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EFFECTS OF PH AND AERATION ON *SCLEROTIUM ROLFSII* SACC. MYCELIAL GROWTH, SCLEROTIAL PRODUCTION AND GERMINATION

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ABSTRACT

Sclerotium rolfsii is one of the devastating soilborne fungus responsible for significant plant losses. The effects of pH and aeration on pathogen mycelial growth, sclerotial production and germination were investigated for three Tunisian isolates. Optimal mycelial growth occurred at pH 6 for Sr2 and Sr3 isolates and at pH 6-7 for Sr1. Dry mycelial growth was optimum at pH values ranging between 4 and 7. Sclerotial initiation started on the 3rd day of incubation at all pH values tested and mature sclerotia were formed after 6 to 12 days. Optimal sclerotial production was noted at pH 5. The dry weight of 100 sclerotia varied depending on isolates and pH and occurred at pH range 4-7. At pH 9, mycelial growth, sclerotial production and dry weight of 100 sclerotia were restricted. The optimum sclerotial germination, noted after 24 h of incubation, varied depending on isolates and pH and occurred at pH 4-9. Mycelial growth was optimum in aerated plates with a significant isolates x aeration treatments interaction. Sclerotial initiation occurred at the 3rd day of incubation and mature sclerotia were observed after 6-9 days. Sclerotial development was very slow in completely sealed plates and dark sclerotia were produced only after 15 days of incubation. The highest sclerotial yields were noted in aerated plates. The highest dry weight of 100 sclerotia for Sr1 isolate was recorded in ½ sealed, no sealed and completely sealed plates, while for Sr2, it was noted in ½ and ⅔ sealed plates. For Sr3, the maximum dry weight of 100 sclerotia was recorded in ½, ⅔ and completely sealed plates. Germination of *S. rolfsii* sclerotia, after 24 h of incubation, did not vary significantly depending on aeration treatments and ranged from 90 to 100% for all isolates.

Keywords: Aeration, germination, mycelial growth, pH, sclerotial production, *Sclerotium rolfsii*

INTRODUCTION

Climate change represents one of the most serious challenges facing humanity in the 21st Century, inducing temperature rise, rainfall variation together with a frequency and an intensity of extreme weather events (Radhouane, 2013). It can affect both crop and livestock production systems in most regions (OECD, 2015). Moreover, changes in environmental conditions are expected to affect the development of plant pathogens and their survival rates and to modify host susceptibility leading to the emergence of new diseases (Anderson *et al.*, 2004; Elad and Pertot, 2014). Thus, new, emerging, and re-emerging plant diseases threatening various crops have been reported in Tunisia within the last years (Hibar *et al.*, 2007; Rhouma *et al.*, 2010; Gaaliche *et al.*, 2017; Jabnoun-Khiareddine *et al.*, 2018). Among those diseases, Southern blight, caused by *Sclerotium rolfsii* Sacc., has become a serious problem causing severe plant and yield losses in a wide range of crops. This pathogen can cause dry-seed yield loss of up to 53.4% on cowpea (Fery and Dukes, 2002) and about 5 to 20% of crop loss on peppermint (Singh and Singh, 2004). Moreover, it is considered as major threat to tomato and artichoke crops leading, respectively, to 10-45% (Banyal *et al.*, 2008) and 60% plant losses (McCarter, 1984).

S. rolfsii is a well-known polyphagous, ubiquitous, omnivorous and most destructive soilborne fungus infecting more than 500 plant species belonging to about 100 botanical families (Aycock, 1966; Anahosur, 2001). The

fungus penetrates, infects plant tissues at or near the soil surface and produces a considerable white cottony thread-like mycelium on basal plant parts (Punja, 1985). Dark brown lesions appear on infected stems just below the soil surface and/or on roots leading to drooping and wilting of leaves and gradual wilting of severely infected plants (Kator *et al.*, 2015). Sclerotia are produced on the mycelium and started out white to tan and become dark-brown to black at maturity. These sclerotia are the main over-wintering structures and represent the primary inoculum for disease development under favorable conditions (Punja, 1985).

This pathogen has been first reported in tropical and subtropical countries and has been recently detected in North Africa. In Tunisia, this pathogen was first observed on olive trees in 2001 (Boulila, 2001), on potato in 2006 (Daami-Remadi *et al.*, 2007) and on apple trees in 2012 (Kalai-Grami *et al.*, 2013). Mycelial growth and sclerotial production of this fungus are influenced by many abiotic factors including temperature, pH, and nutrients (Punja, 1985; Daami-Remadi *et al.*, 2010; Maurya *et al.*, 2010; Ayed *et al.*, 2018). In fact, in old investigations (Aycock, 1966; Sharma and Kaushal, 1979; Punja, 1985), pathogen mycelial growth was reported to be markedly less above pH 8. *S. rolfsii* is also considered as an air-loving organism and sandy soils are, thus, more suitable for its growth (Taubenhaus, 1919). However, some recent studies indicated that pH values varying from 5 to 6 are optimal for its mycelial growth (Sarker *et al.*, 2013).

The influence of some abiotic factors on *S. rolfsii* growth and survival was widely investigated in previous works where mycelial growth, sclerotial production and germination were separately considered in those studies (Hussain *et al.*, 2003; Kumar *et al.*, 2008; Maurya *et al.*, 2010; Basamma *et al.*, 2012; Sarker *et al.*, 2013). However, researches focused on all physiological parameters together and the relationship between environmental conditions, pathogen growth and persistence are still lacking. Therefore, the objective of this study was to provide a more detailed information on the effects of medium pH and aeration on mycelial growth, sclerotial production and germination of three selected Tunisian *S. rolfsii* isolates.

MATERIALS AND METHODS

Pathogen isolates: Three *S. rolfsii* isolates were used in the current investigation. Sr2 and Sr3 were recovered from rotted potato tubers and Sr1 was isolated from artichoke plants showing stem rot symptoms. Identification was based on previously published descriptions of *Sclerotium rolfsii* (Mordue, 1988) as indicated in Ayed *et al.* (2018). Pathogenicity was confirmed by inoculation of healthy potato (cv. Spunta) tubers with an agar plug colonized by the pathogen (Daami-Remadi *et al.*, 2010).

Isolates were held in the Phytopathology laboratory at the Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia. Prior to use, isolates were grown on Potato Dextrose Agar (PDA) medium for one week at 25 °C and in the dark.

Effect of pH on pathogen mycelial growth and on sclerotial production and germination

Effect on radial mycelial growth and sclerotial production: To examine the effect of pH on *S. rolfsii* mycelial growth, sterile double-strength PDA was mixed with an equal volume of buffer to obtain the desired medium concentration. The pH was adjusted to 4-7 and 7-9 with citrate phosphate (0.05 M citric acid, 0.1 M Na_2HPO_4-$7H_2O$) and Tris (hydroxymethyl) aminoethane (0.1 M Tris, 0.1M HCl) buffers, respectively (Gomori, 1955). Mycelial plugs (6 mm in diameter), cut from the margin of 7-day-old colonies, were placed in the center of Petri plates (90 mm in diameter) containing PDA medium modified at the tested pH value and amended with streptomycin sulfate (300 mg/L w/v). Inoculated plates were incubated in the dark at 30 °C for 3 days (Ayed *et al.*, 2018). The diameters of the resulting colonies were measured after 24, 48 and 72 h of inoculation and the radial growth rate (mm/day) was calculated.

The same cultures were further incubated for an additional 21 days. During this period, sclerotial production was monitored and determined at 3-day intervals (Maurya *et al.*, 2010). For the monitoring of sclerotial production, mature sclerotia were removed with a sharp scalpel, placed in fine mesh nylon bags and washed thoroughly with Sterile Distilled Water (SDW) to remove agar debris. They were counted and the average number of mature sclerotia produced per plate was determined. After counting, sclerotia were placed on pre-dried and weighed Whatman grade 1 qualitative filter papers and incubation at 70 °C for 48 h. The dry weight of 100 sclerotia per plate was determined.

For all parameters noted, ten replicate plates were used per individual treatment (per isolate and per pH tested).

Effect on mycelium dry weight: In order to study the effect of pH on mycelium dry weight, 15 ml of Potato Dextrose Broth (PDB) medium adjusted at the different pH values (4-9) were poured into 150 ml-flasks. The

medium was aseptically inoculated with mycelial plugs (6 mm in diameter) cut from 7-day-old *S. rolfsii* colonies. Flasks were incubated in the dark at 30 °C (Ayed *et al.*, 2018). After 5 days of incubation, the growing mycelial mats were filtered through Whatman grade 1 qualitative filter papers, washed thoroughly with SDW, dried at 60 °C for 3 days and weighed immediately on an analytical electrical balance. Ten replicate flasks were used per individual treatment (per isolate and per pH value).

Effect on sclerotial germination: Similarly sized and mature sclerotia (21-day-old) were used in this experiment. Ten sclerotia were placed onto Petri plates (90 mm in diameter) containing PDA medium adjusted at the different tested pH values (4-9) and incubated in the dark at 30 °C (Ayed *et al.*, 2018). Germination was determined after 24, 48 and 72 h of incubation by examining each sclerotium for any outgrowing hyphae when observed under a binocular microscope. A sclerotium was considered germinated when the outgrowing hyphae were equal to or greater than its diameter and the percentage of germinated sclerotia per plate was recorded. Ten replicate plates were used per individual treatment (Ayed *et al.*, 2018).

Effect of aeration on pathogen mycelial growth and on sclerotial production and germination: To assess the effect of aeration on the mycelial growth of *S. rolfsii* isolates as well as their sclerotial production and germination, PDA plates (90 mm in diameter) were inoculated each in their center with a mycelial plug (6 mm in diameter) cut from a 7-day-old colonies, and wrapped with parafilm in the following manners: no sealing, 1/2, ⅔, or complete sealing (Maurya *et al.*, 2010). Petri plates were incubated in the dark at 30 °C (Ayed *et al.*, 2018). The radial growth rate (mm/day), the sclerotial development, production and germination were evaluated as described above. All the treatments were replicated ten times.

Statistical analysis: ANOVA analyses were performed following a completely randomized factorial design where fungal treatment (*S. rolfsii* isolates) and tested factors (pH or aeration) were the two fixed factors. Ten replicates were used per individual treatment and means were separated using Fisher's protected LSD or Students Neuman Keuls tests (at $P \leq 0.05$). All the experiments were repeated twice with 10 replicates per individual treatment for each. For each bioassay, the mean data was presented in the current study. Statistical analyses were performed using SPSS software version 20.

RESULTS

Effect of pH on *S. rolfsii* growth and survival

Effect on radial mycelial growth: After 3 days of incubation at 30 °C on PDA medium, the mycelial growth of *S. rolfsii* varied significantly (at $P \leq 0.05$) depending on pH of the culture medium and pathogen isolates; moreover, a significant interaction was also noted between these two factors. In fact, as shown in Table 1, all *S. rolfsii* isolates were found able to grow over a range of pH from 4 to 9 (Figure 1). Optimal growth occurred at pH 6 for Sr2 and Sr3 isolates with an average growth of 28.93 and 28.23 mm/day, respectively, whereas Sr1 showed its highest mycelial extension at pH 6 and 7 with an average growth rate of 30.19 and 29.71 mm/day, respectively. However, it should be noted that *S. rolfsii* mycelial growth was significantly restricted (at $P \leq 0.05$) at pH 9 for all isolates (15.94-16.89 mm/day) compared to the other pH values (Table 1, Figure 1). For all pH values tested, the mycelial growth of Sr1 was significantly higher than that of Sr2 and Sr3.

Table 1. Effect of pH of Potato Dextrose Agar medium on the radial growth of three *Sclerotium rolfsii* isolates recorded after 3 days of incubation at 30 °C in the dark.

pH value	Radial growth (mm/day)			Mean radial growth per pH value (mm/day)[1]
	Sr1	Sr2	Sr3	
4	28.39 b	27.61 b	27.23 b	27.74 cd
5	28.39 b	28.08 b	27.36 b	27.94 bc
6	30.19 a	28.93 a	28.23 a	29.12 a
7	29.71 a	27.84 b	27.33 b	28.29 b
8	28.06 b	27.89 b	26.46 c	27.47 d
9	16.4 c	15.94 c	16.89 d	16.41 e
Mean radial growth per isolate (mm/day)[2]	26.86 a	26.05 b	25.58 c	-

[1] Mean radial growth per pH value for the three isolates combined, [2] Mean radial growth per isolate for all pH values combined, * LSD (pH × *S. rolfsii* isolates) = 0.53 mm/day at $P \leq 0.05$, * For each isolate and each mean radial growth (per pH value or per

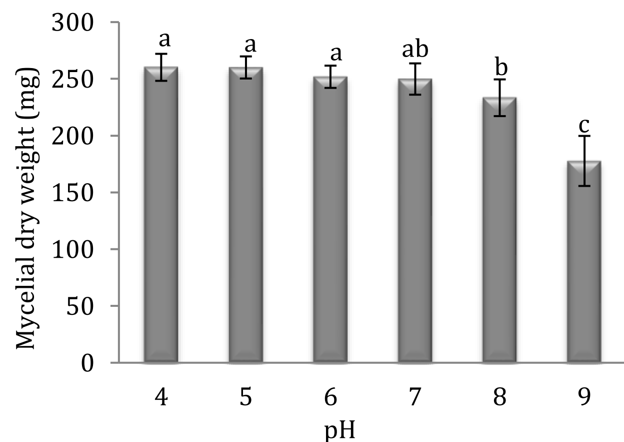
isolate), values followed by the same letter are not significantly different according to Duncan Multiple Range test (at $P \leq 0.05$).

Figure 1. Colonies of *Sclerotium rolfsii* isolate Sr1 formed after 3 days of incubation at 30 °C on PDA medium adjusted at different pH values.

Effect on mycelium dry weight: All tested *S. rolfsii* isolates were shown able to grow on pH-adjusted PDB medium, at pH range 4-9, and their mycelial dry weights varied significantly depending on pH values. As no significant interaction was noted between these two factors, data was presented and commented considering each factor separately as given in Figure 2 (A, B). In fact, for all *S. rolfsii* isolates combined (Figure 2A), fungal dry biomass was optimum at pH values ranging from 4 to 7 with an average mycelial dry weight varying between 249.87 and 260.17 mg, respectively. However, the lowest records were noted at pH 9. For all pH values combined (Figure 2B), the isolate Sr3 showed the highest mycelial dry weight (263.58 mg) followed by Sr2 (249.26 mg) and Sr1 (203.68 mg).

Effect on sclerotial formation and production: As shown in Table 2, pathogen sclerotial formation was affected by pH values. In fact, sclerotial initiation of all isolates started on the 3rd day of incubation from the hyphal branching and anastomosis, followed by hyphal aggregation and network formation at all pH values tested (Figure 1). The network developed to so-called initial and grew to white immature sclerotium on the 6-9th days of incubation at pH 4-8 and after the 9th day for all isolates at pH 9. At pH range 4-6, dark sclerotia were formed quickly after 6 to 12 days of incubation. Nevertheless, at pH 7-9, they were observed on the 12th day (Table 2). The number of mature sclerotia of *S. rolfsii* per plate, noted after 21 days of incubation at 30°C on PDA medium, varied significantly depending on tested isolates and pH values; a significant interaction was also noted between these both fixed factors ($P \leq 0.05$).

As presented in Table 3, all *S. rolfsii* isolates were shown able to produce dark sclerotia when grown on PDA medium adjusted at the different pH values. Sr1 and Sr3 isolates showed optimal sclerotial production at pH 5 which was estimated at 193.2 and 150 sclerotia/plate, respectively. For Sr2, sclerotial production was significantly similar at pH 4 and pH 5 with an average of 137.2 and 168.6 mature sclerotia/plate, respectively. The lowest sclerotial production was noted on cultures grown on PDA adjusted at pH 9 for Sr1, Sr2 and Sr3 isolates with an average of sclerotial production estimated at 63.6, 59.4, and 31.8 sclerotia/plate, respectively (Table 3). For all pH values combined (pooled data of all pH values tested), Sr1 and Sr2 isolates produced more mature sclerotia than Sr3 at the same incubation duration.



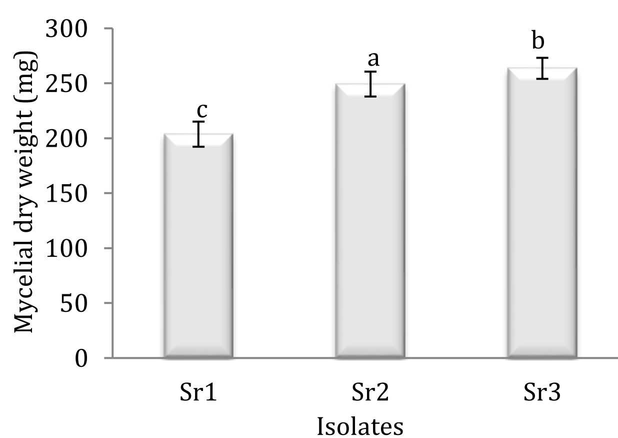


Figure 2. Effect of pH of Potato Dextrose Broth medium on the mycelial dry weight of three *Sclerotium rolfsii* isolates recorded after 5 days of incubation at 30 °C in the dark.
Bars sharing the same letter are not significantly different according to Duncan Multiple Range test (at $P \leq 0.05$).

Table 2. Effect of pH of Potato Dextrose Agar medium on the sclerotial development of three *Sclerotium rolfsii* isolates noted after 21 days of incubation at 30 °C in the dark.

pH value	Isolates	Days after incubation		
		First initials	White sclerotia	Dark mature sclerotia
4	Sr1	3	6	9
	Sr2	3	6	9
	Sr3	3	-	6
5	Sr1	3	6	9
	Sr2	3	6	12
	Sr3	3	6	9
6	Sr1	3	6	9
	Sr2	3	6	9
	Sr3	3	9	12
7	Sr1	3	6	12
	Sr2	3	9	12
	Sr3	3	6	12
8	Sr1	3	6	12
	Sr2	3	6	12
	Sr3	3	6	12
9	Sr1	3	9	12
	Sr2	3	9	12
	Sr3	3	9	12

Table 3. Effect of pH of Potato Dextrose Agar medium on the number of sclerotia produced by three *Sclerotium rolfsii* isolates after 21 days of incubation at 30°C in the dark.

pH value	Number of sclerotia per plate			Mean number of sclerotia per pH[1]
	Sr1	Sr2	Sr3	
4	106.6 c	137.2 ab	107.6 b	117.13 c
5	193.2 a	168.6 a	150 a	170.6 a
6	150 b	150 ab	118.2 b	139.4 b
7	142.2 bc	110.8 b	61.4 c	104.8 cd
8	133.2 bc	107.2 b	39.6 d	93.33 d
9	63.6 d	59.4 c	31.8 d	51.6 e
Mean number of sclerotia per isolate[2]	131.47 a	122.2 a	84.77 b	-

[1] Mean number of sclerotia per pH value for the three isolates combined, [2] Mean number of sclerotia per isolate for all pH values combined, *LSD (pH × *S. rolfsii* isolates) = 25.806 sclerotia at $P \leq 0.05$, *For each isolate and each mean number of sclerotia (per pH value or per isolate), values followed by the same letter are not significantly different according to Duncan Multiple Range test (at $P \leq 0.05$).

The dry weight of 100 sclerotia, noted after 21 days of incubation on PDA medium, was significantly (at $P \leq 0.05$) affected by pH values and isolates tested. A significant interaction was also noted between these two factors. In fact, the highest weight of 100 sclerotia was noted on Sr1 cultures grown on PDA adjusted at pH 6, those of Sr2 cultured at pH 4-7 and those of Sr3 formed at pH 5-6. However, at pH 9, the dry weight of 100 sclerotia was significantly the lowest for Sr1, Sr2 and Sr3 isolates and was estimated at 29.63, 23.21, and 33.37 mg, respectively (Table 4). For all pH values combined, Sr3 isolate showed the highest dry weight of 100 sclerotia as compared to the two others (Table 4).

Effect on sclerotial germination: The germination of *S. rolfsii* sclerotia, recorded after 24 h of incubation at 30 °C on PDA medium, varied significantly (at $P \leq 0.05$) depending on tested pH values and pathogen isolates and on their interactions (Table 5). In fact, the optimum pH range for sclerotial germination was determined as 4-9 for Sr2 isolate. However, the highest percentage of sclerotial germination was recorded at pH values 5, 7, and 9 for Sr1 isolate and at pH 5, 7, 8, and 9 for Sr3. Moreover, a total germination of

sclerotia of all isolates was noted after 72 h of incubation (data not shown). For all pH values combined, the highest sclerotial germination (88.67%) was recorded for Sr3 isolate, followed by Sr2 and Sr1 (77 and 67.33%, respectively).

Table 4. Effect of pH of Potato Dextrose Agar medium on the dry weight of 100 sclerotia produced by three *Sclerotium rolfsii* isolates after 21 days of incubation at 30 °C in the dark.

pH value	Dry weight of 100 sclerotia (mg)			Mean dry weight of 100 sclerotia per pH (mg)[1]
	Sr1	Sr2	Sr3	
4	38.37 b	30.61 ab	36.55 de	35.18 bc
5	39.17 b	31.85 ab	54.86 a	41.96 a
6	45.34 a	34.9 a	50.56 ab	43.6 a
7	34.93 bc	32.67 ab	46.7 bc	38.1 b
8	29.37 c	28.49 bc	42.57 cd	33.48 c
9	29.63 c	23.21 c	33.37 e	28.74 d
Mean dry weight of 100 sclerotia per isolate (mg)[2]	36.13 b	30.29 c	44.10 a	-

[1] Mean dry weight of 100 sclerotia per pH value for the three isolates combined, [2] Mean dry weight of 100 sclerotia per isolate for all pH values combined, *LSD (pH × *S. rolfsii* isolates) = 4.89 mg at $P \leq 0.05$, *For each isolate and each mean dry weight of 100 sclerotia (per pH value or per isolate), values followed by the same letter are not significantly different according to Duncan Multiple Range test (at $P \leq 0.05$).

Table 5. Effect of pH of Potato Dextrose Agar medium on the sclerotial germination of three *Sclerotium rolfsii* isolates noted after 24 h of incubation at 30 °C in the dark.

pH value	Sclerotial germination (%)			Mean sclerotial germination per pH (%)[1]
	Sr1	Sr2	Sr3	
4	54 c	78 a	78 c	70 d
5	70 ab	84 a	88 abc	80.67 bc
6	58 bc	76 a	86 bc	73.33 cd
7	72 ab	70 a	98 a	80 bc
8	66 bc	76 a	88 abc	76.67 bcd
9	84 a	78 a	94 ab	85.33 a
Mean sclerotial germination per isolate (%)[2]	67.33 c	77 b	88.67 a	-

[1] Mean sclerotial germination per pH value for the three isolates combined, [2] Mean sclerotial germination per isolate for all pH values combined, *LSD (pH × *S. rolfsii* isolates) = 10.404% at $P \leq 0.05$, *For each isolate and each mean of sclerotial germination (per pH value or per isolate), values followed by the same letter are not significantly different according to Duncan Multiple Range test (at $P \leq 0.05$).

Effect of aeration on *S. rolfsii* growth and survival

Effect on radial mycelial growth: Mycelial growth of *S. rolfsii* colonies, formed after 3 days of incubation on PDA medium, was significantly (at $P \leq 0.05$) influenced by aeration treatments and fungus isolates. Moreover, a significant interaction was also noted between these two factors. As indicated in table 6, *S. rolfsii* isolates were shown able to grow under all aeration conditions tested. The optimal radial mycelial growth of *S. rolfsii* of PDA medium occurred in unsealed, ½ sealed and ⅔ sealed plates for Sr1, whereas Sr2 and Sr3 exhibited their highest mycelial growth in ⅔ sealed plates, and in ½ and ⅔ sealed plates, respectively. For all aeration treatments combined, the highest mycelial growth was noted on Sr1 cultures.

Effect on sclerotial formation and production: As indicated in table 7, tested aeration treatments affected sclerotial formation. In fact, white sclerotia were formed at the 3rd day of incubation in ½, ⅔ and no sealed plates. They appeared as whitish, tiny and pinhead-like structures, whereas mature sclerotia became brownish at the 6-9th day of incubation. However, sclerotial development was very slow in completely sealed plates where the formation of dark brown sclerotia was noted only after 15 days of incubation (Table 7). After 21 days of incubation, *S. rolfsii* sclerotial production was significantly affected by the tested aeration treatments, pathogen isolates and their interactions. In fact, as indicated in table 8, the highest numbers of sclerotia were noted Sr1 cultures grown in ½, ⅔ and no sealed plates (445.8, 465.4, and 455.4 mature sclerotia/plate, respectively), while those of Sr2 and Sr3 formed significantly more sclerotia in unsealed plates with an average of 405.4 and 323.6 mature sclerotia/plate,

respectively. However, all *S. rolfsii* isolates produced very few mature sclerotia in completely sealed plates. For all aeration treatments combined, Sr1 isolate produced significantly more sclerotia than Sr2 and Sr3 (Table 8).

Table 6. Effect of aeration treatments on the radial mycelial growth of three *Sclerotium rolfsii* isolates recorded on PDA medium after 3 days of incubation at 30 °C in the dark.

Aeration treatment	Radial growth (mm/day)			Mean radial growth per aeration treatment (mm/day)[1]
	Sr1	Sr2	Sr3	
No sealing	23.83 a	22.32 b	22.09 b	22.75 bc
½ sealing	23.77 a	22.38 b	23.07 a	23.07 b
⅔ sealing	23.86 a	24.17 a	23.14 a	23.72 a
Complete sealing	22.68 b	22.57 b	21.89 b	22.38 c
Mean radial growth per isolate (mm/day)[2]	23.53 a	22.86 b	22.55 b	-

[1] Mean radial growth per aeration treatment for the three isolates combined, [2] Mean radial growth per isolate for all aeration treatments combined, *LSD (Aeration treatments × *S. rolfsii* isolates) = 0.54 mm/day at $P \leq 0.05$, *For each isolate and each mean radial growth (per aeration treatment or per isolate), values followed by the same letter are not significantly different according to Duncan Multiple Range test (at $P \leq 0.05$).

Table 7. Effect of aeration treatments on the sclerotial development of three *Sclerotium rolfsii* isolates on PDA medium noted after 21 days of incubation at 30 °C in the dark.

Aeration treatment	Isolate	Days after incubation		
		First initials	White sclerotia	Dark mature sclerotia
No sealing	Sr1	-	3	6
	Sr2	-	3	9
	Sr3	-	3	9
½ sealing	Sr1	-	3	6
	Sr2	-	3	9
	Sr3	-	3	9
⅔ sealing	Sr1	-	3	6
	Sr2	-	3	9
	Sr3	3	6	9
Complete sealing	Sr1	3	9	15
	Sr2	3	9	15
	Sr3	3	9	12

Table 8. Effect of aeration treatments on the number of sclerotia produced by three *Sclerotium rolfsii* isolates on PDA medium after 21 days of incubation at 30 °C in the dark.

Aeration treatment	Number of sclerotia per plate			Mean number of sclerotia per aeration treatment[1]
	Sr1	Sr2	Sr3	
No sealing	455.4 a	405.4 a	323.6 a	394.8 a
½ sealing	445.8 a	261.8 b	251.6 b	319.73 b
⅔ sealing	465.4 a	258.4 b	240.8 b	321.53 b
Complete sealing	147.8 b	212.61 c	73 c	144.47 c
Mean number of sclerotia per isolate[2]	378.6 a	284.55 b	222.25 c	-

[1] Mean number of sclerotia per aeration treatment for the three isolates combined, [2] Mean number of sclerotia per isolate for all aeration treatments combined, *LSD (Aeration treatments × *S. rolfsii* isolates) = 33.92 sclerotia at $P \leq 0.05$, *For each isolate and each mean number of sclerotia (per aeration treatment or per isolate), values followed by the same letter are not significantly different according to Duncan Multiple Range test (at $P \leq 0.05$).

The dry weight of 100 sclerotia, formed after 21 days of incubation on PDA at 30 °C, varied significantly (at $P \leq 0.05$) depending on isolates used and tested aeration treatments; sa significant interaction was also noted between both fixed factors. In fact, Table 9 shows that, for Sr1 isolate, the highest dry weight of 100 sclerotia was recorded in ½ sealed, no sealed and completely sealed plates. For Sr2, the highest records were noted in ½ and ⅔ sealed plates. For Sr3, the greatest dry weight of 100 sclerotia was recorded in ½, ⅔ and completely sealed plates. For all aeration treatments

combined, the highest dry weight of 100 sclerotia was noted on Sr3 cultures (Table 9).

Effect on sclerotial germination: Germination of *S. rolfsii* sclerotia, noted after 24 h of incubation at 30 °C, did not vary significantly depending on isolates and aeration treatments tested. Furthermore, no significant interaction was recorded between these both fixed factors. The noted average of sclerotial germination ranged between 90 and 100% for all treatments and all *S. rolfsii* isolates (data not shown).

Table 9. Effect of aeration treatments on the dry weight of 100 sclerotia produced by three *Sclerotium rolfsii* isolates on PDA medium after 21 days of incubation at 30 °C in the dark.

Aeration treatment	Dry weight of 100 sclerotia (mg)			Mean dry weight of 100 sclerotia per aeration treatment (mg)[1]
	Sr1	Sr2	Sr3	
No sealing	40.9 ab	44.06 b	58.43 b	47.8 a
½ sealing	42.31 ab	51.1 ab	63.46 ab	52.29 a
⅔ sealing	36.11 c	54.22 a	67.72 ab	52.68 a
Complete sealing	47 a	36.08 c	74.37 a	52.48 a
Mean dry weight of 100 sclerotia per isolate (mg)[2]	41.58 c	46.36 b	65.99 a	-

[1] Mean dry weight of 100 sclerotia per aeration treatment for the three isolates combined, [2] Mean dry weight of 100 sclerotia per isolate for all aeration treatments combined, *LSD (Aeration treatments × *S. rolfsii* isolates) = 7.199 mg at $P \leq 0.05$, *For each isolate and each mean of dry weight of 100 sclerotia (per aeration treatment or per isolate), values followed by the same letter are not significantly different according to Duncan Multiple Range test (at $P \leq 0.05$).

DISCUSSION

S. rolfsii is a polyphagous soilborne pathogen attacking extensive plant hosts in the world. Some studies were focused on its morphology, physiology and pathogenicity and others were more concentrated on the effect of diverse environmental factors on its growth and survival. As soil pH is one of these important factors, the present study investigated, in part, the effect of pH of medium culture on mycelial growth, sclerotial formation, production and germination of three Tunisian isolates of *S. rolfsii*.

In the current study, all three *S. rolfsii* isolates were shown able to grow over a range of pH values ranging from 4 to 9 as indicated in previous studies (Deacan, 1984; Hussain *et al.*, 2003). The response of tested isolates was slightly different as optimal growth occurred at pH 6 for Sr2 and Sr3 and at pH 6 and 7 for Sr1. However, for all isolates, mycelial growth was significantly restricted at pH 9. These results are in agreement with those of Kumar *et al.* (2008) and Zape *et al.* (2013), reporting optimal mycelial growth at pH 6-7, and those of Sarker *et al.* (2013) who defined this optimum at pH range 5-6. Moreover, the restriction of pathogen growth at higher pH values (pH 9) confirmed previous studies reporting that *S. rolfsii* mycelial growth was markedly less above pH 8 (Aycock, 1966; Sharma and Kaushal, 1979; Punja, 1985).

In the same sense, dry mycelial growth was optimum at pH values ranging between 4 and 7 but lowest at pH 9. This finding is in agreement with Basamma *et al.* (2012) study reporting that the maximum dry mycelial weight of the fungus was noticed at pH 5, 4 and 6. The effect of pH on fungal dry biomass was also examined in another study where the exposure *S. rolfsii* to pH 5 produced maximum mycelial dry weight as compared to pH 6 (Muthukumar and Venkatesh, 2013b). Furthermore, in another work, vegetative growth of *S. rolfsii* was optimum at pH 3 and was greatly reduced below 2.1 and above 5.2 (Abeygunawardena and Wood, 1957).

Sclerotial initiation started on the 3rd day of incubation at all pH values tested and white immature sclerotia were observed on the 6-9th days and the 9th day of incubation at pH 4-8 and pH 9, respectively. Therefore, mature sclerotia were formed early after 6 to 12 days at pH range 4-6 and on the 12th day at pH 7-9. After 21 days of incubation at 30 °C, all isolates showed optimal sclerotial production at pH 5. A similar sclerotial yield was recorded at pH 4 for Sr3 isolate. Nevertheless, the lowest sclerotial density was observed at pH 9. This finding confirmed a previous study (Sarker *et al.*, 2013) reporting that pH 5 was more favourable to the sclerotial formation of *S. rolfsii* and less at pH 5.5. Furthermore, Abeygunawardena and Wood (1957) mentioned that the highest growth and sclerotial production occurred under the pH range of 2.8-7.8. In another study, pH values varying between 5.5 and 7.5 are optimal for sclerotial

formation where the highest number of sclerotia was noted on cultures grown at pH 7 (Zape *et al.*, 2013). However, sclerotial production was considered as excellent at pH levels 6, 6.5, and 7, good at pH 5 and 5.5, fair at pH 4.5, 7.5, and 8, very poor at pH 8.5, and nil at pH 9 (Kumar *et al.*, 2008).

In the current study, the highest dry weight of 100 sclerotia was recorded at pH 6 for Sr1, at pH 4-7 for Sr2 and at pH 5-6 for Sr3, but at pH 9, sclerotial weight was the lowest for all isolates. This finding confirmed a previous study reporting that dry weight of *S. rolfsii* sclerotia was similar at pH 4.5, 5, 6, and 6.5 with the lowest dry weight noted at pH 7.5 (Sarker *et al.*, 2013).

Furthermore, the optimum sclerotial germination, noted after 24 h of incubation at 30 °C on PDA medium, was noted at pH range 4-9 for Sr2 isolate, at pH 5, 7, and 9 for Sr1 and at pH 5, 7, 8, and 9 for Sr3. For all isolates, sclerotia germinated at 100% after 72 h of incubation. Nevertheless, previous studies reported that *S. rolfsii* sclerotia did not germinate above pH 7 on agar media, but under field conditions (in soil), it occurred at pH values as high as 8.7 (Punja, 1982). Another study also indicated that sclerotial germination for two *S. rolfsii* isolates was greater in acid soils than in alkaline ones (Shim and Starr, 1997).

As well as for pH, the effect of aeration treatments on *S. rolfsii* growth and survival was also investigated. In the present study, the suitability of *in vitro* aeration for the fungus growth at 30 °C was evaluated. In fact, optimal mycelial growth occurred in unsealed, ½ and ⅔ sealed plates for Sr1, whereas Sr2 and Sr3 showed their maximum mycelial extension in ⅔ sealed and in ½ and ⅔ sealed plates, respectively. These results are in agreement with those of Punja (1985) who demonstrated that *S. rolfsii* mycelial extension is limited by low oxygen concentrations to a greater extent on soil than on agar. Therefore, the pathogen was considered as an air-loving organism with a more favourable development in sandy soils (Taubenhaus, 1919).

Sclerotial initiation was observed at the 3rd day of incubation as expressed by the production of white sclerotia in ½, ⅔ and no sealed plates, whereas mature sclerotia became brownish at the 6-9th days of incubation. Nevertheless, sclerotial development was found to be very slow in completely sealed plates where dark brown sclerotia were observed only after 15 days of incubation. These results are in agreement with those of Muthukumar and Venkatesh (2013b) study reporting that sclerotial formation is directly influenced by aeration as completely sealed plates failed to produce sclerotia after 12 days of incubation. Formation of sclerotial initials in cultures was reported to be inhibited by oxygen concentrations below 15% or carbon dioxide above 1% or 4% (Griffin and Nair, 1968; Kritzman *et al.*, 1977). Therefore, a proper aeration is essential for the development of sclerotia. It is already known that sclerotial development and severity of pathogenesis were highest in sandy soils which are more aerated compared to heavy soils as less aeration hinders the formation of abundant sclerotia (Maurya *et al.*, 2010).

As for mycelial growth and sclerotial formation, the aeration was also evaluated for its effect on sclerotial production. In fact, the highest sclerotial yields were noted on Sr1 cultures grown in ½, ⅔ and no sealed plates, while Sr2 and Sr3 isolates formed significantly more sclerotia in unsealed plates. However, all *S. rolfsii* isolates produced very few mature sclerotia in completely sealed plates. These findings are in accordance with those of Maurya *et al.* (2010) and those of Muthukumar and Venkatesh (2013a) who did not note any significant difference in the number of sclerotia between aeration treatments but only when compared to completely sealed plates. However, in this study, the highest dry weight of 100 sclerotia for Sr1 isolate was recorded in ½ sealed, no sealed and completely sealed plates, while that of the Sr2 isolate was noted in ½ and ⅔ sealed plates. For Sr3 isolate, the maximum dry weight of 100 sclerotia was recorded in ½, ⅔ and completely sealed plates. Nevertheless, other researchers reported that the weight of 100 sclerotia was higher in unsealed plates (Maurya *et al.*, 2010).

In the current study, sclerotial germination of *S. rolfsii* did not vary significantly depending on aeration treatments and pathogen isolates. The average sclerotial germination, noted after 24 h, ranged from 90 to 100% for all isolates. However, in another study, germination of sclerotia was considerably increased in aerated soils (Abeygunawardena and Wood, 1957).

CONCLUSION

This study has identified some factors that have an effect on the physiology of Tunisian *S. rolfsii* isolates. The investigation of the trends for mycelial growth, sclerotial production and germination at different pH values and aeration treatments can provide important information about the effect of environmental conditions on the different critical stages of pathogen life cycle. Therefore, our study has contributed to the understanding of the

ecology and physiology of *S. rolfsii* isolates under controlled conditions (pH and aeration). However, further investigations into the effect of other environmental factors on pathogen development under field conditions would provide a greater understanding of the biology of local *S. rolfsii* isolates.

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