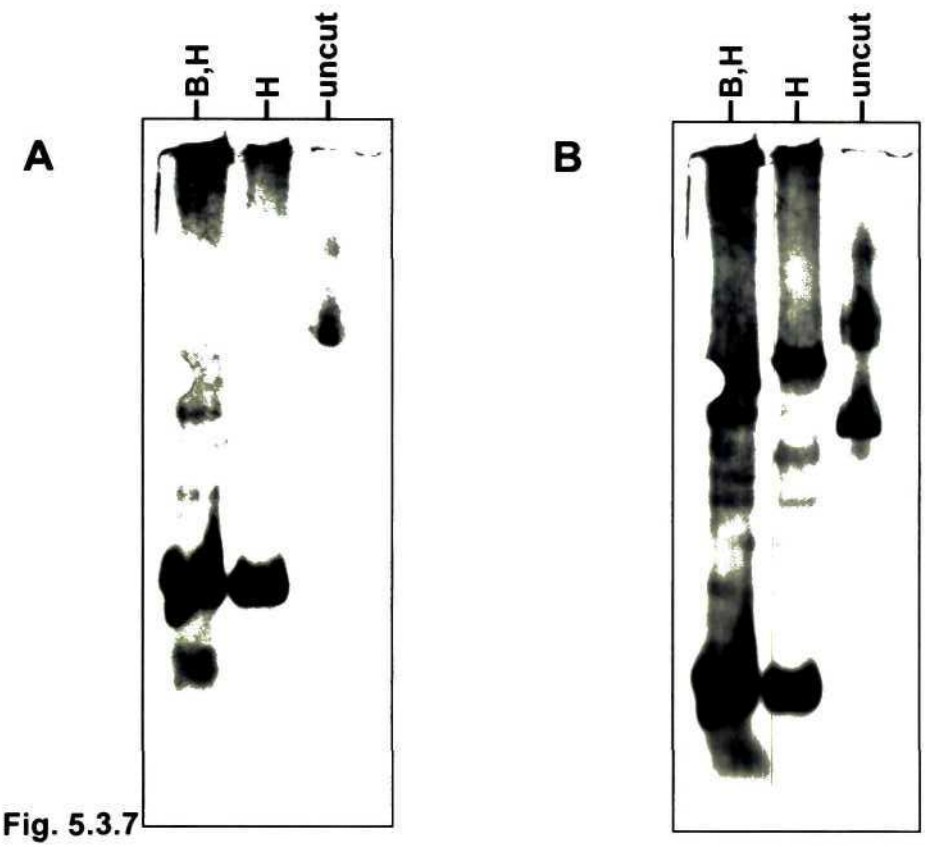


Fig. 5.3.7

5.4 DISCUSSION

Previous reports on differences in hormonal physiology of habituated and non-habituated tissue produced conflicting results. Similar concentrations of hormones in both habituated and non-habituated cell types have been reported (KEVERS, COUMANS, DE GREEF HOFINGER AND GASPAR, 1981). However, it has also been shown that hormone concentration, for which the tissue is habituated, is higher in the habituated tissue than the non-habituated tissue (DYSON AND HALL, 1988). During this study results indicated that a higher concentration of cytokinins are present in the habituated callus than the non-habituated callus (Section 2.3.2.). Although their mode of action is not clearly understood, it is known that cytokinins can activate/repress the expression of several genes (SZABO, KÖVES AND SOMOGY, 1994; SCHMÜLLING, SCHÄFER AND ROMANOV, 1997). Analysis of the proteins of fully habituated sugarbeet callus, however, indicated that the profiles observed in the habituated tissue is specific to the cell type rather than dependent on exogenous factors such as hormonal treatments (TACCHINI, FINK, XUE, GASPAR AND GREPPIN, 1997). In order to fully characterize the phenomenon of cytokinin habituation in soybean callus it is important to study the molecular effects of both habituation and the effects possibly relating to cytokinin overproduction.

Analysis of the total proteins from habituated and non-habituated callus on day 13 of the growth period, revealed the presence of several polypeptides in the habituated callus that were not present earlier in the growth period (day 6). These polypeptides were also not present in the non-habituated callus on day 6 or day 13 of the growth period. Three polypeptides were noted that occurred in both the habituated and non-habituated callus on day 6 of the growth period, but decreased in abundance in the non-habituated callus later in the growth period, on day 13. These differences suggest that the expression of these three polypeptides, of more or less similar molecular weight, are regulated as an effect of cytokinin habituation.

Studies have shown an overall stimulatory effect of cytokinins on protein synthesis as well as the induction of specific new proteins and the repression of others (SCHMÜLLING, SCHÄFER AND ROMANOV, 1997). All these changes may thus imply an altered level of the corresponding mRNA. During this study the total RNA concentration of habituated callus appeared lower than that of the non-habituated callus. In contrast to the non-habituated callus, the RNA concentration of the habituated callus did not vary significantly during the growth period, except for a 12 fold increase on day 13 of the growth period. RNA content of the non-habituated callus increased gradually during the growth period and reached a maximum 24 days after subculturing. The increase in RNA concentration of the non-habituated callus possibly reflect the physiological events associated with the proliferation process induced by exogenous cytokinins present in the media. It would thus appear that the higher concentration of cytokinins present in the habituated soybean callus did not have an effect on the levels of total RNA present in the tissue. In view of the fact that both callus types grow at a similar rate (Section 2.3.1) the striking increase in total RNA content, on day 13 of the growth period, may indicate a potential increase in the translational capacity, which is unique to the habituated cell type. This is supported by an increase in total RNA content concomitantly with an overall increase in the abundance of polypeptides present in the habituated callus, as observed with two dimensional gel electrophoresis.

Whether the overall increased protein synthesis upon cytokinin treatment, is due to *de novo* mRNA synthesis is still unclear from the literature (GWOZDZ AND WOZNY, 1983; CHEN, ERTL, YANG, CHANG, 1987; OHYA AND SUZUKI, 1988; ORDAS, FERNANDEZ AND RODRIGUES, 1992; SCHMÜLLING, SCHÄFER AND ROMANOV, 1997), since cytokinins may also control gene expression at a post-transcriptional level by regulating transcript stability and/or abundance (SCHMÜLLING, SCHÄFER AND ROMANOV, 1997). The specific increase in total RNA levels in the habituated soybean callus, however, appear to be the result of a specific increase in nuclear RNA synthesis. During an ultrastructural study (Section 3.3.1) it was observed that several nucleolar vacuoles were present in the habituated soybean callus cells on day 14 of the growth period. It is well

established that the presence of nucleolar vacuoles are closely related to RNA synthesis in the nucleus (JOHNSON AND JONES, 1967; JOHNSON, 1969). Although the non-habituated callus contained a higher level of total RNA at the corresponding time during the growth period, nucleolar vacuoles were not observed in the cells (Section 3.3.2).

The identification of specific genes and/or their protein products for habituated and non-habituated callus tissue will contribute to the molecular characterization of this phenomenon. Separate cDNA libraries were thus constructed from habituated and non-habituated callus tissue respectively. Each library consisted of 10^6 clones of which most contained inserts of $\geq$ 500 bp. Previous studies have shown that the different phenotypes (habituated and non-habituated) have different patterns of gene expression (EICHOLZ, HARPER, FELIX AND MEINS, 1983; CHRISTOU, 1988; TACCHINI, FINK, XUE, GASPAR AND GREPPIN, 1997). This suggests that the sequences over-expressed in the habituated callus type are most likely to be related to hormone autonomy. Hence, through the use of subtraction techniques 6 different clones were isolated that were over-expressed in habituated callus tissue on day 14 of the growth period. One clone CH7 was chosen for further investigation. The clone had an insert of approximately 10000 bp from which four fragments of approximately 900 bp (Bam HI ends), 1500 bp (Bam HI ends), 3500 bp (Hind III and EcoR I ends) and 4000 bp (EcoR and Hind III ends) were produced when digested with the restriction enzymes EcoR I, Bam HI and Hind III. Genomic hybridization suggested the existence of no other related genes present in the habituated callus tissue.

A comparison of the partial nucleic acid and deduced amino acid sequences of the two fragments (900 bp and 4000 bp respectively) of the insert, showed high similarity to the membrane bound component of a glutamate-aspartate binding protein-dependent transport system of 2982 bp, from *E coli.* The end sequences of the other two fragments (3500 bp and 1500 bp respectively) showed high similarity to the λ KH100 insertion element IS5 and pBluescript cloning vectors.
It appears that the complete insert of CH7 is the result of extensive rearrangements in the plasmid. Prokaryotic transposable elements have been

studied most extensively in *E. coli*. They are generally called insertion sequences (IS), if less than 2 kb in length (KENDREW, 1994). Among the transposable DNA elements that have been detected in bacterial genomes and plasmids, the smaller insertion sequences (IS) have been recognized as a class of autonomous, basic unit of transposition (KRÖGER AND HOBOM, 1982). Insertion sequences (IS) are not only fully competent of transposition but are also able to cause several other related chromosomal aberrations such as deletions of adjacent DNA segments and integration of circular plasmid DNA (KRÖGER AND HOBOM, 1982). Insertion sequences (IS) have also been proved to be the active units of transposition in the larger composite transposons (Tn) elements (KRÖGER AND HOBOM, 1982).

However, not with standing the fact that several rearrangements did occur in the plasmid, the cloning procedure followed (Section 5.2.6) and positive result obtained with the genomic southern blot indicate that the insert cloned is indeed of plant origin.

Plants have a relatively large mitochondrial genome, which range from a minimum of about 200 kbp to at least 2400 kbp (GRAY, 1995). Although the basic function of mitochondrial DNA (mtDNA) is constant, the precise set of genes encoded by mtDNA is not (GRAY, 1995). Given the variation in mtDNA size, it is not surprising that there is also marked diversity in the way in which genes in mtDNA are arranged and expressed (GRAY, 1995). This structural diversity is, however, in sharp contrast with the basic genetic conservatism of mtDNA (GRAY, 1995). Plant mitochondrial rRNAs show a striking structural resemblance (both in primary sequence and potential secondary structure) to their eubacterial homologs (SPENCER, SCHNARE AND GRAY, 1984). Within these conserved regions wheat (*Triticum aestivum*) mitochondrial 18S and 26S rRNA display, respectively, 77% and 73% primary sequence identity with their *E. coli* homologs (GRAY, 1995). Since the insert isolated is of plant origin, its high similarity to a bacterial gene could be explained by its possible mitochondrial origin. Further, it was observed that although plant mitochondrial mRNA contained 5'- and 3'-non-coding regions, these messages did not appear to be polyadenylated (GRAY, 1995).

A possible role for the protein product of the insert isolated, concerns the transport of glutamate and aspartate in and out of the mitochondrion. In contrast to animal mitochondria, plant mitochondria do not exhibit an electrogenic glutamate/aspartate antiporter driving glutamate into the mitochondria and aspartate out of the mitochondria (DOUCE AND NEUBURGER, 1995). This is due to the presence of an NADH dehydrogenase located on the outside of the innermembrane of plant mitochondria (DOUCE AND NEUBURGER, 1995). Consequently plant mitochondria do not require a complex exchange system for the transfer of reducing equivalents from the cytosol to the matrix (DOUCE AND NEUBURGER, 1996). Information on the transport of glutamate and aspartate in plant mitochondria is limited. However, MILLHOUSE, WISKICH AND BEEVERS (1983), reported the exchange of glutamate and aspartate in castor bean mitochondria. It was found that the glutamate/aspartate exchange is completely reversible and that it can vary from tissue to tissue in plants (DAY AND WISKICH, 1983). Fluxes of aspartate and glutamate will respond to the removal of each compound and will be determined by the movement of their corresponding ketoacids and also by the local concentration of each throughout the cell (FREEMAN REICHLING AND BALOGH, 1995).

One hypothesis of why such a glutamate/aspartate transport system might be of great importance to the habituated callus involves the diversion observed in proline metabolism of the habituated soybean callus tissue (Section 4.4). It appears as though ammonium is assimilated at a much slower rate in the habituated callus than the non-habituated callus (Section 4.4). Most of the nitrogen absorbed is assimilated into glutamate and then redistributed to a range of other amino acids. The lack of variation in the content of glutamate and other "precursor" amino acids observed during this study, suggests that the pools of these amino acids present in the cells are under tight metabolic control. The first 14 days of the growth period, when proline is formed from ornithine, consists of actively proliferating callus and consequently high biosynthetic activities. Glutamate will thus be in great demand. In order to keep the glutamate pool stable elsewhere in the cell, glutamate formed by the reaction of OAT catalysing the conversion of ornithine to proline, can thus contribute to the overall glutamate

pool. Therefore glutamate has to be transported out of the mitochondrion, hence the higher level of expression of a glutamate/aspartate transport protein. During the second half of the growth period, when proline is formed from glutamate, the growth rate of the callus decreased and one can envisage that no major rate of primary biosynthetic metabolism is required (Fig. 5.4.1).

However, when we look at the proline content we should not only keep in mind the synthesis of proline, but the degradation of proline is also of key importance. Degradation of proline and/or P5C leads to the production of reducing power in the form of $FADH_2$ and NAD(P)H $+H^+$ during the following enzymatic reactions:

Proline dehydrogenase (E.C.1.4.3) :
Proline + $½O_2$ + FAD $\rightarrow$ Δ^1-pyrroline-5-carboxylate + H_2O + $FADH_2$

Δ^1-pyrroline-5-carboxylate dehydrogenase (E.C.1.5.1.12)
Δ^1-pyrroline-5-carboxylate + $NAD(P)^+$ $\rightarrow$glutamate + $NAD(P)H+H^+$
(as reviewed by HARE AND CRESS, 1997).

Two distinct P5C dehydrogenases have been detected in both mitochondria of *Zea mays* and cultured cells of *Nicotiana plumbaginifolia* (ELTHON AND STEWART, 1982; FORLANI, SCAINELLI AND NIELSEN, 1997). One oxidises P5C derived from proline and the other oxidises P5C derived from ornithine (ELTHON AND STEWART, 1982). FORLANI, SCAINELLI AND NIELSEN, (1997) further demonstrated that one isoform of P5C dehydrogenase is expressed in actively proliferating cultured cells but decreased rapidly during the late logarithmic phase, while expression of the slightly less abundant isoform increased with the onset of the stationary phase (FORLANI, SCAINELLI AND NIELSEN, 1997). Thus the change in proline metabolism observed (Section 4.4) during the growth period might occur in order to correlate with the pathway of degradation. Since glutamate is produced by Δ^1-pyrroline-5-carboxylate dehydrogenase and indirectly connected to the reaction catalyzed by proline dehydrogenase one can envisage the important role of a glutamate/aspartate transporter associated with the mitochondrion (Fig.5.4.1).

The present study demonstrated that the regulation of gene expression in the habituated soybean callus is different from that observed in the non-habituated soybean callus. Total RNA concentrations of the habituated callus tissue is low in comparison to that of the non-habituated callus tissue, except for the striking increase observed on day 13 of the culture period. This increase in total RNA content of the habituated callus appears to be due to *de novo* RNA synthesis. The appearance of several polypeptides in the protein profiles of the habituated soybean callus on the same day of the growth period, corresponds to the significant increase in total RNA content at that time. The change in abundance of three polypeptides from the non-habituated callus' protein profiles further indicate the difference in gene expression that exists between the habituated and non-habituated callus. None of the above described results however, appear to be due to the higher concentation of cytokinins present, but appear to be specific for the habituated phenotype. A subtraction cDNA library was constructed and 6 diferent clones were isolated that were over-expressed in the habituated soybean callus. Only one cDNA clone has been characterized thus far and a further study of genes over-expressed in the habituated callus tissue should prove valuable to the unraveling of the habituation phenomenon.

CONCLUSION

Habituation is defined as the stable heritable loss in the requirement of cultured plant cells for one or more growth factors. The competence for habituation, like habituation itself, has an epigenetic basis. It appears as though the morphological and metabolic characteristics of habituated tissue are neither the result of hormone overproduction nor the result of a general increase of hormone responsiveness. Results from these studies also indicated that the habituated phenotype bears striking similarities to cancerous cells observed in both plants and animals.

During this study a cytokinin habituated soybean (*Glycine* max L.) callus line has been isolated. Results obtained during this study indicated that the habituated soybean callus line exhibited several metabolic and morphological characteristics that was not observed previously in any other habituated callus line investigated. However, as was observed in other habituated callus lines, the habituated soybean callus line produced very little ethylene, had an elevated level of cytokinin, the nitrogen metabolism was different from that of the non-habituated callus and there was a marked difference in gene expression between the habituated and non-habituated callus.

Besides the link between one of the over-expressed genes in the habituated callus and the difference in proline metabolism, no other conclusive link could be made between the different changes in metabolism observed in the habituated and non-habituated callus. The habituated soybean callus may thus represent an early stage of neoplasic progression to full hormone autonomy.

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APPENDIX A

All statistical analysis were done using the SAS (STAT USERS GUIDE RELEASE 6.03, 1988). Greenhouse Geisser adjusted p values were used according to RENCHER, (1995).

T-test LogGrowth (g FW)					
Group	**Time (days)**	**Mean n=4**	**S Dev**	**S Err**	**P-value**
Hab	2	0,178	0,0402	0,020	0,0437
Non-hab	2	0,106	0,039	0,194	
Hab	4	0,200	0,018	0,008	0,0421
Non-hab	4	0,236	0,022	0,011	
Hab	6	0,382	0,064	0,032	0,1023
Non-hab	6	0,490	0,090	0,045	
Hab	8	0,578	0,072	0,036	0,0832
Non-hab	8	0,488	0,33	0,017	
Hab	10	0,524	0,057	0,029	0,1191
Non-hab	10	0,586	0,030	0,015	
Hab	12	0,622	0,077	0,039	0,2501
Non-hab	12	0,686	0,063	0,031	
Hab	14	0,776	0,122	0,061	0,6722
Non-hab	14	0,740	0,100	0,05	
Hab	16	0,953	0,151	0,075	0,0504
Non-hab	16	0,722	0,044	0,022	
Hab	18	0,942	0,089	0,044	0,0354
Non-hab	18	0,740	0,116	0,058	
Hab	20	0,954	0,082	0,041	0,08157
Non-hab	20	0,943	0,011	0,005	
Hab	22	0,985	0,212	0,011	0,7158
Non-hab	22	1,031	0,111	0,056	

Hab	24	0,921	0,189	0,094	0,2841
Non-hab	24	1,072	0,173	0,086	
Hab	26	1,229	0,186	0,093	0,6342
Non-hab	26	1,154	0,236	0,118	
Hab	28	1,039	0,164	0,082	0,0431
Non-hab	28	1,299	0,081	0,041	
ANOVA Time: Greenhouse-Geisser adjusted p value =0,0001					
ANOVA Time*Group: Greenhouse Geisser adjusted p value =0,0526					
T test % Dry Weight					
Group	**Time (days)**	**Mean n=4**	**S Dev.**	**S Err**	**P-value**
Hab	2	3,703	0,367	0,184	0,0094
Non-hab	2	4.785	0,051	0,025	
Hab	4	4,483	0,176	0,088	0,2676
Non-hab	4	4,740	0,364	0,182	
Hab	6	4,813	0,329	0,165	0,8809
Non-hab	6	4,780	0,253	0,126	
Hab	8	4,495	0,205	0,103	0,0280
Non-hab	8	4,865	0,124	0,062	
Hab	10	4,635	0,198	0,099	0,2803
Non-hab	10	4,503	0,069	0,035	
Hab	12	4,805	0,219	0,109	0,0024
Non-hab	12	5,653	0,249	0,125	
Hab	14	5,030	0,289	0,144	0,0534
Non-hab	14	5,478	0,227	0,114	
Hab	16	4,99	0,597	0,298	0,4894
Non-hab	16	5,23	0,171	0,085	
Hab	18	4,885	0,143	0,072	0,3455
Non-hab	18	5,093	0,360	0,180	
Hab	20	4,680	0,220	0,113	0,0028
Non-hab	20	5,410	0,143	0,072	
Hab	22	4,438	0,160	0,080	0,0005

Non-hab	22	5,238	0,171	0,085	
Hab	24	6,855	2,719	1,360	0,1805
Non-hab	24	4,473	1,072	0,536	
Hab	26	4,635	0,693	0,340	0,0895
Non-hab	26	5,488	0,147	0,074	
Hab	28	6,368	1,264	0,632	0,1532
Non-hab	28	5,165	0,1511	0,076	
ANOVA Time p=0.0037					
ANOVATime*Group p=0.0007					
T-test Ethylene Production (nmole.g^{-1} FW. h^{-1})					
Group	**Time (days)**	**Mean n=4**	**S Dev.**	**S Err**	**P-value**
Hab	0	0,000	0,000	0,000	0,0022
Non-hab	0	26,678	5,442	2,715	
Hab	7	1,507	1,579	0,789	0,0001
Non-hab	7	56,030	4,924	2,462	
Hab	14	0,000	0,000	0,000	0,0031
Non-hab	14	39,507	5,277	1,513	
Hab	21	0,000	0,000	0,000	0,0191
Non-hab	21	3,752	1,624	0,812	
Hab	28	0,000	0,000	0,000	..
Non-hab	28	0,000	0,000	0,000	
ANOVA Time : Greenhouse-Geisser adjusted p=0,0001					
ANOVA Time*Group : Greenhouse-Geisser adjusted p=0,0001					

T test Putrescine content (µmole. g^{-1} FW)					
Group	**Time (days)**	**Mean n=4**	**S Dev**	**S Err**	**P-value**
Hab	0	0,000	0,000	0,000	0,0287
Non-hab	0	0,295	0,009	0,005	
Hab	7	0,070	0,007	0,004	0,1043
Non-hab	7	0,224	0,094	0,005	
Hab	14	0,474	0,039	0,022	0,0024
Non-hab	14	0,215	0,019	0,011	
Hab	21	0,132	0,010	0,006	0,0142
Non-hab	21	0,512	0,081	0,046	
Hab	28	1.827	0,389	0,225	0,0627
Non-hab	28	0,974	0,019	0,011	
ANOVA Time : Greenhouse-Geisser adjusted p=0,0003					
ANOVA Time*Group : Greenhouse-Geisseradjusted p=0,0060					
T-test Proline content (µmol g^{-1} Fresh Weight) Method of BERGMAN AND LOXLEY, 1970					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.318	0.0619	0.036	0.182
Non-hab	0	0.523	0.125	0.072	
Hab	7	0.526	0.115	0.066	0.5067
Non-hab	7	0.458	0.113	0.655	
Hab	14	0.458	0.096	0.056	0.2410
Non-hab	14	0.620	0.169	0.098	
Hab	21	0.386	0.089	0.052	0.0196
Non-hab	21	0.644	0.062	0.036	
Hab	28	0.707	0.084	0.048	0.8878
Non-hab	28	0.716	0.060	0.034	
ANOVA Time : Greenhouse Geisser adjusted p=0.0002					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0063					

T-test Proline content (µmol. g^{-1} Fresh Weight) Method of BATES WALDREN AND TEARE, 1973					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.286	0.121	0.070	0.577
Non-hab	0	0.334	0.038	0.022	
Hab	7	0.130	0.028	0.017	0.005
Non-hab	7	0.266	0.032	0.018	
Hab	14	0.091	0.014	0.008	0.004
Non-hab	14	0.222	0.026	0.015	
Hab	21	0.184	0.061	0.035	0.046
Non-hab	21	0.315	0.033	0.019	
Hab	28	0.137	0.034	0.019	0.205
Non-hab	28	0.192	0.051	0.030	
ANOVA Time : Greenhouse Geisser adjusted p=0.003					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.2778					
T-test Proline content (µmol. g^{-1} Fresh Weight) Gabaculine treatment					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.854	0.128	0.074	0.045
Non-hab	0	0.486	0.170	0.098	
Hab	7	0.260	0.054	0.031	0.0081
Non-hab	7	0.465	0.039	0.023	
Hab	14	0.460	0.166	0.096	0.414
Non-hab	14	0.362	0.020	0.012	
Hab	21	0.802	0.132	0.076	0.8978
Non-hab	21	0.788	0.118	0.068	
Hab	28	1.413	0.385	0.222	0.124
Non-hab	28	0.847	0.068	0.039	
ANOVA Time : Greenhouse Geisser adjusted p=0.0002					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0063					

T-test Putrescine content (µmol. g^{-1} Fresh Weight) Gabaculine treatment					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.000	0.000	0.000	0.0497
Non-hab	0	0.0136	0.005	0.003	
Hab	7	0.176	0.099	0.049	0.0178
Non-hab	7	0.408	0.076	0.044	
Hab	14	4.042	0.7098	0.355	0.0007
Non-hab	14	0.584	0.358	0.206	
Hab	21	1.695	0.114	0.057	0.2358
Non-hab	21	1.328	0.38	0.219	
Hab	28	8.312	2.280	1.14	0.0131
Non-hab	28	2.466	0.431	0.249	
ANOVA Time : Greenhouse Geisser adjusted p=0.0004					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0041					
T-test OAT activity (nmol. min^{-1}. mg^{-1} prot)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	6.335	0.740	0.370	0.0213
Non-hab	0	4.701	0.753	0.377	
Hab	7	7.075	2.485	1.242	0.945
Non-hab	7	7.176	1.202	0.601	
Hab	14	53.513	9.265	4.633	0.031
Non-hab	14	17.665	3.026	1.513	
Hab	21	21.848	2.614	1.307	0.2162
Non-hab	21	19.458	2.250	1.125	
Hab	28	26.910	14.786	7.393	0.1984
Non-hab	28	40.291	10.736	5.368	
ANOVA Time : Greenhouse Geisser adjusted p=0.0008					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0078					

T-test OAT activity (nmol. min^{-1}. mg^{-1} prot) Gabaculine treatment					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	2.754	0.896	0.448	0.6337
Non-hab	0	2.463	0.728	0.364	
Hab	7	3.983	3.807	1.904	0.1279
Non-hab	7	7.957	1.777	0.889	
Hab	14	11.480	5.701	2.850	0.9859
Non-hab	14	11.535	0.542	0.271	
Hab	21	20.265	2.648	1.324	0.4185
Non-hab	21	18.678	2.518	1.259	
Hab	28	16.861	5.442	2.7209	0.3871
Non-hab	28	19.620	0.743	0.371	
ANOVA Time : Greenhouse Geisser adjusted p=0.0002					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.3642					
NH_4 content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	1.128	0.127	0.073	0.0385
Non-hab	0	0.837	0.094	0.054	
Hab	7	1.601	0.134	0.077	0.0152
Non-hab	7	0.939	0.207	0.120	
Hab	14	0.587	0.055	0.032	0.0047
Non-hab	14	0.014	0.008	0.005	
Hab	21	0.0723	0.007	0.004	0.0011
Non-hab	21	0.122	0.007	0.004	
Hab	28	0.059	0.003	0.002	0.026
Non-hab	28	0.108	0.0145	0.008	
MANOVA Time : Greenhouse Geisser adjusted p=0.0001					
MANOVA Time*Group : Greenhouse Geisser adjusted p=0.0027					

Glutamate content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.200	0.091	0.053	0.3915
Non-hab	0	0.358	0.249	0.144	
Hab	7	0.138	0.040	0.023	0.083
Non-hab	7	0.213	0.003	0.002	
Hab	14	0.155	0.043	0.025	0.511
Non-hab	14	0.180	0.043	0.025	
Hab	21	0.228	0.024	0.014	0.5142
Non-hab	21	0.264	0.077	0.044	
Hab	28	0.272	0.119	0.069	0.8427
Non-hab	28	0.289	0.067	0.038	
ANOVA Time : Greenhouse Geisser adjusted p=0.1287					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.4328					
Arginine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.255	0.017	0.01	0.002
Non-hab	0	0.123	0.023	0.013	
Hab	7	0.217	0.038	0.022	0.4362
Non-hab	7	0.241	0.029	0.017	
Hab	14	0.256	0.023	0.013	0.1219
Non-hab	14	0.224	0.017	0.010	
Hab	21	0.244	0.016	0.009	0.139
Non-hab	21	0.171	0.054	0.031	
Hab	28	0.181	0.021	0.012	0.2339
Non-hab	28	0.247	0.069	0.040	
ANOVA Time : Greenhouse Geisser adjusted p=0.1819					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0121					

Serine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	2.073	0.508	0.293	0.1197
Non-hab	0	1.345	0.214	0.123	
Hab	7	1.551	0.134	0.077	0.0210
Non-hab	7	1.994	0.053	0.031	
Hab	14	1.567	0.103	0.059	0.6552
Non-hab	14	1.484	0.266	0.154	
Hab	21	0.244	0.016	0.009	0.139
Non-hab	21	0.177	0.054	0.031	
Hab	28	1.658	0.068	0.039	0.3755
Non-hab	28	1.533	0.187	0.108	
ANOVA Time : Greenhouse Geisser adjusted p=0.3485					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0404					
Glycine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.103	0.015	0.008	0.0207
Non-hab	0	0.048	0.004	0.002	
Hab	7	0.07	0.003	0.002	0.4200
Non-hab	7	0.284	0.368	0.213	
Hab	14	0.072	0.053	0.031	0.8792
Non-hab	14	0.077	0.009	0.005	
Hab	21	1.010	0.006	0.003	0.0005
Non-hab	21	0.046	0.004	0.002	
Hab	28	0.59	0.005	0.003	0.0057
Non-hab	28	0.032	0.002	0.001	
ANOVA Time : Greenhouse Geisser adjusted p=0.3531					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.3060					

Aspartate content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.161	0.026	0.101	0.014
Non-hab	0	0.431	0.159	0.172	
Hab	7	0.09	0.029	0.017	0.017
Non-hab	7	0.19	0.033	0.019	
Hab	14	0.127	0.051	0.030	0.0426
Non-hab	14	0.297	0.077	0.045	
Hab	21	0.165	0.041	0.024	0.064
Non-hab	21	0.340	0.091	0.053	
Hab	28	0.076	0.004	0.002	0.9136
Non-hab	28	0.078	0.028	0.016	
ANOVA Time : Greenhouse Geisser adjusted p=0.0129					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.1291					
Lysine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.231	0.086	0.049	0.1957
Non-hab	0	0.137	0.019	0.011	
Hab	7	0.084	0.005	0.003	0.0121
Non-hab	7	0.115	0.009	0.005	
Hab	14	0.241	0.039	0.022	0.0724
Non-hab	14	0.341	0.0717	0.041	
Hab	21	0.319	0.024	0.014	0.0961
Non-hab	21	0.392	0.047	0.027	
Hab	28	0.328	0.019	0.011	0.1148
Non-hab	28	0.427	0.066	0.039	
ANOVA Time : p=0.0001					
ANOVA Time*Group : p=0.0040					

Methionine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.098	0.015	0.009	0.27
Non-hab	0	0.062	0.040	0.023	
Hab	7	0.060	0.026	0.015	0.1369
Non-hab	7	0.026	0.011	0.007	
Hab	14	0.035	0.017	0.010	0.1897
Non-hab	14	0.060	0.022	0.013	
Hab	21	0.080	0.040	0.023	0.9803
Non-hab	21	0.079	0.013	0.008	
Hab	28	0.076	0.004	0.002	0.9136
Non-hab	28	0.078	0.028	0.016	
ANOVA Time : p=0.0147					
ANOVA Time*Group : p=0.1211					
Threonine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.357	0.040	0.023	0.0027
Non-hab	0	0.129	0.025	0.014	
Hab	7	0.108	0.012	0.007	0.0341
Non-hab	7	0.138	0.006	0.003	
Hab	14	0.153	0.0078	0.005	0.025
Non-hab	14	0.229	0.024	0.0137	
Hab	21	0.212	0.022	0.013	0.0602
Non-hab	21	0.165	0.006	0.004	
Hab	28	0.234	0.007	0.004	0.1757
Non-hab	28	0.226	0.002	0.001	
ANOVA Time : Greenhouse Geisser adjusted p=0.0003					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0001					

Isoleucine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.432	0.015	0.009	0.363
Non-hab	0	0.279	0.055	0.032	
Hab	7	0.125	0.021	0.012	0.0854
Non-hab	7	0.160	0.008	0.005	
Hab	14	0.125	0.024	0.104	0.0041
Non-hab	14	0.236	0.022	0.013	
Hab	21	0.251	0.040	0.023	0.0698
Non-hab	21	0.327	0.015	0.009	
Hab	28	0.336	0.009	0.005	0.0053
Non-hab	28	0.219	0.021	0.012	
ANOVA Time : Greenhouse Geisser adjusted p=0.0001					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0004					
Leucine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.177	0.024	0.014	0.0072
Non-hab	0	0.079	0.015	0.009	
Hab	7	0.139	0.025	0.014	0.1716
Non-hab	7	0.110	0.006	0.003	
Hab	14	0.144	0.012	0.007	0.0269
Non-hab	14	0.181	0.003	0.002	
Hab	21	0.235	0.045	0.026	0.2771
Non-hab	21	0.196	0.020	0.012	
Hab	28	0.180	0.010	0.006	0.0079
Non-hab	28	0.135	0.005	0.003	
ANOVA Time : Greenhouse Geisser adjusted p=0.0068					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0372					

Alanine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	1.070	0.493	0.284	0.9907
Non-hab	0	1.075	0.423	0.244	
Hab	7	0.576	0.048	0.027	0.0552
Non-hab	7	0.676	0.043	0.025	
Hab	14	1.226	0.140	0.081	0.0262
Non-hab	14	0.743	0.019	0.011	
Hab	21	0.749	0.028	0.016	0.2108
Non-hab	21	0.945	0.187	0.108	
Hab	28	1.124	0.047	0.027	0.0005
Non-hab	28	0.702	0.035	0.02	
ANOVA Time : Greenhouse Geisser adjusted p=0.1010					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.1255					
Valine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.611	0.049	0.029	0.0008
Non-hab	0	0.230	0.036	0.021	
Hab	7	0.226	0.015	0.009	0.0192
Non-hab	7	0.272	0.008	0.004	
Hab	14	0.341	0.035	0.020	0.0415
Non-hab	14	0.421	0.032	0.018	
Hab	21	0.436	0.102	0.059	0.2040
Non-hab	21	0.326	0.018	0.010	
Hab	28	0.437	0.015	0.009	0.1610
Non-hab	28	0.383	0.044	0.026	
ANOVA Time : Greenhouse Geisser adjusted p=0.0058					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0012					

Tyrosine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.064	0.007	0.004	0.0035
Non-hab	0	0.031	0.006	0.004	
Hab	7	0.111	0.006	0.003	0.2839
Non-hab	7	0.117	0.007	0.004	
Hab	14	0.086	0.100	0.006	0.0405
Non-hab	14	0.060	0.003	0.001	
Hab	21	0.080	0.010	0.006	0.0071
Non-hab	21	0.129	0.012	0.007	
Hab	28	0.081	0.008	0.005	0.0403
Non-hab	28	0.106	0.011	0.007	
ANOVA Time : Greenhouse Geisser adjusted p=0.0001					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0003					
Phenylalanine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.310	0.047	0.027	0.0108
Non-hab	0	0.101	0.018	0.010	
Hab	7	0.130	0.026	0.015	0.2796
Non-hab	7	0.152	0.008	0.005	
Hab	14	0.121	0.014	0.008	0.0116
Non-hab	14	0.187	0.005	0.003	
Hab	21	0.254	0.030	0.017	0.0893
Non-hab	21	0.203	0.009	0.005	
Hab	28	0.195	0.012	0.007	0.5617
Non-hab	28	0.214	0.047	0.027	
ANOVA Time : p=0.0001					
ANOVA Time*Group : p=0.0001					

Histidine content (µmol. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	0.920	0.049	0.029	0.1136
Non-hab	0	0.496	0.274	0.158	
Hab	7	0.690	0.217	0.126	0.2743
Non-hab	7	0.458	0.231	0.133	
Hab	14	0.593	0.142	0.082	0.2608
Non-hab	14	0.455	0.111	0.064	
Hab	21	0.694	0.093	0.054	0.1475
Non-hab	21	0.502	0.149	0.086	
Hab	28	0.680	0.091	0.052	0.0091
Non-hab	28	0.339	0.060	0.035	
ANOVA Time : p=0.0840					
ANOVA Time*Group : p=0.2928					
Total RNA content (mg. g^{-1} Fresh Weight)					
Group	**Time (days)**	**Mean n=3**	**S Dev**	**S Err**	**P-Value**
Hab	0	1.288	0.251	0.145	0.0232
Non-hab	0	3.273	0.630	0.364	
Hab	7	10646	0.266	0.154	0.0443
Non-hab	7	7.590	2.265	1.308	
Hab	14	18.756	4.855	2.803	0.0273
Non-hab	14	5.631	2.361	1.363	
Hab	21	3.893	2.277	1.314	0.0669
Non-hab	21	12.173	4.499	2.597	
Hab	28	0.737	0.121	0.070	0.1251
Non-hab	28	5.061	2.933	1.694	
ANOVA Time : Greenhouse Geisser adjusted p=0.0003					
ANOVA Time*Group : Greenhouse Geisser adjusted p=0.0002					

CURRICULUM VITAE

Sandra du Plessis was born on August 25th 1967 in Kimberley, South Africa. She matriculated in 1985 at the Diamantveld High School in Kimberley.

In 1988 she obtained a B.Sc. degree *cum laude* at the University of the Orange Free State, Bloemfontein, South Africa, majoring in Botany and Microbiology. She obtained both her B.Sc. Honn. and M.Sc. degrees *cum laude* in 1989 and 1991, respectively, at the University of the Orange Free State, Bloemfontein, South Africa. During 1992 she took up a position as research assistant at the Department of Botany, University of Natal, Pietermaritzburg, South Africa and enrolled for a Ph.D. degree in Botany at the same university, during 1993.

During 1996 she was appointed as lecturer at the Department of Botany and Soil Sciences, Potchefstroom University for Christian Higher Education, Potchefstroom, South Africa. She has already presented several papers and posters at national and international conferences. She is a member of the South African Association of Botanists and the South African Academy for Science and Art.