AN INVESTIGATION OF VASCULAR DISCOLOURATION IN POTATO TUBERS (*SOLANUM TUBEROSUM* L.) TREATED WITH IMAZETHAPYR

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ABSTRACT

This thesis describes work undertaken to determine the effect of imazethapyr on the morphology and physiology of stored potato tubers. Imazethapyr is an effective sprout suppressant, providing control at least equivalent to industry standards, and the molecule has a very favourable toxicological profile. However, some treated and stored tubers have displayed increased incidence of vascular discolouration. This work identifies links between imazethapyr treatment and vascular discolouration and the conditions under which it may occur.

Imazethapyr treatment was found to cause an increase in tuber respiration, increases in polyphenol oxidase activity, inhibition of mitotic division, disruption of amyloplast integrity and electrolyte leakage. These effects were also examined when imazethapyr was applied to tubers in adverse conditions. In many cases synergistic relationships were detected when imazethapyr was applied at low temperature, acidic pH, with an ethylene-releaser and with immersion in water. In some cases the formulation of imazethapyr could be contributing to vascular discolouration more than the active ingredient itself.

A novel method was developed to trace ^{14}C-imazethapyr through the treated tuber. This study has illustrated the rapid movement of imazethapyr in the tuber periderm and has demonstrated that only a small percentage of applied imazethapyr enters the tuber cortex. The activity detected in the tuber cortex increased when imazethapyr was applied in acidic conditions, suggesting that the increases in vascular discolouration could be due to increased penetration of imazethapyr into the cortex under stress conditions. It is concluded that imazethapyr, in the present formulation, can cause increased incidence of vascular discolouration, particularly when applied under adverse conditions.

DECLARATION

The author has not been a registered candidate nor an enrolled student for another award of The Nottingham Trent University or other academic or professional institution during this research programme. Material contained in this thesis has not been used in any other submission for an academic award and is entirely the authors contribution. Due acknowledgement is made of assistance received. The author has attended appropriate lectures, seminars and conferences in partial fulfilment of the requirements of the degree.

Signed:.....
(Candidate)

Signed:.....
(Director of Studies)

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PUBLICATIONS

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ABBREVIATIONS

t	tonne
ha	hectare
ai	active ingredient
cv	cultivar
°C	degrees centigrade
PPO	polyphenol oxidase
BAA	branched-chain amino acids
IRGA	infra-red gas analyser
DMN	dimethyl naphthalene
BPC	British Potato Council
FAO	Food and Agricultural Organisation
AHAS	acetohydroxy acid synthase
ALS	acetolactate synthase
TD	threonine dehydratase
2-KB	2-ketobutyrate
2-AB	2-aminobutyrate
DNA	deoxyribonucleic acid
IBS	internal brown spot
fwt	fresh weight
MES	morpholinoethane sulfonic acid
GS	glutamine synthase
AOX	alternative oxidase
mRNA	messenger ribonucleic acid
LAH	lipolytic acyl hydrolase
LOX	lipoxygenase
MSA	methyl sulfonic acid
G_1	gap 1
G_2	gap 2
M	mitosis
LD_{50}	Dose required to kill 50% of population
LC_{50}	Concentration required to kill 50% of population

CONTENTS

LIST OF FIGURES

LIST OF TABLES

1.0 INTRODUCTION

1.1 The potato crop

1.1.1 History of potato use

Potatoes (*Solanum tuberosum*.L.) have been grown in areas of South America for thousands of years and were introduced into Europe by the Spanish in the sixteenth century (Burton, 1989). In Britain, the term potato was first used to describe the tuber that we now know as the sweet potato, *Ipomeoa batata* (Gerard, 1597, cited by Burton, 1948). However, when *S. tuberosum* became available in Britain, the distinction between the two types of tuber was created.

The wild origin of the potato no longer exists as it had been bred out even before its introduction to Europe, and indeed the original cultivation available in Europe only exists as herbarium specimens (Dodds, 1962). The potato has been bred to withstand world-wide climates, to increase yield and to confer increased resistance to diseases.

The real establishment of the potato as a European food crop was in Ireland, although the potato had been grown in other European countries on a small scale. During the eighteenth century it became increasingly popular at the expense of other crops. In the nineteenth century the potato was accepted as a foodstuff throughout Europe and increasing areas of land were devoted to its cultivation. However, in Ireland potato crops were devastated by *Phytophthora infestans* in 1845 and 1846. The famine that followed resulted in 1 million deaths and 1.5 million emigrations from the country (Large, 1940). Following a brief decline, total area devoted to potato cultivation in Ireland increased to a peak of 486,000 ha in 1859 (Burton, 1948). The figure has steadily fallen during this century, but has been counteracted by an increase in yields (van Loon and Crosnier, 1982).

1.1.2 Modern potato production

The potato is one of the most important food crops in the world and is grown in more countries than any other, with the exception of maize (Askew, 1995). The potato has relatively recently become one of the world's major food crops. The

potato crop is ranked fourth in the world in terms of production (after rice, wheat and maize) (Food and Agriculture Organisation (FAO), 1997). World production is 26.5 million tonnes from an area of 18.2 million ha (FAO, 1997). Europe accounts for 29% of global production (3% being produced by the UK). At present, there are 400 cultivars available for use, but only a small proportion (about 30) are used commercially, although different cultivars are becoming more popular.

In the UK between 1960 and 1996 the area of land producing potatoes dropped by about 50% (Figure 1.1), but this was offset by an increase in yield from 22.6 t ha^{-1} to 44.5 t ha^{-1} (British Potato Council (BPC), 1997). Total production of potatoes in the UK during the 1995 season was 6,120,000 t, valued at £0.8 billion, of which the domestic market consumed 5,795,000 t (BPC, 1997).

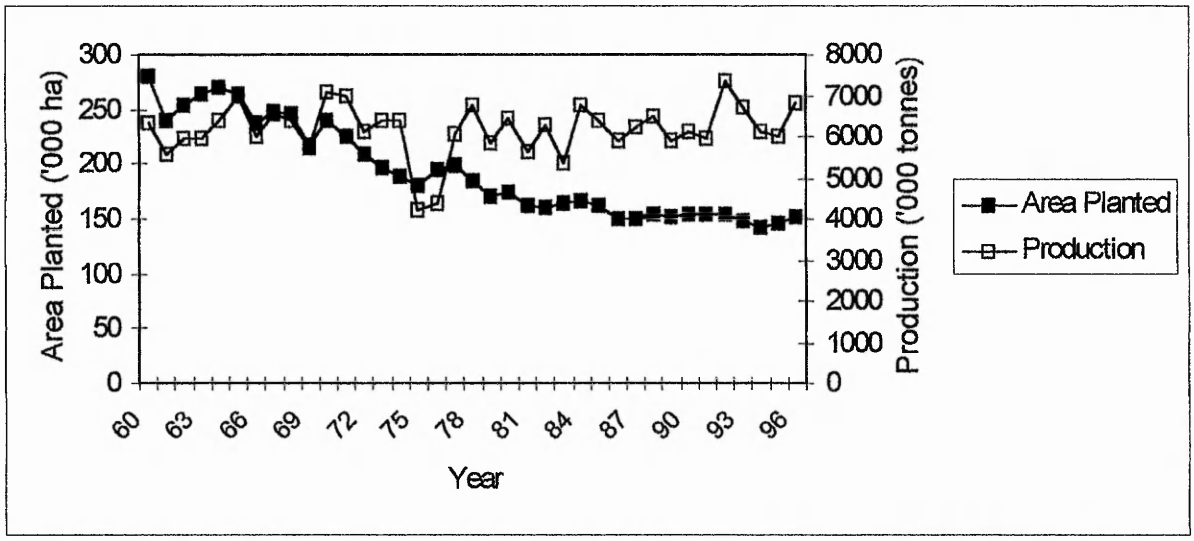


Figure 1.1. Relationship between area and production since 1960. (PMB, 1997).

The most dramatic change in potato usage in recent years is the increase in volume of potatoes sold to the processing industry. In 1995/6 processing accounted for 28.6% of total potato production compared to 18% in 1987/88 (BPC, 1997). This shift in usage has had an impact on both growing and storage practices. Potato use varies in different countries. Typically, in areas where grain production is high, potatoes are unlikely to be used as livestock feed. Similarly, starch and alcohol manufacturers prefer to use grain. Potatoes are used for human consumption in both fresh and processed forms. The U.S.A. is the most developed market in terms of consumption of processed products, with 70% being consumed as processed (BPC,

1997). The UK processed market is increasing as a proportion of total usage, from 30 to 42% of total consumption in 8 years (BPC, 1997). This change is due to altered eating habits and increased use of convenience foods. Frozen French fries and crisps are the two main processed potato products. The remainder are used for canning, dehydration and other products (Dixon, 1995).

1.1.3 Dietary value of the potato

It is a common misconception that the potato is a fattening foodstuff. It is in fact a poor source of calorific energy and is only fattening if eaten to excess. The potato has a high water content and a very low fat content (about 1mg g^{-1}). Many potato cooking methods, especially roasting and frying, will increase the fat content of the potato. The potato is an excellent source of dietary nitrogen and protein. In terms of dry weight, potatoes contain protein in amounts comparable to cereals. The levels of sulphur containing amino acids are only about 70% compared to wheat, but the lysine content is about 3 times higher (Woolfe, 1987). Burton (1966) states that 2 kg per day of boiled potatoes would supply all the nitrogen necessary for an average adult.

Potatoes are an excellent source of vitamins and minerals. Burton (1966) calculated that in the UK potatoes supplied 30% of all vitamin C intake. The vitamin C content of fresh potatoes is about 200 μg g^{-1}. Kolasa (1993) estimated that 15% of the US Recommended Daily Intake of vitamin B_6 was supplied by 1 medium potato, but this potentially useful source of vitamins can be lost by overcooking. In addition, potatoes are a source of the trace elements magnesium, copper, iron and iodine. Although potatoes contain only half the amount of fibre found in other vegetables (Paul and Southgate, 1978), they are consumed in sufficient quantity to make them the most important source of fibre in the UK (Burton, 1989).

The potato crop seems set to increase in yield and continue to be an important part of world agriculture. It is a valuable resource and is relied upon by growers and processors for income and by consumers as an important part of their daily diet. As the processing market expands in the UK and abroad, there is an increasing need for efficient, simple and safe sprout control.

1.2 Storage of the potato crop

There is a demand for fresh potatoes all year round, but the climate in the UK dictates that potatoes can only be harvested from late May (first earlies) to between late October and early November for the maincrop. Consequently, storage is necessary to avoid costly imports of tubers from abroad for the domestic market. In the UK potatoes are usually stored in bulk in box pallets. A large store will contain many pallets and the temperature and humidity can vary greatly across the store. These factors make ventilation an essential part of a well controlled store. During the storage period losses will occur in the crop, for example weight and water loss. To avoid such losses and to avoid sprouting and pathogen development, temperature is finely controlled in store. Potatoes for the pre-pack market are stored at 3-6°C, ware tubers at 7°C and processing tubers at 8-10°C (Potato Marketing Board (PMB), 1992). To prevent the problem of evaporation (and concurrent weight loss), humidity in store should be as high as possible, without causing condensation (Burton, 1989). Tubers are often "cured" prior to entering long term storage. During curing tubers are kept at 12-15°C and high humidity (95%) for about 2 weeks (PMB, 1992). This process allows wound healing and periderm thickening, all helping the tuber to be stored for longer. Another benefit of curing is reduction in skin spot disease if the humidity is reduced (Boyd, 1957). These storage regimes are tailored to each particular store, cultivar and end use.

1.2.1 Sources of loss during storage

There has been extensive study attempting to characterise the physiological changes that the tuber undergoes during storage (Burton, 1963). The work is of great importance to the potato industry as any changes to current knowledge can potentially reduce the losses encountered in storage. At present the total value of potatoes lost through storage is not known. The main areas of loss are bruising, dehydration, greening and sprouting.

Bruising and mechanical damage cost the UK potato industry £30 million per annum (BPC, 1997). Tuber bruising is the result of impact which damages cell membranes allowing oxidation of phenols to melanin, the blue-black pigment which forms 1-3 days after impact (Hughes, 1980). Susceptibility to bruising varies and

depends on cultivar and store temperature. Extensive research is being undertaken to minimise causes of impact to the tuber and to determine the exact nature of the bruising reactions.

The tuber has an effective barrier to dehydration, the periderm. Peeling results in a 300-500% increase in evaporation (Burton, 1955). Despite this, water loss still occurs during storage and causes weight loss and consequently the tuber crop loses value. Dehydration can be minimised by keeping humidity artificially high and altering temperature. The cost of these measures are offset by the increase in the crop value.

Greening and glycoalkaloid formation occur when tubers are exposed to light. The greening is due to the transformation of amyloplasts to chloroplasts and chlorophyll production being initiated (Anstis and Northcote, 1973). Both the chlorophylls and the toxic glycoalkaloids do not degrade once formed and are not destroyed by cooking (Baerug, 1962; Virgin and Sundqvist, 1992). Processors and end users will reject greened potatoes because of the association between greening and glycoalkaloids, although it is likely that greening and glycoalkaloid formation are independent processes (Edwards and Cobb, 1997).

1.2.2 Changes during dormancy

The definition of tuber dormancy has been a source of much debate in scientific literature. For the purposes of this thesis, the terminology in the review of dormancy by Wiltshire and Cobb (1996) will be adopted. Innate dormancy is "the physiological state of the tuber in which autonomous sprout growth will not occur, even when placed under ideal natural conditions for sprouting (darkness, temperature 15-20°C, relative humidity about 90%)" and enforced dormancy "for the period following innate dormancy, when bud growth is suppressed".

The physiological changes that occur during dormancy have been investigated for a limited range of cultivars but the mechanisms that control these changes are not fully understood.

1.2.2.1 Respiration

The metabolic activity of the tuber is measurable throughout dormancy by respiration. Rates are generally high at harvest falling to a low basal level with time. The time lapse associated with this fall is variable and depends on many factors such as cultivar, maturity, harvest conditions and temperature (Burton *et al*, 1992). During storage, there is little change in respiration rates if the crop is held at a constant temperature. Respiration will increase if tubers are stored at temperatures above 10°C. When sprouting starts there is a sharp increase in respiration (Schippers, 1977). Although this increase is not a cause for dormancy break, it is associated with the break (Hemberg, 1985). The respiration of the tuber leads to a slow decline in carbon and consequently weight loss occurs in storage. A review of various studies employing different storage conditions and cultivars, stated losses of 0.5-1% of tuber weight over a 6 month period (Leszczynski, 1989). Respiration has been used as a valuable research tool as an indicator of the metabolic state of the tuber.

1.2.2.2 Sugar accumulation

Reducing sugar accumulation is undesirable because during processing it is a substrate for the non-enzymic Maillard reaction. The Maillard reaction converts amino compounds and reducing sugars to melanin, resulting in browning and nutritional changes in the potato. There are three methods of sugar accumulation. One is the reversible low temperature sweetening. The other two mechanisms are irreversible. They are senescent sweetening and the sweetening linked with sprouting.

Although low temperature storage (below 10°C) is a good way of controlling sprouting, it leads to reducing sugar accumulation, especially when the temperature is below 7 °C (Cunnington, Mawson, Briddon and Storey, 1992). As potatoes used for processing must be of high quality, low temperature sweetening must be avoided. This means that processing potatoes are generally stored at 8-10°C, and will sprout earlier than tubers stored at cooler temperatures, increasing the need for effective sprout control. However, sugar accumulation is highly dependant on cultivar and consequently it is another factor to be considered when using tubers for processing. Low temperature sweetening can be reversed by increasing store temperature to 15-20 °C. This common procedure is known as reconditioning, and allows the reducing

sugars to be metabolised, respired or reconverted to starch (Williams and Cobb, 1992).

The sugar accumulation associated with sprouting is due to the hydrolysis of starch which is necessary to provide carbon and energy for sprout growth. Senescent sweetening is caused by an irreversible loss of subcellular compartmentation and the breakdown of starch, resulting in glucose accumulation.

1.2.2.3 Proteins and nucleic acids

Protein, DNA and RNA synthesis occur throughout dormancy and there is an increase in activity when sprouting begins and dormancy breaks (Macdonald and Osbourne, 1988). The changes at dormancy break are triggered by ethylene and are linked to the regulation of protein synthesis, but not nucleic acid synthesis (Alam, Murr and Kristof, 1994). The Maillard browning reaction involves reaction between free amino acids (normally in excess) and reducing sugars. The deterioration of fry colour has previously been attributed to increases over time of reducing sugar levels. However, research has shown that darker fry colours could not be justified by increased reducing sugars alone, and that the breakdown of storage proteins is implicated (Brierley, Bonner and Cobb, 1997). Glutamine concentration affects the fry colour and, at low concentration of reducing sugars, ascorbic acid and sucrose have been reported to affect the colour quality (Rodriguez-Saona, 1997).

1.2.2.4 Cell integrity

Subcellular compartmentation and intactness is important to the maintenance of dormancy. Membrane damage has been observed as a result of long term storage and low temperature injury (Turnbull and Cobb, 1992). Increased electrolyte leakage is a commonly used indicator of membrane damage. An increased sprouting potential has been observed in conjunction with an increase in electrolyte leakage. The leakage increase often occurs with progressing physiological age (de Weerd, Hiller and Thornton, 1995).

Membranes can also be damaged by the accumulation of oxygen free radicals which cause peroxidative damage and therefore a loss of membrane integrity (Kumar and Knowles, 1993). The presence of antioxidant enzymes will decrease the levels of free radicals thereby protecting the tuber from the loss of compartmentation (Spychella and Desborough, 1990). Endogenous phospholipases catalyse membrane

degradation. One such example is lipolytic acyl hydrolase (LAH) which has been linked to increased electrolyte leakage (Turnbull and Cobb, 1992). LAH hydrolyses the fatty acyl groups of membrane glycolipids and phospholipids, this causes the release of fatty acids, particularly linoleic and linolenic acids. These products can then be utilised as substrates for lipoxygenases (LOX). LOX catalyse the incorporation of molecular oxygen into the fatty acids, the autocatalytic lipid peroxidation may accelerate membrane breakdown.

1.2.3. Dormancy control

Following the period of dormancy, tubers begin to sprout. There are many methods used to control sprouting. Often a chemical treatment is applied to prolong endogenous dormancy (Duncan, Boyd and Muir, 1992). There are other methods available to prolong dormancy, such as cold temperature storage and diffuse lighting, but these alternatives have disadvantages. Low temperature storage will cause increases in reducing sugar concentrations rendering tubers unfit for processing, and a low temperature store is created by using refrigeration equipment, which is often costly or unavailable. Diffuse lighting can be useful for seed potatoes, but store modification is needed to allow the illumination of individual potatoes and also tubers will green unless successful filtering techniques are employed (McGee, 1987).

Consequently, for many potatoes the only method of sprout control is by chemical treatment. Extensive studies have been carried out using different chemicals for sprout suppression as early as 1938 when Guthrie reported the benefits of using indoleacetic acid and the potassium salt of naphthalene acetic acid to control sprout growth.

1.2.3.1. Chemicals used

Most chemicals used for dormancy control are intended to prolong rather than break dormancy. At present the market leaders in sprout suppression are chlorpropham (isopropyl 3-chlorophenylcarbamate), propham (isopropylphenylcarbamate) and tecnazene (1, 2, 4, 5-tetrachloro-3-nitrobenzene), and to a lesser extent maleic hydrazide (6-hydroxy-2H-pyridazine-3-one).

Chlorpropham and propham are mitotic inhibitors and were initially marketed as herbicides but have been used worldwide as sprout suppressants, this new usage was reveiwed by Boyd (1988). Chlorpropham and propham are often used in conjunction with each other. They are frequently used as the standard treatment to compare other sprout suppressants with (Briddon, Cunnington and Storey, 1996). Chlorpropham is normally applied as a fog and can be applied as one dose but is usually applied as multiple doses. Sprouting can be controlled for the duration of storage with multiple doses. However, if the distribution is uneven then some sprouting may occur in tubers not receiving sufficient chemical. In the past, more chemical was applied if sprouting occurred in this way. With the introduction of maximum residue limits (MRL's) for compounds such as chlorpropham and propham, additional applications are not possible.

Tecnazene was initially marketed as a fungicide for control of *Fusarium spp.* It is currently used to provide sprout control as tubers go into store, before the first application of chlorpropham which would take place after wound healing. As with chlorpropham, tecnazene usage may be significantly curtailled with the introduction of MRL's. In addition, environmental concerns exist following the detection of tecnazene and its metabolites in river sediment samples downstream of potato washing plants (Whale, Sheahan and Matthiesen, 1988).

Maleic hydrazide is a mitotic inhibitor that is applied as a sprout suppressant via the foliage. This method of application causes difficulties as the prevailing weather conditions at spray time can affect the efficacy of the compound. Also the timing of the application can affect the success of the treatment. Lack of uniformity in the distribution of the chemical in the tuber can lead to uneven sprout control, thus requiring a subsequent application of an alternative sprout suppressant later on in storage. Once again the toxicology of maleic hydrazide has been questioned, partly due to its mode of action and also due to traces of hydrazine in the formulation.

Many of these compounds were released in the late 1940's and early 1950's, and there is an increasing demand for products with lower chemical residues and improved toxicological profiles from the processing industry and supermarkets.

1.2.3.2. New products

One of the more recent products to be released for tuber sprout control is Carvone, first marketed by Luxan BV as Talent® in 1994 in The Netherlands. It is extracted and purified from the seed of caraway (*Carum carvi).* L.), has been shown in trials to inhibit sprouting and has a degree of anti-fungal activity (Hartmans and Vries, 1996). British Potato Council trials have shown that carvone gives the same degree of control as propham/chlorpropham mixtures. However, tubers stored over 30 weeks required 7 applications as a thermal fog. Commercially and practically, it would be preferable to reduce this frequency of exposure. Wound healing is temporarily inhibited in tubers treated with carvone. The inhibition is linked to the lack of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and phenylalanine ammonia lyase (PAL) in treated tubers (Oosterhaven and Hartmans, 1996).

Other volatiles may have roles in sprout suppression. For example dimethyl naphthalene (DMN) has been shown to control tuber sprouting effectively for 7 months. First used in Idaho, DMN has been registered for use in the USA since 1995. The headspace concentration of DMN is crucial to its activity and subsequent reduction of tuber metabolic rate (Beveridge, Dalziel and Duncan, 1983). A mixture of 1,4- and 1,3- isomers are the most effective for sprout control and this mixture could be registered for use in Europe in the future (Everest-Todd, 1996).

Another new chemical with promising sprout suppressant qualities is imazethapyr (5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinic acid) on which the experimental work in this thesis is based.

1.3 Imidazolinones

Imazethapyr is a member of the imidazolinone family of compounds, a group which was discovered in the 1970's by American Cyanamid. The initial development was initiated as a result of random screening of the phthalimide (Figure 1.2). The herbicidal activity was sufficient to warrant a synthesis programme (Los, 1991).

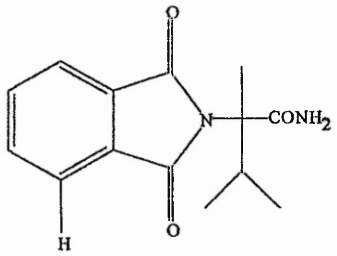


Figure 1.2. Chemical structure of the imidazolinone phthalimide.

1.3.1 Structure

The generic imidazolinone structure is shown in Figure 1.3. The structure is divided into three areas; acid equivalent, backbone and imidazolinone ring.

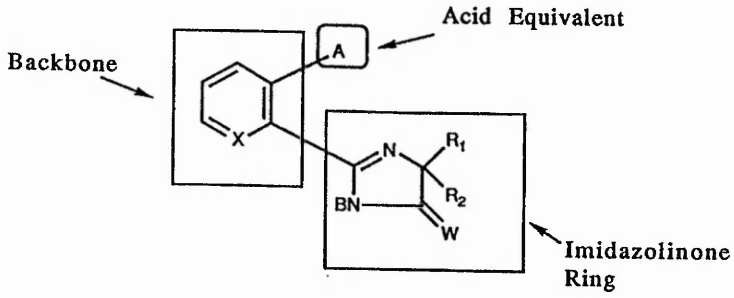
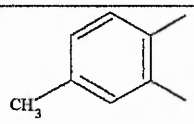
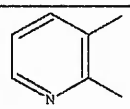
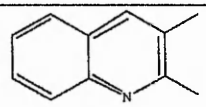
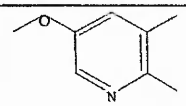
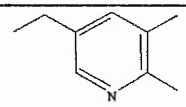


Figure 1.3. Generic chemical structure of imidazolinones.

Table 1.1. Structures of the different commercialised imidazolinone herbicides.

Chemical name	Acid Equivalent	Backbone structure
Imazamethabenz-methyl	$COOCH_3$	
Imazapyr	COOH	
Imazaquin	COOH	
Imazamox	COOH	
Imazethapyr	COOH	

Many imidazolinones have been marketed and Table 1.1 displays the structural differences between them. For sufficient activity the acid equivalent must be a carboxylic acid group or a group that can be easily transformed to a carboxylic acid. Salts of acids are equivalent in activity to the acids themselves and this characteristic is used in many imidazolinone formulations.

Extensive work has been carried out by Cyanamid to investigate the possibility of alterations to the imidazolinone ring structure, whilst still maintaining activity. Many different R_1 and R_2 (Figure 1.3) substitutions have been tested. Methyl and isopropyl as a combination provides the best activity. The oxygen of the carbonyl group appears to be crucial to activity. The nitrogen group can be substituted with easily cleaved groups to give active compounds. Also, total ring substitutions have been tested, but to date no alternative ring system has given an acceptable level of activity, which implies a strong link between the imidazolinone ring structure and the molecular site of activity.

The optimum backbone structure for activity is the benzene ring, despite thiophene often being a successful replacement in other analogue syntheses. The position of the nitrogen relative to the ring and acid equivalent is crucial to high activity. Substituents can be added to the ring, and in general, electron withdrawing substituents cause a loss in activity but electron rich substituents will increase activity, especially at position 3.

Additionally, the acid equivalent and the ring must be *ortho* to each other for activity. This is well demonstrated by two positional isomers of imidazolinyl pyridine carboxylic acids. One is inactive, even at 10kg ha^{-1} and the other is active at 10g ha^{-1}.

1.3.2. Toxicological profile of imidazolinones

The imidazolinones have a very low toxicological profile, because they inhibit a process only found in plants. The compounds are all rapidly excreted in rats, hens and goats as the unchanged product before any accumulation can occur in tissues. No detectable residues (less than 0.001ppm) were found in liver, kidney, muscle, fat or blood (Miller, Fung and Gingher, 1991). The LD_{50} value for all imidazolinones in rats are >5000 $mgkg^{-1}$ body weight, and are considered to be no more than slightly toxic to mammals (Gagne, Fischer, Sharma, Traul, Diehl, Hess and Harris, 1991). Short and long term mammalian studies have been carried out and the no-effect levels in rats are summarized in Table 1.2. The acute toxicity of the imidazolinones to non target organisms is displayed in Table 1.3. In addition the imidazolinones are not genotoxic, metabolism studies in legumes showed no significant residues in the final edible crop, and soil metabolism studies showed that no significant metabolites were formed (Devine, 1991).

Table 1.2. Chronic toxicity study in rats of the imidazolinones. No-effect level (ppm) (Gagne *et al*,1991).

	Imazapyr	Imazamethabenz-methyl	Imazethapyr	Imazaquin
13 week dietry	10,000	1,000	10,000	10,000
24 month dietry	10,000	250	10,000	10,000
2 Generation reproduction	10,000	250	10,000	10,000

Table 1.3. Acute toxicity of imidazolinones to nontarget organisms (Gagne *et al*, 1991).

	Imazapyr	Imazamethabenz-methyl	Imazethapyr	Imazaquin
Mallard duck Oral LD_{50} ($mgkg^{-1}$ body weight)	>2,150	>2,150	>2,150	>2,150
Rainbow trout 96h LC_{50} ($mg\ l^{-1}$)	>100	280	380	320
Honey bee Contact LD_{50} ($\mu g\ bee^{-1}$)	>100	>100	>100	>100

1.3.3. Mode of action of imidazolinones

The imidazolinones inhibit the synthesis of the branched-chain amino acids valine, leucine and isoleucine. These 3 amino acids share 4 common enzymes in their biosynthetic pathway. The first of these enzymes is acetohydroxyacid synthase (AHAS), also known as acetolactate synthase (ALS). Despite being structurally unrelated, 3 other groups of chemical inhibit AHAS as well. These are the imidazolinones, sulfonylureas, triazolopyrimidines and the pyrimidyl-oxy-benzoates.

1.3.3.1. Properties and pathways of plant AHAS

The position of AHAS in the branched-chain amino acid synthesis pathway is shown in Figure 1.4. This Figure shows that AHAS catalyses the condensation of 2 molecules of pyruvate to form 2-acetolactate. It also catalyses the condensation of one pyruvate and one 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate.

In common with the majority of the amino acid biosynthesis enzymes, plant AHAS is nuclear encoded and plastid located (Miflin, 1974). The enzyme requires FAD as a cofactor for stability and occurs in small quantities in plant tissue (Muhitch, Shaner and Stidham, 1987). The other enzymes that are common to the synthetic pathways of valine, leucine and isoleucine are acetohydroxyacid reductoisomerase, dihydroxyacid dehydratase and valine amino transferase. In addition to these, isoleucine requires one additional enzyme, threonine dehydratase (TD) and leucine requires 3 additional enzymes.

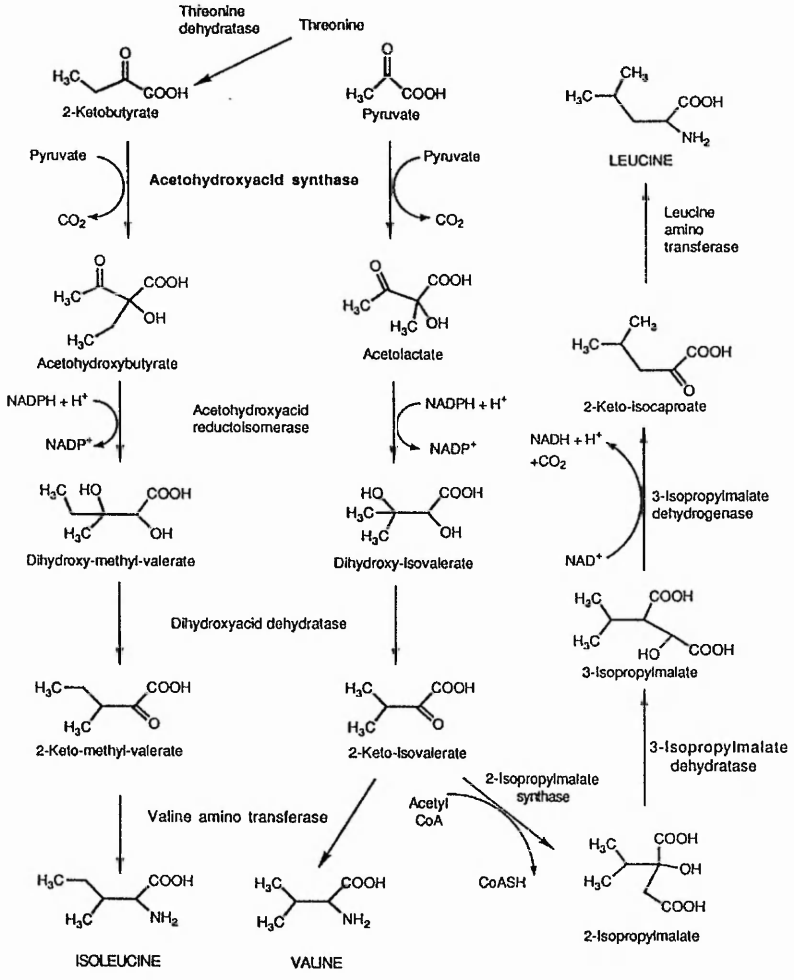


Figure 1.4. The Biosynthetic pathway of the Branched-Chain amino acids (Stidham and Singh, 1991).

The AHAS pathway is regulated in plants at 3 points; isoleucine inhibits TD (Sharma and Mazumder, 1970), leucine inhibits 2-isopropylmalate (Oaks, 1965) and AHAS itself is inhibited by all 3 amino acids (Miflin, 1971). In addition, a co-operative inhibition of AHAS occurs when valine and leucine are present (Miflin, 1974). The inhibition of AHAS by valine *in vitro* is weak, implying that the properties of the enzyme are altered when isolated from the plastid (Schulze-Siebert, Heineke, Scharf and Schultz, 1984).

1.3.3.2. AHAS interactions with imidazolinones

The imidazolinones inhibit the activity of AHAS, shown by 5 definitive experiments carried out between 1984 and 1987. It was shown that susceptible maize cell cultures treated with imidazolinones displayed reduced levels of only 3 amino acids, valine, leucine and isoleucine (Anderson and Hibberd, 1985). Imidazolinone-treated maize supplemented with the branched-chain amino acids shows reduced herbicidal symptoms (Shaner and Reider, 1986). The AHAS enzyme extracted from susceptible plants was inhibited by imidazolinones *in vitro* (Shaner, Anderson and Stidham, 1984). Plants treated with imidazolinones had reduced AHAS activity (Muhitch *et al*, 1987). Finally, AHAS from imidazolinone-tolerant maize (from cell culture selection) was not inhibited by the imidazolinones (Shaner and Anderson, 1985).

The inhibition of AHAS occurs by a slow-binding mechanism. Schloss (1990) showed that the initial enzyme/herbicide binding tightens with time and this binding is very slowly reversible *in vitro*, but there are some differences between *in vivo* and *in vitro* responses. Shaner and Singh (1993) demonstrated that the imidazolinones are more potent inhibitors of AHAS *in vivo* than *in vitro*.

Another interesting aspect of AHAS inhibitors is that although there are several types of AHAS inhibiting herbicides (e.g. imidazolinones, sulfonylureas, triazolopyrimidines and pyrimidyl-oxy-benzoates), the binding sites are not identical (Stidham, 1991). Imidazolinone treatment causes a decrease in the levels of extractable AHAS from plants, the loss being proportional to the log of the internal herbicide concentration. However, this loss is not a feature of treatment with all other herbicides that inhibit AHAS activity. Sulphonylurea treatment does not cause a decrease in extractable AHAS levels, despite being a more potent inhibitor of the enzyme *in vitro*, and indeed some sulphonylureas will behave antagonistically toward imidazolinones with respect to extractable AHAS levels (Stidham, 1991).

The inhibition of AHAS not only causes a decrease in concentrations of valine, leucine and isoleucine but also a build up of 2-ketobutyrate (2-KB). 2-KB can be used in several pathways. Although 2-KB is mainly transaminated to 2-aminobutyrate (2-AB)(Rhodes, Hogan, Deal, Jamieson and Haworth, 1987). It was thought that the accumulation of 2-AB was linked to the phytotoxicity of imidazolinones (LaRossa, VanDyk and Smulski, 1987). However, Shaner and Singh (1993) showed that this was not possible because the accumulation of 2-AB is mediated by isoleucine, and if 2-AB accumulation was important to imidazolinone phytotoxicity then the addition of isoleucine alone should provide some safening against the imidazolinones. This study showed that the addition of isoleucine did reduce pools of 2-KB and 2-AB but did not protect plants from the growth inhibitory effects of the imidazolinone. Also, the exogenous application of high levels of 2-KB and 2-AB (to simulate AHAS inhibitor induced accumulation) did not have a significant effect on growth.

Metabolism of the imidazolinones is relatively unpredictable, varying with type of imidazolinone and species. Studies have been carried out on all the imidazolinones in species such as soybean, maize and peanut (Tecle, da Cuna and Shaner, 1993). The metabolic variations are often dependant on the 5'-substituent of the imidazolinone structure. For example, there are 2 competing metabolic pathways available in soybean. Imazapyr and imazamox take one pathway and imazaquin takes another. The first pathway hydroxylates the 5'-substituent and is then conjugated to glucose. There is rapid accumulation of the inactive glucose conjugate. In the case of imazaquin ring closure is the first step followed by cleavage of the imidazolinone ring, and the pathway results in 2 products with the imidazolinone ring completely open. The 2 products are then further hydrolysed forming quinoline ring structures (Shaner and Mallipudi, 1991). This variation in metabolism is demonstrated throughout the group and provides differing selectivity to many crop species.

Imazethapyr is metabolised in soybean in the same way as imazapyr, through conjugation of a hydroxylated imazethapyr with glucose. In corn there is no conjugation and the hydroxylated imazethapyr accumulates. The different metabolic pathways are represented in Figure 1.5.

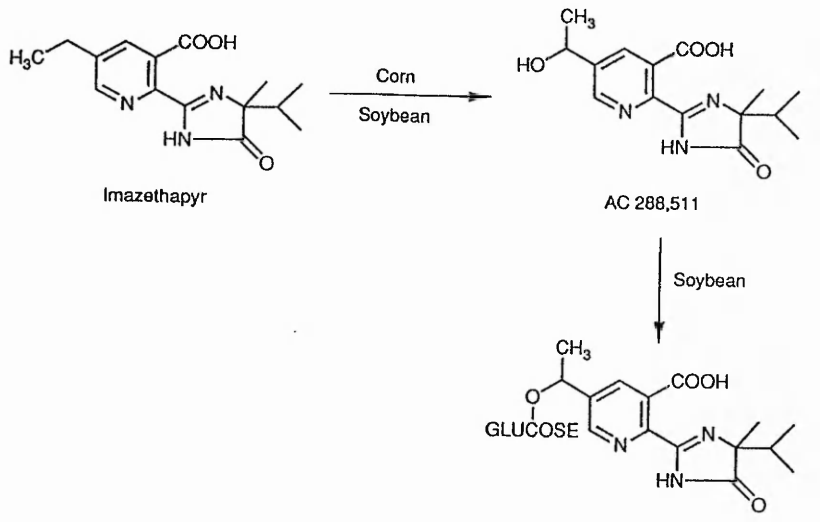


Figure 1.5. Proposed metabolism of imazethapyr in corn and soybean (Shaner and Mallipudi, 1991).

1.3.4. Commercial use of imidazolinones

The imidazolinones are used widely in agriculture and each member of the family is used for a different purpose.

Imazapyr is a broad spectrum herbicide which controls most annual and perennial grasses, broad leaved weeds and deciduous trees. It is registered for use in 42 countries around the world and is mainly used in non-crop and forestry situations, but has also been developed for use in sugar cane and rubber plantations (Beardmore, Hart, Iverson, Risley and Trimmer, 1991).

Imazamethabenz-methyl is a selective, postemergence herbicide used to control weeds in cereal crops. It is used to control many economically important grass and broad leaved weeds and has some soil residual activity so weeds that germinate after application can also be controlled (Bernstein, Kirkland and Youmans, 1991).

Imazaquin was the first imidazolinone used as a herbicide for broad spectrum weed control. It controls many broad leaved weeds and a limited range of grass weeds. It is mainly used for control in soybean but is also registered for use on turfgrasses (Bhalla, Hackett, Hart and Lignowski, 1991).

Imazethapyr is a selective herbicide used to control a wide spectrum of broad leaved weeds and grasses in soybeans and other crops such as peanuts, alfalfa and peas. The application of imazethapyr is extremely flexible as it can be applied at preplant, planting, preemergence or postemergence (Hart, Lignowski and Taylor, 1991).

Imazethapyr tolerant corn has been developed by backcrossing methods, and imidazolinone tolerance is inherited as a single, semidominant gene. Protein extracts from tolerant plants contain AHAS activity insensitive to the imidazolinones. No morphological changes are observed in the absence of herbicide treatment and no deleterious effects attributable to herbicide-insensitive AHAS have been observed (Newhouse, Singh, Shaner and Stidham, 1991).

Imazethapyr, in combination with imazapyr, has been registered for use as a "chemical mower" for cool-season turf grasses and it also controls seed head production in some grass species. Other members of the imidazolinone family have been used as plant growth regulators (PGR's), for example, imazaquin is used as a chemical mower in warm-season turfgrass. Also, imazaquin is used in combination with chlormequat as it has a positive interaction when used to prevent lodging in winter wheat. The addition of imazaquin allows the chlormequat chloride to be more mobile and better distributed in the plant.

Imazethapyr has been proposed as an inhibitor of sprouting in potatoes, with preliminary observations indicating a prolonged suppression time (Tayler, Gussin and Leck, 1996). Table 1.2 shows the application rate and suppression time in comparison to the leading competitor molecules.

Table 1.2. Comparison between imazethapyr and leading competitor molecules.

Chemical name	Application rate (g ai tonne $^{-1}$)	Suppression time (months)	Reference
Imazethapyr	1	7-9	Mawson *et al*, 1996
Chlorpropham	10-20	2-3	Duncan *et al*, 1992
s-Carvone	20-150	1-1.5	Hartmans and de Vries, 1996
Tecnazene	68-135	3-6	Duncan *et al*, 1992

It appears to be an ideal candidate for successful sprout suppression because of the low application rate. In addition, the treatment only needs one application, compared to the numerous applications needed with chemicals such as carvone and tecnazene.

1.3.4.1. Physiocochemical profile of imazethapyr

Imazethapyr ($C_{15}H_{19}N_3O_3$)is an off-white to tan solid with a molecular weight of 289.34 and a melting point of 163-175°C. It has a vapour pressure of $<1 \times 10^{-7}$ mmHg at 60°C and pKa_1=2.1, pKa_2=3.9. It has a solubility at 25°C of 0.14g in 100ml water. The partition coefficient (n-octanol/water) at 25°C was 11 at pH 5, 31 at pH 7 and 16 at pH 9.

1.3.5. Physiological effects of imidazolinone treatment in plants and potatoes

Although many aspects of the physiological responses to AHAS inhibition, plant death and sprout suppression have been examined the entire process is not fully understood. One well documented physiological response is the inhibition of growth and cell division upon treatment. In corn, root application of imazaquin inhibited leaf elongation in only 6 h, due to an inhibition of cell division (Shaner and Reider,1986). Cell division was reported to have stopped at interphase following treatment of *Avena fatua* and *Alopecurus myosuroides* with imazamethabenz-methyl, reducing the number of cells in division by 73 and 55% respectively (Pilmoor and Caseley, 1987). Four different imidazolinones arrested cell division at interphase when tested on pea root tips (Rost, Gladdish, Steffen and Robbins, 1990).

Shaner and Reider (1986) have shown that imazapyr will inhibit the rate of DNA synthesis by 63%, 24 hours after application. Rost *et al* (1990) dosed pea root tips with 3 branched chain amino acids, and by doing this, reversed the inhibition of cell division by imazapyr. Also, Scarponi, Nemet Alla and Martinetti (1995) demonstrated this effect using imazethapyr in soybean. This work indicates that there is a close relationship between inhibition of branched-chain amino acid biosynthesis and DNA synthesis, cell division and growth. A possible connection between DNA synthesis and amino acids was initially proposed by Shaner and Reider in 1986. They hypothesised that the synthesis of a short-lived protein is necessary for DNA synthesis or another aspect of cell division. A mechanism linking the synthesis of amino acids and DNA is not clear. Possibly the branched

chain amino acids are directly involved or required for cell division at a particular stage, for example, to allow the progression of a stage of mitosis. Isoleucine has been shown to initiate DNA synthesis in mammalian systems (Tobey, 1973).

Studies carried out with radiolabelled amino acids showed that imidazolinone treatment had very little effect on rates of protein synthesis. However, after 24h a 30% increase in the free amino acid pool was recorded in corn treated with imazapyr compared to control plants. Also, the soluble protein pool in the root meristem decreased by 36% (Shaner and Reider, 1986). Imazapyr seems to disrupt protein synthesis by altering rates of protein turnover. Another AHAS inhibitor, chlorsulfuron, increased the free amino acid levels in *Lemna minor* and this was due to turnover of existing proteins rather than the synthesis of new ones (Rhodes, Hogan, Deal, Jamieson and Howarth, 1987). Shaner (1991) hypothesised that the response could be due to the plants reaction to nitrogen starvation. The decrease in branched-chain amino acid synthesis after imidazolinone treatment could signal to the plant that nitrogen starvation had occurred, causing an increase in the rate of protein turnover. This hypothesis was confirmed by the observations in many treated plants that mature tissue was less affected than meristematic tissue. Older tissue can obtain amino acids from pools of proteins, and this was easily done as the energy supplied by photosynthesis was not affected. The meristematic tissue had a higher requirement for amino acids for growth and had smaller reserves to draw on.

Another effect of imidazolinone treatment is the increase in total neutral sugars. In corn leaves a 35% increase in sugars was observed within 24h of treatment with imazethapyr (Shaner and Reider, 1986). This increase is probably due to the combination of 2 factors. Firstly, imidazolinones do not affect photosynthesis and consequently photosynthate is still being produced in the leaves. Secondly, imidazolinone treatment affects the transport processes usually employed to move sugars and so the photosynthate remains at the site of synthesis. Consequently, the growing points of the treated plant are deficient in not only amino acids but also photosynthate (Devine, 1989). This inhibitory effect on the photosynthate transport system can be reversed by exogenously applying branched-chain amino acids to the plants (Devine, Bestman and Vanden Born, 1990). This implies that the disruption is linked to the inhibition of branched-chain amino acid synthesis, but the link is as yet unclear.

A further physiological effect of imidazolinone treatment is the inhibition of transpiration. Both imazaquin and imazethapyr cause a 50% decrease in transpiration in *Xanthium strumarium* 2 days after treatment (Shaner, 1992). This reduction in transpiration is not solely as a result of growth inhibition. It also reduces transpiration when calculated on a leaf area basis, implying an effect on the stomata. The mechanism is not known but, as the effect occurs 2 days after treatment, it could be due to any of the processes that are disrupted by treatment beforehand.

Risley (1986) found that imazaquin inhibited the release of auxin-induced ethylene from leaf discs, but treatment did not completely halt the release. The auxin release was restored by the supplementation with valine, leucine and isoleucine.

The herbicidal uses of imidazolinones show that there are many physiological consequences of the inhibition of AHAS including plant death. Understanding the sequence that leads up to the plant death is difficult because of the involvement of so many different processes and pathways. Despite most imidazolinone research focussing on crop plants, some of these plant responses will occur in imidazolinone treated potato tubers.

1.3.6. Imazethapyr treatment of potatoes

Preliminary studies by Cyanamid have indicated that imazethapyr can provide excellent sprout control with very low application rates (1g ai t^{-1}) and can suppress sprouting for up to 9 months. When used in potato treatment, it is applied using a hydraulic sprayer applying 1L t^{-1}. The sprayer is mounted over a roller table, where the potatoes are generally in a single layer and rotating. Trials carried out in commercial stores (both box and bulk) across a range of temperatures (12-35 °C) demonstrated that one application of imazethapyr gave control of sprouting without the need of an application of chlorpropham. In addition, the spray distribution provided even coverage throughout the store (Tayler *et al*, 1996). Sprout control was found to persist for up to 2 weeks following washing and storage under simulated supermarket and domestic conditions (Mawson *et al*, 1996). Imazethapyr was found to be effective when used in conjunction with chlorpropham, and a trial carried out on cv. Record stored at 10°C demonstrated that one application of imazethapyr followed by two applications of chlorpropham granules was as effective

as 8 applications of chlorpropham for control of sprouting up to 40 weeks (Mawson *et al*, 1996).

Despite the excellent sprout suppressant qualities, occasional disorders have been observed with imazethapyr treatment of potatoes. In some populations, up to 80% of treated tubers have been affected by vascular discolouration and cortical pitting. The symptoms of vascular discolouration can vary in severity from shallow speckling near the stolon attachment (Figure 1.6), to a solid ring of deep damage up to 15 mm deep (Figure 1.7). The colour of discolouration can vary from light brown to a dark chocolate brown and is usually more obvious at the stem end. This damage is similar but distinct from the symptoms of Internal Brown Spot (IBS) described by Hiller, Koller and Thornton (1985) and Davies (1998). IBS can be located throughout the tissue, but is commonly found in parenchyma tissue internally from the vascular ring. The vascular discolouration that has occurred in imazethapyr treated tubers is limited to the vascular ring. Hiller *et al* (1985) indicates that when a tuber is under stress any sudden effect, be it mechanical or chemical, could result in vascular discolouration.

The cortical pitting symptoms are equally variable, ranging from slight flecks near the edges of the cortex (Figure 1.8) to larger dense pits which spread out towards the periderm (Figure 1.9). The pits are very dark in colour and are irregular in shape. The symptoms are unusual and there is little mention of them in literature. The pitting does show similarity to the symptoms described as low temperature injury, but the incidence of the symptoms does not correlate with low temperature exposure.

This discolouration has caused potential problems with marketing imazethapyr as a sprout suppressant. The work in this thesis focuses on the treatment of potatoes with imazethapyr, the conditions that cause discolouration to form and ways to minimise its occurrence.

Figure 1.6. Slight vascular discolouration cv. Cara.—— = 1cm.

Figure 1.7. Severe vascular discolouration cv. Cara.—— = 1cm.

Figure 1.8. Slight cortical pitting cv. Cara.—— = 1cm.

Figure 1.9. Severe cortical pitting cv. Cara.—— = 1cm.

1.4. Aims

This study aims to examine the effects of imazethapyr on the morphology and physiology of potato tubers, and identify any possible linkage between imazethapyr treatment and vascular discolouration. It is hoped to define storage regimes which lessen the occurrence of this disorder. Using the techniques of respiration measurements, radiolabel uptake and distribution, ion chromatography, mitotic indices and transmission electron microscopy, symptom development will be examined at morphological and physiological levels.

2.0 VASCULAR DISCOLOURATION FOLLOWING TREATMENT OF TUBERS WITH IMAZETHAPYR

2.1 Introduction

Potato tubers are susceptible to many internal disorders, which can be due to insects, nematodes, infectious diseases or non-infectious physiological disorders. There are also many causal factors, including cultural practices, soil quality and moisture, fertilizer regimes and chemical treatments (Davies, 1998). Their occurrence can also be erratic, depending on the year and the production area (Hiller *et al*, 1985). Disorders resulting from chemical treatments can prove difficult to detect because of other symptoms occurring within the crop. Research tools used to detect them, or the conditions in which the disorder might occur, are wide ranging. Physiological and ultrastructural analysis alongside visual assessment can help to determine the exact nature of a disorder.

Vascular discolouration has been observed in some tubers treated with imazethapyr, in the range of 0-80% of treated populations (Cyanamid, personal communication). The occurance of vascular discolouration is erratic and can be easily mistaken for similar disorders. The nature of the symptoms have been outlined and illustrated in Chapter 1.3.5. The disorder shares some similar symptomology with that of internal brown spot (IBS) (Davies, 1998), but is distinctly defined in Hiller *et al* (1985).

Tuber respiration is a valuable indicator of tuber metabolism (Burton *et al*, 1992). The infra-red gas analyser (IRGA) is a rapid and precise method for measuring respiration, and equipment and methods have been successfully adapted for the measurement of tuber respiration (Williams and Cobb, 1992). Tuber respiration is typically high at harvest and falls to a basal level with time. The length of time for this decline to occur is dependant on cultivar, maturity, temperature and harvest conditions (Burton *et al*, 1992). Respiration rates can change when tuber lifting is carried out in wet weather or from wet soil. There is also an increased risk of water borne diseases (for example, bacterial soft rots) and anoxia (Gunn, 1990).

Ethylene is known to affect tuberization, but is also linked to metabolic changes in damaged cells. Ethylene production is normally negligible in the potato (<5 nl $kg^{-1}h^{-1}$), but Creech, Workman and Harrison (1973) found that under stress

conditions the production increased to 30 $\mu l\ kg^{-1}h^{-1}$. 2-chloroethyl phosphonic acid (CEPA) is an ethylene evolving compound that penetrates into plant tissues and is translocated. In living tissues it progressively decomposes to ethylene, phosphoric acid and chloride ions. However, CEPA is stable in aqueous solutions with pH< 3.5, whilst at higher pH decomposition occurs (The Pesticide Manual, 1994). Thus, CEPA is labile at physiological conditions and so elevated ethylene concentrations can be simulated by treating plant tissues or immersing tubers in CEPA.

Store temperature is a determining factor in the rate of respiration of the stored tuber (Burton, 1963). Consequently, variations in temperature and the effect of temperature on imazethapyr treated tubers, are considered in this chapter. Various environmental conditions were simulated in this study to investigate possible links between changes in respiration and the incidence of vascular discolouration.

Another section of this study was to investigate possible relationships between imazethapyr, the enzyme polyphenol oxidase (PPO) and the internal discolouration of tubers. PPO is an enzyme involved in bruising and oxidative discolouration reactions. It was first described in 1895 by Bertrand, but its biological function is not yet established (Vaughn, Lax and Duke, 1988). It is present in high concentrations in meristematic tissues and the exterior of storage and propagative organs. Concentrations increase upon wounding, and the enzyme seems to be involved in defence, via the octadecanoid signalling pathway (Constabel, Bergey and Ryan, 1995). Chemically, PPO catalyses the hydroxylation of monophenols to *o*-diphenols and the dehydrogenation of *o*-dihydroxyphenols to *o*-quinones. The quinoid products oxidatively polymerize and crosslink various amino acid groups to form melanin (Figure 2.1).

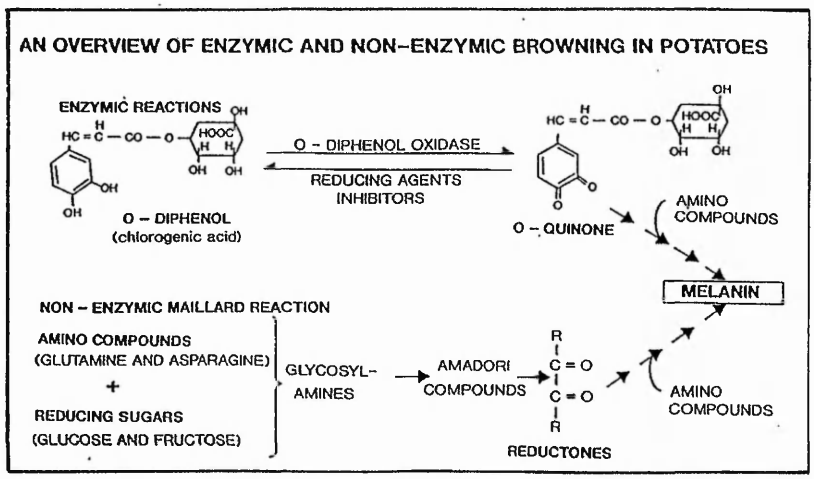


Figure 2.1. Scheme of melanin formation (Cobb, 1996).

This enzymatic browning occurs when tissue is damaged, molecular oxygen enters and the phenolic substrates in the cell vacuole come into contact with the PPO enzymes located in the plastids. PPO activity is increased by various environmental factors, such as acid/alkali shock, low temperature, urea and anionic detergents (Kenton, 1957; Dubernet and Ribereau-Gayon, 1974; Moore and Flurkey, 1990; Sanchez-Ferrer, 1993; Swain, Mapson and Robb, 1966). The possibility that PPO activity in tubers increases after imazethapyr treatment was investigated using crude enzyme extractions.

The overall aim of the work presented in this Chapter was to establish possible interactions between symptom development, tuber physiology (as measured by respiration and PPO assays) and store regime (including temperature, ethylene, water and imazethapyr treatment).

2.2 Materials and Methods

2.2.1 Respiration measurements

Freshly harvested tubers were obtained from various sources. Tubers of cultivar Cara were obtained by Cyanamid UK from QV Foods (Lincolnshire), whilst samples of Maris Piper were purchased from a local supermarket (Safeway).

When treated at TNTU, tubers were completely immersed in 0.1 or 1 g ai L^{-1} aqueous solution of commercially formulated imazethapyr (Formula ref. RLF 11952 Cyanamid, Gosport, Hants. U.K.) for 5 min and allowed to dry at 10°C. To simulate a wet harvest, tubers were immersed in deionised water for 3-24 h, 2 days prior to imazethapyr treatment. To simulate the stress of ethylene production, tubers were immersed in 1 mM 2-chloroethyl phosphonic acid pH 3.0 (CEPA, Sigma-Aldrich Company, Poole, U.K.) for 5 mins, 2 days after imazethapyr treatment.

Treated and control tubers were stored in seed trays (36 x 22 x 5.5 cm) lined with absorbent paper with another upturned tray placed on top. Both trays were wrapped with black plastic bin liners to exclude light and stored in refrigerators at either 2.5, 5, 10 or 20°C. Tuber respiration was measured using an LCA-4 Infra Red Gas Analyser (IRGA) (Analytical Development Company, Hoddeston, Herts. U.K.) as shown in Figure 2.2.

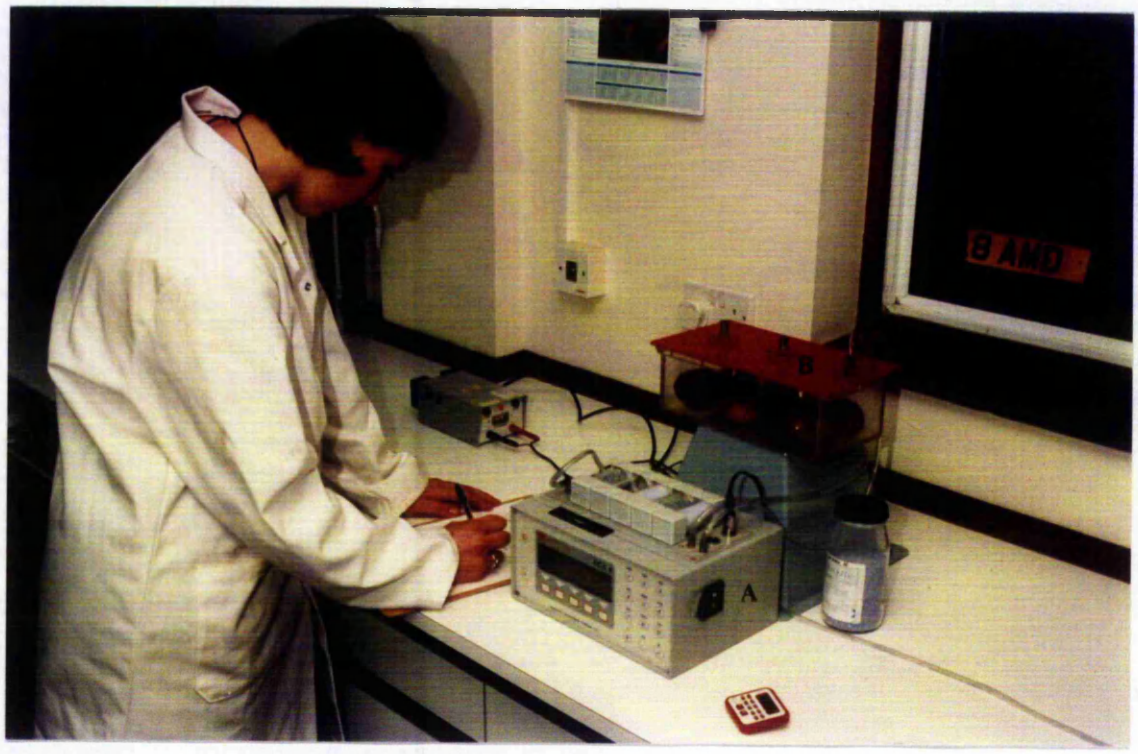


Figure 2.2. Infra-Red Gas Analyser (A) and experimental chamber (B) (in operation at the Potato Marketing Board Experimental Station, Sutton Bridge, Lincs.)

2.2.1.1 IRGA Theory

The analyser measures the gaseous concentrations of CO_2 and H_2O vapour circulating in the experimental chamber in the presence of the tubers. The optical bench contains CO_2 and H_2O cells in series, and a pyro-electric detector is used to measure differences in the infra-red absorption of different gases. The infra-red source produces a modulated wavelength that varies in amplitude according to the energy absorbed by the gases in the cell. The waveform is demodulated to produce a D.C. voltage. Reference and analysis gases are alternated with zero gas during a measurement cycle lasting approximately 16-20 s. This allows the cells to refill and is adjusted automatically to account for the flow rate.

2.2.1.2. IRGA Practice

From each tray of samples, 6 tubers were removed, accurately weighed and placed in an acrylic experimental chamber (26 x 13 x 9.5cm) attached to the IRGA inlet and outlet ports by Nalgene tubing (Sybron Corporation, New York, U.S.A). Dried air was drawn from outside the laboratory into the reference cell and the flow rate was set at 200 μmol s^{-1}. Once the CO_2 differential was constant (this took 0-10 min), 3 readings were taken at 2 min intervals. The CO_2 differential was converted to a mean respiration value as μmol s^{-1} g^{-1} fwt potato. This was achieved using the following equation:

$$\frac{CO_2 \text{ differential x flow rate}}{\text{weight of sample}} = \text{Respiration}$$

This procedure was repeated twice for each tray of tubers, using a further set of 6 potatoes each time. After a tray had been analysed, it was resealed and replaced in the relevant storage regime, allowing continual measurements to be taken.

The data from both samples was meaned and expressed as μmol s^{-1} g^{-1} fwt potato. Statistical analysis in the form of a t-test was carried out using StatMost software package.

2.2.1.3. Assessment of symptoms.

After the analyses had been carried out the tubers were destructively assessed, by cutting each tuber in half and visually recording any vascular damage or cortical damage, using a British Potato Council (BPC) key. This assessment was carried out on 6 tubers per treatment. The scales used were:

Vascular discolouration
- nil
- slight: >25% of vascular ring is grey or brown
- severe: 100% of vascular ring is grey or brown

Pitting / Necrosis
- nil
- slight: small number of small brown spots (<20)
- severe: large number or large spots or cavities

These data were meaned and converted into percentages.

2.2.2 PPO Assays

2.2.2.1 Protein extraction.

A 10.5mm diameter core was taken through a potato tuber, the periderm removed and a 2cm tissue sample cut from the basal end. The tissue was homogenised on ice with 4ml of 0.1M morpholinoethanesulfonic acid (MES) buffer pH 6.0 (Sigma Aldrich Company Ltd, Poole). The homogenate was strained through muslin and the filtrate centrifuged for 5 min at x 13400g in a microcentaur MSE bench centrifuge (Jennings Ltd, Nottingham). The supernatant was decanted and 2.5 ml was pipetted onto a Sephadex® G-25 desalting column (Pharmacia Biotech, Uppsala, Sweden) which had been previously equilibrated with 25ml of 0.1 M MES. Once all of the sample had run into the column, 3.5 ml of buffer was added and the desalted protein extract was collected and stored on ice. The PPO assay was performed polargraphically by O_2 uptake. The oxygen electrode (Hansatech Instruments Ltd, King's Lynn.) consisted of a PTFE membrane, platinum cathode, silver anode and electrolyte of saturated KCl. The apparatus was surrounded by water jackets kept at 25°C. Sodium dithionite was used to provide a zero oxygen point. This value was used in the calibration of the chart recorder. The following reaction mixture (total volume 1ml) was added to the oxygen electrode chamber:

- 800 µl 15mM chlorogenic acid in 0.1 M MES, pH 6.0
- 180µl 0.1M MES
- 20µl de-salted extract

The quantities of buffer and extract were varied if the activity of the enzyme was too low to provide a measurable gradient. The initial gradient of the trace obtained during the enzyme assay represented the amount of oxygen used in the initial reaction per unit time for 1ml of reaction mixture. The specific activity was calculated per mg of protein. Six extracts were made for each treatment and the assay was repeated 3 times for each extract.

2.2.2.2. Quantitative protein assay.

The protein contents of all enzyme extracts were determined by Coomassie Blue dye binding assay (Bradford, 1976). The reagent was made up using 200mg G-250 (Coomassie Brilliant Blue) (Sigma Aldrich Company Ltd, Poole) dissolved in 50 ml 95% (v/v) aq. ethanol to which 100 ml 85% (v/v) aq. phosphoric acid was added forming a stock solution. One ml 0.3% (w/v) SDS was added to 15 ml stock solution, made up to 100ml with distilled water and filtered through Whatman No.1 paper to form working reagent. The assay mixture consisted of:

- 4 ml reagent
- 750µl distilled water
- 50µl sample

The assay mixture was combined in clean glassware and left for 3 min before the absorbance was read at 595nm using a Perkin-Elmer 550-S UV VIS spectrophotometer. All glassware was acid-washed to prevent dye binding to detergents or ethanol. Standard BSA solutions (ranging from 0.05 to 4mg ml^{-1} in 0.1 M MES) were used to provide a regression equation for the protein reagent, relating absorbance to protein content.

2.2.2.3. Calculation of PPO activity.

The distance between the recorded trace before and after the addition of sodium dithionite corresponded to 100% air saturation and 0% air saturation of 1ml of air saturated water at 25°C. Under these conditions 1ml of air saturated water contained 0.253 µmol O_2. The initial gradient of the trace obtained during the enzyme assay was used to calculate µmol O_2 ml^{-1} min^{-1} and the specific activity is calculated by dividing the enzyme activity by the protein content resulting in µmol O_2 min^{-1} mg $protein^{-1}$. It has been assumed that the reaction mixture contains the same amount of oxygen when air saturated as distilled water. Statistical analysis of the data sets in the form of t-tests was carried out using StatMost software package.

2.3 Results

2.3.1 Respiration

The rate of tuber respiration was dependant on storage temperature. When stored at 2.5 °C, treated tubers respired at a significantly higher rate compared to the controls for the first 17 days ($p < 0.05$) (Figure 2.3 a). This was the only significant difference observed between treated and control respiration rates and was not apparent at other temperatures. The rates of respiration altered with temperature. At 5°C the rate was slightly lower than at 2.5 °C (Figure 2.3 b). The lowest rates were observed at 10°C (Figure 2.3 c), whilst the highest were recorded at the highest temperature, 20°C (Figure 2.3 d).

In all cases water immersion of the tubers caused an initial decline in respiration rate. Respiration of tubers treated with imazethapyr fell further immediately after treatment, but after a few days the treated rate had recovered to control rates or higher. The tubers that were immersed in water for less than 8 hours respired at comparable rates to the controls (Figure 2.4 a-c). Immersion in water for 24 hours caused an increase in the respiration rate of treated tubers and this rate was far higher than that of the control (Figure 2.4 d).

The destructive visual assessments were made at the end of the series of respiration measurements (Table 2.1). Control tubers displayed no symptoms, but water immersion increased the incidence of vascular discolouration. The tubers that were not immersed in water, but treated with imazethapyr displayed a low level of vascular discolouration. The incidence of the disorder increased when imazethapyr treatment was combined with 3 hours water immersion, but the symptoms were lower when tubers were immersed for 8 or 24 hours. Severe symptoms of vascular discolouration were not observed in this experiment.

Table 2.1. Effect of water immersion and imazethapyr treatment on symptom development in cultivar Cara after 16 days. Visual destructive assessment of 6 tubers per treatment. Data are mean percentage slight vascular discolouration.

Immersion time (hours)	- imazethapyr	+ imazethapyr
None	0	33.3 ± 21.1
3	33.3 ± 21.1	50 ± 22.4
8	33.3 ± 21.1	33.3 ± 21.1
24	33.3 ± 21.1	33.3 ± 21.1

Figure 2.5 displays the results from tubers exposed to imazethapyr and CEPA. The lowest rates of respiration were recorded by the control tubers (no treatment). The highest rates of respiration were achieved using a mixture of both imazethapyr and CEPA. However, these trends were transient, with all the tubers respiring at a similar rate 2 weeks after treatment.

The visual destructive assessment (Table 2.2) illustrates that the lowest extent of vascular discolouration was in control tubers and the incidence of discolouration increased upon CEPA exposure. The highest levels of vascular discolouration were found in tubers treated with imazethapyr, treatment with imazethapyr resulted in significantly higher incidence of vascular discolouration ($p<0.01$). Severe symptoms of vascular discolouration were not observed in this experiment.

Table 2.2. Effect of CEPA and imazethapyr treatment on symptom development in cultivar Cara after 14 days. Visual destructive assessment of 6 tubers per treatment. Data are mean percentage slight vascular discolouration.

Treatment	- imazethapyr	+ imazethapyr
-CEPA	16.6 ± 16.7	83.3 ± 16.7
+CEPA	33.3 ± 21.7	83.3 ± 16.7

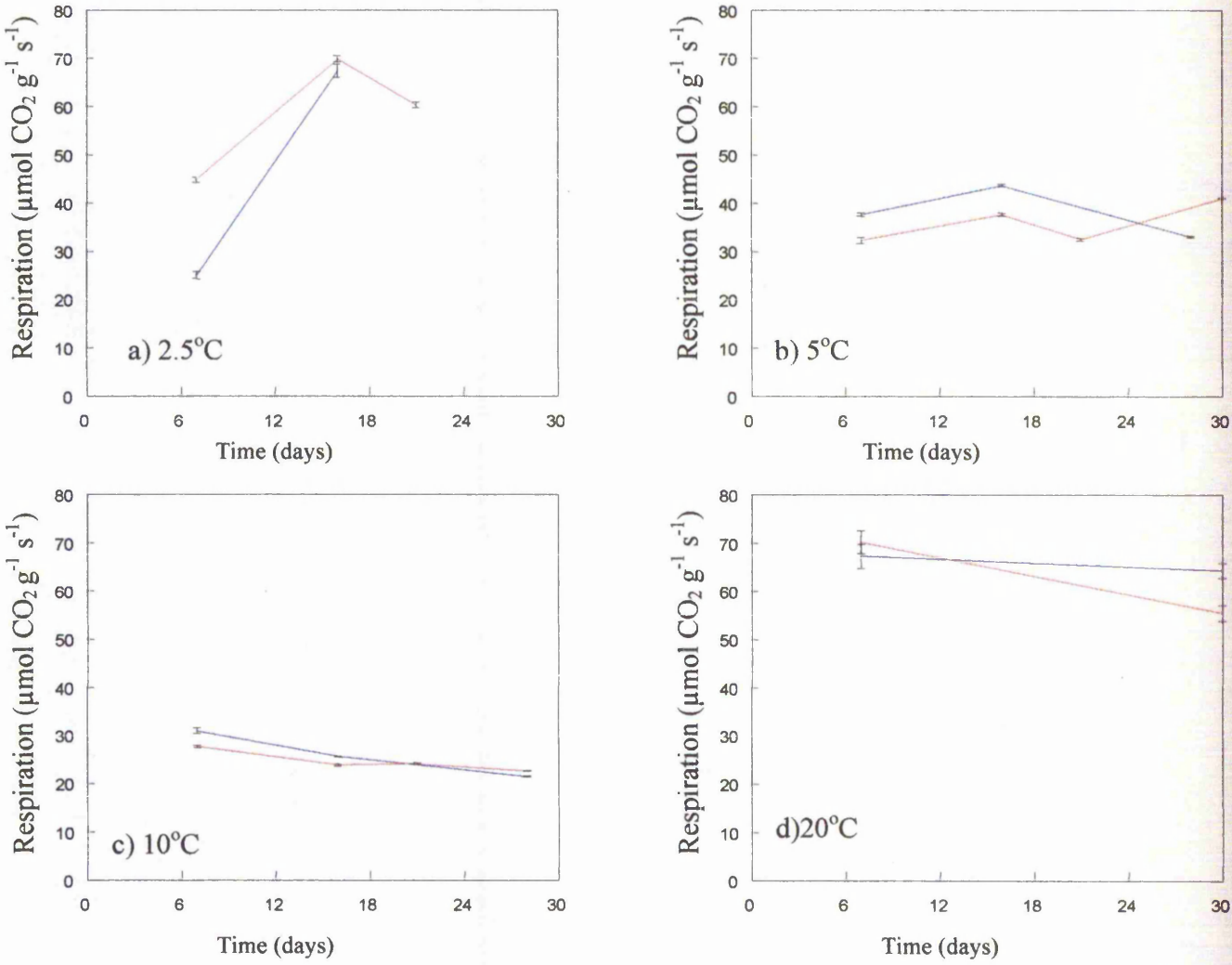


Figure 2.3. Respiration of Maris Piper tubers (± 1 g ai L^{-1} imazethapyr) stored at various temperatures over a period of 30 days. Data are means of 6 values ± standard errors.

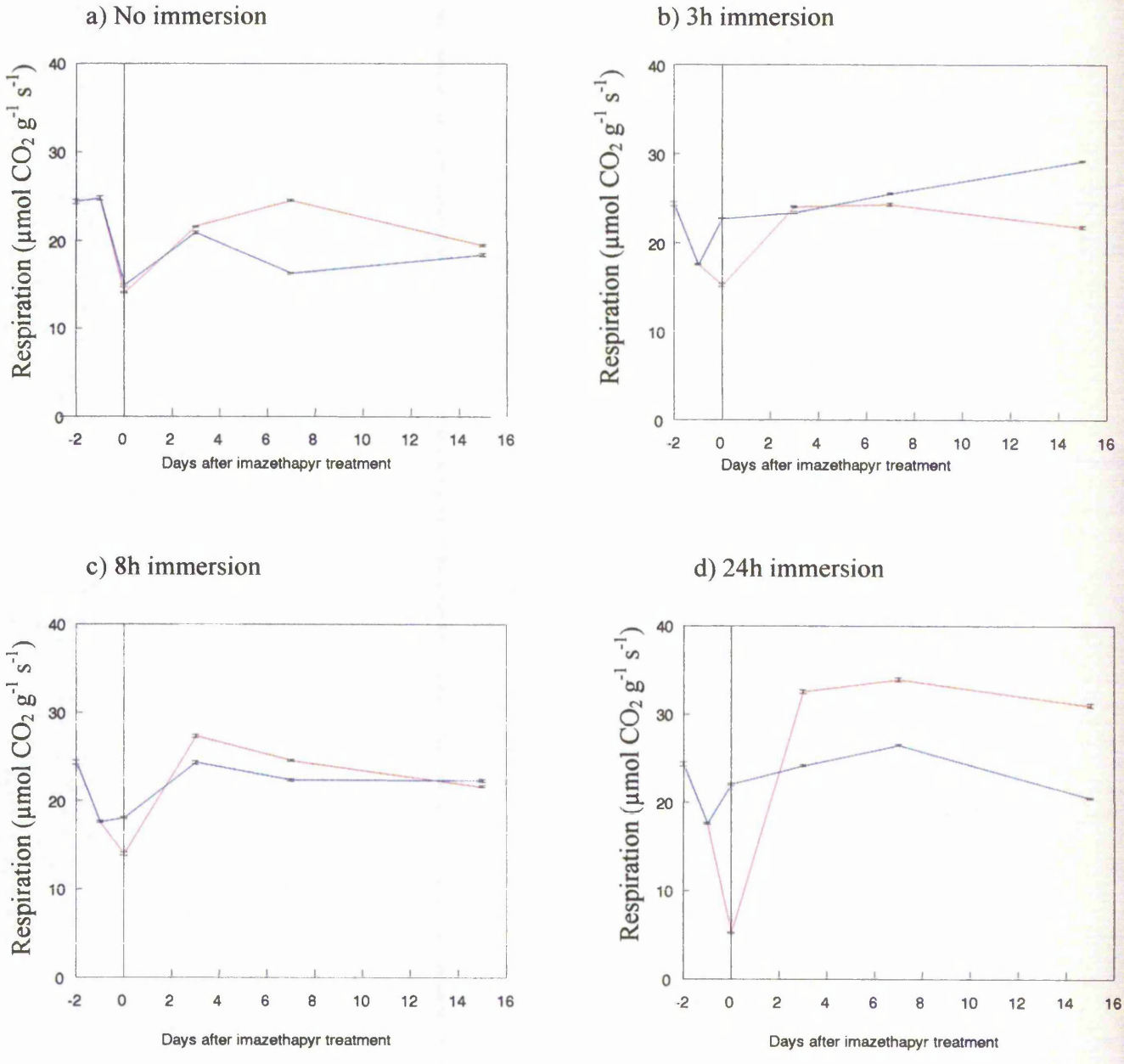


Figure 2.4. Respiration of immersed tubers cv. Cara (± 1 g ai L^{-1} imazethapyr) stored at 10°C. Water immersion took place 2 days prior to imazethapyr treatment. Data are means of 6 replicates ± standard error.

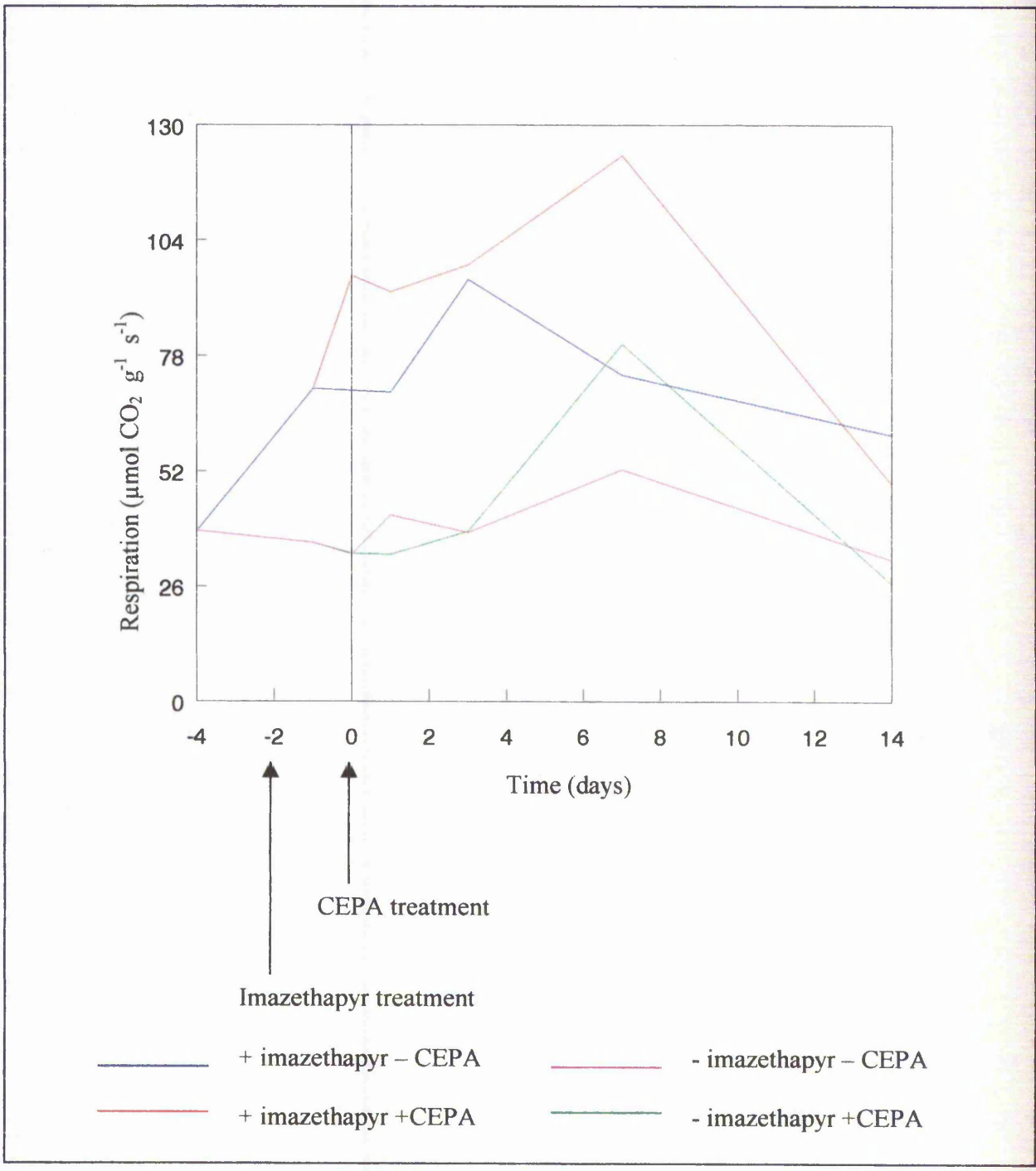


Figure 2.5. Respiration of tubers cv. Cara (±1 g ai L^{-1} imazethapyr and ±1mM CEPA) stored at 10°C. Data are means of 6 replicates and standard errors were less than 5µmol CO_2 g^{-1} s^{-1}.

2.3.2 PPO activity

PPO activity was significantly higher in mature tubers treated with 1g ai L^{-1} imazethapyr compared to untreated tubers, when stored at 2.5°C ($p < 0.05$). PPO activity in tubers stored at other temperatures was not significantly different from the untreated controls (Figure 2.6). Various formulations of imazethapyr and formulation blanks were applied to the tubers prior to crude enzyme extraction at pH 5.0 and 3.5 (Figure 2.7). The only significant result observed was an increase in the activity of the Tween blank at pH 3.5, compared to pH 5.0 ($p < 0.05$). Generally, low PPO activity was recorded using newly harvested tubers. The effect on PPO activity of various chemicals present during extraction is illustrated in Figure 2.8. PPO activity was consistently significantly higher when extracted at pH 3.5 compared to pH 6.0 ($p < 0.05$). At pH 6.0, a significantly greater PPO activity was recorded when extracted in the presence of Agral blank compared to the other treatments at pH 6.0 ($p < 0.05$). Once again, generally low PPO activities were recorded using newly harvested tubers.

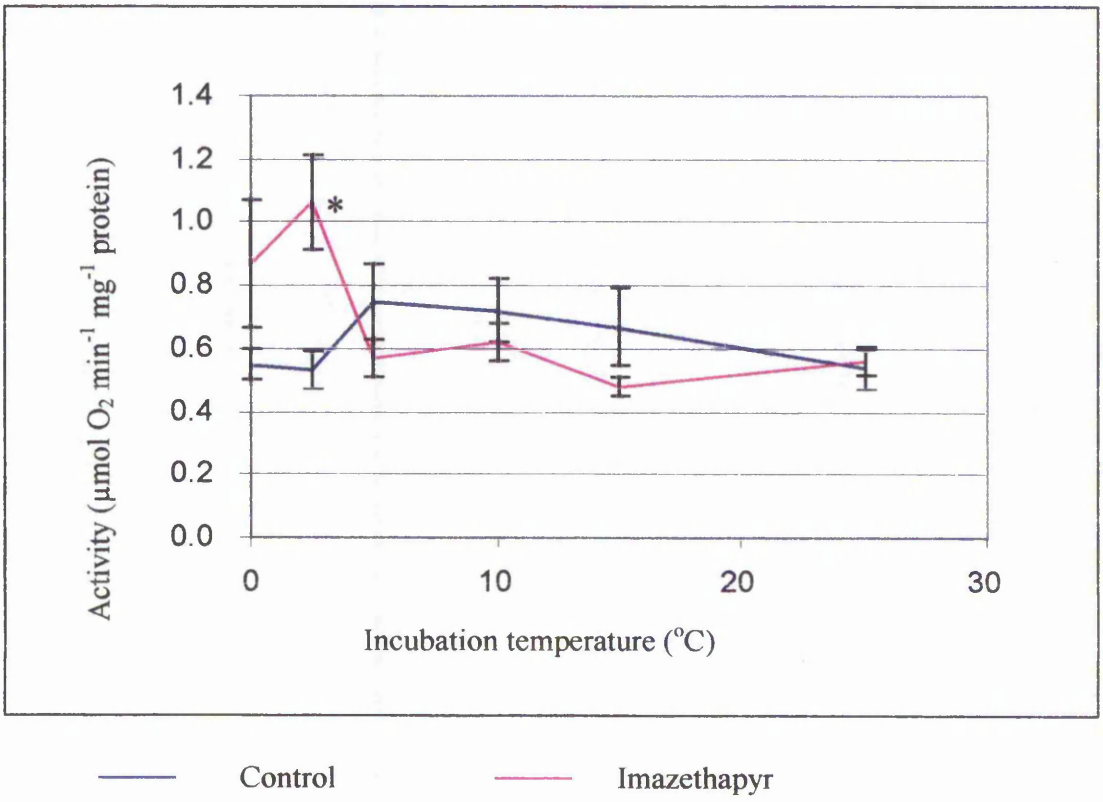


Figure 2.6. Mean PPO activity of mature tubers cv. Cara after 5 min immersion in 1g ai L^{-1} imazethapyr and incubation at various temperatures for 6 days. Data are means of 6 replicates ± standard errors. * = treated sample had significantly higher activity compared to control ($p < 0.05$).

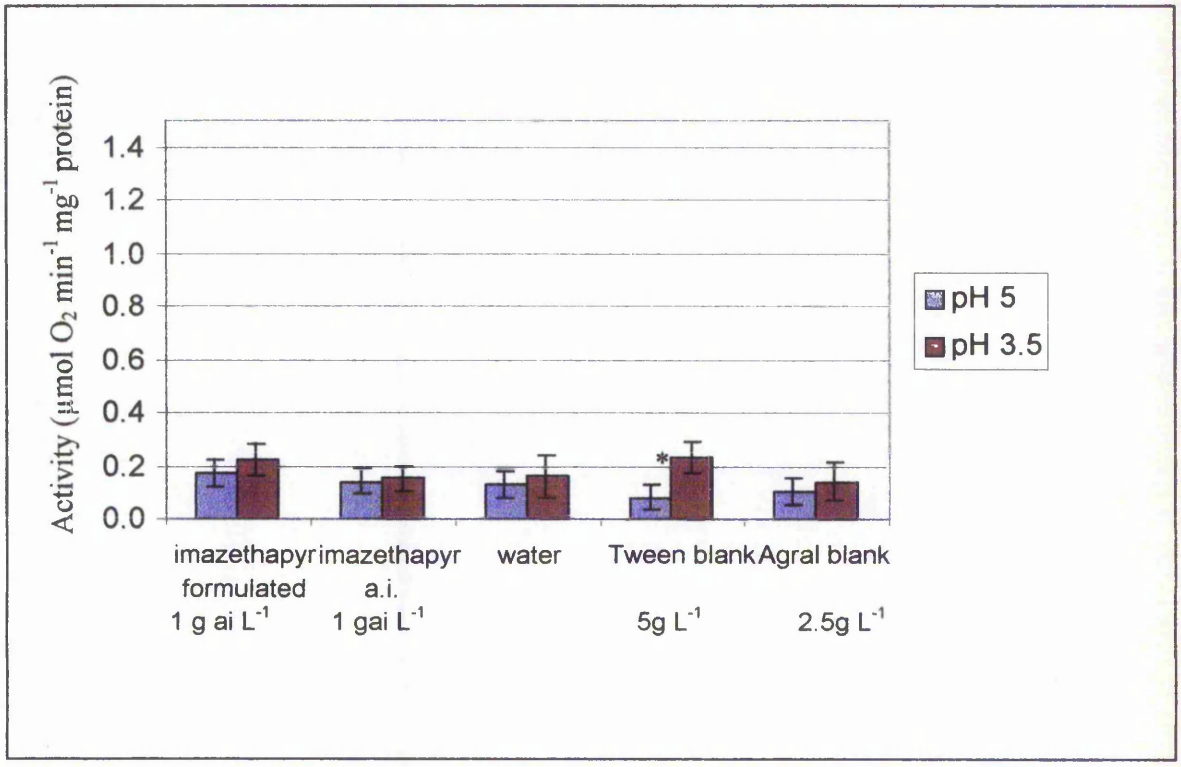


Figure 2.7. PPO activity of newly harvested potato tubers cv. Cara treated with various formulations at pH 3.5 or 5. Tubers were incubated at 2.5°C for 7 days after treatment. Data are means of 10 replicates ± standard error. * = pH 3.5 sample had significantly higher enzyme activity compared to pH 5 ($p < 0.05$).

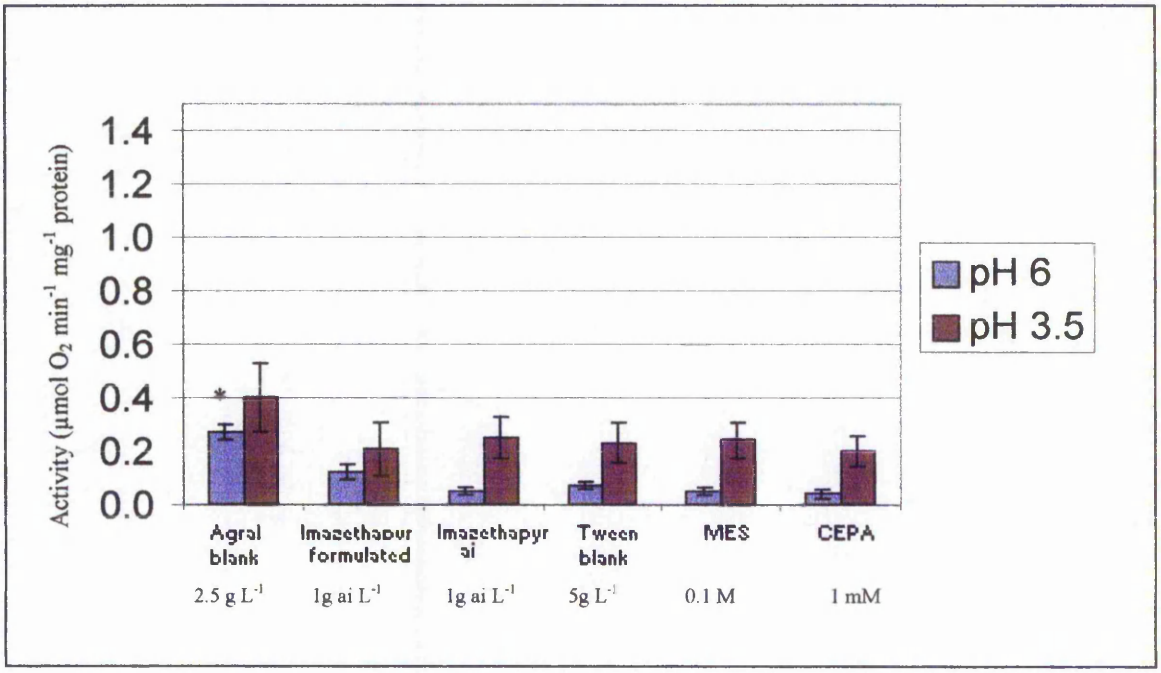


Figure 2.8. PPO activity of newly harvested potato tubers cv. Cara extracted in the presence of various formulations at pH 3.5 or 6. Data are means of 4 replicates ± standard error. * = Agral blank sample had significantly higher activity compared to other pH 6 samples ($p < 0.05$).

2.4 Discussion

This Chapter describes controlled environment experiments undertaken to define the conditions in which vascular discolouration occurs and investigates links between such conditions and changes in respiration and PPO activity of the tuber.

The response of respiration to temperature change was similar to that reported in previous publications (Burton, 1955; Dwelle and Stallknect, 1978) across a temperature range of 1.7-25°C. However, some of these published accounts were quoted as O_2 uptake rather than CO_2 evolution. There has been much debate over the type of measurement used to determine tuber respiration. Respiratory quotient is also frequently quoted (Dizengremel, 1985). The increase in respiration at low temperature has been previously reported (Workman, Cameron and Twomey, 1979) and it was found that low temperature also induced a delayed accumulation of sugar and increase in membrane permeability and an increase in phenolics, in other studies.

Although the specific type of cellular respiration is not distinguished in this study, it is known that imazethapyr and other AHAS inhibiting chemicals trigger the induction of alternative oxidase (AOX). Plant mitochondria transport electrons through the cytochrome (cyt) pathway or the cyanide-resistant respiratory pathway, catalysed by AOX. In thermogenic plants this pathway releases oxidative energy as heat. However, in non thermogenic plants the role of this secondary pathway is unknown at present (Hiser and McIntosh, 1994). AOX activity increases in AHAS inhibited cells (via regulation of AOX gene activity). The induction of AOX activity is not due to the presence of imazethapyr molecules *per se*, but as a function of the inhibition of BAA synthesis (Aubert, Bligny, Day, Whelan and Douce, 1997).

It has been suggested that ethylene stimulation involves the cyanide-resistant respiratory pathway and AOX (Arron, Day, Christoffersen and Laties, 1978). Treatment with ethylene gas resulted in an increase in respiration of whole tubers and isolated mitochondria from tubers (Day, Arron, Christoffersen and Laties, 1978). Ethylene is thought to enhance respiratory capacity in mitochondria. Ethylene pre-treatment of tubers has been shown to reduce the severity of blackspot of tubers (Ilker, Spurr and Timm, 1977). This is thought to be due to the increase in RNA and protein synthesis in ethylene pre-treated tubers, which causes tyrosine to be used in protein accumulation and is unavailable for oxidation into dark phenolic products.

However, in this study increases in vascular discolouration were observed when tubers were treated with CEPA alone, again highlighting the differences between vascular discolouration and blackspot.

Ethylene has dramatic effects on plants and is often produced as a response to external stresses e.g. temperature, anoxia or wounding. It has been used to break tuber dormancy (Alam, Murr and Kristof, 1994) and also to prolong dormancy (Prange, Kalt, Daniels-Lake, Liew, Page, Walsh, Dean and Coffin, 1998). The effect of a gas treatment of ethylene ($30\mu l\ L^{-1}$) has been reported to cause a sharp increase in respiration 24 h after treatment (Solomos and Laties, 1975). The effect of CEPA in this study was similar, causing an increase in respiration rate but the peak was 7 days after treatment. This effect may have been altered because a different concentration was used or because there may have been a lag in the time taken for CEPA to decompose to ethylene. Previous reported uses of CEPA suggest a lag time of up to 14 days before maximum ethylene evolution (Cvikrova, Sukhova, Eder and Korableva, 1994).

Immersion for long periods of time caused respiration in treated tubers to increase significantly above the immersed control tubers, once again highlighting the increase in respiration with imazethapyr treatment combined with an environmental stress. In other plant species, when anoxia was simulated, CO_2 evolution fell significantly within the first 12 hours (Zuckermann, Harren, Reuss and Parker, 1997). It may therefore be of interest to determine tuber respiration hourly for the first day following immersion.

Respiration was found to be consistently higher in treated tubers when the tuber was also subject to other environmental stresses, such as cold temperature storage, water immersion or CEPA exposure. When tubers were treated with imazethapyr and left in ideal conditions, respiration was similar to the untreated controls, indicating that treatment with imazethapyr alone might not damage tubers.

Some background vascular discolouration occurs in control tubers, as confirmed by the visual assessments. However the assessments were carried out on a limited number of tubers, because of difficulties in the supply of tubers. The tubers treated with imazethapyr alone displayed more discolouration than controls, but a larger sample size may confirm this as background discolouration. The tubers that were treated with CEPA or immersed but not treated with imazethapyr contained more vascular discolouration than controls. This indicates that any stresses on the

tuber (chemical or environmental) can increase the possibility of incidence of symptoms, as reported by Davies (1998). Symptoms occurred most frequently in tubers that were both treated with imazethapyr and subjected to an environmental stress, implying an additive effect of both stresses on vascular discolouration.

This highlights the need for correct handling, husbandry and storage of the harvested crop. When treating with imazethapyr, it is vital to avoid harvest in wet weather because tubers can be adversely affected by anoxia, increases in respiration rates and increases in incidence of vascular discolouration. Store conditions should be carefully controlled to avoid any sudden temperature changes, hot or cold, as this could increase respiration rates, potentially causing increases in vascular discolouration and subsequent loss in crop value.

The techniques used in this study are somewhat limited, since there are fundamental differences between these studies and the commercial situation in which imazethapyr would be applied. For example, the method employed to dip tubers, although controlled and accurate, was different when compared to a commercial sprayer, which would coat the tubers in a fine spray, rather than an immersion. The tubers were not sprayed with imazethapyr because of difficulties simulating the conditions of spraying across a roller table. The handling of tubers during the experiment would be less damaging than a commercial store spraying and loading, because each tuber was individually treated and placed in the store. The cultivars used during the experiment were not stored as part of a commercial storage facility and the store was lacking facilities such as humidity controls, and this may affect the reproduction of these results in a commercial store.

In addition, the cultivars used had different basal rates of respiration, so the initial data points of the figures are not the same. However, the rates of change are directly comparable between the cultivars. Another limitation of the method is that the rate of CEPA degradation cannot be calculated and it is assumed that the rate of ethylene evolution is comparable between tubers. As the respiration data was produced during one storage season, the repetition of this study in another season would be valuable, using the same cultivars and harvest times and storage regimes to enable the repetitions to be similar to these studies. Larger sample sizes for the visual assessments should be used in any future studies to define background levels of vascular discolouration more clearly. Image analysis could be used to carry out vascular discolouration assessments in the future this would provide a more

quantitative measurement for the amount of tissue affected and the incidence of the disorder. Autofluoresence has been used to highlight discolouration in other species (Aleemullah and Walsh, 1996) and this technique may be applicable for use in tubers, as it may allow a more precise determination of discolouration, particularly at early stages.

IRGA has been shown to be a highly reliable and sensitive method for measuring tuber respiration and this study demonstrates the value of respiration as an indicator of tuber metabolism and stress when linked with visual observations of discolouration. AOX assays should be undertaken to determine the exact role that this important enzyme plays in the respiration of an imazethapyr treated tuber.

PPO activity can be measured by a variety of techniques, although the use of the O_2 electrode is favoured as the rates are up to 12 times higher than with spectrophotometric measurements (Mayer, Harel and Ben-Shaul, 1966). The inhibitory endogenous phenols were successfully removed from the crude protein extractions using Sephadex® G-25 columns, which was reported to be more successful than dialysis or the addition of polyvinylpyrrolidone (PVP) (Hsu, Thomas and Brauer, 1988).

Initial experiments used mature tubers, cv. Cara and significant increases in PPO activity were recorded following imazethapyr treatment and low temperature storage. Previous studies indicate that PPO activity increases at low temperature regardless of chemical treatment and it was expected that control tubers would also display increased activity (Dubernet and Ribereau-Gayon, 1989; Harel and Mayer, 1968; Parish, 1972). However, the treated tubers may have been more susceptible to the effects of low temperature because of the treatment prior to storage. The control tubers may have displayed an increase in PPO activity if left at low temperature for longer than 6 days. The increase in PPO activity with treated tubers and low temperature is mirrored in the increase in respiration reported under the same conditions.

Newly harvested tubers were used for subsequent experiments. Although the formulation did affect the PPO activity, the activity was not significantly different from formulated imazethapry. Imazethapyr (formulated and active ingredient) provided the highest rates of PPO activity at pH 5.0, but these results were not significantly different from the formulation blanks. A significant difference was recorded using the Tween blank at different pHs, with greater PPO activity at 3.5

compared to 5.0. In all cases, PPO activity was higher at more acidic pH. Latent PPO can be activated by various processes. When the enzyme is activated by pH changes it is accompanied by a change in the Stoke's radius of the protein, suggesting conformational changes during activation (Lerner and Mayer, 1975). In other species, slow pH-induced conformational changes occur during the catalytic cycle of latent PPO (Valero and Garcia-Carmona, 1998). The increased activity of PPO at acidic pH could be due to conformational changes and increased solubilisation of the latent enzyme. As pH decreases, ion trapping of imazethapyr increases and accumulation of imazethapyr molecules in the symplast is more rapid. Ion trapping is discussed in more detail in Chapter 4 (Little and Shaner, 1991).

Both Tween and Agral were used by Cyanamid in initial studies of formulations. Although the Agral formulation was used with imazethapyr the Tween formulation was tested in PPO studies as well. Tween and Agral formulation blanks exerted an effect on PPO activity. Although both of these surfactants are non ionic and were not expected to affect PPO, there may be other components of the formulation that alter the activity of the enzyme. Apart from the main component of the formulation (ie Agral or Tween) the exact ingredients of the formulation were not released. *In vitro* experiments were conducted to attempt to mirror the *in vivo* results, but no significant effects were observed suggesting that the enzyme is not directly affected by imazethapyr formulation or its components.

Low temperature did not appear to affect the newly harvested potatoes in the same way as the mature tubers and the newly harvested control tubers were expected to have a higher rate of PPO activity. The difference in endogenous activity between the 2 supplies of tubers could have been due to many factors in growth and handling before delivery.

The PPO study was conducted over a limited time period and ideally more replications would be performed. Other useful work in this area would include field testing to determine the relative activity of PPO throughout the growing season and at harvest. This could be extended to a field test involving application of substrate to a sliced tuber, resulting in discolouration. Standard measures of the colour obtained over time in relation to PPO activity could be established and linked to likely incidence of vascular discolouration. The effect of formulation could be investigated at other storage temperatures, to determine the effect of imazethapyr on PPO more fully.

For discolouration reactions to proceed, even if there is elevated PPO or substrate levels, the enzyme still has to come into contact with its substrates. Therefore, some disruption of cell compartmentalisation must occur. An investigation into the effects of imazethapyr on enzymes associated with membrane integrity may be useful, for example lipolytic acyl hydrolase (LAH) and lipoxygenase (LOX) (Saravitz and Siedow, 1996). Ultrastructural changes to treated tuber membranes are investigated in Chapter 3.

Other environmental stresses could be further investigated. Infrequent watering has been found to cause an increase in PPO activity in avocado fruit (Bower and van Lelyveld, 1985) and this may also affect PPO activity of potato tubers.

To summarise, the data presented in this Chapter goes some way to defining the environmental conditions which can cause an increase in vascular discolouration. Respiration increased in response to imazethapyr treatment and other stress conditions. Furthermore, PPO activity increased when stress conditions and imazethapyr treatment were combined. The environmental stress conditions that can influence respiration and PPO activity are wide ranging and include acidic pH, ethylene exposure, cold temperature storage and water immersion. These factors can easily combine to cause metabolic changes within the tuber and on occasion disorders such as vascular discolouration may result.

These experiments reveal that the potato tuber is highly metabolically active and highly sensitive to the prevailing chemical and storage environment. Indeed, changes in these parameters can easily divert primary metabolic pathways into secondary metabolism, resulting in the accumulation of phenolic substances appearing as vascular discolouration.

3.0 ULTRASTRUCTURAL CHANGES FOLLOWING TREATMENT WITH IMAZETHAPYR

3.1 Introduction

Examination of tissues at a microscopic level allows further details to be elucidated, especially with regard to the responses of tissues to chemical treatment or its environmental conditions. Light microscopy is commonly used to provide a quick and simple evaluation up to a magnification of x1000.

Electron microscopy allows greater ultrastructural detail to be observed in comparison to light microscopy. Electron microscopy techniques have been adapted for use in plant cells since the 1950's. Initially, specimens were limited to those that were naturally thin or those that could be dried onto specimen holders. The difficulties of observing hard cell walls and large vacuoles were quickly overcome by the use of resins (Roland, 1978). The specialised fixation and sectioning of tissue generates sufficiently thin samples to enable examination of fine structural detail. In addition, the samples are strong enough to withstand the electron beam (Hunter, 1993). Symptoms of damage, such as vascular discolouration, found in some tubers treated with imazethapyr, can therefore be investigated at an ultrastructural level using electron microscopy. The integrity of cellular membranes and organelles can be studied in more depth using electron microscopy and any irregular or unusual features of the tissue can be recorded. These observations can be used to characterise the structural nature of the discolouration.

Light microscopy has been used in many instances to examine the mitotic division of plant tissue and to examine the effects of chemical treatment on the cell cycle. The stages of the cell cycle were defined by Howard and Pelc (1953) in 4 stages: G_1 (Gap 1), S (Synthesis), G_2 (Gap 2) and M (Mitosis). G_1, S and G_2 are collectively known as interphase. The M stage is divided into prophase, metaphase, anaphase and telophase. Chemicals can be grouped into those that effect interphase or mitosis. If the effect is during interphase a decrease in the frequency of mitotic cells will be observed and if the effect is during mitosis, aberrant mitotic figures or binucleate cells will be recorded. Hess (1982) termed these effects as cell division inhibition and cell division disruption, respectively. This terminology is no longer

adequate because disruption of division can sometimes result in no increase in cell number and this can be mistaken for cell division inhibition. In 1987 a refined terminology was proposed. Chemicals which act at interphase and cause a reduction in the frequency of mitotic cells are categorised as causing "inhibition of mitotic entry". Those that affect the mitotic sequence by affecting the spindle apparatus or cell plate formation are causing "disruption of the mitotic sequence" (Hess, 1987).

Imidazolinones are known to act on the mitotic division of cells (Pilmoor and Caseley, 1987) and much work has been carried out using pea and rice root tips, showing certain imidazolinones at various concentrations to inhibit the number of cells found in mitosis. It has been suggested that imazethapyr can cause cells to halt in interphase (Rost *et al*, 1990). The rate of mitosis of root tip meristem cells is an indicator of the growth and stability of the population (Hess, 1987). The technique of mitotic index counting from root tip squashes allows the differentiation between the different stages of mitosis as the number of cell divisions that occur in plant root meristems is very high. For example, the number of cells produced per hour is between 10,900 and 13,700 in peas (Van't Hof, 1967). If inhibition occurs, the stage at which inhibition occurs can be identified by this technique. In addition, any aberrant mitotic figures can be identified, highlighting any unusual divisions.

Using light microscopy is an ideal way to achieve low power images with minimal preparation time. It is also useful for examining tissue that would not withstand the rigours of embedding, fixation and sectioning used in electron microscopy, such as root meristems. Unstable or quickly degraded tissue can be assessed quickly using light microscopy, as the time from sampling to observing is short, although permanent mounting media (e.g. DPX, formulation R.Lamb, Sigma) are available to counteract this problem.

Another method of determining symptoms of imazethapyr treatment at a cellular level is to measure cell integrity by using cation leakage measurements. If the cell has been damaged or the membranes have lost their integrity it is common for cations to leak from the cell. Potato tuber cell membrane function and fluidity are greatly affected by storage temperature (Kuiper, 1984) and cell membranes often lose integrity in mid storage, about 18 weeks after harvest (Turnbull & Cobb, 1992). Ion chromatography can quantify cation leakage and can be used to determine the effect of imazethapyr treatment on cell integrity with storage time. Whilst this

method is less direct, it is useful to link quantitative data with photographic evidence from light or electron microscopy.

The main aims of this Chapter are to define the exact nature of the vascular discolouration observed in some treated tubers, to determine if cell integrity has a role to play in the discolouration process and to define the inhibition of the mitotic process by imazethapyr.

3.2 Materials and Methods

3.2.1 Mitotic index

Various methods have been adapted to achieve the optimum fixation and staining method for the mitotic index determination. The methods consulted include Conn, 1946; Gomori, 1952; Rost & Morrison, 1984; Di Tomaso, 1988; and Yim & Bayer, 1996. The final procedure used is as follows:

Freshly harvested tubers (cv. Cara and Estima) were placed in the dark in trays of vermiculite and watered. After 2 weeks roots began to develop. Root tips of 1 cm length were cut and treated with imazethapyr. Two methods of imazethapyr treatment were used, depending on the length of treatment. When incubation with imazethapyr was less than 2 h, the root tips were placed in a solution of ±1 g ai L^{-1} imazethapyr made up with deionised water. With longer imazethapyr incubations, the tips were placed in 10ml fresh Murashige and Skoog growth medium (Sigma, Poole) without growth regulators, but with 30g l^{-1} Oxoid N° 3 Agar with ± 1g ai L^{-1} imazethapyr. The root tips were left in the growth medium for 2, 4, 16 or 24 h. To counteract any potential anoxia, the experiment was repeated using aerated growth media, using a Whisper 2000 air pump on the lowest setting. The experiment was also repeated using the lower rate of 0.1 g ai L^{-1} imazethapyr.

After the time of exposure to the chemical had elapsed, the root tips were fixed in a 3:1 (v/v) mix of absolute ethanol: glacial acetic acid for 24 h at 4°C. The fixed root tips were washed in distilled water and hydrolysed in 1N HCl at 60°C for 10 min.

After rinsing in distilled water, the samples were stained in periodic acid solution (10g L^{-1}) for 2 h in the dark. The terminal 3 mm of each stained root tip was

excised using a razor blade, placed on a glass slide in a drop of 45% (v/v) aq. acetic acid and macerated thoroughly with a round-tipped glass rod. The slide (with a cover slip) was lightly pressed between 2 sheets of filter paper to spread out the cells and to absorb any excess moisture.

To permanently fix the slide, it was placed on dry ice for several min until the slide and cover slip were completely frozen. The cover slip was removed, with the sample still frozen to the slide, and the slide placed in absolute ethanol and mounted in DPX, formulation R.Lamb (Sigma, U.K.). A new cover slip was placed over the sample and the slide allowed to dry for several h, after which it was viewed and photographed using a light microscope (Dialux 20 E.B, Leitz).

The mitotic index was determined by counting the number of dividing cells in 300 observed at random, i.e. cells in prophase, metaphase, anaphase and telophase. The mean number of dividing cells out of the 300 observed cells was converted to a value of the number of dividing cells per hundred. This figure was used as the mitotic index. The mitotic index was determined from 6 different root tips per treatment. Two different time courses were carried out, one over 2 hours and another over 24 hours. Data are presented as means ± standard errors, where n=6. Statistical analysis was carried out using StatMost software package t-test.

3.2.2 Light and electron microscopy using fixed samples

Control and treated tubers (cv. Cara) were obtained from the Potato Marketing Board Experimental Station, Sutton Bridge, Lincs. Treated tubers were sprayed with 1 g ai L^{-1} imazethapyr + 0.25% (v/v.aq.) Agral upon entering storage. Tissue from vascular and cortical regions of the tuber was removed using a scalpel and cut into 1 mm cubes to allow full penetration of fixation chemicals. Six tubers were used and from each tuber 10 cubes were randomly selected for the light and electron microscopy. The tissue was kept in 3ml capped vials at all time and solutions were changed using disposable plastic pasteur pipettes. The vials were slowly rotated throughout the fixing procedure. After the samples were embedded, each block was sectioned for light and electron microscopy using a Reichert OM U2 microtome. The fixation procedure used is detailed on the flow chart, Figure 3.1.

3.2.2.1 Light microscopy.

Sections (0.5-2μm) were cut with a glass knife and placed in drops of water on a glass slide. The glass slide was placed on a hot plate to ensure water evaporation and adhesion of the section to the slide. The sections were covered with 1% aq. (v/v) alkaline toluidine blue (Trump, Smuckler and Bentitt, 1961) and returned to the hot plate for 60 sec. The slide was washed in hot tap water and dried. The slide was then be mounted permanently with resin, viewed and photographed using a light microscope (Dialux 20 E.B, Leitz).

3.2.2.2 Transmission electron microscopy.

With the information provided by the light microscopy, thin sections could be cut from the blocks. Section thickness was determined by the colours seen through the microtome microscope as the sections float on the surface of the water-filled trough (Hunter, 1993). Gold sections (90-150nm) were used for this low magnification work (x500-x6,000 magnification). The sections were flattened by wafting a cotton tipped applicator of chloroform over the sections. The grids used were 100 mesh, coated with Formvar (0.5% Formvar (v/v) in ethylene dichloride) for support and adhesion. Each grid was held in watchmaker forceps, immersed in the water trough and lifted out of the water from underneath the sections, thereby catching the sections on the grids. The grids were left to dry on blotting paper.

There was a degree of contrast obtained by post-fixing with osmium tetroxide in the block, but for optimum contrast the thin sections required further staining (Roland, 1978). The standard method of uranyl acetate followed by lead citrate was used. Both stains were filtered prior to use, using a Supor Acrodisc 32 0.2μm filter (Gelman Sciences, Michigan, USA.) to avoid any stain precipitate sticking to the grid. A piece of parafilm was placed inside the base of a petri dish and drops of uranyl acetate were placed on the parafilm (the number of drops equal to the number of grids to be stained). Each grid was floated section side down for 15 min and then washed gently in water. The section was then dried using filter paper and the process repeated using lead citrate drops (Reynolds, 1963). Lead citrate staining was carried

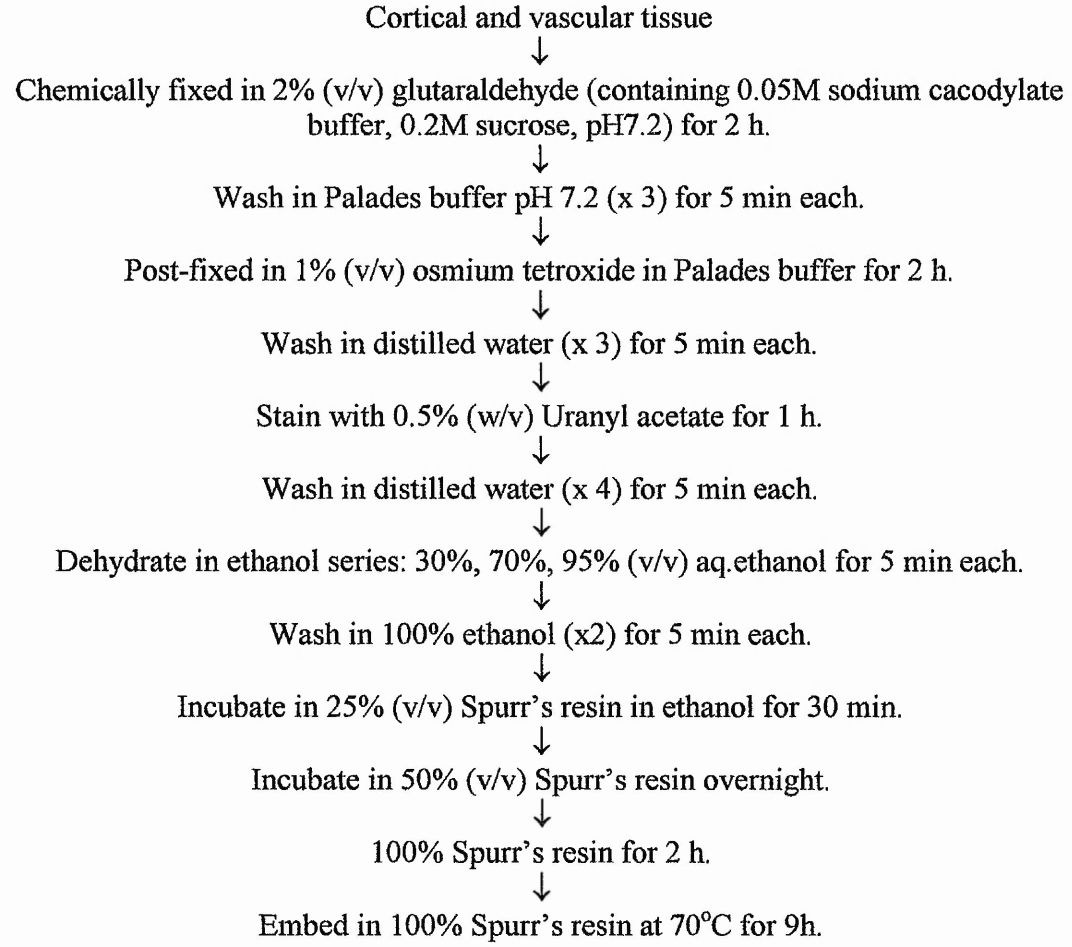


Figure 3.1. Flow chart displaying fixation process for electron microscopy.

out with a small dish of sodium hydroxide pellets within the petri dish, to absorb any CO_2 which reacted with the lead citrate and cause a lead carbonate precipitate on the grids. Once dried, the grids were observed and photographed using a Jeol 2010 electron microscope. From the initial 120 blocks, about 10 sections were cut from each block and approximately 5 sections were supported by one grid, 240 grids were stained. During staining some sections became detatched from the grids and about 200 grids were observed (100 treated and 100 control).

3.2.3. Cation leakage

Tubers (cv. Cara) were stored at 10°C and 12 tubers were sampled 37, 63 and 124 days after harvest. Half of the tubers were treated with a 5 min dip in 1 g ai L^{-1} imazethapyr. The 63 and 124 days after harvest sampling dates were also 31 and 92 days after treatment, respectively. A 12mm diameter core was taken from apex to base through each tuber. The periderm was removed from the top and bottom of each core. The cores were weighed and sliced lengthways into quarters, each quarter was sectioned into approximately 1mm sections. The sections from each core were placed together in a flask containing 20ml of deionised water and incubated in a shaking water bath at 25°C for 30 min, after which the liquid was decanted into McCartney bottles and stored at –70°C.

A standard cation solution was made up using NaCl, NH_4Cl, KCl, $MgCl_2$ and $CaCl_2$ at cation concentrations of 100mg L^{-1} each. The stock was filtered and diluted down to 50, 25, 10, 5 and 1mg L^{-1} and used to check the calibration of the cation chromatography column and was also tested at random times during runs of test samples. A 15mM solution of methyl sulphonic acid (MSA) was made up as the eluent solution and filtered using 0.2µg nitrate cellulose. As the initial 30mM MSA resulted in the peaks overlapping, a reduction to 15mM was used which spread the peaks out over 15 min. Once the standards had been run and the ion chromatography instrument (DIONEX) calibrated, the samples were defrosted, filtered and run. The samples were run through a IonPac CS12 4mm analytical column with an IonPac CG12 4mm guard column and a cation self regenerating suppressor. Data are presented as mean cation concentration (mg g fwt^{-1}) ± standard error, n=6. Statistical analysis was carried out using StatMost software package t-tests.

3.3 Results

3.3.1 Mitotic indices

Photographs were taken of the root tip squashes and Figures 3.2 to 3.7 display the different phases of mitosis observed in cv. Cara. The mitotic indices were calculated as the mean number of cells dividing per hundred and the number of cells in each phase of mitosis were recorded. Aberrant divisions were not observed at any point in the experiment.

Imazethapyr treatment at 1 g ai L^{-1} appeared to inhibit the number of mitotic cells in the population. However, a decline in mitotic divisions was noted in the untreated when using solid media (Figure 3.8). This was probably due to a problem with the incubation conditions of the root tips. Liquid medium was used for the next experiment, which showed that imazethapyr treatment caused a decline in mitotic activity after 1h, but after 2h the activity in the control samples had also dropped (Figure 3.9). It was speculated that the root tips were probably affected by anoxia after 2h incubation. To minimise this possibility subsequent experiments were carried out using aerated media.

In a further experiment a lower concentration of imazethapyr was used (0.1 g ai L^{-1}) and the mitosis in treated samples was inhibited in less than 30 min (Figure 3.10). During this time, the control tissues remained active and were not adversely affected by the incubation conditions. This was also demonstrated over 24 h. The different phases of mitosis were recorded and Figure 3.11 displays some of the data from Figure 3.10 broken down into separate phases. This demonstrates the low incidence of telophase observed and the decline of all phases of mitosis with imazethapyr treatment.

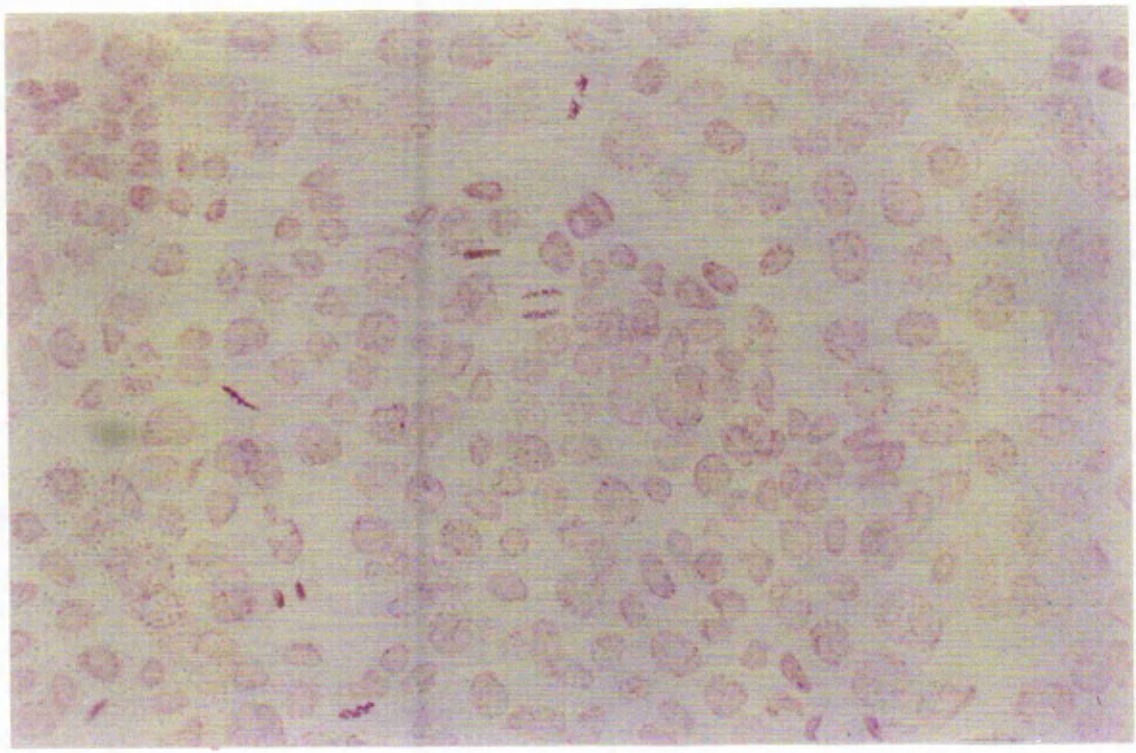


Figure 3.2. Low magnification. A typical population of potato root tip cells in interphase. Stained with Periodic acid solution, x 250.

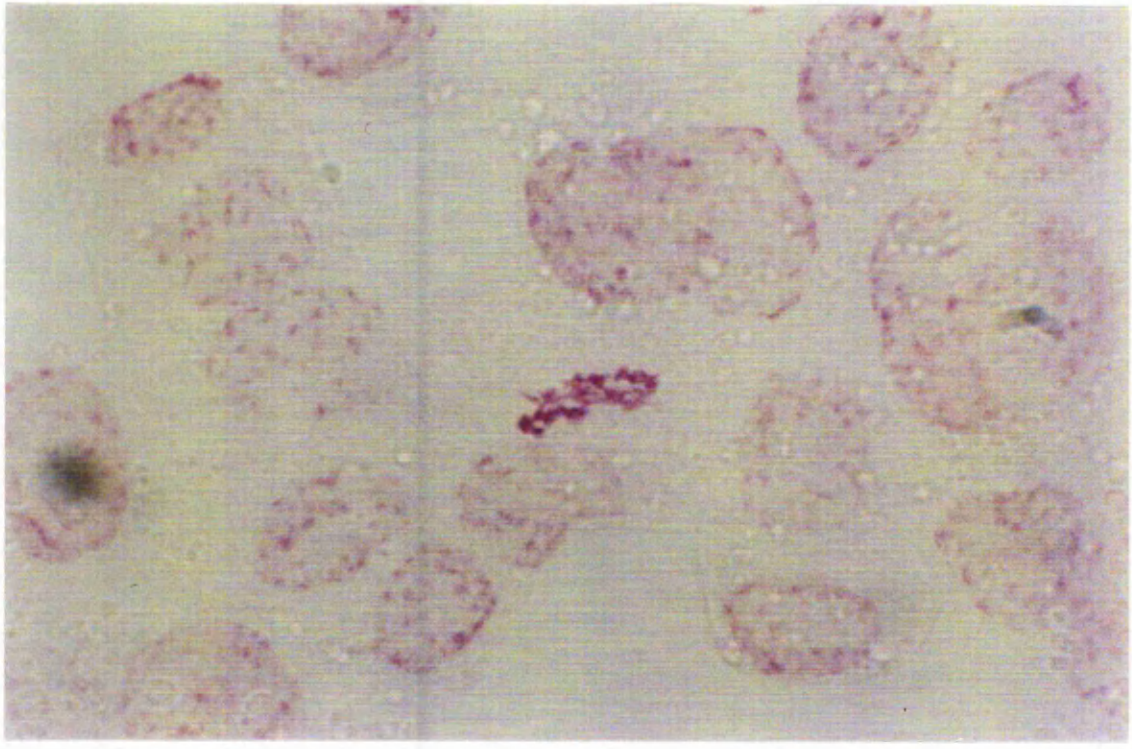


Figure 3.3. Potato root tip cells in pre-metaphase. Stained with Periodic acid solution, x 1000. Note condensed chromosomes moving to cell centre.

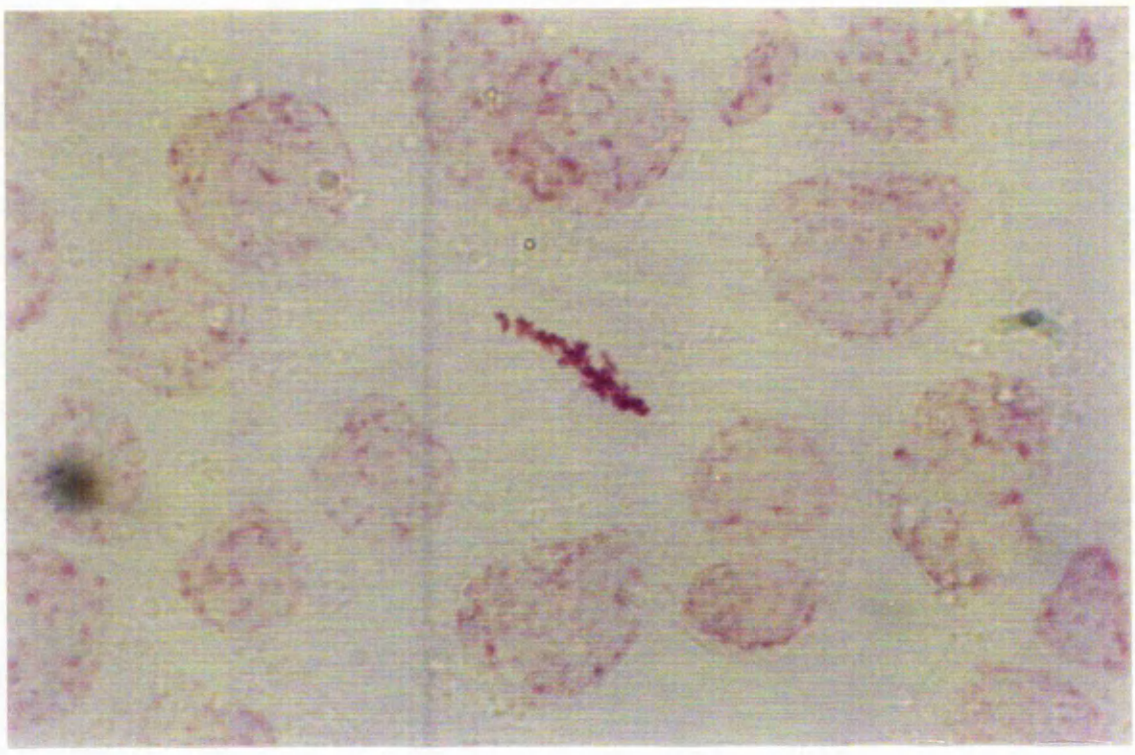


Figure 3.4. Potato root tip cell in metaphase. Stained with Periodic acid solution, x 1000. Note the chromosomes lining up on the metaphase plate.

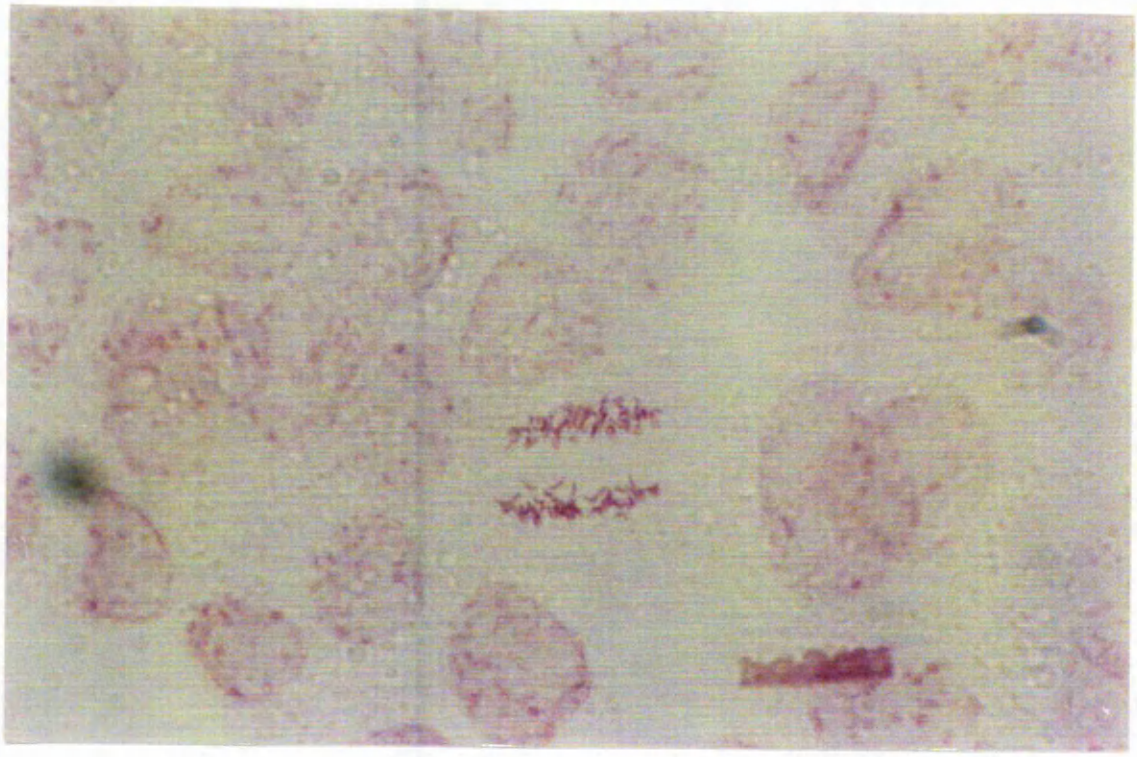


Figure 3.5. Potato root tip cell in anaphase. Stained with Periodic acid solution, x 1000. Note the two sets of daughter chromosomes pulling away from each other.

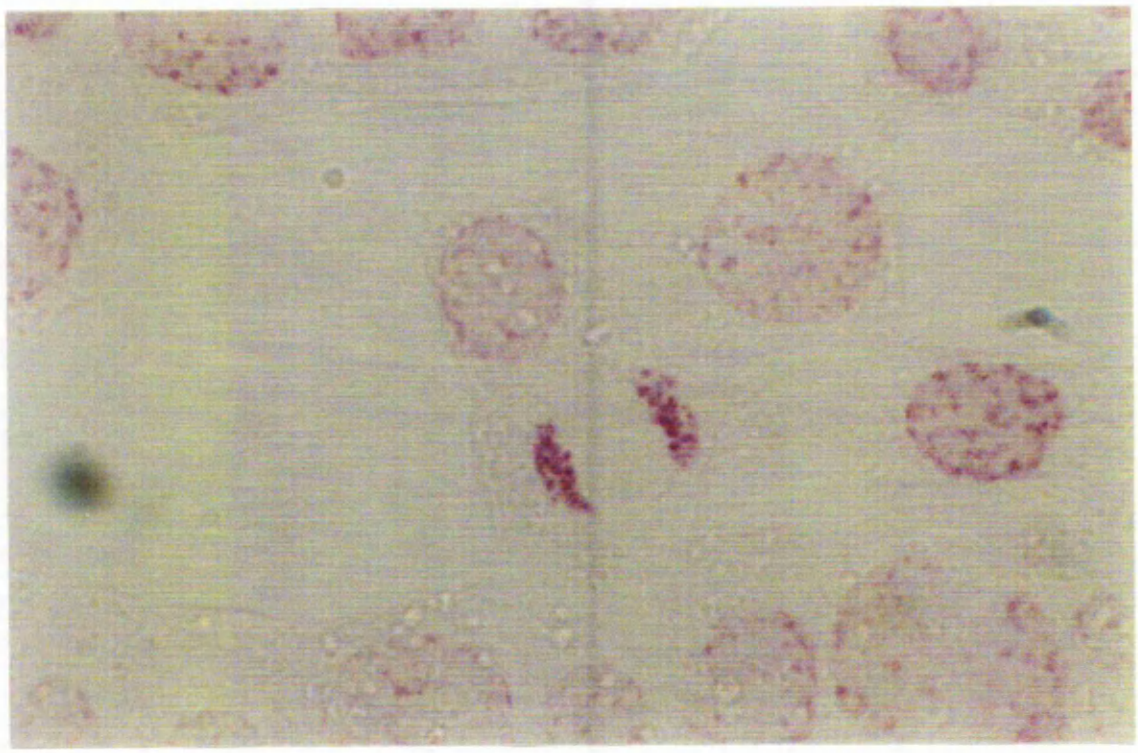


Figure 3.6. Potato root tip cell at the end of anaphase. Stained with Periodic acid solution, x1000. Note each set of chromosomes migrating to opposite poles of the cell.

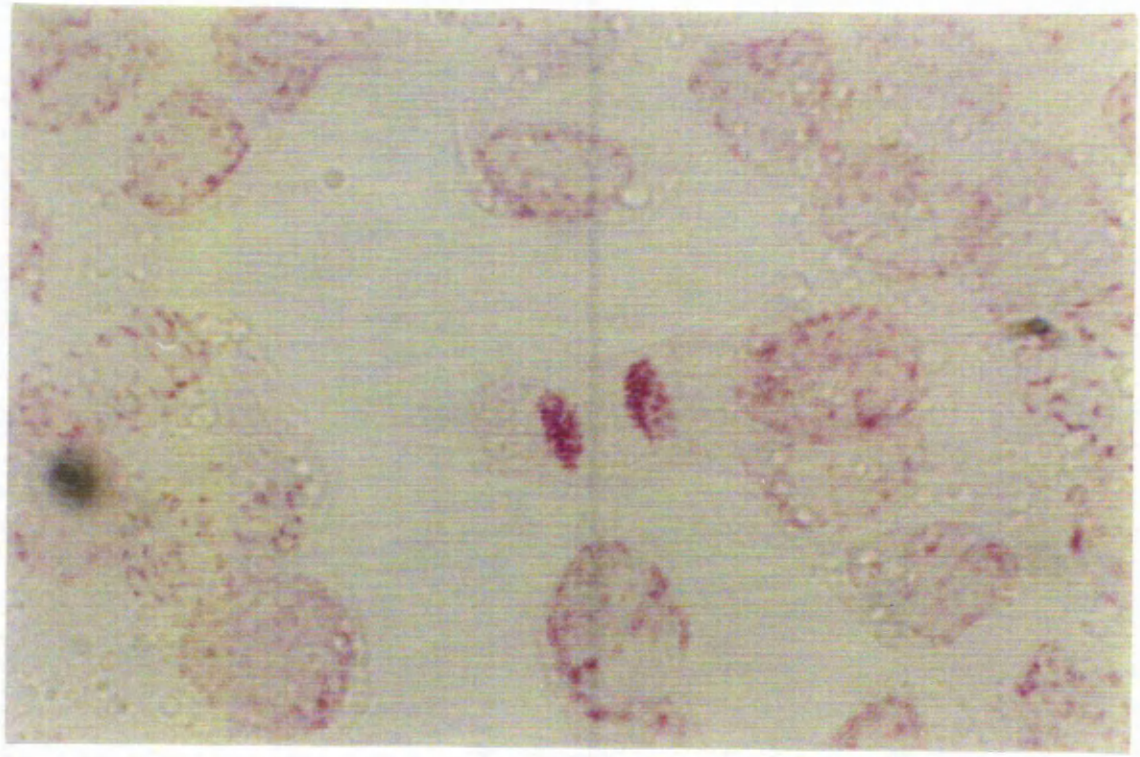


Figure 3.7. Potato root tip cell in early telophase. Stained with Periodic acid solution, x 1000. Chromosomes at each end of the cell are still condensed but are beginning to be separated by new nuclear membranes.

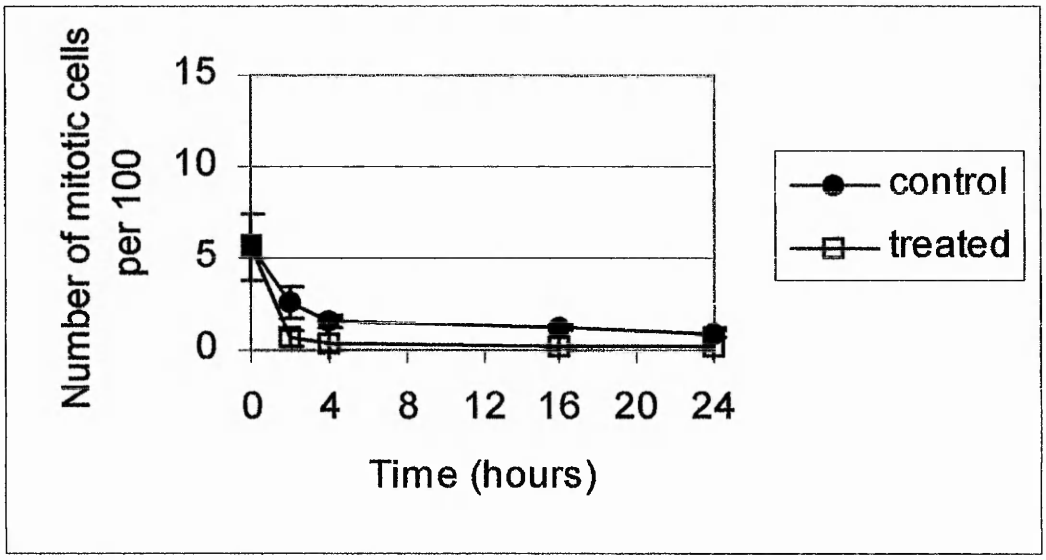


Figure 3.8. The effect of 1g ai L^{-1} imazethapyr on mitotic divisions of cv. Estima root tips using solid media. Data are means of 6 replicates ± standard error. Treated and control samples were not significantly different at any time point.

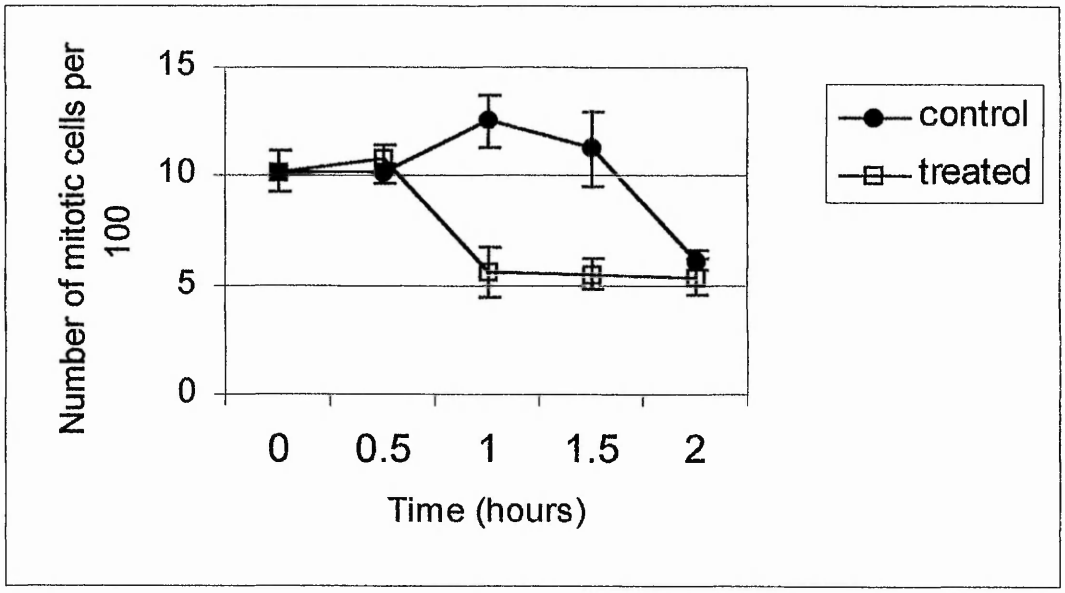


Figure 3.9. The effect of 1g ai L^{-1} imazethapyr on mitotic divisions of cv. Estima root tips using liquid media. Data are means of 6 replicates ± standard error. Control samples displayed significantly greater numbers of mitotic cells than treated samples at the 1 and 1.5h time point ($p < 0.05$).

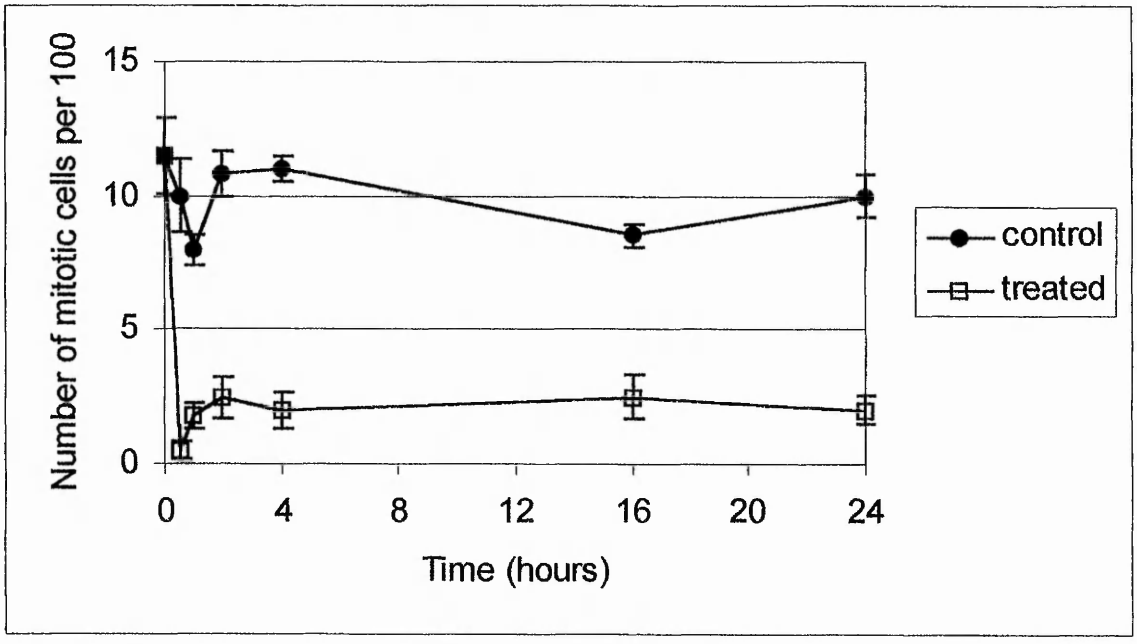
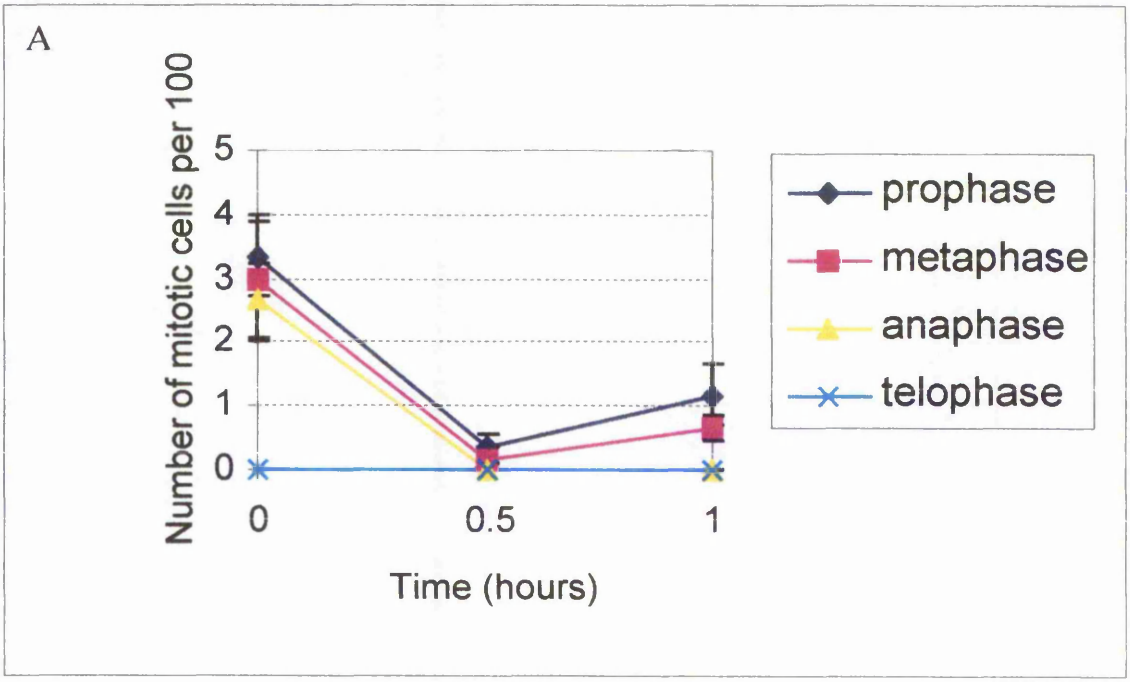


Figure 3.10. The effect of treatment with 0.1 g ai L^{-1} imazethapyr on mitotic divisions of cv. Cara tuber root tips using aerated liquid media. Data are means of 6 replicates ± standard error. Treated and control samples were significantly different at every time point ($p < 0.01$).



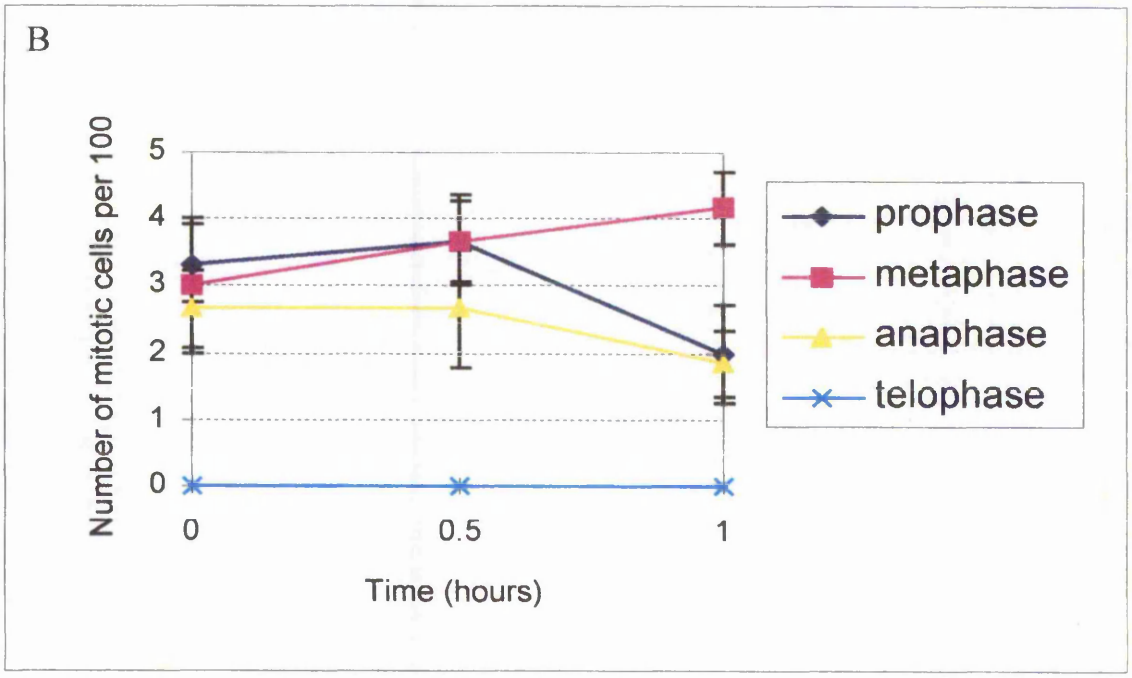


Figure 3.11. The effect of treatment with 0.1 g ai L^{-1} imazethapyr on each phase of mitotic division of cv. Cara tuber root tips. Data are means of 6 replicates ± standard error. A=treated B=control. Treated and control samples were significantly different at 0.5 and 1h in all phases apart from telophase ($p < 0.05$).

3.3.2. Microscopy

Figures are presented of sections taken from blocks of embedded tissues from control and treated tubers. The control tissue displays no sign of amyloplast degradation, tonoplast disruption or cell membrane breakdown, and the cells and organelles appear intact (Figures 3.12 and 3.13). The treated tissues exhibited extensive starch breakdown, with clear gaps between the starch grains and the amyloplast envelope (Figure 3.14). Figure 3.15 shows a ruptured amyloplast envelope. In treated tissue a higher incidence of cytoplasmic vesicles was observed adjacent to or in close proximity with the cell membrane (Figures 3.14, 3.15 and 3.16). Disruption to cell membranes was observed in treated tissues (Figures 3.14, 3.15 and 3.16).

Figures 3.12 was taken using light microscopy and indicates areas of severe vascular occlusion. This section was taken from an area that appeared damaged to the naked eye and light microscopy provides the opportunity for more detailed examination of the damage. The vascular cells have lost integrity and are discoloured. The surrounding cells displayed pitting and have few starch grains.

About 200 grids were observed and these were cut from the 60 blocks, nearly all sections that were cut were observed and photographed. There is no quantitative data associated with the microscopy. However, the selection of Figures shown are typical representatives of the series of sections, and the physiological details noted were common to many of the sections.

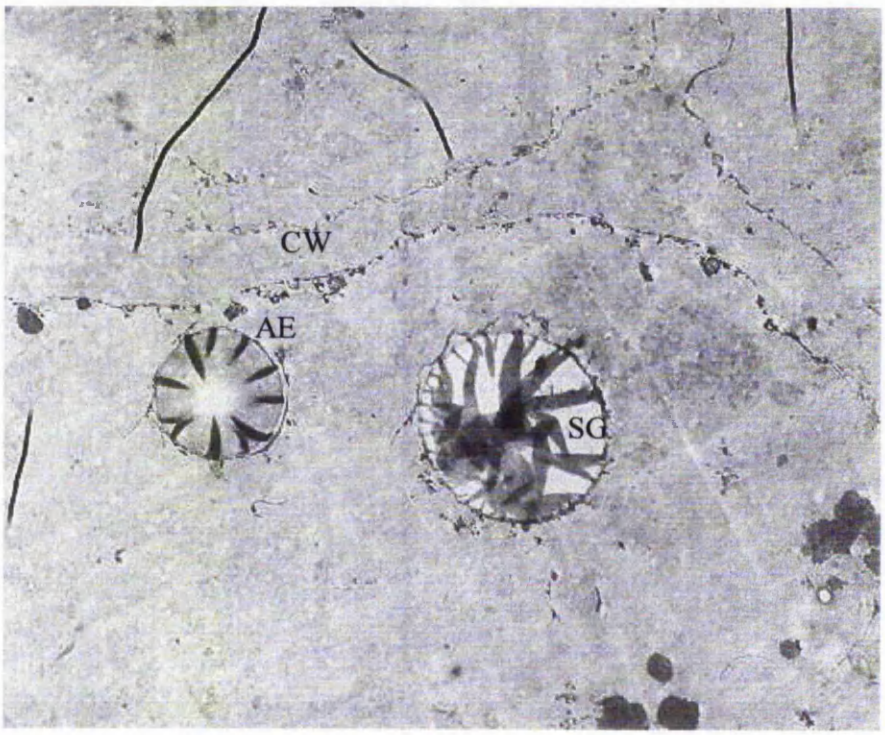


Figure 3.12. Control tissue showing intact cell wall (CW), amyloplast envelope (AE) and starch granule (SG), x2000.

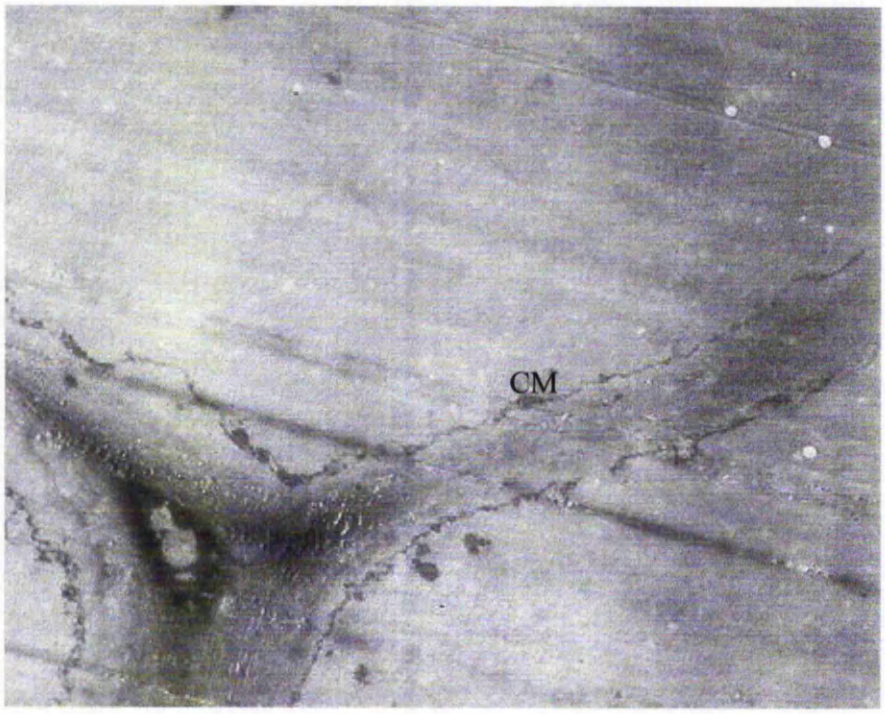


Figure 3.13. Control tissue showing intact cell membrane (CM) and lack of vesicles surrounding the cell membrane, x2000.

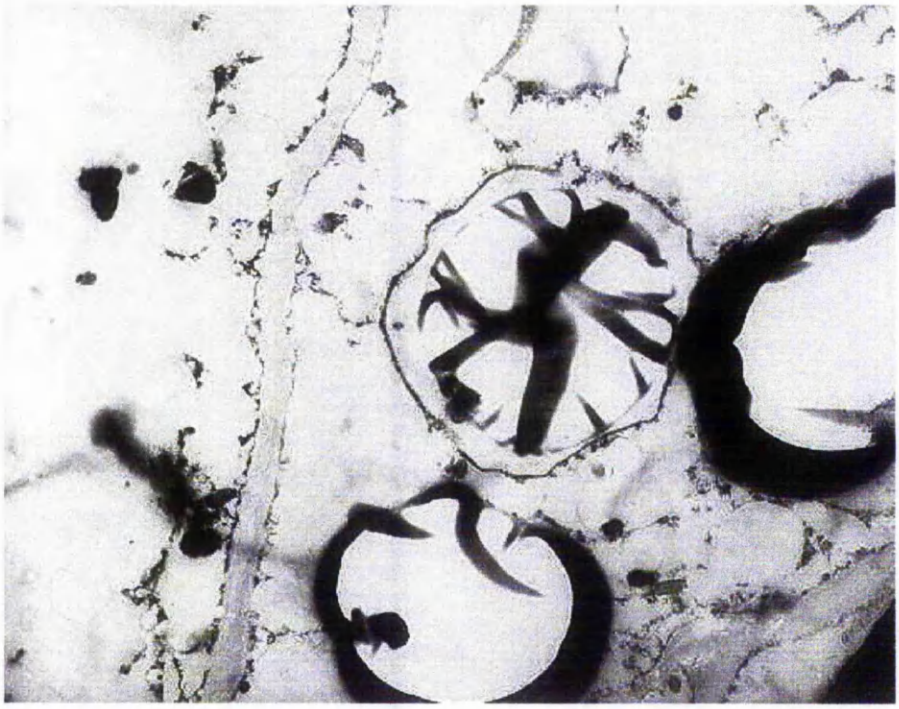


Figure 3.14. Treated tissue showing space between the starch grain and the amyloplast envelope and disruption to the cell membrane, x2000.

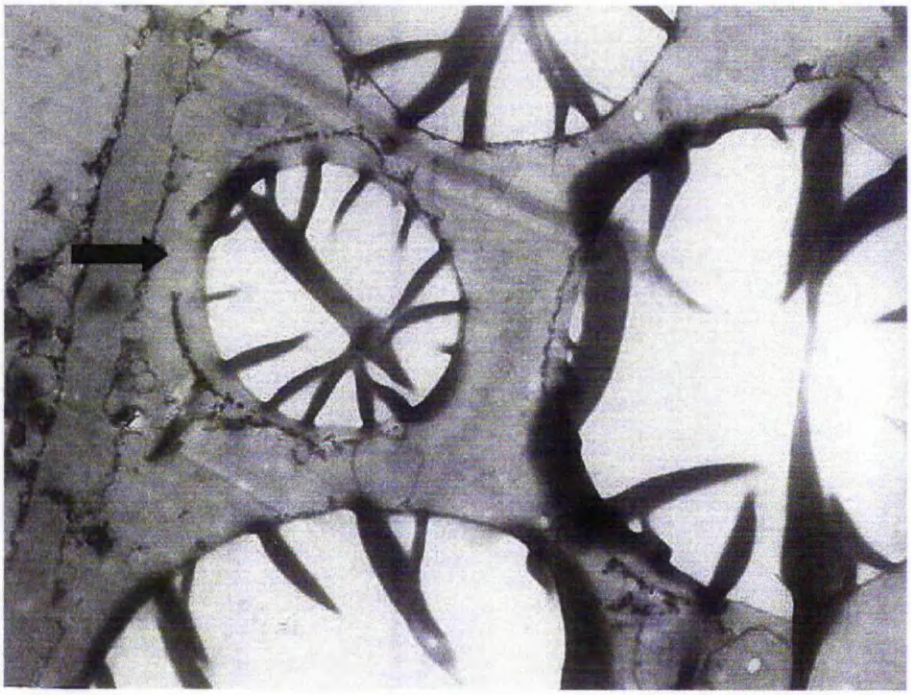


Figure 3.15. Treated tissue showing ruptured amyloplast membrane (arrow) and disruption to the cell membrane, x2000.

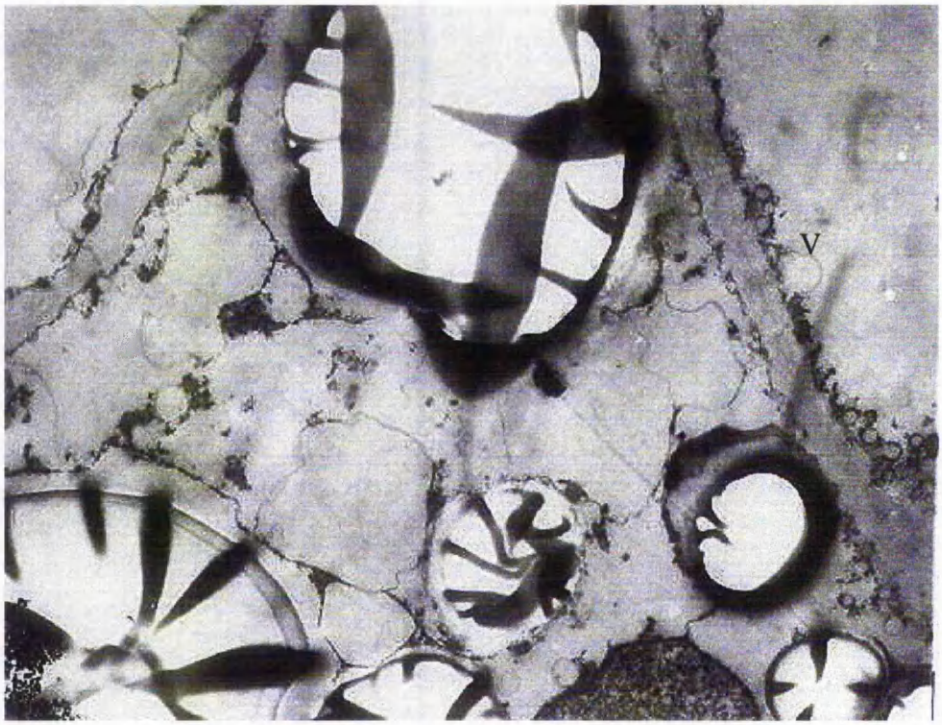


Figure 3.16. Treated tissue showing breakdown of amyloplast envelope, cell membrane and numerous vesicles (V), x2000.

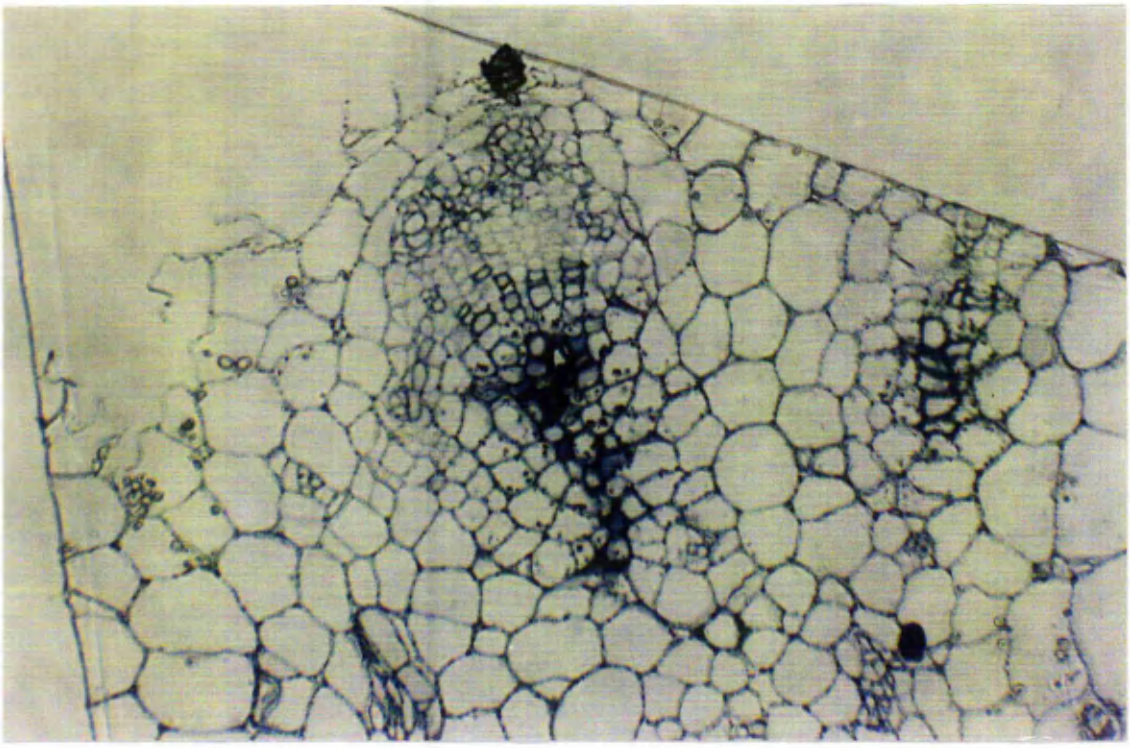


Figure 3.17. Treated tissue displaying vascular occlusions, light microscopy using toluidine blue stain, x 250.

3.3.4. Cation leakage

Cation concentration was calculated on a fresh weight basis (mg g fwt^{-1}) for individual cations and for control and treated samples. Mean data are displayed in Figure 3.18. Calcium, sodium and magnesium concentrations remained constant over the time course (up to 124 days after harvest). Potassium concentration peaked at 37 days after harvest and then slowly fell throughout storage and appeared to decline to a constant level towards the end. Efflux of potassium from treated tissues significantly ($p < 0.01$) increased at the last time point (124 days after harvest). Ammonium ion concentrations decreased during storage until 63 days after harvest, then a significant increase in ammonia concentrations followed in both control and treated samples ($p < 0.01$).

Using StatMost software package, an unpaired t-test showed that the only significant differences between control and treated were observed in ammonium ions at 63 days after harvest and with ammonium, magnesium and potassium concentrations at the end of the experiment at 124 days after harvest.

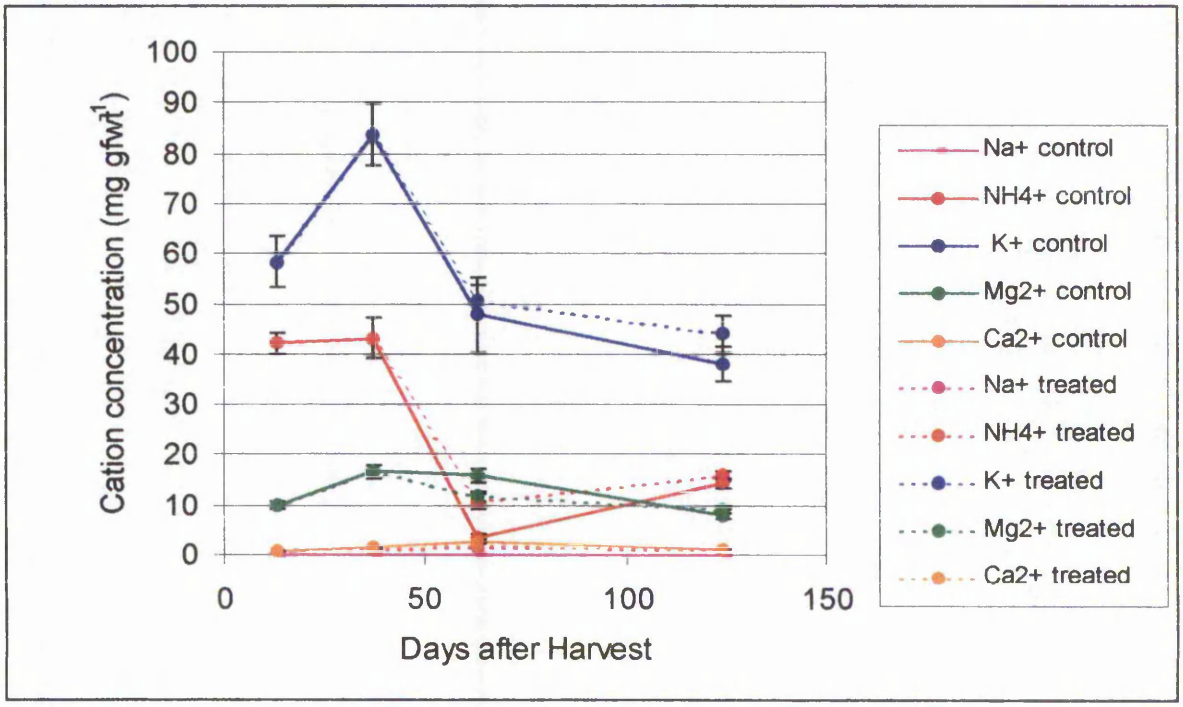


Figure 3.18. Tubers (cv. Cara) treated ± 0.34 g ai L^{-1} imazethapyr for up to 124 days prior to tissue analysis for cation efflux. Data are mean cation concentrations and are means of 6 replicates ± standard errors. Control and treated samples were not significantly different for any cations tested, with the exception of the last K^+ time point ($p < 0.01$) and the last two Mg^{2+} and NH_4^+ time points ($p < 0.001$).

3.4 Discussion

Treatment with imazethapyr has wide reaching effects on the tuber at the ultrastructural level, as demonstrated in this Chapter by the use of various techniques. The data presented shows the ability of imazethapyr to rapidly and sensitively inhibit the mitotic division process. Fewer mitotic cells were observed in treated tissue compared to control, which implies that imazethapyr can be defined as causing an inhibition of mitotic entry (Hess, 1987). Rost *et al* (1990) tested imazethapyr on pea root tips, using a similar technique, and found no inhibition of mitotic entry at comparable concentrations after 12h. However, at higher concentrations (>2μM) inhibition was observed after just 2h of treatment. Lower concentrations of imazethapyr were needed to inhibit the mitotic activity of potato root tips compared to the pea root tip data. The difference is possibly due to a physiological difference in the root tips of the two species. The barrier of the potato root tip could be crossed more easily than that of the pea.

Rost (1984) found that chlorsulfuron (a sulfonylurea herbicide) caused blocks in G_2 G_1 transition points of the cell cycle. It is possible that imazethapyr also blocks at these stages because of the similarity in the sulfonylurea and imidazolinone mode of action. Both chemicals inhibit AHAS and cause an inhibition of the biosynthesis of the branched chain amino acids (BAA) valine, leucine and isoleucine.

The inhibition of the biosynthesis of these amino acids has also been found to disrupt the cell cycle in mammalian cells as a secondary effect. Mammalian chinese hamster ovary (CHO) cells were found to be arrested in G_1 when grown in isoleucine deficient medium (Tobey, 1973). In common with chlorsulfuron treated pea roots, CHO cell RNA synthesis was only slightly inhibited. Protein synthesis was also not totally inhibited, although some reduction in polypeptide translation rate occurred because of the lack of isoleucine (Rost and Reynolds, 1985). Hence, there are similarities in the way mammalian and plant cells react when BAA are not available to them. The actual process linking inhibition of BAA and cell cycle inhibition is not clear at present.

Initial proposals by Shaner and Reider (1986) suggested the presence of a short lived protein that required BAA to function and this protein was involved in the regulation of cell cycle progression and the entry into mitosis. Although Rost *et al*

(1990) hypothesised that whatever protein is involved in the mitotic inhibition may not be solely dependant on a BAA requirement, this conclusion was drawn from studies using imazapyr. Two rates were used, one which inhibited the cell cycle fully and the other only partially. In both cases the BAA pool was reduced by less than 50%. This led to the conclusion that the BAA pool reduction was not the sole reason for cell cycle inhibition, and that a short lived protein may still exist but it may be specifically inhibited by α-ketobutyrate or some other BAA intermediate. The protein has not been identified to date and the connection between the cell cycle and the BAA pathway is still unclear.

The methods of Rost and Morrison (1984) and Yim and Bayer (1996) were successfully adapted to provide an experimental system to observe potato tuber root tips. There was a particularly low incidence of telophase observed, due to the difficulties in distinguishing between telophase and interphase, as the two phases are so similar. Other techniques are available to aid in the distinction between cells in M phase. For example, Ki-67 is an antibody specific for the mitotic spindle apparatus, and also fluorescent dyes can be used to highlight nuclear material.

Another possible improvement to the method would be to grow root tips in aerated media prior to testing. Rost *et al* (1990) grew peas on White's medium for 24 h prior to chemical treatment. Whilst this may equilibrate the root tips, it may cause problems if the tips are separated from the source plant for too long. Obviously the technique is artificial because it only applies chemical to root tips rather than the whole tuber or plant, but it is a useful tool in determining effects of chemical treatment at the cell cycle level.

Further studies to complement the work so far could include an investigation to determine if a reversal of mitotic index inhibition was observed by adding BAA to the media, as has been recorded in other species (Rost *et al*, 1990). Also, radioactive thymidine coupled with autoradiography could be used to determine the duration of each stage of the cell cycle, the location of the block in the cell cycle being identified when a particular stage becomes excessively long.

However, the results from this experiment and previous work shows that there is a dramatic inhibition of the cell cycle with treatment by various AHAS inhibitors. The inhibition of mitotic entry can be considered to be a secondary manifestation of a primary metabolic change induced by imazethapyr.

A detailed examination of the ultrastructure of the tuber was also carried out. The control tissue showed expected features of tissue that had been stored for 12 weeks. There was no starch degradation or tonoplast disruption and the cell membranes appeared intact. These findings correlate well with those of Turnbull and Cobb (1992) using cv. Pentland Dell.

Treated tissue displayed damage to the amyloplast envelope. Starch breakdown resulted in clear spaces between the grain and the amyloplast envelope and in some cases the amyloplast envelope was broken. As imazethapyr treatment causes inhibition of AHAS it also inhibits the transport of photosynthate (Devine, 1989). Consequently the tuber needs to mobilise starch reserves for energy. Amyloplast breakdown is discussed further in relation to the cation leakage results. Cell membranes were disrupted in treated tissue and in many cases formed small vesicles located near the cell membranes. This disruption of cell integrity can lead to reactions between components of the cells that would not normally be able to react, for example the oxidation of phenols to form melanin (Hiller *et al*, 1985). These types of reaction could account for the vascular discolouration that is observed later on in storage.

Light microscopy was used to examine sections that were not stable enough for the electron beam, including some sections of the vascular regions of the treated tubers taken from areas of damage visible by eye (Figure 3.7). These sections were taken later than those used for electron microscopy and the symptoms are more advanced. The vascular regions were very damaged and discoloured. The discolouration is probably the result of the breakdown of the cell contents, allowing the previously separated constituents of the discolouration reactions to come into contact with each other to form melanin. The lack of starch grains suggests that this area of the tuber may have already mobilised much of the available starch.

This investigation could have been improved by using more strongly reinforced grids so all of the samples could have been viewed using electron microscopy. However, the majority of the grids examined provided clear and stable samples. Additional work could involve taking samples from more time points to build up a picture of the way in which the tuber reacts to storage and to imazethapyr treatment during storage. This was not carried out as the fixation process was too time consuming to be carried out at again at harvest time. Other techniques are available for the examination of ultrastructure of tuber tissue, principally the use of

confocal microscopy. Confocal techniques would allow a three dimensional picture of the tuber cell to be created.

Whilst the observations from this study are not quantitative, they provide useful information about the physiological condition of treated and control tubers, and this information allows improved interpretation of the mitotic index and cation leakage data presented in this Chapter.

Cation leakage was studied to provide better understanding of the tuber during storage and to link the findings with electron microscopy results. Previous studies involving cation leakage measurements had been undertaken using cv. Pentland Dell stored at 5 and 10°C (Turnbull and Cobb, 1992). A sharp decline in ammonium ion leakage was observed over time, and was the largest decline in leakage of the cations investigated. This may be due to the incorporation into proteins of ammonium via the glutamine synthetase (GS) pathway. The timing of the drop in ammonium concentrations corresponds to increases in GS activity observed during storage (Brierley *et al*, 1997). When potatoes are not sprouting an increase in GS activity is expected, as the tuber is directing the balance of protein breakdown and synthesis to favour synthesis.

Potassium is the most abundant cation present in potatoes and contributes up to 77% of the total mineral substances (Leszczynski, 1989). The potassium leakage concentrations were the highest of all the cations tested. The initial peak in leakage and subsequent levelling off of the potassium concentrations over time was expected, as the internal membranes of the tuber degrade over time. Sodium, calcium and magnesium together only contribute about 8% of the total mineral substances of the tuber. Previous studies have shown an increase in the concentration of sodium and calcium leakage after 16 weeks of storage (Turnbull and Cobb, 1992). As this experiment only measured up to 18 weeks, these increases may not have been observed. Disruption of cellular membranes such as the tonoplast would account for increases in sodium and calcium leakage relatively late on in storage. These changes are very much related to the function of these cations within the cell. Sodium cations are integral to the functioning of the cytoplasm, both structurally and electrically, whilst calcium is involved in maintaining cytoplasmic structures and also contributes to pectic substances in cell wall synthesis and cohesion.

Magnesium concentration remained stable throughout the storage period with only low concentrations of leakage detected. However, there was significantly more

magnesium leakage detected in the treated samples towards the end of the time-course. Within the cell magnesium is located within organelles, such as amyloplasts and mitochondria, and is an essential cofactor in the numerous enzymatic processes using ATP (Turnbull and Cobb, 1992). Magnesium rarely leaks from the cell even in late storage when many intracellular membranes are losing integrity. However, there was an increase in magnesium leakage corresponding to the time at which amyloplast membranes were disrupted in the treated tissue used for transmission electron microscopy.

There were some differences between the control and treated samples. Following statistical analysis, the only significant differences were seen in ammonium, magnesium and potassium towards the end of the time course, when more leakage of the cations occurred in treated tissue compared to that of the control tissue. Imazethapyr treatment appeared to alter the normal pattern of cation leakage from stored tuber cells, perhaps as a result of the structural changes that occur in treated tissues.

Ion chromatography is a useful and efficient method for determining cation leakage from cells. It can be carried out using only small volumes and with the use of an autosampler, many samples can be run continuously. Detection limits are very sensitive using ion chromatography and concentrations as low as 0.5mg g fwt^{-1} can be reliably traced.

Further work that would be useful to this study would be to extend the time course to determine whether the increases in sodium and calcium leakage occurred after 16 weeks, as seen in Pentland Dell (Turnbull and Cobb, 1992). In addition, differences between treated and control may have become more marked with time. Alternative techniques for the study of cations are available, for example the use of ion localisation and ion specific fluorescing probes. These techniques would allow more accurate investigation into the movement of ions in tuber tissue as well as quantifying the amounts of ions present in different conditions. However, these benefits are offset against the time and cost of using the necessary equipment.

In summary, the data presented in this Chapter illustrates the effect of imazethapyr on tuber tissue ultrastructure. It causes the inhibition of mitotic entry and progression in root tips. It also disrupts cortical cell ultrastructure causing the amyloplast and cell membranes to break down. This in turn may affect the cation

balance and can cause the visible discolouration and vascular damage observed in treated tubers.

4.0 UPTAKE AND DISTRIBUTION OF RADIOLABELLED IMAZETHAPYR

4.1 Introduction

Radioisotopes have been extensively used to study the uptake, movement, mode of action and metabolism of several pesticides in plants (e.g. Thompson, Sanders and Pallett, 1986; Little and Shaner, 1991). The most common isotopes used in plant research are low energy β-emitters, for example ^{3}H, ^{14}C or ^{35}S (Coupland, 1986). They are convenient because they have relatively long half-lives and experiments involving long treatment times are not affected by degradation of the radioisotope. Low energy β-emitters are also poor penetrants which is advantageous to operator safety.

The main stages of radioisotope treatment are application, sampling and counting. Each of these stages requires development, optimisation and adaptation to the individual experimental question under consideration. There are numerous ways to apply the radioisotope to the plant, including topical application or addition to growing media. Topical application can be carried out using micropipette (Thompson *et al*, 1986) or spray simulation (Coggins and Baker, 1983). Depending on the size of plant tissue and final counting method, the tissue can either be sampled whole or sectioned into distinct areas. If autoradiography is to be used and the plant is small, the tissue can be laid intact on a photographic plate for exposure to X-ray film. However, in most cases some sectioning of the plant will be required. The tissue will need to be homogenised if using liquid scintillation counting techniques, so that fragments are broken down sufficiently to allow any radioisotope decay to be detected rather than being absorbed by the tissue itself (Coupland, 1986).

Previously used methods for studying the uptake and distribution of radioisotope in potatoes have relied solely on autoradiography (Cushman, Tibbitts and Sharkey, 1995). Although this method is valuable, it is time consuming and can only be quantified with the use of costly imaging equipment.

In plants the uptake and distribution of imidazolinones is affected by the environmental conditions in which they are applied (Maylefyt and Quakenbush, 1991). Imazethapyr uptake and distribution in plants may be affected by the formulation in which it is applied or by chemicals applied at the same time (Little

and Shaner, 1991). In tubers formulation may affect the uptake and distribution of imazethapyr. The time of treatment of potato tubers with imazethapyr will be as tubers are placed in storage. At this time other chemicals can be applied, for example fungicides. Thiabendazole is a fungicide which is often used in potato stores. It is a non-carbamate member of the benzimidazole family of fungicides. It reduces the damage done to tubers by dry rot, skin spot, silver scurf and gangrene. There are two formulations of thiabendazole, Storite Flowable and Storite Clear. Storite Clear is an acidic formulation (pH 2.9), whereas Storite Flowable is more neutral (pH 6.5). Storite Clear is the formulation used in many potato stores.

This Chapter describes a novel method developed to enable the quantitative analysis of ^{14}C-imazethapyr uptake and distribution in the potato tuber. It also presents an investigation into the effects of changing formulations and environmental conditions on imazethapyr.

4.2 Materials and Methods

Throughout the radioisotope experiments ^{14}C-carboxyl-labelled imazethapyr (98 % purity, Cyanamid USA) was used, as shown in Figure 4.1.

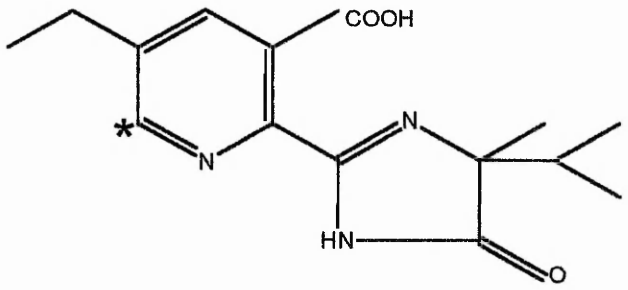


Figure 4.1. Chemical structure of imazethapyr. The position of the ^{14}C-atom is denoted by *.

4.2.1. Tissue incubation

Tubers of cultivar Cara were treated with radiolabelled imazethapyr in the same way throughout the series of experiments. A circle of 1cm diameter was isolated on each tuber using a ring of Vaseline. The isolated area was treated with a combination of labelled and unlabelled imazethapyr. Five µl of labelled imazethapyr

(specific activity 1.33μCi ml^{-1}) was added to 45μl of unlabelled imazethapyr (0.1 or 1 g ai L^{-1}) resulting in a final concentration of 0.34 or 1.14 g ai L^{-1}. Six tubers were placed on moistened tissue in a sealed plastic box (20 x 15 x 15 cm) for the duration of the experiment. Prior to treatment, all tubers were stored at the temperature used in the experiment to allow tubers to equilibrate for at least 48 h. Various temperatures were used during the study ranging from 2.5 – 20°C. After the treatment time had elapsed, a bore of 16mm diameter was taken through the tuber, covering the area of initial treatment, as depicted in Figure 4.2.

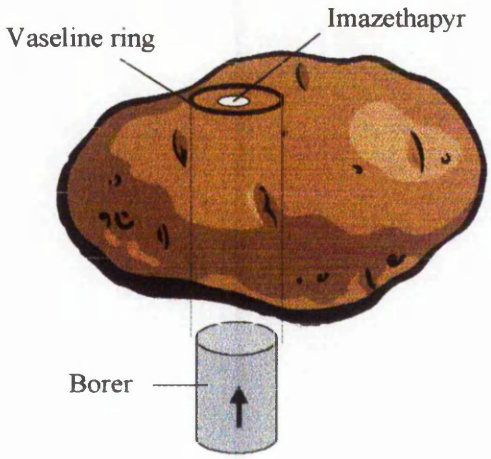


Figure 4.2. Technique used to sample tubers for uptake and distribution studies.

The area of periderm inside the vaseline ring was washed twice with 200μl aliquots of distilled water, the washings were placed in a vial with 10ml of Ultima Gold (Packard, Meriden, USA.) scintillation fluid and counted using a Canberra Packard Tri-Carb 2250CA. The core was sectioned, using razor blades, into periderm, 0-5, 5-10, 10-20, 20-30 mm. The sections were placed in clean scintillation vials and quick frozen using liquid nitrogen and placed at –70°C until they were required.

The effect of applying Storite Clear at the same time as imazethapyr was investigated. Storite Clear was added to the labelled and unlabelled imazethapyr mixture at 0.5% (v/v). Following this, the effect of pH of the imazethapyr mixture

was investigated. The pH of the imazethapyr solution was normally 5.0, but was artificially lowered to pH 3.0 using HCl.

4.2.2. Tissue analysis

Lengthy optimisation of the method of tissue analysis was necessary and the main stages of development are described below and throughout the Chapter. The aim of the method was to provide an homogenous solution for the counting procedure. Initially, sections of the tuber tissue were treated with specific solubilising chemicals such as Soluene 350 and Soluene 100 (Packard, Meriden, USA.) and Fiso solve (Fischer, UK). A 16mm diameter and 2mm deep slice of tissue was added to 1ml of each chemical and was left in a 60°C waterbath overnight. Soluene 350 provided the best solubilisation and Fiso solve was the least successful. However, even Soluene 350 did not completely solubilise the tissue and further combinations of leaving Soluene 350 for longer and at different temperatures were equally unsuccessful and often resulted in caramelization of the sample.

A combination of different acids, bases, detergents and heating were tested, including 4M and 12M nitric acid, 2.ethoxyethanol, and 4M sodium hydroxide. Whilst these treatments were able to solubilise the tuber tissue, the periderm remained difficult to breakdown. The evolution of phenols was another problem with these chemicals and caused the tissue to turn purple.

Alternative methods of achieving a homogeneous mixture for counting were investigated. Firstly, tuber sections were placed in a mortar and pestle with liquid nitrogen and ground up. However, this method was not suitable as there was too much contamination of the mortar and pestle, and the resulting potato tissue did not mix well with the scintillation fluid. The next method was to place the tuber section into the scintillation vial and blend using an Ultraturux. This was not possible as the rotor blades were not small enough to fit inside the vial.

Freeze drying and grinding the tissue inside a scintillation vial provided a fine powder which was more suitable for suspending in solution for scintillation counting. The success of the different methods of suspending the potato powder for counting are detailled in Figure 4.4.

Initially, the powder was added to Ultima Gold scintillation fluid alone, but the sample quickly precipitated out and resulted in extremely low recoveries

(typically 5%). To prevent this, Cab-O-Sil (fumed silica, Sigma, UK) was added to the scintillation fluid as a gelling agent. The optimum concentration of Cab-O-Sil was determined as 0.4g Cab-O-Sil to 10ml Ultima Gold. This successfully suspended the powder but resulted in high quenching and recoveries approaching 73%. Instagel (Packard, Meriden, USA.) was used as an alternative low quench scintillation fluid. This is an organic, solvent-based scintillation fluid with a high xylene content. Although this increases the costs of disposal and risks to the worker, it was ideal for this experimental system because of its gelling properties. Instagel is a clear viscous liquid which clouded upon the addition of 5% (v/v) water, but when more water was added (10-20% v/v) the mixture cleared and gelled. This was ideal for the suspension of the fine freeze-dried tuber powder. The powder did settle overnight and the recoveries were low (typically 52%).

The starch in the freeze dried tuber powder was absorbing the water content of the Instagel mix and causing the Instagel to return to a liquid rather than gel. Hence, the starch in the potato powder needed solubilisation prior to the addition of Instagel and this was done by heating potato powder in 3ml of deionised water at 80°C for 2h prior to the addition of Instagel. This caused the starch grains to rehydrate, swell and burst to form a colloidal solution. There was no additional benefit observed to heating for longer than 2h (Figure 4.5). This meant that the resulting mix could be suspended homogeneously in the scintillation fluid for at least 24h, making the counting process simple and allowing large batches of vials to be prepared and counted, rather than preparing and counting each vial individually. In addition recoveries were higher, typically about 70%. A counting efficiency study was carried out to determine whether the addition of the entire tuber section was causing the counting efficiency to fall (Figure 4.6). This showed that a 95.3% efficiency was achieved using 100µg of tuber compared to 70.3% efficiency using the whole section (approx. 500µg). Only 100µg of each section was used and the weight of the whole section was accounted for in calculations. A quench correction curve for the 100µg was established with an R^2 value of $1 \pm 2.46 \times 10^{-13}$, this was also accounted for in calculations. As the recoveries were still not 100%, experiments were carried out to determine if some of the activity was being transported through the tuber to areas not covered by the cork borer.

The final optimised technique used in this Chapter is summarised in Figure 4.3.

4.2.3. Data analysis

Throughout the study, background readings were always subtracted from the data and this was done automatically by the scintillation counter. The data was transferred to spreadsheets and calculated. Most of the data was expressed as means ± standard error. The number of repetitions of each experiment are stated on the relevant figure legends and in most cases 3 repetitions were performed. Statistical analyses were carried out using StatMost software package.

Tubers which appeared free from disease or insect damage were chosen. A circle of 1cm diameter was isolated on each tuber with Vaseline. Tubers were placed in a plastic box containing paper moistened with 20 ml of deionised water.

↓

Isolated area treated with 45µl imazethapyr (0.1/1g ai L^{-1}) and 5µl radiolabelled imazethapyr (specific activity 1.33µCi ml^{-1}).

↓

The following steps take place at each point in the time-course

↓

Excess chemical pipetted off and added to 10 ml Ultima Gold and counted using a Canberra Packard Tri-Carb 2250CA.

↓

Core of tuber taken with corer of diameter 16mm.

↓

Periderm washed with 2 x 200µl of deionised water, washings added to 10 ml scintillation fluid and counted.

↓

Periderm removed with razor blade and placed in scintillation vial. Liquid Nitrogen added and placed in -70°C freezer.

↓

Sections of tuber core removed (0-5mm, 5-10mm, 10-20mm, 20-30mm). Liquid Nitrogen added and placed in -70°C freezer.

↓

All samples freeze dried and placed in -70°C freezer.

↓

Samples ground to a fine powder.

↓

100µg of powder added to 3 ml of deionised water and placed in an 80°C water bath for 2 h.

↓

Samples cooled on ice and 15 ml of Instagel added. Samples shaken and counted using a Canberra Packard Tri-Carb 2250CA.

Figure 4.3. Summary of the final radiolabel method used.

4.3 Results

The pattern of uptake and distribution of ^{14}C-labelled imazethapyr (0.34 g ai L^{-1}) altered with temperature, as illustrated in Figure 4.4. More activity was recovered from the periderm with increasing temperature. The recovery of activity in the other sections of the tuber was not affected by temperature changes. When the tubers were treated for longer time periods, significant increases in activity recovered from the periderm were observed at 5°C ($p<0.01$) and 10°C ($p<0.05$) when compared to the recovery at 2.5°C.

To confirm that the increase of activity recovered from the periderm with temperature was not a result of metabolic change with temperature, tubers were pre-treated for 2h with or without 10mM NaN_3 (a respiratory metabolic inhibitor), followed by an 8h treatment with imazethapyr (0.34 g ai L^{-1}) (Figure 4.5). No significant difference was recorded between the tubers treated with the metabolic inhibitor followed by imazethapyr and those treated with imazethapyr alone ($p=0.38$).

The effect of increasing the concentration of carrier imazethapyr was determined (Figure 4.6). The activity recovered beneath the periderm indicated that there was no change in activity at concentrations of 0.34, 0.48 and 0.70 g ai L^{-1}, but there was a larger proportion of activity recovered when using 1.14 g ai L^{-1}.

The recoveries of the labelled imazethapyr were not ideal (below 95-100%) and an experiment was carried out using varied bore sizes to determine if the imazethapyr was distributed further than the diameter of the bore used previously. This study showed an increase in the amount of activity detected per mg (Figure 4.7). However, the final data points with the largest sample area, resulted in a decline in detected activity. This was probably because although more label was being sampled, more unlabelled tissue was also sampled with such a large area, and this diluted the mean activity of the whole core. Recoveries approaching 100% were recorded with the final 2 larger sample areas.

Figure 4.8 records the percentage of recovered activity that was detected beneath the periderm layer, a value that declined as the sample area increased. The amount was different for the 2 rates of imazethapyr. When using the higher rate of imazethapyr, less radiolabel was detected beneath the periderm layer.

To determine if the label was spreading further on the surface of the tuber, the experiment was repeated and a peel (2mm thick) was taken from the surface from tip to stolon end, rather than sampling a core (Figure 4.9). Increased activity was recorded in the treated area over time and a decreased activity recorded in the washings with time. Activity above background was detected up to 4cm from the site of treatment, particularly after a long treatment time.

Treatment with 0.5% (v/v) Storite Clear in conjunction with imazethapyr increased the activity recovered from beneath the tuber periderm (Figure 4.10). The addition of the acidic formulation appeared to increase the amount of imazethapyr entering the cortical area of the tuber. The effect of pH alone was investigated by applying imazethapyr buffered to pH 3 or 5 (Figure 4.11). An increase in the percentage of activity recovered from beneath the tuber periderm was noted at the lower pH with time.

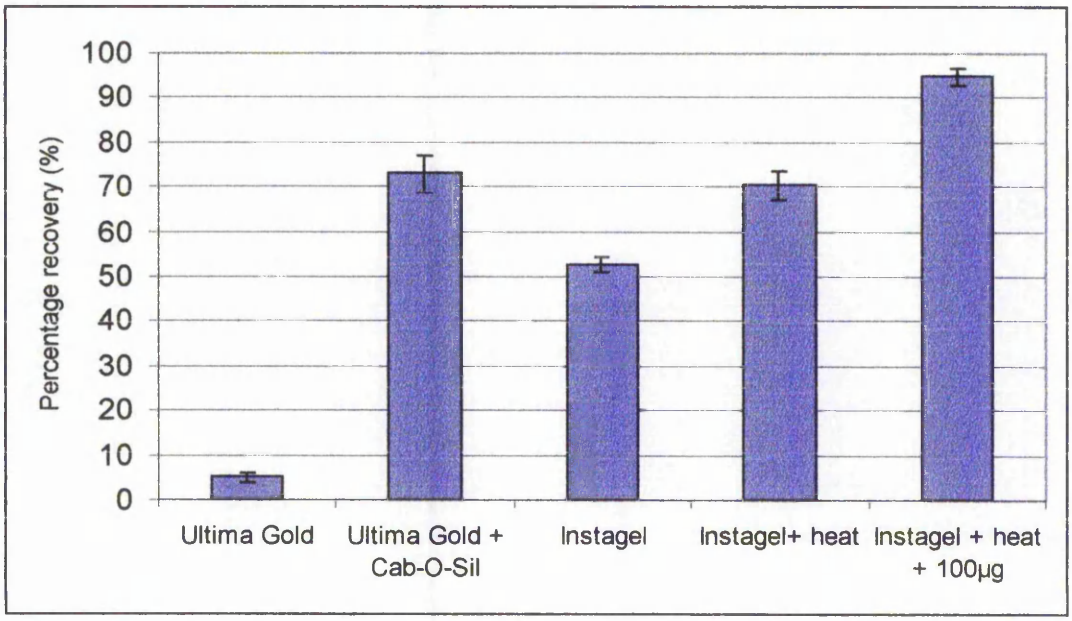


Figure 4.4. The percentage recovery of various methods used to suspend freeze dried potato tuber powder for liquid scintillation counting. Data are means of 3 replicates ± standard error.

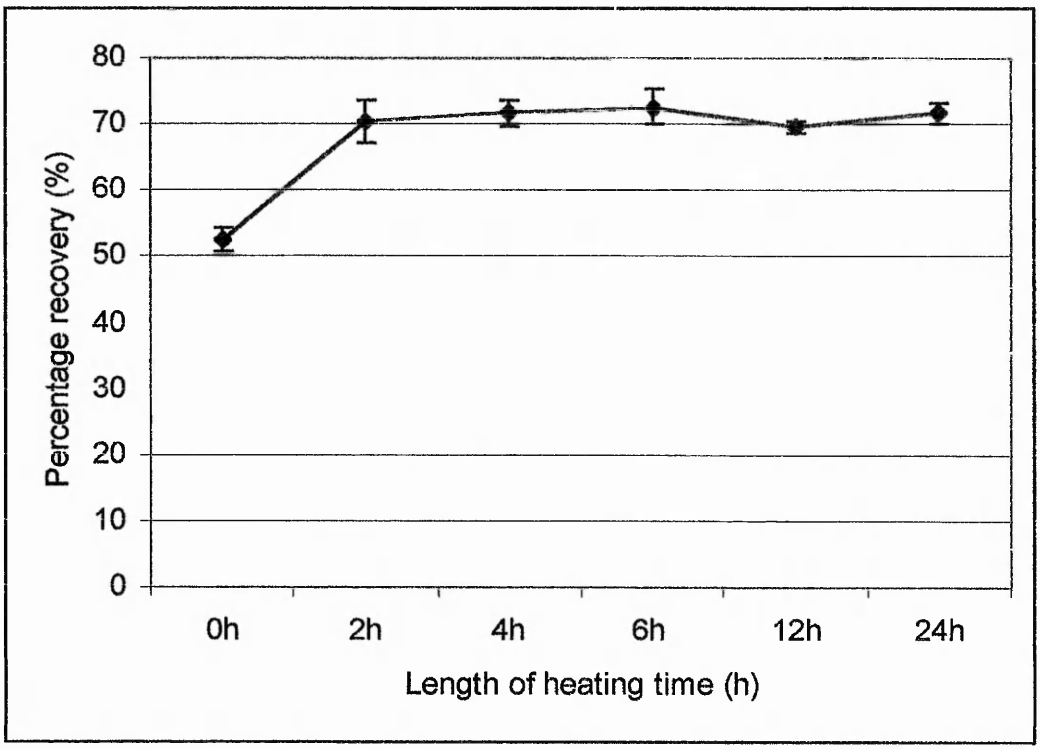


Figure 4.5. Percentage recovery of radiolabel after freeze dried potato tuber was heated for various lengths of time. Solution was subsequently counted in Instagel. Data are means of 3 replicates ± standard error.

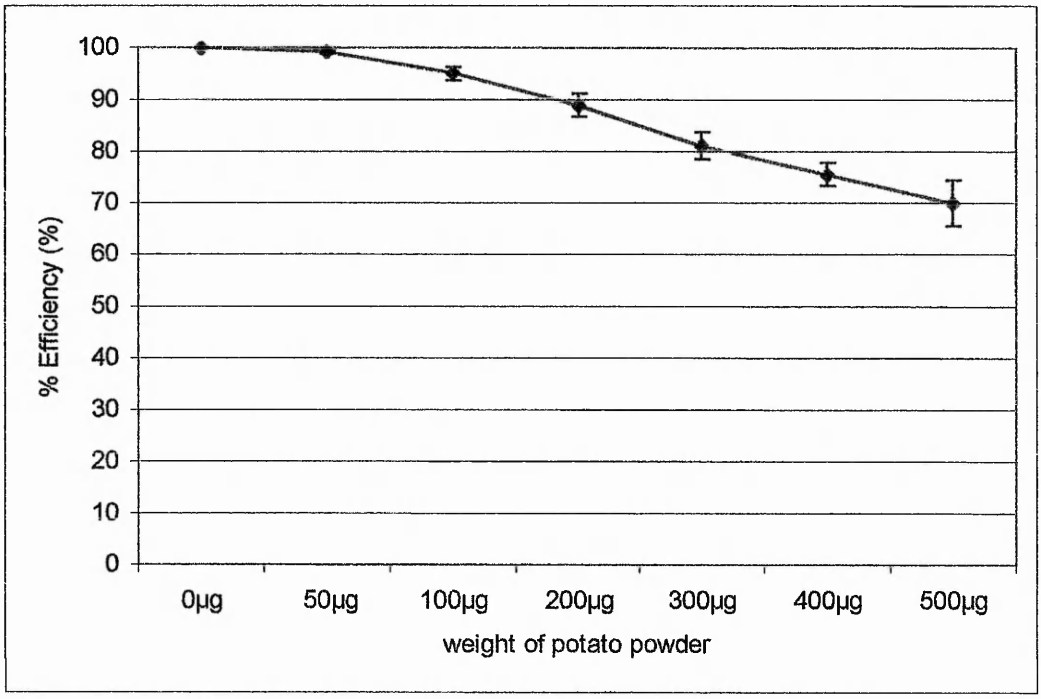


Figure 4.6. Counting efficiency curve displaying the effect of adding increasing weight of freeze dried potato powder to the counting vial. Data are means of 3 replicates ± standard error.

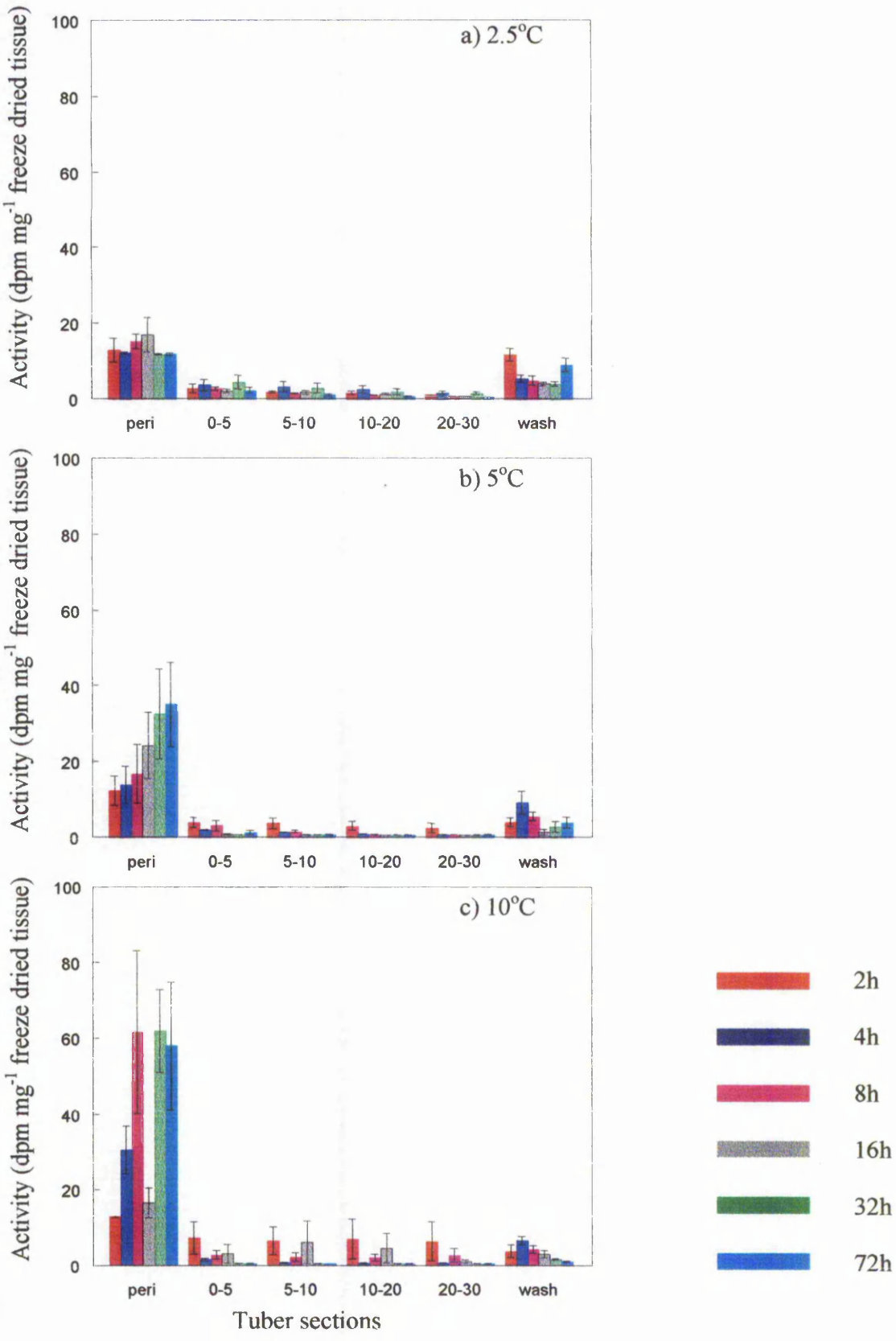


Figure 4.4. Distribution and time-course of ^{14}C-imazethapyr (0.34 g ai L^{-1}) in tubers stored at various temperatures. Samples taken using 16mm bore. Data are means of 3 replicates ± standard error.

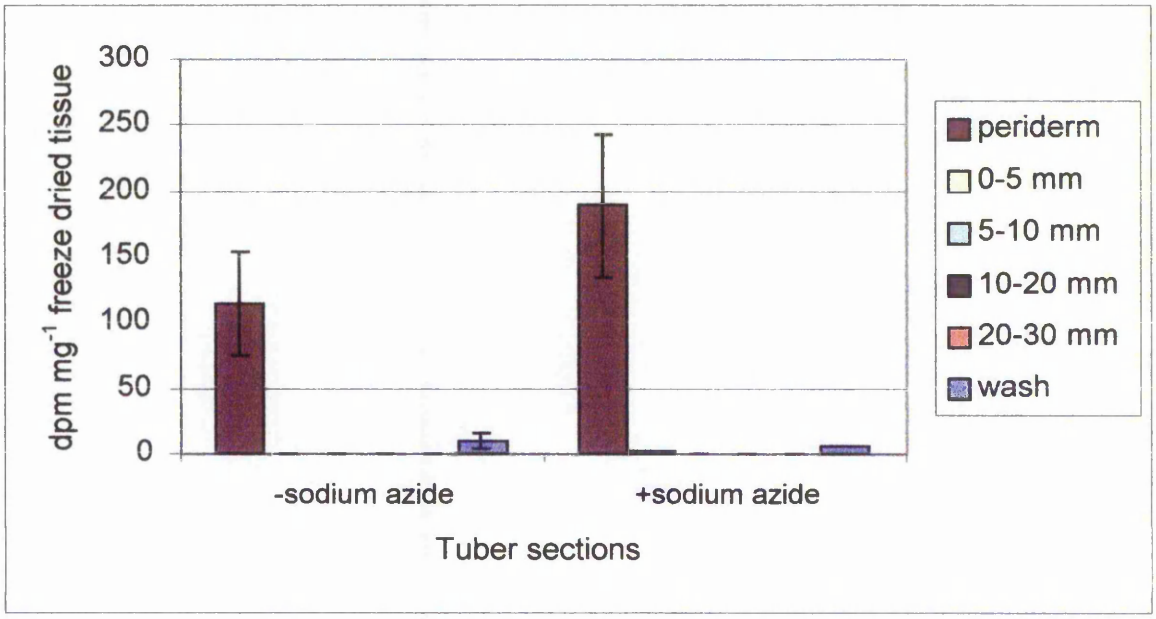


Figure 4.5. Uptake and distribution of imazethapyr (0.34 g ai L^{-1}) ± NaN_3 pre-treatment. NaN_3 pre-treatment was carried out at 10°C for 2h. Imazethapyr treatment was carried out at 10°C for 8h. Data are means of 3 replicates ± standard error. Using StatMost software package unpaired t-test, there was no statistical difference between the two treatments (p= 0.38).

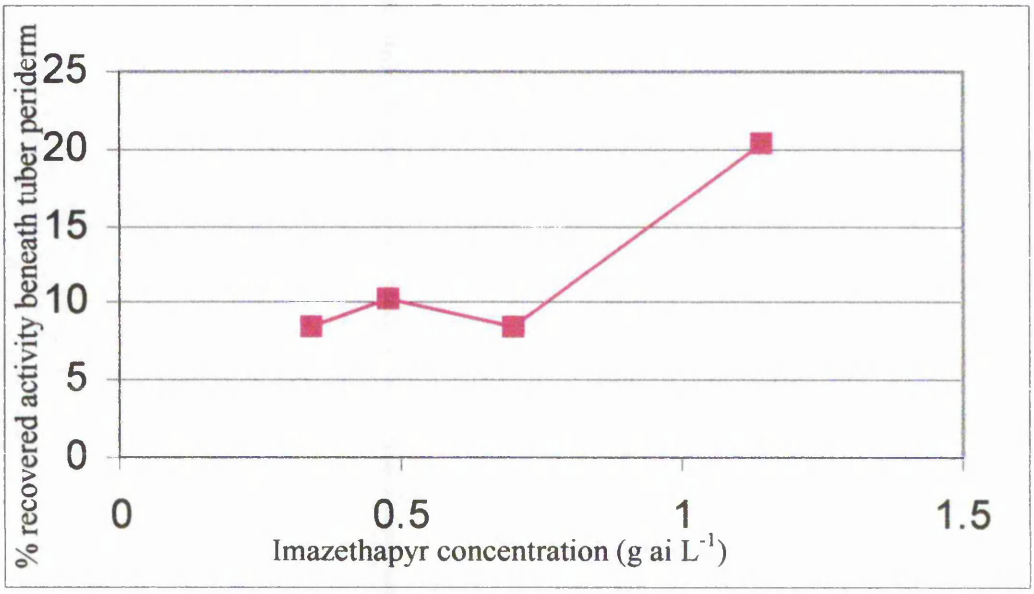


Figure 4.6. Percentage of recovered activity of imazethapyr beneath tuber periderm when using different concentrations. Data are means of 3 replicates, errors were less than 4% of data values.

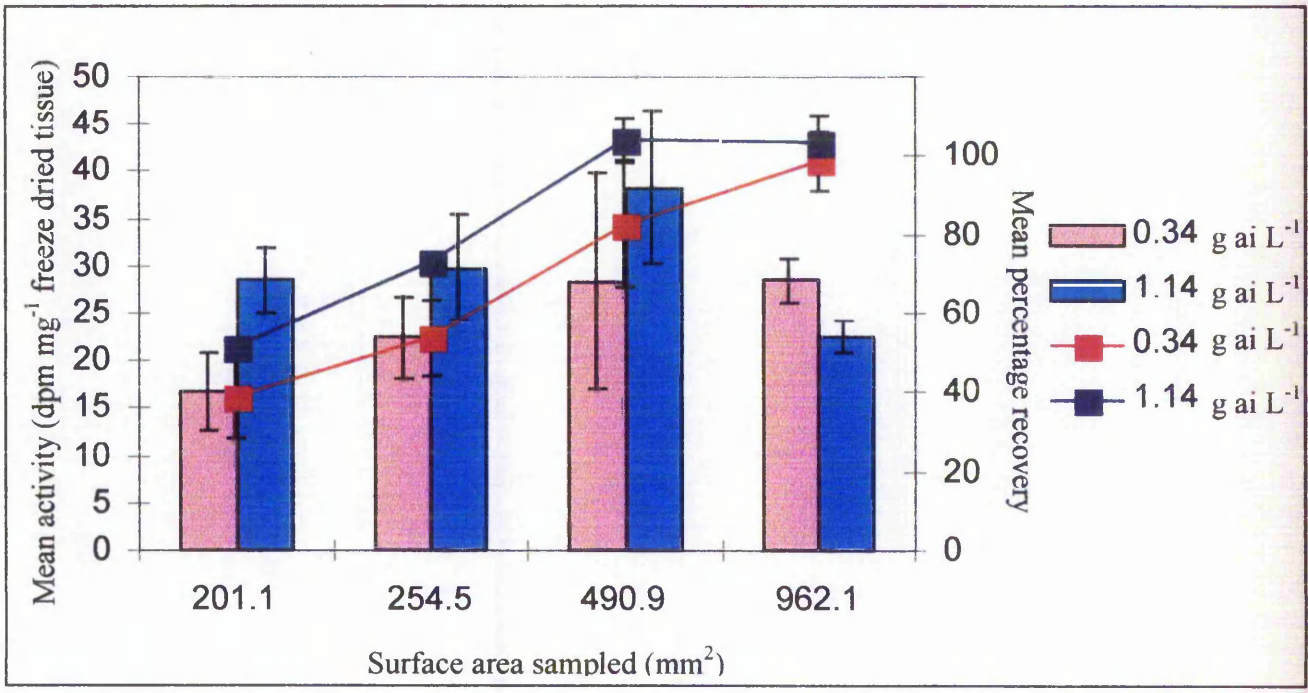


Figure 4.7. Combination chart displaying mean activity (bars) and mean % recovery (lines) using two rates of imazethapyr and sampling various bore sizes. Treatment was carried out at 10°C for 2h. Data are means of 3 replicates ± standard error. Note: non-linear scale on x axis.

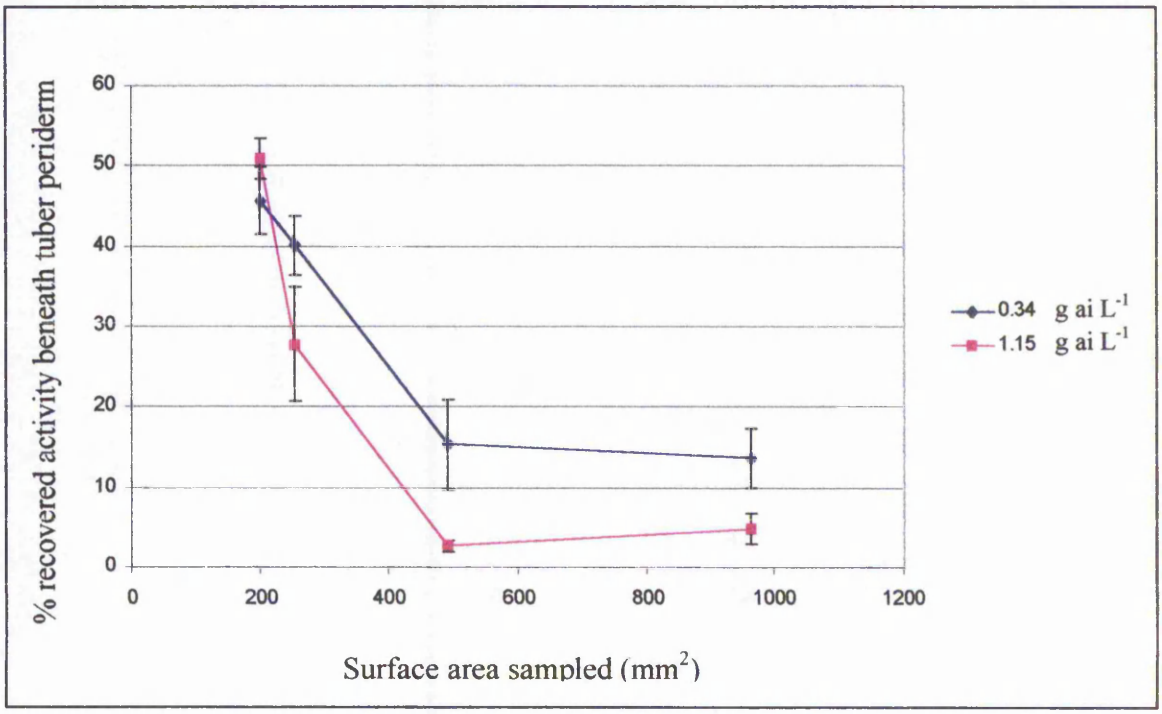


Figure 4.8. Percentage differences in detected activity beneath the periderm using different rates of imazethapyr and varying sampling areas. 8h treatment of imazethapyr. Data are means of 3 replicates ± standard error.

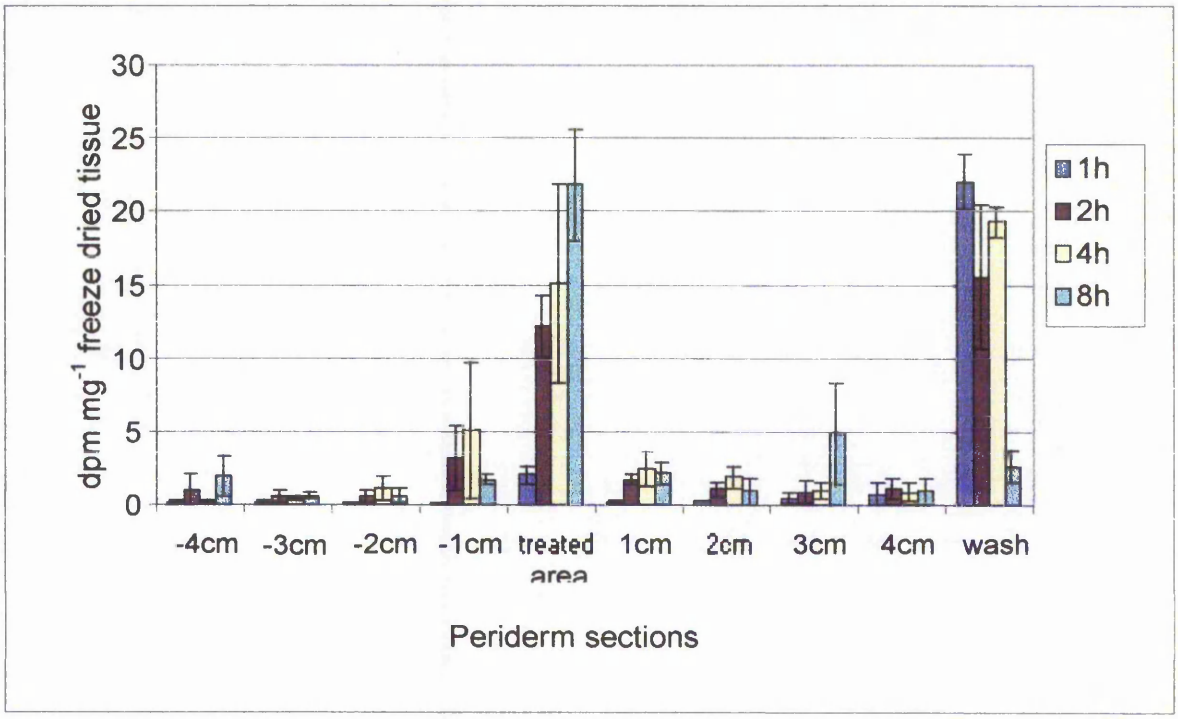


Figure 4.9. Activity recovered from periderm peel sections across the tuber surface using 0.34 g ai L^{-1} imazethapyr at 10°C. Data are means of 3 replicates ± standard error.

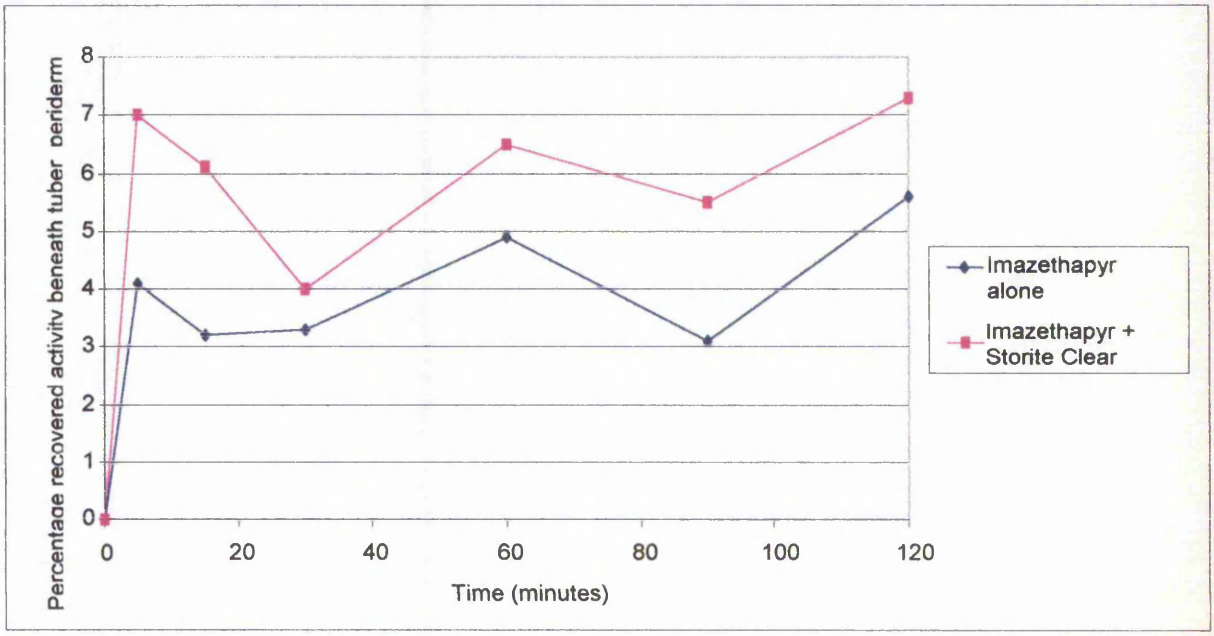


Figure 4.10. A comparison of the percentage recovered activity of imazethapyr recovered from beneath the tuber periderm in the presence or absence of Storite Clear (0.5% v/v) over 2h. Data are means of 3 replicates, standard errors were less than 2% of the means.

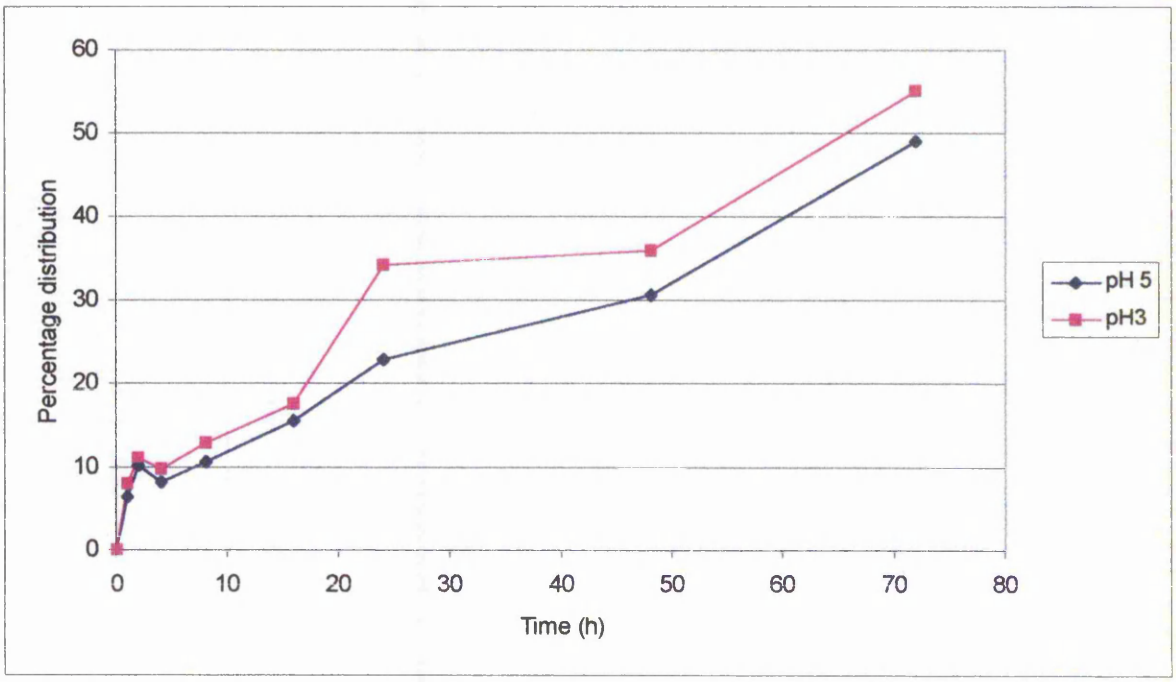


Figure 4.11. Distribution of imazethapyr recovered from beneath the tuber periderm at pH 3 or 5 over 72 h. Data are means of 3 replicates, standard errors were less than 2% of the mean values.

4.4 Discussion

Radiolabelling techniques were used to determine whether imazethapyr application and subsequent uptake and movement in the tuber was affecting the discolouration and damage observed in some treated tubers. With no previous literature relating to the quantitative recovery of radiolabel in potato tubers, a method was successfully developed to trace ^{14}C-labelled imazethapyr.

The novel method enabled the quantitative analysis of distinct sections of tuber, and this provided a profile of movement and distribution of recovered activity through the tuber. The activity was found to be spreading further than the 16mm diameter core originally used and recoveries were improved by increasing core diameter. A peel study demonstrated the ability of imazethapyr to spread within the periderm layer very rapidly. Values above background were recovered from periderm up to 4 cm away from the site of treatment. Minimal activity was detected beneath the periderm and very low recovered activity was detected in the cortical regions. Following refinements to the technique, full recoveries of applied label were achieved.

Proportionately more activity was detected beneath the periderm layer when using a higher concentration of imazethapyr. This was probably due to only a finite number of imazethapyr molecules entering the tuber, and when the radiolabel was applied at 0.34 g ai L^{-1} more of the total molecules would have been labelled in proportion to the amount of molecules labelled at 1.14 g ai L^{-1}. So the same number of molecules were entering the tuber, but a smaller proportion were labelled and therefore the detection was lower, due to the dilution effect of the unlabelled molecules. This must be borne in mind if comparing results using two different rates of imazethapyr.

The amount of recovered activity of ^{14}C-imazethapyr was affected by temperature, with higher periderm recoveries recorded at higher temperatures. The effect of temperature on imazethapyr has been studied in relation to its herbicidal activity in the relevant crops (soybean and alfalfa) by Malefyt and Shaner (1986). In soybean it was found that a cool temperature regime of 15°C (day) / 21°C (night) provided the greatest phytotoxicity. The authors also reported that temperature did not affect absorption or translocation, but did affect metabolism of imazethapyr, with a twofold increase in the rate of metabolism for every 5°C increase. Herrick (1987)

found that temperature did not affect the control of a sensitive species of weed (*Anoda cristata*) by imazethapyr. Despite these studies the periderm of a tuber and the cuticle of a plant are very different, and further studies were carried out on the tuber. To establish whether the increase in periderm activity was a function of increased metabolic rate in the tuber, a respiratory inhibitor was used. The study confirmed that there was no significant difference in recovered activity of ^{14}C-imazethapyr when metabolism was inhibited. To conclude, no clear metabolic component is affecting periderm activity and increases in response to temperature are due to a diffusive or chemiosmotic process, rather than an active process requiring ATP. This is contrary to the finding that absorption was not affected by increases in temperature in soybean. However, a study of imazamethabenz-methyl on *Avena fatua* and *Alopecurus myosuroides* over a cooler temperature range (similar to the range used in this study) found no difference in absorption with temperature variation (Malefyt *et al*, 1983). The effect of temperature on the absorption of different imidazolinones varies greatly according to the species, tissues studied and temperature range investigated. Temperature has greater effect on plants which are able to effectively metabolise imazethapyr. Acid imidazolinones are more active at cooler (18°C) temperatures as they are rapidly deactivated at warmer (35°C) temperatures via metabolism, thereby reducing activity (Wills, 1986). Allen and Caseley (1987) found that frost damage prevented translocation in *A.fatua* and resulted in low activity of imazamethabenz-methyl.

Despite the use of different imidazolinones and crop species, there are parallels between some previous work and the study detailed in this Chapter. Imazethapyr was not very mobile within the periderm at 2.5°C, possibly due to chilling damage and lack of translocation, as observed in *A.fatua* (Allen and Caseley, 1987). At higher temperatures imazethapyr was more mobile in the periderm, but the temperatures were probably cool enough to prevent deactivation via metabolism. The study could be repeated at higher temperatures to determine if similar deactivation would occur, although metabolism in the periderm is unlikely to be possible due to the structure of periderm tissue.

Many other environmental factors have been shown to influence the activity of the imidazolinone family (Musik, 1976; Basham, Levy, Oliver and Scott, 1987; Edmund and York, 1987), but the data presented in this Chapter was produced under controlled

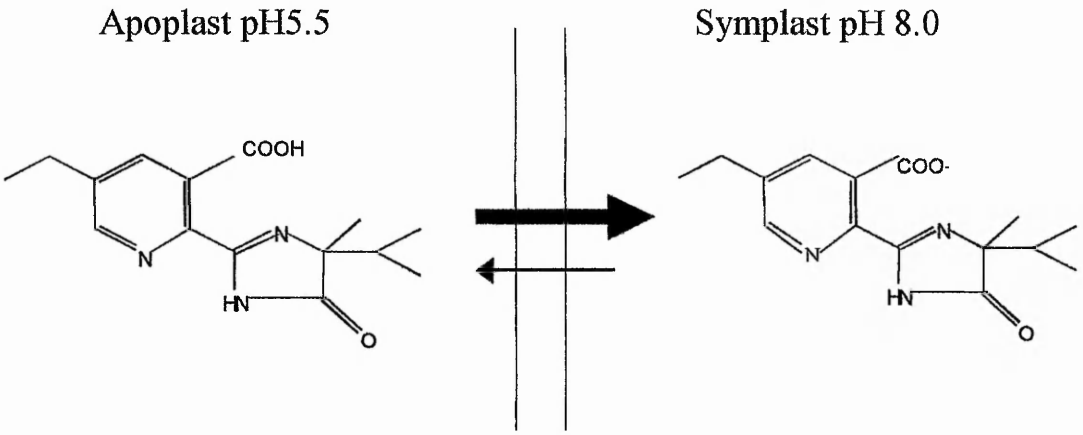


Figure 4.12. A depiction of the ion trap mechanism of imazethapyr in a plant cell (Van Ellis and Shaner, 1988).

environmental conditions and is less likely to be susceptible to such changes. Formulation of imazethapyr greatly affects uptake and distribution within the tuber. When ^{14}C-imazethapyr was applied in the presence of Storite Clear, increased activity was detected within the tuber cortex compared to ^{14}C-imazethapyr alone. This was probably due to the acidity of the Storite Clear formulation. A subsequent study investigated the effect of pH alone and a similar result was obtained. The uptake and distribution of imazethapyr is affected by pH as a result of a process known as ion trapping (Van Ellis and Shaner, 1988). Ion trapping frequently occurs when imidazolinones and other herbicide groups are applied to various plant tissues including potato (Briggs, Rigitano and Bromilow, 1987). Imidazolinones are weak acids and have a pK_a between 3.8 and 4. In the acidic environment outside the plant cell membrane, a significant proportion of the imazethapyr molecules are non-ionised making them sufficiently lipophilic to diffuse through the membranes. Once inside the cell, the alkaline conditions cause the imazethapyr to dissociate. This ionised molecule is then too polar to diffuse out of the cells as it is not lipophilic and becomes trapped within the symplast (Figure 4.12). When imidazolinones are applied as herbicides, the ion trapping occurs in the phloem and the imidazolinone is translocated to cause the death of meristematic cells (Little and Shaner, 1991). The initial penetration of imazethapyr into the periderm is also probably affected by pH. In addition, pH affects the cuticular retention of a herbicide (Kirkwood, 1987).

Tuber tissue displayed increased symptoms of vascular discolouration when commercial applications were made of imazethapyr and Storite Clear together. By applying the Storite Clear, the acidity of the spray solution drops, as imazethapyr has a very poor buffering capacity. The drop in pH will cause the increase in uptake reported in this chapter and will probably induce ion trapping. The increased uptake could be causing the rise in incidence of adverse symptoms observed in some treated tubers. To prevent the increased uptake occurring, imazethapyr must not be applied in conjunction with Storite Clear. If the formulation of imazethapyr was altered to increase the buffering capacity, the acidic compounds may not affect the uptake to such a great extent. Some preliminary studies were carried out to investigate the effect of Agral and Tween formulations on the uptake and distribution of imazethapyr in tubers. This could be continued to establish the ideal formulation for imazethapyr to allow the acidity of the active ingredient to remain constant, whilst maintaining uptake and efficacy.

Criticisms can be made of the methods employed in this Chapter. Firstly, the radiolabel was applied to small areas of individual tubers. This was necessary for the practical execution of the experiment. However this limitation of the method should be considered when extrapolating to the uptake of imazethapyr in whole tubers, or whole storage facilities. Further work could compare the data presented and data from a comparable study carried out using a sprayer, to simulate the commercial situation. However this would require a dedicated sprayer for the use of radiolabel. An alternative method would be to spray the tuber with unlabelled imazethapyr and cover an area for immediate application with radiolabelled imazethapyr. This method was not suitable due to the need to treat a large number of tubers quickly.

Working with radiolabel incurs errors, as radiolabel decay is a random process, but the rate of this decay cannot be influenced by external conditions, for example, light, heat or other radioactive decay nearby. The random process of decay is a source of error but can be minimised by counting and recounting each sample for long periods and by using a radioisotope with a long half-life. Activity is measured from the tuber sections, and whilst the ^{14}C was initially incorporated into imazethapyr, this will not always be the case. There is the possibility of metabolism within the tuber to form, for example, the free acid of imazethapyr or other metabolites.

Another potential source of error was the amount of radioisotope applied to each tuber, this varied with pipetting errors as the volumes applied were small (5µl). Repetitions and relevant controls helped to minimise this source of error. In addition, the grinding of the freeze dried powder was only standardised by eye, as there was no other method employed to standardise the powder size of the samples. Another occasional source of potential error was that tubers could not be obtained from usual sources and supermarket tubers were purchased and used in the study. Whenever this occurred, another repetition was carried out at a later date with tubers direct from growers, results obtained using supermarket tubers were comparable with the results from the grower tubers.

Autoradiography was attempted to provide a comparison to the data presented, however this was not successful. Further investigation of autoradiographic techniques could achieve a useful visual display of uptake and distribution within the tuber.

To summarise, this Chapter has presented a novel method for the quantitative analysis of tuber uptake and distribution of radiolabelled imazethapyr, with typical recoveries of 98.7±7.5%. The study has shown imazethapyr to be rapidly mobile within the periderm. It is susceptible to ion trapping within the symplast of tuber cells, particularly when applied in conditions which lower the pH of the spray solution. The uptake of imazethapyr is affected by temperature in a non metabolic process. Once applied, only a small quantity of active ingredient is required in the cortical regions of the tuber for effective sprout suppression to occur. Discolouration and damage have occurred in tubers treated with imazethapyr, particularly when applied in conditions that allow more imazethapyr to enter the tuber cortex.

5.0 GENERAL DISCUSSION

The morphology and physiology of potato tubers following imazethapyr treatment has been thoroughly examined, and the potential links between treatment and vascular discolouration have been investigated. Imazethapyr application, as tubers enter storage, suppresses sprouting effectively in a range of cultivars and over a range of temperatures (Mawson *et al*, 1996). The suppression achieved using one application of imazethapyr was comparable to the level of control obtained using chlorpropham treatments throughout the storage season. However, in some treated tubers unacceptable levels of vascular discolouration were observed. The levels of discolouration are unacceptable to processors and consumers, and therefore hinders the success of imazethapyr as a potato sprout suppressant.

Imazethapyr application in the current formulation causes increased vascular discolouration in potato tubers. This was demonstrated in Chapter 2 and by large scale storage trials (Cyanamid, personal communication). The work described in this thesis shows imazethapyr to have wide reaching effects throughout the tuber, particularly when application occurs under adverse conditions. These conditions can vary from tubers harvested from wet conditions to application in conjunction with acidic compounds.

Vascular discolouration increased upon treatment with imazethapyr and other environmental and chemical stresses. The stress conditions that induce vascular discolouration are varied. The conditions investigated in Chapter 2 were ethylene exposure, cold temperature storage, water immersion and imazethapyr application. Any of these conditions could affect tuber metabolism individually, but together they can work synergistically to cause more damaging problems to the tuber.

Both ethylene exposure and inhibition of branched chain amino acids (BAA) trigger the induction of alternative oxidase (AOX) in tubers (Day *et al*, 1978; Aubert *et al*, 1997). In Chapter 2 both of these conditions were shown to cause an additive increase respiration and increase the incidence of vascular discolouration. Low temperature storage caused increased respiration, and it is possible that low temperature is another trigger to induce the AOX pathway and could contribute to the increase in respiration rates. Increases in respiration and PPO concentrations in stored treated tubers were recorded. These changes combined with the reported

increase in phenolics at low temperature (Workman, Cameron and Twomey, 1979) affect the incidence of vascular discolouration. The effect of imazethapyr treatment following a simulated wet harvest was investigated in Chapter 2. Prolonged immersion coupled with imazethapyr treatment caused increased vascular discolouration in stored tubers, emphasising the need for dry harvest conditions. The various conditions tested in the respiration studies indicate that imazethapyr treatment alone is not solely responsible for increased vascular discolouration. When imazethapyr is applied in the present formulation in conjunction with other chemical or environmental stresses, vascular discolouration is likely to occur with a frequency that is higher than found in control tubers.

The formulation blanks tested affected tuber PPO activity. The results showed that tubers treated with formulation blanks alone had comparable activities of PPO to imazethapyr treated tubers. In addition, higher activities were recorded when PPO was extracted in the presence of formulation blanks compared to the active ingredient alone. This work would benefit from further research to determine the role of chemical formulation in vascular discolouration, using tubers throughout the storage season, and investigating the individual components of the formulations in turn to determine the precise role of each compound. Further work in other areas would benefit from the use of the formulation blanks, which were only available towards the end of the study. At present the effect of formulation is unclear, but there appears to be an increase in PPO activity upon treatment with formulation or imazethapyr, particularly when applied at acidic pH, suggesting that the formulation of imazethapyr could be contributing to vascular discolouration more than the active ingredient itself. Acidic pH causes increased amounts of imazethapyr to enter other plant cells by ion trapping, a mechanism detailed in Chapter 4 (Van Ellis and Shaner, 1988) and may cause the same effect in tuber cells. The acidic pH caused increases in PPO activity and so provides more available substrate for potential discolouration reactions to take place.

Respiration and PPO measurements have been employed as good model systems. Respiration is an indicator of the metabolic state of the tuber, and the PPO assay focuses on a key enzyme in the discolouration process. Coupling these measurements with visual assessment has gone some way to determining the effect of imazethapyr and its formulation on the stored tuber.

The ultrastructural changes induced by formulated imazethapyr treatment are dramatic. Structural changes were investigated by light and electron microscopy and physiological changes such as mitotic division and cation leakage were also analysed. The structural alterations observed with imazethapyr treatment were related to the metabolic differences that occurred in the treated tuber.

Imazethapyr treatment inhibits the progress of mitotic division in potato tuber root tips. A similar inhibition has been demonstrated using many other species and other AHAS inhibiting compounds (Rost, 1984; Hess, 1987; Shaner and Reider, 1986). The inhibition may be due to the absence of a short-lived protein requiring BAA or BAA intermediates to function (Rost *et al*, 1990). Inhibition of mitosis is a common method used to control sprouting and is the mode of action of propham, chlorpropham and maleic hydrazide.

The work in Chapter 3 was carried out in an artificial system. However, root tips do provide a useful indicator to the whole tuber situation. The technique was successfully adapted to provide an effective staining method and enabled the root tips to remain viable for up to 24h. A possibility for future work would be the use of fluorescent dyes or antibodies to examine the whole tuber for mitotic inhibition using confocal microscopy.

Ultrastructural analysis of imazethapyr-treated cells revealed damage to the amyloplast envelope. Starch breakdown resulted in clear spaces between the grain and the envelope and in some cases the envelope was broken, these features are indicative of starch mobilisation. Amyloplast breakdown was indicated by the cation leakage analysis, when Mg^{++} leakage increased towards the end of study. Mg^{++} is located in amyloplast and mitochondria and therefore not normally released, unless the organelle is disrupted.

Another ultrastructural feature of imazethapyr treatment was cell membrane disruption late in storage. This disruption caused vesicles to form within the cell and also allowed the oxidation of phenols, which had been previously compartmentalised. These types of reaction could account for increases in vascular discolouration, especially when PPO activities were increased by chemical treatment. Whilst increases in sodium and calcium leakage can occur upon tonoplast disruption or other cellular membranes, the leakage tends to occur late on in storage (Turnbull and Cobb, 1992). During the ion chromatography study, sodium and calcium

concentrations remained consistently low, but there may have been increases if the study had continued for longer.

Ion chromatography also indicated that ammonium concentrations declined during storage, probably due to the incorporation into proteins of ammonium via the GS pathway. The tuber directs the balance of protein breakdown and synthesis in favour of synthesis when the tubers are not sprouting (Brierley *et al*, 1997).

Although the microscopy was not quantitative, it serves as a useful illustration of the proposed reactions indicated by other areas in the study. Possible future work would involve the use of confocal microscopy to create a three dimensional image of tuber cell. Ion chromatography is accurate to very low concentrations, the technique is quick and uses small volumes of samples that are easy to obtain. Other techniques are available, such as ion localisation and ion specific fluorescing probes, to determine the movement of ions as well as quantifying the amounts.

Radiolabelling techniques were used to track the movement of imazethapyr through the tuber. A novel method was developed to enable the quantitative analysis of distinct sections of tuber. Imazethapyr was highly mobile in the periderm layer, following ^{14}C-imazethapyr treatment, activity was recovered from up to 4 cm away from the site of treatment. Other findings using this methodology were that the levels of recovered activity of ^{14}C-imazethapyr was affected by temperature, with higher activity recovered from the periderm when stored at higher temperatures. These differences in activity were found to be due to passive uptake rather than any active process.

The acidity of the conditions in which imazethapyr is applied affects the behaviour of the molecule within the tuber. If the imazethapyr is applied in conjunction with acidic compounds such as Storite Clear (pH 3.9), the pH of the spray solution drops. This causes more imazethapyr to enter the tuber cell by ion trapping (Van Ellis and Shaner, 1988). The uptake and distribution of imazethapyr was illustrated to be affected by pH (Chapter 4). When imazethapyr was applied in the presence of Storite Clear, increased radio-activity was detected in the cortex compared to ^{14}C-imazethapyr alone, and a similar result was obtained by applying ^{14}C-imazethapyr at pH 3. PPO activity increased when imazethapyr was applied at acidic pH. These changes may act synergistically, so that more imazethapyr enters the cortex and stimulates PPO activity, causing an increase in potential discolouration reactions.

The temperature response of imazethapyr treated tubers has been detailed. At low temperature storage, a transient increase in respiration was recorded in the week following treatment, PPO concentration increased and less activity was recovered from the periderm. The increase in respiration and PPO are likely to be stress responses and poor translocation and uptake are known to be features of low temperature storage and frost damage.

To enable the practical execution of the work in this thesis, potato tubers were obtained from various sources. Wherever possible cv. Cara was used, as it was found to be a cultivar susceptible to vascular discolouration in preliminary trials carried out by Cyanamid. However, when cv. Cara was unavailable, cv. Estima and Maris Piper were used. These cultivars appeared to behave in a similar manner to each other, but ideally the cultivar would have been the same throughout the study. In addition, there are differences in the tuber populations used because of different harvest times, physiological age and year to year changes in the crop. Also the handling and post-harvest transport of the tubers may have varied between populations. These errors could only be eliminated by growing the same cultivar on the same land each year and restricting the study to those tubers alone. This would not have allowed the same quantity of experiments to be performed.

Future work could be carried out using equipment that was not available at the time of this work, for example confocal microscopy techniques would have been useful in Chapter 3, to determine a three dimensional image of the treated cell, and image analysers could have been used in Chapter 4 to assess autoradiography images. In addition, pH probes could have been employed to determine the intracellular pH of tuber cells in response to applications of imazethapyr whilst under other chemical and environmental stresses.

A summary of the effects are depicted in Figure 5.1. Initially, imazethapyr must cross the barrier of the periderm. From radiolabel studies it is clear that only a small proportion of applied imazethapyr is required at the site of action. The AHAS enzyme is plastid located and the inhibition of this enzyme halts BAA biosynthesis. Mitotic inhibition occurs as a result of BAA inhibition, possibly due to the absence of a short-lived protein that has a BAA (or BAA intermediate) requirement. The cyanide-resistant respiration pathway in mitochondria is also triggered by the inhibition of BAA.

Typically, sprout excision results in a reduced rate of starch degradation as the requirement for sugars of the sprouts is reduced (Davies and Ross,1984). In the same way sprout inhibition would usually result in little starch degradation. However, starch in tubers is frequently converted to sugars as a result of stresses during growth and or storage (Sowokinos, 1990). This provides a substrate for increased respiration, another feature of stress conditions. Different stresses initiate starch breakdown at unique sites of action but influence a common metabolic system. The increased stress to the tuber of imazethapyr treatment could cause increased starch breakdown providing substrates for increased respiration. In turn, glycolysis will provide reducing power and ATP, possibly for use in enzymic browning reactions. This chain of events could be exacerbated by additional environmental or chemical stresses, particularly by low temperature sweetening in storage, providing even more substrate for this potential chain of events.

The GS pathway incorporates ammonia into proteins as the tuber is not sprouting, and consequently the balance of protein synthesis and breakdown favours protein synthesis, to store for the onset of sprouting. Cell membrane disruption causes PPO from the amyloplast and phenolic substrates from the vacuole to come into contact with each other and discolouration reactions take place. As these membranes break down a release of Mg^{++} may occur from the amyloplast.

Finally, these consequences of imazethapyr treatment contribute towards the discolouration occasionally observed when tubers are treated with imazethapyr. Imazethapyr is a highly effective sprout suppressant in potato tubers, with low application rates and a favourable toxicological profile, and at present there is a need for products fulfilling such criteria. The work described in this thesis has gone part way to providing a clearer picture of the behaviour of imazethapyr in the potato tuber and of how the molecule interacts with varied environmental conditions. This work has been achieved by the development and adaptation of novel methodologies for use in potato tubers. However, the formulation and sensitivity to external conditions mean that at present imazethapyr is not a viable option as a commercial sprout suppressant.

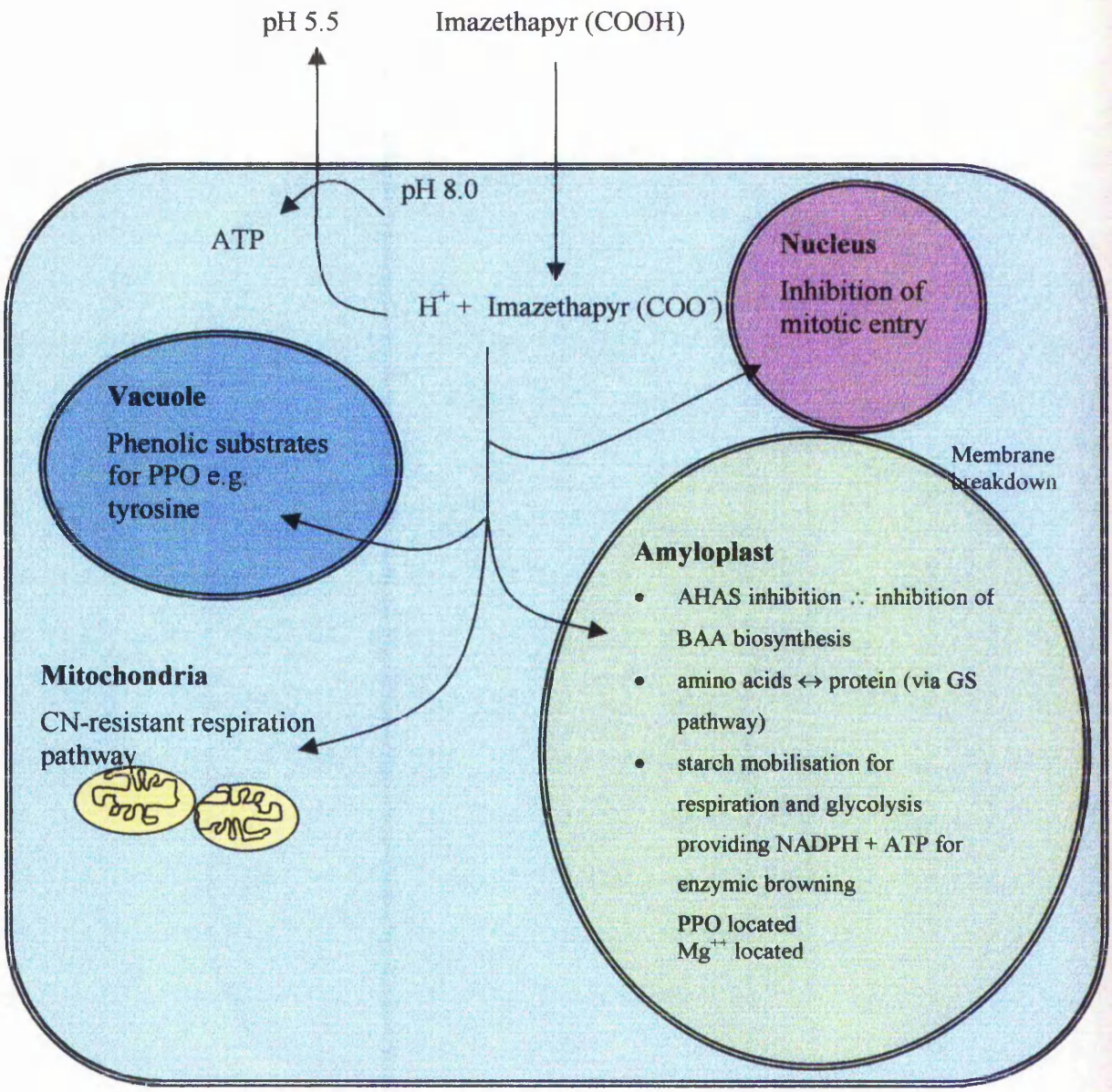


Figure 5.0. Schematic diagram showing the proposed actions of imazethapyr within the tuber cell.

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