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Factors Promoting Pycnidia Production of *Didymella bryoniae*, the Causal of Gummy Stem Blight in Cucurbits

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Gummy stem blight in *Cucurbitacea* caused by the pathogen *Didymella bryoniae* under high humid conditions was previously recorded in many countries. In Egypt, the disease was observed for the first time on cantaloupe grown under sprinkling irrigation system in El-Bostan (Beheira governorate). Percentage of diseased plants ranged from 10 to 15% in the surveyed fields. Many isolation trials were carried out without success in obtaining conidial structures; only undistinguished mycelium was obtained. Among 9 natural media tested, V-8 Agar medium (V-8A) was the fastest in producing the distinguished pycnidia and non-septate pycnidiospores, followed by Bean Dextrose Agar medium (BDA) for number of produced pycnidia. Three concentrations (full strength, ½ and ¼ concs.) of V-8A, Cucumber Dextrose Agar (CDA) and Potato Dextrose Agar (PDA) were also tested, where ¼ conc. of V-8A and CDA produced pycnidia after 7 days only. Near Ultra Violet light boosted pycnidia production when compared with two other lightening systems. In case of incubation materials tested, Pyrex glass plates affected pycnidia production positively. Among several kinds of plant debris evaluated for pycnidia promotion, coriander debris was the best while debris of squash and cucumber, each alone, were the least. Moreover, plant debris affected the shape and number of pycnidia produced. Some bioagents promoted pycnidia production. *Trichoderma harzianum* was the best. The fungus started in producing pycnidia after the 4th day when the surface of mycelial culture was damaged, while no pycnidia were produced if wounding was made before media inoculation**.**

Keywords: *Didymella bryoniae*, gummy stem blight, pycnidia production and *T. harzianum*.

Didymella bryoniae (Auersw.) Rehm, the anamorph of *Mycospharella melonis*, the causal fungus of gummy stem blight of cucurbits is one of the most important diseases of cucurbits causing considerable damage in many countries (Crüger and Schneider, 1964; Fletcher and Preece, 1966; Kagiwata, 1967; Schenck, 1968; Figueiredo *et al.*, 1970; Arny and Rower, 1991; Young *et al.*, 2010; Mason *et al*., 2011; Keinath, 2013 and Basim *et al.*, 2016).

The pathogen commonly attacks members of Cucurbitaceae especially cucumber (*Cucumis sativus* L.), muskmelon (*Cucumis melo* L.), pumpkin (*Cucurbita pepo* L.) watermelon (*Citrullus vulgaris* Shard.) and cantaloupe (*Cucumis melo* var. *cantalupensis*). The disease produces a variety of symptoms on the above ground parts of all cucurbits. In early infection, seedlings die quickly after infection. On older plants, leaf spots, stem canker, vine wilt and black fruit rot

appeared. The fungus is known to be seed-borne on cucumber and watermelon (Chupp and Sherf, 1960 and Richardson, 1979). In Egypt, Ragab *et al.* (1986) mentioned that disease symptoms were observed in watermelon fields, but they did not mention anything about the causal pathogen. El-Wakil and Khalil (2016) observed typical symptoms of gummy stem blight on cantaloupe plants grown under sprinkling irrigation at El-Bostan. Isolation, purification and identification were carried out from the collected diseased plants.

The objective of this study is an attempt to solve the difficulty in obtaining fungal pycnidia by employing some physiological factors promoting mycelia growth and pycnidia production by *D. bryoniae*.

Materials and Methods

1. Collecting infected samples:

Diseased cantaloupe plants showing stem cankers with characteristic red to brown gummy exudates and diseased fruits showing spots of greasy green, brownish and/or brown colors, were obtained from some cantaloupe fields in El-Bostan, Beheira governorate in 2015.

2. Isolation, Purification and Identification:

Infected samples of cantaloupe plants were carefully washed in running tap water to get rid of sand and soil particles. Infected samples were cut into small pieces and soaked in fresh 2% sodium hypochlorite solution for 3 minutes. These pieces were washed four times in sterilized water and dried between two sterilized filter papers. Then samples were transferred into Petri dishes containing Potato Dextrose Agar medium (PDA) and incubated in the dark at 20°C for seven days. The fungal growth was examined using stereo- and compound microscopes. Cultural purification was done using hyphal tip technique (Riker and Riker, 1936). Pure cultures were kept in PDA slants and reserved for further studies. To obtain the pycnidial stage of the isolated fungus, discs of the fungal growth were plated on V-8 medium (Young *et al.*, 2010) and incubated at 25°C in dark. After 10 days, plates were examined for pycnidia formation. Identification of the fungal pycnidia was carried out according to Lee *et al.* (1984).

3. Impact of some factors on D. bryoniae sporulation:

3.1. Effect of media:

Nine different media were used to determine fungal reaction in relation to variable nutrition sources, *i.e*. Sugar Free Carnation-leaves Agar (SFCA), Yeast Extract Agar (YEA), Corn Meal Dextrose Agar (CMDA), V-8 Agar (V-8A), half concentration V-8 Agar (0.5 V-8A), Oat Meal Agar (OMA), Cucumber Dextrose Agar (CDA), Bean Dextrose Agar (BDA) and Potato Dextrose Agar (PDA). Mycelial discs (5 mm) were used to inoculate the above mentioned media in Petri plates. Eight replicates were employed and the average was obtained. Plates were incubated for one month under room conditions and results were obtained on the 10^{th}, 15^{th} and the 30^{th} day.

Four habit characters of fungal growth were evaluated; mycelial color after 7 days of incubation, number of pycnidia in area unit (cm^2), location of pycnidia on medium surface and days to first pycnidium formation.

3.2. Effect of media concentration on mycelial growth and pycnidia production:

The effect of quarter, half and standard concentration of three different media, *i.e.* PDA, CDA and V-8 juice was studied through this experiment. Mycelial discs of 5 mm diameter were cut from the growing margins of fresh fungal culture and placed in the center of 9-cm Pyrex Petri plates. Four replicates were carried out for each treatment. After 7 days of incubation at 22±2^oC under alternating cycle of 12/12 NUV/darkness, results of diameter of radial mycelial growth (mm), mycelial density, pycnidia production and pycnidia location were recorded.

3.3. Effect of light sources on mycelia growth and pycnidia production:

This experiment was designed to study the effect of different light sources on growth habits of *D. bryoniae*. Discs from 7-days old fungal culture were cut using flamed cork-borer and transferred onto PDA in Pyrex Petri plates, then exposed to one of the following light regimes; alternating cycle of Near Ultra Violet light (NUV)/complete darkness (12/12 hrs.), continuous day light (CDL) or complete darkness (CD). Plates were incubated at 22 ± 2^oC for 14 days in quadruplicates for each of the designed lighting regime. Near Ultra Violet was provided by two Philips black light lamps TL 40 w/08, while CDL was supplied by two cool white fluorescent Philips TLF 40 w 143 deluxe tubes, hanged 50 cm above the dishes. Impact of lighting regimes on mycelial color, radial growth and days to first pycnidium formation was evaluated.

3.4. Effect of container material on mycelial growth and pycnidia production:

Permeability to light and material clarity play an important role in introducing light from surrounding climate to in-container limited climate which could affect growth characters of any inoculated organism. Pyrex glass dishes, standard plastic dishes (perspex) and commercial glass dishes were used as the most commonly used plates locally and internationally to study their effects on some major growth habits of *D. bryoniae*. The same light sources mentioned previously were used. Mycelial color, radial growth and number of days to the first pycnidium formation were measured. Four replicates of each treatment were incubated and the averages were presented.

3.5. Effect of plant debris on pycnidia production:

Didymella bryoniae has a wide host range within *Cucurbitaceae*. The pathogen can survive on organic debris from previously infected cucurbits or on wild or volunteer cucurbits. This experiment was conducted to uncover if the fungus can survive on plant debris of cucurbit crops only or on other plant debris as well. Various kinds of plant debris representing 5 different field crops and 4 vegetable crops were used in this experiment. Dead dried main and secondary stems and branches of barley (*Hordeum vulgaris* L.), chickpea (*Cicer arietinum* L.), alfalfa (*Medicago sativa* L.), flax (*Linum usitatissinum* L.), rice (*Oryzae sativa* L.), bean (*Phaseolus vulgaris* L.), coriander (*Cardimum sativum* L.), squash (*Cucurbita pepo* L.) and cucumber (*Cucumis sativus* L.) were dried and autoclaved. Stems and

branches were then cut into 5 cm segments and put on water agar in Pyrex plates and 4 parts in each plate. Each treatment was replicated four times.

Discs from 7-days old fungal culture were aseptically transferred onto the water agar in Petri plates close to plant debris segments and incubated at 22±2°C for eight days under alternating cycle of 12/12, NUV/darkness. Density of mycelium and pycnidia formation were recorded after 4 and 8 days.

4.1 Effect of some antagonists on pycnidia production by D. bryoniae in vitro:

Four well-known bioagents were used as induction factors of *D. bryoniae* for pycnidia production. One isolate of each of *Trichoderma harzianum*, *T. viride* (Martinez *et al.*, 2013) and *Penicillium aurantiogrism* were freshly isolated from native soil, purified, identified and maintained on PDA slants while *Bacillus subtilis* was obtained from Bacterial Diseases Res. Dept., Plant Pathology Res. Institute, ARC. Each treatment (individual bioagent) consisted of four replicates. Discs (5-mm diameter) of *D. bryoniae* culture and each of the bioagents were grown in two opposite-side positions in 9-cm Pyrex dishes containing PDA medium and incubated at 22±2°C for eight days under alternating cycle of 12/12, NUV/darkness. Control treatment was carried out using both discs of *D. bryoniae* only. Inhibition zones and number of aligned pycnidia were estimated. This experiment was ended when mycelial growth from the two discs met in the control treatment.

4.2 Effect of wounding mycelial mat on pycnidia production of D. bryoniae:

This experiment was planned to understand cultural behavior of *D. bryoniae in vitro*. Eight PDA Pyrex plates of 9-cm diameter were divided into two groups, 4 plates each. Random punctures of 5-mm were made in each plate medium of the first group using flamed cork-borer, then inoculation with 5-mm disc of *D. bryoniae* at the center. Plates of the second group were first inoculated and punctured later. Plates of both groups were incubated at 22±2°C for eight days under alternating cycle of 12/12, NUV/darkness. On the eighth day, plates of the second group were fully covered with very light growth of mycelium. Random punctures of 5-mm were made in each plate medium of the second group using flamed cork-borer, then resumed incubation for one more week with daily observation for both groups.

Results and Discussion

Detection of D. bryoniae:

Diseased cantaloupe plants showing stem cankers with characteristic red to brown gummy exudates and diseased fruits showing spots of greasy green, brownish and/or brown colors were collected from El-Bostan (Fig. 1a). At later stages, the pathogen produces typical symptoms on the radicle with numerous pycnidia.

Didymella bryoniae has been shown to cause fruit rot of cucurbits (Waint, 1945; Kagiwata, 1967; Figueiredo *et al.*, 1970; Cardoso *et al.*, 1974 and Sitterly and Keinath, 1996). During the maturation of the seeds and seed harvesting there is ample opportunity for the spores of this pathogen to spread, germinate and invade

seeds. The seed can easily be inoculated artificially with spores of the fungus (Rankin, 1954 and Brown and Preece, 1968).

In the current investigation, most of the conidia in these pycnidia were nonseptate (Fig. 1b), some uniseptate and few biseptate as compared with slightly smaller, generally nonseptate, spores in pycnidia were produced (Lee *et al*., 1984).

The results obtained in this investigation indicated that there was a great chance of introducing the disease to nursery beds. The significance of seed-borne infection by *D. bryoniae* lies large scale development of the disease, but also in the introduction of inocula to previously uninfested areas. For this reason, the presence of *D. bryoniae* on/or in the seed may provide an unsuspected and potentially dangerous source of infection.

Factors affecting pycnidia production by D. bryoniae:

1. Effect of media:

Among media tested in Table 1, three of them gave white mycelial growth, i.e. CDA, BDA and PDA media. While SFCA, YEA, CMDA, V-8A, 0.5V-8A and OMA gave off-white color ranging between dull and pinkish white colors. Regarding the number of pycnidia appeared in square cm, the 0.5V-8A showed the highest number with an average of 17 pycnidia/cm^2 followed by BDA (14 pycnidia/cm^2) and V-8A (8 pycnidia/cm^2). Average number of pycnidia formed on other media was lesser than 5 pycnidia/cm^2.

Only V-8A (full and half- concentrations) were the earliest to show pycnidia formation after 10 days only. However, location of formed pycnidia differed in the two concentrations tested. In case of the full concentration, pycnidia localized around the center, scattered and/or upper surface, while in case of half-concentration, locations of pycnidia developed were toward the edge and/or upper surface only. On the other media tested, pycnidium formation was delayed to 15 – 25 days. These results are in agreement with those reported by Young *et al*. (2010) who stated that pycnidia location varied according to the medium. Alam *et al.* (2001) mentioned that the pycnidia of *Botryodiplodia theobromae* were often found partially embedded in the medium, they were visible from the reverse side of Petri dish. This clearly indicated that the different media; with different nutritive components, affected mycelial color, number and location of pycnidial formation.

Table 1. Physiological impacts of different media on some habit characters of

***D. bryoniae* the cause of gummy stem blight on cucumber plants**

** Pycnidia location:1 = around center, 2 = edge, 3 = scattered, 4 = upper surface,

Media	Habit Character			
	Mycelial color	Av. number of pycnidia/cm^2	**Pycnidia location	Days to first pycnidium formation
SFCA	White beige	2	1 & 4	15
YEA	Pinkish white	4	1 & 5	20
CMDA	White beige	3	5 & 7	20
V-8A	Dull white	8	1 & 3 & 4	10
0.5 V-8A	Dull white	17	2 & 4	10
OMA	Pinkish white	2	6	20
CDA	White	2	1 & 4	20
BDA	White	14	7	20
PDA	White	2	3 & 4	25

5 = reverse surface, 6 = beneath mycelium and 7 = embedded

2. Effect of media concentration on mycelial growth and pycnidia production:

It is clear from the data presented in Table 2 that richest media concentrations did not boost the fungus to produce pycnidia, meantime, least concentrations of media used revealed maximum pycnidial units where V-8 (¼ cons.) and CDA (¼ cons.) gave numerous pycnidia when compared to PDA (¼ cons.) which ranked the least. This is in agreement with Keinath (2013) who mentioned that one-quarter-strength potato dextrose agar produced pycnidia and conidia typical of *D. bryoniae* 3 days after culturing on the surrounding agar. On the contrary, mycelium was very dense on V-8 medium, dense on CDA and 0.5 V-8A media, while other treatments gave light mycelial densities. Figure 2 illustrates variable effects of V-8 medium concentrations on pycnidia production.

Regarding mycelial radial growth, only V-8 and PDA gave distinguished records while others were more or less similar. Data related to location of pycnidia produced on media surface were not markedly affected by media concentration. Alam *et al*. (2001) noticed negative correlation and highly significant effect on formation of pycnidia and reduction of colony diameter when glucose was increased in PDA.

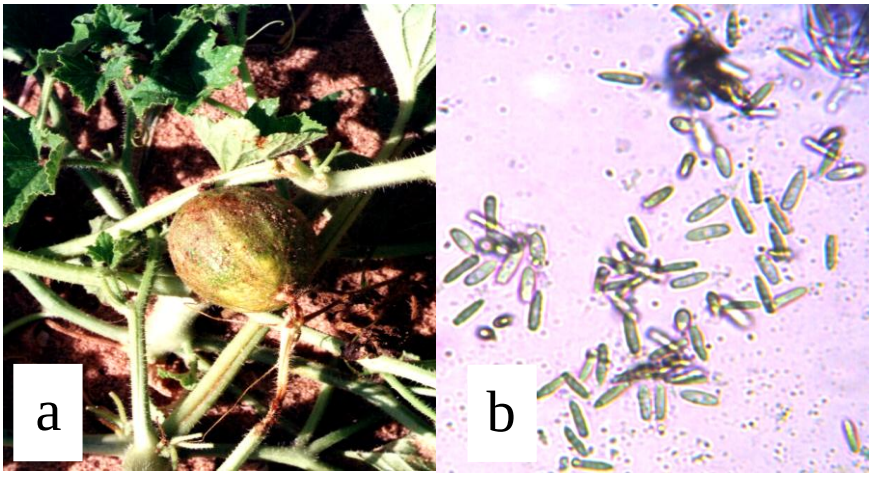

Fig. 1: a = symptoms collected from El-Bostan, Beheira governorate; b = nonseptate pycnidiospores released from pycnidium (1000x)

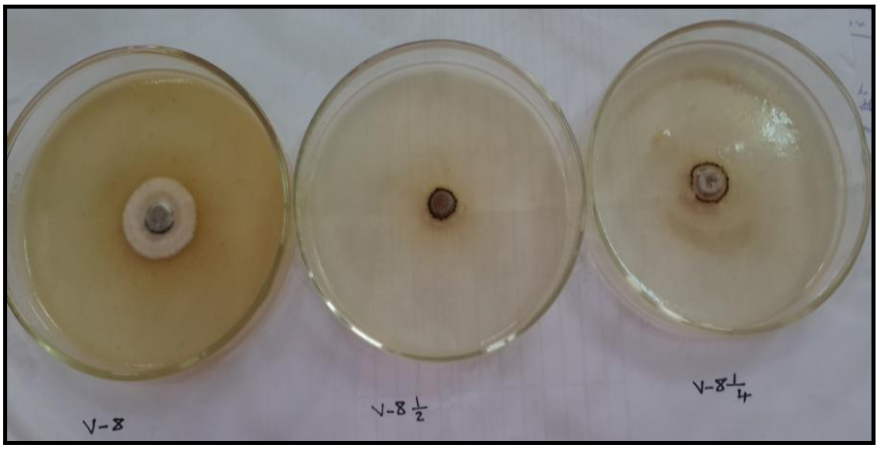

Fig. 2. *D. bryniae* grown on three different V-8 concentrations revealed the maximum number of pycnidia on V-8 (¼ conc.)

Table 2. Effect of media concentration on mycelial growth and pycnidia production after 7 days of incubation at 22±2°C under alternating cycle of

Media concentration	Habit character			
	Mycelial radial growth	Mycelial Density	** Pycnidia production	Pycnidia location
PDA	2.5	Light	None	None
PDA (½ cons.)	1.5	Light	None	None
PDA (¼ cons.)	1.2	Light	+	Around the disc
CDA	1.2	Dense	None	None
CDA (½ cons.)	1.3	Light	++	Around the disc
CDA (¼ cons.)	1.2	Light	+++	Around the disc
V-8	2.6	Very dense	None	None
V-8 (½ cons.)	1.6	Dense	+	Around the disc
V-8 (¼ cons.)	1.5	Light	+++	3mm away from the disc

NUV/darkness (12/12hrs.)

** + = 1 to 10 pycnidia ++ = 11 to 20 pycnidia +++= More than 20 pycnidia

3. Effect of light sources on mycelia growth and pycnidia production:

Data in Table 3 show variable effects of the three lighting regimes applied on mycelial color where NUV gave the most clear and distinguished color of pink, while CW and CD regimes gave immature colors, beige and white, respectively. Regarding number of days till pycnidium formation, NUV was the only regime that boosted the fungus toward pycnidium formation. It seemed that NUV and CW had similar effects on linear growth of the mycelium. Overall, NUV proved its necessity for coloring the mycelial pad and pycnidia formation and missing such an important tool of incubation process, results obtained might be misleading. Current results are in harmony with those obtained by Neergaard (1979) who stated that using monochromatic radiation represented by NUV region of spectrum, 3200 – 4000 Å is very efficient in inducing sporulation. He added that black light fluorescent which emits light mainly at wavelengths near 3650 *Å*, and cool white daylight fluorescent which emits some NUV light had become standard equipment in seed health testing. Moreover, Leach (1967) found that pigmentation of fungi was greatly influenced by the presence or absence of light. Irradiation such as by NUV usually stimulates strong pigmentation.

Table 3. Effect of three light sources on some habit characters of *D. bryoniae* incubated in Pyrex plates for 14 days at 22±2°C

Plate material	Habit character		
	Mycelial color	Av. of radial growth (cm)	Days to first pycnidium formation
Pyrex glass	Pink	7.0	14
Standard plastic	Pink	5.6	14
Commercial glass	Beige	5.8	None

NUV = near ultra violet. CDL = continuous day light CD = complete darkness

4. Effect of container material on mycelia growth and pycnidia production:

Obviously, data presented in Table 4 clarify that Pyrex glass was superior to the other two culturing containers with respect of linear growth (7 cm in 7 days). Surprisingly, the standard plastic plates (perspex) revealed the lowest average of mycelium radial growth of 5.6 cm. Neergaard (1979) mentioned that plastic containers transmit light with the wave lengths stimulating sporulation of fungi and had been recommended by the sixth International Workshop on Seed Pathology in preference to glass containers except Pyrex. Commercial glass material did not promote the fungus to form pycnidium for as long as 14 days of incubation. Most probably, using plates made of commercial glass material was one of the main reasons that delayed the pycnidia recovery in *D. bryoniae* cultures since the disease symptoms were noticed in Egypt on watermelon starting from 1985 (Ragab *et al*., 1986) as it did not allow the transmission of wavelengths needed for sporulation. Recently, El-Wakil and Khalil (2016), completed the picture by obtaining, identifying and recording *D. bryoniae* the causal pathogen of the gummy stem blight on cantaloupe for the first time in Egypt.

Table 4. Effect of plate material permeability on some morphological habit characters of *D. bryoniae* when incubated under NUV light for 7 days at 25+2°C

Light sources	Habit character		
	Mycelial color	Av. radial growth (cm)	Days to first pycnidium formation
NUV	Pink	6.7	14
CDL	Beige	6.7	None
CD	White	7.1	None

5. Effect of plant debris on pycnidia production:

Nutrition is considered one of the most important factors affecting growth behavior of any organism. The pathogen in the current investigation seemed to produce surviving elements, the pycnidia under certain nutritional conditions. Moreover, shape of formed pycnidia differed according to crop species depending on the supporting plant species Shahidul *et al.* (2001).

Data presented in Table 5 reveal that short incubation period of 4 days was not enough for *D. bryoniae* to express sufficient mycelial growth and/or pycnidia production. Where most of the used plant debris did not show any mycelial growth while, alfalfa, squash and cucumber gave light dense mycelial growth. The plant debris of chickpea, flax and cucumber did not help the fungus to produce pycnidia within the period of 4 days. In the current investigation, it was clearly noticed the development of dense pycnidia formed on the node of plant debris while less pycnidia were formed on internode. Beck *et al.* (1983) emphasized that pycnidia development abundantly on nodes could be referred to structural and anatomical factors of nodes cells and tissues; mainly thickness and/or compactness which might lead subsequently to lesser notorious contents in nodes more than internodes (Fig. 3).

Coriander plant debris boosted *D. bryoniae* to produce pycnidia at the maximum number in this experiment, being < 20 and < 30 after 4 and 8 days of incubation, respectively. These results could be explained by coriander plant debris components were the poorest source of nutrition and did not satisfy the fungal needs to grow. On the contrary, squash plant debris was the only nutritional material enhanced the mycelial growth to the level "very dense" after 8 days of incubation. The expression of *D. bryoniae* pycnidial production against flax plant debris (> 10) was the least at the end of incubation period.

Table 5. Effect of 9 plant debris materials on mycelial density and number of pycnidia production after 4 and 8 days of incubation at 22±2°C under alternating cycle of NUV/darkness

Debris	Habit character			
	4 days		8 days	
	Mycelium density	Number of pycnidia/cm^2	Mycelium density	Number of pycnidia/cm^2
Barley	-	> 10	-	10-20
Chickpea	-	-	Light	10-20
Alfalfa	Light	10-20	Light	10-20
Flax	-	-	Light	> 10
Rice	-	10-20	-	10-20
Bean	-	10-20	-	10-20
Coriander	-	< 20	Light	< 30
Squash	Light	> 10	Very dense	10-20
Cucumber	Light	-	Light	20-30

6. Impact of antagonism on pycnidia production:

Data presented in Table 6 show that antagonism between the fungus *D. bryoniae* and the bioagents tested may act as an induction factor for pycnidia production. It was also clear that there was no relation between the developed inhibition zone and pycnidia produced. The bioagent *T. harzianum* was superior in boosting *D. bryoniae* to produce the maximum number of pycnidia (> 10 pycnidia/cm^2) Martinez *et al.* (2013). *Trichoderma viride* and *Bacillus subtilis* gave almost equal and least number produced (3-5 pycnidia/cm^2), while the bioagent *Penicillium aurantiogrisum* moderately induced the pathogen to produce 6-10 pycnidia/cm^2. Aries *et al.* (1997) stated that exudates of *P. aurantiogrisum* might have promoted the fungus to produce pycnidia, while metabolites produced by *T. harzianum* and *T. viride* negatively affected the number of pycnidia.

Table 6. Effect of 4 bioagents on pycnidia production of *D. bryoniae in vitro*

Treatment	Inhibition zone (cm)	Number of pycnidia (cm^2)
Control	Zero	--
T. harzianum	1.2	> 10
T. viride	1.0	3-5
P. aurantiogrisum	1.75	6-10
B. subtilis	2.0	3-5

7. Effect of wounding mycelial mat:

Plates of the first group did not show any pycnidia production of *D. bryoniae*. Figure 4 shows plates of the second group where 6-15 pycnidia on the average were developed on edge of each puncture after 4 – 7 days of wounding. Although James *et al.* (1991) reported that wounding of mycelium did not significantly increase conidial production. Results of the current investigation are in agreement with other researchers who proved the opposite. Campbell *et al.* (2003) obtained similar results related to number of conidia produced by a number of fungi. They mentioned that wounding and exposing culture and medium after 7 days to temperature cycle of 23/19ºC (light/darkness) increased conidia production by 800% or more than the unwounded. They stated that the inhibition of vegetative development through wounding commonly enhances sporulation. They also cited that sporulation of *Pyrenophora tritici-repentis* is routinely enhanced by removal of aerial mycelium, while wounding of mycelium enhanced sporulation of *Pyrenophora graminea* and *Alternaria* species.

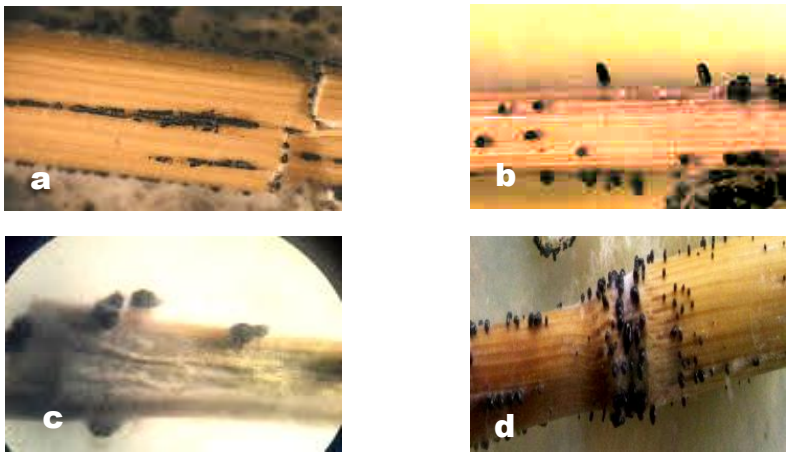

Fig. 3. a, b and c = different shapes of *D. bryoniae* pycnidia formed on different plant debris materials, d= dense pycnidia formed on the node of plant debris while less pycnidia were formed on the internode.

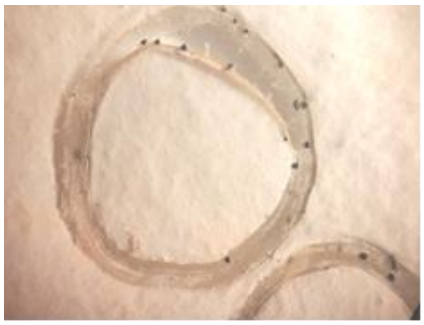

Fig 4. Pycnidia formed on cuttings edges made after the mycelium grew on PDA medium 4-7 days after cutting.

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العوامل المحفزة لإنتاج بيكنيديات الفطر دايدميلا بريوناى مسبب مرض لفحة الساق الصمغية فى الفصيلة القرعية

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سُجل مرض لفحة الساق الصمغية المتسبب عن الفطر *Didymella bryoniae* فى كثير من بلدان العالم حيث يصيب أغلب محاصيل القرعيات خاصة تحت ظروف الرطوبة العالية. وفى مصر ورغم تسجيل الأعراض النموذجية للمرض إلا أنه لم يتم عزل وتسجيل الفطر المسبب لسنوات عديدة حتى تم عزله لأول مرة فى ٢٠١٦ من نبات الكنتالوب تحت ظروف الرى بالرش فى منطقة البستان. حيث ظهرت الاعراض على هيئة تقرحات ذات لون بنى محمر مصحوبة بإفرازات صمغية بينما كانت الأعراض على الثمار عبارة عن بقع ذات لون أخضر لامع فى البداية ثم تتحول الى اللون البنى مؤخرا. تمت محاولات العزل من تلك الاعراض على بيئة PDA إلا أنه لم يتم عزل أى تراكيب جرثومية حيث ظهرت نموات هيفية فقط غير مميزة للفطر، ومن ثم كان حتميا توسيع نطاق الدراسة لتشمل بعض العوامل التى قد تشجع إنتاج البكنيديات ومنها اختبار ٩ بيئات طبيعية كمصادر تغذوية مختلفة فكانت بيئة V-8 أكثر البيئات تحفيزاً على تكوين الأوعية البكنيدية وجراثيمها غير المقسمة المميزة للفطر كما كانت أفضلها فى سرعة تكوين البكنيديات ثم بيئة CDA فى عدد البكنيديات المتكونة. وشملت الدراسة تأثير ثلاث تركيزات لثلاثة بيئات طبيعية هى PDA و CDA و V-8 (تركيز كامل و ½ و¼)، فكان أفضلهم بيئتى V-8 و CDA بتركيز ¼ فى إنتاج البيكنيديات بعد ٧ أيام فقط مقارنة بالبيئات والتركيزات الأخرى. وعند اختبار تأثير نوع الإضاءة فكان استخدام NUV مع ظلام التام فى دورة تبادلية ١٢/١٢ له دورا واضحا فى تكوين البكنيديات المميزة للفطر. كما كان اختبار مادة أوعية التحضين له تأثير أيجابى عند استخدام آطباق الPyrex على تكوين البكنيديات. كما كان لتنمية الفطر على بقايا ٩ أنواع نباتية جافة تأثير كبير على تكوين عدد أكبر من البكنيديات محسوباً فى ١ سم٢، فكانت أفضلهم البقايا الجافة لسوق وأفرع نبات الكزبرة، فى حين كان أقلهم تحفيزا لإنتاج البكنيديات هى السوق والأفرع الجافة لنبات الكتان بينما كانت بقايا الكوسة والخيار متوسطة التحفيز على تكوين البكنيديات. وكان لنوع البقايا النباتية المستخدمة تأثير متباين على شكل وعدد البكنيديات الناتجة. وقد اندفع الفطر إلى تكوين البكنيديات اعتبارا من اليوم الرابع بعد إحداث الجروح فى النموات الميسليومية فى حين لم تتكون البكنيديات إذا تم التجريح قبل تلقيح الأطباق بالفطر.