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Ultrastructural Studies on Penetration Sites of *Neovossia indica*, the Partial Bunt Agent of Wheat

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Abstract: To elucidate the mode of penetration of *N. indica* into the host, five wheat cultivar/lines including a susceptible cultivar (WL711), a morphologically resistant line (R1: Altar 84 CD22344-A-8M-1Y-1M-1Y-2Y-1M-0Y) and three physiologically resistant lines (R2: 6811/RGB-U//WARD/3/FGO/4/RABI/5/CIGM89. 564-0Y; R3: CMH84.1106CMH84.1106-1Y-6B-1Y-2B-2Y-2B-0Y and R4: WEE/KOEL//WEAVER CMBW90M132-138M-010M) were inoculated by three different methods. The sequence of events that took place from post inoculation up to penetration was surveyed by scanning electron microscopy. Germination of allantoid secondary sporidia occurred normally on various spike parts in all three inoculation methods. Directional growth toward the stomata or the bulliform cells of glumes, lemma and rachis was not seen. Direct penetration through the epidermal cells of rachis, lemma and glumes, but not through ovarian walls was sporadically seen only in the susceptible cultivar. Hyphal anastomoses on the glumes and rachis surface of all cultivars were occasionally observed. Penetration of sporidial germ tubes and hyphae through stomata was observed. Entrance of germ tube and hyphae to the bulliform cells was also seen. In most cases, hyphae that tended to penetrate the stomata, actually extruded out instead and frequently true penetration was not observed. This indicates that infection development may take place only when two compatible hyphae are anastomosed in the early stages of penetration. In physiologically resistant lines examined in this study, disease did not develop, even after penetration of the spike by the fungus. This indicates that resistance mechanism to *N. indica* in wheat might become operative only after penetration.

Key words: Karnal bunt, *Neovossia indica*, *Tilletia indica*, penetration sites, ultrastructure, cultivar, wheat

INTRODUCTION

Karnal bunt of wheat is caused by the fungus *Neovossia indica* (*Tilletia indica*) (Mitra) Mundkur, which partially converts kernels into sori filled with teliospores. Despite minor overall yield and quality losses, the disease is of considerable international quarantine concern (Babadoost and Mathre, 2004; Moosawi-Jorf and Farrokhi-Nejad, 2007; Rush and Riemenschneider, 2005; Stein and Maples, 2005).

Different views have been reported concerning the penetration sites of *N. indica* into the host, including (a) direct invasion of hyphae into ovaries through epidermis and funiculus (Munjual and Chatrath, 1976; Aujla *et al*., 1988) (b) penetration of hyphae through stomata of the glumes, lemma and palea and its later growth toward the floret base to the subovarian tissue and then entrance to the ovary through the funiculus (Goates, 1988; Salazar-Huerta *et al*., 1990; Moosawi-Jorf *et al*., 2002) (c) penetration of hyphae through stomata of rachis and rachilla and further systematic growth (Dhaliwal *et al*., 1989) (d) direct invasion of hyphae through the rachis bulliform cells, cavities present between the anticlinal walls of epidermis of glumes and idioblast cell on rachis, glumes, lemma and palea (Rattan, 1988). However, the time and mechanism of primary infection has been a debatable issue.

In this study, to have a better understanding of the mode of penetration of Karnal bunt pathogen into the host, susceptible, morphologically resistant and for the first time physiologically resistant wheat cultivars were inoculated by three different methods and surveyed by scanning electron microscopy.

MATERIALS AND METHODS

Preparation of plants: A wheat cultivar; WL711, highly susceptible to Karnal bunt, a morphologically resistant line, R1 (Altar 84 CD22344-A-8M-1Y-1M-1Y-2Y-1M-0Y), with resistance to the disease under field conditions and three physiologically resistant lines viz., R2 (68111/RGB-U//WARD/3/FGO/4/RABI/5/CIGM 89.564-0Y), R3

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(CMH84.1106 CMH84.1106 1Y-6B-1Y-2B-2Y-2B-0Y) and R4 (WEE/KOEL//WEAVER CMBW90M132-138M-010M) which showed resistance under artificial inoculation, as well (Aujla *et al*., 1990; Alizadeh and Saeedi, 1999) were selected for this study. The plants were grown in pots in a greenhouse at 17-25°C under natural light (about 13 h per day) in cereal pathological lab of Seed and Plant Institute, Karaj, Iran.

Preparation of inoculum: Teliospores from infected kernels collected from Dorz-o-Sayeban village, Lar, in the province of Fars, Iran, were germinated on 2% water agar at 20°C. A culture of germinated teliospores was inverted over a Petri dish of Potato-Dextrose Agar amended with 0.1% Yeast extract (YPDA) for 24 h at 20°C. During this time allantoid secondary sporidia showered onto YPDA. The YPDA plates then were incubated at 20°C. Within 4-5 days a white, floccose colony was formed that actively discharged abundant secondary sporidia (Aujla *et al*., 1990).

Inoculation:

Injection technique: In a completely randomized design (CRD) with three replications, the plants at booting stage (just as awns emerged) were injected with a suspension of 2×105 sporidia per mL by a hypodermic syringe (Fuentes-Davila, 1996; Warham and Burnet, 1990). Inoculated spikes were sprayed with distilled water and covered with transparent nylons. Plants were incubated for 24 h at 20°C under continuous light from two 40 W fluorescent bulbs, then spikelets and various spike parts were excised from inoculated spikes and fixed. Three inoculated spikes from each treatment of WL711, R1, R2, R3 and R4 which were grown in greenhouse to maturity for checks, had 33.34, 9.75, 0, 0, 0 mean infection percent, respectively.

Goates' inoculation method: In a CRD with three replications, the plants before anthesis were used for inoculation. Pots and plants were laid on their side and spikes were placed onto fresh 2% water agar in Petri dishes. Then the YPDA plates containing the fungal colony were inverted over them (Goates, 1988) for 24 h under the above mentioned condition. During this period, sporidia were showered onto the spikes and water agar to the extent that the inoculum was macroscopic on water agar. Then florets that faced the fungal colony during inoculation were fixed. Three inoculated heads from each cultivar of WL711, R1, R2, R3 and R4 which were grown in greenhouse to maturity for checks had 41.17, 14.63, 0, 0 and 0 mean infection percent, respectively.

To place inoculum onto the ovary wall, individual florets on three spikes were injected with suspension of 2×10^5 sporidia per mL and then the plants were incubated for 3 days in an incubator. Developing kernels, 2-5 mm in length, were then excised off the inoculated florets and fixed.

Detached various spike parts inoculation technique: The glumes, lemmas and intact spikelets, each with about 2 mm rachis segments from three of each susceptible and resistant cultivars were excised between their emergence from the boot and anthesis stages and developing grains from three spikes, 3-7 days after anthesis were used for inoculation (Dhaliwal *et al*., 1989). The grains and various spike parts were surface sterilized in 0.25% sodium hypochlorite for 8-10 min, rinsed three times in sterile distilled water and placed on 2% water agar in Petri dishes. These specimens were inoculated with the allantoid secondary sporidia showered from YPDA plates containing the fungal colony that was inverted over them for 22-24 h as above-mentioned conditions and then fixed.

Fixation and preparation for SEM: The inoculated specimens were immersed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) at room temperature for 24 h washed in buffer and post fixed in 1% aqueous OsO4 for 2 h to overnight and washed again with buffer, or were fixed for 5 days in Formalin-Propionic acid-Alcohol (FPA) (Coshion *et al*., 1988; Bauer *et al*., 1997). The specimens were dehydrated with ethanol series and coated with gold in a sputter coater model SCD005. The observations were made with Philips scanning electron microscope model XL30 at 25 Kv of Tarbiat Modarres University, Tehran, Iran. Three pieces from each specimen were studied.

RESULTS

Germination of allantoid secondary sporidia were commonly found on various spike parts of all susceptible, morphologically and physiologically resistant cultivars in all three inoculation methods investigated in this study (Fig. 1-2), suggesting there was no difference among them. In the present study, germinated sporidia on various spike parts inoculated by Goates inoculation method were seen on distal half of the abaxial surfaces also, but in specimens which were inoculated using injection and/or detached methods were seen on all surfaces. In Goates' inoculation method, only the end parts of spikelets faced the fungal colony during inoculation and the basal parts of them were covered with other florets. This explains why the basal surface of specimens inoculated with Goates method had little or no hyphae.

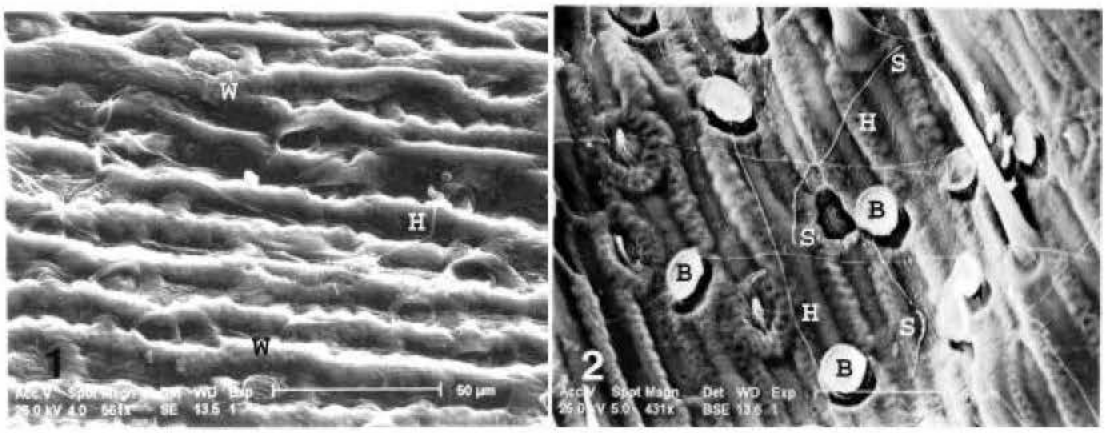

Fig. 1-2: Germination of allantoid secondary sporidia of *Neovossia indica* on various spike parts. 1: Lemma of cultivar WL711 inoculated by detached technique, 2: WL711 rachis inoculated by Goates' method. (H: Hypha, S: Sporidium, B: Bulliform cell, W: epicuticular Wax)

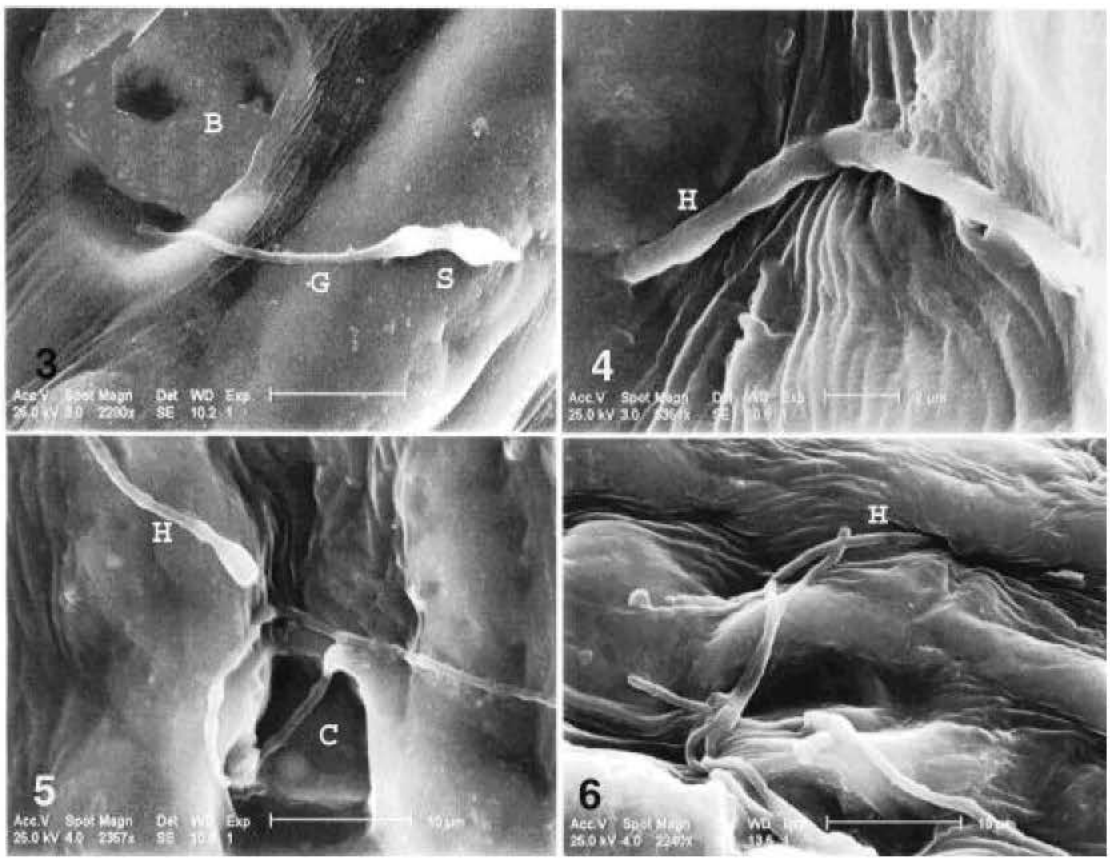

Fig. 3-6: Direct penetration of *Neovossia indica* on susceptible cultivar, WL711, inoculated by detached method. Germ tube or hyphae swollen at the tip, indicative for probably direct penetration of the epidermal rachis bulliform cell Fig. 3 and cavities present between the anticlinal walls of epidermis of glums Fig. 4, 5 and lemma Fig. 6. (S: Sporidium, G: Germ tube, H: Hypha)

Directional growth toward the stomata or the bulliform cells were not seen and hyphae from germinated sporidia grown on surfaces of specimens in all directions.

Direct penetration was seen only sporadically on susceptible cultivar WL711, inoculated by detached inoculation method (Fig. 3-6). Germ tube or hyphal branch swollen at the tip was considered and indicative of probable direct penetration of the epidermal rachis bulliform cell (Fig. 3) and cavities present between the anticlinal walls of epidermis of glumes and lemma (Fig. 4-6), but no direct penetration of the ovary wall was seen in any of the specimens surveyed in this study.

Hyphal anastomosis was occasionally observed on the glumes and rachis surface of all cultivars in each of the three inoculation methods (Fig. 7-10). In Fig. 7, two fungal hyphae were observed in a cavity on the epidermis of susceptible cultivar WL711 which were having hyphal anastomosis.

Penetration of sporidial germ tubes and hyphae through stomata of glumes, lemma and rachis of all cultivars in all three inoculation methods was observed (Fig. 11-24). In most of the penetrations through stomata or entrance to the bulliform cells, hyphae were extruded out from other side of stomata or bulliform cells (Fig. 11, 12, 15, 22, 23, 24 and 27) and true penetration was not observed frequently (Fig. 20, 21). In Fig. 11, making an opening in the guard cell of stomata, the fungal hyphae exits from its other side.

Entrance of germ tubes and hyphae to the bulliform cells on rachis, glumes and lemma were seen also (Fig. 25-31). This type of penetration was reported by Rattan (1988).

Our SEM observation of transverse section of rachis inoculated by injection method (2 days after inoculation) showed that hyphae grew inter-and intracellularly in vessels (Fig. 32).

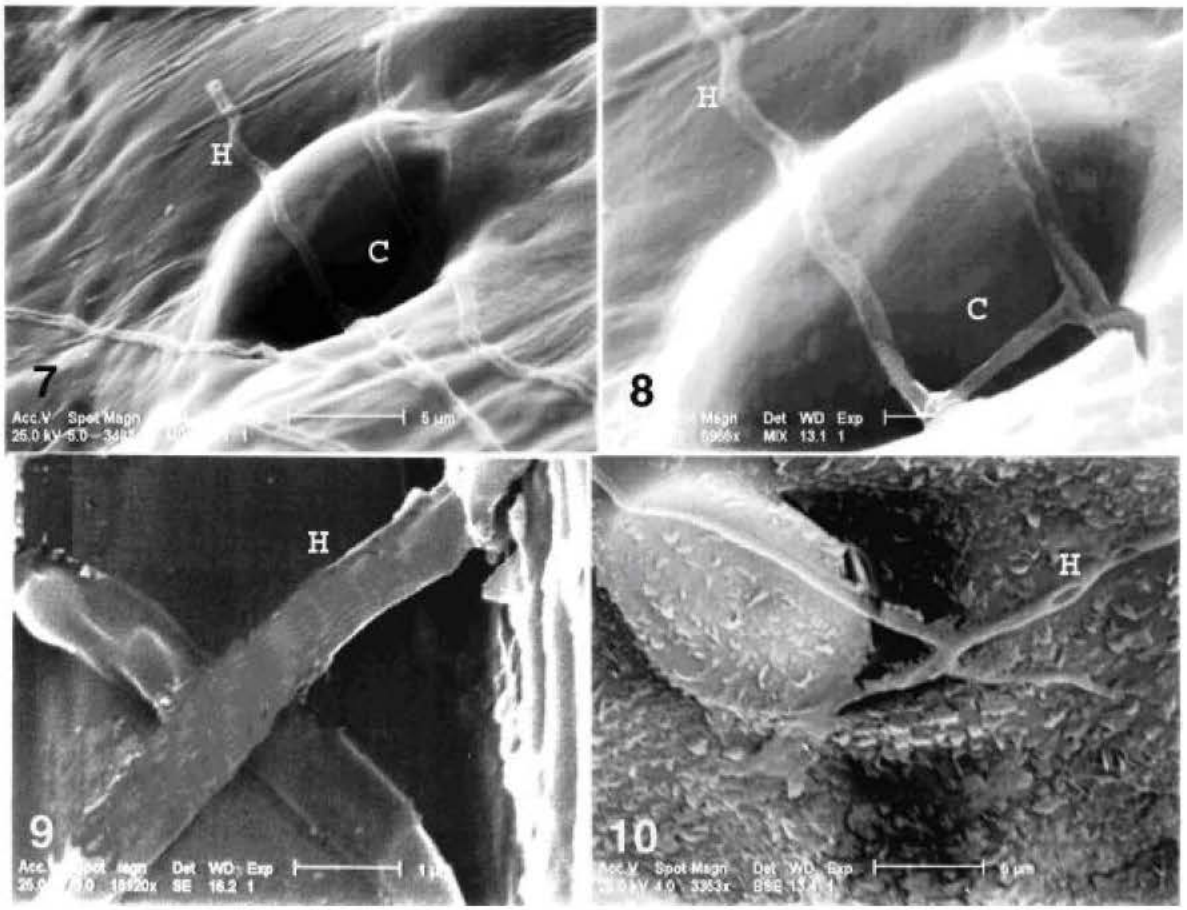

Fig. 7-10: Hyphal anastomosis of *Neovossia indica* on various spike parts. 7: Two hyphae anastomosed in the cavity of WL711 rachis inoculated by injection method, 8: High magnification of Fig. 7, 9: on rachis of line R1 inoculated by detached technique, 10) on glumes of line R3 inoculated by Goates' method. (H: Hypha, B: Bulliform cell, C: Cavity on epidermis)

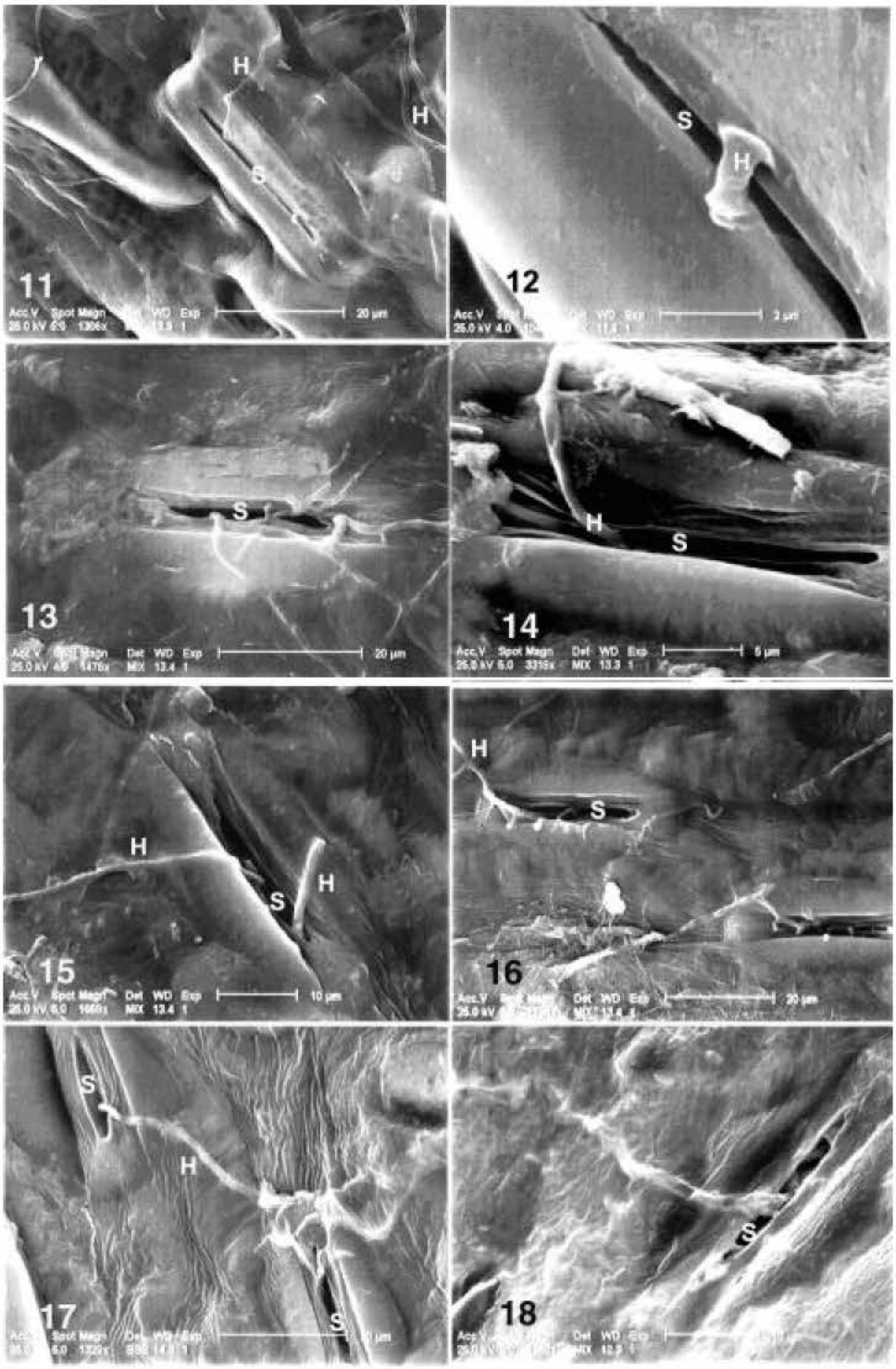

Fig. 11-24: Continued

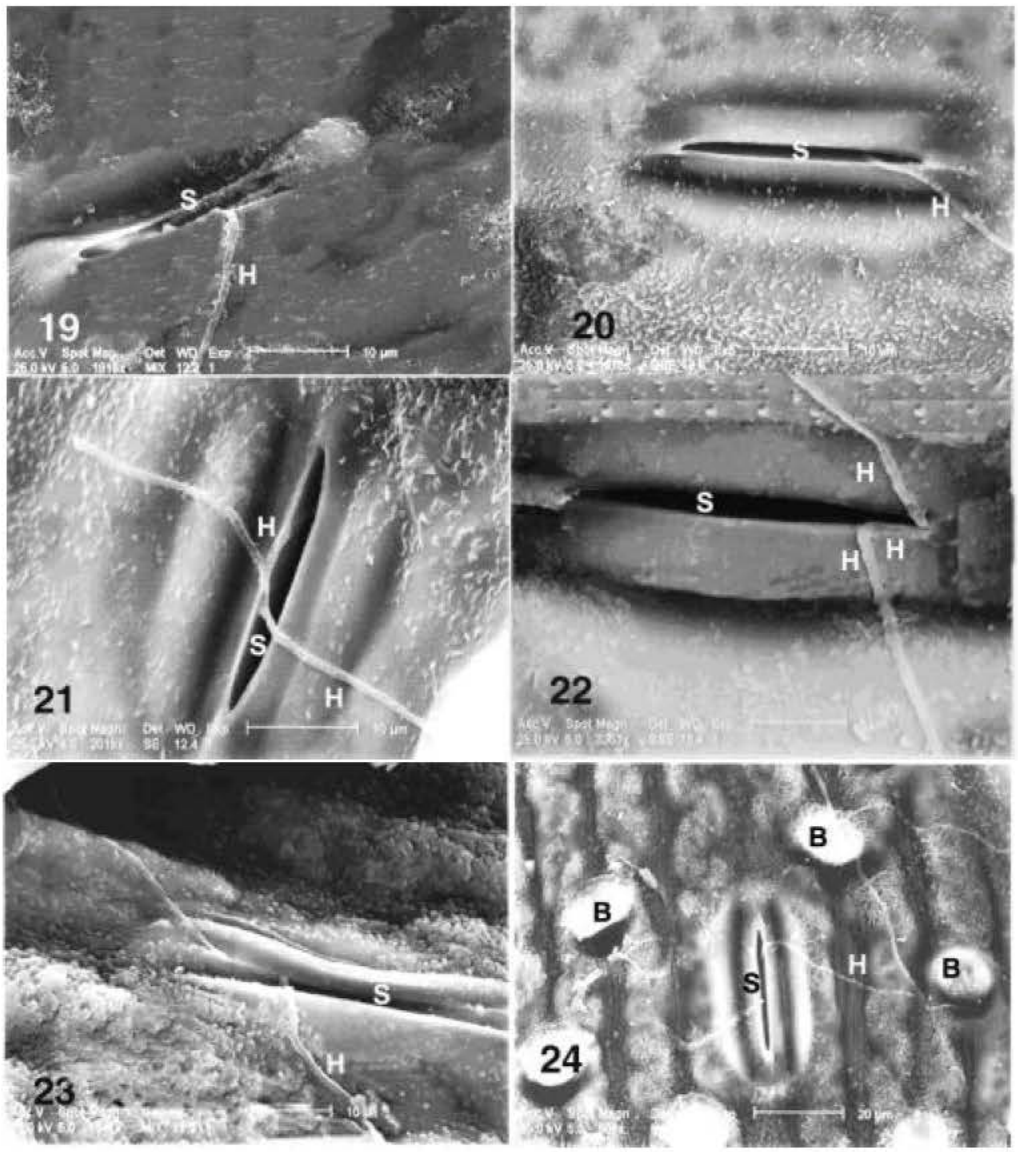

Fig. 11-24: Penetration of sporidial germ tubes and hyphae of *Neovossia indica* through stomata. 11: Penetration in stomata on WL711 glumes inoculated by injection method but direct extraction from guard cell on the other side, 12: High magnification of Fig. 11, 13-16: Stomata on WL711 glumes inoculated by Goates' method, 17: Stomata on WL711 lemma inoculated by Goates' method, 18: Stomata on lemma of line R1 inoculated by Goates' method, 19: on glumes of line R1 inoculated by detached technique, 20-22: on lemma of line R3 inoculated by Goates' method, 23: on glumes and 24: on lemma of line R3 inoculated by detached technique. (B: Buliform cell, H: Hypha, S: Stomata)

DISCUSSION

Mechanism of penetration, type of plant tissue invasion, way of infecting the host and type of colonization of infected plant by *N. indica*, the causal agent of Karnal bunt of wheat, is different from other smuts and bunts of wheat and enjoys a special position (Goates and Jackson, 2006; Marshall and Work, 2003; Nagarajan *et al*., 1997). The agent of this disease unlike other smuts and bunts of wheat is not systemic. Successful infection occurs when the allantoid secondary sporidia could stabilize in the ovarian wall after germination and penetration into the host (Bonde *et al*., 1997; Nagarajan *et al*., 1997; Cashion and Luttrell, 1988; Roberson *et al*., 1987). Various and sporadic studies have been carried out about the mechanism of the penetration

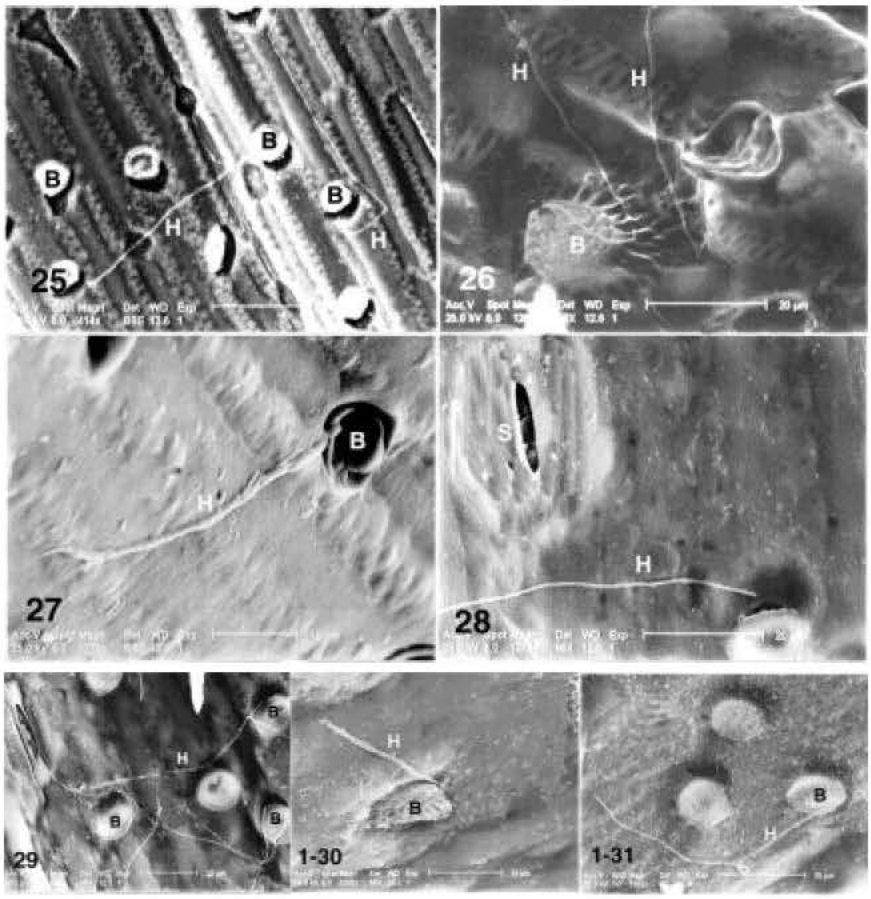

Fig. 25-31: Entrance of germ tubes and hyphae of *Neovossia indica* to the bulliform cells on various spike parts. 25-26: on Wl711 rachis inoculated by Goates' method Fig. 25: and injection method Fig. 26, 27: on rachis of line R1 inoculated by injection method, 28-29: on glumes and 30: on lemma of line R1 inoculated by detached technique, 31: on glumes of line R3 inoculated by Goates' method. (B: Buliform cell, H: Hypha)

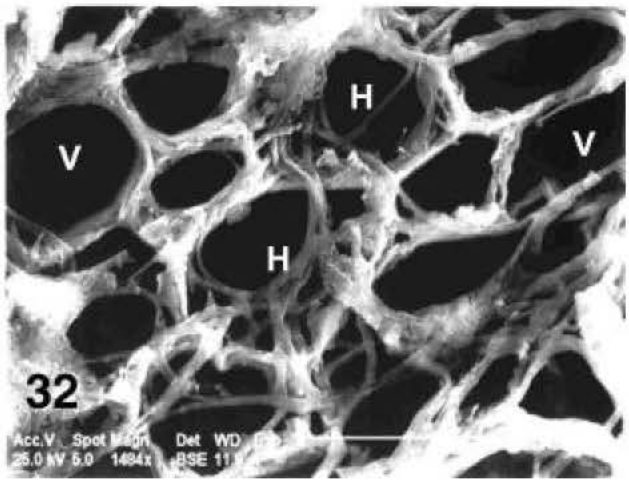

Fig. 32: Transverse section of rachis of line R1, 2 days after inoculation by injection method showed that hyphae of *Neovossia indica* grew inter- and intracellularly in vessels. (V: Vesels of rachis, H: Hypha)

and establishment of *N. indica* hyphae in the host tissue, so different hypotheses have been postulated in this regard. However, the time and mechanism of primary infection is still an important issue. Therefore, to continue relevant basis research is both important to plant pathologists and necessary in practical research for breeding and developing resistant cultivars.

In this study of infection process, first the germination of secondary sporidia was examined on the spike parts of different cultivars. Thus the different parts of spikes of five wheat including susceptible, morphologically and physiologically resistant cultivar/ lines were inoculated in three different methods by allantoid secondary sporidia of the fungus. The results were not showed any significant differences in the germination of sporidia on these cultivars. Dhaliwal *et al.* (1989) showed there was no differences of sporidial germination on detached various spike parts of susceptible bread wheat cultivar Seri 82 and resistant durum cultivar Altar 84. Results indicated that the susceptibility and resistance of cultivars have no effect on germination of secondary sporidia, thus sporidial germination took place at suitable condition on any surfaces such as soil, wheat leaves and wheat spike parts (Smilanick *et al*., 1989; Dhaliwal, 1989).

Scanning electron microscopy studies of Goates (1988) showed numerous secondary sporidia, most of which had germinated, were located primarily on the distal half of the abaxial surface of glumes. To explain this phenomenon, it was mentioned that the existence of electrostatic forces at this part of glum and lemma is probable. In the present study too, in samples inoculated by the above mentioned method (Goates, 1988), the germinated sporidia were observed at the distal half of these surfaces. However, sporidial germination in all samples inoculated by the two methods of injection and detached inoculation method were observed on all surfaces of different spike parts. In Goates inoculation method, only the distal parts of spikelets are subject to fungal colony at the time of inoculation while their basal parts are covered by other florets. This issue is an explanation on why, in Goates inoculation method, the basal surfaces of samples contain no hyphae or rarity of them.

Meanwhile there have been many studies to show whether hyphal growth is of directional or indirectional penetration on the surface of different parts of spike. Scanning electron microscopy (SEM) in this study showed that hyphal growth on different surfaces of spike parts does not follow a particular pattern and that the directional growth of fungal hyphae was not seen towards stomata or bulliform cells. The results have been approved by Dhaliwal *et al.* (1989).

Hyphal anastomosis was occasionally observed. Thus the start of dikaryotic stage in this fungus could not be the outcome of hyphal anastomosis at the stage. Hyphal anastomosis has been reported occasionally on the different parts of spike by other researchers (Salazar-Huerta *et al*., 1990; Dhaliwal *et al*., 1989; Goates, 1988).

In the present study, hyphal anastomosis was occasionally observed on the glumes and rachis surface of all cultivars in each of the three inoculation methods (Fig. 7-10). In previous studies of heterothallism that were based on results of pathogenicity tests, it was shown that each haploid sporidum carries only one of the mating type alleles (Duran and Cromarty, 1977; Moosawi-Jorf *et al*., 2000).

In our exact observation by SEM, direct penetration thorough ovarian wall was not observed. This type of penetration as one way of infecting host among other hypotheses has been introduced by some researchers (Aujla *et al*., 1988; Singh *et al*., 1993; Munjal and Catrath, 1976). Hyphal penetration into the ovarian wall However, was observed neither in this study nor studies by Goates (1988). Therefore, the theory of direct penetration of hyphae through the ovarian wall is apparently rejected. But in the detached inoculation method, some swelling was observed on the terminal of hyphal germ tubes or extensions of the hyphae of susceptible cultivar WL711. It rarely happened on the epidermis of glumes, lemma and rachis that makes probable direct penetration from the surfaces. Direct penetration from these surfaces as reported by numerous workers (Munjal and Chatrath, 1976; Gill *et al*., 1993; Rattan, 1988) was seen only sporadically on susceptible cultivar WL711, inoculated by detached inoculation method (Fig. 3-6). Earlier relevant studies have concluded that fungus penetrates through cuticles and epidermis of glumes, lemma or palea and passes across this organs and then penetrates the ovarian epidermis (Munjal and Chatrath, 1976). Other researchers also believe that the inoculum which exists in floret cavity enables the fungus to penetrate directly through the ovarian epidermis (Aujla *et al*., 1988). Other hypothesis about infection also shows direct penetration of fungus through ovarian epidermis (Singh *et al*., 1993). In other words, infection takes place by direct penetration through ovarian wall. Accordingly, it seems that the fungus penetrates the ovary from random positions which do not correspond to the sporogenous nature of the fungus (Munjal and Catrath, 1976; Mitra, 1931, 1935); because in nature, hyphal invasion in ovary often being from the basal part of them (Bonde *et al*., 1997). To justify the matter, Munjal and Chatrath (1976) believe that the environmental conditions of the basis of the ovaries are more suitable for teliosporogenesis. However, since

Goates (1988) believes that these studies are founded on indirect evidence; and that in the present study and studies by Goates (1988), direct penetration of fungus into the ovarian wall was not observed, direct hyphal penetration through ovarian wall as an infection way seems to be rejected. According to Goates (1988), hyphal penetration has never been observed through the epidermis wall of ovary, not even by direct inoculation. It seems *N. indica* is unable to have a direct penetration through the ovarian wall (Goates, 1988).

Penetration of sporidial germ tubes and hyphae through stomata of glumes, lemma and rachis of all cultivars in all three inoculation methods was observed (Fig. 11-24). This is on a par with the report by Munjual and Chatrath (1976), Goates (1986), Dhaliwal *et al.* (1989) and Salazar-Huerta *et al.* (1990).

Entrance of germ tubes and hyphae to the bulliform cells on rachis, glumes and lemma was also seen (Fig. 25-31). This type of penetration was reported by Rattan (1988).

Our SEM observation and study of Dhaliwal *et al.* (1989) showed that successful penetration takes place rarely through stomata of glumes and lemma. However, penetration through the stomata on the rachis was frequently seen by Dhaliwal *et al.* (1989). Goates (1988) also reported that in about 90% of the cases the hyphae were confined to the vestibule above guard cells of stomata. On the other hand, the entrance of a limited number of hyphae into the substomatal space is another confirmation that, despite the abundance of penetration sites, successful penetrations rarely occurs. This indicates that the infection development may take place only when two compatible hyphae are anastomosed in early stages of penetration. Pathogenicity tests using dikaryotic mycelia that fail to initiate disease development (Moosavi-Jorf and Alizadeh, 2000) confirm this hypothesis. Since dikaryotic mycelia both in culture media and plants are of similar nature, the reason why does not cause infection could only be related to their incapability of penetration. Thus, penetration of dikaryotic mycelia from hyphal anastomosis that were occasionally observed on the glumes and rachis surface, or penetration of germ tube or hyphae from sporidial germination without anastomosis in the early stages of penetration would fail. The possibility is not denied that the hyphae spread systematically in rachis from primary infection sites (Dhaliwal *et al.*, 1989). This possibility was surveyed by our SEM studies. Our SEM observation of transverse section of rachis inoculated by injection method (2 days after inoculation) showed that hyphae grew inter- and intracellularly in vessels (Fig. 32). It shows that infected rachis could play a role in its development as the primary source of infection.

In this investigation, according to different methods of inoculation, the penetration of fungal hyphae through the stomata of glumes, lemma and rachis as well as the bulliform cells on the epidermis of various spike parts was observed. Nevertheless, penetration through stomata of glumes and lemma was observed by Goates (1988) and Salazar-Huerta *et al.* (1990), while direct penetration into bulliform cells of rachis, glumes and lemma by Rattan (1988) were separately studied and confirmed. However, following the studies on mechanism of penetration into the host, Penetration through stomata of rachis has been suggested by Dhaliwal *et al.* (1989).

In this study, we examined all three kinds of wheat germ plus including susceptible, morphologically resistant and physiologically resistant lines/cultivars. Evidences show that in many wheat cultivars, possessing morphological resistance to Karnal bunt, become susceptible upon artificial inoculation (Aujla *et al.*, 1990). This is probably due to a heavy inoculation load and further multiplication of sporidia in the boot (Dhaliwal *et al.*, 1989). On the other hand, physiologically resistant cultivars stay resistant to Karnal bunt even after artificial boot inoculation (Gill *et al.*, 1993; Aujla *et al.*, 1990). Surveying the causes of this phenomenon the observation was that the penetration of germ tubes takes place through stomata and bulliform cells on the glumes, lemma and rachis in all examined cultivars including physiologically resistant cultivars. Thus as there was no infection in grains of physiologically resistant cultivars, despite fungal penetration, it seems that the resistance mechanism to *N. indica* in wheat might become operative only after penetration. As the morphologically resistant cultivar was less infected than the susceptible one in the artificial inoculation, it seems that a level of genetic resistance has been present in the cultivars. Although these findings are based on studies on the mechanism of penetration, it was known that the studies of the resistant mechanism of wheat to this pathogen should focus on factors which follow the penetration.

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