INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 6, No. 4, p. 30-39, 2015

RESEARCH PAPER OPEN ACCESS

Comparison of *Ascochyta rabiei* isolates for cultural characteristics and isozyme

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Key words: *Ascochyta raiei*, Esterase, Acid phosphatase, Superoxide dismutase, Leucine aminopeptidase.

http://dx.doi.org/10.12692/ijb/6.4.30-39 Article published on February 28, 2015

Abstract

The comparison of our isolates on the basis of macroscopic and microscopic criteria reveals differences between 32 isolates of *Ascochyta rabiei,* it is distributed as follows, light brown, dark brown and green (50, 22 and 28%). Pathogenicity is performed on three varieties of chickpea (Flip 90-13, Flip 33-99 and ILC 1799), which showed no aggressiveness with the two varieties Flip 90-13, but with the variety ILC 1799 showed a pathological variability of 10 isolates of *Ascochyta rabiei*. Electrophoretic study of five isoenzymes esterase (Est), acid phosphatase (CAP), leucine aminopeptidase (LAP), superoxide dismutase (SOD), peroxidase (POX) showed that only Esterase and acid phosphatase isoenzyme have an polymorohisme. A dendrogram is established, integrating all the data. It reveals the importance of genome diversification within the species.

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Introduction

Chickpea (*Cicer arietinum*) is the world's third most important pulse crop after bean (*Phaseolus vulgaris*) and peas (*Pisum sativum*), ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labrousse, is a serious foliar disease the infection resulting in severe yield losses in Asian and African countries and all areas of production chickpea in the world (Singh *et. al.* 1997; Ilarslan and Dolar, 2002; Gharbi *et. al.* 2013).

It has been widely studied in term of morphology, pathology, phytotoxin production and DNA fingerprints, but there is currently no information available regarding the use of isozyme analysis in the study of population structure of the fungus.

In order to better understand genetic diversity at *Ascochyta rabiei*, it appeared necessary to us to prospect other tools for characterization that the morphological study and pathogenesis test. For this purpose, we chose to study the polymorphism isoenzymatic of soluble mycelia proteins. The enzymes, coded by different alleles or locus, frequently have variable electrophoretic motilities. This is due to variations in the contents in amino-acids of the molecules, which depend on the nucleotide sequence of the DNA (Micales *et. al.* 1986; Rosendahl and Sen, 1992).

The objective of this article is studied biometric characterization, pathogenicity and isozyme polymorphism to determine variability among various isolates of *A. rabiei* collected from different regions of West Algeria.

Materials and methods

A total of 32 isolates of *Ascochyta rabiei* isolates used in this study were collected from tree different regions (Ain Temouchent, Mascara and Sidi Bel Abbes) of the western Algeria. Isolation of the fungus was performed from stems of plants showing symptoms of Ascochyta blight (Khan *et. al.* 1999; Ilarslan and Dolar, 2002; Chen *et. al.* 2004, Bayraktar *et. al.* 2007; Gharbi *et. al.* 2013).

Morphological study

The study of the macroscopic characters of the isolates is based on the morphological description and on the pigmentation of the colonies incubated during 10 days on the flour medium of chickpea at a temperature of 22 C° and under a 12 hours photoperiod. The microscopic study is carried out in a fresh state; fragments are taken from the superficial part of the 10-day old colonies grown on medium CSMA, then we proceeded to the optical microscopic observation.

Pathogenicity test

This test is carried out with the varieties of chickpea (90-13 Flip, Flip 33-99 and ILC 1799), provided by the Algerian National Institute of Agronomic Research of Sidi Bel Abbes (Algeria). The six-day old seedlings are pulverized with 10 ml of a suspension of 10^5spores/ml, 10 seedlings were used for each isolate, and then the seedlings are covered with a transparent plastic film during 72 hours, followed by several pulverizations with water to maintain a humidification, in order to facilitate the germination of the spores on the organs of the seedling. The degree of the pathogenicity is evaluated using 1- 9 rating scale as suggested by Reddy *et. al.* 1984.

Sample preparation

To obtain mycelia extracts for intracellular enzymes, five 5 mm plugs of mycelia from a 7 day old culture were transferred to 50 ml of growth medium GYP (peptone 1%, yeast extract 1%, glucose 1%) in 250 ml Erlenmeyer flasks and incubated without agitation at 125 rpm at 25°C for 10 days. Mycelium was collected on filter paper by filtration and washed carefully with distilled water then it was crushed in a mortar (maintained cold in the ice) in the presence of sand until obtaining a fine and homogeneous paste, we added phosphate buffer (100 mM; pH 7,1) at 1ml/g, the samples was centrifuged at 10000 g for 20 minutes at 4°C. The supernatant is distributed in Eppondorf tubes by fraction of 100 µl, then, either used immediately for the electrophoresis or preserved at the freezer.

Electrophoresis

Protein extracts were subjected to vertical polyacrylamide gel electrophoresis. Native polyacrylamide electrophoresis was conducted using a Bio-Rad Mini-PROTEAN II (glass plates: 8 x 10 cm, combs: thickness 1,5 mm). The gel of acrylamide includes the stacking gel 5%,the gel buffer was 0.125 M Tris-HCl (pH 6.8), the separating gel 10% with 0.375 M Tris-HCl (pH 8.8)), running buffer was Tris-glycine (pH 8.3). Approximately 10 to 15 µl of protein extract was placed in wells with sample buffer (0.5 M Tris-HCl, pH 6.8, 1% glycerol, and 0.05% bromophenol blue). The gels were run at 120 V, 50 mA per gel for 2 hours at 4°C.

Enzyme assay

After electrophoresis, the gels were stained according to their enzyme system with the appropriate substrate and chemical solutions then incubated at room temperature in dark for complete staining. In most cases incubation for about 1 to 2 hours is enough. The chemical ingredients are listed in Table (1).

Data analysis

Electrophoretic mobility or distance of migration from the origin was recorded for all isozyme bands. In this study the bands were scored 1 (same location) or 0 (absent). A phonetic dendrogram based on the estimated similarity coefficients was constructed by Xlstat (version 2013).

Results

The comparison of isolates on the basis of macroscopic and microscopic criteria reveals differences between isolates (Table 02). In general, we recorded the color of the mycelium is very variable; it can be light brown, brown or green (Fig 1). On the culture medium CSMA, mycelia colonies develop slowly. They are first creamy, and then take extremely varied colors among isolates, after culturing, they generally present very characteristic concentric streaks. This character, we have chosen as a criterion of morphological characterization. The phenomenon appears as zoning of sporulating mycelia bands at alternating with bands of mycelia with low sporulating. The zonation pycnidia observed in all isolates.

Table 1. The staining solutions of isozymes.

Enzyme	Ingredients	Quantity	Authors
Esterase	Fast bleu RR	20 mg	Jonathan and Wendel, 1990
(EC. 3.1.1.1)	100 mM Na-phosphate buffer pH 6.0	50 ml	El-Sharabasy and Wanas, 2008
EST	α- naphthyl acetate 2 % (acetone)	1 ml	Shaw and Prasad ,1970
	β- naphthyl acetate 2 % (acetone)	1 ml	
Acid phosphatase	β-Naphtyl acide phosphate	50 mg	Pan and Chen, 1988
(EC. 3.1.3.2)	Fast Garnet GBC	50mg	
PAC	50 mM Acetate buffer pH 5	100 ml	
Leucine aminopeptidase	L. Leucyl β-Naphtylamide HCl	50 mg	Beckman *et. al.* 1964
(EC.3.4.1.1)	H_2O	30 ml	
LAP	Fast Black K Salt	50 mg	
	20 mM Tris Maleate buffer pH 3,3	50 ml	
	NaOH 20 mM	20 ml	
superoxide dismutase	Solution A :		*Jevremović et. al.* 2010
(EC. 1.15.1.1)	50 mM Na-phosphate buffer pH 7,5	100ml	
SOD	10 mg/ml MTT	1 ml	
	Solution B :		
	50 mM Na-phosphate buffer pH 7,5	100 ml	
	TEMED	0,2 ml	
	Riboflavin	1 mg	
Perioxdase	3 Amino, 9Ethyl carbazole	40mg	Graham *et. al.* 1964
(EC.1.11.1.7)	Diméthyl formamide	5ml	Li, 1981
POX	50 mM acetate buffer pH 5	92ml	
	Ca_2Cl_2 (10 mM)	2ml	
	H_2O_2 (30%)	30µl	

We recorded the color of the colonies of 32 isolates of *Ascochyta rabiei* is distributed as follows, light brown, dark brown and green (50, 22 and 28%) respectively (Fig 1) and no significant difference (P = 0.709) was recorded, distribution according to the aspect of the regions is as follows, the region Ain Temouchent aspect includes three aspects (light brown, brown and green) the percentage is (34, 33 and 33%) respectively (Fig 2), along with the regions of Mascara and Sidi Bel Abbes, with a rate of (43, 14 and 43%) ; (54, 23 and 23%) respectively (Fig 3 and Fig 4).

Table 1. Morphological characters of isolates *Ascochyta rabiei.*

N°	Isolate	region	Morphological aspect
1	A4	Ain Temouchent	Light Brown
2	A6		Brown
3	A32		Brown
4	A1	Sidi Bel Abbes	Light Brown
5	A2		Light Brown
6	A3		Light Brown
7	A8		Light Brown
8	A11		Light Brown
9	A13		Light Brown
10	A14		Light Brown
11	A18		Light Brown
12	A19		Light Brown
13	A20		Light Brown
14	A21		Light Brown
15	A25		Light Brown
16	A30		Brown
17	A31		Brown
18	A33		Brown
19	A34		Brown
20	A36		Brown
21	A37		Green
22	A38		Green
23	A39		Green
24	A41		Green
25	A42		Green
26	A9	Mascara	Light Brown
27	A10		Light Brown
28	A12		Light Brown
29	A16		Brown
30	A17		Green
31	A24		Green
32	A29		Green

Table 2. Test result of the pathogenicity of isolates *Ascochyta rabiei.*

isolat Flip 90	13 Flip 33	99c ILC	1799
A3	0	1	8
A6	1	1	7
A8	1	1	7
A11	3	1	8
A12	0	1	6
A25	1	1	6
A26	1	1	6
A27	2	2	5
A30	1	3	4
A31	2	1	6

On medium CSMA, color Pycnidia varies among isolates. So it varies from pink-brown (Fig 6), but most of the isolates pink-brown color dominance. Pycnidia are globular, dark brown, 65 to 245 µm in diameter and equipped with an ostiole. At maturity, pycniospores out mass pycnidia, mired in mucus. They are hyaline, ovate to oblong straight or slightly curved and rounded at the tip a few are celled but most are unicellular, they measure (8,2-10 x 4,2-4,5 µm).

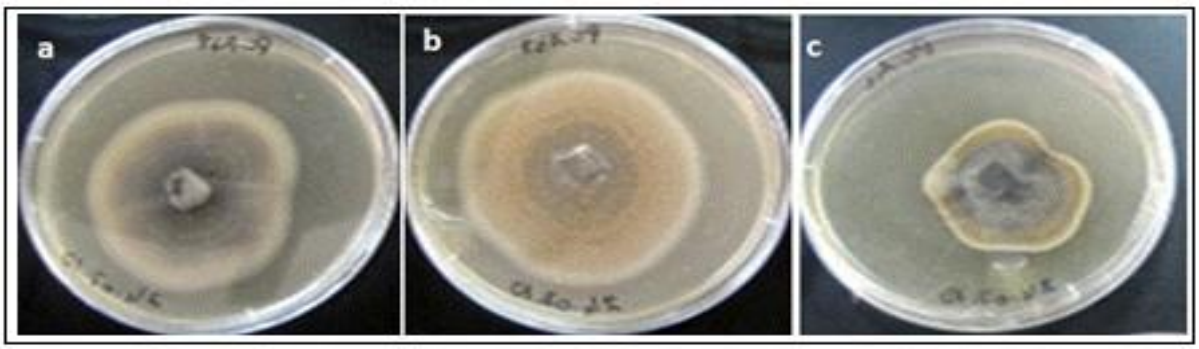

Fig. 1. Different morphological aspects of *Ascochyta rabiei* isolates cultured on culture medium CSMDA (a: light rown; b: brown; c: green).

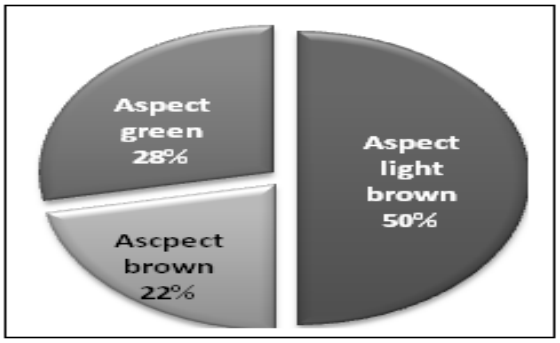

Fig . 2. Different morphological aspects of *Ascochyta rabiei* isolates from western Algeria.

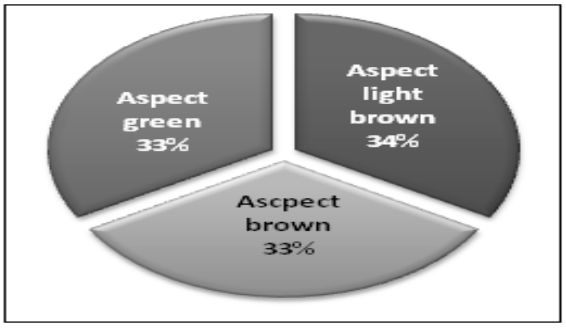

Fig . 3. Different morphological aspects of *Ascochyta rabiei* isolates from Ain Temouchent.

Pathogenicity

The study of pathogenicity is an important passage to confirm the isolates affiliation to species and to evaluate the level of virulence, this study was carried on three varieties of chickpea (Flip 90-13, 33-99 and Flip ILC 1799), after a few days of the inoculation of young seedlings, the isolates behave differently with these three varieties (Table 3), we have found that all isolates exhibit low aggressiveness the varieties of Flip and Flip 33-99c 90-13, we noted the presence of necrosis in leaves as well as stem lesions. The symptoms obtained during the last reading are assessed according to the scale at 9 points; it is primarily based on the response of the host and the development of symptoms on different aerial parts of the plant.

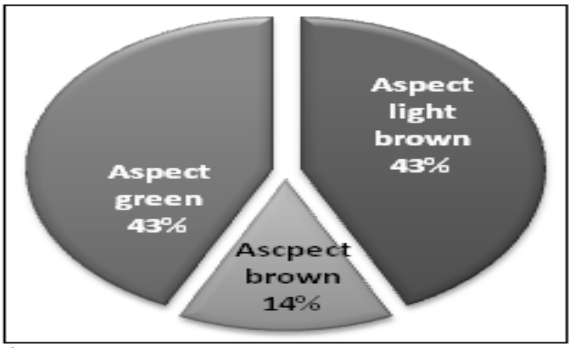

Fig . 4. Different morphological aspects of *Ascochyta rabiei* isolates from Mascara.

Karkachi *et. al.* 2014, they studied the macroscopic variability and the microscopic appearance of *Fusarium oxysporum f.sp albedinis*, they found that there are different aspects in the collection of Foa, they showed that the ras type is majority, it present

50%, the downy type that has 16% other isolates have a compact and cottony mycelium having 33% of the collection. Sarwar *et. al.* 2013, evaluated the pathogenicity of 21 isolates of *Ascochyta rabiei* using three varieties of chickpea differentials, the variety (Aug424 and Pb-1) sensitive variety (Aug480 and CM72) and tolerant varieties (Paidar and CM88) resistant. The pathogenicity test on susceptible cultivars, resulting in the onset of symptoms involving lesions on leaves and stems and even in severe cases have resulted in death of plants (Sayed *et. al.* 2009). Elliott *et. al.* 2013, evaluated the degree of pathogenicity of a collection of isolates of *Ascochyta rabiei* on two growth stages of chickpea on seedlings and growth stages of maturity (P <0.000), they observed significant mean differences, although isolates are similar in degree of pathogenicity, their ranking on this principle was different, this suggests that the genotype-specific expression of genes for resistance to *Ascochyta rabiei* can be related to the stage of growth of chickpea. Three isolates *Ascochyta rabiei* are used to test their degrees of virulence with 52 varieties of chickpea. In addition, almost all chickpea genotypes revealed a high degree of sensitivity to pathogens except some genotypes were moderately resistant (Lamichhane *et. al.* 2012).

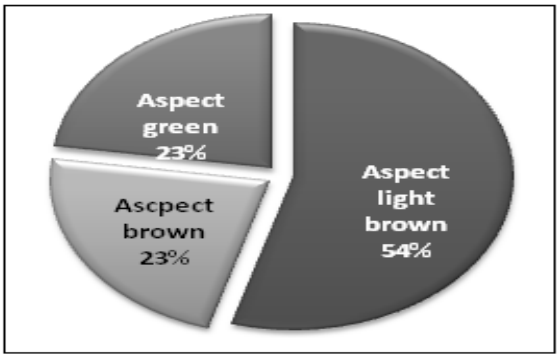

Fig. 5. Different morphological aspects of *Ascochyta rabiei* isolates from Sidi Bel Abbes.

The study of the pathogenicity of sixteen isolates of *Ascochyta rabiei* from seven regions of north-western Algeria was determined pathotypes and physiological races with seven differential chickpea lines. All isolates were classified into three pathotypes and 6 physiological races according to their aggressiveness and virulence, respectively, one less aggressive isolate (6.25%) to pathotype I, 12 moderate aggressive isolates (75%) and three pathotype II aggressive isolates (18.75%) pathotype III (Benzohra *et. al.* 2010 ; Benzohra *et. al.* 2011 ; Benzohra *et. al.* 2013).

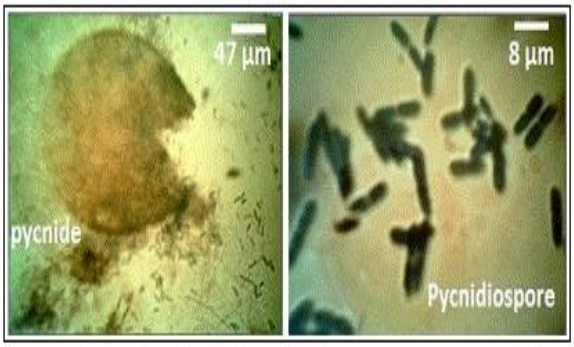

Fig. 6. Microscopic observations of pynides and pycnidiospores of isolates of *Ascochyta rabiei.*

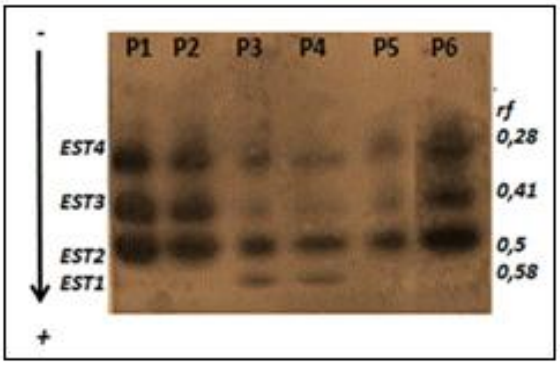

Fig. 7. Esterase isozymes profiles of *Ascochyta rabiei.*

Esterase

Bands of different colors appear on gels, isoenzymes reacting with the beta naphthyl acetate produce a red band, and those reacting with alpha naphthyl acetate produce a purple band. The esterase zymogram of observation is performed after detection of the enzyme by the substrate α-naphthyl acetate (Fig 7). Four bands were revealed. They show the relative motilities of between 0.28 and 0.58, different isolates can be detected by the number and position of the bands identified. The heterogeneity of esterase zymogram gives a fairly good representation of the genetic complexity.

Acid Phosphatase

Zymogram analysis reveals the anode and cathode for a total migration of three active sites (Fig 8) is between 0.16 and 0.73. However, once again, the band frequencies are simultaneous multiple fractions

containing very similar migration and non-separable in the system used. No polymorphism was observed for acid phosphatase to the population concerned. The frequency bands are simultaneous multiple fractions containing very similar and inseparable migration in the system used.

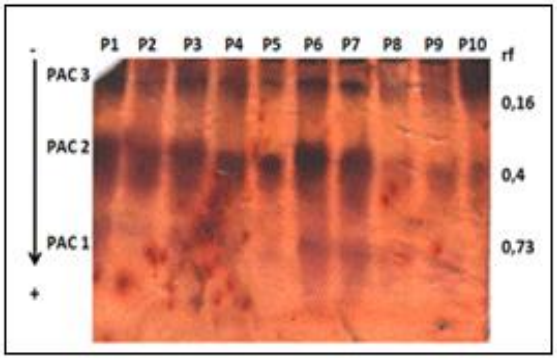

Fig. 8. Acid phosphatase isozymes profiles of *Ascochyta rabiei*.

Superoxide dismutase

Superoxide dismutase in three electrophoretic bands of the activity was detected in the upper region of the gel just after their frequencies gel concentration is between 0.16 and 0.33 and is located in a lower band portion of the separation gel before the migration front in position 0.98 (Fig 9).

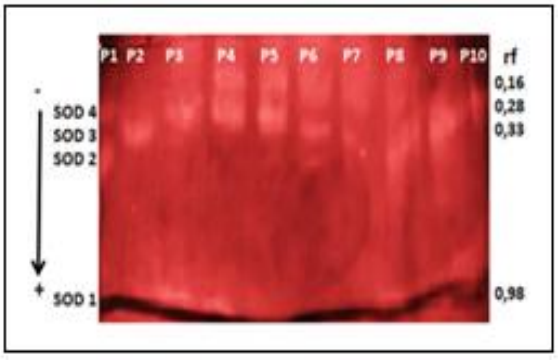

Fig. 5. Superoxide dismutase isozymes profiles of *Ascochyta rabiei*.

Peroxidase

The electrophoretic profile of this system shows a single area of activity with dianisidine. All isolates have isoenzyme (0.14) in common system revealed no isozyme polymorphism (Fig 10).

Leucine aminopeptidase

The electrophoretic profile of LAP is characterized by two bands revealed using the substrate L-leucine-p-naphthylamide. Two bands of are detected different migration: one corresponds to the isoenzyme 1 (0.42) and the other corresponds to this low isozyme by isolate number (Fig 11) appears indeed surprising this result is compared with that of a similar analysis conducted on *Colletotrichum* grasses had revealed an average of four per strain LAP isozymes, one of which was common to both species *Colletotrichum falcatum* et *C. graminicola* (Huguenin *Et. al.*1978).

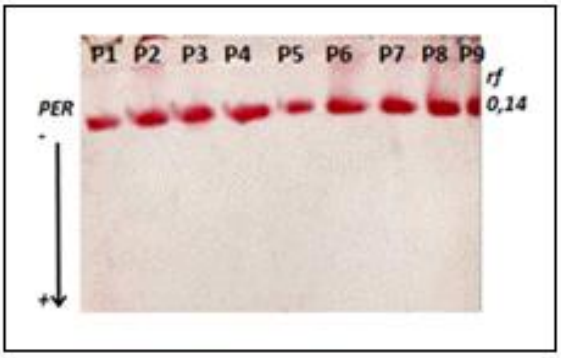

Fig. 6. Peroxidase isozymes profiles of *Ascochyta rabiei*.

Interpretation and data analysis

The combination of different patterns of data obtained with 5 isonezymes isoenzyme systems allowed for the classification of the isolates presented as a dendrogram. This dendrogram constructed from the similarity matrix index Jaccard is shown in (Fig 12), it shows four main branches (A, B, C and D) separated at a rate of 0.49 similarities.

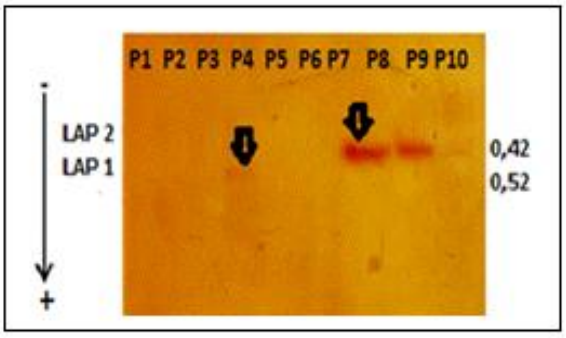

Fig. 11. Leucine aminiopeptidase isozymes profiles of *Ascochyta rabiei*.

The branch A is formed a cluster of 12 isolates (31.25% of isolates), isolates the separate branch 4 branches A1, A2, A3, A4 and A5 for a rate of 0.69 similarity. The branch B groups 10 isolates, it includes

that the isolates which come from two regions of Sidi Bel Abbes and Ain Temouchent, on the other hand the branch C includes 09 isolates of Origin Mascara, Sidi Bel Abbes and Ain Temouchent. The branch D contains only isolate A19 which comes from Sidi Bel Abbes.

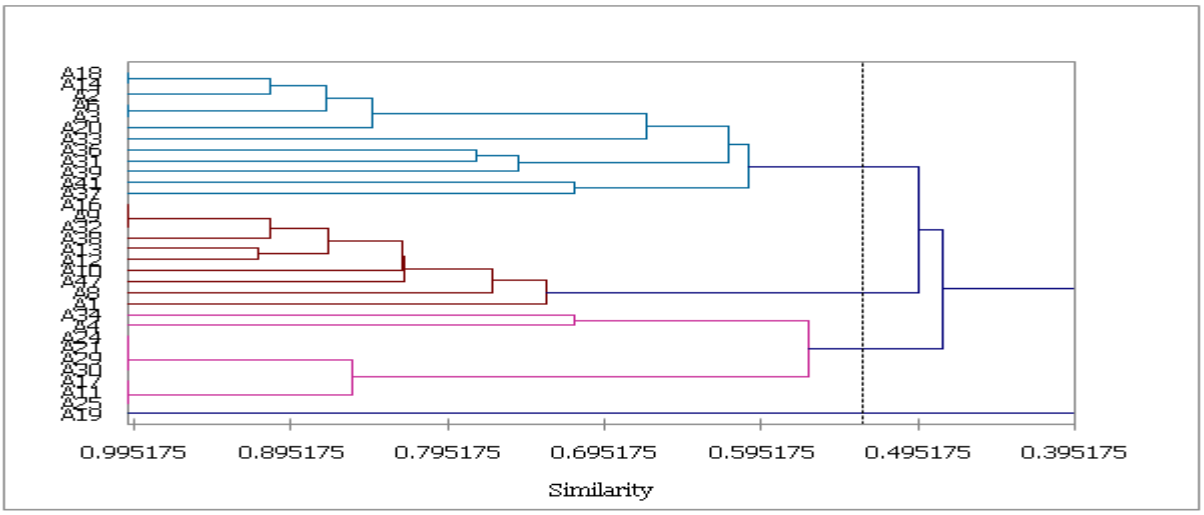

Fig. 12. *Ascending Hierarchical* Classification (AHC) Similarity: Jaccard Index.

(Skovgaard and Rosendahl, 1998) Compared isolates of *Fusarium oxysporum* resulting and coming from different regions with regard to compared with isoenzymes intra - and extracellular. Five intracellular enzymes: esterase superoxidizes dehydrogenase, malate dehydrogenase, dihydrolipoamide dehydrogenase and succinate dehydrogenase were extracted from the mycelium, and oven extracellular enzymes: protease, cellulase (endoglucanase), amylase and lipase were there for obtained from the culture medium. Proteins were separated by intermittent or PAGE or by IEF, and isoenzyme beens are detected by Specific held (coloring). The isolates of *Stenocarpella maydis* isolated from seeds (Dorrance *et. al.* 1999) Analyzed thesis isolates by the study of the polymorphism of isozymes and morphological variability.

The results indicate that the estimated inter-specific variation may be more pronounced with protein markers with isoyzmes when the two approaches are applied to the same population. The level of genetic variability detected, inside and between the population with *Fusarium* spp protein analysis and esterase isoyzmes, suggests that this is a reliable marker technique, efficient, and effective in determine genetic relationships in the genus *Fusarium* (Aly *et. al.* 2003) A total of 13 representative isolates of *Fusarium oxysporum f. sp.melonis* come from different parts of Iran, USA and France, eight isolates of seven special form's Iran and isolate *F.oxysporum f. sp.niveum* and the United States were compared on the basis of isoenzyme analysis and profile of soluble proteins. The isoenzyme of alkaline phosphatase (ALP), catalase (CAT), esterase (EST), malate dehydrogenase (MDH), superoxide dismutase (SOD) and xanthine dehydrogenase (XDH) revealed an isozyme polymorphism between different special form of F. oxysporum 22 wherein phenotypes electrophoretic (EP) were determined (Mohammadi *et. al.* 2004). Polymorphisms of the esterase isozyme, hexose kinase and the malate dehydrogenase are often limited isoenzyme profiles. Analysis of zymograms of the population *Trichoderma harzianum* and *Trichoderma reesei* UPGMA by software, totally separate from those groups except the population of *Trichoderma aureoviride*, this analysis showed this *T.harzianum* high level of genetic diversity over other species of *Trichoderma* (Shafiquzzaman *et. al.* 2007).

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