Polish Journal of Microbiology
2004, Vol. 53, No 3, 167–174

# Comparative Assessment of Genotyping Methods for Study Genetic Diversity of *Fusarium oxysporum* Isolates

KAMEL A. ABD-ELSALAM[1,2], JIAN-RONG GUO[2], FRANK SCHNIEDER[2], ABDEL-MONGY ASRAN-AMAL[1] and JOSEPH-ALEXANDER VERREET[2]

[1] Agricultural Research Center, Plant Pathology Research Institute, 9 Gamaa St., Giza, Egypt
[2] Christian Albrechts Universtät zu Kiel, Institut für Phytopathologie
Hermann-Rodewald-St. 9, D-24118 Kiel, Germany

Received in revised form 12 April 2004

Abstract

In this study, we evaluated three PCR-based methods for the molecular typing of nonpathogenic *Fusarium oxysporum* isolates: random amplified polymorphic DNA (RAPD), polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and amplified fragment length polymorphism (AFLP). The analyses were performed using 64 isolates of *F. oxysporum* collected from cotton-producing areas in Egypt. A number of polymorphic RAPD, PCR-RFLP and AFLP bands were scored in all isolates and the genetic similarity among them was assessed. Clustering analysis separated the isolates into two main groups, with similarities ranging from 87 to 100% for RAPD, 80 to 100% for PCR-RFLP and 88 to 97% for AFLP, respectively. The obtained data suggested that all three types of markers are equally informative, but the three assays differed in the amount of detected polymorphic bands. AFLP fingerprinting was also found to be more differentiating than other techniques for the typing of *F. oxysporum* populations.

Key words: AFLP, RAPD, ITS-PCR-RFLP, polymorphism, *Fusarium oxysporum*

## Introduction

*F. oxysporum* Schlect. emend. Snyd. and Hans is one of the most economically important members of *F. oxysporum*, has a world-wide distribution and is common in a wide range of soils (Gerlach and Nirenberg, 1982). *F. oxysporum* is extremely variable in genetics and its populations containing nonpathogenic strains. Nonpathogenic strains of *F. oxysporum* have received less attention. However, they play an important role in the ecology of *Fusarium* diseases. Indeed, studies on soils naturally suppressive to *Fusarium* wilts have established the role of nonpathogenic *F. oxysporum* populations in the disease suppression (Alabouvette, 1990). Even if their mode of action is not totally understood, experimental evidence indicates nonpathogenic strains that compete with the pathogenic *F. oxysporum* population in soil for nutrients (Couteaudier and Alabouvette, 1990) and on roots for colonization sites (Eparvier and Alabouvette, 1994). Experimental evidence also indicates that nonpathogenic strains vary in their ability to compete with pathogenic strains and to limit disease incidence (Alabouvette and Couteaudier, 1992). Therefore, it would be interesting to characterize the diversity existing among populations of nonpathogenic *F. oxysporum.* Moreover, as stated by Corell (1991), studies of the genetic diversity of nonpathogenic *F. oxysporum* populations should help a better understanding of the diversity among pathogenic strains of *F. oxysporum.* Molecular markers are being increasingly used for the characterization of populations of fungal pathogens in plants. These markers are not subjects of natural selection and can be used to evaluate genetic diversity and phylogenetic relationships within and between species, as well as to identify particular races and pathotypes. One technique with the potential application for population biology is random amplified polymorphic DNA (RAPD) marker (Williams *et al*., 1990). Direct analysis of DNA polymorphism is a general approach for establishing genetic variation within microorganisms. Restriction fragment length polymorphisms (RFLP) analysis of nuclear or mitochondrial DNA has been used to estimate the genetic variation within and between nonpathogenic strains of *F. oxysporum* (Gordon and

* Corresponding author: e-mail: kaabdelsalam@msn.com

Table I
Features of molecular genetic markers used in this study

| Characteristics | PCR-based markers | | |
|---|---|---|---|
| | RAPD | PCR-RFLP | AFLP |
| Abundance | high | low | high |
| Level of polymorphism | high | low | high |
| Locus specificity | no | no | no |
| Type of scoring [1] | dominant | dominant | dominant |
| Reproducibility | low | low | high |
| Labor intensity | low | low | medium |
| Technical demands | low | low | medium |
| Operational costs | low | low | medium |
| Development costs | low | low | low |
| Quantity of DNA required | low | low | medium |
| Amenability to automation | yes | no | yes |
| Data analysis | simple | simple | complex |
| Fragment profile | low-copy | single or low-copy | multi-copy |

[1] Only relevant for diploid fungi

Okamoto, 1992; Edel *et al.*, 1995; Paavanen-Huhtala *et al.*, 1999). In recent years, a novel DNA fingerprinting method, amplified fragment length polymorphism (AFLP), has been proved as a high-resolution genotyping method and a useful tool in taxonomy of fungal pathogens in plants (Vos *et al.*, 1995). Why AFLP data provided better discrimination of intra-specific distances is not known, but we assumed that it could be related to the restriction pretreatment which excluded almost all tandem random amplification? In contrast to what could happen with the RAPD technique (Table I). The goal of the study reported here was to compare the three different types of above-mentioned molecular approaches to establish which of them would be more suitable for characterizing the genetic diversity of *F. oxysporum* isolates.

## Experimental

### Materials and Methods

**Collection and identification of isolates.** A collection of nonpathogenic isolates of *F. oxysporum* was made by direct isolation from nine locations for cotton producing-areas in Egypt (Table II). Sixty four isolates were collected and stored by cryopreservation at –80°C according to Schnieder *et al.* (1998). Isolates were subcultured as single spores and then identified according to morphological criteria in Nelson *et al.* (1983). All F. *oxysporum* isolates were tested on cultivar Giza 74 which has no known resistance to *Fusarium* wilt of cotton. All isolates did not cause any symptoms of disease. These isolates are considered to be nonpathogenic.

**DNA extraction.** For DNA extraction, a modified QIAamp (QIAGEN, Hilden, Germany) DNA extraction protocol was used. A small amount of fungal culture was taken from the culture plates and boiled for 10 min in 100 µl of freshly prepared 25 mM NaOH-0.5% sodium dodecyl sulfate. After cooling and neutralization with 100 µl of 25 mM HCl, 200 µl of buffer AL (QIAamp kit) was added, and the suspension was boiled again for 10 min. Then, the QIAamp protocol was performed according to kit instructions. The amount of DNA was quantified using a fluorescence photometer (DyNA Quant 200, Hofer Corp. Germany), and the DNA was stored in aliquots at –20°C.

**RAPD analysis.** A total of 20 decamer primers of arbitrary base composition (Primer Kit A, Roth, Karlsruhe, Germany) were used. Five following primers were selected based on clearly discernible polymorphic bands: primers 1; (5‘TGCCGAGCTG3’); 2; (5‘AGTCAGCCAC3’); 3 (5‘GGTCCCTGAC3’) 4; (5‘AGGGGTCTTG3’) and 5; (5‘GGTCCCTGAC3’). Those primers were used for the typing of the *F. oxysporum* isolates.

**ITS-PCR-RFLP analysis.** DNA fragments were amplified with ITS1/ITS4, ITS1/ITS5 and ITS4/ITS5 specific primer sets (White *et al.*, 1990). The amplification reaction of genomic DNA was carried out in a volume of 25 ml with 25 pmol of each primer, 50 pg of template DNA, 200 µM of each dNTP, 1 unit of *Pwo* DNA polymerase (Boehringer Mannheim, Germany) and a reaction buffer containing 250 mM KCl, 100 mM Tris-HCl (pH 8.85), 50 mM $(NH_4)_2SO_4$ and 20 mM $MgSO_4$. The PCR condition involved pre-denaturation for 5 min at 94°C, 35 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C, and an extension of 10 min at 72°C in a thermocycler (T-Gradient, Biometra, Göttingen, Germany). PCR products were digested with *Taq*I, *Alu*I, *Rsa*I and *Hae*III. The digested DNA was electrophoresed for 1 h at 70 V in a 1.0% ethidium bromide-staining agarose gel.

**AFLP fingerprint.** AFLP was based on selective amplification of a subset of genomic restriction fragments using PCR (Vos *et al*., 1995). Briefly, restriction fragments for amplification were generated in 40-µl reaction volumes. Genomic DNA (50 to 100 ng) was digested with 5 U EcoRI and 5 U MseI at 37°C for 3 h. Then 10 µl of a solution containing 5 pmol EcoRI and 50 pmol MseI adapters, 1 U of T4 DNA ligase and 1× RL-buffer (50 mM Tris-HCl [pH 7.6]-10 mM $MgCl_2$- 1 mM ATP-1 mM dithiothreitol-5%

Table II
*Fusarium oxysporum* isolates used in this study with indication of cotton-producing areas, previous crops and host genotype

| Isolates No | Geographic origin | Previous Crops | Host Genotype |
|---|---|---|---|
| 1. | Minufiya | Alfaalfa | Giza 89 |
| 2. | Dumyat | Alfaalfa | Giza 45 |
| 3. | Gharbiya | Alfaalfa | Giza 86 |
| 4. | Minufiya | Alfaalfa | Giza 89 |
| 5. | Gharbiya | Onion | Giza 89 |
| 6. | Sharqiya | Alfaalfa | Giza 85 |
| 7. | Daqahliya | Faba-Bean | Giza 86 |
| 8. | Minufiya | Faba-Bean | Giza 89 |
| 9. | Gharbiya | Alfaalfa | Giza 86 |
| 10. | Minufiya | Alfaalfa | Giza 89 |
| 11. | Daqahliya | Onion | Giza 86 |
| 12. | Fayium | Alfaalfa | Giza 83 |
| 13. | Beheira | Alfaalfa | Giza 70 |
| 14. | Beheira | Alfaalfa | Giza 70 |
| 15. | Beheira | Alfaalfa | Giza 70 |
| 16. | Beheira | Alfaalfa | Giza 70 |
| 17. | Beheira | Alfaalfa | Giza 70 |
| 18. | Beheira | Alfaalfa | Giza 70 |
| 19. | Beheira | Alfaalfa | Giza 70 |
| 20. | Beheira | Alfaalfa | Giza 70 |
| 21. | Beheira | Alfaalfa | Giza 70 |
| 22. | Beheira | Alfaalfa | Giza 70 |
| 23. | Minufiya | Alfaalfa | Giza 89 |
| 24. | Daqahliya | Pisum | Giza 86 |
| 25. | Minufiya | Alfaalfa | Giza 89 |
| 26. | Minufiya | Faba-Bean | Giza 89 |
| 27. | Fayium | Alfaalfa | Giza 83 |
| 28. | Minufiya | Alfaalfa | Giza 89 |
| 29. | Daqahliya | Alfaalfa | Giza 86 |
| 30. | Beheira | Alfaalfa | Giza 70 |
| 31. | Beheira | Alfaalfa | Giza 70 |
| 32. | Minufiya | Alfaalfa | Giza 89 |
| 33. | Beheira | Alfaalfa | Giza 70 |
| 34. | Minufiya | Alfaalfa | Giza 89 |
| 35. | Minufiya | Alfaalfa | Giza 89 |
| 36. | Beheira | Faba-Bean | Giza 70 |
| 37. | Beheira | Alfaalfa | Giza 70 |
| 38. | Gharbiya | Onion | Giza 86 |
| 39. | Beheira | Alfaalfa | Giza 70 |
| 40. | Assuit | Alfaalfa | Giza 83 |
| 41. | Assuit | Alfaalfa | Giza 83 |
| 42. | Assuit | Alfaalfa | Giza 83 |
| 43. | Assuit | Alfaalfa | Giza 83 |
| 44. | Assuit | Alfaalfa | Giza 83 |
| 45. | Assuit | Alfaalfa | Giza 83 |
| 46. | Assuit | Alfaalfa | Giza 83 |
| 47. | Assuit | Alfaalfa | Giza 83 |
| 48. | Sohag | Alfaalfa | Giza 83 |
| 49. | Assuit | Alfaalfa | Giza 83 |
| 50. | Assuit | Alfaalfa | Giza 83 |
| 51. | Assuit | Alfaalfa | Giza 83 |
| 52. | Assuit | Alfaalfa | Giza 83 |
| 53. | Assuit | Alfaalfa | Giza 83 |
| 54. | Assuit | Alfaalfa | Giza 83 |
| 55. | Assuit | Alfaalfa | Giza 83 |
| 56. | Assuit | Alfaalfa | Giza 83 |
| 57. | Sohag | Alfaalfa | Giza 83 |
| 58. | Sohag | Alfaalfa | Giza 83 |
| 59. | Assuit | Alfaalfa | Giza 83 |
| 60. | Assuit | Alfaalfa | Giza 83 |
| 61. | Assuit | Alfaalfa | Giza 83 |
| 62. | Sohag | Alfaalfa | Giza 83 |
| 63. | Assuit | Alfaalfa | Giza 83 |
| 64. | Sohag | Alfaalfa | Giza 83 |

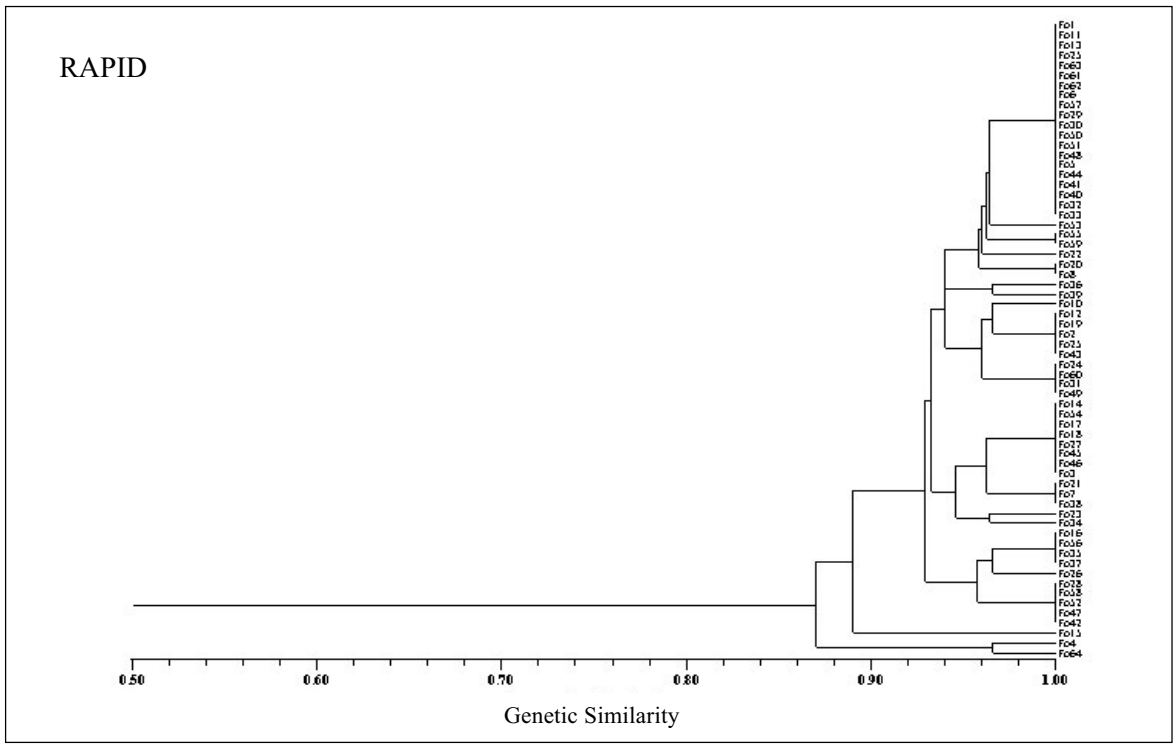
[wt/vol] polyethylene glycol 8000) was added. The ligation reaction was incubated at room temperature for 3 h. After ligation, the reaction mixture was diluted 1:10 with 0.1 × TE-buffer. The following combinations of AFLP primer pairs with two selective nucleotides were used for the selective amplification: EcoRI + AG/MseI + AA, EcoRI + AA/MseI + AG, EcoRI + CC/MseI + AA and EcoRI + CC/MseI + CC (MWG-Biotech, Germany). The amplification reactions were performed in 25 µl volume containing 8 µl of the 1:10 diluted ligation mixture as the template, 1× reaction buffer (10 mM Tris-HCl [pH 8.0] 50 mM KCl and 1.5 mM $MgCl_2$), 200 µM of each dNTP, 1U of *Taq* polymerase, and 30 ng of each primer, and under the following conditions: 36 cycles of denaturation for 30 s at 94°C, annealing for 30 s (see below), and extension for 1 min at 72°C. The annealing temperature of 65°C in the first cycle was subsequently reduced each cycle by 0.7°C for the next 12 cycles and was kept at 56°C for the remaining 23 cycles. Each sample was mixed with equal volume of loading buffer, denatured and loaded on a 5% polyacrylamide sequencing gel in 1 × TBE-buffer (100 mM Tris-base, 100 mM boric acid, 2 mM EDTA). Gel was run for 2 h at 55 W, and then stained by a modification of the silver staining method of Creste *et al.* (2001).

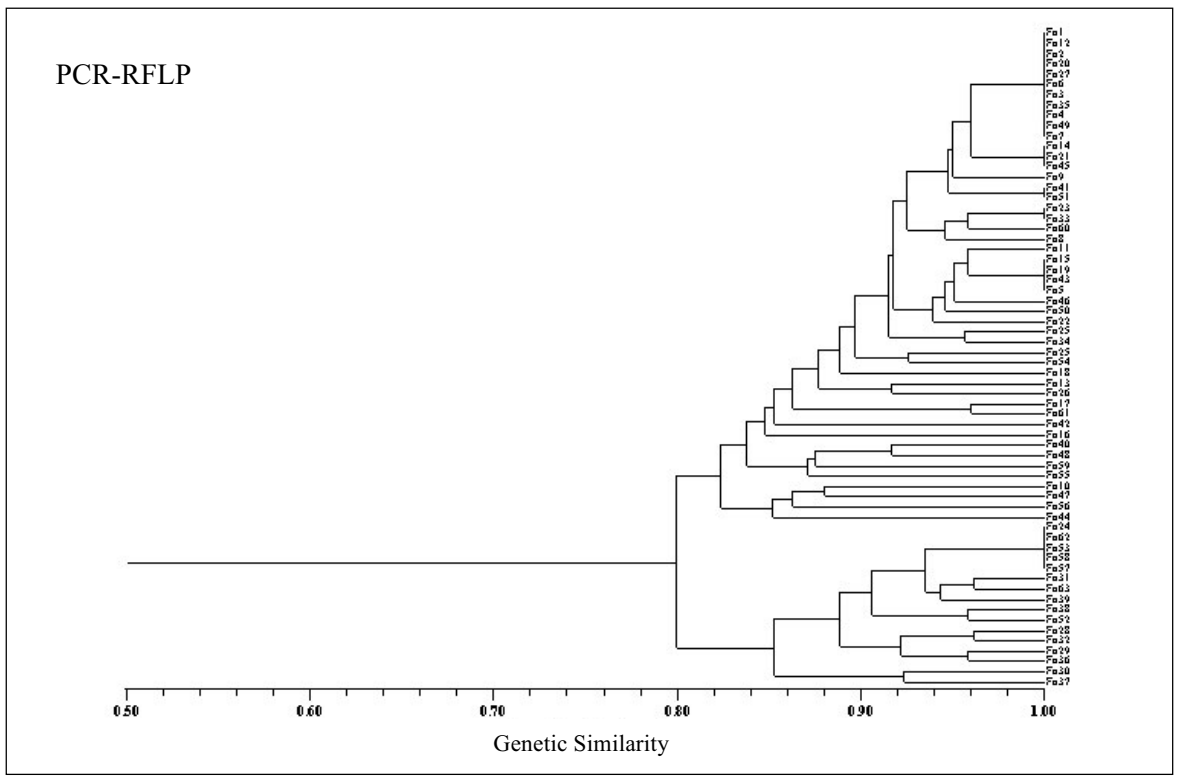
**Data processing.** A matrix was generated, in which each band was scored as a "1" if present and as "0" if absent. The estimate of genetic similarity (GS) among all the *F. oxysporum* isolates was calculated according to the definition of Nei and Li (1979). Mathematically, the formula Sij = 2a/(2a + b + c) was used, in which Sij is the similarity between the two individuals i and j; a is the number of bands present in both i and j; b is the number present in i and absent in j; and c is the number of bands present in j and absent in i. The matrix of similarity was analyzed by the unweighted pairgroup method using the arithmetic average (UPGMA), as indicated by Sneath and Sokal (1973). Computations were done using the procedures of NTSYS-pc statistical package 2.0 (Rohlf, 1998).

## Results

In this study, we assessed the suitability of RAPD, PCR-RFLP and AFLP techniques for rapid molecular characterization of *F. oxysporum* isolates. Various primers which were described in methods and materials were tested for efficiency in the three molecular marker methods.

**RAPD analysis.** Very low genetic variation among *F. oxysporum* isolates was detected using RAPD: a total of 65 bands ranging from 210 to 2000 bp were scored to an average of 13 bands per primer; 38.46% of these bands (25 in total) were polymorphic.



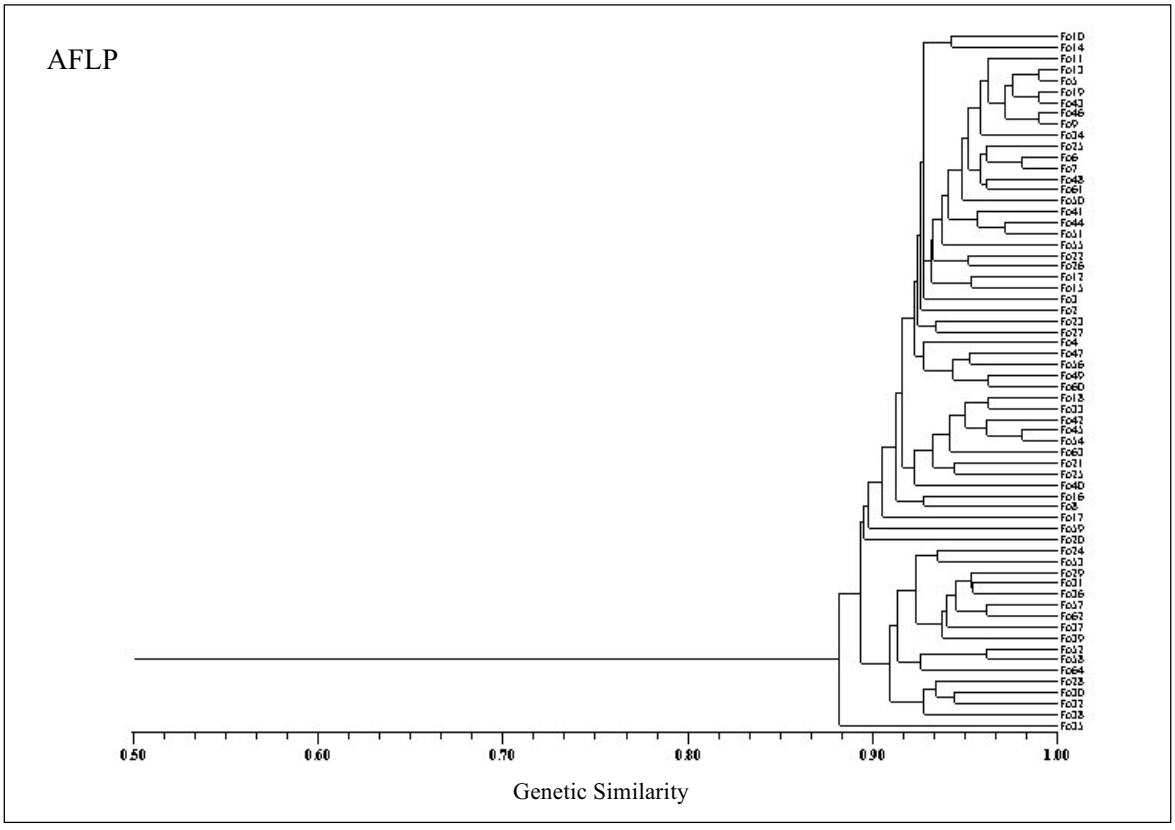



Fig. 1. Dendrograms obtained on sixty four *F. oxysporum* isolates with RAPD, PCR-RFLP and TE-AFLP. Dendrograms were obtained using the tree option on the NTSYS-PC software. The genetic similarity index (GS) with a maximum value of 1.0.

**PCR-RFLP analysis.** AluI showed a higher level of polymorphism than other restriction enzymes. The total number of scored bands was 18, of which six were polymorphic (33.3%). The average number of polymorphic bands per primer/enzyme combination used was 0.50 (Table III).

**AFLP analysis.** A total of 30 primer combinations were used in this study, four of which produced distinct fingerprints. A total of 120 AFLP fragments ranging from 100 to 550 bp were scored, corresponding to an average of 30 bands per primer combination, of which 41.66 % (50 in total) were polymorphic throughout the 64 isolates (Table III).

**Genetic similarity.** Based on the similarity values, UPMGA clustering was conducted for graphical display of the *F. oxysporum* isolates (Figure 1). Very similar clustering pictures with minor exception were obtained with the results of RAPD, PCR-RFLP and AFLP markers. Clustering analysis performed with the three types of markers separated the isolates into two main groups. Between the groups of isolates, similarities ranged from 87 to 100% for RAPD, 80 to 100% for PCR-RFLP and 88 to 97% for AFLP, respectively.

**Correlation with three marker types**. The correlation with three marker types was compared. The values of the Mantel test correlation showed a good fit of data using any of the marker types. The *r* correlation

Table III
Polymorphisms detected at each molecular marker methods

| Number of bands | PCR-based markers | | |
|---|---|---|---|
| | RAPD | AFLP | PCR-RFLP |
| Monomorphic | 65 | 120 | 12 |
| Polymorphic | 25 | 50 | 4 |
| Genetic similarity[a] | 87–100% | 88–95% | 70–86% |

[a] Genetic similarity between groups of isolates

value was 0.88 between PCR-RFLP and AFLP, 0.90 between RAPD and AFLP and 0.86 between RAPD and PCR-RFLP respectively. The results indicated that the genetic similarity index obtained with each marker type in all tested *F. oxysporum* isolates showed a good correlation, and thus the results were commensurate.

## Discussion

In the present study, we assessed the suitability of three different PCR-based methods for rapid molecular characterization of 64 *F. oxysporum* isolates collected from different cotton growing-areas in Egypt. The genetic similarity values obtained with all marker systems were comparable with minor exception. Random amplified polymorphic DNA (RAPD) analysis (Williams *et al.*, 1990) has been applied widely in detection and genetic characterization of phytopathogenic fungi (Miller, 1996; Brown, 1998) including race differentiation in several formae speciales of *F. oxysporum i.e.*, f. sp. *vasinfectum* (Assigbetse *et al.*, 1994) and *cubense* (Bentley *et al.*, 1994). Jimenez-Gasco *et al.* (1998) characterized nonpathogenic strains of *F. oxysporum* isolates by RAPD-PCR. In our study, analysis of the electrophoretic banding patterns of the defined RAPD groups showed that at least 25 polymorphic bands can differentiate nonpathogenic strains of *F. oxysporum*. Cluster analysis of the RAPD data showed a high genetic similarity among the group of isolates, a similar case reported by Jimenez-Gasco *et al.* (2001).

The internal transcribed spacers (ITS) of the rDNA can display variation within genera and are used in the differentiation of species. At the intra-specific level, variability in ITS sequences is generally very low or undetected. An analysis of the molecular variance based on IGS type relationships and frequency revealed that the genetic structure of the populations of *F. oxysporum* varied widely among the soils. Some populations were both highly diverse within the soils and differentiated between the soils. Analysis of the ITS regions of various *Fusaria* has shown that this method was able to discern the species. On the basis, *F. oxysporum* isolates were found as a divergent group, even when isolates could not be discriminated constantly by morphology (Batmen *et al.*, 1996; Edel *et al.*, 1996; O'Donnell, 1996; Waalwijk *et al.*, 1996). Few differences in ITS sequence were found within *F. oxysporum* (Carbone and Khon, 1993). The second method performed in our study was PCR-RFLP analysis of the amplified ITS region, which allowed us to categorize strains within the species *F. oxysporum*. PCR-RFLP of the IGS region of *F. oxysporum* obtained from cyclamen produced polymorphisms which could not separate pathogenic from nonpathogenic isolates (Woudt *et al.*, 1995). Appel and Gordon (1995, 1996) used RFLP and sequence analysis of the IGS region of rDNA to investigate relationships between pathogenic races of *F. oxysporum* f. sp. *melonis* and non-pathogenic *F. oxysporum* isolates. The IGS intraspecific variation was detected in the IGS regions of rDNA.

An analysis of the molecular variance based on IGS type relationships and frequency revealed that the genetic structure of the populations of *F. oxysporum* varied widely among the soils. Some populations were both highly diverse within the soils and differentiated between the soils. A possible relationship between the intrapopulation or interpopulation level of diversity and some external factors such as the soil type or the crop history was evaluated (Edel *et al.*, 2001).

According to Gordon and Okamoto (1992) differences between populations may correlate with adaptations to local conditions. Since along-distance dispersal of conidia is usually limited in soilborne fungi, geographical isolation followed by the genetic drift can be an important factor in the construction of nonpathogenic *F. oxysporum* populations.

We introduced AFLP markers for measuring the genetic similarity in *F. oxysporum* isolates in order to see if this technique was able to substitute RAPD and PCR-RFLP. The UPGMA analysis gave similar results with the three types of markers. The level of polymorphisms (polymorphic bands/total bands) detected with AFLP was substantially higher than that seen with PCR-RFLP (41 vs. 33) but similar to RAPD (41 vs. 38). Leissner *et al.* (1997) used the AFLP fingerprinting methodology to study 18 different *F. graminearum* strains, fifteen of which showed a high degree of similarity in banding patterns. These results confirm the usefulness of AFLP technology for fusaria fingerprinting. The efficiency of the AFLP method is much higher than that of other marker types. Also, Sivaramakrishnan *et al.* (2002) found that AFLP is more informative than RAPD to differentiate more number of the *F. oxysporum* f. sp. *ciceri* isolates with the known races with minimum of outliers. All methods yielded intra-specific polymorphisms, but different levels of discrimination were obtained. Good correlation was found between the groupings obtained by 3 methods. In conclusion, both techniques generated specific genomic patterns which could

differentiate closely related strains. Unique fingerprint profiles generated by the AFLP techniques can be exploited for strain identification.

**Acknowledgments**: This work was supported by a research grant from Institut für Phytopathologie, Christian Albrechts Universtät zu Kiel, Germany.

## Literature

Alabouvette C. 1990. Biological control of Fusarium wilt pathogens in suppressive soils, p. 27–43. In: D Hornby (ed.) Biological control of soil-borne plant pathogens. CAB International, Wallingford, England.

Alabouvette C. and A. Couteaudier. 1992. Biological control of Fusarium wilt with nonpathogenic *Fusaria*, p. 415–426. In: E.C. Tjamos, R.J. Cook and G.C. Papvizas (eds) Biological control of plant diseases. Plenum Press, New York.

Appel D.J. and T.R. Gordon. 1995. Interspecific variation within populations of *Fusarium oxysporum* based on RFLP analysis of the intergenic spacer region of the rDNA. *Exp. Mycol.* **19**: 120–128.

Appel D.J. and T.R. Gordon. 1996. Relationships among pathogenic and nonpathogenic isolates of *F. oxysporum* based on the partial sequence of intergenic spacer region of the ribosomal DNA. *Mol. Plant-Microbe Interact.* **9**: 125–138.

Assigbetse KB., D. Fernandez, M.P. Doubios and J.P. Geiger. 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. *Phytopathology* **84**: 622–626.

Batmen G.L., H. Kwasna and E. Ward. 1996. Relationship among *Fusarium* spp. estimated by comparing restriction fragment length polymorphisms in polymerase chain reaction-amplified nuclear rDNA. *Can. J. Microbiol.* **42**: 1230–1240.

Bentley S.K., G. Pegg and J.L. Dale. 1994. Optimization of RAPD-PCR fingerprinting to analyze genetic variation within populations of *Fusarium oxysporum* f. sp. *cubense*. *J. Phytopathol.* **142**: 64–78.

Brown J.K.M. 1996. The choice of molecular marker methods for population genetic studies of plant pathogens. *New Phytol.* **133:** 183–190.

Carbone I. and L.M. Khon. 1993. Ribosomal DNA sequence divergence within internal transcribed spacer 1 of the Sclerotiniaceae. *Mycologia* **85**: 415–427.

Correll J.C. 1991. The relationship between formallspecies, races, and vegetative compatibility groups in *Fusarium oxysporum*. *Phytopathology* **81**: 1061–1064.

Couteaudier Y. and C. Alabouvette. 1990. Quantitative comparison of *Fusarium oxysporum* competitiveness in relation to carbon utilization. *FEMS Micrbiol. Ecol.* **74**: 261–268.

Creste S., A. Tulmann-Neto and A. Figueira. 2001. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. *Plant Mol. Biol. Rep*. **19**: 1–8.

Edel V., C. Steinberg, I. Avelange, G. Laguerre and C. Alabouvette. 1995. Comparison of three molecular methods for the characterization of *Fusarium oxysporum* strains. *Phytopathology* **85**: 579–585.

Edel V., C. Steinberg and C. Alabouvette. 1996. Evolution restriction analysis of polymerase reaction chain (PCR)-amplified ribosomal DNA for identification of *Fusarium* species. *Mycol. Res.* **101**: 179–187.

Edel V., C. Steinberg, N. Gautheron, G. Recorbet and C. Alabouvette. 2001. Genetic diversity of *Fusarium oxysporum* populations isolated from different soils in France. *FEMS Microbiol. Ecol.* **36:** 61–71.

Eparvier A. and C. Alabouvette. 1994. Use of ELISA and GUS-transformed strains to study competition between pathogenic and nonpathogenic *Fusarium oxysporum* for rot colonization. *Bicontrol Sci. Technol.* **4**: 35–47.

Gerlach W. and H. Nirenberg. 1982. The genus *Fusarium*: a pictorial atlas. *Mitteil. Biolog. Bundesanst Land Forstwirtschaft Berlin Dahlem* **209**:1–406.

Gordon T.R. and D. Okamoto. 1992. Variation within and between populations of *Fusarium oxysporum* based on vegetative compatibility and mitochondrial DNA. *Can. J. Bot.* **70**: 1211–1217.

Jimenez-Gasco M.M., E. Perez-Aarts, J. Rodriguez and D. Jimenez-Diaz. 1998. Characterization of pathotypes and races of *Fusarium oxysporum* f. sp. *ciceris* and of nonpathogenic *F. oxysporum* infecting chickpea by RAPD-PCR. In: Abstr. 3rd European conference on grain legumes, 14–19 November 1998, Valladolid, Spain 96–97.

Jimenez-Gasco M.M., E. Perez-Aarts and D. Jimenez-Diaz. 2001. Identification of pathogenic races 0, 1B/C, 5, and 6 of *Fusarium oxysporum* f. sp. *ciceris* with random amplified polymorphic DNA (RAPD). *Eur. J. Plant Pathol.* **107:** 237–248.

Leissner C.E.W., M.L. Niessen and R.F. Vogel. 1997. Use of the AFLP technique for the identification and discrimination of *Fusarium graminearum*. *Cereal Res. Comm.* **25**: 555–556.

Miller S.A. 1996. Detecting propagules of plant pathogenic fungi. *Adv. Bot. Res.* **23**: 73–102.

Nei M. and W. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* **79**: 5269–5273.

Nelson P.E., T.A. Toussoun and W.F.O. Marasas. 1983. *Fusarium* species: an illustrated manual for identification. Pennsylvania State University Press, University Park.

O'Donnell K. 1996. Progress towards a phylogenetic classification of *Fusarium*. *Sydowia* **48**: 57–70.

Paavanen-Huhtala S., J. Hyvönen, S.A. Bulat and T. Yli-Mattila. 1999. RAPD-PCR, Isozyme rDNA RFLP and rDNA sequence analyses in identification of Finnish *Fusarium oxysporum* isolates. *Mycol. Res.* **103**: 625–634.

Rohlf F.J. 1998. NTSYS-pc. Numerical taxonomy and multivariate analysis system, Version 2.0. Applied Biostatistics, New York.

Schnieder F., G. Koch, C. Jung and J-A. Verreet. 1998. The application of molecular markers for genetic characterization of *Septoria tritici* populations. *J. Plant Dis. Prot.* **105**: 452–461.

Sivaramakrishnan S., S. Kannan and S.D. Singh. 2002. Genetic variability of *Fusarium* wilt pathogen isolates of chickpea (*Cicer arietinum* L.) assessed by molecular markers. *Mycopathologia* **155**: 171–178.

Sneath P.H.A. and R.R. Sokal. 1973. Numerical taxonomy. Freeman, San Francisco.

Vos P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**: 4407–4414.

Waalwijk C., J.R.A. De-Koning and W. Gams. 1996. Ribosomal DNA analyses challenge the status of *Fusarium* sections *Elegans, Liseola* and *Dlaminia* based on ribosomal ITS1 and ITS2 sequences. *Mycologia* **88**: 361–368.

White T.J., T. Bruns, S. Lee and J.W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. In: Innis MA., D.H. Gelfand J.J. Sninsky and T.J. White (eds), PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc., New York.

Williams J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531–6535.

Woudt L.P., A. Neuvel, A. Sikkema, M.Q.J.M. van grinsven, W.A.J. de Milliano, C.L. Campbell and J.F. Leslie. 1995. Genetic variation in *Fusarium oxysporum* from cyclamen. *Phytopathology* **85**: 1348–1355.