

Histopathology of grapevine inoculated with *Lasiodiplodia theobromae*

Abdullah H. Al-Saadoon, Mohanad K.M. Ameen and Eman M.A. Al-Rubaie
Department of Biology, College of Science, University of Basrah, Iraq

Abstract. Histopathological changes of 2-years old grapevine cv. Abbassi inoculated with *Lasiodiplodia theobromae* (syn. *Botryodiplodia theobromae*) were studied. Samples were taken at 7 and 25 days after artificial inoculation. At 7 days, cross and longitudinal sections revealed the presence of disorganized cells, degenerated areas and vascular bundles. The intra and intercellular invasion of the inoculated tissues by the fungus were evident with dark brown color. At 25 days, the fungus caused necrosis in xylem parenchyma and xylem vessels, the hyphae colonized the tissues. Dark deposits appeared in vessels and other tissues as well as abundant production of tyloses. *L. theobromae* has also been found to produce pycnidia on the shoots and petioles of grapevine which yielded 2-celled dark pycnidiospores. After more than 4-weeks, the inoculated grapevine seedlings look flaccid, brown in color and wilting symptoms began to appear and finally the seedlings were dead.

Keywords: grapevine, die-back, histopathology, *Lasiodiplodia theobromae*.

Introduction

Lasiodiplodia theobromae (Pat.) Griffon and Mauble is recognized as an important wood pathogen and has been reported to cause cankers, die-back, and fruit as well as root rot in over 500 different hosts. It is geographically wide spread but is most common in the tropical and sub-tropical regions worldwide (20). Die-back of vitis (*Vitis vinifera* L.) caused by *L. theobromae* is an important disease affecting grapevine all over the world (14).

In Egypt, according to the available literatures, a report concerning the host parasite-interactions of *Botryodiplodia theobromae* (syn. *L. theobromae*) involved in die-back disease of grapevine has been confined to the work of (4), who detected that necrotic xylem parenchyma and xylem vessels, dark inclusions bodies as well as abundant production of tyloses were caused by hyphae of *B. theobromae*. Histopathological studies of die-back

disease incited by *L. theobromae* have been reported in other hosts, including mango (10, 17, 21) and peach (6).

So far, little attention has been given to mycotic grapevine diseases in Iraq. Early research by (16) reported of *Hendersonula toruloidea* to cause branch wilt of grapevine, in central region of Iraq. Recently, based on morphological and molecular data (11) identified *Phaeoacremonium aleophilum* from grapevine decline in Duhok governorate, Northern Iraq. The aim of this investigation was to study the histopathology of grapevine (cv. Abbassi) artificially inoculated with the fungus *L. theobromae*.

Materials and Methods

The artificial infection by *L. theobromae* was done on two years old grapevine seedlings Abbassi cultivars from Abo-Alkhasib vineyards, Basrah, southern Iraq. The isolate of *L. theobromae* was obtained from grapevine shoots with die-back symptoms (3). A wound on shoots, petioles

and leaves was made using sterilized sharp blade and then inoculated by spraying with 40 ml of conidial suspension (5×10^6 conidia ml^{-1}) of *L. theobromae* or just water as a control (1). Inoculated seedlings were covered with plastic bags to provide high moisture contents for two days in green house at 25 C°. Reisolation were done from the inoculated seedlings showed typical symptoms of die-back. Small pieces of necrotic tissues from the edge of each lesion were cut, surface-sterilized for 5 min, in 5% sodium hypochlorite, washed three times with sterile distilled water and transferred into surface of PDA in Petri plates, then incubated at 25 C°. 10 and 25 days post inoculation, samples of shoots, petioles and leaves were cut, washed three times with sterilized distilled water and dried between folds of sterilized filter papers. The artificially inoculated and non inoculated tissues were cut into small portions (5–10 mm long) and fixed in a solution of formalin, acetic acid and ethyl alcohol 5% (5:5: 90%) for 24 hr., washed in ethyl alcohol 50 %, then dehydrated by passage through a series of ethyl alcohol, cleared in xylol and embedded in paraffin (12). Sections of 10–15 μm as thickness were cut using a rotary microtome, then stained with safranin- light green and mounted in Canada balsam (13). The stained sections were examined by a light microscope and photographed by a camera Optika (HD Z600, ITALIA).

Results and Discussion

Microscopical examinations revealed several changes on the tissues of grapevine cv. Abbassi seedlings, artificially inoculated with *L. theobromae*. Transverse and longitudinal sections showed the systemic nature of infection.

A 7 days post inoculation with *L. theobromae*, the transverse sections

showed the site of the initial infection in the shoots (Fig. 1) and the degeneration of the epidermis and parenchyma cells in the cortex (Figs.1, 2).

The intra and intercellular invasion of the parenchymatous cells by fungal mycelia were evident in shoots with dark colour (Figs. 3). Fig. 4 showed that the infection with *L. theobromae* caused serious damage on the cortical layer and the fungal hyphae of the pathogen were observed inside the cortical cells. The infection with *L. theobromae* histological examination of cross sections of the petioles of grapevine cv. Abbassi showed the sites of the initial infection (Fig. 5). Transverse sections of the shoots of the grapevine infected with *L. theobromae* showed serious damage in the vascular bundles (Fig. 6) in comparison with control (Fig. 10).

Also degenerated zones with protolysis symptoms were observed in different tissues, i.e. epidermis, cortex and vascular tissues (Fig. 7). In addition, the longitudinal sections in shoots of grapevine after 7 days of inoculation with *L. theobromae* revealed disorganized epidermal and cortical cells, xylem vessels colonized by hyphae and the formation of dark inclusions were observed (Fig. 8). It is also noticed that, the xylem rays were a suitable path for rapid and easy spreading of the hyphae. Microscopical examination of the sections of the non-inoculated samples showed normal and intact tissues without any visible changes in their histological structures compared to the infected one (Figs. 9, 10).

Sample taken at 25 days old, post inoculation with *L. theobromae* showed a spread of the pathogen through the infected tissues and the sections figured out several histological changes in both shoots and petioles due to *L. theobromae* infection. The fungus spread intra-and inter-cellular

in all tissues particularly in the vascular bundles causing clear destruction of phloem and cambial cells (Fig. 11). Necrotic xylem parenchyma and xylem vessels, colonized by hyphae, and dark deposits appeared in vessels and other tissues as well as a abundant production of tyloses (Figs. 12-15). No hyphae or tyloses were observed in uninfected controls (Fig. 16). The heavy establishment of the fungal mycelium in the various tissues of shoots and petioles led to complete degeneration as seen in (Figs. 17-20). A dense agglomerations of hyphae and pycnidia were found in the infected shoots and petioles of grapevine, which yielded 2-celled dark pycnidiospores (Fig. 21).

Cross sections of 7 days after artificial inoculated leaves of grapevine cv. Abbassi showed that the infection occurred only in the midvein. The epidermal and cortical cells showed plasmolysis and xylem vessel were blocked by gummosis (Fig. 22). While the leaf lamina appeared without any visible change in their histological structure compared with the control (Fig. 23, 24). This result agrees with previous studies conducted in California, Portugal and Mexico, which found that there were no foliar symptoms associated with *L. theobromae*. (19; 22).

After more than four weeks the whole inoculated grapevine cv. Abbassi seedlings became dark brown, flaccid and wilting began to appear and finally the seedlings were dead. These results are consistent with those reported by (4) who found that, 7 days after artificially inoculated grapevine shoots with *B. theobromae* induced disorganized epidermal cells with dark brown color consisting of plasmolysis cells and tissues. The fungal hyphae were clearly noticed after 21 days of inoculation with *B. theobromae* in both xylem parenchyma and xylem vessels causing

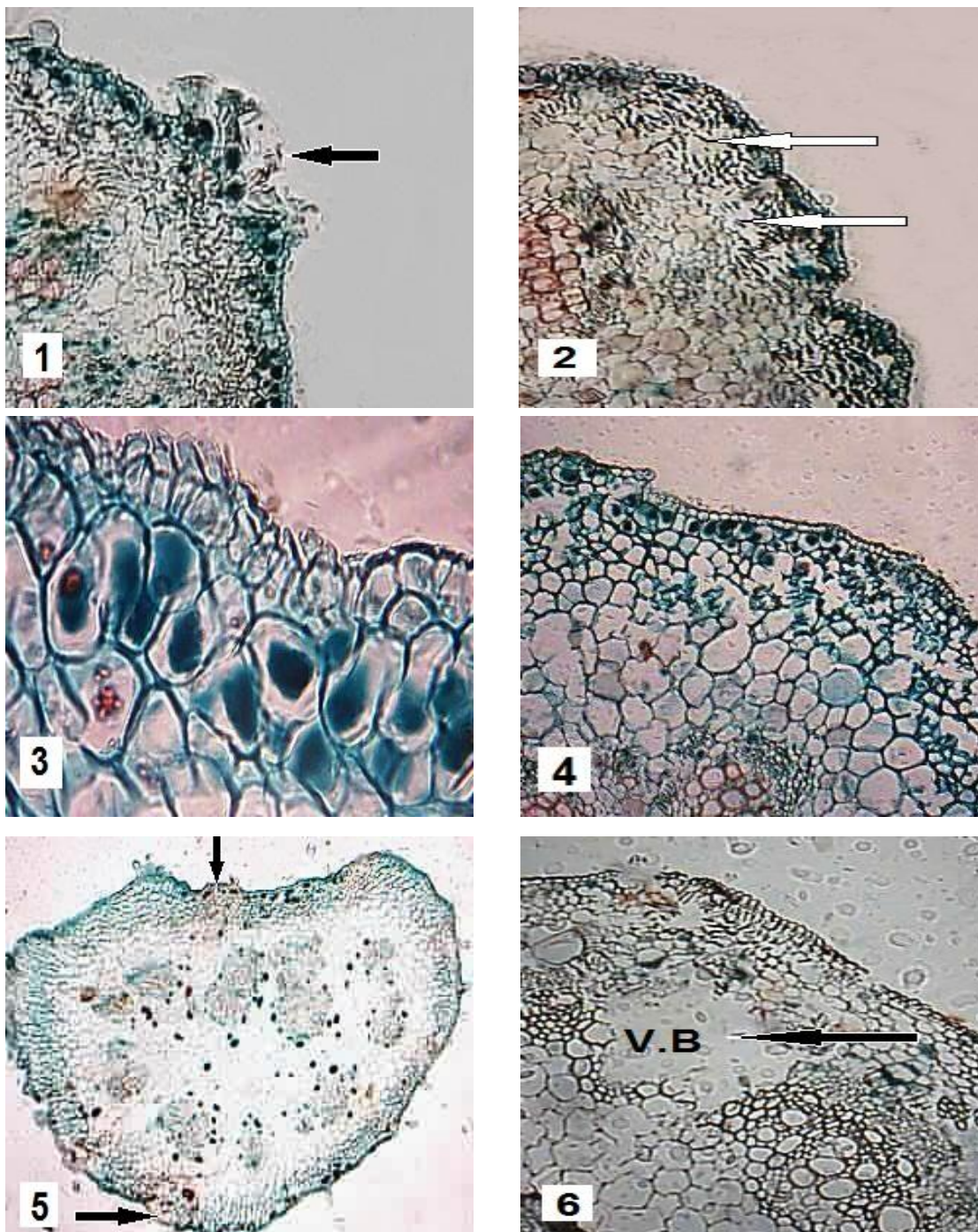
necrosis at these tissues , Also they noticed the xylem tissues were colonized by hyphae and dark inclusions bodies as well as a abundant productions of tyloses. No disorders were noticed in case of *Phomopsis viticola* and *Fusarium solani* after 7 days of inoculation, while, the above mentioned pathogenic fungal hyphae were clearly noticed after 21 days of inoculation in both xylem parenchyma and xylem vessele causing necrosis at these tissues.

The fungal hyphae spread intra and intercellularly in all tissues and the vessels were plugged with gum as well as abundant production of tyloses. Similar findings were obtained by (18) who found that the fungus *Phaeoacremonium chlamydosporum* infects the xylem parenchyma cells of vine shoots as intercellular hyphae and these cells produce tyloses in adjacent xylem vessels. The hyphae also penetrate the vessels, often by way of tyloses. Brown deposits were seen in vessels and cells assumed to be an accumulation of phenolic compounds. The presence of necrotic vessels, hyphae and reduction in the size or collapse of vessels due to infection development of tyloses in the vessels releasing of large molecule compounds in the vessels as a result of cell wall degrading by pathogenic enzymes might bring about dysfunction of the xylem elements with a restriction in the flow of water and minerals (21, 2). The cell walls are consist not only of polysaccharides material but also of condensed tannins and phenols. It appears that these previous two components also accumulate within the tyloses (15), It is known that following attack by micro-organisms, plants sometimes have high levels of condensed tannins because of an increased production of new tannins or the mobilisation of pre-existing tannins towards the infection sites,

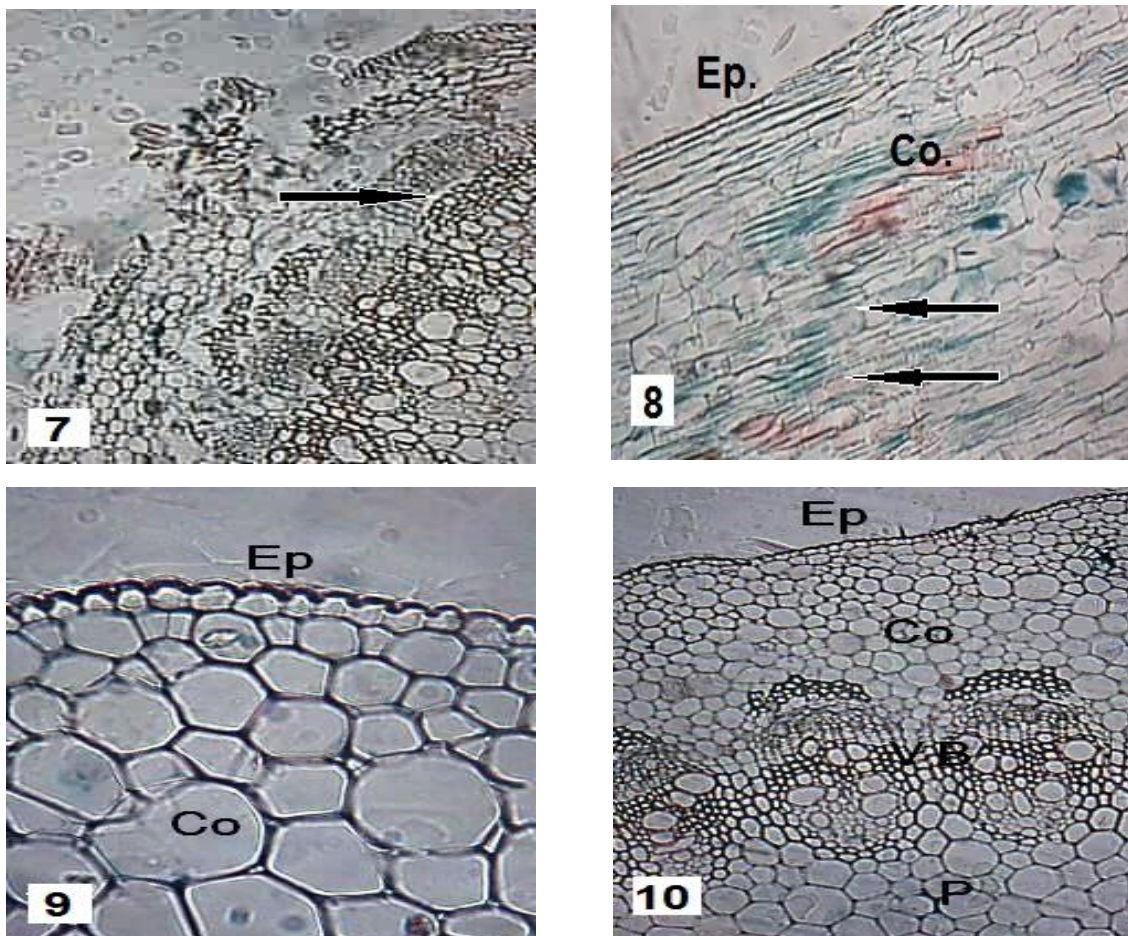
in order for example to inhibit fungal enzymes (8), to reinforce the structural components of the cell walls (5) or to form a chemical barrier to infections (9).

The results of this study are also in agreement with those observed by (17) and (21) for mango, (6) for peach and (7) for apple. Mature pycnidia with pycnidiospores were produced within 25 days after

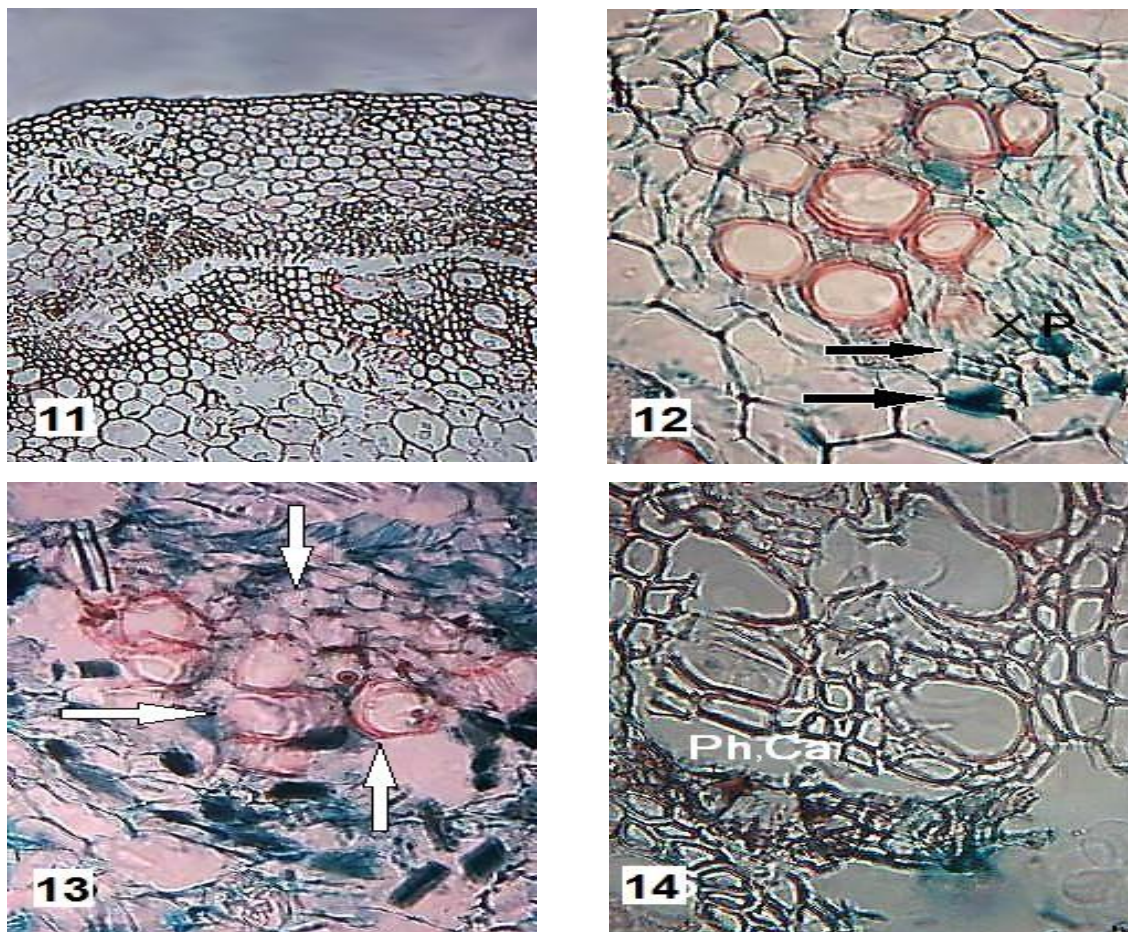
artificially inoculated grapevine shoots and petioles with *L. theobromae*. While the cross sections in shoots inoculated with *B. theobromae* after 21 days showed that pycnidia were embedded in epidermal layer and pycnidiospores in the pycnidial cavity (4).



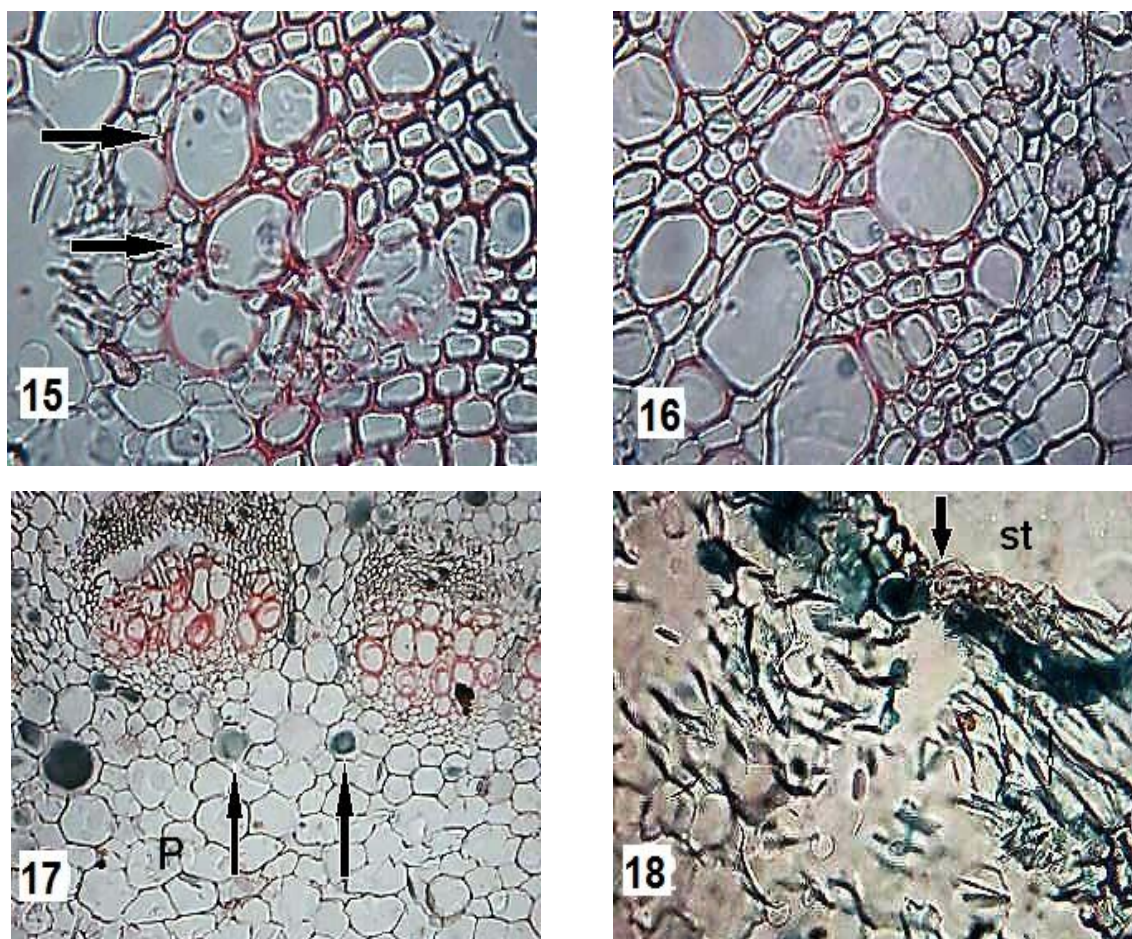
Figures (1– 6): Light micrographs of grapevine cv. Abbassi tissues 7 days after inoculation with *Lasiodiplodia theobromae*. 1 (X10), transverse section in the inoculated shoots showing initial infection in the epidermal cells (arrows). 2 (X10), showing degenerated epidermal and cortical cells (arrows). 3 (X40), infected cortex showing the presence of fungal hyphae colonizing the cortical cells. 4 (X10), development of infection in the cortex . note the degeneration of the parenchyma cells in the cortex and the presence of the fungal mycelia in the cells. 5 (X4.5), transverse section in the infected petiole showing the initial infection in the epidermis (arrows). 6 (X10), transverse sections in the inoculated shoots showing severely damaged of vascular bundle.



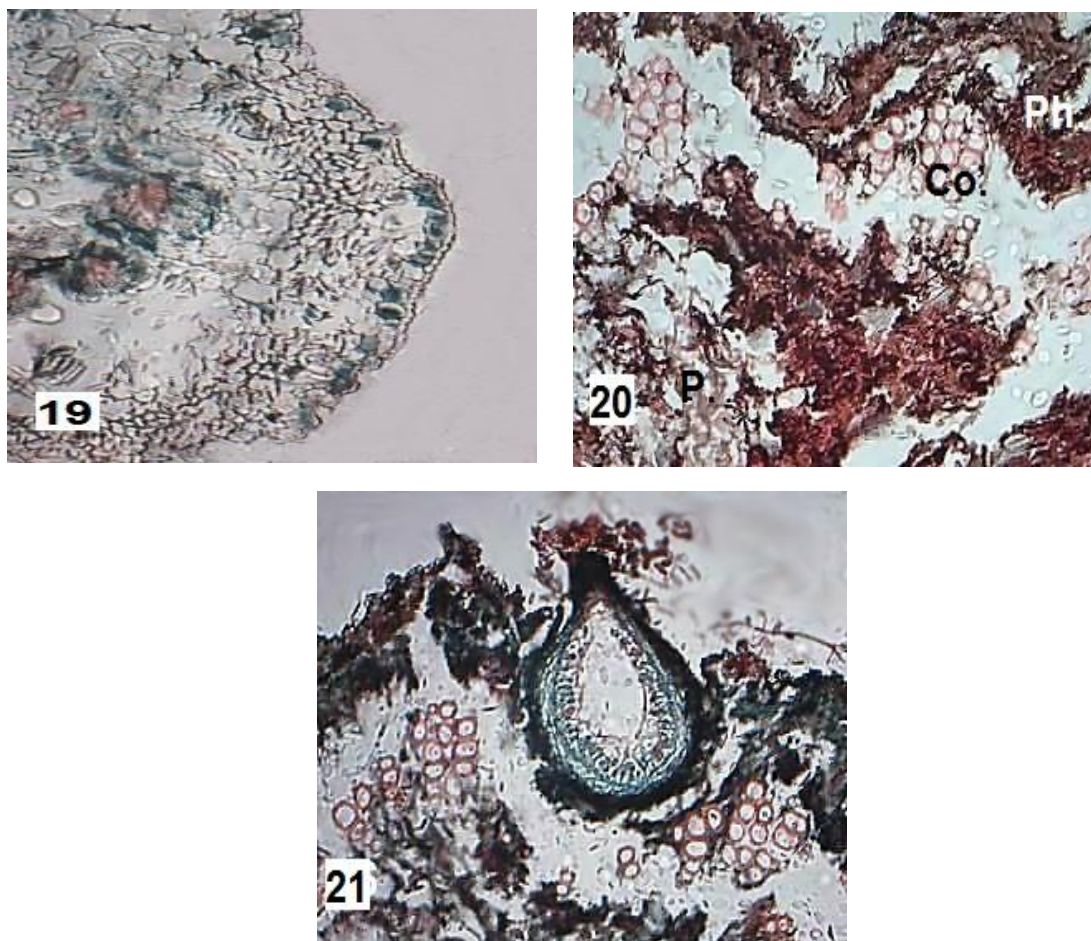
Figures (7 – 10): Light micrographs of grapevine cv. Abbassi shoots 7 days after inoculation with *Lasiodiplodia theobromae* and non – inoculated shoots. 7 (X10), showing apparent break down of epidermal and cortical cells, extending the infection to the vascular tissues. Note cracks in the vascular bundle (arrows). 8 (X10), Longitudinal section of shoots 7 days after inoculation showing disorganized epidermal and cortical cells, xylem (X) necrosis, fungal hyphae in the tissues. 9 (X40), transverse sections in the non-inoculated shoots showing normal epidermis and cortex. 10 (X10), non-inoculated shoots showing intact epidermis (E P), cortex (CO), vascular Bundles (V B), pith (P).



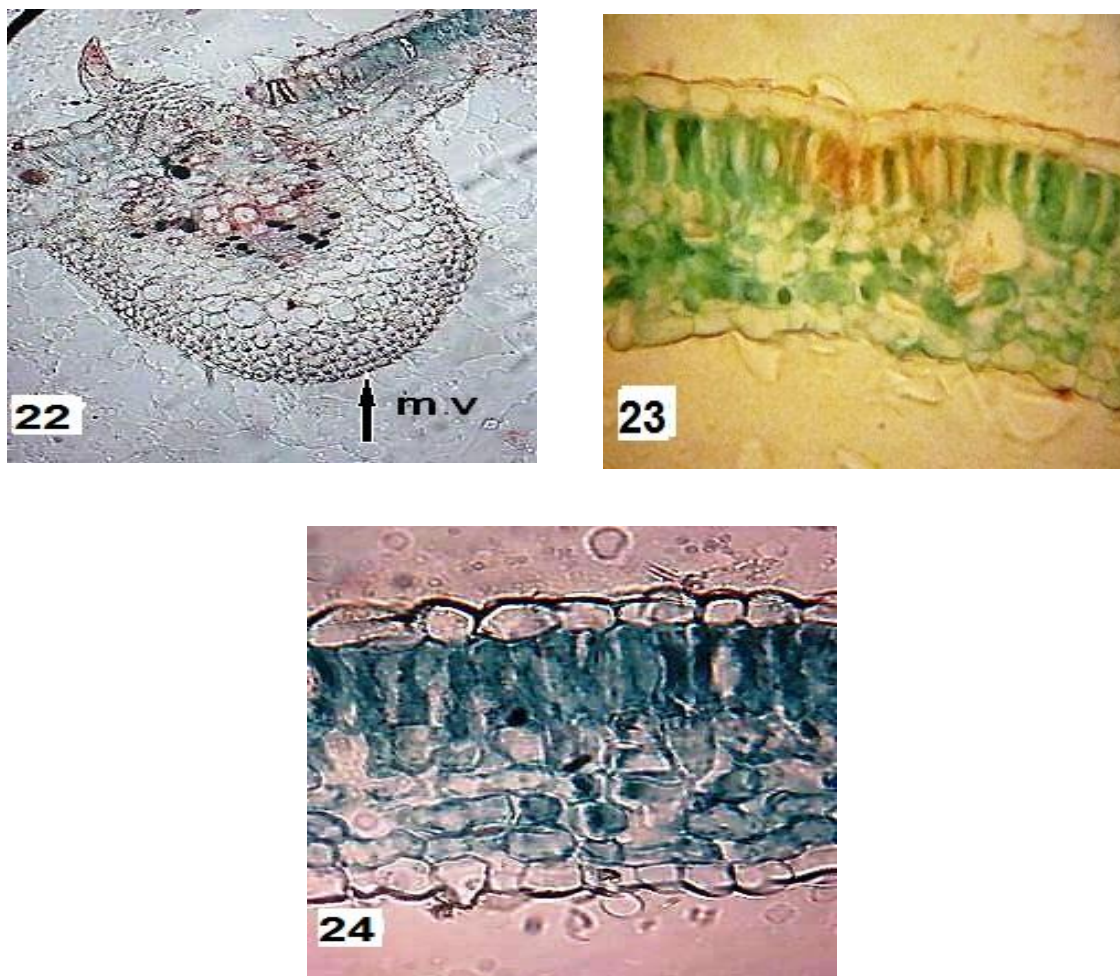
Figures (11-14): Light micrographs of grapevine cv, Abbassi shoots 25 days after inoculation with *Lasiodiplodia theobromae*. 11 (X10), transverse sections in the inoculated shoots showing the infection in the cortex and vascular tissues. note the presence of clear cracks in the vascular bundles, 12 (X40), showing infected vascular bundle. Note xylem parenchyma (x.p) colonized by hyphae (arrows). 13 (X40), showing vascular bundle completely destroyed and colonization of the tissues by fungal hyphae. 14 (X40), showing destroyed phloem and cambium (Ph, Ca) cells.



Figures (15– 18): Light micrographs of grapevine cv. Abbassi 25 days after inoculated with *Lasioidiplodia theobromae*. 15 (X40), transverse sections in the inoculated shoots showing fungus mycelium spread through intercellular spaces (arrows), Xylem vessels blocked with tyloses. 16 (x40), transverse section in the non-inoculated shoots showing normal vascular bundle. 17 (X40), showing fungus mycelium spread through intra- and intercellular spaces, pith tissues colonized by fungal hyphae (arrows). 18 (X40), substomatal (st) infection in the shoots. Note the hyphal invasion of the tissues and destroyed cortex cells.



Figures (19- 21): Light micrographs of grapevine cv. Abbassi 25 days after inoculated with *Lasiodiplodia theobromae*. 19 (X10), transverse section in the inoculated shoots showing severely damaged and disorganized tissues. 20(X10), seriously damaged and degenerated tissues cortex (Co.), phloem (Ph.) and pith(P.) of the inoculated shoots. 21 (X10), Longitudinal sections of shoots showing pycnidium of *Lasiodiplodia theobromae* and 2-celled dark pycnidiospores.



Figures (22–24): Light micrographs of grapevine cv. Abbassi Leaves 7 days after inoculated with *Lasioidiplodia theobromae* and non – inoculated ones. 22 (X10), transverse sections in the inoculated Leaves showing infected midvein (m.v), destroyed epidermal and cortical cells, xylem vessels blocked by gummosis. 23 (X10), transverse sections in the inoculated Leaves showing no signs of infection in the blade (not affected). 24 (X10), transverse sections in non – inoculated Leaves showing normal mesophyll.

Conclusions

There is still little data available especially histopathology about die-back disease of grapevine in Iraq. However, our findings from the present study revealed the systemic nature of infection. Xylem rays were a suitable path for rapid and easy spreading of the hyphae. The heavy establishment of the fungal mycelium, dark deposits as well as abundant production of tyloses led to complete degeneration of various plant tissues.

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التغيرات المرضية النسيجية لنبات العنب الملقحة بالفطر *Lasiodiplodia theobromae*

عبد الله حمود عبدالله، مهند خلف محمد امين، ايمان محمد عبد الزهرة
قسم علوم الحياة، كلية العلوم، جامعة البصرة

المستخلص. درست التغيرات المرضية النسيجية لنبات العنب (صنف عباسي)، بعمر سنتين والملقح بالفطر *Lasiodiplodia theobromae*. اخذت العينات بعد سبعة و 25 يوم من الإصابة الاصطناعية بعد سبعة أيام من الإصابة، أظهرت المقاطع الطولية والعرضية عدم انتظام الخلايا وتحلل الحزم الوعائية. واثبت غزو الفطر داخل خلوي وبين خلوي للانسجة المصابة مع تلون الأنسجة باللون البني الداكن. في اليوم 25 من الإصابة، سبب الفطر تنخر برنكيما ووعية الخشب واستعمار الخيوط الفطرية للانسجة. وشوهدت ترسبات داكنة في الأوعية والأنسجة الأخرى مع كثرة تكوين التايلوزات. ولوحظت بكنيديا الفطر *L. theobromae* على الأغصان وحوامل الاوراق، وقد كونت ابواغا بكنيدية داكنة ثنائية الخلايا. بعد اكثر من أربعة اسابيع، بدت نباتات العنب (صنف عباسي) مترهلة، بنية اللون، وظهرت عليها اعراض الذبول، وفي النهاية موت النباتات.