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Morphological study and Caracterisation of *Fusarium oxysporum f.sp.albedinis*. by Isozymes systems

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Abstract— The characteristics of *Fusarium oxysporium f.sp. albedinis* (F.o.a) isolates were investigated using electrophoretic studies of isozymes systems (esterase and phosphatase). The morphological characteristics of the isolates were very variable to each other. The isoenzymes profiles revealed polymorphic bands or the data were subjected to analysis with the JMP method. The isolates were grouped into 2 main groups A and B, those last were divided into sub-groups. Nineteen (19) isolates creates the group A and four isolates (E1, E2, E3 and M15A) formed the group B. analysis of isozyme banding patterns were found to be a reliable marker technology, efficient and effective tools to find the genetic variability among isolates isolated in different geographical areas.

Keywords— Electrophoresis, *Fusarium oxysporium f.sp. albedinis*, genetic diversity, isozyme analysis.

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1 Introduction

The *Fusarium oxysporum f. sp. albedinis* (F.o.a) is a vascular pathogen that causes a vascular wilt of date palm (Phoenix dactylifera L.) Or Bayoud, it represents the most serious problem for the cultivation of the date palm, particularly in North Africa, which leads to the deterioration of the crop and the lack of quality and this will reflect negatively on the economic and social life of the population of the region [1], [2]. In order to have a better understanding of genetic diversity at Fusarium sp., we felt it necessary to explore other characterization tools that are the morphological study and pathogenicity test. In addition, we chose to study the isoenzyme polymorphism of two systems (esterase and acid phosphatase). Enzymes, encoded by different alleles, often have electrophoretic mobility in polyacrylamide gels. This is due to variations in the amino acid composition of the molecules, which depend on the nucleotide sequence of the DNA [3], [4]. The isoenzyme electrophoresis is used to detect and identify a particular fungus, and the discrepancy in the isoenzymtiques profiles is used to solve problems located at the species [3].
The aim of this work is the study of the pathogenicity and isozyme polymorphism to determine variability among various isolates of F. oxysporium f.sp albedinis collected from different regions of Southern Algeria.

2 MATERIALS AND METHODS

A total of 23 isolates of Fusarium collected from three different regions (Adrar, Ghardaïa and Bechar) of the Southern Algeria were used in this study. Isolation of the pathogen was performed from the date palm rachis presenting the symptoms of bayoud and also from the rhizosphere around infected palms.

2.1 Morphological study

The study of the macroscopic characters of the isolates is based on the morphological description and on the pigmentation of the colony. The isolates were grown on potatos sucros agar (PSA) medium at 25 ± 2 ° C for 7 days °. The microscopic study is carried out from the margin of older colony of seven days on the PSA medium. For best microscopic identification of the isolates, the culture on the blade was used to maintain the shape and mycelium structure.

2.2 Preparation of the sample extract

The isolates of Fusarium were grown on Glucose Yeast Peptone (GYP) medium liquid at 25 ± 2° C in 250 ml Erlenmeyer flasks on a shaker at 100 rpm for 10 days. Mycelia were recovered by filtration through gauze and washed with sterile distilled water, dried with paper towels. Then it is ground into fine paste using phosphate buffer (100 mM - pH 7.1), in a cold mortar and pestle kept in ice, in the presence of sterile sand. The mixture was transferred to centrifuge tubes and centrifuged at 10 000 g for 20 minutes at 4 ° C. The supernatant is rapidly distributed into Eppendorf tubes for each 100 µl and stored at -20 ° C until use.

2.3 Electrophoresis

A total of 23 isolates were then selected to form a collection for electrophoresis on polyacrylamide gel on the basis of the geographical origin.
The electrophoretic profile of the isoenzymes is representative of the structure of the genome of an organism may be used to differentiate between fungal species [5] or subspecies level [6]. The isozyme diversity is related to the expression of genes

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encoding these enzymes are more or less independent of the environment; this approach provides a relatively neutral means for determining genetic variation [7]. Polyacrylamide gel electrophoresis was used for the separation of proteins and isozymes (estérase) band to identify some isolates of Fusarium [8]. The electrophoresis in polyacrylamide gel to separate proteins has been widely used for identification and classification of strains and species [6].

Proteins were separated on polyacrylamide gels according to the method of Laemmli [9].Using dual vertical slab gel electrophoresis apparatus. The dimensions of the gels were 17.5 x 15 cm and 1.0 mm thick. The gel consisted of 10% for separating gel and 5% for stacking gel. The migration of protein is made in a cold room at 4 ° C under a 75 mA amperage and voltage of 180 V. We performed two isozymes systems: Esterase (EST) and acid phosphatase (CAP) (Table I).

2.3.1 Data Analysis

For the collection of isolates converged into one group, we need to collect all the data of each isoenzyme profile in a binary matrix where the number 1 indicates the existence of band and 0 means the absence of the latter [7]. For each enzyme system, All isoenzymes positions of all isolates is listed; these positions are numbered from 1 to n where position 1 corresponding to the band whose migration is the fastest, and n, the lowest. All the data provided by the two enzymatic systems is the "phenotypic profile" characteristic of each isolate.

TABLE I
The staining solution of isozymes

Enzyme	Ingredients	Quantity
Esterase	Fast bleu RR	100 mg
(EC.3.1.1.1)	100 Mm Na-phosphate buffer Ph 7.1	100ml
EST	α - Naphtyl Acetate 2% (Acetone)	1.5 ml
Acidphosphatase	B- Naphtyl acide phosphate	50mg
(EC.3.1.3.2)	Fast Garnet GBC	50 mg
PAC	0.2 M Acetate buffer pH = 5	100 ml

Mm = millimolar; M = molar; mg = milligram; ml = milli Liter

Strains with the same phenotype belong to the same zymodeme [10]. The dendrogram based on phenotypic estimated similarity coefficients were built JMP software.

3 RESULTS

3.1 Morphological study

The study of the macroscopic and microscopic aspect of isolates of Fusarium oxysporum f.sp albedinis, it was based on the aspect and color of the mycelium, the macroscopic observation of isolates showing morphological variability about the appearance of colony in Petri dish. Different morphological aspects of our isolates were observed. These types are the cottony mycelium majority, with 43%, the mucous ras type that represents 26%, the downy type that has 17% other isolates have a mucous mycelium having 13% of the collection. Different morphological aspects of the color of the mycelium of our isolates were observed. This is the White (39%), Whitish (21%), Salmon (21%), and Red (4%) [(Fig. 1) ; (Table II)], and fast growing for most isolates (3.5 to 6.4 cm after 5 days). The isolates were also macrocondia which are few, pediforme at the sharp end, they are curved and septate, usually 2 to 3 septates and rarely 5 (Fig. 1). Microconidia were abundant, generally single celled, globular shapes, elongated, produced only in false heads on short monophialides ported perpendicular to the filaments, finally the chlamydospores one terminal or intermediate position (Fig. 2).

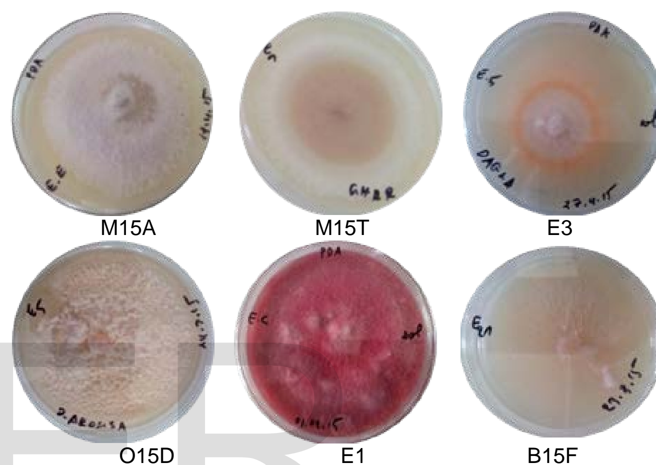

Fig. 1 Different morphological aspects and the color of mycelium of *Fusarium sp.* isolates cultured on culture medium PSA.

E3: Colony cottony and Mycelium Orange; M15T: Colony downy and Mycelium Salmon; M15A: Colony downy and Mycelium Whitish; B15F: Colony Mucous ras and Mycelium Salmon pink; E1: Colony cottony and Mycelium Dark pink and O15D: Colony cottony and Mycelium Whitish.

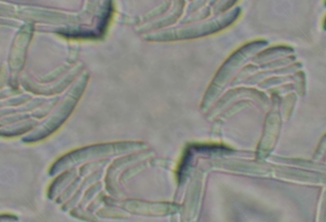

Fig. 2 Different forms of spores of *F. sp*:

A: Microconodia; B: Chlamydospore; C and D: Macroconidia; D: Macro and microconidia of Fusarium sp. isolated from soil.

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TABLE II
Description of twenty three isolates of *Fusarium.sp*.

Designation	Years	Cultivars	Localities	Regions	Morphotype	Color
M15A1	2015	Adala	Metlili	Ghardaïa	cottony	White
M15G	2015	Ghares	Metlili	Ghardaïa	cottony	White
M15D1	2015	Dagla	Metlili	Ghardaïa	cottony	White
E2	2015	Rhizosphère	Metlili	Ghardaïa	cottony	White
B15H	2015	Hmira	Beni Abbes	Bechar	downy	White
M15H	2015	Hmira	Mansor Bouda	Touat	Mucous	Salmon
T15H1	2015	Hmira	Tillilène	Touat	Mucous ras	Salmon pink
A15T	2015	Tegaza	Adgha	Touat	downy	White
E3	2015	Rhizosphère	Metlili	Ghardaïa	cottony	Orange
T15H	2015	Hmira	Timmi	Touat	Mucous ras	White
M15T	2015	Timliha	Mansor Bouda	Touat	downy	Salmon
M15T1	2015	Tazerzait	Metlili	Ghardaïa	Mucous	Salmon
B15F	2015	Feggous	Beni Abbes	Bechar	Mucous ras	Salmon pink
T15D	2015	Dagla	Tillilène	Touat	Mucous ras	Salmon
M15D	2015	Dagla	Metlili	Ghardaïa	cottony	Whitish
O15T	2015	Tilimsou	Ouled Aaroussa	Touat	Mucous ras	Salmon
I08G	2008	Ghars	INPV	Ghardaïa	Mucous ras	Whitish
M15A2	2015	Adala	Metlili	Ghardaïa	cottony	Whitish
E1	2015	Rhizosphère	Mansor Bouda	Touat	cottony	Dark pink
M15A	2015	Aghamou	Mansor Bouda	Touat	downy	Whitish
O15H	2015	Hmira	Ouled Ali	Touat	Mucous	White
O15D	2015	Dagla	Ouled Aaroussa	Touat	cottony	Whitish
M15D2	2015	Dagla	Metlili	Ghardaïa	cottony	White

3.2 Electrophoresis

3.2.1 Esterase

After migration, electrophoresis shows the revelation of ten bands named B1 to B10 light pink or dark pink color appear on the gel, the esterase reacting with beta naphthyl acetate produces dark pink band and the reaction of alpha naphthyl acetate produces a clear band (Fig. 3(A)). A representative diagram of this result is elaborated to facilitate the processing of data (Fig. 3(B)).The Band migration is distributed almost on all different levels. The band went back migration on almost all levels. They show the relative mobility between 0.06 and 0.93. The heterogeneity of esterase zymogram gives a fairly good representation of the genetic complexity [11].

3.2.2 Acid phosphatases

Acid phosphatases (ACP, EC 3.1.3.2) are enzymes that catalysing the cleavage of phosphoric monoester bonds in organophosphorus compounds. ACPs are important for the absorption of phosphate in microorganisms and plants [12] .Many Fusarium species studied has been found to produce at least one or two ACPs: two ACPs in F. moniliforme [13].In our isolates, the zymogram revealed a polymorphism (Fig. 4(A)) and the relative mobility values ranged from 0.02 to 0.96. Detected bands are well separated and exhibits polymorphism a very wearing for phosphatase acid to the population concerned. The total migration of twelve active polymorphic isozyme systems could serve as an indicator of genetic variability, identification and characterization of the pathogen [10]. The electrophoretic profile of acid phosphatase is represented by a diagram for a best interpretation of this result (Fig. 4(B)).

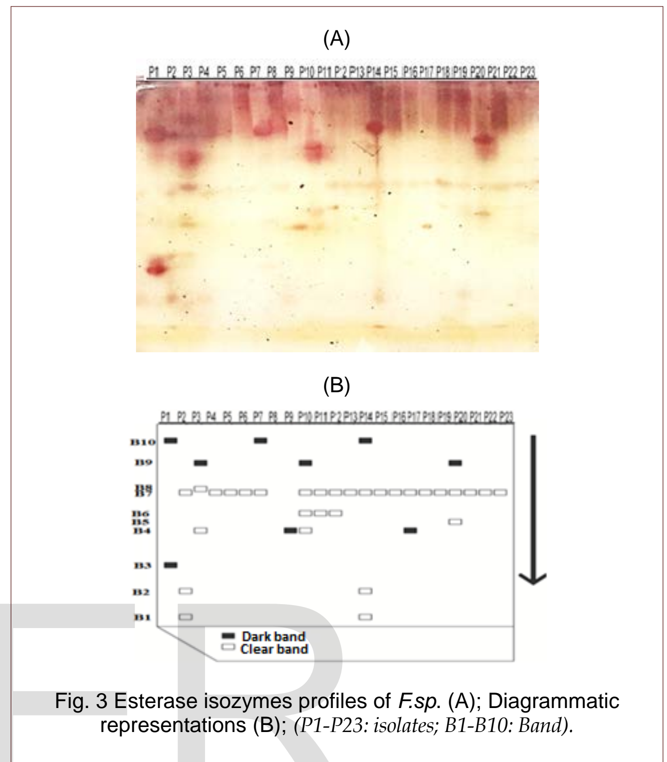

Fig. 3 Esterase isozymes profiles of *F.sp.* (A); Diagrammatic representations (B); *(P1-P23: isolates; B1-B10: Band).*

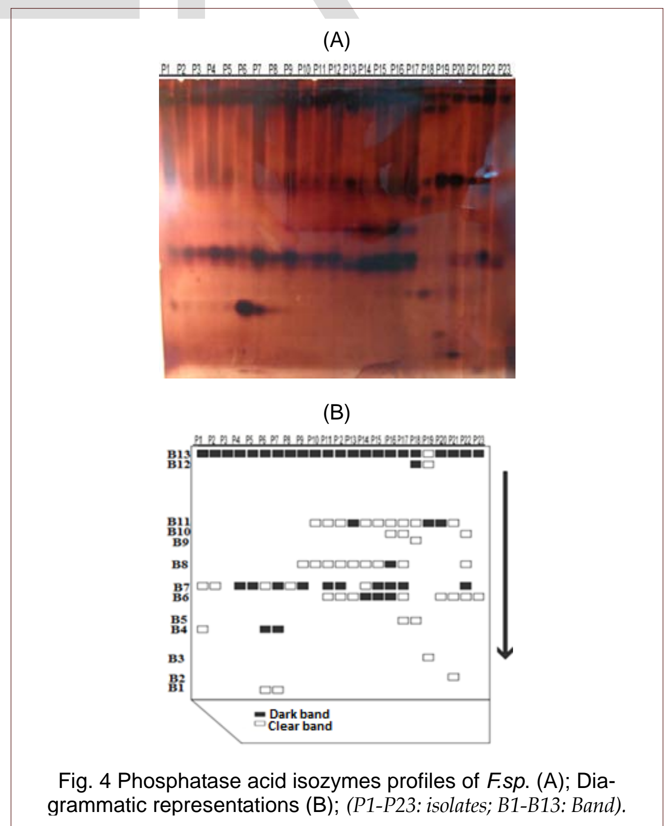

Fig. 4 Phosphatase acid isozymes profiles of *F.sp.* (A); Diagrammatic representations (B); *(P1-P23: isolates; B1-B13: Band).*

3.2.3 Isozyme Cluster analysis

Cluster analysis with the JMP method using genetic distances showed that all 23 isolates formed two main groups, A and B (Fig. 5). The subgroup I closed two isolates (M15A1 and M15D1) with a rate of 94.11% similarity, while fifteen isolates formed the subgroup II who is subdivided into fourteen sub-cluster with a rate of similarity ranged from 94.3% to 100% between Fusarium isolates of origin from the three regions (isolate B15H, M15H, B15F, M15D, O15T, M15A2, O15H, O15D, M15D2, T15H1, A15T, I08G, T15H, M15T and M15T1), two isolates (M15G and T15D) formed the sub-cluster A2 with a rate of similarity 98%. Isolates in the cluster B was further divided into B1 and B2. Two isolates (E1 and E2) formed the sub-cluster B1 with a rate of similarity 94.9%, these last from two regions and isolated from soil, when B2 trained by two isolates (E3 and M15A) with a rate of similarity 93.27%.

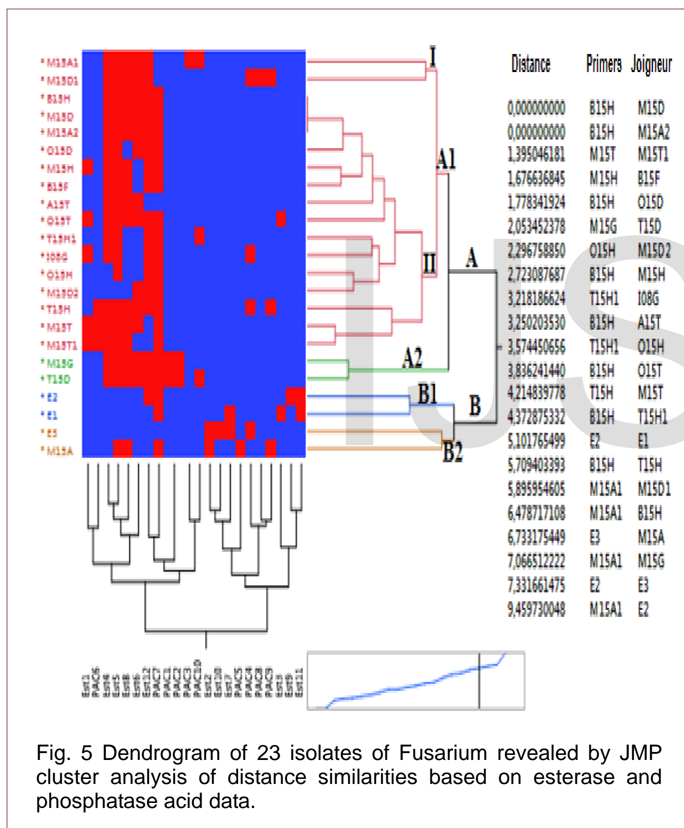

Fig. 5 Dendrogram of 23 isolates of Fusarium revealed by JMP cluster analysis of distance similarities based on esterase and phosphatase acid data.

4 DISCUSSION

The shape of microconidia produced on short phialides is one of the main characteristics defining the species of Fusarium oxysporum and is preferred in identification purpose. Morphological study of isolates has shown morphological and pigmentation variability, Similar studies were also reported by Karkachi et al., (2014) [14] for F.o.a with a difference in the result of pigmentation. Concerning the speed growth of isolates (3.5 to 6.4 cm after 5 days). he is different of which reported by Bonde et al., (2014) in the Fusarium equiseti or they find that All the isolates exhibited similar growth rate of about 7.1-9 cm after 6 days incubation at 25 ± 2° C. The clusters of isozyme analysis of data placed the Fusarium species into two main groups (A and B) and each one consists of several sub – groups. The similarity between the twenty-three isolates of Fusarium varies from 90.55 to 100%. With the formation of a phenotypic tree (constellation diagram) consists of five groups phylogenetic contains each isolates whose distance is close. These results suggested that esterase and phosphatase acid patterns data clearly separated our isolates with a few exceptions. These results of esterase, similar to those obtained by Aly et al., (2003) [7] and Ye and Wu (1985) [15]. Also, a clear cut result was detected by Peroxidase and Protease isozymes in discriminating between Fusarium oxysporum f. sp. Nicotianae (Sumana et al., 2014) [16]. However, no relationship was observed between isozyme patterns and geographic origin or pathogenicity of isolates. Similar studies were also reported by Bosland and Williams, (1987) [17]. While Sumana et al., (2014) [16].recorded a genetic variability in isolates of Fusarium oxysporum f. sp. nicotianae isolated in the same geographic and environmental conditions.

5 CONCLUSION

Our results suggest a phenotypic variation among the isolates of F.o.a. with the use of two isozymes systems. The polymorphic isozyme systems could serve as an indicator of genetic variability in F.o.a and in identifying and characterizing F.o.a. isolates. As we have observed no correlation between the geographical origin and classification of isolates by isoenzymes.

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