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RESEARCH ARTICLE / *ARTIGO*

In vitro production of conidia of *Alternaria solani*

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ABSTRACT

Sporulation of *Alternaria solani* can be scarce and is often reduced when the fungus is cultivated *in vitro*. A series of experiments were conducted to assess the effects of moisture, mycelial wounding, light quality and photoperiod, and culture media on conidial production. A procedure to induce sporulation based on mycelial wounding and dehydration was adapted and validated. Best results were obtained when fungal colonies were grown in V8 medium at 25°C in the dark with agitation for seven days; the mycelium mass was ground, poured into potato dextrose agar (pH 6.5) in plates, and incubated at 25 ± 2°C under near ultraviolet light and 12 h-photoperiod. The procedure was validated with 30 isolates of *A. solani* from different hosts, sampling locations, ages, and storage conditions. Conidial production, germination, and infectivity were quantified. Seventy-five percent of the isolates sporulated and the lowest germination rate was 68%. Inoculations of conidial suspension of all isolates resulted in lesions on tomato and potato plants. The effect of subculturing on *A. solani* sporulation was also assessed. After six subcultures, every seven days, there was no reduction in sporulation of colonies that were induced with the protocol.
Key words: early blight, biphasic technique, subculture, infection frequency.

RESUMO

Produção in vitro de conídios de *Alternaria solani*

A esporulação de *Alternaria solani in vitro* pode ser escassa e tende a reduzir após sucessivas repicagens. Conduziram-se vários experimentos para avaliar o efeito da umidade, injúria da colônia, qualidade de luz e fotoperíodo, bem como do meio de cultura na produção de conídios. Adaptou-se a técnica bifásica para induzir esporulação, por meio de injúria de micélio e desidratação do meio de cultura. Obtiveram-se melhores resultados quando colônias cresceram em meio V8 a 25°C, no escuro, sob agitação durante sete dias; a massa de micélio foi triturada e a suspensão depositada em meio BDA (pH 6,5) em placas de Petri, incubando-se a 25 ± 2°C, sob luz negra com 12 h de fotoperíodo. Validou-se o protocolo para 30 isolados de *A. solani* distintos quanto ao hospedeiro, local de origem, idade e forma de armazenamento. Quantificaram-se a produção, germinação e infectividade de conídios. Dos 30 isolados, 75% esporularam e a germinação mínima de conídios foi de 68%. Todos os isolados foram patogênicos em seus respectivos hospedeiros. Avaliou-se, também, o efeito de repicagens sucessivas na esporulação. Todos os isolados produziram conídios após seis repicagens sucessivas.
Palavras chave: pinta preta, técnica bifásica, subcultura, frequência de infecção.

INTRODUCTION

In vitro experiments are routinely used to study the nature and control of plant diseases caused by fungal pathogens. Sporulation is a key component for several purposes because fungal spores are frequently used as propagules to infect plants. In the case of potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) early blight, caused by *Alternaria solani* Sorauer, conidial suspensions are commonly used as inoculum, but sporulation *in vitro* can be scarce (Rotem, 1994). It is well known that *in vitro* sporulation of *A. solani* requires special conditions and that conidial production tends to decrease after periodic subculturing of the pathogen (Rotem, 1994).

Considerable attention has been directed towards improving conidial production of *Alternaria* species by using unfavorable conditions for vegetative growth (Rotem & Bashi, 1969; Shahin & Shepard, 1979; Walker, 1980; Ávila et al., 2000; Vieira, 2004). Three major factors that influence sporulation *in vitro* are commonly manipulated: nutrition, light spectrum, and temperature. Natural culture media are suitable for sporulation, storage, and to maintain viability of the colony after subcultures (Dhingra & Sinclair, 1995). For instance, the V8 juice medium, PDA (potato-dextrose-agar) and media with parts or extracts of plants are used in protocols to induce sporulation of *Alternaria* spp. (Miller, 1955; Shahin & Shepard, 1979; Dalla Pria et al., 1997; Ávila et al., 2000; Vieira, 2004). Commonly, calcium carbonate ($CaCO_3$) is also added to increase sporulation (Miller, 1955; Shahin & Shepard, 1979), but it is unknown whether its effect is due to the change in pH or due to calcium supplementation.

Light and temperature affect many aspects of fungal development, including the formation of reproductive structures. *In vitro* sporulation of *A. solani* is favored by incubation under continuous fluorescent light (λ = 380 – 775 ηm) at 25°C or 16 h photoperiod at 20°C (Douglas, 1972) and under black light (near ultraviolet – NUV, λ = 320 – 400 ηm) and 12 h photoperiod at 25°C (Fourtouni et al., 1998). However, the spectrum of fluorescent light contains

inhibitory wavelengths, especially the blue wavelengths (Honda & Nemoto, 1984). Fungal sporulation can be either inhibited or stimulated by photoreceptors (Idnurm & Heitman, 2005). In *Neurospora crassa* there are at least five photoreceptor groups, which are related to many aspects of the fungus life cycle, including sporulation (Purschwitza et al., 2006).

The factors described above and stress conditions can be properly combined to induce fungal sporulation. Mycelial injury (wounding) and gradual loss of the culture media moisture content lead to stress conditions that stimulate sporulation in *Alternaria* spp., as found with *A. macrospora* (Walker, 1980), *A. cassiae* (Walker, 1982; Ávila et al., 2000; Pitelli & Amorim, 2003), and *A. euphorbiicola* (Vieira, 2004). In his pioneering work, Walker (1980) described the biphasic technique for massive production of conidia of *A. macrospora*. The technique is called biphasic because initially the mycelium is produced in liquid medium, wounded, and then transferred to a solid medium to allow sporulation. Little information is available about the mechanism that triggers the production of reproductive structures induced by dehydration and wounding.

Although many studies were conducted to investigate sporulation of *A. solani in vitro*, in general the results vary and the viability and infectivity of conidia taken from colonies induced to sporulate were not measured. Another problem related to these studies is the low number of isolates assessed. Usually, only a single isolate is used to determine the favorable conditions to allow *in vitro* sporulation. This can be an important issue for highly variable species such as *A. solani*. Therefore, the objective of the present study was to adapt the protocol developed by Walker (1980) to induce sporulation in *A. solani*, combining the effects of mycelial wounding, loss of culture media moisture, sources of light, photoperiod, culture medium, and subculturing. Additionally, the protocol developed was validated with several isolates of the pathogen.

MATERIALS AND METHODS

Isolates, cultural conditions, and conidia quantification

Thirty different isolates of *A. solani* were used in the experiments (Table 1). Isolates preserved on filter paper were used in all tests to adapt the protocol and to assess the effect of subculturing on conidial production. Mycelial disks of monosporic colonies grown in PDA (200 g of potato, 20 g of dextrose, 16 g of agar, 1 L of distilled water-DW) were transferred to Petri plates with PDA covered with sterilized pieces of filter paper (1 x 1 cm) (Dhingra & Sinclair, 1995). In all experiments, incubation was at 25°C. After full development of the colonies, the pieces of colonized filter paper were removed and stored in paper envelopes at -80°C. Two fragments of colonized filter paper were transferred to Erlenmeyer flasks containing 150 mL of V8 medium (200 mL of V8® Vegetable Juice, 800 mL of DW) supplemented with streptomycin sulfate (50 µg/mL) and chloramphenicol (75 µg/mL). Isolates preserved under different methods were used. In this case, colonies were first grown in PDA, in the dark, for 7 days, then two plugs of mycelium (5 mm diameter) were transferred to V8 medium in flasks, incubating under agitation (110 rpm) at 25°C, in the dark. After 7 days, the mycelium was removed and ground using the Polytron® (System PT 1,200 C, Handheld Homogenizer), at 4,000 rpm, for 60 s. Ten mL of the mycelial suspension was deposited in 15 mL of culture medium, which varied according to the experiment, in 9 cm-diameter Petri plates. Three days after the beginning of the experiments, 10 mL of DW plus 0.01% Tween 80 was used to harvest the conidia with a brush (Foolad et al., 2000). The conidial suspension was collected in beakers; the conidial concentration was determined by counting the number of spores in a 10 µL-drop under the microscope (100X) and multiplying the number counted by 100 to estimate the total number of conidia/mL. The apparatus used to provide light during the incubation of the colonies had six light-bulbs spaced 5 cm apart and located 30 cm above the plates.

Effects of culture medium dehydration and mycelium wounding on sporulation

Five plates with V8 agar-$CaCO_3$ medium (200 mL of V8® Vegetable Juice, 3 g of $CaCO_3$, 16 g of agar, 800 mL of DW) per isolate/treatment were used. The V8 agar-$CaCO_3$ medium was chosen for the first experiment because it is a standard medium for *A. solani* sporulation. Three treatments were compared: plates sealed with Parafilm®; plates without Parafilm®; and open plates (without the lid). Light was provided by three black light bulbs (Sylvania® Luz Negra – 40W - 320-400 ηm) and three fluorescent daylight bulbs (Sylvania® Luz do Dia – 40W) with 12 h photoperiod.

Two isolates (AS073 and AS074) were used in the experiment to quantify the effect of culture medium dehydration on sporulation. Each 20 plates/isolate was weighed before and after pouring the culture medium and mycelial suspension. Plates without lids were kept under black and daylight bulbs, as described above. Every 12 h the plates were weighed, until 84 h after the beginning of the experiment. All subsequent experiments used mycelium wounding and plates without lids, to allow for dehydration and fungal sporulation.

Effects of the source of light, photoperiod, and culture medium on sporulation

Three sources of light were compared: daylight bulbs, black light bulbs, and the combination black / daylight bulbs. In this last treatment, the bulbs were alternated. Each source was tested in a continuous light regime or in a 12 h photoperiod. Three plates with V8 agar-$CaCO_3$ medium per isolate, per treatment (combination of light source and regime) were used. Plates kept under continuous darkness were used as controls.

TABLE 1 - Characteristics of the isolates of *Alternaria solani* used to develop and/or to validate the protocol to induce sporulation

Isolate	Host	Location	Year	Storage[(a)]
AS04-001	Potato	Carandaí MG	2004	FP
AS073	Tomato	Carandaí MG	2004	FP
AS074	Potato	Domingos Martins ES	2004	FP
AS075	Tomato	Viçosa MG	2004	FP
AS076	Potato	Bueno Brandão MG	2004	FP
AS077	Potato	Ibiá MG	2004	FP
AS078	Tomato	Venda Nova do Imigrante ES	2004	FP
AS079	Tomato	Planaltina GO	2006	FP
AS082	Tomato	Muniz Freire ES	2000	Castellani
AS083	Tomato	-	1996	Castellani
AS088	Potato	Ponta Grossa PR	2000	Castellani
AS089	Tomato	Ponta Grossa PR	2000	Castellani
AS090	Tomato	Capão Bonito SP	2000	Castellani
AS091	Tomato	Viçosa MG	2000	Castellani
AS094	Tomato	Coimbra MG	2000	Castellani
AS095	Tomato	Coimbra MG	2000	Castellani
AS117	Tomato	Valão do Barro RJ	2000	Castellani
AS121	Tomato	Itaperuna RJ	2000	Castellani
AS123	Tomato	Tocantins MG	2000	Castellani
AS124	Tomato	Tocantins MG	2000	Castellani
AS130	Tomato	Cajuri MG	2000	Castellani
AS131	Tomato	Cajuri MG	2000	Castellani
AS132	Tomato	Marília SP	2000	Castellani
AS223	Tomato	Castelo ES	2005	PDA
AS250	Potato	Conselheiro Lafaiete MG	2005	PDA
AS259	Potato	Cristalina GO	2005	PDA
AS337	Potato	Itapetininga SP	2005	PDA
AS370	Potato	Ipuiúna MG	2006	PDA
CE20	Tomato	-	1999	SG
UFV22FF	Tomato	Viçosa MG	2000	PDA
COIMBRA	Tomato	Coimbra MG	2004	PDA

[(a)] FP = filter paper (-80ºC), PDA = tube with PDA (4ºC**),** Castellani method (Dhingra & Sinclair, 1995) and SG = silica gel

Four culture media, amended or not with $CaCO_3$, were compared: PDA; V8 agar; Vegetable broth agar – VBA (Pereira et al., 2003) and Carrot agar – CA (20 g of carrot, 16 g of agar, 1 L of DW). The pH was adjusted to 7.5 and 6.5 in treatments with or without $CaCO_3$ (3g/L), respectively. Based on the results of the previous experiment, the plates were kept under black light, 12 h photoperiod. Three plates were used per isolate, per treatment.

Effect of periodic subcultures on sporulation

The isolates AS04/001, AS073, AS074, AS075, AS077 and AS078 were subcultured and at each transfer, sporulation was induced according to the protocol developed in the current study. The colonies were grown in PDA (pH=6.5) in the dark for 7 days. For subculturing two plugs of mycelium taken from the center of each colony to establish a new growth were transferred, one plug to a plate with PDA (pH=6.5) and one plug to a flask with 150 mL of V8 medium. Three plates and three flasks were prepared per isolate at each subculturing. Every 7 days, cultures in plates were subcultured. After colony development, the cultures in liquid medium were obtained and induced to sporulate using the best combination from the previous experiments.

Validation of the protocol to induce sporulation and infection

Two experiments, the first with 20 isolates and the second with 30 isolates, were undertaken to validate the protocol. Conidial production was induced in five plates per isolate. Conidia of three randomly selected plates were harvested and a 10^3 conidia/mL-suspension was used in germination and infectivity tests. To evaluate germination, 50 µL of the conidial suspension from each isolate was deposited on one microscope slide. Three slides were prepared per isolate. In the first experiment, the slides were transferred to a plastic box (11cm length x 11cm width x 4cm height), kept at 25°C, in the dark, for 12 h, until the observation under the microscope (100X). In the second experiment, the incubation period was 6 h. A conidium was considered as germinated when one germ tube was at least as long as the conidium's width. One hundred randomly selected conidia were examined per slide.

To assess the infectivity of the spores, 45 to 60-day-old tomato plants cv. Santa Clara or detached leaflets of potato cv. Monalisa were inoculated. Detached leaflets of potato were used because the number of plants was not enough to conduct the experiment. Each tomato plant was divided into three thirds (upper, middle, and lower) based on plant height. In each third, three leaflets were tagged with a paper tag and a cover glass (1 x 1 cm) was attached on the adaxial surface with double-faced adhesive tape. The conidial suspension was sprayed on the leaflets using a DeVilbiss 15 sprayer. The negative control was sprayed with water and the positive control was sprayed with the isolates AS078/AS079 and AS079, in experiments 1 and 2, respectively. For each isolate, three plants were inoculated and kept for 24 h in a moist chamber (100% relative humidity). Nine detached leaflets of potato were inoculated as above, and kept in a plastic box. After the inoculation, the cover glass was removed and placed upside down on top of a drop of lactophenol on a microscope slide. The number of conidia deposited on the cover glass was counted under the microscope (100X). The number of lesions formed in the leaflets was counted on the third and fourth day after inoculation. Based on the results of experiment 1, only leaflets of the middle third of tomato or potato plants were used in experiment 2. Six leaflets in each plant and four plants/isolate were inoculated.

The infection frequency (IF), considered as the ratio between the number of lesions (L) and the number of conidia deposited on a leaflet, was used to assess infectivity. Leaflet area was measured on the fifth day after inoculation, using an electronic leaf area meter (Li-3.100 – Licor®). The cover glass area (1 cm^2) was subtracted from the total leaflet area to result in a corrected number of spores deposited on a given leaflet (*CN*). The IF was determined as:

$$IF(\%) = (L/CN)x100.$$

Statistical analyses

A two-way analysis of variance was conducted to analyze the data. Whenever the treatment x isolate interaction term was significant, the analyses of the treatment effects were done by isolate. Each experiment was conducted twice (hereafter referred to as assays 1 and 2), except those for the quantification of culture medium dehydration, infection frequency, and subculturing. The Levene test was used to check the homogeneity of variance between assays conducted twice (Kuehl, 1994). Treatment means were compared using the Tukey test ($\alpha = 0.05$). In the experiment with culture media, the pH treatment effect was evaluated by contrasts. In all experiments, the sporulation data were transformed to $\log_{10}$ (number of conidia); when there was no sporulation in a treatment, the data were transformed to $\log_{10}$ (number of conidia + 1). All analyses were done using the SAS® ver. 8.2.

RESULTS

Effects of culture medium dehydration and mycelium wounding on sporulation

Data from each assay were analyzed separately because of the lack of homocedasticity. Aerial mycelium growth with no sporulation was observed in colonies formed in plates with lids, sealed or not with Parafilm. In open plates, medium dehydration occurred and vegetative mycelia were sparse, and produced asexual structures. The sporulation varied among isolates, and ranged from $3.72x10^5$ (SE=$0.74x10^3$) to $11.46x10^5$ (SE=$3.81x10^3$) conidia/mL and from $2.79x10^5$ (SE=$0.74x10^3$) to $5.99x10^5$ (SE=$2.27x10^3$) conidia/mL, in assays 1 and 2, respectively. The highest rate of culture medium dehydration (45%) occurred in the first 12 h. After 60 h, no loss of moisture was observed until the last observation at 84 h.

Effects of the source of light, photoperiod, and culture medium on sporulation

Data from both experiments were pooled and analyzed. Sporulation of all isolates was significantly ($P<0.0001$) affected by light source and photoperiod. Highest number of conidia was observed in colonies kept under black light ($P<0.0001$) and 12 h photoperiod (Figure 1). No isolates sporulated under continuous darkness. The data from the assays of culture media were analyzed separately. The interaction between culture medium (supplemented or not with $CaCO_3$) x pH was significant in three of 16 interactions in assay 1, and in 2 of 16 interactions in assay 2 (Table 2). Although a high number of conidia were produced in all media, either supplemented or not with $CaCO_3$ (Table 3), PDA medium at pH=6.5 was chosen for the subsequent experiments due to its lower cost.

Effect of periodic subcultures on sporulation

Every colony sporulated until the 6th subculture. Different colonies of the same isolate did not differ

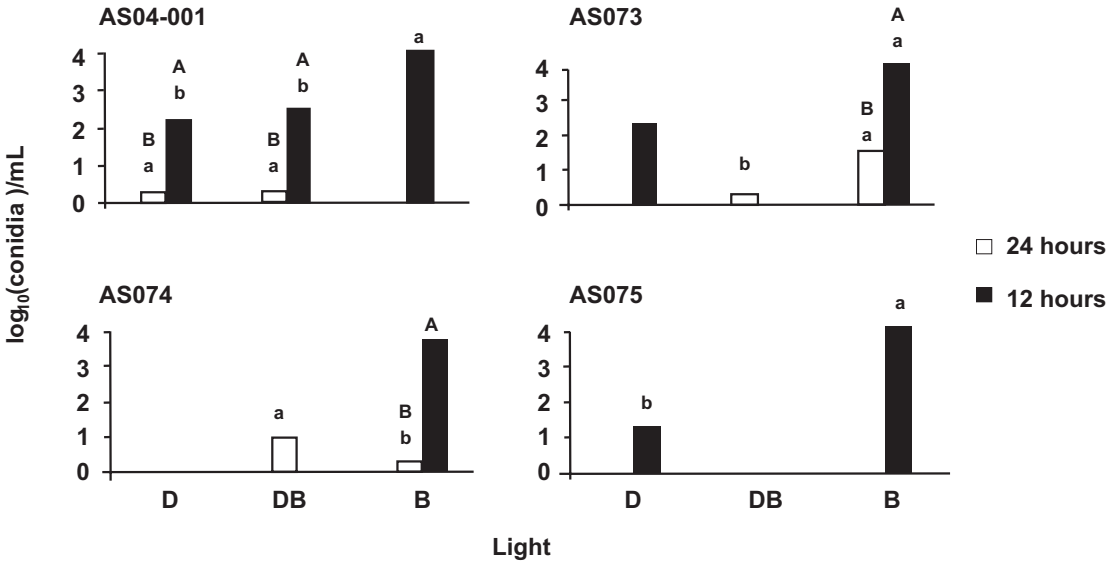

FIGURE 1 – Sporulation [$\log_{10}$ (conidia)/mL] of *Alternaria solani* in different sources of light (D = Daylight, DB = Daylight/Black, B = Black) and photoperiod (24 h and 12 h). For each isolate, bars with the same capital letter, for the same source of light, do not differ according to the Tukey test (α = 0.05). Bars with the same small letter, for the same photoperiod, do not differ according to the Tukey test (α = 0.05).

TABLE 2 – F values and, in parentheses, significance level, for differences between media of four isolates of *Alternaria solani* in different culture media supplemented (pH=7.5) or not (pH= 6.5) with $CaCO_3$, in two assays (1 and 2)

Isolate	Assay	Culture medium				
		PDA	V8 agar	VBA	CA	All
AS04-001	1	12.58* (0.0027)	1.16 (0.2982)	7.11 (0.0169)	0.01 (0.9059)	6.91 (0.0182)
	2	53.60 (<0.0001)	11.10 (0.0042)	4.84 (0.0429)	7.86 (0.0127)	31.68 (<0.0001)
AS073	1	26.28 (<0.0001)	16.63 (0.0009)	10.64 (0.0049)	4.95 (0.0409)	16.72 (0.0009)
	2	17.21 (0.0009)	0.38 (0.5443)	1.12 (0.3070)	14.47 (0.0017)	12.85 (0.0027)
AS074	1	0.67 (0.4246)	0.00 (0.9628)	1.60 (0.2244)	0.12 (0.7297)	0.71 (0.4122)
	2	13.28 (0.0022)	8.97 (0.0086)	0.03 (0.8637)	19.32 (0.0005)	29.49 (<0.0001)
AS075	1	65.54 (<0.0001)	0.73 (0.4064)	6.77 (0.0192)	37.58 (<0.0001)	29.00 (<0.0001)
	2	67.43 (<0.0001)	10.13 (0.0058)	1.38 (0.2570)	14.77 (0.0014)	49.43 (<0.0001)

* Average from 3 replicates.

regarding sporulation. At least one colony of each isolate sporulated until the 9th subculture. For AS077 and AS078, at least one colony produced conidia until the 18th subculture and one colony of the isolate AS074 sporulated until the 20th subculture (Figure 2).

Validation of the protocol to induce sporulation and infection

The following protocol was defined: colonies should be grown in V8 medium under agitation (110 rpm), at 25°C, in the dark, for 7 days. The harvested mycelia should be

TABLE 3. Sporulation [$\log_{10}$(number of conidia+1)/mL] of four isolates of *Alternaria solani* in different culture media supplemented (pH=7.5) or not (pH= 6.5) with $CaCO_3$, in two assays (1 and 2)

		Culture Media							
		PDA		V8		VBA		CA	
	pH	1[*3]	2	1	2	1	2	1	2
AS04/001	**6,5**	5,04[*4]	5,10	4,49	4,87	4,43	4,64	4,35	4,63
		A* a	A a**	A ab	A b	A b	A c	A b	A c
	7,5	4,42	4,71	4,68	4,70	3,96	4,76	4,32	4,49
		B a	B a	A a	A a	A b	A a	A ab	A b
AS073	**6,5**	4,98	5,10	4,73	4,89	4,24	4,98	4,27	4,80
		A a	A a	A ab	A a	A b	A a	A b	A a
	7,5	4,26	4,68	4,15	4,82	4,70	5,10	3,96	4,42
		B ab	B b	B b	A ab	A a	A a	A b	B b
AS074	**6,5**	4,57	4,48	2,85	4,73	2,77	4,58	2,99	4,05
		A a	A ab	A b	A a	A b	A a	A b	A b
	7,5	4,31	3,98	2,87	4,32	2,36	4,60	3,11	3,44
		B a	B b	A b	A ab	A b	A a	A b	B c
AS075	**6,5**	5,24	5,13	4,48	4,58	4,49	3,94	4,77	4,30
		A a	A a	A b	A b	A b	A c	A b	A b
	7,5	3,92	4,40	4,62	4,30	4,91	4,04	3,77	3,96
		B b	B a	A a	A b	A a	A b	B b	B b

***** For the same isolate, culture medium and assay, averages followed by the same capital letter do not differ according to the Tukey test (α = 0.05).
****** For the same isolate, pH and assay, averages followed by the same small letter do not differ according to the Tukey test (α = 0.05).
[*3] Assays 1 and 2.
[*4] Sporulation= $\log_{10}$(number of conidia + 1)/mL. Average from 3 replicates.

ground using Polytron®, at 4000 rpm, for 1 min, and 10 mL of the mycelial suspension transferred to plates with PDA (pH=6.5). The plates should be kept without lids, under six light-bulbs of black light spaced 5 cm apart and placed 30 cm above the plates; 12 h photoperiod, at 25°C, for 60 h.

In experiment 1, all isolates sporulated, and the minimum germination rate was 96%. All isolates were pathogenic to their original host species, but the infection frequency varied. Greater infection frequency occurred with isolates from potato. For most isolates, the highest and lowest values obtained from the middle and upper third of the plants, respectively (Table 4). In experiment 2, 23 out of 30 isolates sporulated, because seven isolates did not grow in liquid medium. In general, the germination rates in experiment 2 were lower than in experiment 1. The methodology used in experiment 2 allowed better observation of the germinated conidia and more accurate counting. In experiment 2 only the middle third of the plants were inoculated and all isolates were pathogenic to their original hosts (Table 5).

DISCUSSION

The adapted and validated protocol allowed efficient induction of sporulation of *A. solani*, *in vitro*. Profuse mycelial growth without the production of conidia is commonly observed *in vitro* conditions. In general, vegetative growth requires different conditions from sporulation (Dhingra & Sinclair, 1995). *In vivo*, conidiophores are formed under high humidity and light, whereas conidial formation is favored by alternating high-low humidity with darkness (Waggoner & Horsfall, 1969). Simulating the *in vivo* conditions that favor sporulation under controlled conditions has not been successful in previous attempts to develop a protocol to induce sporulation in *A. solani*. Other factors, besides light conditions and levels of humidity may be required to stimulate conidial formation in colonies developed *in vitro*.

The dehydration rate and aeration of the mycelia affected sporulation of *A. solani in vitro*. Initially, plates without lid had high humidity, but after 12 h, there was a 10% decrease of the initial mycelium mass and conidiophores were formed under dehydration conditions. Mycelial dehydration may trigger the initial stimulus for sporulation. However, the mechanism involved in this process in *A. solani* is unknown. It was reported that conidiophores in contact with a film of water may revert to vegetative hyphae and halt conidiogenesis (Aragaki et al., 1973). In *Neurospora crassa*, genes associated

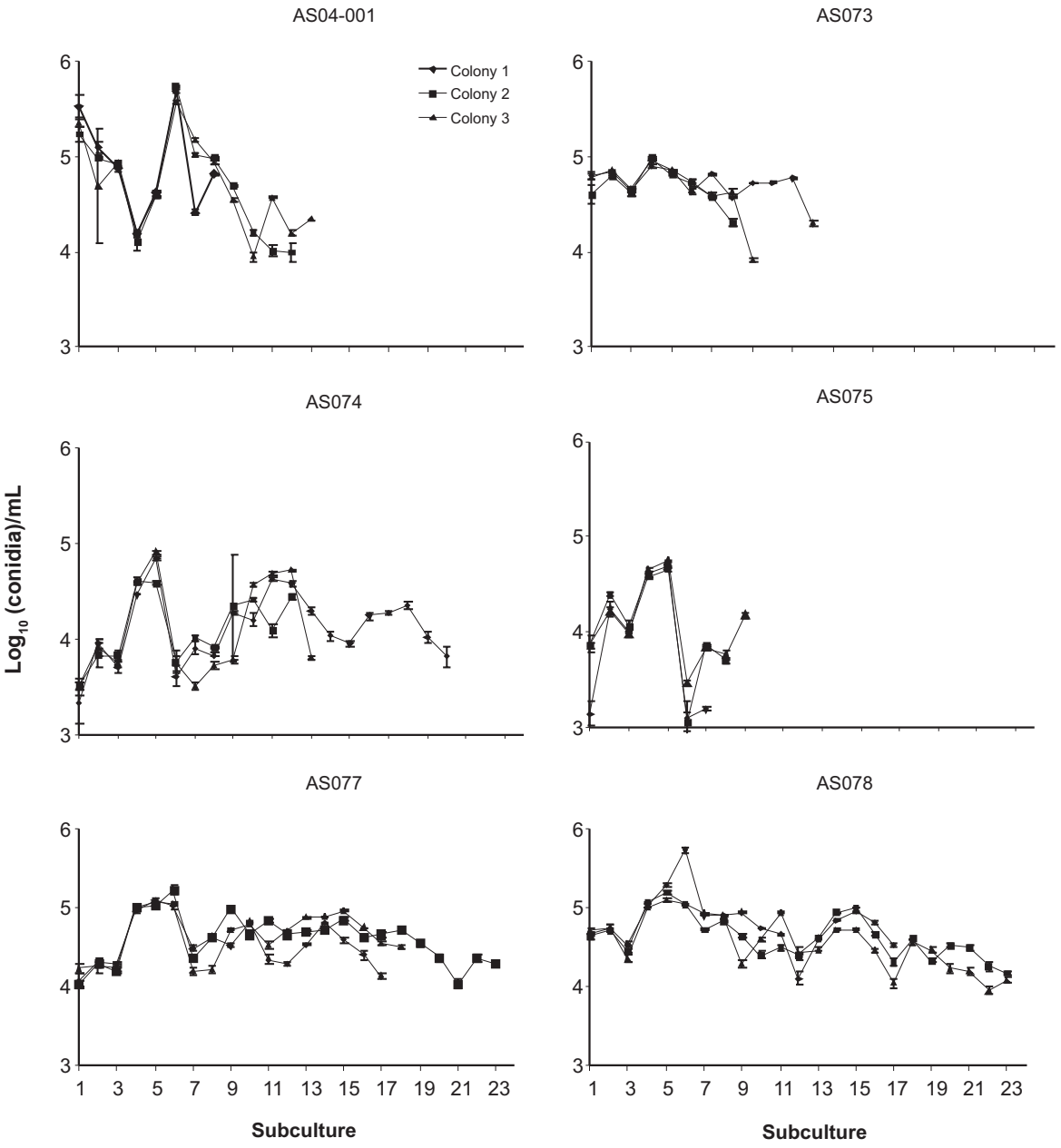

FIGURE 2 - Sporulation [$\log_{10}$ (conidia)/mL] of six isolates of *Alternaria solani* during 24 periodic subcultures. Colonies 1, 2 and 3 are replicates of each isolate. Bars represent standard deviation.

with conidiogenesis were translated only when colony desiccation occurred (Changgong et al., 1997). In the current work, the treatments that did not cause moisture loss promoted mycelial growth and no sporulation. Associated with dehydration, greater aeration of the colonies may also have contributed to pathogen sporulation. Low accumulation of carbon dioxide as well as higher oxygen availability seems to favor sporulation of *A. solani* (Lukens & Horsfall, 1973). The pathogen is more sensitive to the increase of carbon dioxide and the decrease of oxygen during the reproductive rather than the vegetative growth phase (Lukens & Horsfall, 1973). Humidity, CO_2, and oxygen levels affect the sporulation of *A. solani*, but how these factors act on the physiology of the sporulation remains to be elucidated.

The protocol used in the present study also resulted in satisfactory sporulation of other species of *Alternaria* (Walker, 1980, 1982; Ávila et al., 2000, Vieira, 2004). Moreover, it can also be used to induce sporulation in *Colletotrichum graminicola*, *C. malvarum*, *Drechslera sorghicola*, *Helminthosporium* spp. and *Curvularia* spp. (Walker, 1980). This protocol is effective for production of massive amounts of inoculum when strict aseptic conditions are not required (Walker, 1980).

TABLE 4 - Sporulation, percent of conidial germination and infection frequency of 20 isolates of *Alternaria solani* in tomato and potato. Experiment 1, to validate the protocol

Isolate	Sporulation[a]	Germination[b]	Infection frequency[c]		
			Upper third	Middle third	Lower third
AS04/001	8.8 (0.34)	97 (0.67)	0.30[d] (0.18)	13.12 (2.30)	9.26 (3.45)
AS073	10.5 (0.91)	98 (0.33)	0.35 (0.11)	1.06 (0.42)	0.17 (0.06)
AS074	3.1 (0.16)	96 (0.88)	0.10 (0.05)	3.19 (0.47)	6.40 (2.64)
AS075	10.4 (0.53)	98 (0.00)	0.11 (0.07)	0.19 (0.08)	0.07 (0.04)
AS076	21.0 (1.20)	99 (0.58)	2.21 (0.54)	2.47 (0.57)	2.27 (0.38)
AS077	6.3 (0.17)	86 (2.33)	4.30 (1.24)	7.84 (3.35)	5.77 (2.32)
AS078	20.2 (1.35)	99 (0.33)	0.04 (0.02)	0.23 (0.06)	0.13 (0.04)
AS079	53.6 (0.30)	96 (0.58)	5.52 (2.33)	2.84 (0.69)	1.75 (0.42)
AS088	5.34 (0.34)	99 (0.67)	10.08 (5.54)	2.01 (0.36)	2.92 (0.70)
AS089	2.1 (0.15)	97 (1.20)	0.13 (0.08)	0.42 (0.20)	0.05 (0.02)
AS124	13.9 (0.55)	99 (0.33)	1.35 (0.22)	2.72 (0.54)	1.23 (0.42)
AS131	10.2 (0.39)	90 (0.58)	0.04 (0.02)	0.00 (0.00)	0.03 (0.01)
AS132	13.48 (1.00)	96 (1.20)	0.16 (0.06)	0.48 (0.25)	0.56 (0.16)
AS091	2.3 (0.06)	96 (0.00)	0.18 (0.03)	0.29 (0.12)	0.25 (0.15)
AS095	6.6 (0.23)	99 (0.00)	0.18 (0.05)	0.10 (0.07)	0.12 (0.08)
AS121	10.84 (0.49)	99 (0.33)	0.05 (0.02)	0.04 (0.01)	0.12 (0.08)
AS123	6.44 (0.19)	98 (0.58)	0.55 (0.27)	0.55 (0.15)	0.14 (0.05)
CE 20	8.26 (0.48)	92 (0.33)	0.26 (0.11)	0.10 (0.04)	0.07 (0.02)
UFV 22FF	12.48 (1.42)	98 (0.58)	0.02 (0.01)	0.05 (0.02)	0.03 (0.01)
COIMBRA	31.58 (1.21)	98 (0.33)	0.05 (0.03)	0.28 (0.08)	0.16 (0.11)
Control*- 1					
AS078	11.04 (0.34)	98 (0.33)	0.03 (0.02)	0.09 (0.02)	0.07 (0.01)
Control- 1					
AS079	6.84 (0.15)	98 (0.88)	3.54 (0.79)	3.18 (0.63)	1.44 (0.32)
Control- 2					
AS078	9.94 (0.34)	98 (0.33)	0.02 (0.01)	0.07 (0.01)	0.07 (0.03)
Control- 2					
AS079	7.44 (0.19)	98 (0.33)	0.07 (0.02)	0.29 (0.06)	0.20 (0.06)

[a] Average of 5 values (conidia x10^4/mL of distilled water), number in parentheses is the standard error x 10^4; [b] average of 3 replicates; [c] [(number of lesions/ number of conidia in each leaflet) x 100], the isolates were inoculated on their original host, and [d] average of 9 replicates (%).
* Control 1 and 2 = isolates used as positive control.

Both the quality and the amount of light are critical for sporulation of *A. solani* (Leach, 1962; Douglas, 1972). In the present work, greater sporulation was obtained under alternated regime of light (12 h photoperiod). This alternation simulates conditions required for conidial formation, as conidiophore formation occurs under irradiation and conidium formation during the dark period (Lukens, 1960; Leach, 1967; Waggoner & Horsfall, 1969; Douglas, 1972; Walker, 1980). Wavelengths near 310 ηm, provided by black light, stimulate sporulation of *A. solani* and the conidiophores do not revert to vegetative hyphae when exposed to the dark, allowing conidial formation (Aragaki et al., 1973). Some authors have speculated that photoreceptor pigments could be involved in inducing or inhibiting sporulation by certain wavelengths (Leach, 1965; Trione & Leach, 1966; Honda & Nemoto, 1984). A compound called P310 ("Photoreceptor" 310) was obtained from sporulating colonies of *A. chrysanthemi* grown under black light, but not from non-sporulating colonies grown in the dark (Leach, 1965). The authors postulated that this compound could also play a role in sporulation of *A. dauci* (Trione & Leach, 1966). The chemical structure and the function of P310 have not yet been determined.

In *A. solani*, the production of conidiophores has nutritional requirements distinct from the production of conidia. The fungus requires a source of carbon (sugar) to produce high quantity of conidiophores, but high availability of sugar inhibits the production of conidia (Waggoner & Horsfall, 1969). This fact was observed in the present study when sucrose was added to the V8 liquid medium: the fungus grew abundantly, but produced only conidiophores (data not shown). After the pioneering work of Miller (1955), $CaCO_3$ has been added to various culture media, in different protocols, but the results vary according to the medium tested. In this work, as in Vieira's (2004), sporulation was higher when VBA was supplemented with $CaCO_3$, although there were no significant differences from the medium without $CaCO_3$. However, sporulation was lower in PDA supplemented with $CaCO_3$, a fact that has been observed previously on *A. solani* (Moretto & Barreto, 1995). Despite the evidence that the addition of $CaCO_3$ increases sporulation, it is not clear whether the effect is due to the change in pH, or the supplementation of calcium.

Periodical subculturing, commonly used with fungi, can affect sporulation and pathogenicity of fungal plant pathogens (Dhingra & Sinclair, 1995). In *A. solani*,

TABLE 5 – Sporulation, germination and infection frequency (number of lesions/ number of conidia in each leaflet) x 100, of 23 isolates of *Alternaria solani*. Experiment 2, to validate the protocol

Isolate	Sporulation[a]	Germination[b]	Infection frequency[c]
AS078	0.72 (0.07)	82 (3.52)	0.40(0.16)
AS082	2.10 (0.21)	68 (1.20)	0.17 (0.06)
AS083	33.11 (1.25)	79 (1.20)	0.20 (0.05)
AS084	0.74 (0.05)	87 (1.20)	0.44 (0.08)
AS088	3.20 (0.16)	92 (0.88)	0.45 (0.05)
AS089	2.78 (0.13)	80 (1.20)	0.18 (0.02)
AS090	0.76 (0.05)	69 (0.58)	0.75 (0.39)
AS091	0.70 (0.07)	74 (0.88)	0.17 (0.02)
AS094	3.79 (0.15)	79 (3.18)	0.19 (0.04)
AS095	12.88 (0.44)	93 (1.16)	1.17 (0.15)
AS117	4.38 (0.17)	74 (2.03)	0.06 (0.02)
AS121	1.42 (0.09)	82 (0.88)	0.67 (0.08)
AS123	1.12 (0.086)	74 (1.86)	0.31 (0.05)
AS124	0.84 (0.051)	76 (2.52)	1.02 (0.18)
AS130	3.06 (0.09)	90 (0.88)	0.52 (0.12)
AS131	1.40 (0.11)	72 (0.88)	0.77 (0.12)
AS132	2.40 (0.16)	90 (1.20)	0.59 (0.1)
AS223	1.16 (0.12)	87 (1.20)	0.21 (0.05)
AS250	7.74 (0.14)	87 (2.52)	0.28 (0.03)
AS259	3.82 (0.12)	89 (1.77)	0.25 (0.03)
AS337	2.04 (0.09)	89 (4.71)	0.26 (0.03)
AS370	2.32 (0.13)	82 (1.77)	0.51 (0.07)
Control*- 1 AS079	5.62 (0.15)	89 (2.19)	1.08 (0.14)
Control– 2 AS079	6.22 (0.14)	89 (0.58)	0.30 (0.04)
Control– 3 AS079	10.56 (0.56)	88 (2.08)	0.57 (0.09)
Control– 4 AS079	1.20 (0.11)	83 (1.53)	0.15 (0.05)
Control– 5 AS079	3.04 (0.16)	85 (1.86)	0.49 (0.08)

[a] Average of 9 values (conidia x10^4/mL of distilled water), number in parentheses represents the standard error x 10^4; [b] average of 3 values (%); [c] average of 24 values (%).
* Control 1 to 5 = isolate used as positive control.

periodical subculturing affects sporulation (Rotem, 1994). In the present study, the way the fungus was subcultured allowed for all isolates to sporulate for at least six subcultures. In another study, after two or three successive subcultures, the isolates of *A. solani* no longer sporulated (Padhi & Rath, 1973). According to the authors, the isolates that sporulated longer were mutants that emerged in culture after fungal isolation.

The production of a good quantity of quality inoculum is crucial for studying many aspects of fungal species. The evaluated protocol resulted in proper amounts and quality of sporulation of *A. solani*. The high rate of germination found in experiment 1 cannot reflect reality. Due to the long incubation time (12 h), the germ tubes with multiple cells formed a net of mycelia and identifying individual conidia was difficult. In experiment 2, germination rates were lower than in experiment 1, but the methodology allowed better evaluation of germination. Furthermore, conidia were infective to tomato and potato leaflets. Differences in the inoculated material, whether detached leaflets or not, may have influenced the rates of infection frequency. Changes in the physiology of the detached leaflets may have resulted in greater susceptibility, therefore leading to high values of infection frequency being observed for potato isolates. Moreover, *A. solani* has great pathogenic variability, which may be the main cause of the variations of the infection frequencies (Rotem, 1994). Despite the variations, all isolates were infective and the protocol developed was appropriate to induce sporulation of *A. solani in vitro*.

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