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

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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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acquisition of the internal HPLC standards and the production of scintillated ABA and CA, which were needed for the successful solid state extraction protocols. Thanks to Joe Hislop of the Fundamental Science Institute for providing the equipment and direction I needed in that regard.

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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.

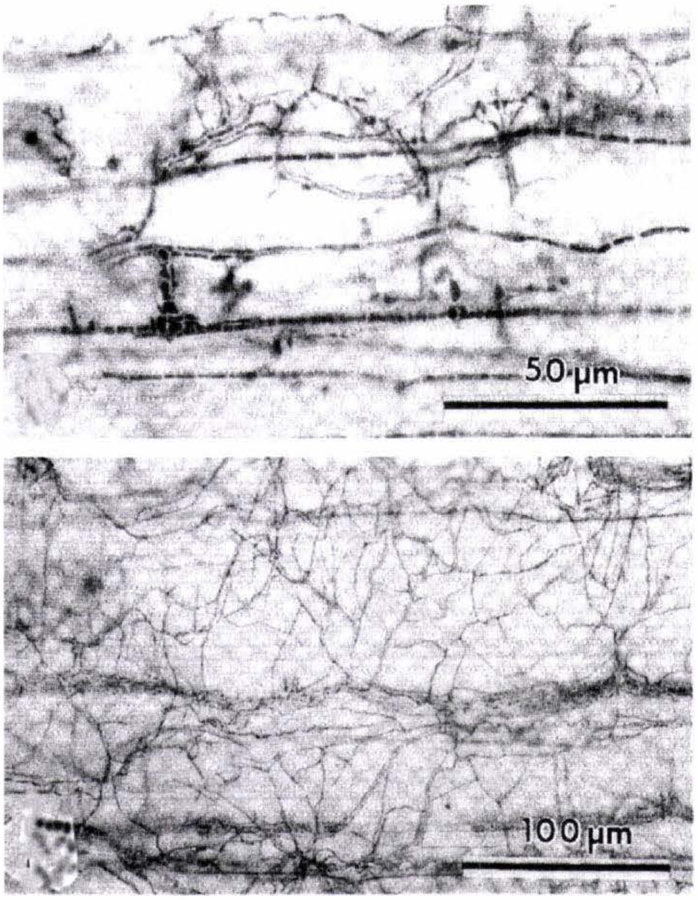

Fig 1.2 The hyphae of highly branched distorted *Neotyphodium lolii* in the outer sheath of *Lolium perenne* of an artificially established association (upper) and the hyphae of a p-endophyte (lower).

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.

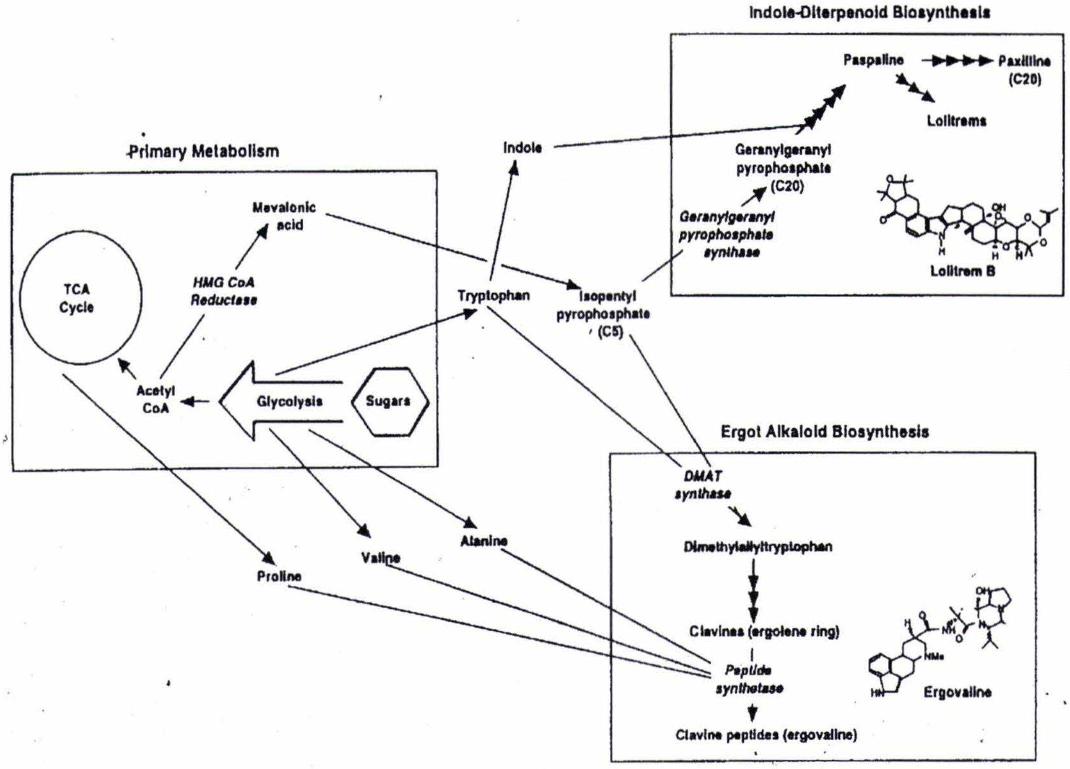

Fig 1.3 The proposed pathway for the biosynthesis of ergot alkaloids and indole-diterpenes in *Epichloë* endophytes.

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).

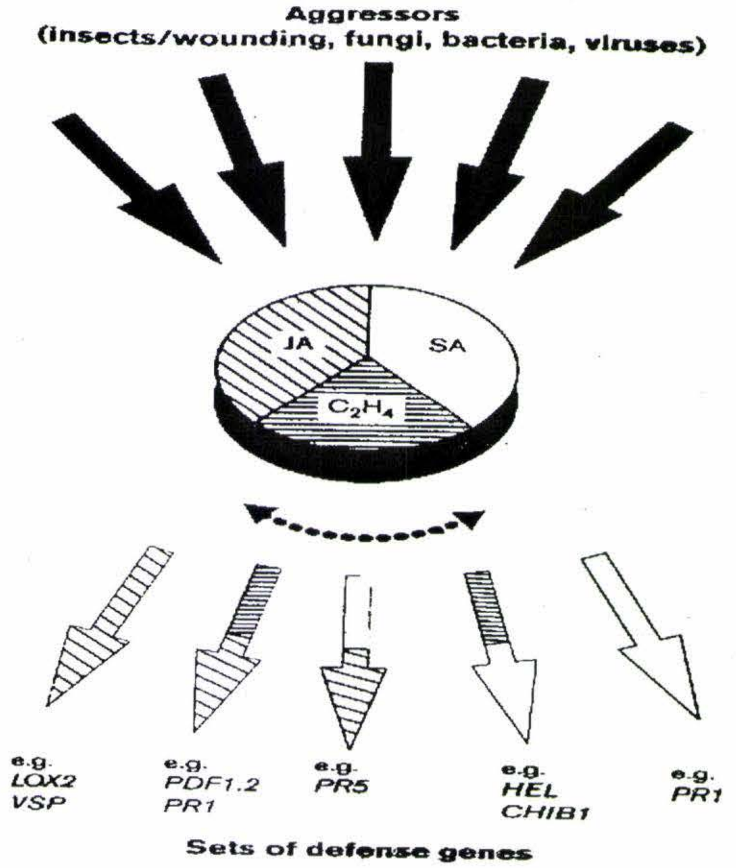

Fig 1.4 Tunable dial model for the regulation of defence gene expression by the three signals JA, ethylene and SA. Depending on the nature of the aggressor, the plant is able to fine-tune (dotted arrow) the induction of the defence genes either by employing a single signal molecule (single-patterned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

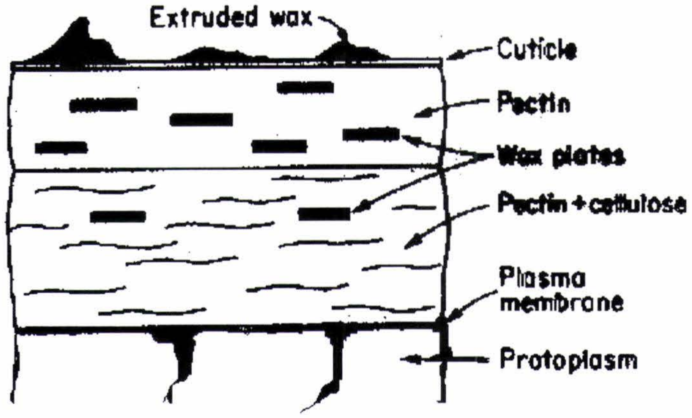

Fig 1.5 The components of the epidermal cell wall, showing extruded wax overlaying a cuticle, and then cell wall layers of pectin and pectin plus cellulose with some wax plates included. Inside the cell walls are tiny protoplasmic connections, called ectodesmata, that allow access into the protoplasm.

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.

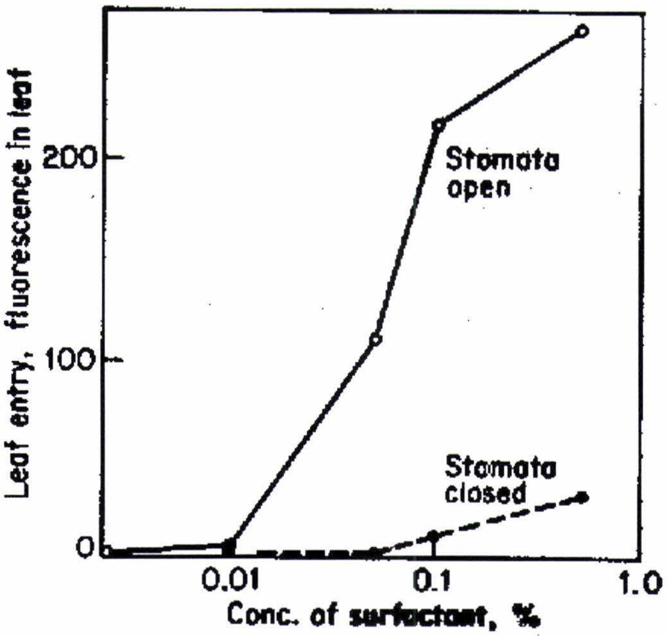

Fig 1.6 The entry of the dye fluorochrome into leaves of *Pyrus communis* as related to the concentration of surfactant (Vatsol OT) and the state of opening of the stomata. Entry of 0.17% before washing and measuring entry by fluorescence in ultraviolet light.

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.

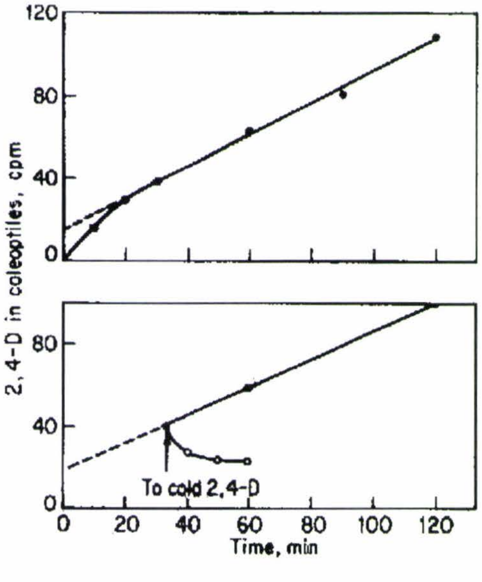

Fig 1.7 The uptake of 2,4-D by oat coleoptile sections, showing the initial rapid uptake phase followed by a steady slower phase (top). Extrapolation of the slower rate to the y ordinate permitted an estimate of the total amount taken up initially. When the sections were moved from the 0.5 mg/L labelled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