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Basal Stem Rot of Vegetables in Controlled Environment Greenhouses in Western Saudi Arabia: (A) Studies on the Causal Agent *Pythium aphanidermatum*

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ABSTRACT. Samples of diseased cucumber (Cucumis sativus L.), cantaloupe (*Cucumis melo var. cantalopensis*) and sweet pepper (capsicum annum L.) exhibiting basal stem rot and damping off were collected from controlled environment greenhouses in the western region of Saudi Arabia. Based on morphological characterization of vegetative and sexual structures (mycelium, zoospores and oospores), the causal agent was identified as *Pythium aphanidermatum* (Edson) Fitsp. Mycelium was hyaline, non-septate. Oogonia were spherical and terminal with dome-shaped, diclenous (antheridia and oogonia from different hyphae) and intercalary antheridia (one antheridium per oogonium). Oospores were spherical and aplerotic (partially filling oogonia). Sporangia were inflated filamentous or lobulate that germinate giving vesicles containing zoospores, which soon be released. Four different culture broth media, V-8 Juice Broth (V-8JB), Oat Meal Broth (OMB), Corn Meal Broth (CMB) and Potato Broth (PB) were quantitatively evaluated for vegetative growth and induction of reproductive structures. The highest mycelial dry weight was significantly (at $P \leq 0.05$) observed on OMB. No significant difference (at $P \leq 0.05$) were found between PB and V-8JB. On the other hand, the lowest mycelial growth was detected on CMB. Oospore density was significantly (at $P \leq 0.05$) the highest on CMB followed by V-8JB then OMB. However, oospores were not formed on PB. Zoospore production, on the other hand was the highest when the fungus was grown in V-8JA, followed by OMA, PA then CMA.

Introduction

Pythium aphanidermatum (Edson) Fitsp., a soilborne plant pathogen distributed worldwide, infects many economically important crops of more than 70 plant species belonging to 59 genera of higher plants (Middleton, 1943). This include grain (Freeman *et al.,* 1966) and vegetable crops (Gottlieb & Butler, 1939; Bates & Stanghellini, 1984; Giammichele & Pill, 1984 and Favrin *et al.,* 1988). The fungus causes damage by inciting damping off (Kaiser *et al.,* 1971, Gullino, 1992 and De & Mukhupadhyay, 1994), root rot (Stanghellini *et al.,* 1984 and Rafin & Tirilly, 1995) and basal stem and crown rot (Stanghellini & Phillips, 1975). It prevails in most of the commercial vegetable producing greenhouses around the world (McArter *et al.,* 1980; Jenkins & Averre, 1983; Bates & Stanghellini, 1984; Gold & Stanghellini, 1985 and Favrin *et al.,* 1988). It also prevails in greenhouses employing a recirculating hydroponic cultural systems (Jenkins & Averre, 1983; Goldberg *et al.,* 1992 and El-Ghaouth *et al.,* 1994). Such diseases are considered as a limiting factor to the production of many crops and often these crops are abandoned.

Widespread of the diseases caused by *P. aphanidermatum* is due to the nature of the reproductive structures. Oospore, the primary source of inoculum, is the sole resistant structure that permits a prolonged survival of the fungus for many years in the soil under adverse conditions (Burr & Stanghellini, 1973; Ayers & Lumsden, 1975 and Ruben *et al.,* 1980). Growth and disease development caused by *P. aphanidermatum* is favored by high temperatures especially during the hot summer days (Litterell & McCarter, 1970; Stanghellini & Phillips 1975; Gold & Stanghellini, 1985).

Diseased samples of cucumber (*Cucumis sativus* L.), sweet pepper (*capsicum annum* L.), and Cantaloupe plants (Cucumis melo var. cantalopensis) were collected from vegetable growing environmentally controlled greenhouses from different parts of the western region. The samples were severely wilted at the fruit-bearing stage. A dark yellow necrosis of the basal part of the stem and the crown area was observed for cucumber and cantaloupe plants. Pepper plants were showing a dark brown to black necrosis of the lower stem extended up to 10-20 cm above the soil line. The disease caused a severe damage and crop loss to the vegetable production. Preliminary studies revealed that the causal agent belong to the *Pythium* group.

Although basal stem rot is widely distributed in the kingdom in environmentally controlled greenhouses (Abu-Jawdah, 1986 and Sharif & Abdeen, 1987), identification of the causal agent to the species level is yet unavailable.

The objectives of this study were to identify the causal agent of the damping off and basal stem rot diseases on vegetables in western Saudi Arabia, char-

acterize its reproductive structures and quantitatively evaluate different culture media for the mycelial growth and induction of reproductive structures (zoospores and oospores) of the fungus.

Materials and Methods

Isolation of the Pathogen

Pure culture of *P. aphanidermatum* was obtained from a diseased mature cucumber plant showing basal stem rot received from the Agricultural Experimental Station at Hada-Sham. Infected lesions were washed thoroughly under running tap water for 30 min, cut into one cm segments and surface sterilized in 0.5% NaOCl for one min, rinsed three times in sterile distilled water, and blotted against several layers of sterile paper towels. Each segment was placed in 9-cm sterile plastic petri plate containing 1.5% water agar amended with 200 mg/l streptomycin sulfate and 50 mg/l rose bengal (Dhingra and Sinclair, 1985). Plates were incubated in the dark at 33ºC for 48 hr. A hyphal tip of the fungus was transferred into plates of freshly prepared potato dextrose agar (200 g fresh peeled potato, 15 g dextrose and 15 g agar in 1000 ml distilled water) (Dhingra and Sinclair, 1985). Plates were incubated at 33ºC for 24 hr and stored in the refrigerator until were needed.

Induction of Reproductive Structures

Oospores were induced and observed under the microscope using the glass slide technique on V-8 Juice Agar (V-8JA) medium containing 20% V-8 juice, v/v, 2% agar, and 0.3% Ca CO_3) Tuite, 1968). Clean glass slides were coated with a film of V-8JA medium by dipping the slides in the molted medium and then wipping out the lower surface with clean sterile tissue paper. Each slide was placed in the bottom of plastic petri plate. A 7 mm agar plug, taken from a 2-day old V-8JA culture of the fungus, was placed in the middle of each slide. A moist piece of tissue paper was placed at the side of the bottom of each plate to maintain moist condition and prevent dessication of the agar film. Slides were incubated at 33ºC for 24 hr and were observed daily under the microscope.

Zoospores were induced by slightly modified procedures of Rahimian and Banihashemi (1979). The glass slide technique was used as described above except that 1% water agar was used to coat the slides instead of V-8JA. A 7-mm agar plugs, obtained from two-day old V-8JA culture of *P. aphanidermatum* incubated at 33ºC under continuous fluorescent light, were placed in a petri plate containing sterile distilled water (sdw) for one hour to wash the plugs out of their nutrients. Agar plugs were then placed in the middle of each slide and five ml of sdw was added for each. For the induction of sporangia, slides were in-

cubated at 33ºC under continuous white fluorescent light inside plastic bags for 24 hr. Induction of zoospore production was achieved by incubating the plates at 20ºC in the dark. Zoospore formation and release was observed under the microscope at two-hour intervals.

Evaluation of Culture Media for Reproduction

Four culture media were compared for quantitative evaluation of mycelial growth, oospore and zoospore production. These were V-8 Juice Broth (20% V-8J and 0.3% Ca CO_3), Oat Meal Broth (6% rolled oats), Corn Meal Broth (4% corn meal), and Potato Broth (20% peeled fresh potato) (Tuite, 1968).

For evaluation of mycelium growth, nine-250 ml Elmayer flasks were used for each culture. Each flask was seeded with four agar discs of *P. aphanidermatum,* obtained from two-day old V-8JA. All media were incubated at 33ºC in the dark for 7 days. Mycelial mats from each culture were washed under running tap water for five min, blotted against several layers of paper towels, let air dry over night, then weighed. Quantitative assay of oospores were achieved by moistening dry mycelial mats in distilled water for one hour. Every three mats were mixed with 100 ml cold distilled water in Warner mixer for three minutes. The mixture was diluted to the 10^{-4} by serial dilution adding distilled water, and enumeration of oospores were acheived using a haemocytometer.

Motile zoospores were produced as described by Rahimian and Banihashemi (1979) using four types of agar media seeded with *P. aphanidermatum* as described above except that 1.7, 1.5, 1.2, and 1.5% of agar were added to V-8JA, OMA, CMA and PA, respectively. All plates were incubated at room temperature for 48 h. Six stripes (5 × 90 mm in dia.) were cut from each culture media and then submerged in sdw for one hour for depletion of nutrients. Stripes were placed in separate plastic petri plates containing 20 ml sdw each. To induce formation of sporangia, plates were placed under white fluorescent light for 24 hr. Water of each plate was discarded to get rid of any trace of nutrients and fresh sdw was added. To induce production of motile zoospores, plates were incubated at 20ºC in the dark for four hr. For the immobilization of the formed zoospores, suspension containing the zoospores was collected by pooling every two plates in 250-ml flasks and refrigerated for two hr. Suspension volume of each medium was adjusted to 100 ml and number of zoospores per ml was determined using a haemocytometer.

Results and Discussion

Characterization of Reproductive Structures

Morphological characteristics, size and formation time of each organ of *P. aphanidermatum* are presented in Table 1. Hypha was fast growing, hyaline and non-septate measuring 3.2-7.1 µ in dia .Oogonia were spherical, terminal with a

size of 23.5-27 µ in dia. They developed 36 hr after seeding the culture with the fungus and could hardly be observed since they were immediately fertilized forming oospores (Fig. 1-1). Antheridia were dome-shaped, one per oogonium, diclenous (antheridia and oogonia from different hyphae), terminal at early stages of fertilization then intercalary (tube of antheridium extended from the base making it lateral), measuring avg 9.6 µ in dia (Fig. 1-1). Oospores are spherical, thick walled, aplerotic (partially filling oogonia) with an average size of 18 µ in dia. (Fig. 1-2).

TABLE 1. Description, size and formation time of *Pythium aphanidermatum* structures.

Structure	Description	Diameter (µ)	Formation time
1. Hypha	hyaline, non-septate	3.2 - 7.1	–
2. Oogonia	spherical, terminal	23.5 - 27.0	> 36 min[a]
3. Antheridia	dome-shaped, diclenous, intercalary	8.3 - 9.6	> 36 min[a]
4. Oospores	spherical, aplerotic	17.4 - 18.8	> 36 h[a]
5. Sporangia	inflated filamentous or lobulate	6.4 - 15.2	18 - 24 h[a]
6. Vesicles	spherical, germinate from sporangia	17.6 - 38.0	2 - 4 h[b]
7. Zoospores	uniform, laterally biciliate	7.5 × 12.4	10 - 15 min[c]
8. Encysted zoospores	spherical	8.3 - 9.7	1 - 2 hr[d]

[a]Formation time after seeding cultures with the fungus.
[b]Formation time after cooling cultures at 20ºC.
[c]Time after vesicle formation.
[d]Formation time after chilling of motile zoospores suspension in refrigerator.

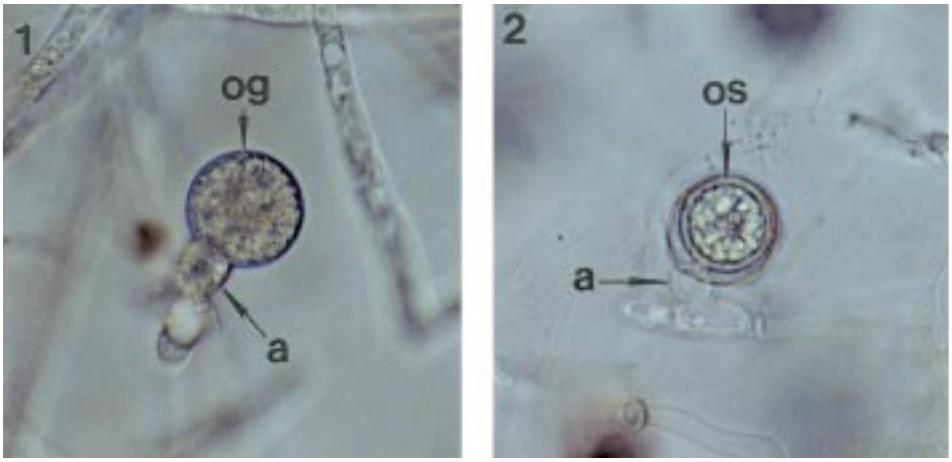


FIG. 1. Oospore formation of *P. aphanidermatum,* 1. og, spherical, terminal oogonium; a, dome-shaped terminal antheridium, 2. os, spherical, aplerotic, thick-walled oospore; a, intercalary antheridium.

Sporangia were developed between 18-24 hrs after seeding (Table 1). They were inflated filamentous (Fig. 2-1) or often lobulate (Fig. 2-2 and 2-3), with lobes formed mostly in complexes with a high variation in size, 6.4-15.2 μ in width (Table 1). Flagellated zoospores were differentiated in vesicles, which were developed from germinating sporangia (Fig. 3-1). Vesicles measured 17.6-38 μ in dia. with 5-17 zoospores (Table 1). They were formed from two to six hr after cooling cultures of the fungus at 20ºC . Vesicles were formed in few seconds after germination of the sporangiun by the fast flow of cytoplasm. Within few minutes, flagellated zoospores were developed and started to rotate inside the vesicle until they were released (Fig. 3-2 and 3-3). The whole process, from germination of sporangia until the release of zoospores, took about 10-15 min. Motile zoospores encysted within 1-2 h measuring about 8.3-9.7 μ (Fig. 3-4) (Table 1).

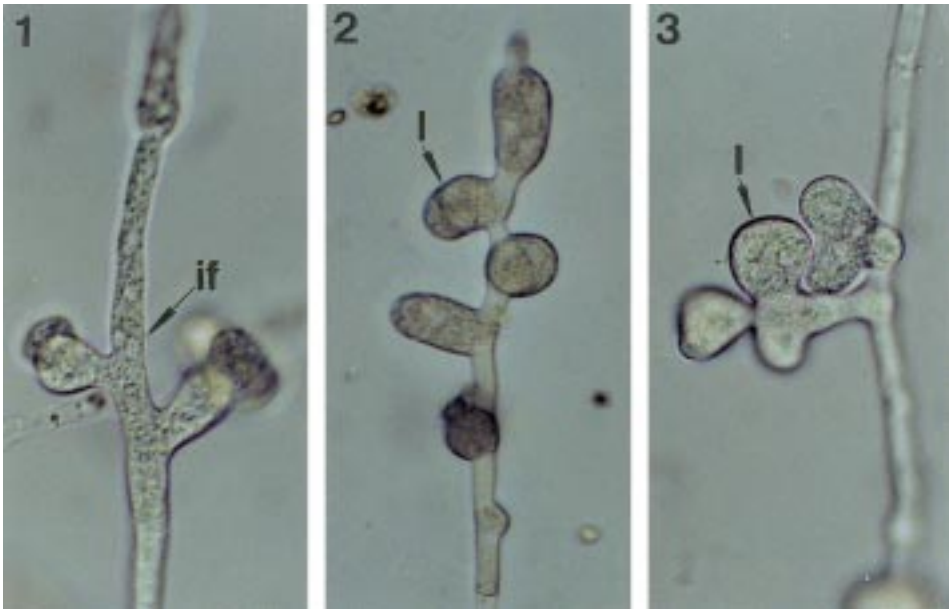


FIG. 2. Different shapes of sporangial formations of *P. aphanidermatum.* 1. if, inflated sporangium. 2. and 3. 1, lobulate sporangia.

All morphological characteristics of mycelia, sporangia, antheridia, oogonia, oospores and zoospores of *P. aphanidermatum* presented by Gilman (1957), Ilag (1976), Middleton (1943), Watanabe (1984) and Watanabe *et al.* (1977) are identical to the characteristics of the isolates obtained from infected vegetable plants showing damping off and basal stem rot. Identification of the causal agent was also confirmed by Dr. Ibtisam Al-Shareef, Plant Pathology Research Institute, Ministry of Agriculture, Cairo, Egypt.

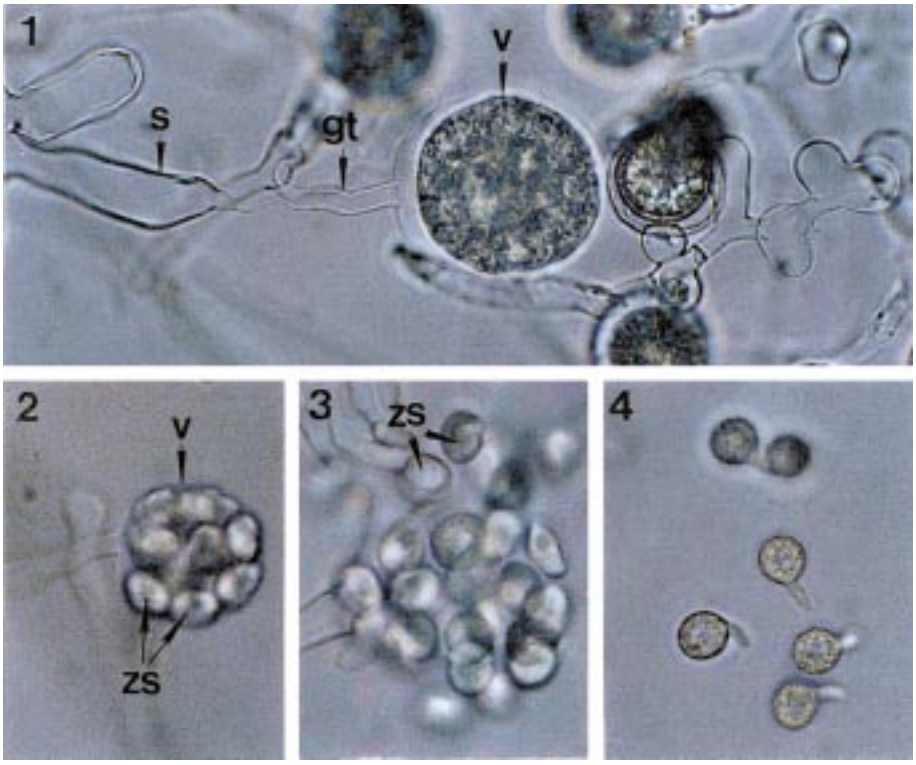


FIG. 3. Zoosporangial formation of *P. aphanidermatum.* 1. s, empty germinated sporangium; gt, germ tube; v, vesicle. 2. v, vesicle; zs, rotating zoospores inside the vesicle. 3. zs, released zoospores. 4. germinating encysted zoospores.

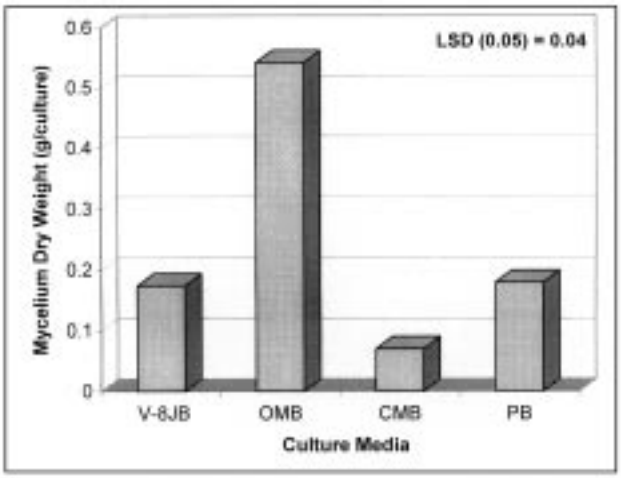
Evaluation of Media for Reproduction

Mycelium growth of *P. aphanidermatum* on V-8JB, OMB, CMB, and PB is shown in Fig. 4. Mycelium dry weight on OMB, significantly at $P \leq 0.05$, recorded the highest (0.54 g/culture flask) over the other culture media. The yield of dry mycelium on V-8JB and PB was not significantly different. The CMB, on the other hand produced the least mycelial dry weight (0.07 g/culture flask) compared to the other culture media.

The density of oospores on the four tested culture media, V-8JB, OMB, CMB, and PB, are presented in Fig. 5. The highest log number of oospores was recorded on CMB producing 8.52 (3.3×10^8), followed by 8.02 (1.06×10^8) on V-8JB then 7.19 (1.6×10^7) oospores/culture flask on OMB. The PB culture media, however, did not induce oospore formation.

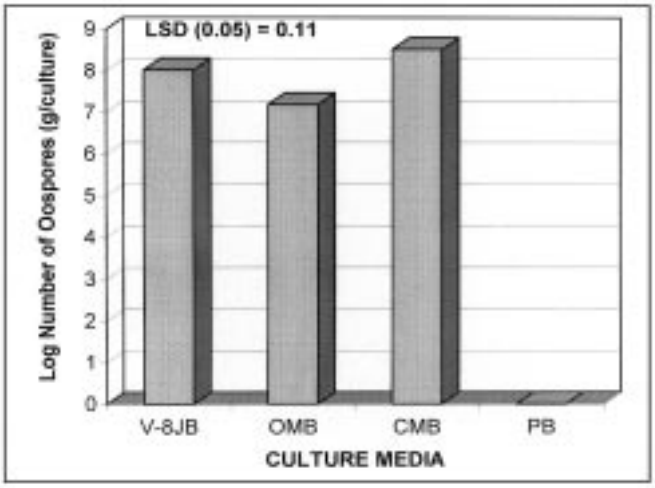
Induction of zoospore formation on different types of culture media is shown in Fig. 6. The highest significant log number of motile zoospores was obtained

using V-8JA, 4.6 (3.8×10^4), followed by OMA, which yielded 4.1 (1.3×10^4) then PA which yielded 2.9 (7×10^2) zoospores/plate. The least log number of motile zoospores, however, was obtained using CMA, which yielded 1.9 (7.9×10^1) zoospore/plate.



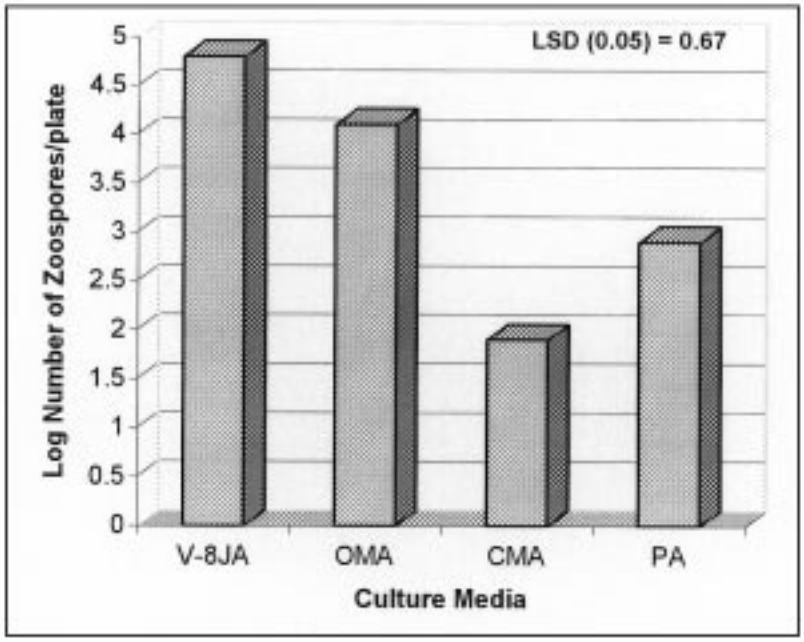
V-8JB : V-8 Juice Broth OMB : Oat Meal Broth
CMB : Corn Meal Broth PB : Potato Broth

FIG. 4. Dry weight of mycelium of *P. aphanidermatum* in four different broth media.



V-8JB : V-8 Juice Broth OMB : Oat Meal Broth
CMB : Corn Meal Broth PB : Potato Broth

FIG. 5. Log number of oospores of *P. aphanidermatum* produced in four different broth media.



V-8JA : V-8 Juice Agar OMA : Oat Meal Agar
CMB : Corn Meal Agar PA : Potato Agar

FIG. 6. Log number of zoospores of *P. aphanidermatum* produced in four different broth media.

Variation in the production of oospores and zoospores by the different culture media might due mainly to the availability of the essential elements for stimulation of sporulation in these cultures. Hendrix (1965b) reviewed the importance of sterols for the stimulation of asexual and sexual reproduction of pythiacious fungi. Ayers and Lumsden (1975) reported a maximum oospore development and maturation of oospores of three *Pythium* species when grown in media supplemented with one mg/l cholesterol or greater concentrations. Other reports have shown an induction of oospore formation by amendment of beta-sitosterol (Harnish and Merz, 1964) ergosterol (Ribeiro *et al.,* 1975) or lipids (Klemmer and Lenney, 1965). The inability of PB to induce oospore production might due to the fact that the medium does not contain enough supplement of these compounds and/or the presence of suppressive compounds.

Other reports also indicated that zoospore production by *Pythium* and *Phytophthora s*pecies is a sterol-induced process (Hendrix, 1965a and Lilly, 1966). Another elements such as Ca^{++} is essential for reproduction in *Pythium* spp. (Yang and Mitchell, 1965). The presence of these products naturally in the media is working as a stimuli for oospore production (Ayers and Lumsden, 1975).

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تعفن قاعدة الساق في الخضر المزروعة في البيوت المحمية في غرب المملكة العربية السعودية : (أ) دراسات عن المسبب *Pythium aphanidermatum*

يحيى حمزة سنبل

قسم زراعة المناطق الجافة ، كلية الأرصاد والبيئة وزراعة المناطق الجافة

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المستخلص. تم جمع عينات مريضة من نباتات خيار ، وشمام ، وفلفل رومي من بعض البيوت المحمية بالمنطقة الغربية من المملكة العربية السعودية وقد ظهرت عليها أعراض تعفن قاعدة الساق والذبول الطري للبادرات. وقد تم تعريف المسبب المرضي *Pythium aphanidermatum* (Edson) Fitsp. حسب المواصفات الظاهرية للأعضاء التكاثرية الخضرية والجنسية (الميسيليوم ، والجراثيم الهدبية ، والجراثيم البيضية). وكانت مواصفات ميسيلوم الفطر أنه شفاف غير مقسم. أما أعضاء التأنيث (oogonia) فهي كروية ، وطرفية ، وملتحمة بها أعضاء التذكير (antheridia) ، لها شكل القبة (dome-shaped) ، كما أن عضوي التذكير والتأنيث يتفرعان من شعيرات ميسيليوم مختلفة (diclenous) ، وعضو التذكير غير طرفي ، حيث إن الأنبوبة التي كونته تنمو ممتدة من قاعدته ، مما تجعله بيني (intercalary). أما بالنسبة للجراثيم البيضية فهي كروية الشكل ، ولا تملأ كل حيز عضو التأنيث (aplerotic). كما أن الأكياس السبورانجية ذات شكل ميسيليوم منتفخ أو مفصص ، والتي تنبت لتعطي حوصلات تتكون داخلها الجراثيم الهدبية ، حيث تتحرر من الحوصلات. كما تم تقييم أربعة أنواع من البيئات الغذائية : مرق الخضار المشكلة (V-8 Juice Broth (V-8JB، وبيئة مرق الشوفان (Oat Meal Broth (OMB ، وبيئة مرق دقيق الذرة (Corn Meal Broth (CMB ، وبيئة مرق البطاطس (Potato Broth (PB ، تقييما وكميا لقياس النمو الخضري وكذلك لقدرتها على تحفيز التكاثر

الجنسي للفطر. فقد تم الحصول على أعلى مستوى للوزن الجاف لميسيليم الفطر في بيئة OMB ، ثم تلتها بيئتي PB ، ثم يليها V-8JB مع عدم وجود فروق معنوية بينهما. أما أقل هذه البيئات إنتاجا للميسيليوم الجاف فكانت بيئة CMB. أما بالنسبة لتحفيز إنتاج الجراثيم البيضية ، فقد وجد أن بيئة CMB أنتجت أعلى كثافة للجراثيم البيضية ، ثم يليها بيئة V-8JB ، ثم بيئة OMB. أما بيئة PB فلم تحفز إنتاج الجراثيم البيضية. وبالنسبة لتحفيز إنتاج الجراثيم الهدبية ، فقد أنتجت بيئة V-8JA أعلى كثافة عن بقية البيئات ، ثم تلتها بيئة OMA ، فبيئة PA ، أما أقلها فكانت بيئة CMA.