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Light and Scanning Electron Microscopy Studies on the Penetration and Infection Processes of Alternaria Alternata, Causing Brown Spot on Minneola Tangelo in the West Mazandaran - Iran

[1]A.A. Dehpour, [2]S.V. Alavi and [3]A. Majd

[1]Department of Biology, Islamic Azad University, Research and Science Branch, Tehran,iran
[2]Department of Plant Pests and Diseases, Mazandaran Agricultural Research Center, Iran
[3]Department of Biology, Islamic Azad University, Tehran - Shomal Branch, Tehran, Iran

Abstract: Alternaria fungi causes two different diseases on citrus in Mazandaran province, North of Iran: Alternaria brown spot of tangerine hybrids and Alternaria black rot of the Navel orange fruit. *A. alternata*, conidium germination, Inoculation, penetration and colonization on the plant surfaces studied using light and electrone microscopies. The tissues cutted to (2×2 mm) pieces and were fixed over night at gluteraldehyde and phosphate buffer, post fixation was osmium tetroxide,after lyophilized,the specimens coated with gold and then studied using a ZEISS DSM_960A SEM. Multiple germ-tubes developed randomly from each conidium and grew in any direction across the leaf and fruit skin surfaces. Penetration in the plant surface, whether directly through the epidermis or via stomata occureed with or without the appressoria formation. The present study showed that occurrence of the infection was through stomata and direct penetration. Hyphal penetration continued through of the substomata cavity and then some of hyphal branches grew in the intercellular space of mesophyll tissue. Hyphal product, especially the toxin, caused cell and cell wall damages.

Key words: Alternaria alternata · Minneola tangelo · Electron microscopy

INTRODUCTION

Alternaria species cause four different diseases of citrus: Alternaria brown spot of tangerine, Alternaria leaf spot of rough lemon, Alternaria black rot of many citrus fruits and mancha foliar on Mexican lime.Mazandaran, with about 90000ha citrus planting areas, is the major citriculture province in north of iran.Alternaria brown spot on Minneola tangelo and Alternaria black rot on Thamson navel orange trees, are two important diseases, caused by Alternaria fungi,in the west and east of mazandran province, respectively [1]. Not any report have puplished about the two of her diseases yet. Alternaria brown spot, caused by 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 minute brown to black spot on young leaves and fruit.

Symtoms can appear as little as 24h after infection. Lesion usually continue to expand and large areas of the leaf may be killed by the host selective ACT-toxine. [2]. On mature leaves, brown spot appears as distinct brown lesion surrounded by a yellow halo. Affected leaves abscise and infected twigs die back, especialy if the leaves have fallen. On fruit, lesion can vary from and can be dislodged, forming apock mark on the surface. Severely affected fruit abscise, reducing yield and blemishes on the remaining fruit diminish marketability. The diseases cycle appears to be relatively simple since no teleomorph has ever been connected with fungus [3].

Conidia are produced primarily on the surface of lesion on mature leaves that remain on the tree or have fallen to the grove floor [3]. Conidia are dispersed by wind currents and are subsequently deposite on the surface of young. Susceptible fruit of leaves.

Conidia germinate quickly if moisture is present and begin to produce toxin even before they penetrate the tissue. penetration of the leaf can occure directly or through stomata. The optimum temperature for infection is 27°C. As temperatures decline, longer wetting periods

Corresponding Author: Dr. A.A. Dehpour, Department of Biology, Islamic Azad University, Research and Science Branch, Tehran, Iran

are needed for infection to occure. Small amounts infection can occur with leaf wetnees duration of 4-8 h, but usually 10-12h. of wetness are need for substantial infection [4, 5]. Sporulation occurs in lesion on mature leaves in the presente of water or even more abundantly when humidity is high [4].

Conidial release is stimulated by the impact of rain drops on the leaf surface and also by sharp drops in humidity. Under fild conditions, conidia are dispersed after rains, were abundant moisture is present to allow infection.Altertnatively, conidial relase may be triggered when the dew dries in the morning and dispersal can occur on winds during the day and infection may fallow with the dew in the evening. The former method of dispersal may be more common in high rainfall areas and the latter in semi-arid regions. There have been no studies on infection of citrus by *A. alternata*. The objective of this investigation was to describe the prepenetration, penetration and infection processes of *A. alternata* on Minneola tangelo using light and electron microscopy.

MATERIALS AND METHODS

The observe the infection process of Alternaria on Minneola tangelo. Sample of leaf pieces and fruit skin after infection collected were prefixed in 2% glutaraldehyde-p-formaldehyde in 0.05 M. Sodium cacodylate buffer at pH 7.2 for 2h, at room temperature. The sample 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. Sample were freez-drayed (-40°C).

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 disks were observed in this experiment.

RESULTS

Conidia of *A. alternata* were small and septate ranging from7 to30 µm invivo with long filiform beaks (Fig. 1). Germinated and ungerminated conidia were not disloged during SEM preparation and so it was concluded that thay adhered strongly to the leaf surface (Fig. 2).

Each conidium produced several germ-tube at random position on the conidium over time and grew profusely in random directions across the surface Mature germ tube were variable in length (10-250 µm) and branched infrequently (Fig. 3) Appressoria not formed directly on the cuticule or on stomata.some germ-tubes grew towards and entered a stoma without forming an appressorium over the stomata. Other germ-tube passed near stomata without appressorium formation and showed on directional growth toward the stomata (Fig. 4, 5).

Stomata passed in this way were found in both open and closed stomata.Occasionally extensive growth of germ tubes formed a hyphal net work on the host tissue (Fig. 6). Conidia are produced primarily on the surface of lesion on mature or senescent leaves and on blighted twigs. Relatively few, if any, produced leaves and on young lesion on leaves or mature lesion on fruit (Fig. 7).

Some of hyphal branches were grown in intercellular space of the mesophyll and paranchyme tissue surrounds (Fig. 8, 9).

The hyphal product,specially toxine (HST and NHST) caused damaged cell and cell wall diuration (Fig. 10).

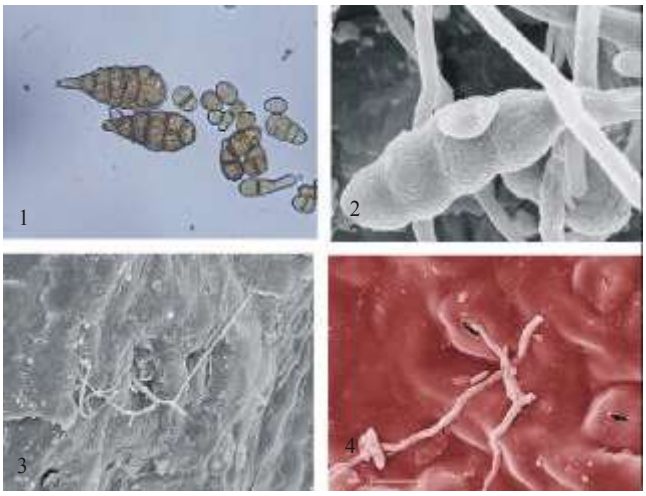
Fungal structure of *A. alternata* on the leaf surface centeral darkned area,represent cell wich have possibly discoloured as a result of infection with fungus (Fig. 11).

The hyphea of *A. alternata* grew extensively abaxial surface and filled the epidermal cell of leaf *Minneola tangelo* (Fig. 12).

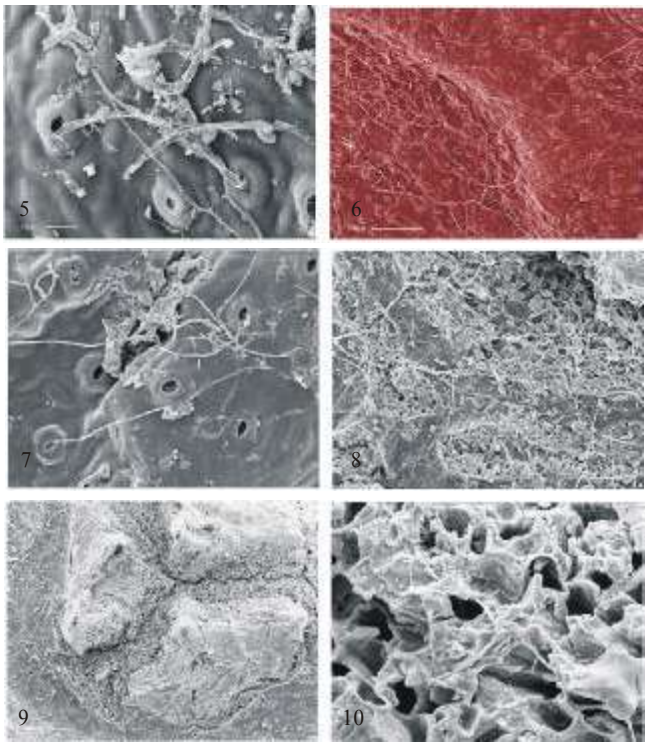
Young conidiophores of *A. alternata* developing and emerging directly through the epidermal leaf of *Minneola tangelo* (Fig. 13).

Spores of other fungi in this side could not the penetration and infection process in open stomata (Fig. 14).

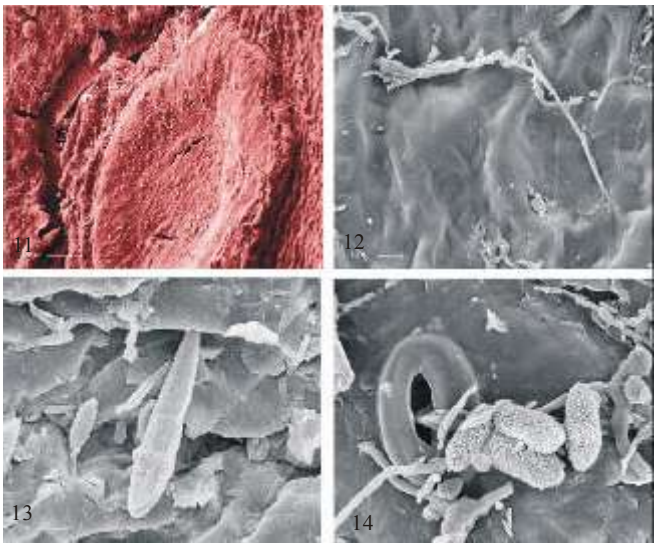
Conidium production is greatest when leaves are lightly moistured or held at high humidity with fewer produced where leaves are very wet.Conidia germinate quickly if moisture is present and begin to produce toxine even befor thay penetrate the tissue. Pentration has been consistently associated with formation of appressoria in studies preformed in Iran. However, in our studies in Iran, penentration occurs through stomates on the abaxial surface of the leaf without the formation appressoria sporulation dos not occur on lesion until the plant tissue mature. Most of the sporulation occurs on leaves whereasa relatively little occurrence fruit or twigs. The optimum temperature for infection is 27°C sporulation occurs in lesion on mature leaves in the presence of water or even more abundantly when humiditly is high.



Figs. 1-4: Light and electron micrographs of conidia and pre-penetration structure formed by *A. alternata* on Minneola tangelo Fig. 1: A mature, beaked conidium of *A. alternata* with multiple septa on surface leaves (×400). Fig. 2: *A. conidium* of *A. alternata* producing several germ-tube at random points on the cinidium body and at the tip of the filiform beak. Bar = 100 µm, Fig. 3: Brannched germ-tubes of *A. alternata* passing open stomata without forming appressoria. Bar = 10 µm, Fig. 4: A germ tube and conidia of *A. alternata* without forming appressoria. Bar=10 µm



Figs. 5-10: Scanning electron micrographs of the penetration events of *A. alternata* on Minneola tangelo leaves. Fig. 5: A germ tube entering a stoma without forming appressorium. Bar = 10 µm, Fig. 6: The leaves surface caverg networking of hyphal on necrotic zone. Bar = 10 µm, Fig. 7: The inner surface of the laef,showing direct penetration of hyphae through the epidermis. Bar = 10 µm, Fig. 8: Hyphae are visible within the mesophyll cells. Bar = 10 µm, Fig. 9: Hyphae growing intercellularly,passing through adjacent epidermal cells. Bar = 10 µm, Fig. 10: The cell wall damaged the affected toxine



Figs. 11-14: Scanning micrographs of the colonization events and structure of *A. alternata* on Minneola tangelo. Fig. 11: A scanning micrograph of fungal structures of *A. alternata* on the leaf surface. Central darkned area represents cells wich have possibly discoloured as a result of infection with the fungus. Bar = 10 µm, Fig. 12: Hyphae of *A. alternata* grew extensively and filled the epidermal cells of Minneola tangelo. Bar = 10 µm, Fig. 13: A young conidiphore of *A. alternata* developing through a stoma of leaf. Bar = 10 µm, Fig. 14: Conidiophores of the other fungi in this side couldn't the penentration in open stomata leaf of Minneola tangelo. Bar = 100 µm.

DISCUSSION

The infection process of *A. alternata* observed on is Minneola tangelo generally similar to that of *A. cassiae* on cowpea [6]. As well as other Alternaria spp.on a renge of hosts [7-11].

Our result confirm those of Van Dyke and Trigino [12] and Mimes *et al*. [13] and Ven Den Berg *et al*. [6], who reported that conidia of *A. cassiae* germinated with in 2-3 hpi. Production multiple germ-tube that grew randomly across the leaf surface. Rotem [10], reported that spores of all Alternaria species germinate in remarkably short time and produce one to several germ-tubes.

Previous SEM studies haw show that extra-cellular material is also associated with germ tube and appressoria of Alternaria helianthi on sunflower (*Helianthus annuus*) [8] and *A. porri* on onions [9] 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*) [14] and *A. cassia* on siklepod [12] and *A. cassiae* on cowpea [6].

Van Dyke and Trigino [12] reported that germ tube 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 cell or over stomata with about equal frequency. Our results also show the dos not formation of both terminal and intercalary appressoria directly on epidermal cell or over stomata.

In the present study, germ tube occasionally entered through a stoma with no appressorium formation, as reported for *A. helianthi* on sunflower [8]. 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 [12] similary found that *A. alternata* entered its specific host through stomata and by direct penetration with or without appressoria. Van Den Berg *et al*. [6] finding that A.cassia on cowpea leaves, direct and indirect penetration with or without the formation of appressoria. Von Ramm [15] also reorted that Alternaria longipes penetrate tobacco (*Nicotiana* sp.) leaves without appressorium formation.

In less pathogenic *Alternaria* spp., The infection court is limited to wounds and stomata [10].

There was no evidence of specific orientation or long-distance attraction towards stomata and germ tubes often passed stomata with on apparent tropic respose. Stomatal penetration appeared to occur by chance.

These results agree with those of A.longipes on tobacco [15] *A. cassiae* on sicklepod [12] *A. porri* on onions [9] and *A. cassiae* on cowpea [6].

The mode of penetration, whetter mechanical or chemical, was not determined in this study. As reported for *A. cassiae* on sicklepod [12] and *A. cassiae* on cowpea [11], darkened areas represnting discoloured cells as a result of interaction with the fungus were also observed in the near vicinity of fungal structure of *A. alternaria* on citrus. The presence of these discoloured cell indicates that the cells have been disrupted. Van Dyke and Trigino [12] 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, secandery hyphae developed from primary hyphae and grew in the intercellular spaces and also penetrated and grew intercellulary with in the epidermal and obtained for *A. cassiae* on sicklepod [12] and *A. cassiae* on cowpea [11].

These authors reported that intera 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 hostes, this is the first study of the infection process of *A. alternata* on Minneola tangelo. This study has broaded our knowledge of the ore-penetration structure, penetration and colonization of *Alternata* spp., especially on citrus.

Alternaria produce such lytic enzymes as polyglacturonase, pectin lyase, pectin methylestrase, cellulose and two categories of toxine, namely, host, specific toxins (HST) and non host specific toxine (NHST). Among the first, toxins such as the AM, AC, AK, AF and AL have been identified and their role in pathogenesis verified [10]. Plant respond by deposition of lignin to the cell wall of infected cells [16].

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