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TOTAL PROTEIN POLYMORPHISM OF SOME ISOLATES OF *FUSARIUM OXYSPORUM F. SP. ALBEDINIS* USING SDS-PAGE

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A B S T R A C T

Fusarium wilt caused by *Fusarium oxysporum* f.sp. *albedinis* (Foa), is one of the main diseases affecting date palm (*Phoenix dactylifera* L.), and poses a constant threat to palm oases, especially in North African countries. In this study, we performed electrophoresis of total proteins extracted from the mycelia of 20 *Fusarium oxysporum* f.sp. *albedinis* and 03 isolates of *Fusarium* sp. Protein was extracted and separated by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis followed by staining with silver nitrate. We noted variations in protein profile between the 23 *Fusarium* strains isolated from three regions Ghardaïa, Bechar and Adrar. These results demonstrate the presence of important phenotypic polymorphism according to the ascending hierarchical classification of total proteins after the analysis of the phonograms constructed by the JMP software (JMP SAS Pro (JMP®, Version <12>. SAS Institute Inc., 2015).

Keywords: Electrophoresis, *Fusarium oxysporum* f. sp. *albedinis*, phenotypic polymorphism, total protein.

INTRODUCTION

The date palm (*Phoenix dactylifera* L.), is a symbol of life in arid and semi-arid regions. It is a dioecious, monocot tree, belonging to the *Arecaceae* family (Salem *et al*., 2001). Palm trees were planted for more than 4000 years ago; this genus is composed of about 14 species distributed in desert, tropical and subtropical regions of the glob (Moussouni *et al*., 2013; Gros-Balthazard, 2013, Al Antary *et al*., 2015; El kinany *et al*., 2017; Bentrad *et al*., 2017). This tree is an essential component of the oasis ecosystem of the pre-saharan and saharan regions (Chakroune, 2005) due to its excellent adaptation to the climatic conditions, nutritional values of its fruits which are so rich in amino acids, vitamins, carbohydrate, and minerals with many other uses as well (Bokhary, 2010; Gros-Balthazard, 2013). It allows to the creation of a favourable and typical environment when practicing other underlying cultivations (arboreal, cereals, vegetables, etc), as well as it ensures a certain degree of oasis economic anatomy (Trifi *et al*., 2000).

Algeria is a phoenicultural country, classed on the third (3rd) worldwide row and in the first (1st) row in Maghreb for its large expanses of culture with of 166900 hectares, this surface is in all the regions that is situated on the Saharan Atlas, from the Tunisian-Libyan frontier Est to the Moroccan frontier in the southwest, with about 18.605.100 palm trees located generally in the oasis of Zibans, Souf, Ouargla, Mezab valley and Touat, from the south-eastern of the country, where the

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productive capacity is 990000, with its annual average of 500,000 tonnes of dates (El-Juhani, 2010; SIDABTECH, 2017). However, 4 regions show 89% of the national phoenicicole heritage: Biskra 41.17%, Adrar 9.19%, El-Oued 24.98% and Ouargla 13%, noting that in these regions are produced the beautiful dates like DegletNour and other commercial varieties, Ghars, MechDegla, DeglaBaida (Anonyme, 2002; SIDABTECH, 2017).

Unfortunately, palm trees has been destroyed during last years by the disease known as vascular fusariosis or Bayoud caused by imperfect Ascomycetes fungus, *Fusarium oxysporum*f. sp. *albedinis* (*Foa*), this disease poses a serious menace to palm groves in North Africa (El Hassni *et al*., 2007; Abohatem *et al*., 2011; Dihazi *et al*., 2012).

This disease is currently widespread in Morocco, in the south and south-east of Algeria, and some localities of Mauritanian palm groves (El-Hadrami *et al*., 2005; Oubella *et al*., 2017). Indeed, since it appearance in 1870 in the North of Africa, it is estimated as the cause of disappearance of 15 million palm trees of palm groves including three millions of Algerian palm groves. Effectively, Bayoud not only causes a reduction in the production of dates “the main food of humans and animals in the desert”, but it also unbalances the oasis ecosystem (desertification, disappearance of underlying crop, cereals, forage, vegetables, and fruit (Trifi *et al*., 2000; Freeman and Maymon, 2002; El Modafar, 2010; Dihazi *et al*., 2012).

For twenty years, the analysis of protein polymorphism has been an important approach in the classification and identification as well as the genetic variability of specific and subspecific taxa of fungi (Bosland et Williams, 1987; Mandeel *et al*., 1994; Huss *et al*., 1996; Nawar, 2016). Several authors have reported that the electrophoretic profiles of total proteins can be used as a criterion for identifying species of the same genus, such as *Fusarium* species (Ibrahim *et al*., 2003; Nawar, 2016). However, the interpretation of band profiles presenting phenotypes under zymograms can be translated in terms of genotypes, genes, alleles, in order to determine phylogenetic relationships between organisms (Micales *et al*., 1992).

Subsequently, the aim of our study focus, on total protein polymorphism of the Foa isolates, arising out of symptomatic palm groves in the three zones: Adrar, Bechar and Ghardaia in the South of Algeria, as well as three isolates of *Fusarium* sp. collected from the rhizosphere. The polymorphism is carried out by electrophoresis on polyacrylamide gel under denaturing condition "SDS-PAGE".

MATERIALS AND METHODS

Fungal Isolates: The 23 isolates used in this study were isolated in our previous works (Sidaoui *et al*., 2017), 20 of Foa isolated from the rachis of palm dates which present the symptoms of the Bayoud, and three isolates (E1, E2 and E3) of *Fusarium* sp. near the rhizosphere of infected palm date in three areas (Adrar, Ghardaia and Bechar) from the south of Algeria (Sidaoui *et al*., 2017; Sidaoui *et al*., 2018).

Protein polymorphism by SDS-PAGE

Preparation of liquid culture: Liquid culture was performed in Erlenmeyer Flasks of 250mL, containing the modified cultural medium of Glucose Yeast Peptone (GYP), this medium was distributed at a rate of 100mL per Erlen and then seeded by 6 discs of 8mm diameter, taken from the margin of each colony aged of 7 day in Synthetischies Nahrstoffarmer Agar medium (SNA). Next, the seeded Erlenmeyer flasks were incubated for 10 days at 27°C (Nawar, 2016).

Extraction of total protein: After ten days of incubation at ambient temperature, the mycelium was recuperated by filtration through gauze, washed with sterile distilled water, wring out by absorbent paper, and transferred in falcon tubes, then frozen at -20°C in order to facilitate the cell membrane rupture. After that, the samples were rehydrated in a trizma-wisteria solution (3g trizma + 14.4g wisteria + sufficient quantity of distilled water for 1L, pH 8.3). For each 500mg of prepared mycelium, 1mL of this buffer was added and the tubes ware well mixed, then centrifuged at 10000g for 40min at 4°C.

Finally, the supernatant containing total cytoplasmic proteins was collected in microcentrifuge tubes, and used directly for SDS-PAGE analysis or stored at -20°C until ulterior utilization (Bent, 1967; Badid *et al*., 2001; Manikandan *et al*., 2018).

Proteins dosage by bradford method quantification or Bradford Protein Assay: The protein concentration in isolates is usually determined according to the Bradford method (Klavons and Bennett, 1986), this method uses the principle of complex formation between Coomassie blue and the aromatic residues of proteins.

The experiment was done by adding 1mL of the reactive Coomassie blue to 100µL of sample. After few minutes of incubation under an ambient temperature and obscurity,

the absorbance was measured at 595nm. In parallel, a solution of bovine serum albumin (Sigma) of 2mg/mL concentration was used as a referential protein in order to elaborate a calibration curve (Table 1 and Figure 1). The obtained optical densities (DOs) allow determining the protein concentration of the isolates according to this curve (Klavons and Bennett, 1986; Weckber and Cory, 1988).

Table 1.The Bradford assay calibration range curve

Tubes	123456
SBA (µl)	200200200200200200
ED (µL)	100100200300400500
RB (ml)	111111

BSA: bovine serum albumin (Sigma); ED: Distilled water; RB: Bradford Reagen

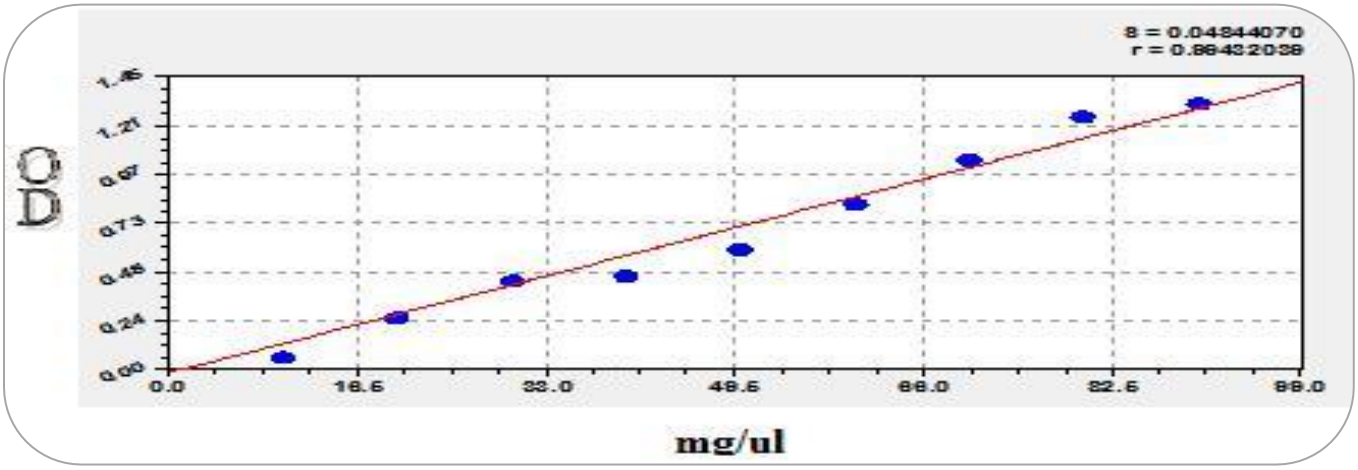


Figure 1. Standard curve

Total protein electrophoresis: The electrophoresis, in denaturing conditions SDS-PAGE, was carried out according to an adapted protocol from that of Laemmli (1979). The electrophoresis gel includes a concentration gel of 4% and a separation gel of 12.5%.

Electrophoresis gel composed of two gels, one concentration gel of 4% and a separation gel of 12.5% in acrylamide / bisacrylamide and containing 0.1% SDS (Table 2) (Westermeier *et al*., 2001; Boriollo *et al*., 2003; Leme *et al*., 2013). The latter linked on proteins, denatures them and gives them negative charges, which will completely hide the native charges of proteins.

Table 2. Electrophoresis gel composition (Boriollo *et al*., 2003; Leme *et al*., 2013)

Composition of the separation gel	
H_2O	3mL
Trizma-Hcl (1.5 M - pH 8.8)	2.5mL
Acrylamide/Bisacrylamide (29.2% - 0.8%)	4 mL
Ammonium persulfate (10%)	50µL
TEMED (N,N,N', N'tetramethylethylenediamine)	30µL
Composition of the concentration gel	
H_2O	6mL
Trizma-Hcl (0.5M - pH 6.8)	4mL
Acrylamide/Bisacrylamide (29.2% - 0.8%)	1.5mL
Ammonium persulfate (10%)	50µL
TEMED (N,N,N', N'tetramethylethylenediamine)	30µL
SDS (sodium dodecyl sulfate)	0.1mL

The concentration and separation gels are prepared, poured into the migration plates and then polymerized for perfect migration. In order to start the migration, samples to be analysed were taken up in the loading buffer in the presence of SDS, and then incubated for 10min at 94°C. Finally, the samples are deposited in the gel wells at a rate of 100µL per well.

Electrophoresis was performed in a buffer trizma-wisteria-SDS- pH 8.3 (trizma- Hcl 25mM, 0.1% SDS, wisteria 192mM) under a tension of 200 V for 4h.

Gel coloring with silver nitrate: At the end of migration, the gel has been separated from the plate and deposited into trough which is submerged with coloring solutions

that allow the revelations of the bands according to the Rabilloud *et al*. (1988) method described below.

The fixations of gel is done with a trichloroacetic acid solution 12%, during 45 min under agitation, then rinsed twice in methanol solution 5% for 20 and in the second time the gel is washing by distilled water during 20 min, directly after that, sodium thiosulfate solution 0.02% (Na2SO3) was poured on the gel and left for one minute.

After that, the gel was washed twice for 20 seconds with distilled water and incubated in a silver nitrate solution 0.2% (AgNO3), added to 76µL of formaldehyde (HCHO) for 20min at 4°C. Subsequently, the gel was rinsed with distilled water twice for 20 seconds when changing the chamber of incubation then, it was developed in a solution of Na2S2O3 (Sodium carbonate) 3%, 100µL of HCHO and some grains of Na2S2O3.

When the development is sufficient, the solution was eliminated rapidly and the gel rinsed with distilled water and the reaction was stopped in HAc 5% solution during min then in ethanol 5% solution for 5min.

Zymogramme analysis: A zymogram reproducing the different phenotypes or electromorphic profiles was constructed for the total proteins. Band profiles are treated as phenotypic traits of the isolate. In which the number "1" was assigned to the presence of a particular band and "0" to its absence in the same gel. Indeed, Bands positions are characterized by occupancy sites Rf for "Retardation factor" and numbered from B1 to Bnwhere the position B1 corresponds to the band whose migration is the quickest and Bnthe weakest. The phenotypic polymorphism data were organized into a binary matrix and a similarity matrix was generated with the JMP software to produce a dendrogram who will determine the genetic homology and allelic diversity of isolates (Boriollo *et al*., 2003; Mohammadi *et al*., 2004; Huss *et al*., 1996).

RESULTS

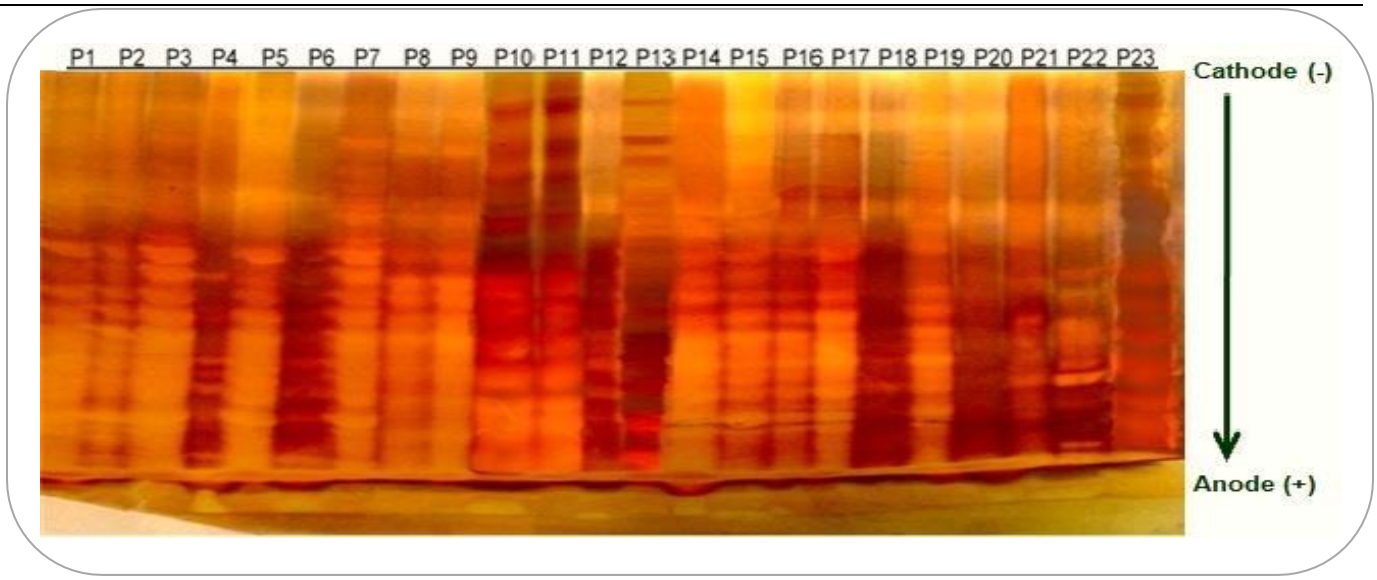
Total protein electrophoresis: In this study, a total of 23 isolates of which twenty belonged to the formae speciales *albedinis* and three isolates of *Fusarium* sp. were compared in terms of total soluble protein profile using electrophoresis on polyacrylamide gel, with the presence of SDS.

There were differences in protein pattern between the 23 isolates according to their profiles, as shown in the figure 2. We have identified heterogeneity of proteins by localization and intensity, using the given data by observation of presence and absence of common band in the 23 isolates (Table 3). In fact, the band B11 is present only on the isolate P13 (E2), while that B2 is present only in the three isolates P12 (M15T1), P4 (B15H) and P22 (O15D), which are isolated from the three regions Ghardaïa, Bechar and Adrar respectively (Figure 2-B-).

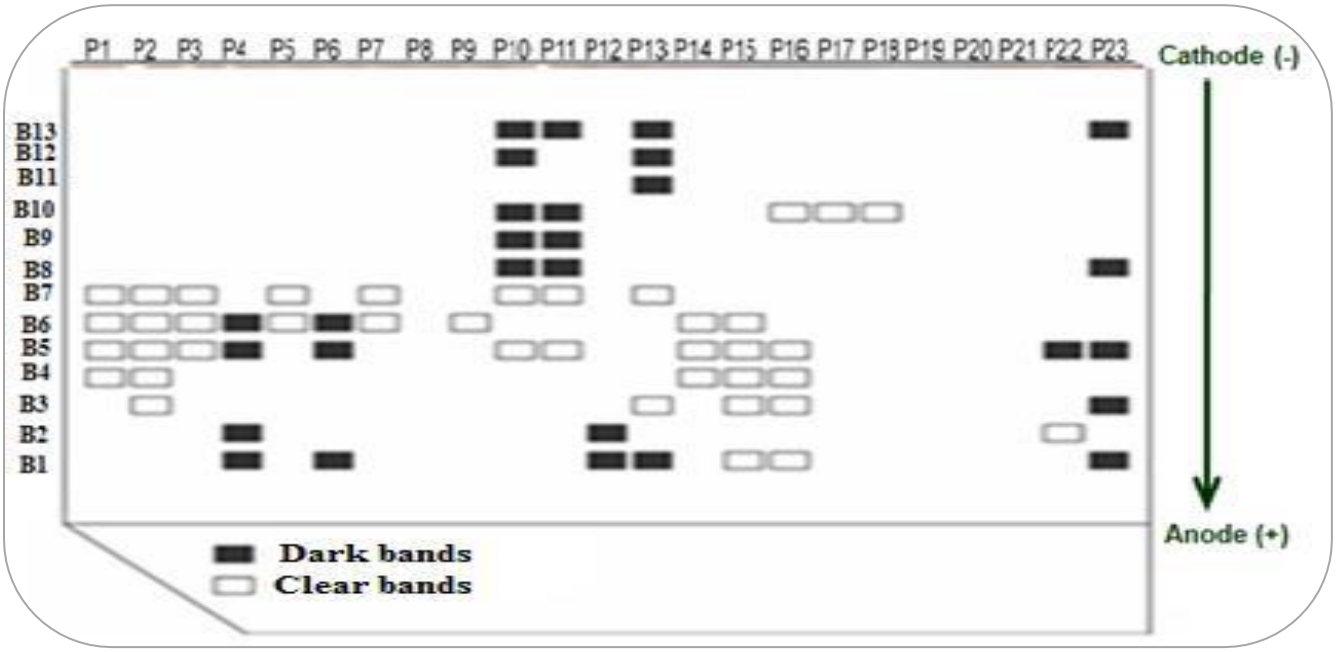
Table 3.Data matrix for total protein.

Isolates		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13
M15A1	P1	0	0	0	1	1	1	1	0	0	0	0	0	0
M15G	P2	0	0	1	1	1	1	1	0	0	0	0	1	1
M15D1	P3	0	0	0	0	1	1	1	0	0	0	0	0	0
B15H	P4	1	1	0	0	1	1	0	0	0	0	0	0	0
E1	P5	0	0	0	0	0	1	1	0	0	0	0	0	0
M15H	P6	1	0	0	0	1	1	0	0	0	0	0	0	0
T15H1	P7	0	0	0	0	0	1	1	0	0	0	0	0	0
A15T	P8	0	0	0	0	0	0	0	0	0	0	0	0	0
M15A	P9	0	0	0	0	0	1	0	0	0	0	0	0	0
T15H	P10	0	0	0	0	1	0	1	1	1	1	0	0	1
M15T	P11	0	0	0	0	1	0	1	1	1	1	0	1	1
M15T1	P12	1	1	0	0	0	0	0	0	0	0	0	0	0
E2	P13	1	0	1	0	0	0	1	0	0	0	1	1	1
T15D	P14	0	0	0	1	1	1	0	0	0	0	0	0	0
B15F	P15	1	0	1	1	1	1	0	0	0	0	0	0	0
O15T	P16	1	0	1	1	1	0	0	0	0	1	0	0	0
I08G	P17	0	0	0	0	0	0	0	0	0	1	0	0	0
M15A2	P18	0	0	0	0	0	0	0	0	0	1	0	0	0
M15D	P19	0	0	0	0	0	0	0	0	0	0	0	0	0
E3	P20	0	0	0	0	0	0	0	0	0	0	0	0	0
O15H	P21	0	0	0	0	0	0	0	0	0	0	0	0	0
O15D	P22	0	1	0	0	1	1	0	0	0	0	0	0	0

M15D2	P23	1	0	1	0	1	1	0	1	0	0	0	0	0



A



B

Figure 2.Total proteins profiles of *Fusarium* sp. isolate (A); Representative Chart (B); (P1-P23: Isolates; B1-B13: Bands).

Protein cluster analysis: An ascendant hierarchical classification based on the establishment of a matrix of similarity coefficient of Jacquard combined to the JMP software method, was used to schematize the relations between the isolates. The phenotypic classifications was prepared in form of a dendrogram, this last show that the 23 isolates form two main groups A and B (Figure 3).

Group B is composed of two subgroups B1 and B2, the subgroup B2 included only one representative isolate of *Fusarium* sp. (E2) isolated from the soil; the B1 is formed by two isolates T15H and M15T corresponding to the special form of *albedinis* at 98% similarity index.

Group A is composed by two subgroups A1 and A2, the subgroup A2 included six isolates A15T, M15D, O15H, M15A, I08G and M15A2 representative of the special form of the *albedinis*, clustered in two single separate branch with high correlation at 100% similarity index, in addition to the isolate E3 which belongs to *Fusarium* sp.

Isolates of subgroup A1 were divided into two sub-sub-groups I and II, the sub-sub-group I included seven isolates

of the special form of *albedinis,* and they are as follows: B15H, O15D, M15T1, M15H, M15D2, B15F and O15T associated between them at levels ranging from 95% to 98.5%. While, the sub-sub-group II included five isolates of the special form of *albedinis*(M15A1, T15D, M15D1, E1, T15H1 and M15G), in addition to the isolate E1 which belongs to *Fusarium* sp. and was connected between them at similarity index from 95.5% to 100% (Figure 3).

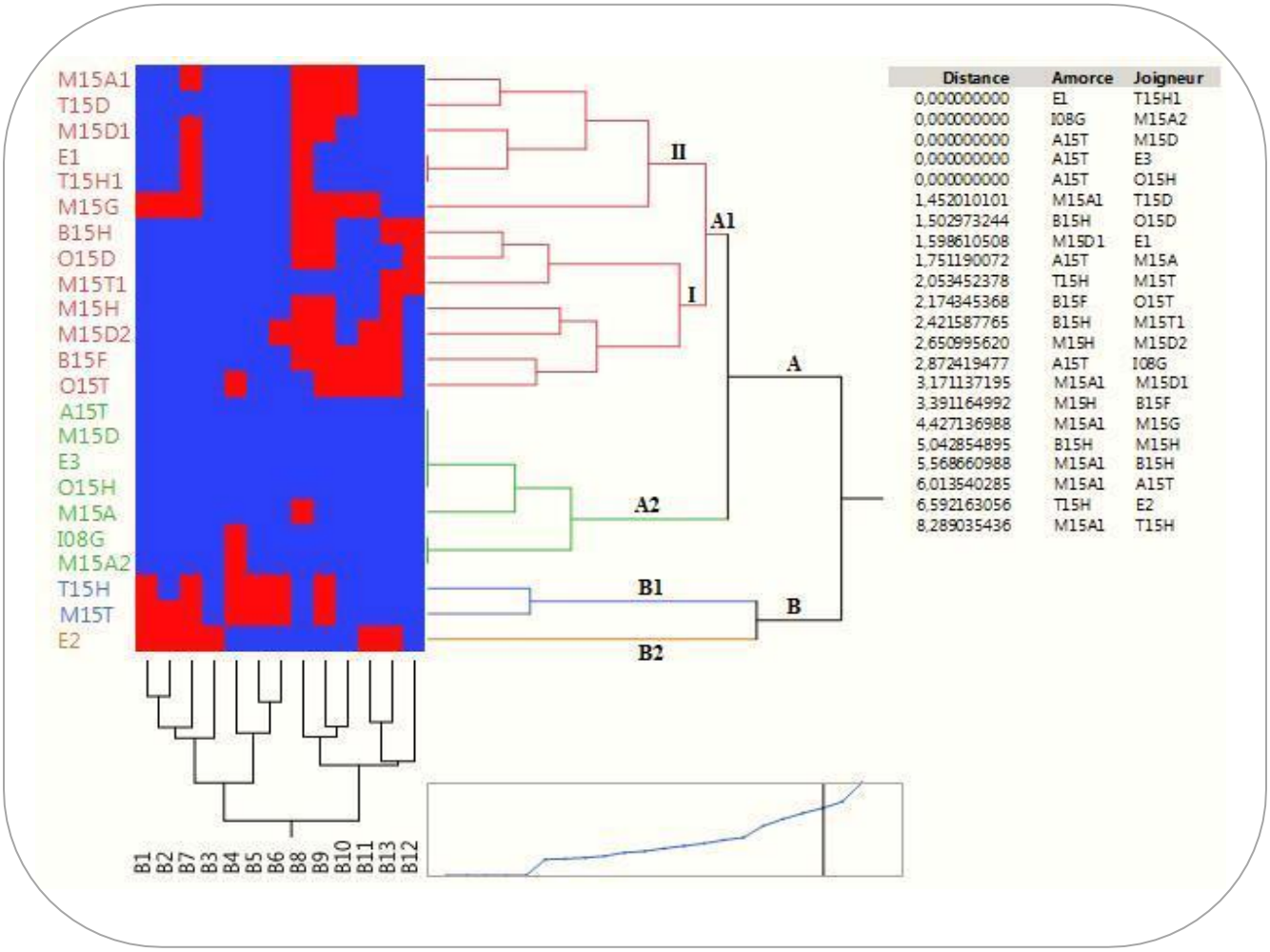


Figure 3.Dendrogram resulting from cluster analysis based on similarity matrix of total protein for 20 isolates of Foa and 03 isolates of *Fusarium* sp. using JMP and Jaccard similarity coefficient.

DISCUSSION

In this study, we analyzed total soluble protein by SDS-PAGE; there were variations in protein profile between 23 *Fusarium strains* isolated from the three regions Ghardaïa, Bechar and Adrar. In addition, soluble protein banding pattern of forma specials *albedinis* under study and the three *Fusarium* sp. isolates showed variations in relative mobility of many protein fractions. These results are consistent in their general scope with those of Al-Khayr *et al*. (2017), who reported that *F. oxysporum* f. sp. *Dianthi* isolated from the four regions of the world USA, Italy, the Netherlands and Colombia, representing variations in the profile of soluble proteins. Similarly, Nawar (2016) has demonstrated that electrophoresis of soluble proteins of *Fusarium* pathogenic species present heterogeneity by location and intensity after their analysis by SDS-PAGE. And well Mohammadi *et al*. (2004) observed slight variations in protein profile between the isolates of *F. oxysporum* isolated from Iran, France and the USA, based on electrophoresis of soluble proteins by SDS-PAGE.

In the same way, the difference in the protein profiles of *Fusarium* species have been reported by several authors, who have studied the morphology of the fungus and the genetic diversity between *Fusarium* sp. isolates (Arie *et al*., 1998; Bhuvanendra *et al*., 2010; Sumana and Devaki, 2014; Manikandan *et al*., 2018). On the other hand, Aly *et al*. (2003) analyzed *Fusarium* spp. proteins by SDS-PAGE

and they showed that the interspecific and intera species genetic similarities were ranged from 21 to 54% and 62 to 97% respectively. Heterogeneity in proteins regarding bands location and intensity on one-dimensional gel SDS-PAGE was observed by Mandeel *et al*. (1994), for *F. oxysporum* isolates obtained from cucumber, therefore soluble protein electrophoresis can be used to distinguish special forms of pathogenic fungi.

On the contrary, Ho *et al*. (1985) showed no variations in the soluble protein profiles between the pathogenic isolates of *Fusarium oxysporum* f. sp. *elaeidis* isolated in Africa from oil palm as well as non-pathogenic isolates of *F. oxysporum* isolated in Malaysia from the rhizosphere of an oil palm plantation.

More of that, the comparison of electrophoretic protein profiles was considered satisfactory taxonomic resolution technique and used in the classification, which may be applicable at the species, subspecies level of pathogenic fungi (Boriollo *et al*., 2003; Abo-Elnaga and Amein, 2011).

CONCLUSION

During this study, we have done an electrophoresis of total protein extracted from mycelium of our isolates; indeed, the ascendant hierarchical classification of total protein provided isolates phenotypic polymorphism and that after the analysis of phonogram constructed by the JMP software. It is important that future studies correlate the protein diversity with the pathogenicity of this pathogen.

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