# Histopathological Studies with Electron Microscopy (SEM & TEM) of *Alternaria alternata,* Causal Agent of Brown Spot and Black Rot Diseases (as Postharvest Diseases) on Citrus in the North of Iran

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**Abstract**
*Alternaria* **species cause two different diseases on citrus in the North of Iran:** *Alternaria* **brown spot of tangerines and** *Alternaria* **black rot of 'Navel' oranges.** *Alternaria* **black rot is also well known as postharvest disease on citrus. In this study infected tissues were collected from leaves, fruits and young stems of the plants. The tissues were cut to 2×2 mm pieces and were fixed over night at 5°C in 0.2 M phosphate buffer including 2% gluteraldehyde. Post fixation was in 1 M osmium tetroxide for 5 h. Specimens were then rinsed and taken through a series of alcohols of increasing concentration and finally lyophilized for 5h. The specimens were coated with gold and then studied using a ZIESS DSM-960A. For TEM, specimens after being fixed in gluteraldehyde and osmium tetroxide, were embedded in spurr's medium and thin and ultrathin (200-500nm ) (70-120nm) sections were stained and shown by ziess transmission electron microscope. The present study showed that the court of infection is through stomata and direct. Penetration conidia were produced and not appressoria formation was seen through stomata. Hypha penetrated through the sub-stomata cavity. Some of the hyphal branches were grown in intercellular space of the mesophyll and paranchyme tissue surroundings. The hyphal product, especially toxine (HST and NHST) caused damaged cell and cell wall duration. This study showed that the hypha did not penetrate in xylem tissues. The albedo part of fruit was the main site of penetration and infection and fruit skin, especially in undeveloped fruit, was completely resistance against hypha of the range. Mycelia did not penetrate in the skin and segregate gland. This study has broaded our knowledge of the penetration and control strategies of** *Alternaria alternata* **especially on citrus.**

## INTRODUCTION

*Alternaria* is a member of imperfect fungi, family *Dematiaceae*. It has different species and induces several diseases on plants, produces toxic substance, is carecenogenic and produces allergic and respiratory disorders. *Alternaria* spp. are also well know as postharvest pathogens on citrus. *Alternaria* was first described by Nees in 1817, with a chain of dark conidia with longitudinal and transverse septa, as morphological characters. *Alternaria* is a cosmopolitan fungal genus that includes saprophytic, endophytic and pathogenic species. Plant pathogenic *Alternaria* spp. have a broad host range including many economically important plants such as citrus (*citrus* spp.), apple (*Malus domestica*), pear (*pyrus pyrifolia*), tomato (*Lycopersicom esculentum*) and potato (*Solanum tuberosum*). The two described *Alternaria* diseases of citrus in Iran include:

### *Alternaria* Stem-End Rot (Black Rot)

Black rot of fruit, a postharvest disease, is caused by *Alternaria alternata.* It occurs through all citrus growing regions, but is rarely abundant enough to cause economic losses in Iran. The disease occurs primarily as a stem-end rot on fruit stored for a long time. It is an important problem in commercial storage of citrus in the North of

Iran. The decay can develop at the stylar end of the fruit, particularly in 'Navel' oranges and cause premature fruit drop. *Alternaria* black rot can also be a problem for the processing by contaminating the juice. The pathogen grows on dead citrus tissue or other substrates and produces air born conidia. Latent infections are established in the button or stylar end of the fruit. The disease can develop further when the button becomes senescent, as in over-mature fruit and during long-term storage.

### *Alternaria* Brown Spot

Brown spot, caused by the tangerine pathotype of *Alternaria alternata* affects many tangerines and their hybrids in the world and infects 'Minneola' tangelo and page mandarin trees in west Mazandaran of Iran. The diseases cause minute brown to black spots on young leaves and fruits. Symptoms can appear as little as 24h after infection. The spots usually continue to expand and large lesions of the leaves may be killed by the host selective ACT-toxin, (Kohmoto et al., 1993). On mature leaves, brown spots appear as distinct brown lesion surrounded by a yellow halo. Affected leaves abscise and infected twigs die back, especially when the leaves have fallen. On the fruits, lesions can vary in form and can be dislodged, forming a pock mark on the surface. Severely affected fruit abscise, reducing yield and blemishes on the remaining fruit diminish marketability. (Timmer et al., 1999). Mazandaran, with about 90,000 ha citrus planting areas, is the major citrus culture province in the North of Iran. Black rot on 'Thomson Navel' orange trees, and spot on 'Minneola' tangelo are two important diseases, caused by *Alternaria alternata* , in the west and east of Mazandaran province, respectively (Alavi et al., 2002).

The objectives of this investigation were to increase our knowledge about the description of morphological and anatomical aspects, antifungal activities of host plants and different types of toxins. Alternate results may cause to differentiate these two species and find a future control strategy.

### MATERIALS AND METHODS

Several isolates of *Alternaria* were collected from the North of Iran (Mazandaran) including isolates from leaves and young fruit lesions of 'Minneola' tangelo and black rot 'Thomson Navel' fruit. Samples of fruit and leaves exhibiting diseases lesion characteristic of symptoms of *Alternaria* black rot and *Alternaria* brown spot were collected from west to east Mazandaran (North of Iran). These included samples of 'Minnoela' tangelo and 'Navel' orange. All samples were returned to the lab for the isolates of *Alternaria* fungi. For SEM, the infection process of *Alternaria* on Minneola tangelo was observed. Samples of leaf pieces and fruit skin after infection that were collected were prefixed in 2% glutaraldehyde -p-formaldehyde in 0.05 M (Enkerli et al., 1997). Sodium cacodylate buffer at pH 7.2 for 2h at room temperature. The samples were then washed three times with a 0.05 M sodium cacodylate buffer solution for 10 min. Samples were post-fixed in 1% osmium tetroxide in the same buffer for 1h. Samples were freeze-dried (-40ºC). The dried materials were adhered on to aluminium specimen mounts with colloidal silver paste and then sputter coated (Balzer SCD004) with gold palladium (approximately 15 nm thickness). The specimens were examined and photographed on a (ZEISS DSM-960A) scanning electron microscope at 15-30 kv. At last 50 leaf discs were observed in this experiment. For TEM, samples were fixed as described above, dehydrated and filtrated in Spurr's resin (Taylor and Mims, 1991). Following resin polymerization, thin sections of samples were cut using an ultra microtome equipped with a glass knife, collected on slot grids, and allowed to dry on form var-coated aluminum racks (Rowley and Moran, 1975). Section were post-stained with uranyl acetate and lead citrate and examined with a Zeiss 902A transmission electron microscope operating at 80 kV.

### RESULTS AND DISCUSSION

Conidia of *A.alternata* were small and septate, ranging from7 to30 μm in vivo with long filiform beaks. Germinated and ungerminated conidia were not dislodged

during SEM preparation and so it was concluded that they adhered strongly to the leaf surface. Each conidium produced several germ-tubes at random positions on the conidium over time and grew profusely in random directions across the surface (Figs. 3-12). Mature germ tubes were variable in length (10-250 µm) and branched infrequently. Appressoria did not form directly on the cuticle or on stomata. Some germ-tubes grew towards and entered a stoma without forming an appressorium over the stomata (Figs. 3, 5, 12). Other germ-tubes passed near stomata without appressorium formation and showed directional growth toward the stomata. Stomata passed in this way were found in both open and closed stomata. Occasionally extensive growth of germ tubes formed a hyphal network on the host tissue (Figs. 6, 8, 10). Conidia are produced primarily on the surface of lesions on mature or senescent leaves and on blighted twigs (Fig. 7). Relatively few, if any, produced leaves and mature lesion on fruit. Some of the hyphal branches were grown in intercellular space of the mesophyll and paranchyme tissue surroundings (Figs. 16, 17). The hyphal product, especially toxine (HST and NHST) caused damaged cell and cell wall diuration (Figs. 16, 17). The fungal structure of *A. alternata* on the leaf surface central darkened area, represent cells wich have possibly discoloured as a result of infection with fungus (Fig. 10). The hyphea of *A. alternata* grew extensively abaxial surface and filled the epidermal cell of leaves of 'Minneola' tangelo. Young conidiophores of *A. alternata* developed and emerged directly through the epidermal leaf of 'Minneola' tangelo (Figs. 9, 10). Spores of other fungi on this side could not penetrate and infect in open stomata (Fig. 11). The conidium production is the greatest when leaves are lightly moistured or held at high humidity with fewer produced where leaves are very wet. Conidia germinate quickly if moisture is present and begin to produce toxine even before they penetrate the tissue (Fig. 6). However, in our studies in Iran, penentration occurs through stomatas on the abaxial surface of the leaf. Without the formation of appressoria sporulation does not occur on lesion until the plant tissue matures. Most of the sporulation occurs on leaves whereas a relatively little occurrence is found on fruit or twigs. The optimum temperature for infection is 27°C. Sporulation occurs in lesions on mature leaves in the presence of water or even more abundantly when humiditly is high. The study showed that the hypha did not penetrate in xylem tissues (Fig. 18). The albedo part of fruit was the main site of penetration and infection and fruit skin, especially in undeveloped fruit, was completely resistant against hypha of the range (Figs. 13, 14). Mycelia did not penetrate in the skin and segregate gland (Fig. 15). This study has broadened our knowledge of the penetration and control strategies of *Alternaria alternata* especially on citrus.

The infection process of *A. alternata* observed on 'Minneola' tangelo is generally similar to that of *A. cassiae* on cowpea (N. Van Den Berg et al., 2003) as well as to other *Alternaria* spp. on a range of hosts (Angell, 1929; Allen et al., 1983; Aveling et al., 1994; Rotem, 1994; Van Den Berg, 2003). Our results confirm those of Van Dyke and Trigino (1987), Mimes et al. (1997) and Van Den Berg et al. (2003), who reported that conidia of *A. cassiae* germinated with in 2-3 hpi producing multiple germ-tubes that grew randomly across the leaf surface. Rotem (1994) reported that spores of all *Alternaria* species germinate in remarkably short time and produce one to several germ-tubes. Previous SEM studies show that extra-cellular material is also associated with germ tubes and appressoria of *Alternaria helianthi* on sunflower (*Helianthus annuus*) (Allen et al., 1983) and *A. porri* on onions (Aveling et al., 1994), and may have an adhesive function. In the present study, germ tubes and their growth were extremely variable, but this is not unusual for *Alternaria* spp. And similar responses have been reported for *A. tenuis* on beans (*Phaseolus vulgaris*) (Saad and Hagedorn, 1969) and *A. cassia* on siklepod (Van Dyke and Trigino, 1987) and *A. cassiae* on cowpea (Van Den berg et al., 2003). Van Dyke and Trigino (1987) reported that germ tubes of *A. cassiae* and their branches terminated in appressoria, and that intercalary appressoria were also occasionally observed. These authors reported that appressoria formed directly on epidermal cells or over stomata with about equal frequency. Our results also show that both terminal and intercalary appressoria do not form directly on epidermal cells or over stomata. In the

present study, germ tubes occasionally entered through a stoma with no appressorium formation, as reported for *A. helianthi* on sunflower (Allen et al., 1983). Results of the present study showed both direct and indirect penetration without the formation of appressoria indicating that appressoria are not always necessary for infection. Van Dyke and Trigino (1987) similary found that *A. alternata* entered its specific host through stomata and by direct penetration with or without appressoria. Van Den Berg et al. (2003) found that *A. cassia* on cowpea leaves, directly and indirectly penetrated with or without the formation of appressoria. Von Ramm (1962) also reorted that *Alternaria longipes* penetrates tobacco (*Nicotiana* sp.) leaves without appressorium formation. In less pathogenic *Alternaria* spp. the infection court is limited to wounds and stomata (Rotem, 1994). There was no evidence of specific orientation or long-distance attraction towards stomata and germ tubes often passed stomata with an apparent tropic response. Stomatal penetration appeared to occur by chance. These results agree with those of *A. longipes* on tobacco (Von Ramm, 1962), *A. cassiae* on sicklepod (Van Dyke and Trigino, 1987), *A. porri* on onions (Aveling et al., 1994) and *A. cassia* on cowpea (Van Den Berg et al., 2003). The mode of penetration, whetter mechanical or chemical, was not determined in this study. As reported for *A. cassiae* on sicklepod (Van Dyke and Trigino, 1987) and *A. cassiae* on cowpea (Van Den Berg, 2003), darkened areas representing discoloured cells as a result of interaction with the fungus were also observed in the near vicinity of the fungal structure of *A. alternata* on citrus. The presence of these discoloured cell indicates that the cells have been disrupted. Van Dyke and Trigino (1987) reported that cells in the substomatal area beneath appressoria were necrotic with no evidence of fungal invasion in the tissue. These authors further stated that hyphal penetrations were seldom observed prior to necrosis of mesophyll cells and that the death of these cells in advance of fungal penetration suggests the action of diffusible toxins. In this study, secondary hyphae developed from primary hyphae and grew in the intercellular spaces and also penetrated and grew intercellulary within the epidermal and were obtained for *A. cassiae* on sicklepod (Van Dyke and Trigiano, 1987) and *A. cassiae* on cowpea (Van Den Berg, 2003). These authors reported that intra- and inter-cellular hyphae were found in the epidermis and plisade mesophylle. Although much is known about the pre-penetration structure and infection processes of other *Alternaria* spp. on their specific hosts, this is the first study of the infection process of *A. alternata* on 'Minneola' tangelo. This study has broadened our knowledge of the ore-penetration structure, penetration and colonization of *Alternata* spp., especially on citrus. *Alternaria* produces such lytic enzymes as polyglacturonase, pectin lyase, pectin methylestrase, cellulose and two categories of toxine, namely, host-specific toxins (HST) and non host-specific toxins (NHST). Among the first, toxins such as the AM, AC, AK, AF and AL have been identified and their role in pathogenesis verified (Rotem, 1994). Plants respond by deposition of lignin to the cell wall of infected cells.

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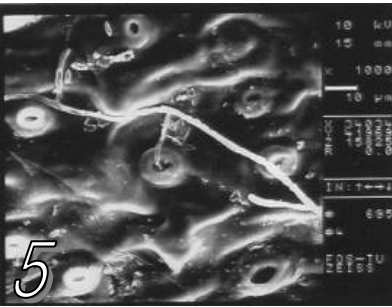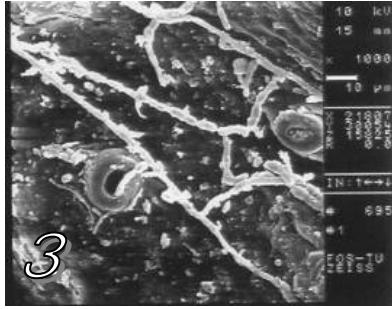
## **Figures**
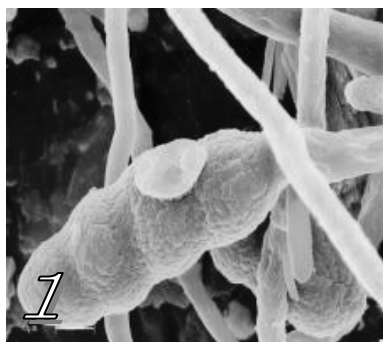


Fig. 1. Scanning electron micrograph of conidiophores of *Alternaria alternata* caused black rot symptoms on 'Navel' orange fruit in North of Iran. bar= 10 μm.



Fig. 2. Scanning electron micrograph of conidiophores of *Alternaria alternata* caused brown spot on mature 'Minneola' tangelo leaves and fruit. bar=10 μm.
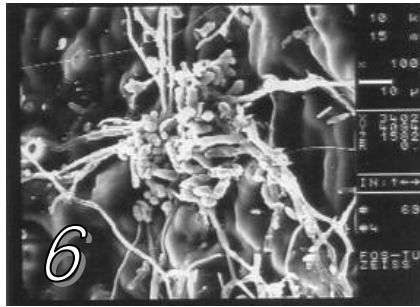
Figs. 3-6.  Scanning electron micrograph of *Alternaria alternata* on 'Minneola' tangelo leaves and fruits. bar=10 μm.

Figs. 3-4.  The germ tubes of fungus entering an opening pore on the sureface of fruit without forming appressorium. bar=10 μm.

Fig. 5.  The germ tubes of *Alternaria alternata* on 'Minneola' tangelo leaves, germ tube direct penetrution of hyphal through the stomata without appressorium. bar=10 μm.

Fig. 6.  The leaves' surface coverage of hyphal network. bar=10 μm.
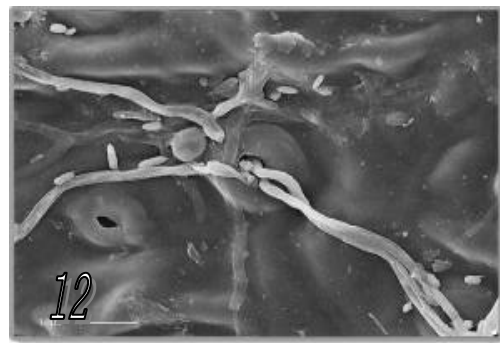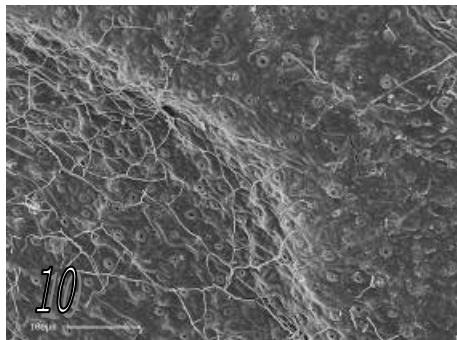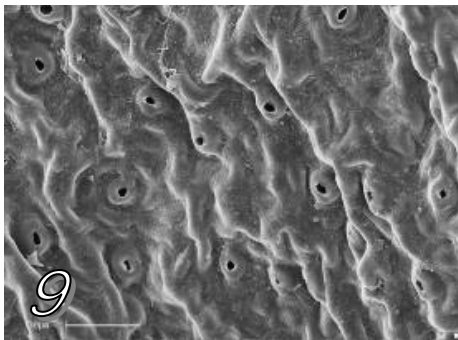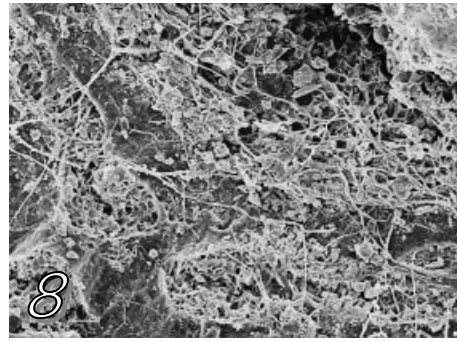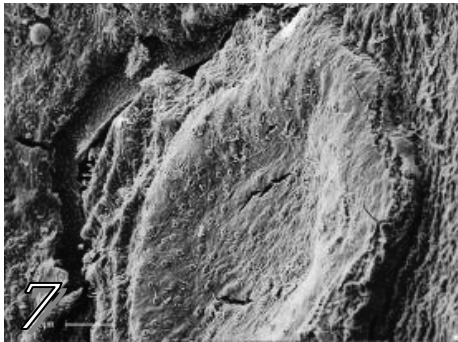
Figs. 7-12.   Scanning electron micrograph of yellow hollow necrotic 'Minneola' tangelo leaves infected by *Alternaria* spp. (Fig. 7):The central darkened area represents cells wich have possibly discoloured as a result of infection with the fungus. Bar=100 μm.

Fig. 8.   The hyphal product, especially toxine (HST and NHSt ) caused damaged cell walls. Bar=100 μm.

Fig. 9.   Any conidia and germ tube not found in healty leaves of minnoela tangelo. bar=100 μm.

Fig. 10.   The yellow halow necrotic 'Minnoela' tangelo leaves. Extensive growth of germ tubes formed a hyphal network on the leaves. bar=100 μm.

Fig. 11.   The spores of other fungi on the leaves' surface could not penetrate and infect in open stomata. bar=10 μm.

Fig. 12.   The germ tube passed near stomata without appressorium formation and showed on directional growth toward the open stomata. bar=10 μm.
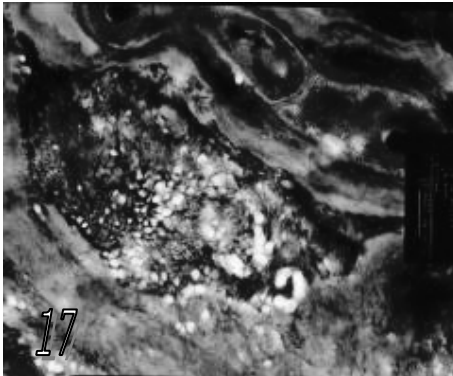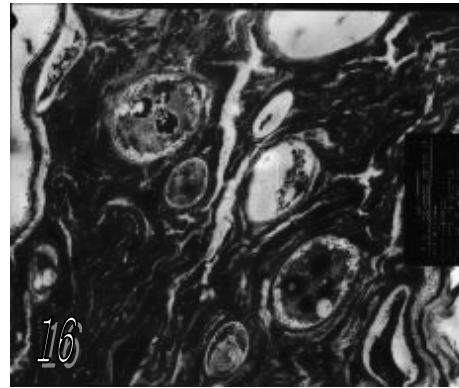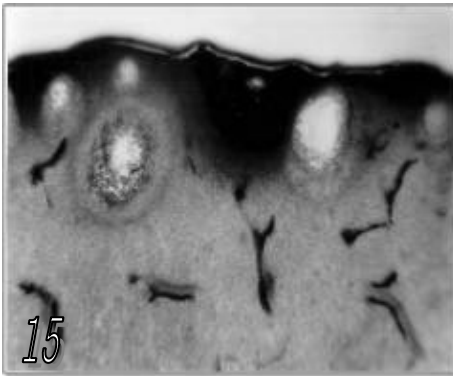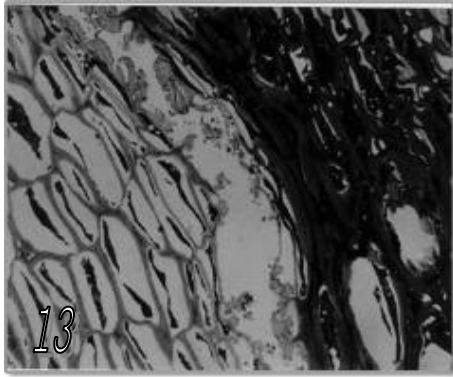
Fig.13-18. The albedo part of 'Navel' orange fruit was the main site of penetration and infection hypha of *Alternaria alternata*, cross section of skin fruit infected by alternaria ×1000.

Fig. 14. The pathogen grows on death citrus tissue or other substrates and produces air born conidia ×1000.

Fig. 15. Cross section of skin fruit, mycelia did not penetrate into segreate glands on skin fruit ×400.

Fig. 16. Transmission electron micrograph of hyphae growing intercellulary passing through adjacent epidermal cells, the mycelia of *Alternaria* are visible within the mesophyll cells of 'Minneola' tangelo leaves. bar=0.75 μm.

Fig. 17. Transmission electron micrograph of hyphae are visible within the mesophyll cells of 'Minnoela' tangelo leaves, germ tubes of conidia extended to the mesophyll cells. bar=0.75 μm.

Fig. 18. Hypha did not penetrate in the xylem. bar=0.75 μm.