Morphological and Genotypic Characterization of Fungi Associated with the *Ascochyta* Blight Complex in Western Regions of Algeria

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Abstract

The study is conducted in two growing areas of garden pea (*Pisum sativum* L.) in northwestern Algeria. Damages caused by *Ascochyta sp* complex are important in particular for the variety of *Kelvedon* Wonder. Observations carried out on the infected plants for several years, indicate the presence of superimposed necrosis of different sizes on all aerial organs. However, these observations do not differentiate symptoms by species. The results of morphological and molecular characterization with sequencing in internal transcribed spacer (ITS) regions and inoculation tests on 32 isolates in the laboratory of symbiosis and plant pathology from Toulouse (France), show a reconciliation of the sequencing by polymerase chain reaction (PCR) products and size necrosis for all *Ascochyta pinodes* and *pinodella*. Alone, *Ascochyta pisi* is distinguished by a smaller size necrosis. On the molecular level, all isolates whose **ITS** regions were amplified by **PCR**, expresses similar size products (550 **bp**). This molecular weight is found on a large set of pathogenic fungi. The three species of *Ascochyta sp* complex do not exhibit polymorphism for *Pisum sativum* species and have an identical molecular weight. The pathogenicity tests performed showed differences in aggressiveness on the host plant. Ascochyta pinodes is the most aggressive than the other two species. As a result, it causes more damage to the crop.

Keywords: *Ascochyta* sp., *Pisum* sativum, PCR, sequencing, inoculation tests

Introduction

Grain legumes such as pea, chickpea, bean and lentil play an important role in human nutrition as a source of protein and carbohydrate. Their worldwide production covers between 22% to 45% of the cultivated areas (Kelley et *al*. 1997; Tivoli, 2006).

However, the cultivation of pea is confronted to some constraints such as

drought that can extend on many consecutive years, the soil salinity and some pests or diseases. Among the pea diseases, one of the most important is anthracnose or ascochytose. It is caused by 3 fungal species: *Ascochyta pisi* Lib., *Phoma medicaginis* (*Ascochyta pinodella* L.K. Jones.) and *Mycosphaerella pinodes* (*Ascochyta pinodes* L.K. Jones), generally known under the generic name "*Ascochyta* complex".

These 3 pathogens cause necrotic lesions on various plant organs that decrease yield and quality of the yield (Allard et *al*. 1993; Davidson *et al.* 2011; Cesnuleviciene *et al.* 2014). The identification of the three species by visual assessment of the symptoms is difficult since all this species may occur on one diseased plant. However, their identification is a necessary step for pest control by fungicides or crop improvement programs by selection of resistant varieties. The present work concerns the occurrence of Ascochyta on pea cultures in western Algeria, in the regions of Mostaganem and Mascara.

Material and methods

Fungal strains

The fungal strains used in this work were sampled from pea plants showing typical anthracnose symptoms, in zone 1 of the coastal region of Mostaganem - Oureah (O) and Stidia (S) – and in zone 2 of the interior plains of Mascara (M). They were cultivated on PDA medium at 24°C. Reference strains of *Ascochyta pisi*, *A. pinodella* and *Mycospherella pinodes* have been obtained laboratory in Germany and were cultured under the same conditions.

Methods

Sampling and symptom scoring.

Seventeen plots have been studied in the two sampling zones and fungal isolates were obtained from various organs of pea plants with necrotic symptoms. In zone 1 isolates were sampled from the variety "Merveille de Kelvedon" Wonder during February when pod filling has been completed, in zone 2 sampling was carried out from the variety "Gros vert" during April when pods were being filled.

15 plots have been assessed for each zone, with 100 plants observed per plot. The level of pathogen attack was estimated as the percentage of the plants showing typical symptoms.

Isolation of fungal strains

The pathogens were isolated from diseased plants by following the method described by Rappilly (1968). Fragments from leaves, stems and pods with typical symptoms were surface-sterilized in a 2% sodium hypochlorite solution, rinsed with sterile water and put onto PDA medium. After 10 days of culture at 21°C, isolates with typical Ascochyta morphology were sub

cultured onto Mathur's medium for spore production (Champion, 1997), and monosporic isolates were prepared with the technique described by Bouznad (1988).

Fungal culture

The fungal isolates were grown on PDA medium for conservation, on Mathur's medium for observations and sporulation, and on PDB medium in Roux bottles for the production of mycelium. Culture conditions were 24°C and a 12 hours photoperiod.

Mycelium was harvested by filtration after 2 weeks of culture, rinsed with distilled water and dried superficially on filter paper, and was then stored at -20°C.

Molecular biology methods

DNA was extracted from fungal mycelium with CTAB-containing extraction buffer, and proteinase K treatment. A spectrophotometer at 260 nm, was been the measurement mean of the DNA concentrations. The ITS region was amplified by PCR with primers ITS1 and ITS 4 in a total volume of 25µl containing from x 2ng-5ng of DNA as a matrix, x 0.50µl dNTPs . The thermocycler was run with the following programme: 5 min at 94°C followed by 30 cycles of 1min at 94°C, 1min at 50°C and 2 min at 72°C, followed by a final extension step of 10 min at 72°C.

PCR product were analyzed by electrophoresis on a 1,5% agarose gel and bands were scored under UV after ethidium bromide treatment of the gel.

The amplified ITS regions (8 µl of reaction) were purified by incubation with exonuclease in a final volume of 12 µl at 37°C for 1 hour, after which the enzyme was inactivated by a treatment at 80°C for 15 minutes. Purified PCR products (2 µl) were sequenced with the ''Big Dye Terminator '' kit using the primers ITS1 and ITS4 in a final volume of PCR product. Indeed, 2 µl of PCR product were added to 18 µl of mastermix and then incubated for 38 cycles (30 seconds at 96 °C, 15 seconds at 55 °C and 4 minutes 60 °C).

Aggressiveness test of *Ascochyta* strains on leaves maintained in survival

The aggressivity test is conducted on sheets placed in Petri dishes and kept in survival. The inoculation is made on sterilized paper soaked by sterile-distilled water by pouring in the center a drop of spore suspension. This test allows to appreciate not only the differences in aggressiveness between species but also the level of resistance of the two pea varieties tested (Onfray and Tivoli, 2005).

The central leaves of the host plant were taken at flowering stage, from on subjects grown in a growing room under controlled conditions. The inoculation by the *Ascochyta* strains is performed at the center of sheets with

10 µl of inoculum concentrated at 10^6 spores.ml^{-1} of sterile distilled water. Infection is subsequently evaluated by measuring the diameter of necrosis that appears on the inoculated leaves.

Results and discussion

Morphological characterization of fungal isolates

The three species *Ascochyta pisi*, *A. pinodella* and *Mycospherella pinodes* often occur together in a field. Lesions caused by *A. pisi* are sharply delineated and distinct from those caused by *A. pinodella* and *M. pinodes*. However, a classification of the species based on symptoms has not been attempted during sampling.

Monosporic isolates were cultivated on Mathur's medium, and colony morphology (texture, colour, presence of chlamydospores) as well as the form and size of conidia were analyzed for a first step of identification of the species (Champion et *al.* 1984; Bouznad, 1988; Maufras, 1996; Champion, 1997; Hafiz *et al*. 2014). The three references strains were grown under the same conditions for comparison.

Isolates with constantly lightly colored, white to pale pink colonies and forming bicellular spores of relatively large size (10,8 to 13,5 µm x 4,2 µm). Chlamydospores were absent. This group contained 23 isolates, obtained from both sampling zones.

Isolates with grey colored dark greenish colonies that produced mono cellular spores of small size (5,4 to 8,1µm x 3,7µm). An abundant production of chlamydospores was observed. Only three isolates corresponded to this type, they were all obtained from one sampling zone.

Isolates with colonies of light grey colour, intermediate between the two described above, with bi-cellular spores of medium size (8,1 to 10,8 µm x 5,1µm), and only sparse occurrence of chlamydospores. This group contained 6 isolates, obtained from both sampling zones.

The characteristics of the first group are well in agreement with the description of *A. pisi*, (those of the second group with *M. pinodella* (refs), and those of the third group with *A. pinodes* (Anselme and Champion, 1962; Punthllingham and Hollyday, 1972; Maufras, 1996; Liu *et al*. 2013).

The two species *A. pinodes* and *M. pinodella* are more closely related and distinction by morphological criteria is sometimes difficult.

Pathogenicity assays on pea leaves showed that *A. pisi* is less aggressive

Algeria and caused the most severe damage to pea culture. *A. pinodes* although limited in occurrence also caused notable yield losses, and *A. pinodella* was found only rarely found.

Molecular identification of isolates

Identification and classification of organisms have been improved greatly by the use of techniques involving characteristic traits of proteins or DNA sequences. Notably PCR amplification of the sequence of Internal Transcribed Spacer (ITS) and Intergenic Transcribed Spacer (IGS) regions within the ribosomal operon is a widely used method for the classification of fungi (White et al. 1990; Henson and French 1993; Gardes and Bruns 1996; and Padder BA *et al*. 2012).

The ITS regions of monosporic isolates were amplified by PCR and analyzed by gel electrophoresis. A DNA band of ca. 550 bp was obtained for all strains. This size is in agreement with the ITS sequence in the genus *Ascochyta*. The absence of a strong polymorphism in the ITS regions of the three *Ascochyta* species on pea (Faris-Mokaiesch, 1995) precludes further classification based either on the size of the PCR products, or on analysis of fragments obtained after digestion with restriction enzymes (Gardes and Bruns, 1996; Faris-Mokaiesch et *al*., 1996). Hence, the amplified ITS sequences were purified and submitted to sequencing by the Sanger method. Sequence analysis by BLASTn confirmed the identity of *Ascochyta* sp. for all isolates and has allowed to clearly to target A. *pisi*). However, the two species *A. pinodes* and *A. pinodella* appear as very closely related and could not be separated on the basis of their ITS sequence. Compared to the morphological characterization of our study, molecular analysis confirmed the identification of *A. pisi* and that this was the dominant specie in both sampling zones

Aggressivity of *Ascochyta* strains

The results of the average diameter of necrosis on leaves, ten days after inoculation, for both varieties ”Merveille de Kelvedon” (V1) and " Gros vert " (V2), show few differences between them.

However, the aggressivity tests are positive for all inoculated leaves that present necrosis well developed and evolutive, five days after inoculation.

Indeed, analysis of variance performed using the StatBox software (version 6), shows a varietal effect not significant. In addition, it shows that the reduced size of necrosis provoked by strains M11, M17, M3, M1, O7 and S10 of *Ascochyta pisi* are significantly different from those induced by strains M12, M13, S5, S13 and S23 of *Ascochyta pinodes and Ascochyta pinodella*

From the fifth day, we observed that some extensions more or less etended accompanies necrosis according to the strain. The fruiting bodies in the form of pycnidia appeared during this sequence. The measurements on the size of necrosis were performed 10th day after inoculation. The results revealed differences in aggressiveness between A. pisi and the other two species. Necrosis caused by strains of the species *A. pisi* have a limited growth and were non-coalescent.

Punthlingam and Hollyday (1972), described the necrosis of *A. pisi* like non-coalescent, first translucent, then light brun in the center surrounded by a dark brown area diffuse, which corresponds to our observations. In the center of each necrosis, many pycnidia brown color more or less grouped are formed .

The central translucency of necrosis is likely due to cellular degradation of the host. We have not observed of secondary lesions on tissue located outside of the brown margin that will can showing their damage. Heath and Wood (1971) think that this is the maceration of enzymes that involved in the genesis of necrosis with limited sizes under effects of *A. pisi* and *A. pinodes*. According to Van Etten et *al*. (1989), *A. pisi* seems more sensitive to pisatin (main phytoalexine of pea) compared to other parasites of the same host.

However, A. *pinodes* and A. *pinodella*, cause necrosis more plated out, with larger sizes, without distinct margin and generating small lesions by coalescence .

The works of Wood (1967) and Heath and Wood (1969 and 1971), reveal a physiological explanation of necrosis genesis caused by *A. Pisi* and *A. Pinodes*, but that it's not yet completely defined. However, Allard *et al.* (1993), having worked on the pea anthracnose, consider in the same way that our results, that *A. Pinodes* and *A. pinodella* are resulting in similar damages (Na Liu *et al*. 2016).

For Tivoli and Banniza (2007), necrotic lesions that develop on the aerial part of the plant under the effect of Ascochyta are generally different but may present, in some cases, resemblances between those caused by *A. pinodes* and *A. pinodella*.

Conclusion

The results of characterization of *Ascochyta sp* complex on *Pisum sativum* obtained in this work show morphological and molecular differences between *Ascochyta pisi* and *Ascochyta pinodes* with *pinodella*. These differences are also evident in terms of virulence and aggressiveness on the pea.

Indeed, *Ascochyta pinodes* and *pinodella* seem more destructive and cause significant damage on the growth and production of peas. The attack begins with *Ascochyta pinodella*, which deteriorates the collar and the first leaves of the plant followed by the *Ascochyta pinodes* rather that affects organs: leaves, stems and pods. *Ascochyta pisi*, being sensitive to the pisatin, intervenes at the end of cycle when pea is weakened by attacking the pods. Due to the size of its relatively large spores, it acts with a low virulence which causes limited necrosis on the leaves.

Conversely, *Ascochyta pinodes* characterized by sizes less large of spores acts with a higher virulence that resulting larger necrosis, compared to these of *Ascochyta pisi*. *Ascochyta pinodella* with spores sizes even smaller, acts with a similar virulence to that of *Ascochyta pinodes*.

The sizes of the spores and the results of (ITS) sequencing appear in relationship to the levels of virulence and aggressiveness of the three species of *Ascochyta sp.* complex, on *Pisum sativum*. In the case of this study, the results of sequencing the ITS are very close to between the two species of *Ascochyta pinodes* and *pinodella* that are most aggressive on the pea. This militates in favor of more thorough molecular characterization of these two species.

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