Addendum

Both examiners have suggested that this thesis be passed and the degree of Doctor of Philosophy be awarded without further examination. They provided several constructive comments regarding the present study for which I am grateful, and to that end, I address the main points raised as follows:-

- pg. 1, line 12: amino propyl is one word.
- pg. 2: SAMDC does not compete only with the ethylene pathway for *S*-adenosylmethionine. The main use of *S*-adenosylmethionine in all eukaryotes is in *trans*-methylation reactions.
- The cloning of the *Arabidopsis* spermine synthase gene should also have been noted in the list of cloned *Arabidopsis* genes at the bottom of page 2. The relevant published reference is as follows:

  HANZAWA, Y., TAKAHASHI, T., MICHAEL, A.J., BURTIN, D., LONG, D., PINEIRO, M., COUPLAND, G. AND KOMEDA, Y. (2000) "*ACAULIS 5*, an *Arabidopsis* gene required for stem elongation, encodes a spermine synthase." EMBO Journal 19:4248-4256.

- pg. 3, line 4: The head-to-tail nature of the *Arabidopsis* arginine decarboxylase was determined in yeast, not *Arabidopsis*.
- pg. 10, line 17: Polyamines can conjugate with acetyl-CoA, not acetic acid.
- pg. 35, line 3: Change the first 'a' to 'α' in the full names of both DFMA and DFMO.
- pg. 57, line 18; pg. 157, line 3; pg. 247 line 8: Although it has been asserted in this thesis and by other researchers (Del Duca *et al.*, 1995; Tassoni *et al.*, 2000) that a polyamine retroconversion pathway may exist in plants, there is still no unequivocal evidence for such a phenomenon. Instead, the increase in putrescine levels following treatment with spermidine is likely to result from inhibition of SAMDC by spermidine, which causes an accumulation of putrescine due to a lack of an aminopropyl donor.
- pg. 60, line 9: Although most plant *ADC* cDNAs are approximately 2·7kb to 3·0kb in length, the *ADC* transcript in the present study was found to be 1·2kb in length. This is in agreement with similar findings by Soyka and Heyer (1999) also working with *Arabidopsis*. *Arabidopsis* is known to have more than one *ADC* gene (Galloway *et al.*, 1998; Watson *et al.*, 1998; Soyka and Heyer, 1999) and it is possible that a shorter version of the gene is responsible for the shorter transcript. Whether this transcript is capable of being translated to produce a functional ADC enzyme remains to be determined.
- pg. 62, line 21: CHA inhibits the enzyme SPDS, thus the anticipated response of the plant to such inhibition may be to increase levels of SPDS enzyme as an attempt to overcome the inhibitory effects of CHA. Therefore, a reduction in levels of SPDS transcript may not be expected following such treatment.
- pg. 96, line 8: Most of the competition for SAM in plant cells involved *trans*-methylase enzymes and not between the polyamine and ethylene biosynthetic pathways. Furthermore, ethylene biosynthesis requires SAM and not decarboxylated SAM.
- pg. 134, line 16: The expected size of the ODC transcript in tobacco is approximately 1·6kb, not 1·3kb as indicated.
- pg. 175: That putrescine application increases the accumulation of SPDS mRNA but not spermidine levels, in *Arabidopsis*, is likely to be due to the rate-limiting nature of SAMDC, not SPDS, for spermidine biosynthesis.
- pg. 181, line 3: The listed positions of SPDS and SAMDC should be reversed.
- pgs. 188, 236-240: References to the published paper by Fritze *et al.* (1995) should be regarded as unreliable, as all papers from that laboratory which used protoplast division assays were subsequently retracted by Prof. Jeff Schell.

[redacted]

Eugene B. Fredericks
8th August 2002

H24/3252

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 6 September 2002 .....
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See. Research Graduate School Committee

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from this thesis.

"Studies involving alterations of polyamine metabolism in *Arabidopsis thaliana*."

A thesis submitted for the degree
of Doctor of Philosophy

December 2001

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Declaration

I declare that this thesis contains no material which has been accepted for the award of any other Degree or Diploma in any university or institute. To the best of my knowledge this thesis contains no material previously published or written by another person, except where due reference is made in the text.

Eugene B. Fredericks

Acknowledgements

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" Facts are the air of scientists.
Without them, we could never fly."
- LINUS PAULING

List of Abbreviations:

ADC	arginine decarboxylase (E. C. 4.1.1.17)
CHA	cyclohexylamine
CHO	Chinese hamster ovary
DAPDC	diaminopimelate decarboxylase
DFMA	DL-α-difluoromethylornithine
DFMO	DL-α-difluoromethylarginine
EST	expressed sequence tag
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
MGBG	methylglyoxal bis-(guanylhydrazone)
NAA	napthalene acetic acid
ODC	ornithine decarboxylase (E. C. 4.1.1.19)
ORF	open reading frame
PA	polyamine
PLP	pyridoxal 5′-phosphate
PUT	putrescine
RACE	rapid amplification of cDNA ends
Ri T-DNA	root-inducing transferred DNA
rol	root locus
SAM	*S*-adenosylmethionine
SAMDC	*S*-adenosylmethionine decarboxylase (E. C. 4.1.1.50)
SPD	spermidine
SPDS	spermidine synthase (E. C. 2.5.1.16)
SPM	spermine
SPMS	spermine synthase (E. C. 2.5.1.22)
STS	silver thiosulphate
T-DNA	transferred DNA

Table of contents

Abstract

Three approaches were used in this study to examine the roles of the polyamines putrescine, spermidine, and spermine, and the regulation of their synthesis, during growth of *Arabidopsis*.

In the first approach, plant tissues were treated with polyamines, as well as chemical inhibitors of key polyamine biosynthetic enzymes, in order to assess effects on overall phenotype. It was noted that addition of polyamines moderately inhibited production of lateral and adventitious roots when added in conjunction with auxin. When added alone however, they had no effect on root growth. Treatments with various inhibitors depressed shoot and root growth, although all inhibitors promoted early initiation of lateral roots relative to untreated controls. Transcript levels of the genes *ADC*, *SAMDC*, and *SPDS* increased or decreased according to specific treatment.

In the second approach, *Arabidopsis* was transformed with genes from the Ri T-DNA of the bacterial plant pathogen *Agrobacterium rhizogenes*, resulting in marked alterations in phenotype analogous to polyamine inhibitor treatment. It has been suggested in the literature that the altered phenotype of similarly-transformed tobacco plants containing the the Ri T-DNA, is due to reduced *in vivo* titres of polyamines. This hypothesis was supported by observations that *Arabidopsis* transformants produced in this study also exhibited reduced levels of polyamines. Exogenous polyamines did not ameliorate the transformed phenotype however, despite the restoration of normal endogenous polyamine titres. Subsequent experiments suggested that factors such as alterations in auxin sensitivity or perception rather than polyamine levels *per se*, are causal to the altered morphology in *Arabidopsis* due to the Ri T-DNA.

Thirdly, in a more direct attempt to study specific alterations in polyamine metabolism mutant lines of *Arabidopsis* resistant to high levels of the inhibitor MGBG were isolated. Genetic analysis suggested that the resistant phenotype segregated as a recessive trait, though some variability in penetrance was observed in offspring. Further characterisation of the mutants suggested that resistance may be due to the high titres of free, conjugated, and bound spermidine detected in these plants.

CHAPTER 1:

GENERAL INTRODUCTION

1·1 BIOSYNTHESIS OF POLYAMINES IN PLANTS

POLYAMINES ARE SMALL, positively charged molecules that are present in all organisms. In plant cells the major polyamines, putrescine, spermidine, and spermine can be found in three forms; (i) as free molecules, (ii) conjugated to hydroxycinnamic acids, or (iii) bound to macromolecules. They have been implicated to play important roles in an array of growth and developmental processes (Galston, 1983; Tabor and Tabor, 1984; Evans and Malmberg, 1989). The synthesis of the diamine putrescine in plants can be via the decarboxylation of either arginine or ornithine by the action of arginine decarboxylase (ADC) or ornithine decarboxylase (ODC), with arginine being first converted by ADC to the intermediate agmatine in this process (Figure 1·1). The decarboxylation of *S*-adenosylmethionine (SAM) by *S*-adenosylmethionine decarboxylase (SAMDC) generates an aminopropyl group which is added to putrescine to produce spermidine via spermidine synthase (SPDS). The subsequent addition of an amino propyl group to spermidine by spermine synthase (SPMS) results in the

production of spermine. SAMDC is recognised as an important enzyme for polyamine biosynthesis as it competes for available SAM with the ethylene biosynthesis pathway.

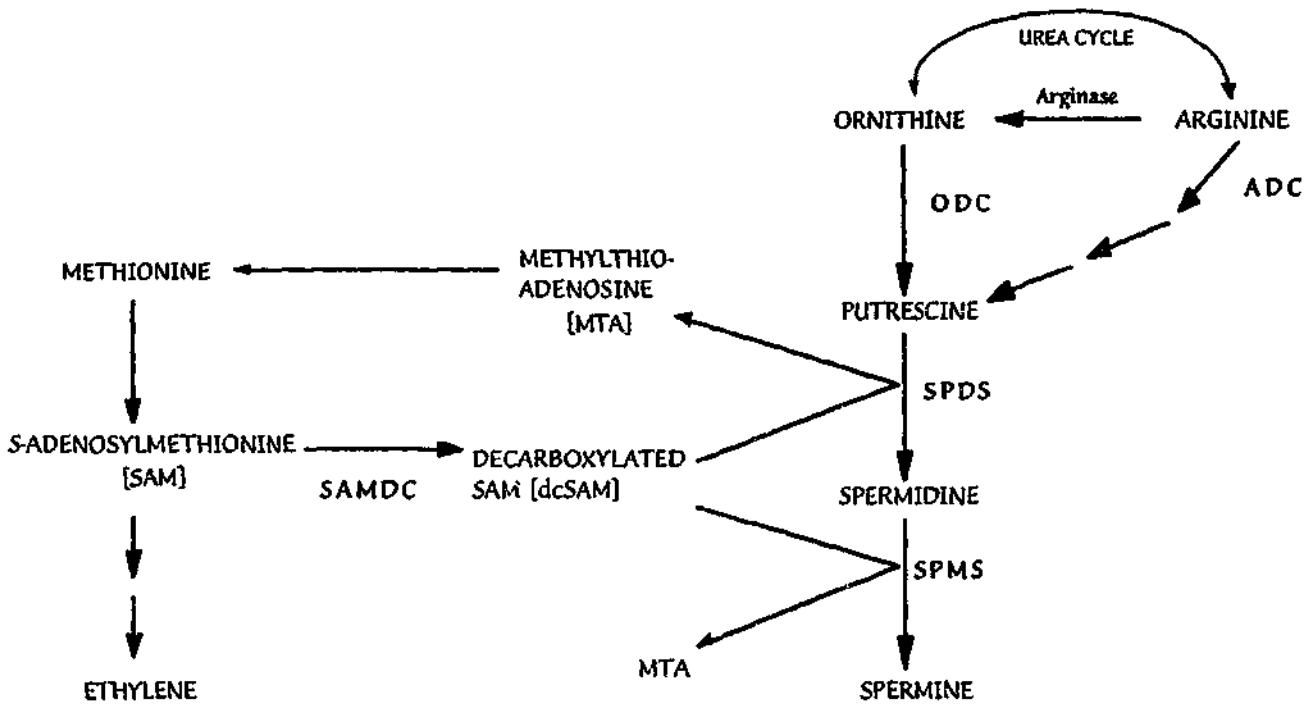


Figure 1·1: The biosynthetic pathway of polyamines in plants

The polyamines putrescine, spermidine, and spermine are produced from ornithine or arginine via the respective enzymes ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). Arginine can undergo conversion to ornithine via arginase. *S*-adenosylmethionine decarboxylase (SAMDC) catalyses the conversion of *S*-adenosylmethionine (SAM) to decarboxylated *S*-adenosylmethionine (dcSAM), which donates an aminopropyl moiety for the production of Spd from Put via spermidine synthase (SPDS), and also for the production of Spm from Spd via spermine synthase (SPMS).

Arabidopsis cDNA sequences representing *ADC* (Watson and Malmberg, 1996), *SPDS* (Hashimoto *et al.*, 1998), and *SAMDC* (Franceschetti *et al.*, 2001), and have recently been cloned, whereas *ODC* has not been isolated and is suggested to be absent from *Arabidopsis* (Hanfrey *et al.*, 2001). It is proposed that the *Arabidopsis* genome has evolved from an ancestral duplication of the whole genome, followed by extensive localised areas of further gene duplication (The *Arabidopsis* Initiative, 2000). This may account for the identification of two active copies of *ADC* in *Arabidopsis* (Watson *et al.*, 1997) and six genes for *SAMDC*, although four of which appear to be defective and not expressed (Franceschetti *et al.*, 2001). Expression of *ADC* is suggested to be regulated by translational and/or post-translational

mechanisms, due to the lack of correlation between ADC activity and mRNA levels (Pérez-Amador *et al.*, 1995; Borrell *et al.*, 1996). One of the two *ADC* genes in *Arabidopsis* has been shown to be inducible by osmotic stress (Soyka and Heyer, 1999). From studies in *Arabidopsis* it is suggested that *ADC* is likely to be a head-to-tail homodimer which is formed before processing (Hanfrey *et al.*, 2001). In animal systems, *ODC* is reported to undergo regulation at both the transcriptional and translational levels (Porter and Bergeron, 1988) and is responsive to feedback regulation by endogenous polyamine levels (Heby and Persson, 1990), which has also been noted in plants (Slocum, 1991). The cloning of *ODC* from *Datura* found that the long 3′ untranslated region involved in rapid turnover of the mammalian enzyme is absent from the plant gene (Michael *et al.*, 1996). Analagous to *ODC*, *SAMDC* was found to have a long leader sequence which may be involved in [illegible] *et al.*, 1996). In plant *SAMDC* genes, a short conserved open reading [illegible] identified in the long 5′ untranslated leader sequence (Mad Arif *et al.*, 1994; [illegible] and Schröder, 1995). It was subsequently demonstrated that there are in fact two highly conserved, overlapping upstream open reading frames within the 5′ leader sequences, which are suggested to be involved in translational regulation (Franceschetti *et al.*, 2001). Spermidine synthase has been suggested to undergo post-translational modification (Hashimoto *et al.*, 1998) or possibly exist within plant cells as a stable multienzyme complex (Bagga *et al.*, 1997). The latter proposal was based on *in vitro* activity leading to the production of spermine and other minor polyamines, in conjunction with spermidine from putrescine (Bagga *et al.*, 1997). The regulation of the key poyamine biosynthetic genes will be discussed in further chapters of this study.

1·2 LOCALISATION OF POLYAMINES AND POLYAMINE BIOSYNTHETIC ENZYMES

While polyamines are synthesised within the cytoplasm, they have also been shown to be localised to several organelles within plant cells (Colombo *et al.*, 1992). Localisation of polyamines to both the mitochondria and vacuole (Colombo *et al.*, 1992) has been reported

in *Arabidopsis*, carrot (Pistocchi *et al.*, 1988) and *Neurospora crassa* (Davis and Ristow, 1991). In addition, two *N. crassa* mutants, *spe3* and *puu1*, which accumulate very high levels of putrescine, both sequester large excesses of putrescine in vacuoles (Davis and Ristow, 1991). It is suggested that localisation of polyamines to the vacuole may act as a storage mechanism (Colombo *et al.*, 1992).

Localisation of polyamines to extracellular spaces has been suggested to be important for the initiation of their long distance transport in plants, or their metabolism by diamine or polyamine oxidases (Colombo *et al.*, 1992). It has been reported that free and conjugated forms of polyamines undergo differential localisation in tobacco (Altamura *et al.*, 1993), and differential transport in *Ricinus communis* (Antognoni *et al.*, 1998). In the latter plant, free polyamines are present in the phloem sap, whereas conjugated forms are sequestered in vacuoles and therefore unable to enter the sieve tube cytosolic fluid.

Gradients of polyamine levels exist within plants, and high titres are found in dividing tissues and developing organs (Altamura *et al.*, 1993). Higher titres have been found in actively growing plant tissues including carrot somatic embryos (Feirer *et al.*, 1984), tobacco thin cell layers (Torrigiani *et al.*, 1987A), cycling cells of *Helianthus tuberosus* (Torrigiani *et al.*, 1987B), *Chrysanthemum* plants during flower initiation (Aribaud and Martin-Tanguy, 1994), potato during tuberisation (Mader, 1995), embryogenic tobacco pollen (Garrido *et al.*, 1995), and differentiating maize callus (Bernet *et al.*, 1998). In addition, treatment with auxin or cytokinin stimulated organogenesis and also increased polyamine accumulation in eggplant cotyledon explants (Scoccianti *et al.*, 2000). A comparable situation has also been observed in mammalian tissues, with several reports demonstrating increased uptake and elevated levels of polyamines in rapidly dividing cancer cells (Moulinoux *et al.*, 1984; Moulinoux *et al.*, 1989; Seemama *et al.*, 1989; Nicolet *et al.*, 1990; Moulinoux *et al.*, 1991; Khan *et al.*, 1994).

In plants, gradients of polyamine levels appear to be the result of activities of polyamine biosynthetic enzymes in various tissues. High activities of ADC and ODC, have been

reported, for example, in growing cells of tobacco (Altamura *et al.*, 1993), and cotyledons of *R. communis* (Antognoni *et al.*, 1998). ODC, ADC, and SAMDC, activities have also been specifically localised to chloroplasts in *Pinus radiata* and mitochondria of *Helianthus tuberosus* (Torrigiani *et al.*, 1985). The authors also noted differences in the activities of each of these enzymes in the different organelles—a factor suggested to reflect the compartmentalisation of the enzymes (Torrigiani *et al.*, 1985). In *Zea mays*, ODC was localised mainly in meristematic zones due to relatively high specific activity in primary and lateral roots (Schwartz *et al.*, 1986). In oat, ADC was localised to the thylakoid membrane of chloroplasts through the use of ADC-specific antibodies (Borrel *et al.*, 1995). The authors suggest that localisation of the ADC enzyme to the chloroplasts may explain why animals do not posses ADC, and hence the ability of plants to use both ODC- and ADC-mediated pathways for putrescine production (Borrel *et al.*, 1995). Activity of SPDS was found to be high in meristematic shoot tip and floral bud tissues in alfalfa, suggesting that polyamines may be involved in cell division (Bagga *et al.*, 1997).

The localisation of enzymes involved in polyamine degradation, including acetylputrescine oxidase, acetamidoaldehyde dehydrogenase A, and acetamidoaldehyde dehydrogenase B, and acetamidoalkanoate deacetylase, have also been found within the peroxisome, mitochondria, and cytosol respectively in the yeast *Candida boindini* (Gillyon *et al.*, 1987). Amine oxidases have also been localised to cell walls in tomato, pea, (D'Orazi and Bagni, 1987), and maize tissues (Slocum and Furey, 1991), and also found in lateral root cap cells, vascular tissues of roots, developing leaves, the hypocotyl, style and stigmatal tissue of *Arabidopsis* (Moller and McPherson, 1998).

1·3 UPTAKE AND TRANSPORT OF POLYAMINES

It is believed that regulation of polyamine levels in mammalian, yeast, and bacterial cells involves an interplay between biosynthesis and uptake and/or transport of polyamines to

various tissues and cell types (Alhonen-Hongisto *et al.*, 1980; Byers and Pegg, 1990; Grillo and Colombatto, 1994).

It has been proposed that at low polyamine concentrations, uptake may be carrier-mediated (Di Tomaso *et al.*, 1992A; Di Tomaso *et al.*, 1992B; Antognoni *et al.*, 1994), whereas at high concentrations, simple diffusion may be the main transport process (Antognoni *et al.*, 1994). Radio-labelled polyamines have been widely used to study cellular polyamine transport. Using carrot protoplasts, such an approach demonstrated that while cell surface binding is important for polyamine uptake, transport does occur across the plasmalemma and tonoplasts (Pistocchi *et al.*, 1988). The existence of a linear polyamine concentration gradient across the plasmalemma and observations that uptake is pH-dependent and saturable at low polyamine concentrations, further suggested that a carrier-mediated process is responsible for polyamine transport into organelles (Pistocchi *et al.*, 1988). In addition, polyamine transport is suggested to be both energy- and temperature-dependent, and in the presence of excess polyamines, is saturable in mammalian cells (reviewed in Seiler and Dezeure, 1989; Nicolet *et al.*, 1990). An analogous situation was also noted in cultured apple inflorescences, in which polyamine uptake was found to be rapid, non-polar, and dependent on both temperature and relative humidity (Pistocchi and Bagni, 1991).

1·3·1 POLYAMINE TRANSPORT SYSTEMS

Several different polyamine transport systems have been found in a range of species. In *E. coli*, for example, three putrescine transport systems exist; one of which is also involved in spermidine transport, and another involved in putrescine excretion (Kashiwagi *et al.*, 1991; Furuchi *et al.*, 1991; Pistocchi *et al.*, 1993). A specific polyamine transport system has also been identified in the fungus *N. crassa* (Davis and Ristow, 1988) and in the yeast *Saccharomyces cerevisiae* (Kakinuma *et al.*, 1992). In mammalian tissues, several transporters specific to cell-type have been discovered (Kumagai and Johnson, 1988; Byers and Pegg, 1989; De Smedt *et al.*, 1989; Kumagai *et al.*, 1989; Seiler and Dezeure, 1989). Interestingly, in most cultured mammalian cells putrescine, spermidine, and spermine are

often taken up from nutrient media via activity of one transport system (reviewed in Seiler and Dezeure, 1989). There are exceptions in which more than one transport system has been identified, however, with each having different affinities for different polyamines (reviewed in Bagni and Pistocchi, 1991). In a rat tumour cell line, for example, two different transporters of putrescine and spermidine exist, one of which was specific for the aminopropyl groups found on spermine and spermidine (Nicolet *et al.*, 1990). In addition, MGBG, a structural analogue of spermidine, has been suggested to share the same uptake system as spermidine in animals (Alhonen-Hongisto *et al.*, 1980; Porter and Sufrin, 1986) and plants (Tassoni *et al.*, 1996), although at low concentrations of MGBG, separate transport systems may operate in plants (Antognoni *et al.*, 1999). There is also evidence, for a lack of specificity in polyamine-transporting systems. Unrelated triamines with chain lengths similar to those of spermidine and spermine have been shown to effectively inhibit the transport of polyamines in a L1210 leukaemia cell line (Porter *et al.*, 1984). Mammalian Chinese Hamster Ovary (CHO) cells lacking a functional polyamine transport system retain the capacity to take up bis(benzyl) polyamine analogues, further demonstrating variation in specificity of polyamine transport (Byers *et al.*, 1990).

It was initially believed that putrescine may pass through inward rectifying K^+ channels of the plasma membrane (Colombo and Cerana, 1991). Subsequent studies investigating the presence of polyamine transport channels across both the plasma membrane and tonoplast in *Arabidopsis* protoplasts and vacuoles found that slow-developing voltage dependent currents are detected when polyamines are present (Colombo *et al.*, 1992). This suggests that ion channels, selective for polyamines, may exist in *Arabidopsis* cells (Colombo *et al.*, 1992).

Interestingly, K^+ is found to stimulate spermidine-binding to the plasma membrane of zucchini hypocotyl cells (Tassoni *et al.*, 1996). Recently, it has been demonstrated that elevated exogenous $[K^+]$ can also induce the excretion of putrescine from the cytoplasm of barley seedling roots into the external media (Tamai, *et al.*, 2000).

Transport of polyamines in animal cells in culture was found to be important in regulating intracellular polyamine concentrations, following observations that the transport pathway is sensitive to changes in polyamine content of cells (Byers and Pegg, 1990; Grillo and Colombato, 1994). DFMO treatment of CHO cells. or polyamine starvation of CHO cells lacking an active ODC, for example, were found to not only decrease polyamine titres, but also increase the transport of polyamines into such cells from surrounding media (Alhonen-Hongisto *et al.*, 1980; Byers and Pegg, 1990). Conversely, polyamine-feeding to CHO cells produced a decrease in polyamine transport, such that polyamine titres were found to be similar to levels noted in untreated controls (Byers and Pegg, 1990). These results suggest that regulation of polyamine transport is rapid, and sensitive to changes in intracellular polyamine titres. It has been proposed that the capacity for a polyamine transport system is an adaptational response of cells to changes in polyamine requirements (Seiler and Dezeure, 1989). It has been demonstrated, for example, that the soil bacterium, *Azospirillum brasilense*, increases polyamine uptake from nutrient media following exposure to 2,4,-Dichlorophenoxyacetic acid (Mori *et al.*, 1995) in order to prevent toxic effects of the treatment by restoring normal intracellular polyamine concentrations.

Treatment of CHO cells with a protein synthesis inhibitor, cycloheximide, demonstrated that while protein synthesis was required to upregulate transport of polyamines in response to polyamine depletion, it was not necessary for the process of down-regulation of polyamine transport (Byers and Pegg, 1989). Control of cellular polyamine titres in mammalian cells may involve an active outward transport system (Seiler, 1987). In addition, in mammalian cells it is reported that regulation of polyamine uptake may be via an inhibitory protein, similar to antizyme, which regulates ODC. Indeed, enhancement of polyamine transport in several different cell types is suggested to be linked to the stimulation of this inhibitory protein by polyamines themselves (Murakami *et al.*, 1992; Mitchell *et al.*, 1994). Furthermore, as the *puu1* mutant of *N. crassa* concentrates putrescine from the growth media due to reduced Ca^{2+} sensitivity of the polyamine transport system, the suggestion has been made that putrescine uptake is normally regulated by a Ca^{2+}-binding protein that restricts polyamine uptake (Davis *et al.*, 1990).

1·3·2 LONG DISTANCE TRANSPORT OF POLYAMINES IN PLANTS

In plants, free polyamines are suggested to be capable of long distance transport and this is supported by observations that the diamines putrescine and cadaverine readily migrate in phloem in rice (Yokota *et al.*, 1994). The polyamines, spermidine and spermine, are more restricted in their movement however, suggesting that the mobility of these molecules is inversely proportional to the number of amino groups (Yokota *et al.*, 1994). Increased polyamine titres are also noted in the phloem of *R. communis* (Antognoni *et al.*, 1998) and also *Sinapis alba* (wild mustard) following floral induction (Havelange *et al.*, 1996). In addition, a delay in flowering of *S. alba* caused by the typical application of DFMO correlates with a decrease in putrescine levels in leaf exudates (Havelange *et al.*, 1996).

1·3·3 BINDING OF POLYAMINES TO CELLULAR COMPONENTS

Subcellular distribution and uptake of polyamines in plants is suggested to involve at least two overlapping process; electrostatic interactions of positively-charged polyamines with negatively-charged cell wall components, and active uptake and transport of polyamines across the plasmalemma of cells (Pistocchi *et al.*, 1988). The capacity of polyamines to bind to various negatively-charged cellular components, such as nucleic acids and phospholipids, has been well documented (reviewed in Bagni and Pistocchi, 1991). In tomato cells polyamines bind to negatively-charged pectin substances in the cell wall (D'Orazi and Bagni, 1987). Furthermore, in tomato and carrot cells, polyamines bind to membrane phospholipids and compete with Ca^{2+} and other cations, for the same anionic sites on membranes (D'Orazi and Bagni, 1987; Pistocchi *et al.*, 1988). Interestingly, uptake into carrot cell cultures is shown to be not only dependent on pH, but also stimulated by elevated $[Ca^{2+}]$ (Pistocchi and Bagni, 1991). In *N. crassa*, virtually all spermidine found in growing cells is sequestered to internal anionic binding sites, with excess, unbound spermidine excreted from cells (Davis and Ristow, 1989). It is suggested that, as the regulation of enzyme levels and activity is a relatively slow process, there is some advantage in

maintaining polyamine pool size via intracellular binding and excretion (Davis and Ristow, 1989). Excretion of polyamines is also thought to be an important regulatory step in maintaining polyamine titres in mammalian cells, despite the fact that eukaryotic cells release only small amounts of these molecules. While the release of polyamines is not thought to be energy-dependent, changes in both [Na^+] and [Ca^{2+}] are thought to be involved in regulating this process (Grillo and Colombatto, 1994).

The presence of primary amino groups within polyamines are suggested to be of critical importance for uptake to occur (Porter *et al.*, 1982; Porter and Sufrin, 1986; Heston *et al.*, 1987; Chang *et al.*, 1988). Hence, the recognition of polyamines by a transport system may be based on the presence of negative charges on cell membranes, spaced at distances corresponding to those of the positively-charged nitrogen atoms of polyamines (reviewed in Seiler and Dezeure, 1989). Studies with spermidine analogues showed that the primary amino groups of spermidine at positions 1 and 8 in particular, are found to be critical for binding specifically to membrane proteins (Tassoni *et al.*, 1996; Tassoni *et al.*, 1998). Furthermore, the four nitrogen atoms of spermine impart a stronger capacity for binding to plasma membrane proteins in zucchini, than the triamine spermidine (Tassoni *et al.*, 1996)

1·4 EFFECTS OF POLYAMINE BINDING IN MEDIATING CELLULAR PROCESSES

In animal cells, polyamines can conjugate with acetic acid to form acetyl derivatives, whereas in plants, polyamines can covalently bind to hydroxycinnamic acids such as ferulic, *p*-coumaric, and caffeic acids (Martin-Tanguy, 1997). Such conjugates occur at high levels in plants, and in some species, they are the predominant form of polyamine (Slocum and Galston, 1985; Tiburcio *et al.*, 1987; Burtin *et al.*, 1990; Aribaud and Martin-Tanguy, 1994B; Michael *et al.*, 1996). The exact roles of conjugated polyamines are unclear, however, they have been correlated with developmental processes including floral initiation (Aribaud and Martin-Tanguy, 1994A; Tarenghi and Martin-Tanguy, 1995; Havelange *et al.*,

1996), germination (Bonneau *et al.*, 1994B), and root formation (Burtin *et al.*, 1990), and are also proposed to be indicators of stress in plants (Campos *et al.*, 1991; Scaramagli *et al.*, 1999A). Conjugated polyamines will be further discussed in Chapters 4 and 5 of the present study.

The capacity of polyamines to bind to several different cellular components, such as nucleic acids, phospholipids, and other negatively-charged proteins residing in cell membranes, as well as pectin in cell walls, may have biological significance (Kashiwagi *et al.*, 1986; D'Orazi and Bagni, 1987; Mizrahi *et al.*, 1989). The binding of polyamines to nucleic acids, for example, is thought to increase stability of mRNA thereby improving translation and protein synthesis (Cocucci and Bagni, 1968; Quigley *et al.*, 1978). In addition, the interaction of polyamines with cell membranes is thought to be an intermediate step in cellular events in animal cells such as aggregation and fusion of liposomes (Schuber *et al.*, 1983), or regulation of Ca^{2+} fluxes in membrane responses (Koenig *et al.*, 1983). A role for polyamines in influencing signal transduction mechanisms is also noted in cultured tobacco, cucumber, and *Arabidopsis* cells, in which addition of polyamines have both marked positive and negative effects on protein phosphorylation at concentrations corresponding to endogenous levels (Ye *et al.*, 1994).

Polyamines have recently been reported to target inward K^+ channels in guard cells of plants, with a possible role in plants of modulating stomatal movements (Liu *et al.*, 2000). Furthermore, cutting injury to root segments of pea and maize causes endogenous polyamines to be released, which in turn inhibit transport processes across the plasma-membrane, specifically K^+ uptake and H^+ extrusion (De Agazio *et al.*, 1988; De Agazio *et al.*, 1989). Interestingly, it was noted that following treatment of cells with aminoguanidine to inhibit diamine oxidase, an enzyme involved in putrescine metabolism, that the degradation products of putrescine oxidation, rather than putrescine itself, are more active in inhibiting K^+ uptake (De Agazio *et al.*, 1989). A further role for polyamines in plant responses to injury is also suggested following observations that application of poly-L-ornithine, or a combination of Ca^{2+} and spermine, activates a 1,3, β-glucan synthase,

producing callose which acts as a transient cell wall material after injury in soybean (Fink *et al.*, 1987). Polyamines also increase efflux of monovalent metal ions such as Na^+, K^+ and Mn^+, which affect photochemical activity of chloroplasts in rice and pea cells (Chattopadhyay and Ghosh, 1990).

1·5 AIMS OF THIS STUDY

The scientific literature contains many reports, some conflicting, of polyamine involvement during plant growth processes. To some extent, these contradictions may be a result of species-specific and cell-specific roles of polyamines. Furthermore, due to antagonism with ethylene biosynthesis, the developmental age of tissues studied may have contributed to the varied results. The ubiquitous nature of polyamines, however, and their established correlations with many aspects of plant development, warrant further experimentation to clarify the *in vivo* roles of polyamines.

The aims of the present study were to appraise three different approaches for examining alterations in polyamine levels and growth and development of *Arabidopsis*. Initially few studies involving polyamine metabolism had focused on *Arabidopsis*, although the number of published reports dealing with this topic has increased recently. The present study seeks to contribute information regarding this model plant, which may also be applicable to other species.

The first approach involved treating plants and plant tissues cultured *in vitro* with chemical inhibitors that target key polyamine biosynthetic enzymes, to determine the extent of depletions of polyamine titres *in vivo* and effects on growth and development. Analogously, effects of excess levels of polyamines were also assessed following application of polyamines themselves to plants. Such treatments of tissues in other species have been shown to cause either negative or positive regulation of a range of growth processes which

can be correlated with altered titres of polyamines. This study sought to clarify the extent of similar responses in *Arabidopsis*.

The second approach involved the generation of transformed *Arabidopsis* plants containing genes from the Ri T-DNA of *Agrobacterium rhizogenes*. An hypothesis exists in the literature (Martin-Tanguy *et al.*, 1990; Burtin *et al.*, 1991) based on experiments involving tobacco, that a direct correlation between transformed phenotype and reduced polyamine content. Analysis of phenotype, polyamine titres, and the expression pattern of polyamine biosynthetic genes in *Arabidopsis* transformants containing Ri T-DNA sought to test this hypothesis.

The third procedure involved attempts at isolating novel mutants of *Arabidopsis* possessing alterations in polyamine metabolism. This was done with a view to using such mutants to gain insights into regulation of polyamine metabolism *in vivo* and effects of genetic perturbations upon growth and development. Screening methods were based on resistance to high levels of polyamines or polyamine biosynthetic inhibitors in order to isolate mutants with deregulated expression of the polyamine biosynthetic genes.

CHAPTER 2:

MATERIALS AND METHODS

The letters (*q.v.*) after a reagent name or method name is an abbreviation of *quod vide* and denotes a cross-reference for that item.

2·1 GENERAL SOLUTIONS AND REAGENTS

Agarose: [ICN Biomedicals Inc.] Dissolved in single-strength TBE buffer; generally, 0·8% agarose was used unless otherwise stated.

Chloroform:Isoamyl Alcohol: 24 parts chloroform [BDH Chemicals] to 1 part isoamyl alcohol [BDH Chemicals].

Denaturer: 1·5M NaCl, 0·5M NaOH. pH adjusted until over 12.

Denhardt's Solution: A 50X stock was made and filtered; 1% (w/v) Ficoll (Type 400), 1% (w/v) soluble polyvinylpyrrolidone, 1% (w/v) BSA (Fraction V).

dNTPs: A dNTP mix was made containing the nucleotides dATP, dCTP, dGTP, and dTTP, each at a concentration of 0·2mM.

EDTA: [Astral Scientific] A 0·5M stock was made. pH adjusted to 8·0 before autoclaving.

Ethidium Bromide: [Sigma] A 1% (w/v) stock was made and stored in the dark at 4°C.

Gel-Loading Buffer: A 6X stock was made; 0·25% (w/v) bromophenol blue, 0·25% (w/v) xylene cyanol FF, 15% (w/v) ficoll type 400.

Hybridisation wash (low stringency): 3X SSC, 0·5% SDS.

Hybridisation wash (medium stringency): 0·5% SSC, 0·5% SDS.

Hybridisation wash (high stringency): 0·1% SSC, 0·5% SDS.

$MgCl_2$: 25mM stock [Promega]; for use with *Taq* DNA polymerase [Promega]

MOPS buffer (1X): 0.2M 3-[N-morpholino]propanesulfonic acid (MOPS); 0.05M NaAc; 0.01M EDTA

Neutraliser: 1·5M NaCl, 1M Tris. pH adjusted to 8·0 with concentrated HCl.

PCR Buffer: A 10X stock of magnesium-free thermophilic buffer [Promega] was used in conjunction with *Taq* DNA polymerase [Promega].

Phenol: 500g of phenol [Wako] was melted and washed twice in 500mL of 0·5M Tris-HCl, 0·05M EDTA (pH = 8·0) and stirred for 60 minutes. A further wash was performed using 1000mL 0·1M Tris-HCl, 0·01M EDTA (pH = 8·0) and stirred for 30 minutes. The pH was checked with indicator paper, and adjusted to 8·0.

pH Standards: [Activon] Capsules for pH 4 and pH 7 solutions were dissolved in MQ water.

Prehybridisation Solution: 5X SSPE, 5X Denhardt's solution, 0·5% (w/v) SDS, 0·04mg/mL salmon sperm DNA.

RNase A: A 10mg/mL stock was made in 10mM Tris-HCl (pH = 7·5) and 15mM NaCl. The solution was boiled for 15 minutes and allowed to cool to room temperature, before storage at -20°C.

RNA loading buffer: For 1.5mL, add 0.72mL formamide, 0.16mL 10X MOPS buffer, 0.26mL 37% formaldehyde, 0.18mL water, 0.1mL 80% glycerol, and 0.08mL of a saturated solution of bromophenol blue.

RNA hybridisation wash 1 (low stringency): 2X SSC; 0.05% (w/v) SDS.

RNA hybridisation wash 2 (medium stringency): 0.1X SSC; 0.1% (w/v) SDS.

Salmon Sperm DNA: A 5mg/mL stock was made.

Sephadex G-50: 5g Sephadex G-50 (DNA grade) [Pharmacia Biotech.] was added to 80mL sterile MQ water. The swollen resin was washed with sterile MQ water several times, and then equilibrated in TE (pH = 7·6) before autoclaving.

Sodium Acetate: 3M stock. pH adjusted to 6·0 with acetic acid before autoclaving.

SSC: A 20X stock was made; 3M NaCl and 3M sodium acetate (or sodium citrate). The pH was adjusted to 7·0 with 10M NaOH before autoclaving.

SSPE: A 20X stock was made of 175g NaCl, 31·2g NaH_2PO_4. The pH was adjusted to 7·7 with NaOH before the addition of 0·5M Na_2EDTA, before autoclaving.

Tris-Borate buffer (TBE): A 10X stock was made; 0·09M Tris-Borate, 0·002M EDTA. pH should be 8·3 without adjustment.

TE: 10mM Tris, 1mM EDTA. pH adjusted to 8·0 before autoclaving.

Tris: A 2M stock solution was made and the pH was adjusted to 7·5 with a Tris-compatible pH electrode before autoclaving.

2·2 BACTERIAL METHODS

2·2·1 SOLUTIONS AND MEDIA

Luria Bertani (LB) Broth: 1% (w/v) tryptone [Oxoid], 0·5% (w/v) yeast extract [Oxoid], 1% (w/v) NaCl. pH adjusted to 7·5

LB agar: As for LB broth, but 1·5% (w/v) agar [Difco Bacto] added before autoclaving

Yeast Mannitol Broth: 0·05% (w/v) K_2HPO_4, 0·02% (w/v) $MgSO_4$, 0·01% (w/v) NaCl, 0·04% (w/v) yeast extract, 1% (w/v) mannitol. pH adjusted to 7·0 with 1M NaOH.

Tryptone Yeast Broth: 0·5% (w/v) tryptone, 0·3% (w/v) yeast extract. pH adjusted to 7·0 with 1M NaOH.

Transformation and storage buffer (TSB): In LB broth (pH = 6·1), make up; 10% PEG (MW = 3350), 10mM $MgCl_2$, 10mM $MgSO_4$, and 5% DMSO (add prior to use).

X-gal: A 100mg/mL stock solution was prepared by dissolving the powder [Progen] in N,N-dimethylformamide (DMFO) [BDH Chemicals]. The solution was filter sterilised with a 0·45µm filter unit and stored in the dark at -20°C.

IPTG: A 0·1M stock solution [Progen] was prepared and filter sterilised with a 0·45µm filter unit before storage in the dark at -20°C.

2·2·2 ONE-STEP MINIPREP FOR THE ISOLATION OF PLASMID DNA

750µL of an overnight culture of *E.coli* was added to a microcentrifuge tube containing an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and vortexed for one minute. The tube was then centrifuged for five minutes, after which 600µL of the upper, aqueous layer was removed and transferred to a fresh tube containing 750µL of isopropanol. This was mixed and immediately centrifuged for a further five minutes. The supernatant was removed and 500µL of 70% ethanol was carefully added to the side of the tube. This initial ethanol was removed before another 500µL was added and was followed by a further five minute centrifugation. The supernatant was discarded and the DNA pellet vacuumed dry before resuspension in 50µL-100µL of TER (TE [*q.v.*] plus 20µg/mL RNase). 5µL-10µL of the plasmid DNA was electrophoresed to check quantity and quality before it was used in a restriction digest.

2·2·3 WIZARD MINIPREP FOR THE ISOLATION OF PLASMID DNA PRIOR TO SEQUENCING

This method is as per "Wizard™ Minipreps DNA Purification System Technical Bulletin", Promega, Revision: 1/94, Sections III and V.

1·5mL of an overnight culture was centrifuged for two minutes. The pelleted cells were resuspended in 200µL of Cell Resuspension Solution before the addition of 200µL of Cell Lysis Solution. The cells were mixed by inverting the tube several times until clear. 200µL

of Neutralization Solution was added, and again the tube inverted several times. This was centrifuged for five minutes, after which, the cleared supernatant was transferred to a fresh tube. 1mL of Wizard Minipreps DNA Purification Resin was added to the supernatant and mixed by inversion. The DNA/Resin mix was pipetted into the barrel of a 3mL disposable syringe and was injected into a Minicolumn. In a similar manner, 2mL of Column Wash Solution was passed through the Minicolumn, before it was removed from the syringe and transferred to a microcentrifuge tube and centrifuged for 20 seconds in order to dry the Resin. The Minicolumn was then removed to a sterile microcentrifuge tube and 50µL of MQ water was added and left for one minute. To elute the DNA, the Minicolumn was centrifuged for 20 seconds. The plasmid DNA was stored at 4°C or -20°C.

2·2·4 PREPARATION OF COMPETENT CELLS

50µL of an overnight culture of *E.coli* DH5α cells was inoculated into a 10mL LB broth (*q.v.*) and grown to the early log phase. After approximately three hours, the growth was checked using a spectrophotometer, until an OD_{600} = 0·6 was reached. The broth was poured into a 10mL tube and centrifuged at 3000rpm or 1000 X g for 10 minutes at 4°C. The pelleted cells were resuspended in one-tenth of the volume of TSB (*q.v.*) at 4°C, and incubated on ice for 10 minutes (the cells were frozen at this stage if required).

2·2·5 TRANSFORMATION

100µL aliquots of competent cells were pipetted into cold 10mL tubes. To these cells, 10ng (2µL-5µL) of the ligation mix was added and incubated on ice for 30 minutes. In order to permit the expression of the antibiotic resistance gene, 900µL of TSB (*q.v.*) containing 20mM glucose was added to the cells, which were then left at 37°C with constant shaking. Aliquots of the cells ranging from 100µL to 400µL were plated onto antibiotic-containing agar plates supplemented with X-gal (40mg/L) (*q.v.*) and IPTG (0·5mM) (*q.v.*) and incubated overnight at 37°C.

2·3 PLANT METHODS

2·3·1 GENERAL MEDIA AND SOLUTIONS

***Arabidopsis* nutrient solution:** For a final volume of 20L, the following solutions were added to tap water; 100mL of 1M KNO_3, 50mL of 20mM Iron EDTA, 40mL of 1M $MgSO_4.7H_2O$, 40mL of 1M $Ca(NO_3)_2.4H_2O$, 20mL of micronutrient solution, and 50mL 1M K Phosphate.

Micronutrient solution: 70mM H_3BO_3, 14mM $MnCl_2.4H_2O$, 0·5mM $CuSO_4.5H_2O$, 1mM $ZnSO_4.7H_2O$, 0·2mM $NaMoO_4.2H_2O$, 10mM NaCl, and 0·01mM $CoCl_2.6H_2O$.

K Phosphate: 1M KH_2PO_4 and 1M K_2HPO_4 were mixed together until the pH was 5·5.

B5 Medium: 3·19% (w/v) Gamborg's B5 basal medium with minimal organics [Sigma], 3% (w/v) sucrose [CSR]. pH adjusted to 6·5 before autoclaving.

Callus-induction medium (CIM): 0·443% (w/v) Murashige and Skoog basal medium [Sigma], 2% (w/v) sucrose, 0·05% (w/v) MES [Sigma], 0·2% (w/v) phytagel [Sigma]. After autoclaving 500µL of a 1mg/mL stock of 2,4-D was added per litre of media.

Chromium trioxide: [BDH Chemicals] A 1% (w/v) solution was made.

Domestos: [Lever] A 10% (v/v) solution was made.

Extraction Buffer (EB): For DNA extractions from plant tissue; 100mM Tris (pH=8·0), 50mM EDTA, 500mM NaCl, 10mM β-mercaptoethanol [Sigma] (added just prior to use), 20% SDS, 5M Potassium Acetate (pH not adjusted).

Germination medium (GM): 0·443% (w/v) Murashige and Skoog basal medium, 1% (w/v) sucrose, 0·05% (w/v) MES, 0·1% (w/v) phytagel.

MS medium: 0·443% (w/v) Murashige and Skoog basal medium, 3% (w/v) sucrose, and 0·18% (w/v) phytagel or 0·8% (w/v) agar.

Shoot-induction medium (SIM): 0·443% (w/v) Murashige and Skoog basal medium, 2% (w/v) sucrose, 0·05% (w/v) MES, 0·2% (w/v) phytagel. After autoclaving, 250µL of a 20mg/mL stock of 2iP was added.

2·3·2 GROWTH REGULATORS

Antibiotics

- Ampicillin: As ampicillin sodium for injection [CSL]; A 25mg/mL stock was made and filter- sterilised using a 0·45µm pore-sized filter.
- Kanamycin: As kanamycin monosulfate [Sigma]; A 25mg/mL stock was made and filter-sterilised using a 0·45µm pore-sized filter.
- Timentin: [SmithKline Beecham] A 200mg/mL stock was made and filter-sterilised using a 0·45µm pore-sized filter.

Hormones

- 2,4-D: 2,4-Dichlorophenoxyacetic Acid [Aldrich Chem. Co.]; dissolved in ethanol as a 1mg/mL stock.
- 2iP: 6-(γ, γ-Dimethyl-allylamino]-purine [Sigma] was dissolved in sodium hydroxide as a 200mg/mL stock and filter-sterilised using a 0·45µm pore-sized filter.
- IAA: Indole-3-Acetic Acid [Sigma]; dissolved in ethanol as a 10^{-1}M stock and then diluted in MQ water and filter-sterilised using a 0·45µm pore-sized filter.
- IBA: Indole-3-Butyric Acid (potassium salt) [Sigma]; dissolved in ethanol as a 10^{-1}M stock and then diluted in MQ water and filter-sterilised using a 0·45µm pore-sized filter.

- NAA: Napthalene Acetic Acid [Sigma] dissolved in sodium hydroxide as a 10^{-1}M stock and then diluted in MQ water and filter-sterilised using a 0·45µm pore-sized filter.

Polyamines and precursors

The three polyamines Putrescine (dihydrochloride) [Sigma], Spermidine (trihydrochloride) [Sigma], and Spermine (tetrahydrochloride) [Sigma] were made up as concentrated stock solutions, typically between 100mM and 500mM. The precursors L-ornithine [Sigma] and L-arginine [Sigma] were made in the same manner as for the polyamines. The pH of the individual solutions were adjusted to that of the growth media (usually pH = 6·2, for MS media). They were then filter-sterilised with a 0·45µm filter before use or storage at -20°C.

Polyamine inhibitors

The inhibitors DL-α-Difluoromethyl ornithine (DFMO) [Merrell-Dow Research Inc.], DL-α-Difluoromethyl arginine (DFMA) [both donated from the Merrell-Dow Research Institute, Cincinnati, Ohio, U.S.A.], methylglyoxal bis-(guanylhydrazone) dihydrochloride (MGBG) [Sigma], and cyclohexylamine CHA [Sigma] were made in the same manner as for the polyamines.

2·3·3 SEED STERILISATION

Seed sterilisation was performed in a laminar air flow cabinet using sterile 1·5mL microcentrifuge tubes and sterile Pasteur pipettes. Seeds were treated with 70% ethanol for two minutes with constant, gentle inversion. Using a Pasteur pipette, the seeds were transferred to another tube, this time containing a 10% (v/v) Domestos solution (*q.v.*), and subjected to constant, gentle inversion for 10 minutes. Thereafter, the seeds were removed and washed in sterile MilliQ® water (hereafter referred to as MQ water) for two minutes (again, with constant inversion). At least four washes in MQ water were required to completely remove all traces of the bleach.

2·3·4 SEED SOWING AND *IN VITRO* GROWTH

In vitro sowing of seed was performed in a laminar air flow cabinet using sterile Pasteur pipettes. After sterilisation (*q.v.*), seeds were left in sterile MQ water at 4°C for at least 16 hours (usually 24 to 48 hours) in order to break the seed dormancy. Prior to sowing, the seeds were aspirated with a pipette to evenly distribute them within the water, immediately after which a small amount of the liquid was drawn up into the pipette shaft. This facilited easy sowing of individual seeds onto the surface of the media. Porous Micropore tape was used to seal the plates, thereby allowing the exchange of gases and also avoided the build-up of condensation. *In vitro* plants were grown under fluorescent light (~60 µE/m^2/s) at a constant temperature (20°C) with a 16 hour photoperiod.

2·3·5 SEED SOWING AND SOIL GROWTH

The soil mixture required for growing plants consisted of equal proportions of Seed-Raising Mix [Debco] and horticultural perlite [Chillagoe]. Punnets were filled with the soil mix and moistened with *Arabidopsis* Nutrient Solution (*q.v.*). After the soil mixture was completely saturated, the seeds were scattered onto the soil and the punnets covered with plastic wrap, before being placed at 4°C for 24 hours to break seed dormancy. After 24 hours, germinating seedlings were placed under constant fluorescent light (~60μE/m^2/s), and maintained at a temperature of 18-21°C in greenhouse conditions. Any *in vitro*-grown plants that were transfered to soil were first gently removed from the media and buried in soil until the roots were completely covered. The tray was again covered with plastic film and immediately transferred to the greenhouse. After seedlings had reached the cotyledon stage, the plastic wrap was removed, and the plants were watered every two to three days with *Arabidopsis* nutrient solution.

2·3·6 *ARABIDOPSIS* CROSSES

Under a binocular microscope, flowers of the recipient were hand-emasculated by removing all six stamens with fine forceps. Only unpollinated flowers of the most apical inflorescence were used. Any pollinated flowers within the inflorescence were removed before proceeding with the crosses. Anthers of mature flowers of the donor were removed, and the pollen applied to the stigmatic surface of the recipients.

2·3·7 *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *ARABIDOPSIS*

This method is based on that of Valvekens *et al.* (1988).

Seedlings were grown on GM medium (*q.v.*) for four-to-six weeks after which the roots were harvested and incubated on CIM medium (*q.v.*) in the dark for two days. The roots were then removed and cut into half centimetre lengths and were added to freshly-grown 10mL cultures of the AGL1 *Agrobacterium* strain containing the gene(s) of interest. The cultures were shaken vigorously to completely mix the roots with the bacteria and then poured through a sterile stainless steel tea strainer. The root fragments were lightly blotted onto sterile filter paper and then roughly dispersed onto fresh CIM plates and incubated in the dark for two days. The roots were then washed in sterile MQ water to remove the surface bacteria, strained, and blotted before their transfer to SIM medium (*q.v.*) in 2cm deep Petri dishes. Selection against *Agrobacterium* was mediated by the inclusion of Timentin at 200mg/L in the SIM media. If required, kanamycin was also included (50mg-100mg/L). After three-to-four weeks, the first shoots appeared. When their growth approached approximately 5mm in length, the regenerated shoots were excised and transferred to 250mL glass jars containing 50mL GM medium (*q.v.*). The raised lids were sealed with Micropore tape and the plants grown until seed set.

2·3·8 LATERAL ROOT PRIMORDIA BIOASSAY

Seedlings were germinated and grown on MS medium (*q.v.*) until four days after sowing, at which they were transferred to MS media containing auxins, polyamines, or polyamine inhibitors. Approximately 40 seedlings were aligned on Petri dishes containing the freshly-made media, in two horizontal rows, each with approximately 20 seedlings. The plates were incubated under fluorescent light at 20°C in racks to maintain a vertical orientation. This allowed the roots to grow down along the surface of the agar, facilitating easy removal of the plant and scoring of primordia. The seedlings were removed at appropriate time-points and treated with 1% chromium trioxide (*q.v.*) for 10 minutes, after which they were subjected to a series of washes in distilled water in order to remove all traces of the stain. The seedlings were then visualised under a microscope and the number of lateral roots and lateral root primordia were counted. In addition, the length of the primary root was measured.

2·3·9 ADVENTITIOUS ROOT PRIMORDIA BIOASSAY

Seven plants were grown per Petri dish containing MS medium (*q.v.*) until day 19 of growth (pre-flowering stage) and/or day 26 of growth (flowering stage). At these time-points, rosette leaves were removed from each plant and dissected longitudinally along the main vein. The resulting two explants from each leaf were placed on MS media supplemented only with the auxin IAA (*q.v.*) or with IAA and either of the polyamines putrescine (*q.v.*) or spermidine (*q.v.*). Several explants were also placed onto hormone-free and polyamine-free control media. The same sets of rosette leaves were taken from all plants when generating explants, in order to remove any developmentally-induced anomalies between different leaf types. No more than 20 explants were placed per plate. The plates were incubated horizontally under fluorescent light at 20°C for approximately 14-to-21 days, at which adventitious roots became visible.

2·3·10 MINIPREP FOR THE ISOLATION OF GENOMIC DNA

3·5mL of 20% SDS was added to 50mL EB buffer (*q.v.*) while a sterile mortar and pestle was cooled with liquid nitrogen. 2g of plant tissue was ground to a fine powder in the mortar and then 3mL of the extraction buffer/SDS mix was added and further ground. The powder was then transferred to a sterile 10mL tube containing 2·5mL of the extraction buffer/SDS mix. This was stored on ice until all tissues were ground. The samples were then incubated at 65°C for 10 minutes, during which the tubes were shaken vigorously several times. 2mL of potassium acetate was then added to the extracts and again mixed by shaking. A further incubation on ice was required for 10 minutes, after which the samples were centrifuged at 10,000rpm for 20 minutes, and then the supernatants were individually filtered through Kimwipe tissues into sterile centrifuge tubes. Three-fifths of the volume of isopropanol was added and the tube gently inverted to mix, followed by another spin at 10,000rpm for 20 minutes to pellet the DNA.

The supernatant was removed and the tube inverted on a paper towel for 5 minutes to briefly dry the pellet. The inside of the tube was wiped with a Kimwipe to remove any excess isopropanol, and the pellet was resuspended in 700µL of TE. This was left for at least 2 hours at 4°C (often overnight) to ensure complete resuspension. The DNA in solution was then transferred to microcentrifuge tubes and centrifuged to remove any insoluble material. The supernatants were then transferred to fresh tubes. RNase was added to each sample to a final concentration of 50µg/mL and the tube was incubated at room temperature for 30 minutes. The DNA was phenol-extracted and then precipitated with one-tenth of the volume of sodium acetate and two-thirds of the volume of isopropanol. The contents were gently mixed and the tube centrifuged at 10,000rpm for 20 minutes. The supernatant was removed and the pellet washed in 70% ethanol, followed by air-drying at 37°C until all ethanol had evaporated. The DNA pellet was resuspended in 50µL-100µL MQ water and the DNA concentration was estimated by electrophoresis with known concentrations of standards.

2·3·11 MINIPREP FOR THE ISOLATION OF TOTAL RNA

RNA manipulation techniques

Gloves were worn for all RNA procedures. A separate set of chemicals, glassware, equipment and consumables were kept for RNA manipulations only. All glassware and vessels to be used for RNA methods were washed successively (with intermittent rinses in sterile MQ water) with the following solutions before being used; 0·1% SDS, 0·1M NaOH (equipment soaked for one hour at this step), chloroform, and finally, absolute ethanol.

Mini prep isolation of RNA

A 50mL centrifuge tube [Falcon] containing 750µL of TLES buffer (100mM Tris [pH 8.0], 100mM LiCl, 10mM EDTA, 1% (w/v) SDS), and 750µL of RNA grade phenol, was prepared for each extraction to be performed and then placed in an 80°C waterbath. 0·5g of plant tissue was then ground under liquid nitrogen, in a mortar and pestle before the ground powder was added to the centrifuge tubes containing TLES-phenol solution. The samples were then vortexed for 30 seconds. 750µL of chloroform-isoamyl alcohol (24:1) was added to the tubes and the samples were again vortexed for 30 seconds. The mixture was then divided into two microcentrifuge tubes and centrifuged at 10,000rpm for five minutes. The supernatant of each sample was subsequently removed to a clean microcentrifuge tube, and an equal volume of 4M LiCl was added. Samples were then kept at 4°C overnight followed by centrifugation at 10,000rpm for 30 minutes at 4°C. The pellet was then resuspended in 250µL of RNase-free MQ water. 25µL of NaAc (pH 6.0) and 500µL of absolute ethanol were added, and the RNA allowed to precipitate overnight at -20°C. Following precipitation, the RNA was pelleted by centrifugation at 10,000rpm for 20 minutes, and was then washed in 70% ethanol. The pellet was resuspended in 50-100µL of RNase-free MQ water.

2·3·12 CHEMICAL MUTAGENESIS OF *ARABIDOPSIS* SEED

Chemical mutagenesis of *Arabidopsis* seeds was achieved by incubating approximately 10,000 seeds (200mg) of the Landsberg *erecta* ecotype in a 40mM solution of ethyl methanosulfonate (EMS) (Sigma). The seeds were stirred in this solution for eight hours before the EMS was neutralised by decanting into a 10M sodium hydroxide solution. The seeds were rinsed 8 times with 100mL of MQ water over a period of 2 hours. To facilitate easy sowing, mutagenised seeds were resuspended in 500mL of cooled 0·15% agar and dispensed with a pipette to a final density of approximately 200 seed per punnet. These M1 seeds were allowed to germinate and grow, and resulting M2 seeds were harvested and used in experiments screening for mutants resistant to high levels of MGBG, CHA, or putrescine. To test the stability of the inhibitors in selection plates, wild-type seed were sown on media that had been stored at 4°C for two weeks. MGBG had the capacity to breakdown or precipitate to some extent, allowing the growth of sensitive wild-type plants. Thus, resistant plants had to be identified during the early growth phase on freshly-made selective media.

2·3·13 GAMMA-RADIATION MUTAGENESIS OF *ARABIDOPSIS* SEED

Irradiation of approximately 10,000 seed of the Landsberg *erecta* ecotype of *Arabidopsis* was carried out in the Gammacell 1000 Gamma Irradiator (Isomedix, Inc. New Jersey) with a cesium^{137} chloride source. The seeds were exposed for an appropriate length of time to achieve doses of 70krads, 75krads, 80krads, 85krads, 90krads, 95krads, and 100krads. To facilitate easy sowing, the mutagenised seed were resuspended in 500mL of cooled 0·15% agar and dispensed with a pipette to a final density of approximately 200 seed per punnet. These irradiated M1 seed were allowed to germinate and grow, and resulting M2 seeds were harvested and used in screening experiments looking for mutants resistant to 10mM ornithine. Screening experiments were performed prior to the report that Arabidopsis lacks *ODC* (Hanfrey *et al.*, 2001), and it was originally anticipated that the *ODC* coding sequence may have been isolated using subractive hybridisation procedures.

2·4 HPLC METHODS

2·4·1 GENERAL MATERIALS AND INSTRUMENTATION

System

- Waters 600E System Controller
- Waters U6K injector
- Waters 717plus Autosampler

- Waters 470 Scanning Fluorescence Detector
- Waters 486 Tunable Absorbance Detector
- Baseline Chromatography Software [Waters]
- Millennium[32] Chromatography Software [Waters]

Columns

- Cartridge column [SGE instruments]; 50mm length, ODS2 (C18) packing, 5µm particle size, and 4mm internal diameter.
- Pre-column [SGE instruments]; 10mm effective length, ODS2 (C18) packing, 5µm particle size, and 4mm internal diameter.

Filters

- For mobile phase; regenerated cellulose [Sartorius] with a 0·45µm pore size.

Chemicals and solutions

- Acetone [BDH chemicals]
- Acetonitrile [BDH chemicals; HiperSolv™ for HPLC]
- Benzoyl chloride [BDH chemicals]
- Dansyl chloride [Sigma]
- Diethyl ether [BDH chemicals]
- Methanol [BDH chemicals; HiperSolv™ for HPLC]
- Perchloric acid [BDH chemicals; 60% stock]
- Sodium carbonate (anhydrate) [Ajax chemicals]
- Toluene [BDH chemicals; HiperSolv™ for HPLC]

Miscellaneous

- High purity compressed helium [BOC gases]
- Vacuum pump [Millipore; model #XX5522050]
- Autosampler vials [Alltech]; 15x45mm clear glass; 4mL WISP vials
- Inserts for vials [Alltech]; 300µL poly inserts for 4mL WISP vials
- Springs for inserts [Alltech]; for 300µL inserts used in 4mL WISP vials

2·4·2 EXTRACTION AND DERIVATISATION OF POLYAMINES FROM PLANTS FOR ANALYSIS BY ABSORBANCE DETECTION

This method is based on that of Flores and Galston (1982).

Plant tissue was homogenised in 5% cold perchloric acid ($HClO_4$) at a ratio of 100mg tissue per mL acid using a hand-held Polytron homogeniser [Selby], and left on ice for one hour. The extract was pelleted by centrifugation at 48,000 X g for 20 minutes. The liberated free polyamines contained within the supernatant fraction were stored at -20°C or derivatised

immediately. Polyamines required benzoylation in order to facilitate their detection by ultra violet absorption. 500µL of the extract was mixed with 1mL of 2M NaOH, followed by the addition of 10µL benzoyl chloride. The solution was vortexed for 10 seconds and incubated at room temperature for 20 minutes. 2mL of saturated NaCl was then added, followed by 2mL of anhydrous diethyl ether to extract the benzoyl polyamines. The sample was centrifuged for 1,500 X g for five minutes before 1mL of the ether phase was collected and evaporated to dryness under a stream of air. The residue was redissolved in 100µL of methanol and stored at -20°C until further analysis. For HPLC analysis, a mobile phase consisting of acetonitrile:water was run isocratically at 52% acetonitrile, at a flow rate of 1mL/minute. The solvent was eluted through a reverse phase C18 column and detected at 254nm.

2·4·3 EXTRACTION OF FREE AND CONJUGATED POLYAMINES FROM PLANTS FOR ANALYSIS BY FLUORESCENCE DETECTION

This method is based on that of Tiburcio *et al.* (1985).

Plant tissue was homogenised in 5% cold perchloric acid ($HClO_4$) in plastic tubes at a ratio of 100mg tissue per mL acid using a hand-held Polytron homogeniser, and left on ice for two hours. The homogenates were then transferred to thick-walled polyallomer tubes for centrifugation in a Beckman ultracentrifuge (75 Ti rotor) at 25,000rpm for 10 minutes at 4°C. 1mL of the supernatant was transferred to a microcentrifuge tube and stored at -20°C until required. The pellet was resuspended in the original extraction volume with freshly-made 1M NaOH by vigorous vortexing and stored at -20°C until required. For the extraction of conjugated and bound polyamines, 200µL aliquots of the supernatant and the resuspended pellet were removed and placed into the bottom of individual glass vials with screw-topped lids. 200µL of concentrated HCl (12M) was then added to each vial before hydrolysing the samples for 16-18 hours at 110°C. After incubating, the vials were pulse-spun in a bench centrifuge at 1500rpm to collect all condensation. The samples were transferred to 1mL disposable syringes containing glass wool plugs and 'filtered' in order to remove the carbonised material. 200µL of the samples was transferred to sterile microcentrifuge tubes and dried in a vacuum centrifuge for one-to-two hours. The pellets were resuspended in 100µL 5% perchloric acid and stored at -20°C until required for dansylation.

2·4·4 DERIVATISATION OF POLYAMINES FOR HPLC ANALYSIS

This method is based on that of Minocha *et al.* (1990).

50µL of each standard solution or centrifuged tissue extract were placed in 2mL screw-topped Sarstedt tubes containing 100µL of a saturated sodium carbonate solution. To each vial, 100µL of freshly-made dansyl chloride (in acetone, at 10mg/mL) was added. The vials were capped tightly and incubated in the dark in a 60°C water bath for one hour. 50µL of a proline solution (100mg/mL) was then added to the reaction mixture to remove excess dansyl

chloride. The vials were incubated for an additional 30 minutes in the dark at 60°C before the acetone was evaporated off by centrifugation under a vacuum for six-to-eight minutes. 400µL of toluene was added and the solution vortexed for 30 seconds. The vials were allowed to sit undisturbed for five minutes in order to separate the organic and aqueous phases, before centrifugation at 2,500rpm in a microcentrifuge for two minutes. Upon separation of the two phases, 200µL of the toluene layer was transferred to a sterile microcentrifuge tube. The toluene was completely evaporated off by centrifugation under a vacuum for 10-15 minutes, after which, the residue was dissolved in 1mL acetonitrile. After vortexing for one minute, the samples were centrifuged for a further five minutes at 4°C before 500µL of the solution was removed to a sterile microcentrifuge tube and stored at -20°C.

2·4·5 HPLC MOBILE PHASE GRADIENT FOR POLYAMINE SEPARATION

A mobile phase gradient using two solvents was set up in order to achieve separation of the three polyamines; putrescine, spermidine, and spermine from *Arabidopsis* tissue extracts. Solvent A was a 9:1 ratio of 10mM Heptane sulphonic acid (pH = 3·4) to acetonitrile. Solvent B was 100% acentonitrile.

The gradient profile was as follows;

Step	*Time (min)*	*Flow rate (mL/min)*	*% Solvent A*	*% Solvent B*
1	0·00	2·5	60	40
2	0·10	2·5	30	70
3	2·10	2·5	5	95
4	2·25	2·5	0	100
5	2·60	2·5	30	70
6	3·00	2·5	25	75
7	3·10	2·5	20	80
8	3·50	2·5	30	70
9	7·40	2·5	60	40
10	10·00	2·5	60	40

The excitation wavelength of the fluorescence detector was set at 340nm and the emission wavelength set at 510nm. 20µL of sample was injected for analysis. The polyamines were generally eluted before 4 minutes, with the remaining 6 minutes of the run allowing the column to regenerate for the next sample. Typically, the elution times for the polyamines were in the order of 2·7 minutes for putrescine, 3·3 minutes for spermidine, and 3·9 minutes for spermine.

2·5 GENERAL MOLECULAR METHODS

2·5·1 PHENOL:CHLOROFORM EXTRACTION OF DNA

An equal volume of phenol:chloroform was added to the nucleic acid sample in a microcentrifuge tube and vortexed until the formation of an emulsion. The tube was centrifuged at 12,000 X g for 15 seconds at room temperature after which the aqueous layer was transferred to a sterile microcentrifuge tube. This extraction was repeated until no protein was visible at the interphase of the aqueous and organic phases. An equal volume of chloroform was added to the aqueous layer before mixing and centrifugation as before to separate the phases. The aqueous phase was removed and the nucleic acid recovered by ethanol precipitation.

2·5·2 ETHANOL PRECIPITATION

To an aqueous solution containing DNA, one-tenth of the original volume of 3M sodium acetate was added, followed by twice the volume of ethanol. The tube was slowly inverted several times to ensure complete mixing and then placed at -20°C for at least three hours. The DNA was pelleted by centrifugation for 30 minutes, followed by a wash in 70% ethanol. The ethanol was removed with a micropipette and the sample air-dried at 37°C for 30 minutes before resuspension in MQ water.

2·5·3 RESTRICTION DIGESTION

1µg of DNA was made up to a final volume of 18µL with sterile MQ water before the addition of 2µL of the appropriate 10X restriction buffer. One-to-two units of enzyme was added and the tube gently tapped to mix the contents. The mixture was incubated at the temperature recommended by the manufacturer of the enzyme for three hours. The enzyme was inactivated by incubation at 65°C for 10 minutes. To check that the digestion was successful, an aliquot was removed and subject to electrophoresis on an agarose gel.

2·5·4 ELECTROPHORESIS OF DNA

DNA samples were electrophoresed on 0·8% agarose (*q.v.*) gels made with single-strength TBE buffer (*q.v.*) for approximately three hours at 60 volts. The gels contained 3µL of a 10mg/mL stock of ethidium bromide (*q.v.*) per 100mL of agarose; a final concentration of 0·3µg per mL.

2·5·5 PURIFICATION OF DNA FRAGMENTS FROM AGAROSE GELS

Phenol freeze method

Agarose bands containing DNA were excised from gels using a sterile blade. The agarose slices were crushed between pieces of parafilm and placed into a 1mL syringe which was

attached to a 27G needle. The agarose was forced through the needle into a sterile microcentrifuge tube and TE buffer was added to make a final volume of 500µL. An equal volume of phenol (*q.v.*) was added and the mixture was vortexed for one minute before freezing at -70°C for one hour. The samples were then centrifuged at 14,000rpm for 20 minutes, before the upper aqueous layer was removed to a sterile microcentrifuge tube followed by the addition of an equal volume of chloroform. The tubes were vortexed for 30 seconds before centrifugation at 14,000rpm for five minutes. This step was repeated, and the DNA was precipitated with ethanol (*q.v.*).

GEL-SPIN™ DNA recovery kit

This method is based on that of the Worthington GEL-SPIN™ DNA Recovery kit protocol. Agarose bands containing DNA were excised from gels using a sterile blade and placed inside a GEL-SPIN unit within a microcentrifuge tube. The tip of a plunger was moistened with sterile water and used to pick up a porous polyethylene filter, which was placed on top of the agarose fragment. The plunger was pushed down on the filter, which forced the agarose into the GEL-SPIN unit. The tube was then centrifuged at 10,000rpm for two minutes. 100µL of GS solution (0·1M KCl) was added to the GEL-SPIN unit and the centrifugation repeated. The GEL-SPIN unit was then discarded and the DNA precipitated with two and a half volumes of ethanol. Following precipitation at -20°C for at least one hour, samples were centrifuged at 14,000rpm for 30 minutes, washed in 70% ethanol, and vacuum dried. The pellets were resuspended in 15µL of MQ water.

2·5·6 QUANTIFICATION OF DNA

Agarose plate

A 1% agarose gel was made containing ethidium bromide (*q.v.*) at a concentration of 0·5µg/mL in a 9cm Petri dish. DNA samples and standards were spotted onto the plate and allowed to dry at 37°C for approximately one hour before visualisation under UV light.

Minigel electrophoresis

The DNA sample was mixed with sterile MQ water up to a volume of 10µL, followed by the addition of 2µL of 6X stop solution. The samples were loaded into the gel in conjunction with a range of uncut lambda DNA standards of known concentrations; 50ng, 100ng, 150ng, 200ng, 300ng, 400ng, and 500ng, and electrophoresed (*q.v.*) for approximately two hours at 60V. The ethidium bromide (*q.v.*)-containing gels were visualised under UV light and the concentration of DNA estimated by a comparison of the fluorescence of the samples with those of the standards.

Spectrophotometer readings

Accurate readings of DNA and RNA concentrations were measured using a Perkin-Elmer Lambda-3 UV/Vis Spectrophotometer. Optical density readings were taken at wavelengths of 260nm and 280nm. The readings measured at 260nm allowed DNA or RNA concentrations to be estimated, while the ratio of the 260nm and 280nm readings provided an estimate of the purity of the sample (an optimum ratio of 1·8 is found for DNA samples, whilst the value for RNA samples is 2·0). The baseline of the spectrophotometer was set to zero at the required wavelength by measuring the absorbance of 1mL of sterile water in a quartz cuvette. 1µL of the DNA or RNA sample resuspended in sterile MQ water was then added the 1 mL cuvette, and the optical density readings at both 260nm and 280nm were recorded. A further 1µL of sample was then placed in the cuvette to check that the readings measured at both wavelengths was twice those initially recorded.

2·5·7 LIGATION OF DNA

DNA ligation reactions were performed in sterile 0·5mL microcentrifuge tubes using either a commercial vector [pGEM-T; Promega] or a 'home-made' vector. The protocol was based on that recommended by Promega [pGEM-T Vector Systems Technical Manual, Part #TM042, USA 4/96, page 7]. The molar ratio of PCR product:vector required optimisation in order to maximise the rate of ligation, consequently, PCR product:vector ratios of 1:1, 5:1, and 8:1 were used with an initial vector amount of 50ng. Also added to the tube were T4 DNA ligase 10X buffer [Promega], control insert DNA [Promega] when required for control tubes, T4 DNA ligase [Promega], and sterile MQ water. The reactions were gently mixed by pipetting and incubated at 15°C for three hours, followed by storage at 4°C.

2·5·8 CONSTRUCTION OF A CLONING VECTOR

A vector for ligation was prepared by the digestion of 5µg of pBluescript DNA with *Eco*RV for 3 hours at 37°C. The DNA was phenol-extracted (*q.v.*) and precipitated (*q.v.*) and then incubated with *Taq* polymerase and 2mM dTTP for 3 hours at 72°C. The DNA was again phenol-extracted and precipitated, and resuspended at a concentration of 50ng/µL. PCR products were directly ligated (*q.v.*) into the vector.

2·5·9 SEQUENCING OF CLONED DNA FRAGMENTS

DNA from plasmids containing inserts to be sequenced was first extracted using the appropriate protocol. Sequencing reactions were then set up in 600µL PCR tubes as follows; 300-500ng of double stranded plasmid DNA, 3·2pmol of the appropriate primer, 8µL of terminator premix, and sterile MQ water to give a final reaction volume of 20µL. The mixture was then overlayed with 40µL of mineral oil and placed in a Perkin-Elmer 480 DNA thermal cycler preheated to 96°C. A denaturation temperature of 96°C was used for 30 seconds, followed by an annealing temperature of 50°C for 15 seconds, and finally an extension

temperature of 60°C for four minutes. This regime was continued for 25 cycles, and after completion, the tubes were stored on ice. The extension products were purified from unincorporated nucleotides by transferring each reaction mixture to separate microcentrifuge tubes containing 2µL of 3M sodium acetate (pH 4·6) and 50µL of 95% ethanol. The tubes were vortexed and left on ice for 10 minutes before centrifugation at 14,000rpm for 30 minutes. As much ethanol as possible was removed, and the DNA pellet was rinsed in 250µL of 70% ethanol. The DNA pellet was then dried under vacuum for three minutes. The dried samples were run on an ABI PRISM Genetic Analyzer. Sequence analysis was performed using the Australian National Genome Information Service (ANGIS) (http://www.mel1.angis.org.au).

2·5·10 FIRST STRAND cDNA SYNTHESIS

First-strand cDNA was prepared using Superscript™ RNase H⁻ Reverse Transcriptase [Gibco BRL]. In a sterile microcentrifuge tube, 1µL of mRNA (≤1µg) was combined with oligo $(dT)_{12\text{-}18}$ at a concentration of 0·5µg/µL. The final volume was made up to 11µL with sterile MQ water, and the tube was incubated in a 70°C water bath for 10 minutes. The tube was chilled on ice before being briefly pulsed in a microcentrifuge to collect the contents. The following reagents were then added to the tube; 4µL of 5X reaction buffer, 2µL of 0·1M DTT, 1µL of a mixed dNTP stock (10mM each of dATP, dGTP, dCTP, and dTTP at neutral pH), and 1µL of RNase inhibitor. The contents were mixed by gently vortexing and the tube was briefly centrifuged to collect the mixture, before a two minute incubation at 37°C to equilibrate the contents. 1µL of M⁻ MLV H⁻ RT (200 units) was added to the tube followed by an incubation at 37°C for one hour. The reaction was terminated by incubation at 65°C for 10 minutes. If second strand cDNA was to be synthesised, the tube was placed on ice.

2·5·11 SECOND STRAND cDNA SYNTHESIS

15µL of first-strand cDNA was added to a sterile microcentrifuge tube containing 42·7µL of sterile MQ water. Using the Superscript™ RNase H⁻ Reverse Transcriptase kit, 16µL of 5X 2nd strand buffer was added, followed by 1·5µL dNTP (10mM), 3µL 0·1M DTT, 1µL *E.coli* DNA ligase, 2µL DNA Polymerase I, and 0·35µL RNase H. The tube was incubated at 16°C for two hours, before the addition of 10 units of T4 DNA polymerase. This was followed by a further 45 minute incubation at 16°C. The reaction was stopped by the addition of 4µL EDTA/glycogen.

2·6 PCR METHODS

Reagents and micropipette tips were set aside for exclusive use for PCR amplification., and were only handled whilst wearing gloves. Amplification reactions were set up using sterile 600µL PCR tubes containing 50-200ng of DNA template; sterile MQ water to adjust the final reaction volume to either 25µL or 50µL; one tenth of the final volume of 10X Thermo buffer (Promega), $MgCl_2$ to a final concentration of between 1 and 3·5mM; dNTP mix (10mM of each dATP, dCTP, dGTP, and dTTP) to a final concentration of 0·2mM of each dNTP and Forward and reverse primers at a final concentration of between 0·2 and 0·4µM. The reaction mix was then mixed and covered with one to two drops of mineral oil. Tubes were placed into a Perkin-Elmer 480 DNA thermal cycler preheated to 94°C. *Taq* DNA polymerase was then added to each tube (1-1·5 Units per reaction) and cycling at the required parameters was immediately commenced.

2·6·1 RAPID AMPLIFICATION OF cDNA ENDS (RACE):

After second-strand cDNA was synthesised, AP1 adaptors [Marathon RACE kit] were ligated onto the ends of the cDNA strands by incubation with T4 DNA ligase at 16°C overnight. The enzyme was inactivated by heating to 70°C for five minutes, before the mixture was diluted in MQ water or TE (at ratios of 1:49 or 1:249). The diluted double-stranded cDNA was heated at 94°C for 2 minutes and then chilled on ice for a further two minutes before storage at -20°C.

2·7 NUCLEIC HYBRIDISATION PROTOCOLS

2·7·1 SOUTHERN BLOT

This method is based on that described in the Amersham life science Hybond™-N+ membrane protocol, version two.

The agarose gel to be blotted was washed in 0·25M HCl until the dyes in the loading buffer had changed to a yellow colour. If fragments larger than 10Kb in size were to be transferred, the gel was left in the acid solution for a further ten minutes. After rinsing the gel in distilled water for five minutes, it was completely covered in denaturation buffer (*q.v.*) for 30 minutes with gentle agitation. The gel was then rinsed in distilled water, and placed in neutralisation buffer (*q.v.*) for 15 minutes, again with agitation. This neutralisation step was repeated, before the gel was removed for capillary blotting. A large dish was then filled with one litre of 20X SSC (*q.v.*) and covered with a perspex or glass platform. A wick was made from three sheets of Whatman 3MM filter paper and was saturated with 20X SSC, and placed across the platform. The two ends of the wick were left dangling in the 20X SSC buffer. The prepared gel was turned upside-down and placed gently on the wick. Any trapped air

bubbles beneath the gel were removed by rolling a clean 10mL glass pipette over the surface, and a sheet of Hybond-N+ membrane was cut to the exact size of the gel and placed on top of the gel. Air bubbles trapped between the gel and membrane were again squeezed out using a 10mL glass pipette. Three sheets of 3MM Whatman paper cut to the size of the gel were then placed on top of the membrane and again, any air bubbles were removed. A stack of absorbent paper towel was placed on top of the Whatman paper, followed by a flat plate, and finally a weight of approximately 500g to 1000g, depending upon the size of the gel. The capillary transfer was allowed to proceed for at least 12 hours. After blotting, the apparatus was dismantled and the position of the wells on the gel were marked on the membrane. The filter was washed briefly in 2X SSC to remove any adhering agarose, and the DNA was then fixed by placing the membrane (DNA side down) on a UV transilluminator for two minutes. Filters were stored at room temperature until required.

2·7·2 PREPARATION OF RADIOACTIVELY LABELLED DNA PROBES.

This method is based on that described in the GIGA prime labelling kit protocol [Bresatec].
100-200ng of double stranded DNA was mixed with sterile MQ water in a sterile microcentrifuge tube to make a final volume of 6µL. The tube was was placed in boiling water for five minutes and chilled briefly on ice. The tube was pulse spun before the addition of: 6µL of decanucleotide solution (Tube 1), 6µL of the nucleotide buffer cocktail (Tube 2A), 5µL of α-^{32}P-dATP, and 1·5µl of Klenow enzyme. The reaction was then incubated at 37°C for 30 minutes. Before the removal of unincorporated nucleotides from the probe, a purification column was prepared by placing a small piece of sterile glass wool at the bottom of a shortened Pasteur pipette. The pipette was then carefully filled with Sephadex G50 (*q.v.*), to within one centimetre of the top, and was covered with parafilm and placed at 4°C until required. The probe reaction was terminated by the addition of 1µL of 0·5M EDTA. 5µL of Orange G dye and 35µL of the dextran blue dye was then added to the reaction. The sephadex column was positioned over a series of sterile microcentrifuge tubes and the parafilm was removed. The reaction was added and allowed to flow through the column by the periodic addition of TE buffer into the top of the column. Since the dextran blue dye co-migrates with large DNA fragments through the sephadex, the fraction containing the blue dye was collected to use as the purified probe. The fractions with the orange dye contained the unincorporated nucleotides and were discarded. Double stranded probes were boiled for 2-3 minutes prior to their use in hybridisation experiments.

2·7·3 SOUTHERN HYBRIDISATION PROTOCOL

This method is based on that described inthe Amersham life science Hybond™-N+ membrane protocol, version two.
Membranes for probing were placed into glass hybridisation tubes with their DNA-sides facing inward. 100µL of a 5mg/mL stock of salmon sperm DNA was denatured by heating

to 100°C for five minutes. The DNA was chilled briefly on ice and mixed with 50mL of pre-hybridisation solution (*q.v.*) in the hybridisation tube containing the membranes. The membranes were pre-hybridised for at least one hour at 65°C in a shaking water bath. Following pre-hybridisation, the double stranded DNA probe was denatured by heating to 100°C for five minutes and added to the tube containing the membrane and pre-hybridisation solution. The probe was left to hybridise to the filters for at least 12 hours at 65°C in a hybridisation oven. Following hybridisation, the filters were washed with low-, medium-, or high-stringency wash solutions (*q.v.*) as required, with constant shaking. The low-stringency washes were performed at room temperature for 10 minutes, the medium-stringency washes took place at 65°C for 15 minutes, and the high-stringency washes were at 65°C for 10 minutes. Following the washes, the filters was wrapped in plastic wrap and exposed to an X-ray film in an autoradiograph cassette at -70°C for the appropriate length of time. The X-ray films were developed in a Agfa-Gevamatic 60 X-ray machine, under NX-914 safety lights.

For successful removal of probes, membranes were never allowed to dry during, or after, the hybridisation and washing procedures. A solution of 0·5% (w/v) SDS was boiled and poured onto filters to be stripped. The solution was then allowed to cool at room temperature. The stripped filter was again exposed to X-ray film to determine if the removal of the probe was successful.

2·7·4 NORTHERN BLOT

This method is based on that described in Davis *et al.* (1986). All gel tanks, trays, and combs were soaked with 1% SDS solution for one hour prior to use, and rinsed thoroughly with RNase free water. To prepare the gel; 1% (w/v) agarose was dissolved in 1X MOPS buffer (*q.v.*)in a clean flask, free of RNase. The agarose was allowed to cool to ~60°C before 5.5% (v/v) of a 37% formaldehyde solution was added. The agarose was then poured into a electrophoresis apparatus and the gel allowed to solidify for at least 1 hour before use. RNA in 1µL to 5 µL aliquots was mixed with 20 µL of freshly prepared RNA loading buffer (*q.v.*)and 0.5µL of ethidium bromide (10mg/mL). The RNA samples were then heated to 65°C for eight minutes before being loaded. Electrophoresis was performed at a voltage ranging from 20-80 volts and RNA bands were visualised using a UV transilluminator. Gels were blotted in a similar manner as for Southern blots (section 2·7·1 [Southern blots]) except that the RNA gels were not washed before beginning the capillary blot transfer. In addition, the filters were not washed following transfer. RNA was fixed onto the filters via exposure to UV illumination for 2 minutes.

2·7·5 NORTHERN HYBRIDISATION PROTOCOL

This method is based on the Clontech ExpressHyb™ Hybridisation solution user manual (PT1190-1). Membranes to be hybridised were placed into plastic bags that were heat-sealed on three sides. Pre-warmed ExpressHyb™ solution (~10mL for a 10cm X 15 cm membrane) was added to the bag containing the membranes, and the bag completely sealed after all bubbles were removed. The membrane was pre-hybridised for 30 minutes at 68°C in a shaking water-bath. Following pre-hybridisation, double stranded cDNA probes were denatured by heating to 100°C for five minutes before being added to 10mL of fresh ExpressHyb™ solution. This solution was then immediately added to the bag containing the membrane (NB. Single stranded cDNA probes were not denatured before adding to the ExpressHyb™ solution). The probes were left to hybridise to the filters for one hour at 68°C in a shaking water bath. After hybridisation the filters were then incubated in RNA hybridisation wash 1 at room temperature for 10 minutes. This wash was repeated three to four times. If necessary, filters were incubated twice in RNA hybridisation wash 2 for 20 minutes at 50°C. Following the washes the filter was wrapped in plastic wrap and exposed to an X-ray film at -70°C for the appropriate length of time. The X-ray films were developed in a Agfa-Gevamatic 60 X-ray machine, under NX-914 safety lights. Following autoradiography membranes were stripped as described in section 2·7·3 (membrane stripping protocol) and re-probed with a *ubiquitin* probe to check for equal loading of RNA samples.

2·7·6 PHOSPHORIMAGING

As an alternative to autoradiography, Northern blot filters were phosphorimaged. Powder-free gloves were always used to handle the phosphorimaging screens and residual background signals were erased from the screen by exposure to bright light for at least 10 minutes. The radioactive membranes were wrapped in plastic and placed RNA-side up in the phosphorimaging cassette. The screen was then placed face down on top of the membrane and left to expose at room temperature for the appropriate length of time. Following exposure, the screen was placed into the STORM phosphoimager and the image scanned into a computer for analysis. The signal intensities for each gene were quantified realtive to those of *UBIQUITIN* using ImageQuant software (Molecular Dynamics).

CHAPTER 3:

EFFECTS OF CHEMICAL PERTURBATION OF POLYAMINE METABOLISM IN *ARABIDOPSIS*

3·1 INTRODUCTION

3·1·1 COMMONLY USED INHIBITORS OF POLYAMINE BIOSYNTHESIS

AN IMPORTANT METHOD FOR studying polyamine involvement in the growth of cells is through the use of irreversible and competitive inhibitors of polyamine biosynthesis. Putrescine production can be inhibited by amino acid analogues DL-a-difluromethylarginine (DFMA) and DL-a-difluromethylornithine (DFMO) which bind irreversibly to the active sites of key polyamine biosynthetic enzymes arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) respectively (Metcalf *et al.*, 1978; Kallio *et al.*, 1981). The enzyme *S*-adenosylmethionine decarboxylase has also been identified as a rate-limiting enzyme in polyamine biosynthesis and can be strongly inhibited by the competitive inhibitor methlyglyoxal bis(guanylhydrazone) (MGBG) (Williams-Ashman and Schenone, 1972). Another competitive inhibitor, cyclohexylamine (CHA) is commonly used to block the

production of spermidine from putrescine via the inactivation of spermidine synthase (Hibasami *et al.*, 1980). Presented in Figure 3·1 is the basic polyamine biosynthetic pathway in plants showing the points of enzyme inhibition by these commonly used polyamine biosynthetic inhibitors.

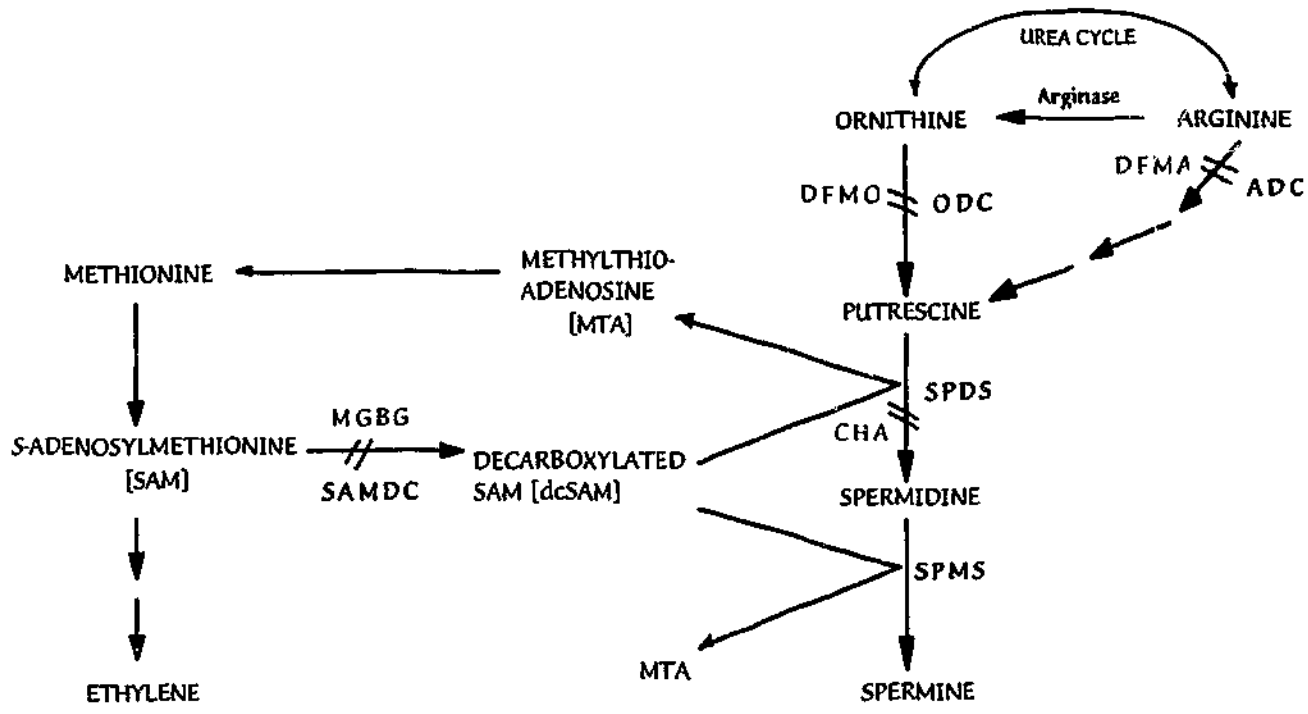


Figure 3·1: Points of blockage in the biosynthetic pathway of polyamines in plants, by inhibitors of key enzymes.

Inhibitors of key biosynthetic enzymes are indicated in red; DL-α-difluoromethylarginine (DFMA) and DL-α-difluoromethylornithine (DFMO) which block ADC and ODC respectively, methylglyoxal bis(guanylhydrazone) (MGBG) which blocks SAMDC, and cyclohexylamine (CHA) which blocks SPDS.

Treatment of whole plants or plant tissues with inhibitors of polyamine biosynthesis has proven useful in establishing correlations of polyamine involvement in various aspects of plant growth. Care must be taken, however, when interpreting results of experiments using such inhibitors. The application of DFMA to plant tissue for example, can lead to the inhibition of both ADC and ODC activities in some tissues as a result of the *in vivo* conversion of DFMA into DFMO through the action of the enzyme arginase (Slocum and Galston, 1985). Similarly, MGBG is not absolutely specific for SAMDC, and treatments

may lead to other unexpected effects such as inhibition of diamine oxidase thereby causing putrescine build up (Pegg and Williams-Ashman, 1987) or vacuolisation of mitochondria (Pleshkewych *et al.*, 1980; Jänne and Alhonen-Hongisto, 1989; Cheng *et al.*, 1990). Due to their similar structures, MGBG and spermidine have been proposed to share the same transport system in animal cells (Alhonen-Hongisto *et al.*, 1980; Porter and Sufrin, 1986), although recent evidence suggests that at low concentrations, this may not be the case in plant cells (Antognoni *et al.*, 1999). It has also been noted that the effects of reversion of MGBG-induced phenotypes by spermidine application may be due to displacement of MGBG from the uptake and transport system, rather than successful competition for SAMDC binding (Pegg and McCann, 1982). Although studies of inhibition of polyamine biosynthesis are correlative by nature, nonetheless they can prove useful in means for providing information on which further molecular characterisation of the polyamine biosynthetic pathway can be based.

3-1-2 TREATMENT OF PLANT TISSUES WITH POLYAMINE BIOSYNTHESIS INHIBITORS

DFMA and DFMO

Inhibition of putrescine accumulation in plant tissue following the application of either DFMA or DFMO has suggested an important role the diamine in promoting root growth. Effects of inhibitor treatment, however, appear to vary according to the specific type, and the developmental age, of the tissues involved. In a study of tobacco using callus tissue, low putrescine titres induced by DFMA were associated with a marked increase in rates of root organogenesis (Tiburcio *et al.*, 1987). In contrast, treatment with either DFMA or DFMO was shown to decrease growth and development of roots in rice seedlings (Bonneau *et al.*, 1994A; Lee, 1997), and when both inhibitors were combined, the same effect was seen in tobacco thin cell layers (Altamura *et al.*, 1991). Studies of embryogenesis have also used DFMA and DFMO to deplete endogenous polyamine titres, again with differing results depending upon the system. During the process of pollen embryogenesis in tobacco,

treatment with these inhibitors at concentrations of between 1·5mM and 12mM suggested that polyamines are required for growth (Garrido *et al.*, 1995). Studies of somatic embryogenesis in carrot cell cultures however, showed that although DFMO inhibited the accumulation of putrescine, cell growth and embryo development were unaffected by treatment with DFMO up to 5mM (Mengoli *et al.*, 1989).

Polyamines have also been suggested to play an important role in the process of flowering in a range of plants. Treatments with DFMO caused reductions in the levels of free and conjugated putrescine, which were correlated with an inhibition of flowering in a variety of plants, including chrysanthemum (Aribaud and Martin-Tanguy, 1994A), strawberry (Tarenghi and Martin-Tanguy, 1995), and wild mustard (Havelange *et al.*, 1996). The lack of inhibition caused by DFMA (Aribaud and Martin-Tanguy, 1994A; Tarenghi and Martin-Tanguy, 1995), led these authors to suggest that the ornithine pathway to putrescine production predominates during flowering.

Stimulation of ADC activity has been shown to be an important mechanism for increasing putrescine in plants following exposure to osmotic stress (Flores and Galston, 1982). Under non-stressed conditions, DFMA application to *Brassica napus* leaf discs decreases endogenous polyamine levels, whereas DFMO has no effect, suggesting a predominant role for the ADC route to putrescine in this system (Aziz *et al.*, 1997). Studies involving both DFMA and DFMO, have also implicated ODC in this phenomenon. The application of either 5mM DFMA or 5mM DFMO to leaf discs exposed to osmotic stress caused by growth in the presence of PEG6000, however, decreased putrescine titres in both cases, suggesting that ODC activation is also involved in perturbing polyamine titres under conditions of osmotic stress in *Brassica napus* (Aziz *et al.*, 1997). Such a response of ODC may be specific to conditions of non-ionic osmotic stress, as it was not seen when tomato leaf discs were subjected to salinity stress and treated with DFMA or DFMO (Aziz *et al.*, 1998). In rice seedlings subjected to chiling, tolerance to cold stress was found to be mediated by high titres of putrescine, both of which were abolished following treatment with DFMA, but were unaffected by DFMO (Lee, 1997).

CHA and MGBG

Treatment of plant tissues with either CHA and MGBG both cause reductions in the levels of endogenous spermidine, with a concomitant increase in putrescine levels. This response has been reported in a variety of plants such as carrot (Minocha *et al.*, 1991B), pea (Villaneuva and Huang, 1993), chick-pea (Gallardo *et al.*, 1994), and *Brassica napus* (Aziz *et al.*, 1997), Following CHA or MGBG treatments, altered polyamine levels have been correlated with reductions in growth processes including the synthesis of cellulose (Berta *et al.*, 1997), the frequency of cell division (Altamura *et al.*, 1993), the formation of root meristemoids (Altamura *et al.*, 1991), the growth of leaves and roots (Bonneau *et al.*, 1994A), seed germination (Bonneau *et al.*, 1994B), and pollen germination and tube growth (Song *et al.*, 2001). Such alterations in growth processes, however, appear to be closely related to tissue type and developmental age. Application of the inhibitors to rice seeds for example, did not inhibit germination in high germination-potential seeds, whereas they were inhibitory in low germination-potential seeds (Bonneau *et al.*, 1994B).

Following from their effects in altering polyamine accumulation in treated plant tissue, treatment with the inhibitors also alters the activities of some of the enzymes involved in polyamine biosynthesis. The increased putrescine accumulation seen with MGBG application resulted in a reduction of ADC activity, probably due to feedback inhibition (Hiatt *et al.*, 1986; Minocha *et al.*, 1991B). An unexpected increase in ADC activity, however, has been noted in CHA-treated carrot cells, possibly contributing to the elevation in putrescine that was observed in these cells (Khan and Minocha, 1991). Most reports note that treatment with MGBG reduces the activity of SAMDC as was demonstrated in treated tissues of pea (Villaneuva and Huang, 1993) and soybean (Yang and Cho, 1991). Occasionally however, binding of MGBG stabilises the enzyme and protects it from proteolytic activity, resulting in increased levels of SAMDC as was observed in tobacco by Hiatt *et al.* (1986).

As both the polyamine and ethylene biosynthetic pathwa compete for the common precursor *S*-adenosylmethionine (SAM), reports of increased production of ethylene following treatment with MGBG (Roberts *et al.*, 1984; Muñoz de Ruedo *et al.*, 1994; Scaramagli *et al.*,

1999B) or CHA (Muñoz de Ruedo *et al.*, 1994; Gallardo *et al.*, 1995) are not unexpected. In chick-pea seeds undergoing thermoinhibition of germination, the production of ethylene was markedly reduced (Gallardo *et al.*, 1991). Upon co-treatment with CHA or MGBG, levels of polyamines within the seeds decreased, whilst ethylene production increased, correlating with a reversal of the thermoinhibition of germination (Gallardo *et al.*, 1994). Thus, alterations in growth following the application of these inhibitors may be due to ethylene-induced effects, rather than the result of perturbed polyamine accumulation.

3·1·3 TREATMENT OF PLANT TISSUES WITH EXOGENOUS POLYAMINES

Exogenous feeding of polyamines to plants may influence growth and development, however, the different effects exerted by the individual polyamines may vary according to the cellular compartment and developmental stage (Ye *et al.*, 1994; Mader, 1995). In conjunction with alterations in phenotype, exogenous polyamine application to plant tissue has been observed to prodcue decreased ADC activity in tobacco (Hiatt *et al.*, 1986), whilst treatment with spermidine alone reduced SAMDC activity (Hiatt *et al.*, 1986; Yang and Cho, 1991; Tassoni *et al.*, 2000). Low levels of exogenous putrescine enhanced root elongation in rice, whereas treatment with spermidine or spermine were inhibitory (Lee, 1997). On the other hand, callus tissue of Scots Pine was unresponsive to exogenous spermidine or putrescine, however, spermine treatment was markedly inhibitory to growth (Laukkanen and Sarjala, 1997). Spermidine treatment altered the phenotype of *Arabidopsis* plants, resulting in stunted shoots with dark green leaves (Tassoni *et al.*, 2000), and was also observed to promote or inhibit flowering, depending upon the time of application (Applewhite *et al.*, 2000). An in-depth study of the application of spermidine and spermine to spinach sub-membrane fractions, found that photosynthetic oxygen evolution was disrupted, with a specific inhibition of electron transport in photosystem II (Bograh *et al.*, 1997).

3·2 RESULTS

3·2·1 ISOLATION OF SEQUENCES ENCODING THE POLYAMINE BIOSYNTHETIC GENES IN *ARABIDOPSIS*

At the commencement of this study, gene sequences representing *ADC*, *ODC*, *SPDS*, and *SAMDC* had not been characterised from *Arabidopsis*. As a result, much effort was devoted to the isolation and cloning of fragments of these genes in order to use them as probes for Northern hybridisations. It was intended to study the transcription of these genes in *Arabidopsis* plants under various conditions, in order to provide insight into regulatory mechanisms involved in the polyamine biosynthetic pathway.

Arginine decarboxylase *(ADC)*

Four *Arabidopsis* EST clones [GenBank accession numbers: Z37204, Z37205, Z33969, and T46784] were identified from the database as possessing similarity at the protein and DNA levels with the arginine decarboxylase sequence from tomato. The nucleotide sequences of the four clones were translated in all six open reading frames, and aligned with the amino acid sequence of ADC from tomato (*Lycopersicon esculentum*; GenBank accession number L16582). Clones Z37204 and Z33969 both exhibited regions of homology to the 3′ region of the tomato *ADC* sequence. Both *Arabidopsis* clones were found to contain a stretch of residues (FPIVPIH) within this region that was conserved in both tomato and pea (*Pisum sativum*; GenBank accession number Z37540) *ADC* sequences. An oligonucleotide primer (5′ TGGATTGGAACAATAGGAAA 3′), to be used in an attempt to amplify *ADC* from *Arabidopsis*, was therefore designed based on the 3′ sequence of the Z37204 clone, which was found to have the highest level of homology (71%) to the tomato *ADC* gene.

As the sequence of the *Arabidopsis* EST clone was located at the 3′ end of the tomato *ADC* gene, a region of homology was required at the 5′ end of the sequence in order to design a

forward PCR primer. The residues LVVRFPD were found to be conserved in tomato and pea *ADC* genes, and were used to design a forward primer (5′ AGACTTGTTGTTCGGTTTCCT 3′). The *ADC* sequence of tomato was used in preference over that of oat (*Avena sativa*; GenBank accession number S12265) when designing PCR primers, as tomato is a dicot and also to allow the possibility of amplification of the gene from *Nicotiana tabacum* for seperate studies in the laboratory. Standard conditions for PCR were used and a fragment of the expected size (1·2kb) was obtained.

The complete sequence of *ADC* from *Arabidopsis* was subsequently reported in the GenBank database [accession number ATU52851]. The DNA sequence of the PCR product was aligned with the reported *Arabidopsis ADC* gene, confirming that the amplified fragment was did represent part of *Arabidopsis ADC* (Appendix 1).

Ornithine decarboxylase *(ODC)*

A fragment of the tobacco *ODC* gene was obtained from PCR amplification using primers based on the *ODC* sequence of *Datura* (Michael *et al.*, 1996). 5′ RACE (rapid amplification of cDNA ends) was used on the *ODC* amplification product in order to obtain a 'virtual' full length cDNA sequence [GenBank accession number Y10472] (Lidgett, 1997). A primary screen was performed in duplicate on a root cDNA library of *Arabidopsis* using *Datura ODC* cDNA (Michael *et al.*, 1996) as a probe. Initial library screens were undertaken by Professor J. D. Hamill, probing at low stringency [55°C, 2 X SSC and 0.1% SDS washes]. Further purification was undertaken in this study. Four potential positives were identified, labelled At01 to At04 respectively, and secondary and tertiary screens performed. Plasmids containing cloned *Arabidopsis* and tobacco fragments were isolated from putative positive plaques using *in vivo* excision (ExAssist/SolR system from Stratagene; Lot#UC121), and inserts were subsequently sequenced. In order to attempt recovery of a *bona fide* full-length cDNA sequence from tobacco, a tobacco cDNA root library was screened in parallel using the *Datura ODC* cDNA. Three positive clones were identified, and designated Nt01, Nt02, and Nt03 respectively.

DNA sequence analysis of the putatively positive *Arabidopsis* clones revealed neither significant homology with each other (Table 3·1) nor with any of the tobacco clones (Table 3·2). When compared with the sequences available in the database, *Arabidopsis* clone At01 showed high homology to aquaporins from a range of plants, and was found to have highest identity with an *Arabidopsis* mRNA for plasma membrane intrinsic protein 1c (99·4% over a 478bp overlap). Clone At02 matched with a *Brassica napus* myrosinase-associated protein (74% over a 371bp overlap), and clone At03 was similar to glutamine synthase from a range of plants, especially the *B. napus* mRNA for glutamine synthase isoform (86% over a 452bp overlap). Clone At04 however, exhibited limited homology to a variety of unrelated genes, and most likely represents an as yet unidentified sequence from *Arabidopsis*. The DNA sequences of the inserts from the positive tobacco clones showed no homology to each other (Table 3·3), however, the insert in clone Nt01 was confirmed to be that of the *ODC* gene from tobacco by comparison with the sequence reported in the database (Table 3·4).

Arabidopsis clone	At02	At03	At04
At01	37%	40%	42%
At02	-	39%	36%
At03	-	-	37%

Table 3·1: DNA sequence homology between positive *Arabidopsis ODC* clones.

Tobacco clones	*Arabidopsis* clones			
	At01	At02	At03	At04
Nt01	32%	42%	36%	41%
Nt02	37%	38%	36%	38%
Nt03	40%	38%	40%	35%

Table 3·2: DNA sequence homology between positive *Arabidopsis ODC* clones and positive tobacco *ODC* clones.

Tobacco clone no.	Nt02	Nt03
Nt01	34%	38%
Nt02	-	34%

Table 3·3: DNA sequence homology between putative positive tobacco *ODC* clones.

Tobacco clone number	Homology with tobacco *ODC* gene
Nt01	94%
Nt02	39%
Nt03	40%

Table 3·4: DNA sequence homology of positive tobacco *ODC* clones with the tobacco *ODC* gene sequence (GenBank accession number Y10472)

That the *Datura ODC* cDNA probe identified a tobacco *ODC* gene during library screening, suggests that the negative result from the *Arabidopsis* screenings were not due to experimental error. Furthermore, these results, indicating that an *Arabidopsis ODC* sequence was not detected by screening an *Arabidopsis* root cDNA library with a tobacco *ODC* probe, contributed to the recent publication by Hanfrey *et al.* (2001) who reported that *Arabidopsis* lacks *ODC*.

S-adenosylmethionine decarboxylase *(SAMDC)*

During this study, the sequence for the *SAMDC* gene from *Arabidopsis* was made available on the database [GenBank accession number: Y07765]. Therefore gene-specific primers were made directly from this sequence (forward: 5′ TCAACTTAATCGTTTCTCTC 3′; reverse: 5 ′ CCAATACAACTTGCTTGTGAAGG 3′). Standard conditions for PCR were used and a fragment of approximately 2·3kb was obtained. Sequence analysis of the amplified product confirmed it to be that of the *Arabidopsis SAMDC* gene (Appendix 2). Recently a second SAMDC gene present in the *Arabidopsis* genome has been identified (GenBank accession number: AJ252212 [genomic], AJ251915 [cDNA]). This smaller gene (2·0kb) share 72% identity with the previously published gene sequence (GenBank accession numbers: Y07765 [genomic], U63633 [cDNA]).

Spermidine synthase *(SPDS)*

Database searches identified an *Arabidopsis* EST [GenBank accession number: T20920] of approximately 350bp in length (Appendix 3), that has limited overall homology to *speE*, the human gene for spermidine synthase [GenBank accession number: P19623]. The translated *Arabidopsis* EST sequence, however, was found to possess several residues which are conserved in tobacco [GenBank accession number: D28506], human, and mouse [GenBank accession number: L19311] SPDS proteins (Appendix 4). A forward oligonucleotide primer for PCR amplification was therefore synthesised, based on this conserved region. This oligonucleotide also contained a *Bam*HI restriction site and a guanine anchor (5′ GGGGGATCCATGTGGCCAGGAGAGGCACA 3′). The design of a degenerate reverse PCR primer was based on the conserved amino acid residues KAAFILP found at the 3′ end of the tobacco, human, and mouse *SPDS* genes. In order to facilitate cloning, this oligonucleotide was also synthesised with a guanine anchor and an *Eco*RI restriction site (5′ GGGGAATTCA(A/G)(A/G)A(C/T)(G/A)AAAGCAGGC(T/C)T(G/A)TG 3′). Standard conditions for PCR with genomic DNA were used . After several trials a fragment of approximately 900bp was amplified, sub-cloned into a TA cloning vector and sequenced. Analysis revealed however, that the clone was 91% homologous at the DNA level to an Athila retroelement, which have recently been identified as a new family of retroelements in *Arabidopsis*. Several other amplification products of less than 500bp in size, were also obtained and by Southern hybridisation these were found to show some homology to a tobacco putrescine methyltransferase (*PMT*) probe, which evolved from spermidine synthase (Hibi *et al.*, 1994). Several attempts were made to re-amplify these smaller PCR products, however, they proved unsuccessful. A comparison of the DNA sequences of the putative tobacco *SPDS* gene subsequently reported in the database [GenBank accession number D28506] and the *Arabidopsis SPDS* EST revealed only 48% identity (Appendix 5).

An alternative approach was taken to isolate the *SPDS* gene based on oligonucleotide primers from the *Arabidopsis SPDS* EST sequence (Appendix 5). Primers SPDSF2·1 and SPDSR2·1 amplified a band of the expected size (310bp) from *Arabidopsis* genomic DNA, and were

then used in a RACE procedure to obtain a larger region of the gene (Appendix 6). Figure 3·2A presents the results of the RACE PCR with varying amounts of cDNA templates from both shoot and root tissue. The autoradiographs following hybridisation with the *SPDS* PCR product (Figures 3·2B and 3·2C) showed hybridisation bands corresponding to both the 5′ and 3′ products. As expected, the size of the 5′ extension product is essentially the same as that of the 310bp PCR product, since the SPDSF2·1 and SPDSR2·1 primer sites are located at the 5′ end of the gene. The single hybridisation band representing the 3′ amplification is approximately 1·3kb in size, corresponding with the sizes of *SPDS* transcripts from human [GenBank accession number: BC000309] and mouse [GenBank accession number: Z67748].

Several attempts were made to reamplify the 3′ RACE PCR products, either directly from the PCR mix, or purified from agarose, in order to obtain sufficient DNA for cloning and to confirm the specificity of the primers. Reamplification however was not successful for reasons unclear as the 5′ RACE product was able to be reamplified using both the SPDSR2·1 and adaptor-specific primers (AP2), or nested primers and AP2.

Given that the 3′ RACE amplification product was of the expected size (1·3kb) and hybridised to the *SPDS* EST probe, and since the *Arabidopsis SPDS* gene was sought only for use as a probe in Northern hybridisations, it was decided that the 310bp *SPDS* PCR product would be sufficient for use as a hybridisation probe. Furthermore, a partial coding sequence for the *Arabidopsis SPDS* gene [Accession number: AB006693] was subsequently made available in the database, which when aligned with the *Arabidopsis SPDS* EST sequence gave 84% homology, confirming that the 310bp probe was part of the gene (Appendix 7).

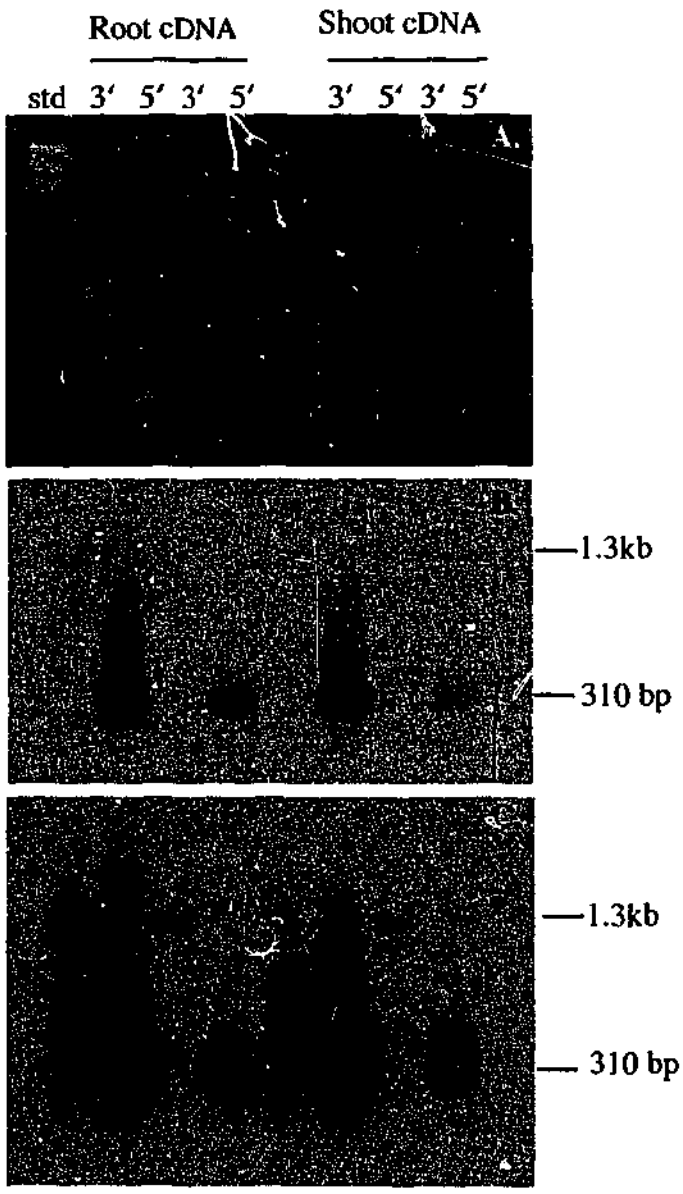


Figure 3. 2 : RACE PCR of the *SPDS* gene from *Arabidopsis*.

A. Ethidium bromide-stained gel of the amplified RACE products. λ*Bst*EII is the size standard. cDNA was made from both shoot and root total RNA.
B. Filter probed with the 310bp *SPDS* PCR product; 30 hour exposure.
C. Filter probed with the 310bp *SPDS* PCR product; 1 week exposure.

3·2·2 BIOLOGICAL EFFECTS OF TREATMENT WITH POLYAMINES OR POLYAMINE BIOSYNTHESIS INHIBITORS

Whole plant morphology

The inclusion of polyamines, or their biosynthetic inhibitors in culture media, at levels ranging from 0·1mM to 3mM was able to alter several growth parameters of whole wild-type *Arabidopsis* plants including inflorescence height, shoot and root weights, endogenous polyamine titres, and the expression of the polyamine biosynthetic genes.

General morphology

The phenotypes of plants grown in the presence of the diamines agmatine and putrescine are shown in Figures 3·3 and 3·4 respectively. Both treatments caused a slight change in shoot morphology, stimulating the production of axillary inflorescences, which was slightly evident at week five and much more so at week ten post germination. Similarly, treatment with spermidine (Figure 3·5) and spermine (Figure 3·6) also resulted in shoots containing more axillary inflorescences than treated controls. Spermidine at1mM and 3mM produced stunted shoots, but interestingly, in the case of 1mM, also markedly stimulated root growth (Figure 3·5). A low level of spermine (0·1mM) was also stimulatory to root growth, however, it did not retard the growth of shoots (Figure 3·6). Heights of primary inflorescences following putrescine treatment are presented in Table 3.5. After nine weeks of treatment post germination, however, any inhibitory effects of polyamines on shoot height were diminished, and the plants exhibited a 'normalising' of the phenotype, and were not visibly different from untreated controls.

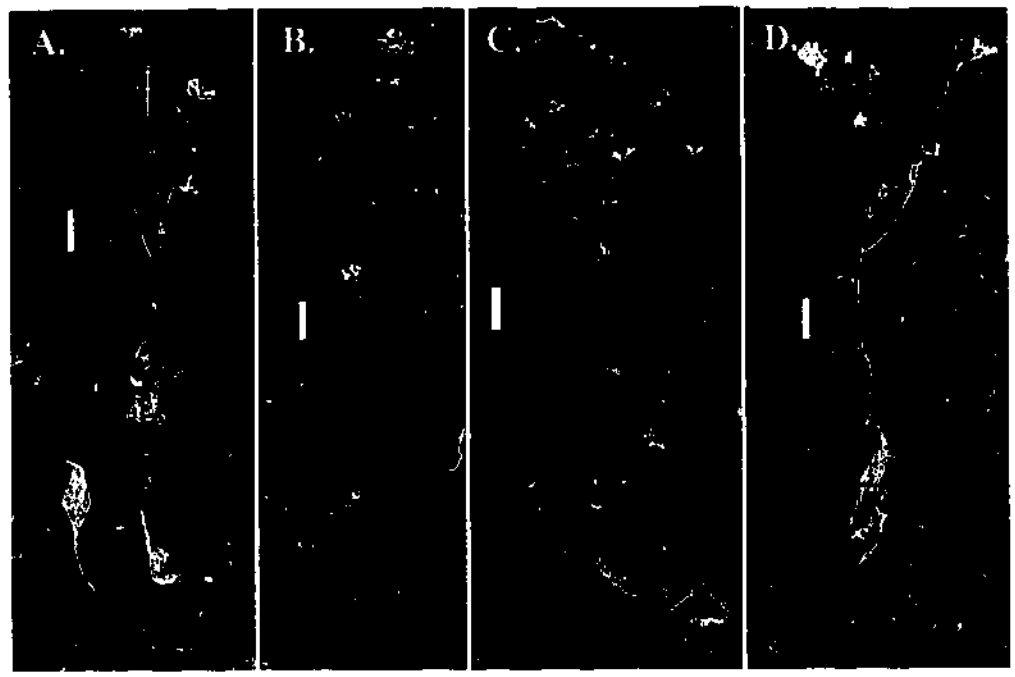


Figure 3.3 : Effects of exogenous agmatine (AGM) on the *in vitro* morphology of wild-type L. *erecta* (week 10 of growth).
A. Untreated **B.** 0.1mM AGM **C.** 1mM AGM **D.** 3mM AGM
(Measurement bars represent 1cm)

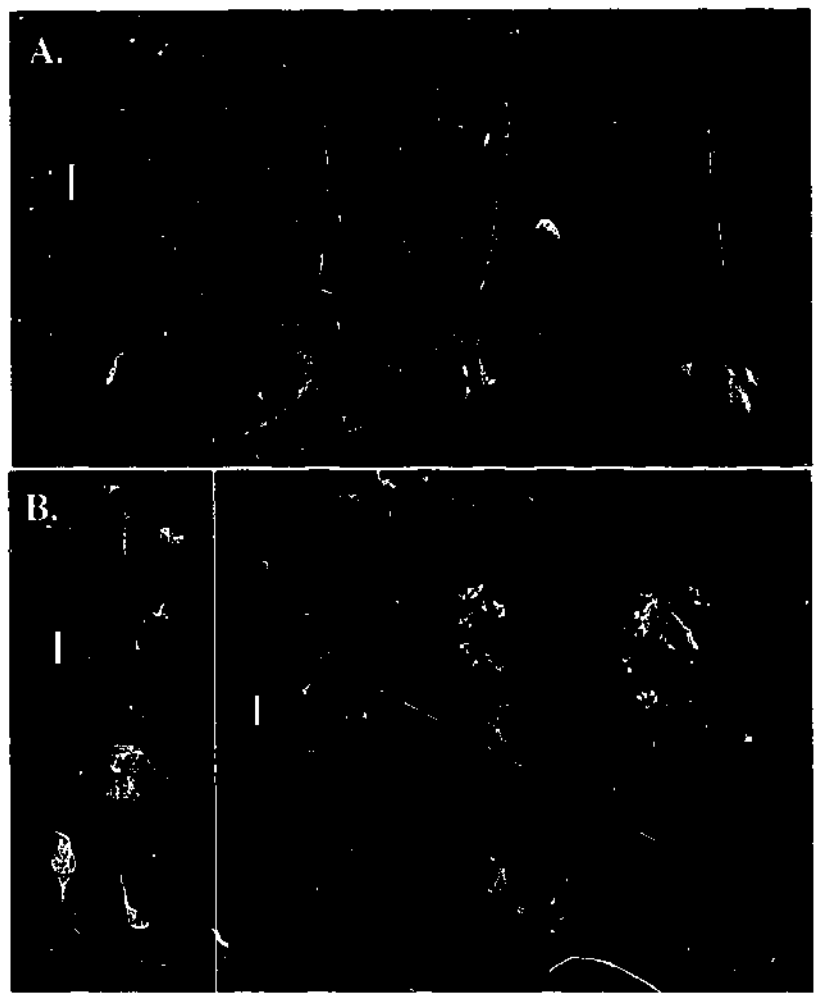


Figure 3.4: Effects of exogenous putrescine (PUT) on the *in vitro* morphology of wild-type L. *erecta*.
A. Week 5. Left to right: Untreated, 0.1mM PUT, 1mM PUT, 3mM PUT
B. Week 10. Left to right: Untreated, 0.1mM PUT, 1mM PUT, 3mM PUT
(Measurement bars represent 1cm)

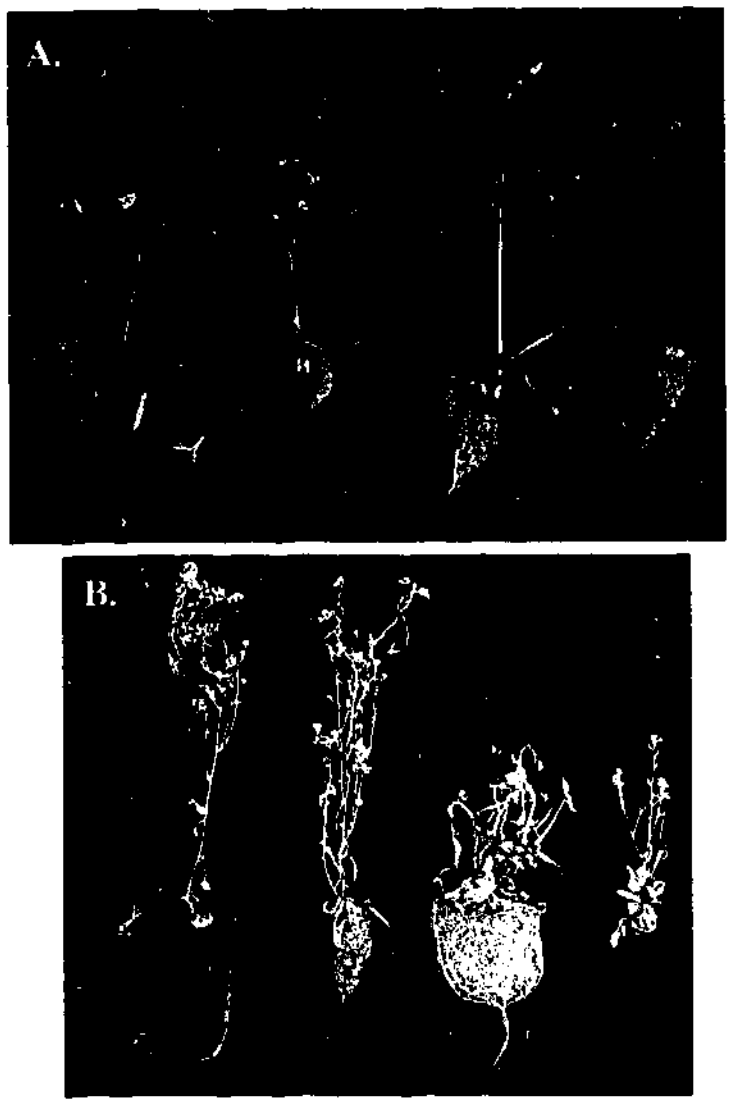


Figure 3.5: Effect of exogenous spermidine (SPD) on the *in vitro* morphology of wild-type L. *erecta*.
A. Week 5. Left to right: Untreated, 0.1mM SPD, 1mM SPD, 3mM SPD
B. Week 9. Left to right: Untreated, 0.1mM SPD, 1mM SPD, 3mM SPD

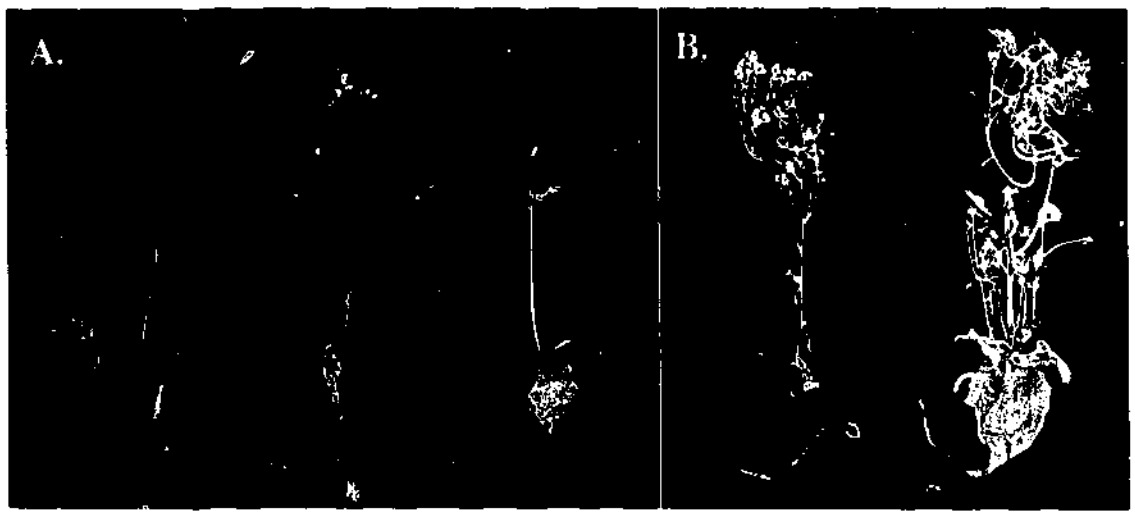


Figure 3.6: Effect of exogenous spermine (SPM) on the *in vitro* morphology of wild-type L. *erecta*.
A. Week 5. Left to right: Untreated, 0.1mM SPM, 1mM SPM
B. Week 9. Left to right: Untreated, 0.1mM SPM

Treatment	*Week 5*	*Week 9*
MS Control	7·00 ± 0·00	11·86 ± 0·58
0·1mM PUT	4·53 ± 0·55	13·27 ± 1·17
1mM PUT	6·86 ± 0·35	13·30 ± 1·84
3mM PUT	6·90 ± 0·48	14·26 ± 1·66
0·1mM SPD	4·10 ± 0·49	11·18 ± 1·44
1mM SPD	6·23 ± 0·84	9·42 ± 1·97
3mM SPD	V	8·95 ± 3·25
0·1mM SPM	5·10 ± 0·29	12·23 ± 2·71
1mM SPM	3·00 ± 1·20	not tested

TABLE 3·5: Primary inflorescence heights after feeding with polyamines Average height in cm ± SEM presented. n ranges from 3 to 8 plants per treatment. V= non-flowering vegetative shoot.

Treatment with the polyamine biosynthetic inhibitors, CHA and MGBG also caused a stunting of the plants which was observable at week five and somewhat diminished by week nine of growth (Table 3.6).

Treatment	*Week 5*	*Week 9*
MS Control	10·26 ± 1·20	18·82 ± 2·69
0·1mM CHA	10·25 ± 1·35	11·93 ± 3·53
1mM CHA	9·02 ± 1·17	18·07 ± 0·92
3mM CHA	8·10 ± 0·76	13·90 ± 3·39
0·1mM MGBG	9·10 ± 1·90	7·63 ± 4·04
1mM MGBG	6·92 ± 0·51	16·13 ± 0·50
3mM MGBG	0·40 ± 0·06	toxic (death)

TABLE 3·6: Primary inflorescence heights after feeding with polyamine biosynthetic inhibitors . Average height in cm ± SEM presented. n ranges from 3 to 8 plants per treatment

Inhibition of spermidine biosynthesis, using the inhibitor CHA, resulted in a slight stunting of shoots which was observable at week five of growth with all concentrations tested (Figure 3·7). By week nine post germination, however, only those plants treated with 3mM CHA remained stunted, whereas all others showed a 'normalising' of the phenotype. At this age, the production of axillary shoots was evident following treatment with all levels of CHA.

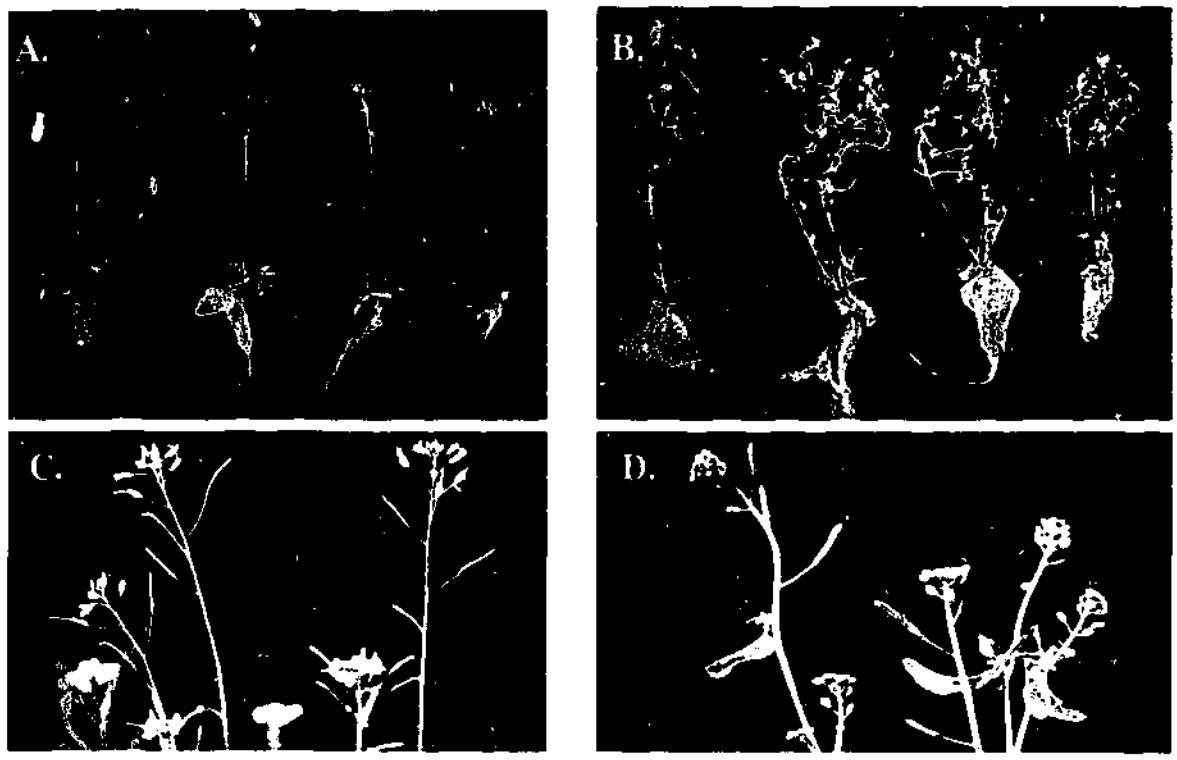


Figure 3.7: Effect of exogenous CHA on the *in vitro* morphology of wild-type L. *erecta*.
A. Week 5. Left to right: Untreated, 0.1mM CHA, 1mM CHA, 3mM CHA
B. Week 9. Left to right: Untreated, 0.1mM CHA, 1mM CHA, 3mM CHA
C. Flowers from untreated L. *erecta* after transfer to soil
D. Flowers from L. *erecta* plants treated with 5mM CHA after transfer to soil

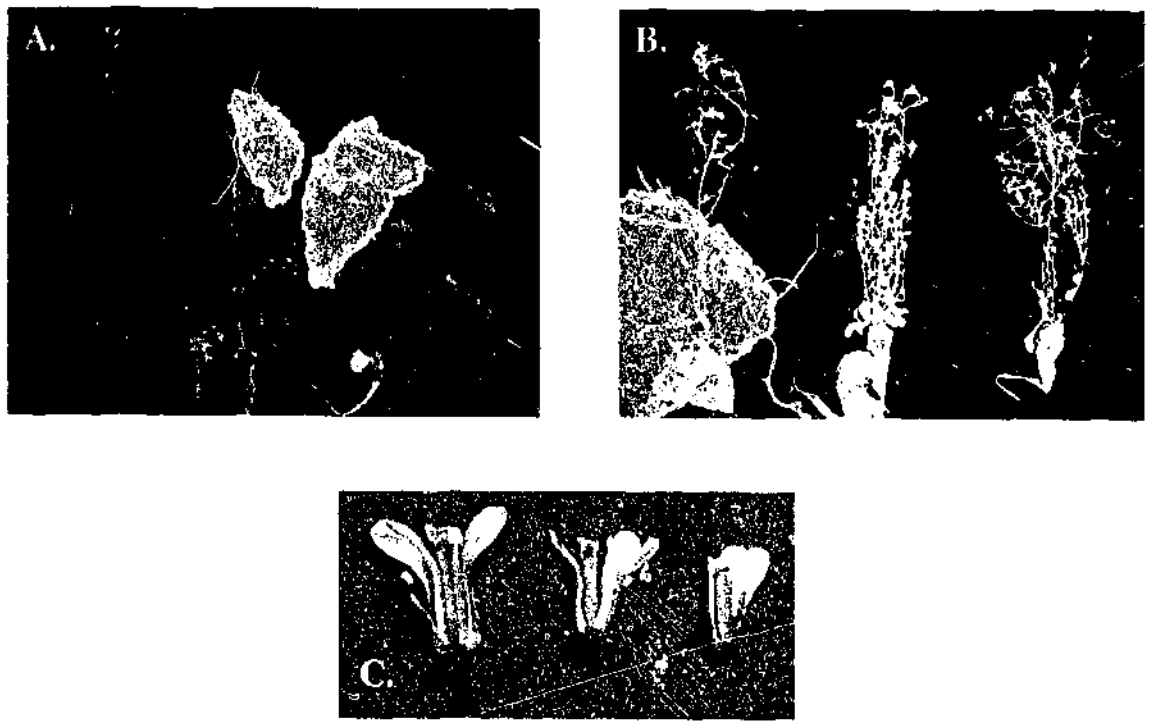


Figure 3.8: Effect of exogenous MGBG on the *in vitro* morphology of wild-type L. *erecta*.

A. Week 5. Left to right: Untreated, 0.1mM MGBG, 1mM MGBG, 3mM MGBG
B. Week 9. Left to right: Untreated, 0.1mM MGBG, 1mM MGBG, 3mM MGBG
C. Flowers from L. *erecta*. Left to right: Untreated, 0.1mM MGBG, 1mM MGBG

Plants treated with the slightly higher level of 5mM CHA, for 14 days *in vitro* before transfer to soil did not exhibit a complete 'normalising' of the treated phenotype. The growth of such plants was delayed, and they possessed small, unopened, green flower buds contrasting with the opened flowers found on untreated control plants (Figure 3·7C and 3·7D). Plants treated with MGBG followed a similar trend, in that five-week old shoots had a stunted phenotype which was dose-dependent. Similarly to CHA-treated plants this phenotype 'normalised' by week nine of growth (Figure 3·8). Again, many axillary shoots were present on the MGBG-treated plants during the later stage of development. Treatments of 0·1mM and 1mM MGBG appeared inhibitory to growth, as seen from the smaller-sized flowers on treated plants compared to controls (Figure 3·8C), and 3mM MGBG was found to be toxic after five weeks exposure. The effects of DFMO were different to the other inhibitors, inasmuch as there was no 'normalising' of the altered phenotype by the latter stage of growth (Figure 3·9). Stimulatory effects of this inhibitor on root growth, were evident after six weeks of treatment (Figure 3·9A), whereas inhibitory effects on shoot growth were not clearly evident until after 10 weeks of growth (Figure 3·9B).

Shoot and root weight

The effects of polyamine treatment on the fresh weights of shoots and roots of whole plants are presented in Figure 3·10. At week five post germination, the only marked difference in shoot weight was seen following treatment with spermine, which caused a reduction compared to the wild-type controls. On average, weights of roots from polyamine-treated plants were, however, lower than those of the controls. In contrast, by week nine of *in vitro* growth post germination , the average weights of all the treated shoots and roots, except for those exposed to 0·1mM putrescine and 3mM spermidine, were greater than the controls (Figure 3·10).

Polyamine biosynthetic inhibitors, particularly MGBG, were effective in altering shoot and root weights during the earlier stage of development (Figure 3·11). At week five of growth for example, only the highest concentration of CHA tested, 3mM, was inhibitory to shoot and root weight, whereas all three levels of MGBG tested were inhibitory. As growth progressed

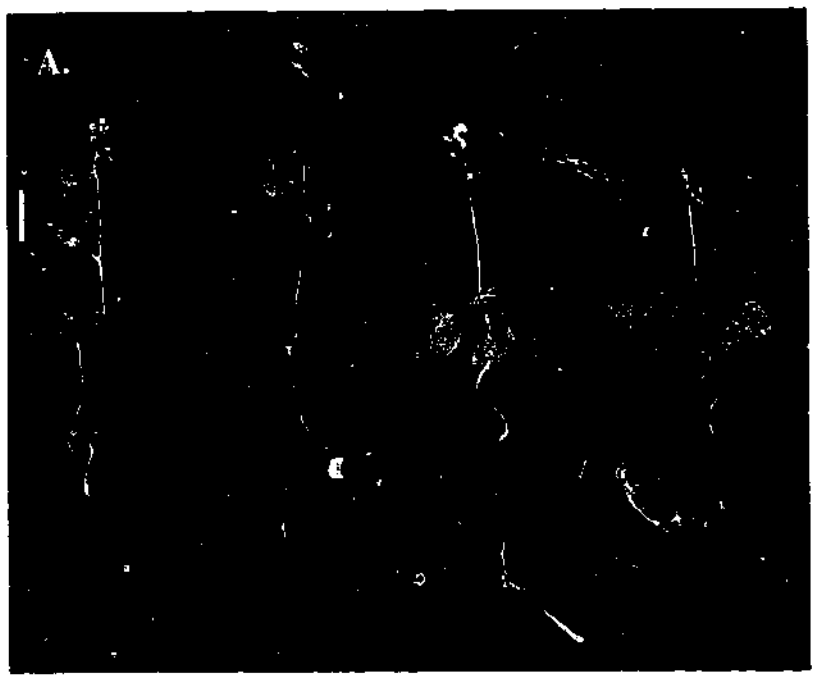
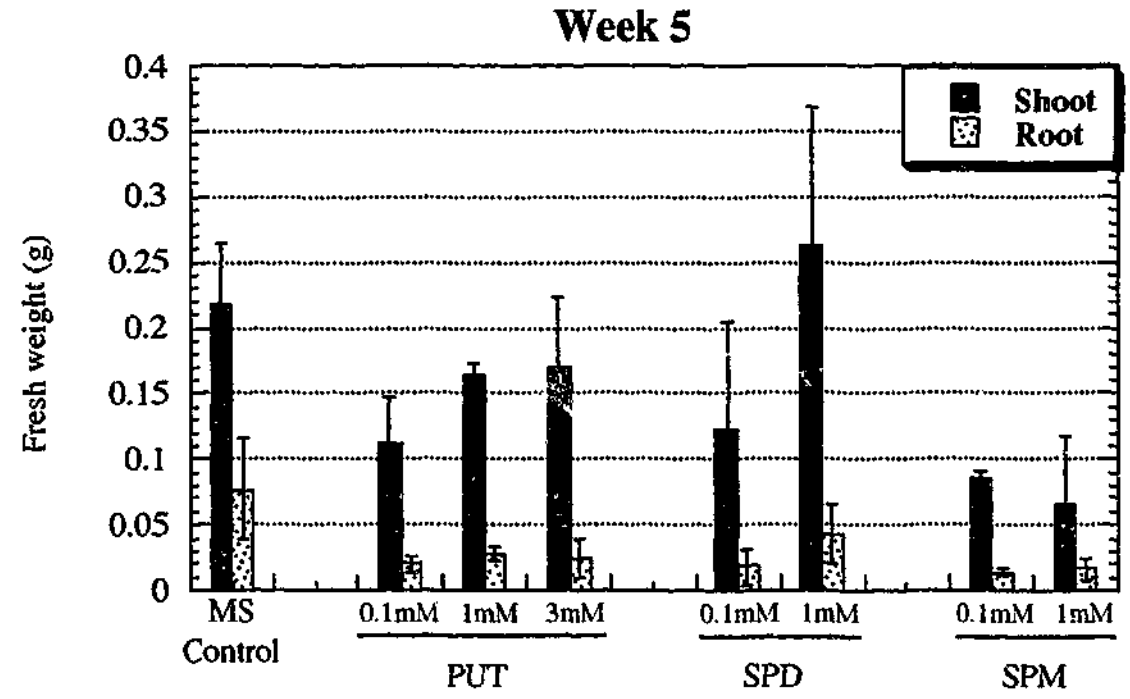


Figure 3.9 : Effects of exogenous DFMO treatment on the *in vitro* morphology of wild-type L. *erecta*.

A. Week 6. Left to right: Untreated, 0.1mM DFMO, 1mM DFMO, 3mM DFMO
B. Week 10. Left to right: Untreated, 0.1mM DFMO, 1mM DFMO, 3mM DFMO

(The measurement bars represent 1cm)



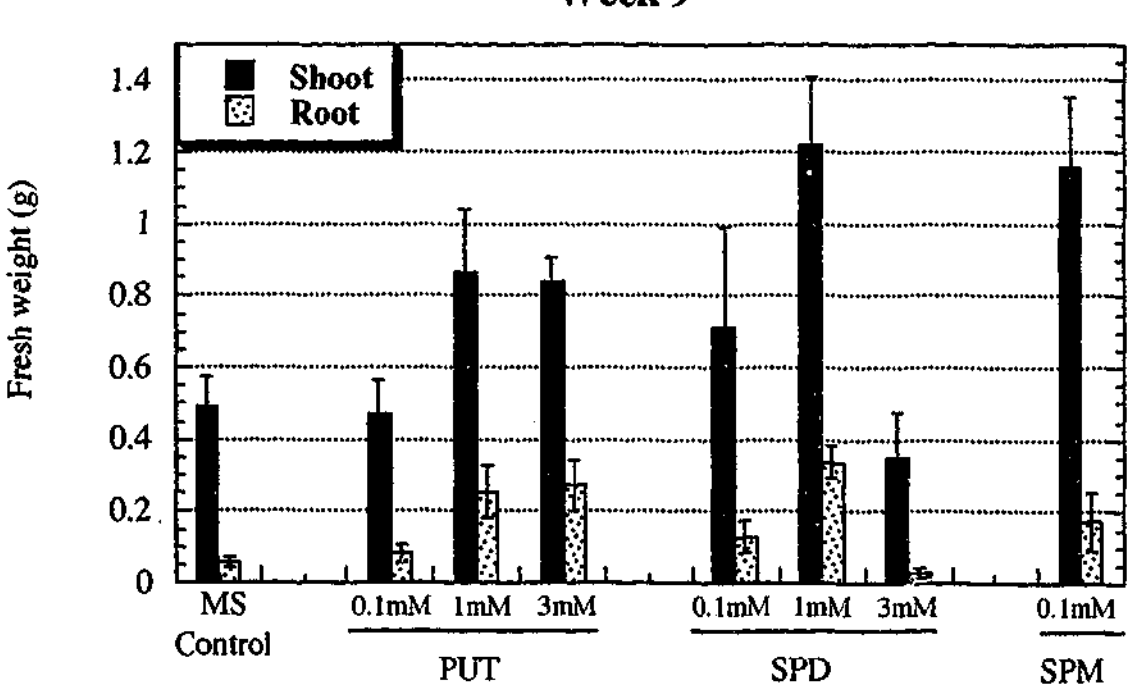


Figure 3.10: Effects of exogenous polyamines on shoot and root fresh weight of wild-type L. *erecta* at days 33 and 60 of *in vitro* growth.

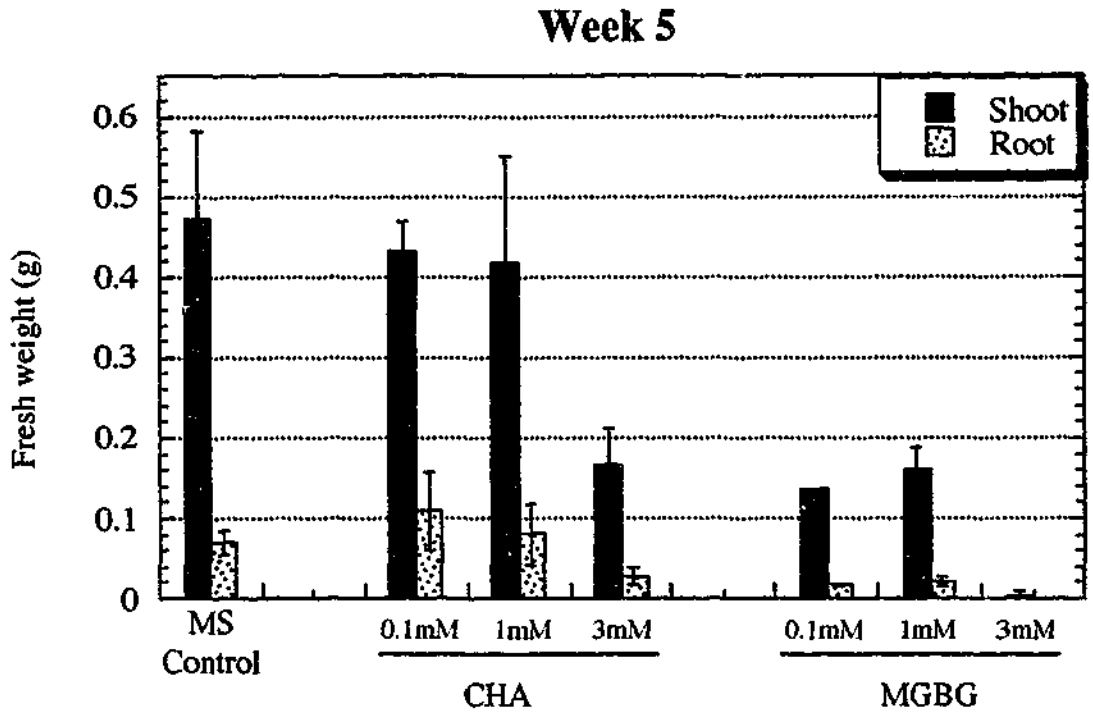


Figure 3.11: Effects of exogenous polyamine biosynthesis inhibitors on shoot and root fresh weight of wild-type L. *erecta* at days 33 and 60 of *in vitro* growth.

however, the efficacy of the inhibitors were reduced, such that by week nine no differences in weights were observed between the treated and untreated plants (Figure 3·10).

Flowering time

Application of the putrescine, spermidine, or spermine, or their biosynthetic inhibitors resulted in minor delays in flowering times of treated plants, as measured by the standard parameter of rosette leaf number at flowering. The histograms in Figure 3·12 show that the spermidine and spermine delayed flowering in a dose-dependent manner, whereas the delaying effects of putrescine did not increase with concentration. Treatment with the inhibitors CHA or DFMO delayed flowering to an equal extent, whilst MGBG was only effective at extending the period of vegetative growth when used at 3mM—a level at which death resulted before the initiation of flowering.

Endogenous free polyamine titres

Treatment with polyamines

When grown in the presence of putrescine, spermidine, or spermine, wild-type *Arabidopsis* seedlings were able to take up the polyamines from the media and translocate them to the shoots, as observed by the altered endogenous titres of the respective free polyamines at week 5 of growth (Table 3·7). Feeding putrescine resulted in a slight increase in free spermidine levels, however, treatment with spermidine did not increase titres of spermine above the levels observed in untreated control plants. Although results obtained were somewhat variable, the increase in putrescine titres after treatment with 0·1mM spermidine indicates a capacity in *Arabidopsis* for conversion of spermidine to putrescine. Analogous to the 'normalising' effects on morphology observed after nine weeks of continual treatment, endogenous free polyamine titres following most treatments are within the ranges observed for the control plants at this stage of development. The only exceptions were plants treated with 3mM spermidine and 0·1mM spermine, which maintained elevated free polyamines even at this later stage of growth (Table 3·7).

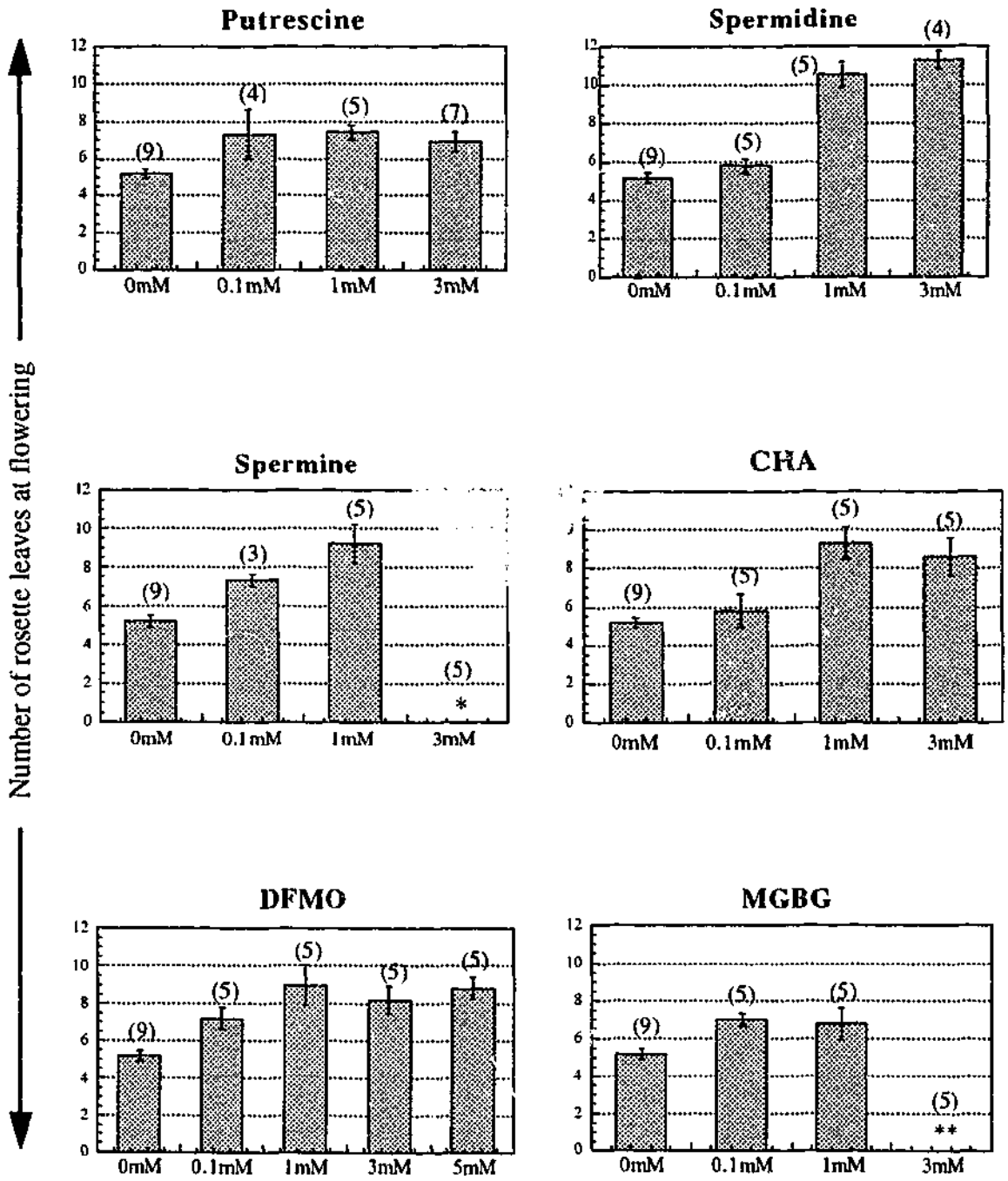


Figure 3.12: Effects of polyamine or polyamine biosynthesis inhibitor treatment on rosette leaf number at the time of *in vitro* flowering.
Average rosette leaf number ± SEM presented.
Sample sizes are indicated in parentheses above each column.

Plants were grown individually in 250mL glass jars (diameter 65mm) with vented lids.
*3mM SPM caused precipitation of the phytagel in the media, detrimentally affecting growth and therefore was not used.
**3mM MGBG was toxic.

	WEEK 5		
Treatment	*PUT*	*SPD*	*SPM*
MS control	25-40	75-110	20-80
0·1mM PUT	47	✓	✓
1mM PUT	65	139	✓
3mM PUT	144	141	✓
0·1mM SPD	116	128	✓
1mM SPD	✓	185	✓
0·1mM SPM	50	249	✓
1mM SPM	137	406	1725

	WEEK 9		
Treatment	*PUT*	*SPD*	*SPM*
MS control	10-20	20-35	30-60
0·1mM PUT	2	4	6
1mM PUT	✓	✓	✓
3mM PUT	✓	✓	✓
0·1mM SPD	✓	✓	✓
1mM SPD	✓	✓	✓
3mM SPD	98	202	205
0·1mM SPM	50	101	✓

Table 3·7: Endogenous free polyamine titres (µg/g) after feeding with polyamines and quantified at weeks 5 and 9 of *in vitro* growth.
(✓ = falls within the range of the controls)
Extractions were performed on pooled shoot tissue; n is between 3 and 6 per treatment. Data presented is the average of 2 independent experiments.

Treatment with polyamine inhibitors

As expected for the inhibitor CHA, which blocks conversion of putrescine to spermidine, treatment resulted in increased accumulation of free putrescine in five-week old plants (Table 3·8). Interestingly, and somewhat strangely, spermidine levels were reduced following treatment with 1mM CHA, whilst treatments with 0·1mM and 3mM caused slight-to-moderate increases in titres of both spermidine and spermine. Application of MGBG was expected to cause reductions in the endogenous titres of spermidine and spermine, with a concomitant increase in putrescine levels. This was not found to be the case, however, at week five of growth after treatment with all three concentrations of MGBG. As expected, putrescine accumulation increased with this inhibitor treatment, but unexpectedly, so too did spermidine and spermine accumulation (Table 3·8). MGBG at 3mM was severely inhibitory to growth, resulting in small, abnormal vegetative shoots which had extremely high levels of all three free polyamines.

	WEEK 5		
Treatment	*PUT*	*SPD*	*SPM*
MS Control	25-40	75-110	20-80
0·1mM CHA	68	118	182
1mM CHA	221	52	82
3mM CHA	217	169	312
0·1mM MGBG	201	330	531
1mM MGBG	72	153	212
3mM MGBG	7691	6935	10496
2mM DFMO	6	150	73

	WEEK 9		
Treatment	*PUT*	*SPD*	*SPM*
MS Control	10-20	20-35	30-60
0·1mM CHA	✓	✓	✓
1mM CHA	132	✓	✓
3mM CHA	721	✓	✓
0·1mM MGBG	✓	✓	✓
1mM MGBG	✓	✓	✓

Table 3·8: Endogenous free polyamine titres (µg/g) after feeding with polyamine inhibitors quantified at weeks 5 and 9 of *in vitro* growth).
(✓ = falls within the range of the controls)
Extractions were performed on pooled shoot tissue; n is between 3 and 6 per treatment. Data presented is the average of 2 independent experiments both of which showed similar trends.

Exposure to the inhibitors for further periods of time resulted in a diminution of their effects, such that free polyamine titres for most treatments were within the ranges of the controls at week nine of growth (Table 3·8). The only exceptions were the two higher levels of CHA treatments which resulted in treated plants maintaining markedly higher putrescine titres than control shoots. As the effects of 3mM MGBG were eventually toxic to growth, no treated plants survived to allow analysis at week nine.

Expression of polyamine biosynthetic genes

ADC

Shoots with unopened flower buds were selected from plants treated with polyamines or polyamine biosynthetic inhibitors for use in RNA extractions. As is clear from Figure 3·13, the 1·2kb *ADC* transcript was high in untreated wild-type shoot tissue which had unopened flower buds, and then decreased as flower development progressed. Treatment with the inhibitors DFMO and MGBG had essentially no effect on *ADC* transcript levels, however, treatment with 3mM CHA produced a slight increase. A low level of exogenous putrescine, 0·1mM, resulted in a decrease in *ADC* transcript, whereas treatment with higher concentrations of 1mM and 3mM had no effect. Spermidine treatment increased transcript

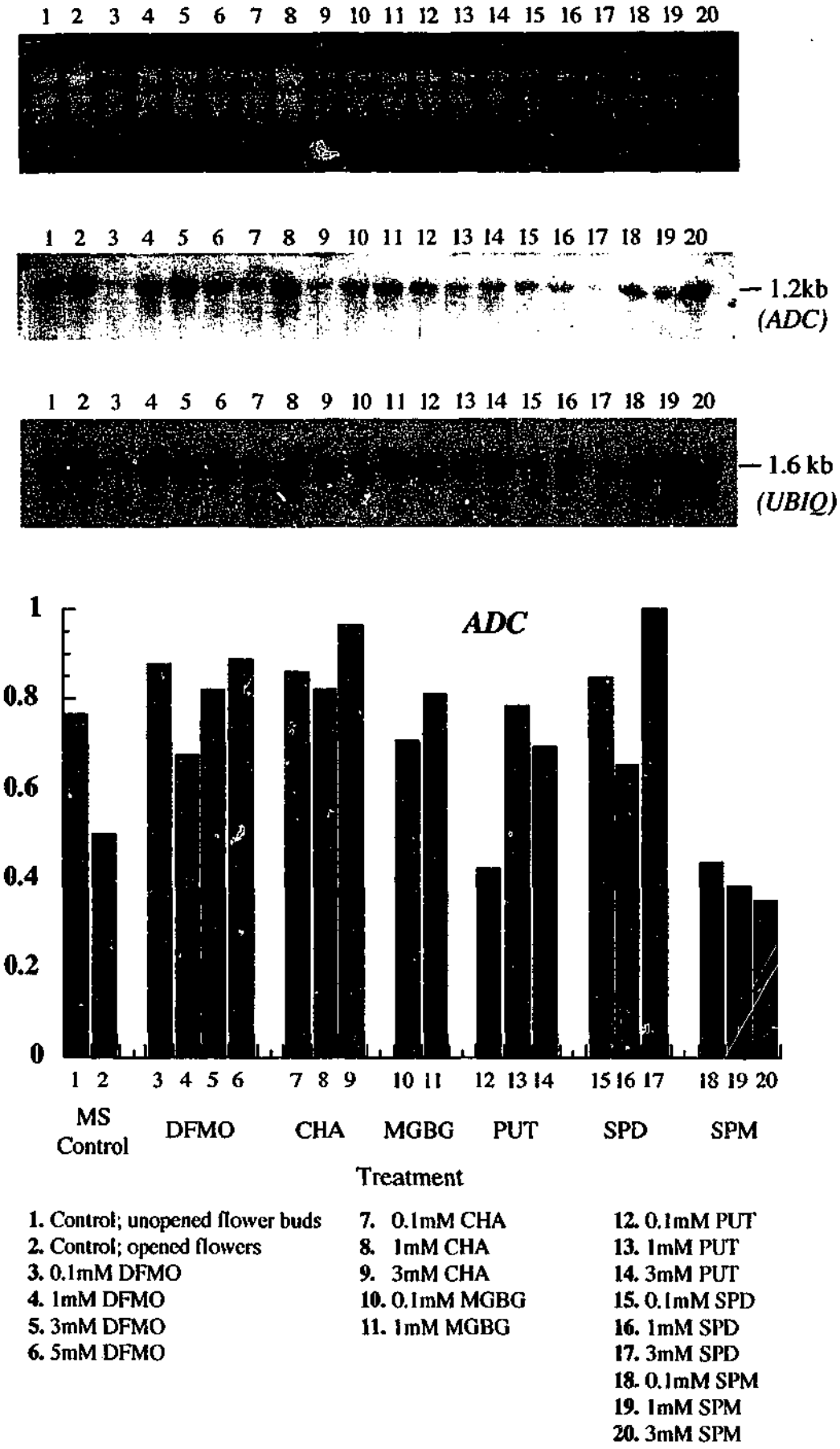


Figure 3.13: Northern blot analysis of *ADC* expression in wild-type *Ler* shoot tissue from 5 week old treated and control seedlings.

Shoots with unopened flower buds were chosen for analysis. Tissues were pooled from each treatment, in order to have a minimum of 0.5g per extraction. On average, this represents approximately 20 plants per treatment. The standardised signal intensities of *ADC* relative to that of *UBIQUITIN* are presented in the histogram. The strongest signal is designated a value of one.

levels of *ADC* only when the highest level tested, 3mM, was used. All three treatments of spermine reduced *ADC* transcript levels to approximately half that of the control shoots.

SAMDC

Probing Northern filters with the genomic DNA PCR product, identified the expected sized transcript of 1·8kb. The expression patterns of *SAMDC* in treated and untreated shoots are presented in Figure 3·14. Unlike *ADC*, no difference was observed in expression of *SAMDC* in shoots having either opened flowers or unopened flower buds. Treatment with each of the three main polyamines resulted in decreased *SAMDC* transcript levels: all concentrations of spermidine and spermine reduced expression by approximately the same extent, whereas putrescine was only inhibitory when applied at low levels. Of the polyamine inhibitors, CHA had no effect on the level of *SAMDC* transcript, whilst MGBG and DFMO caused an increased signal at the higher levels of treatment.

SPDS

The 310bp *SPDS* probe hybridised to a 1·3kb transcript in RNA from *Arabidopsis*. The accumulation of *SPDS* transcripts, like those of *ADC*, were found to be higher in shoots with unopened flower buds (Figure 3·15). Treatment with all three polyamines induced reductions in the levels of *SPDS* transcript. Putrescine caused a reduction in transcript levels of approximately 25% at all concentrations tested, whereas the higher concentrations of spermidine resulted in a 50% reduction in signal compared to the controls. Interestingly, spermine was more effective in reducing transcript accumulation when it was added at the lower concentrations. The results for DFMO treatment were variable, with only slight increases in the levels of *SPDS* message seen with the higher concentrations of inhibitor. CHA acted in a dose-dependent manner to reduce the amount of *SPDS* transcript, which may be expected from its mode of action—to inhibit the synthesis of spermidine from putrescine. Shoot tissue treated with 0·1mM MGBG showed a reduced accumulation of *SPDS* transcripts, whilst a level of 1mM was found to have no effect.

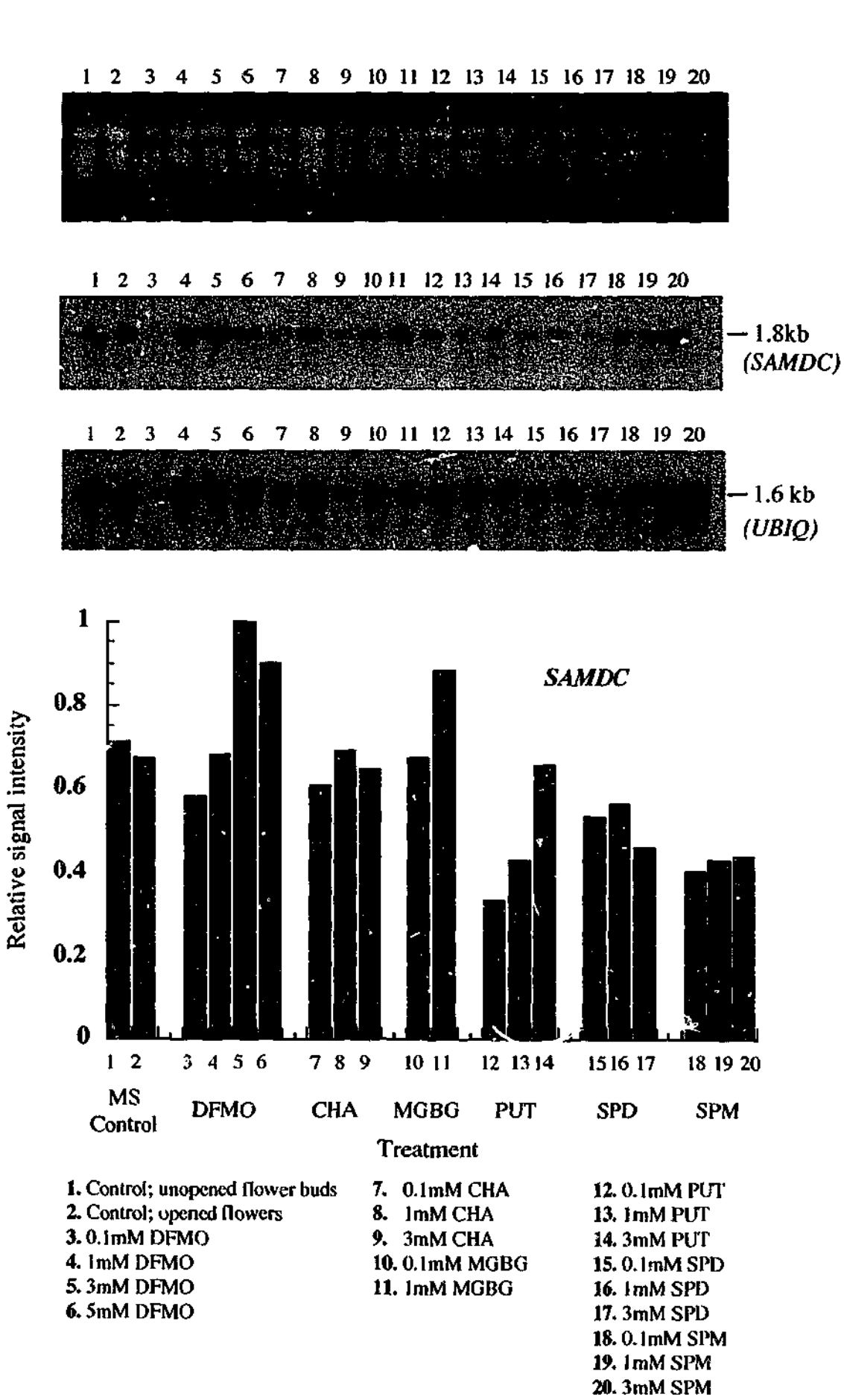
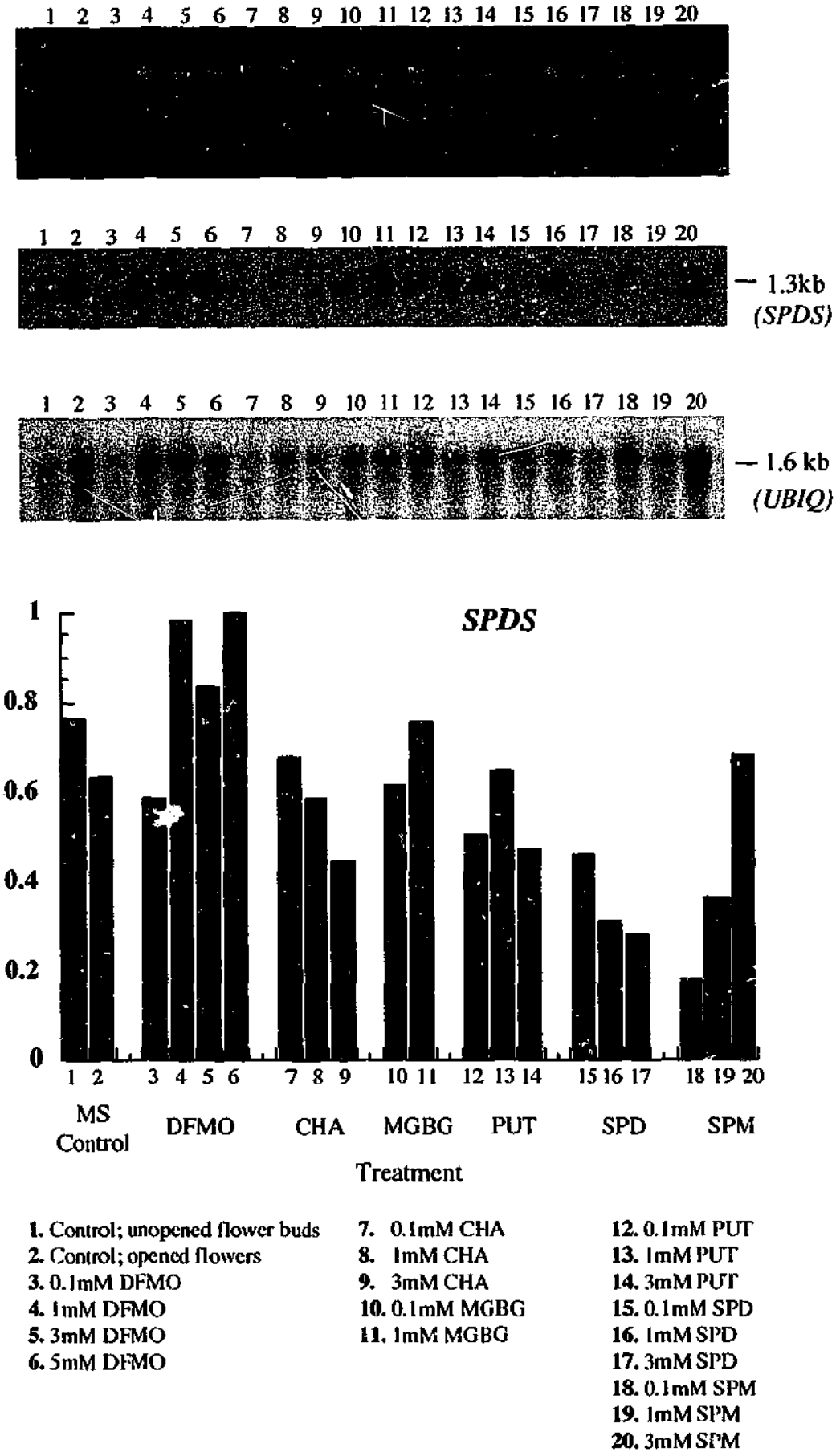


Figure 3.14: Northern blot analysis of *SAMDC* expression in wild-type L*er* shoot tissue from 5 week old treated and control seedlings.

Shoots with unopened flower buds were chosen for analysis. Tissues were pooled from each treatment, in order to have a minimum of 0.5g per extraction. On average, this represents approximately 20 plants per treatment. The standardised signal intensities of *SAMDC* relative to that of *UBIQUITIN* are presented in the histogram. The strongest signal is designated a value of one.



1. Control; unopened flower buds
2. Control; opened flowers
3. 0.1mM DFMO
4. 1mM DFMO
5. 3mM DFMO
6. 5mM DFMO
7. 0.1mM CHA
8. 1mM CHA
9. 3mM CHA
10. 0.1mM MGBG
11. 1mM MGBG
12. 0.1mM PUT
13. 1mM PUT
14. 3mM PUT
15. 0.1mM SPD
16. 1mM SPD
17. 3mM SPD
18. 0.1mM SPM
19. 1mM SPM
20. 3mM SPM

Figure 3.15: Northern blot analysis of *SPDS* expression in wild-type L*er* shoot tissue from 5 week old treated and control seedlings.

Shoots with unopened flower buds were chosen for analysis. Tissues were pooled from each treatment, in order to have a minimum of 0.5g per extraction. On average, this represents approximately 20 plants per treatment. The standardised signal intensities of *SPDS* relative to that of *UBIQUITIN* are presented in the histogram. The strongest signal is designated the value of one.

Transcripts detected by the *Arabidopsis ADC* and the *SPDS* probes used in the present study, corresponded in size to those previously reported (Soyka and Heyer, 1999; Tassoni *et al.*, 2000). The *SAMDC* transcript detected in the present study, however, was slightly smaller (approximately 1·8kb) than the 2·1kb transcript reported by Tassoni *et al.* (2000). It should be noted that the study of Tassoni *et al.* (2000) used the Columbia ecotype, whereas the present study utilised the Landsberg *erecta* ecotype. Further experiments to compare transcript sizes of *SAMDC* from both ecotypes should be undertaken. Northern filters were probed with an 800bp sequence from the 5′ region of the *N. tabacum* ODC cDNA sequence, however no specific signals could be detected in shoots.

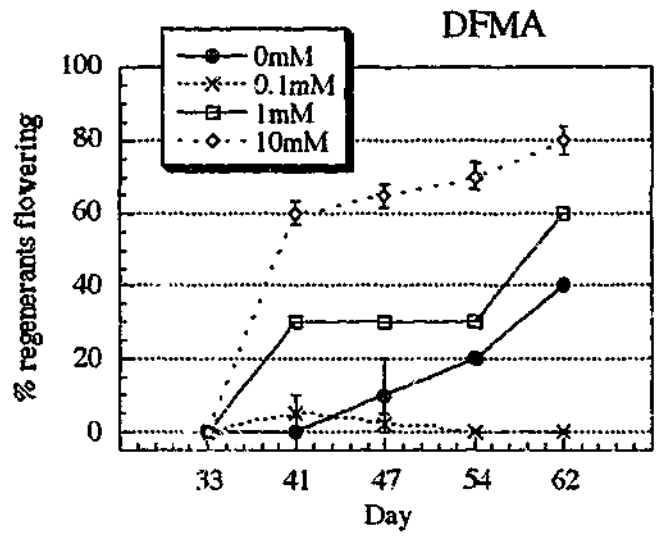
Effects of putrescine biosynthesis inhibitors on *in vitro* regeneration processes

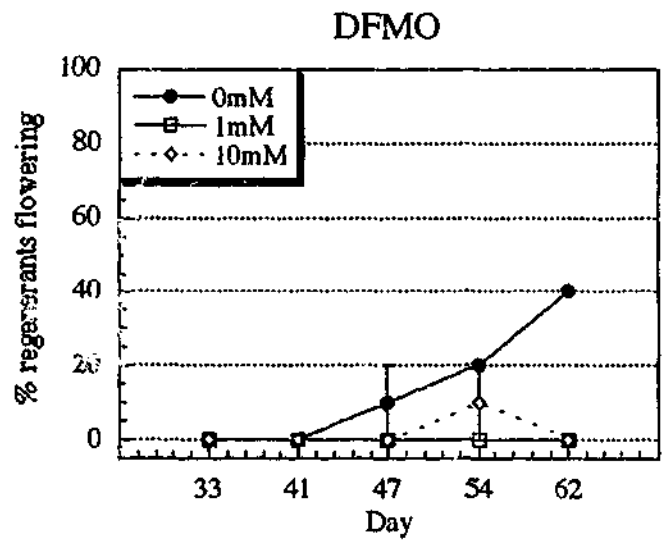
Flower production

Presented in Figure 3·16 are the effects of DFMA and DFMO treatments on flower production of regenerating shoots. When included in the shoot regeneration medium, 0·1mM DFMA inhibited flower production. Rather suprisingly however, 1mM and 10mM DFMA treatments were promotive to flowering whilst DFMO at concentrations of 1mM and 10mM, was inhibitory to flower production. When used in combination, the effects of both inhibitors were additive: levels of both inhibitors at 10mM were promotory to flower production, although not to the same extent as when 10mM DFMA was used alone, while inclusion of both inhibitors at 0·1mM or 1mM reduced the capacity of plants to produce flowers.

Root production

The effects of DFMA and DFMO on the production of roots from the bases of regenerated shoot tissue were also assessed, and results are presented in Figure 3·17. The same trends noted above for flowering were also evident for root growth. That is, the inclusion of 10mM DFMA was stimulatory to root production, both in terms of overall number and the time of initiation, whilst reduced concentrations of DFMA were moderately inhibitory to root





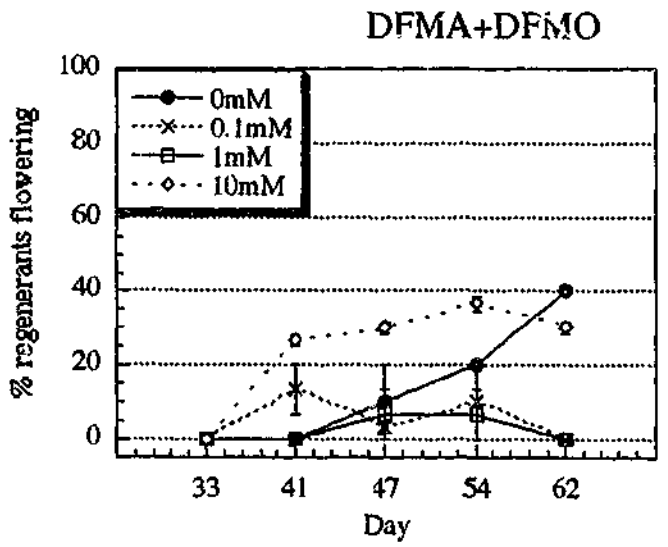
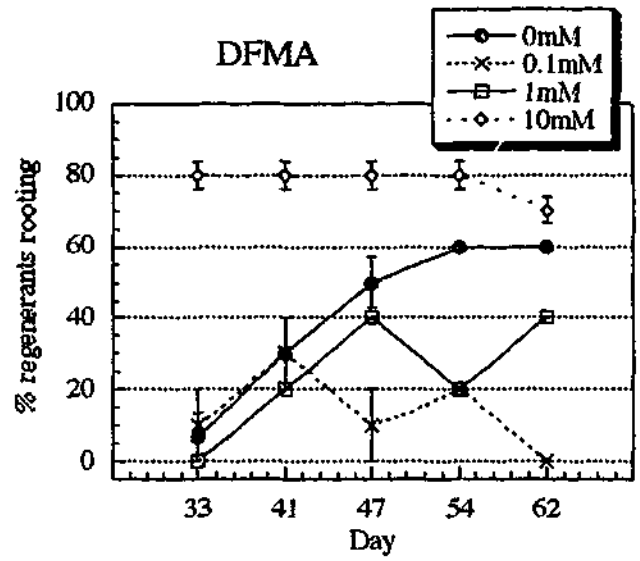
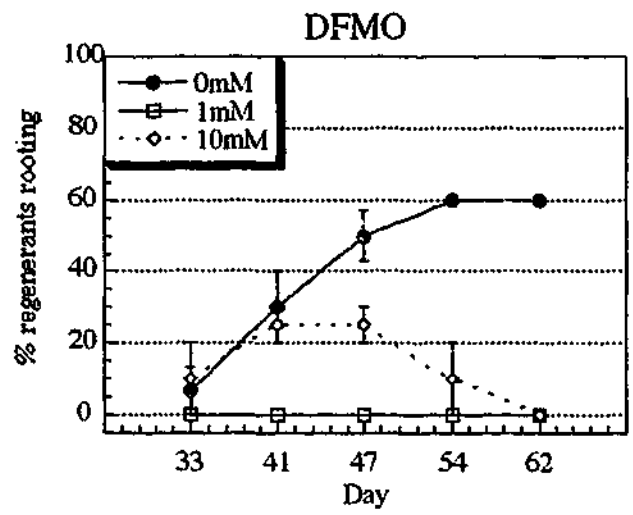


Figure 3.16: Effects of putrescine biosynthesis inhibitors on the flowering process during *in vitro* shoot regeneration.

Inhibitors were included in the shoot regeneration media. Over the time course, new shoots continually formed on the existing callus tissue, and were scored for the presence of floral buds. The number of floral shoots at each time point is expressed as a percentage of the total number of shoots present.





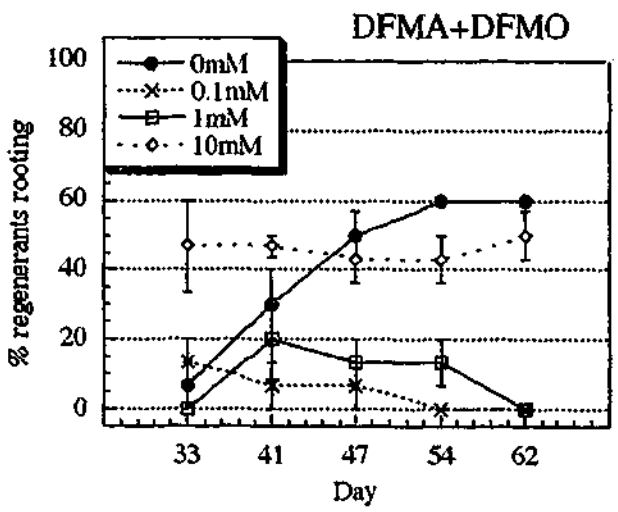


Figure 3.17: Effects of putrescine biosynthesis inhibitors on the rooting process during *in vitro* shoot regeneration.

Inhibitors were included in the shoot regeneration media. Over the time course, new shoots continually formed on the existing callus tissue, and were scored for the presence of roots at the base of the hypocotyl. The number of rooted shoots at each time point is expressed as a percentage of the total number of shoots present.

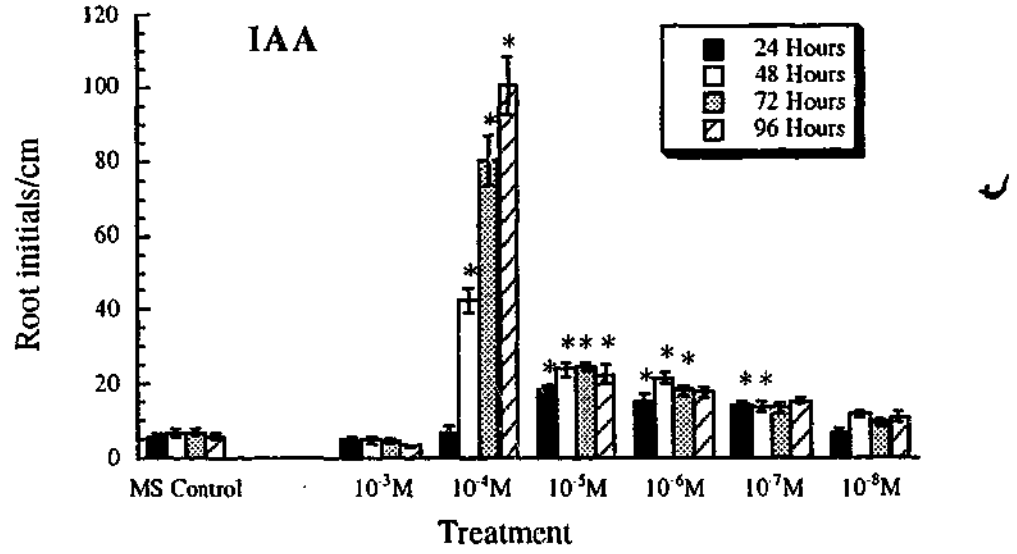
growth. Both levels of DFMO tested, 1mM and 10mM, were found to inhibit the production of roots from the bases of the regenerated shoots. Again, when added in combination, the effects of both inhibitors were additive; 10mM of both DFMA and DFMO resulted in approximately 50% of regenerants producing roots, compared to the figure of 80% when tissue were treated with DFMA alone.

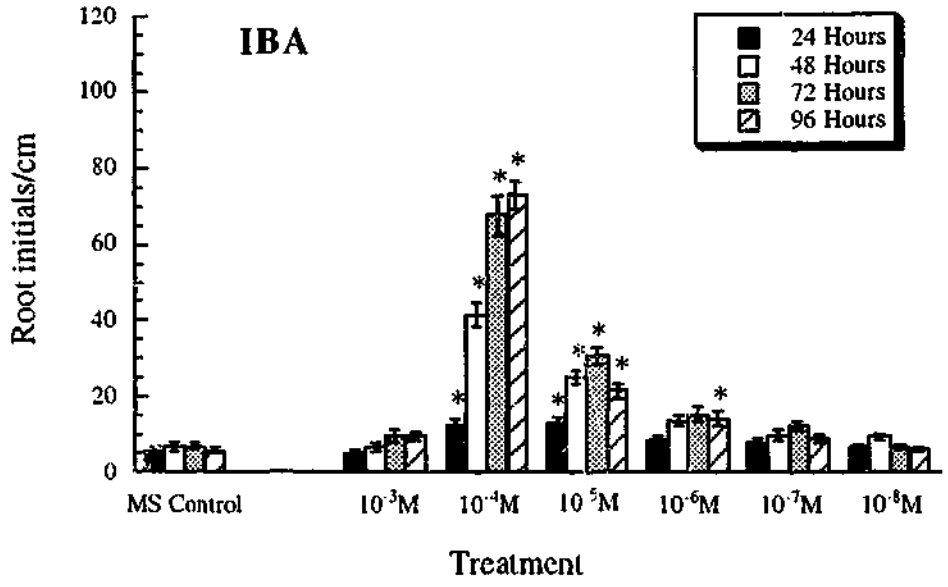
3·2·3 LATERAL ROOT INDUCTION

As mentioned previously, exogenous polyamines were found to moderately stimulate root growth at later stages of development. Therefore, to further examine the roles of polyamines in this process, an appraisal of lateral root primordia (LRP) initiation was performed in response to treatment with the three polyamines and also the polyamine inhibitors. Furthermore, in order to perform a complete appraisal of lateral root initiation in *Arabidopsis* seedlings, the effects of treatment with auxins were also quantified, both when applied alone, or in conjunction with polyamines to determine if a synergistic role exists for polyamines and auxins during root development.

Effects of auxins

The effects of three auxins IAA, IBA, and NAA on rooting of wild-type Landsberg *erecta* seedlings were quantified over a range of concentrations from 10^{-3}M to 10^{-8}M. IAA and NAA significantly stimulated root initiation at levels as low as 10^{-7}M ($p<0.05$), whereas the stimulation caused by IBA was less, such that 10^{-7}M and 10^{-8}M treatments did not significantly alter lateral root primordia production ($p<0.05$) (Figure 3·18). The concentration of auxin which produced the most prolific lateral root stimulation in seedlings was found to be 10^{-4}M for both IAA and IBA, whilst NAA showed an optimal response equally at 10^{-4}M and 10^{-5}M (Figure 3·18). With each hormone treatment, intense proliferation of lateral roots was concomitant with a significant inhibition in the growth of the primary root ($p<0.05$) (Figure 3·19). In the case of the 10^{-4}M treatments for all three hormones, and also 10^{-5}M for NAA, root primordia were initiated along the vascular tissue





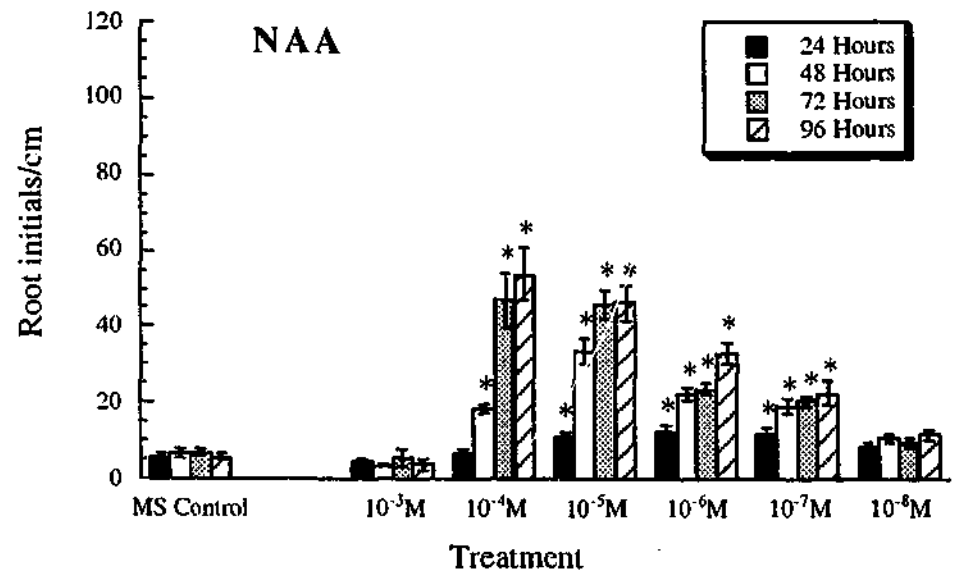
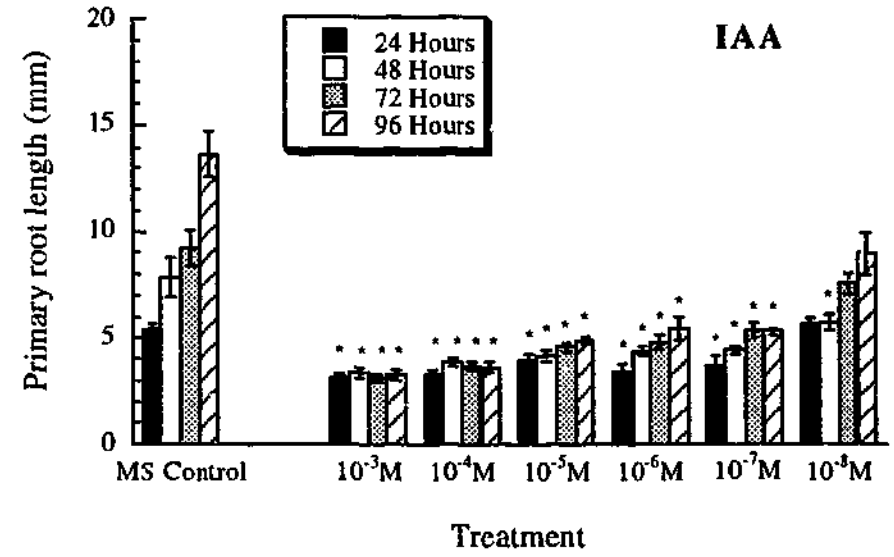
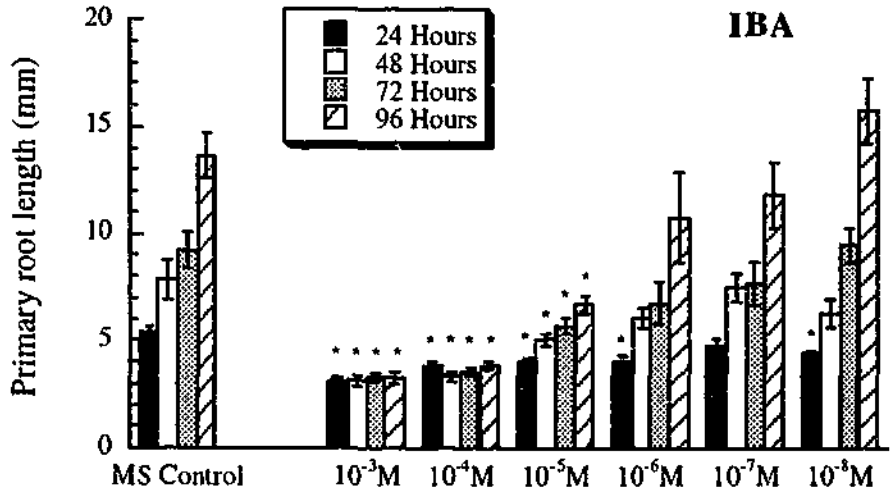


Figure 3.18: Effects of auxin on lateral root primordia initiation in wild-type L. *erecta* seedlings. Average ± SEM presented.
Asterisks indicate data points significantly different from controls ($p<0.05$).
(Sample size is 10-16 seedlings/treatment/timepoint).





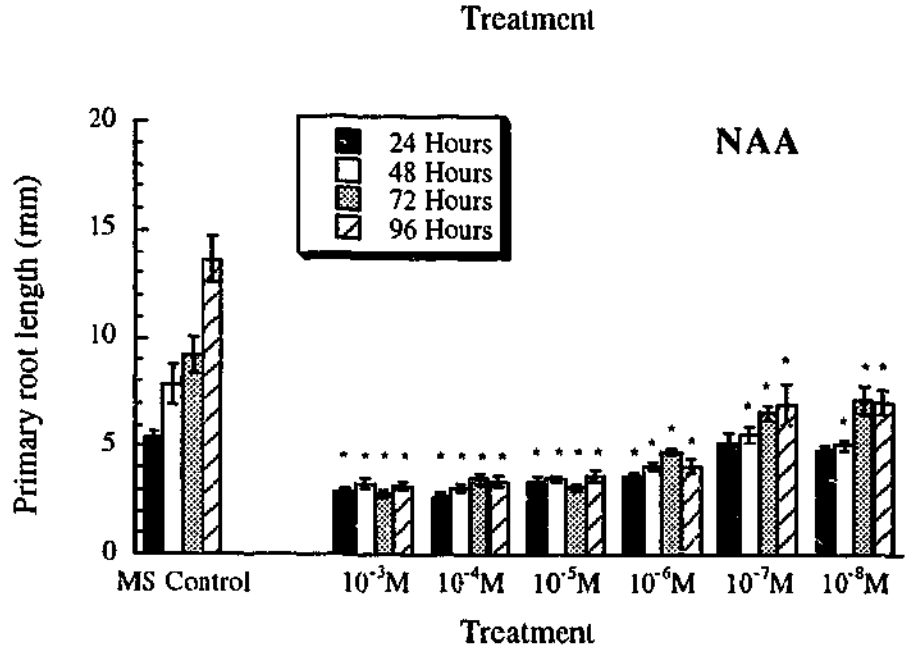


Figure 3.19: Effects of auxin on primary root elongation in wild-type L. *erecta* seedlings. Average ± SEM presented.
Asterisks indicate data points significantly different from controls ($p < 0.05$).
(Sample size is 10-16 seedlings/treatment/timepoint).

of the hypocotyl, resulting in fracturing of the surrounding cortex tissue of the hypocotyl (Figure 3·20 inset). A differential induction of lateral root growth was also occasionally observed on seedling roots, with newer lateral root primordia appearing between other lateral roots that had already started to undergo elongation.

Effects of polyamines

The effects on lateral root primordia production of adding each of the three polyamines, putrescine, spermidine, and spermine, seperately to seedlings are presented in Figure 3·21. It is apparent that exogenous polyamines do not have auxin-like actions and are not stimulatory themselves with respect to lateral root formation. It fact, polyamines have no significant ($p<0·05$) net positive or negative effect on lateral root number at any of the concentrations used. The length of the primary root, however, was significantly stimulated by low concentrations of putrescine (10^{-6}M to 10^{-8}M), a moderate level of spermidine (10^{-5}M), and a high spermine concentration (10^{-3}M and 10^{-4}M) ($p<0·05$) (Figure 3·22). The appearance of the polyamine-treated seedlings are presented in Figure 3·23.

Effects of chemical inhibitors of polyamine biosynthesis

The polyamine biosynthetic inhibitors CHA, DFMO, and MGBG were applied to seedlings to study their effects on the initiation of lateral root primordia formation, at concentrations ranging from 0·1mM up to 10mM for DFMO, and from 0·1mM to 3mM for CHA and MGBG (levels of 5mM or greater were toxic). Figure 3·24 shows the results of such treatments on the number of lateral root initials. In most cases, the presence of inhibitor had little effect on the number of primordia formed. The only significant differences from the untreated controls were noted with the highest concentration of DFMO tested (10mM) which increased root number. Such increases however, were only in the order of two- or three-fold, contrasting with the auxin-induced enhancement of root number up to 20-fold. Interestingly, and possibly of significance root initials were induced to develop very early following exposure to all three inhibitors, and were visible from six hours after treatment,

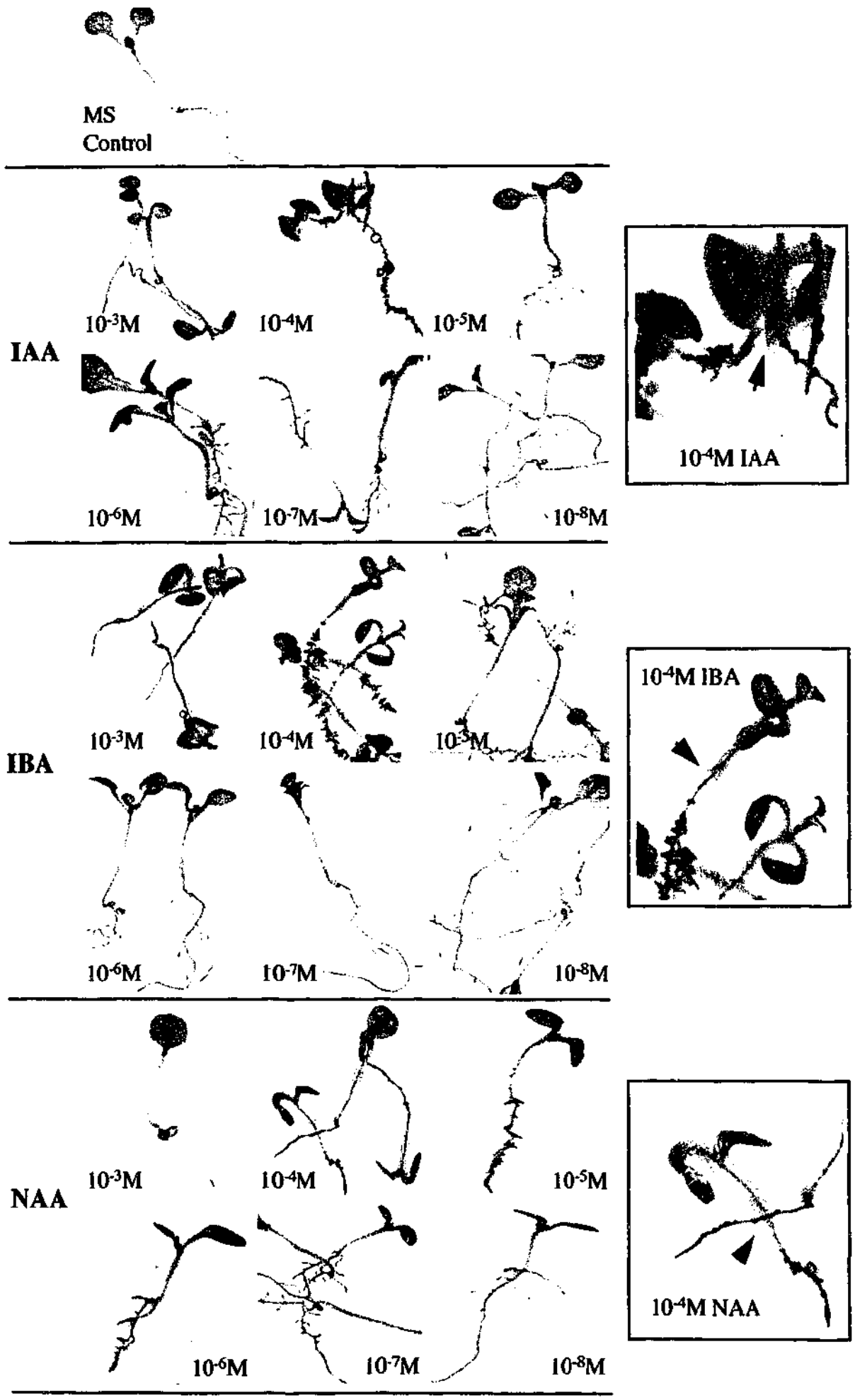
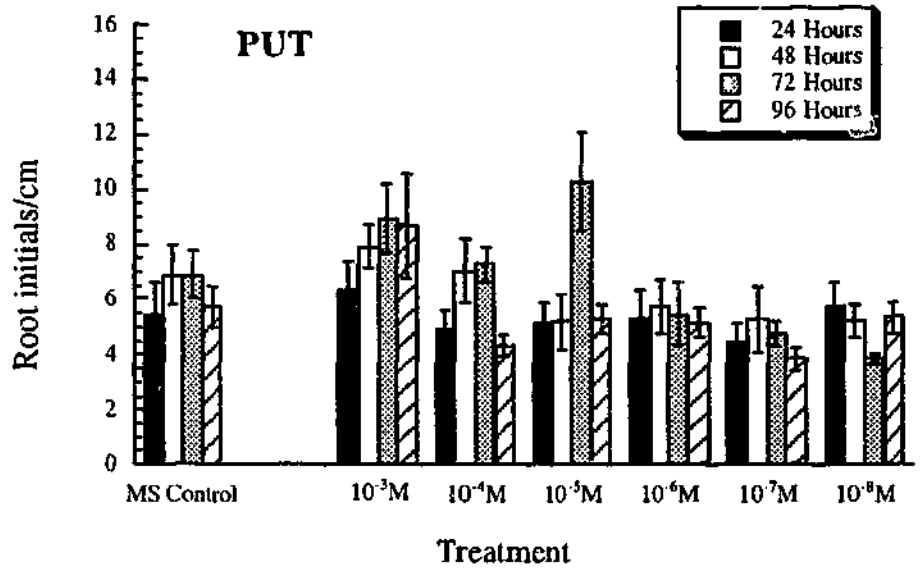
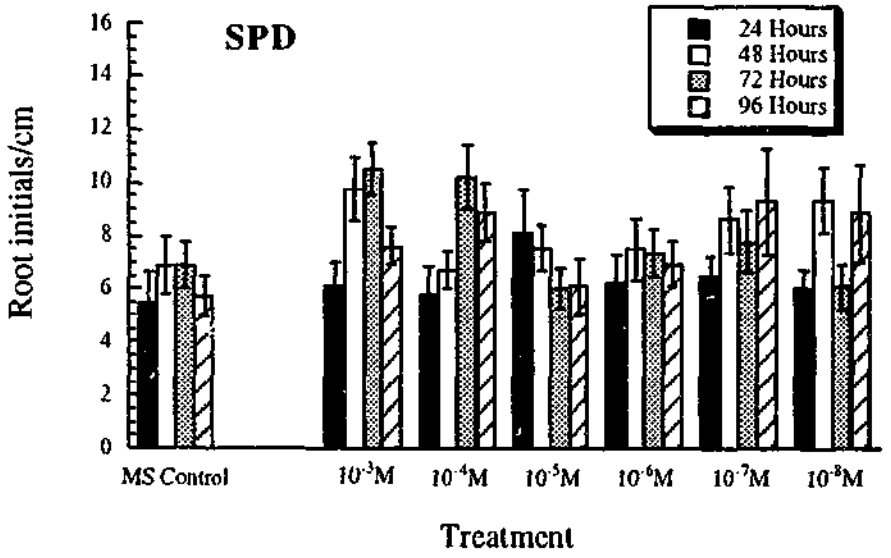


Figure 3.20: Effects of auxin treatment on lateral root initiation in wild-type L. *erecta* (96hrs).

Inset: Seedlings treated with 10^{-4}M of each of the three auxins are enlarged to show the initiation of LRP along the hypocotyl and the fracturing of the cortical tissue (indicated with an arrowhead).





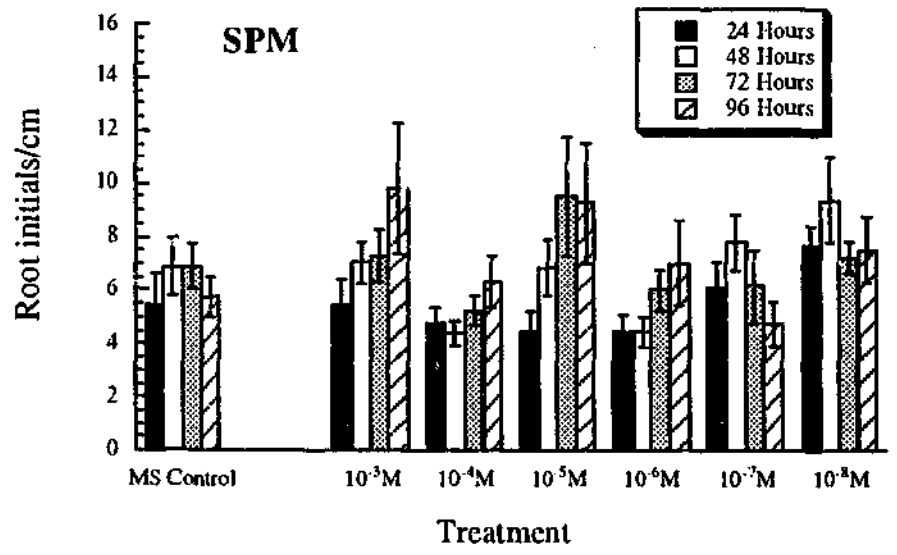
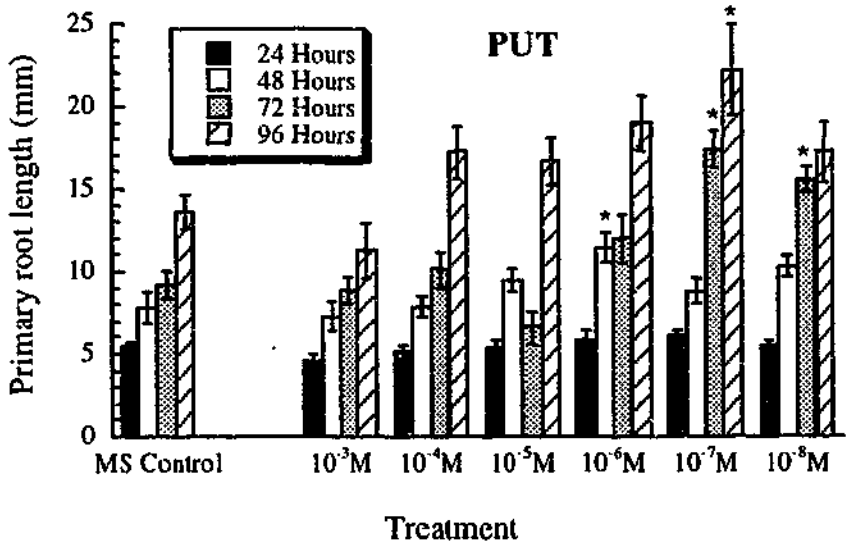
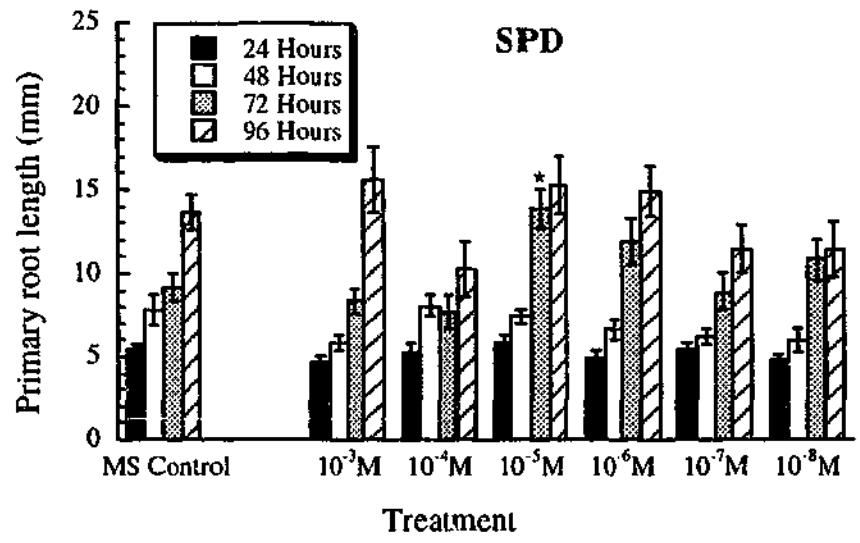


Figure 3.21: Effects of polyamines on lateral root primordia initiation in wild-type L. *erecta* seedlings. Average ± SEM presented. No data points were significantly different from controls ($p<0.05$). (Sample size is 10-16 seedlings/treatment/timepoint)





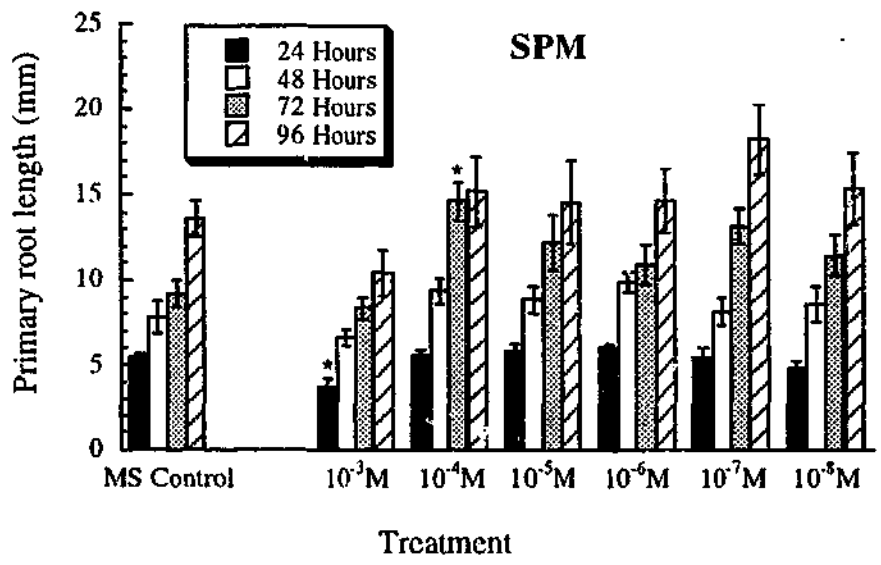


Figure 3.22: Effects of polyamines on primary root elongation in wild-type L. *erecta* seedlings. Average ± SEM presented.
Asterisks indicate data points significantly different from controls ($p<0.05$).
(Sample size is 10-16 seedlings/treatment/timepoint)

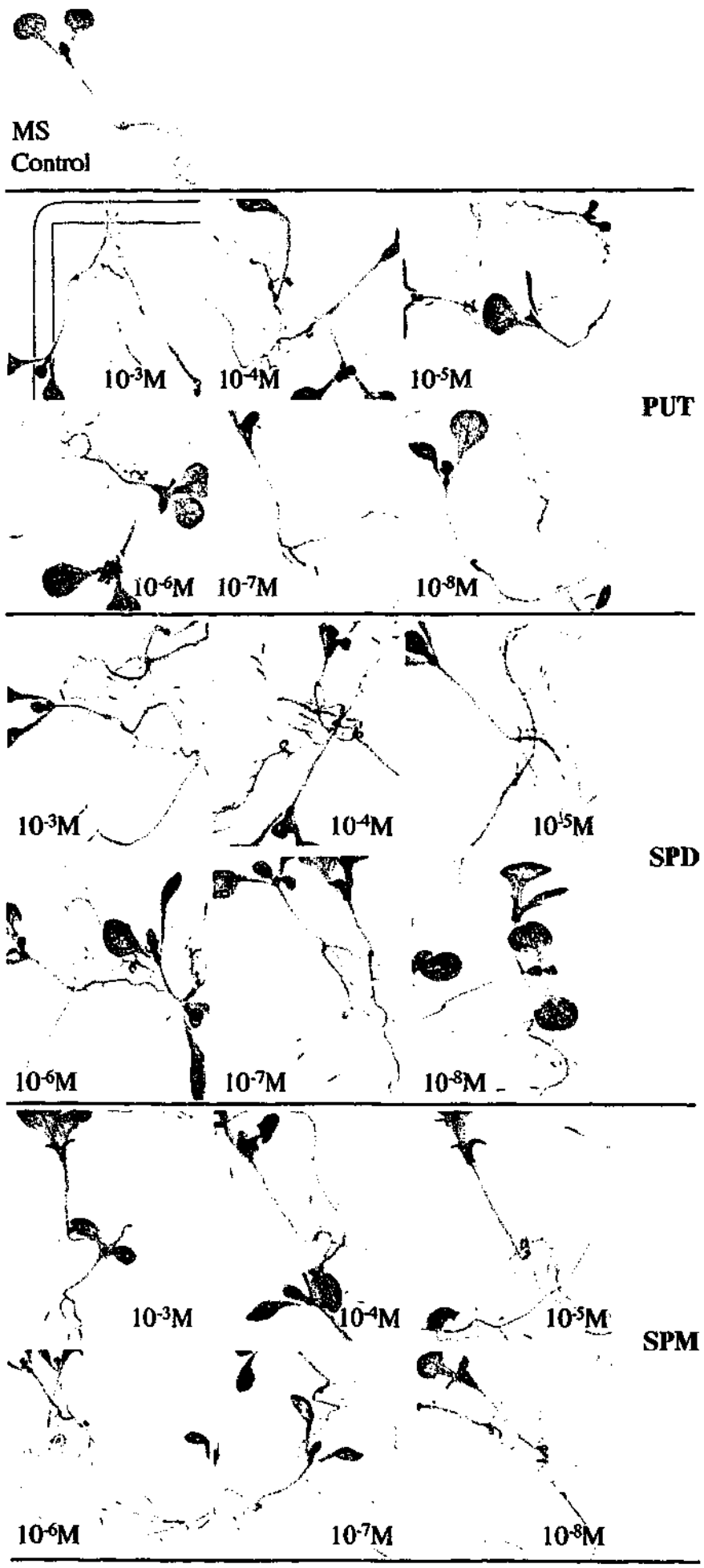


Fig 3.23: Effects of polyamine treatment on lateral root initiation in wild-type L. *erecta* (96 hrs).

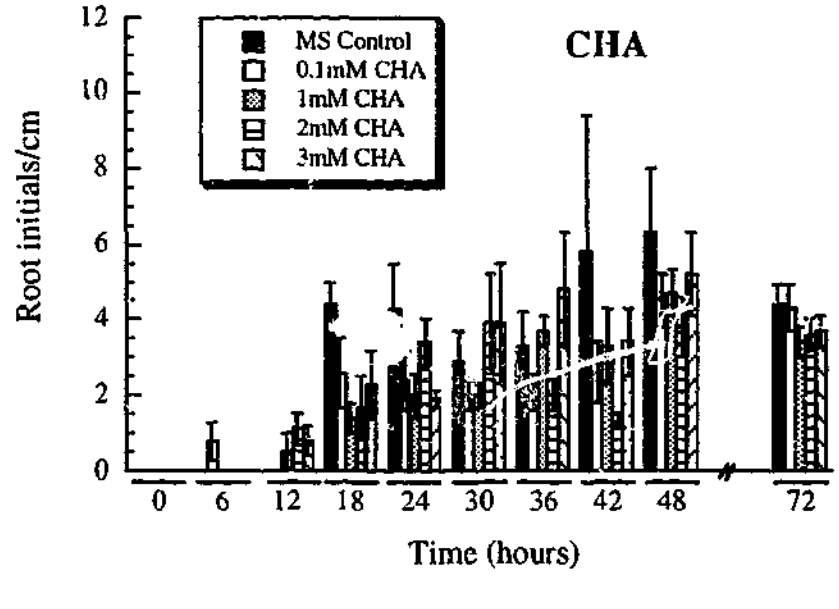


Figure 3.24: Effects of polyamines inhibitors on lateral root primordia initiation in wild-type L. *erecta* seedlings.
Asterisks indicate data points significantly different from controls ($p<0.05$).

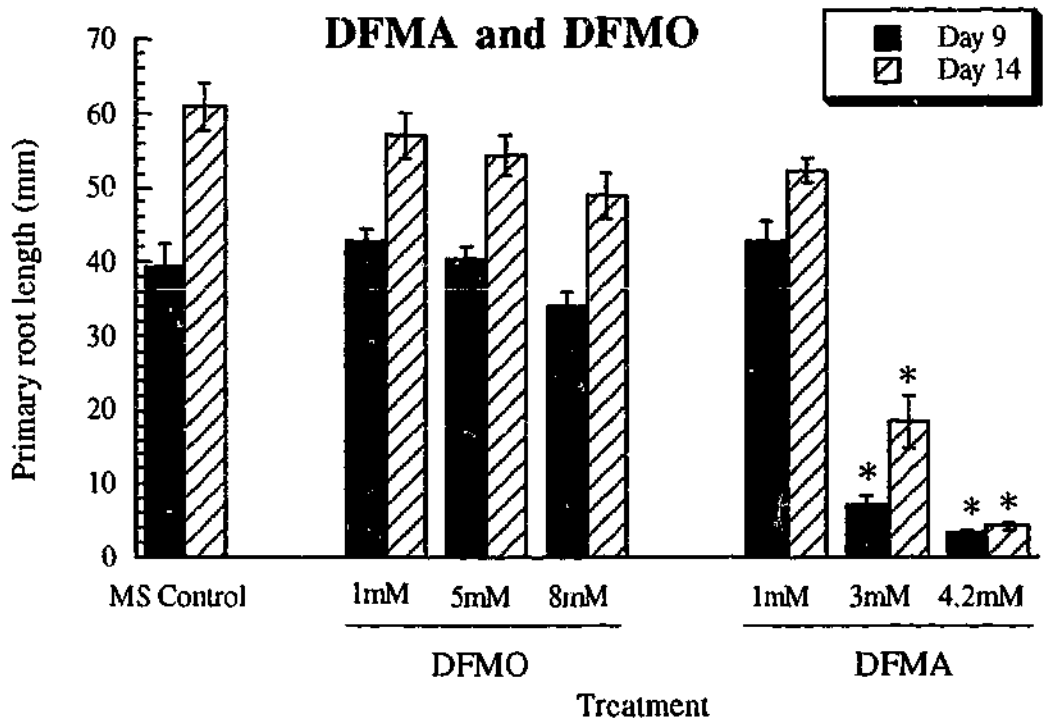
whereas the control seedlings required 18 hours incubation before root primordia were observed (Figure 3·24).

The effects of polyamine inhibitors on the elongation of the primary root are presented in Figure 3·25. CHA, DFMA, and MGBG were all significantly inhibitory to root growth at levels greater than 1mM ($p<0·05$), and slowed development in a dose-dependent manner. DFMO however, exhibited no significant inhibition of root length, even at concentrations as high as 8mM.

Effects of auxins and polyamines when applied simultaneously

To determine whether auxin and polyamines show additive or synergistic effects upon root primordia formation, IAA, IBA, or NAA at concentrations of 10^{-5}M and 10^{-6}M were used in conjunction with either 10^{-3}M putrescine, 10^{-3}M spermidine, or 10^{-3}M spermine. These levels of auxin were previously found to be sub-optimal in their capacity to stimulate lateral root initiation (Figure 3·18), while the polyamine concentration used was considered the optimum for lateral root growth (Figure 3·21). If any positive relationships exist between auxin and polyamines in terms of lateral root initiation, it was thought that the number of roots elicited from such simultaneous applications would increase beyond that expected from a treatment with the sub-optimal IAA levels only.

Figures 3·26A and 3·26B show the results when 10^{-5}M and 10^{-6}M auxins respectively, were used in conjunction with the three polyamines. Also included for comparison are the rooting percentages observed when seedlings were treated only with the auxin, either at the same concentration (shown in blue) or increased (shown in red). In all instances, the rooting values for the combined applications of auxin plus polyamine do not approach those observed when seedlings were treated with a higher concentration of auxin alone. In most cases, the combined treatments reduced the number of lateral root primordia produced below those values seen for the auxin-only treatment (in blue on the graphs), suggesting that the presence of polyamines are inhibitory to the process of auxin-stimulated root primordia formation.



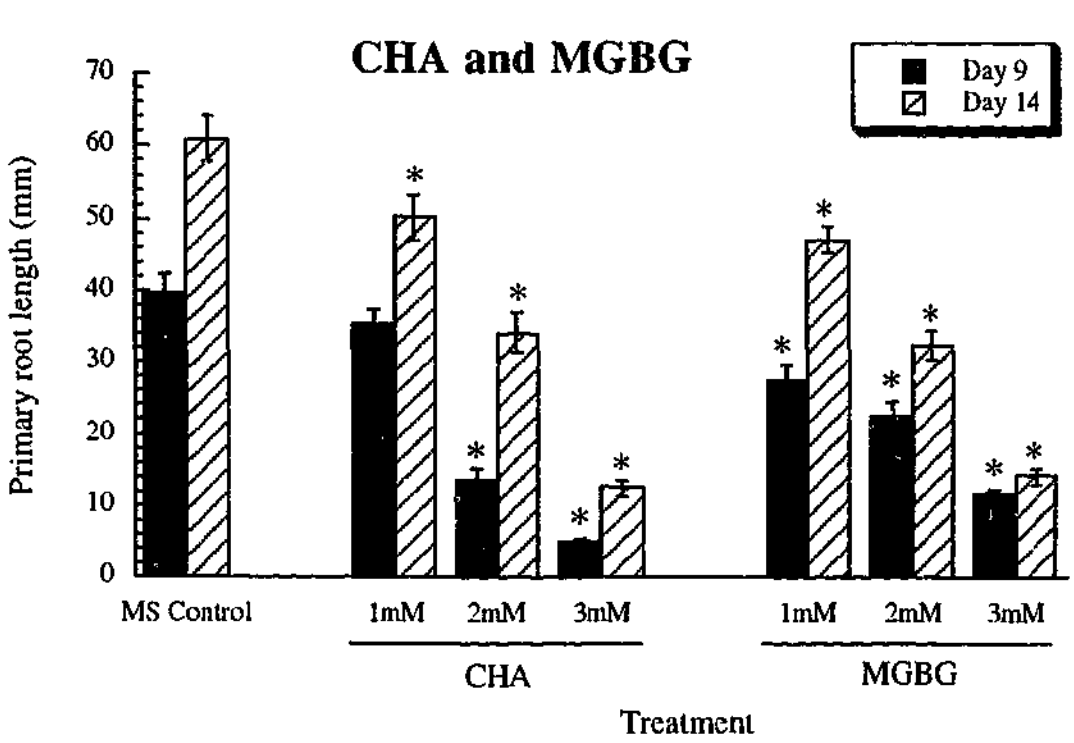


Figure 3.25: Effects of polyamines inhibitors on primary root elongation in wild-type L. *erecta* seedlings. Average ± SEM presented. Asterisks indicate data points significantly different from controls ($p<0.05$). (Sample size is 20-38 seedlings/treatment/timepoint)

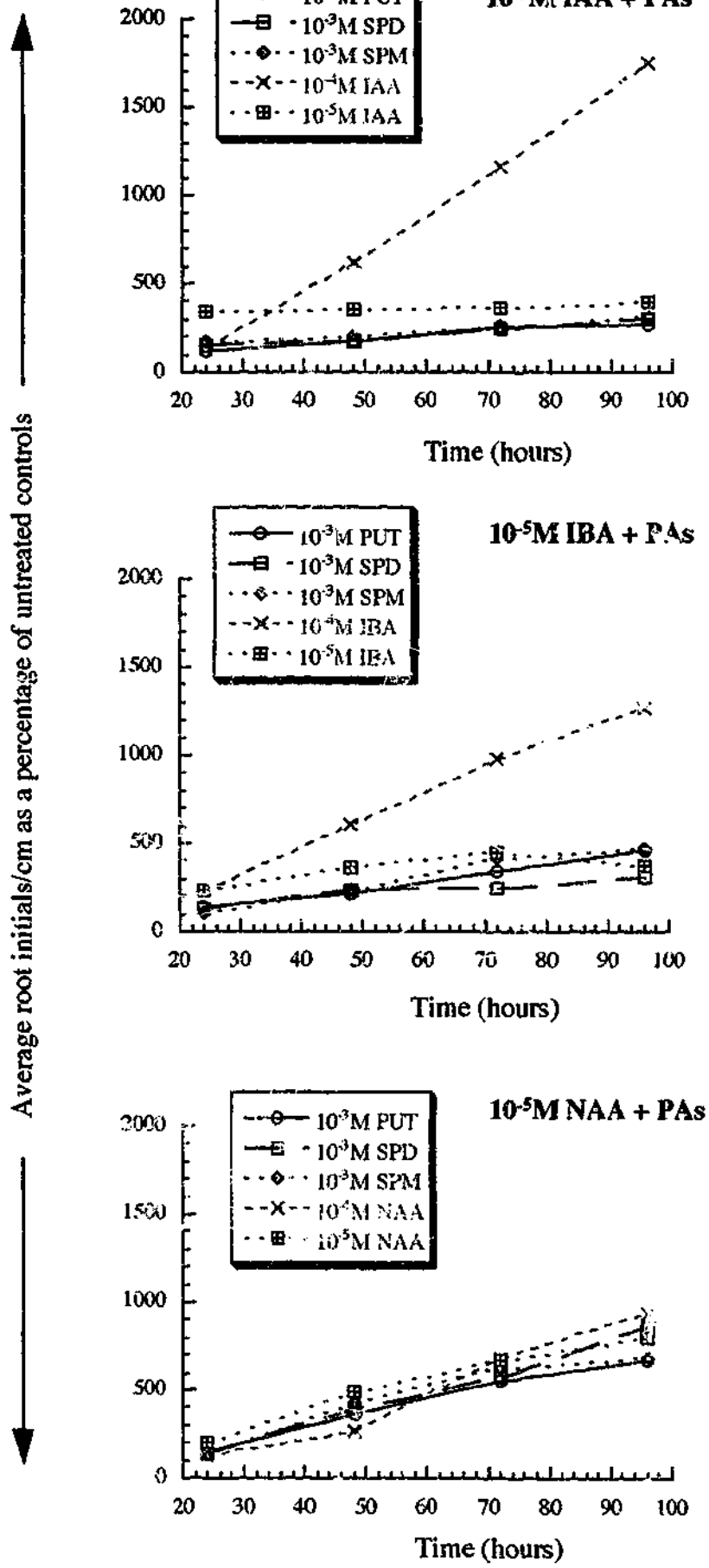


Figure 3. 26A: Effects of simultaneous treatment with auxins and polyamines on the initiation of lateral root primordia.

10^{-3}M of each of the three polyamines, putrescine, spermidine, and spermine were individually combined wih either IAA, IBA, or NAA at 10^{-5}M and applied to seedlings to test for the existence of a synergistic relationship. Controls included to allow a direct comparison are indicated in blue (10^{-5}M auxin-only treatment) and red (10^{-4}M auxin-only treatment). The average number of root initials per cm for each treatment is shown as a proportion of the values for the untreated controls. n is 15-20 seedlings/treatment/timepoint.

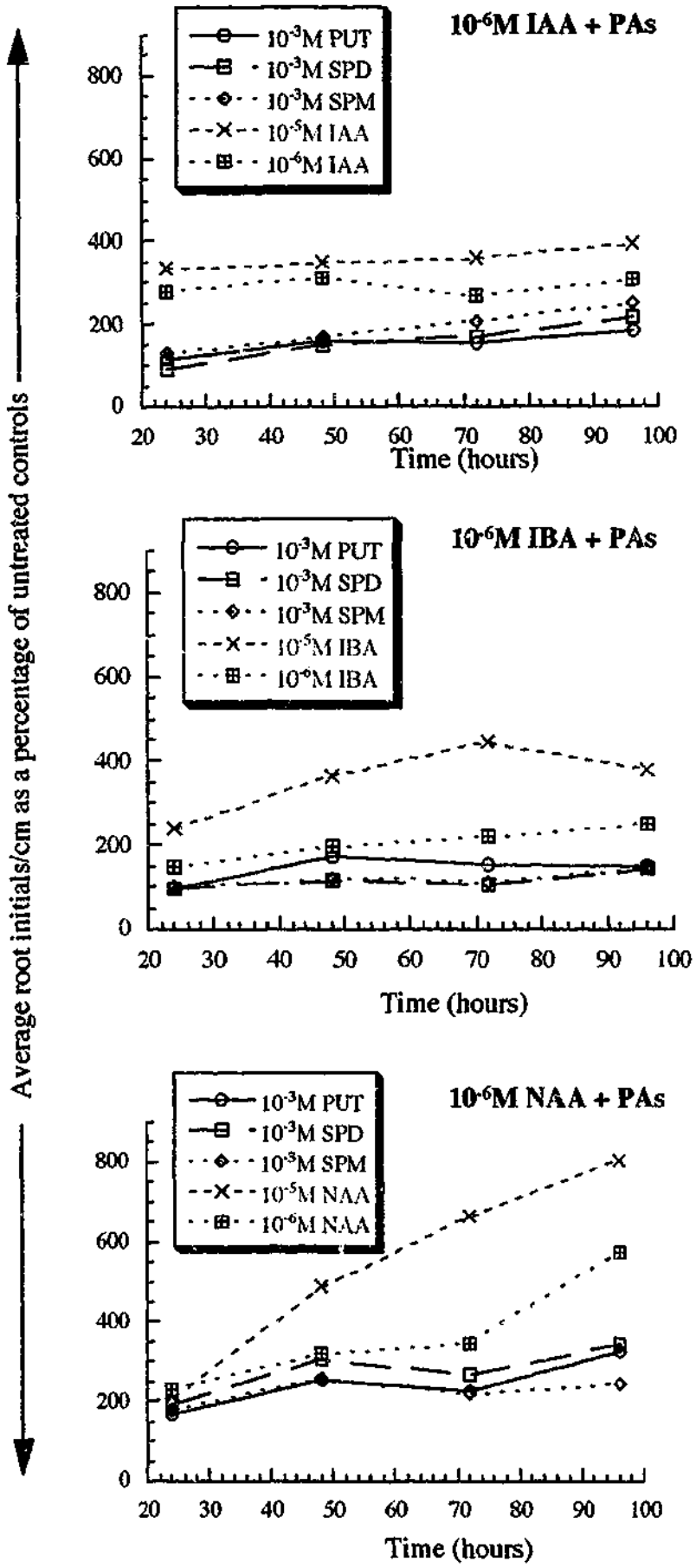


Figure 3. 26B : Effects of simultaneous treatment with auxins and polyamines on the initiation of lateral root primordia.

10^{-3}M of each of the three polyamines, putrescine, spermidine, and spermine were individually combined wih either IAA, IBA, or NAA at 10^{-5}M and applied to seedlings to test for the existence of a synergistic relationship. Controls included to allow a direct comparison are indicated in blue (10^{-6}M auxin-only treatment) and red (10^{-5}M auxin-only treatment). The average number of root initials per cm for each treatment is shown as a proportion of the values for the untreated controls.
n is 15-20 seedlings/treatment/timepoint.

3·2·4 ADVENTITIOUS ROOT INDUCTION

In order to comprehensively study the involvement of polyamines during root formation, it is necessary to also examine the process of adventitious root initiation. The development of such roots from a variety of tissues not normally possessing root meristems may require different signals and cellular processes than those normally involved in the generation of lateral roots from pre-existing root tissue. Hence, although polyamines may not be directly involved in the production of lateral roots, the different processes surrounding adventitious root development may have a requirement for polyamines.

Leaf explants removed from pre-flowering plants

Leaves from soil-grown plants were sensitive to surface sterilisation procedures and did not form any roots when cultured *in vitro*. Therefore basal rosetted leaves of 19 day-old pre-flowering seedlings grown *in vitro* were used in subsequent experiments. No adventitious roots were formed when these leaf explants were cultured on either hormone-free control media, or any of the media containing IAA only. In some explants cultured on hormone-free media, however, shoot regeneration and callus growth occurred at the sites of wounding, indicating that explants were viable and retained some capacity for tissue growth and differentiation (Figure 3·27A). It was also noted that a concentration of 10^{-3}M IAA completely inhibited cellular growth and expansion in the explants, as no sites of callus growth were evident, and the explants did not exhibit an increase in size (Figure 3·27B). Treatment with 10^{-4}M IAA, produced limited callus formation along the exposed vasculature of explants (Figure 3·27C), whilst 10^{-5}M IAA markedly stimulated callus growth in these areas (Figure 3·27D).

To test for a relationship between polyamines and auxin in the generation of adventitious roots, similar leaf explants were incubated on media containing 10^{-4}M IAA supplemented with a range of concentrations of putrescine (Figure 3·28) or spermidine (Figure 3·29). 10^{-4}M IAA was chosen as a concentration that was neither toxic to growth nor stimulatory to

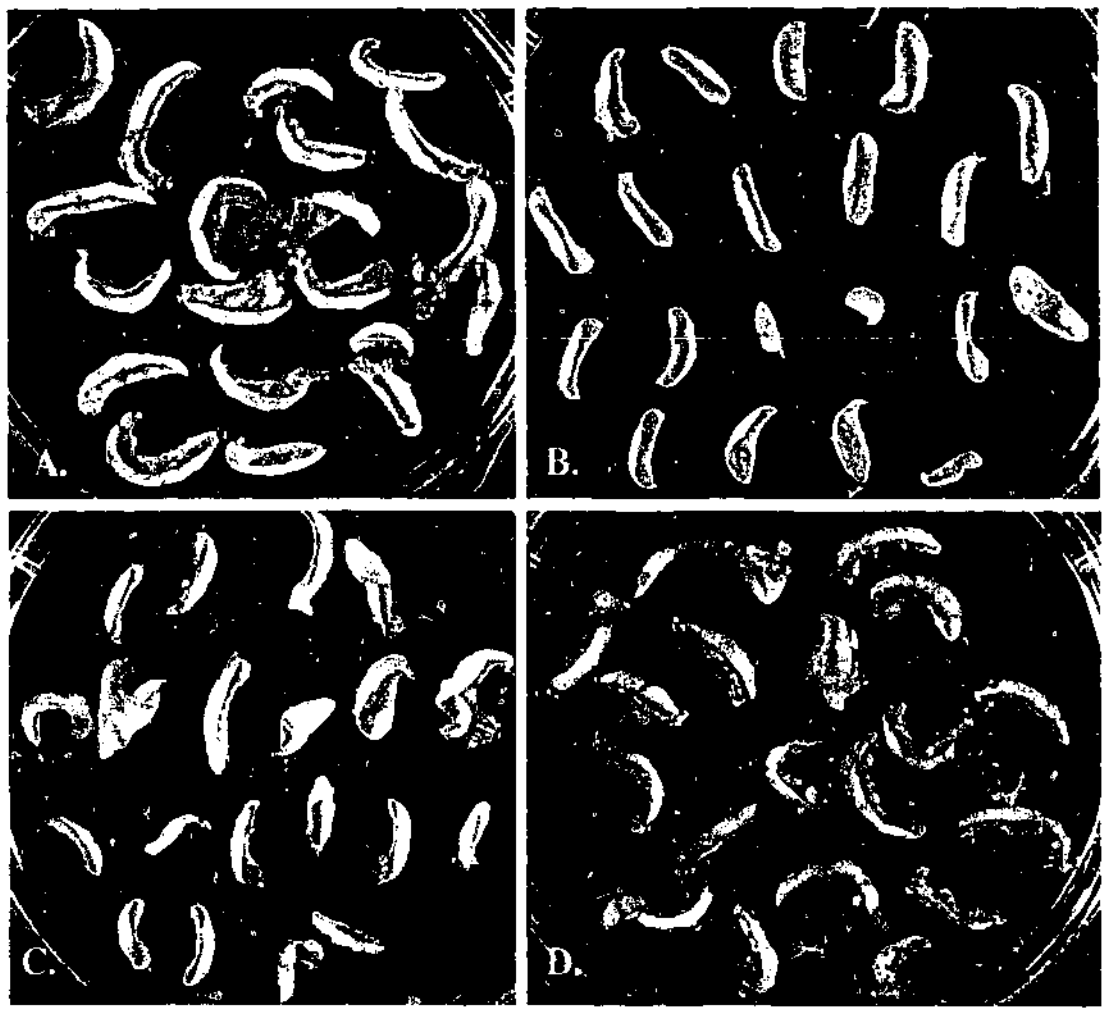


Fig 3.27: Adventitious root formation when explants were excised from pre-flowering wild-type L. *erecta* plants.

A. Hormone free control
B. 10^{-3}M IAA
C. 10^{-4}M IAA
D. 10^{-5}M IAA

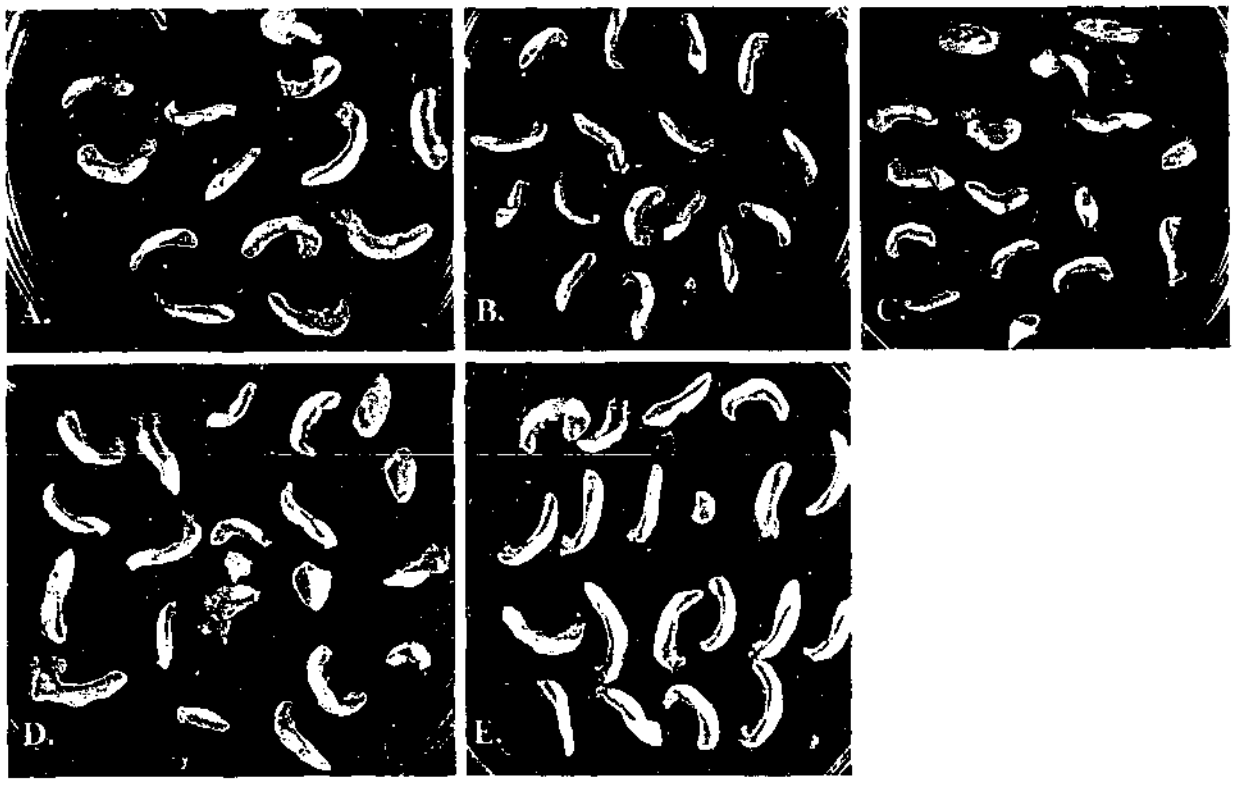


Fig 3.28: Adventitious root formation using explants excised from pre-flowering wild-type L. *erecta* plants. Media was supplemented with 10^{-4}M IAA and:
A. 0.01mM PUT **B.** 0. 1mM PUT **C.** 0.5mM PUT
D. 1mM PUT **E.** 5mM PUT

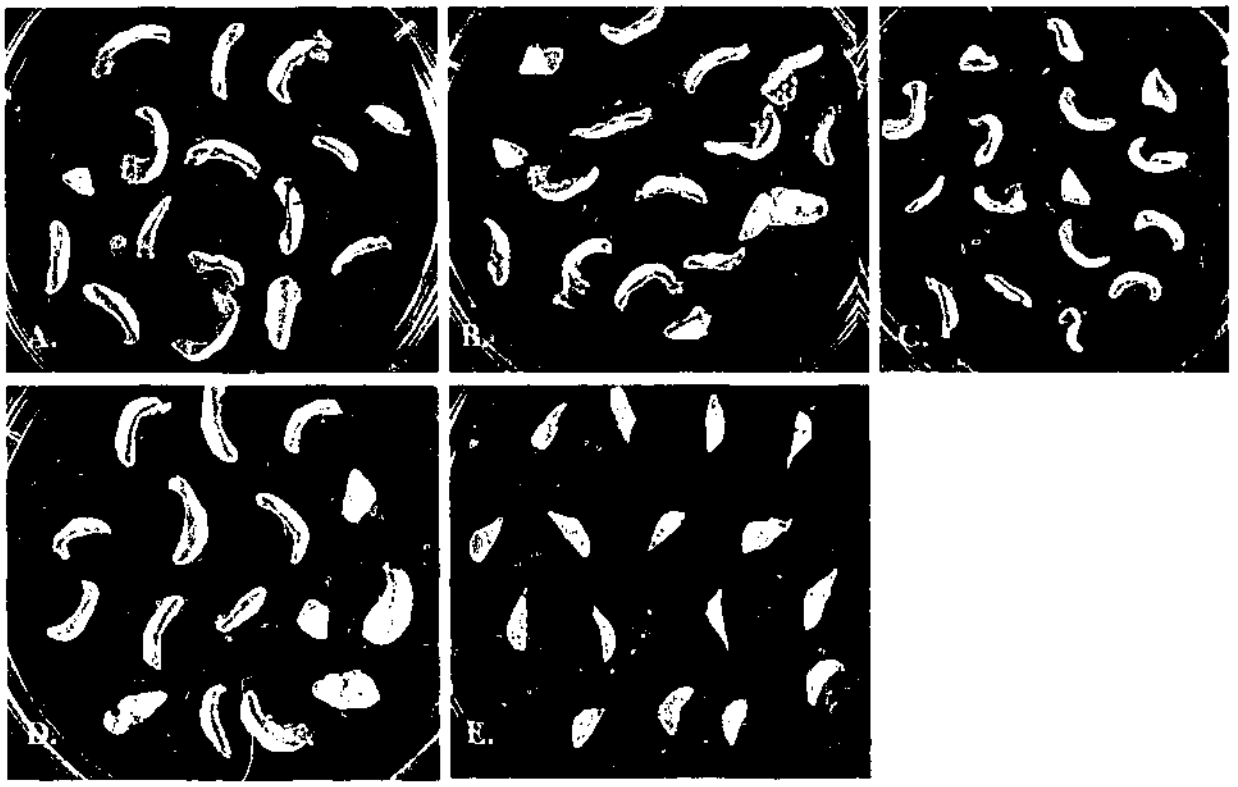


Fig 3.29: Adventitious root formation using explants excised from pre-flowering wild-type L. *erecta* plants. Media was supplemented with 10^{-4}M IAA and:
A. 0.01mM SPD **B.** 0. 1mM SPD **C.** 0.5mM SPD
D. 1mM SPD **E.** 5mM SPD

the production of an excessive amount of callus tissue. No adventitious roots were induced in the presence of either putrescine or spermidine when combined with IAA. Furthermore, a spermidine concentration of 5mM was found to be toxic and resulted in explant chlorosis before any increase in explant size was initiated (Figure 3·29E).

Leaf explants removed from flowering plants

As seen for pre-flowering plants, leaf explants removed from plants that had undergone the transition to flowering did not demonstrate any root growth or shoot regeneration when cultured on hormone-free media (Figure 3·30A). These explants were more responsive, however, to hormonal induction of adventitious root growth, with both 10^{-4}M and 10^{-5}M levels of IAA, proving stimulatory to root formation (Figures 3·30C and 3·30D respectively). Treatment with 10^{-3}M IAA however proved inhibitory to adventitious root formation (Figure 3·30B). When explants from flowering plants were treated with 10^{-4}M IAA and supplemented with either putrescine or spermidine, the IAA-induced rooting response was inhibited by the presence of the polyamines, in a dose-dependent manner (Figures 3·31 and 3·32 respectively). The results for these experiments are presented as histograms in Figures 3·33A and 3·33B. As can be seen the IAA-only control treatments show an even distribution of explants in all the rooting categories, for both the 10^{-4}M and 10^{-5}M levels (Figure 3·33A). The addition of putrescine or spermidine resulted in a skewing of data toward the "zero roots per explant" category (Figure 3·33B).

Effects of inhibition of ethylene biosynthesis

Polyamines and ethylene can have antagonistic roles during plant development (Apelbaum *et al.*, 1985) with exogenous polyamines capable of delaying senescence, whilst ethylene treatment can promote it. This is partly a result of the biosynthetic pathways of these metabolites competing for the common precursor *S*-adenosylmethionine. As polyamines were found to be inhibitory to adventitious rooting, a preliminary experiment was performed to determine the role of ethylene during this process. Leaf explants were removed from flowering *adr* mutant plants, which constitutively produce adventitious roots, possibly

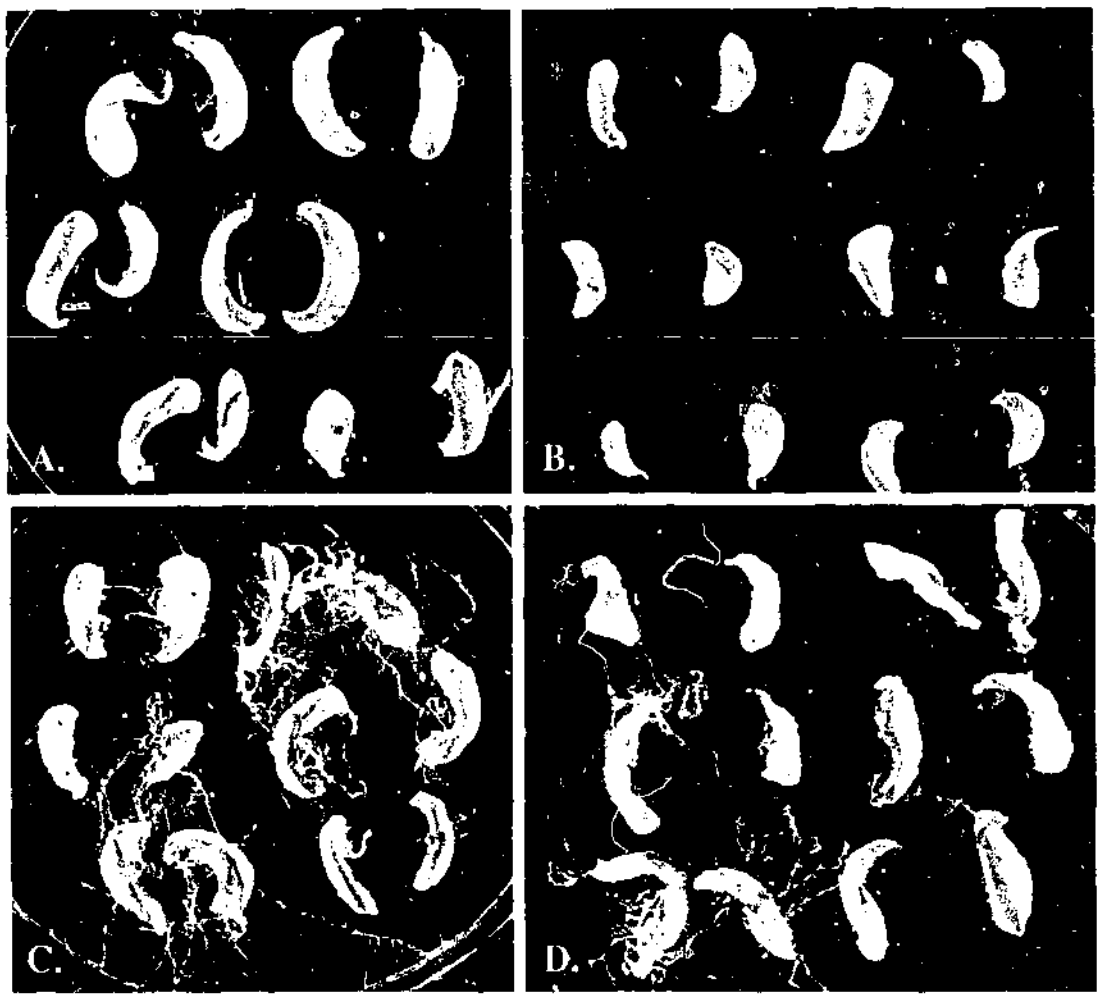


Fig 3.30: Adventitious root formation when explants were excised from flowering wild-type L. *erecta* plants.

A. Hormone free control

B. 10^{-3}M IAA

C. 10^{-4}M IAA

D. 10^{-5}M IAA

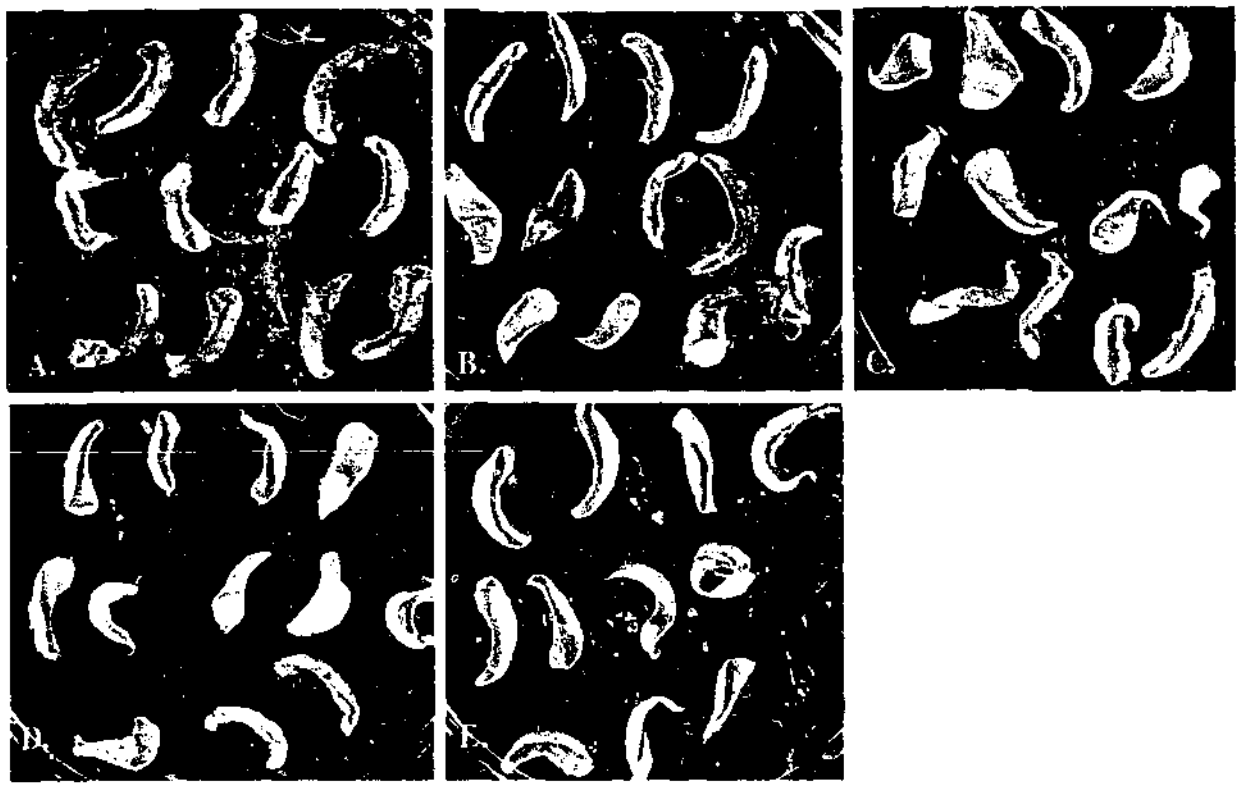


Fig 3.31: Adventitious root formation using explants excised from flowering wild-type L. *erecta* plants. Media was supplemented with 10^{-4}M IAA and:
A. 0.01mM PUT **B.** 0.1mM PUT **C.** 0.5mM PUT
D. 1mM PUT **E.** 5mM PUT

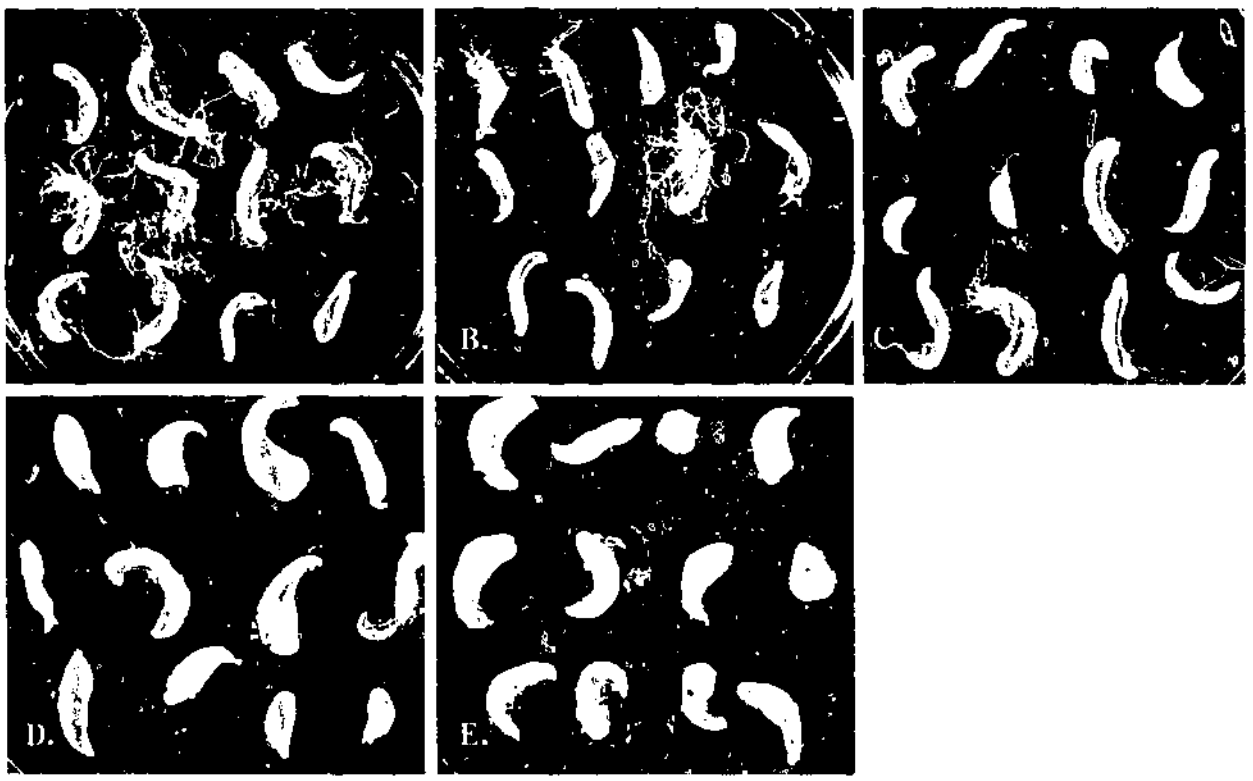


Fig 3.32: Adventitious root formation using explants excised from flowering wild-type L. *erecta* plants. Media was supplemented with 10^{-4}M IAA and:
A. 0.01mM SPD **B.** 0.1mM SPD **C.** 0.5mM SPD
D. 1mM SPD **E.** 5mM SPD

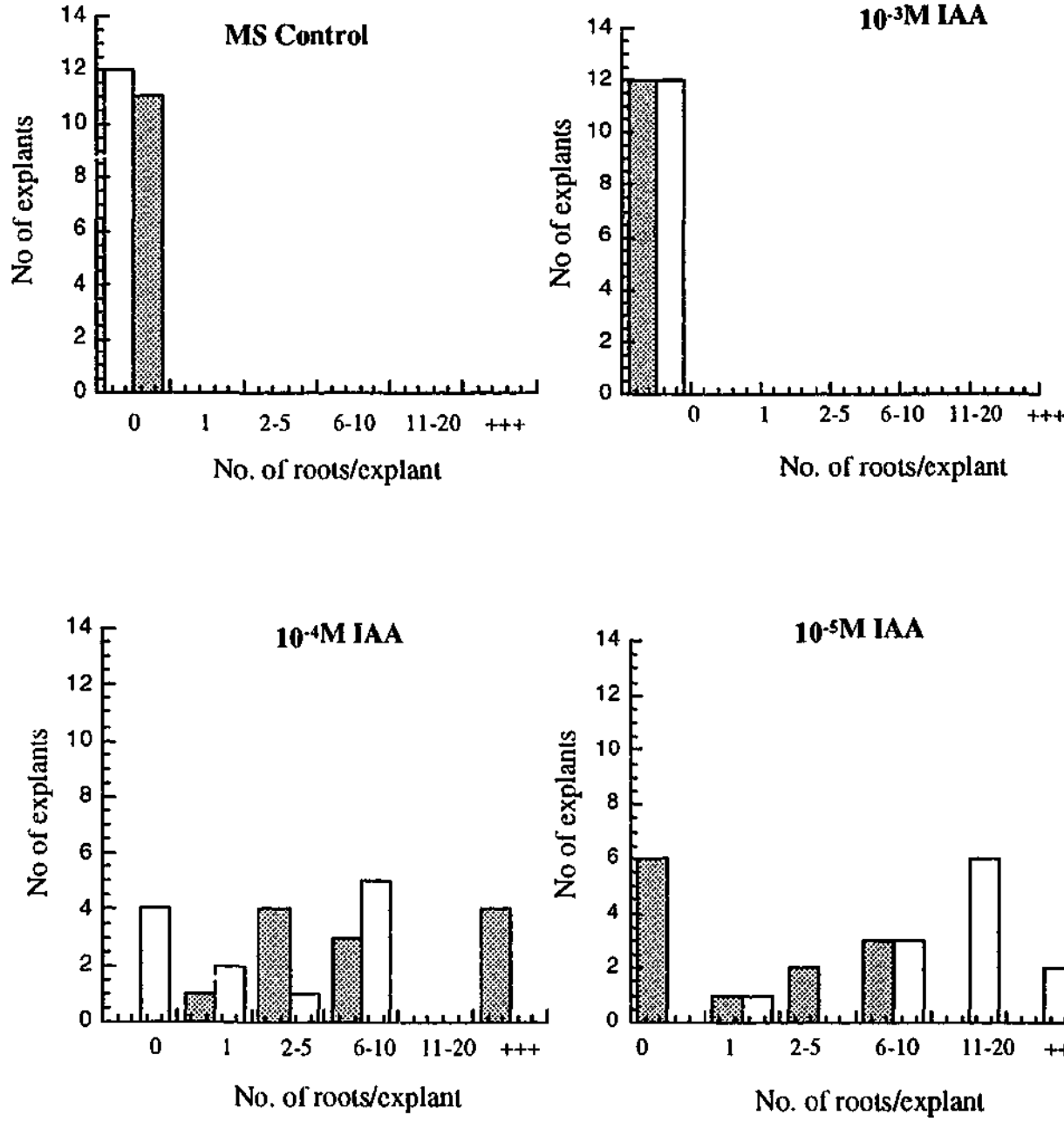
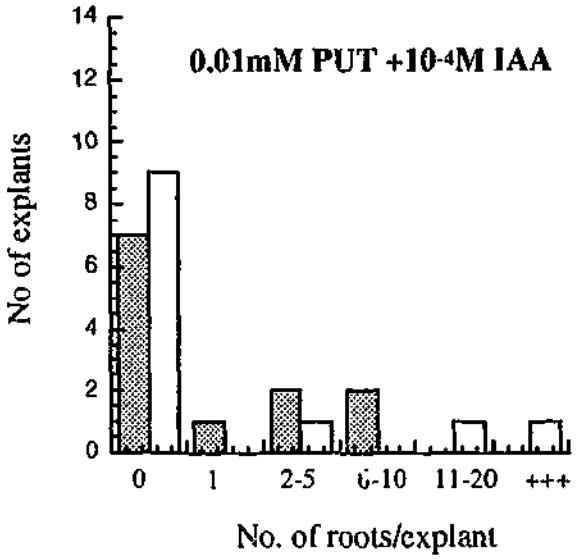
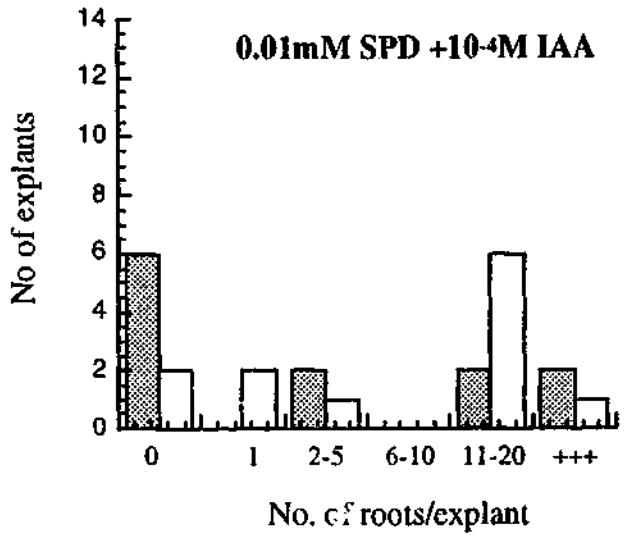
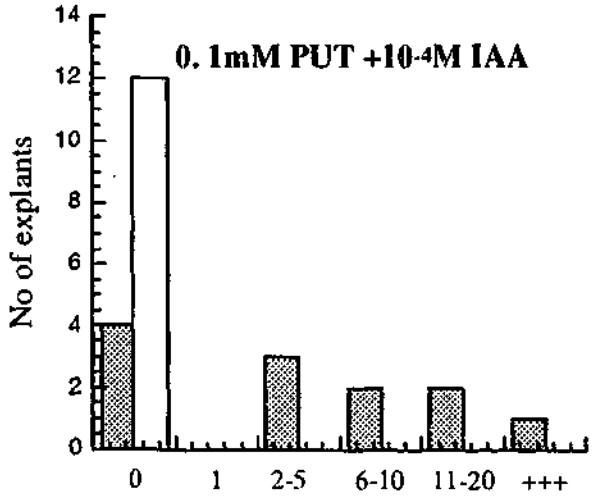


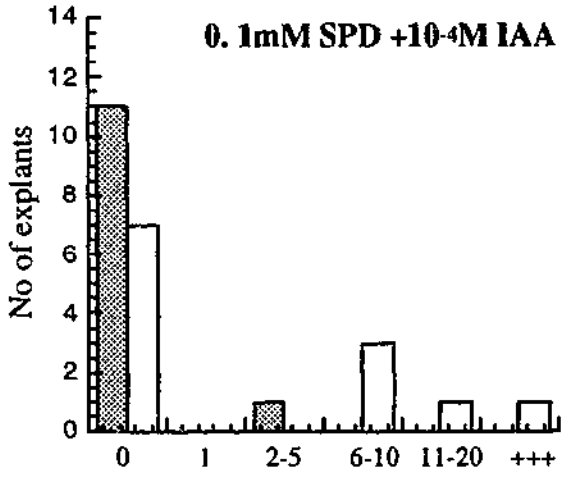
Figure 3.33A: Effects of auxin on adventitious root formation in leaf explants excised from flowering wild-type L. *erecta* plants.

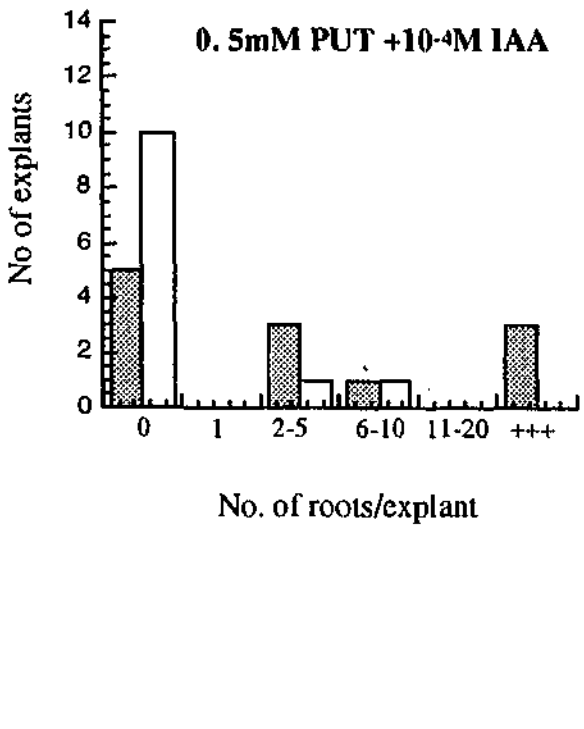
The grey and white columns on the histograms represent two independent experiments; each experiment used 12 explants per treatment.

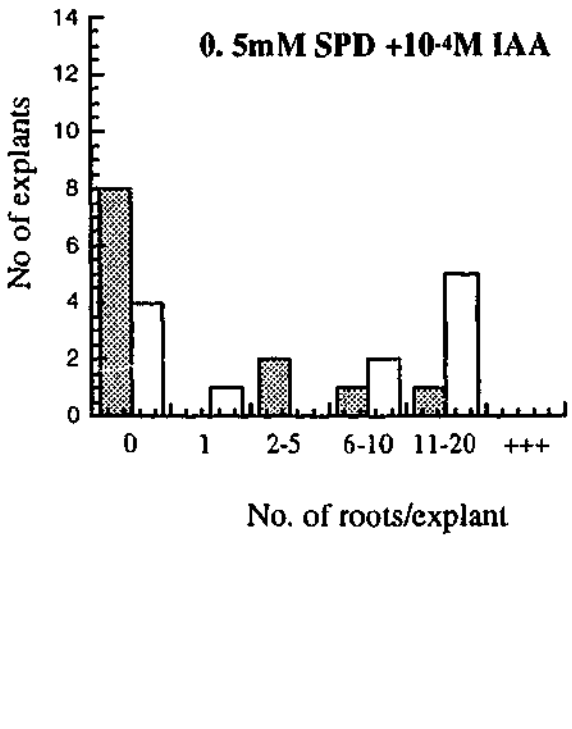

0.01mM PUT +10-4M IAA
No of explants
No. of roots/explant
0
1
2-5
6-10
11-20
+++


0.01mM SPD +10-4M IAA
No of explants
No. of roots/explant
0
1
2-5
6-10
11-20
+++


0. 1mM PUT +10-4M IAA
No of explants
No. of roots/explant
0
1
2-5
6-10
11-20
+++


0. 1mM SPD +10-4M IAA
No of explants
No. of roots/explant
0
1
2-5
6-10
11-20
+++


0. 5mM PUT +10-4M IAA
No of explants
No. of roots/explant
0
1
2-5
6-10
11-20
+++


0. 5mM SPD +10-4M IAA
No of explants
No. of roots/explant
0
1
2-5
6-10
11-20
+++

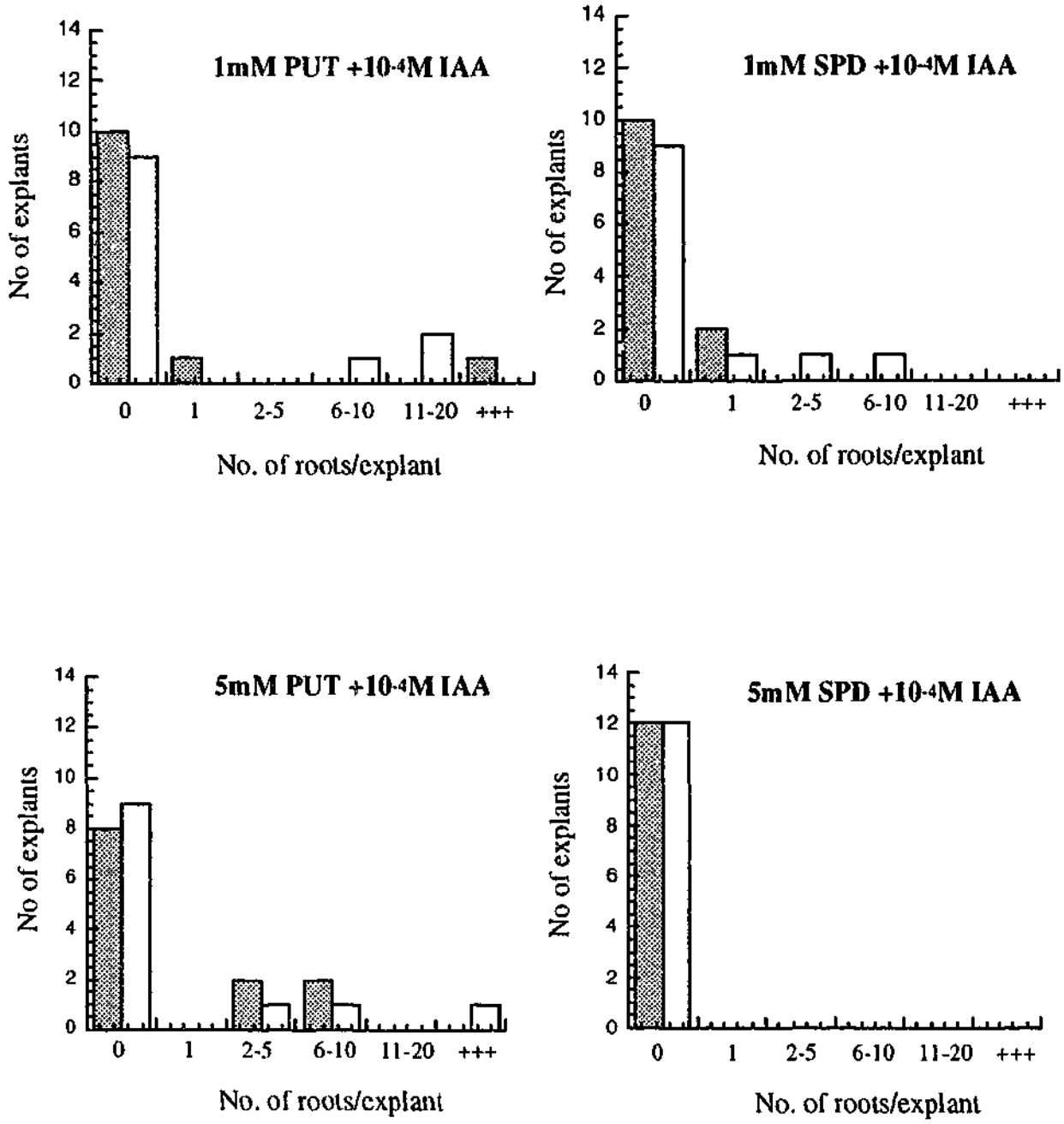


Figure 3.33B: Effects of 10^{-4} M IAA, supplemented with either putrescine or spermidine on adventitious root formation in leaf explants excised from flowering wild-type L. *erecta* plants.

The grey and white columns on the histograms represent two independent experiments; each experiment used 12 explants per treatment.

through disruption in the ethylene receptor or signalling pathway (*adr* mutants kindly provided by Dr. G. Wasteneys; Australian National University, Canberra). Explants were treated with IAA in conjunction with polyamines or the inhibitor of ethylene action, silver thiosulphate. Figure 3·34 clearly shows the enormous capacity of the *adr* explants to produce a mass of adventitious roots in response to both 10^{-4}M and 10^{-5}M IAA treatments. When added in combination with IAA, spermidine at concentrations ranging from 0·01mM to 5mM was inhibitory to root growth (data not shown), as was silver thiosulphate. These observations suggests a role for ethylene in promoting adventitious root production in *Arabidopsis*, providing a further example of the antagonistic roles of polyamines and ethylene in plant development.

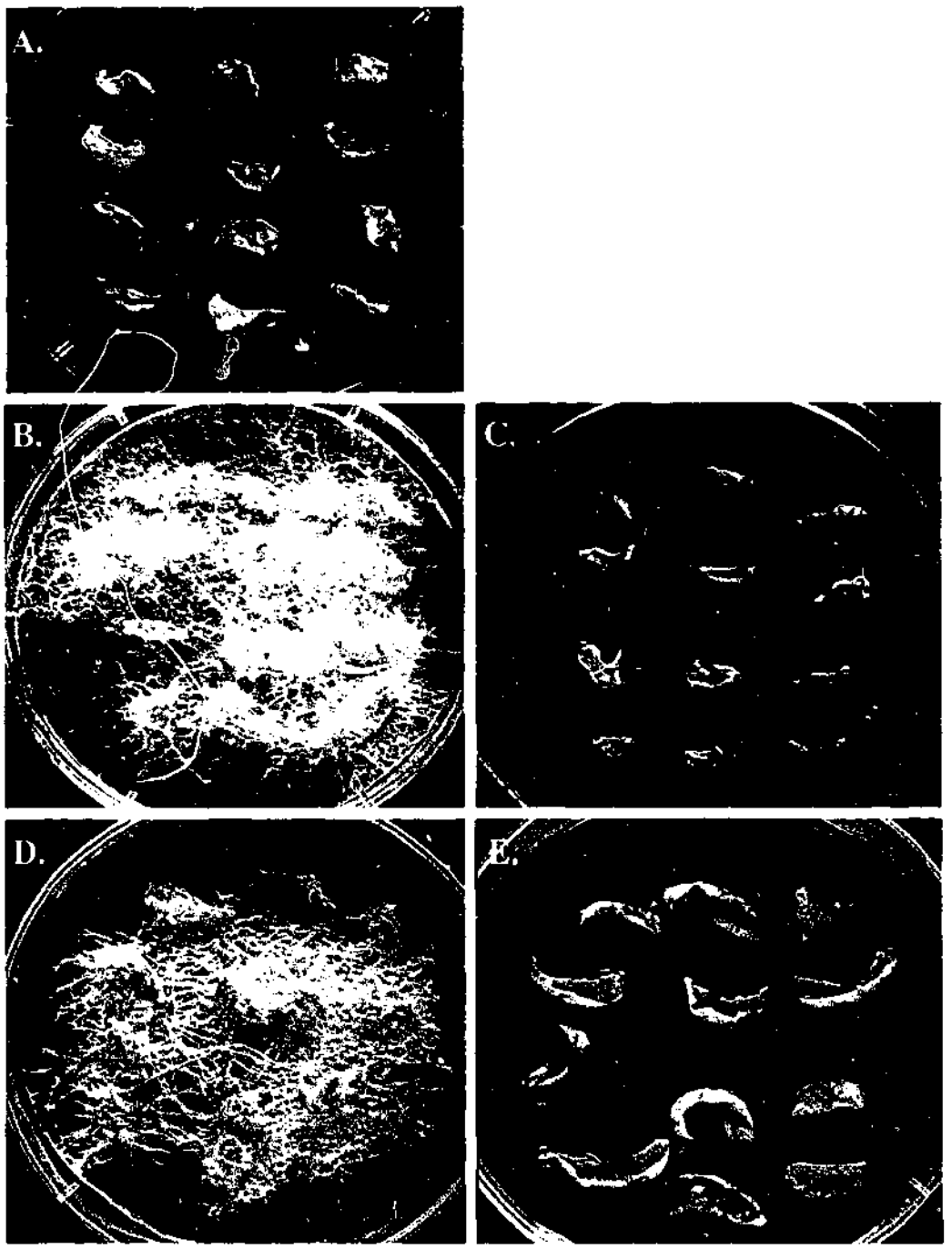


Figure 3.34: Inhibition of adventitious root formation with silver thiosulphate.

Leaf explants from flowering *adr* (*adventitious root*) mutants were exposed to either auxin alone, or in combination with the ethylene inhibitor silver thiosulphate (STS).

A. Unsupplemented MS control
B. 10^{-4}M IAA
C. 10^{-4}M IAA + 1mg/L STS
D. 10^{-5}M IAA
E. 10^{-5}M IAA + 1mg/L STS

adr explants were also treated with 10^{-4}M IAA supplemented with either putrescine or spermidine at concentrations of 0.5mM, 1mM, or 5mM. Spermidine was markedly inhibitory to rooting, whereas the rooting response with putrescine was variable and less effective (data not shown).

3·3 DISCUSSION

3·3·1 ADC AND ODC IN *ARABIDOPSIS*

In *Arabidopsis*, low *in vivo* ODC activity was initially reported, but not found to be reproducible (Watson *et al.*, 1998). It was thought that the use of the inhibitors DFMA and DFMO in enzyme assays however, would provide an accurate quantification of both ADC and ODC activities in *Arabidopsis* leaves (Feirer *et al.*, 1998). A procedure was recently described also reporting quantification of ODC enzyme activity in seedling cotyledons, and leaves from flowering *Arabidopsis* plants (Tassoni *et al.*, 2000). As the genome sequence of *Arabidopsis* is now all but complete, however, it is relevant to note that no coding regions homologous to any plant *ODC* gene have been identified following the screening of diverse *Arabidopsis* cDNA libraries with a Datura *ODC* cDNA probe (Hanfrey *et al.*, 2001). The probable absence of the gene in this species raises the question as to the mode of action of DFMO in altering the phenotype of *Arabidopsis* plants, and queries results claiming the quantification of ODC enzyme activity in this genus.

One possibility is that DFMO itself, or its degradation products, may act on other enzymes unrelated to polyamine biosynthesis in *Arabidopsis* plants. This hypothesis is supported by the report that DFMO can inhibit root formation in leafy spurge hypocotyl segments, without affecting endogenous putrescine levels (Davis, 1997A). Alternatively, it may act on ODC-like molecules to directly or indirectly influence polyamine biosynthesis. In soybean seedlings this was found to be the case, with DFMO inhibiting the activity of lysine decarboxylase, which lead to a reduction in titres of cadaverine, another polyamine found predominantly in the Leguminosae (Gamarnik and Frydman, 1991). The similarity of the *ODC* amino acid sequences from *Datura*, oat, and tomato to the sequences of both *ADC* and the enzyme involved in lysine metabolism, *meso*-diaminopimelate decarboxylase (DAPDC) (Michael *et al.*, 1996), may also suggest that these latter sequences act as potential targets for DFMO action. This is further supported following TBLASTN searches with the *Datura* ODC

sequence and *Arabidopsis* ESTs which detected the C-terminus of DAPDC on chromosome number 5 (Hanfrey *et al.*, 2001). A sequence confirmed to encode lysine decarboxylase in plants has not yet been described in the DNA databases.

In plant species possessing both the arginine and ornithine pathways leading to putrescine biosynthesis, it is thought that the application of DFMA does not necessarily inhibit ADC activity, as it is known that DFMA can be converted into DFMO through the action of arginase (Slocum and Galston, 1985; Burtin *et al.*, 1989). In *Arabidopsis*, while this conversion may still occur, as mentioned above, the mode of action of DFMO remains unclear. The *in vitro* application of 2mM DFMO to *Arabidopsis* seedlings in the present study however, did result in a marked decrease in the accumulation of free putrescine in shoots, with no change in the titres of spermidine and spermine. It remains to be seen whether this effect is merely coincidental, or due to a genuine interaction with the polyamine biosynthetic pathway.

Results from the present study indicate that the alternate *in vivo* conversion of DFMO into DFMA seems unlikely in this species, as low levels of DFMA strongly affected *Arabidopsis* root growth, whereas high levels of DFMO (up to 8mM) were ineffective. This latter observation is in agreement with findings of Mirza and Bagni (1991) who reported that DFMO was only inhibitory to *Arabidopsis* root length when the concentration was increased up to 20mM. The judicious use of DFMA in future work therefore, would appear to be an appropriate means to study the effects on growth of the specific inhibition of putrescine biosynthesis in *Arabidopsis*. It should be noted, however, that experiments performed during the present study to determine the effects of DFMA and DFMO on *Arabidopsis* were limited, due to the lack of availability of the drugs, resulting from a decision in the early 1990s by the manufacturer (Merrell Dow Research Company, Cincinnati, Ohio) to make them available only for cancer-related research activities.

3·3·2 EFFECTS OF POLYAMINE EXCESS OR DEPLETION

Growth of *Arabidopsis* callus tissue on regeneration media containing 10mM DFMA stimulated the production of roots, in agreement with similar findings for tobacco callus (Tiburcio *et al.*, 1987). Furthermore, the inclusion of 10mM DFMA in regeneration media had similar effects in stimulating production of shoots in maize callus (Guergué *et al.*, 1997) as it did in the present study of *Arabidopsis* shoots.

Growth of *Arabidopsis* plants in the presence of the polyamines putrescine, spermidine, or spermine or the biosynthetic inhibitors CHA or MGBG, caused various phenotypic alterations which were ameliorated following prolonged exposure. A similar response has been reported in tobacco thin cell layers treated with CHA or MGBG (Altamura *et al.*, 1991). Such observations may be attributable to either degradation of the additives in the media toward the end of the growth period, or to the compensatory production, metabolism, or transport of endogenous polyamines to overcome the initial excess or depletion during the early phase of growth. Plants treated with DFMO however, did not show such normalising of phenotype after 10 weeks of exposure and remained stunted. This may be due to a variety of factors including non-specific inhibition by DFMO of an enzyme unrelated to polyamine biosynthesis, which once inactivated, results in an irreversible inhibition of growth.

The morphological alterations of *Arabidopsis* plants exposed to CHA or MGBG for up to 5 weeks; included reductions in the heights of inflorescences and a slight delay in the onset of flowering, effects also observed following treatment with each of the three polyamines. The similarity of phenotypes induced by treatments with polyamines, or inhibitors of polyamine biosynthesis, may indicate that any perturbations in the optimal levels of endogenous polyamines affect basic processes, resulting in alterations in morphology and development. In this present study, the application of CHA to *Arabidopsis* plants caused an accumulation of putrescine and a concomitant decrease in spermidine levels. This is in agreement with studies from other plant tissues including carrot cells (Khan and Minocha, 1991; Minocha *et*

al., 1991B), pea seeds (Villaneuva and Huang, 1993); chick-peas (Gallardo *et al.*, 1994), and rape leaf discs (Aziz *et al.*, 1997). In thin cell layers of tobacco, such treatments also lead to inhibitions of cell wall production which were reversible by the application of polyamines (Altamura *et al.*, 1993; Berta *et al.*, 1997).

Although endogenous putrescine levels were increased in *Arabidopsis* shoots by application of CHA or MGBG, little change was observed in accumulation of *ADC* transcripts after treatment with low levels of these inhibitors, suggesting either tight constraints on transcription of *ADC*, or post-transcriptional control operating to alter ADC transcript levels in these plants. An increase in *ADC* signal was induced, however, by treatment with a higher concentration of CHA (3mM). Such positive regulation of gene expression was unexpected, as the increase in putrescine would be expected to cause reductions in expression of ADC via a feedback mechanism. Interestingly, increased putrescine titres after CHA treatment of carrot cells (Khan and Minocha, 1991) and Jerusalem artichoke tubers (Torrigiani *et al.*, 1987B) have also corresponded with elevations in ADC activity. The CHA-induced increases in putrescine and *ADC* expression, and decreases in spermidine, may result from the preferential metabolism of putrescine during growth into other compounds which are not free, conjugated, or bound polyamines (Egea-Cortines and Mizrahi, 1993), rather than its normal use for spermidine production. This may occur via putrescine oxidase in the gamma aminobutyric acid degradation pathway that leads to succinate biosynthesis (Pérez-Amador and Carbonell, 1995). It may therefore be possible that putrescine metabolised in this pathway is replenished by the action of ADC in CHA-treated plants.

As mentioned previously, the application of MGBG to plant tissue has been shown to increase putrescine titres (Minocha *et al.*, 1991B; Villaneuva and Huang, 1993) and decrease spermidine titres (Minocha *et al.*, 1991B; Altamura *et al.*, 1991; Villaneuva and Huang, 1993; Aziz *et al.*, 1998), as well as decreasing the activities of ADC (Hiatt *et al.*, 1986; Minocha *et al.*, 1991B) and SAMDC (Villaneuva and Huang, 1993; Yang and Cho, 1991). In the present study, such treatment resulted in increased accumulation of both putrescine and spermidine in *Arabidopsis*, which correlated with increased *SAMDC* transcript levels.

While the reason for increased spermidine titres is unclear, increased putrescine levels are expected, based on the MGBG-induced inhibition of decarboxylated SAM, the enzyme which is used for the generation of spermidine from putrescine. The elevation in *SAMDC* transcript accumulation observed following MGBG treatment may be a response mechanism to produce more SAMDC and overcome such inhibition. The present work is consistent with the recent report that SAMDC in *Arabidopsis* is at least, partly regulated at the transcriptional level (Tassoni *et al.*, 2000).

Due to competition for decarboxylated SAM by both the polyamine and ethylene biosynthetic pathways (Even-Chen *et al.*, 1982), treatment with MGBG or CHA also has the capacity to increase the production of ethylene, which in turn accelerates plant growth (Roberts *et al.*, 1984; Muñoz de Ruedo *et al.*, 1994; Gallardo *et al.*, 1994; Gallardo *et al.*, 1995). In the current work, *Arabidopsis* plants grown in the presence of both inhibitors, however, demonstrated slight delays in the onset of flowering, possibly suggesting that the rise in endogenous polyamines after inhibitor treatment is sufficiently high so as to negate any effects of increased ethylene production. Alternatively, excess polyamine titres caused by inhibitor- or polyamine-feeding of *Arabidopsis* plants in this study may have inhibited the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) into ethylene—a process also observed in oat leaves treated with exogenous polyamines (Fuhrer *et al.*, 1982). Furthermore, Applewhite *et al.* (2000) reported that while treatment with exogenous spermidine promotes flowering when the endogenous levels are sub-optimal, such treatment delays flowering in *Arabidopsis* when the levels of endogenous polyamines are already optimal.

Interestingly, the activities of ADC and SAMDC can be inhibited by the application of exogenous polyamines (Hiatt *et al.*, 1986; Yang and Cho, 1991) as well as polyamine inhibitors (Hiatt *et al.*, 1986; Minocha *et al.*, 1991B; Yang and Cho, 1991; Villaneuva and Huang, 1993). In addition, both treatments also result in high titres of endogenous polyamines (Minocha *et al.*, 1991B; Villaneuva and Huang, 1993. Both of these factors

may contribute to the overall similarities in the phenotypes of *Arabidopsis* plants treated with polyamines or inhibitors. High levels of endogenous polyamines have also been noted following treatments such as cytokinin-induced stimulation of cotyledon growth in radish (Sergiev *et al.*, 1995) and aluminium treatment in *Catharanthus roseus* (Minocha *et al.*, 1992), suggesting that polyamine levels *per se* reflects growth processes, rather than being the causative factor determining growth. It must also be considered when correlating polyamine titres with stimulations in growth, especially in experiments involving their exogenous application, that these amines may merely function as a source of nitrogen in actively growing tissues (Martin-Tanguy and Carre, 1993).

3·3·3 LATERAL ROOT INDUCTION

Polyamines have often been described as a new class of growth regulator due to their ubiquitous presence in all cell types and correlations of their endogenous levels with the onset of many aspects of growth (Galston and Kaur-Sawhney, 1995). The results of the present study also demonstrate that the application of exogenous polyamines or their biosynthetic inhibitors induce some alterations in root growth and development. In order to further study the involvement of polyamines in the initiation of roots in *Arabidopsis* seedlings, detailed studies of lateral and adventitious root production were undertaken following treatment of plants with polyamines or their inhibitors. Possible interactions of polyamines with auxin during root growth were also appraised.

Effects of auxins

It has been demonstrated that treatments of root tissues with auxin results in an intense stimulation of lateral root initiation in a diverse range of species such as radish (Blakely *et al.*, 1982), pea (Hinchee and Rost, 1986), lettuce (MacIsaac *et al.*, 1989), onion (Lloret and Puglarín, 1992), Eucalyptus (Pelosi *et al.*, 1995), and *Hyoscyamus* (Biondi *et al.*, 1997). The results in this study also indicate that *Arabidopsis* shows this developmental response following treatments with IAA, IBA, or NAA. Each auxin also induced a significant dose-

dependent inhibition of primary root length, which has been reported in several other species including onion (Lloret and Puglarín, 1992), pea (Zeadan and MacLeod, 1984), *Hyoscyamus* (Biondi *et al.*, 1997), and blue honeysuckle (Karhu, 1997).

Treatment with auxin at a level of 10^{-4}M has also been shown to cause regions of sub-apical swelling near the base of root fragments and at the tips of lateral roots in onion (Lloret and Puglarín, 1992). These regions are proposed to be areas which have resumed apical meristem activity after the auxin-induced inhibition of root elongation. Occasionally, such swelling was severe enough to cause fracturing of the surrounding cortical tissue of the treated roots (Lloret and Puglarín, 1992). In *Arabidopsis* seedlings, treatment with auxins at 10^{-4}M had similar effects, such that stimulation of lateral root production was so prolific that primordia were initiated along the vascular tissue of the hypocotyl as well as the primary root. Furthermore, it has been noted that auxin application to onion roots results in a modification of the normal sequence of lateral root formation, resulting in out-of-sequence development of new primordia between roots that had already begun to emerge (Lloret and Puglarín 1992), a trait also evident in the *Arabidopsis* seedlings treated with 10^{-4}M auxin.

Effects of polyamines

In the present study, putrescine, spermidine, and spermine did not exhibit auxin-like actions in terms of stimulating the formation of lateral root primordia, when applied at concentrations ranging from 10^{-8}M to 10^{-3}M. In fact, treatments with the individual polyamines themselves had neither positive nor negative effects on lateral root number.

Numerous studies report that polyamines may have a role, however, in augmenting the root-inducing effects of auxin in a wide variety of species such as mung bean seedlings (Jarvis *et al.* 1983, Friedman *et al.* 1985; Nag *et al.*, 1999), tobacco thin cell layers (Torrigiani *et al.* 1989), wild cherry shoot cultures (Biondi *et al.* 1990), micropropagated poplar shoots (Hausman *et al.* 1994), and hazel microshoots (Rey *et al.* 1994). Therefore the hypothesis that polyamines stimulate *Arabidopsis* lateral root growth in conjunction with auxins, was tested by the individual application of either putrescine, spermidine, or spermine with each of

the hormones IAA, IBA, and NAA. In all experiments conducted with the three auxins at the sub-optimal concentrations of 10^{-5}M and 10^{-6}M and the three polyamines at 10^{-3}M, no evidence of a synergistic or additive relationship between exogenous auxin and polyamines in the case of lateral root growth was observed. In fact, polyamines were found to be somewhat inhibitory to auxin-induced stimulation of lateral root initiation in *Arabidopsis*. Similar inhibitory effects on root elongation and differentiation of lateral roots have been reported in eggplant root cultures following the application of putrescine alone (Sharma *et al.*, 1997). In addition, a study of *Zea mays* root development correlated a decline in endogenous putrescine levels in roots with the development of lateral roots (Schwartz *et al.*, 1986). The inhibition mediated by polyamines, with respect to the intense stimulation of roots by auxins, may be due to the fact that roots are highly active areas of cell division and replication, which are processes known to require polyamines. Therefore, an optimal level of endogenous polyamines may exist within these areas of active growth, such that increases above this value disrupt the normal rate of polyamine turnover and thereby become inhibitory to cell division.

It was also observed that treatment with very low concentrations of putrescine at 10^{-6}M to 10^{-8}M induced a small, but statistically significant stimulation of *Arabidopsis* primary root elongation. This finding is in agreement with the report of Mirza and Bagni (1991) who also demonstrated that levels of putrescine up to 10^{-4}M were stimulatory to *Arabidopsis* root length. The reported effects of exogenous spermidine and spermine on lateral root growth and elongation, however, are somewhat contradictory and therefore suggest that the roles of these higher polyamines during root growth are variable, depending upon factors such as the species, the tissue type, and the growth conditions of the study. In studies of *Zea mays*, for example, contradictory reports exist as to the action of spermidine during root growth. Addition of spermidine at a level of 10^{-3}M, inhibits the growth rate of the primary root by 60% in this species, whereas removal of this polyamine from media allowed the rate of growth of treated seedlings to revert to normal (de Agazio *et al.* 1995). It was suggested by these sauthors that spermidine treatment reduced the mitotic index of the roots, thereby reducing cell elongation. Endogenous spermidine on the other hand, was positively

correlated with root growth in *Zea mays* following the measurement of significant increases in spermidine titres in actively growing roots (Schwartz *et al.*, 1986). In addition, whilst the application of either spermidine or spermine at a concentration of 5 x 10^{-4}M to *Arabidopsis* seedlings has been shown to be sufficient to inhibit root elongation completely (Mirza and Bagni, 1991), in the present study spermidine and spermine application were found to significantly promote *Arabidopsis* root elongation at levels of 10^{-5}M and 10^{-4}M respectively. In support of the latter observation, spermidine was found to have significant promotive effects on lateral root number and elongation when applied to eggplant root cultures at levels up to 10^{-3}M (Sharma *et al.*, 1997).

Despite some uncertainties remaining as to the specific roles of polyamines during root initiation and growth, several general observations can be made. Specifically, in *Arabidopsis* root development, while polyamines had no promotive or hormone like effect on initiation of lateral root primordia, they did appear to stimulate elongation of the primary root, and inhibit the abnormal proliferation of lateral roots in auxin-treated seedlings. This latter point is consistent with a hypothesis that the maintenance of normal growth patterns is a process in which polyamines may play a role.

Effects of chemical inhibitors of polyamine biosynthesis

Treatment with low levels of DFMA inhibited primary root length in *Arabidopsis* seedlings in the present study, however, effects of DFMA treatment on lateral root production was not xamined due to limited availability of the drug. A similar treatment of eggplant root cultures resulted in a strong inhibition of root growth and differentiation of lateral roots (Sharma *et al.* 1997). It was also demonstrated that the application of putrescine was unable to cause reversion of the DFMA-induced effects in that tissue (Sharma *et al.* 1997), suggesting that the inhibition of root growth was not due to reductions in endogenous putrescine content.

As distinct from the effects of DFMA treatment, this study indicates that high concentrations of DFMO stimulate lateral root primordia production, though not to the same extent as auxin, in *Arabidopsis*. While it remains unresolved if such effects of DFMO are the result of altered

polyamine metabolism, given that *Arabidopsis* has been reported to not possess ODC (Hanfrey *et al.*, 2001), it has also been demonstrated that in *Zea mays* plants which possess both ADC and ODC enzymes, the depletion of polyamines resulting from DFMO treatment is correlated with increased production of lateral roots (Schwartz *et al.*, 1986). It should be noted, however, that the effects of DFMO on rooting do appear to be species-specific. In eggplant root cultures for example, the inhibitor did not promote root growth or the differentiation of laterals but rather, was weakly inhibitory to these processes (Sharma *et al.* 1997).

Analogous to DFMA-application, CHA and MGBG treatments, proved significantly inhibitory to primary root elongation in *Arabidopsis* seedlings, a trait similarly noted in eggplant roots (Sharma *et al.*, 1997). In addition, treatment with exogenous spermidine was able to reverse the inhibitory effects induced by MGBG, as well as those induced by DFMA application in these tissues (Sharma *et al.*, 1997). The authors therefore suggest that the effects of the inhibitors are attributable to the decrease in endogenous spermidine titres, rather than the concomitant increase in endogenous putrescine titres (Sharma *et al.*, 1997). It cannot be discounted, however, that the elevated putrescine levels caused by inhibitor treatment is the primary factor correlating with the inhibition of lateral root growth, as putrescine has previously been shown to be inhibitory to rooting (Schwartz *et al.*, 1986).

From examination of the polyamine biosynthetic pathway, the inhibitors used in the present study are expected to have contrasting effects on putrescine accumulation; an inhibition caused by DFMA, and a promotion caused by CHA or MGBG. Despite this prediction, similar root length phenotypes were observed in *Arabidopsis* seedlings treated with any of the three inhibitors. These counter-intuitive observations may be explained, however, by the different modes of actions of these inhibitors in *Arabidopsis*. CHA and MGBG act by competitively inhibiting the enzymes spermidine synthase and SAM decarboxylase respectively, and may inhibit root growth by inducing an elevation in endogenous putrescine titres as well as depletions of spermidine titres. In *Arabidopsis*, an absence of ODC (Hanfrey *et al.*, 2001) effectively means that ADC is the first committed step to polyamine

biosynthesis. Specific inhibition of ADC by DFMA treatment would therefore be expected to reduce total polyamine production—a process that would be expected to have significant repercussions on several aspects of shoot and root growth. Furthermore, treatment with all three inhibitors were found to enhance the timing of root initiation, with lateral root primordia being observed after only six hours of inhibitor treatment, whereas primordia on untreated controls did not appear until 18 hours of incubation. These observations imply a general correlation between reductions in polyamine titres and the onset of lateral root growth in *Arabidopsis*, possibly by triggering a stimulation of G_2 phase pericycle cells near the root tip, which are susceptible to lateral root initiation (Beeckman *et al.*, 2001). Further work to explore possible interactions between auxin-stimulated lateral root production and polyamine inhibitors is warrented and may provide novel insights into the role of polyamines in the process of lateral root primordia induction.

3·3·4 ADVENTITIOUS ROOT INDUCTION

Effects of auxin

Numerous studies report stimulatory effects of auxin in generating adventitious roots in a variety of woody species. Treatment with NAA stimulates adventitious root formation in English ivy de-bladed petioles (Geneve and Kester 1991), micropropagated poplar shoots (Bellamine and Gaspar 1998; Hausman *et al.* 1994), and hypocotyl and epicotyl cuttings from pine seedlings (Goldfarb *et al.*, 1998). In *Taxus* cuttings, root growth requires a combination of both IBA and NAA (Chee 1995), whilst both IAA and IBA are strong inducers of adventitious root production in apple shoots (de Klerk *et al.* 1997). In difficult-to-root micropropagated cultivars of pear, treatment with high IBA concentrations stimulated root formation. Without auxin treatment, however, endogenous IAA levels were found to peak before root induction in easy-to-root cultivars of pear, but not in the difficult-to-root cultivars Baraldi *et al.* (1995), leading the authors to suggest that changes in endogenous IAA levels are critically related to the rooting process in pear cuttings. From the variety of studies described above, it is evident therefore that the inductive effects of different auxins

are likely to be not only species-specific, but also depend on the cell type and the developmental age of the tissue.

Initial experiments examining adventitious root production in *Arabidopsis* leaf explants used plants that did not show signs of floral initiation. Such explants did not develop roots, and exhibited shoot regeneration when incubated in the absence of IAA, or areas of callus growth when in the presence of IAA. These observations may indicate that the ratio of auxin to cytokinin within the untreated 'pre-flowering' explants were low, thus enabling shoot regeneration. In the presence of auxin however, presumably the balance is restored such that the auxin levels are now similar to those of endogenous cytokinin levels, thus stimulating callus growth. When explants were removed from flowering plants, adventitious root growth was initiated in the presence of 10^{-4}M IAA and 10^{-5}M IAA, suggesting that the ratio of endogenous auxin to cytokinin within the 'flowering' explants were at a level sufficient to allow the initiation of root growth.

Effects of polyamines

Reports examining the effects of polyamines of adventitious root generations have mainly focussed on agronomically important or woody plant species. Jarvis *et al.* (1983) first demonstrated the inhibitory effects of high levels of spermidine on the growth of roots from stem cuttings of mung bean seedlings. In the present study of *Arabidopsis*, spermidine inhibited root formation normally induced by exogenous auxin. These results are in agreement with those of Hausman *et al.* (1994 and 1995B) who reported that both spermidine and spermine were inhibitory to the auxin-induced rooting response in micropropagated poplar shoots. Interestingly, Hausman *et al.* (1995A) found that the rooting inhibitions induced by spermidine and spermine were not due to the increased endogenous levels of the two polyamines themselves, but a result of their interaction with endogenous IAA and putrescine. These reports, and the present work, indicate that spermidine has the capacity to inhibit adventitious root formation when applied either alone or in combination with auxin.

In the current study, exogenous putrescine was also found to be inhibitory to adventitious root growth in *Arabidopsis*—an observation which is in agreement with the correlations found by Tiburcio *et al.* (1987) who showed that root organogenesis is inversely related to putrescine titres in tobacco callus cultures. In studies of pear microcuttings, lack of adventitious root formation in difficult-to-root cultivars was suggested to be due to their high levels of endogenous putrescine (Baraldi *et al.*,1995). As noted previously, MGBG application causes an increase in endogenous putrescine titres, which may be the reason for the inhibition of root growth in MGBG-treated mung bean stem cuttings (Jarvis *et al.*, 1983), tobacco thin cell layers (Altamura *et al.* 1991), and poplar shoots (Hausman *et al.* 1994). Other studies have used the putrescine biosynthesis inhibitors DFMA or DFMO, to correlate low putrescine levels with increased root growth. The simultaneous application of DFMA with NAA, for example, resulted in a promotion of root formation in English ivy, which could be inhibited by the addition of 1mM putrescine (Geneve and Kester, 1991). Similarly, a decrease in endogenous putrescine levels in cotyledon explants of the spindle tree, following DFMO application, has been associated with a stimulation of rooting (Bonneau *et al.*, 1995).

On the other hand, numerous studies using polyamine biosynthesis inhibitors correlate a depletion of polyamines with an inhibition of adventitious root generation. Treatment with DFMA and DFMO caused an inhibition of rhizogenesis in tobacco explants, for example, which could be reversed by exogenous putrescine application (Burtin *et al.* 1990). Both inhibitors also strongly reduced root induction in tobacco thin cell layers (Altamura *et al.* 1991), and decreased the auxin-induced rooting response in poplar shoots (Hausman *et al.* 1994). Davis (1997B) used canavanine to block ODC, and canaline to block ADC to decrease the putrescine and spermidine content of leafy spurge hypocotyl segments, a factor which again resulted in an inhibition of root growth.

Several reports have correlated elevated polyamine levels following auxin treatment with the development of adventitious roots in a variety of plants and tissues, such as mung bean stem cuttings (Jarvis *et al.*, 1983), tobacco leaf explants (Burtin, 1990), English ivy petioles

(Geneve and Kester, 1991), and leafy spurge hypocotyl segments (Davis, 1997B). Rey *et al.* (1994) demonstrated, for example, that polyamines act *in concert* with IBA to improve the rooting response of hazel microshoots. Hausman *et al.* (1994) and Hausman *et al.* (1995B) have also shown that putrescine promotes rooting in poplar shoots up to 42% when added to the auxin medium. In conjunction with the proposed roles of free polyamines in the rooting process, Burtin *et al.* (1990) showed that auxin-induced root formation was accompanied by an increase in the levels of putrescine conjugates before the visible appearance of roots.

Although the precise interactions between auxin and polyamines are unclear, the importance of polyamines in the rooting process is supported by the observation that endogenous polyamine levels increase during adventitious root growth even in the absence of auxin treatment. Furthermore, it has been demonstrated that putrescine biosynthesis increases in the organs where roots are formed during root induction in mung bean hypocotyl segments, (Friedman *et al.*, 1985). Similarly, levels of free and bound putrescine and spermidine increase in tobacco thin cell layer explants when root meristemoids appear (Torrigiani *et al.*, 1989). Endogenous polyamine levels also peaked during the later stages of root primordia development preceding root emergence in shoot cultures of wild cherry (Biondi *et al.*, 1990).

Involvement of ethylene in the generation of adventitious roots

A further complication in understanding polyamine involvement during adventitious root growth occurs when the role of ethylene is considered. The pathways for the biosynthesis of ethylene and polyamines are intrinsically linked due to the sharing of the common metabolite SAM. Ethylene treatment of carrot cells for, example, results in reduced activities of ADC and SAMDC, and correlates with inhibition of embryogenesis (Roustan *et al.*, 1994). In the present study, it is concluded that putrescine and spermidine are inhibitory to adventitious root growth in *Arabidopsis*, however, it is not certain that these molecules *per se* cause the inhibition, or whether their application resulted in a downstream down-regulation of ethylene biosynthesis, thereby leading to inhibited root growth. Variable reports of ethylene action during adventitious rooting exist; the inhibition of ethylene biosynthesis stimulates root formation in wild-cherry (Biondi *et al.*, 1990), whereas in

sunflower seedlings, a wound-induced increase in ethylene production has been shown to stimulate the development of root primordia (Liu *et al.*, 1990).

In the present work, a preliminary study of the effects of ethylene on adventitious root initiation was performed by treating explants with an inhibitor for ethylene action, silver thiosulphate. Such experiments used a novel mutant of *Arabidopsis* (*adr*) which produces adventitious roots constitutively, possibly through alterations in the ethylene receptor or signalling pathway (Dr. G. Wasteneys; Pers. Comm.). Adventitious root growth was inhibited in explants from this mutant by silver thiosulphate application, in agreement with similar treatments using sunflower seedlings (Liu *et al.*, 1990). The effects of polyamine treatments on rooting of mutant explants were also noted. That exogenous spermidine also inhibited root growth in the mutant corroborates the proposed anti-ethylene effects of polyamine application (Suttle, 1981; Slocum *et al.*, 1984).

CHAPTER 4:

ANALYSIS OF POLYAMINE METABOLISM IN *ARABIDOPSIS* PLANTS CONTAINING T-DNA FROM *AGROBACTERIUM RHIZOGENES*

4·1 INTRODUCTION

THE PLANT PATHOGENS *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* have a natural capacity to infect a wide range of dicotyledonous plants. The neoplastic growth of tumours or adventitious roots at the infection site occurs as a consequence of the integration and expression of genes contained on tumour-inducing (Ti) or root-inducing (Ri) plasmids respectively, which is transferred from the bacterium to the plant cell (Zambryski *et al.*, 1989). This transferred DNA, or T-DNA, contains genes that undergo regulation by the transcriptional machinery of the plant, leading to the production of opines, which are used by the bacteria as a source of carbon or nitrogen.

Agropine strains of *A. rhizogenes* have been intensively studied and their Ri plasmids contain two regions of T-DNA, designated TL-DNA and TR-DNA respectively, that can be

transferred to the plant cell (White *et al.*, 1985). Eighteen ORFs are found on the TL-DNA and through transposon mutagenesis, four in particular were found to affect root induction (Slightom *et al.*, 1986). These root loci (*rol*) genes were designated *rolA*, *rolB*, *rolC*, and *rolD* and correspond to ORFs 10, 11, 12, and 15 respectively (Slightom *et al.*, 1986). The TR-DNA contains genes functionally homologous to the *iaaM* and *iaaH* auxin biosynthesis genes found on Ti plasmids, and together are referred to as the *aux* genes (White *et al.*, 1985; Cardarelli *et al.*, 1987).

Each adventitious root produced following *A. rhizogenes* infection is thought to represent an individual transformation event (Chilton *et al.*, 1982). Whole plants, often with altered phenotypes can be regenerated from transgenic roots of many species, and the T-DNA and associated phenotype are transmitted to the progeny in a Mendelian fashion as a dominant trait (Tepfer, 1984). This mode of inheritance was first described in the offspring of transformed regenerated tobacco plants that showed three phenotypes upon segregation; normal (N), transformed (T), or super-transformed (T'), with the latter two phenotypes being due to the presence of one copy and two copies respectively, of the Ri T-DNA (Tepfer, 1984; Durand-Tardif *et al.*, 1985). The T-phenotype has characteristics such as reduced apical dominance, leaf wrinkling, reduced flower size and number, delayed flowering, and an intense stimulation of plagiotropic root growth, although some variation is observed between species (Tepfer, 1984). A similar phenotype is also observed in tobacco when only the three main *rol* genes from the TL-DNA, *rolA*, *B*, and *C*, are transformed together (Cardarelli *et al.*, 1987; Jouanin *et al.*, 1987; Spena *et al.*, 1987; Vilaine *et al.*, 1987). The *rol* genes have been implicated in altering hormone metabolism in transformed plant cells, however the precise mechanisms remains unclear. Originally, RolB was proposed to release free auxin from inactive conjugates (Estruch *et al.*, 1991A) and similarly, RolC was thought to release free cytokinin (Estruch *et al.* 1991B). In *rolB*- and *rolC*-transformed plants however, auxin and cytokinin levels respectively were not increased compared to wild-type (Nilsson *et al.*, 1993A & 1993B). A subsequent hypothesis suggested that RolB is not directly or indirectly involved in auxin metabolism, but may have a role in altering the sensitivity of plant cells to IAA (Nilsson and Olsson, 1997). The tissue-specific and auxin-inducible expression of

rolB in tobacco, has since been shown to require binding of the recently identified NtBBF1 (*Nicotiana tabacum rolB* domain B Factor 1) transcription factor (Baumann *et al.*, 1999). In addition, each *rol* gene has been ascribed certain phenotypic alterations when transformed into tobacco individually, either under the control of their respective promoters, or under the control of the CaMV35S promoter. Thus transgenic *rolA* plants exhibit leaf wrinkling, darker leaves and shortened internodes (Schmülling *et al.*, 1988; Sinkar *et al.*, 1988); transgenic *rolB* plants have increased adventitious root production, altered flower morphology, and lesions on leaves (Schmülling *et al.*, 1988); and transgenic *rolC* plants show lighter coloured leaves, reduced chlorophyll content, and reductions in apical dominance, flower size, and pollen production (Schmülling *et al.*, 1988). These phenotypes have been ascribed to alterations in phytohormone metabolism and/or perception (Hamill, 1993; Michael and Spena, 1995; Nilsson and Olsson, 1997).

Correlating with the hairy-root phenotype in tobacco is a reduction of between 30% to 50% in the accumulation of both endogenous free and conjugated polyamine titres, with the magnitude of the reduction being broadly inversely proportional to the degree of alteration in phenotype (Martin-Tanguy *et al.*, 1990). Contrasting somewhat with the hypothesis that conventional hormonal alterations are primarily responsible for phenotypic alterations, these authors proposed that the genes encoded on the TL-DNA act by depressing polyamine biosynthesis, hence causing the transformed phenotype. This hypothesis was further supported by studies using the specific inhibitor of putrescine biosynthesis DFMO, which inhibit the enzyme ornithine decarboxylase (Metcalf, 1978). In tobacco, the application of DFMO to wild-type plants mimics the phenotypic alterations seen following T-DNA transformation (Burtin *et al.*, 1989), and furthermore, the inhibitor is able to accentuate the transformed phenotype of tobacco T segregants (Burtin *et al.*, 1991).

While treatments with DFMO alters the endogenous titres of free and conjugated putrescine in tobacco, and is accompanied by changes in morphology resembling the T phenotype (Burtin *et al.*, 1989), the application of DFMA alone to tobacco plants does not mimic the phenotypic effects induced by transformation (Burtin *et al.*, 1991; Martin-Tanguy *et al.*, 1991). Hence,

the TL-DNA may act by repressing polyamine biosynthesis through inhibitions of the ornithine pathway leading to putrescine production (Burtin *et al.*, 1991). The relationship is further enhanced by the demonstration that putrescine application reverses the stunted shoot phenotype induced by DFMO (Martin-Tanguy *et al.*, 1991), and in combination with another polyamine tyramine, is also able to attenuate the T′ phenotype (Martin-Tanguy *et al.*, 1996). Treatment with putrescine alone, however, has not been shown to reverse the T phenotype in tobacco, which would appear to be a critical requirement of the hypothesis that the T phenotype is caused by a reduction in putrescine synthesis as opposed to be a secondary consequence of transformation.

The purpose of the present study was to address directly the hypothesis that the genes encoded on the Ri TL-DNA, result in a hairy-root phenotype due to inhibition of the biosynthesis of polyamines (Martin Tanguy *et al.*, 1990). The hypothesis of Martin-Tanguy *et al.* (1990) was framed following studies involving tobacco, but should be applicable to other species if correct. As the *rol* genes have strong effects in altering cellular responses to auxin (Maurel *et al.*, 1991), an alternative and likely hypothesis is that depleted titres of polyamines in Ri T-DNA transformants may be a secondary consequence of hormonal alteration within such plants. *Arabidopsis* is an ideal plant in which to clarify the roles of polyamines in transformants as it does not have the metabolic complications of tobacco, in which both spermidine and alkaloids can both be derived from putrescine. Furthermore, the use of *Arabidopsis* allows plants to be grown to flowering aseptically *in vitro*, thus allowing polyamines to be fed to plants via the root system without possible complications due to microbial over-growth in soil.

In the present work, *Arabidopsis* plants were studied that had been regenerated from hairy roots derived from transformation with *A. rhizogenes* or only with the *rolABC* genes from the TL-DNA of Ri plasmid 1855 (which is present in LBA 9402 [agropine type]). As was noted, *rolABC* genes produce hairy-root phenotype in tobacco (Palazon *et al.*, 1988). In an attempt to distinguish whether polyamine depletion is the cause of the altered morphology, polyamines were supplied to the T-DNA-transformed plants cultured *in vitro* in order to test

if they ameliorated the phenotype. Similarly, it was hypothesised that the application of polyamine biosynthesis inhibitors to such transformed *Arabidopsis* plants may deplete endogenous polyamine titres further, and thus exacerbate the transformed phenotype. As well as testing the hypothesis, it was thought that an examination of phenotypes following such treatments, and the quantification of endogenous titres of free, conjugated, and bound polyamines, and the expression of polyamine biosynthetic genes, would also provide some understanding of the regulatory mechanisms operational in the polyamine pathway.

4·2 RESULTS

4·2·1 THE PHENOTYPIC LINK BETWEEN TRANSFORMATION WITH *A. RHIZOGENES* T-DNA AND INHIBITION OF PUTRESCINE BIOSYNTHESIS IN *ARABIDOPSIS*

Arabidopsis plants (Landsberg *erecta* ecotype) were regenerated from hairy roots following transformation with the agropine strain of *Agrobacterium rhizogenes* LBA 9402. Primary transformant plants were stunted with compact inflorescences, and possessed very low self fertility *in vitro*, leading to the production of small quantities of seed which had approximately 50% germination capacity when grown *in vitro*, or in soil. Three distinct phenotypes were observed in the progeny in a 1:2:1 ratio of 'N' (normal or wild-type phenotype) to 'T' (transformed phenotype) to 'T'' (supertransformed phenotype). Such plants are hereafter collectively referred to as 9402 segregants. The 9402 T' segregants exhibited a severe alteration in phenotype, with plants displaying an extreme reduction in both shoot and root apical dominance, a lack of fertility, and a stimulation of root growth. The 9402 T transformants also possessed severe reductions in fertility, allowing only a perfunctory analysis of morphology and quantification of endogenous polyamine levels (Figure 4·1A). Axenic root cultures were readily established from these T lines and whole plant regeneration was attempted on several occasions, however, no additional viable seed were produced.

To further examine the importance of ORFs 10, 11, and 12, in producing the Ri T-DNA transformed phenotype in *Arabidopsis*, and to investigate links with alterations in polyamine metabolism, *Arabidopisis* plants (L*er*) were transformed with an *Eco*RI fragment of T-DNA containing the *rolABC* genes under the control of their own promoters (Durand-Tardiff *et al.*, 1985; Cardarelli *et al.*, 1987). Four independent, pure-breeding, *rolABC* transformant lines were ultimately generated that were resistant to kanamycin and designated as #1, #3, #7, and #9. Line *rolABC*#1 appeared phenotypically normal, whilst *rolABC*#3, *rolABC*#7, and

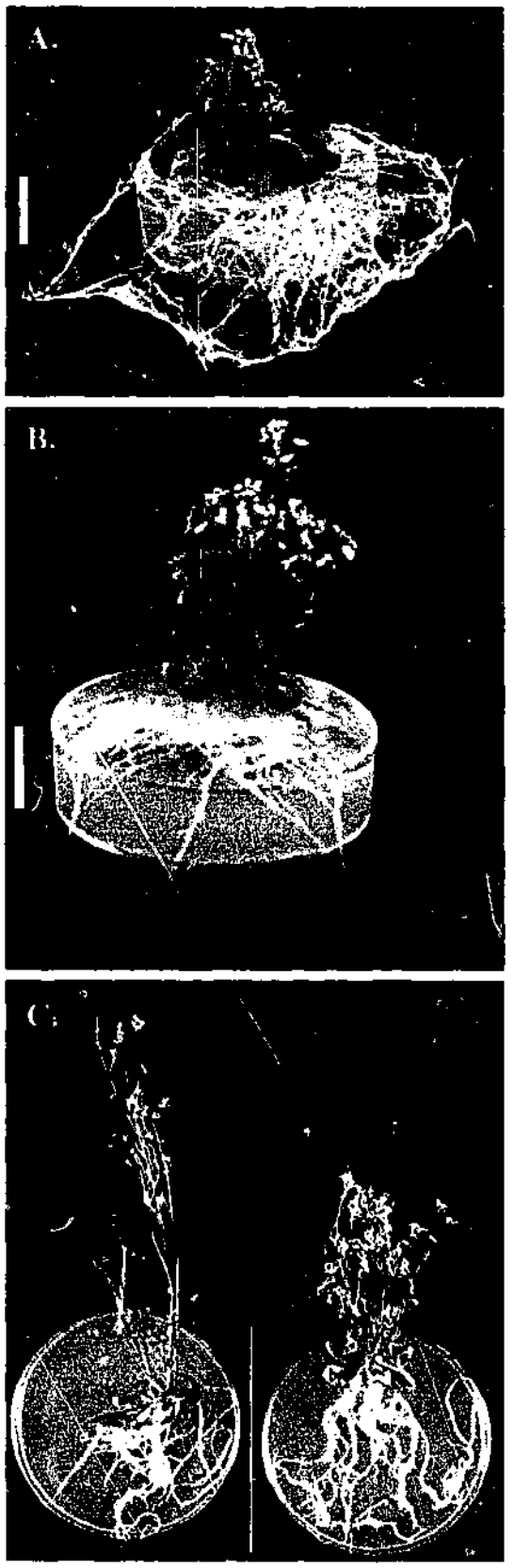


Figure 4.1: *In vitro* plant phenotypes

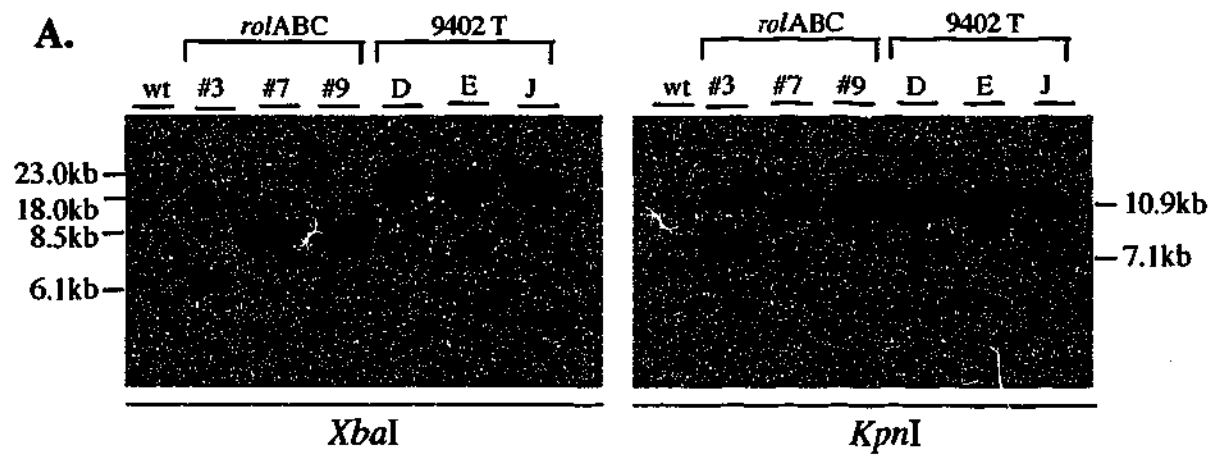
A. 9402 T segregant [Bar represents 1cm]
B. *rol*ABC#3 [Bar represents 1cm]
C. Untreated wild-type L*er* (left) vs. L*er* treated with 1mM DFMA + 1mM DFMO (right) [Bar represents 9cm]

(All plants are at 6 weeks of *in vitro* growth)

rolABC#9, exhibited a broadly similar, though less severe phenotype to that of the 9402 T segregants (Figure 4·1B). Interestingly, the phenotypes of both the 9402 and *rolABC* transformants, which consist of stunted, bushy shoots, increased number of flowers, and prolific root systems resemble those of wild-type plants treated with DFMA and DFMO (Figure 4·1C). Each of the *rolABC* transformants, however, did not exhibit the severe reductions in fertility associated with the 9402 transformants and thus were subsequently used for detailed characterisations of the effects of Ri T-DNA upon morphology, polyamine titres, and expression of polyamine biosynthetic genes in *Arabidopsis*.

4·2·2 CONFIRMATION OF TRANSFORMATION

Southern blots containing genomic DNA digested with *Xba*I and *Kpn*I were probed with the *rolB* coding sequence in order to confirm transformed status and attempt to determine T-DNA copy number of both sets of transformants (Figure 4·2). As expected, the untransformed wild-type control did not show a hybridisation signal. Since *Xba*I and *Kpn*I each cut once in the T-DNA of the binary vector, but not within the *Eco*RI fragment containing the *rolABC* genes, the presence of two hybridisation bands in DNA extracted from the *rolABC*#3 transformant line indicates the presence of two copies of the *rolABC* genes. Similarly, one band in DNA extracted from the *rolABC*#7 and *rolABC*#9 lines indicates the presence of one copy of the *rolABC* gene sequence in these lines. No hybridisation signal was detected in the DNA extracted from the *rolABC*#1 line, even though the seedlings showed resistance to kanamycin. This suggests that only a section of T-DNA, possibly including the right border and sequences up to, and including the *nptII* gene encoding kanamycin resistance, was integrated into the genome of *rolABC*#1. Approximately 10 sibling 9402 T lines from the original transformed parent were generated, and three typical lines (D, E, J) were analysed. Each showed one band under both digestion conditions, suggesting a single copy of TL-DNA in each line (Figure 4·2).



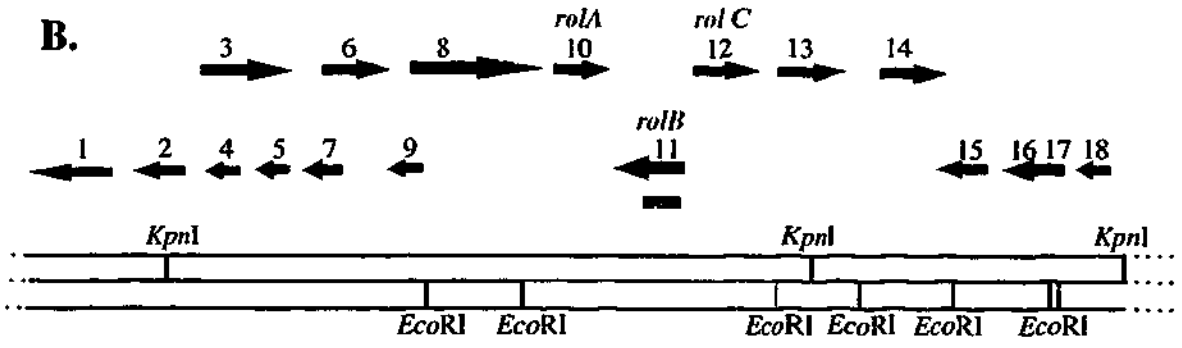


Figure 4.2: **A.** Southern blot using DNA from wild-type and transformed root tissue.

Axenic root cultures were established from wt L*er* control plants and also pure-breeding transformant lines *rolABC*#3, *rolABC*#7, and *rolABC*#9, and sibling lines designated D, E, and J from the original 9402 T parent. DNA was digested with the restriction enzymes *Xba*I and *Kpn*I which do not cut the *rolABC* genes. The membrane was probed with a fragment of the *rolB* gene and washed at high stringency. Line *rolABC*#1 showed resistance to kanamycin but did not hybridise to the *rolB* probe (not shown). As *rolABC*#1 exhibited a wild-type shoot phenotype it was used as a further control in subsequent experiments.

B. Schematic layout of the TL-DNA of pRi9402 from *Agrobacterium rhizogenes*.

The 18 ORFs are depicted by arrows, and a *Kpn*I and *Eco*RI restriction map for that region is presented below. The *rolB* probe is indicated in red. The *Eco*RI fragment containing the *rolA*, *rolB*, and *rolC* genes was used to create the *rolABC* transformants, and is indicated in yellow.

4·2·3 EFFECTS OF POLYAMINE BIOSYNTHESIS INHIBITORS AND T-DNA TRANSFORMATION ON MORPHOLOGY

Inhibition of putrescine biosynthesis

When added together at 1mM, DFMO and DFMA produced a marked change in shoot and root morphology of *Arabidopsis*, resembling the phenotype following transformation with the *rolABC* genes of *A. rhizogenes* (Figure 4·1). Thus DFMO and DFMA treatment decreased shoot height of wild-type L*er* plants by approximately 50% (Figure 4·3A) and also stimulated the number of flowers produced per plant, from an average of 70 flowers in untreated controls, to an average of 140 flowers per treated plant (Figure 4·3B).

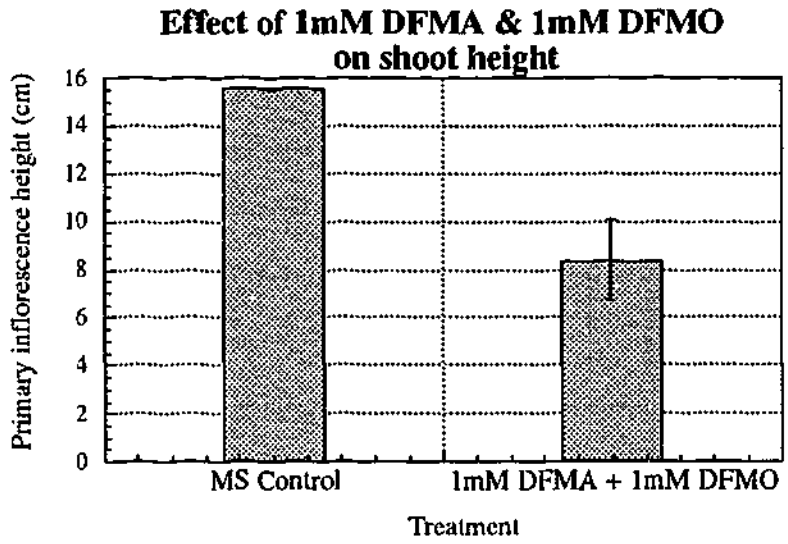
A further experiment using DFMO only, but at the slightly higher level of 2mM (Table 4·1), confirmed these observations with a more pronounced reduction in primary inflorescence height than was the case when both inhibitors were used at 1mM. The overall shoot mass however, increased from an average of 0·69g fresh weight to 1·0g after treatment with 2mM DFMO, due to a stimulation of axillary inflorescence growth (Table 4·1). The length of the primary root was slightly reduced following treatment with 2mM DFMO even though concomitant minor stimulation of overall root mass was observed. A decrease in endogenous free putrescine levels was also observed in plants treated with 2mM DFMO, with levels dropping from an average of approximately 20µg/g fresh weight in controls to an average of less than 6µg/g fresh weight in treated shoot tissue (Table 4·1).

Transformation with *A. rhizogenes* T-DNA

Effects of TL-DNA transformation on weight

A massive stimulation of root growth was observed in 9402 T segregants grown *in vitro* resulting in both the shoot and root tissues having similar weights, whereas in both the 9402 N and 9402 T' segregants, the shoot weight contributed the major proportion of the total dry weight (Figure 4·4). Overall, a dramatic decrease in the shoot and root ratio was observed

(A)



(B)

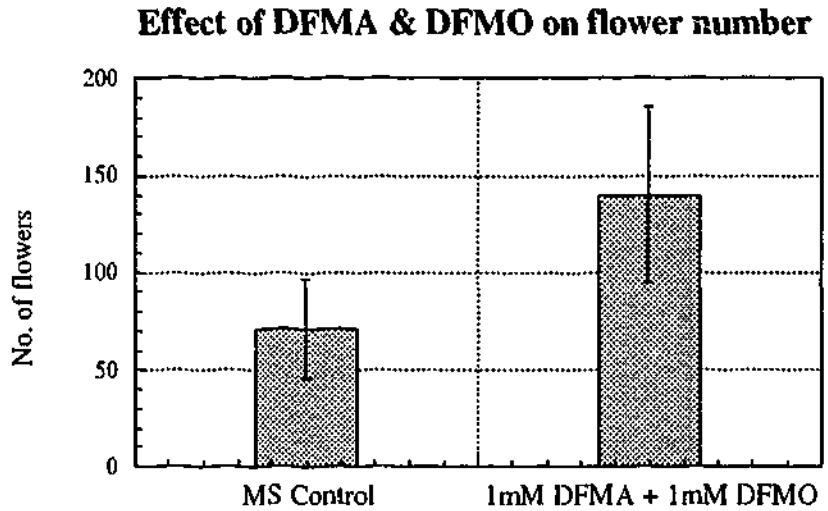


Figure 4.3: *In vitro* effects of putrescine biosynthesis inhibitors on key morphological parameters of wild-type L. *erecta* (n=3).
A. Primary inflorescence height
B. Number of flowers

	Control	2mM DFMO
Shoot height (cm)	16·2 ± 2·0	4·6 ± 0·9
Shoot f.wt. (g)	0·7 ± 0·3	1·0 ± 0·4
Primary Root length (cm)	11·1 ± 1·1	8·9 ± 0·6
Root f.wt. (g)	0·05 ± 0·02	0·08 ± 0·03
Free Putrescine[A] (µg/g f.wt.)	20·2 ± 8·6	5·6 ± 4·1

Table 4.1: Effects of 2mM DFMO on growth parameters of wild-type L. *erecta*.
Values shown are averages ± SEM.

[A] As measured in shoot tissue.

from the 6:1 observed in wild-type L*er*, to an average of 1:1 in 9402 T segregants (Figure 4·4B).

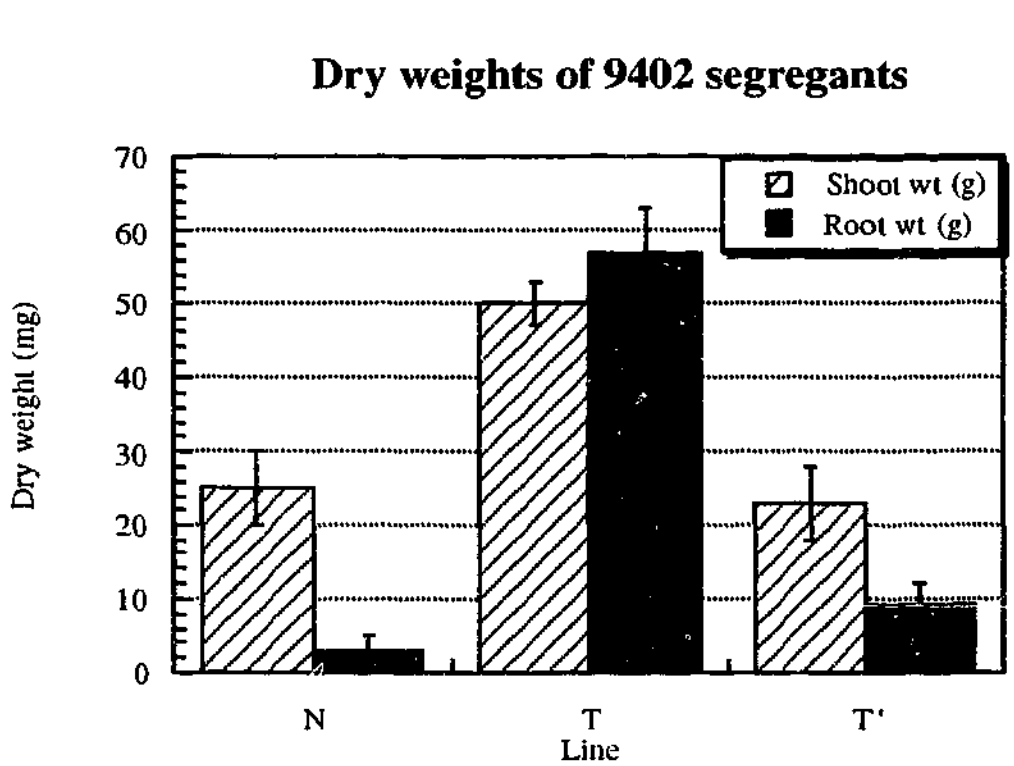


Figure 4·4: Effects of Ri T-DNA on *in vitro* dry weights of 9402 segregants harvested at 10 weeks post-germination. Average ± SEM presented; n=5

Effects of *rol*ABC transformation on root architecture

The root growth of the *rolABC* transformant lines #3, #7, and #9 compared to an untransformed control is shown in Figure 4·5. At eight weeks of *in vitro* growth, a change in root architecture was evident in the transformants compared to the wild-types, whereby the identification of primary roots from surrounding secondary, tertiary, and other higher order lateral roots was difficult. By this age, there were several thick roots all originating from a similar region at the base of the hypocotyl, and each of these 'primary' roots possessed accompanying secondary, tertiary, and quaternary roots, thus contributing to the increased root mass of the transformants.

In an attempt to quantify the observed alterations in root architecture of the *rolABC* transformants, studies of *in situ* root growth on MS medium were performed. Results are

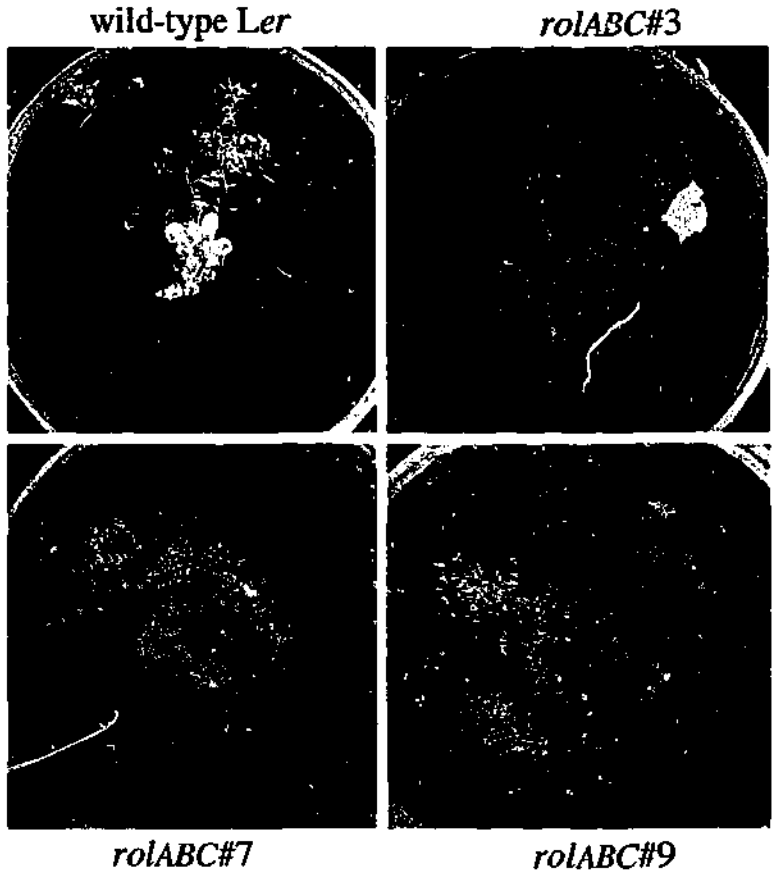


Figure 4.5: *In vitro* growth of control and *rolABC*-transformed plants.
Week 8 of growth presented.

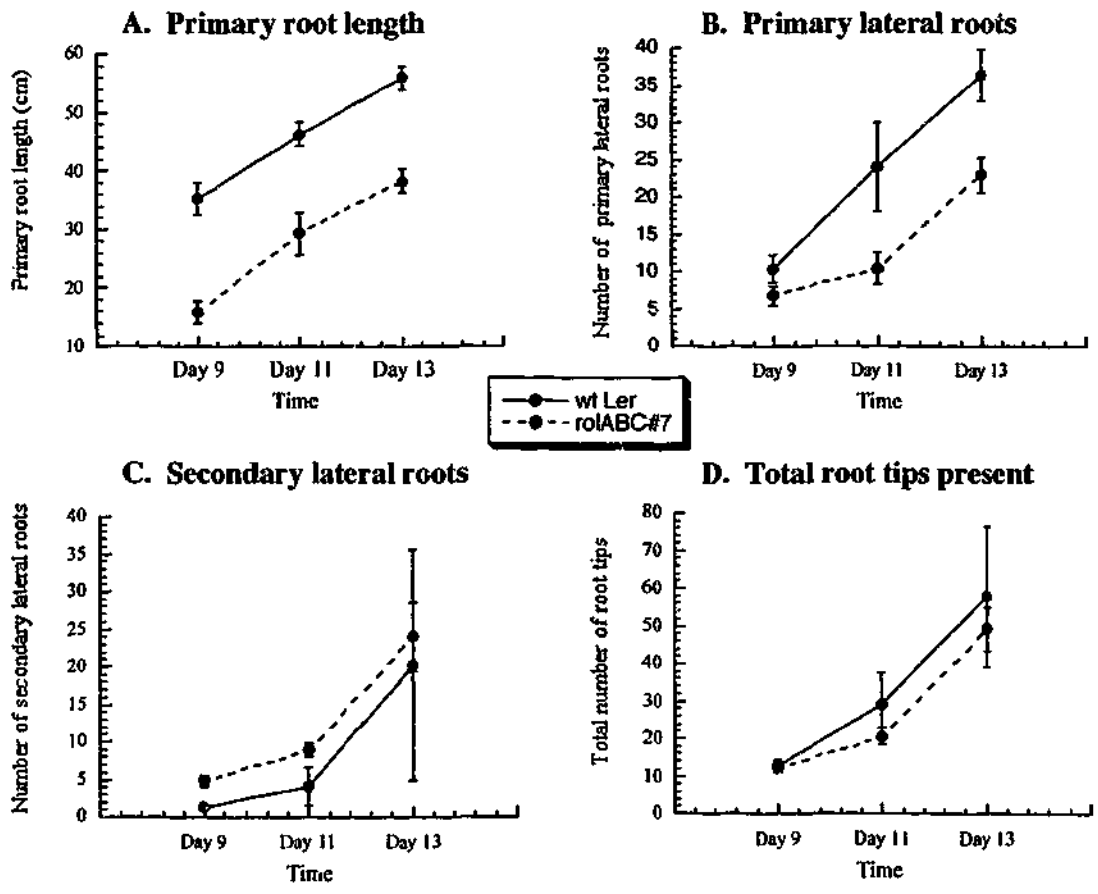


Figure 4.6: Root architecture experiment comparing root growth of wild-type control and *rolABC*-transformants.
A.Primary root length B. Total root tips
C. Primary lateral roots D. Secondary lateral roots
n = 3-to-5 per line per time point

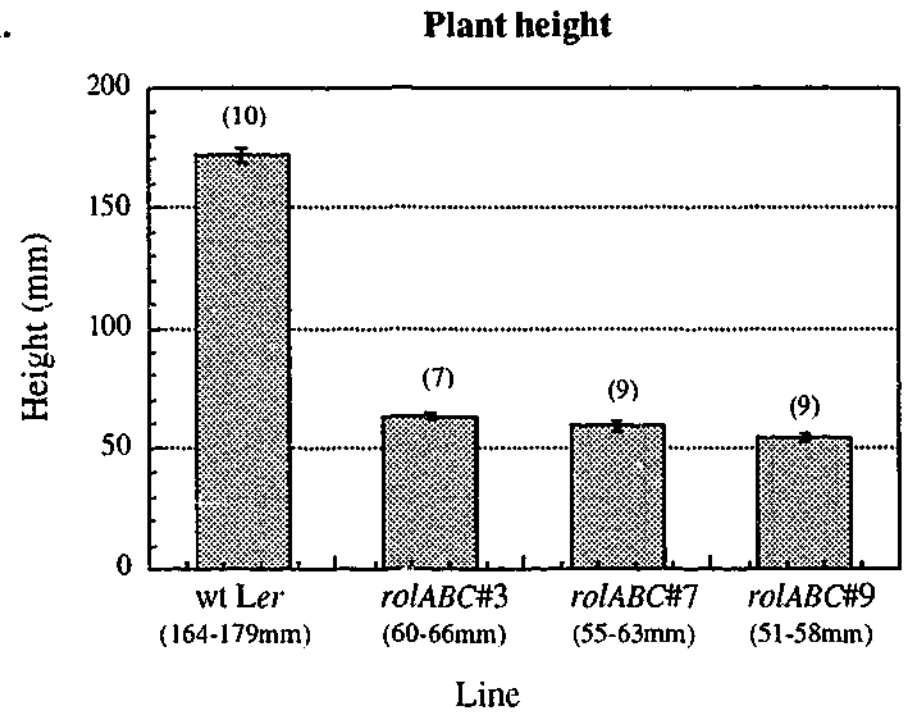
only presented for the *rolABC*#7 line as the extremely complex growth patterns of roots from the *rolABC*#3 and *rolABC*#9 lines were impractical to track accurately for the duration of the experiment. It is clear that a major effect of *rolABC* transformation is to cause a stunting of the primary root compared to untransformed wt *Ler* controls (Figure 4·6A). Although the *rolABC*#7 line exhibited fewer primary lateral roots than wild-type (Figure 4·6B), it was able to maintain a slightly higher number of secondary lateral roots (Figure 4·6C). The total number of root tips per plant however, was not compromised, as both wt *Ler* and *rolABC#7* had approximately 50-55 tips each by day 13 of growth (Figure 4·6D), thereby ensuring overall similarities in total root tip number. From day 15 of growth, the number of lateral roots of the transformants were unable to be accurately counted, due to dramatic increases in the initiation and growth of secondary, tertiary, and quaternary roots from this time onward.

Effects of *rol*ABC transformation on shoot morphology

As presented earlier (Figure 4·1B), transformation of wild-type *Ler* with the three *rol* genes induces a marked change in shoot morphology. The primary inflorescence heights of transformed plants were reduced from an average of 170mm in the wild-type and *rolABC*#1 controls, to approximately 50mm in the three *rolABC*#3, #7, and #9 lines (Figure 4·7A). Such marked differences in inflorescence heights between transformants and controls, however, did not result in shoot weight differences of the same magnitude between the two groups. As presented in Figure 4·7B, the shoot weight still contributed the major proportion of total dry weight in *rolABC* lines, although stimulation of root growth noted in these lines meant that intermediate shoot-to-root ratios of approximately 2:1 were observed. This compares to the 5:1 ratios observed in the wild-type and *rolABC*#1 control lines and the 1:3 ratio for the 9402 T transformant.

The characteristic stunted phenotype of *rolABC* transformants was evident when plants were grown under both *in vitro* (Figure 4·8A) and greenhouse conditions (Figure 4·8B). As previously mentioned, line *rolABC*#1 exhibited a wild-type shoot phenotype when grown *in vitro* (not shown) and in soil (Figure 4·8B, panel F).

A.



B.

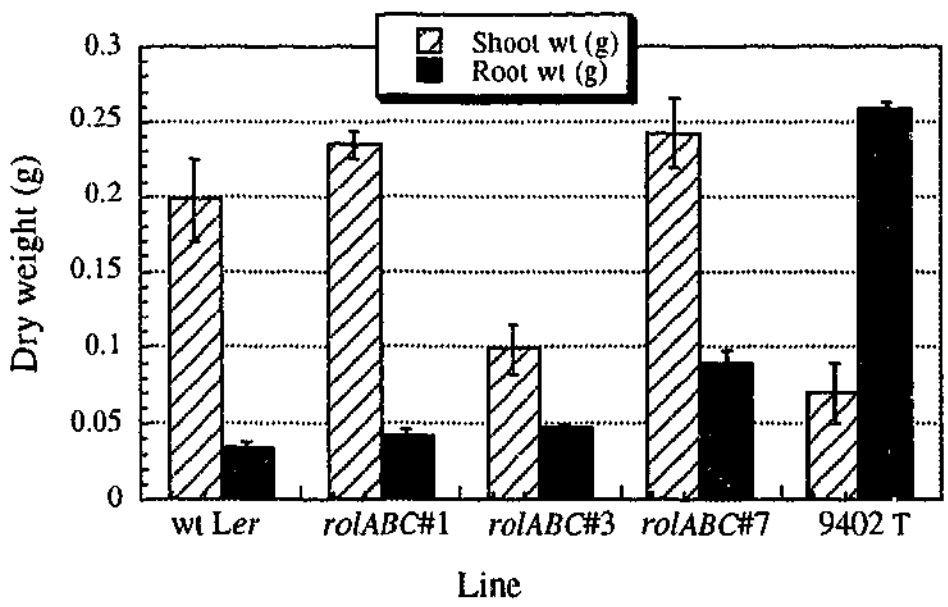


Figure 4.7: Effects of transformation on key morphological parameters *in vitro*.

A. Plant height at seed set of *rolABC* lines (Average ± SEM). The 95% confidence intervals for plant heights (in mm) are shown below the x-axis. The number of samples used per line are indicated above each column.

B. Shoot and root dry weights of *rolABC* lines compared to wt L*er* and the 9402 T segregant. (Average + SEM).

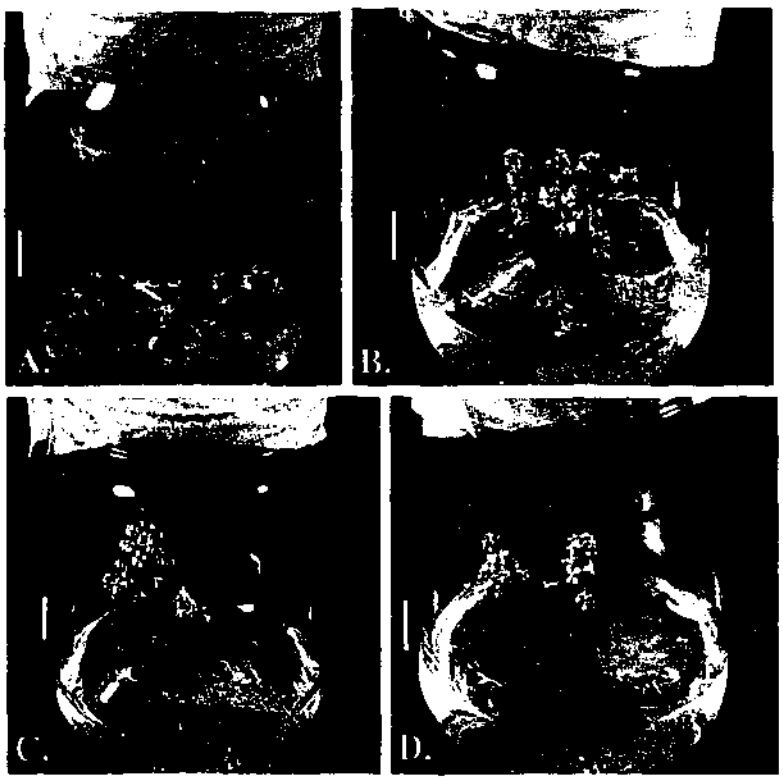


Figure 4.8A: Shoot phenotypes of wild-type and *rolABC* transformants when grown *in vitro*.

A. wild-type L*er* control **B.** *rolABC*#7
C. *rolABC*#3 **D.** *rolABC*#9

The bar in each panel represents 1cm.

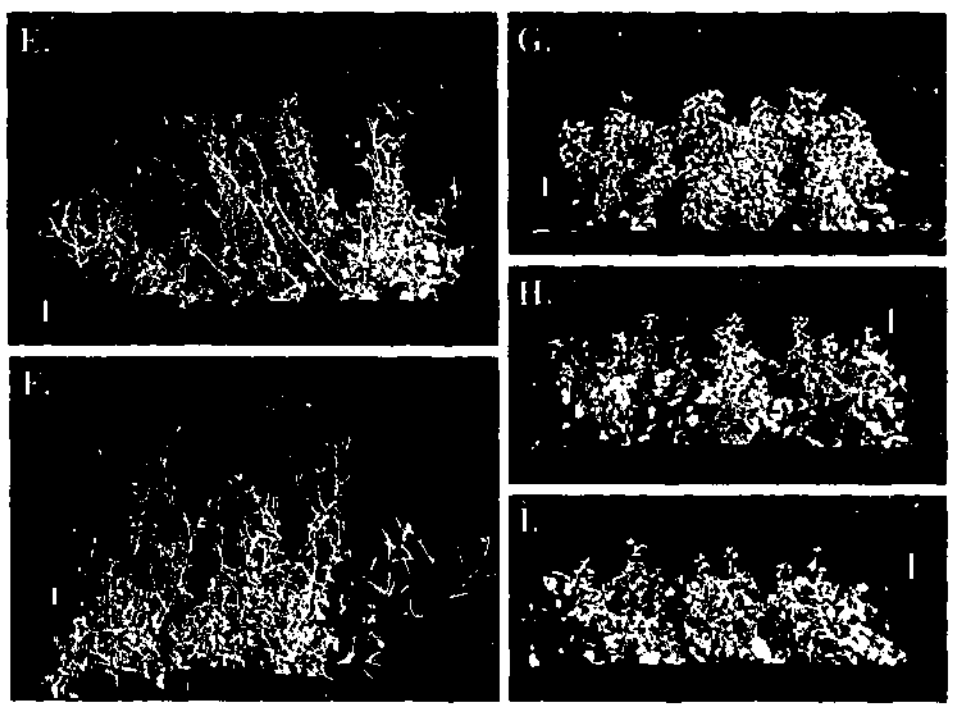


Figure 4.8B: Shoot phenotypes of wild-type and *rolABC* transformants when grown in soil.

E. wild-type L*er* control **G.** *rolABC*#3
F. *rolABC*#1 **H.** *rolABC*#7
I. *rolABC*#9

The bar in each panel represents 1cm.

Effects of transformation with *rol*ABC or Ri TL-DNA on flower structure

The 9402 N segregants have a wild-type shoot phenotype of vegetative and floral structures (Figures 4·9A and 4·9B). Flowers of the 9402 T segregants however, were smaller than wild-type flowers with their stamens approximately half the size of controls (Figure 4·9A and 4·9B). A similar phenotype was also observed in the *rolABC*#3 line (Figure 4·9B), which possessed smaller stamens and also smaller petals and sepals than wild-type L*er* controls and the other transformed lines (Figures 4·10A and 4·10B). The stamens from lines #7 and #9 were slightly reduced in size relative to their respective gynoecia, compared to wild-type L*er* controls and also *rolABC*#1 control flowers. The size of the gynoecium however, was essentially the same in all lines (Figure 4·10B).

Effects of *rol*ABC transformation on silique structure

The phenotype of siliques removed from wild-type and *rolABC* transformant plants grown in soil are shown in Figure 4·11. *rolABC* transformant lines #3, #7, and #9 produced abnormally small siliques which were often bent (Figure 4·11, panels C, D, and E respectively) compared to their wild-type L*er* and phenotypically normal *rolABC*#1 counterparts (Figure 4·11, panels A and B). The reduced size of transformed siliques correlated with a reduction in seed number and also seed size (Figure 4·11, panel F). The total number of siliques produced per plant however, was markedly increased in transformants compared to the wild-type L*er* controls (Figure 4·12) and *rolABC*#1 plants (data not shown). Thus the 95% confidence interval for the wild-type L*er* line is approximately 20-to-30 siliques per plant, and for the *rolABC* transformants the intervals lie between 100-150 siliques per plant for *rolABC*#3, between 55-80 siliques for *rolABC*#7, and between 40-55 siliques for *rolABC*#9.

Effects of *rol*ABC transformation on primary inflorescence structure

To study the cellular organisation of the dwarf shoot phenotype of the *rolABC* lines, cross-sections of the primary inflorescence from wild-type L*er* and the transformants were examined. Figure 4·13 presents cross-sections obtained from the apical and basal nodes of

Figure 4.9: Morphology of flowers from wild-type control and transformed plants.
A. Intact flowers
B. Flowers with petals removed

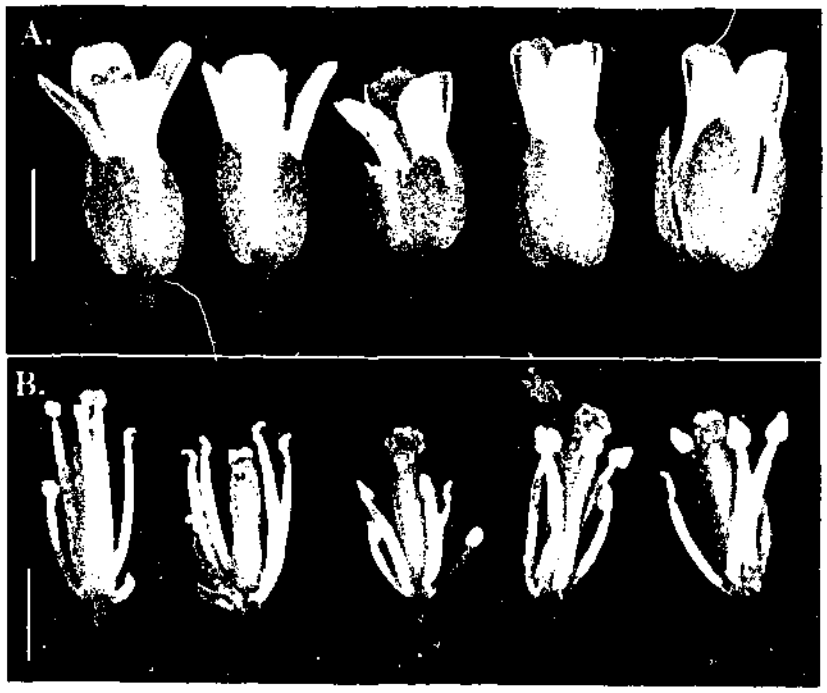


Figure 4.10: Morphology of flowers from wild-type L*er* and *rolABC*-transformed plants.
A. Intact flowers
B. Flowers with petals removed (the withered appearance of *rolABC*#1 anthers is due to age, rather than a transformation effect)

(Bars represent 1mm)

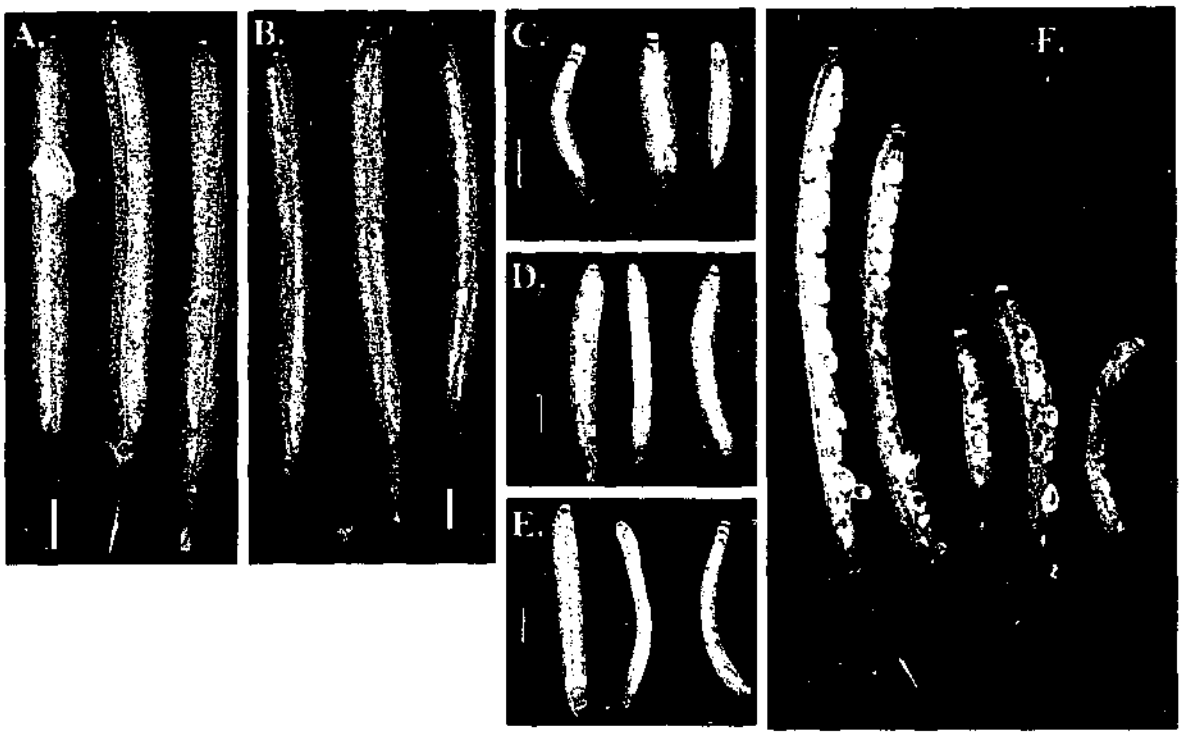


Figure 4.11: Morphology of siliques from wild-type and *rolABC*-transformants.

A. Wild-type L*er*
B. *rolABC*#1
C. *rolABC*#3
D. *rolABC*#7
E. *rolABC*#9
F. Dissected siliques; L. to R.: wt Ler, *rolABC*#1, *rolABC*#3, *rolABC*#7, *rolABC*#9

(Bars represent 1mm)

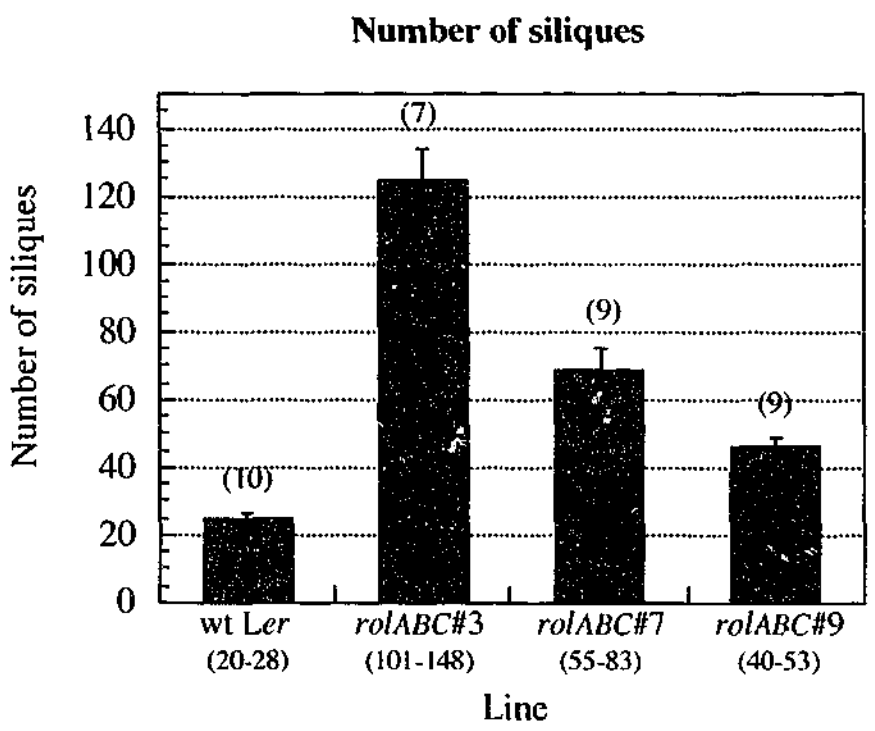


Figure 4.12: The effects of transformation on the number of siliques produced per plant (Average ± SEM).

The 95% confidence intervals for the number of siliques per plant are shown below the x-axis. The number of samples used per line are indicated above each column.

inflorescence axes from wild-type L*er* control (Figure 4·13A) and phenotypically normal *rolABC*#1 (Figure 4·13B) lines, and the *rolABC*#3 (Figure 4·13C) and *rolABC*#7 (Figure 4·13D) transformant lines. The *rolABC*#9 line had the same T-DNA copy number as the *rolABC*#7 line and was not included this study. Close examination of the sections from all four lines revealed that both *rolABC*#3 and *rolABC*#7 were different from control and *rolABC*#1, with *rolABC*#3 plants altered to the greatest extent. Firstly, the spatial positioning of the vasculature was not symmetrical within the *rolABC*#3 line. Instead of a typical pattern of pith cells, having quite evenly spaced, alternating vascular bundles and interfasicular cambium as seen in control and *rolABC*#1 lines (Figure 4·13A), *rolABC*#3 sections, and to a lesser extent the lower section of *rolABC*#7, had an uneven vascular pattern and irregularly-shaped pith zone. Secondly, a lack of uniformity among pith cells, in terms of both their size and shape, was found in both *rolABC*#3 and *rolABC*#7 lines (Figures 4·13C and 4·13D). A further alteration in cellular organisation was seen in the cortical layer of the lower section from the *rolABC*#3 inflorescence. The normally small collenchyma cells in this layer were elongated, with their long axes perpendicular to the epidermal cell layer, rather than the parallel orientation noted for wild-type cortical cells. The increased size of these cells in the *rolABC*#3 lower section, and their changed orientation, resulted in the cortical layer occupying relatively more area of the cross section than in control and *rolABC*#1 lines. This is in spite of the cortical zone of the lower *rolABC*#3 section comprising only two-to-three layers of cells, compared to the four-to-five concentric layers of near-uniformly-shaped collenchyma cells observed in the other lines.

4·2·4 AXENIC ROOT CULTURE ANALYSES

Effects of transformation on growth, polyamine titres, and gene expression

Growth kinetics

To further characterise the effects of transformation on root growth, axenic root cultures were established from both the 9402 T and *rolABC* transformants, and the wild-type *Ler* controls. This allowed the growth kinetics of each line to be determined, and correlated with

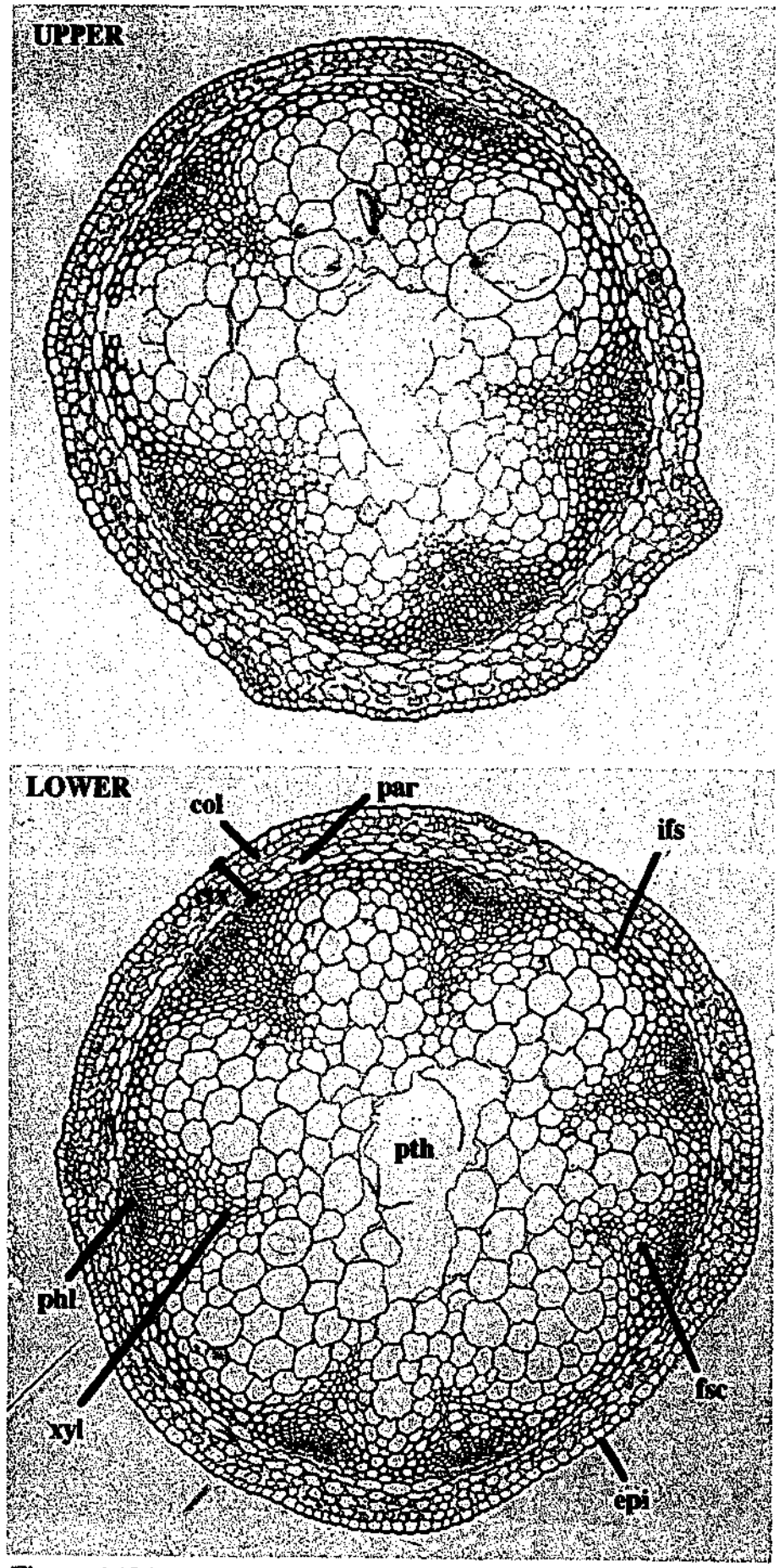


Figure 4.13A: Transverse sections stained with toluidine blue from primary inflorescences of wild-type control (**x25 mag.**)
Upper section; from the apical node
Lower section; from the basal node above the rosette leaves
(Sections were prepared from soil-grown, flowering plants)
ctx - cortex; **col** - collenchyma; **epi** - epidermis; **fsc** - fasicular cambium; **ifs** - interfasicular cambium; **par** - parenchyma; **phl** - phloem; **pth** - pith; **xyl** - xylem

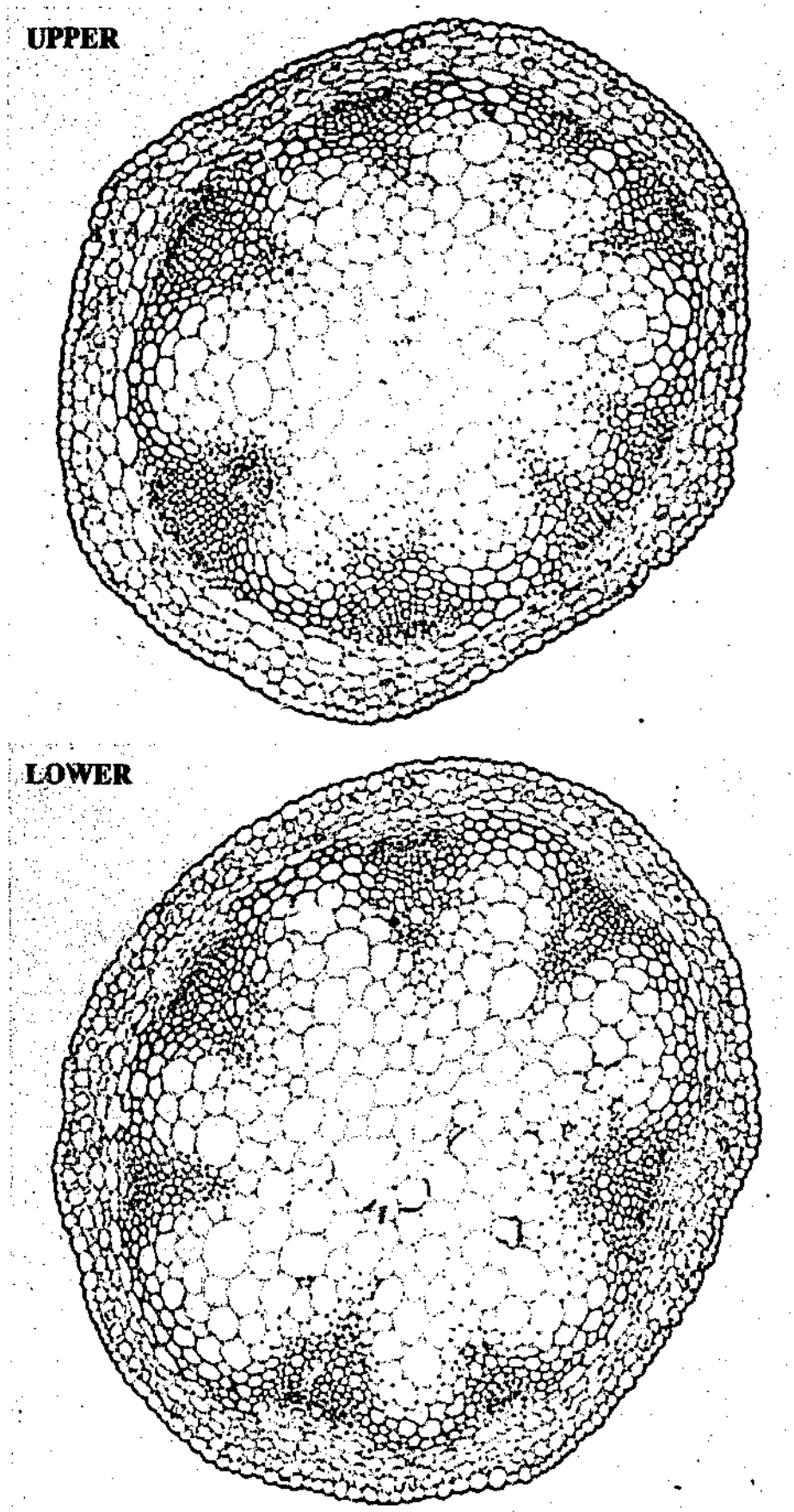


Figure 4.13B: Transverse sections stained with toluidine blue from primary inflorescences of *rolABC*#1 transformants **(x25 mag.)**
Upper section taken from the apical node
Lower section taken from the basal node above the rosette leaves
(Sections were prepared from soil-grown, flowering plants)

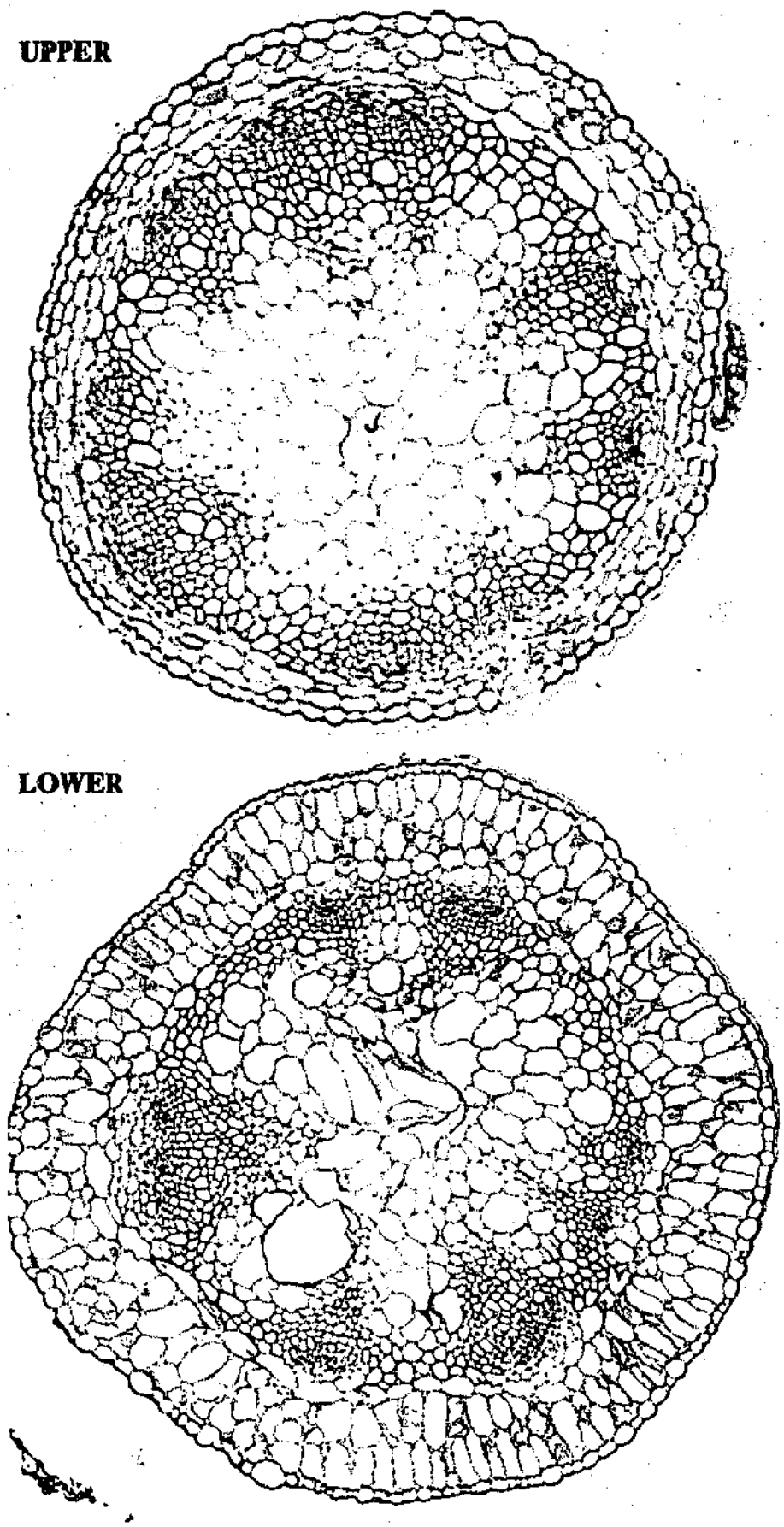


Figure 4.13C: Transverse sections stained with toluidine blue from primary inflorescences of *rolABC*#3 transformants **(x25 mag.)**
Upper section taken from the apical node.
Lower section taken from the basal node above the rosette leaves .
(Sections were prepared from soil-grown, flowering plants)

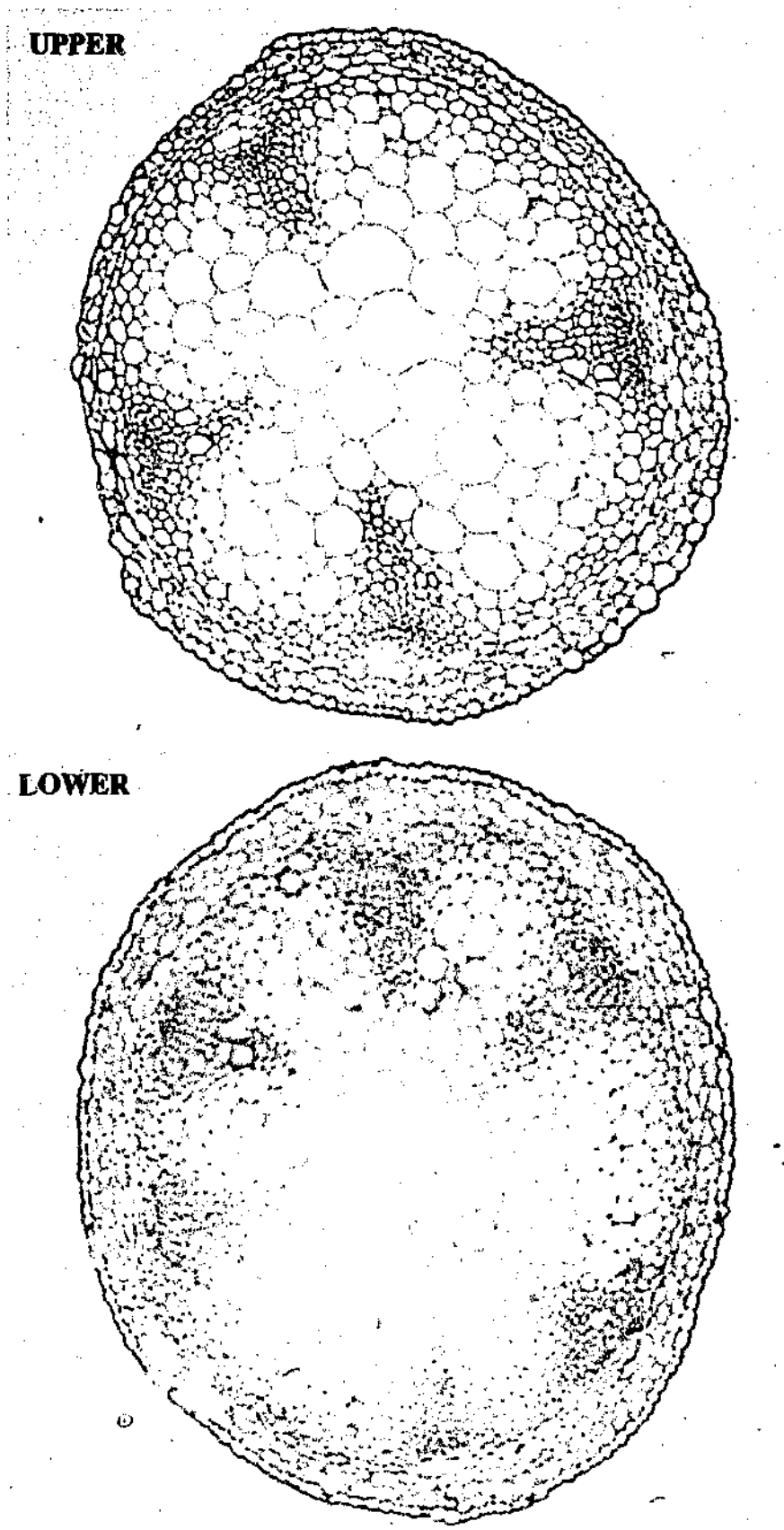


Figure 4.13D: Transverse sections stained with toluidine blue from primary inflorescences of *rolABC#7* transformants **(x25 mag.)**
Upper section taken from the apical node
Lower section taken from the basal node above the rosette leaves
(Sections were prepared from soil-grown, flowering plants)

endogenous polyamine titres and the expression patterns of important polyamine biosynthetic genes during the various phases of root growth over a 42 day period. As could probably have been anticipated, the fastest rate of growth was observed for the 9402 transformants (Figure 4·14A) followed by the *rolABC* transformants (Figure 4·15A). It was observed that all transformed lines entered the rapid growth phase by day 14, whereas the wild-type cultures only did so from day 21. By day 35 however, all lines were observed to be in stationary phase and began to senescence Consequently, day 42 samples were not used in subsequent experiments. Interestingly, all transformant lines, had the capacity to remain alive for several weeks when in the stationary phase, while the wild-type cultures tended to undergo rapid senescence after day 35. This made recovery of wild-type lines difficult or impossible if subcultured after this time.

Endogenous polyamine titres

Quantification of endogenous polyamine titres from root cultures demonstrated that the 9402- and *rolABC*-transformed cultures generally possessed lower levels of free polyamines than wild-type control lines throughout the culture period (Figures 4·14B and 4·15B). Spermidine was the predominant free polyamine in both control and transgenic cultures for all time points analysed except day 14, when putrescine levels were highest. As the age of the cultures increased, the levels of free putrescine and spermidine were generally found to decrease in all lines (Figures 4·14B and 4·15B).

The same trends were evident for the conjugated polyamine titres, with reduced levels evident in cultures of both sets of transformed lines compared to untransformed controls (Figures 4·14C and 4·15C). On the whole, putrescine was the predominant conjugated polyamine in wild-type cultures, whereas in 9402 cultures the levels of conjugated spermidine were slightly higher. In the *rolABC* lines, titres of conjugated putrescine and spermidine were approximately equivalent to one another. As with the free polyamines, titres of conjugates generally were diminished by the end of the growth phase.

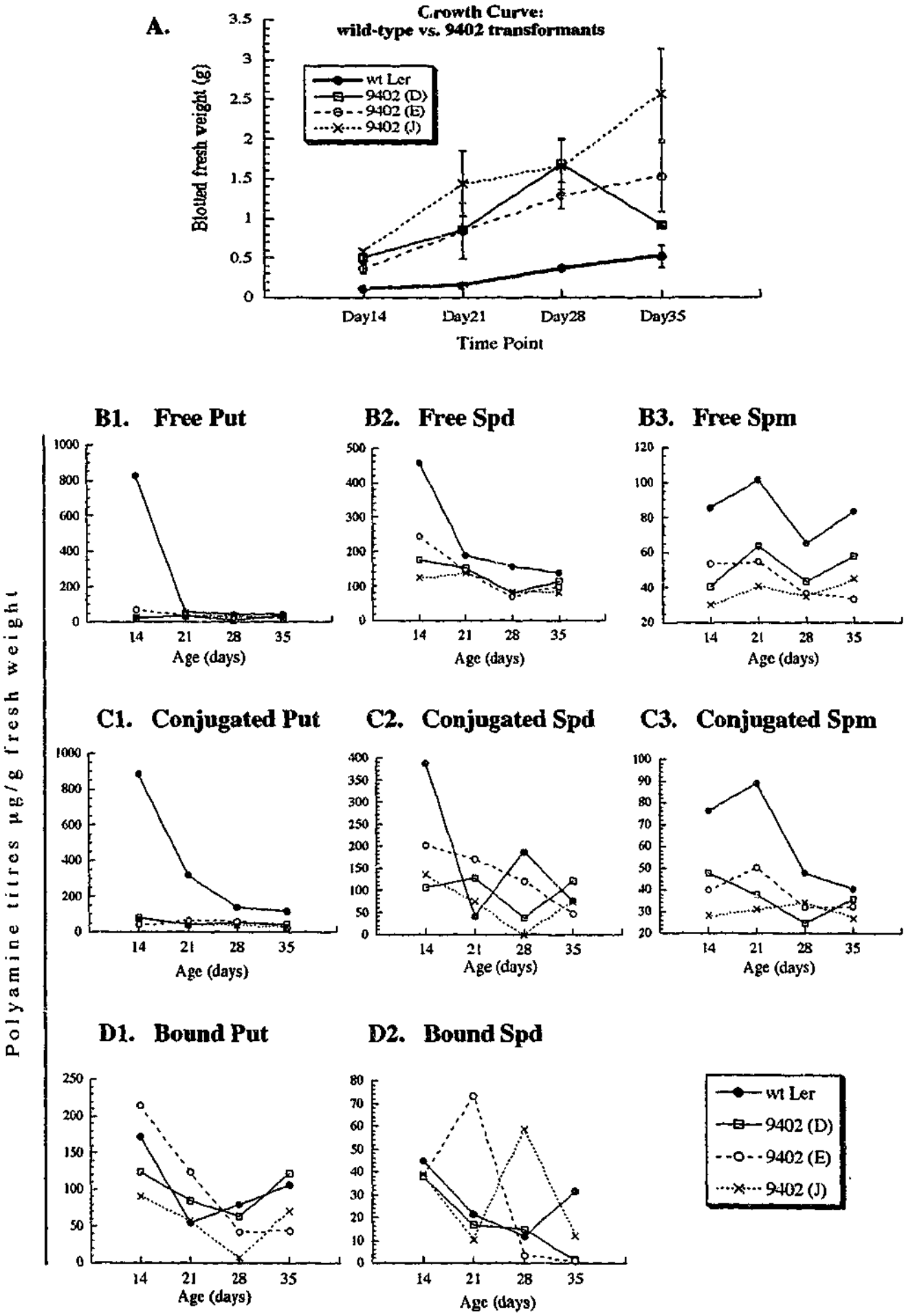


Figure: 4.14: Polyamine titres from wild-type and 9402 T transformant root lines.

A. Blotted fresh weights of 9402 T and wild-type *Ler* root lines

B. Free polyamine titres

C. Conjugated polyamine titres

D. Bound polyamine titres (no bound Spm was detected)

[Free polyamine titres are the average of two independant experiments]

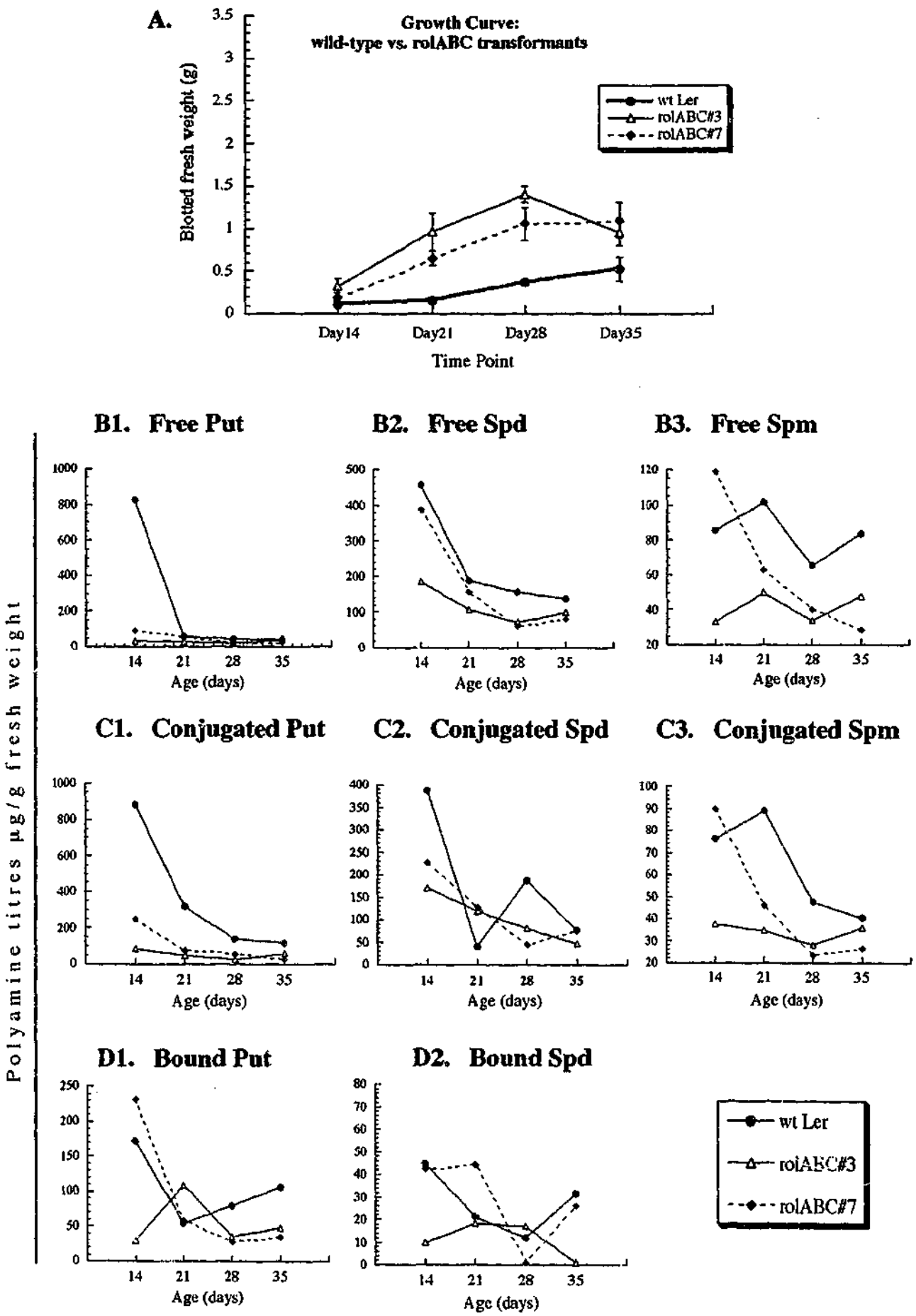


Figure: 4.15: Polyamine titres from wild-type and *rolABC* transformant root lines.

A. Blotted fresh weights of *rolABC* and wild-type L*er* root lines

B. Free polyamine titres

C. Conjugated polyamine titres

D. Bound polyamine titres (no bound Spm was detected)

[Free polyamine titres are the average of two independent experiments]

No marked difference in the titres of bound putrescine or spermidine were observed between control and transformed lines (Figures 4·14D and 4·15D). Bound spermine was not detected in any line.

Polyamine gene expression

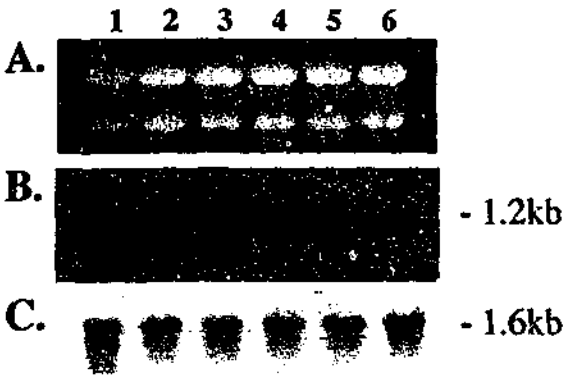
In an attempt to determine whether alterations in polyamine levels were associated with alterations in polyamine gene expression, levels of transcripts for major polyamine genes were compared in wild-type and transformed lines throughout the growth cycle.

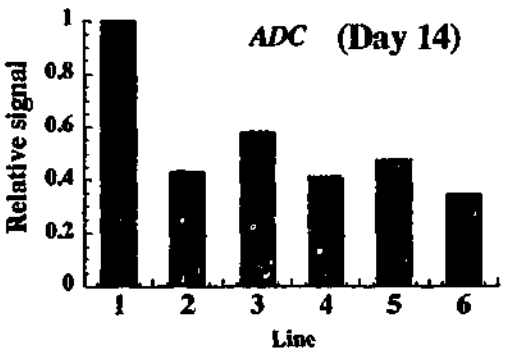
Arginine decarboxylase (ADC)

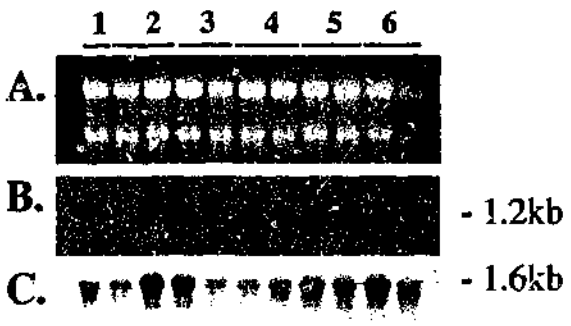
Probing Northern blot membranes with the *ADC* gene from *Arabidopsis thaliana* revealed a 1·2kb transcript in all wild-type and transformant root lines (Figure 4·16B). Accumulation of the *ADC* transcript was high during the early phase of growth, correlating with the high levels of free and conjugated putrescine observed during that stage. By the end of the growth cycle, the expression of *ADC* amongst control and transformed root lines was similar (Figures 4·16C and 4·16D).

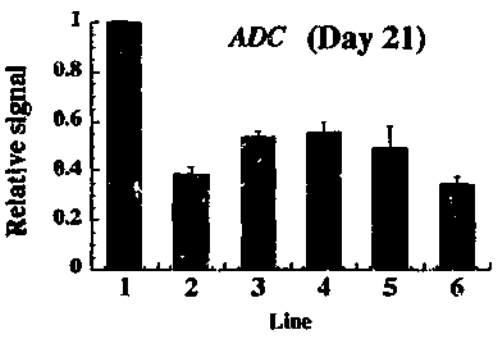
Ornithine decarboxylase (ODC)

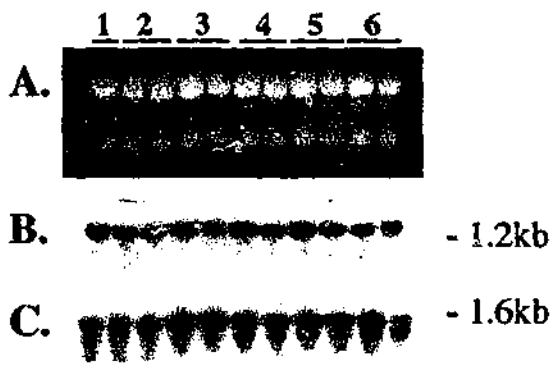
As coding sequence of ornithine decarboxylase (*ODC*) from *Arabidopsis* was not available (see section 3·3·1), a cDNA fragment of 800bp comprising the 5′ region of the *N. tabacum ODC* gene [GenBank accession number Y10472; Lidgett *et al.*, 1997] was used for Northern hybridisations. Following washing at high stringency, a band of the expected size (1·3kb in tobacco was not observed but a single distinct transcript of 0·9kb in size was detected in all root line samples (Figure 4·17B). After standardisation (Figure 4·17D), no distinct differences in expression pattern were evident between the control and the two transformant lines.

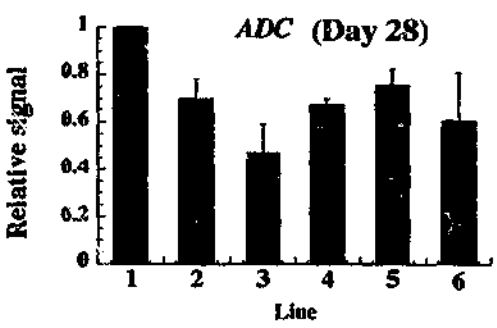


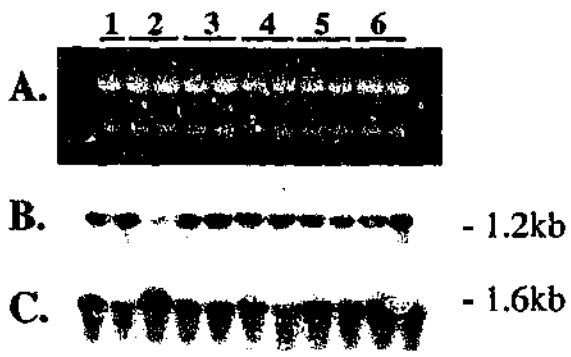












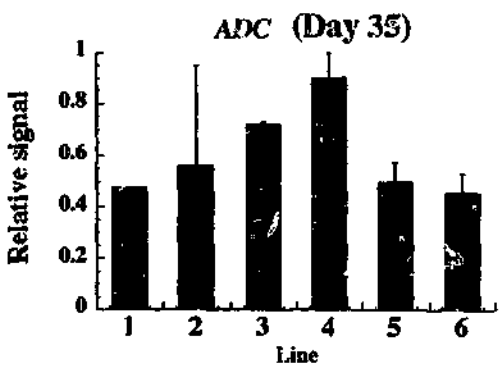
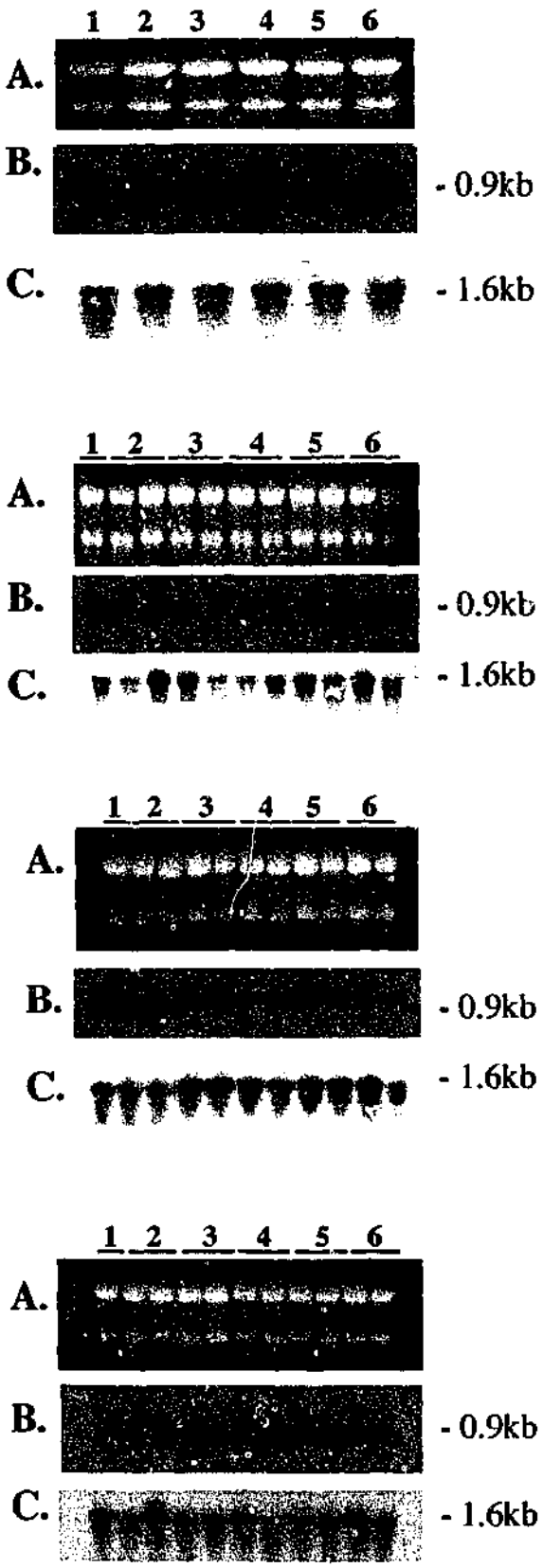
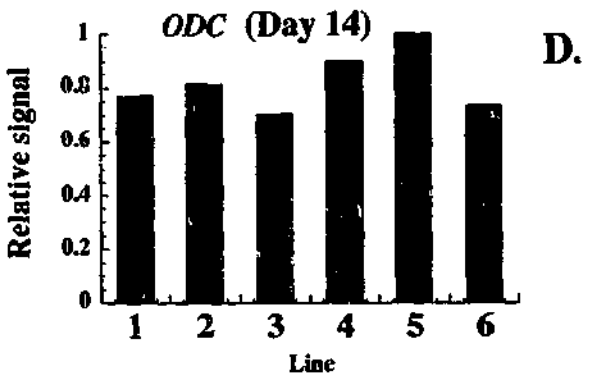
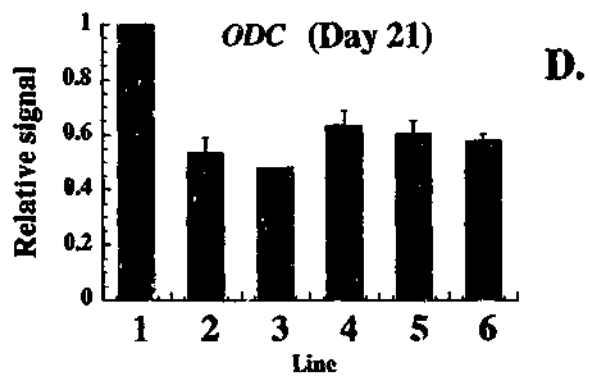
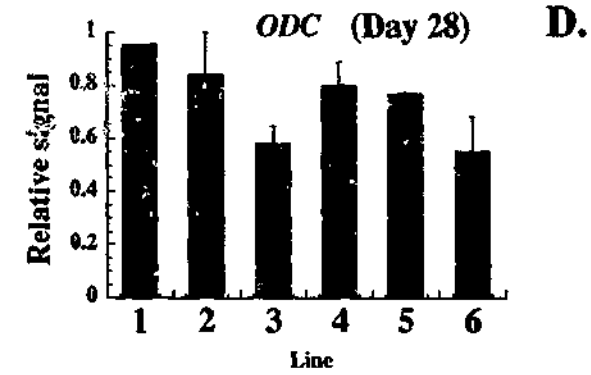


Figure 4.16: *ADC* Northern blots of wild-type control and transformed root lines.
A: EthBr-stained gel (approximately 20µg total RNA loaded per track)
B: Probed with an *Arabidopsis* genomic DNA fragment of *ADC*
C: Probed with an *Arabidopsis* cDNA fragment of *UBIQUITIN*
D: Standardised signal intensity of *ADC* relative to that of *UBIQUITIN*
SAMPLE KEY: **1** = untransformed wild-type **2** = *rolABC*#3
3 = *rolABC #7* **4** = 9402 (D) **5** = 9402 (E) **6** = 9402 (J)









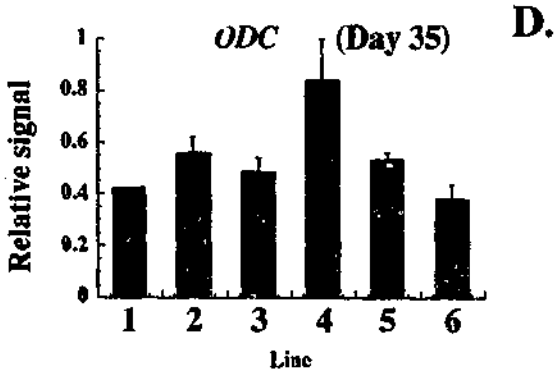


Figure 4.17: *ODC* Northern blots of wild-type control and transformed root lines.
A: EthBr-stained gel (approximately 20μg total RNA loaded per track)
B: Probed with an *N. tabacum* genomic DNA fragment of *ODC*
C: Probed with an *Arabidopsis* DNA fragment of *UBIQUITIN*
D: Standardised signal intensity of *ODC* relative to that of *UBIQUITIN*
SAMPLE KEY: **1** = untransformed wild-type **2** = *rolABC#3*
3 = *rolABC* #7 **4** = 9402 (D) **5** = 9402 (E) **6** = 9402 (J)

Spermidine synthase (SPDS)

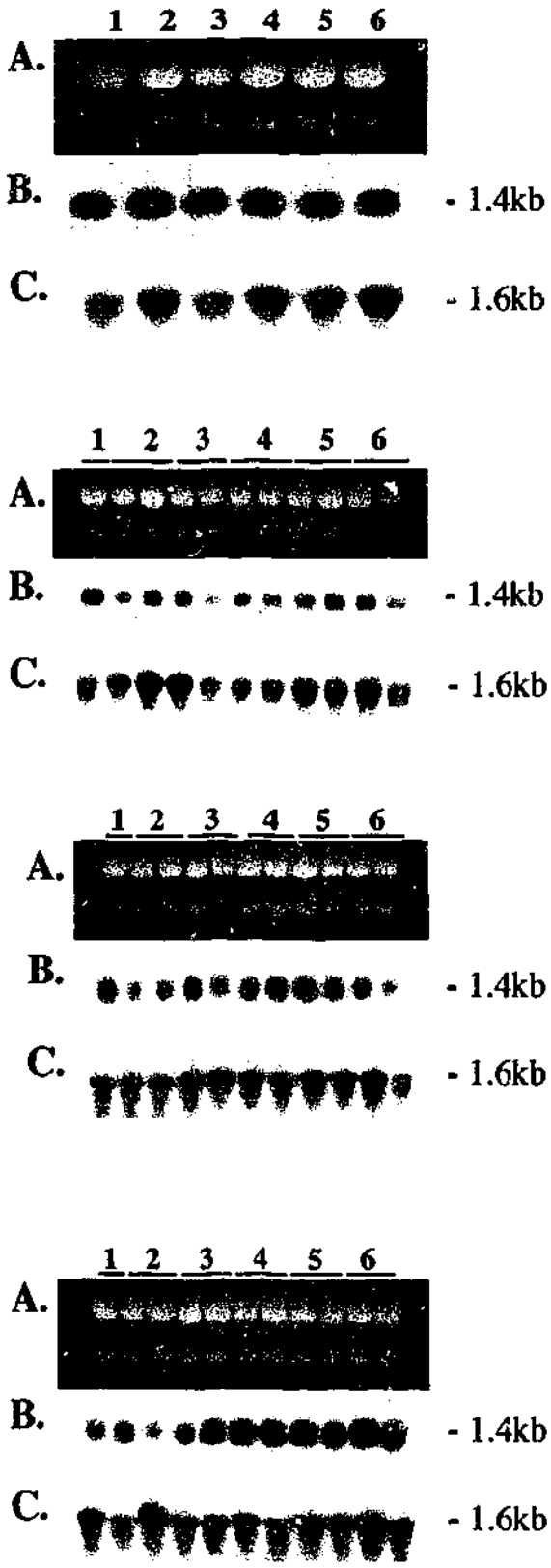
A cDNA fragment of the *Arabidopsis* spermidine synthase (*SPDS*) gene identified a transcript of 1·4kb within the *Arabidopsis* root lines, when used as a probe in Northern hybridisations (Figure 4·18B). The levels of the *SPDS* transcript signal revealed that wild-type root cultures had the highest relative level of expression during the early phase of growth, which progressively decreases with time (Figure 4·18D). The converse pattern was observed for transgenic lines, especially the 9402 cultures, in which the expression of *SPDS* was relatively low at the start of the growth cycle, but increased with age of the culture (Figure 4·18D).

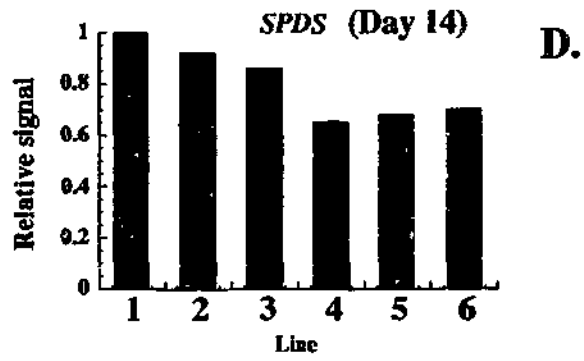
S-adenosylmethionine decarboxylase (SAMDC)

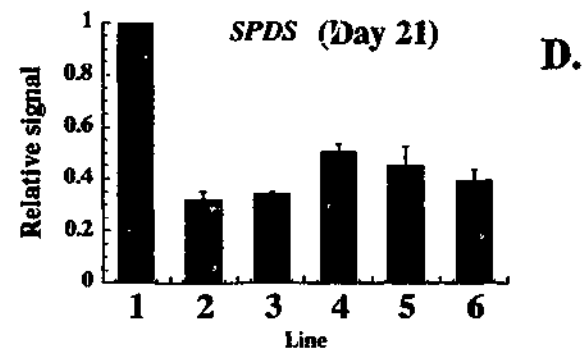
A 1·8kb transcript was detected by an *Arabidopsis* cDNA fragment of the *SAMDC* gene when used as a probe (Figure 4·19B). At day 14 of growth, an inverse correlation between *SAMDC* expression and the growth rates of the three sets of root lines was observed. That is, the slowest-growing wild-type line had the highest relative expression of *SAMDC*; the faster-growing *rolABC* lines showed a decrease in expression to approximately half the level of the wild-types; and the 9402 lines which exhibited the fastest rate of growth had the lowest level of *SAMDC* expression (Figure 4·19D). As the growth cycle progressed, however, the accumulation of *SAMDC* transcripts were observed to be similar for all root lines (Figure 4·19D).

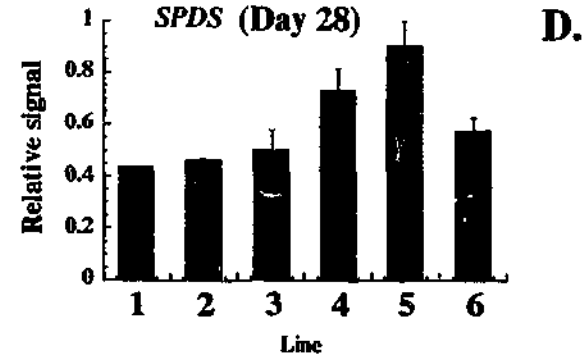
rolB

This sequence was used as a probe, partly to confirm the transgenic status of transformed root lines, and partly to assess whether T-DNA was being actively transcribed in transgenic lines. As expected, no expression of *rolB* was detected in the wild-type L*er* lines although the relative expression of the transgene at days 14 and 21 in the transgenic lines was somewhat variable (Figures 4·20B and 4·20D). By days 28 and 35 of growth, the accumulation of the *rolB* transcript was higher in the 9402 lines compared to the *rolABC* lines (Figure 4·20D).









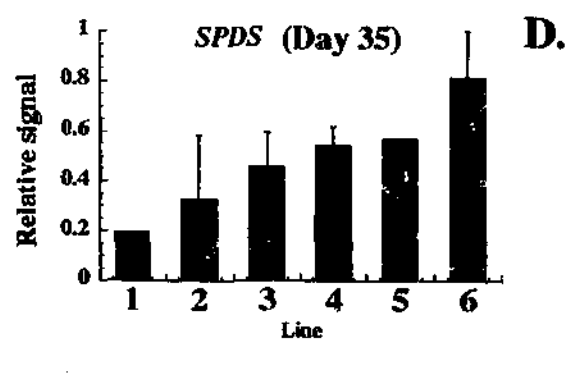
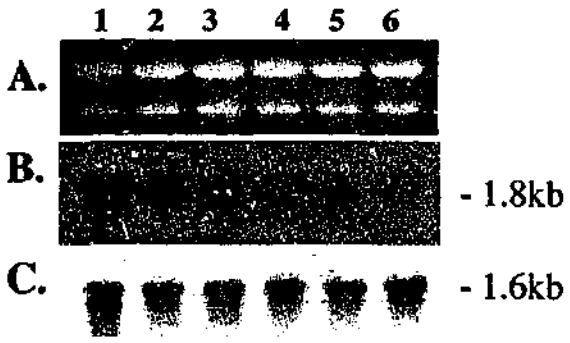
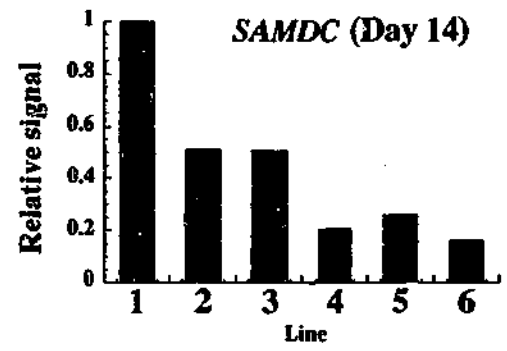
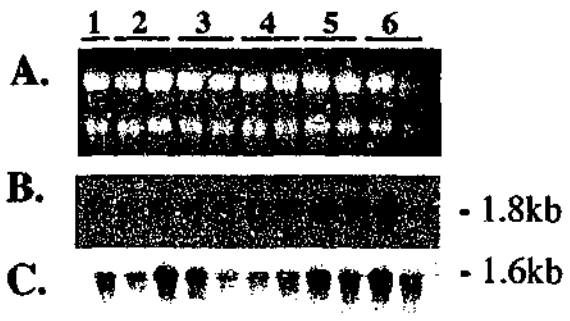
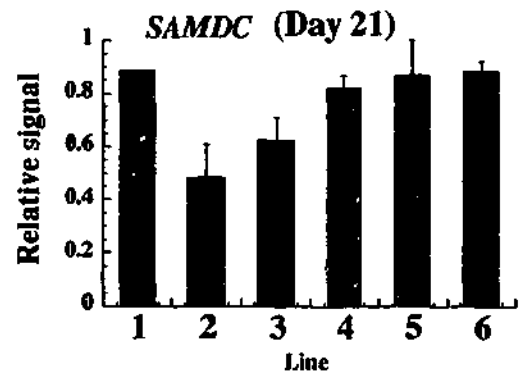


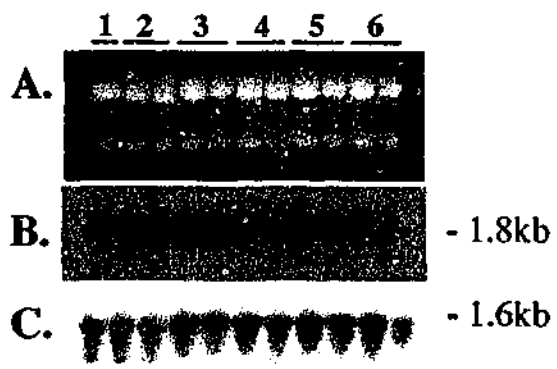
Figure 4.18: *SPDS* Northern blots of wild-type control and transformed root lines.
A: EthBr-stained gel (approximately 20μg total RNA loaded per track)
B: Probed with an *Arabidopsis* cDNA fragment of *SPDS*
C: Probed with an *Arabidopsis* cDNA fragment of *UBIQUITIN*
D: Standardised signal intensity of *spds* relative to that of *UBIQUITIN*
SAMPLE KEY: **1** = untransformed wild-type **2** = *rolABC*#3
3 = *rolABC #7* **4** = 9402 (D) **5** = 9402 (E) **6** = 9402 (J)

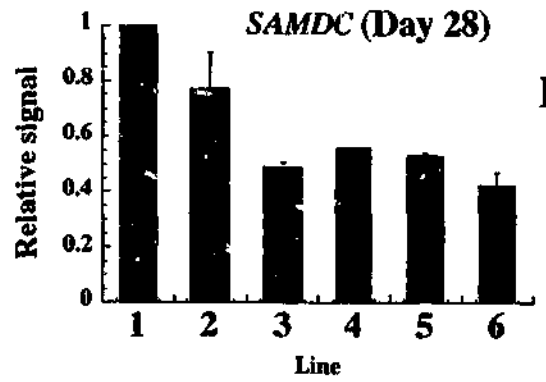


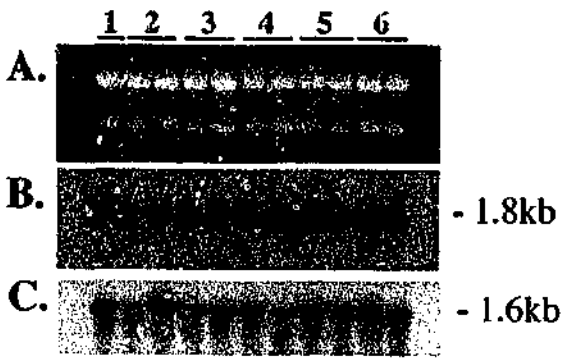












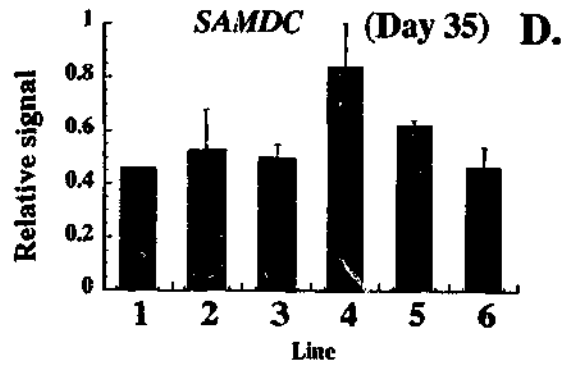
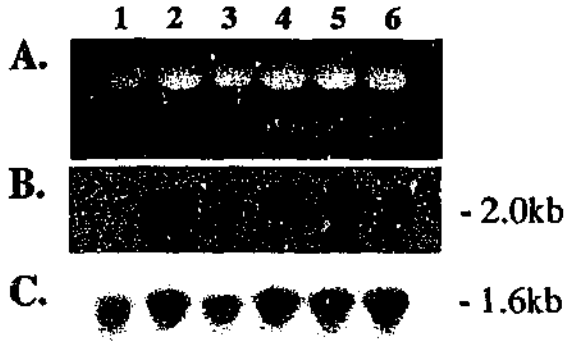
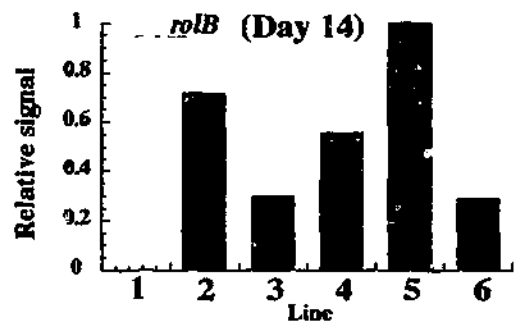
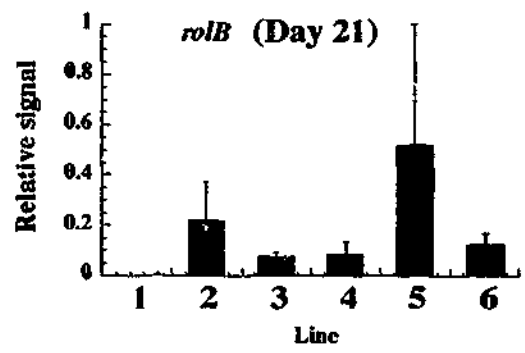
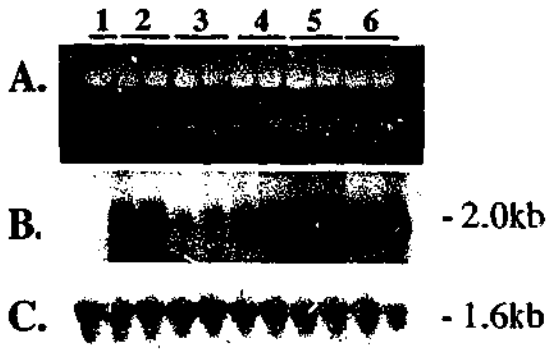


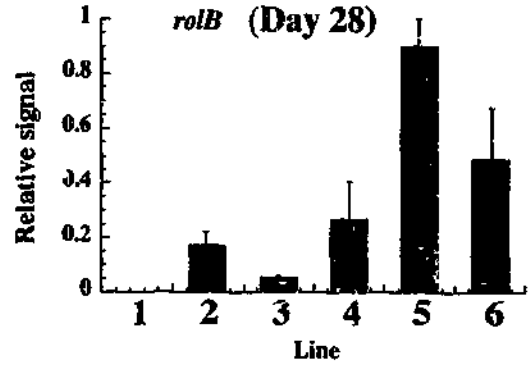
Figure 4.19: *SAMDC* Northern blots of wild-type control and transformed root lines.
A: EthBr-stained gel (approximately 20µg total RNA loaded per track)
B: Probed with *Arabidopsis* cDNA fragments of *SAMDC*
C: Probed with an *Arabidopsis* cDNA fragment of *UBIQUITIN*
D: Standardised signal intensity of *samdc* relative to that of *UBIQUITIN*
SAMPLE KEY: **1** = untransformed wild-type **2** = *rolABC #3*
3 = *rolABC #7* **4** = 9402 (D) **5** = 9402 (E) **6** = 9402 (J)

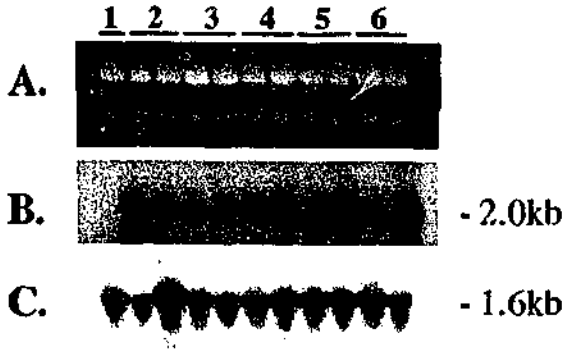












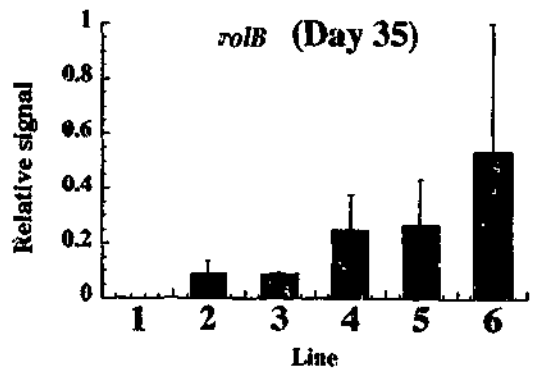


Figure 4.20: *rolB* Northern blots of wild-type control and transformed root lines.
A: EthBr-stained gel (approximately 20µg total RNA loaded per track)
B: Probed with a DNA fragment of *rolB*
C: Probed with an *Arabidopsis* cDNA fragment of *UBIQUITIN*
D: Standardised signal intensity of *rolB* relative to that of *UBIQUITIN*
SAMPLE KEY: **1** = untransformed wild-type **2** = *rolABC*#3
3 = *rolABC*#7 **4** = 9402 (D) **5** = 9402 (E) **6** = 9402 (J)

Effects of putrescine biosynthetic inhibitors on growth and polyamine titres

Exponential growth phase

As has been noted above, transformed root cultures exhibited increased growth rates over wild-type controls, which were correlated with decreased titres of free and conjugated polyamines. In an attempt to directly test the hypothesis that a diminution of endogenous polyamine titres is responsible for the increased growth rate of transformant roots, 5mM DFMO was added to the culture medium of all root lines (experiments were undertaken prior to any suspicion that *Arabidopsis* lacks *ODC*). Analysis was undertaken to determine if depletion of polyamines could be induced by DFMO, and secondly, if reduced polyamine levels correlated with a stimulation of root growth. The inhibitor at 5mM did not adversely affect growth of wild-type control cultures (Figure 4·21). Root growth of transformants in the presence of 5mM DFMO was decreased however, as measured by the average fresh weights of both *rolABC*- and 9402-transformed root cultures. The low titres of free and conjugated polyamines found in untreated transformants however, were further depleted by 5mM DFMO (Tables 4·2 and 4·3) resulting in a marked decrease in accumulation of free putrescine in both transformant and wild-type root lines. Titres of free spermidine in wild-type controls were also reduced by DFMO treatment, however, they were moderately increased in all transformant lines (Table 4·2). On the whole, levels of putrescine conjugates in all root lines were unaffected following DFMO treatment, whereas levels of spermidine conjugates generally increased (Table 4·3).

Stationary growth phase

By day 36 of growth, all root cultures had entered the stationary phase. In general, the inverse relationship between the growth rate and accumulation of free and conjugated putrescine and spermidine seen at day 21 of growth was maintained during the stationary phase (Figure 4·22). Interestingly, by the onset of the stationary phase of root growth, the effect of DFMO was contrary to that observed for the exponential phase, with respect to polyamine accumulation. Thus a slight increase in free and conjugated putrescine

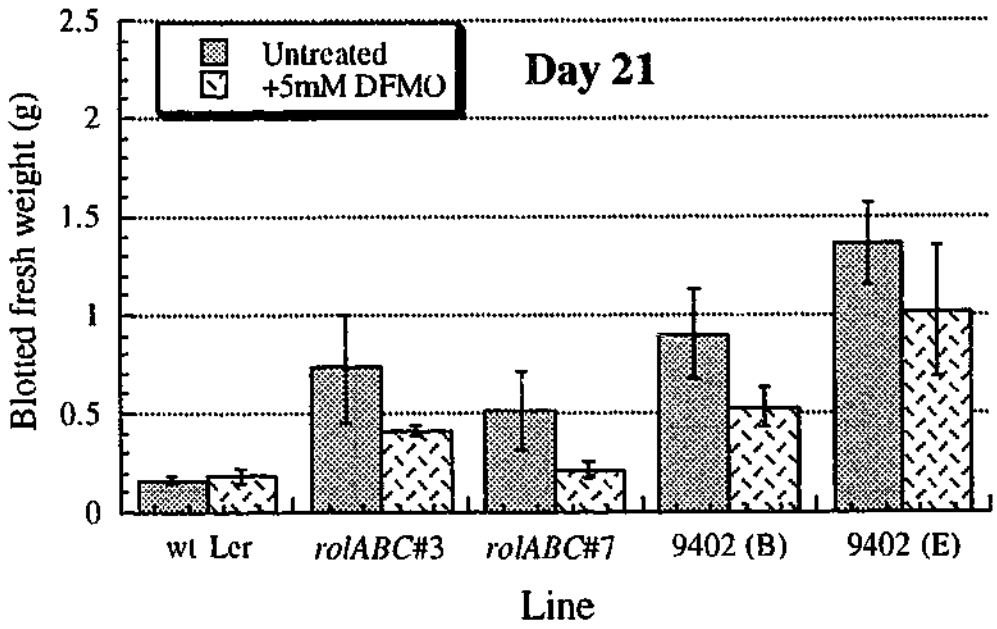


Figure 4.21: Effect of 5mM DFMO on root mass of wild-type and transformed lines at day 21 of growth.

FREE	Untreated controls			Treated with 5mM DFMO		
Line:	Put	Spd	Spm	Put	Spd	Spm
wild-type	158·5	339·7	107·8	80·6	274·7	97·6
rolABC#3	32·6	95·6	31·8	6·0	105·2	35·7
rolABC#7	34·2	111·7	70·8	7·0	159·7	79·5
9402 (B)	31·6	53·0	43·7	4·3	81·1	30·5
9402 (E)	29·6	16·6	38·3	4·4	43·5	26·3

Table 4.2: Effect of 5mM DFMO on free polyamine titres of wild-type and transformed lines at day 21 of growth.

CONJ.	Untreated controls			Treated with 5mM DFMO		
Line:	Put	Spd	Spm	Put	Spd	Spm
wild-type	98·5	296·6	121·5	91·4	313·8	104·7
rolABC#3	30·1	84·7	49·6	31·2	128·4	38·6
rolABC#7	45·2	55·8	44·7	57·0	170·5	54·2
9402 (B)	7·8	21·4	51·1	5·6	11·8	41·3
9402 (E)	26·5	8·7	37·8	6·4	30·5	32·2

Table 4.3: Effect of 5mM DFMO on conjugated polyamine titres of wild-type and transformed lines at day 21 of growth.

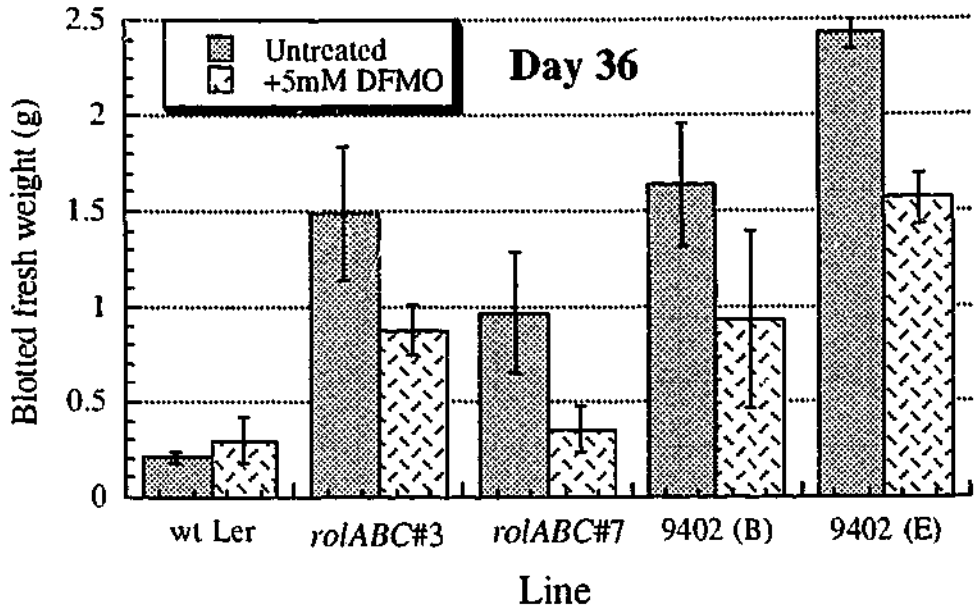


Figure 4.22: Effect of 5mM DFMO on root mass of wild-type and transformed lines at day 36 of growth.

FREE	Untreated controls			Treated with 5mM DFMO		
Line:	Put	Spd	Spm	Put	Spd	Spm
wild-type	96·5	240·7	98·9	101·4	277·3	73·3
rolABC#3	45·3	30·7	47·5	30·4	90·4	50·8
rolABC#7	50·4	102·6	37·3	63·6	181·6	70·2
9402 (B)	27·4	93·9	59·4	29·9	89·0	44·8
9402 (E)	28·4	55·7	38·9	35·9	60·3	45·7

Table 4.4: Effect of 5mM DFMO on free polyamine titres of wild-type and transformed lines at day 36 of growth.

CONJ.	Untreated controls			Treated with 5mM DFMO		
Line:	Put	Spd	Spm	Put	Spd	Spm
wild-type	85·2	135·4	72·1	90·4	401·6	89·5
rolABC#3	26·0	32·7	43·5	27·6	53·8	29·3
rolABC#7	27·9	18·9	42·5	8·5	30·7	55·9
9402 (B)	24·5	6·5	30·0	24·7	0·9	34·0
9402 (E)	6·3	13·7	42·5	25·2	89·1	28·6

Table 4.5: Effect of 5mM DFMO on conjugated polyamine titres of wild-type and transformed lines at day 36 of growth.

accumulation was observed in wild-type and transformed lines. DFMO also induced general increases in the accumulation of free and conjugated spermine (Tables 4·4 and 4·5).

4·2·5 WHOLE PLANT ANALYSES

Effects of transformation on phenotype and polyamine titres

Free polyamines

As mentioned above, transformation with genes from *A. rhizogenes* TL-DNA markedly alters the shoot and root morphology of *Arabidopsis* plants. In the case of axenic root cultures, the resulting stimulation of growth correlates with reduced titres of free and conjugated polyamines. To determine the polyamine profiles of shoots and roots from intact plants, titres of free and conjugated polyamines were quantified in untransformed wild-type L*er*, *rolABC*#3, and 9402 T segregant plants grown *in vitro*. Due to a paucity of seed being available from full Ri T-DNA 9402 transformants, only a limited characterisation was performed on these plants. As presented in Figure 4·23, levels of the predominant free polyamines, putrescine and spermidine, were found not to alter in shoots of any of the three lines. The abnormal 9402 T plant, however, exhibited a severely dwarfed shoot which never flowered and possessed extremely elevated titres of free putrescine and spermidine. The levels of free polyamines within intact roots of whole plants are in agreement with the above findings for axenic root cultures, with an inverse relationship between the degree of morphological alteration and accumulation of free polyamines. Thus roots from wild-type plants had the highest levels of polyamines, followed by roots from the *rolABC* plants, and finally the roots from Ri T-DNA 9402 plants.

Conjugated polyamines

The accumulation pattern of conjugated putrescine and spermidine in shoots was slightly different from that of free polyamines. Again, there were no differences in conjugated putrescine and spermidine profiles of wild-type and *rolABC*#3 shoots. Conjugated putrescine titres in 9402 T shoots however, were markedly elevated, with an approximately

		Free Putrescine	Free Spermidine	Free Spermine
wild-type *Ler*				
	SHOOT:	12.2 ± 1.4	39.3 ± 6.2	12.4 ± 1.9
	ROOT:	75.6 ± 7.3	164.2 ± 12.6	56.6 ± 6.0
rolABC#3				
	SHOOT:	10.4 ± 1.5	37.9 ± 3.2	12.1 ± 1.5
	ROOT:	59.4 ± 5.8	118.7 ± 14.0	37.1 ± 4.7
9402 (T)				
	SHOOT:	14.8 ± 2.8	48.3 ± 3.8	6.4 ± 1.0
	ROOT:	14.0 ± 4.1	65.2 ± 18.5	9.1 ± 2.9
9402 (abnormal T)				
	SHOOT:	91.2 ± 18.6	163.1 ± 42.5	12.7 ± 2.4
	ROOT:	15.1 ± 3.8	52.5 ± 10.1	9.1 ± 2.1

		Conjugated Putrescine	Conjugated Spermidine	Conjugated Spermine
wild-type *Ler*				
	SHOOT:	421.3 ± 176.5	69.2 ± 1.0	23.3 ± 1.7
	ROOT:	3900.3 ± 347.5	196.6 ± 21.9	98.6
rolABC#3				
	SHOOT:	404.3 ± 91.6	64.3 ± 9.7	24.2 ± 6.0
	ROOT:	697.7 ± 473.3	139.9 ± 15.8	53.5 ± 3.7
9402 (T)				
	SHOOT:	1330.7 ± 167.7	89.3 ± 17.6	7.5 ± 0.3
	ROOT:	793.1 ± 379.4	78.3 ± 34.9	9.2 ± 3.6
9402 (abnormal T)				
	SHOOT:	4088.8 ± 785.2	262.1 ± 49.7	20.3 ± 6.1
	ROOT:	714.3 ± 117.5	100.3 ± 26.0	15.6 ± 4.4

Figure 4.23: Titres of free and conjugated polyamines in shoot and root tissue of control and transformed plants, measured in µg/g fresh weight.

three-fold increase over wild-type and *rolABC*#3 shoots (Figure 4·23). The abnormal 9402 T plant had the most severely altered shoot phenotype which correlated with the highest accumulation of conjugated putrescine and spermidine. Titres of conjugated polyamines in roots also followed the same general pattern as that of the free polyamines, with the wild-type roots exhibiting much higher titres than roots from transformants (Figure 4·23).

Effects of feeding polyamines or polyamine biosynthetic inhibitors on phenotype, polyamine titres, and gene expression of transformed plants

Phenotype

Since transformation causes alterations in root and shoot phenotypes, and is correlated with reductions in polyamine titres of intact roots and axenic root cultures, experiments were carried out to test the capacity of polyamines to ameliorate or reverse the transformed phenotype of seedlings. In addition, polyamine biosynthesis inhibitors were also fed to seedlings to determine if the transformed shoot phenotype could be exacerbated by further reducing endogenous polyamine titres. The inclusion of putrescine in the media at levels of 1mM and 10mM, and spermidine at levels of 1mM, 3mM, and 5mM, did not negatively affect the growth of wild-type (Figure 4·24) or *rolABC*#1 (Figure 4·25) control seedlings. Such treatments were clearly unable to ameliorate the transformed shoot phenotype of *rolABC*#7 (Figure 4·26) or *rolABC*#9 (Figure 4·26) seedlings. Furthermore, the simultaneous addition of putrescine and spermidine was also not able to revert the *rolABC*-transformed phenotype. In addition, relatively high levels of the polyamine biosynthesis inhibitors; MGBG (1mM), CHA (10mM), and DFMO (10mM), were able to moderately inhibit the size of all shoots, with DFMO able to induce marked alterations of the shoot by causing severe leaf in-rolling (Figures 4·24 to 4·27).

Polyamine titres and expression of polyamine biosynthetic genes

When grown on media supplemented with either or both putrescine or spermidine, transformed and control plants appeared able to actively take up the polyamines via their roots and translocate them to the shoots. This is evident from the increases in titres of free,

Figure 4.24: Growth of wild-type L*er* on media containing either polyamines or polyamine inhibitors. Day 34 of growth.

A. MS only	**B.** 1mM DFMO	**C.** 10mM DFMO
D. 0.1mM MGBG	**E.** 1mM MGBG	**F.** 1mM CHA
G. 1mM Put	**H.** 10mM Put	**I.** 10mM CHA
J. 1mM Spd	**K.** 3mM Spd	**L.** 5mM Spd
M. 1mM Put + 1mM Spd	**N.** 3mM Put + 3mM Spd	**O.** 5mM Put + 5mM Spd

Seeds were germinated on MS medium solidified with agar and supplemented with the polyamines Put and/or Spd, or the polyamine biosynthetic inhibitors DFMO, CHA, or MGBG. Control media remained unsupplemented.

Figure 4.25: Growth of *rolABC*#1 on media containing either polyamines or polyamine inhibitors. Day 34 of growth.

A. MS only	**B.** 1mM DFMO	**C.** 10mM DFMO
D. 0.1mM MGBG	**E.** 1mM MGBG	**F.** 1mM CHA
G. 1mM Put	**H.** 10mM Put	**I.** 10mM CHA
J. 1mM Spd	**K.** 3mM Spd	**L.** 5mM Spd
M. 1mM Put + 1mM Spd	**N.** 3mM Put + 3mM Spd	**O.** 5mM Put + 5mM Spd

Seeds were germinated on MS medium solidified with agar and supplemented with the polyamines Put and/or Spd, or the polyamine biosynthetic inhibitors DFMO, CHA, or MGBG. Control media remained unsupplemented.

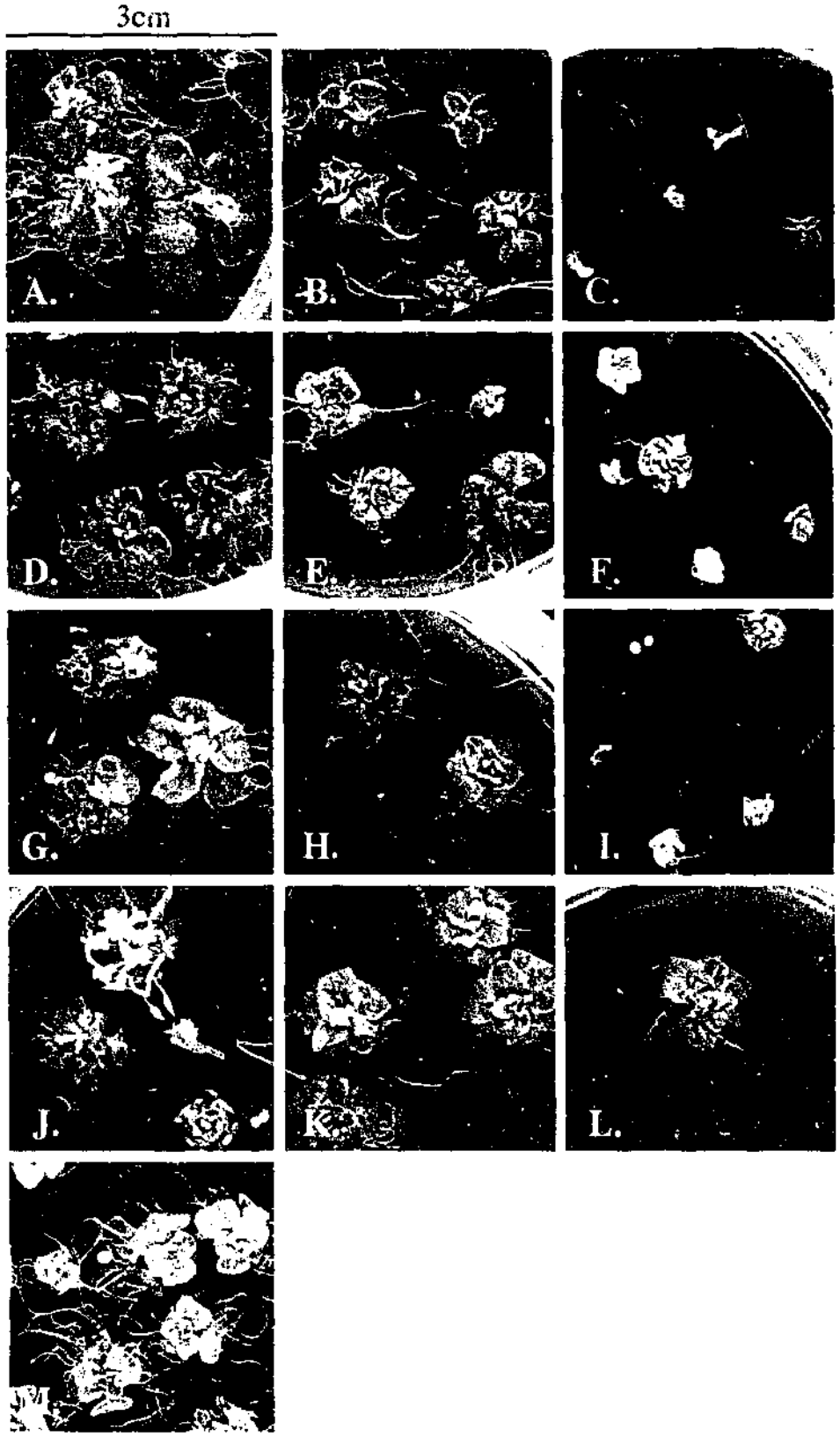


Figure 4.26: Growth of *rolABC#7* on media containing either polyamines or polyamine inhibitors. Day 34 of growth.

A. MS only
B. 1mM DFMO
C. 10mM DFMO
D. 0.1mM MGBG
E. 1mM MGBG
F. 1mM CHA
G. 1mM Put
H. 10mM Put
I. 10mM CHA
J. 1mM Spd
K. 3mM Spd
L. 5mM Spd
M. 1mM Put + 1mM Spd

Seeds were germinated on MS medium solidified with agar and supplemented with the polyamines Put and/or Spd, or the polyamine biosynthetic inhibitors DFMO, CHA, or MGBG. Control media remained unsupplemented.

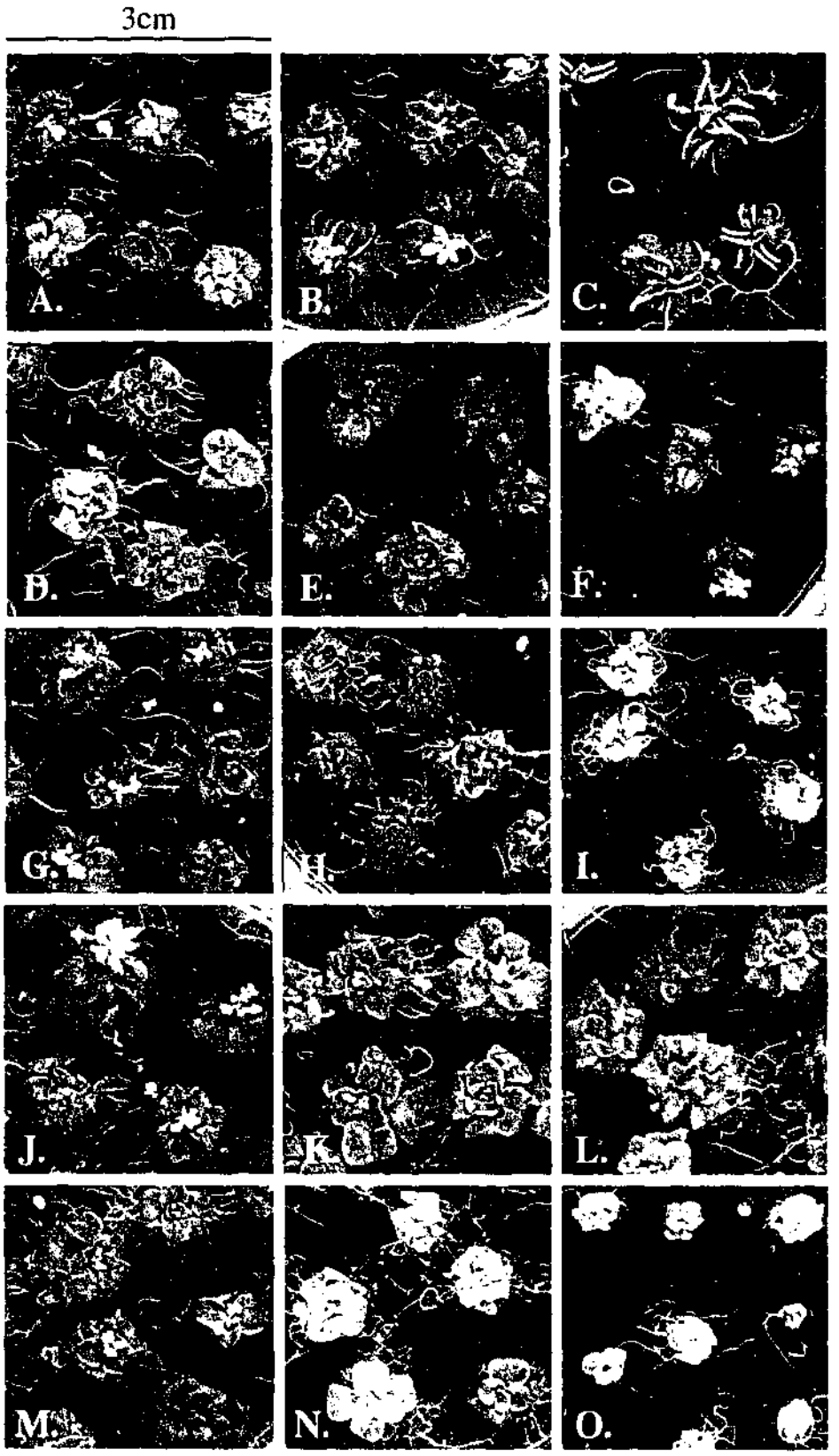


Figure 4.27: Growth of *rolABC*#9 on media containing either polyamines or polyamine inhibitors. Day 34 of growth.

A. MS only	**B.** 1mM DFMO	**C.** 10mM DFMO
D. 0.1mM MGBG	**E.** 1mM MGBG	**F.** 1mM CHA
G. 1mM Put	**H.** 10mM Put	**I.** 10mM CHA
J. 1mM Spd	**K.** 3mM Spd	**L.** 5mM Spd
M. 1mM Put + 1mM Spd	**N.** 3mM Put + 3mM Spd	**O.** 5mM Put + 5mM Spd

Seeds were germinated on MS medium solidified with agar and supplemented with the polyamines Put and/or Spd, or the polyamine biosynthetic inhibitors DFMO, CHA, or MGBG. Control media remained unsupplemented.

conjugated, and bound putrescine when the plants were exposed to putrescine, and similarly, all three forms of endogenous spermidine increased when media was supplemented with spermidine (Tables 4·6, 4·7, and 4·8).

Effects of exogenous putrescine application

Feeding 10mM putrescine stimulated a marked accumulation of the three forms of putrescine in all lines whilst concomitantly increasing *SPDS* mRNA levels. This transcript abundance had little downstream effect, however, on the accumulation of spermidine in the untransformed wild-type and the *rolABC*#1 control shoots. Plants of the *rolABC*#9 line did not show this tight regulation of spermidine levels however, exhibiting a moderate increase in titres of both free and conjugated spermidine following growth on media supplemented with 10mM putrescine. Consequently, free putrescine levels in this line could not be stimulated further than 4000µg/g f.wt. following treatment with 10mM putrescine. Levels of conjugated putrescine in *rolABC*#9 shoots however, were dramatically higher than that of controls following feeding with exogenous putrescine. In the wild-type and *rolABC*#1 control shoots, excess free putrescine does not appear to be used for the synthesis of higher polyamines, and therefore both lines exhibited putrescine titres over 6000µg/g f.wt. compared with approximately 50µg/g f.wt. for untreated controls (Table 4·6). Levels of *ADC* mRNA accumulation generally decreased with putrescine application to all lines. The simultaneous application of putrescine with DFMO did not revert the inhibitory effects of DFMO on Ri T-DNA transformants (data not shown)

Effects of exogenous spermidine application

As mentioned above, spermidine treatment stimulated the accumulation of free, conjugated, and bound spermidine in the controls and the transformed lines, which interestingly correlated with increases in transcript levels of *SPDS*, the enzyme involved in the conversion of putrescine to spermidine, in all lines (Figure 4·28). Such treatment also increased the levels of the three forms of putrescine within those lines, however, it was not found to cause a concomitant elevation of spermine levels (Tables 4·6, 4·7, and 4·8). Although putrescine

FREE POLYAMINE TITRES																
Treatment:	wild-type L*er*				*rolABC*#1				*rolABC*#7				*rolABC*#9			
	n	Put	Spd	Spm	n	Put	Spd	Spm	n	Put	Spd	Spm	n	Put	Spd	Spm
Untreated	7	48·2	100·3	71·3	7	51·5	128·4	60·8	4	39·0	103·6	57·6	10	33·9	95·3	51·3
1mM CHA	7	54·0	29·0	60·7	4	100·5	121·0	79·4	-	-	-	-	8	48·4	107·5	70·8
0·1mM MGBG	7	91·3	17·4	66·2	4	91·6	256·2	118·4	5	40·0	181·0	87·4	8	28·4	140·8	45·8
1mM MGBG	-	-	-	-	-	-	-	-	3	84·7	216·7	192.4	5	36·5	115·5	63·5
1mM DFMO	7	73·7	190·2	60·6	-	-	-	-	6	32·9	195·0	72·1	6	39·1	153·7	100·6
5mM DFMO	3	65·3	214·2	63·2	-	-	-	-	-	-	-	-	-	-	-	-
10mM DFMO	7	50·1	125·4	48·2	6	73·3	272·4	118·6	-	-	-	-	3	49·0	238·4	64·7
1mM Put	7	74·3	113·6	44·5	7	208·0	221·0	61·4	-	-	-	-	6	68·1	184·8	91·0
10mM Put	7	6034	82·4	34·7	5	7082	107·3	28·5	-	-	-	-	5	3783	121·2	57·2
1mM Spd	4	48·8	165·3	73·5	6	85·3	136·6	35·0	-	-	-	-	6	25·2	100·4	33·5
3mM Spd	5	102·8	503·6	71·4	4	82·4	844·4	61·3	-	-	-	-	6	69·7	148·5	55·6
5mM Spd	4	522·3	1618	46·6	8	441.1	1134	38·2	-	-	-	-	6	186·0	833·8	61·6
1mM Put + 1mMSpd	9	130·7	221·7	41·9	-	-	-	-	5	56·9	392·6	87·0	7	73·5	138·3	50·9

TABLE: 4·6: Free polyamine titres (µg/g fresh weight) from shoots of treated and untreated wild-type L*er* and *rolABC* transformants.

Seeds were germinated on MS medium solidified with agar and supplemented with the polyamines Put and/or Spd, or the polyamine biosynthetic inhibitors DFMO, CHA, or MGBG. Untreated plants were grown on MS media with no additives. Polyamine extractions were performed on day 34 of growth using pools of treated shoots. The number of individuals used per extraction are shown in the columns designated 'n'. Treatments without enough tissue for extraction are represented by a dash.

CONJUGATED POLYAMINE TITRES																
Treatment:	wild-type L*er*				*rolABC*#1				*rolABC*#7				*rolABC*#9			
	n	Put	Spd	Spm	n	Put	Spd	Spm	n	Put	Spd	Spm	n	Put	Spd	Spm
Untreated	7	32·1	82·6	21·2	7	46·3	155·0	45·4	4	30·7	101·6	44·5	10	37·1	26·0	37·7
1mM CHA	7	50·3	51·1	73·4	4	114·9	73·2	52·5	-	-	-	-	8	41·4	89·8	64·8
0·1mM MGBG	7	131·0	186·3	49·9	4	65·4	166·0	40·7	5	32·0	32·8	42·3	8	34·5	0·9	30·3
1mM MGBG	-	-	-	-	-	-	-	-	3	153·2	151·3	70·0	5	146·7	16·6	31·4
1mM DFMO	7	47·3	168·5	43·8	-	-	-	-	6	44·9	96·9	53·5	6	2·4	51·1	245·1
5mM DFMO	3	66·0	188·3	52·5	-	-	-	-	-	-	-	-	-	-	-	-
10mM DFMO	7	41·6	20·2	41·2	6	52·8	82·1	94·6	-	-	-	-	3	43·9	6925	55·9
1mM Put	7	103·3	51·1	44·5	7	411·1	225·2	59·5	-	-	-	-	6	2665	2437	142·8
10mM Put	7	4426	102·3	26·5	5	6033	112·7	65·2	-	-	-	-	5	14955	115·9	51·1
1mM Spd	4	41·1	81·6	49·2	6	92·1	125·6	46·4	-	-	-	-	6	45·4	163·6	39·6
3mM Spd	5	195·0	1019	42·1	4	67·3	398·0	40·6	-	-	-	-	6	397·4	714·3	50·2
5mM Spd	4	254·8	1005	42·2	8	859·0	3379	49·0	-	-	-	-	6	775·6	2159	29·9
1mM Put + 1mMSpd	9	125·1	170·7	46·5	-	-	-	-	5	102·4	664·9	71·4	7	162·2	245·4	66·4

TABLE: 4·7: Conjugated polyamine titres (µg/g fresh weight) from shoots of treated and untreated wild-type L*er* and *rolABC* transformants.

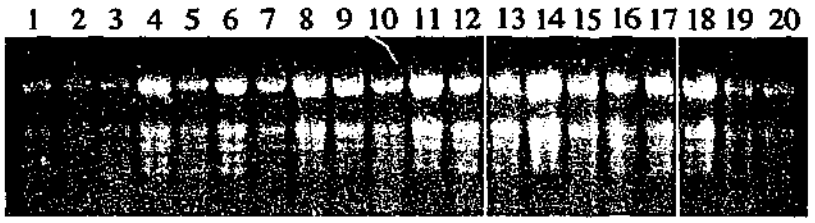
Seeds were germinated on MS medium solidified with agar and supplemented with the polyamines Put and/or Spd, or the polyamine biosynthetic inhibitors DFMO, CHA, or MGBG. Untreated plants were grown on MS media with no additives. Polyamine extractions were performed on day 34 of growth using pools of treated shoots. The number of individuals used per extraction are shown in the columns designated 'n'. Treatments without enough tissue for extraction are represented by a dash.

BOUND POLYAMINE TITRES																
	wild-type L*er*				*rolABC*#1				*rolABC*#7				*rolABC*#9			
Treatment:	**n**	**Put**	**Spd**	**Spm**	**n**	**Put**	**Spd**	**Spm**	**n**	**Put**	**Spd**	**Spm**	**n**	**Put**	**Spd**	**Spm**
Untreated	7	30·0	35·6	N/D	7	38·3	54·3	N/D	4	4·4	16·6	N/D	10	31·6	13·5	N/D
1mM CHA	7	58·3	37·3	N/D	4	44·7	15·2	N/D	-	-	-	-	8	34·9	5·6	N/D
0·1mM MGBG	7	105·3	6·8	N/D	4	48·8	162·7	N/D	5	47·6	10·9	N/D	8	6·8	2·9	N/D
1mM MGBG	-	-	-	-	-	-	-	-	3	42·1	7·4	N/D	5	52·5	34·2	N/D
1mM DFMO	7	76·2	29·8	N/D	-	-	-	-	6	5·9	25·0	N/D	6	35·0	17·0	N/D
5mM DFMO	3	57·3	8·1	N/D	-	-	-	-	-	-	-	-	-	-	-	-
10mM DFMO	7	38·3	79·9	N/D	6	60·9	115·0	N/D	-	-	-	-	3	43·9	1·6	N/D
1mM Put	7	40·7	36·8	N/D	7	69·9	28·2	N/D	-	-	-	-	6	9·0	10·1	N/D
10mM Put	7	133·5	27·8	N/D	5	189·6	31·8	N/D	-	-	-	-	5	519·3	1·1	N/D
1mM Spd	4	49·3	5·4	N/D	6	30·0	33·7	N/D	-	-	-	-	6	40·9	23·2	N/D
3mM Spd	5	75·6	6·5	N/D	4	6·58	25·5	N/D	-	-	-	-	6	28·6	1·0	N/D
5mM Spd	4	114·0	195·4	N/D	8	62·1	107·6	N/D	-	-	-	-	6	40·0	127·4	N/D
1mM Put + 1mMSpd	9	76·8	52·3	N/D	-	-	-	-	5	43·0	1·4	N/D	7	44·5	1·0	N/D

TABLE: 4·8: Bound polyamine titres (µg/g fresh weight) from shoots of treated and untreated wild-type L*er* and *rolABC* transformants.

Seeds were germinated on MS medium solidified with agar and supplemented with the polyamines Put and/or Spd, or the polyamine biosynthetic inhibitors DFMO, CHA, or MGBG. Untreated plants were grown on MS media with no additives. Polyamine extractions were performed on day 34 of growth using pools of treated shoots. The number of individuals used per extraction are shown in the columns designated 'n'. Treatments without enough tissue for extraction are represented by a dash.

A.



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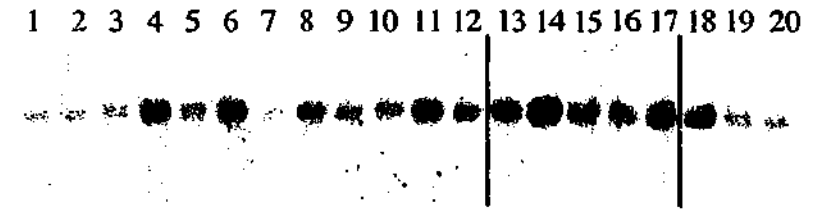

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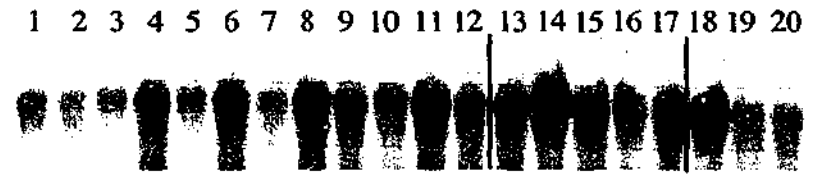

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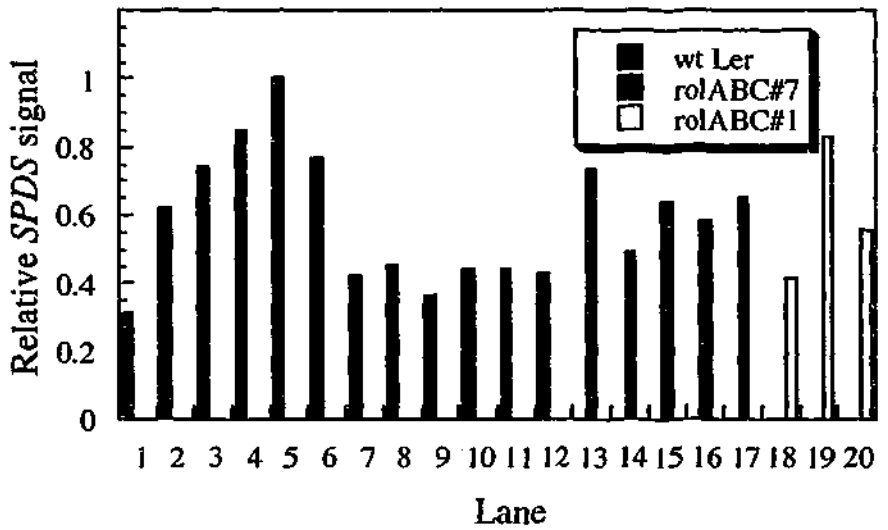
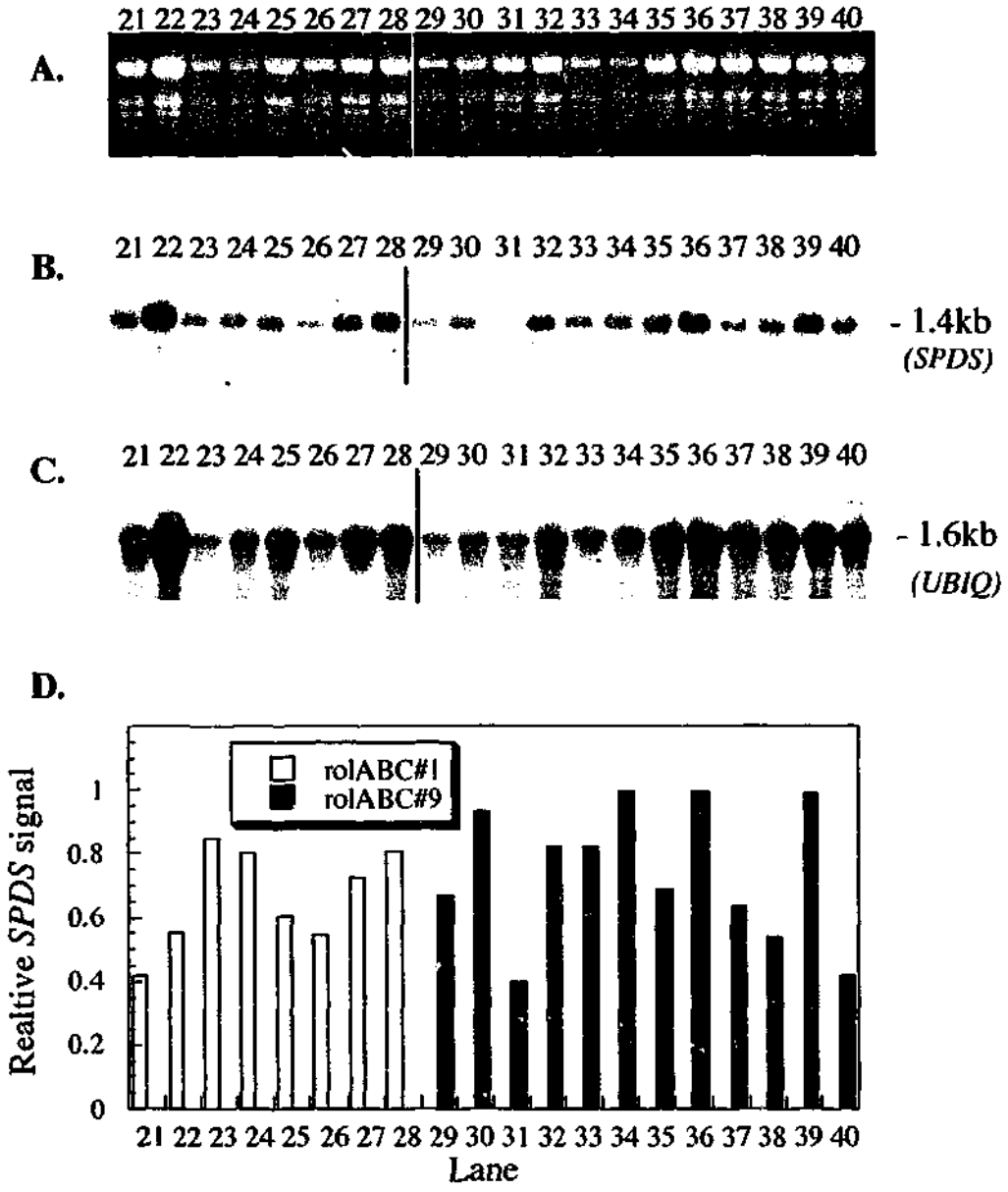


Lane	Sample	Lane	Sample
	[wt *Ler*]		[*rol ABC#7*]
1.	MS control	13.	MS control
2.	1mM CHA	14.	0.1mM MGBG
3.	0.1mM MGBG	15.	1mM MGBG
4.	1mM DFMO	16.	1mv DFMO
5.	5mM DFMO	17.	1mM PUT + 1 mM SPD
6.	10mM DFMO		
7.	1mMPUT		[*rol ABC#1*]
8.	10mM PUT	18.	MS control
9.	1mM SPD	19.	1mM CHA
10.	3mM SPD	20.	0.1mM MGBG
11.	5mM SPD		
12.	1mM PUT + 1mM SPD		



Lane	Sample	Lane	Sample
	|*rolABC#1*|	|*rolABC#9*
21.	MS control	29.	MS control
22.	1mM DFMO	30.	1mM CHA.
23.	10mM DFMO	31.	0.1mM MGBG
24.	1mM PUT	32.	1mM MGBG
25.	10mM PUT	33.	1mM DFMO
26.	1mM SPD	34.	10mM DFMO
27.	3mM SPD	35.	1mM PUT
28.	5mM SPD	36.	10mM PUT
		37.	1mM SPD
		38.	3mM SPD
		39.	5mM SPD
		40.	1mM PUT +1mM SPD

Figure 4.28: Expression of *SPDS* in wild-type and *rolABC* transformant shoots following treatment with polyamines or polyamine inhibitors.

The EthBr-stained gel (~20µg total RNA/track) (**A.**) was blotted and the membrane probed with *SPDS* (**B.**) and *UBIQ* (**C.**). The signal intensities were standardised to those of *UBIQ* (**D.**).

titres increased after spermidine treatment, no down-regulation in transcript levels of the putrescine biosynthetic enzyme, *ADC*, was observed (Figure 4·29). Overall, these observations appear to confirm the existence of an interconversion pathway of spermidine to putrescine in *Arabidopsis*.

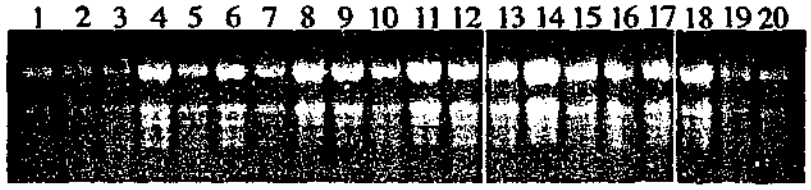
Effects of simultaneous application of exogenous putrescine and spermidine

The simultaneous addition of 1mM putrescine and 1mM spermidine to growth media generally resulted in increases in titres of free, conjugated, and bound forms of the two polyamines in shoots of all lines. The transformed phenotypes of *rolABC*#7 and *rolABC*#9 shoots however, were not ameliorated, even though endogenous polyamine titres were elevated following the various treatments (Tables 4·6, 4·7, and 4·8). The most striking differences in polyamine gene expression between the wild-type control and transformant lines *rolABC*#7 and *rolABC*#9 were in response to this treatment. Whereas a moderate stimulation of endogenous polyamine titres was seen, which correlated with increased transcript levels for *SPDS* (Figure 4·28D), *ADC* (Figure 4·29D), and *SAMDC* (Figure 4·30D) in wild-type shoots, it resulted in reduced transcript signal for those genes in the *rolABC*#7 and *rolABC*#9 shoots.

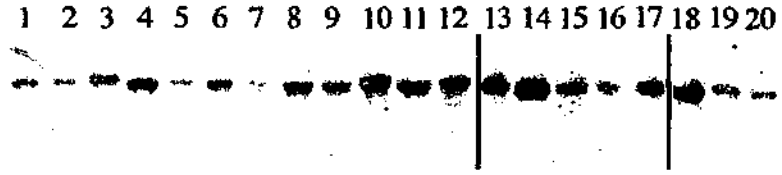
Effects of CHA application

CHA inhibits spermidine synthase, thereby blocking the conversion of putrescine to spermidine. As expected therefore, levels of free putrescine increased and free spermidine decreased in both wild-type and *rolABC*#1 control shoots treated with this inhibitor. In the *rolABC*#9 shoots however, both free putrescine and free spermidine levels increased when grown in media containing 1mM CHA (Table 4·6). Transcript levels of the three polyamine biosynthetic genes after CHA treatment either remained unaltered, or increased in the three lines (Figures 4·28, 4·29, and 4·30).

A.

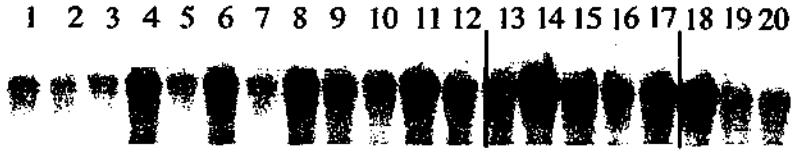


B.



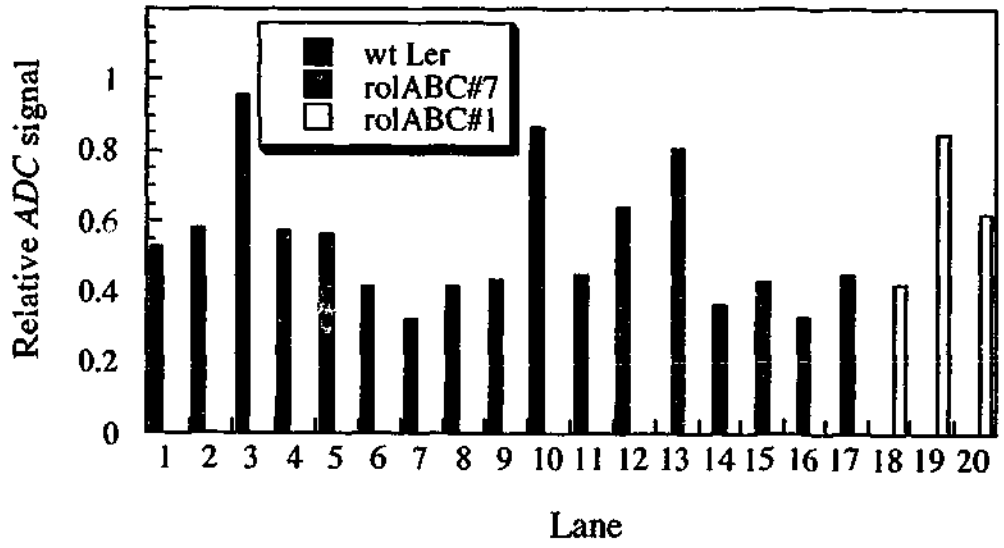
- 1.2kb
(ADC)

C.

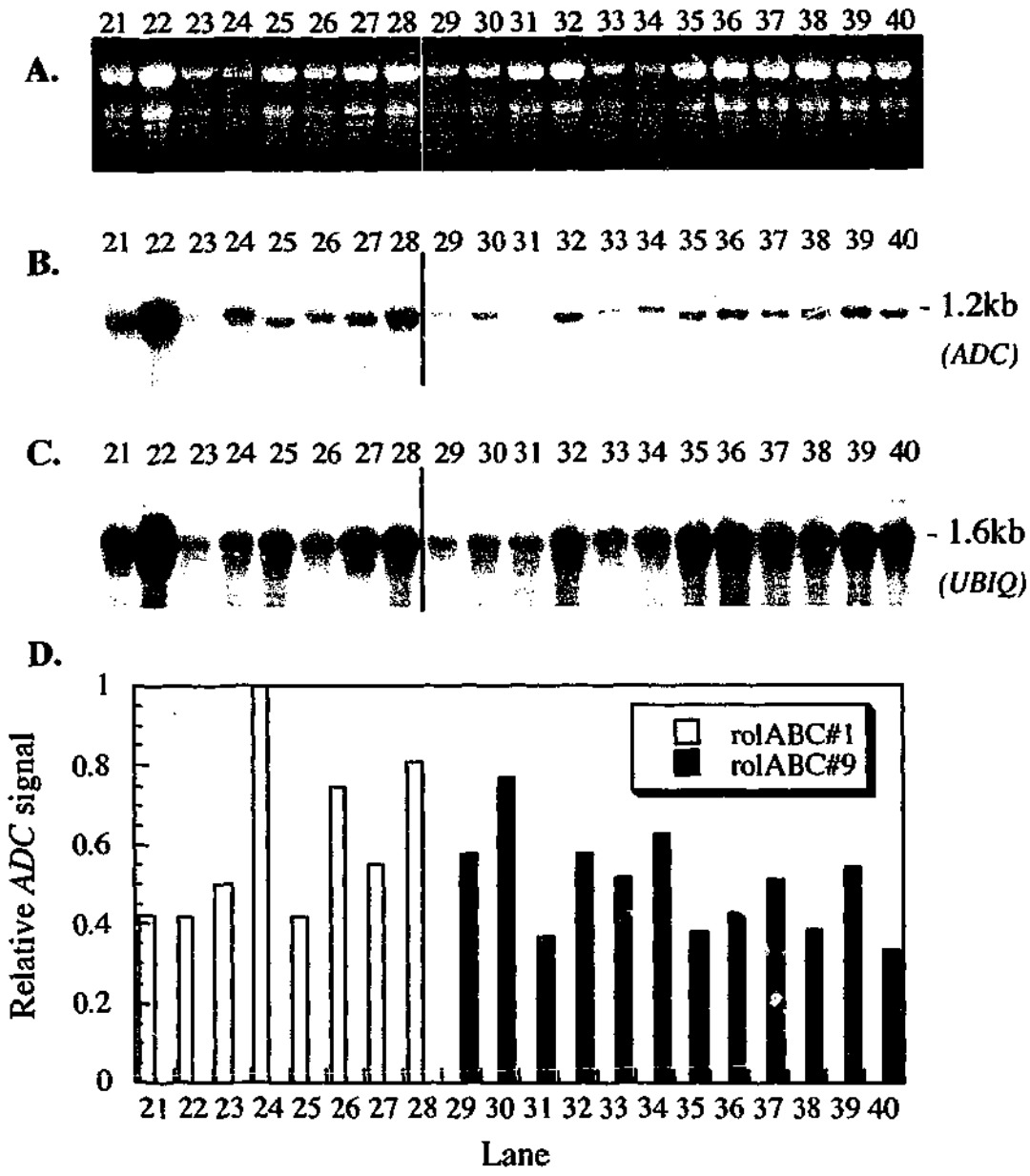


- 1.6kb
(UBiQ)

D.



Lane	Sample	Lane	Sample
	[wt *Ler*]		[*rolABC#7*]
1.	MS control	13.	MS control
2.	1mM CHA	14.	0.1mM MGBG
3.	0.1mM MGBG	15.	1mM MGBG
4.	1mM DFMO	16.	1mM DFMO
5.	5mM DFMO	17.	1mM PUT + 1 mM SPD
6.	10mM DFMO		
7.	1mMPUT		[*rolABC#1*]
8.	10mM PUT	18.	MS control
9.	1mM SPD	19.	1mM CHA
10.	3mM SPD	20.	0.1mM MGBG
11.	5mM SPD		
12.	1mM PUT + 1mM SPD		

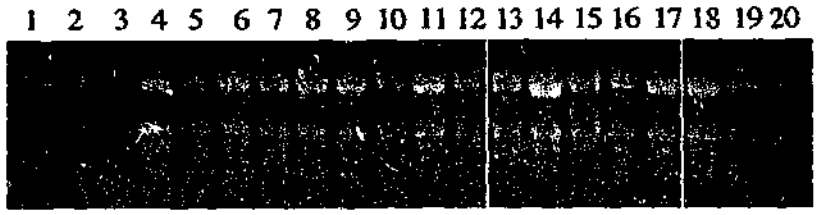


Lane	Sample	Lane	Sample
	|*rolABC*#1|	|*rolABC*#9|
21.	MS control	29.	MS control
22.	1mM DFMO	30.	1mM CHA
23.	10mM DFMO	31.	0.1mM MGBG
24.	1mM PUT	32.	1mM MGBG
25.	10mM PUT	33.	1mM DFMO
26.	1mM SPD	34.	10mM DFMO
27.	3mM SPD	35.	1mM PUT
28.	5mM SPD	36.	10mM PUT
		37.	1mM SPD
		38.	3mM SPD
		39.	5mM SPD
		40.	1mM PUT +1mM SPD

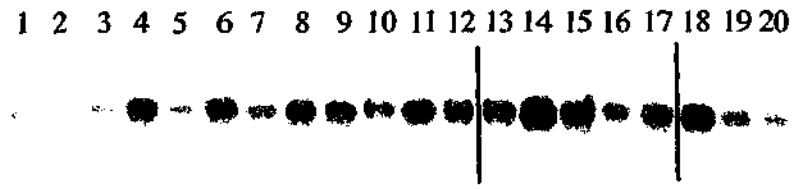
Figure 4.29: Expression of *ADC* in wild-type and *rolABC* transformant shoots following treatment with polyamines or polyamine inhibitors.

The EthBr-stained gel (~20µg total RNA/track) (**A.**) was blotted and the membrane probed with *ADC* (**B.**) and *UBIQ* (**C.**). The signal intensities were standardised to those of *UBIQ* (**D.**).

A.

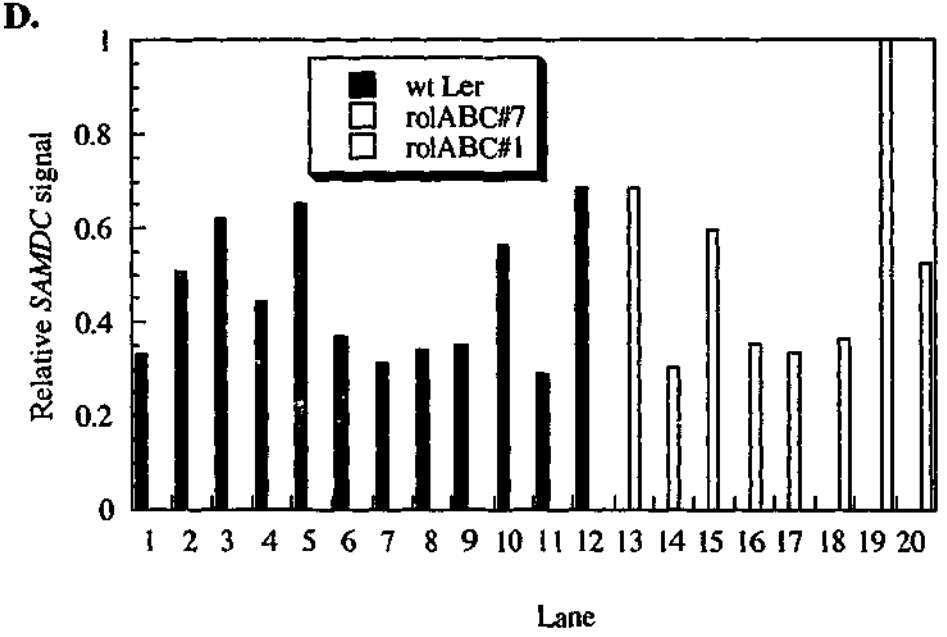


B.

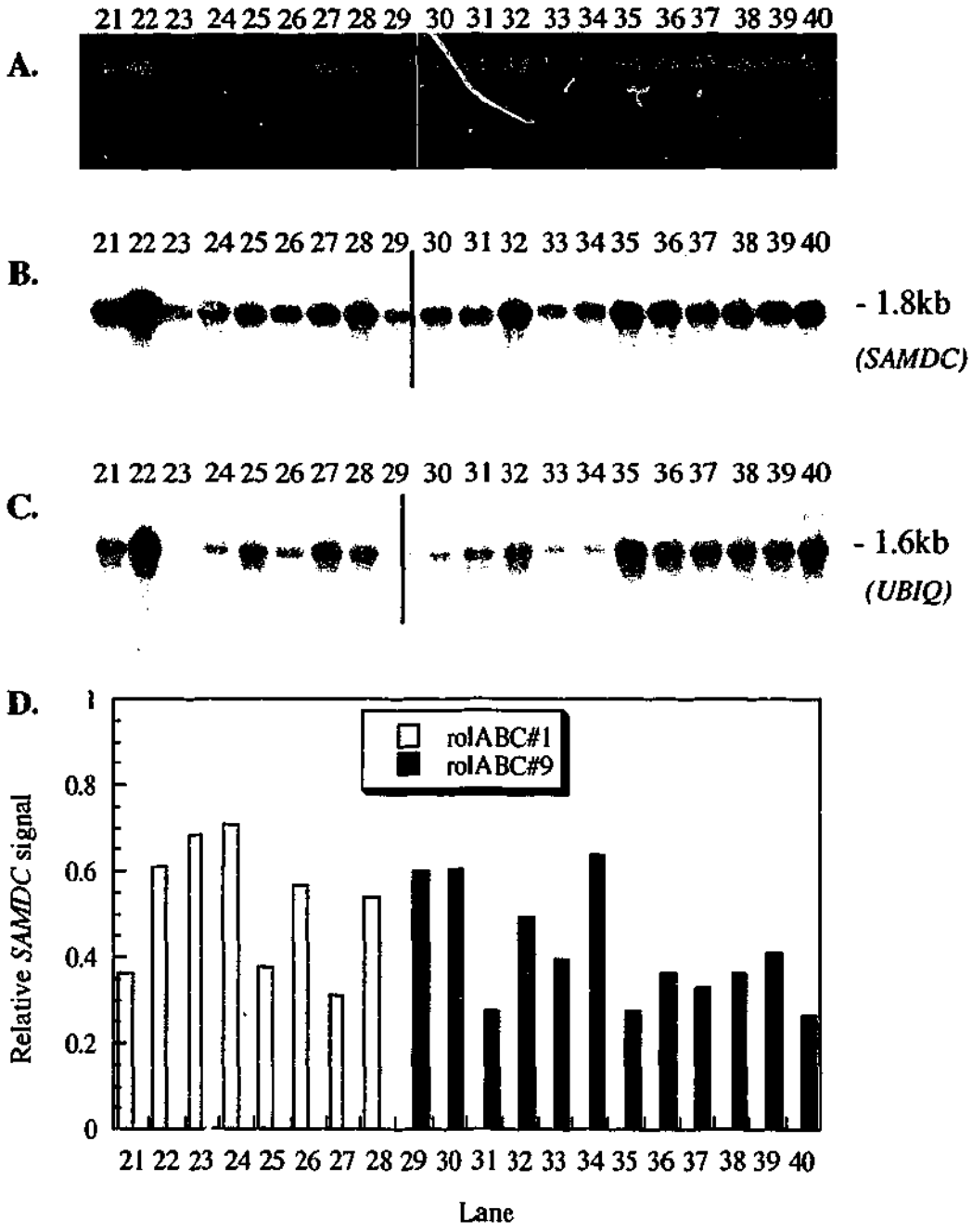


C.

D.



Lane	Sample	Lane	Sample
	[wt Ler]		*[rolABC#7]*
1.	MS control	13.	MS control
2.	1mM CHA	14.	0.1mM MGBG
3.	0.1mM MGBG	15.	1mM MGBG
4.	1mM DFMO	16.	1mM DFMO
5.	5mM DFMO	17.	1mM PUT + 1 mM SPD
6.	10mM DFMO		
7.	1mMPUT		*[rolABC#1]*
8.	10mM PUT	18.	MS control
9.	1mM SPD	19.	1mM CHA
10.	3mM SPD	20.	0.1mM MGBG
11.	5mM SPD		
12.	1mM PUT + 1mM SPD		



Lane	Sample	Lane	Sample
	[rolABC#1]		*[rolABC#9]*
21.	MS control	29.	MS control
22.	1mM DFMO	30.	1mM CHA
23.	10mM DFMO	31.	0.1mM MGBG
24.	1mM PUT	32.	1mM MGBG
25.	10mM PUT	33.	1mM DFMO
26.	1mM SPD	34.	10mM DFMO
27.	3mM SPD	35.	1mM PUT
28.	5mM SPD	36.	10mM PUT
		37.	1mM SPD
		38.	3mM SPD
		39.	5mM SPD
		40.	1mM PUT +1mM SPD

Figure 4.30: Expression of *SAMDC* in wild-type and *rolABC* transformant shoots following treatment with polyamines or polyamine inhibitors.

The EthBr-stained gel (~20µg total RNA/track) (**A.**) was blotted and the membrane probed with *SAMDC*(**B.**) and *UBIQ* (**C.**). The signal intensities were standardised to those of *UBIQ* (**D.**).

Effects of MGBG application

MGBG competitively inhibits the enzyme SAM decarboxylase, thereby reducing the availability of aminopropyl moieties from decarboxylated SAM for the production of both spermidine and spermine. The levels of putrescine would therefore be expected to increase after MGBG treatment, whilst spermidine levels would decrease. Treatment with 0·1mM and 1mM MGBG caused the levels of free (Table 4·6), conjugated (Table 4·7), and bound (Table 4·8) putrescine to increase, or remain essentially unchanged, in both control and transformed shoots. Titres of free spermidine decreased in wild-type plants as expected after MGBG treatment, however, they were also increased in transformants (Table 4·6). Interestingly, conjugated spermidine levels increased substantially in wild-type and moderately in *rolABC*#1 controls, however, the levels decreased markedly in transformant lines *rolABC*#7 and *rolABC*#9 (Table 4·7). A further discrepancy between the control lines and transformant lines was seen at the transcript level following exposure to 0·1mM MGBG. Relative transcript abundance of *SPDS* (Figure 4·28D), *ADC* (Figure 4·29D), and *SAMDC* (Figure 4·30D) increased in the wild-type and *rolABC*#1 control shoots, whereas transcript levels were diminished in the *rolABC*#7 and *rolABC*#9 transformant shoots.

Effects of DFMO application

Application of 1mM and 5mM DFMO induced slight-to-moderate increases in free putrescine levels of plants (Table 4·6), whilst having no observable effect on *ADC* transcript in all lines (Figure 4·29D). Levels of free spermidine levels showed moderate-to-large increases in both DFMO-treated control and DFMO-treated transformant lines (Table 4·6). This correlated with increased *SPDS* transcript levels in all lines, except for *rolABC*#7 at 1mM (Figure 4·28). *RolABC*#9 shoots grown in the presence of 10mM DFMO exhibited a marked elevation in levels of conjugated spermidine, whereas a decrease was observed in similarly-treated wild-type and *rolABC*#1 controls. Furthermore, the accumulation of *SAMDC* transcripts increased in both DFMO-treated control lines, whereas in *rolABC*#7 and also *rolABC*#9 plants exposed to DFMO the levels of *SAMDC* transcript tended to decrease (Figure 4·30). A summary of the Northern results are presented in Table 4·9.

wild-type Ler:

Treatment	ADC signal	SPDS signal	SAMDC signal	Free put level	Free spd level
1mM CHA	no change	++	++	+	---
0.1mM MGBG	++	++	++	++	---
1mM DFMO	no change	++	++	++	++
5mM DFMO	no change	++	++	++	++
10mM DFMO	-	++	no change	+	++
1mM PUT	--	++	no change	++	++
10mM PUT	--	++	no change	+++	--
1mM SPD	--	no change	no change	no change	++
3mM SPD	++	++	++	++	+++
5mM SPD	--	no change	++	+++	+++
1mM P + 1mM S	++	++	++	++	++

rolABC#1:

Treatment	ADC signal	SPDS signal	SAMDC signal	Free put level	Free spd level
1mM CHA	++	++	++	++	--
0.1mM MGBG	++	++	++	++	++
1mM DFMO	no change	++	++	not tested	not tested
10mM DFMO	++	++	++	++	++
1mM PUT	++	++	++	++	++
10mM PUT	no change	++	no change	++	--
1mM SPD	++	++	++	++	++
3mM SPD	++	++	no change	++	+++
5mM SPD	++	++	++	+++	+++

rolABC#7:

Treatment	ADC signal	SPDS signal	SAMDC signal	Free put level	Free spd level
0.1mM MGBG	--	--	--	no change	++
1mM MGBG	--	--	--	++	++
1mM DFMO	--	--	--	--	++
1mM P + 1mM S	--	--	--	++	+++

rolABC#9:

Treatment	ADC signal	SPDS signal	SAMDC signal	Free put level	Free spd level
1mM CHA	--	++	no change	++	++
0.1mM MGBG	decrease	--	--	--	++
1mM MGBG	no change	++	--	++	++
1mM DFMO	no change	++	--	++	++
10mM DFMO	no change	++	no change	++	++
1mM PUT	--	no change	--	++	++
10mM PUT	--	++	--	+++	++
1mM SPD	--	no change	--	--	++
3mM SPD	--	--	--	++	++
5mM SPD	no change	++	--	+++	+++
1mM P + 1mM S	--	--	--	++	++

Table 4.9: Summary of transcript accumulation and free polyamine titres following feeding with polyamines or polyamine biosynthesis inhibitors.

4·2·6 EFFECTS OF TRANSFORMATION ON AUXIN SENSITIVITY

Primary and lateral root growth

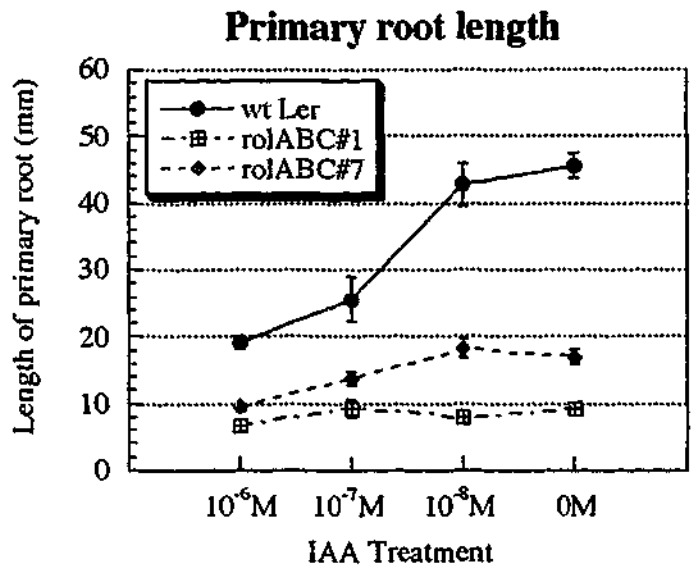
Primary root length

Transformation with *rol* genes alters the response, or production, of auxin in plant cells (Michael and Spena, 1995), although the exact mechanisms involved have not been fully elucidated. To further clarify the response of transformed seedlings to auxin, the effects of treatment with low levels of the natural auxin IAA on the induction of lateral roots in transformants were documented. The *rolABC*#1 line was used as a control in these experiments and as a large number of viable seed were available from *rolABC* #7, this line was used as representative of a line containing Ri T-DNA. Primary roots of the untransformed wild-type control seedlings exhibited the well documented auxin-induced reduction in length (Zeadan and Macleod, 1984; Lloret and Puglarín, 1992; Biondi *et al.*, 1997) (Figure 4·31A), with 10^{-6}M IAA treatment causing a reduction in wild-type primary root length from 45mm to 20mm, whilst 10^{-8}M IAA had no effect on the length of the wild-type primary root (Figure 4·31A). As has been noted plants of *rolABC*#1 line had a completely wild-type phenotype, thought likely to be because of a truncated T-DNA insert. It was therefore somewhat unexpected to find a similar auxin response from both the *rolABC*#1 and *rolABC*#7 primary roots (Figure 4·31A). Untreated primary roots of both transgenic lines were markedly shorter than those of the wild-type controls, ranging between 10mm to 15mm. 10^{-6}M IAA had an inhibitory effect on the two *rolABC* lines, reducing the root length by approximately 5mm in both cases. 10^{-7}M IAA was not markedly inhibitory to root length in either line (Figure 4·31A).

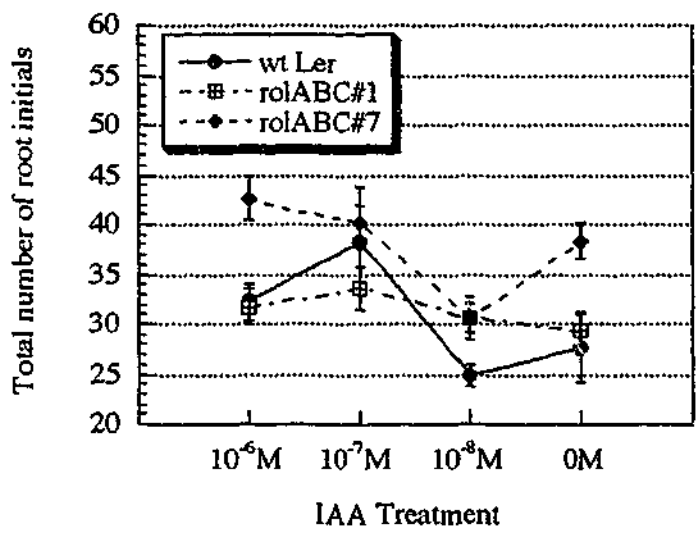
Total number of root initials

When untreated, *rolABC*#7 seedlings showed an increased capacity to produce lateral roots, with approximately 30% more root initials than controls (Figure 4·31B). This made it difficult to determine if the transformed seedlings showed greater sensitivity to low levels of auxin than controls with respect to lateral root primordia. It was observed, however, that

(A)



(B)



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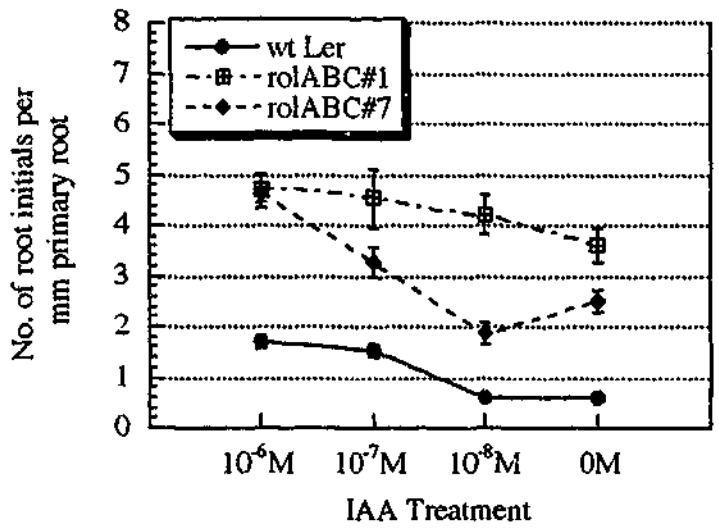


Figure 4.31: Root growth of control and *rolABC* transformants in response to treatment with low concentrations of IAA.
(A) Primary root length
(B) Number of root initials
(C) No. of root initials / mm of primary root

while there was some variance in the number of root initials produced by the untransformed wild-type seedlings, especially at 10^{-7}M IAA, the *rolABC*#7 seedlings consistently generated the greatest number of root initials over all IAA treatments (10^{-6} to 10^{-8}M).

Standardisation of the total number of root initials per seedling, relative to the length of the primary root, revealed that untransformed wild-type seedlings had markedly fewer root initials per millimetre than both *rolABC* transformants, across the four IAA treatments (Figure 4·31C). All lines reached their maximum number of roots per millimetre following 10^{-6}M IAA treatment. As a consequence of the shortened length of the *rolABC*#1 primary roots—possibly due to germination in the presence of kanamycin—the relative numbers of root initials per millimetre for all treatments in this line were as high as that for *rolABC*#7.

Adventitious root growth

Explants derived from flowering plants

To induce growth of adventitious roots from rosette leaf explants of flowering plants, auxin must be supplied, regardless of whether plants are transformed with Ri T-DNA or not (Table 4·10). 10^{-5}M IAA was found to be optimal for induction of adventitious roots, with *rolABC* transformants generally more sensitive than controls, with respect to the root-inducing effects of auxin (Table 4·10). Approximately 10% of explants from wild-type control and *rolABC*#1 lines possessed 20 or more roots on medium containing 10^{-5}M IAA, whereas in *rolABC*#3 and *rolABC*#9 explants the frequency was between 20% to 30%. The frequency of rooting in all lines decreased as the level of IAA was reduced to 10^{-6}M, however, the two transformant lines *rolABC*#3 and *rolABC*#9 again proved to be more sensitive to auxin than controls at this low level.

Explants derived from vegetative plants

Explants originating from vegetative wild-type *Ler* plants could not be induced to produce roots following IAA treatment. Explants from vegetative *rolABC* transformants that showed an altered shoot phenotype, however, demonstrated sensitivity to low concentrations of

Treatment*	No. of roots per explant:				
	0	1-5	6-10	11-20	20+
MS Control					
- wt *Ler* (34)	100·0	-	-	-	-
- *rolABC*#1 (32)	100·0	-	-	-	-
- *rolABC*#3 (32)	100·0	-	-	-	-
- *rolABC*#7 (32)	100·0	-	-	-	-
- *rolABC*#9 (32)	100·0	-	-	-	-
10^{-4}M IAA					
- wt *Ler* (32)	50·0	25·0	15·6	9·4	-
- *rolABC*#1 (32)	96·9	3·1	-	-	-
- *rolABC*#3 (32)	84·4	12·5	3·1	-	-
- *rolABC*#7 (32)	87·5	6·3	3·1	-	3·1
- *rolABC*#9 (32)	87·5	12·5	-	-	-
10^{-5}M IAA					
- wt *Ler* (32)	9·4	34·4	15·6	28·1	12·5
- *rolABC*#1 (32)	21·9	46·9	9·4	12·5	9·4
- *rolABC*#3 (32)	43·8	15·7	6·3	12·5	21·9
- *rolABC*#7 (32)	65·6	6·3	12·5	6·3	9·4
- *rolABC*#9 (24)	8·3	25·0	16·7	20·8	29·2
10^{-6}M IAA					
- wt *Ler* (32)	81·3	18·8	-	-	-
- *rolABC*#1 (32)	90·6	9·4	-	-	-
- *rolABC*#3 (32)	65·6	28·2	3·1	-	3·1
- *rolABC*#7 (32)	93·8	6·2	-	-	-
- *rolABC*#9 (30)	60·0	26·7	6·7	3·3	3·3

*The number of explants per treatment is shown in parentheses

Table 4.10: Adventitious rooting response (%) of *rolABC*-transformed flowering leaf explants to IAA.

Transformed seed were germinated and grown on MSK50 media at a density of approximately 50 seed per plate. Wild-type *Ler* seeds were sensitive to kanamycin, so control experiments were established using *Ler* seeds germinated and grown on MS media. At day 35 of growth, rosette leaf explants were removed from flowering plants using the procedure described in the Materials and Methods.

exogenously applied auxin and were able to produce adventitious roots (Table 4·11). Both *rolABC* lines #7 and #9 produced an optimal rooting response following 10^{-6}M IAA treatment, with 100% of explants from the *rolABC*#7 line and 92% of *rolABC*#9 explants undergoing rooting. Furthermore, *rolABC*#9 explants were also able to root when IAA levels were reduced to 10^{-7}M and 10^{-8}M (Table 4·11).

Treatment	No. of roots per explant:				
	0	1-5	6-10	11-20	20+
MS Control					
- *rolABC*#7	100·0	-	-	-	-
- *rolABC*#9	100·0	-	-	-	-
10^{-6}M IAA					
- *rolABC*#7	-	16·7	8·3	8·3	66·7
- *rolABC*#9	8.3	16·7	25·0	25·0	25·0
10^{-7}M IAA					
- *rolABC*#7	100·0				
- *rolABC*#9	75·0	16·7	8·3		
10^{-8}M IAA					
- *rolABC*#7	100·0	-	-	-	-
- *rolABC*#9	83·3	8·3	8·3	-	-

Table 4.11: Adventitious rooting response (%) of *rolABC*-transformed vegetative leaf explants to IAA.

Transformed seed were germinated and grown on MSK50 media at a density of approximately 50 seed per plate. Wild-type L*er* seeds were sensitive to kanamycin, so control experiments were established using L*er* seeds germinated and grown on MS media. At day 35 of growth, rosette leaf explants were removed from vegetative plants using the procedure described in the Materials and Methods.

4·3 DISCUSSION

Tobacco plants transformed with Ri T-DNA exhibit a hairy-root phenotype (Tepfer, 1984) and reduced titres of polyamines (Martin-Tanguy *et al.*, 1991), both of which can be mimicked by treatment with the ODC inhibitor DFMO (Martin-Tanguy *et al.*, 1990; Burtin *et al.*, 1991; Martin-Tanguy *et al.*, 1991; Tepfer *et al.*, 1994). Together with the observation that simultaneous application of putrescine reverted the stunted shoot phenotype caused by DFMO treatment, led Martin-Tanguy *et al.* (1990) and Burtin *et al.* (1991) to hypothesise that genes carried on the Ri T-DNA may act through interference with polyamine production.

As polyamines are required for normal cell division and proliferation, it has been suggested that in tobacco high DFMO results in cessation of cell division due to a depressive effect on endogenous polyamine accumulation (Berlin and Forche, 1981). Such a phenomenon may have therefore contributed to the stunted shoot phenotype observed when wild-type tobacco plants were treated with DFMO or transformed with Ri T-DNA (Burtin *et al.*, 1991; Martin-Tanguy *et al.*, 1991; Tepfer *et al.*, 1994). Interestingly however, and in contrast to this suggestion, application of DFMO has been reported to have stimulatory effects on other growth processes, including stimulation of cell enlargement in tobacco (Berlin and Forche, 1981) and carrot cell suspensions (Mengoli *et al.*, 1987), enhancement of polyamine content and growth of wheat root cultures (Bharti and Rajam, 1995), promotion of callus growth in tobacco (Burtin *et al.*, 1989) and in sugarbeet (Bisbis *et al.*, 2000), and stimulation of axillary shoot production (Burtin *et al.*, 1991) and root growth in tobacco (Tepfer *et al.*, 1994). In the present study using *Arabidopsis* it was noted that treatment with DFMO reduced putrescine levels in wild-type plants, and also increased production of axillary shoots and root growth, thus resembling the phenotype of T-DNA transformants. This seemingly paradoxical observation that DFMO treatment reduces endogenous titres of polyamines, which are essential for cellular division and proliferation, yet also stimulates various aspects of growth, might be explained by invoking a threshold model for polyamine action whereby reduced polyamine levels are required for several growth processes to occur.

Agreeing with the noted effects of DFMA and DFMO treatment of tobacco (Martin-Tanguy *et al.*, 1990) was the observation in the present study that simultaneous treatment of wild-type *Arabidopsis* with 1mM of both DFMA and DFMO mimicked the transformed phenotype. The application of 2mM DFMO alone was found to reduce the level of putrescine and result in a similar, though less severe phenotype than treatment with both DFMA and DFMO in wild-type *Arabidopsis* plants. In this respect, the results are in agreement with analogous treatments of tobacco reported by Burtin *et al.* (1991). It is noteworthy however, that no coding regions homologous to *ODC* have been identified in *Arabidopsis*. This is consistent with results of experiments involving the screening of diverse *Arabidopsis* cDNA libraries with a *Datura ODC* cDNA probe (including that noted in Chapter 3 of this study) which lead Hanfrey *et al.* (2001) to conclude that *Arabidopsis* lacks ODC. Hence, the similar phenotypes in *Arabidopsis* caused by treatment with DFMO alone, or treatment with both inhibitors simultaneously, may therefore be coincidental and possibly due to general toxicity caused by DFMO. From previously reported ODC enzyme assay results in *Arabidopsis* using DFMO, it is possible that the inhibitor has a high affinity for a protein with some structural similarity to ODC (Ferier *et al.*, 1998).

The *Arabidopsis* transformants containing Ri T-DNA, or *rolABC* genes alone, possessed reduced titres of polyamines compared to controls, and exhibited hairy-root phenotypes analogous to similarly-transformed plants including tobacco (Tepfer, 1984; Palazon *et al.*, 1998), kiwi (Rugini *et al.*, 1991), woody nightshade [*Solanum dulcamara* L.] (McInnes *et al.*, 1991), snapdragon (Handa, 1992), *Begonia* (Kiyokawa *et al.*, 1996), rose (van der Salm *et al.*, 1997), kangaroo apple [*Solanum aviculare*] (Jasik *et al.*, 1997), and *Atropa belladonna* (Bonhomme *et al.*, 2000). Specific morphological features observed in the *Arabidopsis* Ri T-DNA and *rolABC* transformants, have previously been reported for other similarly-transformed plants. The production of axillary inflorescences for example, has been noted following transformation in plants such as woody nightshade (McInnes *et al.*, 1991), *Atropa belladonna* (Kurioka *et al.*, 1992), alfalfa (Frugis *et al.*, 1995), tobacco (Palazon *et al.*, 1998), and rose (van der Salm *et al.*, 1998). An increased production of flowers has been noted in snapdragon (Handa, 1992) and *Atropa belladonna* (Kurioka *et al.*,

1992) transformed with Ri T-DNA or *rolC* respectively. In addition, smaller size and reduced fertility of flowers observed in *Arabidopsis rolABC* transformants has also been reported in T-DNA-transformed snapdragon (Handa, 1992), *Atropa belladonna* (Kurioka *et al.*, 1992), tomato (van Altvorst *et al.*, 1992), tobacco (Sun *et al.*, 1991; Martin-Tanguy *et al.*, 1993), lettuce (Curtis *et al.*, 1996), *Hyoscyamus muticus* (Sevón *et al.*, 1997), and kangaroo apple (Jasik *et al.*, 1997). Reduced fecundity of Ri T-DNA transformed plants has, in some cases, been attributed to the presence of stunted stamens (Sun *et al.*, 1991; Sevon *et al.*, 1997) both of which were also observed in the *Arabidopsis* Ri T-DNA and *rolABC* transformants.

Characteristic of the hairy-root phenotype of *Arabidopsis* lines used in the present study, and those of other species transformed with Ri T-DNA, is an intense stimulation in the production of roots, which are capable of rapid, hormone-autonomous growth as axenic cultures (Amselem and Tepfer, 1992; Handa, 1992, Mengoli *et al.*, 1992; Hamill and Lidgett, 1997; Palazon *et al.*, 1998). In addition, the present study suggests that polyamine levels *per se* may facilitate growth of roots in *Arabidopsis*. This is based on observations that transformed roots have low levels of endogenous polyamines and grow at a faster rate than controls, and that exogenous polyamines inhibit root growth (chapter 3 results). Such results are in agreement with observations of an inverse relationship between polyamine levels and rates of root growth in tobacco transformed with Ri T-DNA (Martin-Tanguy *et al.*, 1990). A correlation between decreased polyamine titres and stimulated root growth is supported by observations that T-DNA-transformed tobacco roots (Ben-Hayyim *et al.*, 1996) exhibited similar patterns of stimulated growth as excised wild-type tobacco roots treated with DFMO (Ben-Hayyim *et al.*, 1994, Tepfer *et al.*, 1994). Furthermore, roots from wild-type *Arabidopsis* used in the present study, exhibited both slow growth in liquid media devoid of phytohormones and relatively high titres of endogenous putrescine. Increased levels of putrescine have also been correlated with slow growth of roots in *rolA* tobacco transformants (Altabella *et al.*, 1995) and non-transformed microcuttings of pear (Baraldi *et al.*, 1995), whilst putrescine treatment has been shown to slow growth of eggplant root cultures (Sharma *et al.*, 1997). Indeed, it has been proposed that titres of endogenous

putrescine may be indicative of the rate of growth of plant tissues, as fluctuations caused by the application of inhibitors are reported to accompany changes in morphology (Burtin *et al.*, 1989). Other studies of tobacco transformants however, report little or no difference in titres of free and conjugated polyamines between N, T, and T′ roots (Mengoli *et al.*, 1992), or between *rolABC*-transformed and control root cultures (Altabella *et al.*, 1995).

The rapid increase in mass characteristic of transformed roots cultured *in vitro* has been shown to be due, at least in part, to increased root branching (Handa, 1992; Mengoli *et al.*, 1992; Akasaka *et al.*, 1998; Bonhomme *et al.*, 2000). Increased production of lateral roots were noted in both the Ri T-DNA and *rolABC* transformants of *Arabidopsis* in the current study, as well as in similarly-transformed tobacco plants (Martin-Tanguy *et al.*, 1993; Ben-Hayyim *et al.*, 1994). As observed in the present study, and reported elsewhere (Ben-Hayyim *et al.*, 1996), with the exception of very young seedlings, the exact nature of the altered lateral root number in transformed tissues is difficult to assess due to the rapid and complex proliferation of the higher order lateral roots. Notwithstanding a lack of an *ODC* gene in *Arabidopsis* (Hanfrey *et al.*, 2001), the present study showed that DFMO treatment reduced putrescine levels in *Arabidopsis* whilst also increasing lateral root number, which further strengthens the suggestion that decreased polyamine levels are stimulatory to increased root growth. To examine this correlation further, additional experiments treating transformed *Arabidopsis* root cultures with putrescine may determine if growth is slowed by addition of this amine, or with any of the higher order polyamines. If the growth kinetics of the transformed lines are maintained following such treatments, it may suggest that the rapid growth of transformants is not a consequence of reduced polyamine levels.

Transformation with T-DNA, or treatment with DFMO, has been reported to reduce levels of conjugated polyamines and cause delays in flowering of tobacco (Martin-Tanguy *et al.*, 1990 and 1991), consistent with the suggestion that observed increases in conjugated polyamines during floral transition are causally associated with this developmental change (Martin-Tanguy *et al.*, 1990). In addition, increases or decreases in levels of free and conjugated polyamines, according to the conditions of growth, have been suggested to play a role in the

flowering process of *Arabidopsis* (Applewhite *et al.*, 2000; Tassoni *et al.*, 2000). In the present work it was interesting to find very high titres of conjugates in shoots of transformed *Arabidopsis* T segregants. Such plants had abnormally compacted, vegetative shoots and underwent senescence *in vitro* without flowering. This may have been due to a diminished capacity to regulate conjugate accumulation in shoot tissue following transformation with Ri T-DNA or *rol* genes. An alternative suggestion, as proposed by Scaramagli *et al.* (1999A), is that a high level of conjugates may be an indicator of stress in *Arabidopsis* plants.

The role(s) of conjugated polyamines during plant development remain unclear. The persistence of high cellular levels of conjugates, following a decline in free polyamines during growth led some researchers to suggest that conjugates do not act as storage forms of polyamines (Burtin *et al.*, 1991). In contrast, other groups have indicated that an important role of conjugates is to act as polyamine reservoirs (Robins *et al.*, 1991). A related suggestion is that formation of conjugates provides a mechanism of reducing high intracellular levels of free polyamines (Protacio and Flores, 1992; Scaramagli *et al.*, 1999C) possibly acting as carriers of free polyamines to the γ-aminobutyric acid (GABA) degradation pathway (Bernet *et al.*, 1998). In transgenic tobacco expressing the oat *ADC* gene, Burtin and Michael (1997) found that high titres of agmatine were not conjugated or converted into polyamines, and suggested the possibility that sequestration to the vacuole may occur in preference to conjugation of agmatine in this species. In the present work, polyamine-feeding experiments to control and *rolABC*-transformed plants stimulated large increases in levels of both free and conjugated polyamines in *Arabidopsis*, suggesting that conjugation may be an active metabolic route for dealing with high titres of endogenous polyamines.

Working with tobacco, Burtin *et al.* (1989) suggested that high titres of putrescine conjugates in foliar explants and roots may inhibit cell proliferation and suppress bud formation. In the present study, levels of putrescine conjugates measured in wild-type shoots treated with 10mM putrescine were dramatically elevated compared to controls, and were comparable to those found in the abnormal shoots of the T segregants grown on MS media without any supplements. Whilst shoots from the latter plants exhibited indeterminate vegetative growth,

however, wild-type plants treated with 10mM putrescine were phenotypically normal, indicating that high levels of conjugated polyamines *per se* cannot be responsible for causing the alterations in shoot phenotype observed in abnormal T segregants.

In the present study, treatment of *Arabidopsis* with DFMO or a combination of DFMO and DFMA, and also transformation with Ri T-DNA, both led to reductions in putrescine titres and comparable alterations in shoot and root morphology, which were also noted in tobacco (Burtin *et al.*, 1991). The phenotypic effects induced by DFMO in tobacco were reversed however, by concurrent application of putrescine (Martin-Tanguy *et al.*, 1991). In *Arabidopsis*, however, the phenotype induced by DFMO was not ameliorated with simultaneous putrescine application (present study; Hanfrey *et al.*, 2001). Moreover, in the present study, treatment of *rolABC* transformants with putrescine or spermidine did not normalise their phenotype, even though HPLC analysis indicated that polyamines were taken up from the media. In addition, the altered phenotype of tobacco plants transformed with *rolA* under the control of the 35S promoter, was not attenuated by application of free polyamines (Sun *et al.*, 1991; Martin-Tanguy *et al.*, 1996). These observations suggest that decreased polyamine levels as a result of transformation with Ri T-DNA are a consequence, rather than the causation, of an altered phenotype. Thus the present study is in agreement with reports in the literature (Mengoli *et al.*, 1992; Altabella *et al.*, 1995) that there is no direct relationship between polyamine levels and expression of genes on the TL-DNA (Mengoli *et al.*, 1992).

In the current work, wild-type *Arabidopsis* plants treated with high levels of putrescine showed a preference to conjugate excess free putrescine, rather than increase production of spermidine *in vivo*. Interestingly, putrescine treatment increased levels of SPDS mRNA transcripts in wild-type and *rolABC*#9 shoots. Following such increases in transcript levels however, titres of spermidine in control shoots did not increase, possibly suggesting that post-transcriptional control of SPDS enzyme activity occurs in wild-type plants. In support of this it is noteworthy that studies utilising animal cell found that increases in *SPDS* mRNA accumulation were not associated with alterations in SPDS enzyme activity or levels of SPDS

protein (Halmekytö *et al.*, 1991; Halmekytö *et al.*, 1993; Kauppinen, 1995). Another possibility for the lack of conversion of putrescine to spermidine in control shoots following putrescine treatment, may be that high levels of putrescine in controls undergo preferential degradation via the diamine oxidase pathway, as also reported in putrescine-fed pea seedlings (Wisniewski and Brewin, 2000).

Several studies aimed at disrupting polyamine metabolism in plant tissues via the overexpression of polyamine biosynthetic genes, have produced varying results. Transformation with heterologous *ODC* (Hamill *et al.*, 1990; Descenzo and Minocha, 1993; Bastola and Minocha, 1995), *ADC* (Masgrau *et al.*, 1997), or *SAMDC* (Noh and Minocha, 1994) genes have been reported to produce modest, if any, increases in levels of polyamines. As previously noted, elevated levels of agmatine in tobacco tissues (Burtin and Michael, 1997), putrescine in rice tissues (Bassie *et al.*, 2000A), or spermidine in *Arabidopsis* tissues (Tassoni *et al.*, 2000), do not necessarily lead to downstream increases in levels of other polyamines *in vivo*. In the current work, however, although *rolABC*#9 transformants treated with putrescine did produce elevated levels of putrescine conjugates, increased production of free and conjugated spermidine was also observed. This may suggest that transformation with *rol* genes has the capacity to alter the controlling mechanisms regulating flux through the polyamine biosynthetic pathway in *Arabidopsis*. Inhibition of diamine oxidase activity may be one possible mechanism in these transformants which may lead to high putrescine titres (Scaramagli *et al.*, 1999A) and a subsequent stimulation of polyamine flux through the pathway, thereby increasing spermidine content (Bassie *et al.*, 2000B).

It has been reported that transcriptional regulation of endogenous polyamine biosynthetic genes is important in modulating polyamine titres in plants (Pérez-Amador *et al.*, 1995; Soyka and Heyer, 1999). At the time the experiments were conducted in this study, an *ODC* gene had not been isolated from *Arabidopsis*, but was thought likely to exist based on published results from ODC enzyme assays (Watson *et al.*, 1998; Feirer *et al.*, 1998; Tassoni *et al.*, 2000). In the absence of an authentic *Arabidopsis ODC* probe, Northern hybridisations were performed using an 800bp sequence from the 5′ region of the *N*.

tabacum ODC cDNA sequence [Genbank Accesion number Y10472]. Under high stringency conditions, a relatively weak transcript of approximately 900bp was detected in roots of *Arabidopsis* using this *ODC* probe. No transcripts were ever detected in *Arabidopsis* shoots, however, even under conditions of low stringency. Following the recent publication proposing that *Arabidopsis* lacks *ODC* (Hanfrey *et al.*, 2001), the identity of the root-specific transcript using the tobacco *ODC* probe remains unclear. It is possible that the probe may have identified an *Arabidopsis* sequence with some homology to the tobacco *ODC* gene, with one candidate being lysine decarboxylase and further work to explore this possibility is warranted.

SPDS transcript levels have been reported to be sensitive to spermidine levels in *Arabidopsis* (Tassoni *et al.*, 2000). In the present study, however, little if any alteration in *SPDS* message was observed following spermidine treatment in control and transformed lines, even though endogenous spermidine titres were increased in both. This suggests that in this case, *SPDS* mRNA is not subject to feedback inhibition, in contrast to observations reported by Tassoni *et al.* (2000). In wild-type *Arabidopsis*, *SAMDC* expression is thought to be regulated by *in vivo* spermidine concentrations (Tassoni *et al.*, 2000). Since spermidine titres have been reported to parallel SAMDC activity in tobacco, it has been suggested that the biosynthetic activity of this enzyme is important in controlling the accumulation of spermidine (Scaramagli *et al.*, 1999C). A lack of ODC in *Arabidopsis* (Hanfrey *et al.*, 2001) may suggest an even more important role for *SAMDC* in regulating polyamines in this species. In the present study, Ri T-DNA- and *rolABC*-transformed *Arabidopsis* hairy-root cultures exhibited similar transcript levels of *SAMDC* as wild-type controls, whereas titres of free spermidine were diminished relative to controls. This suggests that post-transcriptional mechanisms may be contributing to the regulation of spermidine pools in transformed *Arabidopsis* lines.

Spermidine treatment of wild-type and *rolABC*-transformed *Arabidopsis* plants increased endogenous putrescine titres in shoots, confirming the presence of a pathway converting spermidine to putrescine in plants (Del Duca *et al.*, 1995; Tassoni *et al.*, 2000). Little effect

on *ADC* transcript levels were noted following such treatment however, corroborating the results of Tassoni *et al.* (2000). Similarly, the present study found that treatments of *Arabidopsis* with other polyamines or inhibitors made only a slight difference to transcript levels of *ADC* with little impact on shoot phenotype. Further studies could assess effects on ADC activity, as previous reports have indicated post-transcriptional regulation to be important with respect to *ADC* regulation (Malmberg *et al.*, 1992; Malmberg *et al.*, 1994).

The presence of Ri T-DNA in plant cells has been linked to alterations in hormone perception and/or metabolism (Michael and Spena, 1995; Nilsson and Olsson, 1997) The *rol* genes in particular have the capacity to alter the sensitivity or perception of plant cells to endogenous auxin (Maurel *et al.*, 1991), which has been proposed to be involved in the generation of the hairy-root phenotype (Biondi *et al.*, 1997). In the current study, a typical *Arabidopsis rolABC* transformant line (*rolABC*#7) exhibited increased production of lateral roots compared to wild-type controls and *rolABC*#1 lacking part or all of the *rol* gene T-DNA in response to low levels of auxin treatment. In general agreement, increased rates of lateral root formation in *rolABC*-transformed roses (van der Salm *et al.*, 1997) and *H. muticus* hairy roots (Biondi *et al.*, 1997) compared to controls, were observed in response to application of low concentrations of auxin. *Arabidopsis rolABC* transformants generated in the current study also exhibited an increased capacity to form adventitious roots from leaf explants when incubated under conditions of low or zero external auxin concentrations, compared to untransformed controls. This phenomenon has been reported in a variety of other plants transformed with T-DNA including woody nightshade (McInnes *et al.*, 1991), tomato (van Altvorst *et al.*, 1992), lettuce (Curtis *et al.*, 1996), kangaroo apple (Jasik *et al.*, 1997), and rose (van der Salm *et al.*, 1997), suggesting increased auxin sensitivity of Ri T-DNA transformants.

Further strengthening the correlation between altered auxin metabolism and the Ri T-DNA transformed phenotype are studies of cellular anatomy of transformed tissues. The cellular organisation of adventitious roots from transformed apple shoots were found to resemble those of non-transgenic tissues treated with high levels of auxin (Sutter and Luza, 1993).

Transformed apple roots had large, parenchymatous cortical cells which the authors suggested were due to increased levels and/or increased sensitivity of tissues to auxin. Expression of *rolA* and *rolC* have been identified in vascular bundles and companion cells of phloem strands respectively in tobacco transformants, and expression of these genes are proposed to inhibit the elongation and differentiation of parenchyma and pith cells (Guivarc'h *et al.*, 1996A and Guivarc'h, 1996B). In the present study, alterations in structure and organisation of vasculature were found in *Arabidopsis rolABC* transformants following cross-sectional analyses of inflorescence axes. In addition, the degree of cellular alteration in each line was correlated with the number of T-DNA integration events; *rolABC*#3 for example, possessed two T-DNA inserts, correlating with the most severe cellular alterations. Line *rolABC*#1 on the other hand appeared to have a truncated insert, and was phenotypically normal. It has been noted that insertion of multiple copies, or truncation of T-DNA inserts, often follow transformation (Hamill *et al.*, 1987; Visser *et al.*, 1996; Karimi *et al.*, 1999).

Cross-sections of *rolABC* inflorescences produced in the present study appeared similar to published reports of wild-type plants treated with an auxin transport inhibitor, or possessing a mutation in the *AtPIN1* locus (Gälweiler *et al.*, 1998). AtPIN1 is proposed to have a role in polar auxin transport, particularly supporting the efflux of auxin from cells, and has been localised to the basal side of auxin-transport-competent cells within root and shoot vascular tissue (Gälweiler *et al.*, 1998). A related gene, *AtPIN2*, has 64% identity to *AtPIN1* at the protein level and is primarily expressed in root tips (Müller *et al.*, 1998). The AtPIN2 protein is proposed to influence root gravitropism by acting as a transmembrane component of the auxin efflux carrier complex (Müller *et al.*, 1998). It is possible that transformation and expression of *rolABC* genes and possibly others, from the *A. rhizogenes* T-DNA, may somehow perturb the expression of *Arabidopsis* genes involved in hormone metabolism such as *AtPIN1* and *AtPIN2*. Reduced function of *AtPIN1* in the *rolABC* transformants may lead to diminished auxin transport from source tissue including shoot tips and young leaves, and may partly contribute to the stunted transformed shoot phenotype. Altered auxin transport in turn may then lead to enhanced xylem proliferation at sites of high auxin concentrations (Gälweiler *et al.*, 1998) as was seen in the stem cross-sections of the *Arabidopsis rolABC*

transformants. Furthermore, impairment of *AtPIN2* expression or function may alter the normal auxin-regulated gravitropic response of roots, thus contributing to the plagiotropic root phenotype seen in the 9402 and *rolABC* transformants. Expression of T-DNA genes may also perturb other hormonal activities in transformed *Arabidopsis* plants, such as those of gibberellin (Ben-Hayyim *et al.*, 1996) and cytokinin (van der Salm *et al.*, 1998), thus contributing to the overall hairy-root phenotype. Such hypotheses require careful further experimentation to test them adequately, perhaps using a range of *Arabidopsis* mutants impaired in one or more genes involved in hormone metabolism.

The degree of stimulation of root growth following *A. rhizogenes* transformation may be linked to the specific activities of the *rol*, and other, gene promoters. Transformants containing the full-length T-DNA exhibited a strong hairy-root phenotype due to the combined expression of the *rol* genes on the TL-DNA and the auxin biosynthesis genes (*aux*) encoded on the TR-DNA (Amselem and Tepfer, 1992; Moyano *et al.*, 1999). It is therefore possible that the additional expression of other Ri TL-DNA genes or TR-DNA genes in the *Arabidopsis* 9402 transformants accounts for their stronger hairy-root phenotype compared to that of the *rolABC* lines. It should be noted, however, that the presence of the TR-DNA in these 9402 transformants was not confirmed by Southern blot analysis in this study, but was undertaken previously in this laboratory.

In conclusion, this study demonstrated that the transformed phenotype induced in *Arabidopsis* by the Ri T-DNA from *Agrobacterium rhizogenes* is correlated with an alteration in polyamine metabolism. This may not, however, be caused by reduced polyamine levels *per se*, since the application of exogenous polyamines does not ameliorate the phenotype and the expression of polyamine biosynthetic genes is not significantly altered in transformants. The increased sensitivity of leaf explants to auxin and the altered vascular tissue pattern in inflorescence axes of transformants, suggests that the transformed phenotype may be due to perturbations in the sensitivity and/or perception of endogenous hormones. This, in turn, may affect growth and development and as a result, lead to *in vivo* alterations of polyamine levels.

CHAPTER 5:

ISOLATION AND CHARACTERISATION OF *ARABIDOPSIS* MUTANTS EXHIBITING TOLERANCE TO POLYAMINE BIOSYNTHESIS INHIBITORS

5·1 INTRODUCTION

STUDIES OF POLYAMINE METABOLISM perturbation in plants by chemical agents have focussed on the drugs DFMA, DFMO, MGBG, and CHA that block key biosynthetic enzymes, ADC, ODC, SPDS, and SAMDC respectively (Figure 5·1). By searching for variants or mutants with elevated tolerance to normally toxic levels of inhibitor, use of these inhibitors may provide insights into regulatory mechanisms controlling polyamine biosynthesis and metabolism.

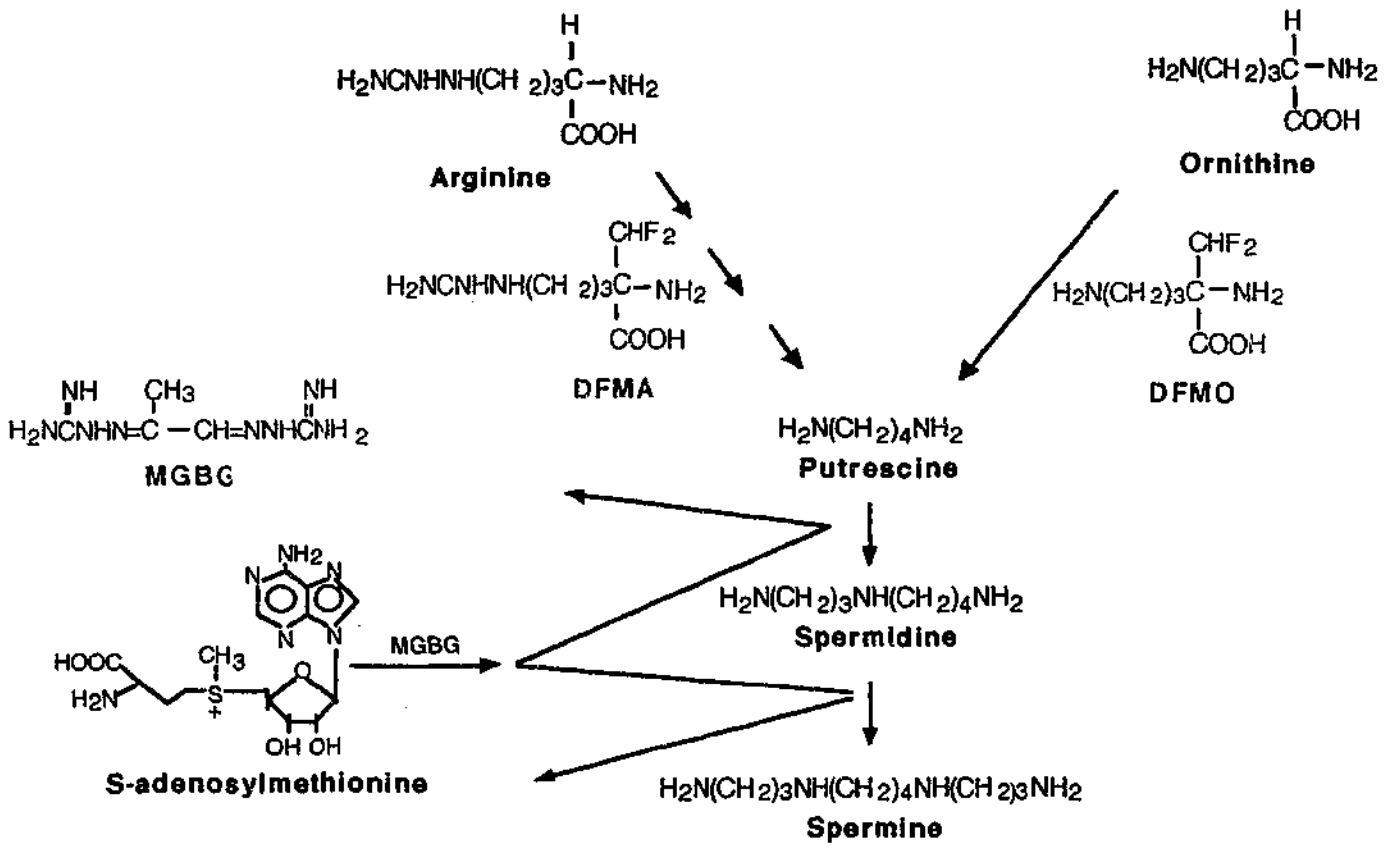


Figure 5·1: Structures of the three major polyamines in plants, their precursor compounds, and some of the key biosynthetic inhibitors.

5·1·1 ODC MUTANTS

Reduced ODC Activity

The enzymes ODC and SAMDC are generally regarded as being the main rate-limiting enzymes in polyamine biosynthesis. Initial studies with Chinese Hamster Ovary (CHO) cells focussed on ODC, and the recovery of lines with mutations in ODC regulation. Steglich and Scheffler (1982) reported the generation of a CHO cell line auxotrophic for putrescine, that possessed only residual ODC activity. The mutant line was isolated by a method of suicide selection using ^{3}H-ornithine treatment after EMS mutagenesis, in which cells failing to incorporate the labelled compound into putrescine escaped the negative effects of radiation and were cultured for further studies. The authors speculated that the primary defect in the mutant was likely to affect the activity of ODC, possibly due to either a mutation in the

structural *ODC* gene, or from one or more mutation(s) affecting other peptides that modify ODC activity. Later, Pohjanpelto *et al.* (1985) provided the first report of higher eukaryotic cells lacking measurable ODC activity, which therefore could not transform ornithine into putrescine. Here, 39 CHO cell lines were isolated and tested for putrescine auxotrophy and two were found to grow in the presence of putrescine but not in the presence of ornithine. Cells exhibited a retarded growth phenotype in normal medium, and a complete cessation of growth in the absence of exogenous polyamines in an otherwise optimal medium. The cell lines contained proteins that were immunoreactive to ODC antibodies, and had similar amounts of *ODC* mRNA as the parental cell line, suggesting that the mutation may have affected the catalytic capacity of the ODC protein.

In studies of ODC variants in plants, Malmberg *et al.* (1985) described a mutant line of tobacco (*Dfr1*) with extremely low ODC activity, that was regenerated from cell lines resistant to DFMO. *Dfr1* plants exhibited a dwarf phenotype and never flowered. A revertant (*Rt1*) was recovered, from MGBG-resistant tissue derived from *Dfr1*, which had wild-type levels of ODC but reduced SAMDC activity. The shoot phenotype of *Rt1* was essentially normal with respect to colour and size, but flowers were both male and female sterile, and showed a slight abnormality in anther development. The authors suggested that *ODC*, along with other metabolically important genes, may be floral-specific in expression and also may be required for flowering. In a subsequent study searching for *Arabidopsis* seedlings with altered *in vivo* ADC activity, Watson *et al.* (1998) also isolated a line which was reported to have very low ODC activity. Analysis of offspring from this line, however, failed to give reproducible segregation of the low activity, and thus was not pursued for further study.

Increased ODC Activity

M^{c}Conlogue and Coffino (1983A) also used DFMO resistance to identify another class of ODC mutant. A mouse cell line (S49), with elevated tolerance to 0·1mM DFMO, was isolated and found to possess an approximately 10-fold increase in ODC activity. Using immunoprecipitation to detect the ODC polypeptide, followed by two-dimensional gel

analysis of [^{35}S]methionine pulse-labelled cell extracts, it was shown that increased enzymic activity in the tolerant cell line was associated with elevated rates of ODC synthesis. The increased rate of ODC synthesis was found to exceed that of any other single protein within the cell line (McConlogue and Coffino, 1983B). Further screening revealed four clones, derived from the original variant cell line, that contained between 2 to 16 times more *ODC* mRNA than the parent cells. Elevated ODC activities were not due to amplification of the *ODC* gene in these four clones. Rather, the rates of synthesis of the ODC polypeptide per *ODC* mRNA molecule in the variant cells was found to be higher than that of wild-type cells (McConlogue *et al.*, 1986). It was thought post-transcriptional regulation of the *ODC* gene therefore, had made an important contribution to the DFMO-resistant phenotype of these variant mouse cells. Similarly, a class of phenotypic variants from a PC12 rat cell line resistant to 1mM DFMO, had wild-type levels of *ODC* mRNA without *ODC* gene amplification, yet elevated levels of ODC activity, also suggesting increased capacity to translate *ODC* mRNA (Marschall and Feinstein, 1995).

In a further study of cellular DFMO resistance, four mouse and two human tumour cell lines exhibiting resistance were characterised in terms of their polyamine biosynthetic enzyme activities and endogenous cellular polyamine titres (Hirvonen *et al.*, 1989). Of the six lines studied, four possessed an amplification of the *ODC* gene which resulted in overproduction of ODC enzyme, and consequently provided tolerance to DFMO. Five of the lines exhibited elevated SAMDC activity, resulting in an enhanced decarboxylation of SAM, suggested by the authors to be closely associated with the overproduction of ODC in the DFMO-resistant tumour cell lines. Enhanced activities of the two rate-limiting enzymes were suggested to be important in the maintenance of normal polyamine pools within the cells (Hirvonen *et al.*, 1989).

Tome *et al.* (1994) studied the regulation of ODC activity in variant rat hepatoma cells resistant to 10mM DFMO. Untreated control cells exhibited a faster growth rate than variant cells grown either in the presence or absence of 10mM DFMO. Polyamine content was correlated with growth rates, as control cells displayed higher endogenous titres of free polyamines than variant cells treated with 10mM DFMO. Upon removal of the inhibitor,

putrescine levels within variant cells were markedly elevated and remained high for several days until the end of the growth period, causing a decrease in cellular viability. Levels of spermidine and spermine were similar to those of the wild-type cells. Elevated putrescine and reduced viability was correlated with an increase in ODC activity and found to be due to alliterated ODC regulation, with the half-life of the enzyme being approximately three hours in the variants, compared to 23 minutes in control cells. Reduced capacity to down regulate ODC therefore appears to cause production and accumulation of toxic levels of putrescine, within mammalian cells cultured *in vitro* (Tome *et al.*, 1994). In a similar study of a *Leishmania donovani* promastigote line resistant to 10mM DFMO, ODC levels were up to 7·5-foldgreater than controls. Putrescine concentration increased approximately 30-fold to reach levels eight times higher than that found normally in wild-type cells, when the selective pressure from DFMO was removed (Coons *et al.*, 1990). Spermidine levels however, were comparable , however, between the sensitive and resistant lines, whereas spermine is not found in *Leishmania* , and was, therefore not monitored (Coons *et al.*, 1990).

5-1-2 ADC MUTANTS

Reduced ADC Activity

Watson *et al.* (1998) developed a novel screening method to isolate mutant *Arabidopsis* seedlings with altered ADC activity, by testing for the ability to decarboxylate ^{14}C-arginine *in vivo*. From 15,000 EMS-mutagenised M2 seedlings, seven independent mutants with altered *in vitro* ADC activity were isolated and categorised into two complementation groups, designated as *spe1* and *spe2*. Plants from both groups demonstrated reduced ADC activity, ranging from approximately 20% to 50% of that of wild-type plants, whilst the level of a double mutant was slightly lower than either mutant alone. Interestingly, no plants with enzyme activity lower than 20% of the wild-type levels were recovered. The major effects on phenotype of reduced ADC activity were manifested in an altered root morphology, with the single mutants showing an increase in lateral root initiation and growth, and the double mutant having a highly kinked and more compact root system. Titres of soluble polyamines

(free and conjugated) were essentially the same in all lines studied, except for the roots of the double mutant, which showed an approximately 50% reduction. From these results, the presence of at least two ADC genes in *Arabidopsis* is inferred, and it is suggested that ADC activity and polyamine levels are particularly important for the function and development of the root system in *Arabidopsis*.

Increased ADC Activity

In a study of polyamine content of four *Petunia hybrida* lines isolated during a search for mutants altered in either floral pigmentation or morphology, Gerats *et al.* (1988) characterised a mutant line, previously designated *alf* (*a*berrant *l*eaves and *f*lowers), showing it to have a two- to three-fold increase in putrescine and over a three-fold increase in ADC activity late in development. This line, thought likely to have originated from the insertion of a transposable element into the gene, exhibited abnormal flower development. Analysis of F1 and backcross progeny from crosses between high and low putrescine strains of *Petunia* suggested that a low putrescine content and low ADC activity was a dominant trait.

5·1·3 SAMDC MUTANTS

Resistance To MGBG And Analogues

MGBG, a competitive inhibitor of the enzyme SAMDC, has been used to generate other types of mutants of the polyamine pathway. Analogues of MGBG possessing novel structures whilst maintaining the potency of the parent compound, are continually being developed for use in antileukaemic studies (Tekwani *et al.*, 1992; Regenass *et al.*, 1994). MGBG has been widely used due to its commercial availability, however the specificity of the mode of inhibition has come under question. It has been suggested that the toxicity of MGBG may involve actions that are independent of those on SAMDC and polyamine biosynthesis, such as antimitochondrial effects in mammalian cells (Pegg and McCann, 1982; Jänne and Alhonen-Hongisto, 1989; Cheng *et al.*, 1990) or effects RNA synthesis in plants (Altamura *et al.*, 1991).

In plants, MGBG has been shown to perturb *in vitro* growth and development, whilst also altering endogenous polyamine titres. For example, mung bean stem cuttings (Jarvis *et al.*, 1983) carrot cell cultures (Minocha *et al.*, 1990), embryonic cultures of *Picea abies* (Santanen and Simola, 1992), pea seeds (Villaneuva and Huang, 1993), potato tubers (Féray *et al.*, 1994) detached soybean leaves (Turano *et al.*, 1997), tobacco thin cell layers (Altamura *et al.*, 1991; Scaramagli *et al.*, 1999B), and white spruce cultures (Kong *et al.*, 1998) all show alterations in polyamine titres in response to MGBG treatment. In some cases, the negative effe on growth have been shown to be reversible by the addition of spermidine (Altamura *et al.*, 1993; Berta *et al.*, 1997), implying that the major toxic effect is due to polyamine perturbation. This must be interpreted with caution however, since MGBG and spermidine may share an active transport system (Antognoni *et al.*, 1993) resulting in competition for uptake between both compounds. The exogenous application of spermidine therefore may not necessarily involve a direct reversal of the MGBG inhibition (Pegg, 1983).

Altered SAMDC structure

Several studies however, have successfully used MGBG to isolate mutant or variant plant lines with increased resistance, due to its strong depressive effects on polyamine biosynthesis. Malmberg and M'Indoo (1983) reported the isolation of UV-mutagenised tobacco cell lines resistant to 10mM MGBG, that upon regeneration, gave rise to plants producing abnormal flowers. One line displayed anthers instead of ovules, whilst another mutant produced male sterile plants. Resistant cell lines derived from the mutant plants were found to exhibit reduced putrescine-to-spermidine ratios when compared to the wild-type controls. Abnormal flower development however, was not observed in plants regenerated from wild-type callus or from plants originating from non-resistant UV-treated cells plated on MGBG (Malmberg and M'Indoo, 1983). Subsequently, Malmberg and Rose (1987) found that SAMDC enzyme activity in two of the mutant lines, unlike in wild-type, was not inhibited *in vitro* by MGBG, suggesting the presence of a mutation in the coding sequence of the SAMDC gene which may have resulted in resistance to MGBG and an altered floral phenotype.

Increased SAMDC activity

Fritze *et al.* (1995) similarly isolated cells derived from tobacco protoplasts that were resistant to 5mM MGBG. Upon regeneration eight resistant lines were isolated which following selfing exhibited abnormal growth of shoots, branches, and leaves, and had modifications in flower development. After selfing, the altered phenotypes of some were lost in subsequent generations, whereas in others it was heritable and ranged in severity. Two lines with severe phenotypic changes were characterised for SAMDC activity and polyamine levels. One line had an increase in both free and conjugated putrescine and spermidine titres with a concomitant increase in SAMDC activity, whereas the other line showed an increase in SAMDC activity but no increase in polyamine levels. Further characterisation of the resistant lines is underway, in order to assess the modifications in polyamine metabolism.

SAMDC gene amplification

In studies using animal tissue, Regenass *et al.* (1994) derived a CHO cell line resistant to toxic levels of an MGBG analogue, CGP 48664. This compound displays high specificity and potency for SAMDC, reduced antimitochondrial activity, and does not utilise the polyamine transport system. Analysis showed that the resistant cell line had a 1000-fold increased resistance to the inhibitor, suggested to be the result of the overexpression of SAMDC mRNA as a result of gene amplification. To date it is unclear whether this inhibitor is available for plant research.

Polyamine uptake

A study of the malignant phenotype of an adenovirus type 2-transformed rat cell line revealed mutants resistant to MGBG following EMS mutagenesis (Rodrigues *et al.*, 1987). Four MGBG-resistant cell lines were isolated which also showed a reversion of the transformed tumour-inducing phenotype, although the relationship between both phenotypes was unclear. The activities of SAMDC in the presence of a low level of MGBG were found to be similarly induced between control and variant revertant lines, ruling out the possibility that

the resistance was due to increased SAMDC activity, or resistance of SAMDC to the inhibitor. From feeding experiments using [^{14}C]-MGBG it was found that the variants exhibited slower rates of drug uptake than the sensitive parental cells, and this was suggested to be the basis of the resistant phenotype (Porter and Sufrin, 1986). Similarly, Heaton and Flintoff (1988) reported the isolation of CHO cell lines resistant to MGBG based on a reduced capacity for the intracellular accumulation of MGBG. Resistance behaved as a genetically recessive trait, and furthermore, complementation tests of the mutant lines resulted in a restoration of a wild-type MGBG-sensitive phenotype in the hybrids, implying that more than one locus is involved in controlling uptake of the inhibitor.

5·1·4 PUTRESCINE MUTANTS

Davis *et al.* (1990) reported the isolation of a *Neurospora crassa* mutant with the ability to concentrate putrescine from the growth medium. Selection was imposed on *spe-1* mutants which lacks *ODC* and requires putrescine on medium containing 0·05mM putrescine, which normally is too low to support growth. A single colony was isolated with an absolute requirement for polyamines, however, growth was severely inhibited by 5mM putrescine—a level which had almost no effect the *spe-1* mutant strain. The new mutation was subsequently designated as *puu-1* (putrescine uptake). The polyamine transport system of *N. crassa* can take up putrescine, spermidine, and spermine effectively, however, the system can be inhibited by the addition of Ca^{2+} to the growth medium. The *puu-1* mutants exhibited a loss in sensitivity to Ca^{2+}, and thus were able to concentrate putrescine from the media, a process suggested by the authors to involve the existence of a cell-surface protein that normally blocks polyamine uptake when bound to Ca^{2+} (Davis *et al.*, 1990). The mutant was further characterised and found to also concentrate spermidine from the medium (Davis and Ristow, 1991). Two other non-allelic *N. crassa* mutants were isolated that accumulated putrescine; *spe*-2 which contained a mutation in *SAMDC* (Pitkin and Davis, 1990), and *spe-3* which most likely contained a mutation in *SPDS* (Davis and Ristow, 1991). The large accumulation of putrescine in *spe-3* and *puu-1* mutants was shown to be due to sequestration in the vacuole (Davis and Ristow, 1991).

5·1·5 SPERMIDINE AND SPERMINE MUTANTS

Deletion-Insertion Mutants

A mutation in the *Saccharomyces cerevisiae SPE2* gene encoding *SAMDC* was generated through the deletion of an 833bp fragment beginning with the ATG initiation codon representing approximately two-third of the gene (Balasundarum *et al.*, 1991). The disruption of the *SAMDC* gene resulted in cells with no detectable SAMDC activity that lacked spermidine and spermine, and which also possessed morphological abnormalities. The complete dependence on exogenous spermidine and spermine for growth, and the structural abnormalities were only observed when the cultures were grown aerobically. Under anaerobic conditions the mutant grew and appeared as wild-type cells under similar conditions, however, SAMDC activity was not observed and spermidine and spermine were still absent, suggesting that the polyamine-depleted mutants were more sensitive to oxygen damage than wild-type cells. Further characterisation revealed that the mutant lacked the ability to use glycerol (GLY$^-$) as the sole carbon source when grown aerobically on polyamine-free media, whereas this function was present when the media was supplemented with spermidine (Balasundarum *et al.* 1993). From crosses with *SPE2*$^+$GLY$^+$ strains, it was observed that the GLY$^-$ phenotype was the result of an additional recessive mitochondrial mutation. Aerobically-grown mutant cells exhibited a permanent loss of mitochondrial function, suggesting that polyamines act in part, by preventing oxidative damage (Balasundarum *et al.* 1993).

Since polyamines have been implicated in a number of cellular roles, including protein synthesis, the *SPE2* mutant of *S. cerevisiae* has also been used to study the roles of spermidine in the control of translational fidelity (Balasundaram *et al.*, 1994). To this end, Balasundaram *et al.* (1994) used an *in vivo* assay in which changes in β-galactosidase activity are dependent upon a -1 or +1 ribosomal frameshift signal induced by the presence of the L-A RNA virus of yeast or the Ty*1* yeast retrotransposable element (Belcourt and Farabaugh, 1990; Dinman *et al.*, 1991). It was noted that spermidine depletion was found to

increase the efficiency of the +1 frameshift but not the -1 frameshift, and to decrease the frequency of Ty1 transposition (Balasundaram *et al.*, 1994). The latter was not due to an inability for cellular growth, rather it suggested that the increased capacity for frameshift generation altered the ratio of fusion protein production, which in turn interfered with the assembly of virus particles. Rom and Kahana (1994) also demonstrated the importance of polyamines in inducing frameshifts, using a mammalian cellular gene encoding ODC antizyme which is the protein that moderates the rate of ODC degradation. Here, spermidine was found to induce expression of ODC antizyme by enabling the ribosomes to shift from one reading frame to another. Low concentrations of polyamines are inferred to prematurely stop translation of antizyme mRNA, whereas at higher concentrations, the +1 frameshift is induced to generate a functional antizyme protein, thereby mediating rapid feedback inhibition to maintain relatively constant polyamine levels.

Resistance To Spermine

In plant studies, 14 mutants of *Arabidopsis* were identified on the basis of their capacity for germination in the presence of a normally toxic level of spermine (Mirza and Iqbal, 1997). Possessing similar developmental abnormalities to the independent MGBG-resistant tobacco mutants studied by Malmberg and co-workers in the 1980s, and by Fritze *et al.* (1995), these spermine-resistant *Arabidopsis* plants exhibited floral abnormalities including larger flowers, altered floral organ numbers, and malformed siliques. The spermine resistance in two lines was further characterised and attributed to single recessive nuclear mutations which were allelic. The mutation did not affect responses to putrescine and spermidine, as mutants and wild-type controls were equally sensitive to increasing concentrations of these polyamines (Mirza and Iqbal, 1997). Interestingly, both mutant lines exhibited a slower germination rate than wild-type controls in the presence of spermine, negating the possibility that resistance was due to faster germination of the mutant seeds.

5·1·6 AIMS OF THIS STUDY

In order to further analyse the regulatory mechanisms that are involved in polyamine biosynthesis and metabolism in *Arabidopsis*, the present study sought to isolate plants containing mutations in the polyamine pathway. The strategy involved the identification of putative mutants on the basis of growth in the presence of normally toxic levels of polyamines, polyamine precursors, or polyamine inhibitors. If so identified, the resistant plants were to be further characterised morphologically and at the biochemical level by measurements of total endogenous polyamine titres. Studies at the molecular level would also be undertaken via Northern analyses using cloned gene fragments of available polyamine biosynthetic genes.

5·2 RESULTS

5·2·1 DEVELOPMENT OF A SCREENING METHODOLOGY

Greenhouse trials

One of the simplest ways to identify variant or mutant plants within a population is to grow the plants under greenhouse conditions. To assess the capacity for the identification of polyamine mutants in *Arabidopsis thaliana* plants grown in compost, a trial using wild-type seeds of the Landsberg *erecta* ecotype was performed. Following germination in soil soaked with nutrient solution, seedlings were regularly sprayed with nutrient solution containing the SPDS inhibitor, CHA, whilst control plants were sprayed with nutrient solution only. This inhibitor is readily available commercially and had previously been shown to be to inhibitory to *in vitro* plant growth whilst also perturbing polyamine levels *in vivo* (see Chapter 3).

Topical spraying of the inhibitor onto seedlings was chosen over direct wetting of the soil, as it was thought that this approach would minimise possible problems of inhibitor breakdown in the soil. It was anticipated that putative mutants would be readily identified on the basis of height, since sensitive plants were expected to be reduced in stature. After several weeks of regular spraying with the inhibitor at levels up to 10mM, however, there was little distinction in height between the control and the inhibitor-treated plants. The fact that under *in vitro* growth conditions, CHA was inhibitory at levels of approximately 5mM, yet in soil 10mM was found to have little effect on growth, may be attributable to the degradation of the inhibitor under the different growth environment, and thus, higher levels of CHA were not trialed.

In vitro trials

Based on the above observations, it was decided that the screening of plants grown *in vitro* in the presence of polyamines or polyamine inhibitors, may be a more effective, albeit a more

laborious method for screening for mutants. Previous results from Chapter 3 showed that both polyamines and their inhibitors could be effectively taken up from the growth media, altering the *in vivo* levels of polyamines, and causing phenotypic changes.

As has been noted, ODC and SAMDC are two rate-limiting enzymes in the polyamine biosynthetic pathway in animal cells. In *Arabidopsis* in particular, ADC may also be a key enzyme in putrescine biosynthesis. To determine suitable screening agents for larger mutant hunts, dose-response experiments were initially performed using the polyamine precursors arginine and ornithine, the diamine putrescine, the SAMDC inhibitor MGBG, and the SPDS inhibitor CHA. Figure 5·2 shows wild-type plants grown on MS media containing these additives at a range of concentrations; from 0·1mM to 20mM. Although all concentrations were tested for MGBG, only the 5mM plate is shown, as this was the level found to be toxic to the early stages of growth. Similarly, CHA was also inhibitory at 5mM. Arginine and putrescine were slightly inhibitory at 20mM but were not considered toxic, whereas 10mM ornithine inhibited the rate of growth. A summary of the set of primary mutant screens is listed in Table 5·1.

Mutagen	Selection Agent	Selection level used	No. of M2 seed screened
EMS	CHA	5mM	15,000
EMS	Put	20mM	20,000
EMS	MGBG	5mM	30,000
GAMMA	Orn	10mM	30,000

Table 5·1: Primary screens for polyamine mutants.

A method for easily visualising putative resistant mutants was investigated in which the Petri dishes were orientated vertically so that roots of seedlings could grow down along the surface of the media. It was anticipated that this method would allow the rapid identification of resistant plants from sensitive ones, based on primary root length in the presence of normally toxic levels of the growth regulator. Similar methods have been reported

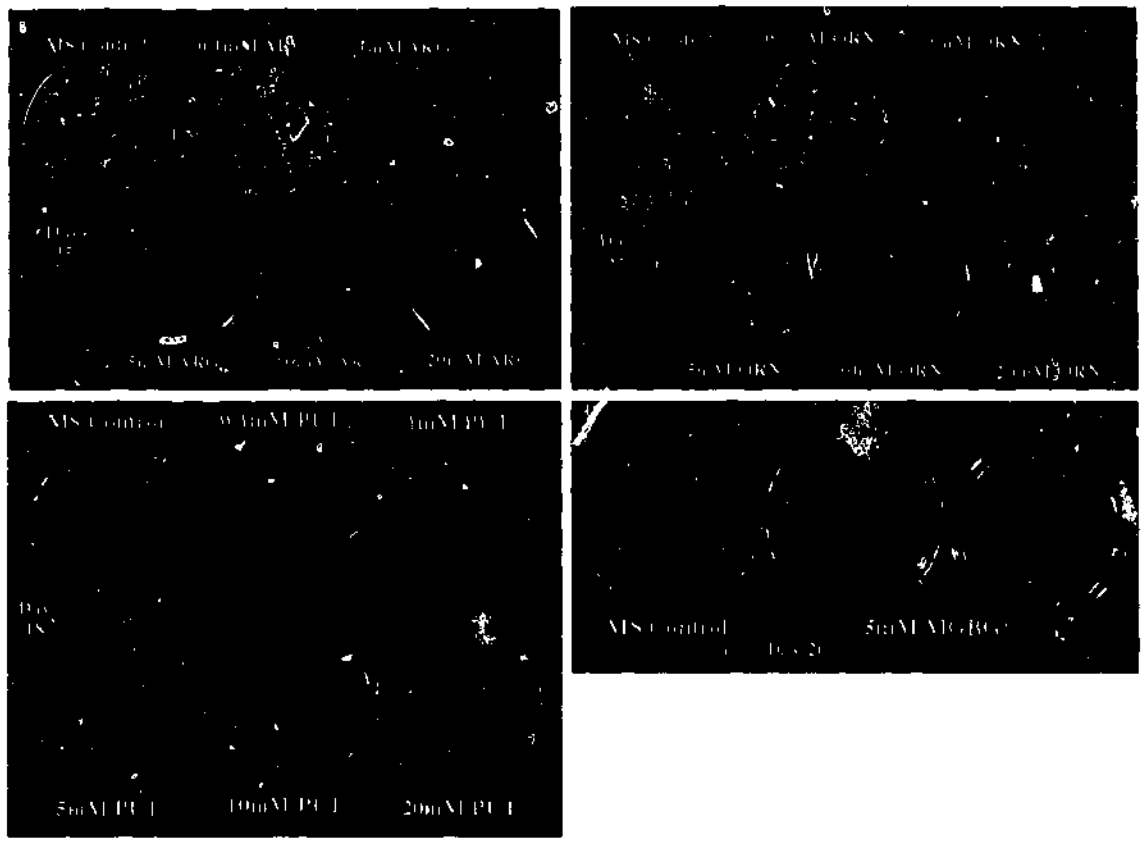


Figure 5.2: *In vitro* growth trials to determine the appropriate levels of growth regulators to allow a mutant screen.

Wild-type L*er* seeds were germinated and grown on varying levels of arginine, ornithine, putrescine, and MGBG. Arginine and putrescine did not effectively cause inhibition of growth except at a concentration of 20mM. Lower concentrations of ornithine (10mM), CHA (5mM) (not shown), and MGBG (5mM) were sufficiently toxic to allow a further screen to be contemplated.

Figure 5.3: Vertical growth of wild-type L*er* seedlings on MS media supplemented with 2mM MGBG.

Plates were orientated vertically to allow the roots to grow along the surface of the media. It is evident however, that this method provided much variation in root growth, hence precluding further use.

previously for the recovery or several *Arabidopsis* mutants possessing altered phytohormone metabolism (Estelle and Somerville, 1987; Bell and Maher, 1990; Su and Howell, 1992; Simmons *et al.*, 1995). From preliminary experiments, however, when wild-type seedlings were grown in this manner on media supplemented with 2mM MGBG which is normally inhibitory, a uniform inhibition of root growth was not observed (Figure 5·3). Thus, this method of screening was not deemed stringent enough to select mutants reproducibly. When the plates were aligned horizontally, allowing the roots to grow into the media and come into direct contact with the selective agent, 2mM MGBG was inhibitory to growth. This eliminated the problem of variation in root growth and was therefore used in subsequent *in vitro* mutant screening experiments.

5·2·2 PRIMARY ORNITHINE SCREEN

Screening for mutant plants resistant to 10mM ornithine was performed using 30,000 gamma-irradiated M2 Landsberg *erecta* seed germinated and grown on horizontally-orientated plates. Putative resistant mutants were selected on the basis of possessing green leaves, and were transferred after 32 days of growth to MS medium supplemented with a slightly lower level of ornithine (7·5mM). This was aimed at reducing the toxic effects of ornithine, thus allowing further growth and seed set, whilst still maintaining selective pressure. A comparison of phenotypes of treated wild-type control and mutagenised seedlings is presented in Figure 5·4. Most wild-type control plants exhibited sensitivity and displayed necrotic lesions or chlorosis of the leaves on plates containing 10mM ornithine. A higher proportion of the mutagenised individuals were more tolerant to ornithine than wild-type plants, and exhibited a larger stature and also greener leaves than controls. Several putative ornithine-resistant mutants also showed strong root growth in the presence of 7·5mM ornithine relative to controls, but only in seven cases underwent flowering and set seed. Progeny seed were re-screened on media containing higher levels of ornithine and results are presented in Tables 5·2, 5·3, and 5·4 below.

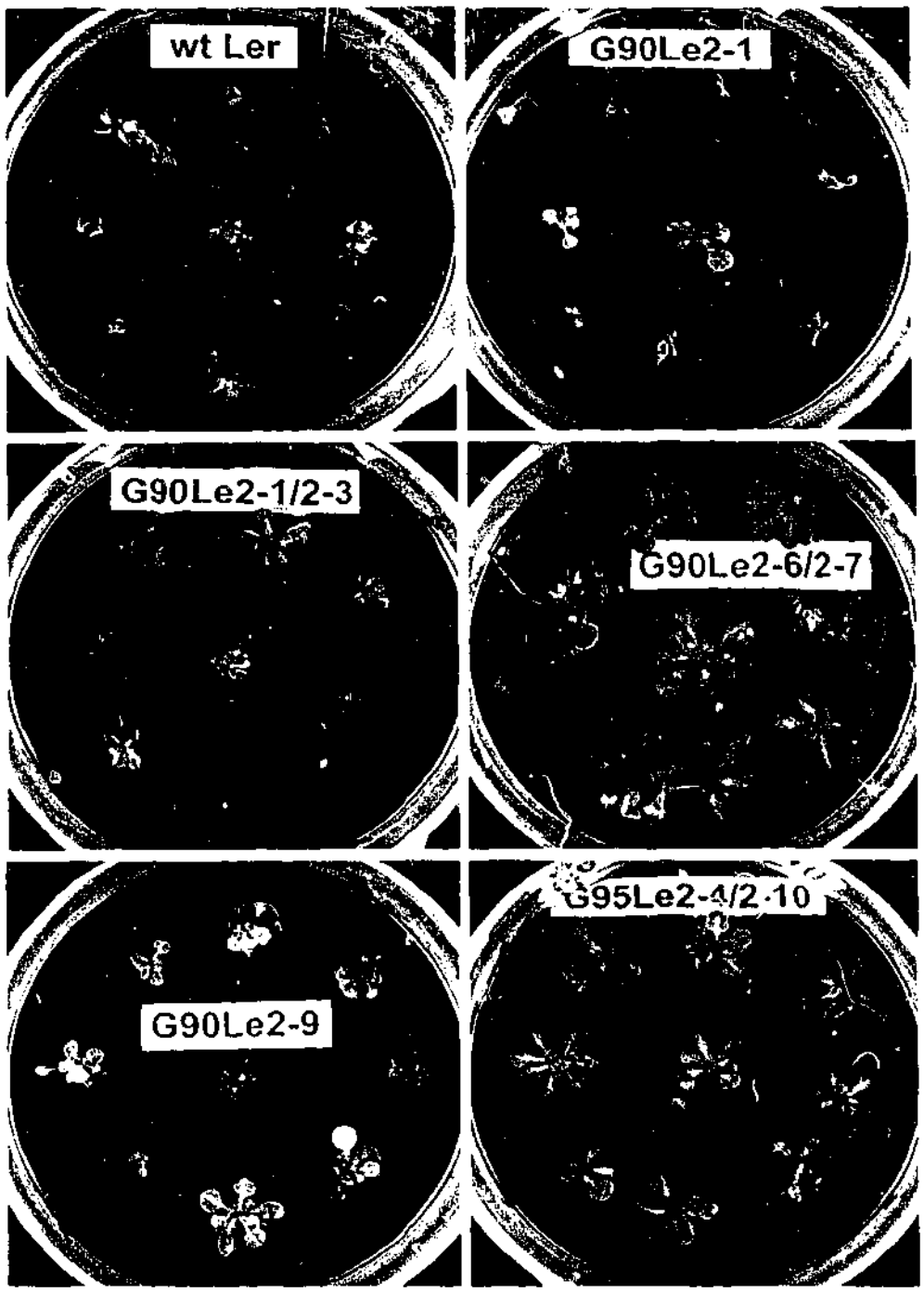


Figure 5.4: Growth of wild-type L*er* and mutagenised L*er* seedlings on MS media supplemented with 7.5mM ornithine.

The primary mutant screen was performed on MS media supplemented with 10mM ornithine. After 32 days of growth, selected individuals from each seed batch were transferred to the lower level of ornithine (7.5mM). This was in order to maintain selective pressure whilst still allowing growth to progress to flowering without being detrimental to seed set. G90 and G95 represent seed batchs derived from exposure to 90krads and 95krads of gamma radiation respectively. Individual pools of seed were designated Le2-1, Le2-2, Le2-3, etc.

Line	% non-germinated	% cotyledon	% 2-leaf	% 4-leaf	% 6-leaf	% 8-leaf
wt *Ler*	-	16·0	40·0	44·0	-	-
wt *Ler* (ORN*)	100·0	-	-	-	-	-
Gamma-2	100·0	-	-	-	-	-
Gamma-6	100·0	-	-	-	-	-
Gamma-8	80·3	12·5	7·2	-	-	-
Gamma-12	100·0	-	-	-	-	-

*'Pre-treated' wt *Ler* seed obtained from non-mutagenised parent plants grown in the presence of 8mM Orn.

Table 5·2: Re-screening of M3 seed on 8mM ornithine.

Line	% non-germinated	% cotyledon	% 2-leaf	% 4-leaf	% 6-leaf	% 8-leaf
wt *Ler*	-	46·2	46·2	7·7	-	-
wt *Ler* (ORN*)	96·2	3·8	-	-	-	-
Gamma-2	100·0	-	-	-	-	-
Gamma-6	100·0	-	-	-	-	-
Gamma·7	100·0	-	-	-	-	-
Gamma-8	88·0	12·0	-	-	-	-
Gamma-10	100·0	-	-	-	-	-
Gamma-11	100·0	-	-	-	-	-
Gamma-12	100·0	-	-	-	-	-

*'Pre-treated' wt *Ler* seed obtained from non-mutagenised parent plants grown in the presence of 10mM Orn.

Table 5·3: Re-screening of M3 seed on 10mM ornithine.

Line	% non-germinated	% cotyledon	% 2-leaf	% 4-leaf	% 6-leaf	% 8-leaf
wt *Ler*	-	23·1	65·5	3·8	3·8	3·8
wt *Ler* (ORN*)	100·0	-	-	-	-	-
Gamma-2	100·0	-	-	-	-	-
Gamma-6	100·0	-	-	-	-	-
Gamma-8	81·5	18·5	-	-	-	-
Gamma-10	100·0	-	-	-	-	-
Gamma-11	100·0	-	-	-	-	-
Gamma-12	100·0	-	-	-	-	-

*'Pre-treated' wt *Ler* seed obtained from non-mutagenised parent plants grown in the presence of 12mM Orn.

Table 5·4: Re-screening of M3 seed on 12mM ornithine.

M3 seeds were collected from putative 10mM Orn-resistant plants and re-screened on media containing 8mM, 10mM, or 12mM Orn. Wild-type Ler seeds were used as controls, and a further control of 'pre-treated' wild-type seeds was used in order to determine if previous exposure to Orn contributed to the resistant phenotype

Subsequent characterisation of the putative ornithine-resistant variants, however, was prevented due to poor germination of the M3 seed, and inability of those that did germinate to grow beyond the cotyledon or 2-leaf stage.

5·2·3 PRIMARY MGBG SCREEN

Isolation of MGBG-resistant plants

M2 seeds from mutagenised L. *erecta* were collected from approximately 1,200 M1 plants that were pooled into 60 groups of 20 plants each. 30,000 M2 seed from a total of 12 M1 pools, were used in the first primary screen on MS medium containing 5mM MGBG. Putative MGBG-resistant variants were identified on the basis of increased shoot growth, and resistance was characterised by the presence of large, green cotyledons and formation of true leaves. MGBG-sensitivity on the other hand, was defined by the presence of small, necrotic and chlorosed cotyledons and no true leaves. Following identification, putative mutants were grouped into two categories, designated as type (i) and type (ii). Type (i) plants exhibited a strong resistant phenotype and were distinctly healthier than treated wild-type control seedlings on all other Petri dishes. Seedlings of type (ii) exhibited a weaker resistance phenotype, but typically, were healthier and larger than other seedlings within the same dish.

The results of the first primary screen are presented in Table 5·5. Only three putative type (i) mutants were found, all derived from EMS M2 seed pool #2, raising the possibility that they were siblings. Thus each plant was isolated and labelled as sub-lines 2(i)a, 2(i)b, and 2(i)c. All three type (i) putative variants flowered *in vitro* and set seed, moderate levels which were collected and stored separately for subsequent characterisation. In addition, 12 putative mutants of type (ii) phenotype were isolated from six other M2 seed pools (Table 5·5). All these plants flowered *in vitro*, however, those from pools #1, #3, and #10 did not produce seed, whilst those from pools #6, #9, and #11 produced very low amounts of viable seed.

Phenotype of MGBG-resistant plants

Following the primary *in vitro* screen on 5mM, all putative mutants were removed from the selective media after 14 days of growth and transferred to soil for self-pollination and seed set (Figure 5·5, panels B to G). Occasionally, severely stunted wild-type plants survived the

in vitro screening, and were transferred to soil along with the putative resistant variants (Figure 5·5, panel A). No phenotypic differences were observed at this stage between treated wild-types and putative MGBG resistant plants. The phenotypes of all treated plants were consistent with the effects of MGBG treatment, with all individuals displayed an overall stunted and bushier shoot morphology, with individual inflorescences also appearing relatively spindly (Figure 5·5, panels A to G). The flowers possessed normal organ number and positioning, however, several siliques from putative mutant plants were small and withered, and lacked seed.

M2 seed pool	No. of putative mutants isolated: Type (i)	Type (ii)	Fertility (*in vitro*)
#1	-	1	sterile
#2	3	-	set seed
#3	-	3	sterile
#4	-	-	-
#5	-	-	-
#6	-	2	set seed
#7	-	-	-
#8	-	-	-
#9	-	2	set seed
#10	-	1	sterile
#11	-	3	set seed
#12	-	-	-

Table 5·5: Primary *in vitro* selection on MS media + 5mM MGBG

15 putative mutants were isolated from the first primary screen of 30,000 M2 seeds on MGBG. The 30,000 M2 seed were screened in 12 separate batches of approximately 2,500 seed. Approximately 400 seed were sown per plate (12cm x 12cm). Three individuals from M2 seed pool #2 had a strong resistance phenotype [Type (i)], and the remaining 12 displayed a weaker phenotype [Type (ii)]. Putative mutants representing four of the 12 families screened exhibited fertility.

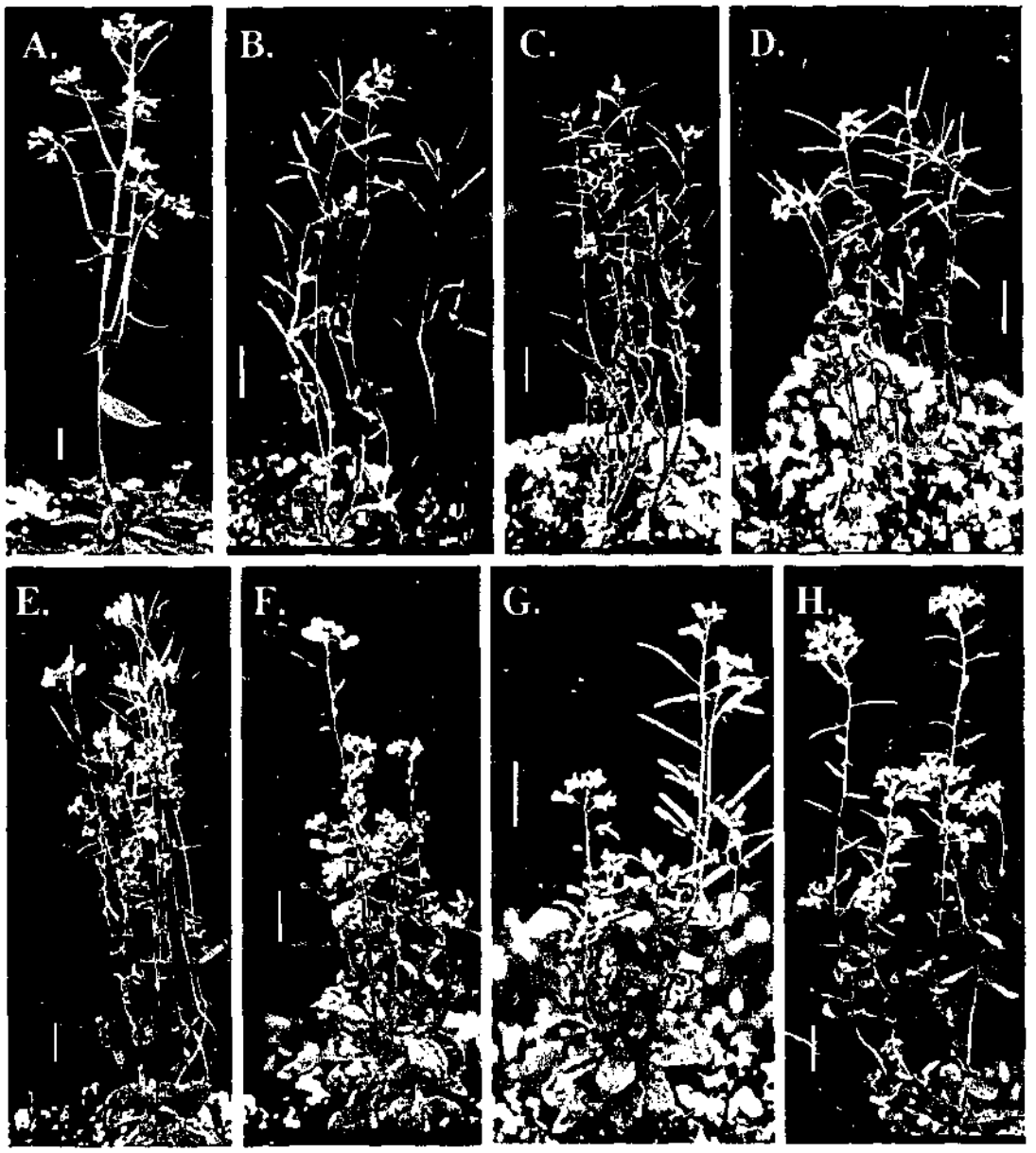


Figure 5.5: Putative MGBG^R mutants after transfer to soil.

Approximately 30,000 EMS-mutagenised M2 generation seed and wild-type Landsberg *erecta* controls were sown *in vitro* on solid MS media supplemented with 5mM MGBG. After 14 days of selection, putative resistant seedlings from the EMS population were transferred to soil for seed set. In order to rescue plants from the wild-type population after the selection, several of these were also transferred to soil, with the aim of using their progeny as subsequent controls when determining the heritability of the resistant phenotype. All treated plants exhibited an overall morphology characteristic of MGBG application; a stunted shoot with several axillary inflorescences. The bar in each panel represents 1cm.

A. Untreated non-mutagenised wt control, **B.** Non-mutagenised wt control treated with 5mM MGBG, **C.** EMS 2(i)a, **D.** EMS 2(i)b **E.** EMS 2(i)c, **F.** EMS 6(ii), **G.** EMS 9(ii), **H.** EMS 11(ii).

5·2·4 HERITABILITY OF THE MGBG-RESISTANT PHENOTYPE

In vitro growth

Following self-fertilisation after growth in soil, seeds were collected from the putative mutant plants and re-screened on MS media containing 5mM MGBG to test for the heritability of the resistant trait (Figure 5·6). Although no resistant wild-type plants similar to class (i) or (ii) variants were observed from the original screen, the largest surviving individuals for wild-type were removed from the selective media and transferred to soil. Seed collected from such 'MGBG-pre-treated' wild-type plants were to be used in subsequent heritability studies to provide evidence that the resistant phenotype of the mutants was not merely due to exposure to the inhibitor in preceeding generations. Such 'pre-treated' wild-type seedlings always displayed sensitivity to MGBG at the cotyledon stage (Figure 5·6, panel B), indicating that several generations of exposure to MGBG conferred no benefit in establishing a resistant phenotype. The heritability of the resistant trait was observed in the progeny of the type (i) putative mutants, which showed several seedlings with elevated tolerance to the inhibitor. Several seedlings from the mutant lines were bigger than wild-type seedlings, and only the biggest, most developed variants were selected from EMS 2(i) lines for further analysis. Inspection of a resistant seedling (Figure 5·6) revealed the developmental stage to be 6- to 8-leaves after 26 days exposure, which was the same as the untreated wild-type plants (Figure 5·6, panel A), although the former were much smaller in stature. In contrast, sensitive seedlings were severely chlorosed and remained at the cotyledon or 2-leaf stage (Figure 5·6, panel F)

After 14 days exposure to 5mM MGBG, seedlings were transferred to inhibitor-free media and allowed to grow for a further seven weeks *in vitro*. Figure 5·7 shows a comparison of the resistant progeny plants from more than one putative mutant with the sole surviving sensitive wild-type plant germinated on MGBG. Progeny from lines 2(i)a, 2(i)b, and 2(i)c clearly exhibited the inheritance of the resistant phenotype (panels A, B, and C respectively) as they were markedly larger than the control individual and produced several flowers and

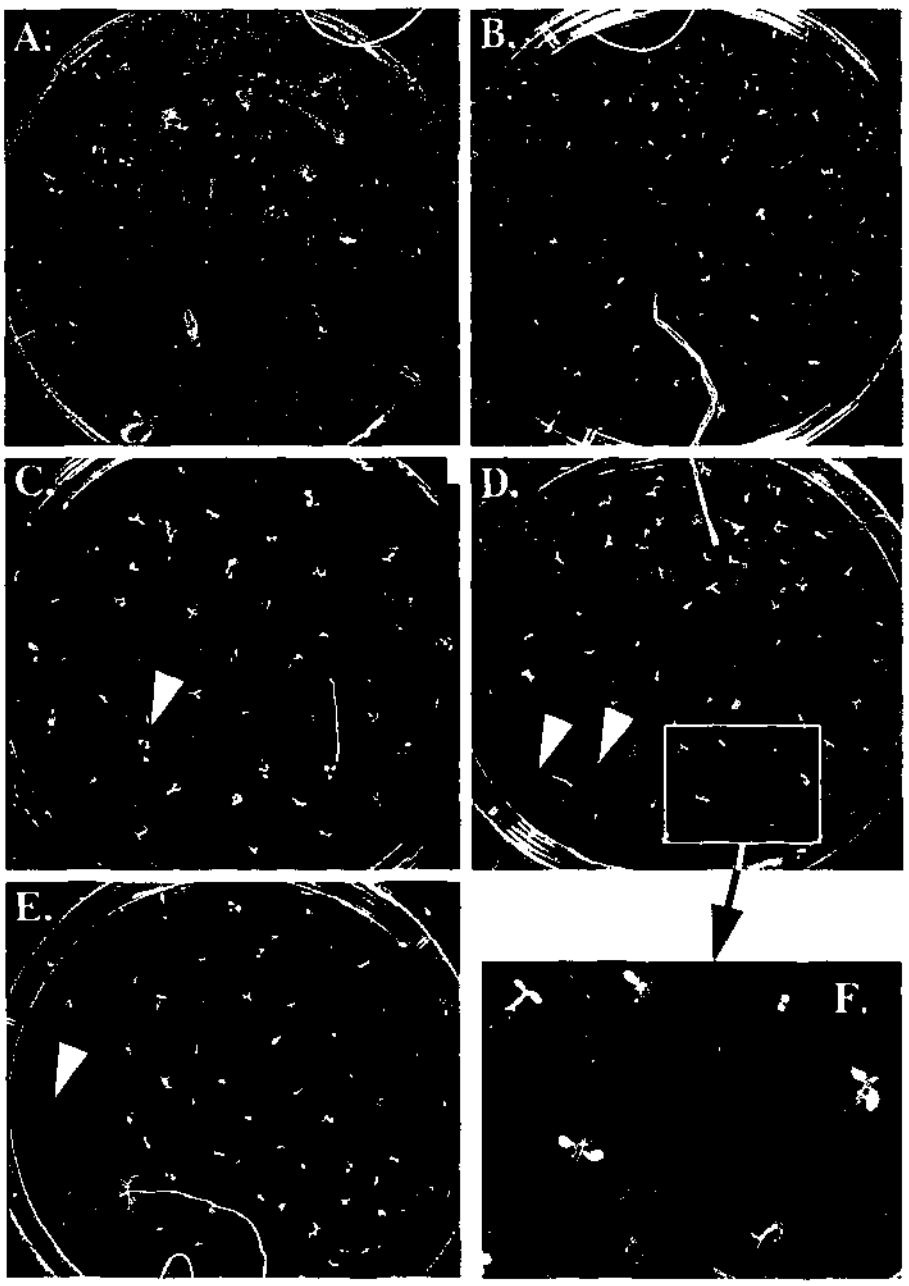


Figure 5.6: Inheritance of the MGBG^R phenotype after self-fertilisation.

Seedlings were germinated and grown on MS media supplemented with 5mM MGBG as appropriate (Day 26 of growth shown).
Some examples of resistant seedlings are indicated with arrows.
A. Wild-type L*er* on unsupplemented MS control media
B. Wild-type L*er*; third generation after exposure to MGBG
C. EMS 2(i)a
D. EMS 2(i)b
E. EMS 2(i)c
F. Magnified view of a resistant seedling compared to surrounding sensitive seedlings.

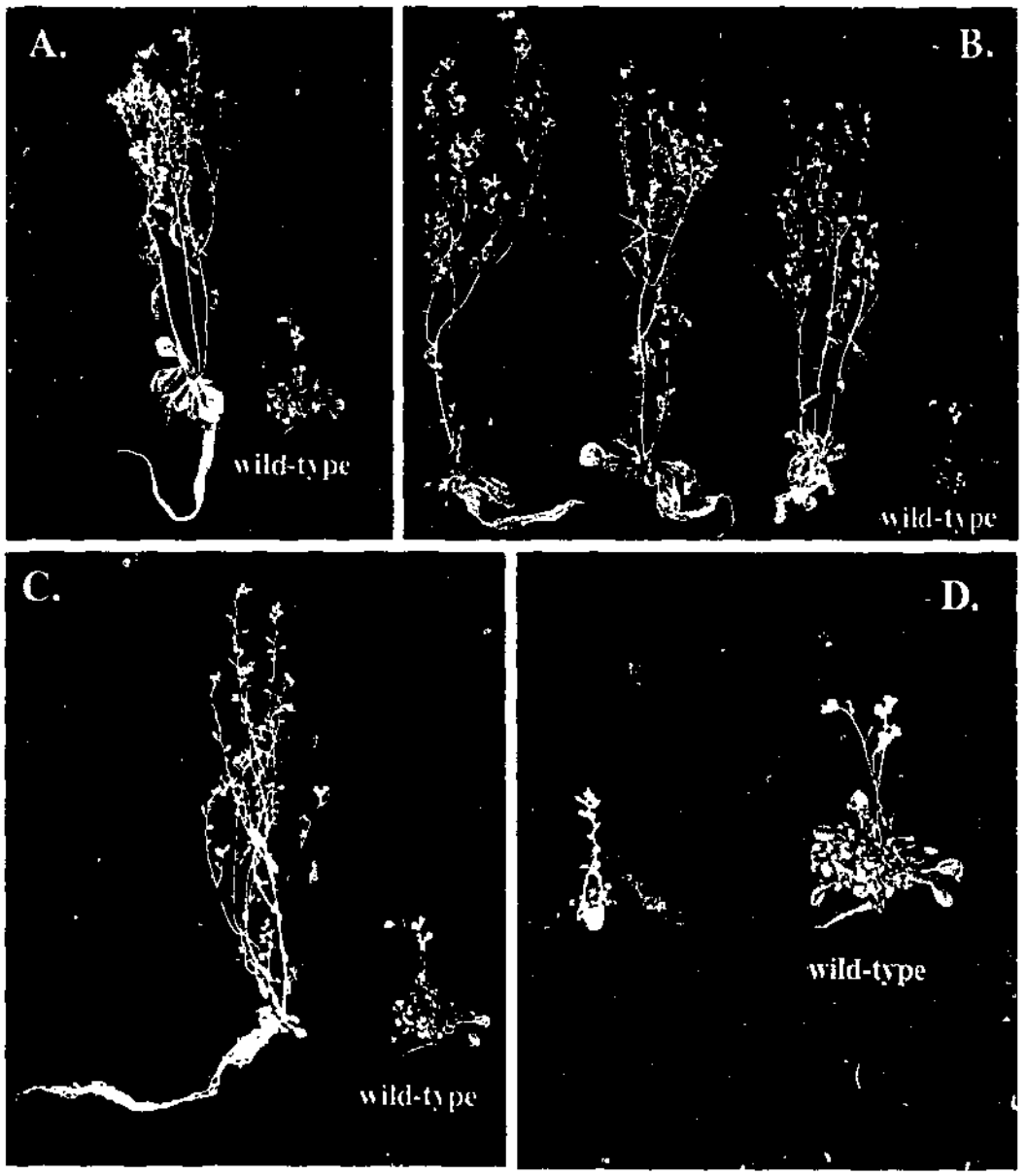


Figure 5.7: Comparison of *in vitro* growth of progeny plants from putative $MGBG^R$ mutants and wild-type controls following initial treatment with 5mM MGBG.

Plants were grown on media containing MGBG for 14 days before transfer to inhibitor-free media. Week 9 of growth shown.

A. EMS 2(i)a vs. wild-type
B. EMS 2(i)b vs. wild-type
C. EMS 2(i)c vs. wild-type
D. EMS 11(ii) vs. wild-type

siliques. From line 11(ii) only one individual was produced that initially appeared to be resistant to MGBG. After further growth, however, the phenotype of this plant showed it to be severely stunted (panel D), and it was subsequently found to be sterile, and no further analysis was undertaken. A similar situation was observed for lines 6(ii) and 9(ii), and details are not presented here.

Soil Growth

After selection *in vitro* on 7mM MGBG for 21 days, putative mutants and surviving wild-type seedlings were transferred to soil for further growth. Representative individuals are presented in Figure 5·8. Soil-grown wild-type plants demonstrated a stunted shoot phenotype characteristic of MGBG treatment (panel A) which ranged in height from approximately 4cm to 10cm as expected for wild-types under these conditions. The putative mutant lines (panels C, D, and E from the M3, M4, and M5 generations respectively) however, were again consistently and markedly larger than the wild-type individuals, and at flowering, were typically 20cm and 25cm in height from soil. In terms of floral morphology, no changes in the organ number or organ position, were evident in any mutants, whilst the flowers of line 2(i)a were distinctly larger than those of the wild-type (Figure 5·8, panel B). The uniform resistant phenotypes of the putative mutant plants compared to the sensitive wild-types following growth either *in vitro* or in soil, confirms the inheritance of the MGBG-resistant trait.

5·2·5 INBREEDING OF THE MGBG-RESISTANT PUTATIVE MUTANTS

After the M2 MGBG-resistant mutants were selfed, seeds were subjected to a screen on medium containing 5mM MGBG (shown in Figure 5·6). Unexpectedly however, the frequency of the resistant phenotype was low in the M3 generation for all lines, although still observable. In an attempt therefore to increase the heritability of the resistant trait, the progeny seeds following self-fertilisation were continually selected on MGBG for several generations. Table 5·6 presents the resistant percentages when the putative mutant lines

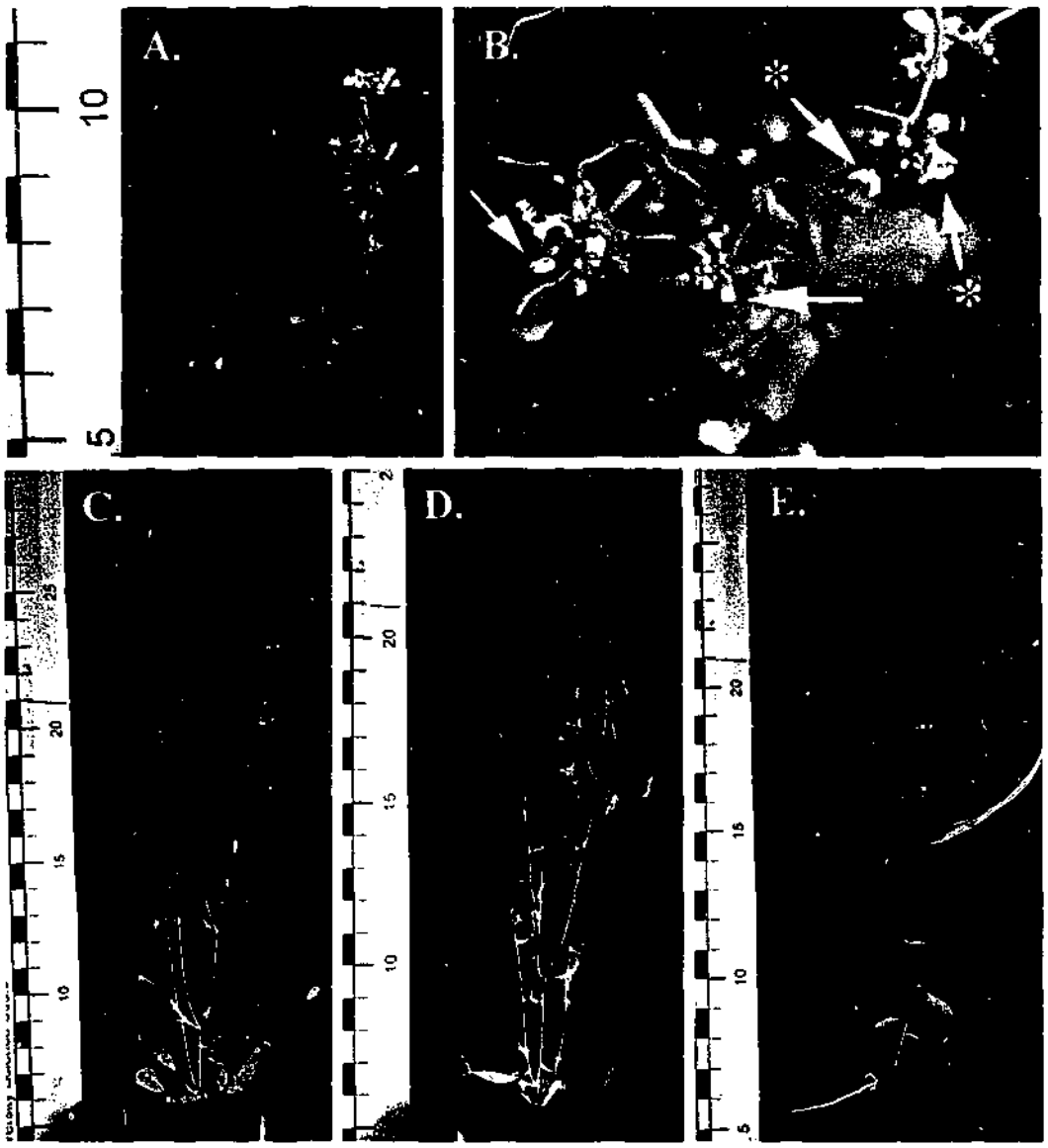


Figure 5.8: Growth of line 2(i) putative mutants in soil following *in vitro* selection on media containing 7mM MGBG for 21 days.

Resistant plants are larger in stature than the sensitive wild-type control, and the flowers from the 2(i)a plant are also larger than controls. A centimetre scale is included to guage inflorescence height

A. Wild-type L*er* (~4cm in height from soil)
B. Magnified view comparing flower sizes from the wild-type L*er* (arrows only) and the 2(i)a mutant (asterisks)
C. $MGBG^R$ 2(i)a (~25cm in height from soil)
D. $MGBG^R$ 2(i)b (~20cm in height from soil)
E. $MGBG^R$ 2(i)c (~20cm in height from soil)

were germinated on media containing 5mM MGBG and 7mM MGBG and scored at day 25 of growth.

MGBG Level	Line and Generation Screened			
	wt Ler	2(i)a	2(i)b	2(i)c
5mM	0 [217]	10% (M3) [405]	1·5% (M3) [196]	27% (M3) [186]
	0 [223]	49% (M4) [127]	13% (M4) [276]	38% (M5) [116]
	0 [72]		18%[a] (M5) [17]	
7mM	0 [109]	53% (M4) [138]	42%[b] (M5) [12]	74% (M5) [125]

Table 5·6: Re-screening putative mutants on 5mM MGBG and 7mM MGBG over several generations (% resistance shown, number of germinating seeds are indicated in square brackets).

The resistance frequency of the 2(i) lines was found to increase under the continual selective pressure. On 5mM MGBG, line 2(i)a showed 49% resistance at the M4 generation, 2(i)b had 18% resistance (though reduced germination) at the M5 generation, and 2(i)c displayed 38% resistance also at the M5 generation. On 7mM MGBG, the resistance frequency increased; 2(i)a was 53% by M4, whilst 2(i)b showed 42% by M4, and 2(i)c was 74% by M4 (Table 5·7). The latter observation was somewhat unexpected and may have been associated with precipitation of some of the MGBG, observed in 7mM plates, and previously reported to be due to interaction with salts (Fritze *et al.*, 1995)

To ensure that false positive resistant phenotypes were not scored on media in which MGBG may have precipitated, seeds were sown on both full-strength and reduced-strength MS media containing 7mM MGBG (Table 5·7). Plants reaching the cotyledon stage of growth were scored as resistant, and were only observed on the full-strength MS media. No resistant plants were seen on the half-strength or quarter-strength MS plates, suggesting that the reduced nutrient supply may adversely affect the growth of the already compromised and stressed putative mutants. The fact that 6% of wild-type were scored as 'resistant' on full

strength medium whereas zero resistance was observed at 5mM does suggest precipitation of MGBG at 7mM effectively reduces the concentration in the medium to levels below that produced by 5mM.

Line	1 x MS	$^1/_2$ x MS	$^1/_4$ x MS
wt *Ler*	6%	0%	0%
MGBG^R 2(i)a·1	51%	0%	0%
MGBG^R 2(i)a·2	58%	0%	0%
MGBG^R 2(i)a·3	27%	0%	0%

Table 5·7: Re-screening putative mutants on reduced-strength MS medium supplemented with 7mM MGBG.

Percentages shown represent the proportion of plants at the cotyledon stage of growth. Putative mutant seed screened were from the fourth generation following continual selection on MGBG followed by self-fertilisation. Wild-type seed were able to germinate on unsupplemented 1/2 x MS and 1/4 x MS media.

5·2·6 CHARACTERISATION OF THE MUTANT PHENOTYPE

I: DETAILED *IN VITRO* GERMINATION STUDY

Seeds were collected separately from 12 plants (designated hereafter as sub-lines 1 to 12), from each of the two lines showing the highest frequency of MGBG tolerance, 2(i)a and 2(i)c, on media containing MGBG (Table 5·6). A detailed study of the germination and growth of the seedlings from each sub-line was performed on MS media containing MGBG at levels of 2mM, 3mM, and 4mM, in order to ascertain whether the resistant phenotype was more clearly apparent on the lower levels of inhibitor. Seedlings were assessed for sensitivity to MGBG in the following categories: non-germinating; radicle only; hypocotyl only; radicle and unexpanded cotyledon; root and unexpanded cotyledon; unexpanded cotyledon only; and chlorotic cotyledon only. Several resistant categories were also devised; green unexpanded cotyledon; one green cotyledon; two green cotyledons; 2-green leaf; 4-green leaf; 6-green leaf; and 8-green leaf. The 2mM MGBG characterisation results are presented as pie graphs in Figures 5·9 and 5.10. Similar characterisation was also

LINE 2(i)a grown on media containing 2mM MGBG

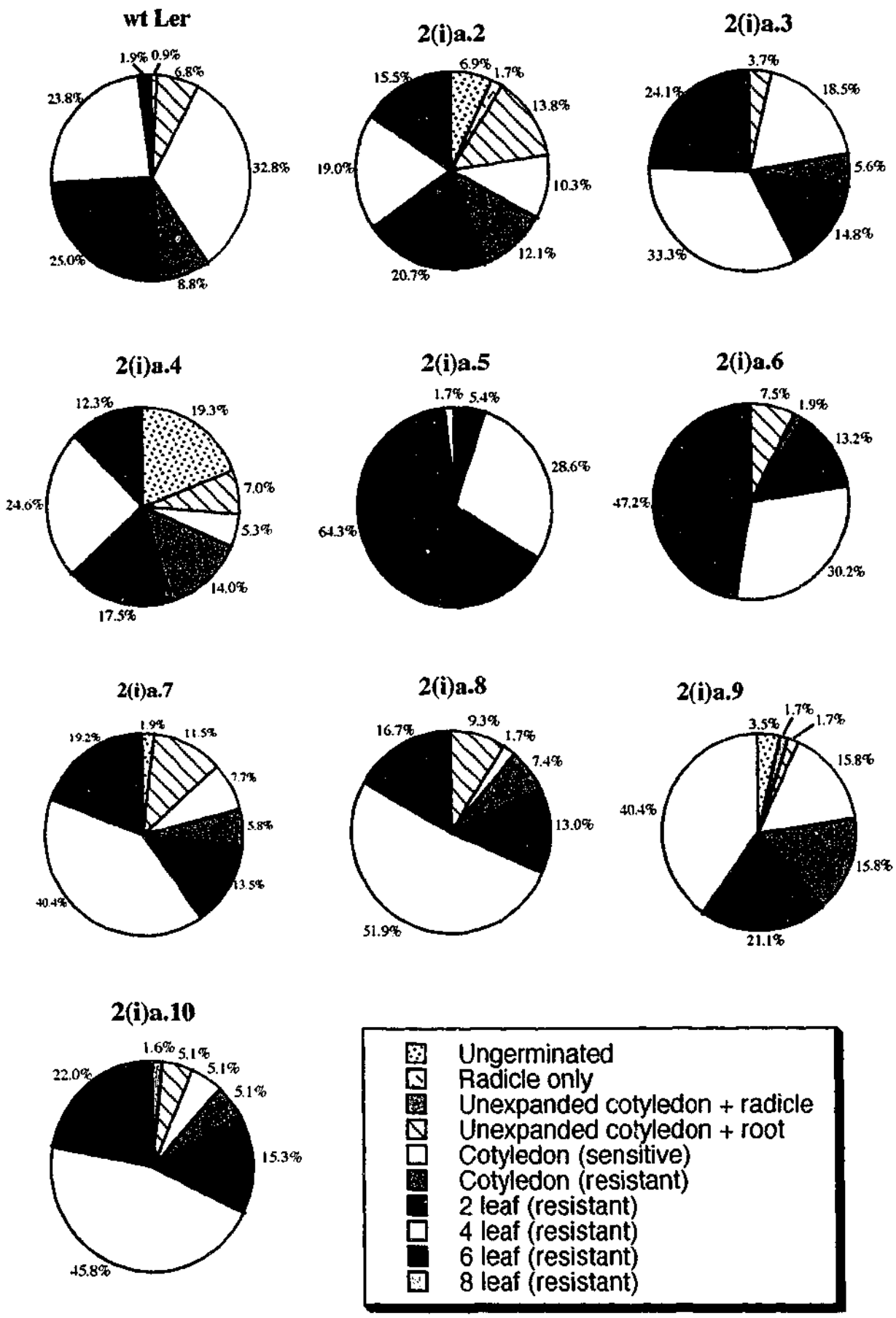


Figure 5.9: Detailed resistance study of line 2(i)a individuals grown on MS media supplemented with 2mM MGBG. Day 18 of growth.

The percentage of plants reaching various developmental stages are shown.

LINE 2(i)c grown on media containing 2mM MGBG

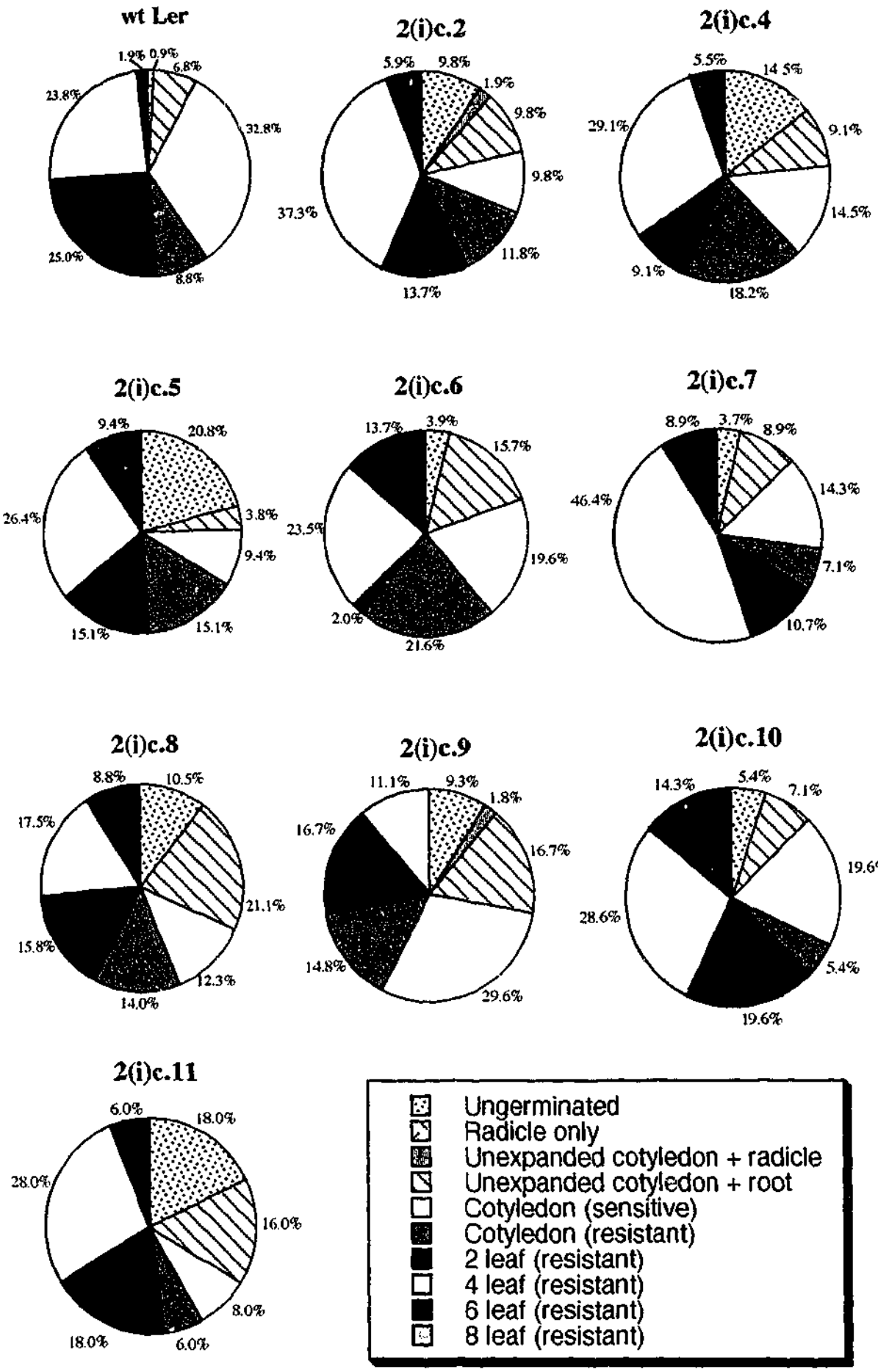


Figure 5.10: Detailed resistance study of line 2(i)c individuals grown on MS media supplemented with 2mM MGBG. Day 18 of growth.

The percentage of plants reaching various developmental stages are shown.

performed on seedlings germinated in the presence of 3mM MGBG and 4mM MGBG with similar trends evident, and are presented in Appendices 10 to 13.

Germination in the presence of 2mM MGBG

Though some sensitive seedlings were observed in each sub-line, the overall observation from the 2mM data presented in Figures 5·9 and 5·10 is that individual sub-lines of 2(i)a display greater MGBG tolerance than that of the wild-type controls. Less than 2% of wild-type seedlings were classed as 6-leaf stage or greater, whilst most sublines (excluding line 2(i)a.9) produced substantial numbers of offspring at 6-leaf or more (ranging from 12% in 2(i)a.4 to 65% in 2(i)a.5) (Figure 5.9). The MGBG tolerance for the individuals from line 2(i)c was not as high, with sub-line 2(i)c·9 exhibiting no greater tolerance than the wild-types, however all other 2(i)c sub-lines showed an increase ranging from 27% for line 2(i)c·8, to 55% for line 2(i)c·7 (Figure 5·10).

Germination in the presence of 7mM MGBG

When the level of MGBG was increased to 7mM in re-screening experiments, the timing of assessment of the phenotype was found to be an important factor determining the accuracy of scoring. At day 8 after germination, the putative mutant sub-lines clearly exhibited precocious germination and displayed a high degree of resistance compared to wild-type. Whereas, by day 18, the distinction between the wild-type and putative mutant seedlings was less clear (Figure 5·11). At day 18 however, the wild-type seedlings were smaller in size and frequently were at delayed stages of growth compared to the putative mutants. Variability in the resistance frequencies however, was still observed within the sub-lines, even though they were all originally derived from the same individual. Overall, the average resistance percentage for line 2(i)a was near 80%, again higher than that observed for line 2(i)c which was just over 50%. The day 8 resistance frequencies for the putative mutant sub-lines in the presence of 7mM MGBG are presented in Table 5·8. As was noted previously, the variability of the MGBG-resistant response on 7mM may be attributable in

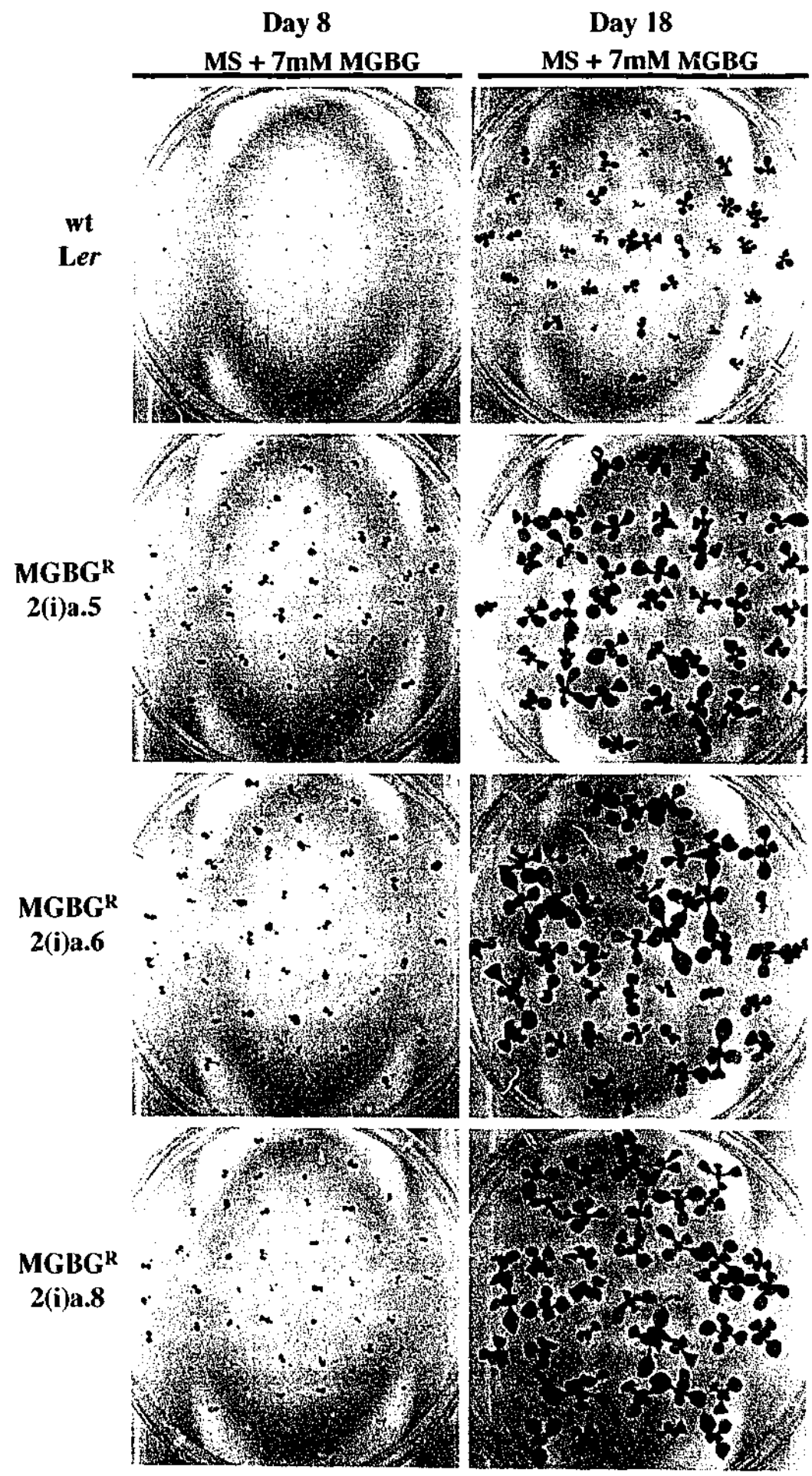


Figure 5.11: Re-screening inbred putative mutants from line 2(i)a on MS media containing 7mM MGBG.

The precocious germination of the mutants is evident at day 8. All sub-lines from 2(i)a and 2(i)c were tested, and representative plates are shown compared to wt L*er*.

part, to the presence of a slight precipitate within the media. For this reason, seeds were not subsequently screened on MGBG levels greater than 5mM.

Sub-line	Resistance Frequency	Sub-line	Resistance Frequency
2(i)a·1	67%	2(i)c·1	91%
2(i)a·2	58%	2(i)c·2	6%
2(i)a·3	63%	2(i)c·3	43%
2(i)a·4	58%	2(i)c·4	67%
2(i)a·5	100%	2(i)c·5	42%
2(i)a·6	93%	2(i)c·6	62%
2(i)a·7	81%	2(i)c·7	63%
2(i)a·8	97%	2(i)c·8	31%
2(i)a·9	81%	2(i)c·9	24%
2(i)a·10	87%	2(i)c·10	80%
2(i)a average:	78·5%	2(i)c average:	50·9%

Table 5·8: Resistance frequencies for the 2(i)a and 2(i)c sub-lines following germination on media containing 7mM MGBG (Day 8 of growth). Wild-type seeds showed no tolerance to MGBG at this age.

From the variability of the *in vitro* screening results, it was clearly not possible to determine if tolerance to MGBG was inherited as a straightforward Mendelian trait, even after several rounds of in-breeding and selection. To gain further information pertaining to the genetic mechanism(s) involved in the $MGBG^R$ phenotype noted above, putative mutant sub-lines 2(i)a and 2(i)c (from generations M4 and M5 respectively) were used as the female recipients in crosses to wild-type plants (reciprocal crosses were also carried out, but failed to yield any seed due to poor pollen production). Although few F1 seed were produced from the crosses, they were germinated and were grown to maturity in a greenhouse, and selfed. Ratios of sensitive-to-resistant F2 seedlings after germination on medium containing MGBG were then noted (Table 5·9).

FEMALE PARENT	TREATMENT			
	2mM MGBG	3mM MGBG	4mM MGBG	5mM MGBG
wt L*er* †	$2^S : 2^R$ (n=38)	$6^S : 0^R$ (n=33)	$6^S : 0^R$ (n=30)	$4^S : 0^R$ (n=38)
2(i)a	$15^S : 14^R$ (n=68)	$34^S : 6^R$ (n=69)	**$28^S : 8^R$ *** (n=60)	$27^S : 0^R$ (n=63)
2(i)c	$23^S : 29^R$ (n=57)	**$45^S : 16^R$ **** (n=65)	$53^S : 0^R$ (n=58)	$52^S : 0^R$ (n=61)

TABLE 5·9: Sensitive-to-resistant ratios of F2 progeny seedlings from crosses of wild-type to the 2(i) putative MGBG-resistant mutants.

† Many wild-type seeds did not germinate in media containing MGBG, unlike MS medium without MGBG where germination frequency was > 85% (data not shown). Seeds were germinated on solid MS media supplemented with different levels of MGBG, and scored at day 18 of growth. Germination was classified as the appearance of the radicle, and resistance to MGBG was classified as the appearance of green cotyledons. The numbers (n) of seeds sown per treatment are designated in parentheses. Chi-squared tests were performed on the observed results, testing for the hypothesis that there is a single gene controlling resistance to MGBG, segregating in a 3:1 ratio of sensitive to resistant in the F2 generation. * 0·50<p<0·70 and ** 0·80<p<0·90 at a significance level of 0·001 shown in bold type

The above results indicate that a mutation in a single nuclear gene is responsible for MGBG tolerance in lines 2(i)a and 2(i)c, but that the level of MGBG used to select seedlings is important to enable the tolerant phenotype to be scored accurately. No resistant F2 seedlings from the cross with line 2(i)a were observed on media containing 5mM MGBG, and in the case of line 2(i)c, none were observed when the MGBG levels were at 4mM or greater. Chi-squared analyses testing the hypothesis that one gene controls resistance to MGBG were performed, using the observed numbers at 4mM MGBG for the 2(i)a seeds, and at 3mM MGBG for the 2(i)c seeds, as these were the highest levels of inhibitor that allowed identification of resistant individuals. The observed ratios of sensitive to resistant seedlings corresponded to the expected 3:1 segregation pattern expected in the F2 generation if the resistant phenotype was controlled by a single recessive allele.

5·2·7 SUMMARY: HERITABILITY AND MGBG TOLERANCE RESULTS

Arabidopsis mutants with the ability to grow in the presence of 5mM MGBG were identified and classified into two groups; three type (i) individuals that exhibited a strong resistant

phenotype, and twelve type (ii) individuals with a weaker phenotype. Only the type (i) plants displayed heritability of the trait but only limited information on the genetics of the tolerant phenotype was obtained. Much more time than originally anticipated had to be devoted to the development of a reliable method of *in vitro* selection of the mutants as it was difficult to achieve consistent results, even following several rounds of in-breeding which improved the frequency of resistance. Several studies were necessary to determine the appropriate levels of MGBG required, and the duration of MGBG treatment, that would allow rapid identification of mutant seedlings. In the putative *Arabidopsis* mutants, it is likely that pleiotropic *in vivo* effects of MGBG contributed to the confusing results of the screening experiments, thus making the identification of mutants a difficult task. Following *in vitro* germination in the presence of MGBG, and transfer either to inhibitor-free media or into soil, the putative resistant mutants consistently displayed a more robust, tolerant phenotype, compared to similarly treated wild-type plants. To understand the genetic basis of the resistant phenotype, wild-type L*er* was crossed to the putative mutants and the germination phenotypes of the F2 seed in the presence of MGBG were noted. Chi-squared statistical tests suggested that the observed results conform to the 3:1 sensitive-to-resistant segregation pattern expected if one gene with two alleles was involved in the MGBG-resistant phenotype. Chi-square analysis was only indicative of a one-gene model at a particular concentration of MGBG, however, indicating that the concentration of MGBG used to identify mutants is of critical importance.

5·2·8 CHARACTERISATION OF THE MUTANT PHENOTYPE

II: HPLC QUANTIFICATION OF POLYAMINE LEVELS

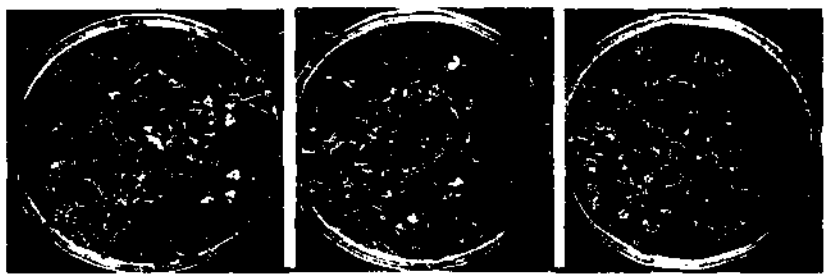
Free Polyamines

Polyamines were extracted from shoots of MGBG-treated and untreated plants, and quantified via HPLC as described in the Materials and Methods. The phenotypes of the wild-type and both lines of mutant plants after 35 days of *in vitro* growth on 0mM, 0·5mM, and 2mM MGBG are presented in Figures 5·12 and 5·13 (note: the same number of seeds were

wt L*er*

MS control 0.5mM MGBG 2mM MGBG

MGBG^R 2(i)a.5

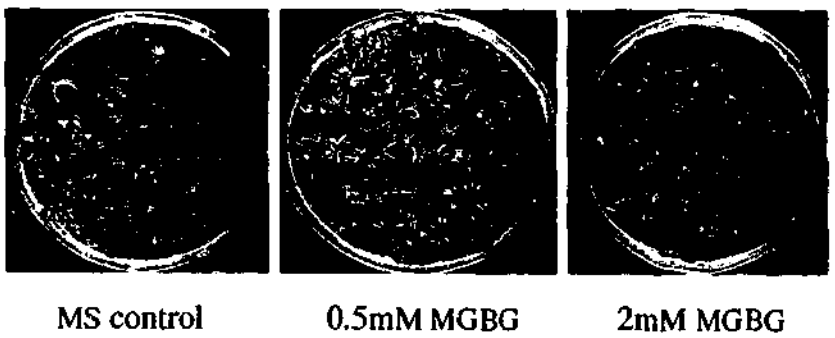


MS control 0.5mM MGBG 2mM MGBG

MGBG^R 2(i)a.6

MS control 0.5mM MGBG 2mM MGBG

MGBG^R 2(i)a.8

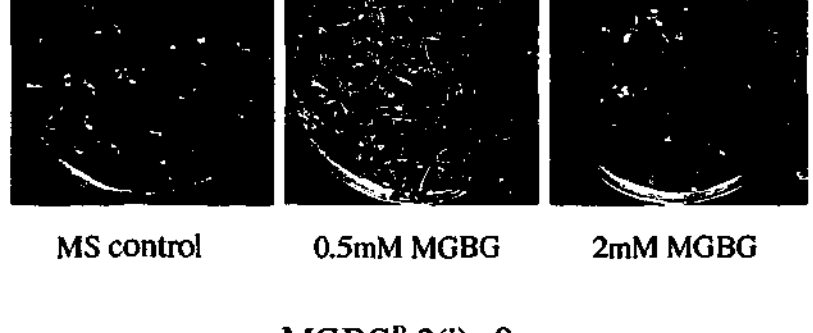


MS control 0.5mM MGBG 2mM MGBG

Figure 5.12: Wild-type and MGBG^R 2(i)a plants grown either in the presence or absence of MGBG.

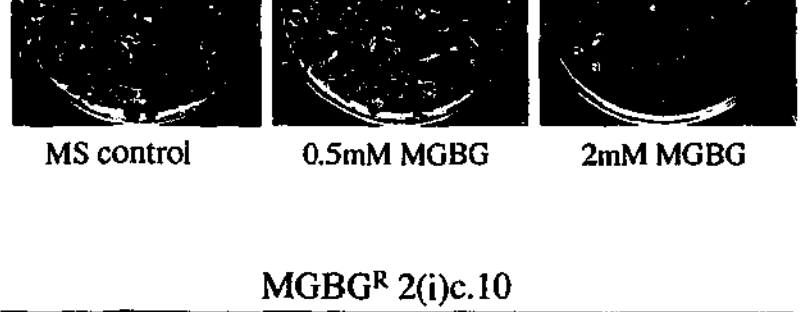
Shoot tissue from these treatments were used for all subsequent RNA and polyamine analyses.

wt *Ler*



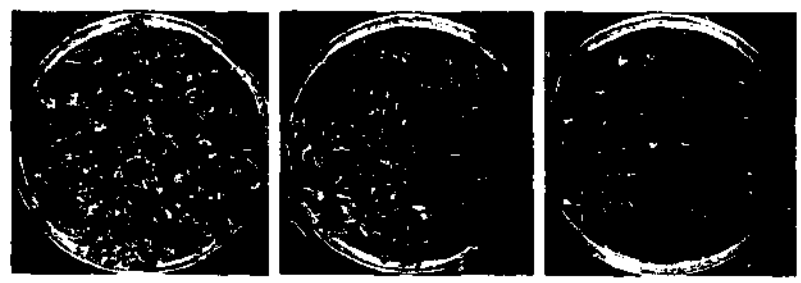
MS control 0.5mM MGBG 2mM MGBG

$MGBG^R$ 2(i)c.8



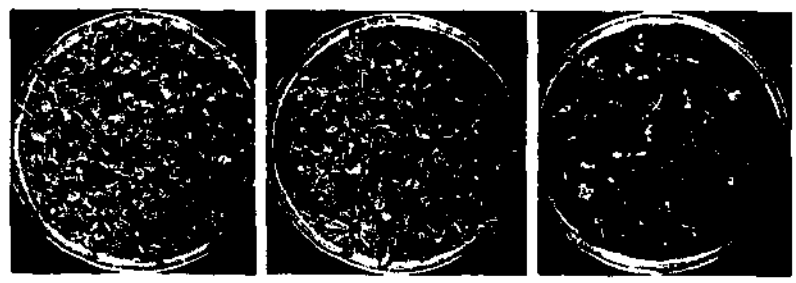
MS control 0.5mM MGBG 2mM MGBG

$MGBG^R$ 2(i)c.10



MS control 0.5mM MGBG 2mM MGBG

$MGBG^R$ 2(i)c.11



MS control 0.5mM MGBG 2mM MGBG

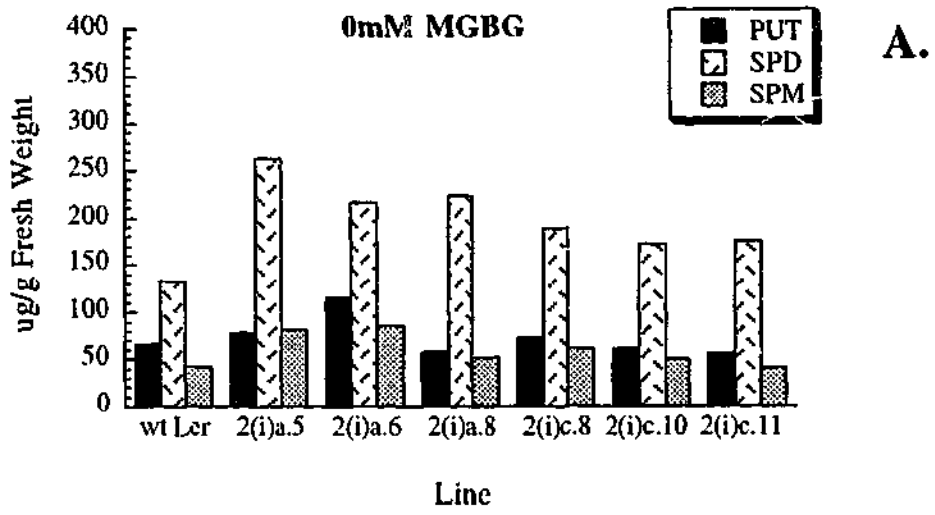
Figure 5.13: Wild-type and $MGBG^R$ 2(i)c plants grown either in the presence or absence of MGBG.

Shoot tissue from these treatments were used for all subsequent RNA and polyamine analyses.

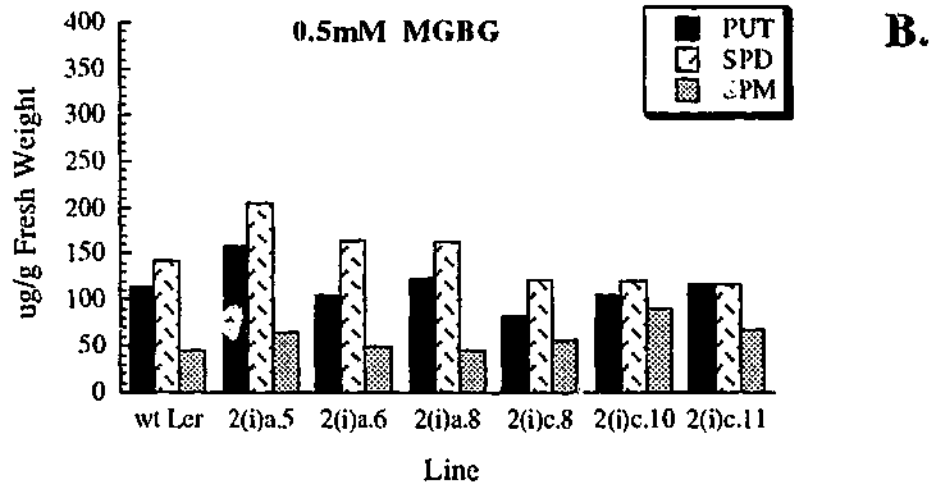
sown on each plate). No flowering was evident amongst any plants grown on media containing 2mM MGBG, and in the case of wild-types, flowering was also delayed on 0·5mM MGBG though plants were healthy (Figure 5·13). Some of the 2(i)a mutants were able to flower in the presence of 0·5mM MGBG (Figure 5·12), however, the 2(i)c mutants did not flower (Figure 5·13). Northern blot analyses of the biosynthetic genes and HPLC quantification and of the endogenous polyamine levels were performed on pools of non-flowering plants from each of these treatments to determine whether any differences existed between wild-type and mutants. The levels of free putrescine, spermidine, and spermine within the wild-type and mutant plants are presented as histograms in Figure 5·14, following the various treatments.

The endogenous levels of free polyamines when plants were grown in the absence of MGBG are shown in Figure 5·14A. Spermidine was found to be the most prominent polyamine within shoots, followed by putrescine, and then spermine. The most striking observation was that all the MGBG-resistant mutants lines analysed displayed a higher level of free spermidine, compared to the wild-type control. The level of spermidine within the pool of wild-type plants was approximately 130µg/g fresh weight, whilst the levels within the mutants ranged from 175µg/g fresh weight to 260µg/g fresh weight. The levels of putrescine showed some variation across the mutant lines, however, there was no marked deviation from that of the wild-type controls where levels were ~70µg/g. The level of spermine within the wild-types was approximately 40µg/g fresh weight, whilst that of the mutants ranged from 40-85µg/g fresh weight.

When grown under the low selective conditions of 0·5mM MGBG, levels of putrescine increased relative to untreated controls in all sub-lines, except for mutant 2(i)a·6, which was slightly lower than its untreated control (Figure 5·14B). It should be noted however, that this sub-line had a high basal level of putrescine before any MGBG treatment. All mutant sub-lines also displayed a decrease in endogenous levels of free spermidine when compared to their untreated controls. Interestingly however, the amount of spermidine within the wild-



A.



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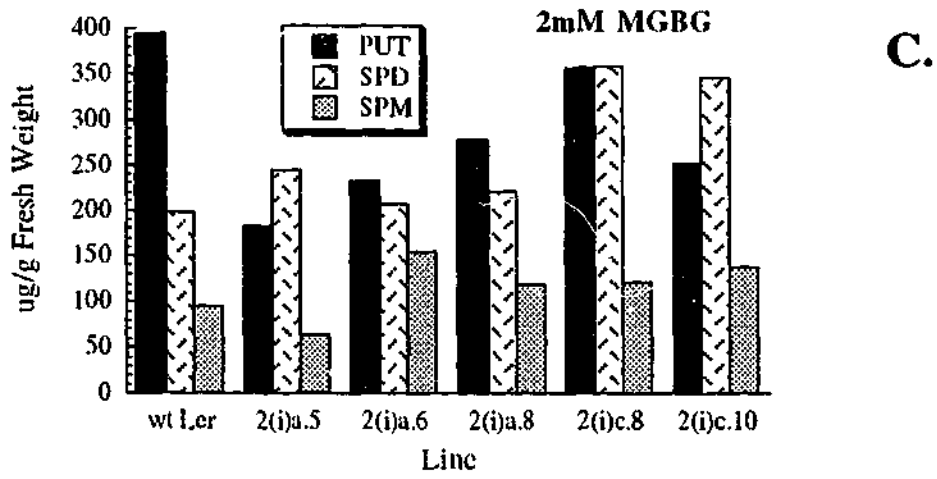


C.

Figure 5.14: Free polyamine titres (µg/g f.wt.) in shoot tissue of selected putative MGBG^R mutants following *in vitro* growth.

A. Unsupplemented MS media (0mM MGBG),
B. MS with 0.5mM MGBG
C. MS with 2mM MGBG

Each column on the histograms represents an extraction from a pool of treated shoot tissue (*n* is between 10 - 20 individual shoots).

types remained similar to untreated controls. Spermidine levels within wild-types were slightly lower than those of the 2(i)a mutants, and somewhat higher than those of the 2(i)c mutants. No difference in the amount of spermine was observed between the untreated and treated wild-type plants. The mutant 2(i)a sub-lines showed a decrease in spermine levels from their untreated controls, whereas the levels of two of the three 2(i)c lines were higher than controls. The treated wild-types had an spermine level of approximately 45µg/g fresh weight, whilst the mutants ranged from 45-90µg/g fresh weight. Under these conditions, differences in polyamine titres between wild-types and mutants were not marked.

Treatment with 2mM MGBG resulted in a large increase in putrescine levels in wild-type seedlings, approximately six times greater than that of untreated controls (Figure 5·14C). Strong increases (between five to six fold) were also observed in the putrescine levels of some mutant sub-lines (2(i)c·8 and 2(i)c·10) whereas others had a smaller increase of 2-fold (2(i)a·5). Wild-type plants exhibited an increase in the amount of spermidine, compared to untreated and 0·5mM MGBG-treated controls. Spermidine levels, however, within 2(i)a mutants were similar to those observed in MGBG-free media. The 2(i)c mutants analysed showed a strong increase in spermidine levels to greater than double that of their untreated controls. The level of spermine was also increased by approximately 2-fold in wild-type compared to the zero or low MGBG treatments. All mutant lines, showed this similar increase in spermine levels, except 2(i)a·5, which displayed no difference between 0·5mM and 2mM MGBG treatments.

Ratios of free putrescine-to-spermidine

The ratios of free putrescine-to-spermidine in the treated wild-types and $MGBG^R$ mutants are graphically presented in Figure 5·15 (A and B). The free putrescine-to-spermidine ratio for all lines was generally found to increase as the selection levels of MGBG increased up to 2mM. No difference in the free putrescine-to-spermidine ratio between the wild-type controls and the mutants were apparent at 0mM or 0·5mM MGBG. A clear distinction however, was evident at the higher level of 2mM MGBG, when all MGBG-resistant mutant

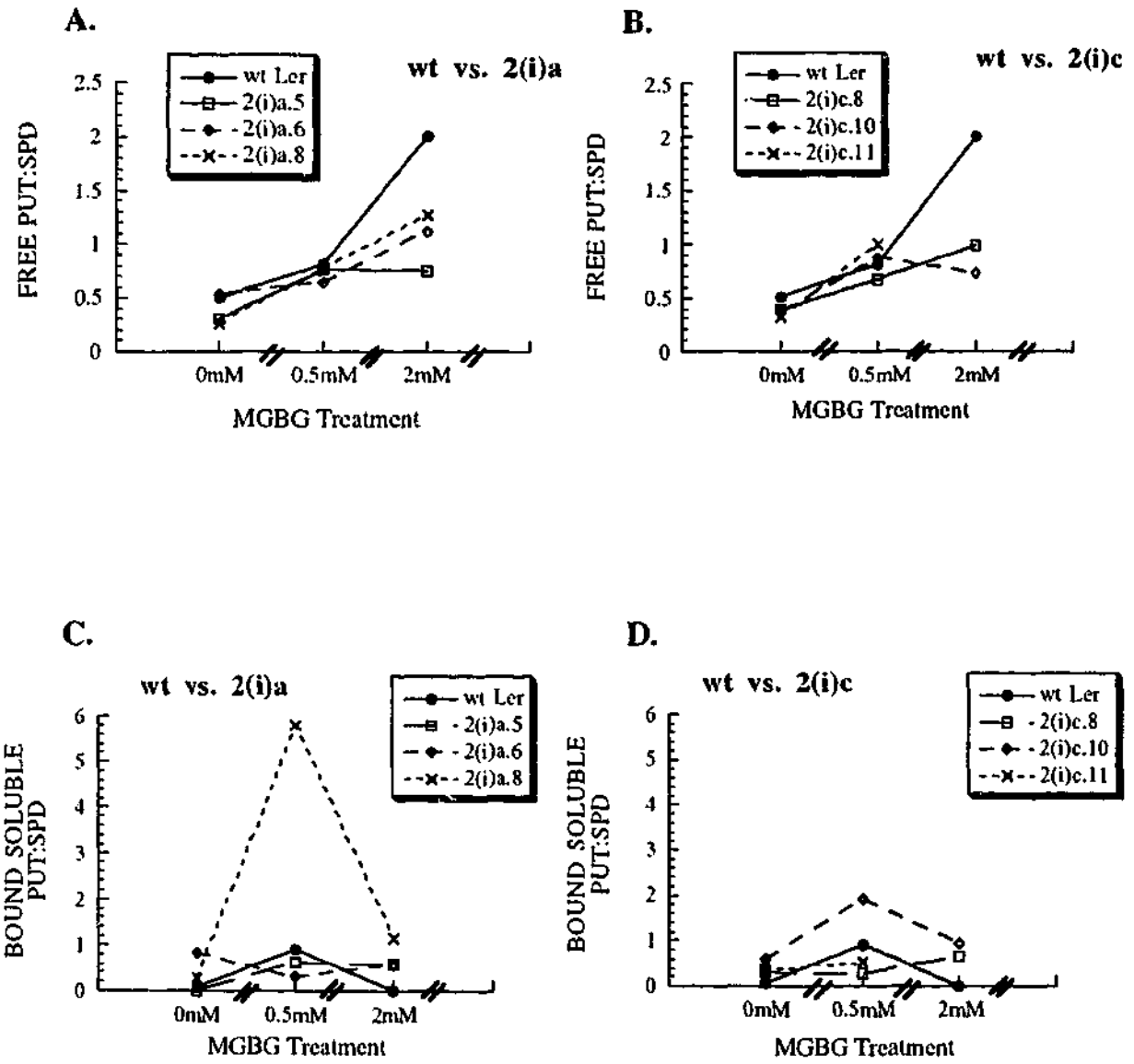


Figure 5.15: PUT-to-SPD ratios of wt L*er* shoots and MGBG^R putative mutant lines 2(i)a and 2(i)c after growth on MS media with or without polyamines.

A. wt vs. 2(i)a - free PUT and SPD
B. wt vs. 2(i)c - free PUT and SPD
C. wt vs. 2(i)a - conjugated PUT and SPD
D. wt vs. 2(i)c - conjugated PUT and SPD

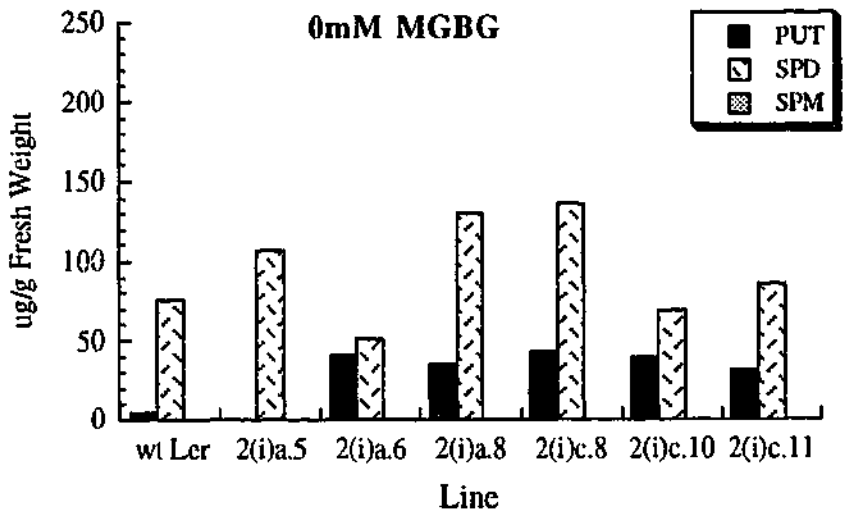
lines displayed a lower free putrescine-to-spermidine ratio than that of wild-type controls. The high ratio observed in wild-type lines was mainly attributable to a large relative accumulation of putrescine in shoot tissues.

Conjugated Polyamines

Compared to levels of free polyamines, the amounts of all three conjugated, or bound-soluble, amines were reduced in the controls and also mutants. Spermidine remained the highest polyamine detected in this fraction, whilst spermine levels in all sub-lines were not detectable. Levels of conjugated polyamines within wild-type and mutant shoots grown in the absence of MGBG are presented in Figure 5·16A. The level of putrescine in wild-type was approximately 5µg/g fresh weight, whereas most of the mutant sub-lines had elevated levels, ranging from 35-45µg/g fresh weight (the exception was sub-line 2(i)a·5, whose putrescine levels were below the detectable limits). There was some variation in the spermidine levels across all sub-lines, but no clear pattern was evident (Figure 5·16A).

When selection was imposed with the low concentration (0·5mM) of MGBG, a clear change in the levels of bound-soluble putrescine was observed in wild-types, but not in mutant sub-lines (Figure 5·16B). Putrescine levels rose dramatically, more than 50-fold, to over 100µg/g fresh weight, whilst levels in mutants remained within the range of 25-to-50µg/g fresh weight—similar to levels observed when MGBG was absent. Conjugated putrescine levels also increased within mutant sub-line 2(i)a·5 (undetectable at 0mM MGBG), and remained within the range of the other mutant sub-lines. Levels of bound-soluble spermidine increased by over 50% in wild-type shoots, whilst they were variable in all mutant sub-lines, ranging from 10-to-120µg/g fresh weight. In line 2(i)a·6, the spermidine levels were the same as that of the treated wild-types, whilst levels of the other mutants were lower. Again, bound-soluble spermine was undetectable in all lines.

Upon treatment with 2mM MGBG, amounts of conjugated putrescine and spermidine were seen to increase in all mutant sub-lines (Figure 5·16C), whilst spermine was not detectable in



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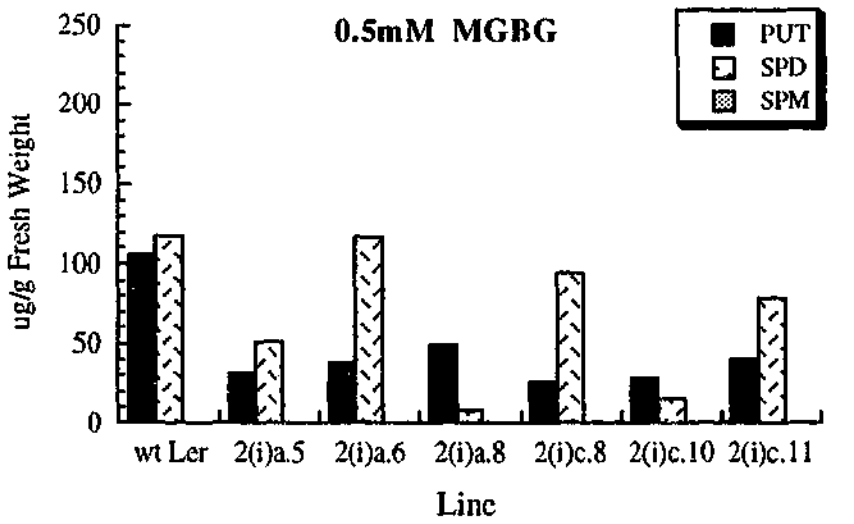

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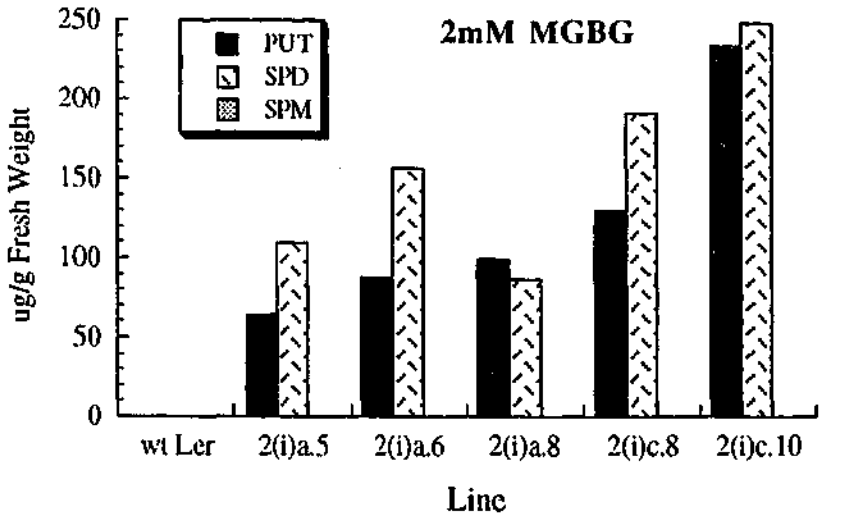


C.

Figure 5.16: Conjugated (bound-soluble) polyamine titres (µg/g f.wt.) in shoot tissue of selected putative MGBG^R mutants following *in vitro* growth.

A. Unsupplemented MS media (0mM MGBG)
B. MS with 0.5mM MGBG
C. MS with 2mM MGBG

Each column on the histograms represents an extraction from a pool of treated shoot tissue (*n* is between 10 - 20 individual shoots).

any of the mutants. The most striking difference in polyamine titres however, was observed between mutants and controls following this treatment, as no bound-soluble polyamines of any sort were detectable in wild-type shoots (note that wild-type plants showed very poor growth at 2mM MGBG). Putrescine and spermidine levels within mutants were markedly higher than those seen for the 0mM or 0·5mM MGBG treatments. Putrescine and spermidine levels of mutant 2(i)a lines were lower than those of 2(i)c lines.

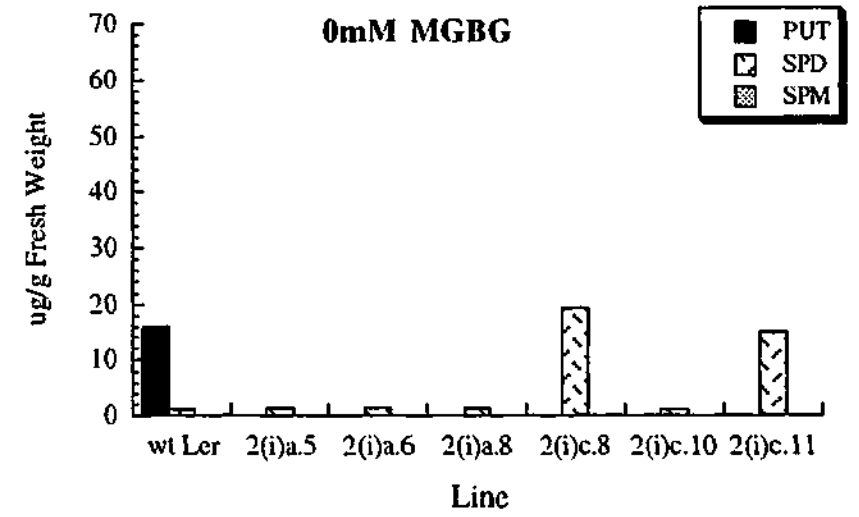
Ratios of conjugated putrescine-to-spermidine

In all mutant sub-lines, except 2(i)a·6, growth on 0·5mM MGBG caused an increase in the putrescine-to-spermidine ratios (Figures 5·15C & 5·15D). At 2mM MGBG, the wild-types had no detectable putrescine or spermidineand therefore did not have a ratio, compared to the mutants, which ranged from 0·56 to 1·14.

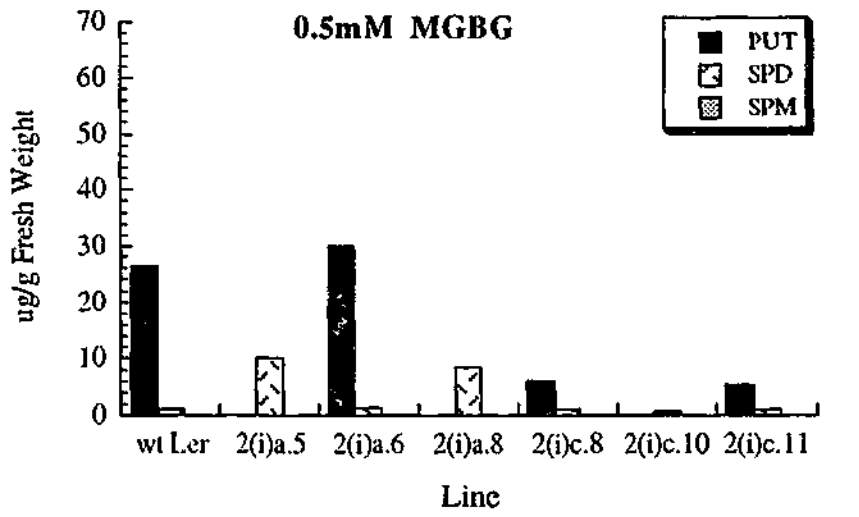
Bound Polyamines

Of the three cellular forms of polyamines, the bound-insoluble class was the least prevalent within shoots of both mutant and wild-type plants. When grown in the absence of MGBG, putrescine was undetectable in all mutant sub-lines. It was however, detectable in wild-type controls (Figure 5·17A). Spermidine was present in all sub-lines in low amounts, except for 2(i)c·8 and 2(i)c·11, whose levels were relatively high (approximately 20µg/g fresh weight and 15µg/g fresh weight, respectively). The amount of bound-insoluble spermine was too low for detection in plants grown in all conditions.

Levels of bound-insoluble polyamines after treatment with 0·5mM MGBG are presented in Figure 5·17B. No trends were evident, but levels of putrescine increased in three of the mutant sub-lines, and wild-type control, following this treatment. The amount of spermidine increased in two of the 2(i)a sub-lines (5 and 8), but decreased in the previously high 2(i)c·8 and 2(i)c·10 sub-lines.



A.



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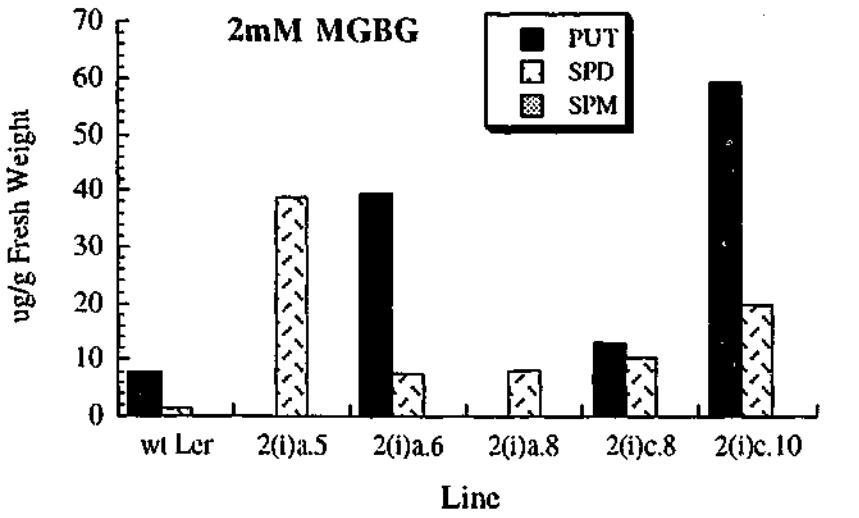


C.

Figure 5.17: Bound (bound-insoluble) polyamine titres (µg/g f.wt.) in shoot tissue of selected putative MGBG^R mutants following *in vitro* growth.

A. Unsupplemented MS media (0mM MGBG)
B. MS with 0.5mM MGBG
C. MS with 2mM MGBG

Each column on the histograms represents an extraction from a pool of treated shoot tissue (*n* is between 10 - 20 individual shoots).

Another difference between the wild-type controls and the mutant sub-lines became evident after selection on 2mM MGBG. Titres of bound-insoluble spermidine were distinctly higher within mutants, compared to wild-type controls whose titres were barely detectable (Figure 5·17C). At this level of MGBG, the amount of putrescine decreased in the wild-types and increased in the remaining mutant sub-lines. Bound-insoluble putrescine was not detectable in mutant sub-lines 2(i)a·5 and 2(i)a·8, regardless of the treatment.

5·2·9 SUMMARY: POLYAMINE TITRE RESULTS

Following growth in the presence or absence of MGBG, quantification of endogenous polyamine titres within the shoots of wild-type or MGBG-resistant putative mutants revealed several major differences between the two groups. When not exposed to MGBG, the putative mutants possessed a higher basal level of free spermidine than wild-type controls. Furthermore, growth on media containing 2mM MGBG resulted in a marked increase in the levels of conjugated putrescine and spermidine within the putative mutants, whereas these polyamines were not detectable in the shoots of wild-types. This level of MGBG also resulted in the putative mutants exhibiting higher titres of bound spermidine than similarly-treated wild-type controls.

5·2·10 CHARACTERISATION OF THE MUTANT PHENOTYPE:

III: NORTHERN ANALYSES

Preliminary studies

To obtain an initial molecular characterisation of the putative mutant lines, Northern analyses were performed using fragments of the *SAMDC*, *ADC*, and *SPDS* genes from *Arabidopsis thaliana* and *ODC* from *Nicotiana tabacum* as probes. *SAMDC* and *ODC* are two rate-limiting enzymes in the polyamine biosynthesis pathway and were thought likely to represent important points controls, regulating polyamine titres within the putative MGBG-resistant mutants (experiments were undertaken before results of Hanfrey *et al.*, 2001 were

published). It was thought possible that regulation of these important genes could be altered in the putative mutants, thus conferring resistance to the effects of MGBG. As ADC offers an alternate route to ODC-mediated putrescine biosynthesis, information regarding the transcriptional regulation of this gene was also sought to provide valuable insight into understanding the relationship between the two arms of the biosynthetic pathway leading to putrescine production, especially under conditions of metabolic stress. The use of an *SPDS* gene probe in conjunction with a *SAMDC* probe, was thought likely to contribute information relating to the direct effects of MGBG on the regulation of spermidine production in *Arabidopsis*.

A preliminary experiment showed that *SAMDC* transcript was increased in *Arabidopsis* shoots following growth on media containing MGBG (Figure 5·18). In this experiment, total RNA was isolated from the shoots of non-flowering wild-type plants grown on MS media containing 7mM MGBG, and also from flowering and non-flowering untreated wild-types as controls. The physiological state of the tissue made little, if any, difference to the induction of the gene, as both the flowering and non-flowering controls contained similarl, quite low levels, of transcript. A more intense signal was observed in the 7mM MGBG-treated sample, even though it contained less total RNA than in the controls (based on ethidium bromide-staining), showing that *SAMDC* expression was clearly increased by treatment with the inhibitor..

SAMDC expression in mutant lines

Total RNA was extracted from pools of plants from both sets of mutant lines and wild-type controls grown on 7mM MGBG, which allowed some growth of wild-type controls. The progeny individuals derived from line 2(i)a and those derived from line 2(i)c (as phenotypically characterised in Section 5·2·6). Comparative levels of *SAMDC* transcript (Figure 5·23B) in the shoots were determined after the growth of the plants on media containing 7mM MGBG, which was previously found to induce transcript levels of the gene (Figure 5·18 above). The *SAMDC* signal intensity from each sample was standardised

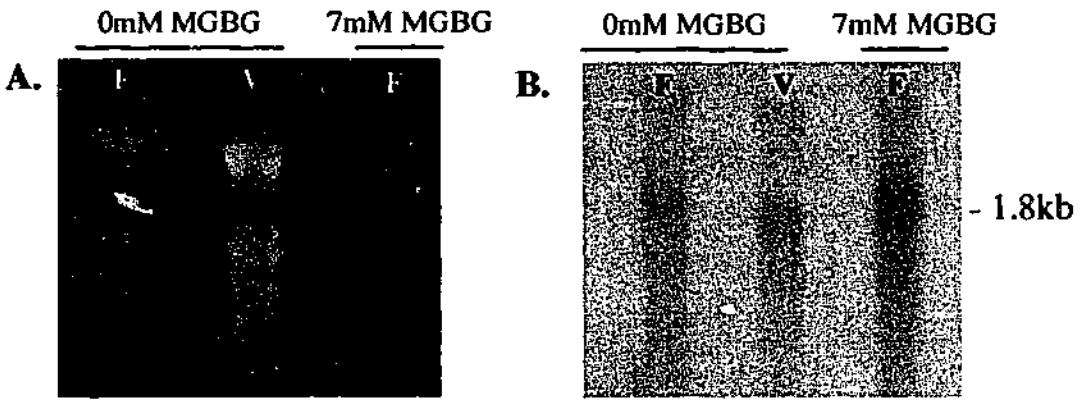


Figure 5.18: Induction of *SAMDC* transcript accumulation in wild-type tissue following MGBG treatment.
RNA was extracted from shoot tissue of flowering (F) and vegetative (V) wild-type L*er* plants which were grown in the presence or absence of 7mM MGBG.
A. Ethidium bromide-stained gel
B. Filter probed with an *Arabidopsis* cDNA fragment of *SAMDC*

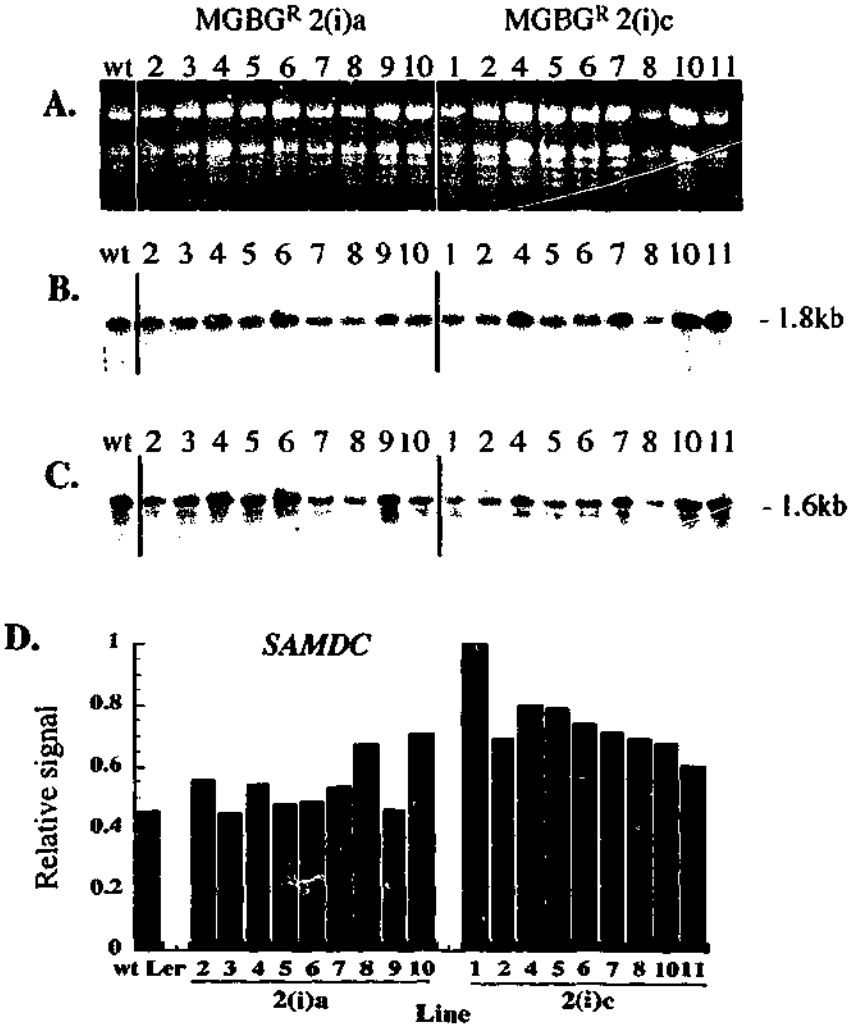


Figure 5.19: Preliminary *SAMDC* Northern blot from putative mutants and wild-type shoots when grown in the presence of 7mM MGBG.
A. EthBr-stained gel **B.** *SAMDC* probe **C.** *UBIQUITIN* probe
D. Standardised signal intensity of *SAMDC* relative to that of *UBIQ*
The strongest signal is designated a value of 1.

relative to its *UBIQUITIN* signal (Figure 5·19D). All mutant samples exhibited *SAMDC* transcripts at either the same level as, or increased from, that of the treated wild-type control. A difference is apparent in the levels of the *SAMDC* transcript between the sub-lines 2(i)a and 2(i)c; the former being similar to the levels of the controls, and the latter all higher than the controls.

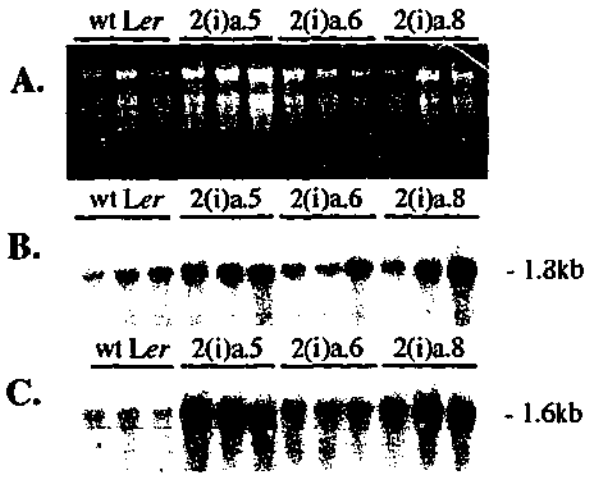
Detailed gene expression study after growth on MGBG

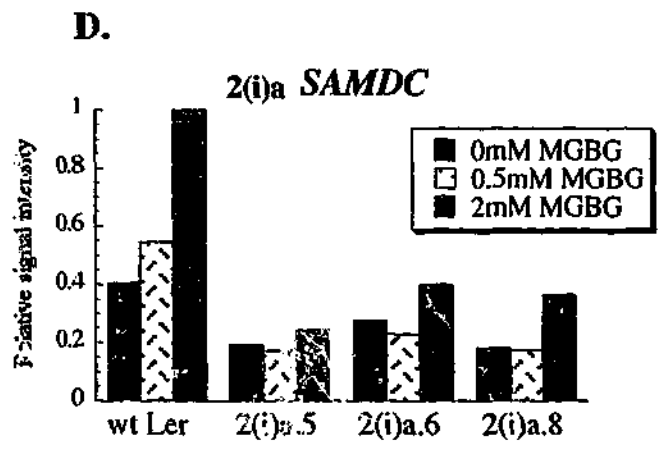
A more detailed study of transcript levels of polyamine biosynthetic genes was performed on selected mutant lines. For this analysis, however, lower levels of the inhibitor (0·5mM and 2mM) were chosen in order to determine if the *SAMDC* trasncript in the mutants was a true reflection of the mutant phenotype and not merely a stress-induced response caused by high levels of MGBG. To determine if the mutants possessed constitutively higher or lower *SAMDC* expression, mutant and wild-type tissue were also analysed following growth in the absence of MGBG.

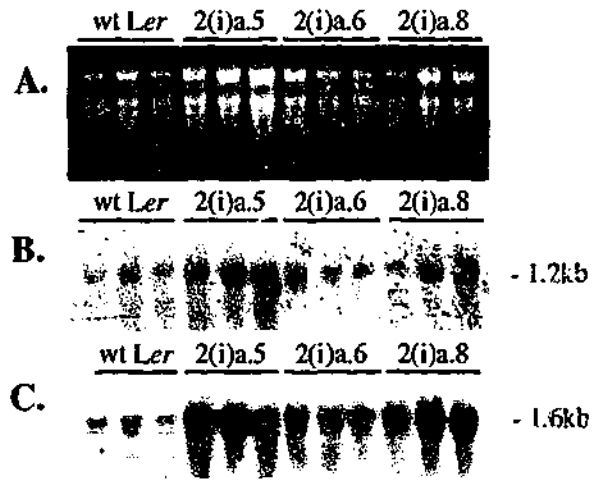
Sub-lines 5, 6, and 8 were selected from line 2(i)a for further analysis, as they exhibited the greatest level of tolerance to 2mM MGBG (from Figure 5·9). The *SAMDC* expression study (Figure 5·19D) revealed quite interesting results for these lines, as it was found that the transcript levels of individuals 2(i)a·5 and 2(i)a·6 were the same as that of the control, whilst 2(i)a·8 was increased by approximately 50%—relative to *UBIQUITIN* in each sample. From line 2(i)c, sub-lines 8, 10, and 11 were selected for further analysis since they too exhibited high resistance to 2mM MGBG (from Figure 5·10) and also had approximately 50% higher *SAMDC* expression than controls (Figure 5·19D).

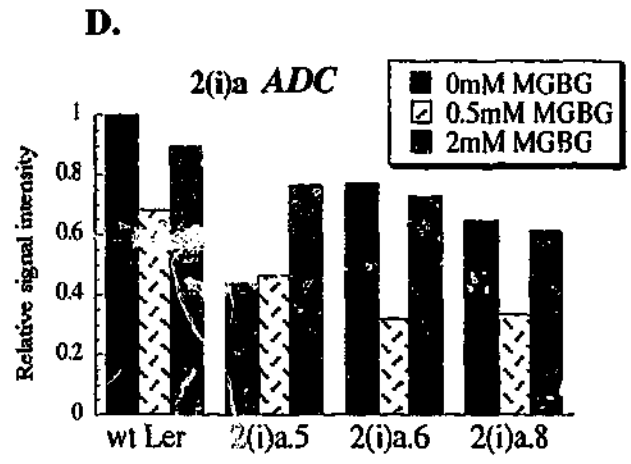
SAMDC

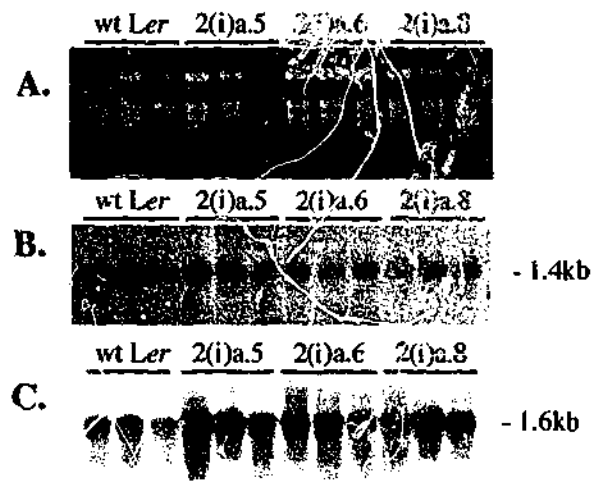
The autoradiographs after probing filters with *Arabidopsis SAMDC* DNA fragments are presented in Figures 5·20 and 5·21 (for mutant lines 2(i)a and 2(i)c respectively). The relative amount of RNA loaded on the gels was standardised through the use of a *UBIQUITIN* DNA probe (Figures 5·20 and 5·21, panels C). The standardised signal intensities, relative











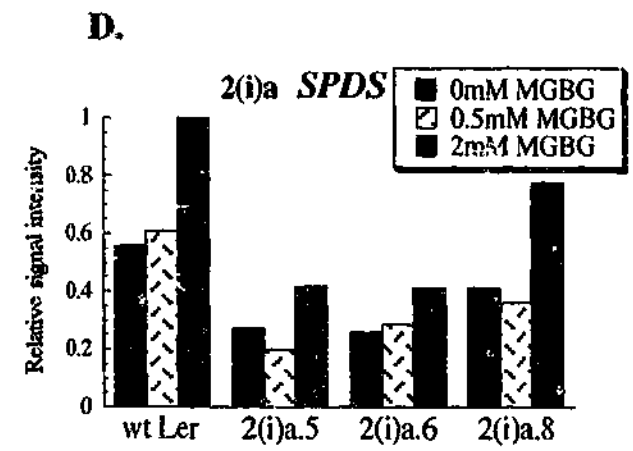
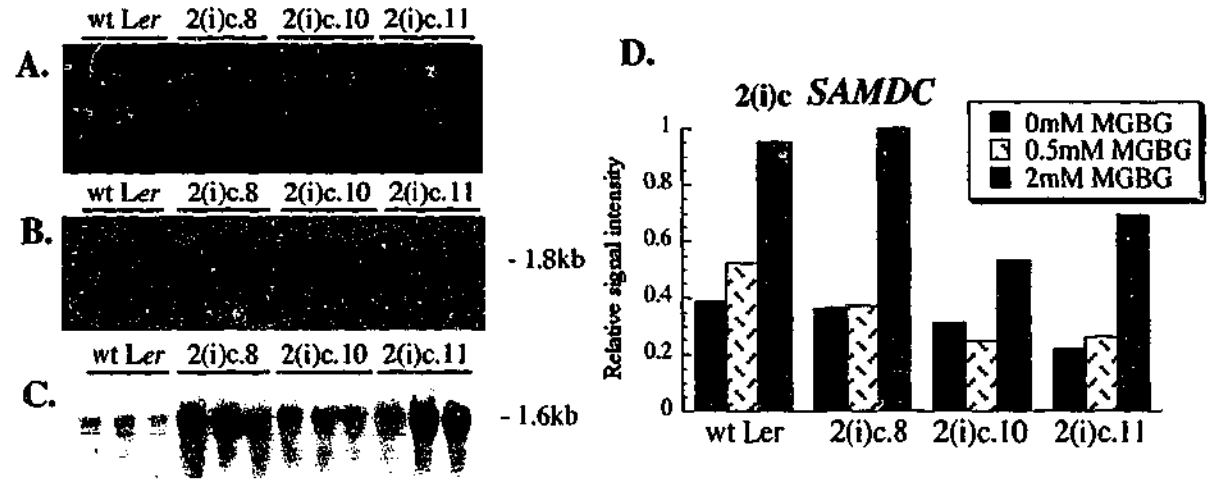
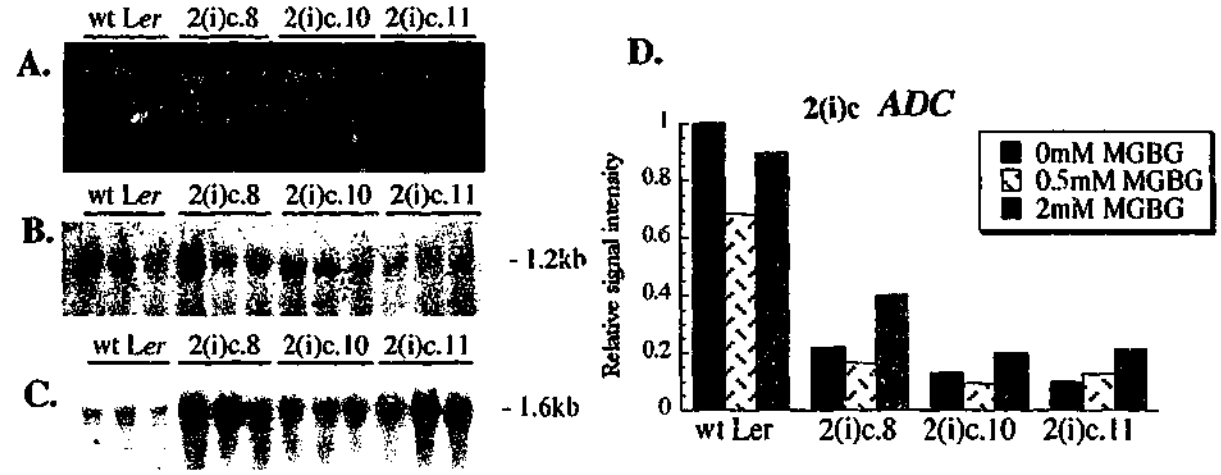


Figure 5.20: Northern blot analyses of wild-type and MGBG^R 2(i)a shoots grown in the presence of either 0mM MGBG, 0.5mM MGBG, or 2mM MGBG.

The EthBr-stained gels are presented (**A.**), as are the autoradiographs after probing with fragments of the specific polyamine biosynthesis genes (**B.**), and the *UBIQ* gene from *Arabidopsis*. (**C.**) (Samples for each line are loaded from left to right on the gel; 0mM, 0.5mM, and 2mM MGBG). The signal intensities for each gene have been quantified relative to those of *UBIQ*, and are presented as histograms (**D.**). A tobacco *ODC* probe was used, resulting in non-specific binding to the ribosomal RNA bands (not presented). Each lane represents an extraction performed from a pool of shoot tissue.





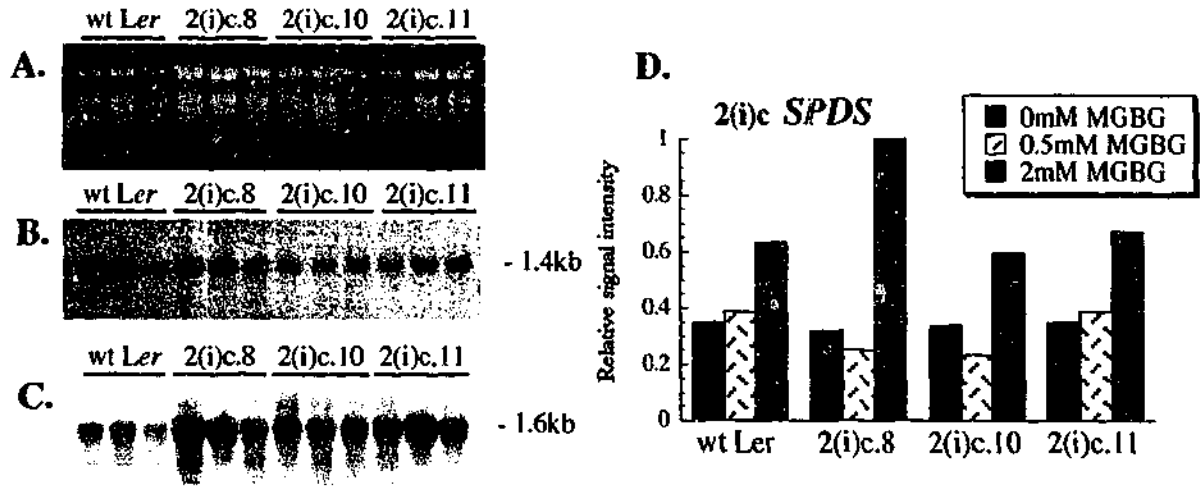


Figure 5.21: Northern blot analyses of wild-type and MGBG^R 2(i)c shoots grown in the presence of either 0mM MGBG, 0.5mM MGBG, or 2mM MGBG.

The EthBr-stained gels are presented (**A.**), as are the autoradiographs after probing with fragments of the specific polyamine biosynthesis genes (**B.**), and the *UBIQ* gene from *Arabidopsis*. (**C.**) (Samples for each line are loaded from left to right on the gel; 0mM, 0.5mM, and 2mM MGBG). The signal intensities for each gene have been quantified relative to those of *UBIQ*, and are presented as histograms (**D.**). A tobacco *ODC* probe was used, resulting in non-specific binding to the ribosomal RNA bands (not presented). Each lane represents an extraction performed from a pool of shoot tissue.

to the amounts of total RNA loaded, are presented as histograms in Figures 5·20 and 5·21 (D panels).

2(i)a mutants showed a different pattern of expression than the wild-types under the three growth conditions. When untreated, the three 2(i)a mutant sub-lines had slightly lower levels of *SAMDC* expression than the wild-type controls. After treatment with 0·5mM MGBG, the expression levels in all three mutants pools decreased marginally from that of their own untreated controls, whereas the expression of the wild-types increased by over 25%. Treatment with 2mM MGBG saw the levels of *SAMDC* transcript reach their peaks in all three mutants pools, however, they again remained markedly lower than the levels observed in the 2mM-treated wild-types. Such wild-types showed a strong induction of over 150% from its untreated control levels (Figure 5·20). The pools of shoots from the three 2(i)c sub-lines when grown in the absence of MGBG displayed essentially similar *SAMDC* transcript levels as untreated wild-types. When grown on 0·5mM MGBG, the expression of *SAMDC* within mutants remained at similar levels, however, expression within the wild-type pool was found to increase by over 25%. Transcript levels in wild-types and mutant sub-line 2(i)c·8 increased following treatment with 2mM MGBG, whilst lines 2(i)c·10 and 2(i)c·11 also increased but to a lesser extent (Figure 5·21).

ADC

The autoradiographs following hybridisation of the filters with a fragment of the *Arabidopsis ADC* gene are presented in Figures 5·20 and 5·21 (for mutant lines 2(i)a and 2(i)c respectively). The relative levels of *ADC* transcript accumulation in the wild-type and 2(i)a and 2(i)c mutant lines were found to be very low.

The 2(i)a mutants exhibited the same expression pattern as wild-types, however, mRNA levels were reduced for all treatments, especially untreated line 2(i)a·5 which was less than half that of the controls. Growth on media containing 0·5mM MGBG resulted in a reduction in expression of *ADC* in wild-type, 2(i)a·6, and 2(i)a·8 lines, and a slight increase in line

2(i)a·5. On 2mM MGBG, *ADC* expression in control and mutant sub-lines 2(i)a·6 and 2(i)a·8 returned to the levels seen for their own untreated controls. Mutant line 2(i)a·5 was an exception, as it had a lower control transcript level to begin with, and thus showed a relative increase (Figure 5·20). Within 2(i)c mutant sub-lines, relative expression of *ADC* was markedly lower than controls across all treatments (Figure 5·21). Growth on media containing 0·5mM MGBG reduced transcript accumulation in wild-types by approximately 25%. In mutant sub-lines, alterations were negligible at this level of inhibitor. When the level of MGBG was increased to 2mM, an increase in *ADC* transcript was seen. The transcripts within the wild-types returned to almost control levels, whereas that of the mutants was slightly increased (Figure 5·21).

SPDS

The autoradiographs following hybridisation of the filters with a fragment of the *Arabidopsis SPDS* gene are presented in Figures 5·20 and 5·21 (for mutant lines 2(i)a and 2(i)c respectively). The relative levels of the *SPDS* transcript within the 2(i)a and 2(i)c mutant lines are presented as histograms within those figures respectively.

When grown in the absence of MGBG, the relative level of *SPDS* expression was reduced in 2(i)a mutant lines compared to the wild-types (Figure 5·20). Exposure to 0·5mM MGBG caused only slight variations in gene expression in both wild-type control and mutant sub-lines, however, the relative levels remained lower in the mutants. When the amount of MGBG was increased to 2mM, expression of *SPDS* reached its peak in both wild-types and 2(i)a mutants, with the wild-type again exhibiting the largest increase. From Figure 5·21, it can be seen that expression of *SPDS* within the three untreated 2(i)c mutants was approximately the same as that of untreated wild-type controls. Growth in the presence of 0·5mM MGBG brought about minor alterations in the transcript levels. Slight decreases were observed in sub-lines 2(i)c·8 and 2(i)c·10, and slight increases in *SPDS* expression for the wild-type control and sub-line 2(i)c·11. Again, 2mM MGBG had the effect of increasing the levels of the *SPDS* transcript in the wild-type controls and all 2(i)c mutant lines. In this

case, the levels of the mutants were the same as that of the wild-types, except for line 2(i)c·8 which exhibited the highest expression.

OTHER PROBES

Northern filters were also probed with fragments of the tobacco and Datura *ODC* genes, as this sequence had not been reported from *Arabidopsis* at the time these experiments were undertaken. Similarly, a tobacco fragment of the SAM synthase gene was also used. These probes proved unsuccessful in generating transcript signals from the wild-type and MGBG-resistant mutant shoot RNA samples, even at low stringency washes. Only non-specific binding to the ribosomal bands were evident, and are not presented here.

Summary: Northern blot results

The primary target of MGBG action in the perturbation of polyamine biosynthesis is inhibition of SAMDC activity. A preliminary experiment demonstrated that *SAMDC* gene expression was markedly induced in wild-type L. *erecta* following treatment with a high level of MGBG. Further analysis of selected mutant sub-lines possessing tolerance to MGBG found that relative levels of SAMDC mRNA were lower in 2(i)a sub-lines compared to wild-types, and also in 2(i)c sub-lines, following all treatments with MGBG. A similar pattern was observed for the accumulation of *SPDS* transcripts in all lines. A converse situation was the case for *ADC* gene expression, as relative levels of transcript were lower for 2(i)c sub-lines than wild-type or the 2(i)a sub-line . Treatment with 2mM MGBG induced the highest level of gene expression in all cases, except for *ADC* in the wild-type and 2(i)a sub-lines.

5·3 DISCUSSION

In summary, this study has isolated two lines of mutant *Arabidopsis* plants that were resistant to the polyamine biosynthesis inhibitor MGBG. These lines were characterised morphologically on the basis of resistance to different levels of the inhibitor, and measurements were also made of the endogenous titres of free, conjugated, and bound polyamines in mutant and wild-type lines. Transcription of polyamine biosynthetic genes was also characterised in these lines relative to controls. Of the two mutant lines, one was found to exhibit a stronger resistance phenotype than the other. The altered phenotypes of the MGBG-resistant mutants correlated with higher titres of free, conjugated, and bound spermidine compared to sensitive wild-type controls. Thus it is presumed that titres of polyamines within plants are important factors in conferring resistance to either MGBG specifically, or to the general metabolic stresses induced within the plant by MGBG.

Screening methodology

In this study, an initial search for *Arabidopsis* plants mutated in polyamine metabolism explored the possibility of screening plants in soil to detect mutants tolerant to polyamine inhibitors, or polyamines themselves. The technique of spraying inhibitor directly onto soil-grown plants has been shown to be effective for studying polyamine transport in response to DFMO treatment in leaves of wild mustard (Havelange *et al.*, 1996). In the present work a similar method with the inhibitor CHA was used, however, concentrations previously shown to be toxic to *in vitro* growth, did not exhibit comparable toxicity when applied to plants grown under greenhouse conditions. Although CHA is rapidly absorbed by excised cotyledons of *Pinus radiata* (Biondi *et al.*, 1986), under greenhouse conditions several reasons may contribute to the lack of inhibition, including variation in uptake from leaves and roots, breakdown of CHA by either microbes or environmental conditions, or different physiological responses of the plants. As a result of this variability, an *in vitro* approach was thought to be a more guaranteed way of avoiding the selection of false positives.

Primary ornithine screen

Plants possessing mutations in the activity or regulation of two rate-limiting enzymes, ODC and SAMDC, were initially sought in order to further understand the roles of these key enzymes during growth and development of *Arabidopsis*. Putative mutants tolerant to 10mM ornithine were isolated, however, heritability of the trait was not determined due to lack of seed set. Future studies here may be instructive if, for example, plants could be transferred to soil after stringent selection, and possibly manually crossed to wild-type plants. As analysis was not undertaken, the reasons for ornithine toxicity and tolerance remains speculative. The isolation of *Arabidopsis* mutants with low ODC activity, however, has been reported, although the trait was not found to segregate reproducibly (Watson *et al.*, 1998). Subsequently however, it has been reported that *Arabidopsis* lacks ODC (Hanfrey *et al.*, 2001). With that in mind therefore, the tolerance of plants to high levels of ornithine noted in this present study, is not thought to be related to polyamine biosynthesis, but may instead result from factors such as altered uptake, transport, or compartmentalisation of ornithine, or possibly altered conversion to arginine via the urea cycle, which could perturb polyamine metabolism.

Primary MGBG screen

Although MGBG has been reported to have toxic effects on plant growth other than depressing polyamine biosynthesis (Jänne and Alhonen-Hongisto, 1989; Cheng *et al.*, 1990), the main reason for treating plants with MGBG has been to study effects on polyamine metabolism (Malmberg and McIndoo 1983, Malmberg and Rose, 1987; Minocha *et al.*, 1990; Villanueva and Huang, 1993; Fritze *et al.*, 1995; Scaramagli *et al.* 1999A , Scaramagli *et al.* 1999B). In the present study, seedlings of L.*erecta* were screened initially on vertically-oriented Petri dishes containing MS media supplemented with 5mM MGBG, a level determined to be toxic in preliminary trials. This approach has proven to be a reliable screening technique for the identification of *Arabidopsis* mutants resistant to toxic levels of phytohormones such as auxin (Estelle and Somerville, 1987), cytokinin (Su and Howell, 1992), and combined resistance to auxin, ethylene, and ABA (Wilson *et al.*, 1990). This

method has also been used to isolate *Arabidopsis* mutants with abnormal gravitropic responses (Bell and Maher, 1990; Simmons *et al.*, 1995). In the present work however, the growth of large numbers of seedlings on vertical plates supplemented with MGBG was found to be very variable, making it impossible to select resistant mutants with any degree of confidence. This may be a reflection of the plasticity of the plant to perturbations in polyamine levels, compared to its more rigorous requirement for an endogenous hormonal balance.

M2 seeds were collected from 1,200 M1 plants, and from that population, approximately 30,000 seeds representing 240 M1 underwent the first primary screen on horizontally-oriented plates containing MGBG. These amounts are within the range used by other workers to isolate *Arabidopsis* mutants (Koornneef *et al.*, 1984; Bell and Maher, 1990; Wilson *et al.*, 1990; Watson *et al.*, 1998). A study by Mirza and Iqbal (1997) reported a similar screening strategy to identify spermine-resistant mutants of *Arabidopsis*, in which 300 EMS M2 seed were placed on each plate of medium containing 0·35mM spermine. The present study to detect MGBG-resistant plants also screened approximately 300 seed per plate, spaced at approximately 1-to-2mm, to ensure that localised depletion of the inhibitor did not occur. This was more laborious than screens for hormone mutants, which can be performed with up to 5,000 EMS-mutagenised M2 seeds per plate of medium containing 5μM of the auxin 2,4-D (Estelle and Somerville, 1987).

Control wild-type *Arabidopsis* seedlings occasionally recovered from the inhibition caused by exposure to 5mM MGBG if removed from the inhibitor at an early stage of growth. When transferred to soil, both the putative tolerant mutants and surviving wild-type controls displayed abnormal shoot development, analogous with MGBG-resistant primary transformants of tobacco (Fritze *et al.*, 1995), although the putative mutants were healthier than controls. The small and feeble shoot stature observed in the MGBG-treated *Arabidopsis* plants may be associated with a perturbation in the polyamine biosynthetic pathway. Cell wall thinning by MGBG has been reported in tobacco thin cell layers, which was reversible

by spermidine application (Altamura *et al.*, 1993; Berta *et al.*, 1997), and may also have contributed to the feeble and stunted phenotype of the putative mutants.

Heritability of the MGBG-tolerant phenotype

The heritability of the MGBG-tolerant trait in the mutants was evident in each line of mutant class (i) which displayed a strong tolerant phenotype, contrasting with wild-type controls consistently exhibiting sensitivity to MGBG. To ensure that exposure to the inhibitor in the preceding generation was not the reason for tolerance to MGBG in putative mutant lines, an inbred wild-type line that was germinated and grown in the presence of inhibitor and allowed to recover, was used as a source of control seeds. This 'pre-treated' control line was found to be sensitive to continued growth on the inhibitor in each generation, and was unable to set seed by the fourth generation of self-fertilisation. When putative MGBG-tolerant mutants were re-screened following several generations of continual selection and self-fertilisation, heritability of the trait was evident, however, the degree of tolerance was not uniform. Whilst overall tolerance frequencies increased in each generation, variable numbers of offspring did show sensitivity to MGBG. In studies of MGBG-resistant CHO cell lines, Heaton and Flintoff (1988) suggested that different genetic loci are involved in determining resistance to MGBG, and that more than one locus may be involved in uptake of the inhibitor in these cells.

Detailed genetic analysis of MGBG-tolerant *Arabidopsis* lines proved difficult however, since crosses of putative mutants to wild-type plants resulted in a very poor seed set, and could only be performed in one crossing direction. This phenomenon of reduced fecundity was also observed by Fritze *et al.* (1995) when crossing MGBG-resistant tobacco plants with wild-type. In the present study, chi-squared analyses of F2 seedlings, grown on appropriate levels of MGBG, from crosses of MGBG-tolerant *Arabidopsis* and wild-type plants suggested that the observed numbers of resistant-versus-sensitive offspring corresponded to the segregation pattern expected if resistance to MGBG was due to a single recessive

mutation. A similar inheritance pattern was also observed in an *Arabidopsis* mutant resistant to high levels of spermine (Mirza and Iqbal, 1997).

The suggestion that resistance to MGBG in *Arabidopsis* mutants is controlled primarily by a single recessive gene is, at first sight, difficult to reconcile with earlier results, where several generations of inbreeding increased the resistance frequency but did not produce plants with 100% tolerance. The possibility exists that the resistant phenotype of the mutants exhibited variable expressivity or penetrance, as reported for an MGBG-resistant tobacco line studied by Malmberg and Rose (1987). The possibility of pleiotrophic effects of MGBG in the mutant lines cannot be ruled out, such as antimitochondrial effects of the inhibitor (Jänne and Alhonen-Hongisto, 1989; Cheng *et al.*, 1990) or other non-specific roles, such as negative effects on plant RNA synthesis (Altamura *et al.*, 1991). Understanding the inheritance patterns involved in the mutant *Arabidopsis* plants may be dependent upon unravelling the full spectrum of MGBG effects in plants, which are yet to be resolved.

Alternatively, the inheritance of the MGBG-resistant trait may in fact follow a simple Mendelian pattern, however, the clear segregation ratios may have been clouded by the inherent variability of the *in vitro* screening methods used. More work than originally anticipated was required in progeny test experiments to ensure that the resistant phenotype was readily identifiable. Since MGBG has a tendency to precipitate in plant tissue culture (Malmberg and McIndoo, 1984), high levels could not be used in re-screening experiments. In an attempt to eliminate this possibility, the strength of the MS media was decreased to avoid precipitate formation, whilst still allowing the use of high levels of MGBG. The growth of both sensitive and resistant seedlings however, was also abolished on reduced-strength media. Reducing the level of MGBG removed the formation of a precipitate, however, proved difficult to clearly separate resistant and sensitive phenotypes of seedlings, especially those derived from crosses to wild-type, as also noted in tobacco (Malmberg and Rose 1987; Fritze *et al.*, 1995).

Phenotype of the resistant plants

In the absence of MGBG, phenotypes of wild-type sensitive plants and MGBG-tolerant mutants were indistinguishable, analogous to the *Arabidopsis ckr1* cytokinin-resistant mutant, which exhibited normal development in the absence of cytokinin (Su and Howell, 1992). The *Arabidopsis* MGBG-tolerant mutant lines however, consistently demonstrated a greater ability to recover from exposure to MGBG when removed from the inhibitor either to unsupplemented MS media *in vitro*, or into soil, than the sensitive wild-type plants. This finding is similar to that reported by Fritze *et al.* (1995) in tobacco regenerants, whereby differences in morphology between MGBG-resistant lines and wild-types only became apparent following growth in soil after initial exposure *in vitro*. After *in vitro* exposure to MGBG and transfer to soil, *Arabidopsis* plants from line 2(i)a possessed larger flowers than those of line 2(i)c and the treated wild-type controls. The observation of altered floral morphology in these mutants is generally consistent with, although not as severe as, those of other *Arabidopsis* polyamine mutants (Mirza and Iqbal, 1997) and tobacco polyamine mutants (Malmberg and McIndoo, 1983; Malmberg and McIndoo, 1984; Fritze *et al.*, 1995).

Polyamine titres

Measurements of endogenous polyamine titres were undertaken to characterise the mutant lines and compare with wild-type controls, following growth in the presence or absence of the inhibitor. Free polyamines were the predominant fraction of the polyamine pool in the Landsberg *erecta* ecotype of *Arabidopsis* used in this study, as also reported for the Columbia ecotype (Tassoni *et al.*, 2000) and for *Petunia hybrida* plants (Gerats *et al.*, 1988). Spermine was the least abundant free polyamine in *Arabidopsis* shoots, in agreement with measurements by Tassoni *et al.* (2000), and in studies of tobacco thin cell layers by Altamura *et al.* (1991). In other species, conjugated polyamines are the predominant form of polyamine, as noted in tobacco (Slocum and Galston, 1985; Tiburcio *et al.*, 1987; Burtin *et al.*, 1990), *Chrysanthemum* (Aribaud and Martin-Tanguy, 1994B), saffron (Jirage *et al.*, 1994), and *Datura* root cultures (Michael *et al.*, 1996).

Free polyamines

In the present study, the MGBG-tolerant *Arabidopsis* mutants exhibited higher titres of free, conjugated, and bound spermidine than wild-type controls when plants were grown either in the absence, or on low levels, of the inhibitor. Analogous high titres of spermidine in tobacco cell lines resistant to MGBG is suggested to offer *in vivo* protection by out-competing the inhibitor (Malmberg and Rose, 1987), due to the similar structures of spermidine and MGBG (Pegg and McCann, 1982). Free spermidine may also have a role in protecting against the general stress induced by MGBG, as it is known that spermidine offers protection to *Arabidopsis* (Kurepa *et al.*, 1998), and rice (Kao, 1997) against toxicity to the herbicide paraquat, whilst *in vitro* experiments have reported that free polyamines are effective scavengers of free radicals (Drolet *et al.*, 1986).

Increased levels of putrescine in both wild-type and mutant *Arabidopsis* plants treated with MGBG, may be expected if spermidine and spermine synthesis was reduced due to inhibition of SAMDC by MGBG. The MGBG-induced decrease in free spermidine levels with a concomitant increase in free putrescine levels observed in this study, has also been reported in mung bean stem cuttings (Jarvis *et al.*, 1983), barley seedlings (Lin, 1985), carrot cells (Minocha *et al.*, 1991B), pea seeds (Villaneuva and Huang, 1993), detached soybean leaves (Turano *et al.*, 1997), and white spruce suspension cultures (Kong *et al.*, 1998) This rise may also be a result of the inter-conversion of spermidine to putrescine, observed in plant tissues including tobacco thin cell layers (Alcamura *et al.*, 1991), *Helianthus tuberosus* chloroplasts (Del Duca *et al.*, 1995), and *Arabidopsis* (Tassoni *et al.*, 2000).

Conjugated polyamines

High levels of conjugated polyamines in the *Arabidopsis* mutants may also be a response to the stress induced by MGBG, as previously noted in tobacco thin cell layers (Scaramagli *et al.*, 1999A), or a general response to stress, as found in *Arabidopsis* (Campos *et al.*, 1991). Their high levels may even act as a signal to arrest further vegetative growth (Scaramagli *et al.*, 1999A), although resistant plants in the present work remained in the vegetative state and

did not undergo flowering when grown on high levels of MGBG. When grown in the presence of 2mM MGBG, high titres of conjugated polyamines in mutants and their complete lack in wild-type plants, are interesting observations. One reason for this discrepancy may be that conjugated polyamines themselves have an important biological function, which in this case may be to ameliorate the inhibitory effects of MGBG. Therefore, the mutation in the tolerant lines may impart a normal capacity to make polyamine conjugates, whereas the capacity of the sensitive wild-types to do so is reduced by MGBG treatment. Conjugated polyamines are implicated in having diverse, biologically active functions including roles in the suppression of cell proliferation and bud formation in tobacco leaf explants (Burtin *et al.*, 1989), the auxin-induced root formation in tobacco thin cell layers (Burtin *et al.*, 1990), the flowering of saffron corms (Jirage *et al.*, 1994), and as carriers of excess polyamines for oxidisation via the GABA pathway in maize callus (Bernet *et al.*, 1998). Conjugated polyamines may also have roles as plant growth regulators themselves, or through synergistic relationships with other plant growth regulators (Martin-Tanguy *et al.*, 1988). Alternatively, it is possible that MGBG causes major perturbations in the polyamine biosynthesis pathway, and that one compensatory response in the sensitive wild-types is to induce an active conversion between the conjugated and free polyamine forms leading to the elimination of conjugated polyamine titres. This phenomenon was originally suggested by Slocum and Galston (1985) in tobacco plants, though it was later challenged (Martin-Tanguy *et al.*, 1988; Burtin *et al.*, 1989). Interconversion between conjugated and bound polyamines in *Arabidopsis* plants however, cannot be discounted at this stage, as it is known that the accumulation of free and conjugated polyamines is species-specific, and may also depend upon the conditions of growth (Kakkar and Rai, 1993).

Bound polyamines

The rise in bound spermidine titres observed in the MGBG-tolerant mutants followed the overall increases in titres of free and conjugated spermidine, which was also seen in tobacco ovary tissues (Slocum and Galston, 1985). It has been suggested that an increase in the bound polyamine fraction in later development is related to the binding of polyamines with

macromolecules such as DNA, RNA, and proteins during the differentiation process (Bernet *et al.*, 1998). The lack of conjugated and bound spermine as found in this *Arabidopsis* study, correlates with similar findings in a range of *Arabidopsis* (Tassoni *et al.*, 2000) and tobacco (Altamura *et al.*, 1991; Burtin and Michael 1997) tissue, suggesting that in these two species at least, spermine may not be as responsive as putrescine and spermidine to changes in growth processes.

Ratio of putrescine-to-spermidine

The ratio of putrescine-to-spermidine within plant tissues has been frequently used as an important biological indicator in studies probing the roles of polyamines during growth. These include MGBG-resistant tobacco lines (Malmberg and McIndoo, 1983; Malmberg and Rose, 1987), MGBG-treated tobacco thin cell layers (Scaramagli *et al.*, 1999A), *Petunia* plants with altered floral morphologies (Gerats *et al.*, 1988), SPDS inhibition in chick pea seeds (Gallardo *et al.*, 1994), effects of pruning on hazelnut trees (Rey *et al.*, 1994), nitrate studies of *Poa* grasses (Van Arendonk *et al.*, 1998), salt stress in tomato (Santa-Cruz *et al.*, 1998), and interactions between putrescine and auxin in maize meristematic callus (Bernet *et al.*, 1998). Furthermore, Gerats *et al.* (1988) found that although there was variability in the polyamine titres per gram of leaf and floral tissue in *Petunia*, the ratio of putrescine-to-spermidine was always constant. The low putrescine-to-spermidine ratio noted after treatment with 2mM MGBG for the *Arabidopsis* MGBG-tolerant mutants, and the high ratio for the wild-types, is consistent with the observation obtained by Malmberg and McIndoo (1983) with their MGBG-resistant tobacco plants.

Northern blot analyses

Measurements of endogenous polyamine titres are important in characterising the metabolic effects of MGBG resistance, however, it is also of interest to gain an understanding of the molecular basis for that resistance. Previous reports of the action of MGBG on plant tissues have focussed on the resulting alterations of polyamine titres (Altamura *et al.*, 1991; Féray *et al.*, 1994; Kong *et al.*, 1998; Scaramagli *et al.*, 1999A), alterations in the levels or activities

of polyamine biosynthesis enzymes (Hiatt *et al.*, 1986; Minocha *et al.*, 1991A), or both parameters (Malmberg and Rose, 1987; Minocha *et al.*, 1991B; Villaneuva and Huang, 1993; Fritze *et al.*, 1995; Watson *et al.*, 1998). Some workers have only discussed the cytological effects of MGBG (Altamura *et al.*, 1993; Berta *et al.*, 1997). In addition, *SAMDC* transcripts were shown to accumulate in tobacco thin layers when treated with 0·5mM MGBG (Scaramagli *et al.*, 1999B), and also in wheat seedlings under salinity stress, possibly as a consequence of an overaccumulation of decarboxylated SAM (Li and Chen, 2000). In *Arabidopsis* plants grown *in vitro*, treatment with 0·5mM spermidine resulted in a decrease in the transcription of *SAMDC*, whereas the expression of *SPDS* remained unaffected (Tassoni *et al.* 2000).

Levels of SAMDC *transcript*

A preliminary Northern blot using RNA from wild-type *Arabidopsis* shoots demonstrated marked differences in *SAMDC* transcript levels in flowering and non-flowering plants following MGBG-treatment. Characterisation of the putative mutants by Northern blots and measurements of polyamine titres, were performed on non-flowering plants, as it has been reported that changes in polyamine levels are correlated with flowering (Kaur-Sawhney *et al.*, 1988; Caffaro and Vicente, 1994; Jirage *et al.*, 1994). Wild-type *Arabidopsis* plants grown on media containing MGBG showed increased *SAMDC* transcript levels relative to untreated controls. Together with reports that the inhibitor has specific effects on polyamine titres in carrot cell cultures (Minocha *et al.*, 1990), and also that it possesses the ability to inhibit SAMDC enzyme activity in pea seeds (Villaneuva and Huang, 1993), this result suggests that whatever other non-specific effects MGBG may have on plant growth, it is undoubtedly a modulator of polyamine biosynthesis.

Further characterisation of the MGBG-tolerant 2(i)a and 2(i)c lines revealed phenotypic difference in sub-lines of both classes, although they were both originally derived from the same seed pool. Untreated 2(i)a sub-lines possessed lower overall levels of *SAMDC* transcript than those of the 2(i)c lines, whilst also displaying a higher overall tolerance to

MGBG. Differences between possible siblings were also observed by Malmberg and McIndoo (1984) in which two dramatically different floral morphologies were reported for separate MGBG-resistant tobacco regenerant lines originating from the same mutagenised cell culture. In that report, and the present work, the possibility remains however, that the siblings may have originated from different mutational events. Future experiments should cross the lines with each other to determine if the mutations are allelic. The differences in *SAMDC* transcript levels for the two *Arabidopsis* mutant lines analysed in detail were confirmed when Northern analyses were performed using plants grown on lower levels of MGBG.

Increased transcription of *SAMDC* may be associated with an elevated capacity to produce decarboxylated SAM, which in turns acts as an aminopropyl donor for the production of spermidine and spermine. High titres of free spermidine measured in untreated mutants however, was not attributable to an increase in *SAMDC* transcript level, as mRNA levels in mutants were the same as, or lower, than those of wild-types. The low level of selection imposed by 0·5mM MGBG was sufficient to stimulate an increase in *SAMDC* transcript levels in sensitive wild-type shoots, whereas in mutant lines it was barely altered. Similarly, treatment of wild-type tobacco thin cell layers with MGBG increased *SAMDC* mRNA levels (Scaramagli *et al.*, 1999B). Although *SAMDC* transcript levels were unchanged, the free spermidine pool in all *Arabidopsis* mutants decreased in response to 0·5mM MGBG-treatment. This may be a function of the high basal level of spermidine in mutant tissue, which overcomes the inhibition of spermidine synthesis caused by MGBG. Furthermore, high levels of spermidine may result in reduced transcription of the *SAMDC* gene as recently reported in *Arabidopsis* (Tassoni *et al.*, 2000). Thus, essentially control levels of transcripts are maintained in the MGBG-tolerant mutants in response to low levels of MGBG.

Levels of ADC *transcript*

MGBG is known to decrease the activity of ADC in tobacco (Hiatt *et al.*, 1986) and carrot cell cultures (Minocha *et al.*, 1991B), however, not when added directly to the ADC enzyme mix

itself *in vitro* (Minocha *et al.*, 1991 B). In the present study, examining the effects of MGBG on transcript levels of the *ADC* gene showed similar patterns of transcript accumulation for the wild-type and mutant plants. Low *ADC* transcript levels in *Arabidopsis* shoots from all lines, regardless of treatment, are in accordance with recent results obtained by Tassoni *et al.* (2000) in *Arabidopsis* where very low levels of *ADC* transcript were reported in plants before and after spermidine treatment. Similarly, Soyka and Heyer (1999) could barley detect *ADC* expression in *Arabidopsis* stem tissue, although they did find expression in leaves. Furthermore, low ADC activity measured in *Arabidopsis* (Watson *et al.*, 1998) compared with other species, may be a reflection of the low *ADC* mRNA levels in this species. Although *Arabidopsis* is known to have more than one *ADC* gene (Galloway *et al.*, 1998; Watson *et al.*, 1998; Soyka and Heyer, 1999) only one band of 1·2kb was observed in the RNA blots in this study, corresponding with the *ADC1* band of the same size observed by Soyka and Heyer (1999). Overexpression of an oat *ADC* gene in tobacco had limited effects in influencing polyamine biosynthesis (Burtin and Michael, 1997), and suggests that major differences may not be expected at the *ADC* transcript level between MGBG-tolerant *Arabidopsis* mutants and wild-type controls due to strict regulation of this gene. Furthermore, the strict control of *ADC* expression in the present study of *Arabidopsis* is expected, due to the importance of ADC as the only step to putrescine production in this plant which lacks ODC (Hanfrey *et al.*, 2001).

Levels of SPDS *transcript*

The *SPDS* gene was found to be easier to detect than *ADC* in Northern blots in both wild-type and mutant shoots. The higher basal level of free spermidine in untreated mutant lines was not attributable to increased *SPDS* transcript levels, as mutants had essentially the same levels as wild-type controls. 2mM MGBG treatment induced the largest accumulation of *SPDS* transcripts in all lines, as may be expected if MGBG acted by inhibiting SAMDC, thereby decreasing pools of free spermidine and spermine. Under such conditions, reductions in spermidine and spermine production by MGBG may be minimised *in vivo* by upregulation of the *SPDS* gene, thus ensuring that SPDS is not a limiting factor in the

synthesis of spermidine from putrescine. Conversely, the down-regulation of *SPDS* expression may be a less sensitive mechanism than that described above, and is only activated when spermidine levels reach cytotoxic proportions, as feeding spermidine to *Arabidopsis* plants was not found to affect the expression of *SPDS* (Tassoni *et al.* 2000). An upper threshold may exist for endogenous spermidine levels within plant cells, based on the finding that attempts to produce transgenic potato plants constitutively expressing the *SAMDC* gene were unsuccessful, and only an inducible promoter expressing the gene resulted in viable plants (Kumar *et al.*, 1996). Furthermore, plant cells possess mechanisms to combat rising endogenous levels of spermidine, by conversting it to putrescine via the action of spermidine/spermine N^1-acetyltransferase (SSAT) (Del Duca *et al.*, 1995; Tassoni *et al.*, 2000). From the present study it appears that a high level of putrescine *in vivo* is not toxic to *Arabidopsis*.

Bases for tolerance to MGBG

The observations of altered polyamine accumulation in MGBG-tolerant lines of *Arabidopsis* are interesting, although it remains to be determined whether they are the causal factors of tolerance to MGBG or simply a response to it. Due to the low level of *SAMDC* transcripts in mutants, one explanation for the basis of resistance that can be probably be excluded, is over-expression of SAMDC mRNA resulting from either gene amplification or increased expression due, for example, to an alteration in promoter structure. This has previously been reported for mammalian cells 1000-fold more resistant than controls, to a potent MGBG analogue, CGP 48664 (Regenass *et al.*, 1994) or resistant to DFMO (Hirvonen *et al.*, 1989). Several possibilities may explain the observed resistance to MGBG in the *Arabidopsis* mutants. Firstly, since all mutant sub-lines analysed displayed a higher accumulation of free, conjugated, and bound spermidine that appeared not to be due to increased transcript levels of either *SAMDC* or *SPDS*, it may be that mutants have an alteration in the regulatory mechanisms governing spermidine biosynthesis or metabolism. The altered regulation of spermidine production is also manifested in mutants as a lower ratio of putrescine-to-spermidine than wild-type controls. Another possibility is that the mutation has altered the

SAMDC enzyme itself, thereby reducing its sensitivity to MGBG, allowing mutants to grow in the presence of the inhibitor. The observations of elevated free spermidine in mutants grown on medium lacking MGBG, and also reduced free putrescine in mutants grown on 2mM MGBG, both relative to wild-type controls, would be compatible with this possibility. A mutation in the *SAMDC* gene was also proposed as the basis for resistance in two MGBG-resistant lines of tobacco (Malmberg and Rose, 1987). Furthermore, it has been suggested that there is a degree of transcriptional regulation of *SAMDC* in *Arabidopsis* (Tassoni *et al.*, 2000) and also in wheat seedlings following drought stress (Li and Chen, 2000). Mutants isolated in the present study exhibiting low transcript abundance in the presence of MGBG, may be useful for correlating gene expression, polyamine levels, and enzyme activities with growth processes. It may be possible that the mutant *Arabidopsis* plants have an alteration in the cellular capacity for polyamine and MGBG uptake since they both may share the same transport system, which was reported for a mammalian cell line exhibiting resistance to MGBG (Rodrigues *et al.*, 1987). Although this is unlikely since the growth of mutants on MGBG resulted in perturbations of the endogenous titres of polyamines and also interfered with the accumulation of *SAMDC* transcripts, it cannot be excluded however, as these alterations may be non-specific, secondary effects of MGBG.

CHAPTER 6:

CONCLUSIONS AND FUTURE DIRECTIONS

THIS STUDY PRESENTS AN examination of the involvement of polyamines during several aspects of *Arabidopsis* growth and development. At the onset of this work, few studies existed reporting the roles of polyamines in *Arabidopsis*, or the regulation of their biosynthesis, thus it was aimed to provide insights about such processes using this plant as a model.

The first approach used in this study was to document the effects of perturbing polyamine biosynthesis on morphology, polyamine levels, and regulation of the key polyamine biosynthetic genes in wild-type *Arabidopsis* by creating excesses or depletions of *in vivo* polyamine titres. Treatment of plants with the polyamines putrescine, spermidine, or spermine, or their biosynthetic inhibitors DFMA, DFMO, CHA, or MGBG perturbed polyamine titres, correlating with morphological alterations such as reduced plant height, altered root growth, and delayed flowering. Further examination of the effects of treatments on root growth found that elongation of the primary root was either stimulated or inhibited by exogenous polyamines or inhibitors, respectively. On the other hand, the overall

numbers of lateral roots produced were unaffected by the three polyamines or the inhibitors CHA or MGBG. Treatment with DFMO, however, caused a minor stimulation of lateral root production when applied at high levels. Interestingly however, treatment with inhibitors appeared to simulate the process of lateral root induction, such that primordia appeared rapidly after treatment, compared to untreated controls which were somewhat delayed. Furthermore, the normal stimulation of lateral and adventitious roots noted with auxin treatment was reduced when polyamines were added simultaneously with auxin. These results agree with the view that polyamines have key roles during root development and suggests that although specific treatments have varied effects, the specific levels of polyamines may be important factors in maintaining normal development. Alterations that increase or decrease such optimal levels may therefore result in perturbations of growth. The mechanisms responsible for root primordia stimulation by inhibitor treatments warrant further examination. A recent study of lateral root development in *Arabidopsis* suggests the existence of signals which trigger quiescent G2 phase pericycle cells to re-enter the cell cycle and undergo *de novo* differentiation into lateral root meristems (Beeckman *et al.*, 2001). It is possibile that a reduction in polyamine levels induced by the inhibitors, acts as such a trigger for cells destined for lateral root meristem formation, hence, resulting in the proliferation of lateral root primordia earlier than untreated control seedlings. The results of the present study are in general agreement with the notion that reductions in polyamine levels facilitate increased root growth, as suggested by findings of Watson *et al.* (1998) that *Arabidopsis* mutants possessing low ADC activity exhibited increased levels of lateral root branching.

Of course there are certain limitations of the application of polyamines or inhibitors to plants in order to study effects of polyamine metabolism on growth and development. Studies using exogenously applied polyamines or inhibitors yield only correlative relationships between polyamines and growth, and are therefore somewhat limited in their scope. Importantly, inhibitors may also have effects *in vivo* other than depleting polyamine levels (Jänne and Alhonen-Hongisto, 1989; Cheng *et al.*, 1990), and hence, the results should be interpreted with caution. Furthermore, inhibitors added exogenously cannot target the

required cell types or developmental events to impair specific growth processes, and results only reflect perturbations at the level of the whole tissue or plant. The application of inhibitors is also reliant on functional uptake and transport processes, which change according to the polyamine status of the tissues (Byers and Pegg, 1990), thus variable efficacies may result following inhibitor treatments.

A second aspect of the present study involved testing the hypothesis that the hairy-root phenotype, noted in tobacco plants transformed with the Ri T-DNA of *A. rhizogenes*, is caused by reduced polyamine titres in transformants (Martin-Tanguy *et al.*, 1990). As expression of the *rol* genes from the T-DNA markedly alters the responses of transformed plant cells to auxin (Maurel *et al.* 1991), speculation exists in the literature that the reduced polyamines levels observed in Ri T-DNA transformants are merely secondary consequences of altered hormonal status (Michael and Spena, 1995). In the present study, *Arabidopsis* transformants containing the Ri T-DNA were used to verify the hypothesis in a species other than tobacco. In addition, *Arabidopsis* transformants containing only the *rolABC* genes were generated, and found to also exhibit a similar, though less severe, phenotype as the Ri T-DNA plants. In agreement with the observations of Martin-Tanguy *et al.* (1990) working with tobacco, wild-type *Arabidopsis* plants treated with the inhibitors DFMA and DFMO exhibited morphological alterations resembling the hairy-root phenotype. Furthermore, polyamine titres were also reduced in tissues of DFMO-fed wild-type plants, and also Ri T-DNA and *rolABC* transformants. Polyamine levels in transformants were inversely related to the degree of phenotypic change in such plants.

Interestingly however, expression of the polyamine biosynthetic genes were unaffected in Ri T-DNA- and *rolABC*-transformed tissues, and enzyme assays are therefore required to determine if any alterations in activity exist relative to controls, which may indicate post-transcriptional regulation of these genes to be operational. That the phenotypes of *rolABC* transformants were not ameliorated or exacerbated following growth in the presence of polyamines or inhibitors, suggests that reduced titres of polyamines are not causal to the

hairy-root phenotype. In addition, *rolABC* plants exhibited stimulated root growth in response to low levels of IAA and also possessed altered internal structures analogous to published observations on auxin mutants, suggesting increased auxin sensitivity in these lines. Thus, alterations in phytohormone metabolism may indeed be the cause of perturbed growth observed in transformants and reduced polyamine levels detected in such plants may therefore be an indirect consequence of the hormone-induced disruptions in growth. To further characterise the *rolABC* transformants generated in this study, complementation tests using a group of auxin mutants displaying similar auxin-sensitive phenotypes may prove useful in understanding the basis of the altered hormone metabolism in such plants.

One aspect of this work which also deserves particular consideration in the light of a recent report that *Arabidopsis* lacks ODC (Hanfrey *et al.*, 2001), is the observation that treatment of these plants with DFMO depleted putrescine titres and induced phenotypic changes analagous to similarly-treated tobacco plants (Martin-Tanguy *et al.*, 1990). Similar observations were also reported by Hanfrey *et al.* (2001) who noted that simultaneous treatment with putrescine did not alter the inhibitory effects of DFMO. It may be that DFMO inhibits another enzyme important in the growth of *Arabidopsis*, which in turn affects growth and development and polyamine metabolism. The identity of such an enzyme(s) remains speculative at this time.

The third approach used in this study attempted to recover novel *Arabidopsis* mutants with altered polyamine metabolism, in order to contribute information regarding this process in plants. An initial method to screen for mutants was based on the identification of soil-grown plants possessing increased tolerance to high levels of the polyamine inhibitor CHA, when applied topically. Plants treated in such a method however, were relatively unaffected, and thus an *in vitro* screening strategy was thought to provide a more accurate inhibition of growth. In an attempt to recover mutants possessing altered regulation of either the two rate-limiting enzymes in polyamine biosynthesis, ODC and SAMDC, respective screening strategies were based on resistance to high levels of ornithine, or the SAMDC inhibitor, MGBG. Several individuals resistant to 10mM ornithine were isolated, however,

characterisation was precluded due to sterility of these plants. In hindsight, screening for such mutants in *Arabidopsis* may not lead to polyamine alterations, due to the reported absence of ODC in that plant (Hanfrey *et al.*, 2001). Characterisation of such mutants however, may reveal whether *Arabidopsis* has a capacity for ornithine uptake similar to other plants which have ODC. Several lines with elevated tolerance to 5mM MGBG were also recovered, and two were further characterised based on inheritance of the tolerant phenotype. Polyamine assays of the variants found that the basis for tolerance to MGBG may be due to elevated basal levels of free spermidine, which was not attributable to increased levels of *SPDS* transcript and/or reduced putrescine in the presence of MGBG. Furthermore, in response to MGBG treatment, levels of conjugated and bound spermidine increased in the variant lines, whereas levels in wild-type controls were undetectable or low. Of the two MGBG-tolerant lines characterised, one exhibited a stronger resistant phenotype, correlating with lower levels of SAMDC transcript when grown in the presence of MGBG.

The results of the mutant screening experiments provide useful information on which to base future studies. Although MGBG has been widely used in the literature for perturbing polyamine biosynthesis in a range of plants (Altamura *et al.*, 1991; Minocha *et al.*, 1991B; Santanen and Simola, 1992; Altamura *et al.*, 1993; Villaneuva and Huang, 1993; Berta *et al.*, 1997; Kong *et al.*, 1998; Turano *et al.*, 1997; Scaramagli *et al.*, 1999A; Scaramagli *et al.*, 1999B, Tassoni *et al.*, 2000) and for the identification of MGBG-resistant mutants in tobacco (Malmberg and McIndoo, 1983; Malmberg and McIndoo, 1984; Malmberg and Rose, 1987; Fritze *et al.*, 1995), MGBG is proposed to have effects other than depressing polyamine biosynthesis in plants (Altamura *et al.*, 1991; Féray *et al.*, 1994) possibly including antimitochondrial activity as noted in animal cells (Jänne and Alhonen-Hongisto, 1989; Cheng *et al.*, 1990). Therefore, the somewhat variable penetrance of MGBG tolerance in offspring of *Arabidopsis* resistant mutants may be attributable to the combined inhibition of polyamine biosynthesis and mitochondial function. Thus, due to the possibility of non-specific effects, the continued use of MGBG in polyamine studies may be limited. Furthermore, the inability to readily identify variant plants when grown in the absence of the

inhibitor, limits future studies determining the basis, or the biological roles, of elevated titres of free spermidine noted in untreated variants. Such variants however, may be useful in studying the biological roles of free spermidine or conjugated polyamines in general, especially as indicators of stress, as suggested in studies of *Arabidopsis* (Campos *et al.*, 1991; Kurepa *et al.*, 1998) and tobacco plants (Scaramagli *et al.*, 1999B).

The methods presented here for the recovery of plants with mutations in polyamine biosynthesis may be useful for similar screening strategies in the future. The development and availability of more specific polyamine biosynthesis inhibitors such as CGP 48664, a potent MGBG analogue (Regenass *et al.*, 1994) may allow additional *in vitro*- or greenhouse-based screening methods in the future. Although more laborious, screening for mutants *in vitro* as described here provides a more sensitive method to detect variations in plant growth.

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APPENDICES

```
1     AGACTTGTTGTTCGGTTTCCTGATGTTCTGAAGAATCGTCTTGAATGTCT
      ||| ||||||| || ||||||||||||||||||||||||| |||||
690   CCGCTTATTGTTCGTTTCCCTGATGTTCTGAAGAATCGTCTTGAGTGTCT

51    TCAATCAGCGTTTGATTACGCGATTCAGAGTCAAGGATATGATTCTCATT
      |||||| ||||||||||||||||||||||||||||||||||||||||||
740   TCAATCCGCGTTTGATTACGCGATTCAGAGTCAAGGATATGATTCTCATT

101   ACCAAGGTGTGTATCCTGTGAAATGTAATCAAGATCGGTTTATTATCGAA
      |||||||||||||||||||||||||||||||||||||||| ||||||
790   ACCAAGGTGTGTATCCTGTGAAATGTAATCAAGATCGGTTTATCATCGAA

151   GATATTGTGGAATTCGGATCCGGTTTTCGATTCGGTTTAGAAGCTGGTTC
      |||||||| ||||||||||||||||||||||||||||||||||||||||
840   GATATTGTCGAATTCGGATCCGGTTTTCGATTCGGTTTAGAAGCTGGTTC

201   CAAGTCTGAGATTCTTCTTGCTATGAGTTGTTTGTGTAAAGGTAATCCTG
      |||| ||||||||||||||||||||||||||||||||||||||||||||
890   CAAGCCTGAGATTCTTCTTGCTATGAGTTGTTTGTGTAAAGGTAATCCTG

251   AAGCTTTTCTTGTGTGTAATGGCCTCAAAGACTCTGAGTATATCTCATTG
      |||||||||||||||||||||||| ||||||||||||||||||||||||
940   AAGCTTTTCTTGTGTGTAATGGTTTTAAAGACTCTGAGTATATCTCATTG

301   GCTTTGTTTGGGAGGAAAC
      |||||||||||||||||||
990   GCTTTGTTTGGGAGGAAAC
```

Appendix 1: DNA sequence alignment comparing the PCR product amplified from *Arabidopsis* using *ADC* primers (upper), with that of the published sequence for *ADC* from *Arabidopsis* (lower) [Accession number: ATU52851].

Sequencing of the PCR product was performed at both the 5′ and 3′ ends. The sequences of both products were aligned with that of the published sequence, and the high level of identity confirmed the isolation of a fragment of the *Arabidopsis ADC* gene. The 5′ sequence alignment is presented above. The forward oligonucleotide primer is indicated in red.

```
1     TCAACTTAATCGTTTCTCTCAAATTTAGGGTTTTCTCTTTTCTCGAAAGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1     TCAACTTAATCGTTTCTCTCAAATTTAGGGTTTTCTCTTTTCTCGAAAGT

51    CTTGCGGTTTTCTGAATCATCTCTATCTGGTGAGTCTCTTTTTTGAATTG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
51    CTTGCGGTTTTCTGAATCATCTCTATCTGGTGAGTCTCTTTTTTGAATTG

101   TAGATCTGCGTTTTCTCTCTGTAGATCTGTTCCGTCGTTGAGGATAATGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
101   TAGATCTGCGTTTTCTCTCTGTAGATCTGTTCCGTCGTTGAGGATAATGT

151   TTTTTAATCGGTGAATTCTGGGTTTTTCTCAGGTTTGAGGGTTTCGTTTG
      |||||||||||||||||||||||||| ||||||||||||||||||||||
151   TTTTTAATCGGTGAATTCTGGGTTTTTTTCAGGTTTGAGGGTTTCGTTTG

201   ATATCTGGAGAAAGGGGTTTCTGGAAACAAGGAGTTCATAATTCGCGATC
      | ||||||||||||||||||||||||||||||||||||||||||||||||
201   AGATCTGGAGAAAGGGGTTTCTGGAAACAAGGAGTTCATAATTCGCGATC

251   TTGATCTATCGATCTTCATTTATATATGTAAGTATCTCTCACGATTGACG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
251   TTGATCTATCGATCTTCATTTATATATGTAAGTATCTCTCACGATTGACG

301   TCGTTCTTCGATTGATTCATTAGCCTAGCTAGGTTAGATAGACATCGTTC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
301   TCTTTCTTCGATTGATTCATTAGCCTAGCTAGGTTAGATAGACATCGTTC

351   TTATCGTCCTAGGTTTTGGTATAAGAGATTGATATTCGCATGCATGGTTA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
351   TTATCGTCCTAGGTTTTGGTATAAGAGATTGATATTCGCATGCATGGTTA

401   GGTTGACTTA
      ||||||||||
401   GGTTGACTTA
```

Appendix 2: DNA sequence alignment comparing the PCR product amplified from *Arabidopsis* using *SAMDC* primers (upper), with that of the published sequence for *SAMDC* from *Arabidopsis* (lower) [Accession number: Y07765].

Sequencing of the PCR product was performed at both the 5′ and 3′ ends. Both sequencing products were aligned with the published sequence, and found to be identical, confirming the isolation of the *Arabidopsis SAMDC* gene. The 5′ sequence alignment is presented above. The forward oligonucleotide primer indicated in red.

```
1     GAAAAACCCT AGTTCCTCCT CTCTCTCATT TCTCGGAGAT ATTCACCAGA
51    GCAATAACCA TGGACGCTAA AGAAACCTCT GCCACCGATT TGAAAAGACC
101   GAGAGAAGAG GATGATAACG GCGGCGCNCT ACCATGGAGA CGGAGAACGG
151   AGATCAGAAA AAGGAACCTG CTTGTTTCTC CACTGTTATT CCTGGGTGGT
201   TCTCTGAAAT NAGTCCTATG TGGCCAGGAG AGGCACACTC ATTGAAGGGT
251   TGAGAAAGTT TTGTTTCAAG GGGAATCAGA TTTATCANGG ATGTTATTGT
301   TTTCCAGTCT TGCAACATAT NGAAAAAGTT TTTGGGTTTT GGGATGGGNG
351   TAAT
```

Appendix 3: DNA sequence of *Arabidopsis* EST clone 8916T7 [Accession number: T20920].

The *Arabidopsis* database was searched using the key word of 'spermidine', and this clone was identified. It has limited amino acid similarity (24%) with *speE*, the human *spermidine synthase* gene [Accession number: P19623]

[T20920]	*SPDS* EST *Arabidopsis*	EMSPMWPGEAHSLK	
[D28506]	A411 tobacco	EFSALWPGEAFSLK	104
[P19623]	*SPDS* human	ETCSLWPGQALSLQ	36
[L19311]	*SPDS* mouse	ETCSLWPGQALSLQ	36

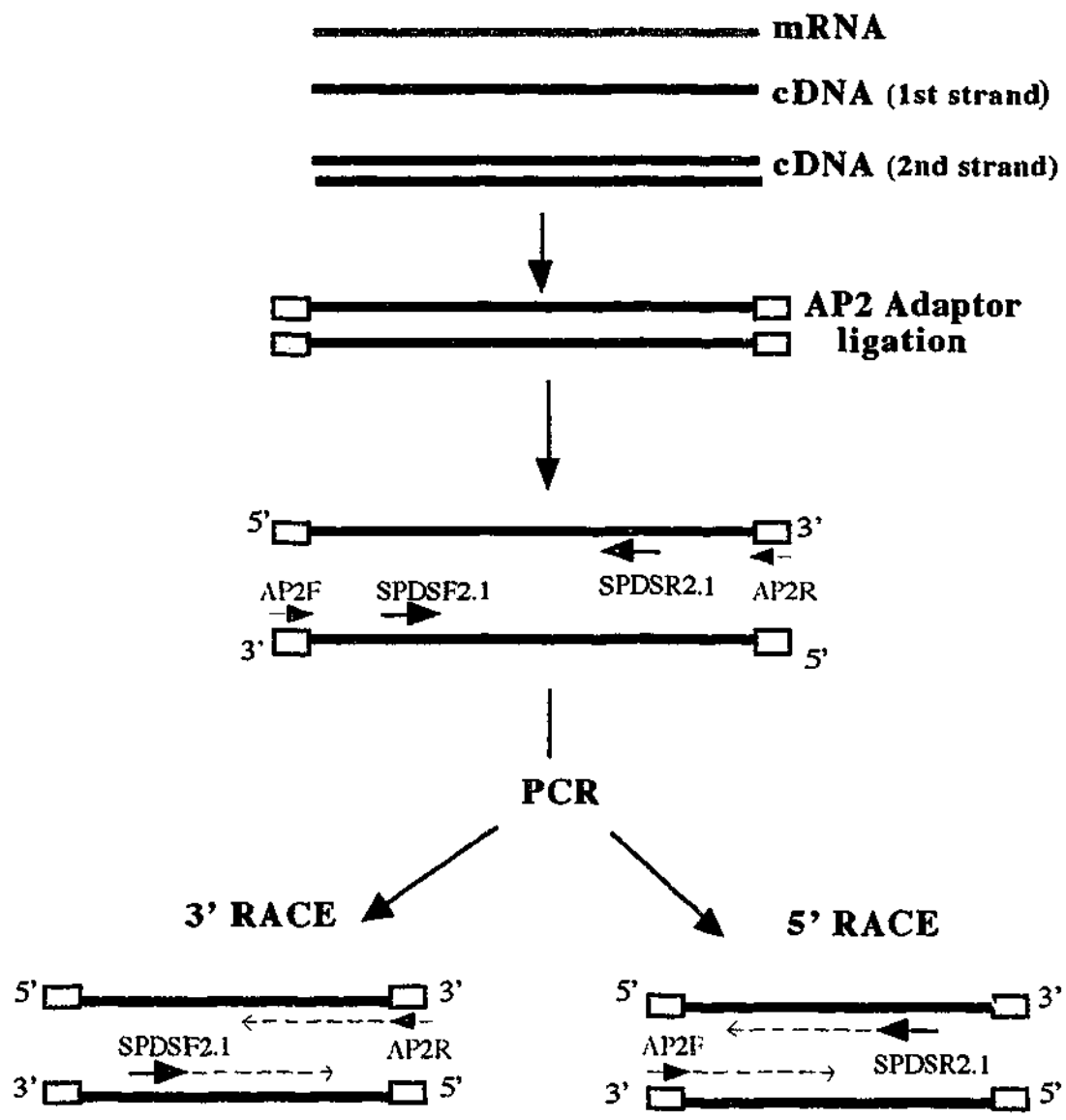
Appendix 4: Partial amino acid sequence comparison of *Arabidopsis* EST clone 8916T7 with a putative SPDS sequence from tobacco and the SPDS sequences from human and mouse. The GenBank accession numbers are listed on the left-hand side, and the conserved amino acid residues across the four species are indicated in red.

The *Arabidopsis* EST sequence with homology to *SPDS* was translated in all six open reading frames. Frame 2 exhibited the highest level of similarity (54%) with the putative SPDS sequence from tobacco, and part of it is presented above. In order to amplify part of the *SPDS* gene from *Arabidopsis* using PCR, a forward oligonucleotide primer was designed based on the *Arabidopsis* EST amino acid residues MWPGEAH.

```
1     ............................ .GA AAAACCCTAG TTCCTCCTCT
                                       ||||     |  |||
101   AGAATGGTGC CATTCCCATG AACGGCCACC AAAATGGCAC TTCTGAACAC

23    CTCTCATTTC TCGGAGATAT TCACCAGAGC AATAACCATG GACGCTAAAG
      |||          |    ||           |   |  |  |  | | | | |
151   CTCAACGGCT ACCAGAATGG CACTTCCAAA CACCAAAACG GGCACCAGAA

73    AAACCTCTGC CACCGATTTG AAAAGACCGA GAGAAGAGGA TGATAACGGC
        |   | |  | |        |      | | |||  |   |  |   |||  |
201   TGGCACTTTC GAACATCGGA ACGGCCACCA GAATGGGACA TCCGAACAAC

123   GGCGCGCTAC CATGGAGACG GAGAACGGAG ATCAGAAAAA GGAACCTGCT
       |  ||  ||  ||        || ||  ||       ||  |  |  ||  |
251   AGAACGGGAC AATCAGCCAT GACAATGG.......CAACGA GCTACTGGGA

173   TGTTTCTCCA CTGTTATTCC TGGGTGGTTC TCTGAAATGA GTCCTATGTG
       | | |  |  || |||  || ||| |||||  || ||  | |  |  |  | ||
295   AGCTCCGACT CTATTAAGCC TGGCTGGTTT TCAGAGTTTA GCGCATTATG

223   GCCAGGAGAG GCACACTCAT TGAAGGGTTG AGAAAGTTTT GTTTCAAGGG
      ||||||  ||  |||  ||||  | || |||||  ||||  |  |  || || |||
345   GCCAGGTGAA GCATTCTCAC TTAA.GGTTG AGAAGTTACT ATTCCAGGGG

273   GAATCAGATT TATCAGGGAT GTTATTGTTT TCCAGTCTTG CAACATATGG
       | ||  ||||    |   |||  ||  ||  | |  |  ||||  |  |||| |||||
394   AAGTCTGATT ..ACCAAGAT GTCATGCTCT TTGAGTC.AG CAACTTATGG

323   AAAAAGTTTT TGGGTTTTGG GATGGGGGTA ATG................
       ||   | |      |    | ||        | |
441   GAAGGTTCTG ACTTTGGATG GAGCAATTCA ACATACAGAG AATGGTGGAT
```

Appendix 5: DNA sequence alignment comparing the *Arabidopsis* EST for *SPDS* (upper) with the putative tobacco SPDS A411 (lower). The forward oligonucleotide primer (SPDSF1) used in the PCR amplification is indicated in blue.

New PCR primers for subsequent amplifications were synthesised and are indicated in red; the upper red sequence is the forward primer SPDSF2·1 and the lower red sequence is the reverse primer SPDSR2·1.



Appendix 6: Schematic representation of the Rapid Amplification of cDNA Ends (RACE) of the *SPDS* gene from *Arabidopsis*.

Following the synthesis of first and second strand cDNA, AP2 adaptors (yellow boxes) were ligated to the ends of cDNA fragments. Both adaptor-specific (AP2F and AP2R) and gene-specific (SPDSF2.1 and SPDSR2.1) primers were used, the latter pair based on an *Arabidopsis* EST fragment. 3' RACE was performed using the AP2R and SPDSF2.1 primers while 5' RACE utilised the AP2F and SPDSR2.1 primers.

```
1     CCACGCGTCC GTGGTTCTCT GAGATTAGTC CTATGTGGCC AGGAGAAGCA
                 |||||||||| || |||||||| |||||||||| |||||| |||
1     ..........GTGGTTCTCT GAAATTAGTC CTATGTGGCC AGGAGAGGCA

51    CATTCTCTCA AGG.TAGAGA AGATTCTATT CCAAGGGAAA TCAGATTACC
      || ||   | | ||| | |||| |  || | ||  |||||| || |||||||
41    CACTCATTGA AGGGTTGAGA AAGTTTTGTT TCAAGGGGAA TCAGATTTAT

100   ..AGGATGTT ATTGTTTTCC AGTCTGC
        |||||||| |||||||||| |||||
91    CANGGATGTT ATTGTTTTCC AGTCTTG
```

Appendix 7: DNA sequence alignment showing the overlapping regions of *Arabidopsis SPDS* (upper) [Accession number: ABO06693] with the *Arabidopsis SPDS* EST (lower) [Accession number: T20920].

By the end of the study, a partial coding sequence for the *Arabidopsis SPDS* gene became available in the database [Accession number: ABO06693]. In order to confirm that the original PCR amplifications were performed on *bona fide* regions of the *Arabidopsis SPDS* sequence, the position of two of the oligonucleotide primers used in the PCR amplifications are highlighted on the *Arabidopsis* EST sequence above; the SPDSF1 primer is in blue, and the SPDSR2·1 primer is in red. [It should be noted that these two primers were never used in combination with each other, and are included here in order to present their location within the published *SPDS* sequence.]

	N. tabacum ODC	*M. japonica LDC*
E. coli LDC - Forward sequence	41%	61%
E. coli LDC - Reverse sequence	37%	35%
M. japonica LDC - Forward sequence	40%	-
M. japonica LDC - Reverse sequence	37%	-

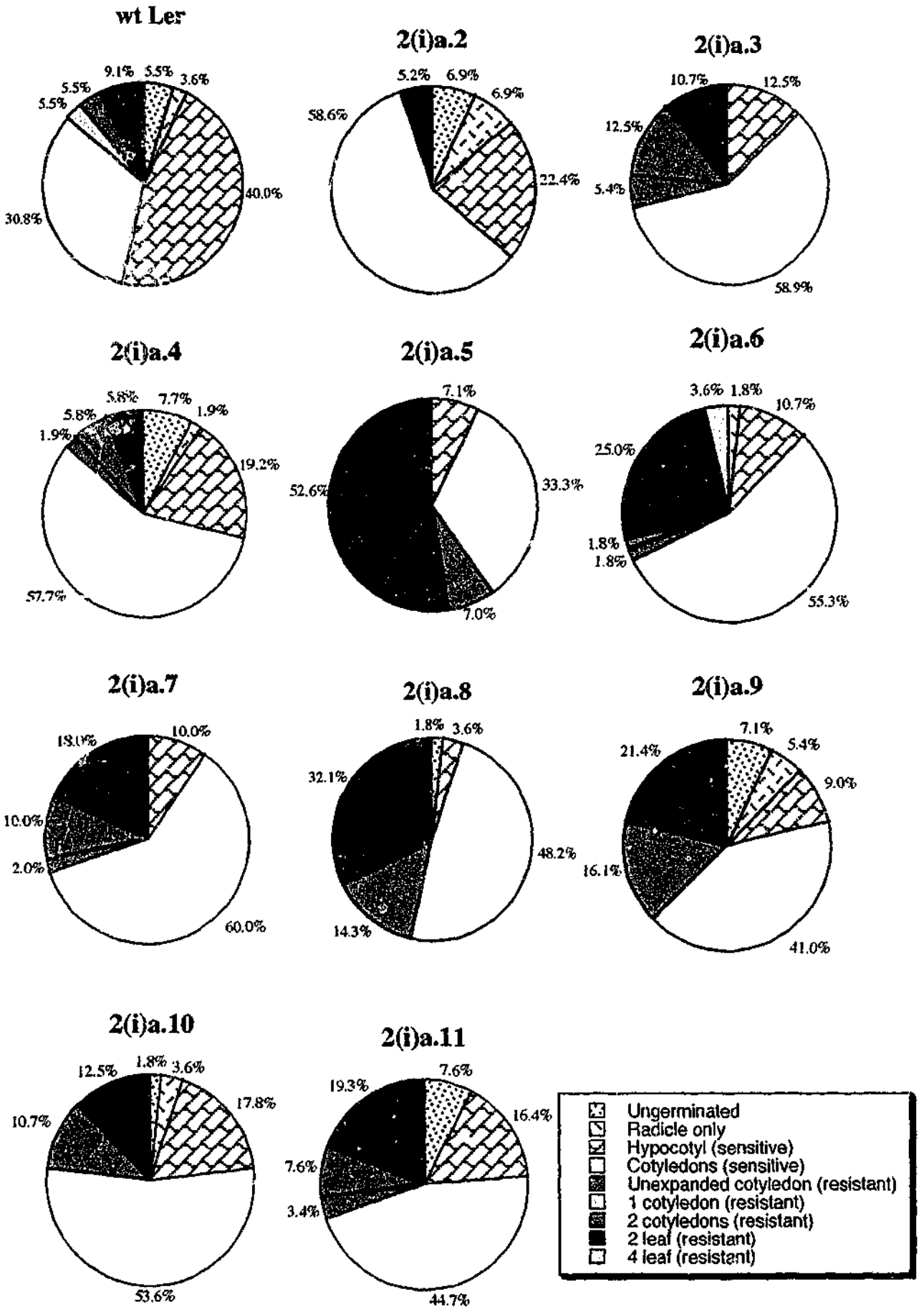
Appendix 8: A comparison of DNA sequence homology between the ornithine decarboxylase gene (*ODC*) from *Nicotiana tabacum*, and the lysine decarboxylase genes (*LDC*) from both *Escherichia coli* and *Moritella japonica*.

[GenBank accession numbers: *N. tabacum ODC* = Y10474
E. coli LDC = BSCADDNA; *M. japonica LDC* = AB041735]

Arabidopsis clone number	*E. coli LDC*	*M. japonicus LDC*
1 - Forward sequence	36%	35%
1 - Reverse sequence	37%	35%
2 - Forward sequence	35%	34%
2 - Reverse sequence	34%	32%
3 - Forward sequence	36%	35%
3 - Reverse sequence	36%	34%
4 - Forward sequence	36%	35%
4 - Reverse sequence	34%	35%

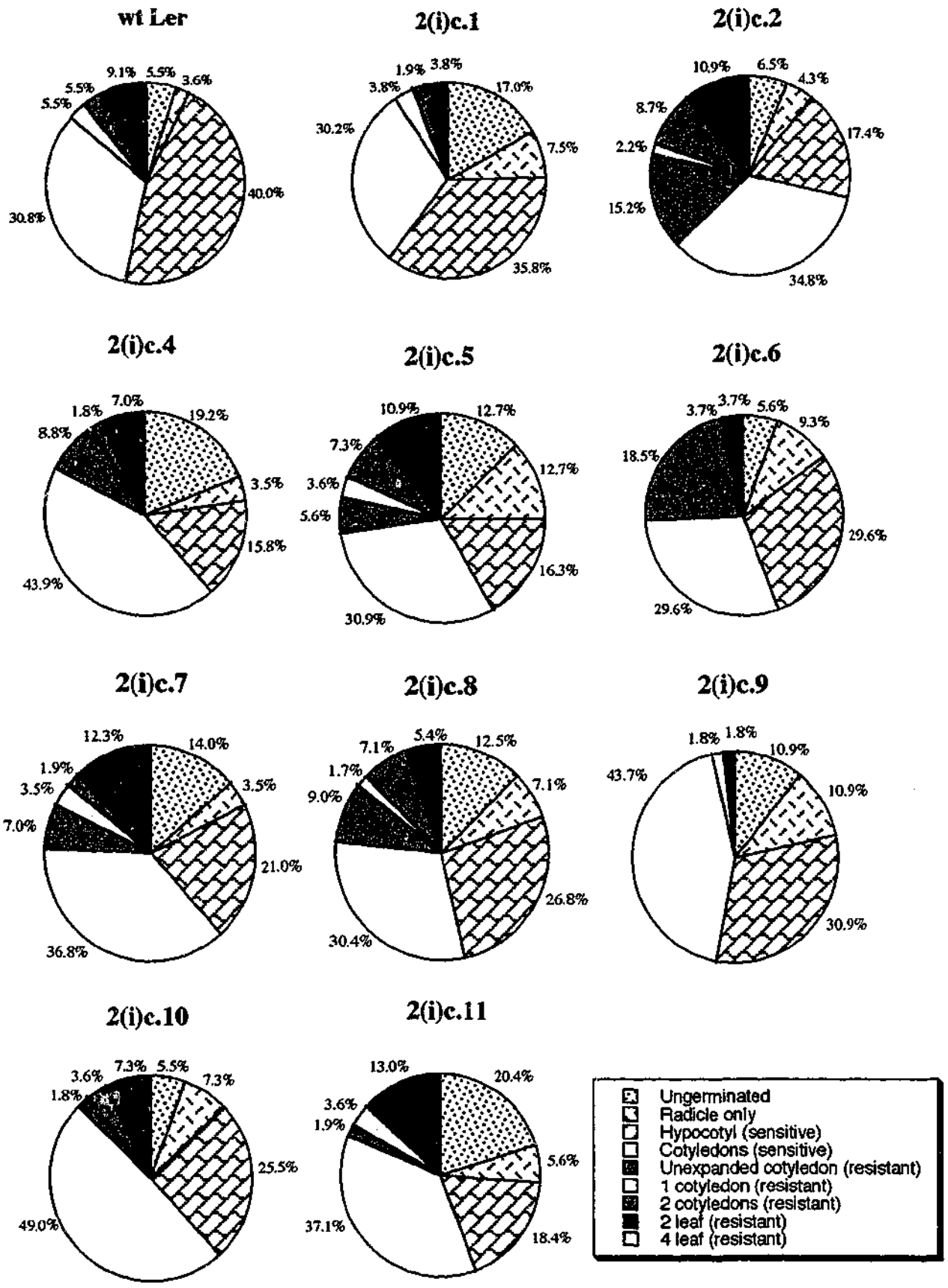
Appendix 9: A comparison of DNA sequence homology between the potential positive clones isolated from screening an *Arabidopsis* root library using a tobacco *ODC* gene fragment, with genes coding for lysine decarboxylase (*LDC*) from both *Escherichia coli* and *Moritella japonica*.

LINE 2(i)a grown on media containing 3mM MGBG



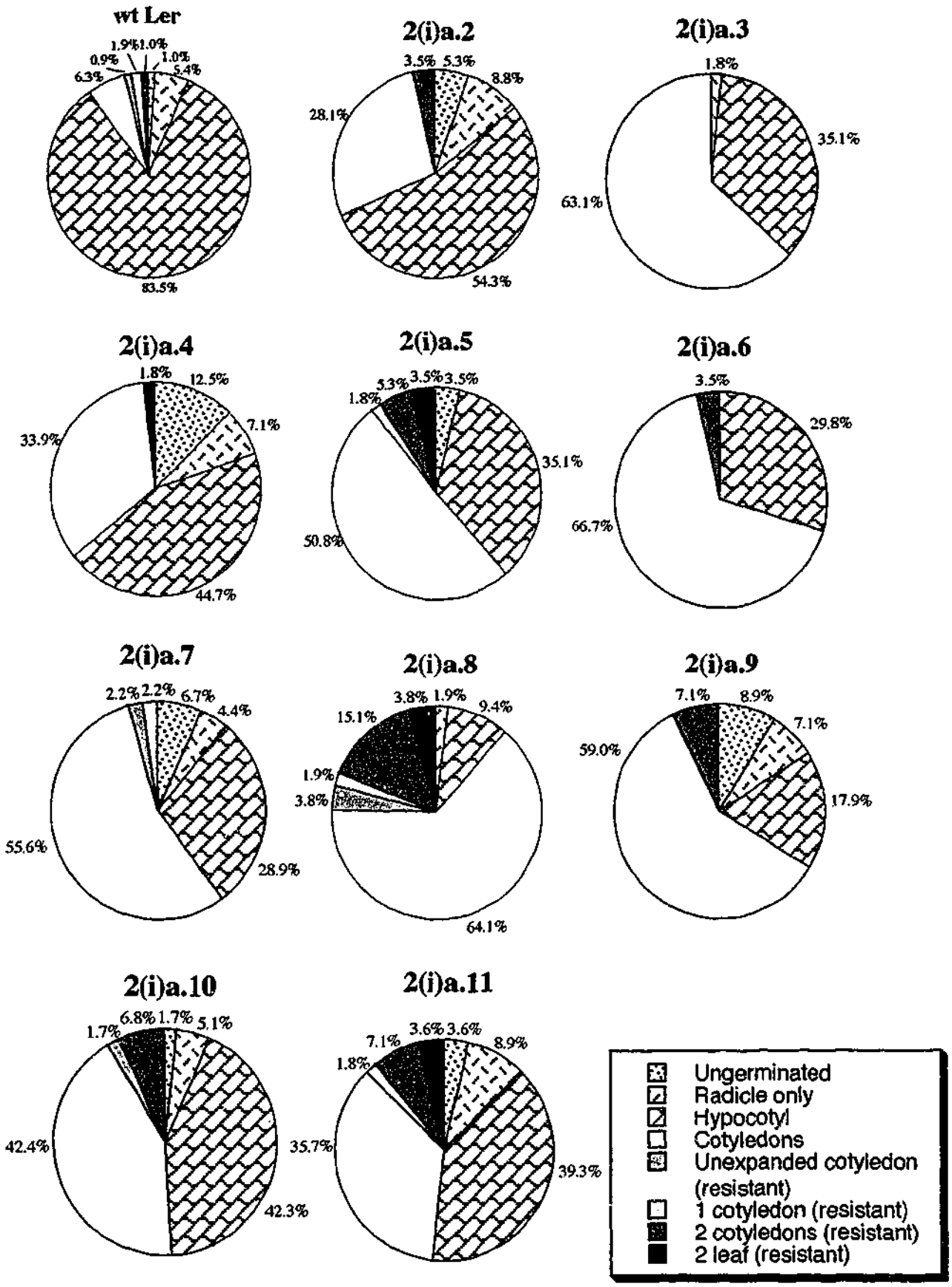
Appendix 10: Detailed resistance study of line 2(i)a individuals grown on MS media supplemented with 3mM MGBG. Day 18 of growth. The percentage of plants reaching various developmental stages are shown.

LINE 2(i)c grown on media containing 3mM MGBG



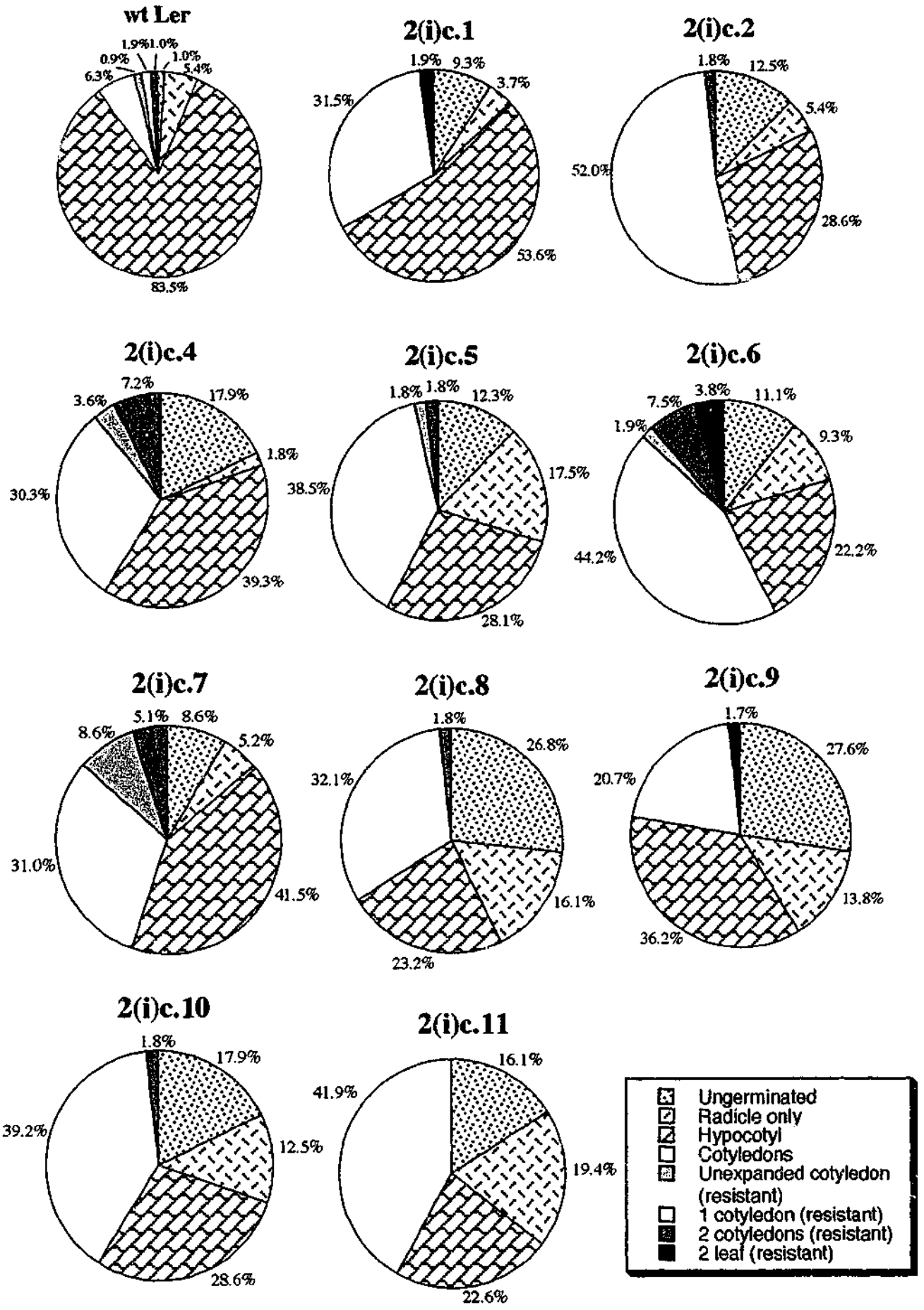
Appendix 11: Detailed resistance study of line 2(i)c individuals grown on MS media supplemented with 3mM MGBG. Day 18 of growth. The percentage of plants reaching various developmental stages are shown.

LINE 2(i)a grown on media containing 4mM MGBG



Appendix 12: Detailed resistance study of line 2(i)a individuals grown on MS media supplemented with 4mM MGBG. Day 18 of growth. The percentage of plants reaching various developmental stages are shown.

LINE 2(i)c grown on media containing 4mM MGBG



Appendix 13: Detailed resistance study of line 2(i)c individuals grown on MS media supplemented with 4mM MGBG. Day 18 of growth. The percentage of plants reaching various developmental stages are shown.