CHARACTERIZATION AND PATHOGENICITY OF SOUTH AFRICAN ISOLATES OF *FUSARIUM OXYSPORUM* F. SP. *MELONIS*

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

SUMMARY

CHARACTERIZATION AND PATHOGENICITY OF SOUTH AFRICAN ISOLATES OF *FUSARIUM OXYSPORUM* F. SP. *MELONIS*

The purpose of this study was to characterize the race and vegetative compatibility of *Fusarium oxysporum* f. sp. *melonis* (FOM) isolates collected in the major melon producing areas, to report on their geographical distribution, and their possible relatedness to isolates from other countries.

Seventy two FOM isolates obtained from 30 fields in 17 melon producing regions were race-typed using the differential cultivars Topmark (susceptible to all races), Doublon (*Fom1*), CM 17187 (*Fom2*) and Perlita (*Fom3*) and grouped by means of vegetative compatibility. All isolates belonged to vegetative compatibility group 0134, indicating a high degree of genetic homogeneity among the South African FOM population. Fifty four isolates were identified as race 0, eight as race 1, and 10 as race 2. Race 0 occurred in 15 of the regions whereas race 1 was sporadically recovered. Race 2, on the other hand, was obtained only from four fields located in one geographical region. Perlita plants (carrying the gene *Fom3*) inoculated with local isolates of race 0 and race 2 and reference isolates of race 0 became stunted, their leaves turned yellow, and became thickened and brittle. These results suggested that *Fom3* in Perlita confers a tolerant reaction compared to the resistant reaction of gene *Fom1* in Doublon. The disease reaction of cultivar Perlita to FOM was therefore reinvestigated. Twenty isolates, including the four FOM races (0, 1, 2, and 1,2) obtained from different countries, were used. The differential cultivars were included to verify virulence of the isolates. Perlita plants inoculated with three isolates of race 2 remained asymptomatic. The remaining race 2 and 0 isolates, induced severe stunting of Perlita plants, but mean percentage stunting values did not differ significantly ($P = 0.05$) and ranged between 25.1 and 50.0. Leaves of stunted plants were chlorotic, thickened and brittle. Disease reaction of Perlita was verified at a lower inoculum concentration with two race 2 (pipette method) and two

race 0 isolates (root dip method). Results proved that *Fom3* does not confer similar resistance towards race 0 and some race 2 isolates as *Fom1* in Doublon. Cultivars possessing *Fom3*, should therefore be considered tolerant to FOM races 0 and 2.

The ability of a *nit* mutant isolate, generated from FOM race 0 which belongs to VCG 0134, to change its virulence during infection of melon plants, was investigated under quarantine. Seedlings of melon cultivars Imperial 45 and Early Sweet (no resistance genes), Amber (*Fom2*) and Fiata (*Fom1*, *Fom2*) were consecutively grown in two cement troughs in a gauzehouse. Each planting was terminated when plants had advanced Fusarium wilt or after the fruit were harvested. In the first planting, Imperial 45 seedlings were transplanted and artificially inoculated with the *nit* mutant isolate. In the consecutive plantings, seeds were sown in the infested soil to enable natural infection. For each crop, representative plants showing Fusarium wilt were selected for isolation. All *F. oxysporum* isolates recovered were single-spored and their *nit* mutant and VCG status verified. Virulence of the labelled isolates was determined using differential cultivars. In trough A, all plants of the susceptible cultivars Imperial 45 and Early Sweet crops showed Fusarium wilt. The labelled isolates recovered from the selected plants were all designated race 0. In the first crop (planting No. 5) of the resistant cultivar Amber, 6.7% of the plants developed Fusarium wilt. In the second Amber crop the disease incidence increased to 56.6%, and to 81.8% in the final crop. Contrary to the susceptible cultivars, only race 2 isolates were obtained from the symptomatic Amber plants. Similar data were found with the susceptible cultivar Imperial 45 and the resistant cultivar Amber in trough B. Planting of Fiata caused a dramatic reduction in Fusarium wilt incidence in trough B. However, 1.2% of plants were affected by Fusarium wilt in the first Fiata crop (planting No. 6), whereas 4% of the plants were symptomatic in the final planting. From these symptomatic Fiata plants only race 1,2 isolates were obtained. These findings, and the fact that the symptomatic plants represented a substantial proportion of the first Amber (approximately 7-15%) and Fiata (approximately 2%) crops, proved that changes in the race structure of this fungal pathogen occurred rapidly when confronted with a resistant cultivar.

The potential of RAPD analysis to differentiate between the isolates displaying virulence changes was evaluated. Four *F. oxysporum* f. sp. *niveum* isolates were included as an outgroup. A histopathological study was conducted to verify whether these isolates retain their ability to behave as true vascular pathogens. The three primers used clearly distinguished the 12 FOM isolates from the four *F. oxysporum* f. sp. *niveum* isolates. However, the primers showed a highly conserved and characteristic banding pattern for the FOM isolates which represented three physiological races (race 0, race 2, race 1,2), indicating that RAPD analysis cannot detect race-specific groupings in FOM. Disease reactions on the three differential cultivars confirmed the virulence of FOM isolates. The histopathological data furthermore proved that the two FOM races (race 2, race 1,2), which derived from the race 0 parent isolate, retained their ability to behave as true vascular pathogens.

OPSOMMING

DIE KARAKTERISERING EN PATOGENESITEIT VAN SUID-AFRIKAANSE ISOLATE VAN *FUSARIUM OXYSPORUM* F. SP. *MELONIS*

Die doel van die studie was om *Fusarium oxysporum* f. sp. *melonis* (FOM) isolate wat in die hoof spanspekproduserende gebiede versamel is, volgens ras en vegetatiewe verenigbaarheid te karakteriseer, en hul geografiese verspreiding en verwantskap met isolate van ander lande aan te dui.

Twee en sewentig FOM isolate afkomstig vanaf 30 landerye wat 17 spanspekproduserende areas verteenwoordig, is gebruik. Die differensiële kultivars Topmark (vatbaar vir alle rasse), Doublon (*Fom1*), CM 17187 (*Fom2*) en Perlita (*Fom3*) is gebruik om die rasbepalings te doen asook om die vegetatiewe verenigbare groepe (VVG) te bepaal. Al die isolate is as VVG 0134 geklassifiseer, wat 'n hoë mate van genetiese homogenesiteit binne die Suid-Afrikaanse populasie aandui. Vier en vyftig isolate is as ras 0, agt as ras 1 en 10 as ras 2 geklassifiseer. Ras 0 is vanaf 15 gebiede afkomstig, terwyl ras 1 sporadies voorgekom het. Ras 2 is vanuit vier landerye binne dieselfde geografiese gebied verkry. Plante van die kultivar Perlita wat met plaaslike isolate van ras 0 en 2, asook verwysings-isolate van ras 0 geïnokuleer is, het verdwerg voorgekom. Die blare van die plante het vergeel, verdik en bros voorgekom. Hierdie siekte reaksie het aangedui dat *Fom3* in Perlita toleransie bewerkstellig in teenstelling met die weerstandbiedende reaksie van geen *Fom1* in Doublon. Die siekte reaksie van Perlita teenoor FOM is dus verder ondesoek. Hiervoor is 20 isolate wat al vier FOM rasse insluit (0, 1, 2, en 1,2), en van verskillende wêrelddele afkomstig is, gebruik. Die virulensie van die isolate is met die differensiële kultivars bevestig. Drie van die ras 2 isolate het geen siektesimptome op Perlita veroorsaak nie. Die ander ras 2 isolate, en al die ras 0 isolate, het egter die Perlita plante aansienlik verdwerg en die blare vergeel en verdik. Laasgenoemde groep isolate het 'n gemiddelde verdwergingsindeks van tussen 25.1% en 50.0% veroorsaak. Die siekte reaksie by Perlita is verder bevestig deur plante

teen 'n laer inokulumdigtheid van twee ras 2 (pipet metode), en twee ras 0 (wortel-doop metode) isolate, te inokuleer. Uit die resultate was dit duidelik dat die weerstand wat *Fom3* teenoor ras 0 en sommige ras 2 isolate verskaf, van *Fom1* verskil. Kultivars wat oor die weerstandsgeen *Fom3* beskik moet dus as tolerant beskou word.

'n Ondersoek is geloods na die vermoë van 'n *nit* mutant isolaat, genereer vanaf die wilde ras 0 isolaat van FOM (VVG 0134), om onder kwarantyn sy virulensie gedurende infeksie van spanspekplante te verander. Saailinge van die spanspekkultivars Early Sweet (geen weerstandsgene), Amber (*Fom2*) en Fiata (*Fom1*, *Fom2*) is opeenvolgens in twee sement trôe in 'n gaashuis verbou. Die afsonderlike aanplantings is beëindig sodra gevorderde Fusarium-verwelksimptome verkry is, of nadat vrugte ge-oes is. Vir die eerste aanplanting is oorgeplante Imperial 45 saailinge kunsmatig met die *nit* mutant isolaat geïnokuleer. Tydens die opeenvolgende aanplantings is saad direk in die besmette grond gesaai ten einde natuurlike infeksie te verkry. Met elke aanplanting is isolasies gedoen vanaf verteenwoordigende plante wat Fusarium-verwelksimptome getoon het. Alle *F. oxysporum* isolate wat verkry is, is ge-enkelspoor en hul *nit* mutant status en VVG is bevestig. Virulensie van die gemerkte isolate is bepaal deur inokulasie van die differensiële kultivars. Alle plante van die vatbare Imperial 45 en Early Sweet kultivars wat in trog A geplant is, het Fusarium-verwelksimptome getoon. Die gemerkte isolate wat vanaf die verteenwoordigende plante verkry is, is almal as ras 0 geklassifiseer. Tydens die eerste aanplanting van die weerstandbiedende kultivar, Amber (aanplanting No. 5), het 6.7% van die plante Fusarium-verwelksimptome ontwikkel. Tydens die tweede en derde aanplanting van Amber het die frekwensie van siektevoorkoms verhoog na 56.6% en 81.8 %, onderskeidelik. In teenstelling met die vatbare kultivars, is slegs ras 2 vanuit die Amber plante met siektesimptome verkry. Soortgelyke resultate is met Imperial 45 en Amber in trog B verkry. Aanplanting van kultivar Fiata het egter 'n dramatiese verlaging in die voorkoms van Fusarium-verwelk bewerkstellig. Tydens die eerste Fiata aanplanting (aanplanting No. 6) het 1.2% plante Fusarium-verwelksimptome ontwikkel, en 4% tydens die laaste aanplanting. Vanaf hierdie plante is slegs ras 1,2 isolate verkry. Hierdie bevindings, en die feit dat 'n aansienlike hoeveelheid van die Amber (ongeveer 7-15%) en Fiata plante (ongeveer 2%) siektesimptome getoon het,

bewys dat FOM vinnig van virulensie verander wanneer die patogeen 'n weerstanbiedende kultivar infekteer.

Die vermoë van RAPD analise om tussen isolate wat in virulensie verander het, te onderskei, is ondersoek. Vier isolate van *F. oxysporum* f. sp. *niveum* is as 'n buite-groep ingesluit. Om te bevestig dat die isolate wat van ras verander het wel egte vaskulêre patogene is, is 'n histopatologiese ondersoek gedoen. Die drie inleiers wat gebruik is, het die 12 FOM isolate duidelik van die vier *F. oxysporum* f. sp. *niveum* isolate onderskei. Die 12 FOM isolate wat drie fiosologiese rasse (ras 0, ras 2, ras 1,2) verteenwoordig het, is egter saam gegroepeer, wat aandui dat hierdie metode nie tussen rasse van FOM kan onderskei nie. Inokulasiestudies met die differensiële kultivars het die virulensie van die isolate bevestig. Die histopatologiese ondersoek het verder bewys dat beide FOM rasse (ras 2, ras 1,2) wat vanaf die wilde tipe ras 0 isolaat ontstaan het, hul vermoë behou het om as egte vaskulêre patogene op te tree.

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CONTENTS

1. THE BIOLOGY OF *FUSARIUM OXYSPORUM* F. SP. *MELONIS*, WITH SPECIAL REFERENCE TO ITS CHARACTERIZATION

FUSARIUM WILT OF MELON

Fusarium oxysporum Schlecht. emend. Snyd. and Hans. f. sp. *melonis* Leach and Currence is a soilborne fungus which causes a vascular wilt of all melon (*Cucumis melo* L.) types, including muskmelon, canteloupe, crenshaw melon, and honeydew melon (Sherf & Macnab, 1986). The disease, called Fusarium wilt, is one of the most important Fusarium vascular wilt diseases attacking cucurbits. Severe seedling losses up to 50% during the first weeks of growth, and 90 to 100% cessation of melon production, are reported for individual fields (Sherf & Macnab, 1986). The disease was first officially reported on melon in New York in 1930 (Chupp, 1930). Three years later it was reported in Minnesota (Leach, 1933) and in 1936 in Ontario (Reid, 1958). However, before these reports several publications indicated earlier disease outbreaks caused by the same fungus, but it was not described in sufficient detail to justify conclusions. In 1899, for example, Sturgis suggested that the wilt disease which had been so prevalent at that time in melon fields may be due to a species of *Fusarium*, possibly the same as that associated with the wilt of watermelons (Sturgis, 1899). In the same year, Selby (1899) reported a wilt of melon, and stated that it appeared to be due to the same, or to a similar fungus to that causing the southern watermelon wilt. Cook (1923) reported a disease of melons in the greenhouse from which he isolated a *Fusarium* sp., very similar to which was then referred to as *F. vasinfectum* Atkinson. Until 1938 the causal organism of melon wilt was designated as form 2 of *F. bulbigenum* Cke. and Mass. var. *niveum* Wr because of its close relatedness to that of the watermelon wilt organism (Leach & Currence, 1938). In spite of the close similarity of the two fungi, Leach (1933) proved that they differ in pathogenicity. Currently Fusarium wilt is present throughout melon producing areas of the world (Brayford, 1992)

Disease symptoms. *F. oxysporum* f. sp. *melonis* can attack melon plants at any stage, even before the plants are sprouted, but especially when the fruit are maturing (Mas *et al.*, 1981; Sherf & Macnab, 1986). Seedlings die very quickly, with symptoms resembling damping-off. On older plants, the first symptom is either a yellowing or wilting of leaves on

one or more laterals. In the case of yellowing, the veins of some leaves turn yellow, then later these leaves become completely chlorotic, thickened, and brittle. Wilting may occur rapidly. Before the plant is completely wilted, necrotic lesions may appear on one side of the stem near the soil surface and extend for some distance as long, narrow, brown streaks from where gum may exude. If moisture conditions are favorable, the fungus sporulates in the necrotic zone and forms pinkish-coloured sporodochia. Plants may also show a sudden wilt without yellowing. Wilting generally starts at the tips of stems, progressing towards the base of the plant. Transverse sections of the stems of diseased plants show clear brown vascular discoloration corresponding to the vessels that have been colonized. Diseased plants may also be severely stunted.

The pathogen can be introduced to new areas on seed (Leach, 1936; Sherf & Macnab, 1986; Brayford, 1992). Once present in soil, the fungus can be spread by wind, equipment, and workers (Sherf & Macnab, 1986). It survives long periods in soil, most likely as chlamydospores, colonizing both host and non-host tissue and competing successfully with other soil fungi (Banihashemi & DeZeeuw, 1973; Banihashemi & DeZeeuw, 1975). The pathogen enters root tips and through tears in the cortex (Sherf & Macnab, 1986). Disease development is favoured by relatively low temperatures of 18-22°C (Leach, 1936; Molot & Mas, 1975), and by high nitrogen and low potassium levels. High nitrogen levels appear to increase susceptibility of melon plants, and high potassium levels the activity of beneficial competitive fungi in the rhizosphere (Stoddard 1947; Wensley & McKeen, 1965; Ramasamy & Prasad, 1975).

Methods of control. Various measures can be applied for disease control. Crop rotation can be useful (Sherf & Macnab, 1986), but may be non-effective due to the capacity of the organism to survive in soil, organic matter, or on the roots of various plants which are symptomless carriers. (Armstrong & Armstrong, 1948; McKeen, 1951; Banihashemi & DeZeeuw, 1973; Banihashemi & DeZeeuw, 1975; Mas *et al.*, 1981). Fumigation with products such as methyl bromide plus chloropicrin has been used, but does not provide complete control, presumably because the fungus reinvades treated soil (Mas & Bouhot, 1974; Marois *et al.*, 1983; Sherf & Macnab, 1986). Products with a therapeutic action, such as the fungicides benomyl and thiophanate applied as drenches, may prevent infection in early stages of plant growth. However, some isolates were found to be tolerant to benomyl and an application giving curative action require very high dosages which might be

phytotoxic (Wensley & Huang, 1970; Maraite & Meyer, 1971; Mas *et al.*, 1981). The use of antagonistic fungi and bacteria (Sherf & Macnab, 1986; Brayford, 1992), the provision of adequate calcium and potassium levels, and avoiding excessive nitrogen levels (Stoddard, 1947; Wensley & McKeen, 1965; Ramasamy & Prasad, 1975; Sherf & Macnab, 1986) as means of control have also been reported. Where the crop is of sufficient value, susceptible plants can be grafted onto resistant rootstocks, including *Cucurbita ficifolia* Bouche' and *Benincasa histida* (Thunb.) Cogn. (Ruggeri, 1967; Benoit, 1974; Sherf & Macnab, 1986).

Because of the persistence of the pathogen in soil, the disease is best managed by using wilt-resistant melon cultivars (Sherf & Macnab, 1986). Resistance is controlled primarily by three independently single dominant genes (*Fom1*, *Fom2*, *Fom3*) (Risser *et al.*, 1976; Zink & Gubler, 1985). Horizontal or non-specific polygenic resistance has been reported in melon from the Far East (Risser, 1973; Risser & Rode, 1973).

CHARACTERIZATION

Formae speciales. *Fusarium oxysporum* Schlecht. was the first of nine species described in the section Elegans, established as one of the six sections of the genus *Fusarium* by Wollenweber in 1913. By 1935, when Wollenweber and Reiking published their monograph of the genus, the number of species, varieties and forms included in Elegans had grown to 40 (Wollenweber & Reiking, 1935). Apart from the difficulty of separating these species, especially after maintenance for some time on artificial media, it was gradually realized that the selective pathogenicity of the Elegans isolates was not necessarily linked to the morphological variant designated by a species name such as "*bulbigenum*" or "*vasinfectum*" (Booth, 1971). The classification of all species and varieties of the section Elegans (Wollenweber & Reinking, 1935) as *F. oxysporum* has been considered in numerous publications (Gordon, 1965; Armstrong & Armstrong, 1968; Messiaen & Cassini, 1968; Booth, 1971; Gordon & Davis, 1997) since the idea was expressed by Hansford in 1926. Considerations such as these led Snyder and Hansen (1940) to amend the description of *F. oxysporum* to agree with that of the section Elegans. This reduced all the other species in the section to synonyms of *F. oxysporum*. They designated 25 forms of this species based on their pathogenic ability to specific hosts, including the Fusarium wilt organism of melon. Formae speciales were specific to one host and were named according to the Latin name of the host plant. Consequently, the form that attacked melons was designated as *F. oxysporum* f. sp. *melonis*.

However, as early as 1940 studies revealed that the initial concept of limited or highly selective pathogenicity of the formae speciales did not always apply. This was suggested by the finding (Armstrong *et al.*, 1940; Armstrong *et al.*, 1942) that *F. oxysporum* f. sp. *vasifectum* (Atk.) could attack plants in more than one family. Later, this was also found to be true for *F. oxysporum* f. sp. *batatas* (Wr.), *F. oxysporum* f. sp. *tracheiphilum* (E.F. Smith), *F. oxysporum* f. sp. *apii* (Nels. & Sherb.), and others (Armstrong & Armstrong, 1975). Amstrong and Amstrong (1978) undertook an investigation to determine whether those formae speciales that cause wilts of the Cucurbitaceae (*F. oxysporum* f. sp. *cucumerinum* Owen, *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *niveum* [E.F.Smith], *F. oxysporum* f. sp. *luffae* Kawai, Suzuki & Kawai, and *F. oxysporum* f. sp. *lagenariae* Matuo & Yamamoto) are sufficiently distinct in pathogenicity to be retained as formae speciales or whether they should be reclassified. They concluded that variations in virulence for a specific host occurred, but each forma specialis exhibited sufficient selective pathogenicity for the host from which it was derived to be retained as a valid forma specialis. However, in cross pathogenicity studies within the Cucurbitaceae, several authors (Reid, 1958; Palti & Joffe, 1971; Martyn & McLaughlin, 1983; Martyn, 1985) reported that one formae specialis could change to another. For example, *F. oxysporum* f. sp. *niveum* changed to *F. oxysporum* f. sp. *melonis* (Bouhot, 1981), *F. oxysporum* from wilted cucumber and watermelon plants were highly pathogenic to all cultivars assayed for cucumber, melon and watermelon (Mc Millan, 1986), and one isolate of *F. oxysporum* f. sp. *cucumerinum* was pathogenic to melon and watermelon (Gerlagh & Blok, 1988). Cross pathogenicity studies (Gerlagh & Blok, 1988) using five formae speciales attacking cucurbitaceous crops showed that cross infectivity regularly occurs, but that the different isolates of *F. oxysporum* from Cucurbitaceae have common characteristics which unite them as a group and distinguish them from isolates from other plants. Gerlagh and Blok (1988) therefore proposed to establish *F. oxysporum* f. sp. *cucurbitacearum* which is defined by the possibility to infect and cause wilting of one or more of the species belonging to the Cucurbitaceae. These authors also suggested that if a crop is considered to be the principal host, it should be indicated with the first two letters of the present forma specialis name, thus "race me" replaces the old "f. sp. *melonis*". Numbers might follow this race indication whenever (sub)races are known, thus *F. oxysporum* f. sp. *melonis* race 0 will become *F. oxysporum* f. sp. *cucurbitacearum* race me 0. However, this proposal was not adopted by mycologists, plant pathologists and breeders who seemed to agree with Armstrong and Armstrong's (1978) statement that although variations in virulence

do occur, sufficient selectivity is present to keep the formae speciales concept within this group.

Physiological races. Historically, isolates of *F. oxysporum* have been divided into formae speciales on the basis of virulence on a particular host or group of hosts. Subdivisions of formae speciales into races are made based on virulence to a particular set of differential host cultivars that vary in disease resistance. Already between 1933 and 1948 various reports regarding the occurrence of natural differences in pathogenicity and cultural characteristics of Fusarium wilt pathogens were published (Snyder, 1933; Sleeth, 1934; Armstrong *et al.*, 1939; Hendrix *et al.*, 1948). However, Miller (1945), who conducted research on the melon wilt organism, indicated that these authors neither purified their cultures by the single spore technique, nor maintained their cultures in a manner which would prevent mutation. Miller (1945) indicated that he could obtain only one type of the melon wilt pathogen from the field. McKeen (1951) supported Miller's contention with respect to the melon wilt pathogen. However, Eide *et al.* (1952) found an isolate with unusual virulence to a resistant melon variety and thought that various pathotypes existed in *F. oxysporum* f. sp. *melonis*. These authors were, however, unable to demonstrate the presence of physiological races. Reid (1958) compared cultural characteristics of 290 isolates of *Fusarium* isolated from melon and described 10 cultural races based on colony morphology on Czapeks' agar. Pathogenicity tests conducted under controlled conditions in a greenhouse revealed inherent differences in pathogenic ability of these races.

The existence of physiological races of *F. oxysporum* f. sp. *melonis* was finally proven in a comparative study of the reaction of three melon cultivars, Charentais T, Doublon, and CM 17187, to various isolates. In this study, Risser and Mas (1965) classified French isolates of *F. oxysporum* f. sp. *melonis* into three races according to their reactions on these cultivars (Table 1). In Doublon, resistance to race 1 was found to be controlled by a dominant gene designated as *Fom1* (Risser, 1973). Resistance of CM 17187 to both races 1 and 2 was attributed to another dominant gene, independent of *Fom1* and designated *Fom2* (Risser, 1973). Banihashemi (1968) showed that American isolates of *F. oxysporum* f. sp. *melonis* differed from the above races by attacking CM 17187, but not Doublon. He designated this as a new race, race 4. Unfortunately Risser *et al.* (1969) used this designation for isolates which, similar to race 3, were able to attack all three differential cultivars but which, in contrast to race 3, induce yellows symptoms instead of wilt. This led to confusion

in race differentiation and a new nomenclature was therefore proposed by Risser *et al.* (1976) (see Table 2). The two independent resistance genes, *Fom1* in Doublon and *Fom2* in CM 17187, were used to characterize four races: race 0, attacking only a cultivar (e.g. Charentais T) which lacks specific resistance genes; race 1, overcoming *Fom1*; race 2, overcoming *Fom2*; and race 1,2, overcoming both *Fom1* and *Fom2*. Race 1,2 was further subdivided into race 1,2 wilt strain and race 1,2 yellows strain (Risser *et al.*, 1976). This nomenclature follows the system of Black *et al.* (1953) for *Phytophthora infestans* (Mont.) Bary races and potato cultivars. Resistance genes in *C. melo* are numbered according to the order of discovery and *F. oxysporum* f. sp. *melonis* races named according to the resistance genes they can attack. Risser *et al.* (1976) stated that this modified nomenclature might not prevent future confusion if additional races are discovered.

Armstrong and Armstrong (1978) indicated that they would rather follow the original proposed numerical system for races of *F. oxysporum* f. sp. *melonis*; i.e., 1, 2, 3, and 4, since they have described three new races; 5, 6, and 7, by including three more differentials. However, this race classification system was never adopted and the modified system proposed by Risser *et al.* (1976), was generally accepted and is still used today. Thus, two independent genes, *Fom1* and *Fom2*, carried respectively by the melon Doublon and CM 17187, enable the classification of the races of *F. oxysporum* f. sp. *melonis*. These resistant genes correspond to the genes governing virulence. The introduction of new independent resistant genes in melon would further increase the chances of diversification within this spectrum of races. This would help to determine the extent of flexibility in virulence.

Allelism tests by Zink and Gubler (1985) showed that gene *Fom1*, which controls resistance to race 0 and 2 in Doublon, was different from the gene controlling resistance to race 0 and 2 in Perlita and that two different genes confer resistance to races 0 and 2 in Doublon and Perlita. The resistant dominant gene in Perlita was designated *Fom3*. Therefore, although a new independent resistance gene was introduced, no changes of diversity within *F. oxysporum* f. sp. *melonis* races were reported since both these genes conferred the same resistance.

Vegetative compatibility grouping and mtDNA restriction patterns. Although virulence has been an extremely useful characteristic for differentiating isolates of *F. oxysporum,* there are some inherent problems associated with characterizing isolates solely on virulence. Groupings based on host-pathogen interaction are dictated by the genetic

makeup of the host or simply the differential cultivars available to distinguish isolates. Pathogenicity tests are influenced by many variables such as environmental conditions, host age, method of inoculation, selection of typical isolates, inoculum concentration, and disease evaluation (Correll, 1991; Windels, 1991) and may also be somewhat subjective. Additional characterization of subspecific groups in *F. oxysporum* was reported by Puhalla (1985), who developed techniques necessary for identifying vegetative compatibility groups (VCGs) within this species. These VCGs correlated to formae speciales which suggested that they may represent genetically isolated populations (Puhalla, 1985). Since then more in-depth studies have been conducted focusing on VCG relationships with formae speciales, races and other molecular traits (Correll, 1991; Kim *et al.*, 1993).

Jacobson and Gordon (1988) did the first vegetative compatibility characterization of a worldwide collection of *F. oxysporum* f. sp. *melonis* isolates. The 65 isolates characterized comprised five distinct VCGs, namely 0130, 0131, 0132, 0133, and 0134. VCGs were numbered according to Puhalla (1985) with each VCG having four digits, the first three refer to the forma specialis and the last digit to the isolates within the forma specialis. Jacobson and Gordon (1990a) added three new VCGs (0135, 0136, 0137) when characterizing an additional 119 *F. oxysporum* f. sp. *melonis* isolates. In 1994 Katan *et al.* identified another grouping, VCG 0138, among 122 isolates from Israel. Although Gennari and D'Ercole (1994) have described three distinct VCGs from 13 *F. oxysporum* f. sp. *melonis* isolates in Italy, they were not matched with the known VCG tester isolates and therefore only nine VCGs are reported so far. However, only eight VCGs are recognized (Zuniga *et al.*, 1997) since VCG 0137 represented only one isolate which was received by Jacobson and Gordon (1990a) as *F. oxysporum* f. sp. *melonis*, but proved to be avirulent (Table 3). Despite the fact that a small number of the isolates used in these studies were vegetatively self-incompatible and therefore could not be assigned to a VCG, this work, together with the integrated use of powerful molecular tools, has greatly facilitated our current understanding of the population biology, and race relationships of *F. oxysporum* f. sp. *melonis*. Contrary to *F. oxysporum* f. sp. *apii* (Correll *et al.*, 1986), *F. oxysporum* f. sp. *conglutinans* (Wr.) (Bosland & Williams, 1987), *F. oxysporum* f. sp. *vasinfectum* (Katan & Katan, 1988), and *F. oxysporum* f. sp. *dianthii* (Prill. & Del.) (Katan *et al.*, 1989), no direct relationship exists between VCG and race in *F. oxysporum* f. sp. *melonis* (Table 3). All four races of *F. oxysporum* f. sp. *melonis* were found in more than one VCG, indicating that no race represents a genetically homogeneous group of isolates. In contrast, all four races were also present in a single VCG.

These worldwide surveys and results indicated that there is substantial genetic diversity in *F. oxysporum* f. sp. *melonis* as reflected by both multiple races and multiple VCGs. Despite the complex race-VCG relationship, vegetative compatibility still can be quite useful in distinguishing pathogens from nonpathogens as well as characterizing genetic diversity within the population.

VCG diversity within and between geographic regions was also found (Jacobson & Gordon, 1988; Jacobson & Gordon; 1990a; Katan *et al.*, 1994; Zuniga *et al.*, 1997) (Table 3). Three VCGs were reported in the United States (0130, 0131, 0134), three in France (0133, 0134, 0135), and two in Israel (0135, 0138). Most of the characterized isolates from France have been assigned to VCG 0134. Although no single trait provides an entirely satisfactory basis for classification of *F. oxysporum* f. sp. *melonis*, Jacobson and Gordon (1991) suggested that *F. oxysporum* f. sp. *melonis* isolates should be described by both virulence and VCG, which is feasible and reveals much more information than either trait alone.

Molecular traits, especially polymorphisms in nuclear and mitochondrial DNA (mtDNA), have been used to assess interspecific and intraspecific relationships in fungi, including plant pathogens. Jacobson and Gordon (1990b) did the initial survey for restriction fragment length polymorphisms (RFLP) in mtDNA with *F. oxysporum* f. sp. *melonis* isolates. These isolates represented seven VCGs, the extent of race diversity and geographical distribution. Using three endonucleases, seven distinct restriction fragment length patterns were visualized (Table 3). These patterns were correlated to VCG. Six VCGs had unique patterns whereas two VCGs (0130, 0131) shared the seventh pattern. However, Zuniga *et al.* (1997) were able to obtain two distinct RFLP patterns for VCG 0130 and 0131 by probing *Eco*R1 digest of total DNA with a repetitive element (Table 3). With mtDNA polymorphisms researchers were able to quantify relationships among VCGs, to clarify the status of self-incompatible isolates within the forma specialis, and provided further insight into the associations between VCG, race, and geographical distribution. The correlation between mtDNA and VCG confirmed the importance of VCG as an indicator of genetically isolated populations within *F. oxysporum* f. sp. *melonis* and served as the foundation from which sub-forma specialis relationships were calculated. However, the race diversity within a VCG or mtDNA framework should not be ignored. For example, multiple races (0, 1, 2, and 1,2) occur within VCG 0134, each with the same mtDNA pattern, indicating that the races most likely originated from a common genetic background. However, a study by

Schroeder and Gordon (1994) revealed variation among *F. oxysporum* f. sp. *melonis* isolates associated with the same VCG when the repetitive element was used to generate the RFLP in nuclear DNA, and thus provide a more sensitive measure of identity than vegetative compatibility. Thus, there probably is no one trait, either physiological, genetic, or molecular, that alone will provide an entirely satisfactory basis for classification at any taxonomic level. Jacobson and Gordon (1991) stated that invoking more than one scenario may, in fact, be the only way to explain the diversity in *F. oxysporum* f. sp. *melonis*. Relationships based on mtDNA polymorphisms suggest that both host specialization and genetic isolation probably occurred repeatedly, but at different levels, in progenitor populations of what is currently recognized as *F. oxysporum* f. sp. *melonis*.

CONCLUSION

A good knowledge and understanding of the genetic diversity of *F. oxysporum* f. sp. *melonis* is essential in any effort to control melon Fusarium wilt effectively. Although the disease occurs annually in fields in the melon producing regions of South Africa, little is known of its origin and the distribution of races. The purpose of this study was to characterize the race and vegetative compatibility of FOM strains collected in the major melon producing areas, to report on their geographical distribution, and their possible relatedness to strains from other countries.

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Table 1. Differential resistance to *Fusarium oxysporum* f. sp. *melonis* as described by Risser and Mas (1965)

	Fusarium oxysporum f. sp. *melonis* isolate		
Melon cultivar	Race 1	Race 2	Race 3
Charentais T	S	S	S
Doublon	R	S	S
CM 17187	R	R	S

R = resistant, S = susceptible

Table 2. A classification of *Fusarium oxysporum* f. sp. *melonis* races according to differential host cultivars of *Cucumis melo* as described by Risser *et al.* (1976)

Race of *F. o. melonis*	Differential hosts and their gene for resistance		
	Charentais T	Doublon (*Fom1*)	CM 17187 (*Fom2*)
Race 0	S	R	R
Race 1	S	S	R
Race 2	S	R	S
Race 1,2	S	S	S

R = resistant, S = susceptible

Table 3. Summary of the variability of vegetative compatibility groups (VCG), physiologic races, mitochondrial DNA restriction pattern, and distribution as indicators of relationships between populations of *Fusarium oxysporum* f. sp. *melonis,* as reported by various authors[a]

VCG[b]	Race[c]	mtDNA pattern	Region
0130	2	A	California, New York
0131	2	A(A1[d])	California, Canada, Indiana, Maryland, Michigan, New York
0132	2	B	Japan
0133	1,2	C	France
0134	0	D	France, Italy, Maryland, Mexico,Texas
	1	D	California, France, Maryland, New York
	2	D	Italy
	1,2	D	France
0135	0	E	Israel, France
	2		Israel
0136	1	F	Mexico
0137	-[e]	G	France
0138	0		Israel
	1		Israel
	1,2		Israel

[a] Gordon and Davis (1997); Jacobson and Gordon (1988); Jacobson and Gordon (1990a); Katan *et al.* (1994); Zuniga *et al.* (1997).

[b] VCG numbered according to Puhalla (1985).

[c] Race classification according to Risser *et al.* (1976).

[d] Isolates used in a survey by Jacobson and Gordon (1990a) revealed the same pattern as VCG 0130, but Zuniga *et al.* (1997), using a revised method, were able to get two different patterns.

[e] Represent one isolate, received as *F. oxysporum* f. sp. *melonis*, but which was avirulent.

2. RACE DETERMINATION AND VEGETATIVE COMPATIBILITY GROUPING OF *FUSARIUM OXYSPORUM* F. SP. *MELONIS* FROM SOUTH AFRICA

ABSTRACT

Seventy two isolates of *Fusarium oxysporum* f. sp. *melonis* (FOM) obtained from 30 fields in 17 melon producing regions in South Africa were race-typed using the differential cultivars CM 17187, Doublon, Perlita and Topmark and grouped by means of vegetative compatibility. Fifty four isolates were identified as race 0, eight as race 1, and 10 as race 2. Race 0 occurred in 15 of the regions whereas race 1 was sporadically recovered. Race 2, on the other hand, was obtained only from four fields located in one geographical region. Perlita plants (carrying the gene *Fom3*) inoculated with local isolates of race 0 and race 2 and reference isolates of race 0 became stunted, with their leaves turning yellow, thickened and brittle. Similar symptoms were induced by reference and local isolates of race 0 on Perlita seedlings using two different inoculation methods. These results indicated that *Fom3* in Perlita confers a tolerant reaction compared to the resistant reaction of gene *Fom1* in Doublon, and should therefore not be used alone in race determination tests. All isolates belonged to vegetative compatibility group 0134, indicating a high degree of genetic homogeneity among the South African FOM population.

INTRODUCTION

Fusarium wilt, caused by *Fusarium oxysporum* Schlecht. emend. Snyd. and Hans. f. sp. *melonis* Leach and Currence (FOM), is an economically important disease of melon (*Cucumis melo* L.) in South Africa and other areas of the world (Sherf & Macnab, 1986; Jacobson & Gordon, 1991). Because of the persistence of the pathogen in the soil, the disease is best managed by using wilt-resistant melon cultivars. Risser *et al.* (1976) has divided four races of FOM based on three differential cultivars: race 0, attacking only cultivars which lack any genes for resistance to FOM (e.g. Charentais T); race 1, overcoming the resistance gene *Fom1* in the cultivar Doublon; race 2, overcoming the resistance gene *Fom2* in the cultivar CM 17187 and race 1,2 overcoming both resistance genes. Race 1,2 is further divided into race 1,2 wilt strain and race 1,2 yellow strain. Inheritance studies

conducted by Zink and Gubler (1985) revealed that similar to Doublon (*Fom1*), resistance in the cultivar Perlita was conferred towards race 0 and race 2 by another dominant gene, *Fom3*.

Isolates of FOM from various geographic regions have been grouped on the basis of vegetative compatibility (Jacobson & Gordon, 1988; Jacobson & Gordon, 1990a; Katan *et al.*, 1994). This approach, initiated by Puhalla (1985), provides a means of distinguishing subspecific groups in *F. oxysporum* based on the genetics of the fungus rather than on the host-pathogen interaction. In FOM, eight vegetative compatibility groups (VCGs) have been characterized worldwide (Jacobson & Gordon, 1988; Jacobson & Gordon, 1990a; Katan *et al.*, 1994). The relationship between race and VCG, however, proved to be complex (Jacobson & Gordon, 1991; Gennari & D'Ercole, 1994). Isolates of all four races of FOM were each found in more than one VCG, indicating that no race represents a genetically homogeneous group of isolates, and in contrast all four races were present in a single VCG. VCG diversity within and between geographic regions also was found although some VCGs were predominantly from specific geographical regions (Jacobson & Gordon, 1990a), with VCG 0138 restricted to Israel only (Katan *et al.*, 1994). Although the race-VCG relationship cannot be used to identify races, it proved to be useful in characterizing genetic diversity within the pathogen population. The correlation between mtDNA and VCG in FOM helped to confirm the importance of VCG as an indicator of genetically isolated populations (Jacobson & Gordon, 1990b). Jacobson and Gordon (1991) therefore suggested that FOM isolates should be described by both virulence and VCG as it reveals more information than either alone.

Although Fusarium wilt occurs annually in fields in the melon producing regions of South Africa, little is known of its origin or the distribution of races. No race determination tests were conducted with South African FOM isolates, and meaningful recommendations for choice of resistant cultivars could not be made. Prior to 1989 most commercial cultivars lacked resistance to Fusarium wilt and those which had resistance were primarily cultivated for their fruit characteristics. The purpose of this study was to characterize the race and vegetative compatibility of FOM isolates collected in the major melon producing areas, their geographical distribution and their possible relatedness to isolates of FOM from other countries. Although isolates of this pathogen are given the classification f. sp. *melonis* because of their host specificity towards *C. melo*, cross pathogenicity of this and other formae speciales among the Cucurbitaceae are reported (Bouhot, 1981; Gerlagh & Blok, 1988).

Consequently pathogenicity tests on other Cucurbitaceae were conducted.

MATERIALS AND METHODS

Selected isolates. During 1989 to 1995, melon plants with typical Fusarium wilt symptoms (Mas *et al.*, 1981; Sherf & Macnab, 1986) were collected from 30 melon fields in the major melon producing regions of South Africa and transported to the laboratory. Diseased stems and roots were washed under a fine spray of tap water, surface-disinfested (1 min in 0.5% NaOCl, rinsed in distilled water, followed by 1 min in 70% ethanol) and air-dried on a laminar flow bench. Stems and roots were split in half, parts were aseptically dissected and plated on Komada's medium (Komada, 1975) amended with 1 ml/l of Tergitol NP-10 (Puhalla, 1984). Plates were incubated at 17-23 °C for 8-14 days on the laboratory bench. Colonies exhibiting the characteristic morphology of *F. oxysporum* (Komada, 1975) were transferred to divided petri dishes containing potato dextrose agar with 0.02% novostreptomycin (Novo Nordisk, South Africa) (PDA+) and carnation-leaf agar (Fisher *et al.*, 1982) with 0.02% novostreptomycin. Divided plates were incubated at 20 °C for 14-21 days under intermittent light (12 h fluorescent plus black light cycles) and single conidial isolates made on water agar as described by Nelson *et al.* (1983). Single-conidial isolates were identified according to Nelson *et al.* (1983) and representative isolates were lyophilised. Seventy two isolates (one to four per melon field) obtained from 17 regions were selected for vegetative compatibility grouping and race determination. Isolates were deposited in the National Collection of Fungi (PREM 51304 - 51353), Private Bag X134, Pretoria 0001, South Africa. FOM isolates ATCC 28856, ATCC 28857, ATCC 28858, ATCC 28861, ATCC 28826, ATCC 32669, Fom 8803, I1, T61, R12w and 466C (Table 1) were used as references in race determination.

Race determination. Virulence was determined by using the pipette inoculation method of Latin and Snell (1986). Lyophilized cultures were grown on PDA+. After 5 days, mycelial plugs (3 mm in diameter) were taken from the actively growing colony margins of each isolate, transferred to 100 ml of a potato dextrose broth (difco) (10 g/l) and incubated at 25 °C for 5 days in a rotary incubator (150 rpm). The contents of each flask was filtered through four layers of sterile cheesecloth, the density of the predominantly microconidial suspension of each isolate determined with a haemacytometer and adjusted to 10^6 conidia/ml. Seeds of the four differential melon cultivars CM 17187 (*Fom2*), Doublon (*Fom1*), Perlita (*Fom3*) and Topmark (no resistance genes) (Jacobson & Gordon, 1988) were surface

disinfested with 5% calcium hypochlorite solution for 5 min, rinsed twice in sterile distilled water and planted into celltype plastic growing trays (one seedling per 60 ml cell) filled with a pasteurized commercial potting mixture (peat:vermiculite:polystyrene [18:12:1 m/m], pH 5.8, Hygrotech Seed, South Africa). Each growing tray contained 128 removeable cells. Seeds were obtained from J.P. Crill, Colorado; T.R. Gordon, University of California; Hollar Seeds, Colorado; and Petoseed, Woodland. The trays were incubated in a growth chamber at 18/25 °C night/day temperatures under cool white fluorescent light with 14 h photoperiod. Seedlings were inoculated 11-12 days (expansion of first true leaf stage) after planting. Five milliliters of the inoculum suspension were delivered to the substrate around each seedling. Control plants received sterile diluted potato dextrose broth. At least five seedlings of each cultivar were inoculated and each isolate was tested at least twice. After inoculation seedlings were watered once a week with a soluble fertilizer (Chemicult hydroponic nutrient, 2 g/l, Chemicult Products, South Africa). Disease development as described by Jacobson and Gordon (1988) (stunting, chlorosis, necrosis and finaly death) was recorded weekly and final assessments were made after 28 days. Isolates were considered virulent on a given cultivar when at least 80% of the seedlings developed symptoms, and avirulent when no symptoms were evident. Isolates were assigned to the appropriate race based on their virulence to differential cultivars.

Cross pathogenicity. Thirty FOM isolates obtained from the different local melon fields were selected randomly and tested for pathogenicity on cucumber (*Cucumis sativus* L. cv. Marketer) and watermelon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai cv. Sugar Baby). Cucumber and watermelon seedlings were grown, inoculated and rated for disease development as previously described.

Vegetative compatibility. VCGs were determined through the complementation of nitrate nonutilizing (*nit*) mutants as a visual indicator of heterokaryon formation (Puhalla, 1985). *Nit* mutants were generated from each of the 72 FOM isolates on potato-sucrose agar containing 1.5% $KClO_3$ (Correll *et al.*, 1986). The concentration of $KClO_3$ was increased to 3% for isolates that were not restricted by 1.5% $KClO_3$ (Jacobson & Gordon, 1988). The fast-growing, chlorate-resistant sectors originating from the initially restricted colony, that grew thinly but expansively on Puhalla's minimal medium (MM) (Puhalla, 1985) were considered to be *nit* mutants. *Nit* mutants were phenotypically classified by their growth on basal medium (MM without $NaNO_3$) amended with one of several different nitrogen sources

(Correll *et al.*, 1987). *Nit* mutants (*nit*1, *nit*3 and *nit*M) generated from each of the FOM isolates were paired with tester strains (*nit*1 and *nit*M) of each of the different established VCGs obtained from T.R. Gordon (P2/6, P2/1, Pt2/1, Pt3/1, T61/1, R12w/13, R12w/14, 660A/1, 660A/17, I1/1, I1/5, K419/2, K419/5, A3/6, A3/4) or generated from wild-type isolates known to belong to a specific VCG. Pairings were made on MM in 9-cm Petri dishes incubated at room temperature (18-23 °C) in the dark and scored for complementation 7 and 14 days later. When a mutant successfully formed a complementary heterokaryon with a given tester, its parent was placed in corresponding VCG.

RESULTS

Race determination. The reference isolates caused the expected disease reactions on the differential cultivars CM 17187, Doublon and Topmark. All were highly virulent on the susceptible cv. Topmark. The race 1 isolates were avirulent on CM 17187 (*Fom2*) but highly virulent on both Doublon (*Fom1*) and Perlita (*Fom3*). Race 2 isolates were highly virulent towards CM 17187 (*Fom2*) but avirulent towards Doublon (*Fom1*) and Perlita (*Fom3*). The race 0 reference isolates were avirulent to both Doublon (*Fom1*) and CM 17187 (*Fom2*). However, all four race 0 reference isolates induced stunting of Perlita (*Fom3*) plants. Leaves of the stunted Perlita seedlings became yellow, thickened and brittle. The virulence test was therefore repeated four times, using Perlita (*Fom3*) seed lots obtained from the four aforementioned sources, and a range of inoculum concentrations (1×10^5, 5×10^5, 1×10^6, 5×10^6 conidia/ml). To further verify this finding the reference isolate ATCC 28856 (race 0) and one of the race 0 isolates from South Africa (PREM 51308) were used to inoculate Perlita seedlings (30 seedlings/treatment, including water treated controls) using the root dip method (Zink & Gubler, 1985). In all experiments, race 0 isolates indiscriminately caused stunting of Perlita, with leaves becoming yellow, thickened and brittle.

Virulence of the FOM isolates, and their geographical and host origin are given (Table 2). All isolates were highly virulent on the susceptible cultivar Topmark. Fifty four isolates, obtained from 15 of the 17 regions surveyed, were avirulent on CM 17187 (*Fom2*) and Doublon (*Fom1*) and therefore assigned to race 0. Forty five of the race 0 isolates were recovered from plants of cultivars with no resistance to FOM, five were recovered from the cultivars Doral (fields 16, 17) and Saticoy (field 6) in which resistance to races 0 and 2 is conferred by *Fom3* (A. Comrie, personal communication), and four from cultivars resistant to races 0 and 1. Three of the latter isolates originated from cultivar Helios (*Fom2*) plants (field

30) infested with root-knot nematode which could contribute to the stunting and yellowing symptoms or reduce the plant's resistance to FOM (Bergeson, 1975). Another isolate was recovered from a Galia 5 (*Fom2*) plant (field 27) from which a race 2 isolate was also recovered. The race 0 isolates, however, all induced similar symptoms on Perlita (*Fom3*) seedlings as was found with the reference race 0 isolates.

Eight isolates avirulent on CM 17187 (*Fom2*), but highly virulent on both Doublon (*Fom1*) and Perlita (*Fom3*), were assigned to race 1. These isolates originated from five regions, in three of which race 0 and race 1 were recovered from the same fields.

The ten remaining isolates were highly virulent on CM 17187 (*Fom2*) but avirulent on Doublon (*Fom1*) and therefore assigned to race 2. These isolates, however, caused the same stunting and yellowing symptoms on Perlita (*Fom3*) as referred to previously with race 0 isolates. These isolates were all recovered from plants of cultivars Galia 5 (*Fom2*), Helios (*Fom2*) and Limor (*Fom2*), all resistant to FOM races 0 and 1, sampled in four separate fields (field 11, 12, 27, 30) in three regions located in one geographical area. All four of these fields had a history of Fusarium wilt, from where FOM race 0 was previously identified. After planting race 0 and 1 resistant cultivars in two successive years on these fields, race 2 isolates were recovered after plants started to wilt in the second year of planting (Table 2).

Cross pathogenicity. The 30 FOM isolates did not induce any Fusarium wilt symptoms on the cucumber and watermelon seedlings.

Vegetative compatibility. The South African FOM isolates readily produced chlorate resistant sectors. At least three *nit* mutants (*nit*1, *nit*3 and *nit*M) were generated from each of the South African isolates and paired with the testers from each of the established VCGs. All 72 South African isolates which represent three distinct virulent phenotypes formed complementary heterokaryons with the testers of VCG 0134.

DISCUSSION

This study demonstrated a high degree of VCG homogeneity among South African isolates of FOM. The 72 FOM isolates comprising three races, recovered between 1989 and 1995 from diseased melon plants sampled from 17 melon producing regions, all belonged to VCG 0134. This VCG is considered to be predominantly a European VCG (Jacobson & Gordon, 1991), suggesting that the South African wilt inducing FOM was introduced from

Europe. The lack of cross pathogenicity to cucumber and watermelon corroborated the host specificity towards *C. melo* of the local isolates and validates the forma specialis concept in cucurbits (Snyder & Hansen, 1940). The data futhermore suggested that the race 2 isolates probably were not introduced to South Africa independently, but derived locally from race 0 isolates. This hypothesis is substantiated by the occurrence of race 2 in melon fields in the production regions Paarl (Field no. 11,12), Wellington (field no. 27) and Worcester (field no. 30). Prior to 1989 most cultivars commercially cultivated in fields in these regions lacked resistance to Fusarium wilt. In 1990 (field no. 11, 12, 27) and 1992 (field no. 30), only race 0 was recovered from diseased melon plants from these four fields. Cultivars resistant to races 0 and 1 (*Fom2*) were introduced in the following years and planted repeatedly in the same fields. Two years after introducing these cultivars producers reported serious yield losses, race 2 was obtained from these specific fields. For comparison, Bouhot (1981) also found that after introducing new resistant melon cultivars into France they were attacked by FOM by the second year of planting. He showed that by using chemical treatments to induce mutations of FOM isolates, one race could mutate into another and that the pathogenicity factors in these forms could coexist in one isolate. Considering this observation and the VCG data, it seems likely that race 2 was derived from the isolates of race 0 previously predominating these fields. The fact that in South Africa race 2 was only found from race 0 infested fields cultivated with resistant cultivars (*Fom2*), that the South African race 2 isolates differed from the race 2 reference isolates in their ability to attack Perlita (*Fom3*), and that both South African phenotypes (races 0 and 2) belonged to the same VCG make it unlikely that race 2 was introduced into these four fields from a foreign source. The host genotype seems to have played a definite role in the establishment and build up of the local race 2 population. This conclusion does not conform with the findings of Bouhot (1981), that race 1,2 existed in France at least 12 years before melon cultivars were bred for resistance to this race.

The susceptible reaction of Perlita (*Fom3*) to the South African race 0 and race 2 isolates, as well as to the reference race 0 isolates, casts doubt on the resistance characteristics of this cultivar. According to Jacobson and Gordon (1988) plants are considered to be diseased if they are stunted and chlorotic. These symptoms were consistently caused on Perlita (*Fom3*) seedlings inoculated with the South African race 0 and race 2 isolates, and reference race 0 isolates. A similar reaction was obtained when a different inoculation method (root dip method) was used. Based on these criteria, our results

demonstrated that when Perlita (*Fom3*) alone is used as differential to determine FOM races, wrong interpretations can be made. It seems that the *Fom3* gene in Perlita rather conferred a tolerant reaction towards race 0 and the South African race 2 isolates and not a resistant reaction similar to the gene *Fom1* in Doublon. Zink and Gubler (1985) also indicated that Perlita (*Fom3*) did not show wilt symptoms when inoculated (root dip method) with a conidial suspension containing less than 5×10^5 conidia/ml of races 0 and 2. However, in our experiments using the pipette inoculation method, isolates of race 0, were able to induce stunting at an inoculum concentration of 1×10^5 conidia/ml. Furthermore, in the commercial fields race 0 was able to induce typical Fusarium wilt symptoms and cause severe yield losses on cultivars possessing the *Fom3* gene (field 6,16,17). Therefore it is not only unreliable to use *Fom3* in race determination tests, but also as a control measure in the field against race 0 and 2. The disease reaction of Perlita (*Fom3*) towards races 0 and 2 is currently under further investigation.

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Table 1. Reference isolates of *Fusarium oxysporum* f. sp. *melonis* representing four physiologic races

Strain	Race[a]	Origin	Source[b]
ATCC 28856	0	France	A
ATCC 28857	1	France	A
ATCC 28858	1,2	France	A
ATCC 28861	2	Japan	A
ATCC 28862	1	Israel	A
ATCC 32669	2	Michigan,USA	A
Fom 8803	0	Spain	B
I1	0	Israel	C
T61	2	Unknown	C
R12w	1,2	France	C
466C	0	Maryland,USA	D

[a]Race designation according to Risser *et al.* (1976).

[b]A = American Type Culture Collection; B = R.M. Jiménez Díaz, Universidad de Cordoba, Spain; C = T.R. Gordon, University of California Davis; D = D.M.S. Spinks, Hollar Seeds, Colorado.

Table 2. Race determination of 72 isolates of *Fusarium oxysporum* f. sp. *melonis* (FOM) recovered from diseased plants in 30 fields from 17 melon producing regions in South Africa

Region	Field no.	Sampling date	Cultivar of origin[a]	Resistance gene[b]	Race(s) recovered[c]	Number of isolates
Citrusdal	1	02/1991	Galia	-	0	1
Clanwilliam	2	10/1990	Galia	-	0	4
	3	11/1990	Lyon Jumbo	-	0,1	3
	4	11/1990	Lyon Jumbo	-	0	1
De Doorns	5	11/1989	Gold King	-	0	4
Douglas	6	11/1992	Saticoy	*Fom3*	0	1
Kimberley	7	02/1992	Saticoy	*Fom3*	1	2
	8	02/1992	Sutter	-	1	1
Klapmuts	9	01/1991	Early Dawn	*Fom1*	1	1
Mooreesburg	10	11/1990	Galia	-	0	2
Paarl	11	09/1990	Galia	-	0	2
	11	11/1992	Galia 5	*Fom2*	2	2
	12	11/1990	Ogen	-	0	2
	12	01/1993	Galia 5,Limor	*Fom2*	2	3
Philipi	13	02/1991	HSR 444	-	0	1
Piketberg	14	11/1990	Lyon Jumbo	-	0	3
Porterville	15	11/1990	Yellow Canaria	-	0	1
	16	11/1991	Doral	*Fom3*	0	2
	17	03/1995	Doral	*Fom3*	0	2
Robertson	18	10/1991	Yellow Canaria	-	0	1
	19	05/1992	Yellow Canaria	-	0	1
	20	05/1992	Yellow Canaria	-	0	1
Stanford	21	03/1993	Galera	-	0	2
Vioolsdrift	22	01/1992	Imperial 45	-	0	2
Vredendal	23	11/1991	Galia	-	0	2
	24	11/1991	Galia	-	0	1
Wellington	25	11/1990	Early Sweet	-	0,1	3
	26	12/1990	Early Sweet	-	0	2

Table 2. (continued)

Region	Field no.	Sampling date	Cultivar of origin[a]	Resistance gene[b]	Race(s) recovered[c]	Number of isolates
	27	12/1990	Lyon Jumbo	-	0	2
	27	11/1993	Galia 5	*Fom2*	0,2	4
Worcester	28	11/1989	Yellow Canaria	-	0,1	5
	29	11/1990	Yellow Canaria	-	0	3
	30	11/1992	Helios	*Fom2*	0	2
	30	01/1993	Helios	*Fom2*	0	1
	30	02/1995	Helios	*Fom2*	2	2

[a] Melon cultivars from where FOM isolates were recovered.

[b] Resistance genotypes of melon cultivars according to South African seed companies (A. Comrie, *personal communication*); - = no resistance.

[c] Race designation according to the differential cultivars CM 17187 (*Fom2*), Doublon (*Fom1*), Perlita (*Fom3*) and Topmark (no resistance genes).

3. REACTION OF MELON CULTIVAR PERLITA, WITH DOMINANT GENE *FOM3*, TO RACES 0 AND 2 OF *FUSARIUM OXYSPORUM* F. SP. *MELONIS*

ABSTRACT

The disease reaction of melon cultivar Perlita, possessing the dominant gene *Fom3*, to *Fusarium oxysporum* f. sp. *melonis* (FOM) was reinvestigated. Twenty isolates, including four races (0, 1, 2, and 1,2) obtained from different countries, were used. Differential cultivars Topmark (susceptible to all races), Doublon (*Fom1*) and CM 17187 (*Fom2*) were included to verify the virulence of isolates. Seedlings were inoculated by delivering 5 ml of a conidial suspension (1.0 x 10^6 conidia/ml) to the substrate around each seedling 11-12 days after planting. Mean percentage stunting (PS), and number of dead plants were recorded 28 days after inoculation. Perlita plants inoculated with three isolates of race 2 remained asymptomatic. The remaining seven isolates of race 2 and all seven race 0 isolates tested induced severe stunting of Perlita plants, but mean PS values did not differ significantly (P = 0.05) and ranged between 25.1 and 50.0. Leaves of stunted plants were chlorotic, thickened and brittle. Disease reaction of Perlita was verified at a lower inoculum concentration (5 x 10^5 conidia/ml) with two race 2 (pipette method) and two race 0 isolates (root dip method). Results proved that *Fom3* does not confer similar resistance towards race 0 and some race 2 isolates as *Fom1* in Doublon. Cultivars possessing *Fom3* should therefore be considered tolerant to FOM races 0 and 2.

INTRODUCTION

Fusarium wilt of melon (*Cucumis melo* L.), caused by *Fusarium oxysporum* Schlecht. emend. Snyd. and Hans. f. sp. *melonis* Leach and Currence (FOM), is an economically important disease in most melon producing countries (Sherf & Macnab, 1986; Jacobson & Gordon, 1991; Schreuder *et al.*, 2000). The pathogen has been divided into four races based on three differential cultivars (Risser *et al.*, 1976): race 0, attacking only cultivars (e.g. Charentais T) which lack any genes for resistance to FOM; race 1, overcoming the resistance gene *Fom1* in cultivar Doublon; race 2, overcoming the resistance gene *Fom2* in cultivar CM

17187 and race 1,2 overcoming both resistance genes. Inheritance studies (Zink *et al.*, 1983; Zink & Gubler, 1985) revealed that similar to Doublon (*Fom1*), resistance in cultivar Perlita was conferred towards race 0 and 2 by another dominant gene, *Fom3*. However, Zink (1991) later showed that although Perlita reacted resistant to races 0 and 2 at low inoculum dosages (0.5×10^6 spores/ml), some plants became stunted or necrotic when inoculated at high dosages (1.0×10^6 spores/ml). Schreuder *et al.* (2000) recorded similar symptoms on Perlita plants inoculated with isolates of races 0 and 2 from a study on race classification and vegetative compatibility of FOM isolates from South Africa. Schreuder *et al.* (2000) furthermore isolated race 0 from plants of the cultivars Doral and Saticoy, which both possess the dominant resistance gene *Fom3* and which showed typical Fusarium wilt symptoms and yield loss in the field. These observations suggest that the *Fom3* gene conferred a tolerant reaction towards race 0 and the South African race 2 isolates, and not a resistant reaction similar to the gene *Fom1*.

Due to the persistence of the pathogen in soil, the disease is best managed by using wilt-resistant melon cultivars (Zink & Gubler, 1986). Douglas (1970) previously suggested that a range of inoculum concentrations should be used in evaluating new melon breeding material for resistance to FOM. His studies indicated that the use of low inoculum dosages are prefereable to detect moderate resistance in cultivars. On the other hand, high inoculum dosages are needed in infection studies to determine the resistance efficacy under favorable disease conditions. It is thus important for melon breeders and plant pathologists to have a thorough understanding of the disease reaction conferred by resistance genes in melon. In the light of the dubious resistance characteristics displayed by Perlita (Zink, 1991; Schreuder *et al.*, 2000), the disease reaction of this cultivar to FOM races 0 and 2 isolates was reinvestigated.

MATERIALS AND METHODS

Isolates. Twenty FOM isolates, consisting of races 0, 1, 2, and 1,2, were used. Races, vegetative compatibility group (VCG), origin and source of the isolates are given in Table 1.

Virulence test. Lyophilized cultures of each isolate were grown on potato dextrose agar (Difco) with 0.02% novostreptomycin (Novo Nordisk, South Africa). After 5 days mycelial plugs (3 mm in diameter) were taken from the actively growing colony margins of each isolate, transferred to 100 ml of a potato dextrose broth (Difco) (10 g/l) and incubated at 25°C for 5 days on a rotary incubator (150 rpm). The contents of each flask was filtered through four layers of sterile cheesecloth, the density of the predominantly microconidial suspension of each isolate determined with a haemacytometer and adjusted to 1.0×10^6 conidia/ml. Perlita seeds were surface-disinfested in 5% calcium hypochlorite solution for 5 min, rinsed twice in sterile distilled water and sown into celltype growing trays (one seed per 60-ml cell) filled with a pasteurized commercial potting mixture (peat:vermiculite:polystyrene [18:12:1 m/m], pH 5.8, Hygrotech Seed, South Africa). Each tray contained 128 removeable cells. The trays were placed in a growth chamber at 18/25°C night/day temperature under cool white fluorescent light with a 14 h photoperiod. Seedlings were inoculated by a pipette method 11-12 days (expansion of first true leaf) after planting (Latin & Snell, 1986). Five milliliters of the inoculum suspension were delivered to the substrate around each seedling with a pipetman (model P5000, Gilson Medical Electronics, South Africa). Control plants received sterile diluted potato dextrose broth only. After inoculation, seedlings were watered once a week with a soluble fertilizer (Chemicult hydroponic nutrient, 2g/l, Chemicult Products, South Africa). The virulence tests were conducted in nine separate trials, each consisting of a control and one to seven treatments (isolates). Each treatment was replicated one to five times (Table 1) and consisted of 20-50 plants per treatment. The differential cultivars Topmark (susceptible to all races), Doublon (*Fom1*) and CM 17187 (*Fom2*) were included to verify virulence of each isolate according to the race classification scheme proposed by Risser *et al.* (1976). Mean percentage stunting (PS), and the number of plants that died after showing typical Fusarium wilt symptoms, were recorded 28 days after inoculation. Mean PS was calculated as follows: PS = 100 (mean vine length of control plants minus mean vine length of a treatment plants)/mean vine length of control plants.

Statistical analysis. The PS data were subjected to one way analysis of variance. Shapiro-Wilk test (Shapiro & Wilk, 1965) was performed to test for non-normality which confirmed no evidence against normality ($P = 0.56$). Student's t-Least Significant Difference ($P = 0.05$) was calculated to compare treatment means.

RESULTS

The 20 FOM isolates each caused disease reactions on the differentials as expected, confirming the race identity of the isolates (data not included). Disease reactions induced by the isolates on Perlita plants are given in Table 1. Plants inoculated with isolates of races 1 and 1,2 developed typical Fusarium wilt symptoms and all were dead 28 days after inoculation. Plants inoculated with three of the reference race 2 isolates (ATCC 28861, ATCC 32669, T61) remained asymptomic, whereas leaves of plants infected by the remaining seven race 2 isolates became chlorotic, thickened and brittle (Fig. 1). These plants were all severely stunted. Similar symptoms were induced on plants inoculated with all seven race 0 isolates. Mean PS values of plants infected by these race 0 and 2 isolates did not differ significantly ($P = 0.05$) and ranged between 25.1 and 50.0. In addition to the stunting symptom, plants infected with five isolates of race 0 (ATCC 28856, Sp206, Sp603, Fom 8803, I-1) and three isolates of race 2 (M126/2, Sp709, Sp717) died, with the incidence of dead plants ranging between 4.4 and 17.5% (Table 1). The mean PS for plants infected by the seven race 0 isolates, which represent VCG 0134 and 0135 (VCG of Fom 8803 not known) from 5 countries, varied between 34.6 and 50.0. The one race 2 reference isolate from California (P-2, VCG 0130) and all six race 2 isolates from South Africa (VCG 0134) caused a mean PS that varied between 25.1 and 45.0.

In order to verify the disease reaction of Perlita, the virulence test was repeated at a lower inoculum concentration (5×10^5 conidia/ml) with race 2 isolates, Sp717 and Sp1020, using the pipette method. Another set of plants were inoculated with race 0 isolates ATCC 28856 and Sp206 using the root dip method (Zink & Gubler, 1985). A similar disease reaction was recorded. Stunting indices for plants inoculated with isolates Sp717 and Sp1020 of race 2 by the pipette method, were 36.2 and 18.0, respectively. Stunting indices for plants that were rootdip-inoculated with isolates ATCC 28856 and Sp206 of race 0 were 63.1 and 66.6, respectively.

DISCUSSION

The disease reaction of Perlita plants to all seven race 0 and seven out of the 10 race 2 isolates tested confirmed the observations made by Schreuder *et al.* (2000) in their race classification and vegetative compatibility studies, and proved that resistance gene *Fom3*

does not confer similar resistance towards race 0 and some race 2 isolates as gene *Fom1* in Doublon. Cultivar Perlita, and those possessing *Fom3* like Doral and Saticoy, should be considered tolerant to FOM races 0 and 2. Tolerance to FOM in these cultivars is also substantiated by field observations (Schreuder *et al.*, 2000) that race 0 induced Fusarium wilt and caused severe yield losses on cultivars possessing the *Fom3* gene. Therefore, not only is it unreliable to use *Fom3* in race determination tests, but also as a control measure in the field against races 0 and 2.

The approach of grouping isolates on the basis of vegetative compatibility provides a means of characterising subspecific groups based on the genetics of the fungus rather than on the host-pathogen interaction (Jacobson & Gordon, 1990b). Correlations between mtDNA and VCG in FOM, on the other hand, help to confirm the importance of VCG as an indicator of genetically isolated populations (Jacobson & Gordon, 1990b). The difference in disease reaction towards Perlita found between the race 2 isolates which belong to different VCG's appears therefore to be an indicator between certain genetically isolated population groups. The seven race 0 isolates, which originated from five different countries and belong to two VCG's (FOM 8803-VCG not known), all caused similar disease reactions on Perlita. The ten race 2 isolates, which originated from three countries (origin of T61 unknown) and which represent four different VCG's, however, behaved differently. Six isolates belonging to VCG 0134 and one belonging to VCG 0130 caused disease symptoms, whereas the other three isolates (two belonging to VCG 0132 and one to VCG 0131) did not. This finding indicated that race 2, which as a group is represented in five different VCG's (Jacobson & Gordon, 1991; Katan *et al.*,1994), does not constitute a genetically homogeneous group, and raises questions concerning the phylogeny of subspecific groups within FOM. It also points to the limitation of the present system of naming races which may not reveal the total virulence diversity within FOM. With the classification system currently in use (Risser *et al.*, 1976) based on two resistance genes (*Fom1*, *Fom2*), the four known races (0,1,2 and 1,2) represent the maximum that can be described. If additional resistance genes were available, more races might have been identified. This could change our perception that no correlation exist between VCG's and races of FOM (Jacobson & Gordon, 1988; Jacobson & Gordon, 1990a; Jacobson & Gordon, 1991; Gennari & D'Ercole, 1994; Katan *et al.*, 1994; Schreuder *et al.*, 2000) and therefore that virulence as a trait seems independent of VCG's and mtDNA (Jacobson & Gordon, 1990b). Differences other than those documented in this study may

exist between isolates of race 2, such as the distinction between the wilting (VCG 0133) and yellow (VCG 0134) isolates of race 1,2 (Jacobson & Gordon, 1991). No such differences were found within the race 0 isolates used, but if more race 0 isolates of different VCG's have been included in the tests similar results might have evolved. Furthermore, these findings ratify the importance that the selection of isolates used in screening germplasm for resistance should be carefully considered. No single isolate may safely be regarded as representative of the pathogenic potential of a race. This supports the suggestion made by Jacobson and Gordon (1991) that *F. oxysporum* isolates should be described by both virulence and VCG, since it is feasible and reveals much more information than either alone.

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Table 1. Races, vegetative compatibility group (VCG), origin and source of *Fusarium oxysporum* f. sp. *melonis* isolates and their disease reaction on Perlita plants

Isolate	Race[a]	VCG[b]	Origin	Source[c]	N[d]	Percentage dead[e]	Percentage stunting[f]	Std Err
ATCC 28856	0	0134	France	A	2	6.7	42.5a	1.6
E-466A	0	0134	Caroline	B	2	0	43.2a	3.3
Sp206	0	0134	South Africa	D	5	9.3	48.7a	8.1
Sp249	0	0134	South Africa	D	2	0	40.6a	3.5
Sp603	0	0134	South Africa	D	4	5.4	34.6a	6.8
Fom 8803	0	--	Spain	C	3	4.4	37.4a	5.4
I-1	0	0135	Israel	B	2	5.1	50.0a	2.9
ATCC 28857	1	013-	France	A	2	100	--	--
ATCC 28862	1	013-	Israel	A	2	100	--	--
P-2	2	0130	California	E	1	0	25.8a	--
ATCC 32669	2	0131	Michigan	A	3	0	-10.4b	10.3
ATCC 28861	2	0132	Japan	A	3	0	-15.0b	8.9
T61	2	0132	Unknown	B	1	0	-14.7b	--
M126/2	2	0134	South Africa	D	2	17.5	38.9a	6.1
Sp709	2	0134	South Africa	D	2	10.0	45.0a	7.9
Sp717	2	0134	South Africa	D	3	8.3	38.1a	3.8
Sp806	2	0134	South Africa	D	1	0	37.0a	--
Sp1020	2	0134	South Africa	D	2	0	25.1a	7.8
Sp1021	2	0134	South Africa	D	1	0	28.5a	--
R12w	1,2	0133	France	B	2	100	--	--

[a] Race of the pathogen according to Risser *et al.* (1976).

[b] VCG as determined by Jacobson and Gordon (1988; 1990a) and Schreuder *et al.* (2000); 013-: self-incompatible isolates (Jacobson and Gordon , 1988); -- = VCG unknown.

[c] A = American Type Culture Collection; B = T.R. Gordon, University of California; C = R.M. Jiménez Díaz, Universidad de Cordoba, Spain; D = W. Schreuder, ARC-Plant Protection Research Institute, South Africa; E = D.M.S. Spinks, Hollar Seeds, Colorado.

[d] Number of replications.

[e] Mean percentage of plants with typical Fusarium wilt symptoms that died.

[f] Mean percentage stunting of vine lengths in terms of the control. Percentage stunting values followed by the same letter are not significantly different according to Student's t-Least Significant Difference test method (P = 0.05); -- = No data, all plants dead 28 days after inoculation.

Fig. 1. Disease reaction of Perlita plants 28 days after inoculation with *F. oxysporum* f. sp. *melonis* race 1 isolate ATCC 28857 (left), control (centre) and race 2 isolate Sp806 (right).

4. VIRULENCE CHANGES IN *FUSARIUM OXYSPORUM* F. SP. *MELONIS* RACE 0 IN MELON INDUCED BY RESISTANCE GENES *FOM2* AND *FOM1*, *FOM2*

ABSTRACT

The ability of a *nit* mutant isolate generated from *Fusarium oxysporum* f. sp. *melonis* (FOM) race 0 (VCG 0134) to change its virulence during infection of melon plants, was investigated under quarantine. Melon cultivars Imperial 45 and Early Sweet (no resistance genes), Amber (*Fom2*) and Fiata (*Fom1*, *Fom2*) were consecutively grown in two cement troughs (A and B) in a gauzehouse. Plantings were terminated when plants had advanced Fusarium wilt or after the fruit were harvested. In the first planting, Imperial 45 seedlings were transplanted and artificially inoculated with the *nit* mutant isolate. In the consecutive plantings, seeds were sown in the infested soil to enable natural infection. Plants showing Fusarium wilt were selected for isolation from each crop. All labelled *F. oxysporum* isolates recovered were single-spored and their VCG and virulence status verified. In trough A, all plants of the two susceptible cultivars developed Fusarium wilt. Isolates recovered from these plants were all designated FOM race 0. In the first crop (planting No. 5) of the resistant cultivar Amber, 6.7% of the plants developed Fusarium wilt. In the second and final Amber crop the frequency increased to 56.6%, and 81.8%, respectively. Contrary to the susceptible cultivars, only FOM race 2 isolates were obtained from the symptomatic Amber plants. Similar data were recorded for cultivars Imperial 45 and Amber in trough B. Planting of Fiata in trough B caused a dramatic reduction in Fusarium wilt incidence with 1.2% of the plants developed Fusarium wilt in the first crop (planting No. 6), and 4% in the final planting. From these symptomatic Fiata plants only FOM race 1,2 isolates were obtained. These isolates have lost their *nit* mutant status, but belonged to VCG 0134. The findings indicate that changes in the race structure of FOM (VCG 0134) occurred rapidly when confronted with resistant cultivars. Changes in virulence were not recorded prior to the planting of resistant cultivars, suggesting that resistance genes are important in bringing about a virulence change.

INTRODUCTION

Fusarium wilt of melon (*Cucumis melo* L.), caused by *Fusarium oxysporum* Schlecht. emend. Snyd. and Hans. f. sp. *melonis* Leach and Currence (FOM), is controlled primarily by the cultivation of resistant cultivars possessing resistance genes *Fom1*, *Fom2* and *Fom3* (Risser *et al.*, 1976; Zink & Gubler, 1985). In most melon producing regions, resistance in cultivars conferred by these genes has been overcome by new physiological races shortly after their introduction. In France field observations (Bouhot, 1981) during the 1960's showed that resistance in newly cultivated melon cultivars was broken after the second crop. In South Africa race 2 was recently isolated from symptomatic plants from four commercial melon fields, known to be infested with race 0, and then planted for two successive seasons with cultivars resistant to races 0 and 1 (possessing gene *Fom2*) (Schreuder *et al.*, 2000). The race 0 and 2 isolates recovered from plants from these fields belong to VCG 0134, indicating a high degree of genetic homology (Schreuder *et al.*, 2000). This finding suggests that the new race 2 isolates either developed from a saprophytic form of the fungus, that race 0 changed in virulence, or that race 2 had been newly introduced into the melon fields. In the case of *F. oxysporum* f. sp. *niveum*, Hopkins *et al.* (1992) concluded that race 2 isolates in the soil increased after mono-cultivation of watermelon cultivars resistant to race 1 of the pathogen.

Hypotheses and models (Jacobson & Gordon, 1990b; Correll, 1991; Jacobson & Gordon, 1991; Katan *et al.*, 1994) on the evolution of FOM are based on host resistance genes (Risser *et al.*, 1976; Zink & Gubler, 1985), vegetative compatibility (Jacobson & Gordon, 1990a; Correll, 1991) and molecular traits (Jacobson & Gordon, 1990b; Schroeder & Gordon, 1993; Zuniga *et al.*, 1997). The first feature defines physiological races based on host-pathogen interaction whereas the latter two characteristics are based on the genetics of the fungus. Findings (Correll *et al.*, 1987) that different races belong to the same VCG (0134), each with a single mtDNA RFLP pattern, for example, suggest that these races most likely originated from a common genetic background. Furthermore, reports that isolates of FOM and other formae speciales altered their virulence when subjected to chemical treatments (Bouhot, 1981), UV irradiation (Kroon & Elgersma, 1991; Kim *et al.*, 1992), and root exudates from a resistant host (Buxton, 1958), substantiate the general belief that selection pressure imposed by resistant genes in the host is involved in the appearance of new races. The fact that chemically induced mutations displayed reduced fitness (Bouhot, 1981;

Kroon & Elgersma, 1991), however, points to a rare occurrence of this kind of mutation in nature.

To investigate virulence changes in FOM, methods should be available to monitor this phenomenon in wild-type isolates in the host. Conventional selective media do not distinguish between pathogenic and nonpathogenic *F. oxysporum* isolates, and once a specific isolate of *F. oxysporum* is introduced into soil, it is morphologically indistinguishable from the natural *F. oxysporum* population (Hadar *et al.*, 1989). One approach to overcome this difficulty is to use isolates labelled with specific markers, for example, mutants that are resistant to antimicrobial agents or auxotrophic mutants obtained through mutagenesis. However, such mutants might not be true representatives of the natural population of the pathogen. This disadvantage can be overcome by using nitrate-nonutilizing (*nit*) mutants, which frequently appear spontaneously as fast-growing sectors from restricted colonies on chlorate-amended media without mutagenic treatments (Puhalla, 1985). *Nit* mutants form a thin expansive mycelium growth on nitrate minimal medium (MM), but are indistinguishable from wild-type isolates on media containing ammonium or organic nitrogen sources (Puhalla, 1985). *Nit* mutants, furthermore, retain their pathogenicity, and their pattern and rate of survival in soil are similar to those of their wild-type parents (Hadar *et al.*, 1989). *Nit* mutants therefore offer a powerful tool to distinguish marked isolates from wild-type isolates.

The aim of this study was to investigate the ability of a *nit* mutant isolate, generated from FOM race 0 which belongs to VCG 0134, to change in virulence during infection of melon plants possessing *Fom2* or *Fom1*, *Fom2* resistance genes. A partial account of this work has been published (Schreuder *et al.*, 1995; Schreuder *et al.*, 1996).

MATERIALS AND METHODS

Induction of a marker isolate. FOM isolate Sp 206, obtained from a naturally infected melon plant collected from a commercial field in the Clanwilliam district, South Africa, was selected as the wild-type (Schreuder *et al.*, 2000). *Nit* mutants were generated on a chlorate containing medium from this isolate (Schreuder *et al.*, 2000). Since some *nit* mutants may be inadequate due to poor growth and difficulty to detect (Hadar *et al.*, 1989), 10 isolates were selected from the generated mutants and verified for *nit* phenotype, VCG and race characteristics. Race classification was conducted by using the differential cultivars

Topmark (susceptible to all races), Doublon (*Fom1*) and CM 17187 (*Fom2*) according to the race classification scheme of Risser *et al.* (1976). The aggressiveness of each of the 10 isolates was then compared to that of the wild-type isolate. For these tests, lyophilized cultures of the selected *nit* mutant isolates, and the wild-type parent were grown on potato dextrose agar (difco) with 0.02% novostreptomycin (Novo Nordisk, South Africa). After 5 days, mycelial plugs (3 mm in diameter) were taken from the actively growing colony margins of each culture, transferred to 100 ml of a potato dextrose broth (difco) (10 g/l) and incubated at 25°C for 5 days on a rotary incubator (150 rpm). The contents of each flask was filtered through four layers of sterile cheesecloth, the density of the predominantly microconidial suspension of each isolate determined with a haemacytometer and adjusted to 1.0 x 10^6 conidia/ml. Seeds of the different cultivars were surface-disinfested with 5% calcium hypochlorite solution for 5 min, rinsed twice in sterile distilled water and sown into celltype plastic growing trays (one seed per 60 ml cell) filled with a pasteurized commercial potting mixture (peat:vermiculite:polystyrene [18:12:1 m/m], pH 5.8, Hygrotech Seed, South Africa). Each tray contained 128 removeable cells. The trays were incubated in a growth chamber at 18/25°C night/day temperatures under cool white fluorescent light with 14 h photoperiod. Seedlings (30 seedlings of each cultivar per isolate) were inoculated by a pipette method 11-12 days (expansion of first true leaf) after sowing (Latin & Snell, 1986). Five milliliters of the inoculum suspension were delivered to the substrate around each seedling. Control plants received sterile diluted potato dextrose broth. Inoculated seedlings were watered once a week with a soluble fertilizer (Chemicult hydroponic nutrient, 2g/l, Chemicult Products, South Africa). Isolations were made on Komada's (Komada, 1975) medium from symptomatic plants infected by *nit* mutant isolates displaying similar aggressiveness as the wild-type isolate. Single-conidial subcultures were prepared and grown on MM to confirm their *nit* mutant status. To verify the stability of the mutants the experiment was repeated with the single-conidial labelled isolates. After the repeated experiment *nit*M mutant isolate Sp 206A was selected to be used as the labelled FOM isolate. The isolate was similar to the wild-type isolate Sp 206 in virulence, aggressiveness and VCG, but reflected mutations at loci that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity (Correll *et al.*, 1987).

Planting schedule and virulence of *nit* mutant isolate Sp 206A. The experiment was conducted under quarantine in two cement troughs (7 m long x 1.5 m wide x 0.7 m deep)

in a gauzehouse to prevent the introduction of other FOM isolates (Fig.1). The cement troughs, which were 17.6 m apart, were filled with a 50 mm layer coarse gravel (19 mm diameter), a 50 mm layer of fine gravel (6 mm diameter), a 50 mm layer of coarse sand, and a 500 mm layer of sandy-loam soil. Both the coarse sand and sandy-loam soil were collected in uncultivated land. The components were independently steam-disinfested at 85°C for 35 min in a pasteurising oven. Irrigation water was obtained from a hot water geyser at 70°C, cooled and delivered by drip irrigation (two lines per trough). Seedlings of melon cultivars Imperial 45 (no resistance genes), Early Sweet (no resistance genes), Amber (*Fom2*) and Fiata (*Fom1*, *Fom2*) were consecutively grown (Table 1) in the two troughs. Each planting was terminated when plants had advanced Fusarium wilt or after the fruit were harvested. The plant debris were removed and the soil cultivated before the next planting to evenly distribute fungal propagules in the soil. In the first planting, Imperial 45 seedlings were grown, transplanted (136 seedlings per trough) and artificially inoculated with *nit* mutant isolate Sp 206A as described previously. In the consecutive plantings, surface-disinfested seeds were sown in the infested soil to enable natural infection. Plants were watered once a week with a soluble fertiliser (Hygrofert, 1g/l, Hygrotech Seed, South Africa.). For each crop, representative plants showing Fusarium wilt were selected for isolation from the vascular system on Komada's medium. All *F. oxysporum* isolates recovered were single-spored and transferred to MM to verify their *nit* mutant status. VCG and virulence of the labelled isolates were determined as described previously.

RESULTS

The percentage plants of each crop showing Fusarium wilt, the number of plants used for isolation and the characteristics of the FOM isolates recovered, are given in Table 1. In trough A, 100% of plants of the susceptible cultivars Imperial 45 and Early Sweet crops showed Fusarium wilt four weeks after planting. Isolates recovered from the selected plants all belonged to VCG 0134 and were designated race 0. In the first crop (planting No. 5) of the resistant cultivar Amber, 6.7% (Fig. 2a) of the plants developed Fusarium wilt. In the second Amber crop the frequency increased to 56.6%, and to 81.8% (Fig. 2b) in the final crop. Final disease assessments were done 16 weeks after planting. Contrary to the susceptible cultivars, only race 2 isolates were obtained from the symptomatic Amber plants. The race 2 isolates all belonged to VCG 0134. Similar results were recorded with the

susceptible cultivar Imperial 45 in trough B. Again, a low percentage (15%) Amber plants developed Fusarium wilt in the first crop (planting No. 3) in trough B. The percentage plants affected by Fusarium wilt increased to 51% in the second Amber crop, and to 100% in the third crop when final assessments were done 16 weeks after planting. Similar to what was found in trough A, only race 2 isolates, belonging to VCG 0134, were recovered from the symptomatic Amber plants. Planting of Fiata, which possesses resistance genes *Fom1* and *Fom2*, caused a significant reduction in Fusarium wilt incidence in trough B. However, 14 weeks after planting, 1.2% of the plants showed Fusarium wilt in the first Fiata crop (planting No. 6), whereas 4% of the plants were symptomatic in the final planting. From these symptomatic Fiata plants only race 1,2 isolates were obtained which all belonged to VCG 0134. These race 1,2 isolates were, however, all unlabelled.

DISCUSSION

This study conducted under controlled conditions in quarantine with a *nit*M mutant isolate of FOM (VCG 0134) proved the natural ability of the pathogen to change in virulence in resistant melon plants. This phenomenon only occurred when the first planting of resistant Amber (*Fom2*) was made in race 0 infested soil, and when resistant Fiata (*Fom1*, *Fom2*) seedlings grew in soil infested with races 0 and 2. The labelled isolates recovered from the selected symptomatic Amber plants were all designated race 2, and isolates recovered from Fiata plants designated race 1,2. The unlabelled race 1,2 isolates are considered to be isolates which have lost their *nit* mutant status since the possibility of introducing race 1,2 was non-existent. The fact that this experiment was conducted under quarantine conditions, that race 1,2 is not recorded in South Africa (Schreuder *et al.*, 2000), and that the recovered race 1,2 isolates belonged to VCG 0134 confirm this statement. These findings, and the fact that the symptomatic plants represented a substantial proportion of the first Amber (approximately 7-15%) and Fiata (approximately 2%) crops, indicate that changes in the race structure of this fungal pathogen (VCG 0134) occurred rapidly when confronted with a resistant cultivar. The fact that no changes in virulence were recorded prior to the planting of resistant cultivars, suggests that resistance genes are important in bringing about a virulence change. Furthermore, strong selection pressure exerted by resistance gene *Fom2* in Amber caused a drastic shift in race structure of the pathogen population and total crop failure in both troughs after three successive plantings. Although the isolate changed in virulence when confronted

with a resistant cultivar during natural infection, it retained its aggressiveness and VCG characteristics. These findings confirmed earlier field observations (Bouhot, 1981; Schreuder *et al.*, 2000) indicating that the pathogen can change in virulence under field conditions and cause severe losses in the second crop of resistant melon cultivars.

A combination of resistance genes (*Fom1*, *Fom2*) in melon is advocated to increase the stability of host resistance (Bouhot, 1981). The phenomenon that race 1,2 already occurred in the first Fiata crop, although at a very low frequency, however indicated that this strategy will not provide long-term disease resistance to FOM. This is also implicated by the data of Cappelli *et al.* (1995) who showed that the cultivation in Italy of cultivars carrying both *Fom1* and *Fom2* resistance genes led to the general occurrence of all four FOM races, with race 1,2 accounting for >70% of the isolates. Changes in cultivar resistance should therefore only be made by producers after disease symptoms have occurred and not only because of the availability of a new resistant cultivar. This strategy may slow down the development of new FOM races.

Confirmation that race 0/VCG 0134 can alter in virulence in resistant melon plants partially clarifies the biodiversity and population dynamics of FOM. Selection pressure exerted by resistant cultivars can therefore contribute to the rapid evolution of FOM, and explain the phenomenon of multiple races within VCG 0134. Studies by Katan *et al.* (1994) in Israel suggest similar race changes within VCG 0135, where race 0 was originally the only race to be associated with this VCG. Katan *et al.* (1994) found four race 2 isolates within this VCG and suggested that these isolates were derived from race 0. These authors also reported three races (0, 1, and 1,2) associated with VCG 0138 in Israel, and suggested that the progenitor race in VCG 0138 could be race 0. Katan *et al.* (1994) furthermore argued that race 1,2 may reflect selection for mutant derivatives of the race 1 isolates, owing to the widespread use of resistant cultivars. This idea is supported by Bouhot (1981) who obtained races 1 and 1,2 in his induced mutation experiments when starting with race 0. In my study race 2 seems to serve as intermediate in the evolution of race 1,2.

The ocurrence of multiple races within a single VCG (0134, 0135 and 0138) suggests that races within a forma specialis may most likely originate from a common genetic background and that virulence, as a trait, is independent of VCG and may have evolved at a different rate (Jacobson & Gordon, 1990a; Jacobson & Gordon, 1991; Katan *et al.*, 1994).

Races in FOM may therefore evolve at a higher rate under the selection pressure of resistant cultivars than VCG. This implies that FOM contains a greater number of genes or combination of genes affecting virulence than resistant genes in the host. Through induced mutation experiments, Bouhot (1981) postulated that the resistant genes in the host act as a sensitive filter revealing within the pathogen the corresponding combination of virulence genes and thus the appearance of new races. Furthermore, the rhizosphere of the melon plant rapidly increases the inoculum level at the end of the first crop so that the second crop is severely attacked. This phenomenon cast doubt on the efficacy of dominant monogenic resistance as practical disease control measure. On the other hand, Bouhot (1981) showed that FOM race 1,2 existed in France at least 12 years before the introduction of resistant melon cultivars. This therefore suggests that FOM can also alter its virulence in the absence of resistant cultivars.

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Table 1. Virulence changes in *Fusarium oxysporum* f. sp. *melonis* (FOM) and percentages wilted plants in successive melon crops grown in soil infested[a] with FOM

Planting No.[b]	Cultivar	Resistance gene(s)[c]	Wilted plants (%)	Number of plants used for isolations	Number of labelled isolates verified	FOM race recovered[d]
Trough A						
1	Imperial 45	none	100	10	15	0
2	Imperial 45	none	100	10	15	0
3	Early Sweet	none	100	10	10	0
4	Early Sweet	none	100	10	12	0
5	Amber	*Fom2*	6.7	6	10	2
6	Amber	*Fom2*	56.6	10	12	2
7	Amber	*Fom2*	81.8	10	8	2
Trough B						
1	Imperial 45	none	100	10	15	0
2	Imperial 45	none	100	10	15	0
3	Amber	*Fom2*	15	10	12	2
4	Amber	*Fom2*	51	10	10	2
5	Amber	*Fom2*	100	10	8	2
6	Fiata	*Fom1*, *Fom2*	1.2	1	4	1,2
7	Fiata	*Fom1*, *Fom2*	4	5	7	1,2

[a] In the first planting, Imperial 45 seedlings were inoculated with a *nit*M labelled isolate of race 0 by the pipette method.

[b] Date of cultivar planting order: 1= 28/08/92; 2 = 22/10/92; 3 = 26/11/92; 4 = 05/04/93; 5 = 05/10/93; 6 = 01/09/94; 7 = 13/02/95.

[c] Cultivar resistance as stated by the marketing seed companies in South Africa, Hygrotech Seed and Starke Ayres.

[d] The isolates all belonged to VCG 0134. All the isolates recovered were labelled, except for those verified as race 1,2.

Fig. 1. Location of gauzehouse used for the successive cultivation of melon crops under quarantine.

(b)

(a)

Fig. 2. The appearance of Amber (*Fom2*) plants (a) 5-6 wk after the first crop was planted in soil infested with a labelled *Fusarium oxysporum* f. sp. *melonis* race 0 isolate, and (b) 14 wk after the third crop was planted in the same trough.

5. RAPD ANALYSIS AND HISTOPATHOLOGY OF *FUSARIUM OXYSPORUM* F. SP. *MELONIS* ISOLATES DISPLAYING VIRULENCE CHANGES AFTER INFECTING RESISTANT MELON CULTIVARS

ABSTRACT

A previous study (Part 4) conducted under controlled conditions in quarantine with a *nit* mutant isolate of *Fusarium oxysporum* f. sp. *melonis* (FOM) race 0 (VCG 0134) provided evidence of the natural ability of the pathogen to change in virulence in resistant melon plants. In this study, the potential of RAPD analysis to differentiate between the isolates displaying virulence changes was evaluated. Isolates used were the original wild-type race 0, the *nit* mutant isolate generated from the wild-type, labelled isolates verified as race 2 after infecting Amber (*Fom2*) plants, and isolates verified as race 1,2 after infecting Fiata (*Fom1*, *Fom2*) plants. Four *F. oxysporum* f. sp. *niveum* isolates were included as an outgroup. A histopathological study was conducted to verify whether these isolates retained their ability to operate as true vascular pathogens. The three primers used clearly distinguished the 12 FOM isolates from the four *F. oxysporum* f. sp. *niveum* isolates. However, the primers showed a highly conserved and characteristic banding pattern for the FOM isolates which represented three physiological races (race 0, race 2, race 1,2), indicating that this method cannot detect race-specific groupings in FOM. Disease reactions on the three differential cultivars (CM 17187, Doublon and Topmark) confirmed the virulence of FOM isolates. The histopathological data furthermore proved that the two FOM races (race 2, race 1,2), derived from the race 0 parent isolate, retained their ability to operate as true vascular pathogens.

INTRODUCTION

In *Fusarium oxysporum* Schlecht. host specialisation has historically been considered the most important trait and, because of its practical application, applied as basis for classification of formae speciales. In *F. oxysporum* Schlecht. emend. Snyd. and Hans. f. sp. *melonis* Leach and Currence (FOM), the agent of Fusarium wilt of melon (*Cucumis melo* L.), a further distinction has been made. Isolates of FOM have been divided into four races (0, 1, 2 and 1,2) based on their disease reaction on cultivars possessing resistance genes *Fom1* and *Fom2* (Risser *et al.*, 1976). The existence of multiple races within FOM suggests

considerable diversity in virulence, but this feature is not necessarily the most meaningful basis for classification. Characteristics other than virulence, that can be used to group isolates within FOM, also provide measures of the underlying diversity. Additional characterization of subspecific groups in *F. oxysporum* was reported by Puhalla (1985), who developed the techniques for identifying vegetative compatibility groups (VCGs) within this species. Eight VCG's were identified within FOM world-wide (Katan, 1999). The substantial genetic diversity in FOM is reflected by both multiple races and multiple VCGs. All four races of FOM were found in more than one VCG, and conversely, all four races were also present in a single VCG (Jacobson & Gordon, 1991). This shows that FOM is more complex than *F. oxysporum* f. sp. *apii* (Nels. & Sherb.) (Correll *et al.*, 1986), *F. oxysporum* f. sp. *conglutinans* (Wr.) (Bosland & Williams, 1987), *F. oxysporum* f. sp. *vasinfectum* (Atk.) (Katan & Katan, 1988), and *F. oxysporum* f. sp. *dianthii* (Prill. & Del.) (Katan *et al.*, 1989), where a direct relationship between VCG and race exists. Molecular traits, especially polymorphisms in nuclear and mitochondrial DNA (mtDNA), have been used to assess interspecific and intraspecific relationships in fungi, including plant pathogens (Jacobson & Gordon, 1991). The restriction fragment length polymorphisms (RFLP) in mtDNA of FOM correlated with the identified VCGs, confirming the importance of VCG as an indicator of genetically isolated populations (Jacobson & Gordon, 1990b; Zuniga *et al.*, 1997). Although this molecular trait could not distinguish between physiological races of FOM (Jacobson & Gordon, 1991), race diversity within a VCG or mtDNA framework cannot be ignored especially because of its practical application.

Different workers (Bouhot, 1981; Cappelli *et al.*, 1995; Schreuder *et al.*, 2000) reported on the appearance of new races of FOM after the introduction of new resistant melon cultivars. A study (Part 4) conducted under controlled conditions in quarantine with a *nit* mutant isolate of FOM (VCG 0134) provided evidence of the natural ability of the pathogen to change its virulence in resistant melon plants. This phenomenon only occurred when resistant Amber (*Fom2*) was planted in race 0 infested soil, and when resistant Fiata (*Fom1, Fom2*)' seedlings grew in soil infested with races 0 and 2. The labelled isolates recovered from the selected symptomatic Amber plants were all designated race 2, and isolates recovered from Fiata plants were designated race 1,2. The labelled isolate only changed its virulence when confronted with a resistant cultivar during natural infection, and retained its aggressiveness and VCG characteristics. These findings (Part 4) partially clarify the biodiversity and population dynamics within FOM, especially within VCG 0134, and

open up new avenues for evaluating methods to distinguish between races. Random amplified polymorphic DNA (RAPD) offers several advantages over other DNA based techniques, including RFLPs, that may be useful in studying races of *F. oxysporum* (Williams *et al.*, 1990). These include rapidness, small amounts of template DNA needed, no requirement of DNA sequence information, or radioisotopes (Tingey *et al.*, 1992). RAPD analysis has provided genetic markers to differentiate races of *F. oxysporum* f. sp. *pisi* (van Hall) (Grajal-Martin *et al.*, 1993), *F. oxysporum* f. sp. *vasinfectum* (Assigbetse, 1994), *F. solani* (Mart.) App. & Wr. f. sp. *cucurbitae* Snyd. and Hans. (Crowhurst *et al.*, 1991), *Gremmeniella abietina* (Hamelin *et al.*, 1993) and aggressive and non-aggressive isolates of *Phoma lingam* (Goodwin & Annis, 1991; Schäfer & Wöstemeyer, 1992).

In this study the potential of RAPD analysis to differentiate between FOM isolates displaying virulence changes after infecting resistant melon cultivars was evaluated. An histological study was also conducted to verify whether these isolates retain their ability to operate as true vascular pathogens.

MATERIAL AND METHODS

Isolates. The FOM isolates were obtained during an investigation (Part 4) on the natural ability of FOM to change in virulence in resistant melon plants. The origin and characteristics of these isolates are given in Table 1. *F. oxysporum* f. sp. *niveum* (E. F. Smith) (FON) isolates (WA 257, WA 552, WA 553 and WA 558), collected from diseased watermelon plants in South Africa (W. Schreuder, unpublished), were included as an outgroup species in the RAPD analysis.

RAPD analysis. For DNA isolation, mycelial plugs from 7-day-old cultures growing on potato-dextrose agar (PDA) were transferred to flasks containing 100 ml of a yeast extract and glucose medium (YEG) [8 g/l yeast extract and 5 g/l glucose]. Flasks were incubated on a rotary shaker at 150 rpm at 25°C for 3 days. Due to excessive polysaccharide production by this fungus in liquid culture, harvesting of mycelia with a Buchner funnel was problematic. Mycelia were therefore harvested by centrifugation at 5000 rpm for 5 min at 4°C. Harvested mycelium was stored at -80°C until required. Mycelia were crushed in liquid nitrogen with a mortar and pestle, transferred to an Eppendorf tube containing 500 µl extraction buffer (50 mM Tris, pH 7.2; 50 mM NaCl; 50 mM EDTA and 3% SDS), 350 µl of phenol were added followed by 150 µl chloroform/isoamylalcohol (24:1 vol/vol). The suspension was mixed

and incubated at room temperature for 15 min with shaking, and subsequently centrifuged at 13,000 rpm for 60 min. The aqueous phase was transferred to a clean Eppendorf tube after which 25 µl RNase (10 mg/ml) (Boehringer Mannheim Chemicals, South Africa) were added and incubated for 30 min at 37°C. An equal volume of chloroform was added followed by centrifugation at 13000 rpm for 10 min. The aqueous phase was transferred to a clean tube and subjected to two more chloroform extraction procedures. DNA was precipitated with 0.54 vol isopropanol, incubated at -20°C for 2 h, and pelleted by centrifugation at 13000 rpm for 5 min. The DNA was subsequently washed twice with 70% ethanol, dried, resuspended in 100 µl TE buffer (50 mM Tris, pH 8.0; 50 mM EDTA) and stored at -20°C for future use. DNA samples that still contained excessive polysaccharides had their volume's taken up to 400 µl with TE buffer. An equal volume of phenol/chloroform/isoamylalcohol (25: 24: 1 vol/vol) was added followed by centrifugation at 13000 rpm for 10 min. The aqueous phase was extracted three more times with an equal volume of chloroform/isoamylalcohol and DNA subsequently precipitated by the addition of 7.5 M NH_4OAc to a final concentration of 2 M and 2 volumes 100% cold ethanol. Samples were washed with 70% cold ethanol, dried and resuspended in 100 µl TE buffer.

Initially 40 primers were screened (Operon Technologies Inc., Alameda, USA) to assess their ability to distinguish isolates of FOM and FON. Amplification reactions were performed in a final volume of 25 µl of reaction mixture. The reaction mixture contained 2.5 µl of 10X *Taq* DNA polymerase buffer (100 mM Tris HCl, pH 8.3; 15 mM $MgCl_2$; 500 mM KCl) (Boehringer Mannheim, South Africa); 200 µm of each dNTP; 10 pmol of oligonucleotide primer, 50 ng genomic DNA and 1.0 U of *Taq* DNA polymerase. The $MgCl_2$ concentration was adjusted to a final concentration of 4 mM. Reaction mixtures were overlaid with 50 µl mineral oil to prevent evaporation during thermocycling. The following three primers were used in the study: OPE 10: 5' CACCACGTGA 3', OPE 15: 5' ACGCACAACC 3', OPY 19: 5' TGAGGGTCCC 3'. Amplifications were conducted in a Biometra TRIO-Thermoblock TB1 (Gottingen, Germany). Reactions underwent an initial denaturation process at 96°C for 120 s, followed by 30 cycles of 92°C for 30 s, 38°C for 30 s and 72°C for 60 s. After the last cycle a final extension step was conducted at 72°C for 120 s. Amplification products were separated through 1.5% (wt/vol) agarose gels in TAE buffer (Sambrook *et al.*, 1989).

Histology. Lyophilized cultures of isolates Sp206A (race 0), Sp 1052A (race 2) and Sp 1204 (race 1,2) were grown on PDA with 0.02% novostreptomycin (Novo Nordisk, South Africa). After 5 days mycelial plugs (3 mm in diameter) were taken from the actively growing colony margins of each isolate, transferred to 100 ml of a potato dextrose broth (Difco) (10 g/l) and incubated at 25°C for 5 days on a rotary incubator (150 rpm). The content of each flask was filtered through four layers of sterile cheesecloth, the density of the predominantly microconidial suspension of each isolate determined with a haemacytometer and adjusted to $1x10^6$ conidia/ml. Seeds of melon cultivars Imperial 45 (no resistance genes), Amber (*Fom2*) and Fiata (*Fom1* and *Fom2*) were surface-sterilised with 5% calcium hypochlorite solution for 5 min, rinsed twice in sterile distilled water and sown into celltype plastic growing trays (one seedling per 60 ml cell) filled with a pasteurized commercial potting mixture (peat:vermiculite:polystyrene [18:12:1 m/m], pH 5.8, Hygrotech Seed, South Africa). Seeds were obtained from D. Millar-Watt, Starke Ayres, South Africa. The trays were placed in a growth chamber at 18/25°C night/day temperatures under cool white fluorescent light with 14 h photoperiod. Seedlings were inoculated 11-12 days (expansion of first true leaf stage) after sowing according to the pipette method (Schreuder *et al.*, 2000). Five milliliters of the inoculum suspension was delivered to the substrate around each seedling with a pipetman (model P5000, Gilson Medical Electronics, South Africa). Forty seedlings of each cultivar were inoculated with one of the FOM isolates. Control plants received sterile diluted potato dextrose broth only. At the same time, each isolate was again tested on the three differential cultivars CM 17187, Doublon and Topmark to verify their race classification (Risser *et al.*, 1976). Seedlings were watered after inoculation once a week with a soluble fertilizer (Chemicult hydroponic nutrient, 2g/l, Chemicult Products, South Africa). Seedlings were regularly evaluated for disease development. For histological studies representative plants were uprooted at 14, 21 and 28 days after inoculation for histology from treatments that showed typical Fusarium wilt symptoms (yellowing, stunting, necrotic streaks on stem), and from treatments that remained asymptomatic.

Hypocotyls of seedlings were excised and washed under running tap water to remove all organic matter. The fresh specimens were fixed in 2.5% glutaraldehyde with 0.5 M phosphate buffer (pH 7.2) and 0.05 % caffein for 2 hours at room temperature (Glauert, 1975; Hayat, 1986). After being washed twice in the same buffer, the specimens were postfixed in 0.5 % osmium tetroxide (OsO_4) for 2 hours (Glauert, 1975; Hayat, 1986). Specimens were dehydrated in an acetone series (starting at 50% acetone and finished with two times 100%

acetone) and inbedded in Spurr medium (Spurr, 1969). Longitudinal and cross-sections (200 µm) were cut with a Sorvall ultramicrotome and mounted on microscope glass slides. The sections were stained with toluidine blue and made permanent (Gabriel, 1982). Observations were made with a Vanox AH2 Olympus light microscope.

RESULTS

Differentiation of isolates with RAPD markers. The three primers listed established RAPD patterns for the 12 isolates of FOM and the four isolates of FON. Profiles were reproducible from one experiment to another. Amplification products generated with primers OPE 10, OPE 15, and OPY 19 are illustrated in Fig. 1a-c, respectively. The size of the amplified DNA fragments generated with the three primers ranged from approximately 0.5 kb. to 3 kb. All three primers gave discriminatory banding profiles between isolates of FOM and FON. Identical RAPD patterns for the 12 FOM isolates were found with primer OPE 10. Polymorphisms were detected in Sp 766 (race 2) and Sp 1138 (race 1,2) using primer OPE 15 and in Sp 206A (race 0) and Sp 1131 (race 1,2) using primer OPY 19. All other FOM isolates were uniform with regard to their DNA profiles. No race-specific grouping of the FOM isolates was detected using these three primers. Between the four FON isolates primer OPY 19 showed identical RAPD patterns while primer OPE 10 and OPE 15 separated them into two and three groups, respectively. Primer OPE 10 separated isolate WA 552, WA 553 and WA 558 from WA 257, and primer OPE 15 grouped WA 553 and WA 558, while WA 552 and WA 257 had both different profiles.

Histopathology. Disease reactions of the three differential cultivars (CM 17187, Doublon and Topmark) confirmed the virulence of FOM isolates Sp 206A (race 0), Sp 1052A (race 2) and Sp 1204 (race 1,2). Susceptible host-pathogen combinations (Imperial 45 vs all three FOM isolates; Amber vs Sp 1052A and Sp 1204; Fiata vs Sp 1204) resulted in clear virulence reactions; at least 80% of the plants of susceptible cultivars were diseased, while resistant cultivars showed no symptoms and were indistinguisable from the controls. In the susceptible host-pathogen combinations the hyphae in the hypocotyl were confined initially to the xylem vessels (Fig. 2a). Tyloses were fairly abundant, distributed randomly in the vessels but not more abundant in areas where hyphae were concentrated. Colonisation became widespread and the fungus not only passed readily from one xylem vessel to another, but also extensively invaded other tissues (Fig. 2b). The cortex often became generally permeated and splitting of the stem occurred. In the resistant host-pathogen combinations

(Amber vs Sp 206A; Fiata vs Sp 206A and Sp 1052A) very few hyphae were detected and those present were confined to the vascular tissue. Abundant tyloses were, however, present in the xylem vessels, and completely obstructed the hyphae (Fig. 3a, 3b).

DISCUSSION

The three primers used in this study clearly distinguished the 12 FOM isolates from the FON isolates. However, the primers amplified a highly conserved and characteristic banding pattern for the FOM isolates which represented three physiological races (race 0, race 2, race 1,2). The fact that the latter isolates belong to VCG 0134 and derived from the same parent, indicated that RAPD analysis cannot detect race-specific groupings in FOM. This finding is in agreement with Jacobson & Gordon (1991) who found a lack of polymorphism in mtDNA within VCG 0134. Grouping in VCG 0134, and not virulence, thus reflects the true genetic relatedness of the FOM isolates, whereas RFLP, RAPD and VCG provide additional means of studying their genetic characteristics and evolution. Virulence tests therefore offer the only means of race classification of FOM isolates. This agree with reports on the occurrence of multiple races (Jacobson & Gordon, 1990a), and the development of new races within VCG 0134 (Part 4). These findings (Jacobson & Gordon, 1990a; Jacobson & Gordon, 1991; Part 4) suggest that, similar to some other fungi (Burdon *et al.*, 1982; Burdon *et al.*, 1983), virulence in FOM evolves at a higher rate than other traits. Adaptation to host resistance may therefore be considered as an explanation of this phenomenon (Jacobson & Gordon, 1990a; Jacobson & Gordon, 1991). In his studies on FOM, Bouhot (1981) indicated that resistance genes in the host act as a sensitive filter revealing within the pathogen the corresponding combination of virulence genes and thus the appearance of new races. The findings in my study, and that FOM race 0 (VCG 0134) displayed virulence changes only after infecting resistant melon cultivars (Part 4), support Bouhot's (1981) statement.

Results from the histopathological study confirmed that the two FOM races (race 2, race 1,2), derived from the race 0 parent isolate, retained their ability to operate as true vascular pathogens. These observations are in agreement with previous histological investigations with FOM on melon (Reid 1958; El Mahjoub *et al.*, 1984), *F. oxysporum* f. sp. *lycopersici* (Sacc.) on tomato (Beckman *et al.*, 1972; Elgersma *et al.*, 1972) and *F. oxysporum* f. sp. *pisi* on peas (Charchar & Kraft, 1989). Our data also substantiated those of Beckman *et al.* (1962; 1972) and El Mahjoub *et al.* (1984) who noticed early tylosis

formation in resistant cultivars. Extensive tylosis formation in the resistant host-pathogen combinations could thus be one of the defence mechanisms conferred by resistance genes *Fom1* and *Fom2*.

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Table 1[a]. Description of *Fusarium oxysporum* f. sp. *melonis* isolates used for RAPD analysis and histopathology

Isolate	Description of isolate
Sp 206	Original wild-type race 0 isolate.
Sp 206A	*Nit*M mutant generated from Sp 206 used to infest soil.
Sp 766 Sp 815 Sp 882A Sp 962A Sp 1020 Sp 1131 Sp 1052A	Isolates recovered from cultivar Amber (*Fom2*) plants after natural infection with *nit*M mutant isolate Sp 206A and which displayed a virulence change. All isolates were verified as race 2.
Sp 1024 Sp 1135 Sp 1137 Sp 1138	Isolates recovered from cultivar Fiata (*Fom1, Fom2*) plants after natural infection with *nit*M mutant isolate Sp 206A and which displayed a virulence change. All isolates were verified as race 1,2.

[a] For more detail on the origin of these isolates see Table 1 in Part 4.

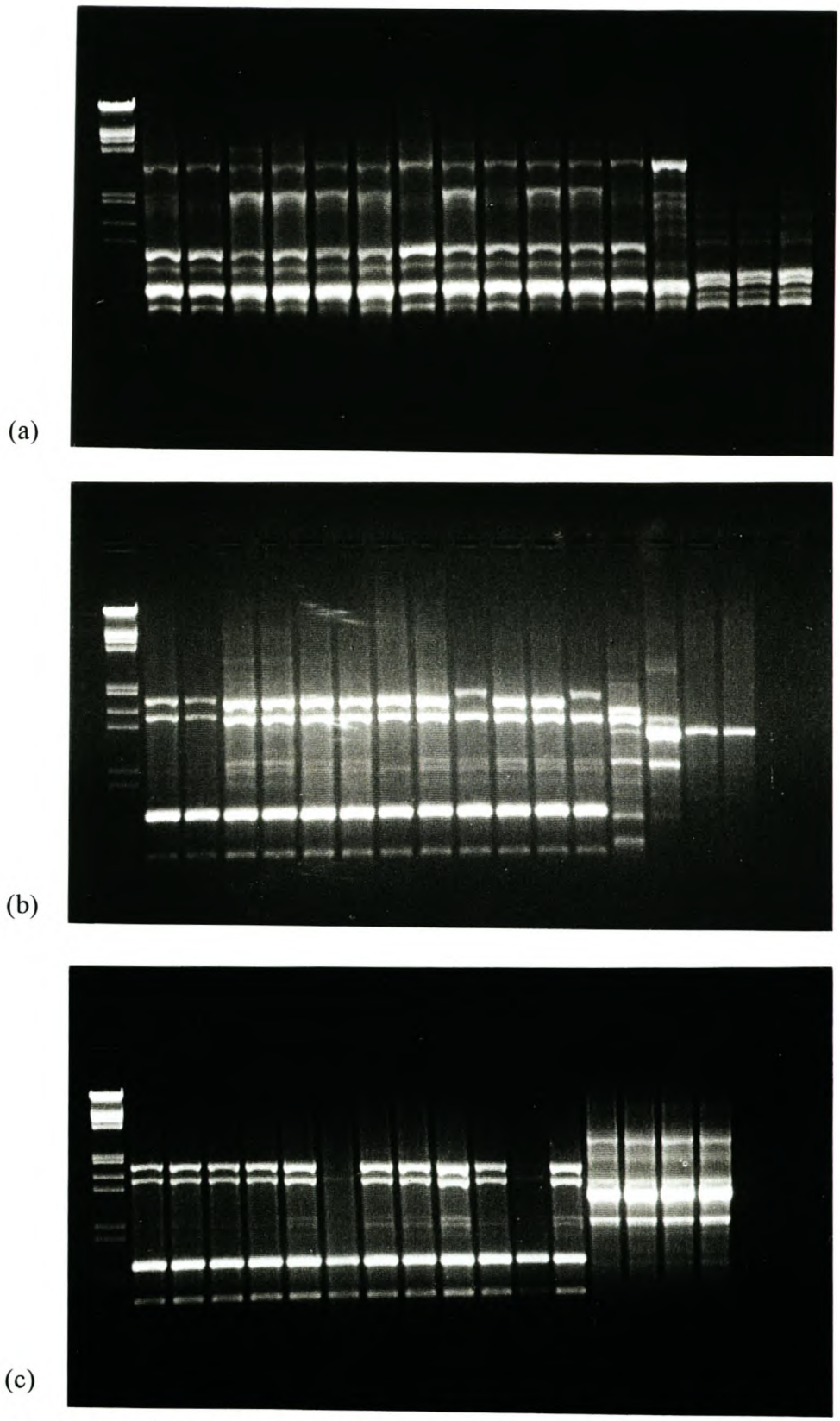
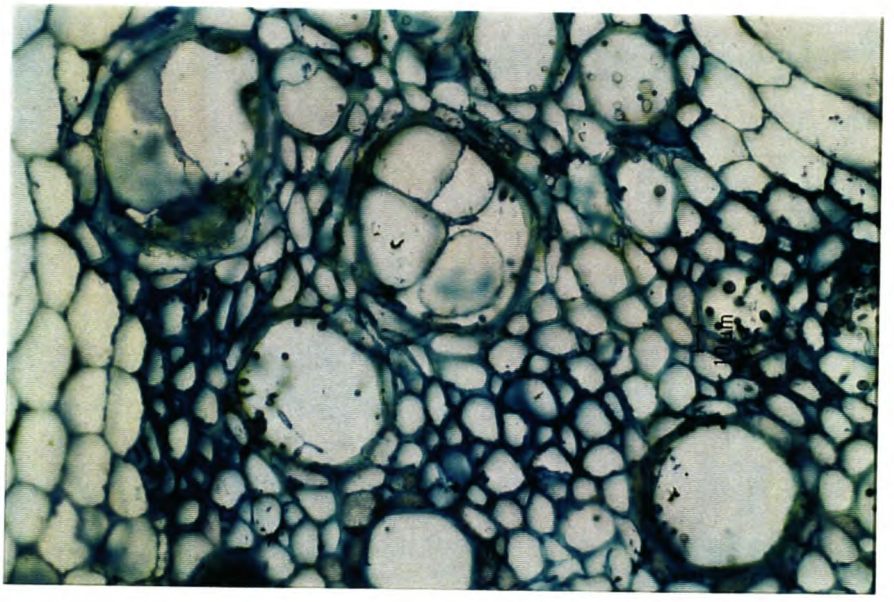


Fig. 1. Banding pattern of 12 *Fusarium oxysporum* f. sp. *melonis* (FOM) and four *F. oxysporum* f. sp. *niveum* (FON) isolates determined by primers OPE 10 (a), OPE 15 (b) and OPY 19 (c). Lane 1 is λ DNA digested with *Eco*RI and *Hin*dIII, and lanes after that from left to right, show amplification products from FOM isolates Sp 815 (race 2), Sp 882A (race 2), Sp 962A (race2), Sp 1020 (race 2), Sp 1052A (race 2), Sp 1131 (race 2), Sp 1135 (race 1,2), Sp 1137 (race1,2), Sp 1138 (race 1,2), Sp 206 (race 0), Sp 206A (race 0), Sp 766 (race 2), and FON isolates WA 257, WA 552, WA 553, and WA 558.

(a)



(b)

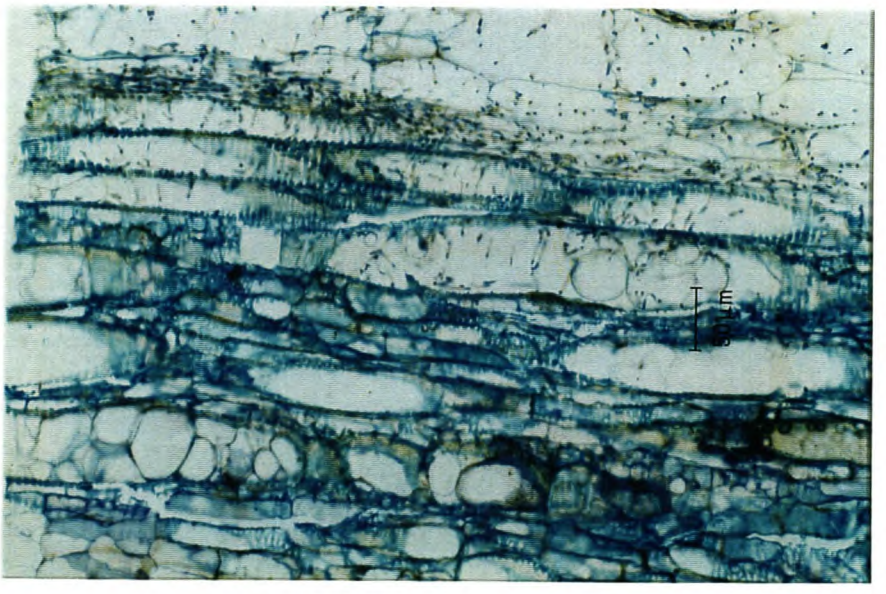
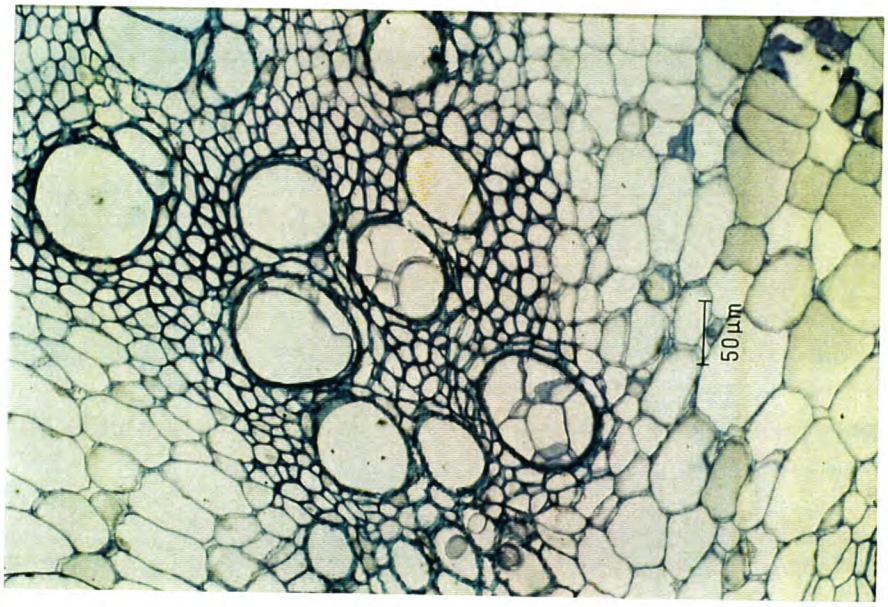


Fig. 2. Cross-section and longitudinal section of the hypocotyl of a susceptible host-pathogen combination (cv Amber, race 2) showing (a) the colonization of xylem vessels and (b) the extensive distribution of the mycelium invading other tissue.

(a)



(b)

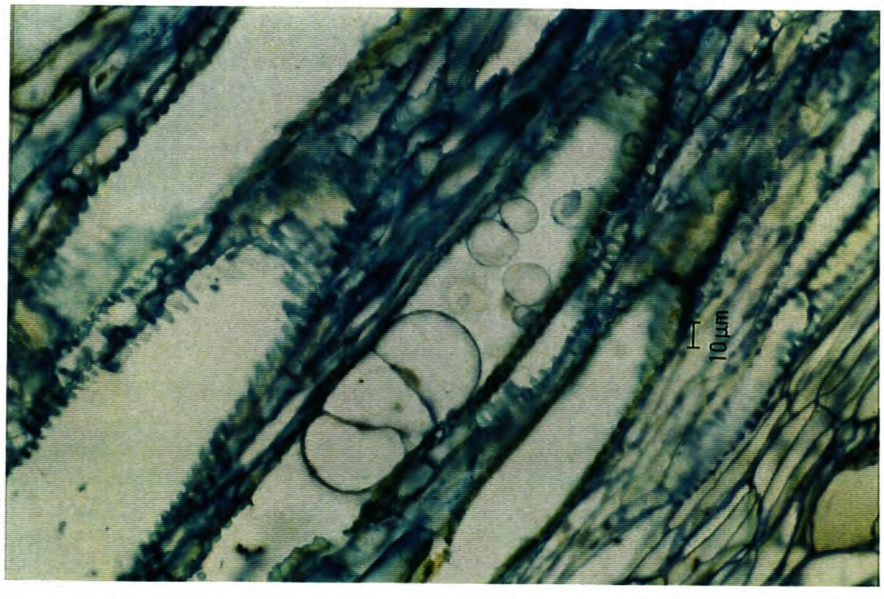


Fig. 3. Cross- (a) and longitudinal (b) sections of the hypocotyl of a resistant host-pathogen combination (cv Amber, race 0) showing abundant tyloses in the xylem vessels.