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Physiological and biochemical response of endophyte infected *Lolium perenne* to water stress and to plant hormone treatment when water sufficient.

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Michael Patrick Seawright

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Abstract

This focus of this thesis was centred on the water stress-induced interactions between the *Lolium perenne* and *Neotyphodium lolii* symbiotum and the *in vivo* biomass relationship between the two species. The aim was to test the hypotheses that endophyte bio-protective metabolites are synthesized at higher levels in water-stressed endophyte-colonised plants than in water-sufficient plants; to carry out ELISA experiments to measure tiller levels of ABA and JA in perennial ryegrass during the application of a controlled water stress and apply these levels, by dipping water-sufficient plants, thereby mimicking the abiotic stress, to test whether the levels of the endophyte-produced bio-protective metabolites, ergovaline, lolitrem B and peramine, increased. The evapotranspiration rate of endophyte hosted and endophyte free perennial ryegrass was measured and no significant difference was found.

The sodium borohydride reduction of ABA and JA, to yield protonated ABA and CA was developed using small quantities of the reducing agent, the products verified by nuclear magnetic resonance (NMR) spectroscopy and high performance liquid chromatography (HPLC). Radioactive sodium borohydride was then used to produce tritiated ABA and CA, the reduction products purified and used as spiking controls in the solid state extraction of ABA and JA from perennial ryegrass. The efficiency of DEAE Sephadex solid state columns at removing ABA and JA from perennial ryegrass leaf and sheath tissue was tested. The spiking methods, the results obtained and their use in adjusting analysed ABA and JA tissue levels are outlined. Hormone dipping regimes, obtained from the literature, were used as the basis for testing hormone uptake, after dipping, by measuring tissue levels by ELISA.

A trial was carried out, that measured by competitive ELISA, the ABA and JA leaf tissue levels in the perennial ryegrass genotypes after they had been progressively water stressed, by re-applying one third of the water lost in the previous 48 h. After 8 days of applied water stress the ABA levels increased substantially, peaking at 2.5×10^4 picomoles/g fresh weight while the JA increase was less dramatic and more sustained,

peaking at 1.68 x 10^3 picomoles/g fresh weight over the same period. This information was used to test the hypothesis that dipping water sufficient perennial ryegrass in known water stress levels of ABA and JA would increase alkaloid output by *N. lolii.*

The main statistically analysed split plot experiment, involved the dipping of water sufficient potted *L. perenne* genotypes in solutions (tissue mass adjusted) of increasing ABA, JA and ABA/JA concentrations from day 8 of an applied water stress, every 48 h for 16 days. The progression of water stress in the control treatments was measured independently. After this period of water stress the leaf and sheath tissue was harvested separately and the tissue levels of lolitrem B, ergovaline and peramine were analysed by HPLC. These dipped replicates were then compared with un-dipped water stressed and water sufficient controls. The data was analysed in three parts. Leaf and sheath combined, leaf only and sheath only data. There was a positive correlation coefficient (r) between the three alkaloids ergovaline, lolitrem B and peramine for the water-sufficient, hormone-treated and water-deficit-treated plants.

Considering ABA, JA and ABA/JA treatments on ergovaline levels in perennial ryegrass for the combined data; ergovaline levels in the sheath were significantly higher than the controls. During water stress where there was a highly significant increase in ergovaline levels in the sheath tissue. There was a significant increase in ergovaline levels in water stressed leaves when using leaf data. A significant increase in ergovaline level occurred in the leaves, but not the sheath, when water sufficient plants were dipped in JA and ABA separately. These levels dropped significantly in leaves when water sufficient plants were dipped in ABA/JA mixed solutions.

Lolitrem B was 3-fold higher in the sheath than the leaf. There was no response to hormone treatments. When sheath data was used, a significant decrease in sheath lolitrem B levels occurred in perennial ryegrass treatments that were water stressed.

Peramine levels were significantly higher in the sheath than the leaves when the combined data was analysed. When leaf data was considered peramine levels fell significantly during water stress while the analysed sheath data showed a significant 1.4-fold increase. Treatments that involved dipping in JA gave significant peramine

increases (1.35 fold). Other hormone treatments increased peramine levels but they were not significant. This study gave strong indications that there is a plant stress hormone communication between *L. perenne* and the mutualistic endophyte *N. lolii* and that water stress does increase endophyte ergovaline and peramine output and decreases lolitrem B levels.

During a confocal microscopic study, *L. perenne* meristems were successfully dissected out, re-hydrated, fixed, stained and 1.3 micron longitudinal sections, viewed. The sections were then digitalized, enhanced and re-assembled into a 3 dimensional rotating image; using computer based confocal microscope software. A stereoscopic anaglyph was made of ryegrass tiller leaf sections, highlighting *N. lolii* colonization patterns.

A non–invasive, means of measuring endophyte to ryegrass biomass ratios was developed using glutaraldehyde based fixing and staining protocols and used to threshold the endophyte mycelium in confocal microscopic images, using off line draw fill software. Once this was done the biomass was estimated for each section, totaled and percentage endophyte occupancy estimated.

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Chapter 1

Introduction

1.1 Taxonomy, agronomy and genetics of perennial ryegrass

Perennial ryegrass *Lolium perenne* is the most widely grown forage crop in New Zealand. This grass is a member of the family Gramineae, sub-family Pooideae within the tribe Poeae. The cultivars used in agriculture are predominantly diploid (2n = 14) but some tetraploid genotypes are also grown, although these have been shown to have low pasture persistence.

Until 1950, imported perennial ryegrass was oversown into pastures that were previously composed of native grass species. Perennial ryegrass has always been favoured as a forage crop because of its pasture persistence, palatability and ability to withstand close grazing due to its basally sited meristem (Soper & Mitchell, 1955). Over the years, ecotypes arose in response to differences in microclimates and grazing regimes. Several of these ecotypes were pooled, interbred and released as New Zealand certified *L. perenne* (Grasslands Ruanui). In the late 1950s, a superior ecotype was identified at Mangere and, in 1962, 1000 randomly selected genotypes from the Mangere site were evaluated against Ruanui in single plot trials. From this trial, nine elite genotypes were selected and polycrossed. The seed from this cross was bulked and released as Grasslands Nui perennial ryegrass (Armstrong, 1977).

1.2 Taxonomy of the endophytes *Epichloë* and *Neotyphodium*

1.2.1 Taxonomy

The *Epichloë* endophytes are a group of clavicipitaceous fungi that form mutualistic intercellular associations (symbiota) with temperate Pooideae grasses (Christensen *et al.*, 2001; Scott, 2001 b). All the *Epichloë* species are sexual and spread both vertically and horizontally, whereas their asexual *Neotyphodium* (previously *Acremonium*) derivatives spread mainly vertically. The life cycle of these fungi is shown in **Fig 1.1**. (Schardl & Philips, 1997).

The life cycle of Neotyphodium lolli

Fig 1.1 Co-ordinated life cycles of *Epichloë festucae* and its host grass *Festuca rubra*. *Neotyphodium* species found in *Lolium perenne* are transmitted vertically in a similar manner.

Currently nine *Epichloë* species are recognized and it is thought that the similar asexual *Neotyphodiun* spp. evolved either from a single *Epichloë* species or more often by an interspecific hybridization {Scott, 2001a & Schardl & Phillips, 1997}. The seed-transmitted species, *Neotyphodium lolii*, is the predominant endophyte found in *L. perenne*. This association of endophytes with *Lolium* was first noted by (Neill, 1940). However, the true significance of this mutualism was not fully appreciated until 1981 when two scientists, Fletcher and Harvey (Fletcher & Harvey, 1981), were able to correlate endophyte levels in Nui with lamb toxicosis, a syndrome known as ryegrass staggers.

The New Zealand strain of *N. lolii* can be considered to be homogeneous with little genetic variation across New Zealand. When the initial ryegrass introductions were made into New Zealand from the UK it is surmised that they were *L. perenne* genotypes that hosted a narrow range of *Neotyphodium* genotypes. This has led to the relatively homogeneous levels of alkaloid found in endophytes today. At the time of the introduction in the late 19th century the nature or existence of the symbiotum was unknown. This was probably due to the manner of its original introduction. Survey

work done at Ruakura has shown that there are no recorded instances of endophytes that do not express alkaloid genes, while in the UK many cases of non-expression have been found in this particular symbiotum. It is proposed that the quantitative differences in ergovaline, peramine and lolitrem B production between *Lolium* genotypes are due to differences in interaction between *N. lolii* with these different *Lolium* genotypes, rather than genetic profile differences in *Neotyphodium*.

1.2.2 Distribution of *N. lolii* in *L. perenne*

The endophyte colonization of grasses is without exception intercellular with no known intracellular invasion. This would be expected for a mutualistic interaction. However, the delicate balance between the endophyte triggering a hypersensitive response from the plant and maintaining compatibility with its host is not well understood (Christensen *et al.,* 2002).

The distribution of an endophyte within its host has also been studied (Keogh *et al.,* 1996) using sectioned plant parts, such as age-related leaf sections, ligules and sheath sections, and analysed for endophyte using enzyme-linked immunosorbent assay (ELISA). This work showed that endophyte biomass was highest in the sheath and lowest in leaf tissue. Older tissue generally had a higher biomass of endophyte than younger tissue.

N. lolii is also known to produce the alkaloids lolitrem B, ergovaline and peramine. Peramine is very soluble whereas lolitrem B and ergovaline are relatively insoluble and thus more restricted in their movement throughout the plant. Analysis of plant sections for these insoluble alkaloids by high performance liquid chromatography (HPLC) confirmed the biomass findings, that lolitrem B and ergovaline, and therefore the endophyte, are found more commonly in the sheath tissue.

Because the concentrations of alkaloids may simply reflect the biomass of endophyte rather than being a direct consequence of the interaction between the endophyte and the plant, a further study was done (Herd *et al.,* 1997) using transformed *Acremonium* (*Neotyphodium*) carrying the *Escherichia coli* β-D-glucuronidase gene (*gusA*) under the control of a constitutive promoter to assess metabolic activity. This study found that

endophyte activity decreased with an increase in plant size and that around 70% of the activity was confined to the leaf sheaths. The results indicated that there is a younger to older basal-apical gradient and that it is established early in leaf development. The study also established that the endophyte in each part of the plant is regulated so that a predetermined threshold of total endophyte activity per plant is not exceeded and a consistent distribution pattern is maintained.
However, a key issue with all these studies is the difficulty in measuring endophyte biomass within the plant. This is an important issue because if there is an active interaction between the participants of the association, then there is a need to correlate endophyte biomass with alkaloid output under different plant stress regimes.

Methods used to determine endophyte biomass include hyphal counts in plant cross-sections (Yong *et al.,* 2001), chitin levels (Roberts & Cabib, 1982; Roberts *et al.,* 1948) and competitive polymerase chain reaction (PCR) (Panaccione *et al.,* 2001; Groppe & Boller, 1997). Confocal imaging has also been used for estimating fungal biomass (Dickson & Kolesik, 1999; Running *et al.,*, 1995).

A more detailed morphological examination of *Neotyphodium* distribution in *Lolium* has shown that vascular invasion can occur in some artificial endophyte associations (*pers comm* Simpson, AgResearch Grasslands Division, Palmerston North, New Zealand 2002; Christensen *et al.,* 2001). However, there is little evidence for vascular invasion that leads to pathogenicity in the wild. There is circumstantial evidence to suggest that the host grass influences the concentration of the hyphae in the leaf sheaths and blades and that hyphal extension and branching occurs for only a short time during the life of a leaf (Christensen *et al.,* 2001). This influence probably occurs as the leaf is growing. The host may also control the concentration of alkaloids produced by the endophyte.

Neotyphodium / Epichloë endophytes, with few exceptions, interact with vegetative tillers of their host grasses in a highly regulated manner (Yong *et al.,* 2001). The hyphae in the leaf sheaths of the host grass seldom branch and tend to align parallel to the leaf axis. The hyphae are randomly distributed and not clustered around the vascular bundles. When found in leaf blades, they tend to cluster around the small un-sheathed vascular bundles, presumably because they have access to nutrients. However, a

benomyl-resistant *N. lolii* mutant and p-endophytes show a different growth pattern when introduced into *L. perenne* (Christensen *et al.,* 2002). In contrast to the normal restrictive endophytic growth pattern, these endophytes show a highly branched growth pattern (**Fig 1.2**) that does not revert to normal growth when leaf growth ceases.

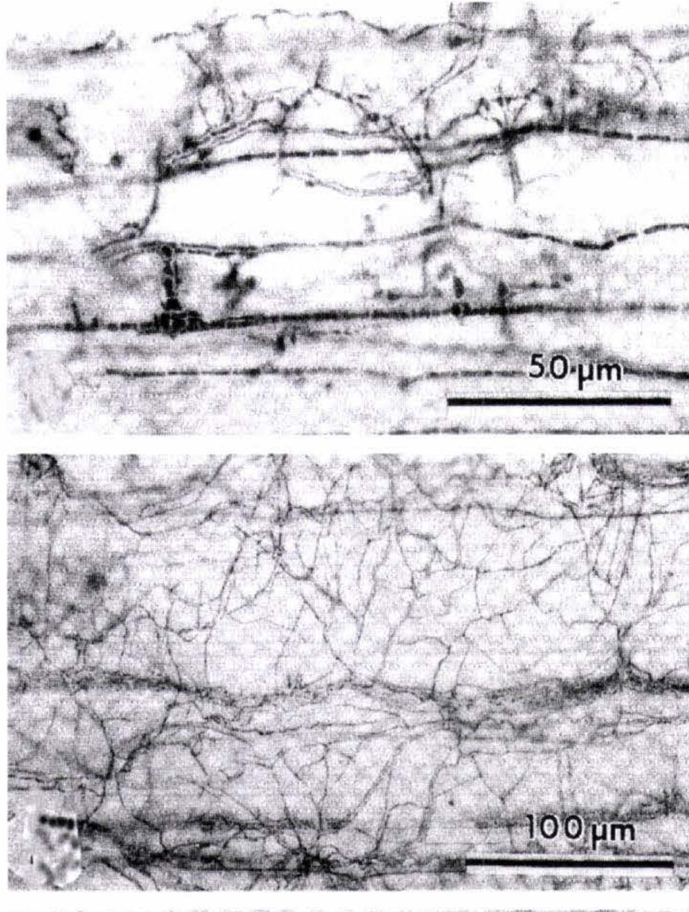


Fig 1.2 The hyphae of highly branched distorted *Neotyphodium lolii* in the outer sheath of *Lolium perenne* of an artificially established association (upper) and the hyphae of a p-endophyte (lower).

For these interactions, the endophyte is acting more as a pathogen than as a mutualistic partner. This unregulated growth of the endophyte could be due to some internal host plant control having been either down-regulated or switched off, resulting in the predetermined threshold of total endophyte activity per plant being exceeded (Herd *et al.,* 1997).

1.3 Endophyte biological effects

Around 1984, scientists in New Zealand and the USA began to appreciate that a number of toxins were being produced by grass containing endophytes and *N. lolii*, formerly

Acremonium lolii, isolated from *L. perenne* was described for the first time (Latch *et al.*, 1984). Farmers also realized that livestock were less productive than would have been expected based on the nutritional dry weight value of the grass. Stock behaviour also pointed to a lack of palatability. This lack of palatability and apparent nutritional value was due to the fungal endophyte producing mammalian toxins. In natural ecosystems these toxins confer major fitness enhancements to the grass host (Prestidge *et al.*, 1993; Gallagher *et al.*, 1985). These toxins (or alkaloids) fall into four major groups: the lolines, peramine, ergot alkaloids and the lolitrems (Bush *et al.*, 1997). The lolines are found in both *Festuca arundinacea* and *F. pratense* associations, whereas all the other toxins are found in ryegrass and tall fescue associations. Given that there was a high correlation between the toxicosis and the presence of the endophyte, it was assumed that the endophyte had the genetic capacity to synthesize the alkaloid toxins. Lolitrems, ergot alkaloids and peramine are commonly found in associations between perennial ryegrass and *N. lolii* (Christensen *et al.*, 1993). Fungal synthesis of peramine (Rowan, 1993), lolitrem (Reinholz & Paul, 2001; Penn *et al.*, 1993) and ergovaline (Bacon *et al.*, 1988; Bacon & Hill, 1979) has been confirmed by culturing the endophytes in axenic culture.

1.3.1 Protection from biotic stress

Lolitrem B is thought to be responsible for the neurotoxic malady known as ryegrass staggers. The correlation of endophyte to this condition was first noted in 1981(Fletcher & Harvy, 1981). Peramine, a pyrrolopyrazine alkaloid has as its primary activity the deterring of feeding insects such as black beetle and Argentine stem weevil (Bush, *et al.*, 1997). Most other insects tested are insensitive to peramine. Peramine has no known activity against mammalian herbivores. Ergovaline is mainly active against vertebrate herbivores although insecticidal activity from ergopeptine alkaloids, including ergovaline, against *Heteronychus arator* (black beetle) has been noted (Ball *et al.*, 1997). The alkaloid ergovaline has been correlated with reduced weight gain, elevated body temperature, restricted blood flow, reduced reproduction and reduced milk production in vertebrate herbivores. Ergovaline is associated with tall fescue toxicosis and gives similar symptoms in ryegrass. At this stage little is known about synergistic interactions between the different classes of metabolites. However, there is evidence

that high levels of ergovaline will enhance lolitrem B toxicosis (pers comm. Hume, AgResearch 2002).

1.3.2 Protection from abiotic stress or abiotic effects

There have been reports of drought and mineral stress tolerance in grasses infected with *Neotyphodium* spp. (Malinowski & Belesky, 2000). Endophytes are thought to affect osmoregulation by indirectly changing root morphology and function. It is thought that these mineral and drought adaptations may arise from a chemical signalling system in the symbiotum. The initial perception of the stress signal occurs in the roots, and affects a range of responses in the host plants that may influence the uptake and transport of water and nutrients. These responses contribute to the adaptability and subsequent persistence in challenging edaphic and environmental conditions. Much of the mineral stress work has been done on tall fescue rather than perennial ryegrass.

1.4 Biosynthesis of *N. lolii* alkaloids

Both ergovaline and lolitrem B are secondary metabolites that are produced from pathways that utilize isoprenoids as precursors (Scott, 2001a). These precursors are then combined in anabolic reactions with metabolites sourced from primary metabolic pathways such as glycolysis and the citric acid cycle to produce the alkaloid intermediates. Isoprenoids are derived from mevalonic acid (MA). This metabolite is phosphorylated and decarboxylated to produce isopentenyl pyrophosphate (IPP), which is then isomerised to produce dimethylallyl pyrophosphate (DMAPP). Both IPP and DMAPP are 5 carbon (C5) compounds.

1.4.1 Biosynthesis of the ergot alkaloid ergovaline

The first step in the ergot alkaloid biosynthesis reaction is a condensation between DMAPP and tryptophan that is catalysed by the enzyme DMAPP synthase to give dimethylallyl tryptophan (DMAT). DMAT is then converted via several intermediates to clavine alkaloids, such as lysergic acid, a member of the ergolene acid group. These intermediates are further transformed into complex ergopeptine derivatives, such as ergotamine produced by *Claviceps purpurea*, by two synthetases, lysergyl peptide

synthetase 2 (LPS2) and LPS1. LPS2 activates lysergic acid, which is transferred to LPS1, which sequentially adds the amino acids alanine, phenylalanine and proline to form ergotamine (Panaccione *et al.,* 2001). The synthesis of ergovaline by endophytes involves similar LPSs that incorporate alanine, valine and proline.

Recently, the peptide synthetase gene from *N. lolii* was cloned and inactivated by making a gene knockout in *Neotyphodium* sp. strain Lp1 (Panaccione *et al.,* 2001). The resulting strain retained full compatibility with its *Lolium* host as assessed by immunoblotting of tillers and quantitative PCR. No ergovaline was detected, as analysed by HPLC (Panaccione *et al.,* 2001). For the first time, this provides genetic proof that *N. lolii* requires a peptide synthetase for ergovaline biosynthesis. This result demonstrated the possibility of ameliorating ergovaline toxicosis in livestock by the genetic manipulation of endophytes.

1.4.2 Biosynthesis of indole-diterpenes

Much less is known about this pathway, and what is known is based on radio-labeling studies (Munday-Finch *et al.,* 1996; Mantle & Weedon, 1994). In *Penicillium paxilli*, the proposed pathway (**Fig 1.3**) for paxilline biosynthesis (Scott, 1999) involves indole that has been derived from tryptophan (or a tryptophan precursor) and IPP from MA as precursors. Indole is condensed with geranylgeranyl pyrophosphate (GGPP), a C20 molecule, to generate the first stable indol-diterpene intermediate, possibly paspaline. Paspaline is subsequently converted to paxilline via paspaline B and 13-desoxpaxilline. In endophytes, paspaline is the proposed intermediate that is converted to lolitrem B via the intermediates α-paxitrol and lolitriol.

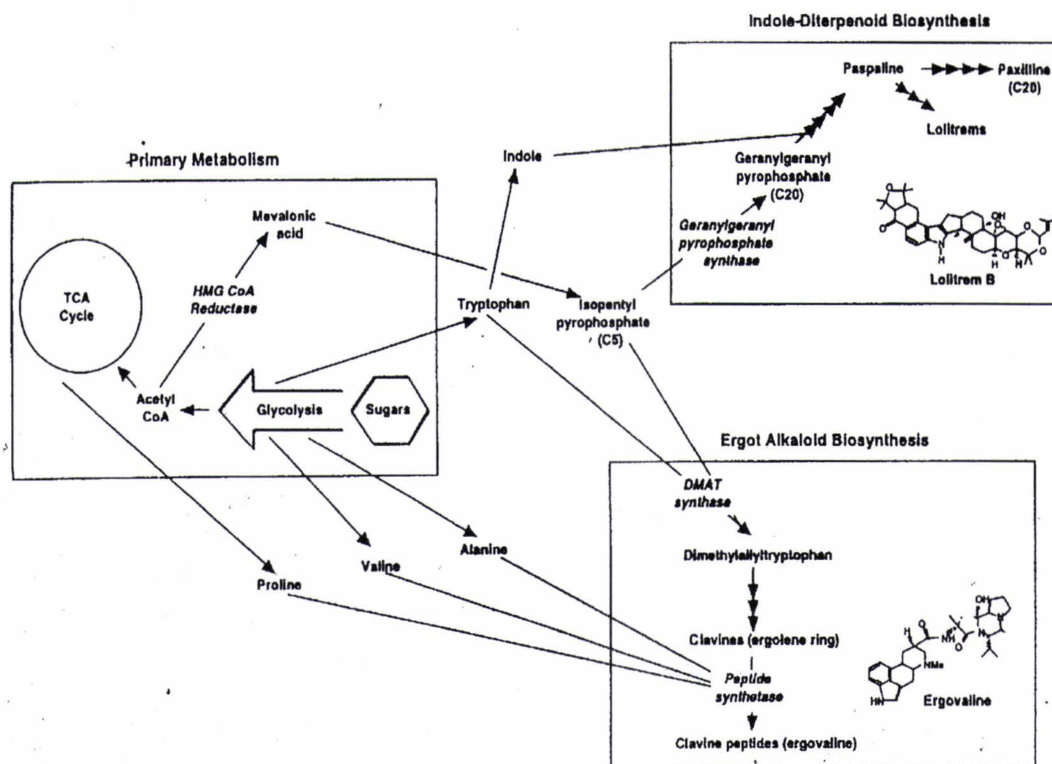


Fig 1.3 The proposed pathway for the biosynthesis of ergot alkaloids and indole-diterpenes in *Epichloë* endophytes.

1.4.3 Biosynthesis of peramine

It has been suggested that peramine is derived from the cyclization and condensation of the amino acids proline and arginine, with the final *N*-methylation carried out by an *N*-methyltransferase using S-adenosylmethionine as a cofactor. The mode of action of peramine is unknown but it is thought to interfere with a microsomal cytochrome P450 enzyme (Siegel & Bush, 1996).

1.5 Plant stresses and hormonal responses

A number of stress conditions are recognized in plants and in many cases the physiological responses have been identified. These include UV light, disease, grazing, nutritional and drought stresses. Hormones are central to the plant responses to these stresses (Davies, 1993).

1.5.1 UV light stress

UV-B radiation had a dramatic effect on the expression of chloroplast proteins in *Pisum sativum*, as demonstrated by severe reduction in chloroplast transcripts (Jordan *et al.*, 1991). However, this effect was reversible and prolonged UV-B exposure tended to reduce the effect (Jordan *et al.*, 1991). UV-B radiation was shown to down-regulate the production of certain photosynthetic transcripts in fully expanded pea leaves. The response was less marked in apical buds and was ameliorated by high-light intensities. Reactive oxygen species (ROS) were highest in low-light leaves and lowest in high-light leaves and buds. The feeding of antioxidants could prevent the UV-B effect (Mackerness *et al.*, 1998). Further work on the effects of UV-B in *Arabidopsis thaliana* showed that, in addition to the down-regulation of the photosynthetic genes, two pathogen-related genes, *PR-1* and *PDF1-2*, were up-regulated and, at the same time, jasmonic acid (JA) and ethylene production increased. It was also shown that there was an increase in ROS. It is well known that salicylic acid (SA) plays a key role in activating the defence pathways that lead to the up-regulation of the pathogen-related *PR-1* and *PDF1-2* genes (Surplus *et al.*, 1999). It is proposed that at least four signalling pathways mediate responses to UV-B. In this scheme

(i) ROS triggers the production of JA, which directly or indirectly triggers *PDF1-2* transcription.

(ii) ROS also triggers the production of ethylene, which then acts on the *PDF1-2* gene. Ethylene may also act indirectly via the SA pathway.

(iii) ROS directly triggers the production of SA, which then acts on the *PR-1* gene.

(iv) ROS acts to down-regulate the photosynthetic genes *Lhcb* and *psbA*.

From this work, it is clear that, although the perception of a stress in plants may be via different response pathways, the transducer signals that respond to the stress are similar, or at least act in parallel cascades (Mackerness *et al.*, 1999; Mackerness *et al.*, 2000).

1.5.2 Heat stress

External application of SA to willow (*Sinapis alba* L) plants enables them to tolerate heat stress up to 55°C. This is known as heat-acclimation (Dat *et al.*, 1997).

1.5.3 Pathogenic stresses and the hormones involved

1.5.3.1 Jasmonates

Jasmonates are derived from the fatty acid linolenic acid via the octadecanoic pathway (Farmer *et al.,* 1998). Most of the at least seven enzymatic steps in the pathway have been extensively characterized (Vick & Zimmerman 1984). The jasmonates are involved in a number of plant processes including fruit ripening, production of viable pollen, root growth and tendril coiling. However, these compounds play a major role in plant defence against pathogenic attack from viruses, fungi, bacteria and from insects. JA is known to act at the gene expression level, modulating transcription, transcript processing and translation (Creelman & Mullet, 1997).

JA and methyl JA synthesis increased when soybean (*Glycine max* L. Merr.) was wounded (Creelman *et al.,* 1992). The JA levels in different tissues within a plant have also been investigated (Voros *et al.,* 1998; Creelman & Mullet, 1995). In soybeans, they were shown to be highest in young tissues. When the leaves were dehydrated by 15%, there was a five-fold increase in JA within 2 h. These levels declined to the control level within 4 h of water restriction. Abscisic acid (ABA) took longer to reach its maximum. In this study, although ABA was shown to reduce transpiration by 72%, JA also reduced transpiration by 22%.

A phytoalexin was shown to accumulate in plants under fungal invasion, but production was triggered by $CuCl_2$, an abiotic elicitor, and not by fungal attack *per se* (Randeep *et al.,* 1996). The production of phytoalexins in rice leaves could be reduced when the leaves were treated with JA biosynthesis inhibitors.

JA does not act alone. Increases in JA are usually accompanied by an accumulation of SA. SA stimulates the production of acidic pathogen response (PR) proteins (Hiroshi *et al.,* 1996). When plants were wounded in the presence of the cytokinin benzylaminopurine, JA production started 6 h earlier than in untreated samples and, when a cytokinin antagonist was applied, the effect was negated. The conclusion was that cytokinins are directly involved in the control of endogenous levels of SA and JA.

JA is a key hormone responsible for the activation of signal transduction pathways in response to predation and pathogen attack. The wound-inducible formation of proteinase inhibitors occurs when JA combines with ABA and ethylene to protect the plant from predation (Wasternack & Parthier, 1997). Recently, the role that JA plays in external plant protection has also been investigated. When plants are attacked, they produce volatiles that attract the natural enemies of the pathogen. The parasitism of caterpillar pests increased two-fold when field-grown tomatoes were induced with JA (Thaler, 1999).

1.5.3.2 SA and stress responses

A plant hormone is a natural compound with the ability to affect physiological processes at concentrations far below those at which either nutrients or vitamins would affect the process. By this criterion SA has recently been classed as a plant hormone. SA biosynthesis is via the chain degradation of cinnamic acid, an important intermediate in the shikimic acid pathway. The final conversion to SA is via either *o*-coumaric acid or benzoic acid. It is not known if there is any connection between the prophylactic effects of salicylates in plants and their therapeutic effect in animals, although their chelating role in animal therapeutics may be utilized in some plant systems. However, there is growing evidence that SA plays a primary role in the induction of PR proteins during systemic and local acquired resistance, although its role as a primary transmission signal has not been established. SA does, however, increase systemically following inoculation with necrotizing pathogens.

The role of SA in dicotyledons has been well documented but little work has been done on its role in monocotyledons. Work on SA in monocotyledons in response to diseases is urgently required. SA is also known to have a role in plant thermogenesis (Dat *et al.*, 1997) and the control of flowering in some species (Raskin, 1992; Klessig & Malamy, 1994). The link between this process and its role in systemic acquired resistance (SAR) is not yet understood. That SA has a role as a signalling molecule in local defence reactions at infection sites and in the induction of the SAR has been confirmed in part by using *Arabidopsis* mutants. However, some work using these mutants has shown that, although SA is required for SAR, it is not absolutely required for the initial hypersensitive response. When a pathogen infection occurs, plants initiate a local

hypersensitive reaction that results in an oxidative burst followed by the induction of genes for pathogenesis-related PR proteins. This is followed by the formation of necrotic leaf spots, and restriction of pathogen growth and spread. Other genes are thought to play a role in the development of SAR. Consequently, should a secondary infection occur a few days later, then infection will result in much smaller lesions. These responses are accompanied by elevated SA levels. By using *Arabidopsis* SA pathway gene *cpri* and *cim3* mutants, it has been shown that the initial hypersensitive reaction occurs but that the ongoing SAR reaction fails to occur, as determined by the smaller lesions occurring in subsequent infections. However, when these plants are treated with SA, the secondary effect can be restored. The role that SA has in plants is similar to pathways found in other eukaryotes; for example, tobacco resistance gene N (Nicotiana) and its product are similar to Toll proteins that activate defence mechanisms in mammals and *Drosophila*. A further comparative analysis of animal and plant defence responses should improve the understanding of plant disease resistance. More recently, methyl SA has been shown to act as an airborne signal that activates defence mechanisms in distal leaves and possibly even on neighbouring plants (Durner *et al.*, 1997).

Finally, although SA is an important signalling molecule in plant defence, plants employ a network of transduction pathways (Pieterse & van Loon, 1999; Reymond & Farmer, 1998), some of which are independent of SA. These independent pathways rely on JA and ethylene but there is 'cross-talk' between them, which provides greater potential for activating multiple resistance mechanisms in various combinations. This potential for cross-talk is shown schematically in (**Fig 1.4**). (Reymond & Farmer, 1998).

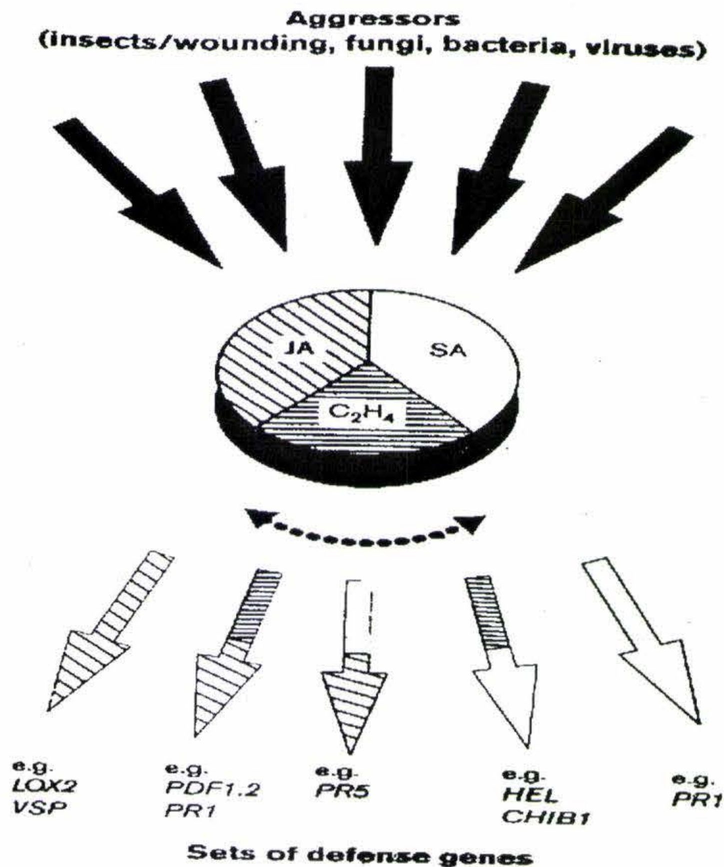


Fig 1.4 Tunable dial model for the regulation of defence gene expression by the three signals JA, ethylene and SA. Depending on the nature of the aggressor, the plant is able to fine-tune (dotted arrow) the induction of the defence genes either by employing a single signal molecule (single-patterned arrow) or by using a combination of these regulators (multi-patterned arrows)

An emerging hypothesis is that, by producing, mimicking or destroying any one of this trio of signals, pathogens and pests can reset the dial and alter the spectrum of the genes induced. Other work in this area using *Arabidopsis* mutants has shown that SA can act to have a negative effect on the activation of gene expression by the signal molecule JA to the extent that SA interferes with JA-dependent signalling (Gupta *et al.,* 2000).

The role of plant hormones in viral defence has been studied by wick feeding plant hormones directly into the plant and then measuring the viral replication titre using ELISA. Under these circumstances, enhancement of the endogenous levels of ABA, IAA (indole-3-acetic acid) and gibberellic acid did not affect virus replication, whereas dihydrozeatin, 1-aminocyclopropane-1-carboxylic acid, SA and JA enhancement all inhibited virus replication (Clarke *et al.,* 1998).

1.5.3.3 ABA and stress responses

ABA is a sesquiterpenoid (15C) synthesized in the chloroplasts and plastids within the palisade cells in the leaves (Sengbush, 2000). Its biosynthesis is closely linked to the isoprenoid pathway and it is synthesized from farnesyl pyrophosphate, a linear C15 molecule. Many of the intermediates in this pathway have been shown to increase under drought conditions (Meyer et al., 1989). There are different isomers and enantiomers of ABA found in the plant and they may have different biological activities. The S-*cis* form is the most biologically active and the form most commonly found in plants. As is the case with most plant hormones ABA is multifunctional with a wide range of biological activities.

Seed and bud dormancy

ABA induces seed maturation in some plant species and enforces seed dormancy in others. During a mild autumn, ABA prevents seed germination by enforced seed dormancy. The seeds will not germinate until a period of winter chilling has occurred. ABA has a similar effect on bud meristems by enforcing their dormancy. For seed maturation, it has been shown that gibberellins antagonize ABA signalling in developing maize embryos, providing temporal control over the maturation phase (White & Rivin, 2000).

General stress tolerance

ABA levels increase when plants encounter saline and increased temperature conditions. Both these conditions are known to cause water deficiency. ABA acts to close the stomata under these conditions. Plant roots are able to sense drought conditions and ABA acts to reduce shoot growth while at the same time stimulating root development and increasing hydraulic conductance (Hartung & Turner, 1997). Prior to the onset of winter, ABA acts to cause the deciduous plants to respond to the upcoming stressful winter temperature by eliciting production of ethylene. Ethylene regulates the genes involved in the abscission process. ABA treatment of some plants at normal

temperatures has been shown to mimic the effect of cold temperature by producing the same set of proteins that are thought to protect the plant against freezing (Skriver & Mundy, 1990).

Cellular transpirational control

This is one of the fundamental actions of ABA in plants. During water stress, the levels of ABA, proline and glycine-betaine rapidly increase (Abernethy & McManus, 1998). Proline is thought to be synthesized *de novo* from glutamate. It has been suggested that proline may serve as a storage compound for reduced carbon and nitrogen during stress. This conclusion was drawn from experiments with New Zealand tussock (*Festuca zealandiae*), which showed proline levels decline, when a drought stress was prolonged for more than 24 h, in the absence of light. Under these conditions proline is the source of respiratory CO_2 under carbohydrate-deficient conditions (Hsiao, 1973). ABA acts to modulate various ion channels, especially calcium channels, in the guard cells to alter their osmotic potential in such a way as to promote their collapse over the stomata (Pei *et al.,* 2000). Previous work has shown that there are key molecules linking ABA to cyclic ADP ribose (cADPR). cADPR has become known as a universal signalling molecule (Pennisi, 1997). The mechanism involves the binding of ABA to the plasma membrane receptors of the guard cells. This initiates a rise in pH in the cytosol, and importantly, the formation of cADPR. The increased pH stimulates the loss of K^+ and organic ions from the cell, while rising levels of cADPR cause Ca^{2+} to move from the vacuole to the cytosol, which blocks the uptake of K^+ into the guard cells. The combined effect results in the loss of solutes in the cytosol and this reduces the osmotic pressure of the cell and thus its turgor and the leaf stomata are closed by the guard cell collapse. The messenger molecule
cADPR appears to be involved in signalling pathways in plants and in animals (Pennisi, 1997).

Other effects

ABA may also inhibit the action of gibberellins by stimulating *de novo* synthesis of α–amylase and inducing gene transcription for proteinase inhibitors in response to wounding. This would explain its role in pathogen defence (Maas, 2000). ABA also plays an important role in the wounding response. When ABA was sprayed on to potato plants, there was an increase in the *Pin*2 gene mRNA transcripts in the absence of wounding. The accumulation was tissue specific and did not occur in the roots. The *Pin*2 gene is known to be involved in plant tissue wounding (Pena-Cortes & Willmitzer, 1993).

1.6 Effects of water stress on *Lolium* hormone levels and *Neotyphodium* alkaloid production

There is some evidence that endophyte-infected tall fescue is more tolerant to drought than endophyte-free plants but the mechanism is unknown. One study showed that infected plants had greater concentrations of fructose and glucose in the leaf blades and higher concentrations of glucose in the sheaths than non-infected plants (Richardson *et al.,* 1992). This was thought to contribute to increased osmotic potential and re-growth capacity of the endophyte-infected grasses. Another study, using detailed sward measurements of clonal material from perennial ryegrass, showed that the endophyte presence may confer advantages to plants only under moderate to severe conditions of drought and/or pest damage (Hume *et al.,* 1993).

A complete picture of the advantages to both participants in the symbiotum has yet to be established. Perennial ryegrass normally spreads asexually by grazing-induced tillering. Mammals grazing low down on to the crowns, as occurs in severe drought, can destroy the low growing meristems of ryegrass causing widespread sward death. Because the endophyte-plant associations are widespread and persistent, a testable hypothesis could be that the mammalian endophytic toxins are not produced in normal growing conditions but only when the plants become stressed. Drought is a major stress of perennial ryegrass. During drought herbivores will begin to graze down on to the crowns, thus irreversibly damaging the very meristems that are facilitating the spread of the plant by tillering. It is at this time that drought stress hormonal signals produced by

perennial ryegrass could result in increased alkaloid production by the endophyte. The effect would be reduced grazing pressure on perennial ryegrass (Barker *et al.,* 1997). One study showed that there was a significant increase in ergovaline levels in drought-stressed perennial ryegrass but that this increase did not occur in the other alkaloids such as peramine and lolitrem B (Barker *et al.,* 1993). Other reasons could be the increased *Neotyphodium* biomass *per se* found in certain *Lolium* genotypes that result in an increased alkaloid output (Easton *et al.,* 2002). Other factors may include environmental conditions such as soil nitrogen levels and ambient temperature (Lane *et al.,* 1997).

1.7 Endogenous levels of plant hormones

To study the effects of endogenously produced plant hormones, these same hormones need to be applied externally in a quantitative manner and the plant responses need to be measured.

Various methods of externally applying ABA and JA to water-sufficient *L. perenne* were investigated. These included spraying, wick feeding and dipping.

The wick feeding method (Clarke *et al.,* 1998) places the hormone directly into the sap stream (phloem vessels) of the plant. This would be comparatively simple in dicotyledonous plants where the vascular bundles containing the phloem and xylem vessels are arranged in cross-sectional rings, but more difficult in the less structured monocotyledonous vascular system of perennial ryegrass where the vascular bundles are scattered individually as discrete units across the stem.

The spray method of hormone application, although universally used for commercial applications of pesticides and herbicides, is far less quantitative than would be needed to carry out a well-designed controlled experiment, involving the application of measured amounts of ABA and JA.

Spraying and wick feeding have various problems whereas modification of foliar application by dipping would seem from the literature to be more manageable. The method that was developed involved up-ending potted perennial ryegrass plants into containers of plant hormone solutions of known concentrations and leaving the leaves

submerged until an equilibrium/active uptake of the hormone occurred, usually around one hour, and then removing the bathing solutions and allowing the up-ended plant leaves to dry before placing them back on the watering trays (**Fig 2.7**).

1.7.1 Pathways of hormonal entry into the plant

The first major barrier to hormone uptake is the cuticle, which is made up of hydroxy-fatty acids that form a three-dimensional polyester network (Martin & Juniper, 1970) of insoluble lipid polyesters called cutin (Holoway, 1980), with its waxy extrusions (see **Fig 1.5**) (Leopold, 1964; Schieferstein & Loomis, 1956). This cuticle becomes thicker with age and not only covers the upper (adaxial) and lower (abaxial) epidermis but also covers, to a lesser extent, the mesophyll and palisade cells that line the sub-stomatal cavities in monocotyledonous plants.

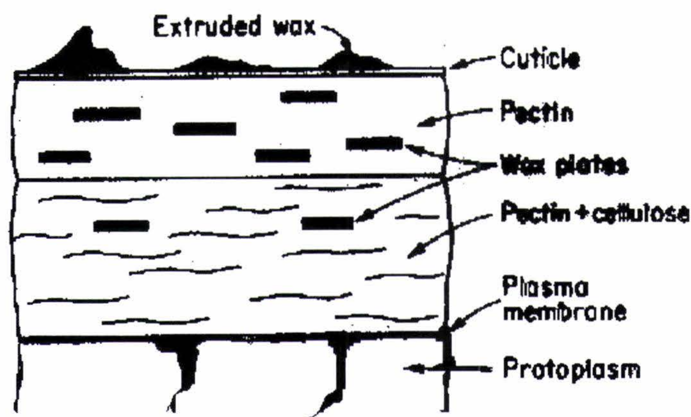


Fig 1.5 The components of the epidermal cell wall, showing extruded wax overlaying a cuticle, and then cell wall layers of pectin and pectin plus cellulose with some wax plates included. Inside the cell walls are tiny protoplasmic connections, called ectodesmata, that allow access into the protoplasm.

The cuticle is composed of polymerized fatty acids, esters and soaps and has ionic properties that allow it to take up water, due to its acidic nature. This gives the cuticle a sponge-like capacity and enables it to expand or contract depending on the availability of water. Wax plates permeate the cuticle and parts of the cell wall. The waxes that make up these plates are made up of fatty alcohols and esters that are produced in the cytoplasm. These plate like deposits are often extruded in large amounts as waxy blooms. Research workers have demonstrated the presence of pectinaceous material in

the cuticle of apple leaves that was shown to be continuous, with similar layers in the walls of the epidermal cells below (Roberts *et al.*, 1948).

The existence of this structure in monocotyledon leaves would make it possible for solutes to move along these pathways into the leaf. The permeability of aqueous sprays through the cuticle is determined by three further cuticular factors: the thickness and the chemical composition of the waxes, where they exist on the leaf, and the physical form of the extruded layer. For instance, if the extruded layer is rod like, this will tend to hold the droplets away from contact with the cuticle. Inside the cuticle is the cell wall with its outer pectin layer and an inner layer composed of cellulose fibres embedded in pectin and other non-cellulosic polysaccharides. Unless the composition of the wall is high in waxy plates, it is usually quite permeable to water.

The plasma membrane is the last barrier. This is composed of a phospholipid bilayer with various protein protuberances. Entry of solutes can be by one of three methods: diffusion through pores in the fatty layer, solubilization into the fatty layer or binding to carrier sites within the membrane. Because of these three options, entry is influenced by the size, shape and charge of a molecule, its solubility in the fatty membrane layer or any other molecular feature that would enable it to become attached to carrier sites in the membrane. Electron micrographs of the cell wall have shown that tiny channels of connecting protoplasm or ectodesmata permeate the cell walls of epidermal cells (Franke, 1961).

Because ABA and JA have hydrophilic acid chains and lipophilic rings they probably enter through the interphase between the hydrophilic aqueous phases of the cuticle and the water-permeable plasma membrane with its lipoidal layer. The wetting of the cuticle is also important as expansion of the sponge-like cuticle will facilitate entry. Drying of the leaves will retard absorption.

The stomata also provide a route for entry of solutes (**Fig 1.6**), (Leopold, 1964; Martin & Juniper, 1970). First reported (Dybing & Currier, 1961). This is especially true for volatile auxin esters or solutions with added surfactants such as the organosilicone Pulse^{TM}. Pulse reduces the surface tension of the spray or bathing solution and allows these molecules to infiltrate directly into the foliage via the stomata.

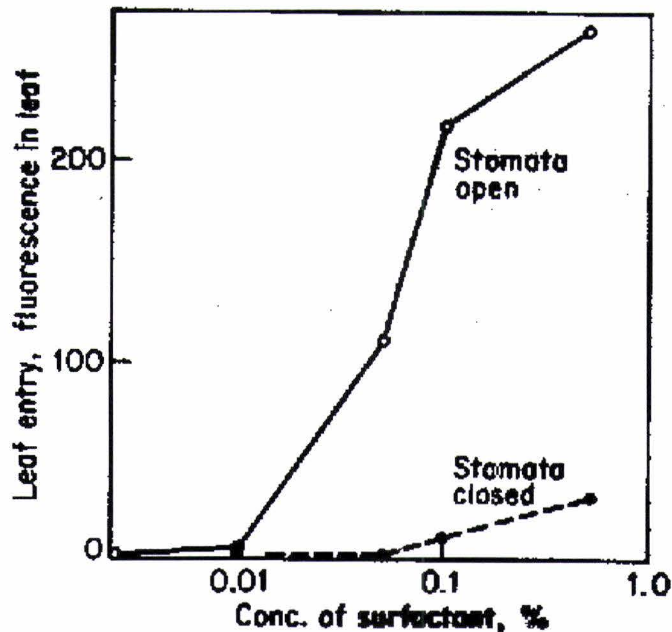


Fig 1.6 The entry of the dye fluorochrome into leaves of *Pyrus communis* as related to the concentration of surfactant (Vatsol OT) and the state of opening of the stomata. Entry of 0.17% before washing and measuring entry by fluorescence in ultraviolet light.

Recent confocal laser scanning microscopy carried out on bean (*Vicia faba* L) and wheat (*Triticum aestivum*) has shown that the uptake of organosilicone solution into the monocotyledon *Triticum* is significantly slower and less than its uptake into *Vicia*. Using confocal, transmission electron and light microscopy, it was shown that *Triticum* has sub-stomatal cavities with discrete thick wall linings (Gaskin *et al.,* 1998) that restrict access and slow movement of organosilicone solutions into the adjacent mesophyll cells. These sub-stomatal cavity walls have been shown to be cuticular in composition. The rapid absorption into *Vicia*, a dicotyledon, in this study was due to the absence of any cuticular-lined sub-stomatal cavities. This allowed direct entry of surfactant solutions into the surrounding mesophyll tissue (Gaskin *et al.,* 1998) as shown in **Fig 1.6** (Dybing & Currier, 1961).

The absorption in *Triticum*, and presumably *Lolium*, has been shown to be predominantly cuticular penetration whereas that in *Vicia* is mainly stomatal in nature (Gaskin, 1995).

Foliar uptake via the stomata is dependent on the concentration of organosilicone surfactant in a spray solution and the carrier volume applied (Gaskin *et al.,* 1996); uptake via the stomata, of ABA and JA plus Pulse (0.125%), for 60 min would ensure substantial uptake into *Triticum* and *Lolium* species (*pers comm.* Gaskin, Forestry Research Institute, Rotorua, New Zealand 2002).

Cuticular penetration of ABA and JA in the absence of a surfactant, such as the organosilicone Pulse, is most likely the form of uptake (Martin & Juniper, 1970). Any cuticular uptake that occurred would be in two phases and would be adsorptive in nature.

When plant leaves are immersed in solutions containing inorganic or organic solutions, there are two stages in the kinetics of entry: an initial stage of rapid entry lasting about 20 min followed by a second steady but slower uptake (**Fig 1.7**) (Leopold, 1964). A dipping period of 60 min would ensure significant uptake of ABA and JA.

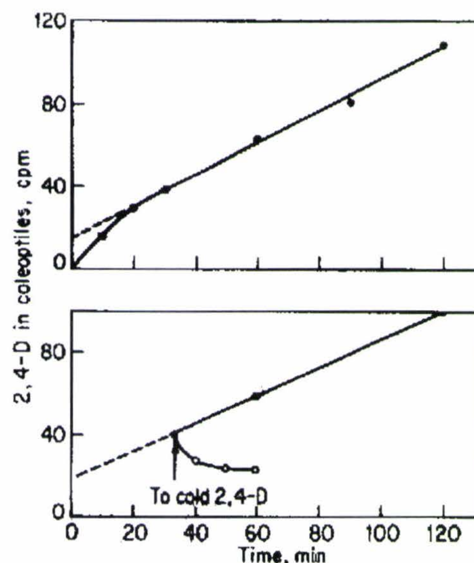


Fig 1.7 The uptake of 2,4-D by oat coleoptile sections, showing the initial rapid uptake phase followed by a steady slower phase (top). Extrapolation of the slower rate to the y ordinate permitted an estimate of the total amount taken up initially. When the sections were moved from the 0.5 mg/L labelled solution to an unlabelled solutions considerable elution occurred (bottom), indicating that the initial uptake was freely exchangeable.

The characteristics of the uptake time curves imply that it would be an adsorption (the taking up of the applied chemical at the surface of the cuticle). This view can be backed

up by the fact that the initial uptake is freely exchangeable (**Fig 1.7**); respiratory inhibitors do not interfere markedly with the initial uptake and the quantitative characteristics are those of an adsorption event (Leopold, 1964).

Further work done on 2,4-D would suggest that penetration occurs largely by preferential sites, including stomata, trichomes and the cuticle above the anticlinal walls of the epidermal cells. However, microautoradiograms of *V. faba* cuticles treated with ^{14}C-2,4-D formulated with a surfactant (HLB 68.6) indicate a more diffuse pattern of penetration (Kirkwood, 1980).

Hypothesis and aims

(1) To test the hypothesis that endophyte bio-protective metabolites are synthesized at higher levels in water-stressed endophyte-infected plants than in water-sufficient plants.

(2) To test the hypothesis that external application of the plant hormones ABA, JA and SA to water-sufficient plants will increase levels of endophyte-produced bio-protective metabolites thereby mimicking abiotic stress.

(3) To carry out HPLC-ms experiments to measure tiller levels of ABA, JA, IAA and SA in perennial ryegrass during the application of a controlled water stress and then to correlate these to *N. lolii* produced alkaloid levels.

(4) To establish non-invasive protocols that would establish endophyte to perennial ryegrass biomass ratios using confocal microscopy.

Chapter 2

Materials and Methods

2.1 Plant Material

The plant cultivars used in this study were Nui ryegrass (*Lolium perenne*) containing wild-type *Neotyphodium lolii*. All wild-type, seed-grown, ryegrass genotypes used in this study were taken from an AgResearch endophyte selection trial at Grasslands Research Centre in Palmerston North.

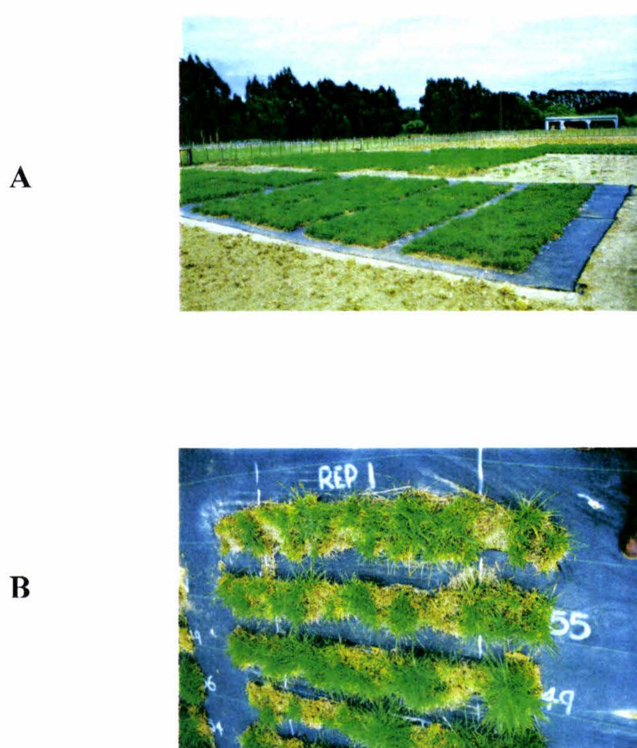


Fig 2.1 Source of perennial ryegrass material used in this work.
(Top) Field trial of different perennial ryegrass genotypes at AgResearch, Grasslands Research Centre Palmerston North
(Bottom) Seventeen perennial ryegrass genotypes were randomly selected for this work.

The highly heterozygous nature of the host contrasts with a very restricted genetic composition of the endophyte (Christensen *et al.,* 1993). Some of the recorded variation in the alkaloid output of the endophyte was thought to be due to the different genetic variations of the host interacting with *N. lolii.* To minimize these host effects, 17 different *L. perenne* genotypes were selected for this work. They were initially labeled A to Q and then subsequently renamed G1060 to G1076 for long term tracking purposes **(Table 2.1)**.

Table 2.1 The conventions used for labeling different genotypes.

Experimental Code	Laboratory Code
A	G1060
B	G1061
C	G1062
D	G1063
E	G1064
F	G1065
G	G1066
H	G1067
I	G1068
J	G1069
K	G1070
L	G1071
M	G1072
N	G1073
O	G1074
P	G1075
Q[a]	G1076

[a]Endophyte-free genotype.

2.2 Growth and maintenance of perennial ryegrass

The field-grown *L. perenne* genotypes were split into clonal masses of three to four tillers, which were potted up into 6 cm propagation tubes of standard AgResearch potting mix to give at least eight plugs for potting on. These plugs were grown on in the Plant Pathology glasshouse at AgResearch for 4 weeks, to allow root establishment and further tillering. Four plugs of different genotypes were then transferred to 18 cm square by 20 cm high black polythene pots containing, by volume, 50% AgResearch potting mix and 50% top soil containing silt with organic matter (Phoenix Garden Centre, Palmerston North). The top soil was air dried for 3–4 h, and then run twice through a peat grinder. The soil and the potting mix were subsequently combined and mixed in a modified concrete mixer. This soil/potting mix was used because of its high water holding capacity, which made it suitable for carrying out water stress experiments.

Each pot was topped up with soil mix to a set weight of 4725 g, as measured on a Mettler Toledo SB32001 Delter Range balance. This was done to facilitate the measurement of water loss and water addition.

The 20 cm high black polythene pots, each containing four different *L. perenne* genotypes, were divided up into five replicates, containing eight treatment pots per replicate. Replicates 1 to 4 were placed in the AgResearch glasshouse (**Fig 2.2A**). Replicate 5 was placed in a growth chamber on the second floor of the AgHort building at Massey University (**Fig 2.2B**). Surplus plants were used to make up Replicate 6.

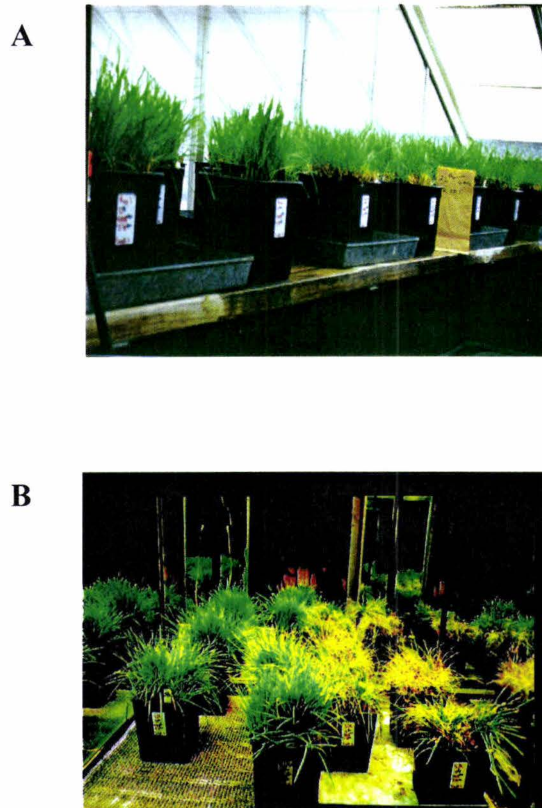


Fig 2.2 Potted perennial ryegrass genotypes used in this work growing in the glasshouse and the growth chamber.
(**A**) Replicates 1 to 4 contained the 16 endophyte-infected perennial ryegrass genotypes. They were potted up, four to a pot, and grown on benches in the glasshouse at AgResearch, Grasslands Research Centre, Palmerston North.
(**B**) Replicate 5 contained four of the perennial ryegrass genotypes. They were potted up, four to a pot, and grown in the growth chamber in the AgHort building at Massey University, Palmerston North.

Environmental conditions in the glasshouse followed the current ambient day length and temperature conditions. The growth chamber was run at a 16 h day with a 2 h dawn and dusk phase, and an 8 h night. Temperatures were kept at 22°C for the day and 15°C for the night.

During the re-establishment period, the pots were watered every 2 days to field capacity. The plants were sprayed with 'Tilt 250 E/C', active ingredient propiconazole (Yates™), mL/L) at the first signs of rust. The plants were maintained in a healthy state by the regular application of liquid 'Thrive' at 12 g/4 L (Yates™). Tilt 250 E/C rather than other fungicides was used because tests have shown that it has no deleterious effects on the systemic biomass of the *N. lolii* endophyte. Promotion of tillering was facilitated by regular cutting of the leaves with scissors just above the ligules (**Fig 2.3**). Cutting into the leaf sheath causes the production of heavily cellulosed re-growth. Because the plants were to be up-ended and the leaves physically dipped in hormone preparations, lush re-growth was preferred to heavily cellulosed leaves.

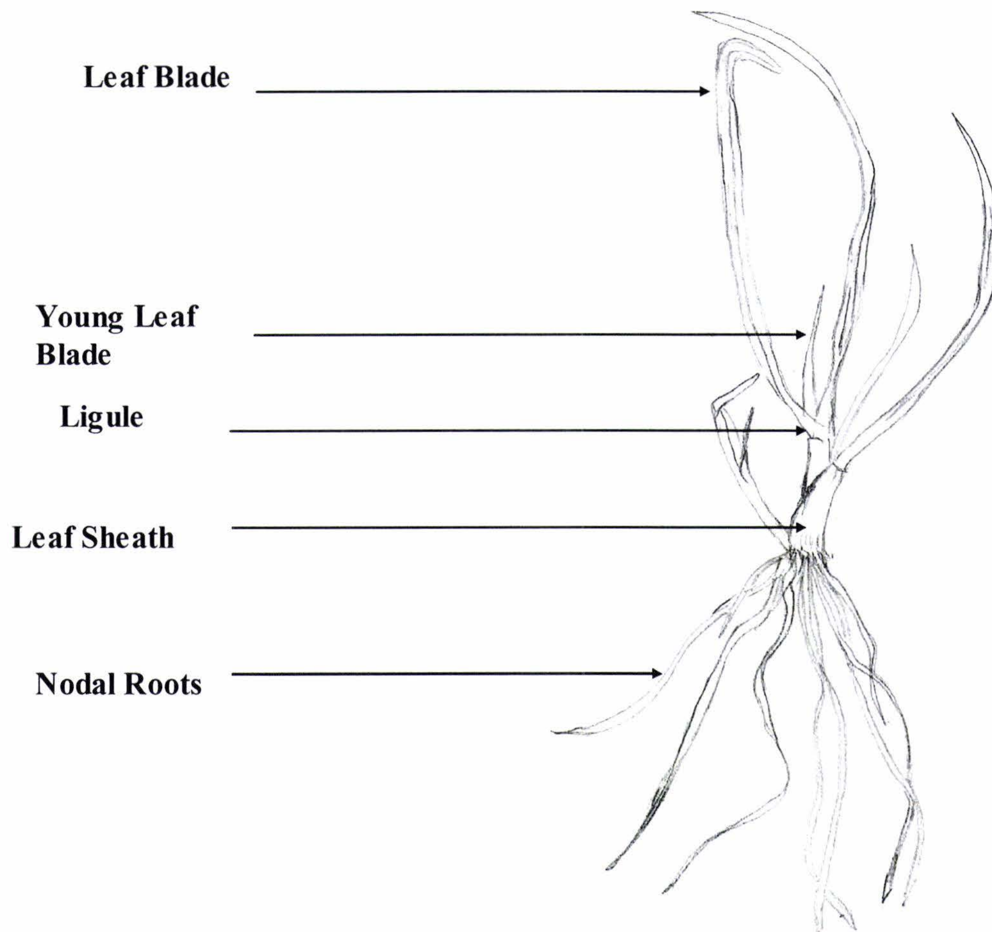


Fig 2.3 Morphological features of a typical *L. perenne* tiller.

2.3 Microscopy

All *L. perenne* plants were checked for the presence of endophyte using the aniline blue test. Leaf sheath epidermal strips, were placed on to a clean microscope slide and were covered in 0.05% (w/v) aniline blue solution. A cover slip was placed on top of the tissue. The preparation was fixed by heating using a methylated spirits burner. The slide was then checked for endophyte using a compound light microscope at 400x magnification (Carl Zeiss). The images were recorded with 400 ASA colour film (Kodak).

2.4 Experimental design

The experimental design used for the drought experiment was a randomized block design using 16 genotypes, with five treatments and five replicates, as shown in **Table 2.2.** Eight treatments were initially planned but this was subsequently modified to five, hence each replicate had 4 replicates of the water stress treatment.

Table 2.2 The selected *L. perenne* genotypes and their allocated replicates.

Reps	Treatments [c]							
	1	2	3	4	5	6	7	8
1[a]	[d] P H L D	P H L D	P H L D	P H L D	P H L D	P H L D	P H L D	P H L D
2	O G C K	O G C K	O G C K	O G C K	O G C K	O G C K	O G C K	O G C K
3	N F B J	N F B J	N F B J	N F B J	N F B J	N F B J	N F B J	N F B J
4	A E I M	A E I M	A E I M	A E I M	A E I M	A E I M	A E I M	A E I M
5[b]	L K D N	L K D N	L K D N	L K D N	L K D N	L K D N	L K D N	L K D N

[a] **Replicates 1** to **4** were grown in the AgResearch, Grasslands Research Centre, glasshouse.
[b] **Replicate 5** was grown in the AgHort growth chamber on the Massey University campus.
[c] **See Tables 2.3**, **2.4** and **2.5** for treatment details.
[d] **See Table 2.1** for the experimental and laboratory codes used to label the different genotypes.

Details of the treatments used are shown in **Tables 2.3**, **2.4** and **2.5**[1].

Table 2.3 Treatments for Replicate 5[a].

	Water Stress[b]	ABA[c]	JA[d]
Treatment 1	+	–	–
Treatment 2	–	+	–
Treatment 3	+	–	–
Treatment 4	–	–	–
Treatment 5	–	+	+
Treatment 6	+	–	–
Treatment 7	–	–	+
Treatment 8	+	–	–

[a] Replicate 5 was grown in the AgHort growth chamber on the Massey University Campus.
[b] The plants were progressively water stressed by adding back to the pots, every 2 days, one-third of the water
lost by evapotranspiration.
[c] See **Table 2.12** for the concentrations of ABA in which the plants were dipped (see also **Fig 2.7**).
[d] See **Table 2.13** for the concentrations of JA in which the plants were dipped (see also **Fig 2.7**).

Table 2.4 Treatments for Replicates 1 and 2[a].

	Water Stress[b]	ABA[c]	JA[d]
Treatment 1	+	–	–
Treatment 2	+	–	–
Treatment 3	+	–	–
Treatment 4	+	–	–
Treatment 5	–	+	+
Treatment 6	–	+	–
Treatment 7	–	–	+
Treatment 8	–	–	–

[a] Replicates 1 and 2 were grown in the AgResearch, Grasslands Research Centre, glasshouse.
[b] The plants were progressively water stressed by adding back to the pots, every 2 days, one-third of the water
lost by evapotranspiration.
[c] See **Table 2.12** for the concentrations of ABA in which the plants were dipped (see also **Fig 2.7**).
[d] See **Table 2.13** for the concentrations of JA in which the plants were dipped (see also **Fig 2.7).**

Table 2.5 Treatments for Replicates 3 and 4[a].

	Water Stress[b]	ABA[c]	JA[d]
Treatment 1	–	+	+
Treatment 2	–	+	–
Treatment 3	–	–	+
Treatment 4	–	–	–
Treatment 5	+	–	–
Treatment 6	+	–	–
Treatment 7	+	–	–
Treatment 8	+	–	–

[a] Replicates 3 and 4 grown in the AgResearch, Grasslands Research Centre, glasshouse.
[b] The plants were progressively water stressed by adding back to the pots, every 2 days, one-third of the water
lost by evapotranspiration.
[c] See **Table 2.12** for the concentrations of ABA in which the plants were dipped (see also **Fig 2.7**).
[d] See **Table 2.13** for the concentrations of JA in which the plants were dipped (see also **Fig 2.7**).

[1] In replicate 5 Treatments 1, 3, 6 and 8 are the same. In replicates 1 and 2 Treatments 1, 2, 3, and 4 are the same.
In replicates 3 and 4 Treatments 5, 6, 7, and 8 are the same.

2.5 Measurement of water loss

The pots were brought to field capacity and were allowed to drain for 2–3 h, to allow the water to drain away by gravity. The pots were then weighed using a Mettler Toledo SB32001 Delter Range balance that was linked into a laptop computer, which was used to download all the data using AgResearch in-house software. The weights of the pots were recorded every 2–4 days and the water lost by evapotranspiration was calculated using the initial field capacity base weight for each pot. Two-thirds of the water lost was replaced for the water stress treatments, whereas the water sufficient treatments were brought back to field capacity at each weighing.

2.6 Preparation of protonated abscisic acid (ABA) and cucurbic acid (CA)

2.6.1 Large scale cold production of protonated ABA and CA by sodium borohydride reduction

Sodium borohydride reduction, as described by (Miersch *et al.,* 1987), was used to produce both CA from jasmonic acid (JA) using (±)-jasmonic acid, 95% (Apex Organics Ltd, Cas No 6894-38-8), and protonated ABA from ABA using (±)-*cis*, *trans*-abscisic acid (Sigma, A1049) (*pers comm* Fielder, HortResearch, Palmerston North, New Zealand, 2000). These procedures were first trialed on a large scale and nuclear magnetic resonance (NMR) was used to verify the synthesis of the correct products. **Fig 2.4** shows the reduction reactions for ABA and JA.

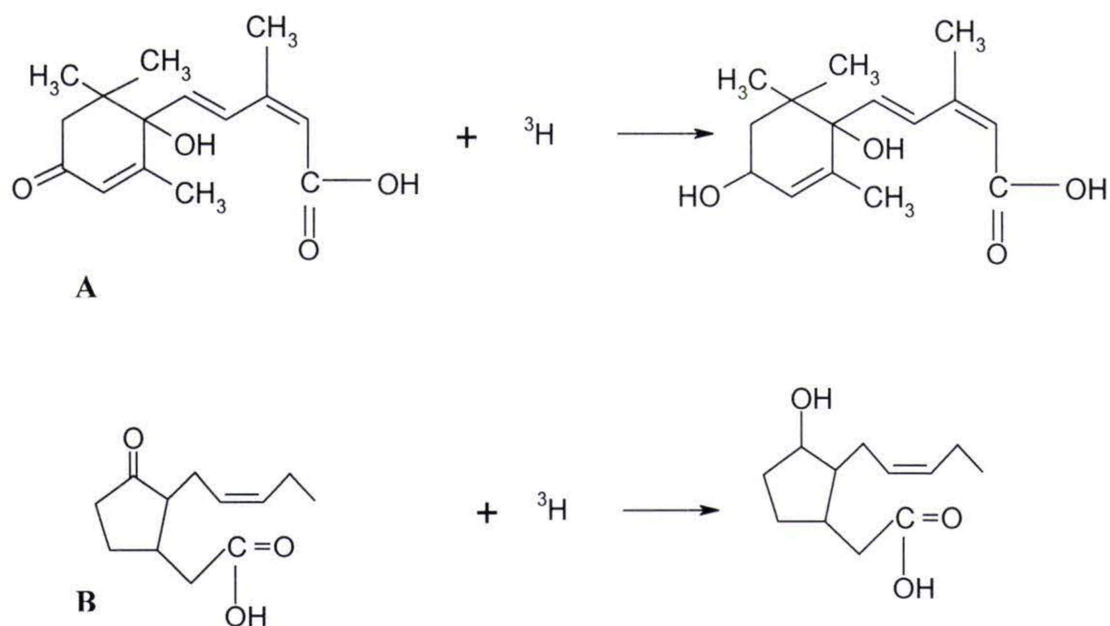


Fig 2.4 The protonation of ABA and JA.
(**A**) Reduction of ABA. The protonation occurs on C4 of the primary benzene ring of ABA.
(**B**) Reduction of JA to CA. The protonation occurs on C6 of the JA molecule.

The reduction of JA was carried out by dissolving 5 mg of JA in 1 mL of 0.2 M sodium bicarbonate followed by the addition of 10 mg of sodium borohydride. The mixture was incubated at room temperature for 30 min, and was then acidified in a fume hood by adding 4 M HCl until the pH was 3.0 using a cut-down 5 mL measuring cylinder, so that the laboratory pH meter electrode could fit inside. An equal volume of chloroform was then added to the mix, which was mixed and partitioned, and the aqueous phase was dried down on a speed vacuum set at 2 torr (2 mm Hg) for 20 min. The dried product was stored in a dessicator.

The same procedure was used to produce the protonated ABA, except that the 5 mg of ABA was dissolved in a small amount of absolute ethanol before adding the 0.2 M sodium bicarbonate. Samples of the reaction products were mixed in deuterated water and placed in glass straws. The success of the reduction reactions was verified by NMR using an AVANCE 400 MHz NMR spectrometer (Bruker).

2.6.2 Small scale cold production of protonated ABA and CA by sodium borohydride reduction

Because of the expense of tritiated sodium borohydride and the need to produce a product with a high specific activity, the reactant quantities in the Miersch et al.,(1997)

protocol were scaled down by about 60-fold so that 50 mCi (0.185 mg) of tritiated sodium borohydride (specific activity 292 mCi/mg) would be used for each reaction. That is, 0.09 mg of JA and 0.07 mg of ABA were used instead of the quantities specified in Section 2.6.1. See **Appendix 2** for the concentrations of the reactants. Dilutions were made to obtain accurate hormone (JA and the speed-vacuum-dried and ABA) and sodium borohydride weights for the cold production runs. For the cold production runs, the sodium borohydride or hormone was added to the sodium bicarbonate buffer. At this time the hormone was also dried down using N_2 gas, to avoided hormonal losses by volatilization. The reactants were combined quickly to allow the reaction to proceed, because the sodium borohydride reducing agent also reduced the buffer. The hormone was dissolved in as small a buffer volume as possible and dried down so that the hormonal residue was at the very bottom of the glass tube. The reactions were carried out in small glass tubes to avoid possible buffer binding. The dilutions for ABA, JA and sodium borohydride are described in **Appendix 2.** The samples were incubated for 30 min. Then using a micro pH meter (in house designed and built), the pH was adjusted to 3.0 ± 0.1 pH units with HCl. All acid additions were done using an A-RN MicroLitre syringe (S.G.E. Scientific Glass Pty. Ltd) with a microtube attached to the needle. Cutting off the used tubing ensured that no cross-contamination of acid could occur. The amount of acid used is recorded in **Appendix 2(d).**

2.7 Small scale hot production of tritiated ABA and CA by sodium borohydride reduction

The hot reaction was carried out on brown paper in a fume cupboard using 100 mCi of ^3H sodium borohydride ± 15%, Code TRK45, Batch 203 (Amersham Pharmacia Biotech). The borohydride had a specific activity of 422 GBq/mmol or 10.8 GBq/mg, 11.4 Ci/mmol or 292 mCi/mg. The by-product of the reaction – tritiated hydrogen – was lost in the fume hood and all plastic and glass tubes used were safely disposed. An aliquot (100 µL) of the product of the reduction, tritiated CA or ^3H ABA, was mixed with 1 mL of scintillation fluid and vortexed to stop partitioning of the solution, and the counts were read using a 1450 Liquid Scintillation and Luminescence Counter (Microbeta Trilux).

2.8 HPLC verification of the tritiated reaction products and their purification

The NMR cold results indicated that the sodium borohydride reduction had occurred but that the yield was low. However, the reaction products gave high counts. Tritiated water contributing to the high counts in the un-purified products had to be removed while retaining the tritiated plant hormones. This was done by high performance liquid chromatography (HPLC) separation, followed by a C18 column purification of the elutants.

2.8.1 HPLC verification of tritiated CA

A Cary I UV Visible Spectrophotometer connected to Cary I application software was used to obtain the ultraviolet (UV) absorption spectra of JA and CA (**Appendix 3(c)**). Then, JA and CA standards were run on a Waters 490E high performance liquid chromatograph with a programmable multi-wavelength detector, with the detector absorbance set at 269 nm. Data previously obtained by Gapper, (1998) was used as a guide as to where the compounds would elute. The composition of the eluting solvents for ABA and JA and their timed application to the column are given in **Appendices 3(a)** and **3(b)**.

2.8.2 C18 column purification of CA

The purification of CA was carried out using a Sep-Pak Plus C18 syringe-driven column (Alphatech Systems Ltd., WATO20515). The C18 column was attached to a 20 mL disposable syringe, pre-conditioned by eluting with 20 mL of 100% (v/v) ethanol and dried with two to three syringes volumes of air. Just before use, the column was flushed with 20 mL of MilliQ water and air dried. Then 100 μL of the CA eluted from the HPLC column was added to 20 mL of MilliQ water and applied to the C18 column. The column was neutralized by applying 20 mL of MilliQ water, flushed to drain and dried with two or three syringes volumes of air. The CA was slowly eluted off the C18 column with 3 mL of 80% (v/v) methanol. A 100 μL sample was added to 1 mL of scintillation fluid, vortexed, and the counts recorded. Finally, a large scale purification using a C18 column only was carried out and the purity of the CA verified.

2.8.3 C18 column purification of tritiated ABA

A C18 column was pre-conditioned by applying 10 mL of methyl alcohol, washing with 20 mL of MilliQ water and air drying. A 40 μL aliquot of tritiated ABA eluted from the HPLC column was then mixed with 200 μL of methyl alcohol and 2 mL of water, applied to the pre-conditioned C18 column and washed with two 10 mL aliquots of water. The tritiated ABA was eluted from the C18 column with three 1 mL aliquots of 80% (v/v) methyl alcohol. Samples of 100 μL of tritiated ABA were added to 1 mL of scintillation fluid, vortexed, and the counts determined on the scintillation counter.

Once the tritiated plant hormones had been prepared, it was then possible to test the efficiency of various solid state procedures for extracting ABA and JA because the tritiated ABA and CA (the JA reduction product) would act in a similar way to their stable parent materials. Thus, a measured input count of tritiated ABA and CA could be compared with the output counts to give a percentage recovery figure.

2.9 Solid state extraction of ABA and JA

2.9.1 Pre-conditioning the DEAE Sephadex 25 and loading the column

The Solid state extraction columns contained a pre-conditioned Sigma anion exchanger on dextran, DEAE Sephadex 25 (diethylaminoethyl Sephadex dry bead size 40–125 μm, with a capacity of 3–4 meq/g (A-25-120)).

To pre-condition the DEAE Sephadex 25 (20 g) was added to 200 mL of 40 mM ammonium acetate and soaked overnight at room temperature. The next day, a further 500 mL of ammonium acetate was added, and the mixture was brought to the boil and then stirred gently to cool to room temperature. This heating was repeated twice. After final cooling, the mixture was placed in a large separating funnel with a glass wool bung attached to a string for ease of removal. The ammonium acetate was decanted off and the DEAE Sephadex 25 was washed with 1 L of 1 M ammonium formate. This step was time consuming and could take some hours to complete. Then the DEAE Sephadex 25 was rinsed with ammonium acetate until the filtrate was at a pH of between 5 and 7. It was covered and stored at 4°C. After this treatment, it could be stored for up to 30 days.

Approximately 10 mL of the conditioned DEAE Sephadex 25 was loaded into a sterile 20 mL disposable syringe with a centre-located needle bayonet fixture, with two layers of filter paper cut to cover the outlet. The plunger was discarded and the syringe was covered with gladwrap until use. Just before use, enough ammonium acetate was passed through until the eluate was running at pH 6–7. Then 10 mL of 50% (v/v) methanol was passed through the syringe.

2.9.2 Pre-conditioning the C18 columns

C18 Sep-Pak columns from Alphatech Systems Ltd (WATO20515) were used to remove chlorophyll, carotenes and xanthophyll from the plant extracts prior to applying them to DEAE Sephadex columns and then to finally remove plant hormones, from the Sephadex columns.

2.9.2.1 To remove chlorophyll, carotenes and xanthophyll

The C18 column was washed with 20 mL of 100% (v/v) methanol and then dried with three to four syringe volumes of air. The C18 column could be stored for up to 1 month in this state.

For the activation step, just before use, 10 mL of MilliQ water was passed through the column, making sure that no air bubbles were trapped. This was followed by 10 mL of 80% (v/v) methanol. The C18 column was now ready to be attached to the syringe.

2.9.2.2 To remove plant hormones

The C18 column was washed with 20 mL of 100% (v/v) methanol and dried with three to four syringe volumes of air, as for the removal of chlorophyll and xanthophyll. For the activation step, just before use, 10 mL of MilliQ water followed by 10 mL of 6% (v/v) formic acid were passed through the column.

The general rule when pre-conditioning any column is that the last wash used should be the same as the eluate, e.g. pre-conditioning with 50% (v/v) methanol when 50% (v/v) methanol is to be used as the carrier for the extracted hormones.

See the summaries of these protocols in **Appendix 4**.

2.9.3 ABA and JA vacuum extraction protocols

Gravity feed systems were compared with vacuum bank extraction. The recoveries were comparable, with the vacuum bank system being considerably faster than the gravity feed systems. The vacuum bank system was used routinely.

2.10 ABA and JA extraction protocols

In the main experiment, the aim was to measure the levels of the plant hormones that were produced to protect the plant against drought, both directly as for ABA and indirectly as for JA, and then to correlate the tissue levels of these hormones with the endophyte levels of lolitrem B, ergovaline and peramine. To do this, leaf and pseudostem samples of *L. perenne* were solid state extracted in preparation for HPLC–mass spectrometry (ms) analysis.

2.10.1 Plant tissue preparation

All plant tissue was lyophilized (freeze dried) in the AgResearch dryer and was stored at –5°C. The plant tissue was ground using a coffee grinder, modified for small quantities. After a number of measurements, it was shown that, on average, 1 g of fresh weight of leaf tissue gave 0.2 g of lyophilized tissue; thus, in most subsequent extractions, 0.2 g of lyophilized tissue was used. See **Table 2.6.**

Table 2.6 The average dry weight of the 16 *L. perenne* genotypes.

Replicate	Wet weight	Dry[a] weight	% Dry weight
1	2.762	0.571	20.7
2	2.329	0.399	17.1
3	1.438	0.278	19.3
4	2.762	0.571	20.7
		Mean	**20.0**[b]

[a]Samples were harvested from the glasshouse using water sufficient plants that were to be used in the main experiment. The samples were collected 3 months after the experiment was set up and the information obtained was used to work out wet to dry weight conversions of perennial ryegrass tissue.
[b]This figure was also used as the dry weight figure for calculating the ABA and JA hormone dipping concentrations in the main experiment.

To 0.2 g of lyophilized tissue was added 10 mL of 80% (v/v) methanol and 10 000 cpm (counts per minute) of tritiated (10 µL of purified material) hormone, and this was extracted overnight at –5°C in the freezer. The following day, this was mixed on a slow shaker for 1 h at 4°C in a cold room and centrifuged at 10 000 rev/min (1593 *g*) for 30 min, and the supernatant was then drawn off immediately to ensure that no solid material had a chance to re-mix with the supernatant. The pellet was re-suspended in 5 mL of 80% (v/v) methanol, shaken for a further hour and centrifuged for 30 min, and the supernatant was immediately drawn off and combined with the first aliquot (approximately 10 mL + 5 mL).

2.10.2 Solid state extraction protocol

The samples were passed through a vacuum-bank-driven pre-conditioned C18 Sep-Pak column to remove the plant pigments chlorophyll, xanthophyll and carotene (**Fig 2.5**).

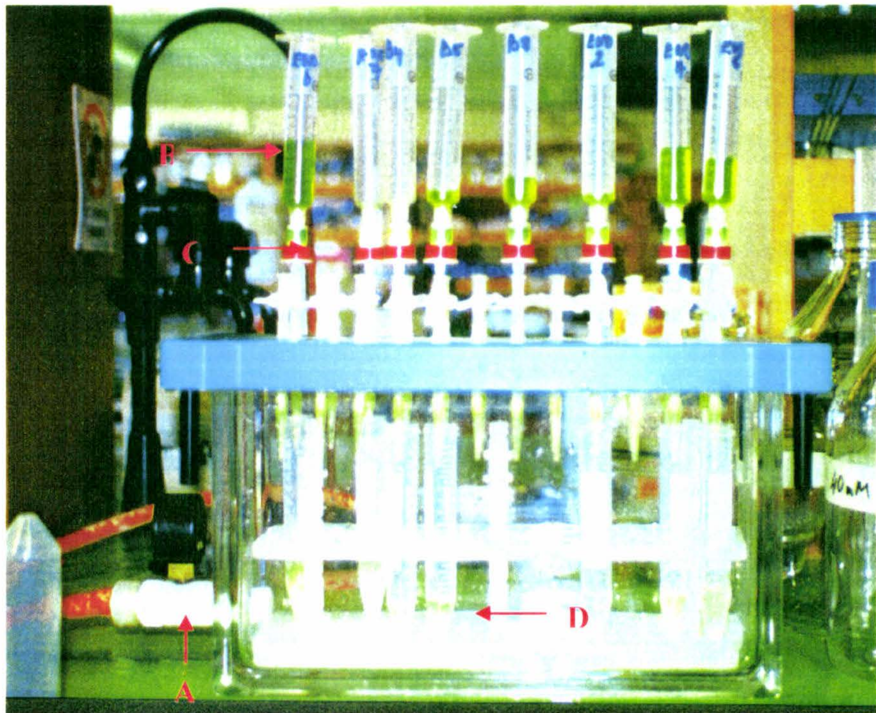


Fig 2.5 Equipment and process used to extract ABA and JA from perennial ryegrass tissue. Stage one: photosynthetic pigment extraction.
(**A**) Vacuum applied and regulated.
(**B**) Previously prepared centrifuged supernatant before being passed through the C18 column.
(**C**) C18 Sep-Pak columns to remove chlorophyll a and b, xanthophyll and carotene.
(**D**) The filtrate ready for stage two of the protocol.

(The C18 columns could be re-used by backwashing with acetone until a clear filtrate was obtained, air drying and pre-conditioning.)

The pigment-free filtrate was suspended in 80% (v/v) methanol, which was then brought to 50% (v/v) methanol by adding MilliQ water, loaded on to a pre-conditioned DEAE Sephadex 25 column (**Fig 2.6**) and the flow rate was adjusted to around 30 drops/min.

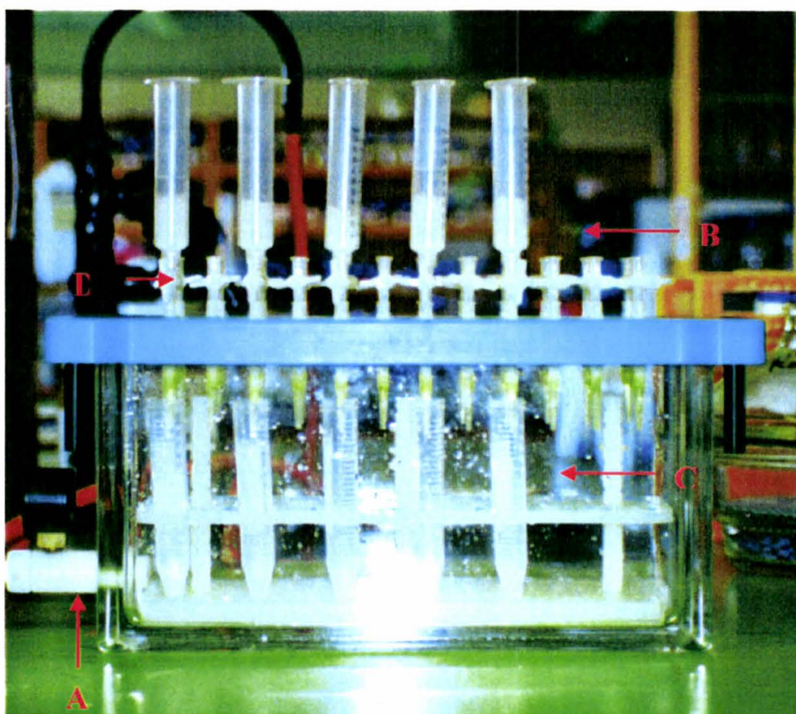


Fig 2.6 Equipment and process used in stage two of the solid state hormone extraction process.
(**A**) Vacuum applied.
(**B**) Prepared 20 mL syringe, DEAE Sephadex ion exchange column for extraction of the plant hormones.
(**C**) The filtrate that was run to waste.
(**D**) Flow rate adjustment valve used to regulate the flow to 30 drops/min.

The column was washed with 20 mL of 50% (v/v) methanol and neutralized by eluting with 20 mL of 40 mM ammonium acetate at pH 6.5. At this point, the plant hormones were on a DEAE Sephadex 25 column and most of the impurities had been flushed to waste. A C18 column pre-conditioned for hormone removal from a DEAE column was then attached and the DEAE column was eluted with 20 mL of 6% (v/v) formic acid to remove the plant hormones from the DEAE column and on to the C18 column. The C18 column was removed and attached to a 20 mL disposable syringe, neutralized by flushing with 20 mL of MilliQ water and dried by syringing air through it. The plant-hormone-loaded C18 column was eluted slowly with 5 mL of 80% (v/v) methanol into a 14 mL Nunc tube, which was labeled, capped and stored at 5°C.

A summary of the procedure can be found in **Appendix 5.**

2.10.3 Calculation of the percentage recovery and sample storage

The percentage recovery was checked by vortexing the sample, removing 100 μL of sample, adding 1 mL of scintillation fluid and reading on a scintillation counter[1].
The remaining samples were speed-vacuumed down until around 100 μL of an oily brown fluid remained and then stored at –18°C.

2.11 Endogenous levels of ABA and JA found in *L. perenne* as measured by ELISA when these hormones were applied by dipping and after the imposition of a prolonged water stress

Before the start of the main experiment, it was necessary to check that ABA and JA would be absorbed from the dipping solutions into *L. perenne* tissue, to see if the endogenous levels of these hormones did actually increase, and to determine the level of that increase as water stress was applied. This was necessary so that the daily dipping concentrations used on the water-sufficient plants in the main experiment were as close as possible to those that actually occurred in *L. perenne* under drought conditions. Water stressing based on a daily two-third water sufficiency (**Section 2.5**) was carried out using all the *L. perenne* genotypes to be used in the main experiment (G1060–G1075). Plant samples were collected for analysis every second day.

The dipping experiments were carried out using replicates of the endophyte-free *L. perenne* genotype (G1076). After the experiments were completed, material was prepared for ABA and JA competitive ELISA by taking 0.2 g of plant material, the equivalent of 1 g of fresh weight, preparing the samples (**Section 2.10.1**), spiking them with tritiated CA, running them through a solid state extraction process (**Section 2.10.2**) and calculating the SAFs (Sample Adjustment Factors). The purified plant extracts adjusted for column losses were then measured using an in-house competitive ELISA for JA (see **Sections 2.11.1, 2, 3,** and **4**) and a commercial Phytodetek™ kit (Agdia) for ABA.

[1] The final hormone loaded C18 column was eluted with 5 separate 1 mL quantities of 80% (v/v) methanol and 100 μL aliquots taken from each for scintillation counting. The calculation of expected counts when a 100 μL aliquot was used was 1 mL/100 μL = 10X. Therefore, multiply all cpm obtained by 10. e.g. 359 cpm x 10 = 3590 cpm. A judgment call was made, but generally the sum of the first 3 readings were used to calculated the recovered counts, the final two sample counts, if very low were assumed to be background counts. Percent recovery was calculated by dividing recovered counts by input counts.

2.11.1 ABA and JA concentrations used to test the absorption rates of these hormones when applied by dipping

To identify the hormone levels to be used, discussions with other researchers were held and a literature search was carried out (**Tables 2.7** and **2.8**).

Table 2.7 A summary of ABA treatments used by various researchers under a variety of different conditions.

ABA Concentration	Fresh/Dry Weight	Water stressed	Mono/ Dicot	Externally applied or measured endogenous level	References	Comments
5-30 ng/g	FW	No	Mono	Endogenous	P.77. (Abernethy, 1996)	
Up to 200 ng/g	FW	Yes	Mono	Endogenous	P.77. (Abernethy, 1996).	ABA concentration in the laminae related to the SWC increase that had occurred on average 12 days after drought was applied (trigger point). When water was added, ABA levels fell rapidly.
25 µg/g	DW	Yes	Dicot	Endogenous	(Creelman *et al.*, 1995).	Soybean leaves dehydrated for 2 h gave 25 µg/g. Maintained at 20 µg/g for 8 h.
100 µM	FW	No	Dicot	Applied	(Creelman *et al.*, 1995).	Reduced transpiration from water stressed plants.
25-600 ng/g	DW	Yes	Mono	Endogenous	(Meyer *et al.*, 1989)	After 24 h 271 ng/g water stressed ; after 48h 604 ng/g.
23 ng/g	FW	No	Mono	Endogenous	(Creelman & Mullet, 1995).	
90 ng/g	FW	No	Dicot		(Creelman & Mullet, 1992).	Soya beans.
10 mg/g	FW	No	Dicot	Applied	(*pers comm* Hussain, Nottingham University UK 2000).	Plants sprayed in growth room to reduce evapotranspiration.
100 µM						

	FW	No	Dicot	Applied	(Pena-Cortes & Willmitzer, 1993).	Sprayed potatoes to switch on the transcription of Pin2 mRNA. Leaves not sprayed also had Pin2 switched on.
3.7 ng/g	FW	No	Mono	Endogenous	(Dathe *et al.*, 1994).	Wheat leaves of 5 day old seedlings. Leaf coleoptiles had 1.4 ng/g.
6.9 ng/g	FW	No	Mono	Endogenous	(Dathe, *et al.*, 1994).	Leaves of 7 day old seedlings. Leaf coleoptiles stayed at the 5 day level.
90 μM	FW	No	Mono	Endogenous	(Voros *et al.*, 1998).	Floated barley leaves on a solution of 1 M sorbitol as a method of inducing water stress.

Table 2.8 A summary of the JA dipping concentrations used by various researchers under a variety of different conditions.

JA Concentration	Fresh/Dry Weight	Water stressed	Mono/ Dicot	Externally applied or measured endogenous level	References	Comments
26 ng/g	FW	No	Dicot	Endogenous	(Creelman & Mullet, 1995).	Levels fell lower.
175 ng/g	FW	Yes	Dicot	Endogenous	(Creelman & Mullet, 1995).	Level rose in 2 h, peaking with the ABA then fell to 55 ng/g within 4 h (spiking)
2.6 ng/g	FW	No	Dicot	Endogenous	(Sibylle *et al* 1998).	Found in poplars plus 1.3 ng/g of methyl-jasmonate.
90 ng/g	FW	No	Dicot	Endogenous	(Creelman *et al.*, 1992).	Similar levels to ABA.
450 ng/g	FW	No	Dicot	Endogenous	(Creelman *et al.*, 1992).	Peaked at 8 h after wounding and held at 400 ng/g for up to 24 h.
10-220 pmol/g	FW	No	Dicot	Endogenous	(Clarke *et al.*, 1998)	
1.5 μM	FW	No	Dicot	Applied	(Thaler, 1999).	The amount applied per 8 week old tomato plant.
250 ppm 2.5 nM 25 nM	FW	No	Dicot	Applied	(Clarke *et al.*, 1998).	Wick feeding concentrations. 2.5 nM, and 25 nM and 250 ppm all suppressed viral multiplication.
1012 ng/g	FW	No	Mono	Endogenous	(Parry *et al* 1994).	5 day wheat seedlings.
588 ng/g	FW	No	Mono	Endogenous	(Parry *et al* 1994).	7 day wheat seedlings.
45 μM	FW	No	Mono	Endogenous	(Voros *et al.*, 1998).	Leaf barley sections floated on solutions of methyl-jasmonate or sorbitol to induce water stress.

ABA/JA concentration levels were used (**Table 2.9**) to investigate leaf absorption of ABA and JA by *L. perenne*. Random leaf samples for ELISA analysis were taken after 1 h of dipping and every hour thereafter for up to 3 h.

Table 2.9 Concentrations of ABA and JA used in the dipping solutions, when *L. perenne* was dipped to test for leaf hormone absorption.

Treatment [a]	Concentration of ABA (mg/L)	Concentration of JA (mg/L)
A	20	10
B	10	5
C	5	2.25
D	2.5	1.25

[a] All treated plants were endophyte free (G1076).

2.11.2 JA ELISA analysis of *L. perenne* after dipping and the imposition of a prolonged drought

An un-purified antiserum was obtained from Dr Sean Clarke of Otago University's Plant Biology Department. It was prepared by inoculating a New Zealand white rabbit with JA conjugated to the immunogen keyhole limpet haemocyanin (KLH-JA). The un-purified Ovalbumin-JA was stored in 50% glycerol (v/v) at 4°C (Clarke, 1996; Gapper, 1998).

2.11.3 Purification of Ovalbumin-JA (OVA-JA) antiserum

One millilitre of blood serum was made up to 10 mL with MilliQ water and stirred. The protein was precipitated by drop wise addition of 10 mL of saturated ammonium sulphate and spun down at 3000 *g* for 25 min. The pellet was re-suspended in half-strength PBS
(phosphate-buffered saline see **Appendix 6**); then the re-suspended protein was dialysed in half-strength PBS for 48 h at 4°C, stirring slowly and replacing the half-strength PBS every 10 h. The protein conjugate was concentrated by washing through a Pall Filtron column with half-strength PBS. Between five and seven 2 mL fractions were collected and the concentration was determined optically, with a Cary I UV Visible Spectrophotometer connected to Cary I application software, using quartz cuvettes at 280 nm (OD 1.4 at 280 nm = 1 mg/mL or a proportion thereof). Purified

antiserum was stabilized by adding 1 mg of sodium azide and 2.5 mg of bovine serum albumin (BSA). A summary of this protocol is given in **Appendix 7**.

2.11.4 JA competitive ELISA

The purified OVA-JA was diluted with coating buffer (ELISA buffers, **Appendix 8**) at 1:1000. Then 150 μL was added to each ELISA well, incubated for 24 h at 4°C and squirt bottle washed five times with PBST (PBS Tween20) buffer. Then 50 μL samples, 0.001 to 100 nM of JA, diluted with ELISA buffer were added to the wells (production of a standard curve). Other wells contained solid state plant extract (**Section 2.10.1**) diluted with ELISA buffer. Fifty microliters of anti-JA IgG (immunoglobulin G) diluted 1:500 with ELISA buffer was then added to all wells and the plates were incubated at room temperature for 18 h. Following the second incubation, the plates were washed five times with PBST buffer and 100 μL of anti-rabbit IgG alkaline phosphatase conjugate, diluted 1:2000 with ELISA buffer, was added to each well and incubated at 37°C for 1 h. Following the third incubation, the plates were again washed and 100 μL of 0.5 mg/mL of freshly made nitrophenol phosphate in substrate buffer was added to each well, and the plates were given a final incubation at room temperature for 1 h to allow colour development. The absorbance of the wells was measured using an ELISA microplate reader at 405 nm. A boxed summary of this protocol is given in **Appendix 8**.

2.11.5 ABA competitive ELISA

The ABA competitive ELISA was done using a commercial Phytodetek™ kit (Agdia; catalogue number PDK 09347/0096) and was carried out as per the instructions that accompany the kits. Four attempts at obtaining a reliable ABA standard curve using their materials were made, (3 of these attempts were made with independent supervision). However we were unable to establish the reasons for the variations between the kits in spite of extensive communications with the production manager of Agdia.

The composite standard curve for ABA was derived by using the combined results from Phytodetek Kit 2 and Kit 3. After four attempts, it was not possible to obtain a

satisfactory standard curve from each kit on its own. To obtain some useful data, the decision was made to combine the best standard curve data from Kits 2 and 3, then to average the OD results from each kit and to read the data obtained against the composite standard curve.

2.12 The main experiment

The objective of this experiment was to measure the alkaloids, lolitrem B, ergovaline and peramine, produced by the endophytic fungi *N. lolii*, in its host *L. perenne*, when water-sufficient *L. perenne* was dipped daily in increasing concentrations of the drought-protecting hormones ABA and JA.

2.12.1 Experimental

The dipping method was the same as that used for the preliminary experiment. The plants were up-ended on pipe racks and their leaves were immersed in various hormone solutions for no more than 1 h (**Fig 2.7**). The concentrations of the ABA and JA hormone solutions are given in **Tables 2.12** and **2.13.**

Fig 2.7 The hormone dipping procedure. The pots of plants were dipped by up-ending the test pots (**A**) on a galvanized pipe rack (**B**) so that the leaves were immersed in the dipping solution (**C**).

The five replicates, the treatments and the genotypes used are given in **Tables 2.3**, **2.4** and **2.5**.

2.12.2 Using the tiller fresh weight to estimate the dipping solution concentrations

Genotype tiller numbers in each replicate had been counted soon after they were transplanted into the 20 cm high black polythene pots. They were recounted prior to the start of the main experiment and their numbers were recorded (see **Table 2.10**). The pots of four *L. perenne* genotypes were then cut back 30 days prior to the start of the main experiment to just above the ligule so that analysis of relatively fresh leaf and sheath tissue would occur during the experiment thereby minimizing any carry over effects from previous growing regimes.

Regular samples were taken during the course of the experiment to update the total fresh weight content of the pots to be dipped, and the concentration of hormone in the dipping solutions was adjusted upwards accordingly to reflect the endogenous sap concentration.

2.12.3 Dipping concentrations deduced from ELISA results

Approximately 0.2 g of lyophilized leaf tissue (1 g of fresh weight (FW), **Table 2.6**) was taken and a solid state (Sephadex/C18) extraction was carried out after spiking with tritiated CA (**Section 2.10.2**). The extract was speed vacuumed down to give a small pellet, which was re-suspended in 1 mL of ELISA buffer. (The hormones in 1 g of FW were now in 1 mL of buffer.) A 100 μL (0.1 mL) aliquot of this solution was added to each ELISA well. As concentration values were given in pM/100 μL on the ELISA standard curves, the values were multiplied by 10 to give pM/mL. The concentration per millilitre was also the concentration per gram of FW of tissue. The FW per pot was estimated by taking tiller leaf samples and counting the number of tillers per pot. **Table 2.10** shows the tiller numbers of the various perennial ryegrass genotypes in August 2000. A recount of the tiller numbers was made for the purposes of FW estimations prior to the start of the main experiment (See **Table 2.11**). From this data can be concluded that there was large differences in tiller production between the various genotypes so in the main experiment certain genotypes such as K would contribute a large number of tillers while genotype I would contribute few tillers to the sample total.

Table 2.10 Tiller numbers in each replicate[a]

Tiller numbers Replicate 1

Rep / Treatment	Genotype L	Tiller P	Numbers D	H	Total	
1/1	18	25	21	23	**87**	
1/2	17	31	21	14	83	
1/3	17	18	29	22	86	
1/4	15	21	18	19	73	
/5	16	16	30	16	78	
1/6	13	23	19	43	98	
1/7	12	19	30	22	83	
1/8	12	27	19	31	89	
Total	120	180	187	190	**84**	**Mean**
Mean	**15**	**23**	**23**	**24**	7	**Stdv**
Stdv	**2**	**5**	**5**	**9**		

Tiller numbers Replicate 2

Rep/ Treatment	Genotype O	Tiller K	Numbers G	C	Total	
2/1	15	43	19	15	92	
2/2	25	33	32	19	109	
2/3	12	31	13	15	71	
2/4	14	47	14	14	89	
2/5	13	47	7	13	80	
2/6	23	44	13	16	96	
2/7	13	41	6	38	98	
2/8	25	36	16	32	109	
Total	140	322	120	162	**91**	**Mean**
Mean	**17.5**	**40.25**	**15**	**20.3**	**12**	**Stdv**
Stdv	**6**	**6**	**8**	**9**		

Tiller numbers Replicate 3

Rep/ Treatment	Genotype N	Tiller J	Numbers F	B	Total	
3/1	20	17	19	11	**67**	
3/2	26	31	16	18	**91**	
3/3	11	14	19	14	**58**	
3/4	9	14	20	15	**58**	
3/5	16	22	19	10	**67**	
3/6	12	12	18	19	**61**	
3/7	9	13	29	23	**74**	
3/8	20	27	42	16	**105**	
Total	123	150	182	126	**73**	**Mean**
Mean	**15**	**19**	**23**	**16**	**17**	**Stdv**
Stdv	**6**	**7**	**9**	**4**		

Tiller numbers Replicate 4

Rep/ Treatment	Genotype E	Tiller M	Numbers A	I	Total	
4/1	18	26	17	8	**69**	
4/2	29	35	29	7	**100**	
4/3	15	32	15	19	**81**	
4/4	6	21	15	12	**54**	
4/5	9	37	15	6	**67**	
4/6	14	30	21	21	**86**	
4/7	15	15	24	14	**68**	
4/8	13	27	11	17	**68**	
Total	119	223	147	104	**74**	**Mean**
Mean	**15**	**28**	**18**	**13**	**14**	**Stdv**
Stdv	**7**	**7**	**6**	**6**		

[a]*Lolium* genotypes were planted, four to a pot on 20/4/2000, cut back on the 1/6/2000, and the 21/7/2000 and the tiller count made one month latter. A further tiller count was made just prior to the start of the main experiment. See **Table 2.11** and **2.13.**

Another tiller count was carried out prior to start of the main experiment in January 2001. This data is shown in **Table 2.11a**. The tillers were counted for the replicate pots that were to undergo hormone dipping. No counts were made for genotypes in pots that were not to be dipped in ABA, JA or ABA, JA mixtures.

Table 2.11a Tiller numbers with averages for each genotype and treatment[a].

Rep/ Treatment number[b]	ABA Treatment[c]	JA Treatment[c]	P	H	L	D		Genotypes
1\5	ABA+	JA+	50	50	70	26	196	
1\6	ABA+	JA-	44	45	36	18	143	
1\7	ABA-	JA+	45	41	35	21	142	Average
							160	tillers/pot
			O	G	C	K		Genotypes
2\5	ABA+	JA+	29	49	22	50	150	
2\6	ABA+	JA-	35	32	22	77	166	
2\7	ABA-	JA+	24	38	74	54	190	Average
							169	tillers/pot
			N	F	B	J		Genotypes
3\1	ABA+	JA+	24	28	19	23	94	
3\2	ABA+	JA-	31	34	19	29	113	
3\3	ABA-	JA+	30	16	44	22	112	Average
							106	tillers/pot
			E	A	I	M		Genotypes
4\1	ABA+	JA+	22	57	15	38	132	
4\2	ABA+	JA-	22	60	16	40	138	
4\3	ABA-	JA+	21	55	17	42	135	Average
							135	tillers/pot

[a]Tiller numbers were counted again before the start of the main experiment and their numbers averaged (shown in red) for the pots that were to undergo hormone dipping.

[b]Replicate 5 tiller and FW data was not used to calculate the dipping solution concentrations.

[c] For the hormone concentrations used see **Tables 2.12** and **2.13**.

Eight estimations of FW increases were carried out during the course of the experiment see **Table 2.11b.** The increase in FW was used to calculate the plant hormone dipping concentrations (See **Table 2.12).**

Table 2.11b Fresh weight increases in hormone treated pots over the experiment

	19/1[a] FW Day 9 11 tillers	Dipping Fresh Weight (g)	22/1 FW Day 11 7 tillers	Dipping Fresh Weight (g)	24/1 FW Day 13 7 tillers	Dipping Fresh Weight (g)	26/1 FW Day 15 7 tillers	Dipping Fresh Weight (g)	28/1 FW Day 17 7 tillers	Dipping Fresh Weight (g)	30/1 FW Day 19 7 tillers	Dipping Fresh Weight (g)	1/2 FW Day 21 7 tillers	Dipping Fresh Weight (g)	3/3 FW Day 23 7 tillers	Dipping Fresh Weight (g)
1[b]	0.6928	10[c]	0.4769	11	0.4675	11	0.7261	17	0.527	12	0.8516	19	0.8844	20	0.8812	20
2	0.5298	8	0.5580	9	0.4949	11	0.5594	13	0.5747	14	0.535	13	0.6282	15	0.6054	15
3	0.9052	8	0.5569	8	1.0102	23	0.8848	20	0.8191	12	0.7193	11	1.0548	16	1.0016	15
4	0.6458	8	0.5264	10	0.8444	19	0.7664	18	0.7819	15	0.657	13	1.077	21	1.0492	20
		9[d]		10		16		17		13		14		18		18

[a]The number off tillers harvested and the dates they were harvested are shown starting from the 19/1/2001.
[b]Gives the replicate number corresponding to the average tillers per pot as highlighted in red in **Table 2.11a.**
[c]The FW of the tillers sampled was used to calculate the FW of the average tillers per pot for each replicate.
[d]The mean FW of the four hormone treated replicates was calculated and highlighted in red. This figure was used to calculate hormone dipping concentrations. See **Table 2.12**.

The ELISA concentration per gram of FW was multiplied by 0.8 of the pot FW to be dipped (the leaves were 20% dry weight and 80% water). The plants were to be immersed in specific hormone solutions so the assumption was made that diffusion would occur until the plant sap concentration equaled that of the bathing solution. This gave the concentration of plant sap that was needed to produce the ABA/JA concentration found at that water-deficient level. The actual amount (mg) of ABA and JA needed per litre of dipping solution was then calculated by proportion (see **Tables 2.11** and **2.12** for ABA and **Tables 2.11** and **2.13** for JA).

Table 2.12 ABA dipping concentrations for the dipping days shown, using Tables 2.11a and 2.11b. (Excel generated)

Date	Predicted ABA picomoles/g FW ($x10^3$) [a]	picomoles / mL for the mean treatment FW ($x10^4$) [b]	picomoles/1500mL of dipping solution. ($x10^6$)[c]	mg ABA/1500 mL of dipping solution[d]
19/1	0.7	0.5	0.8	0.22
22/1	2.5	1.9	3.0	0.78
24/1	3.8	4.8	4.5	1.17
26/1	4.3	5.7	5.1	1.4
28/1	5.0	5.3	6.0	1.6
30/1	5.5	6.1	6.6	1.7
1/2	6.0	8.7	7.2	1.9
3/2	7.5	10.5	9.0	2.4

[a]Estimated from the trend line in **Fig 3.12** then reduced by 30%.
[b] 0.8 of the predicted ABA concentration. 80% water, 20% dry matter $7.0x10^3$ x Dipping leaf fresh weight average 9g (**Table 2.11b**) = $0.5x10^4$ /mL (1g approximately = 1mL)
[c](1500/Dipping leaf fresh weight average 9g) x $0.5x10^4$ picomoles/g FW = $0.8x10^6$ picomoles/1500mL of dipping solution.
[d]picomoles/1500 mL $0.8x10^6$ x 2.65 x 10^{-10} g = 0.22 mg (1 picomole ABA = 2.65 X 10^{-10} g)
[e]Replicate 5, tiller and FW data was not used to calculate the dipping solution concentrations.

The ABA dipping concentrations used in the main experiment are highlighted in red.

Table 2.13 JA dipping concentrations for the dipping days shown, using Tables 2.11a and 2.11b. (Excel generated)

Date	Predicted JA picomoles/g FW ($x10^3$) [a]	picomoles/ mL for the mean treatment FW ($x10^4$) [b]	picomoles/1500mL of dipping solution. ($x10^6$)[c]	mg JA/1500 mL of dipping solution[d]
19/1	0.7	0.5	0.84	0.18
22/1	0.7	0.5	0.84	0.18
24/1	1.0	1.4	1.3	0.27
26/1	1.25	1.7	1.5	0.32
28/1	1.35	1.4	1.6	0.34
30/1	1.4	1.7	1.7	0.35
1/2	1.43	2.1	1.71	0.36
3/2	1.45	2.2	1.74	0.37

[a] Estimated from the trend line in **Fig 3.16** then reduced by 30%.
[b] 0.8 of the predicted JA concentration. 80% water, 20% dry matter $7.0x10^3$ x Dipping leaf fresh weight average 9g (**Table 2.11b**) = $0.5x10^4$ /mL (1g approximately = 1mL)
[c](1500/Dipping leaf fresh weight average 9g) x $0.5x10^4$ picomoles/g FW = $0.8x10^6$ picomoles/1500mL of dipping solution.
[d] pM/1500 mL $0.8x10^6$ x 2.1 x 10^{-10} g = 0.22 mg (1 picomoles JA = 2.1 x 10^{-10} g).

The JA dipping concentrations used in the main experiment are highlighted in red.

2.12.4 Protocol used to independently check the water stress status of the *L. perenne* genotypes as the water stress was imposed in the main experiment

The fresh tissue samples were taken between 11.00 am and 2.00 pm, so that stable and repeatable measurements could be obtained. A complete replicate was sampled on any day, and extremely bright or extremely overcast days were avoided. Samples from plants with free water on the leaves were not taken. From 10 to 20 leaves were taken and immediately stored in a small zip-locked pre-weighed bag to avoid water loss and placed into an ice bucket. Every attempt was made to standardize the time between cutting and weighing. The FW of the leaves was obtained within 30 min of harvest (to 0.1 mg accuracy). The samples were then submerged in a beaker of distilled water and the beaker placed in a refrigerator at 5°C for 24 h. The next day, the leaf tissue was

removed, dried with a paper towel, allowed to air dry on the bench for 2–3 min and then re-weighed (turgid weight, TW). The leaves were then dried for 24 h at 80°C (dry weight, DW). The relative water content (RWC) was calculated as FW – DW/TW – DW. Water-sufficient plants should have an RWC of 0.95 and water-stressed plants will have an RWC of 0.70–0.7 (Barker & Hume, 2000; Turner, 1978).
Six RWC tests were performed for both the glasshouse trial and the growth chamber trial during the course of the main experiment to confirm the drought status of the test plants (see **Fig 3.22**).

2.12.5 Watering regimes used on all trial potted plants

The four pots in every replicate that were maintained in a water-sufficient condition were watered to field capacity every 2 days by watering until excess water poured out of the drainage holes. They were allowed to drain to field capacity and were then weighed. A mean value of the field capacity weight of the four water-sufficient pots in each replicate was calculated and was used as the benchmark against which the four water-stressed pots in each replicate were compared. The four pots in every replicate that were progressively water stressed were placed on galvanized drainage trays so that water tracking through the media could be observed. They were weighed every 2 days and their mean weight was calculated. These water-stressed pot weights and the mean field capacity weights were used to calculate the water deficit. Two-thirds of this water deficit figure was re-applied to the water-stressed pots.
At the completion of the experiment, all the tillers were harvested and divided into leaf (above the ligule) and sheath (below the ligule) samples. This material was placed into identifiable sealable plastic bags and immediately (< 1 min) stored on ice. They were subsequently transferred to a –18°C freezer. The tissue was then lyophilized, ground in a modified coffee grinder, given a bar code sticker for identification and tracking purposes and then returned to –18°C freezer storage.
Four samples were needed from each treatment: one for solid state extraction after spiking with tritiated CA (0.2 g) for ABA and JA HPLC analysis, and three for lolitrem B, ergovaline and peramine HPLC analysis.

2.12.6 Sample preparation for alkaloid HPLC analysis

Lolitrem B was analysed by a modification (Panaccione *et al.,*, 2003) of the method of (Gallagher *et al.,*, 1985). Perennial ryegrass leaf sheath tissue (50 mg; dry weight) was extracted with 1 ml of dichloroethane:methanol (9:1). The sample was agitated in a Savant FastPrep FP 120 (BIO 101 Inc., La Jolla, CA) cell disrupter for 20 s at speed 5, mixed by rotation for 1 h, and then centrifuged for 10 min at 3000 g. Samples (8-20 μl) were analysed by normal phase HPLC (Shimadzu LC-10A system) at 28°C using an Alltima (Alltech Associates, Deerfield, Il) silica gel (150 x 4.6 mm, 5 μm bead diameter) column with a mobile phase of dichloromethane:acetonitrile:water (880:120:1) at a flow rate of 1 mL/min. Eluted products were analysed by spectrophotofluorometry (excitation 265 nm, emission 440 nm) using a RF-10A detector (Shimadzu, Kyoto, Japan). Lolitrem B elutes at ~5 min followed by smaller amounts of other lolitrems. The amount of lolitrem B was estimated by comparison of the integrated peak areas (Class-LC10 software; Shimadzu) of the analyte with an external standard of authentic lolitrem B.

Ergovaline and peramine were analysed by minor modifications of previously described methods (Panaccione *et al.,*, 2003; Spiering *et al.,*, 2002). Perennial ryegrass pseudostem tissue (50 mg; dry weight) was extracted with 1 ml of isopropanol-lactic acid (50% (v/v) propan-2-ol, 1% (w/v) lactic acid) containing internal standards of ergotamine-hemitartrate (1 μg/ml; Sigma Chemical Corp., St Louis, MO) and homoperamine nitrate (2 μg/ml; custom synthesis). The sample was agitated in a Savant FastPrep FP 120 (BIO 101 Inc., La Jolla, CA) cell disrupter for 20 s at speed 5, and then mixed by rotation for 1 h. The extract was incubated at 4°C overnight then centrifuged for 10 min at 3000 g. Samples (8-20 μl) were analysed for ergovaline by reverse phase HPLC at 28°C using a Prodigy ODS3 (Phenomenex, Torrance, CA) C-18 (150 x 4.6 mm, 5 μm) column, fitted with an RP-18 Brownlee Newguard precolumn (Perkin-Elmer Analytical Instruments, Norwalk, CT). The mobile phase, at a flow rate of 1 ml/min, was a multi-linear binary gradient consisting of solvent A (acetonitrile:aqueous 0.1 M ammonium acetate; 1:3 v/v) and solvent B (acetonitrile:aqueous 0.1 M ammonium acetate; 3:1 v/v) in ratios of A:B at programmed time points as follows: 0 min, 95:5; 20 min, 80:20; 35 min, 50:50; 40 min, 30:70; 45 min, 30:70; 47 min, 0:100; followed by a recycling step for 55-58 min, 95:5. Eluted products were analysed by spectrophotofluorometry (excitation 310 nm, emission 410 nm) using a RF551 detector (Shimadzu, Kyoto, Japan). The amounts of ergovaline, together with its natural isomer

ergovalinine, were estimated by comparison of the integrated peak areas (Class-LC10 software; Shimadzu, Kyoto, Japan) with those of the sum of the ergotamine, and its natural isomer ergotaminine, adjusted for relative fluorescences and extraction efficiency.

Chromatography of peramine was carried out by a column-switching procedure with an initial step to remove interfering UV absorbing compounds (Cox and Stout, 1987; Spiering *et al.*,, 2002). Samples (50 μl) were loaded onto a RP-C8 (Alltech Associates, Deefield, IL) silica-based mixed-mode cation exchange cartridge (7.5 x 4.6 mm, 5 μm) in a mobile phase of propan-2-ol:water:ammonium hydroxide (25%) (60:40:1) at a flow rate of 0.4 mL/min for 2 min. The cartridge was then flushed at 1 ml/min with 50 mM ammonium acetate, 5 mM guanidinium carbonate and 0.2% (v/v) acetic acid in water-methanol (4:1, v/v) and analysed by "pseudo-reverse" phase HPLC using a Phenosphere (Phenomenex, Torrance, CA) silica (250 x 4.6 mm, 5 μm) column and the same solvent used for flushing. Eluted products were analysed by UV spectrophotometry at 286 nm with a UV-970 detector (Jasco Corp., Tokyo, Japan). The amount of peramine was estimated by comparison of the integrated peak area (Class-LC10 software; Shimadzu, Kyoto, Japan) of the analyte with the homoperamine internal standard.

2.12.7 Statistical analysis

The experiment was analysed as a split plot design, with five treatments as main plots and plant part as the sub-plots. The main plot treatments were water only (W), ABA + water (AW), JA + water (JW), ABA + JA + W (JAW) and drought (D). The plant-part 'treatment' was leaf or pseudostem (sheath). There were five main plot replicates as whole blocks, four blocks conducted in a greenhouse and one block conducted in a growth chamber. Within each block, the D treatments were replicated four times, but, as the variation was similar to the experimental error, these effects were combined. There were 40 main plots and 80 sub-plots in total. The model tested main-plot (treatment) effects using the block*trt interaction, and the sub-plot (plant-part effects and plant-part*trt interaction) effects using the residual experimental error. Single degree-of-freedom contrasts were used to compare watered versus drought effects, JW (JA) versus W (watered controls) effects, AW (ABA) versus W effects, and the JAW (JA/ABA) interaction within watered treatments. Analysis of variance (ANOVA) was repeated for leaf-only, sheath-only and greenhouse analyses. Multiple ANOVA (MANOVA) was

used to test the combined effects of the three alkaloids together. See **Appendix 10** for the GLM procedure.

2.13 An experiment that produced deuterated methyl jasmonic acid using Pt as a catalyst (*Pers comm* Hislop, Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand and Fielder, HortReseach, Palmerston North, New Zealand)

This material can be used as a stable internal standard when the HPLC analysis of *Lolium perenne* tissue for jasmonic acid is carried out.

Stable deuterated methyl Jasmonic acid was needed because it would finally become necessary to measure by HPLC the drought induced levels of JA and then correlate these with the endogenous level of the alkaloids found in *Lolium.*

Fig 2.8 Deuteration of methyl jasmonate[1]. The reaction was carried out using PtO_2 as a catalyst.
(**A**) Deuterium gas cylinder. (**B**) Graduated water-cushioned gas reservoir.
(**C**) Water reservoir. (**D**) Reaction flask containing methyl JA, the PtO_2 catalyst and ether. (**E**) Magnetic stirrer.

[1] The relative molecular mass (Mr) of methyl Jasmonic acid is 224 and the Mr of deuterium is 4. The number of moles of deuterium needed to hydrogenate the double bond between C_3 and C_4 of methyl jasmonate is given by n = m/Mr. Where 1 mole of A = 1 mole of B. n = 1/224 = 4.6×10^{-3} moles of deuterium (D_2) 1 Mole of gas at STP (Standard Temperature and Pressure) = 24L. Therefore the volume of D_2 needed = $4.46 \times 10^{-3} \times 24L$ = 107mL.

Flush all components with deuterium before fitting the apparatus together then add a small spatular of PtO_2 to the reaction flask **(D)** then flush with D_2 gas (**a** and **b** on **d** off) to activate by pre-reducing the PtO_2.

Once the PtO_2 has turned black 15mL of ether (analar grade) was added and this was followed by the addition of 1 g of Methyl Jasmonic acid that had been dissolved in 10mL of ether.

With the valve of the reaction flask **(b)** turned off (**d**) on 107mL of D_2 gas was dispensed to the graduated water cushioned reservoir **(B).** When this was stabilised the valve **(a)** to the D_2 gas cylinder (A) was turned off and the valve **(b)** to the reaction flask **(D)** was turned on.

At this point the magnetic stirrer **(E)** was turned on.

The end point of the reaction was determined when there was a 107mL difference in water levels in the graduated water tank.

The products were then filtered through a celite filter to remove the catalyst PtO_2 washing the deuterated methyl jasmonic acid through with ether.

Fig 2.9 The celite filter used to remove the PtO_2 catalyst.

This washing ether was then removed using a Rotor vac.

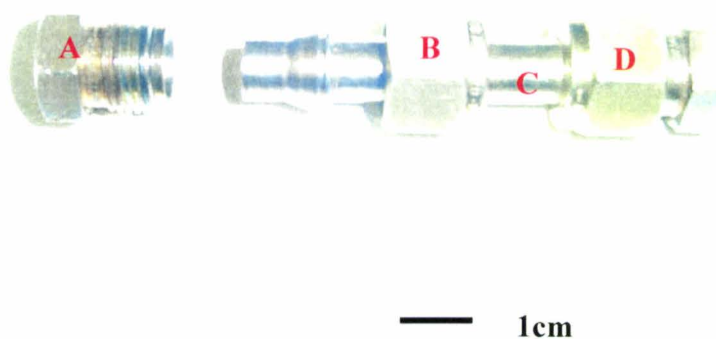
Fig 2.10 The rotary vacuum flask used to remove the ether from the reaction products.

2.14 An experiment to produce deuterated salicylic acid (SA)

This was to be used as a stable internal standard when measuring SA levels in perennial ryegrass by HPLC.

A supercritical exchange reaction (SCR) was carried out using heavy D_2O.

Fifty to 100 mg of SA was added to a SCR bomb **Fig 2.11** 1-2 mL of D_2O added to the bomb and sealed tightly.



1cm

Fig 2.11 Bomb used to carry out the supercritical exchange reaction of SA. **(A)** is screwed into (**B**) on the end of gas copper tube (**C**) to produce a gas fitting **(B)** attachment (**D**).

The loaded tube was then heated in an oven to between 374° and 400°C for one hour. After cooling the contents were rotary-vacuumed and the product taken for NMR analysis. The desired out come is shown in **Fig 2.12.**

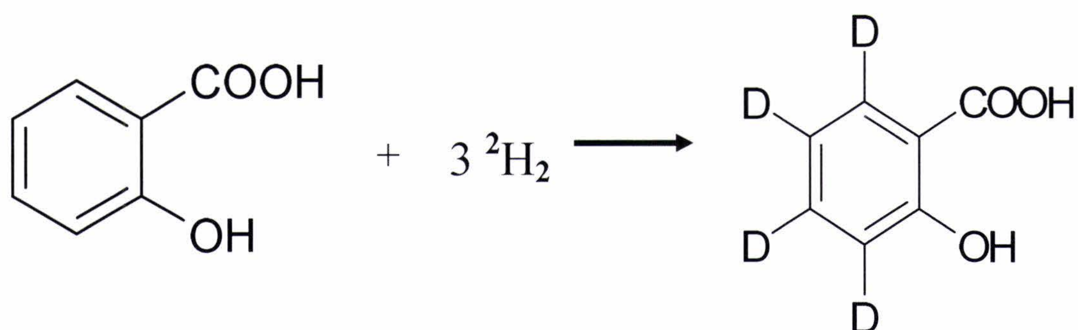


Fig 2.12 The protonation of SA using heavy water (D_2O).

Chapter Three

Results

3.1 Source of plant material for drought experiments

Tillers of 17 distinct genotypes of *Lolium perenne* were randomly selected from a field trial at AgResearch, Grasslands Research Centre (**Fig 2.1**), potted up and grown in a glasshouse as described in **Section 2.2**. After each genotype was established, leaf sheath samples were checked for the presence of endophyte by staining with aniline blue (**Fig 3.1**).

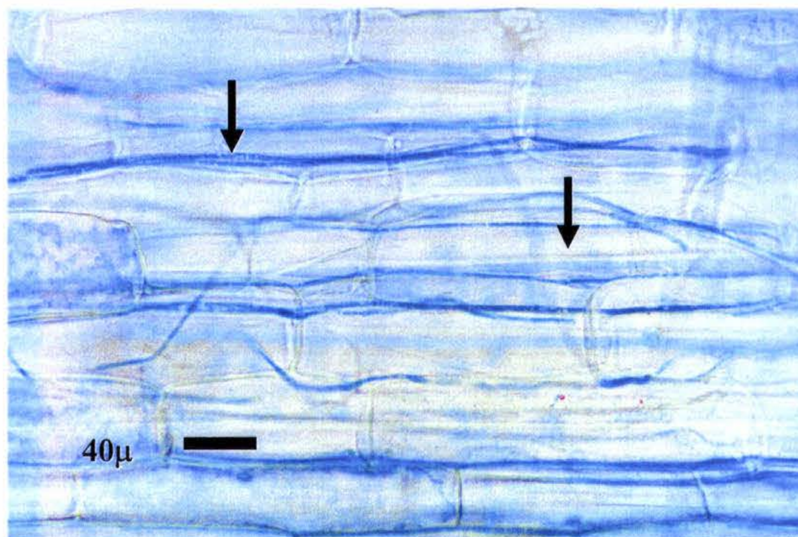


Fig 3.1 Light micrograph of a perennial ryegrass leaf sheath stained with aniline blue. Hyphae of *Neotyphodium lolii* can be observed in the intercellular spaces (**left arrow**) of the cells of *L. perenne* leaf sheath (**right arrow**).

Of the 17 genotypes (G1060–G1076) analysed, 16 (G1060–G1075) were shown to be endophyte positive and one was shown to be endophyte negative (G1076).

3.2 Measurement of water loss to assess drought regime

Having established the experimental design (**Section 2.4**) for measuring alkaloid levels in *L. perenne* plants in response to drought or external application of plant hormones, it was necessary to test whether the drought regime chosen, i.e. replacement of only two-thirds of the water lost by evapotranspiration from the water-sufficient plants, was not so severe that it caused plant death early in the experiment. To overcome the effects of endophyte–plant genotype interactions, 16 genotypes were used and arranged in a randomized block design (**Section 2.4**). Water loss was measured in both endophyte-

positive (E+) and endophyte-negative (E–) plants grown under two different environmental conditions, glasshouse and growth chamber. Four replicates (Replicates 1 to 4) were grown in the AgResearch glasshouse. However, as plants grown in a glasshouse are still subjected to a fluctuating environment, four genotypes were grown under controlled environmental conditions in a growth chamber as a separate replicate (Replicate 5). When the main experiment had been established, surplus plants were included as another replicate (Replicate 6). A separate replicate (Replicate 7) of E– plants was also set up to test for any effects of endophyte on water loss. However, just one genotype that lacked endophyte was available.

The water use over this period was consistently greater for the plants grown in the growth chamber than for the plants grown in the glasshouse (**Table 3.1**), because of the greater temperature and greater air velocity due to forced air circulation.

Table 3.1 Evapotranspiration of *L. perenne* with and without *Neotyphodium lolii* grown under different environmental conditions.

	Evapotranspiration Rate (mL)			
Day[a]	**Glasshouse**[f] **E+ Replicates 1–4**[b]	**Glasshouse**[f] **E+ Replicate 6**[c]	**Glasshouse**[f] **E– Replicate 7**[d]	**Growth Chamber**[g] **E+ Replicate 5**[e]
1	49	57	48	93
2	50	57	48	100
3	50	57	48	111
4	50	57	48	98
5	40	55	46	98
6	71	87	67	98
7	43	59	49	98
8	70	87	80	107
9	70	88	80	107
10	70	88	80	107
11	70	88	80	107
12	89	86	80	
13	82	84	107	
14	92	80	116	

[a]Plants were watered to field capacity, allowed to drain and placed in the glasshouse or the growth chamber, and loss of water was measured daily by weighing.

[b]Average value for the 32 pots that comprised E+ Replicates 1 (G 1075, G1067,G1071, G1063), 2 (G1074, G1066, G1062, G1070), 3 (G1073, G1065, G1061, G1069) 4 (G1060,G1064, G1068, G1072) as described in **Section 2.4**.

[c]Average value for 8 pots of E+ *L. perenne* containing a random selection of all 16 genotypes grown in the glasshouse.

[d]Average value for 5 pots of E– *L. perenne* (all G1076) grown in the glasshouse.

[e]Average value for 8 pots of E+ *L. perenne* comprising Replicate 5 (G1063, G1070, G1073, G1071), as described in **Section 2.4**.

[f]AgResearch, Grasslands Research Centre, Palmerston North.

[g]AgHort building, Massey University.

The use of moving air to eliminate ambient gradients in the growth chamber had the secondary effect of, at times, increasing the relative humidity gradients between the plants and the surrounding air. This increased evapotranspiration. Because the evapotranspiration rates, as determined in this experiment for the plants in the growth chamber, were much higher than for the plants in the glasshouse, the temperature used for subsequent experiments in the growth chamber was reduced 5°C from 20°C to 15°C. On day 7 of the experiment, the glasshouse-grown plants were moved from the south-facing side to the warmer north-facing side, which accounted for the peak in water loss observed on day 7 and the higher average rates of water loss from day 7 until the completion of the experiment. The data from **Table 3.1** was plotted.

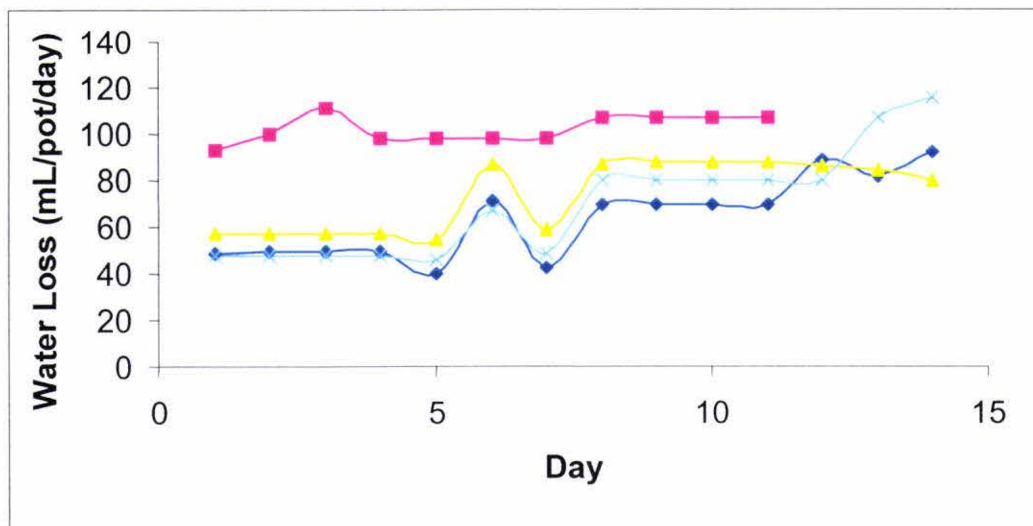


Fig 3.2 Daily water losses as a result of evapotranspiration (mL/pot/day) for endophyte-infected (E+) and endophyte-free (E–) perennial ryegrass plants. Plants were grown under environmental and glasshouse conditions. Average water loss per pot for 32 E+ pots (16 genotypes) from Replicates 1 to 4 (blue), 8 E+ pots (16 genotypes) from Replicate 6 (turquoise) and 5 pots of a single E– genotype (gold) grown in the glasshouse. Average water loss for 8 E+ pots comprising Replicate 5 (four genotypes) grown in a growth chamber (red).

In this experiment, there was no significant difference in water use between the E+ and E– plants both before and after moving the plants. This is in contrast to the observations of some other researchers (Hume *et al.,* 1993), but, in their work, endophyte effects were observed only under conditions of moderate to severe drought (Hume *et al.,* 1993).

3.3 Assessment of endogenous levels of JA and ABA and the uptake of these hormones with foliar application

Various methods of externally applying ABA and JA to water-sufficient *L. perenne* were investigated. These included spraying, wick feeding and dipping. The wick feeding method places the hormones used directly into the sap stream (phloem) of the plant. This would be comparatively simple in dicotyledonous plants but more difficult in the less structured monocotyledonous vascular system of *L. perenne* with their scattered vascular bundles. The spray method of hormone application, although universally used for commercial applications of pesticides and herbicides, is far less quantitative than would be needed to carry out a well-designed, controlled experiment. A foliar application with a dipping system was finally chosen. The system involved up-ending potted plants and dipping the foliage, up to and including the leaf sheath, for a period of 60 min in solutions of ABA, JA and 0.125% Pulse^{TM}, an organosilicone surfactant. See **Section 1.7** for further information on pathways of hormonal entry into the plant.

The literature was searched to determine the levels of abscisic acid (ABA), jasmonic acid (JA) and salicyclic acid (SA) found in a range of plants, with particular emphasis on monocotyledons under drought and water-sufficient conditions (see **Tables 2.7, 2.8** and **Appendix 6**) .

This information was used in conjunction with the drought levels of the hormones measured by enzyme-linked immunosorbent assay (ELISA) in the calibration experiment (**Section 3.7**) to determine the final dipping concentrations of the hormones used in the main experiment.

3.4 Preparation of tritiated plant hormones

For the analysis of hormones in leaf extracts, it was necessary to prepare tritiated protonated ABA and tritiated cucurbic acid (CA) from ABA and JA, respectively (**Fig 2.4**), so that leaf samples could be spiked to determine the efficiency of the solid state plant leaf hormone extraction methods that were developed.

The sodium borohydride reduction protocol was first carried out on a large scale following the protocol of (Miersch *el al.,* 1987) for both ABA and JA (Section 2.6). Whereas the conversion of JA to CA by sodium borohydride reduction is well documented by Miersch and others, the protonation of the ketone group on the

cyclohexane ring of ABA had to be confirmed. The success of the reduction of JA and ABA (**Fig 3.3**) was determined using nuclear magnetic resonance (NMR) spectroscopy.

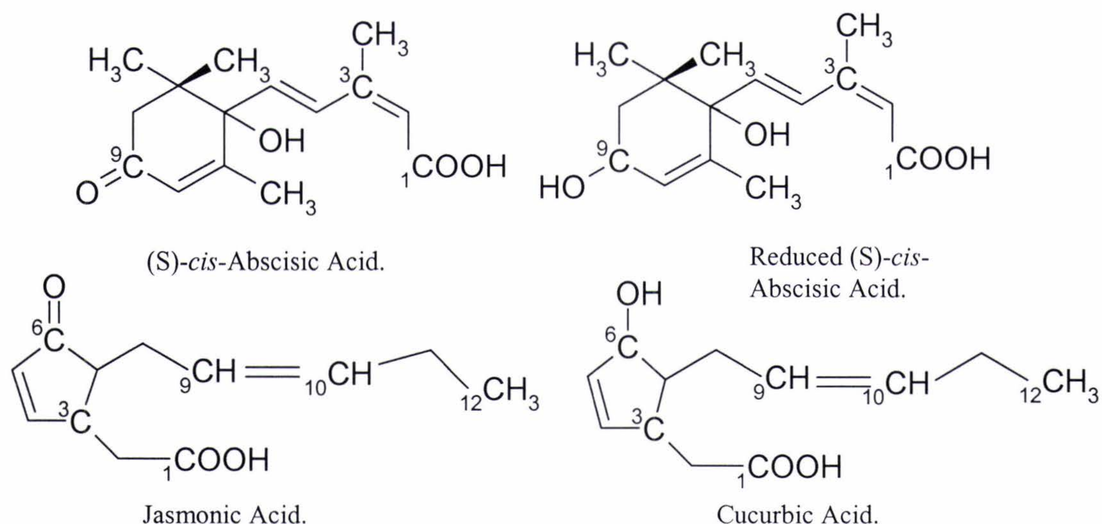


Fig 3.3 Chemical structures of S-*cis*-ABA, reduced S-*cis*-ABA, JA and CA. The carbon atoms are numbered starting from the COOH group.

The reduction of JA was verified by ^{13}C DEPT 90 (distortionless enhancement through polarization transfer 90) (**Fig 3.4**) and ^{1}H NMR (**Fig 3.5)** experiments.

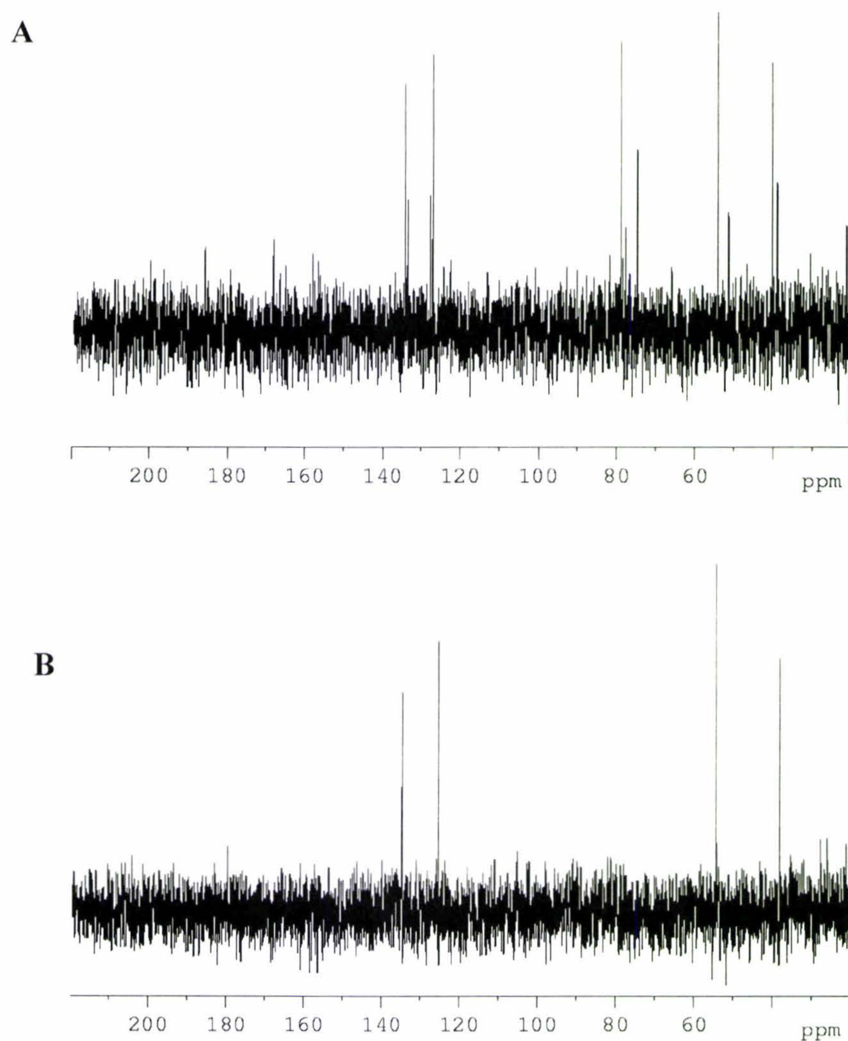


Fig 3.4 ^{13}C DEPT 90 NMR spectra of JA and CA. The analysis was carried out with 5 mg of JA (**B**) and an equivalent amount of its reduction product, CA (**A**).

The ^{13}C DEPT 90 spectrum of JA (**Fig 3.4B**) showed the presence of peaks at 134, 125, 54 and 38 ppm, peaks characteristic of the four methine (CH) groups at positions C3, C7, C9 and C10 of JA (**Fig 3.3**). The spectrum of the reduced form, CA (**Fig 3.4A**), showed an additional pair of methine peaks, one from each of the two isomers, at 75–80 ppm, with chemical shifts consistent with a hydroxyl group bonded to a methine group. This conclusion was further supported by the ^{1}H NMR experiment (**Fig 3.5**).

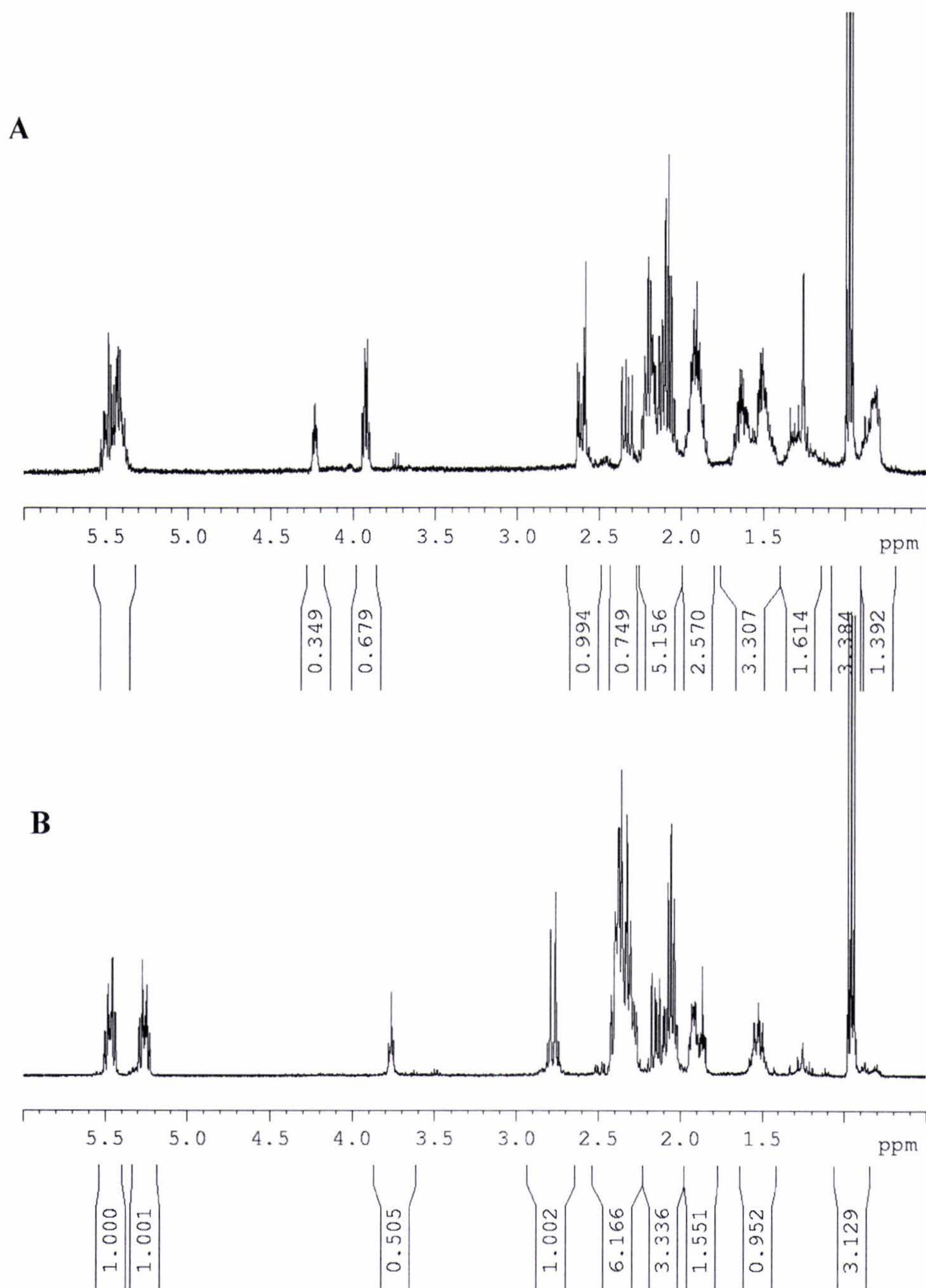


Fig 3.5 ^{1}H NMR spectra of JA and CA. The analysis was carried out with 5 mg of JA (**B**) and an equivalent amount of its reduction product, CA (**A**). The peaks at 3.9 and 4.2 ppm in (**A**) are diagnostic of the two diastereoisomers of CA.

The peaks at 0.92, 5.2 and 5.5 ppm in the ^{1}H NMR spectrum of JA (Fig 3.5B) corresponded to chemical shifts for the terminal methyl, and the C9 and C10 unsaturated carbons. The two additional peaks at 3.9 and 4.2 ppm in the spectrum of the reduced form of JA (**Fig 3.5A**) corresponded to chemical shifts consistent with a proton adjacent to a hydroxyl group for the two diastereoisomers, characteristic of CA.

Initial attempts to obtain a ^{13}C DEPT 90 spectrum for the product of the ABA reduction were unsuccessful, suggesting a low yield of product. Therefore, the product of the ABA reduction was ascertained by one-dimensional ^{1}H NMR (**Fig 3.6**) and two-dimensional ^{1}H–^{1}H COSY (correction spectroscopy) (**Fig 3.7**).

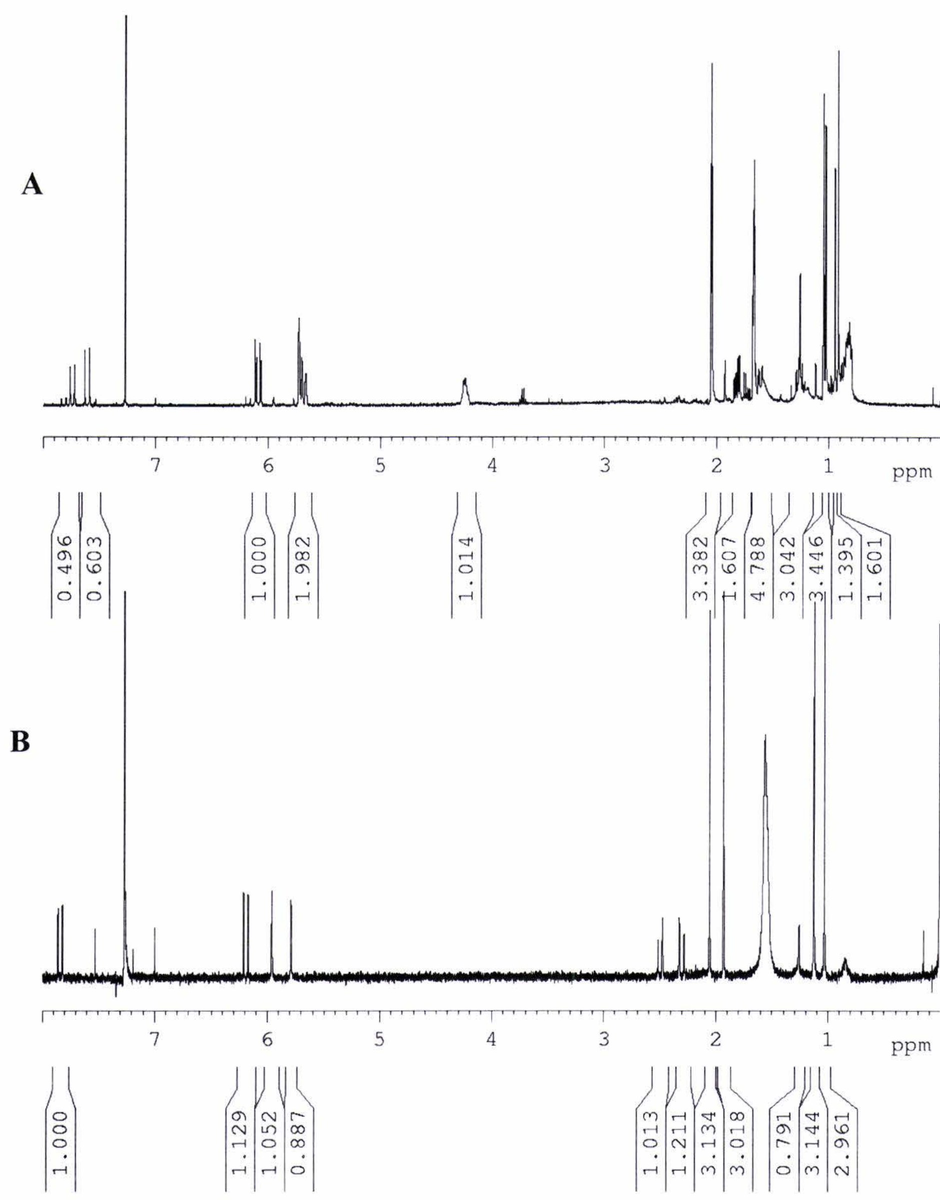


Fig 3.6 ^{1}H NMR spectra of ABA and reduced ABA. The analysis was carried out with 5 mg of ABA (**B**) and an equivalent amount of its reduction product, reduced ABA (**A**). The peak at 4.2 ppm in (**A**) is diagnostic of reduced ABA.

The peaks at 2.3 and 2.5 ppm in the ABA spectrum **(Fig 3.6B)** corresponded to chemical shifts for the ketone group in C9 of the cyclohexane ring of ABA. The additional peak at 4.2 ppm in the spectrum for the reduced form of ABA **(Fig 3.6A)** corresponded to a chemical shift consistent with a proton adjacent to a hydroxyl group,

characteristic of reduced ABA. Because of the small quantities of product, the identity of this compound was verified using very sensitive, two-dimensional ^{1}H–^{1}H COSY **(Fig 3.7).**

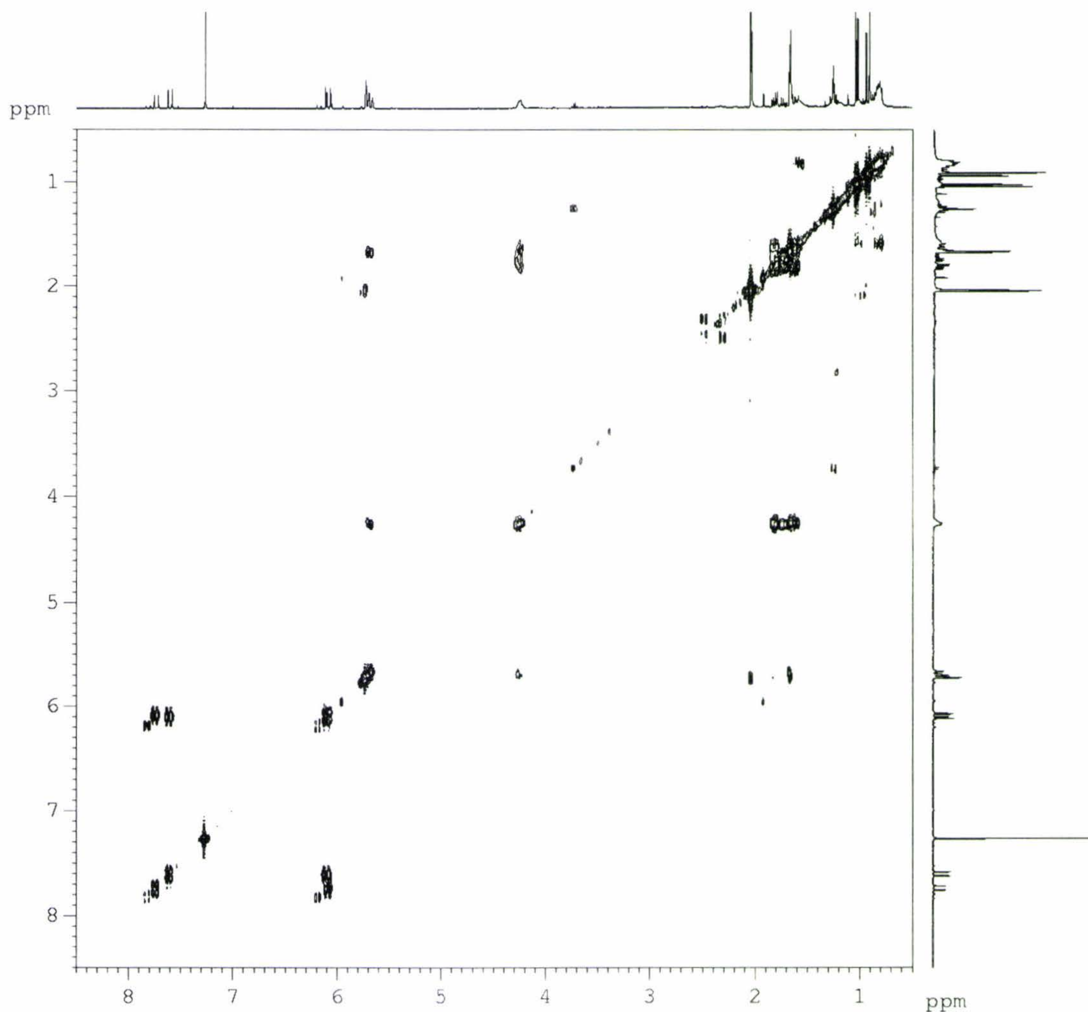


Fig 3.7 Two-dimensional ^{1}H–^{1}H COSY experiment, which was carried out to analyse the reduction product produced from 5 mg of ABA. The spot at 4.2 ppm is diagnostic of an additional proton on C9 of ABA.

This spectrum showed a new proton on C9 of ABA (4.2 ppm) after its reduction using sodium borohydride. This new proton correlated with a methine proton in an unsaturated region (5.7 ppm) of the molecule and a saturated methylene group at C11 (1.8 ppm).

Thus, it was concluded from these NMR spectra that the large scale reduction reaction had produced small, but significant, amounts of both CA and reduced ABA. These experiments confirmed that ABA as well as JA could be reduced by this method.

3.5 HPLC verification of the tritiated reduction product CA and its purification

As outlined in **Appendix 9** the theoretical counts available from 100 mCi of radioactive sodium borohydride were 7.36×10^{11} cpm (disintegrations/minute); of these counts, 3.68×10^{11} cpm were available for the reduction of JA and the balance was available for the reduction of ABA.

Because of the expense of tritiated sodium borohydride, both ABA and JA were subjected to a small scale reduction using tritiated sodium borohydride, as outlined in **Sections 2.6** and **2.7**. The NMR results of the small scale cold reductions of ABA and JA are reported in **Section 3.4**, confirming the reduction to reduced ABA and CA using amounts of sodium borohydride consistent with the tritiated material available. The HPLC verification was carried out in an identical manner for both tritiated ABA and CA, but only the HPLC verification results for tritiated CA are reported here. The small scale (using 30 μL) and the large scale (using 500 μL) purifications of both tritiated ABA and CA were found to be equally successful, but only the purification of tritiated CA is reported here.

3.5.1 HPLC verification of tritiated CA

To identify the HPLC elution times of JA and CA for collection of the appropriate radioactive products, JA and CA standards were analysed using HPLC (**Fig 3.8**).

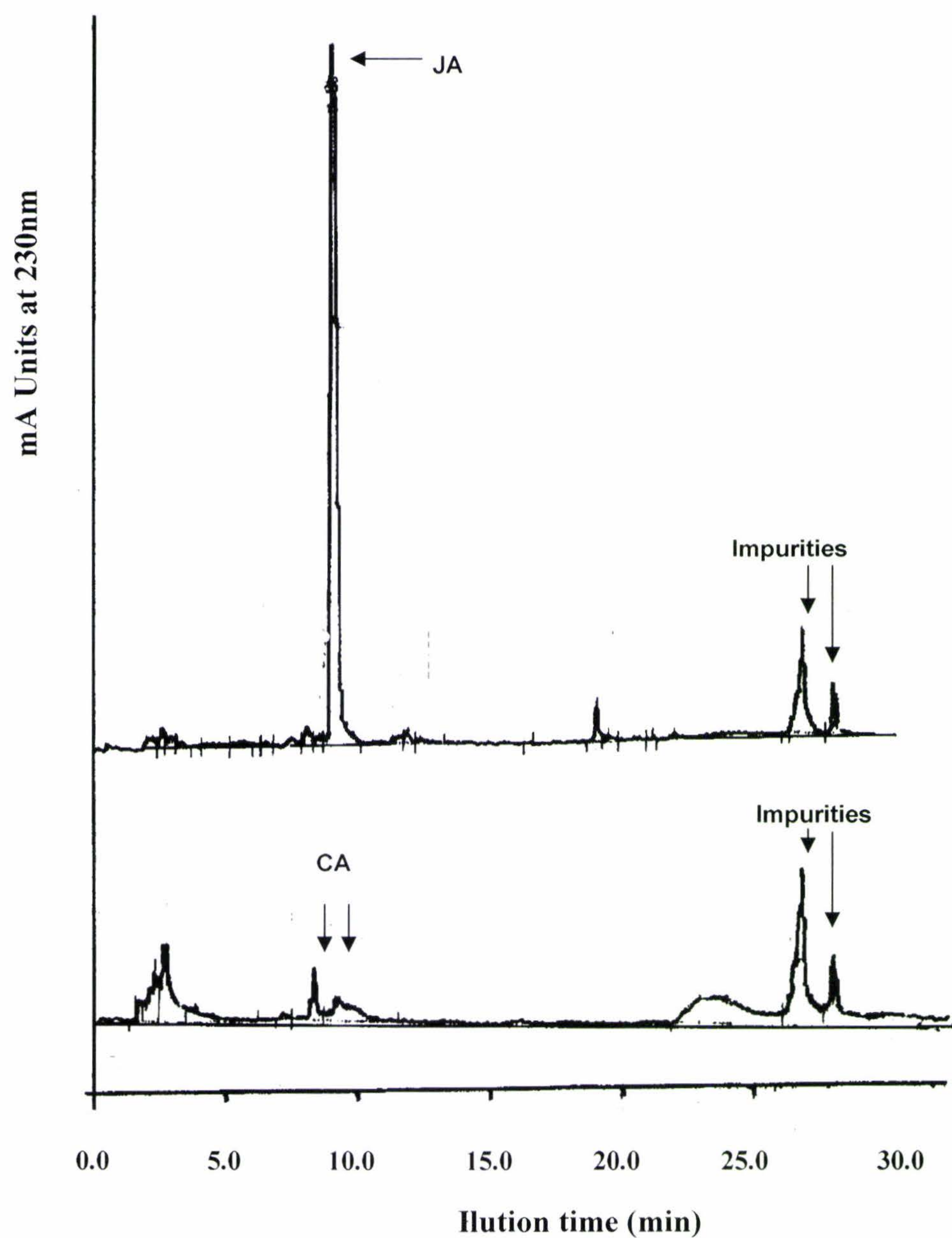
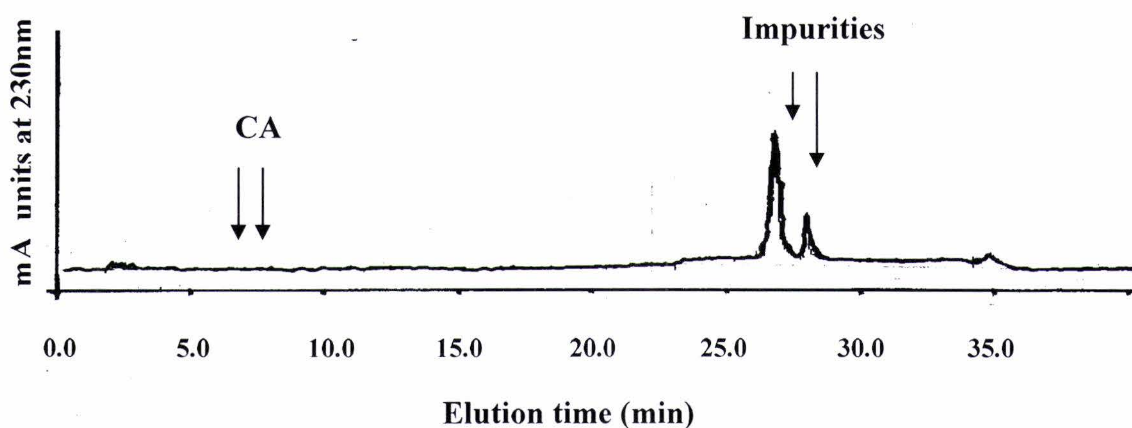


Fig 3.8 Comparison of HPLC separation times for JA (**top**) and CA (**bottom**). JA elutes at 9 min and CA's two diastereoisomers form peaks at 7.3 and 8.4 min. A 10 mg/mL solution of JA was used to provide an injection of 300 μg for JA, and a 1 mg/mL solution of CA was used to provide an injection of 30 μg for CA. The solvents and flow rates used are described in **Appendix 3a.**

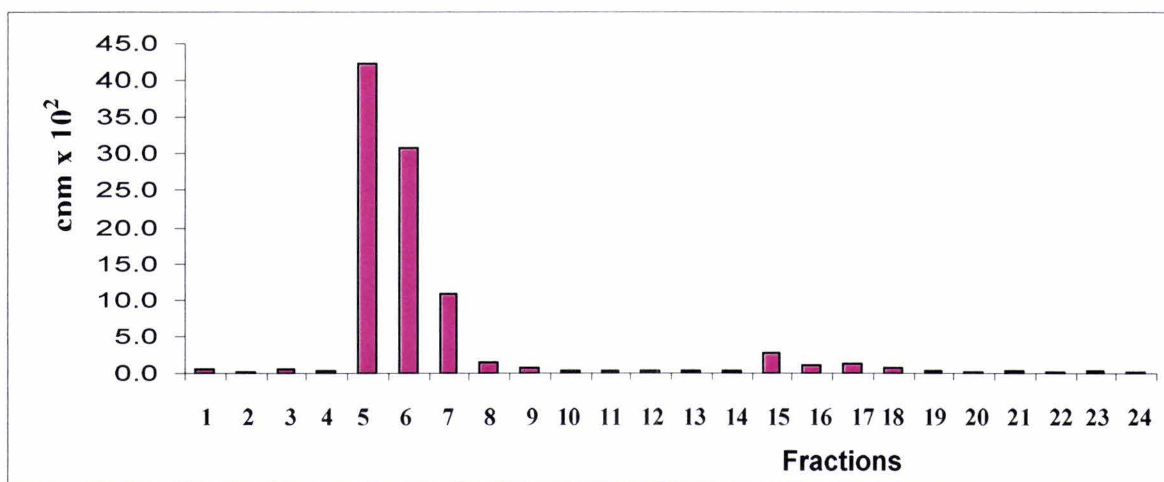
JA was found to elute at 9 min and the two diastereoisomers of CA eluted, and formed twin peaks, at 7.28 and 8.42 min. These elution times were in agreement with those obtained by (Gapper, 1998).

Once tritiated CA had been produced from JA using small scale tritiated sodium borohydride reduction, HPLC was used to separate the tritiated CA from tritiated water and solvents. Because of the small quantities of substrate used (0.09 mg of JA) in the reduction, neither substrate (JA) nor product (CA) could be detected by UV absorbance **(Fig 3.9A)**.

A



B



C

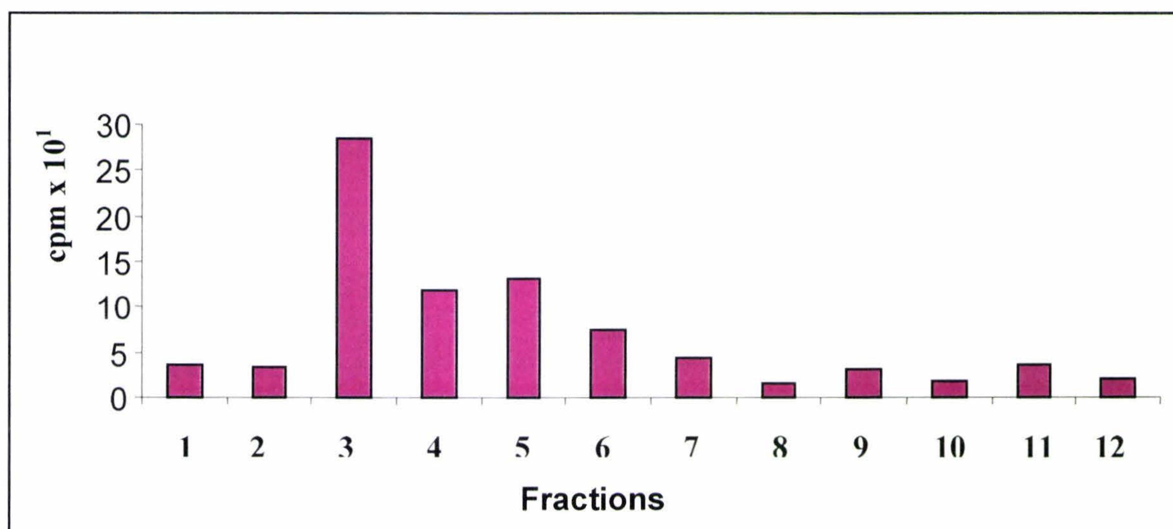


Fig 3.9 HPLC fractionation of the unpurified tritiated JA reduction product.
(**A**) The arrows (CA) indicate the position at which CA would elute based on the analysis of a standard (**Fig 3.8**). A 20 µL sample of the reduction product containing 53 125 cpm was loaded on to the column.
(**B**) Twenty four fractions were collected at 30 s intervals between 0 and 12 min and the radioactivity of each was determined. Peaks of radioactivity corresponding to the two diastereoisomers of CA were observed in fractions 14 to 18 inclusive, corresponding to 7.0–9.0 min. Fractions 5 to 9 were assumed to be tritiated water and solvents.
(**C**) Twelve fractions around and including 14 to 18 were re-collected. Fractions 14 to 18 inclusive, corresponding to 7–9 min, confirmed the presence of tritiated CA. The solvents and flow rates used are described in **Appendix 3a**.

To isolate the radioactively labelled reduction product, 500 µL fractions were collected at 30 s intervals immediately after leaving the column. The radioactivity in each of these fractions was determined and the results are shown in **Figs 3.9B** and **3.9C**.

Fig 3.9B shows the radioactivity of 24 fractions collected at 30 s intervals between 0 and 12 min. There were peaks of radioactivity in fractions 14 to 18, which corresponded to elution times of 7–9 min for CA (**Fig 3.8**). Fractions 5 to 9 were tritiated water and solvents. The 12 fractions around fractions 14 to 18 (7–9 min) were re-collected and the presence of tritiated CA with high specific activity was confirmed (**Fig 3.9C**).

An analysis of the radioactivity was carried out. From **Figs 3.9B** and **3.9C** and given an input count of 53 125 cpm, 16% of the radioactivity was due to unknown solvents and reaction products, 1.2% was due to CA and the balance of 83% was assumed to be due to tritiated water, which was later removed using a C18 solid phase extraction column.

3.5.2 Purification of tritiated CA from the crude extract

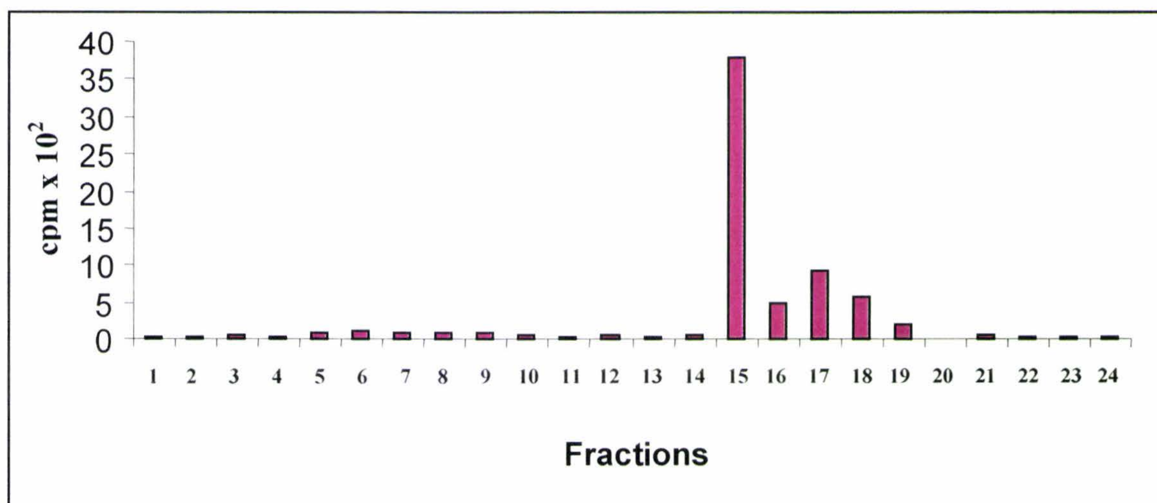
Because of the presence of the tritiated water and solvent fractions, the tritiated CA was purified using C18 solid phase extraction.

One microlitre of the original crude reduction product was placed in 1 mL of 80% methanol. A 100 μL sample had an input count of 1 372 717 200 cpm. A further 100 μL sample was applied to a C18 column for purification (**Section 2.8.2**) and two 1 mL aliquots were obtained. The counts were read and a 9.1% recovery was obtained (counts for aliquot 1 + aliquot 2 / input counts). See **Section 2.10.3** for calculations.

3.5.3 Verification of the efficiency of the small scale purification

A 30 μL sample of the combined aliquots, after solid phase extraction and with an added input count of 40 585 cpm, was injected into the HPLC and samples were collected at 30 s intervals as described previously. Six 500 μL tritiated CA samples were collected at 30 s intervals between 7.5 and 10.5 min. The purity level increased from 9.1% to 71% and the 3–4 min solvent peak showed almost no radioactivity, suggesting that the purification procedure was successful and that large scale purification could be performed. See **Fig 3.10A**.

A



B

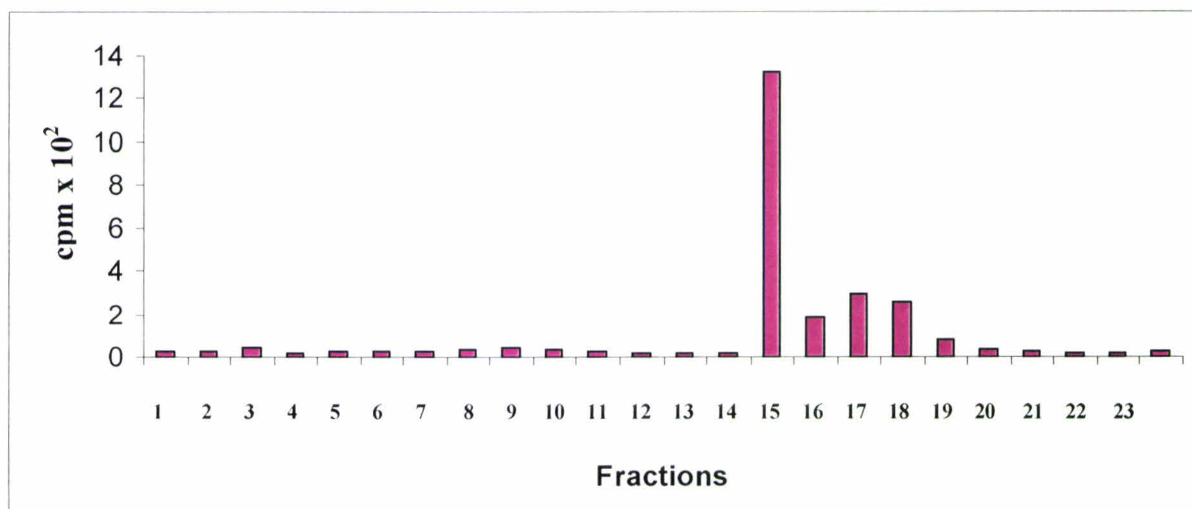


Fig 3.10 Radioactivity found in the HPLC fractions purified by C18 solid state extraction.
(**A**) Radioactivity of HPLC fractions collected at 30 s intervals between 6 and 12 min after small scale C18 purification. A 30 μL sample containing 40 585 cpm was loaded on to the column.
(**B**) Radioactivity of HPLC fractions collected at 30 s intervals between 6 and 12 min after large scale C18 purification. A 500 μL sample containing 14 055 cpm was loaded on to the column.

3.5.4 Large scale purification of tritiated CA

A further 500 μL of unpurified reduction product in methanol was subjected to C18 purification. Six 500 μL tritiated CA samples were collected at 30 s intervals, and the purity increased from 71% to 77% (**Fig 3.10B**). This purified tritiated CA material was used for spiking plant extracts.

3.6 Development, verification and extraction of the plant hormones ABA and JA from *L. perenne*

The purified tritiated ABA and CA were used to spike samples of plant material, to determine the percentage recoveries of the plant hormones ABA and JA obtained using solid state extraction procedures. These experiments allowed the extraction protocols to be verified. The gravity flow method was used to test the extraction efficiency of the solid state C18 and DEAE columns with and without plant material present. Extraction of plant material spiked with ^{3}H-ABA resulted in a 40% recovery (**Table 3.2**). At the time this experiment was carried out, in 2000, the 60% loss of ABA could not be explained. More recent information from a ABA hormone expert at Ohio State University may in part explain the loses of ABA observed in this experiment (**Section 4.3**). The sample spiked with ^{3}H-ABA in the absence of plant material was lost.

Table 3.2 Recovery of tritiated ABA mixed with plant tissue following solid state extraction.

Fraction[a]	Counts (cpm)[b]
AO1	3590[c]
AO2	4550
AO3	1790
AO4	140
AO5	250
Total cpm	9540[d]
Input cpm	25000
% Recovery	40

[a]Five 1 mL aliquots of 80% (v/v) methanol were used to remove the hormone from the C18 column in the final extraction step.
[b]From each of these aliquots, 100 µL was taken, placed in 1 mL of scintillation fluid and then read in the scintillation counter.
[c]Each of the readings obtained was multiplied by 10 (1 mL/100 µL= 10) (**Section 2.10.3**).
[d]Net of background counts(390 cpm/2mL)

Recovery of ^{3}H-CA from a sample extracted in the absence plant material was 100% (**Table 3.3**) The sample containing plant material spiked with ^{3}H-CA was lost.

Table 3.3 Recovery of tritiated CA without added plant tissue, following solid state extraction.

Fraction[a]	Counts (cpm)[b]
DO1	3330[c]
DO2	1040
DO3	580
DO4	1660
DO5	11630
Total cpm	18240
Input cpm	18000
% Recovery	100

[a]Five 1 mL aliquots of 80% (v/v) methanol were used to remove the hormone from the C18 column in the final extraction step.
[b]From each of these aliquots, 100 µL was taken, placed in 1 mL of scintillation fluid and then read in the scintillation counter.
[c]Each of the readings obtained was multiplied by 10 (1 mL/100 µL= 10) (**Section 2.10.3**).

Given the absence of some of the data for the corresponding samples in each of these experiments it is difficult to make firm conclusions about the extraction protocols but it would appear that the presence of plant material reduces the recovery of ABA and probably JA there by emphasizing the need to determine the efficiency of hormone recovery for each experiment.

Different grades and batches of Sephadex were available in the laboratory so an assessment was made of their suitability for the work to be carried out. **Table 3.4** shows the recovery rates of tritiated ABA and CA using these various matrices for samples eluted by gravity flow.

Table 3.4 Effect of sorbent source and type on plant hormone recovery.

Batch	Input Counts	Recovered Counts[b]	Recovery (%)
DEAE Sephadex[c] CA	23 246[a]	22103	95
DEAE Sephadex[c] ABA	11 317[a]	12105	100
DEAE Sephadex [d]CA	23 246	23790	100
DEAE Sephadex[d] ABA	11 317	12980	100
DE52 Cellulose[e] CA	23 246	17890	77
DE52 Cellulose[e] ABA	11 317	12370	100

[a]20 μL of tritiated CA = 23 246 cpm and 20 μL tritiated ABA = 11 317 cpm.
[b]The sum of the counts from five 1 mL aliquot samples that were eluted from the C18 column using 80% methanol and combined; 100 μL was added to 1 mL of scintillation fluid and read, and the background counts were subtracted (**Section 2.10.3**).
[c]Sigma, ex Scott laboratory. Batch number unknown, unopened.
[d]Ex Biochemistry preparation room. Used after conditioned and stored in 40 mM ammonium acetate at 5°C for more than 6 weeks.
[e]Sourced as a possible alternative to DEAE Sephadex.

These experiments showed that dry and unopened Sephadex did not deteriorate even after years of storage and that hydrated Sephadex could be stored for extended periods without any deterioration in its colloidal or binding properties. It was also clear that DE52 Cellulose was not a satisfactory substitute for DEAE Sephadex in its ability to bind CA. Based on these results, the Sigma DEAE Sephadex (ex Scott laboratory) was used for all subsequent plant hormone extractions.

In order to improve the processing rate of samples the protocol for the purification of plant hormones was altered to include a vacuum-assisted elution method (**Fig 2.5** and **Fig 2.6**). The recovery rates obtained were comparable to gravity elution (**Table 3.5**).

Table 3.5 Effect of elution method on plant hormone recovery.

Treatment	Input Counts [c]	Recovered Counts [d]	Recovery (%)
Gravity + ^{3}H-ABA[a]	10 922	6200	57
Gravity + ^{3}H-CA	11 125	8895	80
Vacuum + ^{3}H-ABA[b]	10 922	4789	44
Vacuum + ^{3}H-CA	11 125	8510	77

[a]Solid state filtration under gravity.
[b]Solid state filtration with vacuum assistance.
[c] 20μL ^{3}H-ABA stock gave 10922 cpm and 10μL ^{3}H-CA stock gave 11125cpm.
[d]The sum of the counts from five 1 mL aliquot samples that were eluted from the C18 column using 80% methanol and combined; 100 μL was added to 1 mL of scintillation fluid and read, and the background counts were subtracted (**Section 2.10.3**).

The results shown in **Table 3.5** demonstrate that this alteration to the protocol had little effect on the percentage recovery of plant hormones. As a result, elution of samples using the vacuum bank method was adopted as the preferred method. In addition, these results showed that CA (JA) was more efficiently recovered than ABA, despite their similar ring and carboxylic acid side chain structures.

A further concern was the potential quenching of the light emitted from radioactive samples containing water, given the samples were eluted from the C18 column in 80% methanol compared to the labelled stocks, used as input counts, suspended in chloroform. The presence of water is known to quench light emissions from scintillations in the presence of radioactive samples. To confirm that this was a problem samples of ^{3}H-CA were measured in the presence and absence of water. As can be seen from the data shown in **Table 3.6** the presence of water decreased the efficiency of counting. Consequently an equivalent amount of water to that present in the eluted samples was added to the ^{3}H-CA and ^{3}H-ABA stocks when used as an input samples so that accurate measurements could be made of both input and recovered radioactivity

Table 3.6 The effect of water on the efficiency of counting.

Treatment	Reading 1 (cpm)	Reading 2 (cpm)[c]	Mean Counts (cpm)
20 μL CA stock + 90 μL 80% methanol[a]	15 170	15 660	15 415
20 μL CA stock[b]	18 730	22 234	20 482

[a]A model of the 100 μL sample taken from the five 1 mL eluates removed from the C18 column and combined after solid state extraction of plant tissue for ABA and JA with its 10 μL CA spike.
[b]A model of the 20 μL CA stock purified by C18 extraction in 80% methanol added directly to each plant sample prior to solid state extraction and at the same time added directly to 1 mL of scintillation fluid and then read, and used as the control against subsequent plant extraction readings. Around 23 000 cpm and from which the percentage recovery was estimated using recovered counts.
[c]Readings taken 24 h apart.

3.7 ELISA analysis of endogenous hormone levels of ABA and JA in water stressed plants and uptake of these hormones after dipping

Water-sufficient *L. perenne* plants (a representative sample of the genotypes that were to be used in the main experiment) were dipped in solutions of the hormones at concentrations that could be expected during an imposed water stress and absorption of the hormones was verified using a competitive ELISA.

3.7.1 Plant hormone levels in water-stressed *L. perenne*

This trial was carried out in the glasshouse at AgResearch and in the growth chamber in the AgHort building at Massey University. The pot-grown plants were progressively exposed to water stress. The efficiency of extraction was of the plant hormones was determined in the water stressed plant samples by spiking the sample with ^{3}H-CA (**Table 3.7**).

Table 3.7 Efficiency of hormone extraction from water-stressed samples of *L. perenne.*

Sample Harvest Dates[a]	Counts Recovered (cpm)	Recovery (%)[b]	SAF[c]
13/9	14 520	63	1.6
14/9	17 740	77	1.3
15/9	25 780	111	0.9
18/9	15 060	65	1.5
19/9	18 120	79	1.3
20/9	13 740	60	1.7
21/9	13 600	59	1.7
22/9	11 600	50	2.0
23/9	13 400	58	1.7
24/9	12 680	55	1.8
25/9	11 200	49	2.1
26/9	10 680	46	2.2
27/9	12 560	55	1.9
28/9	11 740	51	2.0
30/9	14 760	64	1.6

[a]To estimate dipping regimes, a preliminary experiment was carried out to determine the leaf levels of ABA
and JA in perennial ryegrass plants that were progressively water stressed. Leaf samples were taken daily from a representative sample of the same ryegrass genotypes that were used in the main experiment. These samples were spiked with tritiated CA and solid state extracted and then analysed using ELISA. The experiment started on 13/9/02 and finished on 30/9/02.
[b]The input counts were 23 216 cpm.
[c]The sample adjustment factor (SAF) was used to adjust the final hormone levels as measured by ELISA

The SAFs were used to adjust the levels of ABA and JA as measured by ELISA (see **Tables 3.9**, **3.13** and **3.16**).

3.7.2 ELISA results for ABA

The composite standard curve used to determine the ABA concentrations is shown in **Fig 3.11.**

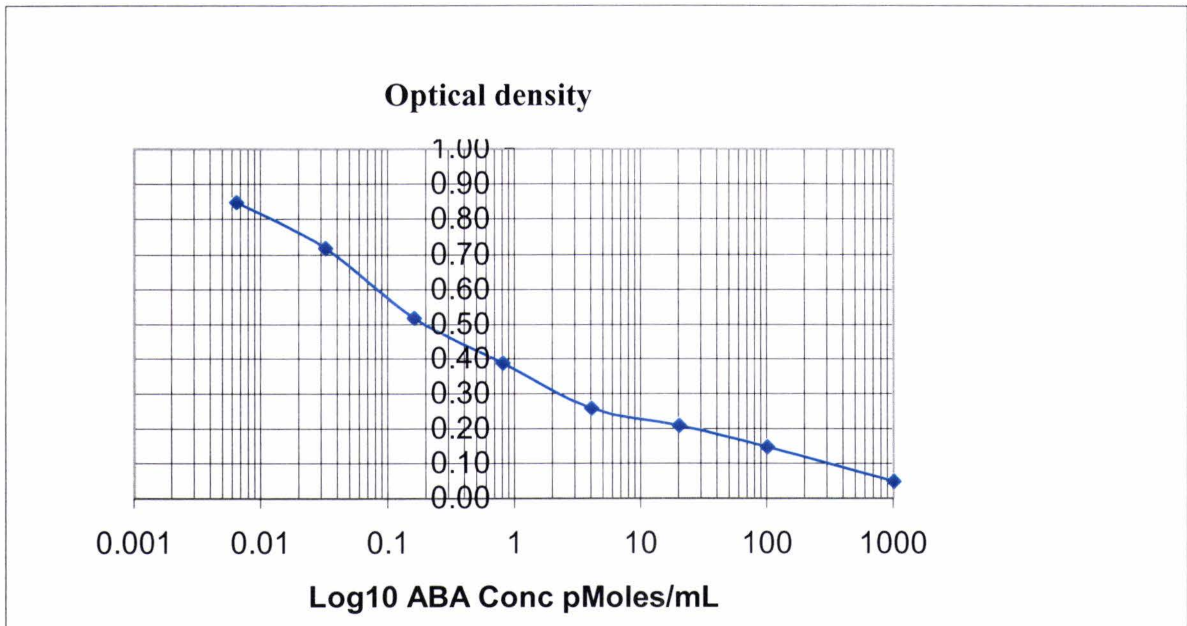


Fig 3.11 The ELISA standard curve used to obtain the concentration of ABA from perennial ryegrass tissue samples.

The calculated ABA concentrations are given in **Table 3.8** and **Fig 3.12**.

Table 3.8 ABA levels in water stressed plants of *L. perenne* grown in the AgResearch glasshouse.

	3rd ELISA absorption	2nd ELISA absorption	Solid state correction factor	Mean 2nd, 3rd absorptions	Conc (pg/well)	g tissue tested [a]	Concentration (pg/wel)l corrected to give 0.2 g dry weight [b]	Concentration (pg/ 0.2 g dry weight) corrected for dilution and extraction losses [c]	3rd ELISA absorption 1 in 10 dilution	2nd ELISA absorption 1 in 10 dilution	Mean 2nd, 3rd dilution	Conc (pg/well)	Concentration (pg/well) corrected to give 0.2 g 1 in 10 dilution [d]	Concentration (pg/ 0.2 g dry weight) Corrected for dilution and extraction losses [e]
13-Sep	0.21	0.37	1.60	0.29	3	0.08	7.5	120						
14-Sep	0.21	0.29	1.30	0.25	4	0.075	11	139						
15-Sep	0.29	1.13	0.90	0.71	0.01	0.08	0.03	0.23						
18-Sep	0.30	0.28	1.50	0.29	4	0.1	8	120						
19-Sep	0.30	0.28	1.30	0.29	4	0.15	5.3	69	0.13	0.46	0.29	4	53	693
20-Sep	0.37	0.28	1.70	0.32	3	0.1	6	102	0.15	0.49	0.32	2	40	680
21-Sep	0.25	0.27	1.70	0.26	8	0.1	16	272	0.16	0.52	0.34	0.5	10	170
22-Sep	0.18	0.24	2.00	0.21	11	0.1	22	440	0.20	0.26	0.23	10	200	4000
23-Sep	0.22	0.39	1.70	0.31	4	0.2	4	68	0.18	0.47	0.33	2	20	340
24-Sep	0.18	0.31	1.80	0.24	10	0.18	11	200	0.17	0.52	0.35	2	22	400
25-Sep	0.19	0.22	2.10	0.20	11	0.05	44	924	0.18	0.33	0.25	5	200	4200
26-Sep	0.20	0.28	2.20	0.24	10	0.1	20	440	0.17	0.44	0.30	3	60	1320
27-Sep	0.20	0.27	1.90	0.24	8	0.2	8	152	0.18	0.33	0.26	7	70	1330
28-Sep	0.27	0.22	2.00	0.25	9	0.15	12	240	0.19	0.26	0.22	11	147	2933
30-Sep	0.33	0.24	1.60	0.28	3	0.2	3	48	0.17	0.33	0.25	11	110	1760

3rd ELISA absorption 1 in 100 dilution	2nd ELISA absorption 1 in 100 dilution	Mean 2nd, 3rd dilution	Conc (pg/well)	Concentration (pg/well) corrected to give 0.2 g 1 in 100 dilution [f]	Concentration (pg/ 0.2 g dry weight) corrected for dilution and extraction losses [g]
0.19	0.42	0.31	3	1200	25200
0.21	0.78	0.49	0	60	1320
0.17	0.58	0.38	1	100	1900
0.18	0.39	0.28	4	533	10667
0.18	0.42	0.30	2	200	3200

[a]Because of the relatively small number of plants used in this experiment, it was not always possible to obtain 0.2 g of freeze-dried (lyophilized) tissue for analysis.
[b]0.2/H9*G9 = 0.2/g tissue tested x concentration per pot.
[c](I9*E9)*10 = Corrected dry weight x solid state correction factor (SCF) x 10
[d] 0.2/H13*N13*10 = 0.2/ g tissue tested on 19th Sept x concentration per pot on 19th of Sept x 10
[e](O13*E12)*10 = Factor to give 0.2 g at 1in 10 dilution x SCF 18th of Sept x 10
[f] 0.2./H19*T19*100 = 0.2/g tissue tested on the 25th Sept x concentration per pot x 100
[g](U19*E19)*10 = Factor to give 0.2 g at a 1in 100 dilution x SCF on the 25th Sept x10
Note: The data given in blue were graphed; see **Fig 3.12**.

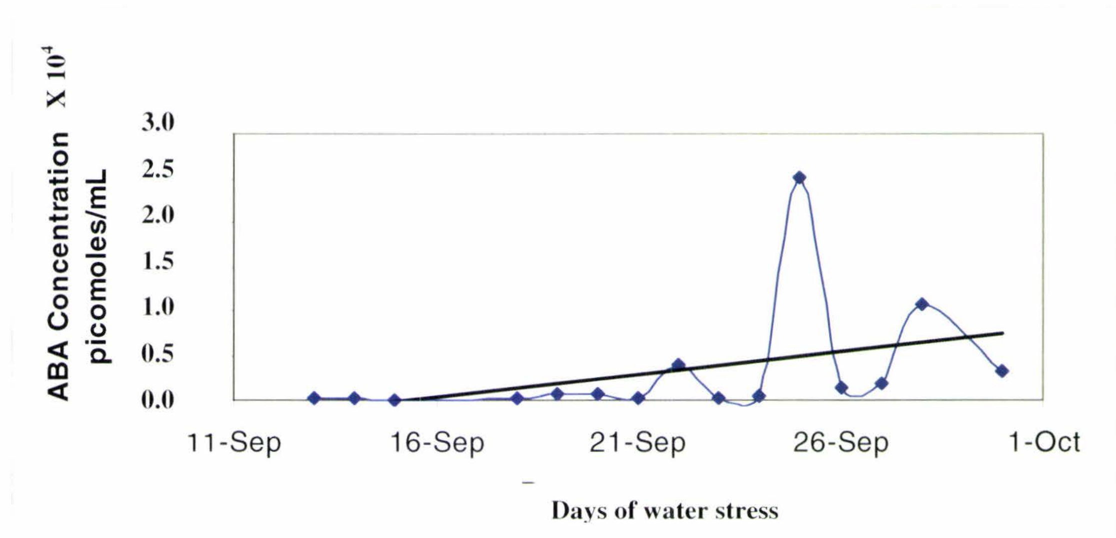


Fig 3.12 The concentration of ABA in perennial ryegrass when subjected to a water stress. The increased concentration of the hormone ABA, measured by ELISA in picomoles/mL (picomoles/g fresh weight), in 16 genotypes of perennial ryegrass during a water stress imposed by replacing only two-thirds of the water lost by evapotranspiration. The trend line is also shown. The genotypes were grown in the AgResearch glasshouses at Grasslands Research Centre, Palmerston North.

The endogenous levels of ABA in *L. perenne* did not increase until a threshold was reached at 8–10 days after the imposition of the water stress. A similar result has been recorded for other monocotyledon grass species (Abernethy, 1996). Abernethy (1996) found that levels of ABA in the New Zealand native grass *Festuca zealandiae* increased once a certain threshold level of water deficit was reached. The peak in ABA around the 25th of September cannot be explained. ABA levels increased significantly from around day 8 after the imposition of water stress (**Fig 3.13**). Consequently the ABA dipping concentrations as shown by the trend line were used for the main experiment. The 25th-Sep spike was not replicated in the main experiment and hormonal dipping did not start until day 9 of the imposed water stress. See **Fig 3.13.** for the re-adjusted trend line.

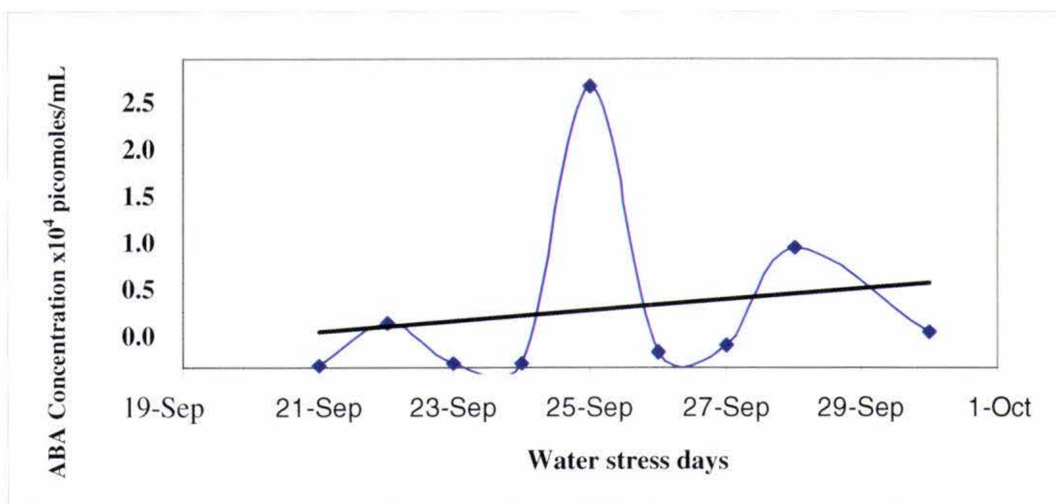


Fig 3.13 The concentration of ABA in perennial ryegrass with the trend line imposed from the 21st September trigger point. An ABA trigger point under water stress conditions is characteristic of ABA increases in many plant species (Abernethy, 1996).

The environment in the growth chamber was different in many respects from that in the glasshouses. The diurnal and nocturnal temperature and relative humidity fluctuations were less severe. A water stress trial was also carried out on Replicate 5 under these conditions. The recoveries and SAFs are given in **Table 3.9**.

Table 3.9 The efficiency of ^{3}H-ABA recovery from water-stressed samples of *L. perenne* grown in a growth chamber.

Sample Harvest Dates[a]	Counts Recovered (cpm)	Recovery (%)[b]	SAF[c]
21/9	9 200	40	2.5
22/9	8 420	36	2.8
25/9	2 900	12	8.0
26/9	8 160	35	2.8
27/9	3 880	17	6.0
28/9	9 560	41	2.4
29/9	10 480	45	2.2
30/9	10 760	46	2.2

[a] To estimate dipping regimes, a preliminary experiment was carried out in the growth chamber to determine
the leaf levels of ABA and JA in perennial ryegrass plants that were progressively water stressed. Leaf samples were taken daily from a representative sample of the same ryegrass genotypes that were used in the main experiment. These samples were solid state extracted and analysed using ELISA. The experiment started on 21/9/02 and finished on 30/9/02.

[b] The input counts were 23 216 cpm.

[c] The SAF was used to adjust the final hormone levels as measured by ELISA.

The calculated ABA data are given in **Table 3.10** and **Fig 3.14**.

Table 3.10 ABA levels in water stressed plants of *L. perenne* genotypes grown in a growth chamber.[a]

	3rd ELISA absorption	2nd ELISA absorption	Mean 2nd, 3rd absorptions	Solid state correction factor	Conc (pg/well)	g tissue used	Concentration (pg/well) corrected to give 0.2 g dry weight [b]	Concentration (pg/.2 g dry weight) corrected for dilution and extraction losses [c]	3rd ELISA absorption 1 in 10 dilution	2nd ELISA absorption 1 in 10 dilution	Mean 2nd, 3rd dilutions	Conc (pg/well)	Concentrati (pg/wel)l correctec to give 0.2 dilution [d]
20-Sep	0.15	0.28	0.22		10.5	0.1	21	21					
21-Sep	0.32	0.24	0.28	2.50	4	0.1	8	20					
22-Sep	0.32	0.27	0.29	2.80	4	0.1	8	22.4					
25-Sep	0.57	0.28	0.42	8.00	0.8	0.1	1.6	12.8	0.30	0.55	0.42	0.7	14
26-Sep	0.33	0.36	0.34	2.80	1	0.1	2	5.6	0.47	0.65	0.56	0.1	2
27-Sep	0.32	0.28	0.30	6.00	3	0.1	6	36	0.58	0.52	0.55	0.1	2
28-Sep	0.18	0.36	0.27	2.40	4	0.1	8	19.2	0.29	0.51	0.40	0.8	16
29-Sep	0.19	0.27	0.23	2.20	10	0.1	20	44	0.25	0.51	0.38	0.7	14
30-Sep	0.15	0.27	0.21	2.20	10.5	0.1	21	46.2	0.21	0.47	0.34	2	40

3rd ELISA absorption 1 in 100 Dilution	2nd ELISA absorption 1 in 100 Dilution	Mean 2nd, 3rd dilution	Conc (pg/well)	Concentration (pg/well) corrected to give 0.2 g dry weight [f]	Concentration (pg/ 0.2 g dry weight) corrected for dilution and extraction losses [g]
0.21	0.762	0.49	0.3	60	360
0.39	0.914	0.65	0.03	6	14
0.29	0.688	0.49	0.3	60	132
0.28	0.67	0.48	0.3	60	132

[a]Because of the relatively small number of plants used in this experiment, it was not always
possible to obtain 0.2 g of freeze-dried (lyophilized) tissue for analysis.
[b]0.2/H9*G9 = 0.2/g tissue tested x concentration per pot.
[c](I9*E9)*10 = Corrected dry weight x solid state correction factor (SCF) x 10
[d] 0.2/H13*N13*10 = 0.2/ g tissue tested on 19th Sept x concentration per pot on 19th of Sept x 10
[e](O13*E12)*10 = Factor to give 0.2 g at 1in 10 dilution x SCF 18th of Sept x 10
[f] 0.2./H19*T19*100 = 0.2/g tissue tested on the 25th Sept x concentration per pot x 100
[g](U19*E19)*10 = Factor to give 0.2 g at a 1in 100 dilution x SCF on the 25th Sept x10

Note: The data given in blue were graphed; see **Fig 3.14**.

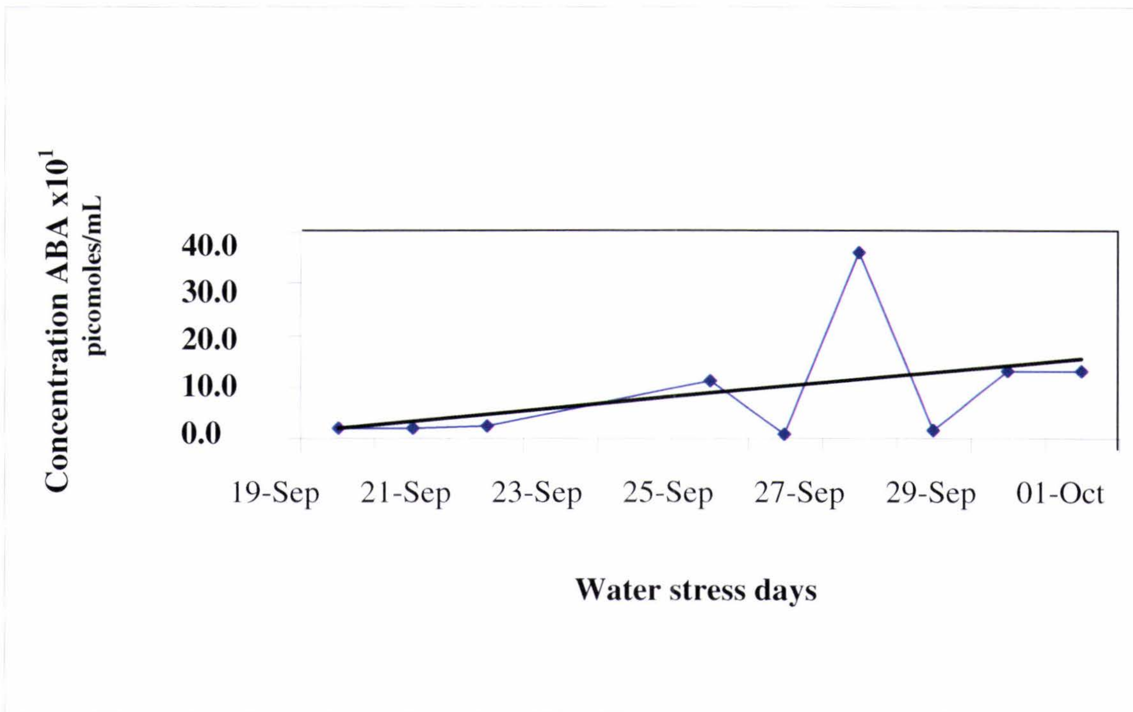


Fig 3.14 The concentration of ABA in perennial ryegrass when subjected to a water stress. The increase in concentration of the hormone ABA, as measured by ELISA in picomoles/mL (picomoles/g fresh weight), in four genotypes of perennial ryegrass (G1063, G1070, G1073 and G1071) during a water stress imposed by replacing only two-thirds of the water lost by evapotranspiration. The trend line is also shown.
The genotypes were grown in the AgHort growth chamber at Massey University.

Fig 3.14 shows that, unlike the glasshouse-grown *L. perenne*, the *L. perenne* in the growth chamber did not have such a defined ABA spike. Rather the ABA concentration tended to increase over the entire water stress period

3.7.3 ELISA results for JA

The standard curve used to determine the JA concentrations for the AgResearch glasshouse trial (**Section 2.11.4**) is shown in **Fig 3.15.**

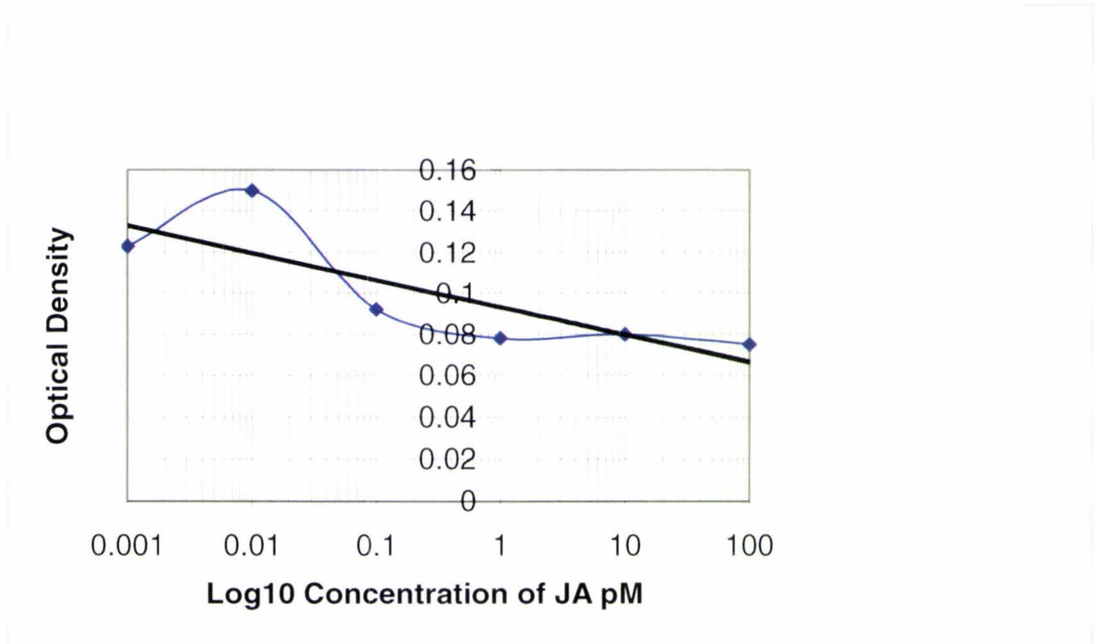


Fig 3.15 The ELISA standard curve used to obtain the concentration of JA from perennial ryegrass tissue samples.

This standard curve was used to determine the concentrations of JA shown in **Table 3.11** and **Fig 3.16.**

Table 3.11 JA levels in water stressed plant of L. perenne grown in the AgResearch Glasshouse.

	ELISA absorption	Solid state correction factor	Conc (pm/well)	g tissue tested	Concentration (pg/wel)l corrected to give 0.2 g dry weight [a]	Concentration (pg/ 0.2 g dry weight) corrected for dilution and extraction losses [b]	ELISA absorbtion 1 in 10 dilution	Conc (pg/well)	Concentration (pg/well) corrected to give 0.2 g dry weight [f]	Concentration (pg/ 0.2 g dry weight) corrected for dilution and extraction losses [g]	ELISA absorption 1 in 100 dilution	Conc (pm/well)	Concentration (pg/well) corrected to give 0.2 g dry weight [e]	Concentratio (pg/ 0.2 g dry weight) corrected fo dilution and extraction losses [f]
13-Sep	0.099	1.6	0.1	0.08	0.25	4.0								
14-Sep	0.106	1.3	0.1	0.075	0.27	3.47								
15-Sep	0.103	0.9	0.1	0.08	0.25	2.25								
18-Sep	0.197	1.5	0.01	0.1	0.02	0.3								
19-Sep	0.106	1.3	0.1	0.15	0.13	1.73	0.16	0.2	2.67	34.67				
20-Sep	0.099	1.7	0.1	0.1	0.2	0.762	0.13	0.3	6.0	102.00				
21-Sep	0.123	1.7	0.01	0.12	0.39	0.914	0.13	0.3	5.0	85.0				
22-Sep	0.128	2	0.01	0.1	0.02	0.688	0.14	0.25	5.0	100.0				
23-Sep	0.107	1.7	0.1	0.2	0.02	1.70	0.16	0.2	2.0	34.0				
24-Sep	0.106	1.8	0.1	0.18	0.1	2.00	0.13	0.3	3.33	60.0				
25-Sep	0.123	2.1	0.1	0.05	0.1	8.40	0.12	0.5	20.0	420.0	0.158	0.2	80	1680
26-Sep	0.101	2.2	0.1	0.1	0.4	4.40	0.12	0.5	10.0	220.0	0.141	0.2	40	880
27-Sep	0.097	1.9	0.1	0.2	0.2	1.90	0.15	0.2	2.0	38.0	0.137	0.45	45	855
28-Sep	0.14	2	0.1	0.15	0.1	2.67	0.18	0.1	1.33	26.67	0.162	0.003	0.4	8
30-Sep	0.128	1.6	0.1	0.2	0.1	1.60	0.17	0.002	0.02	0.32	0.179	0.002	0.2	3.2

[a] 0.2/E9*D9 = 0.2/g tissue tested x concentration per well
[b](F9*C9)*10 = corrected dry weight (DW) to give 0.2 g x SCF
[c] (0.2/E13)*I13*10 = 0.2/g tissue tested x concentration per well on 19th Sept
[d] (J13*C13)*10 = corrected DW to give 0.2 g x SCF on 19th Sept x10
[e] 0.2/E19*M19*100 = 0.2 g/ g tissue tested on 25th Sept x concentration per well on the 25th Sept
[f](N19*C19)*10 = corrected DW to give 0.2 g x SCF per well on the 25th Sept x100
Note: The data in blue were graphed; see **Fig 3.16.**

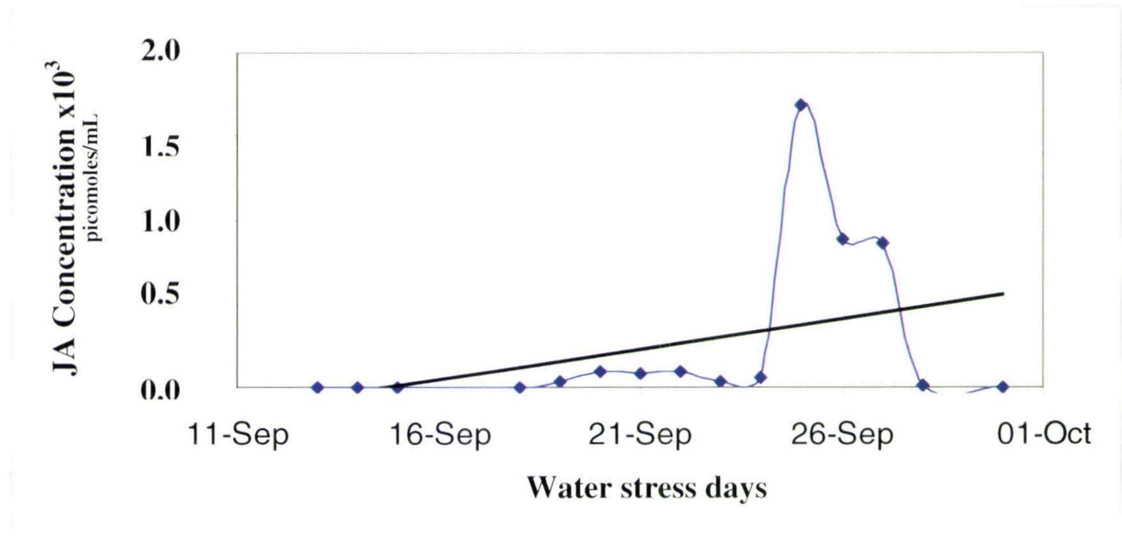


Fig 3.16 The concentration of JA in perennial ryegrass when subjected to a water stress. The increase in the concentration of the hormone JA, as measured by ELISA in picomoles/mL (picomoles/g fresh weight), in 16 genotypes of perennial ryegrass during a water stress imposed by replacing only two-thirds of the water lost by evapotranspiration. The trend line is also shown. The genotypes were grown in the AgResearch glasshouses at Grasslands Research Centre, Palmerston North .

Fig 3.16 shows that the endogenous level of JA did not increase to the same extent as that of ABA. However, there was a spike in the JA level, around the 25th September was similar to that observed for ABA. The lower levels of JA compared to ABA was expected. A number of stress conditions in plants are recognized, and researchers have found that the physiological responses, especially the hormonal cascades of ABA and JA, may be similar (Mackerness *et al.* 1999; Mackerness, 2000). It has been shown that JA can act directly to reduce transpiration, although not to the same extent as ABA. Thus, it is possible that JA or one of the other jasmonates produced under conditions of water stress can act to reduce transpiration in plants (Voros *et al.*, 1998; Creelman & Mullet, 1995). The levels of JA found in this experiment would confirm the previously published findings. The endogenous levels of JA were also determined for *L. perenne* genotypes in the growth chamber. The levels are shown in **Table 3.12,** and have been corrected for extraction losses using previously determined shown in **Table 3.9,** and **Fig 3.17.**

Table 3.12 JA levels in water stressed plant of *L. perenne* grown in the AgHort Growth Chamber.

	ELISA absorption	Conc per pot (nm/L)	Solid state correction factor	g tissue used	Concentration (pg/well) corrected to give 0.2 g dry weight [a]	Concentration (pg/ 0.2 g dry weight) corrected for dilution and extraction losses [b]	ELISA absorption 1 in 10 dilution	Conc per pot (nm/L)	Concentration (pg/well) corrected to give 0.2 g dry weight [c]	Concentration (pg/ 0.2 g dry weight) corrected for dilution and extraction losses [d]	ELISA absorption 1 in 100 dilution	Conc per pot (nm/L)	Concentration (pg/well) corrected to give 0.2 g dry weight [e]	Concentration (pg/ 0.2 g dry weight) corrected for dilution and extraction losses [f]
20-Sep	0.21	0	3.2	0.1	0	0								
21-Sep	0.12	0.09	2.5	0.1	0.18	0.45								
22-Sep	0.21	0	2.8	0.1	0	0								
25-Sep	0.11	0.8	8	0.1	1.6	12.8	0.21	0	0	0				
26-Sep	0.10	0.8	2.8	0.1	1.6	4.48	0.24	0	0	0				
27-Sep	0.18	0	6	0.1	0	0	0.13	0.06	1.2	7.2	0.09	0.9	180	1080
28-Sep	0.15	0.2	2.4	0.1	0.4	0.96	0.12	0.7	14	33.6	0.15	0.01	2	4.8
29-Sep	0.14	0.02	2.2	0.1	0.04	0.088	0.12	0.7	14	30.8	0.16	0.005	1	2.2
30-Sep	0.11	0.9	2.2	0.1	1.8	3.96	0.15	0.15	3	6.6	0.14	0.02	4	8.8

[a] 0.2/E9*D9 = 0.2/g tissue tested x concentration per well

[b] (F9*C9)*10 = corrected dry weight (DW) to give 0.2 g x SCF.

[c] (0.2/E13)*I13*10 = 0.2/g tissue tested x concentration per well on 19th Sept

[d] (J13*C13)*10 = corrected DW to give 0.2 g x SCF on 19th Sept x10

[e] 0.2/E19*M19*100 = 0.2 g/ g tissue tested on 25th Sept x concentration per well on the 25th Sept

[f] (N19*C19)*10 = corrected DW to give 0.2 g x SCF per well on the 25th Sept x 100

Note: The data in blue were graphed; see **Fig 3.16.**

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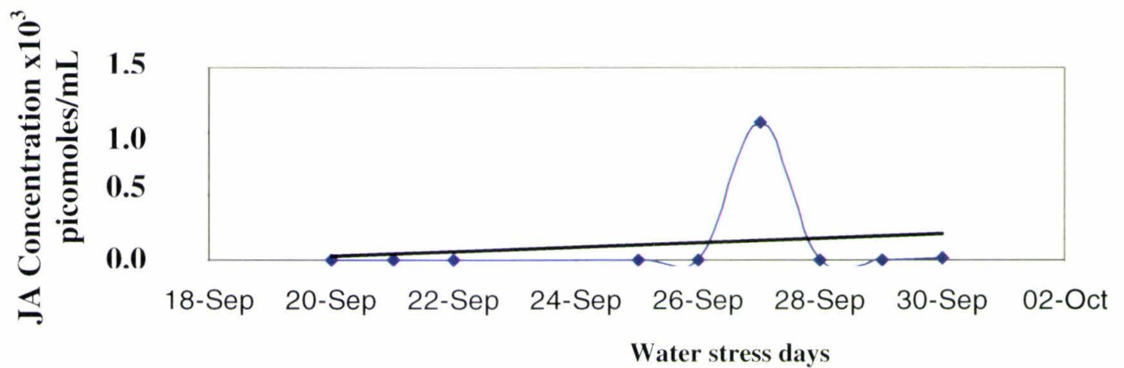


Fig 3.17 The concentration of JA in perennial ryegrass when subjected to a water stress. The increased concentration of the hormone JA, measured by ELISA in picomoles/mL (picomoles/g fresh weight), in four genotypes of perennial ryegrass (G1063, G1070, G1073 and G1071) during a water stress imposed by replacing only two-thirds of the water lost by evapotranspiration. The trend line is also shown. The genotypes were grown in the AgHort growth chamber at Massey University.

Fig 3.17 shows that, apart from the unexplained JA spike (the trend line is based around this spike), the endogenous levels of JA were very low. The reasons are likely to be similar to that discussed above for ABA in the growth chamber **(Section 3.7.2).**

In conclusion, this initial water stress trial provided information on the endogenous levels of ABA and JA in *L. perenne* under conditions of water stress and thereby providing a guide for the levels that needed to be externally applied to water sufficient plants to mimic water stress. This experiment also provided a guide under which water stress effects would be imposed without killing the plants.

3.7.4 Dipping experiments A and D

It was initially proposed that four separate ABA/JA combinations would be used (see **Table 2.9**). However, because some samples were lost, only combinations A and D could be processed.

Dipping experiment A

The SAFs after solid state extraction are given in **Table 3.13.**

Table 3.13 The efficiency of hormone extraction after solid state extraction, of *L. perenne* (G1076) for dipping trial A.

Trial A	Sample Counts (cpm)[a]	Recovery (%)[b]	SAF
A1	15420	66	1.5
A2	4140	18	5.6
A3	8540	37	2.7
A4	6880	30	3.4
A5	18940	81	1.2
A6	8200	35	2.8
A7	11580	50	2.0
A8	2120	9	11.0

[a]Samples were spiked with 20 µL of tritiated CA, which gave an input count of 23 216 cpm.
[b]Sample cpm / input cpm x 100.

The levels of ABA as determined by ELISA assay are given in **Table 3.14** and **Fig 3.18.**

Table 3.14 ABA levels in *L. perenne* tissue after dipping in an ABA/JA hormone solution [d].

		2nd ELISA absorption	3rd ELISA absorption	Mean 2nd, 3rd absorption	Solid state correction factor	Conc (pg/well)	Concentration (pg/ well) corrected for dilution and extraction losses [a]	2nd ELISA absorption 1 in 10 dilution	3rd ELISA absorption 1 in 10 dilution	Mean 2nd, 3rd dilution	Conc (pg/well)	Concentration (pg/ well) corrected for dilution and extraction losses [b]
A1	After dipping for 1 h	0.25	0.33	0.29	1.6	2	32	0.23	0.31	0.27	3	480
A2	1 h after dipping finished	0.26	0.24	0.25	1.7	3	51	0.27	0.26	0.26	5	850
A3	2 h after dipping finished	0.23	0.19	0.21	1.9	10.5	199.5	0.26	0.2	0.23	8	1520
A4	3 h after dipping finished	0.25	0.17	0.21	1.6	10.5	168	0.24	0.19	0.21	11	1760
A5	Undipped leaf after 1 h	0.2	0.17	0.18	1.6	13	208	0.21	0.18	0.19	30	4800
A6	Undipped leaf 1 h after finish	0.21	0.05	0.13	2.5	200	5000	0.24	0.16	0.2	20	5000
A7	Undipped leaf 2 h after finish	0.2	0.16	0.18	1.5	13	195	0.22	0.15	0.19	40	6000
A8	Undipped leaf 3 h after finish	0.24	0.17	0.21	1.7	10.5	178.5	0.34	0.21	0.28	4	680

2nd ELISA absorption 1 in 100 dilution	3rd ELISA absorption 1 in 100 dilution	Mean 2nd, 3rd dilution	Conc (pg/well	Concentration (pg/well) corrected for dilution and extraction losses [c]
0.236	0.202	0.22	6	9600
0.481	0.201	0.34	2	3400
				0
				0
0.396	0.159	0.28	2	3200
0.332	0.186	0.26	4	10000

[a] (H9*I9)*10 = SCF x concentration per well x 10.
[b] (N9*H9*100 = SCF x concentration per well x 100
[c] (S9*H9* 1000 = SFC x concentration per well x 1000
[d] Dipped in a solution of 10mg/ L JA and 20mg/L of ABA
Note: The data in blue were graphed; see **Fig 3.18**

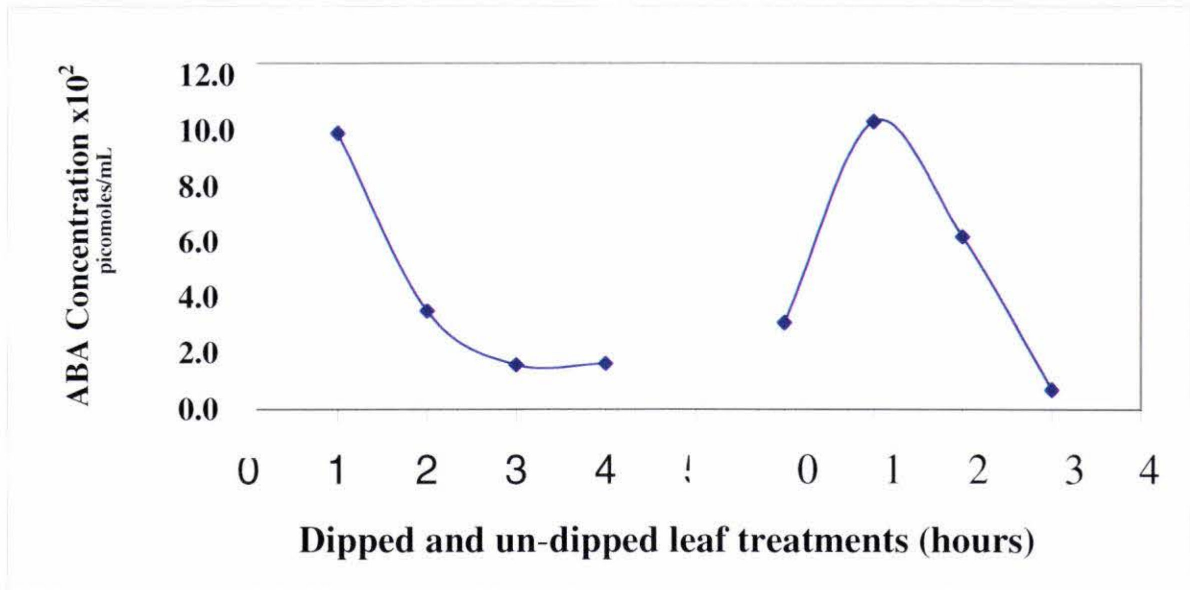


Fig 3.18 Tissue levels of ABA after genotype G1076 (endophyte-free perennial ryegrass) was dipped in a hormone solution containing 10 mg/L JA and 20 mg/L ABA. The plants were dipped for 1 h. The ABA levels were measured by ELISA after dipping, and after another 1, 2 and 3 h (**left**). The tissue levels of ABA in un-dipped G1076 tissue on the same time scale (**right**).

Fig 3.18 shows that ABA was taken up by *L. perenne* after 1 h of dipping in an ABA solution of concentration 20 mg/mL to give an endogenous level of ABA of 1000 picomoles/g fresh weight. The decrease with time would suggest that the initial uptake was rapidly transported out of the leaf tissue or alternatively was rapidly metabolized, starting from the end of dipping. Other research information indicates that the wounding of plant material, another stress, could induce an ABA response. Therefore, the act of cutting the leaf material to obtain a sample for processing may have also induced ABA production. This may account for the ABA spike produced in the un-dipped controls. If this information had been available before the start of the experiment, the four un-dipped controls would have been dipped in water and then used to measure the tissue level of ABA.

This dipping experiment confirmed that ABA is absorbed from a dipping solution into the vascular tissue of *L. perenne*.

The JA levels as determined by ELISA are given **in Table 3.15** and **Fig 3.19.**

Table 3.15 JA levels in *L. perenne* tissue levels after dipping in an ABA/JA hormone solution [c].

		ELISA absorption	Solid state correction factor	Conc (nm/well)	ELISA absorption 1 in 10 dilution	Conc (nm/well)	Concentration (nm/well) after corrected for dilution and extraction losses [a]	ELISA absorption 1 in 100 dilution	Conc per pot (nm/pot)	Concentration (nm/well) after corrected for for dilution and extraction losses [b]
A1	Undipped leaf after 1 h	0.08	1.20	9	0.08	9	1080	0.126	0.05	60
A2	Undipped leaf 1 h after finish	0.09	2.80	2	0.15	0.009	2.52	0.132	0.04	112
A3	Undipped leaf 2 h after finish	0.11	2.00	0.4	0.12	0.08	16			
A4	Undipped leaf 3 h after finish	0.15	11.00	0.2	0.15	0.2	220			
A5	After dipping for 1 h	0.10	1.50	0.7	0.14	0.05	7.5	0.17	0	0
A6	1 h after dipping finished	0.18	5.60	0	0.12	0.09	50.4	0.152	0.008	44.8
A7	2 h after dipping finished	0.11	2.70	0.7	0.14	0.02	5.4			0
A8	3 h after dipping finished	0.13	3.40	0.05	0.18	0	0			0

[a] (I8*100)*F8 = (concentration per well x 100) x SCF
[b] (L8*1000)*F8 = (concentration per well x 1000) x SCF
[c] Dipped in a 10 mg/mL JA and 20 mg/mL ABA solution.
Note: The data in blue were graphed; see **Fig 3.19.**

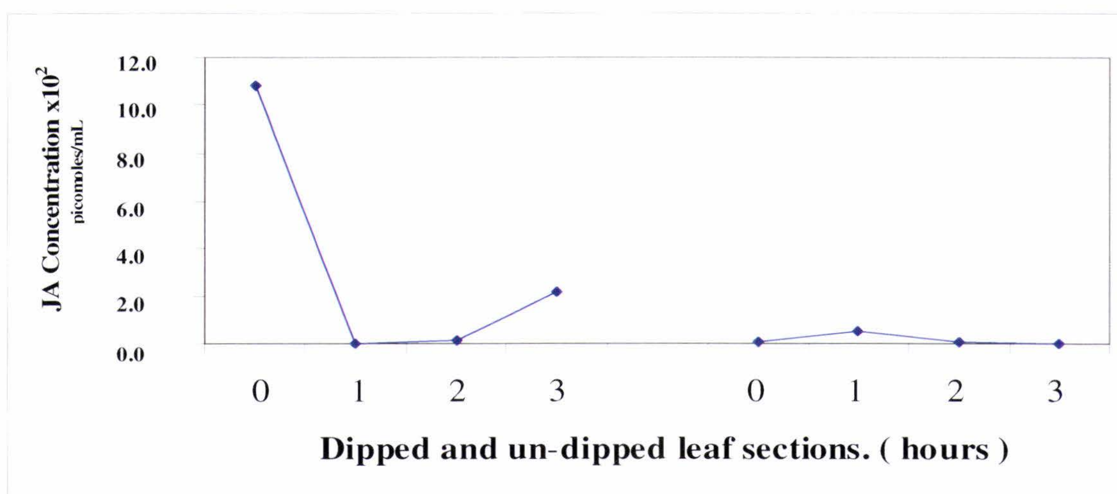


Fig 3.19 Tissue levels of JA after genotype G1076 (endophyte-free perennial ryegrass) was dipped in a hormone solution containing 10 mg/L JA and 20 mg/L ABA. The plants were dipped for 1 h. The JA levels were measured by ELISA after dipping, and after another 1, 2 and 3 h (**left**). The tissue levels of JA in un-dipped G1076 tissue on the same time scale (**right**).

Fig 3.19 shows that JA was absorbed into *L. perenne* tissue and that it was rapidly transported out of the leaf tissue. The information from the un-dipped samples indicates that there was no measurable JA stress response to the cutting of the leaf samples in this case, although JA is known to be involved in the final stress hormonal cascade.

Dipping experiment D.

The SAFs after solid state extraction are given in **Table 3.16.**

Table 3.16 The efficiency of hormone extraction after solid state extraction of *L. perenne* (G1076) for dipping trial D.

Trial D	Sample Counts (cpm)[a]	Recovery (%)[b]	SAF
D1	14820	64	1.6
D2	13700	59	1.7
D3	11980	52	1.9
D4	14420	62	1.6
D5	14680	63	1.6
D6	9420	41	2.5
D7	15860	68	1.5
D8	12540	58	1.7

[a]The original samples were spiked with 20 μL of tritiated CA, which gave an input count of 23 216 cpm.
[b]Sample cpm / input cpm x 100.

The ABA levels as determined by ELISA are given in Table **3.17** and **Fig 3.20**.

Table 3.17 ABA levels in *L. perenne* tissue after dipping in an ABA/JA hormone solution [c].

		ELISA absorption	Solid state correction factor	Conc (pg/well)	Concentration (nm/well) after corrected for for dilution and extraction losses [a]	ELISA absorption 1 in 10 dilution	Conc (pg/well)	Concentration (nm/well) after corrected for for dilution and extraction losses [b]
D1	After dipping for 1 h	0.44	1.6	0.3	4.8	0.53	0.1	16
D2	1 h after dipping finished	0.55	1.7	0.15	2.55	0.72	0.03	5.1
D3	2 h after dipping finished	0.77	1.9	0.02	0.38	0.61	0.08	15.2
D4	3 h after dipping finished	0.42	1.6	0.6	9.6	0.71	0.03	4.8
D5	Undipped leaf after 1 h	0.40	1.6	0.8	12.8	0.65	0.06	9.6
D6	Undipped leaf 1 h after finish	0.53	2.5	0.2	5	0.47	0.4	100
D7	Undipped leaf 2 h after finish	0.30	1.5	2	30	0.44	0.4	60
D8	Undipped leaf 3 h after finish	0.33	1.7	2	34	0.39	0.8	136

[a] (G8*10)*F8 = (Concentration per well x 10) x SCF
[b] (J8*100)*F8 = (Concentration per well x 100) x SCF
[c] Dipped in 1.25 mg/L JA and 2.5 mg/L ABA solution.
Note: The data in blue were graphed; see **Fig 3.20**.

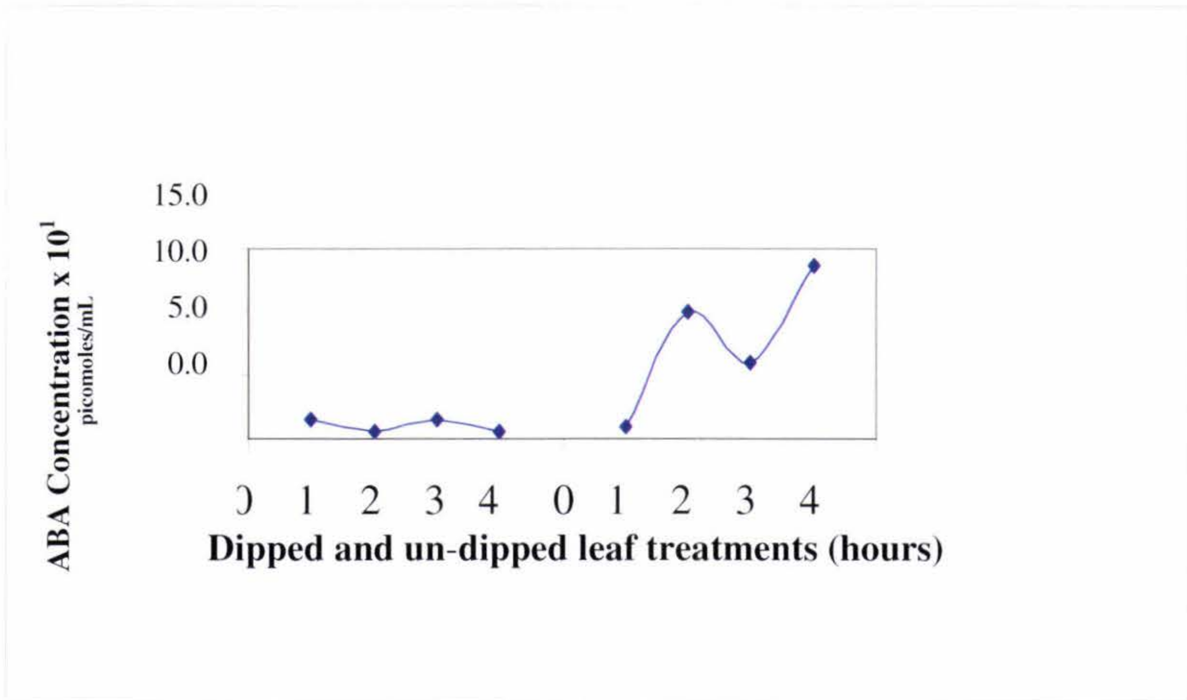


Fig 3.20 Tissue levels of ABA after genotype G1076 (endophyte-free perennial ryegrass) was dipped in a hormone solution containing 1.25 mg/L JA and 2.5 mg/L ABA. The plants were dipped for 1 h. The ABA levels were measured by ELISA after dipping, and after another 1, 2 and 3 h (**left**). The tissue levels of ABA in un-dipped G1076 tissue on the same time scale (**right**).

The concentration of ABA in dipping solution D was 2.5 mg/L. It may well have been too low to record any significant absorption. However, it may be that the stress response was initiated, because cutting the leaf material triggered a response (un-dipped samples).

The JA levels as determined by ELISA are given in **Table 3.18** and **Fig 3.21.**

Table 3.18 JA levels in *L. perenne* tissue after dipping in an ABA/JA hormone solution [a].

		ELISA absorption	Solid state correction factor	Conc (ng/well)[b]	Dilution and extraction losses	ELISA absorption 1 in 10 dilution	Conc (ng/well)	Concentration (nm/well) after corrected for for dilution and extraction losses [c]
D1	After dipping for 1h	0.11	1.6	0.07	1.12	0.14	0.001	0.16
D2	1 h after dipping finished	0.10	1.7	0.06	1.02	0.13	0.002	0.34
D3	2 h after dipping finished	0.07	1.9	100	1900	0.16	0	0
D4	3 h after dipping finished	0.08	1.6	12	192	0.18	0	0
D5	Undipped leaf after 1 h	0.12	1.6	0.015	0.24	0.13	0.002	0.32
D6	Undipped leaf 1 h after finish	0.17	2.5	0	0	0.11	0.07	17.5
D7	Undipped leaf 2 h after finish	0.19	1.5	0	0	0.20	0	0
D8	Undipped leaf 3 h after finish	0.14	1.7	0.009	0.153	0.17	0	0

a Dipping in 1.25 mg/L JA and 2.5 mg/L ABA solution.
b (G9*10)*F9 = (Concentration per well x 10) x SCF
c (J9*100)*F9 = Concentration per well x 100) X SCF
Note: The data in blue were graphed; see **Fig 3.21**.

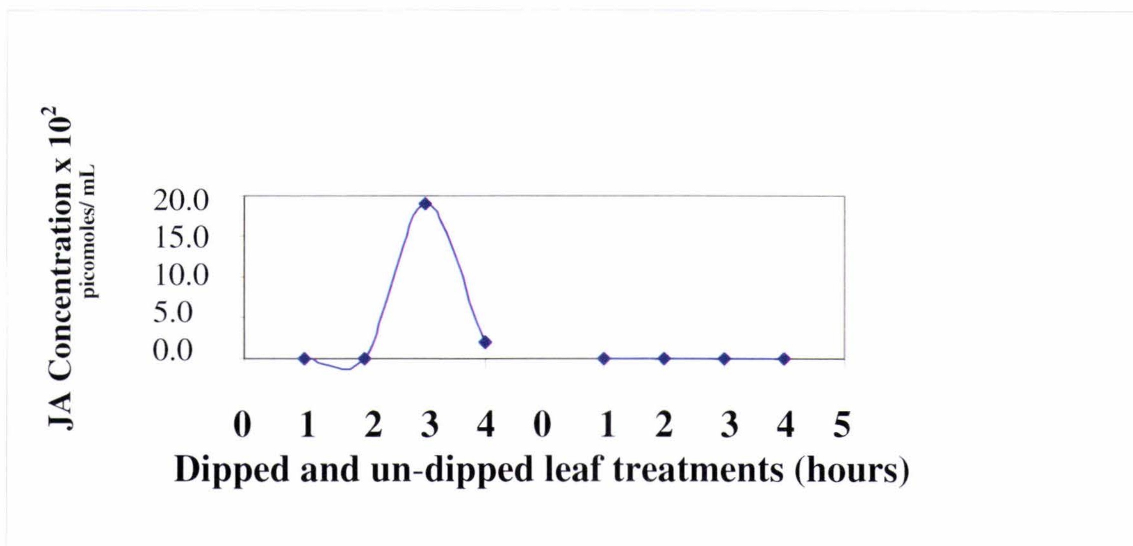


Fig 3.21 Tissue levels of JA after genotype G1076 (endophyte-free perennial ryegrass) was dipped in a hormone solution containing 1.25 mg/L JA and 2.5 mg/L ABA. The plants were dipped for 1 h. The JA levels were measured by ELISA after dipping, and after another 1, 2 and 3 h (**left**). The tissue levels of JA in un-dipped G1076 tissue on the same time scale (**right**).

These data, like those for dipping trial A, show that the 1.25 mg/L of JA was absorbed and that no stress response caused by cutting the leaf material was initiated.

The conclusions that can be drawn from these dipping experiments is that the hormones ABA and JA are absorbed from the dipping solution into the leaves of *L. perenne* by dipping in a hormone solution for no more than 1 h and that the hormones from the dipping solution diffuse into the plant sap until equilibrium is reached. However, the ABA stress response caused by cutting the leaves to obtain the test samples for ELISA needs further investigation; more progress would have been made in this area if the un-dipped samples had been taken from pots that had been dipped in water for 1 h rather than from pots that had not been dipped at all.

3.8 Effect of water deficit and plant hormones on the biosynthesis of alkaloids by endophytes

The aim of these experiments was two fold. Firstly to test the hypothesis that endophyte bioprotective metabolites are synthesized at higher levels in water stressed endophyte infected plants than in water-sufficient plants. Secondly to test the hypothesis that external application of plant hormones ABA and JA to water sufficient plants will increase levels of endophyte produced bio-protective metabolites thereby mimicking abiotic stress.

The aims of this experiment were to

(i) test the effect of water stress on the synthesis of endophyte alkaloids and

(ii) test whether the external application of ABA and JA to water sufficient *L. perenne* would act as signals that would trigger genes in the occupying mutualistic endophyte *N. lolii* to produce or increase the production of the alkaloids lolitrem B, ergovaline and peramine (See **Section 1.6**).

The potted perennial plants that were to be used in the main experiment were cut back to above the ligules on the 23rd December 2000 to ensure that new re-growth leaf tissue was available for the main experiment. The controlled water stress was first applied to the water stress (D +) treatments on the 12th of January 2001 and the water sufficient treatments were first dipped in ABA and JA solutions on the 20th January. The dipping continued every second day, 7 times, (**Section 2.4**) using increasing hormonal concentrations, as dictated by the preliminary experiment (**Section 3.7**). This procedure continued until the experiment ended on the 3rd of February 2001 when plant material was harvested, and then analysed by HPLC for the alkaloids lolitrem B, ergovaline and peramine (**Section 2.12.6**). A relative water content (RWC) method was used as an independent monitor of the application of the water deficit to the trial plants in the glasshouse and in the growth chamber (**Fig 3.22**).

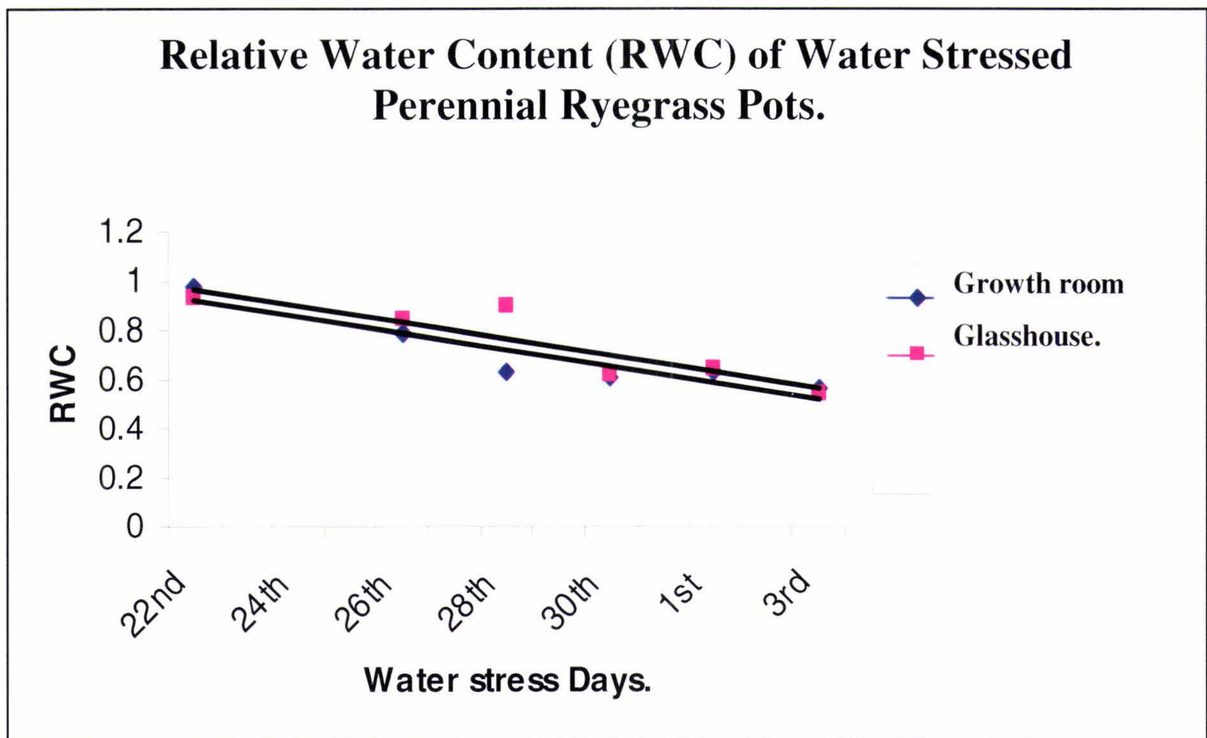


Fig 3.22 RWC of the water-stressed genotypes over the course of the main experiment. They were grown in four replicates in the AgResearch glasshouses (red) and in one replicate in the growth chamber at Massey University (blue). Four of the eight pots in each replicate were subjected to a controlled water stress, imposed by replacing only two-thirds of the water lost by evapotranspiration.

Arbitrarily chosen leaf samples were harvested every 2 days from the potted plants as the water stress was applied from the 22nd January. The method is outlined in **Section 2.12.4**. Using this method 0.95 or higher is normal, 0.7 to 0.8 is high stress and ABA accumulation. (*pers comm* Barker Department of Horticulture and Crop Science, Ohio State University, Columbus, Ohio, USA & Hume AgResearch, Grasslands Division, Palmerston North, New Zealand). **Fig 3.22** shows that the RWC decreased during the course of the experiment, confirming the water deficit status of the plant material that was used in the experiment.

3.8.1 HPLC results and ANOVA analysis for ergovaline, lolitrem B and peramine using the combined leaf and sheath results

The raw data obtained from HPLC analysis of ergovaline, lolitrem B and peramine were combined, sorted and checked using the SAS statistical analysis program (**Table 3.19**). SAS analysis of these data used the average of the combined leaf plus sheath results.

Table 3.19 Ergovaline, lolitrem B and peramine levels found in *L. perenne*[h].

Obs	Locate	Treat	Rep treat nos	JA	ABA	Water	Plant Part	Treat method	ppm Ergovaline	ppm Lolitrem B	ppm Peramine
1	Groom[a]	5	1	No	No	Dry	Leaf	Nil[c]	0.70	1.57	22.9
2	Groom	5	3	No	No	Dry	Leaf	Nil	0.13	0.51	9.7
3	Groom	5	6	No	No	Dry	Leaf	Nil	0.44	1.66	30.1
4	Groom	5	8	No	No	Dry	Leaf	Nil	0.65	1.69	35.0
5	Ghouse[b]	4	5	No	No	Dry	Leaf	Nil	1.50	4.92	76.6
6	Ghouse	4	6	No	No	Dry	Leaf	Nil	0.78	5.36	54.0
7	Ghouse	4	7	No	No	Dry	Leaf	Nil	1.36	6.01	69.3
8	Ghouse	4	8	No	No	Dry	Leaf	Nil	0.61	2.57	35.4
9	Ghouse	3	5	No	No	Dry	Leaf	Nil	1.50	6.41	74.4
10	Ghouse	3	6	No	No	Dry	Leaf	Nil	1.74	6.29	66.3
11	Ghouse	3	7	No	No	Dry	Leaf	Nil	1.12	5.38	59.2
12	Ghouse	3	8	No	No	Dry	Leaf	Nil	0.96	5.73	59.5
13	Ghouse	2	1	No	No	Dry	Leaf	Nil	0.37	2.26	33.4
14	Ghouse	2	2	No	No	Dry	Leaf	Nil	0.38	1.53	23.8
15	Ghouse	2	3	No	No	Dry	Leaf	Nil	0.31	1.60	30.8
16	Ghouse	2	4	No	No	Dry	Leaf	Nil	0.38	2.57	23.6
17	Ghouse	1	4	No	No	Dry	Leaf	Nil	0.52	3.97	41.0
18	Ghouse	1	1	No	No	Dry	Leaf	Nil	0.30	2.44	29.1
19	Ghouse	1	2	No	No	Dry	Leaf	Nil	0.20	1.55	21.5
20	Ghouse	1	3	No	No	Dry	Leaf	Nil	0.08	2.16	22.8
21	Groom	5	5	Yes	Yes	Wet	Leaf	JAW[d]	0.20	0.69	24.3
22	Ghouse	4	1	Yes	Yes	Wet	Leaf	JAW	0.59	5.04	80.6
23	Ghouse	3	1	Yes	Yes	Wet	Leaf	JAW	0.94	5.06	80.1
24	Ghouse	2	5	Yes	Yes	Wet	Leaf	JAW	0.30	5.24	60.9
25	Ghouse	1	5	Yes	Yes	Wet	Leaf	JAW	0.14	2.96	37.6
26	Groom	5	2	No	Yes	Wet	Leaf	AW[e]	0.24	0.63	32.8
27	Ghouse	4	2	No	Yes	Wet	Leaf	AW	1.01	3.64	83.7
28	Ghouse	3	2	No	Yes	Wet	Leaf	AW	1.21	5.98	70.7
29	Ghouse	2	6	No	Yes	Wet	Leaf	AW	0.20	2.03	48.7
30	Ghouse	1	6	No	Yes	Wet	Leaf	AW	0.17	4.70	33.4
31	Groom	5	7	Yes	No	Wet	Leaf	JW[f]	0.28	1.23	68.8
32	Ghouse	4	3	Yes	No	Wet	Leaf	JW	1.06	4.74	78.3
33	Ghouse	3	3	Yes	No	Wet	Leaf	JW	1.10	5.15	80.7
34	Ghouse	2	7	Yes	No	Wet	Leaf	JW	0.33	3.19	64.3
35	Ghouse	1	7	Yes	No	Wet	Leaf	JW	0.26	4.34	31.7
36	Groom	5	4	No	No	Wet	Leaf	W[g]	0.42	1.03	46.0
37	Ghouse	4	4	No	No	Wet	Leaf	W	0.60	5.00	60.3
38	Ghouse	3	4	No	No	Wet	Leaf	W	0.61	3.79	53.5
39	Ghouse	2	8	No	No	Wet	Leaf	W	0.21	2.98	45.0
40	Ghouse	1	8	No	No	Wet	Leaf	W	0.17	2.47	58.3
41	Groom	5	1	No	No	Dry	Sheath	Nil	3.00	4.56	48.0
42	Ghouse	5	3	No	No	Dry	Sheath	Nil	0.85	2.12	25.2
43	Groom	5	6	No	No	Dry	Sheath	Nil	2.87	6.32	66.5
44	Groom	5	8	No	No	Dry	Sheath	Nil	2.63	4.39	54.2
45	Ghouse	4	5	No	No	Dry	Sheath	Nil	5.23	11.46	90.8
46	Ghouse	4	6	No	No	Dry	Sheath	Nil	4.27	9.11	90.3
47	Ghouse	4	7	No	No	Dry	Sheath	Nil	4.64	10.12	99.3
48	Ghouse	4	8	No	No	Dry	Sheath	Nil	2.56	4.16	52.3
49	Ghouse	3	5	No	No	Dry	Sheath	Nil	5.15	19.21	97.4
50	Ghouse	3	6	No	No	Dry	Sheath	Nil	5.45	8.33	64.9
51	Ghouse	3	7	No	No	Dry	Sheath	Nil	4.12	13.44	78.1
52	Ghouse	3	8	No	No	Dry	Sheath	Nil	3.64	10.09	90.4
53	Ghouse	2	1	No	No	Dry	Sheath	Nil	3.37	11.50	56.1
54	Ghouse	2	2	No	No	Dry	Sheath	Nil	3.03	6.43	40.2
55	Ghouse	2	3	No	No	Dry	Sheath	Nil	3.24	8.85	69.0
56	Ghouse	2	4	No	No	Dry	Sheath	Nil	2.94	8.94	61.2
57	Ghouse	1	1	No	No	Dry	Sheath	Nil	2.57	14.15	63.2
58	Ghouse	1	2	No	No	Dry	Sheath	Nil	2.42	19.67	66.0
59	Ghouse	1	3	No	No	Dry	Sheath	Nil	1.00	5.05	31.6
60	Ghouse	1	4	No	No	Dry	Sheath	Nil	0.97	10.24	37.0
61	Ghouse	5	5	Yes	Yes	Wet	Sheath	JAW	0.61	2.77	30.0
62	Ghouse	4	1	Yes	Yes	Wet	Sheath	JAW	1.56	11.22	82.7
63	Ghouse	3	1	Yes	Yes	Wet	Sheath	JAW	1.64	15.18	52.0
64	Ghouse	2	5	Yes	Yes	Wet	Sheath	JAW	1.05	11.66	77.5
65	Ghouse	1	5	Yes	Yes	Wet	Sheath	JAW	0.70	18.80	54.2
66	Groom	5	2	No	Yes	Wet	Sheath	AW	0.69	2.45	35.7

67	Ghouse	4	2	No	Yes	Wet	Sheath	AW	1.95	13.24	79.9
68	Ghouse	3	2	No	Yes	Wet	Sheath	AW	2.39	12.80	58.6
69	Ghouse	2	6	No	Yes	Wet	Sheath	AW	0.84	8.60	60.6
70	Ghouse	1	6	No	Yes	Wet	Sheath	AW	0.49	9.38	34.6
71	Groom	5	7	Yes	No	Wet	Sheath	JW	1.08	7.22	65.0
72	Ghouse	4	3	Yes	No	Wet	Sheath	JW	2.41	12.90	74.7
73	Ghouse	3	3	Yes	No	Wet	Sheath	JW	1.49	13.97	61.8
74	Ghouse	2	7	Yes	No	Wet	Sheath	JW	1.07	14.78	64.8
75	Ghouse	1	7	Yes	No	Wet	Sheath	JW	0.90	13.96	47.5
76	Groom	5	4	No	No	Wet	Sheath	W	0.86	5.19	24.5
77	Ghouse	4	4	No	No	Wet	Sheath	W	1.58	9.09	59.8
78	Ghouse	3	4	No	No	Wet	Sheath	W	1.21	9.39	35.2
79	Ghouse	2	8	No	No	Wet	Sheath	W	0.89	13.89	52.1
80	Ghouse	1	8	No	No	Wet	Sheath	W	0.77	20.87	60.2

[a]Growth chamber.

[b]Glasshouse.

[c]No hormone treatment.

[d]Water-sufficient plants dipped every 2 days in JA/ABA solutions of increasing concentration (**Tables 2.12** and **2.13**).

[e]Water-sufficient plants dipped every 2 days in ABA solutions of increasing concentration.

[f]Water-sufficient plants dipped every 2 days in JA solutions of increasing concentration.

[g]Plants kept in a water-sufficient condition without dipping (controls).

[h]Levels of alkaloids (ppm) in dried leaf and sheath material harvested at the end of the main experiment.

3.8.1.1 Ergovaline

On average, the ergovaline concentration in the sheath was around 3 fold higher than that in the leaves (1.61 and 0.54 ppm, respectively, $P < 0.001$), (**Table 3.20**). Overall, there was a significant treatment effect. However, the single-degree-of-freedom contrasts indicated that this could be attributed to a significant effect of the drought treatment ($P < 0.001$). None of the hormone treatments had a significant effect on the ergovaline levels (**Table 3.20**).

Table 3.20 Ergovaline concentration (ppm) in perennial ryegrass subjected to plant hormone and water deficit treatments.

	Leaves	Sheath	Mean
Water only (W)[a]	0.40	1.06	0.73
ABA + water (AW)[a]	0.57	1.27	0.92
JA + water (JW)[a]	0.61	1.39	1.00
ABA + JA + W (JAW)[a]	0.43	1.11	0.77
Water deficit (D)[b]	0.70	3.20	1.95
Mean	0.54	1.61	
Statistical analysis			
treatment	$F_{4,16} = 25.32$ ***		
plant part	$F_{1,50} = 138.6$ ***		
trt x part	$F_{4,50} = 10.83$ ***		
Single-degree-of-freedom contrasts			
W vs D	$F_{1,16} = 99.4$ ***		
W vs AW	$F_{1,16} = 0.73$ ns		
W vs JW	$F_{1,16} = 1.47$ ns		
ABA x JA	$F_{1,16} = 1.76$ ns		

(ns, $P > 0.05$; ***, $P \leq 0.001$.)
[a]Means of 5 replicates. Pooled glasshouse and growth room data.
[b]Mean of 20 replicates.

A significant treatment * plant-part interaction ($P < 0.001$) was the result of a greater increase (4–6 fold) in ergovaline in the sheath than in the leaves during the water deficit, compared with the other treatments (averaging 2–4 fold).

When the leaf data were analysed separately (i.e. the sheath data excluded), a significant 1.4 fold effect was found for ABA (mean = 0.57 ppm) compared with water only (mean = 0.40 ppm) (Pr > F = 0.0592). There was also a significant negative ABA * JA interaction (Pr > F = 0.0310), in which the ergovaline concentration with both hormones applied (0.43 ppm) was less than that with either ABA or JA applied (0.57 or 0.61 ppm, respectively) and similar to that for water only (0.40 ppm). The effect of water deficit was also highly significant, producing a 1.75 fold increase (0.70 ppm compared with 0.40 ppm) (Pr > F = 0.0011). This was greater in the sheath, producing a 3 fold increase (3.2 ppm for the sheath compared with 1.06 ppm for water only). When the sheath data were analysed separately (i.e. the leaf data excluded), all hormone effects were not significant, and only water deficit effects were significant for all three hormone treatments. However, it was clear that the two tissues, leaf and sheath, responded differently to all treatments ($P \leq 0.001$). The sheath was very responsive to all treatments and the leaf was less responsive.

3.8.1.2 Lolitrem B

On average, the lolitrem B concentration was over 3 fold higher in the sheath than in the leaf (10.98 ppm compared with 3.46 ppm, respectively, $P < 0.001$). The leaves and sheaths (plant parts) responded differently. The sheath was very responsive to drought and the leaf was less responsive ($P \leq 0.001$). None of the hormone treatments had a significant effect on the levels of lolitrem B (**Table 3.21**).

Table 3.21 Lolitrem B concentration (ppm) in perennial ryegrass subjected to plant hormone and water deficit treatments.

	Leaves	Sheath	Mean
Water only (W)[a]	3.05	11.69	7.37
ABA + water (AW)[a]	3.40	9.29	6.35
JA + water (JW)[a]	3.73	12.57	8.15
ABA + JA + W (JAW)[a]	3.80	11.93	7.87
Water deficit (D)[b]	3.31	9.41	6.36
Mean	3.46	10.98	7.22
Statistical Analysis			
treatment	$F_{4,16} = 2.10$ ns		
plant part	$F_{1,50} = 106.4$***		
trt x part	$F_{4,50} = 0.80$ ns		
Single-degree-of-freedom contrasts			
W vs D	$F_{1,16} = 4.63$ ns		
W vs AW	$F_{1,16} = 1.06$ ns		
W vs JW	$F_{1,16} = 0.61$ ns		
ABA x JA	$F_{1,16} = 0.27$ ns		

(ns, $P > 0.05$; ***, $P \leq 0.001$.)
[a]Means of 5 replicates. Pooled glasshouse and growth room data.
[b]Mean of 20 replicates.

3.8.1.3 Peramine

On average, the peramine concentration was significantly greater in the sheath than in the leaves (57.3 and 53.8 ppm, respectively, $P \leq 0.001$). There was a significant treatment * plant-part interaction ($P < 0.0001$) of peramine in the sheath, compared with in the leaf, during water deficit (64.1 and 40.9 ppm, respectively), but this decreased slightly for the other treatments (averaging 57.0 and 55.6 ppm, respectively). There was an increase of 0.45 fold in the peramine concentration for JA dipping compared with the water-only controls (63.8 and 49.5 ppm, respectively, $P \leq 0.1$) (**Table 3.22**).

Table 3.22 Peramine concentration (ppm) in perennial ryegrass subjected to plant hormone and water deficit treatments.

	Leaves	Sheath	Mean
Water only (W)[a]	52.6	46.4	49.5
ABA + water (AW)[a]	53.9	53.9	53.9
JA + water (JW)[a]	64.8	62.8	63.8
ABA + JA +W (JAW)[a]	56.7	59.3	58.0
Water deficit (D)[b]	40.9	64.1	52.5
Mean	53.8	57.3	
Statistical analysis			
treatment	$F_{4,16} = 1.07$ n		
Plant part	$F_{1,50} = 15.48$		
trt x part	$F_{4,50} = 5.11$ **		
Single-degree-of-freedom contrasts			
W vs D	$F_{1,16} = 0.87$ ns		
W vs AW	$F_{1,16} = 0.29$ ns		
W vs JW	$F_{1,16} = 3.12$ †		
ABA x JA	$F_{1,16} = 0.79$ ns		

(ns, $P > 0.05$; †, $P \leq 0.1$ ***, $P \leq 0.001$.)
[a]Means of 5 replicates. Pooled glasshouse and growth room data.
[b]Mean of 20 replicates.

3.8.2 Correlation analysis of the three alkaloids and MAOVA

The concentrations of the three alkaloids were correlated (**Table 3.23**), with weak but positive relationships between them.

Table 3.23 Correlation coefficient (r) between the three alkaloids ergovaline, lolitrem B and peramine for the water-sufficient, hormone-treated and water-deficit-treated plants (n-1 = 79).

	Lolitrem B	Peramine
Ergovaline	**0.51**	**0.61**
Lolitrem B	_	**0.51**

Because of the positive correlation between the three alkaloids, a multiple analysis of variance (MANOVA) was carried out, using the same model as for the ANOVA (**Table 3.24**).

Table 3.24 Multivariate analysis of variance (MANOVA) for a combined analysis of the three alkaloids.

Source of Variation	Degrees of Freedom	Pr > F[b]
Treatment[a]	5	<0.0001 ***
W vs D	1	<0.0001 ***
JA main effect	1	0.2805 ns
ABA main effect	1	0.1069 ns
JA* ABA interaction	1	0.1124 ns
Plant part	1	<0.0001 ***
Trt * Part	5	<0.0001 ***

[a]Main plot effects were tested against the block*treatment interaction.
[b]F statistic calculated using the multivariate, Wilk's Lambda.

This MANOVA analysis confirmed the ANOVA statistics. When all treatments were considered together, overall, there was a highly significant treatment effect (Pr ≤ 0.0001). However, when single degrees of freedom were considered, the main contributors to this result were drought and plant part (Pr ≤0.0001). The treatment * plant-part interaction (Pr ≤ 0.0001) was highly significant and was an indication that the sheath was highly responsive to the treatments but that there was little if any response by the leaves. None of the hormone treatments were significant.

3.8.3 HPLC results and ANOVA analysis for ergovaline, lolitrem B and peramine using only the leaf results

The HPLC results were re-sorted to statistically analyse the leaf and sheath results independently of each other. The mean values of alkaloids in the leaves are shown in **Table 3.25**.

Table 3.25 Ergovaline, lolitrem B and peramine levels found in *L. perenne* leaves[g].

Ob	Locate	Rep	Rep Treat nos	JA	ABA	Water	Plant part	Treat	ppm Ergovaline	ppm Lolitrem B	ppm Peramine
1	Groom[a]	5	1	No	No	Dry	leaf	nil[c]	0.7	1.57	22.9
2	Groom	5	3	No	No	Dry	leaf	nil	0.13	0.51	9.7
3	Groom	5	6	No	No	Dry	leaf	nil	0.44	1.66	30.1
4	Groom	5	8	No	No	Dry	leaf	nil	0.65	1.69	35.0
5	Ghouse[b]	4	5	No	No	Dry	leaf	nil	1.5	4.96	76.6
6	Ghouse	4	6	No	No	Dry	leaf	nil	0.78	5.36	54
7	Ghouse	4	7	No	No	Dry	leaf	nil	1.36	6.01	69.3
8	Ghouse	4	8	No	No	Dry	leaf	nil	0.61	2.57	35.4
9	Ghouse	3	5	No	No	Dry	leaf	nil	1.5	6.41	74.4
10	Ghouse	3	6	No	No	Dry	leaf	nil	1.74	6.29	66.3

11	Ghouse	3	7	No	No	Dry	leaf	nil	1.12	5.38	59.2
12	Ghouse	3	8	No	No	Dry	leaf	nil	0.96	5.73	59.5
13	Ghouse	2	1	No	No	Dry	leaf	nil	0.37	2.26	33.4
14	Ghouse	2	2	No	No	Dry	leaf	nil	0.38	1.53	23.8
15	Ghouse	2	3	No	No	Dry	leaf	nil	0.31	1.6	30.8
16	Ghouse	2	4	No	No	Dry	leaf	nil	0.38	2.57	23.6
17	Ghouse	1	4	No	No	Dry	leaf	nil	0.52	3.97	41.0
18	Ghouse	1	1	No	No	Dry	leaf	nil	0.3	2.44	29.1
19	Ghouse	1	2	No	No	Dry	leaf	nil	0.2	1.55	21.5
20	Ghouse	1	3	No	No	Dry	leaf	nil	0.08	2.16	22.8
21	Groom	5	5	Yes	Yes	Wet	leaf	JAW[d]	0.2	0.69	24.3
22	Ghouse	4	1	Yes	Yes	Wet	leaf	JAW	0.59	5.04	80.6
23	Ghouse	3	1	Yes	Yes	Wet	leaf	JAW	0.94	5.06	80.1
24	Ghouse	2	5	Yes	Yes	Wet	leaf	JAW	0.3	0.3	60.9
25	Ghouse	1	5	Yes	Yes	Wet	leaf	JAW	0.14	0.14	37.6
26	Groom	5	2	No	Yes	Wet	leaf	AW[e]	0.24	0.63	32.8
27	Ghouse	4	2	No	Yes	Wet	leaf	AW	1.01	3.64	83.7
28	Ghouse	3	2	No	Yes	Wet	leaf	AW	1.21	5.98	70.7
29	Ghouse	2	6	No	Yes	Wet	leaf	AW	0.2	2.03	48.7
30	Ghouse	1	6	No	Yes	Wet	leaf	AW	0.17	4.7	33.4
31	Groom	5	7	Yes	No	Wet	leaf	JA[f]	0.28	1.23	68.8
32	Ghouse	4	3	Yes	No	Wet	leaf	JA	1.06	4.74	78.3
33	Ghouse	3	3	Yes	No	Wet	leaf	JA	1.1	5.15	80.7
34	Ghouse	2	7	Yes	No	Wet	leaf	JA	0.33	3.19	64.3
35	Ghouse	1	7	Yes	No	Wet	leaf	JA	0.26	4.34	31.7
36	Groom	5	4	No	No	Wet	leaf	W[g]	0.42	1.03	46.0
37	Ghouse	4	4	No	No	Wet	leaf	W	0.6	5	60.3
38	Ghouse	3	4	No	No	Wet	leaf	W	0.61	3.79	53.5
39	Ghouse	2	8	No	No	Wet	leaf	W	0.21	2.98	45.0
40	Ghouse	1	8	No	No	Wet	leaf	W	0.17	2.47	58.3

[a]Growth chamber.
[b]Glasshouse.
[c]No hormone treatment.
[d]Water-sufficient plants dipped every 2 days in JA/ABA solutions of increasing concentration.
[e]Water-sufficient plants dipped every 2 days in ABA solutions of increasing concentration.
[f]Water-sufficient plants dipped every 2 days in JA solutions of increasing concentration.
[g]Plants kept in a water-sufficient condition without dipping (controls).
[g]Levels of alkaloids (ppm) in dried leaf material harvested at the end of the main experiment.

3.8.3.1. Ergovaline

The levels of ergovaline were significantly higher in the leaves of the water-stressed plants than in those of the water-sufficient plants (0.7 ppm compared with 0.4 ppm, a 1.74 fold increase, $P \leq 0.001$) (**Table 3.26**).

Table 3.26 Ergovaline concentration (ppm) in perennial ryegrass leaves subjected to plant hormone and water deficit treatments.

	Leaves
Water only (W)[a]	0.40
ABA + water (AW)[a]	0.57
JA + water (JW)[a]	0.61
ABA + JA + water (JAW)[a]	0.43
Water deficit (D)[b]	0.70
Mean	0.54
Statistical analysis	
treatment $F_{4,16} = 5.41$ ***	
Single-degree-of-freedom contrasts	
W vs D $F_{1,16} = 15.78$ ***	
W vs AW $F_{1,16} = 2.67$ ns	
W vs JW $F_{1,16} = 4.13$ †	
ABA x JA $F_{1,16} = 5.60$ ‡	

(ns, $P > 0.05$; †, $P \leq 0.05$; ‡, $P \leq 0.01$; ***, $P \leq 0.001$.)
[a]Means of 5 replicates. Pooled glasshouse and growth room data.
[b]Mean of 20 replicates.

There was a 1.51 fold increase in the level of ergovaline when the plants were dipped in JA, with 0.4 ppm for the water-only controls and 0.61 ppm for the dipped pots. This increase was statistically significant (P = 0.05). When the plants were dipped in a mixture of JA and ABA, the levels of ergovaline dropped significantly (P = 0.03), from 0.57 ppm for ABA treatment and 0.61 ppm for JA treatment to 0.43 ppm. This level decreased to the water-only ergovaline level of 0.40 ppm and was statistically significant (Pr = 0.03).

3.8.3.2. Lolitrem B

None of the treatments, including water stressing, had any significant effect on the lolitrem B levels (**Table 3.27**).

Table 3.27 Lolitrem B concentration (ppm) in perennial ryegrass leaves subjected to plant hormone and water deficit treatments.

	Leaves
Water only (W)[a]	3.05
ABA + water (AW)[a]	3.40
JA + water (JW)[a]	3.73
ABA + JA + water (JAW)[a]	3.80
Water deficit (D)[b]	3.31
Mean	3.46
Statistical analysis	
treatment $F_{4,16} = 0 .44$ ns	
Single-degree-of-freedom contrasts	
W vs D $F_{1,16} = 0.29$ ns	
W vs AW $F_{1,16} = 0.25$ ns	
W vs JW $F_{1,16} = 0.95$ ns	
ABA x JA $F_{1,16} = 0.08$ ns	

(ns, $P > 0.05$; †, $P \leq 0.05$; ‡, $P \leq 0.01$; ***, $P \leq 0.001$.)
[a]Means of 5 replicates.
[b]Mean of 20 replicates.

3.8.3.3. Peramine

The application of water stress to the pots greatly decreased the production of peramine by the endophyte. The peramine levels fell from 52.6 to 40.9 ppm on average, i.e. a 0.22 fold decrease (**Table 3.28**). This decrease in production of the alkaloid in the leaves was highly significant ($P \leq 0.001$) during water stress.

Table 3.28 Peramine concentration (ppm) in perennial ryegrass leaves subjected to plant hormone and water deficit treatments.

	Leaves
Water only (W)[a]	52.62
ABA + water (AW)[a]	53.86
JA + water (JW)[a]	64.76
ABA + JA + water (JAW)[a]	56.70
Water deficit (D)[b]	40.92
Mean	53.77
Statistical analysis	
treatment $F_{4,16} = 4.52$ †	
Single-degree-of-freedom contrasts	
W vs D $F_{1,16} = 15.4$ *** (0.0012)	
W vs AW $F_{1,16} = 0.02$ ns	
W vs JW $F_{1,16} = 2.2$ ns	
ABA x JA $F_{1,16} = 0.65$ ns	

(ns, $P > 0.05$; †, $P \leq 0.05$; ‡, $P \leq 0.01$; ***, $P \leq 0.001$.)
[a]Means of 5 replicates. Pooled glasshouse and growth room data.
[b]Mean of 20 replicates.

None of the hormone treatments had a significant effect on the production of peramine in the leaves, either positively or negatively.

3.8.3 HPLC results and ANOVA analysis for ergovaline, lolitrem B and peramine using only the sheath results

The data were sorted into sheath-only results (**Table 3.29**).

Table 3.29 Ergovaline, lolitrem B and peramine levels found in *L. perenne* sheaths[h].

Obs	Locate	Rep	Treat nos	JA	ABA	Water	Plant part	Treat	ppm Ergovaline	ppm Lolitrem B	ppm Peramine
1	Groom[a]	5	1	No	No	Dry	Sheath	nil[c]	3.0	4.56	48.0
2	Groom	5	3	No	No	Dry	Sheath	nil	0.85	2.12	25.2
3	Groom	5	6	No	No	Dry	Sheath	nil	2.87	6.32	66.5
4	Groom	5	8	No	No	Dry	Sheath	nil	2.63	4.39	54.2
5	Ghouse[b]	4	5	No	No	Dry	Sheath	nil	5.23	11.46	90.8
6	Ghouse	4	6	No	No	Dry	Sheath	nil	4.27	9.11	90.3
7	Ghouse	4	7	No	No	Dry	Sheath	nil	4.64	10.12	99.3
8	Ghouse	4	8	No	No	Dry	Sheath	nil	2.56	4.16	52.3
9	Ghouse	3	5	No	No	Dry	Sheath	nil	5.15	19.21	97.4
10	Ghouse	3	6	No	No	Dry	Sheath	nil	5.45	8.33	64.9
11	Ghouse	3	7	No	No	Dry	Sheath	nil	4.12	13.44	78.1
12	Ghouse	3	8	No	No	Dry	Sheath	nil	3.64	10.09	90.4
13	Ghouse	2	1	No	No	Dry	Sheath	nil	3.37	11.5	56.1
14	Ghouse	2	2	No	No	Dry	Sheath	nil	3.03	6.43	40.2
15	Ghouse	2	3	No	No	Dry	Sheath	nil	3.24	8.85	69.0

16	Ghouse	2	4	No	No	Dry	Sheath	nil	2.94	8.94	61.2
17	Ghouse	1	4	No	No	Dry	Sheath	nil	2.57	14.15	63.2
18	Ghouse	1	1	No	No	Dry	Sheath	nil	2.42	19.67	66.0
19	Ghouse	1	2	No	No	Dry	Sheath	nil	1.0	5.05	31.6
20	Ghouse	1	3	No	No	Dry	Sheath	nil	0.97	10.24	37.0
21	Groom	5	5	Yes	Yes	Wet	Sheath	JAW[d]	0.61	2.77	30.0
22	Ghouse	4	1	Yes	Yes	Wet	Sheath	JAW	1.56	11.22	82.7
23	Ghouse	3	1	Yes	Yes	Wet	Sheath	JAW	1.64	15.18	52.0
24	Ghouse	2	5	Yes	Yes	Wet	Sheath	JAW	1.05	11.66	77.5
25	Ghouse	1	5	Yes	Yes	Wet	Sheath	JAW	0.7	18.8	54.2
26	Groom	5	2	No	Yes	Wet	Sheath	AW[e]	0.69	2.45	35.7
27	Ghouse	4	2	No	Yes	Wet	Sheath	AW	1.95	13.24	79.9
28	Ghouse	3	2	No	Yes	Wet	Sheath	AW	2.39	12.8	58.6
29	Ghouse	2	6	No	Yes	Wet	Sheath	AW	0.84	8.6	60.6
30	Ghouse	1	6	No	Yes	Wet	Sheath	AW	0.49	9.38	34.6
31	Groom	5	7	Yes	No	Wet	Sheath	JA[f]	1.08	7.22	65.0
32	Ghouse	4	3	Yes	No	Wet	Sheath	JA	2.41	12.9	74.7
33	Ghouse	3	3	Yes	No	Wet	Sheath	JA	1.49	13.97	61.8
34	Ghouse	2	7	Yes	No	Wet	Sheath	JA	1.07	14.78	64.8
35	Ghouse	1	7	Yes	No	Wet	Sheath	JA	0.9	13.96	47.5
36	Groom	5	4	No	No	Wet	Sheath	W[g]	0.86	5.19	24.5
37	Ghouse	4	4	No	No	Wet	Sheath	W	1.58	9.09	59.8
38	Ghouse	3	4	No	No	Wet	Sheath	W	1.21	9.39	35.2
39	Ghouse	2	8	No	No	Wet	Sheath	W	0.89	13.89	52.1
40	Ghouse	1	8	No	No	Wet	Sheath	W	0.77	20.87	60.2

[a]Growth chamber.
[b]Glasshouse.
[c]No hormone treatment.
[d]Water-sufficient plants dipped every 2 days in JA/ABA solutions of increasing concentration.
[e]Water-sufficient plants dipped every 2 days in ABA solutions of increasing concentration.
[f]Water-sufficient plants dipped every 2 days in JA solutions of increasing concentration.
[g]Plants kept in a water-sufficient condition without dipping (controls).
[h]Levels of alkaloids (ppm) in dried sheath material harvested at the end of the main experiment.

3.8.3.1 Ergovaline

When a water stress was applied to the perennial ryegrass pots, there was a 3 fold increase in the production of ergovaline by the endophyte in the sheath (**Table 3.30**). This was highly significant, with the levels of ergovaline increasing from 1.1 ppm in the water-only controls to 3.2 ppm in the water-stressed plants ($P \leq 0.001$). There were no significant increases for any of the hormone treatments.

Table 3.30 Ergovaline concentration (ppm) in perennial ryegrass sheaths subjected to plant hormone and water deficit treatments.

	Sheath
Water only (W)[a]	1.1
ABA + water (AW)[a]	1.3
JA + water (JW)[a]	1.4
ABA + JA + water (JAW)[a]	1.1
Water deficit (D)[b]	3.2
Mean	1.6
Statistical analysis	
treatment $F_{4,16} = 12.95$ ***	
Single-degree-of-freedom contrasts	
W vs D $F_{1,16} = 117.17$ ***	
W vs AW $F_{1,16} = 0.33$ ns	
W vs JW $F_{1,16} = 0.80$ ns	
ABA x JA $F_{1,16} = 0.88$ ns	

(ns, $P > 0.05$; †, $P \leq 0.05$; ‡, $P \leq 0.01$; ***, $P \leq 0.001$.)
[a]Means of 5 replicates. Pooled glasshouse and growth room data.
[b]Mean of 20 replicates.

3.8.3.2. Lolitrem B

When a water stress was applied to perennial ryegrass, there was a 1.2 fold reduction in the production of lolitrem B by the endophyte in the sheath tissue (**Table 3.31**). This reduction was statistically significant ($P \leq 0.05$). The hormone treatments produced no significant effects.

Table 3.31 Lolitrem B concentration (ppm) in perennial ryegrass sheaths subjected to plant hormone and water deficit treatments.

	Sheath
Water only (W)[a]	11.69
ABA + water (AW)[a]	9.29
JA + water (JW)[a]	12.57
ABA + JA + water (JAW)[a]	11.9
Water deficit (D)[b]	9.40
Mean	10.98
Statistical analysis	
treatment $F_{4,16} = 1.97$ ns	
Single-degree-of-freedom contrasts	
W vs D $F_{1,16} = 4.39$ †	
W vs AW $F_{1,16} = 1.63$ ns	
W vs JW $F_{1,16} = 0.22$ ns	
ABA x JA $F_{1,16} = 0.44$ ns	

(ns, $P > 0.05$; †, $P \leq 0.05$; ‡, $P \leq 0.01$; ***, $P \leq 0.001$.)
[a]Means of 5 replicates. Pooled glasshouse and growth room data.
[b]Mean of 20 replicates.

3.8.3.3. Peramine

All treatments gave relatively large increases in the concentration of peramine in the sheaths (**Table 3.32**). Application of the water stress induced a 1.4 fold increase in the production of peramine in the sheath by the endophyte, with plant tissue levels increasing from 46 to 64 ppm when the water stress was applied. This increase was significant (Pr = 0.07). Treatment with JA gave a 35% increase, from 46 ppm in the water-only controls to 63 ppm (P = 0.08). Other hormone-induced increases were not significant.

Table 3.32 Peramine concentration (ppm) in perennial ryegrass sheaths subjected to plant hormone and water deficit treatments.

	Sheath
Water only (W)[a]	46.36
ABA + water (AW)[a]	53.90
JA + water (JW)[a]	62.70
ABA + JA + water (JAW)[a]	59.28
Water deficit (D)[b]	64.10
Mean	57.3
Statistical analysis	
treatment $F_{4,16} = 1.33$ ns	
Single-degree-of-freedom contrasts	
W vs D $F_{1,16} = 3.55$ (0.0777)	
W vs AW $F_{1,16} = 0.69$ ns	
W vs JW $F_{1,16} = 3.3$ (0.0882)	
ABA x JA $F_{1,16} = 0.74$ ns	

(ns, $P > 0.05$; †, $P \leq 0.05$; ‡, $P \leq 0.01$; ***, $P \leq 0.001$.)
[a]Means of 5 replicates. Pooled glasshouse and growth room data.
[b]Mean of 20 replicates.

3.9 Production and acquisition of stable internal standards

This work was carried out to produce internal standards for the HPLC analysis of JA, ABA and SA. These plant hormones were expected to be found in perennial ryegrass leaf and pseudostem tissue samples collected from the plants that had been subjected to the eight treatment regimes of the main experiment.

3.9.1 Deuteration of methyl jasmonate

The products were checked by thin layer chromatography (**Fig 3.23**). The yield was then measured and was found to be of the order of 94.7% for the deuterated methyl jasmonate.

Fig 3.23 Thin layer chromatogram of the reactants and products used in the reaction that produced deuterated methyl jasmonate. It can be used as an HPLC internal standard in the analysis of perennial ryegrass leaf and pseudostem tissue for JA. The reactants (**A**), the products (**B**) and the reactants plus the products of the reaction (**C**).

A summary of the deuteration reaction is given in **Fig 3.24**.

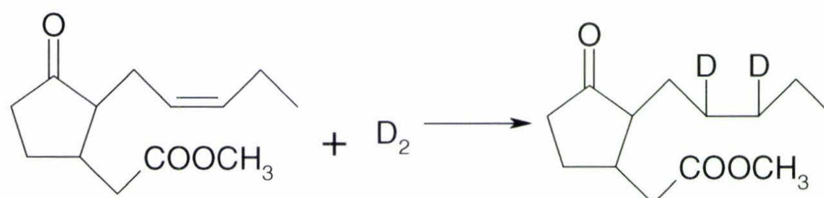


Fig 3.24 Chemical equation of the deuteration reaction. The addition of deuterium is across the double bond between carbon 3 and carbon 4 of methyl jasmonate. This product gives a stable internal standard to be used in the HPLC analysis of JA in plant tissue.

3.9.2 Acquisition of D_6 ABA from Australia

As the synthesis of deuterated ABA is particularly difficult, we were very fortunate to be sent a sample from Dr Brian Loveys of CSIRO Plant Industry, Horticultural Research Unit, Adelaide, Australia. The deuteration reaction is shown in **Fig 3.25**.

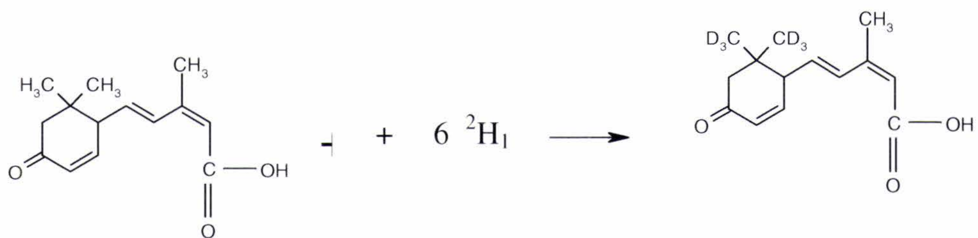


Fig 3.25 Structure of deuterated ABA.

It was pointed out by Dr Loveys that they normally monitor the ions 190, 194, 162 and 166. **Fig 3.26** shows that the sample received was virtually free of D_0 ions.

```
File        : C:\HPCHEM\1\DATA\SUESONLY\ABA10.D
Operator    : SUE
Acquired    : 13 Dec 2000   16:18      using AcqMethod ABASCAN2
Instrument  :    CSIRO HP5
Sample Name: D6aba inject 1ul of 10 ug ml extend run
Misc Info   : save sim as scan repeat
Vial Number: 1
```

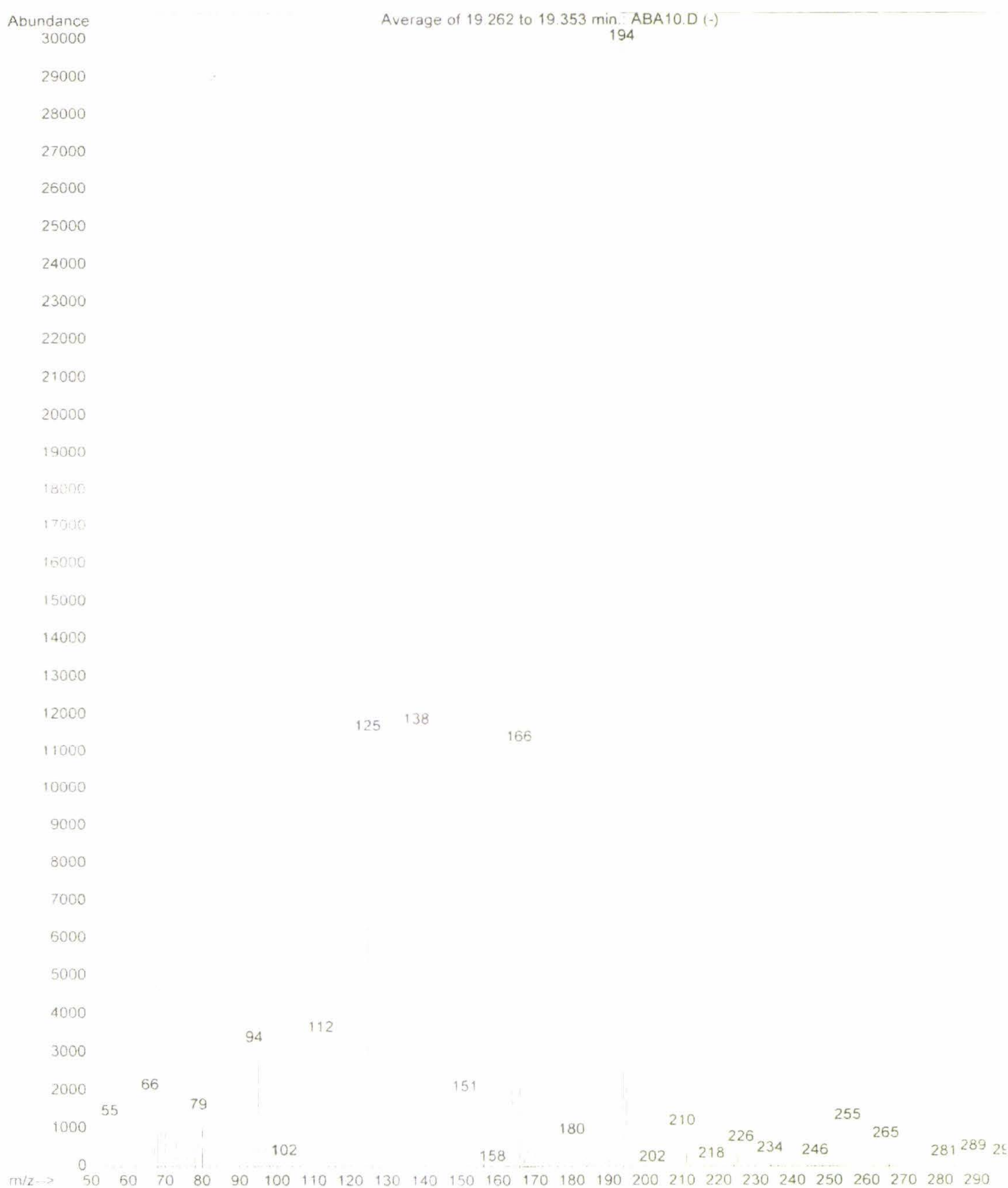


Fig 3.26 Mass spectrum of the methylated sub-sample from the same batch as the sample of deuterated ABA (D_6 ABA) received by the author from Dr Loveys.

This sample has been stored in the Molecular Genetics laboratory at Massey University, Palmerston North.

3.9.3 A supercritical exchange reaction to make deuterated SA

Four attempts were made to obtain deuterated SA using a supercritical exchange reaction (**Fig 3.27**).

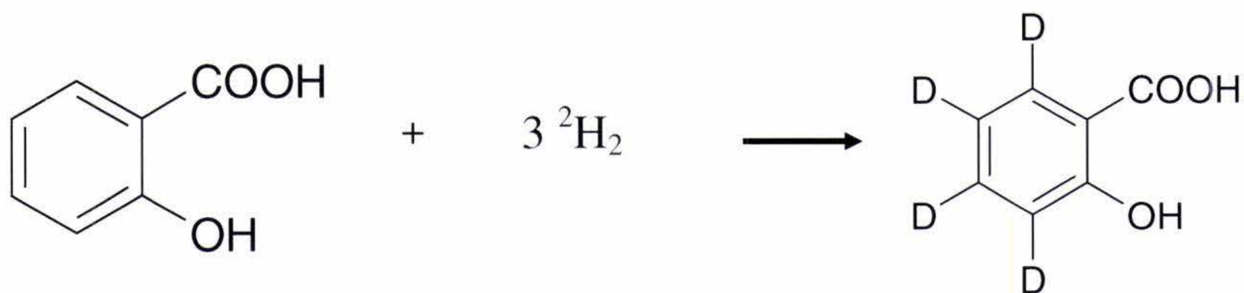


Fig 3.27 Chemical reaction showing the protonation of SA. This reaction was carried out using heavy water (D_2O).

However, after cooling the bomb and opening it (**Fig 2.11**), no reactants and products were found in three trials and no deuterated SA was present in the fourth experiment after NMR analysis.

Through time limitations these experiments were not resumed.

Chapter four

Discussion

4.1 Introduction

These experiments aimed to extend the work that had been carried out by others (Barker *et al.,* 1993; Hume *et al.,* 1993) to test that, under glasshouse and controlled environmental conditions, water stress increased the levels of *Neotyphodium lolii* alkaloids. An additional aim was to test whether this effect could be mimicked by the external application of plant hormones, ABA and JA, to water-sufficient plants.

Evidence supporting this view was found in the course of this study when positive correlations between the three alkaloids produced by the endophyte and the water-sufficient, hormone-dipped perennial ryegrass plants were obtained (**Table 3.23**). This indicated a degree of common control that could in part be dependent on water stress. Other work had indicated that it could in part be due to endophyte biomass (Easton *et al.,* 2002). However, more recent work has suggested that the distribution of *N. lolii* within perennial ryegrass is not a major determinant in the distribution of endophyte alkaloids and that possible factors such as plant genotype, tissue position and tissue age may play a part (Spiering *et al.,* 2004). This study would suggest that water stress could also play a part in fungal alkaloid distribution in perennial ryegrass.

It has been found in another monocotyledon, New Zealand tussock *Festuca-zealandiae*, that, as water stress becomes severe, the plants produce hormones that operate endogenous mechanisms or produce metabolites that will work to ultimately protect or limit any metabolic damage (Abernethy & McManus, 1998). For example, they reduce transpirational losses by closing their stomata. The results from this study suggested that this principle also applies to perennial ryegrass plus *F. arundinaceae*.

The possible advantages of the alkaloids produced by the endophyte to the persistence of perennial ryegrass have already been outlined (see **Section 1.6** of the Introduction).
The production of these bioprotective alkaloids (Bush *et al.,* 1997) by the endophyte and their enhancements to host fitness have been well documented, but the mechanisms responsible for host fitness and how this production is triggered are not well established. The mechanisms responsible for increased host fitness have not been advanced by this study but possible triggering mechanisms have been identified.

It was hypothesized that perennial ryegrass would trigger endophyte alkaloid production by increasing the levels of water stress hormones. These hormones would act as chemical messengers to turn on or up-regulate key endophyte alkaloid genes, leading to an increase in ergovaline, lolitrem B and peramine, commensurate with the water stress (Barker *et al.,* 1993). To support this hypothesis, it has been reported that high ambient temperature and moisture stress induced high alkaloid concentrations in perennial ryegrass (Lewis, 1997).

Possible perennial ryegrass water stress hormone candidates for the role of triggering this change are abscisic acid (ABA) (Skriver & Mundy, 1990; Sengbush, 2000; Meyer *et al.,* 1989), jasmonic acid (JA) (Bush *et al.,* 1997; Creelman & Mullet, 1995) and salicylic acid (SA) (Dat *et al.,* 1997; Durner *et al.,* 1997). ABA is recognized as a drought-protecting hormone that triggers the closure of stomata, and JA and SA are categorized as general stress hormones.

To test the hypothesis that increasing levels of perennial ryegrass stress hormones lead to an increase in endophyte alkaloids, it was necessary to dip water-sufficient, endophyte-colonized perennial ryegrass in solutions of ABA and JA of increasing concentration over a period of 14 days. Support for this approach came from independent sources (Gaskin, 1995; *pers comm* Gaskin, Forestry Research Institute, Rororua, New Zealand. 2002). The dipping concentrations were established by enzyme-linked immunosorbent assay (ELISA) in a preliminary experiment (**Section 3.7**) and were further confirmed by a literature search (**Tables 2.7** and **2.8**). During these experiments it was found that ABA and JA levels produced were significantly lower in the Ag Hort growth chamber at Massey University than they were in the Ag Research glasshouses (**Figs 3.14 and 3.17**). This could in part have been due to the greater evapotranspiration rates observed for plants grown in the growth chamber. The different growing conditions and consequentially different physiological conditions would account for these differences. There is some evidence that fluctuating diurnal and nocturnal environmental conditions are necessary for endophyte toxin synthesis (*pers comm* Tapper, AgResearch, Grasslands Division, Palmerston North, New Zealand 2001 and Barker Department of Horticulture and Crop Science, Columbus, Ohio, USA 2000), so its possible that this is a consequence of the fluctuation of the hypothesised hormone drivers.

Because of time constraints, the hormone levels existing during the main experiment and those obtained by ELISA have not yet been confirmed by high performance liquid chromatography (HPLC) (Anderson, 1985). However, the plant tissue necessary for that confirmation was obtained by solid state extraction and has been stored in a freezer. The internal HPLC standards have been produced or acquired as a first step in that process.
In preparation for the main experiment, measurements to assess transpirational water losses in perennial ryegrass during the time course of applying a water stress were carried out. There is evidence from North America that the presence of endophyte in tall fescue confers a measure of drought resistance to the extent that endophyte-free tall fescue was less persistent during prolonged drought than genotypes containing endophyte (West *et al.,* 1993; Bouton *et al.,* 1993). There is some further evidence that this may also be the case in perennial ryegrass, although the effect was variable and genotype dependent (Hesse *et al.,* 2004; Cheplick *et al.,* 2000). During this experiment, as it was found that perennial ryegrass hosting endophyte was transpiring at the same rate as endophyte-free perennial ryegrass, other factors, apart from factors involving the transpirational stream, are involved.

Methods to produce tritiated cucurbic acid (CA) and ABA on a micro scale were developed. This greatly reduced the amount of radioactive sodium borohydride, and the consequential cost, needed to carry out the reduction for the production of tritiated CA and ABA. These tritiated compounds were used as tracers to obtain estimates of the percentage losses of ABA and JA during the solid state extraction of ABA and JA from perennial ryegrass. It was necessary to carry out these experiments so that we could determine if ABA and JA were taken up by dipped perennial ryegrass. In a separate experiment, an ELISA analysis was carried out to estimate plant hormone levels as the plants were progressively water stressed over a period of 14 days.

4.2 Dipping treatments

The up-ending of plants in hormone solutions proved to be an effective method for the application of ABA and JA. Most studies reported to date have used spraying or wick application methods to test the responses of plants to hormone applications.

Several methods to introduce the plant stress hormones into the perennial ryegrass vascular system were reviewed. No published studies comparing exogenous methods for the application of ABA, JA, indole-3-acetic acid (IAA) and SA were found. However, in a preliminary study, it was determined that spraying and wick feeding were not as effective as up-ending the plants in known concentrations of plant hormone solutions for 60 min.

The levels of plant hormones used in these solutions were those obtained by ELISA in preliminary experiments, in which uptake was measured for water-sufficient trial plants tested with the dipping protocol (**Section 3.7**). This method of applying hormones to plants in a quantifiable manner is not as technically difficult as wick feeding and is more predictable in outcome than applying the hormone by spraying. However, tests to check if the application of ABA and JA by this method resulted in biochemical and physiological changes similar to those brought about by the application of water stress were not carried out.

When using this method, it is important that the pots stay up-ended after the dipping solutions have been removed, until the foliage has dried, so that runoff containing hormone does not enter the soil, because hormones such as ABA can enter the plant via the root system. The dipping method that was developed has much to recommend it and could be used with confidence when a quantitative uptake of hormones by plants is required.

This simple method of applying hormones to plants contrasts with the technically difficult method of wick feeding in monocotyledon plants. As these plants have vascular bundles scattered throughout the stem, locating the conducting vascular tissues in which to implant the wick is difficult. Alternatively, when using the spray method, it is difficult to obtain an even spread of hormone over the leaf. If an even spread did occur, the ambient-temperature-dependent rate of drying would create various hormone uptake rates, depending on distribution and the ambient temperature at the time of application.

4.3 Influences of water stress and hormones on ergovaline levels

It was shown by this study that, as water stress increased, the level of ergovaline in both the leaves and the sheaths increased and the increases were greatest in the sheaths. This result was to be expected from previous research as *N. lolii* colonizes along a basal–apical gradient; thus, there would have been higher endophyte biomass in the sheath regions of the tillers. A recent publication (Spiering *et al.,* 2004) would suggest that biomass per se contributes only 20% to the variation in ergovaline levels. As ergovaline is highly insoluble, it would not have moved from the basal sheath tissue into the leaf in significant amounts.

In spite of increases in ergovaline levels, there were no significant effects of hormone dipping when the combined leaf and sheath data were analysed, except when ABA and JA were used together. In this case, the levels of ergovaline fell. However, when the leaf and sheath data were analysed separately, this decrease was significant in the leaves and dipping in JA alone gave a significant increase in the level of ergovaline.
The lack of an ABA response could have been due to a number of factors. For instance, ABA may not be involved in the response, the levels applied were insufficient to trigger a drought response, the ABA used was rapidly converted to an inactive form, or the period of immersion of the plants in the hormone solution was too short.

Information recently received (*pers comm* Barker/Metzger Department of Horticulture and Crop Science. Ohio State University, Columbus Ohio. USA 2004) from a hormone specialist at Ohio State University would suggest that ABA is rapidly deactivated in air and in the plant. This occurs after a conversion from the active *cis* form to the inactive *trans* isomeric form. As a result, any application of ABA to the plant would have a short term effect lasting up to 8 h. The dipping of plants in hormone solutions does result in hormone uptake but treatment for periods longer than the 60 min used in this experiment may be necessary, because the stomata close quite quickly and this slows the absorption into the plant. This slowed absorption, coupled with the rapid endogenous deactivation of ABA, would lead to a limited hormonal response when hormones were applied in a 60 min dipping protocol. ABA responses occur because of a sustained delivery from the plant roots to the aerial parts of the plant.

Significant increases in the level of ergovaline occurred only in the leaf tissue, with no significant changes in the level of ergovaline in hormone-dipped sheaths. The importance of this leaf result should not be underestimated. It can be concluded that, after having grown through the ligule, a relatively large endophyte biomass in the leaf produced the ergovaline. The fungal endophyte *Neotyphodium coenophialum* has been found in leaf blades of tall fescue (*Festuca arundinacea*) by Christensen et al., (1998). The writer is not aware of any similar reports of *N. lolii* passing through the ligule of perennial ryegrass (*Lolium perenne*) and invading the leaf tissue in high biomass concentrations. However, leaf tissue is colonized because the growth of the endophyte keeps pace with the growth of perennial ryegrass tissue and the growth of the endophyte hyphae in the leaf extends at the same rate as the leaf is extending. Thus, the hyphae growing tip does not pass through the ligular zone. However, there is a high basal to low apical endophyte biomass gradient in all tillers (*pers comm* Christensen, Ag Research Grasslands Division, Palmerston North, New Zealand.).

It was conclusively shown by this study that the leaf and sheath tissues of water-stressed perennial ryegrass had elevated levels of ergovaline; this occurred when the leaf and sheath data were combined and when they were considered separately. Water stress results in a number of physiological changes, ranging from reduced photosynthesis and transpirational activity to increases in proline levels or a reduction in nitrate reductase activity. What effect these changes might have on the endophyte remains to be conclusively determined. However, as we found that perennial ryegrass plants dipped in JA solutions did give an increase in endophyte-produced ergovaline, JA may play a part.

JA is acknowledged in the literature as part of a stress cascade pathway. When soybean leaves were subjected to 15% dehydration, the JA levels increased fivefold, peaking within 2 h of losing 15% of their fresh weight and returning to control levels 4 h after the application of the water stress (Creelman & Mullet, 1997; Voros *et al.,* 1998). Other studies have also shown the involvement of JA in water stress by specifically reducing transpiration by up to 22%. Although this is not as dramatic as the ABA effect of reducing transpiration by 72%, the JA spike does occur in at least some plants. The preliminary experiments in this study showed that JA spiking also occurs in perennial ryegrass (**Figs 3.16** and **3.17**).

4.4 Influences of water stress and hormones on lolitrem B levels

No evidence suggesting that the application of water stress had any effect on the levels of lolitrem B in the leaves was found. This result was not unexpected because lolitrem B is known to be lipophilic (Siegel & Bush, 1996), and its distribution is significantly correlated to *N. lolii* concentrations. As the endophyte is known to have a basal–apical biomass gradient in perennial ryegrass (Keogh *et al.*, 1996), the endophyte biomass will be low in the leaf relative to the sheath. This implies that lolitrem B is retained in the lipid vesicles of the endophyte hyphae and its insolubility in water means that it lacks the mobility necessary to move from its site of production, through the ligule and into the leaf blade proper, to supplement the lolitrem B already there.

Water stress produced a significant reduction in the level of lolitrem B in the sheaths. There are three possible reasons for this: (1) the water-stress-protection hormones in the plants caused the endophyte to reduce production of lolitrem B; (2) the reduction was directly triggered by the water stress impacting on the endophyte and its ability to produce the alkaloid; (3) the water stress was so severe that plant metabolism in general was disrupted.

None of the hormone treatments had any significant effect on the production of lolitrem B. This could have been because lolitrem B is produced in quantities that are sub-threshold in producing toxicosis throughout the annual life cycle of perennial ryegrass and it is the fluctuating endogenous levels of one of the other alkaloids, such as ergovaline, that determines the sensitivity of stock to lolitrem B, with its characteristic toxicosis outcome (*pers comm* Hume, AgResearch, Grasslands Division, Palmerston North New Zealand 2001). Alternatively, it may be that the water stress hormones in the plant do not trigger lolitrem B production but that lolitrem B is produced continually and becomes concentrated as the leaf dries. This would lead to a greater intake by the stock of toxin-contaminated dry matter, which could then lead to the characteristic toxicosis. Or, it could be that some other exogenous factor, such as high ambient temperatures, triggers the production of lolitrem B. Another explanation is that the hormones were not absorbed at all, or the absorption was reduced, during the latter stages of the applied water stress during late January when the ambient glasshouse

temperatures were high. The leaves were dipped for 60 min every 2 days. This treatment re-hydrated the leaves. However, the stomata may not have opened again at each dipping event unless photosynthesis started up again in the guard cells, providing ATP for the active transport of H^+, or unless the photosynthetic light level was sufficient to trigger the K^+ pumps that re-hydrate the guard cells by the osmotic uptake of water, causing the stomata to open. Because the pots were up-ended under the glasshouse benches, where the light intensity was low anyway, and the plant leaves were submerged under water, there was probably not enough light to drive these reactions. Although there is hormone uptake via pathways through the cuticle, by far the majority of the hormone would enter the leaf via the stomata. Hormone uptake through the cuticle could possibly have occurred towards the end of the experiment when the highest dipping concentrations were used.

4.5 Influences of water stress and hormones on peramine levels

This study found that the levels of peramine were significantly higher in the sheath tissue than in the leaf tissue. This result was interpreted as showing that, as the water stress was progressively applied, the production of peramine by the endophyte increased.

When the combined leaf and sheath data were considered, there was a small but significant increase in the level of peramine when water-sufficient perennial ryegrass was dipped in JA. The levels of JA in soybeans increase rapidly but transiently when a reduction in turgor is induced by water deficit (Creelman & Mullet, 1995). This was also found in perennial ryegrass in this study in a preliminary experiment (**Figs 3.16** and **3.17**). JA is thought to act with ABA in abiotic stress biochemical pathway cascades (Creelman & Mullet, 1997), and its correlation with an increase in the level of peramine could suggest that JA mediates signalling between perennial ryegrass and *N. lolii* to facilitate this increase.

The small but significant increase in the level of peramine was not confirmed when only the leaf data were considered. There was a decrease in peramine in the leaf (**Table 3.28**) and a significant 35% increase in the sheath. If there is a hormonal communication pathway between both members of the symbiotum, the sheath-occupying endophyte and

the perennial ryegrass, then this result could be expected. From an evolutionary biology point of view, this complex inter-specific adaptation would have survival merit.

However, the increases in the level of peramine after dipping in ABA and ABA plus JA were not significant. Thus, from these data, the spiked increase in the level of JA, produced by the plant in response to water stress (**Fig 3.16**), triggered the increase in the level of peramine.

4.6 Effects of growth chamber versus greenhouse on the alkaloids

The levels of alkaloids obtained from the plants in the growth chamber were compared with those obtained from the plants in the glasshouse (**Appendix 11**). All hormone dipping treatments produced increases in the alkaloids. There were some large glasshouse increases in ergovaline and in peramine in the sheath (see the data highlighted in orange in **Appendix 11**). The combined growth chamber and glasshouse data are shown (see the data highlighted in blue in **Appendix 11**). When the differences in *L. perenne* genotypes were considered, it was clear that, of the four genotypes used in the growth chamber, two were also used in Replicate 1. Therefore, the data were re-analysed with the Replicate 1 data removed. When this was done, the ergovaline levels were not significantly different among treatments for leaf and sheath. However, there was a large increase in the peramine levels for both the leaf and the sheath (see the data highlighted in mauve in **Appendix 11**). The lolitrem B data appeared to show a progressive removal of the negative effects of the hormones on the production of the alkaloids. These analysis results lacked significance. The combined growth chamber and glasshouse data showed that the responses increased with the growth chamber data removed and increased further when Replicate 1 was removed. However, as the number of replicates analysed decreased, the variation within the remaining data increased, and this produced a larger standard deviation (experimental error). As the larger standard deviation offset the higher means, the results were analysed to obtain the lowest variation (experimental error) and, as a result, the smallest standard deviation. The statistical power of this experiment (i.e. the ability to detect significance) was greatest when all replicates were used.

However, there was a difference between the glasshouse data and the growth chamber data. The removal of the data for Replicate 1 indicated that this difference was not influenced in a major way by the *L. perenne* genotypes used in the experiment, even though data from other studies would suggest the contrary (Easton *et al.,* 2002).
The physiological basis of this difference was likely to be a greater water stress in the growth chamber. Evapotranspiration was greater for the replicates in the growth chamber than for the replicates in the glasshouse. In preliminary experiments in this study on measurements of evapotranspiration in perennial ryegrass with and without an endophyte present, the water loss was on average around 65% higher in the growth chamber plants than in the glasshouse plants (**Fig 3.2**).

Preliminary experiments carried out to measure the increase in the levels of ABA and JA during water stress also showed that, to avoid plant death, the application of water stress needed to be terminated after 10 days in the growth chamber but could be extended to 17 days in the glasshouse (**Tables 3.10** and **3.12**, growth chamber; **Tables 3.8** and **3.11**, glasshouse). This was confirmed by the trend lines for the relative water content measurements made over the course of this experiment (**Fig 3.22**). This difference resulted not because of any major temperature differences but because of the greater air turbulence, increasing the vapour pressure gradient between the inside and the outside of the leaf.

The environmental control in the growth chamber allowed the day (16 h) and night (8 h) temperatures to be different but they were constant and could not be stepped up and down during these times. This was not the case in the glasshouse, where environmental control was not possible. There were major fluctuations in the day and night temperatures and, because the experiment was carried out in late January to early February, the hottest time of the year, the temperatures in the glasshouse were extreme. Daily temperature data were not recorded. Fluctuating ambient and high daily mean temperatures are known to stimulate alkaloid production by *N. lolii* (*pers comm* Barker, Department of Horticultural and Crop Science, Ohio State University, Columbus, Ohio, USA 2003). By contrast, constant temperature, humidity and light were maintained in the growth chamber.

4.7 Limitations of the experimental design

Many of the results obtained in this study were not significant at the 1–5% level, even though some large increases in the levels of the alkaloids were obtained when the plants were dipped in the hormones ABA and JA. There are a number of reasons why they were not significant. It may have been that there was no actual difference, or that differences that could not be detected because of the high variability occurred. Possibly more replicates were needed to take into account the high level of variability. This variability was due to the four different perennial ryegrass genotypes used in each replicate; they interacted with the relatively homogeneous genotype of the endophyte to produce different levels of alkaloids. High variability within a sample coupled with a small sample size produces a large standard deviation and this reduces the likelihood of a statistically significant F value. Of these possibilities, high variability was likely to be a major contributing factor. For example, for the JA and ABA treatments (JAW), the lolitrem B levels ranged from 0.69 ppm in Block 5 in the growth chamber to 5.24 ppm in Block 2 in the glasshouse. Although this variability was accentuated because of the growth chamber data (the growth chamber readings were consistently lower), a wide range of levels was recorded even within the glasshouse data. For example, the lolitrem B levels ranged from 2.96 to 5.24 ppm. This variation was found across the hormone-dipped pots, the un-dipped pots and the water-sufficient controls for all three alkaloids.

As each replicate was made up of four different perennial ryegrass genotypes, it is possible that there was a genotype interaction and that this contributed significantly to the variability (Easton *et al.,* 2002) and/or that the environmental conditions in which the experiment was carried out interacted with the various plant genotypes differently (Spiering *et al.,* 2004).

The analysis of variance (ANOVA) gives the probability that these alkaloid levels could have occurred by chance. As the variability in the results increases, the probability that they did occur by chance and were not real differences also increases, to a level that would make the data not significant. This effect could be isolated by carrying out the experiment again with more replicates and using a mixture of perennial ryegrass genotypes or using a single perennial ryegrass genotype with the same number of replicates.

As well as the variability contribution, the dipping regime had some limitations. The hormone application rates as measured during the preliminary experiment (**Sections 2.11** and **3.7**) were reduced because time constraints did not allow any repeat work. It was not possible to carry out any more than one run. As a result, the hormone dipping concentrations used were very conservative because of the possibility of irreversible foliar damage.

4.8 Limitations of the current research and future research

In the event, the hormone dipping levels used could have been significantly increased to more closely reflect the endogenous hormone levels in the drought treatments without damaging the plants. If the experiment had been repeated, the hormone levels would have been increased to more closely reflect the endogenous hormone levels in the drought treatments. The hormone dipping concentrations used in the experiment were reduced by 20% from those calculated, because of a concern that there would be foliar damage. As there was no sign of leaf damage in these experiments, a 30% increase in hormone concentration could be applied without substantial risk. The ABA and JA ELISA data suggest that perennial ryegrass could sustain high spike levels of these hormones. There would also be a need to monitor biochemical and physiological changes in addition to tracking the course of the water stress.

The small sample size used in some cases for the hormone-treated replicates produced a high standard deviation and this led to a lack of significance even though there were substantial increases in the levels of ergovaline and peramine. Thus, larger sample sizes would be used in any future experiment. As part of any new experimental design, it would be essential that the water-stressed plants were also dipped in hormones and their alkaloids levels measured, to determine if their values were below, the same or above those of the un-dipped water-stressed controls.

In preliminary experiments (**Sections 2.11** and **3.7**), the ABA and JA levels during an imposed water stress and after dipping were analysed. Random tissue samples of all treatment pots were also taken at the end of the experiment and were solid state extracted using protocols outlined in **Section 2.9**. They were dried and stored in a

freezer in preparation for HPLC–mass spectrometry analysis for levels of ABA, JA and SA.

To identify the *Lolium/Neotyphodium* genetic component interaction that clearly led to high variation in this experiment, a novel approach might be to set up a new experiment that used perennial ryegrass replicates made up of a single perennial ryegrass/endophyte genotype. Each pot would have a single genotype and would be given the same five treatments. Thus, for example, 16 different perennial ryegrass genotypes would be looked at individually for their responses to the applied hormones and water stress.

4.9 Conclusions

The application of hormones to plants by dipping was successful with certain provisos involving the hormones used, increased dipping times and frequency of application.
This study showed that there was a weak but positive correlation between the three endophytic alkaloids and the hormone-dipped, water-sufficient perennial ryegrass plants. Suggestions as to the weakness in the correlations have been given and possible solutions have been outlined.

The conclusions previous researchers had reached, that endophyte metabolites, peramine and ergovaline, were produced in water-stressed perennial ryegrass at higher levels than in water-sufficient perennial ryegrass, were confirmed, but this study found that the levels of lolitrem B fell under these conditions, although still retaining the basal–apical gradient.
When water-sufficient perennial ryegrass was dipped in JA, there was a significant increase in ergovaline; however, there was no response to dipping in ABA solutions. Recently acquired unpublished information offers an insight into the possible reasons for this result.
In this study, water stress produced a significant reduction in the level of lolitrem B in the sheaths but a sheath–leaf gradient was retained.

None of the hormone dipping treatments had any significant effect on the production of lolitrem B. A possible explanation may be that lolitrem B is produced in sub-threshold amounts, by the endophyte, and that increasing ergovaline concentrations increase

mammalian sensitivity to lolitrem B. Peramine concentrations increased significantly in water-sufficient perennial ryegrass when plants were dipped in JA solutions. There was no comparable significant effect when they were dipped in ABA and ABA plus JA solutions.

When the different treatments were examined, it was clear that there were differences between glasshouse treatments and growth chamber treatments. Anecdotally, the more uniform growth-chamber environment is known to reduce endophyte alkaloid production. Further to this environmental effect, it was also clear that certain genotype combinations such as in Replicate 1 were less responsive to the treatments carried out in this experiment. The effect of removing Replicate 1 data on the statistical outcomes has been outlined.

Overall, the results were generally consistent with the hypothesis. The data trend supported the hypothesis even if the differences were at times not significant (pers comm Barker, Department of Horticultural and Crop Science, Ohio State University, Columbus, Ohio, USA 2003). There was also an indication that JA produced by *L. perenne* plays a role in the metabolic communication cascade linking perennial ryegrass with *N. lolii* alkaloid production.
The solid state extraction of ABA and JA from perennial ryegrass using Sephadex and C18 columns was developed and improved upon. This extraction procedure requires the production of tritiated ABA and CA (from JA) tracers. A cost-saving methodology was developed for their production and the reduction products were checked by HPLC and nuclear magnetic resonance (NMR).

A trial was carried out to examine the hypothesis that endophyte-colonized perennial ryegrass was less susceptible to evapotranspiration water loss than endophyte-free perennial ryegrass, as maintained by some authors. This study was unable to confirm this finding. Water loss by transpiration was found to be similar in both E+ and E- treatments.

Internal standards for HPLC are now available. Standards for ABA have been obtained and deuterated methyl JA, used as a standard for JA, has been produced. Standards for IAA can be purchased commercially. The production of deuterated SA by a

supercritical exchange reaction was not successful, but problems with the method could be overcome.

The extracted samples that would confirm the tissue levels of ABA and JA are available for HPLC analysis but, because of time constraints, the analysis has not been done. However, from preliminary trials, there was some indication from ELISA analysis that tissue levels changed when perennial ryegrass plants were water stressed.

This research project has at the very least provided a structure and a possible methodology to investigate further the interaction between the two different species that make up this particular symbiotum. Statistical correlations have been established, some significant data have been obtained and the steps in the protocols have been tested. The research to date indicates that water-stress-induced hormonal communication between *N. lolii* and *L. perenne* does exist.

Chapter Five

Confocal microscope study of *Neotyphodium lolii* colonization of *Lolium perenne.*

Section 5.1

Introduction and aims

The endophyte is seed transmitted, colonizing the intercellular basal area of apical and lateral meristems before moving into young leaf sheaths and into leaves formed from these tissues. The endophyte movement within the plant is executed by a combination of mycelium cell division and cell elongation. This produces a basal–apical gradient early in leaf development (Herd, *et al.*, 1997). The hyphae are randomly positioned in the leaf sheath but are associated with small vascular bundles with porous membranes in the leaf blades, which are thought to leak nutrients to the endophyte (Christensen *et al.*, 2002). The highest endophyte biomass is found around the vascular tissue (**Fig 5.3.9**) of young leaf sheath material (Keogh *et al.*, 1996). A delicate balance between the plant and the endophyte biomass occupying the intercellular spaces is maintained (Christensen *et al.*, 2002).

A key issue raised in Chapter 1 is the difficulty in measuring endophyte biomass within the plant. This is an important issue if the metabolic activity (e.g. alkaloid levels, gene expression etc.) of the endophyte in the plant association is to be quantified. Various methods for estimating endophyte biomass are available. These include staining transverse perennial ryegrass leaf sections with aniline blue (**Section 2.3**) and counting the number of hyphae.

A more accurate method for determining biomass is to measure the fungal cross-sectional area by electron microscopy. Transverse sections of plant tissue are fixed in a buffered glutaraldehyde/formaldehyde solution (Karnovsky, 1965) and viewed using an electron microscope. The images are then photocopied and the fungal sections of the micrograph are cut out and weighed. The areas of the magnified hyphal cross-sections are then divided by the square of the magnification factor to obtain the areas of the original hyphal cross-sections (Yong *et al.*, 2001).

Quantitative polymerase chain reaction (qPCR) has also been used to estimate endophyte biomass (Panaccione *et al.*, 2001; Groppe & Boller, 1997). The total plant and fungal DNA is extracted and the fungal component is quantified by PCR using

primers unique to a region of the *Neotyphodium* β-tubulin gene. The PCR products are separated by electrophoresis, stained and photographed under UV light, and the intensity of the fluorescence is quantified densitometrically against standard DNA amplifications of known amounts.

Chitin is found in the cell walls of fungi but not in the cell walls of plants. Methods to quantify the chitin levels of pathogenic fungi in host plants have been developed. These methods could be applied to the perennial ryegrass endophyte (Ride & Drysdale, 1972; Roberts *et al.,* 1998; Roberts & Cabib, 1982).

An alternative approach that has been used is to measure the volume of the fungus by confocal imaging (Dickson & Kolesik, 1999; Running *et al.,* 1995). This method was developed for *Lilium* roots colonized by the mycorrhizal fungus *Scutellospora porrum*. Estimates of the fungal biomass were made by thresholding the fungus fluorescence. The aim of this section of the work was to test whether confocal microscopy could be used to measure the volume of endophyte tissue in perennial ryegrass tissue. The method developed was based on the auto-fluorescence of glutaraldehyde (*pers comm* Vingnani, Institute of Molecular BioScience, Massey University, Palmerston North, New Zealand. 2000; Karnovsky, 1965; Yong *et al.,* 2001). This compound was used to fix the tissue and to enhance endophyte structures against a plant background. Auto-fluorescence of chloroplasts was initially a problem but this was overcome by developing a series of protocols to enhance the images of the endophyte structures, while at the same time reducing the fluorescence from cell inclusions, such as chloroplasts, of the perennial ryegrass host.

Section 5.2
Materials and methods

5.2.1 *Lolium perenne* meristem dissection and preparation for confocal examination

Apical meristems of a perennial ryegrass clone 187JJ (AgResearch) were dissected using a Leica MZ12 dissecting microscope at 80x magnification and dissecting needles. A single tiller with some roots still attached was used. The tiller was laid down and held in place using the attached roots. The outer leaf sheath was removed by splitting the tissue lengthwise and using the needle tip to cut the basal connection, at the same time rolling the shoot on the dissection platform. This procedure was repeated sequentially from outer older leaves to the inner younger leaves until removal of the youngest leaflets exposed the meristematic dome and leaf primordia.

The excised meristem, of around 0.5 mm^3, was then placed in water to re-hydrate for approximately 1 h. This re-hydration was necessary because the large surface area to volume ratio of the explant meant that water was lost rapidly. The meristem was then fixed in glutaraldehyde for a period of between 5 and 6 h (*pers comm* Vingnani, Institute of Molecular BioScience, Massey University, Palmerston North, New Zealand 2000). Samples could be left overnight without adverse effects. Following the glutaraldehyde treatment, the explant was washed three times in a standard phosphate-buffered saline (PBS) solution with sucrose (**Appendix 7**). The solution was changed every 2 h. The explant was then dehydrated using the replacement method by transferring into 1 mL of PBS in a 1.5 mL Eppendorf tube for serial dehydration. Every 20 min, 0.2 mL was removed and replaced with 0.2 mL of absolute ethanol. Five replacements were carried out. The explant was then transferred to a 1% safranin ($GURRS^T$) solution, incubated overnight, washed in absolute ethanol, placed in Fast Green for 10–15 s and removed ready for clearing. The meristem was then placed in a fume cupboard, rinsed in 100% clove oil and then 50% clove oil, washed in 25% ethanol and immersed in 25% Histoclear for 5–10 s. Finally, the explant was placed in a 75% Histoclear solution for 5 min and then washed in a 25% ethanol solution for 5 min.

The meristematic explant was mounted on a slide with three drops of PDX, covered with a large cover slip and left for 48 h. The procedure described above follows the general procedures outlined by Running *et al.*, (1995).

This tissue and all other confocal samples were viewed non-invasively on channel 2 of a Leica TCS 4D confocal laser scanning microscope with an excitation wavelength of 568 nm. The microscope uses a double dichroic (DD) 488 nm/568 nm beam splitter lens to focus laser light through the objective on to the sample and to capture returning fluorescence, back through the objective, from the plane of focus. A relative short pass (RSP) filter of 580 nm then reflects the fluorescence from a mirror on to a BP TRITC band pass filter set at 585–615 nm in a second channel. This filter set rejects all returning fluorescence less than 580 nm and captures 585–615 nm from the plane of focus, channelling it to a detector via an 'out pin hole'.(Rowland, 200; Ladic, 2003)

5.2.2 Protocols for fixing leaf tissue

A series of fixing protocols was trialed to determine the best conditions for visualizing the endophyte within the plant. Initially, four protocols were trialed; one of these, protocol 4, was refined through five further protocols. Protocol 4-6 was then optimized by a series of minor modifications — protocols 4-6A to 4-6R.

Protocol 1

Protocol 1 was the same as the protocol that was used for perennial ryegrass meristem staining (**Section 5.2.1**).

Protocol 2

Perennial ryegrass sheath tissue was soaked in 5% (v/v) glutaraldehyde for 5–6 h, and transferred to PBS buffer containing 6.5% (w/v) sucrose, pH 7.3, for 2 h. The solution was changed three times during this period. Dehydration was carried out in 1.5 mL Eppendorf tubes by progressively increasing the ethanol concentration up to 100% ethanol (**Appendix 13**). Each step was carried out for a minimum of 40 min but could be left for up to 2 h. After 40 min in 100% ethanol, the tissues were transferred to a slide and covered in 100% glycerol, a cover slip was applied and the tissues were viewed under the confocal microscope. Glycerol rather than water was used to mount

the tissues because it has a refractive index closer to those of the glass slide and the oil used on the oil immersion lens of the confocal microscope.

Protocol 3

This protocol was identical to protocol 2 except that, after the glutaraldehyde fixing, the tissue was placed in 2-methyloxyethanol (*pers comm* Vingnani 2000) for 10 min before the serial dehydration steps.

Protocol 4

This protocol was the same as protocol 3 except that the initial step involved soaking the plant tissue in 5% (v/v) glutaraldehyde in PBS for 5–6 h or overnight rather than undiluted glutaraldehyde. The rationale for this change was to improve the definition of the plant cell walls. In addition, the dehydration steps were of 5 min duration instead of the 40 min used in protocols 1 and 2.

5.2.2.1 Refinements to protocol 4

Protocol 4-2

This protocol was identical to protocol 4 up to the end of the dehydration step. At this stage, the plant tissues was placed in a 75% (v/v) absolute ethanol/25% (v/v) Histoclear (1:1) solution for 5–10 s, and then immediately transferred to a 50% (v/v) ethanol/50% (v/v) Histoclear (1:1) solution for 5 min, followed by a 10 min incubation in 100% Histoclear. The tissues were then washed overnight in 100% ethanol and the next day were mounted in 100% glycerol between two cover slips for confocal examination.

Protocol 4-3

In this protocol, the initial bathing was changed from 5% (v/v) glutaraldehyde in PBS to soaking the tissue in 1 M Tris buffer (pH 8.0) followed by incubation overnight. The rationale behind this change was a further attempt to quench the auto-fluorescence from the perennial ryegrass cell walls while still allowing definition of the plant cell walls. The next morning, the tissue was washed in Tris buffer containing 6.5% sucrose. This solution was changed every 40 min for 2 h. This was followed by a 10 min immersion

in 2-methyloxyethanol. The tissue was then dehydrated as for protocol 4-2 and then mounted on a slide in 100% glycerol.

Protocol 4-4

This was identical to protocol 4-3. The tissue was then placed in 75% (v/v) absolute ethanol/25% (v/v) Histoclear (1:1) for 5–10 s, 50% (v/v) ethanol/50% (v/v) Histoclear (1:1) for 5 min and 100% Histoclear for 10 min, and then incubated overnight in 100% ethanol to remove the Histoclear, so that it did not interact with the glycerol to produce bubbles. The tissue was mounted on a slide in 100% glycerol.

Protocol 4-5

This protocol was identical to protocol 4-3 up to the end of the ethanol dehydration step. This was followed by soaking in clearing fluid (**Appendix 14**) for 2 days to remove the chlorophyll, the major source of extraneous auto-fluorescence.

Protocol 4-6

This protocol was the same as protocol 4-3 up to the end of the ethanol dehydration step, but with the additional step of boiling the tissue in a 10% (w/v) KOH solution for 20 min, to remove the chlorophyll, finally washing the tissue in cold water and then mounting it in 100% glycerol.

Further refinements to protocol 4-6 were made with the addition of an autoclaving step in some protocols.

A summary of the key treatments is shown in **Table 5.1**.

Table 5.1 Fixing treatments used for the development of protocol 4-6.

	5[a]	10	15	20	Autoclaved[b]	24[c]
0.3% Glutaraldehyde in Tris buffer[d]	A	B	C	D	E	F
5% Glutaraldehyde	G	H	I	J	K	L
25% Glutaraldehyde	M	N	O	P	Q	R

[a]Time in minutes in 10% (w/v) KOH.
[b]15 psi for 15 min.
[c]Time in hours in 10% (w/v) KOH.
[d]6% (v/v) solution of 5% (w/v) glutaraldehyde in Tris buffer.

Protocol 4-6A

The fixation and dehydration steps were the same as for protocol 4-6. The final steps were similar except that the tissue was boiled in 10% (w/v) KOH for 5 min rather than 20 min. This was done in an attempt to minimize the plant cell wall damage.

Results from Protocol 4-6B were lost.

Protocol 4-6C

This was identical to protocol 4-6A but with reflux boiling for 15 min.

Protocol 4-6D

This was identical to protocol 4-6 but with a 20 min reflux boiling step.

Protocol 4-6E

This was identical to protocol 4-6 but with autoclaving at 15 psi for 15 min after serial dehydration. This was carried out in an attempt to remove the chlorophyll, which was the source of the auto-fluorescence.

Protocol 4-6F

This was identical to protocol 4-6 but with the final stage, after the serial dehydration, of soaking the tissue in KOH for 24 h rather than reflux boiling. This was done in an attempt to reduce cellular damage.

Protocol 4-6G

This was identical to protocol 4-6A except that the tissue was fixed in 5% (v/v) glutaraldehyde rather than the glutaraldehyde–Tris buffer mixture. It was then finished with 5 min reflux boiling in 10% (w/v) KOH.

Protocol 4-6H

This was indistinguishable from protocol 4-6G but had reflux boiling for 10 min in 10% (w/v) KOH as the final step.

Protocol 4-6I

This was the same as protocol 4-6H but finished with boiling in KOH for 15 min.

Protocol 4-6J

This was the same as protocol 4-6I but with boiling in KOH for 20 min as the final step.

Protocol 4-6K

The final KOH boiling was replaced with autoclaving for 15 min.

Protocol 4-6L

The final step was a 24 h soaking in the KOH solution.

Protocol 4-6M

The tissue in this protocol was fixed in a 25% glutaraldehyde solution with all the other steps as for protocols 4-6A and 4-6G.

Protocols 4-6N, 4-6O and 4-6P

These were identical to protocol 4-6M but with 10, 15 and 20 min KOH reflux boiling respectively.

Protocol 4-6Q

This was indistinguishable from protocol 4-6P but finished with a 15 min autoclaving step.

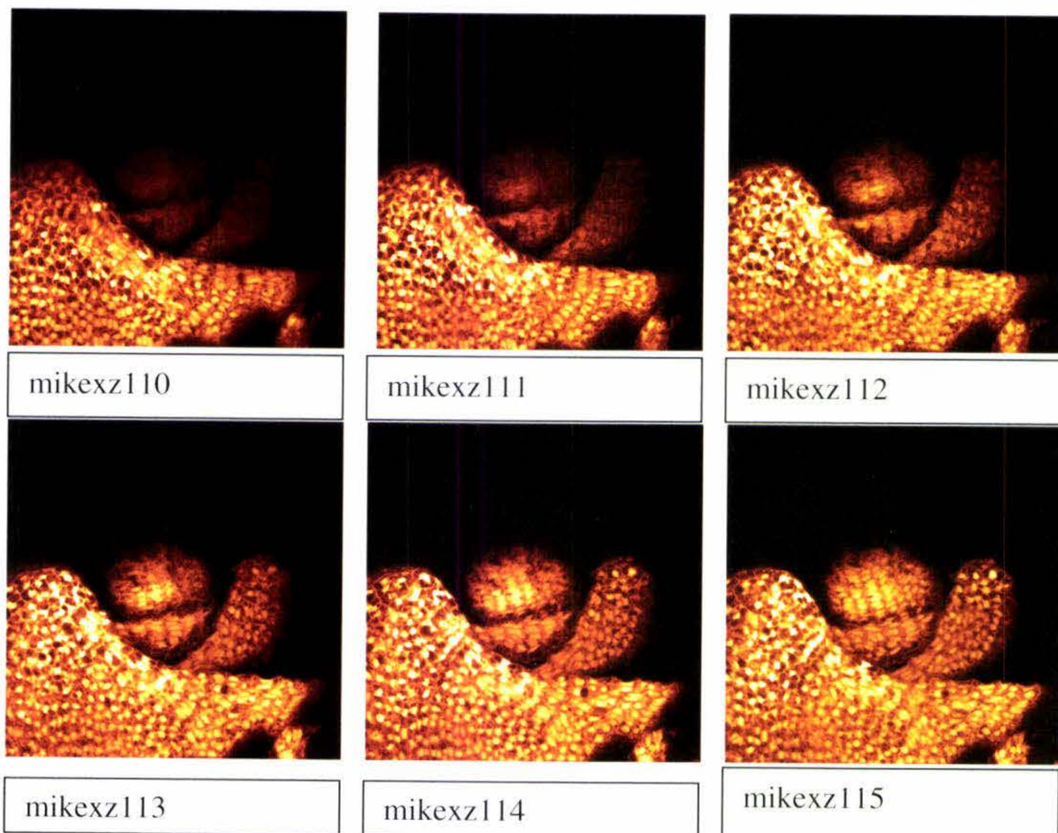
Protocol 4-6R

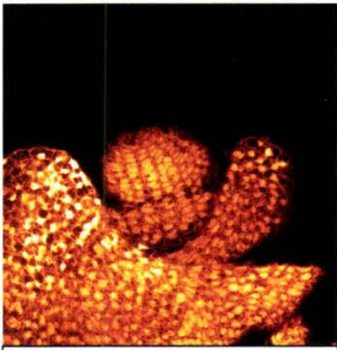
This was identical to protocol 4-6Q but with a final step of soaking the tissue in KOH rather autoclaving it.

Section 5.3
Results

5.3.1 Confocal microscope analysis of perennial ryegrass meristem

This work was carried out to give the basis for a confocal examination of primordial perennial ryegrass tissue (meristematic), which would be carried out to confirm visually that endophyte hyphae must enter primordial tissue for colonization to occur [5]. Forty four sequential confocal micrographs of 1.3 μm sections of a perennial ryegrass meristem are shown in **Fig 5.1** (mikexz110–mikexz152). Images mikexz110–mikexz119 were those taken closest to the surface. With increasing depth, resolution was progressively lost (mikexz120–mikexz152).

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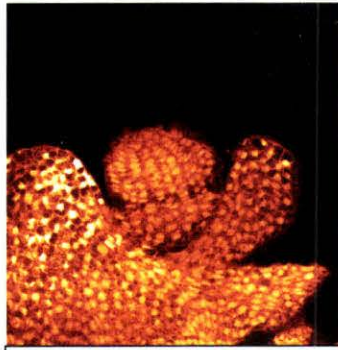
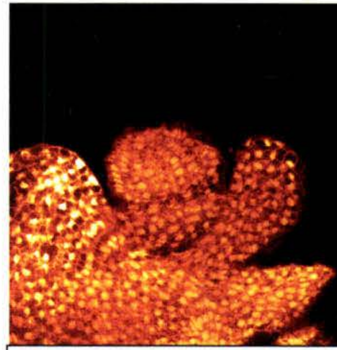

Mikexz117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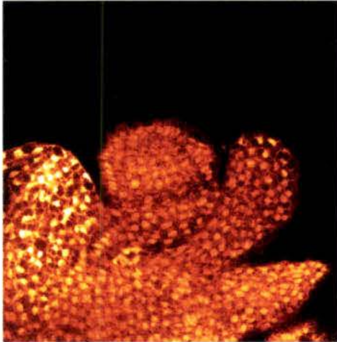
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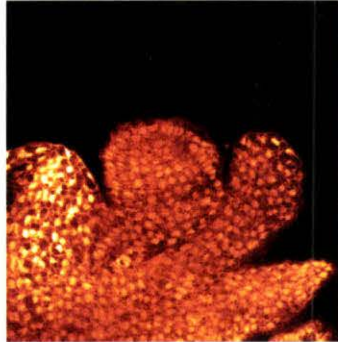
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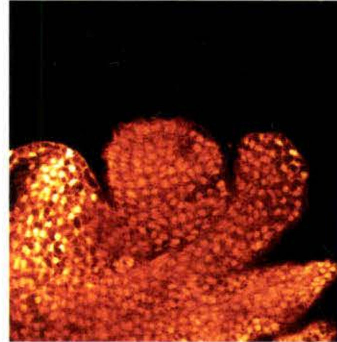
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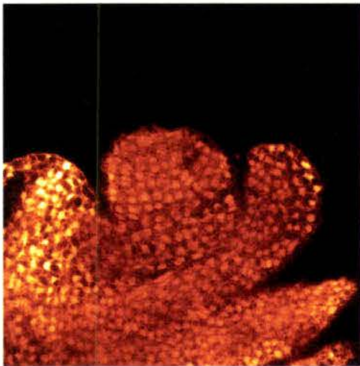
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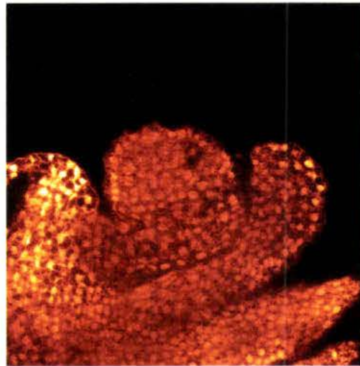
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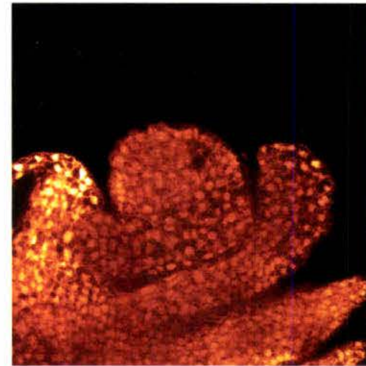
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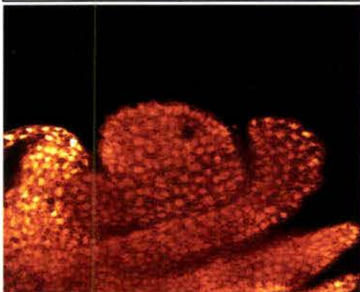
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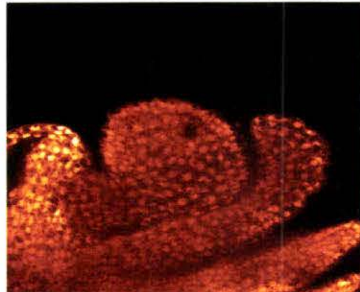
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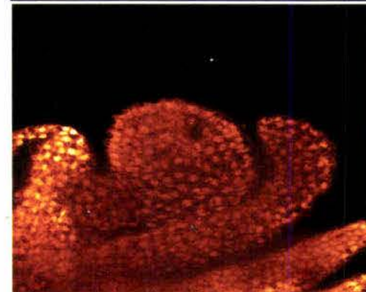
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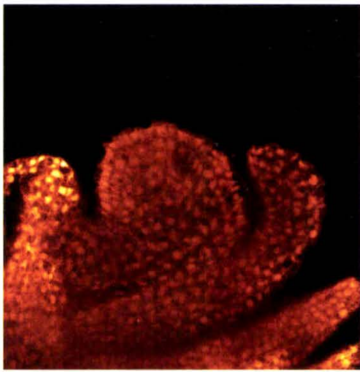

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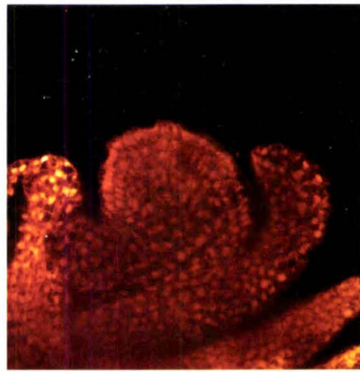

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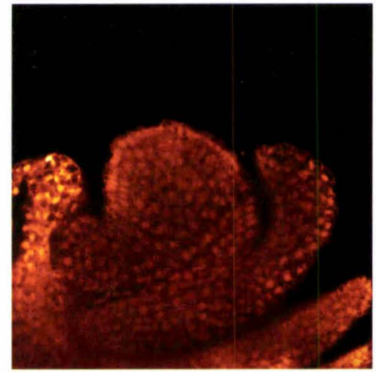

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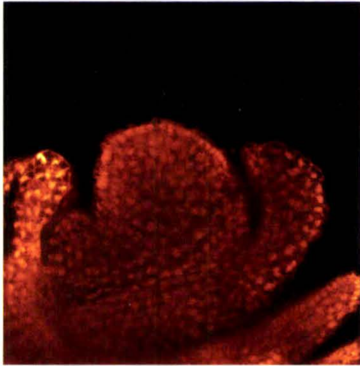

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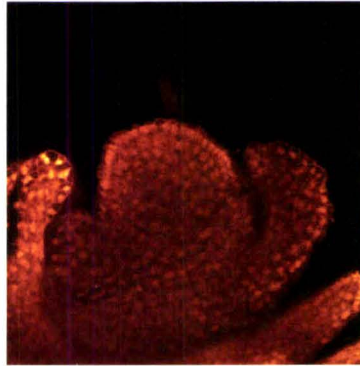

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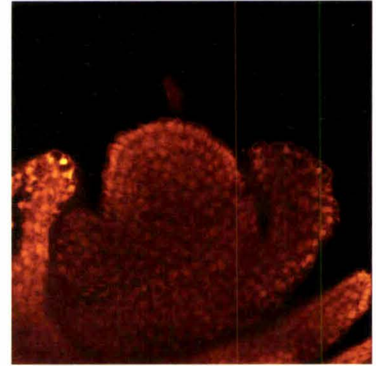

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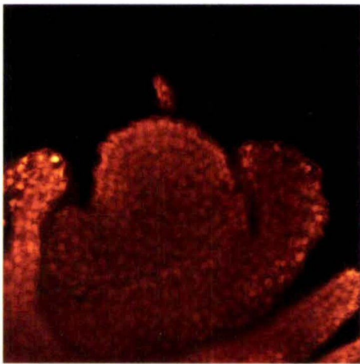

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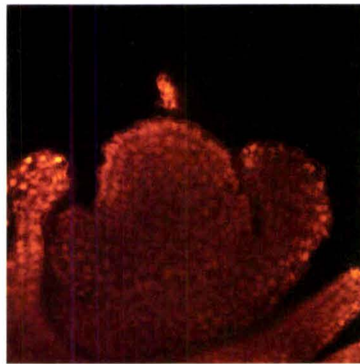

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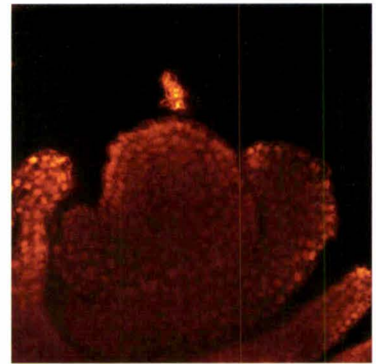

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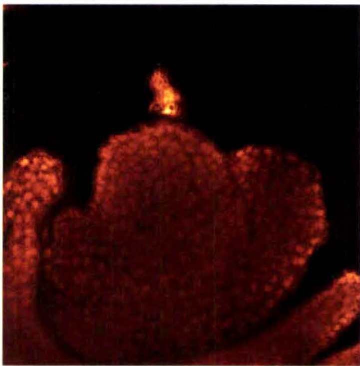

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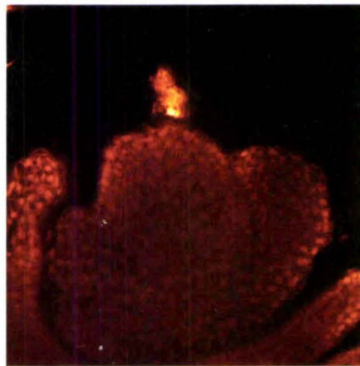

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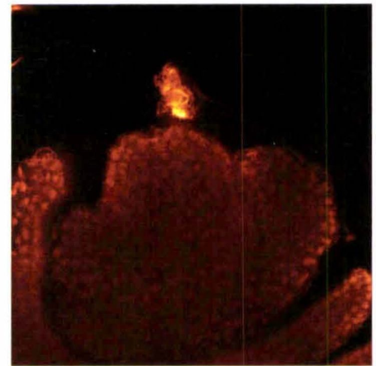

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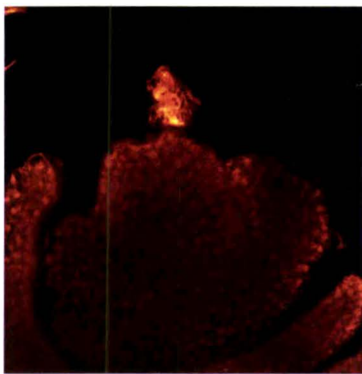
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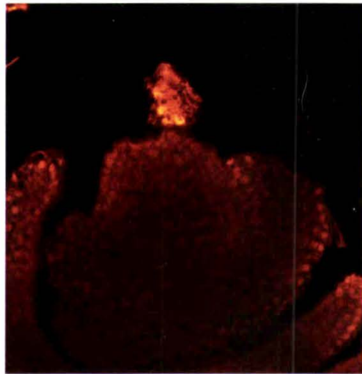
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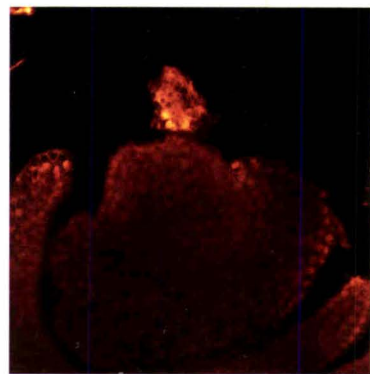
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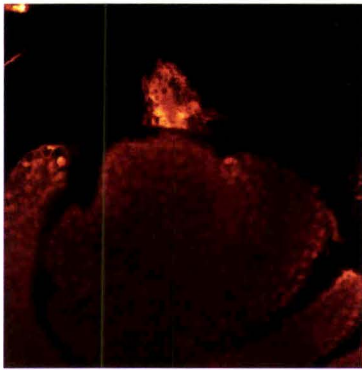
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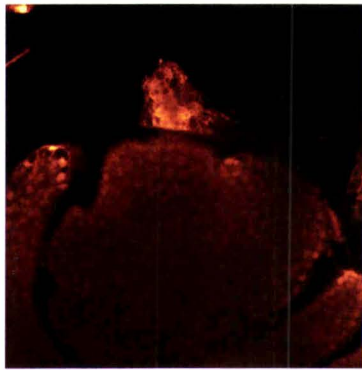
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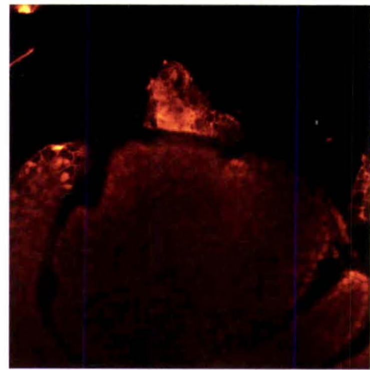
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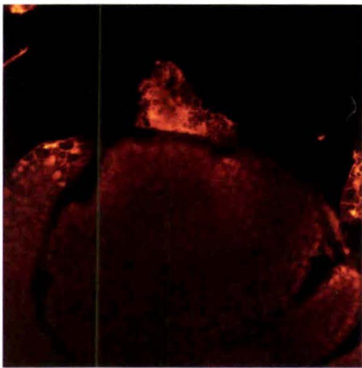
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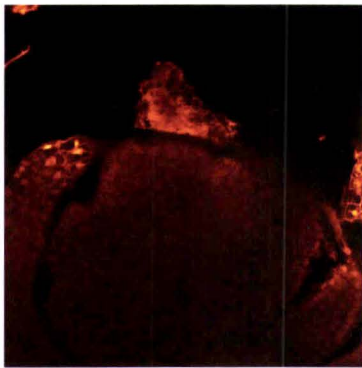
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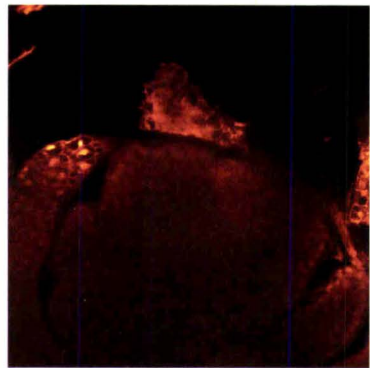
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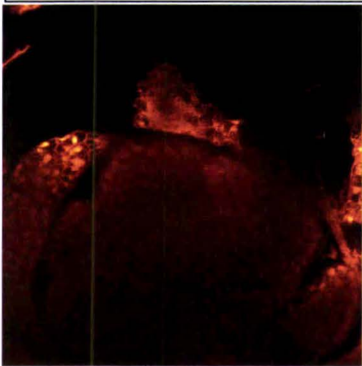
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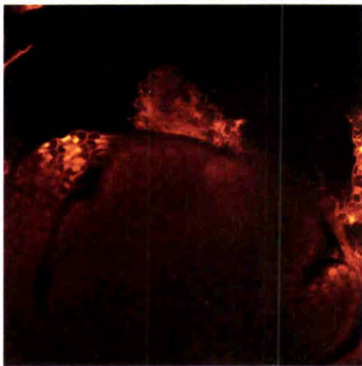
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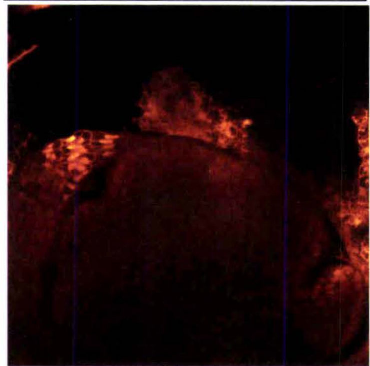
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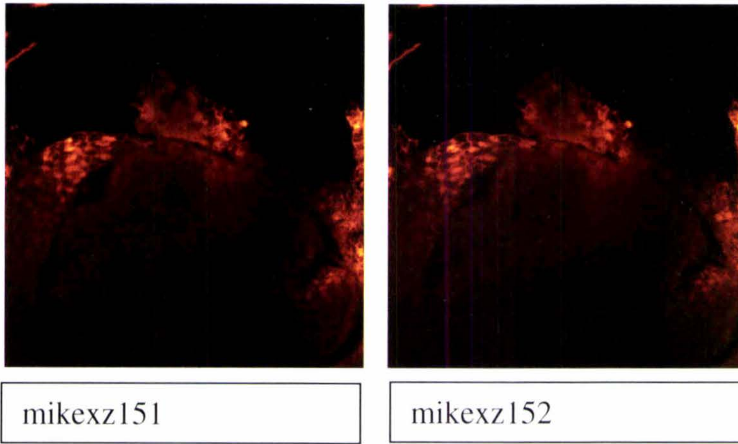


Fig 5.1 Confocal micrographs of 1.3 µm sections through a perennial ryegrass apical meristem.
Forty four images (mikexz110 to mikexz152) were taken at 5.0 µm intervals through the meristem and were examined as outlined in **Section 5.2.1**.

The 44 sequential confocal images were then re-assembled into a three-dimensional image that could be rotated and viewed on three sides using confocal software (**Fig 5.2**). This animated image was recorded on videotape.

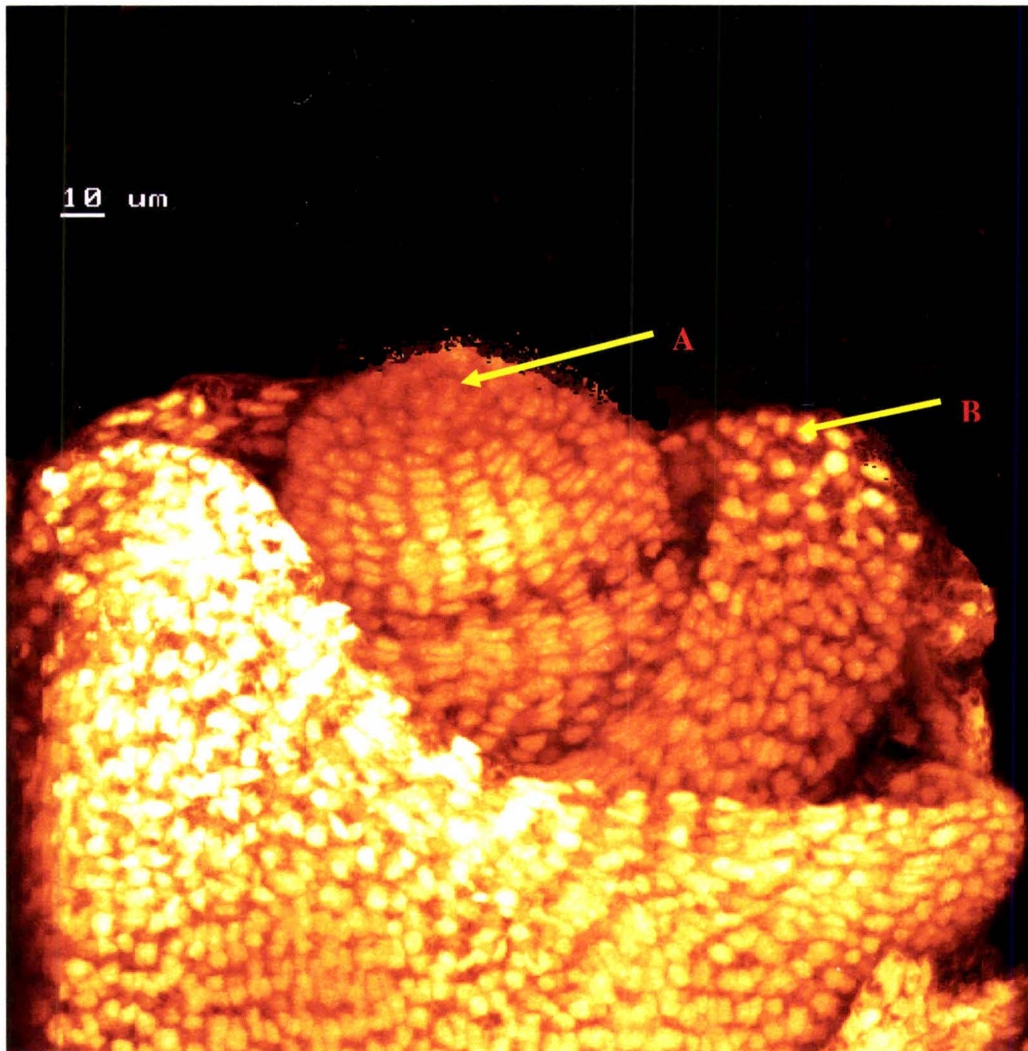
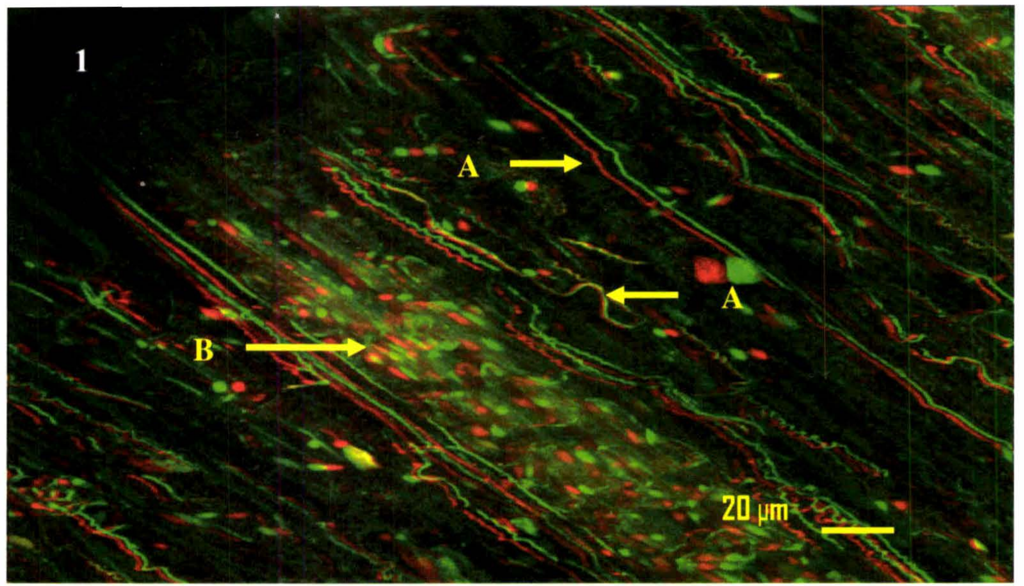


Fig 5.2 Re-assembled confocal images of a perennial ryegrass apical meristem (**A**) with a leaflet wrapped around its base (**B**).

5.3.2 A stereoscopic anaglyph

The anaglyph in **Fig 5.3** was assembled by overlaying a series of confocal images, and then digitally colouring them alternately red and green, so that they could be viewed using red/green cellophane glasses to give a stereoscopic view of the structure. It can be seen that the endophyte (**A**) was abundant in this tissue and was located extra-cellularly. There was no evidence of cellular penetration by the endophyte. The endophyte did appear to be concentrated around the vascular tissue (**B**), which would place the fungus in close proximity to nutritional sources.



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seendo019 seendo021 seendo023 seendo025 seendo027

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Fig 5.3 An anaglyph of 30 confocal images of perennial ryegrass leaf sheath. The anaglyph (**1**) was prepared from confocal images taken at 1.3 µm sections at 5 µm intervals (half of these images are shown (**2**) and the tissue was prepared using protocol 3. The endophyte *N. lolii* (**A**) and perennial ryegrass vascular tissue (**B**) are shown. The sections were overlaid for stereoscopic viewing.

Four fixing protocols (**Section 5.2.2**) were trialed to find the protocol that best enhanced the cell wall auto-fluorescence of the endophyte and at the same time minimized auto-fluorescence from the cell walls of the host plant, so that successful thresholding of confocal sections for biomass estimation could be carried out, (**Section 5.2.2, Protocols 1 to 4**). The suitability of the confocal images produced was judged by the screen image that they produced. No prints of the images were recorded. Of the four fixing protocols trialed, the best, protocol 4 (**Section 5.2.2**, **Protocol 4**), was developed further, producing a series of five new protocols (**Section 5.2.2.1**, **Protocols 4-1** to **4-6**). Of this series, protocol 4-6 was refined further to give **protocols 4-6A** to **4-6R (Section 5.2.2.1)**.

(i) Protocols 1 to 4

Using protocol 1, good staining of the plant cell walls was obtained, as can be seen in **Fig 5.1**. However, staining of the endophyte was poor. The long period of time required to prepare the tissue was also a major disadvantage. Protocol 2 was a considerable improvement on protocol 1. It was clear that the use of the glutaraldehyde enhanced the endophyte cell wall fluorescence, but the plant cell wall fluorescence was too high to allow successful thresholding. Protocol 3 greatly enhanced the fluorescence of the endophyte cell wall over that of the plant cell wall. It was a rapid method but the definition of the cell wall was all but completely lost **(Fig 5.3)**.

It was considered important that the location of the endophyte with respect to the plant tissue was highlighted as well as its overall structure to determine the bio-volume. Therefore a further protocol was tested. The final protocol, protocol 4, was chosen as a basis for further development. It was a relatively rapid protocol and gave good auto-fluorescence of the endophyte with much of the perennial ryegrass cellular tissue able to be observed with little background fluorescence (**Fig 5.4**).

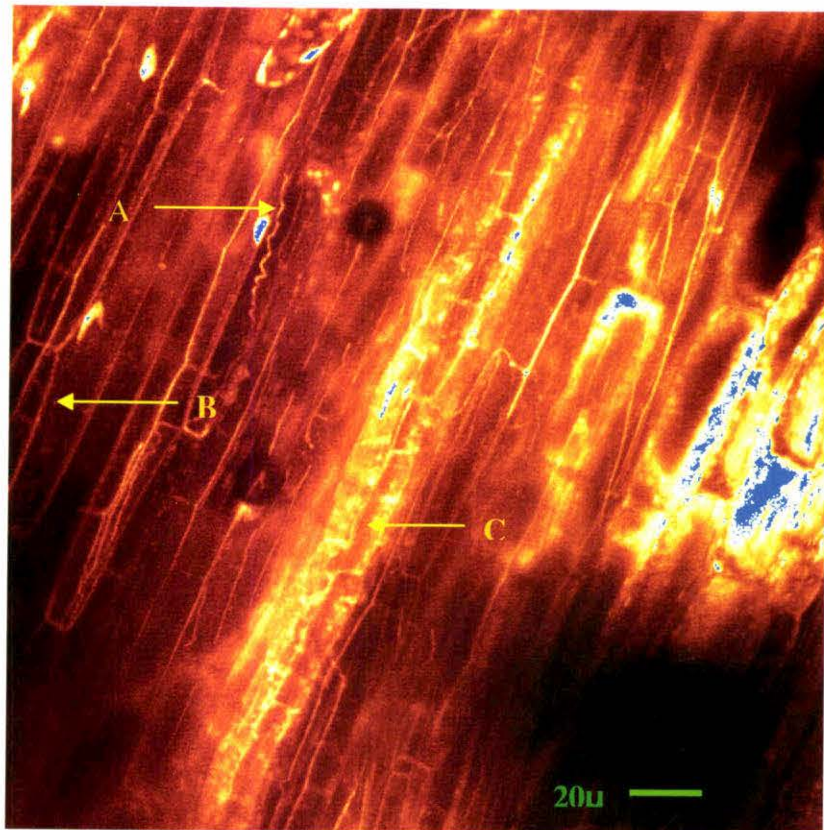


Fig 5.4 Confocal micrograph of a 1.3 µm section of perennial ryegrass leaf sheath tissue prepared using protocol 4-1. The figure shows the endophyte mycelium (**A**), the perennial ryegrass cell walls (**B**) and plant vascular tissue (**C**).

The endophyte was clearly visible (**A**) and there was also clear definition of the surrounding plant cells (**B**). However, the fluorescence of the vascular tissue (**C**) was still a major concern as, at the levels shown in **Fig 5.4**, it would have resulted in thresholding of the endophyte as well as the plant tissue. Despite these limitations, protocol 4 was the most promising protocol and was selected for further refinement.

(ii) Refinements to protocol 4

When protocol 4-2, with basal sheath tissue, known to have an abundance of endophyte hyphae, was used, few hyphae were visible (**Fig 5.5**).

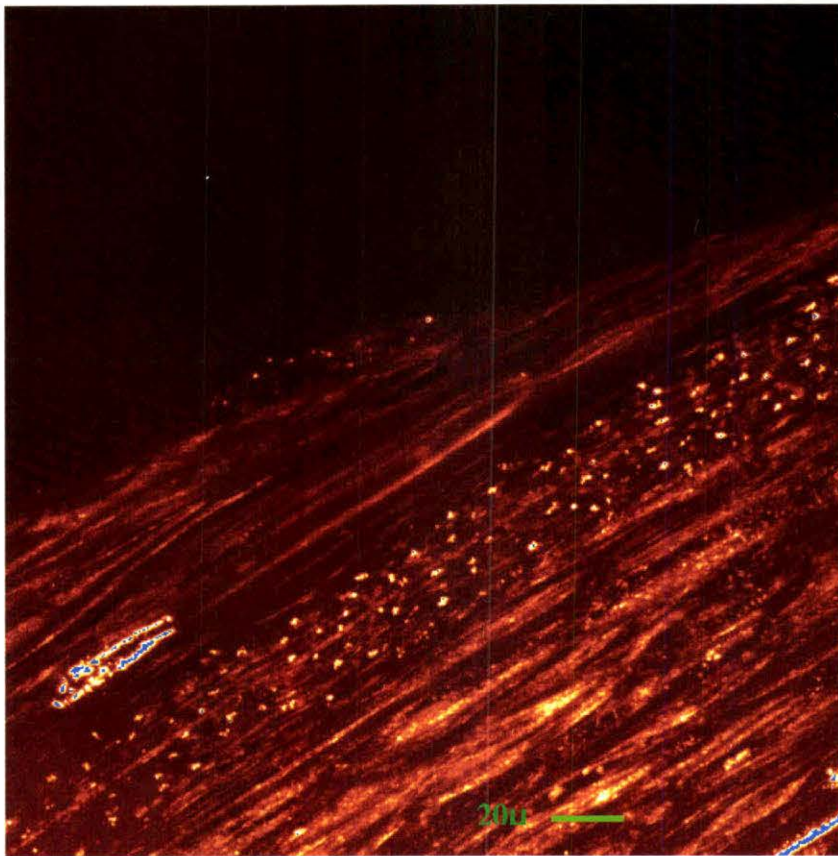


Fig 5.5 Confocal micrograph of a 1.3 μm section of perennial ryegrass leaf sheath tissue prepared using protocol 4-2. The perennial ryegrass tissue used was known to host the endophyte *N. lolii.*

It was clear that the Histoclear solution and the subsequent treatment of the tissue had almost completely depleted any endophyte fluorescence. Because of this problem, Histoclear was removed in subsequent protocols.

No endophyte fluorescence was detected when protocol 4-3 was used (**Fig 5.6**), despite the fact that the tissue used was known to have a large endophyte biomass. Plant cell wall definition was lost completely. However, vascular tissue was clearly visible (**A**).

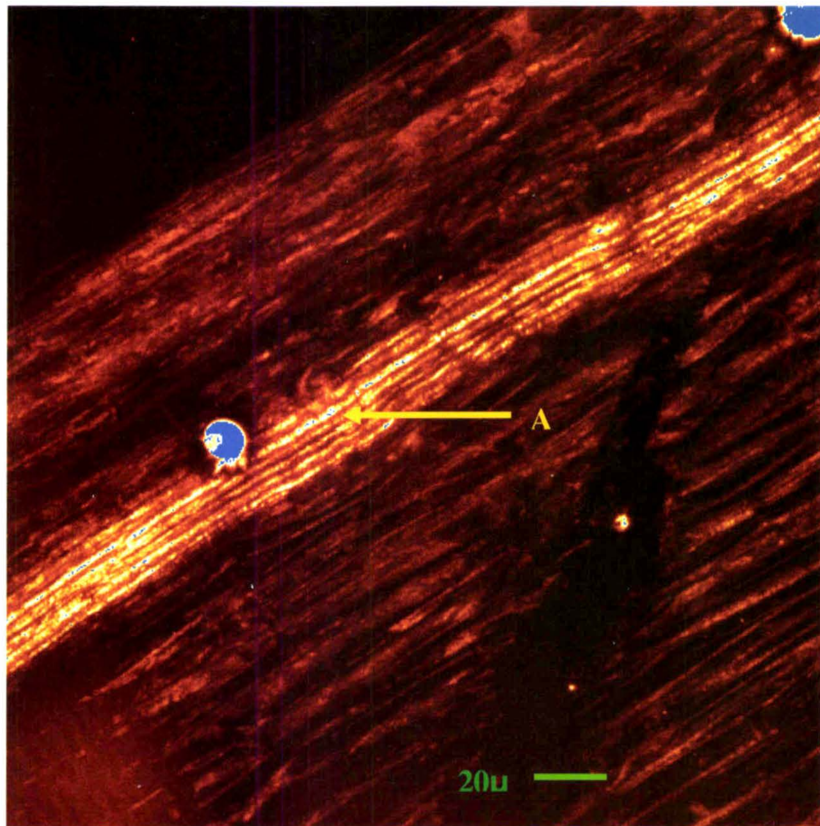


Fig 5.6 Confocal micrograph of a 1.3 μm section of perennial ryegrass leaf sheath tissue prepared using protocol 4-3. This plant tissue was known to host the endophyte and the micrograph shows perennial ryegrass vascular tissue (**A**).

Protocol 4-4 resulted in confocal images that gave good definition of the plant cell walls (**Fig 5.7**). Although endophyte may have been present in the intercellular spaces, hyphal fluorescence was not distinguishable from that of the plant. As a result, further development of this protocol was discontinued.

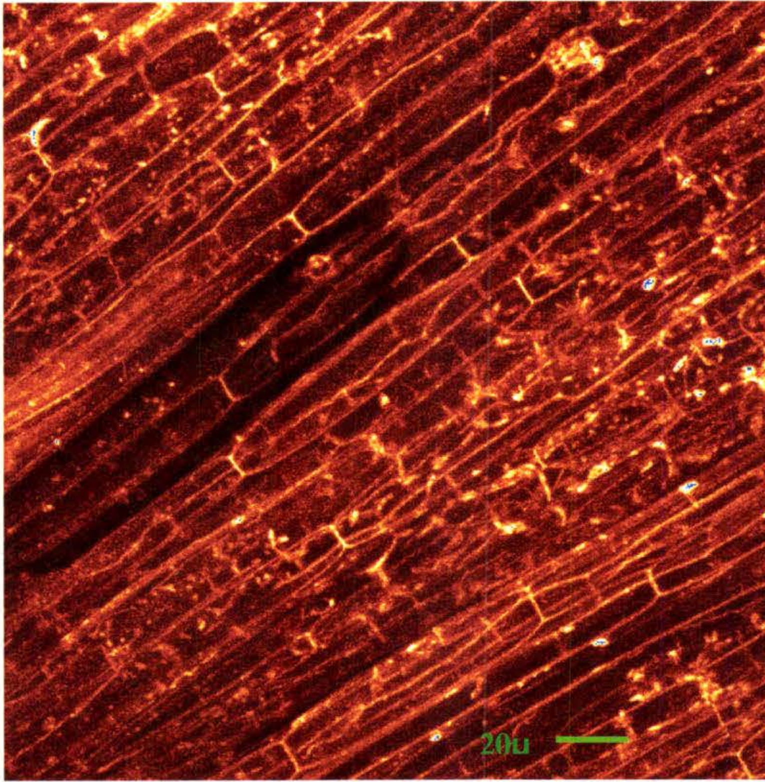


Fig 5.7 Confocal micrograph of a 1.3 μm section of perennial ryegrass leaf sheath tissue prepared using protocol 4-4. This plant tissue was known to host the endophyte.

The image from tissue prepared using protocol 4-5 (**Fig 5.8**) showed clear and enhanced plant cell wall fluorescence (**B**) but completely eliminated auto-fluorescence of the endophyte (**A**). Further development of this protocol was discontinued.

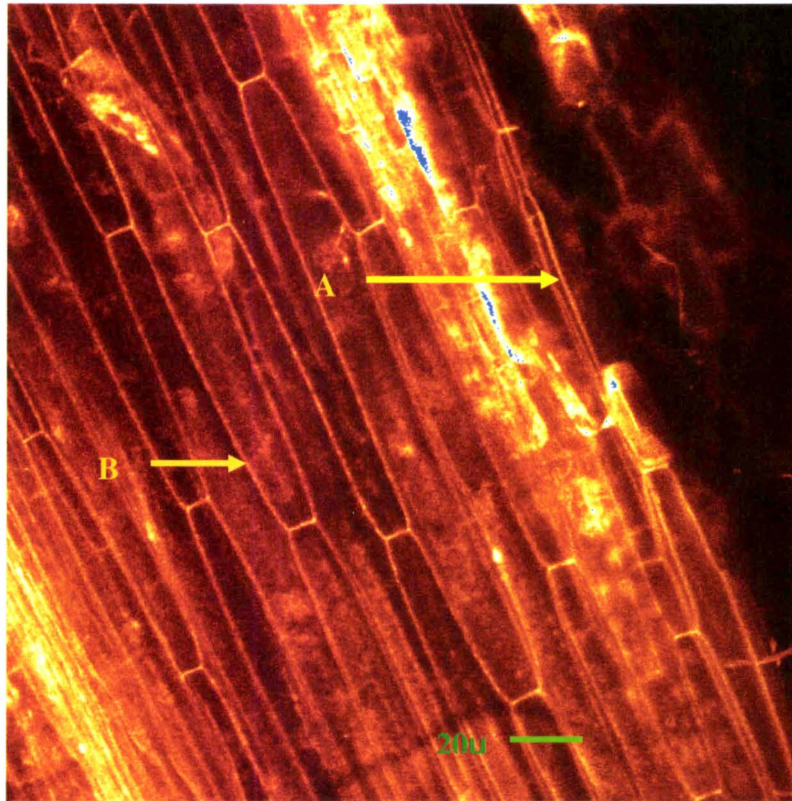


Fig 5.8 Confocal micrograph of a 1.3 μm section of perennial ryegrass leaf sheath tissue prepared using protocol 4-5. This plant tissue was known to host the endophyte. The endophyte mycelium (**A**) and the cell wall of perennial ryegrass (**B**) are shown.

Tissue prepared using protocol 4-6 gave the best confocal images (**Fig 5.9**). This was clearly the best of the six protocols trialed to this point. It not only produced enhanced endophyte fluorescence (**A**) but also eliminated all fluorescence from the surrounding plant cells and at the same time left the more lignified vascular tissue (**B**) intact.

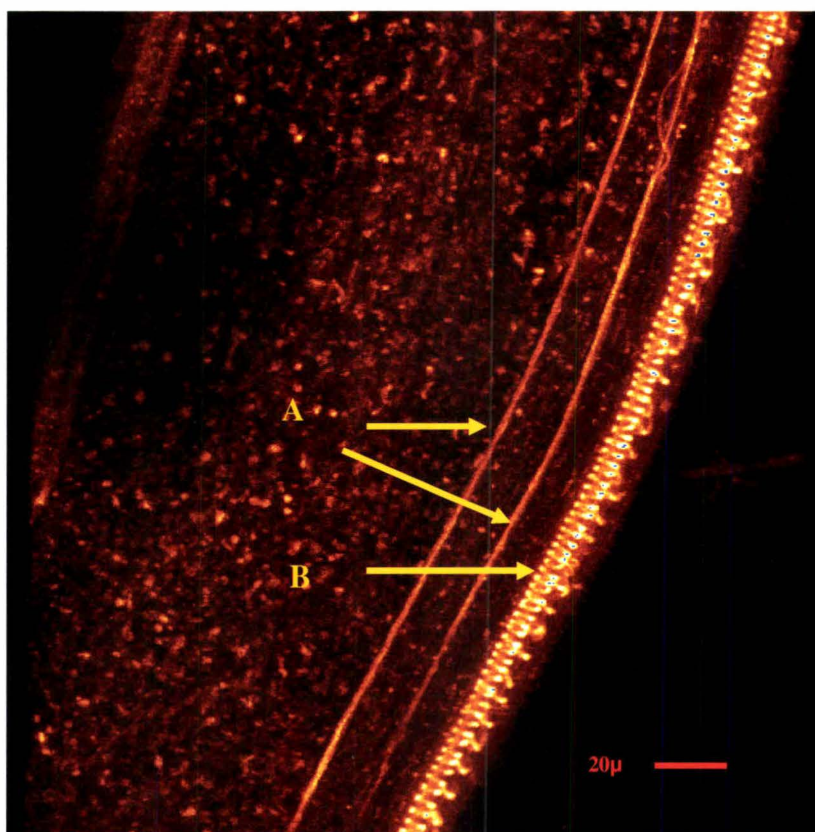


Fig 5.9 Confocal micrograph of a 1.3 µm section of perennial ryegrass leaf sheath tissue prepared using protocol 4-6. This tissue was known to host the endophyte *N. lolii*. The figure shows two endophyte mycelial strands (**A**) running parallel to perennial ryegrass vascular tissue (**B**), against a background of disrupted plant cells that showed relatively low fluorescence.

Fig 5.9 shows the close relationship between the host vascular tissue and the endophyte pattern of growth. The endophyte growth was parallel to the nutrient-providing xylem tissue of the host.

(iii) Further modifications of protocol 4-6

A summary of the further modifications that were trialed is given in **Table 5.1**. The key element in protocols 4-6A to 4-6F was the use of Tris buffer with the glutaraldehyde, in an attempt to enhance the glutaraldehyde effects of fixing and fluorescence, as shown in **Fig 5.10**.

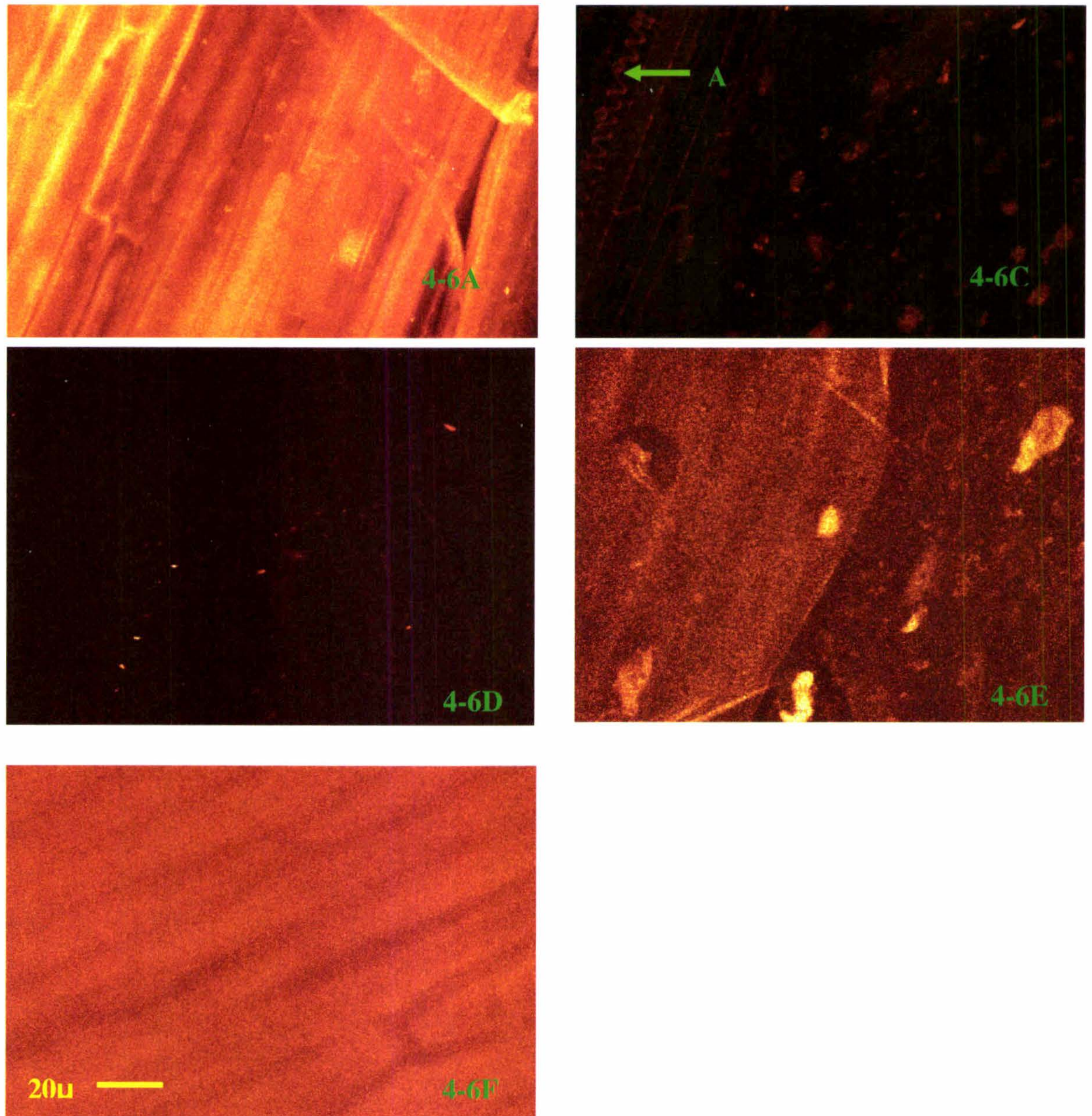


Fig 5.10 Five confocal micrographs of 1.3 μm sections of perennial ryegrass leaf sheath tissue prepared using protocols 4-6A to 4-6F. The protocols are outlined in **Table 5.1**.

These treatments were too harsh. Either there was a complete deterioration in the cell walls of both the perennial ryegrass and the endophyte (4-6C and 4-6D) or definition was lost because of damage (4-6A, 4-6E and 4-6F). *Lolium* vascular tissue is shown (**A**). It was clear that the Tris buffer treatment resulted in the loss of all auto-fluorescence. No endophyte mycelia were visible and the plant cell wall definition was completely lost.

In protocols 4-6G to 4-6L (**Fig 5.11**), the increase in glutaraldehyde concentration increased the resolution of the endophyte. A decrease in the resolution occurred as the

time in KOH was increased, when autoclaving was carried out or when long KOH incubation was implemented. 4-6G had a relatively clear *Neotyphodium* image against a background of a relatively low *Lolium* cell wall definition. All except the 4-6K and 4-6L treatments showed the endophyte. Although the plant cell wall definition was lost in 4-6H, 4-6I and 4-6J, both plant cell wall (**B**) and the occupying endophyte (**A**) were clearly visible in 4-6G. However, it appeared that this treatment caused endophyte mycelium constriction and bursting of the cells on to the surface of the preparation.

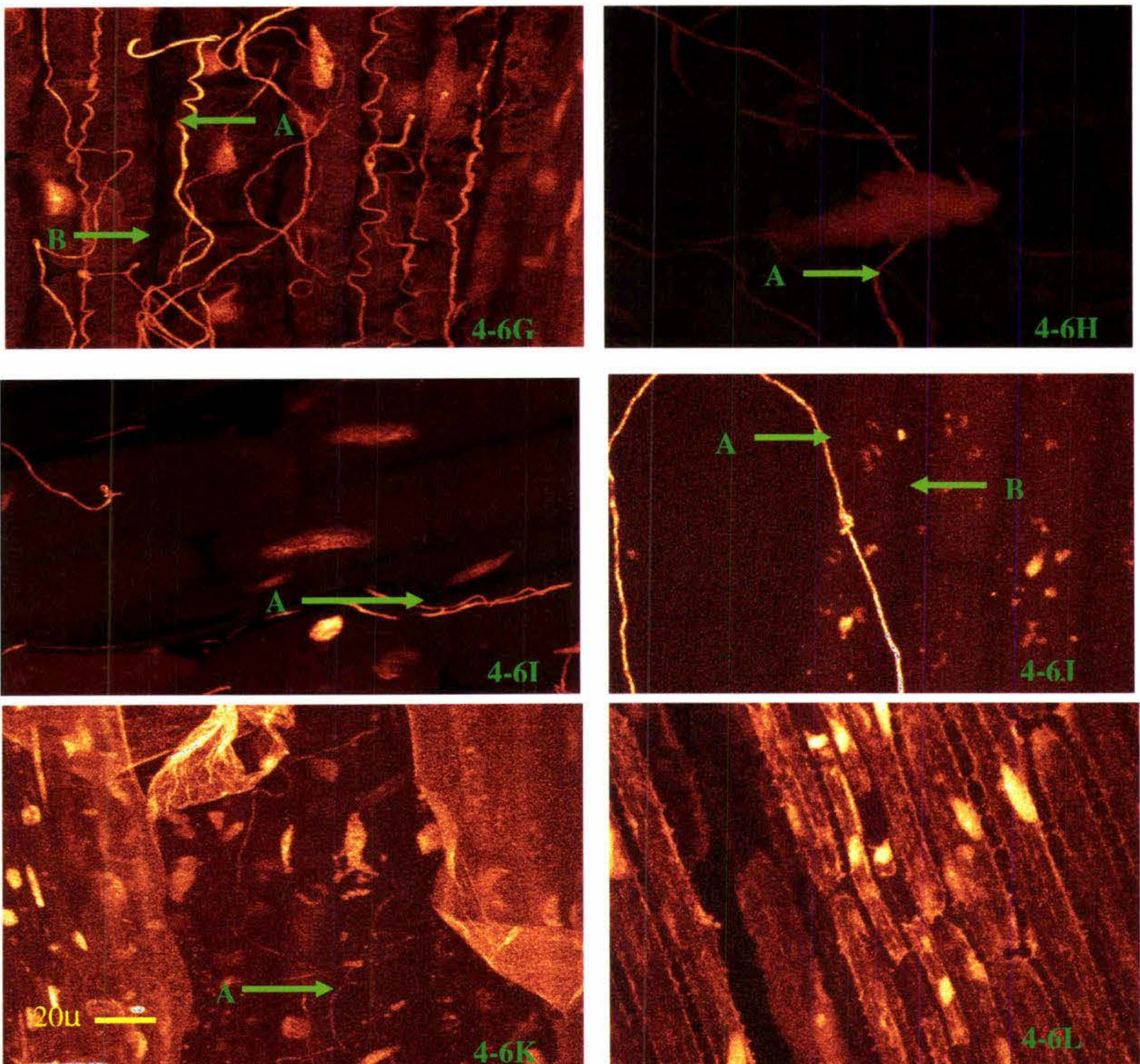


Fig 5.11 Six confocal micrographs of 1.3 μm sections of perennial ryegrass leaf sheath tissue prepared using protocols 4-6G to 4-6L. This plant tissue was known to host the endophyte. The protocols are outlined in **Table 5.1**. The plant leaf sheath cells are shown (**B**), as is the endophyte (**A**).

Tissue prepared using protocols 4-6M to 4-6R gave images with good resolution of the endophyte mycelium (**Fig 5.12**). The use of an increased concentration of glutaraldehyde was the most likely reason for the success of these experiments.

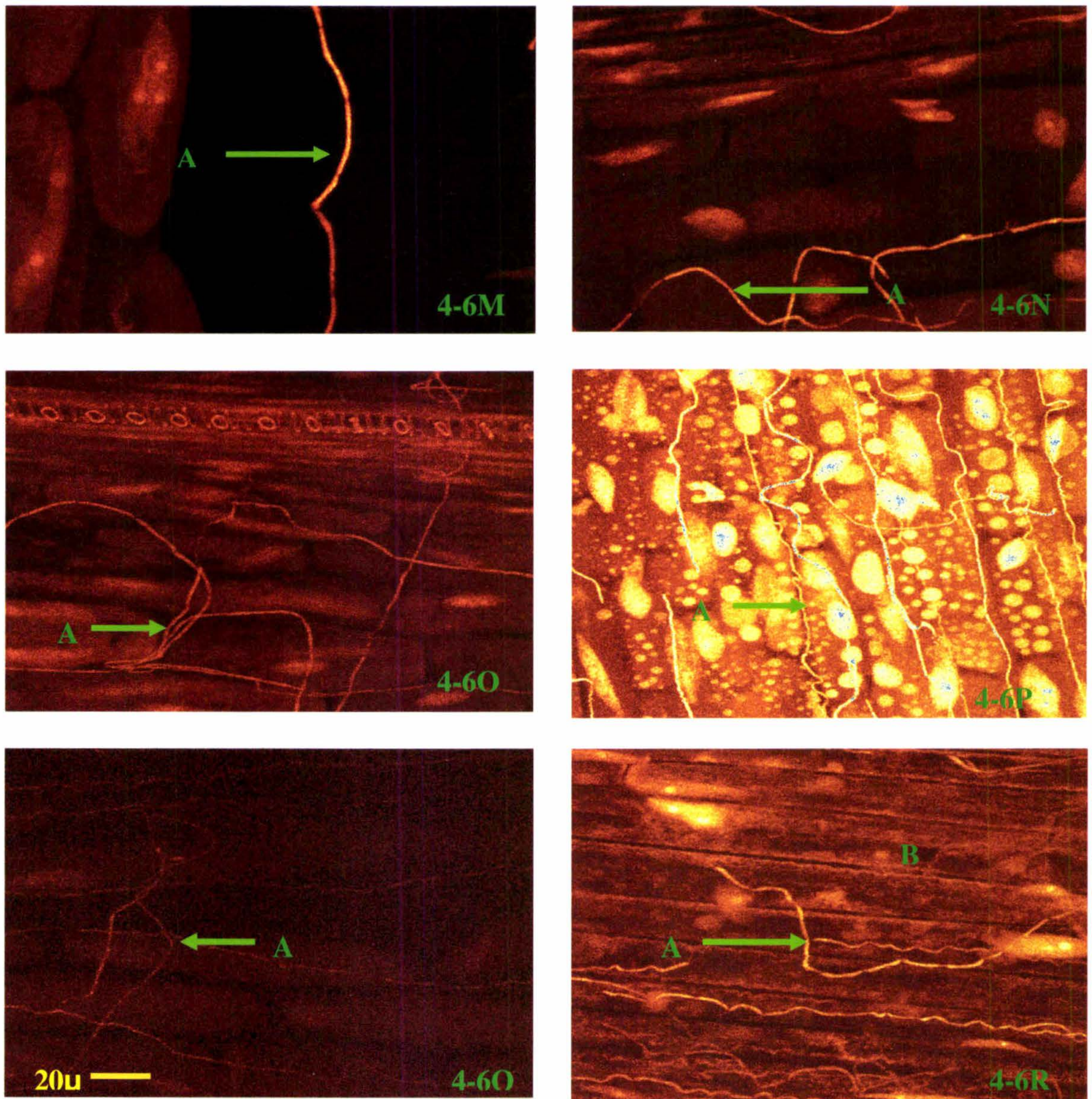


Fig 5.12 Six confocal micrographs of 1.3 μm sections of perennial ryegrass leaf sheath tissue prepared using protocols 4-6M to 4-6R. The protocols are outlined in **Table 5.1**. Perennial ryegrass leaf sheath cells (**B**) and the endophyte *N. lolii* (**A**) are shown.

Having established that the highest endophyte biomass is found alongside the plant 73vascular tissue, a decision was made to move towards a protocol that would clearly define the mycelium of the endophyte and at the same time would quench the

fluorescence from plant organelles and/or would selectively destroy the surrounding plant tissue. Subsequently, all plant tissue prepared for thresholding was treated using protocol 4-6M.

5.3.4 Measurement of endophyte biomass

To determine the endophyte volume, a series of confocal micrograph images was first captured and then assembled. These images were then thresholded individuall 167 example of this procedure can be seen in **Fig 5.13**, where images (A, B, C and D) were overlaid and computer coloured (E) ready for thresholding.

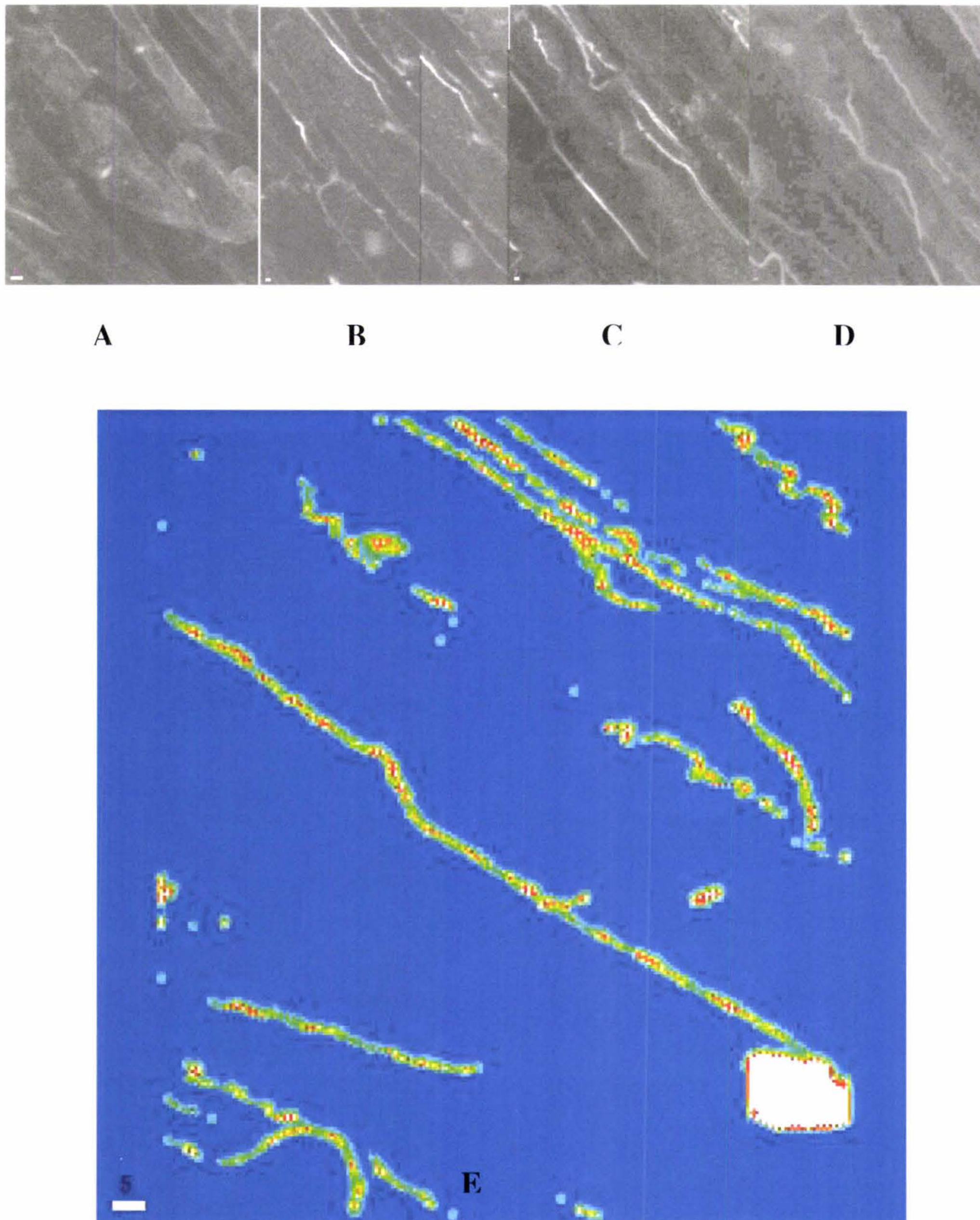


Fig 5.13 Confocal micrographs of 1.3 μm sections (**A**, **B**, **C**, **D**) prepared using protocol 4-6M. The sections were then overlaid to produce a composite image (**E**) that could then be thresholded with Image Space software using an off-line computer.

However, this procedure had a major underlying fault. The final mycelium images in a stack (**E**) could overlay mycelium images immediately below them and thus block that view for thresholding. This would give a low estimate of endophyte biomass.

Because of this, single sectional imaging was carried out. **Fig 5.14** is an example of a single digitally enhanced confocal micrograph of a 1.3 μm section.

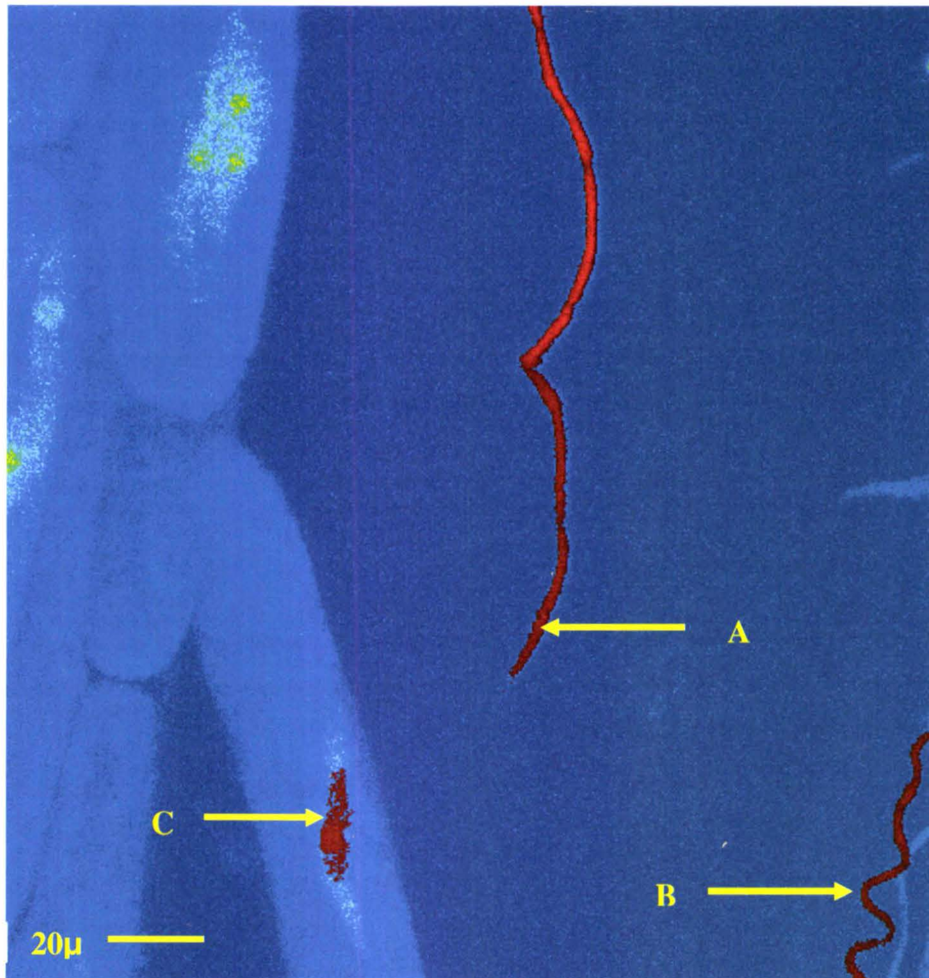


Fig 5.14 Confocal micrograph of a single 1.3 μm section that was prepared using protocol 4-6M. Endophyte mycelium (**A** and **B**) growing in the intercellular spaces of the ryegrass plant is shown along with the nucleus of a perennial ryegrass plant cell (**C**).

Each image was thresholded individually using computer Draw/Fill software to remove obvious flaws. An example of these flaws can be seen, (**C**) in **Fig 5.14**. The individual endophyte volumes were then totalled. The biomass for this section was estimated (**Table 5.2**).

Table 5.2 Estimation of biovolume of a single thresholded confocal section.

Biomass	Volume (μm^3)
A	1880^a
B	580
Total	2460
C	nucleus

^{a}Calculated using a Silicon Graphics Indy Computer using Image Space software.

A summary of the data for six thresholded sections is given in **Table 5.3**. For a complete summary of the results, see **Appendix 16**

Table 5.3 Endophyte biovolume in perennial ryegrass leaf tissue[a].

1.3μm Sections	Endophyte Volume (μm^3)	Endophyte Length (μm)
7	308	4.2
8	274	4.0
9	243	3.9
10	190	3.6
11	181	3.5
12	176	3.5
Total	**1372**	**22.7**
Endophyte occupancy ^{b}	**2.7%**	

[a]Summary of thresholded endophyte volumes estimated from 1.3 μm confocal sections. The complete print out of the data can be found in **Appendix 4.**

^{b}The volume of a confocal section is 190 x 200 x 1.3 μm = 49400 μm^3.

A 'percentage occupancy' can be calculated from these data and, providing a number of samples are taken, randomly, in a balanced way, from leaf and sheath tissue, a significant estimate of endophyte biomass can be obtained for a whole plant genotype.

Section 5.4

Discussion

The use of the confocal microscope to probe plant tissues such as the apical meristem in micron layers, image the sections and then to re-assemble the sections into a three dimensional picture that can be rotated on three planes for viewing has further highlighted the advantages of using confocal microscopy to examine specific plant tissues. This would have potential in work where it was a need to examine meristem development in relation to plant growth and development or the images used in conjunction with the contributing 1.3 μm sections to investigate endophyte colonisation of *L. perenne* (Christensen *et al.,* 2001).

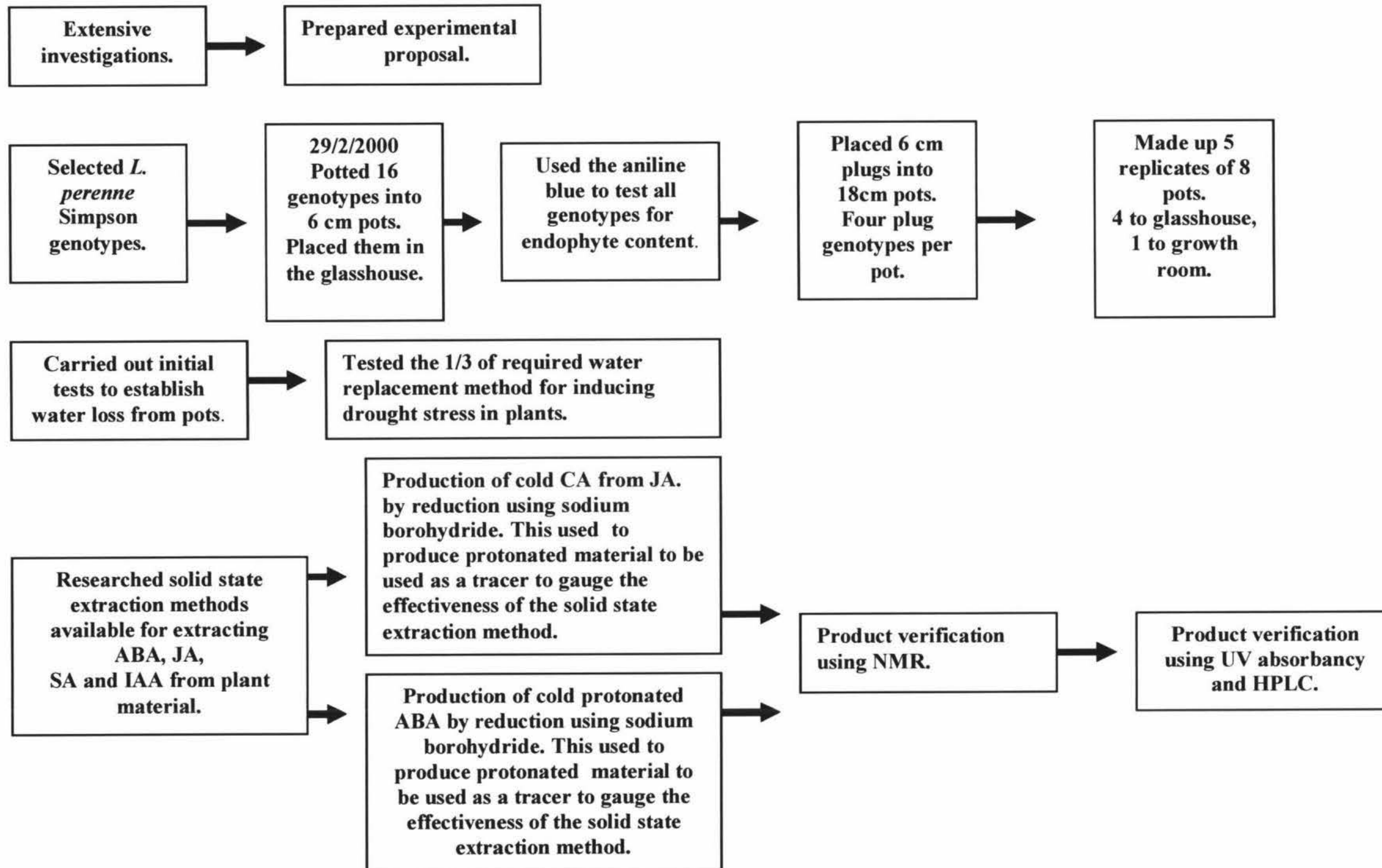
The use of the confocal microscope to produce anaglyphs enables a three dimensional insight into the relationship between the endophyte and the host plants vascular and other tissues. These images are assembled without causing any mechanical damage to either the endophyte or its host.

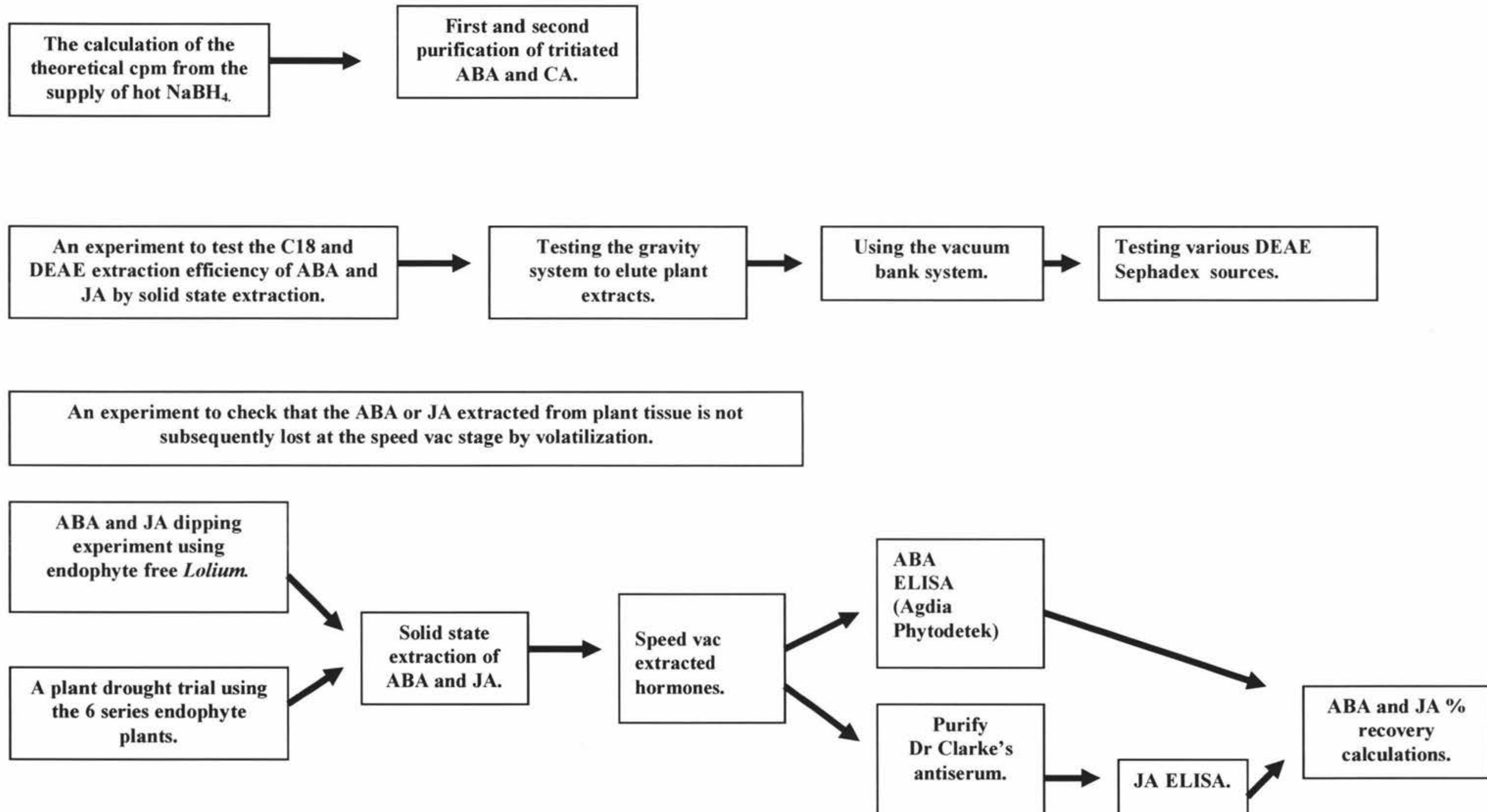
The central aim of this work was to use the confocal microscope to examine *L. perenne* sectional images, in conjunction with staining protocols that would specifically produce an endophyte fluorescence. This endophyte fluorescence was thresholded, using an on line Silicon Graphics Indy computer with Image Space Software, and an estimate of the biomass of *N. lolii* was determined. The relationship of endophyte biomass to specific ryegrass tissues and a measure of endophyte occupancy could then be made because the Image Space Soft ware was able to calculate endophyte biomass volume. The volume of each ryegrass confocal optical section was known so it was possible to calculate a percentage occupancy figure for the endophyte.
This method was not as fast as other methods outlined in the Introduction such as the electron microscope method (Yong. *et al.,* 2001) and the quantitative PCR method (Panaccione *et al.,* 2001; Groppe & Boller, 1997)
Further development is needed to speed up the process reported on here. To increase fungal fluorescence, differentiate fungal fluorescence from plant fluorescence and optimise the sampling of ryegrass, to more accurately reflect normal fungal distribution.

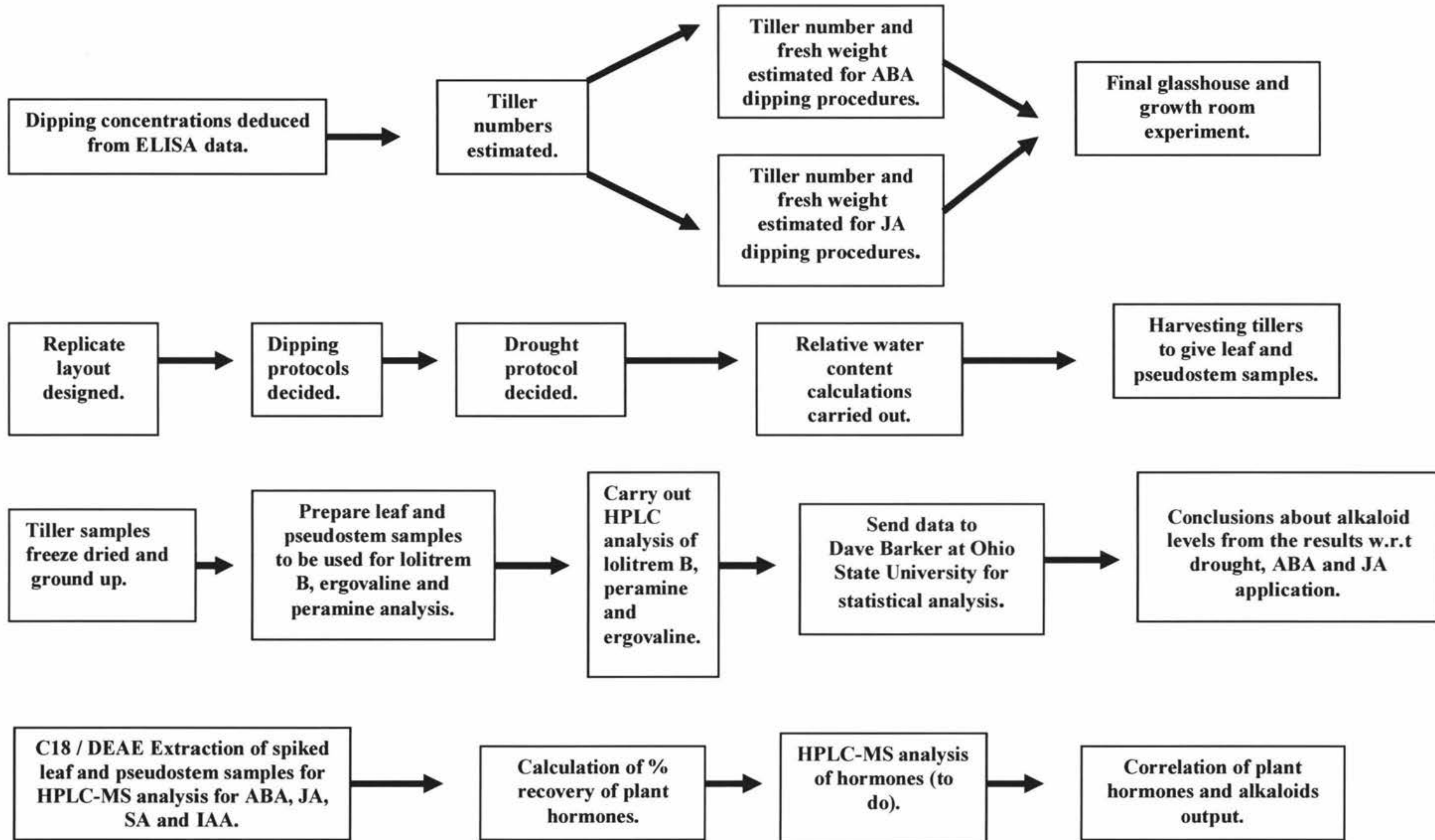
Appendix

Appendix 1

Experimental set up.




The calculation of the theoretical cpm from the supply of hot $NaBH_4$.
First and second purification of tritiated ABA and CA.
An experiment to test the C18 and DEAE extraction efficiency of ABA and JA by solid state extraction.
Testing the gravity system to elute plant extracts.
Using the vacuum bank system.
Testing various DEAE Sephadex sources.
An experiment to check that the ABA or JA extracted from plant tissue is not subsequently lost at the speed vac stage by volatilization.
ABA and JA dipping experiment using endophyte free *Lolium*.
A plant drought trial using the 6 series endophyte plants.
Solid state extraction of ABA and JA.
Speed vac extracted hormones.
ABA ELISA (Agdia Phytodetek)
Purify Dr Clarke's antiserum.
JA ELISA.
ABA and JA % recovery calculations.


Dipping concentrations deduced from ELISA data.
Tiller numbers estimated.
Tiller number and fresh weight estimated for ABA dipping procedures.
Tiller number and fresh weight estimated for JA dipping procedures.
Final glasshouse and growth room experiment.
Replicate layout designed.
Dipping protocols decided.
Drought protocol decided.
Relative water content calculations carried out.
Harvesting tillers to give leaf and pseudostem samples.
Tiller samples freeze dried and ground up.
Prepare leaf and pseudostem samples to be used for lolitrem B, ergovaline and peramine analysis.
Carry out HPLC analysis of lolitrem B, peramine and ergovaline.
Send data to Dave Barker at Ohio State University for statistical analysis.
Conclusions about alkaloid levels from the results w.r.t drought, ABA and JA application.
C18 / DEAE Extraction of spiked leaf and pseudostem samples for HPLC-MS analysis for ABA, JA, SA and IAA.
Calculation of % recovery of plant hormones.
HPLC-MS analysis of hormones (to do).
Correlation of plant hormones and alkaloids output.

Appendix 2

Cold scaled-down sodium borohydride reaction

(a) Dilution for ABA

Add 10 mg of ABA to 100 µL of 100% ethanol and make up to 1 mL with 0.2 M $NaHCO_3$ buffer.

Take 7 µL → 0.07 mg of ABA

(b) Dilution for JA

Add 13 µL of JA to 100 µL of 100% ethanol and make up to 1 mL with 0.2 M $NaHCO_3$ buffer.

Take 7 µL → 0.09 mg of JA

(c) Dilution for sodium borohydride

Add 26.4 mg of $NaBH_4$ to 1 mL of 0.2 M $NaHCO_3$ buffer → 26.4 mg/mL.

Take 7 µL → **0.185 mg** of sodium borohydride

	BUFFER	$NaBH_4$	HORMONE
ABA	**14 µL**	**0.185 mg**	**0.07 mg**
JA	**14 µL**	**0.185 mg**	**0.09 mg**

(d) pH adjustment runs

ABA

10.3 pH, needed 2 x 0.5 µL of 4 M HCl + 2 x 0.5 µL of 2 M HCl → pH 3.18

JA

10.45 pH, needed 2 x 0.5 µL of 4 M + 1 µL of 2 M HCl → pH 3.5

Appendix 3

(a) HPLC solvents for JA.

SOLVENT A	SOLVENT B	SOLVENT C
0.05% (v/v) Trifluoroacetic acid in MilliQ water	0.05% (v/v) Trifluoroacetic acid in acetonitrile in MilliQ water	100% methanol

Solvents used for HPLC analysis of JA at 269 nm.

Time	Flow Rate mL/min	% Solvent A	% Solvent B	% Solvent C	% Solvent D
Initial	1.0	60.0	32.0	8.0	0.0
7.0	1.0	45.0	47.0	8.0	0.0
11.0	1.0	40.0	52.0	8.0	0.0
16.0	1.0	20.0	72.0	8.0	0.0
18.0	1.0	0.0	0.0	100.0	0.0
28.0	1.0	0.0	0.0	100.0	0.0
32.0	1.0	60.0	32.0	8.0	0.0

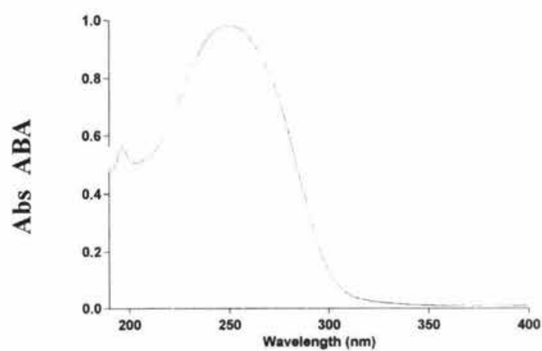
(b) HPLC solvents for ABA.

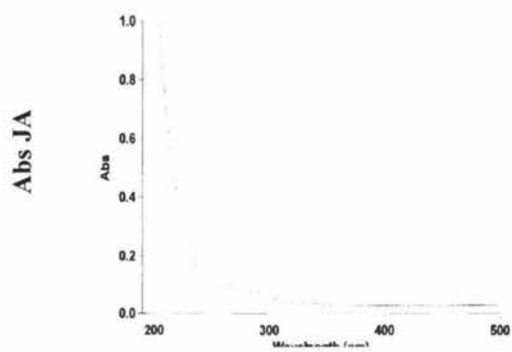
SOLVENT A	SOLVENT B	SOLVENT C	SOLVENT D
HPLC buffer pH 3.35	80% (v/v) methanol 20% HPLC buffer	80% (v/v) acetone 20% HPLC buffer	100% methanol

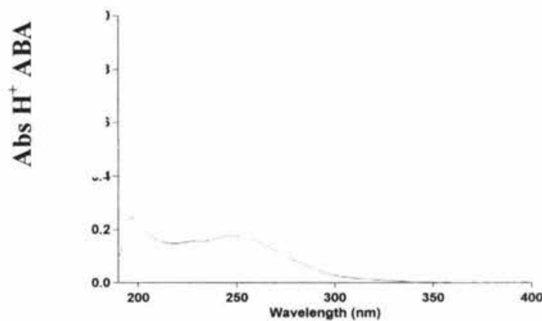
Solvent used for HPLC analysis of ABA at 269 nm.

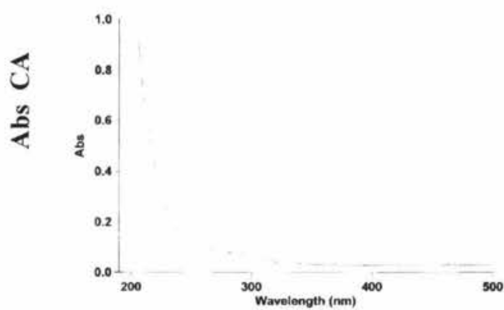
Time	Flow Rate mL/min	% Solvent A	% Solvent B	% Solvent C	% Solvent D
Initial	1.0	80.0	0.0	20.0	0.0
10.0	1.0	45.0	0.0	47.0	8.0
16.0	1.0	20.0	0.0	72.0	8.0
18.0	1.0	0.0	0.0	0.0	100.0
25.0	1.0	0.0	0.0	0.0	100.0
30.0	1.0	80.0	0.0	20.0	0.0

(c) UV absorbances of ABA, ^{1}H ABA, JA and CA

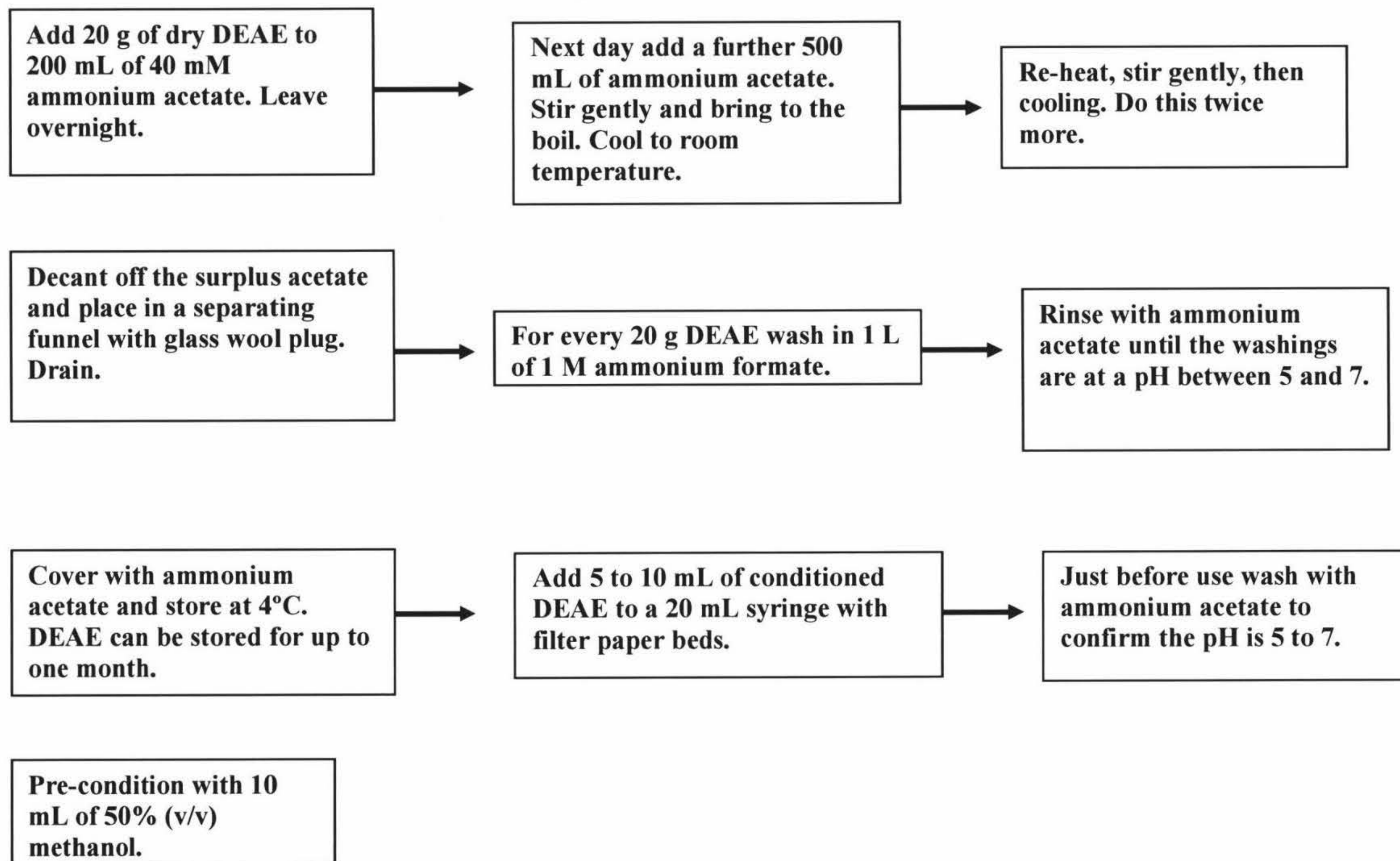






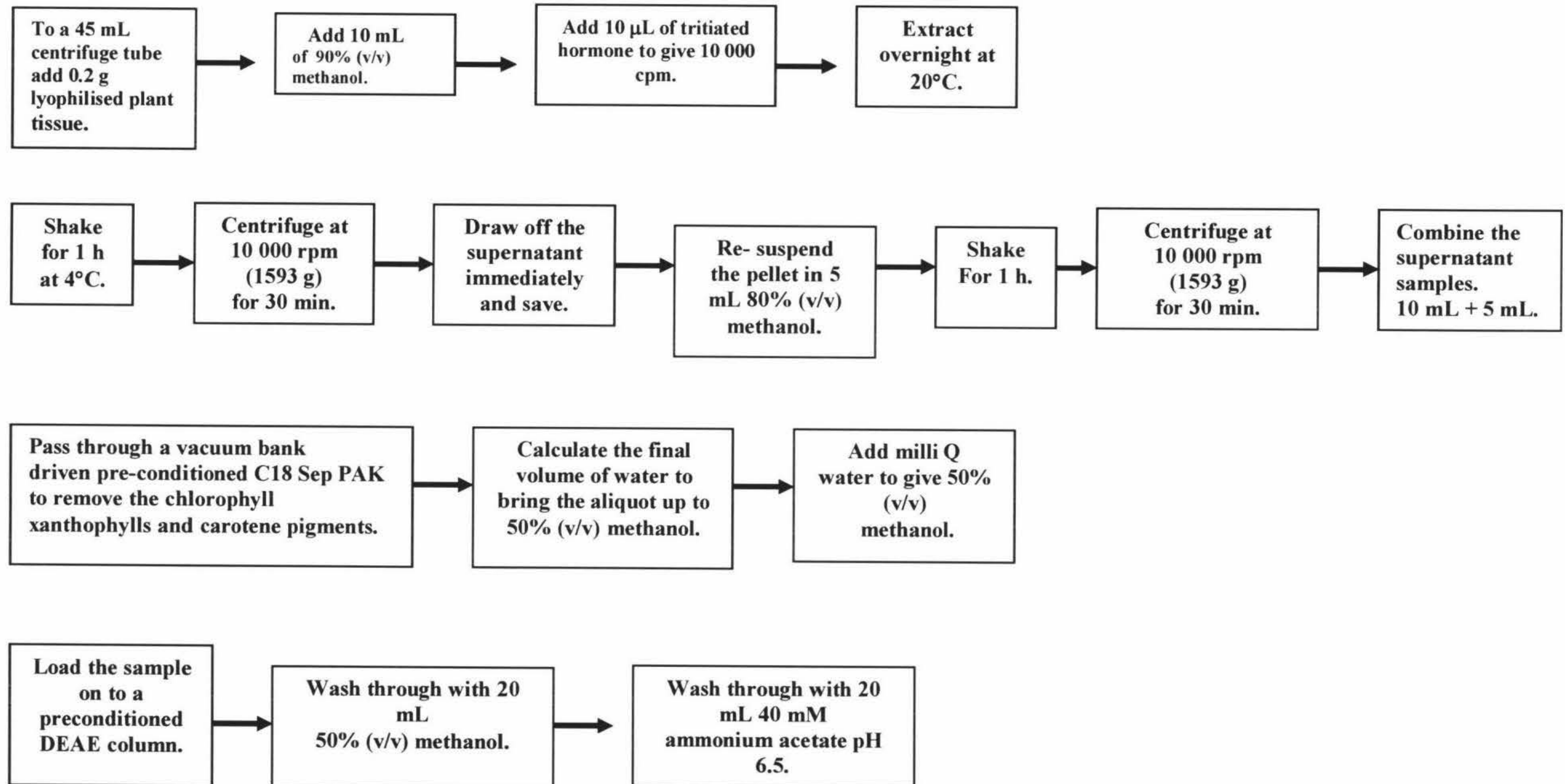


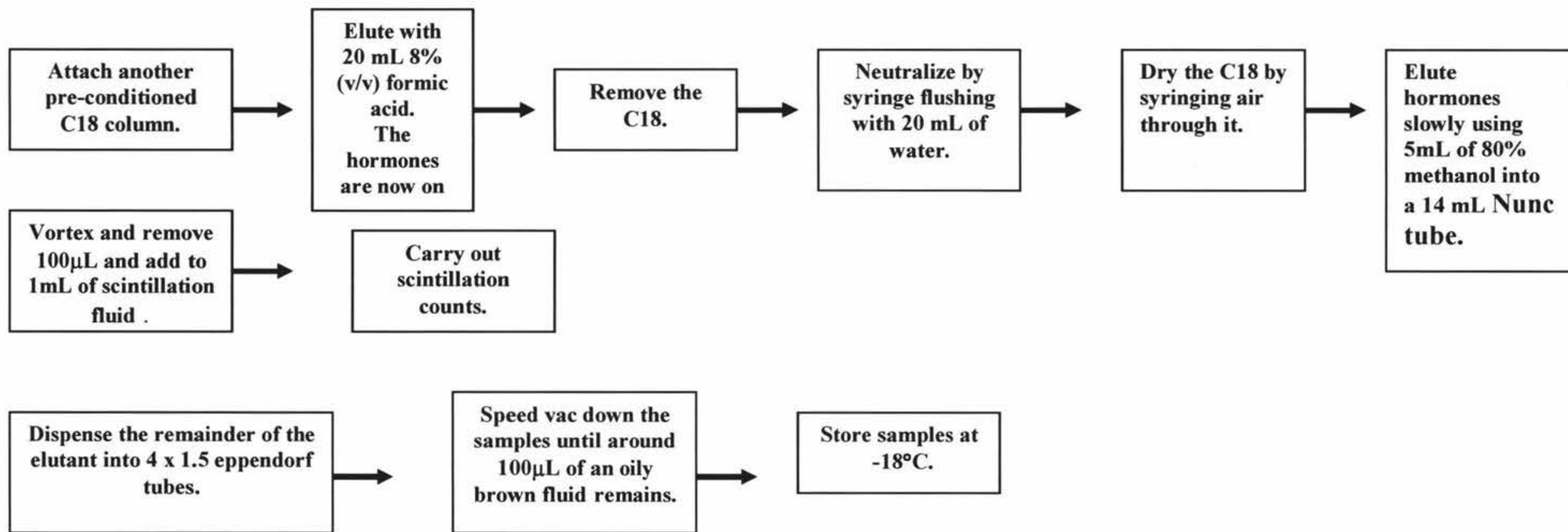
Setting up a DEAE column.



Appendix 5

Protocol for the extraction of the plant hormones ABA, JA and IAA by solid state extraction.
(Note: 1g FW *Lolium perenne* has on average 20% dry weight. The rule for this extraction is 1g FW per 10mL 80% methanol).





C18 pre-conditioning for chlorophyll, xanthophylls and carotene removal.

Syringe through 20 mL of 100% methanol and dry with 3-4 syringe volumes of air.

(a) Pass through 10 mL water.
(b) Pass trough 10 mL of 80% methanol.

C18 Pre-conditioning for the removal of hormones from the DEAE column.

(1) Syringe through 20 mL of 100% methanol.
(2) Dry with 2-3 syringe volumes of air.
(3) Just before use.
  (a) Pass through 10 mL of water.
  (b) Pass through 10 mL 8% formic acid.

DEAE Pre-conditioning.

Just before use

(a) Run through enough ammonium acetate until the elutant runs at pH 6.5.
(b) Pass through 10 mL of 50% (v/v) methanol.

Appendix 6

PBS buffer

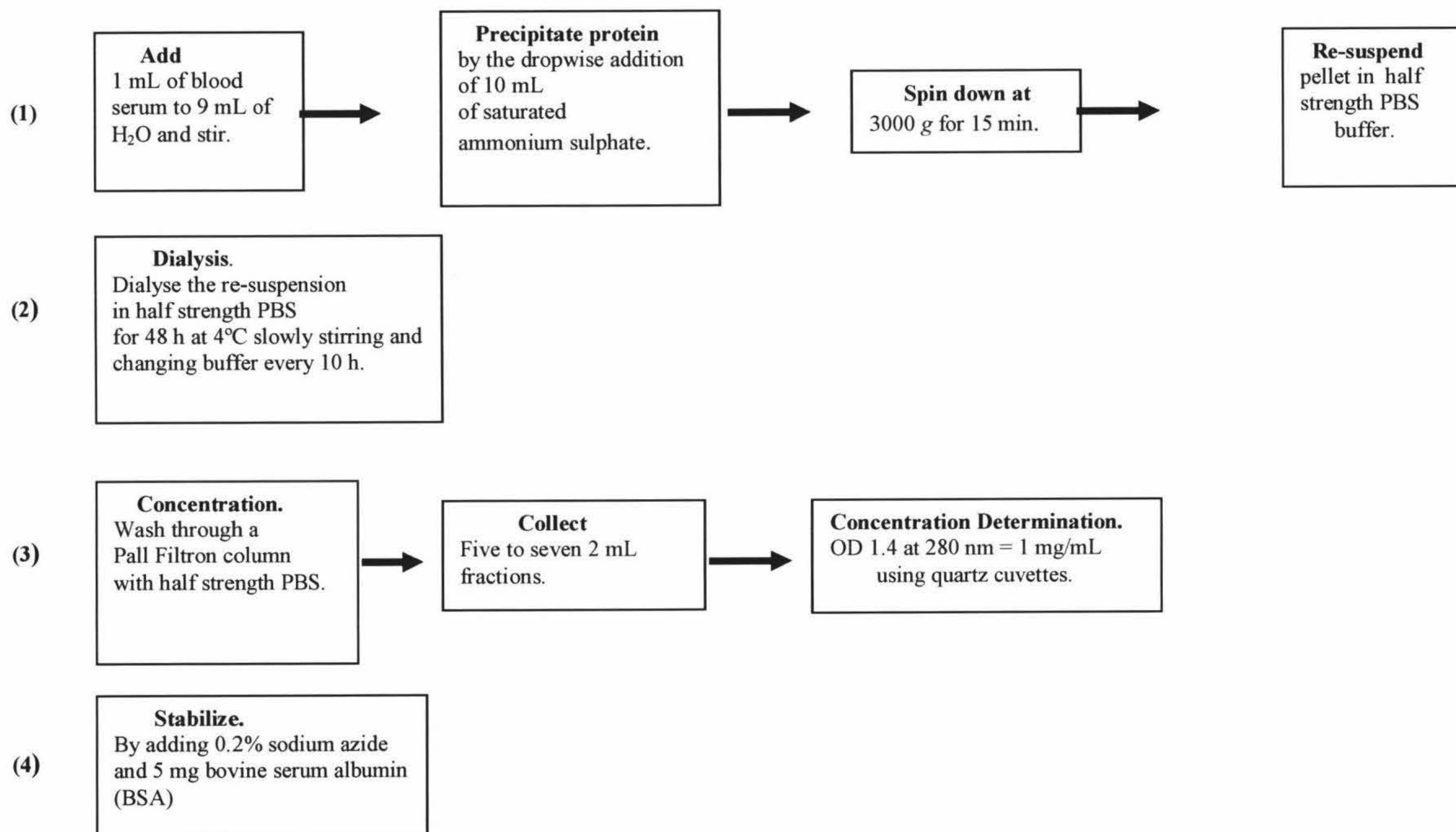
Stock at 10x/L	Per 200 mL	Working Solution (mM)
80 g NaCl	16	109
2 g KCl	0.4	0.05
11.5 g $Na_2HPO_4.7H_2O$	2.3	0.45
2g KH_2PO_4	0.4	0.03

Add 6.5 g of sucrose and adjust pH to 7.2.

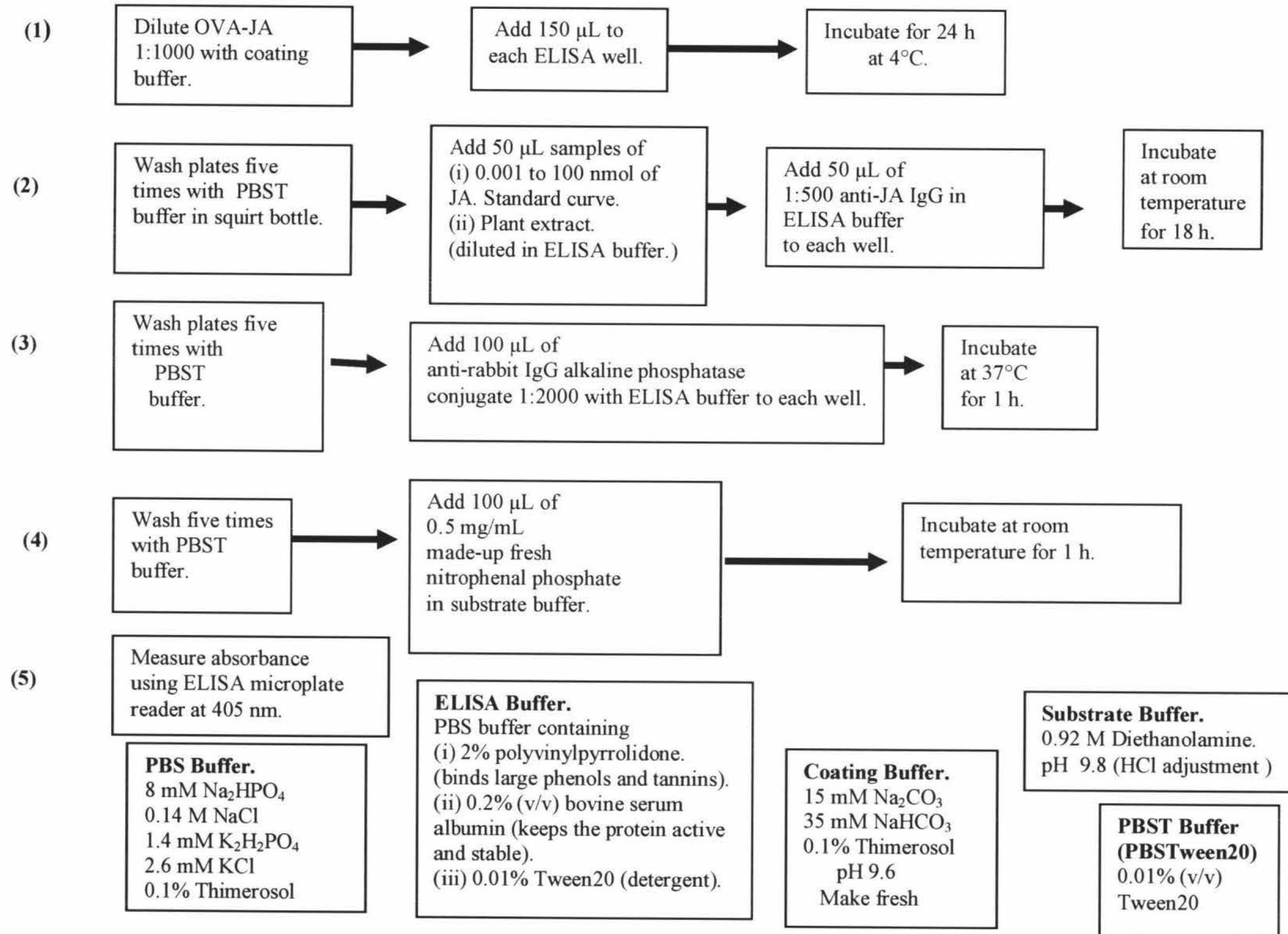
Appendix 7

Purification of antiserum

(The antiserum was in the first instance a gift from Dr Sean Clarke. Some was also kindly donated by Nigel Gapper.)
It was prepared by injecting a New Zealand white rabbit with JA conjugated to the immunogen keyhole limpet haemocyanin (KLH-JA).
The Ovalbumin-JA was stored in 50% glycerol at 4°C. The purification procedure was as follows.



Appendix 8
Protocol for JA ELISA (as per Clarke 1996)



Appendix 9

From the original crude reduction product, 1 µL was placed in 1 mL of 80% (v/v) methanol. From this, 100 µL was used to obtain an input count of 1 372 717 200 cpm. Much of these counts were radioactive water. A further 100 µL sample was applied to a C18 column for purification (Section 2.8.1) and two 1 mL aliquots were obtained. From each of these aliquot samples, 100 µL was taken and combined with scintillation fluid and a scintillation reading of each was obtained. The first aliquot off the C18 column gave a reading of 8 152 592 x 10 = 81 525 920 cpm. The second aliquot gave a reading of 4 296 396 x 10 = 42 63 960 cpm. As a percentage of the input, this gave a 9.1% recovery. (Aliquot 1 + aliquot 2 / input counts.)

Appendix 10

Table of SAS commands

The GLM Procedure.

Class Level Information for 80 Observations.

```
      ;
    proc
   print;
 proc glm;
    class      part block rep trt;
    model evaline lolitrem peramine = block trt block*trt part
                                part*trt;
       test H=trt
      E=block*trt;
        contrast 'water main effect' trt 1 1 1 1 -4
                        /e=block*trt;
      contrast 'JA main effect (wet)' trt 0 0 -1 1 0
                        /e=block*trt;
  contrast 'ABA main effect (wet)' trt 1 0 0 -1 0 /e=block*trt;
        contrast 'JA by ABA interaction (wet)' trt 1 -1 1 -1
                              0/e=block*trt;
   lsmeans trt|part /e=block*trt stderr;
  means trt|part /e=block*trt;
   run;
```

Appendix 11

A comparison between growth chamber data and greenhouse data with and without Replicate 1

Rep	Tissue	Treatment	Ergovaline	% Change	Lolitrem B	% Change	Peramine	% Change
5	leaf	JAW[a]	0.20		0.69		24.3	
4	leaf	JAW	0.59		5.04		80.6	
3	leaf	JAW	0.94		5.06		80.1	
2	leaf	JAW	0.30		0.30		60.9	
1	leaf	JAW	0.14		0.14		37.6	
	Gro+Gho		0.43	7.96	2.25	–26.46	56.70	7.75
	Gho		0.49	23.90	2.64	–25.98	64.80	19.39
	Gho-Rep1		0.61	28.37	3.47	–11.64	73.87	39.55
5	leaf	AW[b]	0.24		0.63		32.8	
4	leaf	AW	1.01		3.64		83.7	
3	leaf	AW	1.21		5.98		70.7	
2	leaf	AW	0.20		2.03		48.7	
1	leaf	AW	0.17		4.70		33.4	
	Gro+Gho		0.57	40.80	3.40	11.20	53.86	2.36
	Gho		0.65	62.83	4.09	14.82	59.13	8.94
	Gho-Rep1		0.81	70.42	3.88	–1.02	67.70	27.90
5	leaf	JA[c]	0.28		1.23		68.8	
4	leaf	JA	1.06		4.74		78.3	
3	leaf	JA	1.10		5.15		80.7	
2	leaf	JA	0.33		3.19		64.3	
1	leaf	JA	0.26		4.34		31.7	
	Gro+Gho		0.61	50.75	3.73	22.13	64.76	23.07
	Gho		0.69	72.96	4.36	22.33	63.75	17.46
	Gho-Rep1		0.83	75.35	4.36	11.13	74.43	40.62
5	leaf	W[d]	0.42		1.03		46.0	
4	leaf	W	0.60		5.00		60.3	
3	leaf	W	0.61		3.79		53.5	
2	leaf	W	0.21		2.98		45.0	
1	leaf	W	0.17		2.47		58.3	
	Gro+Gho	Control	0.40		3.05		52.62	
	Gho	Control	0.40		3.56		54.28	
	Gho-Rep1	Control	0.47		3.92		52.93	
5	Sheath	JAW	0.61		2.77		30.0	
4	Sheath	JAW	1.56		11.22		82.7	
3	Sheath	JAW	1.64		15.18		52.0	
2	Sheath	JAW	1.05		11.66		77.5	
1	Sheath	JAW	0.70		18.80		54.2	
	Gro+Gho		1.11	4.71	11.93	2.05	59.28	27.87
	Gho		1.24	11.24	14.22	6.80	66.60	28.51
	Gho-Rep1		1.42	15.49	12.69	17.58	70.73	44.26
	Sheath	AW	0.69		2.45		35.7	
	Sheath	AW	1.95		13.24		79.9	
	Sheath	AW	2.39		12.80		58.6	
	Sheath	AW	0.84		8.60		60.6	
	Sheath	AW	0.49		9.38		34.6	
	Gro+Gho		1.17	10.55	7.42	–36.52	53.88	16.22

Gho		1.42	27.42	11.01	−17.32	58.43	12.74
Gho-Rep1		1.73	40.76	11.55	7.01	66.37	35.35
Sheath	JA	1.08		7.22		65.0	
Sheath	JA	2.41		12.90		74.7	
Sheath	JA	1.49		13.97		61.8	
Sheath	JA	1.07		14.78		64.8	
Sheath	JA	0.90		13.96		47.5	
Gro+Gho		1.21	13.94	9.77	−16.36	62.76	35.38
Gho		1.47	31.91	13.90	4.45	62.20	20.02
Gho-Rep1		1.66	35.05	13.88	28.67	67.10	36.85
Sheath	W	0.86		5.19		24.5	
Sheath	W	1.58		9.09		59.8	
Sheath	W	1.21		9.39		35.2	
Sheath	W	0.89		13.89		52.1	
Sheath	W	0.77		20.87		60.2	
Gro+Gho	Control	1.06		11.69		46.36	
Gho	Control	1.11		13.31		51.83	
Gho-Rep1	Control	1.23		10.79		49.03	

[a]Water-sufficient plants dipped every 2 days in JA/ABA solutions of increasing concentration.
[b]Water-sufficient plants dipped every 2 days in ABA solutions of increasing concentration.
[c]Water-sufficient plants dipped every 2 days in JA solutions of increasing concentration.
[d]Plants kept in a water-sufficient condition without dipping (controls).

Appendix 12

A dehydration series using ethanol in a 1.5 mL Eppendorf tube

Explant is in 1.5 mL PBS bathing solution. Remove 0.075 mL bathing solution, add 0.075 mL ethanol (5% (v/v) ethanol). Remove 0.225 mL bathing solution, add 0.225 mL ethanol (20% (v/v) ethanol). Remove 0.15 mL bathing solution, add 0.15 mL ethanol (30% (v/v) ethanol). Remove 0.3 mL bathing solution, add 0.3 mL ethanol (50% (v/v) ethanol). Remove 0.375 mL bathing solution, add 0.375 mL ethanol (75% (v/v) ethanol).). Remove 0.375 mL bathing solution, add 0.375 mL ethanol (100% ethanol).

Appendix 13

Heathers clearing fluid for chlorophyll removal.

	Parts by Weight g/100mL
85% (v/v) Lactic acid	2
Chloral hydrate	2
Phenol crystals	2
Clove oil	2
Xylene (xylol)	2

Note: Very toxic. Weigh out in a fume hood.

Appendix 14

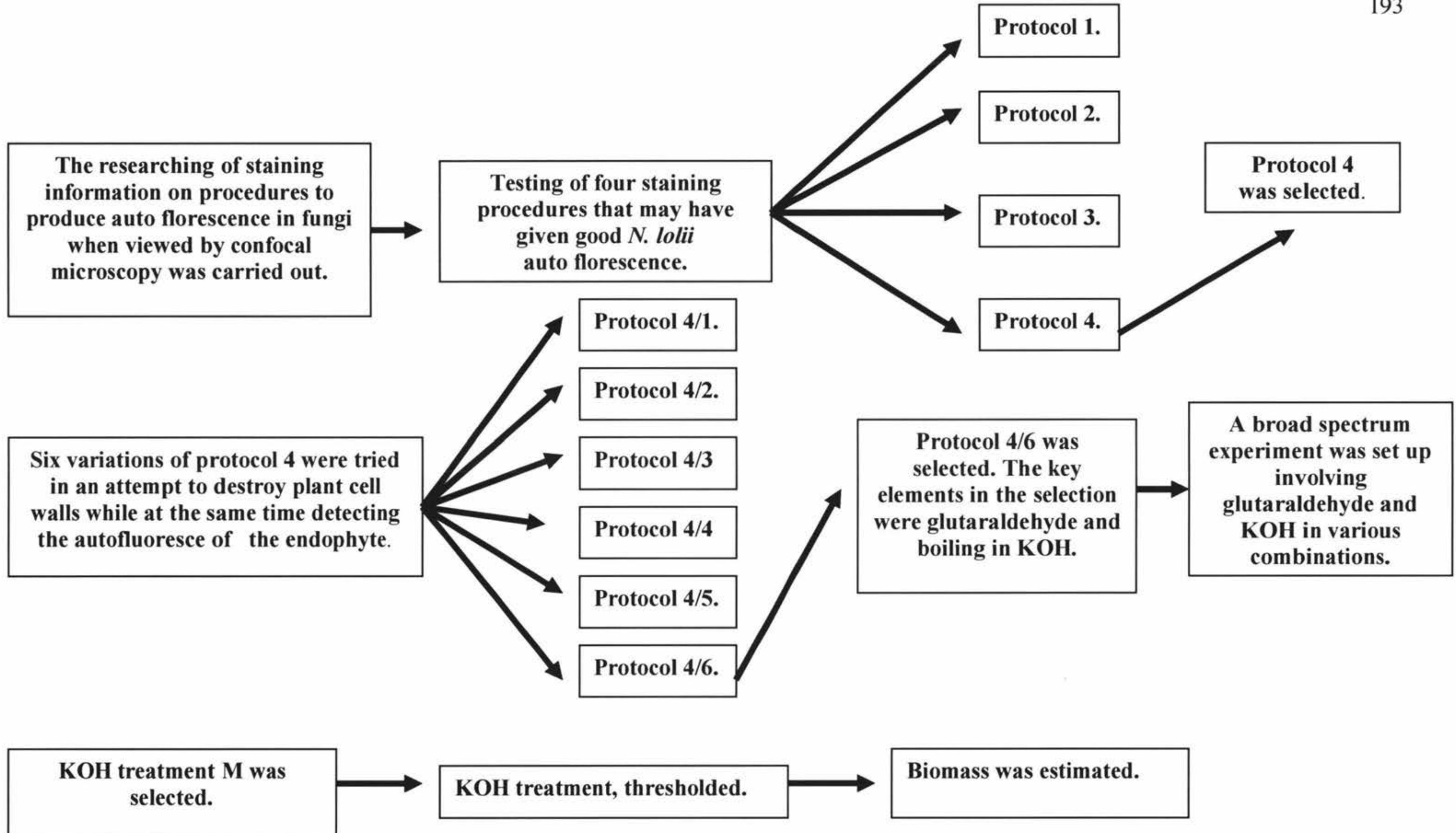
The threshold print out from the Image Space software from which the total volume of the endophyte *Neotyphodium lolii* occupies in the extra cellular tissue of *Lolium perenne* is calculated.

<table>
<tr><td colspan="7">ID-Object Count :</td></tr>
<tr><th>No</th><th>Id Tag</th><th>Centre</th><th>Volume (waxls)[a]</th><th>=|m| (μm^3)[b]</th><th>Radius</th><th>=|m (μm)[b]</th></tr>
<tr><td>7</td><td>84</td><td>65,217,18</td><td>896</td><td>308.18</td><td>6</td><td>4.15827</td></tr>
<tr><td colspan="7">ID-Object Count :</td></tr>
<tr><th>No</th><th>Id Tag</th><th>Centre</th><th>Volume (waxls)</th><th>=|m| (μm^3)</th><th>Radius</th><th>=|m (μm)</th></tr>
<tr><td>8</td><td>129</td><td>20,24,31</td><td>814</td><td>273.62</td><td>6</td><td>4.02734</td></tr>
<tr><td colspan="7">ID-Object Count :</td></tr>
<tr><th>No</th><th>Id Tag</th><th>Centre</th><th>Volume (waxls)</th><th>=|m| (μm^3)</th><th>Radius</th><th>=|m (μm)</th></tr>
<tr><td>9</td><td>70</td><td>208,226,19</td><td>724</td><td>243.37</td><td>6</td><td>3.87308</td></tr>
<tr><td colspan="7">ID-Object Count :</td></tr>
<tr><th>No</th><th>Id Tag</th><th>Centre</th><th>Volume (waxls)</th><th>=|m| (μm^3)</th><th>Radius</th><th>=|m (μm)</th></tr>
<tr><td>10</td><td>34</td><td>194,127,7</td><td>566</td><td>190.26</td><td>5</td><td>3.56793</td></tr>
<tr><td colspan="7">ID-Object Count :</td></tr>
<tr><th>No</th><th>Id Tag</th><th>Centre</th><th>Volume (waxls)</th><th>=|m| (μm^3)</th><th>Radius</th><th>=|m (μm)</th></tr>
<tr><td>11</td><td>166</td><td>27,57,35</td><td>537</td><td>180.51</td><td>5</td><td>3.50592</td></tr>
<tr><td colspan="7">ID-Object Count :</td></tr>
<tr><th>No</th><th>Id Tag</th><th>Centre</th><th>Volume (waxls)</th><th>=|m| (μm^3)</th><th>Radius</th><th>=|m (μm)</th></tr>
<tr><td>12</td><td>158</td><td>197,88,33</td><td>522</td><td>175.47</td><td>5</td><td>3.47297</td></tr>
</table>

[a] A computer based estimate of the volume of the endophyte in waxels.
[b] Volume of the endophyte image in μm^3 of each individual confocal 1.3µm section.
[c] The total length in µm of the fluorescence images that were thresholded in each 1.3µm section image.

Appendix 15

Confocal experimental work.



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