# HOST RESPONSES TO INFECTION IN *FUSARIUM* WILT OF TOMATO

by

Eng Cheng Siong

B.Sc. (Hons) London

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Department of Pure & Applied Biology

Imperial College

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*Abstract*

The responses of near-isogenic lines of tomato cultivars (with regard to the recognition signals which determine resistance or susceptibility) to infection by *Fusarium oxysporum* f.sp. *lycopersici* havebeen investigated.

Experiments on host specificity showed that there was no expression of major gene resistance in parenchymatous tissue and no elicitors of resistance were detected in spore inoculum or culture filtrate.

Following root inoculation, host colonisation was very restricted in resistant isolines compared with susceptible isolines. The quantitative distribution of the pathogen in host tissues was ascertained using Scanning Electron Microscopy and by estimating propagule numbers in tissue homogenates by plating onto selective media. The mode of infection and initial colonisation was similar in young seedlings of both isolines. Root inoculation of hosts was a more efficient technique than stem inoculation.

Elevated levels of the polysaccharidase β -1,3-glucanase were found in infected tissue compared with healthy controls. This increase was progressive with time and was greater in susceptible than in resistant tissue. Enzyme activity and mycelial colonisation were positively correlated for both resistant and susceptible isolines. Isolated petioles treated with culture filtrate or spore inoculum showed an increase in enzyme levels although the response was similar in both resistant and susceptible near-isolines.

Studies on grafted plants showed that resistance or susceptibility was not transferred from the stock to the scion, or vice versa, and that resistance was a feature of both stem and root. Grafts with susceptible stock and resistant scion were the most damaged by infection and scions reacted hypersensitively to "toxins" produced in susceptible stocks.

Four antifungal compounds were extracted with chloroform from the stem and root of infected plants, of which three, rishitin, falcarindiol and falcarinol have been tentatively identified. Phytoalexins were more readily detected in susceptible than resistant tissue.

The implications of these results in relation to the resistance mechanisms operating in wilt-diseased tomatoes are discussed.

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**Abbreviations**

| | |
|---|---|
| AC | Ailsa Craig |
| BB | Bonny Best |
| °C | degree Centigrade |
| CSH | casein sucrose hydrolysate |
| cm | centimetre |
| CMA | corn meal agar |
| *r* | correlation coefficient |
| CN | cotyledonary node |
| CR | Craigella resistant |
| CS | Craigella susceptible |
| cv(s) | cultivar(s) |
| DF | degrees of freedom |
| EDTA | Ethylenediaminetetra-acetic acid |
| f.sp. | forma specialis |
| Fig | Figure |
| *Fol* | *Fusarium oxysporum* f.sp. *lycopersici* |
| g | gram |
| *g* | unit of gravitational field |
| h | hour |
| HS | Homestead |
| HC | hypocotyl |
| kV | kilovolt |
| LSD | least significant difference |

| | |
|---|---|
| l | litre |
| μ m | micron |
| μ g | microgram |
| μ l | microlitre |
| mA | milliamp |
| mg | milligram |
| ml | millilitre |
| mm | millimetre |
| min | minute |
| MM | Moneymaker |
| N | node |
| no(s) | number(s) |
| % | percentage |
| PDA | potato dextrose agar |
| P | Probability level |
| r.h. | relative humidity |
| RT | root |
| RI | root-inoculated |
| RSI | root-shoot inoculated |
| SEM | Scanning Electron Microscopy |
| SED | standard error of difference |
| TLC | thin-layer chromatography |
| UV | ultra violet light |
| v/v | volume/volume |
| WA | Walter |

| | |
|---|---|
| W | Watt |
| w/v | weight/volume |

## 1. INTRODUCTION

In most plant diseases, the interaction between the pathogen and its host is genetically predetermined. These interactions render the host susceptible or resistant to the pathogen, the eventual outcome being moderated by external influences such as weather conditions and host nutrition.

It is a fundamental observation in plant pathology that pathogens exhibit a degree of selectivity in the hosts that they can colonise, and hosts are correspondingly selective in the pathogens that they can "accept". Thus specificity is an integral part of all host-parasite relationships and may take a variety of forms. Host specificity may operate at the species, genus or other taxonomic level and is characteristic for any one particular pathogen. Host specificity is manifest as resistance or susceptibility of individual plants : its operation at the sub-species taxonomic level is of great practical importance in agriculture.

Some pathogens are restricted to parts of their hosts, viz. organ specificity. Examples include various blights restricted to leaves and/or flowers, *Synchytrium endobioticum*, exclusively a pathogen of stems, and *Rhizobia*, exclusively found on roots. Even more locally, specificity may be found at the tissue level, e.g. the restriction of powdery mildews to epidermis and vascular wilt pathogens to the water conducting tissues of vascular plants. A further example is age specificity which describes the situation whereby certain pathogens have a predilection for seedlings, e.g. damping off fungi (*Pythium* species), whereas others are restricted to senescent tissues, e.g. fruit rots (*Monilinia fructicola*) and heart rots of trees (*Armillaria mellea*).

### 1.1. TISSUE SPECIFICITY

As already mentioned, many pathogens exhibit organ or tissue specificity, i.e. they show electivity or selectivity towards specific host organs or tissues (Graniti, 1976). Many parasites are adapted to a preferred habitat within the plant where they depend on the host for nutrition as well as refuge from adverse conditions.

The specific habitat of wilt pathogens is the vascular system (Beckman, 1964; Dimond, 1970, 1972). Although infection eventually progresses to other parts of the plant, restriction to the vascular elements forms the essential part of the parasitic life of the wilt pathogen (Dimond,

1970). The xylem vessel offers the pathogen an environment of least resistance to its subsequent growth and systemic distribution. The nutritional requirements are fulfilled as the pathogen uses the low concentration of nutrients in the xylem sap and degraded wall components (Singh & Smalley, 1969; Dimond, 1970, 1972). The pathogenicity of wilt pathogens is fully expressed as a result of their electivity for vascular elements.

## 1.2. RACE-CULTIVAR SPECIFICITY

Brian (1976) proposed five levels of specificity. The two levels most pertinent here are 1) specificity of pathogens restricted to a single host species or a few closely related species (e.g. rusts, powdery mildews) and 2) specificity of formae speciales or races of pathogens which are restricted to a few genotypes of a host species (which may operate in a gene-for-gene host-parasite relationship). These two types of host range specificity were also recognised by Heath (1981) viz. 1) plant species specificity (which conditions host species range) and 2) cultivar specificity (which conditions the host cultivar range). According to Heath's model (1981), a parasite that can successfully cause infection in a plant species by a variety of morphological and behavioural adaptations and by suppressing or negating the defence mechanism is basically compatible with the host. The race-cultivar level of specificity is developed only after basic compatibility is established. In cultivar resistance, a gene-for-gene relationship between plant and pathogen is generally established.

## 1.3. THE GENE-FOR-GENE CONCEPT

In the study of the inheritance of disease resistance in flax to the rust pathogen (*Melampsora lini*), Flor (1955) concluded that for every gene for resistance in the host, there is a corresponding gene for avirulence in the pathogen and that expression of resistance (incompatibility) requires an interaction between the two genes. The genes for resistance and avirulence are inherited as dominant characters.

This gene-for-gene hypothesis is used to explain the genetic basis of host specificity. Implicit in the hypothesis is the concept that resistance is the active function, which is triggered by a recognition mechanism involving both host and parasite. Susceptibility may be regarded as a passive (non-induced) response due to the absence of resistance genes or their non-activation

(Wheeler, 1975).

The gene-for-gene hypothesis is applicable to most host-parasite systems in which resistance is conditioned by vertical (*sensu* Vanderplank, 1968) resistance genes (Flor, 1971). It assumes that an element of basic compatibility already exists between the host and parasite species.

Examples of host-parasite systems in which the gene-for-gene relationship has been implicated (Flor, 1971) include : Linum-*Melampsora lini* (Flor, 1955, 1971); Triticum-*Puccinia graminis tritici* (Green, 1966; Loegering & Powers, 1962); and Hordeum-*Erysiphe graminis* (Moseman, 1957).

Host-parasite systems that exhibit reciprocal cultivar-race characteristics are assumed to have a gene-for-gene relationship even if it has not been genetically proven (Keen, 1982a).

## 1.4. RECOGNITION IN GENE-FOR-GENE HOST-PARASITE SYSTEMS

Specificity is exhibited very early during infection (Sequeira, 1978) and is determined by both host and pathogen characters. Specificity is dependent on recognition, which may be established as early as the moment of contact between the host cell plasmalemma and the pathogen cell wall (Sequeira, 1980). A "determinative phase" of resistance occurs where there is an initial recognition of the pathogen by plant cells (Talboys, 1957) and which subsequently leads to the "expressive phase" where host cells undergo biochemical changes (e.g. the hypersensitive reaction) which leads to suppression of the parasite (Keen, 1982b). The recognition of surface-located pathogen products (e.g. glycoproteins and polysaccharides) by receptor molecules on host cell walls, is postulated to determine resistance or susceptibility in the host-parasite interaction.

In an incompatible reaction, hypersensitivity is initiated by recognition of the invading pathogen and controlled by race-specific receptor cells in the host (Keen, 1982a). As the hypersensitive reaction is controlled by dominant resistance genes, specificity in host-parasite systems may be controlled by the ability of the pathogen to induce or suppress the hypersensitive reaction. The relationship between the hypersensitive reaction and the induction of phytoalexin accumulation will be discussed later (section 1.7.3.1).

Of the postulated macromolecules involved in the recognition phenomenon, lectins have received the most study (Sequeira, 1978, 1980). These are proteins capable of binding to glycoproteins or polysaccharides free in solution or on cell surfaces through recognition of specific sugar structures (Sequeira, 1978). Even if lectins prove not to be directly involved, molecules of similar behaviour characteristics must be involved in host-parasite signalling. Fungal surface carbohydrates, especially glucans have been suggested to be specific recognition elements because of their high biological activity (Keen, 1982a; Daly, 1984).

Heath (1981) and Bushnell & Rowell (1981) have suggested models in which host-parasite specificity at both species and race-cultivar levels, is apparently controlled by the suppression of the host's defence mechanisms. In an incompatible reaction, the suppressor is unable to attach to the plant's receptor, allowing binding of the elicitor to the receptor which then initiates the defence response (e.g. phytoalexin accumulation). In a compatible reaction, the suppressor is attached to the plant's receptor, negating the defence mechanisms. Thus, the apparently conflicting requirements of non-specific and specific triggers (elicitors) of defence mechanisms and of suppressors of resistance could be reconciled. There is evidence that tomato can respond to non-specific elicitors (section 1.7.3.3) and that other members of the Solanaceae are responsive to resistance suppressors.

In contrast to the voluminous literature (Albersheim *et al*, 1981; Keen, 1982a, 1985; Daly, 1984) on non-specific elicitors, elicitation that is race- and cultivar-specific has remained largely a theoretical possibility. Reports of the production of specific elicitor by pathogens *in vitro* have not been substantiated : Keen (1975) and Bruegger & Keen (1979) detected factors from *Phytophthora megasperma* var. *sojae (glycinea)* and *Pseudomonas syringae* pv. *glycinea* respectively, that appeared to be specific elicitors of glyceollin in soybean; but the evidence was not strong : Wade & Albersheim (1979) detected glycoproteins from *P. megasperma* var. *sojae* that protected soybean from attack by compatible races of the pathogen despite being poor elicitors of phytoalexin accumulation. These experiments were characterised by poor reproducibility of the bioassay and did not survive further scrutiny (Desjardins *et al*, 1982). Keen & Legrand (1980) have indicated that surface glycoproteins from cell walls of *P. megasperma* var. *glycinea* may function as race-specific elicitors of glyceollin.

The most satisfactory report of the existence of specific elicitors is that of de Wit & Spikman (1982) and subsequent papers (de Wit *et al*, 1984, 1985). This group have isolated from *Cladosporium fulvum* (syn. *Fulvia fulva*) race- and cultivar-specific elicitors of necrosis and chlorosis that are postulated to be the primary products of avirulence genes and which probably act by triggering the hypersensitive reaction. This work will probably be seen in the future as constituting a landmark in plant pathology and is especially pertinent to the present study since it indicates that tomato has the ability to discriminate between the avirulence determinants of different races of a fungal pathogen. The technique employs the detection of diffusible elicitors produced by intercellular pathogens. By the same argument one would expect vascular pathogens also to produce water-soluble diffusible elicitors.

## 1.5. AIMS OF THE THESIS

The original aim of this research was to seek specific elicitors using races of *Fusarium oxysporum* f.sp. *lycopersici* and near-isogenic lines of tomato, to obtain a better understanding of the recognition signals that determine resistance or susceptibility to *Fusarium* wilt disease. These hopes have remained largely unrealised, but the opportunity has been taken of investigating a number of separate but related approaches to the question of resistance triggering in tomato *Fusarium* wilt and the mode of suppression of the pathogen. Each approach constitutes a separate chapter of the ensuing text.

## 1.6. LITERATURE REVIEW

### 1.6.1. VASCULAR WILT DISEASES

Since the earliest reports of vascular diseases in the 1880's, the subject has been one of intensive study. Vascular wilt diseases caused by fungi are of major economic importance whether they occur in cultivated crops or in indigenous species. Examples of crops which have suffered severe losses as a result of wilt diseases are cotton by both *Fusarium oxysporum* f.sp. *vasinfectum* and *Verticillium dahliae*, and banana (Panama disease), caused by *F.oxysporum* f.sp. *cubense*. Among non-cultivated species, massive losses to elms have resulted from the highly destructive Dutch elm disease caused by *Ceratocystis (Ophiostoma) ulmi* and to oaks by oak wilt

caused by *C. fagacearum.*

Species of *Fusarium* and *Verticillium* are the most common causes of wilt diseases in higher plants. These include wilt infections of a large number of economically important agricultural and horticultural crops e.g. tomato, cabbage, pea, flax, banana, cotton, muskmelon, carnation, chrysanthemum, gladiolus, and tulip and trees e.g. date palm and oil palm.

Toussoun & Nelson (1975) in their review of *Fusarium* taxonomy state that all modern systems of classification are derived from that of Wollenweber & Reinking (1935). Snyder & Hansen (1940) revised and simplified the taxonomic system in Wollenweber and Reinking's section Elegans, wherein all species of *Fusarium* causing vascular wilts were reduced to a single species *Fusarium oxysporum.* Snyder & Hansen (1940) recognised 25 formae speciales of *F. oxysporum* but the number was expanded to 69 by Armstrong & Armstrong (1968). Booth (1971) listed over 70 formae speciales of *F. oxysporum.* The existence of the many host-specific formae speciales provides *F. oxysporum* with its extensive pathogenic specialisation, making it unique among the vascular pathogens.

Fungal wilt pathogens have a characteristic mode of growth in that directly after penetration, they enter the vascular system of the host and remain in the conductive tissue of the host xylem elements until the disease is well-advanced (Dimond, 1970). The pathogen thrives in this micro-niche using as substrates cellulosic and pectinaceous substance exposed along vessel walls as well as the nutritionally-dilute vascular fluids for growth and sporulation (Wood, 1961; Singh & Smalley, 1969; Dimond, 1970). As infection progresses, familiar wilt symptoms such as foliar chlorosis, leaf epinasty, wilting, stunting, necrosis and defoliation occur (Dimond, 1955). In heavily diseased hosts, death is usually inevitable.

*Fusarium* wilt of tomato has been popularly used in the past as a model for the study of vascular wilt disease as well as in the study of whole plant physiology (Dimond & Waggoner, 1953b; Scheffer & Walker, 1953; Waggoner & Dimond, 1954; Walker, 1971).

### 1.6.2. THE PATHOGEN

*Fusarium oxysporum* f.sp. *lycopersici* (abbreviated henceforth as *Fol*) is a soil-borne pathogen that causes *Fusarium* wilt disease of tomato. This disease was first described by

Massee in 1895 and is of economic importance world-wide. *Fusarium* wilt is most destructive in warm climates, and in acid, sandy soils of temperate regions. In Europe, many cultivars that are susceptible to race 1 (race 0, Gabe, 1975) of the pathogen are still grown because of their select flavours, resulting in the occurrence of wilt in both glasshouse and field-grown tomatoes (Snyder & Smith, 1981).

*Fol* produces three type of asexual spores : microconidia, macroconidia and chlamydospores. The pathogen overwinters as thick-walled intercalary or terminal chlamydospores for long periodsin the absence of a suitable host plant (Agrios,1978). *Fol* is a facultative saprophyte and grows on a variety of synthetic media (Walker, 1971). Growth and sporulation of *Fol* is dependent on a variety of micronutrients (Woltz & Jones, 1968).

### 1.6.3. WILT TOXINS

Among the wilt toxins produced by *Fol*, one of the earliest substances to be implicated in disease was lycomarasmin (Gäumann, 1951). Lycomarasmin is produced in culture only and has not been extracted from diseased hosts. Hence its involvement in disease is controversial. Lycomarasmin has also been isolated from other formae speciales of *Fusarium oxysporum* apart from f.sp. *lycopersici*, namely f.sp. *melonis* and f.sp. *vasinfectum* (Kern, 1972). Tomato shoots treated with pure lycomarasmin exhibit an upward rolling of leaflets, followed by interveinal necrosis, wilting and desiccation (Scheffer & Walker, 1953). Lycomarasmin forms a chelate complex with iron that contributes to its toxicity in host plants. The effects of lycomarasmin have also been thoroughly investigated by Scheffer & Walker (1953) and Dimond & Waggoner (1953a).

Another toxin, fusaric acid, is also produced in pure culture (Gäumann, 1957) but unlike lycomarasmin, has been detected *in vivo* in tomato plants (Davis, 1969). Other formae speciales of *F. oxysporum* also produce fusaric acid and it has been detected in infected banana (Page, 1959), cabbage, flax, watermelon and carnation (Davis, 1969). Davis (1969) reported a possible correlation between the pathogenicity of *F. oxysporum* f.sp. *niveum* and the amount of fusaric acid produced in diseased watermelon. Fusaric acid is not always detected in diseased hosts possibly due to detoxification and metabolism (Gäumann, 1957; Kern, 1972). In higher

plants, it causes necrotic spots on the leaf blade, withering of leaves and desiccation in interveinal areas of leaf tissues (Kern, 1972). Studies on the toxicity of both lycomarasmin and fusaric acid have been extensively reviewed by Walker (1971). In view of the sometimes conflicting evidence, the significance of fusaric acid in pathogenesis has yet to be clarified (Kern, 1972).

### 1.6.4. INFECTION, COLONISATION AND SYMPTOM DEVELOPMENT

The mode of entry of *Fol* is probably through wounds in the root tissue or through intact roots (Snyder *et al*, 1946). The pathogen commonly penetrates between epidermal root tip cells or through intercellular material between root cap cells (Bishop & Cooper, 1983a), or at the point of origin of lateral roots of both resistant and susceptible varieties (Hutson & Smith, 1983). After penetration, the pathogen moves inter- and intra-cellularly through parenchymatous tissue and the primary meristem and invades the tracheal vessels of the protostele. *Fol* does not enter vessel elements through mature stelar cells (Bishop & Cooper, 1983a). The pathogen has apparently similar potential for penetrating xylem vessels of both resistant and susceptible host cultivars (Bishop & Cooper, 1983a).

In susceptible tomato cultivars, hyphae of *Fol* are confined, in the early stages of infection, to the protoxylem and metaxylem vessels in the lower nodal regions (Chambers & Corden, 1963; Mace *et al*, 1971). Lateral spread of the pathogen through vessel pits causes invasion of the secondary vessel elements. Secondary colonisation and vertical spread of the pathogen are attained when spore trappings at perforation plates and end walls allow hyphae to germinate through and grow past these barriers into adjoining vessel elements (Elgersma *et al*, 1972). *Fol* causes extensive degradation of vessel walls in areas in direct contact with hyphae as well as in areas where hyphae are apparently absent (Bishop & Cooper, 1983b). Fungal hyphae and vessel walls are often coated in an amorphous electron-opaque material. Invasion of parenchymatous tissue by *Fol* from vessel elements does not occur until host tissues are moribund (Walker, 1971).

In resistant tomato cultivars, *Fol* is restricted to vascular elements of the root and lower stem (Scheffer & Walker, 1954; Elgersma *et al*, 1972). Large amounts of electron-opaque

material accumulates in the colonised root endodermal cells as well as in vacuoles of xylem parenchyma and in tyloses (Bishop & Cooper, 1984). The vessels in the hypocotyl and lower nodes are occluded by tyloses and gels.

Symptoms of *Fusarium* wilt become apparent 48 h after root inoculation with vein-clearing and petiole epinasty (Dimond, 1955). More conspicuous symptoms appear two to four weeks after inoculation with yellowing of the lower leaves, irreversible wilting, the occas ional formation of adventitious roots, marginal leaf necrosis and finally death (Dimond, 1972). The most prominent internal symptom is vascular browning. Vascular browning appears a few mm in advance of the fungal mycelium, extending from the root up to the stem (Walker, 1971). Browning appears first in the xylem parenchyma and results from the oxidation and polymerization of dihydroxy-phenols (Davis *et al*, 1953; Matta & Dimond, 1963; Mace *et al*, 1972). These melanoid pigments thus formed are later released into the xylem, and may contribute to vascular occlusion (Corden & Chambers, 1966).

Many factors enhance the development of Fusarium wilt. These include high soil and air temperatures (c. 28 °C), low soil moisture, pre-conditioning of plants with low nitrogen and phosphorus and high potassium, low soil pH, short day length and low light intensity (Jones & Woltz, 1981).

### 1.6.5. BREEDING OF RESISTANT CULTIVARS

Before 1940, the most effective resistance to *Fusarium* wilt was the use of polygenically tolerant tomato cultivars, such as Marglobe and Rutgers. These possessed a broad field resistance which was influenced by environmental conditions. In a search for high levels of resistance to tomato wilt, Bohn & Tucker (1940) found a "field immunity" level of resistance in Missouri Accession 160, a red currant species of tomato (*Lycopersicon pimpinellifolium* Miller). This resistance to race 1 of the pathogen was controlled by a single dominant allele of the *I* gene. The first cultivar with this *I* gene resistance was Pan America, which was released in 1941. Many new cultivars featuring this *I* gene have been released since then (Walter, 1967). In 1945, however, a new isolate of *Fol* was discovered that was pathogenic to cultivars bearing *I* gene resistance (Alexander & Tucker, 1945) and which was subsequently named race 2 by

Gerdeman & Finley (1951). Resistance to this new race 2 pathogen was discovered by Stall & Walter (1965) in Accession P.I. 126915, a hybrid between *L. pimpinellifolium* and *L. esculentum*. The gene that was responsible for resistance to race 1 was renamed $I_1$ and that for resistance to race 2 as $I_2$ by Cirulli & Alexander (1966). Stall & Walter (1965) selected the single gene $I_2$, and transferred it to commercial cultivars in which it conferred resistance to race 2 e.g. cv. Walter which has resistance to both races 1 and 2.

Three races of the pathogen are now recognised (Booth, 1975). Race 2 of *Fol* has become widely distributed in areas of tomato cultivation (Snyder & Smith, 1981). Race 3 of *Fol* (Alexander & Hoover, 1955), although overcoming both $I_1$ and $I_2$ genes is apparently rare.

Gabe (1975) suggested that the pathogen virulences should be reclassified as races 0, 1 and 2 to conform with the gene-for-gene terminology (Flor, 1955), a proposal followed hitherto in this thesis. Pathogen race 0 is virulent to cultivars with no resistance genes and those possessing the $I_1$ gene are resistant to race 0 but overcome by race 1.

During the 1960's and 1970's, plant breeders at the Glasshouse Crops Research Institute, at Littlehampton, UK, incorporated the $I_1$ gene into a number of their breeding programmes to give a series of paired near-isogenic lines (Harries, 1965). One of pairs based on Craigella has been used previously in studies on disease resistance (e.g. Tjamos & Smith, 1975; Pegus, 1976 and Hutson, 1978).

### 1.6.6. LOCALISATION OF RESISTANCE

Resistance mechanisms operating in tomato may inhibit any one of a number of stages of pathogenesis, e.g. germination of the pathogen on host surfaces, penetration of the pathogen through the root cortex, growth of the pathogen in the vascular system, escape of the pathogen from vessels to adjoining stem and leaf tissues (Bell & Mace, 1981). Thus, the pathogen confronts different types of host cells and tissues which may exert different degrees of resistance.

The location of resistance expression in tomato has in the past been disputed. In the grafting experiments of Heinze & Andrus (1945), observations on symptom development of the reciprocally grafted plants led the authors to conclude that resistance was localised in the root

system of resistant cultivars. Others (Scheffer, 1957; Keyworth, 1963), also using observations from grafting experiments claimed that resistance was a characteristic of all parts of the resistant plant.

Pegus (1976) reported no differential resistance of cultivars at the penetration stage, *Fol* entering undamaged roots of resistant and susceptible cultivars alike. However, differential resistance of cultivars has been expressed in the vascular development phase e.g. when fungal propagules are taken through severed roots (Elgersma *et al*, 1972) and in stem cuttings (Mace & Veech, 1971). In resistant cuttings, secondary colonisation by the pathogen in the vascular elements is restricted with a slower rate of disease development and a greater discontinuity of the pathogen within the vessels. Resistance which is expressed in many non-host plants can protect against direct penetration by *Fusarium* (Katan, 1971) and *Verticillium* (Levy & Isaac, 1976). Among host species, resistance can protect against infection by avirulent formae speciales (Langton, 1969).

## 1.7. MECHANISMS OF RESISTANCE TO VASCULAR WILT DISEASES

### 1.7.1. PRE-FORMED ANTIMICROBIAL SUBSTANCES

Constitutive antibiotics produced by plants have frequently been implicated as resistance mechanisms because of their toxicity to fungi. These include alkaloids which are synthesized and stored in vacuoles in the cytoplasm (Roddick, 1976). The possible presence of constitutive antibiotics has been demonstrated in the young, upper stems of tomato which were more toxic to *Fol* than the lower, older part of the stem (Mace & Veech, 1971).

#### 1.7.1.1. α-TOMATINE

Irving *et al* (1945) isolated a glycoalkaloid from healthy tomato plants which was subsequently characterised and named α-tomatine and shown to inhibit *Fol in vivo* (Irving *et al*, 1946; Fontaine *et al*, 1948).

α-Tomatine, found in *Lycopersicon* and *Solanum* species (Roddick, 1974), is toxic to a broad range of fungi. However, tomato pathogens are less sensitive to α-tomatine than are most fungi (Arneson & Durbin, 1968). The relationship between tomatine and disease

resistance is unclear. Kern (1952) discounted its importance in resistance to *Fol* on the basis that the fungus primarily colonises roots and xylem cells which are almost devoid of tomatine.

Tomatine has been shown to increase similarly in resistant and susceptible tomato plants infected with *Fusarium* (Langcake *et al*, 1972; McCance & Drysdale, 1975), again suggesting that it is not involved in resistance to *Fusarium* wilt. McCance & Drysdale (1975) reported tomatine in extracts of xylem tissue but not in guttation fluid from severed stems. Tomato cultivars possessing the *I* gene for resistance to *Fol* may have a greater α-tomatine content than susceptible cultivars, as tomatine levels increased most rapidly in wounded resistant roots (Hammerschlag & Mace, 1975).

*Fol* is capable of detoxifying α-tomatine by an extracellular enzyme, cleaving it to a tetrasaccharide, lycotetraose, and the less toxic alkaloid, tomatidine (Ford *et al*, 1977).

### 1.7.1.2. PLANT GLYCOSIDASES

Plant glycosidases, particularly, β-1,3-glucanase and chitinase have been suggested by a number of workers (Netzer *et al*, 1979; Pegg & Young, 1981) to play a role in resistance against fungal pathogens by degrading their cell walls. These enzymes degrade glucan and chitin, major components of fungal cell walls. The lysis of *Verticillium albo-atrum* observed in tomato may be due to autolysis (Dixon & Pegg, 1969) or the action of tomato chitinase (Vessey & Pegg, 1973). Glucanases and chitinases occur in healthy plants (Abeles *et al*, 1970) and their concentrations often increase following infection (Pegg, 1976; Netzer *et al*, 1979). Increased levels of chitinase and β-1,3-glucanase were greater in susceptible tomato cultivars infected with *V. albo-atrum* than in resistant (Pegg & Vessey, 1973; Pegg & Young, 1981). However, in *Fusarium*-infected muskmelon, β-1,3-glucanase increased more rapidly in the resistant than in the susceptible cultivar (Netzer *et al*, 1979). An inverse relationship between host glycosidase activity and *V. albo-atrum* mycelial colonisation in susceptible and resistant hosts has been demonstrated by Pegg & Young (1981). Enzyme extracts from infected stems were found to be more active on purified *Verticillium* cell walls than were those from healthy stems. Mixed enzyme digests including tomato endo-1,3-β-glucanase in combination with fungal exo-1,3-glucanase or β-glucosidase have demonstrated greater activity on fungal cell walls than enzymes

applied individually (Young & Pegg, 1982).

### 1.7.2. RESISTANCE INVOLVING PHYSICAL BARRIERS

#### 1.7.2.1. DEPOSITION OF LIGNIN AND SUBERIN

Structural features in the root often contribute to disease resistance by reducing the direct entry of fungal pathogens. These features include the formation of callose, lignin or suberin in the epidermis, cortex or endodermis.

Lignin or lignin-like materials are deposited in the cell walls of hops infected by *Verticillium albo-atrum* (Talboys, 1958a) and in tomato roots infected with *Verticillium* species (Griffiths & Isaac, 1966). Lignitubers formed within root hairs of hosts infected by *Verticillium* and *Fusarium* species were reported by Griffiths & Lim (1964). The deposition of lignin in cell walls as a response to infection is well-documented and is associated with limited or retarded invasion (Talboys, 1958a; Griffiths & Isaac, 1966; Ride, 1975). Callose deposition has been reported in paravascular parenchyma cells of tomato after inoculation with *Fol* or the root flora (Beckman *et al*, 1982). Callose deposition was more rapid in resistant than susceptible cells as a result of *Fol* infection, and is interpreted as being the response of host cells to infection and physical damage. The formation of host cell wall appositions and papillae, acting as physical barriers to hyphae invading the extra-vascular root tissue has been reported by Bishop & Cooper (1983a).

Suberinization of the endodermis is a normal process occurring in the mature parts of the roots of vascular plants. This process is hastened in the event of infection or physical damage. Suberinization in the endodermis of hop roots occurred as a result of infection by *V. albo-atrum* (Talboys, 1958a).

Extra-vascular mechanisms contribute to resistance by reducing the incidence and intensity of colonisation. This is more apparent at the species than the cultivar level of resistance (Talboys, 1958a).

### 1.7.2.2. VASCULAR OCCLUSION BY GELS, GUMS AND TYLOSES

Colonisation of the vascular system by the pathogen is largely dependent on spore transport within the xylem vessels. The mechanism of resistance to vascular wilts has been suggested by Beckman (1966, 1968) to depend primarily on the physical restriction of the pathogen in resistant hosts by vascular occlusion, thus limiting the build-up and distribution of fungal propagules. In susceptible hosts, it is the secondary colonisation of vessels which results in the systemic spread of the pathogen. This localisation mechanism has been studied in tomato by Beckman (1968), Beckman *et al* (1972) and Elgersma *et al* (1972).

Vascular occlusion is a general phenomenon in which plants respond to damage by sealing-off infections in the vascular system. Gels and gum plugs have been reported in various plant species following vascular infections (Beckman, 1964; Vandermolen *et al*, 1977). Gels originate from the swelling of perforation plates, end walls of vessels and pit membranes and are formed as a result of the distention of primary cell walls and the middle lamella (Beckman & Zaroogian, 1967; Beckman, 1969b; Vandermolen *et al*, 1977). Vascular gels are composed mainly of pectinaceous and hemicellulosic materials (Beckman & Zaroogian, 1967) and constitute a highly persistent membrane structure (Beckman, 1969a).

Tylose formation, as a response to injury, infection or ageing is a normal plant phenomenon. Tyloses arise from the distention of pit membranes between vessels and adjacent xylem parenchyma or ray cells as balloon-like structures. The two processes of gelation and tylosis involving the plasticizing of cell walls appear to be closely similar (Beckman, 1971).

The sealing-off process by gels and tyloses has been studied in detail in banana infected with *Fusarium oxysporum* f.sp. *cubense* (Beckman *et al*, 1962). Three types of physical barriers are involved : firstly, fungal microspores become trapped at perforation plates and end walls. *Fusarium* spores germinate and penetrate the perforation plate and sporulate within 2 - 3 days after inoculation. Next, gels are formed on the upper side of the trapping site. The third barrier, tyloses are last to be initiated, through pit membranes. Tyloses increase in size and number and eventually permanently occlude the vessel above the site of infection at 5 days after inoculation. Vascular occlusion is commonly followed by an infusion of phenols into gels and

tyloses, which apparently strengthens their structure. In susceptible hosts, the fungus is trapped at end walls and perforation plates, but grows through and resporulates at each trapping site, becoming systemic in the process. Gels and tyloses are initiated normally but complete vascular occlusion is delayed until 10 - 12 days after inoculation. When subject to the pressure of the accumulated spores the gels shear, distributing metabolites and spores further along the vessel length.

Talboys (1958b) concluded that the distribution of *Verticillium albo-atrum* in resistant hop cultivars was effectively blocked by tyloses. Dixon & Pegg (1969), however, have questioned the importance of tyloses as a primary resistance factor. They found no significant differences in tylose formation between resistant and susceptible tomato cultivars infected with *V. albo-atrum*.

Beckman *et al* (1972) suggested that the sealing-off of *Fol* infection by rapid tylose development was essential in restricting the pathogen in resistant tomato cultivars, and forms the basis for monogenic resistance to wilt. Tylose development in the susceptible isoline although initiated at the same time, was retarded until two days after inoculation. Beckman *et al* (1972) suggested that both near-isolines possessed the same potential for tylose formation but that *Fol* produced a growth-inhibiting substance that was effective only in the susceptible or that the susceptible isoline was more sensitive to fungal metabolites than the resistant. Elgersma *et al* (1972) observed that the initial growth of *Fol* was similar in both resistant and susceptible near-isogenic tomatoes but secondary colonisation was restricted in the resistant plants.

Occlusion of vessels is more frequent in resistant than in susceptible cultivars of tomato infected with *Fol* (Pegus, 1976; Hutson & Smith, 1980) or with *V. albo-atrum* (Tjamos & Smith, 1975; Hutson & Smith, 1980). Hutson & Smith (1980) concluded that rapid occlusion by tyloses restricted the upward movement of the pathogen. Tjamos & Smith (1975) did not observe any hyphae growing beyond the zone of tylosis, nor were there any in vessels filled with multiple tyloses. Both Pegus (1976) and Hutson (1978) reported little gel formation in vessels of resistant plants and concluded that gels were not significant in limiting the pathogen. Tylosis was also related to vascular browning in that in areas of extensive vascular browning, there was limited tylosis in both cultivars (Pegus, 1976; Hutson, 1978). Although vascular occlusion is an

important factor in resistance to wilt pathogens, it does not preclude alternative resistance factors which may operate at the same time e.g. Mace (1978) and Mace *et al* (1976) have reported the infusion of terpenoid aldehyde phytoalexins into tyloses in xylem vessels of resistant cotton cultivars.

### 1.7.3. POST-INFECTION ANTIFUNGAL COMPOUNDS

#### 1.7.3.1. HYPERSENSITIVE REACTION

The hypersensitive reaction is defined as an extreme cellular response to infection which may lead to a high degree of disease resistance. It results in the sudden death of host cells surrounding an infection site. The growth and subsequent development of the pathogen is believed to be restricted within the moribund host cells. The hypersensitive reaction is a widespread response of plants to infection by fungi, viruses, bacteria, nematodes as well as to non-pathogenic stimuli (Matta, 1971). The possible role of hypersensitivity in resistance and host-parasite specificity has recently been reviewed by Király (1980). The hypersensitive response is frequently associated with phytoalexin production. Accumulation of large quantities of phytoalexins in tissues undergoing hypersensitive cell death (Keen, 1971) contributes to the antimicrobial environment and may be responsible at least in part for host cell death.

#### 1.7.3.2. PHYTOALEXINS

Phytoalexins are defined as low molecular weight antimicrobial compounds that are both synthesized by and accumulated in plants which have been exposed to microorganisms (Mansfield & Bailey, 1982). These stress metabolites, as they are also called (Kuć, 1976; Stoessl *et al*, 1976) accumulate to high levels in damaged tissues for a limited period of time before their concentration declines (Kuć, 1972). Phytoalexins are generally regarded separately from pre-formed antimicrobial compounds which may also be involved in disease resistance (Deverall, 1977).

Members of the Solanaceae mostly produce terpenoid compounds although acetylenic phytoalexins have been isolated from tomato. De Wit & Kodde (1981a) reported the accumulation of falcarindiol and falcarinol from tomato fruits and leaves inoculated with

*Cladosporium fulvum* and described the novel compound *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol from infected fruits. The latter compound was also isolated from tomato stems inoculated with *Verticillium albo-atrum* (Elgersma & Overeem, 1981). Elgersma *et al* (1984) confirmed the presence of falcarindiol and falcarinol from tomato vascular tissue infected with *V. albo-atrum*. Falcarindiol had previously been isolated from carrot root tissue and showed strong fungitoxic activity towards the storage pathogen, *Mycocentrospora acerina* and *Cladosporium cladosporioides* (Garrod *et al*, 1978). Both falcarindiol and falcarinol have also been isolated from young shoots of the ground elder, *Aegopodium podagraria*, but only falcarindiol is fungitoxic towards *Alternaria brassicicola* and *Septoria nodorum* (Kemp, 1978).

The main sesquiterpenoid compound isolated from tomato is rishitin. Rishitin was first isolated from potato infected with *Phytophthora infestans* (Tomiyama *et al*, 1968) and later in tomato infected with the same pathogen (Sato *et al*, 1968). Rishitin has also been isolated from tomato fruit and foliage infected with *Cladosporium fulvum* (de Wit & Flach, 1979).

Phytoalexins were first implicated in resistance to vascular wilts by Bell (1967, 1969) who showed that cotton stems infected with *V. albo-atrum* accumulated gossypol. This accumulation was greater in resistant than in susceptible cultivars early on in infection. Similar qualitative differences in phytoalexin accumulation have also been reported in cotton infected with *V. dahliae* (Bell *et al*, 1975; Mace *et al*, 1976). An indication that antifungal compounds played a role in the resistance of tomato came from observations by Stromberg & Corden (1977). These authors observed that extracts from xylem vessels of a resistant tomato cultivar contained fungitoxic material that suppressed the growth of *Fol in vivo*. Various data on the detection of antifungal compounds in tomato after infection with *Fol* and *Verticillium* have been consolidated in Table 1. In general, only rishitin has been positively identified.

The more rapid accumulation of phytoalexins in the vascular system of resistant compared with susceptible plants implies a role in resistance (Tjamos & Smith, 1974; Hutson & Smith, 1980). It is envisaged that their operation would be enhanced by their impregnation into gums, gels or tyloses, a process facilitating their local accumulation to inhibitory concentrations (Mace *et al*, 1976; Mace, 1978).

Table 1 Antifungal compounds detected in tomato (*Lycopersicon esculentum*) infected with *Fusarium* and *Verticillium* species

(R = resistant cultivar, S = susceptible cultivar) * $ED_{50}$ values of rishitin against eliciting pathogen

| Eliciting pathogen | No. of antifungal compounds | Antifungal compound identified | * $ED_{50}$ $\mu$ g $ml^{-1}$ for conidial germination | germ tube growth | Comments | References |
|---|---|---|---|---|---|---|
| *Fol* | 1 | Rishitin | 50-100 | 8 | In root and stem. Accumulation more rapid in high R cv. than low R cv. Amounts equalled 3 days after inoculation. | McCance & Drysdale (1975) |
| *Fol* | 2 | Rishitin | 10 | 2 | In stem, root and fruit. Initially more rapid accumulation in R than S. | Pegus (1976) |
| *Fol* | 2 | Rishitin | - | 2 | In root and stem. Initially more rapid accumulation in R than S. | Hutson & Smith (1980) |
| *Verticillium albo-atrum* | 5 | Rishitin | 30-100 | 3 | In stem segments and fruit. Four times more rishitin in R than S. | Tjamos & Smith (1974) |
| *V. albo-atrum* | 6 | Rishitin | - | - | In root and stem. Initial accumulation more rapid in R than S. | Tjamos & Smith (1975) |
| *V. albo-atrum* | 6 | Rishitin | - | 2-4 | In root and stem. Initially more rapid accumulation in R than S. | Hutson & Smith (1980) |

Table 1 (cont.)

| | | | | | | |
|---|---|---|---|---|---|---|
| *V. albo-atrum* | 5 | Rishitin | - | - | In stem. No differences in accumulation in R and S. | Elgersma (1980) |
| *V. albo-atrum* | 2 | - | - | - | In seedling roots. More rapid accumulation in R than S. | Hutson & Smith (1983) |
| *V. albo-atrum* | more than 1 | *cis* -tetradeca-6-ene-1,3-diyne-5,8-diol | - | - | In stem vascular tissue of S. | Elgersma & Overeem (1981) |
| *V. albo-atrum* | 4 | Falcarindiol Falcarinol | - | - | In stem vascular tissue of S. | Elgersma *et al* (1984) |
| *V. albo-atrum* *V. dahliae* | 1 | Rishitin | - | - | In fruits, stems and leaves. Higher quantities of rishitin in R. | Grzelinska & Sierakowska (1978) |
| *V. dahliae* | 6 | Rishitin | - | - | In root and stem. Initially more rapid accumulation in R than S. | Emmanouil (1977) |

### 1.7.3.3. ELICITORS OF PHYTOALEXIN ACCUMULATION

Many different types of injury or stress can stimulate phytoalexin production. These factors can include infections with fungi, bacteria, viruses, wounding, ultra-violet irradiation, dehydration, treatment with antibiotics and with phytotoxins (Stoessl *et al*, 1976).

Many fungi are known to produce high molecular weight substances that are capable of eliciting phytolaexin synthesis. These elicitors are non-specific and are produced by both virulent and avirulent races of the pathogens. Albersheim & Anderson-Prouty (1975) have suggested that the surface carbohydrates (glycoproteins) of fungi act as specific defence elicitors by interacting with protein receptors on the surface of host cells. Zaki *et al* (1972) purified a protein-lipopolysaccharide (PLP) complex from *Verticillium dahliae* cultures that induced the synthesis of greater phytoalexin activity in resistant than in susceptible cotton cultivars. The composition of this PLP complex was very similar to the outer heteropolysaccharide wall of *V. dahliae* conidia.

Tomato wilt pathogens have been shown to produce phytoalexin elicitors albeit of a non-specific nature. Culture filtrates from *Fol* and *V. albo-atrum* induced the accumulation of rishitin and other fungitoxic compounds (Hutson, 1978). Woodward & Pegg (1986) also reported the elicitation of rishitin accumulation in whole plants and excised stem segments of both resistant and susceptible cultivars using culture filtrate and cell wall extracts of *V. albo-atrum*. They suggested that the elicitor properties in culture filtrates can be affected by cultural conditions, which would account for the differences reported by various workers in the past (McCance & Drysdale, 1975; Elgersma, 1980; Hutson & Smith, 1980).

Other phytoalexin elicitors such as polyunsaturated fatty acids (viz. arachidonic acid and eicosapentanoic acids isolated from *Phytophthora infestans*) elicited the accumulation of rishitin and lubimin in potato tubers but were not effective in tomato or other hosts (pepper, tobacco, pea, broad bean and French bean)(Bloch *et al*, 1984). This suggests that there is a degree of specificity associated with this elicitation of sesquiterpenes.

Other tomato pathogens also produce elicitors of phytoalexins. De Wit & Roseboom (1980) have reported that high molecular weight compounds from cell-free culture filtrates,

mycelial extracts and cell walls of *Cladosporium fulvum* have elicited rishitin in tomato fruit. Elicitors from mycelial extracts and cell wall extracts appeared to be more active than those from culture filtrates and activity was often dependent on the composition of the growth media and the age of the culture (de Wit & Kodde, 1981b).

At least one tomato pathogen is reported to produce suppressors of resistance (proposed as being important in establishing basic compatibility), although the evidence was obtained with potato rather than with tomato. Suppression of the accumulation of rishitin and phytuberin resulted when potato tuber was inoculated with an incompatible race of *Phytophthora infestans*, subsequent to inoculation with a compatible race of the fungus 12 hours earlier (Varns & Kuć, 1971). A high molecular weight suppressor of the hypersensitive reaction was obtained from zoospores of a compatible race of *P. infestans* (Doke, 1975). Subsequently, water-soluble glucans from mycelia of a compatible race of *P. infestans* werereported to have suppressive action on browning and terpenoid accumulation in potato tuber inoculated with an incompatible race of the pathogen (Garas *et al*, 1979). The hypersensitivity-inhibiting factor was suggested to suppress the reaction between the elicitor and the cell membrane (Doke *et al*, 1979) constituting the determinant factor in potato-*P. infestans* interaction. This suppressive activity of water-soluble glucans has also been demonstrated on potato tuber protoplasts (Doke & Tomiyama, 1980).

## 2. MATERIALS and METHODS

### 2.1. HOST CULTIVARS

Near-isogenic lines of the tomato cultivar Craigella were used. These were developed at Glasshouse Crops Research Institute, Littlehampton. The resistant isoline GCR 152 (referred to as CR) possesses the *I/I* gene for resistance to *Fusarium oxysporum* f.sp. *lycopersici*. The susceptible isoline used was GCR 26 (referred to as CS)(Harries, 1965; Hall & Bowes, 1981).

Other cultivars used were the susceptible Bonny Best (kindly supplied by Dr RM Cooper, University of Bath), Ailsa Craig (Sutton Seeds),Moneymaker (Sutton Seeds) and the resistant American cultivars, Homestead (possessing the *I/I* gene) and Walter (possessing the *I/I* $I_2/I_2$ gene). Except where indicated, seeds were obtained from Imperial College stocks.

#### 2.1.1. GROWTH OF PLANTS

Plants were grown in 100 mm plastic pots (square section) in 'Fisons' Levington potting compost in a temperature-controlled glasshouse (30 °C max, 20 °C min).

Supplementary illumination was provided by mercury lamps at 400 W on a 16 h photoperiod. Plants were given Long Ashton nutrient solution every four weeks. Bio 'Sprayday' containing resmethrin and synergised pyrethrum was used to control whitefly (*Trileurodes vaporariorum*).

#### 2.1.2. NUTRIENT SOLUTION

Long Ashton nutrients were prepared from stock solutions as follows :

| Salt | Stock solution (g $2l^{-1}$ ) | Dilution (ml $l^{-1}$ ) |
|---|---|---|
| $KNO_3$ | 202 | 4 |
| $Ca(NO_3)_2 . 4H_2O$ | 472 | 4 |
| $MgSO_4 . 7H_2O$ | 184 | 4 |
| $KH_2PO_4$ | 362 | 2 |
| *Fe . EDTA* | * | 1 |
| Micronutrients | + | 0.2 |

Where "*" 33 g $Na_2EDTA$ and 4 g $NaOH$ were dissolved in 500 ml distilled water. This was added to a solution containing 24.9 g $FeSO_4 . 7H_2O$ and 0.7 ml concentrated HCl in 500 ml water, aerated for a minimum of 12 h and made up to 1 l.

Where "+"

| Salt | Stock solution ($g\ l^{-1}$) |
|---|---|
| $MnCl_2 . 4H_2O$ | 9.85 |
| $CuSO_4 . 5H_2O$ | 1.25 |
| $ZnSO_4 . 7H_2O$ | 1.45 |
| $H_3BO_3$ | 15.50 |
| $Na_2MoO_4 . 2H_2O$ | 0.60 |
| $CoCl_2 . 6H_2O$ | 0.30 |

### 2.1.3. PLANTS GROWN IN WATER CULTURE

Seeds were wetted with Long Ashton nutrient solution and germinated on absorbent paper in damp sandwich boxes in a growth cabinet (16 h photoperiod, at 20 °C day temperature and 15 °C night temperature, r.h. 60 - 70 %). After 10 days, each seedling was supported with a foam plastic bung and transferred to a boiling tube (diameter 25 mm) filled with Long Ashton nutrient solution. Plants in tubes were grown on in the growth cabinet. Nutrient solution was changed every three days.

## 2.2. ORIGIN OF PATHOGEN

Four isolates of *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) Snyder & Hansen, race O (Gabe, 1975) were initially available and were originally from the following sources :

Isolate 1 Dr RB Drysdale (University of Birmingham)

Isolate 4 & 13 Dr T Hall (Glasshouse Crops Research Institute)

Isolate 14 Dr F Cervone (University of Rome)

After initial pathogenicity tests on cultivars Ailsa Craig, Moneymaker and Homestead, isolate 13 was shown to be the most pathogenic and henceforth was used in all further

experiments.

The pathogen was routinely reisolated from infected plants every six months to prevent possible attenuation of pathogenicity.

### 2.2.1. CULTURE MEDIA

All chemicals used, unless stated, were of Analar grade and sterilised at 121 °C for 15 min.

#### 2.2.1.1. NUTRIENT MEDIA

Potato Dextrose Agar (PDA) (Oxoid) was prepared according to the manufacturer's instructions at 3.9 % (w/v) total solids.

PDA with antibiotics, was used as a selective medium to facilitate isolation of fusaria from host tissues.

| | |
|---|---|
| Streptomycin sulphate | 30 mg $l^{-1}$ |
| Chloramphenicol | 50 mg $l^{-1}$ |

The above antibiotics were dissolved in 5 ml acetone and shaken well. One ml of this was then dispensed into 200 ml of sterilised PDA at 40 °C.

Corn Meal Agar (CMA) (Oxoid) was prepared according to the manufacturer's instructions at 1.7 % (w/v) total solids.

Fungal isolates were maintained on CMA slopes or plates at 20 °C. Stock cultures were stored at 5 °C.

Casein Sucrose Hydrolysate liquid medium (CSH)

| Salt | g $l^{-1}$ |
|---|---|
| Casein hydrolysate (acid)(Oxoid) | 4.6 |
| $KH_2PO_4$ | 1.0 |
| $MgSO_4 . 7H_2O$ | 0.5 |
| Sucrose | 15.0 |
| Glucose | 0.5 |
| * Trace elements | 1.0 ml |

Where "*" :

| Salt | mg $l^{-1}$ |
|---|---|
| $FeSO_4 . 7H_2O$ | 20 |
| $ZnSO_4 . 7H_2O$ | 100 |
| $Na_2MoO_4 . 2H_2O$ | 2 |
| $CuSO_4 . 5H_2O$ | 2 |
| $MnCl_2 . 4H_2O$ | 2 |

250 ml Erlenmyer flasks containing 100 ml of CSH liquid medium were used to prepare and maintain the inoculum. Flasks were inoculated with a 5 mm plug of *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) grown on CMA and incubated in an orbital shake incubator at 200 oscillations $min^{-1}$ at 25 - 27 °C.

## 2.3. INOCULATION TECHNIQUES

### 2.3.1. PREPARATION OF INOCULUM

Inoculum suspension was prepared from 3 day old shake cultures grown on CSH liquid medium. Under these conditions, the fungus grew mainly as micro-conidia and bud-cells.

Shake cultures were filtered through a layer of sterile muslin and the filtrate centrifuged at 700 *g* for 5 min. The spore pellet was resuspended in sterile distilled water and adjusted with a haemocytometer to a spore concentration of $10^6$ propagules $ml^{-1}$ .

### 2.3.2. PREPARATION OF CULTURE FILTRATES

Seven day old shake cultures were filtered through a layer of sterile muslin and the filtrate centrifuged at 700 *g* for 5 min. Centrifugation was repeated at least twice and the final filtrate was sterile filtered (0.22 μ m pore size) to remove any remaining fungal spores.

### 2.3.3. ROOT-DIP INOCULATION OF WHOLE PLANTS

Tomato plants were uprooted and their roots carefully washed free of soil before dipping with the prepared inoculum of *Fol* (concentration $10^6$ propagules $ml^{-1}$ ) (section 2.3.1) for 15

min. Plants were repotted after inoculation. Controls were similarly treated except that they were placed in distilled water.

### 2.3.4. ROOT-DIP INOCULATION OF SEEDLINGS GROWN IN WATER CULTURE

Two week old seedlings grown in water culture (section 2.1.3) were inoculated by cutting 1 mm off their root tips and dipping them in the inoculum for 15 min. After this treatment, they were replaced into boiling tubes containing Long Ashton nutrient solution and incubated in the growth cabinet as before.

### 2.3.5. STEM INOCULATION

Using a sharp blade, an incision was made into the cotyledonary node of the tomato plant under a droplet of inoculum suspension previously positioned on the stem (Elgersma, 1980). This droplet was taken in immediately due to the water potential deficit in the xylem system of the plant. Several more droplets of inoculum were applied until a total of 0.2 ml had been absorbed. Parafilm was used to seal the wound.

### 2.3.6. PETIOLE INOCULATION

Petioles (length 80 - 90 mm) from 6 week old plants (grown as in section 2.1.1) were detached under water and the proximal end attached via a short piece of silicone rubber tubing to a 1 ml syringe. Inoculum and other test fluids were forced through using gentle pressure. Inoculated petioles were then incubated in a damp chamber in the incubator (20 $^{\circ}$C). In an alternative system, petiole leaflets were detached and fluids were forced through until they exuded from the exposed end.

### 2.3.7. INOCULATION OF CUT SHOOTS

Young tomato plants (2 week old), grown as described previously (section 2.1.1), were detached under water just above the roots and the cut shoots dipped into inoculum for 15 min. Subsequently, the cut shoots were placed in boiling tubes containing distilled water and placed in a growth cabinet. Other test fluids (e.g culture filtrates) were introduced into cut shoots in the same way.

### 2.3.8. LEAF INOCULATION

The method used for the inoculation of leaves was adapted from Theodorou & Smith (1979). This injection method is commonly used in studies on bacterial plant pathogens (Klement & Goodman, 1967 ; Klement, 1968).

Using a sterile hypodermic syringe (1 ml) fitted with a fine needle (no. 30 gauge), spore inoculum ($10^6$ propagules $ml^{-1}$ ) was injected into the lamina from the abaxial leaf surface. As the inoculum infiltrated into the intercellular spaces of the leaf, a surface water-soaking effect resulted. Approximately 0.1 ml of inoculum was injected per site on each leaf. Inoculum was applied at two sites on separate leaves of the lower stem region of the plant.

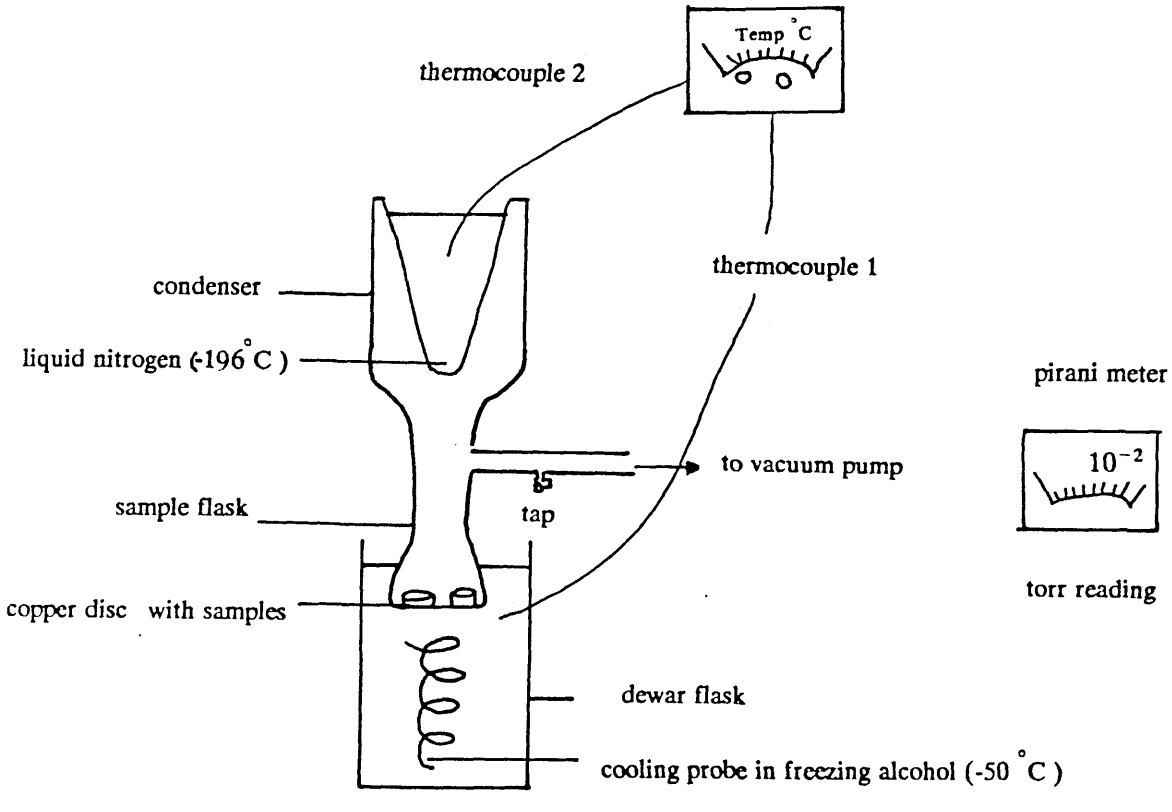
## 2.4. REISOLATION TECHNIQUES

Infected plants were cut into 10 mm sections taken from designated parts of the plant i.e. main tap root, cotyledonary node and alternate nodes up to the apex. The nodal tissue was taken from the area immediately below the node and henceforth referred to as node 1, 3 etc. When petioles (also taken from alternate nodes) were sampled, 10 mm sections were taken from the proximal end and treated as follows. Sections were surface sterilized for 2 min in 5 % (v/v) sodium hypochlorite solution with 0.1 % Tween 20 added. After washing in 3 changes of sterile distilled water, 3 - 5 mm thick discs were sectioned using a sterile scalpel and plated onto PDA with antibiotics (section 2.2.1.1). Plates were inspected for fungal growth after incubation at 20 $^{\circ}$C for 3 days.

### 2.4.1. COLONY COUNT METHOD FOR QUANTIFICATION OF PATHOGEN

The following technique was carried out in a laminar-flow cabinet using pre-sterilised equipment. Plants were grown (section 2.1.1) and inoculated (section 2.3.3) as described previously. One g samples of plant material taken from designated areas of root and nodal regions were surface sterilised and washed with sterile water (section 2.4). Tissue was cut into 1 - 2 mm pieces with a sterile scalpel and homogenised for 2 min in 10 ml of sterile distilled water in a mechanical macerator (MSE)(method adapted from Pegg & Street, 1984). The rim of the homogeniser chamber was wiped with ethanol before pouring the homogenate into

Diagram 1 Diagrammatic representation of the apparatus used for freeze-drying of tissue samples, prior to viewing under SEM



Universal bottles and capping. The chamber was rinsed twice with sterile distilled water between sample blending. Serial 10-fold dilutions of homogenates were made and 0.5 ml aliquots of various dilutions were spread (using a glass spreader) onto plates containing PDA with antibiotics. After incubation at 20 °C for 3 - 5 days, dilution plates (in quadruplicate) yielding colony numbers between 20 - 300 were counted. Dilution plates yielding 10 or less colonies were recorded as trace numbers.

## 2.5. SCANNING ELECTRON MICROSCOPY (SEM)

### 2.5.1. PREPARATION OF TISSUE

Tomato plants were grown (section 2.1.1) and inoculated (section 2.3.3) as described previously. Uprooted plants were washed well and cut under fixative (2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) to avoid air bubbles. Sections were cut (5 mm in thickness) and split longitudinally with a sharp scalpel, and were immersed in fixative for a minimum of 2 h. Specimens were washed 3 times in 0.1 M sodium cacodylate buffer for 10 min each prior to post-fixation treatment (1% osmium tetroxide in buffer) at room temperature for 1 h minimum. Dehydration was accomplished with an ethanol series in 10-min interval steps starting with 10% (v/v) ethanol and finishing with two changes in 100% ethanol at 20 min each. Further dehydration was continued with two changes of 100% acetone for 20 min each. Specimens were then dried in carbon dioxide in a critical point dryer (Polaron E-3000) at 36 - 38 °C. Dried specimens were fixed onto viewing stubs with adhesive and coated with gold using a Polaron Sputter Coater (Polaron E-5000) for 10 min at a voltage of 0.6 kV and a current of 20 mA to give a mean coating thickness of 70 nm. Coated specimens were viewed under a Philips 500 Scanning Electron Microscope and stored in a desiccator when not in use. Photographs were taken using Ilford Pan F film and developed according to the manufacturer's instruction.

### 2.5.2. FREEZE-DRYING TECHNIQUE FOR VIEWING UNDER SEM

As an alternative to critical point drying prior to viewing under the SEM, the following freeze-drying technique was used (Diagram 1). Sample material was first pre-frozen in

"Arcton" (I.C.I) then cooled in liquid nitrogen before freeze-drying under vacuum ($1x10^{-2}$ Torr) at -50 °C for 5 h. Subsequently, the apparatus was left overnight to allow the sample flask to reach room temperature. (This prevents sudden condensation on the material) Once this was achieved, the samples were quickly transferred into a desiccator until required. Sample material was fixed onto viewing stubs with double-sided Sellotape and sputter-coated and viewed as before.

## 2.6. EXTRACTION OF ENZYME (β−1,3− GLUCANASE)

A method adapted from Pegg & Young (1981) was used. Two to three g of freshly harvested stem vascular tissue from either infected or control tissue of both Craigella resistant and susceptible plants was homogenised for 2 min in 0.1 M acetate buffer pH 5.6 (10 ml $g^{-1}$ fresh weight). The macerate was centrifuged at 700 *g* for 5 min. The supernatant was dialysed overnight at 4 °C against 0.1 M acetate buffer, pH 5.6.

### 2.6.1. PREPARATION OF DIALYSIS TUBING

Dialysis tubing (Medicell) was prepared by boiling for 1 h in 10 mM $Na_2EDTA$. This was repeated with fresh EDTA for 0.5 h and then the tubing was rinsed thoroughly with distilled water. The tubing was boiled in the last change of distilled water and stored at 5 °C.

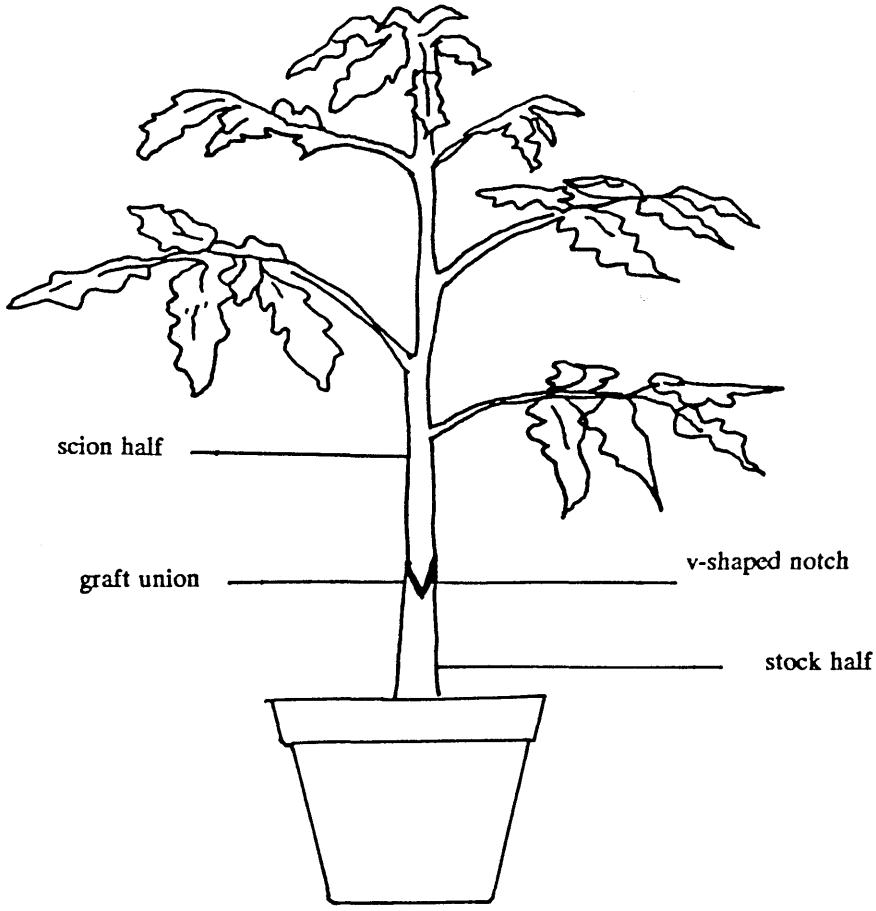
### 2.6.2. PREPARATION OF SUBSTRATE

β−1,3− glucanase activity was determined by measuring the production of reducing sugars from laminarin (Sigma Labs.) (Pegg & Young, 1981). The substrate solution contained 6.25 mg $ml^{-1}$ laminarin (dissolved by heating the solution to 60 °C) in 0.1 M acetate buffer pH 5.6.

### 2.6.3. ENZYME ASSAY

Enzyme and substrate were preincubated at 30 °C. In the assay, 0.2 ml enzyme sample was incubated with 0.8 ml substrate solution for 4 h in a waterbath at 30 °C. Total reducing sugars were assayed by the colorimetric method of Nelson-Somogyi (Nelson, 1944) and expressed as glucose units by reference to a standard curve of 0 - 100 μ g $ml^{-1}$ glucose. One ml

Diagram 2 Method of grafting the tomato plants



of copper reagent was added to the enzyme incubate to stop the reaction by boiling at 100 °C for 10 min. Controls contained a boiled enzyme sample to allow for reducing sugar present in the sample. After cooling to 22 °C, 1 ml of arsenomolybdate reagent was added and mixed well. A fixed amount of distilled water (17 ml) was added to make up to 20 ml and left to stand for 10 min before reading the extinction at 500 nm using a Beckman spectrophotometer. All readings were taken against a blank containing reagents and distilled water only.

One unit of β−1,3− glucanase is the amount of enzyme that produces reducing groups equivalent to 100 μ g glucose in 1 h at 30 °C, pH 5.6.

## 2.7. GRAFTING TECHNIQUES

Tomato plants (4 week old) were used for grafting. Tops of plants were severed just above the cotyledonary node using a sharp blade and a V-shaped point cut into it. A V-shaped notch was made on the lower section containing the rootstock. The two halves (from different plants) were joined and sealed using Parafilm (Diagram 2). To ensure an even union at the graft joint, plants of similar stem girth were used. Grafted plants were kept in a moist chamber for about a week for the grafts to take, after which they were returned to the greenhouse bench. Another week lapsed before the grafted plants were inoculated using the root-dip method (section 2.3.3). After three weeks, plants were harvested and samples examined for distribution of *Fol* by the cultural reisolation method (section 2.4). Symptoms expressed as a result of grafting and inoculation were recorded. Heights of grafted plants at inoculation and just before reisolation were recorded to determine the percentage change in height during the infection period.

## 2.8. PHYTOALEXIN EXTRACTION

### 2.8.1. EXTRACTION OF TERPENOIDS

Stem vascular tissue was excised from control and inoculated plants cv. Craigella and dissected free of excess pith tissue (Hutson and Smith, 1980). Excised tissue was homogenised in methanol (5 ml $g^{-1}$ fresh weight) in a mechanical homogeniser. The homogenate was filtered under suction through Whatman no.1 filter paper. Tissue was re-extracted twice in

methanol and twice in chloroform. The combined filtrates were evaporated to dryness *in vacuo* in a Büchi rotary evaporator at 40 °C. The residue was dissolved in 60 % (v/v) methanol at a rate of 1 ml $g^{-1}$ fresh weight of original tissue and the flask washed out with chloroform. The combined washings were partitioned three times with an equal volume of chloroform and the bulked chloroform fractions were evaporated to dryness and the residue dissolved in a small volume of ethyl acetate for thin-layer chromatography.

### 2.8.2. EXTRACTION OF ALKALOIDS

Methanolic fractions remaining after partitioning with chloroform were combined and evaporated to dryness *in vacuo*. The residue was dissolved in a small volume of 90 % (v/v) methanol for thin-layer chromatography.

### 2.8.3. EXTRACTION OF PHYTOALEXINS FROM ROOTS

Control and infected plants cv. Craigella (section 2.3.3) grown in compost were uprooted and their roots carefully washed and surface-dried (Emmanouil & Wood, 1983). Tissue was comminuted in methanol (5 ml $g^{-1}$ fresh weight) and left for 1 h. The homogenate was filtered through Whatman no. 1 filter paper and the filtrate evaporated to dryness *in vacuo* at 40 °C. The residue was dissolved in 60 % (v/v) methanol at a rate of 1 ml of solvent $g^{-1}$ original fresh weight and partitioned three times in equal volumes of chloroform. The final residue after evaporation of the chloroform fractions was dissolved in a small volume of ethyl acetate for thin-layer chromatography.

## 2.9. THIN-LAYER CHROMATOGRAPHY (TLC)

This was performed on plates of silica gel G (Schleicher & Schüll, West Germany) with an adsorbent thickness of 0.2 mm and containing fluor with maximum emittance at 254 nm.

Aliquots of extracts were loaded onto TLC plates in spots or bands using micropipettes (20 µ l). Bands were applied at a loading equivalent of up to 1 g of extracted tissue $cm^{-1}$ . Chromatograms were developed in Shandon tanks allowing solvent front migration of approximately 150 mm at room temperature, after equilibration in the solvent saturated atmosphere for at least 0.5 h. The following solvent systems were used :

| | |
|---|---|
| cyclohexane : ethyl acetate | 1 : 1 v/v |
| chloroform : acetone | 9 : 1 v/v |
| chloroform : methanol | 95 : 5 v/v |
| butan-1-ol : acetic acid : water | 6 : 2 : 1 v/v |
| diethyl ether | |

### 2.9.1. CHROMOGENIC TESTS

Terpenoids when present were located on chromatograms by the following chemical tests.

Vanillin-sulphuric acid reagent (1 g vanillin, 30 ml methanol , 0.2 ml concentrated sulphuric acid) in which rishitin appears turquoise. (Lyon, 1972).

A saturated solution of antimony (III) chloride in chloroform which renders rishitin red fading to blue. (Varns *et al*, 1971).

Diazotised p- nitroaniline reagent (10 ml, 4 °C of 0.5 % p- nitroaniline in 2 M HCl ; 1 ml,4 °C of 5 % (w/v) sodium nitrite ; 15 ml of 20 % (w/v) sodium acetate and 20 % (w/v) sodium carbonate) which colours rishitin yellow orange (van Sumere *et al*, 1965).

## 2.10. CLADOSPORIUM BIOASSAY

Developed TLC plates were assayed for antifungal compounds by spraying with a heavy spore suspension of *Cladosporium cucumerinum*. Spores were prepared from cultures grown on PDA at 20 °C and suspended in *Cladosporium* nutrient medium (Homans & Fuchs, 1970). Sprayed plates were incubated in a moist chamber at 25 °C for 3 - 5 days. Zones of inhibition appeared white against a dark background of sporulating mycelium.

### 2.10.1. FUSARIUM BIOASSAY

Fungal growth was visualised under ultra-violet light by the metabolic release of fluorescein from the esterase substrate fluorescein diacetate (Woodward & Pearce, 1985).

Developed TLC plates were sprayed with a heavy suspension of *Fol* ($10^6$ propagules $ml^{-1}$ ). Spores were prepared as for inoculation except for resuspending in CSH medium (section 2.3.1). Sprayed plates were incubated in a moist chamber at 25 °C for 3 - 5 days. One drop of

a stock solution of fluorescein diacetate (5 mg $ml^{-1}$ ) in acetone was added to 0.1 M phosphate buffer (pH 5.8) to render the buffer solution cloudy. Incubated TLC plates were sprayed with the buffered solution and further incubated for 2 h at 25 $^{\circ}C$ before examination under UV illumination at 254 nm. Zones of antifungal activity were apparent as dark areas on the plate while fungal mycelium fluoresced green due to the release of fluorescein.

# 3. INITIAL EXPERIMENTS ON HOST SPECIFICITY

## 3.1. INTRODUCTION

The original aims of the project were to define when, where and how resistance is expressed in monogenically resistant plants when infected with a race 0 (Gabe,1975) isolate of *Fol* (i.e. virulent on cultivars which possess no resistance genes). Using a range of test fluids (i.e. spore inoculum and culture filtrates) on cut surfaces of near-isogenic lines of tomato, differential responses were sought, expressed soon after pathogenic invasion, in the determinative phase of resistance. These responses could include any microscopically visible changes occurring in cells directly exposed to the test fluids or in immediately adjacent tissues. Hopefully an enhanced reaction in cells of resistant plants would provide the basis of a bioassay for fungal elicitors of resistance.

Experiments employed tomato cvs Ailsa Craig and Moneymaker (both susceptible to race 0), and Homestead (resistant to race 0, possessing the $I_1$ gene). Later, experiments employed near-isogenic lines of cv. Craigella designated CR and CS, for greater structural uniformity of internal tissues.

## 3.2. PATHOGENICITY TESTS

Four race 0 isolates (nos. 1, 4, 13 and 14) of *Fol* were cultured on PDA. Inoculum suspensions of each isolate of *Fol* were prepared (section 2.3.1). Tomato cvs Ailsa Craig, Moneymaker, Homestead and Craigella at 3 weeks old were inoculated using the root-dip method (section 2.3.3)(Four plants were inoculated per isolate from each cultivar). Controls were sham-inoculated with distilled water. Reisolation of *Fol* from root and lower stem sections of inoculated plants was performed after 14 days (section 2.4).

Susceptible plants showed typical symptoms including vein-clearing in leaflets, foliar chlorosis, stunting, wilting and epinasty 7 days after inoculation. These symptoms became more severe leading to abscission of lower petioles and finally to death of plants. Vascular browning of xylem vessels and adjacent xylem parenchyma became more severe with increasing time after infection, especially in susceptible cultivars. Resistant plants showed no visible

symptoms and were visually similar in vigour to control plants.

The extent of the disease was evaluated and scored using the wilt index shown in Table 2. All plants inoculated with the four different isolates of *Fol* were assessed after 14 days and described using the wilt index. The results (from the average assessment of 4 plants) are shown in Table 3. Among susceptible plants (cvs Ailsa Craig, Moneymaker and Craigella susceptible), those infected with isolate 1 appeared less diseased than those infected with the other isolates. Among the other isolates, isolate 13 appeared the most pathogenic, causing earliest expression of symptoms. All isolates were readily recovered from the root and lower stem sections of both susceptible and resistant plants.

Reisolated fungi were subsequently re-inoculated into healthy susceptible plants, whereupon symptoms were reproduced with a similar range of intensity. Isolate 13 also grew rapidly in culture on PDA and was henceforth used in all further experiments.

Table 2 Disease assessment and wilt index (after Wellman (1939))

| Evaluation of disease | Wilt index | Description |
|---|---|---|
| No disease | 0 | No symptoms. No discolored bundles. |
| Mild disease | 1 | Infection advanced to cotyledonary plate, chlorosis of cotyledons. Discoloured vascular elements extending to cotyledonary plate. |
| Medium disease | 2 | Infection and discoloration of vascular bundles extending to first and second nodes. First leaves chlorotic and wilting. |
| Severe disease | 3 | Infection above the middle to $\frac{3}{4}$ of stem. Older leaves chlorotic. Discoloured vascular bundles extended to middle section of stem. |
| Very severe wilt | 4 | Infection within nodes of tip. All leaves show signs of wilting, yellowing and other symptoms. Darkening of vascular system. |
| Wilt and collapse | 5 | Severe general systemic necrosis and death. |

Table 3 The degree of infection as shown by the wilt index in cvs Ailsa Craig (AC), Moneymaker (MM), Homestead (HS) and Craigella (CS and CR) infected with 4 isolates of *Fol*, assessed 14 days after infection.

| | Wilt index[a] | | | | |
|---|---|---|---|---|---|
| isolate of *Fol* | AC | MM | HS | CR | CS |
| 1 | 2 | 2 | 1 | 1 | 2 |
| 4 | 3 | 3 | 1 | 1 | 3 |
| 13 | 4 | 4 | 1 | 1 | 4 |
| 14 | 4 | 3 | 1 | 1 | 4 |

Legend:

*a* see Table 2

Average assessment from 4 plants in each treatment.

## 3.3. EXPERIMENTS TO INVESTIGATE DIFFERENTIAL HOST RESPONSES

### 3.3.1. SPORE INOCULUM ON EXPOSED STEM SECTIONS

Using cvs Homestead (HS) and Ailsa Craig (AC), 30 mm stem lengths were cut longitudinally on either side to expose the middle section (a thin layer consisting of 4 - 5 cells in thickness). *Fol* inoculum (prepared as in section 2.3.1) of a range of concentrations $10^2 - 10^7$ propagules $ml^{-1}$ was pipetted on to the exposed surfaces and incubated on glass microscope slides in a moist chamber for 3 - 5 days, at 20 °C. Sections were prepared for viewing under the light microscope by fixing in absolute alcohol : glacial acetic acid (3:1) (24 h) clearing in lactophenol at 55 °C (2 - 3 h) and staining in 0.1 % tryphan blue in lactophenol at 55 °C (1h).

After 3 days, sections that were inoculated with the higher spore densities were heavily colonised by mycelium. Sections inoculated with $10^2$ and $10^3$ propagules $ml^{-1}$ *Fol* showed a proliferation of hyphae in cells, collapse of parenchyma with dark areas of discoloration indicating cell death. Control sections showed a few dark areas of discoloration and cell death but the xylem remained intact and there was no fungal growth.

Both HS and AC showed similar responses although there were visibly fewer conidia and hyphae in HS than in AC, at the same spore density. Sections that had been incubated for 5 days were too heavily colonised with surface mycelium to be viewed adequately under the microscope.

The above experiment was repeated using approximately 2 mm thick transverse sections of both HS and AC which were inoculated with $10^3$ propagules $ml^{-1}$ *Fol*, and incubated for 3 days. Controls were similarly treated with distilled water. Results similar to the above were obtained. Both HS and AC showed collapse of parenchyma cells with areas of discoloration. Responses of HS and AC were similar in terms of hyphal colonisation.

### 3.3.2. CULTURE FILTRATE ON EXPOSED STEM SECTIONS

Longitudinal sections of AC and HS were treated with culture filtrates from 10 day old *Fol* shake cultures (prepared as in section 2.3.2). Dilutions ($10^{-1}$ and $10^{-2}$) as well as the neat filtrate were used to flood the stem sections before incubating at 20 °C . Controls were treated

with sterile distilled water.

After 3 days, sections of both HS and AC that were treated with the undiluted culture filtrate showed considerable cell damage and discoloration. Sections treated with diluted culture filtrate were less damaged. There were no visible differences between HS and AC with respect to cell death. Controls showed little change from freshly cut sections.

### 3.3.3. CUT SHOOTS TREATED WITH SPORE INOCULUM

Cut shoots of 2 week old plants (HS and AC) were inoculated by dipping in test tubes containing 25 ml of $10^5$ propagules $ml^{-1}$ *Fol* for 5 h. The inoculum entered the cut shoots by transpirational pull. Subsequently, the cut shoots were transferred to test-tubes containing distilled water and incubated in a growth cabinet at 20 °C for 7 days. Controls were similarly treated in distilled water.

After 7 days, adventitious roots had developed at the cut ends of the shoots. In both inoculated HS and AC, the lower petioles were wilted and yellowing, although upper leaves remained symptomless. Sections taken for viewing under the light microscope showed some discoloration in controls. In inoculated plants of both HS and AC, there was a greater extent of discoloration and cell damage and hyphae were present in cells of the xylem. For both cultivars, sections that were taken from the distal end of the inoculated cut shoots showed less cell damage than those taken at the proximal end.

### 3.3.4. CUT SHOOTS TREATED WITH CULTURE FILTRATE

Cut shoots of 2 week old tomato plants cvs Bonny Best (susceptible), Walter (resistant) and Craigella (resistant and susceptible isolines) were infiltrated (section 2.3.7) with sterile filtrate from 16 day old *Fol* shake culture (prepared as in section 2.3.2) for 4.5 h using transpirational pull, before incubating in distilled water. Controls were similarly treated with distilled water. Sterility of culture filtrates was checked by plating onto PDA (section 2.2.1.1). Sections were taken from filtrate-treated plants after 7 days and plated onto PDA to check for the presence of *Fol* (section 2.4).

After an incubation period of 7 days, all cut shoots treated with culture filtrates showed

necrotic vein-clearing in leaflets which was absent in controls. Culture filtrate and sections taken from cut shoots that were plated on PDA confirmed the absence of the pathogen in the test culture filtrate. Sections that were stained with cotton-blue lactophenol revealed gels present in the vessels of all treatments.

### 3.3.5. ISOLATED PETIOLES TREATED WITH SPORE INOCULUM

Petioles, approximately 90 mm in length, from 6 week old tomato plants cv. Craigella (both isolines) were inoculated (as for cut shoots in section 2.3.7) with 20 ml of $10^6$ propagules $ml^{-1}$ *Fol* for 20 min and subsequently incubated at 20 $^{\circ}$C in distilled water for 5 days. Controls were similarly treated with distilled water.

Inoculated petioles, both resistant and susceptible showed yellowing of the lamina of lower leaflets while controls remained visually healthy. Both resistant and susceptible cuttings showed flaccidity and loss of turgor in the leaflets after inoculation but only the resistant cuttings recovered from the initial wilt.

Transverse sections, taken from the proximal end of the petioles, when viewed under the light microscope showed vessels occluded with gels and gums. The percentage of occluded vessels was much greater in infected CS plants than for the other treatments (Table 4). Reisolation experiments showed *Fol* to be present even at the very distal end of petioles.

Table 4 Percentage of occluded vessels in inoculated and healthy Craigella resistant (CR) and susceptible (CS) petioles.

| Treatment | % Occluded vessels |
|---|---|
| CR infected | 14.7 ± 5.0 |
| CS infected | 72.2 ± 9.3 |
| CR control | 4.8 ± 3.1 |
| CS control | 6.3 ± 3.7 |

Mean of 10 readings with standard error

### 3.3.6. LEAVES TREATED WITH SPORE INOCULUM

Four week old plants cv. Craigella (CR and CS) were leaf-inoculated (section 2.3.8) with 0.1 ml of $10^6$ propagules $ml^{-1}$ *Fol* inoculum per site per leaf. Controls were similarly treated with distilled water.

After 7 days, no obvious wilt symptoms were observed. Inoculated patches on leaves turned necrotic and became desiccated. No visible differences with respect to size and vigour were seen between control and infected plants. After 14 days, sections of the stem and leaf were taken for reisolation of the pathogen (section 2.4). *Fol* was only detected in inoculated leaf sections.

### 3.3.7. COTYLEDONS TREATED WITH SPORE INOCULUM

Cotyledons of 3 week old Craigella plants were inoculated (section 2.3.8) with 0.2 - 0.3 ml of $10^6$ propagules $ml^{-1}$ *Fol* inoculum, by water-soaking the entire cotyledonary area. Controls were infiltrated with sterile distilled water.

No visible wilt symptoms were expressed after 7 days. The infected cotyledons of both CR and CS turned necrotic and eventually were abscised from the stem. Stem sections of all infected plants were examined for the pathogen (section 2.4) but none was detected.

## 3.4. DISCUSSION

The overriding conclusion to be gained from these experiments is that there was no effect of major gene resistance under the conditions employed here. The hope that the $I_1$ gene in cvs Homestead and Craigella resistant might be expressed in parenchymatous tissues remote from the normal site of infection has not been vindicated. This is true, both for spore inoculum and for sterile culture filtrates which might contained putative elicitors of resistance. Specificity in leaf tissues was not exhibited as the effect of the leaf inoculation was mainly localised in the mesophyll areas. As the pathogen is soil-borne , inoculation into leaf tissues would also be an unnatural mode of entry. In the light of these results, there seemed little point in extending trials, originally planned, to include callus culture and protoplast based systems. The only occas ion on which a clear differential response was observed was with inoculated petioles in

which occluded vessels were observed much more frequently in infected susceptible than in resistant plants (Table 4).

The damage to the tissues seen in these experiments must be attributed to non-specific toxins. Whether known *Fol* metabolites such as lycomarasmin (Gäumann, 1951) or fusaric acid (Gäumann, 1957) were present has not been investigated. Lycomarasmin is unlikely to be involved as it has not been detected in diseased tissues and is produced in culture in large quantities only after a long period. External symptoms caused by lycomarasmin injury and *Fusarium* wilt are also different. *Fusarium* inoculated plants showed a gradual disease development with wilting and epinasty followed by leaf blade injury . On the other hand, lycomarasmin caused necrosis of leaf tips and margins which gradually included the entire leaf blade without any flaccidity of petioles (Scheffer & Walker, 1953).

In the present investigation, cut shoots were more sensitive indicators of toxins in the culture filtrates. These showed vein-clearing in leaflets of both resistant and susceptible cultivars after treatment. The initial wilting caused (that was reversible in distilled water) was also an indication of toxic wilting. Gottlieb (1943,1944) showed that tomato plants that had been wilted by a toxin present in the tracheal fluids of plants infected with *Fusarium bulbigenum* v. *lycopersici* regained their turgidity when placed in distilled water. However, Gäumann (1951) has indicated that toxic wilting by lycomarasmin, a peptide toxin produced by *Fusarium lycopersici* in culture, is irreversible. Gäumann observed that various wilting toxins exhibit a marked host specificity, and in a single host, a pronounced tissue specificity. Lycomarasmin, isolated from *Fol* culture filtrates was not observed to cause vascular browning or plugging (Scheffer & Walker, 1953). Kuo & Scheffer (1964) isolated fusaric acid from *Fol* culture filtrates and tested its effects on tomato cv. Bonny Best and subsequently showed that it was not responsible for wilt symptoms, possessing a secondary role in disease development only. More recently, toxins in culture filtrates of *Fol* have been demonstrated by Toyoda *et al* (1984) and Scala *et al* (1985) using tomato callus cells.

Pegus (1976) treated tomato cuttings with filtrates prepared from cultures of *Fol* in which the only carbon source available was isolated tomato cell walls. Both resistant and susceptible isolines responded similarly to culture filtrates.

Fungal metabolites in the culture filtrate induced in cuttings wilting in the leaflets, internal vascular browning and gel formation. Cuttings treated with boiled filtrates did not show vascular browning or tyloses indicating that metabolites are proteinaceous in nature. Corresponding results were obtained when petioles were used in place of cuttings. Pegus (1976) also observed that similar reactions were induced in both resistant and susceptible isolines using spore inoculum. The only differences were the degree of severity i.e. symptoms were more severe in the susceptible than in the resistant tissues. These results correspond to those of the present investigation in that there were no differential responses observed as a result of the treatments. It has been observed that resistant plants although infected always recovered from the initial wilt, as reported also by Scheffer & Gothoskar (1943) and Scheffer & Walker (1954).

Pectolytic enzymes produced by wilt pathogens have long been implicated in symptom induction in wilt pathogenesis. Much evidence in support of this is based on the occurrence of these enzymes in culture filtrates causing wilt symptoms in test plants.

*Fol* grown in liquid culture secretes a variety of polysaccharide-degrading enzymes including polygalacturonase, cellulase, $\alpha$- and $\beta$- galactosidase, arabino-furanosidase (Jones *et al*, 1972). Cooper & Wood (1975) also demonstrated that *Fol* as well as *Verticillium albo-atrum* grown in cultures containing tomato stem cell walls, secreted a similar range of extracellular polysaccharide-degrading enzymes. These enzymes have been demonstrated to degrade cell walls isolated from tomato stems (Cooper *et al*, 1978). Gothoskar *et al* (1955a) treated tomato cuttings with culture filtrates and a commercially prepared pectinase. In both cases similar symptoms developed. The active components in culture filtrates were heat-labile and non-dialyzable implying an enzymic nature (Gothoskar *et al*, 1955b). They concluded that pectinmethylesterase and pectic depolymerase were the cause of wilt and vascular browning in tomato cv. Bonny Best. Pierson *et al* (1955) showed that cuttings treated with culture filtrates from *Fol* developed vascular plugs. These filtrates contained pectinmethylesterase and depolymerase. Pectinmethylesterase and polygalacturonase activities in filtrates have also been reported by Winstead & Walker (1954a), and a cellulase responsible for the release of nutrients from host cell walls was recorded by Husain & Dimond (1960).

# 4. HOST COLONISATION

## 4.1. DISTRIBUTION OF *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) IN HOST TISSUES

Four week old tomato plants cv. Craigella (both resistant CR and susceptible CS isolines) were inoculated by the root-dip method (section 2.3.3) with $10^6$ propagules $ml^{-1}$ . Controls were sham-inoculated with distilled water. At intervals after inoculation, the distribution of the fungus was examined by the reisolation technique (section 2.4).

*Fol* was reisolated from root tissue at all times after inoculation, as it was also for tissue from the cotyledonary node and the first node at 3 days and thereafter (Table 5). From the seventh day onwards marked differences in distribution were apparent. In CS plants *Fol* was recovered from all petioles up to the fifth and all nodes up to the seventh, this representing virtually the entire span of the plants (Table 5). At later times, the fungus was detected in CR at positions up to the third petiole, but only sporadically, and there was no evidence for consistent colonisation of the upper reaches of such plants.

*Fol* was not detected in uninoculated CR and CS, but samples from the root and cotyledonary node did reveal the presence of contaminant soil fungi.

Disease symptoms including chlorosis, necrosis of leaflet margins, petiole epinasty and wilting were visible in CS plants after 7 days. Lower petioles from CS abscised from the stem at 14 days and thereafter. These symptoms were progressive and 21 days after infection, most CS plants were severely stunted and senescing. CR plants did not show any wilt symptoms and were similar in visual symptoms to controls.

This experiment was performed on two separate occas ions with closely similar results.

## 4.2. DISTRIBUTION OF *Fol* IN SEEDLINGS GROWN IN WATER CULTURE

Seedlings of cv. Craigella (CR and CS isolines) were grown in water culture (section 2.1.3) and inoculated at 14 days old (section 2.3.4). Controls were sham-inoculated in distilled water. Plants were observed up to 3 weeks after inoculation for symptoms of the wilt syndrome and reisolation of the pathogen was attempted after 7, 14 and 21 days (section 2.4).

After 7 days, *Fol* could be reisolated only from the root of both CR and CS (Table 6). Samples taken from CR, after 14 days, indicated *Fol* to be restricted to the root whereas in CS, *Fol* was present at all positions up to the first node. After 21 days, sampling showed CS to be totally colonised up to the third petiole whereas in CR, *Fol* was detected only up to the cotyledonary node, with traces in some replicates at the first node. At this stage, there were considerable differences in appearance between resistant and susceptible plants. Inoculated CS were considerably stunted, epinastic and showed yellowing of the lower leaves. Infected CR were similar in height and vigour to controls. Browning of vascular tissues was observed (at plating) in infected tissue of both isolines. *Fol* was usually reisolated from such samples. The frequency of browning in infected cells increased following infection especially in the severely infected susceptible plants.

Legend:

RT root

CN cotyledonary node

N1,3...

first, third... node

P1,3...

first, third... petiole

*a* 4 plants sampled

\+ *Fol* present

\- *Fol* absent

(+) *Fol* present in some but not all replicates

Table 5 Changes in the distribution of *F.oxysporum* f.sp *lycopersici* (*Fol*) in cv. Craigella resistant (CR) and susceptible (CS) isolines with time after infection

(a) CR

| Days | Position on plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | RT | CN | N1 | P1 | N3 | P3 | N5 | P5 | N7 | P7 |
| 1 | +[a] | - | - | - | - | - | - | - | | |
| 2 | + | + | - | - | - | - | - | - | | |
| 3 | + | + | (+) | - | - | - | - | - | | |
| 7 | + | + | + | - | - | - | - | - | | |
| 11 | + | + | + | - | + | (+) | - | - | - | - |
| 14 | + | + | + | (+) | - | - | - | - | - | - |
| 18 | + | + | + | + | (+) | - | - | - | - | - |
| 21 | + | + | + | (+) | + | - | - | - | - | - |
| 23 | + | + | + | - | (+) | - | - | - | - | - |
| 29 | + | + | + | (+) | + | (+) | - | - | - | - |
| 32 | + | + | + | - | (+) | - | - | - | - | - |

(b) CS

| Days | Position on plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | RT | CN | N1 | P1 | N3 | P3 | N5 | P5 | N7 | P7 |
| 1 | + | - | - | - | - | - | - | - | | |
| 2 | + | (+) | (+) | - | - | - | - | - | | |
| 3 | + | + | + | - | (+) | - | - | - | | |
| 7 | + | + | + | + | + | + | + | + | + | - |
| 11 | + | + | + | + | + | + | + | + | + | - |
| 14 | + | + | + | + | + | + | + | + | + | - |
| 18 | + | + | + | + | + | + | + | + | + | - |
| 21 | + | + | + | + | + | + | + | + | + | - |
| 23 | + | + | + | + | + | + | + | + | + | (+) |
| 29 | + | + | + | + | + | + | + | + | + | - |
| 32 | + | + | + | + | + | + | + | + | + | - |

Table 6 Distribution of *Fol* in CR and CS seedings grown in water culture following days after infection

| Days | Isoline | Position on plant | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | RT | CN | N1 | P1 | N3 | P3 | N5 | P5 |
| 7 | CR | +[a] | - | - | - | - | - | | |
| | CS | + | - | - | - | - | - | | |
| 14 | CR | + | - | - | - | - | - | | |
| | CS | + | + | + | - | (+) | - | | |
| 21 | CR | + | + | (+) | - | - | - | - | - |
| | CS | + | + | + | + | + | + | -[b] | |

Legend:

RT root

CN cotyledonary node

N1,3... first, third... node

P1,3... first, third... petiole

*a* 6 plants sampled

*b* apex at N4

\+ *Fol* present

\- *Fol* absent

(+) *Fol* present in some but not all replicates

## 4.3. QUANTITATIVE DISTRIBUTION OF *Fol* IN HOST TISSUES

Both resistant and susceptible isolines of Craigella were used to assess the quantitative distribution of the pathogen in host tissues by the colony count method (section 2.4.1). Plants were root-dip inoculated at 4 weeks old with $10^6$ propagules $ml^{-1}$ (section 2.3.3) and at intervals, samples were taken from designated portions of the plant from which homogenates were prepared (section 2.4.1).

Results obtained from colony counts (Table 7a & b) showed that both the distribution and quantity of *Fol* in CR plants were greatly restricted compared with susceptible CS plants. Numbers of colonies obtained from CR homogenates were much smaller than those from CS homogenates.

In resistant plants, there were two peaks in colony numbers at 3 and 19 days after inoculation in root samples followed by a decline up to the last sample. Colonies obtained from the first node at all samples times with the exception of day 12, were so low as to be recorded as a trace, as were sporadic records from more distal regions (Table 7a).

In susceptible plants, however, samples from the root showed an increase in colony numbers which peaked at day 14, followed by a decline up to the last sample. This pattern was repeated in a general sense for all samples taken throughout the plants. Peak pathogen recoveries were in every instance made at day 12 or 14. This increase in fungal colonisation in susceptible tissues corresponded with the visible symptoms of wilt which were evident after about 14 days. Colony numbers obtained at the seventh node were smaller than those obtained at the lower region but still showed that susceptible plants were almost totally colonised.

Table 7a Changes in the quantitative distribution of *Fol* in CR with time after infection

| Days | Position on plant | | | | | |
|---|---|---|---|---|---|---|
| | RT | N1 | N3 | N5 | N7 | N9 |
| | No. of colonies $x10^{-3}$ $g^{-1}$ fresh weight | | | | | |
| 1 | 2.39[a] ±0.56 | 0 | tr | | | |
| 3 | 10.20 ±0.93 | tr | tr | | | |
| 7 | 9.15 ±2.42 | 0 | tr | tr | | |
| 12 | 2.69 ±0.29 | 1.5 ±0.19 | tr | 0 | 0 | |
| 14 | 8.20 ±2.50 | tr | 0 | 0 | 0 | |
| 19 | 11.05 ±3.46 | tr | 0 | 0 | 0 | 0 |
| 24 | 8.25 ±1.82 | tr | 0 | 0 | 0 | 0 |
| 27 | 4.57 ±1.31 | tr | 0 | 0 | 0 | 0 |

Legend:

RT root

N1,N3,.. first, third.... node

*a* Mean of 4 dilution plates with confidence limits (P=0.05)

tr dilution plate with less than 10 colonies

Table 7b Changes in the quantitative distribution of *Fol* in CS with time after infection

| Days | Position of plant | | | | |
|---|---|---|---|---|---|
| | RT | N1 | N3 | N5 | N7 |
| | No of colonies $x10^{-3}$ $g^{-1}$ fresh weight | | | | |
| 1 | 16.90[a] ±5.04 | 0 | 0 | | |
| 3 | 27.65 ±10.40 | 3.87 ±0.18 | 0 | | |
| 7 | 49.35 ±12.27 | 57.65 ±2.01 | 10.35 ±3.34 | tr | |
| 12 | 207.00 ±66.41 | 414.00 ±106.99 | 137.00 ±10.55 | 46.4 ±10.0 | 22.8 ±4.73 |
| 14 | 247.00 ±23.31 | 317.50 ±37.15 | 178.00 ±24.64 | 27.25 ±3.3 | 3.27 ±0.31 |
| 19 | 199.00 ±3.91 | 189.50 ±52.43 | 39.40 ±8.05 | 11.05 ±4.93 | 1.32 ±0.12 |
| 24 | 43.55 ±4.16 | 30.80 ±12.2 | 44.75 ±3.84 | 2.2 ±0.50 | 0 |
| 27 | 30.90 ±7.25 | 47.75 ±12.10 | 36.90 ±4.94 | 10.30 ±3.16 | 1.2 ±0.29 |

Legend:

RT root

N1,3... First, third.... node

*a* Mean of 4 dilution plates with confidence limits (P=0.05)

tr dilution plate with less than 10 colonies

## 4.4. DISTRIBUTION OF *Fol* IN HOST TISSUES AFTER STEM INOCULATION

### 4.4.1. COMPARISION BETWEEN ROOT- AND STEM- INOCULATED PLANTS

Thirty day old plants cv. Craigella of both isolines were stem-inoculated with 0.2-0.3 ml of a suspension of $10^7$ propagules $ml^{-1}$ *Fol* inoculum as described previously (section 2.3.5). Control plants were root-inoculated using the same inoculum by the root-dip method (section 2.3.3). After a 14 day incubation period plants were sampled by the reisolation technique (section 2.4) and plated on PDA with antibiotics (section 2.2.1.1).

Only the root-inoculated CS plants showed symptoms of wilt. Stem-inoculated plants of both resistant and susceptible isolines were symptomless and visually similar to root-inoculated CR plants. Tissue sections dissected for use in the reisolation method exhibited areas of brown staining in the vascular tissue. This reaction occurred in all segments from which the fungus was reisolated and in no others, in all four host-pathogen interactions.

As shown in Table 8, *Fol* was detected as far up as the first node in both stem- and root-inoculated CR plants. In CS plants, however, *Fol* was detected up to the third and seventh node in stem- and root-inoculated plants respectively.

### 4.4.2. REISOLATION OF *Fol* FROM STEM-INOCULATED PLANTS

The experiment described above (section 4.4.1) was repeated using 4 week old plants inoculated with 0.2 ml of a suspension of $10^7$ propagules $ml^{-1}$ *Fol* inoculum. Controls were similarly treated with 0.2 ml sterile distilled water. Heights of plants from the cotyledonary node to the apex were recorded before inoculation and again just before reisolation as an estimate of growth. This was expressed as a percentage change in growth of plants. Plants were sampled at 14 and 21 days after inoculation.

After 14 days, inoculated plants continued to grow as vigorously as controls and were symptomless save for the presence of adventitious buds on the lower stems of both isolines.

The pathogen was reisolated from infected tissues of both isolines at all positions up to the first node after both 14 and 21 days (Table 9). Susceptible plants were colonised up to the third node with traces of the fungus in the first and third petioles. In CR, however, only erratic

traces of *Fol* were detected above the first node. Samples taken from roots often yielded other soil fungi together with *Fol.* This arose because of the difficulty of keeping root samples completely free of soil particles.

Upon attempted reisolation, internal light browning of vascular cells was observed in infected tissues which often yielded *Fol* upon culturing.

An analysis of variance performed on all results (Table 10) showed no significant differences ($P = 0.05$) between isolines or between inoculated and control plants with respect to height (for details see appendix).

Table 8 Distribution of *Fol* in both root- and stem-inoculated plants cv. Craigella 14 days after inoculation

| | Position on plant | | | | | |
|---|---|---|---|---|---|---|
| Root-inoculated | RT | CN | N1 | N3 | N5 | N7 |
| CR | +[a] | + | (+) | - | - | - |
| CS | + | + | + | + | + | + |
| Stem-inoculated | | | | | | |
| CR | + | + | + | - | - | - |
| CS | + | + | + | + | - | - |

Legend:

RT root

CN cotyledonary node

N1,3.. first,third... node

*a* 4 plants sampled

\+ *Fol* present

\- *Fol* absent

(+) *Fol* present in some but not all replicates

Table 9 Distribution of *Fol* in stem inoculated plants cv. Craigella 14 and 21 days after inoculation.

| Time | Isoline | Position on plant | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| in days | | RT | CN↓ | N1 | P1 | N3 | P3 | N5 | P5 | N7 | P7 | N9 |
| 14 | CR | +[a] | + | + | (+) | (+) | - | - | - | - | - | |
| | CS | + | + | + | (+) | + | (+) | - | - | - | - | |
| 21 | CR | + | + | + | - | - | - | - | - | - | - | - |
| | CS | + | + | + | - | (+) | - | - | - | - | - | - |

Legend:

RT root

CN cotyledonary node

N1,3... first, third... node

P1,3... first, third... petiole

↓ point in the stem where the inoculation was applied

*a* 4 plants sampled

\+ *Fol* present

\- *Fol* absent

(+) *Fol* present in some, not all replicates

Table 10 Comparison of % change in height between stem-inoculated and control plants cv. Craigella after inoculation

| Time in days | Treatment | Isoline CR | Isoline CS |
|---|---|---|---|
| 14 | Healthy | 241.76[a] | 245.21 |
| | Inoculated | 220.53 | 272.73 |
| | | S.E.D(D.F=12) = 24.47 | |
| | | L.S.D(D.F=12,P=0.05) = 53.32 | |
| 21 | Healthy | 404.61 | 475.35 |
| | Inoculated | 408.75 | 469.83 |
| | | S.E.D(D.F=12) = 43.19 | |
| | | L.S.D(D.F=12,P=0.05) = 94.11 | |

Legend:

*a* Mean % height increment of 4 plants

## 4.5. SECONDARY INOCULATION OF ROOT-INOCULATED PLANTS

Craigella resistant and susceptible isolines were root-inoculated (section 2.3.3) at 2 weeks old and some plants sampled (section 2.4) for the distribution of *Fol* after 4 and 7 days. The remaining inoculated plants, at day 4 or 7, were further inoculated as for cut shoots (section 2.3.7) i.e. with the inoculum introduced through the cut stem, and incubated further for another 3 days before reisolation of *Fol* was attempted.

Results (Table 11) show that after 4 days, *Fol* had not advanced beyond the root in CR although traces were detected in the hypocotyl and first node of CS. After 7 days, *Fol* was detected as far up the plant as the first node in CR although plants remained symptomless. CS plants however, were showing wilt symptoms and were stunted with the first petioles wilted and often abscised. *Fol* in these plants were detected as far as the third node.

When inoculum was introduced into the stems hence bypassing the roots, plants showed an initial wilting (during the 15 min of inoculation) but mostly recovered when they were replaced in distilled water and incubated further. CS plants did not fully recover and remained partially wilted with drooping petioles. Such CS plants that had been root-shoot inoculated at day 4 and 7 (i.e sampled at day 7 and 10 respectively) were almost totally colonised by *Fol* up to the third node.

*Fol* was detected mainly in the hypocotyl of CR plants that were root-shoot inoculated at day 4, and had advanced as far up the first node when similarly treated at day 7.

Table 11 Distribution of *Fol* in root-inoculated (RI) and root-shoot inoculated (RSI) Craigella R & S plants.

| Treatment | Reisolation at day | Isoline | Position on plant | | | | |
|---|---|---|---|---|---|---|---|
| | | | RT | HC | N1 | P1 | N3 |
| RI | 4 | CR | + | - | - | - | - |
| | | CS | + | (+) | (+) | - | - |
| RSI | 7 | CR | | + | (+) | - | - |
| | | CS | | + | + | - | (+) |
| RI | 7 | CR | + | + | + | - | - |
| | | CS | + | + | + | + | (+) |
| RSI | 10 | CR | | + | + | - | - |
| | | CS | | + | + | + | (+) |

Legend:

RT root

HC hypocotyl

N1,3... first, third...

P1 first petiole

\+ *Fol* present in all 6 plant samples

\- *Fol* absent

(+) *Fol* present in less than half the samples

## 4.6. SCANNING ELECTRON MICROSCOPY OF HOST TISSUES

Resistant and susceptible isolines of cv. Craigella were sectioned at 4 weeks old and prepared for viewing under SEM (section 2.5) at 1, 4 and 8 days after inoculation (section 2.3.3). Roots of 10 day old seedlings (grown as in section 2.1.3 and inoculated for 30 min as in section 2.3.4) were longitudinally split under a binocular microscope and freeze-dried (section 2.5.2) prior to viewing under SEM. Samples were taken 1, 3 and 8 days after inoculation. The nature of hyphal growth and the structure of cell walls were examined.

There were no structural differences in the cortical cells and vessels between the two isolines. Hyphae were not observed in any of the sections taken 1 day after inoculation. However, hyphae of the pathogen were observed to be growing over the root surfaces of 10 day old seedlings of both isolines, soon after inoculation. They were occasionally accompanied by germinating conidia.

Sections taken from CS roots, 3 days after inoculation showed hyphal colonisation in vessel elements (Plate 1). In some cases, hyphae grew laterally through bordered pit-pairs in the vessel walls (Plate 2). The pathogen was also observed to grow longitudinally along the vessel elements. In sections taken from CS roots, 4 days after inoculation, sporulating hyphae were observed (Plate 3). Fungal colonisation in resistant plants was more limited as seen in CR root section after 8 days, where adjacent vessels remained uncolonised (Plate 4). The advantages of using the two methods, critical point drying and freeze-drying are compared. In critical-point dried sections, although there was some apparent shrinkage (e.g septa rings visible in hyphal strand in Plate 3), hyphae collapsed less than in the freeze-dried material (e.g Plate 4).

SEM also revealed the presence of tyloses. These were frequently found in resistant tissue and rarely in the susceptible. Tyloses could not be found in sections other than the root and hypocotyl regions. They were either present in groups in the vessels (Plate 5) or initially growing singly through pits in the vessel wall (Plate 6). Occasionally, conidia were observed on the vessel walls of susceptible root tissue (Plate 7). Tyloses of different sizes were also observed to be held together in clumps, near the rim of the perforation plate of vessels (Plate

8). This could be the initiation of any occluding mechanism in the host defence system. Plates 7 and 8 also show some deposition of yeast-like particles on the xylem walls.

SEM observations confirm the reisolation studies (section 4.1) showing that colonisation by the pathogen was limited in CR but not in CS with the lower regions being more extensively invaded than the upper regions of the plant (Plates 9 and 10). Heavy growth of the pathogen was apparent in CS root sections after 8 days (Plate 11) with hyphae emerging through pits in some vessels (Plate 12).

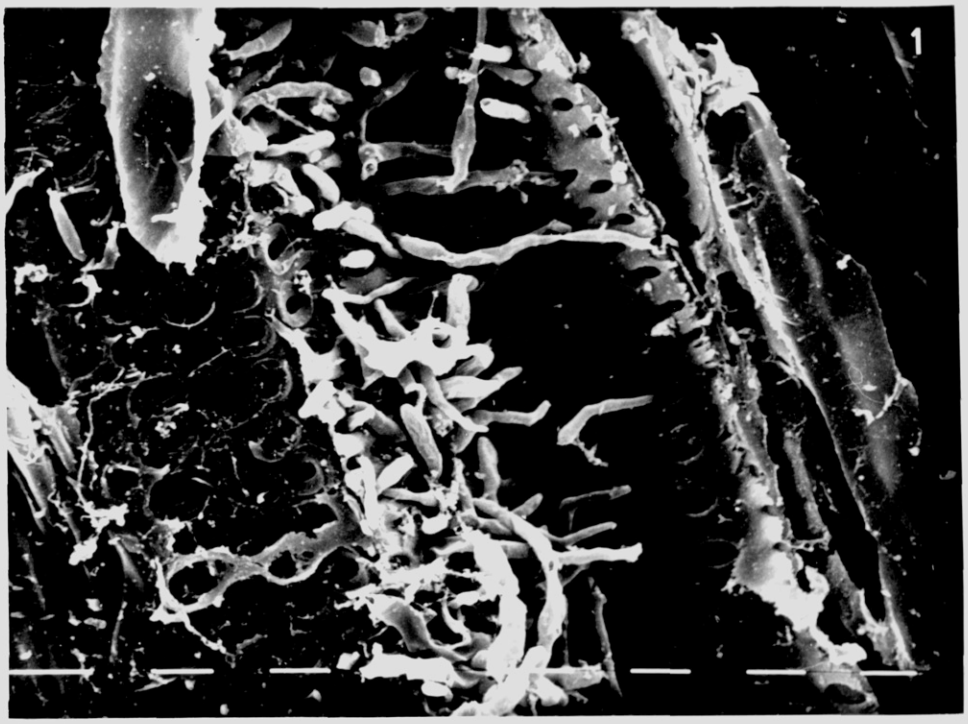
Plates 1-12 Scanning electron micrographs of *Fusarium oxysporum* f.sp. *lycopersici* in tomato plants cv. Craigella, CR & CS isolines. All specimens are critical point dried unless otherwise stated.

Plate 1 Mycelial colonisation by the pathogen in xylem vessels of CS root, 3 days after inoculation. (Specimen was freeze-dried).

X 800 Bar = 10 μ m

Plate 2 Detailed section of Plate 1 showing the lateral growth between adjacent vessels of hypha through pits (arrows).

X 1600 Bar = 10 μ m


1

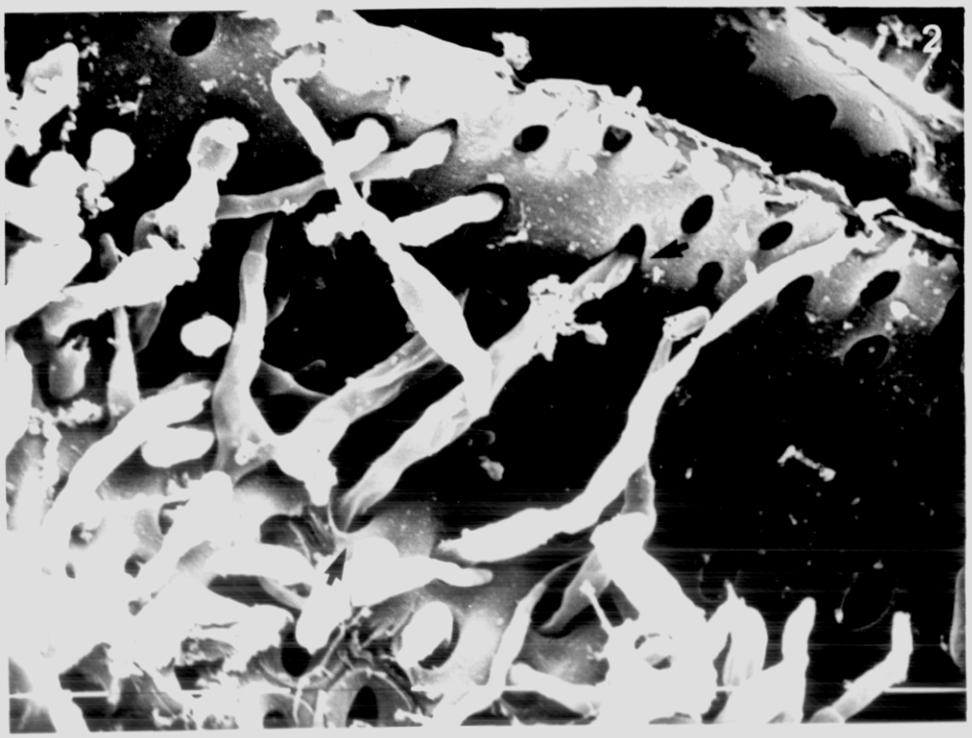
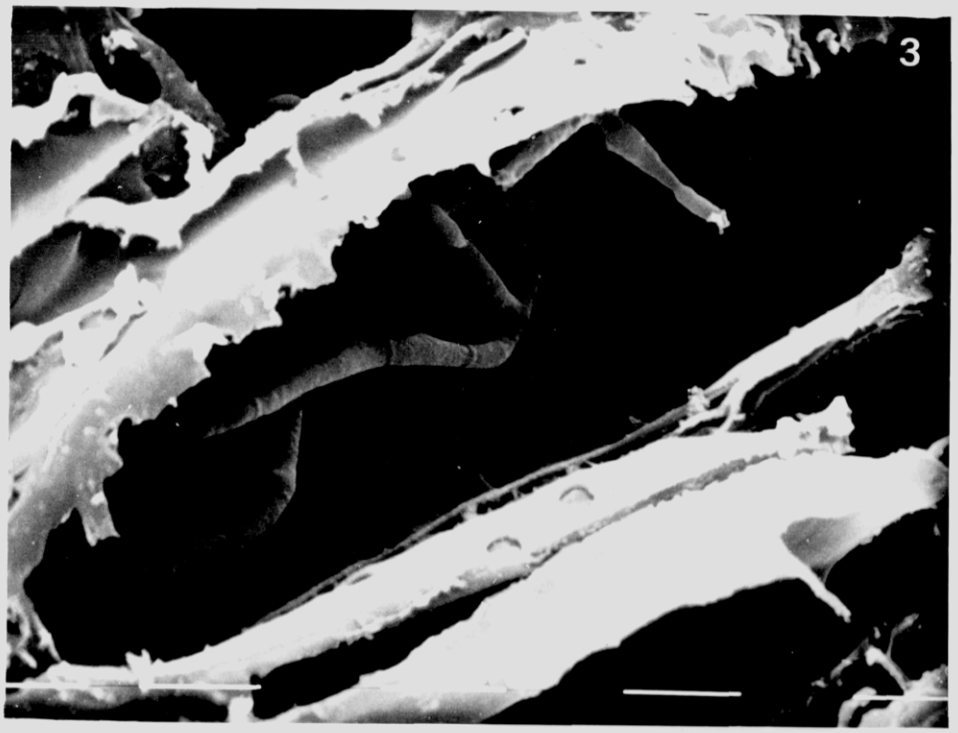

2

Plate 3 Hyphae of pathogen growing longitudinally through a xylem vessel of CS root, 4 days after inoculation. Note the sporulating hyphae and septa rings.

X 1600 Bar = 10 μ m

Plate 4 Longitudinal growth of hyphae in vessel of CR root, 8 days after inoculation. Note the absence of mycelia in adjacent vessels. (Specimen was freeze-dried).

X 800 Bar = 10 μ m


3

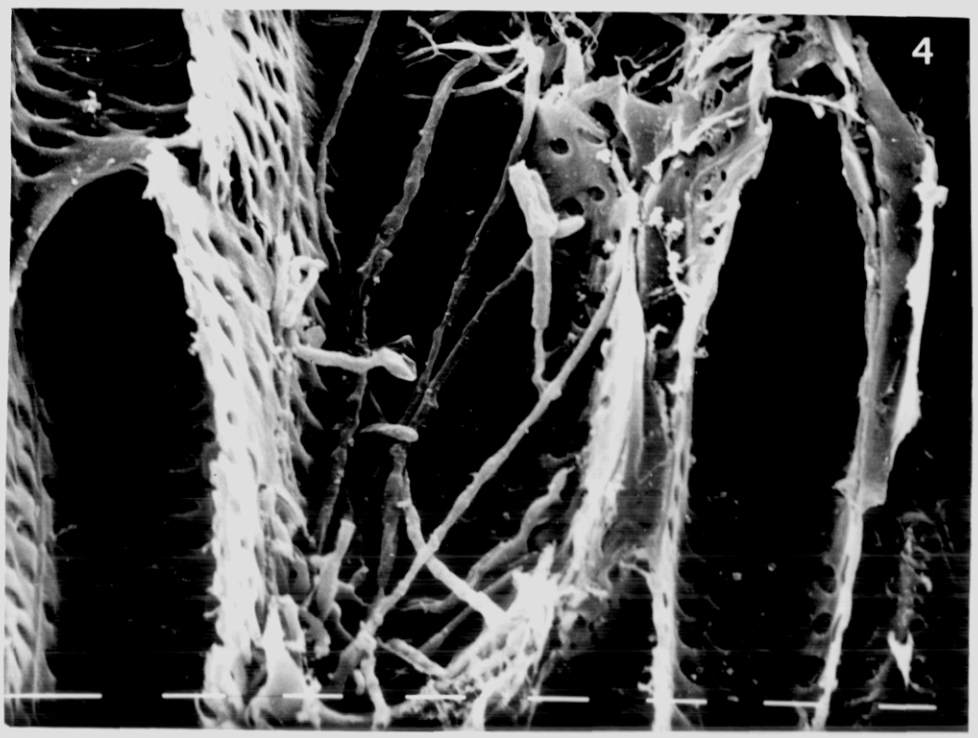
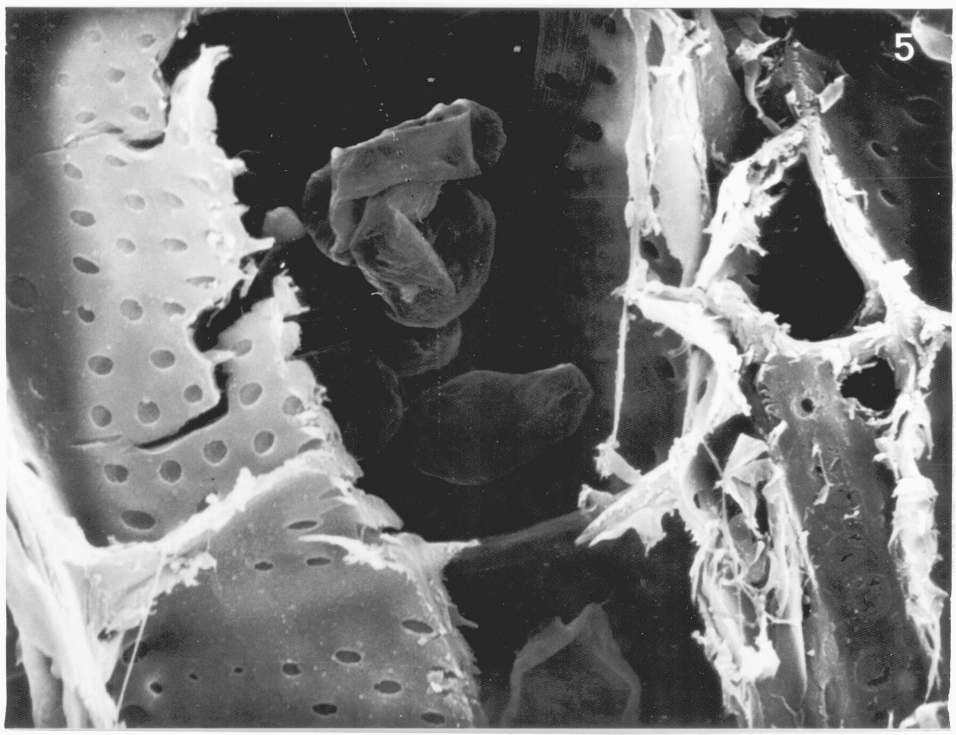

4

Plate 5 A group of tyloses (some showing collapse) in a xylem vessel of CR root, 8 days after inoculation.

X 800

Plate 6 Individual tyloses growing through pits in the vessel wall of CR root, 8 days after inoculation.

X 3200 Bar = 1 μ m


5

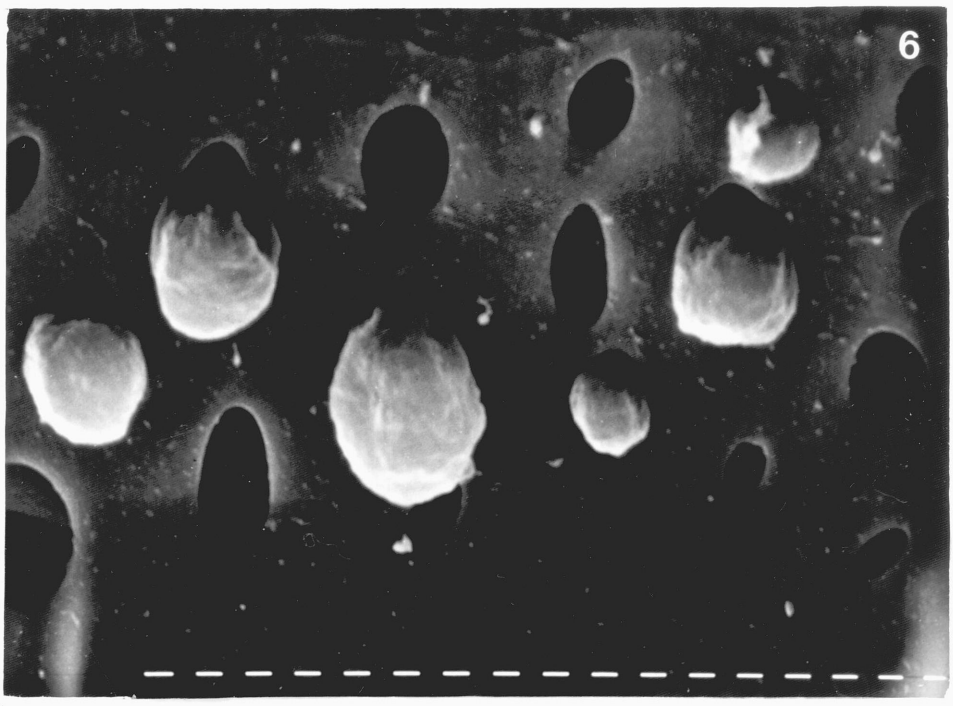
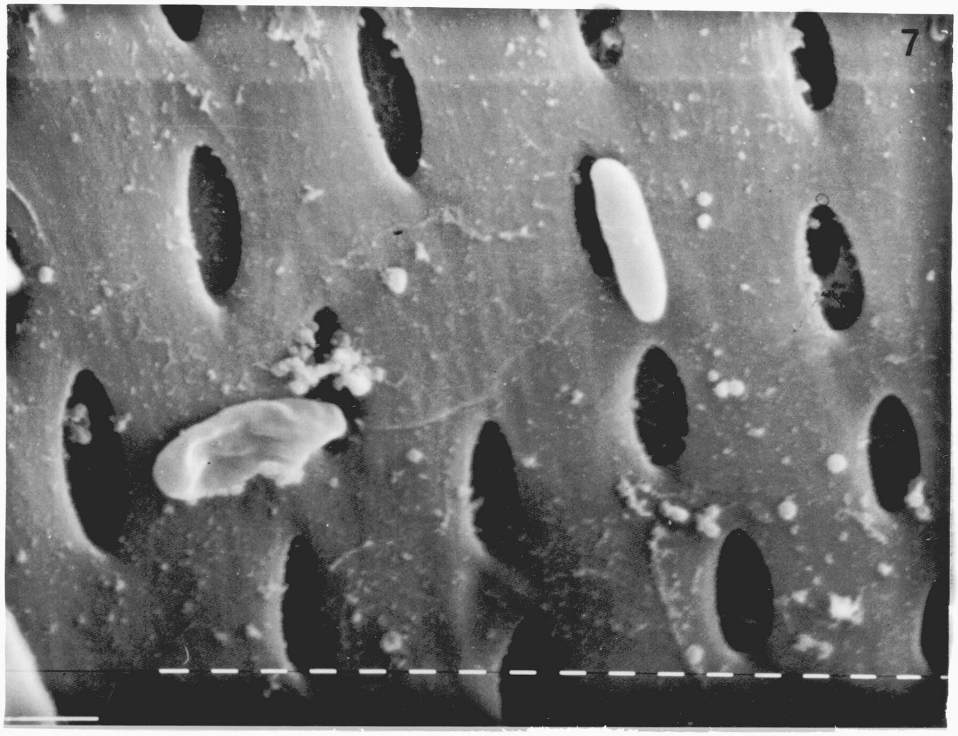

6

Plate 7 Conidia (one collapsed) on vessel wall of CS root, 4 days after inoculation.

X 3200 Bar = 1 μ m

Plate 8 A group of tyloses of varying sizes are clumped near the rim of a perforation plate of an open vessel element, from CR root, 8 days after inoculation.

X 800 Bar = 10 μ m


7

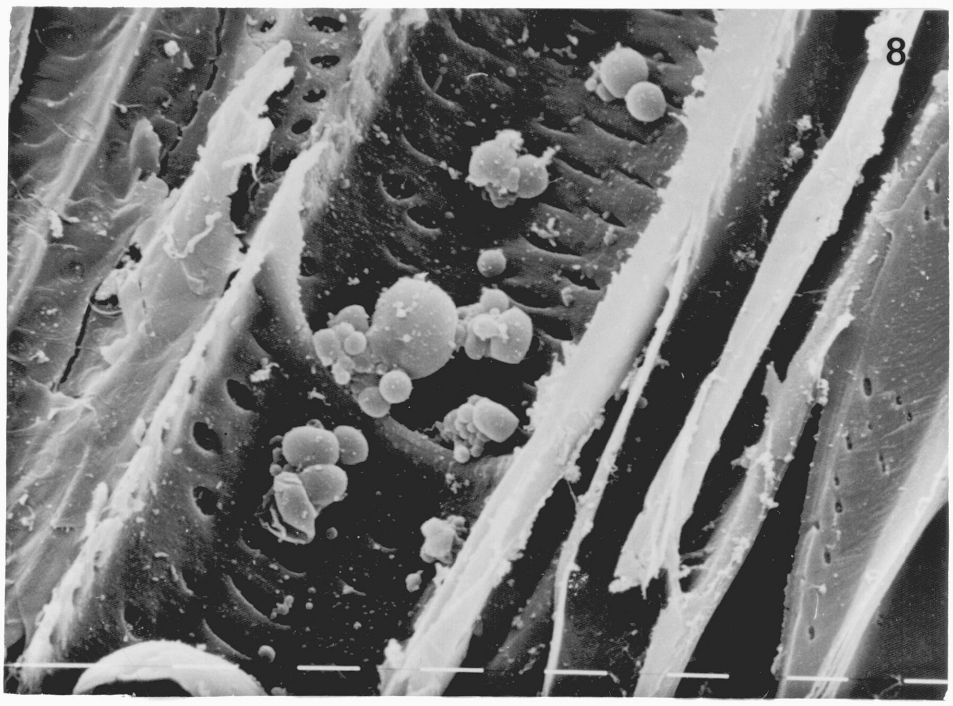
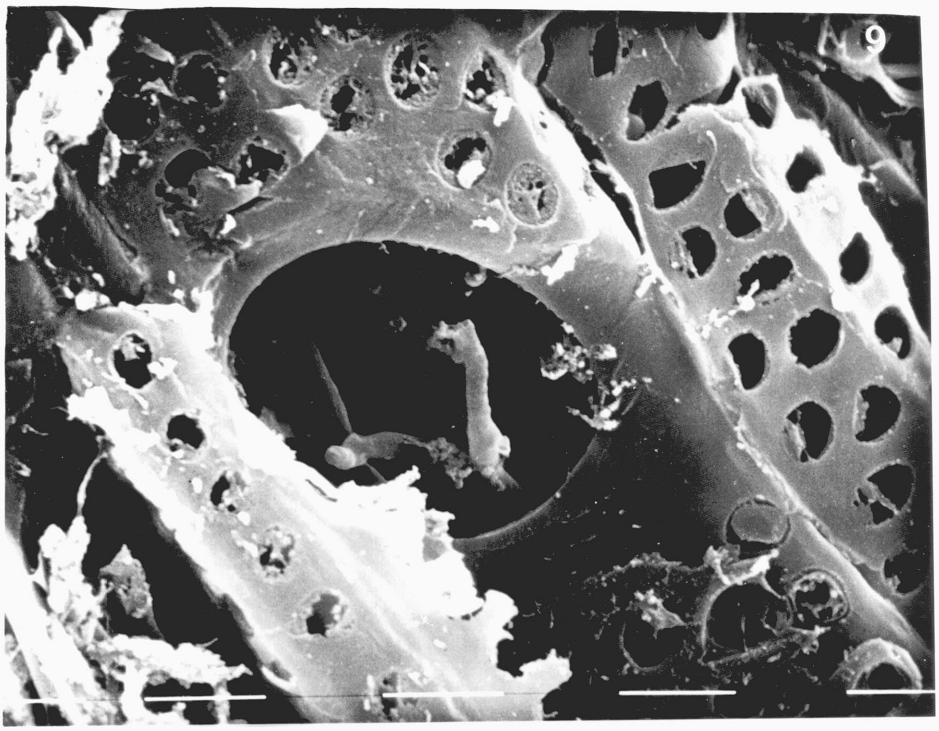

8

Plate 9 Hyphae, some branched, seen through a perforation plate in CS root, 8 days after inoculation.

X 1600 Bar = 10 μ m

Plate 10 A hyphal strand present in a xylem vessel of CS at the first node. Note the absence of mycelia in surrounding vessels.

X 800 Bar = 10 μ m


9

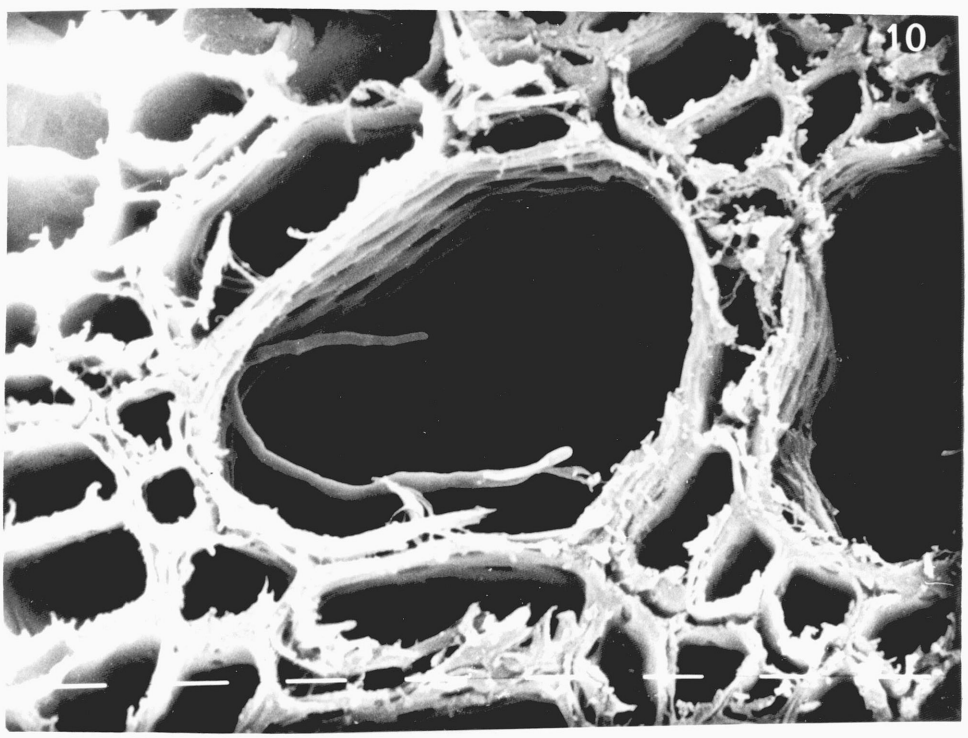

10

Plate 11 Extensive colonisation by mycelia of the pathogen in vessel elements of CS root, 8 days after inoculation. (Specimen was freeze-dried).

X 400 Bar = 10 μ m

Plate 12 Hyphae emerging through pits in the vessel wall of CS root, 8 days after inoculation. (Specimen was freeze-dried).

X 1600 Bar = 10 μ m

11


12

## 4.7. DISCUSSION

Studies of the colonisation of tomato plants by *Fol* introduced by root inoculation showed that the growth and spread of the fungus was limited in resistant but unlimited in susceptible isolines.

In resistant plants, the fungus was present in the root and the lower regions throughout the infection period although it was poorly established in aerial parts above the first node. The fungus was rarely, if at all, detected above the fifth node. The spread of the fungus was also erratic and it was infrequently encountered in lower petioles.

Vertical spread of the fungus, from the root in susceptible plants continued throughout the infection period with the plants eventually becoming totally colonised. The spread of the fungus in petioles was achieved acropetally. There was also more severe vascular browning in susceptible than in resistant tissue, as a result of the greater number of infected bundles. There was also an association between the severity of vascular and foliar symptoms.

These observations broadly agree with those of Mace & Veech (1971) in studies of the colonisation of tomato by *Fol* who showed that mycelium was not observed in resistant stems cv. Pearson VF-11 further than 40 mm above the cotyledonary node. Mycelium, however, was observed in the susceptible cv. Improved Pearson, 70 mm above the cotyledonary node. Scheffer & Walker (1954) also reported that the same pathogen was not detected in the upper stem of inoculated resistant tomato cuttings cv. Jefferson. Pegg (1978), in studies of *Verticillium albo-atrum* infection of tomato cv. Craigella isolines, observed a lesser degree of colonisation in resistant plants than susceptible. The decline in colonisation in resistant tissue was attributed to the death of the fungus or the growth of new tissue in apical regions by Pollock & Drysdale (1976) in their studies on *Verticillium* wilt of tomato.

While plating of tissue sections indicates the spatial distribution of the pathogen, the method of colony counts provides a quantitative estimate of the pathogen in host tissues. The results of the colony counts largely correspond with those obtained by the reisolation method. Colonies obtained from susceptible tissue at all sampling times were significantly greater than those obtained from resistant tissue. Although pathogen populations were initially high in the

roots of susceptible plants, a peak in the colonisation occurred in the region of the first node after 12 days incubation. The extent of colonisation by the pathogen was much reduced in resistant plants with most mycelium being restricted to the roots. There was no significant spread in resistant plants beyond 12 days of infection. Similar observations have been demonstrated by Harrison & Beckman (1982) in the colonisation of resistant and susceptible cotton cvs by *Verticillium dahliae* and *Fusarium oxysporum* f.sp. *vasinfectum*, using the colony count method. There was extensive colonisation in the susceptible cv. Rowden but the distribution of both *Verticillium* and *Fusarium* was restricted to the lower stem of the resistant cv. Seabrook Sea Island. Baayen & Elgersma (1985) using the colony count method in studies of the colonisation of carnation by *Fusarium oxysporum* f.sp. *dianthi*, also demonstrated that hyphae were localised effectively in resistant plants cv. Novada with a much lower level of colonisation than in the susceptible cv. Early Sam. However, Pegg & Street (1984) found greater colonisation in resistant hop plants infected with *Verticillium albo-atrum* than in the susceptible. The use of colony counts has also been popular in studies on *Verticillium* wilt of chrysanthemum (Pegg & Jonglaekha, 1981; Busch & Schooley, 1970).

Although a good estimate of the viable fungus present in host tissues, it has been suggested (Pegg & Jonglaekha, 1981) that the colony count method overestimates the amount of live pathogen present assuming that conidiation occurs in the vessels. Colonies counted may originate from a single microconidium or derive from a substantial mycelial strand. This method of estimating fungal biomass is biased in favour of the conidia present. It is concluded that the two methods (sampling by reisolation and colony counts) complement each other and together give an overall view of the distribution and state of the pathogen within host tissues. Other methods of chemically estimating fungal biomass by measurement of N-acetylglucosamine in chitin have been demonstrated by Ride & Drysdale (1971, 1972), but are open to problems of standardization.

These colonisation patterns for resistant and susceptible isolines are similar in both 2 week old seedlings and 4 week old plants. Growth of the pathogen was a little slower in seedlings grown in water culture.

It is apparent that secondary colonisation via translocated conidia occurs in the compatible host-parasite combination but this process is inhibited in the incompatible combination. Elgersma *et al* (1972) have reported greater colonisation by *F. oxysporum* f.sp. *lycopersici* in susceptible tomato isolines than in resistant ones. Conway & MacHardy (1978) also reported greater colonisation in susceptible tomato cvs infected with races 1 and 2 of the same pathogen, as well as in the resistant cv. infected with race 2. To account for these differences in colonisation patterns, Beckman (1964, 1968) has suggested that monogenic resistance to *Fusarium* wilt is dependent on the physical localisation of infection in vessels through vascular occlusion by gels and tyloses. It is reported (Beckman *et al*, 1972) that there is rapid initiation of tyloses in resistant hosts whereas tylose development is delayed in susceptible plants.

Studies using stem-inoculation did not reveal any significant difference between healthy and diseased plants. Quantities of the pathogen inoculum that do enter directly into the vascular system through the cut in the stem, may not be sufficient to cause infection. Clearly, inoculation through the root system is the more efficient method and the more natural too, the pathogen being soil-borne.

Results from SEM confirm and illustrate the colonisation patterns in resistant and susceptible hosts. Hyphal growth of the pathogen spreads vertically through the perforation plates and xylem end walls. Beckman *et al* (1972) reported spore trappings at perforation plates and end walls which enhanced secondary colonisation by mycelial growth and conidiation in the vessels. The lateral spread of the pathogen was achieved between adjacent vessels through bordered pit-pairs. Similar observations were reported by Stromberg & Corden (1980) in tomato plants cv. Bonny Best infected with *F. oxysporum* f.sp. *lycopersici*, and by Gbaja (1982) in tomato plants cv. Moneymaker. In the present investigation, there were no obvious differences observed in the growth of hyphae between resistant and susceptible isolines. Hutson & Smith (1983) using transmission electron microscopy, reported abnormal growth of *Verticillium albo-atrum* in root epidermal and cortical cells of the resistant tomato seedling only. There was no severe "blistering" of vessel walls as reported by Cooper & Wood (1974).

Tylosis was observed in resistant root vessels after infection and rarely in susceptible tissue. The maximal vascular occlusion by tyloses recorded in resistant hosts by Beckman *et al*

(1972) was not seen in samples taken up to 8 days after inoculation. Stromberg & Corden (1980) and Gbaja (1982) also reported the lack of vascular occlusion and tyloses in stem and root sections. The greater occurrence of tyloses in resistant than in susceptible tissue has also been reported by Hutson & Smith (1980) and Tjamos & Smith (1975) in *Verticillium* wilt of tomato as well as by Pegus (1976) in *Fusarium* wilt of tomato. Talboys (1958b) concluded that the failure of extensive colonisation by *Verticillium* in resistant hop cultivars was related to the extent of tylosis. These tyloses apparently blocked the distribution of the pathogen in vascular elements.

It becomes apparent that the effective localisation of the pathogen in resistant tissues by vascular occlusions is an important feature in the resistance mechanisms to *Fusarium* wilt in tomato plants.

## 5. THE EFFECT OF INFECTION ON β−1,3− GLUCANASE ACTIVITY IN HOST TISSUES

### 5.1. β−1,3− GLUCANASE ACTIVITY IN INFECTED HOST VASCULAR TISSUES

Resistant and susceptible Craigella plants were root-inoculated at 4 weeks old. At intervals after inoculation, enzyme extracts were prepared from excised stem vascular tissue (3 g each pooled from three plants) (section 2.6)

Initial experiments carried out 1 day after inoculation showed no changes in β−1,3− glucanase levels over that in controls. Enzyme assays were repeated at 5, 8, 12, 14 and 15 days after inoculation and show (Fig. 1) that an initial enhancement of activity in CR plants was reversed at day 8. Activity in all plants increased up to day 14, with activity in CS plants eventually reaching almost double that in CR plants. Controls contained negligible activity throughout the experiment.

### 5.2. CORRELATION BETWEEN β −1,3 −GLUCANASE ACTIVITY AND SPATIAL DISTRIBUTION OF *Fol* IN HOST TISSUES

Craigella plants of both isolines were root-inoculated at 4 weeks old (section 2.3.3). Half the total number of plants were used in the enzyme assay and the remainder was used to assess the distribution of the pathogen in parts of the plant by the colony count method (section 2.4.1).

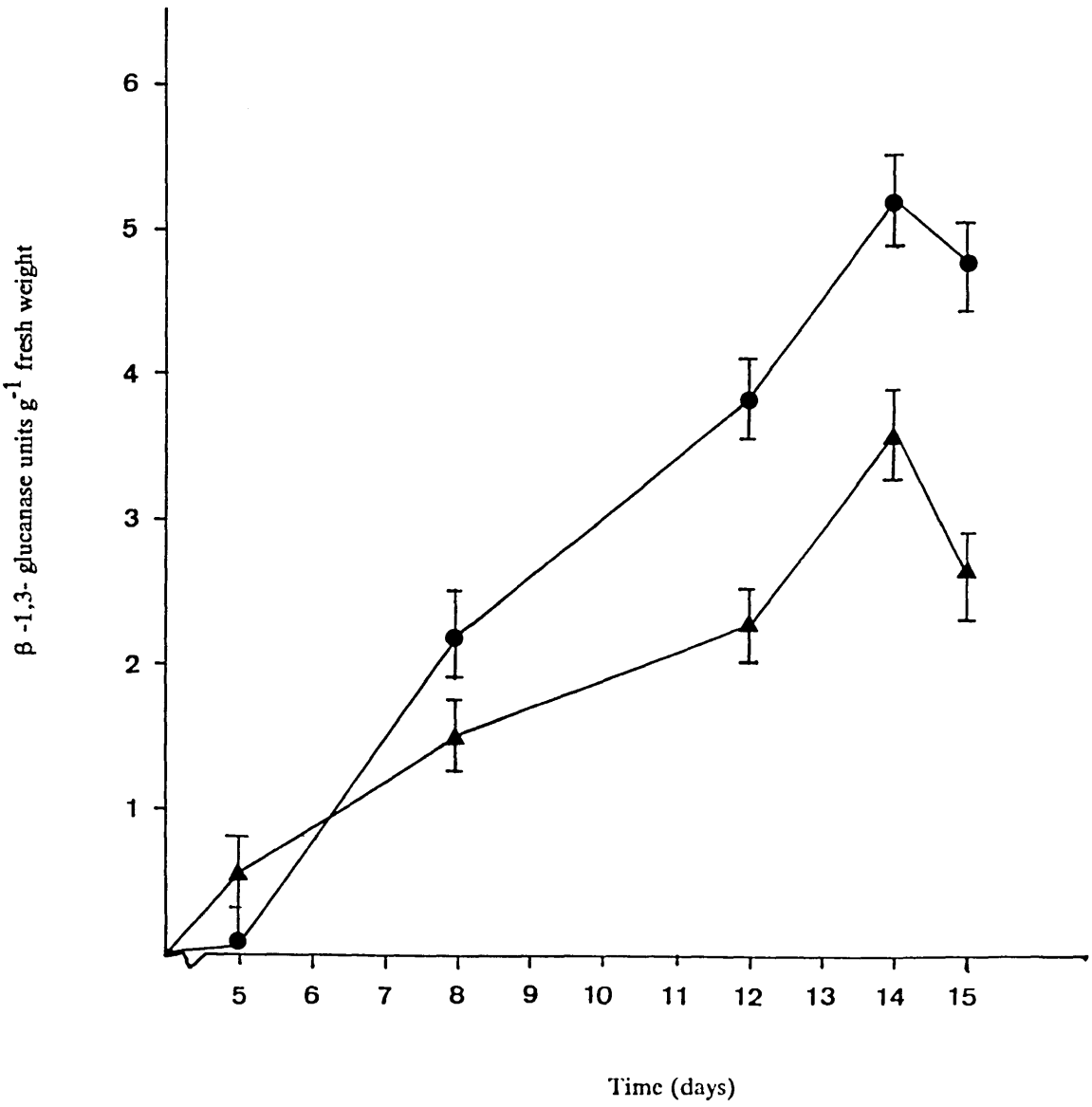
Samples (1 g) were taken from the root, cotyledonary node, first and third nodes of the plant and prepared for colony counts (section 2.4.1) as described earlier. Final homogenates and respective dilutions were plated on PDA plates with antibiotics (section 2.2.1.1) and incubated at 20 °C.

In the enzyme assay (section 2.6.3), preparation of enzyme extracts was carried out using 2 g of excised stem vascular tissue from the above-mentioned regions of the plant. Enzyme extracts after dialysis were stored at -20 °C until assayed.

Results from the colony counts (Fig. 2a & b) show similar trends to results obtained previously (as presented in Tables 7a & b). Much greater numbers of *Fol* were obtained from susceptible than resistant plants.

Fig 1 Changes in β -1,3- glucanase activity following days after inoculation in Craigella R (▲) and S (●) tissues.

(mean of 3 readings with confidence limits at P=0.05)



*Fol* was detected in root samples of both isolines at all sampling times. In CR root tissue, a peak in numbers which occurred at day 2 gradually declined to day 7. In CS, the peak occurred after day 6. In the cotyledonary node of both isolines CR and CS, peaks in colony numbers occurred at day 7 and day 6 respectively. *Fol* was detected in small amounts after day 6 in the first and third nodes of CR, whereas numbers of colonies reached their highest in the first node of CS plants.

Results obtained from the enzyme assay (Fig. 3a & b) show that levels of β−1,3− glucanase activity are greater in roots than in stems. Control healthy plants also show more activity in root tissue than in stem tissue (Fig. 4a & b). Enzyme activity in the stem tissues in CS is greater than those in CR, especially after day 6. In general, enzyme activity increased following time after infection.

Correlation coefficients (*r*) were calculated to assess the degree of correspondence between *Fol* and β−1,3− glucanase activity. Table 12a shows *r* values for both resistant and susceptible isolines at intervals after infection. A high degree of correlation was obtained for CR at days 2, 5 and 6, and at days 2 and 5 only for CS plants.

However, for *r* values calculated at individual regions of the plant (Table 12b), a high degree of negative correlation was obtained for CR root samples only. The other nodal regions showed little correlation between *Fol* and enzyme activity for both resistant and susceptible plants. There was no overall correlation between the number of colonies of *Fol* and β−1,3− glucanase activity.

Table 12a Correlation coefficient, *r* between *Fol* and β -1,3- glucanase activity at individual intervals after infection for Craigella resistant (CR) and susceptible (CS) plants

| | Days | | | |
|---|---|---|---|---|
| | 2 | 5 | 6 | 7 |
| CR | 0.999 | 0.989 | 0.947 | 0.06 |
| CS | 0.994 | 0.999 | -0.297 | -0.394 |

Table 12b Correlation coefficient, *r* between *Fol* and β -1,3- glucanase activity at individual regions for CR and CS plants

| | Regions of the plant | | | |
|---|---|---|---|---|
| | RT | CN | N1 | N3 |
| CR | -0.833 | 0.439 | 0.466 | 0.508 |
| CS | 0.309 | 0.496 | 0.486 | 0.197 |

Legend:

RT root

CN cotyledonary node

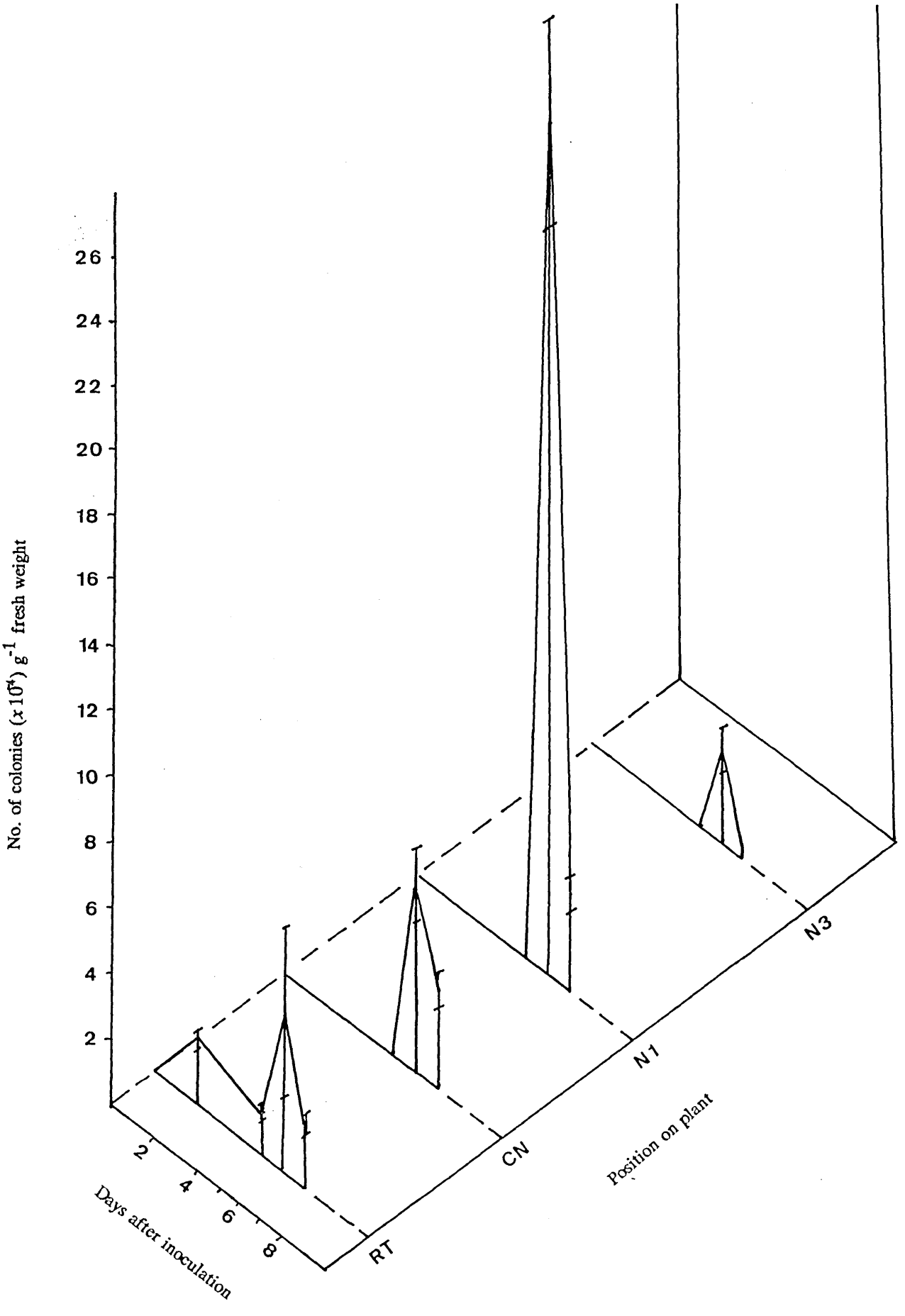
N1 first node

N3 third node

CR Craigella resistant

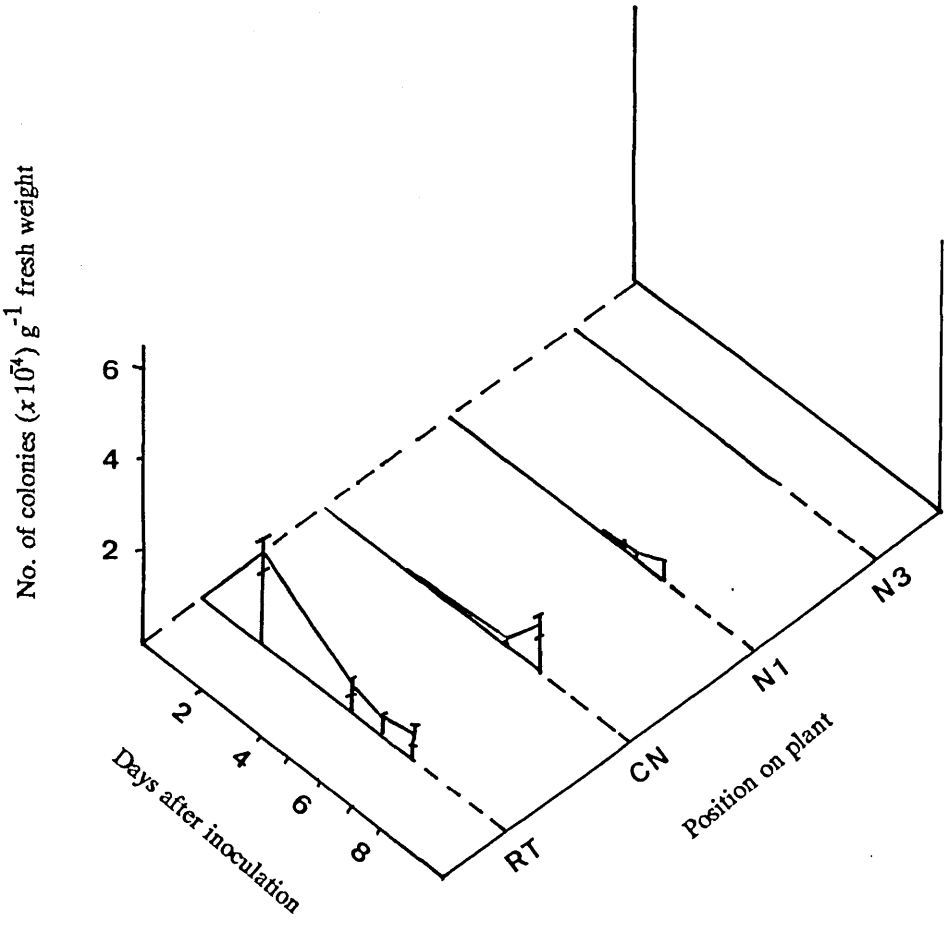
CS Craigella susceptible

Fig 2a The distribution of *Fol* in CS plants



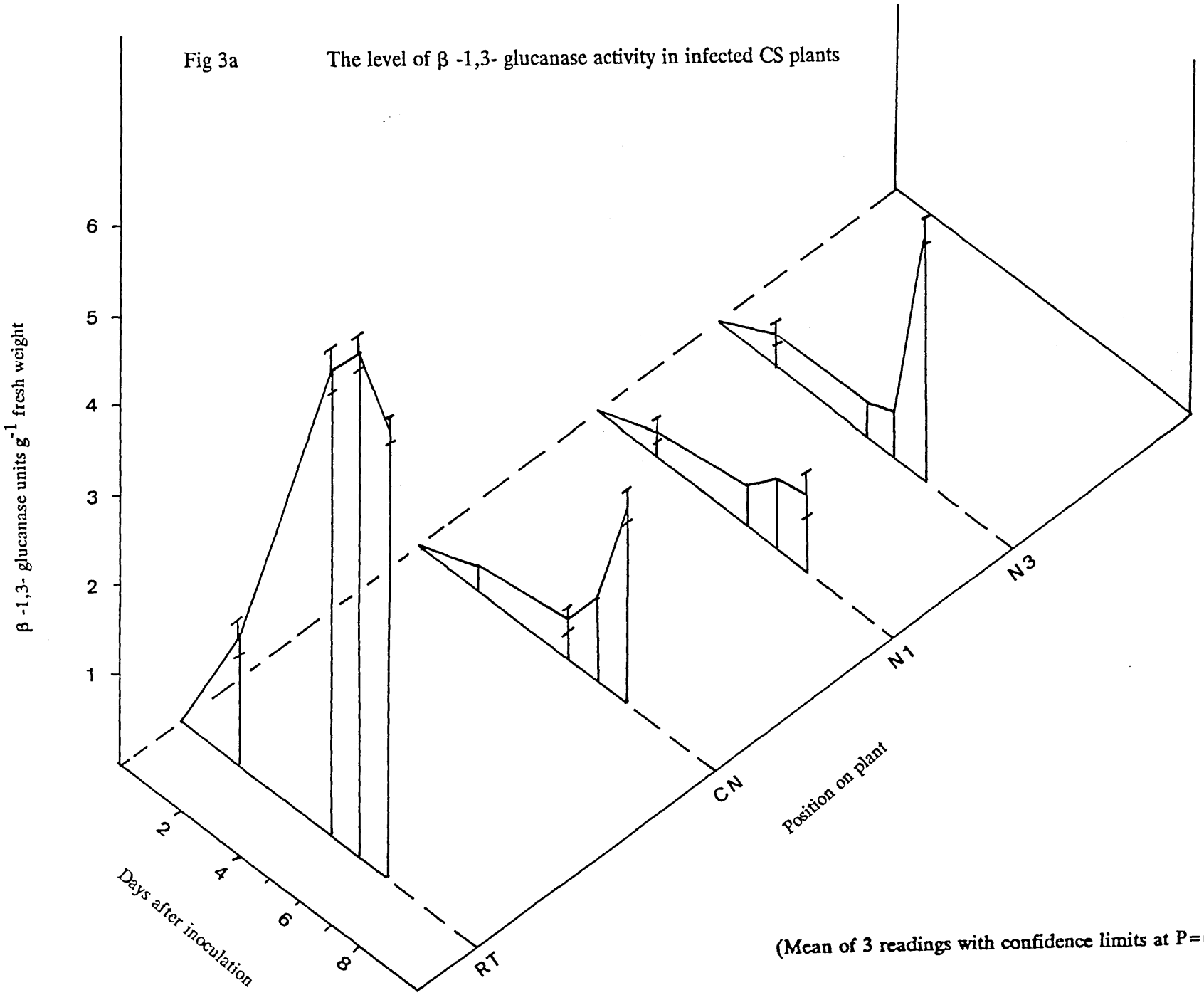

(Mean of 4 dilution plates with confidence limits (P=0.05))

Fig 2b The distribution of *Fol* in CR plants



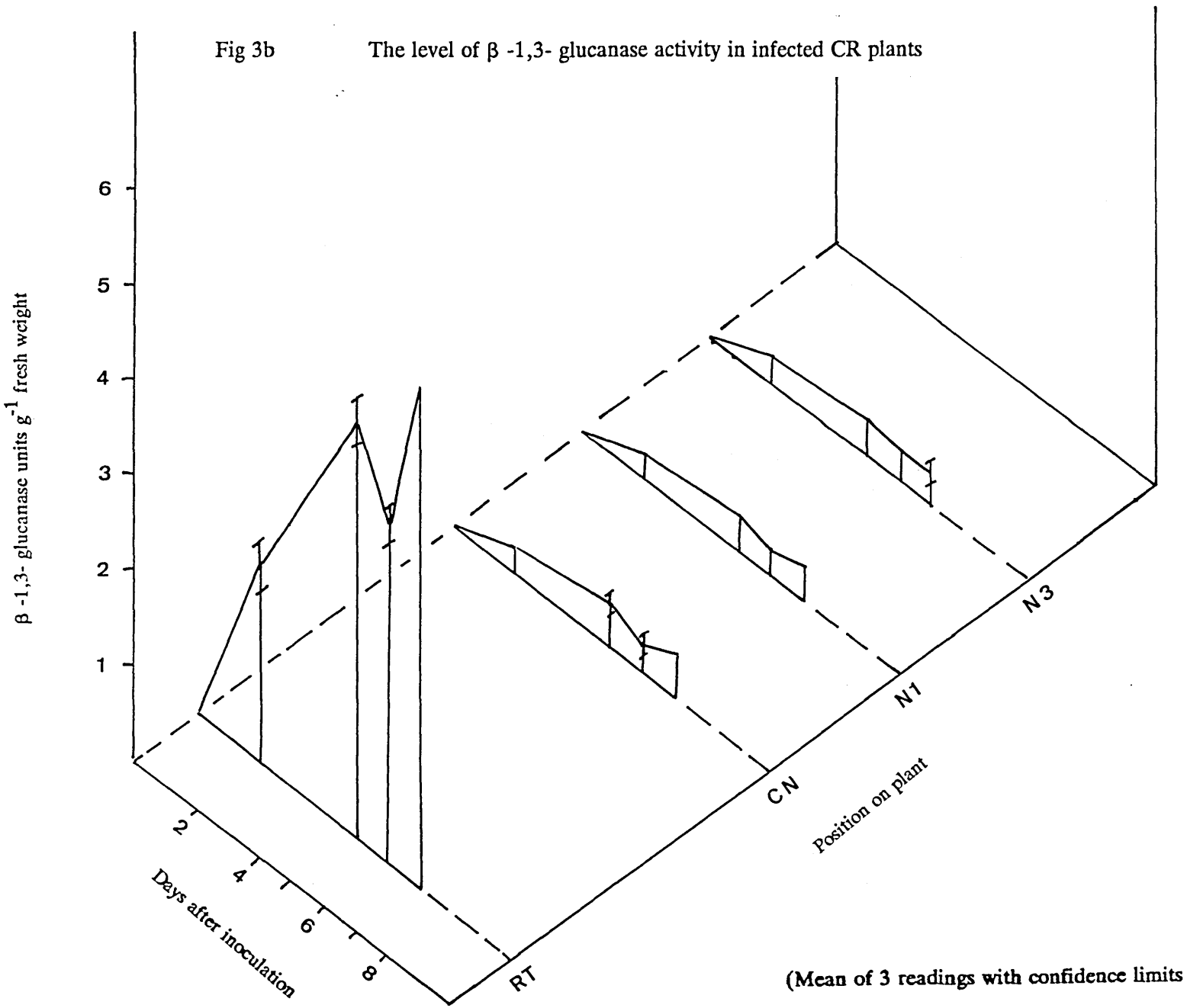

( Mean of 4 dilution plates with confidence limits (P=0.05))

Fig 3a The level of β -1,3- glucanase activity in infected CS plants



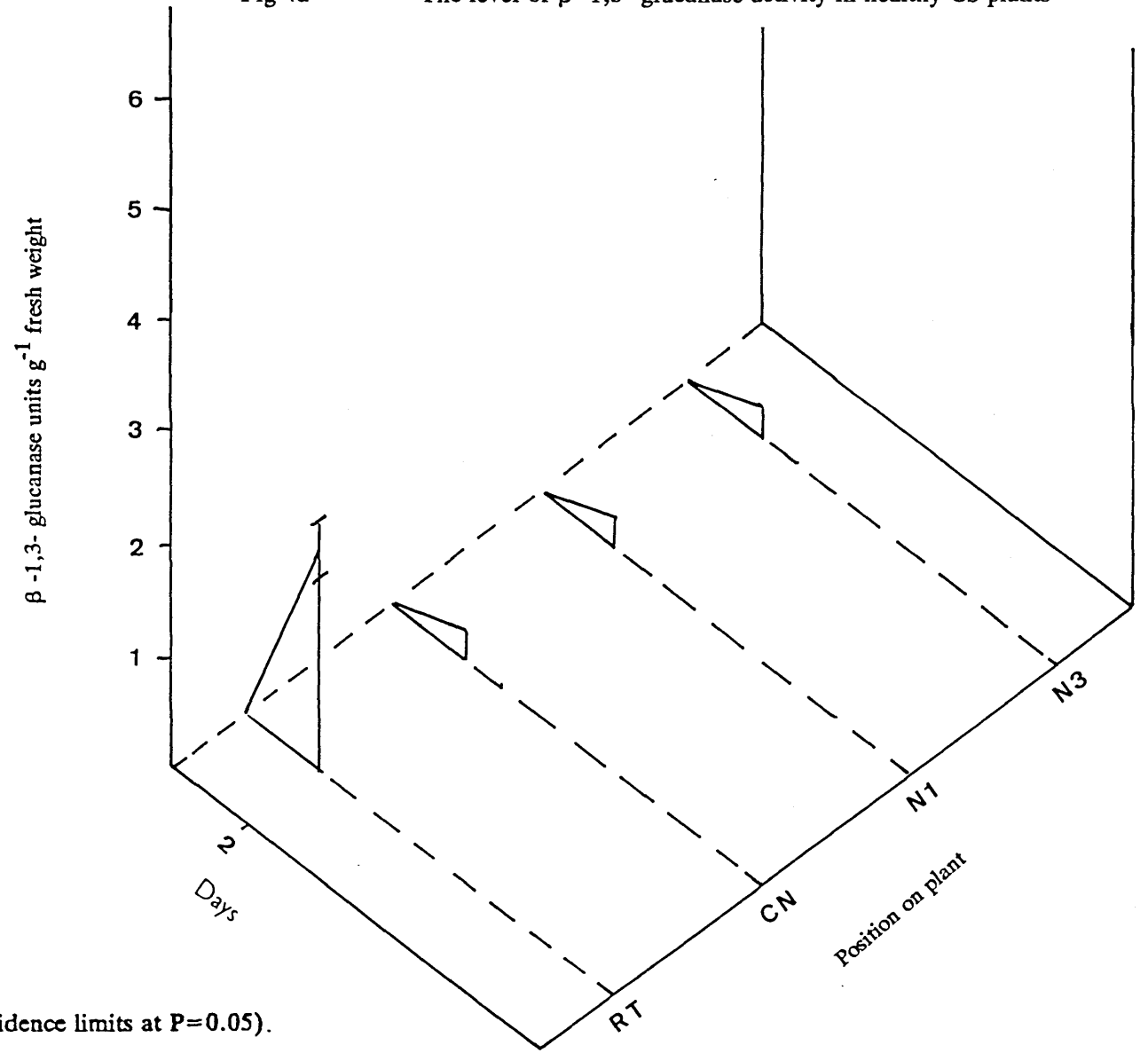

(Mean of 3 readings with confidence limits at P=0.05).

Fig 3b The level of β -1,3- glucanase activity in infected CR plants



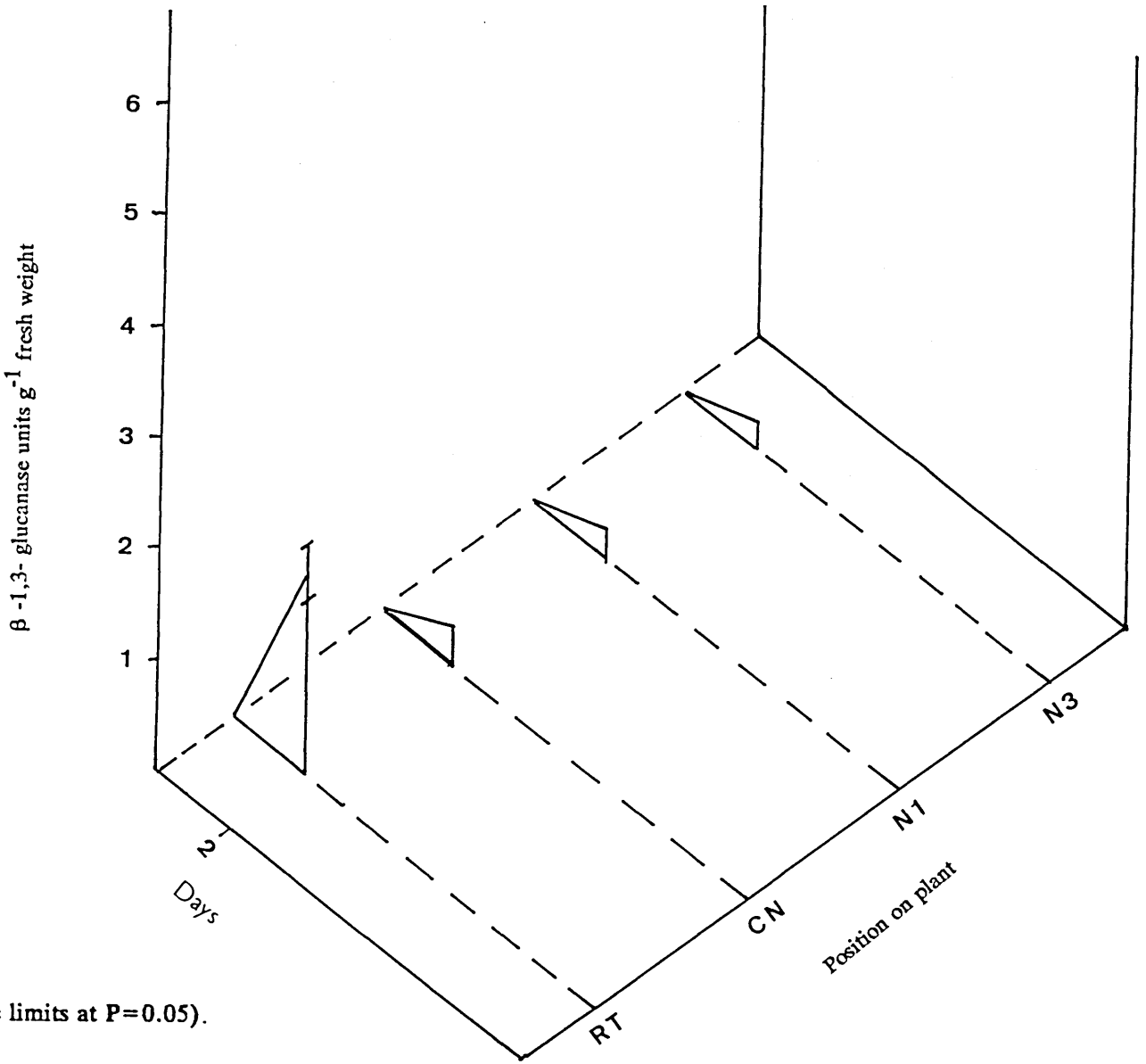

(Mean of 3 readings with confidence limits at P=0.05).

Fig 4a The level of β -1,3- glucanase activity in healthy CS plants




(Mean of 3 readings with confidence limits at P=0.05).

Fig 4b The level of β -1,3- glucanase activity in healthy CR plants




(Mean of 3 readings with confidence limits at P=0.05).

## 5.3. THE EFFECT OF THE TEST FLUIDS ON β−1,3− GLUCANASE ACTIVITY IN DETACHED PETIOLES

Detached Craigella petioles without leaflets from 6 weeks old plants (both CR and CS isolines) were infiltrated with test fluids (as in section 2.3.6). The test fluids used were sterile distilled water; culture filtrate (prepared as in section 2.3.2); and inoculum suspension (prepared as in section 2.3.1). Additional untreated controls were included. All petioles were kept in moist chambers and incubated in a growth cabinet at 20 °C, (16 h photoperiod). Enzyme extraction and assay were carried out after 3 and 6 days using 2 g fresh weight (pooled from five petioles) for each treatment sample.

Results (Fig 5a & b) show that enzyme activity is enhanced when petioles were treated with test fluid as compared with control petioles. Spore inoculum and culture filtrate induced the greatest activity in petioles (approximately double the activity in controls). The responses of both isolines, CR and CS to the different treatments were similar. Samples taken after 6 days showed a similar pattern of response.

## 5.4. β−1,3− GLUCANASE ACTIVITY IN PETIOLES FROM HEALTHY AND INFECTED PLANTS

Craigella R and S isolines were root-inoculated (section 2.3.3) at 4 weeks old. Controls were sham-inoculated with distilled water. Seven days after inoculation, enzyme extracts were prepared from 3 g samples of petioles (from first and third nodes) from healthy and infected plants of both isolines (section 2.6) and subsequently used to assay β−1,3− glucanase activity (section 2.6.3).

Results (Fig. 6) shows that β−1,3− glucanase activity is highest in infected CS tissues, (double the activity recorded in infected CR tissues). Healthy controls of both isolines showed similar but much lower enzyme activity.

## 5.5. β−1,3− GLUCANASE ACTIVITY IN XYLEM EXUDATES

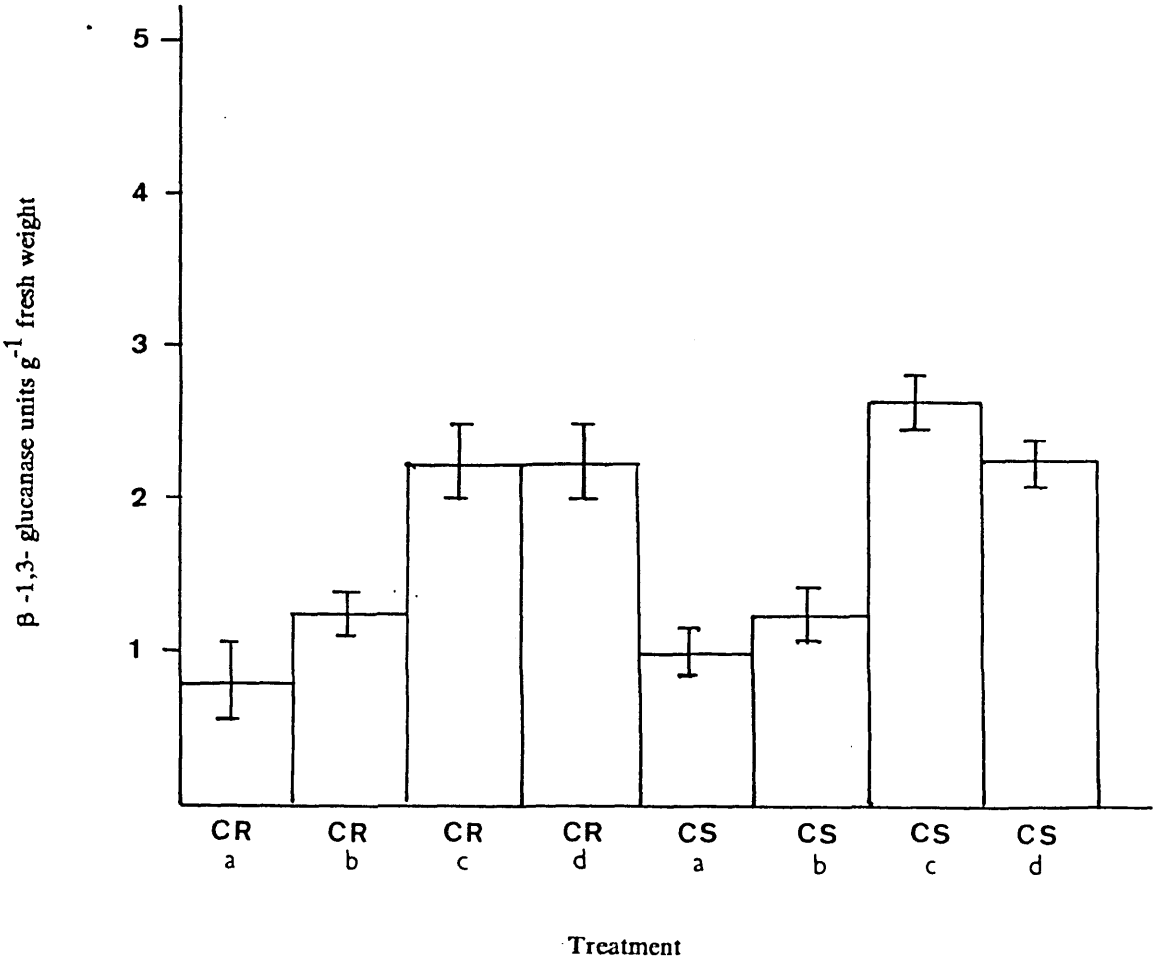
Using intact plants from the above experiment (section 5.4) that had been inoculated for 7 days, plants were each severed at the cotyledonary node (8 plants from each treatment were

Fig 5 β -1,3- glucanase activity in treated detached petioles after a) 3 days, b) 6 days . (Mean of 3 readings with confidence limits at P=0.05).

Legend:

| | |
|---|---|
| CR | Craigella resistant |
| CS | Craigella susceptible |
| a | Control petioles |
| b | Petioles infiltrated with sterile distilled water |
| c | Petioles infiltrated with culture filtrate |
| d | Petioles infiltrated with spore suspension |

Fig. 5 a)


β -1,3- glucanase units g-1 fresh weight
5
4
3
2
1
CR a
CR b
CR c
CR d
CS a
CS b
CS c
CS d
Treatment

b)

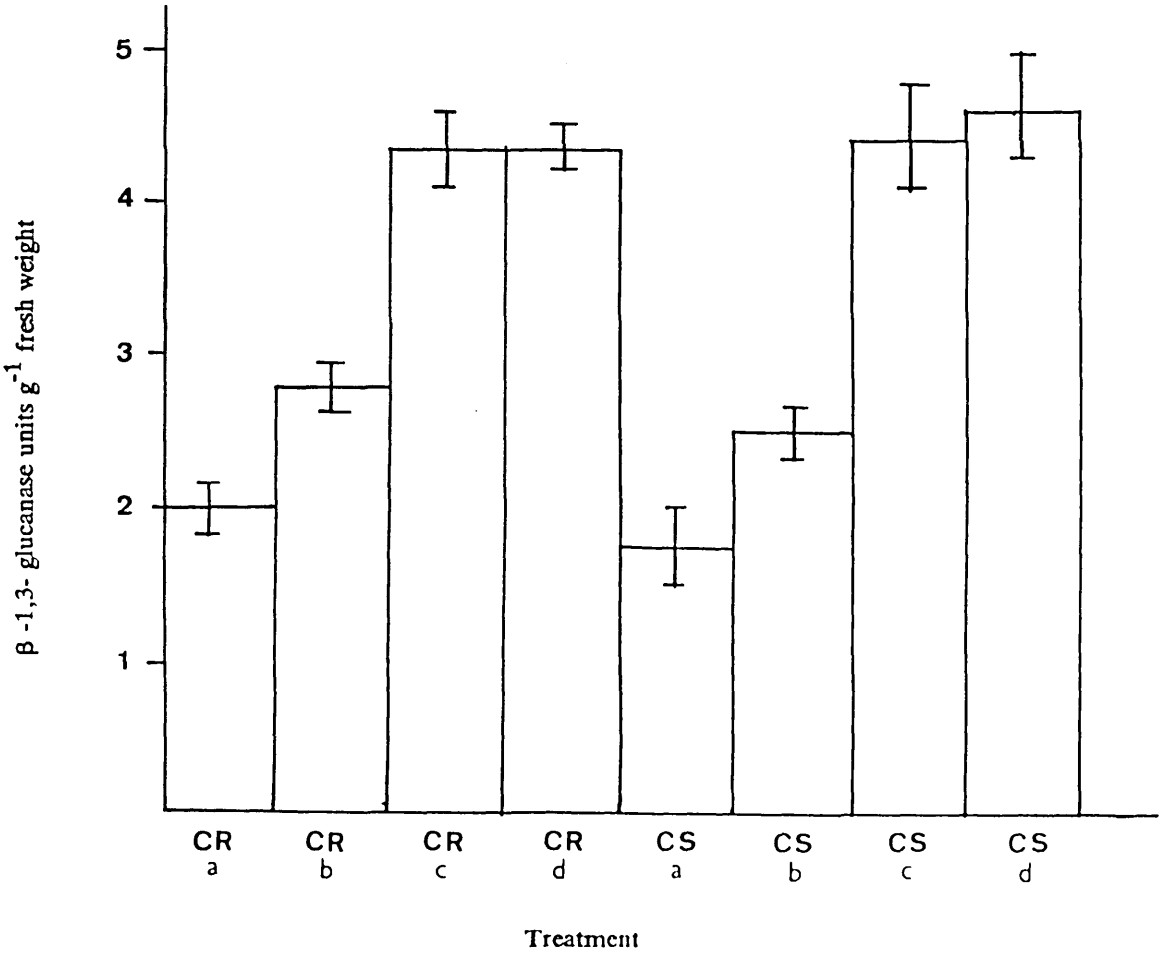

β -1,3- glucanase units g-1 fresh weight
5
4
3
2
1
CR a
CR b
CR c
CR d
CS a
CS b
CS c
CS d
Treatment

Fig 6 β -1,3- glucanase activity in healthy (h) and infected (i) Craigella R and S petioles 7 days after inoculation .

(Mean of 3 readings with confidence limits at P=0.05).

Fig 7 β -1,3- glucanase activity in xylem exudates from healthy (h) and infected (i) Craigella R and S plants 7 days after inoculation .

(Mean of 3 readings with confidence limits at P=0.05).

Fig. 6

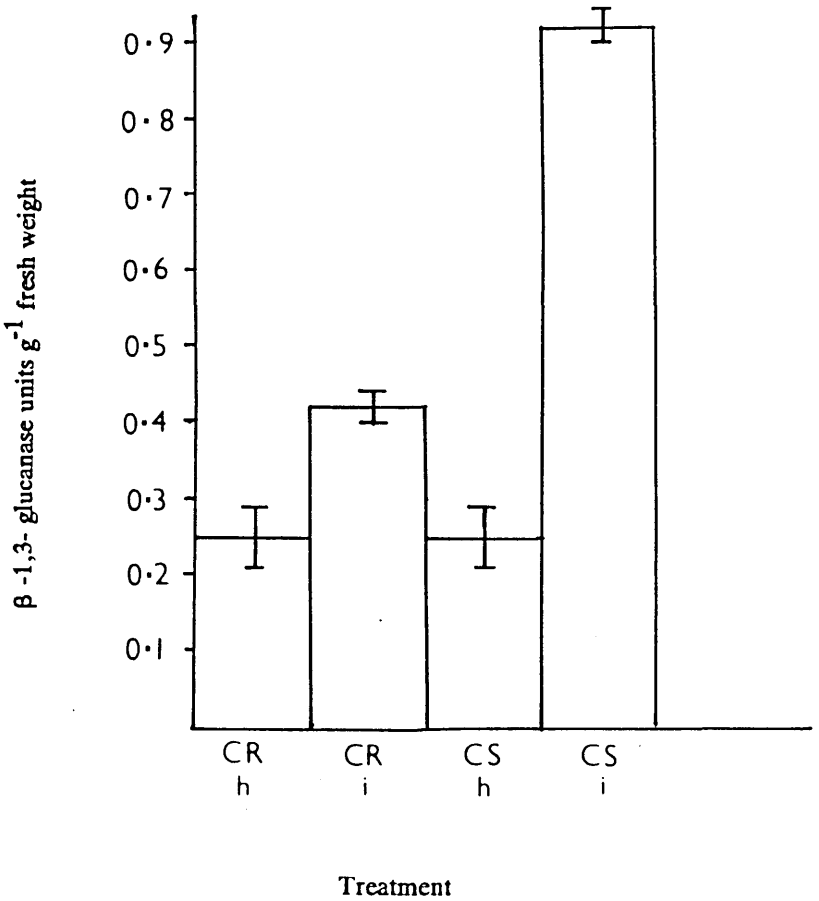
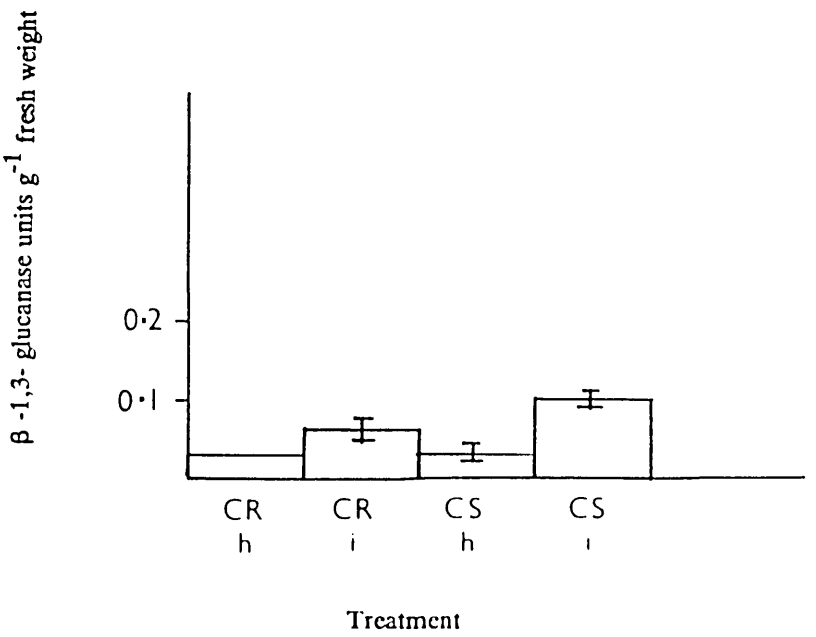

β -1,3- glucanase units g-1 fresh weight
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
CR h
CR i
CS h
CS i
Treatment

Fig. 7


β -1,3- glucanase units g-1 fresh weight
0·2
0·1
CR h
CR i
CS h
CS i
Treatment

used). The exudates were collected by Pasteur pipetting into Universals over a period of 2 - 3 h. The final volume (4 ml) was filter-sterilized (0.22 μ m pore diameter) and kept at -20 °C until assayed. 0.2 ml of the exudate was assayed using the standard procedure (section 2.6.3).

Results (Fig. 7) show that very little β−1,3− glucanase activity was recorded in xylem exudates. The highest activity occurred in the exudate from infected CS plants, (almost double that from infected CR plants). Exudates from control plants again show similar but much lower activity. This pattern of response in term of enzyme activity is similar to results obtained for enzyme activity found in healthy and infected petioles (Fig. 6).

## 5.6. DISCUSSION

Infection of Craigella isolines caused large increases in β−1,3− glucanase activity in the root and stem. These levels were greater in the susceptible isolines than in the resistant, and at least double the base levels present in healthy plants. There were no innate differences in enzyme levels between healthy CR and CS plants although higher levels were recorded in the root than in the stem. Hence, increased enzyme activity following infection could be due to fungal enzyme or an induction of host enzymes in response to the pathogen.

In experiments studying the relationship between host colonisation and increased enzyme activity in infected tissue, the two factors were highly correlated at days 2 and 5 for the susceptible isoline and at days 2, 5, and 6 for the resistant isoline. This relationship is perhaps less evident in susceptible hosts at day 6 when fungal colonisation is at its highest then in the stem. This implies that the amount of pathogen present at this stage greatly exceeds the levels of enzyme produced. Correlation at regional level (i.e. in root, hypocotyl or nodes) was less evident in both isolines. In general, there was an increase in enzyme activity following an increase in the pathogen in plants (except in the roots where the correlation is inverse). As susceptible plants were more colonised than resistant plants, there were higher levels of enzyme produced in CS than CR plants. Enzyme activity recorded in infected plants was higher in the root than in the stem (similarly in healthy plants). It would seem that induced enzyme activity is due to a localised host response to the pathogen i.e. more enzyme is produced to counter the amounts of fungus present (see Fig. 3a).

Pegg (1976) and later, Pegg & Young (1981) reported similar responses to *Verticillium*-infected Craigella isolines in which the levels of glycosidases were greater in susceptible than resistant isolines. Pegg & Young (1981) observed an inverse correlation between host glycosidases activity and *Verticillium albo-atrum* colonisation in both susceptible and resistant. These glycosidases were later confirmed to be of host origin (Young & Pegg, 1981) and have been suggested to play a role in the defence mechanism by fungal lysis. Lysis of *Verticillium albo-atrum* hyphae in tomatoes has been reported by Blackhurst & Wood (1963) as well as by Dixon & Pegg (1969). Fungal autolysis suggesting involvement of β−1,3− glucanase and chitinase has also been reported by Pegg & Vessey (1973).

In *Fusarium* infection of muskmelon, Netzer *et al* (1979) found larger increases of β−1,3− glucanase activity in the resistant isoline than in the susceptible and suggested that the enzyme played a role in the defence mechanism for monogenic resistance to muskmelon wilt.

Mauch *et al* (1984) reported increased levels of β −1,3- glucanase and chitinase in immature pea pods after infection with a pea pathogen, *Fusarium solani* f.sp *pisi* and a non-pathogen of pea, *F. solani* f.sp *phaseoli*. Both enzymes were also induced by autoclaved spores of the two pathogens, as well as by ethylene and known elicitors of phytoalexin in pea pods.

In the present investigation, isolated petioles that were treated with spore inoculum or culture filtrate showed much greater enzyme activity than untreated controls. The levels of enzyme in response to these two treatments were very similar. This could be explained by the localised response of the host to the presence of the pathogen or to its metabolites present in the culture filtrate. As the culture filtrate was sterile, the induced enzyme is likely to be entirely of host origin. Hence, it is concluded that the increase in enzyme activity in spore-treated petioles is also of host origin. The fact that infiltrated petioles showed a greater increase in enzyme activity than that of petioles from healthy and infected plants indicate that the response is more likely to be a localised one. Enzyme activity increased with time, consistent with a host response being the cause. Disappointingly, the responses of CR and CS isolines were identical, frustrating hopes that β−1,3− glucanase induction might constitute an early differential response related to resistance and provide the basis for a bioassay of fungal elicitors of resistance.

Experiments with petioles taken from healthy and infected plants confirm earlier studies using stem vascular tissue showing that enzyme activity was greater in susceptible than in resistant plants. Studies on host colonisation (section 4.1) previously, show that petioles are colonised secondarily to the stem. This limited host colonisation in the petioles, especially in the resistant isoline may also account for the reduced levels of enzyme activity observed in petioles (from healthy and infected plants) compared with enzyme levels observed in infected root and stem tissues. Enzyme activity increases as colonisation progresses showing that enzyme production is a localised response and is not directly involved in any resistance mechanisms. As a result of the higher levels of glycosidase activity present in CS than in CR plants, there is a possibility that host enzymes might release toxic molecules from fungal cell walls. These toxic molecules, concentrated in susceptible tissue, may contribute to the disease by causing severe wilting. This has been demonstrated by Keen *et al* (1975) in studies on mycolaminarins ($\beta-1,3-$ glucans) from *Phytophthora* species.

Very little enzyme activity was found in xylem exudates. This is reasonable since the exudates are not truly representative of the environment in infected vessels. In such studies where the levels of enzyme activity in crude tissue extracts are measured, it is important to consider whether these levels reflect those present in the *in vivo* environment, the colonised vessels. The measured levels of high and low enzyme activities also need to be considered relative to the *in vivo* system, using the adopted assay method.

A number of roles have been proposed for glucanases in plants, including hyphal lysis (Wargo, 1975) and callose removal from phloem in grape vine (Clark & Stone, 1962). Fungal $\beta-1,3-$ glucanases have been reported as constitutive digestive enzymes (Reese & Mandels, 1959) and glucanases of *Claviceps purpurea* are stated to maintain nutrient supply to the pathogen by hydrolysing callose (Dickerson *et al*, 1978). Glucanase probably also has a role in hyphal growth and differentiation, branching or autolysis. Schlumbaum *et al* (1986) reported the antifungal activity of bean chitinase to *Trichoderma viride* and implicated a combined effect with co-induced $\beta-1,3-$ glucanase. These enzymes whether working independently or in combination are efficient inhibitors of fungal growth. Young & Pegg (1982) reported greater activity on fungal cell walls when tomato endo-$\beta-1,3-$ glucanase was mixed with *Verticillium*

*albo-atrum* exo-β−1,3− glucanase or β-glycosidase.

# 6. INFECTION OF TOMATO WITH *Fol* : GRAFTING EXPERIMENTS

## 6.1. THE EFFECTS OF GRAFTING AND INFECTION ON RESISTANT AND SUSCEPTIBLE TOMATO ISOLINES

Near-isogenic lines of tomato cv. Craigella resistant (CR) and susceptible (CS) were grafted (section 2.7) at 4 weeks old using the following scion/stock combinations : resistant/susceptible (CR/CS), susceptible/resistant (CS/CR), and the control combinations resistant/resistant (CR/CR) and susceptible/susceptible (CS/CS). Immediately after grafting, plants wilted slightly but recovered after being placed in a damp chamber.

After 2 weeks when the graft unions had fused successfully, half the total number of plants were inoculated (section 2.3.3), the remainder being similarly treated with distilled water as controls. Additional controls were also included using ungrafted plants, of which half were inoculated in the usual manner (section 2.3.3) and half were sham-inoculated with distilled water. Heights of plants were measured from the cotyledonary node to the apex at inoculation and again after 3 weeks when samples were taken for reisolation of the fungus (section 2.4). This measurement was expressed as a percentage increase in the growth of the plants.

After an incubation period of 2 weeks, disease symptoms such as petiole epinasty, yellowing of leaflets often with necrosis and wilting were observed in the infected CS plants as well as in the grafted plants with susceptible stocks. Lower petioles were often abscised in the more severely infected plants. CR/CS plants were severely stunted and showed the most severe wilt symptoms. Graft combinations with resistant stocks (CR/CR and CS/CR) did not show any wilt symptoms and were visually similar to the infected resistant plants. Some of these grafted plants had the occas ional lower leaf that was yellowed with necrotic leaflet margins but upper leaves remained healthy.

Upon reisolation after 3 weeks incubation, *Fol* was detected in all root and hypocotyl (immediately above the graft union) regions from all 4 graft treatments and also from inoculated ungrafted plants (Table 13a). The ungrafted CS plants were the most heavily colonised with *Fol* detected as high as the seventh node. Grafted CS/CS plants were colonised as far as the fifth node. *Fol* was detected only as far as the hypocotyl with traces in the first

Table 13a Distribution of *Fol* in inoculated grafted and ungrafted plants using cv Craigella resistant (CR) and susceptible (CS) isolines

| Treatment | Position | | | | | |
|---|---|---|---|---|---|---|
| scion/stock[a] | RT | HC | N1 | N3 | N5 | N7 |
| CR/CR | +* | +* | -* | -* | - | - |
| CS/CS | +* | +* | +* | +* | +* | - |
| CR/CS | +* | +* | (+)* | (+)* | -* | -* |
| CS/CR | +* | +* | -* | -* | -* | - |
| ungrafted[b] | | | | | | |
| CR | +* | +* | +* | -* | - | - |
| CS | +* | +* | +* | +* | +* | +* |

Legend:

*a* mean of 3 plants sampled

*b* mean of 4 plants sampled

RT root

HC hypocotyl (immediately above graft union)

N1,3... first, third,.. node

\+ *Fol* present

\- *Fol* absent

(+) *Fol* present in some, not all replicates

* vascular browning

and third nodes of CR/CS grafted plants, although severe wilt symptoms were observed. In grafted plants with resistant stocks (CR/CR and CS/CR) *Fol* was detected as far as the hypocotyl and in ungrafted resistant, it was detected as far as the first node.

Browning of vascular tissue was observed upon sampling during reisolation of the pathogen. Apart from the areas where the fungus could be reisolated, browning was also observed in the upper scion where the fungus was not present. This was often related to the extent of symptoms e.g in CR/CS plants and in CS, the whole of the upper scion showed vascular browning (Table 13a), and this was visible up to the fifth node in CS/CS plants. In grafted plants with resistant stocks as well as with the inoculated ungrafted CR, where there was less fungal colonisation, vascular browning was visible in the upper scion up to the third node although this was sometimes extended to the fifth node in CS/CR plants (where a susceptible scion was involved).

In general, reisolation of *Fol* was not very consistent in the regions above the graft union and often yielded other contaminant fungi. This was often found in samples taken from the hypocotyl zone and above. A *Trichoderma* species was sometimes identified from plated sections. Colonies of yeasts and bacteria were greatly reduced with the use of antibiotic PDA plates (section 2.2.1.1).

The mean percentage increase in height of plants from each treatment are presented in Table 13b. For statistical analysis, the data were transformed into logarithmic form (this was based on the assumption that the proportional increase in each treatment was constant) and confidence limits at $P = 0.05$ were calculated. The standard error of the difference (S.E.D) and the least significant difference (L.S.D) are given in logarithmic form as well. An analysis of variance was performed (for details see appendix) and showed a significant interaction between the treatments (This assumes that the scions and stocks behave independently whether they are resistant or susceptible). This significant interaction in inoculated grafted plants can be seen in Fig. 8a. In the case of un-inoculated grafted plants, there was no significant interaction (Fig. 8b).

The largest percentage increase in growth of inoculated plants was achieved by the CR/CR

plants, followed by CR, CS/CR and CS/CS plants. CR/CS plants grew least (Table 13b). There were significant differences (at P = 0.05) in percentage increase in height between CR/CS plants and the other graft treatments (Table 13b). There were no significant differences in percentage increase in height between the healthy graft treatments.

Comparing the height increments in ungrafted plants, healthy CR and inoculated CS plants differed significantly at P = 0.05, as did healthy and inoculated CS plants. There were no significant differences between healthy CR and CS.

Table 13b Comparison of % increase in height between healthy and inoculated grafted and ungrafted plants using CR and CS isolines

| Treatment | Inoculated | | Healthy | |
|---|---|---|---|---|
| scion/stock[a] | mean | log mean | mean | log mean |
| | % increase in height | | % increase in height | |
| CR/CR | 318.31 (217.27,460.26)[c] | 2.5 | 500.36 (313.33,797.99)[c] | 2.699 |
| CS/CS | 253.24 (172.19,364.75) | 2.399 | 309.07 (184.50,469.89) | 2.469 |
| CR/CS | 97.77 (63.10,133.66) | 1.963 | 420.19 (237.14,603.95) | 2.578 |
| CS/CR | 271.41 (181.13,383.70) | 2.421 | 341.85 (213.80,544.50) | 2.533 |
| | S.E.D[d] (D.F=8) = 0.100 | | S.E.D (D.F=8) = 0.124 | |
| | L.S.D(D.F=8,P=0.05)=0.231 | | L.S.D(D.F=8,P=0.05)=0.287 | |
| ungrafted[b] | | | | |
| CR | 275.83 (185.78,393.55) | 2.432 | 326.41 (221.82,469.89) | 2.509 |
| CS | 197.56 (121.339,257.04) | 2.247 | 376.64 (250.61,530.88) | 2.562 |
| | S.E.D (D.F=12) = 0.106 | | | |
| | L.S.D(D.F=12,P=0.05)=0.231 | | | |

Legend:

*a* mean of 3 plants per treatment

*b* mean of 4 plants per treatment

*c* lower and upper confidence limits (P=0.05)

*d* S.E.D & L.S.D are given in logarithmic form

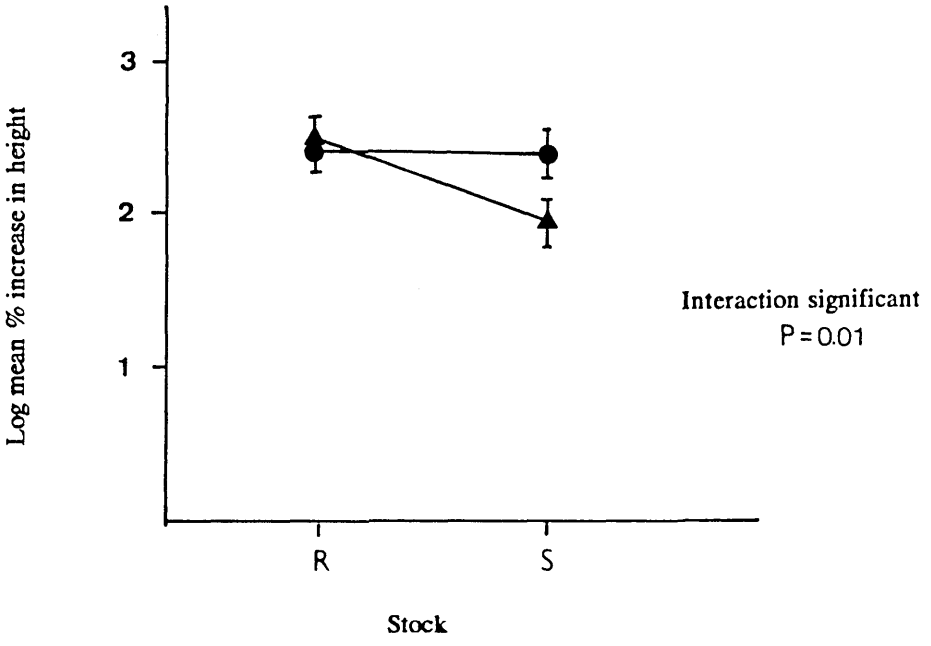
Fig 8 a and b Graphs showing the interaction between all possible scion-stock graft combinations .

Legend:

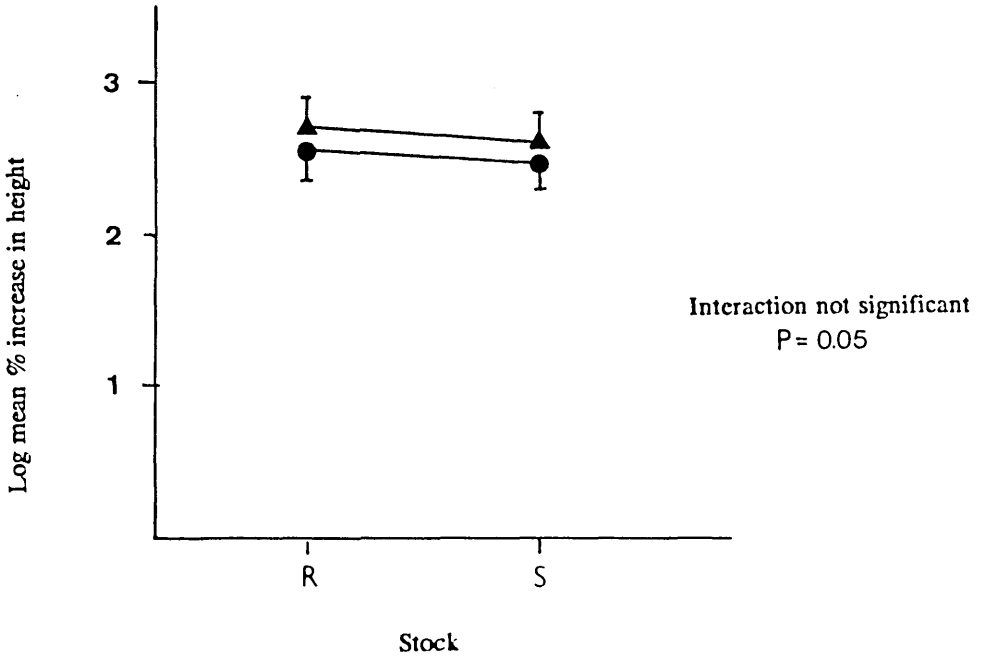
▲ R scion

● S scion

Fig. 8 a) Inoculated Craigella graft combinations in section 6.1




b) Healthy Craigella graft combinations in section 6.1



## 6.2. COMPARISON OF INFECTION UPON GRAFT COMBINATION USING CVS CRAIGELLA, BONNY BEST AND WALTER

All plants were grafted at 4 weeks old and the following scion/stock combination were used : CR/CR, CS/CS, CR/CS, and CS/CR. Also employed were cvs Walter (WA) which is resistant and Bonny Best (BB) which is susceptible to *Fol* race 0, in the following combinations : WA/WA, BB/BB, WA/BB, and BB/WA. Percentage increases in height of the plants were observed for up to 3 weeks after inoculation (section 2.3.3).

Both groups of grafted plants showed similar trends in symptom expression after inoculation. In general, plants with susceptible stocks showed more intense symptoms than those with resistant stocks (Plates 13 & 14). The most susceptible combination was that of resistant scion/susceptible stock (i.e CR/CS and WA/BB). These plants were very stunted and had yellowed petioles with necrotic patches (Plate 15 & 16). Petioles on the WA/BB plants were yellowed with vein-clearing of the leaflets, suggesting a hypersensitive reaction. Grafts involving susceptible cvs (CS/CS and BB/BB) showed mild wilt symptoms with stunting, chlorotic and epinastic petioles (Plate 17). Grafts involving resistant cvs (CR/CR and WA/WA) showed no visible wilt symptoms. The combination of greatest apparent resistance was susceptible scion on resistant stock (i.e CS/CR and BB/WA). These plants showed the greatest percentage increase in height (Table 14b) and were visually symptomless and healthy (Plate 18). Comparing the two groups, the Bonny Best and Walter grafted plants were generally shorter in height than the grafted Craigella plants.

Upon reisolation of the pathogen from plants after 3 weeks inoculation (Table 14a), both groups showed similar trends in pathogen colonisation. The susceptible graft combination, CS/CS and BB/BB were colonised by *Fol* up to the fifth node, although traces only of *Fol* were found in the upper region of the plant. In the resistant combinations CR/CR and WA/WA, *Fol* was detected as far as the first node and hypocotyl region respectively. In the most heavily diseased graft combination, CR/CS and WA/BB, *Fol* was detected at the hypocotyl and first node region only. In the reciprocal graft combination (i.e CS/CR and BB/WA), *Fol* was detected as far as the hypocotyl in both cases, although traces were detected up to the third node in BB/WA.

Browning of vascular cells was also observed in sections taken for reisolation of the pathogen (section 2.4). Grafts having susceptible stocks showed more intense vascular browning, extending from the root to most of the upper scion. Grafts with resistant stocks exhibited less extensive browning, visible as far as the first or third nodes only.

Height increments for the different graft combinations are shown in Table 14b. There were significant differences between all treatments except between CR/CR and CS/CS and also between WA/WA and BB/WA. Interaction between the treatments in each group was not significant (Fig. 8c & d).

Table 14a Distribution of *Fol* in graft combinations using cv. Craigella isolines (CR & CS) cv. Walter (WA) (resistant) & cv. Bonny Best (BB) (susceptible)

| Treatment | Position on plant | | | | |
|---|---|---|---|---|---|
| scion/stock[a] | RT | HC | N1 | N3 | N5 |
| CR/CR | + * | + * | + * | − * | − |
| CS/CS | + * | + * | + * | + * | (+) * |
| CR/CS | + * | + * | − * | − * | − * |
| CS/CR | + * | + * | − * | − * | − |
| WA/WA | + * | + * | − * | − | − |
| BB/BB | + * | + * | (+) * | (+) * | (+) * |
| WA/BB | + * | + * | + * | − * | − |
| BB/WA | + * | + * | (+) * | (+) * | − |

Legend:

*a* mean of 4 plant per treatment

RT root

HC hypocotyl (immediately above the graft union)

N1,3... first, third.... node

\+ *Fol* present

\- *Fol* absent

(+) *Fol* present in some, not all replicates

* vascular browning

Table 14b Percentage increase in height of graft combinations using cv. Craigella (CS & CR), Walter (WA) and Bonny Best (BB) after infection

| Treatment | Mean | Log Mean |
|---|---|---|
| scion/stock[a] | % increase in height | |
| CR/CR | 181.13 (141.25,229.09)[b] | 2.255 |
| CS/CS | 149.35 (116.68,189.23) | 2.172 |
| CR/CS | 93.20 (69.18,112.20) | 1.945 |
| CS/CR | 269.05 (209.41,339.63) | 2.426 |
| | | S.E.D[c] (D.F=12) = 0.068 |
| | | L.S.D (D.F=12,P=0.05) = 0.149 |
| WA/WA | 159.66 (122.74,207.49)[b] | 2.203 |
| BB/BB | 111.64 (84.33,142.56) | 2.04 |
| WA/BB | 54.88 (39.72,67.14) | 1.713 |
| BB/WA | 169.05 (129.12,218.27) | 2.225 |
| | | S.E.D (D.F=12) = 0.074 |
| | | L.S.D (D.F=12,P=0.05) = 0.161 |

Legend:

*a* mean of 4 plants sampled for each treatment

*b* lower and upper confidence limits (P=0.05)

*c* S.E.D & L.S.D are given in logarithmic form

Plate 13 Craigella graft combinations CR/CR, CS/CS, CR/CS and CS/CR 3 weeks after infection with *Fol.*

(Scale 10 mm = 65 mm)

Plate 14 Walter-Bonny Best graft combinations WA/WA, BB/WA, WA/BB and BB/BB 3 weeks after infection with *Fol.*

(Scale 10 mm = 50 mm)

Plate **13**




Plate **14**



Plate 15 Reciprocal graft combinations CS/CR and CR/CS, 3 weeks after infection with *Fol.*

Note the extreme wilt symptoms (epinasty, wilting, stunting, marginal necrosis) in CR/CS compared with the apparently healthy CS/CR plants.

(Scale 10 mm = 40 mm)

Plate 16 Lower leaves from a CR/CS plant showing wilting, chlorosis and necrosis.

Plate 15




Plate 16



Plate 17 A wilting petiole with chlorotic leaves (some with necrotic edges) from a CS/CS plant.

Plate 18 A healthy petiole with leaflets from a CR/CR plant.

Plate 17



Plate 18



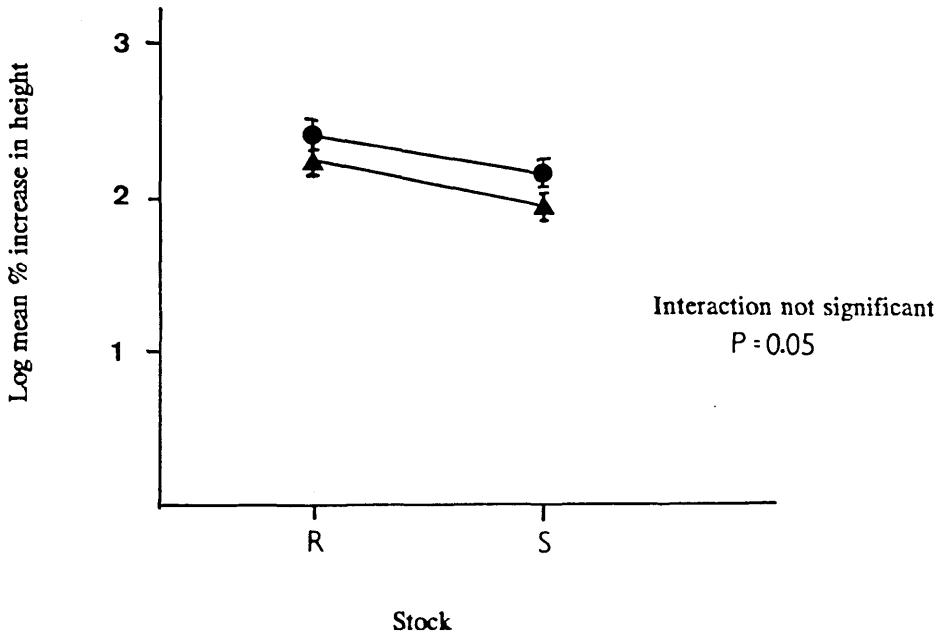
Fig 8 c and d Graphs showing the interaction between all possible scion-stock graft combinations .

Legend:

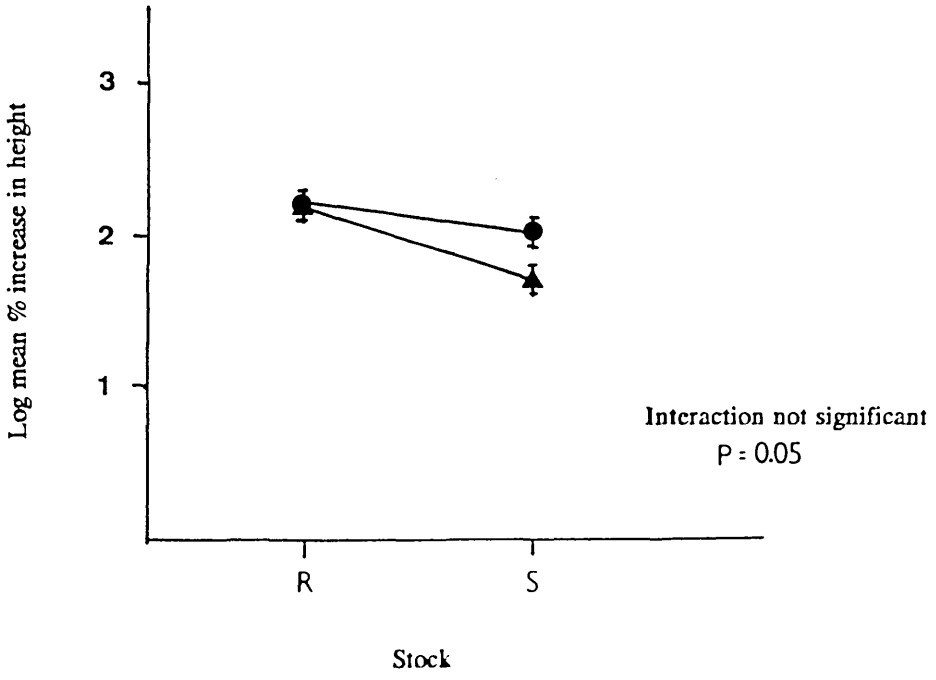
▲ R scion

● S scion

Fig. 8 c) Inoculated Craigella graft combinations in section 6.2




d) Inoculated Walter-Bonny Best graft combinations in section 6.2



## 6.3. THE EFFECT OF PRE-INOCULATING TOMATO GRAFTS WITH A *Trichoderma* SPECIES

Other contaminant fungi were sometimes isolated together with *Fol* from samples taken at the hypocotyl region. Of these, a *Trichoderma* species was frequently detected and its effect on the establishment of *Fol* infection in the host was deemed worthy of assessment. A *Trichoderma* spore suspension was prepared by washing spores off a culture grown on PDA with sterile distilled water and adjusted with a haemocytometer to give a concentration of $10^7$ spores $ml^{-1}$ .

Craigella plants of both R and S isolines were grafted (6 replicates for each treatments) at 4 weeks old, using the combinations as before : CR/CR, CS/CS, CR/CS, and CS/CR. Another set of plants were similarly grafted except that all scion halves were dipped into the *Trichoderma* spore suspension before attaching to the stocks and binding with parafilm. When the grafts had successfully taken (after 3 weeks), plants were root-inoculated with *Fol* (section 2.3.3).

The CS/CS grafted plants that were treated with *Trichoderma* failed to heal. The cut ends at the graft union were very weak and eventually became necrotic and dehydrated, causing the entire plant to wither and die. None of the 6 replicates in CS/CS survived. Among the other treated plants, there were also some losses but the remaining plants had fused at the graft union and were apparently healthy with a few lower petioles with necrotic edges.

Grafting was more successful in the control treatments (without *Trichoderma*) and all surviving plants were apparently healthy, although possessing the occas ional petiole with necrotic edges. CS/CS plants showed more severe wilt symptoms than any of the other graft combinations.

Upon reisolation of the pathogen (Table 15a), *Fol* was detected in the root samples of all treatments. In the *Trichoderma* -treated graft combination CR/CR and CS/CR, *Fol* was just detected in the hypocotyl samples, along with other contaminant fungi. In the treated CR/CS grafts, *Fol* was detected in the scion as far as the third node and *Trichoderma* was reisolated from the hypocotyl sample.

In the control plants, reisolation of *Fol* from CS/CS showed that it was heavily colonised as far up the scion as the seventh node, and in CR/CS, as far as the third node (occassionally

up to the fifth). *Fol* was established as far as the first node in both CR/CR and CS/CS plants. *Trichoderma* was reisolated from the hypocotyl sample of CS/CR.

Browning of the vascular system was similar in both groups, following the pattern of results described earlier in section 6.1. There was a greater degree of discoloration in the more susceptible and more colonised graft combinations.

The plant height data (Table 15b) show that the largest increases were achieved by plants with resistant stocks. Both CR/CR and CS/CR (from control and treated groups) were similar in terms of health and vigour. CS/CS plants grew least and were badly wilted. CR/CS plants, both treated and control groups, showed similar visual symptoms. Statistical analysis show that while in the control group, there was a significant difference at $P = 0.05$ between CR/CR and CS/CS, as well as CS/CR and CS/CS, none of the three *Trichoderma* treatments differed significantly.

Table 15a Distribution of *Fol* in inoculated grafted plants treated with or without *Trichoderma*

| Treatment | Position on plant | | | | | |
|---|---|---|---|---|---|---|
| scion/stock | RT | HC | N1 | N3 | N5 | N7 |
| CR/CR[a] | + | + | (+) | - | - | - |
| CS/CS | + | + | + | + | + | + |
| CR/CS | + | + | + | + | (+) | - |
| CS/CR | + | + | - | - | - | - |
| CR/CR (T)[b] | + | (+) | - | - | - | - |
| CR/CS (T) | + | + | + | - | - | - |
| CS/CR (T) | + | (+) | - | - | - | - |

Legend:

*a* mean of 4 plants sampled

*b* mean of 3 plants sampled

RT root

HC hypocotyl (immediately above the graft union)

N1,3... first, third... node

\+ *Fol* present

\- *Fol* absent

(+) *Fol* present in some, not all replicates

(T) scion half dipped in a suspension of *Trichoderma* ($10^7$ spores $ml^{-1}$ ) before grafting

Table 15b Comparison of % increase in height between grafted plants treated with or without *Trichoderma*

| Treatment | Mean | Log Mean |
|---|---|---|
| scion/stock | % increase in height | |
| CR/CR[a] | 225.85 (149.62,285.10)[c] | 2.315 |
| CS/CS | 66.56 (48.08,91.62) | 1.822 |
| CR/CS | 109.51 (78.16,148.94) | 2.033 |
| CS/CR | 225.69 (158.12,301.30) | 2.339 |
| | | S.E.D[d] (D.F=12) = 0.198 |
| | | L.S.D (D.F=12,P=0.05) = 0.431 |
| CR/CR (T)[b] | 231.13 (161.44,295.12)[c] | 2.339 |
| CR/CS (T) | 144.82 (107.15,195.88) | 2.161 |
| CS/CR (T) | 196.0 (144.21,263.63) | 2.29 |
| | | S.E.D (D.F=8) = 0.080 |
| | | L.S.D (D.F=8,P=0.05) = 0.184 |

Legend:

*a* mean of 4 plants sampled per treatment

*b* mean of 3 plants sampled per treatment

*c* lower and upper confidence limits (P=0.05)

*d* S.E.D & .L.S.D are given in logarithmic form

(T) scion half dipped in a suspension of *Trichoderma* ($10^7$ spores $ml^{-1}$) before grafting

## 6.4. DISCUSSION

These experiments show that graft combinations with susceptible stocks are more susceptible to disease than those with resistant stocks. In general, using percentage height increment as a guide to the severity of infection, a pattern of behaviour in grafted plants was established. Thus, in order of increasing resistance to disease, the scion/stock combinations are R/S, S/S, S/R and R/R. Apart from a few variations where the last two places are interchanged, this pattern was observed in cv. Craigella, as well as cvs Walter and Bonny Best.

Wilt symptoms were expressed later in grafted plants than in ungrafted plants. In part this may result from the greater age of grafted plants compared with earlier experiments (section 4.1); but it is likely that the mechanical act of grafting may itself confer some resistance. Compare for example CS/CS plants with ungrafted CS. As the formation of a successful graft union is important for the organisation in the intact plant (Yeoman & Brown, 1976), if some of the vessels are not completely intact as the graft heals, then the spread of a pathogen through the host vascular system may also be reduced. This could contribute to the slower expression of disease symptoms in grafted plants.

Scheffer (1957) observed that disease developed more slowly in grafted plants because only half the bundles in each stem took up the inoculum, and the other half formed the "tongue" of the graft. Heinze & Andrus (1945) also noticed a slower rate of wilting in grafted plants and attributed it to the greater age of the plants.

In all inoculated graft treatments, *Fol* succeeded in crossing the graft union from the stock to scion. For most graft combinations with resistant stocks, *Fol* was detected a little above the graft union, as far as the first node (although colonisation of a susceptible scion can proceed much farther). CS/CR and CR/CR plants were also visually similar to ungrafted inoculated CR plants. In general, CS/CS plants were the most heavily colonised although symptoms were not as severe as in CR/CS plants. In the latter, the severity of symptoms was similar to that observed in the ungrafted CS plants.

These results were similar to those obtained by May (1930) using tomato cvs Norton (resistant) and Bonny Best (susceptible) that were approach grafted and planted in infected soil.

May described wilting of the resistant scion/susceptible stock (R/S) plants but not the reciprocal combination (S/R). He concluded that as the scion and stock did not confer resistance on each other, the cause of resistance or susceptibility was not transferable. Vascular browning was observed extending about 100 mm above the graft union in R/S plants whereas the vascular system appeared healthy in the S/R plants. In the case of the Craigella grafted plants, presently under investigation, both CR/CS and CS/CR showed vascular browning in the scions. Heinze & Andrus (1945) approach grafted tomato cvs Pan America (resistant) and Bonny Best (susceptible) and demonstrated that upon root inoculation, wilt symptoms were more severe in the R/S graft combination than in the S/S combination, and also that the S/R plants were more vigorous than the S/S plants. They concluded that resistance to wilt was apparently localised in the root of resistant plants, a conclusion later refuted by Scheffer (1957) and Keyworth (1963).

In an attempt to explain why infected CR/CS were the most susceptible, the following hypothesis is suggested : In CR/CS plants, there is an accumulation of the pathogen in the susceptible stock which causes severe vessel blocking below the graft. As the pathogen grows, the production of toxins/elicitors is increased. This influx of toxins/elicitors is transported up the plant to the scions. The resistant scions react sensitively to the toxins/elicitors and hypersensitive responses are induced. These toxins/elicitors may also induce in the resistant scions other reactions such as the formation of tyloses or the production of phytoalexins. This would account for the severe vascular browning and wilting observed in the resistant scion of CR/CS plants. In the CS/CS graft combination, the susceptible scion does not react sensitively to the toxins/elicitors produced, and the progress of infection proceeds in the usual manner. However, in the reciprocal combination CS/CR, the pathogen is largely excluded from the susceptible scion due to restriction in the resistant stock.

Hypersensitive reactions were also observed in the scions of WA/BB plants. These plants were more severely stunted than the corresponding CR/CS. This could be due to the cv. Bonny Best being more susceptible to the disease, e.g when used as a scion, BB/WA tended to be more heavily colonised than CS/CR.

Keyworth (1963) also mentioned a hypersensitive reaction in resistant scions when grafted onto Bonny Best rootstocks. The resistant cultivar Pan America showed different symptoms as

a scion grafted to a Bonny Best stock than a susceptible scion grafted to a Bonny Best stock. It was suggested that metabolic products (which may be produced in the stock during fungal invasion) when transferred to the scion caused a sensitive reaction. He later confirmed (Keyworth, 1964) that resistant scions were hypersensitive to the toxins produced in the Bonny Best stocks on which they were grafted and that susceptible scions were not sensitive to these toxins. Davis (1954) has demonstrated this reaction to toxin in scions of various Solanaceous plants (not susceptible to *Fusarium*) grafted onto Bonny Best rootstocks. After infection with *Fusarium oxysporum* f.sp. *lycopersici*, these scions although not invaded by the pathogen showed vascular discoloration and wilting indicating that a toxin was translocated in advance of the pathogen.

Scheffer (1957) approach grafted tomato plants cvs Jefferson (resistant) and Bonny Best (susceptible) and inoculated them through scion spurs. He reported that resistant scions did not show any wilt symptoms regardless of the stock used (susceptible or resistant) and susceptible scions remained susceptible whether the stock used was resistant or susceptible. This differential stem resistance led Scheffer to conclude that resistance or susceptibility of the scion was not altered by the character of the stock and that resistance is a characteristic of the stem as well as the root. This clearly indicates that the results obtained in grafting experiments are dependent on the type of inoculation used and would account for the differences in conclusions reached by Scheffer (1957) and Heinze & Andrus (1945).

Independent disease reactions from different parts of grafted plants were also reported by Bond (1936). He grafted several varieties of tomato which were resistant or susceptible to *Cladosporium fulvum* Cooke and found that both the stock and scion retained their characteristic reaction to infection regardless of the reaction of the stock or scion grafted to it.

During reisolation of *Fol* from plant sections, *Trichoderma* and other fungi were frequently detected. These fungi could have been introduced into the plant through the graft wound. *Trichoderma*, usually an aggressive soil-borne fungus could be competing with *Fol* for nutrients and may restrict the growth of *Fol* within the host. Mycoparasitic activity of *Fusarium oxysporum* f.sp. *dianthi* has been reported on various isolates of *Trichoderma*, as has similar behaviour of *F. oxysporum* f.sp. *lycopersici* on *Rhizoctonia solani* and *Botrytis cinerea* (Vajna,

1985). Experiments using grafts that were pre-inoculated with *Trichoderma* showed that there was less colonisation in the treated CR/CS plants than in the controls. The presence of *Trichoderma* did not make any difference to colonisation in the other grafts, CS/CR and CR/CR. Treatment with *Trichoderma* did, however, affect the conditions at the graft union, causing a greater proportion of treated plants to die as a result of failure to heal at the graft wound. The susceptible, CS/CS was the most vulnerable.

In conclusion, grafting experiments show that resistance and susceptibility are not transferred between stock and scion although the behaviour of scion is markedly dependent on the stock on which it is grafted. The differential resistance normally shown in stems is somewhat masked when scion and stock interact in a graft combination.

# 7. THE DETECTION OF ANTIFUNGAL COMPOUNDS IN ROOT-INOCULATED PLANTS

## 7.1. EXTRACTION OF TERPENOIDS FROM STEM VASCULAR TISSUES

Five week old tomato plants cv. Craigella R and S isolines were root-inoculated with a $10^6$ propagules $ml^{-1}$ *Fol* (section 2.3.3). Control plants were sham-inoculated with distilled water. The extraction of terpenoids from excised stem vascular tissue was performed at intervals after inoculation (section 2.8.1).

Infected susceptible plants typically showed symptoms of wilt after 7 days. Resistant plants were symptomless and visually similar to controls of both isolines. Dissection of stem tissue revealed internal vascular browning of the xylem vessels. This was observed in stems of susceptible CS, as early as 4 days after inoculation. Vascular browning in CS tissue became more extensive involving xylem parenchyma cells at the later stages of infection, especially after 10 and 15 days. CR tissue showed slight vascular browning which was mainly restricted to lower parts of the plant.

The extracts, when redissolved in ethyl acetate were loaded using 20 $\mu$ l micropipettes onto silica gel plates at a loading equivalent of 1 g $cm^{-1}$ strip and developed in various solvent systems (section 2.9) i.e.(1) cyclohexane : ethyl acetate (1 : 1, v/v) (2) chloroform : acetone (9 : 1, v/v) (3) chloroform : methanol (95 : 5, v/v) (4) diethyl ether. Antifungal compounds were located on the developed chromatograms using chromogenic reagents (section 2.9.1) and the *Cladosporium* bioassay (section 2.10).

Initially, only two antifungal compounds could be detected. One was identified as rishitin, on the basis of $R_F$ values in the different solvent systems and colour reactions with the chromogenic reagents. This was confirmed by co-chromatography with an authentic sample of rishitin which was kindly provided by Dr G D Lyon (Scottish Crop Research Institute). The other compound could not be identified by correlation with the $R_F$ values of known compounds. In later experiments two other antifungal compounds were located. There were tentatively identified as the polyacetylenic compounds, recently described by de Wit and Kodde (1981a) and by Elgersma *et al* (1984), on the basis of their known $R_F$ 's in the relevant solvent systems. The results given below are collective from experiments performed at different times

after inoculation (Tables 16 and 17).

Extracts of stem vascular tissue taken 4 days after inoculation and developed in solvent system (1) did not reveal any inhibition zone when bioassayed with *Cladosporium*. Replicate plates when tested with the locating agent, vanillin-sulphuric acid, revealed the presence of rishitin, given by the characteristic blue colour, with corresponding $R_F$ 's of 0.27 and 0.28 for CR and CS respectively.

Strong antifungal zones were detected in *Cladosporium* bioassays of CS extracts taken 5, 10 and 15 days after inoculation. The fungitoxic zone gave $R_F$ values of 0.22, 0.25 and 0.25 for CS 5, 10 and 15 days respectively in solvent system (2) (Plates 19, 20 and 21). This corresponded to known $R_F$ values for rishitin in that solvent system and was confirmed using the chromogenic tests. Although rishitin was not detected in CR extracts when bioassayed, it was revealed at each infection interval in both CR and CS extracts when sprayed with methanolic vanillin.

The unknown fungitoxic compound, with $R_F$ values of 0.42 in solvent system (2) was strongly inhibitory and detected in *Cladosporium* bioassays of CR extracts after 5 days (Plate 22) and in CS extracts taken after 10 and 15 days. This compound did not give positive reactions with any of the chromogenic reagents used.

Extracts from 20 g of excised stem vascular tissue taken 6 days after inoculation were chromatographed in solvent system (1). Bioassay showed inhibition zones at $R_F$ = 0.32 for both CR and CS extracts, corresponding to the known range for rishitin in this solvent system.

Extracts from 30 g stem vascular tissue taken 10 days after inoculation were developed in solvent system (1) and then bioassayed using *Fusarium* (section 2.10.1). Chromatograms when viewed under UV light (254 nm) showed the presence of antifungal zones at $R_F$ = 0.65 for all extracts. Extracts from control plant tissue also showed very feint inhibition at the same $R_F$ values. This was identical to the $R_F$ value obtained using authentic falcarinol.

Using whole stem tissue from CR and CS plants, extracted 5 days after inoculation, the presence of rishitin was confirmed when inhibition zones at $R_F$ = 0.22 was obtained for both CR and CS extracts on chromatograms developed in solvent system (4), diethyl ether.

Chromatograms developed using solvent system (3), chloroform : methanol (95 : 5, v/v) also revealed inhibition zones at $R_F$ = 0.56 and 0.57 for CS and CR respectively, corresponding to falcarindiol, a polyacetylenic compound.

Table 16 The detection of antifungal compounds in cv. Craigella inoculated with *Fol*, using various solvent systems.

| Solvent system | $R_F$ of antifungal compounds | | | | | |
|---|---|---|---|---|---|---|
| | Authentic rishitin | Authentic falcarinol | Antifungal zones 1 | 2 | 3 | 4 |
| cyclohexane: ethyl acetate (1:1, v/v) | 0.27-0.33 | 0.64 | 0.27-0.33 | 0.63-0.65 | 0.7 | ND |
| chloroform: acetone (9:1, v/v) | 0.22 | NT | 0.22-0.25 | ND | ND | 0.42-0.44 |
| chloroform: methanol (95:5, v/v) | NT | 0.55 | NT | ND | 0.56-0.57 | ND |
| diethyl ether | 0.22 | NT | 0.22 | NT | NT | NT |

Legend:

1 Rishitin

2 Presumed falcarinol

3 Presumed falcarindiol

4 Unknown compound

NT not tested

ND not detected

Table 17 The accumulation of antifungal compounds in cv. Craigella resistant (CR) and susceptible (CS) stem and root tissues following inoculation

| Antifungal zone | Time (days) after inoculation | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Stem | | | | | | | | | | Root | | | | | |
| | 4 | | 5 | | 6 | | 10 | | 15 | | 5 | | 6 | | 10 | |
| | CR | CS | CR | CS | CR | CS | CR | CS | CR | CS | CR | CS | CR | CS | CR | CS |
| 1 | (+) | (+) | (+) | + | + | + | (+) | + | (+) | + | - | - | + | + | - | - |
| 2 | - | - | - | - | - | - | + | + | - | - | - | - | - | - | + | + |
| 3 | - | - | + | + | - | - | - | - | - | - | + | + | - | - | + | + |
| 4 | - | - | + | - | - | - | - | + | - | + | - | - | - | - | - | - |

Legend:

1 Rishitin

2 Presumed falcarinol

3 Presumed falcarindiol

4 unknown compound

(+) inhibition detected with vanillin reagent, not with bioassay

\+ presence of inhibition

\- absence of inhibition

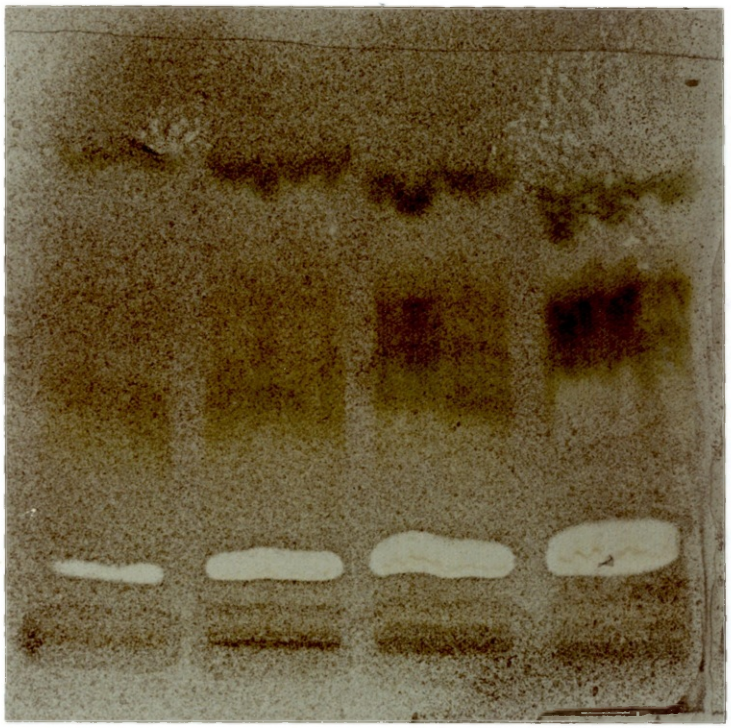
Plates 19-22 Antifungal compounds in stem vascular tissue extract detected using *Cladosporium* bioassay.

Chromatograms were developed in solvent system chloroform : acetone (9:1, v/v)

Plate 19 Rishitin detected in CS stem tissue extract, 5 days after inoculation.

Plate 20 Rishitin detected in CS stem tissue extract, 10 days after inoculation.

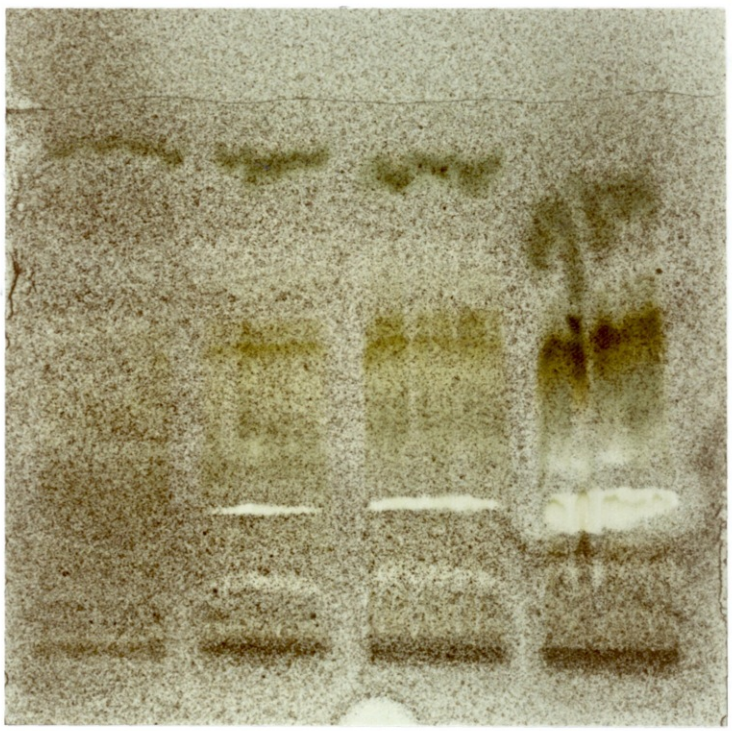
## Plate 19



$R_f$=0.22

Equivalent amount of tissue 0.4 0.8 1.2 1.6 g $cm^{-1}$

## Plate 20



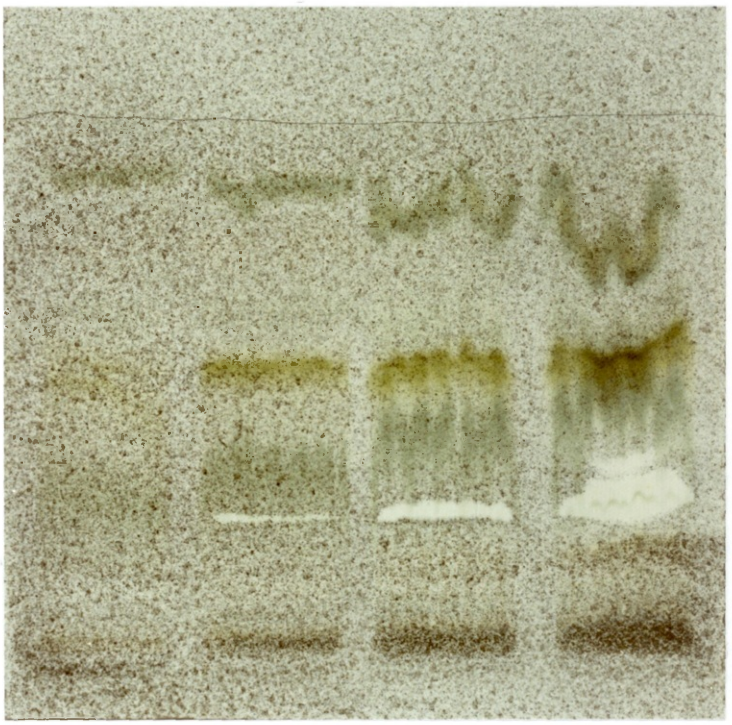
$R_f$=0.25

Equivalent amount of tissue 0.58 0.86 1.14 g $cm^{-1}$

Plate 21 Rishitin detected in CS stem tissue extract, 15 days after inoculation.

Plate 22 Unknown antifungal compound detected in CR stem tissue extract, 5 days after inoculation.

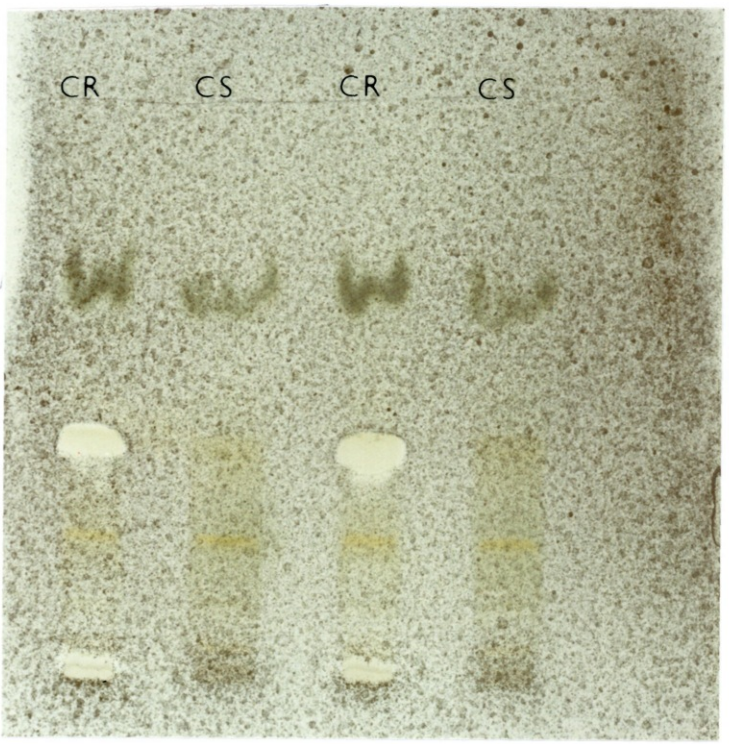
## Plate 21



$R_f$ = 0.25

Equivalent amount of tissue 0.58 0.86 1.14 g $cm^{-1}$

## Plate 22




$R_f$ = 0.42

Equivalent amount of tissue 1.0 1.0 g $cm^{-1}$

## 7.2. THE EXTRACTION OF ALKALOIDS FROM STEM VASCULAR TISSUE

Five week old Craigella plants were root-inoculated as in section 2.3.3. After 4 days, approximately 40 g of stem vascular tissue were excised and extracted as for terpenoids in section 2.8.1. The methanolic fractions remaining after partitioning with chloroform were pooled and used for the detection of alkaloids (section 2.9.1).

Extracts from CR and CS tissue when redissolved in 90 % methanol were located onto silica gel plates at rates equivalent to 0.37, 0.74, 1.11, 1.48 and 1.85 g fresh weight $cm^{-1}$. Authentic tomatine (Koch-Light labs.) was dissolved in 90 % methanol and applied onto silica gel plates at rates between 51.5 $\mu$ g $cm^{-1}$ and 257.1 $\mu$ g $cm^{-1}$. Plates were developed in butan-l-ol : acetic acid : water (6 : 1 : 2, v/v) and bioassayed by spraying with a suspension of *Cladosporium* spores, prepared as in section 2.10.

Results (Table 18) show that antifungal compounds were located on chromatograms with an $R_F$ of 0.22. This migration rate is approximately half that shown by authentic tomatine. The identity of the antifungal material in Craigella extracts is unknown, but the inhibition of *Cladosporium* was intense even at the lowest concentrations tested. Tomatine was inhibitory at concentration of 77 $\mu$ g $cm^{-1}$ of origin and above.

Methanolic extracts from root tissue of both CR and CS taken after various infection times were bioassay with *Cladosporium* but antifungal compounds were not detected.

Table 18 **Antifungal compounds detected in methanolic extracts of stem vascular tissue, 4 days after inoculation with *Fol***

(Chromatograms were developed in butan-l-ol : acetic acid : water, 6 : 1 : 2, v/v)

| Extracts | $R_F$ value |
|---|---|
| CR | 0.22 |
| CS | 0.22 |
| Tomatine | 0.51 |

## 7.3. EXTRACTION OF TERPENOIDS FROM ROOT TISSUE

Craigella plants, CR and CS were root-inoculated (section 2.3.3) at 5 weeks old. Extractions were performed on whole root tissues 5 days after inoculation using the methods described in section 2.8.3. Extracts were redissolved in ethyl acetate and loaded onto silica gel plates at an equivalent of 1 g $cm^{-1}$ strip. Plates were developed using solvent systems (1) cyclohexane : ethyl acetate (1 : 1, v/v) and (2) chloroform : acetate (9 : 1, v/v) and bioassayed using *Cladosporium* (section 2.10).

Antifungal compounds were detected on the developed chromatograms (Table 17 and 19). The $R_F$ value obtained for both extracts in solvent system (1) was 0.7 (Plate 23). Although no authentic standard was available, this $R_F$ value is strongly indicative of falcarindiol (de Wit & Kodde, 1981a; Elgersma *et al*, 1984). No inhibition zones were detected in chromatograms developed in solvent system (2).

Extracts from roots of both CR and CS taken 6 days after inoculation were bioassayed using *Cladosporium*. For both extracts feint inhibition zones were visible at $R_F$ = 0.33 in solvent system (1) (Table 19), corresponding to the known range for authentic rishitin in this solvent system. Falcarindiol was not detected on this occassion.

Further extracts taken 10 days after inoculation (including those from sham-inoculated control plants) were chromatographed in (1) cyclohexane : ethyl acetate (1 : 1, v/v) and (3) chloroform : methanol (95 : 5, v/v). Chromatograms that were developed in solvent system (1) were assayed using *Fusarium* (section 2.10.1) while those developed in solvent system (3) were assayed using *Cladosporium*. Plates included spots of authentic falcarinol (kindly given by Dr R Hoffman, University of London) applied as markers.

Chromatograms that were bioassayed with *Fusarium* contained antifungal compounds with $R_F$ values of 0.63 for all extracts, corresponding to the standard falcarinol (Table 19). Standard falcarinol gave $R_F$ values of 0.64 and 0.55 in solvent systems (1) and (3) respectively. Extracts from control plants also showed very feint inhibition at the same $R_F$ values. The *Cladosporium* bioassay revealed fungitoxic spots at $R_F$ = 0.57 for both CR and CS (Table 19). However, extracts from control plants also showed very feint inhibition at the same $R_F$ values,

corresponding to known values for falcarindiol. The unknown compound, previously detected in stem extracts was not detected in root tissues.

Table 19 Antifungal compounds detected in CR and CS root tissue extracts using bioassay

| Solvent system | $R_F$ value of antifungal compounds Antifungal zones | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| cyclohexane: ethyl acetate (1:1, v/v) | 0.33 | 0.63 | 0.7 |
| chloroform : acetone (9:1, v/v) | ND | ND | ND |
| chloroform : methanol (95:5, v/v) | ND | ND | 0.57 |

Legend:

1 Rishitin

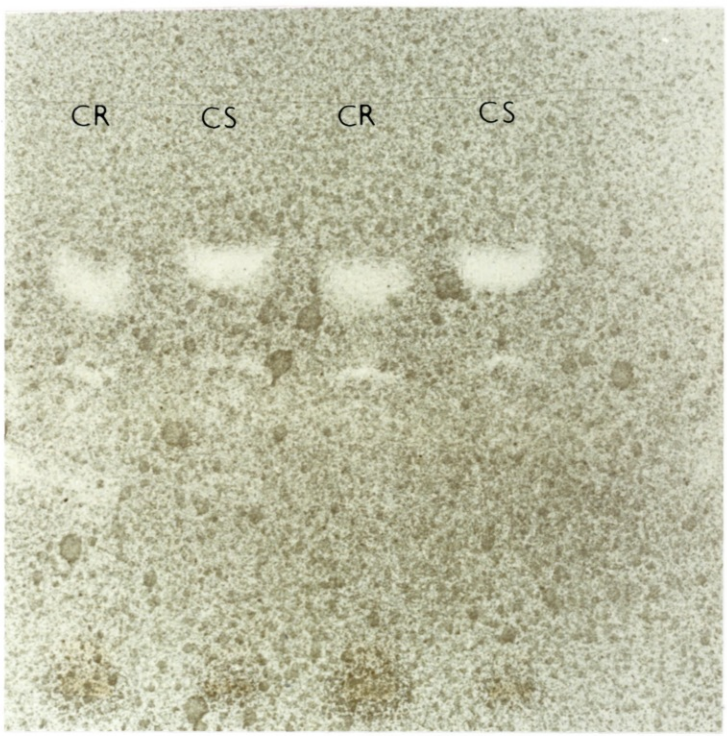
2 Presumed falcarinol

3 Presumed falcarindiol

ND not detected

Plate 23 Falcarindiol detected using *Cladosporium* bioassay in CR and CS root tissue extracts, 5 days after inoculation.

Chromatograms was developed in cyclohexane : ethyl acetate (1 : 1, v/v).




| | | | | | |
|---|---|---|---|---|---|
| Equivalent amount of tissue | 1.02 | 1.02 | 1.02 | 1.02 | g $cm^{-1}$ |

## 7.4. DETECTION OF ANTIFUNGAL COMPOUNDS IN XYLEM EXUDATES

Craigella R and S plants inoculated after 4 days were severed at the hypocotyl. Exuding fluids were collected by absorption on Whatman no. 1 filter paper over a period of 1 h. The moist filter papers were then extracted for terpenoids as in section 2.8.1. The final residues were redissolved in 200 μ l of ethyl acetate and applied onto TLC plates.

Chromatograms were developed in solvent system (1) cyclohexane : ethyl acetate (1 : 1, v/v) and when viewed under UV light showed bands at $R_F$ = 0.71 and 0.77 for CR extract and $R_F$ = 0.71 for the CS extract. Replicate chromatograms were bioassayed, using *Cladosporium* and revealed inhibition zones at $R_F$ = 0.71 and 0.77 for the CR extract only. No inhibition zones were detected in the methanolic fractions after bioassay with *Cladosporium*.

### 7.4.1. THE EFFECT OF XYLEM EXUDATES ON THE VIABILITY OF *Fol*

Four weeks old plants cv. Craigella R and S were root-inoculated (section 2.3.3) together with controls. Seven days after inoculation, stems were severed at 10 mm below the cotyledonary node and the exudates were collected by Pasteur pipetting over a period of 2 - 3 h. Collected exudates were filter-sterilized through 0.22 μ m millipore filters.

0.3 ml of the exudate was mixed with 0.2 ml *Fol* inoculum ( $10^3$ propagules $ml^{-1}$ ) and incubated at 20 °C. At 2-hourly intervals the contents of the test tubes were spread onto PDA plates (with added antibiotics) and incubated for a further 3 days, before counting the number of resulting colonies. As a control, 0.2 ml of spore inoculum was incubated with 0.3 ml of sterile distilled water and plated at the same intervals.

Fig 9 shows that a brief exposure of *Fol* inoculum to xylem exudate has little effect on viability, the recovery being close to the theoretical maximum (viz. 200). However, incubation for 2 h or longer has a marked deleterious effect, the exudates from healthy plants being marginally the more damaging.

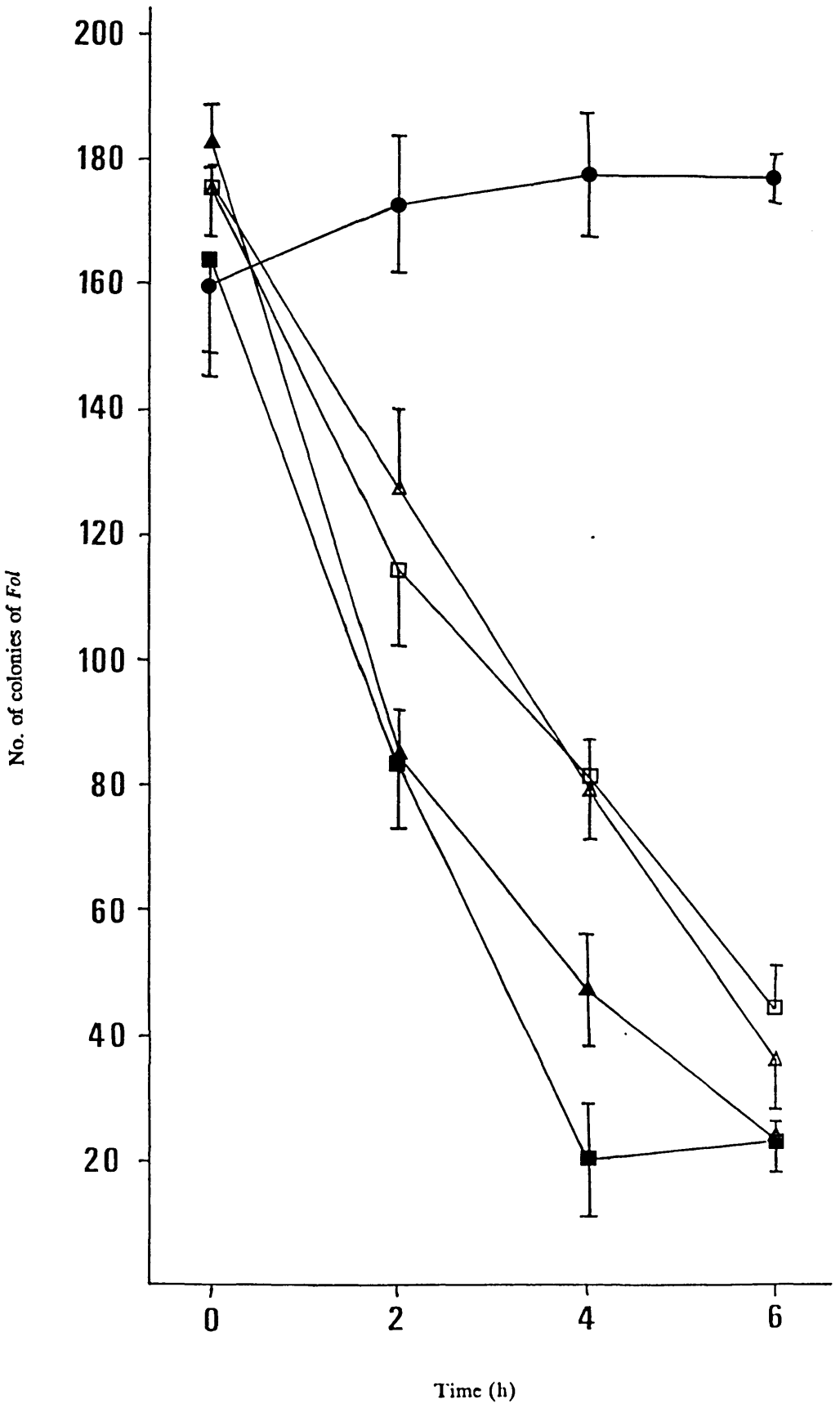
Fig 9 The effect upon *Fol* propagule viability of incubation in the presence of xylem exudates.

(Mean no. of colonies with standard error taken from 3 sample plates, where confidence limits overlap obtrusively, only one half has been drawn to aid visual understanding ).

Legend:

- ■ Healthy CR exudate + *Fol*
- ▲ Healthy CS exudate + *Fol*
- □ Infected CR exudate + *Fol*
- △ Infected CS exudate + *Fol*
- ● Sterile distilled water + *Fol*

Fig. 9


200
180
160
140
120
100
80
60
40
20
No. of colonies of Fol
0
2
4
6
Time (h)

## 7.5. DISCUSSION

These experiments show that antifungal compounds were elicited in both stem and root tissues in response to infection. Four antifungal compounds were detected in host stem vascular tissue. Of these, two were terpenoid phytoalexins : one was positively identified as rishitin, a norsesquiterpene and the other was an unknown compound. The other two were identified as polyacetylenic phytoalexins : falcarindiol and falcarinol.

Rishitin was detected in extracts of stem vascular tissue from both CR and CS at 6 days and from CS only at 5, 10 and 15 days after inoculation, using the *Cladosporium* bioassay. Rishitin was detected at all other times using chromogenic tests. The inhibition was strongest in CS extracts at 5, 10 and 15 days. The unknown compound, at $R_F$ = 0.42 - 0.44 in the solvent system choloroform : acetone (9 : 1, v/v) was also strongly inhibitory in the *Cladosporium* bioassay 5 days after inoculation for the CR extract and again at 10 and 15 days for the CS extract.

The polyacetylenic compounds appeared less frequently and could only be detected by bioassay. Their identification was based on published $R_F$ values in various solvent systems. Falcarindiol was detected in stem vascular tissue 5 days after inoculation and falcarinol, after 10 days. Both compounds were detected in resistant and susceptible plants and trace amounts were present in sham-inoculated controls. Rishitin and the other unknown terpenoid were not detected in control tissues at any time.

Root tissues also contained these phytoalexins but their occurrence was much more sporadic than in stem tissue. Rishitin was just detectable in root tissues 6 days after inoculation. Falcarindiol was detected in extracts of both CR and CS after 5 and 10 days. Falcarinol, was detected as for stem tissues, only after 10 days.

It becomes apparent that most of the extracts were at the limits of fungitoxicity detection e.g. rishitin was detected more readily by chemical assay, but not by bioassay. Due to the insensitivity of the *Cladosporium* bioassay, *Fusarium* bioassay was used as an alternative. This assay was chosen due to its relevance to the *in vivo* situation. Indeed, falcarinol was only detected using the more sensitive *Fusarium* bioassay and not with *Cladosporium* bioassay.

In studies on phytoalexin production in *Fusarium*-infected tomatoes, Hutson (1978) and Hutson & Smith (1980) reported the detection of two antifungal compounds only - rishitin and an unknown compound. This unknown compound had $R_F$ 's of 0.44 - 0.47 and 0.3 - 0.34 in solvents cyclohexane : ethyl acetate (1 : 1, v/v) and chloroform : acetone (9 : 1, v/v) respectively. The compound was not identified as it did not give characteristic colours with locating agents, although it was shown not to be phenolic. Hutson (1978) also reported trace amounts of antifungal compounds at $R_F$ 0.63 and 0.86 - 0.96 in cyclohexane : ethyl acetate. In light of more recent data, the compound at $R_F$ 0.63 is likely to have been falcarindiol.

Previously, two antifungal compounds were reported by Pegus (1976) in *Fusarium*-infected tomato. These were rishitin and an unknown, with an $R_F$ of 0.45 in cyclohexane : ethyl acetate. This unknown was the same compound later obtained by Hutson (1978), but on the basis of $R_F$ values is probably not the same as the unknown found in the present investigation.

Both Pegus (1976) and Hutson (1978) demonstrated greater fungitoxicity in extracts from resistant than susceptible tissue. Pegus (1976) reported an $ED_{50}$ for germination of *Fusarium* spores of 4 - 20 $\mu$ g $ml^{-1}$ for rishitin and $ED_{50}$ for hyphal growth of 2 $\mu$ g $ml^{-1}$. Hutson (1978) observed an $ED_{50}$ for germ tube growth of *Fusarium* of 2 $\mu$ g $ml^{-1}$ for rishitin. Stromberg & Corden (1977) also reported that acetone extracts from xylem vessels of tomato cvs Jefferson and Bonny Best contained fungitoxic material that suppressed the growth of *Fol in vivo*. It was suggested that the highly fungitoxic extracts from the resistant cv. Jefferson contributes to resistant in tomatoes.

Hutson (1978) observed the rapid accumulation of rishitin in susceptible tissue from 7 to 14 days after infection. This was largely due to the extensive colonisation and stimulation of susceptible tissue. The unknown compound accumulated rapidly in resistant stems. He suggested that the compound may be induced in response to low levels of fungal metabolites.

In root tissues, Pegus (1976) reported rishitin in resistant and susceptible Craigella tissues at 3, 7, 14 and 21 days after infection with higher levels in susceptible plants. Hutson (1978), on the other hand, did not detect rishitin in susceptible root tissues at all, only in low levels in the resistant tissue.

McCance & Drysdale (1975) using *Fusarium*-infected tomato cvs Red Top (low resistance) and Homestead (high resistance) detected only rishitin in root and stem vascular tissue. Initially, higher levels of rishitin accumulated in the high resistance cultivar than in the low resistance cultivar. This situation was reversed 4 days after inoculation.

In the present investigation, there was no real evidence for any differences in antifungal compounds produced by CR and CS plants. Due to the insensitivity of the bioassay detection method, it is difficult to conclude whether any of the antifungal compounds accumulated more rapidly in either CS or CR. Only with the unknown compound was it quite clearly shown that the inhibition was stronger initially in the CR extract than in the CS extract. Although rishitin was more readily detected in susceptible tissues at later infection times, there was no real evidence for an increase in the inhibitors with time after inoculation. The increase shown in the case of rishitin is suggested to be more spasmodic than in the form of a conspicuous peak.

Polyacetylenic phytoalexins (falcarindiol, falcarinol and *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol) were first reported in the Solanaceae by de Wit & Kodde (1981a) in tomato fruits and leaves infected with *Cladosporium fulvum*. Mycelial growth of *C. fulvum* and *C. cucumerinum* was completely inhibited by 6 and 18 $\mu$ g $ml^{-1}$ of falcarindiol and *cis*-tetradeca-6-ene-1,3-diyne-5,8-diol respectively. The latter compound has also been reported by Elgersma & Overeem (1981) in tomato infected with *Verticillium albo-atrum*. Elgersma *et al* (1984) isolated and characterised falcarindiol and falcarinol from *Verticillium*-infected tomato vascular tissue. These two fungitoxic compounds were not detected in healthy tomatoes.

In the present investigation, control healthy root and stem tissues also showed very feint inhibition at the same $R_F$ 's as falcarindiol and falcarinol. Without authentic compounds as standards, it is difficult to confirm the presence of these compounds in healthy plants. Falcarindiol has been reported in healthy carrot root tissue (Garrod *et al*, 1978) and in elder (Kemp, 1978). Although shown to be an antifungal compound, the role of falcarindiol as a phytoalexin has yet to be established.

Methanolic fractions of plant tissue extracts were examined for the detection of alkaloids. This potentially would include tomatine, a steroid glycoalkaloid and other related compounds.

Extracts only revealed inhibition zones of antifungal compounds at approximately half the $R_F$ value (0.22) of authentic tomatine (0.51) in butan-1-ol : acetic acid : water (6 : 1 : 2, v/v). This antifungal compound was found in both CR and CS extracts and could be tomatine or a metabolic product of tomatine. No antifungal compounds were detected in control tissues.

Langcake *et al* (1972) reported increasing concentrations of tomatine-like material in tomato stems and roots following infection with *Fusarium*. This increase was similar in both resistant and susceptible plants. The authors reported an $R_F$ of 0.5 for authentic tomatine and 0.3 for tomatine plus plant extracts in butan-1-ol : acetic acid : water (6 : 1 : 2, v/v). They concluded that the inhibitor present in extracts was indeed tomatine as it had an $R_F$ equivalent to 0.3. Assuming that the $R_F$ of tomatine changes in the presence of plant extracts, the antifungal compound detected in the methanolic extracts of the present investigation may indeed be tomatine.

McCance & Drysdale (1975) also reported similar concentrations of tomatine in isolated tomato vascular tissue of all cultivars. They reported an $ED_{50}$ for tomatine of 50 $\mu$ g $ml^{-1}$ for germination of *Fusarium* spores, complete inhibition at 100 $\mu$ g $ml^{-1}$, and an $ED_{50}$ for hyphal extension of 8 $\mu$ g $ml^{-1}$.

The concentration of tomatine in xylem fluids of tomato cultivars infected with *Fol* races 1 and 2 was investigated by Smith & MacHardy (1982). They found no significant differences in the concentration of tomatine in the fluids of any treatments and concluded that tomatine was not a primary determinant of resistance to *Fusarium* wilt.

Differences in the fungitoxicity of extracts from different tomato cultivars were detected by Hammerschlag & Mace (1975). The antifungal activity of resistant root extracts was twice that of the susceptible whether or not the plants were infected. They suggested that the fungitoxic properties of $\alpha$ -tomatine (estimated at 180 $\mu$ g $g^{-1}$ fresh weight of infected wilt-resistant plants) could play a role in resistance.

*Fol* has been shown to detoxify $\alpha$ -tomatine (Ford *et al*, 1977) by producing an inducible extracellular enzyme which cleaves the glycoalkaloid to a much less toxic compound, tomatidine. Pegg & Woodward (1986) have also demonstrated that an inducible extracellular

enzyme, tomatinase produced by *V. albo-atrum*, can hydrolyse α -tomatine to the weakly fungitoxic $\beta_2$ -tomatine. They found no significant differences in tomatine content between inoculated and control, resistant and susceptible plants. Hence, they concluded that tomatine was not involved in the major gene resistance of tomato to *Verticillium*. Kern (1952) had much earlier claimed that tomatine was not involved in the resistance of tomato to *Fol* as the compound was not very inhibitory and did not accumulate in amounts sufficient to inhibit the fungus. It has been suggested (Arneson & Durbin, 1967) that tomatine may be more important in the resistance to leaf-infecting pathogens due to the higher levels recorded in leaves and that the localisation of tomatine within the cells may lead to sufficiently high concentrations to inhibit fungal growth (Arneson & Durbin, 1968).

Results obtained for the detection of antifungal compounds in xylem exudates have been inconclusive. Inhibition zones were obtained using *Cladosporium* bioassay at $R_F$ 0.71 and 0.77 for CR extracts only. The $R_F$ value of 0.71 corresponds to that of falcarindiol in the solvent system cyclohexane : ethyl acetate. This compound was also detected in the CS extract under UV light but did not show as an inhibition zone in the bioassay.

In the present investigation, xylem exudates were not inhibitory to *Fol* propagules when plated directly. There were no differential responses shown in propagules incubated with exudates from healthy or infected, susceptible or resistant plants. However, the number of propagules decreased when incubated directly with xylem exudates over a time period. The established pattern was similar for all treatments. This indicated that there were fungitoxic properties in the exudates which limited the viability of fungal propagules when incubated with the xylem fluids.

Gottlieb (1943) demonstrated the presence of toxins in tracheal fluids of plants wilted by *Fol*. Bugbee (1970) showed that in cotton, occlusion of xylem vessels followed by toxin formation in cotton plants are major mechanisms of resistance to *Fusarium oxysporum* f.sp. *vasinfectum*. Trachael fluids from occluded vessels of infected cotton stems collected by root pressure were toxic to *Fusarium* and *V. albo-atrum*.

Antifungal compounds have been widely reported in tomato plants infected with

*Verticillium albo-atrum*. These vary in number, e.g. Tjamos & Smith (1974) reported four, Elgersma (1980) reported five, Hutson & Smith (1978) and Tjamos & Smith (1975) reported six phytoalexins. In tomatoes infected with *V. dahliae*, Emmanouil & Wood (1983) reported the production of six putative phytoalexins of which only rishitin was identified. Thus, compared with *Fusarium*- infected tomato plants, there seems to be a differential eliciting ability between the two pathogens. Culture filtrates from these two pathogens have also been shown to elicit phytoalexins (Hutson, 1978). Woodward & Pegg (1986) have also demonstrated that culture filtrate and cell wall extracts of *V. albo-atrum* elicited the accumulation of rishitin in whole plants and excised stem segments.

The role of phytoalexins in resistance has been examined repeatedly. Most workers have agreed that phytoalexins, especially rishitin are not found in healthy plants and that fungitoxicity is greater in extracts from resistant than susceptible tissue (Bell, 1967, 1969; Tjamos & Smith, 1974; Pegus, 1976; Hutson, 1978). Elgersma (1980), however, concluded that rishitin is not a factor in resistance as its accumulation appeared not to be greater in resistant than in susceptible tissue. Tjamos & Smith (1974) suggested that phytoalexin synthesis in the root system is an important determinant factor in the restriction of the pathogen in resistant varieties, and linked the production of tyloses to resistance in the roots. Hutson & Smith (1980) implied that phytoalexins are the primary determinant in resistance to vascular wilts in tomato and that the occlusion of vessels by tyloses is a later reaction.

In the present investigation, results show that the production of antifungal compounds was not very consistent and levels were frequently undetectable due to the insensitivity of the bioassay method. The amount of antifungal compounds produced within the plant may not be uniformly distributed thus frustrating efforts at extraction. Rishitin has been shown to be unstable (Shih & Kuć, 1973) and its metabolic breakdown in the plant could account for inconsistency in its detection. The metabolic breakdown of antifungal compounds *in vivo* is also very difficult to assess. Differences in extraction techniques could in part, account for different results obtained in the literature. In view of these differences, the role of phytoalexins as the determinant factor in the resistance mechanism is controversial.

Due to the infrequency in the detection of antifungal compounds, spore germination bioassays were not used to complement the *Cladosporium* and *Fusarium* bioassays in toxicity tests. The use of the bioassay and locating agents can only serve as a qualitative measurement, useful only as an indication of the inhibition zones present. The interpretation of the $R_F$ values is also subjective, serving as a guide to the detection of inhibitors present.

Bearing in mind that infected tomato does not produce phytoalexins in large quantities, it must be concluded that any role played by phytoalexins and other antifungal compounds in the resistance to *Fusarium*- wilt must be secondary.

## 8. GENERAL DISCUSSION

The original aim of this investigation was to obtain a better understanding of the recognition signals that "trigger" the defence mechanisms in *Fusarium*-infected monogenically resistant plants. Many of the results obtained show that resistance is expressed during the phase of vascular colonisation. Experiments on host-specificity sought putative elicitors that would elicit differential responses in host tissues, other than the xylem. This involved the use of test fluids such as spore inoculum and culture filtrate that might contain fungal metabolites acting as elicitors of resistance. Unfortunately, there was no expression of resistance in parenchyma nor were there differential responses exhibited between resistant and susceptible cuttings or petioles when treated with these test fluids. Cut shoots, however, showed a response to non-specific toxins in the culture filtrate, resulting in vein-clearing of the leaflets. Pegus (1976) also reported a lack of differential responses in stem cuttings and petioles treated with culture filtrates.

The induction of β -1,3-glucanase by spore inoculum and by culture filtrate also failed to differentiate between resistant and susceptible petioles. Although β -1,3-glucanase activity was increased after both treatments, the increase was similar in both resistant and susceptible isolines, indicative of a generalised response to infection.

Biochemically, the nature of the response of both isolines to infection was similar. Both Craigella resistant and susceptible near-isolines produced four antifungal compounds extractable with chloroform, and one with methanol. There was no evidence to suggest a differential rate of accumulation of these antifungal compounds although rishitin was more easily detected in susceptible tissue. Both Pegus (1976) and Hutson (1978) reported more rapid accumulation of antifungal compounds in resistant plants following *Fol* infection, although the eventual accumulation in susceptible tissue was greater. Harrison & Beckman (1987) using near-isolines infected with *Fol*, reported rapid accumulation of rishitin in both resistant and susceptible plants at 1 - 4 days after infection, although the level of rishitin continued to increase in susceptible but declined in resistant tissues from 5 - 11 days after infection. In the present investigation, due to the insensitivity of the bioassay method, any qualitative or quantitative differences in the toxicity of resistant or susceptible extracts were not detected. In a separate study, Harrison &

Beckman (1987) found that in their petiole assays, parenchyma of both resistant and susceptible near-isolines showed similar sensitivity to rishitin and other inhibitors.

In grafted plants, resistant scions attached to susceptible stocks (R/S) behaved as if hypersensitive to toxins produced by the pathogen in the stock. Such scions suffered from intense symptoms of vascular browning, wilting and stunting. This situation probably arises because the genetically resistant scion is provoked into massive overaction by the large quantity of *Fol* metabolites translocated up from the heavily colonised stock. It is debatable whether such metabolites should be correctly regarded as "toxins" or "elicitors". The scions of other graft combinations are less affected, each for different reasons. S/S because of the lesser ability of the scion to react to fungal "elicitor"; R/R and S/R because of the more restricted colonisation of the stock results in less "elicitor". This hypothesis adequately explains the superficially worrying observation that resistant scions are more damaged than susceptible ones, but does nothing to explain how the differences in root colonisation arise in the first place.

Colonisation patterns of resistant and susceptible plants by *Fol* showed that growth of the pathogen was severely restricted in plants of resistant cultivars of all ages. The vertical spread of the pathogen was heavily curtailed as it was rarely detected above the third node of resistant plants. Susceptible plants, however, soon became systemically colonised. Quantification of the pathogen in host tissues showed that very high numbers of fungal propagules were recovered from susceptible tissue, especially at the peak colonisation periods of between 12 - 14 days after inoculation. This indicates that sporulation of *Fol* within the xylem vessels is an effective means of rapid spread within host tissues. Resistant plants showed much lower pathogen recoveries and the areas above the first node were practically devoid of viable fungal propagules.

Although wilt symptoms were delayed, colonisation patterns in grafted plants also showed a generally predictable pattern. Resistant scion on resistant stock (R/R) as well as the susceptible scion on susceptible stock (S/S) were colonised in much the same way as ungrafted resistant and susceptible plants. R/S plants, although exhibiting severe wilting and internal vascular browning, were not as heavily colonised as was expected. This suggests that the differential resistance in scions was operating as discussed earlier. S/R plants, although exhibiting a degree of vascular browning, were externally symptomless. Detection of *Fol*

beyond the third node in these plants was rare, due to restriction of the pathogen in the resistant stock. Differential reactions in stems were exhibited after exposure to the pathogen, regardless of whether this arose from direct inoculation of the stem or as a result of secondary colonisation from infected roots.

The localisation of the pathogen in resistant cultivars is an important feature in the wilt resistance mechanism (Beckman, 1968; Beckman *et al*, 1972; Elgersma *et al*, 1972). Vascular occlusion by gels was infrequently observed, as reported also by Pegus (1976) and Hutson (1978) and deemed relatively insignificant in resistance.

Occlusion by tyloses was more obvious, being frequently observed in the resistant and rarely in the susceptible isoline. This confirms previous observations made by Tjamos & Smith (1974), Pegus (1976) and Hutson (1978). Tyloses are important in restricting the vertical colonisation of the pathogen. In the resistant cultivar, the resistance mechanism is triggered early in infection and leads to rapid vascular occlusion and tylosis, thereby reducing the production of secondary propagules. In the susceptible cultivar, however, the delay in tylose formation and the absence or failure of occluding mechanisms facilitates the rapid systemic spread of the pathogen. Beckman *et al* (1972) observed that tylose initiation is delayed about two days after inoculation in the susceptible cultivar. Although rapid vascular occlusion is the basis for single dominant gene resistance (Beckman *et al*, 1972; Elgersma *et al*, 1972), there is no evidence that this gene directly controls vascular occlusion. Furthermore, vascular occlusion alone cannot account for the low intensity of colonisation present in the basal parts of resistant plants.

Tyloses were never observed in association with hyphae and hyphae were also not detected beyond tyloses in vessel elements. Tjamos & Smith (1975) reported similar observations for *Verticillium*-infected tomatoes.

Hutson (1978) suggested that the tylose-inducing factor is related in someway to vascular browning. In areas of extensive vascular browning, relatively few tyloses were observed. This could account for the rare detection of tyloses within susceptible tissue in the present study. Similar observations have been reported by Pegus (1976). The reason for this inverse

relationship between tyloses and vascular browning is unclear. The simplistic explanation that brown cells are dead and hence unable to contribute tyloses fails to explain why the initial reaction of such cells to the pathogen was death rather than tylosis formation.

Harrison & Beckman (1987) have recently postulated that rishitin, an antifungal compound that accumulates in response to infection, contributes to the inhibition of tylosis in susceptible cultivars. The authors attempt to explain this paradoxical situation in the following way. In the susceptible near-isoline, the failure of the temporary sealing-off process in infected vessels by gels causes the stress metabolites to be transported above the site of infection. These in turn affect the growth of the parenchyma cells above the infection site, resulting in the failure of the permanent occluding mechanism and the sealing-off of infected vessels. More rishitin is accumulated due to the elicitation of a larger mass of host tissues. In the resistant cultivar, the resistance responses including the accumulation of rishitin are efficiently sealed-off in the infected vessels, decreasing the amount of free detectable rishitin. Harrison & Beckman (1987) claimed that tylose development may be further suppressed by an unknown metabolite. Fusaric acid was initially thought to be a possible candidate but its subsequent absence in ethyl acetate extracts ruled out its involvement in tylosis suppression (Harrison & Beckman, 1987).

Although the accumulation of antifungal compounds differed little between resistant and susceptible cultivars, differences were apparent between stem and root. Detection of antifungal compounds in the present study was more sporadic in root than in stem tissues. Hutson (1978) reported less phytoalexin accumulation in root tissue, compared with stems, with virtually no rishitin or trace amounts only in response to infection by *Fol* and *Verticillium albo-atrum* respectively. Emmanouil (1977) also noted limited accumulation of antifungal compounds in roots of tomato, pepper and eggplant infected with *V. dahliae*.

The importance of any antifungal compound in the resistance mechanism lies in its ultimate localisation within the infection site, its local concentration and its relative toxicity *in vivo*. If antifungal compounds are not distributed evenly within the host xylem tissue, their effect on the pathogen will be hard to predict. Harrison & Beckman (1987) have indicated that rishitin concentrations obtained in *in vitro* studies have to be corrected by a "distribution factor" to relate to their actual concentrations at the infection sites. In susceptible tissue, a large bulk

of tissue is responsible for phytoalexin synthesis, but the toxic properties may be diluted by the sheer bulk of the tissue concerned. In resistant tissues, phytoalexin synthesis is more highly localised and concentrations at individual sites may be higher.

Results obtained from studies, past and present on antifungal compounds are variable. This may be due to the different inoculation techniques or different extraction methods used. For example, infiltration of spore inoculum directly into stem segments resulted in greater accumulation of phytoalexins (Tjamos & Smith, 1974). The cultural conditions of the pathogen may also affect the phytoalexin eliciting properties. Temperature may also have an effect on the production of phytoalexins. When Hutson (1978) inoculated tomato plants with *Fol* at 25 °C, instead of 28 °C, six antifungal compounds were formed instead of the usual two.

Phytoalexins, although implicated by others (Tjamos & Smith, 1974; Hutson & Smith, 1980) to play a direct role in resistance mechanisms, are seen here as a host response to the stress of infection. Any role played must be of a secondary nature. Antifungal compounds, possibly play a role in association with vascular occlusion, assisting the localisation of the pathogen within these structures. As with the phytoalexins, the pre-formed inhibitor, tomatine, increases following infection but at similar concentrations in both resistant and susceptible cultivars, hence not playing a direct role in resistance.

Selected host glycosidases have been shown to increase following infection (Pegg & Young, 1981; Young & Pegg,1981). Recent reports indicate that the increased enzyme levels are of host origin and are implicated in a generalised resistance response only (Ferraris *et al*, 1987). In accordance with results obtained in the present investigation, Ferraris *et al* (1987) found no inverse correlation between *Fol* mycelial colonisation and glycosidase activity (cf. Pegg & Young (1981) using *Verticillium albo-atrum*), although unlike the present results, they found more enzyme in the stem than in the root. In the present investigation, the initial burst of increased β -1,3-glucanase activity in the resistant cultivar was soon overtaken by that in the susceptible cultivar, probably reflecting the more widespread stimulation of host tissues occurring. Whether β -1,3-glucanase makes a significant contribution to resistance via hyphal lysis is open to debate (Pegg & Young, 1981).

Inoculation through the disturbed root system of uprooted plants seemed the most efficient method of introducing the pathogen. Stem inoculation, although used quite efficiently by Elgersma (1980), was unsatisfactory and did not induce symptoms in inoculated plants. Inoculation into leaf intercellular spaces also did not induce any symptoms, nor allow one to differentiate between resistant and susceptible cultivars. That leaves are capable of a reaction to vascular parasites is suggested by the results of Emmanouil (1977) who injected spores of *Fol* into leaf mesophyll and obtained a degree of systemic protection against *Verticillium dahliae*. In that study, induced synthesis of phytoalexins was suggested to play a role in the induced resistance.

The study of underlying mechanisms of disease resistance in host-parasite relationships should ultimately facilitate the selection of plants for breeding. The physiology and biochemistry of the vascular wilt syndrome is highly complex, and many unresolved questions remain. The search for elicitors of resistance must remain an important objective and the role of phytotoxins needs to be more fully assessed and understood. The original hypothesis with regard to the presence of diffusible elicitors remains intact, as it has not been either proven or disproven. A suitable bioassay has yet to be found to facilitate the search for such putative elicitors. The mechanism of the enhanced sensitivity to toxic metabolites exhibited by grafted genetically resistant scions is worthy of further study. It may be possible using such grafted shoots to devise an appropriate bioassay for the study of specific elicitor molecules in vascular wilt disease.

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# APPENDIX

Table 10 Analysis of variance table to compare heights between stem-inoculated and control plants cv. Craigella R and S

a) 14 days b) 21 days after inoculation

a)

| Source of variation | degrees of freedom | sum of squares | mean square | F value |
|---|---|---|---|---|
| Between isoline | 1 | 2261.48 | 2261.48 | 1.888* |
| Between treatment | 1 | 3.24 | 3.24 | 0.0027* |
| Interaction | 1 | 1455.42 | 1455.42 | 1.215* |
| Error | 12 | 14371.35 | 1197.61 | |

b)

| Source of variation | degrees of freedom | sum of squares | mean square | F value |
|---|---|---|---|---|
| Between isoline | 1 | 17377.83 | 17377.83 | 4.659* |
| Between treatment | 1 | 1.906 | 1.906 | 0.00051* |
| Interaction | 1 | 93.41 | 93.41 | 0.025* |
| Error | 12 | 44759.62 | 3729.67 | |

* No significant differences at P = 0.05 between isolines or treatment

Fig 8a Analysis of variance table to evaluate the differences between scion-stock graft combinations

| Treatment | degrees of freedom | sum of squares | mean square | F value |
|---|---|---|---|---|
| Between scion | 1 | 0.124 | 0.124 | 22.14 |
| Between stock | 1 | 0.574 | 0.574 | 102.5 |
| Interaction | 1 | 0.477 | 0.477 | 84.8** |
| Error | 16 | 0.09 | 0.0056 | |

** Interaction is very significant at less than P = 0.01