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Jamieson, Jennifer Agnes (1988) *Thiabendazole residues and its effects on the storage quality of potatoes.* PhD thesis.

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THIABENDAZOLE RESIDUES AND ITS EFFECTS ON THE STORAGE QUALITY OF POTATOES.

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Thesis presented for the degree of

Doctor of Philosophy

August 1988.

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ACKNOWLEDGEMENTS.

I would like to express my thanks to Dr. H.J. Duncan for his help and guidance throughout the period of research, and especially during the writing of this thesis.

I would like to thank the staff of Agricultural Chemistry for their assistance with field work and other aspects of my research, at both discussion and practical levels.

I am grateful to the staff of the University Mycology Department for their invaluable assistance in the micro-organism studies and for the use of their facilities.

The help and co-operation of Dr. W. Ritchie is also acknowledged in allowing me to use his land for field trial purposes.

My thanks also go to the Potato Marketing Board, who provided me with financial support. The staff of the P.M.B. were also helpful in providing me with statistical data relating to the potato crop.

Finally, I would like to thank my family and friends for encouraging and putting up with me during the preparation of this thesis. It has been worth it.

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SUMMARY.

The objective of this thesis was to gain more information about the post-harvest fungicide thiabendazole (TBZ), in terms of chemical residues in potatoes and in processed products and on factors affecting the storage quality of the crop.

So that TBZ residues could be determined, a sensitive and accurate method for the extraction, clean-up and quantification of TBZ from potatoes, was derived. The recovery of the method was calculated as 93.8% ± 2.1%, and levels in the range of 0.004µg TBZ could readily be detected.

This method was then used to determine the distribution and penetration of TBZ in stored potatoes. The residue was found to associate with the skin of the potato, as its polarity hindered any further penetration of the chemical into the tuber.

TBZ residue analysis was also carried out in processed potato products, including boiled potatoes, baked potatoes and crisps. As little work had been carried out in this area, these findings should provide the basis for future research, so that a complete picture can be built up with respect to residues in processed products.

In general, cooking did not seem to affect either the chemical nature or the absolute residue value of TBZ in the product. The exception to this was the microwaved baked potatoes. In this sample, the residue had decreased after cooking. However, no decomposition products were detected and so the decrease in residue remains unaccounted for.

Factors considered which affected the storage quality of the crop included i) the treatment of seed with TBZ to try to reduce the amount of chemical applied to the ware crop, while still maintaining the desired level of disease control, ii) the effect that TBZ and its formulations had on the wound healing capacity of potatoes and iii) the metabolism of TBZ by fungi and bacteria.

The results of the seed treatment study were very disappointing. It was shown that a seed treatment of TBZ did not affect emergence or yield to any significant extent. The effects on disease control could not be studied satisfactorily though, because a particularly healthy crop was grown and no disease was in evidence (including the controls).

The effect that TBZ had on wound healing was measured by following the development of resistance to water loss in cut potato discs. TBZ was found to promote the development of resistance to water loss in the early stages of wound healing, compared with the control, but after 21 days (the conclusion of each experiment) resistance to water loss was the same in TBZ treated discs, as in the controls. The initial increase in the development of resistance to water loss in TBZ treated discs may be sufficient to inhibit fungal and bacterial pathogens from entering the tubers.

The formulation components of TBZ also influenced the wound healing capacity of discs. Storite, a neutral suspension of TBZ, promoted wound healing to the same extent as TBZ in methanol. However, the acidic formulation of TBZ, Storite Clear, was found to inhibit wound healing and encourage bacterial rotting. The combined formulation of TBZ

and 2-aminobutane, Storite Plus, had mixed effects and therefore no satisfactory conclusions could be made with respect to its effects on the wound healing process.

TBZ was shown to have no significant, beneficial effects on curing in whole tubers. Therefore, although the chemical promotes wound healing, it does not nessesarily follow that it will have the same beneficial effects on curing.

It was important to study the metabolism of TBZ for three reasons :- i) to discover if loss of activity via metabolism was likely to be a problem, ii) to see if any metabolite formed was toxic and iii) to try to ascertain whether the development of resistance to TBZ was likely to become a problem. A number of fungal and bacterial cultures were set up, and resistant strains of the micro-organisms, if they developed, isolated. Of the micro-organisms subjected to TBZ, only soil bacteria, *Erwinia carotovora*, *Phoma exigua* and *Phytopthora infestans* developed resistance. From these isolates, *P. exigua* was the only micro-organism which metabolised TBZ. Very small amounts of TBZ (less than 0.08% of the TBZ initially administered) were metabolised to 5-hydroxy TBZ.

These metabolism studies were carried out under optimum growing conditions for the bacteria and fungi, therefore the likelihood of *P. exigua* developing resistance to TBZ in a store is fairly low.

CHAPTER 1

INTRODUCTION AND THESIS OBJECTIVES.

The objective of this thesis was to gain more information about the effect of the fungicide, thiabendazole (TBZ) on factors affecting the storage quality of potatoes, and TBZ residues in potatoes. In the following sections, some background information is given on i) methods used to improve potato storage, ii) TBZ and iii) some statistics on the flow of potatoes in the U.K..

Today, the potato industry relies on long term cold storage of the product, in general from late September through to the beginning of July, when the first earlies are appearing on the market. However, there are a few problems associated with the long term storage of the crop, including sprouting after dormancy has broken and disease, both fungal and bacterial. At present, two methods of control exist :- i) irradiation and ii) chemical control.

Irradiation.

The possibility of using irradiation to inhibit sprouting and to control disease is currently being evaluated. Hampson (1987) has reported the effects of irradiation on the long term storage quality of potatoes. After potatoes have been irradiated, high levels of reducing sugars were reported, although as the storage season progressed, reducing sugar

levels did decrease to an acceptible level. The tubers were also found to be susceptible to skin spot. Urbain (1986) reports that the levels of irradiation used to inhibit sprouting (100 Gy) are too low to control disease, yet increasing the dose results in unacceptable softening of the potato. There are other problems associated with using irradiation :- i) irradiation inhibits the wound healing process in potatoes, therefore extra costs would be encountered when the store was emptied after wound healing had taken place, so that the potatoes could then be transported to a reactor for irradiation, ii) changes in texture and organoleptic properties of the potato take place after irradiation, iii) lack of public confidence with respect to the safety of the reactors and iv) availability of reactors. Hence, as yet, a number of problems have to be overcome before irradiation is used on a large scale.

Chemical control of sprouting and disease.

At present, the only commercially viable way of achieving sprouting and disease control, is chemically. There are a number of sprout suppressants and fungicides available. However there is not as yet, an effective bacteriocide.

Sprout suppression can be achieved at two levels :- i) administered to the growing crop, where the chemical is translocated into the tuber along with assimilates e.g. maleic hydrazide or ii) applied at some stage after harvest e.g. tecnazene and chlorpropham.

In the U.K., the use of maleic hydrazide is limited, resulting from worries regarding its safety. Although maleic hydrazide has now been given limited clearance, problems with the use of the chemical itself, have now evolved. Uptake poses a major problem due to the unreliable weather conditions found in the U.K., and in connection with poor uptake, varying degrees of sprout suppression are found, as a result.

Tecnazene is applied to potatoes at the beginning of the storage season in the form of granules, as it does not interfere with the wound healing process. However, the cost of the chemical and problems with its application once the potatoes have been loaded into the store means that it is only applied to the ware crop until curing and wound healing have taken place.

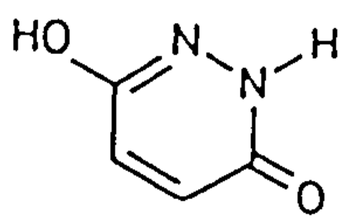
Another problem associated with using tecnazene on the ware crop arises from a processor's point of view. Before potatoes are processed, they are washed, and significant amounts of the chemical are removed with washing. The washings are then released into the sewage system and will eventually become integrated into streams and rivers. As tecnazene is an organochlorine compound, there is increased concern relating to the bioaccumulation of these compounds in fish, and tecnazene may then in fact work its way into the food chain.

Tecnazene is applied to seed stocks, since the sprout suppression is reversible with airing, and the additional costs of the chemical and its application are incorporated into the greater value of the seed.

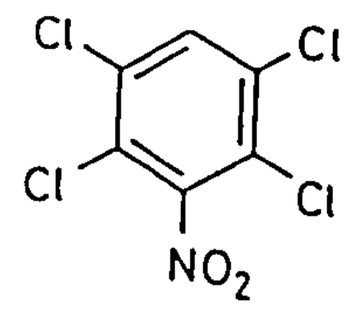
Chlorpropham is applied to the ware crop after wound healing and curing have taken place, as it inhibits both of these processes. It is applied to potatoes in the form of a fog, and can readily be re-applied as and when nessesary, as long as there is a good ventilation system in the store, since chlorpropham is applied to the potatoes by forcing the fog through the ventilation ducts. On a small farm, where the store may be a barn or dicky pie, tecnazene tends to be used, as the ventilation system is insufficient for the application of chlorpropham.

Fungal control can be achieved with the application of thiabendazole (TBZ), 2-amino butane (2-AB), carbendazim, imazalil or tecnazene. TBZ is the most frequently used fungicide for potatoes since the others, with the exception of carbendazim, do not provide the same range of fungicidal control or have some detrimental effects on the skin quality of the potatoes.

Carbendazim belongs to the same class of fungicides as TBZ, i.e. the benzimidazoles. Although a formulation is marketed for use on potatoes, carbendazim is mainly used for the control of fungal diseases on fruit, vegetables, cereals and ornamentals (Anon, 1986). The main problem in using carbendazim on potatoes is that wide spread resistance to it has been reported in a number of places, where the chemical has been used (Hassal, 1982). Cross-resistance with the other benzimidazole fungicides is also a problem as they are all metabolised to carbendazim in order to become active, with the



Maleic Hydrazide



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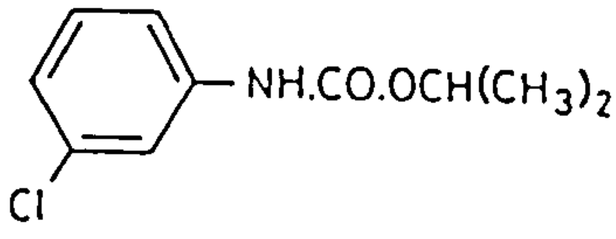

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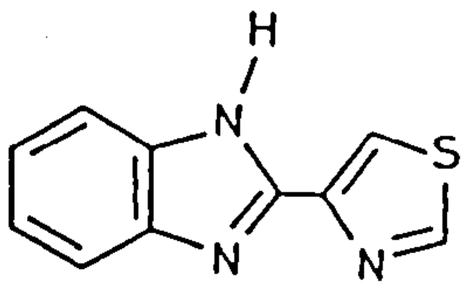

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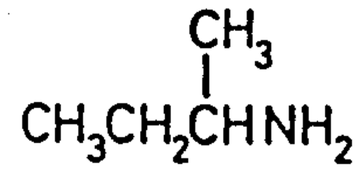
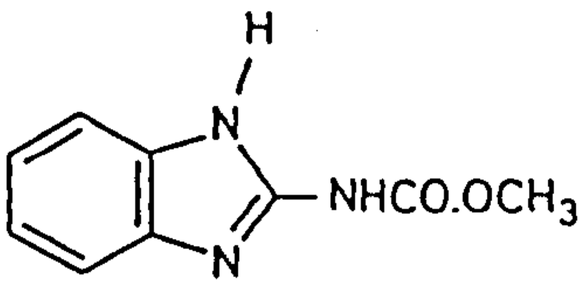
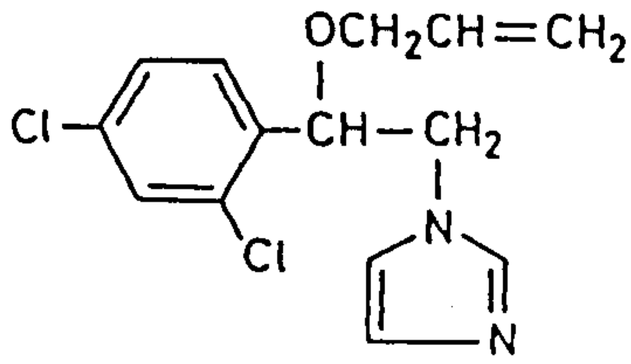


2 - Aminobutane



Carbendazim



Imazalil

exception of TBZ, which is active in its native form.

Hence, TBZ is the most commonly used fungicide that is applied to potatoes.

Uses of thiabendazole.

TBZ (1-H benzimidazole 2,4 thiazoyl) was introduced back in 1962 as an anthelmintic to control roundworm infestations and other gastrointestinal parasites in cattle, sheep, goats and pigs (Brown et al, 1961). In 1968, TBZ was marketed as a broad spectrum fungicide and to date, has been used to control fungal diseases on bananas, citrus fruit (Watkins, 1976), soya beans, apples, pears, sugar beets, sweet potatoes, wheat, rice, carrots and yams (Bardalaye and Wheeler, 1986). TBZ has also been used to control *Ceratocystis ulmi*, the fungus thought to be responsible for Dutch Elm disease (Nishijima and Smalley, 1979).

However, the most common use of TBZ, in its capacity as a fungicide, is to control storage diseases in potatoes. TBZ has been reported to control *Polyscytalum pustulans* (skin spot), *Helminthosporium solani* (silver scurf), *Fusarium spp.* (dry rot) and *Phoma exigua* (gangrene) (Anon, 1984). In order to be effective at controlling these diseases, complete cover of the tuber with the chemical is essential at store loading, as the physical properties of TBZ are very different from the other potato storage chemicals.

Physical properties of thiabendazole.

TBZ is a moderately polar, white odourless powder, which melts at 304°C and sublimes at 310°C. It would appear to have negligible volatility and this is why complete cover of the tuber is nessesary in order to achieve the desired fungal control. TBZ is practically insoluble in water, slightly soluble in organic solvents such as dichloromethane (80 mgl^{-1}) and fairly soluble in methanol (9.3 gl^{-1}) and mineral acids (10.0 gl^{-1} in HCl at pH 2). TBZ has also been shown to be stable to acid, light, heat and hydrolysis (Anon, 1987).

Mode of action of thiabendazole.

To date, the mode of action of TBZ has not yet been fully elucidated, although cell division is affected in some way. TBZ has been shown to inhibit mitosis in *Aspergillus nidulans* by interfering with microtubule assembly (Davidse and Flach, 1978). It has also been found to inhibit cellulase production in *Rhizoctonia solani* (Kannaiyan and Prasad, 1979). It is worth noting that TBZ does not exhibit cross-resistance with the other benzimidazoles. They work by binding to tubulin and thereby inhibit the correct orientation for microtubule assembly at the spindle. Therefore, the mode of action of TBZ is not tubulin binding.

Thiabendazole formulations and usage.

TBZ is applied to tubers at store loading, as a very

fine spray. It is formulated with a number of other storage chemicals such as tecnazene (Storite SS), 2-AB (Storite Plus), iodophor (Tubazole) as well as being marketed with different formulation components e.g. suspension (Storite), clear liquid (Storite Clear) and as a dust (Tecto).

TBZ is used extensively throughout Europe e.g. Austria, Belgium, Poland, Portugal, Spain, Sweeden, Switzerland, U.K. and West Germany, on a variety of produce (Anon, 1986). However, the use of TBZ on the ware crop is only permitted in Belgium, U.K. and West Germany.

Data from the last census, carried out by the Potato Marketing Board in 1981 on the treatment of the crop with potato storage chemicals showed that 14% of the crop was treated with TBZ. The only other fungicide used to any great extent was 2-AB, where only 0.8% of the crop was treated with this fungicide.

At this stage it would be of value to mention potato production and the proportion of the crop which is processed.

Potato production and usage in Great Britain.

Since 1960, the amount of land which has been used to grow potatoes has steadily decreased from 300,000 ha in 1960 to 166,000 ha in 1987. Yet although the area planted has decreased, the yield obtained has infact increased, as a result of improved husbandry and increased usage of chemicals (21·5 tonnes ha^{-1} were lifted in 1960 compared with 38·0 tonnes ha^{-1} in 1987). In 1987, the most popular first early,

second early and maincrop varieties were Maris Bard, Wilja and Maris Piper respectively (Anon, 1988a).

So that production can be related to consumption figures, the supplies and disposals of potatoes for the season June 1986 / May 1987 have been reproduced from Potato Statistics in Great Britain 1983-87 (Anon, 1988b). On average, human consumption of potatoes has increased from 90 kg $head^{-1}$ $year^{-1}$ to 110.6 kg $head^{-1}$ $year^{-1}$.

Supplies and disposals of potatoes for 1986/87.

			Tonnes
Supplies	Home crop		6458,000
	Imports :	Ware from outside U.K.	174,000
		Ware from N. Ireland	17,000
		New potatoes	227,000
		Processed (raw equivalent)	377,000
		Seed for next crop	24,000
TOTAL SUPPLIES			7277,000
Disposals	Human consumption		6105,000
	Exports :	Ware/ new	56,000
		Processed (raw equivalent)	51,000
		Seed	75,000
	Seed for the next crop		553,000
	Carryover into next season		230,000
	Wastage		207,000
TOTAL DISPOSALS			7277,000

Over 24% of potatoes grown in the U.K. are processed. Figures for the tonnage of potatoes used for processing into different products for the season June 1986 / May 1987 are shown below.

Product	Tonnes of raw potato used
Crisped	626,000
Frozen or chilled	530,000
Canned, dehydrated or other	165,000

From all of these data, it is obvious that potatoes are still considered to be a valuable commodity and by improving the quality of the stored crop, greater fin ancial returns will result. In 1981, only 14% of the stored crop was treated with TBZ to improve the storage quality. However, if current trends are anything to go by, then a much greater proportion of the stored crop is being treated with TBZ today.

With the increased usage of the chemical, more information must be sought to find out how TBZ affects the storage quality of the crop. In the next section, the objectives of this thesis are stated.

Thesis objectives.

When a chemical like TBZ is used so extensively on the potato crop, it is important to find out how that chemical will affect the storage quality of the potato. The objectives of this thesis therefore, were to study TBZ residues in potatoes and to ascertain how the presence of the chemical might influence the quality of the stored potato, in terms of disease control and wound healing of the potato.

CHAPTER 2. Development of an analytical technique for thiabendazole residues in potatoes.

Today, eating habits are changing and people are making a conscious effort to eat a healthier diet. As a result, more whole potatoes are being consumed since they contain more fibre and vitamins than peeled potatoes. However, eating more whole potatoes means that more chemical residue will probably be consumed. The work in this chapter describes the development of an analytical method for the analysis of TBZ in potatoes and establishes the distribution of TBZ within the potato.

CHAPTER 3. The effect of thiabendazole seed treatments on fungal disease control in the progeny tubers.

With the consumption of TBZ on the increase, it was interesting to see whether a seed treatment of TBZ could provide the desired disease control in the progeny tubers, and therefore reduce the amount of TBZ applied to the ware crop. A field trial was set up with various seed treatments, and the progeny was stored so that the seed treatment could be related to the storage quality of the yield.

CHAPTER 4. The effect of thiabendazole and its formulations on the wound healing capacity of potatoes.

The storage quality of the yield can be affected in a number of ways, appart from disease. If chemicals are applied to the crop before wound healing and curing have taken place, then substantial losses may be encountered. Therefore it is important to ascertain how a chemical will affect wound healing, before it is applied to the crop. In this chapter, the effect that TBZ and its formulations had on wound healing were studied, so that any beneficial or detrimental effects could be identified.

CHAPTER 5. Thiabendazole residues in processed potato products.

Little information was available with respect to TBZ residues in cooked potato and processed potato products. Unlike other potato storage chemicals e.g. chlorpropham and tecnazene, heating of the raw potato by boiling, frying of baking did not lead to a reduction in the TBZ residue present. In this chapter, residue methods for the extraction of TBZ from processed potato products are described and residue values reported.

CHAPTER 6. Metabolism of thiabendazole.

When a chemical is applied to potatoes, it is important to establish whether or not it is metabolised by the fungi and bacteria present on the tuber surface. It is essential to carry out metabolism studies so that i) possible toxic metabolites can be identified, ii) the rate at which chemical activity is lost can be followed and iii) the likelihood of resistance development to the chemical can be ascertained. In this chapter, the metabolism of, and the development of resistance to TBZ were followed, using a number of fungal and bacterial cultures.

CHAPTER 7. Conclusions and future work.

The conclusions of the research are drawn together and the findings are discussed in terms of potato research today, in this chapter. Some suggestions are also made relating to future work in this field.

CHAPTER 2

DEVELOPMENT OF AN ANALYTICAL TECHNIQUE FOR THIABENDAZOLE RESIDUE ANALYSIS.

Introduction.

With changes in the statutory controls of pesticides based on Part lll of the Food and Environment Protection Act (1985), the Government will now be able to set limits with respect to the maximum permissable residue levels present in food and crops. This information will also become available to the public.

In light of this, and based on the fact that limited information was currently available regarding TBZ residues in potatoes, a study was undertaken to quantify TBZ residues in various fractions of the potato.

A variety of analytical methods have been and still are used to determine TBZ residues in a number of commodities.

Cayley et al (1983) used ultraviolet or fluorimetric spectrophotometry in their work with TBZ. However, this technique lacked the sensitivity required for the type of residue analysis which was to be carried out.

Thin layer chromatography has also been used (Becker, 1984), but as a technique for routine residue analysis, it is time consuming and again, lacks the sensitivity required.

Gas liquid chromatography is sometimes used to determine TBZ residues and suitable methods have been derived

(Bardalaye and Wheeler, 1986). However, it was felt that the necessary stage of derivatisation of the sample resulted in an additional step of the quantification procedure, where losses may be encountered.

The most suitable analytical technique for the quantification of TBZ was found to be high pressure liquid chromatography (HPLC) where a number of methods using either ultraviolet (UV) or fluorimetric detection have been derived e.g. Collinge and Noirfalise (1983), Maeda and Tsuji (1976), from a variety of produce.

Of the extraction and clean-up procedures tested, none were found to be entirely suitable for the extraction of TBZ from potatoes, where poor extraction recovery, low sensitivity and the laborious nature of the technique, being the principal draw backs.

With regard to the HPLC methods studied, poor resolution, tailing and interference from other components extracted from potatoes were the main problems with existing methods and therefore, a modified HPLC method had to be devised.

As TBZ fluoresces, it was decided that fluorimetric detection would be employed when devising a suitable HPLC method as this property provides greater sensitivity and specificity compared with UV detection.

Hence, in this chapter, the development of a suitable extraction, clean-up and quantification method for TBZ from potatoes is discussed, and after this the method is used to determine TBZ residues in potatoes.

Extraction of Thiabendazole from potatoes.

Preparation of sample.

In order to ensure the complete extraction of TBZ from potato tissue, the tissue was first macerated in order to provide as large a surface area as possible, from which the solvent could extract TBZ.

Hence, approximately 500g of potatoes were macerated using a powerful mincer (Bauknect, model AL 2-1) and this was then thc roughly mixed in order to provide a homogeneous potato mix from which 30g sub-samples could be taken for residue analysis (Dalziel, 1978).

In the department, 50g sub-samples of potato are generally taken for residue analysis. However, after preliminary experiments it was found that reproducible results could be obtained using 30g of macerated potato tissue, providing that the initial sample of 500g of tissue had been thoroughly mixed.

Choice of solvent for the extraction of TBZ.

Ethyl acetate was commonly used as an extractant for TBZ (Caley et al (1983), Maeda and Tsuji (1976), Bardalaye and Wheeler (1986)). However, after testing, it was found to give poor extraction recovery unless the potato tissue was re-extracted for a second time.

Methanol was also tried, as TBZ is reasonably soluble in methanol (9.3 gl^{-1}). A soxhlet continuous refluxing system was set up, where approximately 10g of potato tissue was placed in an extraction thimble. This was placed in a soxlet extractor, attatched to a reflux condensor and 100 cm^3 of methanol were added to a flat bottomed flask, containing several boiling stones. The tissue was extracted continuously for 4 hours, after which time the extractant was filtered and the volume made up to 250 cm^3. However, after analysis using fluorimetric HPLC, recoveries of between 46 and 57% (from ten replicate extracts) were recorded, therefore an alternative extractant was sought.

Another solvent commonly used for the extraction of TBZ was dichloromethane (Becker and Fresenius (1984), Analytichem International (Anon)). It has frequently been used for the extraction of TBZ from the peel of citrus fruit.

However, as the potato has a high percentage of water compared with citrus fruit peel, the water had first to be removed so that the dichloromethane could efficiently extract TBZ. This was achieved by adding sufficient anhydrous sodium sulphate to the potato tissue at the extraction stage.

Hence, the extraction procedure involved blending 30g of potato tissue along with 80g of anhydrous sodium sulphate (sufficient to absorb water from 30g of potato tissue) and various volumes of dichloromethane for 1 min in a stainless steel blender. The blender contents and rinsings were then quantitativly transferred to an alumina bottle, and this was shaken on a reciprocating shaker for 1 hour to ensure the complete extraction of TBZ.

The contents were then filtered over suction and washed with 3 x 50 cm^3 portions of dicloromethane. The filtrate was then quantitatively transferred to a 500 cm^3 R.B. flask and the volume was reduced to dryness under reduced pressure.

The residue was redissolved and made up to a final volume of 5 cm^3, using HPLC grade methanol.

In the preliminary analysis, the HPLC method described by Watts et al (1982) was used where a Waters Model 6000A solvent delivery system was connected to an O.D.S. Hypersil C_{18} column (150 x 4.6mm, particle size = 5μm) and a Shimadzu Model RF-530 HPLC fluorimeter (excitation wavelength = 305nm and emission wavelength = 370nm). The mobile phase used was 0.01M K_2HPO_4 (pH 7.0)/ methanol (50:50, v/v).

Under these conditions, 10 mm^3 of the extract were injected into the HPLC system. At a flow rate of 1.5 cm^3min^{-1}, a retention time of 5.6 min was recorded.

Effect of volume on extraction recovery.

Various volumes of dichloromethane were added to potato tissue, which had been "spiked" with 10μg of TBZ at the blending stage, in order to discover the minimum volume of dichloromethane required to yield the optimum extraction of TBZ. The results are shown in table 2.1.

Hence, from the results, it is obvious that by increasing the volume of dichloromethane added, the efficiency of extraction is also increased. However, little benefit is

Table 2.1

Volume of dichloromethane added at blending	% recovery of TBZ*
50 cm^3	50.1% ± 4.4%
100 cm^3	81.5% ± 3.8%
150 cm^3	91.9% ± 2.3%
200 cm^3	97.4% ± 2.1%

* mean of five replicates

achieved in using 200 cm^3 compared with 150cm^3 dichloromethane i.e. only 5% more TBZ is extracted with a 33% increase in the volume of solvent used.

Therefore, in subsequent extractions, 30g of potato tissue was extracted with 80g of anhydrous sodium sulphate and 150 cm^3 dichloromethane.

Clean-up of the residue extract.

It was apparent from the strong yellow colour of the potato extract, that some form of clean-up was nessesary, especially where HPLC is being used, since pigments can be strongly adsorbed onto the column, thereby reducing the resolution of the HPLC column, and hence reducing its working life. Pigments can be desorbed from an HPLC column by

increasing or decreasing the polarity of the mobile phase, however, this is time consuming and not entirely 100% successful, therefore a suitable clean-up method was sought.

Three types of clean-up were considered:-

i) Traditional adsorption column chromatography. This is very common in pesticide residue analysis where different grades of alumina or silica are used to adsorb pigments and other interfering components. However, this type of clean-up was not evaluated as it is very time consuming, and as a result, not suitable for routine TBZ analysis.

ii) Solvent partitioning. This was the most commonly used form of clean-up for TBZ from various components. TBZ was usually partitioned into hydrochloric acid and the acidic layer then washed with several volumes of hexane, to remove any non-polar components. The acidic phase was then made alkaline with sodium hydroxide, before being partitioned with ethyl acetate, where TBZ moves into the organic phase. The organic phase was evaporated to dryness and the residue redissolved in methanol, before being analysed by HPLC (Maeda and Tsuji,1976). In practice, this type of clean-up is time consuming and variable recoveries usually arise, therefore a different type of clean-up was investigated.

iii) Bonded silica sorption extraction columns. The introduction of these "mini chromatography" columns has

revolutionised the process of residue extract clean-up. These columns allow extracts to be cleaned-up and concentrated up on the column, resulting in a much faster clean-up (approximately 5 min) compared with that using a traditional column clean-up (approximately 4 hours). The cost incurred in using a bonded silica sorbent extraction column is much reduced compared with a traditional column clean-up when one considers the cost of solvent used in the elution of components and the number of man hours required for traditional column clean-up.

These columns are prepared with a variety of bonded phases where polarity and charge are the main features considered when choosing a suitable column.

In the clean-up of TBZ from potato extracts, a method developed by Analytichem International (Anon.) for the extraction and clean-up of TBZ from citrus fruit, was adapted.

Throughout this thesis, Bond Elut™ silica sorption extraction columns were used.

After testing a variety of Bond Elut columns and many different elutant systems, the Bond Elut diol column was found to yield the best retention of TBZ, while being able to selectively elute TBZ from the column. The final clean-up procedure for TBZ from potato extracts is detailed below.

A 500 mg Bond Elut diol column was pre-wet with 10 cm^3 of dichloromethane to activate the column. 1 cm^3 of the TBZ extract was quantitatively applied to the diol, and then washed in with a further 10 cm^3 of dichloromethane.

The column was then placed over suction for 5 min in order to completely remove the dichloromethane.

TBZ was eluted from the column with approximately

5 cm^3 of methanol/ 112mM phosphoric acid (50:50, v/v) and the eluate collected into a 5 cm^3 volumetric flask. The sample was made up to 5 cm^3 (using methanol/ phosphoric acid) and quantified using fluorimetric HPLC.

HPLC analysis of Thiabendazole.

In the preliminary development work, the HPLC analysis method of Watts et al (1982) was used. However, there were several draw backs in this method :- i) TBZ was not retained to any great extent. Although this was not a major problem when fluorimetric detection was being used, resolution of TBZ was poor, from interfering components, where UV detection was employed (see later), ii) there was a high degree of tailing of TBZ when the C_{18} column was used and iii) the solvent system used, suppressed the fluorimetric response of TBZ compared with other mobile phases. Hence, using this HPLC method, the overall sensitivity of the extraction and clean-up method would be limited.

Therefore, in the development of an HPLC method for the analysis of TBZ, conditions similar to those used in the clean-up and analysis (Analytichem International) were used i.e. a moderately polar column and a moderately polar solvent system were used.

A nitrile phase HPLC column (150 x 4.6mm, particle size, 5µm) was attached to a Perkin Elmer Series 400 solvent delivery system, a Perkin Elmer ISS-100 sampling system, a Shimadzu model RF-530 HPLC fluorimeter (where the excitation

and emission wavelengths were set at 305nm and 370nm respectively (Watts et al, 1982)) with a Perkin Elmer LCI-100 integrator.

Of the various mobile phases tested, the most suitable phase was found to be acetonitrile/ 0.3mM sodium chloride in 3.5mM phosphoric acic (30:70, v/v).

At a flow rate of 1.5 cm^3min^{-1}, a retention time of 8.0 min was recorded for TBZ. Fig. 2.1 shows a typical trace obtained from a potato extract, using the extraction and clean-up method described previously.

In comparision, Fig. 2.2 shows the trace obtained where the reverse phase HPLC conditions were employed for the same sample. When fluorimetric detection is used, interfering peaks are not a problem, even though TBZ is not retained to any extent. However, it is evident that under these conditions, the TBZ peak has been split, and ionised and uncharged TBZ are resolved from each other. It should be noted that it is the acidity of the mobile phase that has brought about the ionisation, and therefore, this method could not be used to quantify the degree of ionisation of TBZ in a given sample.

The traces shown in Figs. 2.1 and 2.2 also demonstrate the effect that the mobile phase has on the fluorimetric response. Using the normal phase HPLC conditions, a peak of full scale deflection was obtained using an attenuation of 128. In comparision, the peak area obtained using reverse phase HPLC conditions was much less and full scale deflection was obtained with an attenuation of 32.

Hence, using the reverse phase HPLC conditions reduces

Fig. 2.1. HPLC trace of TBZ using a nitrile column and fluorimetric detection.

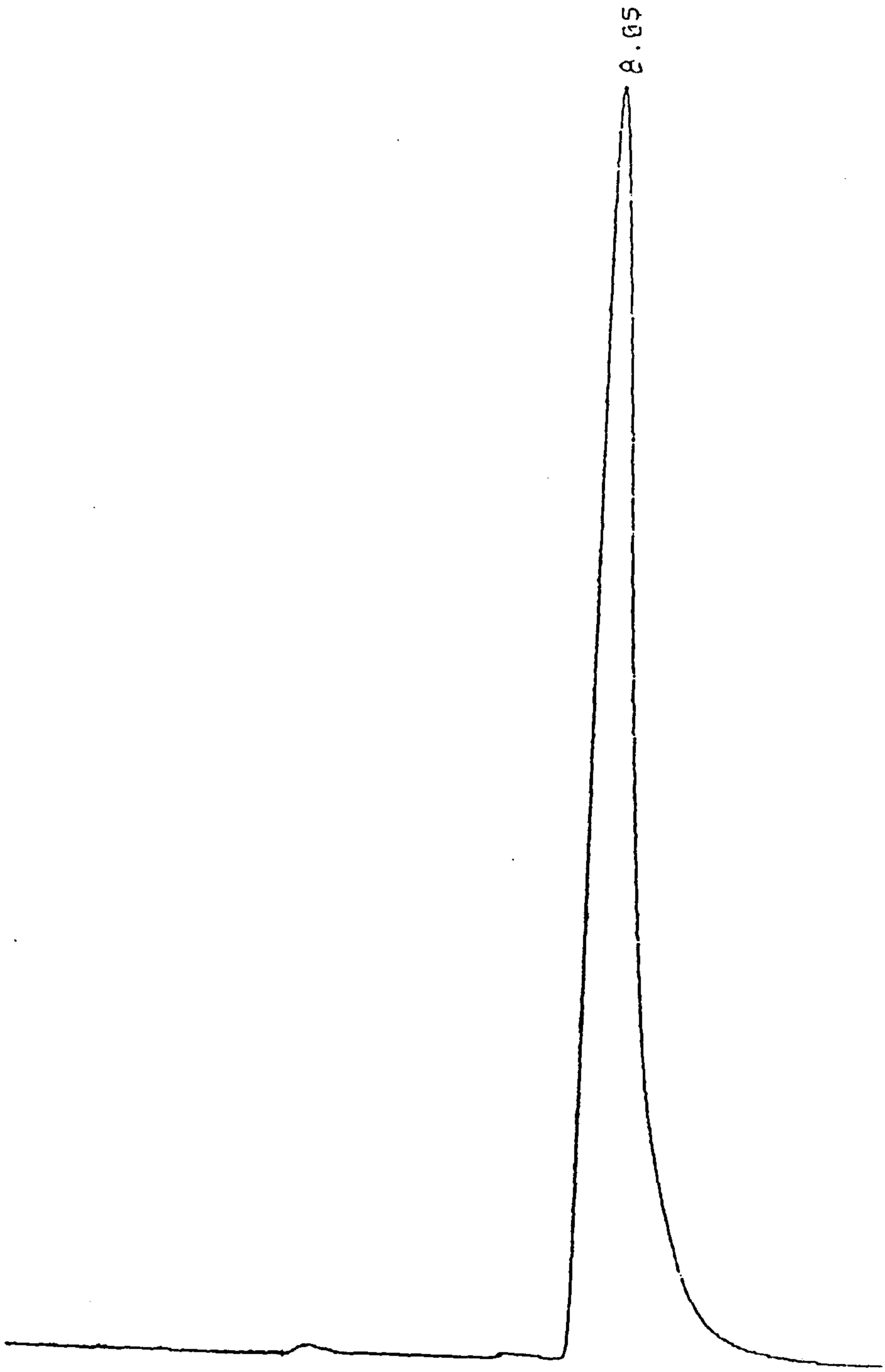


Fig. 2.2. HPLC trace of TBZ using an CDS hypersil column and fluorimetric detection.

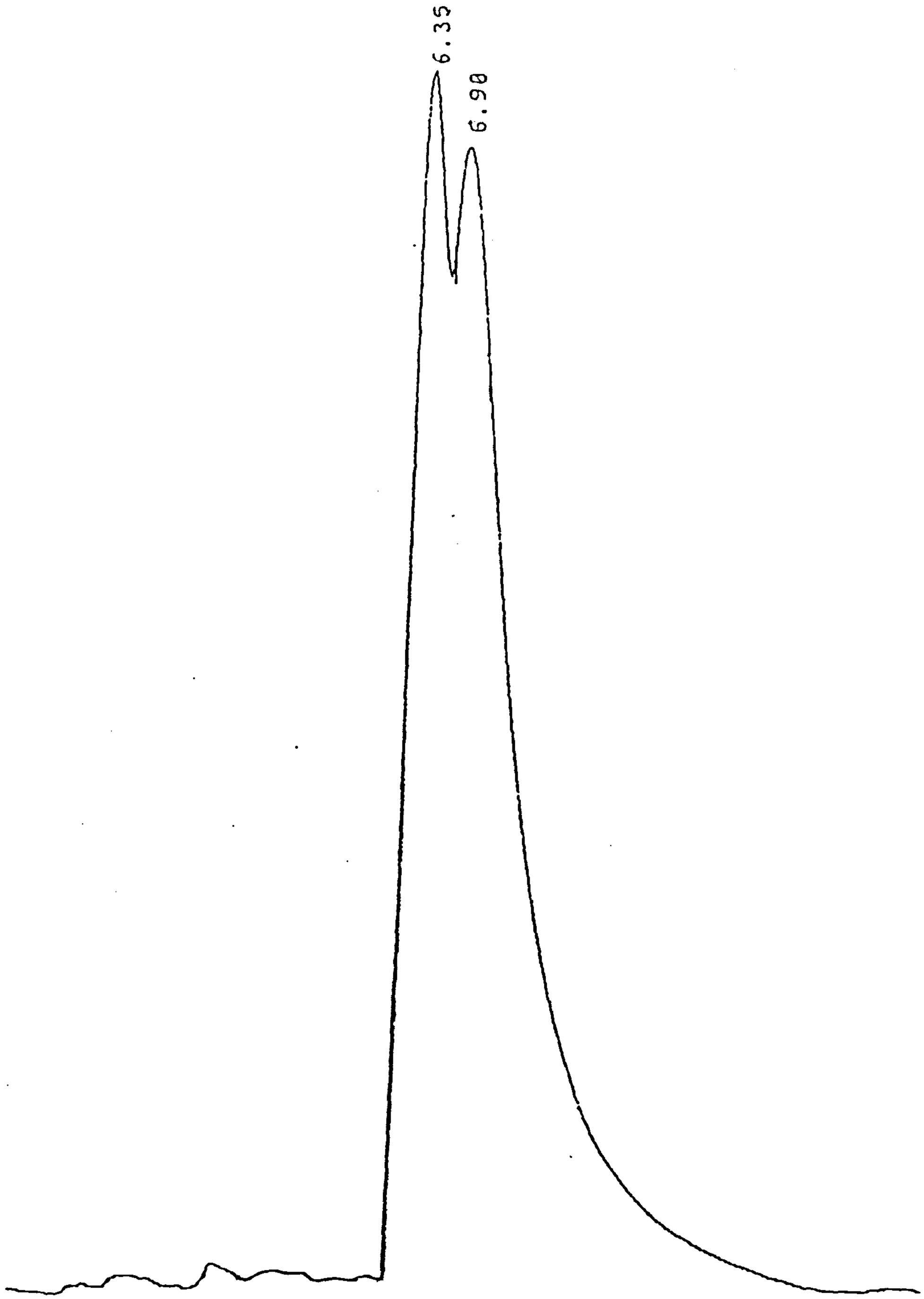


Fig. 2.3. HPLC trace of TBZ using a nitrile column and UV detection.

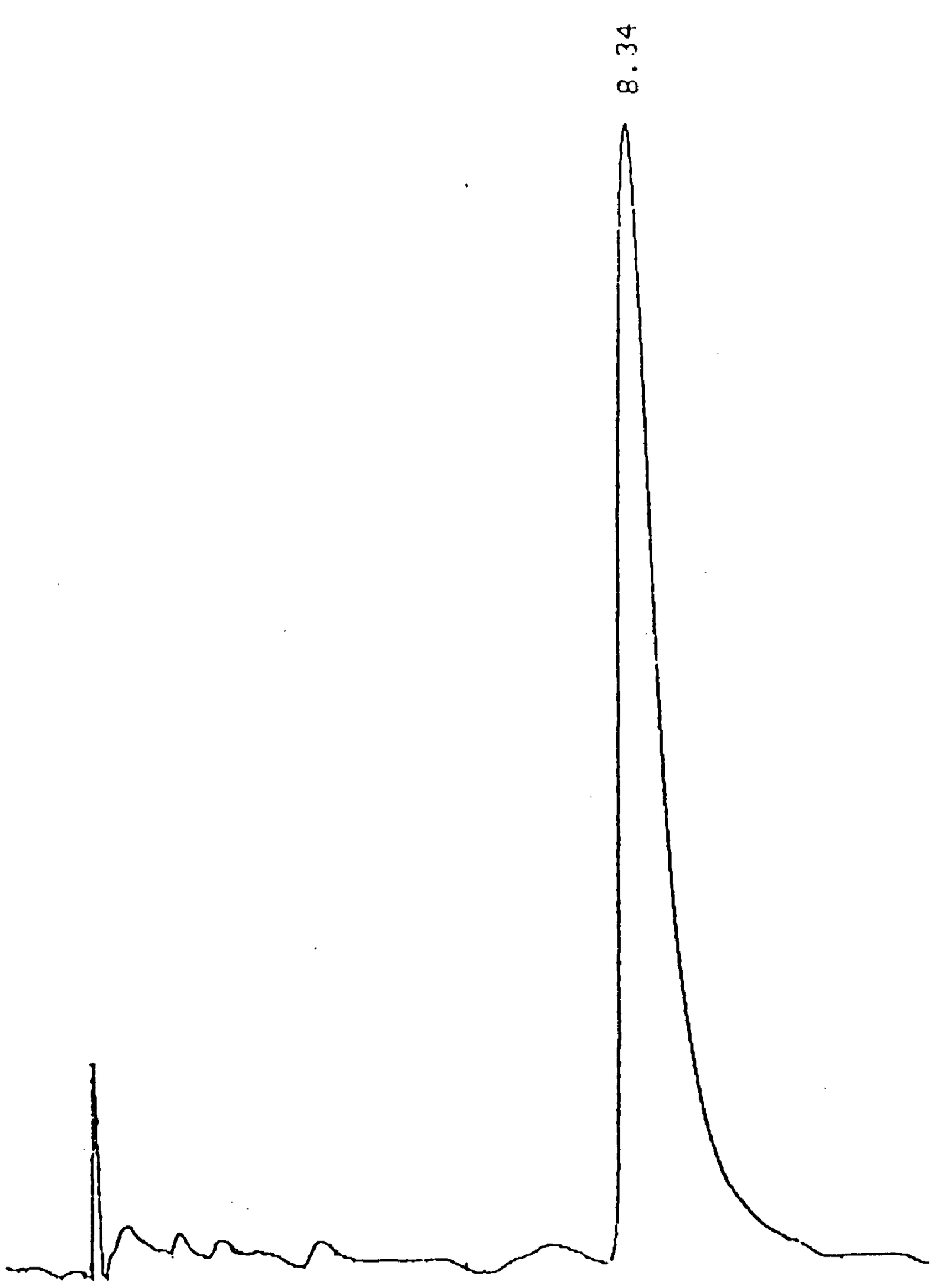
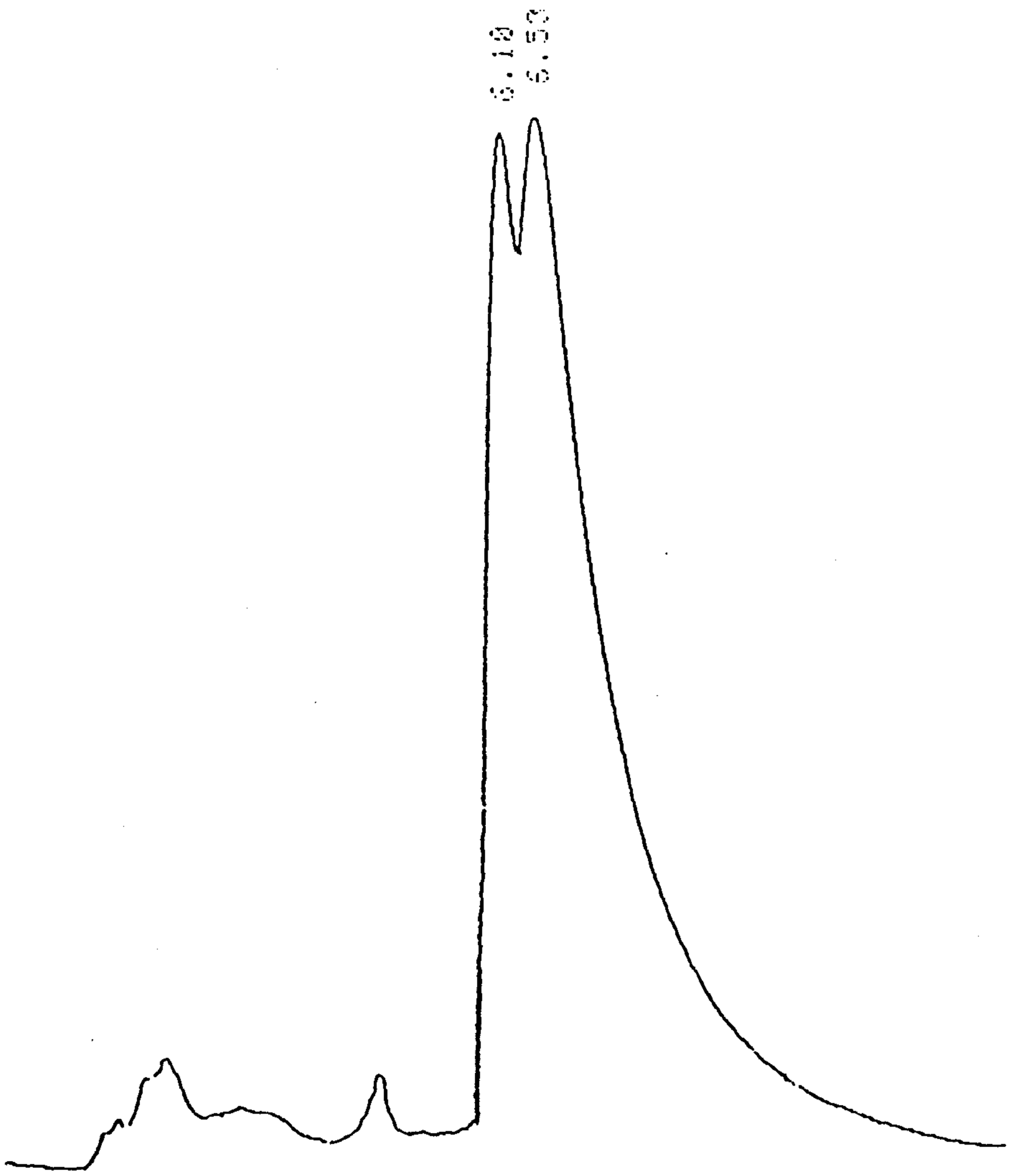


Fig. 2.4. HPLC trace of TBZ using an ODS hypersil column and UV detection.



the fluorimetric response by a factor of four.

Figs. 2.3 and 2.4 show the resulting traces from normal and reverse phase HPLC conditions respectively, where UV detection (302nm) was used. Again it is apparent that the normal phase HPLC conditions allow TBZ to be resolved from any interfering peaks with an increased retention time, compared with the reverse phase HPLC conditions.

Recovery and sensitivity.

Using the method described above, the percentage recovery and sensitivity of the method were measured.

Percentage recovery of TBZ was determined by "spiking" the potato samples with 100µg TBZ and carrying out the subsequent extraction, clean-up and quantitative analysis. The percentage recoveries ranged from 93.8% to 97.1%, with a mean value of 93.8% ± 2.1%, from ten replicates.

The recovery factor of this method was consistent, and higher than those obtained from other methods tested.

Sensitivity of the method was determined, again by "spiking" potatoes with known amounts of TBZ.

It was possible to detect levels of TBZ down to 0.0004µg, which is equivalent to 0.033 $mgkg^{-1}$ potatoes (on a fresh weight basis) using fluorimetric detection. Using UV detection, levels of 0.005µg TBZ (equivalent to 0.42 $mgkg^{-1}$ potatoes) were detected and hence UV detection of TBZ is

twelve times less sensitive than fluorimetric detection.

It should also be noted that there is the capacity to detect even lower levels of TBZ either by reducing the volume of the crude extract, applying a greater volume of crude extract to the diol column or by increasing the HPLC injection volume.

Final method for the determination of Thiabendazole in potatoes.

Extraction of TBZ.

30g of macerated potato tissue was blended in a stainless steel blender along with 80g of anhydrous sodium sulphate and 150 cm^3 of dichloromethane for 1 min at high speed.

This mixture was then quantitatively transferred to an alumina bottle, along with 50 cm^3 of dichloromethane, used to rinse the blender. This was then shaken on a reciprocating shaker for one hour to ensure the complete extraction of TBZ from the potato tissue. After shaking, the mixture was filtered over suction and washed with 3 x 50 cm^3 portions of dichloromethane. The filtrate was then transferred to a 500 cm^3 R.B. flask and evaporated to dryness under reduced pressure. The residue was redissolved in dichloromethane and made up to a final volume of 5 cm^3.

Clean-up of TBZ.

A 500mg Bond Elut diol column was activated by passing 10 cm^3 of dichloromethane through the column, without letting it run dry.

1 cm^3 of the TBZ extract was then quantitatively applied to the column, and washed in with a further 10 cm^3 of dichloromethane. The column was then placed over suction for 5 min in order to completely remove the dichloromethane.

TBZ was eluted from the column with approximately 5 cm^3 of methanol/ 112 mM phosphoric acid (50:50, v/v) and the eluate was collected into a 5 cm^3 volumetric flask. The sample was made up to 5 cm^3 with methanol/ phosphoric acid.

HPLC analysis of TBZ.

TBZ was quantified by HPLC using a Spherisorb nitrile phase column and fluorimetric detection (excitation wavelength = 305nm and emission wavelength = 370nm). The mobile phase used was acetonitrile/ 3.5 mM phosphoric acid in 0.3 mM sodium chloride (30:70, v/v) and under these conditions 10mm^3 of the extract were injected. At a flow rate of 1.5 cm^3min^{-1}, a retention time of 8.05 min was recorded for TBZ.

Hence, now that a suitable analytical method had been derived for the extraction and quantification of TBZ from potatoes, residues were measured in a number of different fractions of the potato. This would then give a complete picture of the penetration and distribution of TBZ in potatoes throughout the storage season.

Distribution of Thiabendazole within the potato.

TBZ is a systemic fungicide applied to a diverse range of commodities including bananas, citrus fruit (Watkins, 1976), soya beans, apples, pears, sugar beets, sweet potatoes, wheat, rice, carrots and yams (Bardalaye and Wheeler, 1986). It is also used as an anthelmintic in cattle and pigs to control parasitic worms (Brown et al, 1961).

However, the primary use of TBZ in the U.K., is that of a post-harvest fungicide applied to seed and ware potatoes, at store loading. As a fungicide, TBZ provides effective control of skin spot (*Polyscytalum pustulans*), silver scurf (*Helminthosporium solani*), dry rot (*Fusarium spp.*) and gangrene (*Phoma exigua*) (Cayley et al,1979).

On a potato, it has generally been accepted that although TBZ has systemic properties, it acts as a contact fungicide and there is therefore little movement of the chemical into the tuber.

However, little information was available with respect to absolute levels of TBZ in treated tubers and of the work published relating to TBZ residues, the sample of potato taken for residue analysis was either not clearly stated or ambiguous. Hence, limited information could be taken from this work.

As a result, several experiments were undertaken to

i) record the distribution of TBZ within the potato and

ii) study the penetration of TBZ into the potato.

Treatment and storage of potatoes.

Newly harvested potatoes (cv. Record) were treated with the TBZ formulation, Storite (M.S.D Agvet) at the commercial application rate of 40 $mgkg^{-1}$, using a Shandon spray gun. The potatoes were then randomly mixed, before being boxed in 4 x 10kg replicates, and stored at $8^{o}C$ (14/10/86).

After curing had taken place, the potatoes were treated with chlorpropham (adsorbed onto neutral grade alumina) at a rate of 20 $mgkg^{-1}$, in order to control sprouting. Earlier preliminary work had shown that the presence of chlorpropham did not affect the distribution or penetration of TBZ into the tuber.

After four months storage, tubers were taken for residue analysis and TBZ was determined using the method described earlier.

Samples for residue analysis were prepared as follows i) whole unwashed tubers were macerated, ii) whole washed tubers were prepared by hand washing in cold water prior to maceration, iii) peeled tubers were prepared using a hand peeler which removes approximately 15% (by weight) of the tuber prior to maceration and iv) unwashed peelings (from (iii)) were macerated prior to extraction.

The TBZ residues present in each sample are reported in table 2.2.

Table 2.2

Sample	TBZ residue ($mgkg^{-1}$)*	reduction in residue
Whole, unwashed	9.89 ± 0.11	-
Whole, washed	1.58 ± 0.09	84.0%
Peeled	NDR**	100%
Peel, unwashed	65.91 ± 10.93	-

* mean of five replicates

NDR** no detectible residue

The penetration of TBZ into the potato was also investigated, where tubers of approximately 80 mm diameter were washed and cored (using a No. 7 cork borer). The cores were subsequently sliced into discs of 10 mm depth. Discs from the same sampling depth were pooled and TBZ was extracted as before. Table 2.3 shows the results obtained.

Hence, from these studies, some conclusions can be made with respect to the distribution and penetration of TBZ into the potato :- i) although TBZ is a systemic fungicide, on the potato it acts as a contact fungicide, where the greatest proportion of the chemical remains on the surface of the tuber, ii) significant amounts of TBZ can be removed with washing which again suggests little movement of TBZ into the tuber and iii) of the residue left after washing, in general the bulk, or all of it can be removed with peeling.

Table 2.3

Sampling depth (mm)	TBZ residue ($mgkg^{-1}$)*
0 - 10mm	5.21 ± 0.12
10 - 20mm	NDR**
20 - 30mm	NDR
30 - 40mm	NDR

* mean of five replicates

NDR** no detectible residue

As TBZ is a moderately polar chemical, it is likely that it has penetrated the outer skin of the tuber. However, the hydrophobic nature of the suberin, which surrounds the tuber and is an integral part of the periderm, has inhibited further movement of TBZ into the polar medullary tissue.

As a consequence, from the toxicological viewpoint, consumption of TBZ is likely to be minimal since most tubers are peeled, or at the very least washed, prior to consumption.

Sampling problems in residue analysis.

In light of the problems encountered in the interpretation of TBZ residues reported by other research workers, some guidelines were established relating to which sample should be taken for residue analysis. The confusion arises from several areas :- i) although the bulk of the

residues reported are extracted from tubers treated with TBZ at the commercial application rate, different samples are used to determine the residue, ii) the sample used for residues is very rarely stated clearly e.g. the distinction between washed and unwashed is seldom made and iii) non-uniformity of the sample taken, e.g. Caley et al (1983) determine TBZ in the peel, yet this relies on uniformity of the peeling.

In order to highlight the problems in using unwashed peel as a sample for residue analysis, the TBZ distribution experiment was repeated using the same batch of potatoes, only analysis was carried out after six months storage. The results are reported in table 2.4.

Table 2.4

Sample	TBZ residue ($mgkg^{-1}$)*	reduction of residue
Whole, unwashed	9.72 ± 1.51	-
Whole, washed	2.29 ± 0.38	76.4%
Peeled	0.30 ± 0.21	96.9%
Peel, unwashed	31.66 ± 16.86	-

* mean of five replicates

If the results shown in table 2.4 are compared with those reported in table 2.2, it is apparent that the residues in the whole, unwashed sample are not significantly different ($P > 0.5$).

However, residues in the peeled and unwashed peel

samples are vastly different.

This highlights sampling problems. As the total residue in the whole unwashed tuber has not changed significantly, the differences between the residues at the two sampling times in the peel and peeled samples is a consequence of non-uniform peeling, and not the result of a large movement of TBZ into the tuber. These observations are substantiated with the high standard deviations obtained from both sets of peeled and unwashed peel sample analyses, indicating that there is a large degree of variability in the sampling.

Further more, a residue in the peel is not of any great value, from the consumer's point of view, as the peel is rarely eaten, and it gives an unreliable value for the whole tuber.

Residues in the peeled tuber are of more value to the consumer, although the sampling problems again give a poor reflection of the actual residue present.

There are also problems associated with using the whole, unwashed tuber for residue analysis, including i) handling of tubers prior to analysis will result in the loss of some of the chemical, ii) removal or not, of soil adhering to the tubers will influence the final residue value and even if one decides to leave the soil on the tuber then accidental loss of soil will greatly affect the end result and iii) evenness of application of the chemical will influence the final residue value. Hence, a residue value in the whole, unwashed tuber is also unreliable and is not of any real value, unless it is being correlated with disease control.

In practice, the most suitable sample for residue analysis would be the whole, washed tuber, where the sample is prepared by lightly washing the tubers in cold water, and any soil is removed. This residue value could also give an indication of the amount of chemical applied initially, since washing removes approximately 87% of the TBZ residue, and therefore a conversion factor could be calculated for each cultivar to relate residues in the whole, washed tuber with the amount of TBZ applied.

Hence, whole, washed tubers provide a uniform sample from which reproducible values for residue analysis can be achieved, and further residue analysis in this thesis have been quantified using whole, washed tubers.

Thiabendazole residues throughout the storage season.

Unlike other chemicals applied to potatoes at the beginning of the storage season e.g. chlorpropham, tecnazene, 2-aminobutane, TBZ is relatively non-volatile, and therefore none of the chemical will be lost as a consequence of air re-circulation. Therefore a storage experiment was set up to investigate whether or not, as time progresses, the amount of residue in the potato increases, since there is a pool of TBZ on the tuber surface.

Potatoes (cv. Cara) were treated at the start of the storage season with the TBZ formulation, Storite, at the commercial application rate of 40 $mgkg^{-1}$. They were stored at $8^{o}C$ and sampled for residue analysis at two month intervals, using the method described previously. In order to inhibit

sprouting throughout the storage period, the tubers were treated with chlorpropham (adsorbed onto neutral grade alumina) at the rate of 20 $mgkg^{-1}$, after curing had taken place. Earlier preliminary work had shown that the presence of chlorpropham did not affect the penetration of TBZ into the tuber.

The TBZ residues present in the whole, washed tubers are reported in table 2.5.

Table 2.5

Period of storage (mth)	TBZ residue ($mgkg^{-1}$)*
0	NDR**
2	1.06 ± 0.08
4	1.36 ± 0.10
6	1.89 ± 0.03

* mean of five replicates

NDR** no detectible residue

As one would expect, at time 0, no TBZ was detected, since not enough time had elapsed in order for the chemical to penetrate through the skin.

However, as time progressed, more TBZ penetrated beyond the skin of the potato into the flesh, giving rise to an increased residue in the whole, washed tuber. TBZ analysis was also carried out in peeled tubers at the corresponding

time intervals, however no significant increase in the residue in the peeled tuber was observed. Any fluctuations were probably a result of sampling problems (as discussed earlier).

Hence these findings would suggest that as time progresses, there is an increase in the uptake of TBZ from the pool of chemical surrounding the surface of the tubers. However, the penetration of TBZ into the flesh of the tuber is in some way inhibited, perhaps by suberin. Therefore, there is an increase in the uptake of TBZ by whole tubers with time, although this increase is not evident in the peeled tubers.

In conclusion, TBZ residues tend to associate with the peel and although the residue increases over the storage season, the residue in the eating part of the potato remains fairly low.

In the next chapter, the possibility of using a seed treatment of TBZ to try to reduce the amount of chemical applied to the ware crop, while still maintaining disease control, is examined. In order to study this, a field trial, where several seed treatments were tested, was set up. The progeny tubers were then stored with various additional treatments of TBZ and disease control was then assessed.

CHAPTER 3

THE EFFECT OF THIABENDAZOLE SEED TREATMENTS ON FUNGAL DISEASE CONTROL IN THE PROGENY TUBERS.

Introduction.

With the increased concern in the usage of pesticides on food, ways have to be sought to reduce the amounts of chemicals used, without a subsequent decrease in quality.

In the case of the potato, the possibility exists of treating the seed prior to planting, in the hope that some disease resistance may be carried forward into the progeny crop.

Several papers have been published, where TBZ seed treatment has been investigated. Hide and Bell (1980) treated seed in January with TBZ and observed that healthier seed produced more and healthier stems per plant and that the incidence of skin spot and black scurf in the stored progeny tubers was decreased. Hide et al (1980) again reported a reduction in skin spot and black scurf, where the seed was treated with 320 mg TBZ kg^{-1} seed. In another experiment, where the seed was treated with 40 mg TBZ kg^{-1} seed, a significant decrease in the incidence of skin spot, silver scurf, black scurf and gangrene was observed. Leach (1985) artificially innoculated tubers with *Fusarium spp*. and noted that progeny tuber contamination was lowest from TBZ treated seed.

Hence, there would appear to be some disease resistance carryover into the progeny tubers, from TBZ treated seed.

However treatment rates, method of application, time of application and disease control all seem to vary. Therefore a field trial and storage experiment of the subsequent progeny was devised in order to try to relate a TBZ seed treatment with disease resistance in the progeny tubers.

The pathway to tuber infection.

In order to see how a seed treatment of TBZ may reduce the incidence of skin spot, silver scurf, dry rot and gangrene, in the progeny tubers, it is important to understand how the fungi contaminate tubers. Therefore, a brief pathway of tuber infection for each of the fungi that TBZ is reported to control is given below (Anon., 1984).

Polyscytalum pustulans (Skin spot). Although skin spot does not affect the eating quality of the potato, it detracts from the appearance of the tuber. It tends to infect the eyes and the areas surrounding them, and as a consequence, the eyes get damaged, which results in late emergence or crop failure.

The chitting of infected seed will improve sprouting. However, if the seed is not chitted, then skin spot will inhibit, or at best delay, emergence.

Infection begins in the seed potato, and in young plants, skin spot causes brown lesions on the shoots, roots and stolons. As the crop dies back, the fungi rapidly multiply, leaving a higher level of innoculum in the soil. Progeny tuber infection can occur through even the smallest injury to the

crop, at or shortly after lifting.

Helminthosporium solani (Silver scurf). With silver scurf, the eating quality is again, unaffected. However, the fungi does have more serious consequences with respect to the quality of tubers, than skin spot. Silver scurf infection causes increased water loss, and a corresponding decrease in weight, particularly during long term storage.

Spores develop on the seed after planting, and then infect the progeny tubers, although the exact pathway of infection is not yet fully understood. Silver scurf is present on most seed stock, but the greatest spore production develops on lightly infected seed. Hence, there is no benefits in planting lightly infected seed.

Symptoms in the ware crop are not obvious at lifting, however, silver scurf develops rapidly in warm, humid conditions, usually present at the beginning of the storage season.

Fusarium spp. (Dry rot). Dry rot inhabits arable soils, where the soil temperature is warm. Partially infected tubers will rot quickly, if planted, but all infected seed produces a weak plant, if a plant develops in the first place.

Infection occurs through minor wounds, and in a few weeks, brown spots on the tuber surface begin to develop. These will eventually develop into cavities, and they then become infected with a white and blue fungal growth. Succeptability to dry rot increases as the storage season progresses, but is at a

peak in early Spring.

Phoma exigua (Gangrene). Gangrene readily colonises on the dying haulm and therefore, any soil adhering to tubers, will be heavily contaminated with innoculum. Infection occurs when the tubers are mechanically damaged during the lifting and storage operations. Further injury and subsequent infections may occur later if the potatoes are graded.

Compared with dry rot, gangrene is less likely to cause the rapid breakdown of seed after planting, and so mildly infected seed will produce normal plants.

Experimental.

Treatments.

In order to ascertain the effects of treatment time and method of application, on disease resistance carryover into the progeny crop, Maris Piper (Grade SE3, 30 mm x 50 mm) were treated in the following ways:- i) single dose before planting, ii) double dose before planting, iii) pre-planting treatment, iv) foliar application to the growing crop and v) a control.

i) Single dose treatment. The seed was sprayed with the TBZ formulation, Storite, at the commercial application rate of 40 $mgkg^{-1}$, and placed in 10 kg cardboard boxes and stored at 8°C for seven weeks , until required for planting. This allowed the

effect that uptake of TBZ, by the tuber, had on the disease resistance of the progeny tubers, to be studied.

ii) Double dose treatment. Seed was again sprayed with Storite, but at the rate of 80 $mgkg^{-1}$, before being boxed and stored at $8^{o}C$, seven weeks prior to planting. Again the uptake effect could be studied, but more importantly, any soil adsorption of TBZ, as described by Cayley and Lord (1980), would play a lesser role. There would be a sufficient reservoir of TBZ surrounding the surface of the tuber even after soil adsorption (by the soil surrounding the tuber in the ground) had taken place.

iii) Pre-planting treatment. Seed was sprayed with Storite at the rate of 40 $mgkg^{-1}$, 24hr prior to planting. This treatment acted as a comparison for the single dose treatment, where the effects of uptake of TBZ, could be investigated.

iv) Foliar treatment. TBZ has been reported to have systemic properties (Pillay and Chatrath (1976), Hide and Cayley (1977)). A foliar application of TBZ may be of some value, as there are problems associated with the strong adsorption of TBZ by soil. Pillay and Chatrath applied 12.5 mg TBZ per plant and TBZ residues of the order of 1000 µg TBZ g^{-1} dry weight of potato were detected, therefore substantial amounts of TBZ were being translocated into the tuber.

An initial experiment was undertaken to study the effect that the way in which the TBZ was formulated, had on the growth of the plant. No commercial foliar application of TBZ was

available and it was decided that applying TBZ in the form of Storite (where the TBZ would be in the form of a suspension), would not facilitate optimum uptake of the chemical. Hence potato plants (cv. Maris Piper) were grown in pots until flowering had ceased (approximately 16 weeks) and 12.5 mg TBZ were applied in 20 cm^3 of i) methanol plus 0.1% Tween 20 (a commonly used surfactant), ii) 0.1 M HCl plus 0.1% Tween 20 and iii) 0.1% Tween 20, to note the effects that the solvent had on the leaves of the plants. After two weeks, each of the treatments of five replicate plants were investigated for leaf develoment and normal healthy growth. Plants treated with methanol had severely shrivelled leaves, whereas the plants treated with HCl or water displayed normal healthy growth.

In order to ascertain how much TBZ had been taken up by the plants, the leaves were washed with water, and the amount of TBZ present in the washings was determined by HPLC. In the methanol/ TBZ treated plants, 23% of the TBZ applied was taken up. 76% of the TBZ applied was taken up by HCl/ TBZ treated plants, whereas only 44% of the TBZ was taken up in the water/ TBZ plants. Obviously these figures were achieved under optimum growing conditions and in the field less TBZ would be expected to enter the plant. However, from these preliminary findings, it was decided to treat the plants with 12.5 mg TBZ in 0.1 M HCl, with 0.1% Tween 20 added. The plants were treated 17 weeks after planting, when flowering had ceased (this is generally thought to be when assimilate movement through the phloem down to the tubers is greatest), using a Shandon spray gun. Two dry days were observed after spraying.

v) Control. Tubers were planted out after being stored in sacks at 8°C.

Planting.

The experimental plot was situated at Arkleston Farm, near Paisley, Renfrewshire. The land had been prepared by the farmer, in accordance with local farming practices. Tubers were planted by hand at 230 mm spacing, in drills 750 mm apart.

The experimental design consisted of four blocks, where each block contained four replicate plots. Each plot consisted of one drill of each treatment, independantly randomised, with 20 tubers planted per drill.

The experimental area was protected by guard drills (cv. Desiree), and a guard drill was planted in between each plot. Two guard tubers were also planted at the end of each plot, in order to distinguish each individual plot, by colour, at harvest.

TBZ residues at planting were measured in whole unwashed tubers, as described in Chapter 2.

Husbandry.

Five weeks after planting, weeds in between the drills were sprayed with a mixture of paraquat and monolinuron (Graminol, I.C.I.). Weeds inbetween the potato plants were

removed by hand, as and when nessesary, until a canopy of foliage had developed.

Blight was controlled using captafol, in accordance with local farming practices.

The crop was left to die back naturally, prior to harvest.

Due to the inclement weather, present throughout the 1987 growing season, harvest was delayed until 3/11/87. Each drill was lifted by hand.

As a result of the short dry period at lifting, all of the mechanical graders were in use by the farmer, and hence the yield could not be graded.

Treatment and storage of the yield.

In order to assess whether or not disease resistance had been carried over into the progeny tubers, a storage experiment of the subsequent yield was devised.

The point of treating the seed with TBZ prior to planting, was to discover if it was possible to reduce the amount of chemical applied to the ware crop, without any reduction in quality. Therefore, the progeny was to be treated in one of three ways :- i) no additional application of TBZ, ii) half dose of TBZ i.e. 20 $mgkg^{-1}$ and iii) full dose of TBZ i.e. 40 $mgkg^{-1}$.

Excess soil was removed from the tubers, and very small and large tubers were discarded. The potatoes were treated with the appropriate amount of TBZ using a Shandon spray gun. The

untreated potatoes were treated with an equivalent amount of water.

For each treatment, 3 x 10 kg replicates were stored at 15°C, in cardboard boxes for the first two weeks of storage, to allow wound healing to take place. The temperature of the store was then reduced to 8°C, and the yield was assessed for disease control, at monthly intervals.

At the beginning of January 1988, the tubers were treated with chlorpropham (at the rate of 20 $mgkg^{-1}$), to control sprouting, so that disease control could still be assessed at the end of the storage season.

Disease assessment was carried out visually and recorded using a scale of 1 - 5, where 5 reflected disease throughout the tuber.

Analysis of results.

Mean emergence time. This was calculated using the following equation :-

$$MET = \frac{\Sigma \text{ interval emergence x days after planting}}{\text{number of tubers planted/replicate drill}}$$

where interval emergence = number of plants which had emerged since the last count.

Total emergence time. This was calculated as the time

when each of the 20 tubers planted, had emerged.

Emergence and yield data. The data was examined for treatment differences, as well as block effects. Significant differences between these parameter's means, were tested using analysis of variance. Comparisons between treatment means were made using Tukey's Honestly Significance test for comparing means (Dowdy and Wearden, 1983). The least significant differences (L.S.D.) between the treatment means were calculated as follows :-

$$L.S.D = q_{\alpha,\ a,\ a(n-1)} \times \frac{MSe}{n}$$

where q_{α} = critical values for the studentised range at a and a(n-1) degrees of freedom

MSe = mean squares on the error

a = number of treatments

n = number of replicates

Results and discussion.

Residues at planting.

In order to check that the correct amounts of TBZ had been applied to the seed, and to try to relate this to any significant emergence or yield data which may come out of the

field experiment, residues at planting were measured. In contrast to the residues reported in Chapter 2, residues at planting were measured in whole unwashed tubers. This was done for two reasons :- i) it is important to know the amount of TBZ which coats the surface of the tuber, as it is this with which the soil fungi come in direct contact with and ii) if the seed was washed prior to residue analysis, very little TBZ would be detected in the pre-planting treatment and would therefore bear little resemblence to the amount of TBZ applied to the tubers before planting.

The residues extracted from the seed, determined using the method described in Chapter 2, are reported in table 3.1.

Table 3.1

Sample	TBZ residue* ($mgkg^{-1}$)
Single dose treatment	13.78 ± 1.62
Double dose treatment	44.65 ± 1.85
Pre-planting treatment	16.25 ± 2.41
Foliar treatment	NDR**
Control	NDR

* mean of five replicates

NDR** no detectible residue

It is worth noting that the residues reported in the single dose treatment are higher than those for ware tubers,

reported in table 2.3. This can be explained in terms of the larger surface area : volume ratio in seed, compared with ware tubers, i.e. the same amount of TBZ will coat a larger surface area per kg of seed potatoes compared with ware potatoes, and hence the higher residue value for seed tubers.

Emergence.

The mean emergence times (MET) and the total emergence times (TET) are reported in table 3.2.

Table 3.2

Treatment	MET (days)	TET (days)
Single dose treatment	24.2	32.4
Double dose treatment	24.8	33.7
Pre-planting treatment	26.2	35.4
Foliar treatment	25.7	35.1
Control	25.9	34.8

The emergence profiles for each treatment, plotted against the control are shown in Figs. 3.1 to 3.4.

In order to establish whether there were any block effects, one way analysis of variance on the emergence data was carried out. No block effects were observed with respect to MET

Fig. 3.1 Emergence profile of single dose and control

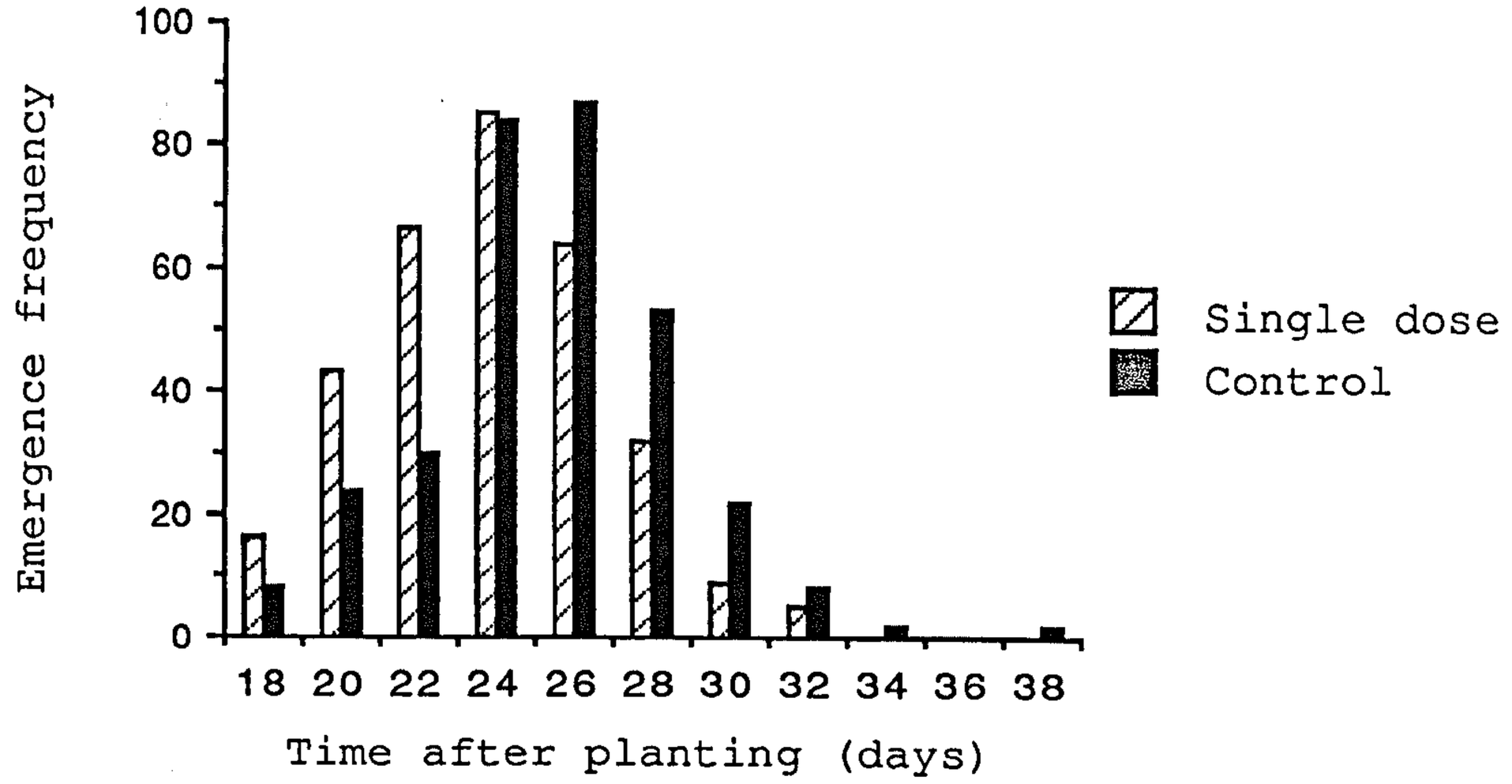


Fig 3.2 Emergence profile of double dose and control

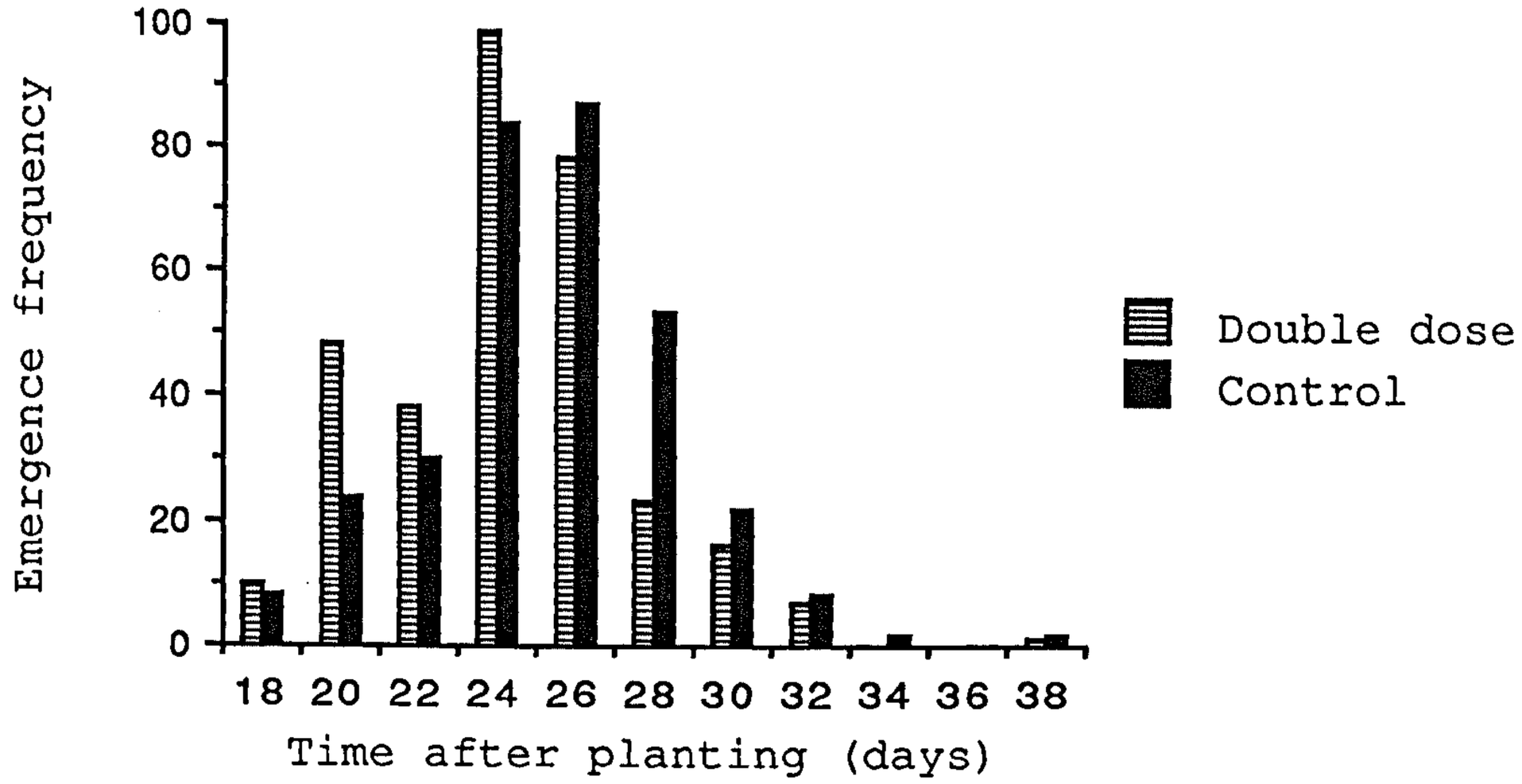


Fig. 3.3 Emergence profile of pre plant and control

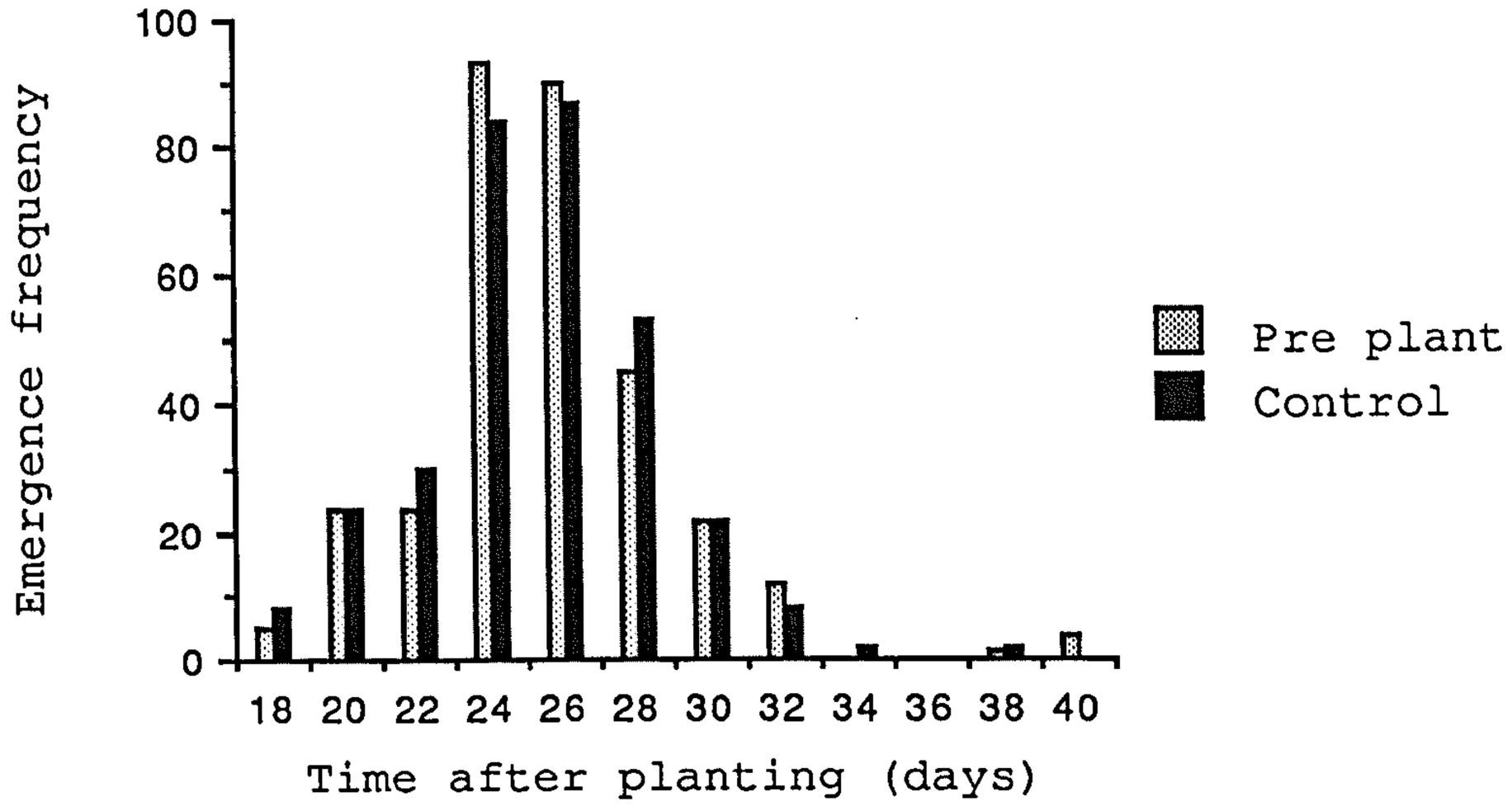
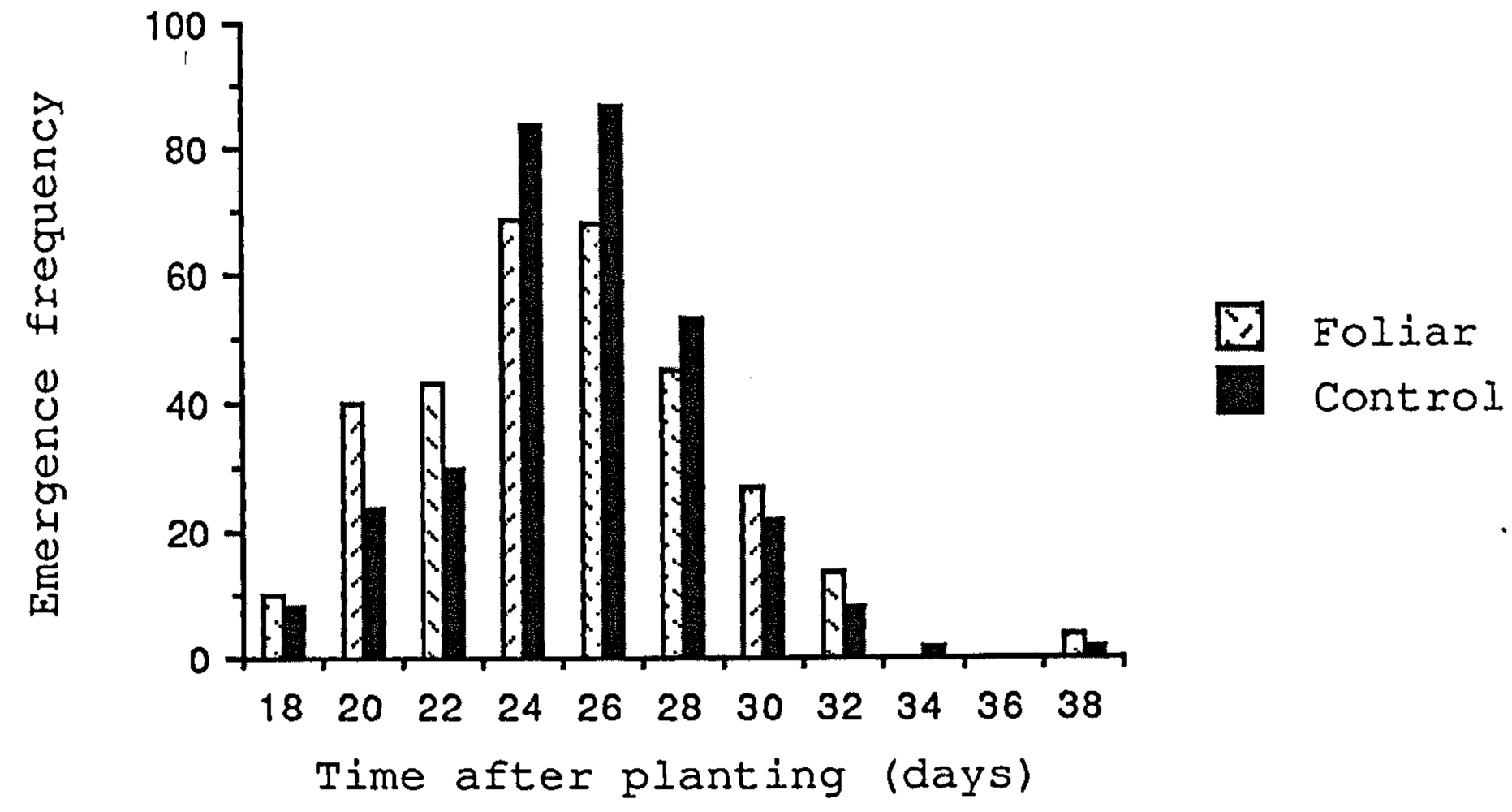


Fig.3.4 Emergence profile of foliar treatment and control



and TET.

By looking at the data and emergence profiles it would appear that the single and double dose treatments resulted in lowering the MET and TET, compared with other treatments. One way analysis of variance was carried out in order to establish whether the difference was significant or not. The F statistic for MET implied that there was a significant difference, and Tukey's Honestly Significance test was applied to calculate the L.S.D.. The L.S.D. was calculated as 1.1 days and both the single and double dose treatments were significantly different from the control at the 5% level.

One way analysis of variance was also carried out on the TET data, however the F statistic implied that any difference present was not significant.

The differences in the MET for single and double dose treatments are not however likely to be a consequence of the chemical treatments, but more likely to have arisen from the storage conditions prior to planting. The single and double dose seed was treated with TBZ seven weeks before planting. It was then stored in 10 kg cardboard boxes at $8^{o}C$ until planting. By contrast, seed for the other treatments was stored in the origi nal paper sacks. It would appear that the weight of the tubers stacked on top of each other, to a depth of approximately 60 cm, was enough to delay chitting, compared with the boxes (depth = 20 cm), where the seed chitted normally. As a result the treated seed was at a more advanced stage than the rest of the seed and this is reflected in the MET, and to a lesser non-significant extent in the TET.

Hence, these sets of data show the importance of looking at both MET and TET. If MET had just been considered, then one may have concluded that the chemical treatments did have a significant effect on the emergence of the crop. However, by also considering TET, one could establish that TET was not affected by the treatments and hence, something else must have been responsible for the earlier MET. Looking at TET alone can also give a false impression, as it just takes a few tubers to be late in emerging, to give a much exaggerated TET. Therefore, in the analysis of emergence data, MET and TET should always be considered together.

Yield.

The total yield obtained from each treatment is reported in table 3.3, along with the average yield per drill and MET.

Analysis of variance was carried out using the data for yield per drill, however, none of the treatments were found to give significantly more or less yield per drill, compared with the control.

In conclusion, although MET was earlier in the single and double dose treated seed, it did not follow that the yield was increased in any way. It is worth noting that in a field trial one must ensure that as many factors as possible are kept equal, and that as many different types of data are recorded e.g. MET, TET, stem numbers (if appropriate), yield, etc., since

Table 3.3

Treatment	MET (days)	Ave. yield drill^{-1}(kg)	Total yield (kg)
Single dose	24.2	21.6 ± 4.3	346
Double dose	24.8	23.5 ± 3.6	376
Pre-planting	26.2	24.4 ± 2.5	391
Foliar	25.7	23.1 ± 3.9	370
Control	25.9	20.6 ± 4.1	330

it has become obvious how a factor such as boxing could influence the interpretation of the results i.e. if MET had just been recorded then the results would imply that single and double dose treatments of TBZ, applied to seed would reduce the MET, and an earlier MET is usually associated with a greater yield.

Storage of the progeny tubers.

In order to see whether any TBZ had been translocated from the seed to the progeny tubers, residue analysis was carried out, using the method described in Chapter 2, with a sample of progeny tubers from each treatment. The results obtained are reported in table 3.4.

Table 3.4

Treatment	TBZ residue* ($mgkg^{-1}$)
Single dose treatment	NDR**
Double dose treatment	0.01
Pre-planting treatment	0.01
Foliar treatment	0.07
Control	NDR

* mean of five replicates

NDR** no detectible residue

From the results it is evident that very little TBZ has been translocated into the progeny tubers. It was interesting to note that the highest residues were found in the progeny of the foliarly treated plants (0.07 $mgkg^{-1}$). Considering the continual unfavourable weather conditions (low temperatures and high rainfall), this would imply that there may be cause for investigating the foliar application of TBZ, further. Obviously one would have to first study disease control and see whether a foliar application of TBZ could control disease in the progeny tubers.

Another problem associated with the foliar application of a chemical is that it is distributed throughout the tuber, e.g. maleic hydrazide is a sprout suppressant which is applied foliarly. McKenzie et al (1987) compared residues in whole tubers with peeled tubers, in potatoes which had been treated

with maleic hydrazide (a foliarly applied sprout suppressant) and chlorpropham (a sprout suppressant which is applied to potatoes in the store by forcing a fog through the ventilation ducts). They reported that peeling maleic hydrazide treated tubers only resulted in a small reduction of the residue, compared with peeling chlorpropham treated tubers.

The purpose of this work was to try to reduce the residues present in the ware crop by treating the seed. However if the residue was to be carried into the ware crop and it could not be reduced with peeling to any extent, then the value of a foliar application must be reconsidered. If the foliar application of TBZ was to be studied further, then the following points should be taken into consideration :- i) can a formulation be developed which will overcome the inclement weather, usually found in this country, ii) will the residues found in the progeny crop actually be reduced compared with treating the ware after harvest, iii) is adequate disease control achieved and iv) what levels of TBZ must get into the tuber in order to provide that disease control?

However, as far as total residues in each of the washed progeny samples is concerned, very little TBZ has been translocated from the seed into the progeny. Hence if disease control is better in the progeny then it has probably arisen from planting healthier seed in the first place. Hide et al (1980), report significantly decreased skin spot, black scurf, silver scurf and gangrene, in progeny tubers which had been grown from TBZ treated seed.

Therefore, in the next section of work, the quality of

the stored progeny in terms of disease, is assessed so that conclusions can be drawn regarding the effect of seed treatment on the storage quality of the progeny.

Quality of the stored progeny.

After the yield had been recorded, sub-samples of progeny from each of the seed treatments were treated at the rate of 40 $mgkg^{-1}$, 20 $mgkg^{-1}$ or with no additional treatment of TBZ. 3 x 10 kg replicates for each treatment were stored at $8^{o}C$ for several months, so that at monthly intervals, each treatment could be visually assessed for disease. On 15/12/87, each replicate was treated with chlorpropham at the rate of 20 $mgkg^{-1}$, to inhibit sprouting. As chlorpropham is a mitotic poison, it will have some fungicidal activity (Ritchie, 1986), but its effects should be ubiquitous since it is applied to eacl of the replicates. In any case, if a sprout suppressant were not applied, then as the tubers sprouted and water loss increased as a result, then disease assessment would prove to be very difficult.

Disease assessment was carried out visually, based on a scale of 1 to 5, where

1 indicated no disease

2 indicated up to 10% of the tuber was diseased

3 indicated up to 25% of the tuber was diseased

4 indicated up to 50% of the tuber was diseased

5 indicated over 50% of the tuber was diseased.

The number of tubers infected, as well as the type of infection present was also recorded.

However, after carrying out disease assessments for four months, very little disease was detected in any of the treatments or controls. Unfortunately for the purposes of this field trial and storage experiment, the potatoes carried minimal infection, and so no conclusions could be drawn regarding disease resistance carryover from treated seed into the progeny crop.

Maris Piper was specifically chosen for this experiment as it is very popular with Scottish farmers (4201 ha of Maris Piper were planted in the 1987 growing season). It was also chosen because, apart from having high resistance to gangrene, it did not posess any natural immunity to any other diseases which TBZ is reputed to control (Anon.,1984). Hence the apparant lack of disease in the progeny must have resulted from i) very high quality seed and ii) low innoculum levels in the soil, and not through resistance bred into the cultivar.

As a result of lack of disease, it was decided to artificially innoculate the tubers with a mixed innoculum of *Phoma exigua*, *Helminthosporium solani*, *Fusarium spp.* and *Polyscytalum pustulans*. The innoculum was obtained by taking swobs of fungi, which were growing on other potaoes (in a different experiment). The fungi were cultured onto potato dextrose agar, and incubated at 15^{o}C. This temperature was not the optimum for the growth of the fungi. However if they were incubated at their optimum temperature of 21^{o}C, then when the

tubers were innoculated with the fungi, the lower temperature of the store i.e. 8^{o}C, would almost certainly affect the growth of the fungi.

After one month (10/4/88), there was sufficent innoculum present to innoculate one replicate from each treatment. The innoculum was divided into 15 batches, and each aliquot made up to 25 cm^{3} with water, before being sprayed onto the tubers (using a hand sprayer).

After three weeks, the tubers were assessed for disease as before. The results are shown on table 3.5, over.

From the results it would appear that there was little pattern to the disease control. The storage treatment of 40 mgkg^{-1} provided the best control of the fungi, as one would expect.

The storage treatment of 20 mgkg^{-1} provided adequate control too, although more tubers were infected than in the 40 mgkg^{-1} treatment, and more of these tubers were infected to a greater extent. This can probably be explained in terms of inadequate cover of some of the tubers with TBZ, and hence the poorer control.

The most diseased tubers were those which received no additional storage treatment of TBZ. The range of infection in these tubers was the greatest too. Additional diseases to those present in the innoculum were also identified. It may be that administering the innoculum resulted in a reduction of the tuber's natural resistance and this allowed secondary infection to occur, with fungi and bacteria present on the soil on the tuber surface.

Table 3.5

Seed treatment	Storage treatment	Range of disease infection	Ave. assessment for the treatment
Single	0 $mgkg^{-1}$	1 - 2	2
	20 $mgkg^{-1}$	1 - 4	2
	40 $mgkg^{-1}$	1 - 2	2
Double	0 $mgkg^{-1}$	1 - 3	3
	20 $mgkg^{-1}$	1 - 3	3
	40 $mgkg^{-1}$	1 - 2	3
Pre-plant	0 $mgkg^{-1}$	1 - 4	2
	20 $mgkg^{-1}$	1 - 5	3
	40 $mgkg^{-1}$	1 - 2	2
Foliar	0 $mgkg^{-1}$	1 - 2	2
	20 $mgkg^{-1}$	1 - 4	2
	40 $mgkg^{-1}$	1 - 3	1
Control	0 $mgkg^{-1}$	1 - 4	3
	20 $mgkg^{-1}$	1 - 3	3
	40 $mgkg^{-1}$	1 - 2	1

The types of fungi found on the tubers were also recorded, in order to discover if any of them were more or less succeptable to the effects of TBZ. The results are reported in table 3.6. The key used, is given below.

Key.

F. spp. - *Fusarium solani*

H. solani - *Helminthosporium solani*

P. exigua - *Phoma exigua*

P. pustulans - *Polyscytalum pustulans*

R. solani - *Rhizoctonia solani*

S. scabies - *Streptomyces scabies*

Table 3.6

Seed treatment	Storage treatment	Fungal infection present
Single	0 $mgkg^{-1}$	*P. exigua, F. spp., P. pustulans*
	20 $mgkg^{-1}$	*P. exigua, H. solani*
	40 $mgkg^{-1}$	*P. exigua*
Double	0 $mgkg^{-1}$	*P. exigua, P. pustulans*
	20 $mgkg^{-1}$	*P. exigua, H. solani*
	40 $mgkg^{-1}$	*P. exigua*
Pre-plant	0 $mgkg^{-1}$	*P. exigua, F. spp.*
	20 $mgkg^{-1}$	*P. exigua, F. spp., H. solani*
	40 $mgkg^{-1}$	*P. exigua*
Foliar	0 $mgkg^{-1}$	*P. exigua, P. pustulans*
	20 $mgkg^{-1}$	*P. exigua, F. spp.*
	40 $mgkg^{-1}$	*P. exigua*
Control	0 $mgkg^{-1}$	*P. exigua, F. spp., R. solani, S. scabies*
	20 $mgkg^{-1}$	*P. exigua, F. spp.*
	40 $mgkg^{-1}$	*P. exigua*

From these results, *P. exigua* was present on all of the tubers to greater and lesser extents. Hence it would appear that TBZ is not entirely successful in the control of *P. exigua*.

Fusarium spp. was the second most commonly found fungi, although it only covered a small area of the tuber.

Tubers which were infected with *H. solani* were found to have a greater coverage of the tuber surface, and a greater predominance of that fungi. Therefore it may be that i) the fungi may grow quickly and cover the tuber surface to a greater extent or ii) play some sort of negative feedback role in the growth of other fungi.

It was interesting to note that in each of the storage treatments of 40 $mgkg^{-1}$, *P. exigua* was the only fungus present on the potatoes. In addition, the extent of the fungal infection was minimal, in general, less than 10% of the tuber surface was infected.

In conclusion, the effects of a seed treatment of TBZ on the quality of the stored tubers appears to have little effect. Obviously this type of work would have to be repeated, since the chances of growing as healthy a crop as was grown in this experiment, are fairly low. From the innoculation studies, again no clear pattern was obtained except that a reduction in the natural resistance of the tuber, caused by fungi present in the innoculum, may in fact lead to the development of other diseases, in the form of secondary infection.

Another point worth noting is that *P. exigua* may develop resistance to TBZ relatively easily. This is discussed

in Chapter 6, in more detail.

Finally, very small amounts of TBZ were translocated from the seed into the progeny. Therefore disease resistance may have resulted by planting healthier seed, rather than by translocation of TBZ in the first place.

In the next chapter, the effects that TBZ and its formulations have on the wound healing capacity are assessed. Wound healing must be allowed to take place after harvest and some chemicals such as chlorpropham inhibit this process. The effects of TBZ have as yet, not been fully elucidated, therefore a study was undertaken to monitor the effects of TBZ on wound healing.

CHAPTER 4

THE EFFECT OF THIABENDAZOLE ON THE WOUND HEALING CAPACITY OF TUBERS.

Introduction.

The harvesting of the potato crop results in a proportion of the crop being damaged, as a result of stone separation, grading and store loading. After damage has occurred, the process of wound healing must be allowed to take place, and in commercial stores it is normal to have a curing period of two to three weeks, where the store temperature is held at 12-15^{o}C, immediately after store loading, to allow skin set and wound healing to take place.

Wound healing can be followed in a number of ways, depending on which of the many components of the wound healing process, one considers to be important. Wound healing has been investigated from several view points including i) headspace analysis of potato volatiles (Waterer and Pritchard, 1985), ii) lipid peroxidation, measured in terms of ethane production (Konze and Elstner, 1978), iii) respiration e.g. carbon dioxide production (Theologis and Laties, 1981), iv) electrolyte release from cells (Lojkowska and Lewosz, 1984), v) RNA, DNA and protein synthesis (Sato et al (1978), Watanabe and Imaseki (1977), Lyubimova et al (1987)), vi) development of a suberised periderm studied in terms of a) disease penetration into the tuber (Hide and Cayley, 1985) and b) the development of resistance to water loss (Jarvis and Duncan, 1979).

Of the aforementioned methods, studying the development of a suberised periderm is probably of most value, as its development indirectly influences the other processes associated with wound healing, yet it is easier to follow than e.g. RNA synthesis.

Methods of studying suberised periderm development.

In the past, the most common method of following the development of resistance to water loss, was histologically (Nielsen, 1973). However this technique involved sectioning and staining, followed by a subjective visual assessment of the degree of staining. As a technique, histological assessment is very laborious and as a consequence, the necessary degree of replication cannot be achieved.

It has been shown that a relationship exists between the development of resistance to water loss from a wounded potato and suberisation and periderm development (Kolattukudy and Dean, 1974). Resistance to water loss can be divided into two components :- i) external resistance to water loss is described as the rate at which water vapour is removed from the surface of the potato, and is influenced by environmental factors such as temperature, relative humidity and the rate of air circulation and ii) internal resistance to water loss which is controlled by the development of a suberised periderm, which influences the free diffusion of water to the tuber surface.

The development of a suberised periderm occurs in three stages where cells at the wound surface suberise, giving rise to a small increase in resistance to water loss, followed by

cell division below the suberised layer, giving rise to a larger increase in resistance to water loss, followed by suberisation of this newly divided layer of cells, providing the largest increase in resistance to water loss, and the basis of a new periderm. This is shown pictorially on Dia. 4.1 (McGee, 1984).

By using the technique described by Jarvis and Duncan (1979) and McGee (1984), it was possible to eliminate the external resistance component, and hence factors which affected the development of internal resistance to water loss i.e. wound healing, could then be studied.

<u>The effect of Thiabendazole on the wound healing capacity of potatoes.</u>

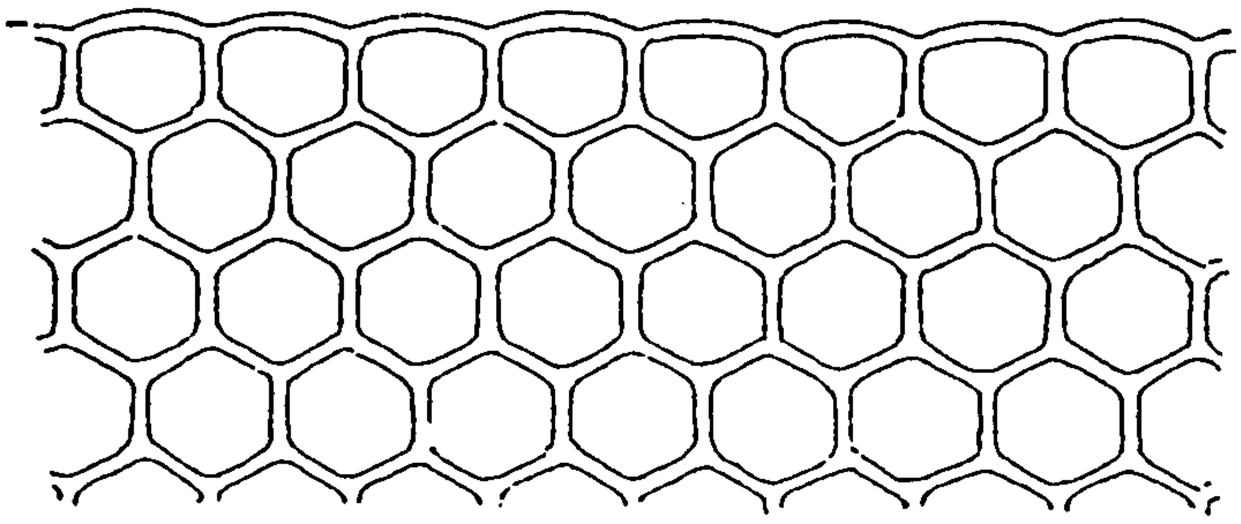
A few studies have been undertaken to monitor the effects of TBZ on aspects of the wound healing process. Elstner et al (1981) reported that treatment of potato slices with TBZ led to enhanced carbon dioxide evolution and increased lipid peroxidation. Hide and Cayley (1985) reported that disease penetration of fusarium dry rot was generally reduced with TBZ, when applied as soon as possible after wounding.

However, very little has been reported relating to the effects of TBZ on the development of resistance to water loss, and it is this component of the wound healing process which directly affects the storage quality of the tubers.

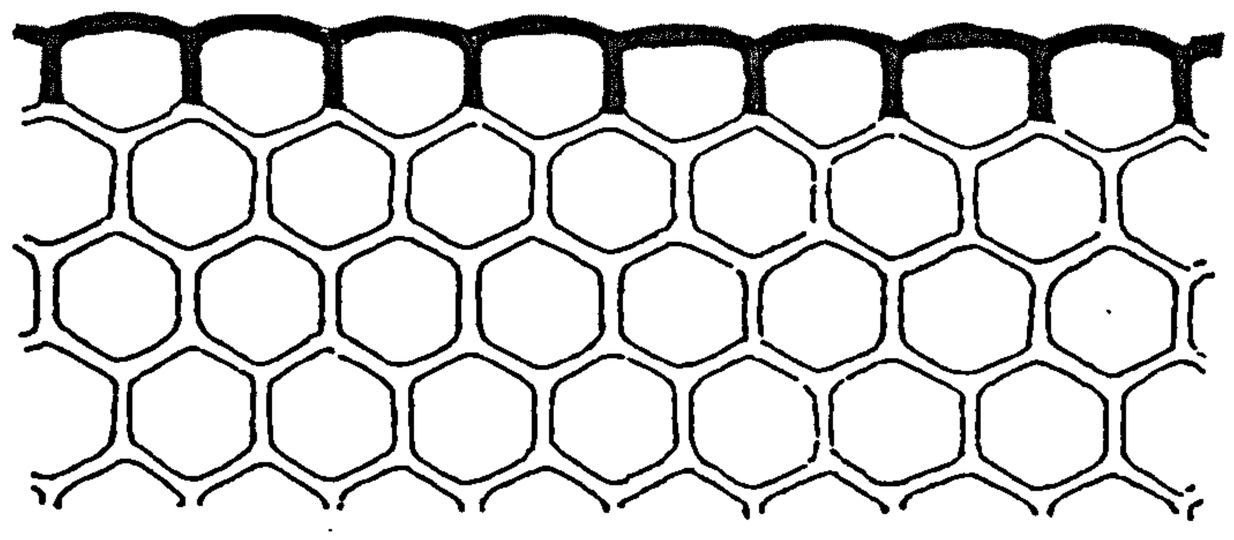
By following the development of resistance to water loss, it was possible to monitor the effects that various concentrations of TBZ had on the wound healing process. In

Dia 4.1. Schematic representation of the wound healing process in potatoes.

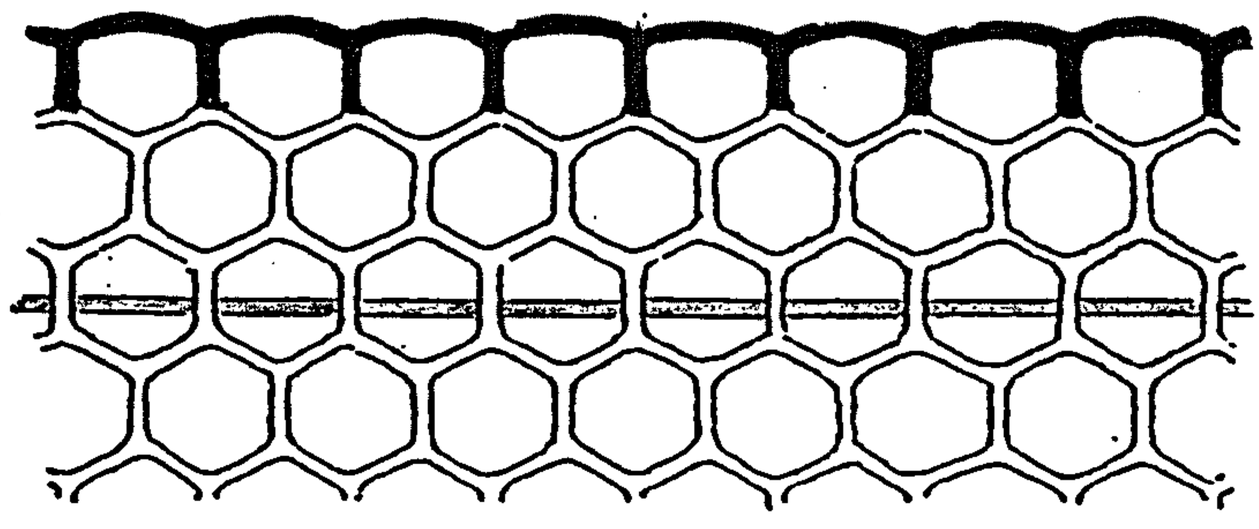
Freshly cut wound surface - minimal internal resistance to water loss.



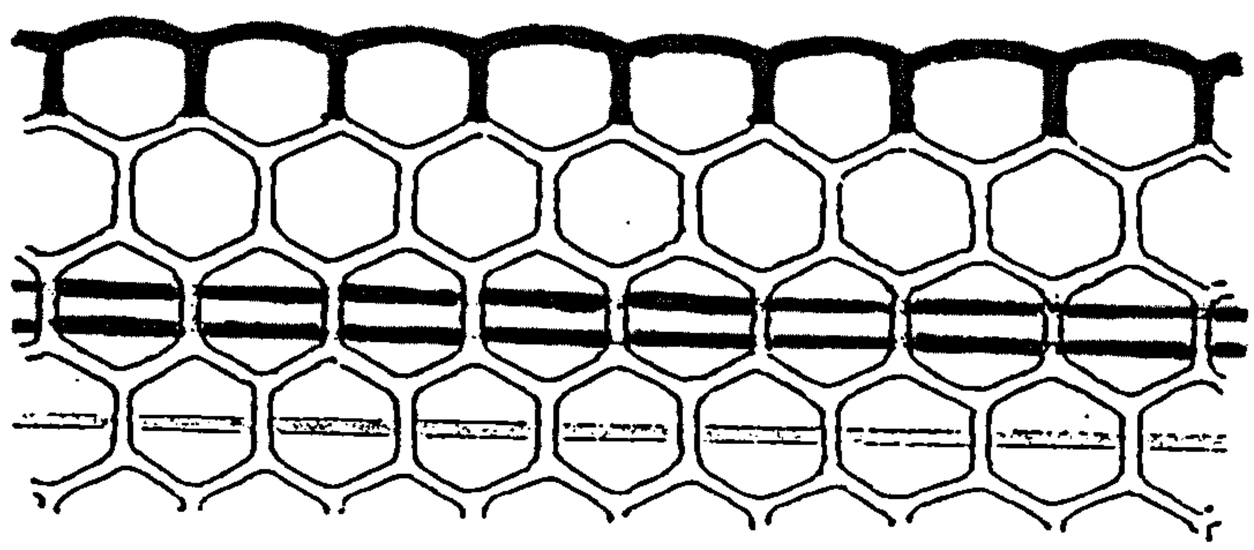
Cells at wound surface suberise - slight increase in internal resistance.



Cells below surface start to divide to form a periderm - beginning of a large increase in internal resistance.



Cells below surface divide and suberise - a large increase in internal resistance.



addition, different formulations of TBZ could be studied for their effect on the development of resistance to water loss.

Hence, this chapter attempts to demonstrate and explain the effects that TBZ has on the development of resistance to water loss.

The effects of low levels of Thiabendazole on the development of resistance to water loss.

A series of experiments were set up to follow the effects of TBZ on the development of resistance to water loss. In each of these experiments, the method described by McGee (1984), with some modifications, was used in order to allow for i) an adequate level of replication and ii) a range of treatment levels and formulations to be investigated, where histological assessment would have proved too laborious, as well as being subjective.

In general, the method entails applying various concentrations of TBZ to cut potato discs, and monitoring water loss (by weight) over a period of time. The water loss values can then be expressed in terms of resistance to water loss.

In the first series of experiments, two levels of TBZ were investigated :- 0.05µg TBZ applied per disc and 5µg TBZ applied per disc. The precise details of the method used are described over.

Preparation of the material for the assessment of the development of resistance to water loss.

Tubers (cv. Record), harvested in October 1985, were stored at 8°C until required for wound healing studies. The tubers were then washed and left to acclimatise at room temperature (21°C) overnight.

They were then surface sterilised by flaming in ethanol three times. Cores of potato were taken using a No. 7 cork borer and from these cores, discs of medullary tissue, 11 mm x 4 mm, were prepared aseptically.

To the surface of each disc, 25 mm^3 of sterile water were applied as a descrete droplet, and onto this droplet, 5 mm^3 of the TBZ solution (made up in methanol) were applied. The purpose of the water was to facilitate the even distribution of TBZ throughout the surface area of the disc.

Six discs were incubated at 21°C ± 2°C in inverted petri dishes, which contained water agar in the base of each dish, in order to maintain 100% relative humidity. Four replicate plates were prepared for each treatment rate, on each of the eight analysis days.

Measurement of the development of resistance to water loss.

The development of resistance to water loss was calculated by measuring the weight loss of the discs, when placed under an air stream for a certain period of time. The precise details are described over.

On the day of analysis, the discs were transferred to a

clean, dry petri dish and were weighed. They were then placed under a cool air stream (provided from an industrial hair drier) for 90 s. The purpose of the air stream was to minimise factors which influence external resistance to water loss, thereby allowing the development of internal resistance to water loss to be studied.

In this first period, water loss is non-linear, and therefore, water loss during this period was discounted. After 90 s, the dish plus discs was reweighed before being subjected to a further three, 20 s periods under the air stream. After each 20 s period, water loss was recorded.

Also, on each day of analysis, water loss was recorded from freshly prepared material (with no additional treatment). This was so that compensations for changes in humidity and temperature could be made, as well as providing a measure for external resistance to water loss.

Internal resistance to water loss could then be calculated using the following equations, although in practice, a computer programme was written to handle the large numbers of data generated.

$$r_{ext} = \frac{d\ (\ 1 - r.h.\ )}{E_o} \qquad \text{and} \qquad r_{int} = \frac{d\ (\ 1 - r.h.\ )}{E - r_{ext}}$$

where

E = rate of water loss per unit area of aged discs

E_o = rate of water loss per unit area of fresh discs

d = saturation vapour density of water vapour in air at the air stream temperature

r.h. = relative humidity in the air stream

Table 4.1, shows the results obtained for total resistance to water loss in sets of discs treated with 0µg, 0.05µg and 5µg TBZ, over a period of 21 days.

Table 4.1

Days after treatment	Total resistance to water loss ($mg^{-1}cm^{-2}s$)		
	0µg $disc^{-1}$	0.05µg $disc^{-1}$	5µg $disc^{-1}$
0	0.22 ± 0.02	0.19 ± 0.02	0.22 ± 0.02
3	0.24 ± 0.16	0.26 ± 0.02	0.24 ± 0.02
6	0.30 ± 0.04	0.34 ± 0.10	0.31 ± 0.06
9	0.40 ± 0.04	0.75 ± 0.09	0.54 ± 0.22
12	0.33 ± 0.03	0.55 ± 0.10	0.45 ± 0.08
15	0.35 ± 0.09	0.40 ± 0.02	0.34 ± 0.03
18	0.33 ± 0.02	0.35 ± 0.10	0.35 ± 0.07
21	0.36 ± 0.06	0.37 ± 0.10	0.36 ± 0.04

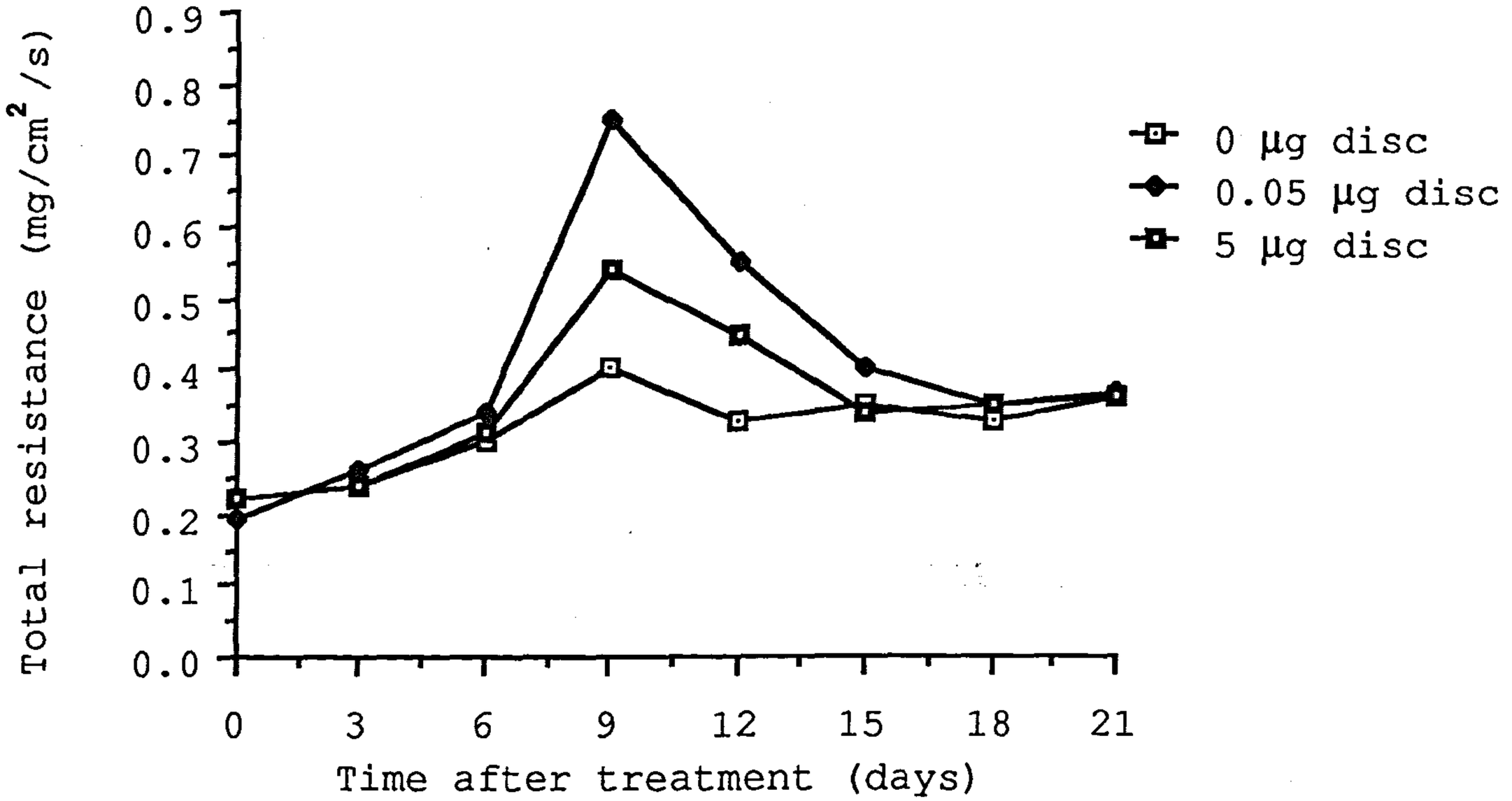
Expressing internal resistance this way has a major drawback in that the equation used to calculate internal resistance uses the function, external resistance and it assumes that external resistance remains constant in a potato disc, as it ages. However, as time progresses, external resistance to water loss decreases and internal resistance to water loss increases i.e. external resistance to water loss is not a constant. Hence, by using external resistance to water loss in the calculation of internal resistance, an error is included.

In order to overcome this problem, total resistance to water loss shall be reported and discussed. Since the development of external resistance to water loss is affected by environmental factors, which in these experiments have been maintained as constant as possible, then any differences in the total resistance figures will be a consequence of changes in the development of internal resistance to water loss.

Fig. 4.1 shows the results obtained for the total resistance to water loss in discs treated with 0µg, 0.05µg and 5µg TBZ per disc, in the form of a graph.

It is apparent from the results that TBZ would appear to promote the development of resistance to water loss to a greater extent than the control. Analysis of variance was carried out to prove that the difference was significant, and using the formula over, the Scheffe method for comparing means was used to calculate the least significant difference (L.S.D.).

Fig. 4.1 Effect of low levels of TBZ on wound healing



$$L.S.D. = (a - 1)\, F_{\alpha,\, a-1,\, a(n-1)} \times \frac{M\,S_e}{n_1} + \frac{M\,S_e}{n_2}$$

where a = number of treatments

n = number of replicates

F = F statistic

α = level of significance

$M\,S_e$ = mean squares on the error

The values obtained for the development of resistance to water loss in TBZ treated discs at days 6 and 9, were significantly different from control discs ($P > 0.05$), and hence TBZ would appear to be beneficial to the wound healing process at this time. This time interval tends to be associated with steps two and three of the wound healing process i.e. cell division and suberisation of the previously divided layer of cells. Therefore, in some way, TBZ promotes these steps. After day 9, however, resistance to water loss then falls to the same level as in the control discs. As yet, this cannot be fully explained. One possibility could be related to the discs themselves. As time progresses, the discs become dried out in appearance, and as the relative humidity within the petri dish is high (nominally 100%), then diffusion of moisture into the discs may result. This water would readily be lost in the determination of resistance to water loss, and so give exaggerated water loss values. Since water loss is inversely proportional to resistance to water loss, then a low resistance value would be recorded, and so this may account for the fall in resistance to water loss after day 9. It is worth noting

that after resistance maxima were reached in both control and TBZ treated discs, resistance always tailed off. Obviously this area requires further investigation.

In conclusion, low levels of TBZ would appear to promote the process of wound healing. In the next set of experiments, commercial levels of TBZ are studied in order to monitor their effects on the development of resistance to water loss. On a surface area basis, an application of 0.05µg TBZ and 5µg TBZ per disc corresponded to 6.25×10^{-4} and 6.25×10^{-2} times the commercial application rate, respectively. A commercial application of 40 $mgkg^{-1}$ potatoes would be equivalent to applying 80µg TBZ per disc, and hence the commercial application rate was investigated.

<u>The effects of commercial levels of Thiabendazole on the development of resistance to water loss.</u>

In this series of experiments, an amount of TBZ equivalent to the commercial application rate (40 $mgkg^{-1}$), on a surface area basis, was applied to each disc. The experimental procedure was the same as before, where the development of resistance to water loss was measured in discs treated with 80µg TBZ (equivalent to the commercial application rate) and 0µg TBZ (acting as a control).

Table 4.2 shows the results obtained for total resistance to water loss.

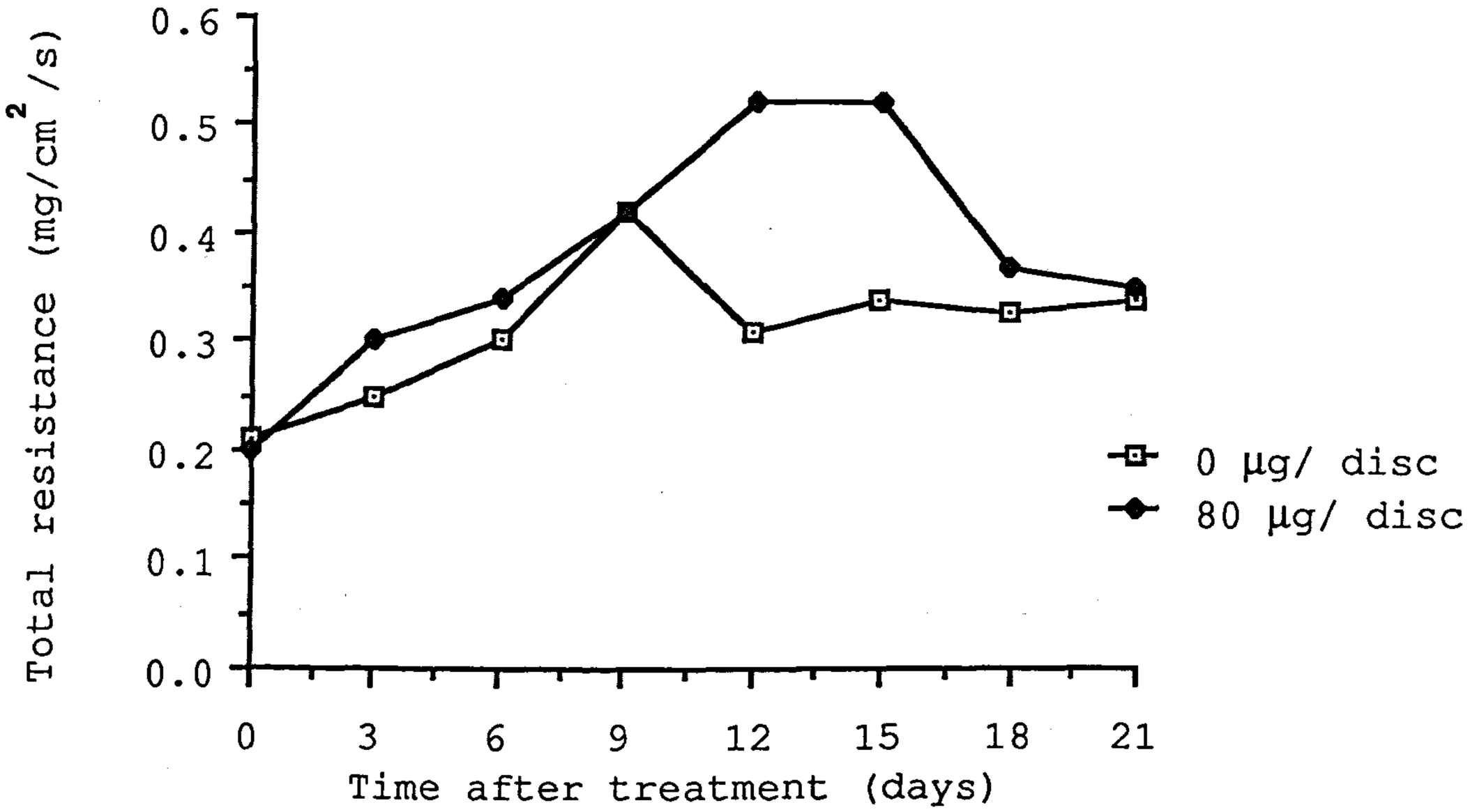
Table 4.2

Days after treatment	Total resistance to water loss ($mgcm^{-2}s^{-1}$) 0µg TBZ	80µg TBZ
0	0.21 ± 0.02	0.20 ± 0.01
3	0.25 ± 0.08	0.30 ± 0.05
6	0.30 ± 0.03	0.34 ± 0.01
9	0.42 ± 0.05	0.42 ± 0.10
12	0.31 ± 0.06	0.52 ± 0.11
15	0.34 ± 0.04	0.52 ± 0.08
18	0.33 ± 0.06	0.37 ± 0.14
21	0.34 ± 0.01	0.35 ± 0.06

These data are also shown in a graphical form on Fig.4.2.

From these results, TBZ again significantly promoted the development of resistance to water loss, but only at days 12 and 15. The resistance maxima was also lower, compared with the maxima obtained when lower levels of TBZ were tested, and in addition, resistance maxima was delayed i.e. maximal resistance was observed at day 9 in discs treated with 0.05µg TBZ and 5µg TBZ compared with day 15 in 80µg TBZ treated discs. Therefore in the event of an application of TBZ higher than recommended, a delay in the development of resistance to water loss may result, and this may be sufficient to allow fungal and bacterial rotting to take place.

. 4.2 Effect of commercial levels of TBZ on wound healing



In the next section of work, the effect that different formulations of TBZ have on the wound healing process is assessed.

The effect of different formulations of Thiabendazole on the wound healing capacity of potatoes.

Many different formulations of the same chemical are available on the market. In general, the mode of application of the chemical determines the formulation used e.g. dusts, sprays, fogs, granules, although the colour of the formulation is an additional criterion being considered, where the same formulation is being marketed in a different colour, more and more.

TBZ is usually applied to potatoes as a very fine spray at store loading, where complete cover of the tuber is essential, so that disease control can be achieved.

Formulations of TBZ are marketed in a white emulsion e.g. Storite, a brown emulsion e.g. Storite SS (contains TBZ + tecnazene), a clear liquid e.g. Storite Clear.

It was decided to follow the effects that the components of the formulation had on the wound healing capacity of tubers. In these sets of experiments, only liquid formulations were considered, because of their ease of application to the discs.

The effect of Storite on the wound healing capacity of tubers.

Storite is the most frequently used formulation of TBZ.

It is a white emulsion applied to tubers as they are loaded into the store.

In order to ascertain the effects that the components of the formulation Storite, had on wound healing, it was nessesary to compare an equivalent amount of pure TBZ with an equivalent amount of TBZ in the formulation. As TBZ is fairly soluble in methanol, a standard solution of TBZ in methanol was prepared so that 20µg of TBZ could be applied per disc. This experiment posed two major disadvantages when comparing a standard with the formulation in that i) the standard was made up in methanol, and the methanol itself may affect the wound healing process itself, as suggested by Wilson et al (1987), who studied the effects of sprout suppressants and methanol on the weight loss from whole tubers, therefore, a control including methanol was also included and ii) in methanol, at that concentration i.e.4000 ppm, TBZ is in the form of a true solution, whereas TBZ in the form of Storite (diluted accordingly with water) formed a suspension. Hence a direct comparison between the effect that the formulation components may have on wound healing cannot be made, but an indication may be obtained.

The development of resistance to water loss was measured in discs (cv. Record) treated with 20µg TBZ in methanol, 20µg TBZ in Storite, 0µg TBZ in methanol and a control (water, as before). The results for the development of resistance to water loss are shown in table 4.3.

Table 4.3

Days after treatment	Total resistance to water loss ($mgcm^{-2}s^{-1}$)			
	Control	0µg TBZ in MeOH	TBZ in MeOH	Storite
0	0.22±0.01	0.22±0.02	0.21±0.02	0.22±0.03
3	0.23±0.04	0.24±0.06	0.29±0.04	0.29±0.04
6	0.29±0.01	0.30±0.04	0.33±0.03	0.33±0.03
9	0.42±0.03	0.40±0.04	0.40±0.02	0.40±0.08
12	0.35±0.03	0.33±0.03	0.58±0.12	0.52±0.12
15	0.35±0.10	0.35±0.09	0.42±0.03	0.51±0.09
18	0.30±0.04	0.33±0.02	0.44±0.06	0.36±0.10
21	0.32±0.02	0.36±0.06	0.44±0.02	0.35±0.12

The above results are shown graphically in Fig. 4.3.

From the graph, it is obvious that the development of resistance to water loss is not affected by the presence of components in the formulation, since there is no significant difference between the results for TBZ in methanol and TBZ in the form of Storite. In addition, the solubility of the chemical would also appear to have little bearing on the development of resistance to water loss.

Another point worth noting is that the presence of methanol appears to have little effect on the wound healing capacity of the discs, compared with the control. This seems to be in direct conflict with the results obtained by Wilson et al (1987). However, they were working on whole tubers, and the

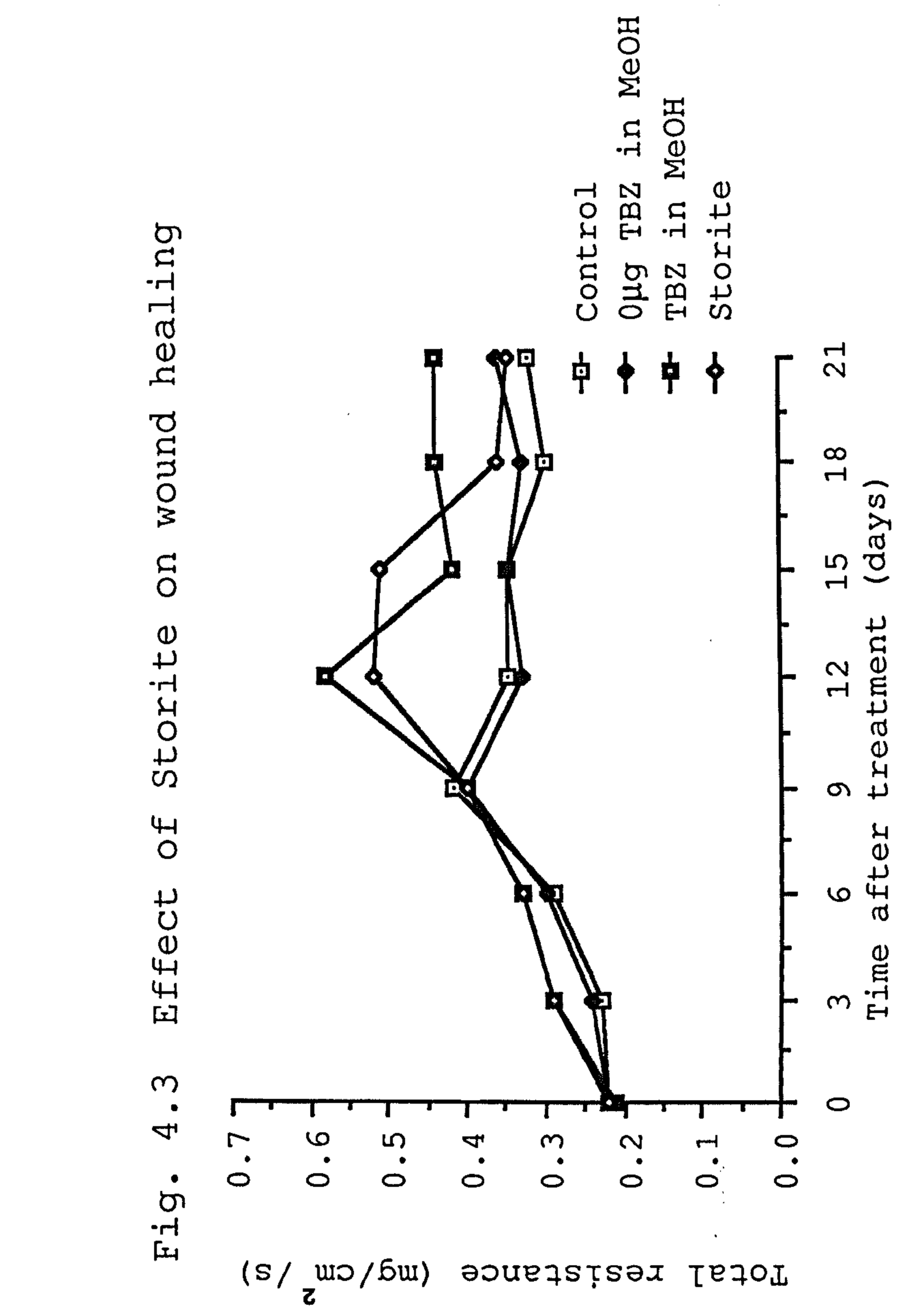


Fig. 4.3 Effect of Storite on wound healing

amounts of methanol which would realistically come into contact with a potato, when used as a solvent for "fogging on" chemicals. In contrast, as far as the discs were concerned,the minimum amount of methanol nessesary to solubilise TBZ was used, and therefore the different results obtained are probably a consequence of the different amounts of methanol applied.

Hence, it would appear that the Storite formulation components are not detrimental to the wound healing process.

In the next series of experiments, the TBZ formulation, Storite Clear, was assessed for its effects on the wound healing capacity of potatoes, as cases have been reported where poor skin setting and the development of a surface blemish have been problems, on potatoes treated with this formulation.

<u>The effects of Storite Clear on the wound healing capacity of tubers.</u>

In order to ascertain the effects of Storite Clear (a formulation of TBZ which is made up in hypophosphorous acid) on the development of resistance to water loss, and subsequently, wound healing, comparative studies were set up using Storite and a control. Skin set and skin quality were assessed on whole tubers, treated with the formulations, and the development of resistance to water loss in cut potato discs, also treated with the formulations, was also monitored.

Skin quality assessment. In order to ascertain the effects that the formulations had on skin setting and skin quality, a storage experiment was set up. Potatoes (cv. Cara) were treated with either Storite or Storite Clear, within 24 hours of lifting, at the commercial application rate of 40 $mgkg^{-1}$, using a Shandon spray gun. A control was also set up, where the tubers were sprayed with an equivalent amount of water. The tubers were then boxed and stored at 8°C.

After 12 weeks storage, tubers were visually assessed for skin set and skin quality.

In tubers treated with Storite Clear, the skin had a flaky appearance, indicating poor skin set (plate 1). This was obvious on all tubers treated with Storite Clear, however it was interesting to note that on tubers which had lumps of soil adhering to them, the skin beneath the soil had set normally (plate 2). This has resulted from the strong adsorption of TBZ by the soil. Therefore, where Storite Clear cannot come into contact with the soil, normal skin setting can take place.

On approximately 25% of the tubers treated with Storite Clear, a surface blemish was observed (plate 3) giving the tubers a scorched appearance. This blemish, at 12 weeks, had just penetrated the tissue beneath the skin.

Hence, the overall skin quality of Storite Clear treated tubers was poor and unattractive.

In comparison, plates 4 and 5 demonstrate the type of skin set evident on tubers treated with Storite and on control tubers, respectively. Skin beneath the lumps of soil was also examined. Throughout these tubers, normal skin set had taken

Plate 1. Storite Clear treated tuber - poor skin set.

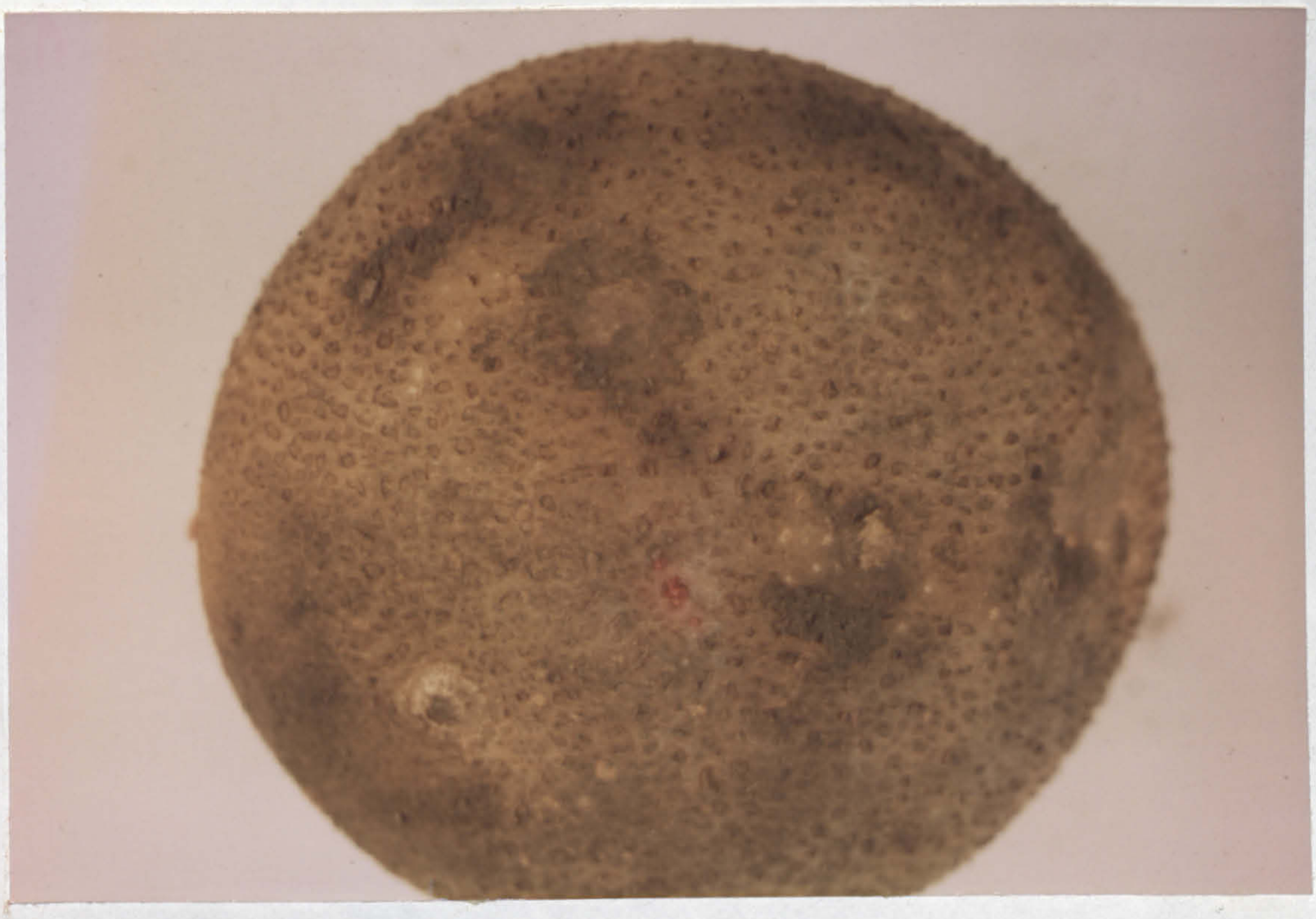


Plate 2. Storite Clear treated tuber - half soil removed.

Plate 3. Storite Clear treated tuber - scorch-like blemish.

Plate 4. Storite treated tuber - normal skin set.

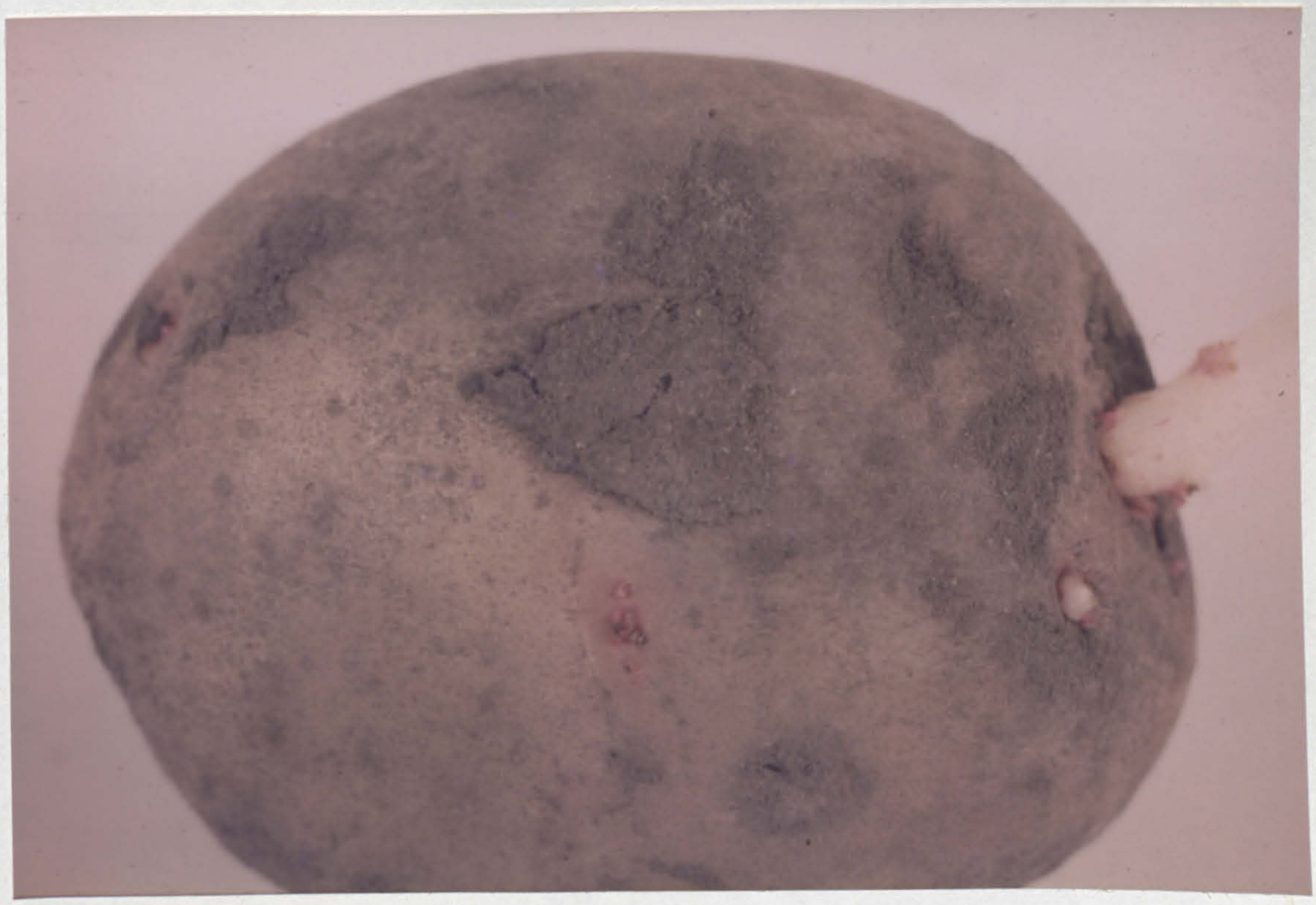
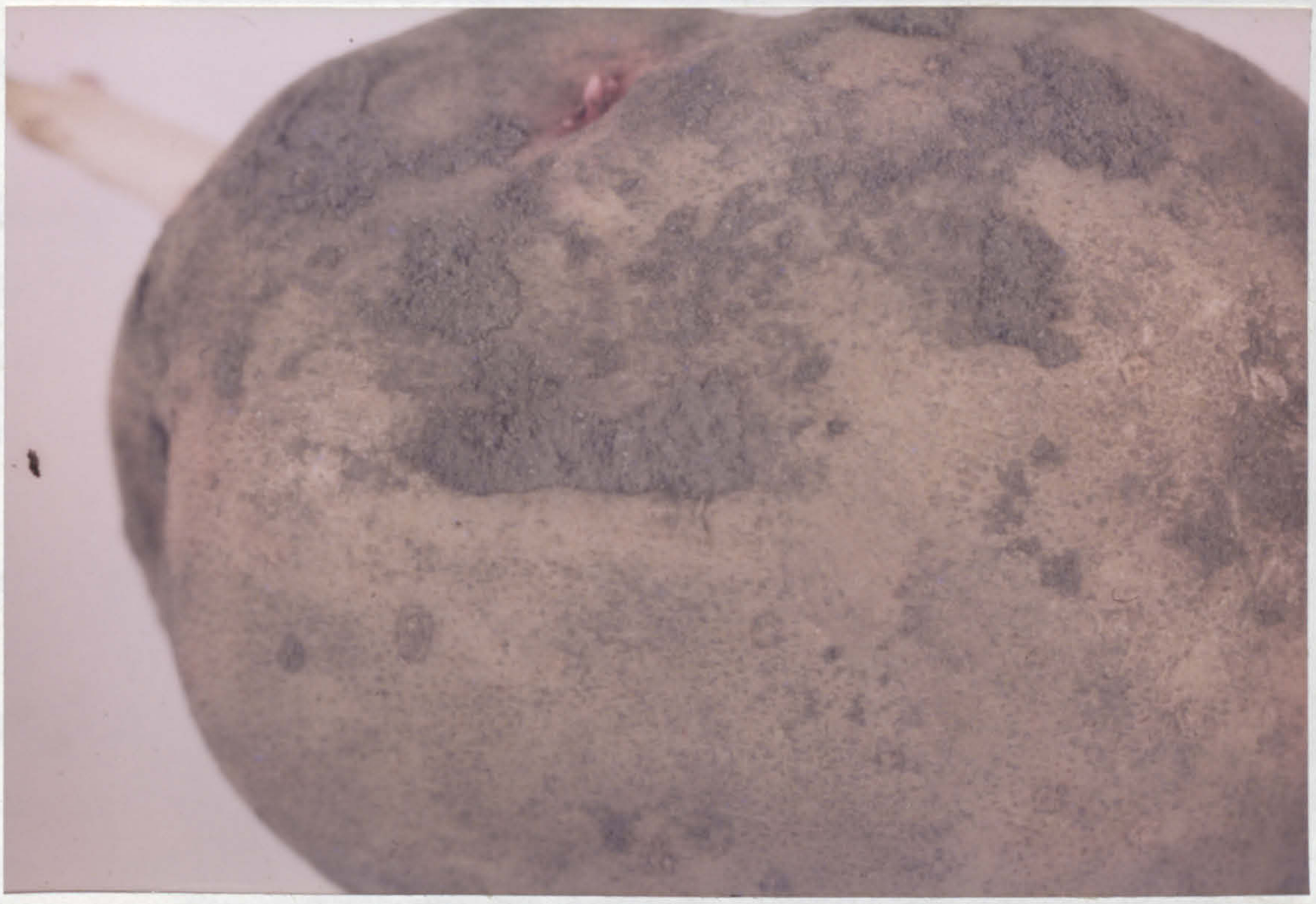


Plate 5. Control tuber - normal skin set.



place and no surface blemish was in evidence. Hence, no detrimental effects to the wound healing process were observed.

Development of resistance to water loss. Discs of potato (cv. Cara) were treated with 80µg TBZ in the form of Storite Clear, 80µg TBZ in the form of Storite and 0µg TBZ, acting as a control, as before.

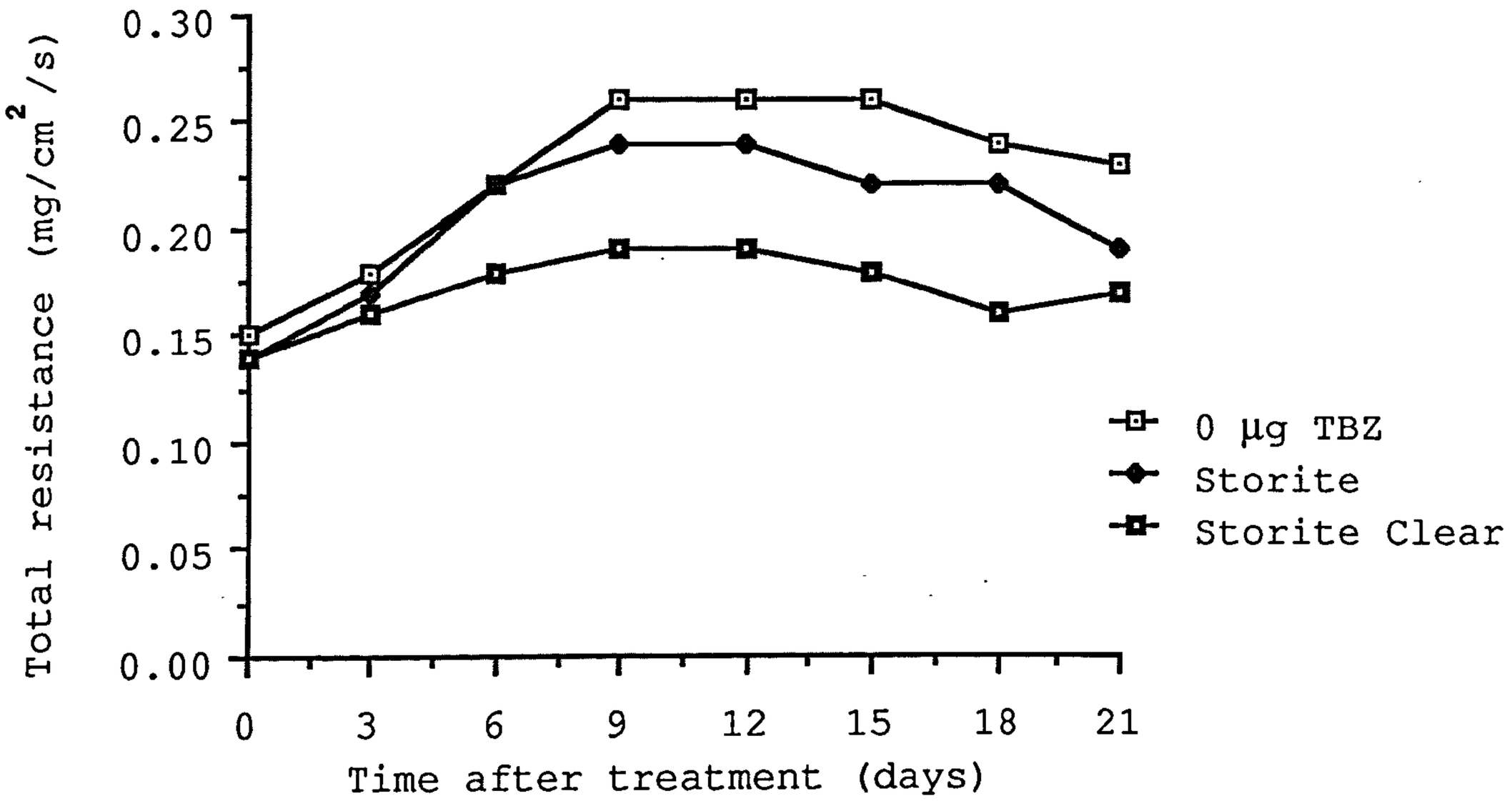
The development of total resistance to water loss was measured and the results are shown in table 4.4.

Table 4.4

Days after treatment	Total resistance to water loss ($mgcm^{-2}s^{-1}$) 0µg TBZ	Storite	Storite Clear
0	0.15 ± 0.06	0.14 ± 0.03	0.14 ± 0.02
3	0.18 ± 0.01	0.17 ± 0.02	0.16 ± 0.002
6	0.22 ± 0.03	0.22 ± 0.02	0.18 ± 0.01
9	0.26 ± 0.04	0.24 ± 0.04	0.19 ± 0.01
12	0.26 ± 0.04	0.24 ± 0.03	0.19 ± 0.02
15	0.26 ± 0.07	0.22 ± 0.02	0.18 ± 0.03
18	0.24 ± 0.02	0.22 ± 0.01	0.16 ± 0.02
21	0.23 ± 0.03	0.19 ± 0.04	0.17 ± 0.004

The results are also shown in a graphical form in Fig. 4.4.

Fig. 4.4 Effect of Storite Clear on wound healing



It was obvious from the results that the development of resistance to water loss in Storite Clear treated discs was impaired compared with Storite and control discs ($P > 0.01$).

Hence, the cut disc experiments reiterate the results obtained from the whole tuber studies in that Storite Clear has a detrimental effect on skin setting and the development of resistance to water loss, i.e. the process of wound healing is certainly impaired, if not altogether, inhibited.

Another point worth noting from the resistance experiments was the increased succeptability to rotting, that discs treated with Storite Clear exhibited. These wound healing experiments were origi nally set up on 1/12/87. However, by day 3, all of the Storite Clear treated discs showed excessive bacterial soft rot, and resistance to water loss could not be measured.

In comparison, only 8% of discs treated with Storite and 5.8% of control discs, showed any signs of bacterial soft rot.

Hence, these experiments were repeated on 12/1/88, where extra tissue was prepared so that sufficient discs could be analysed. In these experiments, the degree of bacterial soft rotting had dropped to 36% in Storite Clear treated discs. However, this was still very high compared with 6.25% in

Storite treated discs and 6.0% in the controls. Therefore, if Storite Clear were applied to tubers directly after lifting, when skin set and wound healing had not taken place, then much soft rotting would be observed.

In conclusion, Storite Clear would appear to be detrimental to the process of wound healing and skin set, as well as encouraging a scorch-like blemish to develop on the tuber surface, and as the period of storage progressed, the blemish penetrated deeper into the tuber. Although this is undesirable in table potatoes, the effect on potatoes used for processing is far more significant, commercially, in that a greater proportion of the potato has to be discarded, before processing can take place.

In the next series of experiments, the effects that the formulation, Storite Plus, had on the development of resistance to water loss was studied. Storite Plus contains both TBZ and 2-aminobutane (2-AB), the latter of which has displayed some detrimental effects on stored tuber quality. Therefore the purpose of studying the effects of Storite Plus was to see if TBZ could overcome the detrimental effects of 2-AB.

<u>The effects of Storite Plus on the wound healing capacity of tubers.</u>

The formulation Storite Plus, contains both TBZ and 2-AB and is applied as potatoes are loaded into the store, to control storage diseases such as skin spot, silver scurf, dry rot and gangrene. The benefits of including 2-AB to control the same storage diseases as TBZ, is that it is volatile and

therefore complete cover of the tuber is not essential, since 2-AB is active in the vapour phase. However, there have been reports that 2-AB is detrimental to wound healing and so a study was set up to investigate the effects that the inclusion of TBZ in the formulation, had on the development of resistance to water loss.

The development of resistance to water loss was measured as before, in discs (cv. Cara) treated with Storite, Storite Plus and 2-AB. Control discs were also set up as before.

The results are shown in table 4.5.

Table 4.5

Days after treatment	Total resistance to water loss ($mgcm^{-2}s^{-1}$) Control	Storite	Storite Plus	2-AB
0	0.14±0.02	0.13±0.04	0.13±0.02	0.13±0.0
3	0.18±0.02	0.17±0.02	0.17±0.05	0.15±0.02
6	0.22±0.01	0.21±0.01	0.19±0.10	0.20±0.02
9	0.25±0.03	0.24±0.01	0.21±0.12	0.25±0.03
12	0.25±0.10	0.24±0.02	0.21±0.05	0.28±0.04
15	0.25±0.05	0.21±0.05	0.20±0.03	0.29±0.05
18	0.24±0.02	0.22±0.09	0.24±0.10	0.22±0.08
21	0.20±0.01	0.19±0.02	0.23±0.07	0.26±0.05

These results are shown graphically in Fig. 4.5.

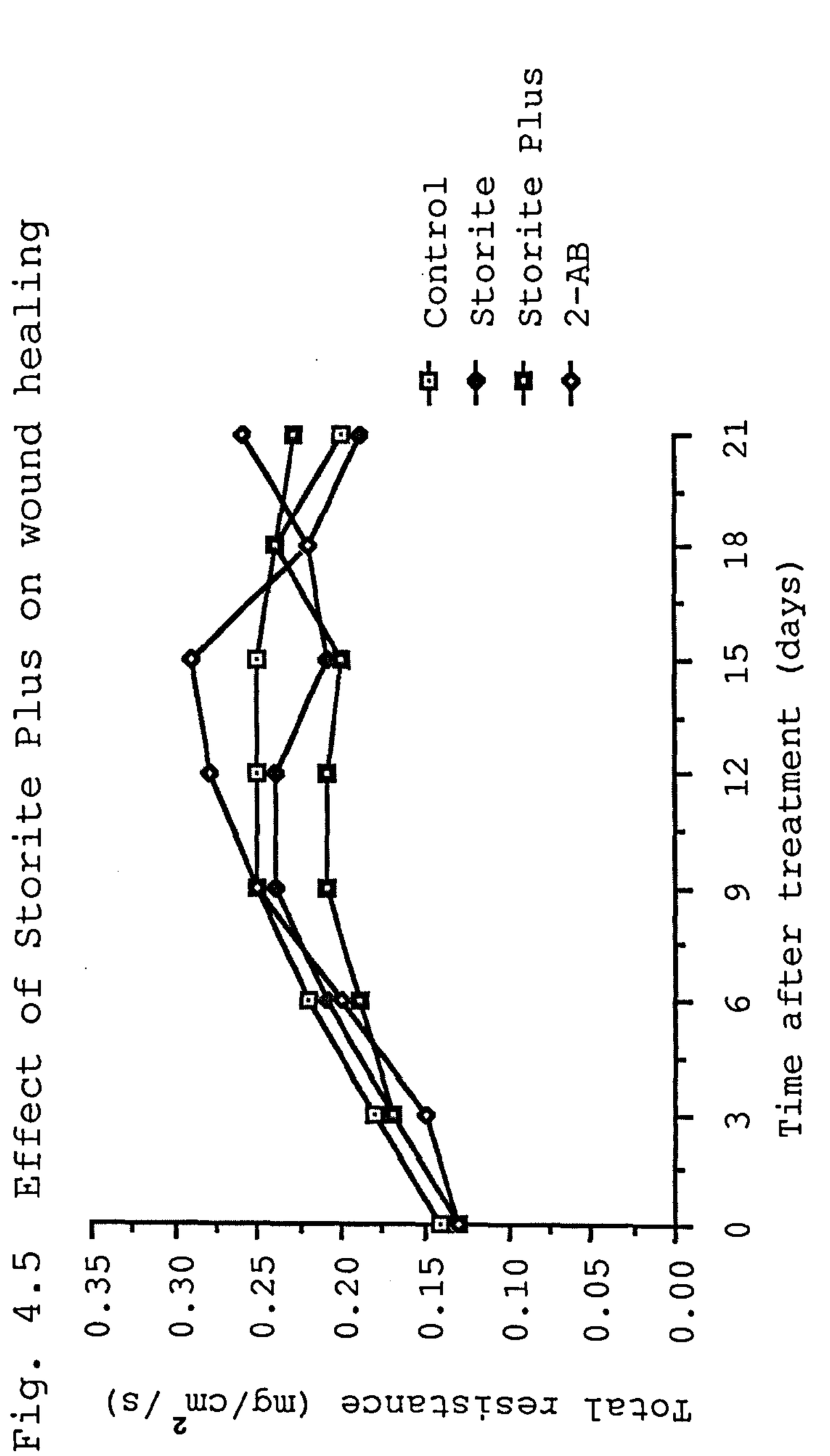

Effect of Storite Plus on wound healing
Control
Storite
Storite Plus
2-AB
Total resistance (mg/cm²/s)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
3
6
9
12
15
18
21
Time after treatment (days)

Fig. 4.5

In discs treated with 2-AB, a surface blackening was observed several hours after treatment. This blackening was also observed on discs treated with Storite Plus, but to a lesser extent. It would appear that the blackening was associated with the presence of 2-AB, however without microscopic investigation, the cause of the blackening could not be determined.

It was obvious from this set of data that there was no pattern to the wound healing of discs which had come into contact with 2-AB, i.e. both 2-AB and Storite Plus treated discs exhibited abnormal development of resistance to water loss.

Hence no statistical analysis was carried out with these results and the experiment was repeated.

However, results similar to those recorded previously, were obtained in that no pattern in the development of resistance to water loss was observed, in discs treated with 2-AB.

This experiment was repeated one more time, only this time, cv. Record was used in case cv. Cara was particularly succeptible to the effects of 2-AB. However, again abnormal resistance to water loss was observed in 2-AB treated discs.

The degree of rotting in discs treated with 2-AB and Storite Plus in each of the three experiments was significantly higher than in the Storite or control discs (10.4% and 9.3% compared with 6.2% and 5.3%, respectively, $P > 0.05$). Hence it may be the case that if a potato is treated with 2-AB in the form of 2-AB or Storite Plus, then the development of

resistance to water loss can take place. However, if the tubers are damaged in any way, then bacterial rotting would take place, and normal wound healing would be affected in some way.

Perhaps using microscopic examination may help to give a clearer picture of the effects that 2-AB was having on the discs i.e. what was causing the blackening, what affected the develpment of resistance to water loss, what happened to the disc that allowed bacterial rotting to take place, in the first place. All of these points need answered, but from the water loss experiments, it would appear that the inclusion of TBZ in the formulation did little to nullify the effects of 2-AB, with the exception of reducing surface blackening, to a degree.

Hence, in conclusion, the formulation components of Storite (TBZ) have no effect on the development of resistance to water loss. However, the acidic formulation of TBZ (Storite Clear) inhibits the development of resistance to water loss, impairs the process of skin setting and encourages bacterial soft rotting. The TBZ + 2-AB formulation, Storite Plus, affects wound healing in some way, but this requires further investigation.

Up until now, wound healing has been assessed from the development of resistance to water loss in cut potato discs. In the next section of work, the effect that TBZ (in the form of Storite) had on moisture loss from whole tubers was studied in order to determine how the discs compared.

Whole tuber weight loss study.

Although the effects that TBZ has on wound healing have been assessed in terms of the development of resistance to water loss in cut potato discs, the effects in a whole tuber will be different. In a newly harvested whole tuber, there may be wounds present and naturally these have to heal. However, another factor which a chemical may affect is curing, the period when skin setting and periderm development take place. Hence, it is important to ascertain how TBZ affects curing.

In order to study this, moisture loss from whole tubers was recorded and the precise details are given below.

Potatoes (cv. Cara) were lifted by hand on 3/11/87, and from these 24 tubers, each weighing approximately 150g, were selected. Water loss over a period of time was to be monitored in wounded and non-wounded tubers, treated with TBZ, before and after curing had taken place.

Weight loss prior to curing. Large lumps of adhering soil were removed from tubers. Four treatments, each of three replicates included i) non-wounded tubers treated with Storite at the rate of 40 $mgkg^{-1}$, ii) wounded tubers (the wound was made by taking a slice off the tuber, approximately 30 mm dia., using a sharp knife) treated with Storite at the rate of 40 $mgkg^{-1}$, iii) non-wounded control tubers, which were sprayed with an equivalent volume of water to that used in the Storite application and iv) wounded control tubers.

The tubers were placed on clock glasses and were weighed. They were then stored at $8^{o}C$ and 94 $\pm$ 2% relative

humidity. At two day intervals, weight loss from each tuber was recorded. Total moisture loss over the 22 day period was expressed as a percentage of the weight of the tuber at day 0, and the results are reported in table 4.6.

Table 4.6

Treatment	Total % moisture loss*
Storite, non-wounded	3.01% ± 0.32%
Storite, wounded	4.96% ± 0.83%
Control, non-wounded	3.1% ± 0.19%
Control, wounded	5.41% ± 0.24%

* mean of three replicates

Weight loss after curing. In order to allow curing to take place, the tubers were stored at 12°C for three weeks, before the same treatments, applied to tubers prior to curing, were carried out.

However, a problem was encountered in this part of the experiment in that after day 6, sprouting had begun and therefore exaggerated percentage moisture losses were observed. The early sprouting is probably a combination of late harvesting, followed by a relatively high temperature, when left to cure.

Total percentage moisture loss up to day 6 for tubers treated after curing is shown in table 4.7.

Table 4.7

Treatment	Total % moisture loss*
Storite, non-wounded	0.43% ± 0.03%
Storite, wounded	1.38% ± 0.15%
Control, non-wounded	0.34% ± 0.02%
Control, wounded	1.43% ± 0.06%

* mean of three replicates

One way analysis of variance was carried out on the data from both cured and non-cured moisture loss results to see if any differences were significant. However no statistical differences were observed between TBZ and control tubers in each experiment.

Hence, although TBZ would appear to promote the development of resistance to water loss in cut potato discs, no significant effect was observed on whole tubers.

In conclusion then, low levels of TBZ promote the development of resistance to water loss in cut potato discs, to a greater extent than commercial levels, but in each case, a treatment of TBZ significantly promoted the development of resistance to water loss, compared with the control.

The effect that the formulation components had on the wound healing capacity, was varied. The white emulsion, Storite, had the similar beneficial effects on wound healing that a standard solution of TBZ exhibited. However, the acidic formulation, Storite Clear, had extreme detrimental effects on

the wound healing process, and excessive rotting may be encountered when this formulation is used.

The effect of TBZ and 2-AB, in the form of Storite Plus, was also assessed, but requires further investigation.

And finally, whole tuber weight loss was monitored. In the whole tuber, TBZ was found to have no beneficial or detrimental effects on moisture loss and therefore, it may be that TBZ can promote wound healing, but has little effect on curing.

In the next chapter, methods for determining TBZ residues in processed products are discussed, and residues in the processed products are reported.

CHAPTER 5

THIABENDAZOLE RESIDUES IN PROCESSED POTATO PRODUCTS.

Introduction.

Today, the term processed potato products can mean anything from canned potatoes to crisps. In fact the range of processed potato products is constantly increasing e.g. canned potatoes, chipped potatoes, baked potatoes, crisps, reformed potato snacks e.g. "Hula Hoops", potato waffles, re-constituted potato e.g. "Smash", potato salad, as well as plain old boiled potatoes. In order to manufacture all of these products, potatoes must undergo a number of cooking processes including boiling, frying, dehydration and extrusion (where dry matter is mixed with heat and moisture and the mixture is then forced through a die to form different shapes e.g. potato rings, sticks and other reformed potato snacks such as "Smash").

It would be of interest to discover what happens to chemicals which have been applied to potatoes during storage, in the cooking process. Very little has been published on TBZ residues in processed products. However, information was available regarding other potato storage chemicals such as chlorpropham, tecnazene and maleic hydrazide.

The fate of chlorpropham in crisps and frier oil has been discussed by Ritchie (1986). He reports that most of the

chlorpropham present on potato slices, prior to frying, remains in the crisp after frying, and that some partitioning of chlorpropham into the frier oil does take place. It would appear that insignificant amounts of chlorpropham are lost through volatilisation. This is partially explained in terms of i) condensation of the vapour on the sides of the frier, where this vapour falls back down into the oil after cooking has ceased and ii) chlorpropham has a greater affinity for oil than water. If volatalisation did occur, then it is unlikely that the high levels of chloropropham, reported in the frier oil, would have resulted in the first place. Ritchie acknowledges that more work needs to be carried out so that the lack of loss of chlorpropham through volatilisation, can be understood more fully.

Contamination of untreated potatoes is also reported. Untreated potatoes were fried in oil which had been used to fry treated potatoes, and a chlorpropham residue was detected in the crisps. Therefore, some chlorpropham from treated potatoes must have partitioned from the potato into the oil (or by condensation of the chlorpropham vapour), and when untreated potatoes were fried, they picked up the chlorpropham along with the oil, and hence became contaminated.

A similar picture has been shown with tecnazene (Dalziel and Duncan, 1980). The tecnazene residue was found to associate with the skin of the potato, as substantial amounts of tecnazene are lost with peeling the tubers. Similarly to chlorpropham, the frier oil becomes contaminated with

tecnazene. Again, losses through volatility appear to be minimal, as a direct consequence of its solubility in oil.

Dalziel (1978) also reported the effects of boiling potatoes, on the tecnazene residue. He reported that although losses of the chemical did result, these were minimal and that the annual U.K. consumption of tecnazene for that year would only be approximately 0.5g.

Hence, although chlorpropham and tecnazene are both volatile, losses through heating are minimal. Also the residue of both chemicals associates with both the potato and the frier oil.

In contrast, maleic hydrazide is non-volatile and reasonably polar. McKenzie et al (1987) report that as the maleic hydrazide residue is distributed throughout the potato, peeling of the tubers does little to reduce the residue in the processed product, unlike chlorpropham and tecnazene residues. The maleic hydrazide residue tends to associate with the polar potato tissue, and therefore negligible amounts of the chemical were detected in the frier oil. Boiling maleic hydrazide treated potatoes has little effect on the residue, although McKenzie (Private communication) reports that he was unable to account for 100% of the residue found in uncooked potato.

Therefore, it would appear that the polarity of the chemicals influence their distribution in potatoes, oil and water i.e. chlorpropham and tecnazene are relatively non-polar

chemicals and therefore have an affinity for the frier oil and can thus contaminate it, yet little is found in water used to boil these potatoes. By contrast, insignificant amounts of maleic hydrazide are found in the frier oil, but the residue tends to remain with the polar potato tissue.

No information was available regarding TBZ residues in processed potato products. With changing eating habits and increased public concern relating to pesticide residues in food, it was decided to investigate the distribution of TBZ in processed potato products.

TBZ is different from the other potato storage chemicals in that it is moderately polar, virtually non-volatile, unaffected by heat and is applied as a surface coating to the crop at the commencement of storage. Hence it was of interest to discover how various cooking procedures affected the chemical residue.

As cooking will affect the moisture content of the potato, where possible, residues are reported on a fresh weight basis (unless otherwise stated).

Treatment and storage of potatoes.

Newly harvested potatoes (cv. Record) were treated with the TBZ formulation Storite, at the commercial application rate of 40 $mgkg^{-1}$, using a Shandon laboratory spray gun. The potatoes were then randomly mixed before being boxed and stored at $8^{o}C$. After curing had taken place, the tubers were treated with chlorpropham at the rate of

20 $mgkg^{-1}$, in order to inhibit sprouting throughout the period of storage.

After a period of storage (approximately four months), the potatoes were cooked in a number of ways, and TBZ was then determined in each of the cooked samples.

Thiabendazole residues in boiled potatoes.

Today, more potatoes are being boiled with their skins still intact, as the skin is thought to provide a valuable source of vitamins and fibre (Hampson, 1976). However, as reported in Chapter 2, the bulk of the TBZ residue associates with the peel, and so it may be possible that higher levels of TBZ may be ingested. Hence it was of interest to discover what, if anything happens to the TBZ residue after boiling.

Approximately 300g of potatoes were washed, before being boiled, in accordance with normal domestic practice. After boiling, the potatoes were mashed, and TBZ was extracted from them, as described in Chapter 2.

TBZ was also determined in the water in which the potatoes were boiled. The water was filtered and then evaporated to dryness. The residue was redissolved in methanol/ 112mM phosphoric acid (50:50, v/v), and the concentration of TBZ present in the water was determined using HPLC.

Residue analysis was also carried out in uncooked,

washed potatoes, so that the effects of cooking could be established. The results are shown in table 5.1.

Table 5.1

Sample	TBZ residue ($mgkg^{-1}$)*
Washed, uncooked	0.86 ± 0.12
Washed, boiled	0.52 ± 0.08

* mean of five replicates, expressed on a fresh weight basis

Hence, it would appear that when 1kg of potatoes are boiled, approximately 0.3mg of TBZ are lost. When the water was analysed, it was found that an amount equivalent to 0.3 $mgkg^{-1}$ was detected. Therefore some TBZ is lost from the potatoes during boiling. It may be possible that the heat of the process has caused some TBZ to dissipate into the water, but it is more likely to be a consequence of the potatoes partially disintegrating during cooking, where some cells may have burst, and as a result, the surface area from which the water can solubilise TBZ has increased. Therefore boiling does reduce the TBZ residue slightly.

Thiabendazole residues in baked potatoes.

The baked potato is becoming more popular with consumers due to its versatility, and with the growers, since

higher premiums can be demanded for first class bakers, compared with ware potatoes. More fast food outlets are adding baked potatoes to their menus and even the frozen food manufacturers are including baked potatoes in their lines. With the number of households owning a microwave oven, also on the increase, baked potatoes are becoming a popular alternative to boiled or chipped potatoes.

There are two main methods for cooking baked potatoes, i.e. conventional oven baking and microwaving. TBZ residues were measured in potatoes using both methods, so that the effects of each could be assessed.

Large washed tubers (cv. Record) weighing approximately 250g were either i) oven baked at 220^{o}C for 1.5 hours or ii) microwaved in a 650 Watt oven for 10 minutes. Residue analysis was carried out as described in Chapter 2 in each of the samples, as well as in uncooked, washed tubers. The results are shown in table 5.2.

Table 5.2

Sample	TBZ residue ($mgkg^{-1}$)*
Uncooked	0.78 ± 0.08
Oven baked	0.78 ± 0.13
Microwaved	0.55 ± 0.12

* mean of five replicates, expressed on a fresh weight basis

It is obvious from the results that the residue in the microwaved sample is lower than in the uncooked or oven baked sample. Hence, analysis of variance was carried out to prove that the difference was significant, and an L.S.D. of 0.18 was calculated, using Tukey's Honestly Significant Difference Test. Therefore microwaving did reduce the TBZ residue significantly.

The extract from the microwaved sample was subjected to mass spectrometry to see if any breakdown products could be detected, however none were identified. It may be that the extraction procedure used to extract TBZ, was inefficient at extracting other components present in the microwaved sample, and as a result, no other decomposition products of TBZ were detected.

One additional point worth noting is that although baked potatoes are associated with healthy eating, since they are considered to be natural. However, people tend to eat the whole potato, including the skin, yet work in this thesis has shown that TBZ (and other chemicals) concentrate around the skin of the potato. Therefore, by eating the skins as well, the amount of chemical ingested is also increased.

<u>Thiabendazole residues in crisps.</u>

Today, crisp manufacturing is changing with the introduction of thicker cut crisps, crisps with their skins intact and more reconstituted potato snacks. As a result of

these changing practices, more chemicals are being "fried" along with the potato, and it is therefore important to discover what happens to the residue after frying. As discussed earlier, chlorpropham and tecnazene can concentrate up in the frier oil and contaminate untreated potatoes.

However, the picture with TBZ is unclear, since no work has been published relating to the distribution of TBZ in crisps. The chemical properties of TBZ are very different from chlorpropham and tecnazene, as it is more polar, virtually non-volatile and reasonably insoluble in oil, and as a result very little could be postulated relating to the distribution of TBZ in crisps.

In the following sections, methods are described for the extraction and quantification of TBZ from crisps and oil. Residues present in laboratory treated samples are also reported. Firstly though, a brief description of commercial crisp manufacturing is given.

Crisp manufacturing.

Incoming tubers, either from the factory's own store or from commercial stores, are washed and then peeled, using an abrasive peeler. The peeled tubers are then sliced (2 mm thickness) and the slices are washed to remove any reducing sugars and starch grains from ruptured cells.

The slices are then fried in vegetable oil at 180^{o}C for five to six minutes. The actual composition of the oil can vary, but it is usually a mixture of palm oil and soya oil.

After frying, the crisps are then passed under a bank of infra-red heaters to reduce the oil content of the crisps. The crisps are then salted, flavoured and finally packaged.

In general, crisps contain approximately 30% of their weight, as oil.

For the purposes of these experiments, "home-made" crisps were prepared in the laboratory, as access to a processing line proved to be difficult.

Extraction of Thiabendazole from oil.

So that TBZ could be determined in the oil surrounding the crisp, as well as in the frier oil, a method had to be developed for the extraction of TBZ from oil. In each of these experiments, a blend of rape and soya oil was used (Bejam Pure Vegetable Oil).

In order to extract TBZ from oil, the oil must first be diluted using a suitable solvent, as it is too viscous to extract directly. Methanol, dichloromethane and hexane were all tested for their ability to dilute the oil. Various ratios of "spiked" oil (yielding a concentration of 5 $\mu g cm^{-3}$ TBZ in the oil) and solvent were mixed and applied to a 500 mg Bond Elut diol column, which had been activated with the appropriate solvent. The sample was then run into the column, and washed with 10 cm^3 of the appropriate solvent. The column was then placed over suction so that all of the oil/ solvent mixture was removed. After five minutes, TBZ was extracted

from the diol column with methanol/ 112mM phosphoric acid (50:50, v/v). The eluate was made up to a volume of 5 cm^3 and the recovery of TBZ determined by HPLC (as described in Chapter 2).

The percentage recoveries for each of the solvents and ratios are shown in table 5.3.

Table 5.3

Solvent	Volume of oil:solvent (cm^3)	% recovery of TBZ*
Methanol	5:10	26.4 ± 3.6%
	5:25	29.1 ± 8.5%
	5:50	29.0 ± 10.8%
Dichloromethane	5:10	74.9 ± 10.3%
	5:25	69.9 ± 8.2%
	5:50	83.6 ± 6.4%
Hexane	5:10	77.0 ± 6.1%
	5:25	89.9 ± 4.9%
	5:50	99.2 ± 2.6%

* mean of five replicates

In general, as the ratio of oil to solvent was increased, the recovery of TBZ from the oil was also

increased. The recovery of TBZ from oil which had been diluted with methanol was very low. This is probably a consequence of the poor retention of TBZ on the diol column, as methanol is used to desorb the TBZ.

The greatest recovery was obtained using hexane to dilute the oil, at the ratio of 5:50. Hence in each of the subsequent experiments, hexane was used to extract TBZ from oil.

Extraction of Thiabendazole from crisps.

TBZ treated potatoes (cv. Record) were washed and sliced (2mm thick), before frying in vegetable oil. They were fried at 180^{o}C for approximately five minutes, until the crisps were a light golden colour. After frying, excess oil was removed by blotting the crisps with absorbent paper.

30g of crisps were then placed in a Waring blender along with 100 cm^3 of hexane, and blended at high speed for 60 seconds.

The contents of the blender were then transferred to a Buchner flask and filtered over suction. The residue was washed with 3 x 50 cm^3 portions of hexane, and the filtrate was then transferred to a round bottomed flask.

Enough hexane was evaporated (using a rotary evaporator) so that the ratio of oil to solvent was 1 : 10. The extract was finally transferred to a diol column, and extracted as before. A value for the amount of TBZ present in

the oil surrounding the crisps, could then be determined.

The crisp residue left after extracting the oil, was also analysed for TBZ. The residue was blended up with 150 cm^3 of dichloromethane and 80g of anhydrous sodium sulphate, in a Waring blender for 60 seconds. The contents of the blender and the rinsings were then transferred to an alumina bottle and shaken on a reciprocating shaker for one hour.

After shaking, the contents were filtered and washed, before the filtrate was evaporated, and redissolved in dichloromethane. The extract could now be cleaned up and analysed for TBZ using the method described in Chapter 2.

The amount of TBZ left in this crisp residue provided an indication of the degree to which TBZ was adsorbed by potato tissue.

Finally, the oil in which the potatoes were fried was also analysed. Using the method described earlier for the extraction of TBZ from oil, the amount of TBZ present in the oil sample was determined. This would give an indication of whether contamination of the oil was likely to be a problem.

The results for TBZ present in each of the aformentioned samples are shown in table 5.4, where the residues are expressed in terms of the sample, and not on a fresh weight basis.

Table 5.4

Sample	TBZ concentration in the sample*
Washed potatoes	2.71 ± 0.10 $mgkg^{-1}$
Crisp residue	10.79 ± 0.08 $mgkg^{-1}$
Oil extracted from crisps	3.40 ± 0.02 μgg^{-1}
Frier oil	NDR**

* mean of five replicates

NDR** no detectible residue

These results can be expressed in terms of a residue of TBZ on a fresh weight basis, by using a series of conversion factors.

a) TBZ theoretically present in 1 kg of crisps. The conversion factor used to convert a weight of fresh, washed potato into a weight of crisps was calculated as 3.02.

The TBZ residue present in washed potatoes was equal to 2.71 $mgkg^{-1}$. Hence, in theory, 1kg of crisps should contain 3.02 x 2.71 = 8.18 mg TBZ kg^{-1} crisps.

b) TBZ detected in the crisp residue. The conversion factor used to convert a weight of crisps into a weight of crisp residue was calculated as 0.62, i.e. in 1 kg of crisps there are 620g of crisp residue (and by difference, 380g of oil).

Hence, the residue value of 10.79 $mgkg^{-1}$ in the crisp residue is equivalent to 0.62 x 10.79 = 6.69 mg TBZ kg^{-1} crisps.

c) <u>TBZ detected in the oil extracted from the crisps.</u> The amount of TBZ extracted from the oil surrounding the crisps was calculated as 3.4 μgg^{-1} oil.

In 1kg of crisps, there are 380g of oil. Therefore, the amount of TBZ extracted from the oil surrounding 1kg of crisps is equal to 380 x 3.4 = 1.29 mg TBZ kg^{-1} crisps.

d) <u>Comparison of the theoretical residue with the actual residue.</u> From the experimental values obtained, there are 6.69 mg TBZ kg^{-1} crisps present in the crisp residue and 1.29 mg TBZ kg^{-1} crisps in the oil surrounding the crisps. Therefore, in 1kg of crisps, there is a total of 6.69 + 1.29 = 7.98 mg TBZ.

This figure is very close to the theoretical value of 8.18 $mgkg^{-1}$, extrapolated from the TBZ residue value found in fresh potatoes. Hence, the majority of the TBZ residue in washed potatoes has been accounted for. The slight discrepancy between the two figures, is almost certainly a consequence of small variances in the the conversion factors used.

It would appear that the bulk of the TBZ residue remains associated with the crisp residue, and that only small amounts of TBZ move into the oil surrounding the crisp. It may be possible that the hexane actually removes some of the TBZ

from the crisp residue, during the oil extraction, and that this is where the TBZ residue in the oil comes from. Preliminary work showed that hexane was inefficient at extracting TBZ from potato tissue. The reason for this was thought to be related to the fact that the hexane could not wet up the potato tissue sufficiently, due to the high water content of the potato. However, in crisps the water content is much reduced, and it may now be possible for hexane to extract TBZ from the crisp residue.

The likelihood of contamination of the frier oil is very low. Since the oil is non-polar, the TBZ would have a greater affinity for the polar potato tissue, where some adsorption onto cell walls may take place.

Obviously these results were achieved in the laboratory. Access to a commercial processing line would have been of value, since differences in the production of "home-made" crisps and commercially produced crisps, may affect the distribution of the TBZ residue i.e. in the production of the "home-made" crisps, the potatoes were not i) cut as uniformly as they would have been in a commercial operation, ii) washed prior to frying and iii) passed under infra-red heaters to reduce the oil content of the crisps, all of which may have influenced the TBZ residue in the final product. Another advantage of studying the TBZ residues present in crisps produced commercially is that the effect of frying tonnes of TBZ treated potatoes, on the residue in the oil, could be studied in more detail.

In conclusion, the effects of cooking TBZ are minimal, on the final residue value present in the cooked product, with the exception of microwaving, where some loss of the chemical in its native form takes place. Therefore in general, the amount of chemical ingested after cooking is approximately the same as the residue in the raw potato.

Although TBZ is not considered a highly toxic chemical (LD_{50} = 3300 $mgkg^{-1}$ in rats), ways should be sought to try to reduce the levels ingested, since more and more chemicals are being found to play a role in the development of less serious diseases such as allergies and dermatological problems.

In the next chapter, the metabolism of TBZ by fungi and bacteria is discussed. It is important to follow the metabolism of a chemical so that the development of any toxic metabolites can be observed, as well as noting how quickly loss of chemical activity occurs and to see whether the development of resistance to the chemical is likely to become a problem.

CHAPTER 6

METABOLISM OF THIABENDAZOLE.

Introduction.

So that a pesticide can be registered, extensive research must be carried out in areas including toxicology of target and non-target organisms, residues in the crop and in the environment, persistence and metabolism. Recently, the study of the metabolic pathway of a chemical has become increasingly important, as registration requirements demand that the primary metabolites of a chemical are identified.

All living organisms possess some type of defence mechanism, so that toxic substances can be detoxified or eliminated in some way. The different defence mechanisms vary from organism to organism, and there are a number of factors which will influence i) the uptake of a pesticide and ii) its detoxification. In plants, the polarity of the chemical is very important, since polar chemicals will not be able to penetrate the waxy cuticle on the leaf surface to any great extent, without the addition of surfactants and humectants. In a similar vein, if a non-polar chemical is ingested, then as a consequence of its polarity, the chemical will be readily excreted from mammalian tissues. However, if any of the non-polar chemical is absorbed, then levels will accumulate in the fatty tissues.

There are three principle reasons for studying the

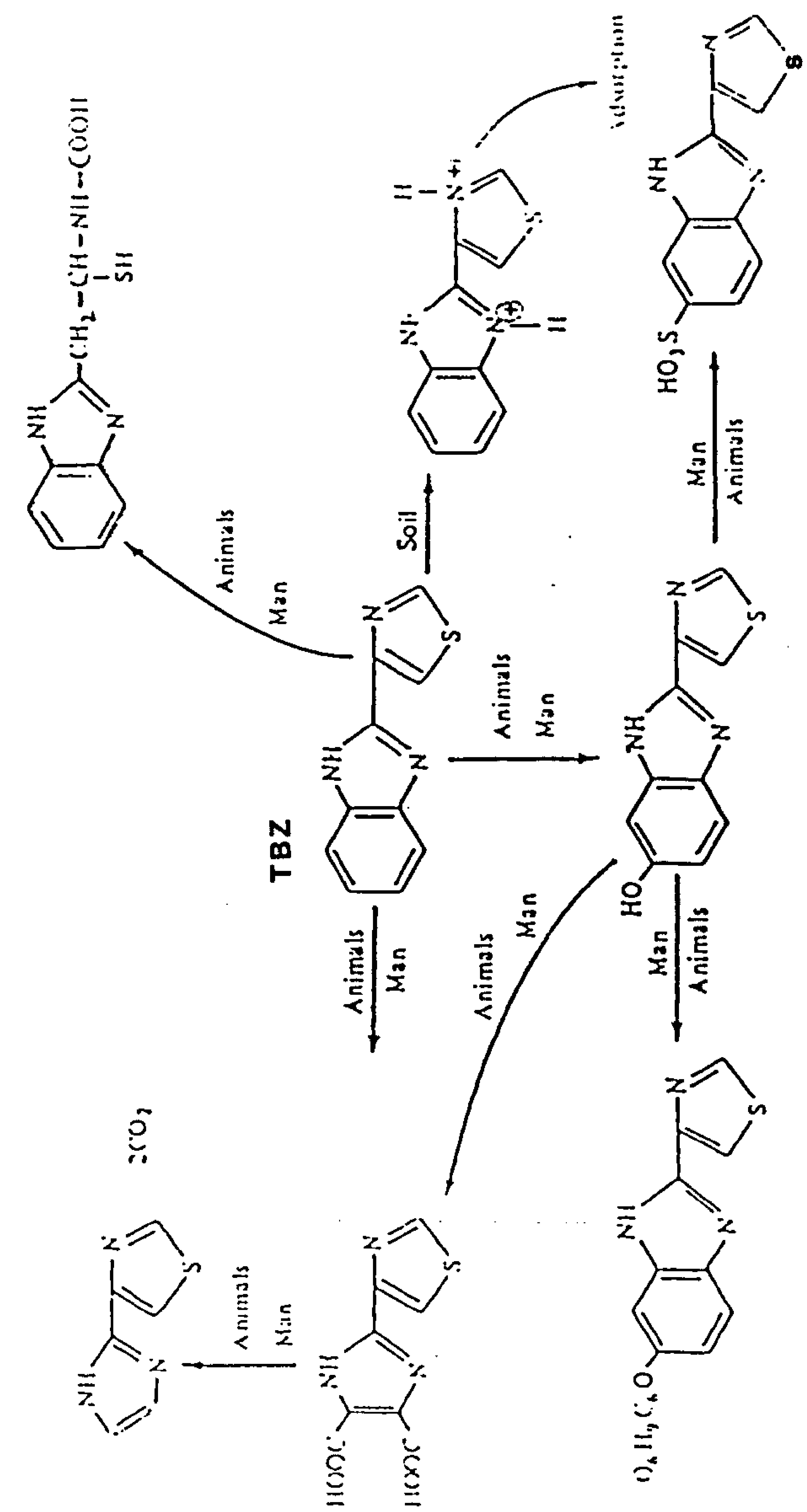
metabolism of a chemical :- i) to discover if the metabolite produced, is toxic, ii) to monitor how quickly the chemical is metabolised and as a consequence, work out how frequently re-application of the chemical is nessessary and iii) to find out whether the development of resistance to the chemical, is going to be a problem.

In the case of TBZ, metabolism may be a result of reduction or hydroxylation, followed by conjugation with sulphuric or glucuronic acid. These reactions increase the polarity of TBZ, and therefore make its excretion easier. The site of action of TBZ will also influence the metabolism e.g. micro-organisms such as bacteria and fungi can use the carbon skeleton as their source of energy.

Fig. 6.1 shows some of the possible transformations of TBZ (Zbozinek, 1984).

The objectives of this chapter were to try to identify any microbially produced metabolites of TBZ, and to ascertain the likelihood of the development of resistance to TBZ by the fungi which TBZ is reported to control. In these experiments, the principle involved was to try to get various micro-organisms, both fungal and bacterial, to grow in the presence of TBZ. Resistant strains were then cultured onto agar containing higher levels of TBZ, and any metabolic products of TBZ were then analysed for.

Fig. 6.1 Transformations of thiabendazole.



The metabolism of Thiabendazole by bacteria.

Although TBZ is not renowned for having any bacteriocidal activity, bacteria will constantly be in contact with TBZ. The bacterial populations will arise from infected potatoes e.g. *Erwinia carotovora* or from soil bacteria. Therefore it is important to study the possible metabolism of TBZ, resulting from bacterial breakdown.

Bacteria use the carbon skeletons of many compounds as their source of energy, and are capable of breaking bonds, for which the chemical energetics are normally unfavourable. The bacterial population of the soil will largely depend on soil factors e.g. anaerobic or aerobic soil, pH, soil temperature and soil type. Hence many different bacterial populations could be used in bacterial metabolism studies for TBZ.

In the following sections, two bacterial populations were used for TBZ metabolism studies : i) *Erwinia carotovora var. carotovora* and ii) a mixed bacterial population taken from the soil on which the field trial potatoes (described in Chapter 3) were grown.

Metabolism of Thiabendazole by *Erwinia carotovora var. carotovora*.

E. carotovora is the organism responsible for the soft rot of potatoes in the ground and in the store. The bacterium can infect the crop directly or by adhering to the

soil surrounding the tubers. Therefore the likelihood of *E. carotovora* coming into contact with TBZ is reasonably high. It was because of this that a series of metabolism studies were set up using *E. carotovora*.

Isolation of a resistant strain. In order to discover whether or not TBZ could be metabolised by *E. carotovora*, the bacterium was incubated in the presence of different amounts of TBZ, over a period of time.

A suspension of bacteria was prepared (from stock cultures), and then vortexed to ensure an even distribution of bacteria, before 0.2 cm^3 was transferred to Universal containers. These contained 10 cm^3 of nutrient broth, and 0.25 cm^3 of TBZ in 0.1M HCl, at the appropriate concentration. Four replicates were prepared for each treatment, and the cultures were then incubated at 26^oC for 26 hours.

After the incubation period, the optical density of the cultures was measured using a spectrophotometer (Pye Unicam, Model SP1800) at 520nm. Earlier a standard curve had been prepared, so that optical density (at 520nm) could be related to bacterial numbers, using the counting technique described by Collins and Lyne (1984). Measuring optical density eliminated the need for cell counting, and in so doing, removed any bias, as well as the tedium involved in counting bacteria. The results obtained are shown in Table 6.1.

Table 6.1

Concentration of TBZ	O.D. at 520nm	Number of bacteria* ($x10^4$)	% inhibition of growth
0ppm	0.250	140	-
0.1ppm	0.238	135	3.6
0.25ppm	0.240	136	2.9
0.5ppm	0.220	129	7.9
1.0ppm	0.220	129	7.9
5.0ppm	0.220	129	7.9
10.0ppm	0.224	130	7.1
50.0ppm	0.180	111	20.7
100.0ppm	0.160	103	26.4

* mean of four replicates

From the results, it would appear that TBZ was eliciting some type of inhibitory effect on the growth of *E. carotovora*, yet did not inhibit its growth altogether. Bacteria from the 100ppm treatment were pooled, and sub-samples cultured onto nutrient agar slants, which had been prepared with TBZ incorporated into them giving a concentration of 100ppm TBZ in the agar. The bacteria were cultured onto agar slants as they are easier to maintain than liquid cultures. Although the growth of *E. carotovora* was not as prolific on the agar slants, it was observed that the shape of the resistant bacteria had changed and that

they were more crystalline in nature, compared with the smooth, non-resistant *E. carotovora*. This indicated that some physiological changes were taking place in bacteria that were growing in the presence of TBZ.

Isolation of Thiabendazole metabolites. Now that a TBZ resistant strain of *E. carotovora* had been isolated, a time course study was set up to monitor the growth of the bacteria, and to provide samples from which TBZ and its metabolites could be extracted. Liquid cultures were prepared as before, at a concentration of 100 ppm TBZ. They were incubated at 26^{o}C for 0, 24, 48, 72, and 168 hours. Controls (0 ppm TBZ) and blanks (*E. carotovora* plus nutrient broth) were also set up.

After each incubation period, the optical density of each replicate was recorded and the liquid cultures were autoclaved, to inhibit any further growth or metabolism.

TBZ was extracted from the liquid cultures by partitioning with ethyl acetate (recovery = 100%). The ethyl acetate was evaporated to dryness and the residue redissolved in 2cm^3 methanol. The amount of TBZ present in the extract was determined by HPLC (described in Chapter 2). HPLC analysis was also carried out to see if any 5-hydroxy TBZ could be identified in the extracts, as it is the primary metabolite of TBZ. 5-hydroxy TBZ fluoresces at different wavelengths from TBZ (excitation = 305nm, emission = 470nm (Watts et al, 1982)), and so its presence could be readily detected. The samples were also analysed using UV

detection at 254nm, since most compounds will elicit some response at this wavelength, and therefore any other metabolite would be detected. Hence the dissappearance of native TBZ and the appearance of 5-hydroxy TBZ and any other metabolite of TBZ could be followed using HPLC.

The results for bacterial growth, dissappearance of TBZ and appearance of TBZ metabolites are reported in table 6.2.

From the results, it would appear that the presence of TBZ and 0.1M HCl were having an inhibitory effect on the growth of *E. carotovora*. Also, although the bacteria developed some resistance to TBZ, no metabolites of TBZ or loss of TBZ was detected. Therefore one would conclude that the development of resistance to TBZ by *E. carotovora* had resulted via some mechanism, other than metabolism.

Table 6.2

Incubation time (hr)	Sample	Number of bacteria*	% of TBZ remaining	Presence of metabolites
0	Blank	100×10^4	0	-
	Control	98×10^4	0	-
	Sample	98×10^4	100	ND**
24	Blank	140×10^4	0	-
	Control	126×10^4	0	-
	Sample	108×10^4	100	ND
48	Blank	160×10^4	0	-
	Control	140×10^4	0	-
	Sample	122×10^4	100	ND
72	Blank	148×10^4	0	-
	Control	152×10^4	0	-
	Sample	131×10^4	100	ND
168	Blank	148×10^4	0	-
	Control	152×10^4	0	-
	Sample	135×10^4	100	ND

* mean of four replicates

ND** None detected

Metabolism of Thiabendazole by soil bacteria.

Since there were a number of different bacteria which could be chosen for metabolism studies with TBZ, it was decided to use a bacterial population which would be in direct contact with TBZ and potatoes. Hence bacteria were isolated from the soil on which the field trial was planted.

Isolation of soil bacteria. Two replicate soil samples (approximately 1g) were taken from the field trial site at Arkleston Farm, Paisley. The soil was added to sterile water in Universal containers, and the contents shaken vigorously. The washings from each were pooled, and samples were transferred to nutrient agar slants. These were incubated at 21°C.

After 24 hr, the slants showed copious growth of a mixture of bacteria, and to aid identification, the cultures were streaked onto plates of nutrient agar. After 24 hr, six different types of small rods, and one type of large rod were identified. (One yeast was also identified.)

For each of the following experiments, bacteria were cultured from the soil suspension, as fitness and natural selection influence the bacterial population present on the slants.

Isolation of a resistant strain. Since the bacterial population was mixed and different types would have

different optical densities, it was decided to culture onto agar and measure the radial growth, rather than measure the optical density in liquid cultures. The mixed culture was scraped off the surface of the slant using a disposable innoculum needle and added to a Universal container, which contained 10 cm^3 of sterile water. The mixture was vortexed to ensure an even distribution of bacteria. 0.2 cm^3 of the suspension was placed in the centre of a petri plate, which contained various amounts of TBZ suspended in 20 cm^3 of nutrient agar. Four replicates were prepared for each treatment and the plates were incubated at 21^oC. At two day intervals, radial growth was measured. However, in each of the treatments i.e. 0, 10, 20, 30, 40 and 50µg TBZ per plate, no inhibition of growth was observed. Therefore the experiment was repeated using 0, 100, 200 and 500µg TBZ per plate, but again, no inhibition of growth was observed.

In conclusion, it would appear that TBZ has little or no bacteriocidal activity, and that the chemical is not metabolised by micro-organisms. It may be that no metabolism of the chemical took place because the nutrient agar/ broth provided a readily available energy source for the bacteria, and if a restricted nutrient source had been used, then some metabolism of TBZ may have resulted.

In the next sections, the metabolism of, and the development of resistance to TBZ, by fungi, is discussed.

The metabolism of Thiabendazole by fungi.

In Britain, the primary agricultural use of TBZ, is as a fungicide. It is used to control many other fungi, other than those found on potatoes. The more frequently a fungus comes into contact with a chemical, then the greater the likelihood that the fungus will develop resistance to that chemical. Georgopopoulos (1977) has reviewed the genetics and the biochemical mechanisms of fungicide resistance, and has reported 41 instances in 23 different fungi, where TBZ resistance developed.

As TBZ is the most commonly used fungicide on potatoes, then the possibility exists that fungal resistance may develop. In the next sections, the likelihood of fungal resistance developing, as well as the isolation of TBZ metabolites, is discussed.

Metabolism of Thiabendazole by soil fungi.

Since TBZ was likely to come into contact with soil fungi, present on the surface of the potato, metabolism studies using fungi isolated from soil were carried out.

Isolation of soil fungi. Fungi were isolated from soil (using the same soil suspension, used for the isolation of soil bacteria) by transferring aliquots of the soil suspension onto petri plates, containing 2% Malt agar. After five days incubation at 21^{o}C, three distinctive organisms

were isolated by serial dilution plating (10^5). They were identified as *Phytopthora infestans*, a *Penicillium* (family Moniliaceae (Fungi Imperfecti)) and a third organism (F1), the thallus of which was coenocytic, but was not identified further. Each of the isolates were maintained on 2% Malt agar slants.

Isolation of resistant strains. Each of the three fungi were incubated in the presence of TBZ in order to try to isolate resistant strains of fungi. A suspension of each fungi was prepared using a disposable innoculum needle and sterile water. 0.2 cm^3 of the suspension was placed on the centre of a petri plate containing 0, 10, 20, 30, 40 and 50µg TBZ per plate in 20 cm^3 of 2% Malt agar. Four replicates were prepared for each treatment and were incubated at 21°C. At two day intervals, the radial growth in each plate was measured.

Growth of the F1 isolate and of *Penicillium spp.* were completely inhibited by TBZ, and therefore TBZ was displaying fungistatic activity on both of these fungi. The innoculum which was transferred to the plate initially remained alive for 14 days, but no further growth of either organisim was observed.

By contrast, TBZ showed no inhibitory effects on the growth of *P. infestans* at the levels tested. Therefore, the experiment was repeated using higher levels of TBZ (0, 100, 200 and 500µg TBZ per plate). At these levels, degrees of restriction of growth were observed. Table 6.3 shows the

radial growth of the fungi at different levels of TBZ, expressed as a percentage of the fungal growth in the

Table 6.3.

Amount of TBZ per plate (µg)	% Growth with time* Day 2	Day 4	Day 6
0	92%	100%	100%
100	51%	76%	100%
200	48%	70%	100%
500	0%	38%	75%

* mean of four replicates

control.

By day 10, *P. infestans* in the 500µg TBZ plate treatment, had covered the whole plate (100% growth). Full plate cover in the control was observed at day 4. Hence the presence of TBZ was restricting the growth of *P. infestans* but growth was not arrested altogether.

The fungi were left to grow on the plate until the agar had dried up, and no further growth could take place. The residue was extracted with 100 cm^3 methanol and then filtered before the sample was injected into the HPLC. However, again no loss of TBZ or appearance of 5-hydroxy TBZ was observed. Hence it would appear that *P. infestans* was using some route other than metabolism, to overcome the

effects of TBZ.

Hence, it would appear that the breakdown of TBZ by soil fungi and bacteria is negligible. These were short term experiments, in relation to the time that a chemical may persist in the soil. However, the most likely reason for the lack of metabolism of TBZ is that there was an alternative, more readily available energy source present, in the nutrient agar, and this was used by the organisms, rather than TBZ.

Breakdown of TBZ in the soil is discussed in a review article by Zbozinek (1984). It would appear that physical and chemical soil factors such as pH, soil type, cation exchange capacity, organic matter content and aeration affect the breakdown of TBZ, since these factors will affect the availability of TBZ to the micro-organisms as well as influencing the microbial populations present in the soil in the first place. In general, the persistence of TBZ is temperature dependant, where the breakdown of TBZ increases with temperature.

The experiments in this review were carried out in whole soil, and the disappearance of TBZ was followed over a period of months, where environmental factors would play an important role. However, by carrying out *in vitro* studies with individual bacteria and fungi, this made it easier to follow the development of resistance to and the metabolism of, TBZ.

In the following sections, the metabolism of TBZ by fungi which the chemical controls, is assessed. The ease at which the fungi developed resistance to TBZ was also studied.

Metabolism of Thiabendazole by *Polyscytalum pustulans*.

P. pustulans is the fungus responsible for the disease known as skin spot. It has been reported that chlorpropham is thought to promote the incidence of skin spot on tubers (French, 1976), yet TBZ is reported to control the disease.

A series of experiments were set up where the growth of *P. pustulans* in the presence of different amounts of TBZ was recorded. The presence of metabolites, as well as the development of resistance was also investigated.

Isolation of a resistant strain. *P. pustulans* was transferred from a 2% potato dextrose agar (PDA) slant onto a plate containing 2% PDA and incubated at 21^{o}C for seven days. After this time, cores of *P. pustulans* / agar were taken from the advancing edge of the fungi, using a sterile No. 1 cork borer. Each core was then placed in the centre of a petri plate, which contained 0, 10, 20, 30, 40 or 50µg TBZ per plate, in 20 cm^{3} of 2% PDA. Four replicates were set up for each treatment and the plates were incubated at 21^{o}C. At two day intervals, radial growth of the fungus was measured.

After 14 days, no subsequent growth of *P. pustulans* had taken place on the plates which contained TBZ. The fungi

did continue to grow on the cores of agar, used to innoculate the plates, as this agar did not contain any TBZ. It was difficult to conclude whether TBZ was displaying a fungitoxic or a fungistatic effect on the development of the fungi, because the core did not contain any TBZ. However, two things were apparent in that i) metabolism of TBZ by *P. pustulans* is highly unlikely and ii) the development of resistance of the fungi to the chemical does not appear to be feasible.

Metabolism of Thiabendazole by *Phoma exigua var.foveata.*

P. exigua is the fungus responsible for the development of gangrene in potatoes. A similar experimental set-up, to that used for *P. pustulans* was adopted.

Isolation of a resistant strain. Resistant strains of *P. exigua* were isolated in a similar way to *P. pustulans*, except that 2% Malt agar was the growing medium used.

At two day intervals, the radial growth on each plate was measured, and it became apparent that resistance to TBZ was developing to TBZ. The results are reported in table 6.4, where growth is expressed as a percentage of the daily growth in the control.

It is obvious from the results that as the amount of TBZ present in each plate increased, the growth of *P. exigua* decreased. Growth is not inhibited completely, therefore some type of resistance to TBZ must be developing.

Table 6.4

Amount of TBZ per plate (µg)	% growth with time*				
	Day 2	Day 4	Day 6	Day 8	Day 10
0	100%	100%	100%	100%	100%
10	96%	95%	95%	100%	100%
20	61%	67%	79%	89%	70%
30	53%	51%	52%	55%	52%
40	39%	24%	20%	22%	17%
50	42%	19%	17%	13%	10%

* mean of four replicates

It was decided to culture on the fungi which were growing on the 30µg TBZ per plate treatment, as there was sufficient growth of fungi, yet some resistance to the chemical was developing.

Cores of the resistant *P. exigua* were taken from the advancing front, and transferred onto plates containing 0, 30, 40 and 50µg TBZ per plate, and incubated and measured as before. The percentage growths are shown in table 6.5.

Table 6.5

Amount of TBZ per plate (µg)	% growth with time* Day 2	Day 4	Day 6	Day 8	Day 10
0	100%	100%	100%	100%	100%
30	78%	76%	77%	84%	79%
40	29%	33%	38%	40%	39%
50	28%	31%	27%	27%	30%

* mean of four replicates

As before, radial growth decreases, as the amount of TBZ increases. However, it was interesting to note that the radial growth of the resistant *P. exigua* was greater at given TBZ level, compared with the radial growth in plates innoculated with non-resistant *P. exigua*, at the same TBZ level. Hence it would appear that the ability of the resistant strain to overcome the effects of TBZ, was increasing as the time of exposure to the chemical, increased. Therefore problems with resistance of *P. exigua* to TBZ may result with frequent usage of TBZ, and if an innoculum of *P. exigua* which had been exposed initially to TBZ, remains in the soil, or in the store, then a second encounter with the chemical will almost certainly increase the fitness of the resistant strain.

Isolation of Thiabendazole metabolites. As the greatest

resistant fungal growth was observed in petri plates containing 30µg TBZ, extractions were carried out to discover whether any of the TBZ had been metabolised by resistant *P. exigua*.

The plates were left to dry for four weeks, and the residue from each of the four replicates was pooled, and dissolved in 100 cm^3 of methanol. The solution was filtered and then evaporated down to 10 cm^3. The solution was analysed by HPLC (as described in Chapter 2).

Of the 120µg TBZ initially present in the extract, only 100µg TBZ were detected in the fungal extract. Therefore 20µg TBZ remain unaccounted for, and may have been metabolised. HPLC analysis for 5-hydroxy TBZ (as described earlier) was also carried out. In the development of the HPLC method for the analysis of 5-hydroxy TBZ, the minimum amount of 5-hydroxy TBZ which could be detected in "spiked" fungal extracts was 10 $\mu g cm^{-3}$. Baseline noise was too great to detect levels of 5-hydroxy TBZ lower than this.

When the *P. exigua* extract was analysed by HPLC, no 5-hydroxy TBZ was detected. If 20µg TBZ had been metabolised to 5-hydroxy TBZ, then the concentration in the fungal extract would have been 10 $\mu g cm^{-3}$, but since none was detected, then the amount of 5-hydroxy TBZ present must have been lower than the detection limits of the HPLC method.

Therefore, although one could not be precise in the quantitation of 5-hydroxy TBZ, it is fair to say that the concentration of 5-hydroxy TBZ in the *P. exigua* extract was less than 10 $\mu g cm^{-3}$.

In order to discover whether any of the TBZ unaccounted for, had been metabolised into 5-hydroxy TBZ, the fungal extract was subjected to mass spectrometry. The fungal extract was concentrated up, and an aliquot of it subjected to an M.S. 12 Single Focusing Mass Spectrometer (ionisation voltage = 70eV, running temperature = 180°C). The spectrum obtained is shown on Fig. 6.2.

A peak characteristic of TBZ was seen at 201 AMU (and at 174 AMU, characteristic with the loss of HCN), and another peak at 217 AMU, characteristic of 5-hydroxy TBZ (and at 190 AMU, characteristic with the loss of HCN).

Therefore *P. exigua* had metabolised some TBZ to 5-hydroxy TBZ. Unfortunately, due to the compounds having different response factors, the relative amounts of TBZ and 5-hydroxy TBZ could not be quantified. However, it is reasonable to assume (from the minimum detection limits of the HPLC method for 5-hydroxy TBZ) that of the 120µg TBZ added to the cultures initially, only levels less than 0.1 µg TBZ (equivalent to 10 $\mu g cm^{-1}$) were metabolised, and hence less than 0.08% of the native TBZ was being metabolised.

With the hydroxylation of TBZ now having taken place, many other metabolites may be formed e.g. conjugation with glucuronic acid, sulphuric acid etc., and these can readily be excreted or metabolised further.

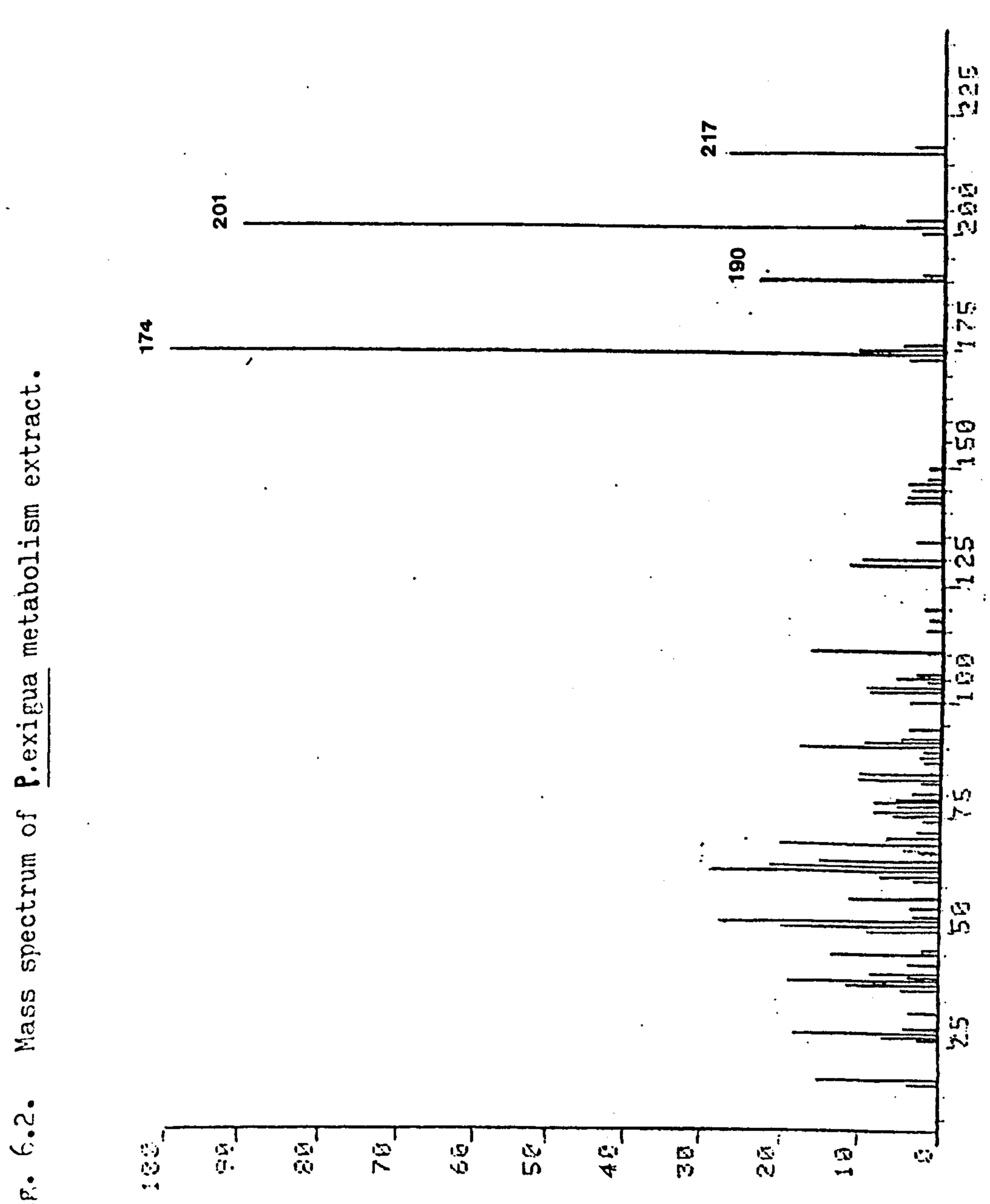


Fig. 6.2. Mass spectrum of P.exigua metabolism extract.

It would appear that *P. exigua* could easily develop resistance to TBZ, and that the development of resistance is a consequence of the fungal metabolism of TBZ.

Metabolism of Thiabendazole by *Helminthosporium solani*.

H. solani is the fungus responsible for the silver scurf infection in potatoes. It can result in large water losses from tubers and therefore reduce the storage quality of the crop. TBZ is used in Britain to inhibit the development of *H. solani*.

Isolation of a resistant strain. *H. solani* was transferred from slants onto petri plates containing 20 cm^3 of 2% oatmeal agar and 0, 10, 20, 30, 40 and 50µg TBZ, using the stab innoculum technique. The plates were incubated at 21^oC for two weeks, and radial growth recorded at two day intervals.

The organism developed very slowly, as full coverage of the control plates took two weeks. Fungal growth in the plates containing TBZ was greatly inhibited, although the inhibition observed differed from that seen with other micro-organisms. Up until Day 6, the radial growth in each treatment was uninhibited compared with the control. However, after Day 6, any further growth of *H. solani* in plates containing TBZ was inhibited. Therefore, although some resistance did develop, it was very short lived,

therefore resistance in a store situation is not likely to be a problem.

Metabolism of Thiabendazole by *Fusarium solani*.

Fusarium is the fungi responsible for the dry rot fungus in potatoes. There are numerous strains of the fungi, and in these experiments, *Fusarium solani* was used.

Isolation of a resistant strain. The choice of growing media for *F. solani* will affect its growth and the metabolites produced. Therefore two different media were chosen in the hope that a TBZ metabolite was produced. *F. solani* was transferred to petri plates containing either i) 2% Malt agar or ii) 2% PDA which is a nitrogen deficient nutrient source, along with 0, 10, 20, 30, 40 or 50µg TBZ. The transfer was made using the stab innoculum technique. The plates were then incubated at 21^{o}C, and radial growth measured at two day intervals.

However, growth in each of the plates containing TBZ was completely inhibited. Therefore the likelihood of *F. solani* developing resistance to TBZ was very low.

In conclusion, the probability that TBZ resistance will develop in *P. pustulans*, *H. solani* or *F. solani* is very low, as little or no subsequent growth of the fungi took place. In practice the levels of TBZ present on potatoes

would be higher, and the growth of any of these fungi would be inhibited. By contrast, *P. exigua* developed resistance to TBZ relatively easily, and TBZ metabolites were detected.

It is worth noting that resistant strains of *P. exigua* were grown at optimum temperature and where there was a readily available source of nutrients. In a potato store, the temperature is much lower and the source of nutrients not so readily available. In addition, the levels of TBZ with which the fungus would come into contact, will be much higher in a store. Hence, although resistance did develop readily under optimum conditions, a different picture would probably exist under store conditions and it would therefore be of value to carry out metabolism studies in a store, in order to ascertain whether or not the development of resistance of *P. exigua* to TBZ was likely to become a real problem.

It would appear that only very small amounts of TBZ were metabolised to 5-hydroxy TBZ. By using radiolabelled TBZ, the metabolism or degradation of the compound could be studied in more depth and the identification of metabolic products would be easier. However, loss of chemical activity would be minimal via metabolism.

In the final chapter, the conclusions of this thesis are drawn together.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK.

The objective of this thesis was to gain more information about thiabendazole (TBZ), in its capacity as a post-harvest fungicide, applied to potatoes. The work followed two general paths :- i) TBZ residues in both potatoes and potato products and ii) a study of factors which may influence, directly or indirectly, the storage of treated potatoes.

The work in Chapter 2 describes the development of an analytical technique, used to determine TBZ residues in potatoes. Although there were a number of methods in the literature, none were found to be easily adapted for the quantification of TBZ in potatoes or proved too laborious for routine residue analysis.

TBZ has been extracted from a variety of produce, including yams, bananas and citrus fruit. However, the only method reported for the extraction of TBZ from potatoes was that of Cayley et al (1983). These workers used ethyl acetate to extract TBZ and partitioned the extract with hydrochloric acid, before quantifying the TBZ using UV or fluoresence spectroscopy. However, this technique was found to be laborious and lacked the sensitivity required. Therefore, an extraction method was developed where TBZ was extracted from macerated potato tissue by blending it with dichloromethane and anhydrous sodium sulphate.

The extract was then cleaned up by passing it through a Bond Elut diol column. The clean-up procedure developed was much faster and more quantitative than traditional column clean-ups or solvent partitioning and a clean-up could be carried out in a matter of minutes, rather than hours. In addition, the the volume of solvent required to elute TBZ from the Bond Elut column was much reduced, compared with the other clean-up procedures. A clean sample was now available for quantification.

TBZ has been quantified using thin layer chromatography (TLC), gas chromatography (GC), spectrophotometry and HPLC. TLC and spectrophotometry lacked the desired sensitivity, and GC required derivitisation of the sample before analysis. Hence HPLC was evaluated for the quantification of TBZ. When using HPLC, the type of detector used, depends on the physical properties of the chemical, and in the case of TBZ, fluoresence was chosen, as it is very specific and problems with interfering peaks is less likely to create problems. The conditions developed utilised a moderately polar phase column and a polar mobile phase. Under these conditions, a retention time of approximately 8 minutes was recorded for TBZ.

Hence, a satisfactory method was derived for the extraction, clean-up and quantification of TBZ from potatoes. The recovery factor for this method was calculated as 93.8% ± 2.1%, and levels down to 0.004µg TBZ could readily be detected from potato extracts.

The remainder of Chapter 2 was concerned with the distribution of TBZ residues in potatoes. Although Cayley and

Hide have carried out a number of research projects on TBZ, they have mainly been concerned with storage quality, rather than chemical analysis, and as a result, little has been reported on the distribution of TBZ within the potato. Of the work reported on residue levels in potatoes, ambiguity often resulted when comparing different workers results, since the sample chosen for residue analysis was seldom stated clearly. Hence the objective of this work was to report the distribution of TBZ in the potato, study the penetration of TBZ into the potato and to try to make some suggestions as to which sample would be of greatest value when carrying out residue analysis in potatoes.

Residue analysis was carried out in various fractions of TBZ treated potatoes, at different periods of the storage season. From the results it was obvious that there was little movement of TBZ into the tuber, even though TBZ is classed as a systemic fungicide. Large quantities of the residue were removed with washing, and in general, all of the residue can be removed with peeling. The poor penetration of TBZ into the tuber is probably a consequence of its polarity. Suberin is a non-polar component of the periderm, and surrounds the tuber surface. Hence, due to the polarity of TBZ, the chemical cannot penetrate through this barrier, and so associates with the skin of the potato.

Although the penetration of TBZ was hindered by suberin, it was observed that as time progressed, the TBZ residue present in the whole, washed tuber increased, but a corresponding increase of the residue in the peeled tuber did not result. Therefore, it would appear that the uptake of TBZ

from the surface of the tuber into the skin increased with time, yet the suberin barrier impeded its path into the flesh of the tuber.

A fundamental problem in trying to compare one's research work with that of others, is the non-uniformity of the sample taken for residue analysis. Part of the work in this chapter was related to trying to establish which sample would provide the most meaningful residue value. After taking a number of factors into account, it was decided that a residue in the whole washed tuber provided the most information and was less succeptible to other factors which may influence the final residue figure, such as handling, peeling, etc.. TBZ residues in the whole washed tuber could also provide a value for the amount of TBZ likely to be consumed, as well as giving an indication of the amount of chemical which had been applied in the first place.

The objective of Chapter 3 was to discover whether a seed treatment of TBZ would improve fungal disease control in the progeny tubers. Unfortunately, the expectations of this work were not met. Seed was planted with various TBZ treatments applied, and the crop was grown and harvested in accordance with local farming practices. The progeny were then treated with various amounts of TBZ before being stored. Residue analysis and disease assessment were carried out at intervals throughout the storage season. However, the principle problem with this experiment was that perfectly healthy progeny had been grown in the first place and so comparisons in the disease control between treatments and

controls could not be made. It was appreciated that this was not normal, otherwise there would not be any need for post-harvest fungicides such as TBZ.

An attempt was made at artificially innoculating the seed with cultures of fungi which TBZ is reported to control. The most diseased tubers were those which received no additional treatment of TBZ at the commencement of storage, and the greatest disease control was achieved in the tubers treated with 40 $mgkg^{-1}$ TBZ, the commercial application rate. The most predominant fungus present on the tubers was *P. exigua*, which was present on each of the tubers in each treatment. Work in Chapter 6 showed that *P. exigua* readily developed resistance to TBZ (under optimum conditions) and that the chemical was metabolised. However, it was surprising that the fungus grew so readily in less than optimum conditions.

The other conclusions drawn from the disease assessment study were general microbiological observations :- i) *H. solani* tended to spread throughout the tuber and thereby restrict the growth of other fungi, ii) *F. spp.* only infected a small proportion of the tuber and iii) where fungal infection did result, secondary infection from fungi already present in the soil surrounding the tuber, resulted.

There is plenty of scope available in this area of research. If the amount of chemical applied at the commencement of storage was reduced with the aid of a seed treatment, the amount of chemical ingested by the consumer would also be reduced. Unfortunately, disease resistance carryover from the seed into the progeny could not be properly

assessed, and using an artificial innoculum didn't really shed much light in this area.

One point of particular interest was the ability of the chemical to be translocated from the leaves, down into the tubers, when applied foliarly. Scottish weather conditions are by no means ideal for the foliar application of chemicals, yet some TBZ was translocated. Again, by using a foliar application, the amount of TBZ required for contol in the progeny tubers may be reduced although i) a suitable formulation of TBZ would have to be developed and ii) as the chemical is translocated in the phloem, along with the assimilates, then the TBZ may be distributed throughout the tuber, like maleic hydrazide and hence the amount of chemical eaten may not actually be reduced (compared with eating a peeled potato which had been treated at the commencement of storage).

Therefore, there is a great deal of research which could be carried out into the area of seed treatments as a means of reducing the amount of chemical applied to the ware crop.

The objective of Chapter 4 was to gain more information about the effects that TBZ and its formulations had on the wound healing capacity of tubers. In the majority of these experiments, wound healing was measured in terms of the development of resistance to water loss, which was directly related to periderm formation. Although there were many different parameters which could be studied to follow the process of wound healing, measurement of the development of

resistance to water loss allowed an adequate degree of replication to be achieved, which was quantitative, yet not as laborious to measure as other parameters. Using this method also meant that many different experiments could be set up relatively easily.

The first series of experiments were set up to examine the effects that TBZ had on the wound healing capacity of cut potato discs. The results showed that very low levels of TBZ (0.05µg and 5µg TBZ applied per disc) promoted the development of resistance to water loss, during the first nine days of the study, compared with the control discs. However, at the end of the 21 day study, the development of resistance to water loss in both treatments and in the control, had risen to the same level. When commercial application rates were examined (80µg TBZ applied per disc), the development of resistance to water loss was again promoted, compared with the control, but both treatment and control had reached the same level at the end of the study.

It was interesting to note that the resistance maxima in the 80µg TBZ per disc study was delayed and lower, compared with the 0.05µg and the 5µg per disc studies. This may imply that higher levels do reduce the ability of the disc to wound heal, but not to the same extent as the ability of control discs. It might be that if even higher levels of TBZ were applied e.g. by mistake when preparing the formulation, then this may reduce the ability of the discs to wound heal, and could actually be detrimental to the wound healing process. Obviously more work would have to be carried out into this area.

The next area of research was concerned with studying the effects that the formulation components had on the wound healing process. Two TBZ formulations were considered, namely Storite, a neutral suspension of TBZ, and Storite Clear, an acidic formulation.

TBZ and the formulation Storite, have been associated with the idea that they promote the wound healing process, with very little evidence to back this up. Therefore a study was undertaken to try to establish whether or not Storite had any effects on the wound healing capacity of tubers, measured in terms of the development of resistance to water loss. In carrying out this experiment, the effects that the formulation components had on wound healing could also be assessed.

Wound healing was measured in discs treated with TBZ in methanol, TBZ in the form of Storite, a methanol and a water control. The presence of the formulation components did not seem to affect the wound healing capacity of the discs to any extent. However, it is worth noting that this was not a direct comparison since TBZ in methanol was in the form of a true solution, yet TBZ in the form of Storite was in the form of a suspension, but it was as good a comparison as could be made.

There have been incidents reported where potatoes treated with Storite Clear have developed a scorch-like blemish on the surface of the tubers. A skin quality assessment study was set up where tubers were treated with Storite Clear and Storite, at the commercial application rate, and stored for several months. After three months storage, the Storite Clear treated tubers had a flaky appearance,

indicating that poor skin setting had taken place, yet the skin which had not come into direct contact with the Storite Clear i.e. skin beneath clumps of soil adhering to the tuber, had set normally. Also approximately one quarter of the tubers treated with Storite Clear had developed a scorch-like blemish, which at three months, had just penetrated beyond the tuber surface. Neither the Storite or the control tubers showed any evidence of this blemish or of poor skin setting.

Hence it was obvious that the Storite Clear formulation was affecting skin setting in some way. Therefore a resistance study was set up to see if Storite Clear also affected the development of resistance to water loss. The development of resistance to water loss was measured in discs treated with Storite, Storite Clear and in control discs. The results showed that Storite Clear was indeed inhibiting the development of resistance to water loss, and that excessive rotting also took place.

Hence it would appear that Storite Clear does not posess any of the beneficial effects of Storite, and this would probably be a result of the acid present in the formulation. From a storage point of view, the effects of using Storite Clear as a post-harvest fungicide could prove disasterous. When the resistance deveopment experiments were initially set up, 100% of the Storite Clear treated discs rotted. In a store, at the beginning of the storage season, there is a large *E. carotovora* population present, and so anything which may reduce the tuber's nataural resistance to this bacterium would certainly reduce the storage quality of the crop.

Rotting aside, the appearance of the Storite Clear treated tubers is unattractive and so lower premiums would be paid for the crop. However, the scorch blemish has a more significant impact on the potato processors, since a larger proportion of the tuber would have to be discarded, to remove all of the blemished tissue, before processing could take place.

Overall, there appears to be little benefit of using Storite Clear to control post-harvest fungi.

The effects that a combined formulation of TBZ and 2-aminobutane, Storite Plus, had on the development of resistance to water loss was also studied. There have been reports of tubers, which were treated with 2-aminobutane, that had poor skin quality. Therefore, it was of interest to discover if the inclusion of TBZ in the forumlation, could overcome the detrimental effects of 2-aminobutane.

After carrying out the series of experiments, it was clear that Storite Plus was affecting wound healing in some way. What effect, however, was unclear. Unfortunately, no matter how often the experiments were repeated, no pattern to the development of resistance to water loss could be established. The strange results may have had something to do with the volatility of 2-aminobutane, in that unequal headspaces may have resulted. A significantly higher degree of rotting was observed in discs treated with Storite Plus, yet some of the other discs appeared to wound heal normally. It could be possible that if the discs get damaged in any way, then bacterial rotting can occur, and the formulation may accelerate this. But if the discs are relatively undamaged,

then normal wound healing will take place.

This area obviously requires further investigation, but it does seem strange that a formulation of TBZ and 2-aminobutane is marketed in the first place. It is generally accepted that 2-aminobutane does little for the appearance of the tuber, yet no additional fungicidal activity is achieved, compared with using TBZ on its own. The only benefit of having 2-aminobutane in the formulation would appear to be its volatility, an advantage if full coverage of the crop is not achieved. It would be of value to see how this formulation behaves in a storage situation.

Now that the effects that TBZ had on wound healing had been ascertained, the effects that it had on skin setting and periderm development, i.e. curing, was studied. This was done by following the moisture loss, by weight, in individual tubers which had been treated with Storite before and after curing, as well as on wounded and non-wounded tubers. From the data, moisture loss from Storite treated tubers was not significantly different from control tubers, in each experiment. However, comparisons between moisture loss data from tubers before and after curing could not be made because the tubers which had been cured prior to treatment had begun to sprout, six days after treatment, and so exaggerated moisture losses were recorded. Sprouting in these tubers was probably a combination of late harvesting followed by a relatively high temperature during curing.

Hence, although TBZ appears to promote wound healing, it has no beneficial or detrimental effects on curing.

Chapter 5 describes work carried out to try to quantify TBZ residues in processed potato products. Very little information was available on TBZ residues in processed products and so this research was to provide the basis for future research in this area. A number of residue methods were developed and cooking methods were also standardised.

Residues were measured in potatoes which had been boiled, baked and crisped. On the whole, the TBZ residue was not affected by the cooking procedure, in terms of the absolute residue and the nature of the residue. The exception to this was baked potatoes which had been prepared by microwaving. The residue in these potatoes was significantly lower than the residue in oven baked potatoes. The extract was subjected to mass spectrometry to see if any decomposition products could be detected. However none were found, and it could be that the decomposition products were not extracted in the first place.

TBZ could hardly be described as a toxic chemical to mammals since it has a very high LD_{50} value of 3300 $mgkg^{-1}$ in rats, and the levels detected in these processed potato products are of the order of approximately 1 $mgkg^{-1}$ product. Therefore, the annual consumption of TBZ is going to be fairly low. However, one should not disregard residue levels as being insignificant. It is common knowledge that people eat the skins of potatoes more frequently, as they are considered to be a valuable source of fibre and vitamins. Yet it is in the skin of the potato where the residues concentrate up. Therefore, the consumption of the chemical may be higher than first anticipated.

Also, the acute toxicity of a chemical is not the only criterion to be considered. The medical profession seem to be linking allergies and hyperactivity to the diet, in terms of additives and pesticides, in particular. Therefore, now matter how innocuous a chemical or its residue may appear, one must always be able to explain how much is present, where it associates, what happens to the residue during cooking and identify the form in which the chemical is present.

The aim of the metabolism chapter was to see whether TBZ was metabolised and whether the development of resistance to the chemical was likely to become a problem. A number of fungi and bacteria were grown in the presence of TBZ to see if growth was restricted. The only fungi which were found to grow in the presence of TBZ were *P. infestans* and *P. exigua*. Both of the bacterial cultures were found to have degrees of resistance, however, the only micro-organism found to metabolise TBZ, was *P. exigua*. The metabolite formed was 5-hydroxy TBZ, and now that hydoxylation had taken place, it is possible that other metabolites may in fact form, including sulphuric and glucuronic acid conjugates. It would be interesting to discover whether the metabolites produced, had any fungicidal activity.

P. exigua is one of the fungi which TBZ is reported to control, and so it may appear that the development of resistance to the chemical, by the fungus, may become a problem. However, resistance was induced in fungi grown under optimum temperature and where the nutrient source was readily available. In a store, the development of resistance is not

likely to be as much of a problem as one might have concluded from the *in vitro* studies, since the store temperature is considerably lower, the nutrient supply is not so readily available and resistant fungi lack the fitness of non-resistant fungi, therefore the likelihood of the carryover of a resistant strain from one storage season into the next, is fairly low.

One point worth noting came from the innoculation studies, carried out in Chapter 3. After innoculation, the most predominant fungus present on the tubers was *P. exigua*, even though the tubers had been treated with TBZ. Although a large innoculum was used, it does seem strange that this particular fungus should grow so readily. Obviously more research should be carried out at the store to see if *P. exigua* is better at adapting to less than optimum conditions, than other fungi, and to discover just how big a problem this could become.

In a summary, the following conclusions were drawn from this research :-

i) a suitable extraction, clean-up and analysis method was derived for the quantification of TBZ from potatoes,

ii) the distribution and penetration of TBZ was ascertained. The residue associates with the skin of the potato, and most of the residue can be removed with peeling,

iii) cooking of the potato did little to affect the residue value or the nature of TBZ, with the exception of microwaving, where a reduction in the amount of TBZ present initially, was observed,

iv) very small amounts of TBZ are translocated from treated seed into progeny tubers. However, due to the fact that all of the progeny, including control progeny tubers, were disease free, no conclusions could be made with respect to the carryover of disease resistance into the progeny crop,

v) TBZ would appear to promote the development of resistance to water loss, during the initial stages of wound healing, compared with the control,

vi) the formulation components of TBZ affect the ability of cut potato discs to wound heal. The formulation components of Storite promoted the wound healing capacity of cut potato discs to the same extent as TBZ. However, Storite Clear inhibited the development of resistance to water loss in cut potato discs and excessive bacterial rotting was observed,

vii) no satisfactory conclusions were reached regarding the effects of Storite Plus (TBZ plus 2-aminobutane) on the wound nealing capacity of cut potato discs, except that wound healing was affected in some way,

viii) Storite does not appear to affect the tuber's ability to cure, even though it does promote wound healing and

ix) in general, of the micro-organisms tested, TBZ either inhibited their growth, or if resistance to the chemical did develop, then it was not via metabolism. The one exception to this was the metabolism of TBZ by *P. exigua*, where 5-hydroxy TBZ was detected.

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