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The histopathological trend towards infection of wheat plants by *Septoria tritici* in province of Khuzestan, Iran

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Wheat *Septoria* leaf blotch is one of the most important diseases of wheat in Khuzestan province. It has been spreading widely and making severe damages specially on sensitive wheat cultivars in the recent years. Greenhouse raised seedlings of wheat Falat cultivar at two-leaf stage have been inoculated with 10^6spore/ml of spore suspension. Inoculated seedlings were then stained and examined after 24 hours. Histopathological studies showed that stomata are the main entrance point of the fungus into the host cell. Following germination, each pycnidiospore produces germ tube which penetrates to stoma through producing a fine appressorium. A thin hyphal branch then emerges from each appressorium which penetrates into the cell. There is also evidence of extension some branches of hypha to mesophyllic cells. Fungal developing hypha in substomal chamber produces a mass of mycelia which gradually change toward initiation of pycniduim walls. Mature pycnidia appear in substomal chamber 20-28 days after inoculation. Thus, each diseae cycle of *Septoria* leaf blotch takes about 25-30 days according to climatic condition of Khuzestan province.

Key words: *Septoria tritici*, wheat, histopathology, Iran

Introduction

Septoria leaf blotch is among the most important diseases of wheat throughout the world. The causal agent, *Septoria tritici* Robex. Desm. belongs

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to order Sphaeropsidales, Class Deutromycetes. In sexual stage it is associated with an ascomycetous fungus *Mycosphaerella graminicola* (Fuckel.) Schrocter. that is classified in family Dothideaceae under the order of Dothideales. *Septoria* leaf blotch of wheat was first reported from France by Desmazires in 1842 and subsequently from other parts of the world (Cohen and Eyal, 1993). Petrak and Esfandiari (1941) reported the disease from Iran. Presence of *Septoria* leaf blotch then reported by Ershad (1995) from different wheat growing areas of the country. Under favorable weather conditions, epidemic of the disease can cause losses up to 40 percent of the yield per year (Eyal and Ziv, 1974). In its anamorphic stage, *Septoria tritici* produces dark brown to black globose to eliptical pycnidia on leaf blades, sheats as well as other foliar tissues of the host plant. Inside the pycnidia there are a large numbers of long, filiform, three to several-celled conidia (pycnidiospores). Dissemination of the diseases is carried out by wind born and rain splashed conidia. The teleomorphic stage belongs to Ascomyceteous fungi which produces asci within pseudothecia that each of them usually contains eight ascospores (Eyal *et al*., 1987). Both ascospores and conidia have been reported as primary source of inocula in different countries. The severities of disease on short stem high-yield wheat cultivars are much more important than those of long stem cultivars. The fungus is capable of affecting wheat host plant at each of its growing stages and can cause notorious epidemics under favourable conditions. Appearance of the disease varies greatly depending on the region and environmental condition. The first symptoms are usually seen on the leaves that are in contact with soil as small pale spots wherever gradually extend to become chlorotic lesions which then with the death of affected cells turn to necrotic blotches. Soon after the fungus established, the blocthes are speckled with small, submerged, black pycnidia. Under favourable weather the spots may coalesce and produce larger blotches that result in partly or entirely death of the leaf blades. Pycnidia are generally produced in lines alongside with leaf veins. In favourable weather conidia germinate after releasing from pycnidia. Germination of conidia occurs either through direct germination of cells at both ends of spores or through budding. In laboratory condition germination of conidia as well as penetration occur after 12 and 24 hours following the inoculation, respectively. The fungus finds its way into the host via stomata or by direct penetration through epidermal tissue (Eyal *et al.*, 1987). Evidence of the first disease symptoms varies depending on environmental conditions. In 18-24ºC and high relative humidity the first chlorotic leaf spots appear 5-6 days and for necrotic spots 3-6 days after inoculation. Appearance of disease symptoms is directly affected by weather conditions, as in Khuzestan province leaf blotchs may be seen in early

December while it may be delayed to late December for Golestan province located in north of Iran (Torabi, 1979). Within pycnidia, conidia are being merged into a slimy or sticky matrix that is yellow to creamy colored, containing carbohydrate and protein materials. The matrix helps the survival of conidia in dry weather condition. When the pycnidia become wet, they swell and the conidia and their matrix exude out in long tendrils.

The sticky matrix is also maintaining the moisture by occluding the pycnidia ostiole in low humid condition (Eyal *et al*., 1987). Among the most important factors that affects penetration of the fungus are temperature and relative humidity (Hess and Shaner, 1985). Presence of moisture is a prerequisite for all development stages of the disease like germination, penetration and invasion as well as production of pycnidia (Hooker, 1957; Browning, 1979). There are several methods for isolation of the fungus from leaf samples using exuded pycniospores. Wheat seedlings are inoculated by spore suspensions applying quantified or non-quantified methods based on objectives of the investigation. Inoculation can be done by gently rubbing a piece of cotton, soaked with spore suspension on to host leaves. Adding one or two drops of a surfactant material like Tween 20 helps for better spreading of the inocula on the leaf surface. In this method which is very simple and proper for primary examinations, the amounts of inocula are not quantifiable and controllable. On the contrary quantifying methods allow the researcher to determine the amount of propagule which is used for inoculation precisely (Eyal *et al*., 1987). Mycelia of *S. tritici* grow into intercellular spaces of epidermal and mesophyllic tissuse as well as substomotal chambers of infected leaves. The hyphae often spread from an infected stoma to the adjacent ones and occupy all the substomatal spaces. Inside the substomatal chamber the fungus produce masses of hyphae which later pycnidia and their sporogenous hyphae initiate from them. Pycnidia only form in stomata in a shape that their arrangements are analogous to the linear distribution of stomata on the leaf surface. Torabi (1979) showed that *Septoria* leaf blotch of wheat is not a seedborn disease and at least three volunteer weed species namely *Poa annua*, *Hordeum murinum* and *Secale cereal* with their constant presence in farm lands involve in survival of fungus from one season to another in Iran.

Materials and methods

Sampling and isolation of causal agent from infected tissues

In 2001, field number 310, a field with severe infection background with Septoriose, located in Safiabad Agricultural Research Center of Dezful selected for sampling and performing field studies. The field was prepared for sowing and

then sowed with wheat Falat cultivar seeds that is sensitive to septoriose in fall of 2002. After emergence, plants studied to record the stages of disease development. Also, several samples were taken from different fields in North of Khuzestan province. Samples kept in paper bags and tagged separately for studies.

Identification of the species was carried out by using morphologic and morphometric characteristics of the isolated fungi from different infected leaf samples. Using water resistant glue, segments of infected leaves were sticked on the microscopic slides and examined by stereomicroscope (Olympus ZSH10) and bright field microscope (Olympus BH-2). Identification made by comparing the characteristics with different identifying key references (Eyal *et al.,* 1987). Isolations were carried out directly from infected tissues or indirectly in different culture media as well as single-spore subculture method.

Growth and reproduction of the fungus under experimental condition

Both liquid and solid culture media like Potato Dextrose Yeast Liquid Media, Yeast Sucrose Broth, Potato Dextrose Agar, Yeast Malt Agar and Czapek Dox V8 Agar were used for growth and reproduction of *S. tritici* (Eyal *et al.,* 1987).

Greenhouse inoculation of wheat seedlings

Six isolates of *S. tritici*, isolated from different hosts, were cultured in PDY Broth on a rotary shaker flasks for ten days (Eyal *et al.*, 1987). Spore suspensions of each flasks then strained with cheesecloth and diluted to prepare 10^6/ml spore suspensions. Then five pots of two-leaf stage seedlings of wheat plants were inoculated by 100ml of the spore suspension from each fungal isolate with a hand sprayer. The control treatment containing five pots was sprayed by distilled water. Pots covered with plastic bags and transferred to greenhouse at 20 ºC and 90% relative humidity.

Histopathological surveys

This research has been conducted to elucidate the pattern of penetration, temporal processes of disease development and production of pycnidia. To understand the manner of penetration, sampling from the inoculated seedlings was carried out 24 hours after inoculation by removing three leaves from each treatment. The leaves were segmented in to 2 centimeter equal pieces and then placed into vials containing 3:1 Ethanol 95% and Acetic acid glacial mixture for 48 hours. Then the leaves removed from the vials and after rinsing by distilled water placed in vials containing cotton blue lacto-phenol and mounted on glass

slides in a drop of glycerine and examined by a bright field microscope (Olympus BH-2). The process repeated 2, 3, 4 and 7 days after inoculation and all the results were recorded. To study events that occur after penetration and also production of pycnidia in host tissues, samples of a week-inoculated leaves were taken every second day, segmented in to 2-3cm pieces and stained by Cohen and Eyal (1993) method. The samples were cleared using Chloral hydrate solution and after 24 hours they were examined microscopically to investigate manner of haustorium development and colonization of host tissues as well as production of pycnidia by the fungus. The specimens were again mounted on glass slides containing a drop of glycerine 50% and examined by 400X magnitude.

Results and discussion

Isolation of the fungus from infected tissues

Among techniques which were used for isolation of *S. tritici*, indirect method that is suggested by Dadrezaee (1999) was distinguished more effective than others. Both PDA and YMA were proper for culture of the fungus where all the isolates had a similar growth rate in both latter media. Accordingly a week after incubation, white creamy to pinkish creamy colony of fungus appeared in both above mentioned media. Microscopic examination showed that there were wefts of loose mass of hyphae similar to pycnidia containing a large number of hyaline elongated conidia. Without any antibiotics, subcultures were prepared from colony of the fungus and incubated under same conditions for a week. In theses cultures, the fungus first produced fine light hyphae with elongated conidia resembling pycnidiospores but to some extent smaller than them. Two weeks later, dark green to dark blue mycelia which were thicker and wider than primary hyphae appeared. In fact they were secondary mycelia which initiated from primary fine hyphae. The secondary mycelia were probably sterile because no spore which could be associated with them was found. After three weeks, the mycelia changed in to dark brown to black leathery colonies protruded from culture media surface. Leather colonies were seldom capable of regrowing in fresh culture media and they apparently had lost their vitality after remaining for a few months in old cultures (Fig. 1a).

Culture media suitable for the fungus growth in laboratory

In this research two kinds of solid and two kinds of liquid culture media were used for growth and reproduction of the target fungus. Most of the isolates had an equal growth rate in both liquid media and they could produce a

large number of secondary conidia with concentration of 10^6 spore/ml, 7-10 days after incubation. Successfully growth of all the isolates was also observed in both soild media used in this study appeared as growth of pinkish creamy colonies of the fungus in PDA and Czapek Dox V8 Agar media 7 days after incubation. In the both solid media, after 7 days the diameter of colonies measured equally and around 2 mm.

Results of histopathological surveys

Inoculation and penetration of the pathogen

Penetration of the fungus was well documented through microscopic observations of stained leaf samples which were prepared 24 and 48 hours after inoculation of seedlings in greenhouse conditions. Results showed that germination of the conidia occur 12 hours after inoculation either through direct germination of cells at both ends of spores or through budding of middle cells. In each case, the emerging germ tube start moving on the leaf surface toward stomatal cell where it produces a fine appresorium which in turn gives out a penetration peg that reaches itself in to substomatal chamber. Some direct penetrations, less than 10 percent, through epidermal tissues and leaf surface corky hairs was also observed. Thus, 90 % of penetrations took place via stomata and indicated that the leaf stomata seem to be the main entrance way for the *S. tritici* in order to gain access inside its host (Fig. 1b).

Growth and development of pathogen into the host tissues

Hyphal clusters of *S. tritici* were observed inside the substomatal chamber in pieces of leaves stained by trypan blue. Microscopic examinations showed that one week after inoculation, some fungus hyphal branches also extended to adjacent stomata and leaf mesophyl tissues. There are no haustoria production were observed in examined infected tissues, so it seems that the fungus grows intercellular through host leaf cells.Ten days after inoculation, hyphal branches were completely developed in to several adjacent stomata. The infected tissues stained dark blue in trypan blue. Two weeks after inoculation, fungus developed by producing a mass of dark compact inseparable short hyphae which turned to spherical or elliptical shapes in infected stoma and hyphal branches continued to grow individually in leaf mesophyllic tissues. It was precisely coincided with appearance of necrotic spots on the leaf surface. The size of necrotic spots directly correlated with the number of infected stomata and mesophyllic tissues. Occurrence of infection in two adjacent stomata due to germination of two distinct spores were also

observed. In cases that the disease contracted from a stoma to adjacent ones, the symptoms were restricted to interveinal spaces, concluded the limitation of fungus spread by leaf veins (Fig.1c).

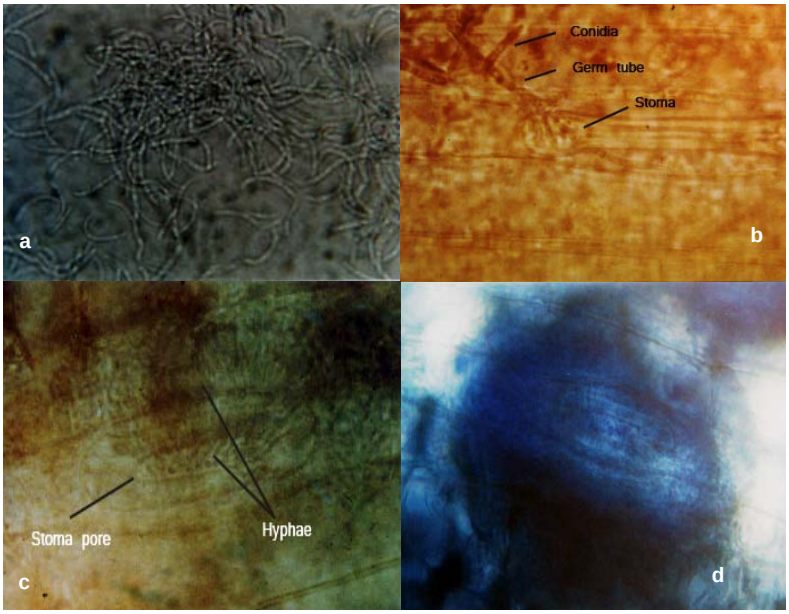

Fig. 1. Growth of *Septoria tritici* on medium (400X), a) germination and penetration of *S. tritici* through a stoma (400X), b) colonization of the substomatal chamber by the fungus hyphae (400X), c) formation of pycnidia in the host tissues (400X), d) formation of pycnidia in the host tissue (400X).

Development of pycnidia

The first symptoms of *S. tritici* pycnidia development were observed two weeks after inoculation in examined leaf samples as premature pycnidia in a shape of massive light to dark brown pseudoparenchymatous structures. Mature pycnidia could be observed about 18 days after inoculation which were brown to black spherical to elliptical structures with an apical ostiole that precisely took shape under the stomatal guard cells. The pseudoparenchymatous walls of pycnidia took shape from polyhederal dark brown cells. As a pycnidium is to be fully developed, other pycnidia begin to take shape close to it. Thus development of *S. tritici* pycnidia occurs alternatively. It means that in an infected tissue mature and immature pycnidia may be found at the same time (Fig.1d). In greenhouse conditions pycnidia appeare in the form of black specks on necrotic leaf spots of inoculated seedlings 3-4 weeks after inoculation. Disease cycle takes about 4 weeks in

Khuzestan province. It means that in favourable weather conditions, the fungus is capable to complete its life cycle in approximately one month. In this study, we found that less than 10 percent of infection occurred by direct penetration through intact epidermal cells and stomata are the main points of entry for the fungus to get access inside the leaf tissues. Following the penetration, substomatal chambers are the major sites for accumulation of infection hyphae where after colonization, the fungus produce its pycnidia in a shape that the pycnidium ostiole precisely locates under the stomatal pore. In this research, infections of leaf veins were not observed and all the blotches were restricted to interveinl spaces of leaves. The results of this study are in agreement with other researchers (Cohen and Eyal, 1993) which they believed that the stomata are the main entrance for the pathogen as well as the only production site of pycnidia. Among two different penetration ways, Torabi (1979) believed that direct penetration is more important than penetration through stomata and this is in contrary with the results of Cohen and Eyal (1993). The fungal hyphal branches also invade mesophyllic tissues and grow alongside the leaf veins where reaches itself to adjacent stomata and colonize them. Thus a single pycnidiospore can cause the infection of several stomata and subsequently production of several pycnidia in an infected tissue. Occurrence of the fungus hyphae in mesophyll cells of leaf also have been reported by Cohen and Eyal (1993). Studying the histopathology of *Mycosphaerella graminicola* on two different sensitive and resistant wheat cultivars, Kema *et al*., (1996) found out that the pathogen caused 89% necrosis and produced 86% pycnidia on leaf surface of sensitive cultivar while it affected 5% and 1% of those observed quantities on resistant cultivar, respectively. Whereas, there were no apparent differences between two cultivars until the time of penetration and colonization of stomata by the pathogen. Germination of conidia took place on the leaf surface from both ends and penetration happened via stomata and guard cells toward substomal spaces. No direct penetration was observed. Accumulation of pathogen mycelia in substomal spaces was also observed. More over using electron scanning microscopy to study the penetration process, they concluded that penetration could take place by producing appressorium or occasionally by forming appressorium like structures over stomata. These are in accordance with our investigation especially in terms of the penetration process. They also showed that the apparent feature of chloroplast of infected spongy parenchymal cells begins to alter i.e., they became concentrated and along with nuclei were driven toward cell wall presumably because the enlargement of central vacuole, 48 hours to 14 days following the infection. Collapse and drying of the mesophyllic cells also occurred 12 days after infection.

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