ROTTING OF POTATO TUBERS BY THE BLACKLEG BACTERIUM, ERWINIA ATROSEPTICA

by

DAVID ERIC STEAD, B.Sc. (Hons.)

A thesis submitted for the Degree of

Doctor of Philosophy

Imperial College of Science
and Technology,
Silwood Park,
Sunninghill,
Ascot,
Berkshire.

December, 1973

ABSTRACT

Pectolytic bacteria were isolated from soil and potato tubers using a single-layer pectate gel and Erwinia spp. were determined from these by their negative oxidase reaction. The results were sometimes unreliable because other bacteria grew in close association with the erwinias.

These methods indicated that the pectolytic bacteria (mainly Erwinia spp.) are present in some seed tubers and that when these tubers decay they are present in the surrounding soil and on the new, developing tubers. Treatment of young tubers with 0.25% 2-3,5 triphenyl tetrazolium chloride (sodium salt) showed that Erwinia spp. were present both on the tuber surface and within lenticels. In storage these bacteria usually remained latent within the lenticels but they initiated a rot when the tuber surface was covered with a moisture film. The further development of this rot was favoured by increasing the turgidity of tissue and restricting aeration.

The enzyme involved (an endo-pectate trans-eliminase) had optimum activity at pH 9. It was induced by pectic compounds and by cell wall material from potato tubers and its synthesis was repressed by glucose, fructose and sucrose. These sugars are present in tuber tissue in concentrations above those required for the repression of trans-eliminase synthesis in vitro, but they are contained within cells and thus their availability is limited. Consequently, the rate of rotting may depend on the concentrations of free sugars.

There was little evidence from experiments that phenols and associated enzymes were involved in resistance to rotting. Physical barriers such as suberised cells may be important in restricting the soft rot bacteria to lenticels.

Experiments using various newly-produced anti-bacterial chemicals as dips gave some promising results. The work is discussed in relation to present and to future control practices.

ACKNOWLEDGEMENTS

My sincere thanks are due to my supervisor, Professor R.K.S. Wood for his invaluable guidance throughout this project.

I would also like to express my thanks to the following for their assistance,

Mr. Cywinski (Wingfield Industries) for supplying a sample of Tricidal RE-10.

Dr. R.T. Fox (Plant Protection) for supplying a sample of DS 9073.

Dr. T. Bent (Plant Protection) for supplying a sample of Sanspor.

Buckman Laboratories Limited for supplying samples of Bunema, HPMTS, and WSCP.

Professor A. Kelman for his invaluable suggestions.

Dr. G. Murdie and Miss L. Rushton for statistical advice.

Mr. E.E. Green, Mrs. P. Tyler, Miss C. Davies, and Mr. A. Yeardley for technical assistance.

I am especially grateful to fellow students Mrs. A.J. Prickett (nee Phear), Mr. D.K. Martin and Mr. N.P. Summers for providing invaluable assistance in obtaining results for work involving eradication, sugar changes of tubers during dormancy break, and a comparison of selective media, respectively, and to Miss V.A. Taylor who has afforded me constant encouragement during the last two years.

This work was carried out during the tenure of an award donated by the Potato Marketing Board, the receipt of which is most gratefully acknowledged.

CONTENTS

	Page
TITLE PAGE	1
ABSTRACT	2
ACKNOWLEDGEMENTS	4
CONTENTS	6
INTRODUCTION	10
REVIEW OF THE LITERATURE	12
MATERIALS AND METHODS	27
EXPERIMENTAL WORK	
SECTION I. ISOLATION AND IDENTIFICATION OF THE CAUSAL AGENT	41
A comparison of selective and semi-selective media for the isolation and identification of *Erwinia atroseptica*	41
The effect of the media on growth of *E. atroseptica*	41
The selectivity of the media	44
Recovery of *E. atroseptica* from artificially infested soil	45
The use of Cuppels and Kelman's medium to estimate population densities of *E. atroseptica* during rotting	46
SECTION II. THE INFECTION PROCESS	49
The incidence of infection of tubers in the field	49
Observations on heel end rots (1972)	56
The origin of hell end rots	56
The infection in relation to soil water content and tuber size	57
Development of VTSC seed tubers in the field	62
Infection of lenticels	63
Incidence of infection in VTSC potato tubers	63
The effect of tuber turgidity in relation to infection	67

Page

Identification of infected areas by the use of 2,3,5 triphenyl tetrazolium chloride 68

The lenticel as the major point of entry of the pathogen into tubers 71

SECTION III. OBSERVATIONS ON THE INFECTION DURING TUBER STORAGE 75

The position of the pathogen in or on the tuber 75

The process of soft rotting 75

Formation of dry limited rots 77

Methods for inducing infected tubers to rot 78

Reduction of the number of sites of infection during storage 79

Viability of soft rot bacteria within lenticels 83

Contamination of VTSC tubers 84

Effect of dormancy on the incidence of infection 85

Examination of the degree of infection of old and new tubers 87

SECTION IV. FACTORS AFFECTING THE SUSCEPTIBILITY OF TUBERS TO ROTTING 89

Water content of tubers 89

Effect of restricted aeration on rotting 93

Effect of non-aqueous films on rotting 93

Effect of different gaseous conditions on rotting 95

Effect of surface moisture on rotting 100

Formation of limited lesions from lenticel infections 103

Importance of surface moisture in relation to the absence of air, on rotting 104

SECTION V. BACTERIAL PHYSIOLOGY IN RELATION TO ROTTING 107

Growth curves of E. atroseptica 107

Effect of temperature on growth of E. atroseptica 109

Effect of pH on growth of E. atroseptica 109

Page

SECTION VII. ERADICATION OF THE PATHOGEN 167

Effect of antibiotics on the growth of the pathogen 167

Effect of streptomycin on rotting 168

The use of Tricidal RE-10 to eradicate E. atroseptica and other soft rot bacteria from potato tubers 170

Investigations into the use of four chemicals for eradicating soft rot bacteria from potato tubers 171

Miscellaneous observations 180

DISCUSSION 181

REFERENCES 207

APPENDIX 214

APPENDIX TABLES 217

Page

Mechanisms of bacterial soft rotting 110

Preparation of pectic enzymes 111

Optimum temperature for PTE activity 112

Effect of temperature on stability of PTE 113

Effect of pH on PTE activity 115

pH optima for enzymes produced on pectic media at pH 8.4 116

Effect of pH on the growth and enzyme production in E. atroseptica A13 cultured on different pectic substrates 120

Effect of potato tuber cell walls as inducing agents for PTE synthesis 125

Induction of PTE by tuber tissues 128

Induction of PTE in various Erwinia spp. 131

Induction and catabolite repression of pectic enzyme synthesis 131

Effect of different carbohydrates on the induction of PTE synthesis 137

SECTION VI. TUBER PHYSIOLOGY IN RELATION TO RESISTANCE 140

Effect of dormancy on tuber resistance to rotting 140

Effect of natural breaking of dormancy 144

Rotting of cold stored, Fusarex treated, and sprouting tubers 145

Effect of major sugars within potato tubers on resistance to rotting 146

Resistance of tuber tissue to maceration by PTE 157

Phenolic substances in relation to resistance 158

Effect on rotting of phenolic substances and certain enzymes 158

Phenols and polyphenol oxidase in potato tubers 163

Prescence of bacteriotoxic substances in potato tuber tissue 165

INTRODUCTION

The gradual introduction of packaged tubers over the last 25 years has readily become accepted by the consumer with the result that many potatoes are now pre-packed, usually in polythene bags. The special environment of the polythene bag favours the onset of rotting by the soft rot coliform bacterium, E. atroseptica, and to a lesser extent, by the related species, E. carotovora and E. aroideae. Furthermore, apparently non-pathogenic bacteria may become pathogenic under the new environmental conditions in polythene bags.

It is known that most tubers are infected during growth in the field (Perombelon, 1973), and that the bacteria remain latent within tubers. Infected tubers stored in dry, cool, aerated conditions rarely rot, but one rotting tuber may result in rotting of many.

The methods used to eradicate the bacterium from potato stocks have had only limited success. A stem cutting programme developed in Scotland has not eliminated the pathogen from potato stocks, although the reasons are obscure. This technique involves the growth of stem cuttings of young potato plants and avoids the carry-over of the bacteria, normally present in seed tubers. As yet, no methods of chemical eradication are available. Such studies are difficult since the infection is not fully understood. The latently infected tuber is certainly different from one inoculated artificially, and it is this difference with which part of this thesis is concerned.

The physiology of the potato tuber is complex. Growth of tubers, from their initiation at the end of a stolon, with rapid accumulation of starch, through a dormant and resting period to a final period of activity in the formation of a new plant, is a process which cannot be viewed as that of a homogenous mass of inactive storage tissue.

Thus, the purpose of this investigation was to study the infection of tubers of the potato plant, Solanum tuberosum L. c.v. Majestic, by the blackleg bacterium, Erwinia atroseptica (van Hall) Jennison, the nature of the resultant latent infection, and the physical, physiological, and biochemical conditions that might determine the development of actively spreading rots in potato tubers.

REVIEW OF THE LITERATURE

Blackleg of potatoes is caused by Erwinia atroseptica, a bacterium belonging to the family Enterobacteriaceae, order Eubacteriales (Bergey, 1948), the name of the disease referring to the blackening and subsequent collapse of the stems of potato plants. The causal organism is probably of greater economic importance in this country as a post harvest pathogen, causing soft rotting of tubers in storage.

Classification and Nomenclature of the Pathogen

There has been some discussion concerning the classification and nomenclature of the pathogen. Burkholder and Smith (1949) envisaged three separate species, E. atroseptica, E. carotovora, and E. aroideae. However, other authors, including Leach (1930), Bonde (1939), Rudd Jones (1950), and Hellmers and Dowson (1953), believe that the first two are varieties of a single species. Thus, the accepted nomenclature is given as, E. carotovora var. carotovora, E. carotovora var. atroseptica, and E. aroideae.

Overwintering of the Pathogen

The method of carry-over of the infection from year to year has long been problematical and currently, three possible means are recognised.

(1) Overwintering of the pathogen in the soil.

(2) Overwintering in diseased tissue in the soil.

(3) Overwintering as a latent infection in the seed tuber.

Jennison (1923) and McKay (1955) suggested that the disease could be controlled by roguing infected plants as the disease symptoms appeared, thus effectively eliminating the possibility of any overwintering.

Leach (1930), Kerr (1953), and Van den Boom (1967) inoculated tubers with soil and obtained rots from which they were able to recover the pathogen, suggesting that it is present in the soil at all times. However, Rosenbaum and Ramsey (1918) and Binianskaite (1963) were unable to find the blackleg organism overwintering in the soil, and Graham (1958) and Binianskaite (1963) concluded that it did not survive in rotting or infected material, although many other highly virulent soft rotting bacteria, often Pseudomonas spp., were isolated. The more recent work of Betencourt and Prunier (1965) and Perombelon (1973) suggests that such results may mean little, since these authors isolated the pathogen from lenticels of tubers, and thus potatoes used in the tests for isolating soil borne bacteria are probably already infected.

The isolation of Erwinia spp. from soil around potato crops during the growing season, by Harper, Boyd, and Graham (1963), led these authors to conclude that the pathogen is a 'soil invader' and able to infect neighbouring tubers after moving some distance through soil. However, survival of the pathogen in the soil for any length of time is questioned by Webb (1971), and work by Logan (1968) on this problem has shown that although it frequently could not be found at all, if conditions in the soil were favourable, it could persist for up to seven weeks. In parallel

laboratory experiments, six weeks was the maximum survival time under a variety of temperature and other conditions, although in buried diseased stem tissue the longevity of the organism increased to twenty seven weeks, but the pathogen was apparently confined to the vascular tissue.

It would, therefore, seem that the carry over of the infection for the relatively long period between potato crops must be explained by a tuber borne latent infection, as suggested by the work of Betencourt and Prunier (1965), and Perombelon (1973) on the isolations of E. atroseptica from lenticels of stored tubers.

Infection of Potato Tubers

Having dealt with the method of carry-over of the infection, the infection of the lenticels should now be discussed. Several authors, Ramsey (1919), and Stapp (1958) cited by Van den Boom (1967) trace the origin of the disease to the seed tuber. Jennison (1923), showed that infection occurred through the stolon, the bacterium being isolated from the heel end of the tuber.

The planting of infected seed does not necessarily result in such a corresponding incidence of the disease in the subsequent crop (Jennison, 1923; Bonde, 1950; and Van den Boom, 1967) but the degree of infection of the seed tuber was found by Logan (1968) to be important. Van den Boom (1967) found considerable variability in the expression of the disease according to growing conditions. Conversely, apparently healthy tubers may be induced to soft rot under certain conditions (Van den Boom, 1967), and Smith (1950),

and Davidson (1948), found that invasion may occur through water soaked lenticels. In addition, Logan (1964), and Rudd Jones and Dowson (1950), observed arrested lesions and Logan (1964) also reported that a hard, black non-spreading rot beneath lenticels may be induced to form a soft rot. Since bacterial infection is not affected by surface sterilisation with mercury compounds or streptomycin (Graham and Volcani, 1961), it appears that it is either systemic, or established below the suberised layers around lenticels.

The foregoing shows that the appearance of symptoms in store or field is not an essential part of the life cycle of the pathogen. Perombelon (1973) has shown that almost all tubers from the field are infected with the blackleg organism. Thus, a symptom free crop is not a disease free crop.

Perombelon (1973) also states that current publications still claim that bacteria mainly overwinter in the vascular tissues of tubers which become infected from stolons.

There is relatively little work on latency, probably because although the process of infection of tubers is now understood in some detail, the period of latency is not. It has been shown (Perombelon, 1973) that blackleg symptoms in the field are caused by E. atroseptica. However, relatively little work has been done on the pathogen's relationship with tubers, during their storage phase.

Classically, the term blackleg refers to a stem disease caused by E. atroseptica, and found to be closely associated with storage rotting. Many authors still

consider storage rotting in terms of E. atroseptica alone. Perombelon (1973) states that the only soft rot coliform bacteria found in tubers and soil around them in Scotland are E. carotovora var. atroseptica, and E. carotovora var. carotovora, the former being detected four times more frequently. Lund and Nicholls (1970) and Rudd Jones (1950) refer to Clostridium infections as well as E. carotovora infections. Graham (1958) has identified Pseudomonas spp. in rots from which no Erwinia spp. could be isolated, whereas Betencourt and Prunier (1965) isolated several strains of E. carotovora from lenticellular dry rots, similar to the varieties, typicum, aroideae, and probably atroseptica.

Isolation and Identification of Erwinia spp.

Techniques for isolating and identifying the three varieties of Erwinia spp. have been studied by Graham (1966) and Dowson (1941), using for example, the ability to ferment various sugars. Logan (1963), Stewart (1962), Kado and Heskett (1970), Cuppels and Kelman (1971) and Perombelon (1971), have devised isolation media for the selective isolation of Erwinia spp. The media formulated by Stewart (1962), Logan (1963), and Perombelon (1971) consists of a layer of pectate above a basal layer of agar containing calcium, which causes gelling of the pectate. Cuppels and Kelman (1971) devised a single layer pectate agar. On Kado and Heskett (1970) medium, Erwinia spp. are selectively isolated on the basis of membrane permeability to high concentrations of lithium chloride. Kelman (personal communication) claims that his medium is very suitable for

recovering large proportions of populations of Erwinia spp. added to soil, and in eliminating other bacteria.

In any field study, an estimation of the number of tubers infected with soft rot bacteria requires fairly rapid and efficient isolation and identification tests. Legg (nee Whitehead, personal communication) used two tests for the identification of Erwinia spp. Any bacteria showing pectolytic activity on Cuppel and Kelman's medium, and a negative reaction in an oxidase test, was assumed to be an Erwinia sp.

The Location of the Pathogen in and on Tubers

Entry of the pathogen into lenticels is usually followed by a period of latency, in which the bacterial infection does not normally affect the tuber until it is replanted as a seed tuber the following year. However, environmental conditions during tuber storage may occasionally cause a premature end to the period of latency, resulting in rotting of tubers. It is therefore essential to investigate the location of the bacterial infection within tubers, and to determine the environmental conditions which favour soft rotting by Erwinia spp.

Perombelon (1973) found Erwinia spp. on the surfaces of tubers and within lenticels; they were rare in the vascular ring and never located in the cortex. The numbers of infected lenticels were not markedly reduced by long storage, whereas tuber surface contamination by Erwinia spp. was. Betencourt and Prunier (1965) have isolated Erwinia spp. from lenticels during the storage phase. However, they included

a pathogenicity test on tuber tissue in their identification procedure, and it must be realised that since any tuber tissue may be infected by soft rot bacteria, such tests must be treated with caution.

Tuber Physiology in Relation to Resistance

The presence of cork formation has often been described as a barrier to infection of tubers by E. atroseptica. Prevention of cork formation by placing tubers in anaerobic conditions (Leach, 1930), and in atmospheres of carbon dioxide and nitrogen (Nielsen,1964), results in increased rotting of tubers. Audia et al. (1962) showed that isopropyl n-(3 chlorophenyl) carbamate delayed suberin formation at cut surfaces, and increased susceptibility of tubers to attack by soft rot bacteria. However, Murant and Wood (1957) found little evidence that enhancement or delay in suberin formation affected rotting. The surface cork barrier which forms on cut surfaces exposed to air for a few days is, however, an effective barrier to rotting (Smith and Smart, 1955; Murant and Wood, 1957). Betencourt and Prunier (1965) claim that entry of bacteria into lenticels is probably aided by the lack of suberisation in lenticels, particularly in tubers which are immature at harvest. Logan (1964) described hard rots which were limited by a suberin barrier, although incubation in moist conditions led to typical soft rotting.

Rudd Jones and Dowson (1950) consider it likely that dry storage conditions decrease the rate of advance of bacterial soft rots. It seems likely that existing cork barriers formed as a response to wounding of tubers, may be effective

against later soft rot attack under normal storage conditions. However, cork formation as a response to infection is not normally an effective method of resistance (Logan, 1964).

Turgid conditions made by water injection of tubers, or sealing with vaseline, can increase rotting (Murant and Wood, 1957). In both cases increase in susceptibility was attributed to partially anaerobic conditions within tubers. Scholey (1968), working with tubers in polythene bags, found that the incidence of rotting increased, and that when tubers were first washed in water contaminated with E. carotovora, respiration of tubers increased the carbon dioxide and decreased the oxygen concentrations in the bags. Leach (1930) stated that under aerobic conditions, rotting of tubers was very slight, whereas Lund and Nicholls (1970) state that incubation of wound inoculated tubers in atmospheres of carbon dioxide and of nitrogen increased rotting. They also found that rotting in 1% oxygen in nitrogen was greater than in air but not so great as in anaerobic conditions. However, Perombelon (personal communication) has said that anaerobic conditions although favourable for rotting, are less effective than a film of water on the surface of tubers.

Phenolic substances have been implicated in resistance of tubers to infections by Phytophthora infestans (Rubin and Aksenova, 1957). Pectinase produced by E. carotovora stimulated production of phenolase in potato tuber tissue (Lovrekovich, Lovrekovich, and Stahmann, 1967). Oxidised phenols, formed by phenolase activity, produced a zone of blackened tissue which functioned as an infection

barrier. As the bacterium spread in the tuber tissue, however, the formation of the zone of dark tissue was progressively suppressed. Presumably, the bacteria produced a system which reduced the oxidised phenols and prevented them from forming the infection barrier. Earlier, the relationship of reducing power and pathogenicity was investigated by Kelman (1954), who used a triphenyl tetrazolium medium to select virulent isolates of Pseudomonas solanacearum. However, colonies which strongly reduced the dye were either weakly virulent or avirulent. Thus, in this case, lack of reducing power was associated with virulence. Held, Kedar and Birk (1965) claim that susceptibility of tubers to infection by P. infestans is associated with an increase in dehydrogenase activity of the host during invasion.

Murant and Wood (1957) found that E. aroideae grew and produced pectic enzymes in oxidised potato sap. Lund and Mapson (1970) have shown that cauliflower tissue treated with pectic enzymes produces ethylene. The biosynthesis of ethylene involves methionine as a precursor, and is thought to depend on the activities of a transaminase, a glucose oxidase and a peroxidase, together with their substrates and cofactors. Lund (1973) also states that acceleration of ethylene biosynthesis has not yet been implicated in potato tissue infected with Erwinia cartovora. If it is, the possible effects on respiration of tubers and phenolic biosynthesis, could be responsible for changes in resistance of tubers to rotting.

Jaffe (1965) found that the electrical conductance of potato tissue inoculated with E. carotovora, showed a

positive correlation with rot produced. Red light increased lignin deposition and decreased the rate of rotting in tuber tissue, and he suggested that this may be due to increased concentrations of lignin precursors in the tissue. A zone of scopolin, fluorescing in U.V. light, is found surrounding E. atroseptica lesions in tubers, and Hughes and Swain (1960) suggest that this may be active in disease resistance.

Changes in the physiology of tubers during storage have been implicated in their resistance to rotting. Van den Boom (1967) stated that tubers became progressively more susceptible to rotting by E. atroseptica during storage. Tubers treated with the sprout repressant 'Agermin' and stored for eight months were inoculated with E. atroseptica and the number of rots developing was compared with those from untreated tubers. Approximately four times as many rots developed in untreated tubers as did in 'Agermin' treated tubers. This was related to the turgidity of tubers, those treatment with 'Agermin' being more turgid. However, this result differs from Murant and Wood (1957) findings that turgid tubers are more susceptible to rotting than normal sub-turgid tubers. L'vova (1965) noticed that in cold storage, resistance of tubers increased for up to four weeks and then decreased rapidly. This change was associated with changes in ascorbic acid levels, total acidity and respiration.

Jordana (1958) found a correlation between resistance and the physiological state of tubers, and that dormant tubers were more susceptible than sprouting tubers to soft rot. He suggests that the state of physiological activity of the tuber when high produces a bacteriostatic effect.

He also found that central tissue was more susceptible to rotting than the outer cortex tissue. Although his conclusions do not take into account the drastic nature of some treatments, his work does emphasise the importance of the biological and physiological changes in tubers, in resistance to rotting.

The Role of Pectic Enzymes in Rotting

It is known that the enzyme involved in the virulence of the soft rotting Erwinia spp. is a trans-eliminase (Dean and Wood, 1967). This is probably the same enzyme found by Wood (1955) and Echandi et al. (1957), called then a depolymerase. Some Erwinia spp. have been found to produce a polygalacturonase (Seiichi and Starr, 1966; Dean and Wood, 1967).

The trans-eliminase enzyme causes maceration of tuber tissue by its ability to split α 1-4 glycosiduronic linkages in polygalacturonic acid and other pectic substances, the main constituents of the middle lamella (Wood, 1967).

The biochemistry of the cell wall in relation to infection has been discussed by Albersheim et al. (1969). They claim that there is an interaction between the pathogen and the carbohydrates of the cell walls of the host, which determines the ability of the pathogen to produce enzymes capable of host cell wall degradation. The trans-eliminase enzymes produced by E. chrysanthemi have been studied by Garibaldi and Bateman (1971) who obtained from cultures grown on pectic acids, six enzymes having similar properties with respect to molecular weight, calcium requirement, pH

optima, cell death and maceration, the only differences being in isoelectric points.

Lang and Knosel (1970) demonstrated that several strains representing three species of Erwinia, E. carotovora, E. atroseptica and E. aroideae, produced pectate trans-eliminases with similar properties, and in no case did the enzyme have any influence on virulence. However, Friedman (1962) demonstrated that virulence of E. carotovora was directly related to the amounts of enzyme produced.

It has been demonstrated that production of many fungal and bacterial pectic enzymes is induced by their substrates. Starr and Nasuno (1967) state that most strains of Xanthomonas campaestris studied, inducibly produced pectin methylesterase, and pectin trans-eliminase on pectin whereas, few strains produced the latter on a glucose medium. Tejerina and Serra (1969) have demonstrated induction of pectates trans-eliminase in E. carotovora, and have also shown that production of this enzyme is subject to catabolite repression by glucose at concentrations greater than 2.5×10^{-3}M; evidence which agrees with that of Keen and Horton (1966) for catabolite repression of polygalacturonase synthesis in Pyrenochaeta terrestris. The inducing agent in this case was found to be galacturonic acid,its polymers, and structural relatives.

Schwimmer et al. (1954) have detected three major sugars in potato tissue, glucose, fructose, and sucrose, and it seems possible that catabolite repression of pectic enzymes by sugars within tubers may be of importance in resistance, during the latent phase of infection. It has

been demonstrated that sugars increase during cold storage (Denny and Thornton, 1941) and that sucrose concentration increases during break of dormancy, chemically induced by ethylene chlorhydrin. However, reducing sugar concentrations showed no fixed trends during the break of dormancy.

The dependence of certain diseases on the levels of sugars present in plant tissue, has led Horsfall and Dimond (1957) to suggest that high sugar content may function to repress enzyme production in certain host parasite interactions, a supposition which agrees with that of Keen and Horton (1966) who propose that high sugar content of onion roots imparts resistance to Pyrenochaeta terrestris by catabolite repression of the pectic enzymes of the pathogen.

Tribe (1955) has suggested that death of higher plant cells accompanying maceration by Botrytis cinerea and E. aroideae may be explained by direct hydrolysis of pectic materials in them, and accessible in unplasmolysed living cells. The more recent work of Mount et al. (1970) shows that trans-eliminase activity causes maceration, accompanied by cellular death. Hall and Wood (1970) have also demonstrated this phenomenon and it seems that death is not due to toxicity of products released during maceration (Fushtey, 1957). However, Bateman and Millar (1966) found difficulty in understanding how purified pectic enzymes can induce death of protoplasts unless the cellular membranes contain pectic substances.

Eradication of the Pathogen From Tubers

Since the nature of resistance of tubers to the pathogen is obscure, breeding for resistance is difficult and eradication of the pathogen from tubers seems the most likely method for mitigating the problem. Although rotting from latent infections can be prevented by good storage practice, the pathogen is always present within lenticels, and although blackleg in the field is not serious in the United Kingdom, it can be in warmer soils, as in the United States (Fox, personal communication). However, Scholey (1968) has expressed concern in connection with the increased incidence of rotting in polythene bag-packed tubers, since the conditions in the bags are favourable for the onset of rotting, and one such tuber spoils the whole bag.

Many workers have envisaged successful eradication in the field by simply roguing plants showing blackleg symptoms, but since symptom expression is not related to the incidence of infection, such eradication is ineffectual. Therefore, new methods for successful eradication must be sought. Graham (personal communication), working in Scotland has devised such a method by taking Erwinia free stem cuttings from young plants. The method involves the removal of the seed tuber from a young plant. Two pieces of stem each about on inch in length, removed aseptically from the base of the cutting are macerated individually and a sample of each is streaked out on a pectic medium. If no pectolytic bacteria are found, the cutting is planted in 'Erwinia-free' soil. The new tubers produced are then grown the following year, in soil in which potatoes have never been grown before.

Although E. atroseptica can be eradicated by this technique, use of these tubers under normal field conditions may be limited by the difficulty in preventing E. carotovora from entering the crops. Perombelon (personal communication) has found that many stocks obtained from E. atroseptica-free soil contain E. carotovora, and that virulent strains of E. carotovora could destroy the potential effectiveness of the stem cutting programme.

It has been demonstrated by Lund and Nicholls (1970) that bacteria other than Erwinia spp. can cause soft rotting and thus any successful eradication scheme should take this into account. It seems more likely that a successful method will be of chemical nature, either as a disinfectant which can penetrate the lenticels, or a systemic bactericide applied to the soil during growth of the crop.

Graham and Volcani (1961) failed to obtain successful eradication with streptomycin or mercury compounds and in fact increased the incidence of successful rots simply because they were in effect applying a liquid film. The activity of the disinfectants seems to be removed from the site of infection by the tissue surrounding it. Personal communications with Hampson and Graham, indicated that no successful methods of chemical eradication are yet known, although Paton (personal communication) has found some evidence for biological control using certain strains of Arthrobacter spp. This may be applied to flume water at Sutton Bridge to control further contamination of tubers during washing.

MATERIALS AND METHODS

Bacterial Cultures

Bacterial cultures were as follows:

Erwinia atroseptica culture No. A13 (Webb, 1970)
Erwinia atroseptica culture No. A12 (Webb, 1970)
Erwinia atroseptica NCPPB culture No. 1277
Erwinia atroseptica E14 obtained from Professor Kelman
Erwinia carotovora NCPPB culture No. 1280
Erwinia carotovora NCTC culture No. 1747
Erwinia carotovora MCTC culture No. 1774
Erwinia aroideae NCPPB culture No. 1276

E. atroseptica A13 was used for pathogenicity tests. Cultures were prepared by incubating for 2 d on nutrient agar slopes at 25°C. Slope cultures were stored at -18°C. A standard suspension used for inoculation was prepared from a 24 h nutrient broth shake culture at 25°C. Cells were removed by centrifugation at 17000 x g for 2 min and resuspended in sterile distilled water at 10^9 cells/ml.

Growth and Storage of Potatoes

Potato plants and tubers used were cv. Majestic. Seed came from Scotland and was of two types, virus tested and virus tested stem cutting (VTSC). Crops grown at Silwood Park were regularly inspected and treated for potato blight and mineral deficiencies, and were harvested in late September or early October. Tubers were stored in open trays in a shed where the temperature did not fall below 8°C. To prolong dormancy, tubers were kept in closed dustbins containing 'Fusarex', or stored between 2 and 3°C in paper sacks.

Isolation and Identification of the Pathogen

Agar media for isolation and identification were as follows:

Kado and Heskett (1971) (D_3 for Erwinia spp.)

10 g	sucrose
10 g	arabinose
5 g	casein hydrolysate
7 g	$LiCl_4$
3 g	glycine
5 g	NaCl
0.3 g	$MgSC_4 \cdot 7H_2O$
50 mg	sodium dodecyl sulphate
60 mg	bromothymol blue
100 mg	acid fuchsin
15 g	agar (Difco Bacto)
1 ℓ	water.

The pH was adjusted to 8.2 with sodium hydroxide before autoclaving, and 6.9-7.1 afterwards. The medium is said to be selective for Erwinia spp., which produce a red colouration in the medium. The intensity of the colour depends upon the species of Erwinia. Members of the soft rot group, E. aroideae, E. carotovora, and E. atroseptica, characteristically produce a more intense colour than those of the E. amylovora group. Growth of other bacteria is said to be suppressed usually, although some saprophytes eg. Escherischia spp. will grow on it. Xanthomonas spp. produce a dark blue reaction.

Cuppels and Kelman medium (1971)

300 ml of boiling water were placed in a preheated Waring blender. The following were added and the mixture blended at high speed for 15 sec:

4.5 ml	IN NaOH
3.0 ml	10% $CaCl_2 \cdot 4H_2O$ solution in water
1.5 g	Difco Bacto agar
1.0 g	$NaNO_3$.

15 g of sodium polypectate (Sunkist Growers Ltd., Exchange Brand number 6074) were added with constant stirring, and the whole then blended at high speed for a further 15 sec. After autoclaving for 25 min at 15 psi in 2 litre flasks, 1 ml of 0.075% crystal violet solution was added.

Logan medium (1963)

23 g	Simmon's citrate agar
3 g	$CaCl_2 \cdot 4H_2O$
5 g	bile salts
0.001 g	crystal violet
1 ℓ	distilled water.

1 g of calcium carbonate was added to obviate adjusting the pH after autoclaving (10 psi/10 min). 15 ml samples of agar were pipetted aseptically into Petri dishes and allowed to set. 5 ml of a sterile 1.5% solution of sodium polypectate, adjusted to pH 7.0, and autoclaved for 10 min at 10 psi, were pipetted on to the layer of agar. The upper layer gelled within 2 h.

Stewart medium (1962)

34.7 g	McConkey agar No. 3
2.7 g	$CaCl_2 \cdot 4H_2O$
500 ml	distilled water.

The agar was autocalved at 10 psi for 10 min. 15 ml aliquots were poured into Petri dishes and allowed to set before adding 5 ml of the pectate solution as above.

Perombelon's modification of Stewart's medium (1971)

10 g	bacteriological peptone (Oxoid L37)
5 g	lactose
2.5 g	NaCl
0.015 g	neutral red
2.7 g	$CaCl_2 \cdot 4H_2O$
7.5 g	Difco Bacto agar
500 ml	distilled water.

The agar was autoclaved at 10 psi for 10 min before adding 5 ml of 1.15×10^{-1} g/l gentian violet to a final concentration of 1.15×10^{-3} g/l. 5 ml of pectate solution was added to a 15 ml basal layer in Petri dishes.

The pectate solution was prepared by suspending 2 g sodium polypectate in 6 ml absolute alcohol, and making up to 100 ml with 0.01% aqueous ethylenediaminetatra-acetic acid disodium salt solution. The pH was adjusted to 7.4 with sodium hydroxide solution before autoclaving at 10 psi for 10 min.

Cuppels and Kelman's medium was chosen for work on isolation and identification of Erwinia spp. Bacteria producing pitted colonies on this medium were streaked out on to fresh plates of nutrient agar. After 24 h (25°C) incubation, all single colonies were tested for oxidase activity using a freshly prepared solution (1%) of NNN'N' tetramethyl-p-phenylene diamine dihydrochloride. Pectolytic and oxidase negative colonies were assumed to be Erwinia spp.

Most isolations were from active rots. Artificially or naturally infected tubers were made to rot by a technique developed by Perombelon cited by Hirst (personal communication).

Tubers were placed individually in 9" x 7" polythene bags (Transatlantic Plastics Ltd.) with 10 ml water. Each bag was tied after expelling as much air as possible. All bags in a particular treatment were placed inside a 30" x 12" polythene bag which was knotted at the top. The bags and contents were stored at room temperature (19-21°C) in the dark for 12 to 14 d. Rotting had usually commenced by day 6, and almost always by day 12, so that after 12 d any tubers not rotting were assumed not to be infected with soft rot bacteria.

The degree of infection of samples of tubers was also determined by staining with 2,3,5-triphenyl tetrazolium chloride sodium salt. Tubers were dipped in or sprayed to run off with an 0.25% aqueous solution of the salt and then allowed to air dry before they were placed individually in polythene bags with 10 ml water. Tubers were incubated in the dark for 48 h at room temperature, after which time infected areas of the tuber stained pink. These two tests will be referred to respectively as the polythene bag and tetrazolium tests.

Inoculation of Tubers

Two methods were used.

1. Wound inoculation

Washed and air dried tubers were wounded with a cork borer (No. 4), internal diameter 5 mm to depths of 2.5 and 10.0 mm, one of each per tuber 0.005 or 0.02 ml, respectively, of a freshly prepared bacterial-suspension suspension (10^9 cells/ml) were added to the wounds. Controls were inoculated

with sterile water instead of the bacterial suspension. The wounds were sealed with a molten mixture of vaseline:paraffin wax (50:50). After incubation for 4 d on moist tissue paper in plastic boxes, at room temperature (19-21°C), the rotten tissue was removed, and the volume of the cavity was measured by filling with water from a burette.

2. Lenticel inoculation

Tubers were washed individually under running tap water and placed either in polythene bags with 10 ml of water, or in buckets of water for 24 h to initiate lenticel proliferation. Field infected tubers treated in this manner were first soaked for 6-12 h in a bacterial suspension (10^6 cells/ml). Preliminary studies indicated that tuber infections obtained during tuber growth, could be transferred from one tuber to another. Tubers were individually sprayed to run off with a bacterial suspension (10^6 cells/ml) above a mesh grid, and allowed to air dry overnight. This reduced the possibility of infection by wild strains of Erwinia spp. between tubers.

VTSC tubers were soaked for 6-12 h in a bacterial suspension as above, before air drying overnight in open plastic bins.

Dormancy of Tubers

Dormancy of tubers was broken with ethylene chlorhydrin (2 chloro-ethanol) (Denny, 1929). Tubers were placed in a desiccator just large enough for the tubers and treated with ethylene chlorhydrin at 0.8 ml/kg of tubers for 4 d at room temperature in the dark. The ethylene chlorhydrin was applied

to a cotton wool pad to enable more rapid evaporation throughout the container. After treatment, tubers were washed and air dried in a fume cupboard.

Quantitative Estimations of Sugars

Total soluble carbohydrates were determined in 0.3 ml samples using anthrone reagent (Hewitt, 1958). 3 ml of reagent was added to the sample, and heated for 7 min in a boiling water bath followed by immediate cooling. Readings were taken in a Beckman DB spectrophotometer at 620 mμ against a water blank and were compared to a standard cure prepared with glucose.

Glucose was determined by the glucose oxidase method of Marks (1959), a modification of that of Middleton and Griffith (1957). 'Fermcozyme', a stable liquid preparation of glucose oxidase containing 750 μg/ml was obtained from Hughes and Hughes Ltd., London. The glucose oxidase reagent was prepared by adding 0.5 ml Fermocozyme to about 80 ml of 0.15 M acetate buffer pH 5.0. To this was added 5 ml of peroxidase solution (20 mg/100 ml acetate buffer) and 1 ml of o-tolidine (1% in absolute alcohol). The solution was made up to 100 ml with buffer and stored at 2°C in a dark bottle. 0.5 ml samples were added to 3 ml reagent, mixed gently and the colour read at 625 mμ, after exactly 10 min. Glucose standards were prepared in saturated benzoic acid.

Individual sugars were determined by gas liquid chromatography by a modified method of Sweeley, Makita and Wells (1963).

Gas Chromatograph Details

Instrument	Pye series 104.
Column	Coiled glass tube 1.5 m x 4 mm internal diameter.
Liquid phase	6% SE52 silicone gum rubber methyl phenyl on CQ72-85 support.
Detector	Flame ionisation.
Gases	Nitrogen 10 psi. Hydrogen 15 psi. Air 25 psi.
Injection block temperature	150°C for monosaccharides. 210°C for disaccharides.
Recorder	Smith's sensitometer pen recorder 30 in/h.
Sample size	2 µl.

Extraction of Sugars from Tuber Tissue

Two methods were used.

1. Aqueous extraction

Tubers were peeled and ground in a meat mincer. The macerate was placed in several layers of muslin and the aqueous extract squeezed out. The extract was immediately centrifuged at 17000 x g for 2 min. The volume of extract was measured, and a known aliquot dropped into boiling water of equal volume. The extract was re-centrifuged at 17000 x g for 5 min and the soluble proteins removed by the addition of 3 ml of a neutralised, freshly prepared 10% lead acetate solution. After precipitation was complete, the residue was spun down and discarded. A few more drops of the lead

acetate solution were added to ensure total precipitation of the proteins, and the extract was then deleaded by addition of 2 ml of a saturated solution of sodium oxalate. The precipitation was removed by centrifugation at 17000 x g for 5 min. After measuring the final volume of the extract, 1 g of Permutit 'Biodeminrolite', a mixed bed resin was added to the flask and shaken for 30 min to remove most ionic material. 5 ml of the extract were then pipetted into a 10 ml ampoule and freeze dried.

2. Alcoholic extraction

Tubers were washed, peeled and chopped and after weighing the total were minced. The mince was mixed thoroughly and a 31 g sample was rapidly placed into 50 ml of 95% ethanol to give a final ethanol concentration of 70-75%. The alcoholic mixture was heated in beakers in a boiling water bath for 5 min. The straw coloured liquid was decanted. The residue was just covered with 70% ethanol and reboiled. This procedure was repeated 8 times after which time the supernatent was colourless. Extractions were pooled and starch removed by centrifugation (17000 x g for 2 min). Alcohol was removed by rotary evaporation at 50°C and 350 mm Hg pressure after the solution had been neutralised. On removal from the bath proteins were removed as above, the extract deleaded and deionised, before freeze-drying 5 ml samples.

This method was chosen for quantitative sugar determinations although both methods were used for qualitative assessments.

The freeze dried extracts were dissolved in 1 ml pyridine dried over KOH pellets, and converted to their trimethylsilyl (TMS) derivatives by the addition of 0.2 ml hexamethyl disilazane and 0.1 ml trimethyl chlorosilane at room temperature. The heavy precipitate formed was not removed. Manual injections of 2 µl of the reaction mixture were made using 1 µl hexane (A.R.) as a front marker.

The peaks obtained were traced on to 1 mm graph paper and their areas determined. Standard sugar preparations (α-glucose, β-glucose, β-fructose, sucrose, inositol, ribose, mannose, and maltose) at various concentrations from 0 to 40 mg per ml sample of pyridine were mixed with the TMS reagents and run through the column to provide standard curves. From the known weight of tuber material used and the volume of deionised aqueous precipitate obtained, the sugars were calculated as mg/gm of tuber tissue.

Polyphenol Oxidase Activity

Polyphenol oxidase activity was measured for crude enzyme extracts by its ability to oxidise catechol to its coloured quinone, o-benzoquinone (Fehrmann and Dimond, 1967). The reaction mixture was prepared as follows. To 1 ml of 0.02 M catechol was added 1 ml 0.1 M citrate buffer (pH 6.0) followed by 1 ml of crude enzyme preparation in 0.1 M citrate buffer (pH 6.0). Immediately after enzyme addition, the increase in colour was recorded against time at 405 mµ against a no enzyme blank. The first linear part of the reaction was used to determine the reaction rate expressed as increase in optical density units per min. Measurements were made at room temperature (19-21°C).

Total Phenol Estimations

Total phenols were estimated by the method of Folin and Denis ( ). Folin-Denis reagent was prepared as follows. To 75 ml of water was added 10 g of sodium tungstate, 2 g phosphomolybdic acid and 4 ml of phosphoric acid. The mixture was refluxed for 2 h, diluted to 100 ml with distilled water and stored in a dark bottle.

An aliquot of an alcoholic preparation of tuber tissue, containing not more than 0.5 ml of alcohol was diluted to 7 ml in a 10 ml test tube. After mixing, 0.5 ml of Folin-Denis reagent was added. The tube was shaken and left for 3 min exactly, before adding 1.0 ml saturated sodium carbonate. The whole was made up to 10 ml. After 1 h the optical density at 752 μm was read against a reagent blank. Total phenol concentration was determined by use of a calibratic curve (10-100 μg catechol per aliquot).

Preparation of Pectic Enzymes on Pectic Substrates

The liquid medium (basic salts medium) for production of pectic enzymes consisted of:

4.6 g	Bacto vitamin-free casimono acids (Difco).
1.0 g	K_2HPO_4.
0.5 mg	$MgSO_4 \cdot 7H_2O$.
10.0 g	sodium polypectate.
1 ℓ	water.

To this was added 10 ml of minor elements solution.

0.02 g	$FeSO_4 \cdot 7H_2O$
0.1 g	$ZnSO_4 \cdot 7H_2O$
0.002 g	$NaMoO_4 \cdot 2H_2O$
0.002 g	$MnCl_2 \cdot 4H_2O$
1 ℓ	water.

100 ml of medium were sterilised in 250 ml conical flasks by autocalving at 10 psi for 10 min. 0.5 g sterile calcium carbonate was added to each bottle after autoclaving.

Sodium polypectate and pectin were purified before use as substrate for enzyme preparation as follows. 25 g substrate per 150 ml 70% ethanol containing a final concentration of 0.5 N hydrochloric acid was agitated for 1 h by a magnetic stirrer. The substrate was then collected on No. 541 Whatman filter paper under suction on a Buchner funnel. The solid residue was washed several times in 70% ethanol until no more chloride could be detected in the washings with silver nitrate solution. The substrate was then washed once more with 95% ethanol and dried at 70°C for 48 h. The powder was allowed to equilibrate with air at room temperature (c. 20-23°C) for 48 h.

Buffers

The following buffers were used in the extraction and assay of enzymes, and prepared as described by Long (1961).

Citric acid-sodium citrate (0.1 M), pH 3.0-6.2

di-sodium hydrogen phosphate-sodium dihydrogen phosphate (0.1 M), pH 5.8-8.0

Tris-(hydroxy methyl)-amino methane-hydrochloric acid (0.1 M), pH 7.2-9.1

Glycine-sodium hydroxide (0.1 M), pH 8.6-10.6.

Partial Purification of Pectic Enzymes

Cell free filtrates of culture were obtained by centrifuging at 17000 x g at 4°C for 20 min, collecting the

supernatent and centrifuging again for 20 min. The filtrates were kept in 10 ml lots at -18°C. When required the filtrates were thawed and pooled as necessary before bringing to 60% saturation with solid ammonium sulphate at 10°C. The precipitate was collected by centrifuging at 17000 x g for 20 min at 4°C, and discarded. The supernatent was then brought to 90% saturation with ammonium sulphate and left at 10°C for the precipitate to form. This was collected by centrifugation at 17000 x g for 20 min, dissolved in one-tenth the original culture filtrate volume of 0.5 mM phosphate buffer at pH 7.0 before dialysing against 0.002 M calcium chloride solution for 24 h at 4°C (Visking tubing, 6 mm diameter). Unless stated otherwise, semi-purified enzyme preparations were diluted ten times in distilled water before use.

Estimation of Pectic Enzymes

Two enzyme systems were assayed.

Polygalacturonase at pH 5.0.

Endo-polypectate trans-eliminase at pH 9.0.

Ostwald viscometers were used and assays done at 25°C. Reaction mixtures contained:

3.5 ml of 3% sodium polypectate (unless otherwise stated),

3.5 ml buffer (0.1 M),

3.0 ml pectic enzyme preparation.

Viscosity reducing activity was expressed as 1000/T, where T was the time in min for 50% reduction in viscosity of the reaction mixture after the flow time for water had been subtracted. Measured in this way activity was found to be directly proportional to enzyme activity at high enzyme

concentrations where T_{50} values did not exceed approximately 3 min. Below this activity, the linear relationship did not exist and a standard curve of enzyme dilution against $1000/T_{50}$ was prepared to calculate such activities in enzyme units (referred to in the text as Arbitrary Enzyme Units).

Staining Reactions

Suberin in lenticels and periderm

Tuber sections were cut with a razor and heated to boiling in a saturated solution of Sudan IV (Gam Ltd.) in 70% ethanol. Sections were left in the stain for 2-3 h before inspecting under a binocular Olympus microscope.

E X P E R I M E N T A L W O R K

SECTION I

ISOLATION AND IDENTIFICATION OF THE CAUSAL AGENT

A Comparison of Selective and Semi-selective Media for the Isolation and Identification of Erwinia atroseptica

The isolation and identification of Erwinia atroseptica is a necessary, but difficult procedure. The ultimate method for isolation and identification is a single selective medium, but so far this has not been achieved. Several media are available and are discussed in the Materials and Methods. A good medium must have a high population recovery rate for Erwinia spp., and high selectivity. The project was designed to compare the efficiency of four selective and semi-selective media at present used for isolation of Erwinia spp.

1. Cuppels and Kelman's medium (1971)
2. Logan's selective medium (1963)
3. Stewart's selective-diagnostic medium (1962)
4. Perombelon's semi-selective medium (1971).

The Effect of the Media on Growth of Erwinia atroseptica

Before use agar plates were dried overnight at 25°C, the lid of the Petri dish being separated from the base by a sterile filter paper. Just before inoculation, eight rings were marked on the surface of the medium with a heat sterilized culture tube cap, 18 mm in diameter.

A freshly prepared suspension of E. atroseptica A13 (10^9 cells/ml) was serially, ten-fold diluted; 0.02 ml of the appropriate dilution was pipetted into the marked areas, each

plate having eight spots of the same dilution of the inoculum. The plates were incubated at 25°C for 48 h (72 h for Logan's medium), after which the number of E. atroseptica colonies in each spot were counted. These counts are shown in Table 1.

Table 1. Counts of E. atroseptica A13 colonies on various media, at two dilutions

Medium		Theoretical number of cells applied/ml	
		10	100
		Mean count per circle (mean count/ml of inoculum)	
Nutrient agar	a[1]	7.06 (353)	67.93 (3396)
	b[2]	9.66 (483)	57.33 (2876)
Perombelon	a	7.25 (362)	60.56 (3028)
	b	4.87 (222)	54.40 (2650)
Cuppels and Kelman	a	6.25 (312)	69.00 (3450)
	b	6.25 (312)	45.00 (2250)
Stewart	a	0.25 (12)	58.67 (2933)
	b	4.34 (217)	38.50 (1925)
Logan	a	0.25 (12)	20.12 (1006)
	b	0.94 (47)	8.44 (425)

[1,2]a and b are results obtained on separate occasions.

The results in Table 1 show a constant discrepancy between the number of bacteria applied to the medium, and the number recovered, approximately a thirty-fold increase, which cannot be explained.

An analysis of variance showed very significant differences ($p < 0.001$) between the dilutions and the media. From this, a Tukey test was carried out to find the significance between the media at $p < 0.05$. The results were then expressed as a percentage of the Erwinia atroseptica cells

which were prevented from growing by each of the media as compared to nutrient agar. These results are shown in Table 2.

Table 2. Percentage of cells prevented from growing on each medium as compared to growth on nutrient agar

Medium	% 'kill' at a dilution of 10 cells/ml		% 'kill' at a dilution of 100 cells/ml	
	a	b	a	b
Perombelon	0	*54	11	7
Kelman	12	*35	0	*22
Stewart	*96	*55	13	*33
Logan	*96	*90	*60	*85

a,b are results from separate experiments.
* significantly different ($p = 0.05$) from growth on nutrient agar.
} no significant difference between growth of E. atroseptica A13 on the media.

The results in Table 2 suggest that the percentage 'kill' of E. atroseptica A13 is highest on Logan's medium and lowest on Perombelon's and Cuppels and Kelman's media. The results from the repeat experiment differ in that the numbers of colonies obtained from the lower dilution of bacteria (10 cells/ml) were generally higher than in the first experiment while those obtained from the higher dilution of bacteria (100 cells/ml) were lower. However, dilutions theoretically containing as little as 10 or 100 bacteria/ml can rarely be expected to be accurate, and clumping of cells will markedly affect results from such high dilutions of bacteria.

The Selectivity of the Media

To test the selectivity of the media, serial ten-fold dilutions of Erwinia-free soil suspensions were plated onto the media. The influence of these bacteria in the soil on the recovery rate of E. atroseptica was tested by adding known concentrations of E. atroseptica cells to soil suspensions and repeating the plate counts.

Soil suspensions were prepared by shaking 10 g of soil fresh from the field, in 200 ml of distilled water for 20 min and serial dilutions of this were made as for E. atroseptica suspensions. Soil suspension dilutions containing E. atroseptica were prepared by adding appropriate E. atroseptica suspensions to the various soil suspensions. The results are shown in Tables 3 and 4.

Table 3. Mean counts of bacteria in field soil, growing on the media

Mean count/0.02 ml sample of various soil suspensions[1]

Medium		Dilutions (10^X)				
		10^1	10^2	10^3	10^4	10^5
Perombelon	a	-	9.12	0.25	-	-
	b	-	18.40	2.38	-	-
Cuppels and Kelman	a	-	4.12	0.25	-	-
	b	-	9.87	1.67	-	-
Stewart	a	-	5.62	0.50	-	-
	b	-	12.40	1.50	-	-
Logan	a	35	3.37	0.125	-	-
	b	-	11.10	0.12	-	-
Nutrient agar	a	-	-	-	6.50	7.37
	b	-	-	10.00	3.62	1.50

[1]Each count is a mean of 16 spots.
- indicates that no count was made.
a,b are results from two experiments.

An analysis of variance on the results in Table 3 excluding those on nutrient agar and on Logan's medium for 10^1 dilutions, since no comparison was available, showed significant differences between the 10^2 and 10^3 dilutions ($p = 0.05$), but none between the media. The great difference between these media and nutrient agar is obvious, since they seem to reduce the populations of bacteria other than Erwinia atroseptica by 90-95%. However, the insignificant differences between the media made it difficult to choose the most selective medium.

Recovery of Erwinia atroseptica from artificially infested soil suspensions

A soil suspension was seeded with E. atroseptica A13 (10^7 cells/g of soil). The counts obtained on the media are given in Table 4.

Table 4. Mean counts of E. atroseptica from seeded soil suspensions

Medium	Mean count of pectolytic bacteria per ml of suspension[1]		
	Dilution of soil suspension (10^x)		
	10^1	10^2	10^3
Perombelon	-	2168	212
Cuppels and Kelman	-	2406	269
Stewart	-	1856	400
Logan	2031	750	44

[1]Each count is a mean of 8 spots.

An analysis of variance showed that growth on Logan's medium was significantly less that that on the other three media. Nutrient agar was not assayed in this experiment,

because it was impossible to identify E. atroseptica colonies in the presence of other bacteria.

The evidence for the choice of one medium for future experiments involving isolation of Erwinia spp., is not good, since there were no significant differences between the media with respect to selectivity. Growth of Erwinia atroseptica A13 on Logan's medium was poor, and often restricted on Stewart's medium. Some difficulty with Perombelon's medium was found in deciding whether some soil bacteria were pecto lytic or not. The ease of preparation and use of Cuppels and Kelman's medium together with the above limitations of the other media influenced its final choice for use in further experiments involving isolations of Erwinia spp.

The Use of Cuppels and Kelman's Medium to Estimate Population Densities of E. atroseptica During the Progress of a Rot

New potatoes were dug up, washed and put in polythene bags with 10 ml of water. Samples of three tubers were removed periodically. 1 g of tissue was removed from each tuber. The pooled tissue was then macerated in 10 ml of water under aseptic conditions in a Sorval microhomogeniser. Serial ten-fold dilutions were made and 0.02 ml samples of each dilution were plated onto Cuppels and Kelman's medium. Where rotting was present, the 1 g sample of tissue was taken from the rot. The numbers of pitted colonies present on the medium after incubation for 48 h (25°C) are shown in Fig. 1.

The results in Fig. 1 show that populations of pecto-lytic bacteria were in excess of 10^5 cells/g fresh weight of

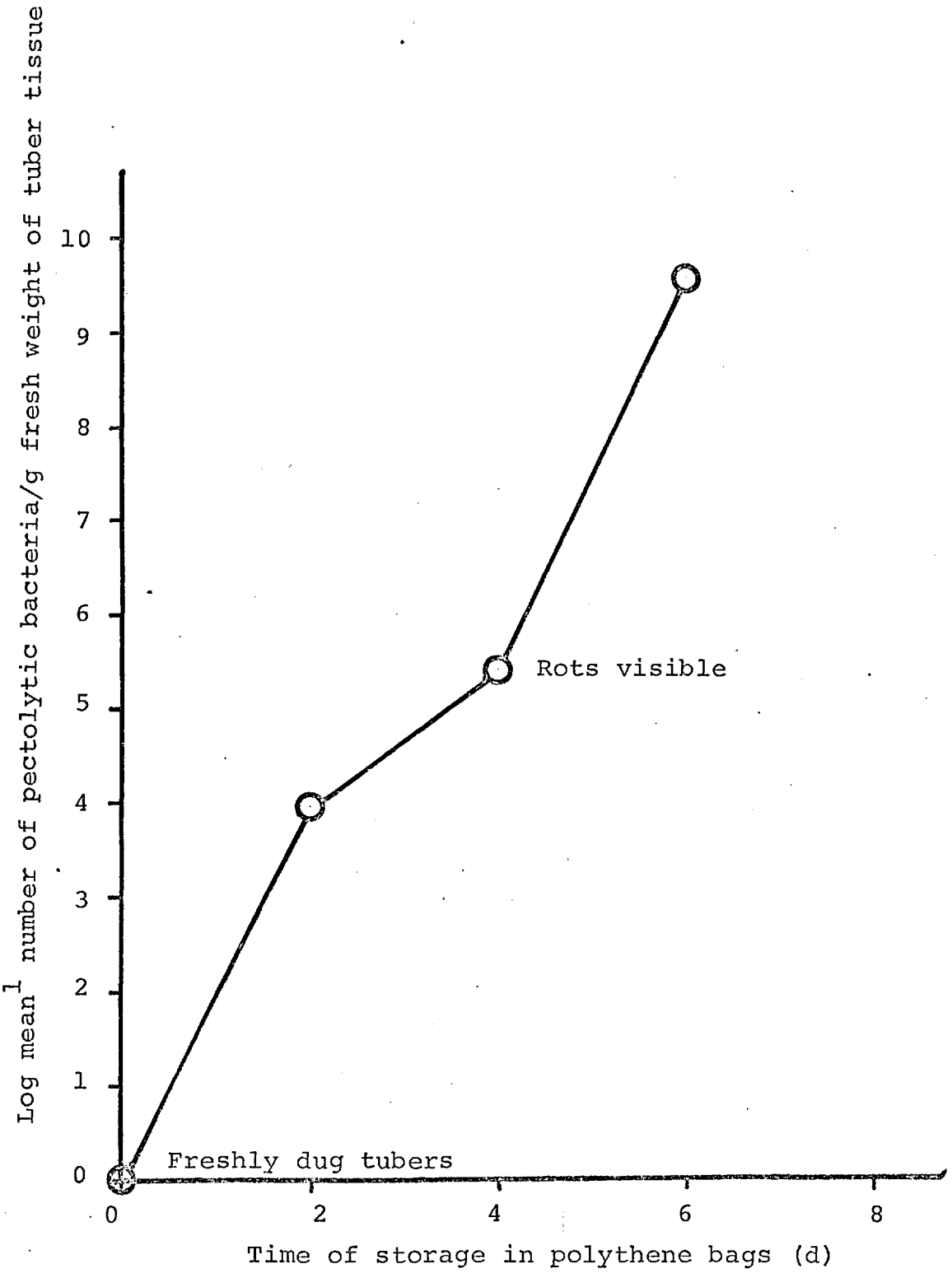


Figure 1. Bacterial growth in an active rot

[1]Mean of 8 spots.

tuber tissue, when rotting was first detectable, and within 6 d after placing the tubers in polythene bags with water, populations of approximately 10^9 cells/g fresh weight of tuber tissue were found.

SECTION II

THE INFECTION PROCESS

The Incidence of Infection of Tubers in the Field

Developing tubers were taken from symptomless Majestic plants during the 1971 and 1972 growing seasons and tested for rotting and presence of *Erwinia* spp. by using the polythene bag test in conjunction with pectolytic and oxidase tests on rot isolates cultured on Kado and Heskett's medium for the 1971 crop and Cuppels and Kelman's medium for the 1972 crop. Eight plants were sampled for each treatment at each date.

During sampling of the 1971 crop, tubers were found to rot within six days. Tubers were therefore tested for the presence of *Erwinia* spp. after this time. Later in the season it was found that some tubers did not rot until twelve days after putting in polythene bags. Consequently, in 1972, the period of incubation of tubers in polythene bags was extended to twelve days. The numbers of tubers rotting and those infected with *Erwinia* spp. in 1971 and 1972 are shown respectively in Tables 5 and 6.

Table 5. % developing tubers rotting in polythene bags, 1971 (% rotted tubers containing *Erwinia* spp.)

Sampling date	Seed tuber rotted	Seed tuber not rotted
5/7/71	34 (34)	13 (11)
9/7/71	65 (59)	23 (23)
17/7/71	54 (52)	49 (47)
2/8/71	59 (54)	37 (25)

Table 6. % developing tubers rotting in polythene bags, 1972 (% rotted tubers containing Erwinia spp.)

Sampling date	Seed tuber rotted	Seed tuber not rotted
7/7/72	92 (77)	93 (62)
14/7/72	75 (73)	61 (56)
21/7/72	64 (32)	60 (39)
2/8/72	83 (71)	
21/8/72	85[1] (-)	

[1]No assessment made.

In 1971, the method of identification of Erwinia spp. on Kado and Heskett's medium was not found to be so selective as hoped, since Bacillus subtilis produced colonies indistinguishable from those of Erwinia spp. The results for the percentage rotted tubers containing Erwinia spp. cannot be considered an accurate estimation of the incidence of infection of tubers by Erwinia spp. Before this trial was begun, all rotting was thought to be attributable to Erwinia spp. This proved not to be the case, since the drastic nature of the polythene bag test appears to allow bacteria other than Erwinia spp. to rot potatoes.

By mid-August 1971 no plants with entire seed tubers were found. The results in Table 5 show that early in the season, 34% of tubers from 'rotted seed tuber' plants, rotted in polythene bags, compared with 13% for tubers from 'non-rotted seed tuber' plants. Both proportions increased until mid-August, when the field trial was completed. Although the results from isolation on to Kado and Heskett's medium must be treated with caution, they did suggest that a high percentage of rots contained Erwinia spp.

If one visualises the infection by Erwinia spp. as being tuber borne, passing from seed to new tubers through the soil after onset of seed tuber decay, then the results obtained support this hypothesis. At three out of four sampling dates, the degree of infection is higher in tubers from plants whose seed tuber had rotted.

The results obtained from the 1972 trial, shown in Table 6, show that rotting of developing tubers and incidence of infection by Erwinia spp. was little affected by seed tuber decay. This was surprising and poses the question as to the origin of the bacteria that infected the developing tubers.

In early July 1972, the incidence of tubers rotting in polythene bags was high in both treatments, between 90 and 95%. By late July, the number of tubers infected with soft rot bacteria had decreased to 60-65% in both treatments. After this, no plants with non rotted seed tubers were found, and the number of tubers infected with soft rot bacteria increased until in late August, 85% of developing tubers rotted in polythene bags.

The number of rotted tubers containing Erwinia spp. also decreased from early to late July and after no more plants with entire seed tubers were found, an increase was found. Developing tubers from 'non-rotted seed tuber' plants, which rotted in polythene bags, contained fewer Erwinia spp. infections than those from 'rotted seed tuber' plants.

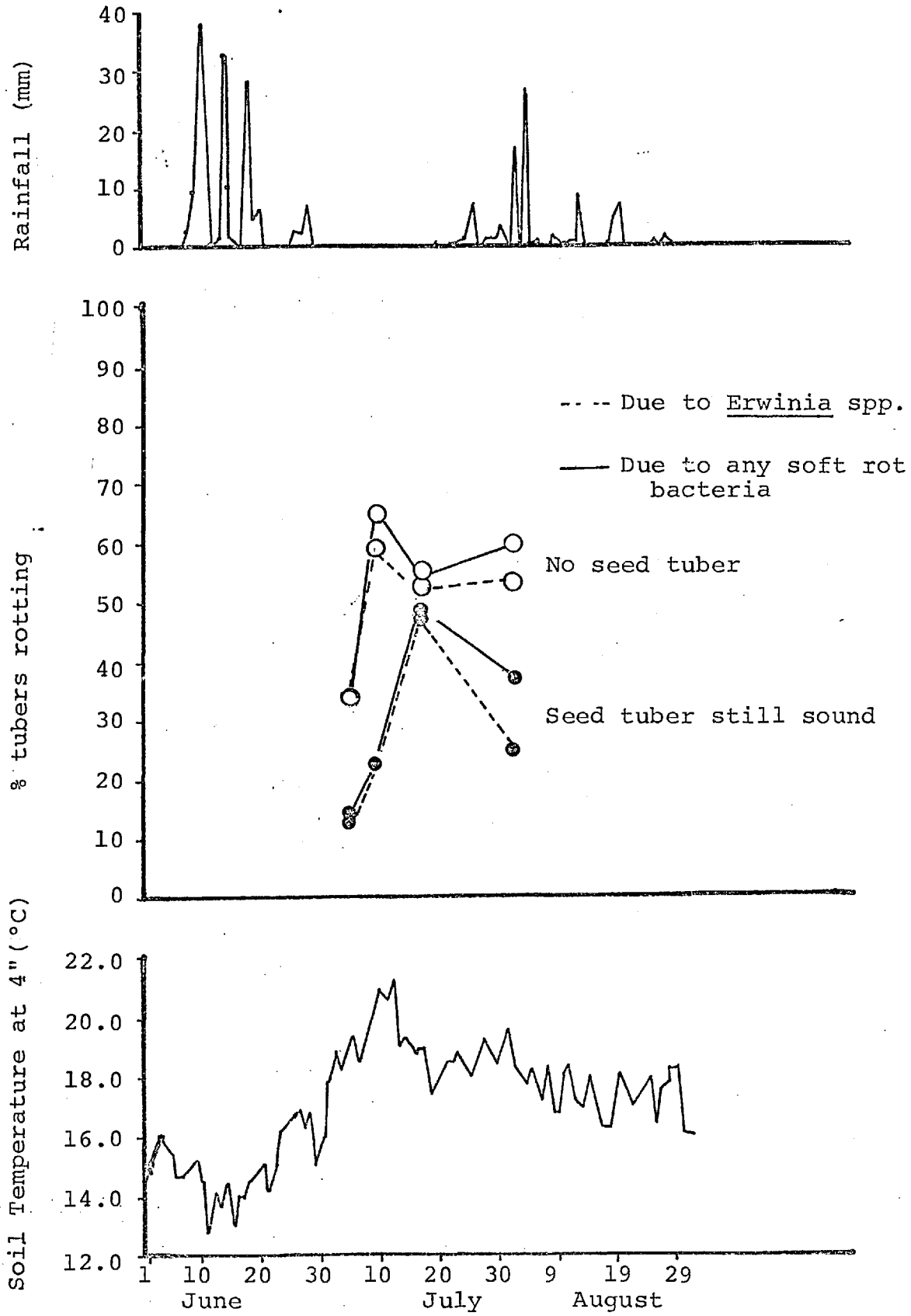
The use of two diagnostic criteria, pectolytic activity and a negative reaction in an oxidase test, for the

identification of Erwinia spp. from rots obtained in polythene bags during the 1972 trial proved inadequate, since some pectolytic colonies transferred to nutrient agar for the oxidase test, showed no pectolytic activity when placed back on Cuppels and Kelman's medium. This was not known until early August 1972, and the problem was not resolved. Therefore the results in Table 6, showing the percentage number of tubers rotting with Erwinia spp. must again, as in the 1971 trial, be treated with caution.

During this trial, the incidence of heel end infections in developing tubers was recorded, and the results are shown in Table 7. Such rots were found only occasionally and even then, rots were found on other parts of the same tubers. Infection of tubers through stolons is undoubtedly much less frequent than infection through soil.

The unexpected differences in the results in 1971 and 1972 are difficult to explain. The effects of soil moisture, determined by rainfall, and of soil temperature, on the infection of tubers in the field is illustrated in Figs. 2 and 3 for the data obtained in 1971 and 1972, respectively. Rainfall and soil temperature data were obtained for the period June 1st to August 31st for both years. Although tubers were not sampled in June due to the lack of tuber initiation until the third week in June, the amount of rainfall and changes in soil temperature were thought to be important during this period, since some decaying seed tubers had been found. By mid-July 1971 approximately 50% of seed tubers had decayed and there had been very little rain since the end of June. It is interesting to note that 'rotted mother tuber' samples taken

Figure 2. Field infection in relation to rainfall and soil temperature 1971



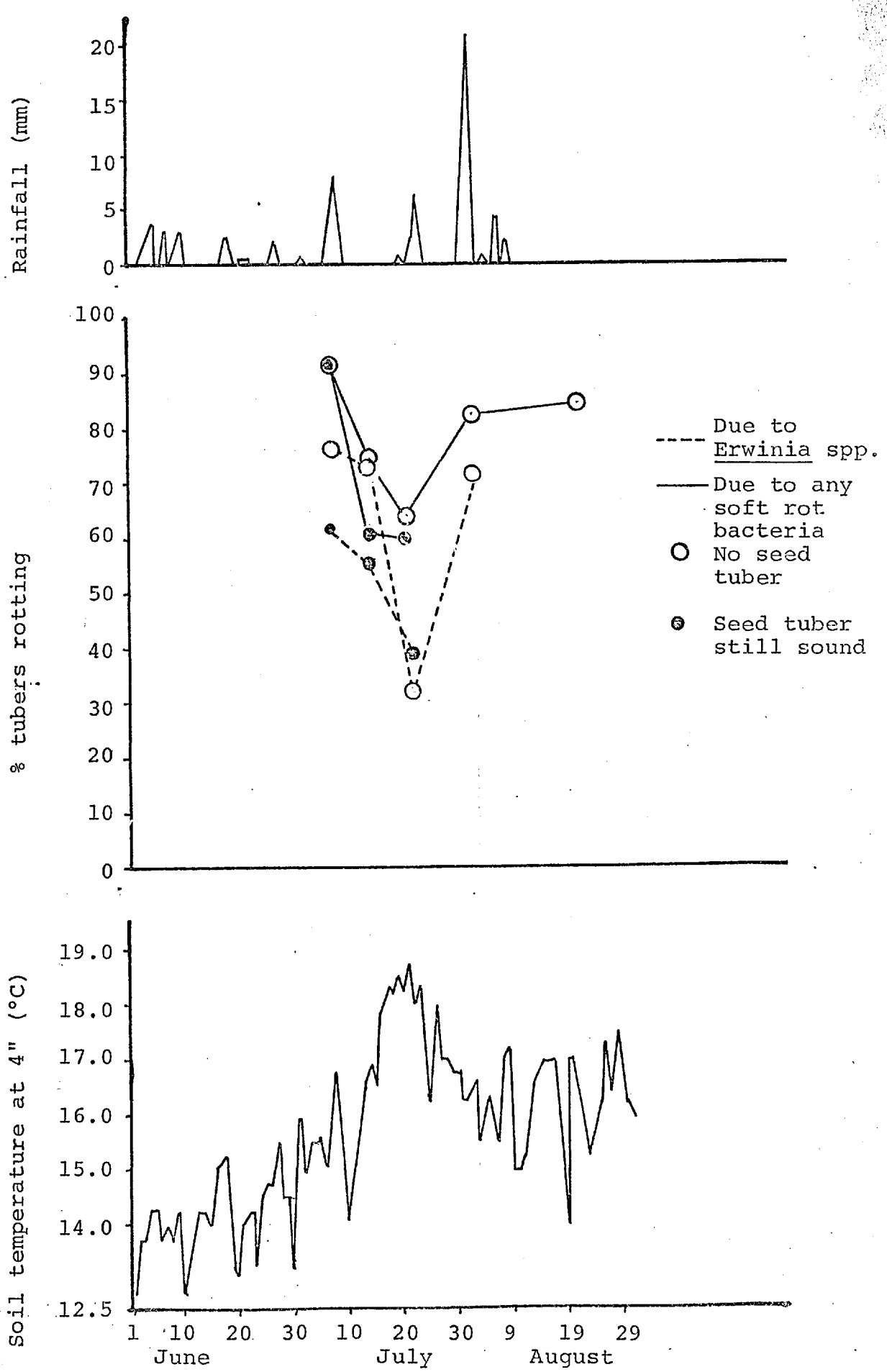


Figure 3. Field infection in relation to rainfall and soil temperature 1972

on July 7th 1971 accompanied by 5 mm of rain showed higher percentage of infected tubers than those taken on July 14th during a dry spell. Although there was a large difference between the proportions of infected tubers harvested from both treatments on July 7th, the additional rain on July 8th and 9th may have caused the lack of difference in those tubers harvested on July 14th.

One could argue that various trends could be explained by the meteorological data shown in Figs 2 and 3, but high rainfall and high soil temperature between June and August in 1971 were accompanied by initially low numbers of tubers infected by soft rot bacteria, whereas low rainfall and low temperatures over the same period in 1972 were accompanied by initially high numbers of infected tubers. For bacteria to migrate through soil the presence of free water would appear to be essential. The results expressed in Figs. 2 and 3 appear to show the opposite, an unexplainable result and unfortunately soil moisture content in the field was not determined in either trial. This may have helped to explain the problem.

No Erwinia spp. were found in soil around the tubers until late June and early July in 1971 and 1972, but no quantitative bacterial population counts in the soil were made for either year.

In conclusion, it would appear that although the infection is initially present in the seed tuber (data given in Appendix 3), infection of tubers by bacteria passing through soil from the decaying seed tuber is complex and does not appear to depend on rainfall and soil temperature as major

factors. It is also obvious that bacteria other than Erwinia spp. may be responsible for rotting.

Observation on Heel End Rots During the 1972 Field Trial

During the 1972 trial in the field, the incidence of heel end rot was assessed after incubation in the polythene bag test. The results are shown in Table 7.

Table 7. Incidence of heel end infections in developing tubers in 1972

Sampling date	Number of tubers sampled	% showing heel end rot
7/7/72	46	4
14/7/72	59	8
21/7/72	49	6
2/8/72	67	6
21/8/72	65	9

Although heel end rots were found, other rots occurred on the same tubers. Consequently, it appears that although infections may be carried over from the seed to the new tuber via the stolon, infection can occur in other ways.

The Origin of Heel End Rots

An attempt to determine the origin of heel end rots was made by taking whole plants from the field, removing the soil from the sub aerial parts, which were then enclosed in polythene bags containing 25 ml of water. Out of ten plants five had their seed tubers removed. The plants were stored at room temperature in the dark.

Some new tubers rotted but none showed heel end rots although a few stolons collapsed. An interesting observation was that after incubation for twelve days, even very small tubers (5 g) had begun to sprout while still attached to the plant. This happened only with tubers from those plants whose seed tuber was still present. Seed tuber decay had started in most tubers and it is suggested that ethylene released during rotting may have broken the dormancy of these immature tubers.

The Infections in Relation to Soil Water Content and Tuber Size

Possible factors affecting infection are tuber size and the water content of soil. Tuber size could be a factor expressed in two ways; the surface area of tuber exposed to the bacteria, and the physiological state of the lenticels. This will be dealt with in more detail in a later section. The experiment was also intended to give some information on the effectiveness of UTSC stocks to eradicate E. atroseptica as a pathogen of potatoes. This will also be discussed in more detail in a later section.

Large black polythene bags containing about 60 lb of soil were treated with 5 g 13:13:40 potato fertilizer and limed to about pH 7.0. The soil came from a field in which potatoes had not been grown for at least 12 years. VTSC seed tubers were planted in the soil which was later treated in one of four ways.

a. Wet soil - E. atroseptica added (WI)
b. Wet soil - killed E. atroseptica added (WNI)
c. Dry soil - E. atroseptica added (DI)
d. Dry soil - killed E. atroseptica added (DNI).

E. atroseptica A13 was used for treatments (b) and (d), and killed by autoclaving at 15 psi for 30 min for use in treatments (a) and (c). 'Wet soil' received one litre of water per day for two weeks after tuber initiation and then every three to four days. 'Dry soil' was watered only sufficiently to prevent wilting of the plants. Samples of soil were removed from one bag in each treatment which consisted of five replicate bags. The water contents of the soil samples, pooled from five samples per bag, were calculated from the soil weights before and after drying to constant weight (Appendix 5).

Bacteria were added to soil as 50 ml (10^8 cells/ml) of E. atroseptica A13, known to be virulent. Cells were obtained from 24 h broth cultures suspended in sterile water after washing, and added to the soil down two glass tubes inserted in the soil with their lower openings adjacent to and at the level of the middle of the seed tuber. This method of inoculation was intended to simulate bacteria coming from a rotting seed tuber. Inocula were added at two dates to separate bags and tubers sampled at four dates after the first inoculation and twice after the second. Sampling involved removal of all tubers from each bag of soil, and weighing each tuber after washing and drying individually. Tubers were then placed in the polythene bag test, each batch consisting of the tubers from one plant. The numbers of tubers rotting in polythene bags are shown in Table 8. The data presented in Table 8 show that rotting even occurred in tubers grown under 'Erwinia-free' conditions, and that some of this rotting was caused by Erwinia spp., a puzzling result which cannot easily be explained.

Table 8. % developing tubers rotting in polythene bags (% tubers rotting due to Erwinia atroseptica)

Sample Date	Treatment			
	Wet Soil		Dry Soil	
	Inoculated[1]	Control[2]	Inoculated[1]	Control[2]
Inoculated 28/7/72				
2/8/72	88 (76)	71 (29)	78 (54)	67 (16)
20/8/72	92 (57)	66 (29)	71 (41)	47 (21)
14/9/72	96 (71)	58 (22)	85 (70)	37 (8)
27/10/72	99 (84)	56 (24)	89 (70)	50 (12)
Inoculated 30/8/72				
4/9/72	97 (67)	68 (20)	69 (48)	48 (15)
14/10/72	78 (62)	52 (23)	73 (41)	50 (13)

[1]Living cells added to soil.

[2]Heat-killed cells added to soil.

The data also suggests that more new tubers are infected under wet conditions than under dry conditions. However, the differences were not so large as expected. This applied both to infection by Erwinia spp. and by other bacteria. Since bacteria must migrate from the rotting seed tuber to new tubers, the presence of free moisture in soil would appear to be an important factor in this process. The soil water content data shown in Appendix 5 show large differences between 'wet soil' and 'dry soil' throughout the experiment. It appears that a soil water content of 7.5 to 9.0%, found in 'dry soil', which just maintained non-wilting conditions, is sufficient for successful migration of bacteria through soil and consequent infection of tubers.

Erwinia atroseptica inoculations added to soil in late August did not produce substantially different numbers of tubers rotting, to those produced from soil inoculations made in late July. The progress of the infection with time shows some variation in numbers of tubers rotting and in those containing Erwinia spp., but this may have been caused by the limited size of the trial, with only five replicates of each treatment.

A major criticism of the experiment, was found in the method of isolation and identification of Erwinia spp. from rotted tissue. Bacteria were identified as Erwinia spp. if single pectolytic colonies from Cuppels and Kelman's medium were oxidase negative when transferred to and cultured on nutrient agar. However it was found that when such oxidase negative colonies, supposedly taken from pure culture, were transferred back to Cuppels and Kelman's medium, some were not pectolytic. The purity of pectolytic bacterial colonies isolated from tubers rotting in polythene bags is in doubt. No investigations as to whether Erwinia spp. have symbiotic relationships with other bacteria have been made, but this experiment does point out the difficulties involved in sampling bacteria for isolation and identification. Every method tried has failed to yield a satisfactory procedure when used to isolate and identify Erwinia spp. present in rots. Even Koch's postulates cannot be applied since almost all tubers are infected in the field, and any tissue slice used for the ability of a particular bacterial culture to cause rotting, will almost certainly rot due to its own populations of soft rot bacteria. Inoculations of the bacteria under test into wounds in whole tubers are mostly unsuccessful for use in

identification of Erwinia spp., since the conditions under which one Erwinia spp. culture will rot may not favour rotting by another. These results are shown in Table 16. Under the very favourable conditions for rotting to occur, for example, the presence of free moisture on the tuber surface, and low oxygen concentrations in the atmosphere around tubers, many infections already established within tubers also cause rotting.

Any series of biochemical tests for the identification of Erwinia spp. must involve too many tests to be of use in the identification of bacteria isolated from the rots obtained throughout the duration of a field experiment conducted in the same manner as this one.

The results in Table 9 show the relation of infection incidence of tubers to their weight at the time of harvesting.

Table 9. % tubers infected in relation to tuber weight

Sampling Date	Inoculation Date	Treatment	Weight of tubers (g) 0-30	30-70	>70
28/7/72	2/8/72	WI[1]	91(88)*	63(58)	33(0)
		DI[2]	84(64)	50(25)	-
	28/8/72	WI	81(61)	96(52)	57(0)
		DI	70(43)	77(38)	50(25)
	14/9/72	WI	100(79)	100(81)	80(30)
		DI	91(78)	89(67)	25(25)
	27/10/72	WI	100(93)	100(83)	91(64)
		DI	95(78)	95(74)	25(0)
30/8/72	4/9/72	WI	100(83)	100(68)	70(46)
		DI	76(59)	63(37)	0(0)
	14/10/72	WI	87(79)	75(54)	50(25)
		DI	37(17)	76(48)	25(0)

[1]WI = Wet, inoculated soil.

[2]DI = Dry, inoculated soil.

*Numbers in brackets are % tubers containing Erwinia spp.

The results in Table 9 suggest that larger tubers are less likely to be infected than are smaller tubers. This may depend on rates of differentiation of lenticels as tubers mature and will be discussed in a later section.

In conclusion, the results obtained in this experiment suggest that infection of tubers is affected by water content of the soil, but not markedly so. The size of the tuber appears to be an important factor in their infection by soft rot bacteria including Erwinia atroseptica. The methods used for isolation and identification of Erwinia spp. from rotting tubers have proved inadequate.

It had been intended to repeat this experiment in 1973 with marked (antibiotic resistant), mutant strains of E. atroseptica but such mutants as were obtained had lost their virulence to potatoes, so further experiments with polythene-container grown plants were not done in 1973. Information on the production of antibiotic resistant mutant strains of E. atroseptica is presented in Appendix 2.

Development of VTSC Seed Tubers in the Field

VTSC tubers were planted between groups of ten non-VTSC seed tubers along four peripheral rows in an acre plot of Majestic potatoes. New tubers were taken at two dates from VTSC plants and from plants next to them and placed in polythene bags with 10 ml of water without prior washing for 12 d. The results are given in Table 10.

The results in Table 10 show that new tubers from VTSC seed tubers planted close to non-VTSC seed tubers became infected but to a lesser extent than did tubers from plants

grown from non-VTSC tubers.

Table 10. Rotting of tubers from VTSC and non-VTSC plants

Sampling Date	Mean % tubers infected with soft rot bacteria[1]		
	VTSC plants	Field infected plants[2]	
		Sample 1	Sample 2
1/8/72	38	49	63
1/9/72	53	74	63

[1] 8 plants per treatment.

[2] Samples 1 and 2 are from plants on either side of VTSC plant.

The incidence of rotting of tubers from VTSC plants was also found to be less than that found in the polythene-container grown plants discussed in the previous experiment. It was also lower in early August in non-VTSC plants in the peripheral rows than in tubers from plants taken at the same time from within the plot (see Table 6). This suggests that new tubers become infected by bacteria coming from rotting seed tubers of other plants as well as from the seed tubers of the parent plants.

Infection of Lenticels

The incidence of soft rot bacteria in lenticels and on the tuber surface

In November 1972, lenticels and scrapings of equivalent size to lenticels from non-lenticellular areas of the tuber surface were removed aseptically with a sterile scalpel, from tubers stored under different conditions. Ten lenticels or non-lenticellular scrapings were pooled and macerated in 1 ml

of sterile water in an alcohol-flamed pestle and mortar. 0.1 ml aliquots of each treatment were streaked on to Cuppels and Kelman's medium (10 plates/treatment, each from separate macerations), and after incubation for 48 h at 25°C, all pectolytic colonies were transferred to freshly prepared VTSC tuber tissue slices. These were prepared by slicing the tubers with a heat sterilized knife after first flaming alcohol-dipped tubers. Each slice was placed in a Petri dish containing 5 ml of sterile water. A groove was cut into the upper surface of the slice with a sterile scalpel and the bacteria streaked into this groove. These slices were incubated together with non-inoculated control tissue slices, for a further 48 h at 25°C before observing for the presence of soft rotting. The numbers of isolates that contained soft rot bacteria are shown in Table 11.

Table 11. Isolation of soft rot bacteria from the surfaces of tubers stored in different ways

	Number of isolates (out of 10) containing soft rot bacteria	
	Part of tuber surface sampled	
Tuber treatment	Lenticels	Periderm
VTSC stock stored in sacks at normal temperatures (8-15°C)	0	0
Field infected tubers stored in open trays at normal temperatures (8-15°C)	7	2
Field infected tubers stored at 2-3°C	9	6

The results in Table 11 show that VTSC tubers obtained in November 1972 contained few if any soft rot bacteria. An interesting observation is that the incidence of infection

both on the surface and within lenticels was greater in tubers stored at 2-3°C, although in both these storage treatments lenticels showed a greater incidence of soft rot infections than the tuber surface.

Infection of the lenticels

To investigate the entry of the pathogen into tubers, lenticels in various positions on the tuber surface were ringed with a wax seal before applying 0.02 ml of different concentrations of cells of E. atroseptica NCPPB 1277 or E. atroseptica A13. The tubers were incubated above moist vermiculite in closed boxes for up to a week before putting them in the polythene bag test. They were inspected daily to determine what proportion of rots originated from treated lenticels. The nature of the rots and the rapidity with which they developed in and around the inoculated areas made it difficult to determine their origin, so the results were inconclusive.

In December 1972 the experiment was repeated using VTSC tubers. Cultures of E. atroseptica NCPPB 1277 and E. atroseptica A13 were used. Loopfulls of rots developing from lenticels treated with E. atroseptica NCPPB 1277 were streaked on to nutrient agar and after 48 h incubation at 25°C, single colonies were tested for agglutination with antisera to E. atroseptica NCPPB 1277 produced by Webb (1971), by a slide agglutinin test. After seven days incubation over moist vermiculite in closed boxes the tubers were placed in the polythene bag test for twelve days. The results are given in Table 12.

Table 12. Lenticel inoculations from droplets (0.02 ml) containing Erwinia atroseptica

Culture	Cell concentration (cells/ml)	% treated lenticels rotting	% treated lenticels rotting in which bacteria agglutinated anti-E. atroseptica NCPPB 1277 serum were found
Erwinia atroseptica NCPPB 1277	0	0	0
	10^2	0	0
	10^3	30	30
	10^4	25	25
	10^5	55	55
	10^6	80	75
Erwinia atroseptica A13	0	0	-
	10^2	0	-
	10^3	20	-
	10^4	40	-
	10^5	75	-
	10^6	75	-

The results in Table 12 show that the threshold concentration for infection by both bacterial strains was 10^3 cells/ml, and that Erwinia atroseptica can infect potato tubers through lenticels. Occasionally, rots other than those beneath treated lenticels were found but were not typical soft rots. The use of antisera as a method of identification of bacteria has many limitations, although it was useful in this experiment. Many non-pectolytic bacteria were isolated from rots and none of these agglutinated the antiserum. However, E. atroseptica A13 did, and therefore isolations from tubers inoculated with this strain were not tested.

Incidence of Infection in VTSC Potato Tubers

VTSC tubers were washed individually and placed in the polythene bag test. The results are shown in Table 13.

Table 13. Soft rot contaminants in VTSC stock

Year	Number of tubers sampled	Number infected by soft rot bacteria
1971	40	0 (3)*
1972	50	4 (7)

*The figures in brackets show the number of tubers showing rotting due to fungi, after 12 days.

Although some rots were found, no typical soft rots developed with 1971 VTSC tubers. However, 1972 tubers from the same farm occasionally gave typical soft rots and, pectolytic bacteria isolated from these rots on Cuppels and Kelman's medium when reintroduced into tuber wounds 1 cm deep, caused soft rotting.

The bacteria obtained from the soft rots in 1972 were found to have pectolytic activity on Cuppels and Kelman's medium and one culture was found to be oxidase negative.

The Effect of Tuber Turgidity in Relation to Infection

VTSC tubers were washed individually and half were placed in the polythene bags with water (10 ml) for 24 h. The other half were placed in polythene bags containing no water. An inoculum of E. atroseptica A13 (10^7 cells/ml) was then applied to the surface of the tubers by dipping for a few seconds, and allowing them to dry overnight. Tubers were

then placed in the polythene bag test after a further 48 h. The results are expressed in Table 14.

Table 14.

Day	Tuber Treatment	% tubers rotting (out of 50)	Mean number of rots per tuber
4	Soaked	40	3
	Non-soaked	32	3
8	Soaked	92	5
	Non-soaked	90	4
12	Soaked	100	5
	Non-soaked	96	5

A 2 x 3 contingency table on the percentage number of tubers rotting showed no significant differences between the tubers. Thus it appeared that the state of turgidity of the tubers had little effect on the infection.

Identification of Infected Areas by the Use of 2,3,4-triphenyl tetrazolium chloride

From the results in Table 11 it is evident that soft rot bacteria may be found both on the tuber surface and within lenticels. However, the results obtained were to some extent misleading since it is difficult to remove an entire lenticel without removing other tissue. The use of redox reagents has been explored to provide a more suitable method by which infected sites may be identified. The following chemicals were used:

Benzyl viologen (coloured in the reduced state)

Phenylindo-2,6-dichlorophenol (coloured in the oxidised state)

Safranine (coloured in the oxidised state)

Methylene blue (coloured in the oxidised state)

2,3,5-triphenyl tetrazolium chloride sodium salt (tetrazolium salt) (coloured in the reduced state).

Tetrazolium salt is a useful redox reagent since the reduced product, a formazan, is insoluble in water.

Erwinia-free tubers (VTSC) were well washed and placed in the polythene bag test for 24 h, before dipping into a 10^7 cell per ml suspension of E. atroseptica A13. After air drying overnight and storing for 48 h in the dark at room temperature (20-23°C) tubers (25 per sample) were sprayed to run off with the above salts at a concentration of 0.25%. After 48 h incubation in the polythene bag test tubers were inspected for stained or non-stained areas.

Of those reagents giving staining in the oxidised state, only phenylindo-2,6-dichlorophenol gave any indication of a colour change in the tubers. The liquids in some bags remained turquoise, others light green or cream. Those tubers giving cream colouration rotted within 6 days, whereas those showing a light green colouration rotted within 12 days. Any giving no colour change after 6 days did not rot.

Of the reagents staining in the reduced state, only tetrazolium salt gave differential staining. It was noted that many lenticels stained, showing a localised pink colouration around and within lenticels. Many wounds and growth cracks gave a similar staining reaction. After twelve days in the polythene bag test, rots had developed from most of

lenticels, and almost all rots that did develop, originated from stained lenticels. Soft rots often developed from stained wounds and lenticels.

Optimal Concentration of Tetrazolium salt

Washed field infected tubers were dipped into a variety of tetrazolium salt concentrations (0.05%, 0.1%, 0.25%, 0.5% and 1.0% aqueous solutions). After a 5 sec dip, tubers were air dried and placed in the polythene bag test. Examination at 24 h intervals, indicated that the optimum concentration was 0.25%. Above this value, weak tissue staining other than that of bacterial staining occurred, while below 0.25%, a very weak lenticellular staining reaction was found.

The use of a 0.25% aqueous solution of tetrazolium salt was thus found to be an efficient method for identifying infection sites on a tuber. A spray was considered more useful than a dip, since contamination from tuber to tuber was minimised.

The theory behind its action is explained by the fact that tetrazolium salts are efficient redox indicators. The colour change is noticeable when the respiratory rate changes to such an extent that the insoluble reduced conjugate is obvious by its degree of accumulation in that area of respiratory change. The conditions of the polythene bag test cause an increase in the populations of those bacteria capable of rapid multiplication on or within a potato tuber. Some bacteria, Erwinia spp., for instance, possess high levels of cell-bound dehydrogenase enzymes. Thus the reducing power

may be increased. It is possible that the only bacteria capable of achieving such a rapid rate of multiplication within the environment of the lenticel are those which possess the ability to produce pectic enzymes. The evidence does not indicate whether the change of respiration rate is due to the increased bacterial population or its effect upon the tuber tissue or both. However a bacterial culture (10^8 cells/ml) did stain when treated with 0.25% tetrazolium salt solution.

The Lenticel as the Major Point of Entry of the Pathogen into the Tuber

25 VTSC tubers were washed, dried and sprayed to run off with 0.25% tetrazolium salt solution, before drying and treating in polythene bags. After 12 d very few areas had stained. These areas were limited to growth cracks, wounds and lenticels, and some water soaked areas eventually stained, but no rotting was found. It appeared that these VTSC tubers contained few if any soft rot bacteria.

A batch of 50 VTSC tubers were treated with tetrazolium salt and placed in the polythene bag test. No staining occurred after 48 h. The tubers were washed and dried, and one half of each tuber was dipped into a suspension of either *E. atroseptica* A13, or *E. atroseptica* NCPPB 1277 (10^7 cells/ml), and allowed to air dry overnight. The tubers were replaced in polythene bags with water and observed for staining and rotting.

After 24 h only the inoculated halves contained stained lenticels, but after 4 days many lenticels in non-inoculated areas stained due to contamination from other

lenticels *via* the water in the bags. At this time rotting was observed in the inoculated halves, always beneath stained lenticels, but not until after another 3 d in the non-inoculated halves.

This result gives good evidence for lenticels being the major entry sites for infection by the pathogen. It was also interesting to note that no soft rot bacteria were found in the VTSC stock and it seems highly likely that the soft rot infections in VTSC seed used in the polythene container field trial (see Table 8), were infected by soil bacteria, when planted under *Erwinia*-free conditions.

The results in Tables 15a and 15b show typical numbers of lenticels staining in artificially inoculated VTSC stock and naturally infected tubers grown in the field at Silwood Park and stored for 1 month after harvest.

Table 15. Typical numbers of lenticels staining in tetrazolium treated tubers

a. VTSC stock dipped into a suspension of *E. atroseptica* A13 (10^7 cells/ml)

	Sum of stained areas on 25 tubers				
Incubation time(d)	Stained lenticels	Rotted stained lenticels	Rotted non-stained lenticels	Stained growth cracks and wounds	Rotted stained growth cracks and wounds
1	63	0	0	10	0
2	77	2	0	21	1
4	96	53	0	25	14
6	≈103	71	0	28	18
8	≈110	100	0	28	19

Results obtained on days 6 and 8 were difficult to assess because rots had begun to merge. The number of stained lenticels per tuber ranged from 0 to 14.

b. Field infected tubers taken from storage in late October

Sum of stained areas on 25 tubers

Incubation time (d)	Stained lenticels	Rotted stained lenticels	Rotted non-stained lenticels	Stained growth cracks and wounds	Rotted stained growth cracks and wounds
1	59	0	0	4	0
2	71	5	0	8	0
4	78	51	0	12	2
6	≈81	76	0	12	5
8	≈89	80	0	12	6

The number of stained lenticels per tuber ranged from 0 to 11. Lenticel staining and rotting from results in Table 15a are illustrated in Fig. 4 which shows that after 8 d almost all stained lenticels had rotted.

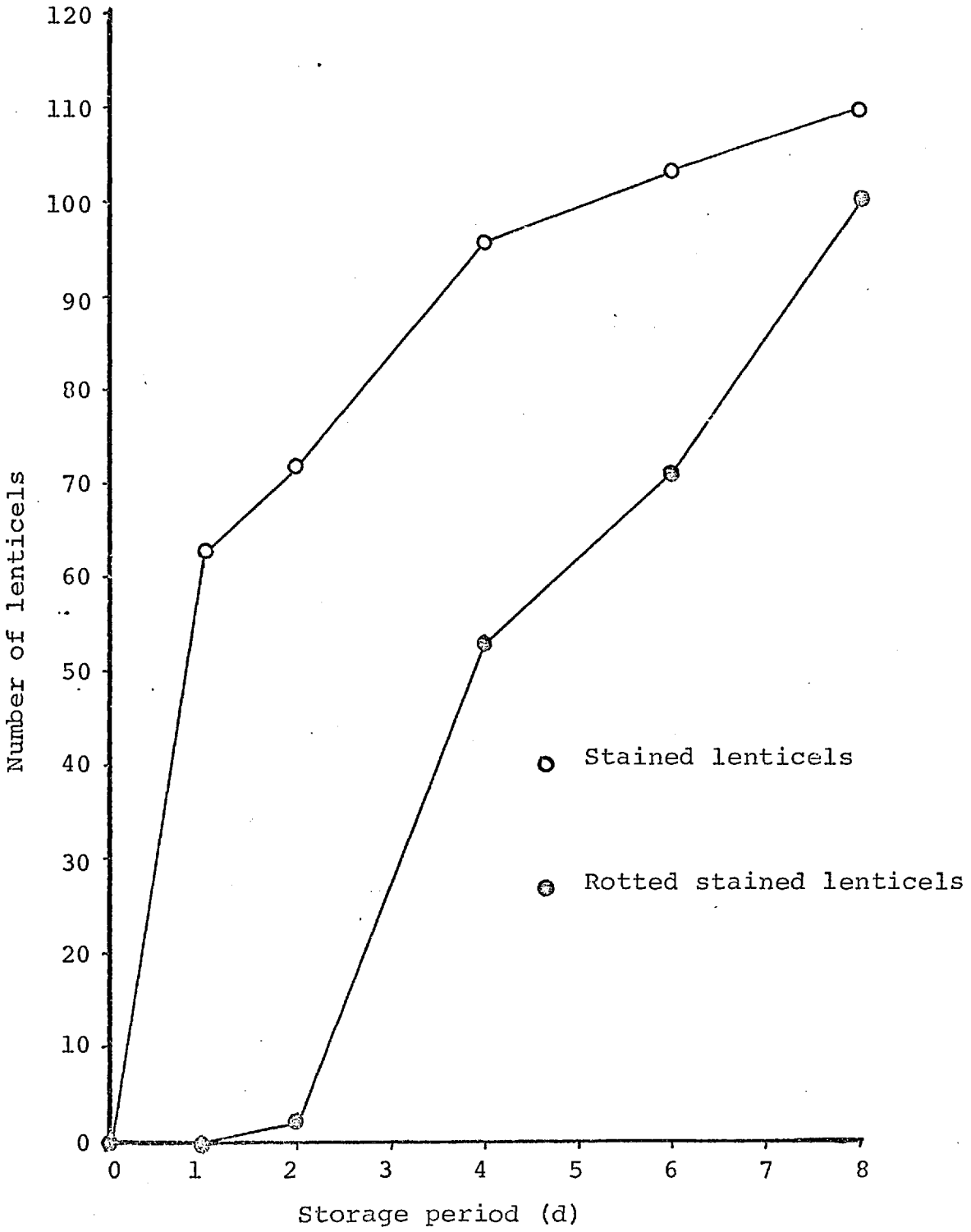


Figure 4. Staining and rotting in tetrazolium salt treated tubers

SECTION III

OBSERVATIONS ON THE INFECTION DURING TUBER STORAGE

The Position of the Pathogen in or on the Tuber

This section, as did parts of Section 2, deals with the establishment of infection during the growing season, once the pathogen has entered the tuber, and with its life history throughout tuber storage.

The Process of Soft Rotting

Soft rots whether found in storage or induced by polythene bag treatments always develop from a point source on the tuber surface. Erwinia spp. have been shown to infect tubers mainly via the lenticels (Section 2), and it is in these areas in which they appear to remain.

Rotting clearly could depend upon the environmental conditions of storage. Accordingly, washed and air dried tubers were inoculated with 10^9 cells/ml suspensions of different Erwinia spp. strains (10^9 cells/ml), into cork borer wounds 0.25 and 1.0 cm deep. The wounds were sealed and the tubers incubated in closed boxes in either dry or humid conditions for 4 days at room temperature (c. 20-21°C). Humid conditions were produced by placing tubers above moist vermiculite. The volumes of rots produced are presented in Table 16.

A multi-factorial analysis of variance showed significant differences at $p = 0.001$ for inoculation depth. No significant differences between strains of species and humidity were found. Since the replication could not be

Table 16. Mean volume of rot (10 tubers) (ml) at two inoculation depths

Culture	Treatment			
	10 mm		2.5 mm	
	Dry	Humid	Dry	Humid
E. atroseptica NCPPB 1277	1.8	2.7	0.3	0.6
E. atroseptica E14	2.1	2.7	0.4	0.6
E. atroseptica A13	2.2	2.9	0.4	0.7
E. carotovora NCTC 1747	0	0.9	0	0
E. carotovora NCTC 1774	0	0	0	0
E. carotovora NCPPB 1280	0	1.0	0	0
E. aroideae NCPPB 1276	0	0.7	0	0.2

included as a fourth factor, the statistical test has been ignored, and replaced by simple observation. In further, similar experiments, the same pattern emerged, and the conclusions were drawn as follows.

1. Increase in humidity of the storage atmosphere increased rotting. With strains *E. carotovora* 1774 and 1747 and *E. aroideae* 1276, high relative humidity is a critical factor in their ability to cause soft rotting.
2. Different *Erwinia* spp., have very different virulence.
3. Rots in humid conditions were found to be odourless, wet, white rots, while those in dry conditions were dry, limited brown rots.

Tubers incubated in polythene bags with water almost always rotted. Such rots always originated at the tuber surface and were always white in colour, often spreading rapidly along the surface, not rotting to any depth below the vascular

tissue until two to three days after rotting had commenced. A blistered effect was often noted on the periderm above the rot and surrounding this, watermarking was a common feature. Rots arising from different point sources eventually met and within seven to eight days the whole tuber had often rotted. Occasionally rots did not spread throughout the whole tuber, thus leaving an area of normal healthy tissue, where sprouts formed rapidly. In juvenile tubers, lenticel proliferation was a common occurrence. This phenomenon was also found on non-infected tubers treated in the same manner. VTSC tubers placed in polythene bags with 10 ml of water for 48 h before being dipped in a suspension of E. atroseptica A13 (10^5 cells/ml), rotted in the same manner as field infected tubers, when re-subjected to the polythene bag test.

Formation of Dry Limited Rots

Inoculated VTSC tubers, and field infected tubers when taken out of the polythene bag test after 3 d before storing in dry conditions for a further 4 d, contained rots that were dry and limited. Such tubers were either replaced in polythene bags with 10 ml water or incubated above moist vermiculite in closed boxes to increase humidity. These conditions caused typical white non-limited soft rotting to develop from the dry limited lesions.

Tubers showing lenticellular dry rots, stored dry and well aerated for two months could also be induced to form wet rapidly spreading rots within 4 d of placing them in the polythene bag test. It appeared that the pathogen, although limited in these dry rots was still viable, its virulence

being restricted by dry aerobic storage, and effective only in moist anaerobic storage.

Methods for Inducing Infected Tubers to Rot

From the field experiment on rotting of VTSC seed tuber offspring grown under '*Erwinia*-free' conditions, it was noted that rots were commonly induced during polythene bag storage, and that few were associated with *Erwinia* spp. The polythene bag test is a stringent test, in which soft rotting is not restricted to that caused by *Erwinia* spp. On the suggestion of Mrs. Legg (nee Whitehead) an attempt to develop a less stringent test was made. Mrs. Legg had developed a 'bucket test' intended to allow only *Erwinia* spp. to cause soft rotting. A modification of this was tested.

A 7 litre polythene bucket with a mesh platform at its base was lined with soft tissue paper. Ten tubers were placed on the mesh, and more tissue paper placed above them. Another 'pocket' of tubers was placed above this and after the addition of 250 ml of water, the bucket was flushed out with carbon dioxide, and sealed with a tight fitting lid. Tubers were examined for rotting at four day intervals after incubation at room temperature. Fig. 5 shows the arrangement of tubers in the bucket test.

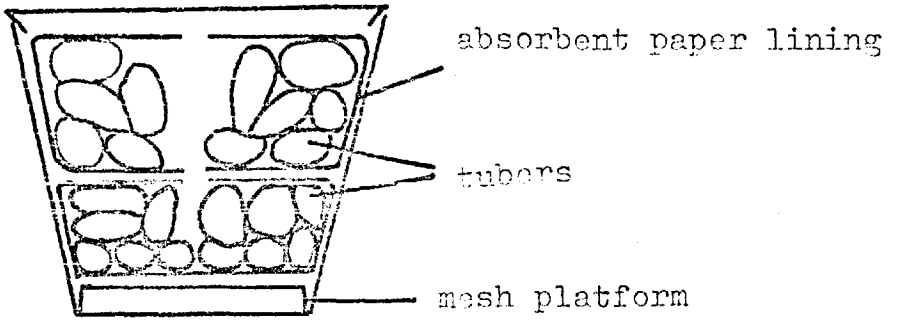


Figure 5. Diagramatic representation of the bucket test.

In one trial, tubers (20 per bucket) of both field infected tubers taken from store in January 1973, and VTSC tubers dipped in a suspension of E. atroseptica A13 (10^5 cells/ml) were compared to equivalent samples incubated in polythene bags containing 10 ml of water. The results are shown in Table 17.

Table 17. A comparison between rotting of tubers stored in the bucket test and the polythene bag test

	Number of tubers rotting				
	Tubers stored in buckets		Tubers stored in polythene bags		
Period of incubation (d)	Field infected tubers	Inoculated VTSC tubers	Field infected tubers	Inoculated VTSC tubers	Non-inoculated VTSC tubers
4	0	0	2	4	0
8	0	0	5	10	0
12	0	0	10	18	0
16	4	5	10	16	1
20	3	8	12	19	0

It was evident that the incidence of rotting was much greater in tubers stored in polythene bags than those in buckets. Comparison of the numbers of inoculated VTSC tubers in the two treatments shows large differences in the numbers of tubers infected only with Erwinia atroseptica which rotted, and it is obvious that the bucket test is not an efficient method for obtaining the maximum number of rots from the number of sites of infection.

Reduction of the Number of Sites of Infection During Storage

These experiments used tubers from both the 1971 and 1972 crops grown at Silwood Park. Tubers were taken from

growing plants in the field and from store over the period August to March. Some were untreated, others were well washed and air dried. Others were placed for 15 min in a 10% aqueous solution of 'Chloros' before air drying. The rots developing during storage in the polythene bag test are shown in Tables 18 and 19, and are illustrated in Fig. 6.

Table 18. Effect of various tuber surface treatments on rotting of tubers taken from storage in 1971-1972

% tubers rotting in polythene bags with 10 ml water

Sampling date	Time of incubation (d)	Surface Treatment		
		Untreated	Washed	'Chloros'
2/8/71[1]	4	80	85	85
	7	95	100	90
	8	100	100	95
20/11/71[2]	4	40	48	8
	9	88	96	84
12/12/71[2]	4	50	50	8
	6	80	70	45
	9	88	90	83
13/1/72[2]	4	28	32	0
	6	40	48	8
	9	68	80	52

[1] 20 tubers per treatment.

[2] 25 tubers per treatment.

The results in Table 18 show that in 1971, surface treatment of immature tubers with 10% chloros did not markedly reduce the numbers of tubers which rotted. However, it did reduce the numbers of mature tubers which rotted in the successive samples. Further, the results show that washing tubers increases the number of rots even when care is taken

to prevent contamination from tuber to tuber.

Table 19. Effect of various tuber surface treatments on the rotting viability of tubers taken from storage in 1972-1973

% tubers rotting in polythene bags with 10 ml water[1]

Sampling date	Time of incubation (d)	Surface treatment		
		Untreated	Washed	'Chloros'
10/9/72	4	28	32	16
	6	56	68	36
	12	76	80	68
21/10/72	4	32	40	24
	6	48	60	60
	12	76	88	72
8/12/72	4	4	8	0
	8	44	60	16
	12	56	84	56
3/2/73	4	0	0	0
	8	12	24	4
	12	32	40	32
12/4/73	4	0	0	0
	8	4	8	4
	12	24	32	20

[1] 25 tubers per treatment.

The results in Table 19 show that in 1972, Chloros treatment did not markedly reduce the numbers of mature tubers which rotted, and that the incidence of rotting was lower than in 1971. During storage in both years, the number of tubers which rotted decreased, although the rate of decrease in store was greater in 1972 than in 1971.

Another point of interest in the 1972 data, was that the numbers of tubers which rotted increased during harvest.

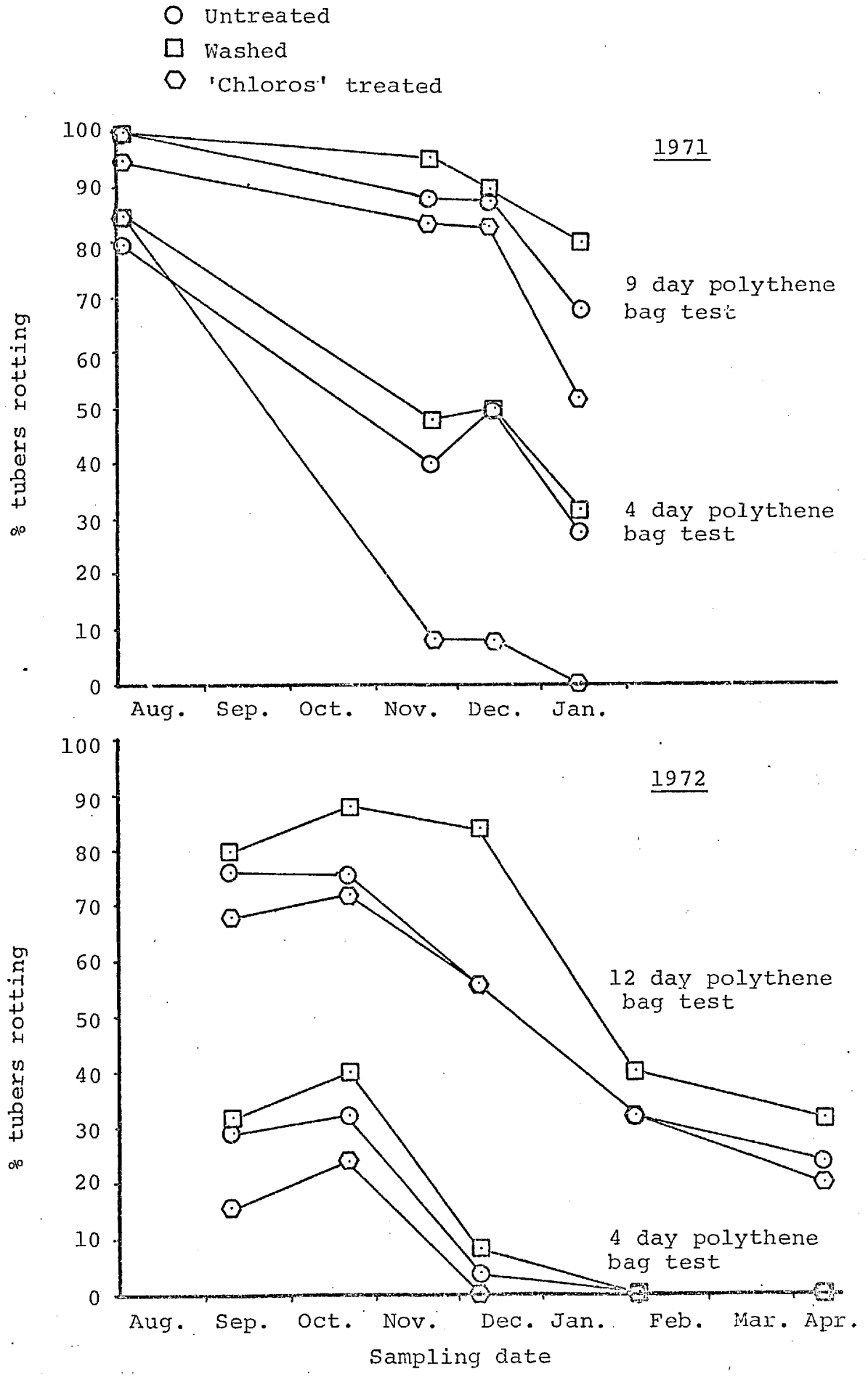


Figure 6. Surface treatment of tubers during storage

Differences between treatments are often best shown with the 4 d polythene bag test data and Fig. 6 illustrates this quite well.

Viability of Soft Rot Bacteria Within Lenticels

Tubers were taken at intervals throughout the growth and storage of the 1972 crop, and after washing tubers individually, they were treated with tetrazolium salt. The staining and rotting developing after storage for twelve days in the polythene bag test is shown in Table 20 compared with non-treated tubers.

Table 20. Rotting from lenticel infections during storage[1]

Sampling date	Treatment	Mean No.[2,3] of stained lenticels per tuber	Mean No.[4] of rots per tuber	% of tubers staining[3]	% of tubers rotting[4]
27/6/72	Tetrazolium	6	5	80	100
	None	-	4	-	84
15/8/72	Tetrazolium	5	5	84	80
	None	-	6	-	72
15/10/72	Tetrazolium	4	4	88	88
	None	-	3	-	84
12/12/72	Tetrazolium	4	4	76	76
	None	-	3	-	84
5/2/73	Tetrazolium	2	2	28	36
	None	-	1	-	28
24/3/73	Tetrazolium	0-1	1	12	12
	None	-	1	-	12

[1] Results from tubers incubated in polythene bags with water.
[2] 25 tubers per treatment.
[3] 48 h reading.
[4] 12 d reading.

The first two samples of immature tubers taken from the field showed many stained areas other than those in and

around lenticels possibly due to bacterial contamination on the tuber surface. Occasionally rots developed from these areas. This was probably due either to splitting of the periderm caused by the increased turgidity of the tubers, or bacterial entry into the tissue via lenticels.

Tubers breaking dormancy taken during the last two samples showed occasional rots developing from non-stained areas, and it was difficult to determine whether these rots had developed from lenticels. It appears that tetrazolium treatment of immature tubers increases the number of rots per tuber and also the number of tubers rotting.

The decline in numbers of infected tubers parallels that of the previous experiment (Tables 18 and 19). Also, the widespread staining reaction on immature tubers indicates that tubers in the soil have large populations of soft rot bacteria on their surfaces, these populations declining rapidly after they were harvested as mature tubers in early October.

Contamination of VTSC Tubers

In the experiment dealing with surface treatment of tubers throughout growth and storage, it was noted that washing tubers individually, increased the incidence of infection, possibly by the transferrance of bacteria from one tuber to the next, or from one infection to form another on the same tuber.

Five field infected tubers were placed in a bucket of water containing 25 VTSC tubers. After two hours, tubers were removed and placed in polythene bags with water and the

percentage number of tubers rotting compared to that found in VTSC tubers soaked in water along. The results are expressed in Table 21.

Table 21. Contamination of VTSC tubers

% number of tubers rotting

Period of incubation(d)	Treatment: Contaminated VTSC stock	Treatment: Non-contaminated VTSC stock
4	4	0
8	12	0
12	20	4

It is obvious that contamination of clean stocks is difficult to avoid when handling field stocks at the same time. It appears that successful lenticel infections at the time of contact with the contaminant bacteria is not essential. Infected water in the polythene bags is probably sufficient to cause rotting within four days.

The Effect of Dormancy on the Incidence of Infected Lenticels

In January 1973 tubers stored in various ways were treated with tetrazolium salt and placed in the polythene bag test. The results in Table 21 show the mean number of stained lenticels per tuber, and the number of tubers rotting for each of the three storage treatments used, which were, Fusarex treated tubers still dormant, tubers stored at 3°C, and sprouting tubers stored in open trays in a darkened shed.

From the results in Table 22, it is apparent that lenticel infections in tubers kept in a dormant state either

Table 22. Tuber storage and the effect of dormancy upon the incidence of infections in lenticels

Tuber treatment	Mean number of lenticels per tuber staining after 48 h	Number of tubers (out of 10) rotting in the polythene bag test with time (d)		
		3	5	12
Fusarex treated tubers	5	1	5	9
Sprouting tubers	2	0	0	4
Cold-stored tubers	7	0	1	9

by chemical treatment or by cold storage do not decrease as rapidly as do those in normal storage. However, the rate at which tubers rotted in polythene bags was not the same in these two treatments. The non-dormant tubers showed little lenticel infections and a very slow rate of rotting with a final incidence of 40% of tubers infected. Fusarex treated tubers appeared to be infected to a lesser extent than cold stored tubers but they rotted more rapidly. After 12 days, 90% of tubers in both of these treatments rotted. One suggestion for this anomaly is that whereas under normal storage conditions, surface contamination on the periderm is eradicated, leaving infections in the lenticel only, the tubers stored in closed bins containing Fusarex retained their surface populations of soft rot bacteria. However, this explanation was less persuasive after noting that condensation had formed on the inside surface of the bind containing Fusarex treated tubers. Thus the relative humidity in the bin must have been

greater than that in bins of tubers stored at 3°C, and this could well have been the determining factor.

Examination of the Degree of Infection of Old and New Tubers

This experiment was performed by an M.Sc. student, Miss J. Phear, working with the author for a short period and describes the progression of staining and rotting in tubers treated with tetrazolium salt.

Ten tubers from each of the following storage treatments were treated with tetrazolium salt and placed in the polythene bag test. They were examined periodically for staining and for rotting:

a. field infected tubers stored in open trays for 8 months,
b. field infected tubers stored at 3°C for 8 months,
c. VTSC stock stored in sacks for 8 months,
d. VTSC stock stored at 3°C for 4 months after storage at 4-6°C for 4 months, and
e. immature tubers taken from the field in May 1973.

The results are shown in Table 23.

A striking result is the difference between VTSC tubers stored at normal temperatures and those stored at 3°C. Before receiving VTSC tubers they had been stored in a thick stone-walled building in 10 cwt batches at temperatures between 4 and 6°C. This evidence indicates that fresly harvested tubers are surface contaminated with bacteria other than Erwinia spp. which may rot potato tubers when incubated in polythene bags with water, and these populations are reduced by storage for 7 months above 8°C. Rotting found in cold-stored VTSC tubers

Table 23. Condition of tubers, staining with tetrazolium and incidence of rotting of old and new tubers

Period of incubation (d)	Storage treatment				
	a	b	c	d	e
0	Flaccid and sprouting	Sub-turgid and dormant	Flaccid and sprouting	Sub-turgid and dormant	Sub-turgid
1	Pink around shoots	Pink around lenticels	No staining	No staining	Pink patches and lenticels
3	20 stained lenticels	113 stained lenticels	9 stained lenticels	7 stained lenticels	9 tubers rotted
12	No change	Some rotting	No change	Some rotting	No change
14	Rotting commenced	Rotting commenced	No change	Some rotting	No change
20	2 tubers rotted	8 tubers rotted	2 tubers rotted	7 tubers rotted	No change

was not characteristic of the rapidly spreading rots caused by E. atroseptica. It was limited and maceration in the area of rotting was not complete, so that tissue was partially intact.

The staining on new tubers indicated heavy contamination on their surfaces. Whether this was due to pectolytic bacterial spp. is not clear but 90% of the tubers rotted.

SECTION IV

FACTORS AFFECTING THE SUSCEPTIBILITY OF TUBERS TO ROTTING

Water Content of Tubers

Tubers used for experimental purposes in this investigation were stored in single layers in open wooden trays. Under these well aerated conditions, very few soft rotted tubers were found. The bulk of tubers harvested from the field at Silwood Park were, however, stored in a shed to a depth of approximately six feet, and covered with straw. 'Pockets' of soft rotted tubers were often found, more so with the 1972 crop than the 1971 crop. Pectolytic, oxidase negative bacteria were isolated from these rots, and it was noted that rotted tubers were invariably associated with moist conditions. Some 'pockets' of rotted tubers were found directly beneath leaks in the roof of the shed but in other cases the predisposing conditions could not be determined.

Results from a previous experiment (Table 16) indicated that humid conditions were favourable for rapid rotting, and it was known that incubation of tubers in polythene bags with water predisposed them to rotting. Thus, the possibility that water content of tuber tissue affected the susceptibility of tubers to rotting was investigated.

VTSC tubers were halved and one half of each was placed for 48 h with the cut surface on a glass plate. The other halves were treated as follows.

a. 48 h above moist vermiculite in a closed container,

b. 48 h with the cut surface in a shallow layer of water

which was changed at 12 h intervals,

c. 24 h as (b) followed by 24 h with the cut surface on a glass plate.

Each half was then inoculated at some distance from the cut surface at a depth of 2.5 or 10.0 mm with a suspension of E. atroseptica A13 (10^9 cells/ml). The volumes of rots obtained after a 4 d incubation period at room temperature (20-22°C) are shown in Table 24.

Table 24. Effect of water content of tubers on rotting

Mean volume of rot (ml)[1]

Tuber Treatment	Depth of wound (mm)	
	2.5	10.0
48 h moist vermiculite	0.4	1.8
Control (glass plate)	0.4	2.1
48 h water	0.5	2.8
Control (glass plate)	0.1	1.9
24 h water, 24 h glass plate	0.4	2.1
Control (glass plate)	0.2	1.6
Whole tubers	0.5	3.2

[1]Mean of 10 tubers.

Storing in a saturated atmosphere over moist vermiculite did not increase rotting whereas there was considerably more rotting after tubers had been allowed to absorb water through the cut surface. The amount of rot in the whole tubers was unexpectably large.

A similar experiment was set up as follows. VTSC tubers were halved and one half of each was placed for 72 h with the cut surface on a glass plate. The other half of each tuber was placed for 72 h with the cut surface in a shallow

layer of water, changed at 12 h intervals to reduce the chance of soft rotting at the cut surface. Each half was then inoculated and incubated as above, before measuring the volumes of the rots. The results are shown in Table 25.

Table 25. Effect of water content of tubers on rotting

	Mean volume of rot (ml)[1]	
	Depth of wound (mm)	
Tuber treatment	2.5	10.0
72 h water	1.2	3.6
72 h glass plate	0.2	0.9

[1]Mean of 25 tubers.

A multi-factorial analysis of variance showed significant differences (at $p = 0.05$) between both inoculation depth and tuber treatments. Thus the results show that increase in tissue turgidity increases the susceptibility of tubers to rotting.

The Effect of Tissue Turgidity on Rotting of Tubers by Various Strains of Erwinia spp.

VTSC tubers were treated as in the previous experiment, but inoculated with various isolates of Erwinia spp. The results are shown in Table 26.

As in the previous experiments, increased turgidity predisposes the tissue to more rapid rotting, and with all cultures except E. carotovora NCTC 1774, E. atroseptica A13, and E. atroseptica NCPPB 1277 rotting took place only in turgid tissue. Only E. atroseptica strains rotted whole tubers under normal storage conditions.

Table 26.

	Mean volume of rot (ml)[1]			
	Tuber treatment			
	Water		Glass Plate	
	Inoculation depth (mm)			
Strain of *Erwinia* spp.	2.5	10.0	2.5	10.0
E. atroseptica A13	0.7	2.2	0.1	0.7
E. atroseptica NCPPB 1277	1.0	2.6	0.1	1.2
E. atroseptica E14	0	1.4	0	0.1
E. aroideae NCPPB 1276	0	0.7	0	0
E. carotovora NCPPB 1280	0	0.4	0	0
E. carotovora NCTC 1747	0	0.7	0	0
E. carotovora NCTC 1774	0	0	0	0
Non-inoculated control	0	0	0	0

[1]Mean of 10 tubers

Effect of Tissue Turgidity on Rotting from Lenticels

An attempt was made to investigate the above phenomena under less artificial conditions.

VTSC tubers were soaked overnight (8 h) in a bucket of water before adding a suspension of *E. atroseptica* A13 to a final concentration of 10^5 cells/ml. After 12 h the tubers were removed and allowed to air dry overnight. Tubers were halved, one half being placed in a shallow layer of water, changes frequently, and the other half on a glass plate.

The tuber halves were kept under these conditions at room temperature (20-23°C) for 12 d and the developing rots were counted every four days. The results are given in Table 27.

Table 27. The effect of tissue turgidity on rotting from inoculated lenticels

Period of incubation (d)	Mean number of rots per tuber half[1]	
	Treatment	
	Water	Glass plate
4	0-1	0
8	2	0
12	3	0

[1]Mean of 20 tuber halves.

Tissue turgidity is an important factor in rotting from lenticels. Whether this predisposition to rotting is caused by the increased turgidity or by anaerobic conditions imparted upon the tissue by the presence of water-filled intercellular spaces, is not elucidated by the results.

The Effect of Restricted Aeration on the Susceptibility of Tubers to Soft Rotting

The effect of non-aqueous films

Field infected tubers and VTSC tubers infected with *E. atroseptica* A13 as described in Materials and Methods, were entirely covered with thin films of vaseline or a mixture comprising 50% paraffin wax and 50% vaseline. The sealed tubers were stored in closed boxes at room temperature (20-23°C) for one month. Samples of ten tubers were removed at weekly intervals and examined for rotting, which is shown in Table 28.

Some rotting was found in sealed lenticel inoculated VTSC tubers but to a much lesser degree than in tubers made turgid by soaking the cut surface in water, even after storage for 4 weeks.

Table 28. The effect of non-aqueous films on rotting from lenticels

Period of storage (wk)	Number of tubers out of 10 rotting					
	Treatment					
	Vaseline sealed		Vaseline/Paraffin wax sealed		Non-sealed	
	A[1]	B[2]	A[1]	B[2]	A[1]	B[2]
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	1	0	0	0	0
4	0	3	0	1	0	0

[1]A = field infected tubers.

[2]B = E. atroseptica lenticel inoculated VTSC tubers.

Effect of non-aqueous films on rotting from E. atroseptica infected wounds

Field infected tubers were washed, air dried, and inoculations of E. atroseptica A13 (10^9 cells/ml) made into cork borer (No. 4) wounds 2.5 mm and 10.0 mm deep. Half the batch of tubers were sealed with a mixture of 50% paraffin wax and 50% vaseline. The other half were sealed only above the site of the cork borer wound. After storage for 4 d at room temperature (20-23°C) in closed boxes, the volumes of the rots produced were measured, and are shown in Table 29.

Unpaired t-tests on the data for inoculated tubers showed a significant difference ($p = 0.05$) between the rot volumes produced on sealed and non-sealed tubers at both inoculation depths, and the results from these experiments clearly show a difference between lenticel inoculations and wound inoculations. Sealing with vaseline increased rotting

Table 29. The effect of a non-aqueous film upon rotting from infections made into cork borer wounds

Mean volume of rot produced[1]

Tuber treatment							
Sealed tubers				Non-sealed tubers			
Inoculated		Non-inoculated		Inoculated		Non-inoculated	
Depth of inoculation (mm)							
2.5	10.0	2.5	10.0	2.5	10.0	2.5	10.0
0.9	2.7	0	0.4	0.3	1.8	0	0

[1]Mean of 10 tubers.

from inoculated wounds but had much less effect on rotting from lenticels though after four weeks, 3 out of 10 tubers sealed with vaseline had rotted compared with none in the controls.

The effect of different gaseous conditions on rotting

Since the effect of sealing tubers did not promote rapid rotting from infected lenticels, a further investigation into the effect of anaerobic conditions on rotting was made, by incubating samples of lenticel infected VTSC tubers and cork borer (No. 4) wound infected tubers under a variety of gaseous conditions.

Tubers were inoculated as described in Materials and Methods and placed in desiccators. Treatments were as follows, vacuum, CO_2, 10% CO_2 in air, 100 ppm ethylene in air, 1000 ppm ethlene in air, and 10% CO_2 in hydrogen. They were achieved as follows.

a. Vacuum. The vessel was evacuated using an electric pump for 1 h.

b. CO_2. After evacuation for 15 min the vessel was flushed with CO_2, until the lid of the desiccator lifted. This procedure was repeated twice.

c. 10% CO_2 in air. With the desiccator lid placed acentrically on the vessel to allow equilibration of pressure, 10% of the volume of air in the vessel was replaced by CO_2 delivered by a Luer Lok syringe. This was taken from a narrow necked flask, evacuated and filled with CO_2. with a constant stream of CO_2 passing through it. The desiccator lid was replaced immediately after addition of the CO_2.

d. Ethylene

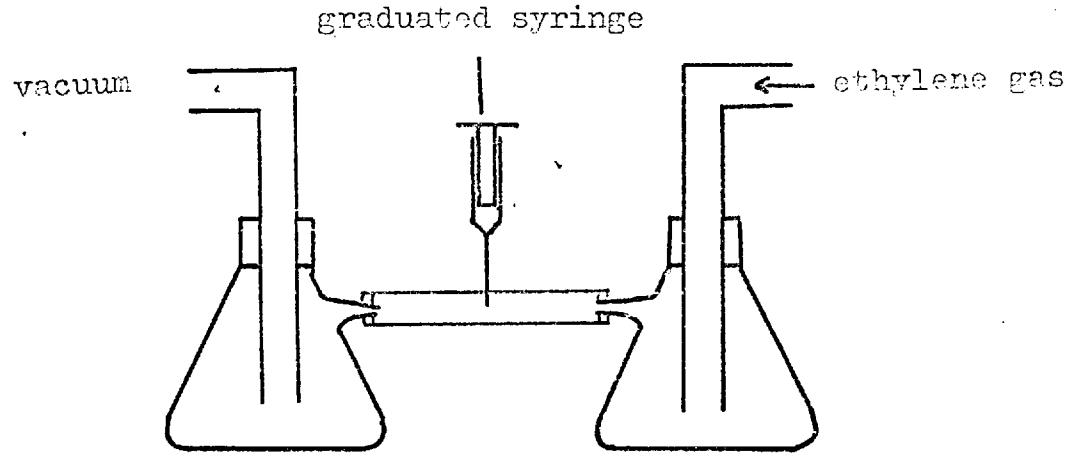


Figure 7.

The system illustrated in Fig. 7 was sealed and evacuated, before filling with ethylene and allowing a constant stream to flow through. The needle of a graduated syringe was inserted into a piece of rubber tubing joining the two flasks, and the required volume of gas was removed. This was injected into the desiccator with its lid acentrically placed as before. The vessel was then sealed. The operation was performed in a fume cupboard.

e. 10% CO_2 in hydrogen. A Bekton-Dikinson 'Gas Pack' jar was filled with tubers. A 'Gas Pack' generator was placed down the side of the tubers together with a methylene blue indicator strip. The prescribed catalyst was placed in the lid of the jar, and 10 ml of water were added to the generator, and the lid clamped on. Within 3 h the indicator strip turned colourless, the oxygen having been reduced to water, and the atmosphere reputedly 10% CO_2 in hydrogen.

All containers were stored at room temperature (20-23°C) in the dark. Lenticel infected tubers were stored for up to six weeks before removing them from the container and counting the number of rotting tubers. Cork borer wound infected tubers were stored for 4 d before measuring the volumes of the rots produced. The results are shown in Tables 30 and 31.

Table 30. Effect of different gases on rotting from lenticels

Treatment	Number of tubers (out of 25) which rotted	Length of storage (weeks)
Air	1	5
Vacuum	3	5
CO_2	6	6
10% CO_2 in air	7	6
100 ppm ethylene	6	6
1000 ppm ethylene	5	6
10% CO_2 in H_2[1]	2 (5)	3

[1]Container size limited samples to ten tubers (5 ≡ 25 tubers).

All rots found were white, spreading rots, although rots in two tubers incubated in 100 ppm ethylene in air and in one tuber in 1000 ppm ethylene in air, were limited by a

dark zone of tissue. This dark zone was widest around the vascular system of the tuber, and tissue around the vascular system was often still intact.

Although these results suggest a pronounced effect of each treatment, they must be interpreted with caution because each rot originated from the surface of the tuber in contact with a film of moisture on the inside of the container.

Table 31. Effect of different gases on rotting from cork borer wounds

	Mean rot volume (ml)[1]	
	Depth of inoculation (mm)	
Treatment	2.5	10.0
Air	0.6	1.8
Vacuum	0.9	2.9
CO_2	1.2	3.0
10 CO_2 in air	1.3	3.9
100 ppm ethylene	0.7	2.1
1000 ppm ethylene	0.8	2.3
10% CO_2 in hydrogen	1.1	3.6

[1]Mean of 10 tubers.

Analysis of variance showed that each treatment except 100 ppm ethylene caused a significant increase in rotting, compared with controls in air ($p = 0.001$). It appears that absence of air is more important than the presence of the gas itself, although altering the conditions can be sufficient to give an effect as shown by 10 CO_2 in air and 1000 ppm ethylene in air treatments. This may be due to a change in the respiration rate of the tubers leading to a subsequent reduction in resistance to rotting.

The effect of varying the gaseous environment in the polythene bag test

Lenticel inoculated VTSC tubers were placed individually in polythene bags with 15 ml water. Air was expelled from the bag and replaced with either CO_2 or ethylene. The gases were expelled from the bags again and the bags were refilled with the gas before tying a knot in the top of the bag. The bags were then placed in a larger bag which was filled with the same gas and sealed. Tubers were observed for rotting at intervals throughout a twelve day incubation at room temperature (20-23°C) in the dark. The numbers of tubers rotting are shown in Table 32.

Table 32. Effect of varying the gaseous environment in the polythene bag test

Storage time (d)	% number of tubers rotting[1]		
	Treatment		
	CO_2 filled	Ethylene filled	Air filled
6	50	65	15
9	75	85	40
12	100	85	90

[1]Each treatment contained 20 tubers.

Although the polythene bag test normally causes rotting of all infected tubers within 12 d the presence of ethylene or CO_2 in high concentration increased the rate of rotting. The results do not indicate, however, whether the prevailing factor was the presence of the gases or the absence of air. No dark zone was found in tubers rotting in atmospheres of ethylene. All rots found were white and rapidly spreading. The origin of the rots was not elucidated

due to the rapidity with which rotting spread after initiation.

Effect of surface moisture on rotting

Since anaerobic conditions alone cannot simulate the drastic conditions in the polythene bag test, or even the onset of rotting from lenticel infections, the effect of a moisture film on the tuber surface was investigated.

Inoculated VTSC tubers, approximately 100 g in weight were placed in polythene bags containing different volumes of water. The bags were sealed, and incubated for sixteen days during which time the tubers were investigated for rotting. The numbers of tubers rotting are shown in Table 33.

Table 33. The importance of water in the polythene bag test

Storage time (d)	% number of tubers rotting (out of 25)					
	Volume of water in the bag (ml)					
	0	2	4	6	8	10
4	0	0	0	20	40	44
8	0	0	0	40	76	76
12	0	0	8	76	88	96
16	0	0	32	80	92	96

Bags containing eight or more ml of water, contained tubers which were completely covered with a film of water, and these tubers rotted rapidly. The main criterion for the rapidity of rotting in the polythene bag test appears to be the presence of an entire moisture film continuously surrounding the tuber surface.

The effect of submerging tubers in water

Inoculated VTSC and field infected tubers (stored for 1 month) were totally submerged singly in beakers of water. At 4 d intervals they were removed and observed for rotting. Since VTSC tubers had previously been shown to possess few, if any, populations of soft rot bacteria, no uninoculated controls were prepared. The results are shown in Table 34.

Table 34. Effect of submerging tubers in water

% number of tubers rotting (out of 25)

	Tuber treatment			
	Incubated wet		Incubated dry	
Storage time (d)	Inoculated VTSC	Field infected	Inoculated VTSC	Field infected
4	28	12	0	0
8	80	36	0	0
12	84	48	0	0

Total immersion in water appears to be as efficient as the polythene bag test for inducing tubers to rot. All rots found were white and rapidly spreading.

The effect of a constantly changing film of water on rotting

Inoculated VTSC tubers and field infected tubers stored for 3 weeks after harvesting in October 1972 were placed on a wire mesh platform beneath plastic trays drilled with holes in a uniform distribution. A constant head of water was applied to the trays, causing a shower effect on the tubers. The sink containing the apparatus was shielded

from light and tubers were inspected for rotting at intervals of 4 d.

The results in Table 35 show that the constantly changing water film stimulated the onset of rotting just as in the polythene bag test, although the numbers of field infected tubers rotting are surprisingly low compared with those from polythene bag tests at the same time (Table 33).

Table 35. Effect of a constantly changing film of water on rotting

	Number of tubers rotting (out of 10)			
	Tuber treatment			
	Wet tubers		Dry tubers	
Storage time (d)	Inoculated VTSC	Field infected	Inoculated VTSC	Field infected
4	2	0	0	0
8	6	3	0	0
12	9	4	0	0

The effect of a partial moisture film on rotting

Inoculated VTSC tubers were placed on a wire mesh platform in a plastic box. Areas of the tubers which touched the sides of the container were wetted with water and the box sealed. These areas were kept wet by daily addition of more water. After 24 d incubation at room temperature only these areas had rotted. Non-wetted control tubers did not rot. Partially wet tubers do not rot as rapidly as do totally wet tubers.

The formation of limited lesions from lenticel infections

Inoculated VTSC tubers and field infected tubers taken from store in November after almost two months storage were placed in polythene bags individually with 10 ml of water for 4 d and removed. Storage was continued in closed boxes for a further 8 d. All rots developing after 4 d were noted and the surface area of the rot was marked. Some rots were cut open and investigated for browning. The numbers of rots per tuber and the numbers of browned rots found after the initial incubation for 4 d in polythene bags are shown in Table 36.

Table 36. Production of limited lesions

Storage time after 4 d polythene bag test (d)	Number of rots[1]		Number of browned rots[1]	
	Method of storage			
	Closed box	Polythene bags	Closed box	Polythene bags
0	19	21	0	0
4	15	29	13	0
8	18	52	18	0

[1]Ten tubers per treatment.

All rots from tubers incubated in polythene bags with water produced rapidly spreading white rots, but this could be altered by incubating tubers having such rots in dry conditions, where rots did not markedly increase in surface area or remain white. Under the dry conditions little further rotting occurred.

The effect of a film of water on tubers with cork borer wound infections

Field infected tubers were washed and air dried. *E. atroseptica* A13 inoculations were made into 2.5 and 10.0 mm wounds made by a bork borer (No. 4). Tubers were incubated individually in polythene bags with or without 10 ml water for 4 d at room temperature in the dark, before measuring the volumes of the rots which are shown in Table 37.

Table 37. Effect of a film of water on rotting from infected wounds

Mean volume of rot (ml)[1]

Treatment			
Tuber surface wet		Tuber surface dry	
Inoculation depth (mm)			
2.5	10.0	2.5	10.0
1.2	2.5	0.5	1.2

[1] 25 tubers per treatment.

Significant differences ($p = 0.01$) between the tuber treatments were found, and the results indicate that a film of water around the tuber increases the rapidity of rotting from infected wounds. Once again it was noted that rots from tubers incubated in wet conditions were white, whereas those from tubers incubated in dry conditions were brown and often limited by a dry edge to the rot.

The importance of surface moisture in relation to the absence of air on rotting

The conditions present in the polythene bag test initially thought to be responsible for rotting from infected

lenticels were the absence of air and the presence of a film of moisture, but it was difficult to think that the two factors were very closely related, since covering the tuber surface with water might establish anaerobic conditions within the tuber. This close relationship was made more evident in preliminary experiments which used wound inoculated tubers. However, the use of lenticel inoculated tubers, showed that there was a difference between the two factors. A final experiment in this series was set up to clarify the situation.

Two desiccators each containing 25 lenticel inoculated tubers, were prepared. One contained a beaker containing 100 ml of water, the other an empty beaker. Both vessels were evacuated and flushed twice with CO_2, to remove the air. The tubers were stored for 2 weeks at room temperature in the dark, and the numbers of rotted tubers were counted. The vessels were set up again, the water now being distributed around the tubers to maintain a continuous film of their surfaces. After a further 4 weeks storage, the numbers of rotting tubers were again counted. The results are given in Table 38.

Table 38. Effect of water and anaerobic conditions on rotting

Storage time (weeks)	Numbers of tubers rotting (out of 25)	
	CO_2 + water	CO_2
2	0	0
6	21	9

In the vessel containing only CO_2, all rots developed from parts of tubers in contact with films of moisture on the inside of the desiccator. This and other results show clearly the importance of moisture and that in its absence rotting does not normally occur in anaerobic conditions.

SECTION V

BACTERIAL PHYSIOLOGY IN RELATION TO ROTTING

Although it is essential to study pathogenesis in terms of the host parasite interactions on a physiological and biochemical level, much useful information may be obtained by studying relevant features of both host and pathogen. This section deals with certain aspects of the physiology of the bacterium.

Growth Curves of E. atroseptica on Nutrient Broth

Flasks containing sterile nutrient broth (100 ml) were inoculated with a loopful of E. atroseptica A13, and were incubated for up to 72 h at 25°C on an orbital incubator at 200 rpm. Two flasks were removed after intervals, the contents collected by centrifugation (17000 x g for 10 min), washed, recollected and resuspended in 10 ml of water. The bacterial population was then determined by turbidimetric techniques in conjunction with the standard curve in Appendix 1. Bacterial numbers were also obtained in two experiments by plate counts of viable cells. The growth curves from these data are illustrated in Fig. 8.

Although more extensive data could have been obtained, those illustrated show typical rapid growth in the logarithmic phase after a relatively brief or no apparent log phase, the end of the log phase being after 36 h under these conditions at which time depletion of the medium or conditions of culture became limiting factors. The turbidimetric assay showed a maximum population of more than 10^9 cells/ml in the culture

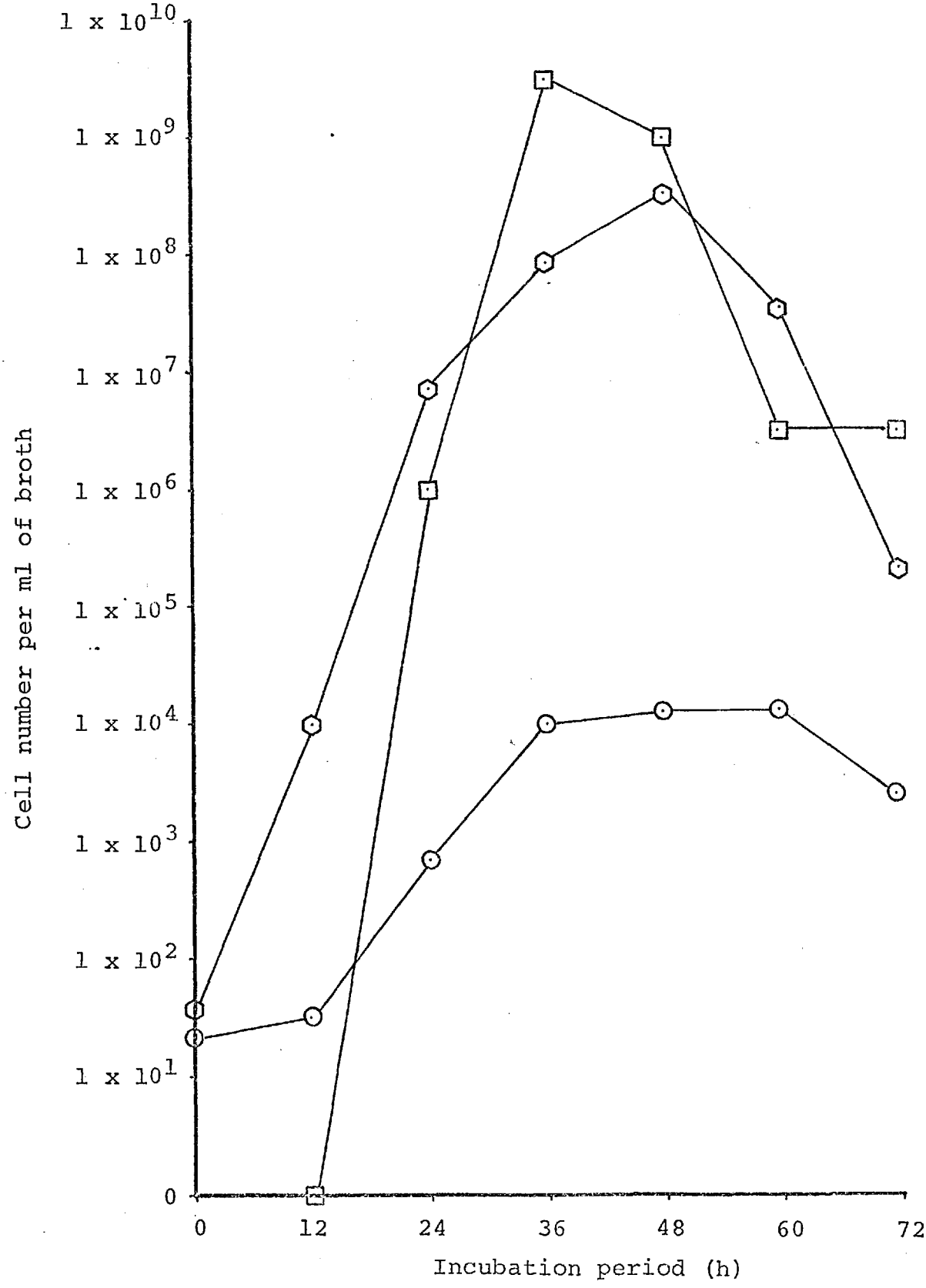


Figure 8. Growth curve for E. atroseptica A13

medium, although this would include non-viable cells. The viable plate count assays showed maximum population numbers of 10^8 cells/ml and 10^4 cells/ml. This apparent lack of rapid growth rate in the latter assay has been an almost constant feature with several of the Erwinia cultures used. The reasons are not known.

Effect of Temperature on the Growth of Erwinia atroseptica

Test tubers containing 5 ml of nutrient broth were inoculated in triplicate for different temperatures. The inoculum was a loopful of E. atroseptica A13 taken from a 10^9 cells/mo suspension. The tubers were incubated at different temperatures, and the degree of growth was estimated on a scale of + and - values to a maximum of 5+, after 48 h growth.

Table 39. Effect of temperature on growth of E. atroseptica

Incubation temperature (°C)						
0	5	10	15	20	25	30
-	+	+	++	++++	+++++	++++
-	+	+	+++	+++++	+++++	++++
-	+	+	++	++++	+++++	+++++

Rapid multiplication is limited below 20°C although growth still occurs at 5°C. There was an optimum at 25°C.

Effect of pH on the Growth of Erwinia atroseptica

Test tubers containing 10 ml of nutrient broth buffered at different pH values as described in the Materials

and Methods, were inoculated in triplicate with a loopful of E. atroseptica A13 (10^9 cells/ml). After incubation at 25°C for 48 h, the degree of growth was recorded on a sliding scale of - to 5+ as above.

Table 40. Effect of pH on growth of E. atroseptica

pH value of broth									
3.5	4.1	4.8	5.7	6.2	7.3	7.9	9.0	10.1	10.7
-	-	+	++++	+++++	+++++	+++++	++++	+	+
-	+	+	++++	+++++	+++++	+++++	+++	+	+
-	-	+	+++	++++	+++++	+++++	++++	+	+

Growth was limited outside the pH range 5.7-9.0. Within this range there was no well defined optimum value.

Mechanisms of Bacterial Soft Rotting

Upon entering potato tubers, the pathogen usually exists as a latent infection. When the environmental conditions are favourable for rotting, the bacterial cells multiply and secrete enzymes which cause death and maceration in the surrounding tissue. As the bacteria utilise the contents of the cells they multiply rapidly, filling the macerated area. More enzymes are produced, and a rapidly spreading soft rot is produced. The nature of the cell-killing and macerating factor has been determined and it has been shown to be an endo polypectate trans-eliminase (Hall, 1970).

Experiments in this section studied the mechanisms of enzyme production during bacterial multiplication from the latent infection within lenticels.

Preparation of Pectic Enzymes

Rotting tuber tissue, taken 4 d after inoculation with E. atroseptica A13 (10^9 cells/ml) into cork borer (No. 4) wounds 1 cm deep, was squeezed in a muslin bag, and gave an extract that was centrifuged at 17000 x g for 5 min to remove bacteria and other debris. After saturating to 60% with ammonium sulphate and discarding the precipitate, the supernatent was saturated to 90% with further ammonium sulphate addition. The precipitate was retained by centrifugation (17000 x g) for 5 min, resuspended in distilled water (10% of the original volume of the sap) and dialysed against 0.01 M $CaCl_2$ solution for 24 h at 10°C. This semi-purified enzyme preparation was then tested for the presence of polygalacturonase and endo polypectate trans-eliminase activities by visometric techniques described in the Materials and Methods section. Enzyme activity was expressed in the arbitrary units described in Appendices 13 and 14. The number of units of the two pectic enzymes present in the rot extract are given in Table 41.

Also given in Table 41 are the numbers of units of the enzymes obtained from growth of E. atroseptica A13 on sodium polypectate. The enzyme preparation was obtained as follows. Flasks containing basic salts medium were supplemented with 0.5% sodium polypectate as the sole carbon source. The medium was autoclaved at 10 psi for 10 min and cooled before inoculating with loopfuls of E. atroseptica A13. After incubation at 25°C for 48 h, bacteria were removed by centrifugation at 17000 x g for 5 min. The enzymes were recovered from the 60-90% ammonium sulphate saturation

fraction as described above, and dialysed against 0.01 M $CaCl_2$ for 24 h at 10°C. Enzyme activity of this semi-purified preparation was estimated and expressed as above.

Table 41. Units of pectic enzyme present in the extracts

Enzyme	Rotting tissue extract	Sodium polypectate medium
Polypectate trans-eliminase (PTE)	1000 units/g fresh weight	725 units/ml
Polygalacturonase (PG)	0	50 units/ml

The results in Table 41 show that PTE was present in both extracts, whereas PG was found only in sodium polypectate cultures. The results agree with those of Hall (1970) who worked in the Department of Plant Pathology at Imperial College. Since this worker and others in the same department were concerned with the action of the enzymes on potato tissue, no further investigations into this aspect of rotting were made.

In conclusion, it appears that the enzyme responsible for rotting potato tubers by E. atroseptica is an endo polypectate trans-eliminase enzyme.

Optimum Temperature for PTE Activity

A large quantity of PTE was prepared from growth of E. atroseptica A13 on sodium polypectate as previously described. Samples of this were tested for their activity at different temperatures by viscometric techniques. The viscometers were held in water baths at different temperatures,

allowing the enzyme and substrate buffered at pH 9.0 to equilibrate before mixing and determining the $1000/T_{50}$ values. These values were converted to arbitrary units and are given in Table 42.

Table 42. Units of activity for PTE activity at different temperatures

	Temperature of incubation (°C)						
	5	10	15	20	25	30	35
Units of activity	10	50	750	1000	1250	1250	750

The optimum temperature for PTE activity under the conditions of this experiment lies between 25-30°C, and activity is low at temperatures below 15°C.

Effect of Temperature on Stability of PTE

Semi-purified PTE preparations were incubated for 30 min in water baths at different temperatures, before removing and determining the activities on sodium polypectate buffered at pH 9.0, and at 25°C, by viscometric techniques. The results are given in Table 43.

Table 43. Effect of temperature on stability of PTE

	Incubation temperature (°C)						
	5	10	15	20	25	30	40
Activity at 25°C[1]	1800	1800	1800	>2000	>2000	1000	125

[1]PTE activity is expressed as arbitrary units.

PTE activity is little affected by incubating in the absence of substrate at temperatures below 30°C. Above this the enzyme appears to be denatured.

Stability of a Semi-Purified PTE Preparation

A semi-purified PTE preparation from potato tissue containing 50 ppm streptomycin, to reduce bacterial growth was left in a screw-capped container at room temperature (20-23°C) for up to 14 d. During this time, 3 ml samples were removed, and their activities on sodium polypectate were measured by viscometric techniques, at pH 9.0. The activities given in Table 44 are expressed as units of enzyme.

Table 44. Stability of PTE activity during storage at room temperature

Period of incubation (d)	T_{50} (min)	$1000/T_{50}$	Enzyme units
0	1	1000	>2000
1	1	1000	>2000
2	1½	670	1800
3	2	500	1000
4	2½	400	800
6	3	333	650
7	3	333	650
11	1	1000	>2000
14	1	1000	>2000

Although the results are somewhat erratic, the enzyme appears to be stable even if the activities obtained on days 11 and 14 are disregarded.

Effect of pH on PTE Activity

3 ml samples of a semi-purified PTE preparation were placed in viscometer tubes containing 3.5 ml of 3% sodium polypectate and 3.5 ml of buffer. Buffers were prepared as described in Materials and Methods. Controls not tested for viscosity reduction were prepared to test the final pH of the reaction mixtures. PTE activity (Table 45) is expressed in arbitrary enzyme units.

Table 45. Effect of pH on PTE activity

	pH of reaction mixture						
	3.6	4.4	5.6	6.2	7.8	9.0	10.2
Units of enzyme activity	0	0	0	473	1000	>2000	>2000

Rather surprisingly, pH values below 6.2 gave no activity. Above this value high activity was found with no reduction in activity at the high pH values. This result is odd since the pH of a tuber tissue macerate is between 5.0 and 6.5.

An experimental control with no enzyme present showed some slight activity on sodium polypectate degradation at pH 10.2, but none at lower pH values.

Effect of pH on PTE Activity on Pectin and Sodium Polypectate

The enzyme was produced on 0.5% sodium polypectate in a basic salts medium buffered at pH 8.4. After incubation of *E. atroseptica* A13 on this medium for 48 h at 25°C, a semi-purified PTE was prepared as previously described. Samples

of this extract were tested for viscosity reduction activity on pectin and sodium polypectate. Reaction mixtures consisted of

3.5 ml buffer
3.5 ml 3% pectin or sodium polypectate (NAPP)
3.0 ml PTE preparation.

PTE activity was measured over a wide range of pH values at 25°C. The results are illustrated in Fig. 9.

The viscosity for boiled enzyme controls were similar for both substrates and no non-enzymic degradation of either pectin or sodium polypectate was found.

From Fig. 9 it can be seen that PTE activity on sodium polypectate was greater than on pectin, although the pH optima were the same, at pH 9.0.

pH Optima for Enzymes Produced on Sodium Polypectate and Pectin at pH 8.4

0.5% sodium polypectate and pectin in basic salts media were buffered to pH 8.4 and inoculated with E. atroseptica A13. After incubation for 48 h at 25°C the enzyme was harvested and semi-purified as previously described. Enzyme activity was determined by measuring the percentage loss in fresh weight of potato discs 2 mm thick and 7 mm diameter. Four such discs were placed in a 7 ml McCartney bottle containing

2 ml 0.02 M buffer
2 ml distilled water
0.5 ml 0.02 M $CaCl_2$
0.5 ml enzyme preparation.

Measurements were taken as described in Materials and Methods. Autoclaved (15 psi for 15 min) enzyme controls were also prepared. The results are illustrated in Figs. 10 and 11,

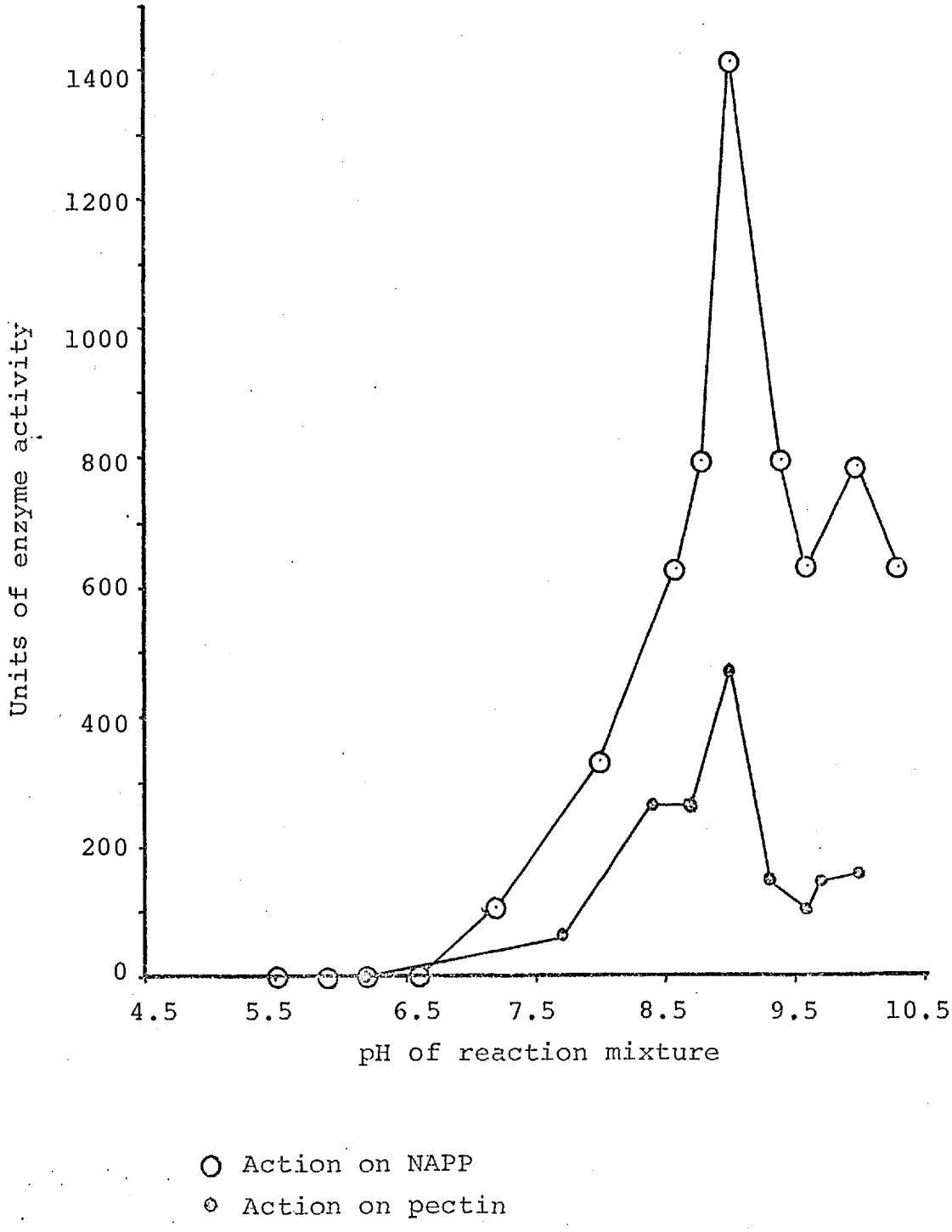


Figure 9. pH optima for pectate trans-eliminase activity on pectin and sodium polypectate (NAPP) substrates

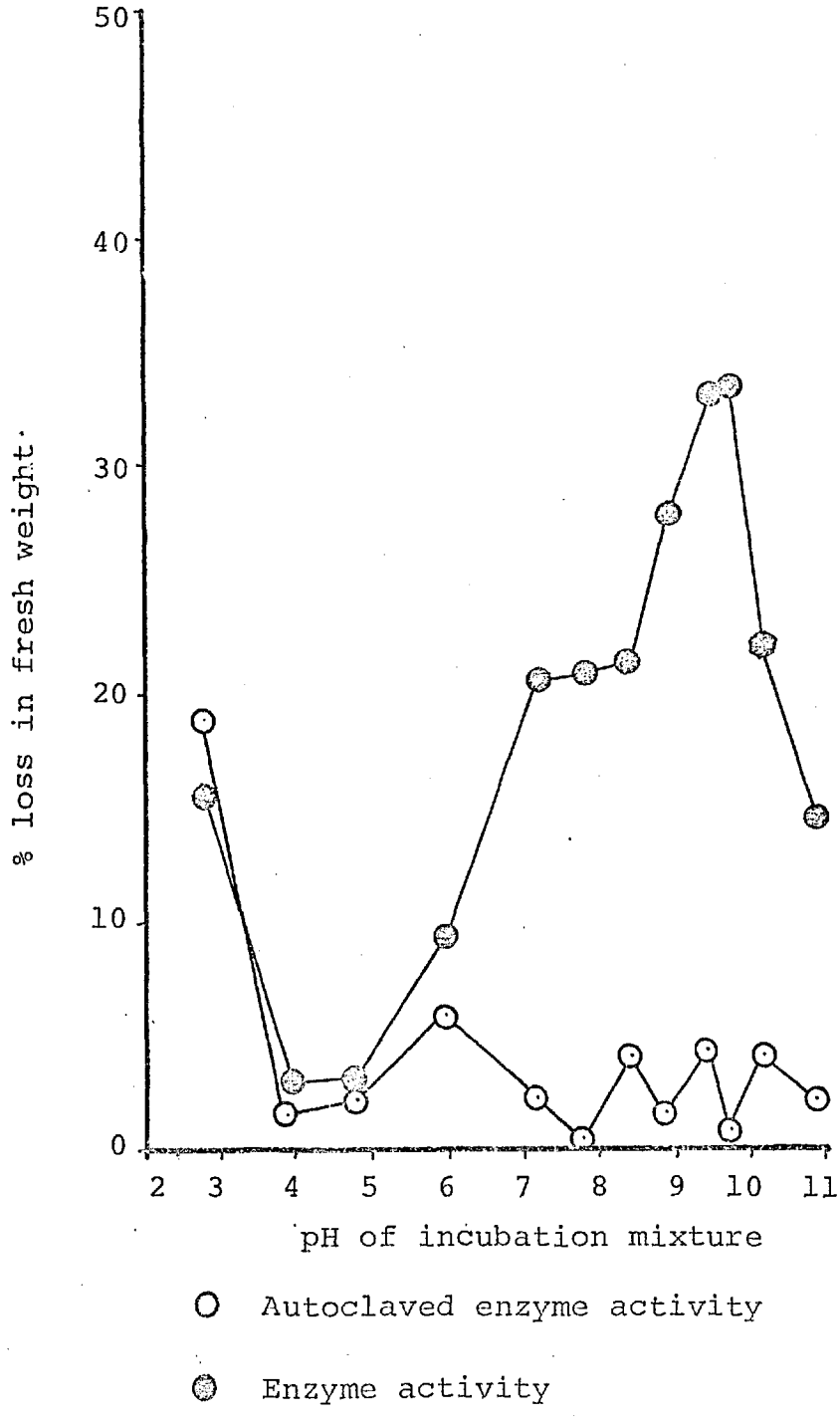
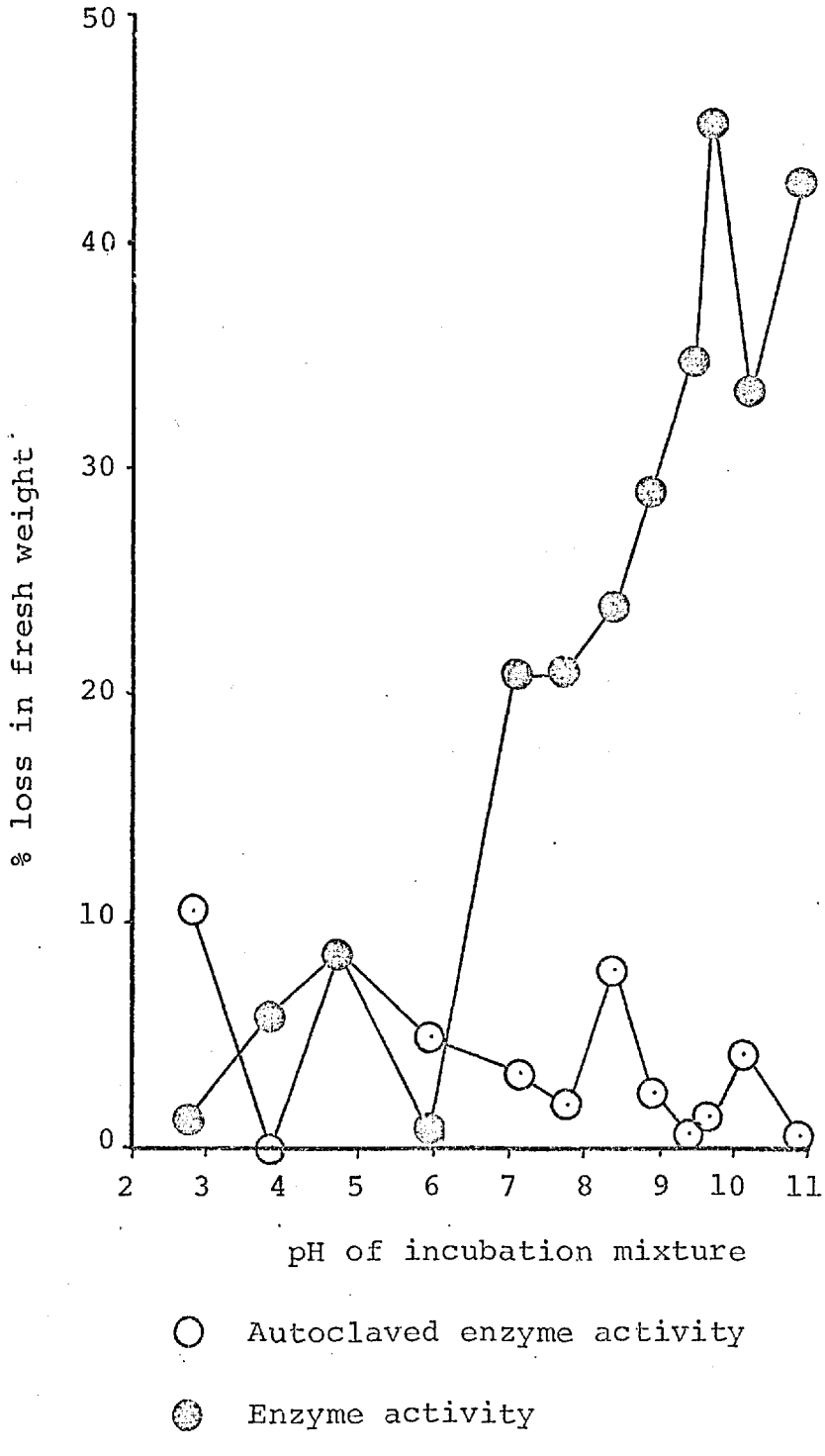


Figure 10. pH optimum for enzyme produced on pectin

Figure 11. pH optimum for enzyme produced on sodium polypectate



which show that the optimum pH for tissue degradation by both enzymes is 9.6. Below this value the curves are similar but at pH 9.6 and above, enzyme produced on sodium polypectate has a greater activity than that produced on pectin. Some non-enzymic tissue degradation was found at pH 2.7, but this may have been due to water loss.

In conclusion to these experiments concerning the effect of pH on PTE activity, it appears that the optimum pH is between 9 and 10, although above these values activity may still be high. PTE activity is found to be markedly reduced below the optimum pH and is very low at pH 5 to 6, the pH of tuber tissue.

Effect of pH on the Growth and Enzyme Production in Erwinia atroseptica A13 Cultured on Different Pectic Substrates

1 ml samples of E. atroseptica A13 (10^7 cells/ml) were pipetted into flasks containing 250 ml of buffered 0.5% pectin or sodium polypectate in a basic salts medium. 1 ml of sterile 0.05 M $CaCl_2$ solution was added to each flask with the bacterial inoculum. Pectic media were buffered at pH values from 3 to 11. The flasks were shaken on an orbital incubator (200 rpm) at 25°C and 10 ml samples removed periodically. Each sample was immediately tested for pH, bacterial cell number and viscosity. The samples were centrifuged at 17000 x g for 5 min. The bacteria were resuspended in 10 ml of water and the cell concentration determined by turbidimetric techniques in a nephelometer. The pH of the supernatent was read before placing the 10 ml sample in a viscometer tube and measuring the viscosity at 25°C. The supernatent was then stored at -18°C until at a future date,

the enzyme activity was determined using sodium polypectate as a substrate buffered at pH 9.0.

Figures 12a and 12b, and 13a and 13b, show changes in pH with incubation time, and changes in the viscosity of the media with time for growth on pectin and sodium polypectate respectively. Fig. 14 shows the optimum pH for degredation of pectin and sodium polypectate. Fig. 15 shows increases in PTE production during bacterial growth, measured by endo polypectate trans-eliminase activity on sodium polypectate buffered at pH 9.0. Fig. 16 shows the pH optimum for multiplication of E. atroseptica A13 after incubation for approximately 24 h.

The results in Figs. 12a and 13a show little difference in changes in pH throughout bacterial multiplication and enzyme production. The pH of media buffered at 6.0 increased to about 8.0 although the pH change was more rapid on pectin than on sodium polypectate. Below pH 6.0 little growth was observed. The optimum pH for reduction of viscosity of the media was 6.2-7.9 for sodium polypectate and 7.9 for pectin (figs. 12b and 13b).

Fig. 14 shows that pectin may be unstable at pH 7.1 and is consequently less affected by PTE activity at this value than at pH values above and below. The optimum pH for sodium polypectate degradation is 7.3 although activity is found between pH 5.7 and 9.0 for both substrates.

The results in Fig. 15 show the effect of pH on PTE production. The optimum pH lies between pH 6.2 and 7.9. No activity was found at pH 4.1 and activity was markedly reduced

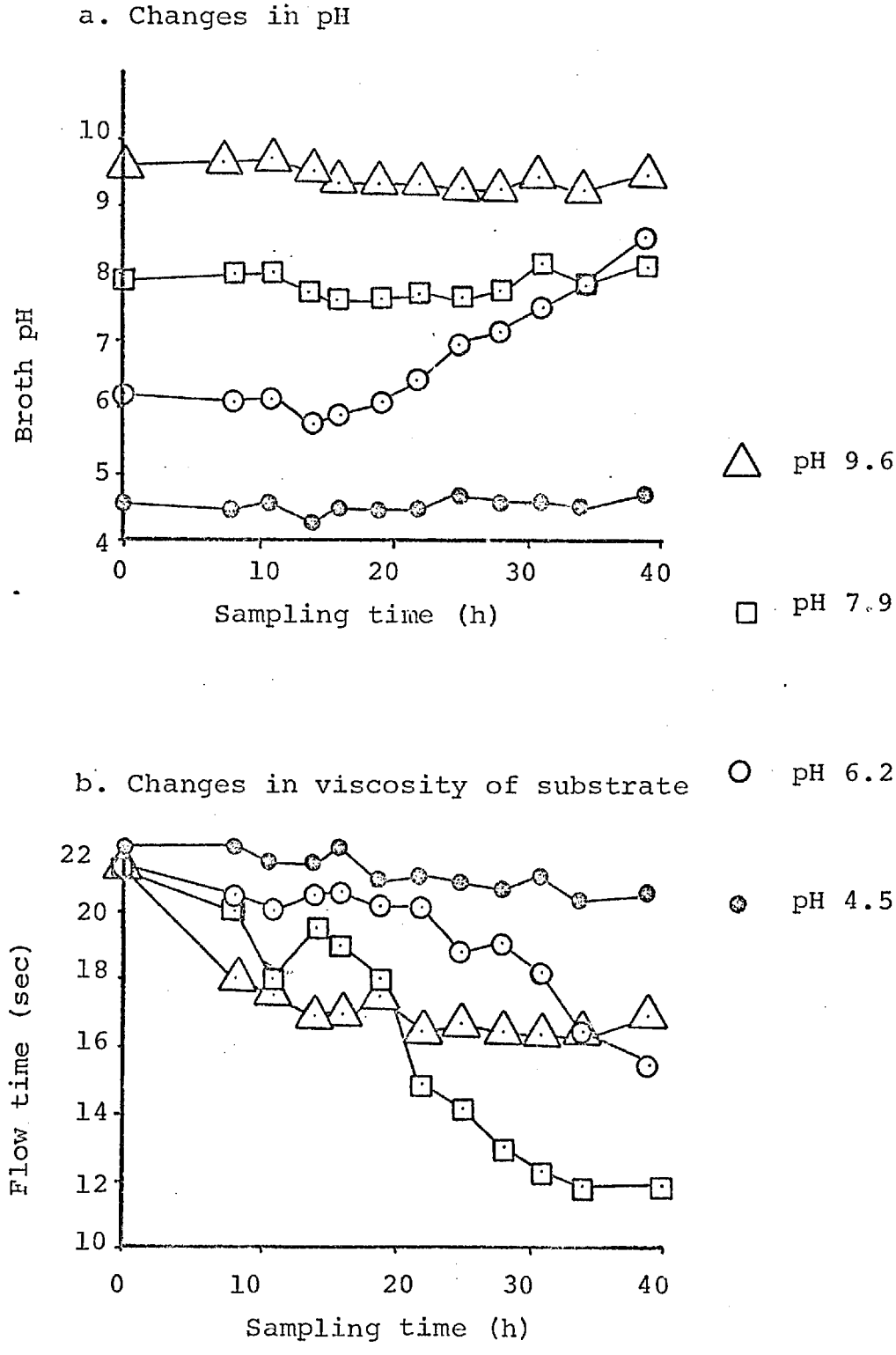
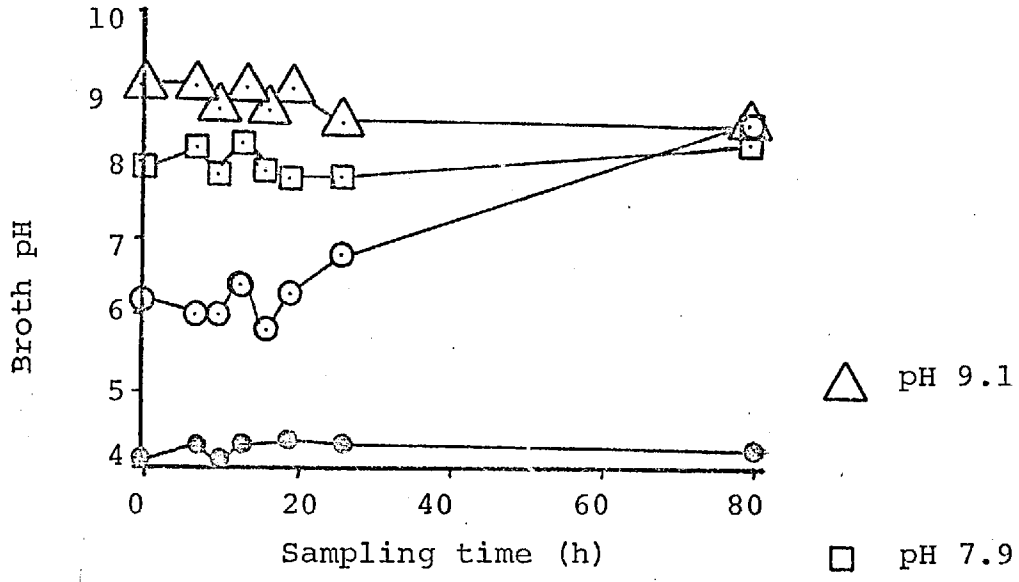


Figure 12. Growth on pectin.

a. Changes in pH



b. Changes in viscosity of substrate

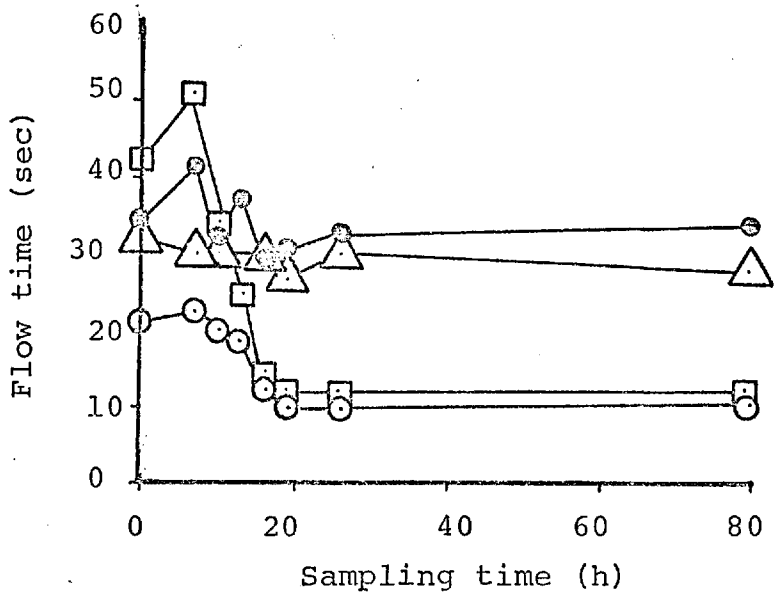


Figure 13. Growth on sodium polypectate

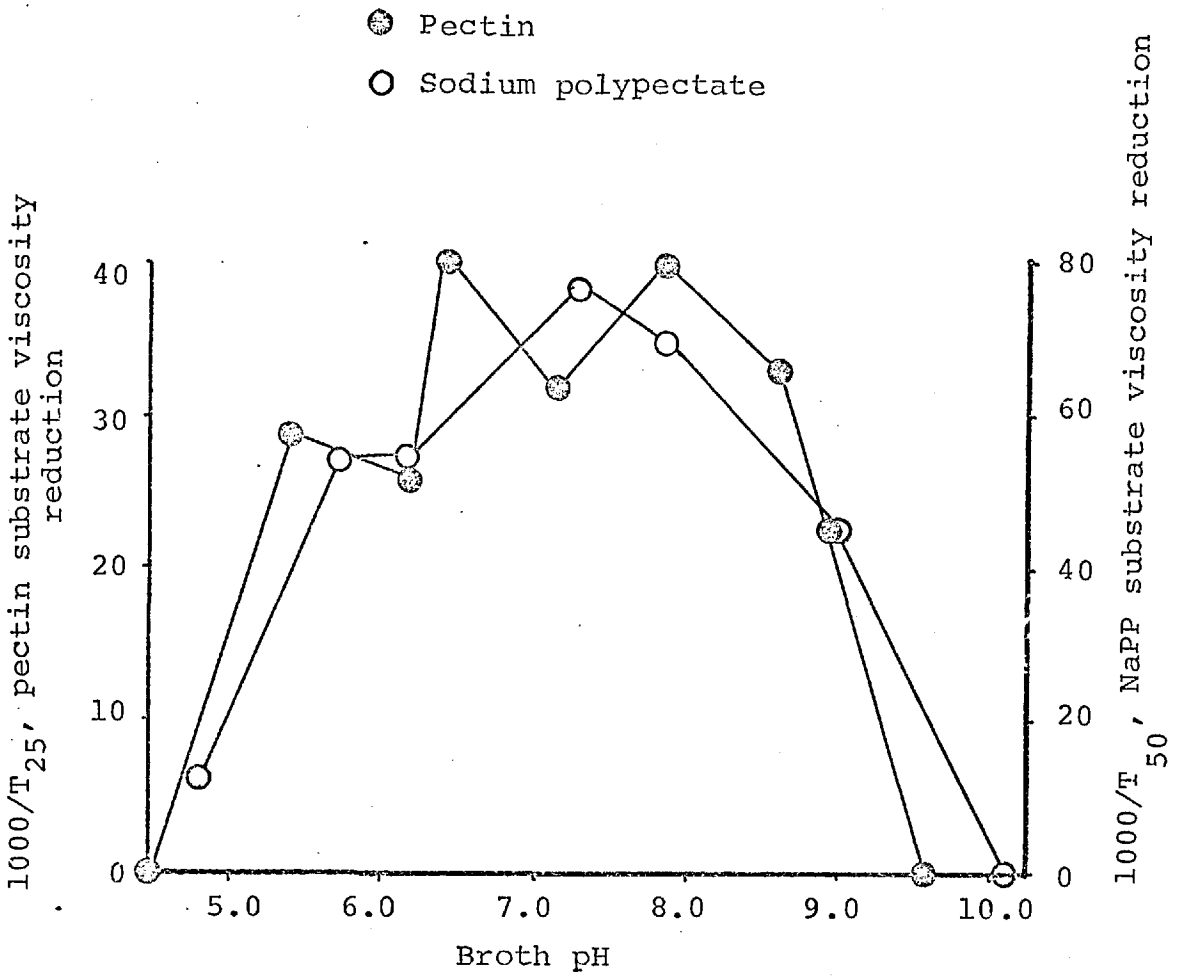


Figure 14. Optimum pH for substrate degradation

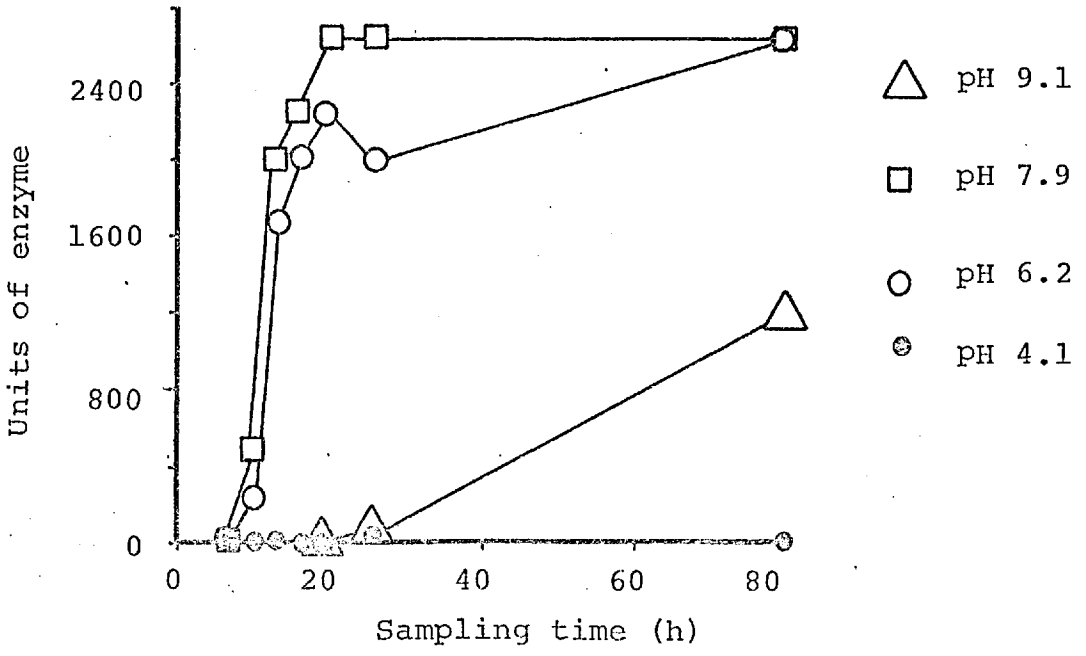


Figure 15. Trans-eliminase production during growth on sodium polypectate, measured by viscometry pH 9.0

at pH 10.1. The results do not distinguish between PTE and PG, since at the time of performing the experiment it was not realised that Erwinia atroseptica could produce PG. However, Fig. 16 shows two peaks for growth on both sodium polypectate and pectin. The peaks for growth after approximately 25 h on sodium polypectate were found at pH 5.6 and 7.5, those for pectin being found at pH 6.2 and 10.1. The peaks obtained at the lower values could be due to PG activity.

In conclusion, it appears that E. atroseptica A13 can utilise sodium polypectate more efficiently than pectin. The optimum pH for growth and pectic enzyme production on sodium polypectate is between 6 and 8, which is lower than the optimum pH for PTE activity. However, once growth on a pectic substrate has started, the pH of the medium changes to a more favourable value for PTE activity.

Effect of Potato Tuber Cell Walls as Inducing Agents for PTE

Having shown that PTE production can be affected by the substrate, its production may be assumed to be by induction. This fact is now accepted by many workers. The effect of cell walls and their constituents as inducing agents included two methods of extraction of cell wall material.

Cell Disintegration in Liquid Nitrogen

Washed and peeled tubers were diced and the pieces dipped into liquid nitrogen in a mortar. After grinding, the fine powder was suspended in 10 vol 0.1 M potassium phosphate buffer (pH 7.0), and ground for 5 min in a Waring blender. The residue was collected on a coarse sintered glass funnel,

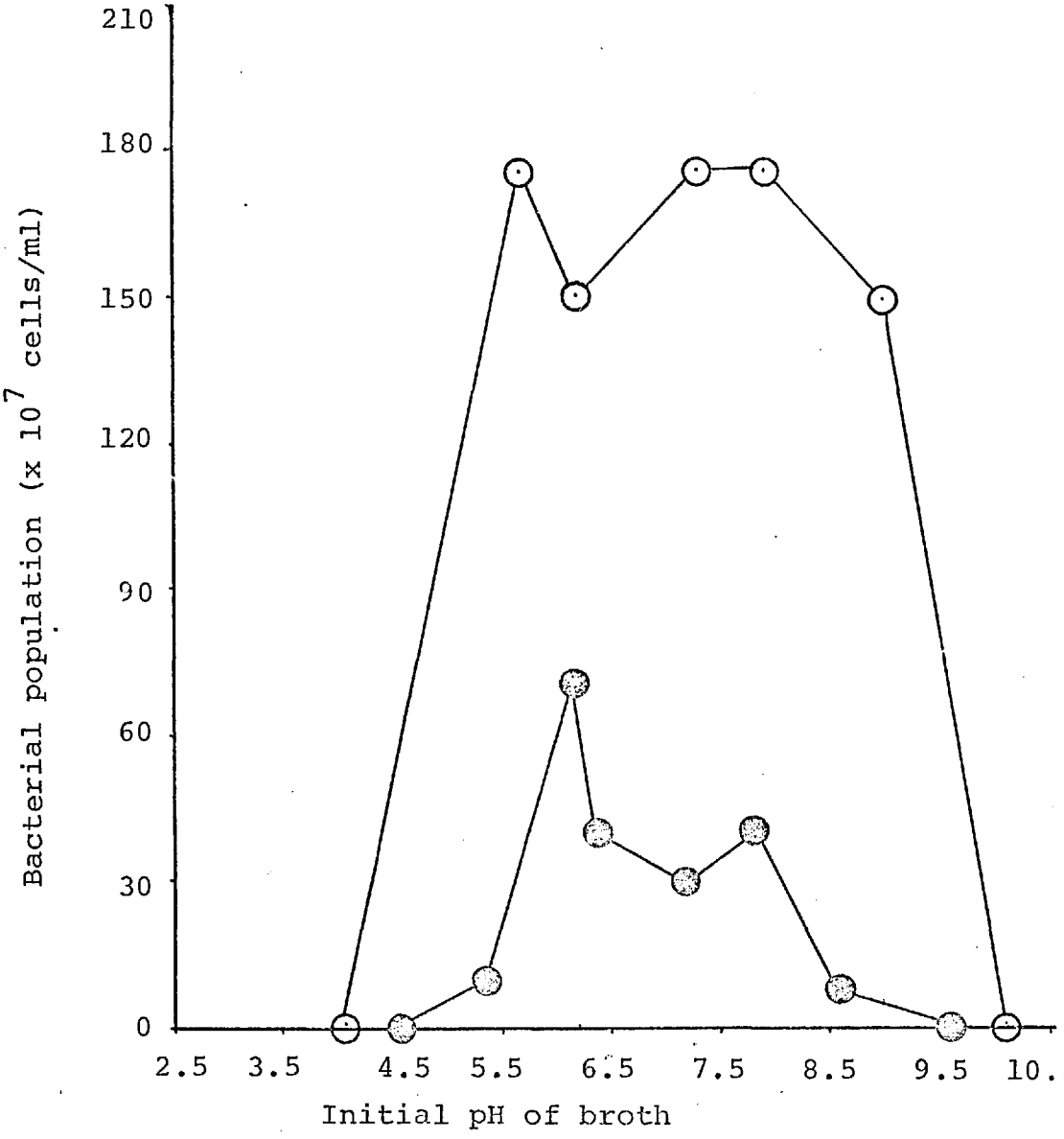


Figure 16. Optimum pH for bacterial multiplication

washed five times with 10 vol of the suspension buffer and resuspended in more buffer. After grinding again the residue was collected, washed with buffer and finally washed in distilled water. The insoluble fraction of cell wall material was suspended in 10 vol of chloroform-methanol mixture, washed with acetone and dried in a Petri dish at room temperature (19-21°C). All extracts were carried out under 5°C.

Comminuting in Ethanol

Tubers were washed, peeled and chopped before comminuting 2 kg in ethanol (1 g/ml) in a Waring blender. The homogenate was filtered on cheese cloth and washed with distilled water (10 vol). The residue on the cheese cloth was suspended in 1 vol of distilled water and boiled for 10 min to gelatinize the remaining starch granules. The suspension was brought to pH 7 with NaOH solution, before adding 4 g of sodium chloride, and the volume was made up to 5 litre. 20 ml of freshly prepared human saliva was added, together with 2 drops of toluene and digestion was allowed to take place for 24 h at 10°C. The whole was then filtered under suction and the filtrate discarded. The residue was suspended in 10 vol. of chloroform-methanol mixture, washed with acetone and dried in Petri dishes at room temperature.

Flasks of nutrient broth, and basic salts medium containing 0.1 and 0.5% of each cell wall preparation distributed into the medium by slow addition and rapid stirring, were inoculated with a loopful E. atroseptica A13. After 48 h incubation at 25°C, the culture was cleared by centrifuging at 17000 x g for 5 min. PTE activity was determined by viscometry and compared to that produced on sodium polypectate.

The results for nutrient broth cultures are given in Table 46.

No growth or enzyme production was obtained in cultures on cell wall preparations in basic salts medium; it thus appears that E. atroseptica cannot utilise cell wall material as a sole carbon source. The results in Table 46 show that per bacterial cell, E. atroseptica produced more enzyme on both cell wall preparations than on sodium polypectate although the results are somewhat erratic. However results from other experiments (see Table 49) indicate that maximum enzyme production occurs during the early part of the log phase of growth. In this experiment the bacterial populations were highest in those broths containing sodium polypectate, and after 48 h growth on this substrate had probably reached the stationary phase, whereas the cell wall preparation broths still showed rapid multiplication. Consequently it is difficult to compare PTE production on different media unless the rates of multiplication are similar.

Induction of PTE by Tuber Tissue

Tubers of different physiological nature were washed, dried, and soaked in absolute alcohol. After flaming, a core (4 cm x 2.5 cm diameter) was removed aseptically from each tuber. Two such tissue cylinders were placed in each flask containing 100 ml of sterile distilled water. The flasks were inoculated with E. atroseptica A13; 10 ml samples were removed from each flask after 48 h incubation at 25°C on an orbital incubator (200 rpm). The bacteria and tuber debris were removed by centrifuging at 17000 x g for 5 min. The pH of the supernatent was measured and 3 ml samples were

Table 46. Production of pectate trans-eliminase by E. atroseptica[1]

Substrate	Substrate concn	Replicate Number	Enzyme activity (T_{50})[2]	Enzyme activity (Units)[3]	Cell number (10^7 cells/ml)	Units of enzyme per 10^7 cells/ml
Cells disintegrated in liquid N_2	0.1%	1	-	-	No growth	-
		2	10	475	9	53
		3	26	133	7	19
	0.5%	1	5	1190	50	24
		2	4	1413	80	18
		3	9	430	60	9
Ethanol extraction	0.1%	1	21	178	20	9
		2	13	335	9	37
		3	19	200	15	13
	0.5%	1	15	250	90	3
		2	5	1190	100	12
		3	11	470	80	6
Sodium polypectate	0.1%	1	3	1880	350	5
		2	2	2000	300	7
		3	1	2650	500	5
	0.5%	1	1	2650	800	3
		2	2	2000	500	4
		3	1	2650	800	3

[1] No growth obtained in cell wall preparations in basic salts medium.
[2] Time (mins) for 50% decrease in viscosity of test solution.
[3] Arbitrary enzyme units.

tested for PTE activity at pH 9.0 by viscometry. The results are given in Table 47.

Table 47. Induction of PTE by various tuber tissues

Physiological state of tuber	Replicate number	pH of broth	Enzyme activity	
			$(T_{50})^1$	(Units)[2]
Immature	1	7.6	60	45
	2	7.4	45	118
	3	7.3	0.5	3750
Dormant tubers kept in Fusarex	1	7.1	5	422
	2	7.3	28	190
	3	7.2	1	2600
One year old flaccid tubers	1	7.7	57	200
	2	7.1	36	89
	3	7.2	1	2600

[1]Time (minutes) for 50% decrease in viscosity of test solution.

[2]Arbitrary enzyme units.

No differences between the treatments can be seen due to the large differences in enzyme activity within the treatments. It is obvious however that the production of pectic enzymes can be induced by potato tuber tissue, and that the enzyme produced often has extremely high activity on sodium polypectate. All tissue cylinders showed some rotting, but this was limited in some replicates. Blackening of tissue cylinders from one year old flaccid tubers together with blackening of the liquid in the flasks was found, but this did not seem to effect pectic enzyme production.

In conclusion, the physiological state of tubers does not appear to affect PTE production on tuber tissue.

Induction of PTE in Various Erwinia spp.

Loopfuls of cultures of Erwinia spp. (10^9 cells/ml) were inoculated into flasks containing 100 ml of nutrient broth. Three replicates per culture of Erwinia spp. were prepared. 10 ml samples were removed from each flask after 24 and 48 h incubation at 25°C in an orbital incubator (200 rpm). These were cleared by centrifugation at 17000 x g for 5 min, and the residues were resuspended in 10 ml of water and a bacterial count made by nephelometry. The PTE activities of the supernatents were measured at pH 8.8 by viscometry, and these are given in Table 48.

The results summarised in Table 48 show that some isolates of Erwinia spp. can produce relatively large amounts of enzyme when grown in the absence of pectic compounds. However, E. atroseptica A13 does not and is therefore a useful strain to use in induction experiments. Erwinia carotovora NCPPB 1280 which is particularly efficient in producing constitutive enzymes, is much less virulent than E. atroseptica A13 in rotting potato tubers (Table 16).

Again as in the results in Table 46, the reduced production of PTE relative to the bacterial cell number during the growth in the log phase can be seen.

Induction and Catabolite Repression of Pectic Enzyme Synthesis

In a potato tuber rot, bacteria are exposed to pectic compounds present in the middle lamellae, which have been

Table 48. Constitutive synthesis of pectate trans-eliminase

Isolate	Period of incubation (h)	Replicate Number	Cell number (10^6/ml)	Enzyme activity	
				Units[1]	Units/10^6 cells/ml
E. atroseptica A13	24	1	2	32	16
		2	4	28	7
		3	2	40	20
	48	1	50	35	0.7
		2	40	32	0.8
		3	8	32	0.4
E. atroseptica NCPPB 1277	24	1	7	266	38
		2	1	299	299
		3	7	237	34
	48	1	10	631	63
		2	20	422	21
		3	100	473	5
E. carotovora NCPPB 1280	24	1	0.9	211	234
		2	0.6	141	235
		3	0.5	427	859
	48	1	2	335	167
		2	5	266	53
		3	0.9	531	590
E. aroideae NCPPB 1276	24	1	40	531	13
		2	40	1189	30
		3	800	1189	1.5
	48	1	200	56	0.3
		2	70	1000	14
		3	90	1680	19

[1]Arbitrary units of enzyme.

shown to induce synthesis of the enzymes required to degrade them, resulting in maceration of tissue (Table 46). Maceration is accompanied by cell death and subsequent leakage of cell contents into the intercellular spaces of tuber tissue. Large amounts of soluble sugars are released and utilised by the bacteria. It is known that induced pectolytic enzyme synthesis in many fungi can be catabolite repressed by soluble sugars (Cooper and Wood, 1973).

The following experiment aims to determine to what extent soluble sugars present in potato tissue can catabolite repress synthesis of PTE in the presence of pectic compounds.

Nutrient broth solutions 1.3 g/100 ml were supplemented with sodium polypectate (0.5 g/100 ml). The pH was adjusted to 7 to simulate more natural conditions found within potato tuber tissue. Controls contained nutrient broth (1.8 g/100 ml). Some flasks contained α-glucose at concentrations of 0.1, 0.8 and 1.5%. Appropriate nutrient broth controls were prepared. Three replicates per treatment were inoculated with a loopful of E. atroseptica A13 (10^9 cells/ml) after autoclaving the broths at 7 psi for 7 min. The flasks were shaken at 200 rpm at 25°C, and samples (10 ml) were removed after 18 and 48 h. Each sample was centrifuged at 17000 x g for 5 min to remove cells. The supernatents were decanted, the pH of each determined and assayed for PTE activity at pH 9.0 and PG activity at pH 5.0 by viscometric techniques. The cell residue was resuspended in 10 ml of water and the population numbers determined by turbidimetric techniques. After 48 h incubation, the concentrations of glucose remaining in the supernatents were estimated by the

Table 49. Induction and end repression of PTE synthesis

Treatment	Replicate Number	Final pH	Cell number (10^9 cells/ml)	Enzyme activity	
				Units[1]	Units/10^7 cells/ml
NaPP	1	6.1(8.6)*	0.78(3.3)	2100(3750)	290(11)
	2	6.2(8.2)	0.64(4.3)	50(2650)	0.8(6)
	3	- -	- -	- -	- -
NaPP + 0.1% glucose	1	5.2(5.2)	0.92(1.5)	0(75)	0 (0.5)
	2	5.7(5.4)	0.55(1.6)	0(3)	0 (0.02)
	3	5.8(5.2)	0.67(1.6)	0(3)	0 (0.02)
NaPP + 0.8% glucose	1	5.3(4.8)	0.66(1.6)	0(0)	0 (0)
	2	5.7(4.7)	0.48(2.4)	0(0)	0 (0)
	3	5.5(4.7)	0.62(1.7)	0(0)	0 (0)
NaPP + 1.5% glucose	1	5.2(4.7)	0.66(1.8)	0(0)	0 (0)
	2	4.9(4.7)	0.73(1.6)	0(0)	0 (0)
	3	5.2(4.7)	0.62(1.8)	0(0)	0 (0)
1.8% nutrient broth	1	6.8(7.5)	0.47(1.6)	0(22)	0 (0.1)
	2	7.4(8.1)	0.67(1.5)	16(30)	0.02(0.2)
	3	7.4(8.1)	0.18(1.4)	0(32)	0 (0.2)
2.6% nutrient broth	1	7.4(8.2)	0.69(2.4)	0(32)	0 (0.1)
	2	7.3(7.9)	0.52(1.7)	0(475)	0 (2.8)
	3	7.5(8.2)	0.61(2.2)	0(170)	0 (0.8)
3.3% nutrient broth	1	7.5(8.2)	0.53(2.1)	0(89)	0 (0.4)
	2	7.5(8.2)	0.69(3.4)	0(170)	0 (0.5)
	3	7.5(8.2)	0.67(2.6)	0(100)	0 (0.4)

[1]Arbitrary enzyme units.

*Figures in brackets denote 48 h readings; figures not in brackets denote 18 h readings.

Table 50. Induction and end repression of PG after incubation for 48 h

Treatment	Replicate Number	Final pH	Cell number (10^9 cells/ml)	Enzyme activity	
				Units[1]	Units/10^7 cells/ml
NaPP	1	8.6	3.3	530	1.6
	2	8.2	4.3	475	1.1
	3	-	-	-	-
NaPP + 0.1% glucose	1	5.2	1.5	0	0
	2	5.4	1.6	0	0
	3	5.2	1.6	0	0
NaPP + 0.8% glucose	1	4.8	1.6	0	0
	2	4.7	2.4	0	0
	3	4.7	1.7	0	0
NaPP + 1.5% glucose	1	4.7	1.8	0	0
	2	4.7	1.6	0	0
	3	4.7	1.8	0	0
1.8% nutrient broth	1	7.5	1.6	15	0.1
	2	8.1	1.5	30	0.2
	3	8.1	1.4	32	0.2
2.6% nutrient broth	1	8.2	2.4	42	0.2
	2	7.9	1.7	1680	10.0
	3	8.2	2.2	140	0.7
3.3% nutrient broth	1	8.2	2.1	32	0.15
	2	8.2	3.4	30	0.1
	3	8.2	2.6	22	0.1

[1]Arbitrary enzyme units.

glucose oxidase method. The results are given in Tables 49 for PTE and 50 for PG.

The results in Tables 49 and 50 show that synthesis of both PTE and PG is induced by sodium polypectate (NaPP) and that both enzymes are catabolite repressed by the presence of glucose at concentrations above 5.05×10^{-3} M. The rate of glucose utilisation is shown in Table 51, where it is obvious that induction of PTE occurred after most of the available glucose had been utilised. The final pH values of the incubation media were indicative of the process of substrate utilisation. Fermentation of glucose produces acids, and this is reflected in the low pH values obtained; these may not have favoured PTE synthesis once the glucose had been removed (see Fig. 14).

Replicate number 2 in 2.6% nutrient broth had extremely high PTE and PG activity. It appears that occasionally both enzymes may be produced constitutively in large amounts. All other nutrient broth controls showed little constitutive production of either enzyme, and no PG was detected until after 48 h incubation.

Table 51. Concentration of glucose present in the media after incubation for 48 h

Treatment	Replicate Number	% Glucose concentration remaining
0.1% glucose	1	0
	2	0
	3	0
0.8% glucose	1	0.67
	2	0.63
	3	0.64
1.5% glucose	1	1.35
	2	1.37
	3	1.39

The Effect of Different Carbohydrates on the Induction of PTE Synthesis

Since PG was not found in active potato rots, no further assays were made for this enzyme. The carbohydrates used in a further experiment were chosen on the basis of their possible occurrence in potato tuber tissue. They included representatives from most types of carbohydrates, keto sugars, aldo sugars, disaccharides, monosaccharides, polysaccharides, hexoses, pentoses, sugar acids and their polymers.

Sodium polypectate was distributed into basic mineral solutions to final concentrations of 0.5%. Carbohydrates were added to a final concentration of 0.1% and the broths were adjusted to pH 7.0 and autoclaved for 7 min at 7 psi. Sample flasks were removed and incubated at 25°C for 48 h to test for contamination. Controls contained no sodium polypectate, the carbohydrate under test being the sole carbon source. One set of replicates also contained sodium polypectate as the sole carbon source. Three replicates for each treatment were prepared. The flasks were inoculated with 0.1 ml of an *E. atroseptica* A13 culture (10^8 cells/ml), before being incubated at 25°C and 200 rpm in an orbital incubator. 10 ml samples were removed aseptically from each flask after 24, 48, and 72 h incubation, and assayed for PTE activity at pH 8.8 by viscometry. pH and bacterial cell number were determined as previously described. No assays were made for flasks whose controls showed no utilisation of that sugar as sole carbon source. The results are presented in summary in Table 52. Further data are given in Appendix 16.

Table 52. Catabolite repression of PTE synthesis by different carbohydrates[1]

Carbohydrate	Incubation time (h)	Growth	pH	Enzyme activity Units/10^7 cells/ml[2]	Cell number (10^7/ml)
α-D-glucose + NaPP	24	+	6.8	0	1.3
	48	+	5.2	0.7	18.3
	72	+	5.7	1.9	540
β-D-fructose + NaPP	24	+	5.6	0	1.6
	48	+	5.2	0	25.3
	72	+	6.0	0.5	390
Sucrose + NaPP	24	+	6.0	0	1.0
	48	+	5.4	0	7.3
	72	+	5.2	0.05	387
Inositol + NaPP	24	+	6.3	15	1.1
	48	+	5.9	11	9.4
	72	+	5.6	11	95.3
Raffinose + NaPP	24	-	-	-	-
	48	-	-	-	-
	72	-	-	-	-
Lactose + NaPP	24	+	5.3	65	1.1
	48	+	5.1	71	15.0
	72	+	6.2	20	128
Galacturonic acid[3] + NaPP	24	+	6.8	0	-[4]
	48	+	6.7	0	-[4]
	72	+	6.1	0	1.2
Starch (potato) + NaPP	24	-	-	-	-
	48	-	-	-	-
	72	-	-	-	-
Sodium polypectate (NaPP)	24	+	7.7	411	1.7
	48	+	8.0	211	17
	72	+	8.4	9.6	376

[1]Each result given is a mean of 3 replicates.

[2]Arbitrary PTE units.

[3]Mean of 2 replicates since one did not show bacterial growth.

[4]Bacterial multiplication was too low to measure.

The results in Table 52 show that most sugars which can be utilised as a sole carbon source by E. atroseptica A13 can also catabolite repress PTE synthesis. Two compounds used did not, namely, lactose and inositol. Catabolite repression can occur in biochemical pathways which are closely linked to another pathway. The 'glucose effect' has been described by Loomis and Magasanik (1964). Since catabolite repression was first found by these authors in glucose biosynthesis, the term 'glucose effect' was used as another name. Any compound interconvertable with glucose can cause the effect. However in this assay, lactose did not, and it is suggested that lactose, in the presence of sodium polypectate is not utilised by the normal fermentation pathways of glycolysis. It may be fermented directly to lactic acid before it is oxidized in the TCA cycle.

Inositol is an allicyclic compound and is probably not assimilated in the biochemical pathways which glucose is.

Raffinose and potato starch were not utilised as sole carbon sources, and galacturonic acid allowed only slow growth of E. atroseptica A13.

Again as in the latter experiment PTE was produced when all or most of the catabolite repressant had been removed. This production was accompanied by an increase in pH. The highest bacterial populations were found with glucose utilisation, and this was reflected in the increase in PTE activity after 48 h, whereas sucrose and fructose showed no PTE activity until 72 h incubation.

SECTION VI

TUBER PHYSIOLOGY IN RELATION TO RESISTANCE

Effect of Dormancy on Tuber Resistance to Rotting

As tubers age in store, the populations of Erwinia spp. on the tuber surface and in lenticels decrease. Observations on the physiological state of the tubers during this period indicate a possible link between break of dormancy and the incidence of infection. These experiments were designed to investigate connexions between the incidence of infection and tuber tissue resistance with the break of dormancy.

Ethylene chlorhydrin (2-chloroethanol) has been found to break the dormancy of potato tubers within 4 d (Denny, 1926).

Potato tubers were treated with ethylene chlorhydrin as described in the Materials and Methods. Treated and non-treated tubers were stored at room temperature (19-21°C) in the dark in plastic bins. Samples of five tubers from each treatment were removed periodically and inoculated with E. atroseptica A13 (10^9 cells/ml) into cork borer wounds (2.5 and 10.0 mm deep). After storage at room temperature for 4d in plastic bins, the volumes of rots were measured. The results are illustrated in Fig. 17. As sprouting developed, the sprouts were removed and weighed. Fig. 18 shows the development of sprouting after treatment with ethylene chlorhydrin.

Fig. 17 shows that chemically induced break of dormancy does reduce tuber resistance to rotting. An interesting point

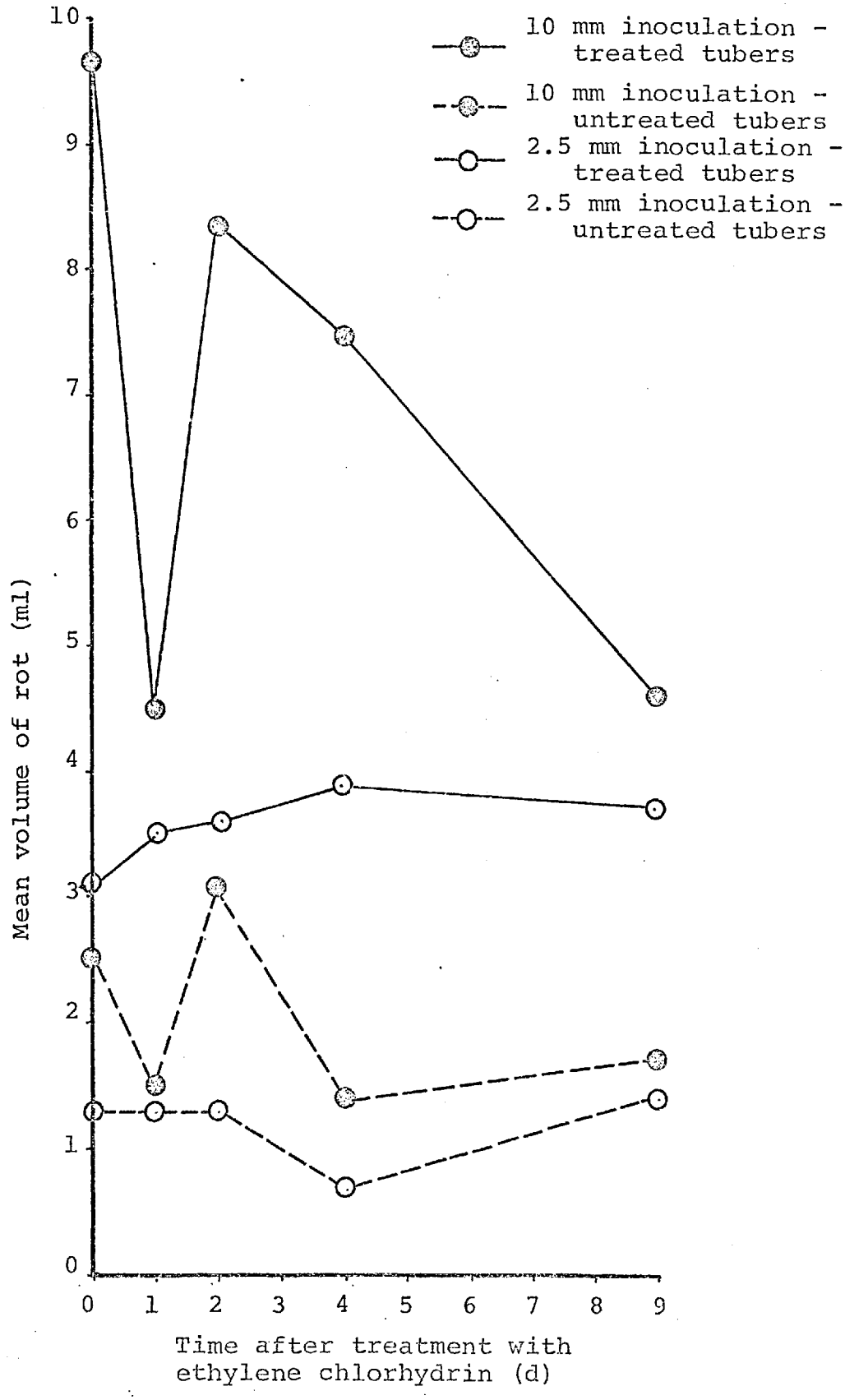


Figure 17. Effect of break of dormancy on resistance of potato tubers to rotting

was that all rots found in the treated tubers were white, whereas, brown rots were often found in untreated tubers.

Except after 1 day, when no differences were found, treatment with ethylene chlorhydrin caused increased rotting. However, large differences in rotting in the treatments were found after a further day, but these differences decreased, and by 9 d after removal from the treatments, no differences in rotting were again found. During this gradual change in response to treatment, sprouts appeared in treated tubers (Fig. 18). There was no sprouting in untreated tubers until 9 d after removal from the treatments.

An analysis of variance for the rot volume data are given in Appendix 17. This particular experiment gave significant differences between the treatments ($p > 0.05$) at days 0, 2, and 4 after removal of tubers from the treatments. However, in many repeats of the experiment there were no differences between rotting in treated and non-treated tubers. Table 53 shows the results from one such experiment.

Table 53. Effect of break of dormancy on rotting

	Volume of rot (ml)[1]			
	Treatment			
	Ethylene chlorhydrin		Control	
	Inoculation depth (mm)			
Days after treatment	2.5	10.0	2.5	10.0
0	0.7	2.5	0.8	2.2
1	0.1	2.8	0.8	2.6
2	0.6	3.1	0.6	2.4
4	0.6	3.4	0.5	3.0
5	0.6	3.3	0.6	2.3

[1]Mean of tubers.

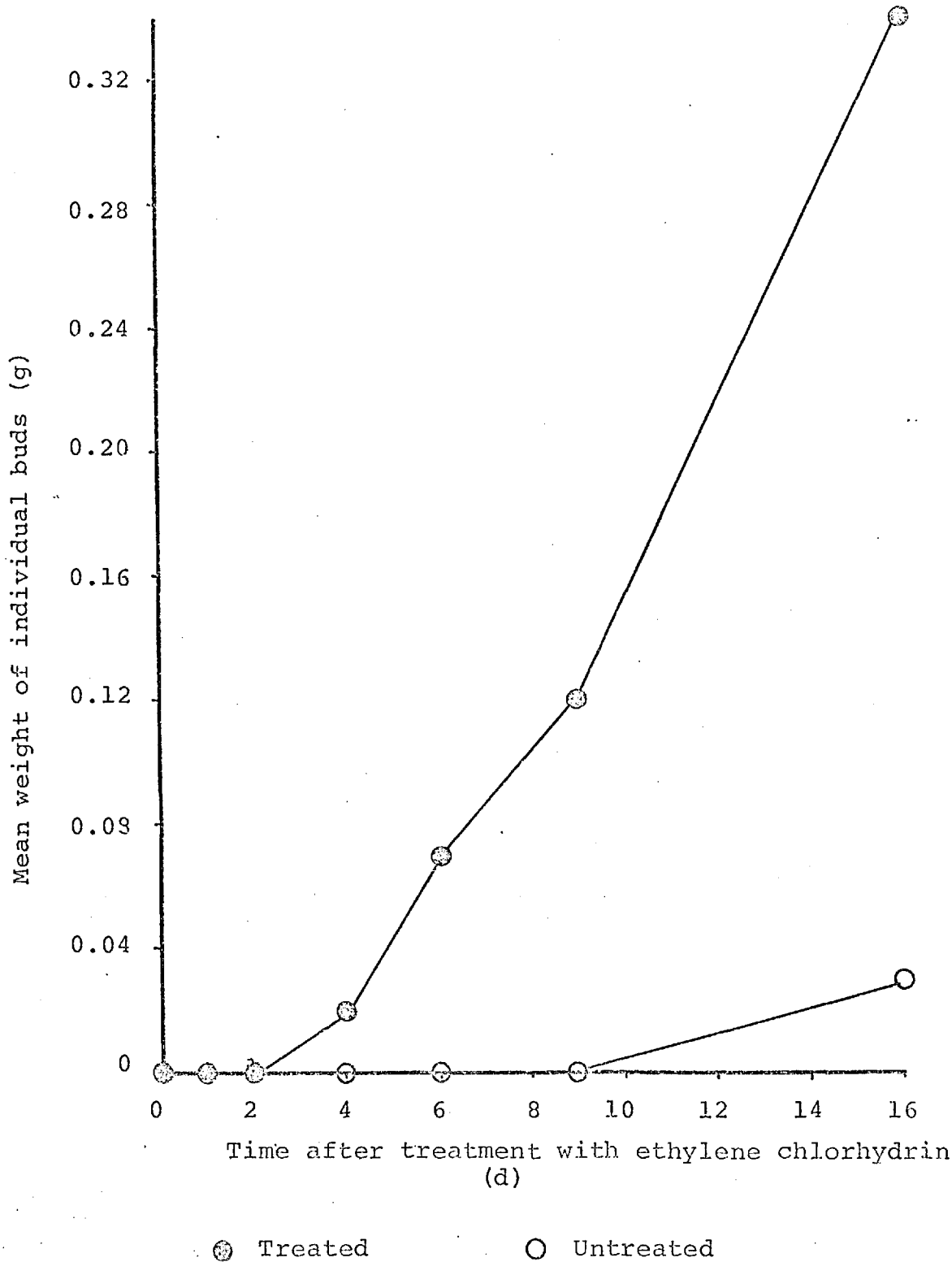


Figure 18. Sprouting development after ethylene chlorhydrin treatment

The effect of break of dormancy on rotting (Fig. 17 and Table 53) is not easily explained. The differences in rotting in treated and untreated tubers may be due to the sudden changes in tuber physiology caused by the chemical treatment. The only conclusion that can be drawn from this series of experiments is that resistance of potato tubers to rotting may be affected by the state of dormancy of the tubers, and that any effect produced involves initial loss of tuber resistance to rotting, which is usually regained as sprouting ensues.

The Effect of Natural Breaking of Dormancy

Field infected tubers were stored in open trays in a darkened shed and sampled periodically. After washing they were inoculated with a suspension of E. atroseptica (10^9 cells/ml) into cork borer wounds and stored for 4 d at room temperature (19-21°C). The mean volumes of rots produced are shown in Table 54.

Table 54. Rotting in tubers of increasing age

	Mean rot volume (10 tubers)	
	Depth of inoculation (mm)	
Sampling date	2.5	10.0
10/10/72	0.6	3.2
5/11/72	0.5	1.8
11/12/72	0.7	2.6
23/1/73	0.1	2.1
18/3/73	0.6	2.2

A linear regression on the data showed no change in resistance to rotting as tubers aged, and there seems to be

little to suggest that susceptibility to rotting increases during storage. Statistical data is presented in Appendix 19.

Rotting of Cold-Stored, Furarex Treated, and Sprouting Tubers

Tubers stored at 2-3°C, treated with the sprouting inhibitor 'Fusarex', or sprouting were washed, air dried, and treated with tetrazolium salt before placing them in polythene bags with 10 ml of water. The mean numbers of lenticels staining (after 48 h) and the numbers of tubers rotting are given in Table 55.

Table 55. Rotting of cold-stored; Fusarex treated, and sprouting tubers[1]

Tuber treatment after storage for 4 months Oct.72-Jan.73	Mean number of lenticels per tuber staining (after 48 h)	Number of tubers (out of 10) rotting in polythene bag test		
		3 d	5 d	12 d
Fusarex	5	1	5	9
Cold stored	7	0	1	9
Sprouting	2	0	0	4

[1]This experiment has already been described in Section III but is included here since it is of relevance in this section.

Fusarex treated tubers rotted most rapidly probably because they were kept at a somewhat higher temperature than were cold-stored tubers and because of the higher humidity within the closed containers. It is of some interest that the incidence of rotting was lowest in the sprouting tubers which had been stored at the same temperature as the Fusarex treated tubers.

Rotting of Cold Stored, Fusarex Treated, and Sprouting Tubers from Inoculated Wounds

Tubers were removed from the above storage conditions in February 1973, washed, air dried and inoculated with E. atroseptica A13 (19^9 cells/ml) into cork borer wounds, 2.5 and 10.0 mm deep. After storage for 4 d at room temperature (19-21°C) in closed bins, the volumes of the rots produced were measured. The results are given in Table 56.

Table 56. Rotting of cold stored, Fusarex treated and sprouting tubers from inoculated wounds

	Mean volume of rot (ml)	
	Inoculation depth (mm)	
Tuber treatment	2.5	10.0
Fusarex treated	0.20	1.8
Cold stored	0.25	2.0
Sprouting	0.50	2.4

Analysis of variance showed significant differences ($p > 0.05$) between dormant and sprouting tubers at both inoculation depths. It appeared that dormant tubers were more resistant to rotting than were sprouting tubers. Statistical data is presented in Appendix 20.

Effect of Major Sugars Within Potato Tubers on Resistance to Rotting

It has been shown (Fig. 17) that dormant tubers may be more resistant to rotting than non-dormant tubers. Denny (1926) has shown that sugar concentrations within potato tubers may change during break of dormancy. This project, done in collaboration with D.K. Martin in part fulfillment for his M.Sc. in the Department of Botany at Silwood Park,

attempted to relate sugar changes in potato tuber tissue to resistance to rotting during break of tuber dormancy.

Sugars were extracted from tuber tissue by two methods as described in Materials and Methods. The alcoholic extraction procedure was chosen for this assay.

Alcoholic extracts were evaporated slowly at 50°C in a rotary evaporator under reduced pressure, to remove alcohol. After removing soluble proteins and deionising, 5 ml samples of the extracts were freeze dried and stored at -18°C until required for further assays.

The residues were treated with 1 ml of anhydrous pyridine (kept above KOH pellets), 0.2 ml hexamethyldisilzane, and 0.1 ml of trimethyl chlorosilane (TMS reagents), in a small stoppered phial. Occasionally it was noted that extracts did not dissolve in the pyridine. Several procedures to overcome this were attempted. Heating the extracts in pyridine before and after addition of the TMS reagents gave non-uniform dissolution. The best results were obtained by mixing all the reagents and allowing them to stand at room temperature (19-21°C) for 30 min with occasional shaking.

Trials involving the determination of the types of soluble sugars present in the extracts were based on retention times comparative to those obtained from a series of sugar standards. The retention times were classed on a time scale with α-D-glucose as 1.0 at 150°C and sucrose as 1.0 at 210°C. All sugars and extracts were tested as a 2 µl sample injected into an SE52 column. Peaks corresponding to the sugar concentration in the sample were obtained on a Smith's sensitometer pen recorder. Standard curves of sugar concentration

against the area beneath the peaks were used to calculate the quantities of the sugars present. Table 57 shows the relative retention times with α-D-glucose and sucrose as standards described above.

Table 57. Relative retention times of TMS derivatives with those of α-D-glucose (150°C) and sucrose (210°C) being 1.0

Sugar	Retention time (min)	Temperature of the column (°C)
α-D-glucose	1.00	150
D-arabinose	0.25	150
D-ribose	0.29	150
β-D-fructose	0.67	150
β-D-glucose	1.70	150
Inositol	2.43	150
Sucrose	1.00	210
D-mannose	0.54	210
α-maltose	1.17	210
β-maltose	1.38	210
α-glucose	0.08	210

Mixtures of sugars were prepared at predetermined concentrations and extracted in the same manner as potato tissue. The recovery of each sugar present was generally 100%, although some sucrose was occasionally lost, possibly due to its poor solution in pyridine.

Potato tissue extracts were shown to possess four major sugar components, sucrose, glucose, fructose, and inositol. The α- and β-glucose concentrations were combined as some mutorotation appeared to take place. Minor sugar components found were ribose, maltose, and one other component

not identified. Major and minor sugars were distinguished by their occurrence in potato tissue. Minor sugars were often found in concentrations too low to estimate. Major sugars were found at concentrations of about 1 mg/g of tuber tissue. On the basis of these preliminary trials only the major sugars sucrose, fructose, glucose and inositol were assayed during break of tuber dormancy.

Tubers taken from store in December 1970 were found to have broken dormancy. Preliminary tests with ethylene chlorhydrin treatment showed no differences between treated and untreated tubers with respect to rotting from inoculated cork borer wounds. Consequently tubers were sprayed to run off with naphthalenacetic acid (NAA) at a rate of 75 mg/kg of tubers. Denny (1941) recommended the use of 50-100 mg of NAA/kg of tubers to inhibit sprouting, which also induced dormancy in the tubers.

After spraying, the tubers were left for 24 h in buckets in a fume cupboard, before air drying and storing for 3 weeks. For the main trial four tuber treatments were used as follows.

1. Tubers stored for 3 months (October-January) in closed bins containing Fusarex, treated with ethylene chlorhydrin.
2. Tubers stored for 3 months (October-January) in closed bins containing Fusarex, not treated with ethylene chlorhydrin.
3. Tubers stored in open trays, treated with NAA 3 weeks before use, treated with ethylene chlorhydrin.

4. Tubers stored in open trays, treated with NAA 3 weeks before use, not treated with ethylene chlorhydrin.

100 tubers per treatment were prepared. Half in each batch received an inoculum of E. atroseptica A13 (10^9 cells/ml) delivered into cork borer (No. 4) wounds 2.5 and 10.0 mm deep, on days 0, 1, 2, 4 and 8 after removal of the tubers from the ethylene chlorhydrin treatment. The other half was assayed for amounts ofthe four major sugars present at the same times as above. Inoculated tubers were incubated at room temperature (19-21°C) for 4 d, before measuring the volumes of rots produced. The sound tissue was assayed immediately for the presence and amounts of sugars. A summary of the results obtained is illustrated in Figs. 19, 20, 21, and 22. More complete data are presented in Appendices 21 and 22.

Glucose, fructose and sucrose have been found to catabolite repress PTE synthesis by E. atroseptica A13 (Table 52); consequently, the concentrations obtained for each have been added together and will be referred to as sugar concentrations. Inositol was not found to cause catabolite repression of PTE synthesis and is illustrated separately.

Sugars were extracted from tubers at the same times as batches from the same population of tubers were inoculated, and from the sound tissue of these inoculated tubers immediately after the volumes of rots had been measured. Measurement of sugar concentrations from the inoculated tubers at

Figure 19. The effect of changes in sugar concentrations in tubers during break of dormancy on rotting

1. Fusarex treated tubers not treated with ethylene chlorhydrin

a. Effect of sugar concentrations of inoculated tubers after incubation for 4 d, on rotting

b. Effect of sugar concentrations at the time of inoculation with E. atroseptica, on rotting[1]

[1]Tubers assayed on these dates were not inoculated with E. atroseptica.

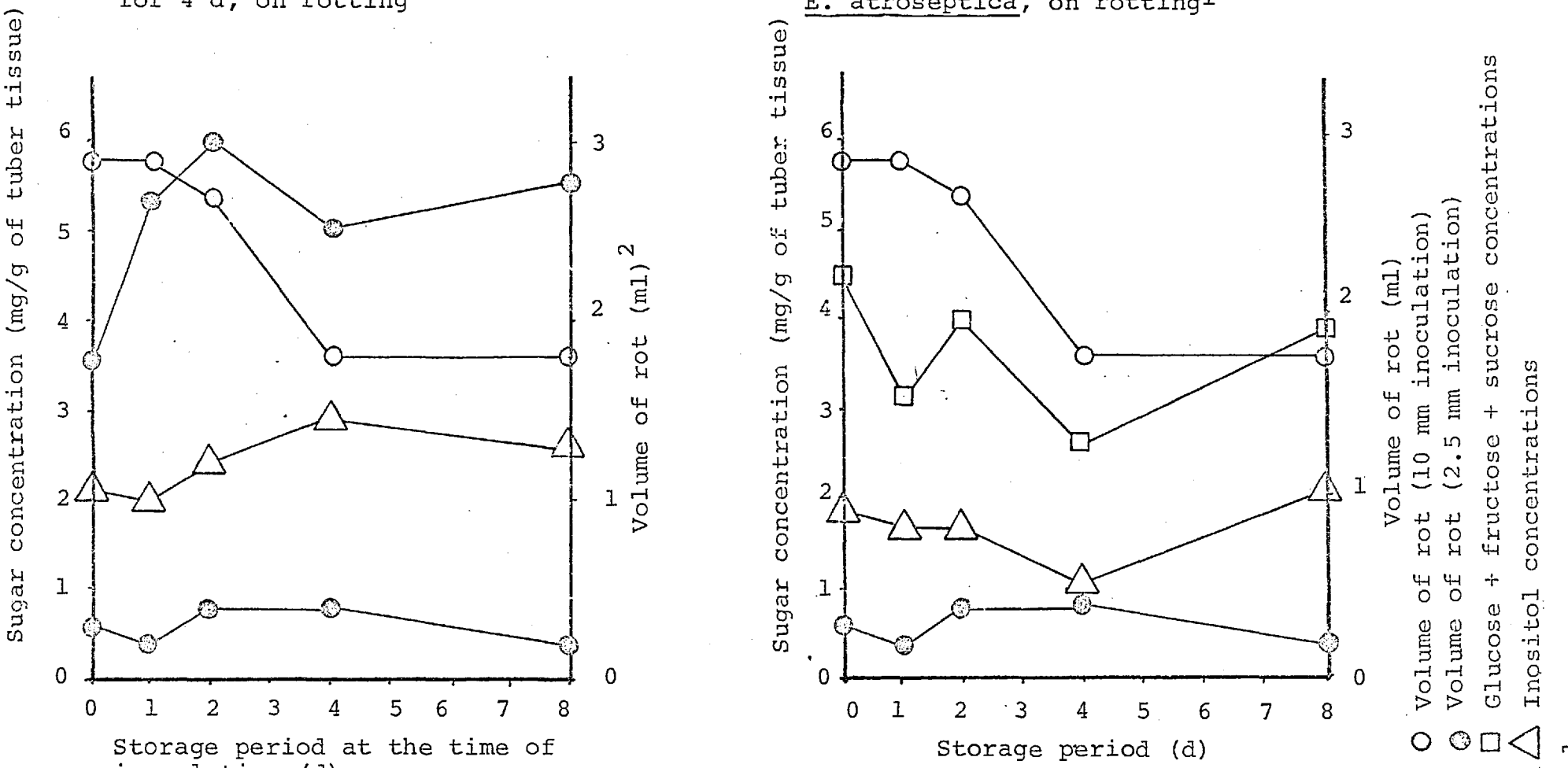
[2]The volumes of rots produced and shown in Figs. a and b are the same.

Figure 20. The effect of changes in sugar concentrations in tubers during break of dormancy on rotting

2. Fusarex treated tubers treated with ethylene chlorhydrin

a. Effect of sugar concentrations of inoculated tubers after incubation for 4 d, on rotting

b. Effect of sugar concentrations at the time of inoculation with E. atroseptica, on rotting[1]



[1]Tubers assayed on these dates were not inoculated with E. atroseptica.

[2]The volumes of rots produced and shown in Figs. a and b are the same.

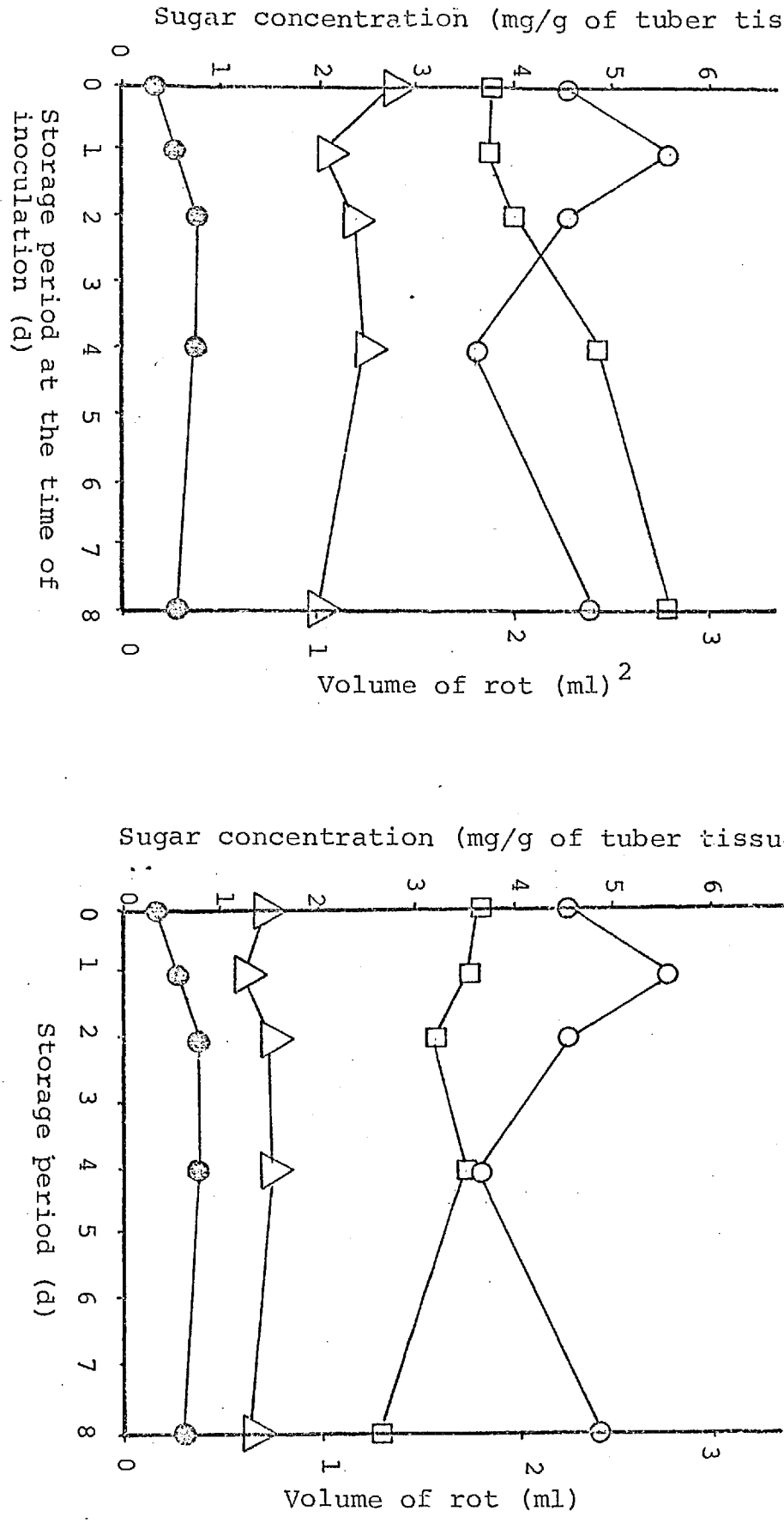


Figure 21. The effect of changes in sugar concentrations in tubers during break of dormancy on rotting

3. NAA treated tubers not treated with ethylene chlorhydrin

a. Effect of sugar concentrations of inoculated tubers after incubation for 4 d, on rotting

b. Effect of sugar concentrations at the time of inoculation with E. atroseptica, on rotting[1]

[1]Tubers assayed on these dates were not inoculated with E. atroseptica.

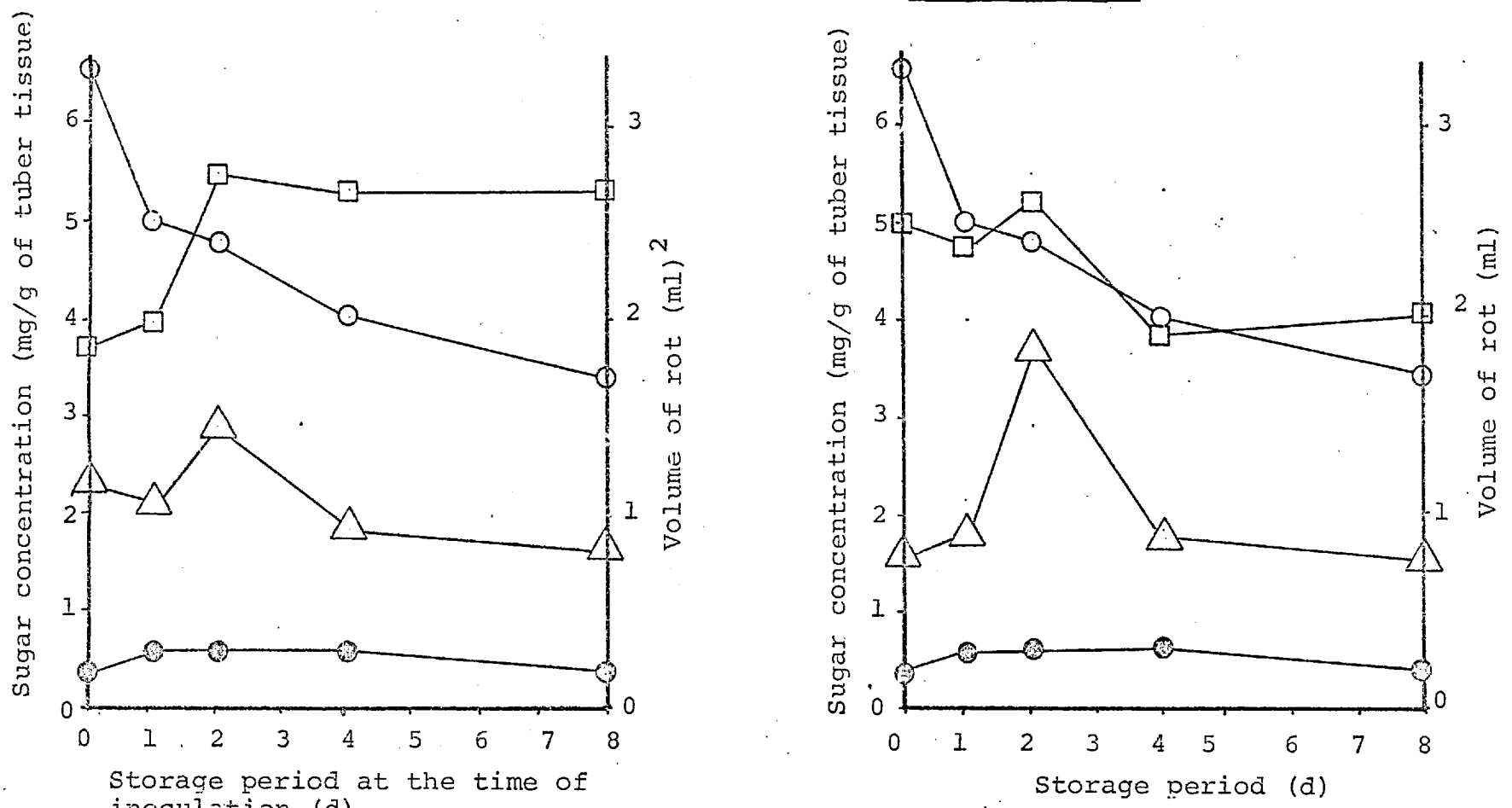
[2]The volumes of rots produced and shown in Figs. a and b are the same

Figure 22. The effect of changes in sugar concentrations in tubers during break of dormancy on rotting

4. NAA treated tubers treated with ethylene chlorhydrin

a. Effect of sugar concentrations of inoculated tubers after incubation for 4 d, on rotting

b. Effect of sugar concentrations at the time of inoculation with E. atroseptica, on rotting[1]



[1]Tubers assayed on these dates were not inoculated with E. atroseptica.

[2]The volumes of rots produced and shown in Figs. a and b are the same

the time of inoculation, would possibly have given more reliable results than those obtained, but removal of tissue from these tubers may have affected rotting since slicing tubers is known to break dormancy. Tubers were peeled, chopped and minced. This was mixed well before removing the sample.

Fig. 19a does not reveal any relationship between sugar or inositol concentrations of the tubers, measured after rotting was assessed, and the rapidity of rotting at either depth. An effect between the same parameters can be seen in Fig. 19b. Initially, a rapid decrease in sugar concentrations was accompanied by a decrease in the rapidity of rotting, and as the sugar concentrations increased at day 4, the decrease in rapidity of rotting lessened.

The results in Fig. 20a show no relationship between sugar concentration and rotting, but ethylene chlorhydrin treated tubers (Fig. 20b) rotted more slowly from 10 mm inoculations as the concentrations of sugars decreased. This effect was not observed at 2.5 mm inoculations, but since the differences in rotting in treated and non-treated tubers at both depths of inoculation, were negligible, it is difficult to assess the effect of sugar changes accompanying break of dormancy on rotting. Tubers treated with ethylene chlorhydrin sprouted twelve days after removal fromthe desiccators, but controls did not. It is concluded that tubers treated with Fusarex retain dormancy, and that this state can be broken by treatment with ethylene chlorhydrin. However, the changes in sugar concentrations were complex and had little effect on rotting.

Rotting in tubers treated with NAA (Fig. 21a) was not affected either by sugar concentrations measured at the times of rot volume assessment, or at the times of inoculation (Fig. 21b).

Rotting (10 mm inoculation) in ethylene chlorhydrin treated tubers previously treated with NAA (Fig. 22a) was greater than in tubers not treated with ethylene chlorhydrin inoculated immediately after removal from the desiccators, but decreased after day 0, and by day 8, rotting was more rapid in non-treated tubers. Sugar concentrations measured at the time of inoculation at day 8 in ethylene chlorhydrin treated tubers were greater than those in non-treated tubers measured at the same time (Fig. 22b). An opposite effect is seen in Fig. 22a. Sprouting of tubers previously treated with NAA was not found 12 d after treatment with ethylene chlorhydrin, and by the time sprouting did occur (21 d) sprouting was also found in NAA treated tubers not treated with ethylene chlorhydrin. Thus the results in Figs. 19 and 20 cannot be used to assess the changes in sugar concentrations during break of dormancy in relation to rotting.

In none of the assays expressed in the figures was a relationship between inositol and rotting found.

In conclusion, the effects of changes in concentrations of sugars able to catabolite repress PTE synthesis, on rotting of potato tubers by E. atroseptica A13, are obscure. Figs. 19b, 20b, and 22b, indicate that the rapidity of rotting may depend onthe amount of sugar present in the tuber tissue at the time of inoculation; as sugar concentrations decreased so did the rapidity of rotting. From the data obtained in

the previous section (Table 52) it was expected that rotting would decrease as the sugar concentrations increased, and vice versa, due to catabolite repression of PTE synthesis. This effect was not found.

The difficulty experienced with sucrose dissolution in pyridine, may have influenced the results in that sucrose concentrations obtained may not be accurate. However, since the three major sugars (glucose, fructose, and sucrose) which cause catabolite repression of PTE synthesis, were present within tuber tissue at concentrations (0.3-0.7% w/w) in excess of those required to cause catabolite repression in the in vitro assays shown in Table 50 (0.1%), the possible inaccuracy of the sucrose concentration determinations is of little importance in this respect.

Resistance of Tuber Tissue to Maceration by PTE

Discs of potato tuber tissue (1 mm thick) were removed from each of ten cores (5 mm diameter) taken at right angles to the periderm to a total depth of 1 cm. The discs were separated, and placed in flasks of water each containing discs at one depth of tissue. The periderm was removed from the uppermost discs. After allowing the discs to become turgid (1 h), 5 discs per sample were removed, blotted dry and weighed immediately in aluminium foil cups, before placing them in a reaction mixture consisting of 2 ml water, 2 ml 0.2 M glycine-NaOH buffer at pH 9.4, 0.5 ml 0.002 M $CaCl_2$ and 0.5 ml polypectate trans-eliminase preparation. After incubation at room temperature (19-21°C) for 3 h with occasional shaking, the discs were removed and placed

on a piece of muslin lining the inside of a large funnel to form a concave screen onto which water was sprayed gently (6-9 litre per min) through pinched rubber tubing (5 mm internal diameter). It was applied for 20 sec. After a further 15 min in distilled water the sections were again gently blotted and reweighed. Boiled enzyme was used in controls. This technique, an adaptation of Mussel and Morre's technique (1969) was used for tuber tissue taken from beneath the periderm and sprouts. The % losses in fresh weight at each depth are shown in Fig. 23.

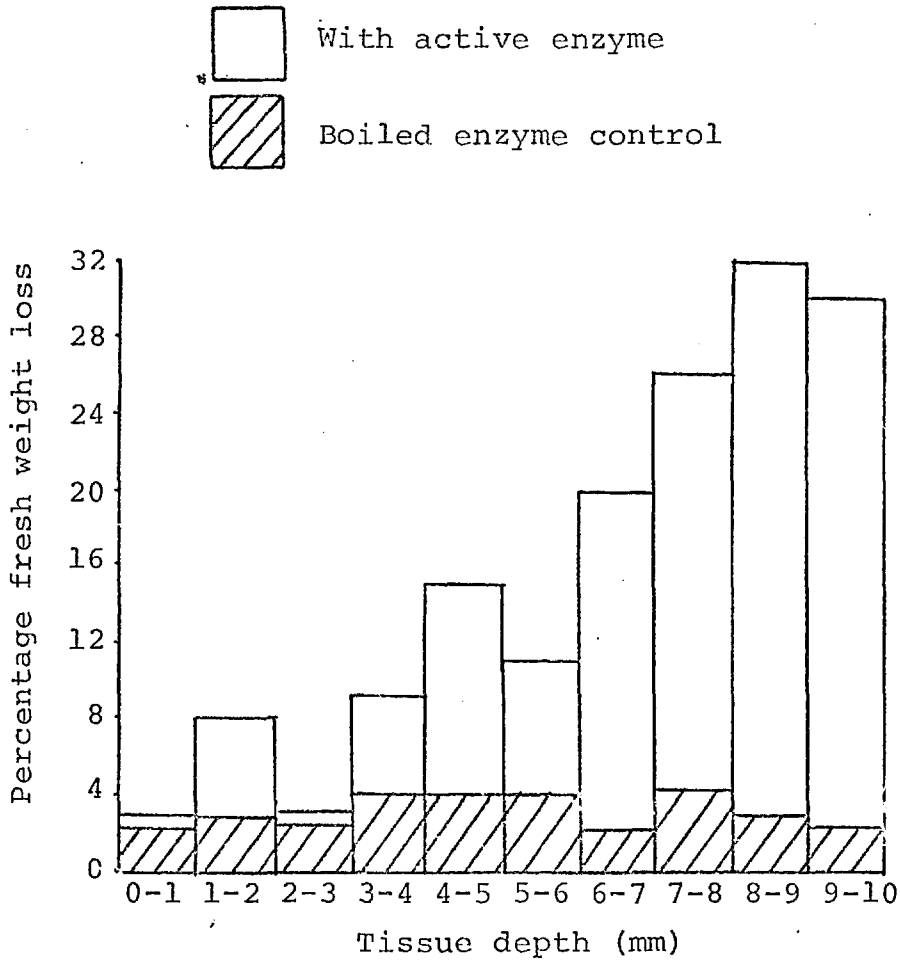
Tissue beneath sprouts containing vascular tissue running longitudinally within it, shows little difference in resistance to maceration with depth. There is, however, a steady increase in resistance to maceration from the outside to the inside cores obtained from non-sprouting parts of tubers. It has been shown in Section III p. that the vascular tissue can remain intact in an active rot. Tissue at a depth of 5-6 mm was more resistant to maceration than was tissue at a depth of 4-5 mm. Vascular tissue was found in discs at this depth and it appears that the vascular tissue of a tuber is more resistant to maceration than inner cortex tissue is, but is not so resistant as the tissue adjacent to the periderm. This resistance of the outer tissues may explain in part why bacteria in these tissues remain latent.

Phenolic Substances in Relation to Resistance

Effect on rotting of phenolic substances and certain enzymes

Preliminary tests involved the addition of phenolic substances and enzymes concerned with phenolic compound

a. Normal tissue



b. Tissue beneath sprout

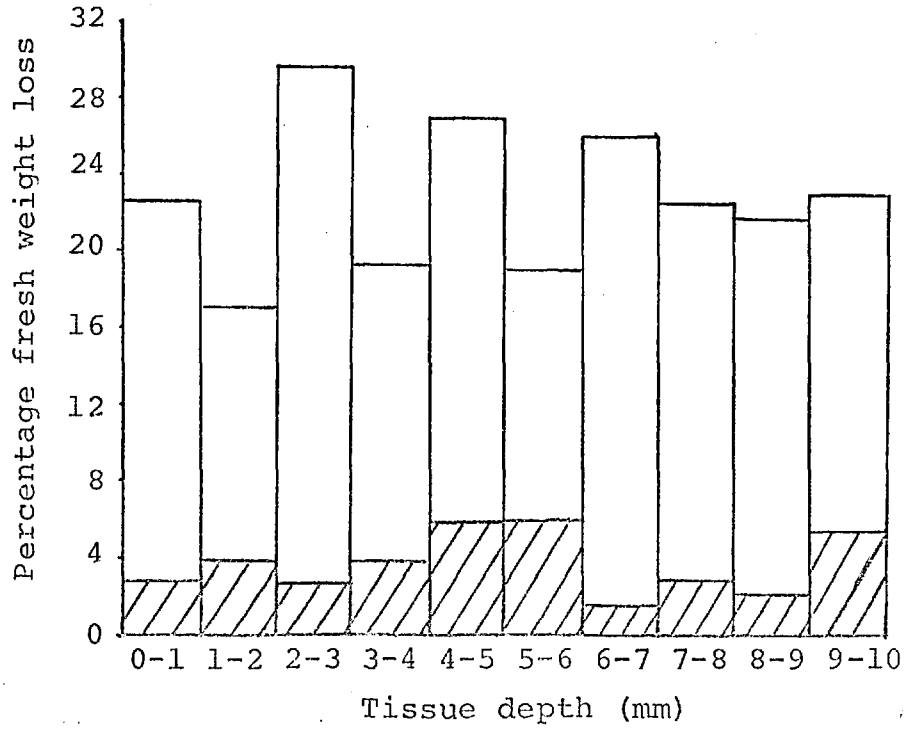


Figure 23. Resistance of tuber tissue to maceration by pectic enzymes.

metabolism to cork borer (No. 4) wounds in whole tubers at depths of 2.5 and 10.0 mm. 0.005 and 0.01 ml respectively of solutions of the phenolic compounds (3 mg/ml) enzymes (1 mg/ml) and H_2O_2 (0.1%) singly and in mixtures (1:1:1 ratio) were added to the wounds using an Agla syringe. After 1 h, Erwinia atroseptica A13 (10^9 cells/ml) was added in the same quantities to the wounds. After sealing the wounds, tubers were incubated at room temperature (19-21°C) for 4 d in closed containers. The volumes of rots obtained are given in Table 58.

Table 58. Effect on rotting of solutions of phenolic substances and certain enzymes

Mean volume (ml) of rot (10 inoculations)

	Method of application of treatment				
	A		B		C
	Depth of inoculation (mm)				
Treatment[1]	2.5	10.0	2.5	10.0	5.0
Ascorbic acid	0	0	0.3	1.8	2.5
Indole acetic acid (IAA)	0	0	0.6	1.6	2.3
Hydrogen peroxide	0	0	0.2	1.9	2.2
Tyrosine	0	0	0	1.8	1.9
Ascorbic acid + peroxidase + H_2O_2	0	0.6	0.6	2.1	2.3
IAA + peroxidase + H_2O_2	0	0	0.5	2.0	2.2
Tyrosine + peroxidase + H_2O_2	0	0.4	0.1	1.9	1.8
Ascorbic acid + Tyrosinase	0	0	0.3	1.8	2.3
IAA + tyrosinase	0	0	0.4	2.0	2.4
Tyrosine + tyrosinase	0	0	0	1.4	2.2
Tyrosinase	0	0.3	0	2.0	2.4
Peroxidase + H_2O_2	0	0	0.2	1.8	2.4
Tyrosinase + H_2O_2	0	0	0.2	1.8	2.3
Water	0	0.2	0.3	1.9	2.4

[1]Phenolic compounds 3 mg/ml, enzymes 0.1 mg/ml, H_2O_2 0.1%, present in mixtures in 1:1 ratios.

A = Test solution introduced into cork borer wound 1 h before bacterial inoculum.

B = Test solution applied to cork borer wound at the same time as the inoculum was added to an inner concentric wound.

C = Test solution added to wound 24 h before bacterial inoculum.

Few tubers rotted and it was assumed that tuber tissue wound reactions had prevented rotting. This posed the problem of distinguishing between the host's response to the pathogen and to wounding.

Another attempt to study the effect of phenolic compounds and associated enzymes on rotting was made as follows.

Inocula of E. atroseptica A13 (10^9 cells/ml) were added to cork borer (No. 4) wounds 2.5 and 10.0 mm deep. Immediately, 0.2 ml solutions of mixtures of phenolic compounds and associated enzymes at the same concentrations described above, were added to cork borer (No. 8) wounds placed concentrically around the first wound to a depth of 1.5 cm. The phenolic compound/H_2O_2/enzyme mixtures contained equal volumes of each chemical. The area of wounding was sealed and the tubers were incubated at room temperature (19-21°C) for 4 days in closed containers. The volumes of rot obtained are given in Table 58.

Although rotting did occur, no rotting beyond the outer wound was found even in water treated controls. Again it appears that the normal reaction of a tuber to wounding was more effective in resistance to rotting than were the treatments. The reaction of tubers to wounding probably involved phenolic compounds, but was possibly more mechanical than chemical in nature, in the form of suberin, lignin or melanin type compounds.

An attempt to investigate the effect of phenolic compounds and associated enzymes in tubers under conditions which minimized the wounding reaction was made as follows.

Plugs of tissue, 5 mm deep and 0.4 cm diameter, were removed from tubers and a glass tube 5 cm high, external diameter 0.4 cm was placed inside the well. These tubers were filled with solutions of various test substances and left for 24 h in the dark, at room temperature (19-21°C). The tube was then replaced by a tube filled with an E. atroseptica suspension (10^7 cells/ml). After incubation for 3 d under the conditions described above, the volumes of the rots produced were measured; they are also shown in Table 58.

An analysis of variance showed that the presence of tyrosine alone or in combination with peroxidase and hydrogen peroxide, in potato tuber tissue increases resistance ($p = 0.20$), but to an extent that cannot be regarded as significant. All other treatments produced no significant differences in rotting compared with water treated controls. The difficulties found during the preliminary trials pose the question as to whether the reaction of tubers to wounding is greater than that to rotting. It is also difficult to introduce phenolic compounds into a potato tuber without wounding. The third method used in this group of experiments enabled the tissue infiltrated with the test compounds to be kept flooded before addition of the bacterial inocula, thus reducing oxidation reactions in the wounded area to a minimum. This was done in the hope that it would also reduce the wounding reaction of the tuber to a minimum. However, it is probable, that phenolic compounds are involved in the process of rotting, since in unrestricted aeration, rots often are limited and brown whereas in restricted aeration,

rapidly spreading white rots are found. Further experiments attempted to determine the changes in phenolic compounds and associated enzymes during rotting.

Phenols and Polyphenol Oxidase in Potato Tubers

Washed and air dried VTSC tubers were halved. One half of each was kept for 72 h with its cut surface in a shallow layer of water, changed every 12 h. The other was kept for 72 h with its cut surface on a glass plate. Both sets of tubers were then inoculated by placing E. atroseptica (10^9 cells/ml) in a 1.0 cm deep cork borer (No. 4) wound. After sealing, the tubers were incubated in closed boxes above moist vermiculite. Tubers were assayed after 0, 1, 2, and 4 days. Approximately 1 g samples were removed and pooled to give a total sample of 10 g from sound and from inoculated tissue. Phenols were extracted in 50 ml ethanol and tyrosinase (polyphenol oxidase) was extracted in 10 ml water. The samples were homogenised for 2 min in a Sorval Microhomogeniser. Polyphenol oxidase activity was measured by its ability to oxidise catechol to its coloured quinone, O-benzoquinone, after first removing the cellular components of the homogenate. Enzyme preparations were stored at 4°C before use or at -20°C if not used immediately. Phenols were estimated by the method of Folin and Denis ( ) and expressed as mg of catechol per g fresh weight. The results are given in Table 59.

From the results in Table 59 it can be seen that polyphenol oxidase activity was higher in turgid than in sub-turgid tissue and increased in both as rots developed.

Table 59. Phenols and polyphenoloxidase (PPO) in potato tubers

Incubation period (d)	Treatment							
	Inoculated[1] turgid tissue		Control turgid tissue		Inoculated sub-turgid tissue		Control sub-turgid tissue	
	Phenols[2]	PPO[3]	Phenols[2]	PPO[3]	Phenols[2]	PPO[3]	Phenols[2]	PPO[3]
0	0.415	14.00	0.500	15.25	0.350	9.75	0.350	13.00
1	0.360	15.25	0.510	14.75	0.375	12.25	0.310	11.25
2	0.390	16.50	0.395	17.00	0.310	13.00	0.360	12.75
4	0.215	19.25	0.430	13.75	0.400	17.00	0.325	13.50

[1]The terms sub-turgid and turgid tissues refer to treatments involving tuber halves kept on glass plates and in water, respectively.

[2]Phenols are expressed as mg catechol per g fresh weight of tuber tissue.

[3]PPO, polyphenol oxidase activity is expressed as increase in optical density units/min with catechol as substrate.

Amounts of phenolic compounds decreased with rotting in turgid tissue probably through the action of oxidases. Whether phenolic substances are significant in resistance to rotting remains problematical from the data in Table 59 because there was much more rotting in turgid tissue in which concentrations of phenolic substances, were initially higher than in sub-turgid tissue.

It must also be noted that rots developing in sub-turgid tissue were brown, limited and apparently drier than the adjacent sound tissue, whereas rots in turgid tissue were white, rapidly spreading and often apparently wetter than adjacent sound tissue.

The Presence of Bacteriotoxic Substances in Potato Tuber Tissue

Ethanol extracts of inoculated and untreated, turgid and sub-turgid tissues prepared as in the previous experiment, were streaked on to thin layer chromatography plates (3.0 ml per 10 cm). Chromatograms were run in butanol-acetic acid-water (4:1:1 v/v) over 10 cm. Three plates were prepared for each treatment. One plate was marked at the points of fluorescence under UV light while the others were sprayed with a suspension of E. atroseptica A13 (10^2 cells/ml) in nutrient broth. The plates were incubated for 18 h at 25°C in closed boxes maintained at a relatively high humidity by lining the insides with moist blotting paper, before spraying with 0.25% tetrazolium salt solution. After 1 h the plates were examined for pink staining due to bacterial growth.

No bands of inhibited growth were found, although the intensity of the pink colouration was less in areas

corresponding to pronounced fluorescence. Such bands were found on chromatograms from each of the four treatments.

Suberisation in and Around Lenticels

Tubers of different size taken from field and from store were washed, and sections through lenticels were cut with a razor. The sections were treated with Sudan IV as described in Materials and Methods.

Suberised cells which took up the stain were found adjacent to the periderm. Cells produced by the lenticel meristem did not stain. A weak staining reaction was observed in cells immediately beneath the meristematic region. Whether these cells were less suberised than those adjacent to the periderm or did not take up the stain is not known.

SECTION VII

ERADICATION OF THE PATHOGEN

Eradication of E. atroseptica from potato tubers has been approached in two ways. One involves the use of stem cuttings which are used to produce tubers free from the pathogen, since the seed tuber is removed while still entire and not rotting. The tubers produced from such plants are then grown in soil in which potatoes have never been grown before. These tubers are then used commercially as seed tubers. The aim of the stem cutting programme is to rid all stocks of potatoes of the blackleg organism.

The other approach involves the use of bacteriotoxic chemicals which are able to eradicate E. atroseptica infections within tubers.

This experimental section attempts to investigate the use of various bacteriotoxic chemicals for this purpose.

Effect of Antibiotics on the Growth of the Pathogen

0.1 ml aliquots of each of three isolates of E. atroseptica (10^6 cells/ml) were seeded on to two plates of nutrient agar which had been dried for 5 h at 40°C, with a sterile filter paper separating the lid of the Petri dish from its base. After 1 h (to allow the inocula to be absorbed by the media), sterile Multidiscs (Oxoid Ltd.) U_1 and S_1 were placed aseptically on to the surfaces of the media. After incubation at 25°C for 36 h, the plates were observed for zones of growth inhibition. The diameters of these were measured. The results are given in Table 60.

Table 60. Effect of antibiotics on the growth of the pathogen

Antibiotic	Concentration (units) in the paper disc	Diameter of inhibition zone (mean of two) (cm) Isolate of E. atroseptica A13	A12	NCPPB 1277
Cloxacillin	5	0	0	0
Penicillin G	1.5	0.25	0	0
Ampicillin	2	1.2	1.2	1.3
Streptomycin	10	1.5	1.3	1.8
Tetracycline	10	0.8	0.8	0.9
Chloramphenicol	10	1.0	0.9	1.1
Erythromycin	10	0	0	0
Sulphafurazole	10	0	0	0
Nalidixic acid	30	1.4	1.5	2.0
Nitrofurantoin	200	2.1	1.9	2.1
Colistine methane sulphonate	200	1.4	1.2	1.5

Several antibiotics were found to be active against E. atroseptica, streptomycin, ampicillin, nalidixic acid and nitrofurantoin being the most active.

Effect of streptomycin on rotting

Field infected tubers were washed and air dried before dipping them in a solution containing 50 ppm streptomycin. After air drying again, they were placed individually in polythene bags with 10 ml water. The numbers of tubers rotting compared to untreated tubers are shown in Table 61.

Although a streptomycin dip (50 ppm) did decrease the number of tubers rotting, it did not eradicate the bacteria responsible for rotting. It was thought that the

Table 61. Effect of streptomycin on rotting

Tuber treatment	Number of tubers rotting (out of 25) in polythene bags with 10 ml water		
	3 d	5 d	12 d
Streptomycin	5	9	15
Control	4	13	19

activity of the antibiotic was reduced by its inability to enter the lenticel.

Use of dimethylsulphoxide (DMSO) to aid the uptake of streptomycin into potato tubers

DMSO has the capacity to penetrate tissues rapidly. Washed and air dried tubers were dipped for 5 sec into one of the following solutions:

1. Water,
2. 50 ppm streptomycin,
3. 50 ppm streptomycin in 2% DMSO,
4. 2% DMSO.

After air drying, tubers were placed individually in polythene bags with 10 ml of water, and incubated for 12 d at room temperature in the dark. The numbers of tubers (out of 25) in each treatment are shown in Table 62.

Table 62. The use of DMSO to aid streptomycin uptake into tubers

Tuber Treatment	Number of tubers rotting (out of 25) in polythene bags		
	3 d	8 d	12 d
Water	3	12	16
50 ppm streptomycin	3	11	12
50 ppm streptomycin in 2% DMSO	1	9	14
2% DMSO	0	11	12

DMSO did not aid streptomycin uptake by potato tubers in a way in which bacterial populations were eradicated. Also DMSO had no bacteriotoxic activity within potato tubers.

Further tests with DMSO showed no preferential uptake by tubers of dyes dissolved in DMSO compared with water. The use of this chemical was not investigated further.

The use of Tricidal RE-10 to eradicate E. atroseptica and other soft rot bacteria from potato tubers

Tricidal RE-10 (Wingfield Industries Ltd.) is a liquid containing:

5,5'-dichloro-2,2'-dehydroxy-diphenyl methane

4 bis-(p-/dimethyl-amino/-phenyl)-methylene-2,5-cyclohexadine-dimethyl ammonium chloride

iso-propyl alcohol

water.

VTSC tubers were soaked for 12 h in water before placing in a suspension of E. atroseptica A13 (10^6 cells/ml), for 6 h. After air drying overnight, tubers were dipped into one of three treatments:

1. 1% Tricidal RE-10,
2. 10% Tricidal RE-10,
3. water.

After air drying they were stored in sealed buckets and tested 1 day and 1 month later. Tubers were placed in polythene bags with 10 ml of water. The number of tubers rotting are shown in Table 63.

Table 63. Eradication of E. atroseptica A13 from tubers by Tricidal RE-10

Number of tubers rotting (out of 25) in polythene bags with 10 ml water

Storage period (months)	Treatment								
	1% Tricidal RE-10			10% Tricidal RE-10			Water		
	4 d	8 d	12 d	4 d	8 d	12 d	4 d	8 d	12 d
0[1]	1	7	17	0	6	14	3	11	19
1	4[2]	4	6	8[3]	9	9	0	6	12

[1]Tubers sampled 1 d after treatment.

[2]3 tubers had rotted in the bucket

[3]6 tubers had rotted in the bucket

The results in Table 63 show that treatment with Tricidal RE-10 (1% and 10%) for 1 day before placing tubers in the polythene bag test, retards rotting and possibly reduces the populations of Erwinia atroseptica, but does not eradicate them. Results obtained after storage for 1 month show some eradication. However, three tubers in 1% and six tubers in 10% Tricidal RE-10 treatments rotted in the buckets. Whether this was due to phytotoxic effects of the chemical on tubers was unclear. However, condensation had formed on the inside of the buckets, and after incubation for 1 month, some rotting from infected tubers might be expected. The trial was not continued because on investigation of the remaining stored tubers, more rotting was found.

Investigation into the use of four chemicals for eradicating soft rot bacteria from tubers

This project was organised for an M.Sc. research project and I am grateful to Mrs. J.C. Prickett (nee Phear) for the results.

The chemicals used were:

1. Tricidal RE-10 (Wingfield Industries Ltd.)
2. DS 9073 (Plant Protection)
3. Bunema (Buckman Laboratories Ltd.)
4. HPMTS (Buckman Laboratories Ltd.)

The active principles of DS 9073, Bunema and HPMTS were not known.

Bacteriotoxic effect of the chemicals on *E. atroseptica* A13

Surfaces of nutrient agar plates (15 ml) were seeded by distributing 0.1 ml of a suspension of *E. atroseptica* A13 (10^5 cells/ml). A central well was made in each plate with a sterile cork borer (No. 4). 0.2 ml of four, ten-fold, serial dilutions of each chemical were pipetted into the wells. The plates were incubated for 24 h at 25°C before measuring the diameters of zones of growth inhibition. The results are given in Table 64.

Table 64. Bacteriotoxic effect of the chemicals on *E. atroseptica* A13

Diameter of inhibition zones (mean of 3 replicates) (mm)

Chemical	Dilution of chemical			
	10^3	10^4	10^5	10^6
Tricidal RE-10	4	2.5	0	0
Bunema	4.5	0	0	0
DS 9073	4	1	0	0
HPMTS	12	5	1.5	0.5

The results in Table 64 show that all four chemicals were bacteriotoxic at the concentrations used, HPMTS being

the most active. It is difficult to compare the chemicals since the concentrations of the active principles in the solutions obtained were not known.

DS 9073 was received at a concentration of 20% active principle in water.

Bacteriotoxic Effect of Tricidal RE-10 Within Potato Tissue

The effect of local applications of Tricidal RE-10 on growth of E. atroseptica in potato tissue

VTSC tubers were surface treated by soaking in a 10% aqueous solution of 'Chloros' for 15 min. After slicing into 4 mm thick sections with a sterile knife, a central 2 mm deep well was cut into each slice with a cork corer (No. 2). 0.02 ml of a suspension of E. atroseptica A13 (10^9 cells/ml) were added to each well. 0.02 ml of 10^3, 10^4 dilutions of Tricidal RE-10 or water were then added to the wells. The potato tissue slices were incubated at 25°C and investigated for rotting at daily intervals. The diameters of the macerated areas were measured and are presented in Table 65.

The results given in Table 65 show that application of the chemical to cut tuber surfaces causes greater rotting than in water treated controls. It is suggested that Tricidal RE-10 at concentrations of 10^{-3} and 10^{-4} causes phytotoxic effects in tuber tissue. This test was not repeated for the other chemicals.

Table 65. Effect of Tricidal RE-10 on growth of *E. atroseptica* within potato tuber tissue

Incubation period (d)	Surface of slice	Range of diameters of macerated area (mm)[1] Chemical treatment concentration		
		Control (water)	10^{-4}	10^{-3}
1	Upper	0	0	0
	Lower	0	0	0
2	Upper	0-5	0-12	1-8
	Lower	5-10	2-15	0-10
3	Upper	2-12	1-12	4-10
	Lower	10-16	10-40	15-30
4	Upper	4-12	8-20	10-26
	Lower	17-30	17-48	20-40

[1]Each treatment had 5 replicates

Effect of Tricidal RE-10 on non-inoculated tissue slices

VTSC tuber tissue slices were prepared as in the previous experiment and sprayed to run off with one of the following:

1. Tricidal RE-10 (10^3 dilution)
2. Tricidal RE-10 (10^4 dilution)
3. Water.

The slices were then placed individually in Petri dishes on sterils moist filter papers, and incubated at 25°C, investigating for rotting at intervals. The results are given in Table 66.

As in the previous experiment, application of Tricidal RE-10 increased rotting. Rotting in Tricidal RE-10 treated discs was accompanied by more water loss from the discs than

Table 66. Effect of Tricidal RE-10 on non-inoculated tissue slices

Maximum and minimum percentages of rotted tissue in discs[1]

Period of incubation (d)	Tuber surface	Treatment		
		Water	10^4 dilution of Tricidal RE-10	10^3 dilution of Tricidal RE-10
1	Upper	0	0	0
	Lower	0	0	0
2	Upper	0	0-40	5-50
	Lower	0	0-60	5-80
3	Upper	0	0-60	5-100
	Lower	0-40	70-100	40-100
4	Upper	0-30	30-100	30-100
	Lower	0-50	70-100	60-100

[1]Five discs per treatment

in water treated discs. As to whether water treated discs rotted from contaminant bacteria or bacterial infections within the tissue is not known. However, it appears that Tricidal RE-10 is phytotoxic to potato tuber tissue.

Effect of Application of the Chemicals on Rotting of Whole Tubers

VTSC tubers were well washed, and soaked in water for 24 h before soaking for 12 h in a suspension of *E. atroseptica* A13 (10^6 cells/ml). After air drying overnight the tubers were treated in one of three ways.

1. 5 second dip in chemical (10^3 dilution),
2. 5 second dip in chemical (10^4 dilution),
3. 5 second dip in distilled water.

After air drying again, tubers were placed individually in polythene bags with 10 ml water. The numbers of

tubers rotting (out of 10) and the numbers of rots per 10 tubers after 12 d in polythene bags with 10 ml of water are given in Table 67.

Table 67. Effect of application of chemicals on rotting after 12 d in polythene bags with water

Chemical treatment	Number of tubers rotting (out of 10)	Number of rots/ten tubers	% reduction in the number of rots
Tricidal RE-10			
Water control	10	58	
10^4 dilution	9	21	64
10^3 dilution	7	16	72
Bunema			
Water control	8	41	
10^4 dilution	7	35	14
10^3 dilution	5	29	29
DS 9073			
Water control	10	43	
10^4 dilution	7	18	58
10^3 dilution	6	14	67
HPMTS			
Water control	9	28	
10^4 dilution	8	23	18
10^3 dilution	7	17	39

The results in Table 67 show that all chemicals reduced the numbers of infected tubers and also the numbers of infections per tuber. The % reductions in the number of rots are not comparable since each chemical treatment involved tubers inoculated on different dates. This is illustrated by the fact that the numbers of rots per tuber in the

water treated controls, are very different. It is also difficult to assess whether the reductions of populations of E. atroseptica A13 are from contaminant or infection populations. Contaminant bacteria on the surface of tubers can cause rotting when the tubers are incubated in polythene bags with water. Any chemical which has a bacteriotoxic effect on E. atroseptica A13, would reduce the populations of contaminant bacteria on the surfaces of tubers.

Effect of Application of Chemicals on the Reduction of Infections of E. atroseptica A13 and other Soft Rot Bacteria, During Storage

At the time of this trial, no VTSC or field infected Majestic tubers were available. Ulster Premier tubers were purchased and soaked for 24 h in water, before soaking in a suspension of E. atroseptica A13 (10^6 cells/ml) for 12 h. Tubers were air dried and treated in one of two ways.

1. 1 min dip in water,
2. 1 min dip in one of the chemicals (10^3 dilution).

After air drying overnight, tubers were stored in buckets lined with absorbent paper. Samples of ten tubers were removed periodically and the tubers were placed individually in polythene bags with 10 ml of water. The numbers of rots per 10 tubers after incubation for 12 d in polythene bags with water, are given in Table 68.

All chemicals at a dilution of 10^3, reduced the number of rots per ten tubers, compared with water treated control tubers. Bunema and DS 9073 caused large initial reductions in the numbers of rots. However, the number of rots in water treated control tubers also decreased

dramatically during the 16 d storage period.

Table 68. Effect of application of chemicals on rotting in polythene bags with water, during storage

Number of rots/10 tubers after incubation in polythene bags with water for 12 d

Storage period (d)	Chemical treatment (10^3 dilution)					
	Water control		Tricidal RE-10	Bunema	DS 9073	HPMTS
0	48	48	36	11	11	38
4		21	9	6	3	3
7		14	10	5	0	6
16		4	3	5	2	1

A similar experiment using freshly harvested tubers is in progress at time of writing.

Field infected tubers were washed individually, and air dried, before dipping for 5 seconds into one of the following treatment:

1. water,
2. 100 ppm DS 9073 (original solution contained 200 g DS 9073 per litre of water)
3. 500 ppm DS 9073[1]
4. 1000 ppm DS 9073
5. 5000 ppm DS 9073
6. 0.1% Bunema + % WSCP (a sticker provided by Buckman Laboratories Ltd.)
7. 0.4% Bunema + % WSCP[1]
8. 0.05% HPMTS + % WSCP
9. 0.2% HPMTS + % WSCP[1]
10. 0.5% Tricidal RE-10
11. 2.0% Tricidal RE-10[1]

[1]Concentration recommended by supplier.

After air drying overnight, tubers were stored in buckets covered with absorbent paper, at room temperature (18-20°C). Samples of 15 tubers from each treatment were removed periodically and placed individually in polythene bags with 10 ml of water. The numbers of tubers rotting at 4 day intervals during incubation at room temperature, are given in Table 69.

Table 69. Effect of chemicals on rotting of field infected tubers stored for 1 month (October-November 1973)[1]

Number of tubers rotting(out of 15) in polythene bags with water

Chemical treatment	Storage period (wk)					
	0[2]			2		
	[Period of incubation in polythene bags with water (d)]					
	[4]	[7]	[12]	[4]	[8]	[12]
Water	9	12	15	1	4	5
100 ppm DS 9073	7	10	13	0	4	5
500 ppm DS 9073	8	13	15	0	0	0
1000 ppm DS 9073	2	11	14	0	0	1
5000 ppm DS 9073	1	9	15	0	0	1
0.1% Bunema + WSCP	5	12	13	0	4	5
0.4% Bunema + WSCP	9	14	15	0	2	4
0.05% HPMTS + WSCP	12	15	15	0	2	3
0.2% HPMTS + WSCP	0	9	15	0	1	2
0.5% Tricidal RE-10	4	11	14	0	0	1
2.0% Tricidal RE-10	3	12	14	0	0	0

[1]The results presented are of the first two sampling dates only; the experiment is still in progress.

[2]Tubers were stored for 1 day after treatment with the chemicals before placing in polythene bags with water.

The results obtained so far, indicate that storage of tubers at room temperature may cause more marked

eradication of the pathogen, than the chemical treatments themselves. Rotting of tubers, placed into polythene bags with water 1 day after treatment with the chemicals, was markedly retarded by DS 9073 (1000 and 5000 ppm), Tricical (0.5 and 2%) and HPMTS + WSCP (0.2%). Bunema produced odd results, in that a 0.4% solution produced more rotted tubers than 0.1% solution. However, at this time, the treatments did not eradicate the pathogen.

After storage of tubers in buckets for 2 weeks, DS 9073 (500, 1000, and 5000 ppm), and Tricidal (0.5 and 2%) had reduced the number of tubers which rotted to a very low level. However the effect of the chemical treatments is not shown clearly since storage of tubers at room temperature reduced the numbers of tubers rotting in polythene bags to a greater extent than did the chemicals themselves.

Miscellaneous Observations

Incidence of blackleg plants in the field

The numbers of plants developing blackleg symptoms during growth in the field at Silwood Park in 1971 and 1972, are given in Table 70.

Table 70. The Number of plants developing blackleg symptoms

1971	1972
18	12

The incidence of plants developing blackleg symptoms was approximately 0.05%.

DISCUSSION

Before considering the disease, the methods available for the isolation and identification of Erwinia spp. must be discussed. The ideal method is a single selective and diagnostic medium. Several media have been formulated for this purpose. Cuppels and Kelman's (1971), Logan's (1963), Stewart's (1962), Perombelon's (1971), and Kado and Heskett's (1970) media have been used, and qualitative and quantitative assessments of the first four were made.

Although difficulties in obtaining uniformity in dilution series were not overcome, the results did indicate that none of the media could be used selectively to isolate and identify Erwinia spp. Logan's medium was the best, but growth of Erwinia atroseptica A13 on it was poor. Growth was often restricted on Stewart's medium, although on Cuppels and Kelman's and Perombelon's media, it was good. No significant differences between the media with respect to selectivity were found. Perombelon (1971) claims that his medium is semi-selective and diagnostic. However, some difficulty was found in deciding whether or not certain bacteria isolated from soil were pectolytic. No such difficulties were found with Cuppels and Kelman's medium and because it was easy to prepare and use, it was chosen for most isolations from soil and rotting tubers. However, in experiments which used this medium to isolate from rotting tubers, its use was found to be limited. Pectolytic colonies which were oxidase negative when cultured on nutrient agar, were often not pectolytic when recultured on Cuppels and Kelman's medium. It appeared

that oxidase negative, non-pectolytic bacteria grew in the pits formed by E. atroseptica A13. These organisms were not identified.

The use of Kado and Heskett's medium was abandoned after the first field trials in 1971, as its selectivity for Erwinia spp. appeared poor.

A personal communication with Graham (1972) indicated that successful identification of an Erwinia sp. involved the use of a large number of biochemical tests. This was considered impractical for the field trials at Silwood Park, since the number of rots from tubers incubated in polythene bags with water was large (approximately 800 per sampling date). Consequently, two tests, pectolytic activity and a negative oxidase reaction was chosen for the identification of the genus Erwinia. Species determinations were not attempted. Various authors have attempted to distinguish between Erwinia spp. by the use of single tests. Dowson (1957) suggested the use of ethyl alcohol agar, and Logan (1966), the use of maltose. All have been tried but none have given satisfactory results, since named cultures obtained from the NCPPB have given positive reactions for the separate species. Erwinia spp. isolated from rotting tubers have proved to be E. atroseptica by one test and E. carotovora by another. No emphasis has been placed on these results and as to whether a particular culture is E. atroseptica or E. carotovora, seems of little importance if there is little or no difference in their virulence to potato tubers. Cultures obtained from the NCPPB and the NCTC have shown that E. atroseptica strains are usually more virulent than E. carotovora

strains, but this is dependent upon the environmental conditions of tuber storage. However, Perombelon (1973) states that E. atroseptica can be isolated four times more frequently from potato tubers than E. carotovora.

Results from the field experiments which determine the numbers of tubers infected by soft rot bacteria agree with those of Webb (1971) in that the number of plants showing blackleg symptoms, is not indicative of the number of plants bearing infected tubers. In contrast, Jennison (1923) and McKay (1955) suggested that the disease could be eradicated by roguing plants showing symptoms of blackleg.

0.5-1.0% of the plants in the field in 1971 and 1972 had typical blackleg sumptoms, but almost all plants sampled had tubers which were infected with soft rot bacteria, although not all such bacteria were Erwinia spp. Up to 92% of all tubers sampled from the field, rotted in the polythene bag test, and according to the isolation and identification procedure used in 1972, up to 77% of these were infected with Erwinia spp., although for reasons already stated this is not an accurate estimate. Perombelon (1973) states that virtually all tubers are infected with Erwinia spp. during growth in soil.

From the position of the rots on the tuber and the low incidence of heel end infections obtained in 1972, it seems that infection of tubers by bacteria passing through stolons from the stems of the parent plant contribute little to infection of tubers during growth. The pathogen appears to overwinter as a seed tuber borne infection. When seed tubers rot in the soil, the causal organisms may pass in to

the soil and migrate randomly through it. Any new tubers within this seeded soil may become infected. Soil containing freshly planted seed tubers contained few pectolytic bacteria until after seed tubers had begun to rot.

Possible factors, namely rainfall and soil temperature, which may affect the migration of, and subsequent tuber infection by Erwinia spp. were investigated. No correlations between these factors and infection were found.

The incidences of infection of tubers found during growth in 1971 and 1972 were different. In 1971, the numbers of tubers infected with soft rot bacteria were low initially (30%) increasing during July and August to approximately 60%. In 1972, samples taken in early July showed that 90% of tubers were infected with soft rot bacteria. During July and August the numbers of tubers which rotted decreased before increasing again to 85% approximately.

The results from these experiments showed that the differences in numbers of infected tubers obtained from plants with and without non-rotted tubers, were smaller in 1972 than in 1971. It appears that bacteria from a rotting seed tuber can migrate through the soil and infect tubers from other plants. However, VTSC tubers grown in Erwinia-free soil produced tubers which rotted in polythene bags with water. Although some bacteria isolated from these rots were identified as Erwinia spp., many rots contained pectolytic, oxidase positive bacteria, capable of rotting potato tissue slices, but not whole potato tubers when inoculated into cork borer wounds. The polythene bag test

is a drastic test and this field trial gives evidence to support this statement, in that normally non-virulent pectolytic bacteria can rot potato tubers. It is also possible that under the soil conditions present around tubers grown in polythene sacks, the ecology of soil bacteria is different from that found in normal field conditions. Pectolytic isolates have been found to be anaerobic spore formers. Some aerobic spore formers were also found. These were assumed to be Clostridium and Bacillus spp., respectively.

The results from the polythene sack grown tuber trial indicate that water content of soil may affect infection of tubers, but the increased number of infections in wet soil was not so great as expected. However, according to Hellmers and Dowson (1953) and Lazar and Burcur (1964), cool wet summers would retard the growth of the plant which would allow the pathogen to multiply and cause the development of a greater percentage of plants with blackleg. Dry summers would prevent the spread of the bacterium through the soil which would result in only a small proportion of new tubers becoming infected. Davidson (1948) found that if plants were grown in soil with a very high water content, soft rots developed at the lenticels of the tubers. The results fromthe trials carried out at Silwood Park have not shown conclusively that water content of soil or soil temperature are major factors in infection of tubers by Erwinia spp.

The data also indicated that the size of tubers at the time of infection was important. Tubers heavier than

70 g had a lower incidence of infection than did smaller tubers. However, growth of individual tubers in polythene sacks was not so great as those growing in the field, and little increase in size occurred after mid-July. Consequently the numbers of tubers above 70 g in weight was much less than of those in the other two weight categories.

The incidence of infection of tuber samples taken at intervals during growth in the polythene containers, did not change markedly after initial infection had occurred at the time of the addition of E. atroseptica inocula.

Having shown that soft rot bacteria including Erwinia spp., are not present in large numbers in soil before planting seed tubers, and that these bacteria are present in seed tubers, it seems highly probable that the disease overwinters as a seed tuber borne latent infection. This agrees with the conclusions of Webb (1971) and Perombelon (1973). The data obtained is confusing and in 1972, the use of labelled mutants of E. atroseptica, was investigated in the hope of being able to detect changes in population numbers during the carry over of the infection from seed to new tuber, and to determine the position of the infection within new tubers. Unfortunately, all mutants obtained to streptomycin lost virulence to potatoes and pectolytic activity on Cuppels and Kelman's medium.

Another point arising from this trial, is the role of the seed tuber in non-Erwinia infections of new tubers. VTSC tubers grown in Erwinia-free soil, produced new tubers which rotted in polythene bags with water. Many tubers were rotted by bacteria other than Erwinia spp. Although in these

containers, some seed tubers rotted, the presence or absence of a rotted seed tuber did not affect the numbers of infected new tubers. Consequently, it seems that these bacteria were initially present in soil, and their increases in population after tubers were planted must be related to the plant rhizosphere within the polythene sacks.

The low incidence of heel end infections has been mentioned. Rots on tubers in the polythene bag test, and found naturally were randomly distributed over tuber surfaces. All rots found appeared to originate at the surface of tubers and initial observations indicated some evidence for the claims developed by Ruehele (1940), Smith and Ramsey (1947) and Davidson (1948), that the pathogen could enter tubers via lenticels.

Isolations from lenticels and other areas of the tuber surface yielded Erwinia spp. Lenticels often contained larger populations of Erwinia spp. than did similar quantities of periderm tissue. The tetrazolium test has shown clearly that both lenticels and the periderm of freshly harvested tubers stain and therefore contain bacteria in relatively large numbers. During storage, contamination on the periderm decreased. Infection was limited mainly to lenticels, although growth cracks and wounds often rotted in polythene bags with water.

VTSC tubers treated with tetrazolium salt did not stain, but VTSC tubers soaked in a suspension of E. atroseptica (10^6 cells/ml), air dried and stored for 48 h did stain when treated with the salt and incubated in polythene bags with water. Staining was confined to lenticels. This evidence

suggests that infection of tubers by Erwinia atroseptica is via lenticels.

Incubation of tubers with 0.02 ml droplets of Erwinia atroseptica suspension at different concentrations, showed that the threshold value for successful infection was 10^3 cells/ml for two cultures of E. atroseptica (i.e. 2 cells theoretically).

Smith and Ramsey (1947) and Davidson (1948) claimed that entry into lenticels was facilitated by increased tissue turgidity around the lenticel. Studies involving dipping water soaked and non-water soaked tubers into a suspension of E. atroseptica showed no differences between the treatments. However, the use of the polythene bag test to determine the numbers of infected tubers, may not have distinguished between infection of lenticels and contamination of the periderm, since contaminant bacteria may have gained access to tuber tissue via lenticels. Storage of tubers treated as above for 14 days at 8°C before incubating them in polythene bags with water, indicated that water soaked tubers were more easily infected by E. atroseptica, although not markedly so.

Results from tetrazolium salt studies have shown that tubers taken from store shortly after harvest normally contain between 1 and 11 stained lenticels, the mean being 7. These figures may decrease to 0 during storage.

The presence of dry limited lesions in infected tubers could be induced by removing tubers from polythene bags with water after 3 days and storing in dry, aerobic conditions. Rots already present after incubation in

polythene bags with water did not increase in size, but dried out slowly, becoming brown. Such rots were obvious by the wrinkled appearance of the periderm above them. Dry limited lesions could be induced to rot rapidly by replacing them in polythene bags with water. This observation agrees with those of Leach (1930), Rudd-Jones and Dowson (1950) and Logan (1968), in that formation of dry limited lesions is not a mechanism by which tubers become free of infection as in other disease responses in plants. The development of an arrested lesion is probably only a delaying mechanism by tubers stored in unfavourable conditions for rotting. The pathogen however remains viable.

It has been stated that the polythene bag test is a very drastic test, since tubers are incubated in low oxygen concentrations at relatively high temperature, surrounded by a constant entire film of moisture. Burton and Wigginton (1971) found that the presence of a persistant film of water of average thickness 3×10^{-2} mm, around tubers about 6 h at 10°C, or 2 h at 21°C, caused anaerobic conditions within the tubers. This may be of importance when considering the effect of a film of water on bacterial soft rotting, or breaking of tuber dormancy. These authors also found that tubers partly immersed in water did not become anaerobic although the oxygen tension, determined at the centre of the immersed part was reduced to an extent related to the area of periderm covered by water. It must be remembered that E. atroseptica is a facultative anaerobe and that these conditions may favour its further growth.

A bucket test developed at Rothamsted Experimental Station, was tested as a less drastic method of rotting than

the polythene bag test. Unfortunately, only tubers under similar conditions (in contact with a water film) to those present in the polythene bag test rotted. No account of incubating infected tubers individually is made. Consequently contamination from tuber to tuber may occur during tuber storage in the buckets.

Results fromthe tuber surface treatment tests in 1971 and 1972 indicate that the incidence of infection decreases throughout storage. Washing tubers appears to increase the incidence of infection, whereas, surface treatment by 'Chloros' decreases it. It is doubtful whether such sterilisation can be used to eradicate the pathogen, evidence which agreed with that of Perombelon (1973). It appears that infections in immature tubers are less easily affected by 'Chloros' than are mature tubers. The results obtained from the tests showed this more clearly in 1971 than 1972. It was noted that the incidence of infection was much lower in 1972, when tubers were infected much earlier in the field than in 1971. Results from the trial involving growth of tubers in polythene containers, showed that the incidence of tuber infection did not increase markedly after the first sampling date. Trials in the field suggest that the incidence of infection may even decrease before harvest. There is also some evidence from a more detailed study of infected tubers and their lenticels that harvesting of tubers is accompanied by an increase in the number of infected tubers but not the mean number of infected lenticels per tuber. These results also agreed with the surface treatment tests in that the numbers of infected lenticels, and the number of infected tubers decreased during storage. By March in 1971 and 1972,

the percentage of infected tubers decreased to about 10-15%, and the mean number of infected lenticels per tuber decreased to about one per tubers. The greatest decrease occurs between December and February. This coincides with break of dormancy of the tubers, and is not fully understood. Decreases in temperature have been shown to reduce the rate of decline of infected lenticels. It seems likely that the rapid decrease in infection at temperatures greater than 3°C must be due either to desiccation of tubers or changes in the tubers' metabolism, associated with break of dormancy. Tubers stored at 3°C for 8 months after harvest retained dormancy and infection incidence was not markedly reduced during this time.

It is evident that under the climatic conditions of this country that the disease exists mainly as a latent infection within lenticels of tubers. Good aeration, dry conditions, and low temperatures minimise rotting in storage. The conditions under which rotting occurs in the polythene bag test are opposites to these in every respect, and it is obvious that storing infected tubers in restricted aeration, moist conditions, and at high temperatures (19-21°C) allows the bacteria to rot potato tubers.

Water content of tuber tissue has been shown to be an important factor in rotting. Turgid tissue is more susceptible than normally sub-turgid tissue, and this phenomenon has been found to be of importance when considering the virulence of various cultures of Erwinia spp. Cultures that will rot tuber tissue slices, but not whole tubers under normal storage conditions, often rotted tubers incubated in conditions of high relative humidity. Lenticellular infections

of E. atroseptica A13 rotted only when tubers were made turgid.

Burton and Wiggington (1970) demonstrated that a persistent film of water 3 x 10^{-2} mm caused anaerobic conditions within these wet tubers. An attempt to distinguish the turgidity effect from an anaerobic effect, by covering infected tubers with films of wax and vaseline, showed that anaerobic conditions alone did not cause rotting to the same extent as did turgid conditions. It did show however, that there is a marked difference between infections in lenticels and inoculated cork borer wounds, with respect to rotting. Sealing tubers with Erwinia atroseptica inoculations made into cork borer wounds increased rotting compared to non-sealed controls. Lund and Nicholls (1969) have stressed the importance of anaerobic and restricted aeration conditions in rotting from inoculated wounds. The results discussed above and of experiments involving storage of infected tubers in different gaseous atmospheres indicate that such conditions are insufficient to cause rotting from latent infections. This phenomenon suggests that the pathogen is not present in tissue in a latent infection, in the same way that it is when introduced into cork borer wounds. However, once rotting has commenced, anaerobic and restricted aerations conditions increase the rate of rotting.

Perombelon (personal communication, 1973) has stated that the presence of a water film on the tuber surface is usually necessary for rotting. Results in this research programme agree with this statement. A minimum of 4 ml of water in the polythene bag test appears necessary for rotting

from infected tubers, approximately 100 g in weight. Below this volume, no rotting was found. Tubers in bags containing less than 4 ml of water were not completely covered by a moisture film. However, this test was not continued after 16 days, but preliminary tests involving tuber incubation in glass containers of varying gaseous environment, showed that all rotting that did occur after 5 to 6 weeks, was associated with condensation on the vessel surface.

Other tests on the effect of moisture films on rotting included the presence of a constantly changing water film, and tubers immersed in water. All proved the effectiveness of water films in predisposing tubers to rotting.

The results from cork borer wound inoculated tubers showed that the presence of a film of water increased the rapidity of rotting compared with dry controls.

The final experiment in this series involved lenticel inoculated VTSC tubers stored in CO_2, with and without the presence of a moisture film. Many more tubers rotted in the presence of water, and it was again noted that virtually all 'dry' stored tubers rotted only when in contact with condensation on the surface of the container.

The conclusions from this section are that both anaerobic or restricted aeration conditions, and the presence of a moisture film on the tuber surface predispose tubers to rotting from inoculated wounds, but only the latter factor predisposes tubers to rot from lenticel infections. It is suggested that the bacteria reside in the moribund tissue of the lenticel and do not normally come into contact with

living parenchyma tissue. The presence of a water film on the tuber surface allows this to happen since the water status of even apparently fully turgid tubers taken from the soil at field capacity is always at a deficit (Perombelon, 1973). As water is taken in through lenticels, the bacteria are taken in with it. Once bacteria are present within parenchyma tissue, rotting rapidly ensues, and is aided by environmental conditions which favour rapid bacterial growth and may restrict the defence mechanisms of tubers.

Preliminary studies into suberisation in and around lenticels have been made and although the results obtained are somewhat confused, there is reasonable circumstantial evidence that latent infection is limited to lenticels by the nature of suberised cells around the lenticel. The fact that the meristem of the lenticel is composed of closely packed cells with very small intercellular spaces may also help to explain why the infection is normally confined to lenticels.

Studies into the biochemistry and physiology of the pathogen, have shown it to be a typical member of the family Enterobacteriaceae.

The bacterium shows a normal growth curve in in vitro studies. Frequent sub-culturing on synthetic media causes a reduction in virulence to potatoes. The use of deep frozen master cultures (-18°C) has avoided this loss of virulence. The optimum temperature for growth is 25°C, and although rapid growth is limited below 15°c, the bacterium will grow at 5°C. Rapid growth is limited to a range of pH between 5-9.

The macerating factor has been found to be an endo-pectate trans-eliminase enzyme (Hall, 1971). Polygalacturonase enzymes are produced in vitro, but not in vivo. This agrees with results obtained by Hall (1971). Pectate trans-eliminase enzyme obtained from growth of E. atroseptica A13 on sodium polypectate had optimum activity at 25°-30°C and at pH 9.0 to 10.2. Above 30°C, denaturation occurred, whereas below 15°C and pH 7.8 activity was greatly reduced. The pH of potato tissue macerates is 5.5-6.5, and it was surprising to find such low activity at these values.

Activity of polypectate trans-eliminase had an optimum at pH 9.0 with pectin and sodium polypectate substrates, although activity was greater on sodium polypectate. Pectate trans-eliminase had greater activity than pectin trans-eliminase prepared similarly, on maceration of potato tissue discs. The pH optimum for both enzyme preparations was 9.6, on potato tissue. Maceration of tuber tissue with respect to pH optimum was investigated by a modification of the technique devised by Mussell and Morre (1969), which was initially adapted for potato tissue maceration by pectate trans-eliminase enzymes by Cronshaw and Wood (1973).

The effect of pH on growth and enzyme production by E. atroseptica A13 was studied. Optimum pectate trans-eliminase production was at pH 7.5, as was optimum growth, although another peak at pH 5.5 was found, possibly due to the production of a polygalacturonase. Optimum pectin trans-eliminase production was found at pH 6.5 and 8.0 as was optimum growth. Cultures of filtrate for growth on sodium polypectate buffered between pH 7 and 8.5, after incubation

for 26 h, had good activity on sodium polypectate.

The method of pectate trans-eliminase production has been studied by many workers, and it is generally agreed that most pectic enzymes, including trans-eliminase may be induced. Tribe (1955) and Murant and Wood (1957) have shown that E. aroideae will secrete pectic enzymes into culture solutions which do not contain pectic substances. Such production is termed constitutive synthesis of trans-eliminase. Cooper (1973) has demonstrated that pectate trans-eliminase production by two tomato pathogens, Verticillium albo-atrum, and Fusarium oxysporum f.sp. lycopersici, is induced, the true inducer being mono-galacturonic acid.

In this study, trans-eliminase has been shown to be induced by pectin and sodium polypectate, compounds related to the components of the middle lamellae of potato tuber cells. However, the amount that appears to be induced, is dependent upon the culture of Erwinia spp. used. E. atroseptica A13 produced much more pectate trans-eliminase on nutrient broth supplemented with sodium polypectate, than on nutrient broth alone. E. carotovora NCPPB 1280 produced large amounts of trans-eliminase in nutrient broth alone. However, E. carotovora NCPPB 1280 was not virulent on potato tubers. Thus the ability to produce large amounts of constitutive trans-eliminase is not directly associated with virulence.

At the time of performing these experiments, it was realised that it was difficult to distinguish between constitutive and induced syntheses of trans-eliminase enzymes.

No account in such experiments is made for the fact that the products of trans-eliminase activity may catabolite repress further synthesis of the enzyme. Catabolite repression has been demonstrated in E. carotovora by Tejerina and Serra (1969). Glucose at concentrations greater than 2.5 x 10^{-3} M will catabolite repress trans-eliminase synthesis until glucose concentrations in the culture medium are reduced to this value. This effect has also been demonstrated for V. albo-atrum and F. oxysporum f.sp. lycopersici, by Cooper (1973). The use of diffusion capsules, whereby the inducer, or catabolite repressor can be delivered into culture media containing starved cells, enabled this worker to determine the effects of the inducers and repressors in the absence of other metabolic by-products.

Until such studies are made for E. atroseptica, induction and constitutive production of trans-eliminase synthesis, cannot be wholly distinguished.

Studies have shown that the major sugars (glucose, sucrose, and fructose) of potato tubers catabolite repress trans-eliminase synthesis. Lactose and inositol do not, and although lactose does not occur in large quantities in tubers, it is obvious that its utilisation by E. atroseptica A13 does not occur by the biochemical pathways by which other sugars eg. glucose, are. Inositol is an allicyclic compound, and no reference to its utilisation by bacteria has been found.

Two anomalies with respect to trans-eliminase production have been found. Growth of E. atroseptica on sodium polypectate buffered at pH 7.0 approximately, was accompanied

by an increase in pH. It has been stated previously, that trans-eliminase activity below pH 8 is restricted. The pH of potato tissue appears to be 5.5 to 6.5. It is suggested that utilisation of pectic compounds of the middle lamellae may increase the pH of the surrounding medium, thus allowing increased trans-eliminase activity. The second anomaly is that trans-eliminase production per bacterium decreases during the log phase of bacterial multiplication. Thus, it can be misleading to compare enzyme production in two cultures, growing simultaneously but not at the same rate. The use of diffusion capsules, whereby large relatively static samples of bacteria from the same population can be studied simultaneously could remove the confusion obtained by the methods used in this study.

It is not possible to suggest the true pattern of events in the initiation of a rot, by pectic enzymes but it does seem that the enzyme responsible is a trans-eliminase not unlike those produced on pectic substances in vitro. The presence of Ca^{2+} ions is essential for its activity (Starr and Moran, 1962; and Hall, 1970). These are probably present in the pectate fraction of the middle lamellae. Evidence from Hall (1970), Stevens (personal communication, 1972), and Mount, Bateman and Basham (1970) indicates that the trans-eliminase enzyme kills cells during or before maceration, causing leakage of the contents of cells into the intercellular spaces.

It is evident from the work on the effect of major sugars of potato tubers during break of dormancy, that the concentration of sugars in the cells is large enough to

cause catabolite repression of trans-eliminase synthesis. 0.1% glucose in the presence of sodium polypectate causes in vitro catabolite repression, whereas, the concentration of soluble sugars (glucose, fructose, and sucrose) within potato tissue was 0.3 to 0.7%. This poses the question as to how rotting can take place, and must be explained by the fact that the sugars are compartmentalised within cells. The system of rotting is possibly analagous to growth of bacteria in flasks containing diffusion capsules. The rate of release of nutrient from the capsules would determine the rate of growth ofthe bacteria. In the same way, the rapidity with which rotting takes place must be dependent upon the rate of release of soluble sugars from killed potato tuber cells. This in turn is related to the rate at which trans-eliminase synthesis takes place, and although catabolite repression may function as the sugars are released, these sugars will be the limiting factor in the rate of rotting, and consequently, will be utilised rapidly by bacterial cells during their logarithmic growth phase. Rapidly changing sequential induction and catabolite repression may occur at the advancing edge of the rot.

Initially it was thought that sugar changes during break of tuber dormancy may account for the changes in resistance of tubers during this period. Denny (1926) states that as the dormancy of tubers is broken by treatment with ethylene chlorhydrin, sucrose concentrations and often, reducing sugar concentrations increase. Increases in sugar concentrations could support increased and consequently, more rapid growth of E. atroseptica, and since catabolite repression does not appear to be related to any loss in virulence

due to inhibition of trans-eliminase synthesis, this may be the case. The results obtained from experiments which involved rotting of tubers during break of dormancy, were varied. However, Denny (1926) states that sugar changes found during break of dormancy also vary, even with tubers from the same population. Also, results from the experiments which attempted to relate sugar changes to rotting during this period, often showed a correlation between decreases in sugar concentration and decreases in rotting. In conclusion, it is suggested that rotting may depend upon the availability of sugars within tuber tissue.

The above conditions affecting rotting do not take into consideration tuber defence mechanisms against rotting. No correlation between natural break of dormancy and rotting was found. It may be that the loss of tuber resistance obtained during chemically induced break of dormancy, is a consequence of the rapid physiological and biochemical changes occurring within tubers during this short period. Burton and Wiggington (1969) claim that water films causing anaerobic conditions within tubers, may affect the state of dormancy, and it is quite possible that these conditions not only favour the pathogen's ability to multiply as a facultative anaerobe, but also to cause rapid physiological changes in tubers, resulting in loss of resistance to rotting.

It is known that physiological changes in tubers affect their rates of respiration. Such changes are caused by wounding, increased turgidity, anaerobiosis, dormancy break, and it has been shown that these treatments increase tissue susceptibility to rotting. It appears likely that

the natural resistance of rubers to rotting is affected by any sudden change which affects the rate of tuber respiration.

Oxidases have been implicated in resistance to rotting, and Lovrekovich et al. (1967) have shown that oxidised phenols, formed by phenolase, produced a zone of blackened tissue which functioned as an infection barrier. As the bacteria spread in the tuber tissue, however, the formation of the zone of dark tissue was progressively suppressed, possibly due to the presence of NADPH systems on the surface of E. carotovora.

Although no significant effects were obtained by adding phenolic compounds and associated enzymes to wounds in potato tissue before adding E. atroseptica A13 inocula, the results may be misleading due to the undetermined nature of the natural process of the tuber's reaction to physical wounding.

A correlation between browning of rotting tissue and rapidity of rotting has been found. White rapidly spreading rots were invariably obtained from inoculated tubers incubated in conditions of restricted aeration, whereas in strongly aerobic conditions, brown limited rots were found. Whether this is cause or effect of the resistance found under aerobic conditions is not known, but the browning reaction of tubers is caused by phenolase activity, oxidising polyphenols to their corresponding quinones. Benzoquinone obtained from catechol oxidation was not toxic to E. atroseptica A13, and no antibacterial compounds were isolated from either limited or rapidly spreading rots, although a

UV light fluorescent compound may have restricted growth of E. atroseptica A13, although not markedly.

In conclusion, it appears that any change in the rate of respiration of tubers is accompanied by increased rotting, if rotting has already started. Incubation in aerobic conditions limits the rate of rotting although the exact reasons are not understood. However it must be noted that aerobic conditions do not stop rotting, and therefore the resistance of tubers is not absolute. The main criterion in resistance to rotting is that the infection must be maintained in its latent state. Whether the pathogen is present within living tuber tissue is not known, but the factors already discussed do not appear to account for the tubers' resistance to rotting if the latent infection is within living tuber tissue.

Resistance in terms of physical barriers to rotting were studied, and it is evident that tissue adjacent to the periderm is resistant to maceration probably due to the nature of suberisation in these areas. Staining demonstrated the presence of suberin in the periderm but not in lenticels possibly due to the lack of intensity of staining in the compact cells above the meristem or the dead cells above these.

Areas resistant to maceration around the vascular system of tubers have been found, and since rotting from cork borer wounds 2.5 mm deep was often restricted, even when tubers were incubated in conditions of restricted aeration, it seems that latent infections in lenticels may be limited by the nature of the surrounding tissues.

Eradication of the Pathogen

It is evident from results of the trial involving VTSC tubers grown in Erwinia-free soil in polythene sacks, that the problem of soft rotting does not involve only Erwinia spp. The extent to which bacteria from other genera can rot tubers during storage appears to depend on the environmental conditions of storage. Lund and Nicholls (1969) have demonstrated the presence of Clostridium spp. which are able to rot and assist in rotting potato tubers. Isolates possibly representing the genera, Clostridium, Bacillus, and Pseudomonas, which were pectolytic on Cuppels and Kelman's medium and which rotted potato tissue slices, have been obtained from potatoes grown at Silwood Park.

The breeding programme in Scotland has aimed at obtaining disease-free seed tubers by the stem cutting procedure. This method relies on the fact that certain pathogens are carried over from year to year only in seed tubers. Coupled with adequate crop rotation this technique enables a drastic reduction in the incidence of several diseases of potatoes. However, the fact that E. carotovora is present in other crops and can easily be transmitted to a field of VTSC tubers via farm machinery, has created problems with blackleg (Graham, personal communication, 1973). Although E. carotovora cultures are often non-virulent on potato tubers, virulent cultures do exist and eradication of these by the VTSC programme would be extremely difficult. Paton (personal communication, 1973) has expressed the possibility that L-forms of E. atroseptica may be present within tubers, and if so could escape detection in supposedly Erwinia-free stem cuttings.

A more obvious approach to eradication of the pathogen is by the use of antibacterial compounds. These could eradicate not only Erwinia spp. but also other soft rot bacteria, if used on ware tubers as well as seed stocks. At the time of writing this thesis no chemical products for eradication of E. atroseptica are marketed.

Surface sterilisation of tubers by chlorine and antibiotic application, did not eradicate the pathogen. The problem lies in the fact that the bacteria within lenticels appear to be inaccessible to these chemicals. Graham and Volcani (1961) found such treatments increased rotting, possibly due to liquid films in which the chemicals were applied.

The use of dimethyl sulphoxide, a chemical which penetrates plant tissues rapidly, in conjunction with streptomycin did not increase the ability of the antibiotic to eradicate the pathogen.

The use of Tricidal RE-10 as a dip, reduced the number of tubers rotting in the polythene bag test, but several rotting tubers were found after storage of the treated tubers for 1 month. Initially this was thought to be due to moisture films on the tuber surfaces caused by condensation in the sealed buckets. However, this was not established, and it was suggested (Fox, personal communication, 1973) that this may have been a phytotoxic effect of the chemical. Later studies showed that application of Tricidal RE-10 on potato tissue slices caused increased rotting in inoculated slices and rotting in non-inoculated slices.

A comparison of four chemicals shown to be toxic in low concentrations to E. atroseptica, was made with respect to eradication of the pathogen from tubers. All chemicals (Tricidal RE-10, Bunema, DS 9073, HPTMS), were shown to retard rotting during incubation in polythene bags with water, immediately after dipping tubers in the chemicals. The active principle of Tricidal RE-10 was a diphenyl methane. Those of the other chemicals were not known. However, none of the chemicals eradicated the pathogen. The greatest reductions in rotting compared to water-treated controls, were found in Tricidal RE-10, and DS 9073 treated tubers.

A similar experiment involved sampling tubers at intervals after treatment, when tubers were placed in polythene bags with water. The tubers used in this assay were Ulster Premier, since at that time (April-May) no Majestic tubers were available. Bunema and DS 9073 markedly reduced the number of rots per tuber immediately after treatment. However, the water treated controls showed a 90% reduction in the number of rots per tuber after storage for 16 d at room temperature (19-21°C). Thus the effects of the chemicals, although reducing the number of rots per tuber more rapidly than the conditions of storage alone, were not fully understood.

A repeat of this experiment in October 1973, using freshly harvested tubers grown in the field at Silwood Park, is as yet incomplete. The rapid decrease in the number of rots in Ulster Premier tubers was thought to be due to the lack of infection by the E. atroseptica treatment applied. Tubers were soaked in water for 24 h before soaking them in

a suspension of E. atroseptica A13 (10^6 cells/ml) for 12 h. These tubers were not VTSC tubers and consequently were infected with soft rot bacteria at the time of purchase, shown by tetrazolium salt treatment.

Results obtained from the repeat experiment in October 1973, indicate that storage at room temperature reduces infection in water treated tubers, and it appears that the effect of the chemicals will again be not fully realized. If storage at relatively high temperature (c. 19°C) does markedly reduce the incidence of infection, the use of chemicals to eradicate the pathogen may be of little further use. Ideally the experiment should be carried out at normal storage temperatures (6-10°C) to investigate the effect the chemicals have on eradication of soft rot bacteria from tubers.

REFERENCES

Adelberg, E.A., Mandel, M. and Chen, G.C.C. 1965. Optimal conditions for mutagenesis by N-Methyl-N'-Nitro-N-Nitrosoguanidine in E. coli K12. Biochemical and Research communications, 18, 788-795.

Albersheim, P., Jones, P.T. and English, P. 1969. Biochemistry of the cell wall in relation to infective processes. Ann. Rev. Phytopath., 7, 171-194.

Albersheim, P. and Killias, U. 1963. Histochemical localisation at the electron microscope level. Amer. J. Bot., 50, 732-745.

Audia, W.V., Smith, W.L. and Craft, C.C. 1962. Effects of isopropyl n-(3-chlorophenyl) carbamate on suberin, periderm and decay development by Katahdin potato slices. Bot. Gaz., 123, 255-258.

Bateman, D.F. and Millar, R.L. 1966. Pectic enzymes in tissue degradation. Ann. Rev. Phytopath., 4, 119-146.

Bergey, D.H., et al. 1923-48. Manual of Determinative Bacteriology. Williams and Williams Co., Baltimore.

Bétencourt, A. and Prunier, J.P. 1965. A propos de la pourriture séche lenticellaire des tubercules de pommes de terre provoquee por Erwinia carotovora (Jones) Holland, Eur. Potato J., 8, 230-235.

Binianskaite, I. 1963. Spread of 'blackleg' of potato and some measures for its control in the conditions of the Lithuanian S.S.R. Rev. Appl. Mycol., 42, 568-569.

Bonde, R. 1939. Comparative studies of the bacteria associated with potato blackleg and seed piece decay. Phytopathology, 29, 847-848.

Bonde, R. 1950. Factors affecting seed piece decay and potato blackleg. Maine Agricultural Research Station, Bulletin 482.

Boom, T. van den. 1967. Untersuchungen über die Vorassetzung für das Auftreten der Schwarzkeinigbeit der Kartoffel. Phytopath. Z., 58, 239-276.

Burkholder, W.H. and Smith, W.L. 1949. Erwinia atroseptica (Van Hall) Jennison and E. carotovora (Jones) Holland. Phytopathology, 39, 887-897.

Burton, W.G. and Wiggington, M.J. 1970. Effect of a film of water upon the oxygen status of a potato tuber. Potato Research, 13, 180-186.

Cooper, R.M. and Wood, R.K.S. 1973. Induction of synthesis of extracellular cell-wall degrading enzymes in vascular wilt Fungi. Nature, London, 246, 309-311.

Cronshaw, D.K. and Wood, R.K.S. 1973. An analysis of the Mussel and Morre Quantitative Bioassay for polygalacturonases using pectate trans-eliminase from E. atroseptica. Ann. Bot., London, 37, 463-471.

Davidson, R.A. 1948. Factors affecting the development of bacterial soft rot of potato tuber initials. Phytopathology, 38, 673-687.

Dean, M. and Wood, R.K.S. 1967. Cell wall degradation by pectate trans-eliminase. Nature, London, 214, 408-410.

Denny, F.E. 1926. Hastening of sprouting of dormant potato tubers. Contr. Boyce Thompson Institute, 1, 59.

Denny, F.E. 1929. Chemical changes induced in potato tubers by treatments that break the rest period. Contr. Boyce Thompson Institute, 2, 131-142.

Denny, F.E. 1941. The use of the methylester of α Naphthalenacetic acid for inhibiting sprouting of potato tubers, and an estimate of the amount of chemical retained by tubers. Contr. Boyce Thompson Institute, 12, 387-403.

Denny, F.E. and Thornton, N.C. 1941-2. Sugar forming characteristics of tubers in cold storage and suitability for production of potato chips. Contr. Boyce Thompson Institute, 12, 405-429.

Dowson, W.J. 1941. Identification of bacteria commonly causing soft rot in plants. Ann. appl. Biol., 28, 102-106.

Dowson, W.J. 1957. Plant Disease Due to Bacteria. Cambridge University Press, p.172.

Echandi, E. and Walker, J.C. 1957. Pectolytic enzymes produced by Sclerotinia sclerotiorum. Phytopathology, 47, 303-306.

Emilsson, B. 1949. Studies on the rest period and dormant period in the potato tuber. Acta agric. suec., 3, 189-284.

Fehrmann, H. and Dimond, A.E. 1967. Peroxidase activity and Phytophthora resistance in different organs of the potato plant. Phytopathology, 57, 69-72.

Folin and Denis.

Friedmann, B.A. 1962. The physiology differences between a virulent and weakly virulent radiation induced strain of Erwinia carotovora. Phytopathology, 52, 328-332.

Fushtey, S.G. 1957. Studies in the physiology of parasitism. XXIV. Further experiments on the killing of plant cells by fungal and bacterial extracts. Ann. Bot., Lond., 21, 273-286.

Garibaldi, A. and Bateman, D.F. 1971. Pectic enzymes produced by Erwinia chrysanthemi and their effects on plant tissue. Physiol. Pl. Path., 1, 25-40.

Graham, D.C. 1958. Occurrence of soft rot bacteria in Scottish soils. Nature, Lond., 181, 61.

Graham, D.C. 1966. Taxonomy of the soft rot coliform bacteria. Ann. Rev. Phytopath., 2, 13-42.

Graham, D.C. and Volcani, Z. 1961. Experiments on the control of blackleg disease of potato by disinfection of seed tubers with mercury compounds and streptomycin. Eur. Potato J., 4, 129-137.

Hall, J.A. 1971. The maceration of tissues and killing of cells by Erwinia carotovora and Corticium praticola. Ph.D. Thesis, University of London.

Hall, J.A. and Wood, R.K.S. 1970. Plant cells killed by soft rot parasites. Nature, Lond., 227, 1266-1267.

Harper, P.C., Boyd, A.E.W. and Graham, D.C. 1963. Growth cracking and bacterial soft rot in potato tubers. Pl. Path., 12, 139-142.

Held, A., Kedar, N. and Birk, Y. 1965. Dehydrogenase activity of potato tuber tissue infected with Phytophthora infestans. Phytopathology, 55, 970-976.

Hellmers, E. and Dowson, W.J. 1953. Further investigations of potato blackleg. Acta Agric. Scand., 3, 103-112.

Hewitt, B.R. 1958. Spectrophotometric determination of total carbohydrate. Nature, Lond., 182, 246.

Horsfall, J.G. and Dimond, A.E. 1957. Interactions of tissue sugar, growth substances, and disease susceptibility. Z. PflKrankh. PflPath. PflSchutz, 64, 415-421.

Hughes, J.C. and Swain, T. 1960. Scopolin production in potato tubers infected with Phytophthora infestans. Phytopathology, 50, 398-400.

Jaffe, M.J. 1965. Chlorophyll production, lignin decomposition, synthesis of phenolic compounds and inhibition of bacterial soft rot by photoactivation in the potato tuber. Diss. Abstr., 25, 4927-4928.

Jennison, H.M. 1923. Potato blackleg with special reference to the etiological agent. Ann. Mo. bot. Gdn., 10, 1-72.

Johnson, G. and Schaal, L.A. 1957. Accumulation of phenolic substances and ascorbic acid in potato tuber tissue upon injury and their possible role in disease resistance. Am. Potato J., 34, 200-209.

Jordana, R. de Vicente. 1958. Cytoarjesis in potato tubers. IV. Importance of maturity of tubers and presence of sprouts on the arrest of infection. Microbiol. esp. 11, 219-241.

Kado, C.I. and Heskett, M.G. 1970. Selective media for isolation of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas and Xanthomonas. Phytopathology, 60, 969-976.

Keen, N.T. and Horton, J.C. 1966. Induction and repression of polygalacturonase synthesis by Pyrenochaeta terrestris. Can. J. Microbiol., 12, 443-453.

Kelman, A. 1954. The relationship of pathogenicity in Pseudomonas solanacearum to colony appearance on a tetrazolium medium. Phytopathology, 44, 493-495.

Kerr, A. 1953. A method for isolating soft-rotting bacteria from soils. Nature, Lond., 172, 1155.

Lang, and Knosel, 1970. On the importance of pectolytic, cellulolytic and proeolytic enzymes for the virulence of phytopathogenic bacteria. Phytopath. Z., 69, 315-328.

Lapwood, D.H. 1957. Studies in the physiology of parasitism. XXIII. On the parasitic vigour of certain bacteria in relation to their capacity to secrete pectolytic enzymes. Ann. Bot. Lond., N.S. 21, 167-184.

Lazar, I. and Burcur, E.L. 1964. Recent research in Roumania on blackleg and bacterial soft rot of potatoes. Eur. Potato J., 7, 102-111.

Leach, J.G. 1930. Identity of the potato blackleg pathogens. Phytopathology, 20, 743-751.

Logan, C. 1964. Bacterial hard rot of potatoes. Eur. Potato J., 7, 45-56.

Logan, C. 1963. A selective medium for the isolation of soft rot coliforms from soil. Nature, Lond., 199, 623.

Logan, C. 1966. A simple method of distinguishing Erwinia carotovora var. atroseptica from Erwinia carotovora var carotovora and Erwinia aroideae. Nature, Lond., 212, 1584-1585.

Logan, C. 1968. Survival of the Potato Blackleg Pathogen Overwinter. 1st. International Congress of Plant Pathology, London, 14-26th July.

Long, C.I. Biochemist's Handbook. E. and F.N. Spon Ltd., 22, Henrietta St. London, WC2.

Loomis, W.F. and Magasanik, K. 1964. The relation of catabolite repression to the induction system for galactosidase in E. coli. J. Mol. Biol., 8, 417-426.

Lovrekovich, L., Lovrekovich, H. and Stahmann, M.A. 1967. Inhibition of phenol oxidation by Erwinia carotovora in potato tuber tissue and its significance in disease resistance. Phytopathology, 57, 737-742.

Lund, B.M. 1973. The effect of certain bacteria on ethylene production by plant tissue. Fungal Pathogenicity and the Plant's Response, Academic Press, 64-84.

Lund, B.M. and Mapson, L.W. 1970. Stimulation by Erwinia carotovora of the synthesis of ethylene in cauliflower tissue. Biochem. J., 119, 251-263.

Lund, B.M. and Nicholls, J.C. 1970. Factors influencing the soft-rotting of potato tubers by bacteria. Potato Res., 13, 210-214.

L'vova, N.M. 1965. Effect of temperatures near 0°C on the microflora of potatoes and their resistance to micro-organisms during storage. Sb. Trud. Leningr. Inst. Sov. Torgovli, 23, 47-56.

McKay, R. 1955. Potato Diseases. Irish Potato Marketing Co. Ltd., Dublin.

Marks, V. 1959. An improved glucose oxidase method for determining blood, C.S.S. and urine glucose levels. Clin. Chim. Acta., 4, 395.

Middleton, J.E. and Griffiths, W.J. 1957. Rapid colorimetric micro-method for estimating glucose in blood and C.S.S. using glucose oxidase. Brit. med. J., 2, 1525.

Mount, M.S., Bateman, D.F. and Basham, H.G. 1970. Induction of electrolyte loss, tissue maceration, and cellular death of potato tissue by an endopolygalacturonate trans-eliminase. Phytopathology, 60, 924-931.

Murant, A.F. and Wood, R.K.S. 1957. Factors affecting the pathogenicity of bacteria to potato tubers. II. Ann. appl. Biol., 45, 650-663.

Mussel, H.W. and Morré, D.J. 1969. A quantitative bioassay specific for polygalacturonases. Ann. Biochem., 28, 353-60.

Nielsen, L.W. 1964. Pathogenesis of 3 Erwinia spp. to potato tuber tissue in a carbon dioxide-nitrogen atmosphere. Phytopathology, 54, 902.

Perombelon, M.C.M. 1968. The Scottish Horticultural Research Institute, 15th Annual Report, p.34.

Perombelon, M.C.M. 1971. A semi-selective medium for estimating population densities of pectolytic Erwinia spp. in soil and plant material. Potato Res., 14, 158-160.

Perombelon, M.C.M. 1973. Sites of contamination and numbers of Erwinia carotovora present in stored seed potato stocks in Scotland. Ann. appl. Biol., 74, 59-65.

Ramsey, G.B. 1919. Viability of the potato blackleg organism. Phytopathology, 9, 285-288.

Robertson, N.F., Friend, J., Aveyard, M., Brown, J., Huffee, M. and Homans, A.C. 1968. The accumulation of phenolic acids in tissue culture pathogen combinations of Solanum tuberosum and Phytophthora intestans. J. gen. Microbiol., 54, 261-268.

Rosenbaum, J. and Ramsey, G.B. 1918. Influence of temperature and precipitation on blackleg. J. Agr. Res., 13, 507-513.

Rubin, B.A. and Aksenova, V.A. 1957. Participation of polyphenolase systems in defense reactions of potato against Phytophthora infestans. Biokhimiya, 22, 191-197.

Rudd Jones, D. 1950. On the nomenclature and identity of the coliform soft rot bacteria. Trans. Brit. mycol. Soc., 33, 73-81.

Rudd Jones, D. and Dowson, W.J. 1950. On the bacteria responsible for soft rot in stored potatoes and the reaction of the tuber to invasion by Bacterium carotovorum. Ann. appl. Biol., 37, 563-569.

Ruehele, D.G. 1940. Bacterial soft rot of potatoes in Southern Florida. Tech. Bull. U.S. Dep. Agric., 348, 1-36.

Scholey, J. 1968. Pathological problems associated with pre-packing potato tubers. Unpublished.

Schwimmer, S., Bevenue, A., Weston, W.J. and Potter, A.L. 1954. Survey of the major and minor sugar and starch components of the white potato. J. Agric. and Food Chem., 2, 1284-1290.

Seiichi, Nasuno and Starr, M.P. 1966. Polygalacturonase of Erwinia carotovora. J. biol. Chem., 241, 5298-5306.

Smith, W.L. 1950. Pathogenic differences manifested by Erwinia carotovora and Erwinia atroseptica. Phytopathology, 40, 1011-1017.

Smith, M.A. and Ramsey, G.B. 1947. Bacterial lenticel infection of early potatoes. Phytopathology, 37, 225-242.

Smith, W.L. and Smart, H. 1955. Relation of soft rot development to protective barriers in Irish potato slices. Phytopathology, 45, 649-654.

Starr, M.P. and Moran, F. 1962. Eliminative split of pectic substances by phytopathogenic soft rot bacteria. Science, 135, 920-921.

Starr, M.P. and Nasuno, S. 1967. Pectolytic activity of phytopathogenic Xanthomonads. J. gen. Microbiol., 46, 425-433.

Stewart, D.J. 1962. A selective-diagnostic medium for the isolation of pectolytic organisms in the Enterobacteriaceae. Nature, Lond., 195, 1023.

Sweeley, R.B., Makita, M. and Wells, W.W. 1963. Gas liquid chromatography of trimethylsilyl derivatives of sugars and related compounds. J. Am. Chem. Soc., 85, 2497.

Tejerina, S. and Serra, T. 1969. Response of Erwinia carotovora to different carbon sources. Microbiol. esp., 22, 251-261.

Tribe, H.T. 1955. Studies in the physiology of parasitism. XIX. On the killing of plant cells by enzymes from Botrytis cinerea and Bacterium aroideae. Ann. Bot., Lond., 19, 351-368.

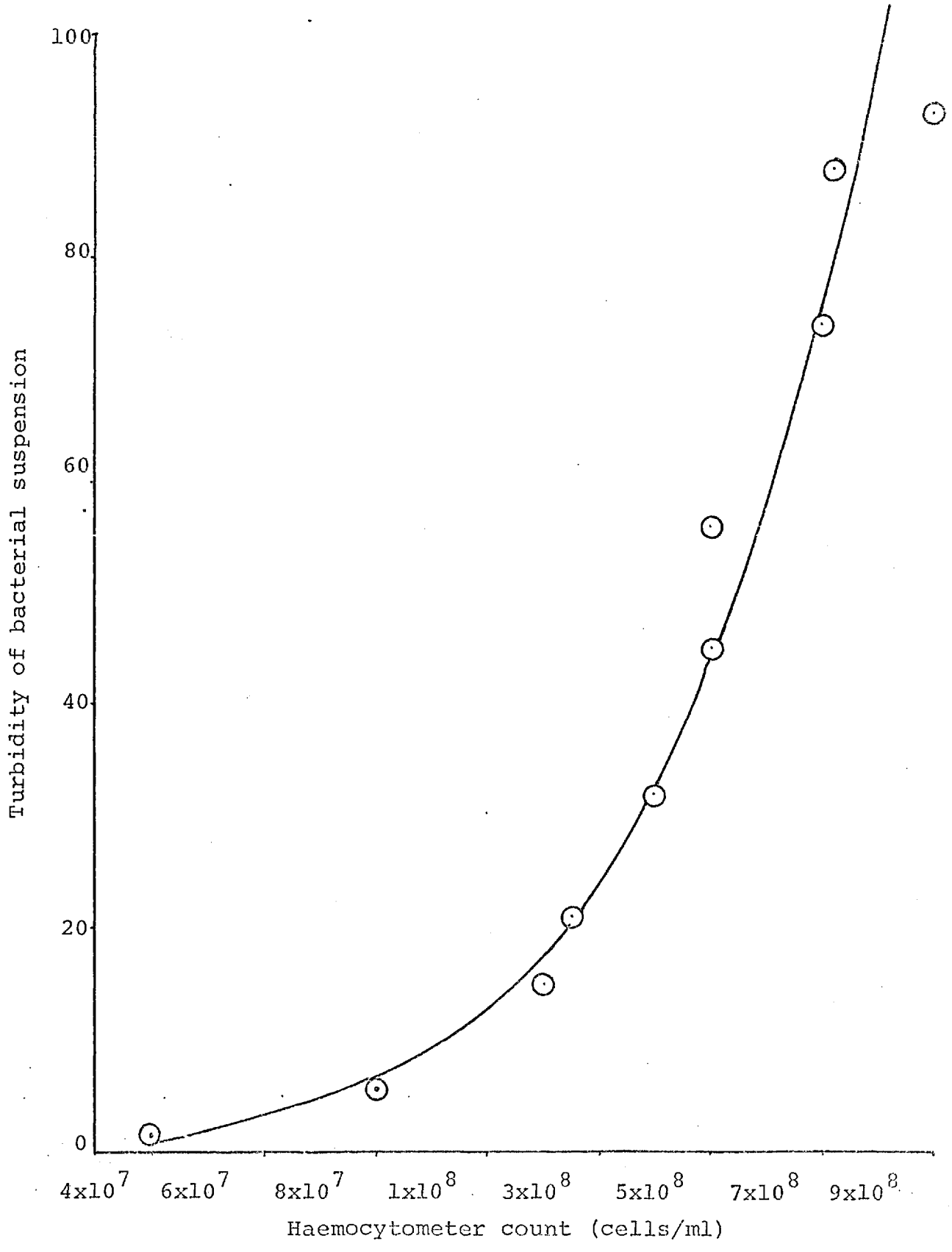
Webb, L.E. 1971. Infection of potato plants and tubers by Erwinia atroseptica. Ph.D. Thesis, University of London.

Wood, R.K.S. 1955. Pectic enzymes secreted by pathogens and their role in plant infection. Symposium Soc. Gen. Microbiol., 5, 263-293.

Wood, R.K.S. 1967. Physiological Plant Pathology. Blackwell, Oxford and Edinburgh.

A P P E N D I C E S

Appendix 1. Determination of Bacterial Numbers by Nephelometry.



Appendix 2. Preparation of Streptomycin-resistant Mutants of *E. atroseptica*

1. Mutagenesis by N-methyl-N'-nitro-n-nitrosoguanidine (NTG)

The method used is a modification of that of Adelberg *et al.* (1965). A refrigerated (4°C) log phase culture of *E. atroseptica* A13 was diluted with nutrient broth solution (10^8 cells/ml), and incubated at 25°C with shaking for 2 h to allow bacteria to pass into the log phase again. NTG (100 and 200 ml/ml) was added tc flasks containing the bacteria. After incubation for 30 min, the cells were collected by centrifugation (17000 x g) for 5 min, washed in sterile distilled water, collected, and diluted in fresh nutrient broth solution for 1 h. 0.2 ml aliquots were then plated out on to nutrient agar and Cuppels and Kelman's medium each containing streptomycin (50 and 100 ppm). After incubation for 48 h at 25°C, the plates were investigated for the presence of mutants.

2. Mutagenesis by U.V. irradiation

Washed bacteria taken from a log phase culture of *E. atroseptica* A13, were plated on to nutrient agar containing 50 ppm streptomycin. The plates were placed under a Chromatolite Ultra Violet Lamp and removed at ten minute intervals. The Petri dish lids were replaced and the plates incubated at 25°C for 48 h, before investigating for the presence of mutants.

All streptomycin resistant colonies found in both tests were non-pectolytic and avirulent on whole potato tubers.

Inoculations were made (10^9 cells/ml) into cork borer (No. 4) wounds 10 mm deep as described in Materials and Methods. All colonies found produced cells with more rapid multiplication rates than the type strain.

Appendix 3. The Incidence of Infection of Tubers in the Field, 1971.

Sampling date	Plant replicate	Number of developing tubers rotting in polythene bags (number of rotting tubers containing *Erwinia* spp.)	
		Seed tuber rotted	Seed tuber not rotted
5/7/71	1	4/10 (4)	3/10 (2)
	2	3/13 (3)	2/6 (2)
	3	3/9 (3)	0/9 (0)
	4	4/11 (4)	0/10 (0)
	5	6/14 (6)	1/11 (1)
	6	3/8 (2)	1/10 (0)
	7	4/11 (4)	2/10 (2)
	8	3/10 (3)	1/9 (1)
9/7/71	1	10/10 (10)	0/8 (0)
	2	8/11 (7)	1/7 (1)
	3	8/11 (7)	6/12 (6)
	4	5/9 (5)	0/11 (0)
	5	4/9 (4)	4/9 (4)
	6	3/9 (1)	3/9 (3)
	7	7/10 (7)	3/12 (3)
	8		1/9 (1)
17/7/71	1	2/8 (2)	4/14 (4)
	2	9/9 (9)	9/11 (9)
	3	6/13 (6)	7/13 (6)
	4	7/11 (7)	4/11 (4)
	5	5/10 (4)	
	6	6/13 (6)	
	7	6/12 (6)	
	8	7/13 (7)	
2/8/71	1	15/19 (9)	4/15 (4)
	2	1/8 (1)	5/9 (2)[1]
	3	12/18 (12)	
	4	10/13 (10)	
	5	11/22 (10)	
	6	11/15 (11)	
	7	7/14 (7)	
	8	10/19 (10)	

[1]Seed tuber did not rot in the polythene bag test.

Appendix 4. The Incidence of Infection of Tubers in the Field, 1972

Plant replicate number	Number of tubers/ plant	Number of tubers rotting 4d	Number of tubers rotting 12d	Number of tubers containing pectolytic bacteria	Number of tubers containing pectolytic, oxidase negative bacteria
Sampling date 7/7/72			Seed tuber rotted		
1	11	11	11	11	9
2	8	5	7	7	4
3	15	9	12	12	8
4	10	7	9	9	6
5	5	4	5	5	5
6	5	5	5	5	5
7	6	3	5	5	5
8	10	10	10	10	10
			Seed tuber not rotted		
1	10	6	9	9	6
2	13	10	13	6	4
3	15	15	15	11	9
4	13	10	11	10	10
5	14	10	14	13	10
6	12	8	10	10	5
7	6	4	4	4	4
8	8	4	8	8	7
Sampling date 14/7/72			Seed tuber rotted		
1	5	3	4	4	4
2	7	4	5	5	3
3	6	3	4	4	3
4	8	3	5	5	4
5	7	4	5	4	3
6	7	4	5	4	4
7	5	5	5	5	5
8	6	3	5	5	5
			Seed tuber not rotted		
1	7	3	4	4	3
2	5	3	3	3	3
3	6	2	5	5	3
4	8	4	5	5	4
5	5	3	4	3	3
6	5	2	3	3	2
7	6	3	5	5	4
8	6	4	6	5	5

Appendix 4. Continued.

Plant replicate number	Number of tubers/ plant	Number of tubers rotting 4d	Number of tubers rotting 12d	Number of tubers containing pectolytic bacteria	Number of tubers containing pectolytic, oxidase negative bacteria
Sampling date 21/7/72			Seed tuber rotted		
1	6	3	4	3	2
2	7	2	3	3	3
3	7	3	7	7	2
4	7	2	5	4	3
5	5	0	3	1	0
6	5	2	2	1	1
7	8	3	4	4	2
8	9	8	9	6	6
9	7	1	3	2	2
			Seed tuber not rotted		
1	6	0	4	3	3
2	6	3	3	3	3
3	5	2	3	2	2
4	6	4	5	2	2
5	5	1	2	2	1
Sampling date 2/8/72			Seed tuber rotted		
1	5	2	4	4	3
2	6	4	4	4	3
3	4	2	4	4	4
4	6	6	6	6	6
5	6	3	3	2	2
6	7	4	6	6	5
7	7	4	7	7	5
8	6	4	5	5	5
			Seed tuber not rotted		
No such plants available					
Sampling date 21/8/72			Seed tuber rotted		
1	5	2	5	-[1]	-
2	6	1	6	-	-
3	8	1	7	-	-
4	8	3	7	-	-
5	7	4	5	-	-
6	7	1	5	-	-
7	4	2	4	-	-
8	5	1	3	-	-

[1] not tested.

Appendix 5. Soil Moisture Contents for Polythene Container Grown Plants

Date of sample	% soil moisture content[1]	
	Tuber treatment	
	Wet Soil	Dry Soil
29/7/72	14.3	7.7
20/8/72	16.2	8.2
14/9/72	13.9	8.9

[1]Mean of 20 bags taken randomly throughout the plots.

Appendix 6. Identification of Erwinia spp. in Rots

Date: 2/8/72 Inoculation date: 28/7/72

Treatment		Number of tubers/ plant	Number of tubers rotting with time(d) 4	8	12	% tubers containing soft rot bacteria	% tubers containing pectolytic bacteria	% tubers containing pectolytic & oxidase negative bacteria
WI	1	12	11	12	12	100	100	100
	2	11	7	7	7	64	64	64
	3	12	9	10	11	92	92	75
	4	12	8	9	10	83	75	75
	5	9	9	9	9	100	100	67
Mean						88	86	76
WNI	1	8	1	1	3	37	25	0
	2	13	5	11	13	100	92	54
	3	8	2	5	7	87	87	62
	4	7	3	3	4	57	57	0
	5	11	3	6	8	73	54	27
Mean						71	63	29
DI	1	8	4	6	6	75	50	50
	2	6	5	5	5	83	75	67
	3	4	2	2	3	75	75	25
	4	6	3	4	4	67	67	67
	5	10	8	9	9	90	70	60
Mean						78	67	54
DNI	1	9	1	1	5	55	55	11
	2	10	1	5	5	50	50	10
	3	9	3	5	6	67	67	44
	4	8	1	2	6	75	63	0
	5	7	3	4	6	86	71	14
Mean						67	61	16
Date: 20/8/72						Inoculation date: 28/7/72		
WI	1	9	3	9	9	100	100	44
	2	15	6	15	15	100	100	73
	3	11	2	10	10	90	90	82
	4	12	5	11	11	92	92	54
	5	12	2	9	9	75	75	33
Mean						92	92	57
WNI	1	12	0	7	9	75	58	36
	2	15	2	5	6	40	40	13
	3	8	3	7	7	89	89	25
	4	10	1	8	8	80	80	50
	5	9	2	4	4	44	44	22
Mean						66	62	29

Appendix 6. Continued

Treatment		Number of tubers/ plant	Number of tubers rotting with time (d) 4	8	12	% tubers containing soft rot bacteria	% tubers containing pectolytic bacteria	% tubers containing pectolytic & oxidase negative bacteria
DI	1	8	1	4	5	62	50	12
	2	9	1	4	4	44	33	33
	3	3	0	3	3	100	100	67
	4	6	2	5	6	100	67	50
	5	14	2	7	7	50	50	43
Mean						71	60	41
DNI	1	10	0	4	4	40	40	10
	2	12	1	9	10	83	83	67
	3	7	0	1	2	29	28	14
	4	12	0	3	5	42	25	8
	5	14	1	3	6	43	21	7
Mean						47	39	21
Date: 14/9/72						Inoculation date: 28/7/72		
WI	1	13	4	9	13	100	100	69
	2	12	5	11	12	100	100	75
	3	15	5	12	14	93	87	53
	4	9	3	7	8	89	89	56
	5	11	6	11	11	100	100	100
Mean						96	95	71
WNI	1	13	2	4	6	36	38	15
	2	14	4	5	8	57	50	14
	3	7	3	3	4	57	57	14
	4	9	2	3	6	67	67	33
	5	11	2	7	7	64	64	36
Mean						58	55	22
DI	1	9	3	7	9	100	100	100
	2	11	4	7	9	82	73	63
	3	8	4	4	6	75	75	75
	4	7	0	5	7	100	100	71
	5	10	4	7	7	70	70	40
Mean						85	84	70
DNI	1	9	0	2	4	44	44	0
	2	9	2	2	3	33	22	11
	3	9	3	5	5	56	56	22
	4	7	1	2	2	29	29	0
	5	12	1	3	3	25	25	8
Mean						37	35	8

Appendix 6. Continued

Date: 27/10/72 Inoculation date: 28/7/72

Treatment		Number of tubers/ plant	Number of rubers rotting with time (d) 4	8	12	% tubers containing soft rot bacteria	% tubers containing pectolytic bacteria	% tubers containing pectolytic & oxidase negative bacteria
WI	1	15	5	10	14	93	93	87
	2	9	6	9	9	100	100	89
	3	14	9	13	14	100	100	86
	4	12	4	9	12	100	100	83
	5	12	5	11	12	100	100	75
Mean						99	99	84
WNI	1	16	0	4	5	31	25	13
	2	9	2	3	6	67	56	22
	3	14	3	5	8	57	57	21
	4	13	1	6	6	46	46	31
	5	13	4	9	10	77	69	31
Mean						56	51	24
DI	1	10	5	9	9	90	80	80
	2	9	2	6	8	89	89	67
	3	9	4	5	7	78	78	56
	4	8	6	8	8	100	88	88
	5	10	2	5	9	90	90	60
Mean						89	85	70
DNI	1	7	0	4	5	71	57	0
	2	10	2	4	4	40	40	10
	3	8	1	3	4	50	38	13
	4	8	3	5	5	63	63	25
	5	8	0	0	2	25	25	13
Mean						50	45	12
Date: 4/9/72						Inoculation date: 30/8/72		
WI	1	11	2	10	10	91	82	54
	2	16	6	12	15	94	87	69
	3	9	3	8	9	100	67	56
	4	13	3	10	13	100	92	69
	5	13	4	10	13	100	92	85
Mean						97	84	67
WNI	1	14	4	9	11	79	64	21
	2	12	3	5	8	75	58	33
	3	8	2	6	6	75	37	0
	4	17	2	9	11	65	47	29
	5	11	2	5	5	45	46	18
Mean						68	50	20

Appendix 6. Continued

Treatment		Number of tubers/ plant	Number of tubers rotting with time (d) 4	8	12	% tubers containing soft rot bacteria	% tubers containing pectolytic bacteria	% tubers containing pectolytic & oxidase negative bacteria
DI	1	4	4	4	4	100	100	100
	2	9	2	5	6	67	67	56
	3	9	1	4	5	56	33	22
	4	7	3	4	5	71	71	29
	5	6	2	3	3	50	50	33
Mean						69	64	48
DNI	1	7	0	3	3	43	29	0
	2	7	1	3	3	43	29	0
	3	8	0	1	2	25	25	13
	4	10	2	4	6	60	40	30
	5	3	0	1	2	67	67	33
Mean						48	38	15
Date: 14/10/72					Inoculation date: 30/8/72			
WI	1	12	4	10	12	100	100	100
	2	12	4	9	10	83	75	58
	3	10	3	5	7	70	70	50
	4	13	4	7	8	61	61	46
	5	13	5	9	10	77	77	54
Mean						78	77	62
WNI	1	12	1	6	6	50	50	25
	2	12	2	3	5	42	42	17
	3	11	3	7	7	64	64	27
	4	13	2	5	6	46	46	15
	5	9	2	4	5	56	56	33
Mean						52	52	23
DI	1	8	2	3	5	63	50	38
	2	6	0	0	3	50	50	50
	3	5	3	4	5	100	100	60
	4	10	4	7	8	80	70	30
	5	7	1	2	5	71	71	29
Mean						73	68	41
DNI	1	7	0	3	4	57	57	0
	2	8	1	3	3	37	37	13
	3	8	0	2	4	50	25	13
	4	11	2	4	5	45	45	18
	5	5	0	3	3	60	60	20
Mean						50	45	13

Appendix 7. Field Contamination of VTSC Tuber Progeny (1972)

Aug. 1 1972 Number of tubers infected by soft rot bacteria

Plant No.	VTSC			Normal 1			Normal 2		
	4 d	6 d	12 d	4 d	6 d	12 d	4 d	6 d	12 d
1	0/9	1/9	2/9	0/7	3/7	3/7	4/5	4/5	4/5
2	2/10	3/10	4/10	1/9	2/9	5/9	1/7	3/7	3/7
3	1/6	2/6	2/6	2/6	5/6	5/6	2/9	3/9	6/9
4	1/8	3/8	4/8	5/10	7/10	7/10	5/8	5/8	6/8
5	4/5	4/5	4/5	1/5	2/5	2/5	2/10	3/10	5/10
6	0/3	0/3	0/3	0/5	0/5	0/5	1/6	3/6	3/6
7	2/7	2/7	3/7	4/6	4/6	4/6	4/9	5/9	7/9
8	2/9	3/9	3/9	2/8	3/8	3/8	2/5	2/5	3/5
Sept. 1 1972									
1	2/11	3/11	5/11	4/7	6/7	7/7	5/8	7/8	8/8
2	2/10	3/10	5/10	4/7	6/7	6/7	2/9	4/9	5/9
3	2/10	3/10	4/10	3/7	4/7	5/7	3/7	4/7	5/7
4	2/4	2/4	3/4	2/7	4/7	5/7	4/7	6/7	6/7
5	3/8	5/8	5/8	0/9	1/9	4/9	3/11	5/11	7/11
6	2/7	4/7	5/7	7/9	9/9	9/9	5/8	5/8	6/8
7	0/4	0/4	1/4	5/9	7/9	8/9	4/9	6/9	7/9
8	3/6	4/6	4/6	4/5	0/5	1/5	6/10	5/10	8/10

Appendix 8. Effect of Water Content of Tubers on Rotting

Individual volumes of rots (mm)

Tuber treatment			
72 h water		72 h glass plate	
Inoculation depth (mm)			
2.5	10.0	2.5	10.0
1.3	4.0	0	1.1
1.1	3.2	0	1.1
1.0	3.3	0	0.7
1.4	3.3	0.4	0.4
1.0	3.8	0	0.7
1.4	3.8	0	0.9
0.8	3.8	0	0.9
1.6	4.4	0	1.0
1.0	3.6	0.6	1.1
1.0	3.2	0.7	0.7
1.6	3.3	0	0.8
1.0	3.4	0	1.1
0.9	3.8	0.4	1.5
1.1	3.9	0	1.2
1.8	4.2	0.5	1.2
1.7	2.9	0	1.0
1.0	2.9	0	0.8
0.9	3.6	0.4	0.4
1.4	3.5	0.3	0.7
1.3	4.0	0.5	1.0
1.3	3.6	0.4	1.0
2.0	4.0	0	0
1.2	3.8	0	1.2
1.0	3.0	0.4	0.9
1.4	3.6	0.4	1.1

Analysis of Variance

Factor	Level	Grand mean = 1.5
A	2	
B	2	
C	25	

Source	DF	F-test
A	1	430.9
B	1	596.9
AB	1	115.9
ABC	17	
Error	0.102	

Necessary difference between means for a difference between treatments ($p = 0.001$) is 0.204.

Appendix 9. Effect of Non-Aqueous Films Upon Rotting from Infections Made into Cork Borer Wounds

Individual volumes of rot (mm)

Sealed tubers				Non-sealed tubers			
Inoculated		Non-inoculated		Inoculated		Non-inoculated	
Depth of inoculation (mm)							
2.5	10.0	2.5	10.0	2.5	10.0	2.5	10.0
1.0	2.9	0	0.3	0	1.6	0	0
0.8	3.5	0	0	0	1.6	0	0
0.7	2.4	0	1.1	0.5	2.2	0	0
0.6	1.9	0	0	0.6	1.8	0	0
1.3	2.7	0	0	0	1.3	0	0
1.3	2.9	0	1.1	0	2.0	0	0
1.0	3.3	0	0	0.5	2.0	0	0
0.8	2.7	0	0.4	0.6	1.8	0	0
0.7	2.1	0	0.4	0.4	2.1	0	0
0.8	2.8	0	0	0.5	1.6	0	0

Unpaired t-tests for inoculated tubers

a. 2.5 mm

Sealed tubers: Mean = 0.9
Variance = 0.06
Std. deviation = 0.244
Std. error = 0.07

Non-sealed tubers: Mean = 0.3
Variance = 0.074
Std. deviation = 0.273
Std. error = 0.08

t = 5.09 for 19 degrees of freedom

b. 10.0 mm

Sealed tubers: Mean = 2.7
Variance = 0.24
Std. deviation = 0.49
Std. error = 0.15

Non-sealed tubers: Mean = 1.8
Variance = 0.077
Std. deviation = 0.278
Std. error = 0.08

t = 5.146 for 19 degrees of freedom.

Appendix 10. One-way Analyses of Variance on Data from Tubers Inoculated with Two Depths and Stored in Different Gaseous Atmospheres

Treatment	ID* (mm)	Individual volumes of rots (cc)									
Air	2.5	0.6	0.3	0.6	0.9	0.5	0.8	0.6	0.7	0	1.1
control	10.0	1.5	2.0	2.3	2.3	1.8	1.6	1.5	2.0	1.3	1.8
Vaccuum	2.5	1.3	1.2	0.7	0.9	1.0	0.6	0.7	0.8	0.7	0.9
	10.0	3.3	3.1	3.0	2.5	2.8	2.8	2.9	3.2	2.1	3.2
10% CO_2	2.5	0.9	0.7	1.5	1.3	1.9	1.1	0.9	0.9	0.9	1.7
	10.0	3.0	2.9	3.3	2.6	2.5	3.0	3.0	2.9	3.3	3.5
100% CO_2	2.5	1.5	1.1	1.3	1.2	1.0	0.9	1.8	1.6	1.3	1.3
	10.0	4.6	4.0	3.5	3.2	3.5	3.2	4.0	3.8	4.6	4.4
100 ppm	2.5	0.5	0.8	0.8	1.0	1.0	0.5	0.6	0.7	0.4	0.5
ethylene	10.0	2.0	2.3	2.3	1.9	1.8	1.9	2.4	2.5	2.0	1.9
1000 ppm	2.5	0.6	0.5	0.6	0.9	0.9	1.0	0.9	1.1	0.1	0.8
ethylene	10.0	2.3	2.5	2.5	1.9	2.1	2.3	3.5	2.7	2.0	2.2
10% CO_2	2.5	1.5	0.9	0.9	1.0	1.3	0.9	1.3	0.9	1.1	1.2
in H_2	10.0	3.9	3.9	3.7	3.4	3.0	2.9	3.8	3.6	4.0	3.8

*ID = Inoculation depth

a. 2.5 mm inoculation depth

Factor	Level
A	7
B	10

Grand mean

Source	Sum of squares	DF	Mean sq.	F-test
A	4.183	6	0.695	9.6
B	0.493	9	0.054	
AB	4.098	54	0.0759	
Error	0.073			Significance level
Total	8.763			0.001

Difference between means necessary for difference between treatments at p = 0.001 = 0.15.

b. 10.0 mm inoculation depth

Grand mean = 2.79

Source	Sum of squares	DF	Mean sq.	F-test
A	35.84	6	5.95	46.14
B	3.49	9	0.277	
AB	5.64	54	0.104	Significance level
				0.001
Error			0.13	
Total	43.87			

Difference between means necessary for difference between treatments at p = 0.001 = 0.26.

Appendix 11. The Effect of a Film of Water on Rotting from Infected Wounds

Individual volumes of rot (ml)

Wet tubers		Dry tubers	
Depth of inoculation (mm)			
2.5	10.0	2.5	10.0
1.0	2.6	0.5	1.2
1.0	2.8	0	1.2
1.5	3.0	0.8	1.5
1.3	2.1	0.7	1.4
1.2	2.3	0.6	1.0
1.2	2.3	0.3	1.0
1.6	2.5	0.2	0.9
1.0	2.4	0.3	1.4
0.8	2.4	0.4	1.5
0.9	2.3	0.8	1.4
1.0	2.0	0.6	1.2
1.3	2.7	0.7	0.8
1.3	2.7	0.7	1.2
1.4	2.5	0	1.1
1.5	2.8	0.6	1.1
1.2	2.6	0.6	1.6
1.1	2.5	0.7	1.2
1.0	2.5	0.3	1.1
0.2	2.3	0.5	1.1
1.5	2.5	0.5	1.5
1.6	3.0	0.6	1.1
1.2	2.1	0.6	1.0
1.1	2.6	0.5	1.2
1.1	2.6	0.4	1.1
1.0	2.6	0.7	1.2

Unpaired t-tests analyses

a. 2.5 mm

Wet tubers

Mean = 1.20
Variance = 0.05
Std. deviation = 0.221
Std. error = 0.04

Dry tubers

Mean = 0.5
Variance = 0.05
Std. deviation = 0.220
Std. error = 0.04

t = 11.0 for 49 degrees of freedom

b. 10.0 mm

Wet tubers

Mean = 2.5
Variance = 0.066
Std. deviation = 0.258
Std. error = 0.05

Dry tubers

Mean = 1.2
Variance = 0.04
Std. deviation = 0.202
Std. error = 0.04

t = 19.8 for 49 degrees of freedom

Appendix 12.

Mean Viable Plate Count Assay (No. of Colonies/Plate)

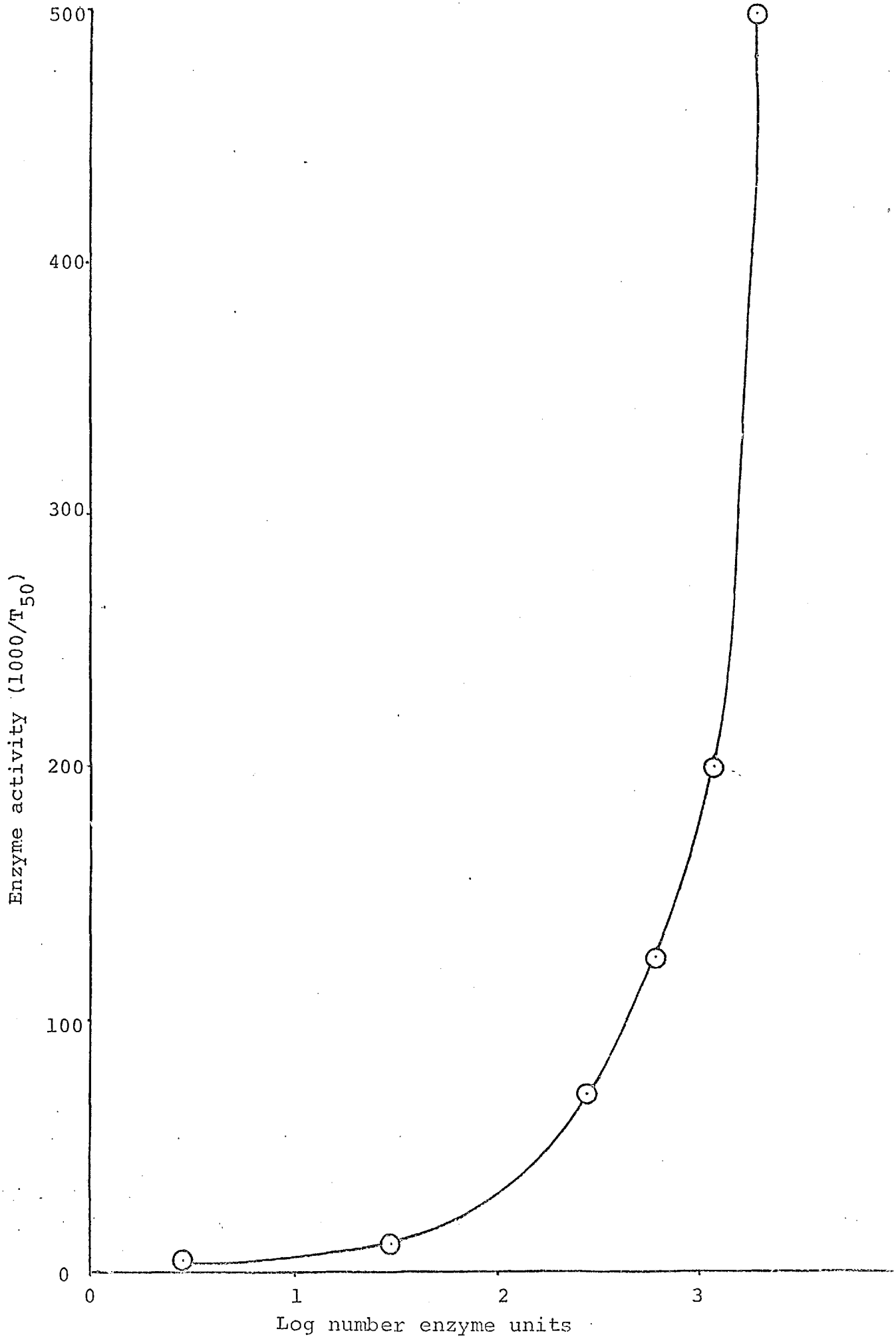
Incubation Period	Dilution of broth culture								
	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9
0	*(3)5	(0)0	(0)0	(0)0	(0)0	(0)0	(0)0	(0)0	(0)0
12	(5)∞	(1)110	(0)23	(0)2	(0)0	(0)0	(0)0	(0)0	(0)1
24	(8)∞	(11)∞	(1)∞	(0)210	(0)78	(0)18	(0)2	(0)0	(0)2
36	(111)∞	(110)∞	(11)∞	(2)∞	(0)∞	(0)96	(0)29	(0)3	(0)0
48	(∞)∞	(156)∞	(19)∞	(3)∞	(1)∞	(0)270	(0)46	(0)10	(0)0
60	(∞)∞	(154)∞	(20)∞	(2)∞	(0)182	(0)46	(0)16	(0)14	(0)1
72	(102)∞	(36)∞	(6)∞	(1)27	(0)9	(0)6	(0)0	(0)0	(0)0

*Figures in brackets indicate results from another assay.

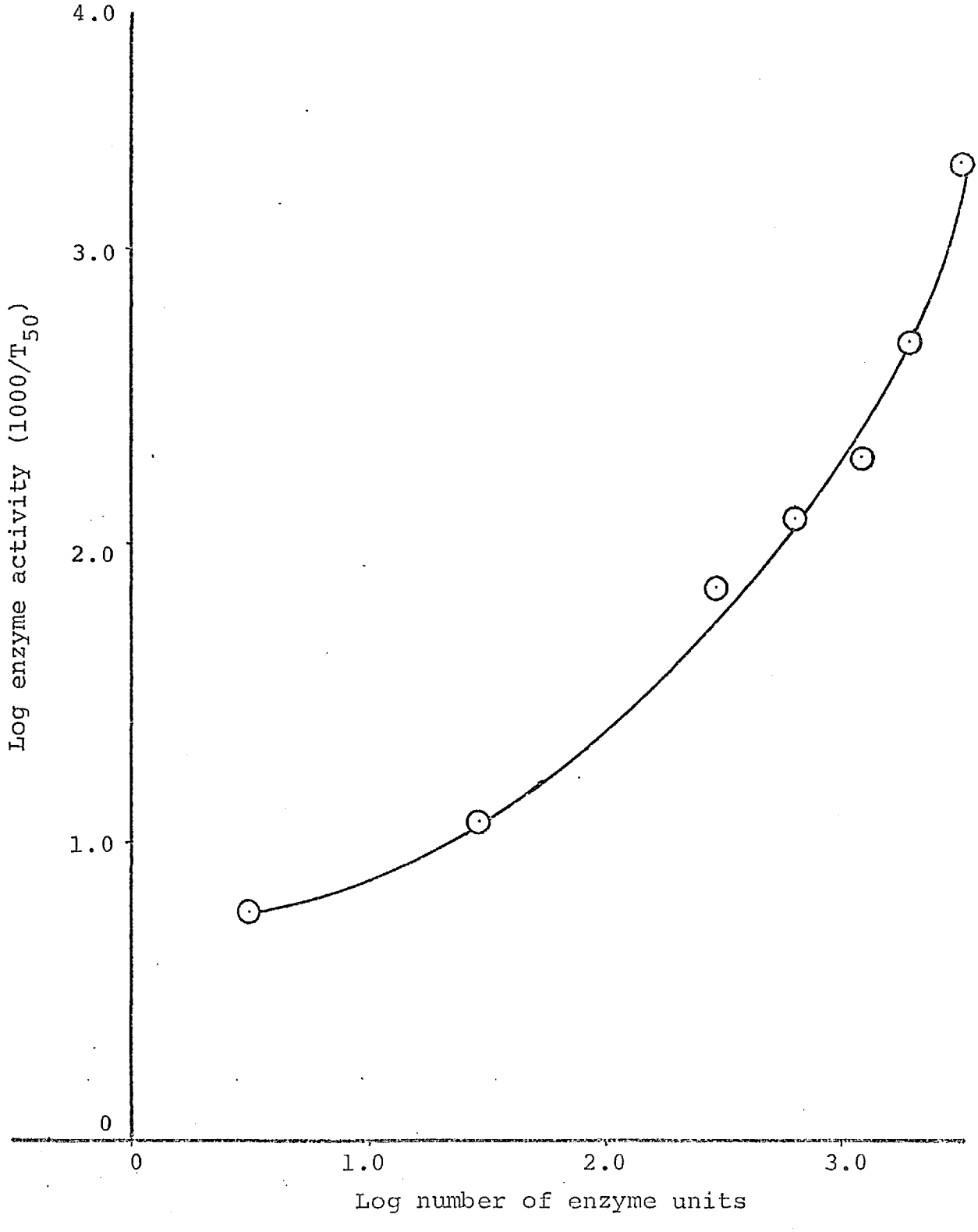
Estimation of Cell Numbers per ml for Growth Curves

Incubation period (h)	Viable plate count assay		Turbidimetric assay
	a	b	
0	3×10^1	5×10^1	0
12	5×10^1	1×10^4	0
24	8.5×10^2	8×10^6	1×10^6
36	1×10^4	9×10^6	9×10^9
48	2×10^4	5×10^8	1×10^9
60	2×10^4	5×10^7	5×10^5
72	4×10^3	3×10^5	5×10^5

Appendix 13. Determination of Arbitrary Units of Enzyme Activity



Appendix 14. Determination of Arbitrary Units of Enzyme Activity



Appendix 15. Effect of pH on the Growth and Enzyme Production in *E. atroseptica* A13 Cultured on Different Pectic Compounds

1. Sodium polypectate

a. pH change

Sampling time (h)	Broth pH									
0	3.5	4.1	4.8	5.7	6.2	7.3	7.9	9.0	10.1	10.5
7	3.3	4.3	5.0	5.9	6.0	7.4	8.2	9.0	10.1	10.4
10	3.3	4.1	4.9	5.7	6.0	7.0	7.8	8.7	9.5	9.8
13	3.3	4.3	4.8	6.0	6.4	7.2	8.3	9.0	9.9	10.2
16	2.9	3.7	4.6	5.5	5.8	6.4	7.9	8.7	9.6	10.0
19	3.6	4.4	5.1	6.0	6.3	6.8	7.8	9.0	9.9	10.1
26	3.4	4.3	5.0	6.3	6.8	7.8	7.9	8.5	9.8	10.1
80	3.3	4.2	5.7	8.5	8.6	7.9	8.3	8.5	9.1	9.5

b. Viscosity reduction

Flow time (sec)

Sampling time (h)	Initial pH of Broth									
	3.5	4.1	4.8	5.7	6.2	7.3	7.9	9.0	10.1	10.5
0	∞	33.6	22.0	46.1	21.2	32.2	42.7	26.0	32.2	29.2
7	∞	41.6	20.5	38.2	23.0	34.0	51.0	26.9	29.6	28.8
10	∞	32.4	30.0	42.5	20.1	27.2	33.1	26.0	30.2	29.0
13	∞	37.5	22.8	41.5	19.2	15.0	25.2	24.0	29.5	28.0
16	∞	29.5	21.0	38.6	12.7	11.0	14.3	24.5	27.1	26.5
19	∞	30.2	21.0	14.8	10.0	10.8	11.3	22.2	30.3	30.4
26	∞	32.6	16.1	11.2	10.0	10.9	10.3	10.6	28.1	27.4
80	∞	34.0	11.0	12.0	11.0	11.0	11.1	10.5	28.1	27.0

c. Cell multiplication after 26 h

Bacterial population (x 10^7 cells/ml)

Initial pH of Broth									
3.5	4.1	4.8	5.7	6.2	7.3	7.9	9.0	10.1	10.5
0	0	-	175	150	175	175	150	0	0

d. Units of enzyme activity during growth

Sampling time (h)	Initial pH of Broth			
	4.1	6.2	7.9	9.0
0	0	0	0	0
7	0	0	0	0
10	0	230	500	0
13	0	1660	2000	0
16	0	2000	2240	0
19	0	2200	2650	0
26	0	2000	2650	50
80	0	2650	2650	1180

Appendix 15. Continued

2. Pectin

a. pH change

Sampling time (h)	Broth pH										
0	2.8	3.65	4.6	5.4	6.2	6.4	7.2	7.9	8.7	9.6	10.3
8	2.8	3.6	4.5	5.4	6.1	6.4	7.1	8.0	8.8	9.7	10.3
11	2.9	3.6	4.6	5.5	6.3	6.4	7.3	8.0	8.8	9.7	10.0
14	2.8	3.5	4.3	5.2	5.8	6.5	7.0	7.7	8.4	9.5	10.1
16	3.0	3.8	4.5	5.3	5.9	6.1	6.7	7.6	8.4	9.3	10.0
19	2.8	3.5	4.4	5.2	6.1	6.4	7.0	7.6	8.3	9.3	9.9
22	3.0	3.6	4.5	5.4	6.4	6.7	7.2	7.7	8.3	9.4	9.9
25	3.0	3.8	4.7	5.7	7.0	6.7	7.1	7.6	8.2	9.2	9.8
28	3.0	3.7	4.6	5.5	7.2	6.8	7.2	7.7	8.3	9.2	9.8
31	3.0	3.8	4.6	5.7	7.5	7.0	7.5	8.1	8.6	9.4	
34	3.0	3.8	4.5	5.9	7.8	7.4	7.3	7.8	8.3	9.2	
39	3.0	3.8	4.7	7.0	8.6	7.1	7.6	8.2	8.4	9.4	

b. Viscosity reduction

Flow time (sec)

Sampling time (h)	Initial pH of Broth									
	2.8	3.65	4.6	5.4	6.2	6.4	7.2	7.9	8.7	9.6	10.3
0	21.1	21.1	22.0	21.0	21.5	19.6	22.5	21.2	20.4	21.0	19.0
8	21.2	21.0	22.0	21.3	20.5	20.1	21.2	20.0	19.0	18.0	17.0
11	20.7	20.7	21.5	21.2	20.0	19.1	21.0	18.0	18.5	17.5	16.0
14	20.4	20.5	21.5	20.8	20.5	20.0	21.2	19.5	18.0	16.9	16.4
16	21.7	20.3	22.1	21.7	20.6	19.5	18.1	19.0	18.5	16.9	16.5
19	20.0	20.5	20.9	20.7	20.2	17.1	19.9	18.0	18.6	17.4	16.7
22	20.2	20.6	21.0	20.0	20.1	14.8	18.2	14.9	16.9	16.4	16.2
25	19.8	19.7	20.8	20.3	18.7	14.1	16.7	14.2	16.4	16.6	16.3
28	20.4	20.0	20.6	20.8	19.0	14.2	16.1	13.0	16.4	16.3	16.3
31	20.0	21.0	21.0	20.1	18.1	13.5	14.8	12.4	13.3	16.2	16.2
34	20.0	21.0	20.3	15.9	16.4	12.9	14.0	11.9	12.9	16.2	16.3
39	20.1	20.7	20.5	14.0	15.7	13.7	13.8	11.9	11.5	16.8	16.3

c. Cell multiplication after 25 h

Bacterial population (x 10^7 cells/ml)

Initial pH of broth										
2.8	3.65	4.6	5.4	6.2	6.4	7.2	7.9	8.7	9.6	10.3
0	0	0	9	70	40	30	40	8	0	0

Appendix 16. Catabolite Repression of PTE Synthesis by Different Carbohydrates

a. 24 h incubation (3 replicates per treatment)

Carbohydrate	Growth	Cell No/ml (x 10^7/ml)	pH	Enzyme activity T_{50}	Units	Units/cell No/ml
α-D+glucose	+	1.3	5.8	∞	0	0
+NaPP	+	1.4	5.8	∞	0	0
	+	1.1	5.7	∞	0	0
β-D-fructose	+	1.3	5.7	∞	0	0
+NaPP	+	1.8	5.6	∞	0	0
	+	1.6	5.6	∞	0	0
Sucrose+NaPP	+	0.9	6.0	∞	0	0
	+	1.1	5.9	∞	0	0
	+	1.0	6.1	∞	0	0
Inositol	+	0.9	6.2	125	15	17
+NaPP	+	1.2	6.3	161	10	8.3
	+	1.1	6.3	96	22	20
Raffinose	-	-	-	-	-	-
+NaPP	-	-	-	-	-	-
	-	-	-	-	-	-
Lactose+NaPP	+	1.0	5.3	92	25	25
	+	1.1	5.4	56	53	48
	+	1.3	5.1	24	158	21
Galacturonic	-	-	-	-	-	-
acid+NaPP	+	-	6.9	∞	0	0
	+	-	6.6	∞	0	0
Starch+NaPP	-	-	-	-	-	-
	-	-	-	-	-	-
	-	-	-	-	-	-
NaPP	+	1.6	7.8	8	631	394
	+	2.1	7.7	4	1334	635
	+	1.3	7.5	15	266	205

Appendix 16. Continued

b. 48 h incubation

Carbohydrate	Growth	Cell No/ml (x 10^7/ml.)	pH	Enzyme activity T_{50}	Units	Units/cell No./ml
α-D+glucose	+	11	5.3	∞	0	0
+NaPP	+	18	5.2	88	25	1.4
	+	26	5.2	100	21	0.8
β-D-fructose	+	27	5.2	∞	0	0
+NaPP	+	30	5.1	∞	0	0
	+	19	5.2	∞	0	0
Sucrose+NaPP	+	3.2	5.4	∞	0	0
	+	7.8	5.4	∞	0	0
	+	11	5.4	∞	0	0
Inositol	+	6.7	6.0	75	34	5
+NaPP	+	12	5.9	53	67	6
	+	9.6	5.9	21	211	22
Raffinose	-	-	-	-	-	-
+NaPP	-	-	-	-	-	-
	-	-	-	-	-	-
Lactose	+	21	4.9	2½	1900	90
+NaPP	+	11	5.1	18	224	20
	+	13	5.1	4	1330	103
Galacturonic	-	-	-	-	-	-
acid+NaPP	+	-	6.9	∞	0	0
	+	-	6.5	∞	0	0
Starch+NaPP	-	-	-	-	-	-
	-	-	-	-	-	-
	-	-	-	-	-	-
NaPP	+	10	8.2	½	3750	375
	+	23	7.9	1	2600	113
	+	18	7.9	1	2600	144

Appendix 16. Continued

c. 72 h incubation

Carbohydrate	Growth	Cell No./ml (x 10^7/ml)	pH	Enzyme activity T_{50}	Units	Units/cell No./ml
α-D-glucose	+	210	5.4	10	473	2.2
	+	790	5.7	6	1120	1.4
	+	620	6.1	4	1330	2.1
β-D-fructose	+	340	6.0	54	56	0.2
+NaPP	+	420	6.1	18	266	0.6
	+	410	6.0	17	281	0.7
Sucrose+NaPP	+	190	5.3	154	10	0.05
	+	540	5.3	∞	0	0
	+	430	5.1	90	63	0.10
Inositol	+	90	5.8	10	473	5.3
+NaPP	+	110	5.6	8	631	5.7
	+	86	5.4	3	1780	20.7
Raffinose	-	-	-	-	-	-
+NaPP	-	-	-	-	-	-
	-	-	-	-	-	-
Lactose+NaPP	+	110	5.9	1	2600	23.6
	+	93	6.4	2	2000	21.5
	+	180	6.4	1	2600	14.4
Galacturonic	-	-	-	-	-	-
acid+NaPP	+	1.5	6.3	∞	0	0
	+	2.1	5.9	∞	0	0
Starch+NaPP	-	-	-	-	-	-
	-	-	-	-	-	-
	-	-	-	-	-	-
NaPP	+	330	8.3	½	3750	11.3
	+	310	8.4	½	3750	12.1
	+	490	8.5	1	2600	5.3

Appendix 17. The Effect of State of Dormancy Upon the Resistance of Potato Tuber Tissue to Soft Rotting

Rot volumes (cc) at two depths of inoculation (mm)

Ethylene chlorhydrin treatment										Control treatment									
2.5					10.0					2.5					10.0				
Day					Day					Day					Day				
0	1	2	4	9	0	1	2	4	9	0	1	2	4	9	0	1	2	4	9
2.6	1.4	5.7	1.6	2.4	9.4	3.1	13.0	9.5	6.5	2.1	1.3	1.3	0.3	0.9	3.4	4.1	4.6	3.8	3.2
1.9	1.3	2.5	0.7	0.5	8.2	5.8	8.2	5.8	5.1	2.3	1.6	1.4	1.8	1.3	2.4	2.8	5.0	4.1	2.3
1.9	1.7	1.8	0.9	1.8	2.8	3.5	6.5	5.9	3.6	0.9	0.4	1.3	0.7	1.7	1.9	2.9	4.1	4.9	5.1
2.1	1.5	2.4	1.6	1.1	15.2	3.1	8.5	7.3	4.5	0.8	1.4	0.5	0.9	1.3	2.3	3.4	3.3	3.2	3.8
4.0	1.4	3.2	2.0	1.8	13.0	6.7	5.6	8.7	3.2	0.4	1.7	2.0	0.6	1.8	4.4	3.5	0.9	3.5	4.2

Analysis of variance for complete multifactorial design

Factor	Level	
A	2	A = difference between treatments
B	5	B = difference between storage times
C	5	C = replication

Grand mean = 1.614 (5.196)

Source	Sums of squares	DF	Mean square	F-test
A	8.73 (146.55)*	1	8.73 (146.55)	17.04 (33.53)
B	8.40 (48.42)	4	2.101 (12.10)	4.09 (2.77)
AB	4.57 (63.74)	4	1.14 (15.93)	2.23 (2.65)
C	3.57 (20.55)	4	0.89 (5.14)	
AC	3.24 (25.93)	4	0.81 (6.48)	
BC	5.03 (79.61)	16	0.31 (4.97)	
ABC	8.67 (48.72)	16	0.54 (3.04)	
Error	0.512 (4.37)			
Total	42.22 (433.54)			

*Figures in brackets denote 10 mm inoculated wounds.

Appendix 18. The Effect of State of Dormancy Upon the Resistance of Potato Tuber Tissue to Soft Rotting

Rot volumes (cc) at two depths of inoculation (mm)

Ethylene chlorhydrin treatment										Control treatment									
2.5					10.0					2.5					10.0				
Day					Day					Day					Day				
0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8
0.9	0	0	0.9	0.3	2.4	2.2	2.8	3.1	3.6	0.7	0.5	1.0	0.3	0.3	2.0	1.8	1.9	2.0	2.5
1.0	0	0.7	1.0	0.4	3.9	3.3	2.2	4.3	2.9	0.8	0.3	0.3	1.0	0.6	1.7	2.2	2.6	4.8	2.2
0	0	0	0	0.5	3.9	5.1	5.1	2.5	3.4	0.4	0.7	0.8	0.2	1.0	2.1	3.0	1.9	3.5	2.9
0.7	0.4	0.5	0	0.8	1.3	2.2	3.9	3.1	3.1	0.9	1.1	0.9	0.4	0.5	2.3	2.3	2.0	1.9	2.4
0	0	0.6	0.8	0.7	1.8	2.3	1.7	4.3	2.6	0.7	1.0	0.3	0.7	0.3	1.5	3.0	1.8	2.2	2.4
1.1	0	1.0	1.5	0.5	1.5	1.4	2.2	2.8	3.0	0.8	0.8	1.4	1.0	0.6	2.2	1.7	3.2	3.6	2.6
1.3	0.4	1.0	0.7	0.8	3.4	2.2	3.1	2.3	3.8	1.1	0.7	1.2	0.5	0.6	3.0	2.1	2.9	2.3	2.0
0.7	0	0.6	0.6	0.6	2.6	4.3	3.0	3.7	4.0	1.0	1.0	0.7	0.2	0.9	2.2	3.2	1.8	3.4	2.4

Analysis of variance for complete multifactorial design

Factor	Level	
A	2	A = difference between treatments
B	5	B = difference between storage times
C	8	C = replication

Grand mean = 0.615 (2.697)

Source	Sums of squares	DF	Mean square	F-test
A	0.648 (7.688)*	1	0.648 (7.688)	5.652 (11.329)
B	0.955 (5.568)	4	0.239 (1.392)	2.081 (2.051)
AB	1.533 (0.843)	4	0.383 (0.210)	2.343 (0.310)
C	1.166 (10.907)	7	0.166 (1.558)	
AC	0.244 (4.532)	7	0.034 (0.647)	
BC	3.730 (18.263)	28	0.133 (0.652)	
ABC	2.884 (13.796)	28	0.103 (0.492)	
Error	0.115 (0.678)			
Total	11.16 (61.59)			

*Figures in brackets denote 10 mm inoculated wounds.

Appendix 19. Rotting in Tubers of Increasing Age

Linear regression analysis: a. 0.25 mm inoculation

Storage period (d)	Volume of rot (ml)
0	0.6
26	0.5
52	0.7
95	0.1
149	0.6

Regression line equation

$$Y = B_0 - B_1X$$

Variant	Mean	Standard deviation
X	64.40	58.88
Y	0.50	0.23

Parameter	Regression coefficient	Computed T value
B_0	0.559	3.0023
B_1	-0.0009	-0.4091

For all values $Y \simeq B_0$.

Linear regression analysis: b. 10.0 mm inoculation

Storage period (d)	Volume of rot (ml)
0	3.2
26	1.8
52	2.6
95	2.1
149	2.2

Variant	Mean	Standard deviation
X	64.40	58.88
Y	2.38	0.54

Parameter	Regression coefficient	Computed T value
B_0	2.645	6.712
B_1	-0.004	-0.869

For all values $Y \simeq B_0$.

Appendix 20. Rotting of Cold Stored, Fusarex Treated and Sprouting Tubers from Inoculated Wounds

Volumes of rots (ml) at two depths (mm)

Tuber treatment					
Fusarex treated		Cold stored		Sprouting	
2.5	10.0	2.5	10.0	2.5	10.0
0	1.9	0.4	1.6	0.3	2.9
0	1.9	0	2.1	0.7	2.5
0.4	1.9	0	2.0	0.3	2.6
0	1.5	0.3	1.9	0.5	1.6
0.3	1.4	0.4	2.1	0.4	1.6
0.3	1.4	0.6	2.0	0.4	2.6
0	1.7	0.5	1.8	0.4	2.4
0	2.0	0.3	1.9	0.7	2.1
0.3	2.4	0	2.6	0.4	2.7
0.7	2.1	0	2.0	0.3	3.1

Multivariant analysis of variance

a. 2.5 mm inoculation depth

Factor	Level	
A	3	A = difference between treatments
B	10	B = difference between replicates

Grand mean = 2.96

Source	Sum of squares	DF	Mean square	F-test
A	0.32	2	0.160	3.58
B	0.13	9	0.014	
AB	1.08	18	0.059	
Error			0.044	
Total	1.53			

Difference between the means necessary for a difference between the treatments at p = 0.05, is 0.08.

b. 10.0 mm inoculation depth

Factor	Level
A	3
B	10

Grand mean = 2.96

Source	Sum of squares	DF	Mean square	F-test
A	1.83	2	0.914	6.454
B	2.09	9	0.233	
AB	1.73	18	0.096	
Error			0.142	
Total	5.65			

Difference between the means necessary for a difference between the treatments at p = 0.01, is 0.28.

Appendix 21. Effect of Major Sugars Within Potato Tubers on Resistance to Rotting.

Volumes of rot produced by E. atroseptica A13 after incubation for 4 d at room temperature

SP (d)	Treatment	ID (mm)	Volume of rot (ml)									
0	NAA control	2.5	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2
		10.0	3.8	2.8	1.8	2.1	2.0	2.8	2.0	1.3	2.3	2.4
0	NAA ethylene	2.5	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
		10.0	3.5	3.7	3.8	2.5	3.0	3.0	2.0	3.0	4.1	4.2
0	Fusarex control	2.5	0.5	0.2	0.2	0.2	0.3	0.5	0.2	0.2	0.2	0.2
		10.0	3.8	2.0	3.6	3.1	2.7	2.9	3.0	2.3	2.9	2.5
0	Fusarex ethylene	2.5	0.6	0.5	0.5	0.3	0.5	0.5	0.5	0.3	0.2	0.2
		10.0	3.5	3.5	3.1	2.4	2.7	3.1	2.1	3.4	1.6	2.8
1	NAA control	2.5	0.9	0.3	0.2	0.3	0.2	0.3	0.3	0.2	0.2	0.2
		10.0	3.3	0.7	3.2	2.3	1.7	4.1	2.4	2.3	1.6	1.7
1	NAA ethylene	2.5	0.3	0.2	0.2	0.3	0.6	0.3	0.3	0.2	0.3	0.2
		10.0	2.8	2.9	3.8	2.7	3.2	3.7	2.5	1.5	2.0	2.2
1	Fusarex control	2.5	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.3	0.2	0.2
		10.0	3.5	3.2	2.8	4.0	1.2	2.8	2.3	2.4	3.2	2.1
1	Fusarex ethylene	2.5	0.6	0.6	0.2	0.2	0.3	0.3	0.9	0.3	0.3	0.2
		10.0	2.5	2.5	1.3	2.2	1.8	3.2	2.6	3.2	1.6	3.3
2	NAA control	2.5	1.1	0.4	0.2	0.2	0.2	0.2	0.6	0.2	0.2	0.2
		10.0	2.9	1.5	2.3	3.0	2.0	3.1	2.0	2.0	2.8	2.4
2	NAA ethylene	2.5	0.6	0.2	0.5	0.7	0.2	0.5	0.2	0.2	0.2	1.0
		10.0	3.2	2.0	1.9	1.7	2.0	1.8	2.6	2.5	2.2	2.9
2	Fusarex control	2.5	0.6	0.2	0.2	0.5	0.2	0.6	0.8	0.5	0.2	0.6
		10.0	3.3	2.7	1.8	3.2	2.5	3.3	3.2	2.9	2.1	2.0
2	Fusarex ethylene	2.5	0.5	0.6	0.8	0.6	0.4	0.3	0.3	0.2	0.6	0.7
		10.0	1.5	2.1	3.0	1.8	1.1	3.1	1.6	1.6	1.8	3.2
4	NAA control	2.5	0.2	0.5	0.2	0.6	0.2	0.2	0.4	0.3	0.3	0.2
		10.0	2.2	2.2	1.8	1.4	2.1	2.6	2.0	1.8	2.1	2.1
4	NAA ethylene	2.5	0.3	0.4	0.4	0.2	0.2	0.3	0.5	0.3	0.7	0.2
		10.0	1.9	1.2	1.3	2.5	1.3	1.9	2.3	1.7	2.5	1.3
4	Fusarex control	2.5	0.3	0.7	0.2	0.2	0.4	0.3	0.5	0.3	0.5	0.4
		10.0	1.4	2.3	1.3	2.0	2.1	2.4	1.3	1.3	2.3	1.7
4	Fusarex ethylene	2.5	0.2	0.3	0.2	0.2	0.5	0.4	0.4	0.2	0.5	0.2
		10.0	2.0	1.5	2.3	1.2	0.8	1.7	2.0	2.6	2.5	1.7
8	NAA control	2.5	0.3	0.2	0.2	0.2	0.4	0.2	0.2	0.2	0.2	0.3
		10.0	1.7	2.0	1.3	1.2	1.7	1.6	1.9	2.1	1.8	2.1
8	NAA ethylene	2.5	0.3	0.2	0.2	0.5	0.2	0.4	0.2	0.6	0.3	0.4
		10.0	3.0	2.7	3.0	2.2	1.1	2.8	2.5	2.6	2.5	2.2
8	Fusarex control	2.5	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
		10.0	1.5	2.0	2.4	1.7	1.4	2.5	1.4	1.6	1.8	1.9
8	Fusarex ethylene	2.5	0.2	0.3	0.3	0.2	0.2	0.3	0.3	0.3	0.4	-
		10.0	1.4	2.8	1.3	1.5	1.1	1.2	1.4	2.4	3.2	-

SP = Storage period in days
ID = Inoculation depth (mm)

Appendix 22. Comparison of Mean Volumes of Rot (ml) and Sugar Concentrations (mg/g fresh weight of tuber tissue)

Treatment	SP (d)	Vol of rot 2.5 mm	Vol of rot 10.0 mm	Sugar			
				Fructose	Glucose	Inositol	Sucrose
NAA control	0	0.2	2.3	1.47(0.99)*	2.09(1.42)	2.76(1.51)	0.11(1.24)
	1	0.3	2.8	1.31(1.08)	2.32(1.42)	2.10(1.28)	0 (1.07)
	2	0.4	2.3	1.32(1.00)	2.56(1.52)	2.41(1.53)	0.09(0.66)
	4	0.4	1.8	1.32(1.49)	2.59(1.77)	2.50(1.56)	0.96(0.24)
	8	0.3	2.5	1.51(0.83)	2.76(1.56)	2.02(1.31)	1.34(0.23)
NAA ethylene	0	0.2	3.3	1.33(1.48)	1.70(2.04)	3.34(1.56)	0.68(1.96)
	1	0.3	2.5	1.24(1.18)	1.94(2.51)	2.15(1.79)	0.77(0.96)
	2	0.3	2.4	1.74(1.56)	3.20(2.49)	2.86(3.65)	0.56(1.17)
	4	0.3	2.0	1.67(1.34)	3.45(2.33)	1.81(1.65)	0.15(0.06)
	8	0.2	1.7	1.48(1.27)	2.20(2.16)	1.63(1.53)	1.61(0.60)
Fusarex	0	0.4	3.1	1.26(1.09)	1.95(1.84)	1.86(1.71)	0.03(1.25)
	1	0.4	2.5	1.20(1.03)	1.63(1.33)	1.67(1.26)	1.19(1.07)
	2	0.5	2.1	1.20(0.83)	1.48(1.16)	2.44(1.20)	0.80(0.23)
	4	0.3	1.9	1.20(1.01)	1.95(1.16)	2.06(1.42)	0.65(1.06)
	8	0.3	1.8	1.11(0.78)	1.57(1.14)	1.90(1.18)	0.37(0.36)
Fusarex ethylene	0	0.3	2.9	1.39(1.03)	1.98(1.95)	2.13(1.90)	0.17(1.54)
	1	0.2	2.9	1.51(1.03)	2.83(1.26)	2.04(1.67)	1.05(0.86)
	2	0.4	2.7	1.39(1.72)	2.93(2.28)	2.40(1.64)	1.71(0)
	4	0.4	1.8	1.36(1.17)	2.76(1.44)	2.90(1.06)	0.92(0)
	8	0.2	1.8	1.65(1.54)	2.67(2.13)	2.55(2.05)	1.22(0.14)

*Figures in brackets are for non-inoculated tubers.

Appendix 23. Resistance of Tuber Tissues to Maceration by PTE

% fresh weight loss

Tissue depth (mm)	Treatment: Tissue under sprout		Tissue not under sprout	
	Test	Control	Test	Control
0-1	22.0	2.5	3.5	3.7
1-2	17.5	4.0	8.2	3.7
2-3	29.0	2.4	3.2	1.9
3-4	19.5	2.7	9.4	3.9
4-5	26.5	3.8	15.0	4.0
5-6	18.6	5.8	10.7	3.7
6-7	25.8	1.8	20.4	2.1
7-8	22.5	2.9	26.2	3.9
8-9	21.7	2.1	32.0	2.4
9-10	23.0	5.1	30.2	1.9

Appendix 24. Effect on Rotting of Solutions of Phenolic Substances and Certain Enzymes (Method C)

Treatment	Volume of rots (ml)									
Ascorbic acid	2.1	2.1	2.3	2.4	2.1	2.2	1.4	2.2	2.5	2.1
Indole acetic acid (IAA)	2.2	2.3	2.1	2.8	2.6	1.7	2.1	2.1	2.1	2.4
Hydrogen peroxide	1.8	2.1	2.0	1.9	1.9	2.8	2.5	2.5	2.1	2.1
Tyrosine	1.6	1.8	1.9	2.0	1.4	2.8	1.9	2.0	1.8	1.9
Ascorbic acid + peroxidase + H_2O_2	2.7	2.8	2.5	2.4	2.9	1.8	2.2	0.9	1.9	2.6
IAA + peroxidase + H_2O_2	2.1	2.2	2.1	1.3	3.1	2.6	2.7	1.9	1.9	2.3
Tyrosine + peroxidase + H_2O_2	0.9	1.7	1.7	1.5	2.1	1.5	2.8	1.9	1.7	2.1
Ascorbic acid + tyrosinase	3.0	1.9	2.1	2.2	2.1	2.2	3.5	2.8	1.9	1.9
IAA + tyrosinase	2.0	2.0	1.8	2.5	2.3	2.4	2.7	3.0	3.0	1.9
Tyrosine + tyrosinase	2.1	2.6	2.8	2.6	2.2	1.6	1.5	1.8	2.7	2.3
Tyrosinase	2.0	1.8	1.6	2.4	2.9	3.1	2.4	2.4	2.6	2.0
Peroxidase + H_2O_2	1.8	2.1	2.9	3.1	1.7	2.2	2.4	2.1	2.7	2.7
Tyrosinase + H_2O_2	2.1	2.4	3.1	1.6	1.9	2.1	2.6	2.3	2.8	2.9
Water	1.7	2.6	1.9	2.9	2.6	2.4	2.1	2.5	2.7	2.1

Multifactorial analysis of variance

Factor	Level
A	14
B	10

Grand mean = 2.22

Source	Sum of squares	DF	Mean square	F-test
A	4.03	13	0.309	1.59
B	0.96	9	0.107	
AB	23.54	118	0.201	
Error			0.1944	
Total	28.54			

Difference between the means required for difference between treatments at p = 0.20, is 0.39.