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Phylogenetic Analyses of *Verticillium Fungicola* Varieties Based on Molecular and Secondary Metabolite Profiles

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Abstract: The variety *flavidum* of *Verticillium fungicola* is receiving little research attention compared to the varieties *fungicola* and *aleophilum*. Molecular analyses based on ITS-RFLP (restriction digestion of the ITS fragment) and RAPD (random amplified polymorphic DNA) as well as secondary metabolite analysis were used to study phylogenetic variations among type strains of *Verticillium fungicola* (*V. f.* var. *fungicola*, one type strain; *V. f.* var. *aleophilum*, one type strain; *V. f.* var. *flavidum*, three type strains) the common causal agent of dry bubble disease of the cultivated mushroom *Agaricus bisporus*. The Molecular phylogenetic techniques confirmed the uniqueness of the type strain *V. f.* var. *flavidum* 195 which was delineated by the five used primers and was the only one showing a different restriction pattern using EcoR1. Tree diagrams based on secondary metabolite profiles and RAPD patterns showed that the investigated type strain of *V. f.* var. *aleophilum* is of closer relationship to the two *flavidum* type strains, *V. f.* var. *flavidum* 133 and 194, than to the investigated strain of *V. f.* var. *fungicola*. RAPD patterns revealed the heterogeneity among the three investigated *V. f.* var. *flavidum* type strains.

Key words: Secondary metabolite profile; RAPD; ITS-RFLP; Phylogenetic relationship; Genetic variation.

INTRODUCTION

The genus *Verticillium* has been reported to be heterogeneous and polyphyletic (Bidochka *et al.,* 1999; Pegg and Brady, 2002). Until recently, *Verticillium* is still a polyphyletic entity that requires resolution into more natural units (Zare and Gams, 2008).

Verticillium fungicola belongs to *Verticillium* sect. *Albo-erecta*. This section was introduced to accommodate *Verticillium* species with white to yellowish colonies, erect conidiophores, and mainly fungicolous habit (Gams and Zaayen,1982). *V. fungicola*, the common causal agent of dry bubble disease of the cultivated mushroom *Agaricus bisporus*, was redescribed by Gams (1971) and was defined more narrowly by Gams and van Zaayen (1982) according to optimum and maximum temperatures for growth.

Verticillium fungicola constitutes three varieties (*fungicola*, *aleophilum* and *flavidum*). The variety *flavidum* differs from the other two varieties mainly in its lower optimum temperature, presence of sclerotia, yellowish colonies, pungent odor, and nonpathogenic abilities on *Agaricus bisporus*. However, the only consistent difference between *V. fungicola* var. *aleophilum* and *V. fungicola* var. *fungicola* is the temperature maximum for optimal growth (Gams and Zaayen, 1982).

Recent research reported that isolates inside each variety (*Verticillium fungicola* var. *fungicola* and *V. f.* var. *aleophilum*) differ genetically and in pathogenicity (Larsen and Frisvad, 1995; Collopy *et al.,* 2001; Juarez *et al.,* 2002;). Owing to its nonpathogenic characters, very few research studies have been conducted on var. *flavidum* (Collopy *et al.,* 2001).

There are different opinions on the use of secondary metabolites in taxonomy. Some workers claim that they are strain-specific (Engel *et al.,* 1982; Vining, 1992), whereas others believe that the secondary metabolites present or produced in culture are very sensitive to environmental factors (Bu'Lock, 1980; Monaghan *et al.,* 1995) in contrast to molecular genetic data. These criticisms have been rejected in many major studies

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involving several strains of each taxon (Culberson and Culberson, 1994; Larsen and Frisvad, 1995; Smedsgaard and Frisvad, 1997). The problem of the influence of the environment on phonetic features, including micromorphology and secondary metabolites can be solved by careful attention to optimization and standardization of culture conditions and to work with "normkultur"(Apple and Wollenweber, 1910): one in which all the forms characteristic of a fungus are present and of good development" (Hawksworth *et al.,* 1995).

More and more secondary metabolites have been shown to have possible ecological functions, and probably all of them have functions (Gloer, 1995; Sterner, 1995). It should be noted that if all secondary metabolites have ecological functions, their significance in taxonomy must be very high (Frisvad *et al.,* 1998).

The development of molecular nucleic acid techniques has revolutionized fungal systematics. The analysis of random amplified polymorphic DNA (RAPD) (Williams *et al.,* 1990) has been used to resolve genetic variations between fungal strains (Edel, 1998). It has been reported that in fungi, most RAPD analyses concern intraspecific studies (Feisvad *et al.,* 1998). The RAPD method has been used successfully to differentiate isolates within fungal species belonging to different fungal genera such as *Aspergillus* (Bayman and Cotty, 1993)*, Colletotrichum* (Guthrie *et al.,* 1992), *Fusarium* (Assigbetse *et al.,* 1994), and *Rhizoctonia* (Duncan *et al.,* 1993), or arbuscular-mycorrhizal fungi (Wyss and Bonfante, 1993).

RAPDs have also been used successfully to look at genetic variation in a variety of populations including those of fungal pathogens (Bonnen and Hopkins, 1997). RAPD technique has been used to investigate genetic variability as well as homogeneity or heterogeneity among isolates of *Verticillium fungicola* varieties (Collopy *et al.,* 2001; Hawksworth, 2002; Juarez *et al.,* 2002; Largeteau *et al.,* 2006).

The ITS region is considered a powerful taxonomic indicator at the species level and variability in the ITS sequences is generally very low or undetected at the intraspecific level (Lee and Taylor, 1992; Carbone and Kohn, 1993; Frisvad *et al.,* 1998). The nuclear ITS2 region of five plant-pathogenic *Verticillium* species (*V. albo-atrum*, *V. dahliae*, *V. longisporum, V. nigrescens* and *V. tricorpus*) was sequenced and was able to discriminate among them (Fahleson *et al.,* 2004).

The goal of the current study was to determine the phylogenetic relationship among five type strains of *V. fungicola* varieties that cause dry bubble disease of the cultivated mushroom *Agaricus bisporus* on the basis of ITS-RFLP and RAPD patterns as well as their secondary metabolite profiles.

MATERIALS AND METHODS

This work was carried out at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt.

- Verticillium Fungicola Type Strains:

The five type strains investigated were *V. fungi*cola var. *fungicola* MTB 7046*, V. fungicola* var. *aleophilum* CBS 357.80, *V. fungicola* var. *flavidum* MTB 7038/1 (designated 195), *V. fungicola* var. *flavidum* MTB 5936 (designated 194), *V. fungicola* var. *flavidum* CBS 342.80 (designated 133). They were kindly provided by Dr. Gert Bachmann, University of Vienna, Vienna, Austria to The Regional Center for Mycology and Biotechnology.

-DNA - based techniques:

a- Fungal DNA Extraction Using Qiagen kit:

The mycelial growth from 5–7 day old cultures on Malt Extract Agar (MEA) slopes were scraped by using 2 ml of sterile distilled water. The two ml of spore suspension were used to inoculate a 100 ml of Yeast Extract Sucrose (YES) medium in a universal 250ml flask and incubated with gentle shaking (180 rpm at 22˚C for 48hrs). The mycelia from the flasks were harvested by filtration under aseptic conditions using a microcloth, washed with sterile distilled water and stored at -20 overnight in a sterile Petri dishes. The mycelia were lyophilized in a Heto lyophilizer system model Maxi Dry. The freeze-dried mycelia were ground in a mortar using a sterile pestle, and the powdery samples were placed in eppendorf tubes (1.5 ml). DNA extraction was conducted using DNeasy kit (Qiagen-Germany).

b- RAPD-PCR:

Ready to go PCR beads kit (purchased from Amershambioscience) was used to amplify DNA genomic fragments using a thermal cycler machine (gradient Robocycler 96 Stratagene, USA) by combining the lyophilized bead, 25 pmole of each primers, 100 ng DNA as a template in 25ml of total reaction volume. The mixture was then placed to the thermal cycler machine directly to start the appropriate PCR program including

a universal denaturation cycle (5 min at 94°C), 45 cycles of annealing/extension reactions (1 min at 94°C, 1 min at an optimum annealing temperature 36°C for each used universal primer and 2 min at 72°C) and cycle of final extension step (5 min at 72°C) was followed by soaking at 4°C.

The primers used in this study were supplied with the Ready to go kit and are of the following sequences: Primer1: 5'-d {GGTGCGGGAA}; Primer3: 5'-d {GTAGACCCGT}; Primer4: 5'-d {GTAGACCCGT}; Primer5: 5'-d {AACGCGCAAC}; Primer6: 5'-d {CCCGTCAGCA}.

c- ITS-PCR:

Ready to go PCR beads kit (purchased from Amershambioscience) was used to amplify ITS fragment using a thermal cycler machine (gradient Robocycler 96 Stratagene, USA) by combining the lyophilized bead, 50 pmole of the primer, 100 ng DNA as a template in 50ml of total reaction volume. The mixture was then placed to the thermal cycler machine directly to start the appropriate PCR program including a universal denaturation cycle (5 min at 94°C), 30 cycles of annealing/extension reactions (30 sec at 94°C, 1 min at an optimum annealing temperature 56°C for the primer {the forward sequence of the primer: 5'-TCCTCCGCTTATTGATATGC-3', while the sequence of the reverse primer: 5'-GGAAGGAGAAGTAACAAGG-3'} and 1 min at 72°C) and cycle of final extension step (5 min at 72°C) was followed by soaking at 4°C.

d- Restriction Digestion of the ITS Fragment (ITS-RFLP):

ITS fragments were purified by phenol-chloroform extraction and ethanol precipitation. The purified products were digested with each of six restriction endonucleases; ApaII, BamHI, HindIII, XhoI, EcoRI and XbaI. The digestion products were analyzed by electrophoresis on agarose gel as described below.

e- Agarose Gel Electrophoresis:

The desired amount of agarose 2% was added to 100 ml (1X) of electrophoresis buffer (10X TBE; Tris-Borate EDTA; tris-base, 108g/l; boric acid, 55g/l; 40 ml of 0.5M EDTA (pH8)). The gel was boiled and ethidium bromide solution (10 mg/ml) was added at 55°C, then poured into sealed gel tray and the appropriate comb was inserted.

All the molecular biology techniques were carried out according to Sambrook *et al.,* (1989).

f- Secondary Metabolite Analysis:

I- Fungal Strains Cultivation:

The 7-day old fungal mycelia were harvested by filtration from YES broth through microcloth under aseptic conditions. The mycelia were washed several times with sterilized distilled water while the excess water was removed by plotting on dry filter papers. Ten g of fungal mycelia (wet weight) were placed in sterile Petri-dishes, stored at -20°C overnight and then freeze-dried. The freeze-dried mycelia were disrupted using high-speed homogenizer and grounded with an approximately equal weight of clean cold sand using an appropriate amount of chloroform: methanol (2:1) in a sterile cold mortar. The homogenate was filtered through Whatman No.1 filter paper and then vigorously shaken after adding an equal volume of chloroform: methanol (2:1) in an appropriate separating funnel, while the residue was re-extracted twice for complete extraction. The chloroform: methanol (2:1) extract was concentrated in a rotary evaporator till dryness then dispensed to a clean dry 1.5ml Eppendorph tubes and centrifuged at 3000 rpm for 10 min. The supernatants were reconstituted in 1 ml of methanol prior to chromatographic analysis.

II- Instrumentation and Chromatographic Conditions:

Loading, development and analysis of secondary metabolites of the investigated extracts were carried out using fully automated HPTLC system (CAMAG-Switzerland).

According to Paterson and Bridge (1994), griseofulvin was used as the reference standard dissolved in chloroform: methanol (2:1, v/v) and 15 ml of each extract were loaded using the automatic applicator unit (LINOMAT 5, CAMAG) onto the TLC G F 60 plate (Merk). The TLC plate was developed in chloroform: acetone: isopropanol (85:15:20, (v/v/v); CAP) using the automatic developing chamber (ADC2, CAMAG). The plate was scanned under white light and long UV light (365 nm) using automatic scanner (TLC scanner 3, CAMAG) and all the spots were recorded. The plate was sprayed with 1% ceric sulfate in 3 M sulfuric acid and scanned as previously. Then, it was heated for 8 min at 110 °C and scanned as previously again. The identity of the metabolites was then achieved by comparing colors and R_f values (Paterson and Bridge, 1994).

g -Statistical Analyses:

The role of PCR-RAPD patterns and secondary metabolite profiles in studying phylogenetic relationships of the investigated *Verticillium fungicola* type strains were evaluated by using statistical cluster analysis with joining (tree clustering) being the clustering method. The DNA profile was amalgamated by unweighted pair-group average method analysis (UPGAMA). UV visualization, imaging and cluster analysis of the DNA bands were carried out using Quantity one 4.0.3 software of the gel doc.2000 system (Bio-Rad). However, complete linkage was the method for studying secondary metabolite profiles using Statistica for windows, release 4.5f, state Soft, Inc.1993 software. Euclidean distances (similarity matrix) were used as the distance metric in both as well as dice coefficient as the calculation method.

RESULTS AND DISCUSSION

Secondary metabolite profiles of the investigated *V. fungicola* type strains are illustrated in fig. (1). Results are tabulated in table (1) which shows the detection of ten metabolites in the cell free extract of *V. f.* var. *aleophilum* (citrinin, carlosic acid, xanthocillin, psoromic acid, 2-pyrovoylaminobenzamide, griseofulvin, chaetoglobosin C, cytocelacin C, pentrim A and patulin). However, only seven metabolites were detected in case of *V. f.* var. *fungicola* (citrinin, carlosic acid, 2-pyrovoylaminobenzamide, chaetoglobosinC, cytocelacinC, genitisic and mycophenolic acid). The three investigated *V. f.* var. *flavidum* type strains possessed the same secondary metabolite pattern; citrinin, carlosic acid, xanthocillin, psoromic acid, 2-pyrovoylaminobenzamide, griseofulvin, chaetoglobosinC, cytocelacinC, pentrimA, genitisic and dehydrocarolic acid, with the last being specific for only the three *flavidum* type strains and were not detected in the other two strains. Additionally, patulin was only detected in *V. f.* var. *fungicola* while mycophenolic acid was only detected in *V. f.* var. *aleophilum*.

The phylogenetic relationship among the investigated type strains based on their secondary metabolite profiles was illustrated through a tree diagram (Fig. 2). For possessing the same profile, the three *V. f.* var. *flavidum* type strains were linked by a linkage distance of zero indicating they are so close as being treated as one (clade one). *V. f.* var. *aleophilum*, representing clade 2, was linked to clade one by an Euclidean distance of 2. Clade three (*V. f.* var. *fungicola*) was linked to clade two by a distance of 3.3.

ITS-RFLP using six enzymes and RAPD using five primers were performed. The six investigated restriction enzymes (Xho, Xba, BamH1, EcoR1, Hind III and ApaII) failed to cut the ITS region of the five type strains with the exception of EcoR1 which discriminated *V. f.* var. *flavidum*195 from the other strains by the development of digestion profile by EcoRI which was different for the remaining profile of the other strains (Fig. 3).

Banding patterns of RAPD- PCR of the five *V. fungicola* type strains using five primers are illustrated in plate (1). Phylogenetic relationship of the investigated type strains based on their RAPD patterns was represented through dendrograms illustrated in plate (2). The highest DNA polymorphism was through primer 1 where 0.35 represented the maximum Euclidean distance reached through primer 1, hence primer 1 grouped *V. f.* var. *flavidum* 194 and *V. f.* var. *aleophilum* in one clade but are still fairly related to each other for being joined by a distance of 0.35 (Plate 1a).

The closest relationship was through primer 4, which grouped *V. f.* var. *flavidum* 133 and *V. f.* var. *flavidum* 194 in one clade and joined them by a linkage distance of 0.64 (Plate 1c). This relationship was confirmed by the rest of the investigated primers (primer 3 at a distance of 0.63, primer 6 at a distance of 0.6 and primer 5 at a distance of 0.52) constituting clade 1 in all of them (Plate 1b, 1e and 1d respectively). The second close relationship was between clade 1 (*V. f.* var. *flavidum* 133 and *V. f.* var. *flavidum* 194) and clade 2 (*V. f.* var. *aleophilum*) through primer 6 which joined them by a distance of 0.55 (Plate 1e). Also, *V. f.* var. *aleophilum* constituted clade 2 in case of primer 3 (plate 1b), primer 4 (plate 1c) and primer 5 (plate 1d).
V. f. var. *fungicola* was distantly related to the investigated strains except to *V. f.* var. *aleophilum* (clade 2) through primer 6 (plate 1e) being linked to it by a distance of 0.54. Despite being a *flavidum* variety, *V. f.* var. *flavidum* 195 represented the most distant type strain where all the investigated primers confirmed this result (Plate 1).

Discussion:

Verticillium fungicola (Pruess) Hassebrauk is the causal agent of dry bubble disease of the cultivated button mushroom *Agaricus bisporus* (Lange) Imbach. Taxonomic revisions have divided it into three different varieties (*fungicola*, *aleophilum* and *flavidum*) based on morphological taxonomy and pathogenicity studies (Collopy *et al.,* 2001).

Table 1: Intracellular secondary metabolites of the five *Verticillium fungicola* type strains.

Type strains 2ry Metabolites	*V. fungicola* var. *aleophilum*	*V. fungicola* var.*flavidum 133*	*V. fungicola* var. *flavidum 194*	*V. fungicola* var. *flavidum 195*	*V. fungicola* var. *fungicola*
Citrinin	+	+	+	+	+
Carlosic acid	+	+	+	+	+
Xanthocillin	+	+	+	+	
Psoromic acid	+	+	+	+	
2-pyrovoylamino-benzamide	+	+	+	+	+
Grisofulvin	+	+	+	+	
Chaetoglobosin C	+	+	+	+	+
Cytocelacin C	+	+	+	+	+
Pentrim A	+	+	+	+	
Patulin	+				
Genitisic		+	+	+	+
Mycophenolic acid					+
Dehydrocarolic acid		+	+	+	

Fig. 1: TLC plate for the five *Verticillium fungicola* type strains. 1, reference standard; 2, *V. fungicola* var. *aleophilum*; *3*, *V. fungicola* var. *flavidum* 133; 4, *V. fungicola* var. *flavidum* 194; 5, *V. fungicola* var. *flavidum* 195 and 6, *V. fungicola* var. *fungicola Verticillium* extracts.

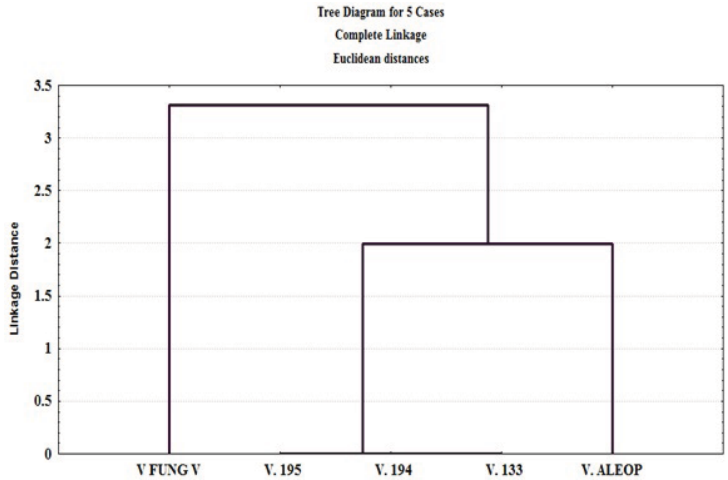


Fig. 2: Tree diagram of the five *V. fungicola* type strains based on their intracellular secondary metabolite production.

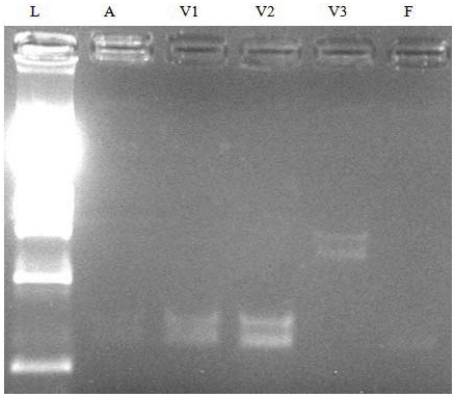
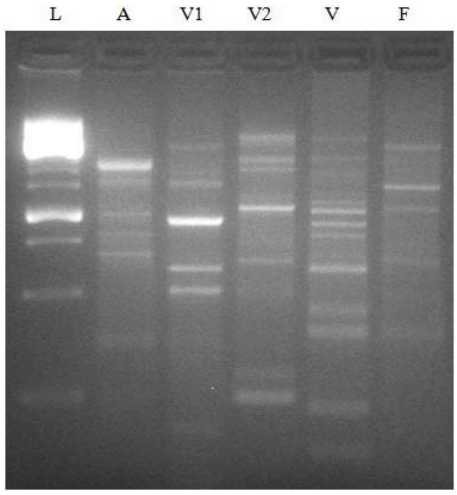
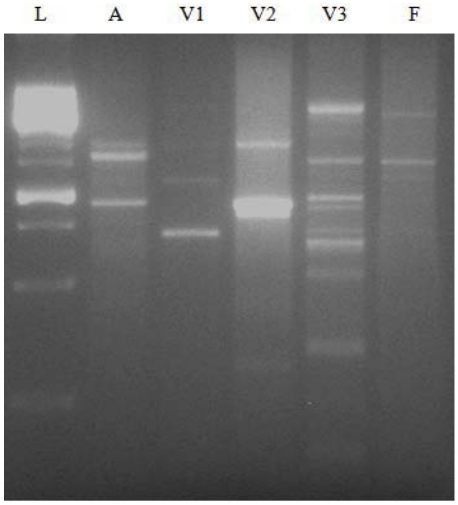


Fig. 3: ITS-RFLP of the five investigated type strains using EcoR1. L, 250 bp ladder; A, *V. f.* var. *aleophilum*; V1, *V. f.* var. *flavidum* 133; V2, *V. f.* var. *flavidum* 194; V3, *V. f.* var. *flavidum* 195; F, *V. f.* var. *fungicola*.

(A)



(B)



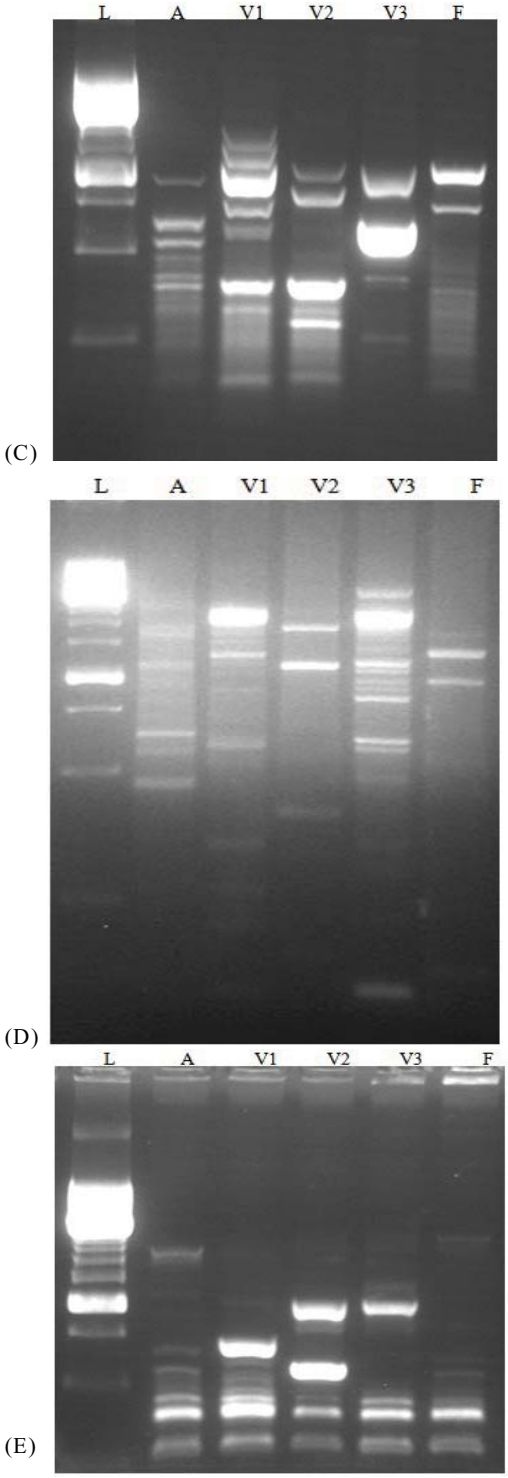


Plate 1: RAPD patterns of *V. fungicola strains* using primers 1(a), 3(b), 4(c), 5(d), 6(e). L, 250 bp ladder; A, *V. fungicola* var. *aleophilum*; V1, *V. fungicola* var. *flavidum* 133; V2, *V. fungicola* var. *flavidum* 194; V3, *V. fungicola* var. *flavidum* 195; F, *V. fungicola* var. *fungicola*.

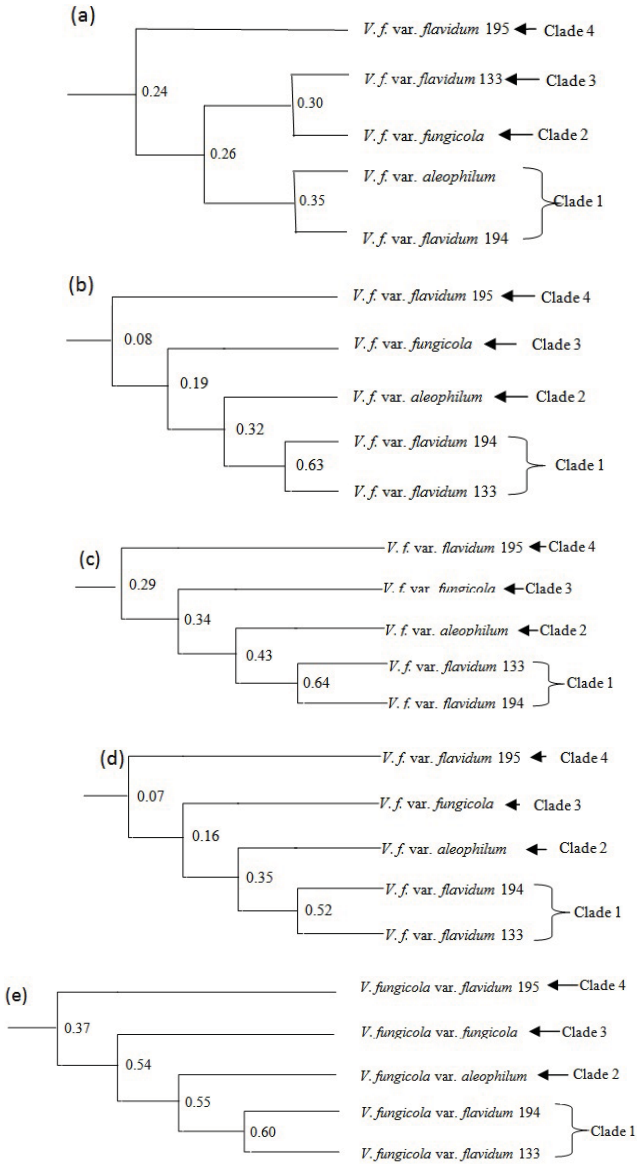


Plate 2: Dendrograms of *Verticillium fungicola* type strains based on the similarity matrix of RAPD analysis using primer 1 (a), 3 (b), 4 (c), 5 (d) and 6 (e).

The worldwide distribution of the pathogen incites questions regarding its variability. A few studies have been carried out on the *V. fungicola* species complex and all of them pointed out to genetic variability among its strains while were different in settling the homogeneity or heterogeneity among isolates of each strain. It has been reported that *V. f.* var. *fungicola* isolates were homogeneous (Collopy *et al.,* 2001). Another study reported that isolates of the var. *aleophilum* were more homogeneous, whereas var. *fungicola* is more heterogeneous through studying their RAPD fingerprinting (Largeteau *et al.,* 2006).

In the current study and taking the advantage of using type strains, genetic variability and phylogenetic relationship of representatives of the three different *V. fungicola* varieties (*V. f.* var. *fungicola*, one type strain; *V. f.* var. *aleophilum*, one type strain; *V. f.* var. *flavidum*, three type strain) were studied on the basis of their RAPD patterns (using five primers), ITS-RFLP patterns (using six restriction enzymes) and secondary metabolite profiles.

The role and applications of secondary metabolites in the taxonomy of filamentous fungi have been reviewed where conclusively it has been reported that they have been valuable, occasionally even indispensable, in classifications that are expected to be unequivocal and stable (Frisvad *et al.,* 1998). According to the available research, the current work is considered the first in studying the use of secondary metabolite profile (SMP) in *Verticillium* taxonomy.

Tree diagram of the five investigated type strains based on their SMP gathered the three *V. f.* var. *flavidum* type strains in one clade for possessing the same profile. However, more discrimination was achieved by RAPD technique where polymorphism was observed among the three *flavidum* type strains with the strain 195 possessing a unique RAPD pattern making it of the highest polymorphism and thus the most distantly related strain acquiring clade 4 in case of the five investigated primers. The uniqueness of the DNA of strain 195 was confirmed by ITS-RFLP where it was the only strain possessing a single restriction site for EcoR1.
RAPD patterns of the other two *flavidum* strains (133 and 194) gathered them in one clade (clade 1) in case of all primers (except for primer 1).

Both RAPD patterns and SMFs related *V. f.* var. *aleophilum* to *V. f.* var. *flavidum* 133 and 194 with a linkage distance more than that to *V. f.* var. *fungicola.*

It has been reported that the only consistent difference between *V. fungicola* var. *aleophilum* and *V. fungicola* var. *fungicola* is the temperature maximum for optimal growth van (Zaayen and Gams, 1982). The same study reported that *V. f.* var. *flavidum* is non-pathogenic to *Agaricus* species van (Zaayen and Gams, 1982). However, the pathogenicity of *V. f.* var. *fungicola* and var. *aleophilum* to *Agaricus* species causing dry bubble disease has been reported by many authors (Largeteau *et al.,* 2006). Additionally, the analyses of ex-type strain *V. fungicola* var. *flavidum* suggested that this fungal isolate should not be considered a variety of *V. fungicola*, but rather a distinct species and should no more be part of the *V. fungicola* species complex (Collopy *et al.,* 2001).

The previous reports reveal that *V. f.* var. *fungicola* and var. *aleophilum* are closer to each other more than to var. *flavidum*, however the current results (SMP and RAPD patterns) showed the opposite where *V. f.* var. *aleophilum* was more related to *V. f.* var. *flavidum* than to *V. f.* var. *fungicola*. This could be attributed to the heterogeneity of isolates of *V. f.* var. *fungicola* which has been confirmed by many authors (Largeteau *et al.,* 2006; Zare and Gams, 2008). Additionally, it has been reported that RAPD analysis and physiological tests on *V. f.* var. *aleophilum* and var. *fungicola*, showed that the former variety have the greater pathogenic potential, but the latter was genotypically more variable (Hawksworth, 2002). Also, the var. *fungicola* isolates were placed into a distinct cluster, remote from the var. *aleophilum* isolates and also reported that isolates of the var. *aleophilum* were more homogeneous in that study, whereas var. *fungicola* is more heterogeneous (Largeteau *et al.,* 2006).

The current work also highlights the result that the isolates of the var. *flavidum* were heterogeneous as reflected from the polymorphism of the RAPD fragments and the fact that despite the grouping of 133 and 194 in one clade by four of the five investigated primers, the shortest distance joining them was 0.64 reflecting the existence of polymorphism. In addition to the distantly related strain *flavidum* 195.

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