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Characterization of *Ascochyta* as Pathological Species of Pea (*Pisum sativum* L.) at the North-West of Algeria

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Abstract: It is the weakness of works on this pathogenic agent that pushed us to characterize *Ascochyta* on the plans morphological and molecular. Thirty seven samples of biological materials were isolated from three zones i.e., Mostaganem, Relizane and Mascara all located at North-West of Algeria. The samples are the isolated strains and are obtained from various organs of pea seedlings attacked by *Ascochyta* sp. According to the morphological aspect, the results allowed to distinguish between three categories of strains representing the three species. Nevertheless, the distinction between these three categories is not very reliable for cause of the extreme variability of these species according to their middle of culture. According to the molecular data, all the isolated-strains who's Internal Transcript Space (ITS) regions in DNA have been amplified by Polymerase Chain Reaction (PCR) have expressed identical results with the same size of the PCR products (550 Pb). No polymorphism is noted at the set of the tested strains. On the other hand, the molecular sequencing permitted to distinguish *Ascochyta pisi* from *Ascochyta pinodes* and *Ascochyta pinodella*. These last two species remain similar in their ITS1 and ITS4 regions and don't show any difference. This study was done at the North-West of Algeria between 2004 and 2008. The prospection of other DNA regions with other molecular markers is to be planned in order to detect polymorphism between *Ascochyta pinodes* and *Ascochyta pinodella*.

Key words: *Ascochyta* sp., *Pisum sativum* L., polymerase chain reaction, sequencing, internal transcript space

INTRODUCTION

The food leguminous plants as pea (*Pisum sativum*), chickpeas (*Cicer arietinum*), broad bean (*Vicia faba*) and lenses (*Lens culinaris*) take very significant roles in the agricultural systems of the World. Their importance in cultivated area varies between 22 and 45% (Tivoli *et al*., 2006).

In Algeria, as other cultures, the pea (*Pisum sativum* L.) is subjected to several constraints such as drought, salinity and disease problems prevailing many consecutive years (Bouznad, 1989). Among these, the anthracnose or *Ascochytose* occupies an important place. It is caused by three major species: *Ascochyta pisi* Lib., *Phoma medicaginis* (*Ascochyta pinodella* L. K. Jones) and *Mycosphaerella pinodes* (*Ascochyta pinodes* L. K. Jones) generally called Complex *Ascochyta* (Tivoli and Banniza, 2007).

These three pathogenic agents cause on the organs of various plantation of Pea, the maculae necrotic that affect the yield and the quality of their harvests (Allard *et al*., 1993). There are very few works in the molecular characterization of this species in the Algerian North-West (Tivoli and Banniza, 2007). However, the diagnosis of the three species by simple visualization of the symptoms remains random because of their frequent superposition on the same plant host (Onfroy *et al*., 2007). To this effect, their characterization remains necessary for the elaboration of any control of the disease program so much for the resistant cultures selection that at the time during the seeds analysis, witch it is the principal way of contamination. In reference to the literature (Faris and Fares, 1995; Bouznad *et al*., 1996; Onfroy *et al*., 1999) on the characterization by RAPD (Random amplified polymorphic DNA) of the *Ascochyta* species, this type of studies require to test a greater number of isolated strains of various geographical origin with various molecular markers.

The objective of this survey is for to characterize, on the morphological and molecular plans, the species of *Ascochyta/Pisum sativum* and also to research on a molecular polymorphism between three species of pea pot culture, located at three different zones of the North-West of Algeria. The aim of this study is to determine the taxonomy of the Ascochyta three species on *Pisum sativum* for serving like basis to a epidemiological approach of these pathogenical agents.

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MATERIALS AND METHODS

Biological materials: The used isolated strains were obtained from different organs of pea (*Pisum sativum* L.) that show characteristic symptoms of the anthracnose. Samples of the isolated strains were selected on several agricultural years: 2004-2005, 2005-2006, 2006-2007 and 2007-2008 in north-west zone of Algeria represented by three departments (Fig. 1): Mostaganem (Stidia and Ouréah cities), Relizane (Yellel and Bel-hacel cities) and Mascara (Mascara city). In the other hand, the setting *in vitro* culture of selected contaminated stumps, took place at the laboratory of the plants protection at the University of Mostaganem. Thereafter, we have made the morphological and molecular analysis of the stumps to the laboratory of symbiosis and pathology of the plants of the Superior National School of Agronomy (ENSAT) at Toulouse (France) between 2006 and 2008.

Analytical methods

Technique of isolation: The pathogenic isolation consists in taking the fragments of leaves, stems and pods presenting the symptoms characteristic of the disease. After their disinfection with sodium hypochlorite at 2°C during 5 min, they are rinsed successively 3 times with distilled water, than drying on sterile blotting paper, the fragments are deposited in Petri dish in Potato Dextrose Agar (PDA) middle with a rate of 5 fragments per Petri dish. Each organ of the plant is treated separately (Rappilly, 1968).

After incubation, the aseptic transplanting was accomplished in the sound cultures that are taken on the level of the peripheral zone of the colonies. The operation is repeated until obtaining a healthy culture. Later, during the microscopic observation, we have isolated the strains having the *Ascochyta* properties for cultivating them in Mathur middle in order to promote the *Pycniogenesis* (Champion, 1997). In order to limit the risks of the strain variability, we have chosen the method of single spore's cultures according to the technique recommended by Bouznad (1989).

Characterization of the single-spores isolated strains is accomplished on the morphological criterion basis, like aspect and color of the colony, presence or absence of *Chlamydospores* and size and form of the *Conidia* (Bouznad, 1989; Maufras, 1997; Champion, 1997). To

Fig. 1: Studied locations

compare the studied strains of our three *Ascochyta* species, we have take like strains reference some strains acquired at the biotechnology laboratory of ENSA at Toulouse (France).

Molecular characterization

Amplification by PCR (Polymerase Chain Reaction): The number of isolated strains providing from respectively, Ouréah-Stidia (coastal zones) and Bel-Hacel-Yellel-Mascara (interior zones) were 15 and 16. The 3 referential strains of *Ascochyta* species retained were: *Ascochyta pisi*, *Ascochyta pinodes* and *Ascochyta pinodella*. After 15 days of incubation on Potato Dextrose Agar (PDA) middle (in continuous light at 25°C) (Rapilly, 1968), the thallus bits of each strain with approximately 1 cm^2 of surface were sown in roux flasks containing 150 mL of Potatoes dextrose brood (PDB) middle. Obtained *myceliums* are collected by a filtration system under vacuum pump while taking care to eliminate all traces from culture middle in particular, the agar-agar of the transplanting implants (Gill and Zentmyer, 1978). Three distilled water rinsing were made during the filtering time. For each strain, the *mycelium* is dried with sterile filter paper, then is weighed and finally is preserved at -20°C in foil (Gill and Zentmyer, 1978). The DNA extraction is conducted according to a protocol based on the use of the K proteinase (Saiki *et al*., 1988). This last is the degradation of the proteinic composition of the cellular walls and inactivation of DNAases (enzyme molecule who specifically recognizes DNA or RNA) and RNAases during cells bursting.

The CTAB use (Saiki *et al*., 1988) (powerful detergent) allows the solubilization of the cellular membranes and denatures proteins. One deproteinisation of the extract is obtained by a phenol/chloroform mixture (Saiki *et al*., 1988). At the end of purification protocol (Saiki *et al*., 1988), the DNA is made soluble in Tris-EDTA buffer solution. DNA concentration of our strains is obtained by dosage in spectrophotometer (Le Pennec, 1991).

Polymerase chain reaction analysis was done with the ITS1 and ITS4 markers (Saiki *et al*., 1988). In this case, each middle of reaction has a final volume of 25 µL, 5 µg mL^{-1} of DNA concentration and 30 cycles (Saiki *et al*., 1988). The amplification reactions are obtained by using a thermo-cycle apparatus programmed for 30 successive cycles: 5 min at 94°C (DNA denaturation), 1 min at 94°C; 1 min at 50°C; 2 min at 72°C and 10 min at 72°C (elongation) (Saiki *et al*., 1988). The obtained PCR products are separated according to the method of DNA electrophoresis on Agarose gel at 1.5% (Sambrook and Russel, 2001) and the amplified bands are visualized under UV rays and then photographed (Dubose and Hrtl, 1990).

Sequencing of ITS products: The sequencing preparation for the purification of the strains PCR products is made with the exonuclease enzyme, the final volume is equal to 12 µL (8 µL of PCR product and 4 µL of master mix) (White *et al*., 1992). The operation is realized by using a thermo-cycle apparatus with a cycle of 60 min at 37°C and 15 min at 80°C (White *et al*., 1992). Purified PCR products are prepared by sequence reaction with the Kit of Sequence « Big Dye Terminator» for the marker F-ITS1 and the marker R-ITS4 (respectively 2 µL of PCR and 18 µL of master-mix) for 38 cycles of 30 sec at 96°C , 15 sec at 55°C and 4 min at 60°C.

RESULTS

Morphological characterization: The characterization led to three categories of isolated strains:

- Enough clear colonies, white with pink blade (Fig. 2a) with presence of bicellular spores with big size included between (10.8 and 13.5)×4.2 µm (Fig. 2b) and absence of *Chlamydospores* characterizing the *Ascochyta pisi*
- Colonies of the gray color (Fig. 3a) with an abundance of *chlamydospores* (Fig. 3b) that generates the presence of dark green to gray aspect colonies and single spore conidia (Fig. 3b) of included size between (5.4 and 8.1)×3.7 µm characterizing the *Ascochyta pinodella* species
- Colonies of clear gray color intermediate between the two quoted categories previously (Fig. 4a), characterizing the bicellular conidia (Fig. 4b) whose the size is between (8.1 and 10.8)×5.1 µm and a weak presence of chlamydospores as typical form of the species *Ascochyta pinodes*

Molecular characterization

Amplification by PCR: All the isolated strains whose ITS region is amplified by PCR gave identical results with of the same size PCR products (550 Pb). No polymorphism is thus noted at together of tested isolated strains (Fig. 5).

ITS products sequencing: The preparation for the sequencing of the PCR products of the ITS regions of the DNA of each isolated strains has been achieved to the laboratory of symbiosis, improvement and protection of the plants at ENSA of Toulouse (France). The sequencing

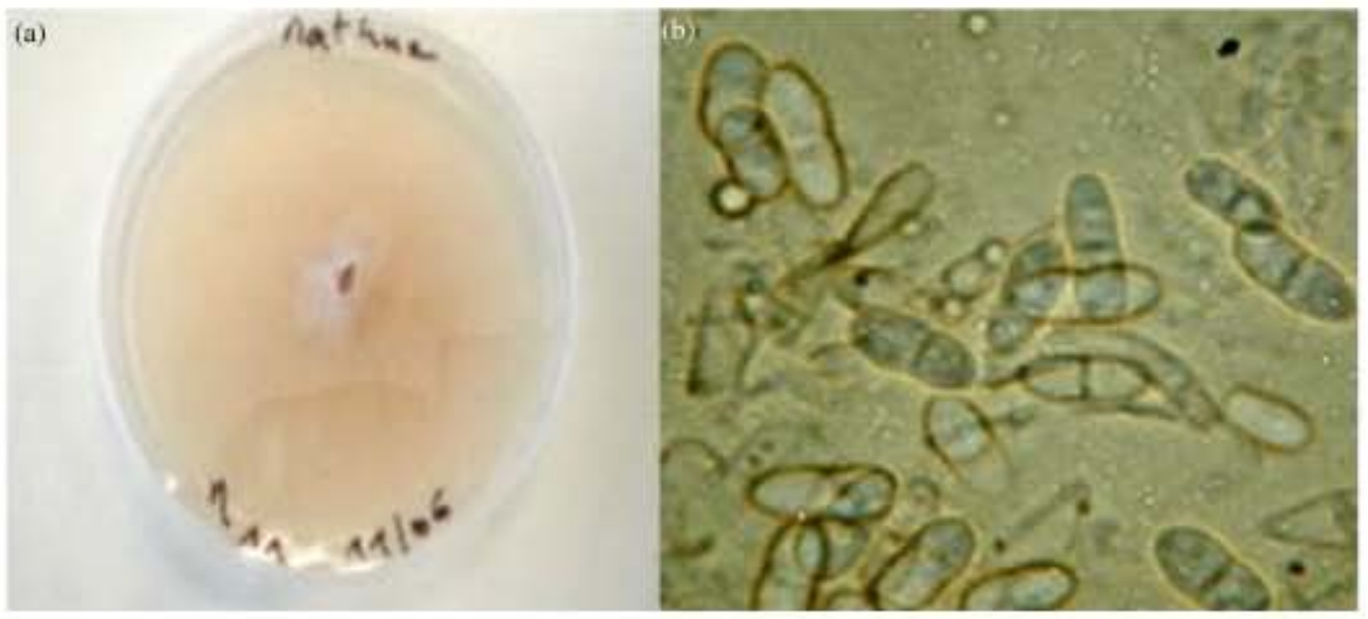

Fig. 2: (a) Color and aspect of the *Mycelium* colonies on Mathur middle and (b) Microscopic sight (x400) of *Ascochyta pisi* conidia

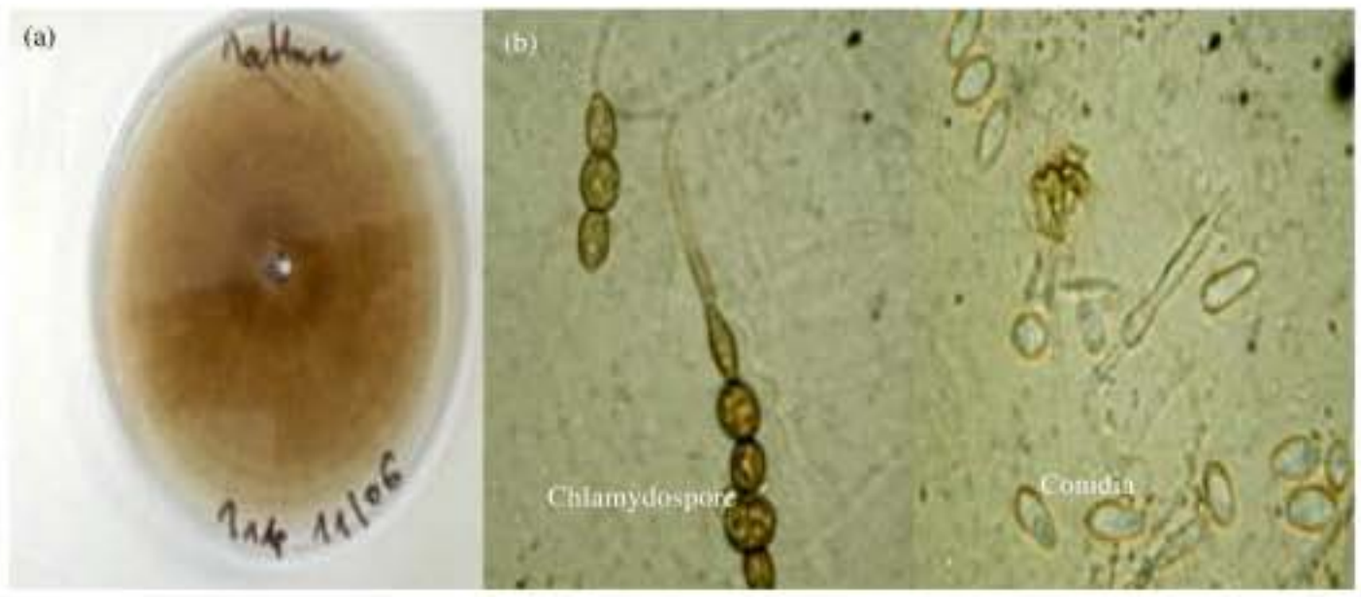

Fig. 3: (a) Colonies aspect on Mathur middle and (b) single cellular spore and chlamydospores (x400) of *Ascochyta pinodella* conidia

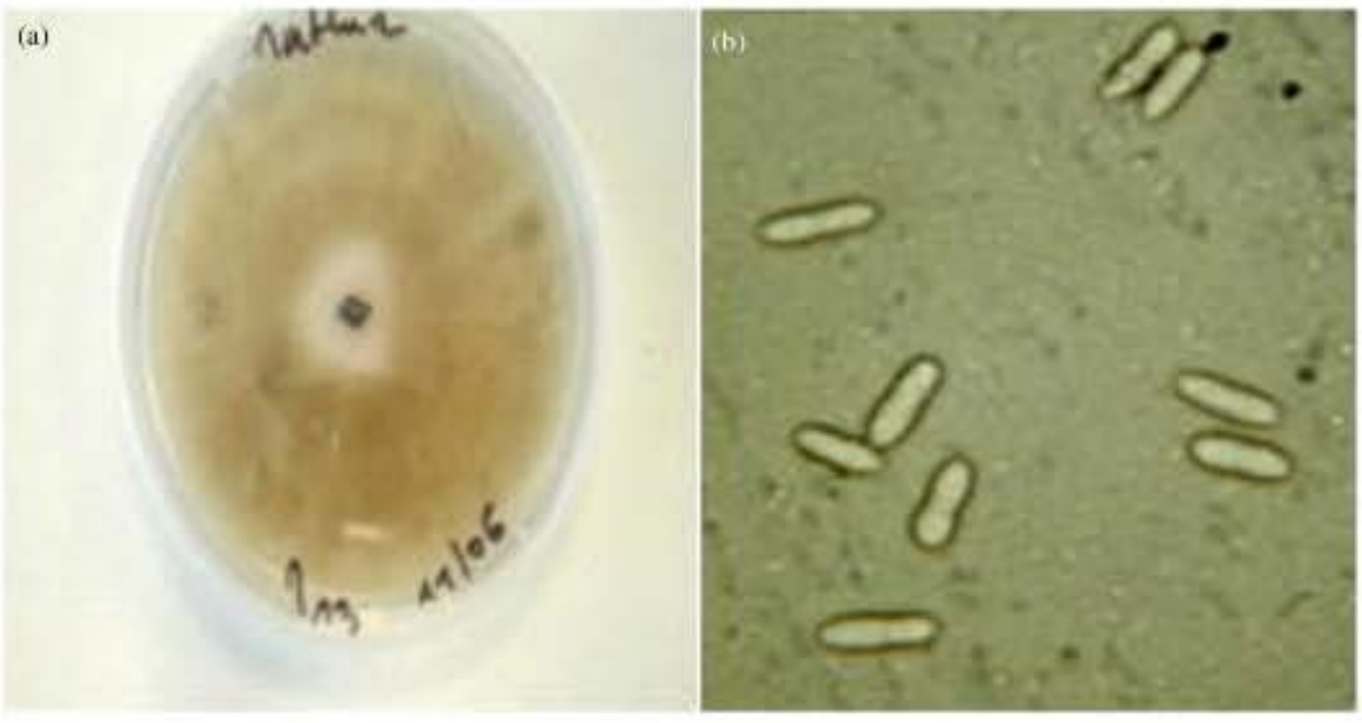

Fig. 4: (a) Colonies aspect on Mathur middle and (b) microscopic sight of *Ascochyta pinodes* conidia (x400)

results are analyzed by the software Blast Search Results, that shows the strains: S_{17}, S_7, S_2, S_6, M_{17}, O_6, M_{18}, S_{10}, S_{30}, M_4, M_{21}, M_6 , M_{10}, M_{21}, M_3 , M_8, M_2, O_7, belong all to the species of *Ascochyta pisi*. Nevertheless, the other strains: S_{18}, M_{13}, M_{14}, S_{13}, BH_2, Y_1, S_4, belong to the two species: *Ascochyta pinodes* and *Ascochyta pinodella*.

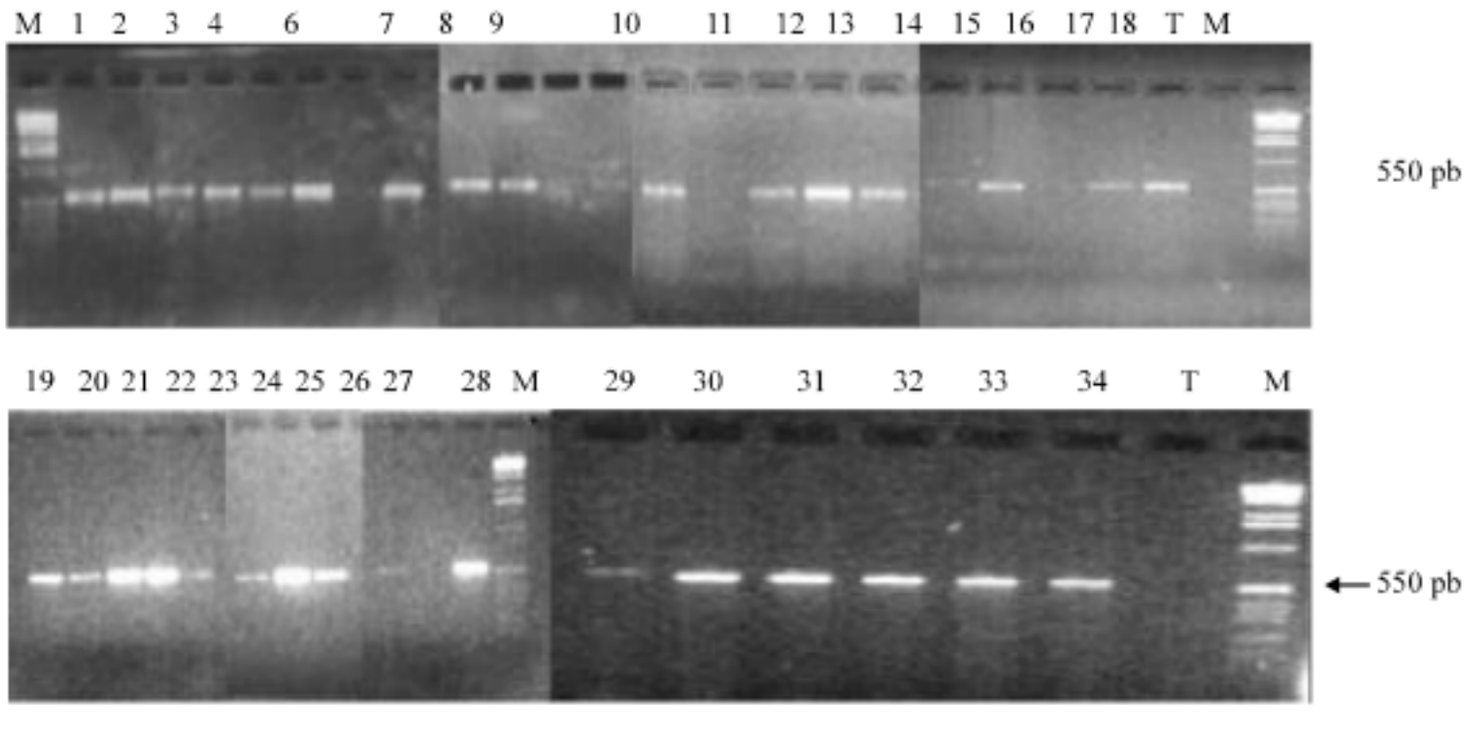

Fig. 5: Electrophoresis patterns on Agarose freezing showing amplification regions of DNA ITS1 and ITS4 by PCR for isolated *Ascochyta* species strains, M: Molecular Marker (1 Kb); T : Witness. Isolated strains are identified by their number as Reference Strains: 20 and 23, Mostaganem: Stidia: 13, 24, 25, 26, 27, 28, 29, 30, 31, 33, 34, Ouréah: 5, 18, 19, 21, Relizane: Bel-hacel: 1 and 2, Yellel: 3 and Mascara: 4, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 22, 32

DISCUSSION

One of the first comments of these results, is that our measurements are included among the interval (11, 5 to 14 µm) of results founded by Sutton (1980). At the most, *Ascochyta pisi*, is remained dissociated with any presence of chlamydospores according to our observations, but very present only on the two other species. What confirms the quotations of Maufras (1997), where the presence or absence of chlamydospores, remains a pertinent differentiation criteria for only *Ascochyta pisi* where we notes a total absence of chlamydospores. On the other hand, our observations made on *Ascochyta pinodes* showed the presence of chlamydospores equipped in bicellular spores with a size of 8.1 to 10.8 µm. This size is intermediate between those *Ascochyta pisi* and *Ascochyta pinodella*. Also for *Ascochyta pinodes*, of other measurements found by Bouznad (1989): 6.25 to 16, 25 µm and Maufras (1997): 8 to 16 µm, are very near of those that we found. Concerning *Ascochyta pinodella*, our measurements and observations gave weak spores sizes between 5.4 and 8.1 µm but with single-cellular form, what corresponds to sizes reported by Bouznad (1989) who vary between 3.75 and 10 µm.

Some small divergences exist on the ITS of the three pathogenic agents of the complex *Ascochyta/Pisum sativum* and were reported by Faris and Fares (1995). In this order, always according to this author, some non-negligible differences exist between the IGS (Internal Genic Space) region of *Ascochyta pisi*, *Ascochyta pinodes* and *Ascochyta pinodella*. These two last species present some small differences but cannot, until now, being differentiated with certainty. We also mentioned that the use of the RAPD marker in *Mycosphorella pinodes* and *Phoma médicaginis* var. *pinodella* could give no distinction between them (Bridge and Arora, 1998). According to the works of Gardes and Bruns (1996), the species whose features are closely linked, are not sufficiently different to the interior of their ITS for to discount to see a polymorphism. What agrees with the absence of polymorphism observed in the ITS1 and ITS4 regions of our studied species of *Ascochyta pisum* and *Ascochyta sativum*.

According to Henson and French (1993), the ITS allow to differentiate the species inside a kind or among a population. Thus they are largely used, for the manufacture of specific markers of gender identification and/or detection, but only for small percentages (< 2%) of genetic variability observed on the mushrooms (Gardes and Bruns, 1996). In a study on *Verticilium dahlia*, Bellahcène *et al*. (2005), also shows that the PCR amplification of the ITS region of ribosomal DNA by two universal markers reveal the presence of a single fragment with same length of 550 pb. Otherwise, it is important to recall that the works of Bellahcène *et al*. (2005), led to the similar results to ours although they concern another species: *Verticilium dahlia*.

When we study the very close organisms, the polymorphism becomes insufficient. It's the case of the two species of *Ascochyta pinodes* and *Ascochyta pinodella*. Bouznad *et al*. (1996) reported that the *Ascochyta pisi* species is very different from *Ascochyta pinodes* and *Ascochyta pinodella* with the RAPD marker and also with five other markers (results not published

again). The kinds taxonomy of *Ascochyta* and *Phoma* remains complex although these two kinds are relatively close.

Consequently, the ITS regions of our studied strains that were taken at the three geographical places remain preserved and do show no polymorphism variability. Nevertheless, it be necessary to consider the use of other molecular markers in order to prospect other DNA regions and consequently to distinguish the differences between and intra specific.

The sequencing of ITS products results shows the existence of a sequencing homology between *Ascochyta pinodes* and *Ascochyta pinodella* species. Otherwise, only the *Ascochyta pisi* species differentiates itself distinctly of the two other species. That agrees the works of Faris and Fares (1995) and Bouznad *et al*. (1996), who affirm that *Ascochyta pinodes* and *Ascochyta pinodella* are possessing the very near taxonomies. We recall that the (no-polymorphism) observed between these two species, in the case of our survey, is mainly due to a homology of their nucleotide sequences in the ITS1 and ITS4 regions. Our study showed that the DNA amplification through ITS1 and ITS4 regions did not give a possible way to differentiate between these two last species. We recall that our results show that the three studied *Ascochyta* species are present on the cultures of green pea in the region covered by the survey. *Ascochyta pisi* is especially, the most widespread specie during the stages of formation and filling of plant cloves. In this survey we have characterized these three species on the plans morphological and molecular.

CONCLUSIONS

In order to characterize the studied strains for this work, we initially chose traditional methods based on morphological characters. One of the first morphological results shows that our strains measurements are included through the intervals reported by the literature works. In the other hand, the three studied species *Ascochyta pisi*, *Ascochyta pinodes* and *Ascochyta pinodella* are separated in three classes and constitute potential agents of contamination of the pea (*Pisum sativum* L.) but their distinction through the symptoms observations, remains random. It is probably linked to, the extreme variability of strains according to the culture middle that we have observed it in laboratory. Otherwise, all the isolated strains whose ITS regions were amplified by PCR expressed identical results with the same size of PCR products (550 Pb). No polymorphism is thus noted for all tested strains. On the other hand, the Sequencing allowed distinguishing *Ascochyta pisi* from *Ascochyta pinodes* and *Ascochyta pinodella*. These two last species remain very similar in their ITS1 and ITS4 regions and do not show differences. All molecular markers that we have used for polymorphism study of *Ascochyta pisum/Sativum* like the RAPD, ITS1, ITS4, RFLP and sequencing of ITS1and ITS4 don't have permit to show a polymorphism between *Ascochyta pinodes* and *Ascochyta pinodella*.

We think that it's thus necessary to pursue the molecular analysis study with other markers, particularly the microsatellites and SNP in order to search a polymorphism between the complex *Ascochyta/Pisum sativum* and principally between *Ascochyta pinodes* and *Ascochyta pinodella*. Nevertheless, we recall that we didn't find in the literature, some molecular survey on these three studied species in the Algerian north-west, in spite of their damages on the pea culture. The relative difference of our results with the other results of the literature, resides in the survey of the molecular aspect of the ITS1 regions and ITS4 of the DNA non exploited and insufficiently studied at this time. The previous studies were about other molecular scorers: RADP and RFLP.

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