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EFFECT OF CULTURE MEDIA ON MYCELIAL GROWTH AND SPORULATION OF TWO ISOLATES *OF Alternaria solani*, THE CAUSAL AGENT OF EARLY BLIGHT DISEASE OF TOMATO

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ABSTRACT

Sporulation of *Alternaria solani* (Ellis and Martin) Jones and Grout, can be scarce and is often reduced when the fungus is cultivated *in-vitro*. An experiment was conducted to assess the effect of culture media on mycelial growth and sporulation of two isolates of *A. solani* namely Badr and Al-Tawfiqiyah, *in-vitro*. Badr isolate gave the highest mycelial linear growth when grown on potato dextrose agar (PDA) and V-8 media. However, it produced maximum yield of conidia on V-8 medium. Al-Tawfiqiyah isolate was best grown but least sporulated on S-medium and PDA. However, its maximum yield of spores was obtained on V-8 medium. Thus, V-8 medium was the best among all media tested for sporulation of both *A. solani* isolates.
Keywords: *Alternaria solani*, sporulation, *in-vitro*, early blight, tomato, potato.

INTRODUCTION

Early blight caused by *Alternaria solani* (Ellis and Martin) Jones and Grout is a serious and important plant disease in warm and humid regions (Hooshyari *et al.,* 2013). *Alternaria solani* infects solanaceous crops including tomato, potato, eggplant, and pepper producing leaf blight (Kemmitt, 2002).

Sporulation of *A. solani* can be sparse and is often rare when the fungus is cultivated *in-vitro* (Rodrigues *et al*., 2010). It is well known that *in-vitro* sporulation of *A. solani* needs special conditions and that conidial production tends to decrease after periodic sub culturing of the pathogen (Rotem, 1994).

Many researches have been conducted on the possibility of growth and sporulation of *Alternaria* species by using unfavorable conditions for vegetative growth (Foolad *et al*., 2000; Rodrigues *et al*., 2010 and Shahbazi *et al*., 2011).

The major factors that influence sporulation *in-vitro* are usually nutrition and temperature as well as, adding calcium carbonate to increase sporulation (Shahin and Shepard, 1979). Dhingra and Sinclair (1995) showed that natural culture media are suitable for sporulation, storage, and to maintain viability of the colony after subcultures. Somappa *et al.* (2013) revealed that sporulation of *A. solani* was maximum on potato dextrose agar (PDA). They added that pathogen also sporulated maximum at temperature 25^{o}C. Results from different studies (Krishna*et al*., 1998; Arunakumara, 2006 and Jaggal somappa *et al*., 2013), showed that, the V-8 Juice medium, and media with parts or extracts of plants are used in protocols to induce sporulation of *Alternaria* spp.

Due to the spread and the significance of the early blight disease of tomato, present investigation was undertaken to determine the best medium for sporulation of the pathogen *in-vitro*. Our developed protocol was validated with two pathogenic isolates.

MATERIALS AND METHODS

The present investigation on *A. solani*, was conducted at Plant Pathology Department, and Seed & Tissue Pathology Lab (SEPA), Central Laboratory, Faculty of Agriculture, Mansoura University, Egypt. The materials used and methods followed are described below.

Source of the pathogen

Two pathogenic isolates of *A. solani* namely Badr and Al-Tawfiqiyah were obtained from Plant Pathology Research Institute, Agricultural Research Centre, Giza, Egypt.

Pure cultures of the isolates under study were transferred onto PDA slants and kept in refrigerator at 4^{o}C for further investigation.

Preparation of mycelial suspension (inoculum)

Two isolates of *A. solani* were used in this study. Each isolate was cultured on potato dextrose broth (PDB) medium for 10 days at 25°C, and then filtered through sterilized Whatman No.1 filter papers. Mycelial mats were washed several times with sterilized distilled water, and then blended for 3 min., using an electric blender. Mycelial fragment were diluted by sterilized distilled water (1:2, w/v) and used as inoculum.

Pathogenicity test

Tomato seedlings were grown in earthen pots filled with mixed soil (25% sand and 75% peat moss), which was autoclaved twice at 121°C for 30 min in two consecutive days and air dried. Growing seedlings (40-days-old) were sprayed using hand-operated atomizer, with the mycelial suspension of each isolate (Badr and Al-Tawfiqiyah), which was prepared in sterile water from 10-day-old culture. The mycelial suspension was sprayed on leaves, then the inoculated plants were covered with polyethylene bags. The control treatment was sprayed with sterile water. Five replicates were used for each treatment. After 75 days from inoculation, incidence and severity of early blight were recorded by visual observation of symptoms based on method described by (Watterson, 1986). The early blight incidence was calculated by the following formula:

$$\% \text{ Early blight incidence} = \frac{\text{Number of early blight symptoms bearing plants}}{\text{Total number of plants}} \times 100$$

Early blight severity was assessed following standard severity scale: 0 (No disease symptom), 1 (1-10% leaf area infected), 2 (11-25% leaf area infected), 3 (26-50% leaf area infected), 4 (51-75% leaf area infected), and 5 (76- 100% leaf area infected) (Volkalounakis, 1983).

Fungal pathogen was re-isolated from infected plants and microscopically investigated.

Re-isolation of pathogenic isolates

Tomato blighted leaves and stems from artificially-inoculated plants showing typical early blight symptoms were washed carefully under tap water to remove the adhering soil particles. These plant materials were cut into small pieces and surface-sterilized by immersing pieces into 1% sodium hypochlorite solution for 2-5 min., and then washed several times in sterilized distilled water to remove any residues of sodium hypochlorite. Then, leaf and stem pieces were dried between two sterilized filter papers, then transferred onto PDA amended with rose Bengal (0.003%) and streptomycin sulfate (0.01%) in Petri dishes and incubated at 25±2^{o}C for 4-7 days. The growing fungi were individually transferred onto PDA medium. Pure cultures of the recovered fungi were obtained using single spore or hyphal tip technique. The fungal isolates were then identified. Pure cultures of the isolated fungi were transferred onto PDA slants and kept in refrigerator at 4^{o}C for further uses.

Effect of culture media on mycelia growth and spore production of *Alternaria solani*

Seven types of culture media prepared from three main media; PDA, V-8 medium (V-8) and S-medium and their possible combinations were evaluated for their effect on mycelial growth and sporulation of the two isolates of *A. solani* (Badr and Al-Tawfiqiyah) as follows:

1. Potato dextrose agar (PDA): 200 g peeled potato pieces were boiled in a pot with lid with about 800 ml aqua distilled for 1 hour. The remaining cooking liquid is filtered through two layers of cheese-cloth, mixed with 20 g agar and 20 g dextrose, filled up to 1 liter and autoclaved (Rotem, 1994).
2. V-8 medium (V-8): 100 ml of Campbell's V-8 juice; 1.5 g $CaCO_3$; 15 g agar; and 900 ml distilled water (Gudmestad *et al*., 2013).
3. S-medium: 20 g sucrose; 3 g $CaCO_3$ and 20g agar per liter of distilled water (Shahin and Shepard, 1979).
4. Mixture of (PDA + V-8): prepared as 1:1(v/v).
5. Mixture of (PDA + S-medium): prepared as 1:1(v/v).
6. Mixture of (V-8 + S-medium): prepared as 1:1(v/v).
7. Mixture of (PDA+V-8+S-medium): prepared as 1:1:2 (v/v).

Media were poured into sterilized 9-cm Petri dishes (20 ml per plate). Five replicates were used for each treatment. After that all plates were inoculated with 5-mm-diameter discs from 10-day-old cultures of each isolate. All plates were ncubated at 25±2^{o}C. The colony diameters (cm) were measured 7 days after inoculation.

Cultures for sporulation trial were kept for 30 days after inoculation and then conidia were removed by repeatedly flooding plates with 10-ml of sterile distilled water. Resultant spore suspensions were filtered through two layers of sterile cheesecloth, combined and centrifuged at 10 ml^{-9} for 5 min. Resultant pellets were diluted to the desired concentration(s) with sterile distilled water. The concentration of conidia was counted with a haemocytometer (Chohan *et al*, 2015).

Statistical analysis

A one-way analysis of variance was conducted to analyze the data, by completely randomized design (CRD). Data collected from all experiments

were statistically analyzed using the Statistical Analysis System package (SAS institute, Cary, NC, USA). Differences between treatments were determined using Fisher's least significant difference (LSD) test by Duncan's multiple range test (Duncun, 1955). All comparisons were performed at $P \leq 0.05$.

RESULTS

Pathogenicity test

Two *A. solani* isolates were tested for their pathogenicity, under greenhouse conditions, on tomato plants, Carmen F1 c.v., (Nongwoo-Bio, KANZA CO. Ltd) which is highly susceptible to early blight disease. Data presented in Table (1) show that after 75 days, the disease incidence caused by Al-Tawfiqiyah isolate was significantly higher than that of Badr isolate (86.67 vs. 60.33%, respectively).However, there was no significant difference between both isolates with regard to their disease severity(Table 1).

Table (1): Pathogenicity test of two *Alternaria solani* isolates (Badr and Al-Tawfiqiyah) on tomato under greenhouse conditions.

Treatment	DI (%)[a]	DS (%)[b]
Control	0.00 c[c]	0.00 c
Badr isolate	60.33 b	54.00 a
Al-Tawfiqiyah isolate	86.67 a	51.00 a

[a] **DI = Disease incidence (%)**
[b] **DS = Disease severity (%)**
[c] **Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (*P*=0.05).**

Effect of culture media on mycelia growth of*Alternaria solani*

Mycelail growth of two *A. solani* isolates was determined after 7 days of growing on seven culture media, *i.e*. PDA, V-8, S-medium and the combinations(PDA+V-8), (PDA+S-medium), (V-8+S-medium) and (PDA+V-8+S-medium). Data show that PDA andV-8 media were the best for mycelial growth of Badr isolate (Table 2). These were followed by S-medium and then all mixed media (Table 2). On the other hand, PDA, V-8 and all combinations that are containing V-8 induced the highest growth rate for Al-Tawfiqiyah isolate (Table 3).

Effect of culture media on spore production of *Alternaria solani*

In general, it was noticed that sporecount per ml of Badr isolate over all media was more than Al-Tawfiqiyah. Data presented in Tables (2&3), show that Badr and Al-Tawfiqiyah isolates gave the highest yield of spores on V-8 medium (1.8×10^7 and 7×10^5 spores ml^{-1}, respectively), followed by mixture PDA+ S-medium (1.2×10^7 and 4×10^5, respectively) then mixture PDA+V-8+S-medium (1×10^7 and 6×10^5 spores ml^{-1}, respectively), whereas minimum sporulation of both isolates was observed on mixture V-8+S-medium (3×10^5 and 2×10^4 spore ml^{-1}, respectively).

Table (2) : Effect of seven culture media on colony growth and spore production of *Alternaria solani in-vitro*.

Medium	*Alternaria solani* (isolate Badr)		
	Mean colony diameter (cm)[a]	Growth rate (cm day^{-1})	Conidia ml^{-1} [b]
PDA	8.00	1.14 a[c]	6×10^{6} c
V-8	7.55	1.08 a	1.8×10^{7} a
S-medium	7.06	1.01 b	3×10^{6} d
Combinations (mixtures):			
PDA+V-8	6.53	0.93 c	0.9×10^{7} bc
PDA+S-medium	6.21	0.89 c	1.2×10^{7} ab
V-8+S-medium	6.28	0.90 c	3×10^{5} e
PDA+V-8+S-medium	6.36	0.91 c	1×10^{7} bc

[a] Radial growth was measured 7 days after inoculation.
[b] Spores were counted 30 days after inoculation.
[c] Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (*P*=0.05).

Table (3): Effect of seven media types on growth and sporulation of *Alternaria solani* (isolate Al-Tawfiqiyah) in-*vitro*.

Medium	*Alternaria solani* (isolate Al-Tawfiqiyah)		
	Mean colony diameter (cm)[a]	Growth rate (cm day^{-1})	Conidia ml^{-1}[b]
PDA	7.65	1.09 ab[c]	5×10^{4} c
V-8	6.50	0.93 b	7×10^{5} a
S-medium	7.83	1.12 a	5×10^{4} c
Combinations (mixtures):			
PDA+ V-8	7.00	1.00 ab	4×10^{5} b
PDA+ S-medium	6.46	0.92 b	4×10^{5} b
V-8+ S-medium	6.57	0.94 ab	2×10^{4} d
PDA+ V-8+ S-medium	7.00	1.00 ab	6×10^{5} ab

[a] Radial growth was measured 7 days after inoculation.
[b] Spores were counted 30 days after inoculation.
[c] Values within a column followed by the same letter are not significantly different according to Duncun's multiple range test (*P*=0.05).

DISCUSSION

Culture media and other factors play main role in growth and sporulation of fungi and other microorganisms. Many fungi like *A. solani* often grow *in-vitro* with vegetative growth, but produce no or sparse conidia (Dhingra & Sinclair, 1995). In this study, the effect of different media on the linear growth and spore production of two isolates of *A. solani* (Badr and Al-Tawfiqiyah) was tested. Both Isolates could grow on a wide range of culture media. It is well known that sporulation of *A. solani* can be limited and is often reduced when the fungus is cultivated in-*vitro* (Rodrigues *et al*., 2010). Production of conidia need nutritional requirements and fungus requires a source of carbon (sugar) to produce conidia, but however high concentration of sugar may reduce sporulation. Our results showed that the two isolates

produced spores on different media. Sporulation was occurred on all tested culture media when tested isolates were incubated under continuous darkness. This result agreed with that reported by Kishore Varma *et al*. (2014). Spletzer and Enyedi (1999), Foolad *et al*. (2000) and Rodrigues *et al*. (2010) stated that maximum sporulation of *A. solani* cultures was recorded when grown on V-8 agar medium. Tested media containing $CaCO_3$ showed vary results, as Vieira's (2004) found that sporulation was higher when V-8 was supplemented with $CaCO_3$, although there were no significant differences from the medium without $CaCO_3$. However, sporulation was moderate on PDA amended with $CaCO_3$. It is not yet clear whether the effect is due to the change in pH, or the amending of calcium.

CONCLUSIONS

The seven selected media (*i.e*. PDA, V-8, S-medium, PDA+ V-8, PDA+ S-medium, V-8+ S-medium and PDA+ V-8+ S-medium) gave good mycelial growth for the two isolates, Badr and Al-Tawfiqiyah. Among the different culture media tested, V-8 medium was found to be the best for sporulation of the two isolates, Badr and Al-Tawfiqiyah.

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تاثير بيئات المزارع على النمو الميسيليومي وإنتاج الجراثيم لعزلتين من الترناريا سولاني في المختبر

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تكوين الجراثيم لفطر ألترناريا سولاني غالبا ما يكون نادرا عند تنميته في المختبر. ولذلك أجريت تجربة لتقييم تأثير البيئات المختلفة على النمو الميسيليومى وإنتاج الجراثيم لعزلتين من الفطر الممرض ألترناريا سولاني (بدر والتوفيقية) في المختبر. وقد أعطت العزلة بدر أعلى معدل للنمو الميسيليومي عندما نمت على بيئتي آجار البطاطس والدكستروز (PDA) و آجار مستخلص الخضروات (V-8). أما عزلة التوفيقية فقد أعطت نمو ميسيليومي جيد عندما نمت على S-medium و PDA ومـــــزيج (V-8+PDA) ومزيج (S-medium+V-8+PDA) ومزيج (S-medium+V-8) و V-8 ومزيج (S-medium+ PDA) على التوالي. وكانت البيئة V-8 أفضل البيئات وأكثرها تشجيعا لتكوين الجراثيم للعزلتين بدر والتوفيقية حيث بلغ عدد الجراثيم (1.8×10^6 و 7×10^5 جرثومة /مل على التوالي) عند التحضين على درجة 25 °م بالمقارنة بالأوساط الغذائية الاخرى.