Scarification and Environmental Factors that Enhance Carpogenic Germination of Sclerotia of *Sclerotinia sclerotiorum*

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ABSTRACT

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Ascospores of *Sclerotinia sclerotiorum* are the primary source of inoculum for disease epidemics in many economically important crops. Mass production of ascospores under laboratory conditions is required to prepare inoculum for use in selection of genotypes with resistance against *Sclerotinia* diseases. A study was undertaken, first, to investigate the effect on carpogenic germination of scarifying sclerotia from two *S. sclerotiorum* isolates taken from canola (*Brassica napus*) and, second, to identify environmental factors that enhance carpogenic germination. Seven different environmental treatments were applied to scarified and unscarified sclerotia: (i) sterilized distilled water for 4 months at 15°C, (ii) aerated water for 4 months at 4°C, (iii) constant rinsing with tap water for 8 weeks at 4°C, (iv) cold-conditioning for 4 weeks at 4°C and subsequent transfer into moist unsterilized compost at 15°C, (v) incubation in sterilized river sand at 15°C, (vi) air drying for 2 weeks followed by subsequent transfer into sterilized moist river sand at 15°C, or (vii) placed into 0.5% water agar and incubated at 15°C. Carpogenic germination of scarified sclerotia was significantly greater ($P < 0.05$) than for unscarified sclerotia. There was significant interaction ($P < 0.001$) between scarification and the different environmental treatments in relation to the carpogenic germination. Carpogenic germination of scarified sclerotia was enhanced by incubation of sclerotia in compost or in sterilized river sand. Further, overall carpogenic germination of both scarified and unscarified sclerotia occurred to the greatest extent when sclerotia of either of the two isolates were subjected to constant rinsing with tap water. We believe this to be the first report of both the enhanced carpogenic germination by scarification in *S. sclerotiorum* and the environmental factors we report that enhance carpogenic germination of scarified sclerotia. The progression of carpogenic germination in all the environmental treatments was also monitored as a part of this study across the two consecutive years for the same two isolates. The majority of sclerotia of both isolates germinated between the months of June and September in both years, a period which coincides with the main part of the cropping season when *Sclerotinia* stem rot is normally observed in rainfed canola in Western Australia. These data suggested the existence of a seasonal rhythm-like pattern in relation to the carpogenic germination of this pathogen.

Sclerotinia sclerotiorum is an important pathogen of soybean (*Glycine max*), sunflower (*Helianthus annuus*), and canola (*Brassica napus* L.) in North America, Europe, Australia, India, and China (6,27,30,32,33,41,47). Although sclerotia of this pathogen can germinate to produce infective hyphae, myceliogenic germination generally plays only a minor role in disease epidemiology (2). An exception is *Sclerotinia* stem rot of oilseed *Brassica* crops in India, where myceliogenic germination is considered to be important (47). More commonly, sclerotia germinate car-

pogenically, producing apothecia that release windborne ascospores (33), which are generally considered the primary inoculum source for *Sclerotinia* disease epidemics on many economically important crops (1,26,38,41,49,59). For this reason, artificial production of ascospore inoculum is of prime importance, both in selecting genotypes with resistance against *S. sclerotiorum* (18) and for studies to better understand aspects of the epidemiology of this disease (1).

Sclerotia of most isolates of *S. sclerotiorum* are known to possess constitutive and exogenous dormancy that must be overcome before they can be germinated carpogenically to produce ascospores (15,40). The roles of temperature (11,12,28,36,43,49), soil moisture (35,39,44,60) and aeration, and burial depth (40,60) as conditioning treatments to induce carpogenic germination or to break the dormancy of sclerotia have been well docu-

mented. The sclerotial rind is also known to impose constitutive dormancy, even though it is considered to be permeable to water, gases, and some molecules such as carbohydrates (15). Sclerotia of both *S. sclerotiorum* and *S. trifoliorum* are reported to germinate more rapidly when the sclerotial rind is broken or damaged (31), even though rind is known to regenerate (15,61). There have also been a few studies, for other pathogens, on this aspect. For example, Scott (46) observed that artificial abrasion of the rind was required for immediate germination of sclerotia of *Sclerotium cepivorum*, while Boswell (7) and Chet (9) reported increased germination of sclerotia of *S. rolfsii* following mechanical damage of the rind. These studies suggested that, in an undamaged state, the sclerotial rind may play a role in inhibition of sclerotial germination. However, most published studies on the role of damage to sclerotial rind relate to myceliogenic germination, and the role of damage to the sclerotial rind in stimulating carpogenic germination of *Sclerotinia sclerotiorum* is not clear. Further, the effect of various environmental treatments on scarified sclerotia to stimulate the carpogenic germination of scarified sclerotia has not been investigated to date.

A great deal of work has been reported on environmental factors such as temperature, light, moisture, and burial depth required to stimulate carpogenic germination of sclerotia of *S. sclerotiorum* (57). However, findings frequently differ among different studies and can even be contradictory (40,60). For example, although there is general agreement that favorable apothecial production occurs at 10 to 20°C (57), specific temperatures of 7 to 25°C are reported by others (40). Moreover, reports on specific temperature requirements for preconditioning of sclerotia to stimulate carpogenic germination are also incongruous among different studies; for example, 0°C (28), 3°C (44), 4°C (12), 5°C (39), 7°C (14), 8°C (18), and 10°C (25). In contrast, some isolates of *S. sclerotiorum* have also been reported to germinate without any chilling requirements (4,42). The length of the preconditioning period suggested to stimulate carpogenic germination is also highly variable, ranging from 7 to 10 days to 8 weeks (1,34,40). Such differences in germination behavior of the scler-

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rotia of the *S. sclerotiorum* isolates could partially be due to the geographical origin (25) or the temperature conditions under which sclerotia were formed (24).

Moisture is also considered to be a vital requirement for carpogenic germination, and even a small osmotic stress can inhibit apothecial formation (2,22,52,58,60). Additionally, light is also reported to be essential for the development of mature apothecia but not for the stipe formation (50,54). The substrate used for production of sclerotia (49,57) and burial depth (60) can also influence apothecial production of *S. sclerotiorum*. Because a large number of different variables can influence carpogenic germination, it is not surprising that reproducibility of different individual treatments or methods used to induce apothecial production in different locations has often been reported to be very poor (18,21,36,49). Such variability makes it particularly difficult to relate published findings on carpogenic germination of sclerotia of *S. sclerotiorum* to particular geographical locations, such as the Mediterranean environment of southwest Western Australia.

Inconsistencies in the results reported to date in relation to the carpogenic germination of *S. sclerotiorum* prompted us to evaluate the effects of scarification and environmental factors on carpogenic germination of *S. sclerotiorum* and to test for the presence of any seasonal patterns associated with their germination. The objectives of our study were first, to determine whether carpogenic germination of the sclerotia of *S. sclerotiorum* can be enhanced by scarification; second, to evaluate whether certain environmental factors can enhance carpogenic germination; and, third, to determine the influence of season or time of the year upon induction of carpogenic germination.

MATERIALS AND METHODS

***S. sclerotiorum* isolates and production of sclerotia.** Two isolates of *S. sclerotiorum* (MBRS-1 and WW-3) were collected from the Mount Barker (MBRS-1) and Walkaway (WW-3) regions of Western Australia (WA) in 2004, in each instance from infected tissue of *B. napus* and from a site where there was significant disease on a canola crop (29). Initial isolation and sterilization of sclerotia were carried out as described by Clarkson et al. (12) and Sansford and Coley-Smith (45). Briefly, a single sclerotium each of *S. sclerotiorum* isolates MBRS-1 and WW-3 was surface sterilized in 50% (vol/vol) sodium hypochlorite and 70% ethanol for 4 min followed by two washes in sterilized distilled water for 1 min. The sclerotium was then cut in half and placed on potato dextrose agar (PDA). Sclerotia obtained from this culture were removed and air dried.

Bulk production of sclerotia of the two isolates (MBRS-1 and WW-3) was under-

taken by growing each isolate on sterilized wheat grain. We used wheat media for mass production of the sclerotia of the two isolates, primarily to ensure uniformity among all the treatments and to mitigate any substrate influence on the carpogenic germination as reported previously (49). Erlenmeyer flasks (250 ml) containing 25 g of wheat grain and 60 ml of distilled water (autoclaved three times at 120°C for 20 min) were inoculated by using four agar plugs from the edge of 3-day-old *S. sclerotiorum* colonies (12,36). Flasks were placed in incubator at 20°C for 4 weeks to obtain mature sclerotia. During this period, these flasks were shaken three times a week to even the colonization of wheat grains. After 4 weeks, sclerotia were harvested by sieving under running water to obtain sclerotia of a uniform size of 2 to 5 mm in diameter and to remove any remnants of colonized and uncolonized wheat grains.

Experimental design. A three-way asymmetric factorial design (i.e., two by two by seven) was used for this study. For each isolate of *S. sclerotiorum*, sclerotia were separated into two groups: scarified and unscarified sclerotia (1,400 sclerotia/isolate and 700 sclerotia/group). Each isolate and sclerotial treatment group was then divided into seven separate lots (each of 100 sclerotia) for each treatment applied in relation to carpogenic germination. Each lot was further divided into four units to obtain four replications with 25 sclerotia/replication/treatment. Each replication unit (25 sclerotia) was then placed in a transparent plastic container (650 ml; Bunzl Australasia) with airtight lids containing either river sand, water, or unsterilized organic compost to 2 cm in depth according to each treatment tested for its ability to stimulate carpogenic germination. However, round plastic autoclavable containers (6.5 by 7.0 cm, diameter by height) with airtight lids were used where sclerotia were placed in water agar. Sclerotia that were composed of each replication (together for scarified and unscarified sclerotia) were taken from each flask separately for each treatment. All plastic containers were then randomly placed in a controlled environment incubator (Thermoline, Australia) maintained at 15°C with a 12-h light-and-dark cycle as described by Clarkson et al. (12). At saturation, gravimetric moisture content of the sand and compost was 22.1 and 64.5%, respectively. Water potential of the compost was maintained at -10 kPa by first measuring the initial weight of every container containing the appropriate amount of water. Subsequently, water was added each week to maintain the weight of each container at the original weight (12). However, containers with sand were watered up to saturation (0.1 kPa) and water in these containers (with holes at the bottom) was freely drained, maintaining moisture content at near to field capacity. All the plastic

containers were randomly redistributed within the incubator every month in order to ensure uniform exposure to light and temperature. No treatment showed any sign of carpogenic germination (i.e., no initiation of apothecial stipe) for the first 4 months of incubation in the controlled environment cabinet (except for sclerotia in 0.5% water agar, where a few sclerotia of WW-3 carpogenically germinated). Plastic containers were then transferred onto the benches of an air-conditioned laboratory where the maximum temperature was maintained between 23 and 25°C. Redistribution of the containers on the lab benches was undertaken weekly to ensure uniform light and temperature exposure. Sclerotia were monitored for stipe formation over 2 years (i.e., 2006–07 and 2007–08) on a weekly basis.

The experimental design comprised sclerotia subjected first to scarification (or maintained as unscarified sclerotia), followed by conditioning with seven different environmental factors (for both scarified and unscarified sclerotia). The description of all seven treatments and their respective temperature regimes used are summarized in Table 1. The procedure for scarification and other details of the different environmental factors tested, by application of seven treatments in this study, are described below.

Scarification. Scarification of the two isolates was performed using sandpaper (medium-course, grit size no. 80). Sclerotia were placed between two 8-by-8-cm sandpaper sheets and were uniformly rubbed by hand for approximately 30 s, by which time the light, whitish medulla layers of sclerotia were just visible. Scarification of sclerotia incubated on water agar was done using autoclaved sandpaper (120°C for 20 min) in a laminar flow cabinet.

Treatments. *Treatment 1.* Sclerotia were incubated in sterilized distilled water in a plastic container containing 150 ml of sterilized distilled water (SDW) at 15°C as described by Grogan and Abawi (22) and Ekins et al. (20). The water in plastic containers was changed every week. Because no sclerotial germination was recorded for 4 months (January to April 2007) in this treatment, these preconditioned sclerotia were then transferred into sterilized river sand at 15°C, because some sclerotia in one replication were disintegrating from having been in water for this period.

Treatment 2. Sclerotia were loosely packed in a cheesecloth bag and each replication was placed in a separate container containing water with constant aeration (using an aquarium pump) at 4°C as described by Dillard et al. (18), Nelson et al. (37), and Cobb and Dillard (14). Water in the containers was changed every week. No carpogenic germination was recorded in this treatment up to 4 months, when sclerotia were then transferred into sterilized river sand at 15°C.

Treatment 3. Sclerotia were loosely packed in a cheesecloth bag and evenly distributed at the bottom of a plastic container (36 by 20 by 17 cm) having holes (2 mm in diameter). Each box (with each replication) was placed at a 45° angle and positioned separately underneath the tap water; the end of the box with the holes was furthest away from the stream of tap water falling from a height of 80 cm. Sclerotia were then subjected to constant rinsing with tap water at a flow rate of approximately 3 liter/h while being maintained at 4°C for 8 weeks (18), followed by subsequent placement of sclerotia into sterilized river sand at 15°C.

Treatment 4. This treatment was according to the procedure described by Clarkson et al. (12), Mylchreest and Wheeler (36), and Sansford and Coley-Smith (45), but with slight modifications. Mature sclerotia were cold conditioned by transferring the Erlenmeyer flasks (250 ml) with sclerotia to 4°C for 4 weeks. After this period, sclerotia were removed from the flask and sieved under running tap water as described above. Sclerotia were air dried overnight in a laminar flow cabinet and then evenly distributed into a 2-cm layer of unsterilized organic compost at 15°C (Richgro, Jandakot, Western Australia) in plastic containers. Plastic containers were then placed in a controlled environment incubator as described above.

Treatment 5. Sclerotia were incubated in sterilized river sand at 15°C, directly after sieving with water and without any pre-conditioning treatments, using the method described by Grogan and Abawi (22).

Treatment 6. Sclerotia were air dried for 2 weeks on a laboratory bench (23) followed by incubation in sterilized river sand at 15°C, using the methods described by Grogan and Abawi (22) and Steadman and Nickerson (51).

Treatment 7. Sclerotia were sterilized with 70% ethanol for 30 s in a laminar flow cabinet, then pressed 0.5 cm deep into

0.5% sterilized water agar (total water agar depth = 4 cm) contained in a round plastic container (6.5 by 7.0 cm, diameter by height) with the help of sterilized forceps (51,53). Plastic containers with sclerotia and water agar were then incubated at 15°C.

Data collection and data analysis. Sclerotia were observed weekly for stipe formation. Sclerotia for which stipe initiation (up to 3 to 4 mm in size) was observed were removed from their respective plastic containers in order to avoid any duplication of data. The total number of sclerotia germinated in each replication of every treatment was compiled at the end of the 2-year period. Square root transformation of the total number of sclerotia germinated in each replication was undertaken to obtain a more normal distribution of the observed data. The carpogenic germination data were then analyzed by three-way analysis of variance (ANOVA) using GENSTAT (9th edition; Lawes Agricultural Trust, Rothamsted, UK). Fisher's least significant differences at $P < 0.05$ were used to test for differences between the treatments. The comparison of scarified and unscarified

sclerotia in relation to carpogenic germination when subjected to constant rinsing with tap water was also made separately in the month of June at different time intervals by using a single-factor ANOVA using GENSTAT (9th edition; Lawes Agricultural Trust). Progression of sclerotial germination in each month of each isolate was obtained by adding the total number of sclerotia germinated for each replication of each treatment to identify any evidence of seasonal rhythm in relation to the carpogenic germination.

RESULTS

Effect of scarification, environmental treatment, and isolate on carpogenic germination of *S. sclerotiorum*. Overall, scarification enhanced the carpogenic germination of sclerotia of both isolates used. There was significantly greater ($P < 0.05$) carpogenic germination in scarified than unscarified sclerotia for both isolates (MBRS-1 and WW-3) of *S. sclerotiorum* used in this study (Table 2).

Significant differences ($P < 0.001$) were also observed among different environmental treatments used to induce carpo-

Table 2. Three-way analysis of variance showing significance of effect of isolate, scarification of sclerotia, and the seven environmental treatments applied in relation to the carpogenic germination of the sclerotia of *Sclerotinia sclerotiorum*

Significance of	P values ^a	LSD ^b
Isolate (MBRS-1 and WW-3)	<0.001	0.17
Scarification (± scarification)	<0.05	0.17
Environmental treatment ^c	<0.001	0.33
Isolate × scarification	n.s.	...
Isolate × environmental treatment	<0.001	0.46
Scarification × environmental treatment	<0.001	0.46
Isolate × scarification × environmental treatment	n.s.	...

^a Values: n.s. = not significant at $P \leq 0.05$.

^b Least significant difference.

^c Seven environmental treatments were used to induce carpogenic germination: (1) sclerotia incubated in sterilized distilled water, (2) sclerotia placed in water with constant aeration at 4°C, (3) sclerotia subjected to constant rinsing with tap water, (4) sclerotia cold-conditioned for 4 weeks at 4°C and then transferred into moist unsterilized compost, (5) sclerotia incubated in sterilized river sand, (6) sclerotia air-dried for 2 weeks on a laboratory bench and then incubated in sterilized moist river sand, and (7) sclerotia incubated in 0.5% water agar.

Table 1. Different environmental treatments and the respective temperature regime (T) used to stimulate carpogenic germination of scarified and unscarified sclerotia of two isolates (MBRS-1 and WW-3) of *Sclerotinia sclerotiorum* (all environmental treatments established in January 2007)

No. ^b	January 2007–April 2007		May 2007–December 2008		Stipes ^a	
	Treatment	T (°C)	Treatment	T (°C)	MBRS-1	WW-3
1	Sclerotia incubated in sterile distilled water	15	Sclerotia transferred into sterilized river sand	23–25	– ^c	August 07
2	Sclerotia incubated in aerated water	4	Sclerotia transferred into sterilized river sand	23–25	July 08	August 07
3	Constant rinsing with tap water at 4°C for 8 weeks and subsequently transferred into sterile river sand at 15°C	4, 15 ^d	Sclerotia in sterilized river sand	23–25	June 07	June 07
4	Cold-conditioning at 4°C for 4 weeks without sieving and then transferred to unsterilized compost at 15°C after sieving sclerotia with running water	4, 15 ^d	Sclerotia in unsterilized compost	23–25	October 08	September 07
5	Sclerotia incubated in sterile river sand	15	Sclerotia in sterilized river sand	23–25	October 08	August 07
6	Air drying of sclerotia for 2 weeks on lab bench and then incubation in sterilized river sand	15	Sclerotia in sterilized river sand	23–25	November 08	July 07
7	Sclerotia pressed into 0.5% sterilized water agar	15	Sclerotia in 0.5% sterilized water agar	23–25	June 08	April 07

^a Time at which first apothecial stipes were visible.

^b Treatment number.

^c No carpogenic germination was recorded in this instance.

^d Temperatures of 4°C for 4 weeks and then 15°C.

genic germination of the two isolates of *S. sclerotiorum* (Table 2). Carpogenic germination of the sclerotia of isolate MBRS-1 tested in this study was greatest when sclerotia were subjected to constant rinsing with tap water at 4°C. For isolate WW-3, whereas the total number of sclerotia germinated was greatest when subjected to constant rinsing with tap water, two other treatments (sterilized distilled water and sterilized river sand) produced similar high levels of germination (Table 3; Fig. 1). However, sterilized distilled water and sterilized river sand treatments resulted in sporadic germination patterns, with the majority of sclerotia germinating only after 18 months of incubation. In contrast, the treatment involving constant rinsing with tap water ensured reliable carpogenic germination of sclerotia of both the isolates after only 5 months of incubation of sclerotia, with 90% of the sclerotia germinating within the month of June itself. Further, overall carpogenic germination of isolate WW-3, as observed across the different environmental treatments, was significantly greater ($P < 0.001$) compared with the isolate MBRS-1 (Table 2; Fig. 1).

The number of stipes observed per sclerotia varied from 1 to 5, with a median value of 1.0. Moreover, for treatment 1, approximately 5% of the sclerotia of isolate MBRS-1 (when both scarified and unscarified sclerotia were taken together) appeared to have rotted.

Environmental treatment-scarification, isolate-scarification, and isolate-environmental treatment interactions. There was significant overall interaction between scarification and environmental treatments ($P < 0.001$) in relation to the carpogenic germination. Scarification significantly enhanced carpogenic germination of sclerotia for both isolates but only for certain environmental treatments, such as for sclerotia incubated in compost and in sterilized river sand. However, for WW-3, carpogenic germination of unscarified sclerotia was improved, compared with scarified sclerotia, when sclerotia were either placed into 0.5% water agar or when air dried for 2 weeks followed by their

placement into sterilized river sand. It is noteworthy that carpogenic germination of the scarified and unscarified sclerotia was comparable across the three treatments where sclerotia had continuing exposure to water (i.e., sclerotia incubated in sterilized distilled water, sclerotia placed in water with constant aeration at 4°C, and sclerotia subjected to constant rinsing with tap water). However, of these three treatments, with the constant rinsing with tap water treatment, carpogenic germination of scarified sclerotia occurred 1 week earlier compared with unscarified sclerotia. There was no significant interaction observed between isolates and the scarification treatment in relation to the carpogenic germination, suggesting that enhanced carpogenic germination in scarified sclerotia was independent of the isolate used.

There was a significant isolate-environmental treatment interaction ($P < 0.001$) with respect to the carpogenic germination of the two isolates, suggesting that carpogenic germination of the two

isolates was influenced by the type of environment factor or treatment (Fig. 1).

June 2007 was the period when maximum carpogenic germination of WW-3 was obtained; therefore, the effect of scarification on sclerotia subjected to constant rinsing with tap water was also analyzed at different time intervals in this month. There were significant differences between scarified and unscarified sclerotia in relation to the carpogenic germination for this isolate, showing a peaking of carpogenic germination in scarified sclerotia approximately 1 week earlier than in unscarified sclerotia.

There was no significant three-way interaction between isolate, scarification, and environmental treatment with regard to the carpogenic germination of the sclerotia.

Seasonal rhythm of the two isolates in response to the time of the year. A seasonal rhythm-like pattern for carpogenic germination of the sclerotia of both isolates (MBRS-1 and WW-3) was observed (Fig. 2). Maximum carpogenic germination of the two isolates occurred from June

Fig. 1. Mean value of sclerotia germinated (scarified and unscarified sclerotia taken together) for two isolates of *Sclerotinia sclerotiorum*. Error bars indicate the standard errors associated with the mean values for sclerotia germinated in each treatment. The X-axis represents the various environmental treatments for stimulating carpogenic germination: (1) sclerotia incubated in sterilized distilled water, (2) sclerotia placed in water with constant aeration at 4°C, (3) sclerotia subjected to constant rinsing with tap water, (4) sclerotia cold-conditioned for 4 weeks at 4°C and then transferred into moist unsterilized compost, (5) sclerotia incubated in sterilized river sand, (6) sclerotia air dried for 2 weeks on a laboratory bench and then incubated in sterilized moist river sand, and (7) sclerotia incubated in 0.5% water agar.

Table 3. Carpogenic germination of scarified and unscarified sclerotia from two isolates (MBRS-1 and WW-3) of *Sclerotinia sclerotiorum* when preconditioned with seven environmental treatments

Isolate	Scarification	Number (percent germinated) for each treatment ^a						
		1	2	3	4	5	6	7
MBRS-1	Scarified	0 (0)	2.68 (30.0)	4.74 (90.0)	1.14 (7.00)	0.71 (4.0)	0 (0)	0.60 (3.0)
	Unscarified	0 (0)	2.21 (22.0)	4.69 (88.0)	0 (0)	0 (0)	0.35 (2.0)	0.43 (3.0)
WW-3	Scarified	4.51 (82.0)	3.58 (52.0)	4.87 (95.00)	4.30 (74.0)	4.74 (90.0)	3.43 (48.0)	2.36 (23.0)
	Unscarified	4.82 (93.0)	3.66 (54.0)	4.84 (94.0)	3.08 (41.0)	2.76 (32.0)	4.53 (83.0)	3.38 (46.0)
LSD ^b	...	0.25	0.76	0.14	0.58	0.61	0.46	0.60

^a In all, 25 sclerotia were tested for each replication of each treatment. Numbers in the table represent the mean values for carpogenic germination per treatment after square root transformation, and numbers in parenthesis represent the percentage for carpogenic germination of sclerotia for the two isolates for each environmental treatment. Treatments: 1, sclerotia incubated in sterilized distilled water; 2, sclerotia placed in water with constant aeration at 4°C; 3, sclerotia subjected to constant rinsing with tap water; 4, sclerotia cold-conditioned for 4 weeks at 4°C and then transferred into moist unsterilized compost; 5, sclerotia incubated in sterilized river sand; 6, sclerotia air-dried for 2 weeks on a laboratory bench and then incubated in sterilized moist river sand and; 7, sclerotia incubated in 0.5% water agar.

^b Least significant difference at $P < 0.05$.

to September for the two consecutive years. There was little germination of sclerotia for MBRS-1 outside of this period, with only a few germinating over the period of October to December in the second year (Fig. 2). In contrast, there was relatively more germination outside the months from June to September for isolate WW-3 compared with MBRS-1, with some sclerotial germination also occurring from January to May 2007 and from September 2007 to May 2008 (Fig. 2).

DISCUSSION

To our knowledge, this is the first report, both of the enhanced carpogenic germination following scarification and for the environmental factors we report that enhance carpogenic germination of scarified sclerotia of *S. sclerotiorum*.

Overall, carpogenic germination of scarified sclerotia was significantly better compared with unscarified sclerotia, possibly for one or both of the following reasons. First, sclerotia of *S. sclerotiorum* are mainly composed of two layers, an outer pigmented rind and a medulla of prosenchymatous tissue (28,43,57). The primordia which originate from the medulla rupture the rind and form young apothecial stipes (43). Artificial abrasion of the rind in our study may have encouraged carpogenic germination of sclerotia because primordia developing directly underneath the ruptured rind may have had greater opportunity to grow as apothecial stipes. Second, the rind can also impose a constitutive dormancy of the sclerotia because of being thick-walled and highly melanized (15). The act of physical scarification of the sclerotia may help in breaking this dormancy by facilitating increased diffusion of oxygen and moisture through the scarified surface, because these factors have been reported as essential for carpogenic germination in *S. sclerotiorum* (3,35,60).

There were significant interactions ($P < 0.001$) between scarification and the vari-

ous environmental factors tested or applied to stimulate carpogenic germination of sclerotia of *S. sclerotiorum*. For instance, scarified sclerotia had higher levels of carpogenic germination when incubated in compost or in sterilized river sand. In contrast, carpogenic germination of unscarified sclerotia was improved compared with scarified sclerotia when they were either placed into 0.5% water agar (WW-3 only) or air dried for 2 weeks followed by their placement into sterilized river sand (particularly for isolate WW-3).

It is noteworthy that carpogenic germination of the scarified and unscarified sclerotia was comparable in three treatments where sclerotia had continued exposure to water; in these treatments, sclerotia were incubated in sterilized distilled water, placed in water with constant aeration at 4°C, or subjected to constant rinsing with tap water. However, of these three treatments, it was with constant rinsing with tap water that carpogenic germination of the scarified sclerotia occurred 1 week earlier compared with unscarified sclerotia. Moreover, it is interesting that the overall carpogenic germination for both isolates tested was greatest for this same treatment (constant rinsing with tap water). Although germination of sclerotia of isolate WW-3 incubated in sterilized distilled water and in sterilized river sand was similar to that with constant rinsing with tap water (Fig. 1), sclerotia in the former two treatments showed an inconsistent germination pattern, with most sclerotia germinating only after 18 months of incubation. In contrast, sclerotia of both isolates, when placed under constant rinsing with tap water, germinated after only 5 months. Our findings are consistent with Dillard et al. (18), where carpogenic germination of the sclerotia was observed to be the greatest when they were subjected to constant rinsing with water for 8 weeks. The need for adequate oxygen and continuous exposure to water in order to break down the sclerotial reserve material (i.e., required for

carpogenic germination; 8) could explain the enhanced germination we observed from constant rinsing with tap water. Moreover, sclerotia of *S. sclerotiorum* are known to excrete many soluble carbohydrates, salts, amino acids, proteins, lipids, and enzymes during the development and maturation phase (10,57). These may also include a range of sugars (e.g., glucose, sucrose, and trehalose) and other organic and inorganic compounds that have been reported to inhibit sclerotial germination (51). Therefore, the enhancement of germination we observed with constant rinsing with tap water could also be a consequence of the leaching of the inhibitory compounds present in the form of dried exudates on the sclerotial surface, as suggested by Dillard et al. (18).

Carpogenic germination of sclerotia of both isolates of *S. sclerotiorum* placed in water with constant aeration and in sterilized distilled water was much less effective in comparison with constant rinsing with tap water. This was despite all three methods involving continual exposure of sclerotia to water. This difference could perhaps be due to the presence of the inhibitors of carpogenic germination being contained within the container for the first two treatments but leached away in the treatment involving constant rinsing with tap water. This may also be related to the substrate used for bulk production of the sclerotia in comparison with previous studies (14,49).

A seasonal rhythm-like pattern in relation to the carpogenic germination from both isolates of *S. sclerotiorum* tested was observed in this study (Fig. 2). The majority of sclerotia for both isolates germinated between the months of June and September during the two consecutive years. The presence of a "biological clock" relying on the accumulated information of daily photoperiodic changes and other physical cues such as temperature, moisture, and humidity has been suggested for insects (16), with its proposed function being to

Fig. 2. Mean values of sclerotia showing carpogenic germination for two isolates (MBRS-1 and WW-3) of *Sclerotinia sclerotiorum* from January 2007 to December 2008. Numbers presented represent the mean values of total sclerotia (scarified and unscarified) germinated across all the environmental treatments for each isolate separately.

provide an internal measure of the external time (5). This is suggested in our study where the majority of carpogenic germination of sclerotia was initiated at and following the arrival of appropriate seasonal timing and climatic conditions coinciding with the availability of the host crop, including the flowering period. It is possible that the exposure of sclerotia to extremely hot dry summers and, subsequently, to cool, wet cropping seasons in the Mediterranean regions of southern Australia could be related to the development of a seasonal rhythm-like pattern as we observed, because this could offer a significant competitive advantage to the pathogen. Ideally, further studies on carpogenic germination that examine the effects of age of sclerotia and include setting experiments across different periods of the year, using sclerotia from different climatic zones and monitoring carpogenic germination under a range of different controlled environment conditions, are needed to confirm our findings. Further, studies at a molecular level, such as those conducted for the *Neurospora crassa* circadian system (17,19), could then help to elucidate the mechanisms involved.

As indicated above, overall, scarification or constant rinsing with tap water enhanced the carpogenic germination of sclerotia under the environmental conditions that we deployed in this study. Similarly, in the Mediterranean climate of southern Australia, it may be possible that the opening rainfall events in the early winter period after the hot, dry summer period could provide a somewhat similar "scrubbing effect" as that from the treatment conditions we imposed in our study. Further, the unique soil environment of the Mediterranean regions of southwest Western Australia affects the saprophytic and parasitic phases of fungal plant pathogens (48). This environment has also been shown to affect ageing and dormancy of seed in unusual ways (55,56), with their timing of germination coinciding with the breaking rains following a hot summer. However, it remains a challenge to correlate our findings on factors influencing carpogenic germination directly with the influence of the complex combinations of variable environmental factors operating under field conditions. Future work based on confirming the results of conditioning treatments similar to those we studied but involving burial of variously conditioned sclerotia in the field under different climatic conditions could not only help in developing prediction models for carpogenic germination but also could lead to development of a disease forecasting system for *Sclerotinia* disease for *Brassica* spp. in Australia, as reported for lettuce by Clarkson et al. in the UK (11,13).

Overall, our study has shown not only that carpogenic germination of scarified sclerotia was significantly improved com-

pared with the unscarified sclerotia but also that carpogenic germination was further enhanced by some environmental factors, such as incubation of sclerotia in compost or in sterilized river sand. Moreover, although carpogenic germination of scarified and unscarified sclerotia was comparable within the treatments where a continual supply of the water was maintained, scarified sclerotia germinated slightly earlier than unscarified sclerotia when subjected to constant rinsing with tap water. Of all the environmental treatments, constant rinsing with tap water maximized the carpogenic germination of the two isolates used in this study. The seasonal rhythm-like pattern we obtained in relation to the carpogenic germination of *S. sclerotiorum* has not been previously reported and further investigations into this aspect may offer valuable insights into pathogen behavior in the field.

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