CONTRACTILE GROWTH OF THE HYPOCOTYL IN WHITE CLOVER SEEDLINGS (*Trifolium repens* L.)

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ABSTRACT

White clover (*Trifolium repens* L.), like many epigeal emerging legumes, displays a phenomenon known as contractile growth. This process occurs in the weeks following seedling emergence and involves the longitudinal shortening and radial expansion of tissues in the hypocotyl and upper primary root. During routine trials at AgResearch Grasslands, Palmerston North, it was noted that some seedlings of cv. Grasslands Huia displayed an aberrant growth form characterized by a failure to display normal contractile growth. These seedlings were intermated for one to two generations to form the 'non-contractile' populations used in this thesis. The 'contractile' populations used were formed through the random selection and intermating of plants which showed normal contraction. The non-contractile phenotype was found to occur naturally in populations of white clover at a frequency of 2-22%. When compared to tissue from normal contractile seedlings, hypocotyl tissue from non-contractile seedlings showed reduced radial expansion which in turn led to decreased contraction.

Since the plant hormone ethylene has been implicated in the promotion of radial expansion in many plant tissues and systems (Abeles 1973 b), the effects of applying silver thiosulphate and the ethylene-releasing compound Ethrel, were examined in contractile and non-contractile seedlings. Endogenous ethylene production by contractile and non-contractile seedlings was also examined. Seedlings from the contractile line showed an overall higher production of ethylene during early seedling growth than did seedlings from the non-contractile line. When treated with silver thiosulphate (which blocks ethylene perception), hypocotyls from seedlings of the contractile line failed to display contractile growth and hypocotyls from seedlings of the non-contractile line displayed non-contractile growth to a greater degree. When treated with Ethrel hypocotyls from seedlings of the non-contractile line displayed contractile growth but hypocotyls from seedlings of the contractile line failed to display contractile growth. These results indicate that ethylene is required for normal contractile growth of the hypocotyl in white clover seedlings, and that non-contractile growth of the hypocotyl is caused by a decreased production of, rather than a decreased sensitivity to, the plant hormone ethylene.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 : INTRODUCTION

1.1 White clover.

In many temperate regions of the world white clover (*Trifolium repens L.*) is the most important pasture legume. It shows wide adaptation to pasture situations in environments ranging from cool-temperate uplands in north-western Europe and New Zealand to warm-temperate regions of the USA and even to sub-tropical parts of Australia (Williams 1987). In New Zealand, white clover is grown in pastures for four main reasons - 1) as a legume it fixes nitrogen, 2) it improves sward quality, 3) it complements seasonal growth patterns of commonly used grass species, and 4) it improves forage intake and utilisation rates of animals (Caradus *et al.* 1995).

White clover is a tetraploid (2n = 4x = 32) and highly variable species. The glabrous, shallow-rooted plants spread by stolons that root at the nodes. Leaves are trifoliate, have long petioles, and are usually marked with a white V that is simply inherited (Carlson *et al.* 1985). Flowers are white, with occasionally pinkish variants, and are borne in an almost globose head on peduncles that are slightly longer than the petioles. Petiole length and leaf size vary with growing conditions and are greatest during cool, moist periods (Carlson *et al.* 1985). Seeds are small, and seedling emergence is epigeal (Metcalfe and Nelson 1985). During germination, the primary root breaks the seed coat and rapidly penetrates the soil, forming a slender taproot. The hypocotyl then elongates with further elongation and straightening of the hypocotyl raising the cotyledons above the ground. The seedling develops an upright primary stem with very short internodes. The first epicotyledonary leaf is unifoliate, whereas the second and subsequent leaves are trifoliate. Within six to eight weeks stolons may begin to grow radially from the lower axillary buds of the primary stem, and elongation of the primary stem ceases soon after. The stolon, in contrast to the primary stem, has elongated internodes and a vegatative apical bud that remains active. Growth is rapid after stolon initiation and leaves are borne alternately on the stolons, with one leaf per node. One or more roots may develop at each node and contraction of these roots causes stolon burial (Cresswell 1995). If growth occurs at the axil of a leaf, it gives rise to either a branch stolon or a

flower. Growth of axillary buds depends upon environment and genotype. The primary root branches and does not develop into a deep, long-lived perennial root. Root development is promoted by cool weather, which helps the plant survive periods of moisture stress (Metcalfe and Nelson 1985).

Like many epigeal emerging legumes, white clover displays a phenomenon known as contractile growth. This process places the growing crown in a less exposed microenvironment and may thus afford protection (Fujita and Humphreys 1992). During routine trials at AgResearch Grasslands, Palmerston North, some seedlings of cv. Grasslands Huia displayed an aberrant growth form characterized by seedlings which failed to display normal contractile growth. In these seedlings the crown and first node were not pulled beneath the soil surface, the hypocotyl remained extended and the crown developed some distance above the ground. The resulting plants developed normally in every way other than their failure to display contractile growth. The aim of this thesis was to investigate contractile growth by comparison of these 'non-contractile' seedlings to seedlings which display normal contractile growth, and to investigate a possible role for ethylene in the process of contraction.

1.2 Contractile growth.

Depending on the growth activity of the hypocotyl, dicotyledonous seedlings emerge in two different ways. Hypogeal emergence occurs when the hypocotyl remains inactive throughout germination and the cotyledons remain below the ground. Epigeal emergence occurs when the hypocotyl is active and pulls the cotyledons above the ground. More than ninety-percent of dicotyledonous species seedlings - including white clover, emerge in this way. Advantages of this system over hypogeal emergence include the terminal bud being protected by the cotyledons while it is drawn through the soil, and cotyledons being photsynthetically active soon after emergence (which provides the seedling with more energy than that which can be provided by storage alone). However, epigeal-emerging seedlings have no buds or stored food below the point of cotyledonary attachment (the 'first node') and die if the tops are removed below that point (Nelson and Larson 1984).

In many epigeal species, several weeks after seedling emergence the hypocotyl and upper primary root begin to shorten in a process known as contractile growth. Contractile growth is regarded as the difference between the maximum extension of the hypocotyl above ground surface and the subsequent position of the cotyledons (Gardener 1980). In species such as alfalfa, sweet clover, red clover and white clover; this process occurs six to eight weeks after emergence and places the first node (where the cotyledons were attached) at or below the soil surface (Nelson and Larson 1984). This may act to protect the node from hazards such as grazing, treading, defoliation or fire, and generally places plant organs in a less exposed microenvironment (Pott *et al.* 1983, Fujita and Humphreys 1992). Studies also show that the 'winter hardiness' of many species is directly proportional to contractile growth (Nelson and Larson 1984). Thus the process of contractile growth offers protection to the young seedling and developing plants. As seedlings which display the 'non-contractile' phenotype described above do not contract their crown beneath the soil surface, it is assumed that few survive in the natural environment due to lack of protection from the described hazards.

A literature survey revealed contractile growth of the hypocotyl and upper primary root to have been observed in a variety of epigeal emerging species (Pott *et al.* 1983, Nelson and Larson 1984, Metcalfe and Nelson 1985, Fujita and Humphreys 1992). Studies of sweet clover, alfalfa, red clover and white clover all propose that contractile growth is caused by a lateral growth of cells in the hypocotyl and upper primary root (Nelson and Larson 1984, Metcalfe and Nelson 1985). The cells within these structures which grow long and narrow during seedling emergence, are thought to expand laterally, causing the entire structure to shorten and thicken (Nelson and Larson 1984, Metcalfe and Nelson 1985). However, there appears to have been no material published containing cellular details of how this process occurs in any of these species.

Although no details regarding the exact nature of contractile growth in hypocotyls are available, many reports concerning contractile growth in the roots of plants have been published. Contractile roots are observed in many plant families and groups, including monocots, dicots, and even some ferns (Stevenson 1975). In white clover contractile

growth of adventitious roots has been shown to cause stolon burial (Cresswell 1995). Three main types of root contraction have been described. In many monocotyledons, root contraction is associated with tangential and radial enlargement of inner cortical cells and the collapse of outer cortical parenchyma. Examples of plants with roots which contract in this way include *Hyacinthus* (Liliaceae; Wilson and Honey 1966, Wilson and Anderson 1979, Jernstedt 1984 a, Cyr *et al.* 1988), *Gladiolus* (Iridaceae; Sterling 1972, Halevy 1986), *Narcissus* (Amaryllidaceae; Chen 1969), *Chlorogalum* (Liliaceae; Jernstedt 1984 b) and *Eucomis* (Eucommiaceae; Reyneke and Van der Schiijff 1974). This type of contraction is usually, but not always (e.g. *Freesia*) accompanied by longitudinal shortening of inner cortical cells. The roots of *Freesia* (Iridaceae) shorten through radial growth of middle cortical cells without an associated shortening of the same cells (Ruzin 1979). Accompanying these related changes in radial and longitudinal dimensions, cell volumes of inner or middle cortical cells ("active" cells) increase some two- to six-fold during the contraction process (Wilson and Honey 1966, Ruzin 1979, Jernstedt 1984). Because of the increase in cell volume, the necessity for turgor maintenance (Wilson and Honey 1966) and the continuation of cell wall synthesis during contraction (Wilson and Honey 1966), shortening of roots in this way is generally considered to be a true growth phenomenon, manifested as reorientation of specific cortical cell expansion (Jernstedt 1984 b).

The second type of root contraction observed involves the collapse of transverse discs of cortical cells which are crushed longitudinally by layers of turgid cells above and below (Jernstedt 1984). Plants with roots which contract in this way include *Oxalis* (Oxalidaceae; Thoday 1926), *Brodiaea* (Liliaceae; Smith 1930), and *Zamia* (Zamiaceae; Stevenson 1980). A third pattern of contraction has been reported in the fern *Botrychium* (Stevenson 1975), in which lysigenous cavities which form in the cortex subsequently collapse, resulting in a shorter root (Jernstedt 1984 b).

However, all the plants (except the fern *Botrychium*) described above are monocotyledonous and although contractile roots have been reported in many dicotyledonous species a thorough search of the literature has revealed no reports which explain the cellular basis of contraction in these plants. Thus, this thesis contains information on contractile growth which is unique in two ways. Firstly, it investigates

the process of contractile growth at a cellular level in the hypocotyl instead of the root; and secondly, it investigates contractile growth in a dicotyledonous plant rather than a monocotyledonous one.

1.3 Why implicate ethylene?

Ethylene (C_2H_4) is considered to be a plant hormone which can affect plants at almost every phase of their development. Despite its chemical simplicity, it is a potent regulator and has been shown to affect the growth, differentiation and senescence of plants at concentrations as low as 0.01 $\mu l\ l^{-1}$ (Reid 1987). The effects of C_2H_4 have been observed in practically all aspects of plant growth and development, including seed germination, seedling growth, root growth, growth of leaves, stress phenomena, and ripening, ageing, and senescence (Arshad and Frankenberger 1988).

Ethylene has been shown to inhibit longitudinal growth and induce radial swelling (growth in the lateral direction) in many plant tissues and systems including the shoots, petioles, hypocotyls and roots of various species (Abeles 1973 a, Burg 1973, Lieberman 1979, Eisinger 1983). In fact, ethylene has been shown to inhibit shoot elongation in most terrestrial dicotyledonous plants and, with few exceptions (Suge 1971, Cornforth *et al.* 1973), in monocotyledonous plants as well. Such radial expansion is also usually accompanied by marked increases in ethylene production and evolution (Vreugdenhil *et al.* 1984, Zheng and Inouye 1990, Sarquis *et al.* 1991, Zheng *et al.* 1997). It is this ability to inhibit elongation and induce lateral growth in plant tissues (especially roots and stems) which suggests a possible role for ethylene in the contractile growth of white clover.

Although ethylene retards the elongation of tissue in most land plants, the stems and/or petioles of some plants (such as *Callitriche platycarpa, Ranunculus sceleratus,Nymphoides peltata,* and most species of rice) which grow at least part of the time with their roots and stems underwater respond to ethylene in the opposite way - with enhanced elongation (Salisbury and Ross 1992). However, studies of stem growth in various species of rice indicate that ethylene does not cause elongation in these

tissues *per se*. Instead, ethylene is believed to remove the block to gibberellin synthesis and/or cause the tissues to become more sensitive to various forms of the hormone - thus inducing elongation (increased levels of and/or sensitivity to gibberellins induce the elongation of cells and tissues in most plant systems) (Khan *et al.* 1986, Suge 1987, Blom *et al.* 1994, Furukawa *et al.* 1997). This is in contrast to the situation which occurs in the stem tissue of most land-based plants where exposure to ethylene decreases the level of endogenous gibberellins and induces radial growth (Pearce *et al.* 1991).

Studies at the cellular level indicate that in most plant systems the radial expansion of tissues is due to a change in direction of cell growth from longitudinal to lateral, rather than due to increased cell divisions. Similar patterns of radial expansion are seen (and indeed lateral growth can be induced) when plant cells and tissues are treated with ethylene or chemicals which either break down to form ethylene e.g. Ethrel, or induce ethylene synthesis itself e.g. auxin (Burg and Burg 1966, Eisinger and Burg 1972, Burg 1973, Linkins *et al.* 1973, Biro *et al.* 1980, Jackson 1991, Ortuno *et al.* 1991, Abeles *et al.* 1992, Baskin and Williamson 1992, Bravo *et al.* 1992, Zacarias and Reid 1992, Petruzzelli *et al.* 1995). Thus ethylene is implicated as a causal factor in the lateral expansion of cells in many plant tissues.

The direction in which plant cells expand is generally accepted to be dependent upon the primary orientation of cellulose microfibrils in the cell wall (particularly those most recently deposited), and their association with plasma-membrane associated cortical microtubules (Roelofsen 1965, Ridge 1973, Richmond *et al.* 1980, Maclachlan 1977, Eisinger 1983, Duckett and Lloyd 1994). These two fibrillar elements often show parallel patterns of deposition (Duckett and Lloyd 1994) and it has been proposed that the deposition of cellulose microfibrils is controlled by the orientation and deposition of cortical microtubules (Newcomb 1969, Hepler and Fosket 1971, Giddings and Staehelin 1991, Cyr 1994). Cellulose microfibrils are extremely strong (their tensile strength and elastic modulins are similar to steel; Niklas 1992) and are generally deposited at right angles to the direction of expansion. The deposition of microfibrils in cell walls prevents expansion parallel to the microfibrils, but allows expansion perpendicular to them (Salisbury and Ross 1992). Thus a predominantly lateral or transverse

arrangement of microtubules and microfibrils favours cell expansion in the longitudinal direction, whereas a predominantly longitudinal arrangement favours expansion in the lateral or radial direction. In many plant tissues, treatment with ethylene causes a net change in microtubule (Steen and Chadwick 1981, Lang *et al.* 1982, Roberts *et al.* 1985) and microfibril (Veen 1970 a, Veen 1970 b, Apelbaum and Burg 1971, Eisinger and Burg 1972, Ridge 1973, Lang *et al.* 1982, Mueller and Brown 1982) orientation from predominantly transverse to predominantly longitudinal. As previously stated, this orientation of microtubules and microfibrils favours cell expansion in the radial direction, which in turn leads to radial expansion of the tissues involved. Therefore, the lateral expansion of plant tissues is thought to occur (at least partly) due to the effect of ethylene on microtubule and microfibril orientation, and the subsequent effect on cellular expansion.

However, microtubule and microfibril orientation is not determined solely by ethylene. Abscisic acid and cytokinins, such as kinetin, have also been shown to induce longitudinally oriented microtubules and lower stem elongation rates (Shibaoka 1974, Sakiyama and Shibaoka 1990, Ishida and Katsumi 1992, Sakiyama-Sogo and Shibaoka 1993). Conversely, auxins and gibberellins have been shown to stimulate the realignment of microtubules and microfibrils to a more transverse arrangement and promote cell expansion in the longitudinal direction (Bergfield *et al.* 1988, Shibaoka 1993, Zandomeni and Schopfer 1993). In fact the radial expansion of root cells (via changes in the orientation of microtubules and microfibrils) in both citrus and tomato plants have been proposed to be regulated by a competitive interaction between ethylene and gibberellins. In these tissues, ethylene is thought to act as the ultimate activator of expansion in the radial direction, whereas gibberellins are thought to act as repressors of the expansion process (Nakielski and Barlow 1995, Tadeo *et al.* 1997).

The ability of cells to contract in response to ethylene seems to be at least partially dependent on the type of cells involved (Osborne 1982). Poovaiah (1974) stated that the radial increase observed in the first internodes of Ethrel-treated bean was due to swelling of the cortical cells only. Work done by Isaac in 1938 and cited by Abeles (1973 a), also indicated that the swelling observed in bean hypocotyls treated with ethylene was due to cortical enlargement only. Further, when treated with ethylene,

cortical cells in the petiole and stem sections of *Phaseolus vulgaris* L. (Linkins *et al.* 1973), the roots of *Arabidopsis thaliana* (Benfrey *et al.* 1993), and the hypocotyls of *Lupinus albus* L. (Ortuno *et al.* 1991, Bravo *et al.* 1992) expanded radially to a greater extent than other cells. That certain cell types (such as cortical cells) appear to be more affected by ethylene than others supports the concept that the location of individual cells within tissues and their sensitivity to ethylene drastically affects their responsiveness to ethylene (Osborne 1976, Reid 1987, Ortuno *et al.* 1991). Work with *Lupinus albus* L. hypocotyls (Bravo *et al.* 1992) concluded that ethylene-induced cell expansion in cortical cells varied according to the location of the cells in the tissue: the central and sub-epidermal layers showed little change, whereas the innermost layers exhibited the greatest increase. Various ideas as to why this occurs have been proposed including the suggestion that the cortical cells may act as 'target cells' for ethylene (Biro *et al.* 1980), or that microtubules in cortical cells may be particularly sensitive to changes in ethylene levels (Aeschbacher *et al.* 1995). As all three patterns of root contraction discussed in Section 1.2 involve the radial enlargement and/or collapse of cortical cells only, and as the contraction of such tissues is at least partially caused by the reorientation of microtubules by ethylene, ethylene is further proposed as playing a role in the contractile growth of white clover.

1.4 Ethylene/auxin interactions and cellular growth.

Investigations regarding interactions between ethylene and auxin with respect to cell enlargement, reveal plant tissues to contain three distinguishable cell types, namely Type I, Type 2, and Type 3 respectively (Osborne 1982). Type I cells have been studied the most extensively and occur in the elongating parts of immature dicotyledonous shoots as well as the mesocotyls and coleoptiles of monocotyledonous plants. These cells enlarge or elongate in response to auxin but they do not do so in response to ethylene. Rather, ethylene regulates the *direction* of the auxin-controlled cell expansion, not the *extent* of expansion. As a result, exposure to ethylene leads to lateral growth (rather than elongation) of the cell and the final shape tends to an isodiametric rather than an elongate form (Osborne 1982). The second type of cell described (Type 2 cells) is most commonly found in the abscission zones of plants where zone cell expansion is often a visible prelude to the separation event. In these cells auxin *suppresses*

expansion but ethylene can *induce* it. The third cell type, Type 3, is common to the stems and petioles of semiaquatic plants (Osborne 1982). These cells elongate upon shoot submergence and exhibit large increases in overall extension in response to either auxin *or* ethylene. Significantly, this ethylene-induced enlargement has an absolute requirement for the presence of adequate levels of endogenous (or supplied) auxin to permit the enlargement to occur (Walters and Osborne 1979). The response of such tissues to ethylene in the presence of auxin is more than additive and has been termed the 'supergrowth' effect of auxin with ethylene. Although Type 3 cells are more common to semiaquatic plants, some are found in limited clusters in land plants. It is to groups of these cells, positionally differentiated along the upper adaxial flanks of the bases of leaf petioles that we may attribute the auxin *or* ethylene induction of epinastic responses by the leaves of certain plants (Osborne 1982). However, exactly how auxin and ethylene are differentially perceived by the three cell types mentioned remains largely unknown.

Interactions between ethylene and auxin are also thought to affect the size of plant cells by causing changes in the orientation of microfibrils in the cells walls. As discussed previously in Section 1.3, ethylene and auxin cause opposing effects on microfibril orientation in many plant tissues. For example, in pea (*Pisum sativum*) concentrations of auxin that result in the deposition of transversely oriented cell wall microfibrils enhance elongation of stem segments but inhibit lateral expansion. By contrast, higher concentrations of auxin, which induce much greater ethylene production and result in deposition of longitudinally oriented microfibrils, bring about considerable radial growth and swelling of the stem segments (Raven *et al.* 1986).

Another ethylene/auxin interaction which has been studied extensively is the ability of ethylene to inhibit the polar transport of auxin (Burg and Burg 1966, Morgan and Gausman 1966, Beyer and Morgan 1970, Suttle 1988, Sagee *et al.* 1990). This interaction causes the differential distribution of ethylene and auxin in many plant

tissues and is suggested to be the mechanism by which hypocotyl hook maintenance is regulated in emerging seedlings (Schierle and Schwark 1988, Schwark and Schierle 1992).

1.4 Biosynthesis, regulation and perception of ethylene

The developmental changes which occur in plant tissues in response to plant hormones are brought about via 1) changes in the biosynthesis and/or regulation of the hormone, and/or 2) changes in the sensitivity of the tissues to the hormone. Therefore, in order to further understand and suggest a possible role for ethylene in the contractile growth of white clover, a basic understanding of how this plant hormone is synthesized, regulated, and perceived within plant tissues is needed.

Biosynthesis

The pathway for ethylene biosynthesis was elucidated by Shang Fa Yang and his collaborators in the late 1970's (Yang and Hoffman 1984) and has provided the basis for all subsequent biochemical and molecular genetic analysis of the pathway. The final critical steps of the biosynthetic pathway leading to ethylene production are shown in Figure 1.1.

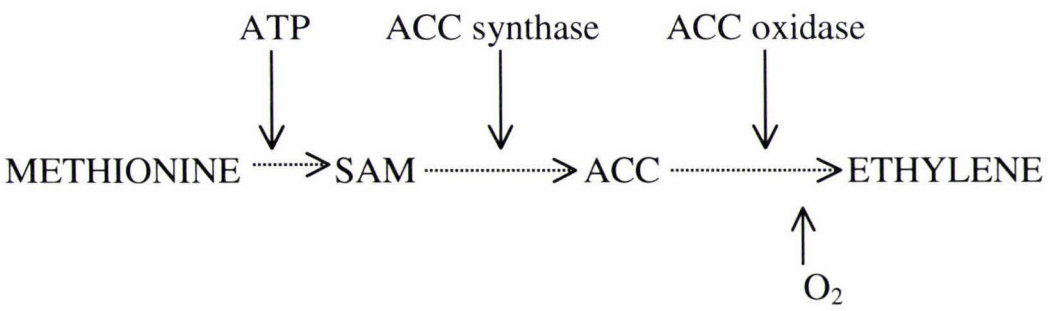


Figure 1.1 : Final steps in the biosynthetic pathway of ethylene.

Regulation

The regulation of ethylene synthesis is known to occur via both hormonal and non-hormonal factors. Of the hormonal factors, one of the strongest regulators of ethylene

synthesis is ethylene itself. This regulation has been shown to occur in both positive and negative manners.

Positive regulation of ethylene synthesis by ethylene has been demonstrated in certain ripening fruits and senescing tissues when a massive increase in ethylene production is triggered by exposure to ethylene (Mattoo and White 1991). This auto enhancement is known as the *autocatalytic effect*. In 1972, McMurchie and co-workers proposed the now widely accepted model of two regulatory systems which control ethylene synthesis. In this model, System I produces low base levels of ethylene and is controlled by the level of ACC synthase. It is present in both climacteric and non-climacteric tissues. System II is only operational in climacteric tissue and involves the production of high levels of ethylene in the autocatalytic response of exposure to increased levels of ethylene from System I (Yang and Hoffman 1984). This involves increases in both ACC oxidase and ACC synthase, and it is this system which can be inhibited by Ag+ (Veen 1987).

The negative regulation of ethylene synthesis by ethylene itself is a phenomenon known as *autoinhibition*. This process has been shown to occur in a number of both fruit and vegetative tissues (Vendrell and McGlasson 1971, Zauberman and Fuchs 1973, Zeroni *et al* 1976, Saltveit and Dilley 1978, Yang and Hoffman 1984) and is suggested to occur partly via feedback repression of the synthesis of ACC synthase. The conjugation of ACC to MACC by malonyl transferase which increases when certain tissues are exposed to ethylene also suggests another mechanism by which ethylene regulates its own production.

Of the other plant hormones shown to influence ethylene biosynthesis, auxins (such as IAA) appears to have the most effect - and as such have been most studied. In vegetative tissues, which normally produce quite low amounts of ethylene, auxin markedly increases ethylene biosynthesis (Abeles and Rubinstein 1964, Burg and Burg 1966, Kang *et al* 1971, Abeles 1973 b, Lau and Yang 1974) via an increase in the expression of the ACC synthase gene (Sato and Theologis 1989). Such increases in ethylene production have been extensively studied in etiolated mung bean and pea seedlings where auxin stimulates ethylene production several hundred-fold (Imaseki 1983, Yang and Hoffman 1984). IAA conjugates may also play an important role in the

regulation of ethylene biosynthesis by auxin. Auxin conjugates are generally thought to act as components for the general transport, storage, and subsequent reuses of IAA within plant tissues (Cohen and Bandurski 1982). In fact, observations by Aharoni and co-workers in 1984 (Aharoni *et al* 1984) led them to believe that the natural regulation of ethylene biosynthesis in some plant tissues is primarily dependent on the type and levels of the endogenous IAA conjugates formed in the tissue and their susceptibility to hydrolytic enzymes.

Perception and signal transduction

Recent advances in understanding these processes have come from pursuing a genetic approach with *Arabidopsis thaliana*. Mutants have been identified which are either insensitive to applied ethylene (Bleecker *et al* 1988, Guzman and Ecker 1990, Van der Straeten *et al* 1993, Roman *et al* 1995) or express the ethylene-induced phenotype constitutively (Kieber *et al* 1993). Several classes of mutants obtained by screening affect a range of ethylene responses throughout the life cycle of the plant, indicating that these responses share a primary signal transduction pathway (Bleecker and Schaller 1996). In particular, multiple mutant alleles at the *ETR1* and *EIN2* loci confer global insensitivity to ethylene (Guzman and Ecker 1990, Chang *et al* 1993). In contrast, mutations at the *CTR1* locus lead to constitutive activation of ethylene-regulated pathways (Kieber *et al* 1993), indicating that *CTR1* is a negative regulator of the ethylene response. Double-mutant analysis indicates that *CTR1* is epistatic to *ETR1* and that *EIN2* is epistatic to *CTR1* (Kieber *et al* 1993). Biochemical characterization of the ETR1 protein has taken place (Schaller *et al* 1995) in *Arabidopsis* and cellular fractionation studies indicate that it is membrane associated.

From these and many other observations, a model for the ethylene-response pathway in plants has emerged (Figure 1.2). This model involves the perception of ethylene at the plasma membrane by the ETR1 protein and/or its homologues such as ETR2, ERS, ERS2 and EIN 4 (which are thought to serve redundant functions in ethylene signalling) (Bleecker and Schaller 1996, Hua and Meyerowitz 1998). This binding has been hypothesized to be mediated through a transition metal coordinated within the hydrophobic region of ETR1, which then induces a conformational change

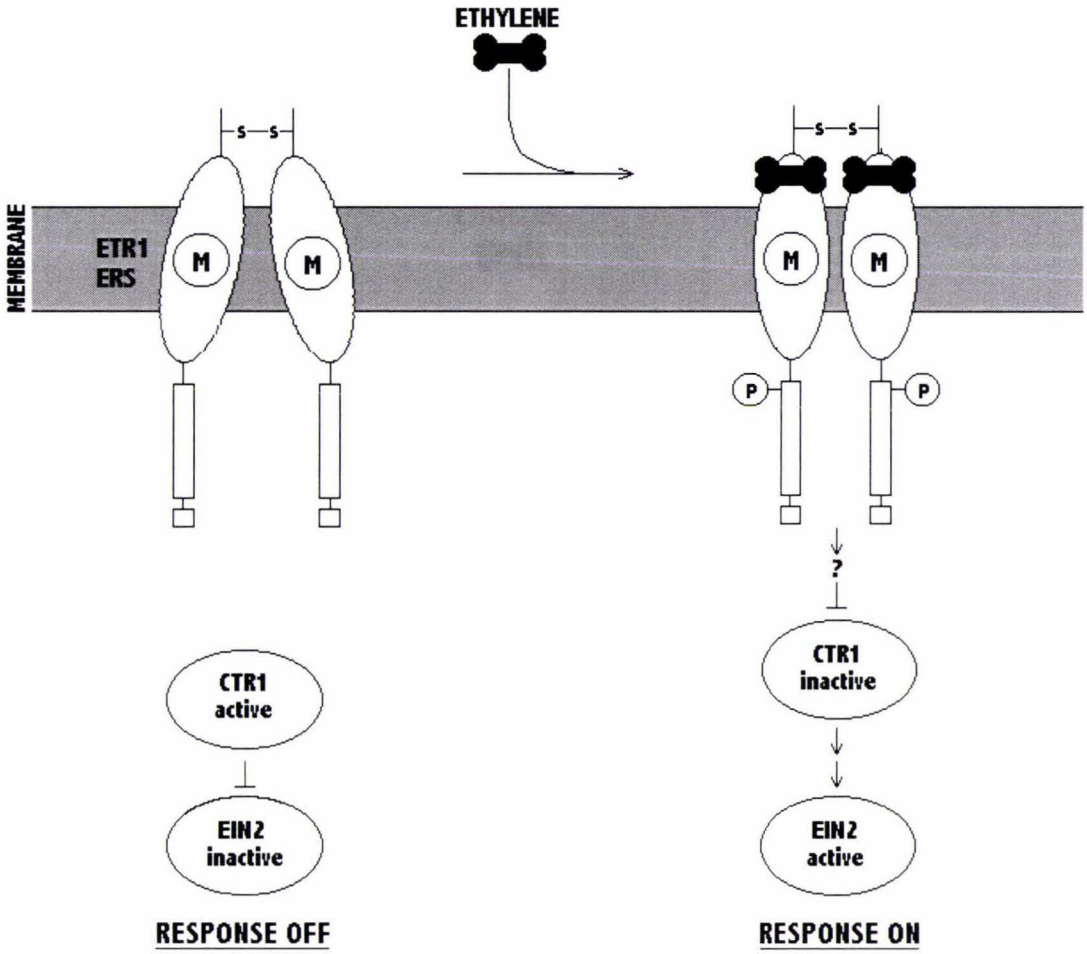


Figure 1.2 : Proposed model for the mechanism of signal transduction in *Arabidopsis*. Arrows represent positive regulatory steps, and the flat symbols represent negative regulatory steps. Ethylene is thought to bind to a transition metal (**M**) chelated in the hydrophobic domain of the receptor and that binding alters the kinase activity through conformational changes in or between monomers. CTR1 is thought to repress the signal transduction pathway which is constitutively active, and that ethylene relieves this inhibition. Each arrow may represent several steps in the pathway and the question mark indicates the position of a postulated response regulator protein. **s-s**, disulphide bond; **P**, phosphohistidine. (Drawn from figures by Bleecker and Schaller 1996, and Kieber 1997).

of ETR1 that may alter the rate of *trans*-phosphorylation between subunits (Bleecker and Schaller 1996). The altered conformation of ETR1 then results in the shutting off of the kinase activity of CTR1, either directly or indirectly (Kieber 1997). Recent work has indicated that ETR1 and CTR1 can physically interact, and that the activation of CTR1 could be via a direct phosphorylation or dephosphorylation by the receptor-

related proteins or through localization of CTR1 by physical attachment to the proteins (Clark *et al.* 1998). Thus, CTR1 is thought to repress the ethylene signal transduction pathway which is constitutively active, and that ethylene relieves this inhibition (Zarembinski and Theologis 1994). There is also some evidence that ETR2, ERS2 and EIN4 do not bind ethylene directly, but instead form receptor complexes with other ethylene binding proteins ((Hua and Meyerowitz 1998). The occurrence of more than one means of ethylene reception, i.e. by the various homologues of ETR1 (including ERS), suggests that these sensors may serve different, but overlapping roles in ethylene signaling (Chang 1996). It makes sense for plants to have multiple switches leading to the same responses, as it would allow for the fine tuning of complex responses that require the integration of diverse and/or opposing signals (Chang 1996). Also, the different homologues have been shown to be differentially expressed in tissues during development. For instance, in tomato the *eTAE1* gene product (Zhou *et al.* 1996), which contains all of the structural elements found in the *Arabidopsis ETR1* gene product and has an overall amino acid sequence similarity to ETR1 of 81%, mRNA is expressed during flower and fruit senescence (Theologis 1995); whereas the *nr* gene product (Wilkinson *et al.* 1995), which lacks a response regulator domain and is structurally similar to the *Arabidopsis ERS* gene product, mRNA is developmentally regulated during fruit ripening (Theologis 1995). Thus, the perception, signal transduction, and subsequent action of ethylene in various plant tissues during various stages of growth and development, may largely depend on the ethylene sensing homologue/s being expressed in the tissue at the time.

The end of the ethylene signal transduction chain is likely to involve alterations in gene expression. Indeed, ethylene has been shown to induce the synthesis of new mRNA's and proteins in several plant systems (reviewed in Broglie and Broglie 1991, Christoffersen and Laties 1982). However, exactly how the signaling events described above (as well as other components known to affect ethylene signal transduction) act to cause such changes remains unknown (Chang 1996).

Over the years studies have indicated that changes in sensitivity of tissues to plant hormones are as important (if not more important) than changes in the concentrations of the hormones themselves. In fact, Trewavas suggested the idea that only sensitivity was involved (Trewavas and Cleland 1983). However, Cleland disagreed and suggested that

all options, including changes in hormone concentration, changes to hormone sensitivity, or a combination of both, should be considered. Changes in sensitivity to ethylene are well documented, especially during fruit ripening and in the abscission zones of leaves (Suttle and Hultstrand 1991). Such changes in sensitivity may come about by means of a change in the number or type of receptors, a change in the affinity of the receptors, or a change in the subsequent chain of events. If ethylene does in part cause the contractile growth of white clover, then the 'non-contractile' phenotype described in Section 1.1 may be caused by decreased sensitivity to ethylene. One of the purposes of this research was to investigate this possibility.

1.6 The ethylene-mediated 'triple response' and ethylene mutants

Contractile growth of the hypocotyl and upper primary root in white clover is hypothesized to occur via the radial expansion of individual cells within these tissues (see Section 1.2). When attempting to implicate ethylene in this process, the changes in growth which occur when etiolated (dark grown) dicotyledonous seedlings are exposed to ethylene, and the mutants obtained by the screening of such plants, are of interest.

The characteristic changes in growth observed when etiolated dicotyledonous seedlings are exposed to ethylene were first described by the Russian physiologist Dimitry Neljubow in 1901, and are collectively referred to as the 'triple response'. They include inhibited stem elongation, increased stem thickening, and the development of a diageotropic (horizontal) growth habit (Goeschl *et al* 1966). Furthermore, expansion of leaves is inhibited, and normal opening of the epicotyl hook is retarded. It is this ability to inhibit stem elongation and increase stem thickening in seedlings which is of interest when suggesting ethylene as a causal factor in contractile growth.

The growth changes which occur during the triple response have been demonstrated and discussed for many plant species, including pea (Knight *et al* 1910) and of course *Arabidopsis* (Kieber and Ecker 1993). However, as early as the seventeenth century, Haberlandt (cited in Ecker 1995) had suggested that these dramatic changes may be a

stress-induced adaptation that allows seedlings to penetrate the soil without damage to the apical meristem. This early hypothesis has been substantiated by both physiological (Goeschl *et al* 1966, Zheng and Inouye 1990) and genetic experiments (Harpham *et al* 1991). Physical obstruction of seedling growth leads to dramatic increases in ethylene biosynthesis, which in turn induces the development of the triple response morphology (Goeschl *et al* 1966). The idea that ethylene-induced swelling of seedling hypocotyls and roots acts to protect the delicate apical meristem during its journey to the soil surface is interesting as (ethylene-induced?) swelling of the same organs has been suggested as a possible cause of contractile growth (which aids protection of the apical meristem by placing it in a less exposed microenvironment).

Induction of the triple response must rely on the ability of the plant to perceive and respond to ethylene, as inhibitors of ethylene perception or biosynthesis and mutations that eliminate all ethylene responses prevent this morphological transformation (Kang *et al* 1967, Beyer 1976, Bleecker *et al* 1988, Guzman and Ecker 1990). *Arabidopsis* mutants which either fail to respond to exogenous ethylene, or constitutively display the response in the absence of ethylene, have been isolated using the triple response as a screening method. The triple response in *Arabidopsis* was used because of its high reproducibility, ease of screening of large numbers of individuals ($>10^6$) and the early stage at which screening can be done (three days) (Kieber and Ecker 1993).

Ethylene biosynthesis mutants

A class of *Arabidopsis* mutants which display the triple response in the absence of exogenous ethylene, were found to be ethylene overproducers. A number of these mutants which produce significantly increased amounts of ethylene have been isolated in *Arabidopsis* (Guzman and Ecker 1990, Kieber *et al.* 1993). The ethylene overproduction (Eto^-) mutants *eto1, eto2,* and *eto3* all display the triple response in the absence of exogenously applied ethylene (Ecker 1995). The recessive *eto1* mutant produces tenfold more ethylene than wild-type dark-grown seedlings (Guzman and Ecker 1990, Kieber *et al* 1993). Two dominant mutants have been isolated: *eto2*, in which ethylene production is 20-fold higher than in wild-type dark-grown seedlings, and *eto3*, in which levels of the hormone are enhanced 100-fold (Kieber *et al* 1993).

Interestingly, the mature rosette leaves of these mutants do not produce such high levels of ethylene, suggesting that ethylene biosynthesis may be regulated independently in seedlings and adult plants, or in light- and dark-grown plants (Kieber and Ecker 1993). Treatment of Eto$^-$ seedlings with inhibitors of ethylene biosynthesis or antagonists of ethylene action cancels the constitutive triple response phenotype, indicating that the Eto$^-$ mutants are defective in ethylene biosynthesis (Kieber *et al.* 1993, Guzman and Ecker 1990). The proposed relative position of these (and other) loci in the ethylene response pathway as established by epistatic relationships in *Arabidopsis* are shown in Figure 1.3.

Ethylene response mutants

The triple response in *Arabidopsis* has also been used to isolate mutants affected in the perception of ethylene. Three classes have been identified : mutants that are insensitive to ethylene, mutants that display ethylene responses in the absence of the hormone, and mutants that are affected only in specific tissues (Kieber and Ecker 1993). Ethylene-insensitive (*ein*) mutants fail to display the triple response in the presence of ethylene. These mutant (tall) seedlings are readily identified protruding above a "lawn" of wild-type (short) seedlings when mutagenized populations are plated in the dark in the presence of ethylene (Ecker 1995). Eight ethylene-insensitive (*ein/etr/eti/ain*) loci have been characterized genetically: *etr1* (Bleecker *et al* 1988, Chang *et al* 1993), *ein2* (Guzman and Ecker 1990, Roman *et al* 1995), *ein3* (Kieber *et al* 1993, Roman *et al* 1995), *ain1* (Van Der Straeten *et al* 1992), *ein4, ein5, ein6,* and *ein7* (Roman *et al* 1995). Each of the mutant seedlings show varying degrees of insensitivity to ethylene as defined by a complete deficiency or reduction in the magnitude of the triple response (Roman *et al* 1995). *etr1*, the first characterized

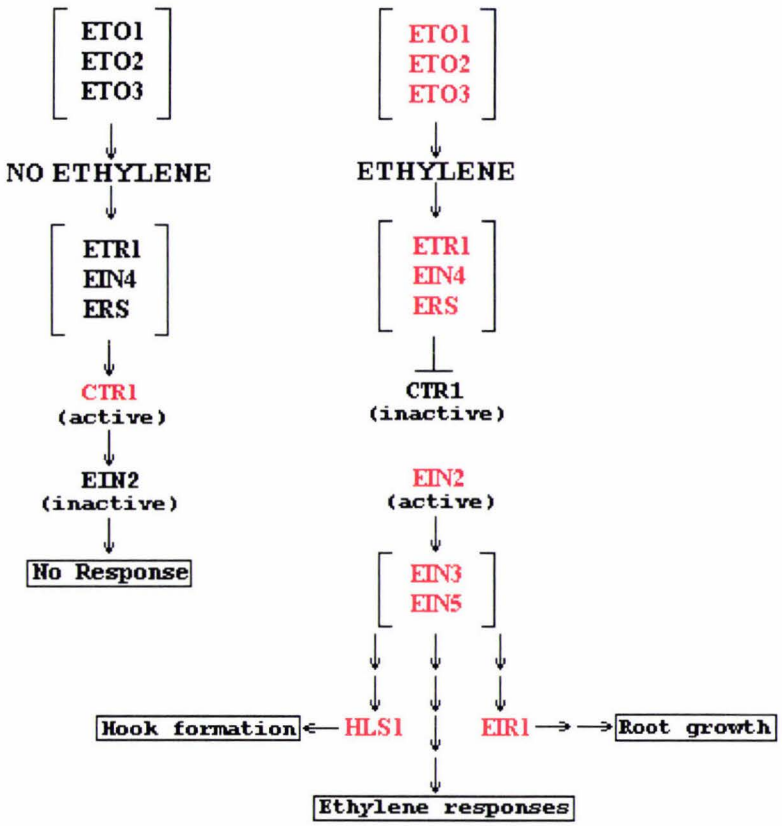


Figure 1.3 : Position of loci in ethylene response pathway as established by epistatic relationships in *Arabidopsis*. Loci listed together are nonallelic but may encode for similar functions and bracketed loci are not distinguishable epistatically. Arrows represent positive regulatory steps, and the flat symbol represents a negative regulatory step. Each arrow may represent several steps in the signal transduction pathway as direct interactions have not yet been demonstrated. The gene symbols in red correspond to the active state, whereas those in black correspond to the inactive state. (Compiled from figures drawn by Kieber and Ecker 1993, Ecker 1995, Fluhr and Mattoo 1996, and Kieber 1997).

ethylene-insensitive mutant, is inherited as a single gene, dominant mutation (Bleecker *et al* 1988). *etr1* was identified on the basis of its high degree of insensitivity to ethylene-mediated inhibition of hypocotyl elongation in etiolated seedlings, but is also

defective in a number of other ethylene responses including promotion of seed germination, enhancement of peroxidase activity, acceleration of senescence of detached leaves, and negative feedback of ethylene biosynthesis (Bleecker *et al* 1988). *etr1* plants bind only one-fifth as much ethylene as wild-type plants. Furthermore, the ability of ethylene to induce the transcription of target genes is blocked in *etr1* plants (Samac *et al* 1990, Lawton *et al* 1994). These, and other effects have caused researchers to suggest that *etr1* may encode an ethylene receptor or act at an early step in the signal transduction pathway (Figure 1.3) (Ecker 1995, see also Section 1.5). A second dominant ethylene-insensitive mutant called *ein4* has also been identified, and shows many of the characteristics of the *etr1* mutant (Roman *et al* 1995).

Another well characterized mutant, *ein2,* is insensitive to high amounts of ethylene (Guzman and Ecker 1990, Roman *et al* 1995). This recessive mutant is similar in phenotype to *etr1* and *ein4* in that strong alleles of *ein2* are pleiotropic, lacking all known ethylene responses (Guzman and Ecker 1990, Roman *et al* 1995). Like *etr1* (Bleecker *et al* 1988), *ein2* plants have larger rosette leaves (Guzman and Ecker 1990) and larger cells (Ecker 1995) than wild-type plants, perhaps because of a failure to respond to a basal level of ethylene. These plants also show increased ethylene production relative to wild-type plants (Guzman and Ecker 1990) which suggests that auto-inhibition of ethylene biosynthesis may be affected by the defect in ethylene perception (Bleecker *et al* 1988). Five additional ethylene-insensitive mutants have been genetically characterized (Roman *et al* 1995). The *ain1, ein3, ein5, ein6,* and *ein7* mutants have a significantly less severe phenotype than *etr1, ein4,* or *ein2* (Ecker 1995). Interestingly, members of the *ain* class of mutants retain their apical hook unlike those of *ein* class of mutants. In light-grown adult plants, *ain* mutants show decreased sensitivity with respect to leaf senescence but show no differences compared to wild-type plants when exposed to biotic and abiotic stresses known to elicit ethylene production (Zarembinski and Theologis 1994). Unlike the *ein* loci, the *ain1-1* allele produces three-fold less ethylene which suggests that the AIN1 protein positively regulates ethylene production (Zarembinski and Theologis 1994). In another study, five ethylene-insensitive seedlings were isolated that were referred to as *eti* (Harpham *et al* 1991). These plants have only been partially characterized genetically, so it is unclear whether they represent independent or previously unidentified loci. It is also likely that additional ethylene-insensitive loci remain to be

detected because there is no evidence that the mutant screens are saturated and only limited attempts have been made to recover mutants that are weak, lethal, or infertile (Ecker 1995).

The ethylene response mutants which constitutively display the triple response in the absence of ethylene are all recessive and fall into a single complementation group called *ctr1* (constitutive triple response). The phenotype of these mutants cannot be reversed by inhibitors of ethylene biosynthesis or action suggesting that this mutant is defective in ethylene perception or signal transduction (Kieber *et al* 1993). In fact, experimental evidence suggests that CTR1 may repress the ethylene signal transduction pathway which is constitutively active, and that ethylene relieves this inhibition (Figure 1.3) (Zarembinski and Theologis 1994, refer also to Section 1.5). The *ctr* mutation has dramatic effects on the morphology and development of seedlings and adult plants: etiolated *ctr1* seedlings take longer than wild-type seedlings to open their apical hook and expand their cotyledons when shifted to light. The rosette leaves of *ctr1* plants are smaller, their root system less extensive, their inflorescence is much more compact than that of wild-type plants, and mutant plants bolt unusually late (Kieber and Ecker 1993). These characteristics can be phenocopied by growing wild-type plants in the presence of ethylene, suggesting that *ctr1* mutants constitutively display adult ethylene responses (Kieber and Ecker 1993). Epidermal cells from *ctr1* leaves are 5-fold smaller than those from wild-type plants. This reduction in cell size may account for at least part of the decrease in leaf size observed in *ctr1* and may underlie some of the other *ctr1* phenotypes such as the shortened hypocotyl, compacted inflorescence and reduced root system. As discussed previously in Section 1.3, ethylene has been shown to inhibit cell elongation in other plant species perhaps due to a reorientation of the cytoskeleton and/or cell wall (Apelbaum and Berg 1971, Steen and Chadwick 1981, Lang *et al* 1982, Roberts *et al* 1985, Shibaoka 1994, Yuan *et al* 1994). In contrast to *ctr1* mutants, ethylene insensitive mutations such as *etr1* and *ein2* described above have larger rosette leaves than wild-type plants (Bleecker *et al* 1988, Guzman and Ecker 1990, Hua *et al* 1995). This suggests that they may have larger cells than wild-type plants (Bleecker *et al* 1988, Guzman and Ecker 1990) - possibly because they cannot respond to basal levels of ethylene (Kieber and Ecker 1993).

Examples of ethylene-response mutants which affect only specific tissues include the *nr (never ripe), rin (ripening inhibitor)* and *nor (nonripening)* mutants in tomato, and the *hls1 (hookless1)* and *eir1* mutants in *Arabidopsis*. In tomato, fruit ripening is ethylene-mediated and a number of tomato mutants which affect the ripening process are known (Abeles *et al* 1992). As their names suggest, fruit ripening in the *rin (ripening inhibitor)* and *nor (nonripening)* mutants is delayed (Lanahan *et al* 1994) and experimental evidence strongly suggests that mutations in these loci affect ethylene sensitivity specifically during fruit ripening (Ecker 1995). The *hls1 (hookless)* mutant in *Arabidopsis* is one of several mutations isolated that affect the differential elongation of cells in the hypocotyl hook region (Figure 1.3). Interactions between ethylene and auxin are suggested to cause maintenance of the hypocotyl hook (see Section 1.4), and as such these mutations may help to identify genes that control these interactions (Ecker 1995). A final example of a "tissue"-specific mutation is the ethylene insensitive root (*eir1*) mutant which affects ethylene sensitivity specifically in the root Figure 1.3) (Roman *et al* 1995).

1.7 Light and the triple response

The effect of light on the hypocotyl growth of dicotyledonous seedlings is also of interest when investigating a possible cause of contractile growth and white clover. Both red (Rubinstein 1971) and blue (Liscum and Hangarter 1993) light have been shown to inhibit hypocotyl elongation, and suppress maintenance of the hypocotyl hook in many dicotyledonous seedlings. Opening of the hook in these seedlings occurs when cells on the inner side of the hook begin to elongate at a greater rate relative to those on the outer side (Silk and Erickson 1978). Etiolated *ctr1 Arabidopsis* mutants (see Section 1.6) show a drastic reduction in rapidity of light-mediated de-etiolation responses such as cotyledon expansion and hook opening (Ecker and Theologis 1994). Consequently, one might expect that mutants which demonstrate constitutive light phenotypes in the absence of light (*cop* and *det*) may overlap those of the apical hookless class (Chory *et al* 1989, Deng *et al* 1991). Unlike the other Cop$^-$ mutants, *cop3* and *cop2* seedlings show normal regulation of light-related genes, normal

elongation of the hypocotyl and root, and normal chloroplast development. These results suggest that these mutations (*cop2* and *cop3*) may affect ethylene or auxin regulation of cell expansion in the apical region of the hypocotyl (Ecker and Theologis 1994). In support of the hypothesis, genetic complementation studies have indicated that *cop3* is an allele of *hls1* (see Section 1.6) (Hou *et al* 1993, Roman *et al* 1995) indicating a requirement of this allele in expression of two different environmental inputs (light and ethylene). With regard to the regulation of differential growth in the apical hook region by both ethylene and light, it will be interesting to examine the effects of combinations of mutations that cause constitutive suppression of the hook (such as *det1* or *cop1*) with those that cause constitutive hook formation (such as *ctr1* or *eto1*). Such studies may allow positioning of the point(s) of intersection between light and ethylene signaling pathways in the control of skoto-/photo-morphogenic development (Ecker and Theologis 1994).

Cellular growth in plants may also be affected by light via changes in microtubule orientation. Irradiation with white, red or blue light has been shown to cause the reorientation of microtubules from transverse to oblique or longitudinal in etiolated oat and pea tissue (Iwata and Hogetsu 1989). Evidence that this effect may be mediated by decreased levels of IAA has also been provided (Iwata and Hogetsu 1989). Similarly, Nick *et al* (1990) and Zandomeni and Schopfer (1993) have shown that blue light induces microtubules to realign from transverse to longitudinal in the outer wall of maize coleoptile epidermal cells. Evidence that ethylene also causes the reorientation of microtubules from transverse to oblique or longitudinal (see Section 1.3) and the interactions which occur between ethylene and auxin (see Section 1.4), indicate that these light effects may be linked to the production or perception of ethylene in some way.

1.8 Aims

The aims of this thesis were to investigate contractile growth in the hypocotyl of white clover seedlings, and to determine what role, if any, ethylene may play in this process. In order to achieve these objectives the following were conducted:

- Observation of hypocotyl growth in a number of different lines and cultivars of white clover (including the 'contractile' and 'non-contractile' lines) to gain an understanding of the natural frequency of non-contractile growth and a general concept of the patterns and timing involved in contraction.
- Observation of contractile and non-contractile seedlings treated with solutions of Ethrel (which breaks down within plant tissues to release ethylene) and silver thiosulphate (silver ions block ethylene perception) to investigate whether non-contractile growth may be caused by a decrease in ethylene production or a decrease in ethylene sensitivity.
- Measurement of ethylene production by contractile and non-contractile seedlings to establish and quantify any differences in ethylene emanation between the two lines.
- Observation and measurement of hypocotyl cells in contractile and non-contractile seedlings to investigate the physical means of contraction.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Contractile growth in a range of cultivars.

Overview: Ten cultivars of white clover were chosen to represent a full range of leaf size (as classified by the Organisation for Economic Co-operation and Development 1993) and origin. For each leaf size classification one cultivar of New Zealand origin, and one of overseas origin were chosen. The hypocotyl height of each seedling was measured and recorded every 4 d until 65 d after sowing.

Plant material: The cultivars chosen were:

Small-leaved : Grasslands Tahora (New Zealand), Gwenda (United Kingdom)

Small/Medium-leaved : Grasslands Prestige (New Zealand), Hajek (Czech Republic)

Medium-leaved : Grasslands Huia (New Zealand), Avoca (Ireland)

Medium/Large-leaved: Grasslands Sustain (New Zealand), Milkanova (Denmark)

Large-leaved : Grasslands Kopu (New Zealand), Tillman (United States)

Breeding backgrounds of the cultivars (as cited in Caradus & Woodfield 1997) are as follows:

New Zealand cultivars –

* Grasslands Tahora - developed from ecotypes collected from New Zealand moist hill country. Purpose-bred for hill country to supersede Grasslands Huia.

* Grasslands Prestige – bred for sheep grazing systems from clover ecotypes collected and evaluated in Northland, New Zealand.

* Grasslands Huia – selected from New Zealand wild white No. 1 strains collected in Hawkes Bay and North Canterbury for yield and persistence under grazing.

* Grasslands Sustain – selected from crosses between New Zealand and overseas germplasm, predominantly from the Mediterranean and the United States.

* Grasslands Kopu – based on crosses between 'Pitau' and selected plants from three ladino cultivars i.e: 'Regal', 'Pilgrim', and 'Ladino gigante lodigiano'. Bred for use in intensive lowland farming.

Overseas cultivars –

* Gwenda – selected for persistency under continuous grazing. Bred from Spanish germplasm.
* Hajek – bred at the breeding station Slavice, Czech Republic from 'Pastevec', 'Blanca', 'Daeno', 'Milkanova', and 'Retor'.
* Avoca – bred from 'Kersey'(developed from a single plant from Suffolk in 1924), 'S.184'(selected from New Zealand 'White Dutch' and 'English Wild' white clover), and 'Kent'(ecotype collected from registered fields of white clover in England and Wales.
* Milkanova – selected from 'Pajbjerg Milkanova' which was in turn selected from 'Morso' which is an old Danish ecotype.
* Tillman – bred from six white clover clones of diverse origin for persistence, high stolon density and disease resistance in South Carolina. Three of the six clones are identical to parent clones of 'Regal' (bred for increased summer production in Alabama).

Method: Seeds were scarified between two pieces of fine sandpaper until they acquired a 'scuffed' appearance. They were then placed in petri dishes lined with a double layer of moistened filter paper and pregerminated in a dark refrigerator at 4 °C for 48 h. The seeds were then placed in an incubator set at 25 °C for 48 h. Seed trays were filled with regular potting mix (6:4 peat to sand ratio with beads of Nutracote slow release fertilizer added) and seedlings placed in 3-4 mm deep depressions before being covered with a fine layer of sand. Four trays of each cultivar were sown, each tray containing 30 seedlings.

The trays were then placed in a semi protected glasshouse environment under prevailing daylength and temperature (June-August 1997) at AgResearch Grasslands, Palmerston North. Trays were placed in a random block arrangement and rotated every four days due to conditional differences in areas of the glasshouse. The trays were watered from the bottom to prevent the disturbance of soil levels surrounding the emerging plants and the hypocotyl height of each seedling (from crown to soil surface) measured using vernier calipers every four days. Measurements were recorded from 4 d after sowing to 65 d from sowing. At day 65 the length and width of the central

leaflet from the largest leaf of each plant was measured and recorded. Mean leaf lengths and widths were recorded for each cultivar, and mean leaf areas calculated (leaves were assumed to be ellipsoid in shape).

2.2 Comparison of contractile vs non-contractile growth.

Overview: Seedlings of the contractile and non-contractile lines (bred from cv. Grassland Huia) were grown and the hypocotyl height (from crown to soil surface) of each seedling measured up to 39 d after sowing.

Method: The two white clover lines used (contractile and non-contractile) were obtained from stocks held by AgResearch Grasslands, Palmerston North. Non-contractile seedlings were identified among more than 20,000 cv. Grasslands Huia seedlings screened for abnormal phenotypes. Seedlings displaying the non-contractile phenotype were re-screened 3-4 times over a twelve month period. Eight plants with non-contractile hypocotyls were selected and polycrossed to give the Cycle 1 population. These seeds were termed 'non-contractile' and used for Experiments 1, 2 and 3. The 'contractile' control line for these experiments came from the randomly selected plants which showed normal hypocotyl contraction. Thus, the two lines were selected from the same base population, but are not isogenic.

Seeds of the contractile and non-contractile lines were scarified, pre-germinated and sown as described in Section 2.1. Three trays (each tray containing 48 seedlings) of each line were prepared, and arranged, rotated and watered as described in Section 2.1. The trays were placed in a semi protected glasshouse environment under prevailing daylength and temperature (March-May 1995) at AgResearch Grasslands, Palmerston North. The hypocotyl height of each seedling was measured (see Section 2.1) and recorded every 2-3 d until 39 d after sowing.

2.3 Application of Ethrel and silver thiosulphate to contractile and non-contractile seedlings.

2.3.1 Experiment one

Overview: Seedlings of the contractile and non-contractile lines (see Section 2.2 for selection of lines) were grown and treated with various concentrations of Ethrel or silver thiosulphate. The hypocotyl height of each seedling was measured up to 50 d after sowing.

Method: Seeds of the contractile and non-contractile lines were scarified, pre-germinated and sown as described in Section 2.1. Twenty trays (each tray containing 20 seedlings) of each line were prepared, and arranged, rotated and watered as described in Section 2.1. The trays were placed in a semi protected glasshouse environment under prevailing daylength and temperature (April-June 1995) at AgResearch, Grasslands Palmerston North. The hypocotyl height of each seedling was measured (see Section 2.1) and recorded every 2-3 d until 50 d after sowing. The twenty trays of each line were divided into five treatment lots and treated weekly with the first treatment 7 d after sowing. The following treatments were applied :

Treatment 0 : Controls, water

Treatment 1 : Ethrel 48, 50 $\mu l\ l^{-1}$

Treatment 2 : Ethrel 48, 500 $\mu l\ l^{-1}$

Treatment 3 : Ethrel 48, 5000 $\mu l\ l^{-1}$

Treatment 4 : Silver thiosulphate, 1mM

The Ethrel 48 (May and Baker agrochemicals; active ingredient Ethephon, 480 g per litre) was diluted with distilled water to give the concentrations used. The silver thiosulphate solution was prepared by mixing equal volumes of (analytical grade) 16 mM sodium thiosulphate solution and 2 mM silver nitrate solution (Veen and van de Geijn 1978). Fresh solutions were prepared and the silver nitrate added slowly to the sodium thiosulphate to avoid precipitation. The Ethrel solutions were applied to seedlings by spraying to incipient run-off, whilst approximately 2 ml of silver thiosulphate solution was applied to the sand plug directly surrounding each seedling.

The plants in the control trays (Treatment 0) were sprayed to incipient run-off with distilled water as a reference.

2.3.2 Experiment two

Overview: Experiment one (Section 2.3.1) was repeated using different concentrations of Ethrel and silver thiosulphate.

Method: The general methods used were the same as those described above in section 2.3.1. However, the concentrations of Ethrel 48 and silver thiosulphate used varied to that of the previous experiment as did the time of year when the experiment was performed. Twenty trays of each line (each tray containing 20 seeds) were grown under prevailing daylength and temperature (July-September 1995) at AgResearch Grasslands, Palmerston North, divided into five treatment lots and treated weekly with the first treatment 7 d after sowing. The following treatments were applied :

Treatment 0 : Controls, water

Treatment 1 : Ethrel 48, 50 $\mu l\ l^{-1}$

Treatment 2 : Silver thiosulphate, 1mM

Treatment 3 : Silver thiosulphate, 5mM

Treatment 4 : Silver thiosulphate, 20mM

The hypocotyl height of each seedling was measured and recorded every 2-3 days up to 48 d from sowing. In addition, the average petiole length for each plant was recorded at 26, 29, 32, 35, 38 and 41 d.

2.4 Ethylene production by contractile and non-contractile seedlings grown in culture.

Overview: Seeds of the contractile and non-contractile lines (see Section 2.2 for selection of lines) were grown in culture in contained glass vials. Gas samples were

obtained from the headspace of each vial and analysed for ethylene using gas chromatography.

Method: Seeds of the contractile and non-contractile lines were rubbed between two pieces of fine sandpaper until they aquired a 'scuffed' appearance (scarification) then placed in acidified mercuric chloride solution (0.2 % + 0.5 % HCl) for 5 min (surface sterilisation). They were then rinsed five times with sterile water and allowed to imbibe for 1 h. Next they were placed in petri dishes containing 0.8 % water agar and grown for 2 d in a growth room situated at AgResearch Grasslands, Palmerston North (approximately 25 °C, 16 h daylength and 310 $\mu Em^{-2} s^{-1}$, November-December 1996). The seedlings were then transferred under sterile conditions to 5 ml Venoject blood sample vials containing 2 ml of half strength CRO media (White & Voisey 1994) and covered with sterile foil caps. One seedling was placed in each vial. Fifteen seedlings from each line were then grown for a further 4 d at 24 °C before the vials were sealed with sterile rubber bungs for 48 h. Duplicate gas samples were then taken (100µl volume) from the vials and analysed using the Photovac situated at the Plant Growth Unit, Massey University (day 7 samples). Vials containing media but no seedlings were also sampled as controls. The remaining seedlings were grown, prepared and sampled in a similar manner to give Day 14 and Day 21 samples respectively. Once sampled the seedlings were removed from the vials and cut into shoot, hypocotyl and root sections before being weighed. At each time period (i.e. at days 7, 14 and 21) duplicate gas samples were taken from four vials containing media but no seedlings to act as controls.

Although fifteen seedlings of each line were prepared, not all of these were able to be sampled due to experimental and logistical problems. Each vial was sampled in duplicate. The number of seedlings sampled and the total number of samples taken at each time period is shown in Table 2.1.

Table 2.1 : Number of vials sampled and total number of samples taken 7, 14, and 21 d after imbibition.

	Contractiles		Non-Contractiles	
Day	Number of vials sampled	Total number of samples	Number of vials sampled	Total number of samples
7	9	18	9	17
14	15	30	14	28
21	14	28	14	28

2.5 Measurement of cortical and epidermal hypocotyl cells from contractile and non-contractile plants grown in culture.

Overview: Hypocotyls from the seedlings used in Section 2.4 (ethylene production) were fixed, sectioned, stained and examined using light microscopy. Measurements of cortical and epidermal cell heights and widths were recorded.

Method: Seedlings grown in culture (November-December 1996) were separated into root, shoot and hypocotyl sections and the fresh weight of each determined. Once weighed the hypocotyls were immediately placed in 70 % ethanol to fix and preserve them until required. When removed from the ethanol, longitudinal sections (L.S sections) of the hypocotyls were prepared by hand (with a razor blade) and stained briefly (1-2 min) with Toluidine Blue (0.1 % in 0.1 % disodium tetraborate). These sections were rinsed, tranferred onto slides, mounted in glycerine and the coverslips sealed with clear nail varnish. The sections were then observed using a Zeiss compound light microscope. Sections from three hypocotyls of each line (contractile and non-contractile) for each time period (i.e. days 7, 14 and 21) were observed and the cell height and width of approximately 20 cortical and 20 epidermal cells from each hypocotyl (from the same region of the hypocotyl) were measured using an eyepiece micrometer and the area of each cell calculated.

2.6 Examination of hypocotyls from contractile and non-contractile plants grown in a glasshouse - light microscopy part I.

Overview: Seeds of the contractile and non-contractile lines were grown and hypocotyl samples taken 14, 21, 35 and 56 d after sowing. Longitudinal sections of these samples were prepared and counter-stained with Safranin O and Fast Green before being observed using light microscopy.

Method: In 1995 two hundred progeny of Cycle I seed (see Section 2.2 for Cycle I selection) were grown in a glasshouse (situated at AgResearch Grasslands, Palmerston North). Two genotypes which most strongly expressed the non-contractile phenotype after eight months were pair-crossed to provide the Cycle 2 non-contractile population. Eight plants which expressed the contractile phenotype as seedlings (after 42 days) were polycrossed in isolation to provide a contractile control population. One tray of seeds per line (each tray containing 20 seeds) were scarified, pre-germinated and sown as described above in Section 2.1. The trays were then placed in a semi protected glasshouse environment under prevailing daylength and temperature (September-November 1997) at AgResearch Grasslands, Palmerston North. Hypocotyls of each line were taken 14, 21, 35 and 56 d after sowing and fixed in Bouin's fluid (70ml saturated picric acid solution, 25ml formalin, 5ml glacial acetic acid) for 6-8 h at room temperature. The hypocotyls were then rinsed with 70% ethanol until no yellow colour remained and transferred to 70% ethanol and stored until paraplast wax processing. Paraplast processing involved the tissue undergoing dehydration (70% ethanol 1h, 95% ethanol + 0.1% eosin stain overnight; absolute ethanol x3 changes 1h each; absolute ethanol overnight), clearing (25% histoclear 1h, 50% histoclear 1h; 75% histoclear 1h; 100% histoclear x3 changes 1h each), and infiltration (50:50 histoclear/paraplast 4h; 4-6 changes of paraplast 4h each) before being embedded in blocks of pure paraplast. A microtome was used to cut the tissue into 10 μm thick sections.

The sections were then rehydrated (100% histoclear 1-3 min; 75 % histoclear 1-3 min; 50 % histoclear 1-3 min; 25 % histoclear 1-3 min; 100 % ethanol 1-3 min; 95 % ethanol 1-3 min; 75% ethanol 1-3 min; 50% ethanol 1-3 min; 25 % ethanol 1-3 min;

ddH_2O 1-3 min) and left to stain overnight in a 1% aqueous solution of Safranin O. Slides were rinsed with water to remove excess stain, dipped in 95% ethanol 15-20 times, and dipped for 30-40 secs in acidified 95% ethanol (1.9 ml hydrochloric acid sp. gr. 1.18, per litre of 95% ethanol) to differentiate the Safranin O. The action of the acid was stopped by dipping the slides in 95% ethanol to which 4-5 drops of ammonia (sp. gr. 0.89) per 100 ml had been added. Slides were then rinsed in 95% ethanol and dipped in a 0.1% solution of Fast Green in 95% ethanol, for 2-6 min. The slides were then rinsed twice in absolute ethanol and histoclear, mounted using DPX and a No.1 thickness glass coverslip.

At 14 and 21 d, sections from three seedlings of each line were examined (using a Zeiss compound light microscope). The height and width of twenty cortical and twenty epidermal cells from the same region of each seedling (between 1 and 3 mm below the cotyledonary node) were measured using an eyepiece micrometer. At 35 and 56 d, sections from each three seedlings of each line were also examined, but the height and width of twenty ray parenchyma cells per seedling was measured as cortical and epidermal tissue no longer existed. The mean height, width and area of all cell types were then calculated.

The overall height (from soil level to the base of the cotyledonary node) and width (3 mm below the cotyledonary node) of each hypocotyl were also measured, and the type and number of cells across each tissue layer recorded. The number of cells (epidermal and cortical, or parenchyma depending on the age of the seedling) along the length of each hypocotyl (from the cotyledonary node to the soil surface) were also measured and means and Least Significant Differences (LSD's) calculated.

2.7 Examination of hypocotyls from contractile and non-contractile plants grown in a glasshouse - light microscopy part II.

Overview: Seeds of the contractile and non-contractile lines (see Section 2.6 for selection of lines) were grown and hypocotyl samples taken 49 d after sowing.

Transverse sections of these samples were prepared and counter-stained with Safranin O and Fast Green before being observed and photographed using light microscopy.

Method: Seedlings were grown (July-September 1997) and sections prepared as described above in Section 3.6, but transverse sections instead of longitudinal sections were prepared, and only hypocotyls from 49 d old seedlings were observed. Sections taken from 3 mm below the cotyledonary node from representative seedlings displaying typical contractile and non-contractile growth were observed and photographed using light microscopy. For each seedling, the total hypocotyl width, and the width of the central core containing xylem was measured and recorded. The type and number of cells in each tissue layer were also noted.

2.8 Statistical analysis

Statistical analysis of all experiments was performed using Proc. GLM of Statistical Analysis System (SAS Institute, 1988). Data normality was tested using the univariate procedure and data transformed using log or square root transformations where appropriate. F values were determined from expected means squares for mixed models (Steel and Torrie, 1980) and calculated using Type III sums of squares to account for unbalanced replication and missing values. Standard errors (SE's), least significant differences (LSD's), correlation coefficients (R values), and coefficients of variance (CV's) were calculated and are shown where appropriate.

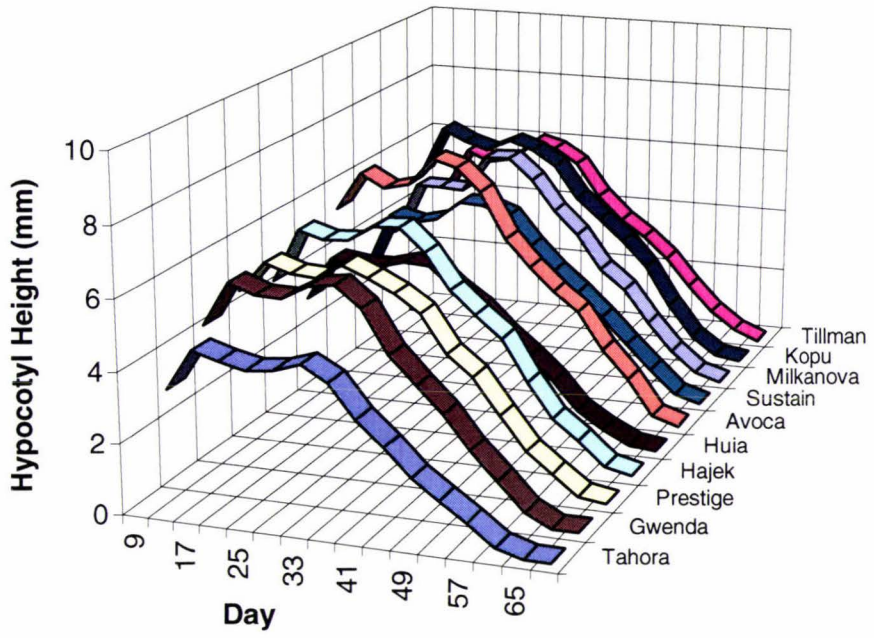
CHAPTER 3 : RESULTS

3.1 Contractile growth in a range of cultivars

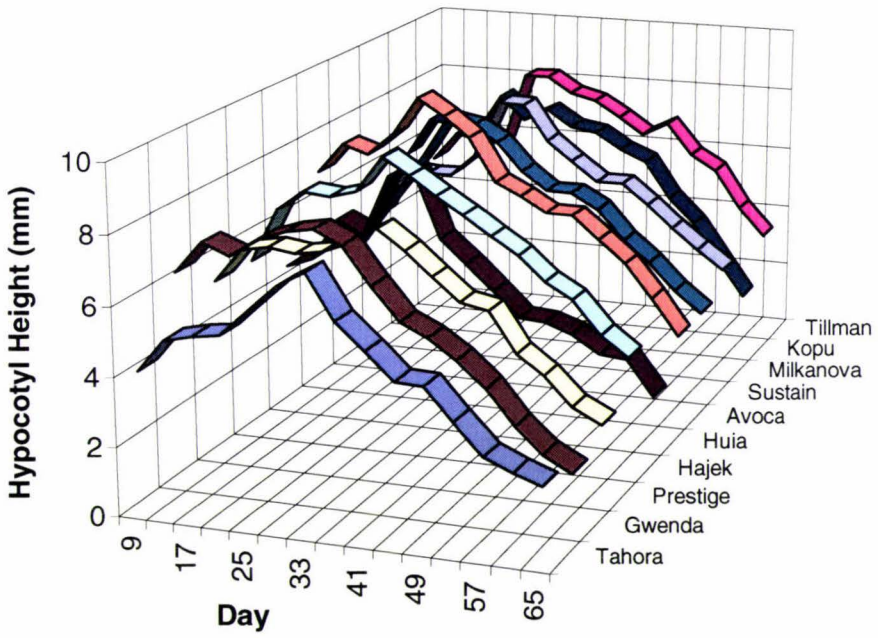
The hypocotyl growth of ten different white clover cultivars (*Trifolium repens* L.), chosen to represent a full range of leaf size and origin, was measured, analysed and compared over time. The mean length, width and area of mature leaves were also measured and calculated for each cultivar.

Figure 3.1A shows the hypocotyl growth trends displayed by plants from the various cultivar populations that displayed contractile growth. All of the cultivars showed similar trends of hypocotyl growth over time. Hypocotyl height (measured from the crown to the soil surface) increased rapidly until 13 d after sowing when the rate of growth decreased slightly for several days. Hypocotyl height increased again over the next 7-10 d until a maximum was reached 25-29 d after sowing. Hypocotyl height then steadily decreased until 61-65 d (by this time the hypocotyls had contracted to such an extent that the crown of the plant lay at or below the soil surface). Figure 3.1B shows the hypocotyl growth trends displayed by plants from the various cultivar populations that displayed non-contractile growth. Again, the trends in hypocotyl growth displayed by the various cultivars over time were very similar. Hypocotyl height rapidly increased until reaching a maximum at around 25 d, then decreased steadily from this time until 65 d. Although the hypocotyl height of these plants was still decreasing at 65 d, none of the hypocotyls had contracted to or below the soil surface (as was the case with the contractile plants).

The percentage of plants from each cultivar population that 1) survived, and 2) displayed non-contractile growth are shown in Table 3.1. The percentage of plants from each population that survived until 65 d ranged from 46.7% (Huia) to 95.8% (Kopu), and the percentage of plants that displayed non-contractile growth ranged from 1.8% (Huia) to 21.8% (Avoca).



(A)



(B)

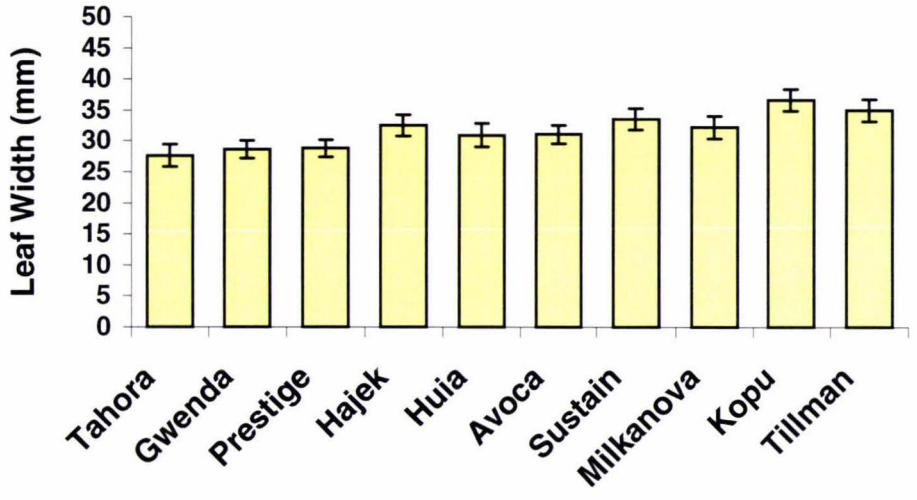
Figure 3.1 : Hypocotyl growth trends for seedlings of different cultivars which (**A**) displayed contractile growth, and (**B**) displayed non-contractile growth. Values shown are means calculated after data from plants displaying non-contractile growth were removed from the contractile data set, and data from plants displaying contractile growth were removed from the non-contractile data set.

Table 3.1 : Percentage of plants (from an initial population of 120 plants per line) that survived by 65 d, and percentage of surviving plants that displayed non-contractile growth.

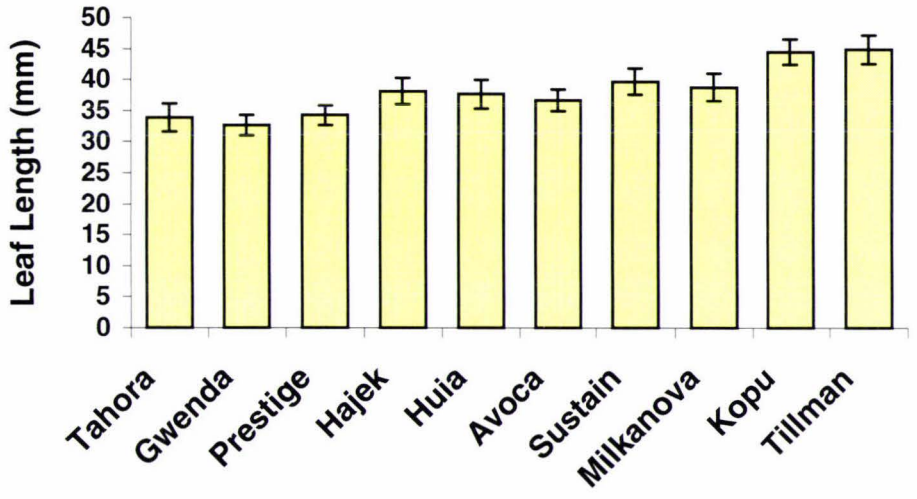
Line	Plant survival (%)	Surviving plants that displayed non-contractile growth (%)
Tahora	47.5	10.5
Gwenda	81.7	12.2
Prestige	93.3	10.7
Hajek	61.7	17.6
Huia	46.7	1.8
Avoca	81.7	21.8
Sustain	67.5	16.0
Milkanova	70.0	11.9
Kopu	95.8	5.2
Tillman	78.3	11.7
Mean	72.4	11.9

The mean length and width of mature leaves from each of the cultivars are shown in Figure 3.2 A and B. Significant differences in both leaf length and width can be seen between cultivars classified by the OECD (Organisation for Economic Co-Operation and Development 1993) as small, small/medium, medium, medium/large, or large leaved. The mean maximum hypocotyl height attained by each cultivar, and the day at which that maximum was attained, were also calculated and are shown in Table 3.2.

To investigate a possible relationship between leaf size and maximum hypocotyl height, mean leaf areas were calculated for each cultivar, and plotted against the mean maximum hypocotyl height attained by that cultivar. This correlation is shown in Figure 3.3. The correlation coefficient ('R' value displayed on Figure 3.3) for the cultivars of New Zealand origin was 0.69, indicating a positive relationship between hypocotyl height and leaf area for this material (i.e. the larger the leaves the longer the hypocotyl). This relationship was almost linear for all of the New Zealand cultivars except Prestige (which had a much longer hypocotyl than expected for a cultivar with such small leaves). The 'R' value for the cultivars of various overseas



(A)



(B)

Figure 3.2 : Leaf length **(A)**, and leaf width **(B)**, in a range of cultivars. Values shown are means ± SE, calculated after data from plants displaying non-contractile growth were removed from the contractile data set, and data from plants displaying contractile growth were removed from the non-contractile data set.

Table 3.2 : Maximum hypocotyl height ±SE attained by contractile and non-contractile seedlings of white clover (*Trifolium repens* L.) cultivars. Data from plants displaying non-contractile growth were not included in calculations for the 'contractile' population means and vice versa.

	Line	Maximum mean hypocotyl height ±SE (mm)	Days after sowing that maximum height was achieved
Contractiles	**Tahora**	4.58 ± 0.24	29
	Gwenda	6.11 ± 0.24	29
	Prestige	5.99 ± 0.26	25
	Hajek	6.56 ± 0.24	29
	Huia	4.88 ± 0.26	25
	Avoca	7.46 ± 0.26	25
	Sustain	5.60 ± 0.26	25
	Milkanova	6.55 ± 0.24	29
	Kopu	6.57 ± 0.26	25
	Tillman	6.06 ± 0.26	25
Non-contractiles	**Tahora**	7.32 ± 0.85	33
	Gwenda	7.80 ± 0.44	29
	Prestige	7.28 ± 0.36	33
	Hajek	8.73 ± 0.39	29
	Huia	7.74 ± n/a	29
	Avoca	9.42 ± 0.28	25
	Sustain	8.24 ± 0.45	25
	Milkanova	8.35 ± 0.51	29
	Kopu	8.00 ± 0.41	25
	Tillman	8.23 ± 0.50	29

origins (see Chapter 2, Section 2.1 for descriptions and origins of cultivars) was 0.23. Thus the correlation between hypocotyl height and leaf area for this material was extremely weak. Amongst the overseas cultivars Tillman stood out as having much larger leaves in relation to hypocotyl height than the other overseas cultivars.

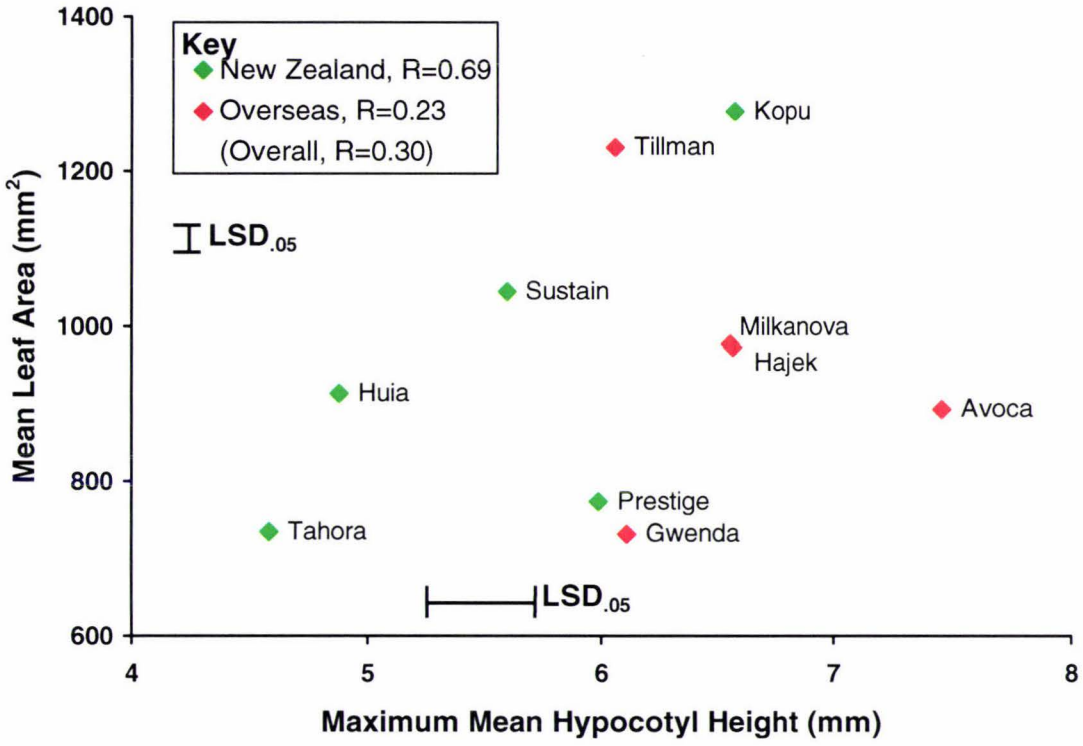


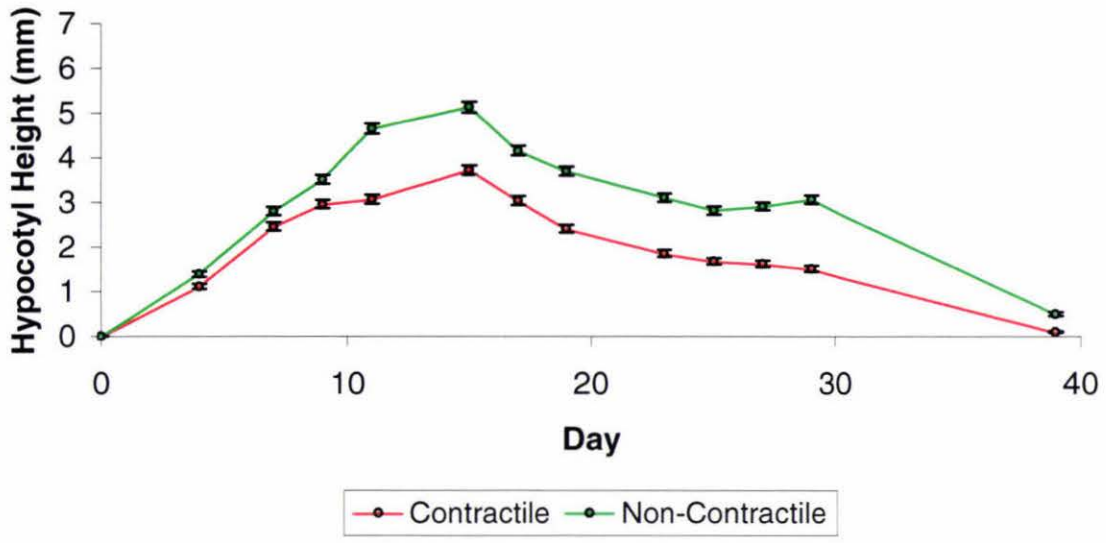
Figure 3.3 : Maximum mean hypocotyl height versus mean leaf area for ten cultivars of i) New Zealand and ii) Overseas, origin. Values shown are means calculated after data from plants which did not display contractile growth had been removed from the data set.

3.2 Comparison of contractile *vs* non-contractile growth

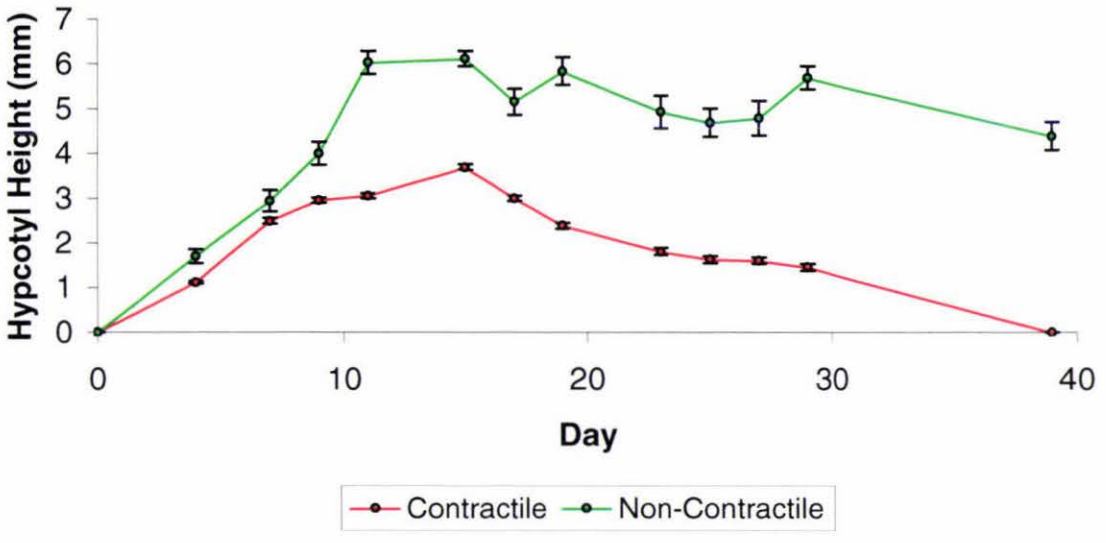
The hypocotyl heights of seedlings from the contractile and non-contractile lines (cycle I selection) were measured and growth comparisons made.

Hypocotyls of the two lines followed the same general growth trend with hypocotyl height increasing steadily until a maximum around 15 d after sowing, then decreasing steadily (Figure 3.4 A). However, there were significant differences in the mean hypocotyl heights attained by the two lines. The maximum mean height attained by the contractile line (at 15 d) was 3.7 mm whereas the maximum mean height attained by the non-contractile line was 5.1 mm. This 1-2 mm height difference was maintained until the last sample point at 39 d where the difference between the contractile and non-contractile lines was only 0.5 mm. Also, after 39 d the mean hypocotyl height of the contractile line was almost zero (0.1 mm) indicating that by this time almost all of the seedlings had fully contracted, whereas the mean hypocotyl height of the non-contractile line was still 0.5 mm.

Figure 3.4 B also displays the hypocotyl growth of the two lines as observed and recorded during this experiment. As such the data follows the same general trends as those displayed in Figure 3.4 A. However, in this graph data from plants in the contractile population which displayed non-contractile growth, and data from plants in the non-contractile population which displayed contractile growth, were removed before the mean values were calculated. The maximum mean hypocotyl height attained by the non-contractile line in Figure 3.3 A was 5.14 mm at 15 d, and the mean hypocotyl height attained after 39 d was 0.5 mm. However, once contractile data was removed, these values changed to 4.38 mm after 39 d, and a maximum height of 6.11 mm at 15 d. The percentages of plants in each population which i) survived, and ii) displayed non-contractile growth, are shown in Table 3.3.



(A)



(B)

Figure 3.4 : Hypocotyl growth of seedlings from the contractile and non-contractile lines with data from plants in the contractile population which displayed non-contractile growth and data from the non-contractile population that displayed contractile growth included in the analysis **(A)**, and **(B)** removed before analysis. Values shown are means ± SEs.

Table 3.3 : Percentage of plants (from an initial population of 144 plants per line) from the contractile and non-contractile lines which survived at 39 d, and percentage of surviving plants that displayed non-contractile growth.

Line	Plant survival (%)	Surviving plants that displayed non-contractile growth (%)
Contractile	56.9	2.4
Non-contractile	59.7	11.6

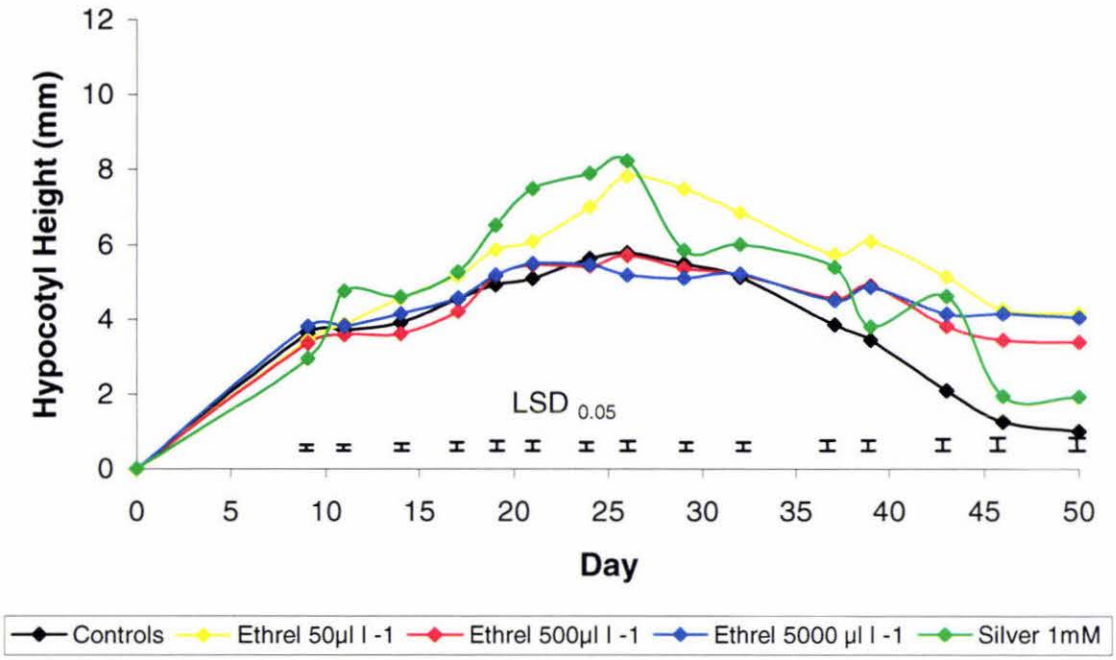
3.3 Application of Ethrel and silver thiosulphate to contractile and non-contractile seedlings

3.3.1 Experiment one

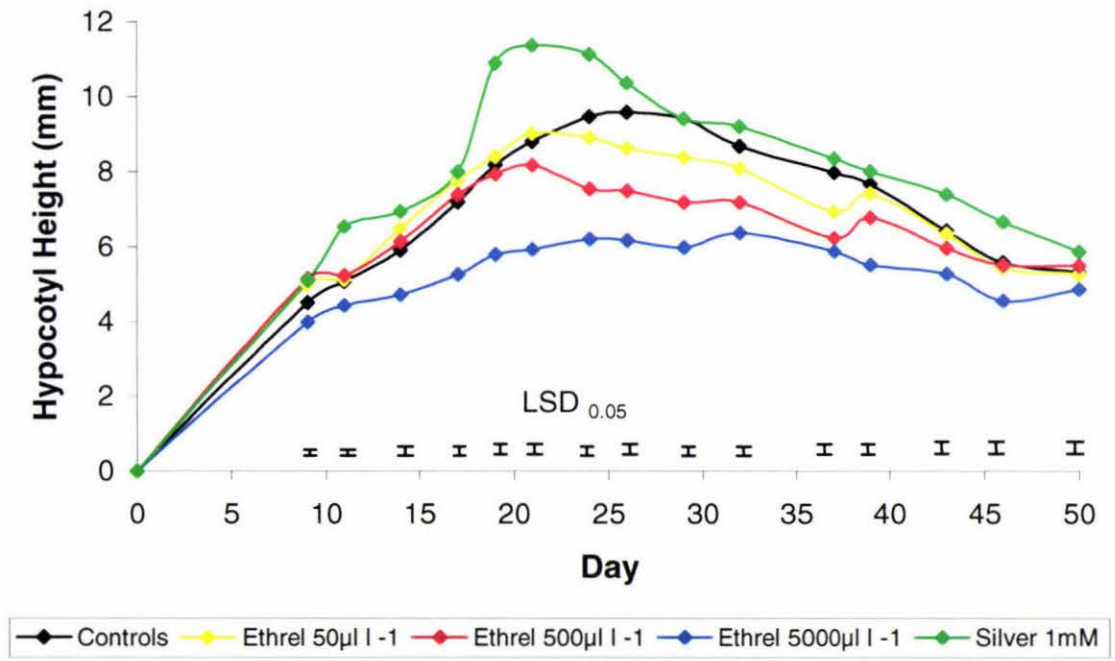
Seedlings of the contractile and non-contractile lines (cycle I selection) were treated weekly with solutions of silver thiosulphate or Ethrel and hypocotyl growth measured.

Hypocotyl growth of both the contractile and non-contractile lines followed the same general trend regardless of the treatment applied (Figure 3.5 A and B). That is, hypocotyl height increased steadily until reaching a maximum around 23-26 d after sowing, then decreased steadily. However, some significant differences in mean hypocotyl heights were obtained (both within the lines and between them) when seedlings were treated with Ethrel or silver thiosulphate.

The mean hypocotyl height measurements attained by seedlings from the contractile line are shown in Figure 3.5 A. Treatment of contractile seedlings with both Ethrel (particularly at 50 $\mu l\ l^{-1}$) and silver thiosulphate solutions resulted in longer hypocotyls than those of the control group. When treated with 50 $\mu l\ l^{-1}$ Ethrel, seedlings produced hypocotyls that were significantly longer than those of the control group from 15 d onwards. Seedlings treated with 500 and 5000 $\mu l\ l^{-1}$ also had longer hypocotyls than the control group, but these differences only became significant after 37 d.



(A)



(B)

Figure 3.5 : Hypocotyl growth of seedlings from the **(A)** contractile, and **(B)** non-contractile lines, treated with various concentrations of Ethrel and silver thiosulphate. Values shown are means.

Throughout the experiment contractile seedlings treated with 1 mM silver thiosulphate solution produced hypocotyls which were significantly longer than those of the control group.

The mean hypocotyl height measurements attained by seedlings of the non-contractile line are shown in Figure 3.5 B. Treatment of non-contractile seedlings with all three concentrations of Ethrel (particularly 5000 $\mu l\ l^{-1}$) resulted in shorter hypocotyls than those of the non-contractile control group, whereas treatment with 1 mM silver thiosulphate solution resulted in longer hypocotyls than those from the control group. Hypocotyls of seedlings treated with 500 $\mu l\ l^{-1}$ Ethrel were significantly shorter from 21 d to 39 d, and hypocotyls of seedlings treated with 5000 $\mu l\ l^{-1}$ Ethrel were significantly shorter than those of the control group throughout the experiment. Seedlings treated with 50 $\mu l\ l^{-1}$ Ethrel produced hypocotyls that were significantly shorter than those of the control group from 24 d to 36 d.

Direct comparisons of the effects of the various treatments between the two lines are displayed in Figure 3.6 A-E. The mean hypocotyl height measurements attained by seedlings from both the contractile and non-contractile lines when treated with water only (controls) are shown in Figure 3.6 A. The mean hypocotyl heights attained by the non-contractile seedlings were significantly greater than those attained by contractile seedlings throughout the time frame examined (0-50 d from sowing). In fact, from 26 d to 50 d a difference of approximately 4 mm remained between the two lines. Maximum mean heights of 5.78 mm and 9.60 mm were reached at 26 d by the contractile and non-contractile lines respectively. By 50 d the mean hypocotyl height for the contractile line was 1.00 mm whereas the mean height for the non-contractile line was 5.33 mm. This is because by 50 d almost, but not all, of the 'contractile' seedlings had fully contracted while most of the 'non-contractile' seedlings continued to display non-contractile growth.

When treated with Ethrel at 50 $\mu l\ l^{-1}$ the mean hypocotyl heights attained by seedlings from the contractile line were significantly greater than those attained by the non-contractile line (by approximately 1-2 mm) until 29 d after which the differences became non-significant (Figure 3.6 B). The maximum height achieved by the contractile line was 7.84 mm (at 26 d) and the maximum height achieved by the

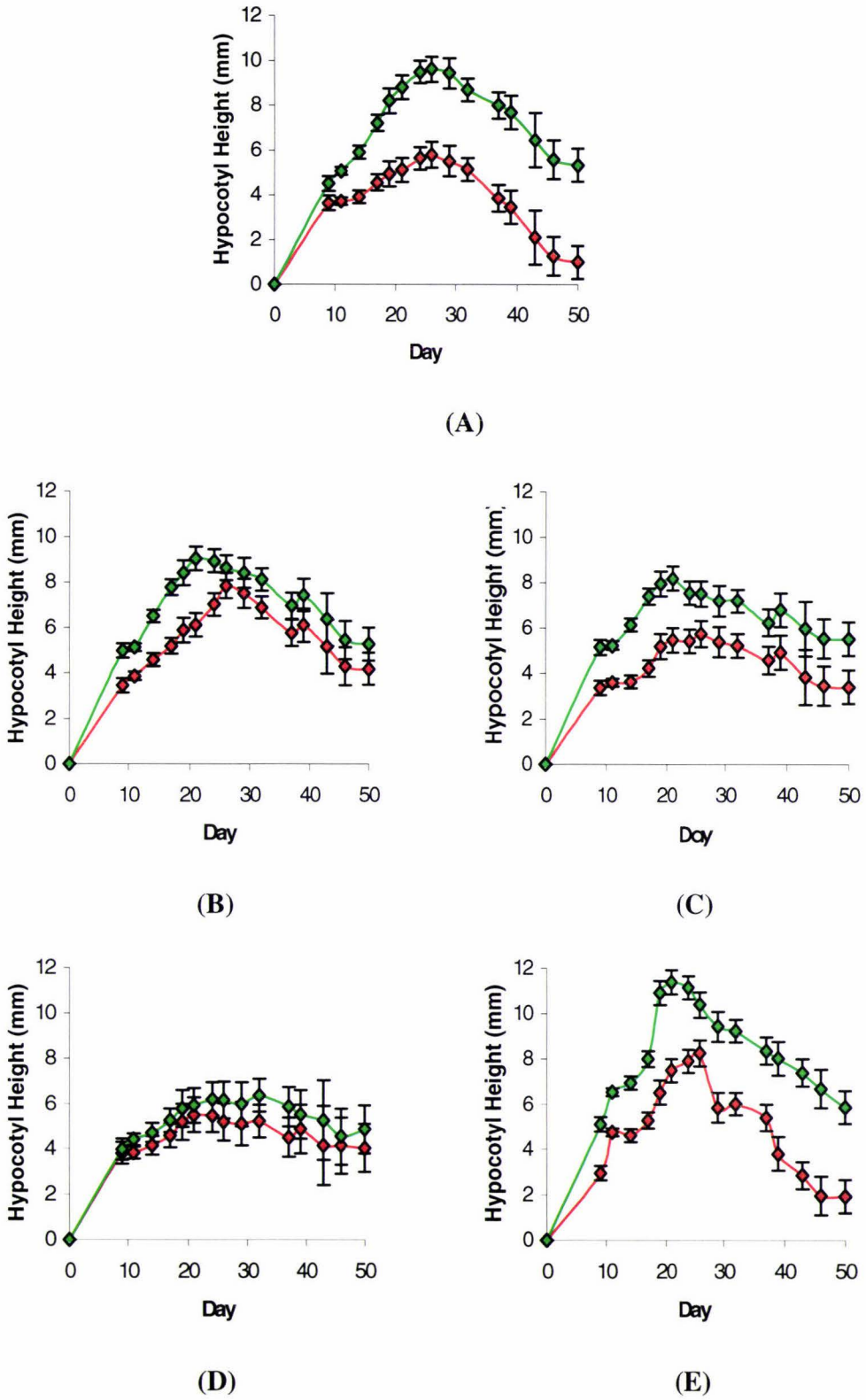


Figure 3.6 : Hypocotyl growth of seedlings from the contractile (shown in red) and non-contractile (shown in green) lines treated with various concentrations of Ethrel and silver thiosulphate. Values shown are means ± SEs.
(A) – Control plants (water).
(B) – Ethrel 50 $\mu l\, l^{-1}$.
(C) – Ethrel 500 $\mu l\, l^{-1}$.
(D) – Ethrel 5000 $\mu l\, l^{-1}$.
(E) – Silver thiosulphate 1mM.

non-contractile line was 9.03 mm (at 29 d). At 50 d the mean hypocotyl height of the contractile line was 4.18 mm as a high proportion of the seedlings had not contracted (Table 3.4). The mean height of the non-contractile line at 50 d was 5.27 mm.

The mean heights attained by non-contractile seedlings treated with Ethrel at 500 $\mu l\ l^{-1}$ were significantly greater than those attained by contractile seedlings treated in the same manner (Figure 3.6 B). In fact, a difference of approximately 2-3 mm remained throughout the experiment with contractile hypocotyls reaching a maximum height of 5.72 mm at 26 d, and non-contractile hypocotyls reaching a maximum of 8.18 at 21 d. At 50 d the mean hypocotyl heights attained by seedlings of the contractile and non-contractile lines were 3.4 mm and 5.5 mm respectively.

When treated with Ethrel at 5000 $\mu l\ l^{-1}$ the mean hypocotyl heights attained by non-contractile seedlings were greater than those attained by the contractile seedlings – but not significantly. Hypocotyls from the contractile line reached a mean maximum of 5.5 mm at 21 d, and hypocotyls from the non-contractile line reached a mean maximum of 6.2 mm at 24 d. By 50 d the mean hypocotyl heights were 4.04 mm for the contractile line and 4.87 mm for the non-contractile line.

When treated with 1 mM silver thiosulphate solution, the mean hypocotyl heights attained by non-contractile seedlings were significantly greater than those attained by contractile seedlings from as early as 10 d (Figure 3.6 E). In fact, a difference of approximately 4-5 mm in height remained from 19 d until the end of the experiment at 50 d. Seedlings from the contractile line attained a mean maximum of 8.25 mm after 26 d, and seedlings from the non-contractile line attained a maximum of 11.39 mm after 21 d. By 50 d the contractile hypocotyls had reached a mean height of 1.93 mm and the non-contractile hypocotyls a mean height of 5.87 mm.

The percentage of plants from the original populations sown that survived and the percentage of surviving plants which displayed non-contractile growth, are shown in Table 3.4. When treated with Ethrel at 5000 $\mu l\ l^{-1}$, less than 50% of either line survived whereas treatment with 50 $\mu l\ l^{-1}$ Ethrel , 500 $\mu l\ l^{-1}$ Ethrel, or 1 mM silver thiosulphate solutions had little or no effect on plant survival (when compared to the percentage of control plants which survived).

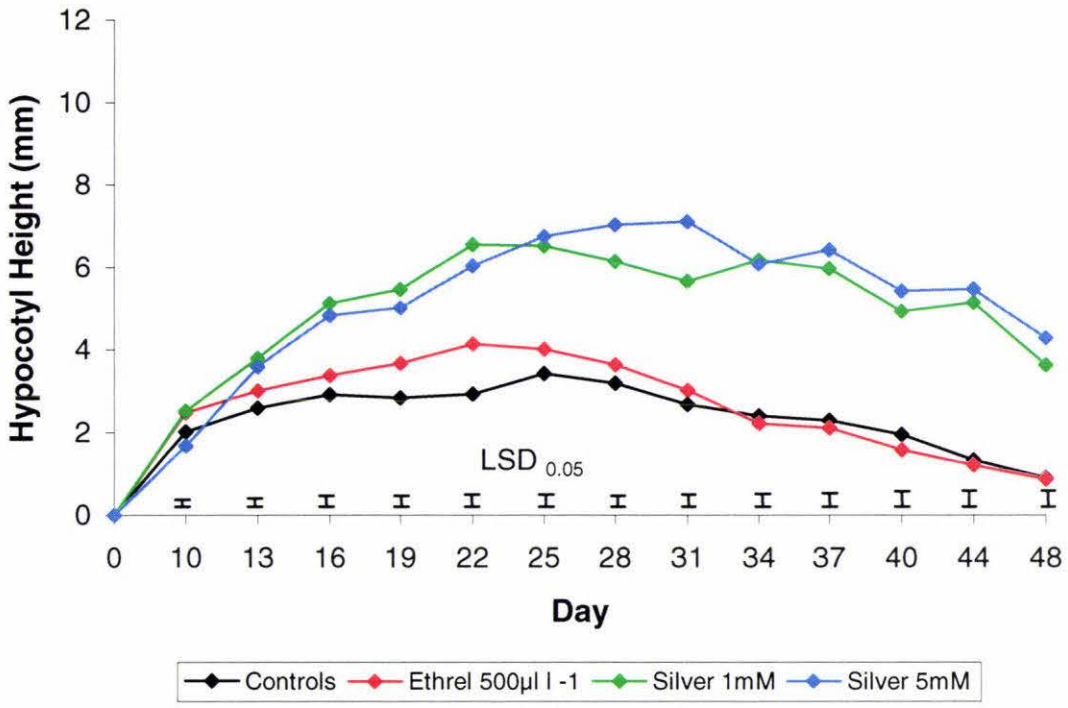
Table 3.4 : Percentage of plants (from an initial population of 80 plants per treatment lot per line) from the contractile and non-contractile lines that survived by 50 d, and the percentage of surviving plants that displayed non-contractile growth.

Treatment	Line	Plant survival (%)	Surviving plants that displayed non-contractile growth (%)
Controls, (water)	Contractile	78.7	22.2
Ethrel, (50 µl l^{-1})	Contractile	77.5	72.6
Ethrel, (500 µl l^{-1})	Contractile	76.2	68.9
Ethrel, (5000 µl l^{-1})	Contractile	42.5	94.1
Silver, (1 mM)	Contractile	71.2	35.1
Controls, (water)	Non-contractile	83.8	79.1
Ethrel, (50 µl l^{-1})	Non-contractile	95.0	82.9
Ethrel, (500 µl l^{-1})	Non-contractile	85.0	85.3
Ethrel, (5000 µl l^{-1})	Non-contractile	35.0	89.3
Silver, (1 mM)	Non-contractile	96.2	87.0

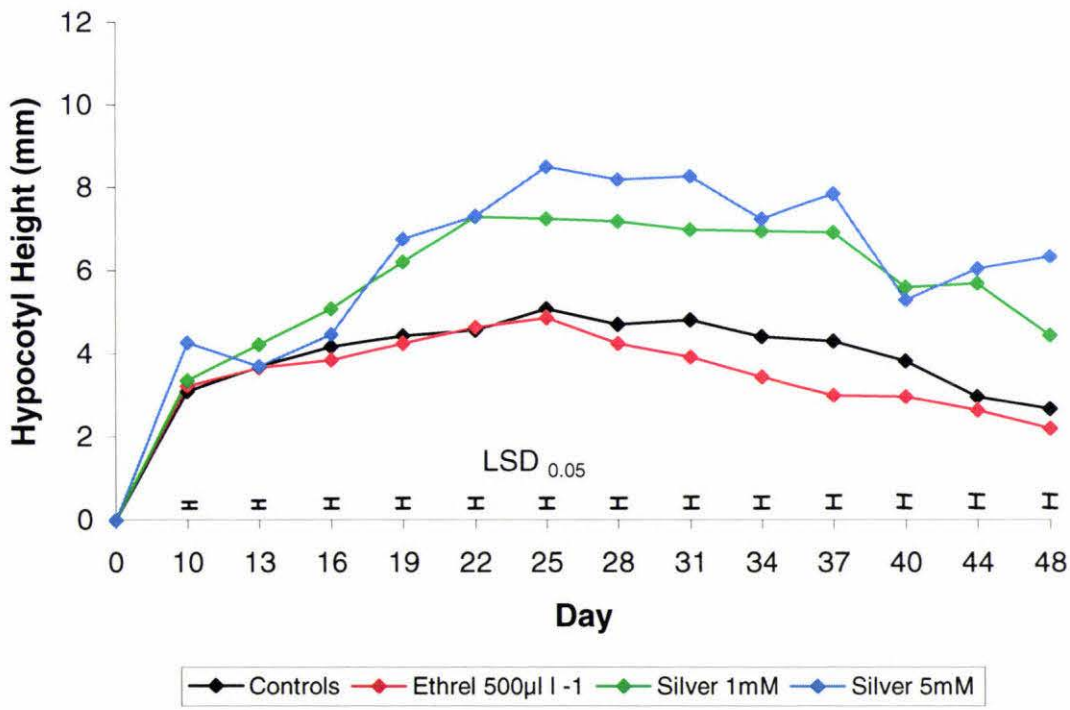
3.3.2 Experiment two

As in experiment one, seedlings of the contractile and non-contractile lines (cycle I selection) were treated weekly with solutions of Ethrel or silver thiosulphate and hypocotyl growth observed. However, for this experiment silver thiosulphate at 1 mM, 5 mM and 20 mM concentrations, and Ethrel at 500 µl l^{-1} were used. Data from plants treated with 20 mM silver thiosulphate are not presented as all plants died within 10 d of the first application.

The effects of the various treatments on hypocotyl growth of the two lines are shown in Figure 3.7 A and B. Growth trends for the two lines were similar, with hypocotyl height increasing steadily until reaching a maximum 22-31 d after sowing, then decreasing steadily. As in experiment one significant changes in hypocotyl growth occurred in both lines when seedlings were treated with Ethrel and the various silver thiosulphate solutions. When treated with either Ethrel or silver thiosulphate, the



(A)



(B)

Figure 3.7 : Hypocotyl growth of seedlings from the **(A)** contractile, and **(B)** non-contractile lines, treated with various concentrations of Ethrel and silver thiosulphate. Values shown are means.

hypocotyl heights attained by contractile seedlings were greater than the heights attained by the control plants of the same line (Figure 3.7 A). However, treatment with 500 µl l^{-1} Ethrel only caused a significant increase in hypocotyl length until 31 d. The hypocotyls of contractile seedlings treated with 1 mM silver thiosulphate were longer than the controls from 10 d on, and those of seedlings treated with 5 mM were longer than controls from 13 d on.

Treatment of seedlings from the non-contractile line with Ethrel resulted in hypocotyls that were shorter than those of the control group, whereas treatment with silver thiosulphate resulted in hypocotyls that were longer than those of the control group (Figure 3.7 B). Differences in height were significant from 28 d onwards after treatment with 500 µl l^{-1} Ethrel (with the exception of 44 d). When treated with 1 mM silver thiosulphate the hypocotyls of non-contractile seedlings were longer than control hypocotyls from 13 d on, while those treated with 5 mM silver thiosulphate were longer than the control hypocotyls from 19 d on.

Direct comparisons of the effects of the various treatments between the two lines are displayed in Figure 3.8 A-E. The mean hypocotyl height measurements attained by seedlings from both the contractile and non-contractile lines when treated with water only (controls) are shown in Figure 3.8 A. The mean hypocotyl heights attained by the non-contractile seedlings were significantly greater than those attained by contractile seedlings throughout the time frame examined (0-48 d from sowing). In fact, from 22 d to 48 d a difference of approximately 2 mm remained between the two lines. Maximum mean heights of 3.43 mm and 5.08 mm were reached at 25 d by the contractile and non-contractile lines respectively. By 48 d the mean hypocotyl height for the contractile line was 0.90 mm whereas the mean height for the non-contractile line was 2.67 mm. This is because by 48 d almost all of the contractile seedlings had fully contracted while most of the non-contractile seedlings continued to display non-contractile growth.

The mean hypocotyl height measurements attained by seedlings treated with Ethrel at 500 µl l^{-1} are shown in Figure 3.8 B. The mean heights attained by the non-contractile line were significantly greater than those attained by the contractile line from 25 d onwards. The amount by which the non-contractile hypocotyls were longer than the contractile hypocotyls continued to increase until 44 d when the difference was

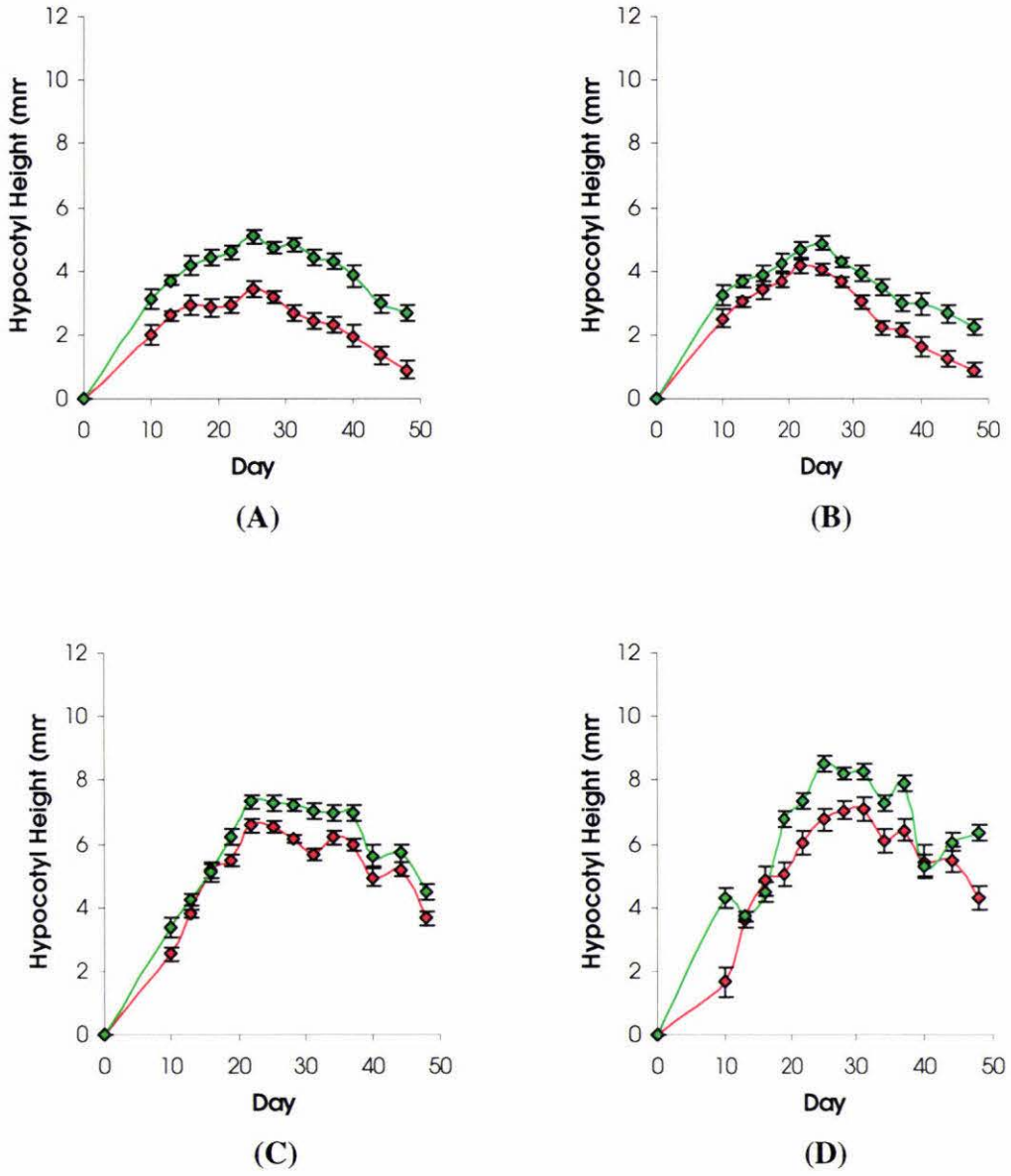


Figure 3.8 : Hypocotyl growth of seedlings from the contractile (shown in red) and non-contractile (shown in green) lines treated with various concentrations of Ethrel and silver thiosulphate. Values shown are means ± SEs.

(A) – Control plants (water).

(B) – Ethrel 500 $\mu l\ l^{-1}$.

(C) – Silver thiosulphate 1mM.

(D) – Silver thiosulphate 5 mM.

approximately 1.5 mm. Hypocotyls from the contractile line reached a maximum mean height of 4.15 mm by 22 d, and the non-contractile hypocotyls reached a maximum height of 4.87 mm by 25 d. The mean measurements obtained 48 d after sowing were 0.89 mm and 2.22 mm by the contractile and non-contractile lines respectively.

The hypocotyl heights of seedlings treated with 1 mM silver thiosulphate are shown in Figure 3.8 C. The non-contractile hypocotyls were significantly longer than the contractile hypocotyls from 22 d onwards and maximum mean heights of 6.56 mm and 7.31 mm were attained by the contractile and non-contractile lines 22 d after sowing. By 48 d after sowing the mean hypocotyl height for the contractile line was 3.65 mm, and the mean height for the non-contractile line was 4.46 mm.

When treated with 5 mM silver thiosulphate hypocotyls of the non-contractile seedlings were significantly longer (by 1-2 mm) than those of the contractile seedlings - except at 16, 19, 40 and 44 d (Figure 3.8 D). A mean maximum of 7.11 mm was attained by the contractile seedlings at 31 d, and a mean maximum of 8.51 mm was attained by the non-contractile seedlings at 25 d. By 48 d the respective hypocotyl heights of the contractile and non-contractile lines were 4.29 mm and 6.36 mm.

A large number of control seedlings, and a large number of non-contractile seedlings treated with 1 mM or 5 mM silver thiosulphate, died in this experiment due to excess algal growth and black fly infestation (Table 3.5). These factors were therefore taken into account when interpreting these results.

Table 3.5 : Percentage of plants (from an initial population of 80 plants per treatment lot per line) from the contractile and non-contractile lines which survived at 48 d, and percentage of surviving plants that displayed non-contractile growth.

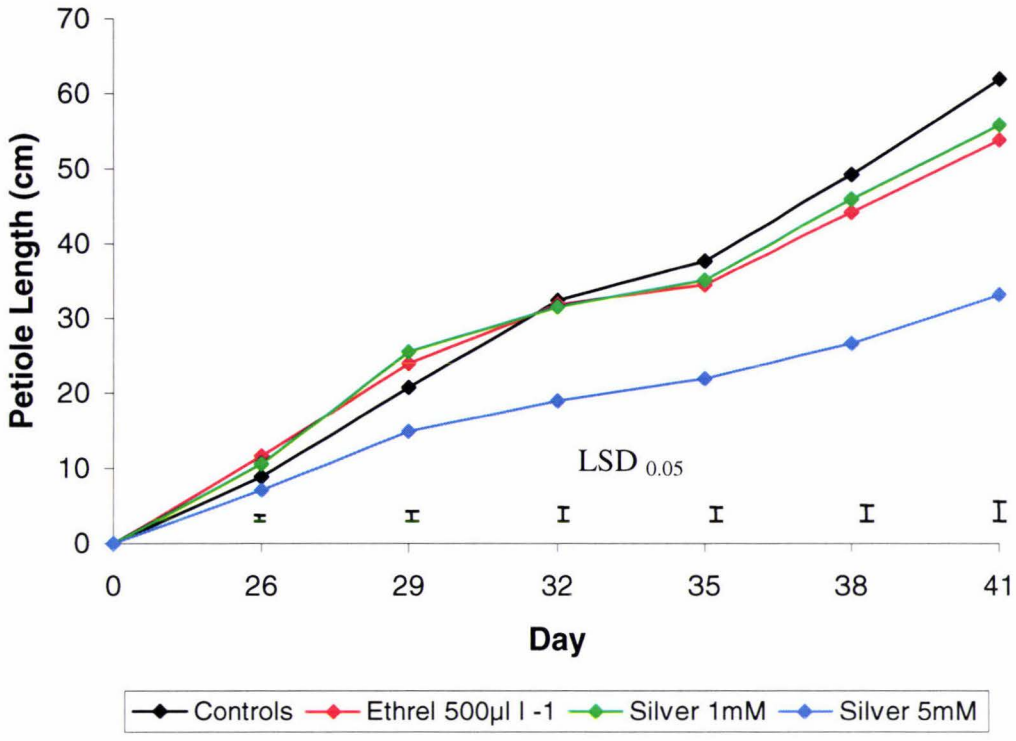
Treatment	Line	Plant survival (%)	Surviving plants that displayed non-contractile growth (%)
Controls, (water)	Contractile	30.0	25.0
Ethrel, (500 µl l^{-1})	Contractile	68.7	58.2
Silver, (1 mM)	Contractile	70.0	19.6
Silver, (5 mM)	Contractile	57.5	47.8
Controls, (water)	Non-contractile	75.0	70.0
Ethrel, (500 µl l^{-1})	Non-contractile	81.2	78.5
Silver, (1 mM)	Non-contractile	23.7	55.9
Silver, (5 mM)	Non-contractile	23.7	100.0

3.3.3 Experiment two (petiole comparisons)

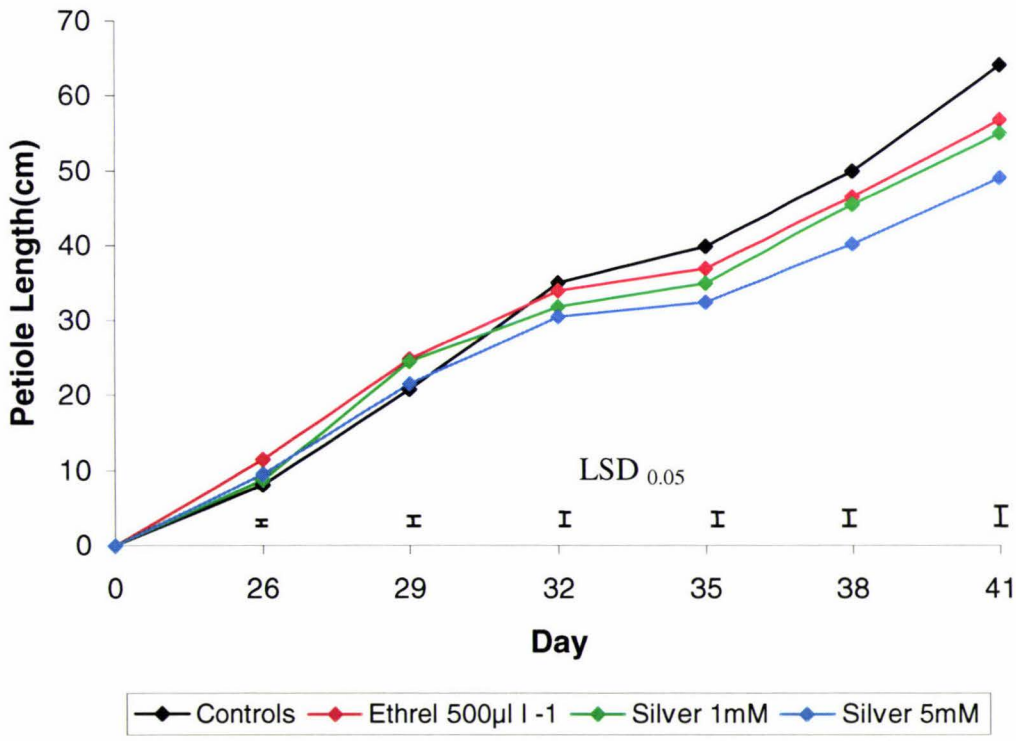
The mean petiole length of each plant studied in Section 2.3.2 was measured at 26, 29, 32, 35, 38 and 41 d.

The petiole growth of plants from both the contractile and non-contractile lines followed the same general trend regardless of the treatment applied (i.e. the petioles continued to increase in length from 26 d to 41 d) (Figure 3.9 A and B). However, significant differences in petiole length were obtained (both within the lines and between them) when plants were treated with Ethrel or silver thiosulphate.

The mean petiole length measurements obtained from plants of the contractile line are shown in Figure 3.9 A. The petioles of contractile seedlings treated with 500 µl l^{-1} Ethrel were significantly longer than those of seedlings treated with water (controls) 26 and 29 d after sowing. However, by 32 and 35 d there was no significant difference, and by 38 and 41 d they were significantly shorter than those of the control plants. The petioles of contractile plants treated with 1 mM silver thiosulphate were also



(A)



(B)

Figure 3.9 : Petiole lengths of plants from the **(A)** contractile, and **(B)** non-contractile lines treated with Ethrel and various concentrations of silver thiosulphate. Values shown are means.

significantly longer than those of the control plants at 26 and 29 d, and significantly shorter by 41 d. The petioles of plants treated with 1 mM silver thiosulphate displayed a similar trend, whereas, the petioles of plants treated with 5 mM silver thiosulphate were significantly shorter than those of the control plants at each of the six sample dates.

The mean petiole lengths of non-contractile plants treated with Ethrel at 500 $\mu l\ l^{-1}$ were significantly greater than those of plants treated with water (controls) at 26 and 29 d (Figure 3.9 B). However, by 41 d they were significantly less than those obtained from the control group. At 32, 35 and 38 d there was no significant difference in petiole lengths between the control plants and plants treated with 500 $\mu l\ l^{-1}$ Ethrel. The petioles of plants treated with 1 mM silver thiosulphate were significantly longer at 29 d, but significantly shorter at 35, 38 and 41 d, than those of the control plants. Plants treated with 5 mM silver thiosulphate had significantly shorter petioles than the control plants at 35, 38 and 41 d.

Direct comparisons of the effects of the various treatments between the two lines are displayed in Figure 3.10 A-D. When treated with water only (controls) the petiole lengths of both the contractile and non-contractile plants increased from 26-41 d, although there was no significant difference in length between the two lines (Figure 3.10 A). The petioles of plants treated with 500 $\mu l\ l^{-1}$ Ethrel and 1 mM silver thiosulphate also followed this trend - increasing in length from 26-41 d but showing no significant difference in length between the two lines (Figure 3.10 B and C). When treated with 5 mM silver thiosulphate the petioles of the non-contractile plants were significantly longer than those of the contractile plants by as much as 10 cm (Figure 3.10 D).

3.4 Ethylene production by contractile and non-contractile seedlings grown in culture

Seedlings of the contractile and non-contractile lines (cycle II selection) were grown in culture. Gas samples were taken from the headspace above the seedlings and analysed for ethylene using a Photovac.

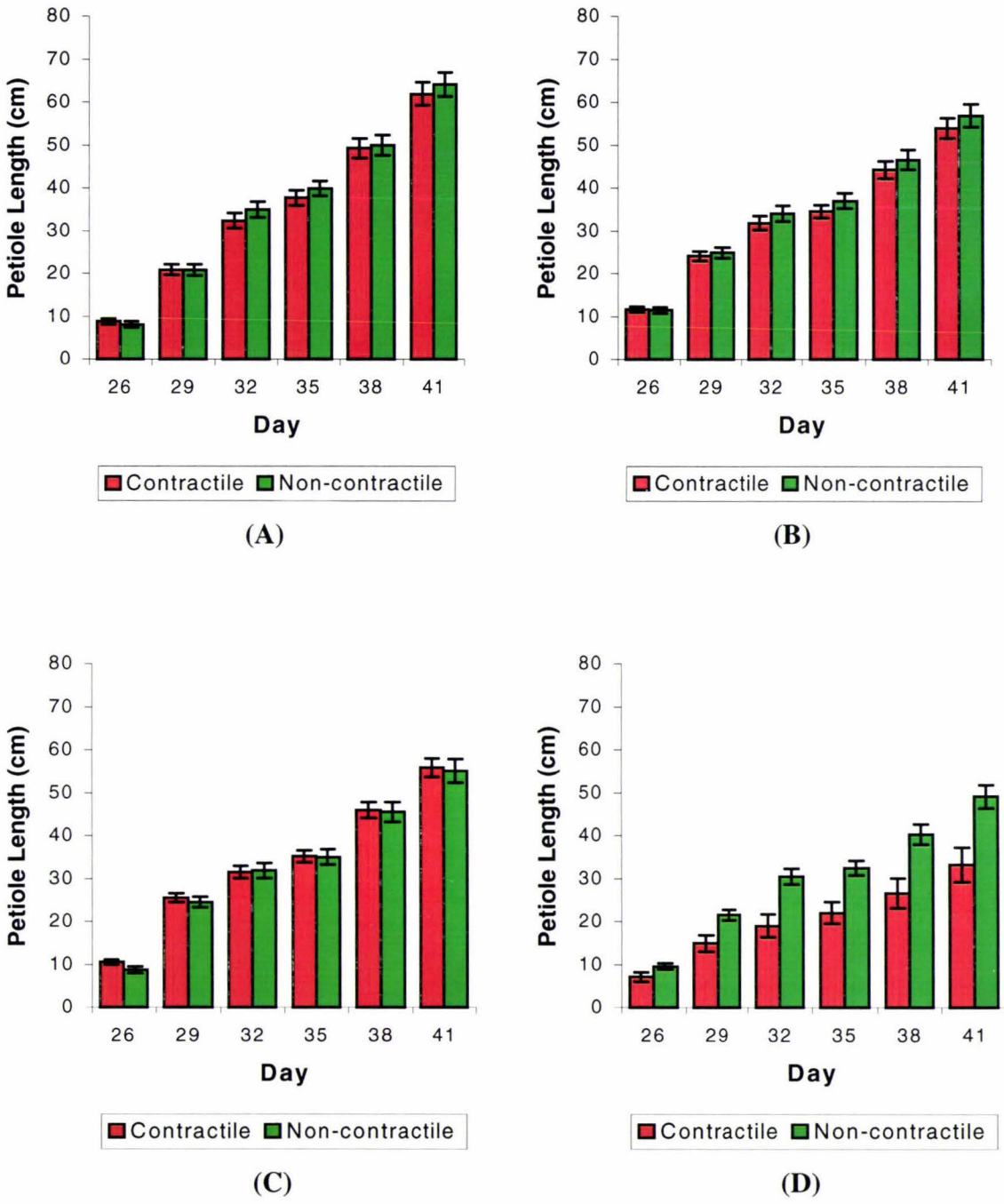


Figure 3.10 : Petiole lengths of plants from the contractile and non-contractile lines treated with various concentrations of Ethrel and silver thiosulphate. Values shown are means ± SEs.

(A) – Control plants (water).

(B) – Ethrel 500 $\mu l\ l^{-1}$.

(C) – Silver thiosulphate 1mM.

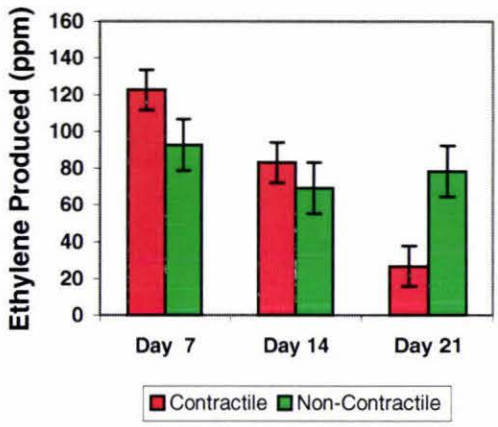
(D) – Silver thiosulphate 5 mM.

The mean amount of ethylene produced (per seedling) by contractile and non-contractile seedlings 7, 14 and 21 days after imbibition are shown in Figure 3.11 A. After 7 d the amount of ethylene produced by the contractile seedlings was significantly greater than the amount produced by the non-contractile seedlings. By 14 d there was no significant difference in ethylene production between the two lines, and by 21 d the contractile seedlings were producing significantly less ethylene than the non-contractile seedlings.

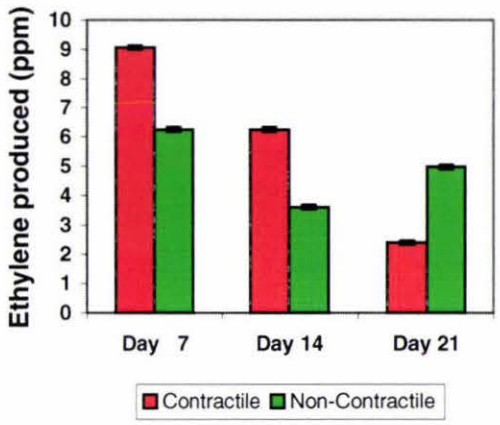
The amount of ethylene produced (per seedling) by contractile and non-contractile seedlings in relation to the height and/or weight of various seedling parts are shown in Figure 3.11 B-E. The mean amount of ethylene produced per millimetre of hypocotyl height (per seedling) 7, 14 and 21 d after imbibition are shown in Figure 3.11 B. After 7 and 14 d the contractile seedlings were producing significantly more ethylene per millimetre of hypocotyl height than the non-contractile seedlings. However, by 21 d this pattern had reversed and the contractile seedlings were producing significantly less ethylene per millimetre of hypocotyl height than the non-contractile seedlings. The amount of ethylene produced (in relation to hypocotyl height) by the contractile seedlings decreased significantly from 7-21 d whereas the amount of ethylene produced by the non-contractile seedlings decreased from 7-14 d, then increased significantly by 21 d. These patterns were similarly repeated when ethylene production was graphed in relation to hypocotyl weight (Figure 3.11 C).

The amount of ethylene produced by seedlings per milligram of shoot weight 7, 14 and 21 d after sowing are shown in Figure 3.11 D. At 7 and 14 d there was no significant difference in the amount of ethylene produced between the two lines. However, by 21 d the contractile seedlings were producing significantly less ethylene than the non-contractile seedlings. As before, the amount of ethylene produced by the contractile seedlings decreased from 7-21 d, whereas the amount produced by the non-contractile seedlings decreased from 7-14 d (but remained constant from 14-21 d).

By 7 and 14 d after sowing the amount of ethylene produced by the two lines in relation to root weight was not significantly different (Figure 3.11 E). By 21 d, however, the contractile seedlings were producing significantly less ethylene than



(A)



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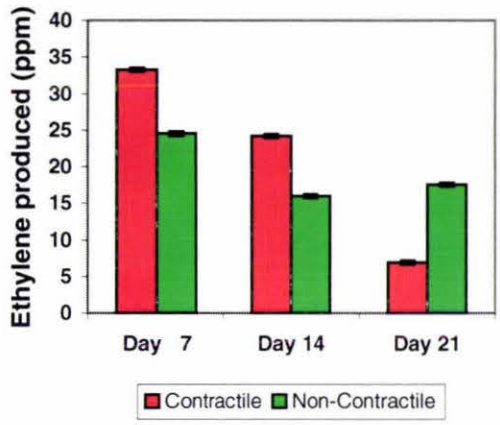

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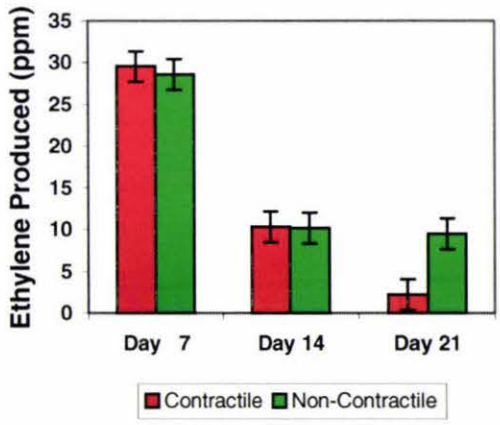
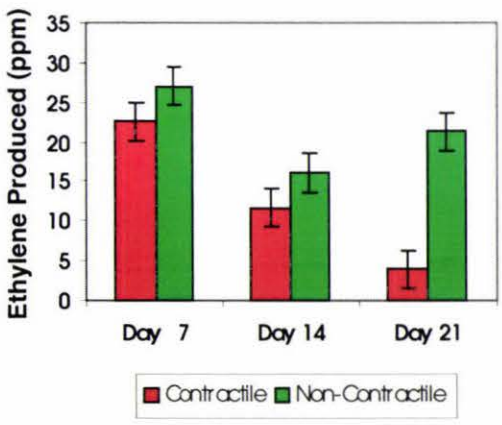


(D)



(E)

Figure 3.11 : Ethylene produced by contractile and non-contractile seedlings. The mean amount of ethylene produced by blank controls (containing media but no plant tissue) was subtracted from each sample value before the overall means were calculated.
(A) Ethylene produced per seedling
(B) Ethylene produced per millimetre of hypocotyl height
(C) Ethylene produced per milligram of hypocotyl weight
(D) Ethylene produced per milligram of shoot weight
(E) Ethylene produced per milligram of root weight

the non-contractile seedlings. Again, the amount of ethylene produced by the contractile seedlings decreased significantly from 7-21 d, while the amount produced by the non-contractile seedlings decreased from 7-14 d (then increased from 14-21 d).

3.5 Measurement of cortical and epidermal hypocotyl cells from contractile and non-contractile plants grown in culture.

Cortical and epidermal cells from the hypocotyl region of contractile and non-contractile seedlings (cycle II selection) grown in culture were examined and measured using a compound light microscope.

The mean height of cortical cells from the contractile line increased significantly from 7-14 d, but did not increase significantly from 14-21d, whereas the mean height of non-contractile cells increased significantly from 7-21 d (Table 3.6 A). The height of cells from the non-contractile line was significantly greater than the height of cells from the contractile line at all three sample dates. The widths of cells from the contractile line increased significantly from 7-21 d, but the widths of cells from the non-contractile line only increased significantly from 7-14 d. The cell areas (calculated by multiplying cell height by cell width) of both lines increased significantly from 7-21 d and approximately doubled during this time.

The height of epidermal cells from the contractile line significantly increased from 7-14 d, but did not significantly increase from 14-21 d. The height of non-contractile epidermal cells also increased from 7-14 d, but much more significantly, and did not change significantly from 14-21 d (Table 3.6 B). The width of epidermal cells from the contractile line increased significantly from 14-21 d but not from 7-14 d, whereas the width of epidermal cells from the non-contractile line did not change significantly at all during the given time frame. Cells from the contractile line were significantly wider than cells from the non-contractile line by 21 d, but there was no significant difference in cell width between lines at 7 and 14 d. The mean cell area of the contractile line increased significantly from 7-21 d, but the mean cell area of the non-contractile line only increased significantly from 7-14 d. The cell area of both lines approximately doubled from 7-21 d.

Table 3.6 : Height, width and area of **(A)** cortical, and **(B)** epidermal, cells from the hypocotyl region of contractile (C) and non-contractile (NC) seedlings 7, 14 and 21 days after sowing. Values shown are means and LSD's. Three seedlings of each line were examined at each time frame, and twenty cells from each seedling measured.

(A)

	Cell Height (μm)		Cell Width (μm)		Cell Area (μm^2)	
	C	NC	C	NC	C	NC
Day 7	138.43	201.74	32.18	37.67	4496	7664
Day 14	177.70	251.88	40.71	44.76	7153	11221
Day 21	189.61	287.52	48.88	43.63	9105	12557
$LSD_{0.05}$	13.99		2.83		820	

(B)

	Cell Height (μm)		Cell Width (μm)		Cell Area (μm^2)	
	C	NC	C	NC	C	NC
Day 7	141.16	194.37	17.70	17.81	2480	3478
Day 14	188.44	334.60	17.79	18.00	3361	6078
Day 21	199.30	317.04	21.33	17.91	4188	5746
$LSD_{0.05}$	18.92		0.94		424	

3.6 Examination of hypocotyls from contractile and non-contractile plants grown in a glasshouse - light microscopy part I.

Seedlings from the contractile and non-contractile lines (cycle II selection) were grown in a glasshouse and longitudinal sections of hypocotyls from each line examined with a light microscope 14, 21, 35, and 56 d after sowing.

The primary structure of the hypocotyl 14 d after sowing was the same for both the contractile and non-contractile lines (Table 3.7 A). Each hypocotyl consisted of a single layered epidermis, followed by three layers of cortical cells, a single layered endodermis, a single layered pericycle, and a central stele of vascular tissue

approximately 0.22 µm wide. The structure of the hypocotyls 21 d after sowing was similar to the structure observed 14 d after sowing – except that the pericycle was 3-4 cell layers thick and the central stele had increased in width to approximately 0.40 µm (Table 3.7 A). In both cases cells within the central stele were a mix of protoxylem, protophloem, gelatinous fibres and parenchyma.

By 35 d the structure of the hypocotyl was completely different to that observed in 14 and 21 d old seedlings (Table 3.7 B). Each hypocotyl consisted of an outer layer of periderm (8-9 cells thick), followed by a layer of phloem interspersed with parenchyma and gelatinous fibres (16-18 cells thick), a layer of recently divided cells (6-7 cells thick), a vascular cambium (2-3 cells thick), and a central core of xylem interspersed with parenchyma and gelatinous fibres. In the case of the contractile hypocotyls the xylem layer was approximately 0.64 µm wide, whereas in the non-contractile hypocotyls it was only 0.53 µm wide. In either case the structure of the xylem and surrounding tissue made counting of cells in this layer impossible.

By 56 d the overall structure of the hypocotyls was similar to the structure observed at 35 d (Table 3.7 B). However, the number of cells in the phloem layer had increased (to 24-26) and the xylem layer had also increased in width - in the case of the contractile hypocotyls to 1.23 mm, and in the case of the non-contractile hypocotyls to 0.82 mm.

The total height (from cotyledonary node to the soil surface) and width of each hypocotyl was also measured and means and LSD's calculated for each line (Table 3.8). There was no significant difference in the height or width of hypocotyls from the contractile and non-contractile lines at 14 or 21 d, but by 35 d and 56 d, hypocotyls from the contractile line were significantly shorter and wider than hypocotyls from the non-contractile line. The height of hypocotyls from both lines decreased significantly from 14 –21 d, but the width of the hypocotyls did not change significantly during this time. From 21-35 d, and again from 35-56 d, the contractile hypocotyls decreased significantly in height, but increased significantly in width. The width of the non-contractile hypocotyls also increased significantly from 21-35 and again from 35-56 d, but the height of these hypocotyls did not change significantly from 21 d onwards.

Table 3.7 : Number and type of cells in each layer of the hypocotyl in contractile and non-contractile seedlings, **(A)** 14 and 21 d, and **(B)** 35 and 56 d, after sowing. Values shown are means of three seedlings.

(A)

	Day 14		**Day 21**	
	Contractile	**Non-contractile**	**Contractile**	**Non-contractile**
Epidermis	1	1	1	1
Cortex	3	3	3	3
Endodermis	1	1	1	1
Pericycle	1	1	3-4	3-4
Central stele	0.22 μm	0.22 μm	0.41 μm	0.39 μm

(B)

	Day 35		**Day 56**	
	Contractile	**Non-contractile**	**Contractile**	**Non-contractile**
Periderm	8-9	8-9	8-9	8-9
Phloem layer	16-18	16-18	24-26	24-26
Recently divided cells	6-7	6-7	24-26	24-26
Vascular cambium	2-3	2-3	2-3	2-3
Central core of xylem	0.64 μm	0.53 μm	1.23 mm	0.82 mm

The mean number of cells along the height of contractile and non-contractile hypocotyls are shown in Tables 3.9 and 3.10. At 14 and 21 d, the number of cortical and epidermal cells were counted – but at 35 and 56 d cells in the outer layer of parenchyma were counted as cortical and epidermal tissues no longer existed (Table 3.9). By 14 d after sowing there were significantly more cortical and epidermal cells along the length of the non-contractile hypocotyls than along the length of the contractile ones. By 21 d there was no significant difference between the lines in the number of cells along the

Table 3.8 : Height and width of hypocotyls from contractile and non-contractile seedlings (cycle II selection). Values shown are means and LSD's of three seedlings.

	Height (mm)		Width (mm)	
	Contractile	**Non-contractile**	**Contractile**	**Non-contractile**
Day 14	6.75	6.80	0.84	0.75
Day 21	4.66	4.83	0.99	0.88
Day 35	3.68	4.80	2.04	1.56
Day 56	2.79	4.82	2.93	2.16
$LSD_{0.05}$	0.22		0.27	

Table 3.9 : Number of cortical and epidermal cells along the length of hypocotyls from contractile and non-contractile seedlings 14 and 21 d after sowing. Values shown are means and LSD's of three seedlings.

	Number of cells			
	Cortical		Epidermal	
	Contractile	**Non-contractile**	**Contractile**	**Non-contractile**
Day 14	67.67	70.00	72.67	89.33
Day 21	85.33	85.33	81.33	83.67
$LSD_{0.05}$	2.37		4.05	

hypocotyl. From 14 –21 d the mean number of both cortical and epidermal cells in contractile hypocotyls increased significantly. From 14 –21 d the number of cortical cells in the non-contractile hypocotyls increased significantly, but the number of epidermal cells decreased significantly. At both 35 and 56 d, the mean number of cells along the non-contractile hypocotyls was significantly greater than the number of cells along the contractile hypocotyls (Table 3.10). From 35 – 56 d the number of cells in the contractile hypocotyls decreased significantly as did the number of cells along the non-contractile hypocotyls.

Table 3.10 : Number of parenchyma cells along the length of hypocotyls from contractile and non-contractile seedlings 35 and 56 d after sowing. Values shown are means and LSD's of three seedlings.

	Number of cells	
	Contractile	**Non-contractile**
Day 35	117.33	139.67
Day 56	65.33	90.00
$LSD_{0.05}$	3.24	

The mean heights, widths and areas of cortical cells from 14 and 21 d old seedlings are shown in Table 3.11 A. At 14 d cells from the contractile line were significantly taller than those of the non-contractile line, but by 21 d this pattern reversed. There was no significant difference in either cell width or area between the lines at 14 d, but by 21 d cells from the non-contractile line were significantly wider and had a significantly greater cell area than cells from the contractile line. From 14 –21 d cells from both the contractile and non-contractile lines decreased significantly in height, but increased significantly in width and cell area.

The mean heights, widths and areas of epidermal cells from 14 and 21 d old seedlings are shown in Table 3.11 B. At 14 d cells from the contractile line were significantly taller than those of the non-contractile line, but by 21 d there was no significant difference in cell height between the two lines. There was no significant in the width of cells from either line at either 14 or 21 d, and no significant difference in cell area at 21 d. From 14 –21 d cells from both the contractile and non-contractile lines decreased significantly in height, but increased significantly in width and cell area.

Table 3.11 : Height, width and area of, **(A)** cortical, and **(B)** epidermal, cells in the hypocotyl region of contractile (C) and non-contractile (NC) seedlings 14 and 21 days after sowing. Values shown are means and LSD's (three seedlings of each line and twenty cells per seedling were measured).

(A)

	Cell Height (µm)		Cell Width (µm)		Cell Area (μm^2)	
	C	NC	C	NC	C	NC
Day 14	99.95	97.50	27.76	27.64	2774	2695
Day 21	54.91	57.05	54.24	55.21	2998	3170
$LSD_{0.05}$	1.28		0.70		81	

(B)

	Cell Height (µm)		Cell Width (µm)		Cell Area (μm^2)	
	C	NC	C	NC	C	NC
Day 14	92.81	76.34	13.88	13.59	1285	1047
Day 21	58.05	58.86	19.33	19.33	1122	1139
$LSD_{0.05}$	3.16		0.43		61	

In the case of 35 and 56 d old hypocotyls, the mean heights, widths and areas of parenchyma cells were measured and calculated as cortical and epidermal tissue no longer existed (Table 3.12). Between 35 and 56 d the mean height, width and area of cells from the contractile line increased significantly. During the same time the height and area of cells from the non-contractile line increased significantly, but the width of these cells decreased significantly. At both 35 and 56 d, cells from the contractile line were shorter and wider than cells from the non-contractile line.

Table 3.12 : Height, width and area of ray parenchyma cells in the hypocotyl region of contractile (C) and non-contractile (NC) seedlings 35 and 56 days after sowing. Values shown are means and LSD's (three seedlings of each line and twenty cells per seedling were measured).

	Cell Height (µm)		Cell Width (µm)		Cell Area (µm^2)	
	C	NC	C	NC	C	NC
Day 35	31.31	40.79	30.12	25.14	944	1025
Day 56	43.48	54.00	35.38	20.95	1537	1132
LSD $_{0.05}$	0.45		0.31		19	

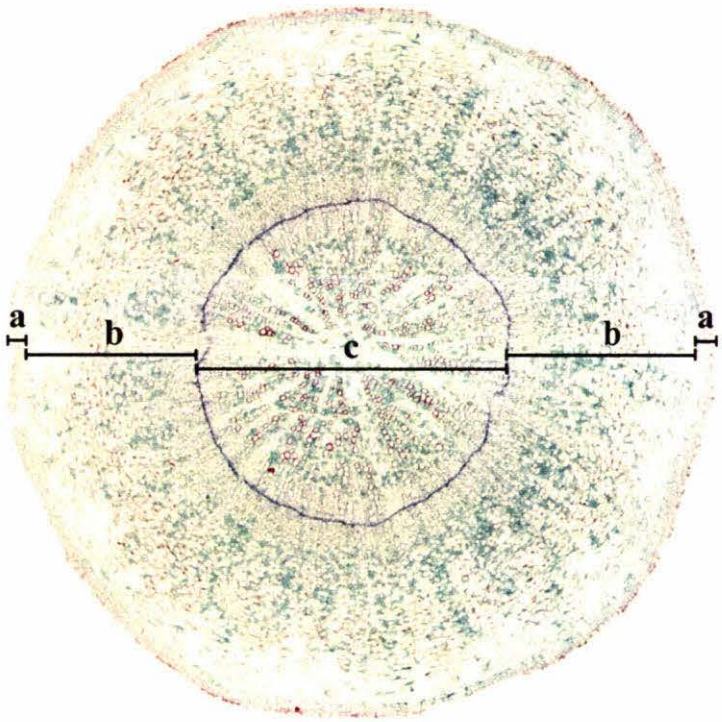
3.6 Examination of hypocotyls from contractile and non-contractile plants grown in a glasshouse - light microscopy part II.

Seedlings from the contractile and non-contractile lines (cycle II selection) were grown in a glasshouse and transverse sections of 49 d old hypocotyls from each line examined using a light microscope.

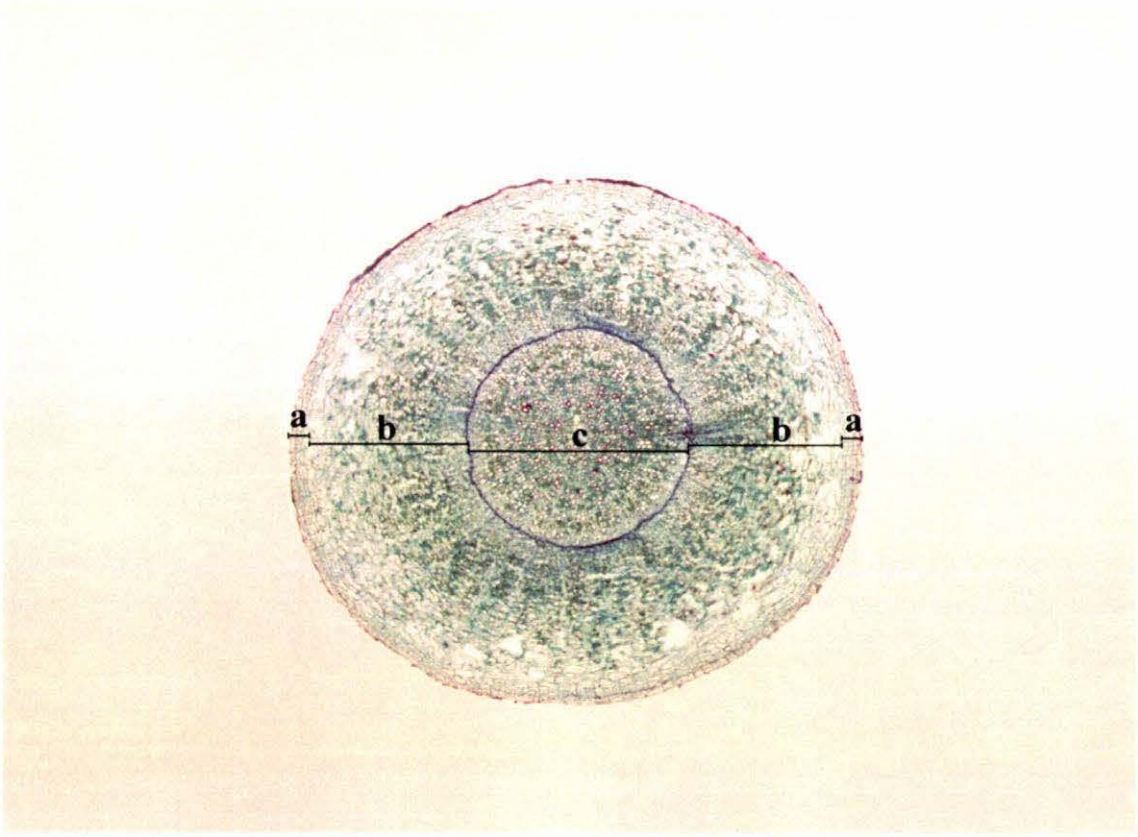
The general structure of both the contractile and non-contractile hypocotyls was the same as that described for 56 d old seedlings (see Table 3.7 B). Each hypocotyl consisted of an outer layer of periderm (8-9 cells thick), followed by a layer of phloem interspersed with parenchyma and gelatinous fibres (24-26 cells thick), a layer of recently divided cells (6-7 cells thick), a vascular cambium (2-3 cells thick), and a central core of xylem interspersed with parenchyma and gelatinous fibres. In the contractile hypocotyl examined (Table 3.13, Figure 3.12 A) this layer containing xylem was approximately 1.43 mm and 48 -50 cells wide, whereas in the non-contractile hypocotyl examined (Table 3.13, Figure 3.12 B) there were the same number of cells (48-50) but the area was only 0.86 mm wide. This central xylem layer comprised 44.6 % of the total hypocotyl width in the contractile hypocotyl, and 42.0 % of the total hypocotyl width in the non-contractile hypocotyl (Table 3.13). The periderm layers of both the contractile and non-contractile hypocotyls were approximately 8-9 cell layers, and 0.08 mm thick.

Table 3.13 : Width of hypocotyl tissue layers in contractile and non-contractile seedlings 49 d after sowing.

	Total width of hypocotyl (mm)	**Width of periderm (mm)**	**Width of phloem layer (mm)**	**Width of central xylem core (mm)**
Contractile	3.20	0.08	0.80	1.43
Non-contractile	2.05	0.08	0.51	0.86



(A)



(B)

Figure 3.12 : Transverse sections of hypocotyl tissue from **(A)** contractile, and **(B)** non-contractile seedlings (x50). Both sections were taken approximately 3 mm below the cotyledonary node.

a: periderm layer
b: phloem layer
c: xylem layer

CHAPTER 4 : DISCUSSION

Contractile growth of the hypocotyl in white clover and other epigeal emerging seedlings is thought to protect the cotyledonary node from hazards such as grazing, treading, defoliation or fire (Pott *et al.* 1983, Fujita and Humphreys 1992). However, observations (Derek Woodfield, pers. com.) suggested that up to 10 % of seedlings in normal populations of white clover either contract more slowly, or contract to a lesser degree than the bulk of the population. The results of this study support this estimate, as the mean percentage of plants that displayed non-contractile growth (over all of the cultivars studied) was 11.9 % (Table 3.3). However, the frequency of plants which displayed non-contractile growth, varied widely amongst the cultivars. For example, 1.8 % of seedlings in the Huia population displayed non-contractile growth, whereas 21.8 % of seedlings in the Avoca population displayed such growth (Table 3.3). The non-contractile phenotype should be deleterious, since in the natural environment seedlings which display epigeal emergence have no buds or stored food below the cotyledonary node, and die if the plants are defoliated below this point (by natural hazards such as grazing, treading, etc) (Nelson and Larson 1984). However, these results indicate that this trait is maintained at relatively high frequencies in populations of white clover. That this trait has been maintained in a species grazed intensively by animals, even after thousands of years of evolution and more than 100 years of breeding, suggests a possible evolutionary advantage. The possession of a non-contractile hypocotyl may aid survival during competition for light during seedling germination. Another possibility is that the gene(s) responsible for the non-contractile trait are maintained due to genetic linkage with a gene(s) responsible for other essential traits. Future genetic analysis may allow this distinction to be made.

The hypothesis that overall growth of the hypocotyl in white clover seedlings may be affected by the overall seedling size was investigated by relating maximum hypocotyl height to leaf size in a range of cultivars. With the exception of the large-leaf category, the cultivars which were bred overseas had much longer hypocotyls than the New Zealand-bred cultivars (Figure 3.3). Perhaps in New Zealand's cool, moist climate there has been selection for plants with hypocotyls which do not extend as much as their

overseas counterparts. This may reflect greater selection pressure during grazing since pastures in New Zealand are intensively grazed, with grazing by sheep predominating. Sheep graze plants to a much lower height than cattle (Collins and O'Toole 1963, Harris 1987, Brock and Hay 1993), which may cause additional selection pressure for seedlings with shorter hypocotyls by actively removing plants which display non-contractile growth. Selection pressure may also explain why the large leaved varieties had longer hypocotyls than expected, and why the relationship between maximum hypocotyl height and leaf size was positive and almost linear for all of the New Zealand bred cultivars except for Prestige (which had a much longer hypocotyl than expected) (Figure 3.3). Cultivars bred for large leaves (such as Kopu and Tillman) are generally grown in pastures which are grazed by cattle rather than sheep (Caradus and Woodfield 1997). Cattle do not graze the plant as low as sheep and this may reduce the selection pressure for seedlings with shorter hypocotyls in these pastures. Prestige was bred from clover ecotypes collected from Northland where the climate tends to be hot and dry (Caradus and Woodfield 1997). In sub-tropical environments such as this the growth rate of most plants is increased and a longer than usual hypocotyl may be necessary if emerging clover seedlings are to compete with the surrounding pasture plants for light.

In the past, several authors have proposed that the contractile growth of white clover (and many other epigeal emerging legumes such as sweet clover, alfalfa and red clover) is caused by a lateral expansion of cells within the hypocotyl and upper primary root (Nelson and Larson 1984, Metcalfe and Nelson 1985). This hypothesis probably arose since in many monocotyledonous species contractile growth of the root(s) is caused by the radial expansion and longitudinal shortening of cells, particularly cortical cells (Wilson and Honey 1966, Chen 1969, Sterling 1972, Reyneke and Van der Schiijff 1974, Wilson and Anderson 1979, Jernstedt 1984, Halevy 1986, Cyr *et al.* 1988). However, until now no work showing the cellular basis of contraction in a dicotyledonous species has been published. As such, one of the main aims of this thesis was to investigate some of the morphological changes which occur during contractile growth of the hypocotyl in white clover. This was achieved by comparing the growth and development of hypocotyls from contractile and non-contractile seedlings (see Sections 2.2 and 2.6 for selection of the two lines). However, comparisons of the contractile and non-contractile populations were not perfect since there was segregation

for both phenotypes within each of the lines and further selection is required to fully stabilise this trait.

In each of the experiments, the hypocotyls of both contractile and non-contractile seedlings reached maximum extension approximately 14 to 21 d after sowing (Section 3). These hypocotyls consisted of a central stele (which contained fibres and developing vascular tissues) surrounded by 1 to 4 layers of pericycle cells, one layer of endodermal cells, three layers of cortical cells, and an outer layer of epidermal cells. However, by the time these seedlings were 35 d old no epidermal or cortical cells were present and the entire structure was surrounded by a thick layer of periderm (Table 3.7 A and B). A similar change in structure occurs during the secondary growth of roots in white clover seedlings. Secondary growth of the root starts in the normal manner through the meristematic activity of the cells of the conjunctive parenchyma internal to phloem groups (Erith 1924, Thomas 1987). These cambial groups are soon joined by meristematic cells of the pericycle outside the protoxylem, and a complete cambium layer is formed. Cells produced by this cambium layer form secondary xylem to the inside of the root and secondary phloem to the outside of the root, as well as parenchyma and fibre cells throughout (Erith 1924, Thomas 1987). This secondary growth results in an increase in root thickness causing the cells of the primary cortex to become stretched tangentially and somewhat ruptured. However, even before the first cambium layer is complete, a second cambium, the phellogen, arises from the pericycle and, by its activity, forms a periderm consisting of several layers of phellem (cork) externally and several layers of phelloderm (secondary cortex) internally. Thus, by the time the primary outer cortex is sloughed off, a protective layer (the periderm) surrounds the central cylinder (Erith 1924). That the hypocotyl tissue of white clover seedlings should undergo a similar change in development is not surprising, as the hypocotyl is a transitional region between the root and the shoot. In fact, an increase in the meristematic activity of cambial tissue could account for the increase in the number of cells in the phloem layer, and the overall increase in hypocotyl width which occurred in both contractile and non-contractile hypocotyls between 35 and 56 d (Tables 3.7 B and 3.8).

The cellular basis of contraction in white clover was further investigated by measuring the heights (above soil surface) and widths (3 mm below the cotyledonary node) of hypocotyls from contractile and non-contractile seedlings; and (depending on the age of the seedling) the heights and widths of cortical and epidermal, or parenchyma, cells within the hypocotyls. The widths of the hypocotyls from both contractile and non-contractile seedlings increased significantly from 14 to 56 d, as did the widths of the cortical and epidermal, or parenchyma cells within the hypocotyl tissue (Tables 3.8, 3.11 A and B, and 3.12). However, there was no significant difference between two the lines in either the widths of the hypocotyls, or the widths of the cells within the hypocotyls, until 35 d (Tables 3.8, 3.11 A and B, and 3.12). After 35 d, both the hypocotyls and cells from contractile seedlings were significantly wider than the hypocotyls and cells from non-contractile seedlings (Tables 3.8, 3.11 A and B, and 3.12). These results indicate that the hypocotyls from contractile seedlings were wider than hypocotyls from the non-contractile seedlings due to increased cell expansion, rather than increased cell division, since the number of cells in each of the hypocotyl tissue layers was the same in hypocotyls from contractile and non-contractile seedlings for seedlings of the same age,

Hypocotyls from both contractile and non-contractile seedlings reached maximum heights 14 to 21 d after sowing (Table 3.8). Hypocotyls from the contractile seedlings then decreased in height until the cotyledonary node lay at or below the soil surface. The hypocotyls from the non-contractile seedlings also decreased in height, but not to the same extent as those from the contractile seedlings (Table 3.8). The cortical and epidermal cells within these tissues also decreased in height from 14 to 21 d, whereas cells from the non-contractile hypocotyls were significantly taller than those from the contractile hypocotyls at 14 and 21 d (Table 3.11 A and B). However, the height of the parenchyma cells in the hypocotyls of both contractile and non-contractile seedlings increased between 35 and 56 d even though the total amount of hypocotyl tissue above the soil surface decreased (Tables 3.12 and 3.8). Hypocotyls of most seedlings which failed to display full contractile growth failed to contract even after prolonged periods (i.e. more than six months).

Overall, these results indicate that during the first 14 to 21 d, contractile growth of the hypocotyl in white clover seedlings is caused by the radial expansion and longitudinal

shortening of epidermal and cortical cells. This type of contraction also occurs in the contractile roots of many monocotyledonous species. After 21 d, increases in hypocotyl width occur by means of cell division in the cambial layer to form fibre, parenchyma, secondary xylem and secondary phloem cells, along with the radial expansion of these cells. In comparison, cells from the hypocotyls of non-contractile seedlings do not expand radially. The results also indicate that contractile growth may occur via different mechanisms before and after 21 d, since hypocotyl height decreases from 35 to 56 d, but the height of the cells within the hypocotyls increases.

Another aim of this thesis was to investigate what, if any, role ethylene may play in contractile growth of the hypocotyl. Ethylene was implicated as it has been shown to inhibit longitudinal growth and induce radial swelling in many plant tissues (Abeles 1973, Burg 1973, Lieberman 1979, Eisinger 1983). This radial swelling is usually caused by the radial expansion of cells within these tissues due to increases in ethylene production (Burg and Burg 1966, Eisinger and Burg 1972, Burg 1973, Linkins *et al.* 1973, Biro *et al.* 1980, Vreugdenhil *et al.* 1984, Zheng and Inouye 1990, Jackson 1991, Sarquis *et al.* 1991, Ortuno *et al.* 1991, Abeles *et al.* 1992, Baskin and Williamson 1992, Bravo *et al.* 1992, Zacarias and Reid 1992, Petruzzelli *et al.* 1995, Zheng *et al.* 1997). Once again various aspects of growth and development in the hypocotyls of contractile and non-contractile seedlings were investigated.

If ethylene does play a major role in somehow inducing or controlling the process of contraction, then seedlings which do not display contractile growth may either produce less ethylene, or be less sensitive to ethylene, than seedlings which do contract. To investigate whether or not contractile growth was promoted by ethylene, seedlings from the contractile and non-contractile lines were treated with various concentrations of silver thiosulphate. Silver thiosulphate blocks the perception of ethylene by interfering with the receptor mechanism. When compared to hypocotyls from untreated control plants, hypocotyls from both the contractile and non-contractile seedlings increased in height significantly when treated with 1 mM silver thiosulphate (Figures 3.5 A and B, and 3.7 A and B). This increase in height was even more pronounced when seedlings were treated with 5 mM silver thiosulphate (Figures 3.5 A and B, and 3.7 A and B). These results support the hypothesis that ethylene plays a major role in causing

contractile growth of the hypocotyl in these seedlings, as blocking the perception of ethylene caused the hypocotyls of both contractile and non-contractile seedlings to extend.

The possibility that non-contractile seedlings are less sensitive to ethylene than contractile seedlings was investigated by treating cells from both lines with various concentrations of Ethrel. Ethrel is broken down within plant tissues to form ethylene so has the effect of increasing the amount of ethylene present. In several experiments, treatment with Ethrel caused the hypocotyls from non-contractile seedlings to decrease in height (Figures 3.5 A and B, and 3.7 A and B). This indicates that a lack of sensitivity to ethylene is probably not the main cause of non-contractile growth because if these seedlings were defective in their ability to perceive ethylene we would not expect to observe any changes in hypocotyl growth after treatment with Ethrel. These results, however, do support the hypothesis that non-contractile growth may be caused (at least in part) by a lack of ethylene production, since the hypocotyls of non-contractile seedlings displayed greater contraction than usual when ethylene levels were increased by Ethrel treatment.

If ethylene indeed plays a major role in contractile growth, then we might also have expected the hypocotyls of contractile seedlings to contract either faster or to a greater degree when treated with Ethrel. However, in both of the experiments performed, treatment of contractile seedlings with Ethrel at either 50 μl^{-1} or 500 μl^{-1} actually caused the hypocotyls of contractile seedlings to extend significantly (Figures 3.5 A and B, and 3.7 A and B). Ethylene usually inhibits elongation in the stems and roots of most plants, but cases where it induces elongation have also been shown - including the stems and petioles of plants such as *Callitriche platycarpa, Ranunculus scleratus, Nyphoides peltata,* and most species of rice (Salisbury and Ross 1992). However, all of these plants grow at least part of the time with their roots and stems underwater, and ethylene is not thought to induce the elongation effect *per se*. Instead, ethylene is thought to either remove the block to gibberellin synthesis or enhance the sensitivity of these tissues to gibberellin (Khan *et al.* 1986, Suge 1987, Blom *et al.* 1994, Furukawa *et al.* 1997). Ethylene has also been shown to cause elongation in the hypocotyl of light grown *Arabidopsis* seedlings (Smalle *et al.* 1997) and the stem of the prairie ecotype of

the weedy carophyllaceous plant *Stellaria longipes* (Emery *et al.* 1994, Emery *et al.* 1997, Kathiresan *et al.* 1998). However, stem growth in the alpine ecotype of *S. longipes* is inhibited by ethylene – indicating that stem elongation in response to ethylene is a highly plastic response in this species. Thus, elongation of the hypocotyl in contractile seedlings treated with Ethrel may have occurred due to the interaction of ethylene with, or the independent action of another plant hormone (e.g. gibberellin); or high levels of plasticity with regard to the effect of ethylene on hypocotyl elongation in such plants.

The concept that hypocotyl elongation and subsequent contraction in white clover seedlings are highly plastic responses was reinforced by the results of the various experiments comparing hypocotyl growth in the contractile and non-contractile seedlings. When the seedlings were grown during the summer months (when light intensity was high and the photoperiod was long) 2.4 and 11.6% of the contractile and non-contractile seedlings respectively, displayed non-contractile growth (Table 3.3). When the seedlings were grown during the winter months (when light intensity was low and the photoperiod was short) 22 to 25 % of contractile seedlings and 70 to 80 % of non-contractile seedlings displayed non-contractile growth (Tables 3.4 and 3.5). The amount of time taken for contractile growth to occur also changed. In the experiment run during the summer months contractile growth of the hypocotyl began 14 to 21 d after sowing and was completed 35 to 42 d after sowing (Table 3.3). However, in the experiments run during the winter months contractile growth of the hypocotyl began 21 to 28 d after sowing and was still not complete 49 to 56 d after sowing (Tables 3.4 and 3.5). In each case, these changes were most probably caused by differences in the amount and/or quality of light available to the seedlings. Light has been shown to negatively influence hypocotyl extension and positively increase contractile growth in *Lotononis bainesii* seedlings (Fujita and Humphreys 1992). Other parameters which affect seedling growth (such as water availability and positioning in the glasshouse) were constant across the experiments.

Many instances have been reported where tissues within the same plant display differential sensitivity to plant hormones according to the developmental age or posistion of the tissue (reviewed by Abeles *et al.* 1992). As discussed previously, when

contractile and non-contractile seedlings were treated with silver thiosulphate the hypocotyls of both lines increased in height, whereas the hypocotyls of contractile seedlings treated with Ethrel extended, and the hypocotyls of non-contractile seedlings treated with Ethrel contracted. In comparison, the petioles of both contractile and non-contractile seedlings decreased significantly in length after treatment with either Ethrel or silver thiosulphate (Figure 3.9 A and B). Perhaps basal levels of ethylene are needed for petiole elongation and removing ethylene from the system (with silver thiosulphate), causes the inhibition of this process. On the other hand, perhaps supraoptimal concentrations of ethylene inhibit petiole elongation by inducing radial growth. However, unfortunately petiole width was not measured. Either way, these results indicate it is likely that different ethylene response mechanisms operate in the hypocotyl and petiole tissues of white clover seedlings.

The possibility that ethylene may play a major role in contractile growth of the hypocotyl was also investigated by comparing the amount of ethylene produced by contractile and non-contractile seedlings grown in culture. The seedlings were grown under sterile conditions to avoid ethylene production by microbes contaminating samples. Unfortunately, growing seedlings in culture also caused them to become highly etiolated, so neither type of seedling displayed any form of contractile growth which in turn may have had some effect on ethylene production. The amounts of ethylene produced on a per seedling basis were calculated for each sample date, as were the amounts of ethylene produced in relation to hypocotyl height, hypocotyl weight, shoot weight, and root weight. In general the amount of ethylene produced by seedlings from the contractile line decreased from 7 to 21 d (Figure 3.11 A-E); whereas the amount of ethylene produced by seedlings from the non-contractile line either decreased from 7 to 14 d then increased from 14 to 21 d (Figures 3.11 B, C and E), or did not change significantly (Figure 3.11 A and C). Also, at 7 and 14 d the amount of ethylene produced by contractile seedlings was greater than the amount produced by non-contractile seedlings, but by 21 d the non-contractile seedlings were producing more ethylene. Under normal growing conditions (plants grown in soil) the processes involved in contractile growth of the hypocotyl have already been initiated by 21 d. Therefore, these results may suggest that contractile seedlings produce more ethylene than non-contractile seedlings until the time at which contractile growth would be

initiated, but that the amount of ethylene produced by the contractile seedlings decreases once contractile growth is initiated. These results may also indicate that the amount of ethylene produced by the non-contractile seedlings either increases or remains stable due to contractile growth not being initiated.

Conclusions

During the first two to three weeks of seedling growth, contractile growth of the hypocotyl in white clover is caused by the radial expansion and longitudinal shortening of epidermal and cortical cells with no accompanying change in the number of cells present. After this time increases in hypocotyl width are associated with the division and expansion of cells in the radial direction. However, although the height of the hypocotyl above the soil surface decreases during this time, the height of the cells within the hypocotyl increases, indicating that the mechanism of contraction is different to that in younger seedlings. Non-contractile growth is caused by reduced expansion of cells in the radial direction and increased expansion of cells in the longitudinal direction leading to hypocotyls which are taller and thinner than those that undergo full contraction.

The hypothesis that ethylene plays a role in contractile growth of the hypocotyl was supported by the results of this study, since hypocotyls from contractile seedlings treated with silver thiosulphate (which blocks ethylene perception) failed to display contractile growth, and hypocotyls from non-contractile seedlings displayed non-contractile growth to a greater degree. In addition, the amount of ethylene produced by contractile seedlings was greater than that produced by non-contractile seedlings during the first two weeks of seedling growth. However, by the third week of seedling growth the amount of ethylene produced by the contractile seedlings had decreased significantly whereas the amount produced by the non-contractile seedlings remained the same. These results may indicate that in normal contractile seedlings ethylene production decreases once contractile growth of the hypocotyl has been initiated. In contrast, ethylene production by non-contractile seedlings may remain high as contractile growth is not initiated. These results also support the hypothesis that non-contractile growth is caused by a lack of ethylene production.

Further support for a lack of ethylene production causing non-contractile growth came from the experiments where non-contractile seedlings were treated with Ethrel. Increasing the amount of ethylene present caused non-contractile seedlings to display contractile growth. However, interpretation of the results obtained when contractile seedlings were treated with Ethrel is less straightforward since the addition of extra ethylene caused contractile growth to be inhibited rather than enhanced. This may indicate that optimal concentrations of ethylene are needed to cause the radial expansion and longitudinal shortening of hypocotyl tissues during contractile growth, but that supra-optimal concentrations of ethylene cause the opposite effect. Another alternative is that ethylene affects hypocotyl growth differently according to the age and/or developmental status of the seedling. However, as the results of this study indicate that contractile growth is a highly plastic response, other explanations are also possible.

Although this study provides a good basis as to the processes which occur during contractile growth of the hypocotyl in white clover, it is clear that much additional work will be required before contractile growth, the role of ethylene in this process are completely understood. Further work that could prove helpful regarding this matter includes the investigation of :

- the heritability and stability of the non-contractile trait within populations (particularly in response to grazing pressure)
- changes in either the shapes or numbers of cells in the hypocotyls of contractile and non-contractile seedlings treated with Ethrel or silver thiosulphate (including the orientation of microfibrils and microtubules in the cell walls)
- the cellular nature of contractile growth in the root, and
- ethylene production by contractile and non-contractile seedlings grown in soil and under glasshouse conditions.

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