ETHYLENE PRODUCTION BY *BOTRYTIS CINEREA* AND INFECTED KIWIFRUIT

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بسم الله الرحمن الرحيم

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والصلاة والسلام على خاتم الأنبياء والمرسلين

In the name of Allah

the compassionate, the merciful,

praise be to Allah, Lord of the universe,

and peace and prayers be upon

his final Prophet and Messenger

ABSTRACT

Botrytis cinerea is an important fungus causing serious losses to field and glass house grown fruits and vegetables and it is also an important postharvest pathogen. As a postharvest pathogen it is responsible for significant quality and economic losses to stored fruits and vegetables on a global scale. In New Zealand, infection by *B. cinerea* is one of the major causes of postharvest losses to the kiwifruit industry. This may be direct loss of infected fruit or an indirect loss due to secondary effects from the production of ethylene (C_2H_4) which causes softening of other non-infected fruit in the same tray.

Several fungi are known to produce C_2H_4 but *B. cinerea* has not been reported to do so. One objective of this study was to establish whether *B. cinerea* is capable of producing C_2H_4 *in vitro*. To achieve this objective, 4 potential precursors of C_2H_4 (methionine, glutamate, α-ketoglutarate and 1-aminocyclopropane-1-carboxylic acid (ACC)) were added to Pratts modified medium at a range of pH's using two different systems of incubation (shake and static culture). Methionine was shown to be the most efficient precursor of C_2H_4 under both shake and static culture systems, with optimum pH being 3.5 and 4.5 respectively. ACC is known to be a precursor of C_2H_4 in higher plants but it did not result in C_2H_4 production in *B. cinerea*, either alone or when added with methionine. Although methionine was a substrate of C_2H_4 production by *B. cinerea*, this production was significantly inhibited by α-aminooxyacetic acid (AOA), indicating that a pyridoxal phosphate (PLP) mediated reaction might be involved. This inhibition was not reversed by addition of ACC suggesting that ACC is not the immediate precursor of C_2H_4 in *B. cinerea*. Cobalt ions (Co^{++}) added to a culture medium supplemented with methionine, had a temporary inhibitory effect on C_2H_4 production by *B. cinerea* compared with methionine alone. This inhibitory effect soon disappeared, with the C_2H_4 peak in the Co^{++} treatment reaching the same level as for methionine, only delayed by 2-4 days. This suggests that the ethylene-forming enzyme (EFE) complex in *B. cinerea* is different from that in higher plant. These results have shown that under defined conditions *B. cinerea* is capable of producing C_2H_4 from methionine but that the biosynthetic pathway appeared to be different from that present in higher plants.

Increased C_2H_4 production in response to stress is a common feature of plants. In an experiment at 20°C, kiwifruit infected with *B. cinerea* produced more C_2H_4, than uninfected fruit, even when the latter were physically damaged, or wounded, by drilling a hole through the stem scar. At 0°C, no ethylene was produced by wounded or healthy fruit and only infected fruit were shown to produce C_2H_4. Healthy fruit stored with infected fruit in the same tray did not produce C_2H_4. These results suggest that at low temperature C_2H_4 production by infected fruit may not trigger an autocatalytic response from healthy fruit in the same tray. At 0°C, wounding of fruit or C_2H_4 in the environment did not trigger the autocatalytic response in kiwifruit but infection caused by *B. cinerea* did trigger this response. This suggests that infection may have activated the ACC synthase and ACC oxidase genes of the C_2H_4 pathway which consequently caused an autocatalytic response by the fruit.

A few reports have suggested that the increased C_2H_4 production in response to infection may arise from noninfected tissue at the periphery of infection. Use of slices from different parts of infected kiwifruit has shown that most ethylene was produced by the healthy tissue immediately ahead of the infection front. This suggests that in these tissues a transmissible signal was produced which could be acting as an elicitor of C_2H_4 production. Such an elicitor may have been a compound produced by the fungus itself, or it may have been produced as a result of secreted fungal enzymes acting on cell wall polysaccharides. Pectic and xyloglucan oligomers derived from polysaccharides are known to induce C_2H_4 in other plant systems. The nature of the C_2H_4 elicitor in *B. cinerea* infected kiwifruit tissue has not been determined, but some possibilities have been discussed.

Little or no ethylene was produced by infected kiwifruit tissue while ACC and ACC oxidase levels were no less than in healthy tissue. This suggests that the entire ethylene biosynthetic pathway was intact in these infected tissues. While all the individual components necessary for C_2H_4 synthesis were present the biosynthetic pathway could not operate in infected tissue. The reason for this is not known but could include inadequate oxygen (O_2) levels for C_2H_4 production in water soaked tissue; presence of a fungal produced toxin which inhibited the action of C_2H_4 enzymes or receptors; or lack

of EFE activity in tissue where membrane integrity was destroyed as a result of infection.

This work has provided an opportunity to study in more detail the effect of *B. cinerea* infection on localized kiwifruit tissue. Although this study did not answer all the questions it has answered some difficult and interesting ones.

This study has shown that *B. cinerea* can form ethylene from methionine using a non ACC pathway and that ethylene production is enhanced ahead of the infection front but ceases in diseased tissue. The questions raised by this study which requires further research are the steps involved in ethylene production by *B. cinerea* and the mechanism by which ethylene production is enhanced ahead of the infection front.

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CHAPTER 1

INTRODUCTION

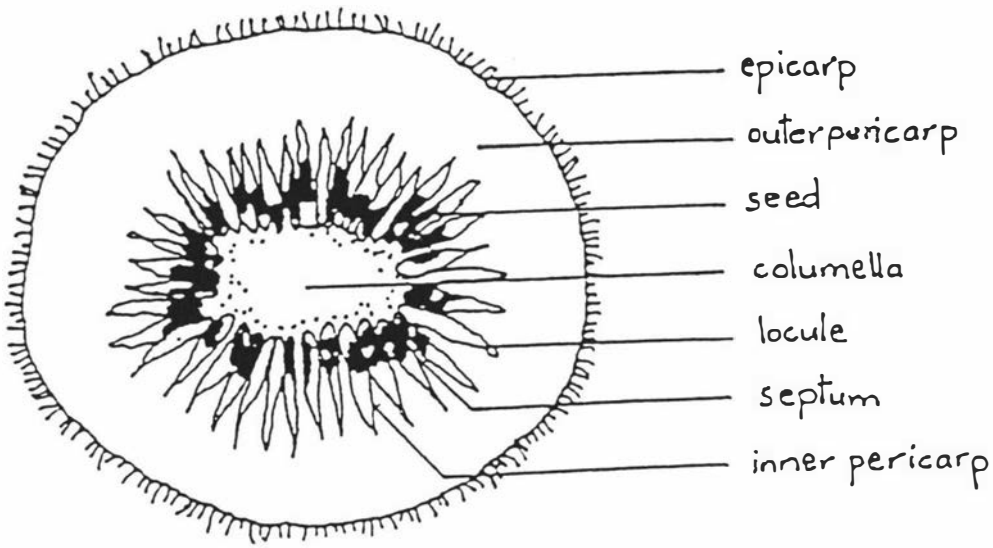
The kiwifruit {*Actinidia deliciosa* (A.Chev.) C.F Liang & A.R. Ferguson, formerly *Actinidia chinensis* Planch.} is one of four new fruit successfully introduced into commercial trade this century (Janick, 1992). In a period of 30 years it has become an important commercial crop grown in many temperate countries in the world. Originally from China, plants and seeds of *A. deliciosa* were sent to Europe, the United States of America and to New Zealand during the period 1900-1915. In New Zealand commercial kiwifruit can be traced back to two female plants and one male plant which originated from a single introduction of seed in 1904 (Ferguson and Bollard, 1990). Hayward has emerged as the most popular commercial cultivar in New Zealand and in most of the commercial plantings throughout the world.

Kiwifruit has brown skin which is densely covered with long, stiff hairs (Ferguson, 1984). The pericarp or the edible portion of kiwifruit is green which makes it unusual and it has commercial importance because it is considered to be an attractive attribute. This green colour is due to the presence of chlorophyll in the pericarp cells (Robertson and Swinburne, 1981). Concentrations of chlorophyll found in the chloroplast are similar to those of other fruits such as muskmelon (Reid et al, 1970) and tomato (Simpson et al, 1976) that are green when immature. The kiwifruit retains a bright green colour when ripe, with chlorophyll levels falling only slowly during prolonged storage, in a similar manner to that observed in senescing leaves (Possingham et al, 1980; Ben-Arie et al, 1982).

A transverse section across a mature fruit (Fig 1.1) shows that the edible part is made up of three different portions: the core, the seed area (inner pericarp) and the flesh (outer pericarp). The shape of individual cells and the assembly of cells in the three tissue types is different; this heterogeneity is considered to give rise to physiological differences during ripening of the fruit (MacRae, 1989a). The levels of sugars and organic acids differ in each of these portions (Redgwell et al, 1988). Fruit maturity affects the relative amount of sugars and acids present

FIG. 1.1

MID CROSS-SECTION OF MATURE 'HAYWARD' KIWIFRUIT (1).



(Wright and Heatherbell,1967; Heatherbell, 1975) and differences in chemical composition occur between the portions as maturity of the fruit progresses. Fruit taste is known to be influenced by sugars and acids, the levels of which change during long term storage. The extent of these changes depend on maturity at harvest and on the conditions under which fruit is stored (Ferguson, 1984).

B. CINEREA AS A PATHOGEN

Pests and diseases in general were not a problem during the early stages of industry development (Bailey, 1950, 1961; Bailey and Topping, 1951; Schroeder and Fletcher, 1967). As planting become more intensive, incidence of pests and diseases increased requiring application of comprehensive spray programmes (Sale 1980; Brook 1990b). Hawthorne et al, (1982; Hawthorne and Otto, 1986) and Pennycook (1984; 1985a; b) identified a few storage diseases in stored kiwifruit (Table 1.1), but these were not a severe commercial problem initially. As the kiwifruit industry expanded an increasing proportion of the crop was kept in store at 0°C, to spread out the marketing season especially in the Northern hemisphere. As storage time increased it was found that *B. cinerea* became a serious problem in some years, causing direct losses because of

Table 1.1

FUNGAL DISEASES OF KIWIFRUIT

Causal Pathogen	Major Diseases
Botrytis cinerea	Botrytis storage rot
Botryosphaeria dothidea	Ripe rot
	Minor Diseases
Alternaria alternata	Superficial storage blotch
Fusarium acuminatum	Storage rot
Cryptosporiopsis spp.	Storage rot
Phomopsis spp.	Storage rot
Diaporthe actinidiae	After storage, rots appear *Diaporthe* sp.I
at ambient temperature	
D. perniiciosa.	

infected fruit and creating additional costs to the industry because of the need to repack trays prior to export. *B. cinerea* was only one of several fungi found to be present. This has caused serious commercial problems for stored kiwifruit in New Zealand (Beever et al, 1984).

The grey mould pathogen, *B. cinerea*, is responsible for causing serious pre and postharvest losses on numerous fruits, vegetables, flowers and ornamental plants. Although normally regarded as a weak pathogen, it is non-specific in its host range and is responsible for considerable economic losses on a global scale (Heale, 1992). In kiwifruit *B. cinerea* storage rot causes significant losses in Italy (Bisiach et al, 1984), California (Weet, 1979; Sommer et al, 1983), and New Zealand (Pennycook, 1984; 1985a). In New Zealand, after fruit softening, storage rot is the most important factor in losses to stored kiwifruit (Pak and Manning, 1994).

Coley-Smith (1980) has described several reasons why *B. cinerea* is such a successful pathogen. *B. cinerea* produces many spores and wind can carry them a great distance. These spores do not need free water, but can germinate in conditions of high humidity. Because it is multi nucleate it can adapt to diverse conditions, such as a wide range of hosts growing under different conditions and range of temperatures. As *B. cinerea* is a non specific pathogen it can attack almost any plant and can even germinate on dead plant parts. Sclerotia are structures of *B. cinerea* which can survive for more than one season.

Detailed information on the initial infection process is necessary to allow application of appropriate and effective control measures against *B. cinerea*. A well known pattern of *B. cinerea* infection occurring in several other fruits, is known as "quiescent", or "latent" infection (Burton, 1994; Jarvis, 1994). Verhoeff (1974) defined 'latency' as inhibition in the growth of fungi between the arrival of inoculum and progressive disease development. In strawberries, invasion by *B. cinerea* takes place at flowering when the fungus establishes a small focus of mycelium in tissue at the base of the fruitlet receptacle. This stays quiescent until fruit reach a certain stage of maturity, and then "explodes" to colonise the ripe strawberry (Jersch et al, 1989). In New Zealand, Beever

(1979) suggested that *B. cinerea* infection in kiwifruit was a quiescent infection, initiated at flowering. In Italy Bisiach et al, (1984) were definite in stating "... that *B. cinerea* contamination occurs through the flower residues from the end of the blossoming until harvest. Penetration through the wound of the stem, at the moment of harvest, is of secondary importance". However Manning and Pennycook (1985) and Pennycook (1985b) found that stem-end *Botrytis* rot originated from contamination and infection of the picking wound at harvest, not from a quiescent infection established at blossom (Brook, 1990a). It has now been confirmed that *B. cinerea* infection in kiwifruit is not a quiescent infection; rather it is a slow and continuous progression of hyphal growth through the stem scar of kiwifruit resulting from spores depositing on the wound of the fruit stem scar at or shortly after harvest (Hallett at al, 1991). Although kiwifruit are contaminated at harvest, the first symptoms of *B. cinerea* infection may not appear until the fruit has been stored at 0°C for 6-8 weeks (Pennycook, 1984). Once the fungus is established, and hyphae have penetrated through the stem scar, the rot advances evenly along the fruit length towards the distal end. External symptoms of *B. cinerea* infection can be seen as dark green colour of the skin compared with the light green healthy part of the fruit. The extent of the internal progression of the disease down the fruit is often indicated by the presence of a dark ring. As the disease progresses, mycelium emerges from the rotten fruit, and often it can be seen on the stem end of the diseased fruit.

Incidence of Botrytis storage rot in kiwifruit is unpredictable and highly variable. This variability occurs from year to year and from orchard to orchard, and is observed between samples of fruit picked from the same vines on different days (Pennycook 1984). It appears however, that the main factor influencing severity of *B. cinerea* storage rot is the weather at the time of harvest. It is generally accepted that, in kiwifruit, epidemics caused by *B. cinerea* occur during warm, wet and humid weather conditions. Such conditions are widely known to be favourable for sporulation and infection. Kiwifruit picked during the first one or two days after rain are reported to be more susceptible to *B. cinerea* storage rot than fruit picked before or during the rain (Brook, 1990a).

It has been shown that infected kiwifruit in a tray induce softening in healthy fruit.

Pennycook and Manning (1987, unpublished work, cited by Brook, 1990a) found that when 4 infected kiwifruit were placed inside a tray with healthy fruit, the softening rate of healthy fruit was faster than when 1 infected kiwifruit was used. Although ethylene levels inside these trays was not measured, it is possible that higher concentrations of ethylene were produced from the 4 infected kiwifruit than from a single infected fruit causing more rapid softening of noninfected fruit.

Infection often causes increased ethylene production by plants (Archer and Hislop, 1975; Elad, 1990; Graham and Linderman, 1980; Tongthieng and Sangchote, 1994). The relationship between infection and ethylene production is still unclear (Abeles, 1973; Archer and Hislop, 1975; Sequeira, 1973). In kiwifruit it is not known whether the pathogen somehow induces the host to produce ethylene. Ethylene is induced as a result of infection and subsequent tissue damage caused by *B. cinerea*, whether the pathogen itself produces ethylene, or whether a combination of all these factors occurs following infection.

ETHYLENE BIOSYNTHESIS IN HIGHER PLANTS

Ethylene, one of the simplest organic molecules with biological activity, is a plant hormone that regulates many aspects of plant growth, development and senescence (Yang and Hoffman, 1984; Mattoo and White, 1991; Abeles et al, 1992). It plays an important regulatory role in the physiology of plants and in particular, the senescence and postharvest physiology of fruits and vegetables. Therefore, depending on where and when it occurs, it may be beneficial or harmful to harvested horticultural crops (Yang, 1985; Yang, 1987a,b). Because of its potent effects on induction of plant senescence, large losses of fruits and vegetables have incurred in both developed and developing countries, if ethylene production and ethylene levels in storage environment were not controlled. Losses are often high in developing countries because of lack of postharvest handling systems and of adequate temperature control (Theologis et al, 1992). The effectiveness of horticultural postharvest systems, therefore, can be improved by the ability to regulate ethylene synthesis and/or responses to suit specific practical needs (Yang and Hoffman, 1984; Yang, 1985; Matto and White, 1991). In order to control and manipulate plant susceptibility and response to ethylene, a fundamental understanding

of ethylene biosynthesis and action is required.

Ethylene synthesis in higher plants is known to occur through a pathway involving, L-Methionine ——— *S*-adenosyl methionine (SAM) ——— 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979). The enzymes involved in this pathway are ACC synthase and ACC oxidase (Kende, 1989; 1993) which catalyze the conversion of SAM to ACC and ACC to ethylene, respectively (Yang and Hoffman, 1984; Imaseki, 1991; Kende, 1993). In addition to ACC, ACC synthase also produces 5'-methylthioadenosine (Boller et al, 1979; Yu et al, 1979) which is utilized for the synthesis of new methionine via the modified methionine or Yang cycle (Miyazaki and Yang, 1987; Abeles et al, 1992; Theologis, 1992). This makes it possible for high rates of ethylene biosynthesis to be maintained even when the free methionine pool is small (Kende, 1993).

ACC synthase is a key enzyme and is the main site of control of ethylene production (Yang, 1980; Yang and Hoffman, 1984). It was first identified in an homogenate of tomato pericarp tissue (Boller et al, 1979; Yu et al, 1979), and has subsequently been found in many ethylene producing plants such as winter squash (Hyodo et al, 1983), mungbean (Tsai et al, 1988) and apples (Bufler and Bangerth, 1983; Yip et al, 1991). Its activity is enhanced by factors that promote ethylene formation, including auxin and different stress conditions (Kende, 1993; Osborne, 1989; Thomas and Yang, 1987; Yang and Hoffman, 1984).

In addition to serving as a precursor of ethylene, ACC can be metabolized to a presumably biologically inactive end-product, 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) (Hoffman et al, ,1982; Yang and Hoffman, 1984; Imaseki, 1991), a reaction catalyzed by ACC malonyltransferase (Liu et al, 1984). This malonylation of ACC participates in the regulation of ethylene biosynthesis (Liu et al, 1984; Liu et al, 1985a; Abeles et al, 1992).

The last step in the ethylene biosynthetic pathway is the conversion of ACC to ethylene. This is an oxygen (O_2) dependent reaction catalysed by ACC oxidase (Abeles et al,

1992). Early work noted that disruption of plant tissue, leading to the destruction of membrane integrity, resulted in an inhibition of ethylene production (Burg and Thimann, 1960; Inaba and Nakamura, 1986). This suggested that ACC oxidase was highly structured and was located on a cellular membrane (Lieberman, 1979). Recently, extraction and assay procedures have been developed to maintain the *in vitro* ACC oxidase activity in extracts from ripening melon (Ververidis and John, 1991). Following this discovery, significant progress has been made in the purification and characterization of ACC oxidase (Dong et al, 1992; Dilley et al, 1993; Fernandez-Maculet et al, 1993; Hyodo et al, 1993; Mathooko et al, 1993; Poneleit and Dilley, 1993; Smith and John, 1993; Yang et al, 1993; Finlayson and Reid, 1994). These reports have indicated that while O_2 is necessary for activity of this enzyme in the tissue, it's elimination during extraction is critical for *in vitro* activity and that ACC oxidase is activated by CO_2. The presence of ascorbate and Fe^{++} was required for stability (Ververidis and John, 1991).

PRODUCTION OF ETHYLENE BY MICRO-ORGANISMS

While a great deal of detailed information is now available on ethylene synthesis in higher plants, there is much less certainty about the production of ethylene from lower plants (Osborne, 1989). A wide range of lower plants are known to produce ethylene (Fukuda et al, 1993) including unicellular algae (Maillard et al, 1993), lichens (Lurie and Garty, 1991), and mosses (Rohwer and Bopp, 1985). Ethylene is a common metabolic product of bacteria (Freebairn and Buddenhagen, 1964, Goto et al, 1985), fungi (Ilag and Curtis, 1968) and yeasts (Thomas and Spencer, 1977); but there is little consensus on the nature of ethylene precursors, intermediates and enzymes involved in ethylene synthesis in such plants.

Fukuda et al (1984b) screened a wide range of microorganisms (including 80 genera, 150 species and 137 strains which were clearly distinct taxonomically) for ethylene producing capacity. Of these 30% produced ethylene (60% were fungi, 20% yeasts, 11% were bacteria and 6% were actinomycetes) and many ethylenogenic microbes are pathogenic (Fukuda et al, 1993).

Production of ethylene by a fungus was first recognized by Gane (1934) who found that growth of pea seedlings was inhibited by the atmosphere from bakers' yeast when the cultures were grown aerobically. Later Biale (1940), and Miller et al, (1940), showed independently that the common green mould of citrus, *Penicillium digitatum*, produced a physiologically active emanation that had the same effect on plant tissues as that caused by ethylene gas. Subsequently it was shown that this fungus produced the emanation whether grown on its natural host or in culture (Biale and Shepherd, 1941). Ilag and Curtis (1968) carried out detailed studies with various groups of fungi reporting that 58 of the 228 species examined produced ethylene. The ratio of ethylene producing to non-producing species in different groups of fungi was: 1:31 in phycomycetes; 1:10 in ascomycetes; 1:6 in basidiomycetes; and 1:4 in fungi imperfecti. Within the fungi imperfecti, the ratio in the class Moniliales was 1:3. *Botrytis spectabilis*, a member of the class Moniliales, did produce ethylene, but *B. cinerea* was not included in this survey.

Various fungi have been shown to produce ethylene from a range of precursors (Fukuda et al, 1993) such as glucose (Thomas and Spencer, 1978), glutamate (Chou and Yang, 1973), or methionine (Chalutz and Lieberman, 1977; Axelrood-McCarthy and Linderman, 1981; Wilkes et al, 1989).

Methionine, the precursor of ethylene in higher plants, was found to be the precursor in fungi under some conditions. *P. digitatum* used methionine to produce ethylene in shake but not in static cultures (Chalutz and Lieberman, 1977), but methionine was not a substrate in a cell-free ethylene-forming system from *P. digitatum* (Fukuda et al, 1986). Chalutz and Lieberman (1977) suggested that production of ethylene in shake cultures, in response to methionine, involved induction of an ethylene-synthesising enzyme system(s) in the fungal cell. They indicated that methionine was a precursor of ethylene produced by the fungal cell, but not by the filtrate, suggesting that the fungus took up methionine from the medium and, presumably, released a metabolite into the medium, which, in turn, was converted to ethylene. While *Verticillium*, *Fusarium* and *Colletotrichum* produced ethylene in the presence of methionine in both shake and static cultures (Tzeng and DeVay, 1984), this induction was concentration dependent; with

P. digitatum (Chalutz and Lieberman, 1977) there was a linear increase in ethylene production as methionine concentration increased from 0.07 m*M* to 7m*M* above which there was a steady decline in ethylene production.

Considerable efforts have been made to determine the pathway for ethylene biosynthesis in lower plants, particularly in fungi. Currently ethylene production in fungi is thought to occur by more than one pathway. Fukuda and Ogawa (1991) and Fukuda et al, (1993) divided ethylene forming microbes into two groups: (a) those synthesising ethylene from methionine, and (b) those synthesising it from glutamate or α-ketoglutarate. Ethylene production in *P. digitatum* was closely associated with the Krebs cycle as 2-oxoglutarate and glutamic acid were found to be efficient precursors (Chou and Yang, 1973). These findings were confirmed by Chalutz and Lieberman (1977) who found that glutamic acid and α-ketoglutarate were precursors of ethylene in static cultures of *P. digitatum.*

It appears that ethylene is produced from different precursors, depending on whether the fungus is cultured under static or shake conditions. Glutamate was the precursor in *P. digitatum* under static conditions, while methionine could act as a precursor in shake cultures of the fungus (Chalutz and Lieberman, 1977). Hottiger and Boller (1991) have also reported glutamate as an ethylene precursor in *Fusarium oxysporum* f.sp. *tulipae*. They showed that cultures grown in liquid produced high amounts of ethylene during the stationary phase of fungal growth and confirmed that glutamic acid was a precursor of ethylene. Interestingly, kainic acid, a cyclic analogue of glutamic acid, which inhibits glutamate metabolism (Schmidt et al, 1976), had a stimulatory effect on ethylene production by *P digitatum*. It was suggested that perhaps by inhibiting alternate uses, the availability of glutamate for ethylene biosynthesis was increased (Chalutz and Lieberman, 1978).

Miyazaki and Yang (1987) demonstrated that an ATP dependent methionine salvage process occurs in higher plants, with α-Keto-γ-methylthiobutyric acid (KMBA), a precursor of methionine, being an intermediate of this cycle. Although there is no proof that such a system occurs in microorganisms, KMBA has been shown to increase ethylene production by microorganisms. Primerose (1977) suggested that KMBA might

be an intermediate in ethylene production. Billington et al, (1979) showed that KMBA is a common metabolic product of several microoganisms when cells are grown in the presence of methionine. Ince and Knowles (1986) found that KMBA is an intermediate of ethylene biosynthesis in *Escherichia coli*. Tzeng and DeVay (1984) reported that KMBA added to cultures of *Verticillium dahlia* Kleb stimulated ethylene production.

ACC is the direct precursor of ethylene in higher plants (Adams and Yang 1979), but its role in lower plants is not clear. Osborne (1989) has suggested that higher and lower plants may have different biosynthetic pathways for ethylene production based on feeding studies with ACC. As ethylene was not produced by a wide range of lower plants when ACC was added, it was suggested that the ethylene pathway of higher plants is a modern adaption to land. Working with a cell-free ethylene-forming system from *P. digitatum*, Fukuda et al, (1986) showed that ACC added to the mycelium did not result in ethylene formation. Similarly, Rupp et al, (1989) found that ACC was not a precursor of ethylene formation by *Laccaria laccata*. Ethylene production was actually inhibited when ACC was added to cultures of *V dahlia* (Tzeng and DeVay, 1984) or *Laccaria* spp. (Livingston, 1991) and ACC was not detected in a mycelial extract of *F. oxysporum* f.sp. *tulipae* (Hottiger and Boller, 1991), However, ACC did promote ethylene formation in sterile cultivated protonema of the moss *Funaria hygrometrica* (Rohwer and Bopp, 1985). Similarly, when ACC was added to the medium in which the unicellular green alga, *Haematococcus pluvialis* was growing, it enhanced ethylene production (Maillard et al, 1993) suggesting the existence of an ACC dependent pathway.

In higher plants it is possible to control ethylene production by using chemicals which block the synthesis and/or activity of ACC synthase or ACC oxidase. Rhizobitoxine and its synthetic analogues, α-aminooxyacetic acid (AOA) & aminoethoxyvinylglycine (AVG), inhibit the action of ACC synthase in higher plants markedly reducing but not entirely eliminating ethylene production (Yang and Hoffman, 1984). Addition of AOA to methionine containing medium had no effect on in vitro ethylene production by *V dahlia* (Tzeng and DeVay, 1984). In *P syringae* pV *phaseolicola*, ethylene production from glutamate was not inhibited by AVG, and was slightly inhibited by AOA,

irrespective of concentration. However, methionine and ACC were not precursors of ethylene production in this system (Goto et al, 1985). In the lichen *R duriaei*, stimulation of ethylene occurred when glutamate, methionine or ACC were provided. However, when AVG was added to the medium with methionine, it did not inhibit ethylene production suggesting that ACC was not an essential intermediate (Lurie and Garty, 1991). It seems that an alternate pathway of ethylene production, not including ACC may exist in lower organisms since they do not respond to some of those precursors used by higher plants and conventional inhibitors of this pathway are usually ineffective.

The ethylene forming enzyme (ACC oxidase) which catalyses the final step in ethylene biosynthesis from ACC to ethylene was recovered and purified only recently from higher plants (Ververidis and John, 1991). However, partial purification of an ethylene forming enzyme from a fungus was achieved by Fukuda et al, (1986) from *P. digitatum* which produced ethylene from α-ketoglutarate, but not from methionine. α-Ketoglutaric acid was found to be the immediate precursor of ethylene production in *P. digitatum* (Fukuda et al, 1986). Interestingly, the purification of EFE from *P. digitatum* was the first instance of purification from an ethylene-producing microorganism or from higher plants (Fukuda et al, 1986). Although EFE is important in fungi using glutamate as a precursor it may not be in fungi using methionine as a precursor of ethylene, as in the latter case, the last step conversion of KMBA to ethylene may be non enzymatic and based on a chemical reaction (Fukuda et al, 1993).

Cobalt ions (Co^{++}) have been shown to inhibit a range of reactions in plants (Paliti et al, 1994). In plants, the final step in the biosynthesis of ethylene can be inhibited by Co^{++}. Application of Co^{++} resulted in endogenous ACC accumulation in mung bean (*Vigna radiata* [L.]Wilczek), while ethylene production declined (Yu and Yang, 1979). In the unicellular green alga, *Haematococcus pluvialis*, ethylene production increased when ACC was added; this ACC dependent pathway was further stimulated by Co^{++} but inhibited by Cu^{++} and salicylhydroxamic acid. This suggests that the enzymatic complex involved in the last step of ethylene synthesis in lower plants may be different from that in higher plants where Co^{++} inhibits ethylene production (Maillard et al, 1993). Fukuda

et al, (1986) suggested that in cell free system of *P. digitatum* some kind of Fe^{++} complex might be required for the formation of ethylene. The divalent transition metal ions Co^{++}, Cu^{++} and Mn^{++} inhibited ethylene-forming activity to a certain extent probably by competing with Fe^{++} for formation of such a complex. It is suggested that there is a pathway of ethylene production which does not involve ACC and it is possible that the immediate precursor of ethylene in fungi may be either KMBA or α-ketoglutarate (Fukuda et al, 1993).

Ethylene production in microorganisms is affected by several factors such as presence of phosphate, reducing agents, Fe^{++}, pH of the incubating medium and phase of fungal growth (Arshad and Frankenberger, 1992). Ferrous ions (Hottiger and Boller, 1991) and ascorbate (Fukuda et al, 1989a) markedly stimulated the rate of ethylene production by *F. oxysporum* f.sp. *tulipae* and *P. digitatum* grown on glutamate or 2-oxoglutarate. Ethylene biosynthesis was strongly inhibited by the heavy metal chelator α,α'-dipyridine; this effect was fully reversed by Fe^{++} ions but not by supplying glutamate or 2-oxoglutarate (Hottiger and Boller, 1991). This indicates that a step in the ethylene biosynthetic pathway downstream of 2-oxoglutarate is dependent on Fe^{++}. This requirement for Fe^{++} ions and a reducing agent (ascorbate) to convert α-ketoglutarate to ethylene in a fungus, is similar to that reported for obtaining ACC oxidase activity in extract from higher plants (Smith et al, 1992). This suggests that there may be some similarity between EFE from higher plants and that from microorganisms. Since fungal (*P. digitatum*) EFE did not transfer ACC into ethylene these two EFE may not be identical.

It is not clear whether ethylene production is always dependent on rate and phase of fungal growth. Pratt (1944) showed it to be related to active growth and respiration in *P. digitatum* but Spalding and Lieberman (1965) found that the period of greatest ethylene production occurred as growth rate started to decline.

The pH of culture media could be a critical factor influencing ethylene production by lower organisms. It is important in higher plants where ACC dependent ethylene production occurs. McGarvey and Christoffersen (1992) and Smith et al, (1992) found that pH 8.5 was optimal for ACC oxidase activity in avocado and melon. With fungi

there has been little work to determine the optimum pH level for ethylene production. The fungus *P digatitum* has been shown to produce ethylene over a range of pH's, from 3 to 6 (Chou and Yang (1973); Jacobsen and Wang (1968); Spalding and Lieberman (1965); Chalutz and Lieberman (1978); Young et al (1951); Fukuda et al, (1986)). In studies with other fungi such as *Verticillium*, *Fusarium* and *Colletotrichum* species the media used was adjusted to pH 5.0 and ethylene was produced (Tzeng and de Vay, 1984). With the bacteria *Pseudomonas syringae* pv. *phaseolicola* (Goto et al, 1985), and *E coli* (Ince and Knowles, 1986), the pH's were adjusted to 6.8 and 7.0 respectively and ethylene was produced. The influence of other pH's was not reported. There appear to be no published reports suggesting optimal pH's for ethylene production from any fungi regardless of precursors or growing conditions used.

Ethylene production in fungi has been shown to be influenced by phosphate level in the medium. In *P. digitatum* low levels of phosphate (0.001 m*M*) induced 200-500 times more ethylene production than high phosphate (100 m*M*) levels; the low level phosphate stimulation was reversed by addition of phosphate to the culture medium (Mattoo et al, 1979). Increase in ethylene production by *P. cyclopium* was related to depletion of phosphate content in the medium (Pazout et al, 1982). Later this ethylene production was found to occur in two phases depending on phosphate content in the medium and on the stage of fungal growth. For example *P. cyclopium*, grown in static culture with 2-oxoglutarate or glutamate, produced ethylene at two stages of the growth cycle. The first peak was associated with aerial mycelium formation, whereas the second, higher, peak occurred during formation and maturation of conidia (Pazout and Pazoutova, 1989).

Ethylene production by many groups of fungi has been reported but there is little published information relating to *B. cinerea.* Early reports (Smith et al, 1964) suggested that *Botrytis* spp. isolated from carnations, did not produce ethylene *in vitro*. Elad (1990) confirmed that *B.cinerea*, grown on various nutrient sources, did not produce ethylene, although application of methionine to inoculated tissues did accelerate disease development.

STRESS ETHYLENE IN HIGHER PLANTS

A number of factors influence ethylene biosynthesis in higher plants. An understanding of these factors may be useful in controlling ethylene synthesis and ultimately allowing manipulation of crop response. A number of stress conditions promote ethylene production by plant tissues and this has proven to be universal among plants. Such stresses include exposure to chemicals, chilling temperature, drought, flooding, radiation, insect damage, disease and mechanical wounding (Abeles et al, 1992; Mattoo and Suttle, 1991). Ethylene produced in response to infection in diseased tissue as a result of host-parasite interaction, is thought to be a result of wounding and mostly of host origin (Mattoo and Suttle, 1991), being produced partially or entirely by the plants as a response to infection (De Laat and Van Loon, 1983). A large amount of ethylene is produced by detached leaves of tomato, pepper, french-bean and cucumber infected with *B. cinerea* compared with noninfected leaves (Elad, 1990). *Pseudomonas solanacearum* itself does not produce ethylene, but infection by this pathogen induced ethylene production in the host plant (Primerose, 1979). However ethylene may originate from the pathogen, as has been shown for a range of bacteria and fungi (Chalutz and Lieberman, 1978; Ilag and Curtis, 1968; Fukuda et al, 1993). At different stages of infection the major portion of ethylene may be produced by either fungus or plant. In grapefruit (*Citrus paradisi*), the early relatively low rate of ethylene production in *P. digitatum* infected fruit seemed to originate mostly from the fruit tissue. Since grapefruit tissues in advanced stages of infection were incapable of converting ACC to ethylene (Achilea et al, 1985a), the large increase in ethylene production from the grapefruit infected tissue which occurred in later stages of infection probably originated from the fungus. Exogenously applied ACC led to a marked increase in ethylene production by healthy citrus peel tissue (Achilea et al, 1985b; Hyodo and Nishino, 1981), but had no effect on ethylene production by *P. digitatum* infected peel discs. This suggested that ethylene production from infected peel was not of plant origin (Achilea et al, 1985b) but originated from the fungus which may produce ethylene by a pathway not involving ACC (Fukuda et al, 1986). A wide range of fungi have been reported to induce hosts to produce ethylene in response to infection (Table 1.2).

HARMFUL EFFECTS OF ETHYLENE ON STORED KIWIFRUIT

The presence of ethylene in the storage environment is harmful to stored kiwifruit. Although kiwifruit do not produce a significant level of ethylene at 0°C, they are responsive to ethylene above a threshold level that may be present in the storage environment. Hyodo and Fukasawa (1985) have shown that at 21°C individual kiwifruit began to produce ethylene at an increasing rate after reaching a minimum threshold production of 0.1 µl/kg/h. It is not known if a threshold level exists for other temperatures. Softening of kiwifruit held at 0°C occurs in the continuous presence of ethylene at concentrations of 0.03 to 0.1 ppm (Harris 1981; Harris and Reid, 1981; McDonald and Harman 1982; Billing 1986; Arpaia et al, 1985). Therefore, kiwifruit are regarded as being sensitive to the continued presence of ethylene during storage (Fletcher et al, 1959; Pratt and Reid, 1974; Lallu et al, 1989).

In kiwifruit, there is a relationship between concentration of propylene and fruit softening at both 0°C and 20°C. The low temperature (0°C) blocked autocatalytic production of ethylene but not fruit softening (Sfakiotakis et al, 1989).

A relationship was observed with ethylene at 20°C; a concentration of 3 ppm ethylene was required for a minimum of 5 to 6 hours to trigger a permanent softening response from freshly harvested kiwifruit. A longer exposure time is required for lower ethylene concentrations. A relatively short exposure time of 2 hours had no effect on softening even at 10 ppm. (Lallu and Elgar, Pers. com).

PLANTS DEFENCE MECHANISM

Plants have a range of defence mechanisms which may be induced in response to fungal invasion (Bell, 1981; Hahn et al, 1989). These defencer esponses include induction of enzymes such as chitinase (Grando and Boller, 1993) and 1,3-β-glucanase (Wubben et al, 1993). Chitin is commonly a component of the fungal cell wall (Collinge, 1993). Chitinase inhibits growth of many fungi *in vitro* by causing lysis of hyphal tips, especially when applied in combination with 1,3-β-glucanase (Broekaert et al, 1988; Broglie et al, 1991; Mauch et al, 1984). Chitinase produced by infected plant tissue has been shown to accumulate around fungal hyphal material within plants

Table 1.2

C_2H_4 production by plants fungi in culture medium and by plants in response to fungal infection.

Pathogen	Host	Specific response	Reference
Agaricus bisporus	-	Mushroom produced ethylene.	Lockard and Kneebone (1962)
Alternaria and *Gloeosporium* spp.	Navel orange	C_2H_4 and fungal infection were associated with blossom-end yellowing of navel orange.	Southwick et al, (1982)
Aspergillus parasiticus	Culture medium	Dimedone, an aldehyde trapping agent, was found to stimulate C_2H_4 evolution without any effect on the growth of fungus.	Sharma et al, (1988)
Bipolaris sorokiniana	Poa pratensis leaves	Enhanced C_2H_4 was responsible for much of the chlorophyll loss	Hodges and Coleman (1984)
	Poa pratensis leaves	Stimulated ACC synthase activity and C_2H_4 production during pathogenesis	Coleman and Hodges (1984)
	Poa pratensis leaves	C_2H_4 was produced when methionine; added ACC was not efficiently converted to C_2H4.	Coleman and Hodges (1986)
Blastomyces dematitidis	-	Fungus produced C_2H_4 causing epinasty.	Nickerson (1948)
Botryosphaeria and/or *Phomopsis*	Kiwifruit	Fungal infection caused increased C_2H_4 production and ACC activity.	Hasegawa and Yano (1990) Yano and Hasegawa (1993)
Botrytis cinerea	Black currant	Substantially higher concentration of C_2H_4 produced by inoculated flowers than uninoculated controls.	McNicol et al, (1989)
	Carrot root slices	C_2H_4 biosynthesis is required for the accumulation of 6-methoxymellein, and for the fungal resistance response.	Hoffman et al, (1988)
	Kiwifruit	Infection caused increased C_2H_4 production	Niklis et al, (1992)
	Rose leaves, petal and flowers	C_2H_4 production was correlated with the severity of disease STS, AOA and AVG decreased this severity but C_2H_4 production diminished when host become completely macerated.	Elad (1988)
	cucumber, pepper, Tomato and French-bean leaves	Fungal infection increased C_2H_4 production but AOA and AVG decreased severity of disease.	Elad (1990)

	Tomato and Pepper leaves	C_2H_4 production was inhibited in tomato leaves treated with propyl gallate, ascorbic acid and benzoic acid, but not in pepper leaves.	Elad (1992)
Cercospora arachidicola	Peanut leaves	Increase in C_2H_4 production coincided with disease symptoms, i.e., defoliation and abscission	Ketring and Melouk (1982)
Ceratocystis fimbriata	Sweet potato root tissue	Enhanced C_2H_4 production in response to fungal infection.	Hirano et al, (1991)
	Potato infusion medium	C_2H_4 biosynthesis pathway of fungus involved methionine but not ACC	Hirano et al, (1991)
Chaetomium globosum	Carrot cells	Fungi-infected carrot cells generated an appreciable amount of C_2H_4 that seemed to be sufficient for induction of chitinase activity	Kurosaki et al, (1990)
Colletotrichum musae	Banana	Diseased fruit produced early and more C_2H_4 than control fruit.	Tongthieng and Sangchote (1994)
Colletotrichum lagenarium	Cucumber seedlings	Pretreatment with C_2H_4 increased the incidence of anthracnose.	Biles et al, (1990)
	Melon seedlings	C_2H_4 and cell wall hydroxyproline-rich glycoprotein biosynthesis was greatly enhanced	Toppan et al, (1982)
	Melon tissue	Infection led to early stimulation of C_2H_4	Toppan and Esquerre-Tugaye (1984)
	Melon hypocotyl	Infection triggered C_2H_4 production, may have involved hydroxyproline-rich glycoprotein (HRGP) synthesis.	Roby et al, (1985)
	Melon leaves or seedlings	Increase in chitinase activity with a simultaneous increase in C_2H_4 production	Roby et al, (1986)
Diplodia natalensis	Citrus	C_2H_4 enriched environment caused stem-end rot.	Brown and Lee (1993)
Endothia gyrosa and *Cytospora eucalypicola*	Eucalyptus maculata	C_2H_4 production on inoculated sapwood was observed. Both fungi produced C_2H_4 on artificial medium.	Wilkes et al, (1989)
Endothia parasitica	Bark of American and Chinese Chestnut and Scarlet Oak	C_2H_4 production was stimulated in expanding initial lesion, before lignification.	Hebard and Shain (1988)
Fusarium spp.	Tomato	Increased C_2H_4 production: reached peak within 9-10 days, coincident with marked foliar wilting and basal leaf abscission.	Gentile and Matta (1975)

Fusarium spp. pathogenic to tulips	Tulip bulb	Host produced 2000 times more C_2H_4 than when inoculated with nonpathogenic *Fusarium* strains	Swart and Kamerbeek (1976)
Fusarium oxysporum f.sp. *tulipe*	Tulip bulb	Increased C_2H_4 production, caused gummosis and bud necrosis	de Munk (1971)
Fusarium solani f.sp. *phaseoli* (nonpathogenic) *Fusarium solani* f.sp. *pisi* (pathogenic)	Pea pods	Fungal infection strongly increased C_2H_4 production, but C_2H_4 and fungal infection were independent signals for the induction of chitinase and β-1,3-glucanase	Mauch et al, (1984)
Laccaria bicolor *Laccaria laccata*	Culture medium	Enhanced C_2H_4 production when methionine added to media. ACC in media had a tendency to reduce C_2H_4 production.	Livingston W H (1991)
	Culture medium	AOA did not inhibit methionine induced C_2H_4. ACC was not the precursor of C_2H_4.	Rupp et al, (1989)
Mycorrhizal fungi and *Fusarium*	Douglas fir	Enhancement of C_2H_4 formation coincided with development of mycorrhizae and stimulated lateral root development. *Fusarium*-infected seedlings produced more C_2H_4	Graham and Linderman (1980)
Mycosphaerella citri	Rough lemon and grapefruit leaves	Infection induced C_2H_4 production and caused leaf chlorosis and abscission	Graham et al, (1984)
Penicillium corymbiferum	Bulb of Iris	Treatment with ethylene increased incidence of infection in wounded bulb.	Doss et al, (1989)
Penicillium digitatum	Grapefruit	Fungal invasion was associated with increase in both ACC and C_2H_4 production but ability of tissue to convert ACC to C_2H_4 decreased with the development of infection.	Achilea et al, (1985a)
	Culture medium	fungus produced a gas causing epinasty. Gas believed to be C_2H_4.	Miller et al, (1940)
	Culture medium	C_2H_4 production by fungi growing on phenolic acids.	Considine and Patching (1975)
	-	Gas released by fungi conclusively shown to be C_2H_4.	Young et al, (1951)
	Citrus	Emanations of fungus promote degreening.	Biale (1940)
Penicillium italicum	Orange fruit	Fungal inoculation increased C_2H_4 production. Treatment with C_2H_4 (1000 µl/ litre) and inoculation enhanced respiration rates of fruit	El-Kazzaz et al, (1983)

Peronospara tabacina	Tobacco	Pathogen induced increase in C_2H_4 release, causing growth retardation and accumulation of scopoletin in upper stem	Reuveni and Cohen (1978)
Phytophthora citrophthora	Citrus	C_2H_4 production by the infected stem tissues was a direct factor influencing duct development	Gedalovich and Fahn (1985)
Phytophthora megasperma var. *sojae*	Soybean cotyledons	Increased C_2H_4 formation, phenylalanine ammonia lyase (PAL) activity and glyceollin accumulation	Paradies et al, (1980)
Phytophthora megasperma	Parsley cells	Peak of ACC synthase activity preceded maximal PAL activity	Chappell et al, (1984)
Puccinia coronata f.sp. *avenae*	Oat leaves	Increased C_2H_4 production was observed following inoculation.	Yamamato and Tani (1986)
Pyricularia oryzae	Rice	Considerable increase in C_2H_4 evolution, creating stunting of blast	Kozaka and Teroka (1978)
Rhizopus stolonifer	Non ripening tomato mutant	Infection markedly stimulated C_2H_4 production followed by accelerated climacteric-like pattern of respiration	Barkai-Golan and Kopeliovitch (1983)
Trichoderma viride	Tobacco leaf tissue	An application of ,β-1,4-endoxylanase, a fungal protein induced C_2H_4 formation and hypersensitive-type necrosis as well as other plant defence responses.	Anderson et al, (1990); Bailey el al (1990; 1992)
Uromyces phaseoli	Beans	Out burst of C_2H_4 caused differentiation of uredosori and large, brown necrotic spots	Montalbini and Elstner (1977)
Ustilago maydis	Maize seedlings	Reduction in elongation growth of leaves and shoots, increase in basal diameter, and decrease in weight preceded by a 4-fold increase in C_2H_4 synthesis	Andrew et al, (1981)
Verticillium dahlia Kleh	Cotton	Accelerated C_2H_4 production in defoliating plants	Tzeng and De Va y (1985)
White-rot fungi *Coriolus hirsutus* *Coriolus versicolor* *Flammulina veltipes* *Hirchioporus abietinus* *Irpex lacteus* *Lenzites betulina* *Phanerochaete chrysosporium* Brown-rot fungi *Gloeophyllum trabeum* *Lentinus lepideus* *Pholiota adiposa*	Agar medium	These fungi produce ethylene in agar medium containing α-oxo-γ-methylthiobutaric (KTBA) acid and were involve in lignin degradation. This suggest that lignin degradation and ethylene-generating system from KTBA is similar in these fungi.	Tanaka et al, (1986)

(Benhamou et al, 1990; Wubben et al, 1992) and is present in stem end tissue of freshly harvested inoculated kiwifruit (Poole et al, 1993). Recently, Wurms and Sharrock (Pers. Com) have found higher chitinase activity in the pericarp of inoculated cured kiwifruit than in uninoculated kiwifruit.

Another important defence mechanism is the formation of a lignin layer in response to infection (Vance et al, 1980; Ride, 1983) or wounding (Poole et al, 1993). This biopolymer is resistant to degradation by most microorganisms (Kirk, 1971; Kirk et al, 1979). In kiwifruit a lignin layer starts to develop in the wounded stem scar soon after harvest and this may prevent or reduce natural infection and reduce effectiveness of infection by inoculation in laboratory studies (Poole et al, 1993). Drilling a hole through the kiwifruit stem scars before inoculation removed this physical defence barrier and resulted in higher fruit infection following inoculation (*Long, Pers. com.*).

CELL WALL DEGRADING ENZYMES BY *B. CINEREA*

B. cinerea has long been known to produce chemicals that can kill host tissues. Nordhausen (1899) thought that *B. cinerea* killed leaf tissues of *Vicia faba* by secreting both cell-wall dissolving enzymes and toxins. Brown (1915) detected an enzyme in *B. cinerea* culture filtrates which macerated the middle lamella of cucumber cell walls. Blackman and Welsford (1916) speculated that the death of leaf cells of *V. faba* in front of advancing hyphae of *B. cinerea* may have been due to diffusion of an enzyme from the fungus. Some information suggests that *B. cinerea* can produce enzymes which are known to be involved in tissue disintegration, such as polygalacturonase (Leone, 1990; Johnston and Williamson, 1992; Tobias et al, 1993; Van den Heuvel and Waterreus, 1985), laccase (Marbach et al, 1984; Viterbo et al, 1992, 1993 & 1994; Roudett et al, 1992), pectin methyl esterase (Marcus and Schejter, 1983), pectinase (Miura et al, 1994; Garcia-Romera et al, 1990), proteinase (Movadehi and Heale, 1990) and cellulase (Verhoeff and Warren, 1972; Heiler et al, 1993). Xyloglucanase has not been identified from *B. cinerea* but is produced by *Trichoderma viride* (Vincken et al, 1994). It is possible that in kiwifruit storage rot caused by *B. cinerea*, all these enzymes, either singly, or in combination, may contribute to cellular disintegration.

ELICITORS OF ETHYLENE

Pectins and hemicelluloses are important components of cell walls, being comprised of a diverse range long chain polysaccharides, which have an important function in maintaining strength/ coherence of walls (Carpita and Gibeaut, 1993). They are readily hydrolysed to smaller fragments or oligosaccharides by hydrolyic enzymes produced during ripening and by invading pathogens .

The idea that biologically active oligosaccharides (oligosaccharins) exist is about 18 years old (Ayers et al, 1976; Albershiem and Valent, 1978). Oligosaccharides can be released from the plant cell wall following treatment with pectic-degrading enzymes such as polygalacturonase (Nothnagel et al, 1983; Leone, 1990), pectinase and cellulase (Fry et al, 1993). An oligosaccharin is defined as any short chain of sugar residue interconnected by glycosidic linkages (Fry et al, 1993) which can exert a "signalling" effect in plant tissue other than as carbon or energy source (Darvill et al, 1992; Aldington and Fry, 1993). Thus, while all oligosaccharins are oligosaccharides, not all oligosaccharides are oligosaccharins. Most of the known oligosaccharins are derived from cell wall polysaccharides. Any biologically active oligomers that, although rich in sugar residues, also contain some non-carbohydrate material, e.g, phenolics or peptides can be considered as oligosaccharins (Aldington and Fry, 1993).

A number of oligosaccharides, when applied to healthy or wounded tissue, have been shown to induce ethylene production. Oligosaccharides isolated from cell walls of fungal and plant tissue are potent elicitors of ethylene (Anderson, 1989) and other cellular responses (Fry et al, 1993; Aldington and Fry, 1993). Addition of pectic oligomers of tomato to tomato discs, induced ethylene production (Campbell and Labavitch, 1991). Some glycopeptides found in the mycelium, the cell wall, and filtrate of *Colletotrichum lagenarium*, stimulated ethylene production by melon (*Cucumis melo* cv Cantaloup charentains) tissues (Toppan and Esquerre-Tugaye, 1984). This elicitation of ethylene is not fungal species specific. For example, elicitors of phytoalexins, obtained from three *Phytophthora* species, enhanced ethylene biosynthesis in melon tissues (Toppan and Esquerre-Tugaye, 1984).

In culture, the phytopathogenic fungus *Phytophthora cryptogea* secretes a protein called cryptogein, which when added at sub-lethal concentrations to tobacco cell suspension cultures, elicited production of ethylene (Bellin et al, 1991). Similarly, a protein β-1,4-endoxylanase isolated from *Trichoderma viride* (Fuchs et al, 1989; Dean and Anderson, 1991), induced ethylene formation in tobacco leaf tissue (Anderson et al, 1990; Bailey et al, 1990, 1992; Sharon et al, 1993). In tobacco cell suspension cultures treated with elicitors obtained from *Phytophthora parasitica nicotianae*, ethylene production followed a surge in ACC synthase activity (Rickauer et al, 1990). These results indicate that the increase in ethylene production in response to pathogen attack probably occurs as a result of an increase in ACC-synthase activity somehow induced by pathogen elicitation (Chappell et al, 1984). There is also evidence to indicate that the enhanced ethylene production in response to elicitor treatment may be caused by induction of ACC oxidase activity. Induction of this enzyme was observed in cultured tomato (Bouzayen et al, 1991; Felix, 1991; Spanu et al, 1991) or parsley (Chappell et al, 1984) cells treated with an elicitor. Anderson et al, (1993) suggested that the increase in ACC-synthase and ACC oxidase activity from fungal elicitation is thought to result from the direct activation of ACC-synthase and oxidase genes (Anderson et al, 1993). There is no published work suggesting the activation of these genes in response to *B. cinerea* infection in kiwifruit or in any other plant. As MacDiarmid and Gardner, (1993) and Gardner (Pers. com) have identified the ACC synthase and ACC oxidase gene in kiwifruit and Niklis et al, (1993) have shown an increase in ACC activity before the increase in ethylene, it is possible that there might be an activation of an ACC synthase and ACC oxidase gene in kiwifruit in response to *B. cinerea* infection.

POSTHARVEST CONTROL OF *B. CINEREA*

The losses in perishable food crops due to postharvest diseases has become a major international concern. Although the losses in developing countries are often at unacceptable levels, the adverse economic impact of postharvest diseases is much greater in developing countries (Dennis, 1983).

A number of fungi from several genera cause problems with fruit and vegetables in storage. Among these are species of *Botrytis*, *Penicillium*, *Mucor*, *Alternaria*, *Aspergillus*, *Monilina*,

Rhizophus, *Gloeosporium* and *Geotrichum* (Helgeson, 1989). Amongst all these *B. cinerea* is the most important postharvest pathogen (Komeon, 1992). It is the most serious postharvest pathogen causing losses in stored kiwifruit in New Zealand (Pak and Manning, 1994).

Traditionally fungicides have been widely used to control postharvest diseases. Recently such procedures have come under close scrutiny because of potential health risks when they are applied to fresh food. Even when acceptable fungicides are applied for the control of postharvest diseases, it is possible that pathogens will develop resistance to them. Consequently much effort is being directed towards developing effective non chemical methods of preventing diseases on food crops which pose less risk to human health and to the environment.

Much attention has been devoted towards alternative methods of controlling postharvest losses. These include biological control (Esterio et al, 1993), enhanced host resistance through breeding and biotechnology (Helgeson, 1989), postharvest treatment with hot air and/ or hot water (Cheah et al, 1992), storage at low temperature (Lallu and Manning, 1994), in controlled atmospheres (Romo-Parada et al, 1989), or in high CO_2 and/ or low O_2 atmosphere (Goulart et al, 1992).

High CO_2 and/ or low O_2 often cause anaerobic respiration which has been shown to increase the acetaldehyde (AA) levels in fruit (Kader, 1986). AA is known for its fungicidal and insecticidal properties. Avissar et al, (1990) reported that AA vapour induced electrolyte leakage from *B. cinerea* and *Rhizopus stolonifer* membranes and this leakage was positively correlated with the vapour concentration applied. Prasad (1975) has shown that AA vapour can have a fungicidal effect on postharvest pathogens of citrus fruit. Similarly, Pesis and Avissar (1989) reported that short term anaerobic conditions of 99% CO_2 or 97% N_2, reduced fungal development during both cold storage and subsequent shelf life of oranges. However CO_2 treatment caused some injury, while N_2 caused almost none. Lockhart et al, (1971) also found that a N_2 atmosphere for a brief storage duration was effective in suppressing microorganisms in cranberries. Several other workers have also demonstrated the ability of AA to inhibit the development of postharvest rots on various

crops. For example, significant reduction of *B. cinerea* and *Rhizopus stolonifer* on strawberries (Prasad and Stadelbacher, 1974; Morris et al, 1979), and grapes (Avissar et al, 1989), and of *Penicillium expansum* on apple (Stadelbacher and Prasad 1974) was achieved by AA treatment. AA has also been reported to retard the *in vivo* growth of postharvest pathogens (Aharoni and Barkai-Golan, 1973; Aharoni and Stadlbacher, 1973; Prasad, 1975; Avissar et al, 1990) and to inhibit food spoilage yeasts (Barkai-Golan and Aharoni, 1976).

The original thrust of this thesis was to evaluate high CO_2 and/or N_2 effects on kiwifruit storage rots. Experiments carried out in the 1990 and 1991 seasons, resulted in severe physiological damage such as core damage, development of off flavours and enhanced levels of decay in kiwifruit, indicating a high degree of sensitivity to elevated (95%) CO_2. Because of this lack of success, the thrust of this thesis was redirected to investigate the relationship between *B. cinerea*, ethylene production and the infection process in kiwifruit.

Therefore, the objectives of this study were to determine:

(1) Whether *B.cinerea* is capable of producing ethylene *in vitro*, (there is no published record of *B. cinerea* producing ethylene). If *B. cinerea* does produce ethylene then an attempt will be made to identify the nature of precursors and intermediates in the biosynthetic pathway.

(2) Whether kiwifruit infected with *B. cinerea* produce more ethylene than uninfected fruit by using tissue slices along the fruit to:

(a) ascertain the magnitude and location of the ethylene response.

and (b) measure ACC levels and ACC oxidase activity in different zones of kiwifruit.

CHAPTER 2

GENERAL MATERIALS AND METHODS.

Materials and methods used throughout this study are described in this section. Details of specific techniques are given in each chapter.

PLANT MATERIAL

FRUIT SUPPLY

Kiwifruit were obtained from six year old vines (T-bar training system) growing on a commercial property, owned by Mr. B. Kirkwood, Wanganui, twenty meters above sea level (39° 56′ latitude and 175° 03′ longitude,) and 45 kilometres North West of Palmerston North. The kiwifruit were harvested at a commercial stage of maturity. Total soluble solids (TSS) were determined using a refractometer. In 1992 kiwifruit were also harvested from the Massey University orchard, thirty meters above sea level (40° 23′ latitude and 175° 37′ longitude) .

FRUIT HARVEST

Kiwifruit harvested in 1991 were mainly 36 count size (98-101 g mean weight), and those in 1992 were mainly 33 count (107 g mean weight). Kiwifruit were harvested in the morning between 9-11 am. They were harvested at random from throughout the orchard, except that fruit on canes adjacent to shelter belts were not harvested as they were thought to have a potentially high incidence of *B. cinerea* infection (Manning and Pak, 1993). Kiwifruit were harvested by snapping the fruit off the stem; they were placed in a picking bag and emptied into cartons before being transported to Massey University, Palmerston North.

FRUIT INOCULATION

In 1991, kiwifruit were inoculated with *B cinerea* within 12 hours of harvest, and in 1992 within 8 hours of harvest. After inoculation, fruit were packed in single layer trays containing a pocket pack of appropriate size surrounded by a plastic liner. Kiwifruit

(inoculated and uninoculated) were kept at the same temperatures which unless described otherwise, were at 0±0.1°C or 20±1°C. Kiwifruit to be used later for experimental purposes were maintained at 0±0.05°C.

ESTABLISHMENT OF BOTRYTIS ISOLATES

SINGLE SPORE ISOLATES

Single spore isolates of *B. cinerea* were obtained from naturally infected kiwifruit collected from six different growers in the Wanganui area. Fruit were stored at 0°C until infection was obvious. Conidial masses of *B. cinerea* were lifted from fruit using a sterilized needle and placed in deionized autoclaved water inside McCartney bottles to make dilute spore suspensions. The bottles were shaken to separate spores. A needle was dipped in to the dilute spore suspension and streaked over a plate of water agar and left at 20°C for four hours. Single spore isolations were made onto malt agar (MA) with a sharp needle, under a binocular microscope. Of 7 isolates only one (K3) sporulated freely. The remainder were either sclerotial or mixed sclerotial/ sporulating strains. The K3 isolate was maintained by monthly sub-culturing onto 13 ml of MA in 8.5 cm plastic petriplates, transfers being done under aseptic conditions using a laminar flow cabinet (model Oliphant HLF 3/L).

GROWTH MEDIUM

The basal medium used for all cultures was MA which contained 15 gl^{-1} agar (Difco agar) and 30 gl^{-1} malt (Maltexo (NZ) LTD). The agar medium was prepared by adding malt to agar powder and deionised water, bringing it to the boil while stirring, then autoclaving at 120°C for 15 minutes.

INCUBATION CONDITIONS

Single spore isolates of *B. cinerea* were incubated at 20°C±1 either under near-UV light (Sylvania black light blue F40/BLB, 40W) or under light bank of 3 30 W fluorescent tubes (No 31 white De-Luxe)

using a 12 hour photo period. UV-light was used to obtained good sporulation. Distance of cultures from the lights was approximately 35cm.

SPORE SUSPENSION

Spore suspensions used in this study were prepared from 15-20 day old colonies of *B cinerea* by flooding colonies with 0.02% Tween_20 (Polyoxyethylene sorbitan monolaurate by SERVA, Feinbiochemica) and agitating with a glass hockey stick. This crude spore suspension was filtered through sterile glass wool to McCartney bottles under sterile conditions. Spore concentrations were measured using a HAEMACYTOMETER (American Optical Corporation) and adjusted to the required concentration.

GAS MEASUREMENTS

Ethylene production was estimated using either a Varian 3400 or a Pye Unicam Series 104 gas chromatograph (GC) fitted with flame ionisation detector (FID) and an activated alumina column (80-100 mesh, 1.8m long and 0.32cm diameter) in stainless steel. Temperatures of injector, column and detector were 150°C, 100°C and 100°C respectively. Nitrogen was used as carrier gas with a flow rate of 30 cm^3 min^{-1}, hydrogen and air for the detector (flow rates 30 cm^3 min^{-1} and 300 cm^3 min^{-1} respectively). A Photovac, 10S55 gas chromatograph, fitted with a photoionization detector (PID) was used for detection of low ethylene concentration (< 0.01 ppm). It was fitted with both a pre-column (0.15 m) and an analytical column (0.240 m), type XE-60, made of teflon. Column and detector were maintained at ambient temperature (22-27°C). Clean air was used as a carrier gas for both the analytical and pre-columns at a flow rate of 15cm^3 min^{-1} .

Fruit or slices were weighed and ethylene production determined by sealing single fruit or slices in individual air_tight gas jars (approximately 600 ml volume) fitted with a rubber septum for taking samples of gas from inside the jar.

Fungal ethylene production was determined by incubating *B. cinerea* and sealing in 25 ml Ehrlenmeyer conical flask with a rubber suba seal.

One ml gas samples were extracted from the head space of each jar/ flask or vial and samples were injected manually into the GC using a gas-tight disposable syringe.

MEASUREMENT OF QUALITY PARAMETERS

The following methods were used to assess fruit quality:

FIRMNESS

Using a potato peeler, approximately 1 mm thickness of skin was removed from two opposite areas on the equatorial surface of the kiwifruit. Fruit firmness (the force required to penetrate the kiwifruit in Newtons) was measured on both areas using a hand-held Effegi penetrometer fitted with a 7.9 mm diameter probe. Fruit firmness was obtained as the mean of the two measurements taken and converted to Newtons (N) (Kgf multiplied by 9.807).

SOLUBLE SOLIDS

Unless described otherwise soluble solids concentration (%) of juice from two opposite ends (proximal & distal) of Kiwifruit was estimated using a hand-held Atago N-20 refractometer (Model N, McCormick fruit Tech. brix range from 0~20% at 20°C). The refractometer was zeroed using distilled water. The prism surface and the light plate were thoroughly washed and dried with a clean soft tissue paper between each reading. The two readings taken on each fruit were averaged prior to data analysis.

pH AND TITRABLE ACIDITY

Total titrable acidity (TTA) and pH were measured using a Mettler DL21 auto-titrator. A one ml sample of fruit juice was mixed with approximately 40 ml of distilled water in a 100 ml plastic container connected to the machine. The machine measured the sample pH then titrated the sample against 0.1 N sodium hydroxide (NaOH) to an end point of pH 8.2. The volume of alkali required was used to calculate the titrable acidity as percent citric acid v/v.

CHEMICALS

All chemicals used in this study were of ANALAR grade and were obtained from Sigma or BDH.

CHAPTER 3

ETHYLENE PRODUCTION BY *B.CINEREA*

INTRODUCTION

Ethylene is a plant hormone which regulates many aspects of plant growth (Abeles et al, 1989). It is produced not only by higher plants but also by a number of microorganisms. Since 1934 (Gane, 1934) there have been many reports of production of ethylene by aerobic heterotrophs. The major ethylenogenic micro-organisms are overwhelmingly bacteria and fungi (Fukuda et al, 1993). Freebrain and Buddenhagen (1964) reported that a pathogenic bacterium, *Pseudomonas solanacearum*, produced ethylene using peptone, glucuronic acid, glutamic acid or fumaric acid as the substrate. Subsequently, Primerose (1976a,b, 1977) and Primerose and Dilworth (1976) showed that many soil bacteria have an ethylene-producing ability added to which they demonstrated that *Escherichia coli* could produce ethylene using methionine as the substrate. Shipston and Bunch (1989) examined the physiology of the catabolism of methionine in an ethylene producing strain of *E. coli*, and Mansouri and Bunch (1989) studied the ethylenogenic capabilities of a number of selected bacteria during growth in media supplemented with methionine and α-keto-γ-methiolbutyric acid (KMBA).

Production of ethylene by a fungus was first reported in 1934 from bakers yeast (Gane, 1934). Later it was reported from *Penicillium digitatum*, the cause of green mould of citrus fruit (Biale, 1940; Miller et al, 1940) and *Blastomyces dermatitidis* (Nickerson, 1948). Ilag and Curtis (1968) reported that 58 of 228 species of fungus examined produced ethylene, and they concluded that the hydrocarbon is a common metabolic product of fungus. Chalutz and his colleagues observed activation of ethylene production under phosphate-limiting conditions in shaken cultures of *P. digitatum* (Chalutz and Lieberman, 1977, 1978; Mattoo et al, 1979). Several review articles including Lieberman (1979), Arshad and Frankenberger (1992) and Fukuda et al (1993) have summarized the available data on ethylene production by fungi and other microbes but no one has mentioned ethylene production by *B. cinerea*, a ubiquitous postharvest pathogen. An attempt was made by Elad

(1990) to determine the ethylene production by *B. cinerea*. He added methionine in potato dextrose agar medium or to autoclaved leaves of pepper, french bean and cucumber but negligible amounts of ethylene were produced.

Lower plants do not use the ethylene biosynthetic pathway of higher plants (Osborne, 1989). It is possible that an alternative, and as yet unknown pathway for ethylene biosynthesis exists in lower plants, where ACC-oxidase is not an essential intermediate enzyme. Osborne (1989) suggested that ethylene biosynthesis via methionine, SAM and ACC evolved as a modern adaption to the land.

Fukuda et al (1993) have shown the typical ethylene biosynthetic pathways in microorganisms. Microorganisms and plants synthesize ethylene by different biosynthetic pathways. In microorganisms, there are two ethylene biosynthetic pathways. One pathway derived ethylene via methionine with KMBA being an immediate precursor in *E. coli* (Ince and Knowles, 1986), *Cr. albidus* (Fukuda et al, 1989b,c) and probably in most ethylenogenic microorganisms (Fukuda et al, 1993). In the second pathway ethylene is derived from glutamic acid with 2-oxoglutarate being an immediate precursor, as for example in *P. digitatum* (Chou and Yang, 1973; Fukuda et al, 1986, 1989a) and *Ps. syringae* (Goto et al, 1985, Goto and Hyodo, 1987; Nagahama et al, 1991a,b, 1992; Fukuda et al, 1992a,b). Nagahama et al, (1992) classified 229 strains of ethylene-producing bacteria. They identified 225 as being methionine dependent while only two were 2-oxoglutarate dependent.

Following a series of experiments on production of ethylene by *E. coli*, Primerose (1977) proposed that KMBA might be an intermediate in the production of ethylene by *E. coli*, and suggested two possible mechanisms for its conversion to ethylene: (a) by the action of light and excreted flavin; (b) by an enzymic process, possibly via a peroxidase. Billington et al, (1979) described methods for identifying KMBA and showed that this compound is a common metabolic product of micro-organisms such as *E. coli*, *Pseudomonas pisi*, *Bacillus mycoides*, *Acinetobacter calcoaceticus*, *Aeromonas hydophila*, *Rhizobium trifolii*, and *Corynebacterium* sp., when cells are grown in the presence of methionine. It seems that formation of ethylene by most ethylenogenic micro-organisms is through a methionine-

dependent ethylene-forming system: methionine ----KMBA ---- ethylene. The enzyme methionine adenosyltransferase may be involved in the conversion of methionine to KMBA, but the conversion of KMBA to ethylene may be based on a chemical reaction with EFE not being important or necessary (Fukuda et al, 1993).

The objectives of the work described in this chapter were to determine: (a) whether *B. cinerea* is capable of producing ethylene under *in vitro* conditions. If so then (b) to determine the nature of the ethylene biosynthetic pathway in *B. cinerea* by using known precursors and/ or inhibitors of ethylene production.

MATERIALS AND METHODS

Single spore isolates of *B. cinerea*, obtained from kiwifruit, strawberry, blueberry, camellia and grapes, were maintained on Malt agar (MA) at 20°C with bimonthly subculturing. When required a 0.5ml spore suspension was transferred to Pratt's modified medium (Spalding and Lieberman, 1965). Because a wide range of pH's (2.5-8.5) were to be tested, universal buffer was used in Pratt's modified medium (Dawson 1969). The KH_2PO_4 concentration (3.9 g l^{-1}) was less than the 13.61 g l^{-1} used in the original Pratt's medium to compensate for the phosphate in the Universal medium. The composition of media used in this study was (g l^{-1}):
Glucose 20.0, KH_2PO_4 3.9, NH_4NO, 4.0, $MgSO_4.7H_2O$ 1.23, $FeCl_3.6H_2O$ 0.02, $ZnSO_4.7H_2O$ 0.5, citric acid 6.0, H_3BO_3 1.77 and diethylbarbituric acid 5.2. Spore suspensions were prepared as described in Chapter-2.

Unless stated otherwise, all precursors (L-methionine, α-ketoglutarate, L-glutamate, and 1-aminocyclopropane-1-carboxylic acid {ACC}) and the inhibitor (aminooxy) acetic acid (AOA) were used at a concentration of 35 m*M*. Cobalt chloride (Co^{++}) was used at 0.5 m*M*. Media were sterilised by autoclaving at 104 KPa (120°C) for 15 minutes. The potential precursors, methionine and glutamate, were added to the medium before autoclaving. The AOA, ACC and α-ketoglutarate were sterilised by filtration through a 0.45μ millipore filter and added to the appropriate media cooled to 45°C. Media were adjusted to pH 3.5, unless otherwise stated. Cotton wool plugs enclosed in muslin cloth, were fitted in the flask neck

before autoclaving.

PREPARATION OF CULTURES.

Spore suspensions were prepared and adjusted to 10^5 spores per ml as described in Chapter 2. Experimental cultures were prepared by adding 0.5 ml of the spore suspension to 10 ml of medium in 25 ml Erhlenmeyer flasks. All cultures were grown at 22°C.

In shake cultures flasks were agitated on a rotovator shaker (200 r.p.m.) from 4 to 22 days depending on the experiment. At the end of each experiment, unless otherwise stated, the pH of medium in each flask was recorded before harvesting mycelium; mycelium from each replicate of each treatment was than placed on filter paper which had been dried overnight at 80°C, and weighed. Mycelium and filter paper were then dried overnight at 80°C and weighed. Dry weight of the mycelium was calculated by subtracting the original filter paper weight from the combined dry weight of mycelium and paper.

One experiment was designed to determine the relationship between fungal growth in shake culture and ethylene production. Destructive harvests were carried out to obtain dry weights during growth of the cultures. There were two treatments: 1. Control (basal medium) 2. basal medium plus 35 m*M* methionine. Flasks were separated into two series:
Series a: Flasks from which ethylene production was measured every day for the duration of the experiment (non-destructive harvest). Series b: Flasks from which ethylene production was measured immediately prior to removing mycelia for dry weight measurement and measurement of pH and phosphate content of the medium (destructive harvest).

Five replicate flasks per treatment were used for series a and four replicate flasks per treatment for series b. The aliquots of medium for phosphate determination were stored at -20°C for subsequent analysis.

DETERMINATION OF ETHYLENE

To determine ethylene production, flasks were first sealed with a rubber suba seal and cellotape wrapped around the edges to prevent leakage. Flasks were sealed in batches of

12 at half hour intervals to enable gas samples to be analyzed within 30 minutes of extraction from flasks. Before sealing a 1 ml gas sample was removed from each flask to determine the presence and concentration of any initial residual ethylene. One hour after sealing a 1 ml sample of gas was removed from each flask using a gas tight syringe and injected onto a Photovac gas chromatograph for ethylene determination (details in Chapter-2). Ethylene production was calculated by subtracting initial concentration from final concentration.

All experiments had 3 to 5 replicates. Some experiments were repeated more than once and results were averaged for presentation in this chapter. Gas samples were taken at regular interval (either daily or on alternate days) throughout the experiment

With some experiments only the peak ethylene values are presented, while in other experiments the area under the curve (see Appendix 1) was calculated. This provided an estimate of the total amount of ethylene produced during an experiment, and allowed ready comparison between treatments. Standard errors were calculated as a measure of variability between treatments.

RESULTS

In the initial shake culture experiment, ethylene was produced by *B. cinerea* isolates from strawberry (Str), kiwifruit (K3), blueberry (BB), grapes (Gr) and camellia (Cam). Less than 0.02 µl/l/h ethylene was produced when the basal modified Pratt's medium was used as substrate, but in flasks where 35 m*M* methionine was included, ethylene production by all isolates was considerably enhanced. Peak ethylene production from different isolates grown with methionine were (in µl/l/h): 0.71 from K3, 0.52 from strawberry, 0.30 from grapes, 0.28 from camellia and 0.25 from blueberry. The pattern of ethylene production was similar for all isolates with a peak being produced after about 4 days incubation followed by a decline to low levels after 7 days. This typical pattern is illustrated by 'K3' isolate from kiwifruit (Fig 3.1).

To obtain a comparison of the total ethylene production over 7 days by each isolate, the

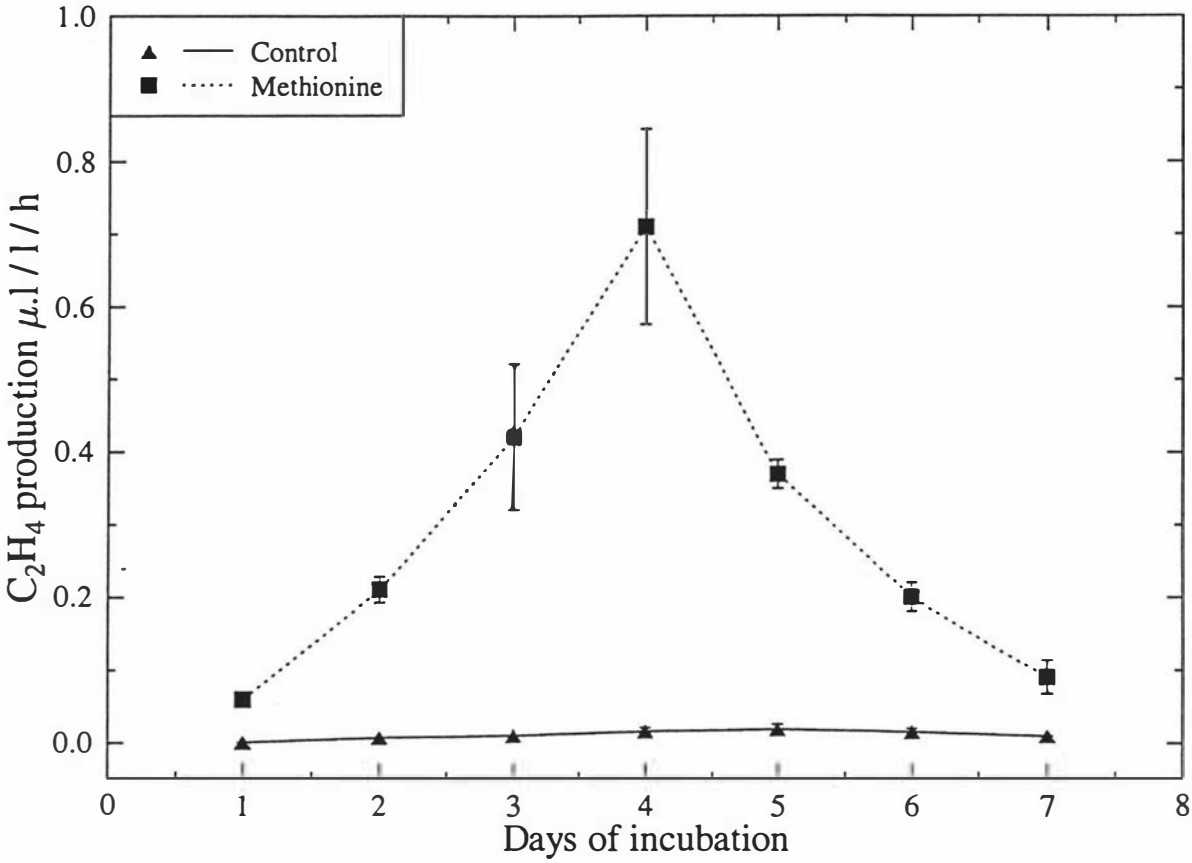


Fig 3.1 Ethylene production by the 'K3' kiwifruit isolate of B. cinerea on shake culture at 22°C with basal medium supplemented with 35 mM Methionine. Bars represent standard errors.

area under the production curve was calculated. Although the camellia isolate grown on basal medium produced more ethylene than the other four isolates, when grown in the presence of 35 m*M* methionine, isolates 'K3' and 'Str' produced approximately 50% more ethylene over 7 days incubation than did 'BB' 'Gr' and 'Cam' (Fig 3.2). As kiwifruit was the experimental crop studied in this thesis, and because the 'K3' isolate produced maximum ethylene only in the presence of methionine, this isolate was used for subsequent experiments reported in this chapter.

Ethylene production by *B. cinerea* was dependent on concentration of methionine in the medium, reaching a maximum at 35 m*M* with no further increase at 50 m*M* methionine (Fig 3.3). Mycelium dry weight (Table 3.1) was lower on media containing methionine than in control but this effect was not concentration related. There was no consistent change in pH of the medium during incubation (Table 3.1).

Three chemicals (methionine, glutamate and α-ketoglutarate) were tested for their ability to act as ethylene precursors for *B. cinerea* grown on shake and static cultures with a range of media pH. In shake cultures, ethylene was produced by *B.cinerea* at a range of pH (2.5-8.5) levels when methionine was added to the media (Fig 3.4). Some ethylene was produced at pH 2.5 in the methionine treatment, but there was a more than 3 fold increase to 1.1 μl/l/h at pH 3.5 which was the optimal pH for ethylene production when methionine was used as precursor. Ethylene production gradually decreased as medium pH increased, until at pH 8.5 it was 0.14 μl/l/h. There was no detectable ethylene produced from glutamate and α-ketoglutarate at pH 2.5, 7.5 and 8.5 and only trace amounts occurred at pH 3.5, 4.5 and 6.0 (Fig 3.4). There was no difference in ethylene production between control and α-ketoglutarate, however, there was an indication that glutamate slightly increased ethylene production (0.02μl/l/h) at pH 3.5 compared with control (0.01 μl/l/h). These increases were very slight compared with the methionine effect in this experiment (Fig 3.4).

When *B.cinerea* was inoculated in basal medium at pH 3.5 in shake culture, there was very little ethylene produced during the 21 days of the experiment (Fig 3.5). Methionine was the most effective putative precursor for inducing ethylene production. Within 24 h of

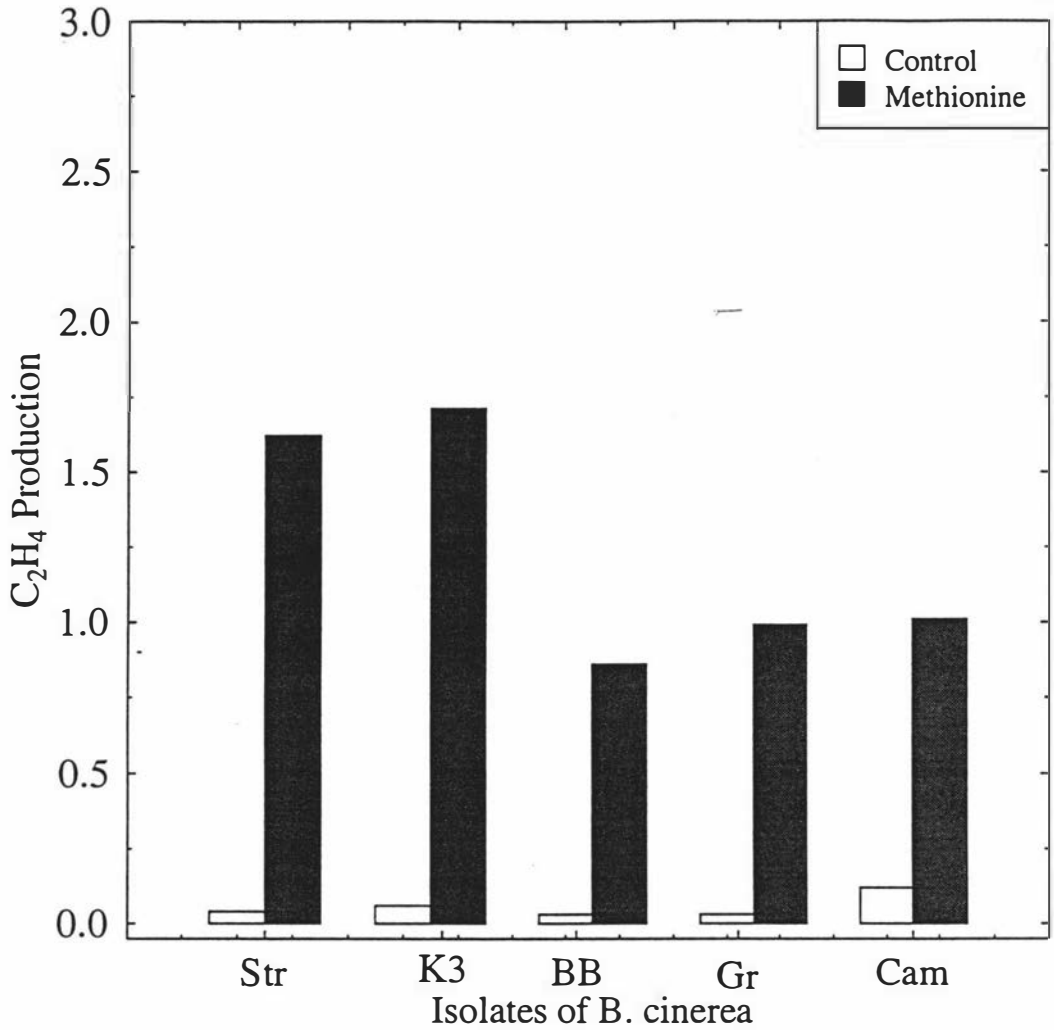


Fig 3.2 Ethylene production by B. cinerea in shake culture, grown in media supplemented with 35mM methionine, over 7 days of incubation at 22°C. Data expressed as area under a 7 days production curve, the isolates from different crops are: Str=strawberry, K3=kiwifruit, BB=blueberry,Gr=grape and Cam=camellia.

NB. The value for C_2H_4 production as area under the curve, was derived from daily headspace measurements of C_2H_4 production by B. cinerea during 1 hr each day calculated as μ. l / l / h, over the period of incubation. For detail of the method used see appendix 1

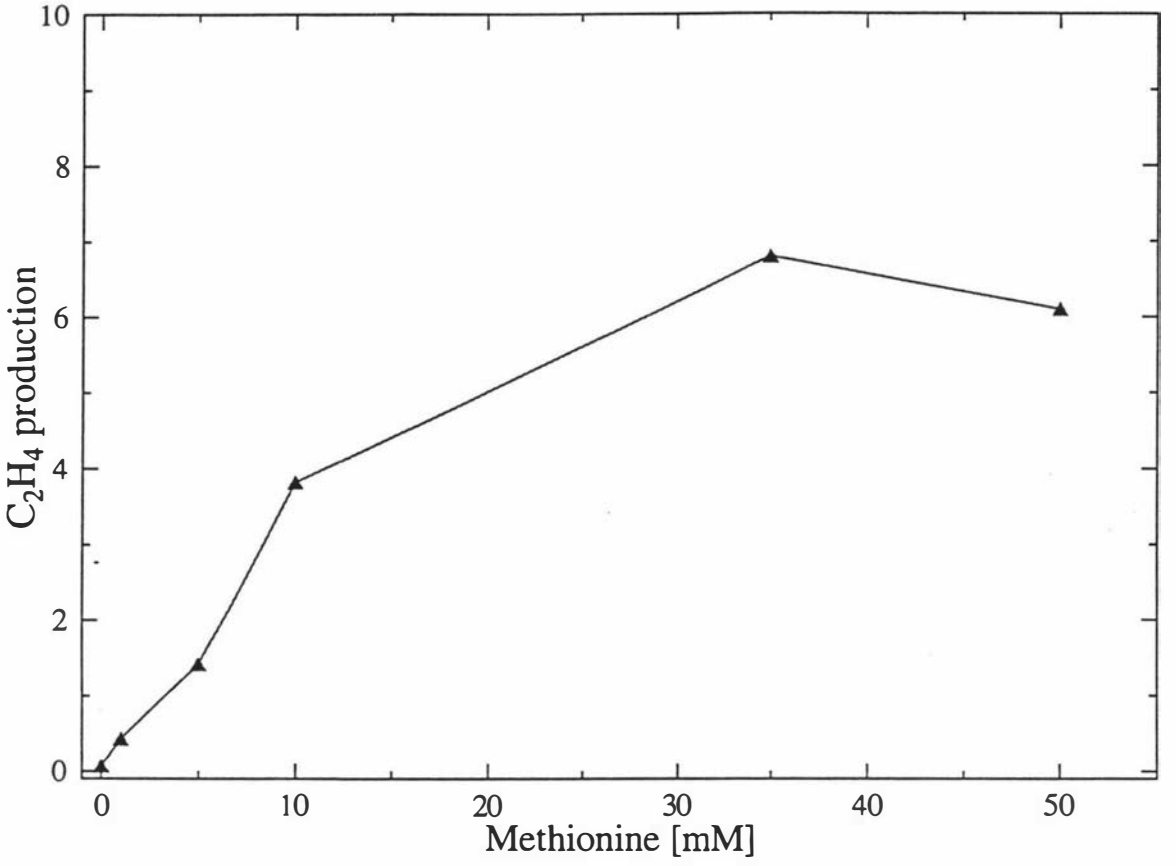


Fig 3.3 Ethylene production by B. cinerea grown in a range of methionine concentrations over 7 days of incubation in shake culture at 22°C (shown as area under the 7 days production the curve).

NB. Procedure for deriving C_2H_4 production as area under the curve (see Fig 3.2 and Appendix 1).

Table 3.1

Mycelial dry weight (g) of *B. cinerea* and final pH in
basal medium supplemented with different methionine concentrations in shake culture after 7 days of
incubation at 22°C. Initial pH was 3.5

Treatments	Mycelial dry weight ± SE	Final pH ± SE
Control	0.29 ± 0.012	3.00 ± 0.00
1 m*M*	0.21 ± 0.01	3.35 ± 0.08
5 m*M*	0.16 ± 0.12	2.25 ± 0.02
10 m*M*	0.16 ± 0.02	2.52 ± 0.06
35 m*M*	0.18 ± 0.03	2.87 ± 0.02
50 m*M*	0.14 ± 0.03	3.15 ± 0.05

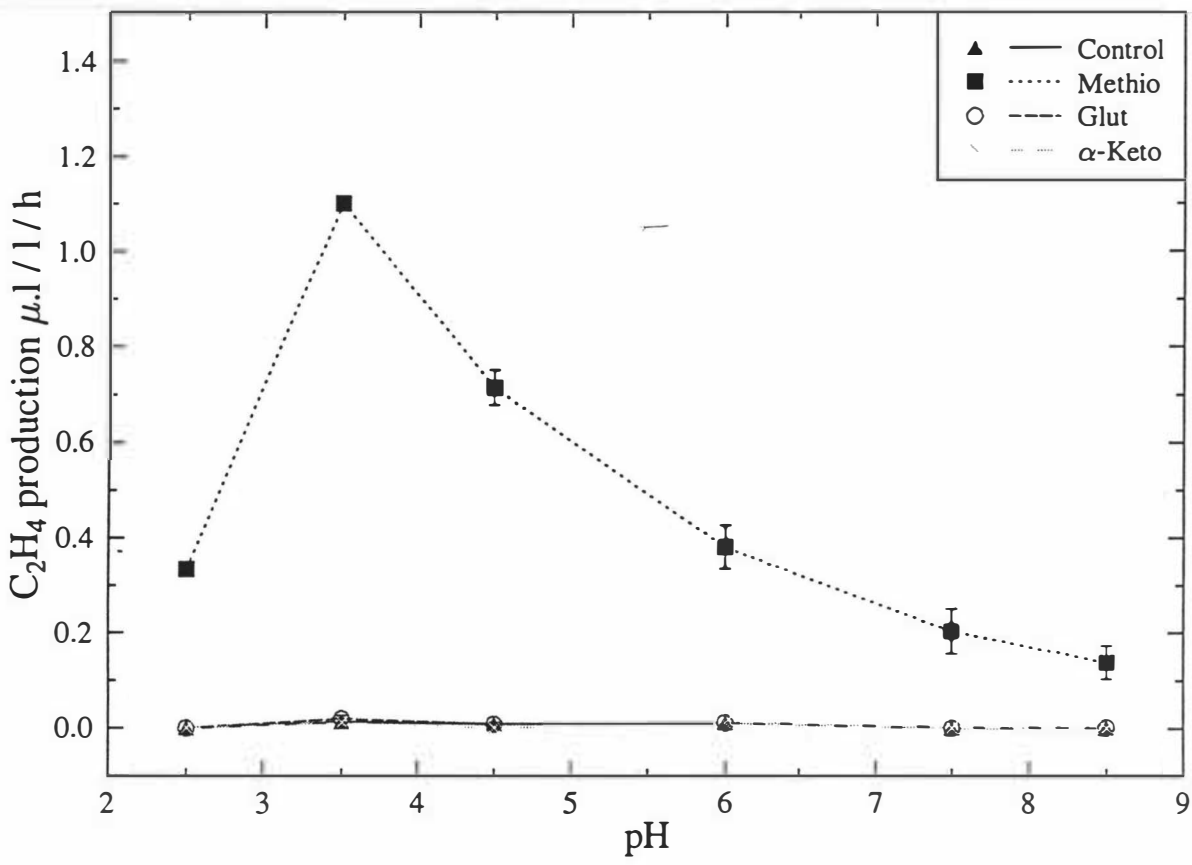


Fig 3.4 Peak ethylene production by B. cinerea in shake cultures at 22°C grown in media supplemented with 35 mM methionine, glutamate or α-ketoglutrate at a range of pH values. Bars represent standard errors.

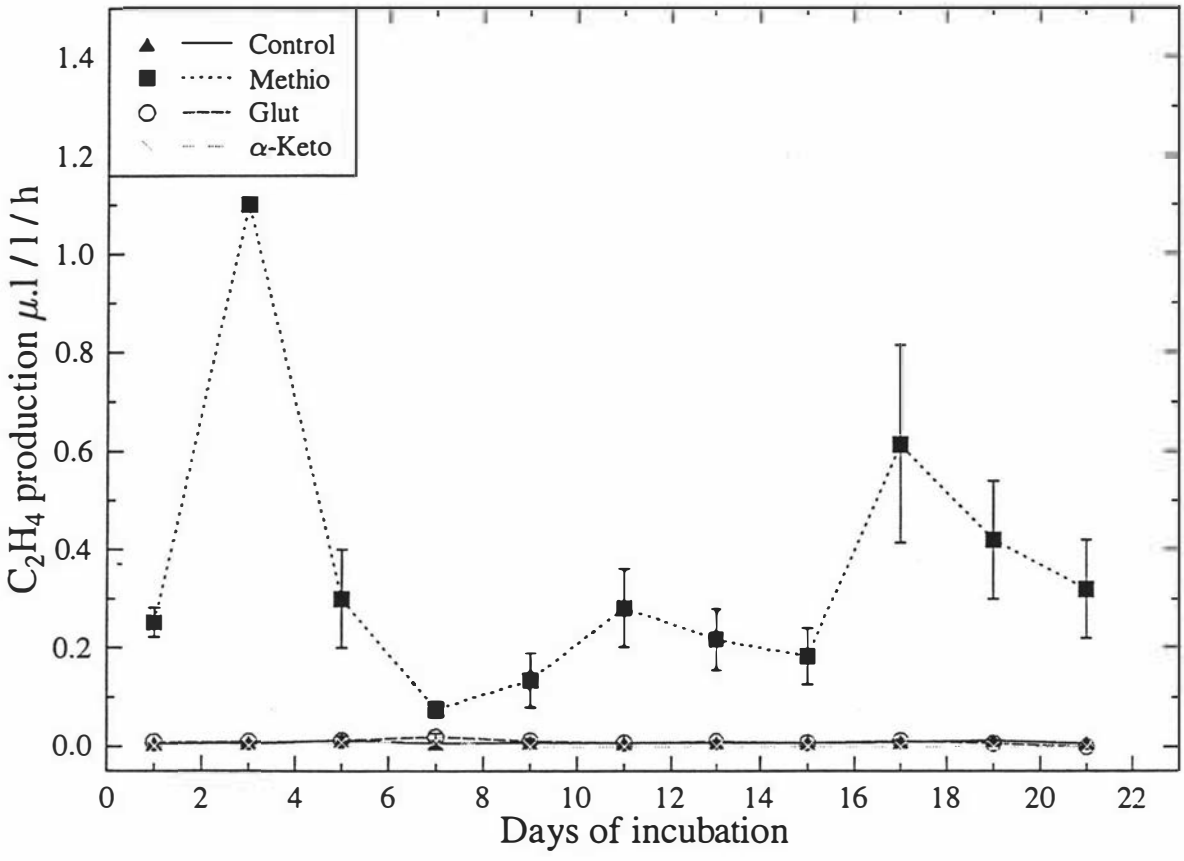


Fig 3.5 Ethylene production by B. cinerea grown in shake culture at 22°C using basal medium (pH 3.5) supplemented with 35 mM methionine, glutamate or α-ketoglutarate. Bars represents standard errors.

commencing incubation ethylene was detectable ; a peak of ethylene production (1.1 µl/l/h) was reached after 3 days incubation, after which production decreased to a minimum at 7 days. A slight increase occurred between days 7 and 15, after which there was a further increase to 0.6 µl/l/h at day 17, before a gradual decline through day 21. This bimodal pattern of ethylene production was not found when glutamate or α-ketoglutarate was added to the medium. Neither of these chemicals resulted in increased ethylene production (Fig 3.5). Under shake culture conditions, there was no major difference in final dry weight of mycelium between treatments (Table 3.2), although visual observation suggested a decreased growth of *B.cinerea* when α-ketoglutarate was added to the medium. Major changes in pH of medium occurred during incubation in this experiment (Table 3.3). When initial pH was 3.5 or above, there was a tendency for pH's to drift towards neutrality; those initially at pH 3.5, 4.5 and 6.0 increased while those initially at pH 7.5 and 8.5 decreased. There was a slight increase in final pH on those cultures which commenced at pH 2.5, except for the control which decreased slightly to 2.4. These results suggested perhaps there may have been inadequate buffering capacity at media pH's above 2.5.

B.cinerea grown in static culture conditions did not respond in exactly the same way as when grown on shake cultures. The same potential ethylene precursors (methionine, glutamate and α-ketoglutarate) were tested in static culture at a range of media pH's. Under these conditions methionine was the most efficient precursor among the three tested (Fig 3.6); ethylene was produced at all pH's from 2.5-7.5 but there was no detectable ethylene produced at pH 8.5. Some ethylene was produced at pH 2.5 and 3.5, but maximum ethylene was produced at pH 4.5, indicating that pH 4.5 was the optimal pH for ethylene production under static culture conditions. Ethylene production decreased at pH 5.5 but again there was a slight increase at pH 6.0. Glutamate was the second most efficient precursor with maximum ethylene production (0.22 µl/l/h) at pH 4.5. There was no detectable ethylene produced from glutamate at pH 2.5, 3.5 and 8.5 and only traces of ethylene were found at pH 6.0 and 7.5. When α-ketoglutarate was added to the medium, cultures produced traces of ethylene in the pH range 2.5-6.0 only on the 3rd day of incubation with no detectable ethylene being produced at pH 7.5 and 8.5. There was an indication that α-ketoglutarate slightly inhibited ethylene production compared with the basal medium (Fig 3.6). When *B.cinerea* was inoculated onto the basal medium at pH 4.5

Table 3.2

Mycelial dry weight (g) of *B. cinerea* grown in basal medium with or without possible ethylene precursors at a range of pH levels on shake cultures after 24 days of incubation at 22°C

Treats / pH	Mycelial dry weight (g) ± SE						
	2.5	3.5	4.5	6.0	7.5	8.5	Mean
Control	0.59 ± 0.00	0.60 ± 0.00	0.63 ± 0.01	0.58 ± 0.01	0.60 ± 0.01	0.58 ± 0.01	0.60
Meth	0.60 ± 0.01	0.57 ± 0.00	0.56 ± 0.01	0.56 ± 0.01	0.58 ± 0.01	0.58 ± 0.00	0.58
Glut	0.65 ± 0.02	0.63 ± 0.02	0.64 ± 0.03	0.58 ± 0.00	0.60 ± 0.01	0.60 ± 0.00	0.62
α-Keto	0.71 ± 0.07	0.62 ± 0.01	0.62 ± 0.00	0.57 ± 0.00	0.56 ± 0.01	0.56 ± 0.01	0.61
Mean	0.64	0.61	0.61	0.57	0.58	0.58	

Meth= methionine 35m*M*

Glut= glutamate 35m*M*

α-Keto= α-ketoglutarate 35m*M*

Table 3.3

Final pH of basal medium in which *B cinerea* was grown at a range of pH levels with or without possible ethylene precursors in shake culture after 24 days of incubation at 22°C.

Treat	Final pH ± SE						
Initial pH	2.5	3.5	4.5	6	7.5	8.5	Mean
Control	2.40 ± 0.00	6.20 ± 0.02	6.53 ± 0.10	6.96 ± 0.05	6.96 ± 0.02	7.20 ± 0.00	6.04
Meth	3.00 ± 0.05	5.06 ± 0.19	5.63 ± 0.17	6.86 ± 0.02	7.20 ± 0.03	7.4 ± 0.02	5.86
Glut	3.60 ± 0.04	6.60 ± 0.00	6.63 ± 0.03	6.83 ± 0.07	7.20 ± 0.12	7.33 ± 0.10	6.36
α-Keto	3.30 ± 0.46	6.83 ± 0.05	6.70 ± 0.03	6.90 ± 0.05	7.33 ± 0.07	7.10 ± 0.12	6.36
Mean	3.10	6.17	6.37	6.88	7.17	7.26	

Meth= methionine 35m*M*

Glut= glutamate 35m*M*

α-Keto= α-ketoglutarate 35m*M*

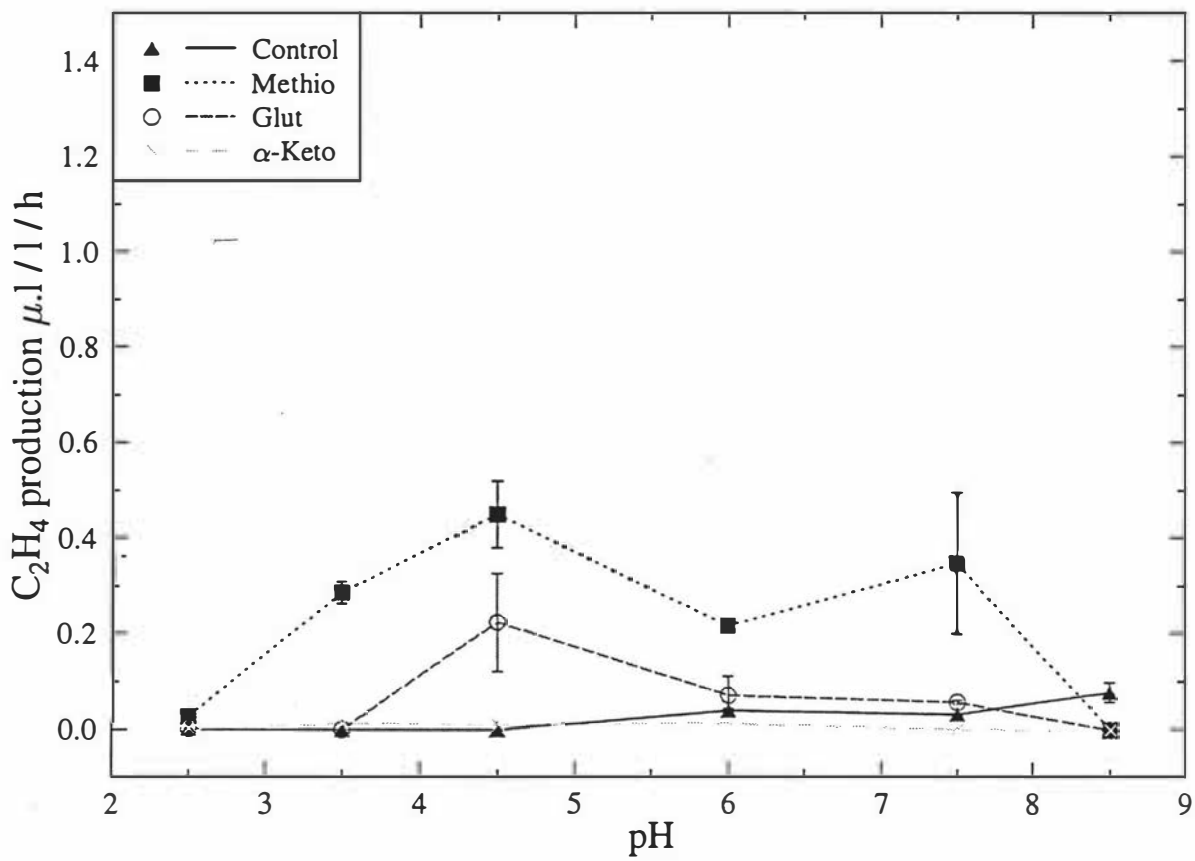


Fig 3.6 Peak ethylene production by B. cinerea in static culture at 22°C grown on media supplemented with 35 mM methionine, glutamate or α-ketoglutrate, at a range of pH values. Bars represents standard errors.

in static culture, there was very little ethylene produced during the 22 days of the experiment (Fig 3.7). Methionine was the most efficient precursor for inducing ethylene production. Within 24 h of inoculation ethylene was detectable and ethylene production increased to a peak (0.45 µl/l/h) after 9 days, after which ethylene levels decreased through 15 days. A slight increase occurred between day 15 to 19, with a peak of 0.22 µl/l/h at day 17 followed by a steady decline through day 22. The bimodal pattern of ethylene production that occurred when methionine was added to the medium was not found with glutamate or α-ketoglutarate (Fig 3.7).

Under static culture conditions there was no major difference between methionine and glutamate on final dry weight of mycelium but α-ketoglutarate appeared to have a slight inhibitory effect on growth (Table 3.4). Unlike shake culture, there was a gradual decrease in final dry weight of mycelium with increasing pH of the medium except between pH 2.5 and 3.5 where there was no major difference.

Major changes in medium pH did not occur during incubation in static culture (Table 3.5). When the initial pH was 3.5 and 4.5, there was a tendency for pH's to drift towards pH 6.0, those initially at pH 2.5, 3.5 and 4.5 increased while those initially at pH 6.0 and above decreased. There was a slight increase in final pH in those cultures which commenced at pH 2.5. These results again suggested that there was inadequate buffering capacity at pH's above 2.5.

In an attempt to relate ethylene production from *B.cinerea* to mycelial growth throughout its growth cycle, ethylene was measured immediately before destructive harvesting and mycelial dry weight measurement. Dry weight of cultures grown in basal medium increased rapidly to a maximum after about 8 days, remained constant until day 14, after which it declined steadily through day 22 (Fig 3.8). In contrast, dry weight of cultures grown in the presence of 35 m*M* methionine increased to a maximum at day 6 but declined rapidly after day 8 to reach a low but relatively constant dry weight at day 12 through day 22.

In this experiment ethylene production was measured from those cultures which were grown throughout the 22 days of incubation (series a, non-destructive; Fig 3.9) and from

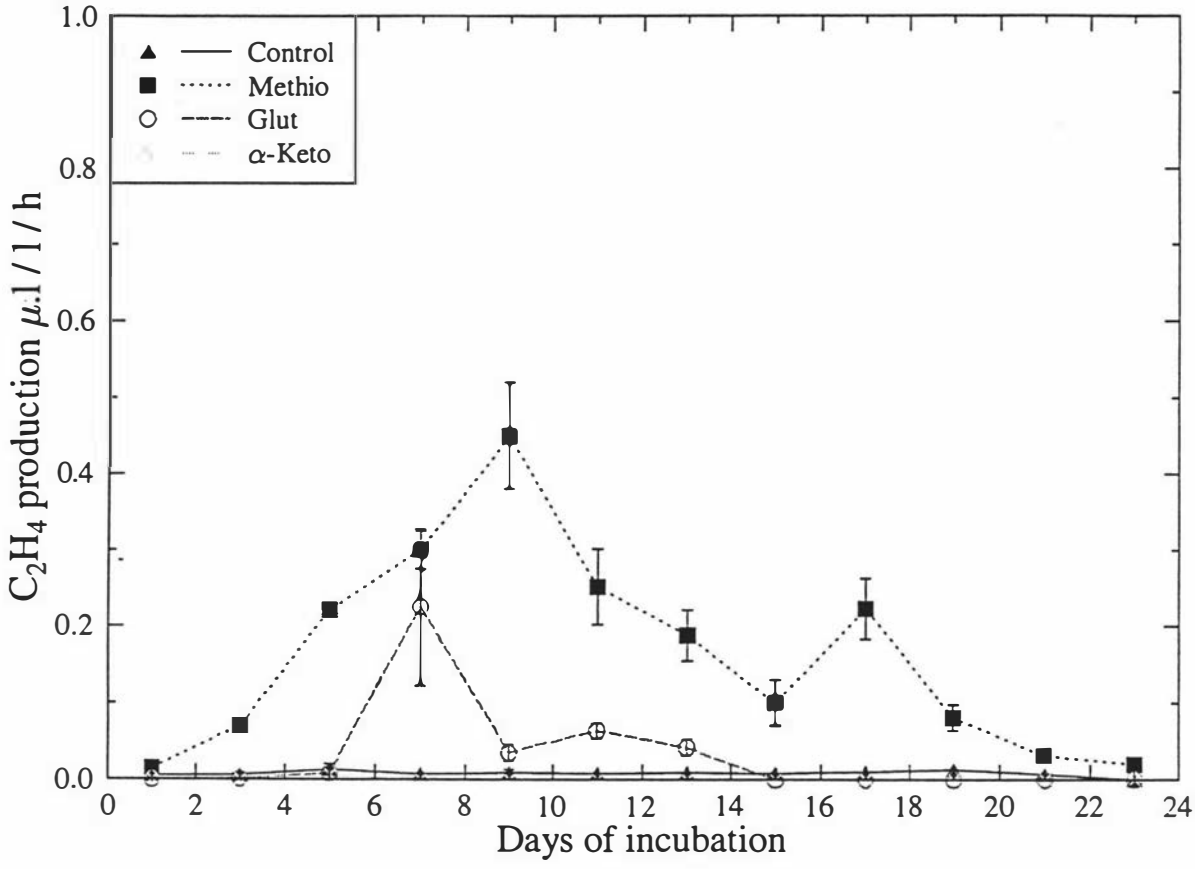


Fig 3.7 Ethylene production by B. cinerea grown in static culture at 22°C using basal medium (pH 4.5) supplemented with 35 mM methionine, glutamate or α-ketoglutarate. Bars represents standard errors.

Table 3.4

Mycelial dry weight (g) of *B.cinerea* in basal medium at a range of pH levels with and without possible ethylene precursors in static culture after 24 days at 22°C.

Treat	Mycelial dry weight (g) ± SE						
Initial pH	2.5	3.5	4.5	6.0	7.5	8.5	Mean
Control	0.13 ± 0.02	0.15 ± 0.00	0.12 ± 0.01	0.11 ± 0.01	0.10 ± 0.03	0.07 ± 0.01	0.11
Meth	0.16 ± 0.01	0.15 ± 0.02	0.12 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.02	0.10
Glut	0.16 ± 0.01	0.17 ± 0.01	0.12 ± 0.01	0.08 ± 0.00	0.09 ± 0.00	0.08 ± 0.01	0.12
α-Keto	0.10 ± 0.01	0.15 ± 0.01	0.09 ± 0.01	0.08 ± 0.00	0.06 ± 0.01	0.05 ± 0.00	0.08
Mean	0.14	0.15	0.11	0.08	0.08	0.07	

Meth= methionine 35m*M*

Glut= glutamate 35m*M*

α-Keto= α-ketoglutarate 35m*M*

Table 3.5

Final pH of basal medium in which *B cinerea* is grown at a range of pH levels with and without possible ethylene precursors in static culture after 24 days of incubation at 22°C.

Treat	Final pH ± SE						
Initial pH	2.5	3.5	4.5	6.0	7.5	8.5	Mean
Control	2.70 ± 0.06	3.20 ± 0.16	5.23 ± 0.34	5.16 ± 0.08	5.60 ± 0.13	5.00 ± 0.00	4.48
Meth	2.70 ± 0.06	3.90 ± 0.11	5.93 ± 0.08	5.73 ± 0.12	7.00 ± 0.08	5.40 ± 0.23	5.11
Glut	2.60 ± 0.03	4.46 ± 0.21	6.36 ± 0.31	6.03 ± 0.18	5.86 ± 0.03	5.66 ± 0.12	5.16
α-Keto	2.50 ± 0.06	5.00 ± 0.56	6.60 ± 0.32	5.90 ± 0.12	5.56 ± 0.03	5.80 ± 0.20	5.23
Mean	2.62	4.14	6.03	5.70	6.00	5.46	

Meth= methionine 35m*M*

Glut= glutamate 35m*M*

α-Keto= α-ketoglutarate 35m*M*

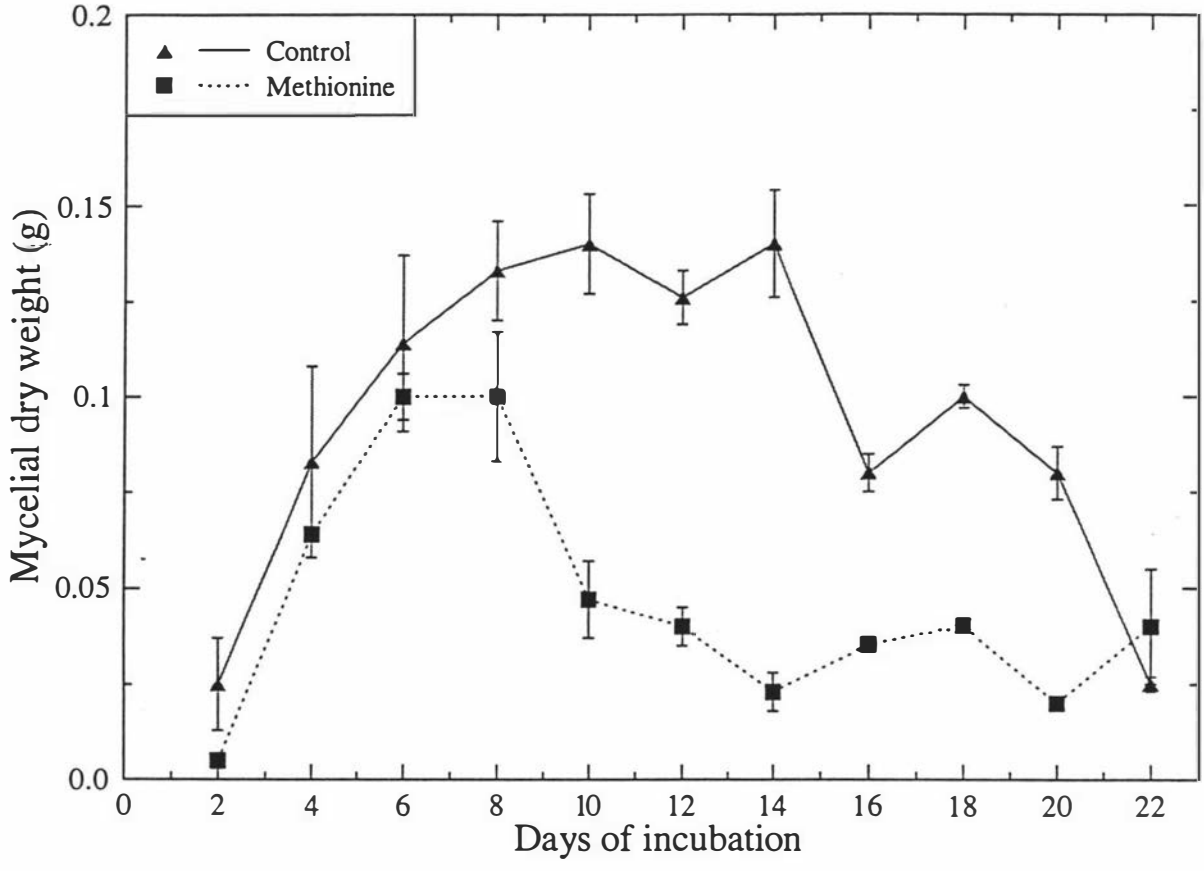


Fig 3.8 Dry weight of B. cinerea mycelium grown in shake culture at 22°C in basal medium with or without 35mM methionine. Bars represents standard errors.

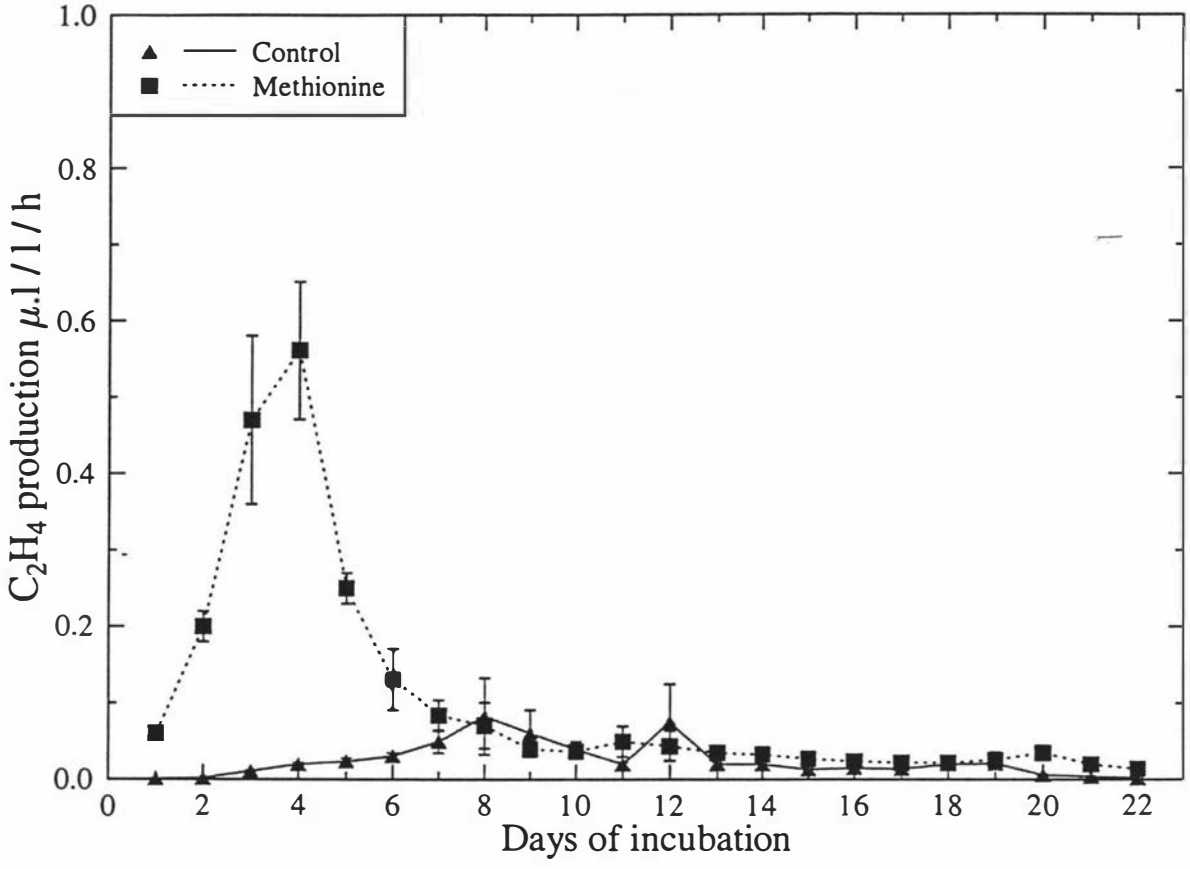


Fig 3.9 Ethylene production by B. cinerea grown in shake culture in basal medium at 22°C with and without 35 mM methionine (Series a: non-destructive). Bars represents standard errors.

those which were harvested for mycelial dry weight determination at 2 day intervals (series b, destructive; Fig 3.10 & 11). From series a ethylene production started within 24 h of incubation in the cultures containing methionine, reaching a peak on day 4 before declining to control levels on day 8, thereafter remaining at low but detectable levels, similar to those of controls, through 22 days. (Fig 3.9) There was no second peak of ethylene production as found in the previous experiment. In control cultures, without methionine, there was a slight increase in ethylene production to 0.08 µl/l/h at day 8, after which there was low but declining level of ethylene detected through day 22. A similar trend was found when ethylene production from series b was measured; there was a peak in ethylene production on day 4 (0.36 µl/l/h), after which there was a steady decline to the same level as the control media after 9 days. (Fig 3.10) A slight but significant increase did occur on day 10. From day 11 through day 22, ethylene production was low, but at the same level of control cultures. There was no second peak of ethylene production.

Data from the destructive harvest allowed determination of rate of ethylene production per unit dry weight of mycelium through the 22 day incubation period (Fig 3.11). Within 48 h of inoculation large amounts of ethylene were being produced, approximately 780 µl/g/h per dry weight unit of the mycelium ($10^{2.9}$ µl/g/h on log scale). Production per g dry weight decreased gradually until day 8, after which it remained relatively constant at about 12 µl/g/h through day 22. The pH of medium remained unchanged through day 8 after incubation in both control and methionine treatments after which it drifted up to 5.1 in control and 5.3 in methionine treatment until day 16 when there was a slight decline in pH of the methionine medium while in the basal medium pH remained constant (Fig 3.12). The phosphate content of both media remained constant throughout the incubation period of 22 day and did not differ between treatments (Table 3.6).

The preceding results have shown that 35 m*M* methionine added to basal media can induce ethylene production in both static and shake cultures of *B. cinerea*. It is accepted that methionine is a precursor of ACC, and that ACC is a critical intermediate in the ethylene biosynthetic pathway in higher plants (Adams and Yang 1979). The rate limiting step in this pathway is SAM to ACC catalysed by the enzyme ACC synthase. Activity of this enzyme is inhibited by AOA (Murr and Yang 1975). The other important enzyme in this

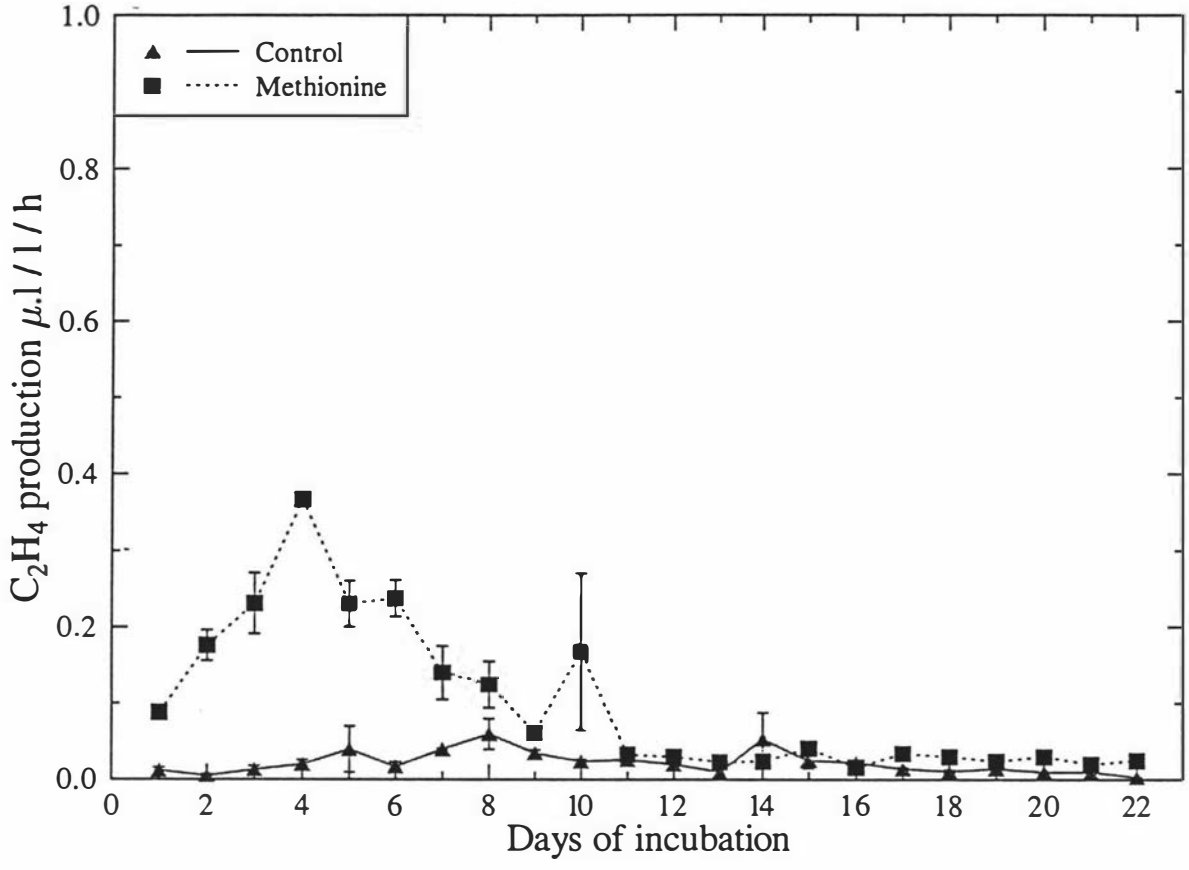


Fig 3.10 Ethylene production by B. cinerea in shake culture, grown in basal medium supplemented with 35 mM methionine at 22°C (Series b: destructive harvest). Bars represents standard errors.

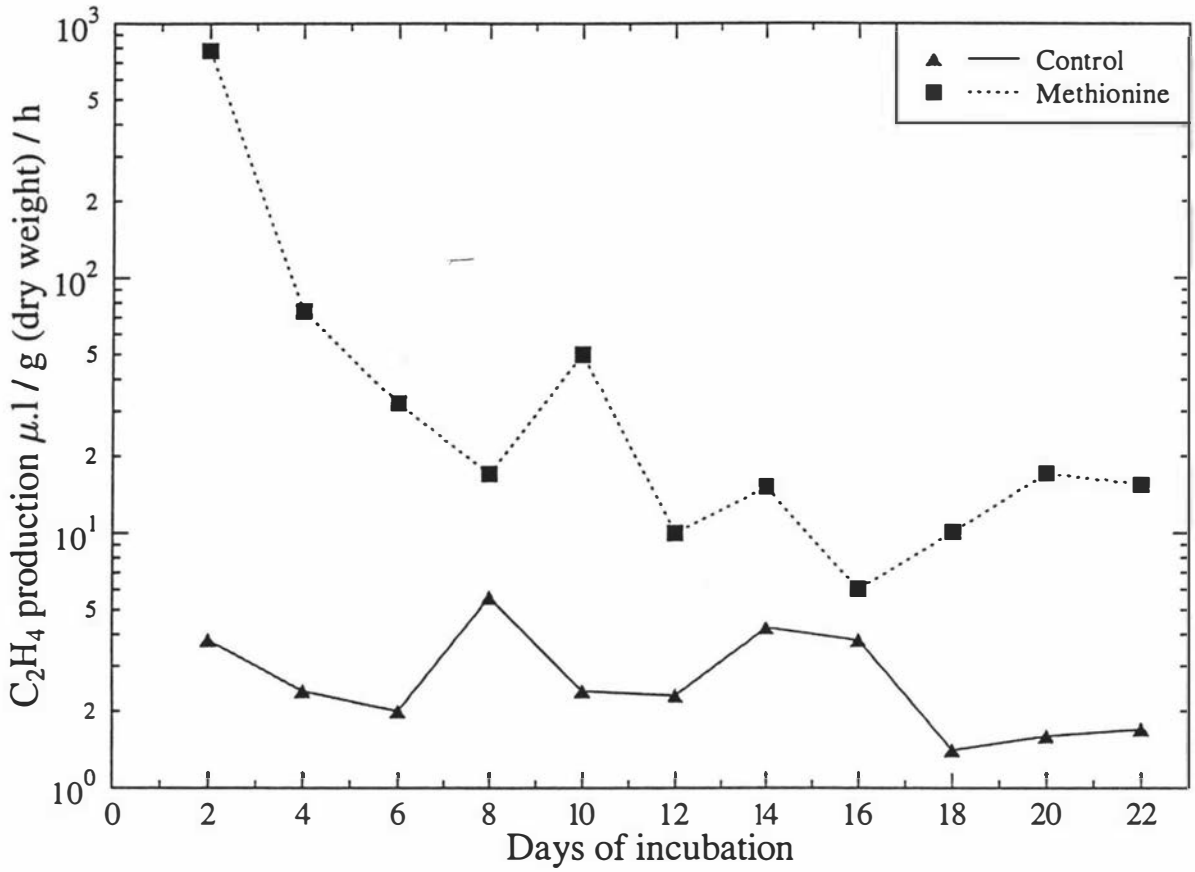


Fig 3.11 Ethylene production by B. cinerea in shake culture, grown in basal medium supplemented with 35 mM methionine at 22°C. (Series b:destructive harvest). NB. Use of logarithmic scale on x axis

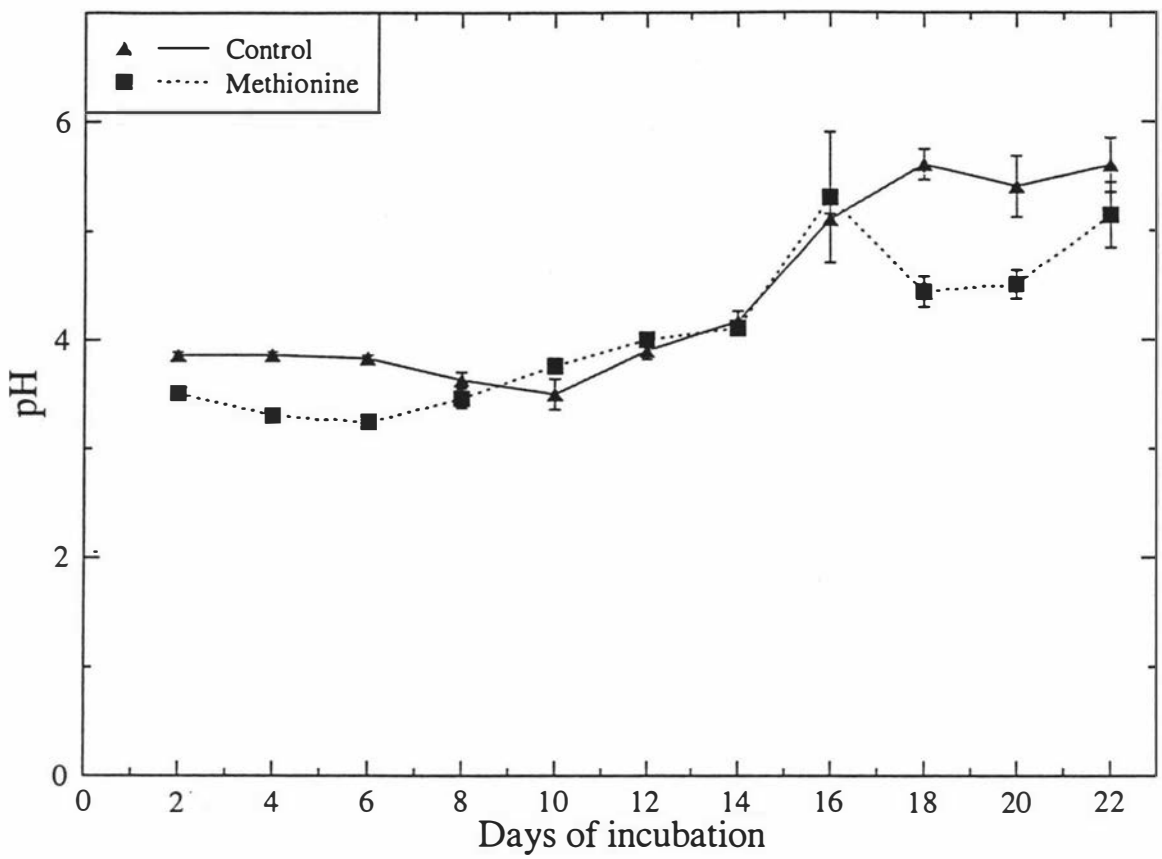


Fig 3.12 pH changes in medium when B. cinerea was grown in shake culture at 22°C with and without 35 mM methionine. Bars represents standard errors. (Series b: destructive harvest)

Table 3.6

Phosphate concentration (m*M*) in basal medium with or without 35m*M* methionine after 24 days incubation on shake culture at 22°C.
Initial phosphate concentration was 28.67m*M*.

Phosphate concentration ± SE		
Days	Control	Methionine
2	26.8 ± 3.2	27.0 ± 3.7
4	24.8 ± 2.8	25.7 ± 2.8
6	27.0 ± 3.6	25.9 ± 3.7
8	25.7 ± 2.5	27.0 ± 2.4
10	26.0 ± 3.5	25.0 ± 3.2
12	24.0 ± 3.1	26.8 ± 1.7
14	26.0 ± 3.1	25.0 ± 2.4
16	27.0 ± 2.5	26.8 ± 3.0
18	25.5 ± 2.2	26.7 ± 3.3
20	26.0 ± 1.8	25.0 ± 2.6
22	26.7 ± 2.4	27.4 ± 3.2

pathway is ACC oxidase which catalyses the formation of ethylene from ACC; this reaction is oxygen dependent and is partially inhibited by Co^{++} ions (Yu and Yang 1979).

The following series of experiments were designed to:

a) determine if ACC could act as a precursor of ethylene for *B. cinerea.*

b) evaluate the effect of inhibitors AOA and Co^{++} on ethylene production

c) attempt to elucidate whether or not methionine was metabolised according to the commonly accepted ethylene pathway or whether an alternate pathway could exist in *B. cinerea.* This was attempted by variously combining methionine, ACC, AOA and Co^{++} in a series of experiments.

A series of three experiments with different combinations of precursors and/or inhibitors were carried out in shake culture. Similar data were obtained from each of these experiments so the results were combined. In the control treatment only a trace of ethylene was produced by *B. cinerea* (Fig 3.13); all other treatments produced detectable levels of ethylene. Maximum ethylene production occurred when methionine was added to the medium. This production was 120 fold greater than control and was approximately 6% higher than the combination of methionine and ACC. When ACC, the immediate precursor of ethylene in higher plants, was added to the basal medium alone, it stimulated ethylene production slightly, but only 5 fold more than the basal medium and approximately 20 fold less than the methionine treatment. Addition of AOA plus methionine to the medium resulted in a small amount of ethylene being produced but 8 fold less than when methionine was used alone. When ACC was included with methionine and AOA, the inhibitory effect was not overcome and ethylene production was approximately the same as for methionine and AOA together. Addition of Co^{++} and methionine to the medium resulted in 20% less ethylene being produced than when only methionine was present. Addition of Co^{++} to the ACC containing medium had no effect on ethylene production (Fig 3.13). Cobalt had no further inhibitory effect on ethylene production when added to methionine+AOA; similarly ACC added to methionine+AOA+Co^{++} did not effect ethylene

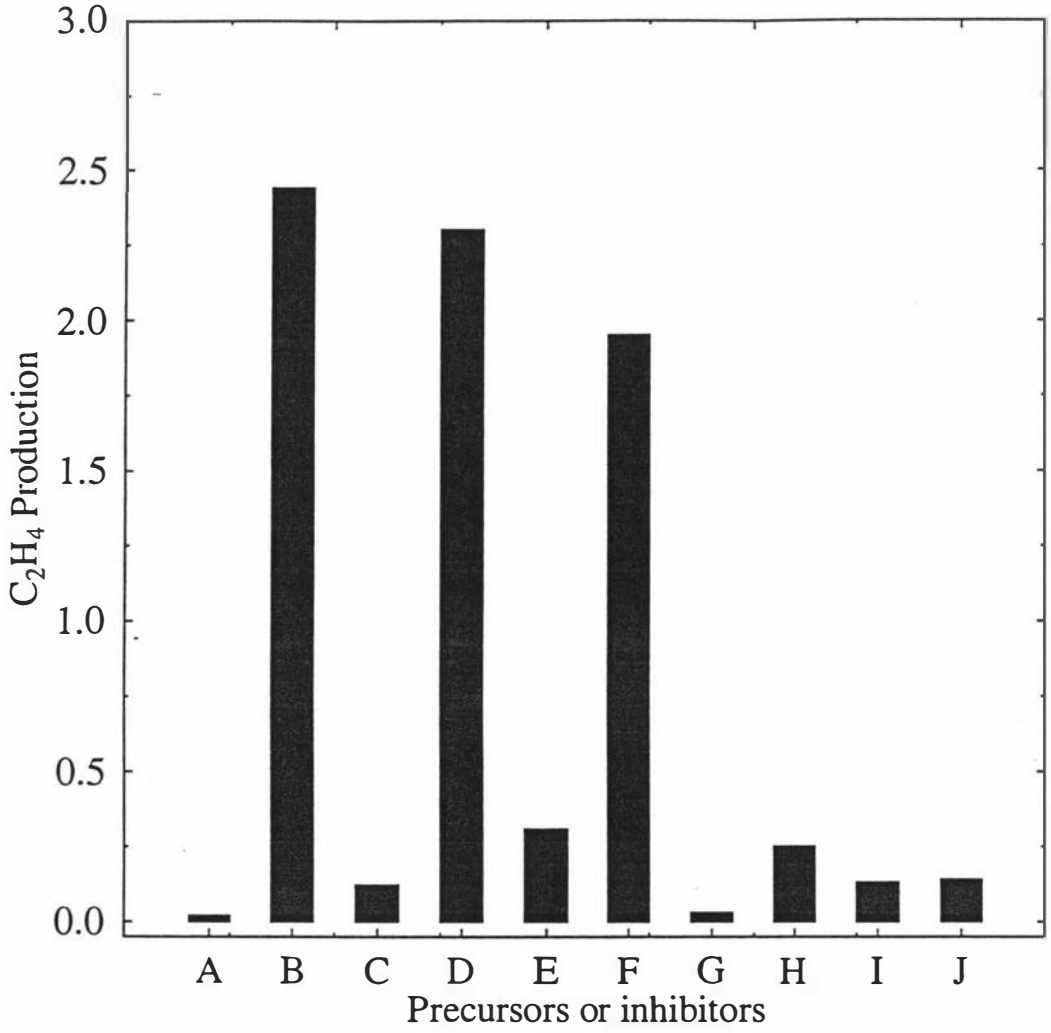


Fig 3.13 Ethylene production by B. cinerea grown in shake culture medium supplemented with range of 35mM precursors and/ or inhibitors (35mM) except Co [0.5mM] after 8 days of incubation at 22°C. Data expressed as area under the curve. A= Control, B= Methionine, C= ACC, D= Met+ACC, E= Meth+AOA, F= Meth+Co, G= ACC+Co, H= Meth+AOA+ACC, I= Meth+AOA+Co, J= Meth+AOA+ACC+Co

NB. Procedure for deriving C_2H_4 production as area under the curve (see Fig 3.2 and Appendix 1).

production which was 20 fold less than when methionine alone was added (Fig 3.13). There was no apparent consistency in final dry weight of mycelia (Table 3.7) grown in treatments with different combinations of methionine, ACC, AOA, and Co^{++}. Mycelia grown in methionine+Co^{++} had the highest mycelium weight, 100% more than in control cultures. Four treatments, ACC, ACC + Co^{++}, methionine+AOA+Co^{++} and methionine+AOA+ACC+Co^{++} had an inhibitory effect on mycelial weight while mycelia from the other treatments were heavier than those in basal medium (Table 3.7). The final pH of the treatment containing AOA and methionine+Co^{++} was less than the control, but there was no major difference in final pH of media from other treatments (Table 3.7).

A further experiment was carried out to confirm the previous findings. In this experiment a combination of methionine, ACC and AOA was used. Traces of ethylene were produced from cultures grown in the basal medium, (Fig 3.14). Methionine induced ethylene production when added to the basal medium. With methionine there was a steady increase in ethylene production to a peak (0.67 µl/l/h) on day 4, followed by a decline. When ACC was added to the basal medium, only traces of ethylene were produced through 8 days (maximum 0.03 µl/l/h), but when methionine and ACC were combined the ethylene peak obtained was the same as with methionine alone, (0.7 µl/l/h after 4 days) although production declined more rapidly than with methionine alone (Fig 3.14). When AOA was added with methionine, ethylene production increased slowly to 0.06 µl/l/h and remained at this level through 8 days (Fig 3.14).

There were no major differences in final dry weight of mycelium between treatments except with ACC where final dry weight was lower than that of the control (Table 3.8). The final pH's of all media were higher than the control, but there were no major differences between treatments except for ACC where the pH had increased during incubation (Table 3.8). When various concentrations of ACC were added to shake cultures very slight, and inconsistent responses occurred. Traces of ethylene were produced in all treatments (Fig 3.15) but ethylene production was many fold less than when methionine was added. Addition of ACC to the medium had little effect on final dry weight of mycelium although there appeared to be a slight inhibition of growth of 5 and 10 m*M* ACC (Table 3.9). There was no consistent change in final pH of the medium (Table 3.9).

Table 3.7

Mycelial dry weight (g) of *B. cinerea* and final pH in the medium with and without a range of possible ethylene precursors and/or inhibitors in shake culture after 8 days of incubation at 22°C. Initial pH was 3.5. These are average of 3 experiments.

Treatments	Mycelial dry weight ± SE	Final pH ± SE
Control	0.16 ± 0.01	3.40 ± 0.03
Methionine	0.29 ± 0.01	3.10 ± 0.03
Meth + ACC	0.22 ± 0.01	3.35 ± 0.34
ACC	0.09 ± 0.01	3.05 ± 0.06
Meth + AOA	0.25 ± 0.02	2.67 ± 0.03
Meth + Co^{++}	0.33 ± 0.01	2.95 ± 0.02
Meth + AOA + Co^{++}	0.11 ± 0.01	2.70 ± 0.00
Meth + AOA + ACC	0.25 ± 0.02	2.72 ± 0.03
Meth + AOA + ACC + Co^{++}	0.09 ± 0.01	2.80 ± 0.00
ACC + Co^{++}	0.12 ± 0.01	3.15 ± 0.02

Meth= methionine

ACC= 1-Aminocyclopropane-1-carboxylic acid 35m*M*

AOA= (Aminooxy) acetic acid 35m*M*

Co^{++}= Cobalt 0.5 m*M*

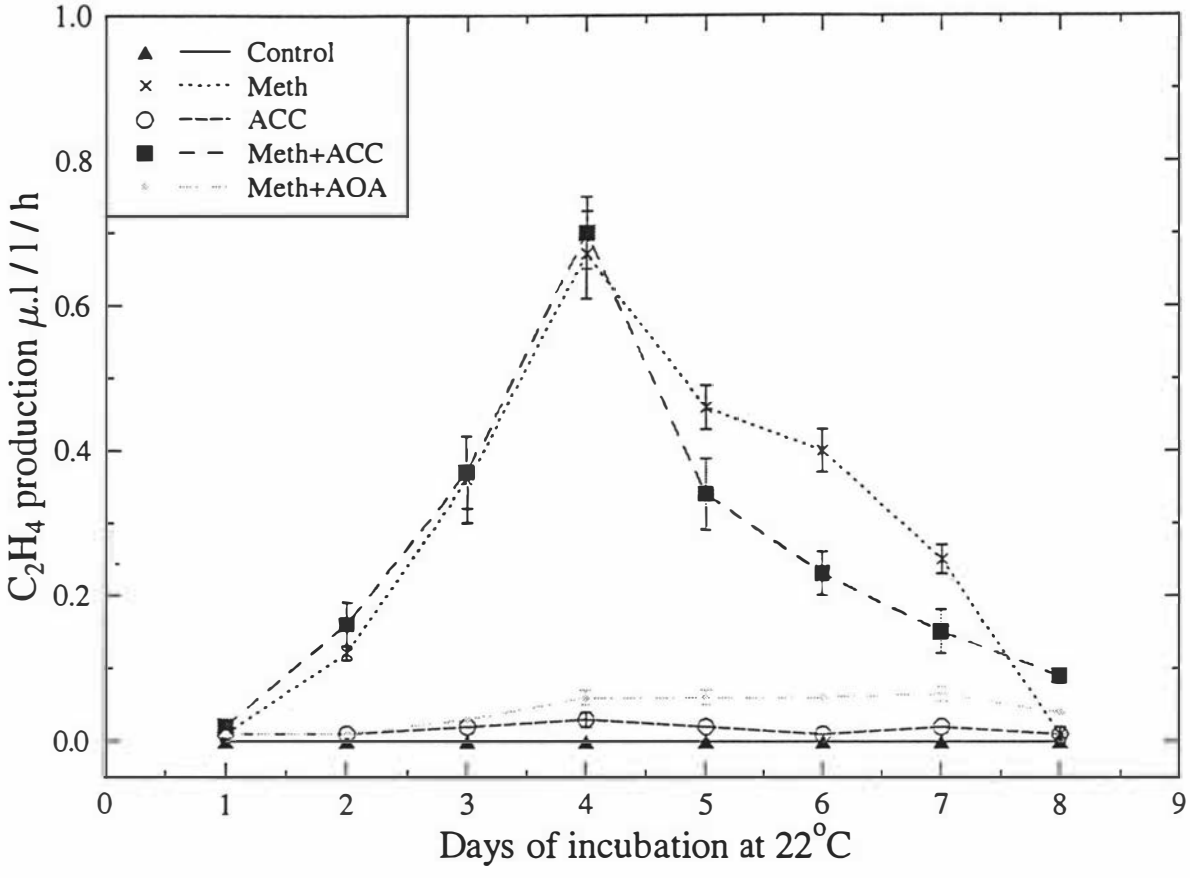


Fig 3.14 Ethylene production by B. cinerea grown in shake culture at 22°C supplemented with 35mM precursors and/or inhibitor. Data represents average of 4 experiments. Bars represents standard errors.

Table 3.8

Mycelial weight (g) *B. cinerea* and final pH in the basal medium with and without a range of possible precursors and/or inhibitore on shake culture after 8 days of incubation at 22°C.
Initial pH was 3.5

Treatments	Mycelial weight ± SE	Final pH ± SE
Control	0.15 ± 0.01	2.70 ± 0.07
ACC	0.09 ± 0.01	4.30 ± 0.07
Meth	0.16 ± 0.00	3.40 ± 0.13
Meth + AOA	0.15 ± 0.01	3.54 ± 0.02
Meth + ACC	0.15 ± 0.01	3.87 ± 0.03

Meth= methionine 35m*M*

ACC= 1-Aminocyclopropane-1-carboxylic acid 35m*M*

AOA= (Aminooxy) Acetic acid 35m*M*

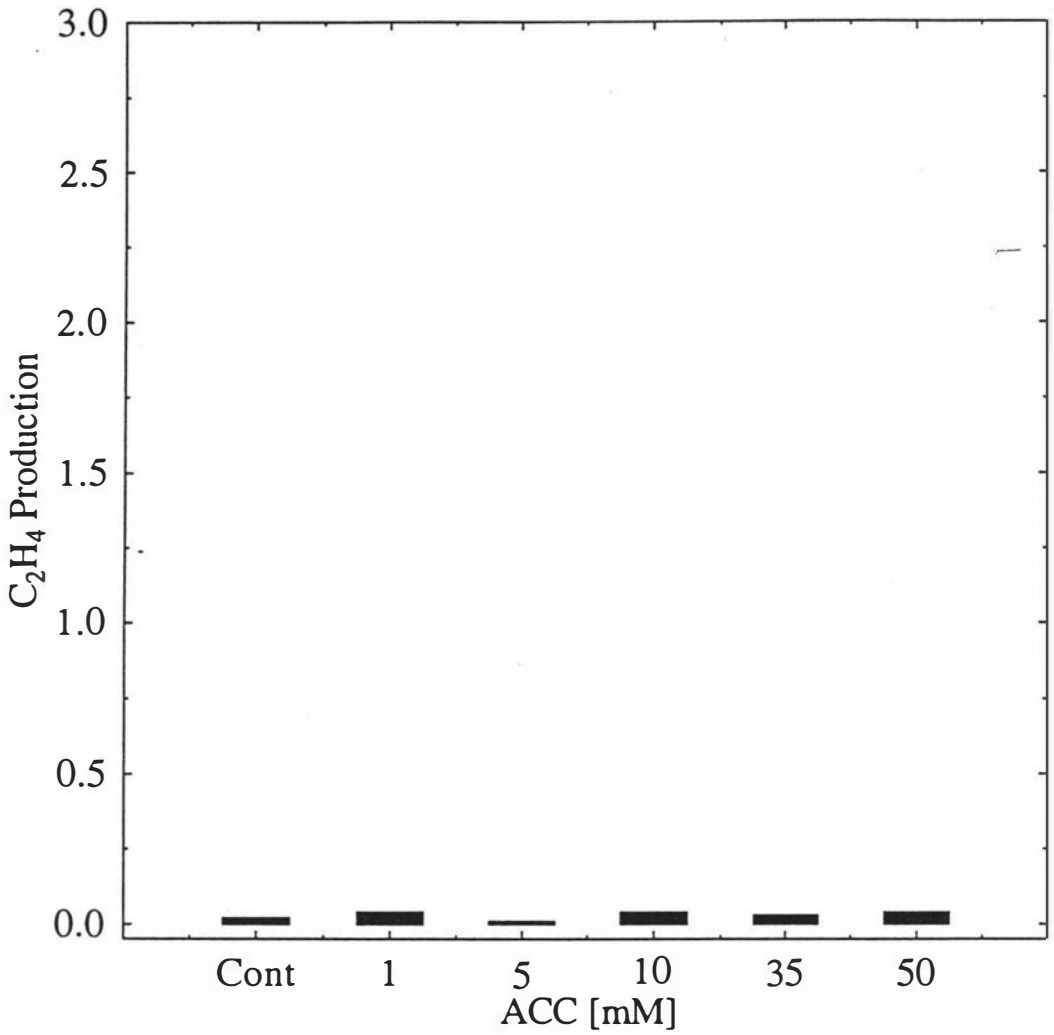


Fig 3.15 Ethylene production by B.cinerea in shake culture grown in media containing different ACC concentrations, after 8 days of incubation at 22°C. Data expressed as area under the curve

NB. Procedure for deriving C_2H_4 production as area under the curve (see Fig 3.2 and Appendix 1).

Table 3.9

Mycelial dry weight (g) of *B. cinerea* and final pH in basal medium following growth at a range of ACC concentrations in shake culture after 8 days of incubation at 22°C. Initial pH was 3.5

Treatments	Mycelial weight ± SE	Final pH ± SE
Control	0.29 ± 0.01	3.00 ± 0.00
1 m*M* ACC	0.26 ± 0.02	3.40 ± 0.23
5 m*M* ACC	0.22 ± 0.03	3.02 ± 0.06
10 m*M* ACC	0.24 ± 0.04	3.20 ± 0.14
35 m*M* ACC	0.27 ± 0.01	3.15 ± 0.12
50 m*M* ACC	0.30 ± 0.02	3.20 ± 0.11

An attempt was made to explore the interaction between ACC and Co^{++} in a medium of pH 5.0. Slight production of ethylene was detected in the control medium and this was not affected by the presence of either Co^{++} or ACC. The combination of ACC and Co^{++} resulted in a slight increase in ethylene production compared with that produced with either ACC or Co^{++} alone (Fig 3.16). The effect of Co^{++} on inhibition of ethylene production was concentration dependent (Fig 3.17). In the presence of methionine, ethylene decreased as Co^{++} concentration increased from 0.05 to 3 m*M* (Fig 3.17). Mycelial dry weight was not affected by Co^{++} concentrations up to 0.5m*M* but decreased at 1 and 3 m*M* (Table 3.10).

Two separate experiments were undertaken to investigate the interaction between methionine and Co^{++} on ethylene production. Little or no ethylene was produced in control treatments. The pattern of ethylene production was similar although displaced in time over the first 8 days of incubation with methionine or methionine plus Co^{++} in the medium. With methionine alone there was a steady increase of ethylene production reaching a peak of 0.7 µl/l/h after 4 days after which production gradually decreased. When Co^{++} was combined with methionine, ethylene production between day 1 and 4 increased slowly and at a lower rate than when methionine alone was used. However in experiment 1 after day 4 and experiment 2 after day 5 ethylene production increased rapidly to a peak of 0.7 µl/l/h on day 5 and 7 for experiments 1 and 2 respectively, after which it declined to a similar level to that of methionine after 8 days (Fig 3.18a & b). The peak height obtained was the same as for methionine alone.

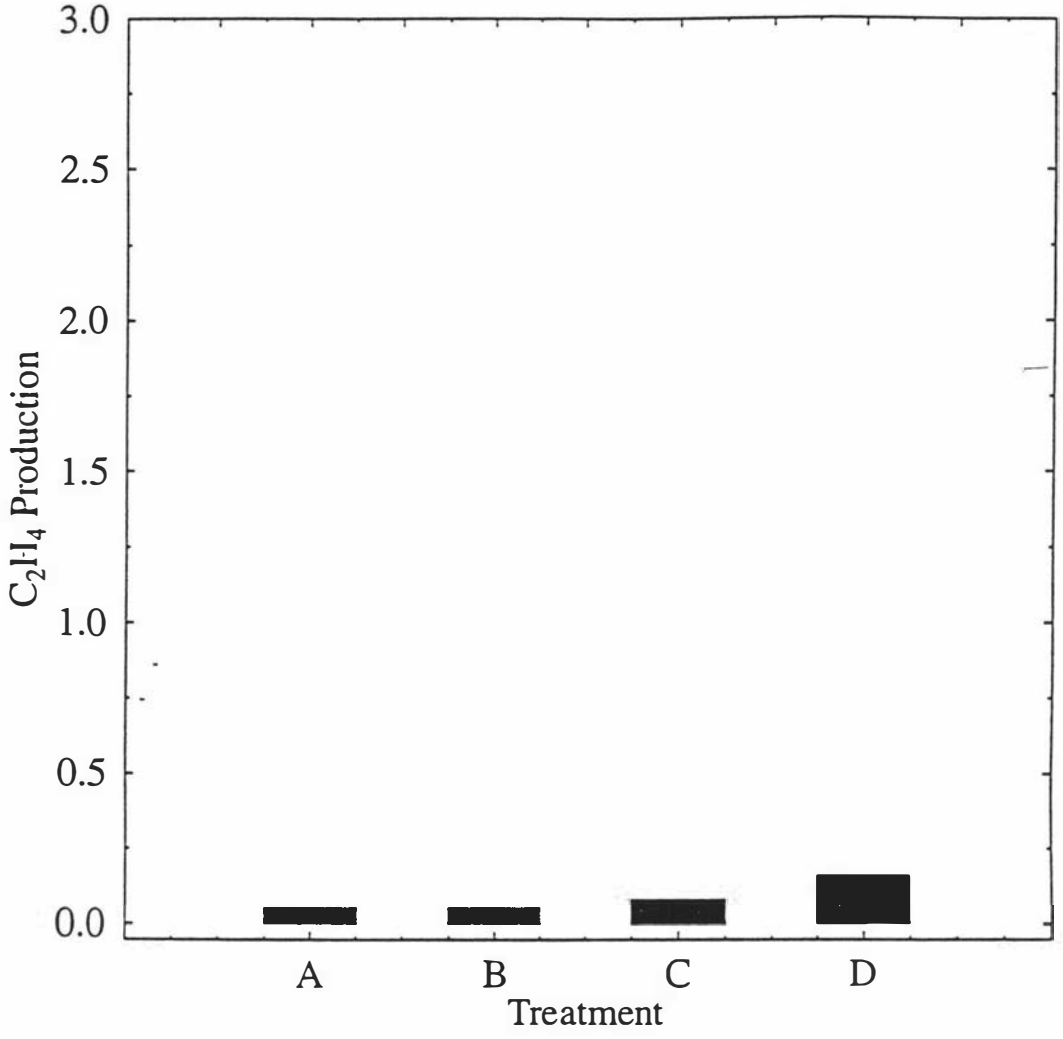


Fig 3.16 Ethylene production by B. cinerea in shake culture expressed as area under the curve, grown in media supplemented with 35 mM ACC and/ or 0.02 mM Cobalt after 4 days of incubation at 22°C A= Control,B= Cobalt, C= ACC, D= ACC+Cobalt

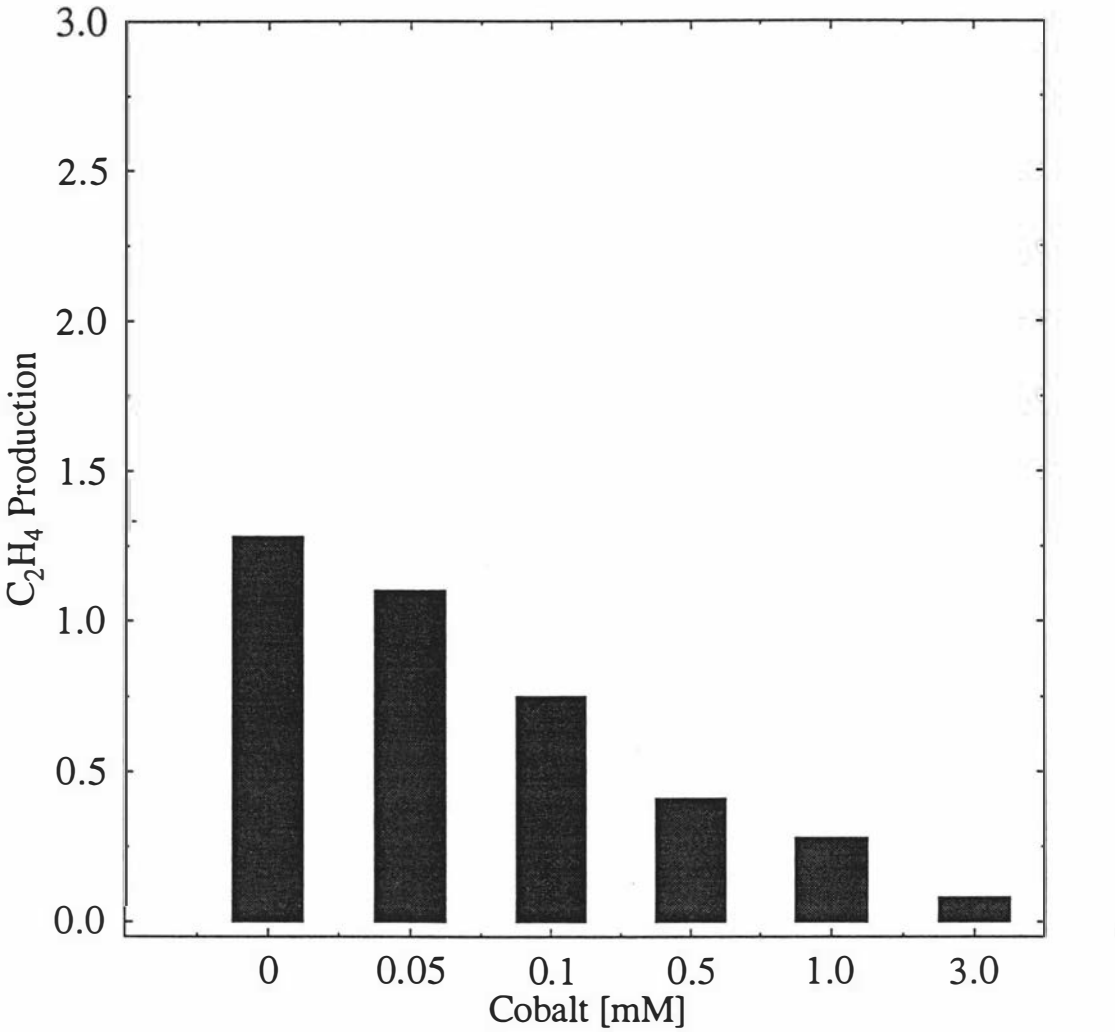


Fig 3.17 Ethylene production by B. cinerea in shake culture, grown in media supplemented with 35 mM methionine and different concentrations of Cobalt, after 4 days of incubation at 22°C. Data expressed as area under the curve.

NB. Procedure for deriving C_2H_4 production as area under the curve (see Fig 3.2 and Appendix 1).

Table 3.10

Mycelial dry weight of *B. cinerea* in basal medium supplemented with 35m*M* methionine and range concentrations of $CoCl_2$ in shake culture after 4 days of incubation at 22°C.

Treatments	Mycelial weight ± SE
Methionine	0.10 ± 0.01
Meth+ 0.01 m*M* Co^{++}	0.10 ± 0.01
Meth+ 0.05 m*M* Co^{++}	0.10 ± 0.01
Meth+ 0.10 m*M* Co^{++}	0.10 ± 0.00
Meth+ 0.50 m*M* Co^{++}	0.10 ± 0.02
Meth+ 1.00 m*M* CO^{++}	0.06 ± 0.01
Meth+ 3.00m*M* CO^{++}	0.05 ± 0.01

Meth= methionine

Co^{++}= Cobalt

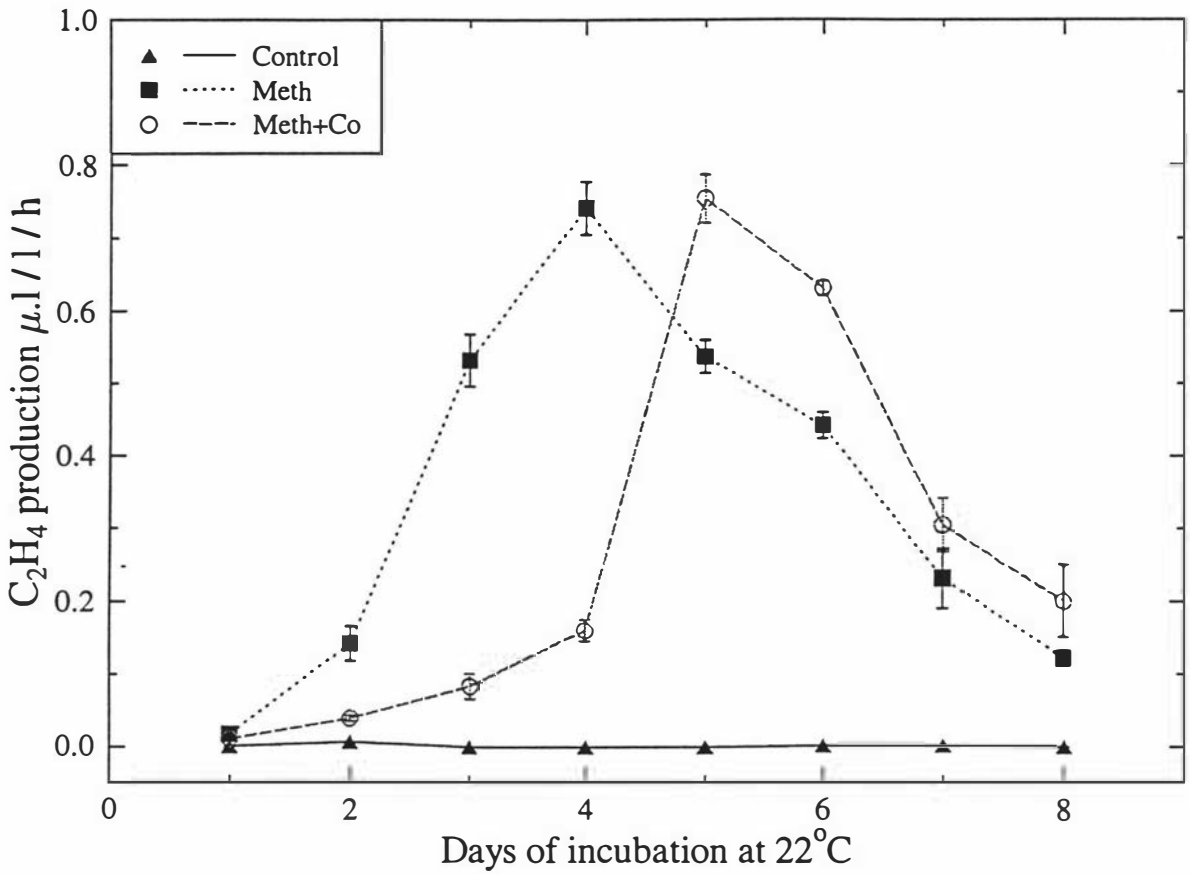


Fig 3.18a Ethylene production by B. cinerea in shake culture, grown in medium supplemented with 35mM methionine and/ or 0.5mM Cobalt at 22°C. Bars represents standard errors. Co= Cobalt, Meth= Methionine

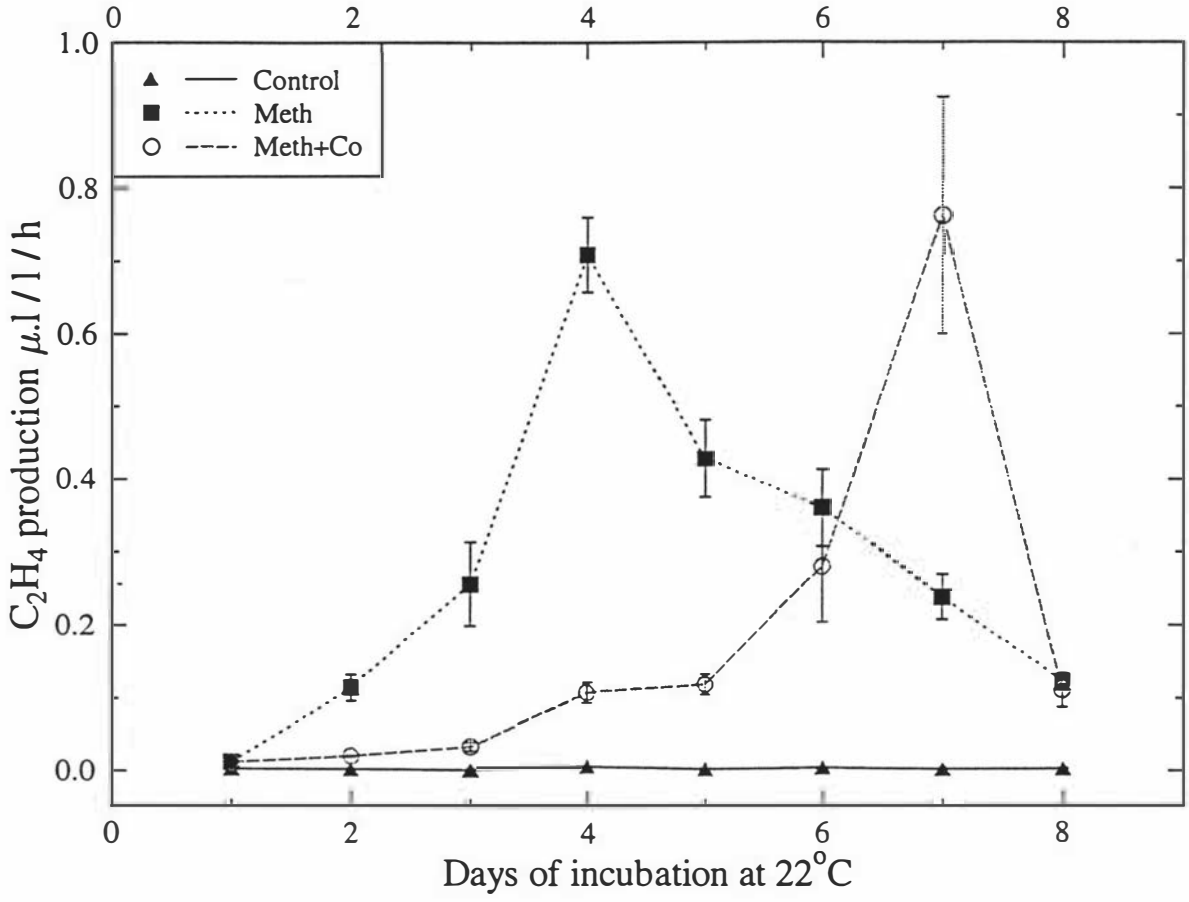


Fig 3.18b Ethylene production by B.cinerea in shake culture, grown in medium supplemented with 35mM methionine and/ or 0.5 mM Cobalt at 22°C. Bars represents standard errors. Co= Cobalt, Meth= Methionine

DISCUSSION

Ethylene is produced by *P. digitatum* an important postharvest pathogen. It is also produced by a range of plant pathogenic bacteria and fungi but there are no reports that ethylene is produced by *B. cinerea* a widespread ubiquitous and commercially important postharvest pathogen (Smith et al, 1964; Elad, 1990).

B. cinerea grown on a modified Pratt's basal medium has been shown to produce only trace amounts of ethylene when incubated for up to 22 days in both shake and static cultures. Addition of methionine to the basal medium consistently resulted in physiological levels of ethylene being produced by *B. cinerea.* This effect was concentration dependent with the maximum and saturating effect occurring at 35 m*M* methionine.

B. cinerea is a pathogen with a wide host range. Isolates of this fungus can exhibit extreme variations in morphology both on the host plant and in culture (Stewart, 1986). Conidia and hyphal cells of *B.cinerea* may contain up to 18 nuclei and the morphology of any one isolate may be determined in part, by the arrangement and number of nuclei containing either of the two "elements", responsible for mycelial or conidial development (Hansen, 1938). The expression of these "elements" can be modified to a certain degree by the environment in which the fungus is growing (Paul, 1929).

Production of ethylene by *B. cinerea* isolates showed some variability when grown on basal media. A camellia isolate produced more ethylene than isolates from kiwifruit, strawberry, grape and blueberry. Methionine added to the basal media induced marked increases in ethylene production from all isolates tested with those from strawberry and kiwifruit producing about 50% more than those from camellia, grape and blueberry. This variability of ethylene production between different isolates of fungus was also observed in *E. gyrosa* and *C. eucalypticola* (Wilkes et al, 1989). The isolate 'K3" which was used for the majority of the experiments reported in this chapter, varied in its capacity to produce ethylene from one experiment to another under the same cultural conditions; peak production with 35 m*M* methionine in the medium ranged from 0.36 to 1.1 µl/l/h in different experiments. Such variability has been demonstrated in *P. digitatum* where ethylene production by cultures

derived from individual spores varied markedly from zero (Prince et al, 1988) to 500 µg/day/culture (Spalding and Lieberman, 1965). The present results show that in *B. cinerea*, all isolate tested has the capacity to produce ethylene in the presence of methionine.

In *B. cinerea* methionine was the most efficient ethylene precursor tested in both static and shake cultures over a range of pH's. Neither glutamate nor α-ketoglutarate were able to induce ethylene production in the culture conditions used and are therefore unlikely to be effective precursors for ethylene in *B. cinerea.* Most reported work on ethylene production by fungi has measured production during the culture period rather than relating it to growth of the fungus itself (eg. mycelial weight). However, there are a few reports which do relate ethylene production to mycelial weight. In shake culture, ethylene production by *P.digitatum* was about 280 nl/mg F.wt/h (Chalutz and Lieberman, 1977) and in *V dahliae* this rate was 3.11 nl/mg/h (Tzeng and DeVay, 1984). In lower plants such as the unicellular green alga, *H.pluvialis*, ethylene production was 151 nmol/g (dry weight)/h (Maillard et al, 1993), and in the lichen, *R.duriaei*, it was 3.4 nl/g/h (Lurie and Garty, 1991). None of the above indicated that large amounts of ethylene were produced soon after inoculation. However in *B.cinerea* within 2 days of inoculation, the rate of ethylene production was high at about 780 µl/(dry weight) g/ h after which it declined to 74 µl/g/h and after 3 days ethylene production was about 32 µl/g/h. In *P.digitatum* a surge in ethylene production (about 280 nl/mg F.wt/h) was reported to occur after 3 days of incubation (Chalutz and Lieberman, 1977). That *B. cinerea* is capable of producing such large amounts of ethylene in shake culture conditions soon after inoculation indicates that provided methionine is present, *B. cinerea* contains the necessary constitutive enzymes present to allow biosynthesis of ethylene. As this level of ethylene production is not sustained during the remainder of the incubation period this suggests that in culture *B. cinerea* may secrete some kind of ethylene inhibitor in to the medium. Lieberman (1977) reported that microorganisms secrete a number of alkyl-substituted enol ether amino acids into their growth media which inhibit ethylene production. It may also suggest that ethylene production was surplus to requirements and this initiated a feedback mechanism to reduce production. In higher plants ethylene production can exhibit both positive and negative feedback. Under experimental conditions, externally applied ethylene can either increase

(autostimulation) or decrease (autoinhibition) ethylene production. The large rise in ethylene production observed during fruit ripening and floral senescence of some species is called autocatalytic ethylene production, and is the result of increased ACC synthase and EFE activity (Sawamura and Miyazaki, 1989). It has been suggested that the autostimulation of ethylene production is due primarily to an increase in EFE activity (Gupta and Anderson, 1989; Ievinsh et al, 1990; Schierle et al, 1989). The autoinhibition of ethylene production is due to a decrease in the levels of ACC synthase (Hyodo and Fujinami, 1989; Liu et al, 1985a; Nakajima et al, 1990; Philosoph-Hadas et al, 1985) or EFE (Ketsa and Herner, 1989; Sawamura and Miyazaki, 1989), or to an increase in conjugated ACC (Gupta and Anderson, 1989). However there have been no reports which indicate the existence of autostimulation or autoinhibition of ethylene in fungi.

The pathway of ethylene production in higher plants is well established (Abeles et al, 1992; Konze and Kende, 1979; Lieberman, 1979; Yang and Hoffman, 1984; Yang, 1985; Lieberman et al, 1966; Yang, 1974). Methionine is accepted as the precursor which is converted to SAM, with ACC being the immediate precursor of ethylene (Adams and Yang, 1979; Konze and Kende, 1979). Many lower plants also produce ethylene, but the biosynthetic pathway for these organisms is not at all clear. Osborne (1989) has suggested that ACC may not be a critical precursor in lower plants. Addition of ACC to such plants often did not result in increased ethylene production (Osborne 1989; Fukuda et al, 1986; Goto et al, 1985; Hottiger and Boller, 1991; Coleman and Hodges, 1986), as found for many higher plants (Lieberman, 1979; Adams and Yang, 1979; Lurssen et al, 1979; Konze and Kwiatkowski, 1981; Guy and Kende, 1984).

A number of compounds have been shown to act as ethylene precursors in fungi including glutamate in *P. digitatum* (Chalutz and Lieberman, 1978), methionine in *P. digitatum*, (Chalutz and Lieberman, 1977) and *E. gyrosa* and *C. eucalypticola*, (Wilkes et al, 1989), glucose in *Mucor* sp. (Dasilva et al, 1974) and vanillic and syringic acid in *Penicillium* sp. (Considine and Patching, 1975). Fukuda and Ogawa (1991) have proposed that ethylene production in fungi may come from two different pathways: either it is produced from a pathway starting with methionine or it is derived from a pathway closely related to the Kreb's cycle with glutamate or α-ketoglutarate as the initial substrate (Chou and Yang,

1973). The particular pathway for production of ethylene may depend on cultural conditions being used in different experiments. In shake cultures of *P. digitatum*, ethylene is produced from methionine (Chalutz and Lieberman, 1977) but in static cultures glutamate or α-ketoglutarate are precursors (Chalutz and Lieberman, 1978). In other fungi such as *Verticillium*, *Fusarium* and *Colletotrichum*, ethylene is produced in the presence of methionine under both shake and static conditions (Tzeng and DeVay, 1984). This indicates that these fungi possess the ethylene-forming enzyme system necessary to catalyse the various intermediates which would be involved in such a methionine dependent ethylene pathway but the nature of these intermediates have not been identified. However in *P. digitatum* shake cultures production of ethylene in response to methionine seems to involve induction of an ethylene-synthesizing enzyme system(s) in the fungal cell. The lack of ethylene production in the absence of methionine, the requirement of viable fungal cells for ethylene production to occur and the existence of a lag period all support this view (Chalutz and Lieberman, 1977). Evidence to support this suggestion was produced by Fukuda and Ogawa (1986) who demonstrated that methionine did not act as a precursor of ethylene formation in a cell free system of *P. digitatum*.

The enzyme methionine adenosyltransferase (ATP:methionine S-adenosyltransferase, EC 2.5.1.6), which catalyses the conversion of methionine to SAM, has been widely studied (Chou et al, 1977). This enzyme was purified from *E coli*, (Markham et al, 1980; Boyle et al, 1984) and yeast (Chiang and Cantoni, 1977). Hoffman and Kunz (1980) purified this enzyme for the first time from mammalian (rat liver). Konze and Kende, (1979) indicated that it may be related to ethylene biosynthesis in plant tissues. It is possible that this enzyme is present in *B. cinerea*, although no reports exist to indicate that it has been detected. If it was found in *B. cinerea* this might suggest that methionine could be converted to SAM before being converted into ethylene. If it is not present, then this might indicate that in *B. cinerea* methionine is converted into an as yet unknown precursor before being converted into ethylene. Fukuda et al, (1993) suggested that in some microorganisms methionine converts into KMBA which is then converted into ethylene. It is possible that the same pathway also exists in *B. cinerea*.

In an attempt to determine whether the ethylene biosynthetic pathway in higher plants

(involving methionine, SAM and ACC) exists in *B. cinerea*, a series of experiments were carried out with different combinations of possible ethylene precursors, such as methionine and ACC, and/or inhibitors such as AOA and Co^{++} ions, inhibitors of ACC synthase and ACC oxidase respectively (Yang and Hoffman, 1984; Yu and Yang, 1979). AOA is a known inhibitor of pyridoxal phosphate mediated enzyme reactions (Yu et al, 1979) and strongly inhibits the conversion of methionine to ACC (Amrhein and Wenker, 1979). Co^{++} is an effective inhibitor of ethylene synthesis (Yu and Yang, 1979; Lau and Yang, 1976) and it is thought to exert its inhibitory effect by complexing with protein sulfhydryl groups (Thimann, 1956; Yu and Yang, 1979).

In *B. cinerea* addition of ACC to the basal medium slightly but inconsistently induced a small increase in ethylene production. No consistent effect of ACC concentration on ethylene production was observed. When ACC was added to media containing methionine, the overall ethylene production was no greater than that of methionine alone. However ethylene production was reduced by about 70% when AOA was added to media containing methionine; addition of ACC to this medium did not reverse the inhibitory effect of AOA, which has been demonstrated in higher plants (Mekers et al, 1983). AOA inhibited ethylene production from *P. syringae* pV *phaseolicola*, a bacteria in which glutamate but neither methionine nor ACC were found to induce production of ethylene (Goto et al, 1985). In the lichen *R. duriaei*, the ACC synthase inhibitor AVG, did not prevent the stimulation of ethylene production induced by glutamate, methionine or ACC (Lurie and Garty, 1991).

These results show clearly that ethylene production is induced in *B. cinerea* with methionine, that ACC added to the culture media is not able to induce ethylene production and that AOA strongly inhibits ethylene production, an inhibition which is not reversible by addition of ACC. This suggests that while methionine can be metabolised to form ethylene in *B. cinerea*, it does not occur through the biosynthetic pathway which exists in higher plants, via ACC. Wilkes et al, (1989) also found that methionine induced ethylene production in *E. gyrosa* and *C. eucalypticola* where ACC was not an intermediate of the ethylene pathway. It is possible that in *B. cinerea* methionine metabolised to ethylene through a pathway in which a pyridoxal phosphate mediated enzyme reaction exists, which

is different from ACC synthase. The pyridoxal phosphate dependent enzymes are known to be involved in a number of important metabolic reactions such as the formation of vitamin B6 (Delport, 1991), and in higher plants the conversion of SAM to ACC (Adams and Yang, 1979). However in *B. cinerea* the identity of the enzyme(s), the nature of the intermediate(s) between methionine and ethylene and rate limiting steps in this pathway are not known and cannot be inferred from the present results.

Addition of 0.5m*M* Co^{++} ions to a medium containing 35m*M* methionine did not inhibit ethylene production over an 8 day period. The main effect of the addition of Co^{++} ions was to delay the increase of ethylene production by 1-3 days when compared to cultures grown in media supplemented with methionine alone; peak ethylene production was the same in both treatments. This delay in ethylene production might indicate that either: (1) Co^{++} could suppress the usual ethylene forming enzyme system(s) in *B.cinerea* and after 4-6 days when this suppression diminished there was a surge in ethylene production, or (2) With the suppression of the natural ethylene-forming enzyme there was induction of an alternate ethylene-synthesizing system(s). In shake culture of *P. digitatum* there was a delay in ethylene production in response to methionine indicating the induction of an ethylene-synthesizing system (Chalutz and Lieberman, 1977). These results suggest possible differences in the nature of the ethylene forming enzyme complex between lower and higher plants. In higher plants the immediate precursor ACC is metabolised to ethylene by ACC oxidase (Ververidis and John, 1991) and its activity is inhibited by addition of Co^{++} (Yu and Yang, 1979). However, in lower plants Co^{++} appears to have little or no effect on ethylene production as with *B. cinerea* or it can stimulate ethylene production, as in unicellular green algae. Thus there appear to be major differences between the ethylene-forming enzymatic complexes that occur in higher and lower plants (Maillard et al, 1993).

Results presented in this chapter clearly indicate that methionine can act as a precursor for ethylene production from *B. cinerea* grown on a defined medium in both static and shake cultures. However ethylene does not appear to be produced through the biosynthetic pathway commonly accepted as occurring in higher plants. ACC does not seem to be involved in *B. cinerea*, even though AOA does inhibit ethylene production. The immediate precursor of ethylene in this *B. cinerea* system is not known; that ethylene production is

only delayed rather than being inhibited when Co^{++} ions are added to a methionine containing medium raises the possibility that multiple forms of ethylene forming enzymes might exist in *B. cinerea*. ACC synthase is known to occur as several isozymes, different forms being activated depending on the environmental signal received and specific cell response required (Mori et al, 1993). ACC oxidase can also exist present in multiple forms (Bouzayen et al, 1993); if this happened in *B. cinerea* than it is conceivable that the nature of the isozyme present may depend on the stimulus being received by the fungus and perhaps may vary according to the isolate being used.

CHAPTER 4

ETHYLENE PRODUCTION BY *B. CINEREA* INFECTED KIWIFRUIT.

INTRODUCTION

Kiwifruit is a major export horticultural crop of New Zealand (Jenks, 1994). At the beginning of commercial development, this crop was regarded as virtually disease-free (Bailey 1950, 1961; Bailey and Topping, 1951; Schroeder and Fletcher, 1967), but with the rapid expansion of production and a prolonged period of monoculture, disease problems have become more numerous and important (Sale, 1980). In 1978, storage rot caused by *B. cinerea* was recognised as a problem in New Zealand kiwifruit (Beever et al, 1984), when overall estimated loss to the industry was about 2-3% (Pennycook, 1985a). In addition to grower losses due to infected fruit there are three further serious implications for fruit quality. Firstly fruit infected with *B. cinerea* produce ethylene. The presence of infected fruit in a tray hastened softening of other fruit in the tray reducing storage life (Manning and Pak, 1993). Secondly, fruit infected with *B. cinerea* may result in an increased incidence of "ripe rot", caused by *Botryosphaeria* spp, in fruit at ambient temperature after storage (Brook, 1990a; b). Thirdly, Botrytis rot generally did not appear in fruit until 4-6 weeks after they were packed into trays and stored at 0°C before exporting. Costly systems had to be developed to ensure removal of infected rot fruit either before export or in overseas markets (Manning and Pak, 1993).

Lines of fruit from different orchards develop differential amounts of Botrytis rots in storage (Manning and Pak, 1993). This may relate to differences in growing conditions between orchards, to varying amounts of Botrytis inoculum present prior to and at harvest and also to differences in postharvest handling of fruit. Infection occurs through the picking wound at harvest (Manning and Pak, 1993) and incidence of *B. cinerea* infection is proportional to the spore load present on the stem scar *(Long, Pers. com.)*.

Several mechanisms have been suggested as being responsible for the initial postharvest infection in kiwifruit; dying petals may provide a suitable substrate for the growth and

production of *B. cinerea* spores (Brook, 1990a), and when kiwifruit are harvested these senescent parts could provide a major source of inoculum (Pak and Manning, 1994). *B. cinerea* is known as weak pathogen requiring an open wound site through which to gain entry (Heale, 1992). The picking wound of kiwifruit stem provides an ideal infection site for *B. cinerea* entry. When *B. cinerea* spores were applied to the picking wounds of freshly picked fruit, 85-100% of the fruit became infected and rotted during cold storage (Pennycook, 1984). In the same study when *B. cinerea* spores were placed in the picking bags there was an increase in incidence of storage rot from 11% to 28% (Pennycook, 1984). The development of high levels of storage rot after direct application of *B. cinerea* spores to the picking wound provided convincing evidence of the importance of the postharvest mechanism and it is now generally accepted that *B. cinerea* contamination occurs at harvest or soon after harvest during grading and packing (Brook, 1990a).

The presence of ethylene undoubtly accelerates the rate of softening of kiwifruit (Wright and Heatherbell, 1967; Pratt and Reid, 1974; Matsumoto et al, 1983). At 20°C continuous treatment of kiwifruit with 5 ppm of ethylene has been shown to cause rapid flesh softening, from 6.3 kgf to 1.1 kgf in 4 days (Matsumoto et al, 1983). Even at 0°C, kiwifruit are susceptible to ethylene and concentrations as low as 0.03 ppm have been shown to affect fruit softening, although the minimum threshold limit of kiwifruit sensitivity to ethylene has not been determined (Harris and Reid, 1981; McDonald and Harman, 1982).

Several studies in postharvest and wound physiology of plant tissue have been carried out using tissue slices (ap Rees, 1969; Palmer and McGlasson, 1969). Slices can be used to study a range of metabolic (physiological/ biochemical) effects following treatments, with results being able to be applied to whole tissues. Pesis et al (1991) studied the effect of *B. cinerea* infection on compositional changes of kiwifruit using slices from different zones of the fruit. Slices have also facilitated experiments on the effect of exogenous substances on tissue (Campbell and Labavitch, 1991), as well as metabolic pathway and reaction mechanisms (Frenkel et al, 1969). Hirano et al (1991) used tissue slices to investigate the ethylene production in sweet potato root tissue infected by *C. fimbriata*. Studying the wound effect, Meight et al (1960) showed that ethylene production by tomato fruit was greatly stimulated by cutting the pericarp wall into slices of 4-mm cubes. The rate of

ethylene production in slices was more than 23 times that in whole fruit. Wounding induced ethylene production in *rin* mutant tomato fruit that produce little or no ethylene when intact (Herner and Sink, 1973). McGlasson and Pratt (1964) found striking differences in the rate of ethylene production between whole fruit and tissue slices (4 and 6 mm thick) of cantaloupe melon. The rate of ethylene production by the slices was at least 10 times greater than that produced by whole fruits. Similarly, a rapid rate of ethylene production was induced in discs of sweet potato root (Imaseki et al, 1968b), green banana (McGlasson, 1969) and grapefruit flavedo (Riov et al, 1969).

Various environmental factors such as wounding, physical compression, disease, and chilling temperatures induce ethylene production in plants. These kinds of ethylene production are collectively called "stress ethylene" (Abeles, 1973). Ethylene production in plants induced by stress (Kimmerer and Kozlowski, 1982; McKeon et al, 1982; Wang and Adams, 1982), mechanical wounding (Boller and Kende, 1980; Jackson and Campbell, 1976; Kende and Boller, 1981; Konze and Kwiatkowski, 1981; Saltveit and Dilley, 1978; Yu and Yang, 1980), and disease (De Laat and Van Loon, 1982, 1983; Hodges and Coleman, 1984; Koch et al, 1980; Montalbini and Elstner, 1977; Reuveni and Cohen, 1978; Sakai et al, 1970; Spanu and Boller, 1989; Canfield and Moore, 1983), has been studied extensively. Biosynthesis of disease induced ethylene is believed to differ from that induced by wounding and from that in plants or fruit undergoing normal physiological development (Koch et al, 1980; Montalbini and Elstner, 1977; Sakai et al, 1970). Details of these different ethylene biosynthetic pathways involving different ACC or ACC oxidase gene triggers have been outlined in chapter 1.

Ethylene evolved by infected plants is a stress response to pathogen attack (Abeles, 1973; Archer and Hislop, 1975; Sequeira, 1973), and the rise in ethylene production is an early event which happens soon after inoculation (Montalbini and Elstner, 1977; Pegg, 1976). In tomato, induction of ethylene occurred within 24h of inoculation with *Colletotrichum gloeosporioides* at which stage infection was barely detectable visually (Cooper et al, 1993). Similarly, in infected melon seedlings, ethylene was detected 30 h after inoculation about 2 days before appearance of symptoms (Roby, 1982; Toppan et al, 1982). At that time, the main hydrolytic activities usually displayed by pathogens were not detectable

(Albershiem and Valent, 1978; Esquerre-Tugaye, 1972).

The unpublished work of S.R Pennycook (cited by Brook, 1990a,b) investigated the relationship between ethylene production from *B. cinerea* infected fruit and fruit softening in kiwifruit. He inoculated and maintained kiwifruit at either 0°C or 20°C, after 24 weeks of storage at 0°C. No detectable ethylene was produced by healthy fruit at either temperature, whereas ethylene was detectable in infected fruit at both temperatures before rot symptoms appeared. Ethylene production was detectable after 8 days at 20°C in inoculated infected fruit but rots were not visible until 15 days. At 0°C ethylene was detectable 20 days after inoculation and rots were visible after 34 days. Further work (unpublished results of Pennycook and Manning, cited by Brook, 1990a,b) to determine the relationship of ethylene evolved by infected kiwifruit on fruit softening, indicated that during storage at 0°C, the presence of *Botrytis* infected fruit reduced the storage life of healthy fruit packed in the same tray. Their fruit firmness measurements (Fig 4.1) showed that healthy fruit in a tray containing infected fruit initially softened more rapidly than fruit in the control treatment. However by the 8th week, there was little difference between treatments. As fruit with firmness below 1-1.2 kgf at the pack house are not acceptable for shipping, it would appear that even a few infected fruits could cause a line to be rejected for softness.

It is generally accepted that once cells are killed as a result of fungal infection they cease to produce ethylene (Williamson 1950). Similar conclusions were drawn from work on black rot disease of sweet potato caused by *Ceratocytis fimbriata* (Imaseki et al, 1968b), brown rot of apple caused by *Sclerotinia fructigena* (Hislop et al, 1973), and *B. cinerea* infected leaves of tomato, pepper, bean and cucumber (Elad, 1990). In all cases ethylene generation was concentrated at the margin of the lesion. However some ethylene was produced from the body of diseased sweet potato (Imaseki et al, 1968b), or apple (Hislop et al, 1973) tissue where host cells were dead but the mycelium was still viable. It is possible that this ethylene was produced by the fungus rather than the diseased tissue.

The elevation in ethylene production in response to pathogen attack is one of the earliest chemically detectable events in pathogen-infected plants

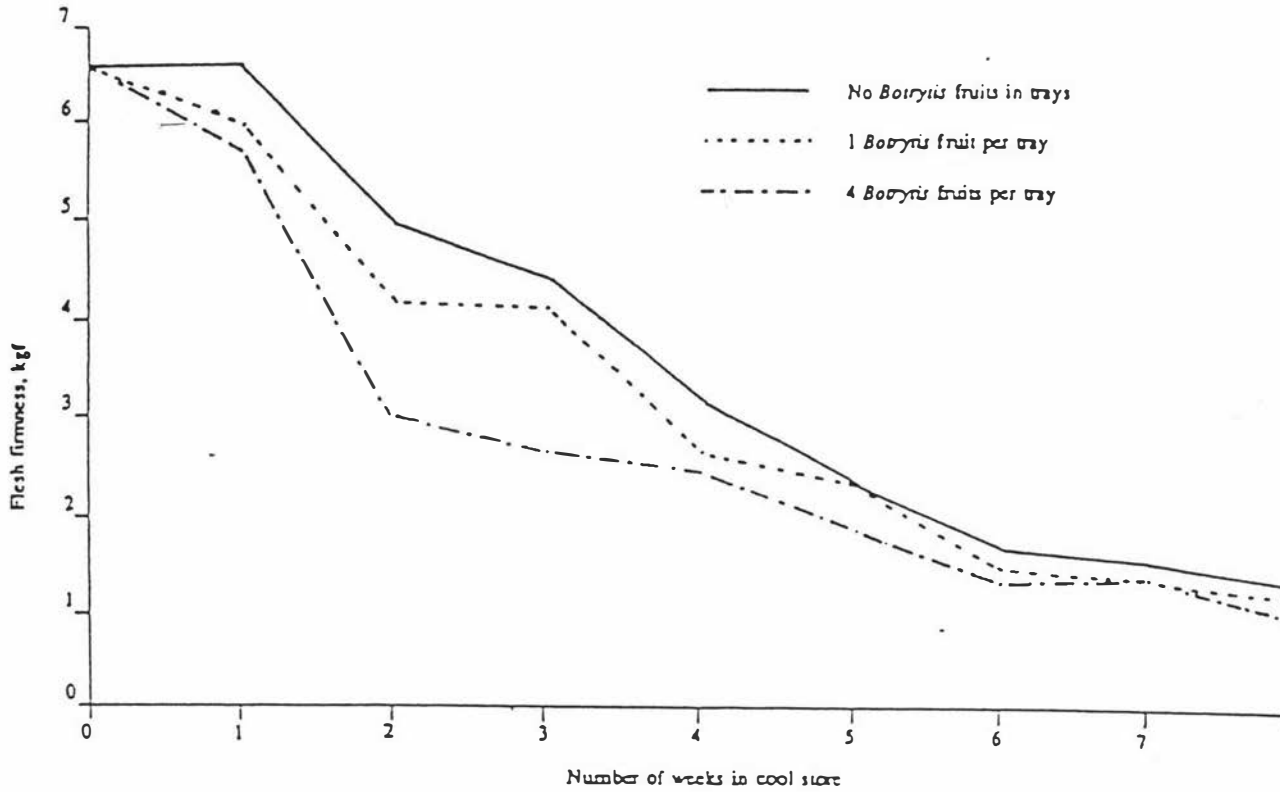


Fig. 4.1 Flesh firmness of healthy fruit during the first 8 weeks in cool store. Fruit was packed in trays. Differential treatments were: 1 inoculated fruit packed in each tray; 4 inoculated fruits packed in each tray; controls - no inoculated fruits in trays. Five trays (of 33fru its) per treatment at each examination date.

(Data: S.R. Pennycook & M.A. Manning, 1987).

(Paradies et al, 1979; Pegg, 1976; Toppan et al, 1982). This stress-induced rise in ethylene production is caused primarily by the enhanced 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity (Boller and Kende, 1980; Yu and Yang, 1980; De Laat and Van Loon, 1982; Fuhrer, 1982). This enzyme has been described as a rate limiting step in biosynthesis of ethylene (Hoffman and Yang, 1980; Sitrit et al, 1986; Yang, 1985; Yang, 1987b). In *Poa pratensis* the peak ACC-synthase activity in response to infection was more than five times greater than that due to wounding (Coleman and Hodges, 1987). ACC content of kiwifruit infected with *B. cinerea* (Niklis et al, 1993) or soft rot (*Botryosphaeria* and/ or *Phomopsis* spp.) (Hasegawa and Yano, 1990) was higher than that in noninfected fruit. Within 10 days after inoculation, there was a sharp accumulation of ACC content followed closely by an increase in ethylene production in the infection front of infected kiwifruit (Nikilis et al, 1993). Similarly, ACC content of *B. cinerea* infected tomato fruit was much higher than in control fruit (Takeda and Nakamura, 1990). However this increase in ACC has not always been detected following fungal infection. In infected sweet potato root tissue there was no increase in ACC synthase activity, but ethylene production was greater than in healthy tissue (Hyodo and Uritani, 1984). This suggests that a possible alternate pathway of ethylene production, not involving ACC, may be activated in response to infection in some plants.

ACC-oxidase or ethylene forming enzyme (EFE) catalyses the final step in ethylene biosynthesis. Until recently this enzyme could not be recovered *in vitro* because of the complete disappearance of activity when tissue was homogenized (Yang and Hoffman, 1984; Kende, 1989). However, some activity was retained by vacuoles isolated from leaf mesophyll (Guy and Kende, 1984) and by membrane vesicles in juice squeezed from kiwifruit (Mitchell et al, 1988). These systems retained only 1% (Porter et al, 1986) or less (Mitchell et al, 1988) of the *in vivo* activity, and it disappeared completely when membrane integrity was destroyed (Porter et al, 1986; Mayne and Kende, 1986). This ACC oxidase activity was finally recovered and purified from higher plants (Ververidis and John, 1991). However, Fukuda et al (1986) reported the partial purification of an ethylene forming enzyme from a fungus (*P. digitaum*) which did not produce ethylene when ACC was added suggesting that in this fungus ACC was not involved as a precursor of ethylene.

Tetrazolium dye is commonly used to determine whether plant tissues are alive or dead (Busso et al, 1993). Dehydrogenase enzymes present in living tissue reduce the colourless, water soluble tetrazolium salt to the red, water insoluble formazane, which is precipitated within living cells; in dead cells no reaction takes place and dead tissues usually remain colourless (Perry, 1981; Grabe, 1970). Lindenbein (1965) found that sometimes dead tissue showed a pink colour due to the presence of organisms such as bacteria which continue to grow in the dead tissues. In this situation it would be the organisms not the plant tissue which reduced some of the tetrazolium salt. Similarly, if the pinkish colour occur in *B. cinerea* infected tissue it would be the *Botrytis* hyphae.

Fungal invasion causes breakdown of sugars in mature kiwifruit (Pesis et al, 1991). The predominant sugars in mature kiwifruit are glucose and fructose with sucrose being present in lesser amounts (Heatherbell 1975; MacRae et al, 1989a). The levels of total soluble solids are higher towards the blossom end than towards stem end of the fruit (MacRae et al, 1989a). In *B. cinerea* infected kiwifruit there is a significant reduction in sugars in the infected zone (stem end), compared with the non-infected zone (blossom end) because of carbohydrate breakdown (Pesis et al, 1991).

Carbohydrates, including sucrose, galactose, sorbitol, fructose, glucose and mannitol have been shown to enhance ethylene production by stimulating both ACC synthase activity and conversion of ACC to ethylene in various vegetative tissues, such as leaf discs (Meir et al, 1985; Philosoph-Hadas et al, 1985; Riov and Yang, 1982) and mung bean hypocotyls (Colclasure and Yopp, 1976). Galactose stimulated ethylene production by tomato fruit, but sucrose, fructose, glucose and sorbitol did not (Kim et al, 1987). Although the mechanism of the stimulative effect of sugars on ethylene production is unknown, it has been suggested that sugars stimulate the enzymic hydrolysis of indole-3-acetyl-l-alanine to produce IAA which in turn stimulates ACC synthase (Meir et al, 1989). It is not known if infection by a pathogen such as *B. cinerea* influences production of ethylene in kiwifruit tissue as a result of carbohydrate breakdown.

The objectives of the work described in this chapter were to determine: (a) ethylene production by *B. cinerea* infected kiwifruit at temperatures of 0°C and 20°C. (b) The

contribtion of tissues from different zones of infected kiwifruit to total ethylene production.
(c) ACC and ACC oxidase activity in the various zones of *B. cinerea* infected kiwifruit.

MATERIALS AND METHODS

B CINEREA GROWTH ON KIWIFRUIT AND ON MALT AGAR

In 1992 kiwifruit were harvested from the Massey University Fruit Crops Orchard by snapping fruit off the vine at three different maturities: {total soluble solids (TSS) 7.1, 8.6 and 10.8%}. Sepals on the harvested kiwifruit were removed using a soft nail brush. Stem scars of the fruit were inoculated with an 18µl droplet containing 5,000 spores of *B. cinerea*. To ensure consistent infection, stem scars were drilled, using a 4.5mm diameter bit, to a depth of 5mm using a Black & Decker drill. Malt agar (MA) plates to determine growth rate of *B. cinerea* on media, were inoculated with 5mm diameter MA plugs cut from 10-12 day old cultures of *B. cinerea*. Inoculated fruit and agar plates were incubated at 0°C, 5°C, 10°C, 15°C, 20°C and 25°C. There were ten replicate plates for the MA media and 10 inoculated fruit for each maturity and each temperature. *B. cinerea* growth was measured weekly at 0°C and 5°C, every 3 days at 10°C and 15°C, and daily at 20°C and 25°C using digital callipers (Mitutoyo Corporation, model CD-6"). In kiwifruit, lesion development was measured from the outer edge of the stem scar to the margin of the dark green soft area which marked the extent of lesion development. Fungal growth on MA plates was measured from the edge of the inoculum plug to the edge of the colony.

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Kiwifruit were harvested from a commercial orchard in the Wanganui area on 10 May 1991 when the average TSS of picked fruit was 6.7%. Fruit were 36 count as defined by the New Zealand Kiwifruit Marketing Board (NZKMB) with an average fruit weight of 99.5 g (range 98-101 g). Fruit were transported to Massey University and inoculated with *B. cinerea* within 12 hours of harvest using four different methods:

a) Control: no inoculation.
b) Spores suspension: stem scars inoculated with an 18µl droplet of spore suspension containing 1 X 10^4 spores per droplet.

c) Spore dust: stem scars were inoculated with spores using a small paint brush. Spores were collected from a well sporulating plate of *B. cinerea* with a gentle touch of a paint brush which was then touched onto the stem scar.

d) Spore mixed with talc powder: three plates of *B. cinerea* spores were diluted with 70g Johnson baby talc powder. This mixture was applied to the stem scar using a small paint brush. The spore load per stem scar for treatment (c) and (d) was not known.

Fruit were individually weighed and numbered at the beginning of the experiment and than placed on their sides in plastic pocket packs with plastic liners in wooden trays (3.3kg). Trays of fruit were placed at either 0±0.1°C or at 20±1°C and treatments were arranged in a completely randomized block design.

Each treatment comprised three replicate trays each containing 36 fruit. To determine ethylene production, ten fruit were taken at random from each treatment, with at least three fruit from each replicate. At 20°C, ethylene production was measured every day following inoculation up to day 15, and then at two or three days intervals for the duration of the experiment (21 days). At 0°C the same fruit were used to measure ethylene production and to assess lesion development. Ethylene production was measured two and four weeks after inoculation. After 4 weeks ethylene production was detected only from infected fruit, and for subsequent measurements only obviously infected fruit were chosen for ethylene determination, which was measured weekly until fruit had become completed infected with *B. cinerea* and no further ethylene could be detected. At 0°C only about 30% of inoculated fruit were infected after 8 weeks. Ethylene production was also measured from ten inoculated, but uninfected kiwifruit. After ethylene determination and lesion assessment, fruit were replaced in the tray in a horizontal position. Levels of ethylene inside each tray and in the room environment were measured before opening trays.

Ethylene production was determined by sealing single kiwifruit in a 600 ml jar at the treatment temperatures. Rubber bungs, fitted in the lid of each jar, were filled with water to prevent leakage from the small hole made when inserting the syringe needle through the bung. After 60 min at 20°C, or 24 hours at 0°C, a 1 ml gas sample was removed from

each jar for ethylene determination.

After 8 months at 0°C uninfected control kiwifruit were transferred to 20°C for 20 hours before measuring ethylene production. Ten fruit (at least three fruit from each replicate) were taken for measuring ethylene production.

At 20°C assessment of lesion development was not carried out because fruit went soft quickly. At 0°C inoculated infected kiwifruit lesions were measured with callipers as described previously. Lesion development was assessed weekly on fruit which developed lesions in the first 4 weeks of cool storage. After 4 months storage at 0°C the final assessment of *Botrytis* incidence on the fruit was made.

KIWIFRUIT SLICES 1991

To measure the ethylene production from different zones along the length of the kiwifruit, ten fruit from the above treatments were selected on the basis of lesion development when there was enough infected tissue from which to excise a transverse slice. At 0°C fruit were cut into slices after 70 days. The firmest uninfected fruit in the control treatment, and only those infected fruit with a clearly visible infection 'front', located about 25 mm from the stem scar, were selected for slicing. Lesion development on fruit infected with *B. cinerea* was clearly visible externally as a dark green and soft area. Diseased flesh was glossy and had a water soaked appearance in contrast to the uniform light green colour and firm texture of uninfected fruit.

Fruit were divided into 4 zones (Plate-4.1.1 to 4.1.3) and a 7 mm thick transverse slice of fruit was taken from each of the following zones:

(1) infected zone- slice taken from midway between the proximal end of the fruit and the infection 'front'.

(2) Invasion zone- slice taken from the infection 'front' containing both infected tissue and tissue immediately ahead of the infection front.

(3) Adjacent non-infected zone- slice taken from ahead of invasion zone (called mid non-infected zone in plate 4.1.3).

(4) Distal non-infected zone- slice taken from non-infected tissue at the distal end

of the fruit.

Slices were taken from equivalent zones of non-infected control fruit.

Ethylene production was measured from each of 10 selected intact noninfected and infected kiwifruit immediately before slicing at either 0°C or 20°C. Kiwifruit at 20°C were divided into three zones: infected, invasion and adjacent non-infected, while fruit at 0°C were divided into four zones: infected, invasion, adjacent non-infected and distal non-infected. Fruit were weighed prior to slicing. Batches of three or four fruit were sliced at a time, with a one hour interval between batches, to allow ease of handling and to reduce the time involved in holding gases in syringes between sampling and injection onto the GLC. Slices were weighed and ethylene production determined by sealing a single slice in a 600 ml glass jar. Jars were maintained for 3h at 20°C or 72h at 0°C before a 1 ml sample of gas was taken from each jar for ethylene measurement by GLC.

INTACT KIWIFRUIT-1992

Kiwifruit were harvested on 13 May 1992 from the same commercial orchard as in 1991. Fruit were 33 count, with an average fruit weight of 107g (range 104-110g) and with an average TSS of 6.8%.

Three treatments were applied:

1) Undrilled, uninoculated

2) Drilled, uninoculated

3) Drilled inoculated (inoculation method was used as described in previous section).

Five replicate trays per treatment were stored at each of two temperatures (0±0.1°C & 20±1°C). At 20°C, trays containing un-drilled uninoculated fruit were stored in a separate room from drilled inoculated and drilled uninoculated fruit which were placed at a distance apart on separate shelves in the same room. This was to minimise the chances of ethylene contamination. At 20°C Purafil in small plastic containers was spread around individual trays of all treatments to absorb any ethylene present. At 0°C, trays of all three treatments were stored in a completely randomized block design in one cool room.

Ethylene was analyzed from 10 fruit per treatment (two per replicate) by the same method

Zones of infected Kiwifruit

Plate 4.1.1

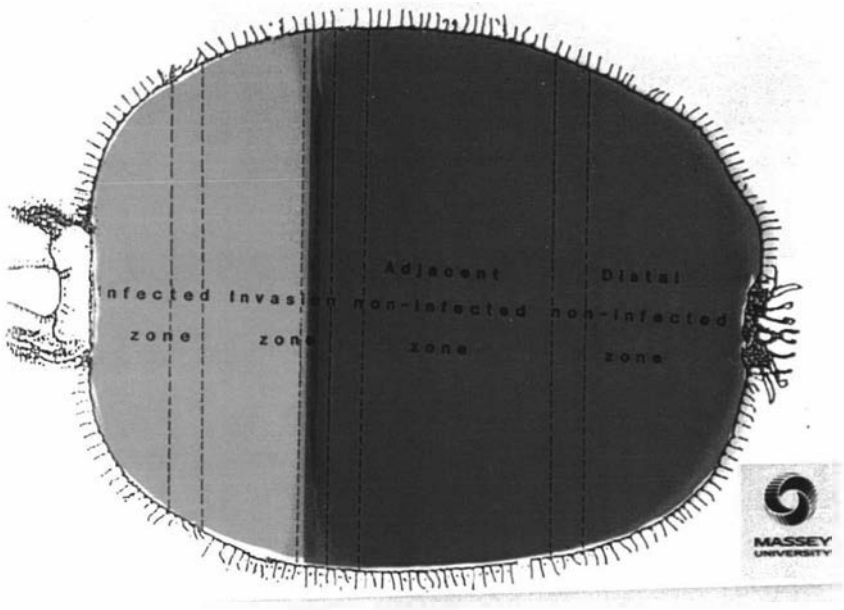


Plate 4.1.2

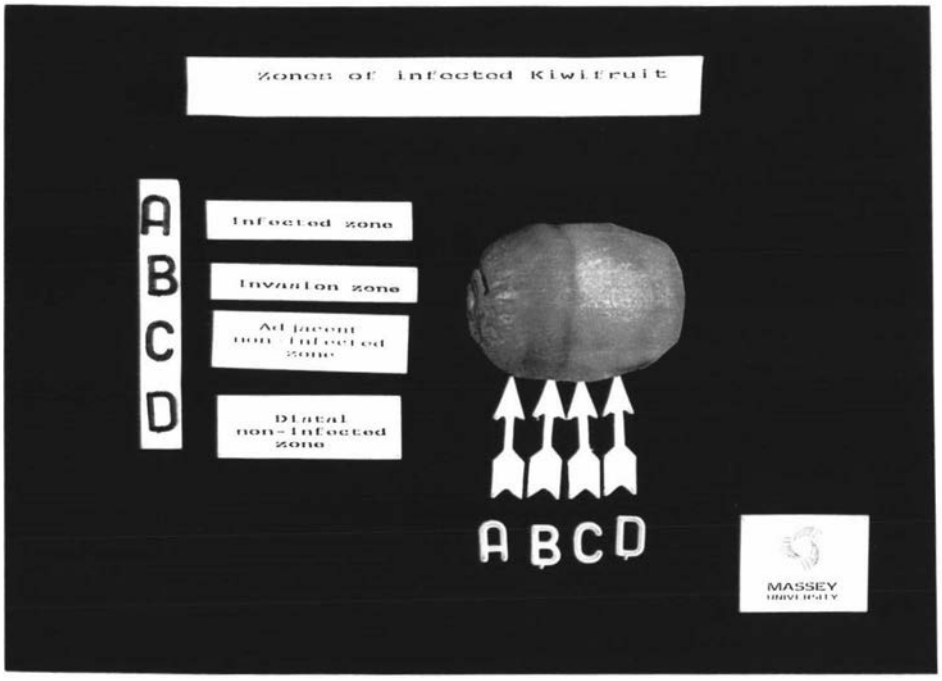
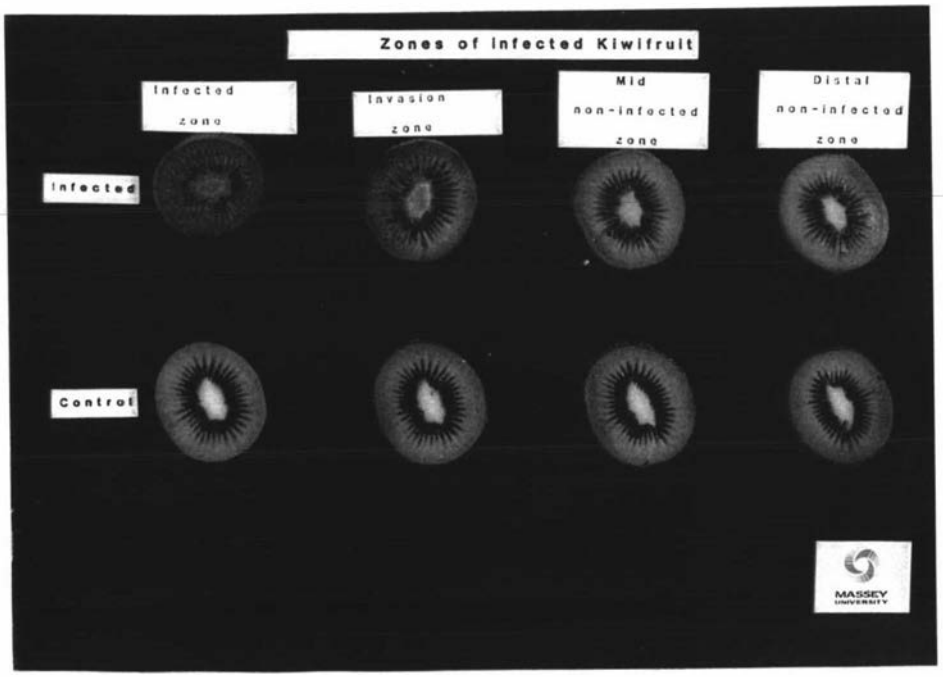


Plate 4.1.3



as described earlier. Ethylene production from treatment 1 fruit at 20°C was measured at weekly intervals for 95 days at which time ethylene was detected and subsequent measurement were made every 2 days. In fruit from treatments 2 and 3 at 20°C ethylene production was measured every alternate day after inoculation until 36 and 28 days respectively when ethylene production declined to approximately 4.5 and 3 µl/kg/h respectively. On the basis of 1991 results, measurements of ethylene production on fruit at 0°C commenced after four weeks incubation and were done at two weekly intervals thereafter for 14 weeks.

KIWIFRUIT SLICES 1992

Ethylene production from different zones of the fruit was analyzed from ten fruit taken from the above treatments, using the same method as described earlier. Fruit from both 0°C and 20°C were divided into four zones (Plate 4.1.1 - 4.1.3).

TETRAZOLIUM TEST.

The tetrazolium staining technique allows detection of living and dead plant tissue (Moore 1985). Longitudinally cut healthy and infected kiwifruit were immersed in a 1% solution of tetrazolium for 4 hours at 25°C to determine the extent of dead tissue in *B. cinerea* infected zones of kiwifruit (Plate 4.2).

A square of agar with well sporulating *B. cinerea* was immersed in a 1% solution of tetrazolium for 8 hours at 25°C to determine whether *B. cinerea* itself reacted to this stain (Plate 4.3).

HISTOCHEMICAL STUDIES

The outer pericarp of *B. cinerea* infected kiwifruit was longitudinally cut into approximately 2 cm slices and fixed in formalin: acetic acid (60%) ethyl alcohol (FAA); 1:1:20 (Hopping, 1976). After fixation slices were processed in labelled embedding cassettes in a Shandon Hypercenter tissue processor. The full cycle of the tissue embedding process took about 14 hours, and was carried out as follows:

70 % ethanol	1 hour
95 % ethanol	1 hour
100 % ethanol	1 hour

100 % ethanol	1 hour
100 % ethanol	2 hours
100 % ethanol	2 hours
Xylene	45 minutes
Xylene	45 minutes
Xylene	45 minutes
Wax	2 hours
Wax	2 hours

Prior to wax embedding the tissue was evacuated to remove air. The wax used was Paraplast (Medium), melting point 56 °C, and it was applied under a vacuum. Then the tissues were embedded in a mould in a Shandon Hypercenter tissue processor. A Heitz 1512 microtome was used to cut 8 and 16 μm sections which were dried overnight at 60 °C before staining.

The process of staining was as follows:

Dewax sections in xylene (2 x 5 minutes)
Hydrate through alcohol 70 % to water
Stain in Gills haematoxylin (3 minutes)
Wash in tap water (30 seconds)
Wash in Scotts tap water (30 seconds)
Wash in tap water (60 seconds)
Dehydrate through alcohol 95 %
Clear in Xylene
Mount in DPX mountant (BDH)

The section of the stained kiwifruit were observed using a compound microscope at 100 and at 400 x magnification.

ACC CONTENT IN *BOTRYTIS CINEREA* INFECTED KIWIFRUIT

PLANT MATERIAL.

B. cinerea infected kiwifruit were removed from cool store after 2 months at 0°C and

maintained for 48h at ambient temperature. Three infected and three healthy fruit were taken and divided into 4 zones as described earlier (Plate 4.1.1 to 4.1.3); each zone of the fruit represented a replicate.

PREPARATION OF TISSUE EXTRACTS

ACC was extracted using a modification of the method described by Nieder et al, (1986). Outer pericarp tissue (2.5 g), from each zone of infected and healthy fruit was homogenized in an Ultra-Taurrax type 18/10 homogenizer, fitted with 1 cm probe, then extracted in 20 ml 95% ethanol for 30 minutes at 80°C. Extracts were centrifuged at 10,000 rpm for 20 minutes at 2°C then the supernatant was decanted into 250 ml round bottomed flasks through mira cloth. After removal of ethanol under vacuum at 40°C, 2 ml of glass distilled water was used to rinse flasks. Two ml of chloroform was added and the mixture centrifuged at 3500 rpm for 4 minutes at 4°C in a bench centrifuge. The supernatant was then transferred to Bijoux bottles and stored at -20°C until required for assay of ACC.

ASSAY METHOD

The effiency of the conversion of ACC to ethylene was about 73% (Table 4.1)

Table 4.1.

Effiency of conversion of ACC to C_2H_4.

ACC added (nmol)	C_2H_4 (nmol)	Percentage conversion to C_2H_4
1	0.72	73
5	3.64	73
10	7.30	73
20	14.98	75

ACC was assayed using the method described by Lizada and Yang (1979). The assay was conducted in an ice bath as follows:

400 µl of extract, or 100 µl of ACC solution was placed into a 32 ml flat bottom vial containing 300 µl of 1 µ*M* $HgCl_2$ and was made up to 800 µl with distilled water. Reaction vessels were then sealed with rubber suba-seals and placed in ice. Hypochlorite (200 µl, containing 3.15% of active ingredient, sodium hypochlorite) was injected through the suba-seal. The vial was then agitated on a vortex mixer for 5 seconds, incubated on ice for 5 minutes, and then agitated for a further 5 seconds. A 1 ml gas sample was then taken from the head space and injected into a Photovac gas liquid chromatograph for ethylene determination.

ACC OXIDASE ACTIVITY IN *B. CINEREA* INFECTED KIWIFRUIT

PLANT MATERIAL.

Kiwifruit, which had been inoculated with an 18µl droplet containing 5,000 spores of *B. cinerea* onto the stem scar that had been drilled with of 4.5mm diameter bit to a depth of 5mm, were removed from cool store and kept for 48h at 20°C. Ten fruit from each treatment (infected & non-infected) were taken, fruit were divided into 4 zones as described earlier (Plate 4.1.1-3), with each zone of each fruit being a replicate. The outer pericarp from each zone of the same fruit was used for both *in vivo* & *in vitro* studies.

IN VIVO ACC OXIDASE ACTIVITY

To measure activity *in vivo* a modified version of the method described by Mitchell et al, (1988) was used. 0.3 ml of 10 m*M* ACC was added to weighed discs of outer pericarp tissue (about 1.5 g) which were suspended in 2.7 ml of buffered mannitol solution (0.7 *M)* and 10 m*M* potassium phosphate (pH 7.5), in 25 ml Erhlenmeyer flasks which were incubated in a water bath at 25°C with gentle shaking at 70 rpm. After 4 h a 1 ml gas sample was taken from the head space for ethylene determination using a Photovac gas liquid chromatograph.

IN VITRO ACC OXIDASE ACTIVITY

EXTRACTION PROCEDURE

The extraction procedure was modified from the method described by Fernandez-Maculet and Yang (1992). The outer pericarp tissue was removed and frozen in liquid N_2. After removal from -70°C approximately 2 g tissue was extracted in 2ml g^{-1} of extraction medium containing 0.3 *M* Tris-HCl (pH 7.5), 30 m*M* sodium ascorbate, 30% glycerol, 0.05 m*M* $FeSO_4$ and 10% insoluble polyvinylpolypyrrolidone (PVPP). After the slurry had thawed in buffer, tissues were homogenized (Ultra-Taurrax homogenizer type 18/10 fitted with a 1 cm probe). Samples were then placed in an ice bath for 2 hours, filtered through two layers of muslin cloth, pH readjusted to 7.5, then centrifuged at 28,000g for 30 min. The crude supernatant was used for enzyme assay.

ASSAY OF *IN VITRO* ACC OXIDASE ACTIVITY

Except where indicated in legends, ACC oxidase activity was assayed as follows: 0.3 ml of crude extract was added to 2.7 ml reaction mixture containing 0.3 *M* Tris-HCl (pH 7.5), 30 m*M* sodium ascorbate, 30% (v/w) glycerol, 0.05 m*M* $FeSO_4$, 15 m*M* ACC and $NaHCO_3$ (in powder form to give 600 m*M*) in a 25 ml Erhlenmeyer flask. After 1 hour of incubation at 25°C a 1 ml gas sample was removed and injected onto a Photovac GLC, for ethylene determination.

ACC OXIDASE ACTIVITY IN SLICES OF KIWIFRUIT

Kiwifruit were taken after 2 months at 0°C and sliced into 4 zones (Plate 4.1.1 to 4.1.3) and their ethylene production was measured after 10, 12, 14, 16 and 18 hours. To determine when ACC oxidase activity was initiated in kiwifruit slices, 1 ml of 0.05 m*M* ACC solution was applied to the surface of kiwifruit slices immediately after slicing or 16-18h after slicing (when these slices were producing ethylene).

COMPOSITIONAL CHANGES IN KIWIFRUIT INFECTED BY *BOTRYTIS CINEREA*.

In 1991 infected kiwifruit were selected after 2 months at 0°C and 7 mm thick slices were cut from the four zones of individual fruit. Slices from the same position on five different kiwifruit were bulked to give a composite sample and juice was extracted by squeezing

through three layers of muslin cloth. Fresh juice was analyzed for TSS (using a hand refractometer), pH and titratable acidity. All measurements were done in triplicate on juice samples from individual zones of the composite sample.

Aliquot of the same batches of juice were used to prepare ethanol extracts for separation and estimation of sugars and organic acids (Pesis et al, 1991). Aliquot were centrifuged at 12,000g. The supernatant was mixed with 95% ethanol (1:5 v/v), heated to boiling in a microwave oven to inactivate enzymes, filtered and stored at -20°C for 2-3 months to allow various cell components to precipitate before analysis (Paull et al, 1984). One ml of extract was dried under a stream of N_2 at ambient temperature (18-20°C) and redissolved in 400 µlitres of deionised water for analysis. To measure sugars (glucose, sucrose and fructose) 20 µl samples were injected onto a Biorad HPLC Aminex HPX-87C carbohydrate column in a Waters high pressure liquid chromatography system, eluted with water at 80°C (0.6 ml/min) and detected using an Optilab 5822 refractive index detector with sensitivity set to x100. Organic acids were analyzed using a modified C18 column (ODS-220 x 4.6 mm). Samples (20 µlitres) were injected and eluted isocratically with 0.013*N* sulphuric acid at a continuously increasing flow of 0.4-0.7 ml/min for 20 min, at 20°C. Carboxylic acids were detected at 210 nm using a 490 E UV/VIS detector. Peak area measurements from a Waters 745 integrator were used to quantify fructose and the organic acids. Results were expressed in g/ 100 ml juice relative to known standard solutions.

RESULTS

Rates of *B. cinerea* growth on fruit from harvest 1 (initial TSS 7.1%) increased as incubation temperature increased, reaching a maximum of 1.7 mm/day at 25°C (Fig 4.2). Fungus grew on fruit from harvest 2 & 3 at the same rate as harvest 1 fruit at 0°C, but retained the same growth rate at 5°C and 10°C, before increasing to a maximum (1.8 mm/day) at 20°C and declining slightly at 25°C (Fig 4.2). *B. cinerea* growth rate on MA was approximately the same as on fruit at 0°C but was much greater than on fruit as temperature increased with a maximum of ≈ 10 mm/ day at 20°C, thereafter declining at 25°C (Fig 4.3).

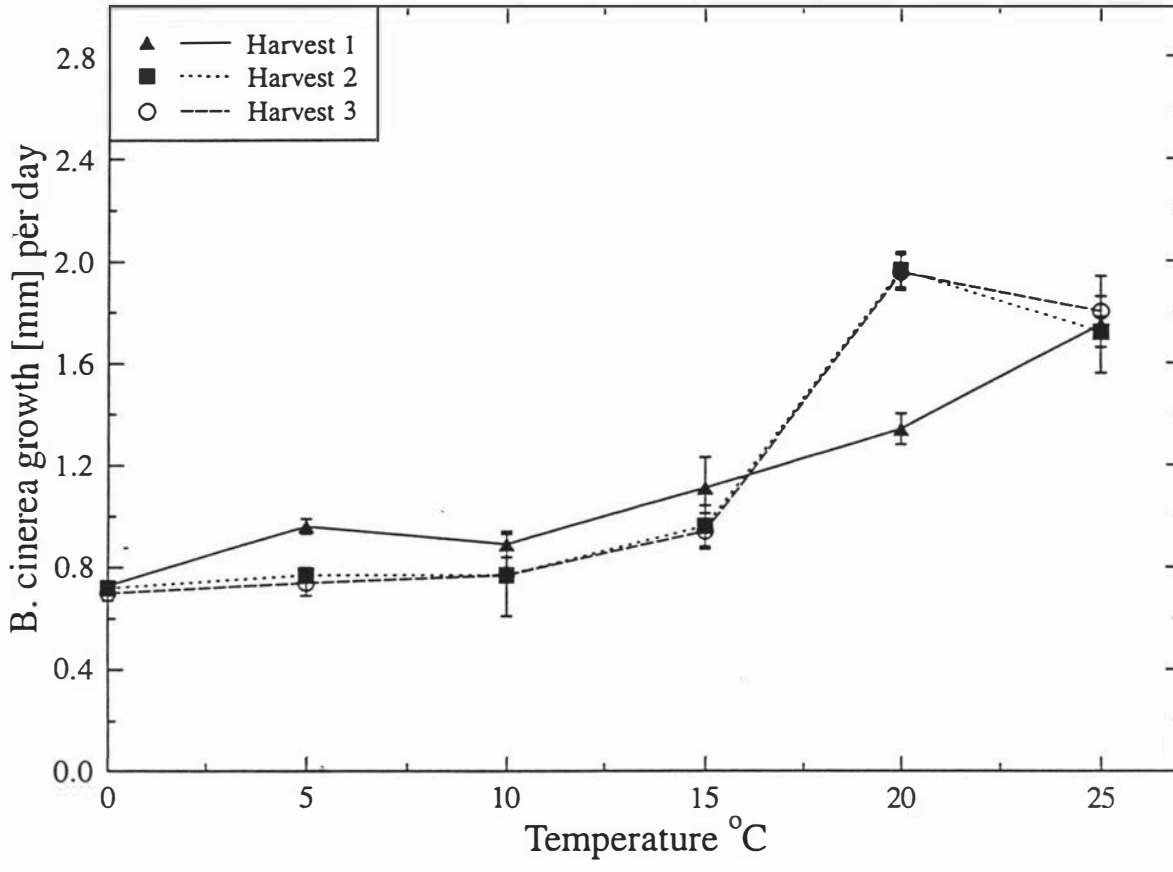


Fig 4.2 B. cinerea growth rate at a range of temperatures on kiwifruit of differing maturity (harvest 1, 2 & 3= TSS of 7.1, 8.6 & 10.8 % respectively). Bars represents standard error.

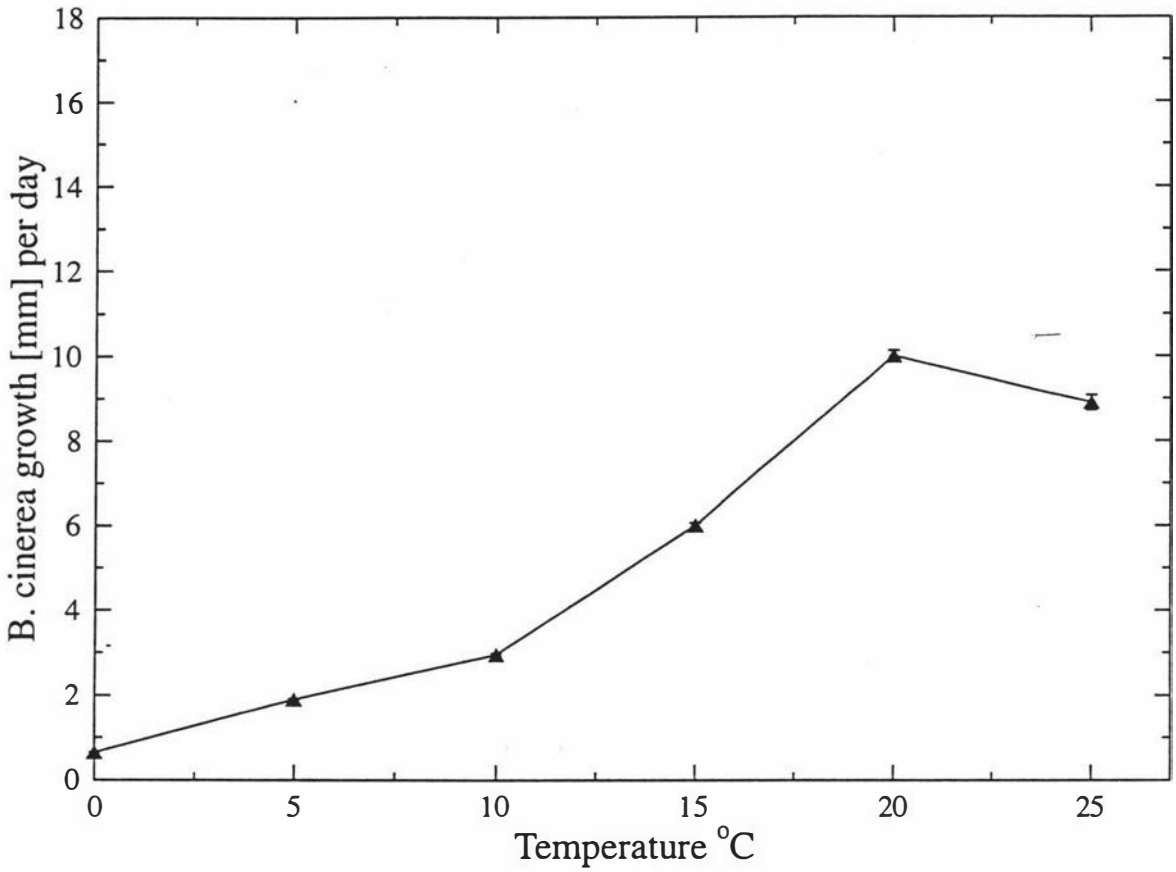


Fig 4.3 B. cinerea growth on malt agar, at a range of temperatures. Bars represents standard error.

1991 EXPERIMENT

In 1991 ethylene production from intact uninoculated (control) kiwifruit commenced after 13 days incubation at 20°C, reaching a peak of 140 µl/kg/h on day 18 after which there was a sharp decline to 70 µl/kg/h at day 20 (Fig 4.4).

In inoculated treatments all fruit (infected or noninfected) started to produce ethylene after 8-9 days of incubation, reaching approximately the same peak value as control fruit on day 10-11 for dust and powder treatments and on day 12 with the spore suspension treatment; ethylene production then declined rapidly through day 15 followed by a steady decline to 10-15 µl/kg/h at day 20 (Fig 4.4). There was no significant difference in peak height or rate of ethylene production between fruit inoculated with *B. cinerea* spores in dust or powder. Fruit inoculated with spore suspension began to produce ethylene 1 day later than other inoculated treatments, but the subsequent pattern of ethylene production was the same as the other treatments, delayed by 1 day.

It was not expected that the ethylene production from control fruit would be the same as fruit from inoculated treatments. That this occurred may have been caused by exposure of control fruit to ethylene produced earlier by inoculated fruit stored in the same store. In subsequent experiments control fruit were stored in a separate rooms.

In fruit at 0°C there was no detectable ethylene produced by uninoculated (control) and inoculated uninfected kiwifruit during the entire storage period of 8 months. Ethylene production began simultaneously in inoculated infected fruit from all treatments after 4 weeks at 0°C (Fig 4.5). In all treatments there was a steady rise in ethylene to a peak 8 to 11 weeks after inoculation, followed by steady decline reaching undetectable levels after 14-16 weeks. At this time *B. cinerea* had infected entire fruit. Ethylene production from fruit inoculated with spores in talc powder was the highest of all treatments, reaching a peak of 367 nl/kg/h after 10 weeks before declining to undetectable levels at week 14. Initial ethylene production was slightly faster for dust and slightly slower for suspension treatments than the powder treatment. Dust treated fruit reached a peak of ethylene production (237 nl/kg/h) after 8 weeks, remaining at approximately at the same level until week 11, while the spore suspension treated fruit reached a peak of 265 nl/kg/h after 11

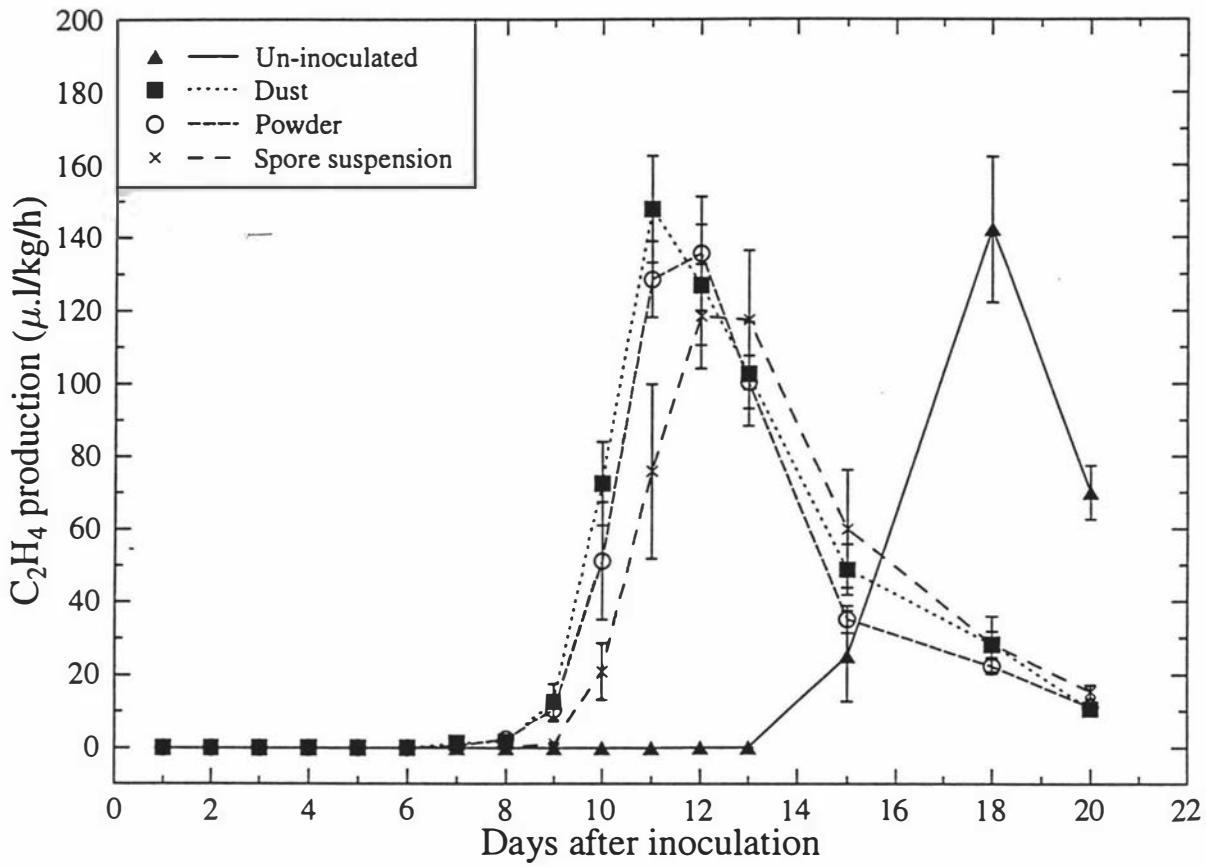


Fig 4.4 Ethylene production by intact kiwifruit at 20°C inoculated with B. cinerea using different methods of inoculation in 1991. Bars represents standard error.

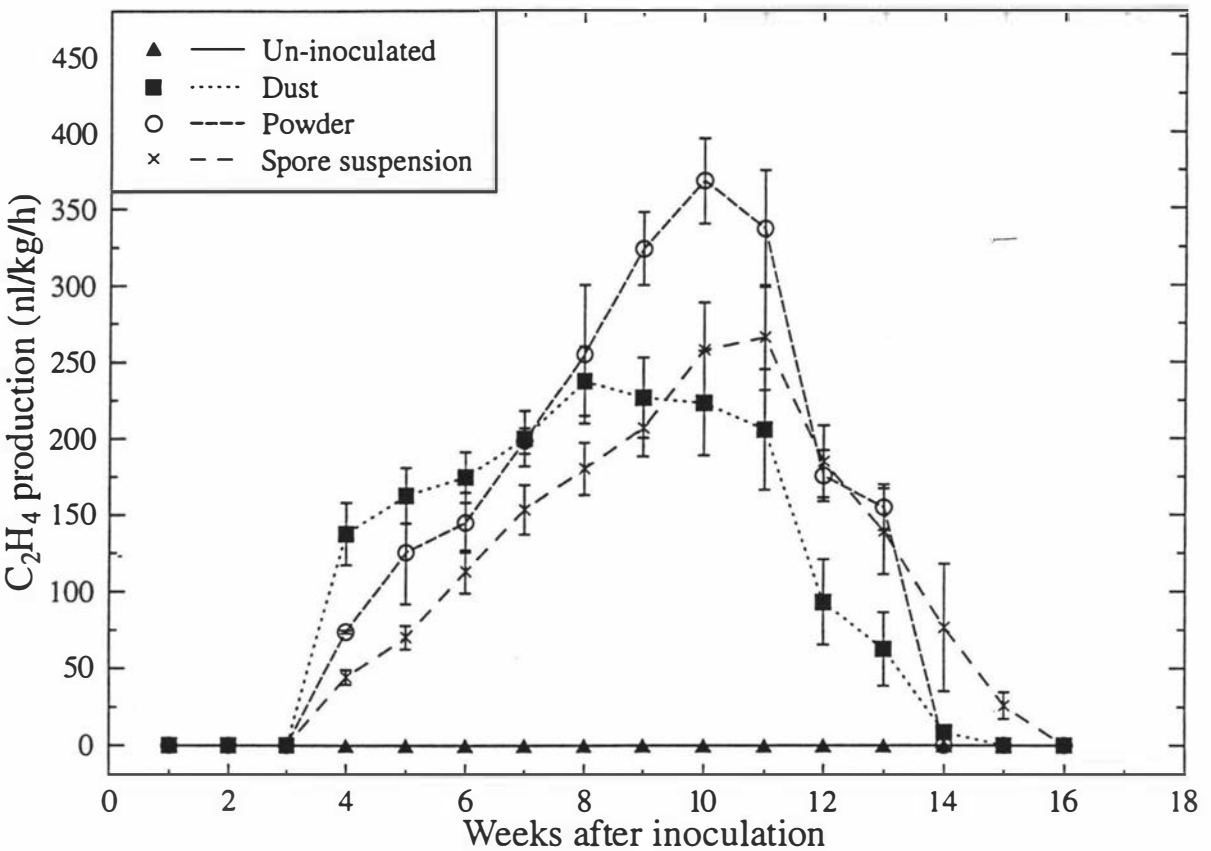


Fig 4.5 Ethylene production by intact kiwifruit at 0°C inoculated with B. cinerea using different methods of inoculation in 1991. Bars represents standard error.

weeks; both declined to base levels after 14-16 weeks. After 8 months at 0°C uninoculated uninfected fruit were removed to 20°C; ethylene was measured within 24 h, reaching a peak of 20 µl/kg/h after 5 days before declining steadily through 15 days (Fig 4.6).

The appearance of lesions and commencement of ethylene production occurred simultaneously. In all inoculation treatments, lesions became visible after 4 to 5 weeks incubation at 0°C regardless of method of inoculation. Rate of lesion development along fruit was 1 mm per day, approximately the same for all treatments (Fig 4.7). At 0°C inoculation with dust, spore suspension and powder gave 30%, 21% and 18% of fruit infected respectively (Fig 4.8), although there was no significant difference between inoculated treatments.

In an attempt to determine the extent of ethylene production from parts of a single kiwifruit which were infected with *B. cinerea*, fruit were divided into separate zones, representing infected tissue, tissue at the invasion front (i.e at the interface between the infected and apparently non-infected tissue), tissue immediately distal to the invasion front and un-infected tissue at the distal end of the fruit. Ethylene production was measured from both healthy (control) and infected fruit before slicing these fruit into the above zones. In this experiment control intact fruit at 20°C produced approximately 4 fold more ethylene than infected fruit (Fig 4.9). Slicing fruit resulted in an increased ethylene production compared with intact fruit, probably caused by wounding. Tissue from the centre of control fruit, zones corresponding to the invasion front and the tissue immediately distal to this, produced the same amount of ethylene as the proximal end of the fruit. In infected fruit, maximum ethylene production occurred in the tissue distal to the invasion front (adjacent noninfected zone) and production was approximately the same as for healthy fruit. There was a marked reduction in ethylene produced in the infected tissue (approximately 4 fold less) and in the invasion front (43% less) compared with control fruit.

In fruit stored at 0°C ethylene was also measured in tissue at the distal end of the fruit in advance of tissue where hyphae were present, as well as in the same tissue zones as for the fruit at 20°C. There was no detectable ethylene produced by intact control fruit while infected intact fruit produced 139 nl/kg/h (Fig 4.10). Ethylene production was

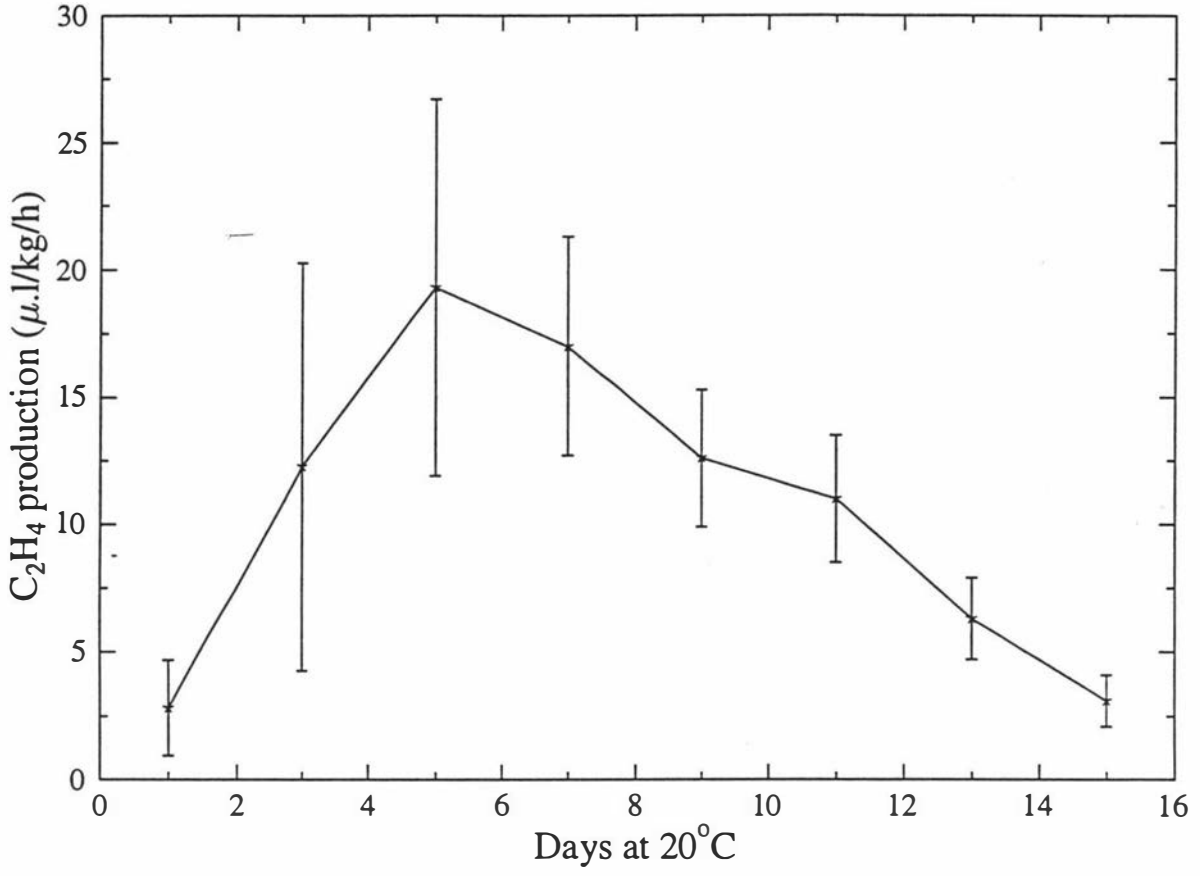


Fig 4.6 Ethylene production by healthy kiwifruit at 20°C after 8 months storage at 0°C in 1991. Bars represents standard errors.

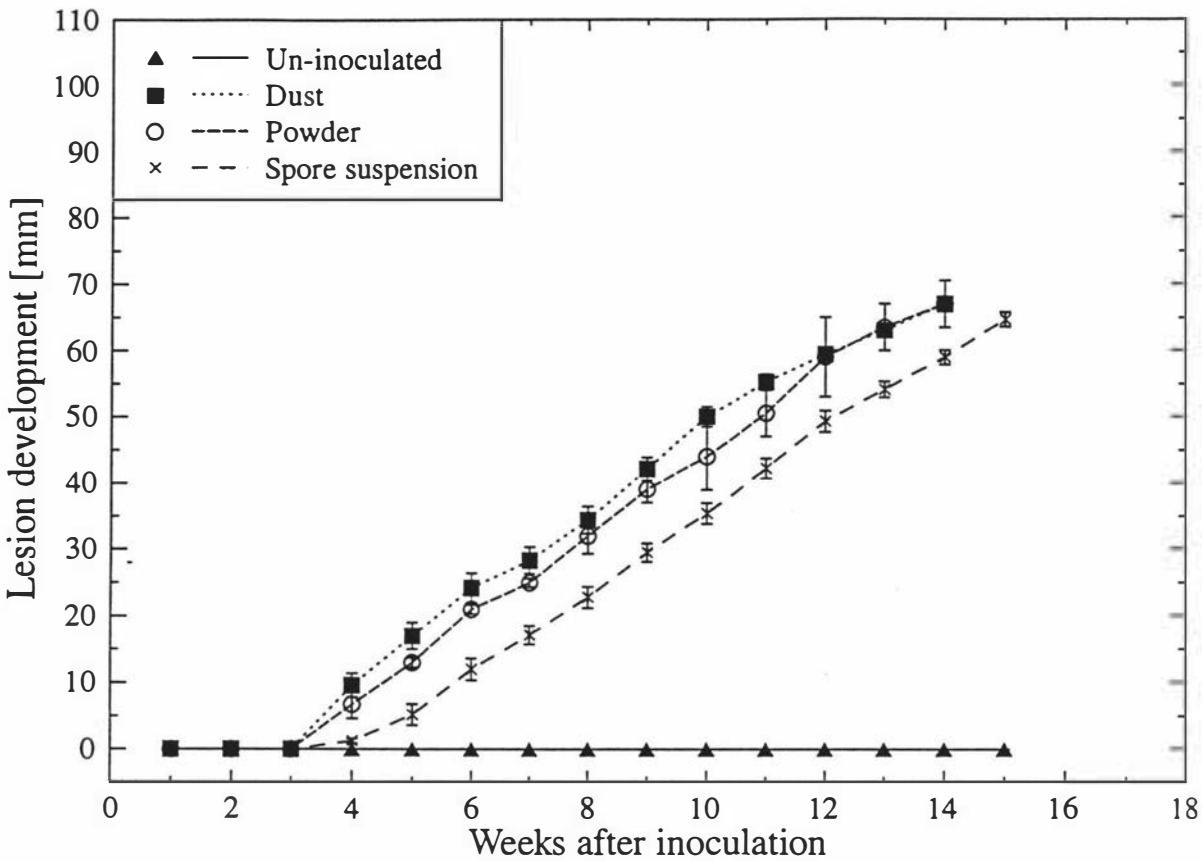


Fig 4.7 Lesion development on kiwifruit stored at 0°C inoculated with B. cinerea using different methods of inoculation in 1991. Lesion development was the distance "in mm" between the stem scar and the visible invasion front along the longitudinal axis of the fruit. Bars represents standard errors.

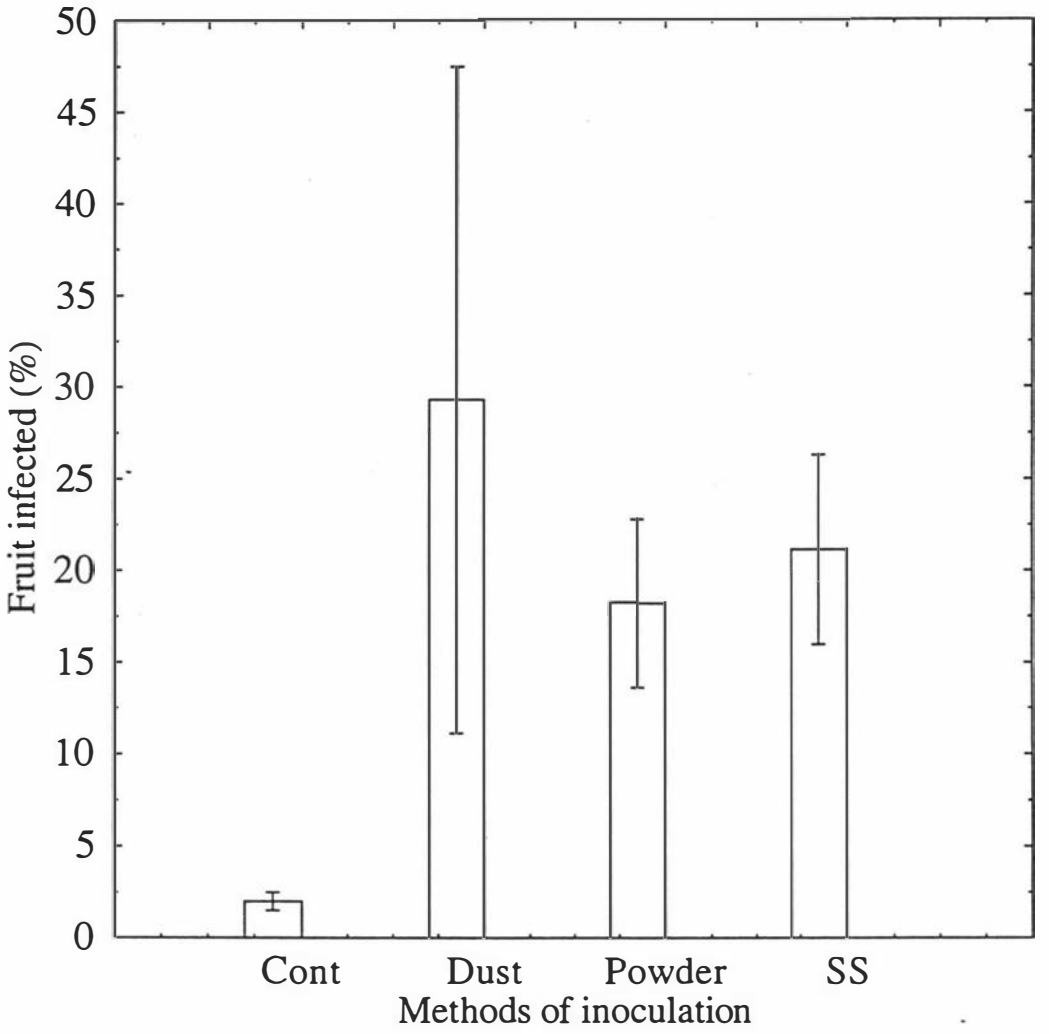


Fig 4.8 Percent infected kiwifruit, inoculated with B. cinerea, using different methods of inoculation, after 4 months at 0°C in 1991. Bars represents standard errors. Cont= control, SS= spore suspension.

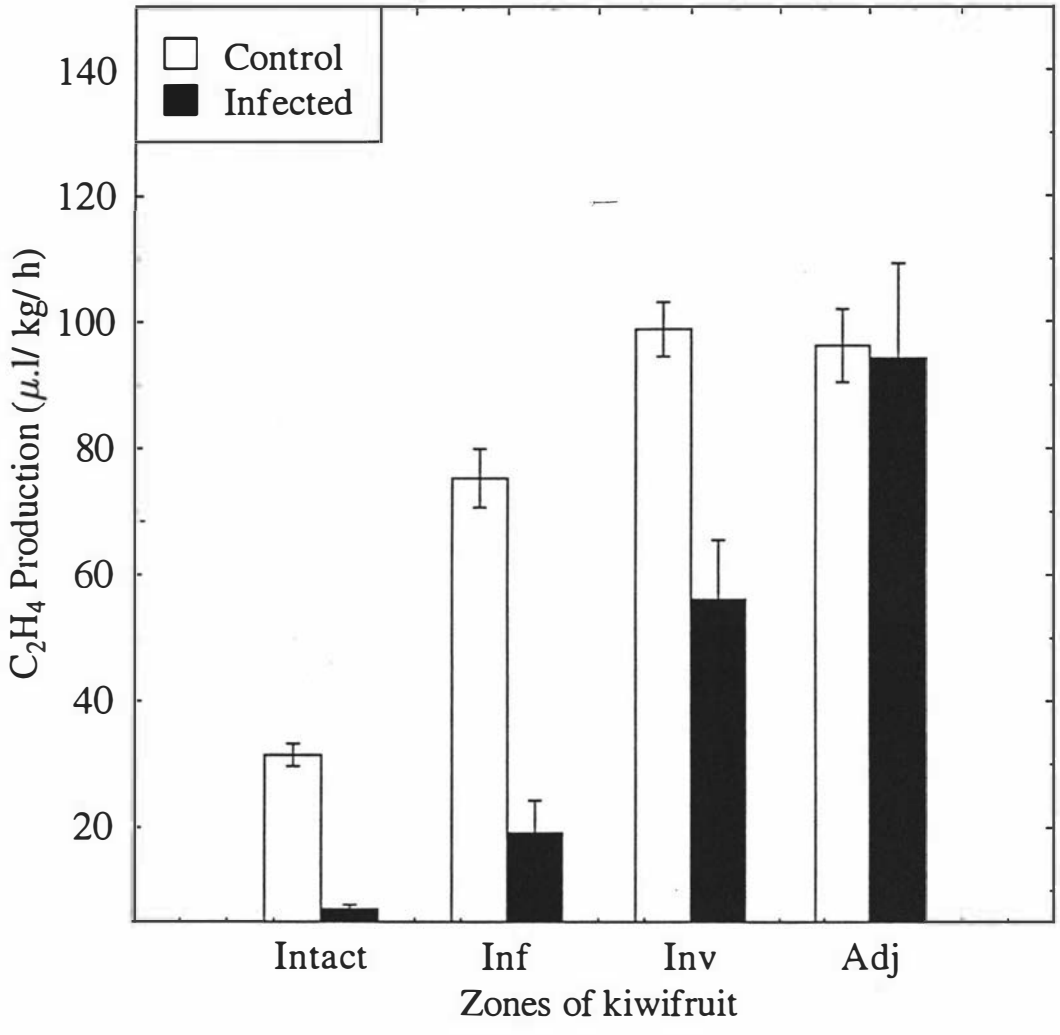


Fig 4.9 Ethylene production from intact B. cinerea infected and control fruit and from zones of the same kiwifruit stored at 20°C in 1991. Inf= infected, Inv= invasion and Adj= adjacent noninfected zone, Intact= intact fruit prior to slicing. Bars represents standard errors.

approximately the same (about 80 nl/kg/h) from all four zones of control fruit. In contrast, no detectable ethylene was found in the infected zone, some ethylene was found in the invasion zone and maximum ethylene production (348 nl/kg/h) occurred in the adjacent non infected zone, while lesser (171 nl/kg/h) ethylene was produced in the distal non-infected zone of infected fruit (Fig 4.10). In this latter zone infected fruit produced 50% more ethylene than did control fruit.

1992 EXPERIMENTS

In 1992 the pattern of ethylene production from the intact fruit at 20°C was not the same as in 1991 (Fig 4.11). There was no detectable ethylene from un-drilled un-inoculated control fruit until 95 days after inoculation. At this time ethylene production started and increased to a maximum of 35 µl/kg/h after about 100 days; it stayed at this level for about one week and then declined (Fig 4.11). Ethylene production in drilled uninoculated fruit started after 20 days of incubation. There was a sharp increase to a peak of 50 µl/kg/h on day 23 after incubation after which there was a steady decline over the next 10 days. In fruit that had been drilled and inoculated, ethylene production started 7 days after inoculation increasing rapidly to a peak of 96 µl/kg/h on day 12, and before declining sharply until day 15 followed by a steady decline to base level at day 29 (Fig 4.11).

At 0°C there was no detectable ethylene measured in intact fruit that had not been inoculated with *B. cinerea* irrespective of whether they had been drilled through the stem scar or not. In drilled inoculated infected fruit ethylene production had already commenced when sampling began after 4 weeks at 0°C. Maximum ethylene production occurred after 6 and 8 weeks at 0°C, after which production decreased through 14 weeks when production was only slightly higher than from uninoculated fruit (Fig 4.12). At 0°C 92% of fruit which had a 4.5 mm hole drilled through the stem scar before inoculation with *B. cinerea* were infected. Only 2% and 5% of non drilled and drilled uninoculated fruit respectively were infected with *B. cinerea* after 14 weeks at 0°C (Fig 4.13).

In an attempt to determine the extent of ethylene production from parts of a single kiwifruit which were infected with *B. cinerea*, fruit were divided into separate zones as described earlier. In this experiment no detectable ethylene production was measured from control

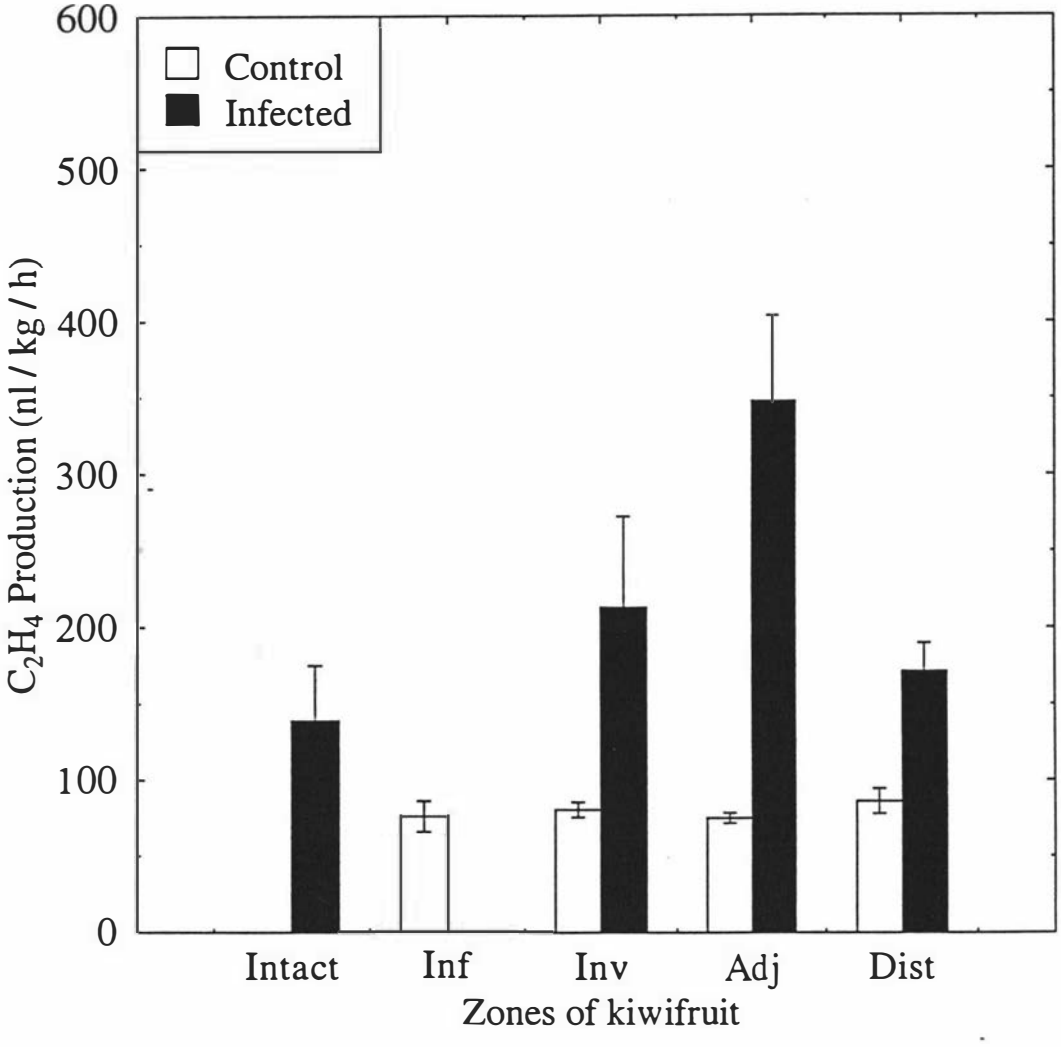


Fig 4.10 Ethylene production from intact B. cinerea infected and control fruit and from zones of the same kiwifruit stored at 0°C in 1991. Inf= infected, Inv= invasion, Adj= adjacent noninfected and Dist= distal non-infected zone, Intact= intact fruit prior to slicing. Bars represents standard errors.

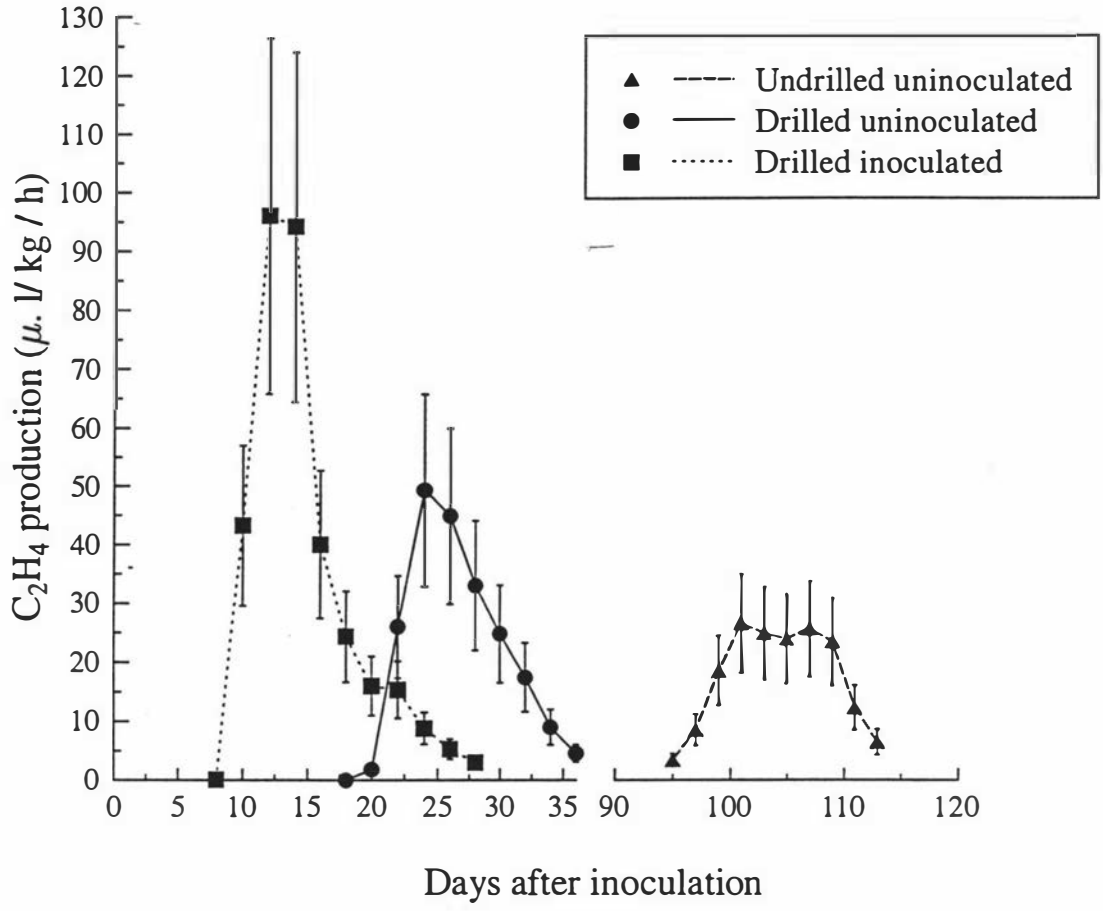


Fig 4.11 Ethylene production by undrilled noninfected, drilled noninfected and drilled infected kiwifruit at 20°C in 1992. Bars represents standard errors.

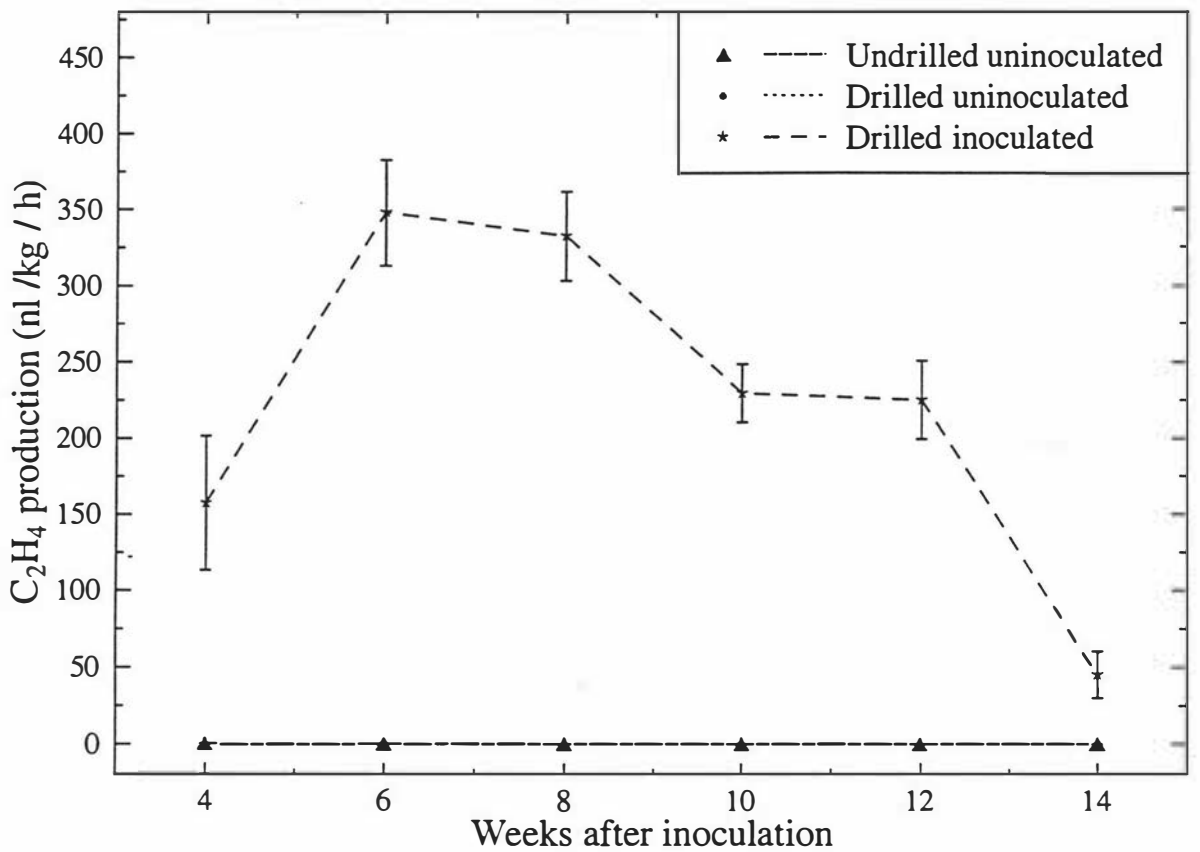


Fig 4.12 Ethylene production by undrilled noninfected, drilled noninfected and drilled infected kiwifruit at 0°C in 1992. Bars represents standard errors.

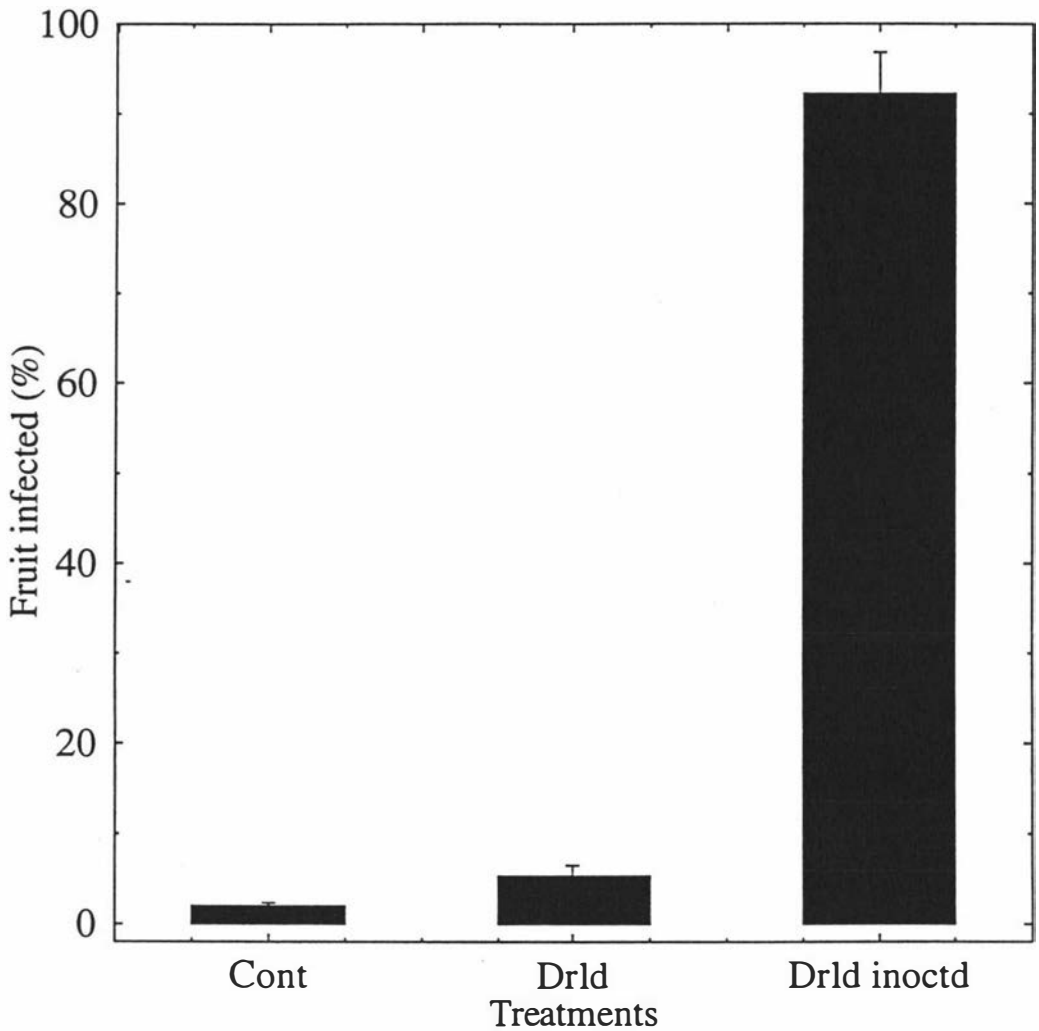


Fig 4.13 Percent infected kiwifruit in different treatments after 4 months at 0°C in 1992. Cont.= undrilled uninoculated, Drld.= drilled uninoculated, Drld inoctd.= drilled inoculated. Bars represents standard errors.

intact fruit at 20°C, drilled uninoculated fruit produced 60 µl/kg/h, approximately 7 fold more ethylene than drilled inoculated infected fruit (Fig 4.14). Slicing fruit resulted in an increased ethylene production compared with intact fruit. Tissues from all zones of control fruit produced approximately the same amount of ethylene (91 µl/Kg/h). Tissues from drilled uninoculated fruit had the same ethylene production rate as control fruit from each of the zones along the fruit. In infected fruit tissues there was very little ethylene production in the proximal infected zone (4 µl/kg/h) while in the invasion zone it was slightly higher at 20 µl/kg/h; this was only 25% of that produced by the equivalent zone in control and drilled uninoculated fruit. Slices from the distal end of the fruit produced ethylene at the same rate as slices from control and drilled noninfected fruit; slices from the adjacent zone produced slightly less ethylene than the equivalent zone in fruit from the other treatments but the difference was not significant (Fig 4.14).

At 0°C no detectable ethylene was produced by intact control or uninoculated drilled fruit while infected intact fruit produced 172 nl/kg/h (Fig 4.15). Ethylene production was approximately the same in slices from all tissue zones but tended to gradually decrease towards the distal end of control and drilled uninoculated fruit (Fig 4.15). In contrast no detectable ethylene was found in the infected zone from drilled inoculated fruit, some ethylene was found in the invasion zone (125 nl/kg/h) and in the distal noninfected zone (237 nl/kg/h) while maximum ethylene production (455 nl/kg/h) occurred in adjacent non infected zone (Fig 4.15).

There are difficulties in interpreting these data from tissue slices from fruit maintained at 20°C. Fruit used to take slices were at different physiological states when excision occurred. In order to take slices from different zones along the length of infected fruit, the infection zone had to progress to 30-40% of the fruit length in order to obtain an adequate sized slice from the infected tissue. However in intact infected fruit ethylene production commenced after 7 days in 1991 and after 10 days in 1992 and reached a peak of ethylene production after a further 4 or 2 days respectively. The corresponding peaks in uninoculated fruit occurred after 14 days, later than in inoculated fruit in both seasons 1991 and 1992. Slices were taken on the same day for both inoculated and uninoculated uninfected or drilled uninoculated uninfected fruit. In 1991 this was 8 days after peaked

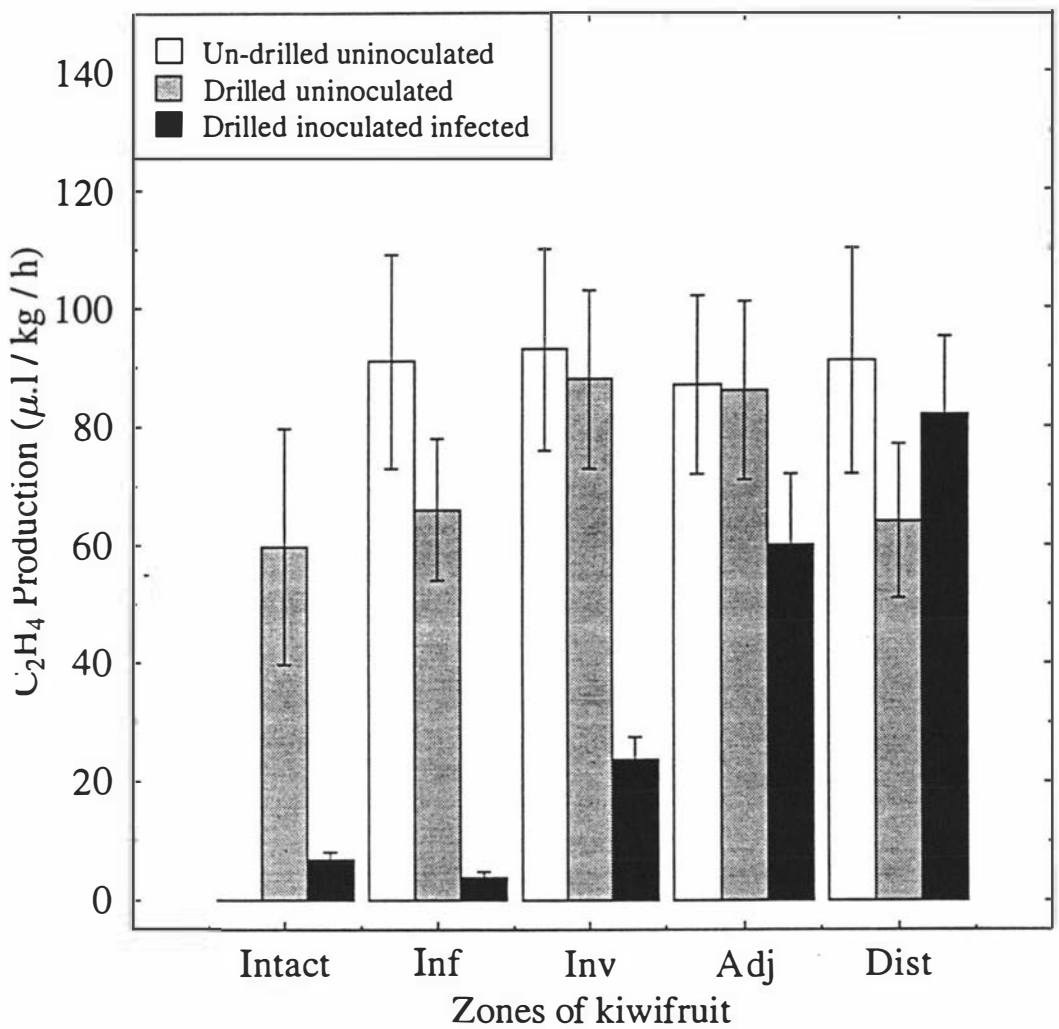


Fig 4.14 Ethylene production from intact fruit and from slices of same kiwifruit stored at 20°C in 1992. Inf=infected, Inv= invasion, Adj= adjacent noninfected and Dist= distal noninfected zone Intact= intact fruit prior to slicing. Bars represents standard errors.

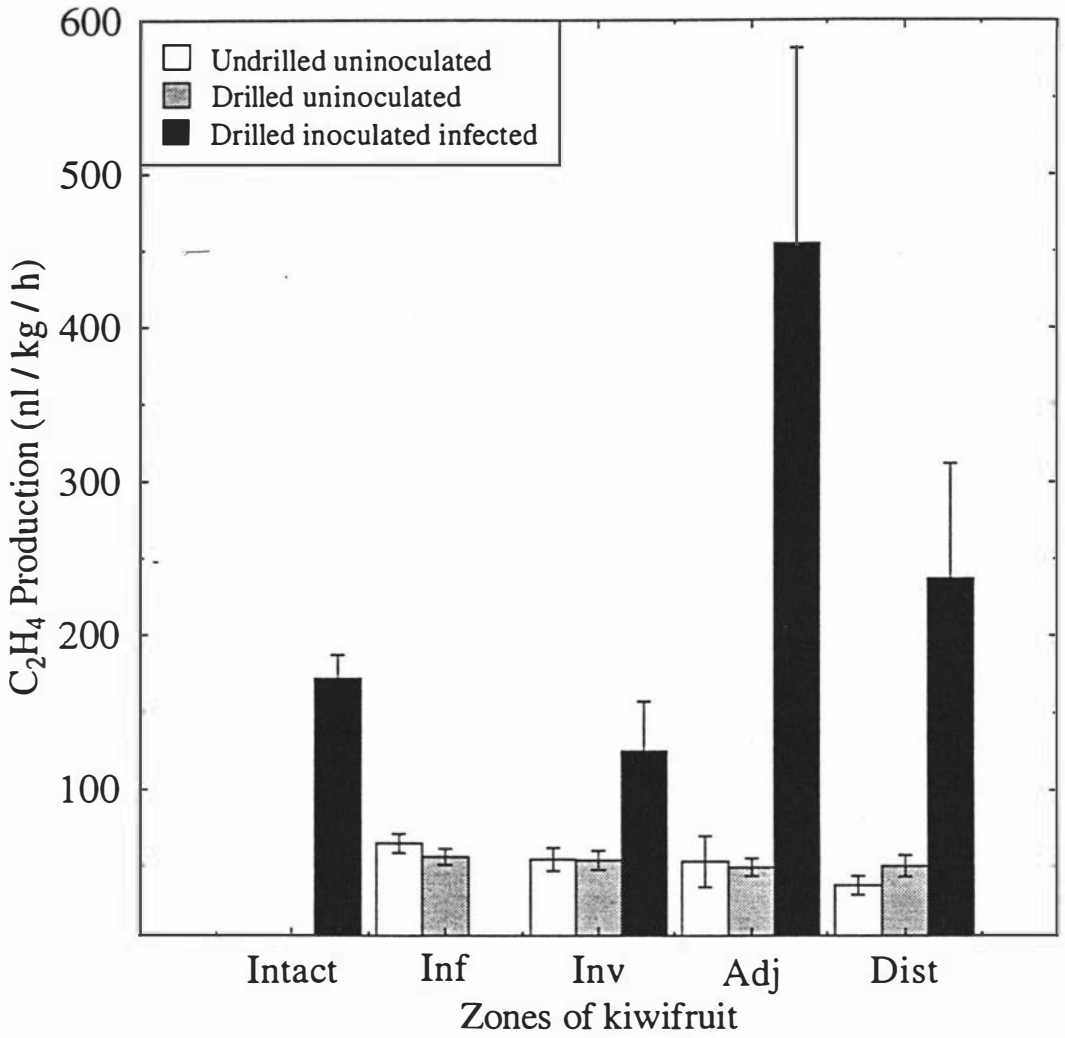


Fig 4.15 Ethylene production from intact and from slices of the same kiwifruit stored at 0°C in 1992. Inf= infected, Inv. invasion, Adj.= adjacent non-infected and Dist= distal non-infected zone Intact= intact fruit prior to slicing. Bars represents standard errors.

ethylene production when rate had reduced to 7 µl/kg/h but only 5 days after the peak for uninfected fruit where production was still high (31µl/kg/h). In 1992 this was 13 days after peaked ethylene production when rate had reduced to 7 µl/kg/h but only 5 days after the peak for uninfected fruit where production was still high (60 µl/kg/h). Because of these differences in timing, the ethylene production from uninoculated uninfected or drilled uninoculated uninfected intact fruit in figures 4.9 and 4.14 appear much higher than that in infected fruit. An attempt was made to normalize or adjust ethylene production of the infected fruit for both 1991 (Fig 4.9) and 1992 (Fig 4.14) by using the following assumptions: that the pattern of ethylene production from both lots of fruit was similar; that the response of the uninfected zone would be in direct proportion to the difference in production between infected and uninfected intact fruit; that infected tissue was dead and would produce minimal or no ethylene, so infected tissue was not adjusted; and that the invasion front had 50% of dead tissue (which was not adjusted) and 50% live tissue (adjusted similarly to distal zones). This meant that ethylene production from the invasion zone, adjacent zone, and the distal zone from infected fruit were multiplied by 2.5 or 5 (Fig 4.16) and 4 or 8 or 8 (Fig 4.17) respectively, to obtain adjusted values of ethylene production for Fig 4.9 and Fig 4.14. These normalized data suggest that if slices had been taken at the same physiological stage then difference between the infected and noninfected tissue would have been approximately 5 & 8 times greater in the adjacent and distal uninfected slices of infected fruit in 1991 and 1992 respectively than was actually measured (Fig 4.9 and Fig 4.14).

The tetrazolium test showed that only the noninfected part of infected fruit developed a deep pinkish stain which indicated that it was actively respiring. Infected tissue developed a very slight colour compared with the infected fruit (Plate 4.2). *B. cinerea* hyphae grown on agar developed a pinkish colour when stained with tetrazolium dye (Plate 4.3). A histochemical study of *B. cinerea* infected fruit tissue showed that hyphae could penetrate and spread inside the xylem vessels (Plate 4.4) and also could penetrate tissue both inter- and intra-cellularly (Plate 4.5).

The ACC content in healthy kiwifruit was approximately 10 times less than that in infected kiwifruit (Fig 4.18). Some variation in ACC content (nmole/g) occurred from infected and

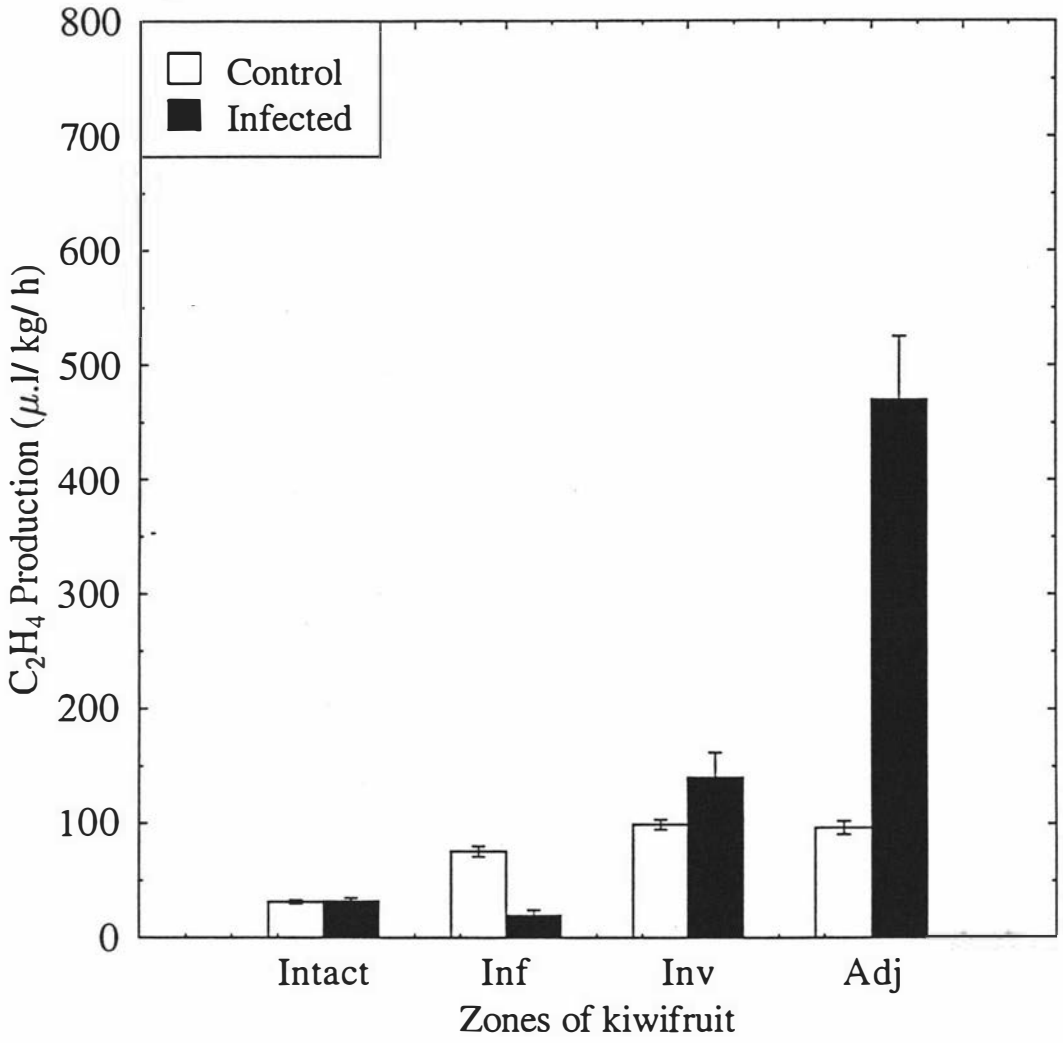


Fig 4.16 Normalized graph (Fig 4.9) of ethylene production from intact fruit and from zones of kiwifruit stored at 20°C in 1991.Inf= infected, Inv= invasion and Adj= adjacent noninfected zone, Intact= intact fruit prior to slicing. Bars represents standard errors.

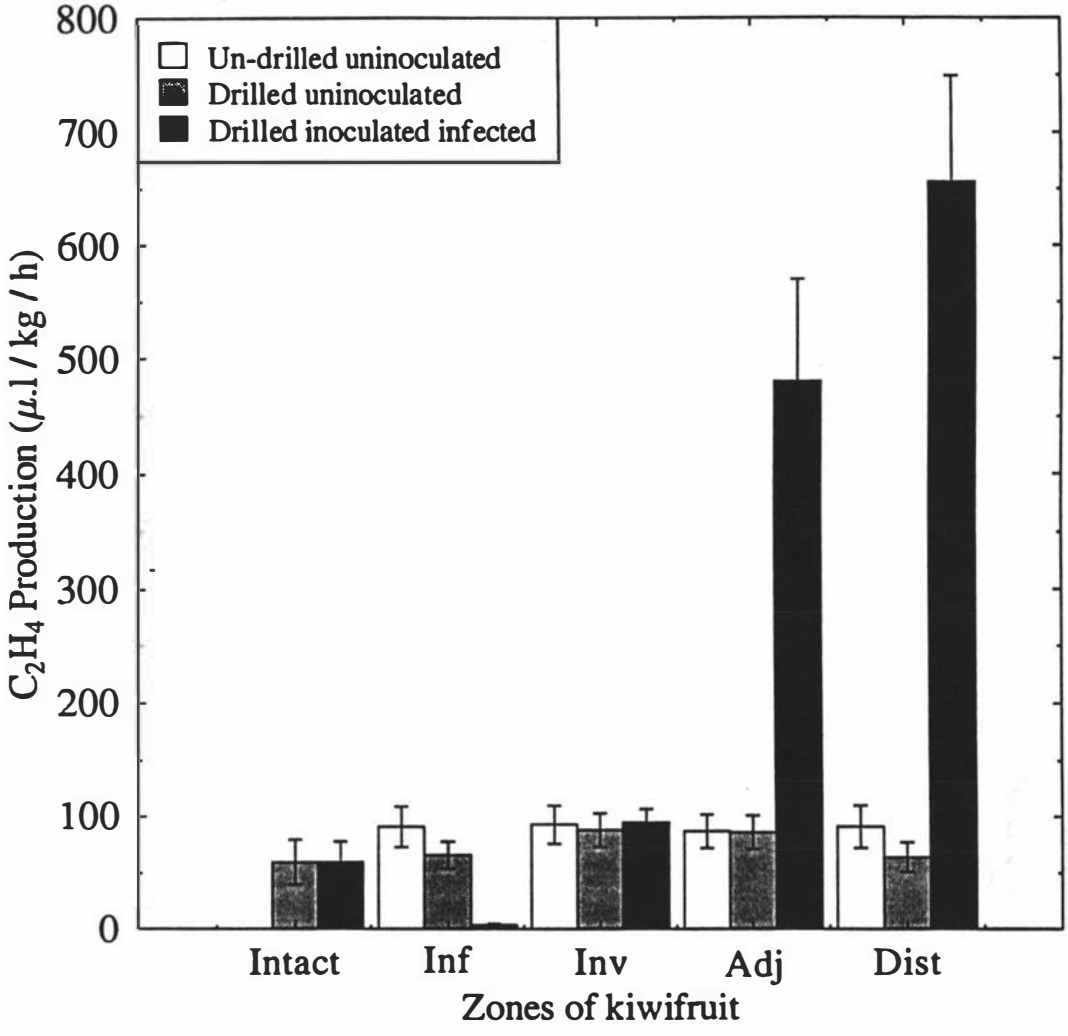


Fig 4.17 Normalized graph (Fig 4.14) of ethylene production from intact fruit and from zones of kiwifruit stored at 20°C in 1992. Inf=infected, Inv= invasion, Adj= adjacent noninfected and Dist= distal noninfected zone Intact= intact fruit prior to slicing. Bars represents standard errors.

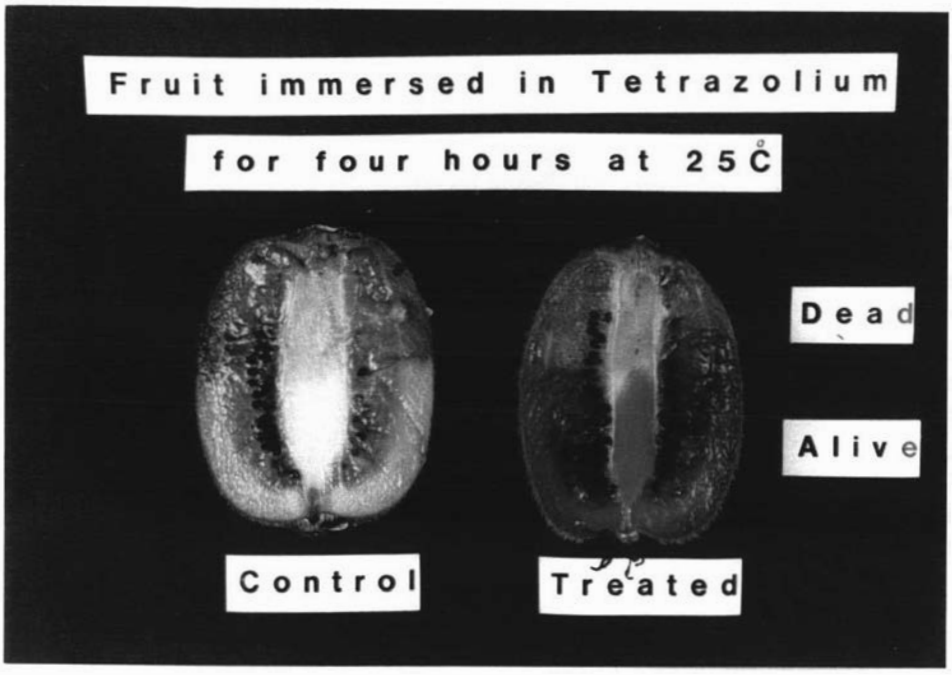


Plate 4.2 *B.cinerea* infected kiwifruit after two months at 0°C

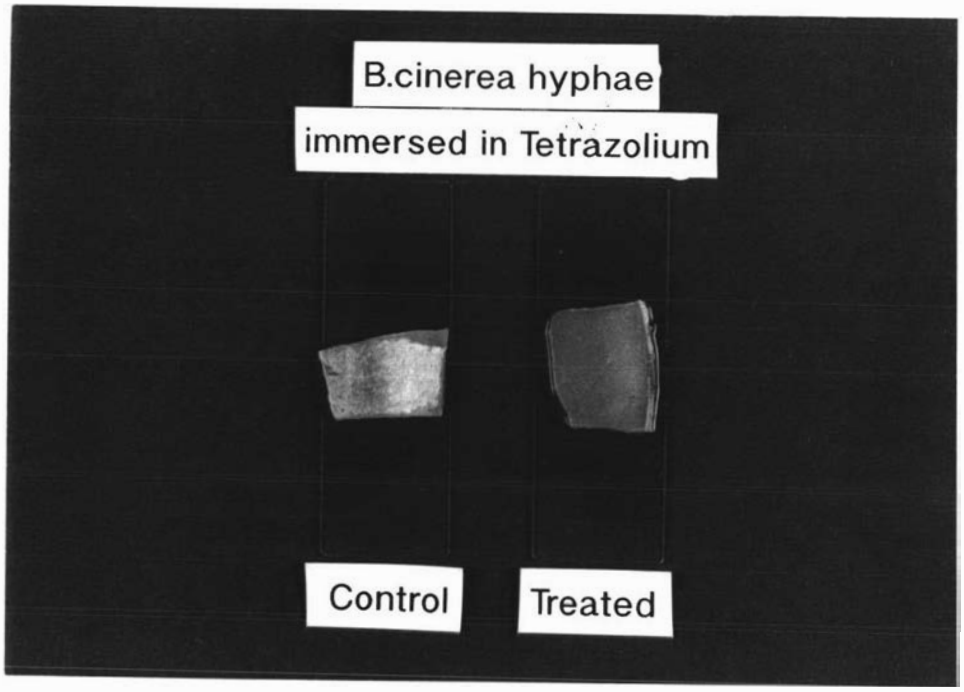


Plate 4.3 Botrytis cinerea hyphae immersed in tetrazolium for 10 h at 20 C

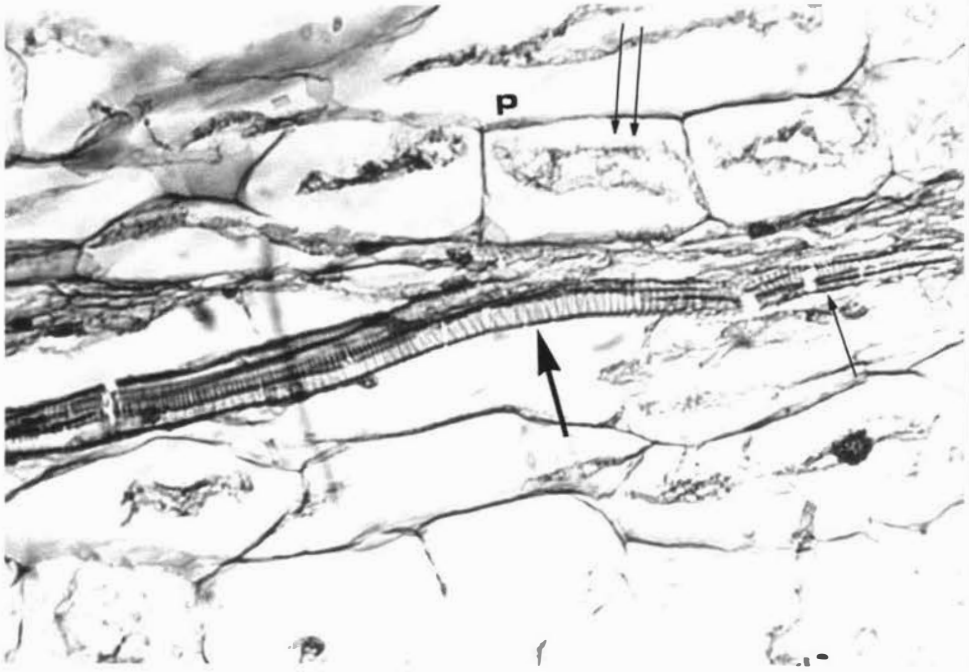


Plate 4.4 Longitudinal section through infected fruit pericarp tissue showing hyphae of *B. cinerea* in both xylem vessels and parenchyma tissue. p: parenchyma. Thick arrow: xylem vessel. Thin arrow: hyphae inside xylem vessel. Double thin arrow: hyphae inside parenchyma (x100).

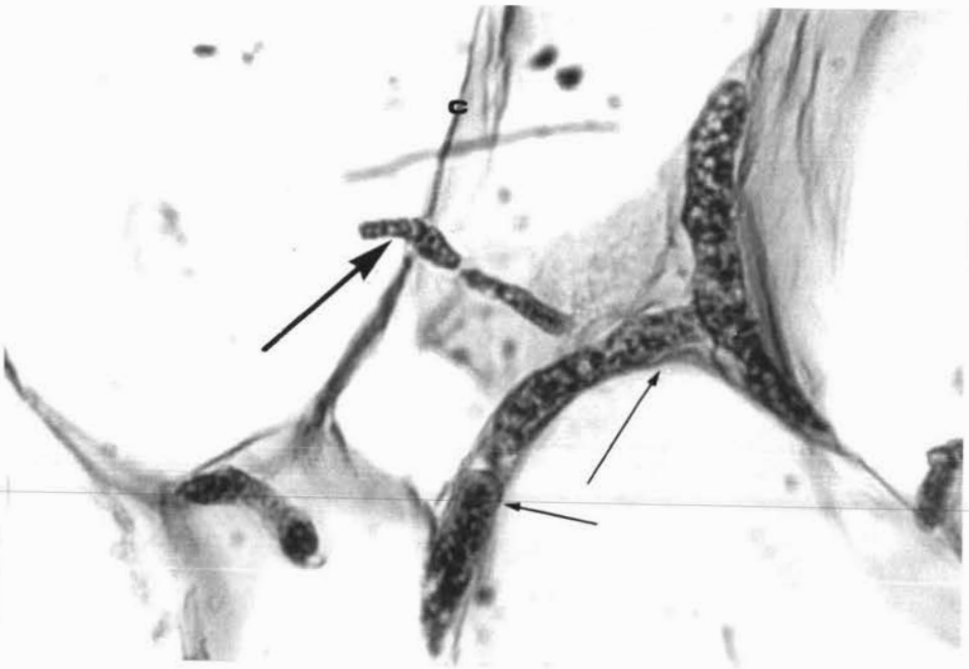


Plate 4.5 Longitudinal section through infected fruit pericarp tissue. Hyphae of *B. cinerea* have grown both inter- and intra-cellularly. c: plant cell wall. Thick arrow: intra-cellular hyphae at point where it passes from one cell to another. Thin arrows: inter-cellular hyphae (x400).

invasion fruit tissue slices. ACC content was highest in the infected zone of infected fruit and declined gradually towards the distal end of the fruit. There was no significant difference in ACC content between tissue from the infected and invasion front tissue. However ACC content was about 23% higher in the infected zone than in distal end tissue (Fig 4.18).

To measure the ACC oxidase activity in the slices from uninfected kiwifruit stored at 0°C for 2 months, kiwifruit were divided into 4 zones (Plate 4.1.1-4.1.3) and ACC was applied exogenously to excised slices from each of these zones. In the absence of exogenous ACC, ethylene production was detectable after 16 h incubation with approximately the same amount being produced from each zone, (Fig 4.19). Addition of exogenous ACC, resulted in an approximately 4-5 fold increase in ethylene production from all zones of the fruit. This increase in ethylene production commences about 4 h after incubation with ACC (Fig 4.19).

A further experiment was carried out to determine the time ACC takes to activate ethylene production from freshly cut kiwifruit slices. ACC was added to the slices from different zones of kiwifruit. In the absence of exogenous ACC, ethylene production was not detectable after 4 h of incubation (Fig 4.20). Addition of exogenous ACC, resulted in an increase in ethylene production in all zones after 4 h of incubation with no significant differences between zones (Fig 4.20).

When the reaction mixture containing 10 m*M* ACC was added to outer pericarp tissue from different zones of non-infected kiwifruit, ethylene production was between 30-37 µl/g/h from all zones (Fig 4.21). However when ACC was added to equivalent zones from infected fruit, major differences in ethylene production occurred. Very little ethylene (1 µl/g/h) was produced from infected tissue, a 37 fold reduction compared with the equivalent zone in healthy fruit. Tissue from the invasion zone produced about 15 times more ethylene than tissue from the infected zone, but it was only 50% of that produced from the equivalent zone in healthy tissue. Tissue from the zone immediately adjacent to the invasion front produced 32% more than that produced from the invasion front but 26% less than equivalent healthy tissue. Tissue from the distal end of infected fruit produced

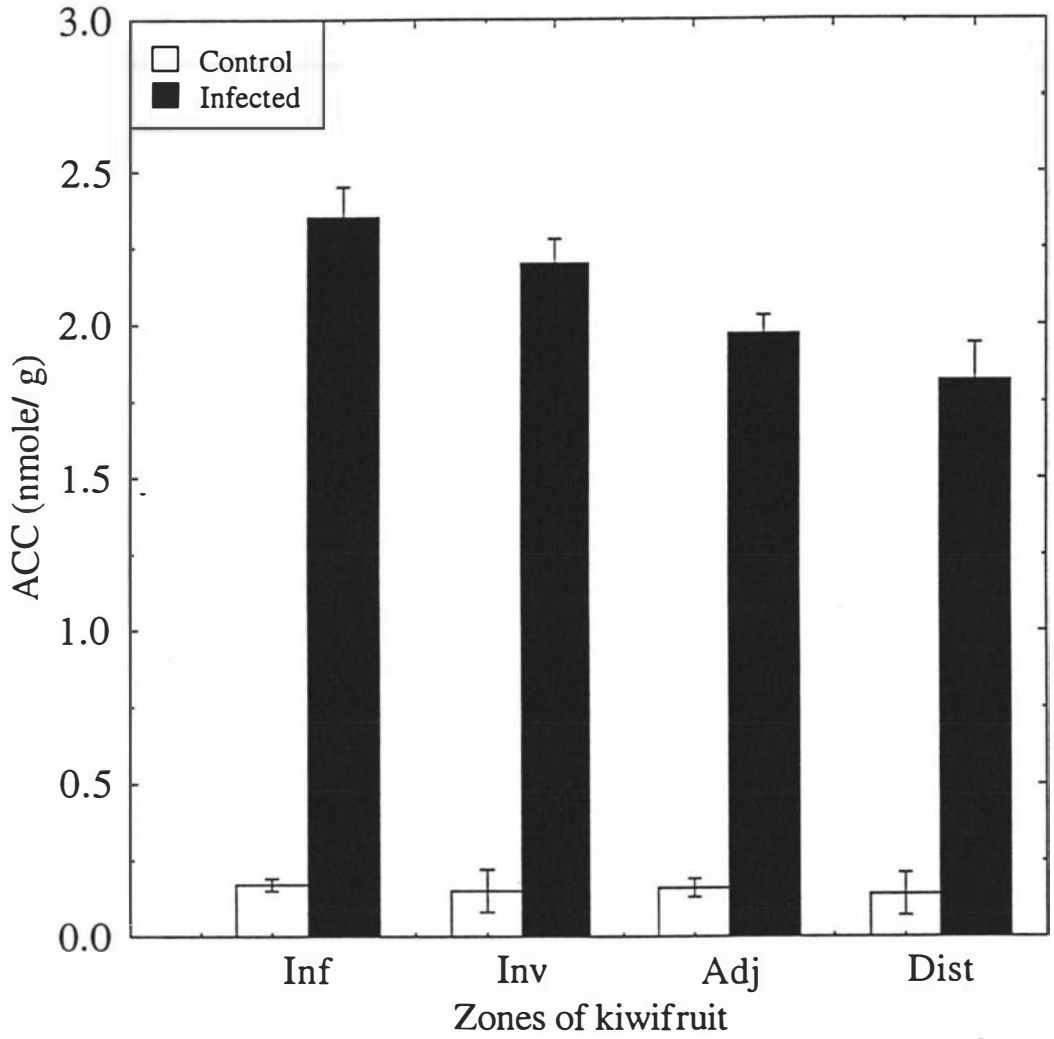


Fig 4.18 ACC levels from zones of infected and noninfected kiwifruit after 2 months storage at 0°C in 1992. Inf= infected, Inv= invasion, Adj= adjacent noninfected and Dist= distal noninfected zones. Bars represents standard errors.

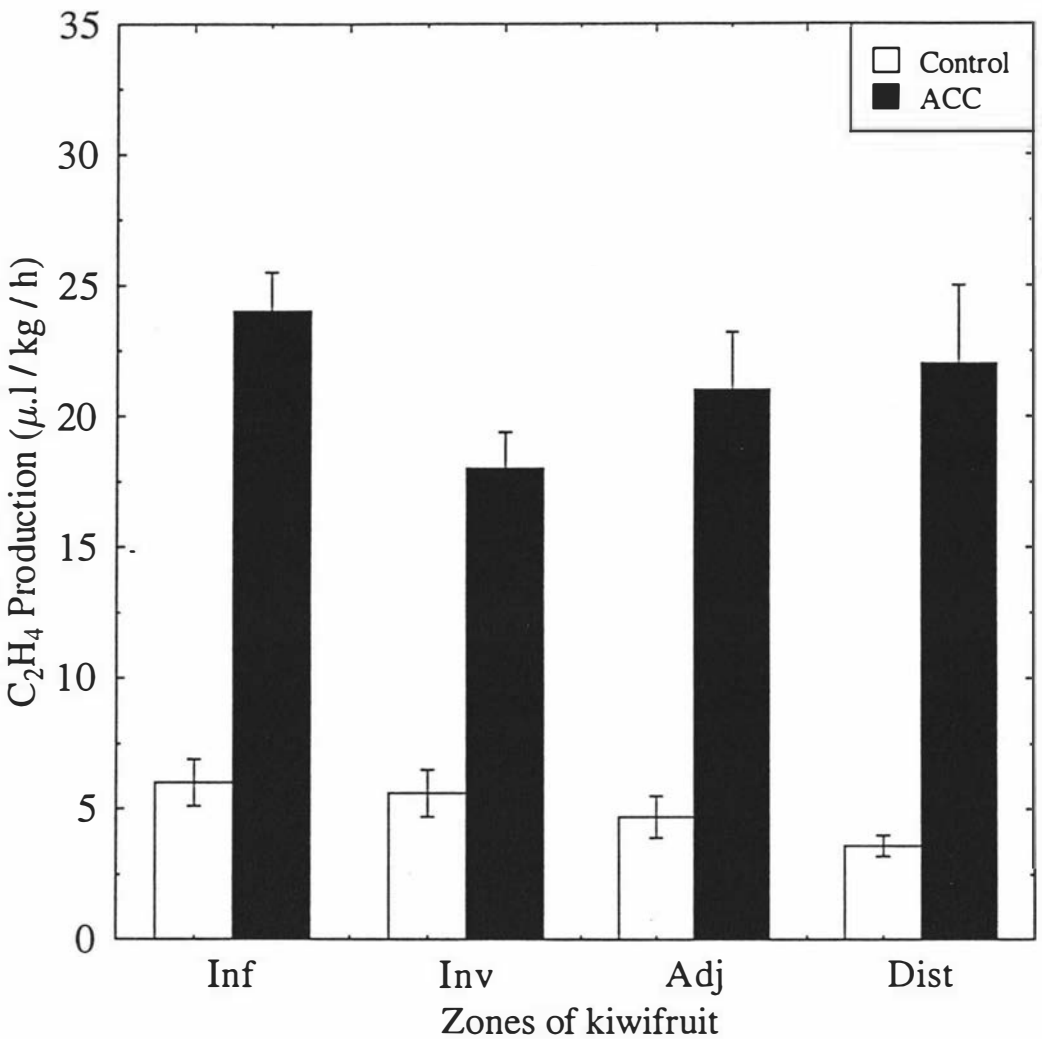


Fig 4.19 Ethylene production from slices of uninfected kiwifruit 16 h after slicing at 20°C with or without exogenous ACC. These fruit were stored at 0°C for 2 months before slicing. Inf= infected zone, Inv= invasion zone, Adj= adjacent noninfected zone and dist= distal noninfected zone. Bars represents standard error.

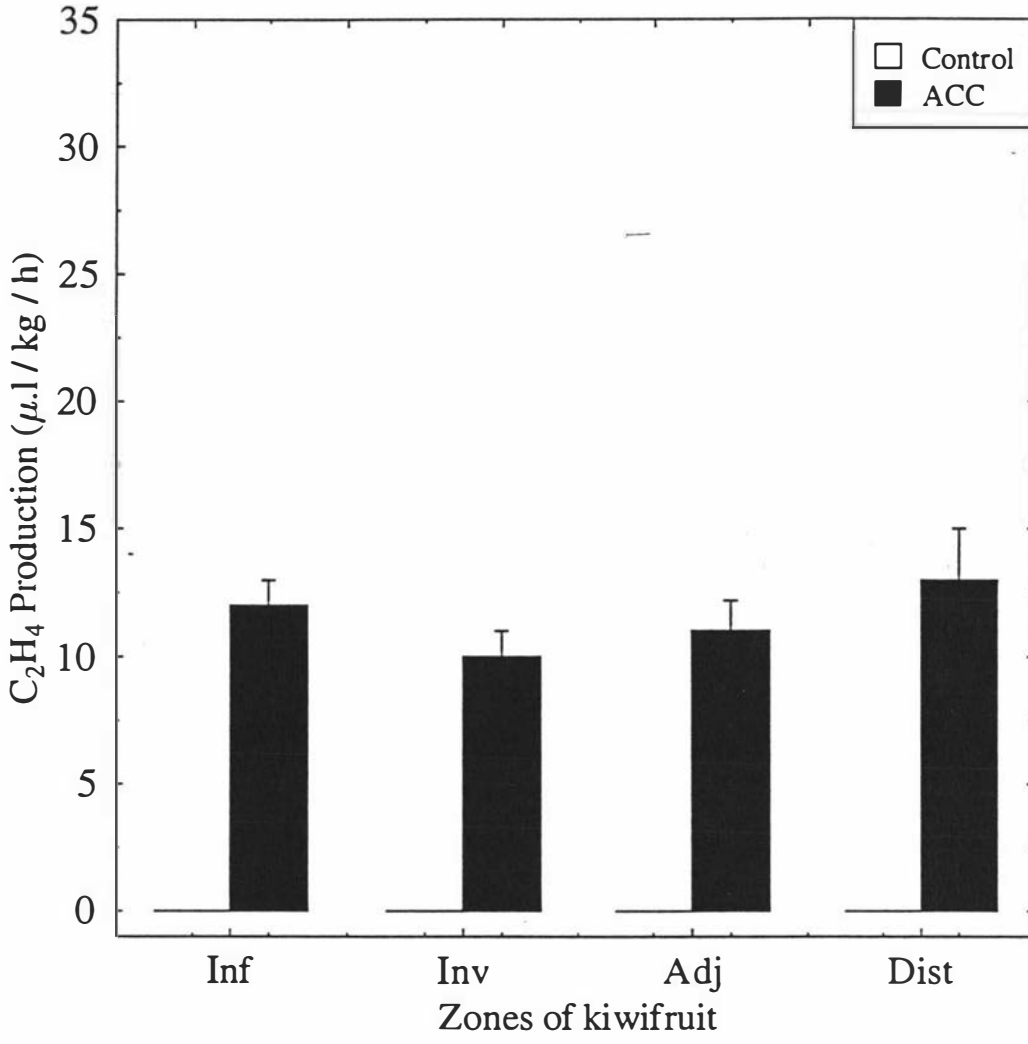


Fig 4.20 Ethylene production from slices of uninfected kiwifruit at 20°C with or without 1 ml of 0.05 [mM] exogenous ACC. ACC was applied soon after slicing and ethylene production measured after 4h of incubation. These fruit had been stored at 0°C for 2 months. Inf= infected zone, Inv= invasion zone, Adj= adjacent noninfected zone and Dist= distal noninfected zone. Bars represents standard error.

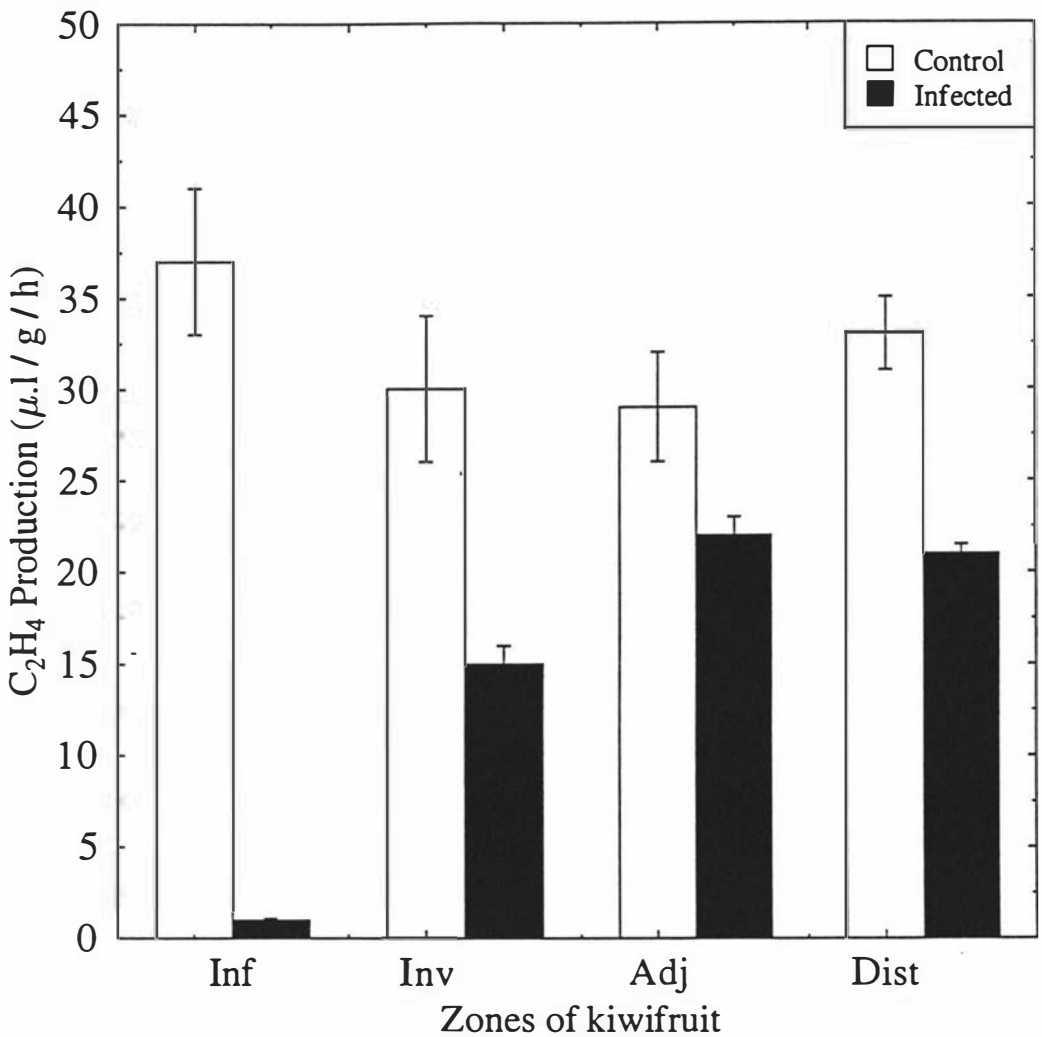


Fig 4.21 In vivo ACC oxidase activity from outer pericarp of different zones of infected and noninfected kiwifruit at 25°C. Inf= infected; Inv= invasion; Adj= adjacent non infected zone, Dist.= distal noninfected zone. Bars represents standard errors.

approximately the same amount of ethylene as the adjacent noninfected zone but 34% less than equivalent healthy tissue (Fig 4.21).

Extracts of kiwifruit pericarp tissue were incubated with varying concentrations of $NaHCO_3$ in an incubation medium containing 10 m*M* ACC. Ethylene production increased in a linear relationship with $NaHCO_3$ concentration between 50 and 400 m*M*; no further increase was measured at higher concentration (Fig 4.22).

In a further experiment extracts of kiwifruit pericarp tissue from healthy fruit were incubated with different concentration of ACC in an incubation medium containing 600 m*M* $NaHCO_3$. Ethylene production increased in a curvilinear manner as ACC concentration increased to reach a maximum at 16 m*M* ACC (Fig 4.23). Extracts were prepared from the previously described zones of healthy and infected fruit and ACC oxidase activity estimated after incubating in a medium containing 16 m*M* ACC and 600 m*M* $NaHCO_3$. ACC oxidase activity was similar in all zones of healthy fruit and was slightly higher in healthy fruit than in similar zones of infected fruit (Fig 4.24). In infected fruit, maximum ACC oxidase activity was present in the infected zone; ACC oxidase activity in the invasion zone and in the zone immediately distal to it was the same being about 40% less than in equivalent healthy tissue. In the distal zone of the infected fruit, ACC oxidase activity was about 17% less than in the equivalent zone in healthy fruit (Fig 4.24).

TSS of pericarp tissue from the distal (noninfected zone) of infected fruit was 14.2%, the tissue from invasion zone had an intermediate TSS value of 11.8%, while TSS of infected tissue was 9.4% (Fig 4.25). Fructose concentration was approximately 6% in all zones, but both glucose and sucrose concentrations decreased distally. Noninfected tissue in the distal zone contained 6.5 g/100 ml glucose and 2.0 g/ 100 ml sucrose, but this was reduced more than 50% in infected tissue. Tissues from the invasion zone had intermediate concentrations of both glucose and sucrose (Fig 4.25). The pH of pericarp tissue from the infected zone was slightly less than that in healthy and invasion zones of infected fruit, and this was reflected in a corresponding increase in titratable acidity in the infected and invasion zones compared with the healthy distal zone (Fig 4.26).

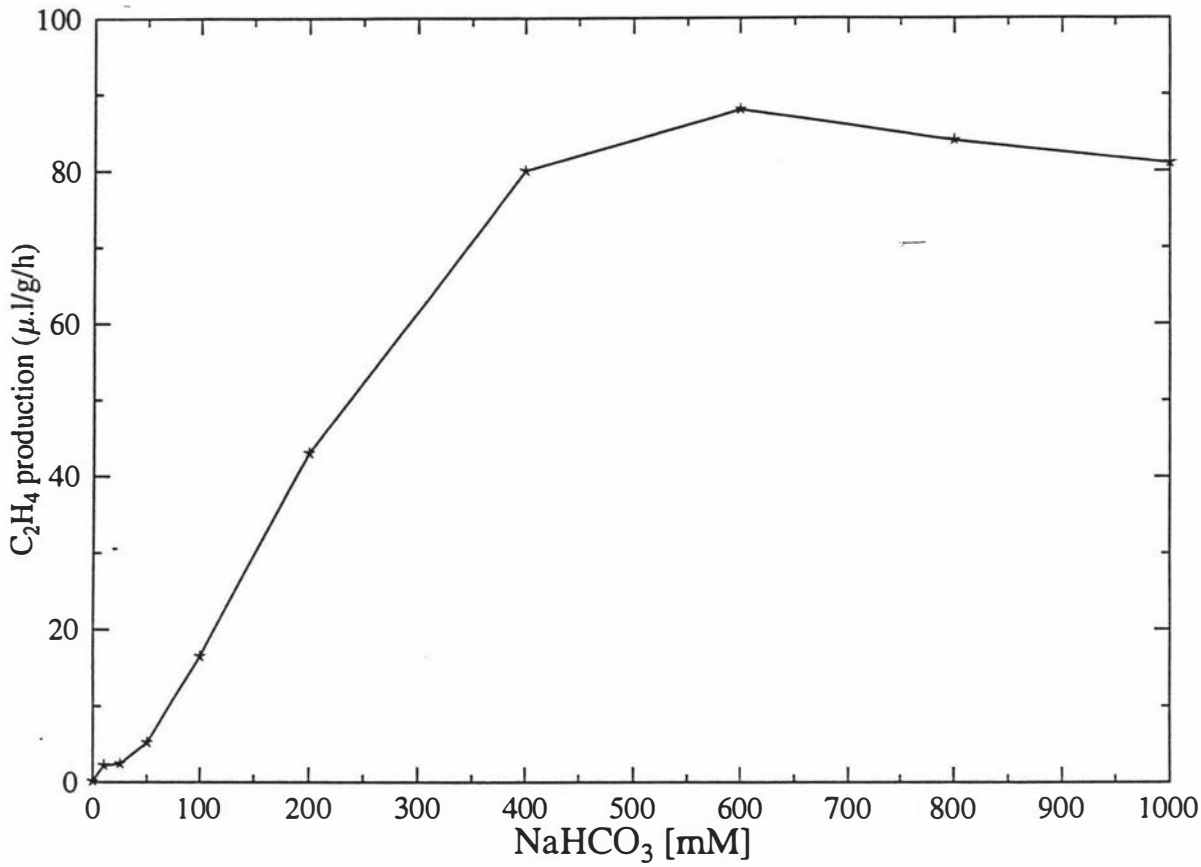


Fig 4.22 In vitro ACC oxidase activity from outer pericarp of kiwifruit incubated with a range of $NaHCO_3$ levels and 10 mM ACC at 25°C.

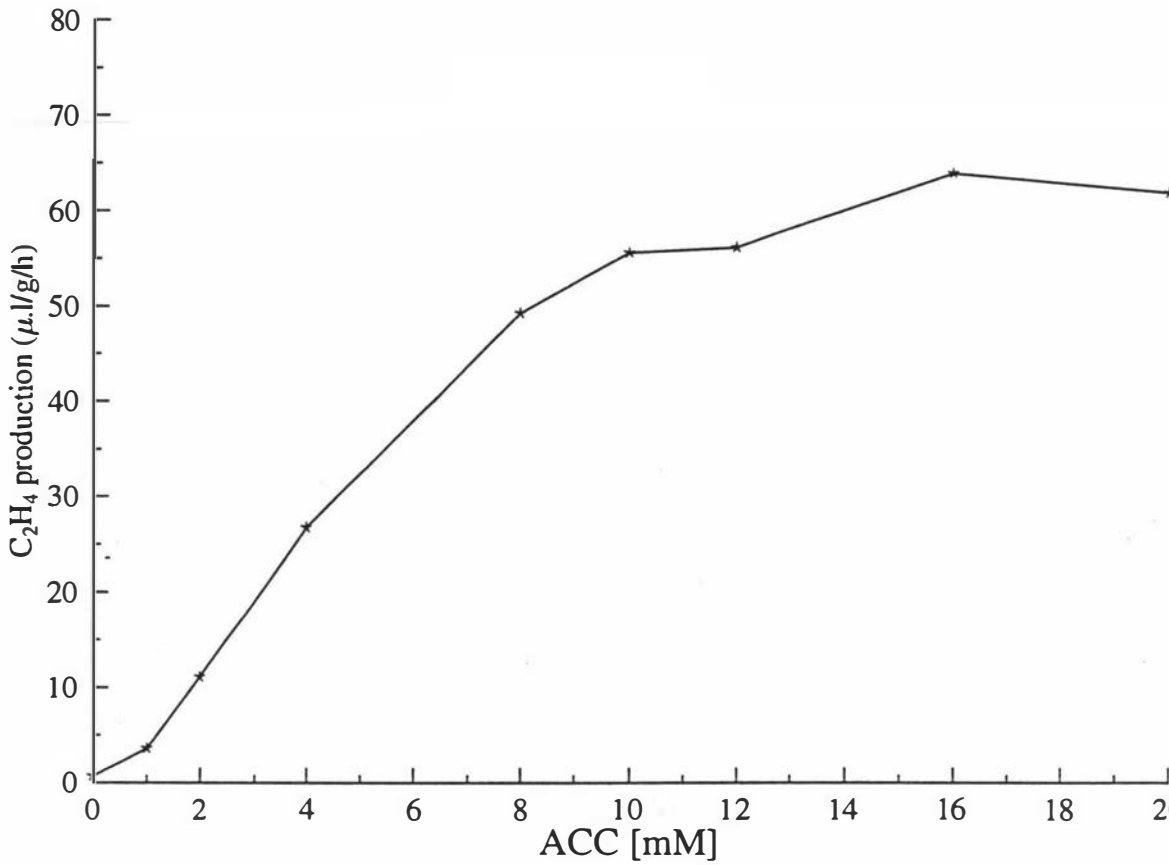


Fig 4.23 In vitro ACC oxidase activity from outer pericarp of kiwifruit with the range of ACC levels and 600 mM $NaHCO_3$ at 25°C.

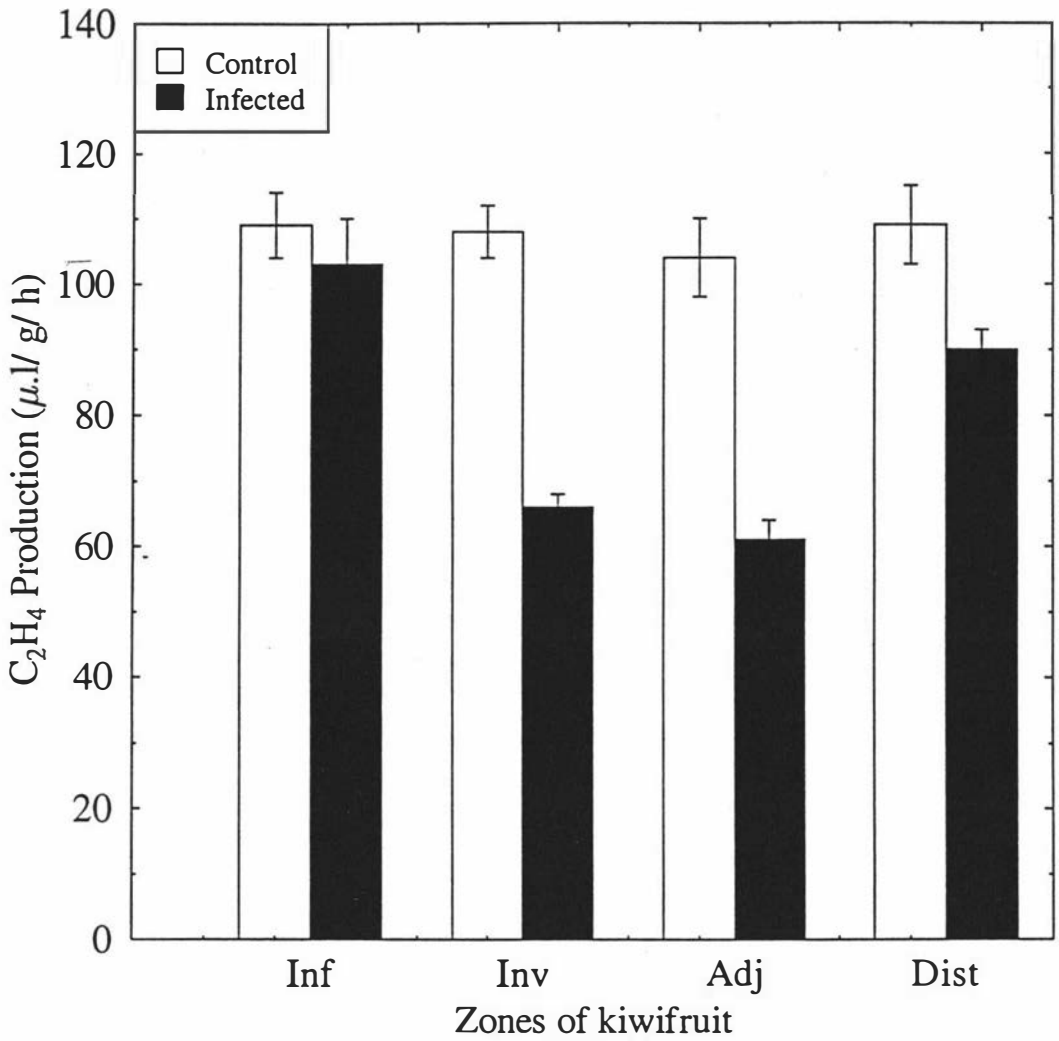


Fig 4.24 In vitro ACC oxidase activity from different zones of infected and noninfected kiwifruit. These fruit were stored at 0°C in 1992. Inf= infected, Inv= invasion; Adj= adjacent noninfected and Dist= distal noninfected zone. Bars represents standard errors.

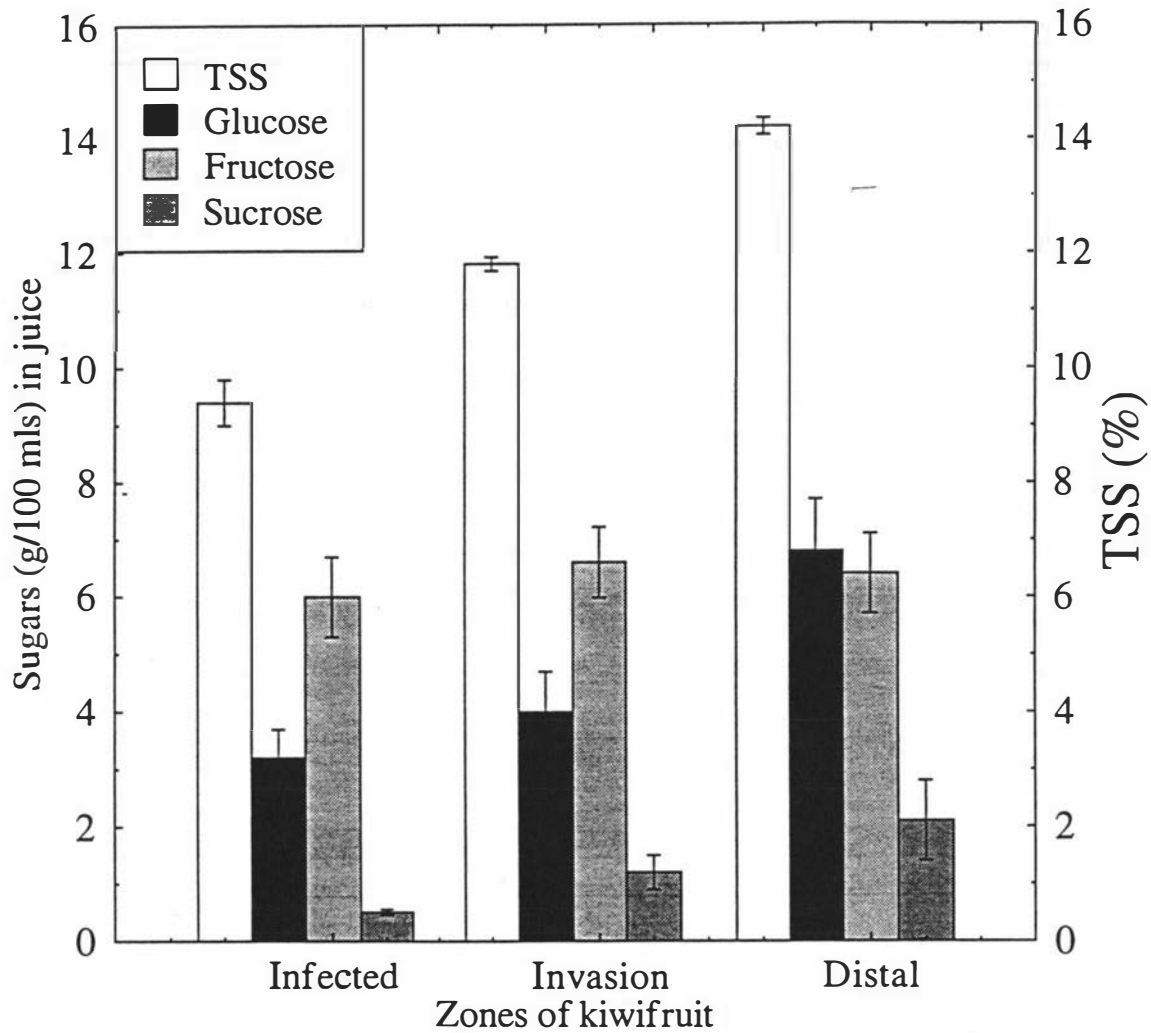


Fig 4.25 TSS, Sucrose, Glucose and Fructose concentration in the zones of infected kiwifruit stored at 0°C for 2 months. Bars represent standard errors.

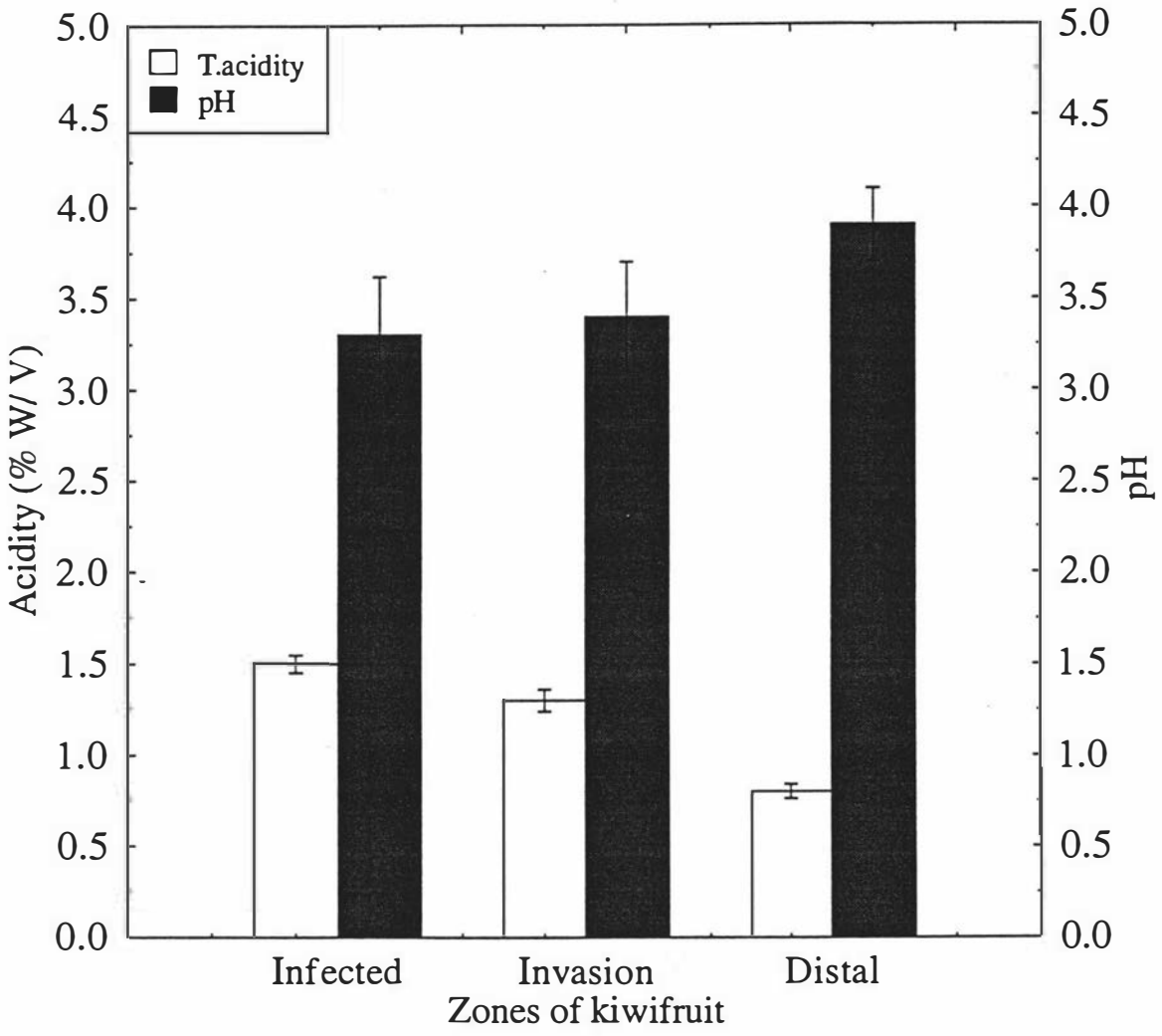


Fig 4.26 Titratable acidity and pH levels from zones of infected kiwifruit stored at 0°C for 2 months in 1991. Bars represent standard errors.

The increase in titratable acidity in the infected and invasion zones was caused by an increase in several organic acids. There were major increases in citric acid (the major acid in kiwifruit) concentration of approximately 1.5 and 2 fold in the infected and invasion zones respectively compared with the distal end of infected kiwifruit. The second most abundant acid was quinic acid; its concentration approximately doubled in both the invasion and infected zones compared with the healthy distal zone (Fig 4.27). Malic acid, which was present at lower concentrations than either citric or quinic acid was the same in all zones (Fig 4.27). Both of the minor acids (oxalic and fumaric) accumulated significantly in infected tissues (Fig 4.28). Oxalic acid concentrations were 2 and 3 fold higher, while the fumaric acid concentrations were 5 and 8 fold higher in the invasion and infected zones respectively, compared with tissue from the distal zone (Fig 4.28).

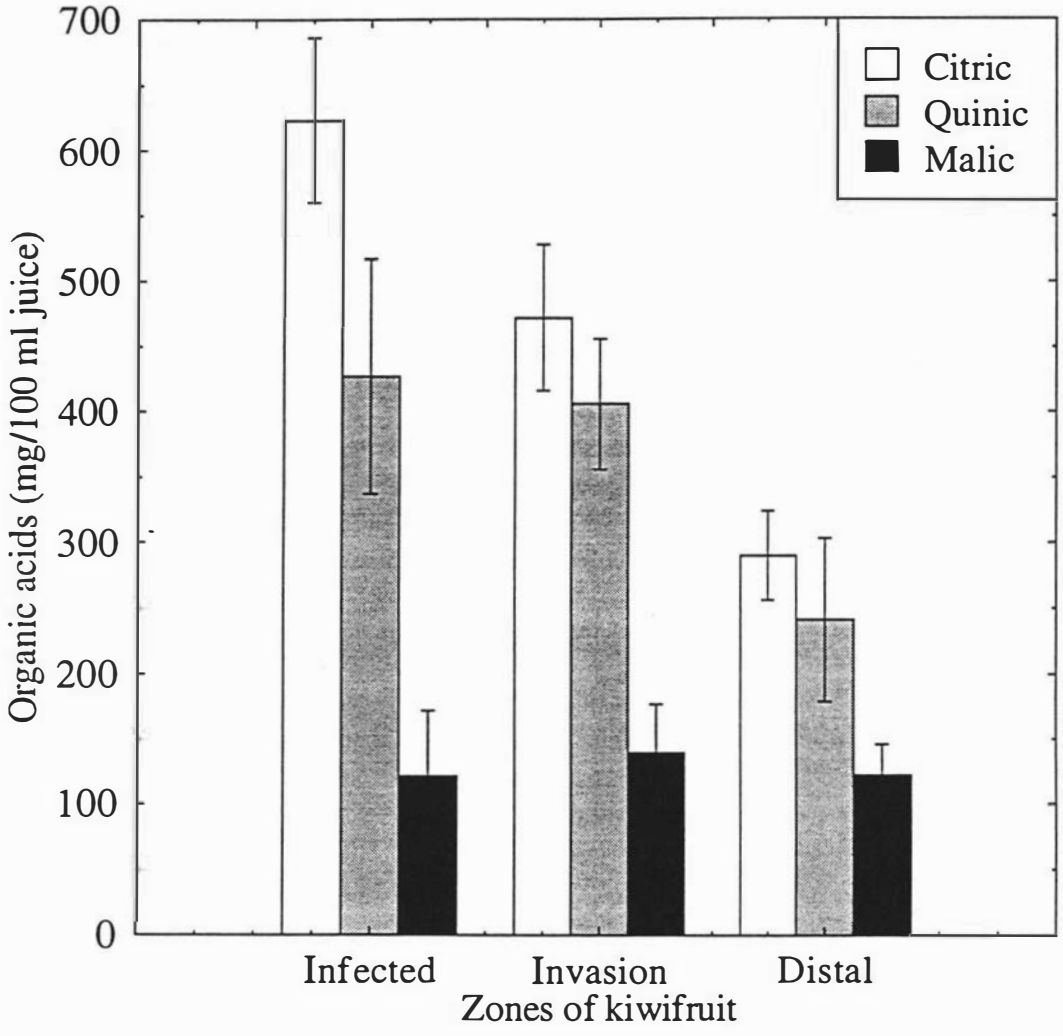


Fig 4.27 Quinic, Malic and Citric acid concentration in the zones of infected kiwifruit stored at 0°C for 2 months in 1991. Bars represents standard errors.

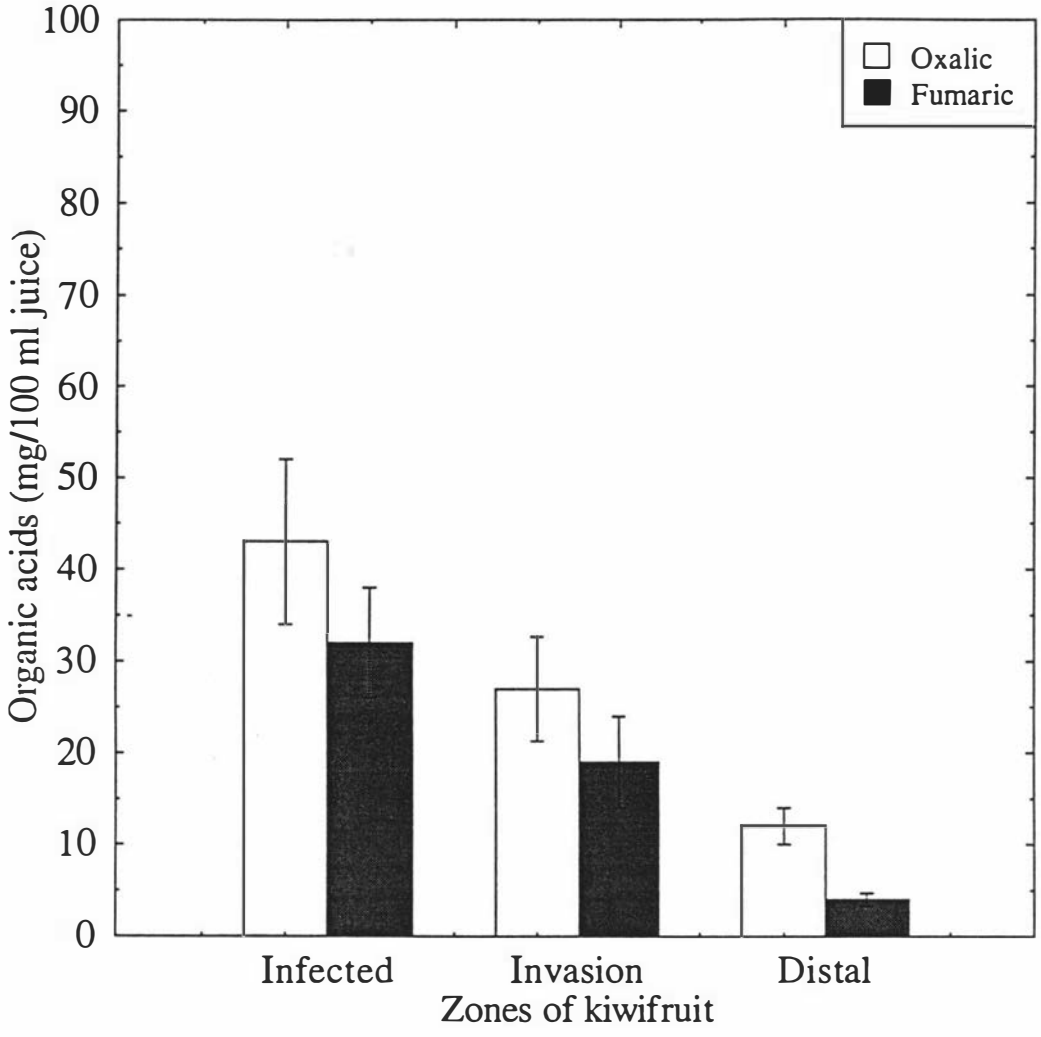


Fig 4.28 Oxalic and fumaric acid concentration in zones of infected kiwifruit stored at 0°C for 2 months in 1991. Bars represents standar errors.

DISCUSSION

Maturity of fruit at harvest has a significant impact on the incidence of *B. cinerea*, a postharvest pathogen causing serious losses to kiwifruit in storage. A decline in disease incidence with increased harvest maturity has been reported (Brook, 1990b). Fruit harvested at 6.2%, 7.8% and 10.8% TSS had 15%, 1.2% and 1.3% *B. cinerea* infection after 15 weeks storage at 0°C (Hopkirk et al, 1990). Rate of *B. cinerea* growth in kiwifruit inoculated at three different maturities and subsequently maintained at different temperatures, showed no consistent differences between harvests. Rate of lesion development on these infected fruit was similar between 0°C and 10°C in all harvests. Lesion development on the least mature (7.1% TSS) fruit was slightly faster at 5-15°C than on later harvested fruit, and reached a maximum in these experiments at 25°C rather than 20°C. These results confirm that *B. cinerea* is an aggressive postharvest pathogen at low temperatures, even though there appears to be little differences in rate of disease progression along the fruit between 0°C and 10°C. They also suggest that endogenous fruit factors, which change with maturity (Hopkirk et al, 1990; Pratt and Reid, 1974) may influence the ability of the fungus to grow in kiwifruit tissue.

Hyphal growth of *B. cinerea* inoculated onto malt agar and grown at the same temperatures as for inoculated inact fruit was approximately the same as in intact fruit at 0°C but was 2 to 5 times faster at higher temperatures. There are a number of possible reasons why these differences in growth rate of the fungus occurs between intact fruit and on agar. First, the physical barriers imposed by stem scar tissue and walls of parenchyma cells may impose a physical restraint on hyphal growth. More energy would need by the fungus to produce the enzymes required for breaking down cell walls and/or membranes to allow hyphal penetration (Kameon, 1992); this could well slow down the rate of progression of the infection front. Second, in the infected kiwifruit because of high rate of respiration there may be high CO_2 which could reduce fungal growth. Fungi in general are sensitive to CO_2 and an atmosphere of 10% CO_2 checks the growth of most fungi (Brown, 1922). Third, *B. cinerea* requires a readily available energy substrate to sustain rapid growth (Blakeman, 1975). While this is available on malt agar, the preferred carbohydrate source in kiwifruit may only become progressively available as starch is converted to soluble sugars (Wright

and Heatherbell, 1967; Okuse and Ryugo, 1981; Reid et al, 1982). Fourth, plants usually respond to fungal attack by invoking a variety of biochemical defense mechanisms. One of these responses is the synthesis of chitinase which is capable of inhibiting *Trichoderma reesei* growth *in vitro* (Verburg and Huynh, 1991). The level of this fungal inhibitor is higher in inoculated cured kiwifruit than in uninoculated kiwifruit (Wurms and Sharrock, Pers. com.). It is possible that this enzyme or some other fungal inhibitors may have been induced in *B. cinerea* infected kiwifruit and reduced fungal growth. Any or all of these factors could influence the development rate of *B. cinerea* along the fruit from the initial infection point at the stem scar and explain the difference in growth rate between *B. cinerea* on MA and on intact fruit. Currently available information does not enable specific characterisation of the factor influencing rate of infection progress along *B. cinerea* infected kiwifruit.

The incidence of *B. cinerea* differs from year to year (Pennycook, 1984) and from orchard to orchard (Manning and Pak, 1993). Differences in the incidence of *B. cinerea* have also been observed between samples of fruit picked from the same vine on different days (Pennycook, 1984). Environmental conditions during the growing and harvesting season are critical, as they influence the concentration of airborne spores (Manning and Pak, 1993). Investigations on *B. cinerea* infection and its effect on postharvest physiology of kiwifruit, usually use fruit inoculated artificially under laboratory conditions. In New Zealand, scientists investigating *B. cinerea* infection have used different methods of inoculation eg, spore dust and spore suspension, and have also inoculated at different times after harvest. Different laboratories have achieved different results from similar or identical experiments. At the time this research commenced there was considerable discussion amongst scientists as to the most effective and appropriate method for inoculation of kiwifruit with *B. cinerea*. Spores applied to the stem scar of kiwifruit within 12h of harvest in three different ways (dusting of spores, spores in liquid medium or spores diluted in talc) caused approximately the same degree of infection at 0°C regardless of application method. There was a considerable degree of variation within treatments, and the maximum infection was only 30%, indicating that natural barriers to infection were present in the fruit stem scar. The inoculum potential of a pathogen is increased as the number of infective units per unit area of host surface area increases (Garrett, 1960). Other research in this laboratory has

demonstrated a positive relationship between *B. cinerea* spore load on the stem scar and percentage infection of kiwifruit after storage (Long, Pers. Com). It has also been shown that percent infection can be consistently increased by inoculating through a small hole drilled through the stem scar into the top of the columella of the kiwifruit. Subsequent experiments utilized this technique of drilling the stem scar and using spore suspension to obtain consistent, high levels of infection.

Wounding is known to induce ethylene production through the accepted ethylene biosynthetic pathway in a wide range of plant tissue (Boller and Kende, 1980; Jackson and Campbell, 1976; Kende and Boller, 1981; Konze and Kwiatkowski, 1981; Saltveit and Dilley, 1978; Yu and Yang, 1980). Ethylene is also produced as a result of pathogen infection (Southwick et al, 1982) but biosynthesis of ethylene in host-pathogen interactions is considered to be different from that of wounded plants (Koch et al, 1980; Montalbini and Elstner, 1977; Sakai et al, 1970). Drilling through the stem scar of kiwifruit prior to inoculation, to facilitate infection caused wounding to the tissue. Therefore, in such drilled, inoculated tissue it would be expected that the ethylene produced would result from both wounding and infection perhaps with different ethylene biosynthetic pathways operating. In the present experiment at 20°C (Fig 4.11) ethylene production in drilled inoculated fruit was 4 times that in intact control fruit and approximately twice that in drilled, uninoculated fruit. This increased ethylene production in kiwifruit infected with *B. cinerea* compared with that in wounded fruit is similar to the results of Southwick et al, (1982) who found 6 times more ethylene production in navel oranges infected with either *Alternaria* or *Gloeosporium* compared with the control fruit and twice as much compared with wounded fruit. Similarly, Coleman and Hodges (1987), measured a five fold increase in ethylene production from *Poa pratensis* leaves infected with *Bipolaris sorokiniana* compared with leaves that had been wounded.

Tissue slices have been widely used to study aspects of postharvest physiology because of the ease of undertaking replicated experiments which are less convenient to do with intact fruit (Palmer and McGlasson, 1969; McGlasson et al, 1971; Laties, 1987; Dominguez and Vendrell, 1993). Apart from the wounding response, results are thought to represent what happens in intact fruit. Pesis et al, (1991) used slices to investigate the effect of *B. cinerea*

infection on chemical composition of kiwifruit. In the current work, tissue slices were used to examine ethylene production from different zones along the length of infected and noninfected kiwifruit. In general ethylene production from slices of control fruit was approximately similar, at a given temperature, in all the zones along the length of fruit in both 1991 and 1992. There was no ethylene measured from intact uninfected fruit at 0°C in either year or from fruit at 20°C in 1992. Slices from fruit which had a hole drilled through the stem scar, but which were not inoculated, produced approximately the same amount of ethylene as slices from undrilled fruit, indicating that the wound effect induced by slicing, overshadowed the wound-effect caused by drilling. At 20°C intact fruit with a drilled stem scar, did produce more ethylene than control undrilled fruit, indicating that wounding did induce ethylene. However this may have been a reflection of the physiological stage of the experimental fruit (see later).

Temperature influenced ethylene production. At 20°C ethylene production from slices of fruit was about 90 µl/kg/h whereas at 0°C ethylene production was about 400 nl/kg/h. Ethylene production is known to increase in a linear fashion in apples (Nakayama and Ota, 1983) and in kiwifruit (Niklis et al, 1992) as temperatures increase from -5 to 30°C.

Tissue from infected zones produced little if any ethylene at 20°C. This tissue was regarded as dead since it did not stain when exposed to tetrazolium dye. It was concluded that dead cells cease to produce ethylene (Williamson, 1950). Biosynthesis of ethylene was inhibited in citrus fruit tissue infected by *P. digitatum* (Achilea et al, 1985b). Sweet potato tissues infected by *Ceratocytis fimbriata* (Imaseki et al, 1968b; Hirano et al, 1991), apple infected by *Sclerotina fructigena* (Hislop et al, 1973) or leaves of tomato, pepper, bean and cucumber infected by *B. cinerea* (Elad, 1990), produce no or little ethylene from infected tissue. The small amount of ethylene that was measured from these infected tissues and from infected zones in this study may have been due to a non-enzymatic production of ethylene (Axelrood-McGarthy and Linderman, 1981; Swanson et al, 1979), or may have been produced by hyphae which were undoubtly still present in the water soaked infected tissue. Measurable ethylene production has been found in *B. cinerea* hyphae when given methionine as a precursor (see chapter-3).

At 0°C ethylene production was greater in the invasion zone, in the zone adjacent to the invasion zone and in the distal zone than in equivalent tissue in uninfected fruit in both 1991 and 1992. This higher rate of ethylene production occurred in tissue, which was not water soaked and presumably did not contain any *B. cinerea* hyphae. The data from tissue slices of fruit maintained at 20°C was adjusted to take account of the differential physiological stages of tissue development in relation to the 'developmental' age of tissue with respect to peak ethylene production (climacteric). A process of normalization of data was carried out which attempted to indicate the likely response of tissues which were at the same developmental stage with respect to ethylene production. Results of these adjustments indicated a similar pattern of ethylene production at 20°C, in both 1991 and 1992, to that found at 0°C. Ethylene production was greatest in the zone immediately ahead of the invasion zone, i.e, the 'adjacent noninfected zone' in 1991 and in both the adjacent noninfected and distal zones in 1992. This apparent stimulation of ethylene production ahead of infection front suggests that some signal was being produce by tissue in the process of being becoming infected, that it moved ahead of the front, being perceived by the as yet uninfected tissue, which somehow reacted to produce ethylene. The nature and/or mode of action of such a signal has not yet been determined, although it is known that some oligosaccharides, derived from the enzymatic breakdown of cell walls can induce ethylene production in a number of systems (Nothnagel et al, 1983; Heale, 1992). If such a signal was being transmitted ahead of the infection front, some changes in the enzymes involved in ethylene synthesis or the amount of one or more central intermediates in the ethylene biosynthetic pathway should be detectable in the zones distal to the infection front.

In the current experiments there were no major differences in ACC content from the tissue of infected or noninfected zones along the length of kiwifruit. It appears that, in these experiments at least, availability of ACC was not a limiting factor for ethylene production by the infected tissue since the ethylene biosynthetic pathway was at least partially intact and able to produce ACC. Niklis et al, (1993) found that ACC accumulated in *B. cinerea* infected kiwifruit tissue but ethylene production was decreased. Similarly, Achilea et al, (1985a) found that ACC accumulated in *P. digitatum* infected grapefruit peel tissue but the ability of the tissue to convert ACC to ethylene decreased with development of the infection.

The above results suggest that in the infected tissue ACC synthase was active and forming ACC but a further reaction which converts ACC into ethylene was not active. In infected tissue where ACC is being synthesised but not being converting into ethylene, the ACC level should have been significantly higher than in uninfected tissue. In the present study ACC levels were not significantly higher in the infected zone. It is possible that ACC was synthesised in the infected tissue but was being converted into the conjugated form, malonyl ACC (MACC). The reaction to convert ACC into MACC is catalyzed by the enzyme ACC malonyltransferase (Yang et al, 1990). The presence of the ACC malonyltransferase may serve as an alternative means of converting high levels of ACC into an inactive product (Hoffman et al, 1983). In the present study MACC levels were not determined, but this topic would be worth investigating in future work.

While *B. cinerea* infection did not prevent the accumulation of ACC, it may have disrupted the activity of the enzyme ACC oxidase which converts ACC to ethylene, since ACC did not appear to be a limiting factor but the ability to produce ethylene was diminished in *B. cinerea* infected kiwifruit tissue. In citrus fruit ethylene biosynthesis in *P. digitatum* infected tissue was inhibited (Achilea et al, 1985b), as indicated by the impaired ability of the infected tissue to convert ACC to ethylene (Achilea et al, 1985a). In kiwifruit a possible reason for a reduction in the ability of infected tissue to convert ACC to ethylene may be due to water soaking which may have created anaerobic environment in the infected tissue and reduced the availability of oxygen. The inhibition of ethylene production under anaerobic conditions has been observed by many workers for a variety of tissues. Low O_2 atmosphere inhibited ethylene production from climacteric tissues of apples (Burg and Thimann, 1959; Zhen-guo et al, 1983), banana (Mapson and Robinson, 1966) and pear (Blankenship and Richardson, 1986) and non climacteric tissues of strawberries (Li and Kader, 1989) and lettuce (Ke and Saltveit, 1989b). An anaerobic atmosphere also reduced ethylene production by 96% in *F. oxysporum* (Swart and Kamerbeek, 1977).

Overall ACC oxidase activity from healthy kiwifruit was higher than from infected fruit in both *in vivo* and *in vitro* experiments. The *in vivo* ACC oxidase activity increased with increased softening in kiwifruit (Mitchell et al, 1988) and during the post climacteric stage, there was a decline in EFE activity in both, kiwifruit (Mitchell et al, 1988) and avocado

(Hoffman and Yang, 1980; Sitrit et al, 1986). In the present experiments ACC oxidase activity was not measured over time, but it is possible that the higher ACC oxidase activity in control treatments of both *in vivo* and *in vitro* experiments may have been because control fruit were at a pre-climacteric stage and at this stage ACC oxidase activity may have been higher than at the post climacteric stage.

The trend of *in vivo* ACC oxidase activity from different zones of infected kiwifruit was similar to that of ethylene production from different zones along the length of fruit at 20°C. *In vitro* ACC oxidase activity was higher than that measured *in vivo*, reason for this is not known. Although no major difference was found from the tissue in the invasion front and the zone immediately ahead of the invasion front, slightly higher ACC oxidase levels occurred in the infected zone and the uninfected zone at the distal end of *B. cinerea* infected kiwifruit (Fig 4.24). In the *in vitro* experiments, $NaHCO_3$ was added as a CO_2 source, whereas in the *in vivo* experiment CO_2 was not added in the reaction mixture. Carbon dioxide was found to be required for, and stimulated, ACC oxidase activity extracted from apple fruit (Poneleit and Dilley, 1993; Smith and John, 1993; Tian et al, 1993). A reason for lower *in vivo* ACC-oxidase activity could be due to insufficient CO_2 in the system. A second reason could be due to a pH effect. The optimum pH for ACC-oxidase activity was 7.5-8.0 for melon (Smith et al, 1992) and avocado fruit (McGarvey and Christoffersen, 1992). In the *in vitro* experiment, the pH of the extraction mixture decreased after extraction and was re-adjusted to 7.5 for maximum ACC oxidase activity. In the *in vivo* experiment, although initial pH was adjusted to 7.5, it is not certain whether this pH level was maintained and it is possible that it changed to give a lower pH than in the *in vitro* system with a consequent effect on ACC-oxidase activity. The present results suggest that ACC oxidase activity was not a limiting factor in ethylene production by *B. cinerea* infected tissue of kiwifruit. It does seems likely that the complete ethylene biosynthetic pathway was intact in infected tissue, but due to some unknown reason the last step of ethylene production, conversion of ACC to ethylene, was impaired. It is possible that the higher acid levels in infected tissue had an inhibitory effect on the ACC-oxidase activity.

Simple sugars have been shown to induce the production of ethylene in plant tissues. A

range of sugars including sucrose, galactose, sorbitol, fructose, glucose and mannitol increase ethylene production in several vegetative tissue and were thought to somehow stimulate ACC synthase and ACC oxidase activities. Meir et al, (1989) have postulated that some sugars stimulate the enzymic hydrolysis of IAA alanine to IAA which in turn stimulate ACC synthase. However there has been little or no work carried out to determine the relationship between carbohydrate breakdown caused by fungal infection and increase ACC-synthase activity. Kamoen et al, (1978) obtained galactose from cultures of *B. cinerea* grown on high glucose (10%) for 10-30 days i.e long term culture. In tomato fruit, increased ethylene production was obtained when galactose was applied compared with control fruit (Kim et al, 1987).

In the work described here, an attempt was made to investigate the ethylene production by *B. cinerea* infected kiwifruit at 0°C and 20°C. The large amount of ethylene produced by the tissue immediately ahead of the infection front supports work by Ketring and Melouk (1982) who found that the major ethylene production in infected leaves occurred in the cells immediately adjacent to the periphery of the disease lesion. These experiments also gave a better understanding of the ethylene pathway in infected tissues where it seems that the ethylene pathway is intact and functional except for ACC oxidase activity which is present, but due to some unknown reason, does not convert ACC into ethylene.

CHAPTER 5

FINAL DISCUSSION

Botrytis cinerea attacks a wide range of plants in temperate regions. It infects field-grown and glasshouse-grown fruits and vegetables and is also an important postharvest pathogen (Verhoeff, 1992). *B. cinerea*, as a postharvest pathogen of many fruits and vegetables, is responsible for significant quality and hence economic losses in many countries through out the world (Heale, 1992). Storage rot caused by *B. cinerea* is one of the major causes of postharvest losses of kiwifruit internationally, and has been reported from Italy (Bisiach et al, 1984), California (Sommer et al, 1983), Greece (Niklis et al, 1993) and New Zealand (Pennycook, 1984; 1985a,b). In New Zealand, storage rot is regarded as the second most important cause of losses in stored kiwifruit after fruit softening (Pak and Manning, 1994; Davies et al, 1994). *Botrytis* storage rot originates from contamination and infection of the kiwifruit picking wound at harvest (Brook, 1990a). Wounds generally favour the penetration of *B. cinerea* and the subsequent development of lesions by providing ready access to the internal tissues of the fruit when the damaged cell contents are available as an energy source for the fungus. *B. cinerea* which is normally regarded as a weak pathogen, can then proliferate within the tissue, producing enzymes and/ or toxins which may defuse into adjacent healthy tissue causing development of typical disease symptoms (Kamoen, 1992).

Infection is known to induce ethylene production by plants (Ross and Willamson, 1951) with infected plant tissues producing increased ethylene compared to healthy tissue (Archer and Hislop, 1975; Graham and Linderman, 1980; Elad, 1990; Tongthieng and Sangchote, 1994). There is still considerable uncertainty as to the relationship between infection and ethylene production (Abeles, 1973; Archer and Hislop, 1975; Sequeira, 1973). It is not known whether the pathogen itself produces ethylene, whether all the ethylene induced in the plant tissue is as a result of infection and subsequent tissue damage caused by the pathogen; whether the pathogen somehow induces the host to produce ethylene in tissue remote from the damaged centre; or whether a combination of all these occurs following infection.

It is well established that increased ethylene production is a common consequence of stress in plants (Abeles et al, 1992), particularly wounding. Infection by plant pathogens is a form of wounding , as tissue disintegration almost always occurs. During the infection process, hyphae penetrate the tissue and induce breakdown of cell walls and membranes, destroying compartmentalization of cellular structure. Ethylene is generally produced at a higher rate in infected tissue than in comparable healthy tissue, but how much is produced by the pathogen itself and how much is produced by the wounded tissue is unclear.

In higher plants stress ethylene has been shown to occur through the same pathway as for normal ethylene (Abeles et al, 1992), which comes from L-methionine --- *S* -adenosyl-L-methionine (SAM) ---- 1-aminocyclopropane-1-carboxylic acid (ACC) ---- ethylene (Adams and Yang, 1979; Konze and Kende, 1979). Stress ethylene results from an increase in the synthesis of ACC synthase, which leads to the accumulation of ACC and subsequent conversion into ethylene (Yu and Yang, 1980; Boller and Kende, 1980; Kende and Boller, 1981; Konze and Kwiatkowski, 1981).

Higher plants are not the only producers of ethylene. It is a common metabolic product of a range of lower plants and microorganisms, including marine algae, *Codium latum* (a green alga) and *Porphyra tenera* (Wantanabe and Kondo, 1976), a unicellular algae, *Haemetococcus pulvialis* (Maillard et al, 1993), a lichen *Ramalina duriaei* (Lurie and Garty, 1991), moss *Funaria hyrometrica* (Rohwer and Bopp, 1985) and bacteria *Pseudomonas solanacearum* (Freebairn and Buddenhagen, 1964, Goto et al, 1985). Fungi are also known to produce ethylene (Ilag and Curtis, 1968; Fukuda and Ogawa, 1991). However *B. cinerea*, an ubiquitous postharvest pathogen (Heale, 1992), has not been shown to produce ethylene (Elad, 1990), although plants infected with this fungus do produce substantial amounts (McNicol et al, 1989; Elad, 1990).

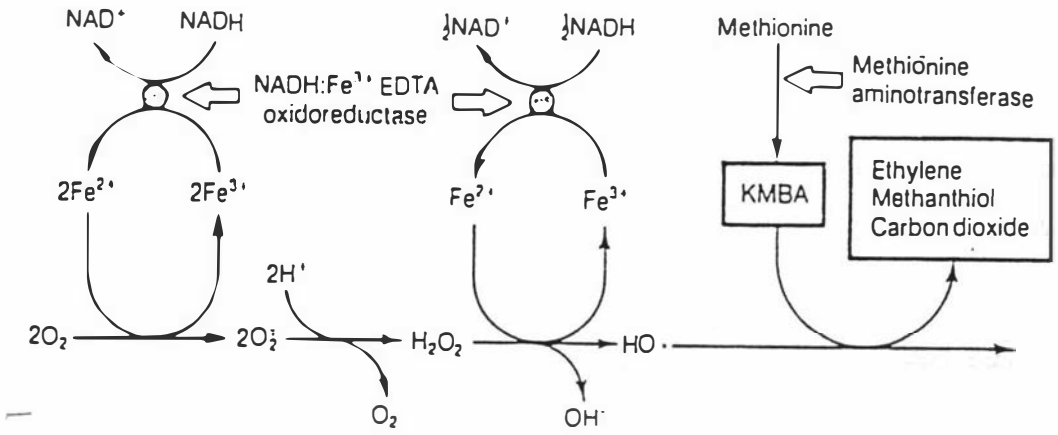
While there are many published reports of infected tissue producing ethylene (Table 1.2), there are only a few documented examples of specific fungi producing ethylene grown *in vitro* isolated from host tissue, and/ or grown on defined media. Notably among these are *P. digitatum* (Chalutz and Lieberman, 1977), *Verticillium*, *Fusarium* and *Colletotrichum* (Tzeng and DeVay, 1984; Hottiger and Boller, 1991) all of which are well known plant

pathogens.

The biosynthetic pathway for ethylene in higher plants has been closely defined over the past 25 years, since the discovery that methionine was the predominant precursor (Lieberman and Mapson, 1964) and that ACC was a critical and immediate precursor of ethylene (Adams and Yang, 1979). However, there is much less certainty about the pathway of ethylene synthesis in microbes. There is no general acceptance of the main precursors, the intermediates in the pathway, or the nature of the enzymes involved in catalysing the various reactions leading to the production of ethylene.

Various fungi have been shown to produce ethylene while growing on defined synthetic media amended with a variety of possible precursors including glucose (Thomas and Spencer, 1978), glutamate (Chou and Yang, 1973), or methionine (Chalutz et al, 1977; Axelrood-McCarthy and Linderman, 1981; Wilkes et al, 1989). Knowledge about these precursors and intermediates as well as enzymes involved in the ethylene biosynthetic pathway(s) is less clear. It was proposed that ethylene production in fungi may come from two different pathways: it may be produced from a pathway starting with either methionine or glutamate (Fukuda and Ogawa, 1991), with KMBA (Ince and Knowles, 1986; Ogawa et al, 1990a) and 2-oxoglutarate (Fukuda et al, 1986) respectively being immediate precursors. Interestingly, KMBA is a precursor of methionine in higher plants through the ATP dependent methionine salvage pathway (Miyazaki and Yang, 1987).

Fukuda et al (1993) have undertaken an extensive review of the literature on ethylene production by microorganisms, and provided the most recent suggestions for ethylene biosynthesis in microorganisms. They suggested that most ethylenogenic microorganisms use methionine for the ethylene-forming system. The enzyme involved in this pathway is methionine aminotransferase which converts methionine to KMBA (Ogawa et al, 1990b). The last step of ethylene formation was studied by Fukuda et al (1989c) and Ogawa et al (1990b); they concluded that the ethylene-forming enzyme is an NADH: Fe^{+++} -EDTA oxidoreductase, which reduces 2 mol of Fe^{+++} EDTA with 1 mol of NADH to give 2 mol of Fe^{++} EDTA and 1 mol of NAD^{+}, and proposed the following mechanisms of formation of ethylene from methionine.



After Fukuda et al, 1993.

This reaction requires the generation of hydroxyl radicals (HO·) in their system. The hydroxyl radical is capable of serving as an oxidizing agent in the oxidation of KMBA to ethylene (Diguiseppi and Fridovich, 1980; Tauber and Babior, 1980).

The essential enzymes involved in the ethylene biosynthetic pathway of higher plants are SAM adenosyltransferase (Chou et al, 1977), ACC synthase (Adams and Yang, 1979) and ACC oxidase (Ververidis and John, 1991). The presence of all these enzymes in fungi has not been established. Some of them occur in lower plants and there are some reports suggesting the presence of similar, but not identical, kinds of enzymes in fungi. In plants, the first enzyme of the ethylene pathway, (S-adenosylmethionine transferase) converts methionine into SAM. This enzyme has been reported in *E. coli* (Markham et al, 1980; Boyle et al, 1984) and in yeast (Chiang and Cantoni, 1977), but SAM has never been reported as a precursor of ethylene in fungi (Fukuda et al, 1986). Although SAM was not tested as a precursor of ethylene in the present experiments, on the basis of results reported for other microorganisms it is possible that SAM is not involved in the ethylene pathway of *B. cinerea*.

The critical importance of ACC synthase and ACC in the biosynthesis of ethylene in higher plants has been shown in a number of different studies (Abeles et al, 1992). Compounds such as rhizobitoxin and its synthetic analogues, AVG and AOA, inhibit although do not

completely eliminate, ACC synthase activity and reduce the formation of ACC (Hoffman and Yang, 1984; Owens et al, 1971). Treatments which block the metabolism of ACC to ethylene, such as chilling temperature (Guye et al, 1987), and low O_2 (Burg and Thimann, 1959), leading to the accumulation of ACC in affected tissue, can be reversed by removal of the treatment conditions resulting in eruption of ethylene production (Abeles et al, 1992). Addition of ACC to a range of tissue eg, apple (Saftner and Baker, 1987) results in increased ethylene production compared to control where ACC was not applied.

Supplying ACC to culture media in which *B. cinerea* was growing did not result in increased ethylene production. Even when combined with methionine, the resultant ethylene production could be attributed to methionine alone. It is possible that ACC was not available to the fungus, perhaps because it was not able to be taken up by the hyphae, but this is unlikely. Many other tissues can readily utilize applied ACC with a consequent increase in ethylene production. When AOA was added to the *B. cinerea* culture medium with methionine present, ethylene production was substantially reduced. ACC did not reverse this inhibition which it can do in higher plants.

Inhibitors of pyridoxal phosphate mediated (PLP) enzymes have not been widely used to investigate ethylene production by microorganisms. The few reports that have appeared indicate that AVG or AOA have little if any effect on ethylene synthesis regardless of the nature of precursors used in the medium. Tzeng and DeVay (1984), have used AOA and AVG in a methionine amended medium in an attempt to inhibit the ethylene production from *V. dahlia* but they had no effect. Similarly, Goto et al (1985) have found no effect of AVG when added to glutamate amended medium, while AOA had only a small inhibitory effect on ethylene production by *P. syringae* pV *phaseolicola*. This suggests that in different microorganisms the same precursor, methionine, may be metabolised through different biosynthetic pathways to produce ethylene. Fukuda et al (1993) have shown that an ethylene-forming system which exist in microorganisms, uses methionine as a substrate which is converted into KMBA by methionine aminotransferase. Taylor and Metzler (1990), have shown that aspartate aminotransferase depends upon PLP and the activity of aspartate aminotranferase and glutamic-oxalacetic transaminase can be inhibited by AOA (Amagasa et al, 1992; Taylor and Metzler, 1990). It is possible that methionine

aminotransferase may also be dependent upon PLP and hence it is sensitive to AOA.

It has been clearly shown that *B. cinerea* can produce ethylene under defined conditions and that methionine is the most efficient precursor of ethylene. Addition of ACC to the culture medium did not increase ethylene production, while addition of AOA to methionine containing medium significantly inhibited ethylene. This suggests that ACC was not the precursor of ethylene and that there is a PLP mediated enzyme reaction downstream from the methionine originated ethylene biosynthetic pathway in *B. cinerea*.

The final step of ethylene biosynthesis in higher plants is the conversion of ACC to ethylene. This reaction is dependent on EFE, which has now been purified and identified as ACC oxidase from higher plants (Ververidis and John, 1991; Smith et al, 1992). However, Fukuda et al (1986, 1989a) reported the existence of EFE in a cell free system of *P. digitatum.* It had similar properties to the ACC oxidase from higher plants in having specific requirement for Fe^{++}, O_2 and reducing agents such as ascorbate or dithiothreitol (Fukuda et al, 1986 & 1989a; Ververidis and John, 1991; Smith et al, 1992). This suggests that EFE from fungi and ACC oxidase from higher plants have similar characteristics. However, the EFE from fungi fails to convert ACC into ethylene (Fukuda et al, 1986), so while it may be similar to ACC oxidase it is not identical.

ACC oxidase from higher plants is responsive to a number of inhibitors, including Co^{++} ions, sodium caprylate (Hyodo and Fukasawa, 1985), fatty acids (Hyodo and Tanaka, 1982), salicylic acid (Leslie and Romani, 1988) and Ni^{++} (Lau and Yang, 1976). Cobalt has been shown to inhibit ethylene production (Hyodo and Fukasawa, 1985; Kang et al, 1967; Samarakoon et al, 1985) at the step in which ACC is converted to ethylene (Yu and Yang, 1979; Leforestier et al, 1993) in a number of plants. However in lower plants and in infected tissue this inhibitory effect of the Co^{++} ion does not always occur. Application of Co^{++} to a unicellular green alga (Mailard et al, 1993) stimulated ethylene production, where ACC stimulated ethylene production. This suggests that the EFE in unicellular green alga is different from that in higher plants (Maillard et al, 1993). Hirano et al (1991) reported that Co^{++} inhibited ethylene production in healthy sweet potato root tissue and in a cell free EFE system from the fungus *P. digitatum* (Fukuda et al, 1986 & 1989a), but stimulated it

in tissue infected by *C. fimbriata.* The addition of Co^{++} to *B. cinerea* culture medium supplemented with methionine resulted in a temporary reduction in ethylene production. After a 4 day lag period a stimulation in ethylene production occurred with the same amount produced as for methionine alone. This temporary reduction in ethylene production by the addition of Co^{++} suggests that the EFE complex of *B. cinerea* is different from the ACC oxidase system in higher plants.

In higher plants ACC oxidase is controlled by a small multi gene family *eth1*, *eth2*, and *eth3* (Cooper et al, 1993). Further analysis on tomato using *eth2*, *eth3* specific probe revealed no detectable levels of expression in infected fruit, which suggested that in tomato fruit *eth1* is the pathogen induced EFE gene (Coooper et al, 1993). Similarly it is possible that in microorganisms there could be a multi gene EFE family present, and under specific conditions different genes may be activated to produce EFE. In *P. digitatum*, where methionine was not the precursor of ethylene in static culture, the addition of methionine resulted in ethylene formation, suggesting the induction of an enzyme (Chalutz and Lieberman, 1977). Similarly, in *B. cinerea* it is possible that the addition of Co^{++} may have activated an alternate EFE.

After establishing that under *in vitro* conditions *B. cinerea* can produce significant amounts of ethylene, it was essential to establish the pattern of ethylene production during the progress of infection by *B. cinerea* in kiwifruit. One way of establishing this relationship was to divide the kiwifruit into specific zones and to remove these zones as dissected slices, measure the ethylene produced from the slices as well as ACC and ACC oxidase activity, and build up a picture of the changes in ethylene that occurred as disease progressed along the length of the fruit.

Use of slices is a legitimate and useful tool for biological studies. Tissue slices have been used for a wide range of physiological and biochemical experiments on kiwifruit and other tissues. These include research on pigment changes and colour composition in canned kiwifruit (Cano and Marin, 1992), changes in chemical compositions of *B. cinerea* infected kiwifruit (Pesis et al, 1991), induction of enzyme (such as chitinase) in pumpkin fruit (Esaka et al, 1993), ethylene induction in preclimacteric avocado fruit (Starrett and Laties,

1993), mRNA in tomato and potato (Roberts et al, 1988), O_2 affinity of ACC and ethylene production by apple and banana slices (Banks et al, 1985; Banks, 1985), sugar efflux in senescensing ephemeral daylily petal slices (Bieleski and Reid, 1992) and ion uptake in bean, barley and maize leaves (Nissen, 1974; Luttge and Ball, 1973).

Slicing obviously wounds tissue and causes subsequent ethylene production, but results obtained are thought to represent what happens in intact tissue as long as appropriate corrections are made to account for this wounding effect. Wounding does have a significant effect on ethylene production. Meight et al (1960) showed a more than 23 fold increase in ethylene production from 4 mm slices of tomato fruit, and McGlasson and Pratt (1964), found a 10 fold increase in ethylene production from slices of cantaloupe melon, compared with intact fruit.

This slicing technique allowed determination of the localized effect of infection on ethylene production in different zones of the fruit. Ethylene production varied according to the presence or absence of *B. cinerea* infection and the slice position along the length of the fruit. Little or no detectable ethylene production was found in the infected tissue, while an enhancement in ethylene production was found ahead of the infection front. This enhancement in ethylene production from slices ahead of the infection front may be due to some kind of transmissible signal which is induced by infection and moves into uninfected tissue to somehow promote ethylene production.

It is possible that the enhanced ethylene production which occurs ahead of the infection front in kiwifruit infected with *B. cinerea* could have resulted from direct or indirect elicitation of ethylene synthesis. Direct elicitation may occur following the release of a fungal compound which somehow interacts specifically with the host tissue to induce the production of ethylene. Proteins such as cryptogein, secreted by the phytopathogenic fungus *Phytophthora cryptogea* (Bellin et al, 1991) or glycopeptides found in the mycelium, cell wall, and filtrate of *Colletotrichum lagenarium* (Esquerre-Tugaye and Toppan, 1977; Esquerre-Tugaye et al, 1979; Toppan et al, 1982; Esquerre-Tugaye et al, 1983; Toppan and Esquerre-Tugaye, 1984; Esquerre-Tugaye et al, 1985), have been shown to induce ethylene. Such, an elicitor of ethylene has never been purified from *B. cinerea*. Indirect elicitation

may be caused by the production of cell wall degrading enzymes which break down polysaccharides into oligomers which have been shown to act as 'secondary messenger (s)' triggering active responses in adjacent healthy cells. It is also possible that both of these factors could be involved simultaneously, each contributing to the enhancement of ethylene production which occurs in tissue distal to the infection front. Ketring and Melouk (1982) postulated that a large part of the ethylene produced by peanut leaves, infected with *Cercospora arachidicola* Hori., originated immediately adjacent to the periphery of the lesion.

Fungal invasion of plant tissue is accompanied by secretion of a number of enzymes that degrade plant cell walls (Cooper, 1976); this probably facilitates penetration of host tissue by the fungus. However, it also results in breakdown of cellular integrity and probably makes available from the cytoplasm, an energy and nutrition source (carbohydrates and amino acids) required by the fungi for its growth (Hall and Wood, 1973; Dickman et al, 1982; Marcus and Schejter, 1983; Van Etten and Kistler, 1984). Like other pathogens, *B. cinerea* produces a range of hydrolytic enzymes in the infection process, including those which can break down cell walls and membranes eg, polygalacturonase (Leone, 1990; Johnston and Williamson, 1992; Tobias et al, 1993), laccase (Marbach et al, 1984; Viterbo et al, 1992, 1993 & 1994; Roudett et al, 1992), pectin methyl esterase (Marcus and Schejter, 1983), pectinase (Miura et al, 1994; Garcia-Romera et al, 1990), proteinase (Movadehi and Heale, 1990) and cellulase (Verhoeff and Warren, 1972; Heiler et al, 1993). Recently *Trichoderma viride* has been shown to produce xyloglucanase, an important enzyme for breaking down hemicellulose (Vincken et al, 1994) and although not reported, it is possible that this enzyme may also be produced by *B. cinerea* during infection of plant tissue.

Cell wall degrading enzymes or their products are known to stimulate ethylene biosynthesis (Anderson et al, 1982). For example, the increase in ethylene production was observed when partially digested citrus pectinase was injected into orange (Labavitch, 1983). Similarly, when pectolyase (pectinase, endo pectin lyase and xylanase) was injected into orange fruit, production in ethylene was observed and levels of ACC also increased (Baldwin and Pressey, 1988). Tomato pectinase injected in citrus had similar effects

(Baldwin and Biggs, 1988).

Cell wall degrading enzymes can break down cell wall polysaccharides into short chain oligomers (Aldington and Fry, 1993). A few select oligosaccharides which have sugar residues interconnected by glycosidic linkages, can at very low concentrations, exert "signalling" effects on plant tissue (Fry et al, 1993). Such oligosaccharides are termed "oligosaccharins" or "elicitors" and they can induce a number of quite different responses in plants (Darvill et at, 1992; Aldington and Fry, 1993). For example, oligosaccharins derived from xyloglucan by partial digestion with cellulase (Fry et al, 1993) can antagonize growth promotion induced by the auxin 2,4-D (York et al, 1984) or promote elongation of pea stem segments in the absence of 2,4-D (McDougall and Fry, 1990). Pectins are another primary cell wall polysaccharide and digestion of pectic polysaccharides with pectinase yields oligosaccharins (Fry et al, 1993) which have been shown to induce defence responses such as the synthesis of phytoalexins (Fry et al, 1993), and to influence plant growth (LoSchiavo et al, 1991; Filippini et al, 1992). Some oligosaccharides are thought to act as anti-auxins (Filippini et al, 1992), affecting plant growth and development, including the control of organogenesis in thin cell layers of tobacco (Mohnen et al, 1990), the inhibition of auxin-stimulated induction of new auxin binding sites (LoSchiavo et al, 1991), and rooting in tobacco leaf explant (Fillipini et al, 1992).

Leone et al, (1990) have suggested that polygalacturonase has a double role in the infection process; firstly it assists penetration by *B. cinerea* and secondly, it triggers the onset of the chain production of other isozymes for pectin catabolism (Leone et al, 1990). This latter function could be mediated by the release of monomers and oligomers from the pectic portion of the cell wall (Leone et al, 1990). In kiwifruit, the pectin degrading enzymes endopolygalacturonase and β-galactosidase may play a role in the breakdown of pectic molecules during kiwifruit ripening, (Redgwell et al, 1992). Although β-galactosidase, which is capable of degrading a number of cell-wall pectic polysaccharides *in vitro*, may solubilize cell-wall polysaccharides, it is not known if these pectic fragments are capable of inducing ethylene production, or whether these polysaccharides breakdown into oligomers in the case of infection (Redgwell, Pers. com.). It is possible that the enhanced ethylene production that consistently occurs in the tissue immediately ahead of the infection

front, may have been due to the secretion of cell wall degrading enzymes by the fungus which caused hydrolysis of kiwifruit cell wall polysaccharides to oligomers. If these were of the appropriate specificity it is possible that they in turn, could induce enhanced ethylene production by the same, as yet unknown, mechanism that is thought to occur in other tissues.

Regardless of the nature of the eliciting signal and whether it moves intercellularly or intracellularly to penetrate the plasmalemma of cells to interact with some form of receptor(s), it somehow leads to the activation of genes responsible for ethylene production. Such genes could clone either or both ACC synthase and ACC oxidase. Anderson et al, (1993) proposed a model of ACC synthase gene activation in response to as an yet unidentified water soluble protein (EIX) isolated from a filtrate of the fungus *Trichoderma viride*. They suggested that this elicitor moved throughout the leaf in the xylem. Once the elicitor reached sensitive cells it perhaps interacted with (as yet unknown) binding sites or specific receptors. The nature of the various steps involved in this process are not yet understood: it is not known whether specific cells are involved and there is no information on the nature or location of specific receptors or binding sites in the cell. Details of the movement of the receptor/elicitor complex to the nucleus and DNA, the nature of the initiation/ activation of gene responsible for the production of ACC synthase and ACC oxidase have not yet been resolved. According to Anderson et al, (1993) the gene responsible for ACC-synthase activity must be activated in response to infection before production of ethylene from infected plant tissue. When tobacco cell suspension cultures were treated with an elicitor from *Phytophthora parasitica nicotianae*, a surge in ACC synthase activity occurred before ethylene production started (Rickauer et al, 1990). Niklis et al (1993) measured an increase in ACC content in zones of kiwifruit infected with *B. cinerea* before increased in ethylene production. It is likely that this was the result of induction of ACC synthase synthesis or activity in the fruit. Both ACC synthase and ACC oxidase genes have been identified in kiwifruit tissue (MacDiarmid and Gardner, 1993; Gardner pers. com.) and it is possible that the putative transmissible signals which are thought to precede *B. cinerea* infection in kiwifruit somehow switches on the appropriate genes resulting in the autocatalytic production of ethylene in healthy tissue ahead of the infection front.

The model presented by Anderson et al (1993) could be valid for attached leaves where there is a mass flow of water driven by transpiration. However it is unlikely to be the same for detached fruits, because after harvest there is little if any water movement inside the fruit, provided the relative humidity of the storage environment is high and transpiration is limited. Therefore, only localized effects at low temperature should be expected in response to infection. It is not yet known how these transmissible signals are transported in harvested fruit. Kamoen (1992), who studied the factors influencing development and expansion of lesions, suggested that signal(s) must precede the hyphae to kill the cells ahead. Such signals could be transferred either in the symplast (Kahl, 1982) or in the apoplast by intercellular diffusion (Kamoen, 1992). Since *B. cinerea* penetrates kiwifruit tissue both inter-and intracellularly, it is possible that any transmissible signal produced could travel by either route to cells ahead of the infection front and elicit ethylene production.

No major differences in ACC content were measured from tissues of infected or noninfected zones along the length of kiwifruit. It appears that, in these experiments at least, availability of ACC was not a constraint for ethylene production by the infected or noninfected tissue. Achilea et al (1985a) found an accumulation of ACC content in *P. digitatum* infected grapefruit peel tissue, although in this example, ACC dependent ethylene production decreased with progressive development of the infection. These combined results suggest that in infected tissue, at least part of the ethylene biosynthetic pathway was intact and able to produce ACC. The fact that very little ethylene was produced in *B. cinerea* infected zones of kiwifruit, even though there seemed to be adequate ACC present to produce large amounts of ethylene, suggests that the small amount of ethylene from the infected zone may have been of fungal origin, as *B cinerea*, is capable of producing ethylene (Chapter 3) provided that methionine and essential cofactors are present. Alternatively ethylene could have been produced nonenzymatically (Wilkes et al, 1989), if a source of hydroxyl free radicals was available to convert KMBA to ethylene (Diguiseppi and Fridovich, 1980; Tauber and Babior, 1980).

If there is enough ACC present to sustain ethylene production in infected tissue, yet it does not occur, then there must be an inhibition of the enzyme catalyzed conversion of ACC to

ethylene. Inhibition in ethylene production has been found in the sweet potato root infected by black rot (Imaseki et al, 1968b) and apple fruit with brown rot (Hislop et al, 1973). This inhibition in ethylene production could have been due to destruction of ACC oxidase activity. In citrus fruit tissues infected with *P. digitatum*, addition of exogenous ACC did not increase ethylene production, and it was concluded that ACC oxidase activity was impaired in the infected region (Achilea et al, 1985a,b). Prior to 1991 it was considered that in higher plants ACC oxidase was membrane bound. Extraction techniques which caused disruption of plant tissue, destroyed membrane integrity, and resulted in inhibition of ethylene production (Burg and Thimann, 1960; Inaba and Nakamura, 1986). However, Ververidis and John (1991) clearly demonstrated that EFE or ACC oxidase was present in a soluble form in the cell and did not need to be membrane bound; *in vitro* ACC oxidase activity was successfully recovered from homogenated melon tissue provided the correct extraction procedure was used and appropriate cofactors were present. This technique was further improved by Smith and John (1993). However in 1986, Fukuda et al, had reported the recovery of EFE from cell free system of *P. digitatum* and had shown that both Fe^{++} and ascorbate were essential components of the extraction mixture. The technique developed by Ververidis and John (1991); Smith and John (1993) was used to measure *in vitro* ACC oxidase activity from infected kiwifruit. It was shown that ACC oxidase activity in both the infected zone and in the uninfected zone at the distal end of *B. cinerea* infected kiwifruit was slightly higher than that in the invasion zone and in the zone immediately ahead of the invasion front. Up to 109 µl/g/h of ethylene was produced in the *in vitro* experiments to which ACC was added, indicating that ACC oxidase was not a limiting factor in ethylene production by *B. cinerea* in the infected tissue of kiwifruit. It can be concluded that in infected tissue of kiwifruit, the components required for ethylene biosynthesis were present (ACC and ACC oxidase), but for some unknown reason ethylene production was limited in the infected zone.

FINAL CONCLUSION AND FUTURE DIRECTION

An important aspect of this study was to find out whether the ethylene which is produced from infected kiwifruit is produced by *B. cinerea* itself, if the fungus produces ethylene that in turn induces the kiwifruit to produce ethylene, or if the kiwifruit responds to infection with *B. cinerea* by producing ethylene. Prior to this investigation there was no

evidence to suggest that *B. cinerea* was capable of producing ethylene. It has now been shown that *B. cinerea* is capable of producing ethylene under defined conditions. Of four potential precursors of ethylene tested (methionine, ACC, glutamate and α-ketoglutarate) methionine was found to be the most efficient precursor under both static and shake culture conditions. Although *B. cinerea* is capable of producing ethylene *in vitro* this research was not able to determine whether *B. cinerea* did in fact produce ethylene *in vivo*.

Although methionine was found to be the most efficient precursor of ethylene, the pathway of ethylene production in *B. cinerea* is not at all clear. Ethylene was not produced when ACC was added to the incubation medium containing methionine yet addition of AOA resulted in an inhibition of ethylene production.

As AOA is known to inhibit PLP mediated enzymes including a number of aminotransferases, then it is likely that it may inhibit methionine amino transferase which is thought to be involved in metabolising methionine to KMBA (Fukuda et al, 1993). If this is so then it is possible that KMBA could be an immediate precursor of ethylene in *B. cinerea*. This could require the presence of methionine aminotransferase and hydroxyl free radicals, neither of which have been shown to occur in *B. cinerea*. It would be of interest to provide KMBA to *B. cinerea* cultures *in vitro*, with appropriate cofactors, to ascertain whether the pathway proposed by Ogawa et al (1990) for ethylene production in microbes, does in fact occur in *B. cinerea*.

The increased ethylene production in plant tissue as a result of pathogen attack is a common phenomenon but only a few detailed experiments have been carried out to determine which tissue of the infected plant produces more ethylene. The present detailed study of *B. cinerea* infected kiwifruit, allows a reasonably confident conclusion that the increased ethylene production is mainly confined to few mm area ahead of the infection front. Perhaps increased ethylene production caused by pathogen attack of fruit in general is produced mainly by cells on the periphery of the infection front. If this zone is beyond tissue that contains actively growing hyphae, then this implies the existence of a transmissible signal which is capable of inducing ethylene in uninfected tissue. The nature of this signal is unknown in *B. cinerea*. However, specific proteins and glycoproteins have

been isolated in certain host/ pathogen combinations and oligosaccharins arising from pathogen induced breakdown of cell walls have been shown to act as mobile elicitors of ethylene production in some systems. It is probable that a similar phenomenon is occurring in kiwifruit infected with *B. cinerea*. Further research will be necessary to establish whether breakdown products of cell walls from *B. cinerea* infected kiwifruit can indeed act as oligosaccharins and hence as ethylene elicitors.

It is not known why ethylene is not produced in kiwifruit tissue infected with *B. cinerea*, even when there seems to be enough ACC and ACC oxidase activity present. It remains to be determined if this is due to inhibition of endogenous ACC oxidase arising from a breakdown of cellular compartmentalization from the breakdown of cell membranes caused by the infection or presence of specific EFE inhibitors.

While ethylene is known to regulate a number of physiological reactions in plants, its role in the process of infection by postharvest pathogens is still far from clear. The knowledge that *B. cinerea* is capable of producing ethylene *in vitro*, given the appropriate precursors and cofactors is important new knowledge. This should encourage further research in kiwifruit to determine the contribution to ethylene production either made by the pathogen directly or made indirectly as a secondary response in host tissue to physiological changes induced by the fungus. The fact that enhanced ethylene production occurred at or ahead of the infection front, improves understanding of the host-pathogen interaction between *B. cinerea* and kiwifruit, and indicate that further research is required to elaborate the nature of the transmissible ethylene signal postulated to occur in infected kiwifruit.

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Appendix-1

AREA UNDER THE CURVE

A number of experiments reported in chapter 3 involved several treatments and measurements of ethylene production at regular intervals for the period of incubation. Ethylene was measured in the headspace of *B. cinerea* cultures after enclosing cultures for 1 hour.

Presentation of the time course of ethylene production from all the treatments on any one graph made interpretation of results difficult, and daily peak height did not necessarily reflect the total ethylene production by the cultures during incubation. In an attempt to present the result in a clear manner, with one value representing the response for a given treatment, allowing easier comparison between treatments, area under the response curve was calculated by using the following formulae:

$$\frac{C_2H_4 \text{ production on day 1 + day 2}}{2} + \frac{C_2H_4 \text{ production on day 2 + day 3}}{2} \ldots.$$

For example:

Days	C_2H_4 Prodn.	Area under the curve
1	0.012	
		0.063
2	0.114	
		0.187
3	0.260	
		0.484
4	0.708	
		0.568
5	0.426	
		0.394
6	0.360	
		0.299
7	0.238	
		0.180
8	0.122	
Total C_2H_4 production=		2.175

APPENDIX-2

Calculation of ethylene production for intact kiwifruit/ slices or outer pericarp was calculated using the formula:

$$\text{prodn.} = \frac{\{\text{volume of a jar(ml)} - \text{volume of the fruit (ml)}\} * C_2H_4 \ \mu l l^{-1}}{\text{Fruit weight (grams)} * \text{hours}}$$

($\mu l/Kg/h$)

Calculation of nmol. of ethylene/ACC was calculated by using following formula:

$$\frac{C_2H_4\ (\mu l\ l^{-1}) \times \text{Free volume (L)} \times \text{ethylene density g/l at 0°C (1.26)} \times 1000}{\text{Mol. wt of ethylene (28.05)}}$$

Or

$$\frac{C_2H_4\ (\mu l\ l^{-1}) \times \text{Free volume (L)} \times P\ (101325\ Pa) \times (10^{-9})\ M^3\ \mu l\ l^{-1}}{R\ (8.3143) \times \text{Temperature (273.15) Kelvin}}$$

Calculation of titrable acidity was calculated as %(w/v) of citric acid in juice is as follow:

$$\%\ \text{citric acid} = \frac{\text{ml NaOH} * N(0.1) * 64}{1\ \text{ml juice} * 10}$$

Where 64 = molecular weight of citric acid divided by 3