Hyaline mutants from *Verticillium dahliae*, an example of selection and characterization of strains for host–parasite interaction studies

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The objective of the present work was to select stable well-characterized strains of *Verticillium dahliae* that could be used as biological tools in genetic and plant–microbe interaction studies. Hyaline mutants, known for their stability in pathogenicity, were chosen for the study. Diversity in pathogenicity was found among hyaline subclones obtained from a defoliating wild-type clone, but not within those from nondefoliating ones. Most subclones from the defoliating clone had parental pathotypes, but one (V7-2) exhibited weak pathogenicity. This subclone (V7-2), together with a highly virulent one (V7-7) deriving from the same defoliating parent clone (7), were selected for further characterization, because of their differences in pathogenicity. When studied on the basis of their growth requirements, the two subclones expressed marked differences. V7-7 grew better than V7-2 over a wider range of temperature conditions. Both subclones grew similarly in media supplemented with NH\textsubscript{4} as nitrogen source, but in those with NO\textsubscript{3}, V7-7 grew more vigorously than V7-2 and only the former could grow when NO\textsubscript{2} was used. In spite of these differences, the two subclones were found to belong to the same vegetative compatibility group, confirming their genetic proximity. These results highlight the physiological and genetic complexity inherent in *V. dahliae*. In view of their characteristics, the clones obtained in this study should prove to be valuable tools in furthering the understanding of genetic and host–*V. dahliae* interactions.

**Introduction**

Diverse behaviour has been reported within the genus *Verticillium*, including parasitism of animals (Samson \textit{et al.}, 1988; Jun \textit{et al.}, 1991) and other fungi (Barron \& Fletcher, 1970; Lim \& Nik, 1983), although it is as a pathogen of higher plants that this genus is better known (Gubler \textit{et al.}, 1978; Schnathorst, 1981b; Heale, 1985). Even within the same species, namely *V. albo-atrum* and *V. dahliae*, there may be a wide host range. The latter can infect over 160 plant species belonging to more than 40 different families (Schnathorst, 1981a), including economically important crops all over the world (Gubler \textit{et al.}, 1978; Heale, 1985; Follin, 1986; Koroleva \& Kasyanenko, 1987; Gu \textit{et al.}, 1988; Bejarano-Alcazar \textit{et al.}, 1995). In cotton crops, *V. dahliae* limits production in several countries (Tjamos \& Kornaros, 1978; Follin, 1986; Koroleva \& Kasyanenko, 1987; Bejarano-\textit{Alcazar} \textit{et al.}, 1990). Current procedures for management of this pathogen, such as the use of resistant cultivars and crop rotations, are often not durable, because of the lack of specificity of the parasite and its extreme variability. In addition, *V. dahliae* displays a wide spectrum of variation in morphology (Tolmsoff, 1972), ecology (Heale, 1985) and biochemistry (Heale \& Isaac, 1963; Wheeler \textit{et al.}, 1978), leading to interference with studies on genetic diversity and on plant–microbe interactions. The choice of fungal strains to be compared for the reactions they induce in host plants is often based solely on differences in virulence. Such variability has been shown to be high in wild-type strains of *V. dahliae*, even among conidial progeny of clones. However, this seems to be exclusive to microsclerotial wild-type strains (Hadasutrisno, 1987). On the other hand, hyaline variants appear to possess a certain stability in pathogenicity (Heale \& Isaac, 1965; Boisson \& Lahlu, 1982). The hyaline character is a result of loss of the capacity to produce microsclerotia (Brandt \& Roth, 1965) or the melanin pigment (Typas \& Heale, 1976). These mutants may be obtained spontaneously or by mutagenesis (Bell \textit{et al.}, 1976; Typas \& Heale, 1976, 1979; Wheeler \textit{et al.}, 1978). The objectives of this study were: (i) to analyse intraclonal diversity within
V. dahliae, using spontaneous hyaline variants and (ii) to select hyaline subclones showing differences in pathogenicity and to characterize these according to pathogenicity on cotton, vegetative compatibility (VC) and growth on several nitrogen sources and under different temperatures. Fulfilment of these objectives should lead to the selection of well-characterized, stable subclones differing from one another in pathogenicity. Such biological material would help to circumvent some problems associated with V. dahliae variability.

Materials and methods

Plant material

Gossypium hirsutum Lsa-205, G. barbadense Ashmouni, G. hirsutum Tashkent 1 and G. arboreum Xiao were used, because of their reported different levels of resistance to Verticillium wilt. G. hirsutum is more susceptible than G. barbadense and G. arboreum (Barrow, 1970, 1973; Wilhelm et al., 1974; Bell & Mace, 1984). After a 5-min surface disinfection with 5% calcium hypochlorite, seeds were placed for 24 h at 25°C in Petri dishes containing sterile filter paper moistened with sterilized distilled water. Germinated seeds were then sown in a potting mixture (loam and sand, 6:1, v/v).

Fungal material and generation of subclones

The three microsclerotial wild-type clones used in this study originated from single spores obtained from pure cultures of isolates 6, 8 (nondefoliating) or 7 (defoliating), provided by IRCT-CIRAD, Montpellier, France. A drop of a conidial suspension from each clone was deposited in the centre of a new Petri dish and grown for 2 weeks. Two hundred subclones were isolated from each cotton cultivar to be inoculated, 25 2-week-old plants were carefully uprooted and the roots immersed for 15 min in 100 mL of a conidial suspension containing 10⁶ spores/mL. Twenty-five plants were immersed in sterile distilled water to serve as controls. All plants were replanted in separate plastic pots (9 cm diameter) and placed in a growth chamber with a 12-h photoperiod, at 27 ± 2°C and 70–90% relative humidity.

Mode of infection

For each cotton cultivar to be inoculated, 25 2-week-old plants were carefully uprooted and the roots immersed for 15 min in 100 mL of a conidial suspension containing 10⁶ spores/mL. Twenty-five plants were immersed in sterile distilled water to serve as controls. All plants were replanted in separate plastic pots (9 cm diameter) and placed in a growth chamber with a 12-h photoperiod, at 27 ± 2°C and 70–90% relative humidity.

Estimation of disease severity

Disease severity was estimated by observing plant stunting and foliar damage 23 days after inoculation and vascular discoloration 1 day later. Quantification of these symptoms was made as follows. To estimate stunting, the length of the epicotyl (Epl) was measured, or the stunting index (SI) was calculated from SI = 100(Epl - Eplₐ)/Eplₐ, where Eplₐ is the mean Epls for control plants and Eplₐ is the epicotyl length for each treated plant. To evaluate foliar damage on each plant, a score (xi) was attributed to each cotyledon and leaf according to the scale (0) no foliar symptoms; (1) yellowing or partial necrosis of cotyledon; (2) cotyledon scar; (3) yellowed leaf; (4) wilted or necrotic leaf; (5) leaf scar (Daayf et al., 1995). A 'Foliar Alteration Index' (FAI) was then calculated for each inoculated plant as FAI = 100 (xi)/ (4+5n), where 4 is the maximum score for cotyledons (2×2), 5 is the maximum score for each leaf and n is the number of leaves of each plant. Vascular discoloration was evaluated on each plant according to a modified method of Erwin et al. (1976); discoloration was scored (yi) for each internode based on the scale (0) no discoloration, (1) 2–5 localized brown regions within the vascular tissue of the same internode; (2) browning of long stretches of the vascular tissue; (3) browning of all the vessels but not the adjacent tissues; (4) browning of both vessels and adjacent tissues. Browning Index (BI) was then calculated for each plant as BI(%) = 100×yi/ (4d), where d is the total number of seedling internodes including hypocotyl and 4 the maximum score for an internode. For each treatment, a mean was calculated for SI, FAI and BI.

Effect of temperature and nitrogen source on the growth of V. dahliae

Four replicates of culture pieces were deposited in separate Petri dishes containing potato dextrose agar (PDA) for temperature studies, or a minimal medium (Daayf et al., 1995), where the nitrogen sources were: ammonium tartrate (0.8 g L⁻¹) (MM₁), sodium nitrate (2 g L⁻¹) (MM₂), ammonium tartrate (0.8 g L⁻¹) amended with calcium carbonate (0.5 g L⁻¹) (MM₃), or sodium nitrate (0.5 g L⁻¹) (MM₄). The Petri dishes were placed in incubators at 21 and 27°C for temperature studies and 25°C for nitrogen effects studies. Growth was estimated 15 or 20 days later, by measuring two diameters of each colony.

Vegetative compatibility studies

Selection of mutants

Mutants affected in the nitrogen assimilation pathway, called nitrate-nonutilizing mutants or 'nit' mutants, were used to assess VC relationships among strains of V. dahliae. They were produced using a modified procedure (Daayf et al., 1995) of Cove (1976), adapted for P. oxysporum by Puhalla (1983) and for V. dahliae by Joaquim & Rowe (1990). Agar pieces (approximately 15 × 2 mm) were cut from the edge of wild-type colonies of V. dahliae clones growing on PDA and placed on MM amended with 30 g L⁻¹ potassium chloride in 9-cm Petri dishes. After incubation for 10 days at 25°C, a fragment (1 cm diameter) was cut from the advancing margin of a...
chlorate-resistant colony, placed in a tube containing 8 mL of sterile water and then shaken to release the spores. The spore suspension was adjusted to a concentration of $10^6$ conidia/mL and 50 µL of this suspension was placed on MM: Each spore that germinated was transferred to a new MM plate.

**Characterization of nit mutants**

A piece of each chlorate-resistant mutant culture was transferred to basal medium (nitrogen-free) (Correll et al., 1987) amended with one of the following nitrogen sources: sodium nitrate (0.2 g L$^{-1}$), sodium nitrite (0.4 g L$^{-1}$), hypoxanthine (0.5 g L$^{-1}$) or ammonium tartrate (0.8 g L$^{-1}$) buffered with calcium carbonate (0.5 g L$^{-1}$). Assignment of nit mutant phenotype designations, nit1, nit3 and nitM, corresponded to those used for *V. albo-atrum* (Correll et al., 1988), *F. oxysporum* (Correll et al., 1987) and *V. dahliae* (Joaquim & Rowe, 1990). Nit1 mutants originate from a mutation at the structural locus of nitrate reductase, Nit3 mutants from a mutation at a specific regulatory locus of the nitrate assimilation pathway and NitM mutants from a mutation at one of the loci controlling synthesis of a molybdenum-cofactor necessary for nitrate reductase and purine dehydrogenase activities (Correll et al., 1987).

**Pairing of mutants**

To determine VC grouping among the strains of *V. dahliae*, different nit mutants were paired by placing two agar culture pieces (1 mm$^3$) 7–8 mm apart on MM. Four separate pairings were carried out in the same plate without inter-pairing interactions. Aerial mycelium in the meeting zone was visible as early as 4–5 days when heterokaryosis occurred. The trial was concluded 10 days after the pairing of mutants.

Different growth types occurred at the mycelial interface as follows: r1, formation of microsclerotia.

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**Figure 1**

Epicotyl length (Epl) (a), Foliar alteration index (FAI) (b) and Browning index (Bl) (c), estimated on *Gossypium hirsutum* cv. Iss-205, 23, 23 and 24 days, respectively, after inoculation with clones 6 (V6), 7 (V7) and 8 (V8) of *Verticillium dahliae* and their subclones.
Intraclonal diversity in pathogenicity of hyaline mutants of *V. dahliae* on cotton

Among the 200 subclones isolated from each of the three microsclerotial wild-type single-spore clones, a few were hyaline variants (seven, seven and five from clones 6, 7 and 8, respectively). Most hyaline subclones produced a few microsclerotia after 5–6 weeks, but some (V6-1, V6-4, V7-2, V7-6, V7-7, V8-1 and V8-2) did not.

The three wild-type microsclerotial clones and 18 of their hyaline subclones were tested for pathogenicity on *G. hirsutum* cv. Isa-205. Results recorded after 23 days are shown in Table 1. Only those plants inoculated with clones 6 and 8 and subclones V6-4, V6-5 and V8-5 showed a significant reduction of epicotyl length (Fig. 1a). Clone 7 and its subclones exhibited wide differences in reducing plant growth, from the least aggressive, V7-2, to the most aggressive, V7-6 and V7-7. Leaf alterations (FAI) caused by clones 6 and 8 and their subclones were quite low, never exceeding 10% (Fig. 1b); those caused by clone 7 and its subclones V7-3, V7-6 and V7-7 were severe, sometimes leading to death, but subclones V7-1, V7-2 and V7-4 caused relatively weak foliar symptoms. All tested strains caused browning of vessels and adjacent tissues (Fig. 1c), but the browning index (BI) showed a pattern of severity among clones and subclones that was variable, similar to that recorded by the FAI index. The lowest BI values were recorded for clones 6, 8 and their subclones, with very little differences among them (Fig. 1c).

Results of pairings between nit-mutants selected from Epls and FAIs, the BI for clone 7 and its subclones were variable, 7, V7-3, V7-6 and V7-7 being the most aggressive. Subclone V7-7 caused the least severe internal symptoms, similar to those of nondefoliating strains. Among the subclones with which BI reached 100%, V7-6 and V7-7 caused plant death the most rapidly. Further experiments using most of these subclones (V6-1, V6-2, V6-4, V8-1, V8-2, V7-2, V7-3, V7-6 and V7-7) yielded the same results (data not shown). Thus, in general, the subclones obtained from clone 7 were the most diverse and appeared to be the most interesting for selection of hyaline subclones possessing wide differences in pathogenicity.

Based on these results, the subclones V7-2 and V7-7 were selected as the least and most aggressive, respectively.

### Intraclonal diversity in vegetative compatibility

Results of pairings between nit-mutants selected from the hyaline subclones are presented in Table 2. Mutants obtained from clone 8 and its subclones were compatible with aerial mycelium; r2, visible reaction in the medium matrix only; r3, late formation of loose aerial mycelium in the contact line; r4, high reaction with differentiation of a dense aerial mycelium in localized areas at the contact line; r5, elaboration of a dense aerial mycelium in the confrontation line with differentiation of microconidia-containing spherules; and r6, production of mucoid thalli identical to those described with some wild-type strains and often forming microsclerotia. Types r1 to r4 were regarded as weak reactions and scored (±). Only r5 and r6 reactions were scored (+) (Daayf et al., 1995). To determine whether our VCGs corresponded to those defined by Joaquim & Rowe (1990), pairings between their testers (T9 from VCG1, WM from VCG2 and BB and S39 from VCG4) and om strains were conducted.

### Table 1

<table>
<thead>
<tr>
<th>SI (%)</th>
<th>FAI (%)</th>
<th>BI (%)</th>
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<tbody>
<tr>
<td></td>
<td>V7-2</td>
<td>V7-7</td>
</tr>
<tr>
<td>G. <em>hirsutum</em> 108F</td>
<td>6 ± 1</td>
<td>64 ± 15</td>
</tr>
<tr>
<td>G. <em>hirsutum</em> Tashkent-1</td>
<td>12 ± 2</td>
<td>72 ± 35</td>
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<tr>
<td>G. <em>barbadense</em> Ashmouni</td>
<td>15 ± 5</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>G. <em>arboreum</em> Xiao</td>
<td>3 ± 3</td>
<td>81 ± 11</td>
</tr>
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</table>

SI, stunting index; FAI, foliar alteration index; BI, browning index; ± represents standard deviation.
Verticillium dahliae
hyaline mutants

Table 2: Vegetative compatibility pairings between nit mutants of Verticillium dahliae

<table>
<thead>
<tr>
<th></th>
<th>V6-1</th>
<th>V6-4</th>
<th>8</th>
<th>V8-1</th>
<th>V8-2</th>
<th>7</th>
<th>V7-2</th>
<th>V7-4</th>
<th>V7-6</th>
<th>V7-7</th>
<th>T9</th>
<th>WM</th>
<th>BB</th>
<th>S39</th>
</tr>
</thead>
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<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>±</td>
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<tr>
<td>V6-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>V6-4</td>
<td>+</td>
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<td>8</td>
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<td>V8-1</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>V8-2</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>7</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>V7-2</td>
<td></td>
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<td>V7-4</td>
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<td>V7-6</td>
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<tr>
<td>V7-7</td>
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</tbody>
</table>

Every (+, − or ±) represents the interpretation of all reactions observed resulting from pairings of nit, nit and nitM mutants of one strain with complementary nit mutants of another strain; (+), strong reaction; (±), weak reaction; (−), no visual reaction observed; (), not paired.

with each other and also with nit-mutants deriving from clone 6 and its subclones and with nitM from WM (VCG2). Mutants from clone 7 and its subclones were also compatible with each other and with nitM of T9 (VCG1), but were never compatible with nit mutants from clones 6 and 8 or their subclones. These results confirmed that clones 6 and 8 and their subclones belong to the same VCG and that they are not compatible with strains from VCG1. They also confirmed that V7-2 and V7-7, produced from the same defoliating clone 7, are genetically close, although they differ in pathogenicity on cotton.

Effects of temperature and nitrogen source on the growth of V7-2 and V7-7

Twenty days after incubation of clones V7-2 and V7-7 at two different temperatures (21 and 27°C), growth reduction at 27°C was greater in clone V7-2 than in clone V7-7 (Table 3). Of the nitrogen sources, NH₄ plus CaCO₃ was the most favourable for growth of both clones. A difference in growth was found between V7-2 and V7-7 on NO₂- and NO₃-supplemented media, both more favourable to V7-7 (Table 3).

Table 3: Effects of temperature and nitrogen source on the growth of Verticillium dahliae subclones V7-2 and V7-7

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Colony diameter (mm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>V7-2</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td></td>
</tr>
<tr>
<td>21°C</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>27°C</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>Nitrogen</td>
<td></td>
</tr>
<tr>
<td>NH₄</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>NH₄+CO₃</td>
<td>71 ± 3</td>
</tr>
<tr>
<td>NO₂</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>NO₃</td>
<td>4 ± 4</td>
</tr>
</tbody>
</table>

± represents standard deviation.

Discussion

This study has characterized two subclones of V. dahliae that showed large differences in pathogenicity and growth. Intraclonal diversity in pathogenicity was particularly common in subclones from defoliating clone 7. Strains from nondefoliating clones 6 and 8 also differed pathogenically, but they always remained nondefoliating as previously shown for V8-2 and V8-5 (Daayf et al., 1995). Within the progeny of defoliating clone 7, where differences in pathogenicity were most noticeable, subclone V7-2 produced weak symptoms. Results obtained from vegetative compatibility studies, a powerful tool for genetic proximity determination (Leslie, 1993), confirmed that all tested subclones from clone 7, including V7-2, belonged to the same group.

Comparison of these results with those previously obtained with V7-2, V7-4, V7-6, V7-7, V8-2 and V8-5 (Daayf et al., 1995) confirmed the stability in pathogenicity of these hyaline mutants. Because of the wide range in pathogenicity among subclones from clone 7, V7-2 (the least aggressive) and V7-7 (the most) were chosen for further characterization studies.

Differences in pathogenicity among strains of V. dahliae may be partly related to their different adaptation to high temperatures, as reported by Bell (1992) and other workers (Heale, 1985) and may be true for distribution of defoliating and nondefoliating strains of V. dahliae throughout the world. Results confirmed the tendency of the most virulent strains to grow better than the less virulent ones over a wider range of temperature conditions. Temperature may also affect parasitism of the fungus in the plant as observed in V7-2-infected cotton plants, which expressed more symptoms when they were first left 3 days in a cooler growth chamber (21°C) before being transferred to 24°C (data not shown).

Growth of V7-2 and V7-7 was similar on media containing buffered or unbuffered NH₄. On the other hand, V7-7 grew more vigorously than V7-2 on NO₃. V7-2 failed to grow on NO₂ whereas V7-7 grew well. This correlates well with differences in pathogenicity of
such subclones and supports the statement by Bell (1992) that pathogenicity may be related to the ability of *V. dahliae* to disturb hormonal and nitrogen metabolism. The same author has also shown a relation between pathotype and capacity to use ammonium and nitrate nitrogen in diseased plants (Bell, 1991).

The accumulation of fungitoxic compounds in cotton plants in response to inoculation with *V. dahliae* subclone V7-2 was greater than in those inoculated with V7-7 (data not shown). This indicates that in spite of their genetic proximity, the two subclones either elicit different responses in the host plant or have different capacities for phytoalexin detoxification.

In conclusion, using hyaline mutants, it was possible to show intrACLonal diversity in pathogenicity of *V. dahliae* and to characterize two subclones differing in their behaviour, although they were obtained from the same clone. Comparable hyaline mutants are in use by some breeders for selection of tomato cultivars resistant to *Verticillium* wilt (C. Boisson, personal communication, ORSTOM, France, retired) and their stability is of great interest for assessment of interactions of different responses in the host plant or have different capacities for phytoalexin detoxification.


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Verticillium dahliae hyaline mutants

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