Thaxtomin A toxicity in plant cells
(studies associated with common scab disease of potato)

by

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Declaration

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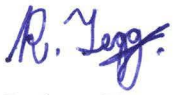

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Publications

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Abstract

Common scab, a bacterial disease of potato causes significant losses to the Australian potato industry through rejected seed and/or increased processing costs. Disease symptoms caused by plant pathogenic *Streptomyces* sp., are attributable to a key phytotoxin it produces, namely thaxtomin A. The development of resistance to this disease, through selection of somaclonal varieties tolerant to thaxtomin A is a current key industry supported program. However, mechanisms relating to resistance and patterns of toxicity produced by thaxtomin A are not well understood and represent the key objectives of this project.

The purpose of this study was to initially assess the impact of thaxtomin A on various plants and plant cell systems from an electrophysiological, morphological and pathological perspective. The effect of thaxtomin A in combination with various auxin sources and analogues was also examined. Further work aimed to quantify resistance to thaxtomin A within mutant strains of potato plants and calli, and whether any specific change to a known thaxtomin A susceptible gene may be responsible for altered levels of resistance to thaxtomin A.

Electrophysiological data obtained using ion-selective microelectrode ion flux estimation (the MIFE) technique showed that interaction between plant and toxin was characterised by a rapid and short-lived Ca^{2+} influx and activation of the plasma membrane proton pump. Thaxtomin A was more effective in young, physiologically active tissues (root elongation zone or pollen tube apex), suggesting higher density of thaxtomin A-binding sites in these regions. This provided the first evidence that thaxtomin A triggers an early signalling cascade, which may be crucial in plant-pathogen interactions.

Glasshouse trials showed that foliar application of sublethal concentrations of 2,4-D sprays on potato foliage reduced severity and occurrence of common scab. Lenticel numbers, lenticel external dimensions and periderm structure (key features critical to *S. scabiei* entry and penetration into a tuber) were generally not changed by 2,4-D sprays, suggesting no direct effect of 2,4-D on these morphological structures. In contrast, tubers harvested from 2,4-D treated plants had a decreased sensitivity to thaxtomin A compared with the controls, which may explain enhanced resistance.

This suggests an indirect effect of 2,4-D impacting on the toxin, thaxtomin A, rather than morphological changes to the developing tuber.

Further experimental evidence confirmed an interaction between thaxtomin A and auxin. Inhibition of tomato pollen tube growth by thaxtomin A was ameliorated by addition of NAA. Also, auxin/auxin transport inhibitor sensitive *'ucu2-2/gi2' A. thaliana* mutant showed significantly greater sensitivity to thaxtomin A, observed as root growth suppression, increased rates of necrosis (chlorosis), plant death, and more severely altered H^+ flux profiles (electrophysiological data) in the mutant compared to the wild-type. Moreover, inhibition root growth assays with the thaxtomin A-resistant *'txr1' A. thaliana* mutant showed a 3 fold increase in resistance to the polar auxin transport inhibitor, 1-NPA, suggesting an interaction between thaxtomin A and the auxin efflux carrier associated with the NPA binding protein. Cross-resistances to 1-NPA and isoxaben of *'txr1'* and the isoxaben resistant *'ixr1' A. thaliana* mutant suggests a similarity of function between isoxaben and thaxtomin A, and also *'txr1'* and *'ixr1'*.

The *'txr1'* gene homolog from potato has been successfully cloned and sequenced from a series of mutant potato lines, selected for resistance to thaxtomin A. Compared with the parent (control) there were no mutations within the *'txr1'* gene examined suggesting the resistance phenotype is due to some other genetic change.

These studies have contributed to a better understanding of mechanisms of toxicity of thaxtomin A in plant cells and advanced our knowledge of pathogen: host interactions within the common scab disease pathosystem.

Table of Contents

Chapter 1. Review of literature

1.1 Introduction

Potato (*Solanum tuberosum* L.) is an economically important vegetable crop grown throughout the world (Anon 2005a,b,c). It is a source of fresh food as well as a range of processed products with farmers relying on potatoes as an economically important and reliable component of crop rotation. Within Australia, potatoes are the most valuable vegetable crop produced comprising approximately 50% of total Australian vegetable production (Anon 1996). Potatoes are cropped in all six Australian states (Fig. 1.1) with Tasmania, Victoria and South Australia accounting for the majority of production (Anon 1996, 2000; Cirillo 2001, 2002). The latest annual production yield figure (2002/03 season) was approximately 1.3 million tonnes (P. Hardman, Simplot Australia, *pers. comm.*) and the farm-gate value of the national industry has oscillated around A$400-450 M in recent years. The processing industry (french fry and crisping) account for approximately 75% of the national potato industry.

Common scab, caused by pathogenic *Streptomyces* spp. is an economically important bacterial disease of potato found throughout the world (Labruyère 1971; Hooker 1981; Slabbert *et al.* 1994; Loria *et al.* 1997; Galal *et al.* 1999). Symptoms of the disease include superficial blemishes on tubers and roots that most usually reduce tuber quality rather than crop yield. In severe infections however crop yields may be reduced, and deep scabs increase wastage from double peeling (Agrios 1997; Wilson 2005). The typical symptoms of shallow, brown raised or sunken corky lesions conferred by the pathogen (Labruyère 1971) can be reproduced with the application of a phytotoxin, thaxtomin A, to the developing tuber (Lawrence *et al.* 1990). Thaxtomin A is a dipeptide phytotoxin produced by all plant pathogenic *Streptomyces* sp. responsible for common scab disease (King *et al.* 1989; Loria *et al.* 1997). Indeed, mutation within thaxtomin A biosynthesis genes eliminating thaxtomin A production by the pathogen *Streptomyces acidiscabies* rendered the strain non-pathogenic (Healy *et al.* 2000) indicating a central role of thaxtomin A in common scab pathogenesis.

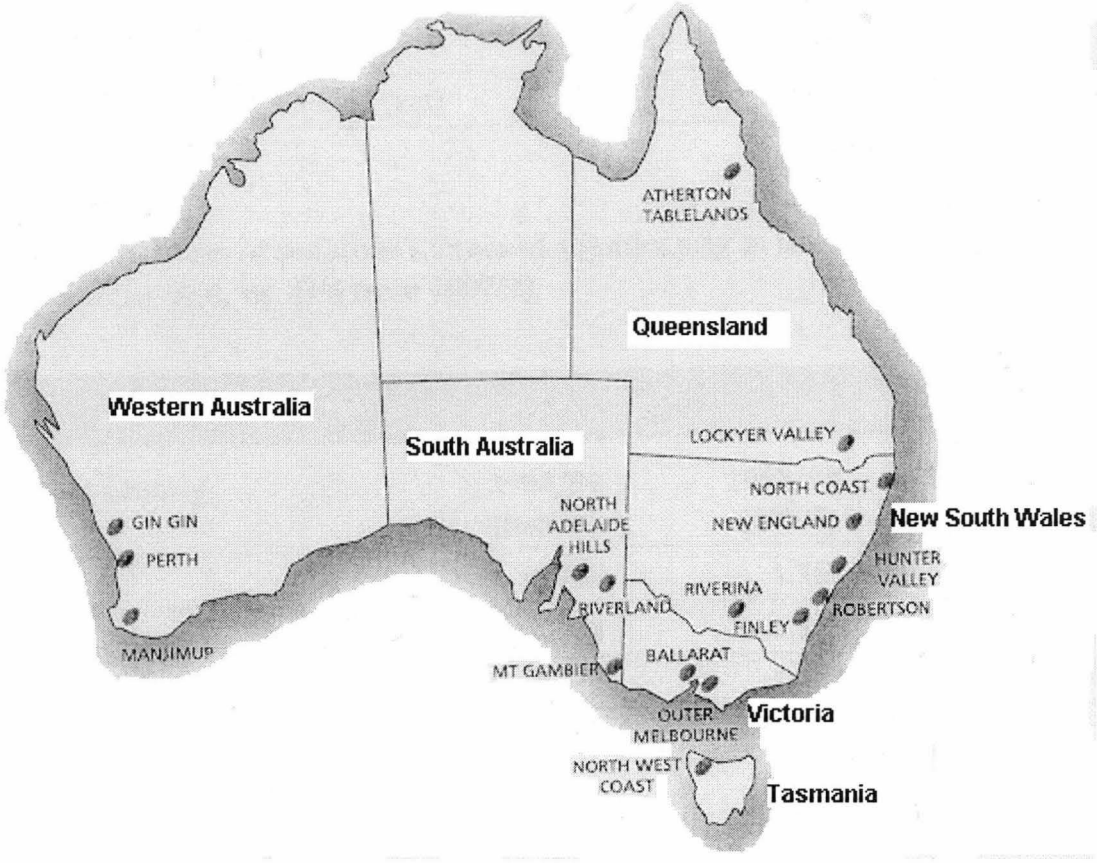


Fig. 1.1. Major potato production regions in Australia (Figure adapted from Cirillo 2001).

Thaxtomins are therefore crucial and unique toxins implicit in symptom expression. The importance of thaxtomin and its primary role in eliciting scab symptoms has seen researchers targeting thaxtomin as a possible control target in scab control (Acuna *et al.* 2000; Wilson *et al.* 2004; Wilson and Eyles 2004; A. Conner, *pers. comm.*). Developing resistance through cell selection technique directly to the toxin thaxtomin A represents a current and novel approach to combating this disease (Wilson *et al.* 2004; Wilson and Eyles 2004). Within this framework there exists a need to better define mechanisms of thaxtomin A toxicity and resistance. This will involve identifying critical responses to thaxtomin A.

1.2 Plant-pathogenic Streptomycetes

The Actinomycete family of bacteria, which represent a high proportion of the soil microbial biomass, produce a wide variety of phytotoxins, whether it be antibiotics or extracellular enzymes (Doumbou *et al.* 2002). Phytotoxins, such as the thaxtomins are secondary metabolic products, which play beneficial roles to the producing organisms in their various ecological niches, and contribute significantly to the pathogenic life-style of many bacteria (Mitchell 1991). Microbial secondary metabolites, are by definition, substances that are not needed for the growth or other essential processes in the cell and are produced by an organism undergoing morphological differentiation (Vining 1990). The streptomycetes are potent producers of secondary metabolites. Of the approximately 10 000 known antibiotics, 45-55 % are produced by streptomycetes (Demain 1999; Lazzarini *et al.* 2000).

Streptomycetes are aerobic, Gram-positive, filamentous prokaryotic bacteria, which are distinguished by the production of nonfragmenting substrate mycelium that colonize and penetrate organic matter in the soil; achieved through the production of extracellular hydrolytic enzymes (Loria *et al.* 1997; Loria *et al.* 2003). *Streptomyces* species have DNA with a high proportion of guanine and cytosine (GC-content is 69-78%) (Williams *et al.* 1989). They are immobile bacteria, and under environmental cues (Kutzner 1986; Williams *et al.* 1989) produce spores that aid in their colonization and dispersal (Loria *et al.* 1997). At the same time, the production of pigments, antibiotics and other secondary metabolites is initiated (Kutzner 1986; Williams *et al.* 1989; Loria *et al.* 1997; Demain 1999). The production of antibiotics combined with their differentiating life cycle improves the survival ability of the *Streptomyces* sp. under the fluctuating growth conditions that occur in a highly competitive soil environment (Kutzner 1986; Loria *et al.* 1997).

Within soil, streptomycetes are found associated with surfaces, such as plant residues or fungal hyphae (Mayfield *et al.* 1972) and the largest concentrations of streptomycetes can be found in the organic horizon (Hagedorn 1976). The streptomycetes are able to utilise a wide range of organic compounds as a carbon source, including complex biological materials, such as cellulose, lignocellulose, chitin and lignin (Loria *et al.* 2003), and can also utilise an inorganic nitrogen source (Kutzner 1986; Morosoli *et al.* 1997). Interestingly, only a very small proportion of

the described *Streptomyces* species are known to be plant or animal pathogens (Locci 1994; Loria *et al.* 1997).

Those Streptomycetes currently regarded as causing disease of roots and other underground plant structures (primarily common scab of potato) *include S. scabiei* (Lambert and Loria 1989a), *S. acidiscabies* (Lambert and Loria 1989b), *S. caviscabies* (Goyer *et al.* 1996), *S. turgidiscabies* (Miyajima et al. 1998), *S. europaeiscabiei* (Bouchek-Mechiche *et al.* 2000a), *S. stelliscabiei* (Bouchek-Mechiche *et al.* 2000a), *S. luridiscabiei* sp. nov., *S. puniciscabiei* sp. nov. and *S. niveiscabiei* sp. nov. (Park *et al.* 2003a). These species are important causal agents of common scab disease of potato and are further discussed in Section 1.3.3.

There are a variety of other diverse *Streptomyces sp.* (Doering-Saad *et al.* 1992) that may cause superficial lesions on potatoes although their importance as pathogens is somewhat questionable (Loria *et al.* 1997).

In addition to the aforementioned *Streptomyces* spp. known to promote common scab like symptoms other important pathogenic streptomycetes include *S. ipomoeae* (soil pox of sweet potato) (Person and Martin 1940) and *S. reticuliscabiei* (netted scab of potato) (Bouchek-Mechiche *et al.* 2000a). They both cause extensive fibrous root rot that decreases the yields of their hosts (Loria *et al.* 2003). One other little-known *Streptomyces* disease is root tumor of melon (Yoshida and Kobayashi 1991) caused by a new *Streptomyces* pathogen and characterised by root galls on the fibrous roots and the lower stems of cucurbits (Loria *et al.* 2003).

1.3 Potato Common Scab

1.3.1 The disease and infection process

Common scab disease, caused by pathogenic strains of *Streptomyces* spp., has a major impact on potato (*Solanum tuberosum* L.) production, throughout the world (Lambert and Loria 1989a; Wilson 2005). It is characterised by corky lesions on the tuber surface, which may be superficial, raised or sunken (Labruyère 1971; Goth *et al.* 1995; Loria *et al.* 1997) (Fig. 1.2). Symptoms first appear as necrosis around the infection site (Fellows 1926) and lesions subsequently develop a corky appearance due to suberisation of surrounding tuber tissue. Individual lesions are normally circular but when many are present they may coalesce to form irregular shaped scabbed areas that may completely cover infected tubers (Emilsson and Gustafsson 1953). Beside tuber symptoms, brown necrotic spots have been noted on roots and stolons of affected plants (Dutt 1979; Hooker 1981), but no disease symptoms are found on aerial parts of the plant (Smith 1968).

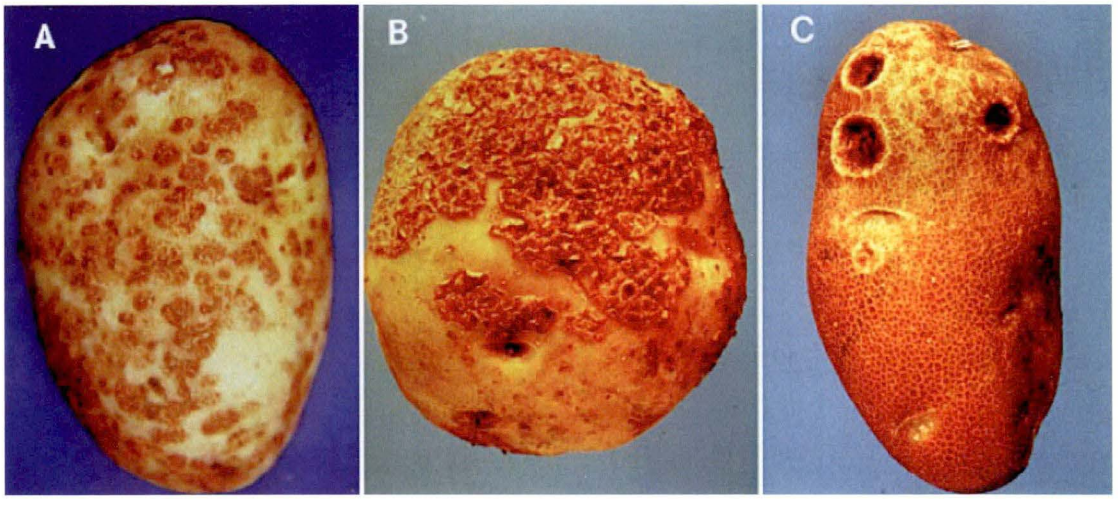


Fig. 1.2. Typical symptoms induced by the common scab pathogen *Streptomyces scabiei*, include A) superficial, B) raised, and C) deep-pitted lesions (from Loria *et al.* 1997).

Common scab is believed to be caused predominantly by infection of lenticels in young, rapidly expanding tubers (Fig. 1.3), by pathogenic strains of *Streptomyces* sp. (Adams & Lapwood 1978). It is suggested that internodes that form successively at the apex of a growing tuber, each pass through a period of susceptibility based on stomata-lenticel maturity (Lapwood and Adams 1973, 1975;

Adams 1975a). Usually the youngest one or two internodes at any time bear stomata, which are resistant to infection. These stomata then develop into young lenticels, with unsuberised filling cells, which appear to be susceptible. On older internodes, lenticels become suberised and resistant (Fellows 1926; Jones 1931; Lapwood and Adams 1973). Based on physiological development (Lapwood and Hering 1970), each internode should pass through its susceptible phase of about 10 days in duration from 1-2½ wk after formation (Adams and Lapwood 1978). Whilst immature lenticels appear to be the most likely entry or infection point (Adams and Lapwood 1978), some evidence suggests stomata may also be an infection site (Fellows 1926), while Loria *et al.* (2003) has also reported direct penetration by the pathogen through the periderm.

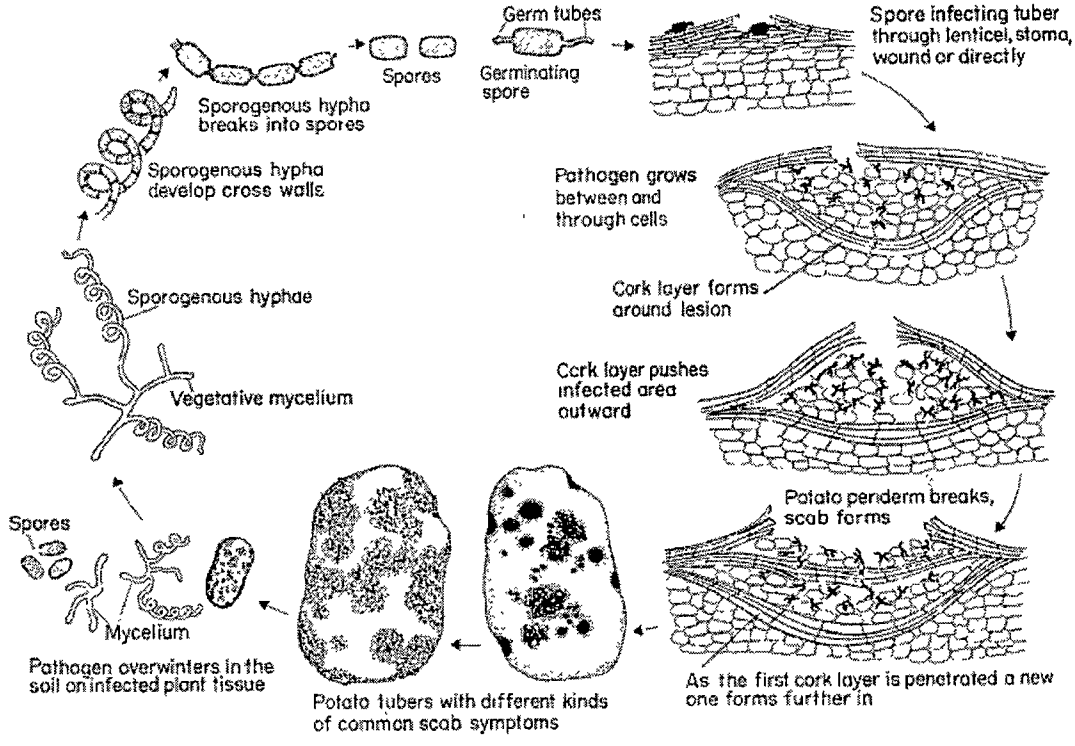


Fig. 1.3. Disease cycle of the common scab of potato caused by *Streptomyces scabiei* (from Agrios 1997).

After penetration the pathogen is believed to grow between or through a few layers of cells, the cells die, and the pathogen then derives food from them (Agrios 1997). In response to infection, meristematic tissues surrounding the lesion divide rapidly producing a cork wound tissue layer, which isolates the pathogen (Fig. 1.3). The pathogen may then penetrate this wound periderm which results in a second or

third wound periderm layer to be formed, allowing large scab lesions to develop (Lapwood 1973; Agrios 1997). Depth of the lesion is related to soil conditions and cultivar (Hooker 1981) and also the invasion of scab lesions by other organisms. Insect larval feeding can damage the wound periderm layers allowing the pathogen to penetrate to greater depths into the tuber (Afanasiev 1937; Agrios 1997).

The pathogen is both soil- and tuber-borne, although the relative contribution to final disease levels of either source is unclear (Goss 1937; Labruyere 1971; Lapwood 1972; Adams and Hide 1981; Singh *et al.* 1987; Wilson *et al.* 1999) and it is likely that the relative role of each varies with ecosystem. For example, Pavlista (1996) concluded that no significant contribution to the disease level within a crop comes from seed-borne inocula whilst Wilson *et al.* (1999) reported that under Tasmanian growing conditions planting of visibly infected seed led to a 10-fold increase in disease levels. Recent work reported by Wang and Lazarovits (2005) indicated a correlation between planting scabby mother tubers and an increased incidence of scab in progeny tubers, supporting the findings of Wilson *et al.* (1999).

The pathogen is a highly successful saprophyte (Loria *et al.* 2003) with a great ability to adapt to varying soil conditions and may survive on soil organic matter or alternative hosts in absence of potatoes for extended periods of time (Loria *et al.* 1997; Wang and Lazarovits 2004). There are reports that *S. scabiei* can survive in soil and (or) plant residues for over a decade (Kritzman and Grinstein 1991). The pathogen is disseminated on infected seed tubers and in infested soil transported by vehicles or stock, or carried by wind or water (Sharma and Sharma 1989; Agrios 1997).

1.3.2 Industry and economic importance

Common scab disease affects potato production throughout the world (Hooker 1981; Loria *et al.* 1997; Wilson 2005) and causes economic loss within fresh, processing and seed markets. This may include the down grading of seed stocks to ware quality, consumer resistance to blemished tubers reducing retail sales, and restrictions to export markets for seed. With deep-pitted scab, there are losses to potato processors through the necessity for double peeling or tuber rejection (Agrios 1997; Wilson 2005).

A 1991 survey of North American growers ranked common scab as the fourth most important disease of potato (Slack 1992). The disease is one of the three most significant in Australia (Wilson *et al.* 1999) and within Tasmania common scab has been widely regarded as the most economically important disease of potatoes in recent years (P. Hardman, Simplot Australia, *pers. comm.*; Pung 1997; Wilson 2005).

Industry figures for Tasmania in 2003 estimate that common scab costs the Tasmanian french fry processing industry A$3.66 M p.a. or approximately 4% of the annual industry value (P. Hardman, Simplot Australia, *pers. comm.*). Seed growers face the largest losses (A$2.31 M p.a. or 31% of this sectors value) attributed to seed not meeting certification standards and the need to contract ~30% more seed than required to cope with anticipated loss of certified seed. Processing losses (A$1.35 M p.a. or 1-2% of this sectors value) are attributed to lower yields from severely affected crops harvested early or rejection of whole or part crops at the factory (P. Hardman, Simplot Australia, *pers. comm.*).

1.3.3 Common scab causing organisms

Thaxter first described the casual organism of potato scab as *Oospora scabies,* isolated from Connecticut in 1890 (Thaxter 1891; 1892). It is now designated as *Streptomyces scabiei* (Thaxt.) Lambert and Loria, after being renamed and redescribed throughout the 1900's before being accepted and included on the list of approved bacterial names in 1989 (Lambert and Loria 1989a). A strain offered by Lambert and Loria (ATTC 49173) is now accepted as the neotype. It is characterised by its production of melanin and of smooth grey spores borne in spiral chains (Lambert and Loria 1989a). *Streptomyces scabiei* is the most widespread of the scab producing streptomycete pathogens, being found wherever potatoes are grown worldwide.

Whilst *S. scabiei* is primarily associated with common scab of potato tubers, it does impact on other crops (Leiner *et al.* 1996; Goyer and Beaulieu 1997). Infection has been noted on roots of beet, *Beta vulgaris* L. (Hoffmann 1991), carrot, *Daucus carota* L. (Janse 1988; Hanson and Lacy 1990), radish, *Raphanus sativus* L. (Levick *et al.* 1985; Tashiro *et al.* 1990) and turnip, *Brassica rapa* L. (Jones 1953; Embleton *et al.* 2004). Pathogenic *S. scabiei* also infect fibrous roots of seedlings

including soybean (*Glycine max* Merr.), pea (*Pisum sativum* L.), wheat (*Triticum vulgare* Vill.), radish and beet (Hooker 1949). Shoot growth of seedlings including radish, alfafa (*Medicago sativa* L.), cauliflower (*Brassica oleracea* L.), colza (*Brassica napus* L.) and turnip are also inhibited by *S. scabiei* (Leiner *et al.* 1996).

Whilst *S. scabiei* is the species historically associated with common scab disease of potato (Lambert and Loria 1989a), there are a number of other distinct species that have been reported to cause common scab or other scab-like diseases of potato. These to date include *S. acidiscabies* (Lambert and Loria 1989b), *S. caviscabies* (Goyer *et al.* 1996), *S. turgidiscabies* (Miyajima *et al.* 1998), *S. europaeiscabiei* (Bouchek-Mechiche *et al.* 2000a), *S. stelliscabiei* (Bouchek-Mechiche *et al.* 2000a), *S. luridiscabiei* sp. nov., *S. puniciscabiei* sp. nov. and *S. niveiscabiei* sp. nov. (Park *et al.* 2003a).

Streptomyces acidiscabies Lambert and Loria causes common scab in soils with pH values below 5.2 (Lambert and Loria 1989b). Whilst the bacterium *S. acidiscabies* is physiologically and morphologically distinct from *S. scabiei*, the symptoms produced are indistinguishable from common scab. *Streptomyces acidiscabies* was first reported in Maine, USA, in 1953, and was initially restricted to northeastern parts of the United States and Canada (Lambert and Loria 1989b). There are reports of it now occurring in northern Asia (Park *et al.* 2003b). It does not appear to survive very well in soil and is spread by infected seed (Manzer *et al.* 1977). It can also produce scab symptoms on other root crops (Lambert 1991; Loria *et al.* 1997).

Streptomyces caviscabies was reported as a recently described species associated with deep pitted scab lesions of potatoes grown on irrigated soil in Québec, Canada (Faucher *et al.* 1992; Faucher *et al.* 1995; Goyer *et al.* 1996). *Streptomyces turgidiscabies* has been listed as causing potato scab in the Hokkaido region of Japan (Miyajima *et al.* 1998), although strains have also been recently reported in Finland (Kreuze *et al.* 1999), Korea (Park *et al.* 2003b) and Sweden (Lehtonen *et al.* 2004), with strains in Australia, requiring further classification, likely to be included in this species (C. Wilson, *pers. comm.*). *Streptomyces europaeiscabiei* and *S. stelliscabiei* are reported to have been isolated from common

scab lesions on potato tubers in France (Bouchek-Mechiche *et al.* 2000a). Some isolates of *S. europaeiscabiei* can produce netted scab (Bouchek-Mechiche *et al.* 2000a,b), which is distinct from common scab and characterised by superficial, brown lesions on the tuber skin and roots, resulting in both skin alteration and yield reductions (Bang 1979, 1995; Scholte and Labruyère 1985; Scholte 1989). *Streptomyces luridiscabiei* sp. nov., *S. puniciscabiei* sp. nov. and *S. niveiscabiei* sp. nov. represent three new distinct species from Jeju Island, Korea that have been reported to cause raised corky potato common scab lesions (Park *et al.* 2003a).

1.3.4 Detection and Quantification

A reliable and rapid method for determining the presence of pathogenic common scab forming *Streptomyces* in tuber and soil is highly desirable. A number of techniques have been developed with varying rates of success (Wilson 1995; Conn *et al.* 1998; Lazarovits *et al.* 2001; Bukhalid *et al.* 2002; Lehtonen *et al.* 2004; Wang and Lazarovits 2004). Conn *et al.* (1998) developed a semi-selective culture (STR) medium specific for isolating *Streptomyces* populations. Whilst it was useful for reducing the growth of unwanted bacteria and fungi, it did not differentiate saprophytic (non-pathogenic) from pathogenic strains (Conn *et al.* 1998). Therefore further tests were required to determine pathogenicity (Wang and Lazarovits 2004). This is most readily done by assessing thaxtomin A production (King *et al.* 1991) which is both time consuming and expensive. Recently, polymerase chain reaction (PCR) assays have been developed (Lazarovits *et al.* 2001; Bukhalid *et al.* 2002; Wang and Lazarovits 2004) and they have found to be very effective. The first assay developed targeted the detection of the *nec1* gene that confers a mild necrogenic phenotype (Lazarovits *et al.* 2001; Bukhalid *et al.* 2002; also see Section 1.4.7). Whilst the presence of *nec1* was assumed to correlate with pathogenicity some studies have identified the occassional strain where this criteria is not met (Lazarovits *et al.* 2001; Bukhalid *et al.* 2002; Park *et al.* 2003b; Wang and Lazarovits 2004; Wilson 2005). However, the number of such isolates appears to be small, and any errors, using the *nec1* gene for quantification of the pathogens, would not make a significant difference in an ecological study, given the very high populations of pathogens present in the lesion tissues (Wang and Lazarovits 2004). Further PCR assays targeting the *txtA* gene, one of several essential to thaxtomin biosynthesis

(Healy *et al.* 2000; also see Section 1.4.7) may provide a tighter correlation with pathogenicity (Wang and Lazarovits 2004).

1.3.5 Management

Irrigation, chemical usage, low soil pH (acid-producing fertilisers or sulfur applications), green manures, organic amendments, biological control, use of resistant potato varieties and altered cultural practices (Hooker 1981; Loria *et al.* 1997; Waterer 2002) have been used to manage common scab disease. Some methods are quite costly and impractical and the degree of success can quite often be site specific. There is no universally acceptable solution for controlling common scab.

Levels of common scab disease control required depend on which markets (seed or processing) the tubers are destined for and also what regions of the world they are grown in, imported to or exported to. Within Australia, seed growers face some of the tightest certification levels for common scab disease infection. Seed crops fail certification if infection levels exceed the national guidelines of ≤4% tubers possessing lesions (Pitt 1998; I. Kirkwood, Primary Industries Tasmania, *pers. comm.*). Certification tolerance levels vary throughout the world. As an example, for certified seed production in France and Canada, tuber crops cannot have ≥ 5% infection with common scab (Anon 2002; Barkley 2005).

1.3.5.1 Irrigation

Increasing irrigation or soil moisture levels over the 6 week period of tuber susceptibility to pathogen attack (see 1.3.1) has aided in disease control of scab in susceptible varieties (Lewis 1970; Lapwood *et al.* 1970, 1973; Lapwood and Adams 1973, 1975; Adams and Lapwood 1978; Wilson *et al.* 2001).

The mechanism of scab control was thought to be through some form of microbial antagonism, where the wet conditions favour the build up of microbial populations antagonistic to the pathogen (Lewis 1970; Adams and Lapwood 1978). Evidence for this was provided by the proportions of different microorganisms isolated from lenticels under varying soil moisture conditions. Frequencies of the

pathogenic actinomycetes were much higher in dry than wet soils, with other bacteria replacing the pathogen as soil water potentials increased, indicating an antagonistic inter-relationship between the pathogenic actinomycetes and other bacteria (Lewis 1970; Adams and Lapwood 1978). Adams (1975a) also describes how lenticel morphology changes markedly in wet soils, with a proliferation of filler cells, which may influence pathogen entry. Other mechanisms are also believed to play a part in the alleviation of scab symptoms under irrigation (R. Loria, *pers. comm.*).

The fact that water is a limited resource has resulted in the use of irrigation for scab control, in certain parts of the world, but not in others. Hence in the United Kingdom irrigation is a popular control strategy whilst in North America it is regarded as impractical and uneconomical (Loria *et al.* 1997). Also, where irrigation or unseasonal rainfall occurs over the tuber initiation period, and effectively controls common scab, tubers become more susceptible to another scab disease. Powdery scab, caused by *Spongospora subterranea* (Wallr.) Lagerheim, is favoured by wet, moist conditions early in the growing season (Adams *et al.* 1987; de Boer *et al.* 2005; Thomson and Waterer 2005; van de Graaf *et al.* 2005).

1.3.5.2 Chemical means of controlling common scab

Seed treatment

Seed treatment aims to prevent disease introduction into fields and/or provide some suppression of disease if the field is already infected. Wilson *et al.* (1999) when testing a range of seed treatments concluded that mancozeb and pentachloronitrobenzene (PCNB), although effective, represented potential health hazards. They identified a few alternative (safer) potential fungicides which all gave good to very good disease control when used as seed spray or dip on diseased seed (Wilson *et al.* 1999). A range of other seed chemical treatments continue to be used worldwide (Davis and Callihan 1971; Hooker 1981; Singh *et al.* 1987; Mishra *et al.* 1991; De and Sengupta 1993), however they are generally not used in Australia, where safer alternatives are being sought (Wilson *et al.* 1999).

Soil treatment

Control of this disease has been achieved by soil treatment with fungicides including PCNB (Potter *et al.* 1958; Wilson *et al.* 1999) and regulating pH of the soil with acid producing fertilizers or sulfur applications (Doyle and MacLean 1960; Soltani *et al.* 2002; Mizuno *et al.* 2003; Sturz *et al.* 2004; Pavlista 2005). Concerns over potential human health hazards of PCNB and related substances has seen these treatments either banned or severely restricted (McIntosh 1973, 1976; Locci 1994; Wilson *et al.* 1999), with alternatives needed to be sought. As was the case for seed treatments there are still a number of chemicals in use worldwide for soil treatment (Davis and Callihan 1971; Hooker 1981; Singh *et al.* 1987; Mishra *et al.* 1991; De and Sengupta 1993) that are no longer utilised in Australia (Wilson *et al.* 1999). The long-term practice of altering soil pH is also not environmentally sustainable (Liu *et al.* 1995). Wilson *et al.* (1999) concluded that further work is required to fully investigate the benefits of a soil treatment perhaps in combination with a seed treatment for common scab disease control under Australian conditions.

Foliar treatment

Foliar sprays have previously been tested as alternatives to direct soil applications, with varying rates of success (McIntosh 1979; McIntosh and Bateman 1979; McIntosh and Burrell 1980; McIntosh *et al.* 1981, 1982, 1988; Tuomola *et al.* 1996). Whilst not currently commercially used their modes of action in controlling common scab are quiet novel and worthy of discussion, some of the more interesting and successful ones are described.

Daminozide and ethionine were two compounds found to decrease the incidence of common scab (McIntosh 1979; McIntosh and Bateman 1979; McIntosh and Burrell 1980). However, as their effectiveness is well below that conferred by quintozene soil treatment these chemicals do not give commercially acceptable control. In spite of this, their varying mechanisms of decreasing common scab are interesting: Ethionine appears to be active against the causal organism *S. scabiei*, and decreases scab by its toxicity to *S. scabiei* (McIntosh and Burrell 1980). Daminozide is however only weakly toxic to *S. scabiei*; its reduction in scab is thought to be related to physiological changes in the plant, reducing the occurrence of scab

symptoms (McIntosh and Bateman 1979), rather than the prevention of primary infection as observed with ethionine usage.

The alteration of the physiological state of the plant and subsequent protection from common scab symptoms as observed with daminozide application, has led to the identification of other compounds that control scab, with similar modes of action. McIntosh *et al.* (1981, 1982) found the phenoxyacetic acids, particularly 3,5-dichlorophenoxyacetic acid (3,5-D) had an outstanding effect against scab, reducing it by about 90%. However, the herbicidal and auxinic effects of 3,5-D which result in decreased yields and increased proportions of deformed tubers, preclude the practical use of these types of chemicals.

McIntosh *et al.* (1988) was able to identify compounds from the substituted benzoic and picolinic acid groups (mainly 2,5-disubstituted acids), that had disease suppressive action with no negative effects on yield or shape of tubers. These compounds were not as effective as 3,5-D, but they showed promise in giving satisfactory control of soil-borne diseases without damaging the plants or decreasing yields. Although none of these foliar sprays are used for commercial production, their modes of action provide a useful insight into this disease pathosystem (see Chapter 3).

1.3.5.3 Biological control

Biological control is a strategy aimed at reducing or suppressing pathogenic, scab forming streptomycete strains. This may be through the use of naturally suppressive soils or the inoculation of nonpathogenic suppressive *Streptomyces* strains into disease-conducive soil (Liu 1992; Liu *et al.* 1995; Bowers *et al.* 1996) or onto seed potato pieces prior to planting (Kloepper and Schroth 1981; Wilson 2005). Suppressive nonpathogenic streptomycete strains are sourced from around the lenticels of potatoes grown in a naturally occurring disease suppressive soil (Lorang *et al.* 1989; Liu 1992). These suppressive soils have been shown to develop over long periods of sustained potato monoculture. Suppressive strains have been isolated from potato nurseries or fields after 13 (Liu *et al.* 1995) and 23 years of monoculture (Lorang *et al.* 1989).

Suppression of disease is thought to occur mainly through the production of antibiotics by the suppressive strain, which has been shown to inhibit pathogenic *S. scabiei in vitro* (Liu 1992; Lorang *et al.* 1995). This ability has been utilised in a biofertilizer product containing *S. albidoflavus* strain CH-33 that produces an antibiotic lethal to *S. scabiei.* (Hayashida *et al.* 1989). The physical competitive ability and colonization characteristics of the suppressive strain may also play a part in successful biological control (Ryan and Kinkel 1997). One possible constraint to biological control is the development of antibiotic resistant mutants in the pathogenic strain, which arise spontaneously at a relatively high frequency. Fortunately, they are generally less pathogenic than the parent strain they evolved from (Neeno-Eckwall and Schottel 1999). Other suppressive bio-control agents, effective against pathogenic *Streptomyces* sp. are being sourced and include bacteria from *Bacillus* sp. (Han *et al.* 2005).

Whilst the production of antibiotics by the suppressive strain which inhibits S. scabiei is a key cause of disease suppression (Liu 1992; Lorang *et al.* 1995) in some cases part of this suppression effect may be linked to the ability of the biocontrol agent to transform, detoxify or utilise the toxin, thaxtomin (Dombou *et al.* 1988; King *et al.* 2000; Lazarovits *et al.* 2004).

A study by Dombou *et al.* (1998) demonstrated that thaxtomin A-utilizing bacteria, isolated from potato tubers, have protected growing plants against common scab. *Ralstonia pickettii*, and two non-pathogenic *Streptomyces* sp. strains were the bacteria that utilised or reduced thaxtomin A concentrations in artificial media cultures. More recently King *et al.* (2000) was able to demonstrate the *in vitro* biotransformation of thaxtomin A and B into much less phytotoxic glucosidic compounds. This was accomplished using cultures of *Bacillus mycoides*, which were initially identified as contaminants in cultures, but were later identified as the organism transforming the thaxtomins into the less toxic, more polar glucosides (King *et al.* 2000). Similarly Lazarovits *et al.* (2004) were able to identify a fungal isolate *Aspergillus niger* that could biotransform thaxtomin A. Under optimized *in vitro* conditions the fungus converted thaxtomin A into two major and five minor metabolites. The major metabolites proved to be much less phytotoxic than thaxtomin A when assayed on aseptically produced mini-tubers. Although these

studies did not prove a direct linkage between thaxtomin A utilisation and protection against common scab, the evidence of these trial supports the theory that phytotoxin utilisation or detoxification is a mode of action of some biocontrol agents (Dombou *et al.* 1988; King *et al.* 2000; Lazarovits *et al.* 2004).

Biological control is a unique challenge because the suppressive strain needs to effectively colonize the soil prior to the existence of the tubers to prevent infection by the pathogen (Ryan and Kinkel 1997), as tubers are most susceptible to scab disease 2-5 weeks after tuber initiation (Davis *et al.* 1974). A better knowledge of the relative roles of antibiotic production and rhizosphere colonization in success of the suppressive agent will help in selecting more efficient strains (Liu *et al.* 1995; Ryan and Kinkel 1997). Ideally, a final biological control product will be composed of several complementary suppressive strains to reduce the chances of the development of resistance in the pathogen (Neeno-Eckwall and Schottel 1999), and to enhance the range of environmental conditions in which biocontrol is successful (Liu *et al.* 1995). Biological control of potato common scab is likely to be difficult to achieve (Ryan *et al.* 2004), but in combination with other well managed control methods, may aid in the further improvement of disease control.

1.3.5.4 Organic Amendments

With environmental concerns over excess inorganic fertiliser use and the restriction of harsh fumigants such as methyl bromide for disease control, interest in organic amendments is re-emerging in their usage of controlling soil-borne pathogens, such as *S. scabiei*, and in their role as a fertilizer source (Lazarovits 2001). This is partly due to the fact that the role these organic amendments play in disease control can now be better predicted and utilised as their modes of action are now being elucidated (Lazarovits *et al.* 2000; Lazarovits 2001). Organic amendments containing high levels of nitrogen (chicken manure, soymeal, meat and bone meal), have been shown to significantly reduce populations of a wide spectrum of soil-borne plant pathogens, including *S. scabiei* (Conn and Lazarovits 1999; Lazarovits *et al.* 1999). Pathogen control was shown to arise from the ammonia and/or nitrous acid generated from the biological degradation of these high-nitrogen amendments (Lazarovits *et al.* 2000; Lazarovits 2001). The rich nutrient source also favours the multiplication of rapidly growing saprophytic organisms, at the expense

of the pathogens, which are quickly displaced (Lazarovits 2001). Increases in overall microorganism population numbers are between 100 to 1000-fold (Conn and Lazarovits 1999; Lazarovits *et al.* 1999). The usage of these high-nitrogen organic amendments which selectively enrich beneficial microorganisms serve as an alternative to inundative bio-control (Lazarovits 2001).

1.3.5.5 Planting and harvest dates

Over several recent seasons in commercial potato seed crops within Tasmania there has been a consistent outcome that planting later in the season (late December instead of early November) has led to an approximate 10-fold reduction in seed rejection rates attributable to common scab disease (Table 1.1) (M. Heap, Simplot Australia, *pers. comm.*). Whilst the mechanism is unknown a similar study in the USA demonstrated that tubers harvested early, reducing the time the crop was in the ground, reduced excessive grade-out due to common scab (Waterer 2002). Manipulating planting and harvest dates may therefore aid in management of common scab disease.

Table 1.1. Effect of planting date on rejection rate of seed potato crops due to common scab infection in Tasmania (2002/03 season) (M. Heap, Simplot Australia, *pers. comm.*)

Planting Date	Area planted (ha)	Rejected crops (ha)	Loss (%)
4th Nov	1850	1250	67
18th Nov	4725	2950	62
25th Nov	3440	1120	32
9th Dec	4480	490	11
23rd Dec	2175	150	7

1.3.5.6 Resistance to common scab

Host resistance is generally the most effective tool in management of common scab disease as potato varieties possess varying degrees of resistance to the disease (McKee 1958; Anon 1990; Goth *et al.* 1995). No commercial varieties currently have complete resistance to the disease, however there is one report from

Russia detailing a resistant, immune clone, however it has poor agronomic performance (Yakauleva and Gancharova 1990). The building of one effective single wound barrier localising infection is a key physical attribute of common scab resistant varieties, susceptible varieties require several layers of wound periderm to form resuling in greater disfigurement of tubers (Mishra and Srivastava 1991). Other physical attributes, such as lenticel density and size and periderm thickness and ultrastructure, which are important determinants of susceptibility to other potato diseases such as *E. carotovora subsp. carotovora* (Zhang *et al.* 1991) are not well understood (Adams 1975a) in context with the common scab pathogen. Further study is required to better understand key physical attributes that are critical to common scab susceptibility and therefore important selection criteria within breeding programs (Loria *et al.* 1997).

Direct resistance to the toxin, thaxtomin A, has been reported to correlate with resistance to common scab disease (Delserone *et al.* 1991; Acuna *et al.* 1998), providing another useful attribute for selection within breeding programmes (Acuna *et al.* 2000). The ability to detoxify thaxtomin A has been shown to be related to scab resistance and susceptibility in potato grown *in vitro* (Acuna *et al.* 2001). Specifically, the ability to transform or glucosylize thaxtomin A into a glucose conjugate, thaxtomin *A*-β-di-*O*-glucoside (TAG), was correlated with increased scab resistance as TAG is 6 times less toxic than thaxtomin A. The relative activity of the enzyme that catalyses this detoxification, glucosyl transferase, in a scab-resistant cultivar such as 'Nooksack' has been shown to be twice that of a susceptible cultivar selection of 'Ranger' (Acuna *et al.* 2001). The increased presence of this enzyme and the observed greater quantities of detoxified thaxtomin (TAG) in scab-resistant cultivars shows a linkage in the ability to glucosylize thaxtomin A and improved scab resistance (Acuna *et al.* 2001). King *et al.* (2000) suggested that these processes resulting in the production of less toxic derivatives of thaxtomin A, might explain cultivar susceptibilities to thaxtomin A and also provide a venue for enhancement of resistance to the scab phytotoxins. They also stated that no direct evidence for the presence of thaxtomin A glucosides in either field or greenhouse scab-infected potatoes had yet been demonstrated (King *et al.* 2000, 2003).

Within the Australian french fry processing industry cultivar choice is largely determined by processing qualities rather than disease resistance, this has led to the dominant usage of 'Russet Burbank' which possesses excellent processing qualities and moderate resistance to common scab (Anon 1990; Goth *et al.* 1995). However, within Tasmania the disease has steadily increased since the late 1980s, suggesting local factors (Wilson *et al.* 1999) and the possibility that novel pathogenic *Streptomyces* spp. (Lacey 2000) are both increasing disease incidence. Clonal variability to common scab disease has been demonstrated within Russet Burbank and these clones provide some selection choices for increased resistance (Wilson 2001). Other approaches, including genetic engineering, although trialed (Wilson and Conner 1995; Wilson *et al.* 1996) require further work and there are questions regarding the market acceptability of the use of this technology. Nevertheless, in recent times a thaxtomin resistant *A. thaliana* mutant has been selected from an EMS mutagenised population (Scheible *et al.* 2003). A corresponding TXR1 homolog has been silenced within potato providing a genetically modified plant with potential disease resistance (A. Conner, *pers. comm.*).

As there are no currently available commercial genotypes that exhibit complete resistance to common scab, breeding programs are still endevouring to attain such aims. Within the Australian potato industry one key breeding program using a novel approach has successfully developed common scab resistant potato plants using cell selection techniques utilising thaxtomin A (Wilson *et al.* 2004; Wilson and Eyles 2004). As the resistance targets the toxin that is central to disease induction, both extreme and durable resistance is likely, which makes this technique a sustainable commercially acceptable disease management option (Wilson *et al.* 2004).

1.4 Thaxtomin

1.4.1 Toxin Discovery

Since the 1920s researchers have studied the host-parasite interaction during development of common scab disease of potatoes. Fellows (1926) was the first to note darkening of the tuber cell walls in advance of colonization by the pathogen, concluding that this symptom was a response by the tuber to the action of a toxin or enzyme produced by the scab organism. Jones (1931) provided evidence that the pathogen was localized to the periphery of developing lesions, which indirectly supported Fellows (1926) conclusions.

Direct evidence to implicate toxin action first came from Shoemaker (1952) who induced scab lesions by transferring small agar blocks taken from the proximity of nonsporulating colonies of *S. scabiei* to the surface of tubers maintained under sterile conditions. Subsequent scab lesion development provided evidence for the induction of common scab by "a diffusible metabolic substance" originating in the pathogen. However the conclusion was questioned when it was revealed that contaminants had grown from the 'sterile' tubers (Lawrence *et al.* 1990). Sakai *et al.* (1984) noted the production of extracellular substances produced by potato scab forming *Streptomyces* spp. These substances were found to inhibit the root growth of rice seedlings suggesting pathogenicity may be conferred by toxin production (Sakai *et al.* 1984).

However, the first definitive evidence including isolation and identification of a new group of phytotoxins associated with plant pathogenic streptomycetes, the thaxtomins (King *et al.* 1989), occurred in 1989. Thaxtomins were shown to be capable of inducing complete common scab disease symptoms when applied to developing tubers in the absence of the pathogen itself (Lawrence *et al.* 1990). These key findings have provided a key insight into the nature of the pathogenesis reaction and have allowed scientists to target and focus on the toxins role in disease development.

1.4.2 Types of thaxtomin

Thaxtomins are modified dipeptide molecules which may arise biosynthetically from tryptophan and phenylalanine (King 1997) by as yet uncharacterized pathways. Essential to the phytotoxic activity is the presence of the 4-nitroindol-3-yl and phenylalanine groups linked in an L, L configured cyclodipeptide (King *et al.* 1992). Production of organic compounds containing nitro (NO_2) groups is relatively unusual in nature (Loria *et al.* 1997), adding interest to the study of these bioactive compounds.

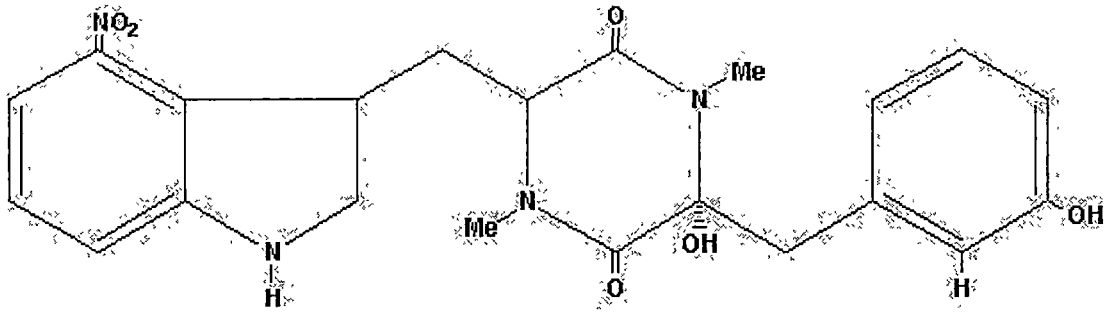


Fig 1.4. Structure of thaxtomin A

All plant pathogenic strains of *Streptomyces* sp. have been found to produce a group of up to 11 related thaxtomins (King *et al.* 1989) whilst non-pathogenic strains do not (King and Lawrence 1996). Thaxtomin A (Fig. 1.4) and thaxtomin B were the first identified, with the original isolation and characterization undertaken by King *et al.* (1989). Production of thaxtomin A normally exceeds thaxtomin B by a ratio of approximately 20:1 *in planta* (King *et al.* 1989, 1991); thaxtomin A also has greater biological activity than all thaxtomin analogues (King *et al.* 2001). Many of the 11 isolated thaxtomin compounds may be precursors to, or breakdown products of thaxtomin A. Thaxtomin A is the primary toxin associated with *S. scabiei*, *S. acidiscabies*, *S. caviscabies* and *S. turgidiscabies*; all of whom produce scab-like symptoms on potato tubers. Thaxtomin C (a precursor/derivative of thaxtomin A) is the key toxin associated with infection of sweet potato (*Ipomoeae batatas* (L.) ham.), by the bacterium *Streptomyces ipomoeae* (Person and Martin) Waksman and Henrici (King *et al.* 1994; King 1997).

1.4.3 Effect of Thaxtomin A

Thaxtomin A has been shown to affect all higher plant species to which it has been applied (Leiner *et al.* 1996), promoting injury symptoms similar to those caused by known inhibitors of cellulose biosynthesis such as dichlobenil and isoxaben (King *et al.* 2001). Phytotoxicity evaluations with thaxtomin A revealed growth $rate_{50}$ values on *Lemna minor*, LEMMI and *Agrostis palustris*, AGRPA of 12 and 5 ppb, respectively. This level of activity is greater than that of many commercial herbicides, including many inhibitors of acetolactate synthase (King *et al.* 2001). *In vitro* agarose-based symptomology tests of *Arabidopsis thaliana*, Columbia showed 50 % inhibition (I_{50}) values of approximately 10 ppb, for thaxtomin A. These tests also confirmed that thaxtomin A had greater biological activity than all its analogues. Some, including thaxtomin B, thaxtomin C, hydroxy-thaxtomin C and hydroxy-thaxtomin A were relatively active in the assay with I_{50} values of $\leq$50 ppb. In contrast, thaxtomin A p-isomer, thaxtomin A o-isomer and des-N-methylthaxtomin C were relatively inactive in the assay, with I_{50} values of $\geq$100 ppb (King *et al.* 2001). Scheible *et al.* (2003) in their Arabidopsis seedling growth assays found I_{50} values for *A. thaliana* of between 25 to 50 nM, under their growth conditions. Whilst these values are slightly higher than those of King *et al.* (2001), which may be a factor of assay technique and growth conditions, they demonstrate that thaxtomin A has biological activity within the nano-molar range. However, thaxtomin A is not regarded as a useful herbicide, as it has poor phloem mobility and systemic activity (King *et al.* 2001).

The key morphological responses to thaxtomin A are common across a range of seedling types with seedling growth decreasing as concentrations of thaxtomin A increase (Leiner *et al.* 1996). General symptoms associated with application of sublethal concentrations of thaxtomin A below 1.0 μM include cell hypertrophy and growth reduction. At higher lethal concentrations of thaxtomin A, plant cell growth is inhibited with no hypertrophy, seedlings become necrotic and are killed (Lawrence *et al.* 1990; Leiner *et al.* 1996; Loria *et al.* 1997; Fry and Loria 2002).

1.4.4 Disease severity is controlled by thaxtomin levels

The amount of thaxtomin produced by a pathogenic strain of Streptomyces during infection correlates with the severity of disease induced (King *et al.* 1991; Loria *et al.* 1995; Goyer *et al.* 1998; Kinkel *et al.* 1998; Toth *et al.* 1998; Natsume *et al.* 1998). Highly virulent strains are able to produce relatively large amounts of thaxtomin, less virulent strains produce less thaxtomin whilst non-virulent strains produce no thaxtomin. Kinkel *et al.* (1998) showed that there are only a few high level producing isolates, presumably reflecting the fitness costs and subsequent selection pressure against high level thaxtomin A producers in the pathogenic *Streptomyces* population. Kinkel *et al.* (1998) also demonstrated for the first time that thaxtomin A production in culture was significantly positively correlated with the percentage of tuber surface infected but not with the number of lesions per tuber. Whilst virulence or disease severity is correlated with thaxtomin A production, growth of the strains was not (Loria *et al.* 1995).

1.4.5 Regulation of toxin production (Thaxtomin biosynthesis regulation)

Thaxtomin biosynthesis is known to be regulated by several factors including developmental stage of the bacteria and temperature (Babcock *et al.* 1993), plant compounds (Beausejour *et al.* 1999), glucose (Babcock *et al.* 1993; Loria *et al.* 1995), and aromatic amino acids (Babcock *et al.* 1993; Lauzier *et al.* 2002).

1.4.5.1 Developmental stage and environment

Babcock *et al.* (1993) showed that yellow-pigmented compounds (thaxtomins) were only identified and isolated during the late exponential to early stationary phases (24 to 33 h) of pathogenic *Streptomyces sp.* culture growth, identifying thaxtomin as a secondary metabolite. They also found that the optimum temperature for thaxtomin production in oatmeal broth was 28°C (Babcock *et al.* 1993). Recently, evidence has been produced to suggest that ultraviolet light inhibits thaxtomin production from the pathogenic *Streptomyces* sp. (R. Loria, *pers. comm.*).

1.4.5.2 Amino acid amendments

Lauzier *et al.* (2002) demonstrated the inhibition of thaxtomin A biosynthesis by tryptophan and phenylalanine, yet these two aromatic amino acids are the biosynthetic building blocks of thaxtomin (King 1997). Babcock *et al.* (1993) also observed significant inhibition of thaxtomin A biosynthesis with the addition of tyrosine and tryptophan to cultures. The specificity of this inhibition has also been demonstrated. Babcock *et al.* (1993) showed that only some aromatic amino acids, and no aliphatic amino acids inhibited thaxtomin A production. Secondly, Lauzier *et al.* (2002), demonstrated that methylation of tryptophan prevented or reduced the inhibitory effect on thaxtomin A biosynthesis, further demonstrating structural specificity. The inhibition of a metabolic pathway by biosynthetic precursors is rather unusual (Lauzier *et al.* 2002), and in the case of thaxtomin production, not fully understood. It is anticipated that some feedback pathway is operational, possibly linking thaxtomin A regulation back to the aromatic amino acids or their precursors (Babcock *et al.* 1993; Lauzier *et al.* 2002).

1.4.5.3 Glucose

Whilst glucose stimulates the growth of the pathogen, thaxtomin A production is suppressed by up to 130-fold, when a typical medium such as oatmeal broth is supplemented with 0.5% or more additional glucose (Babcock *et al.* 1993; Loria *et al.* 1995). The inhibition of secondary metabolite (thaxtomin) production by glucose is common among streptomycetes (Okami and Hotta 1988; Demain 1989). Babcock *et al.* (1993) have related the repression of thaxtomin A production by glucose to the infection process of the tuber itself. They suggested that *S. scabiei* infects young tubers through immature lenticels when glucose levels in the potato peel are low, so that thaxtomin production is not suppressed by glucose, during the early stages of infection.

1.4.5.4 Plant extracts

Loria *et al.* (1995) have shown that thaxtomins are not produced in minimal or rich media without plant extracts. In a minimal medium, the addition of suberin was enough to support the recovery of thaxtomin A from culture supernatant (Beauséjour *et al.* 1999). Suberin is thought to be critical in promoting extracellular

esterase activities in *S. scabiei*, which may be important for thaxtomin A production (Beauséjour *et al.* 1999). Whilst suberin appears to be sufficient to allow thaxtomin A synthesis, the low amount of toxin produced in the minimal starch-suberin medium suggests that other compounds are required for the production of appreciable quantities of thaxtomin A (Beauséjour *et al.* 1999). Media based on the nutritional composition of oatmeal, oatbran and potato peels contain common components that support successful production of thaxtomin (Babcock *et al.* 1993; Beauséjour *et al.* 1999). Recommended broths for thaxtomin A production include oatmeal broth (Loria *et al.* 1995), and for optimal production oat bran broth (Beauséjour *et al.* 1999).

1.4.6 Modes of action

Whilst the exact mechanism and site of thaxtomin action on plant cells is not known a number of sites have been tentatively identified; some more likely candidates than others. The dramatic cell hypertrophy response to thaxtomin A (Leiner *et al.* 1996; Loria *et al.* 1997; Fry and Loria 2002) may suggest interaction with microtubular or other cytoskeletal components, or alternatively an impact on cell membrane function (Fry and Loria 2002). Fry and Loria (2002) discounted the cell membrane as a site of action as there was no evidence of increased hydrogen ion (H^+) pumping in the plasma membrane following addition of thaxtomin A (Fry and Loria 2002). If the cell membrane was a site of action; a stimulation of H^+ pumping as observed with the application of the toxin fusicoccin, or the plant bioregulator, IAA, would be expected. These substances are known to activate cell expansion by stimulating H^+ pumping in the plasma membrane, thus leading to cell wall acidification and loosening (Marre 1979; Peters and Felle 1990).

Other recent evidence suggest that the most likely toxin-plant cell interface resides in the cell wall (Goyer *et al.* 2000; King *et al.* 2001; Fry and Loria 2002; Scheible *et al.* 2003). Fry and Loria (2002) demonstrated that isolated protoplasts from thaxtomin-sensitive plants are insensitive to thaxtomin until replication and reformation of cell wall structures occurs, indicating a probable target for toxicity. Goyer *et al.* (2000) provided indirect evidence of an interaction between thaxtomin A and the cell wall. They showed that mature potato cells treated with thaxtomin A showed no hypertrophy, yet the common response of young seedlings to thaxtomin A

is cell hypertrophy (Leiner *et al.* 1996). They hypothesised that this reflects the nature of the cell walls in mature tissues. Since these mature cells were not dividing at the time of thaxtomin A treatment, no effect on mitotic activity would be expected (Goyer *et al.* 2000).

Fry and Loria (2002) also noted responses to thaxtomin A in tobacco cell culture that were specific to cell cycle phases of rapid activity, dramatic hypertrophy occurred from 13.5 to 26 h after exposure to thaxtomin A. This suggests that typical responses to thaxtomin A, such as hypertrophy (Leiner *et al.* 1996), may be dependent on the physiological state of the plant tissue (Fry and Loria 2002).

Thaxtomin A produces some analogous responses to the inhibitors of cellulose biosynthesis, including the herbicides dichlobenil and isoxaben (King *et al.* 2001). Cell wall regeneration experiments with tobacco protoplasts, in the presence of thaxtomin A, indicated that abnormal cellulose deposition may be the cause of hypertrophic or swelling cells. The observance of abnormalities at cytokinesis, including the occurrence of cell plate deformities in thaxtomin A treated meristematic cells, supports a cell wall target for thaxtomin A (Fry and Loria 2002).

The recent identification of a thaxtomin A resistant (*txr1*) *Arabidopsis thaliana* mutant (Scheible *et al.* 2003) has also confirmed an effect on cell wall production. Their work further supported that of King *et al.* (2001) showing that thaxtomin A inhibits cellulose synthase. Amongst a range of evidence, thaxtomin A specifically inhibited the incorporation of ^{14}C-glucose into the cellulosic (i.e. acid-insoluble) cell-wall fraction of dark-grown wild-type seedlings (Scheible *et al.* 2003), much like the related toxin, isoxaben (Heim *et al.* 1990). Glucose was shunted into the hemicellulose fraction, rather than cellulose fraction. Whilst the exact mode of action of thaxtomin A remains unknown Scheible *et al.* (2003) proposed that the increased resistance to thaxtomin A in the *A. thaliana 'txr1'* mutant was as a result of a decrease in the rate of toxin uptake and that TXR1 is a regulator of a transport mechanism.

Further recent work elucidating key responses at the cell membrane to thaxtomin A are providing further insight into possible modes of action of thaxtomin A (Tegg *et al.* 2005) and are described in Chapter 2.

1.4.7 The genetic basis of thaxtomin production

Thaxtomin was identified as recently as the late 1980's (King *et al.* 1989) and work to understand thaxtomin production and regulation from a genetic perspective is progressing well. Since 1997 researchers have identified critical genetic components controlling thaxtomin biosynthesis and virulence (Bukhalid and Loria 1997; Bukhalid *et al.* 1998; Healy *et al.* 1999, 2000, 2002; Joshi *et al.* 2004; Kers *et al.* 2004b), which provide an added perspective to the role of thaxtomin in plant pathogenicity. All of these components have been found on a large, mobile pathogenicity island (PAI) from plant pathogenic *Streptomyces* species (Kers *et al.* 2005) and they are discussed in the chronological order that they were discovered.

Bukhalid and Loria (1997) identified two genetic components from *S. scabiei* that were found to play a possible role in thaxtomin production; Open Reading Frame1 (designated ORF*tnp*) and the 0.67-kb ORF3 (designated *nec1*). Cloned fragments, that included *nec1*, were able to induce the nonpathogenic *Streptomyces lividans* to necrotize tuber tissue and produce scab-like symptoms. ORF*tnp* was shown to have evolved in '*Streptomyces*', whereas *nec1* evolved elsewhere, indicated by a low G + C content (54%) atypical of protein coding and noncoding sequences within the *Streptomyces* genome. It has been suggested that ORF*tnp* improved the survival of the organism it inhabited (*Streptomyces*) by transposing *nec1* from another genus (Bukhalid and Loria 1997).

Another closely associated insertion sequence (IS*1629*) adjacent to *nec1* was identified by Healy *et al.* (1999) in all pathogenic *S. acidiscabies*, *S. turgidiscabies* and some *S. scabiei* (Type II). These authors suggested that the ORF*tnp-nec1*-IS*1629* region was transferred horizontally in a unidirectional manner through dissemination of a "pathogenicity island" from IS-*1629*-containing (Type II) *S. scabiei* isolates to *S. acidiscabies* and *S. turgidiscabies*. The transposition of these virulence genes mediated by ORF*tnp* and a study of their genetic properties reveals a potential mechanism for the evolution of pathogenicity within *Streptomyces* species.

Because of the higher genetic diversity of *S. scabiei* it is likely that it first acquired the virulence gene before transposing it into *S. acidiscabies* and *S. turgidiscabies*. This is reflected in the time (year) when each species was initially described and their current geographic distributions (Bukhalid *et al.* 2002). *Streptomyces scabiei* was first isolated in 1890 in Connecticut, USA (Thaxter 1891) and occurs worldwide. *Streptomyces acidiscabies* and *S. turgidiscabies* were first reported as a cause of potato scab much later, 1953 and 1991 respectively (Lambert and Loria 1989b; Miyajima *et al.* 1998). Bukalid *et al.* (1998) believed that transfer of pathogenicity asssociated genes from *S. scabiei* to *S. turgidiscabies* occurred during the last 50 years. These 2 species were also initially geographically limited: *S. acidiscabies* to the northeastern United States and *S. turgidiscabies* to the island of Hokkaido in Japan (Bukhalid *et al.* 2002). However, recent evidence suggests that these 2 species are now being found in regions across the world (Kim *et al.* 1998a, b; Kim *et al.* 1999; Park *et al.* 2003b; Wilson 2005).

Whilst the correlation between the presence of *nec1* and thaxtomin A production (Bukhalid and Loria 1997) appeared conclusive, a pathogenic strain of *S. scabiei* from South Africa, CEK-018, that produced thaxtomin A, but did not possess a *nec1* homolog was found (Bukhalid *et al.* 1998). On the basis of this one strain, the authors suggest that *nec1* is not directly involved in thaxtomin A biosynthesis, but is perhaps, a virulence factor in plant-pathogenic streptomycetes. Other studies have since identified additional strains whereby the *nec1* gene is present but no thaxtomin A is produced or *nec1* gene not present but thaxtomin A is produced (Lazarovits *et al.* 2001; Bukhalid *et al.* 2002; Park *et al.* 2003b; Wang and Lazarovits 2004; Wilson 2005). Nevertheless, high correlation between *nec1* and thaxtomin A production does suggest physical linkage between *nec1* and the thaxtomin A biosynthetic genes (Bukhalid *et al.* 1998).

Healy *et al.* (2000) made major progress in understanding thaxtomin production when they identified two peptide synthetase genes (*txtA* and *txtB*). Thaxtomin A production was abolished in *txtA* disruption mutants, whereas pathogenicity and thaxtomin A production was restored when the thaxtomin synthetase cosmid was inserted into the *txtA* mutant. The probe used to hybridize to the probable peptide synthetase genes was specific to the thaxtomin A-producing

bacteria; *S. scabiei*, *S. acidiscabies* and *S. turgidiscabies*, and did not hybridize to the thaxtomin C-producing *S. ipomoeae*, which has a different aetiology and host range.

Further progress in elucidating biochemical pathway synthesis was achieved with the identification of the monooxygenase gene homolog *txtC* (Healy *et al.* 2002). It encodes an enzyme that catalyzes at least one, and probably two, reactions in the thaxtomin biosynthetic pathway. It catalyzes the hydroxylation of the non-phytotoxic thaxtomin A precursor, thaxtomin phenylalanyl (thaxtomin D) to thaxtomin B. It may also function in the conversion of thaxtomin B to thaxtomin A, another hydroxylation reaction (Healy *et al.* 2002). Recently, a bacterial nitric oxide synthase, encoded by *nos*, was identified (Kers *et al.* 2004b). It carries out a highly specialized nitration event that is essential for the production of normal levels of thaxtomin A (Kers *et al.* 2004b; Wach *et al.* 2005).

More recently, the genetic organizaton of PAI from pathogenic *Streptomyces* has been elucidated further revealing other important virulence factors (Kers *et al.* 2005). As well as containing multiple virulence-associated genes, including the thaxtomin biosynthetic genes, *nec1*, and a tomatinose homologue, the PAI from *S. turgidiscabies* contains cytokinin biosynthetic genes encoded by the *fas* operon. Of the strains examined *S. scabiei* and *S. acidiscabies* do not have a *fas* operon within their PAI. This suggests that the *fas* operon has been independently acquired, probably from the plant pathogen *Rhodococcus fascians*, following acquisition of the PAI. The *fas* operon is required for the production of leafy galls and is just one more virulence factor that enables a better understanding of the PAI from pathogenic *Streptomyces* (Joshi *et al.* 2004; Kers *et al.* 2005). The importance of the PAI has been demonstrated by the conjugal transfer of the 660 kb PAI from the pathogenic *S. turgidiscabies* into the non-pathogenic *S. diastatochromogenes*, giving this strain a pathogenic phenotype (Kers *et al.* 2004a). The expected complete sequencing of the *Streptomyces* genome shortly will aid in further understanding of the genetics of pathogenicity (R. Loria, *pers. comm.*).

1.5 Research Aim

1.5.1 Research outline

The key aim of this project was to gain a better understanding of thaxtomin A toxicity in plant cells. This study focused on the response of specific individual ions, molecular changes up to responses at the whole plant level to identify critical responses to thaxtomin A.

The initial interaction between plant and the toxin thaxtomin A was studied using non-invasive microelectrode ion flux estimation (MIFE) technique (Shabala *et al.* 1997). This relatively new technique enabled the characterization of electrophysiological responses of plant cell systems to application of thaxtomin A. It enabled the identification of specific ion flux parameters and ion channels effects, thus providing new evidence regarding inducement of thaxtomin A toxicity and possible mode of action.

At the whole plant level the role of the pathogen *S. scabiei* and the toxin thaxtomin A in promoting common scab symptoms was examined in combination with foliar auxin sprays. These sprays have been shown to reduce common scab symptoms (McIntosh *et al.* 1981, 1982) and these pot trials aimed to replicate these results but also identify possible mechanisms of disease suppression conferred by auxin foliar sprays.

The possible interaction between thaxtomin A and auxin sources was further determined using an array of different plants and tissue types. The usage of *Arabidopsis thaliana* plants with a range of well characterized mutations to auxin sources, auxin transport inhibitors, other toxins and signalling pathways enabled pin-pointing of specific interactions with thaxtomin A. Likewise, the response of these plants to auxin sources, auxin transport inhibitors and other toxins provided further evidence of interactions. Tomato pollen systems also provided a better alternative to root-based assessment systems for clarifying possible auxin-thaxtomin A interactions.

Thaxtomin A-resistant potatoes and calli have been developed using cell selection techniques, a key commercial objective of the broader project. Within this

framework there exists a need to quantify key morphological or physiological mechanisms that provide thaxtomin A and potentially common scab resistance to a given potato cultivar. The molecular characterization of these thaxtomin-resistant potatoes was examined with the objective to firstly identify and then characterize a thaxtomin resistant (*txr1*) gene homolog from potato, and search this gene for important mutations. Whether these thaxtomin resistant plants have or have not undergone genetic changes will provided valuable understanding of the mechanisms behind scab resistance, and facilitate the transfer of this resistance to a wide-range of potato cultivars.

1.5.2 Research objectives

Having defined current priorities for research through a critical evaluation of the literature, the objectives of the work presented in this thesis are:

1. to identify ion flux and ion signalling responses to thaxtomin A, with characterisation of specific, useful flux parameters;
2. to determine responses of both the pathogen and the toxin to foliar applied auxin sprays within potato systems;
3. to determine critical responses of various plant systems to thaxtomin A and examine the role auxin may play in mediating these responses; and
4. to determine morphological and physiological properties that can distinguish thaxtomin resistant vs thaxtomin susceptible potato selections; and to identify and characterise genes associated with thaxtomin resistance.

These form part of a longer-term research objective to understand the morphological and physiological basis of thaxtomin A toxicity and resistance in potato systems and the ongoing incorporation of resistance into important commercial potato varieties.

Chapter 2. Plant morphological & ion flux responses to thaxtomin A

2.1 Introduction

Thaxtomin A is a dipeptide phytotoxin produced by all plant pathogenic *Streptomyces* sp. responsible for common scab disease (King *et al.* 1989; Loria *et al.* 1997). The major cellular targets and mode of action of thaxtomin A toxicity in plant cells are not fully understood. Initial studies have suggested it ultimately targets the plant cell wall, causing inhibition of cellulose biosynthesis or deposition (Fry and Loria 2002). Also, injury symptoms of thaxtomin A are similar to herbicides such as dichlobenil and isoxaben which work by inhibiting cellulose biosynthesis (King *et al.* 2001). The ability of *Arabidopsis thaliana* thaxtomin resistant mutants (*txr1)* to lay down a cell wall in the presence of thaxtomin A with altered cell wall components (Scheible *et al.* 2003) also implicates a cell wall-mediated response.

Whilst these and others studies have contributed to a greater understanding of how thaxtomin A may operate, they have all focused on the later stages of plant responses to toxin (e.g. specific gene activation, synthesis of specific metabolites and enzymes and, ultimately, a morphological response). The events preceding these responses have not been studied, yet they are an essential component of the disease induction response. The mode of action of thaxtomin A will be more clearly elucidated with the identification of ion channels or candidate receptor sites mediating the interaction between the toxin and a host plant plasma membrane.

Calcium signalling in response to plant pathogenic extracts, (including phytotoxins and elicitors) has been reported across a range of species including tobacco (Lecourieux *et al.* 2002; Kadota *et al.* 2004), soybean (Ebel *et al.* 1995), carrot (Bach *et al.* 1993) and parsley (Nürnberger *et al.* 1994; Jabs *et al.* 1997; Blume *et al.* 2000). With plant defence elicitors, transient increase in cytosolic Ca^{2+} level is crucial for the induction of the oxidative burst and thus defense responses (Clough *et al.* 2000). This has been demonstrated by removing Ca^{2+} from the culture medium or blocking Ca^{2+} with ion channel blockers, preventing pathogen-induced

Ca^{2+} influx and subsequent downstream defense reactions, such as defense gene activation and phytoalexin synthesis (Jabs *et al.* 1997). The magnitude, frequency and duration of the Ca^{2+} influx is also critical in determining specific downstream events (Jabs *et al.* 1997; Lecourieux *et al.* 2002). In the case of thaxtomin A, it is unknown as to whether interaction between toxin and plant is characterized by a rapid Ca^{2+} flux change, and whether this recognition is responsible for subsequent downstream toxic effects.

A range of techniques has been used to study Ca^{2+} signalling events in response to plant pathogenic extracts. These include $^{45}Ca^{2+}$ uptake experiments (Bach *et al.* 1993; Nürnberger *et al.* 1994), pharmacological evidence obtained by using lanthanides (La^{3+} and Gd^{3+}) to block Ca^{2+} influx (Atkinson *et al.* 1990; He *et al.* 1993; Nürnberger *et al.* 1997), aequorin technology (Knight *et al.* 1991; Blume *et al.* 2000; Lecourieux *et al.* 2002; Grant *et al.* 2000; Felix *et al.* 1999), and patch-clamp experiments (Gelli *et al.* 1997; Zimmermann *et al.* 1997). Whilst providing valuable knowledge, there are some deficiencies associated with each of these techniques, outlined in Tegg *et al.* (2005). Direct quantification and resolution of Ca^{2+} fluxes to plant pathogenic extracts, in vivo, are lacking and further studies are required to better understand this crucial early interaction between the host and pathogen.

Fluxes of other ions such as H^+, K^+ and Cl^- are also implicated in early pathogen recognition (Zimmermann *et al.* 1999; Clough *et al.* 2000; Lecourieux *et al.* 2002). For H^+, literature reports are rather controversial, suggesting that effects may be plant or tissue specific. In most cases, elicitor-induced H^+ influx or extracellular alkalinization is reported (Atkinson *et al.* 1990; Nürnberger *et al.* 1994; Scheel 1998; Kuchitsu *et al.* 1997). However, Vera-Estrella *et al.* (1994) found a four-fold increase in plasma membrane H^+-ATPase activity in elicitor treated tomato cells. This is consistent with the acidification of the extracellular medium (increased H^+ efflux), observed elsewhere (Blumwald *et al.* 1998) and suggests that the mode of action of this specific elicitor may differ from that observed with nonspecific elicitors or pathogenic toxins (Vera-Estrella *et al.* 1994). It remains to be answered if the above specificity is also a case for thaxtomin A.

A convenient way of measuring early electrophysiological responses at the plasma membrane to pathogens or toxin action is the use of non-invasive microelectrode ion flux estimation (MIFE). This technique has previously been successfully applied to study plant adaptive responses to various abiotic stresses such as salinity (Shabala 2000; Shabala *et al.* 2003), cold (Shabala and Shabala 2002), osmotic (Lew 1998; Shabala *et al.* 2000; Shabala and Lew 2002) and acid stress (Shabala *et al.* 1997; Babourina *et al.* 2001).

In this work, the MIFE technique has been applied to study early events associated with thaxtomin A perception and signalling in *Arabidopsis thaliana* roots and tomato roots and pollen tubes. This work provides the first evidence that thaxtomin A triggers an early signalling cascade, causing rapid and tissue-specific changes in net Ca^{2+} and H^{+} ion flux profiles, which are crucial in plant-pathogen interactions. The physiological significance of this data and possible ionic mechanisms involved in mediation of thaxtomin A-induced ion fluxes in plant root and pollen tissues are discussed. This data will be critical for the elucidation of possible cellular targets and toxicity mechanisms for thaxtomin A.

2.2 Materials and Methods

2.2.1 Thaxtomin A production and purification

Thaxtomin A production and purification methods were similar to those of Loria *et al.* (1995). Oat meal broth (OMB) was prepared by boiling rolled oats (20g/L) in distilled water for 12 min and straining though fine mesh cheesecloth, amended with trace elements (1g/L each of $FeSO_4.7H_2O$, $MnCl_2.4H_2O$ and $ZnSO_4.7H_2O$) and adjusted to pH 6.8 with 0.1 N NaOH. The OMB was inoculated with 10 ml of spore suspensions and incubated at 22C for 7 days on a rotary shaker (125 rpm). The liquid cultures (500 mL) were extracted three times with 250 mL chloroform. Chloroform extracts were concentrated with rotary evaporator and combined residues fractionated by chromatography column on silica gel with elution by chloroform:methanol (9:1 vol/vol) to collect a bright orange fraction. The fraction was evaporated to dryness and dissolved in chloroform (1 mL) for further purification. Crude extracts were loaded onto a silica gel 60 thin layer chromatography plates and run in chloroform:methanol (9:1 vol/vol). The orange area (Rf = 0.3) was scraped from the plate and eluted with methanol. The methanol was removed and the residue re-dissolved in chloroform. The eluate was filtered through a 0.2 m syringe filter (Whatman) to remove any ash. After storage at –20C for approximately 10 days, bright orange crystals formed in the cold chloroform which were recovered by filtration. The crystals were analysed with high performance liquid chromatography (HPLC) combined with mass spectroscopy (LC-MS). Purity of thaxtomin A was ≥98% (G. Luckman, *pers. comm.*) and stock solutions were made by dissolving 2.5 mg thaxtomin A in 5 mL acetone and making up to 100 mL with distilled water.

2.2.2 Plant material, media and root growth experiments

For all root experiments, plants were grown in a culture room at ambient temperature 22° ± 1° C and 16 h day length (60 $\mu mol/m^2/s$). *Arabidopsis thaliana* seeds were obtained from Arabidopsis Biological Resource Center (ABRC), Ohio State University, Columbus, Ohio USA and the Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham, Loughborough, Leicestershire UK. Tomato seeds (*Lycopersicon esculentum var. esculentum* cv. 'Chandler's English') were kindly supplied by E. Chandler (Chandlers Nursery, Hobart, Tasmania). Potato

plants (*Solanum tuberosum* 'Russet Burbank') were obtained from Department of Primary Industry, Devonport, Tasmania.

Arabidopsis thaliana seeds were surface-sterilized for 15 min in bleach solution with gentle agitation every 5 min (available Cl: 1.5% m/v). Twenty seeds were plated directly in 2 rows into Petri dishes containing Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 8 g/L agar and 10 g/L sucrose, and poured under aseptic conditions into sterile 90 mm Petri plates. Thaxtomin A (thermolabile) was filter-sterilized (0.2 μM Supor® Membrane syringe filter – Gelman Laboratory, Ref 4612) and added after autoclaving (120 kPa., 121°C, 20 min.).

After a vernalization period of 2 days at 4°C, plates were transferred into the culture room and oriented at an upright angle of about 85^0, enabling roots to grow along the agar surface essentially without penetrating it. After 5 days, root length was measured and plants were transferred to the above media containing thaxtomin A. After 3 days of incubation root length was re-measured, and the effect of thaxtomin A on root growth quantified. Each treatment had two replicates with 20 seeds per plate (n = 40 in total). The effect of thaxtomin A on root hair growth and development, was assessed using *A. thaliana* seeds germinated directly onto treatment media, following a 2 day vernalization period (4°C). Root characteristics were measured after 5 days growth. Five representative plants per plate were assessed over 2 replicates (n = 10 in total).

Tomato seeds were germinated in Petri dishes on a sterile filter paper moistened with distilled water. After 5 d evenly germinated seedlings were selected and exposed to various concentrations of thaxtomin A. Root lengths were measured twice: immediately prior to treatment, and 24 h after thaxtomin A application. Each treatment had 2 replicates with 10 seeds per plate (n = 20 in total).

Potato plantlets grown in tissue culture were used. Single-node sections, containing one leaf and a lateral bud, were excised from plantlets and placed in Petri dishes, with filter paper moistened with Potato Media Solution (PMS) containing MS, 3% sucrose, 0.05% casein hydrolysate and 0.004% ascorbic acid. After 5 d,

cuttings with a single root were selected and transferred to Petri dishes with PMS ameliorated with thaxtomin A. Potato root lengths were measured twice: immediately prior to treatment, and 24 h after thaxtomin A application. Each treatment had 2 replicates with 5 plants per dish (n = 10 in total).

2.2.3 Pollen growth experiments

Pollen was collected from glasshouse grown tomato plants 40-60 days after planting and dried overnight. The dried pollen was transferred to 0.2 mL of growth solution (containing 15% sucrose, 0.003% H_3BO_3, 1 mM $CaCl_2$ and 0.2 mM KCl) with thaxtomin A treatments and placed in 96-well microtitre plate (Selby Biolab, Australia). The homogenous distribution of pollen grains was achieved by shaking the wells for several seconds using a vortex. Then the plate was inverted and pollen grains germinated at 29 ^{0}C for 3 h in the dark (the hanging drops method; Melian and Balashova 1994). At the end of that period, 50 μL of 10% formalin was added to each well to stop the growth process and fix germinated pollen tubes. Germinated pollen for each treatment was pooled (8 wells per treatment) and examined microscopically (Olympus CH biological microscope, Tokyo, Japan). The length of 50 to 100 randomly chosen pollen tubes for each treatment was measured and averaged.

2.2.4 Non-invasive ion flux measurements

Net fluxes of H^+ ,K^+ and Ca^{2+} were measured non-invasively using ion-selective vibrating microelectrodes (the MIFE technique; University of Tasmania, Hobart, Australia), generally as described by Shabala *et al.* 1997, 2000). Briefly, electrodes with a tip diameter of about 2 μm were pulled from borosilicate glass capillaries, dried in an oven, and silanized with tributylchlorosilane (Sigma-Aldrich, Milwaukee, WI, USA). Electrodes were first back-filled with an appropriate solution, and then the electrode tips were front-filled with commercially available ion-selective cocktails (H^+, 95297; K^+, 60031; Ca^{2+}, 21048; all from Sigma-Aldrich). After conditioning, the electrodes were calibrated in a known set of pH buffers (from 5.1 to 7.5) and standard Ca^{2+} solutions (0.1-1.0 mM range). Electrodes with a response of less than 50 mV per decade for H^+ and K^+ and 25 mV per decade for Ca^{2+}, and correlation $R<0.999$, were discarded. The reference electrode was a glass capillary filled with 500 mM KCl in 2% agar.

The microelectrodes were mounted on an electrode holder (MMT-5; Narishige, Tokyo, Japan) that allowed precise 3-dimensional positioning of electrode tips. The electrodes were initially positioned 20 μm from the root surface. During measurements, electrodes were moved in a slow square-wave manner between two positions - close (20 μm) and distant (50 μm) - radially outwards from the measured surface. The duration of each half-cycle of the electrode movement was 5 s; the actual electrode movement between two positions took about 0.4 s. The first 2 s after the movement began were ignored to allow both the movement and the electrochemical settling of the electrodes (Newman 2001). The recorded voltage gradients between positions were then converted into concentration differences using the calibrated Nernst slopes of the electrodes. Net flux values (influx positive) were calculated assuming cylindrical diffusion geometry as described in Shabala *et al.* (2000). No mixing of the bath medium occurred due to electrode movement as indicated by zero net fluxes measured in the absence of plant tissue in the chamber (data not shown).

2.2.5 Experimental protocols for ion flux measurements

Both growth conditions and media used to grow plants for ion flux experiments were as described previously (2.2.2 and 2.2.3). All experiments with *A. thaliana* were conducted on 5-day old plants. Short-term transition experiments were conducted on excised root segments as described by Demidchik *et al.* (2003). Briefly, excised roots were immobilized on liquid agar in a holding chamber placed in a bathing medium (0.2 mM KCl, 0.1 mM $CaCl_2$). Experiments commenced 15 to 30 mins after immobilization, after steady state conditions were reached. Net ion fluxes were measured (prior to treatment) for 5 to 10 min, and then thaxtomin A was added to the chamber to give a final concentration of 3 μM thaxtomin A in the chamber. When H^+ and Ca^{2+} channel blockers were used, these were added into the bathing medium at the time of immobilization. Long-term experiments involved the transfer of whole plants onto MS media ameliorated with thaxtomin A (0.2 μM for *A. thaliana*; 0.5 μM for tomato), followed by incubation and subsequent growth for 24 hrs. Roots were then excised and fluxes in different root zones (meristem, elongation and mature) measured for 3-5 minutes.

For ion flux measurements on pollen, a small amount of freshly collected and dried pollen was immobilized on a surface of the thin (1 mm OD) glass capillary essentially as described by Shabala *et al.* (2001). The capillary was then mounted in a horizontal position in the Perspex holder within the measuring chamber filled by solution, used for pollen growth experiments. Depending on the aim of the experiment, the chamber was either left in the growth cabinet (at 29 ^{0}C) for 1-2 h (when fluxes were measured from already germinated tubes), or placed in the Faraday cage for measurements immediately after pollen imbibition. In both cases, the room temperature during MIFE experiments was maintained at 29 ± 1 ^{0}C to provide optimal conditions for pollen growth. Ion fluxes were measured from either tip of the germinated pollen tube, or pollen grain base, depending on experimental purposes. Due to the small size of the pollen tube, only H^+ flux was measured. All measurements were taken from single pollen cells, with no others tubes present in the field of view (x 400 magnification) to ensure that ion fluxes were measured from the single tube only.

2.2.6 Data analysis

Data were subjected to analysis of variance using Genstat 6 (Rothamsted Experimental Station, Harpenden, Herfordshire, UK). For root growth data significance was calculated at P = 0.05 and least significant difference (LSD) was used for comparison of mean values. For ion flux data the standard t-test was used for comparison of mean values at various P values, as stated in the results.

2.3 Results

2.3.1 Thaxtomin A induced effects on root growth

Thaxtomin A reduced the total root length of *A. thaliana*, tomato and potato (Fig. 2.1) in a dose-dependant manner. Tomato and potato were less sensitive than *A. thaliana*, with higher doses required to inhibit root growth by 50%. At higher thaxtomin A concentrations all species showed necrosis and eventual death (data not shown). In addition to reducing total root length, thaxtomin A treatment had a significant effect on other morphological root parameters (Table 2.1). A progressive increase in root diameter in both the elongation (data not shown) and mature zone (Table 2.1) was observed. Thaxtomin A treatment also increased (up to 10-fold) the root hair density in *A. thaliana* roots, reduced the length of the root hair-free apical zone and significantly (>50%) shortened the length of the elongation zone (Table 2.1). The mean length of the root hair was not significantly (*P>0.05*) affected.

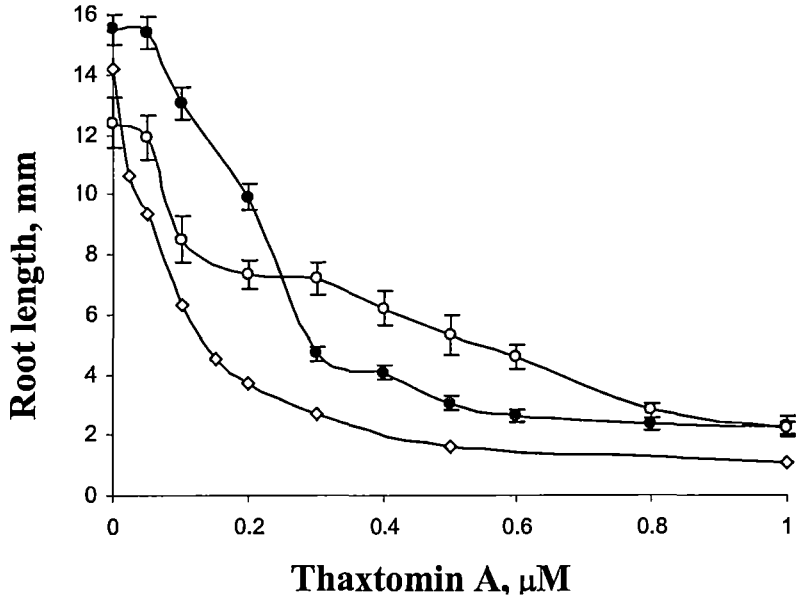


Fig. 2.1 Effects of exposure to thaxtomin A on growth of *Arabidopsis thaliana* (WT Columbia; open diamonds), tomato (closed circles) and potato (open circles) roots. Arabidopsis plants were treated for 3 days; tomato and potato plants – for 24 h. Data are mean ± SE (n = 40 for *A. thaliana*, n = 20 for tomato, n = 10 for potato).

Table 2.1. Effect of thaxtomin A treatment on growth and development of *A. thaliana* (WT Columbia) root hairs. Data is mean ± SE (n = 10).

Thaxtomin A concentration (μM)	Root length, mm	Root diameter, μm	Length of elongation zone, μm	Root hair density (hairs mm^{-1})	Tip zone length free of root hairs (μm)
0	22.55 ± 0.44 a*	135.0 ± 4 d	251.5 ± 8 a	16.7 ± 1.2 g	1178 ± 43 a
0.025	15.58 ± 0.28 b	139.5 ± 5 cd	250.5 ± 10 a	19.6 ± 1.1 fg	912 ± 28 b
0.05	13.53 ± 0.21 c	141.0 ± 5 cd	221.5 ± 7 b	27.4 ± 2.0 ef	804 ± 25 c
0.1	7.75 ± 0.19 d	150.0 ± 4 bc	196.5 ± 6 c	37.7 ± 1.9 de	580 ± 30 d
0.15	4.83 ± 0.11 e	159.0 ± 5 b	177.0 ± 6 c	41.8 ± 2.2 d	542 ± 29 d
0.2	2.90 ± 0.11 f	161.5 ± 5 b	125.5 ± 8 d	67.6 ± 2.3 c	220 ± 12 e
0.3	1.83 ± 0.07 g	203.5 ± 7 a	108.5 ± 7 d	72.7 ± 3.4 c	199 ± 11 e
0.5	1.13 ± 0.08 h	203.0 ± 10 a	N/m	174.0 ± 7.1 a	N/m
1.0	0.93 ± 0.08 h	N/m**	N/m	159.0 ± 6.3 b	N/m
LSD (0.05)	0.55	14.7	20.4	10.3	72.2

* Means followed by same letter within the same column are not significantly different at the 0.05 probability level using Fisher's LSD test.

** N/m – not measured

2.3.2 Effects of long-term (24 hrs) thaxtomin A incubation on root H^+ fluxes

Twenty four hours incubation with 0.2 μM thaxtomin A enhanced the active H^+ export in all functional root zones (meristematic, elongation, and mature) of *A. thaliana* (significant ($P<0.05$) reduction in net H^+ influx; Fig. 2.2A). Functionally more active zones, such as elongation and meristem regions, were more sensitive to thaxtomin A (Fig. 2.2A). Treatment of tomato with 0.5 μM thaxtomin A also resulted in enhanced H^+ extrusion within the elongation zone, but not in the meristem or mature zones (Fig. 2.2B).

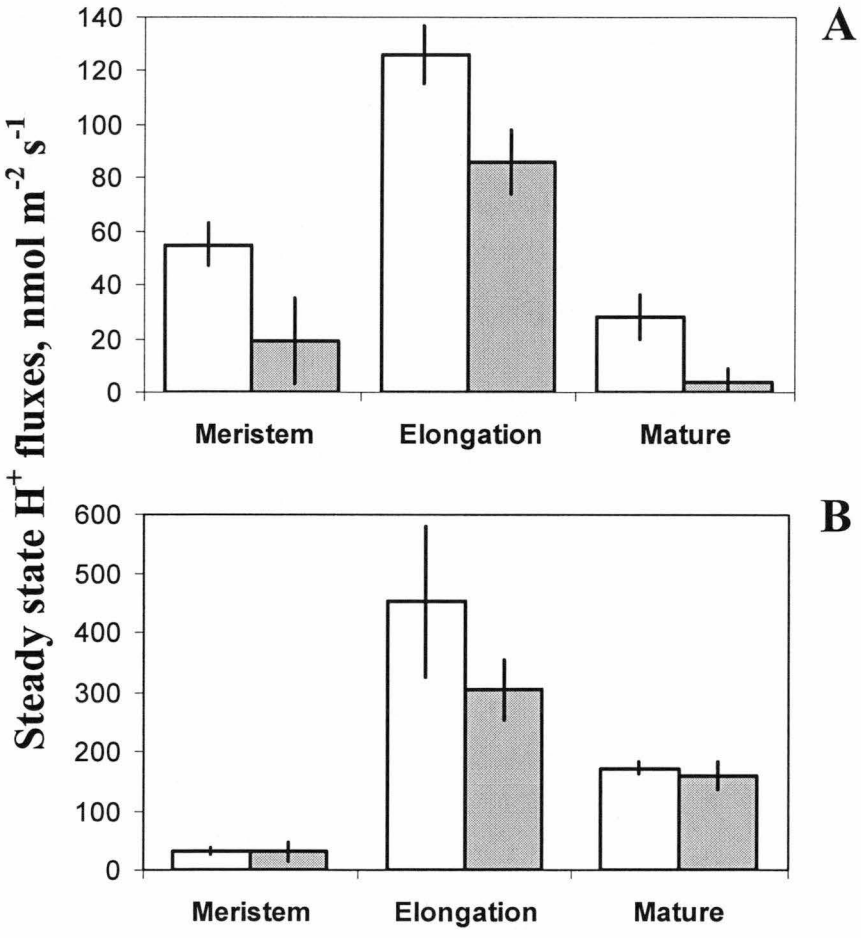


Fig. 2.2. Effect of 24 hr exposure to thaxtomin A on net H^+ flux (inward positive) measured near the surface of *Arabidopsis thaliana* and tomato roots (both for meristematic, elongation and mature zones). **A** – *A .thaliana* (0.2 μM thaxtomin A); **B** – tomato (0.5 μM thaxtomin A). Control (clear), treated with thaxtomin A (shaded). Data are means ± SE (n = 5).

2.3.3 Effects of long-term (24 hrs) thaxtomin A incubation on root K^+ and Ca^{2+} fluxes

Twenty four hours incubation with 0.2 μM thaxtomin A enhanced the passive K^+ extrusion from meristematic and elongation root zones of *A. thaliana* (significant ($P<0.05$) increase in net K^+ efflux; Fig. 2.3A), but not in the mature zone. In contrast, a 24 hr incubation with thaxtomin A enhanced the net Ca^{2+} uptake in the elongation zone (significant ($P<0.05$) increase in net Ca^{2+} influx; Fig. 2.3B), but not in the meristem or mature zones.

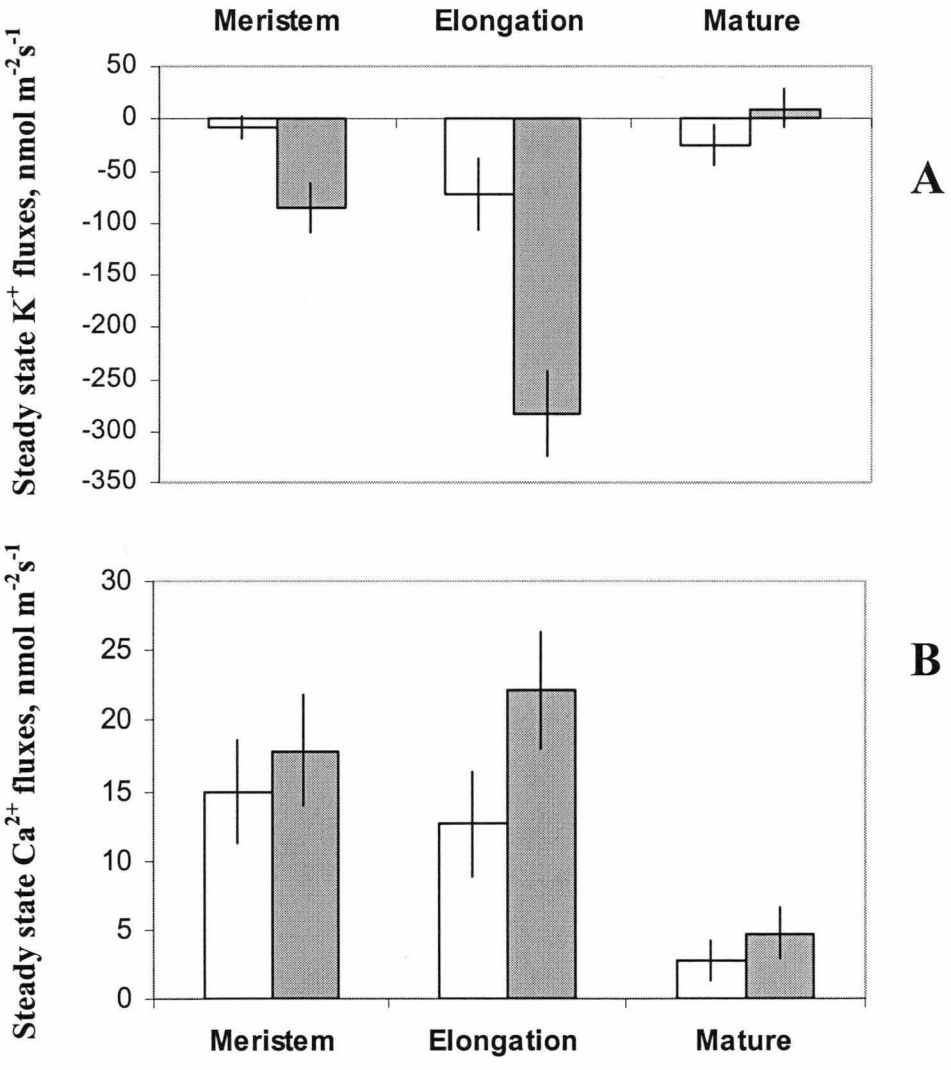


Fig. 2.3. Effect of 24 hr exposure to thaxtomin A (0.2 μM thaxtomin A) on **A**) net K^+, and **B**) net Ca^{2+} fluxes (inward positive) measured near the surface of *Arabidopsis thaliana* roots (for meristematic, elongation and mature zones). Control (clear), treated with thaxtomin A (shaded). Data are means ± SE (n = 5).

2.3.4 Short-term transient ion flux responses to thaxtomin A

Transient H^+ flux responses within the elongation root zone to thaxtomin A application were rather variable and essentially split into 2 distinct groups (Fig. 2.4A). For *ca.* 50% of plants tested net H^+ influx was unchanged or even slightly increased (two upper traces in Fig. 2.4A), while plants in the other group showed a significant ($P<0.05$) shift towards net efflux after addition of thaxtomin A (final concentration = 3 μM) to the bath (two lower traces in Fig. 2.4A).

Overall, within the elongation root zone, the average net H^+ flux was barely changed after thaxtomin A application (closed circles in Fig. 2.4B). At the same time, there was a pronounced and significant ($P<0.01$) shift towards net Ca^{2+} efflux (Fig. 2.4B, open circles) the magnitude of which progressively decreased 8 to 10 min after the treatment.

Within the mature zone, the average net H^+ flux was also barely changed after thaxtomin A application (closed circles in Fig. 2.4C). In contrast to results from the elongation zone, there was no significant effect on Ca^{2+} fluxes either (Fig. 2.4C).

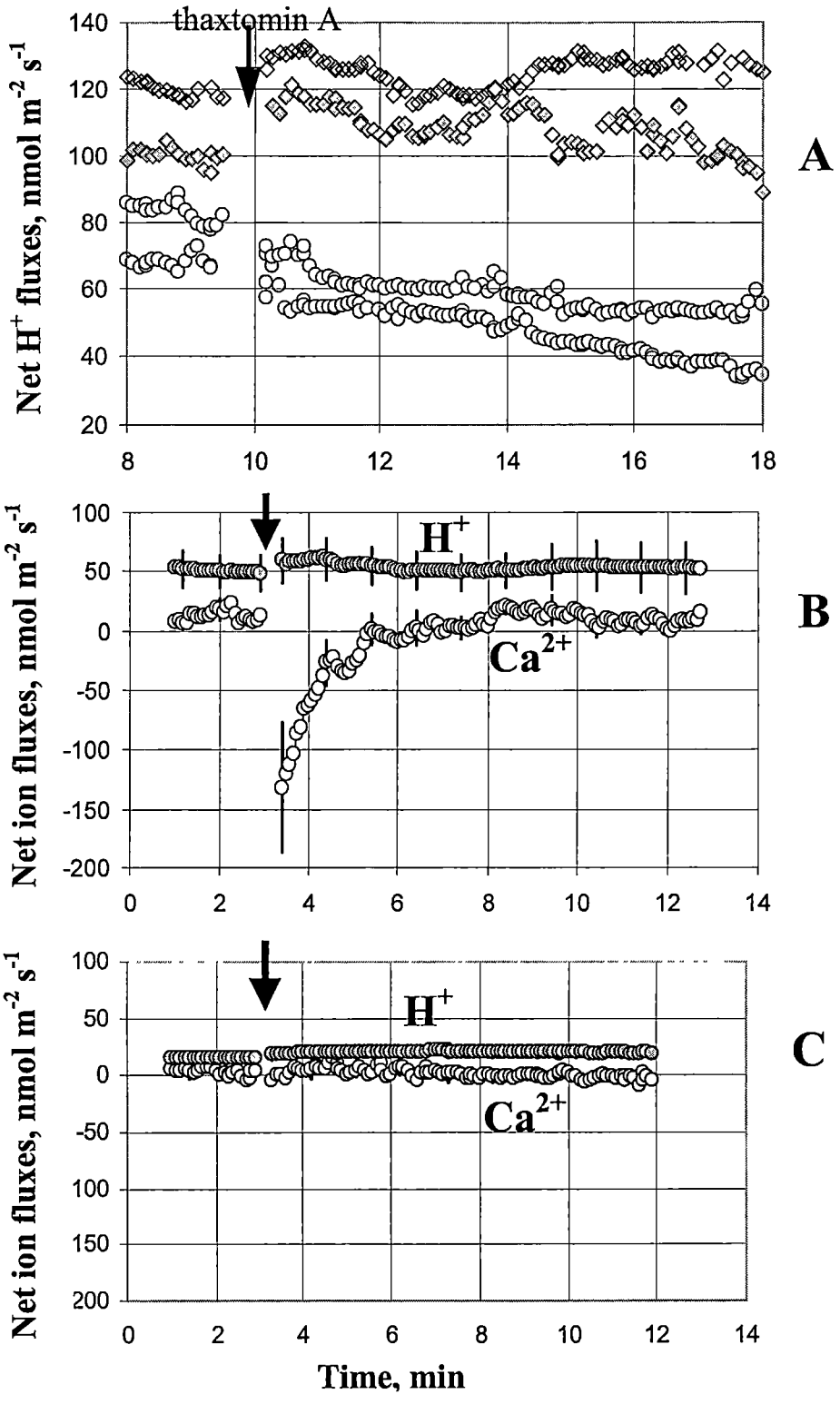


Fig. 2.4. Short-term transient ion flux responses to thaxtomin A (3 μM final concentration in the chamber). **A** - typical examples of net H^+ flux transition in response to thaxtomin A application, measured from elongation zone of *Arabidopsis thaliana* (WT Columbia) roots. Average transient H^+ and Ca^{2+} flux responses to thaxtomin A in non-buffered solution from elongation (**B**) and mature (**C**) zones. Data are mean ± SE (n = 6).

Whilst there was variability in the H^+ flux transition in response to thaxtomin A treatment, approximately half of the plants showed a significant shift towards efflux (Fig. 2.4A). In these plants, root pretreatment in either orthovanadate and carbonyl cyanide m-chlorophe-nyl hydrazone (CCCP) significantly ($P < 0.05$) reduced the magnitude of thaxtomin A-induced shift towards net H^+ efflux (Fig. 2.5).

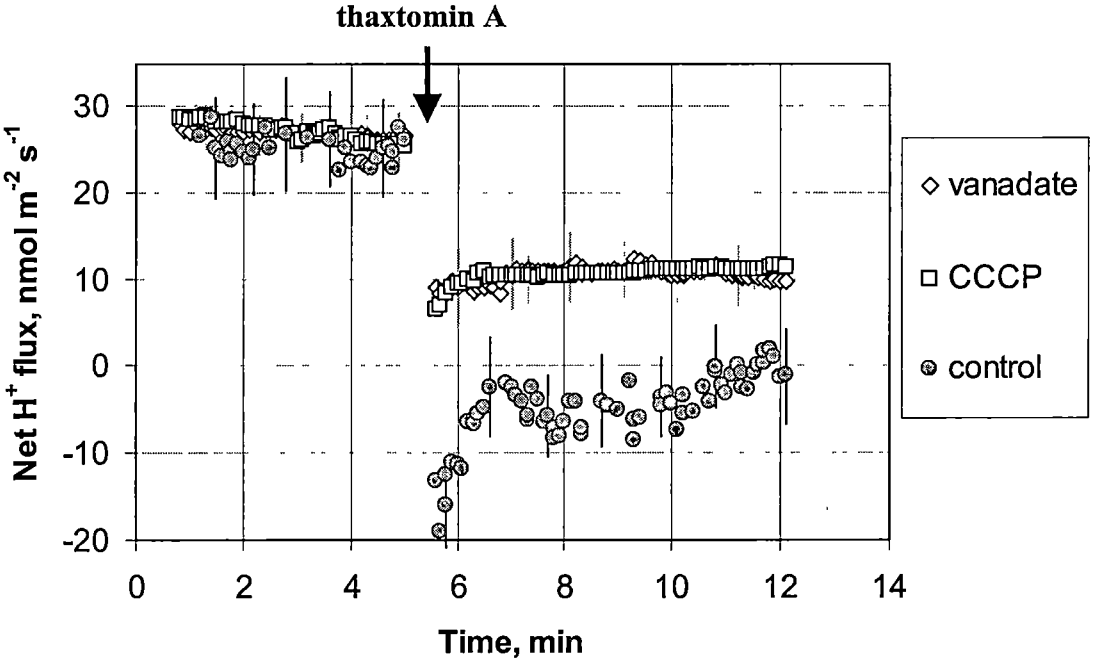


Fig. 2.5. Effect of the H^+ pump inhibitors; orthovanadate – 1mM (open diamonds), CCCP -50μM (open squares), control (closed circles), on thaxtomin A induced efflux. All measurements were taken from the elongation zone of *Arabidopsis thaliana* (WT Columbia) roots. Data are means ± SE (n = 5).

2.3.5 Rapid Ca^{2+} signaling in response to thaxtomin A

The unpredictable H^+ flux transition necessitated the examination of Ca^{2+} flux transients in a pH- buffered solution. This was to prevent H^+/Ca^{2+} exchange in the Donnan system and separate any thaxtomin A-induced changes in Ca^{2+} transport across the plasma membrane from the Ca^{2+} fluxes, originating from the cell wall (Shabala and Newman 2000). Accordingly, net Ca^{2+} flux responses were measured in a pH-buffered (2 mM MES + 4 mM TRIS, pH 5.7) solution. Under these conditions, application of thaxtomin A (final concentration in bath equal to 3 μM) caused an immediate and rapidly diminishing spike of Ca^{2+} influx (Fig. 2.6A). This thaxtomin A-induced Ca^{2+} uptake was fully suppressed by known blockers of Ca^{2+} -permeable channels at the plasma membrane, such as La^{3+} (Fig. 2.6B) and Gd^{3+} (Fig. 2.6C).

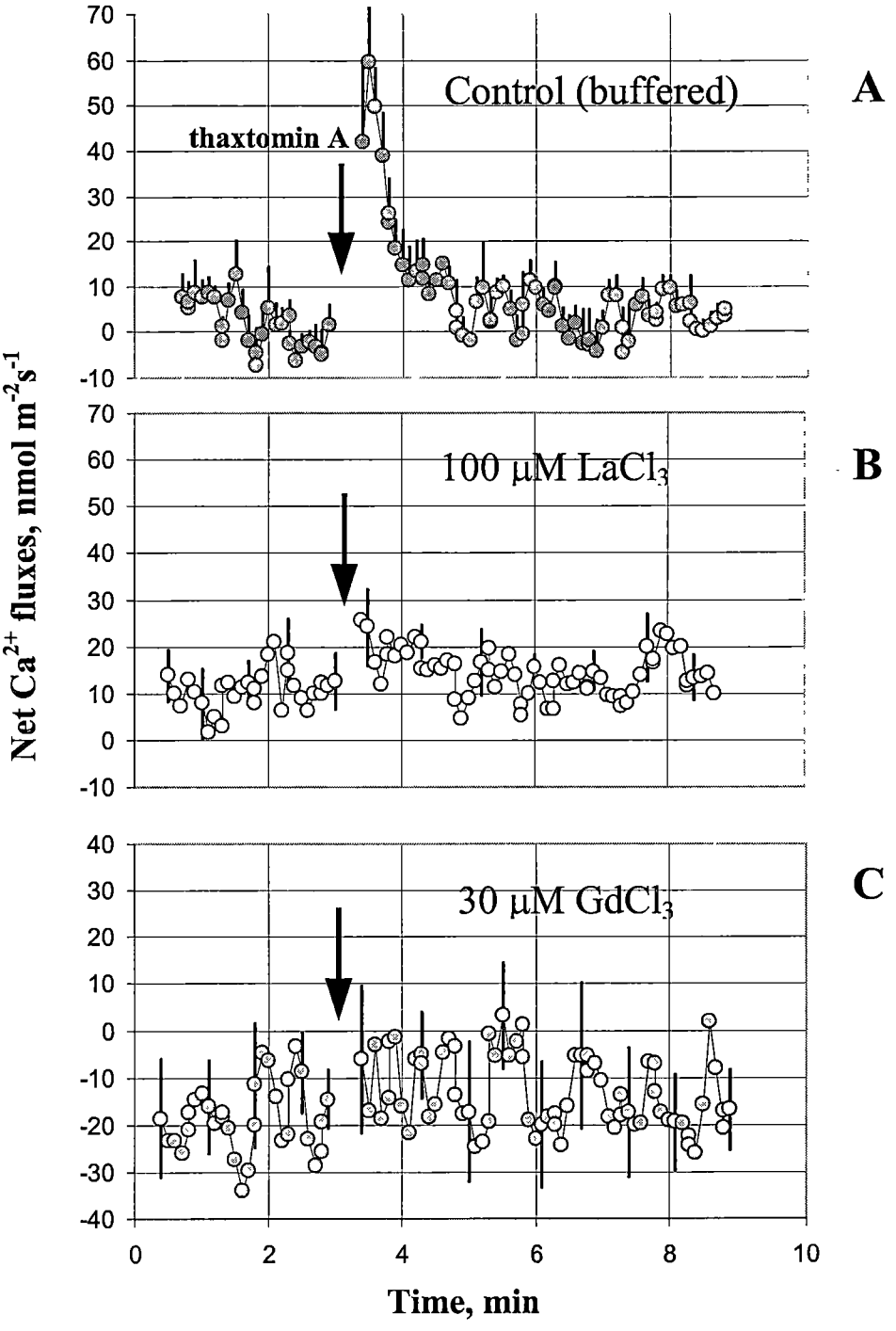


Fig. 2.6. Evidence for rapid Ca^{2+} signaling in response to thaxtomin A. **A** – average net Ca^{2+} flux transients in a H^+ buffered media (MES + TRIS); **B** - effect of the $La3^+$ block (100 μM); **C** – effect of $Gd3^+$ block (30 μM). All measurements were taken from the surface of *Arabidopsis thaliana* (WT Columbia) root in the middle of elongation zone. Data are means ± SE (n = 5).

2.3.6 Effect of thaxtomin A on pollen tube growth and ion fluxes

Tomato pollen tubes are a convenient model system for studying effects of thaxtomin A on the tip-based mechanisms of growth of plant axial organs. Pollen tube growth was reduced with the application of increasing levels of thaxtomin A (Fig. 2.7A), with 50% reduction observed in presence of 0.5 μM thaxtomin A. When net hydrogen fluxes were measured near the tip of growing pollen tube, large oscillating H^+ influx was observed (Fig. 2.7B, from 3 to 6 min). Such ion flux oscillations were present in every growing tube and had a period within 1 to 2 min range (Fig. 2.7B). Application of thaxtomin A caused a significant shift towards net H^+ efflux (Fig. 2.7B) consistent with previous data on roots (Fig. 2.2).

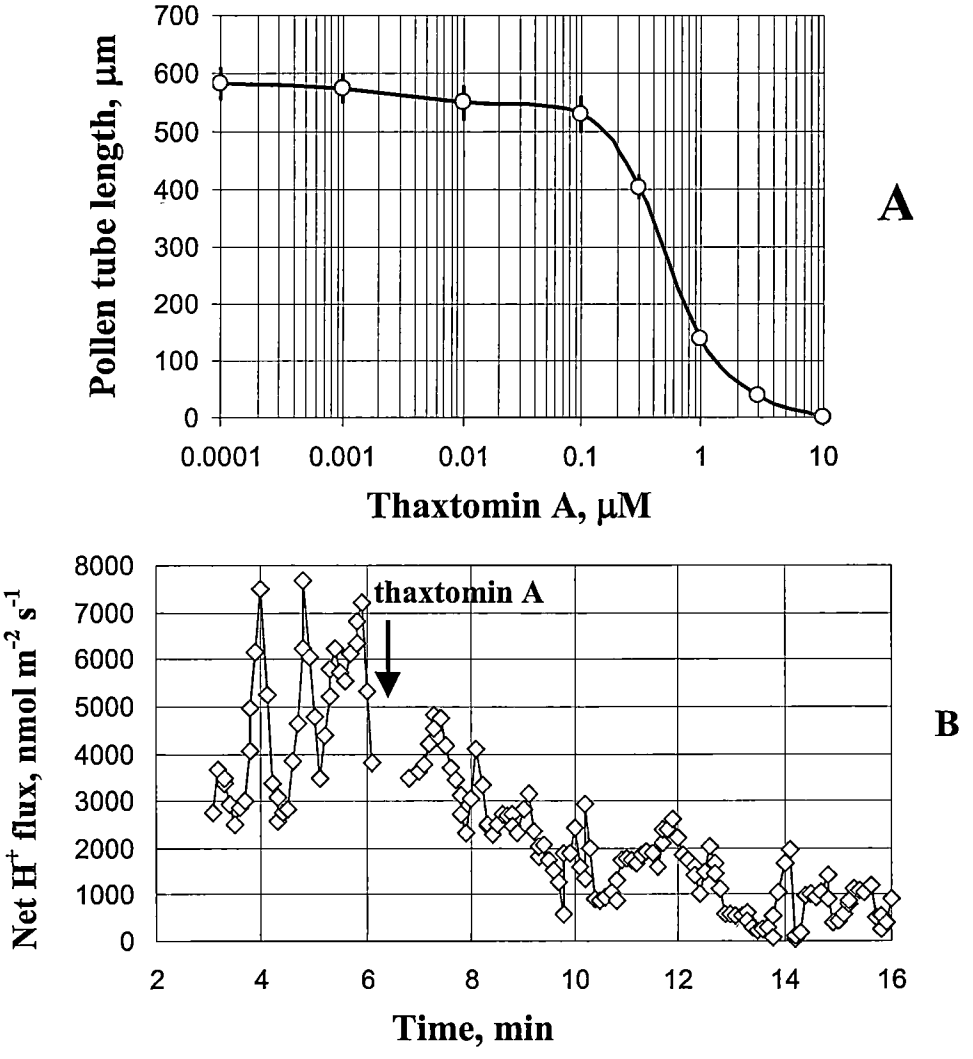


Fig. 2.7. Effect of thaxtomin A on ion fluxes from tomato pollen tube. **A** – dose-response curves showing reduction in tube growth rate as a function of thaxtomin A concentration. Data are mean ± SE (n = 50); **B** - transient H^+ flux responses to thaxtomin A (3 μM) from pollen tip. One (out of 5) typical examples is shown.

As the pollen tube growth is limited to the tip only (Hepler *et al.* 2001), studies of thaxtomin effects on ion flux kinetics, measured from various parts along the growing pollen tube was an efficient way of locating potential thaxtomin A targets in plant cells. Accordingly, net H^+ fluxes were measured from the tip (T) and base (B) regions of growing (G) and non-growing (N-G) pollen tubes (Fig. 2.8). In control (open bars), growing pollen tubes were characterized by polarized ion flux patterns (large net H^+ influx at the tip, and large net H^+ efflux at the base; both significantly different from zero at $P < 0.01$)(Fig. 2.8). In contrast, only small net H^+ efflux was measured from non-growing pollen grains (Fig. 2.8). Application of thaxtomin A had a significant, but inverse effect in both apical and basal part of the pollen cell (dark bars in Fig. 2.8), but only in growing cells. No significant effect of thaxtomin A on non-growing pollen grains was found, although non-zero fluxes were still measured from the imbibed but not growing pollen grains (Fig. 2.8).

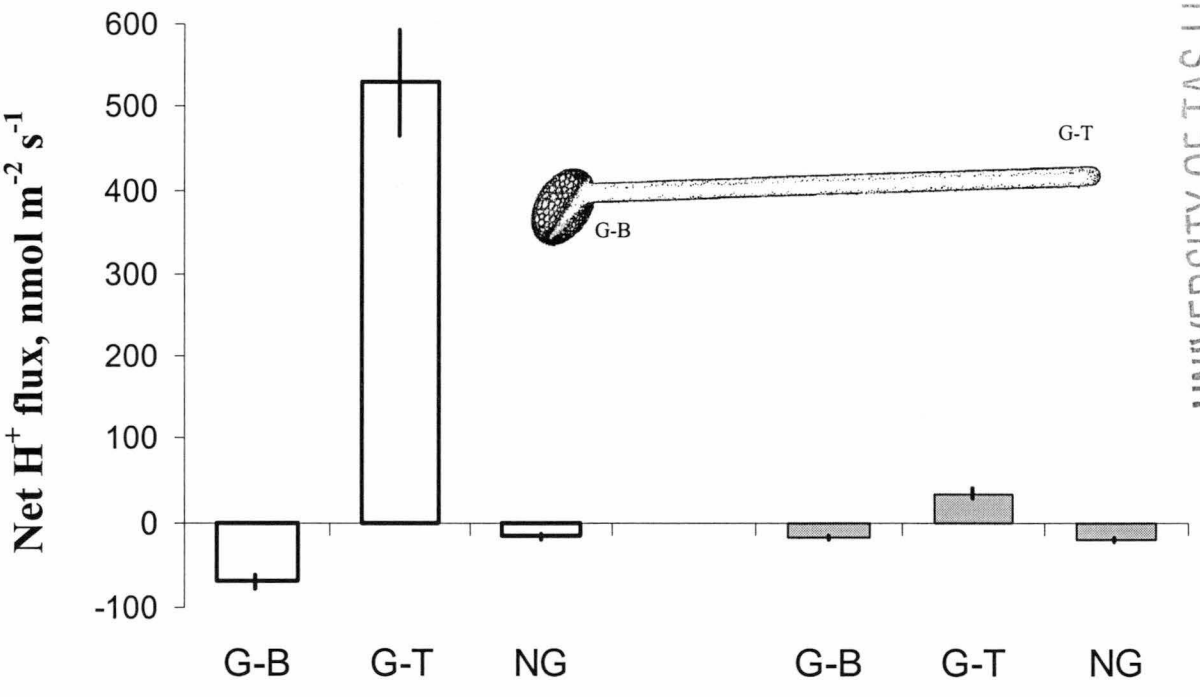


Fig. 2.8. Spatial variation of thaxtomin A effects on ion fluxes measured from pollen cells. Steady state H^+ fluxes from pollen tip (T) and base (B) as affected by incubation with thaxtomin A (3 μM). Open bars – control; closed bars – thaxtomin A treatment. Data is mean ± SE (n = 7 to 12). Legend: G-B = base region of growing pollen tube; G-T = tip region of growing pollen tube, as indicated on schematic pollen tube; NG = base region of non-growing pollen tube.

2.4 Discussion

2.4.1 Influence of thaxtomin A on root morphology

The effect of thaxtomin A on the growth and development of roots in *A. thaliana*, tomato and potato plants was consistent with that previously described (Leiner *et al.* 1996, Loria *et al.* 1997, Fry and Loria 2002, Scheible *et al.* 2003). This information was used to derive effective working concentrations of thaxtomin A (0.2 and 0.5 μM respectively for *A. thaliana* and tomato), for subsequent experiments.

The described significant increase in root hair density suggests a pivotal role of thaxtomin A in root development. Root hair growth is tip-based and is achieved by deposition of new plasma membrane and cell wall material at the expanding tip (Peterson and Farquhar 1996). Regulation of the direction in which the secretory apparatus operates appears to be linked intimately to the cytosolic free Ca^{2+} concentrations in the apex (Gilroy and Jones 2000). This fact provided good justification for further electrophysiological experiments using the MIFE technique, in which Ca^{2+} fluxes were measured in response to thaxtomin A.

2.4.2 Physiological active plant tissue has increased sensitivity to thaxtomin A

The observed enhancement of H^+ extrusion after 24 h incubation in thaxtomin A (Fig. 2.2) suggests that root growth inhibition may be partially mediated by H^+ flux patterns. The greater effect of thaxtomin A on physiologically active plant tissues, such as the elongation zone of both *A. thaliana* and tomato roots (Fig. 2.2) or tips of the growing pollen tubes (Fig. 2.8) are consistent with this idea. These findings are also supported by the K^+ and Ca^{2+} flux data (Fig. 2.3) as following 24hr treatment of roots with thaxtomin A, greater effects were also seen within the elongation zone. The observed enhancement of K^+ extrusion and Ca^{2+} uptake reflects the concentration differences between external bath solution and the cytosolic levels of these ions. This combined with a loss of membrane integrity and ability to compartmentalise ions (caused by thaxtomin A) results in the passive, unregulated movement of the ions. Potassium therefore moves from the cytoplasm (K^+_{cyt} ~ 150mM, Maathuis and Amtmann 1999) into the buffer solution (K^+_{bath} ~0.2 mM) and Ca^{2+} moves from the bath solution (Ca^{2+}_{bath} ~0.1 mM) into the cytoplasm (Ca^{2+}_{cyt} ~ 0.1 μM, Marschner 1995). This reflects a breakdown in control of solute

movement, possibly attributable to the toxin, and was most significantly demonstrated in the elongation zone.

These electrophysiological findings support the morphological studies of Fry and Loria (2002) who found that tobacco cell cultures going through the active expansion phase of cell cycle growth were much more susceptible to thaxtomin A-induced hypertrophy than other phases of the cell cycle. The reported findings are also supported by other electrophysiological data, suggesting that elongation and mature root zones respond differently (both qualitatively and quantitatively) to other stresses such as salinity (Shabala *et al.* 2004), reactive oxygen species (Demidchik *et al.* 2004) and signalling molecules (Ludidi *et al.* 2004), with a higher sensitivity attributed to the elongation zone. Thus, it is logical to suggest that the primary perception site for thaxtomin A action in plants may be within these physiologically active zones.

2.4.3 Electrophysiological aspects of thaxtomin A signalling

As mentioned in the Introduction and in Tegg *et al.* (2005), despite a vast bulk of literature, no direct measurements of Ca^{2+} flux through the plasma membrane in response to phytotoxins or elicitors has been reported *in vivo* at the single cell level. This work was tailored to these intact plant systems as thaxtomin A is proposed to have an end target in the cell wall (Fry and Loria 2002; Scheible *et al.* 2003).

In non-buffered solution, H^+ flux responses to thaxtomin A were variable (Fig. 2.4A) while Ca^{2+} flux was always pushed to net efflux (Fig. 2.4B). This reflects an interaction between H^+ and Ca^{2+} (Donnan effect; Richter and Dainty 1989; Shabala and Newman 2000) whereby thaxtomin A induction of the plasma membrane H^+- ATPase causes acidification of the extracellular space and a release of Ca^{2+} from the cell wall as a result of the shift in chemical equilibrium (Fig. 2.9). This also explains variability of measured thaxtomin A-induced transient H^+ fluxes in non-buffered solution: depending on the "buffering patterns" of the cell wall, some H^+ ions were able to pass through (two lower traces in Fig. 2.4A), or the extruded H^+ was exchanged for Ca^{2+} (top two traces in Fig. 2.4A). This may also explain the failure of detection of an acidification response in prior studies (Fry & Loria 2002).

When experiments were carried out in a pH-buffered solution, it enabled the resolution of the rapid thaxtomin A-induced transient Ca^{2+} spike (Fig. 2.6A) which rapidly decayed (within 1.5-2 min). Notably the thaxtomin A-induced Ca^{2+} influx peak occurs 1 or 2 min earlier than other reported $[Ca^{2+}]_{cyt}$ peaks for a range of plant species in response to various elicitors (Grant *et al.* 2000; Blume *et al.* 2000; Lecourieux *et al.* 2002; Kadota *et al.* 2004). That may indicate that initial and rapid Ca^{2+} influx across the plasma membrane may be required to trigger further Ca^{2+} release from some internal stores (such as ER or vacuole; Sanders *et al.* 1999) via Ca^{2+}-induced Ca^{2+}-release mechanism (Fig. 2.9).

The specific nature of the Ca^{2+}-permeable channel mediating thaxtomin A-induced Ca^{2+} influx measured in these experiments (Fig. 2.6A) remains to be answered. A series of recent publications suggested that several Arabidopsis mutants, such as *dnd* (Clough *et al.* 2000; Jurkowski *et al.* 2004) or *hlm* (Balague *et al.* 2003), that fail to produce the hypersensitive response, are defective in proteins, encoding cyclic nucleotide-gated non-selective ion channels (CNG). Such CNG channels are known to be permeable to all physiologically relevant cations, including Ca^{2+} (Very and Sentenac 2002) and sensitive to Gd^{3+} and La^{3+}. The fact that in these experiments thaxtomin A-induced Ca^{2+} influx was completely inhibited by $La3^{+}$ and $Gd3^{+}$ (Fig 2.6B and 2.6C) suggests CNG may be likely candidates mediating thaxtomin A interaction with the cellular membrane (Fig. 2.9).

From this data, the observed Ca^{2+} influx signature to thaxtomin A application is likely to be the most rapid event, triggering the cascade of events, leading to the plant responses observed, including root growth inhibition and plant death. Specific details of this signalling remain obscure and require further experimentation. At the same time, the observed thaxtomin A-induced activation of H^{+} pump (see 2.4.4) and resultant Donnan exchange between H^{+} and Ca^{2+} in the cell wall might be one of the reasons for increased plant susceptibility to pathogen. Exchange of H^{+} for Ca^{2+} will significantly acidify the cell wall and activate a large number of wall enzymes (primarily, expansins; Cosgrove 2000) involved in the cleavage of molecular links between cell wall components, weakening cell wall structure. This may provide a partial explanation for the observed cell hypertrophy phenotype (Fry & Loria 2002), and reported disruption of cellulose synthesis/deposition (Scheible *et al.* 2003). It

also may explain the ability of the pathogen to successfully penetrate through plant tissue, a rare attribute of bacterial pathogens (Loria *et al.* 2003).

Given the effect of thaxtomin A in mediating Donnan exchange in the cell wall, and the measurement of cell wall acidification, which would be critical for pathogen/toxin efficiency it would be interesting to note whether known phytotoxins or elicitors (whereby a Ca^{2+} signature has been noted) would promote similar phenomena. With little or no other work being reported under *in vivo* conditions this represents another area of future work which would improve our understanding of the plant-toxin interface in physiologically intact systems.

2.4.4 Thaxtomin A activates the plasma membrane proton pump in plant root epidermis

The activation of the energy-requiring plasma membrane proton pump has been reported previously in a few cases when elicitors have been applied to plant tissues (Vera-Estrella *et al.* 1994). The long-term effects of root exposure to thaxtomin A, with observed significant shift towards net H^+ efflux in all the structures and plant types studied (*A. thaliana* roots; Fig. 2.2A), tomato roots (Fig. 2.2B) and pollen tubes (Fig. 2.8) suggests that this H^+ efflux might be mediated by H^+-ATP pump (Fig. 2.9). This was further confirmed in direct pharmacological experiments, when H^+ pump activity was suppressed by orthovanadate and CCCP (Fig. 2.5). As the primary mode of action of both orthovanadate and CCCP is on the plasma membrane H^+-ATPase, these results implicate H^+-pump involvement in observed root responses to thaxtomin A. Thus, this study suggests that plasma membrane H^+-ATP pump is either a primary target of thaxtomin A itself or, more likely, mediates rapid cell responses to thaxtomin A treatment via a Ca^{2+} -mediated pathway (Fig. 2.9).

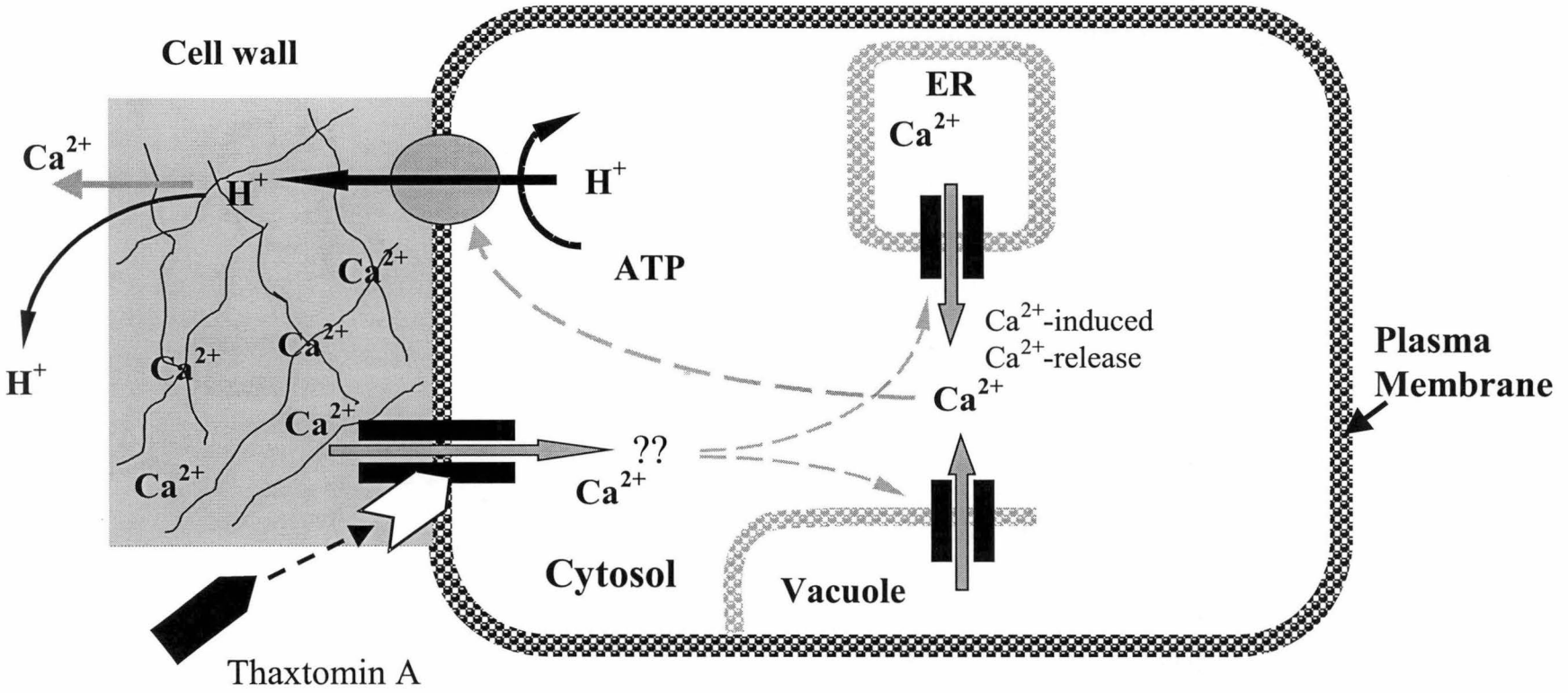


Fig. 2.9. A possible model of thaxtomin A binding and signalling. Thaxtomin A triggers Ca^{2+} influx, leading to an increase in $[Ca^{2+}]_{cyt}$. This process is then amplified by the positive feedback (Ca^{2+} -induced Ca^{2+}-release from internal stores such as the endoplasmic reticulum or a vacuole). Elevated $[Ca^{2+}]_{cyt}$ then enhances activity of the plasma membrane H^+-pump, causing cell wall acidification and displacement of wall-bound Ca^{2+} ions for H^+. The cell wall will be weakened, increasing the ability of the pathogen to penetrate through plant tissue successfully.

2.4.5 Tomato pollen tube: a convenient model system that improves understanding of thaxtomin A action

The pollen tube is a highly specialized cell type that delivers the sperm cell to the ovule for fertilization. Being one of the fastest growing plant cell types (Holdaway-Clarke and Hepler 2003), pollen tubes represent a very convenient system to study mechanisms of cell growth and their regulation by both abiotic and biotic factors.

In this work, pollen tube growth was reduced in a dose-dependent manner by increasing thaxtomin A concentrations (Fig 2.7A), demonstrating that not only vegetative (Fig. 2.1) but also reproductive structures are affected by toxin. This is consistent with other studies showing that thaxtomin A toxicity is observed across all vegetative and reproductive structures studied (Fry and Loria 2002; Scheible *et al.* 2003).

It is well known that both intracellular and extracellular profiles of Ca^{2+} and H^+ are crucial for pollen tube growth (Hepler 1997; Feijo *et al.* 1999; Hepler *et al.* 2001; Holdaway-Clarke and Hepler 2003). Because both a tip-based Ca^{2+} gradient (Pierson *et al.* 1994; Malhó *et al.* 1995) and the plasma membrane H^+ pump (Briskin and Hanson 1992) are implicated in tube growth, the pollen tube represents an ideal model system for studying thaxtomin A (toxin) action.

In the growing pollen tube, there is a pronounced pH gradient between apical (acidic domain) and basal (constitutive alkaline) parts of the cell (Feijo *et al.* 1999; Holdaway-Clarke and Hepler 2003). When vibrating ion-selective microelectrodes were used in lily (Feijo *et al.* 1999), the extracellular H^+ fluxes measured from growing pollen tubes almost perfectly matched the cytoplasmic H^+ distribution, with a large H^+ influx in the tip, and marked H^+ efflux from the basal part. The results on tomato pollen tubes are consistent with these observations (Figs 2.7 and 2.8).

The logical explanation for the observed polar H^+ fluxes in growing pollen tubes is that there is a significant difference in distribution of key H^+ transporters between cell apex and base, with higher density of H^+-ATP pumps in the base region, and larger number of non-selective cation channels (NSCC; presumably H^+

permeable), in the cell apex (Holdaway-Clarke and Hepler 2003). The H^+-ATPase is the primary electrogenic pump responsible for generating proton gradients, with evidence indicating that ATPases (or their activity) are intrinsically polarized (Feijó *et al.* 1999). They are richly expressed in the plasma membrane of the pollen grain, but with the possible exception of the tip domain, only weakly, if at all, over the pollen tube itself (Weisenseel and Jaffe 1976; Feijó *et al.* 1999). Both inward and outward H^+ fluxes were affected by thaxtomin A treatment in these experiments (Fig. 2.8). However, effect on H^+ flux from the root apex was much more pronounced, indicating that other transporters (presumably, those for Ca^{2+}) were affected. The tip-based Ca^{2+} gradients are considered to be the major driving force behind the pollen tube growth and originate from Ca^{2+} influx across the plasma membrane in the cell apical part through high-density Ca^{2+} channels (Malhó *et al.* 1995). These channels become closed in non-growing tubes, leaving no Ca^{2+} gradient (Pierson *et al.* 1994; Malhó *et al.* 1995). The absence of any effect of thaxtomin A on H^+ flux patterns from non-growing pollen cells (Fig. 2.8) is consistent with these observations. Together with other data, this suggests that, rather than having a direct impact on H^+ pump activity, thaxtomin A effects on H^+ fluxes in pollen cells are mediated by intracellular Ca^{2+} changes (Fig. 2.9). This is consistent with the root data. Therefore, it is suggested that the thaxtomin A binding site is associated with a plasma membrane Ca^{2+} channel (Fig. 2.9).

Overall, the data shows that the effect of thaxtomin A on pollen tube growth may be mediated by regulation of both Ca^{2+} channels and H^+ pump activity (albeit via different mechanisms). Also, the experiments on pollen localise thaxtomin A perception to the tube tip. The usefulness of the pollen tube for studying thaxtomin A action is not surprising. In tomato, pollen tube growth in the presence of the toxins from *Stemphylium solani* and *Alternaria solani* were used to detect resistance to these pathogens (Marquez Bell 2001; Melian and Balashova 1994). Indeed, pollen selection techniques proved to be an efficient breeding and selection tool for detection of toxin tolerance in tomato (Melian and Balashova 1994).

2.4.6 Conclusions

The use of non-invasive ion flux measuring technique allowed the characterisation of specific ion responses to thaxtomin A in plant tissues. The evidence presented suggests that thaxtomin A triggers an early signalling cascade, causing rapid and tissue-specific changes in net Ca^{2+} and H^+ ion flux profiles, which may be crucial in plant-pathogen interactions. In a non-buffered system, the production of the Ca^{2+} flux signature and proton pump activation was masked by a Donnan exchange in the cell wall. The observance of cell wall acidification and therefore increased pathogen susceptibility was also of note. Thaxtomin A was more effective in young, physiologically active tissues, suggesting higher density of thaxtomin A-binding sites in these regions. The pollen tube and H^+ ion flux patterns measured from it were an ideal model system for studying thaxtomin A action.

Chapter 3. Common Scab-Auxin Glasshouse Trials

3.1 Introduction

Common scab, caused by infection of developing tubers with *Streptomyces scabiei* (Thaxt.) Lambert and Loria, is an economically important disease of potatoes with no current commercially acceptable control strategy. Whilst traditional breeding programs endeavour to enhance disease resistance, alternative cost-effective strategies that can be developed to control or reduce the impact of common scab on the Australian potato industry would be desirable.

In the 1980s McIntosh *et al.* (1981, 1982, 1985, 1988) demonstrated that foliar application of auxin or certain auxin analogues on potatoes significantly reduced occurrence of common scab disease. Auxin is a key plant hormone (Leyser 2002) essential for cell elongation and differentiation, amongst an array of other essential functions (Swarup *et al.* 2002; see Chapter 4). Glasshouse trials showed that foliar sprays such as the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 3,5-dichlorophenoxyacetic acid (3,5-D) reduced scab by 50 and 90% respectively, when sprayed at or just before tuber initiation (McIntosh *et al.* 1981, 1985). However, associated disadvantages of using 3,5-D and 2,4-D included decreased yields, increased numbers of small tubers and an increased proportion of deformed tubers (McIntosh *et al.* 1981). Field trials with 3,5-D resulted in scab reduction of only 30% and significant decreases in yield and mean tuber weight precluding the commercial use of these chemicals for disease control (McIntosh *et al.* 1982). Benzoic acids, including 5-chloro-2-nitrobenzoic acid (CNB) were later identified as also possessing disease inhibiting activity, and were more attractive, because in contrast to the phenoxyacetic acids, their impact on yield, tuber number and distortion was less severe (McIntosh *et al.* 1988).

Chemical induced disease resistance has also been demonstrated in a range of different plant-pathogen systems but of particular interest are the phenomena of herbicide-induced disease resistance and/or indole (including auxins) related compounds that induce resistance, noting that 2,4-D and 3,5-D have both auxinic and herbicidal action. Studies undertaken so far have identified that the chemicals

(herbicides or indole compounds), at the concentrations used to induce resistance, have no apparent toxicity to the disease-causing organisms, suggesting that protection results from activation of indirect mechanisms (Grinstein *et al.* 1984; Cohen *et al.* 1986, 1987, 1996; Bolter *et al.* 1993; Starratt and Lazarovits 1996, 1999; Ueno *et al.* 2004). These mechanisms include physiological and anatomical changes (Cohen *et al.* 1996), but in many cases mechanisms are unknown and are classified as unidentified plant defence mechanisms (Starratt and Lazarovits 1999).

Within herbicide-induced disease resistance, mechanisms postulated include changes in hormone balance, particularly giberellic acid biosynthesis inhibition (Cohen *et al.* 1987) and suppression of ethylene production (Cohen *et al.* 1986); increases in free amino acid levels (Bolter *et al.* 1993; Starratt and Lazarovits 1996, 1999); increases in sugar content (Cohen *et al.* 1996); and stimulation of phytoalexin production (Grinstein *et al.* 1984), all indirect mechanisms.

Indole related compounds that have been recently reported to induce disease resistance include indole-3-acetic-acid (IAA), tryptamine and tryptophan (Ueno *et al.* 2004), key compounds within the IAA biosynthesis pathway. These indole compounds were identified as plant activators, as they increased phenylalanine ammonia-lyase, peroxidase and chitinase activities, whilst also promoting H_2O_2 generation (Ueno *et al.* 2004), all key plant defence mechanisms (Chamnongpol *et al.* 1998; Ueno *et al.* 2004).

Consistent with the aforementioned studies on herbicide and indole compounds, McIntosh *et al.* (1981, 1988) demonstrated through *in vitro* trials that the auxin sources, at the rates applied, neither directly inhibit nor severely disrupt growth of the pathogen, *S. scabiei*. McIntosh *et al.* (1981, 1988) suggested that the protective effect induced by these auxin sprays was due to their ability to modify the response of the host to the infection, so that scab symptoms did not develop. Whilst the mechanism of disease suppression was not investigated, McIntosh's work provides clues to the success of auxin-based sprays as they were only found to be effective at key physiological stages, 2 – 6 weeks after tuber initiation. Rapid tuber expansion, and morphological changes in the putative pathogen entry sites, (stomata transformation into lenticels), occur at this time (Adams 1975a).

Lenticels are natural openings in the tuber periderm used for gas exchange (Wigginton 1973) with immature lenticels representing the key entry sites into tubers for *S. scabiei* (Lapwood and Adams 1973, 1975). However there is also evidence that both *S. ipomoeae* and *S. scabiei* hyphae can also directly penetrate the tuber periderm and infect potato tissue (Clark and Matthews 1987; Loria *et al.* 2003). In potatoes, cultivars resistant to various diseases have been characterised as having low lenticel density, more cell layers in the periderm and intensive cuticularization in lenticel tissues (Weber and Bartel 1986; Zhang *et al.* 1991; Mahajan *et al.* 2004). Whether auxin affects the morphological development of lenticels or periderm, during early tuber growth (the phase of susceptibility to infection) (Adams 1975a; Adams and Lapwood 1978), may help to determine how auxin sprays confer a protective effect against common scab disease. Furthermore, from our studies it is known that auxin sources inhibit plant cell toxicity of thaxtomin A, the phytotoxin produced by pathogenic *Streptomyces sp.* (Tegg *et al.* 2004b; Chapter 4).

This work aims to confirm the results of McIntosh by demonstrating that auxin foliar sprays reduce common scab disease in 'Russet Burbank', a commercially important cultivar used in the potato processing industry in Tasmania. In addition, possible mechanisms of activity were investigated by examining the impact of 2,4-D on lenticel development, gross periderm structure and tuber sensitivity to thaxtomin A.

3.2 Materials and methods

The effects of foliar auxin sprays on tuber and scab development were studied in four pot trials. Sprays tested included the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) and the synthetic weakly auxinic 5-chloro-2-nitrobenzoic acid (CNB), sprayed once (s), twice (b) or three (m) times during the period of rapid tuber expansion. These sprays were chosen based on promising results previously obtained (McIntosh *et al.* 1981, 1985, 1988), and the novel interest in the modes of action of these compounds. Further details of auxin sprays, formulations, application times, general media conditions and assessments undertaken are given in Table 3.1.

3.2.1 Pathogen culture and plant establishment

The pathogen *Streptomyces scabiei* (strain G#32), a highly pathogenic and high thaxtomin producing strain, initially isolated and purified in 1990 from a common scab diseased tuber from the NW-coast of Tasmania was cultured and maintained on ISP2 agar slopes (Appendix 1) at 30°C in the dark. Inoculum was prepared with spores from a two-week-old slope culture of *S. scabiei* which was then suspended in 5 ml of sterile water, added to a sterilized mixture of 100 g vermiculite and 500 ml SAY solution (Appendix 1) and incubated at 24°C in the dark for 14 days. The observance of grey powdery coating (*S. scabiei* spores) over the vermiculite mixture was indicative of good colonization of the substrate and therefore a suitable inoculum mix ready for use.

Pots (25 cm diameter) were filled with a mixture of sand, peat and pine bark (10:10: 80; pH 6.0) premixed with Osmocote 16-3.5-10 NPK resin coated fertiliser at the rate of 6 kg/m^3. A soluble fertiliser was applied monthly (Miracle-Gro® Water Soluble all purpose plant food, 15-13.1-12.4 NPK, Scotts Australia Pty Ltd.). In treatments where the pathogen was included, a 3cm deep, and 15cm wide, band (approx. 10 g) of inoculum was incorporated in the surface and covered by an additional 5 cm of potting mix. Two-week-old tissue culture plants of 'Russet Burbank' (2.2.2) were transplanted into these pots and hand-watered for the first week. For the remainder of the experiment pots were watered by an automatic overhead watering system every 2nd day with 5 ml delivered to each pot at each separate irrigation. This regime allowed soil to dry substantially between waterings.

Additional plants were grown alongside the trial to monitor tuber initiation. All trials were undertaken within a glasshouse environment with daily temperatures maintained at 22 ±2°C.

3.2.2 Experimental design and key treatment dates

Treatments and experimental design within each trial were tailored and altered year-to-year based on the outcomes of the previous trials.

Trial 1: Five separate foliar spray treatments; control, 2,4-D (s), 2,4-D (m), CNB (s) and CNB (m) (Table 3.1) were applied to Russet Burbank plants and all pots were amended with inoculum of the pathogen, *S. scabiei*. The treatments were replicated (n=6) in a randomized block design and there were 30 pots (plants) total in this trial.

The first foliar spray was applied on 7th April 2003 and tubers were harvested 8 wks later and assessed for common scab disease.

Trial 2: Two separate foliar spray treatments, control and 2,4-D (m), were applied to Russet Burbank plants. Fifty pots were amended with *S. scabiei* inoculum, and a further fifty were unamended. Five sequential assessments (see 3.2.4 and 3.2.6) were made throughout the trial. The treatments were replicated (n=5) in a randomized split-split plot design and there were 100 pots (plants) total in this trial.

The first foliar spray was applied on 5th April 2004 and tubers were harvested 8 wks later and assessed for common scab disease.

Trial 3: Two separate foliar spray treatments, control and 2,4-D (m), were applied to Russet Burbank plants. No pots received pathogen inoculum. There were five sequential harvest assessments (see 3.2.4 and 3.2.6) made throughout the trial. The treatments were replicated (n=4) in a randomized split plot design and there were 40 pots (plants) total in this trial.

The first foliar spray was applied on 10th March 2005.

Trial 4: Two separate foliar spray treatments, control and 2,4-D (m), were applied to Russet Burbank plants. No pots received pathogen inoculum. There was one harvest assessment (see 3.2.4 and 3.2.6) made. The treatments were replicated (n=5) in a randomized block design and there were 10 pots (plants) total in this trial.

The first foliar spray was made on 10th May 2005.

3.2.3 Spray treatments

Spray treatments were timed to coincide with the critical infection period, which occurs 2-6 wks after tuber initiation (Lapwood and Adams 1973, 1975). This was determined by monitoring the additional plants for tuber initiation. The single spray was made 14 d after tuber initiation while multiple spray treatments were made at 14, 24 and 34 d after tuber initiation. Spray treatments were applied to leaves until run-off with a hand held sprayer that delivered a fine mist. Absorbent cotton towels were placed on the soil surface during spraying, to ensure soil was protected from direct spray contact, and towels were removed immediately after treatment.

Table 3.1. Treatments and key assessment criteria for pot trials assessing the impact of auxin foliar sprays on common scab and tuber development. (s) = 1 single spray, (d) = 2 sequential sprays, (m) = 3 sequential sprays.

Trial (Year)	Auxin foliar sprays	Formulation of single spray (all sprays contained 0.2% ethanol + 0.5 g/L Tween 80 and were made to 1L volume with distilled water)	Treatment time (No. days after tuber initiation)	Potting media		Disease assessment at harvest	Tuber physiological assessment^
				Pathogen amended	unamended		
Trial 1 (2003)	Control		14				
	CNB (s),	5-chloro-2-nitrobenzoic acid (0.32 g/L)	14				
	CNB (m),	5-chloro-2-nitrobenzoic acid (0.32 g/L)	14, 24 and 34				
	2,4-D (s),	2,4-dichlorophenoxyacetic acid (0.20 g/L)	14	✓		✓	
	2,4-D (m)	2,4-dichlorophenoxyacetic acid (0.20 g/L)	14, 24 and 34				
Trial 2 (2004)	Control		14				
	2,4-D (m)	2,4-dichlorophenoxyacetic acid (0.20 g/L)	14, 24 and 34	✓	✓	✓	✓
Trial 3 (2005)	Control		14				
	2,4-D (m)	2,4-dichlorophenoxyacetic acid (0.20 g/L)	14, 24 and 34		✓		✓
Trial 4 (2005)	Control		14				
	2,4-D (d)	2,4-dichlorophenoxyacetic acid (0.20 g/L)	14 and 24		✓		✓

^ Tuber physiological assessment involved the partial (trial 2 – see 3.2.4) or complete harvest (trials 3 and 4 – see 3.2.4) of tubers from pots at various tuber development stages.

3.2.4 Tuber harvests

In pot trial 1, tubers were harvested at senescence and scored for disease and agronomic performance (3.2.5).

In pot trial 2, two typical tubers were sequentially harvested from each pot immediately prior to the first foliar spray, and then at 1, 2 and 4 wks after the first spray, and at harvest. Harvesting was undertaken with care so as not to damage other tubers, the two tubers were placed in 70% ethanol and stored at 4°C, before being prepared for sectioning and microscopic examination (3.2.6.1). The pot was returned to the trial and grown through to harvest for scoring of disease and agronomic performance (3.2.5).

In pot trial 3, all tubers from selected pots were sequentially harvested immediately prior to the first auxin foliar spray (control), and then at 1, 2, 3 and 4 wks after the initial spray treatment. Typical tubers were kept for physiological assessment, which included measurement of lenticel numbers and external dimensions (3.2.6.2) and measurement of tuber slice sensitivity to thaxtomin A (3.2.6.3).

In pot trial 4, all tubers were harvested at 2 wks after the initial spray treatment. Typical tubers were kept for physiological assessment which included measurement of tuber slice sensitivity to thaxtomin A (3.2.6.3) and quantification of 2,4-D levels in tubers (3.2.6.4).

3.2.5 Disease Assessment

After plants had fully senesced (completely died back), tubers were harvested, rinsed gently under running cold tap water and allowed to air dry. The number of tubers per pot and individual weight of each tuber was recorded. Each tuber weighing more than 2.0 g was assessed for disease. The percentage of scab on the surface of each tuber was estimated using the method of Richardson and Heeg (1954). Scab lesion severity was assessed using the modified rating scale of Bjor and Roer (1980) whereby the most severe lesion on an individual tuber was rated as:

0 = no disease

1 = superficial lesions < 1 mm deep

2 = typical star cracked lesions of 1-3 mm deep

3 = deep pitted lesions > 3 mm deep

3.2.6 Tuber physiological assessments

3.2.6.1 Microscopic examination of periderm and lenticular development

Tissue blocks approximately 50 mm^3 were cut from selected tubers and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (Sörensen 1909) under vacuum for 24 hr at ambient temperature. Following two buffer washes (each for 20 min), the samples were dehydrated in an ascending acetone series in 20% increments and taken to 3 changes of 100% acetone (each for 30 min), finishing with two changes (each of 20 min) of propylene oxide. The leaf blocks were slowly infiltrated with Spurr's resin of medium hardness (Spurr 1969) and cured for 24 hr at 70°C.

Polymerised blocks were hand-trimmed with a razor blade and semi-thick sections (4-5 µm thick) were cut with a glass knife fitted to a Reichert Om U2 ultramicrotome (Vienna, Austria). The sections were transferred to a drop of distilled water on a clean glass microscope slide and gently heat-fixed to the glass. The slides were then immersed in 1% (w/v) Toluidine Blue O in 1% (w/v) sodium borate solution for 30 s, rinsed in distilled water, decolourised in 70% ethanol for 30 s, rinsed again in distilled water and air dried. The sections were mounted in Euparal (Australian Entomological Supplies, NSW, Australia) beneath a coverslip and cured on a cool hotplate. Sections were examined with a Leica DMLB (Type LB 30T) compound microscope (Leica Microsystems Wetzlar GmbH, Germany) and photographs of sections were taken with a Nikon E995 Coolpix (Nikon Corp., Japan) fitted with a microscopic adaptor.

3.2.6.2 Lenticel number and dimensions

Tubers from each pot that met weight and size specifications (Table 3.3) were examined both by eye and using a Leica MZ12 dissecting microscope (Leica Microsystems Wetzlar GmbH, Germany) at 20 X magnification. Three tubers from each pot were assessed and the number of lenticels visible counted. Under magnification, the external dimensions of lenticels were measured from 2 tubers per pot. The maximum length (L) and width (W) of 15 individual randomly selected lenticels per tuber were recorded, 5 from the stem or stolon end, 5 from the mid-section and 5 from the bud or rose end (Fig. 3.1).

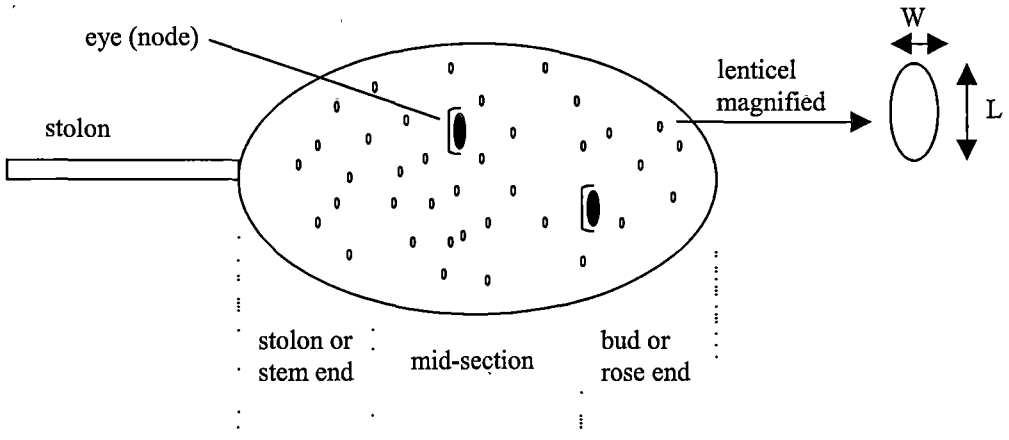


Fig. 3.1. Schematic of external surface of potato tuber showing key sections and features used for selection and measurement of lenticels.

3.2.6.3 Sensitivity of tubers to thaxtomin A using tuber slice assay

At each sequential assessment three tubers within the appropriate size specifications (Table 3.3) were selected from each pot and tested for thaxtomin A sensitivity using a modification of the method described by Acuña *et al.* (2001). Whole tubers were surface-sterilized with 0.5% sodium hypochlorite for 10 min and air-dried before being cut into 0.5 cm thick-slices and placed in 90 mm petri dishes with moist sterile filter paper (Whatman # 1, Whatman Int., Maidstone, UK). Filter paper disks of 6 mm diameter (Whatman # 1) were immersed in thaxtomin A solution (57 µM) for 1 hour, air-dried and placed on the potato slices (1-3 disks per slice) with disks immersed in 5% acetone solution used as controls. After placement

on the potato slices, 10 µL of sterile distilled water were applied to each disc. Plates were incubated at 24°C in the dark and evaluated after 7 d for necrosis in the defined area under the filter paper disk: 0= no necrosis, 1= few brown flecks, 2= brown flecks in determined necrotic area, 3= brown necrosis and 4= brown to black necrosis.

3.2.6.4 Extraction and quantification of 2,4-D levels from potato tubers

Methods to extract and quantify 2,4-D from tubers were developed based on a modification of the methods used for IAA analyses from various plant tissues (Ross *et al.* 1995). Harvested tubers were cut into 1 cm cubes and 10 g of tuber tissue immediately immersed in cold (-20°C) 80% methanol, containing butylated hydroxytoluene (Sigma, 250 mg/L). Samples were stored for 1-2 wks at -20°C. Samples were then homogenized using a barmix blender, the homogenate was left overnight at 4°C. After 20 hours insoluble material was removed by filtration (Whatman no. 1, Whatman, Clifton, NJ). Based on preliminary trial extractions, 90 ng of 2,4-D internal standard ([$^{13}C_6$] 2,4-D, 100 ng/µl in Acetone, Catalog no. XA11940 200AC., Dr. Ehrenstorfer Laboratories, Augsburg, Germany) was then added to an aliquot (control – 8 ml; 2,4-D sprayed – 2 ml) of the filtrate, and left overnight at 4°C.

Extracts were then dried using a rotary evaporator. A VacRC C18 Sep-Pak (Waters SA, Guyancourt, France) was pre-conditioned with 15 mL 100% methanol followed by 15 mL 0.4% acetic acid in dionised water. The dried samples were transferred in 2 mL wash of 1% acetic acid, and 2 x 2 mL washes of 0.4% acetic acid to the VacRC. 2,4-D was eluted from the sep-pak using 15 mL methanol in 0.4% acetic acid, directly into a small round bottom flask. This was reduced to dryness in a rotary evaporator. The samples were then transferred in 2 x 1 mL washes of 100% methanol into a small vial and dried under N_2; 400 mL of 100% methanol and 1.5 mL of diazomethane was added and again dried under N_2. One mL of distilled water was added to the samples, followed by 3 x 400 uL aliquots of ethyl ester, with the top layer (ether partition) transferred to a collection vial after each addition. This was dried under N_2 and 40 µL chloroform was added. The vial was then stored in

aluminium foil at room temperature (overnight) before gas chromatography-mass spectrometry (GC-MS) analysis.

Levels of 2,4-D were analysed by GC-MS-selected ion monitoring with internal standards (Ross 1998). The GC-MS system consisted of a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970B mass selective detector. A 25x0.3 mm diameter HP1 (0.17 μm film) fused silica GC column was used, with the oven temperature programmed from 60°C to 150°C at 30°C min^{-1} and then at 10°C min^{-1}, with a column head pressure of 15 p.s.i. (Ross 1998). Calculations of 2,4-D levels were performed as described by Lawrence *et al.* (1992).

3.2.7 Data analysis

Data were subjected to analysis of variance using Genstat 6 (Rothamsted Experimental Station, Harpenden, Herfordshire, UK). Significance was calculated at either $P = 0.05$ or $P = 0.01$ as noted, and least significant difference (LSD) was used for comparison of treatment means. Where data had unequal variance (3.3.2.4), natural log transformation was used to compare the data. Data are presented as mean values for each treatment combination.

3.3 Results

3.3.1 Disease and agronomic performance

In pot trial 1, untreated control tubers had obvious substantial common scab disease symptoms while those treated with 2,4-D showed negligible disease symptoms (Fig. 3.2). Common scab surface coverage (%) of 2,4-D treated plants was significantly reduced ($P<0.05$) (Table 3.2). Whilst common scab disease severity was reduced following 2,4-D treatment, the reduction was not significant ($P>0.05$) from the control. The impact of the sprays on mean potato weight and the number of potatoes were not significant although there was a trend suggesting that 2,4-D may have decreased potato weights. The effect of CNB (s) and (m) was similar to the control (Table 3.2).

In pot trial 2 a similar effect of 2,4-D treatment was shown (Table 3.2). Common scab surface percentage and common scab lesion severity were significantly reduced ($P<0.05$) with the application of 2,4-D (m), compared to the control (Table 3.2). Whilst the number of tubers per plant and mean weight of individual tubers were not affected significantly by treatment, the total weight of potatoes per plant was reduced significantly ($P<0.05$) with the application of 2,4-D (m).

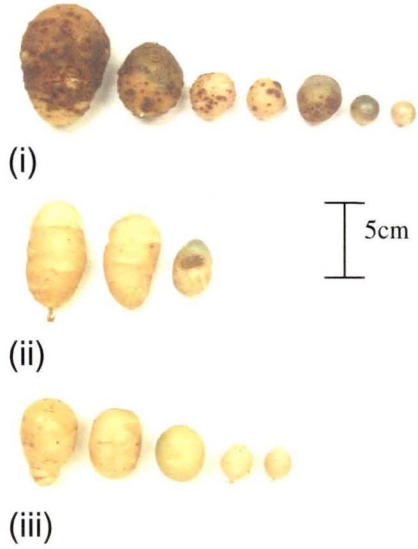


Fig 3.2. Russet Burbank grown in soil inoculated with *S. scabiei* isolate G#32 and treated with 2,4-D foliar sprays. Spray treatments included (i) control, (ii) 2,4-D single (s) spray and (iii) 2,4-D multiple-3 (m) sequential sprays. Initial sprays were made at 14 d after tuber initiation, and subsequent sprays made at 10 d intervals.

Table 3.2. Impact of 5-chloro-2-nitrobenzoic acid (CNB) and 2,4-dichlorophenoxyacetic acid (2,4-D) foliar sprays on common scab disease and tuber properties of Russet Burbank grown in soil inoculated with *S. scabiei* isolate G#32. Spray treatments included single (s) or multiple-3 (m) sequential sprays, with initial sprays made at 14 d after tuber initiation, and subsequent sprays made at 10 d intervals.

Treatment	Scab surface %	Scab severity^ (0-3)	No. of tubers/plant	Total weight of tuber/plant (g)	Mean tuber weight (g)
		Pot trial 1			
Control	30.0 a	1.26 ab	3.8	24.9 ab	8.8
CNB (s)	40.5 a	1.82 a	3.2	32.6 a	11.1
CNB (m)	27.8 a	1.18 ab	2.8	17.1 ab	6.0
2,4-D (s)	3.8 b	0.81 b	2.7	13.0 b	4.9
2,4-D (m)	2.2 b	0.50 b	4.0	14.9 b	5.1
LSD (P=0.05)	23.1	0.83	1.9	17.04	7.1
		Pot trial 2			
Control	24.1 a	2.15 a	9.64	190.7 a	22.55
2,4-D (m)	5.6 b	0.93 b	8.48	164.5 b	19.55
LSD (P=0.05)	4.5	0.27	1.4	21.9	3.2

Means followed by same letter within the same column are not significantly different at the 0.05 probability level using Fisher's LSD test.

^ Scab lesion severity was assessed using the scale: 0 = no disease, 1 = superficial lesions < 1 mm deep, 2 = typical star cracked lesions of 1-3 mm deep, 3 = deep pitted lesions > 3 mm deep.

Whilst the aim of pot trial 3 was to examine the impact of 2,4-D foliar sprays on tuber development in unamended media, some pathogen amended pots were run alongside this trial. They confirmed the findings of trials 1 and 2, that foliar 2,4-D application suppressed common scab disease development (data not presented).

3.3.2 Tuber physiological development

In pot trials 2 and 3 tubers were harvested for assessment sequentially from a period of 2 weeks after tuber initiation through to 6 weeks after tuber initiation, corresponding with the phase of rapid tuber development. These tubers were then subjected to further measurements and tests (see 3.2.5 and 3.2.6). Table 3.3 provides a guide to the size and dimension ranges of typical tubers assessed in pot trials 2 and 3.

Table 3.3. Weight and size ranges of tubers used in sequential assessments for pot trials 2 and 3.

Weeks after 1st spray	Weeks after tuber initiation	Weight range (g)	Length dimensions (mm)	Width dimensions (mm)
0	2	7.5 ± 2.5	30 ± 4.0	22 ± 3.0
1	3	12.5 ± 7.5	34 ± 8.0	25 ± 5.0
2	4	22.5 ± 7.5	42 ± 7.0	32 ± 5.0
3	5	32.5 ± 7.5	53 ± 7.0	37 ± 3.0
4	6	42.5 ± 7.5	57 ± 7.0	40 ± 3.0

3.3.2.1 Periderm and lenticel observations

In pot trial 2 microscopic examination of tuber samples showed no visually discernible differences in periderm and lenticular morphology between control tubers and those tubers whose foliage had been sprayed with 2,4-D. In both cases, early tuber development (tubers of 1-3 weeks of age) was characterised by a thin periderm layer (Fig 3.3, plates 1a,b) with layer numbers increasing with age. Lenticular development was generally not seen in tuber tissue of 1-2 weeks of age, by 3 weeks of age lenticular activity was sometimes evident. As the tuber aged from 4-6 weeks, higher cell density characteristic of meristematic activity and lenticular development was observed frequently within the phellogen (Fig 3.3, plate 2). Also, predominantly in 4-6 week old tubers, regions of torn outer periderm were observed as the inner rapid meristematic tissue fractured the outer periderm, a characteristic typical of lenticel formation (Fig 3.3, plate 3).

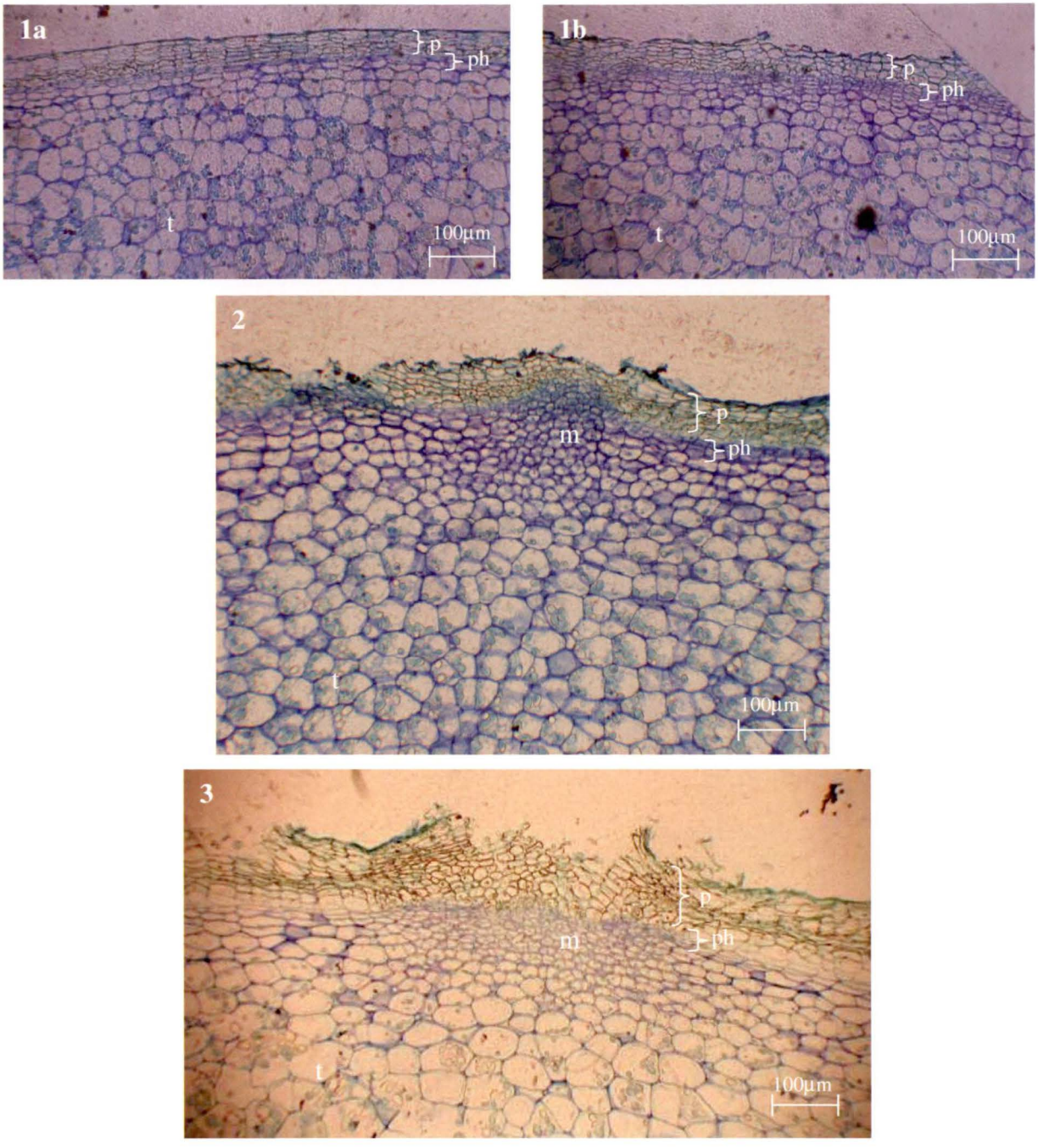


Fig. 3.3. Transverse sections of gross periderm structure and lenticular development in potato tuber tissue. Plates **1** show gross periderm structure before the onset of lenticular development in **a**) a tuber harvested from plant whose foliage had been sprayed with 2,4-D, and **b**) a tuber harvested from control plant. Both tubers were harvested 7 days after spray and are approximately 2-3 week old tubers. Plate **2** shows a control tuber approximately 4 weeks old with intense meristematic activity occuring in the phellogen, typical of lenticular development. Plate **3** shows a control tuber approximately 5 weeks old with a torn outer periderm (phellum) as the result of the high meristematic activity. m – meristematic activity, p – phellum, ph – phellogen, t – tuber parenchyma (storage) cells containing starch granules.

3.3.2.2 Lenticel number and dimensions

Similarly, in pot trial 3 little obvious effect of 2,4-D treatments on lenticel development was shown as foliar sprays of 2,4-D had no significant effect (*P>0.05*) on lenticel numbers (Table 3.4). Lenticel numbers increased from approximately 70 per tuber to 120 per tuber over the 3-week assessment period.

Table 3.4. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) foliar sprays on lenticel numbers from tubers harvested at 1, 2, 3 and 4 weeks after first foliar spray. Spray treatments were multiple-3 (m) sequential sprays, with initial sprays made at 14 d after tuber initiation, and subsequent sprays made at 10 d intervals. Values are means ± standard errors (n = 12).

Treatment	Lenticel numbers per tuber			
	weeks after first foliar spray			
	1	2	3	4
Control	68 ± 4.1	91 ± 4.7	109 ± 5.3	125 ± 5.9
2,4-D (m)	69 ± 3.7	88 ± 4.8	106 ± 4.6	117 ± 6.3
F probability	0.81	0.60	0.60	0.28

Foliar sprays of 2,4-D did have a significant effect (*P<0.05*) on lenticel dimensions on some occasions, most evident at the mid-section of the tuber (Table 3.5). Lenticel dimensions on the stolon and bud-end of the tuber were not significantly affected (*P>0.05*) by 2,4-D sprays in all but one assessment. This occurred in the bud-end, 3 weeks after the initial foliar spray, whereby lenticel width was significantly larger from those plants treated with 2,4-D.

From the mid-section of the tuber, lenticel width from 2,4-D treated plants was significantly smaller (*P<0.05*) on 3 of the 4 assessment dates (1, 3 and 4 weeks after the initial foliar spray). Also from the mid-section of the tuber and on the 1st assessment date, lenticel length of 2,4-D-treated plants was significantly less (*P<0.05*). It is of note that these significant results generally only represented an approximate 10% change in lenticel size or width. As the size of the tuber increased (week 1 through to week 4 after 1st spray), external lenticel dimensions generally increased (Table 3.5).

Table 3.5. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) foliar sprays on lenticel dimensions (μm) from tubers harvested at 1, 2, 3 and 4 weeks after first foliar spray. Spray treatments were multiple-3 (m) sequential sprays, with initial sprays made at 14 d after tuber initiation, and subsequent sprays made at 10 d intervals. Measurements represent the mean maximum length (L) and width (W) of a lenticel from either the stolon, mid-section or bud-end of a tuber (n = 40).

Location	Treatment	Lenticel dimensions (μm)							
		weeks after first foliar spray							
		1		2		3		4	
		L	W	L	W	L	W	L	W
Stolon end	Control	415	358	478	409	485	418	514	448
	2,4-D (m)	406	326	465	418	478	416	491	420
	LSD (0.05)	ns	ns	ns	ns	ns	ns	ns	ns
Mid-section	Control	525 a	436 a	518	439	578	475 a	585	503 a
	2,4-D (m)	477 b	372 b	515	449	564	450 b	573	469 b
	LSD (0.05)	31.1	27.2	ns	ns	ns	14.8	ns	20.5
Bud end	Control	400	321	448	365	494	384 b	529	422
	2,4-D (m)	401	319	441	360	500	410 a	515	406
	LSD (0.05)	ns	ns	ns	ns	ns	24.4	ns	ns

Means followed by same letter within the same column are not significantly different at the 0.05 probability level using Fisher's LSD test.

3.3.2.3 Tuber slice assay

In pot trial 3 the tuber slice assay showed that on 3 of the 4 different assessment weeks there was a significant reduction ($P<0.01$) in necrosis rating (caused by thaxtomin A) for tubers that had been harvested from plants sprayed with 2,4-D (m), compared to tubers from control plants (Table 3.6).

In pot trial 4 the tuber slice assay showed that 2 weeks after the first foliar spray there was a significant reduction ($P<0.01$) in necrosis rating (caused by thaxtomin A treatment) for tubers that had been harvested from plants sprayed with 2,4-D (m), compared to tubers from control plants (Table 3.6). This is visually demonstrated in Fig. 3.4 where typical tubers harvested from 2,4-D sprayed plants have more discrete brown necrotic flecks compared to control tubers that have larger areas of brown necrosis after being exposed to thaxtomin A-containing discs for 7 days.

Table 3.6. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) foliar sprays on mean necrosis rating from tuber slices exposed to 57 μM thaxtomin A. Spray treatments included two-2 (d) sequential sprays or multiple-3 (m) sequential sprays, with initial sprays made at 14 d after tuber initiation, and subsequent sprays made at 10 d intervals. Tubers were harvested and tested at 1, 2, 3 and 4 weeks after first foliar spray in pot trial 3, and at 2 weeks only after first foliar spray in pot trial 4. (n = 36, pot trial 3; n =20, pot trial 4).

	Necrosis rating^ from tuber slice assay					
	Pot trial 3				Pot trial 4	
	weeks after first foliar spray					
	1	2	3	4		2
Treatment					Treatment	
Control	2.78 a	2.81	2.78 a	3.11 a	Control	3.05 a
2,4-D (m)	1.92 b	2.72	2.00 b	2.28 b	2,4-D (d)	2.40 b
LSD (0.01)	0.422	0.382	0.533	0.427	LSD (0.01)	0.520

^ Necrosis rating was assessed after 7 days exposure to thaxtomin A, using the scale: 0= no necrosis, 1= few brown flecks, 2= brown flecks in determined necrotic area, 3= brown necrosis and 4= brown to black necrosis.

Means followed by same letter within the same column are not significantly different at the 0.01 probability level using Fisher's LSD test.

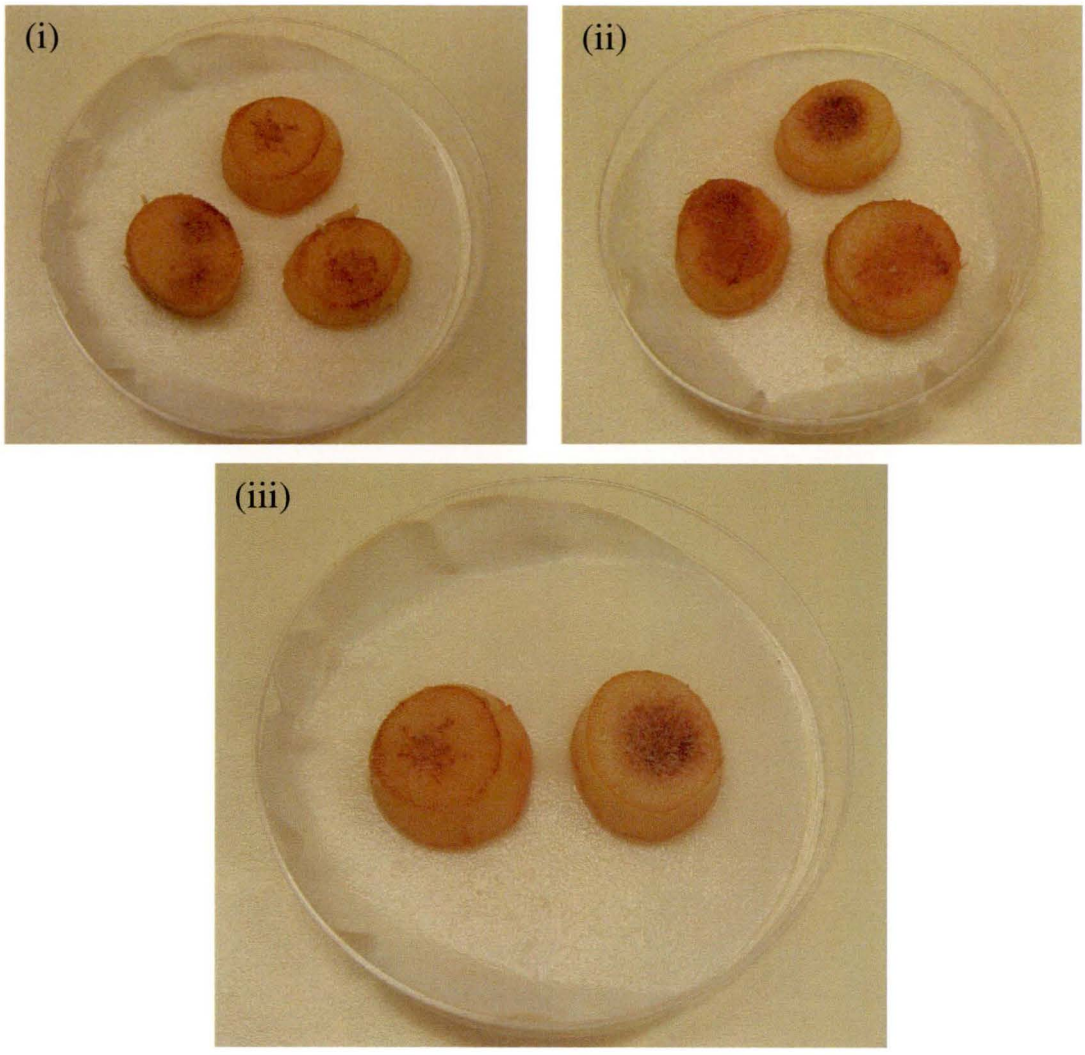


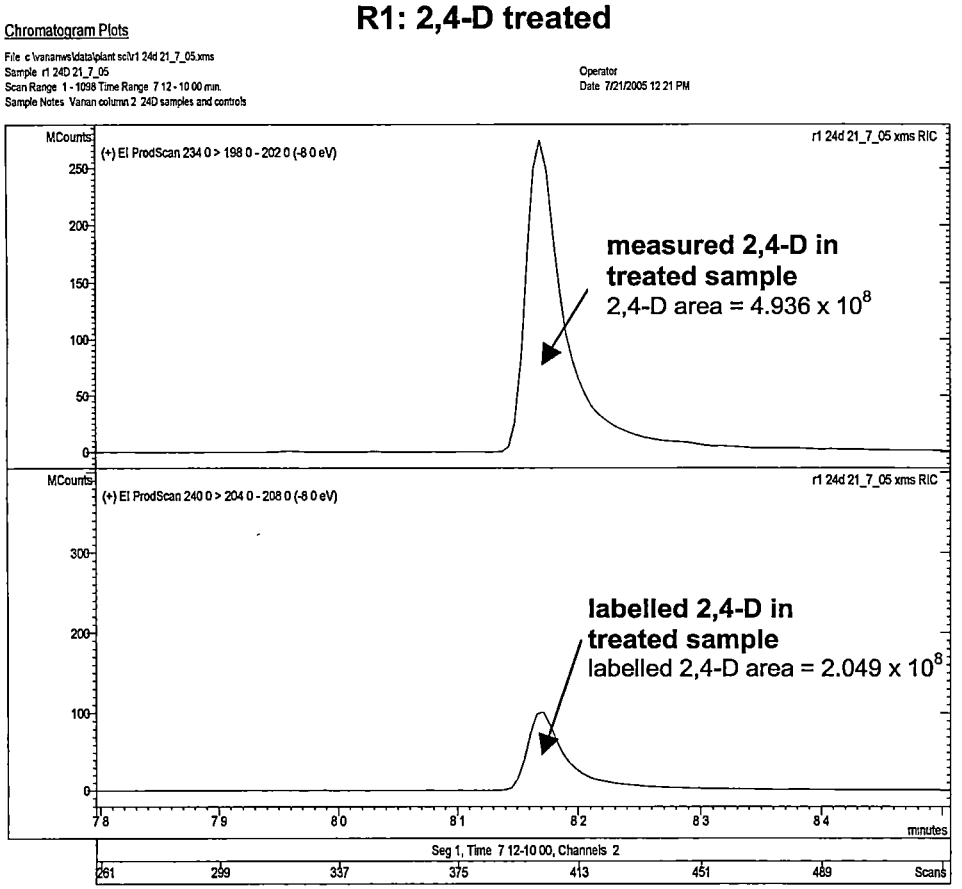
Fig 3.4. Necrotic lesions on tuber slices after 7 days exposure to 57 uM thaxtomin A. Tubers were harvested from plants that had their foliage sprayed twice with either (i) 2,4-D, or (ii) control. Plate (iii) shows a close up: left tuber slice was 2,4-D-treated and has discrete brown necrotic flecks, right tuber slice was control treated and has larger brown necrotic lesions. Petri dishes are 7cm in diameter.

3.3.2.4 Quantification of 2,4-D levels from tubers

To determine that 2,4-D had made its way from the sprayed foliage into the tuber, levels of 2,4-D from within the tuber were quantified using GC-MS ion monitoring. Typical GC-MS scans from treated and control tubers are shown in Fig. 3.5. Tubers that had their foliage sprayed with 2,4-D recorded levels of 992 ng 2,4-D/g FW of tuber tissue (Table 3.7). In the controls trace levels of 2,4-D were recorded: 20 ng 2,4-D/g FW of tuber tissue. This represents a 50-fold difference in 2,4-D levels assayed from the two treatments. When the data was analysed using log transformation because of the unequal variance in the data set, it corresponded to highly significant (*P=0.0002*) differences between the treatments.

Table 3.7. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) foliar sprays on levels of 2,4-D ± standard error, extracted from tubers. Spray treatments were two-2 (d) sequential sprays, with initial sprays made at 14 d after tuber initiation, and subsequent spray made 10 d later. Tubers were harvested and tested at 2 weeks after first foliar spray. (n = 3).

Treatment	2,4-D (ng/gFW)
Control	19.644 ± 2.5
2,4-D	992.030 ± 342.9



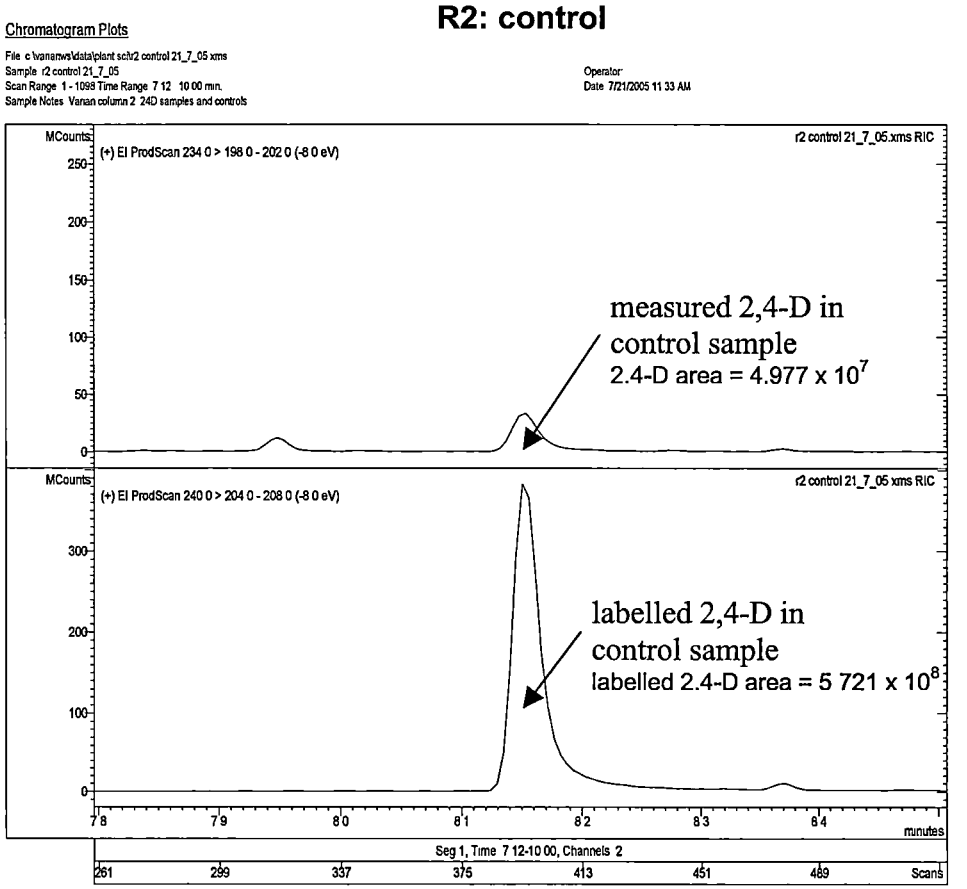


Fig. 3.5. Representative GC-MS scans produced on tubers harvested from plants that had foliage sprayed twice with either 2,4-D or control treatments. Note that scan for 2,4-D treated sample was from a 2mL aliquot, scan for control was from an 8mL aliquot (4 fold dilution). This combined with the ratio of measured to labelled 2,4-D in the above scans (2.5: 1 for 2,4-D treated; 1:11.5 for control) equates to an approximate 100-fold measured difference in 2,4-D levels extracted from tubers in these specific samples.

3.4. Discussion

3.4.1 Common scab disease levels reduced by 2,4-D foliar sprays

The suppression of common scab symptom development by foliar auxin sprays has been previously demonstrated by McIntosh in a series of glasshouse and field trials (McIntosh *et al.* 1981, 1982, 1985, 1988). McIntosh *et al.* (1981) had particular success with the phenoxyacetic acids 3,5-D and 2,4-D in reducing scab by 90 and 50% respectively. Trials 1 and 2 using 2,4-D from this thesis (80-90% reduction in scab surface coverage) provided even greater disease suppression than that recorded by McIntosh *et al.* (1981). However the disease suppression McIntosh *et al.* (1988) achieved with CNB (50% scab surface reduction) was not found in this thesis (trial 1). The major benefit according to McIntosh *et al.* (1988) of using the di-substituted benzoic acids, was that yield and shape of the tuber was not affected, in contrast to the dichlorophenoxyacetic acids which deform and reduce tuber yield (McIntosh *et al.* 1982, 1985). Deformation in tuber shape was not obvious in these trials with 2,4-D application but yields, although not significantly affected by the treatment, tended to be lower following 2,4-D treatment. Lack of significant detrimental effects on tuber production may be partially due to production in pots whereby tuber growth will not be optimal and could mask deformation and major yield effects. This was alluded to by McIntosh whereby field trials with 3,5-D (McIntosh *et al.* 1982) produced greater tuber deformity and yield reductions than their glasshouse work with 3,5-D (McIntosh *et al.* 1981). Also, their field trials with 3,5-D produced weaker effects on scab (30% reduction) compared to their glasshouse trials (85% reduction), questioning the applicability and practicality of using auxin sprays in commercial field situations.

Whilst the effect of 2,4-D reducing scab symptoms has been demonstrated both in these pot trials and those of McIntosh *et al.* (1981), the modality behind 2,4-D conferring a protective effect against the pathogen *S. scabiei* is not known. *Streptomyces scabiei* is not affected directly by 2,4-D at the rates used in these experiments (McIntosh *et al.* 1981), with the protective effect conferred by some indirect mechanism (McIntosh *et al.* 1981, 1988).

3.4.2 Morphological characteristics not altered by 2,4-D foliar sprays

Lenticels, natural openings in the periderm of most plants (Royle 1975), which function as sites for gas exchange (Wiggington 1973), are key entry points for several potato pathogens (Adams 1975b). They include those causing common scab (*S. scabiei*), late blight (*Phytophthora infestans* (Mont.) de Bary) (Lohnis 1925) and bacterial soft rot (*Erwinia carotovora* (Jones) Bergey *et al.*) (Smith and Ramsey 1947). Immature lenticels are suggested to represent the key physiological stage most susceptible to infection by the common scab pathogen (Lapwood and Adams 1973, 1975).

There are no reports on the impact of auxin sources on lenticel structure and numbers in potato tubers. Indeed, the only related work documenting auxin action on lenticels is from work on tree species, using relatively high physiological concentrations (0.1-1.0 mg/ml) of the naturally occurring IAA (Singh and Paliwal 1985a,b; Badola *et al.* 1987). These authors found that IAA activated lenticel meristem activity, with Singh and Paliwal (1985a) documenting an increased lenticel density of up to 1.7-fold and an increased area of a single lenticel up to 2-fold, with IAA application. These reports contrast with the results obtained in trials 2 and 3 in this thesis using the synthetic auxin 2,4-D, whereby there was no effect on lenticel density (numbers) and an occasional small (approx. 10%), decrease in lenticel size in the mid region of tubers. Part of the reason for this is probably attributable to the high IAA concentrations used compared to the 2,4-D concentrations that were approximately 10-fold less in our trials. Also, in the trials on woody tree species IAA was directly applied to the stem cuttings whereas in the foliar spray trials, 2,4-D was applied to the foliage with the effect measured below ground in the tubers. The physiological significance of 2,4-D reducing particularly the width of the lenticel is not clear. The small reduction (10%) would obviously reduce the size of the colonization entry site for the pathogen, but the amount is perhaps physiologically insignificant when comparing the structural differences of resistant and susceptible plant cultivars to other diseases.

There are no reports relating lenticel density to common scab and few conclusive reports relating lenticel structure or size directly to common scab of potato. Those that have related lenticel structure to common scab resistance have

produced conflicting results (Longree 1931; Darling 1937; Adams 1975a). Longree (1931) concluded that the shape and size of the surface of lenticels are unimportant in scab resistance, rather the compact (in resistant varieties) or loose (in susceptible varieties) nature of the complementary filling cells was important. Darling (1937) concluded that the size of lenticels was important, larger lenticels associated with susceptible cultivars. Like Longree (1931) however, Darling (1937) concluded the open or closed nature of the complementary cells reflected scab susceptibility. Adams (1975a) further contradicted and disputed both these reports by noting no differences in lenticel formation between resistant and susceptible varieties. Further clarification of this work is required but there is no dispute that the nature of this work is difficult and conclusions derived still not completely clear (Adams 1975a).

However there are reports of other potato and crop diseases in which resistance has been related more conclusively to structural physical properties including lenticels. In potatoes, cultivars resistant to *E. carotovora subsp. carotovora* had fewer lenticels/unit area of tuber surface, thicker cuticle in the epidermis, more cell layers in the periderm and intensive cuticularization in lenticel tissues (Zhang *et al.* 1991). Other studies with *E. carotovora subsp. atroseptica* (Weber and Bartel 1986) and *P. infestans* (Mahajan *et al.* 2004) also found that resistant potato cultivars had fewer lenticels compared to susceptible cultivars. High lenticel density has also been correlated with greater disease incidence in a number of other crop types including apples (Li *et al.* 2004, Liu *et al.* 2003), and sweet potato (Bajit and Gapasin 1987). Lenticel structure has also been reported to affect disease resistance in apple cultivars. Susceptible cultivars had an open lenticel structure with no filling cells, resistant cultivars had a closed lenticel structure with filling cells below (Li *et al.* 2004).

The periderm represents a direct barrier to pathogens and its properties also correlate with disease susceptibility in many crop types. In potato, more periderm layers were associated with cultivar resistance to *P. infestans* (Mahajan *et al.* 2004). Increased periderm thickness has also been associated with disease resistance in other crops including sweet potato (Bajit and Gapasin 1987) and grapes (Gabler *et al.* 2003). For common scab, the role of the periderm in disease resistance is not well defined. Hooker and Page (1960) reported that the suberized periderm cannot be

penetrated by *S. scabiei*. However there are rare exceptions whereby *S. ipomoeae* and *S. scabiei* hyphae have been shown to penetrate the tuber (Clark and Matthews 1987; Loria *et al.* 2003), implying the periderm may perhaps play a minor role in common scab resistance.

The results from these trials showed no obvious change in gross periderm structure with cell layers in periderm and its thickness consistent across 2,4-D and control spray treatments. Likewise, there were no discernable differences in lenticular development (filling cells etc.) and overall lenticel architecture. It must be noted that these studies may require further validation as Adams (1975a) stated that studies relating to lenticular development and common scab need to be precise. Indeed, Adams (1975a) questioned the relevance of the findings of Longrée (1931) and Darling (1937) stating that studies of the physiology of scab-resistance must concentrate on susceptible lenticels i.e. those passing through their 10d susceptibility growth stage (Adams and Lapwood 1978). Our studies did include and focus on this susceptible phase, and although further replication would be useful, our studies suggest no impact on these morphological features at the rates of 2,4-D applied in these experiments.

3.4.3 Thaxtomin A: key to identifying modality of 2,4-D – based disease suppression

Given the evidence aforementioned in that auxin (2,4-D) does not modify or alter morphological features of the tuber such as lenticels and periderm, by a physiologically significant amount, other evidence is required to explain how auxin sprays confer a protective effect against common scab disease. A possible mechanism is suggested in the results of trials 3 and 4 whereby thaxtomin A toxicity in tubers has been partially ameliorated with 2,4-D treatment. This indicates that auxin interacts with thaxtomin A, rather than directly with the pathogen itself. Within the tuber environment this excess auxin (2,4-D) may reduce or prevent the efficacy of thaxtomin A action and this may correlate with a reduced efficiency of pathogen movement, as thaxtomin aids this penetration from cell to cell as it degrades tissue (Loria *et al.* 1997, 2003). This supports our other studies, which have clearly shown an inhibitory affect of auxin treatments on thaxtomin A toxicity in a number of plant species and tissue types (Chapter 4, Tegg *et al.* 2004b). It is

worthy of note that the thaxtomin concentration used in the tuber slice assay is likely to greatly exceed that present at the infection front during disease establishment.

The phenomena of herbicide/indole induced disease resistance has been widely reported in a number of plant-pathogen systems with indirect or unknown mechanisms postulated as enhancing resistance (Grinstein *et al.* 1984; Cohen *et al.* 1986, 1987, 1996; Bolter *et al.* 1993; Starratt and Lazarovits 1996, 1999; Ueno *et al.* 2004). Within the common scab pathosystem, 2,4-D (a herbicide and indole compound) also induces disease resistance. A unique and feasible mechanism of action, whereby 2,4-D induces resistance to the toxin that *S. scabiei* produces, thaxtomin A, is postulated. This mechanism of modality appears novel and at least within this pathosystem provides some insight into understanding the phenomena of herbicide/indole induced disease resistance.

3.4.4 Conclusions

The usage of 2,4-D sprays represents a novel way of reducing scab symptoms although its direct applicability for commercial potato production is perhaps limited due to the negative effects of yield reductions and tuber deformation. Nevertheless, the identification of a potential mechanism of alleviation of scab by auxin sources is useful and provides a better knowledge of the disease and perhaps with some refinement an option for incorporation into an integrated disease management strategy for common scab. Likewise, the identification of potential modality of these auxin sprays whereby they interact with the key pathogenicity determinant, thaxtomin A, provides further insight into the disease process and a greater understanding of herbicide/indole induced disease resistance.

Chapter 4. Thaxtomin A toxicity and interaction with auxin

4.1 Introduction

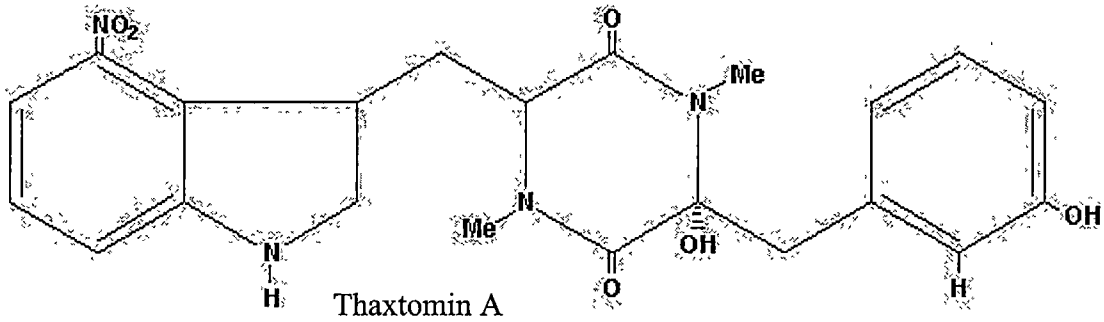
Foliar auxin sprays have been shown to ameliorate symptoms of common scab disease (McIntosh 1980; Chapter 3). Furthermore, qualitative evidence exists demonstrating that foliar-applied auxin (2,4-D) reduces thaxtomin A-induced toxicity in potato tissue (Chapter 3). This novel interaction between auxin and thaxtomin A warrants further investigation, firstly to confirm the interaction across a number of plant cell systems and secondly to better pinpoint the specificity and nature of this interaction.

An understanding of auxin and its mode of transport around the plant may be essential in defining an interaction with thaxtomin A. Auxin is an essential yet unique multifunctional plant hormone (Leyser 2002) known to influence many processes of growth and development, including cell elongation, apical dominance, gravitropism, and root initiation (Swarup *et al.* 2002). Indole acetic acid, the most common auxin, is synthesized primarily in apical meristems and young leaves and is moved basipetally through the polar auxin transport system. Polar auxin transport is specific for active free auxins, occurs in a cell-to-cell manner and has a strictly unidirectional character (Friml and Palme 2002; Cooke *et al.* 2002). An elaborate system of uptake and efflux carrier proteins regulates polar auxin transport although the process is not completely understood (Muday and DeLong 2001; Friml and Palme 2002; Muday and Murphy 2002).

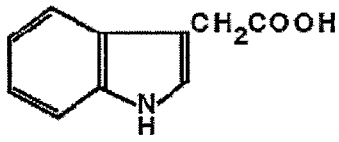
The movement of auxin out of a cell, efflux, and the processes that regulate it are perhaps better understood than inward movement, due to the availability of compounds that inhibit this polar efflux transport (Lomax *et al.* 1995; Friml and Palme 2002). Two such utilised and well-defined compounds include 1-napthylphthalamic acid (NPA) and 2,3,5- Triiodobenzoic acid (TIBA). Although both compounds block polar auxin transport efflux, NPA, classed as a phytotropin (Rubery 1990) is chemically and functionally distinguishable from TIBA, a morphactin (Lomax *et al.* 1995; Friml and Palme 2002). TIBA is reported to inhibit auxin efflux by directly binding at the efflux carrier site (Lomax *et al.* 1995).

However, NPA is postulated to interfere with the cellular efflux of auxin from anion channels by binding to a distinct regulatory site (NPA-binding protein) rather than the auxin channel pore/auxin efflux carrier site itself (Muday 2001; Cooke *et al.* 2002). These differences may enable differentiation of specific modality if different responses from for example, *A. thaliana* mutants, are observed to these polar auxin transport inhibitors.

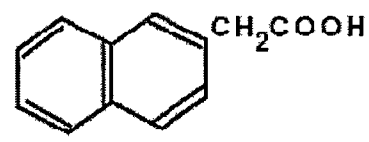
The structural makeup of thaxtomin A (Fig. 4.1) and its precursors and indeed natural and synthetic auxins share some similarity and may provide a clue to a possible thaxtomin A/auxin interaction.



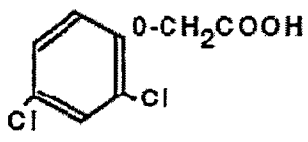
Thaxtomin A



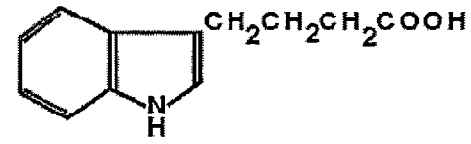
IAA Indole acetic acid



NAA Naphthaleneacetic acid



2,4-D 2,4 dichlorophenoxyacetic acid



IBA Indole butyric acid

Fig. 4.1. Structure of Thaxtomin A and the natural auxin, IAA (Indole-3-acetic acid), and synthetic auxins including NAA (1-Naphthalene acetic acid), 2,4-D (2,4-dichloro-phenoxyacetic acid, and IBA (indole-3-butyric acid).

Thaxtomins are modified dipeptide molecules which may arise biosynthetically from tryptophan and phenylalanine (King 1997) by as yet uncharacterised pathways. Essential to the phytotoxic activity is the presence of the 4-nitroindol-3-yl and phenylalanine groups linked in an L,L configured cyclodipeptide (King *et al.* 1992). The indole ring of thaxtomin A shares considerable homology with IAA and IBA structures, perhaps implying structural similarity in terms of recognition and binding at receptor sites.

In elucidating and understanding the impact of thaxtomin A on plant response, plants such as *Arabidopsis thaliana*, radish and tobacco cell cultures have been utilised (Leiner *et al.* 1996; Fry and Loria 2002; Scheible *et al.* 2003). The usage of well characterised plants such as *Arabidopsis thaliana* may provide a model system to allow a better understanding of the interaction between auxin and toxins such as thaxtomin A. A range of Arabidopsis mutants with varying resistances to the naturally occurring auxin IAA, and synthetic auxin herbicides such as 2,4-D, based on the mutation of a single gene (Maher and Martindale 1980; Lincoln *et al.* 1990; Pickett *et al.* 1990; Wilson *et al.* 1990; Hobbie and Estelle 1995) may aid in this process. Other mutants resistant to auxin transport processes (Ruegger *et al.* 1997, 1998) or toxin related signaling and perception processes (Petersen *et al.* 2000; Innes 2001) may also be useful. The recent identification of a thaxtomin resistant (*txr1*) mutant (Scheible *et al.* 2003) provides an invaluable tool, as do other related mutants resistant to cell wall biosynthetic inhibitors, such as the isoxaben resistant (*ixr1*) mutant (Heim *et al.* 1989).

Other groups of compounds, specifically those that inhibit polar auxin transport may also help in better defining (or pinpointing) the thaxtomin/auxin interaction. Likewise, other key compounds acting similar to thaxtomin A such as the cell wall biosynthetic inhibitors, dichlobenil and isoxaben, (King *et al.* 2001) serve as useful substances for comparison. In defining common modality between toxins, patterns of resistance across *A. thaliana* mutants are useful. For example, a more recently identified cell wall biosynthetic inhibitor, 5-tert-butyl-carbamoyloxy-3-(3-trifluromethyl) phenyl-4-thiazolidnone (TZ) has a similar effect on plants to isoxaben. The *ixr1-1* mutant of *Arabidopsis* exhibits resistance to both isoxaben and

TZ, indicating that isoxaben and TZ share a common mode of action (Sharples *et al.* 1998; Scheible *et al.* 2001).

This work aims to further understand the phenomena of herbicide or auxin induced disease resistance by studying independently the predominant toxin within the common scab pathosystem, thaxtomin A. By using a range of plant and plant cell systems including *A. thaliana* mutants and tomato pollen tubes treated with auxin sources and/or transport inhibitors, in combination with specific toxins it is hoped that progress can be made in pinpointing the thaxtomin A/auxin interaction. In addition, by comparing the performance of similarly related toxins to thaxtomin A, the relatedness of the modes of action of these compounds may be better defined.

4.2 Materials and methods

4.2.1 Root growth of A. thaliana lines in response to thaxtomin A treatments

Fifteen different *A. thaliana* lines were chosen based on their reported responses to auxin/auxin transport/cellulose targeting toxins/signaling and pathogen defence (Table 4.1). Their root growth on thaxtomin A-containing media was examined. Plant growth conditions and media conditions were as described earlier with root growth measured after 3 days on treatment media (thaxtomin A containing plates) as detailed in 2.2.2. Each treatment had two replicates with 6 plants per plate (n = 12 in total).

4.2.2 Root growth of A. thaliana lines in response to auxin treatments

The root growth response of selected *A. thaliana* lines on media containing the auxins (2,4-D, IAA or NAA) was examined. Selected lines chosen were: WT *'Col'*, *'KOR'*, *'aux1-7'*, *'ucu2-2/gi2'*, *'txr1'*, and *'ixr1'*. The auxin sources IAA (Indole-3-acetic acid; Sigma I-2886), 2,4-D (2,4-Dichloro-phenoxyacetic acid; Sigma D-8407) and NAA (1-Naphthalene acetic acid; Sigma N-0640); which are all thermostable were added before autoclaving to the basal MS media (120 kPa., 120°C, 20 min.), but otherwise the plant, media and treatment conditions were as described earlier (2.2.2). Each treatment had four replicates with 5 plants per plate (n = 20 in total).

4.2.3 Hydrogen flux responses of A. thaliana lines Col (WT) and ucu2-2/gi2 in response to thaxtomin A incubation for 24h

The H^+ flux magnitude from these two *A. thaliana* lines was examined from the meristem, elongation and mature root zone of these plants in response to 24h incubation in 0.2 µM thaxtomin A. Plant growth and media conditions were as detailed in 2.2.2 while experimental protocols for ion flux measurements are detailed in 2.2.5. Measurements were made for each treatment from at least 5 individual plants.

Table 4.1. *Arabidopsis thaliana* lines used for thaxtomin A screening.

Allele/s	Line number/source[%]	Ecotype[$]	Group^	Mutation properties	Reference/s#
Col	N1093	Col		Wild-type	
aux40	N116	C24	A	Auxin resistant, Auxin overproduction	van der Graaff *et al.* 2003
abp-1S	CS16148	Col	A	Endoplasmic reticulum auxin binding protein 1 (involved in cell division and elongation)	Jones *et al.* 1998; Chen *et al.* 2001
AIR1-8	CS224	Col(gl1)	A	auxin induced kanamycin/GUS reporter line	Van der Kop *et al.* 1999
aux1-7	CS3074	Col	A	Auxin resistant, also ethylene (associated with auxin influx carrier)	Bennett *et al.* 1996; Swarup *et al.* 2004
aux1-7, ein2	N8843	Col	A	Auxin resistant, also ethylene (associated with auxin influx carrier)	Bennett *et al.* 1996; Swarup *et al.* 2004
tir1	N3798	Col	B	Transport inhibitor response 1, resistant to auxin inhibition of root elongation.	Ruegger *et al.* 1998
tir3-101	CS3928	Col(gl1)	B	Auxin transport inhibitor resistant (specifically 1-Napthylphthalamic acid resistant), reduction in polar auxin transport.	Ruegger *et al.* 1997
pin1-1, ttg-1	N8065	En-2	B	Auxin transport mutant (defective in polar auxin transport), encodes putative auxin efflux carrier	Blilou *et al.* 2005; Paponov *et al.* 2005
ucu2-2/gi2	N3397	Col-1	B	Appearance similar to rol gene of Agro. rhiz.	Perez-Perez *et al.* 2004
txr1	Courtesy of R.Loria	Col	C	Thaxtomin A resistant	Scheible *et al.* 2003
KOR1	N298	Ws	C	Cellulose deficiency	Nicol *et al.* 1997; Zuo *et al.* 2000
ixr1	CS6201	Col	C	Isoxaben resistant	Heim *et al.* 1989
gpa1-1	CS3910	Ws-2	D	G-protein signaling	Ma *et al.* 1990; Ullah *et al.* 2003
mpk4-1	CS5205	Ler	D	Mitogen-activated protein kinase, mediating responses to pathogens	Petersen *et al.* 2000; Innes 2001
pgp5	N57506	Ler	D	Putative protein kinase	Anon 2001

% N - Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham, Loughborough, Leicestershire UK; CS - Arabidopsis Biological Resource Center (ABRC), Ohio State University, Columbus, Ohio USA.

[$] Col – Columbia, En – Enkheim, Ws – Wassilewskija, Ler – Landsberg (er).

^ *A. thaliana* lines with similar mutation properties were grouped: A – auxin resistant/affected; B – auxin transport inhibitor mutant; C – cellulose associated mutant; D – signalling, defence related, miscellaneous mutants.

Either original reference or definitive reference for given mutant allele.

4.2.4 Root growth of A. thaliana lines Col (WT) and ucu2-2/gi2 in response to alternariol and fusaric acid treatments

The root growth of these two *A. thaliana* lines was examined on media containing the fungal toxins, alternariol (Sigma A-1312) or fusaric acid (Sigma F6513). These toxins were added to the basal MS media before autoclaving. Plant, media and treatment conditions were as described earlier (2.2.2). Each treatment had four replicates with 5 plants per plate (n = 20 in total).

4.2.5 Toxicity and survival of A. thaliana mutants in response to thaxtomin A and auxin treatments

The toxicity and survival of selected *A. thaliana* lines on media containing both thaxtomin A and the auxins (2,4-D or IAA) was examined. Selected lines chosen were: WT *'Col'*; *'aux 1-7'*; *'aux 1-7/ein 2'*; *'ucu2-2/gi2'*; and *'axr 1-3'*. The auxin sources IAA and 2,4-D (thermostable) were added before autoclaving to the basal MS media, with thaxtomin A added after autoclaving. Seeds were plated directly onto treatment media, plant and media conditions were as described earlier (2.2.2). Lines were split into three thaxtomin A treatments (concentrations) of 0, 0.10, 0.20 μM. These were further split to contain the auxin treatments (0, 0.10, 0.20, 1.0 μM of IAA; 0.10, 0.20, 1.0 μM of 2,4-D) in a split-split plot design. Plates were examined after 25 d incubation with numbers of seedlings actively growing (seedling survival %) recorded. Actively growing was defined as having green active meristematic growth (including organised tissue or unorganised callus growth) and/or visually obvious root growth. Callus growth activity and colour was also recorded as well as patterns of necrosis. Each treatment had four replicates with 10 seeds per plate (n = 40 in total).

4.2.6 Pollen tube growth in response to the toxins (thaxtomin A, alternariol and fusaric acid) and 1-Napthalene acetic acid (NAA)

Tomato pollen tube growth was examined in media containing the toxins (thaxtomin A - 1 μM, alternariol - 30 μM, or fusaric acid - 10 μM) in combination with NAA (1 – 100 μM). Toxin concentrations used were those that inhibited pollen tube growth by approximately 50% as determined by growth inhibition curves for thaxtomin A (Fig. 2.7.a), and alternariol and fusaric acid (data not presented). All other experimental protocols used in this experiment were as described in 2.2.

4.2.7 Root growth of A. thaliana lines in response to the auxin transport inhibitors, 1-napthylphthalamic acid (NPA) or 2,3,5-Triiodobenzoic acid (TIBA)

The root growth response of selected *A. thaliana* lines on media containing the auxin transport inhibitors, 1-NPA (Naptalam®; Riedel-deHaën 3371) or TIBA (2,3,5- Triiodobenzoic acid; Sigma T5910) was examined. Selected lines chosen were: WT *'Col'*, *'KOR'*, *'aux1-7'*, *'ucu2-2/gi2'*, *'txr1'* and *'ixr1'*. The transport inhibitors were added after autoclaving to the basal MS media, but otherwise the plant, media and treatment conditions were as described earlier (2.2.2). Each treatment had four replicates with 5 plants per plate (n = 20 in total).

4.2.8 Root growth of A. thaliana lines in response to the herbicides, dichlobenil or isoxaben

The root growth response of selected *A. thaliana* lines on media containing the herbicides, dichlobenil (Dichlobenil®; Riedel-deHaën 45431) or isoxaben (Isoxaben®; Riedel-deHaën 36138) was examined. Selected lines chosen were: WT *'Col'*, *'KOR'*, *'aux1-7'*, *'ucu2-2/gi2'*, *'txr1'*, and *'ixr1'*. The herbicides were solubilized in < 1% dimethyl sulfoxide (DMSO) for stock solutions; and in media had a maximum level of < 0.1%, which did not effect plant growth. The herbicides were added before autoclaving to the basal MS media, but otherwise the plant, media and treatment conditions were as described earlier (2.2.2). Each treatment had four replicates with 5 plants per plate (n = 20 in total).

4.2.9 Data analysis

Data was analysed as described previously (2.2.6 and 3.2.7) and as described in the results section (4.3). Comparisons of root growth parameters between *A. thaliana* lines under different treatment regimes were analysed using two methods. Lines were either compared separately at each specific treatment concentration or where data could be easily summarised the RG_{50} (treatment concentration that inhibits root growth by 50%) provided one summary measure across the data set for effective comparison.

4.3 Results

4.3.1 A. thaliana *mutants root growth in response to thaxtomin A treatments*

All *A. thaliana* lines tested showed variable root growth rates under control conditions (0 μM thaxtomin) ranging from 13 to 27 mm over 3 days (data not presented). The results presented in Fig. 4.2.a-d demonstrate the impact of thaxtomin A relative to control root growth rates for each line and show a trend of decreasing root growth with increasing thaxtomin A concentrations. Two factor ANOVA revealed a significant thaxtomin A by line interaction ($P<0.001$). This broad test separated the *'ucu2-2/gi2'* mutant from all other lines having a significantly ($P<0.05$) lower root growth rate at 0.05, 0.10 and 0.15 μM thaxtomin A. At 0.2, 0.3 and 0.5 μM thaxtomin A *'ucu2-2/gi2'* still had the lowest root growth rate, although not significantly less than all other lines. At 0.15 μM thaxtomin A the *'txr1'* mutant had significantly greater root growth rates than all other lines. At higher concentrations of thaxtomin A (≥ 0.20 μM; linear portion of graph), a one factor ANOVA was used to identify root growth differences between lines, at separate thaxtomin A concentrations. At 0.2 ($P<0.01$), 0.3 ($P<0.05$) and 0.5 ($P<0.01$) μM thaxtomin A, *'txr1'* had significantly greater root growth rate than all other lines. At the highest thaxtomin A concentration of 1 μM, *'txr1'* had a comparable root growth rate with the *'pgp5'* mutant; both these mutants had significantly ($P<0.01$) greater root growth rates than all other lines at this treatment level.

4.3.2 A. thaliana mutants root growth in response to various auxin sources

The mutant *'aux1-7'* had a greatly enhanced resistance to both 2,4-D and IAA, but not to NAA; when compared with all other lines tested (Fig. 4.3.). The thaxtomin resistant line *'txr1'* had a comparable sensitivity to the WT *'Col'*, as recorded across all three auxin sources. Mutant line *'ucu2-2/gi2'* had increased sensitivity to 2,4-D which was significant ($P<0.05$) when comparing values of 2,4-D that inhibited root growth by 50% (RG_{50}) to WT *'Col'*.

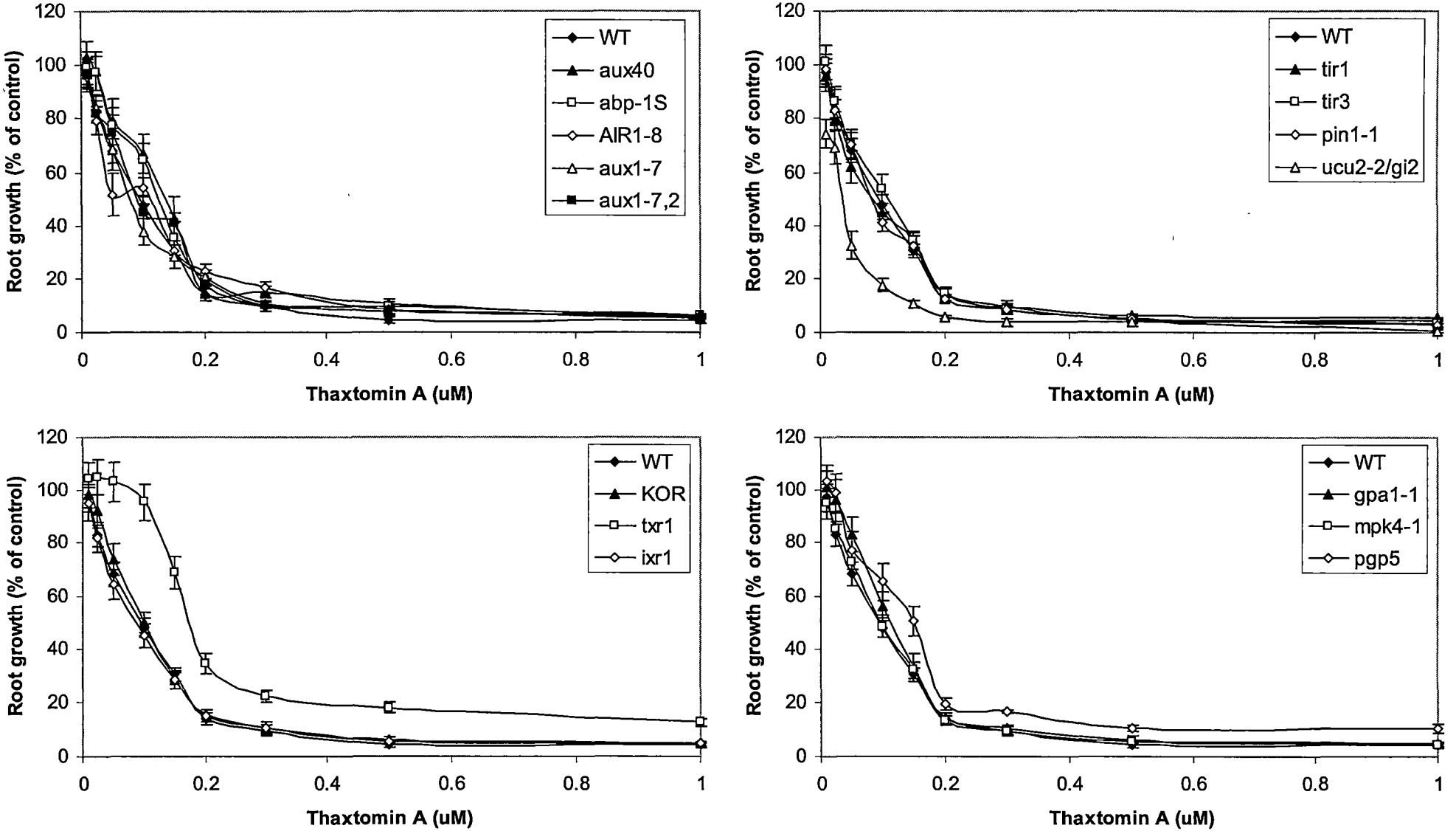


Fig. 4.2. Effect of thaxtomin A on the root growth of various *Arabidopsis thaliana* mutant lines resistant or susceptible to a) auxin; b) auxin transport inhibitors; c) cellulose synthesis; and d) cell signaling processes. Data are expressed as mean percentages ± SEM (n = 12) of control root growth on medium with no thaxtomin A applied.

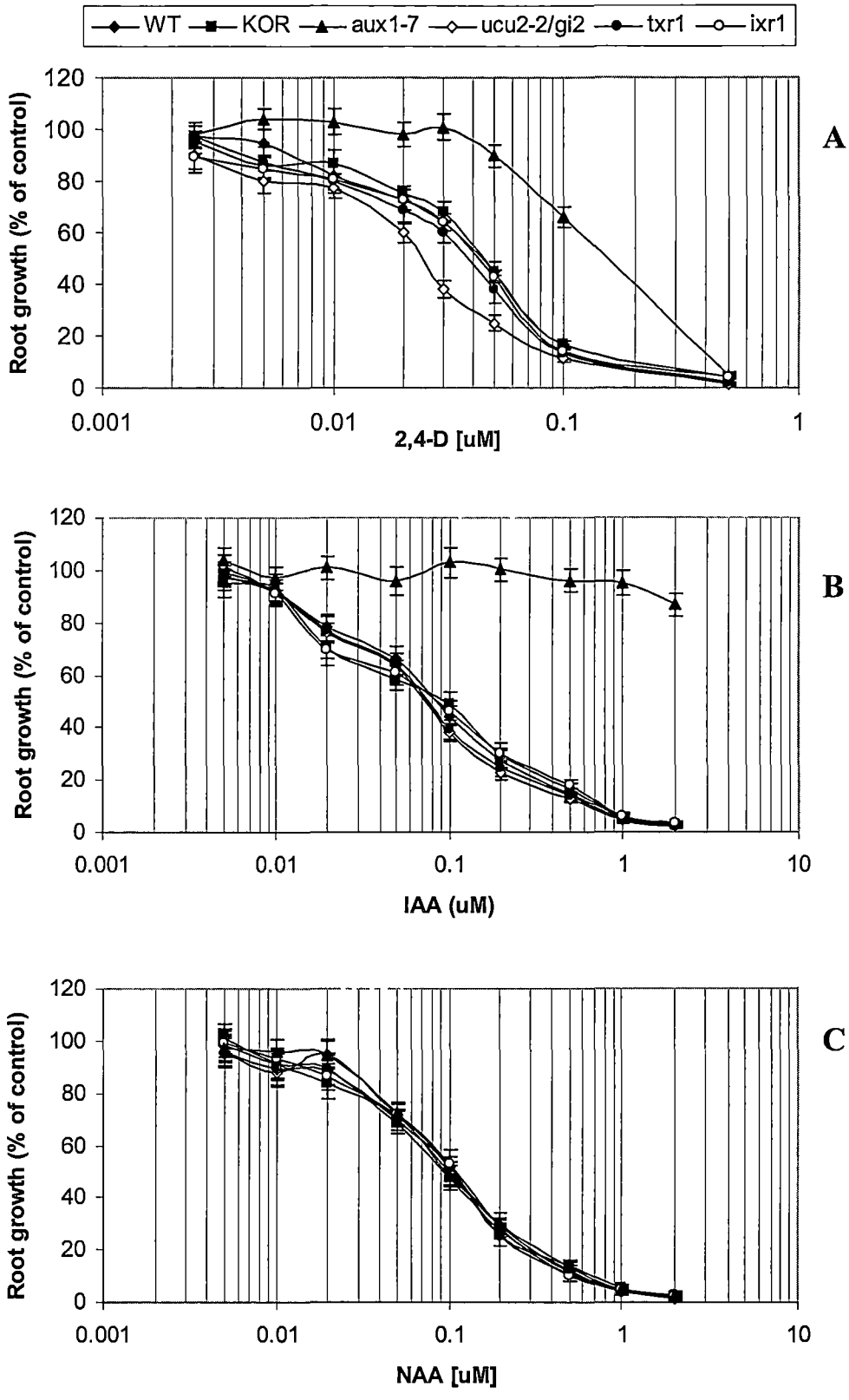


Fig. 4.3. Effect of the auxin sources; **A**) 2,4-D; **B**) IAA; or **C**) NAA on root growth suppression of various *A. thaliana* lines. Data are expressed as mean percentages ± SE (n = 20) of control root growth on medium with no auxin applied.

4.3.3 Increased sensitivity of A. thaliana auxin sensitive mutant to thaxtomin A incubation

The *A. thaliana* double mutant (*ucu2-2/gi2)* was compared to wild-type Columbia genotype by measuring net H^+ flux responses after 24 h exposure to 0.2 μM thaxtomin A (Fig 4.4). While both WT and *ucu2-2/gi2* mutant species showed significant shift towards net H^+ efflux in both meristematic and elongation zones (the most sensitive zones to thaxtomin A application; Fig. 2.2), the magnitude of reduction was 4 - 5 fold greater (significant at $P < 0.05$) for *ucu2-2/gi2* plants (Fig. 4.4).

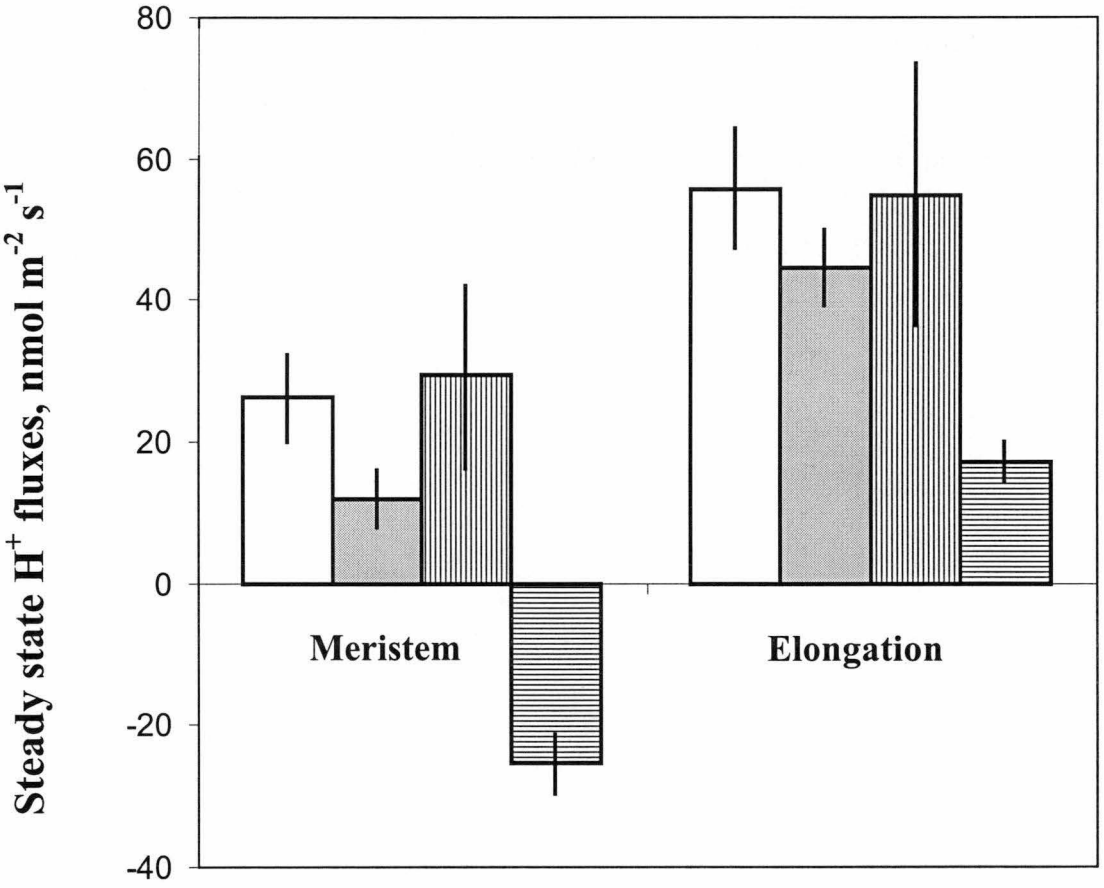


Fig. 4.4. Effect of 24 hr exposure to 0.2 μM thaxtomin A on net H^+ fluxes measured from the elongation and meristematic zones of *Arabidopsis thaliana* WT Columbia and auxin-sensitive *ucu2-2/gi2* mutant roots. WT control (clear), WT treated with thaxtomin A (shaded), *ucu2-2/gi2* control (vertical lines), *ucu2-2/gi2* treated with thaxtomin A (horizontal lines). Data is mean ± SE (n = 5 to 9).

4.3.4 Sensitivity of A. thaliana auxin sensitive mutant to other toxins

The auxin/auxin transport sensitive *A. thaliana* mutant *ucu2-2/gi2* was also highly sensitive to thaxtomin A incubation in comparison to WT *'Col'* (Fig. 4.2, Fig 4.5.a). However, it showed comparable resistance to the toxin, Alternariol (Fig. 4.5.b) and a higher resistance ($P<0.05$) to Fusaric acid (Fig. 4.5.c) when comparing RG_{50} values with WT *'Col'*.

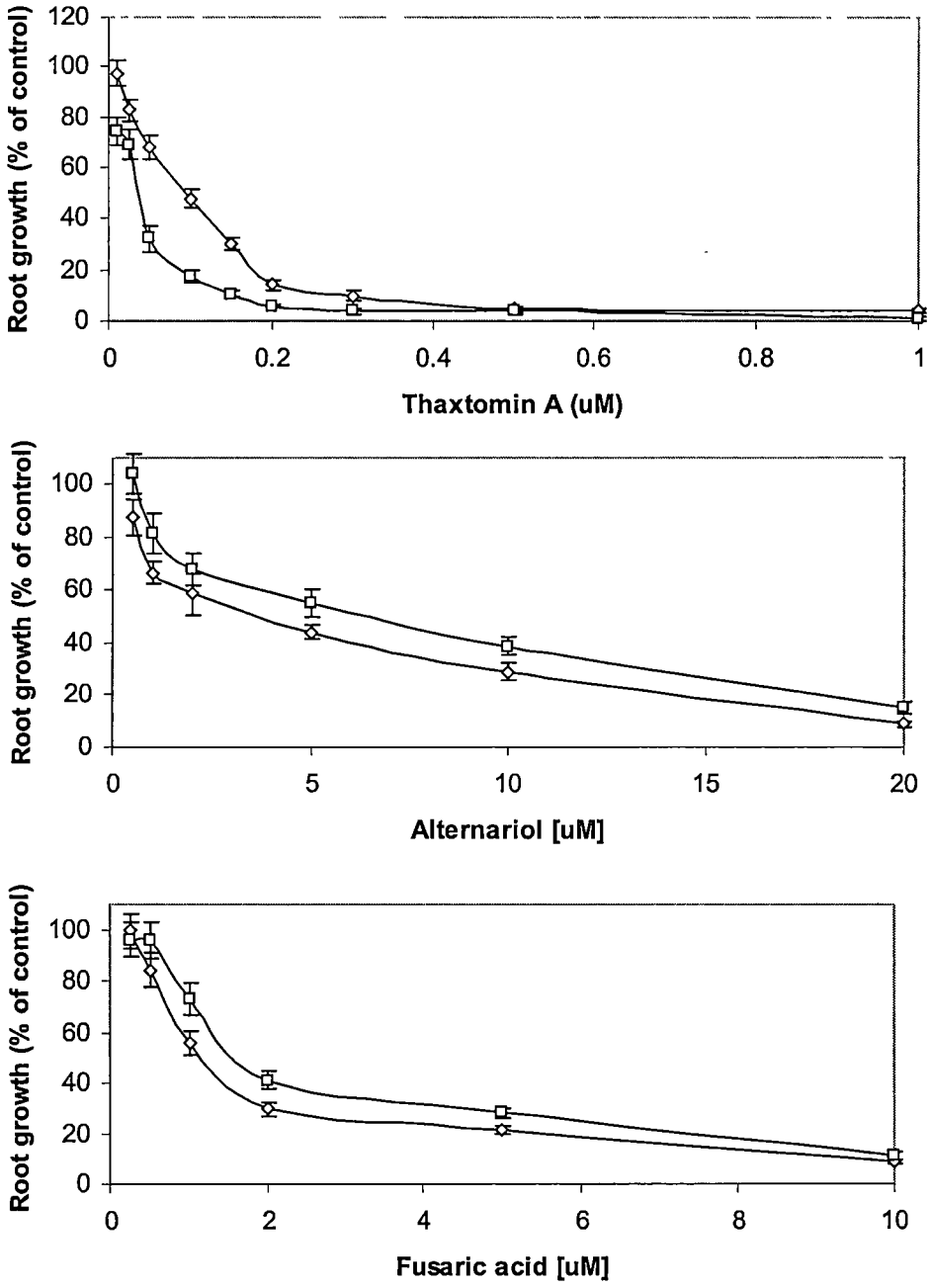


Fig. 4.5. Root growth of *A. thaliana* 'Col' wild-type (open diamond) and *A. thaliana* *'ucu2-2/gi2'* (open square) seedlings after 72 h on medium containing (A) thaxtomin A, (B) Alternariol, or (C) Fusaric acid. Data are expressed as mean percentages ± SE (n = 20) of control root growth on medium with no toxin applied.

4.3.5 A. thaliana *mutants responses to thaxtomin and auxin treatments*

The response of the various *A. thaliana* lines to thaxtomin and the auxin 2,4-D is summarized in Table 4.2; with percentages of seedling survival for each treatment presented. Results for IAA are not presented in this table as their usage at concentrations between 0.1 and 1.0 μM had no effect at all when compared with the control. Chlorosis ratings over the 25 day duration of the experiment showed similar patterns across all lines apart from line *'ucu2-2-gi2'* which showed a faster rate of bleaching and subsequent death when compared to other lines (data not presented).

Seedling survival percentages at day 25 (Table 4.2) show that when the media contains thaxtomin A alone (at either 0.1 or 0.2 μM) all seedlings, across all lines have died by day 25. When the synthetic auxin, 2,4-D is added to the media some of the seedlings still remain alive. This is particularly obvious across *Arabidopsis thaliana* lines *'aux1-7'*, *'aux1-7/ein2'* and WT *'Col'* where for example 54.0, 46.0 and 26.5 % of the seedlings respectively, are still alive when incubated with 0.1 μM thaxtomin A and 1.0 μM 2,4-D. Visually, these plants have obvious green cell growth observed coming from the meristem and hypocotyl region, which contrasts with the completely bleached and necrotic symptoms of a dead seedling (Fig. 4.6).

Table 4.2. Seedling survival percentages of selected *A. thaliana* lines 25d after plating onto media containing 2,4-D and thaxtomin A treatments. Shaded area represents increased percentage survival compared with control (n = 40).

Auxin (μM)	Thaxtomin (μM)	WT *'Col'*	*'aux1-7'*	*'axr1-3'*	*'aux1-7/ein2'*	*'ucu2-2/gi2'*
0 (control)	0	100	100	100	100	100
	0.1	0	0	0	0	0
	0.2	0	0	0	0	0
0.1 2,4-D	0	100	100	100	100	100
	0.1	25.0	72.5	0	47.5	0
	0.2	0	40	0	0	0
0.2 2,4-D	0	100	100	100	100	100
	0.1	47.5	62.5	0	65.0	0
	0.2	0	47.5	0	27.5	0
1.0 2,4-D	0	90	100	100	100	50.0
	0.1	27.5	55.0	0	45.0	0
	0.2	5.0	25.0	0	7.5	0

Fig. 4.6. Directly plated *Arabidopsis thaliana* plants (Line *'aux1-7'*), 20 days after plating as effected by addition of 0.2 uM thaxtomin (dead plant on left) and 0.2 µM thaxtomin plus 0.2 µM 2,4-D (greener plant on right).

4.3.6 Pollen tube growth in response to the toxins (thaxtomin A, alternariol and fusaric acid) and 1-Napthalene acetic acid (NAA)

Pollen tube growth was inhibited when incubated with the toxins alone such that they were significantly less than the control ($P<0.01$) (Fig. 4.7a,b,c). When NAA was added to the growth solution containing thaxtomin A it was able to ameliorate this pollen tube growth reduction (Fig. 4.7a). This was demonstrated with the addition of 3 and 10 µM of NAA which produced pollen tube growth to the same levels ($P>0.05$) as the control and significantly greater ($P<0.05$) than when treated with thaxtomin A alone (Fig 4.7.a). The effect of NAA alone, in absence of the toxin, showed that it produced pollen tube growth comparable to the control from 1-10 µM NAA ($P>0.05$) (Fig. 4.7.d). At 30 and 100 µM NAA, pollen tube growth was severely inhibited ($P<0.01$) when compared with the control (Fig. 4.7.d).

When NAA was added to the growth solution containing either alternariol (Fig. 4.7.b) or fusaric acid (Fig. 4.7.c) it did not ameliorate pollen tube growth reduction. In both cases, NAA concentrations of 1-10 µM did not significantly ($P>0.05$) alter pollen tube growth when compared to treatments with the toxins alone (Fig. 4.7.b,c).

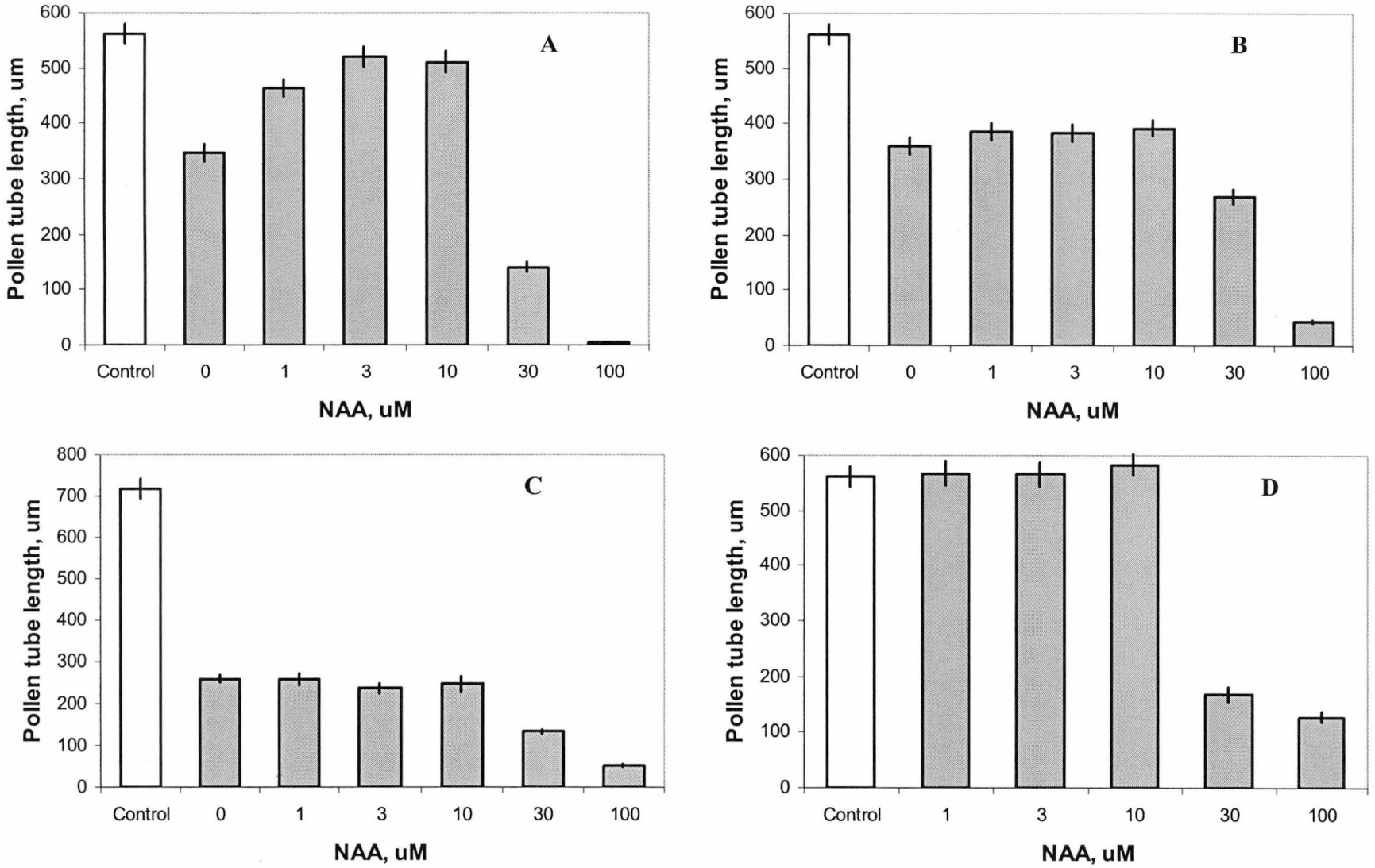


Figure 4.7. Tomato pollen tube length ± se (μm) as effected by 3 hrs incubation with the toxins, a) Thaxtomin A (1 μM), b) Alternariol (30 μM) or c) Fusaric acid (10 μM) in combination with the synthetic auxin, 1-NAA (1-100 μM). open: control; shaded: toxin treated. Plot d) shows NAA alone (n=50).

4.3.7 Root growth of A. thaliana lines in response to the auxin transport inhibitors, 1-napthylphthalamic acid (NPA) or 2,3,5-Triiodobenzoic acid (TIBA)

Both lines *'ixr1'* (isoxaben resistant) and *'txr1'* (thaxtomin resistant) showed an increased resistance to 1-NPA when compared to WT *'Col'* (Fig. 4.8.a). Concentration levels of 1-NPA required to inhibit root growth by 50% (RG_{50}) were approximately 10 (*P<0.01*) and 3-fold (*P<0.05*) higher for *'ixr1'* and *'txr1'* respectively, compared to WT *'Col'*. Line *'ucu2-2/gi2'* showed enhanced sensitivity to 1-NPA with a RG_{50} value approximately 0.6-fold (*P<0.05*) compared to WT *'Col'*. All other lines tested were similarly comparable to WT *'Col'*.

When grown under varying concentrations of TIBA, all lines showed a similar pattern of root growth suppression such that TIBA concentrations that inhibited root growth by 50% were not significantly different (*P>0.05*) across all lines examined (Fig. 4.8.b).

4.3.8 Root growth of A. thaliana lines in response to the herbicides, dichlobenil or isoxaben

Root growth responses of the selected lines to dichlobenil showed a similar pattern of root growth suppression such that dichlobenil concentrations that inhibited root growth by 50% were not significantly different (*P>0.05*) across all lines examined (Fig. 4.9.a).

Root growth responses to isoxaben showed that line *'txr1'* and *'KOR'* had an approximate 3-fold (*P<0.01*) and 1.8-fold (*P<0.05*) higher level of resistance respectively than WT *'Col'* as determined by levels of isoxaben that inhibited root growth by 50% (Fig. 4.9.b). Line *'ixr1'* had an approximate 100-fold higher level of resistance (*P<0.001*) than WT *'Col'*. Line *'ucu2-2/gi2'* had a 0.6-fold (*P<0.05*) lower level of resistance than WT *'Col'* when comparing RG_{50} (Fig. 4.9.b).

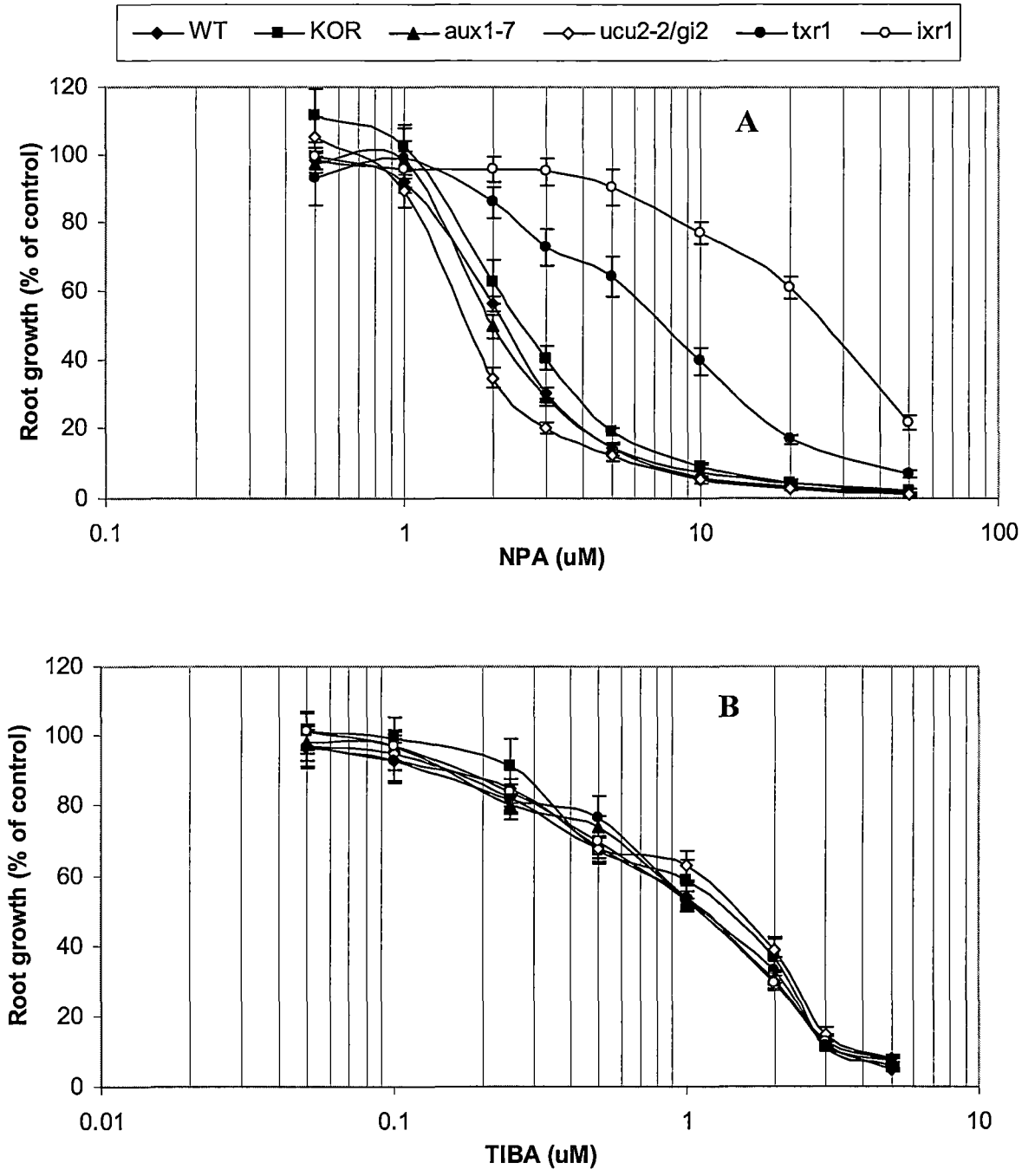


Fig. 4.8. Root growth of various *A. thaliana* seedlings after 72 h on medium containing (A) 1-napthylphthalamic acid – NPA, or (B) 2,3,5-Triiodobenzoic acid - TIBA. Data are expressed as mean percentages ± SE (n = 20) of control root growth on medium with no exogenous transport inhibitors applied.

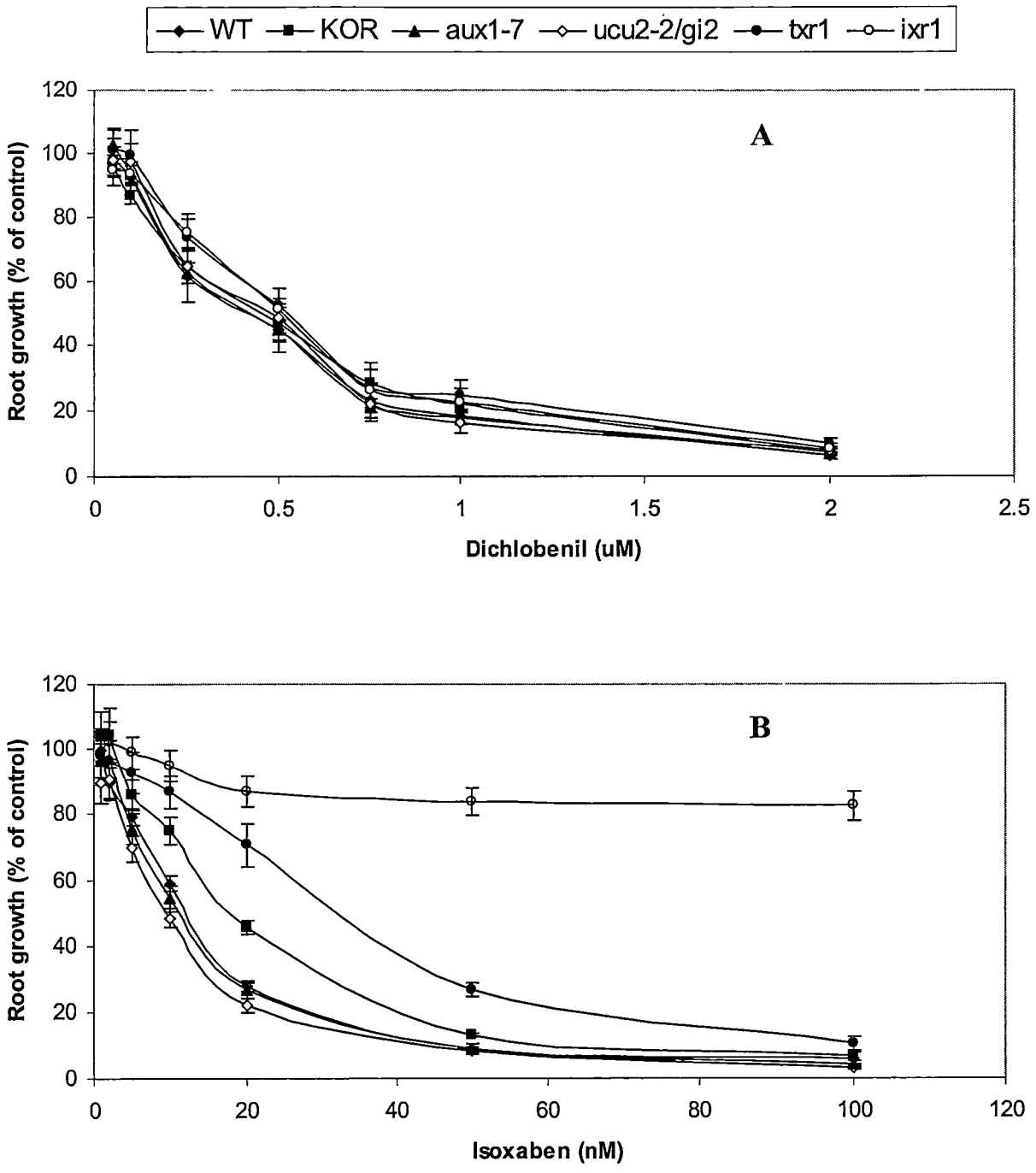


Fig. 4.9. Root growth of various *A. thaliana* seedlings after 72 h on medium containing (A) Dichlobenil, or (B) Isoxaben. Data are expressed as mean percentages ±SE (n = 20) of control root growth on medium with no exogenous herbicides applied.

4.4. Discussion

4.4.1 Root growth response to thaxtomin A and common auxin sources

The root growth suppression patterns of a range of *A. thaliana* mutants, in response to thaxtomin A, served to quantify whether any specific mutation tested effected thaxtomin A toxicity. The key and expected resistant mutant identified was *'txr1'*, as this had been selected for enhanced resistance to thaxtomin A (Scheible *et al.* 2003). That nearly all other mutants studied showed a comparable resistance to WT *'Col'* indicated that mutations or resistance to auxin sources, signalling processes or other related cellulose inhibiting toxins does not confer any enhanced resistance to thaxtomin A. This suggests that thaxtomin A targets a specific receptor site. This is supported by the *'ixr1-1'*, isoxaben resistant (Heim *et al.* 1989) mutant that showed no enhanced resistance to thaxtomin A even though both compounds have a similar mode of action as cell wall biosynthesis inhibitors (King *et al.* 2001). Whilst no mutants, other than *'txr1'*, showed enhanced resistance to thaxtomin A, one mutant *'ucu2-2-gi2'* did show enhanced susceptibility to thaxtomin A and this has provided further evidence as to how thaxtomin A may operate (see 4.4.2).

Root growth suppression patterns in response to the three auxin sources (2,4-D, IAA and NAA) confirmed that *'txr1'* nor any other mutant resistant to cellulose inhibiting toxins (*'ixr1'* or *'KOR'*) had altered responses to auxin indicating that a direct interaction between these *A. thaliana* lines and auxin was not present. However, these studies did show a slightly enhanced susceptibililty of the *ucu2-2-gi2* mutant to particularly 2,4-D; this provided a partial linkage between increased 2,4-D sensitivity and increased thaxtomin A sensitivity. It should be stated that these findings may partially conflict with those of Perez-Perez *et al.* (2004) who stated that growth of homozygous *ucu2/ucu2* plants was similar to wild-type when grown in media augmented with 2,4-D. Nevertheless, the work in this thesis was with a line including the *gi2* mutation, which was different to that used by Perez-Perez *et al.* (2004). Other findings of Perez-Perez *et al.* (2004) on this mutant confirm results we observed with auxin transport inhibitors confirming this mutant to be at least hypersensitive to these inhibitors (see 4.4.5).

4.4.2 The ucu2-2-gi2 A. thaliana mutant provides evidence of a thaxtomin A/auxin interaction

The identification of a sensitive mutant to thaxtomin A was perhaps the most significant outcome obtained from screening a small population of *A. thaliana* lines (Table 4.1). The '*ucu2-2-gi2*' mutants increased sensitivity to thaxtomin A was evidenced by severe root suppression at low concentrations of thaxtomin A, with rapid rates of chlorosis and seedling plant death at higher thaxtomin A concentrations. Although all other mutants (including *'txr1'*) tested in this thesis showed the same negative responses to thaxtomin A, albeit at higher concentrations of thaxtomin A, the changes were not as dramatic or rapid as that observed for '*ucu2-2-gi2*'.

Electrophysiological data comparing *'Col'* wild-type and the '*ucu2-2-gi2*' mutant also supported the root growth suppression data and the scenario of increased susceptibility to thaxtomin A of the '*ucu2-2-gi2*' mutant. Hydrogen flux patterns after incubation in thaxtomin A are a useful tool for comparing the sensitivities of different root zones to thaxtomin A as this toxin may target the proton pump, with an effect on H flux kinetics (Tegg *et al.* 2005; Chapter 2). Indeed this tool was used to map hydrogen flux patterns to compare the intraspecific differences between *'Col'* wild-type and the '*ucu2-2-gi2*' mutant. That '*ucu2-2-gi2'* mutant had more severely reduced H^+ fluxes than *'Col'* roots after thaxtomin A incubation suggests a greater susceptibility of the '*ucu2-2-gi2*' mutant to thaxtomin A.

One question that remained to be answered about the '*ucu2-2-gi2*' mutant was whether it was just a weak under performing *A. thaliana* mutant sensitive to a range of compounds and toxins or was this sensitivity specific to thaxtomin A. The finding that '*ucu2-2-gi2*' had comparable or slightly higher tolerances to two other unrelated plant-pathogenic toxins, alternariol and fusaric acid, as *'Col'* wild-type indeed confirmed that '*ucu2-2-gi2*' sensitivity to thaxtomin A was specific to this molecule and not a whole range of other toxins.

The susceptibility of *'ucu2-2-gi2'* to the auxin source, specifically 2,4-D, indicates that '*ucu2-2-gi2'* is potentially both an 'auxin' and 'thaxtomin A' sensitive mutant, linking auxin sensitivity with thaxtomin A sensitivity. Increases in root hair density with thaxtomin A treatments (Chapter 2) and root hair production patterns in

the *'ucu2-2-gi2'* mutant may also provide evidence of a thaxtomin A – auxin interaction. It is known that only some root epidermal cells, called trichoblasts, are destined to develop root hairs (Peterson and Farquhar 1996; Gilroy and Jones 2000). Recent molecular studies have suggested that there are at least two factors that might control the cell decision to become a trichoblast or not. One is the degree of vacuolation (Galway *et al.* 1997), and the other is cell hormonal status (specifically, ethylene and auxin balance)(Masucci and Schiefelbein 1994). Given the fact that the auxin-sensitive mutant *'ucu2-2-gi2'* was affected more by thaxtomin A is supportive of the "hormonal" scenario in mediating thaxtomin A effects on root hair initiation.

4.4.3 Auxin sources (2,4-D) reduce thaxtomin A-induced seedling mortality in A. thaliana

Thaxtomin A, which is structurally quite similar to IAA (Fig. 4.1) and also has a proposed similar biosynthetic pathway (King and Lawrence 1996), may interact with an auxin-receptor molecule. Qualitative evidence whereby 2,4-D reduced specific lines of *A. thaliana* mortality caused by thaxtomin A provided initial evidence of auxin negating thaxtomin A toxicity. Once again, the susceptibility of *'ucu2-2-gi2'* to both thaxtomin A and/or 2,4-D was demonstrated. The differential saving of particularly the *'aux'* based lines and to an extent the Col line contrasts with the inability of auxin to save the *'ucu2-2-gi2'* and *'axr'* based lines. Previously in our lab we have also observed the amelioration of thaxtomin A toxicity using IAA and NAA in *A. thaliana* and tobacco cells using a subjective chlorosis and necrosis scoring scale (Wilson 2005). Both sources of evidence, whether in this thesis with 2,4-D or reported elsewhere with NAA and IAA (Wilson 2005) suggest amelioration of thaxtomin A toxicity with auxin sources, although quantitaive data or a better model system (see 4.4.4) may better demonstrate these outcomes.

4.4.4 Pollen assay quantifies amelioration of toxic effect by thaxtomin A

The tomato pollen tube growth assay represents a quick, efficient and sensitive test for assaying the toxic effect of thaxtomin A (Chapter 2; Tegg *et al.* 2005). Using this assay in combination with the synthetic auxin, NAA, provided further substantial and definitive evidence that thaxtomin A toxicity can be ameliorated by auxin treatment (Fig. 4.10). NAA, which is not as powerful as 2,4-D was able to restore or prevent pollen tube growth inhibition caused by thaxtomin A,

in a quantifiable manner without the added confounding effects (callus etc...) that may be induced by a compound like 2,4-D. The specificity of this interaction was confirmed when pollen tube growth inhibition induced by two unrelated toxins, alternariol and fusaric acid, couldn't be restored by NAA treatment. The findings of this assay presented in this thesis chapter represent the first quantitative proof of a thaxtomin A/auxin interaction.

4.4.5 Differential responses to two key auxin transport inhibitors links thaxtomin A interaction with NPA binding protein

The resistance of particularly *'ixr1'* and *'txr1'* mutants to NPA implies a major interaction between these two similarly related toxin resistant mutants and this auxin transport efflux inhibitor (Fig. 4.10). The specificity of this interaction was confirmed with the finding that *'ixr1'* and *'txr1'* had no enhanced resistance to TIBA, another transport inhibitor that acts at the auxin efflux carrier (Lomax *et al.* 1995). This helps to pinpoint further auxin-thaxtomin interactions as TIBA and NPA have differing modes of action reflected by their structural make-up (Lomax *et al.* 1995; Friml and Palme 2002).

As stated earlier, auxin efflux carrier sites nor the mode of action of inhibitors acting on these sites are fully understood (Muday and DeLong 2001; Cooke *et al.* 2002; Friml and Palme 2002; Muday and Murphy 2002). However, Cooke *et al.* (2002) was able to conclude that NPA may function as a general inhibitor of secretory processes (by binding to a putative NPA-binding protein) associated with the auxin efflux carrier site. This contrasts to TIBA which has its binding site directly associated with the auxin efflux carrier site (Lomax *et al.* 1995) and distinct from the putative NPA-binding protein. It is important to note that auxin transport and binding studies indicate that all types of auxin transport inhibitors act at a site distinct from the auxin binding site on the efflux carrier i.e. they are non-competitive with auxins (Lomax *et al.* 1995). This combined with the earlier lack of response to general auxin sources suggests that both *'ixr1'* and *'txr1'* have a modified response to polar auxin efflux rather than auxin itself, potentially similar to that of the 1-Napthylphthalamic acid resistant mutant such as *'tir3'* (Ruegger *et al.* 1997). This may suggest that the interaction of thaxtomin A may be specific to the NPA-binding protein of the auxin efflux carrier (Fig. 4.10).

NPA is known and used as a herbicide, inhibiting polar auxin transport it causes an abnormal accumulation of IAA in plant meristems, leading to growth inhibition, loss of tropic responses, and plant death (Grossmann 2003). That both *'ixr1'* and *'txr1'* have resistance to this compound may suggest that both the herbicides, isoxaben and thaxtomin A share some common mode of action with NPA.

4.4.6 Comparison of other cellulose inhibiting compounds with thaxtomin A

Testing given *A. thaliana* mutants and identifying common resistances across toxins, can aid in clarifying or differentiating common modes of action between toxins. Indeed, similarity of responses of *'txr1'* and *'ixr1'*, with enhanced multiple resistance to both isoxaben and NPA suggests that thaxtomin A and isoxaben share a common mode of action. However, *'txr1'* was the only line found to have resistance to thaxtomin A. It is known that *'txr1'* lacks a TXR1 gene product, with the product suspected to be involved in a transport system (Scheible *et al.* 2003). Therefore, both isoxaben and NPA must utilise this transport system in some manner. In contrast, the *'ixr1'* mutant has an altered component of a cellulose synthase gene (Scheible *et al.* 2001). It is probable that this altered target enzyme may not be a target of thaxtomin A, hence the lack of cross-resistance of *'ixr1'* to thaxtomin A. Nevertheless, it is probable that there is a commonality in the modes of action of thaxtomin A, isoxaben and NPA. Other researchers have postulated similar outcomes with other cellulose synthesis inhibitors observed to have a common mode of action, specifically TZ and isoxaben (Sharples *et al.* 1998; Scheible *et al.* 2001). Furthermore, the common theme of enhanced sensitivity of *'ucu2-2-gi2'* to thaxtomin A, isoxaben and NPA may also suggest a linkage in the modes of action of these compounds.

That given *A. thaliana* mutants responded differently to two known cellulose synthesis inhibitors, isoxaben and dichlobenil (King *et al.* 2001), confirmed that these toxins may have different modes of action. Whilst *'txr1'* and *'ixr1'* had enhanced resistance to isoxaben and NPA, and *'ucu2-2-gi2'* had enhanced susceptibility to isoxaben and NPA, none of these mutants had altered resistance to dichlobenil. It therefore appears that isoxaben is more intimately linked to thaxtomin A than with the mode of action of dichlobenil (Fig. 4.10).

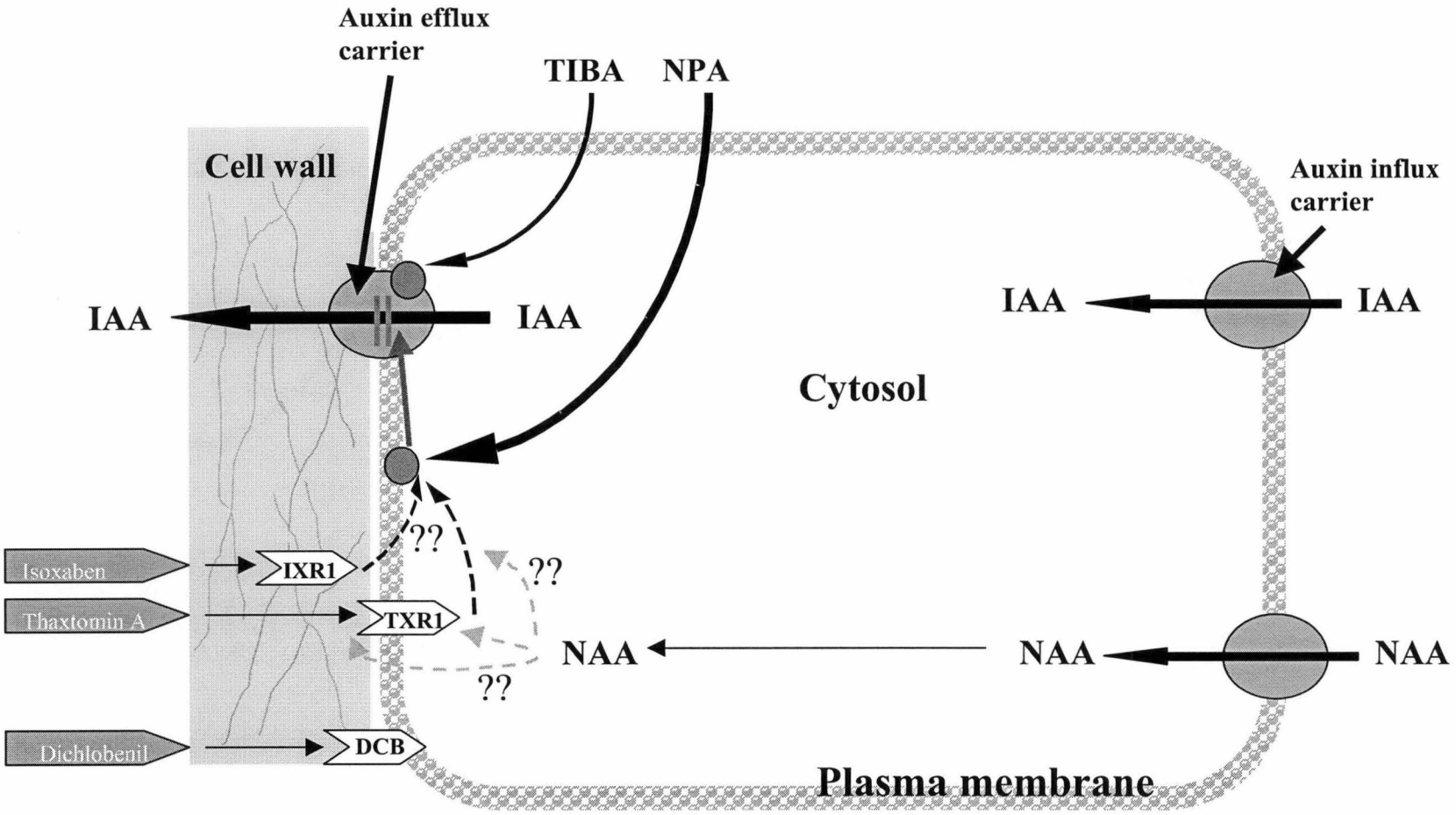


Fig. 4.10. Proposed cellular model demonstrating interaction of cellulose inhibiting compounds (thaxtomin A, isoxaben and dichlobenil) and their associated receptors (TXR1, IXR1, DCB) with the auxins (IAA and NAA) and compounds inhibiting polar auxin transport efflux (NPA and TIBA). Two key interactions are 1) IXR1 and TXR1 with the NPA binding site; and 2) NAA interaction with thaxtomin A, either by impacting on thaxtomin A directly, thaxtomin A receptor (TXR1) and/or subsequent cascades after binding. Also note the physical closeness of IXR1 and TXR1 receptors indicating potential similarities in mode of action, in contrast to DCB.

4.4.7 Conclusions

Evidence presented in this chapter has further substantiated the findings of Chapter 3 whereby the phenomena of herbicide/auxin induced disease resistance within the common scab pathosystem was demonstrated. Further, it confirmed the initial findings of auxin suppressing thaxtomin A toxicity within potato tissues using a series of both qualitative and quantitative measures. A key interaction was identified linking thaxtomin A resistance with increased resistance to the auxin transport inhibitor, NPA.

Chapter 5. Thaxtomin Resistance

5.1 Introduction

Potato calli and plantlets with enhanced resistance to thaxtomin A have been developed through cell selection techniques and they may be crucial in reducing the impact of common scab on the Australian potato industry (Wilson *et al.* 2004; Wilson and Eyles 2004). However, the mechanism and magnitude of resistance to thaxtomin A are respectively not understood or have not been accurately quantified.

In the model plant species, *Arabidopsis thaliana*, mutants resistant to thaxtomin A have been selected using ethyl methanesulfonate-mutagenized populations. Increased thaxtomin A resistance has been characterised by a stop (point) mutation in the TXR1 gene that can be readily identified from a genetic perspective (Scheible *et al.* 2003). In potato, homologs to *A. thaliana* TXR1 have been found by pairwise comparison of sequences (A. Conner, *pers. comm.*).

Potatoes with enhanced resistance to both common scab and thaxtomin A have been developed in our laboratory using a novel *in vitro* cell selection technique (Wilson *et al.* 2004). However, the random nature of the cell selection technique may make characterisation of any subsequent mutation difficult. A mutation may or may not have occurred in a TXR1 homolog, other changes may have occurred in other genes that may alter thaxtomin perception, transport or degradation e.g.glucosyl transferase (Acuna *et al.* 2001). Looking at a related toxin such as isoxaben, point mutations in the IXR1 gene are responsible for modification of cellulose synthase which confer resistance to isoxaben (Scheible *et al.* 2001).

There is already underlying variation in cultivar sensitivity to common scab disease that have been widely reported (Bjor and Roer 1980; Gunn *et al.* 1983; Calgari and Wastie 1985; Marais and Vorster 1988; Goth *et al.* 1995). However, these cultivars sensitivities to thaxtomin A, a major pathogenicity determinant of common scab (Healy *et al.* 2000) are not definitively known. What little work that has been done has concluded a correlation exists between resistance to thaxtomin A and resistance to common scab disease (Delserone *et al.* 1991; Acuna *et al.* 1998), making thaxtomin A sensitivities a useful attribute for selection within breeding

programmes (Acuna *et al.* 2000). Further knowledge within this area would enable more detailed focus by plant breeders on what properties need to be enhanced within breeding programmes, whether that be general physical skin based resistance to the pathogen or specific physiological enhancement of resistance to thaxtomin A.

Measuring and accurately quantifying resistance to toxins can be undertaken using whole plant or plant cell systems. Heim *et al.* (1989) when measuring resistance across a mutagenized derived population of *A. thaliana* to the cellulose synthase inhibiting herbicide isoxaben (Scheible *et al.* 2001) was able to effectively determine different levels of resistance to isoxaben using both shoot and callus growth curves (Heim *et al.* 1989). Likewise, in bean and soybean calli resistance levels to isoxaben has been quantified (Diaz-Cacho *et al.* 1997, 1999; Corio-Costet *et al.* 1991). Measuring differences in sensitivity to the toxin thaxtomin A across a population of potato regenerants, Wilson *et al.* (2004) used a number of different assays including necrosis leaf assays and tuber slice assays, however both are subjective (qualitative) measures. Calli growth curves on thaxtomin A media may more accurately quantify resistance levels across selected thaxtomin resistant potato calli and plantlets.

This work aims to quantify the level of resistance to thaxtomin A between potato plant cultivars, within cultivars of specially bred thaxtomin A-resistant potato plants and calli, and identify whether any genetic change within the putative TXR1 homolog gene in potato may be responsible for altered levels of resistance to thaxtomin A in these mutant lines.

5.2 Materials and methods

5.2.1 Sensitivity of potato cultivars to thaxtomin A using tuber slice assay

In trial 1, mini tubers including the varieties 'Bismark', 'Coliban', 'Desiree', 'Maris Piper', 'Pontiac', 'Russet Burbank', 'Shepody' and 'Tasman', were grown and supplied by Department of Primary Industries, Water and Environment, Devonport. In trial 2, mini tubers including the varieties 'Desiree', 'Russet Burbank', 'Shepody' and selected disease resistant mutant lines derived from 'Russet Burbank' and 'Desiree' (Wilson *et al.* 2004) were grown on site at Department of Primary Industries, Water and Environment, NewTown. In both trials tubers were stored at 4°C for four-six weeks, before being tested for thaxtomin tolerances using the tuber slice assay test (3.2.6.3). For each cultivar (or mutant line) being tested, eight tuber slices were cut from two tubers, and 1-3 disks infiltrated with either 28 or 57 μM thaxtomin A, were placed onto these slices; which were then placed into petri dishes. A necrosis rating assessment (3.2.6.3) was carried out after 7 days and the diameter of the circular necrosis lesion was also measured with a metal ruler.

5.2.2 Potato calli resistance to thaxtomin A

Callus cultures of 'Russet Burbank', 'Shepody' and thaxtomin resistant regenerates derived from 'Russet Burbank' and 'Shepody' were used to conduct tissue culture growth tests. The procedure, modified from Heim *et al.* (1989), involved weighing callus pieces (90-120 mg) and their placement on PCM5 media (Appendix 1), which is specific for maintaining and growing potato callus. The media was amended with different concentrations of thaxtomin A.

Five replicates in individual 5 cm Petri dishes were run for each test point. These dishes were incubated in the light at 26°C for 21 d; the callus was then removed and again weighed. The increase in weight divided by the initial weight was calculated, averaged over the five replicates, and normalized to the value obtained for the same genotype without thaxtomin A.

5.2.3 Molecular characterization of thaxtomin resistance

5.2.3.1 Nucleic acid extraction from plant tissues

Total nucleic acid extracts of samples were used as templates for PCR. The extraction procedure as described by Smith *et al.* (1992) was modified slightly and used in this study. Leaves (approximately 100 mg) were placed in a ceramic mortar (10 cm diameter) and ground in liquid nitrogen with a ceramic pestle, and the powder transferred into a 2.0-ml sterile Eppendorf tube. Then, 500µl of nucleic acid extraction buffer (25mM Tris-HCl (pH 8.0), 75mM NaCl, 1mM EDTA, 5% polyvinylpolypyrrolidone, 1% SDS), was added to each sample, and this was mixed briefly by inverting. Then, 500µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added, briefly vortexed, and centrifuged (13,000 rpm, 4°C) for 10 min. The aqueous supernatant was transferred to a 2.0-ml sterile Eppendorf tube and re-extracted by adding an equal volume of chloroform/isoamyl alcohol (24:1), and centrifuged (13,000 rpm, 4°C) for 10 min. The aqueous phase was transferred to a 2.0-ml sterile Eppendorf tube and if still discoloured it was extracted again in chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated from the aqueous layer with addition of 0.1 volume 3M sodium acetate (pH 5.2) and 2 volumes ice-cold 100 % ethanol. These tubes were placed in freezer (-80°C) for ~2 hrs. Pellets were recovered by centrifugation at 15,000 rpm (4°C) for 10 min. The pellet was washed with 250 µL ice-cold 75% ethanol, briefly spun, and ethanol removed carefully with pipette. The Eppendorf tube was left open to air-dry (15 min) and the pellet was resuspended in 50 µL sterile deionized water (DEPC treated), and stored at -20°C.

5.2.3.2 Primer development

Three primer sets were initially developed and used based on the complete genomic sequence of the *Arabidopsis thaliana* thaxtomin resistance protein TXR1 (At3g59280) gene (Table 5.1).

Table 5.1. Primers used for polymerase chain reaction (PCR) to detect *Arabidopsis thaliana* thaxtomin resistance gene TXR1 (At3g59280).

Primers[#]	Polarity	Sequence	Position
txr1-1S	+	5′-TGGTCATCGGTGTTCC-3′	53-68
txr1-1A	–	5′-ATGGCCGTTTTGTAAG-3′	1605-1590
txr1-2S	+	5′-TCGTATTCGCAGTG-3′	554-567
txr1-deg1A	–	5′-TYADSWWGGDGTWCC-3′	1499-1485
txr1-deg1S	+	5′-GCTGGWMGNYTNGC-3′	795-811

[#] Predicted product size of the primers txr1-1S & txr1-1A is 1553 bp; txr1-2S & txr1-1A is 1052 bp; txr1-deg1S & txr1-deg1A is 650 bp.

Further primer sets were developed based on the cDNA sequences from potato (*Solanum tuberosum*) (Table 5.2). The specific sequence targeted was from the SGN database (SGN-U267789) as this was identified as a homolog to *A. thaliana* TXR1 by pairwise comparison of sequences. Primers both within and external to the described coding region of the gene were developed (Table 5.2).

Table 5.2. Primers used for polymerase chain reaction (PCR) to detect thaxtomin A resistance homologs from *Solanum tuberosum*. Primers developed based on identified TXR1 homolog - SGN-U267789.

Primers[#]	Polarity	Sequence	Position
		External primers (outside the coding region)	
mag -1S	+	5′-AAAAATCATTTGAATGGAC-3′	1-19
mag -1A	–	5′-GACAAAAGATCCAAGG-3′	564-549
mag -2S	+	5′-ATCATTTGAATGGACAT-3′	5-21
mag -2A	–	5′-CAAGGAACTAACAAGGT-3′	553-537
mag -3S	+	5′-TTCGCGTTCCCTCT-3′	79-92
		Internal primers (within the coding region)	
MAG -2S	+	5′-TCAGCAATGGCTGC -3′	141-154
MAG -2A	–	5′-TTATTTTGTTTCTGGTTC -3′	491-474
MAG -3S	+	5′-ATGGCTGCAAAAATTCTTG-3′	147-165
MAG -3A	–	5′-TTCCAAACACTCTTTAGC -3′	455-438

[#] Predicted product size using the external primers of mag-1S & mag-1A is 564 bp + nc; mag-2S & mag-2A is 549 bp + nc; mag-3S & mag-2A is 475 bp; and for the internal primers of MAG-2S & MAG-2A is 351 bp + nc; MAG-3S & MAG-2A is 345 bp + nc; MAG-3S & MAG-3A is 309 bp + nc. nc: non-coding (intron) region of unidentified length.

5.2.3.3 Polymerase chain reactions (PCR's)

All PCR reactions (Amplifications) were carried out in a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Wellesly, MA, USA). Reaction mixtures and cycling profiles used for PCR amplification varied with plant species used as described.

Arabidopsis thaliana

For *Arabidopsis thaliana* the PCR amplification was performed in 25 µl of reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM each of the four dNTPs, 3.0 mM $MgCl_2$, 2% dimethyl sulfoxide and 1.0 unit of *Taq* DNA Polymerase (Qiagen Inc., Valencia, CA, USA). Concentrations of the primer pairs used were 0.5 µM and for each reaction 1.0 µl of template DNA was used. The PCR cycling profile was one cycle at 90°C for 2 min, followed by 40 cycles of 90°C for 30 sec, 42°C for 2 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. Manual hot-starts were sometimes undertaken to optimise reactions i.e. prevent non-specific primer binding, and this was achieved by placing the template DNA into the reaction mixture only when it has reached 90°C, at the start of the PCR cycle.

Solanum tuberosum

For *Solanum tuberosum* the PCR amplification was performed in 50 µl of reaction mixture containing 25 µl of HotStarTaq Master Mix (Qiagen, CA, USA) which provided a final concentration of 2.5 units HotStarTaq DNA polymerase, 0.2 mM each of the four dNTPs, 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.7), and 50 mM KCl. Concentrations of the primer pairs used were 0.5 µM and for each reaction 0.5 to 1.0 µl of template DNA was used. The PCR cycling profile was one cycle at 95°C for 15 min, followed by 35 cycles of 94°C for 1 min, 42°C for 30 sec, and 72°C for 1 min, with a final extension step at 72°C for 10 min.

5.2.3.4 Electrophoresis

Aliquots (2 µl) of PCR-amplified fragments were electrophoresed at 100V (BIO-RAD PowerPac Universal™) in 1% agarose gels containing 0.5 µg/ml ethidium bromide in Tris-acetate (TAE) buffer (0.04 M Tris base, 0.04 M glacial acetic acid, 0.001 M EDTA, pH 8.0). A 100-bp (Bio-Rad, California) and/or 1-kb DNA ladder

(BioLabs, New England) was used as a nucleic acid marker. After electrophoresis, gels were analysed using a 2011 Macrovue Transilluminator (LKB Bromma). Gels were photographed with a Kodak DC290 Digital Zoom camera with a 49 mm ultraviolet lens, images were viewed and manipulated with Kodak 1D Limited Edition v.3.5.4 Scientific Imaging Systems software (New Haven, CT, USA) and Adobe® Photoshop® Version 6.0 (Adobe Systems Incorporated, USA).

5.2.3.5 Sequencing of amplified fragments

PCR reactions that produced a single band of the correct size were purified directly from the PCR reactions (QIAquick® PCR Purification Kit, Catalog no. 28106, Qiagen). Where multiple distinct bands were produced, DNA was excised and purified from the agarose gel (QIAquick™ Gel Extraction Kit, Catalog no. 28704, Qiagen).

For PCR reactions from *Solanum tuberosum*, cloning was necessary to obtain suitable clean sequence readings (TOPO TA Cloning® Kit containing pCR®2.1-TOPO® vector and TOP10 OneShot® Chemically Competent cells, Catalog no. K4500-40, Invitrogen™) with Plasmid DNA then purified (QIAprep® Spin Miniprep Kit, Catalog no. 27104, Qiagen).

5.2.3.6 Sequencing reactions

In preparation for sequencing, DNA quantities of the products were determined by running them in a gel alongside a Precision Molecular Mass Standard (Catalog no. 170-8207, Bio-Rad). The purified PCR products (2.5-5 µl) were then sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), with a Sodium acetate/ethanol based clean up. Precipitated dry samples were posted to Griffith University DNA Sequencing Facility (Griffith University, Queensland) where they were sequenced using a 3130xl Capillary Electrophoresis Genetic Analyser (Applied Biosystems, USA).

Sequences were edited, aligned and characterised using the Bioedit Sequence Alignment Editor, version 7.0.5.2. (Hall 1999). Sequences were compared to known

sequences by utilising the BLAST searches of the National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894 and the SOL Genomics Network (SGN), Cornell University, USA.

5.2.4 Data analysis

Data were subjected to analysis of variance using Genstat 6 (Rothamsted Experimental Station, Harpenden, Herfordshire, UK). Significance was calculated at either $P = 0.05$ or $P = 0.01$ as noted, and least significant difference (LSD) was used for comparison of mean values. Data are presented as mean values for each treatment combination.

5.3 Results

5.3.1 Tuber slice assay for detection of cultivar susceptibility to thaxtomin A

In trial 1, tuber slice assays detected significant cultivar differences to thaxtomin A (57 µM) sensitivity ($P<0.05$) as measured through necrosis ratings and necrosis diameters (Table 5.3). 'Maris Piper' recorded the lowest necrosis rating of 2.93, this was significantly less ($P<0.05$) than 'Shepody', 'Tasman' and 'Russet Burbank'. 'Russet Burbank' recorded the highest necrosis rating of 3.48 and this was significantly higher ($P<0.05$) than all other cultivars tested. 'Shepody' recorded the smallest necrosis diameter of 8.67 mm (Fig. 5.1), this was significantly less ($P<0.05$) than all other cultivars. 'Russet Burbank' recorded the highest necrosis diameter of 11.00 mm (Fig. 5.1) and this was significantly higher ($P<0.05$) than all other cultivars tested.

Table 5.3. Effect of cultivar on necrosis rating and necrosis diameter from tuber slices exposed to 57 µM thaxtomin A for a period of 7 days. (n =15).

Cultivar	Necrosis rating from tuber slice assay	Necrosis diameter (mm)
Bismark	3.10^ab	10.00 b
Coliban	3.07 ab	9.93 b
Desiree	3.17 ab	10.20 b
Maris Piper	2.93 a	9.45 b
Pontiac	3.07 ab	9.80 b
Russet Burbank	3.48 c	11.00 c
Shepody	3.20 b	8.67 a
Tasman	3.20 b	10.07 b
LSD (0.05)	0.258	0.756

^ Necrosis rating was assessed using the scale: 0= no necrosis, 1= few brown flecks, 2= brown flecks in determined necrotic area, 3= brown necrosis and 4= brown to black necrosis.

In trial 2, tuber slice assays detected significant cultivar/selected mutant line differences to thaxtomin A (28 µM) sensitivity ($P<0.05$) as measured through necrosis ratings and necrosis diameters (Table 5.4). When comparing the three control cultivars, 'Russet Burbank' had the highest necrosis rating of 3.96 which was

significantly greater ($P<0.05$) than both 'Desiree' and 'Shepody' (Table 5.4, Fig. 5.2). 'Desiree' had a necrosis rating of 3.01 which was significantly higher ($P<0.05$) than the 2.82 rating of 'Shepody'. Consistent with trial 1, necrosis diameters of both 'Desiree' and 'Shepody' were significantly smaller ($P<0.05$) than that of 'Russet Burbank'.

A comparison of a small number of selected mutant lines showed variability in their response to 28 µM thaxtomin A, when compared to the controls (Table 5.4). Looking specifically at the two mutants that were used in the molecular studies (see 5.3.3.2), namely 'Russet Burbank' '22C' and 'A380', both showed some enhanced resistance to thaxtomin A compared with the control. In the case of '22C' it had a significantly lower ($P<0.05$) necrosis rating than the control, whilst for 'A380', it had a significantly smaller ($P<0.05$) necrosis diameter than the 'Russet Burbank' control (Table 5.4).

Table 5.4. Effect of cultivar/selected mutant lines on necrosis rating and necrosis diameter from tuber slices exposed to 28 µM thaxtomin A for a period of 7 days. (n =18).

Cultivar/selected mutant line	**Necrosis rating from tuber slice assay**	**Necrosis diameter (mm)**
Desiree (control)	3.01 b	9.64 ab
Desiree 15a	3.01 b	10.00 bc
Desiree A44	3.00 b	9.43 a
Russet Burbank (control)	3.96 d	10.78 e
Russet Burbank 17A	3.89 cd	10.32 cd
Russet Burbank 22C	3.85 c	10.47 de
Russet Burbank 24B	3.78 c	10.50 de
Russet Burbank A355a	3.89 cd	10.25 cd
Russet Burbank A365	3.99 d	10.22 cd
Russet Burbank A375	3.89 cd	10.56 de
Russet Burbank A380	4.00 d	10.01 bc
Shepody (control)	2.82 a	9.25 a
LSD (0.05)	0.106	0.417

^ Necrosis rating was assessed using the scale: 0= no necrosis, 1= few brown flecks, 2= brown flecks in determined necrotic area, 3= brown necrosis and 4= brown to black necrosis.

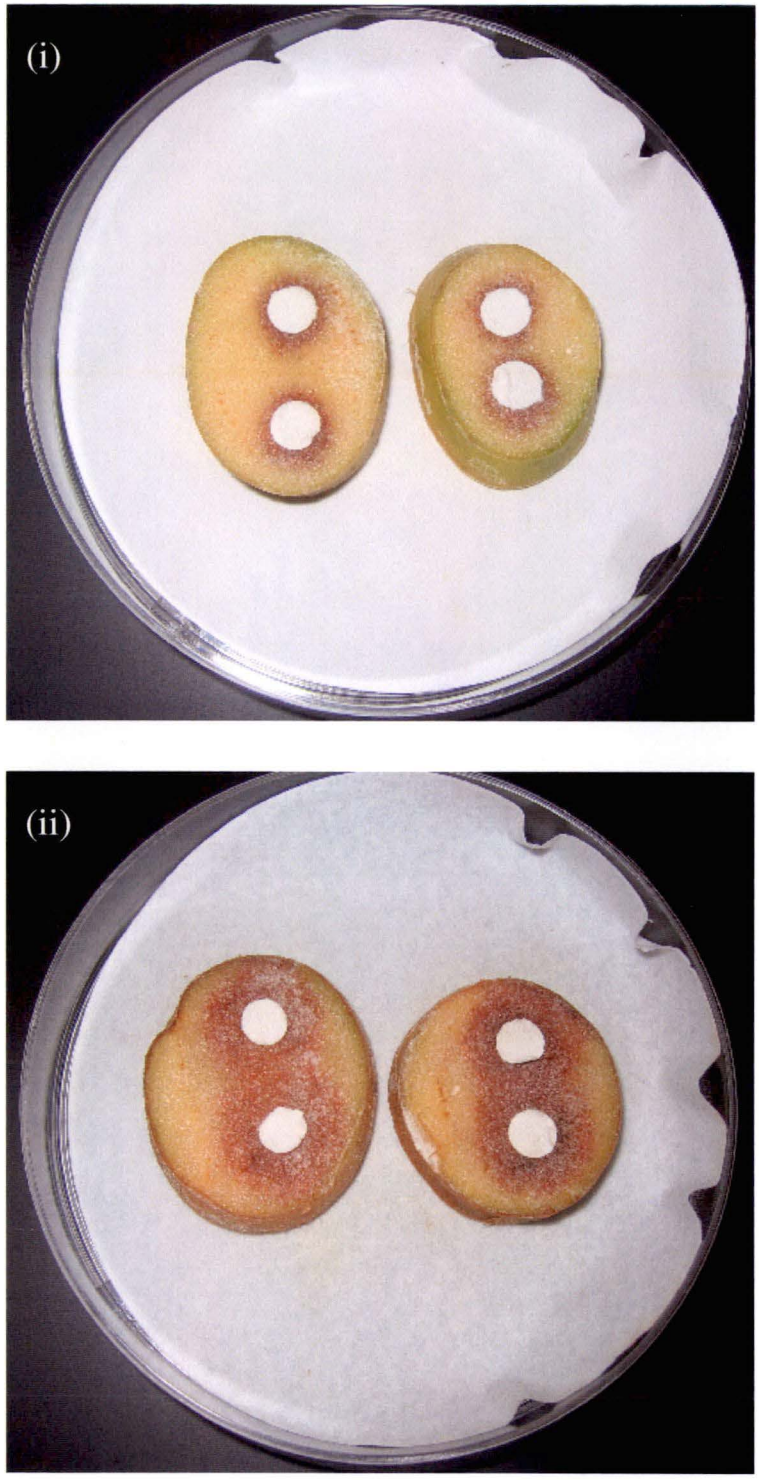


Fig. 5.1. Effect of incubation with thaxtomin A (57 µM) containing discs for 7 days on necrosis patterns from tubers of (i) 'Shepody', and (ii) 'Russet Burbank'. Petri dish diameter = 7cm.

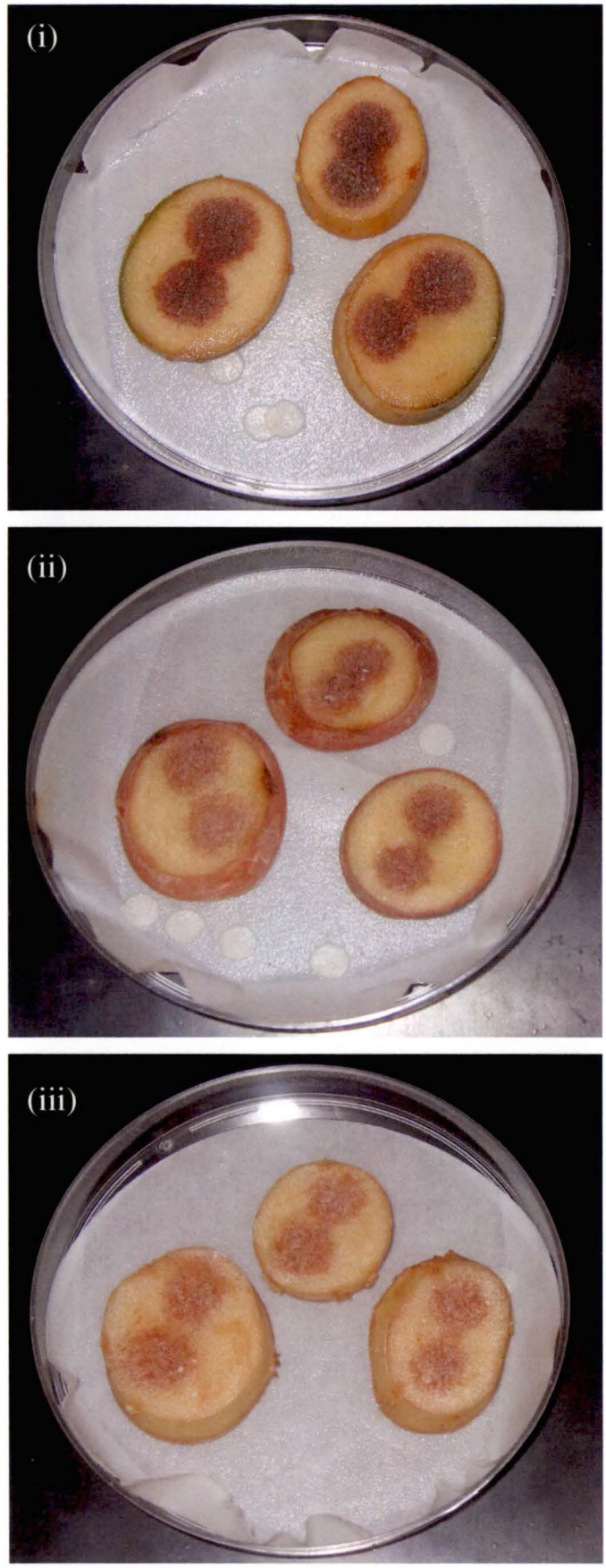


Fig. 5.2. Effect of incubation with thaxtomin A (28 μM) containing discs for 7 days on necrosis patterns from tubers of (i) 'Russet Burbank', (ii) 'Desiree', and (iii) 'Shepody'. Petri dish diameter = 7cm.

5.3.2 Potato calli growth susceptibility to thaxtomin A

Selected potato calli lines showed differential growth inhibition patterns when incubated on thaxtomin A containing media (Fig. 5.3). At relatively low thaxtomin A concentrations of 0.1 and 0.2 μM the 'Russet Burbank' control 'parent' produced the lowest callus growth which was significantly ($P<0.05$) less than some of the other lines. At 1 μM thaxtomin A, the 'Shepody' derived line '7A' produced significantly ($P<0.05$) greater growth than the 'Shepody' parent and the 'Russet Burbank' derived lines ('7X' and 'Russet Burbank' parent). At 2 μM thaxtomin A, the 'Shepody' derived lines ('7A', '7C' and 'Shepody' parent) produced significantly ($P<0.05$) greater callus growth than the 'Russet Burbank' derived lines ('7X' and 'Russet Burbank' parent). At 5 μM thaxtomin A, callus growth was inhibited severely such that there were no significant differences across all lines examined (Fig.5.3).

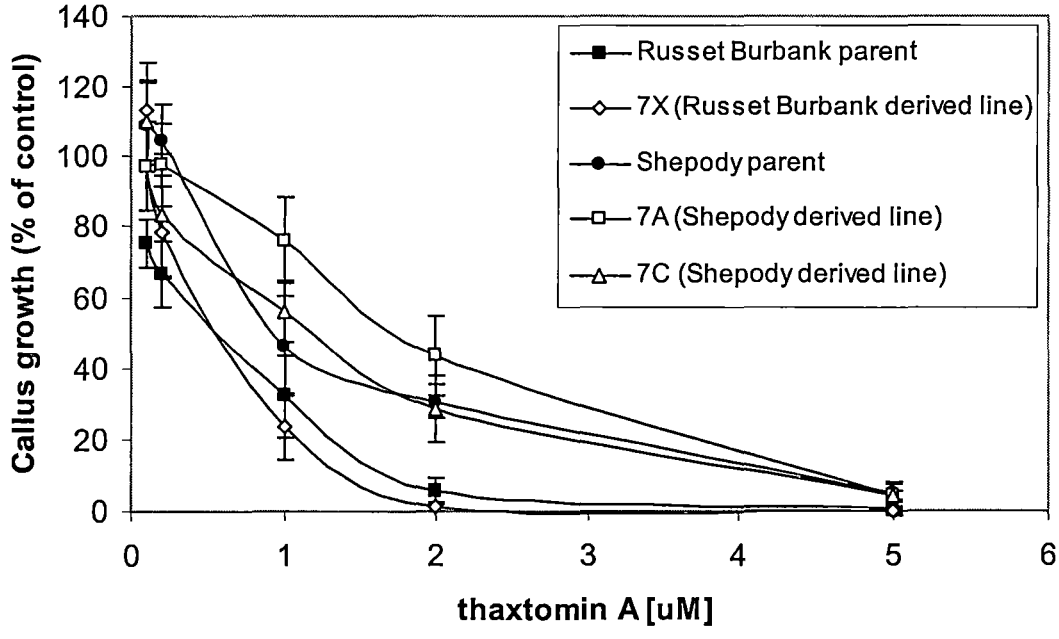


Fig. 5.3. Growth inhibition curves of tissue culture callus lines derived from *Solanum tuberosum*. Calli was grown on thaxtomin A amended media for 21d. Data are expressed as mean percentages ±SEM (n = 5) of control calli growth on medium with no thaxtomin A applied. Mean weights of control callus growth per plate are: 'Russet Burbank' parent – 840 mg, '7X' – 585 mg, 'Shepody' parent – 650 mg, '7A' – 620 mg, and '7C' – 605 mg.

5.3.3 Molecular characterisation of thaxtomin resistance

5.3.3.1 Arabidopsis thaliana

Running a nested PCR (with outer primer set txr1-1S : txr1-1A, followed by an inner primer set txr1-2S : txr1-1A, Table 5.1) produced 4 distinct bands from *A. thaliana* WT *'Col'*, 5 distinct bands from *A. thaliana 'txr1'* and 5 semi-distinct bands from *S. tuberosum* 'Russet Burbank' (Fig. 5.4).

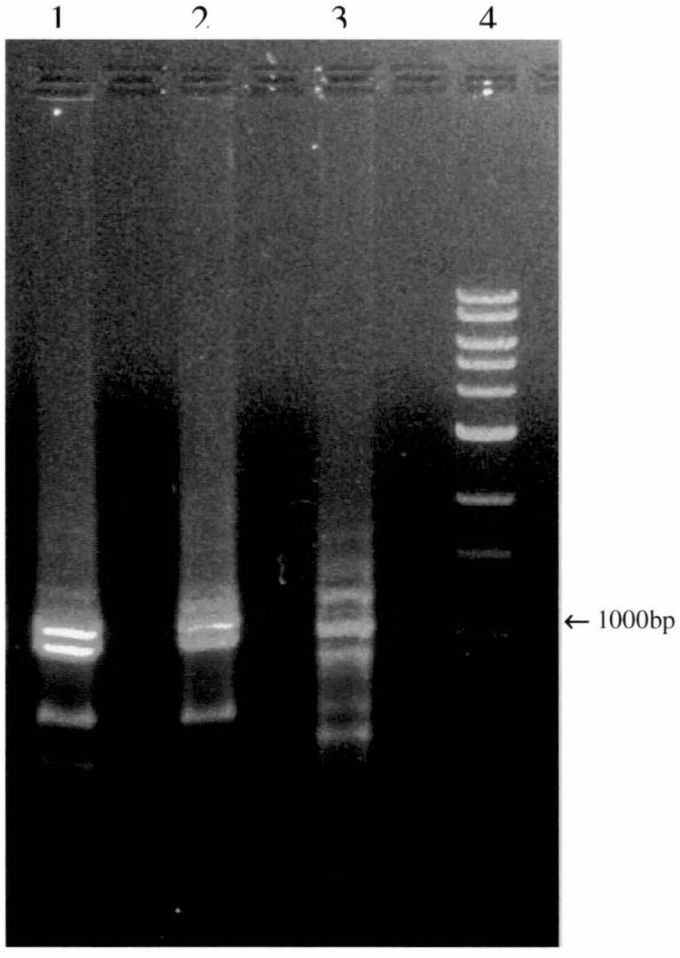


Fig. 5.4. Analysis of polymerase chain reaction (PCR) products of *Arabidopsis* and potato plant tissue run as a nested PCR (Primer set txr1-1S : txr1-1A followed by primer set txr1-2S : txr1-1A). Lane 1: *A. thaliana* WT *'Columbia'*; lane 2: *A. thaliana 'txr1'*; lane 3: *S. tuberosum* 'Russet Burbank'; lane 4: 1 kb + 100-bp DNA ladder.

These bands were excised, purified and amplified with primer set txr1-2S : txr1-1A, producing single distinct bands (Fig. 5.5). Partial sequencing of these products showed that lanes 1 and 2 (~1 kb from *A. thaliana* WT *'Col'*, Fig 5.5) and lanes 5 and 6 (~1 kb from *A. thaliana 'txr1'*, Fig. 5.5) were the *A. thaliana* TXR1

gene, with characteristic stop codon mutation (Scheible *et al.* 2003) in *A. thaliana* *'txr1'* (sequences not presented). Sequencing of similar size products from *S. tuberosum* (e.g. lanes 11 and 12, ~1 kb, Fig 5.5) gave DNA sequence data homologous to published potato DNA sequences but unrelated to the *A. thaliana* TXR1 sequence. The degenerate primer set txr1-deg1S : txr1-deg1A (Table 5.1) produced no clear distinct bands of the appropriate size from any plant species.

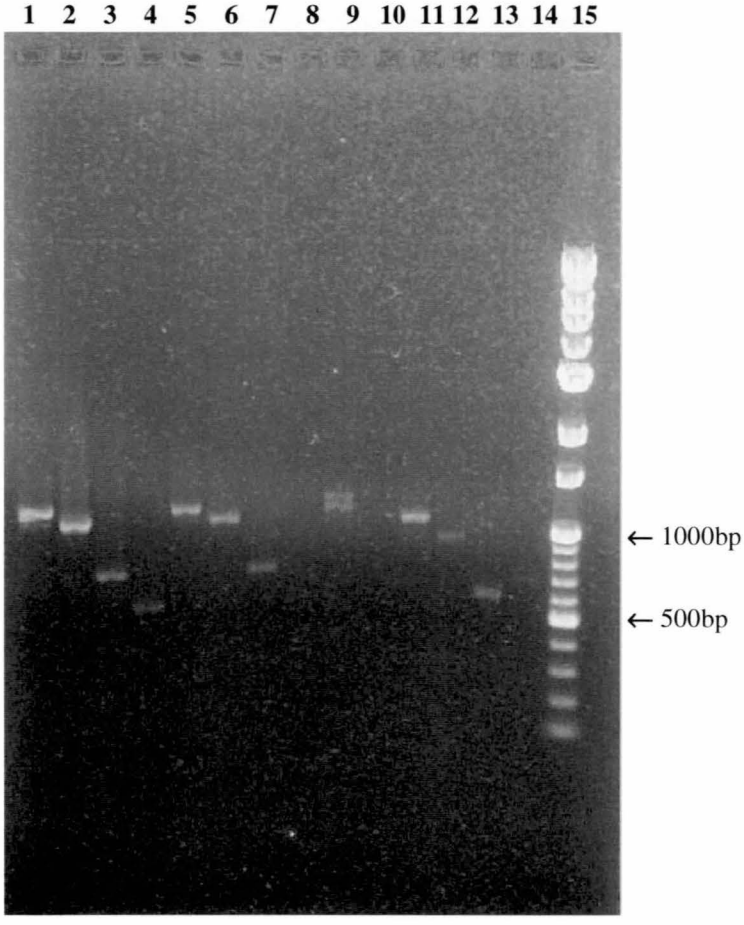


Fig. 5.5. Analysis of polymerase chain reaction (PCR) products of *Arabidopsis* and potato plant tissue. Bands are those cut out and purified from Fig. 5.4 and then run as a PCR with primer set txr1-2S : txr1-1A. Lanes 1-4: *A. thaliana* WT *'Columbia'*; lanes 5-9: *A. thaliana 'txr1'*; lanes 10-14: *S. tuberosum* 'Russet Burbank'; lane 15: 1 kb + 100-bp DNA ladder.

5.3.3.2 Solanum tuberosum

Using a potato homolog (SGN-U267789) to the *A. thaliana* TXR1 gene, external primer combinations (Table 5.2) were unable to amplify the desired gene product (SGN-U267789). Internal primers, specifically primer set MAG-3S : MAG-

2A (Table 5.2) were able to amplify the desired gene product (Fig. 5.6). The desired gene product consisted of known exon sequence (345 bp) plus intron region (unknown bp) giving a gene product of ~600 bp (Fig. 5.6, lanes 1-4). The amplified DNA was cloned producing a 2500 bp vector construct (Fig. 5.6, lanes 5-8) and sequenced. Five distinct allelic forms were identified from all lines examined, this included the parent line 'Russet Burbank' and the two somatically selected thaxtomin (scab) resistant lines designated '22C' and 'A380' (Fig. 5.7 and 5.8). All five allelic forms in all Russet Burbank lines examined (parent, '22C' and 'A380') were identical at the nucleotide (Fig 5.7) and amino acid level (Fig. 5.8). The most likely ratio of alleles present for allele numbers 1 thru to 5 are 3 : 2 : 1 : 1 : 1 respectively based on the 36 sequences obtained.

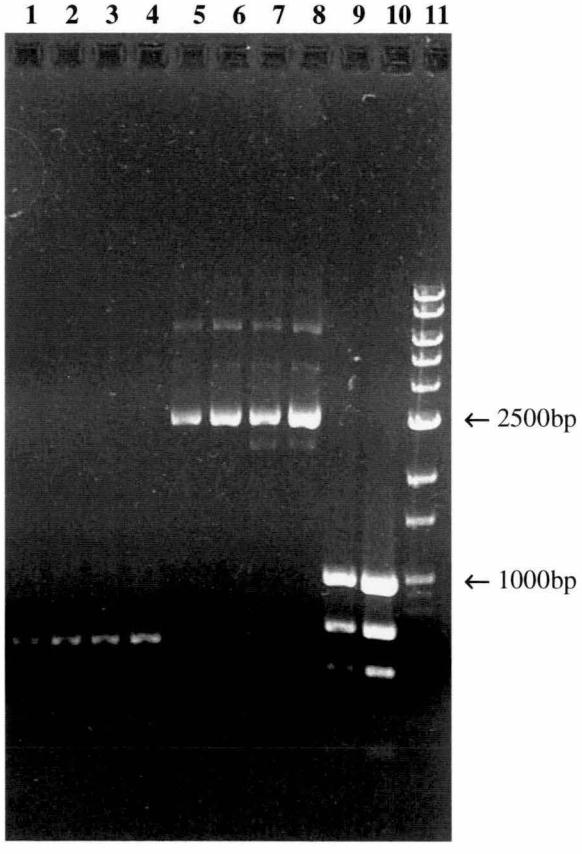


Fig. 5.6. Analysis of polymerase chain reaction (PCR) products of *Solanum tuberosum* 'Russet Burbank' using primer set MAG-3S : MAG-2A. Lanes 1 and 2: *S. tuberosum* 'Russet Burbank' control plants; lanes 3 and 4: *S. tuberosum* 'Russet Burbank' 'A380'; lanes 5 – 8: clones of lanes 1-4 respectively; lane 9: 1.25 µl molecular ruler; lane 10: 2.50 µl molecular ruler; lane 11: 1kb + 100-bp DNA ladder.

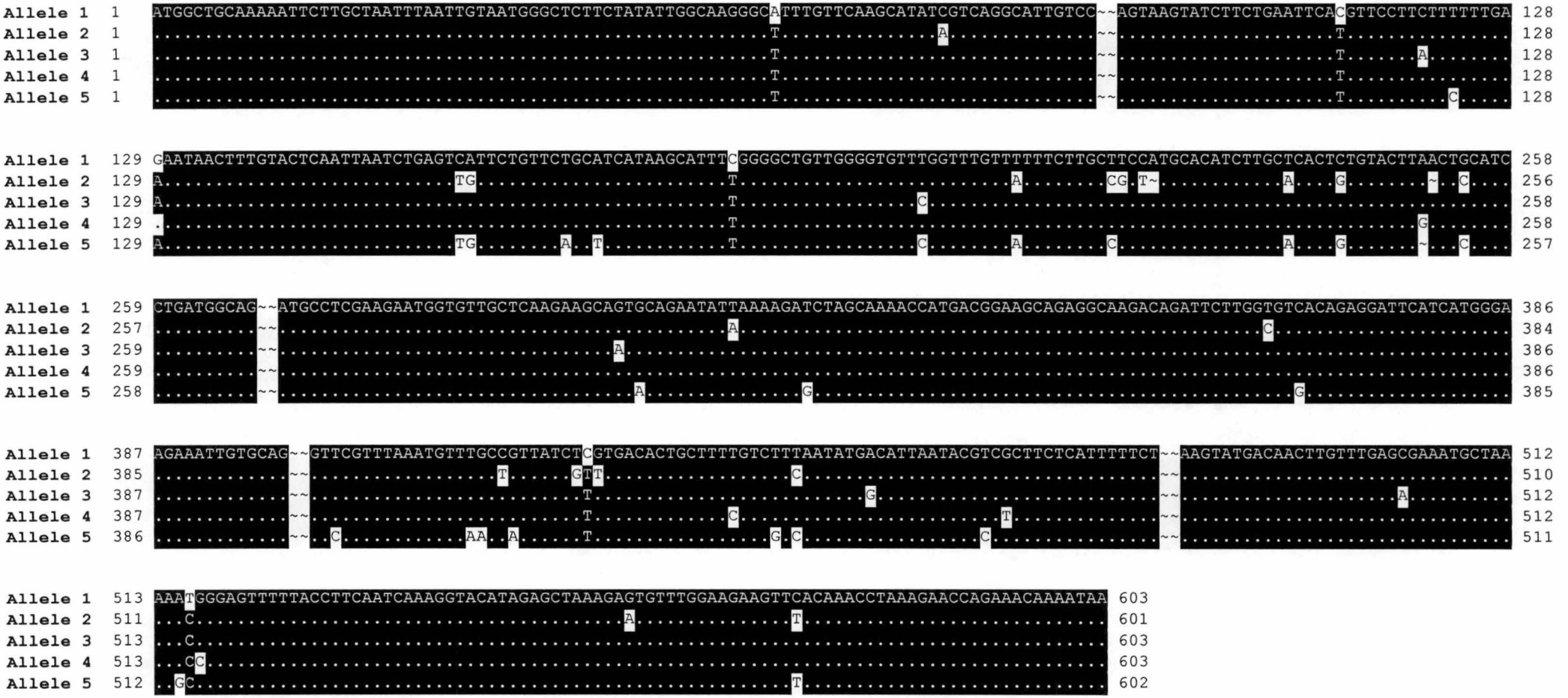


Fig 5.7. Allelic variants in Nucleotide Alignment of the putative *S. tuberosum* TXR1 homolog. The sequences were amplified using the primer set MAG-3S : MAG-2A (see Table 5.2) and subsequent cloning. Sequences consist of 3 exon and 2 intron regions separated by ~~. In the above sequence exon region 1 is 90 nucleotides (position 1 – 90); intron region 1 is 177 nucleotides (position 91 – 267); exon region 2 is 132 nucleotides (position 268 – 399); intron region 2 is 81 nucleotides (position 400 – 480); exon region 3 is 123 nucleotidess (position 481 – 603). These gap regions have been identified using SGN-U267789 (Cornell University, USA) with the 1st intron region identified between exon nucleotides 237 and 238 (SGN-U267789) and the 2nd intron region identified between exon nucleotides 368 and 369 (SGN-U267789).

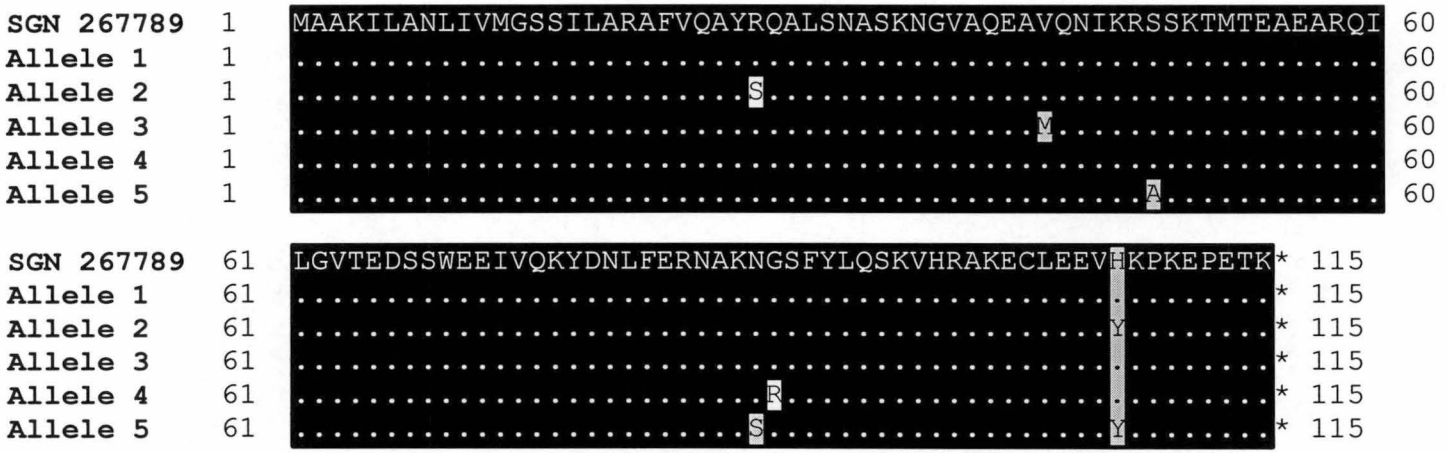


Fig. 5.8. Allelic variants in Amino Acid Alignment of TXR1 homolog from potato. This sequence corresponds with SOL Genomics Network (SGN) -U267789 (Cornell University, USA) from nucleotides 147-491 inclusive i.e. 115 amino acids. These sequences were amplified using the primer set MAG-3S : MAG-2A (see Table 5.2) and subsequent cloning. Two intron gap regions were identified from within this sequence, they are presented in Fig. 5.7.

5.4 Discussion

5.4.1 Cultivar susceptibility to thaxtomin A

Varietal or cultivar susceptibilities to thaxtomin A toxicity have rarely been studied or reported (Delserone *et al.* 1991; Acuna *et al.* 1998) so the data presented in this thesis chapter provides a novel insight into the common scab pathosystem. In contradiction to the previous two small published reports on this topic (Delserone *et al.* 1991; Acuna *et al.* 1998), the levels of resistance to common scab disease (Darling 1937; Lapwood *et al.* 1973; Goth *et al.* 1995; Park *et al.* 2002; Pasco *et al.* 2005; P. Hardman, *pers. comm.*) did not correlate with the levels of resistance to thaxtomin A (as measured through tuber slice assay and callus growth inhibition) reported in this thesis.

This is most readily demonstrated by the important processing cultivar 'Russet Burbank' which is regarding as having moderate common scab disease resistance (Darling 1937; Goth *et al.* 1995; Park *et al.* 2002; P. Hardman, *pers. comm.*) yet in these trials it was highly sensitive to thaxtomin A. This contrasts with the fresh market variety 'Desiree' and processing variety 'Shepody' which are regarded as highly susceptible to common scab disease (Lapwood *et al.* 1973; Park *et al.* 2002; Pasco *et al.* 2005; P. Hardman, *pers. comm.*) yet in these trials it showed high resistance to thaxtomin A. These findings are very interesting and may provide guidance for the development of future potato breeding programmes aiming to combat common scab disease. As an example, in 'Russet Burbank' its high sensitivity to thaxtomin A means improving this trait would be highly beneficial. On the contrary, 'Desiree' may have considerable thaxtomin A resistance already yet poor physical resistance to the pathogen itself, therefore a breeding programme aimed at improving physical resistance properties (skin russeting etc.) may be beneficial for overall common scab resistance for that cultivar.

The two key assays that were used to determine thaxtomin A sensitivity, callus growth inhibition and the tuber slice assay, produced consistent results and were able to distinguish significant differences between cultivars assessed, notably 'Shepody' and 'Russet Burbank' which were the focus of this study. These assays therefore serve as highly useful in future project needs (Wilson *et al.* 2004; Wilson

and Eyles 2004) to assess high numbers of regenerant plantlets for thaxtomin resistance.

There are however benefits and weaknesses of both assays. The callus growth inhibition assay does not require the bulking of tuber material, rather the callus is derived directly from a leaf (midrib) cutting off the plantlet, saving time in growing plants through to tubers. This assay also has the benefit of not being subjective, as callus is quantified by weighing. The negatives of this assay include difficulty in standardising callus growth. Also, as this assay occurs on undifferentiated plant callus there may be questions as to whether resistance measures are reflective of whole plant conditions. According to Heim *et al.* (1989) and their results with isoxaben resistant *A. thaliana* callus cultures and plantlets, sensitivity to isoxaben was not dependant on a differentiated plant state, indicating that this assay is highly effective and representative of the whole plant response to the toxin, isoxaben. The tuber slice assay has a couple of weaknesses, firstly the time required to bulk tubers and secondly the subjective nature of the assay. The major benefit is that this assay is carried out on the same plant material thaxtomin A targets, tuber tissue, so provides a true characterisation of toxin sensitivity.

As an aside, a limited number of root growth inhibition assays carried out (data not presented) proved to be ineffective at characterising thaxtomin A resistance, largely due to the sporadic and inconsistent nature of root production patterns by potato plantlets making this assay ineffective. In summary, the callus growth inhibition assay and the tuber slice assay proved to be useful and used in combination should be able to effectively screen and provide an accurate representation of thaxtomin A resistance across a population of potato plantlets.

5.4.2 Is disease/thaxtomin A resistance associated with mutation in a potato homolog of Arabidopsis *TXR1*

The TXR1 gene within *A. thaliana*, conferring resistance to thaxtomin A, was first characterised by Scheible *et al.* (2003). This gene has homologs from a wide ancestral range including all fully sequenced eukaryotes and many other plants (Scheible *et al.* 2003). A probable TXR1 homolog in potato has also been identified (A. Conner, *pers. comm.*). Although not fully understood, it is proposed that TXR1

is a regulator of a transport mechanism (Scheible *et al.* 2003). That *A. thaliana 'txr1'* is characterised by poor growth, when compared to its' parental stock (Scheible *et al.* 2003) suggests that the TXR1 gene may have an important and potentially essential function.

Part of this thesis chapter successfully cloned and characterised a related homolog of *A. thaliana* TXR1 from the potato, *Solanum tuberosum*. Of the lines tested, which included 'Russet Burbank' parent and two putative scab (thaxtomin A) resistant, regenerant lines, there was no differences in the TXR1 gene homolog found. This indicates that the random nature of the cell selection technique used to regenerate thaxtomin A resistant plants (Wilson *et al.* 2004) did not target this specific gene. There are further thaxtomin resistance genes reported (Scheible *et al.* 2003; R. Loria, *pers. comm.*) that have not been characterised. Perhaps the mutations in the regenerant lines exist within these other genes and therefore do not appear in the TXR1.

Of note, it was apparent that 5 allelic forms were characterised from both the parent (control) and the two putative thaxtomin A resistant lines. This may indicate that the TXR1 gene characterised could have more than 1 copy per genome and therefore ploidy might be eight. If there were only one copy of the gene per genome then it would be expected that only 4 alleles would be identified, typical of the tetraploid potato. The ratio of the 5 alleles identified (based on a ploidy of 8) served to provide a measure of the relative abundance of each allele. It is of note that allele number 1 which was the most common allele (⅜) corresponds with that presented in the SOL Genomics Network (SGN) database (SGN-U267789).

It is worth noting that the PCR reactions were not carried out with high fidelity polymerase with proof reading activity suggesting that sequencing errors may be a factor. However given that multiple PCR's and multiple lines examined provided identical sequence the likelihood of sequencing errors is low.

Whilst this work failed to identify any differences within the TXR1 gene homolog it was able to fully characterise all intron and exon regions within the amplified region of the gene. Introns are non-coding, intervening sequences of DNA

that are transcribed, but are removed from within the primary gene transcript and rapidly degraded during maturation of messenger RNA (Anon 2005d). The intron sequences for the TXR1 homolog in potato was not published within the SGN database as the published sequence was derived from analysis of cDNA only. The intron sequences for this specific TXR1 homolog (SGN-U267789), to our knowledge, have never been elucidated so this thesis has at least identified and sequenced such regions.

5.4.3 Conclusions

Differences in thaxtomin A resistances were detected intraspecifically within potato. This intraspecific or cultivar resistance to thaxtomin A did not mirror published resistances to common scab disease indicating other mechanisms (and not just thaxtomin A resistance) may be important in measuring overall resistance to the disease. Differences in resistance to thaxtomin A were also observed between parent lines and putative thaxtomin A (common scab) – resistant lines tested. This disease/thaxtomin A resistance did not coincide with any molecular change in an identified TXR1 homolog, suggesting increased resistance may be associated with other unknown genes and/or physical/physiological parameters.

Chapter 6. General Discussion

This thesis has provided a better understanding of thaxtomin A toxicity and resistance mechanisms by undertaking electrophysiological, pathological, physiological and molecular based studies.

Whilst this thesis provided answers to hypotheses presented in specific areas, it also identified scope for future work which may enable a greater understanding of the role of thaxtomin A in the common scab pathosystem. In this general discussion, key points and questions arising from some aspects of this thesis are identified with potential future experiments that may further address these queries discussed.

6.1 Ion signalling

Ion signalling produced some interesting outcomes and has opened up the door for future work (Tegg *et al.* 2004a; Tegg *et al.* 2005; Chapter 2). Whilst work in this thesis was associated with whole plant or intact roots response to thaxtomin A, the response of protoplasts to thaxtomin A would be worthwhile. This may provide further evidence for the candidate receptor site in the plasma membrane.

Notwithstanding, the response of whole plants to thaxtomin A was extremely important and the signature flux responses, Ca^{2+} spike (short-term) and the magnitude of H^+ flux suppression (long-term) may be effective markers for screening large populations of potato plantlets for sensitivity to thaxtomin A. Indeed MIFE has been used effectively for screening large populations of plants (barley selections) to other stresses (salt) (Chen *et al.* 2005). With the *A. thaliana 'txr1'* mutant which became available later in the project it would be interesting to observe whether it had the characteristic Ca^{2+} spike and H^+ flux suppression patterns as observed in the *'WT'*. Given that *'txr1'* only has moderate resistance to thaxtomin A, i.e. resistance is not total, these signature responses would probably be still observed, perhaps their magnitude may be altered.

6.2 Common scab – 2,4-D effects

The herbicide and auxin related compound 2,4-D significantly reduced common scab with no apparent effect on tuber morphological features. An effect of 2,4-D on thaxtomin A toxicity was demonstrated and it is tempting to suggest this may be the mechanism of resistance shown (Chapter 3). Whilst 2,4-D concentrations used were quite low it would be interesting to find out whether concentrations could be reduced still further, such that the sprays still remained effective in reducing common scab symptoms. A trial using a dilution series of 2,4-D concentrations may answer this hypothesis. On the contrary it would also be interesting to increase 2,4-D concentrations to see what levels are required to start affecting morphological (specifically lenticel and periderm) development significantly, as the auxin rates used in this thesis did not affect these features. Auxin does effect lenticular density and size if applied at a high enough rate (Singh and Paliwal 1985a,b; Badola *et al.* 1987), and undoubtedly would effect tuber development at higher rates.

6.3 Thaxtomin A – auxin interaction

The amelioration of thaxtomin A toxicity in tomato pollen by the synthetic auxin, NAA, was a key finding but its specificity may need to be further investigated. Whilst NAA did not ameliorate alternariol or fusaric acid treated pollen (Chapter 4), isoxaben or dichlobenil treated pollen was not investigated. Given the relatedness of responses of *A. thaliana 'txr1'* and *'ixr1'*, particularly cross-resistance to isoxaben and NPA, it is probable that isoxaben treated pollen could be saved by NAA. On the contrary, it is less likely that dichlobenil treated pollen would be saved by NAA, as based on studies in this thesis, dichlobenil does not appear as closely related as thaxtomin A and isoxaben.

6.4 Understanding mode of action of toxins using A. thaliana *mutants*

Whilst the response of various *A. thaliana* mutants to various toxins and transport inhibitors served to quantify similarities and/or differences between the modes of action of these compounds and mutants, certain mutants and compounds were not tested because of availability or time constraints. Of the mutants tested, the transport inhibitor response (TIR) mutants represent a large group of plants with various mutations effecting the outward polar movement of auxin from plant cells. Whilst *tir1* and *tir3* were sourced for this thesis, when tested against various transport inhibitors they failed to demonstrate documented resistance and thus further studies were not possible. Sourcing more recently characterised mutants from this group or related groups (now referred to as BIG mutants) may enable better characterised mutants to be tested against thaxtomin A and other cellulose inhibiting compounds.

Whilst the key cellulose synthesis inhibiting toxins tested within this thesis were thaxtomin A, isoxaben and dichlobenil, other related compounds would be well worth testing. Such compounds include 5-tert-butyl-carbamoyloxy-3-(3-trifluromethyl) phenyl-4-thiazolidnone (TZ) which is reported to share a common mode of action with isoxaben (Sharples *et al.* 1998; Scheible *et al.* 2001), other compounds include triazofenamide and quinclorac (Heim *et al.* 1998; Vaughn 2002). Testing the relatedness of theses compounds to thaxtomin A may provide further clues as to how thaxtomin A may act.

Likewise, there are an array of other auxin transport inhibitors that could potentially be tested, particularly some of the related phytotropins to NPA to see if there is a similar pattern of resistance as detected with the *'ixr1'* and *'txr1'* mutants.

6.5 Varietal differences in susceptibility to thaxtomin A

The cultivar variation in susceptibility to thaxtomin A was an interesting conclusion derived from a series of simple experiments measuring callus growth inhibition and tuber slice sensitivity to thaxtomin A (Chapter 5). That 'Shepody' had greater thaxtomin A resistance than 'Russet Burbank' was interesting, given that 'Russet Burbank' has undisputedly greater common scab resistance than 'Shepody'. It implies that additional resistance parameters, other than to thaxtomin A toxicity, are important in common scab disease resistance.

The mechanism or mechanisms underlying this intraspecific variation in thaxtomin A sensitivities were not investigated. Researchers have observed varietal or cultivar differences in the ability to glucosylate thaxtomin A into less toxic forms (Acuna *et al.* 2001) and this may be a potential mechanism of resistance worthy of further investigation. Another documented mechanism of resistance is provided by the *A. thaliana 'txr1'* mutant, which is presumed to have a reduced rate of thaxtomin A uptake (Scheible et al. 2003). However, given the major findings of key interactions presented in this thesis between thaxtomin A and auxin provides another area worthy of study.

Could thaxtomin A resistance be related to different levels of endogenous auxins produced by different varieties, for instance does 'Shepody' produce higher levels of auxin during the tuber initiation phase than 'Russet Burbank'. Monitoring auxin levels at tuber initiation may provide an explanation for other phenomena observed. Late planting within Tasmania has been correlated with decreased common scab occurrence (A. Pitt, *pers. comm.*). Late planting would result in an earlier onset of tuber initiation and this could potentially correlate with increased levels of auxin in the tuber at initiation compared to an early planting where plants would remain longer in the ground before initiating tubers. Experiments monitoring auxin levels whilst looking at parameters that may change tuber initiation (long photoperiod vs short photoperiod) may provide a definitive answer as to the mechanisms behind the success of late plantings.

6.6 Physical properties responsible for common scab resistance

Physical properties of the tuber itself responsible for common scab resistance are poorly understood but lenticel size, density and structure, and periderm properties have been correlated with disease resistance/susceptibility in other common potato diseases. Perhaps these interspecific differences in common scab resistance may be a function of these physical properties, given that thaxtomin A resistance does not necessarily correlate with common scab resistance. Future experiments detailing these physical parameters, although difficult, may enable a greater understanding of common scab resistance parameters, and therefore important traits useful in breeding programmes.

6.7 Genetic, proteomic and morphological/physiological basis of scab (thaxtomin A) resistant plantlets and callus

Resistant potato plants and callus cultures studied within this thesis were developed using a somatic cell selection technique with thaxtomin A (Wilson *et al.* 2004). Whilst resistance was characterised (Chapter 5) the nature of the resistance was not elucidated, possibly due to the random nature of the cell selection technique. It was shown that a TXR1 homolog was not altered between parent and a small selection of mutant progeny potato lines (Chapter 5). Future study may need to target other potential genes/proteins that may have been altered in the resistant lines. These may include uncharacterised thaxtomin resistance genes (Scheible *et al.* 2003; R. Loria, *pers. comm.*) and/or proteins such as glucosyl transferase (Acuna *et al.* 2001) which are reported to be associated with thaxtomin A resistance. Alternatively, a more general genetic analysis (e.g. microarray study) may be necessary to map changes that may have occurred in the resistant lines.

The potato calli lines studied in this thesis had a range of sensitivities to thaxtomin A (Chapter 5) but the morphological/physiological basis of this resistance was not studied. With other related cellulose-inhibiting herbicides such as isoxaben the basis of enhanced resistance has been studied, and there appears to be two distinct mechanisms. Firstly, habituation has been reported whereby resistant bean (*Phaseolus vulgaris* L.) calli alter the composition of their cell walls, decreasing cellulose content and increasing hemi-cellulose/other pectin content (Diaz-Cacho *et al.* 1999). The second mechanism is whereby the physiological change is caused by

an altered recognition (receptor) event (Scheible *et al.* 2001), and there is no alteration in cell wall compositions as reported in *A. thaliana* (Heim *et al.* 1989) and soybean calli (Corio-Costet *et al.* 1991).

Future work in characterising calli generated within our project (Wilson *et al.* 2004) may determine if the resistances to thaxtomin A are through habituation or altered receptor (target) mechanisms. On the basis that our calli have been maintained off 'thaxtomin A-containing' media for some time, yet they still maintain thaxtomin A resistance, it is assumed the mechanism of resistance is not habituation, but some other yet to be identified resistance mechanism.

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Appendix 1

ISP2 medium for cultivation of *Streptomyces*

Malt extract	10 g
Yeast extract	4 g
Glucose	4 g
Agar	15 g
Water: make up to	1 L

Adjust pH to 7.4 and autoclave

SAY Solution

Sucrose	20 g
L-asparagine	1.2 g
K_2HPO_4	0.6 g
Yeast extract	10 g
Water: make up to	1 L

Adjust pH to 7.2

PCM5 medium

MS salts and vitamins	4.43 g
Thiamine	0.40 mg
Nicotinic acid	0.45 mg
Folic acid	0.50 mg
Biotin	0.05 mg
Glutamine	200 mg
MES	500 mg
PVP	500 mg
Adenine	40 mg
Casein hydrolysate	100 mg
Mannitol	20 g
Glucose	20 g
Kinetin (1 mg/mL solution)	0.1 ml
NAA (1 mg/mL solution)	3.0 ml
GA_3 (1 mg/mL solution)	0.2 ml (add after autoclaving)
Final Volume	1 L
pH	5.8
Agar	6 g/L