

## Microscopic Study on Expression of *Yr-18* Gene Related to Adult Plant Resistance in a Near-isogenic Line of Spring Wheat (*Triticum aestivum* L.) to the Stripe Rust (*Puccinia striiformis* f. sp. *tritici*)

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### ABSTRACT

In this study, isolate SR99-UA (Race 70E 128) of wheat stripe rust (*Puccinia striiformis* West. f. sp. *tritici* Eriks) was used to infect susceptible spring wheat (cultivar Thatcher) and its near isogenic line possessing *Yr-18* adult-plant resistance gene. Samples were taken at the flag leaf stage and observed using light, transmission and scanning electron microscopes. No major differences in the infection process were detected prior to haustorium formation, but sub-stomatal vesicles appeared to be formed after penetration, slightly earlier in susceptible than in the resistant genotypes. Higher numbers of haustoria were formed at any particular time in the susceptible cultivar than in the resistant near isogenic line 2-3 days after inoculation. In the early stages of infection, host cell necrosis was not observed in the susceptible host, but was occasionally seen in the resistant isogenic line. Fourteen days after inoculation, the number of necrotic cells of hypersensitive response were higher on the resistant genotype than on the susceptible cultivar at this time when many of the infected mesophyll cells of the resistant host had collapsed. The first clearly developing pustules were usually observed 12 days after inoculation on the susceptible cv. Thatcher and 14 days after inoculation on its resistant near isogenic line. Eighteen days after inoculation, the mean number of pustules produced, per unit of infected leaf area, was high on the susceptible cultivar but on the resistant genotype the pustules were much less in number, diminutive and poorly sporulating.

**Keywords:** Histological expression, Resistance, Stripe rust, Wheat.

### INTRODUCTION

Stripe rust of wheat caused by *Puccinia striiformis* West. f. sp. *tritici* Eriks (Stubbs, 1985) is one of the major diseases of wheat (*Triticum aestivum* L.) in various regions in the world (Roelfs *et al.*, 1992) such as Northern Europe and other cool temperate climates, although its range has become extended to warmer and more arid regions through the world (Spehar, 1966; Bamdadian, 1972).

The annual yield losses due to wheat stripe rust have been estimated up to 8-75% of total production (Stubbs, 1985). Stripe rust

infection in western Canada has decreased yield, by as much as 79% (Conner and Kuzyk, 1988). In 1993, yield losses due to yellow rust in some parts of Iran were estimated at about 1.5 million tones (Torabi *et al.*, 1995)

Various strategies for the control of cereal rusts, especially stripe rust, have been considered. The most important control strategy is the use of resistant cultivars. The ability of plants to resist rust infection has been known for many years. The first investigation into the inheritance of resistance to *P. striiformis* was made by Biffen (1905, 1912) who showed that the resistance of Rivet wheat

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(*Triticum turgidum*) was controlled by a single recessive gene. Genetic resistance is the most widely used and is an environmental friendly mean of epidemic control for the three wheat rusts, including stripe rust (Johnson and Law, 1975). Several resistance genes effective at the seedling and/or adult-plant stages exist in wheat germplasm (McIntosh *et al.*, 1998). A break down in the resistance to cereal rusts has frequently been reported in the world. During a period of ten years, the accidental introduction of a single race of *P. striiformis* in Australia occurred, where eleven new races have now been detected (Wellings and McIntosh, 1990). The in most recently detected virulence to the wheat stem rust gene *Sr31*, was reported from Uganda, which is responsible, in part, for the stem rust resistance in many growing regions of the wheat in the world (Pretorius *et al.*, 2000).

The effect of use of resistance genes (*Yr* genes) in Europe on the evolution of corresponding pathogen virulence is well documented has been discussed by Stubbs (1985). Johnson (1981) reported that resistance of some cultivars to stripe rust disease was durable, remaining effective over a considerable period of time while in significant commercial usage. Stripe rust durable adult-plant resistance as described by Johnson and Law (1975) and controlled by minor or additive genes is often described as slow rusting and is generally known as being more durable (Wellings and McIntosh, 1990; Singh and Rajaram, 1994).

Many microscopic studies of rust infection, on a range of host species have been reported and the histological effect of host-pathogen interaction in the cereal rust was reviewed by Rohringer and Heitefuss (1984). In particular, the infection of wheat by *Puccinia graminis* has been intensively studied by several authors including Harder *et al.* (1978, 1979a, b); Samborski *et al.* (1977a), and Skipp *et al.* (1974), but fewer studies have been concerned with *P. striiformis* infection. However, Goddard (1974) investigated the histology of wheat yellow rust interaction on seedlings displaying re-

sistance and the susceptible reaction type. Mares (1979a, b) produced a light and electron microscopic study of susceptible interaction. Mares and Cousen (1977); and Mares (1979b) reported the development of stripe rust in wheat cultivars possessing adult-plant resistance genes and compared it with susceptible hosts at different leaf stages. Both Goddard (1976) and Mares and Cousen (1977) described the production of infection structures of *P. striiformis* and recorded that, following penetration, the pathogen produced a substomatal vesicles. The latter authors reported that such vesicles were first observed between 8-36 hours after inoculation in both susceptible and resistant cultivars. Following vesicle production, these authors observed on all cultivars studied that the pathogen achieved little growth for a period of 2 or 3 days and suggested that the vesicle may be a resting organ, as described by Rothman (1960) for *Puccinia coronata*. Goddard (1974) observed some penetrations on cv. Maris Nimord without substomatal vesicle formation.

The most common reaction of resistant plants to infection by micro organisms is host cell death and this is probably of primary importance, in the obligate biotrophic pathogens which require living host cells for the survival. Stakman (1915) observed cell death in reaction to *P. graminis* and suggested the term "hypersensitivity" to describe this reaction. Since 1915, the role of host cell necrosis has been widely debated and this term was widely used to describe all such reactions. The actual role that hypersensitive cell death plays in resistance is often debated. Crute *et al.* (1985), in an extensive review of the mechanisms of resistance to fungi and bacteria, listed five possible explanations of occurrence of the hypersensitive response (HR) and its relation to disease resistance.

Several authors have discussed the mechanisms conferring compatibility or incompatibility to the host/pathogen interaction. Heath (1980, 1981a, b) distinguished between two types of incompatibility as "non-host resistance" and "cultivar resistance".

Many studies have characterized the resistance of plants to diseases by the various stages of the development of host-pathogen interaction at which incompatibility is expressed. Heath (1986) reviewed methods of resistance-towards fungal and bacterial pathogens in general and suggested that they can be divided into two overlapping categories. Firstly, those which act externally to the pathogen and interfere with tissue colonization and, secondly, those which directly disrupt the pathogens' integrity or metabolism.

According to observations by some researchers in recent years, active genetical defense against a pathogen is usually accompanied by the rapid death of one or more plant cell (Xu and Heath, 1998). This rapid localized cell death associated with disease resistance is known as the "hypersensitive response" (HR); increasing evidence has indicated that HR is a form of programmed cell death (Greenberg *et al.*, 1994; Heath, 1998) that has some features in common with mammalian apoptosis (Ryerson and Heath, 1996). Recognition of a pathogen by the plant presumably leads to a signal transduction cascade in the plant cells (Ebel and Cosio, 1994). The aim of this investigation was to study histologically the defense responses conditioned by the Yr-18 gene.

## MATERIALS AND METHODS

### Plant Material

This investigation was performed on Canadian spring wheat (*Triticum aestivum* L.) Thatcher, and its near isogenic line possessing Yr-18, the adult-plant stripe rust resistance gene. These lines were originally developed by Dyck and his co-workers and kindly provided to me by Ketuz, A., Agriculture Canada, Winnipeg. Thatcher carried the Yr-7 seedling resistance gene in addition to Yr-18, but this did not interfere with the results since the pathotype used in this study was virulent on Yr-7.

### Pathogen

Stripe rust isolate SR99-UA, designated 70E 128, multiplied on seedlings of the susceptible cv. Avocet and Keep. In our previous seedling and adult-plant evaluation (unpublished), both isogenic and near-isogenic lines showed compatible interaction with SR99-UA at the seedling stage. However, at the adult-plant stage, Thatcher showed high disease severity while its near-isogenic line possessing the Yr-18 gene had very low severity of the disease.

To establish the experiment at the adult-plant stage, 18 seeds of each genotype were sown in 12.5 cm diameter pots containing compost (Metro-Mix 292, Ltd, Terra), and inoculated at about 8-9 weeks after sowing, when, the flag leaf was fully expanded. Suspensions of fresh urediniospores in a light mineral oil (Soltrol-170) at a concentration of 5 mg ml<sup>-1</sup> were sprayed on the leaves. A light coating of oil was enough to ensure good infection. Inoculated plants were left at least one hour in order for the oil evaporate off the leaves. Then, the plants were sprayed with a little water, covered in plastic bags to maintain high humidity, and kept in the dark at 10°C for one day. After inoculation, the plants were moved to a growth chamber, set at 15°C with a 16 hours photoperiod and at a relative humidity ranging between 60-70%. The light intensity was approximately 8,000 Lux at seedling height.

For observation with light microscope, samples were taken two days after inoculation and then at 2-day intervals until 18-20 days after inoculation when active sporulation occurred. At each sampling time, 4-cm long leaf segments were taken 5-9 cm from the leaf tips.

The clearing and staining technique by Bruzzese and Hasan (1983) was used. The leaf segments were placed in boiling tubes containing a mixture (2:1) of ethanol:lactophenol anylin blue. Tubes were then placed in boiling water bath for two minutes and, then, allowed to cool. They were kept for at least two days at room temperature. This



treatment ensured that the stain penetrated the leaves. Then, leaves were removed from the staining solution, rinsed in water, blotted dry and immersed in a saturated solution of chloral hydrate (5 g in 2 ml distilled water) for 30 to 50 minutes. This procedure removed excess stain from the leaf tissue. Segments were then mounted on a microscope slide in 50% glycerin and covered with a coverslip for observation. Cells which showed the anylin blue stain, and became dark blue in color, were considered as necrotic, irrespective of any sign of collapse (Wright and Heate, 1984).

For studies with a transmission electron microscope (TEM), four segments were sampled 3, 8, 12 and 15 days after inoculation. These segments were further divided into 2×2 mm pieces and fixed for one hour in cold gluteraldehyde in a 0.1M phosphate buffer (pH 7.0) with 2% sucrose added. They were rinsed two times (5 minutes for each) with 0.1M phosphate buffer, and fixed for 1.5 hours in osmium tetroxide (OsO<sub>4</sub>) in 0.1M phosphate (1-2%). The leaf pieces were then dehydrated using a 30, 50, 70, 90 and 100% acetone series with 10 minutes for each concentration.

Finally, the pieces were transferred for two further periods of 10 minutes in 100% acetone. The preparation for TEM sample proceeded with the immersion of leaf pieces in a mixture (1:1) of spur resin with low viscosity and acetone and sample was rotated for approximately 1.5 hours on a rotator at 4rpm. The material was then transferred to 100% spur resin and rotated overnight. This resin was replaced with fresh resin and again rotated for 24 hours after which the small leaf pieces were transferred to rubber embedding blocks containing fresh resin. The rubber blocks were then placed in a polymerizing incubator at 60°C overnight.

After embedding was complete, the specimens were sectioned, parallel to the longitudinal axis of the leaf, using ultra microtome. Sections were mounted on copper grids and stained for 15-20 minutes with an aqueous uranyl acetate solution (Watson, 1957) and lead citrate (Reynolds, 1963), washing with

distilled water after each stain. Specimens were observed using transmission electron microscopy (TEM) at 60 kv.

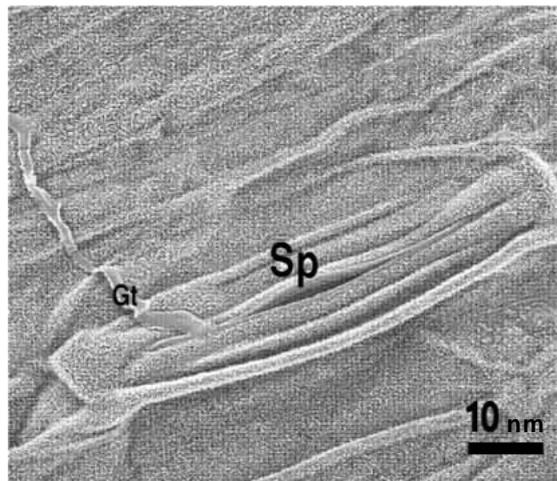
For studies with the scanning electron microscope (SEM), segments of leaves from plants susceptible to resistant hosts were sampled 18 days after inoculation. They were then mounted onto coverslips using double-sided sticky tape. The samples were then vaporfixed with 1% osmium tetroxide in water and air-dried in a fume hood at room temperature for about two days. Samples were mounted onto SEM metal stubs using double-sided sticky tape and secured with Marivac colloidal carbon paint. The specimens were then coated in a Nanotek, SEM Prep 22 with an approx. 15 nm gold layer and observed in a Jeol JSM 6301 XV SEM operated at 5 to 20 kV.

## RESULTS AND DISCUSSION

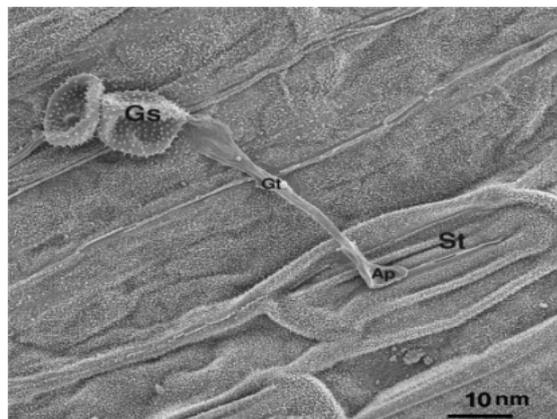
Spore germination on both susceptible and resistance hosts was generally high (over 60%). This result was in contrast to the findings of Gaddard (1974) and Mares and Cousen (1977) who reported germination levels of 12-25% and 0-30%, respectively.

In most observations, the germ tube penetrated the stomatal pore directly (Figure 1) but, in few cases germ tub penetrated after forming a small apresorium (Figure 2), similar to that described by Mares and Cousen (1977) for *P. striiformis*.

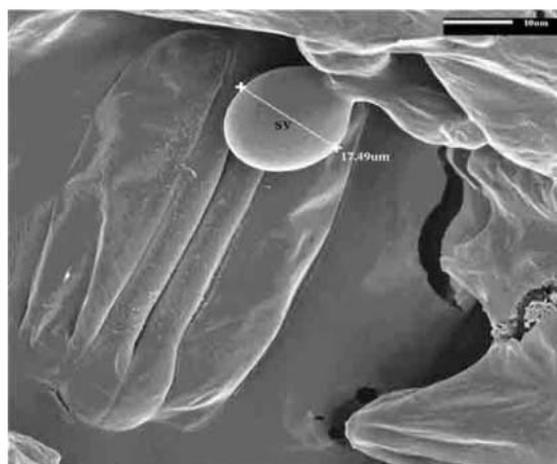
Following penetration, the pathogen usually produced a substomatal vesicle (Figure 3) however, in the resistant genotype, it took longer than in the susceptible host. In this study, haustorium mother cells were initially seen as terminal and separated from the infection hyphae by a cross wall. The first haustoria were observed three days after inoculation on the susceptible host and, occasionally, 4 days after on the resistant near isogenic line. Haustoria penetrated the host cell wall and invaginated, but did not penetrate into the host membrane (Figure 4). In the resistant host, haustoria formation was rear and formed over a longer period than



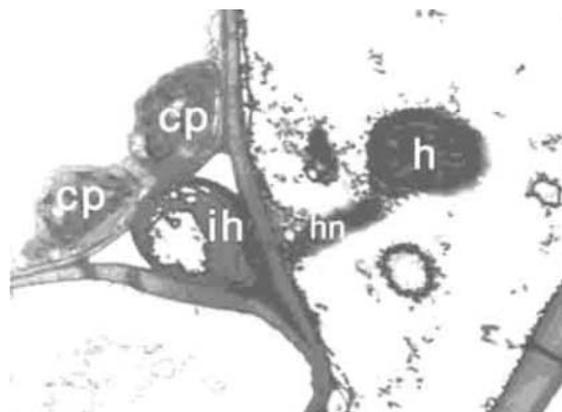
**Figure 1.** SEM micrograph of germ-tub penetration of *Puccinia striiformis* via the stomatal pore (SP) directly without aporus formation. Gt= Germ tube.



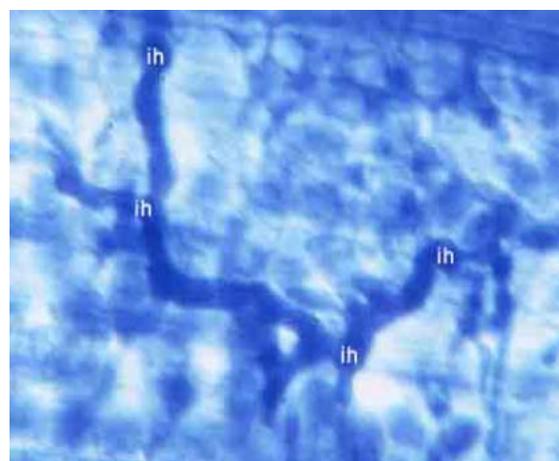
**Figure 2.** SEM micrograph of germ-tub penetration of *Puccinia striiformis* via the stomata (St) after aporus formation (Ap). (Gt)= Germ tube.



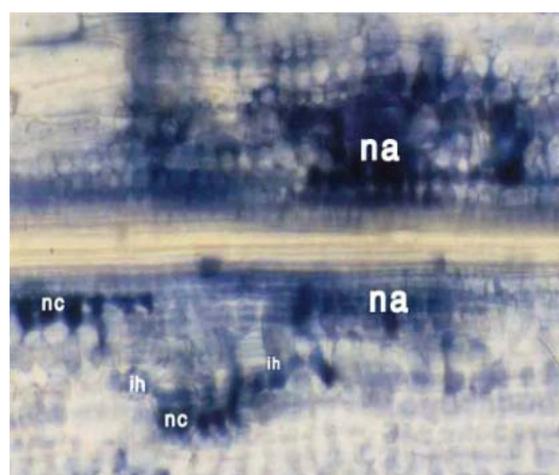
**Figure 3.** SEM micrograph of an infection site of *Puccinia striiformis* with a substomatal vesicle (Sp). (Sp)= Stomatal pore.



**Figure 4.** TEM micrograph of a spherical shaped body (h) within the mesophyll cell of the susceptible cv. Thatcher infected with *Puccinia striiformis* and intercellular hyphae (ih). (hn)= Haustorial neck, (Cp)= Chloroplast  $\times 3500$ .



**Figure 5.** Light microscopy of infected area of leaf on the susceptible host to *Puccinia striiformis* showing intercellular hyphae (ih) and absence necrotic cells.



**Figure 6.** Light microscopy of infected area of leaf on the resistant host to *Puccinia striiformis* showing large number of necrotic cells (nc) from hypersensitive response conditioned by the *Yr-18* gene. (ih)= Infection hyphae, (na)= Necrotic area.

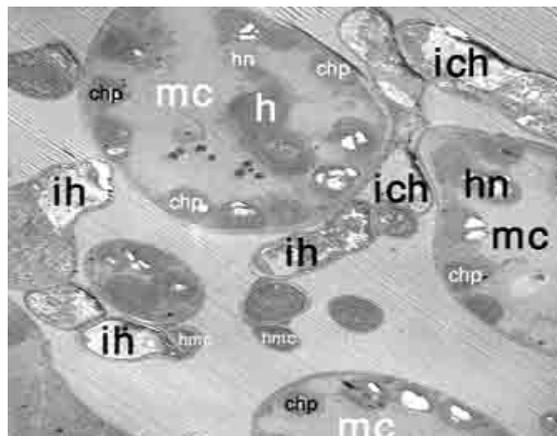
the susceptible genotype. Also, most of the haustoria were smaller than on a susceptible host or necrotic. However, haustoria formation was not common until 3 or 4 days after inoculation on susceptible and resistant hosts, respectively. Mares and Cousen (1977) recorded that haustorial formation started between 36 and 48 hours after inoculation. The longer period to haustorial observation in this study may be the result of using mature plants and, also, it might be a variation conditioned by variety.

Haustoria formation and the growth of hyphae through the mesophyll cell lead to necrosis or collapse of the tissue in a resistant host (Figures 6 and 8). These results were similar to the findings of Jørgensen *et al.* (1993) on barley infected with scald (*Rhynchosporium secalis*), and also similar to the observations of Xu and Heath (1998) on cowpea infected with rust fungus (*Uromyces vignae*). In resistant hosts, cell necrosis was more obvious and common than the growing of infection hyphae. In the susceptible genotype, this growth does not cause the death of any host cells (Figure 5). This observation was similar to that of Heath (1998). Various stages in hypersensitive cell death have been recognized cytologically in the resistance

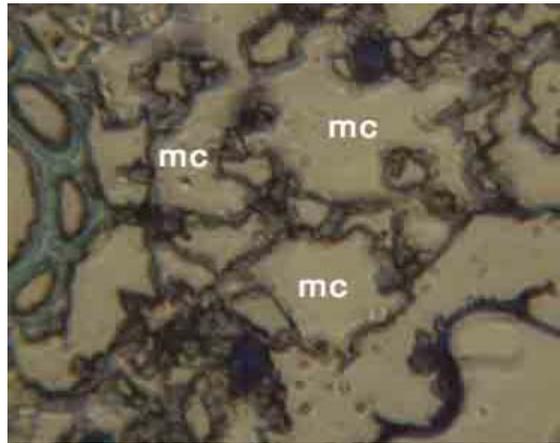
response in this study. This observation was similar to Chen and Heath (1991).

Twelve to fifteen days after inoculation, dead or necrotic tissue or pustules were frequently seen on the resistant host. Pustule development appeared to be inversely correlated with the levels of host cell necrosis, were much less in number, diminutive and showed poor sporulation in the near-resistant isogenic line (Figure 10). However, the susceptible host revealed copious development of pustules and spores therein (Figure 9). These observations were similar to those of Mares and Cousen (1977).

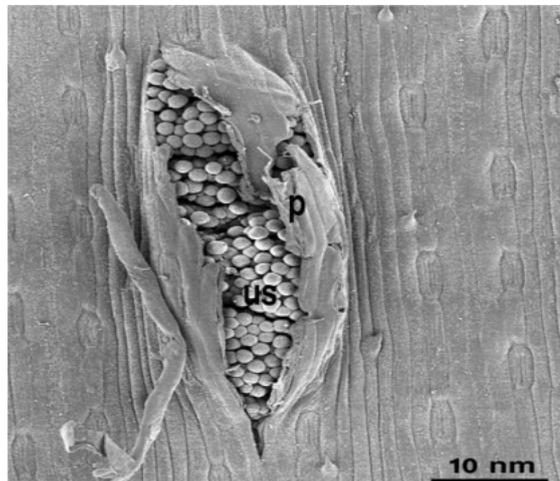
As mentioned above, Heath (1976) considered hypersensitive necrosis to be a complex phenomenon whose role in host resistance is far from clear. Brown *et al.* (1966) and Ogle and Brown (1971) reported that host cell necrosis is not always correlated with reduced fungal growth related in the wheat/stem rust interaction. However, as reported here, Mares and Cousen (1977) noted that the number of necrotic cells per colony were related to the resistance genotype. Nevertheless, these authors recorded that the portion of penetration cells which become necrotic was low even on the most resistant genotype. In this study, it was in



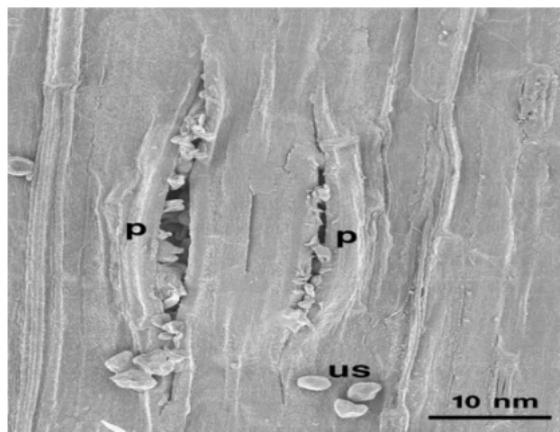
**Figure 7.** TEM image of mesophyll cells of susceptible genotype infected with *P. striiformis*. Note: (chp)= Chloroplast of normal host cells; (ih)= Intercellular hyphae; (h)= Haustorium; (hn)= Haustorium neck; (mc)= Mesophyll cell; (ich)= Intercellular hyphae; (ih)= Infection hyphae, (hmc)= Haustorium mother cell.



**Figure 8.** Micrograph of ultra section of a unit infected leaf area in the resistant genotype infected with *P. striiformis* showing crumpling of mesophyll cells (mc).  $\times 3000$ .



**Figure 9.** SEM image of pustules (p) on the susceptible genotype, infected with *P. striiformis* showing high amount of urediniospores (us), 18 days after inoculation.



**Figure 10.** SEM image marked on the surface of the necrotic area/dead pustules (p) with few shrinkaged urediniospores (us) of resistant genotype infected with *P. striiformis*.

teresting to record that some infected areas of resistant genotype had few necrotic cells, whereas other infected areas frequently had cells and necrotic pustules. Also, within many of infected cells in the resistant host, a big body of spherical or nearly oval shapes was observed by light microscopy and TEM observations. This might be a result of protoplasm accumulation in the mode of action of the Yr-18 gene in mediating stripe rust resistance in spring wheat.

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### مطالعه میکروسکوپی بیان ژن Yr-18 عامل مقاومت در زمان بلوغ به بیماری زنگ‌نواری (*Puccinia striiformis f.sp. tritici*) در یک لاین ایزوژنیک گندم بهاره

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#### چکیده

در این مطالعه از جدایه SR99-UA (Race 70E 128) زنگ زرد برای تلقیح گندم بهاره حساس به نام تاچر (Tatcher) و لاین ایزوژنیک حاوی ژن Yr-18، عامل مقاومت در زمان بلوغ به بیماری زنگ زرد (*Puccinia striiformis f.sp. tritic Eriksi*) استفاده شد. نمونه‌ها در زمان برگ پرچم از رشد بوته برداشت شدند و با میکروسکوپ نوری، الکترونی و الکترواسکن مشاهده شدند. اختلاف بارزی تا قبل از تشکیل مکینه (هوستوریم) تشخیص داده نشد. اما کیسه‌های زیر روزنه در ژنوتیپ حساس کمی زودتر از ژنوتیپ مقاوم تشکیل گردیدند دو تا سه روز بعد از مایه‌زنی، تعداد زیادی مکینه در رقم حساس در مقایسه با لاین ایزوژنیک مقاوم تشکیل شدند. در مراحل اولیه آلودگی، مرگ سلولها در میزبان مشاهده نشد. ولی گاهی در لاین ایزوژنیک شد. چهارده روز بعد از مایه‌زنی، مرگ سلول ناشی از واکنش فوق حساسیت در ژنوتیپ مقاوم بیشتر بود. در این زمان بسیاری از سلولهای پارانشیمی میزبان مقاوم دچار فروریختگی شدند. اولین تاوولهای زنگ معمولاً ۱۲ روز بعد از مایه‌زنی در رقم حساس تاچر و ۱۴ روز بعد در لاین ایزوژنیک مقاوم آن تشکیل شدند. هیجده روز بعد از مایه‌زنی میانگین تعداد تاوولهای زنگ در واحد سطح برگ آلوده در رقم حساس بالا بود ولی در ژنوتیپ مقاوم تاوولهای زنگ از نظر تعداد بسیار کمتر، کوچکتر و با اسپورزایی ضعیف‌تر بودند.