VARIATION AMONG ISOLATES OF *PHIALOPHORA GREGATA*

Baraka; M.A [1]; A.M. Hassanein[2];
A.M. El-Garhy[2] and M. A. Abdel-Al[2]

(1) Fac. of Agric. Seuz Canal University.
(2) Plant Pathology Res. Inst., ARC, Giza Egypt.

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Abstract

Six isolates of *Phialophora gregata* from soybean were collected from six geographic areas in Egypt. Single spore cultures were studied for their variation in cultural characteristics and virulence to soybean. All isolates varied in their growth rate and colony characteristics on PDA medium at 28°C . Maximum growth of the six isolates was at 28°C . The isolates varied from highly to moderately virulent and were grouped into two pathotypes based on their ability type(I) or inability type(II) to cause chlorosis , necrosis and wilt of foliage. Both pathotypes caused internal stem discoloration. All isolates of *P. gregata* produced toxic metabolites in culturel filtrates, which caused reduction of seed germination and length of radicles. Immersing cut ends of seedlings in autoclaved and unautoclaved undiluted crude culture filtrates of different isolates of *Phialophora gregata* ,caused the small leaves to become flaccid, browning in petiole and veins, then interveinal necrosis was noted , followed by wilting , complete drying and defoliation of the leaves in type (I). Seedlings in culturel filtrate of Type(II) showed, no symptoms.

INTRODUCTION

Phialophora gregata (Synonym *Cephalosporium gregatum*) is considered an important pathogen causing brown stem-rot disease of soybean in Egypt (Sayed Ahmed, 1983 and 1988) and in many parts of the world uncluding U S A, Canada and Mexico, affecting both yield and quality of seeds (Sinclair, 1982 and Gray, 1972). The disease has been reported to severely attack soybean plants at seedling stage causing severe damage with internal and external symptoms (Schneider *et al.*, 1971) *Phialophora gregata* was the most predominant fungus and high frequency was obtained from all inspected localities in the surveyed Governorates (Abdel-Al, 1999).

Isolates of *P . gregata* vary widely in cultural characteristics, growth rate in culture , colony color, colony surfaces (Allington and Chamberlain, 1948; Abo El- Dahab, 1968; Sinclair, 1982 and Mengistu and Grau, 1986). Also, isolates of *P. gregata* differed in virulence and ability or inability to cause chlorosis , necrosis and wilt of foliage (Phillips , 1973 and Mengistu and Grau, 1986). The optimum temperature for growth of *Cephalosporium maydis*, the causal organism of late wilt disease of maize, was between 27-30°C, while *C. gregatum* was 18°C (Sabet *et al.,* (1966).

The culture filtrate of *C.gregatum* causes wilting of leaves and death and vascular browning of the Adzuki beans (Kobayashi and Ui, 1977). This investigetion aimed to study the variation among isolates of the fungus and the effect of different temperaturses as well as the effect of culture filtrate on soybean seed germination.

MATERIALS AND METHODS

Mycelial radial growth and characteristies:

The *In vitro* variability in radial growth and colony characteristics of the isolates were studied on PDA medium . The characters included colony margin , colony surface, colony colour , conidial size and radial growth after 12 days of incubation at 28°C (Mengistu and Grau, 1986).

Variation in pathogenicty:

Plants of H_2L_4 , a susceptible soybean genotype were grown in pots(25cm) containing sterilized soil. Six isolates of *P. gregata* were cultured in soybean stem broth in (SSB) to produce inoculum for pathogenicty test (Sills et al, 1991). Flasks (250 ml)were incubated at 28°C. for two weeks. Cultures were filtered through Whatman No.1. The suspensions were adjusted to 3×10^7 conidia / ml. Fifteen days old plants were inoculated by pipetting 8.0 ml of inoculum into the periphery of taproots 3 cm below the soil line. Control (untreated) plants received filtrate of the (SSB) alone . Four weeks after inoculation , plants were examined for severity of foliar symptoms and the percentage of internal stem discoloration (PISD) was used to assess stem rot severity as the following :

$$PISD = \frac{\text{height of internal stem discoloration cm}}{\text{Plant height cm}} \times 100$$

to determine the relative virulence of each isolate . Severity of foliar symptoms was evaluated first , then stem was split longitudinally and examined for discoloration of pith and vascular tissues of stems (Mengistu and Grau, 1986).

Effect of different temperatures:

Sterilized Petri dishes, each containing 15 ml potato dextrose agar (PDA) medium, were separately inoculated with 4.0 mm disks obtained from the six different isolates of *P.gregata*. Four replicates for each isolate were used and incubated at 10,15,20,25 and 30°C . Eight days later, colony diameter was measured twice in two directions at right angles and were averaged for each replicate.

Culture filtrate studies:

On soybean seeds germination:

Culture filtrates from different isolates of *P.gregata* were tested for their effect on seed germination and length of radicles of H_2L_4 genotype and Crawford cultivar. Ten sterilized seeds were plated on sterilized filter paper with 5ml of fungal filtrate per dish. Sterilized distiled water (5ml per dish) were used for the control (untreated) and ten dishes were used per treatment. Data were recorded after incubation for 8 days at 28°C.

On soybean seedlings:

Ten days old soybean seedlings of H_2 L_4 and Crawford cultivar were cut ,with a sterile scalpel, just above the soil line with cotyledons removed, were kept in sterile distilled water for 3-4hrs. before being exposed to test culture filtrates . Ten seedlings were immersed separately in 10 vials containing culture filtrate for each isolate. Control (untreated) cut seedlings were immersed in vials containing noninoculated Czapek's medium . Observations were recorded as the following:

Bv = Browning in petioles and veins .

N = leaves become , flaccid , yellowing and interveinal necrosis .

CM = Inward or outward of leaf margins.

BS = stem browning .

+,++,+++ degrees of severity (Gray and Chamberlain, 1975) .

This experiment was repeated using autoclaved culture filtrate (Gray and Chamberlain, 1975).

RESULTS

Mycelial radial growth and characteristics:

Radial growth, colony margin, colony surface and colour of cultures of the six isolates of *P.gregata* are summarized in Table (1). Data show that isolates . 1,2,4 and 5 grew faster at 28°C. (78mm average) than isolates 3 and 6 (63mm average). Colony margin of isolates 1,2,4 and 5 were lobed, while isolates No. 3 and 6 were smooth. The colony surface was raised and rough in isolates No. 1,2,4 and 5, while aerial mycelium was abundant in isolate 3 and 6 . Colour of the cultures ranged form dark brown as in isolates. 1,2,4 and 5, to gray white or light brown as in isolates 3 and 6 .

Table 1. Mycelial radial growth and colony characteristies of *Phialophora gregata* isolates, on PDA.

Isolate No.	Colony margin	Colony surface	Colony Colour	Radial growth at 28°C after 12days (mm).
1	Lobed	Raised and rough	Dark brown	79
2	Lobed or smooth	Raised and rough	Dark brown	75
3	Smooth	Aerial mycelium	Gray white to light brown	62
4	Lobed	Raised and rough abundant and smooth	Dark brown	86
5	Lobed	Raised and rough	Dark brown	71
6	Smooth	Aerial mycelium abundant and smooth	Gray white to light brown	64

Pathological characters:

Data presented in Table (2) show that isolates of *P.gregata* were grouped into two pathotypes based on their ability or inability to cause chlorosis, necrosis and wilt of foliage. Both pathotypes caused internal stem discoloration. Isolates 1 and 4 were more virulent than other isolates showing more intensive internal discoloration, while isolates. 3 and 6 were less virulent as expressed by the absence of foliar symptoms and less internal discoloration. Based on these reactions, the six isolates can be grouped into two types; isolates,1,2,4 and 5 in type(I) and isolates 3 and 6 in type (II).No concrete trend characterizes the spore of the two types.

Table 2. Spore characteristics and virulence of six isolates of *Phialophora gregata* recovered from soybean plants showing symptoms of brown stem rot disease.

Isolate No.	Conidial size (micron)				Foliar symptoms severity	Internal stem discoloration % (PISD)
	$\bar{X}$ Length	Range	$\bar{X}$ Width	Range		
1	4.1	2.9-5.6	2.7	1.8-3.2	+++	90.1
2	4.3	3.3-5.9	2.6	2.2-3.4	+++	78.2
3	4.2	3.1-5.7	2.8	2.2-3.6	- - -	58.1
4	4.3	2.9-6.8	2.7	2.1-3.4	+++	90.0
5	3.9	3.0-6.5	2.3	2.1-3.6	+++	67.7
6	4.4	3.7-7.1	2.4	2.1-3.6	- - -	60.0

Effect of different temperatures:

The effect of different temperatures; 10, 15, 20, 25, 28, and 30°C, on the linear growth of *P. gregata* isolates was studied on PDA medium. Data in Table (3) indicate that the minimum and maximum temperatures are below 10°C and above 30°C, respectively. The optimum temperature ranged from 25 to 30°C. Also, the rate of growth differed in different isolates. Rate of radial growth increased when incubation temperature increased above 10°C reaching the maximum at 28°C, then decreased with no growth at 40°C. Isolates. 1,2,4 and 5 were faster in growth than other isolates.

Table (3): Effect of temperature on linear growth of *P.gregata* isolates, on PDA

Isolate No.	Mean linear growth (mm)						
	10°C	15°C	20°C	25°C	28°C	30°C	40°C
1	1	12	18	31	79	39	0.0
2	2	11	16	29	75	41	0.0
3	2	11	19	39	62	41	0.0
4	7	17	33	44	86	45	0.0
5	1	12	16	21	71	45	0.0
6	9	28	30	37	64	45	0.0

L.S.D at 5 %

Temp. (T) 0.07

Isolates (I) 0.52

Tx I n. s

Culture filtrates studies:

Culture filtrate of four isolates of *P.gregata* type (I) and (II) grown on Czapek's liquid medium were tested for their effect on the percentage of seed germination and length of radicles and on 10 days old cut seedlings of H_2L_4 and Crawford soybean cultivars . Data in Table (4) indicate that no significant differences were observed between the two cultivars in seed germination and length of radicles. In general, all culture filtrates of the different isolates reduced seed germination and length of radicles when compared with the control (untreated).The highest reduction in percentage of seed germination of the geno-type H_2L_4 was obtained with isolates 2 and 4, giving 20 and 25% equivalent to 78.40% and 72.97% reduction, respectively. The least effect was exerted by isolates 3 and 6, where 57.0 % and 72.0% germination occurred equal to 38.38% and 22.16% reduction, respectively. Also, the highest reduction in seed germination of cultivar Crawford was obtained with isolates 4 and 5 at 69.23% and 73.79%, respectively, The least reduction was evident with isolates 1 and 3, where 62.5% and 67.5% of the seeds germinated at 35.90%and 30.77% reduction.

Radicles showed a pronounced reduction in length as a response to culture filtrates . The highest reduction in genotype H_2L_4 where obtained with filtrates of isolates 4 and 2, where radicle lengths were 3.6 and 4.6 mm equal to 96.11% and 95.03% reduction compared with the control (92.55 mm). The least reduction was experienced with isolates 1 and 6 where radicles reached 6.2 and 7.5 mm at reduction percentages of 93.30% and 91.96% respectively, Also, the highest reduction and percentage reduction in length of radicles of cultivar Crawford was obtained with isolates. 2,3 and 5 where they reached 6.6,9.6 , 9.9mm equal to 89..05%,84.07%and 83.57% reduction respectively, while the lowest effect was obvious with isolates 4, 6 and 1 as redicles reached 11.3, 12.5 , 13.0 mm representing 81.24% , 79.25% ad 78.42 reduction respectively.

Table 4. Effect of culture filtrate of six isolates of *Phialophora gregata* on seed germination and length of radicles (mm)

Isolate No.	seed germination %				Length of radicles (mm)			
	H_2L_4	% reduction	Crawford	% reduction	H_2L_4	% reduction	Crawford	% reduction
1	47.5	48.60	62.5	35.90	6.2	93.30	13.0	78.42
2	20	78.40	42.5	56.41	4.6	95.03	6.6	89.05
3	47	38.38	67.5	30.77	5.7	93.84	9.6	84.07
4	25	72.97	30.0	69.23	3.6	96.11	11.3	81.24
5	35.0	62.16	27.5	71.79	5.6	93.95	9.9	83.57
6	72.0	22.16	57.5	41.03	7.5	91.90	12.5	79.25
Control (untreated)	**92.5**		**97.5**		**92.55**		**60.25**	

L.S.D at 5 % for

Isolates (i)	9.85	3.4
Cultivar (c)	n s	ns
I x c	13.95	ns

Data in Table (5) show that cut seedlings of cultivar Crawford immersed in undiluted crude culture filtrates of different isolates (Type (I), were flaccid within few hours, browning in petioles and veins and interveinal necrosis was noted, followed by wilting and drying of the leaves within 48hrs. The use of culture filtrate of isolates of type (II)

did not produce symptoms on the leaves. Both culture filtrates of type (I) and type (II) caused vascular browning in the stem. The leaves affected by isolate of type (I) twisted inward or outwards depending on the severity of symptoms designated as +,++ and +++. Severity of these symptoms depended on the type of *P.gregata* isolates and exposure time to culture filtrate. A repetition of this experiment, including non-autoclaved culture filtrate for comparison indicate that autoclaving did not influence the symptoms produced on the leaves. These results indicate that culture filtrate contained heat stable materials (Toxins), which may play a role in pathogenesis. Cut seedling in the control (normal Czapek's medium) remained green and healthy.

Table 5. symptoms of soybean cut seedlings immersed in culture filtrates of six isolates of *Phialophora gregata.*

Isolate No.	Type	Symptoms after (hour)			
		6	12	24	48
1	I	BV, N	BV, N, CM	BV^{++}, N^{+}, CM^{++}, BS^{+++}	Wilt and drying
2	I	BV, N	BV, N, CM	BV^{++}, N^{+}, CM^{++}, BS^{+++}	Wilt and drying
3	II	---	---	--- BS++	BS++
4	I	BV, N	BV, N, CM	BV^{++}, N^{+}, CM^{++} ,BS^{+++}	Wilt and drying
5	I	BV, N	BV, N, CM	BV^{+}, N^{+}, CM^{++}, BS^{+++}	Wilt and drying
6	II	---	---	--- BS^{+}	BS^{+}
Cont.		---	---	---	---

BV: Browning in petioles and veins

N: leaves flaccid, yellowing and interveinal necrosis.

CM: Inward or outward rolling of leaf margins.

BS: stem browning.

+, ++, +++, degrees of the severity of symptoms.

DISCUSSION

In recent years, soybean (Glycine max (L)Merrill) has become a very important leguminous crop in Egypt and many other countries. Its importance stems from the high value and amount of oil and protein content in the seeds, which are

used for both human and animal consumption. Morphological and cultural characteristics of *P.gregata* varied among isolates. These characters included, radial growth, colony margin, colony surface, color of culture and conidial size (Philips, 1973 and Mengistu and Grau, 1986).In this study, mycellium of six isolates ranged from white to dark brown, with light tan being most common. Allington and Chamberlain (1948) and Abou El-Dahab (1968) found that mycelial mat is dense, white later becoming putty colored or gray or brown. Colony margin was lobed or smooth, but colony surface was raised and rough or aerial mycelium abundant and smooth, These characteristics were reported by Philips (1973). Isolates 1, 2, 4 and 5 were faster in growth than isolates No. 3 and 6. Gray (1971) reported that the isolates in type (I) grew faster in culture than isolates of type (II). Variation in pathogenicity in isolates of *P.gregata* within a given geographical area has been demonstrated (Gray, 1971; Philips, 1973 and Mengistu and Grau, 1986).In the represent study, isolates of *P.gregata* from soybean stems were classified into two pathotypes based on their ability or inability to cause chlorosis, necrosis and wilt of foliage. Both pathotypes caused internal stem discoloration. The six isolates of *P.gregata* grew at different temperatures ranging from 10°C to 30°C, then growth decreased with no growth at 40°C. Sinclair (1982) reported that optimal temperatures for growth on soybean stem agar were 22-24°C (maximum 30°C, minimum 8°C). Type (I) isolates were more vigorous than type (II) isolates (Gray1971). However, there was no significant difference in radial growth between pathotypes above 28°C. Mengistu and Grau (1986) reported that, no significant difference in radial growth between pathotypes above 24°C. *Phialophora gregata* types (I) and (II) produced heat stable toxic substance(s) in culture filtrate that reduced seed germination and length of radicles. However, type (I) isolate may produce additional substance (s) that contribute to browing the petioles and veins, interveinal necrosis, drying of the leaves in absence of physical contact between the host and pathogen. These results agree with results of Hassanein *et al.* (1996) who mentioned that different isolates of *M. phaseolina* produced metabolites in culture filtrates which caused reduction in seed germination and a wide range of symptoms on seedlings of soybean and that severity of symptoms was correlated with the virulence of isolates. The above mentioned symptoms characterizing type (I) and type (II) were reproduced using culture filtrates and cut seedlings, where it was obvious that type (II) culture filtrates produced no foliage

symptoms. Such results conform with Gray and Charmberlain (1975) and Gray (1971) who also reported on the heat stable properties of toxic metabolites produced in culture. Based on the results obtained, it was possible to differentiate among different pathotypes of *P.gregata* on the basis of symptoms produced in susceptible soybean cultivars.

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الاختلاف بين عزلات الفطر فيالوفورا جريجاتا

أحمد محمد حسنين(٢) متولى على بركة(١)
محمد عبد العظيم عبد العال(٢) عبد الله متولي الجارحى(٢)

(١) كلية الزراعة – جامعة قناة السويس .
(٢) معهد بحوث أمراض النباتات مركز البحوث الزراعية جيزة

جمعت ست عزلات من الفطر فيالوفورا جريجاتا المسبب لمرض عفن الساق البني في فول الصويا لدراسة الاختلافات المزرعية والقدرة المرضية وإنتاج المواد السامة في البيئة . وقد وجد أن هناك اختلاف بين العزلات في معدل النمو والصفات المز رعية على بيئة الـ PDA وكان أقصى نمو للعزلات الستة على درجة حرارة ٢٨مْ درجة مئوية كما وجد أن كل العزلات لها القدرة على أحداث المرض ولكن بدرجات مختلفة (قوية – متوسطة) . وقد قسمت هذه العزلات إلى طرازين مرضيين على أساس مقدرتها (الطراز الأول) أو عدم مقدرتها (الطراز الثانى) لأحداث تدهور في لون النبات أو مناطق ميتة أو ذبول المجموع الخضري وقد وجد أن كلا من الطرازين احدثا تلونا داخليا في الساق ووجد أن كل عزلات الفطر فيالوفورا جريجاتا أنتجت سموما في راشح المزرعة والتي أحدثت نقصا في نسبة انبات البذرة وطول الجذير وعمد غمر النهايات المقطوعة للبادرات في الراشح المعقم أو غير المعقم الخام غير المخفف لعزلات الفطر فيالوفورا جريجاتا المختلفة لوحظ أن الأوراق الصغيرة في بادرات منماه في راشح الطراز الأول أصبحت مسترخاه وبنية العنق والعروق وبعد ذلك لوحظت مناطق ميتة بين العروق أعقبها ذبول ثم جفاف كامل وسقوط للاوراق أما البادرات المغموسة في راشح الطراز الثاني فلم تظهر أي من الأعراض السابقة إلا أن الطرازين أعطيا تلونا داخليا بالساق .