Relationship between the drying of the sclerotia of *Phymatotrichopsis omnivora* and its survival

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Relación entre la desecación de los esclerocios de *Phymatotrichopsis omnivora* y su sobrevivencia

Resumen. *Phymatotrichopsis omnivora* es un importante hongo fitopatógeno de plantas, fue reproducido en laboratorio y sus esclerocios separados por tipo: grandes, medianos, pequeños y albinos. Todos los tipos de esclerocios fueron desecados hasta 60 minutos o después de ser desecados sometidos a tratamientos de estrés: a) ninguno (control); b) esclerocios enterrados en suelo arenoso húmedo (25% p/p); c) esclerocios sumergidos cuatro horas en solución de hipoclorito de sodio al 0.3%; d) esclerocios enterrados en suelo inundado y adicionado con glucosa a razón de 1 mg g^{-1} (glucosa/suelo). El peso inicial, la pérdida de peso (después de desecarse) y la sobrevivencia de todos los tipos de esclerocios fueron estadísticamente diferentes ($p = 0.001$). La sobrevivencia de los esclerocios fue similar en los tratamientos de estrés y solo al ser desecados, esto sugiere que los esclerocios principalmente mueren antes de aplicar tratamientos de estrés. La proporción y rapidez de la pérdida de peso de los esclerocios desecados fue inversa a su tamaño. Cada tipo de esclerocio correlacionó su pérdida de peso con su sobrevivencia R^2 0.60-0.94. Los esclerocios albinos desecados sobrevivieron más que el resto de los esclerocios evaluados. Este trabajo indica que los esclerocios de *P. omnivora* mueren rápidamente al desecarse.
Palabras clave: hongos del suelo patógenos de plantas, melaninas.

Abstract. *Phymatotrichopsis omnivora* is an important plant pathogen, which was reproduced in the laboratory, and its sclerotia were separated by type: large, medium, small and albinos. All type of sclerotia were dried up to 60 minutes or after being dried, were applied stress treatments: a) none (control), b) sclerotia buried in moist sandy soil (25% wt / wt), c) sclerotia submerged four hours in sodium hypochlorite solution at 0.3%, d) sclerotia buried in flooded soil and supplemented with glucose at 1 mg g^{-1} (glucose / soil). The initial weight, weight loss (after dried) and the survival of all type of sclerotia were statistically different ($p = 0.001$). The survival of sclerotia was similar in treatment of stress and where they only were dried, this suggests that the sclerotia are die on drying before applying the stress treatments. The proportion and rate of weight loss of dried sclerotia was inverse to its size. Each type of sclerotia lost weight correlated with survival R^2 from 0.60 to 0.94. Albino sclerotia that were dried died less than other type of sclerotia. This work indicates that the sclerotia of *P. omnivora* dried, quickly die.
Key words. soil fungi plant pathogens, melanin.

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Introduction

Phymatotrichopsis omnivora (Duggar) Hennebert, may origin root rot in more than 2000 species of plants, causing significant economic losses by destroying crops in Mexico as well as United States (Uppalapati *et al*., 2010). The sclerotia of this fungus survive naturally years in the soil; but sclerotia that are reproduced in the laboratory, it is unknown how is affect their survival after they are dried. Sclerotia from the laboratory have been used in various researches, such as determining the number needed to kill a population of cotton plants *Gossypium hirsutum* L. (Lyda and Burnett, 1970), evaluation of anhydrous ammonia as a fumigant (Rush and Lyda, 1982), the effect of pH on the survival (Samaniego-Gaxiola, 2008a) and being used as inoculum to evaluate the resistance of the pistache plant *Pistacia atlantica* Betoum (Tarango-Rivero and Herrera-Pérez, 1997). However, not all the researches were detailed the type of sclerotia that was used, or if there were evaluated different type of sclerotia.

Some sclerotia such as *Macrophomina phaseolina* (Tassi) Goid (1947), *Sclerotium cepivorum* Berk, *S. rolfsii* Sac., *Sclerotinia minor* Jagger and *Verticillium dahliae* Klebs can be drying since days to months without affecting their viability (Maiti and Sen, 1988; Hawke and Lazarovits, 1994; Olaya *et al*., 1996; Harper *et al*., 2002). In contrast, sclerotia of *P. omnivora* recovered from the soil appear to be very susceptible to drying.

When the sclerotia of *Sclerotium rolfsii* Sac. lost weight, they also lost viability (Hyakumachi and Lockwood, 1989). Other fungi that produce sclerotia loss weight and viability when are subjected to cycles of wetting – drying (Coley-Smith, 1979). The loss weight of sclerotia can occur when its metabolism increase, excreted nutriments, loss water or combination of all them (Hyakumachi and Lockwood, 1989; Coley-Smith, 1979). Sclerotia of *P. omnivora* reproducing in laboratory, can suffer drying in soil, e.g. when the fungus is placed as inoculum on root of plants; but, it is unknown if drying of sclerotia during little times can affect their viability. The effect of drying of natural sclerotia of *P. omnivora* in respect to its survival has been studied, so the sclerotia that were dried during one month caused a 20% of survival, and null survival after three months (King and Eton, 1934). In this research, were used sclerotia recovered into soil; its size was large, medium and small; however, did not record the weight according to the size of the sclerotia, were only used pigmented sclerotia (reddish brown color), and it was not clear the use of individual or clusters of sclerotia. Additionally, King and Eaton (1934) mentioned that whitish (albino) sclerotia were not possible to obtain in soil, and the sclerotia produced in cultures (laboratory) were not considered desirable. In the laboratory, sclerotia reproduced and harvest of *P. omnivora*, allows us to recover sclerotia of several arrangements, shapes, sizes, and pigmentation. There are individual sclerotia or in clusters from two to more than fifty; their form can be cylindrical to almost round; the size varies from <1 to >10 mm and can be pigmented or albino.

Soil moisture may be a stress factor for plant pathogens fungi, which affects their survival (Mondal and Hyakumachi, 1998), other factors are: flooding the soil (Crowe and Debons, 1992); flooded soils with added carbohydrates (Samaniego-Gaxiola, 1994) and keep them in hypochlorite solution sodium (Samaniego-Gaxiola, 2008b). Consequently, drying of sclerotia together with other stress factors may increase the loss of survival of *P. omnivora*. Accordingly, in this study were used individual sclerotia of *P. omnivora* with different sizes produced in the laboratory (large, medium, small, and albino) to determinate the relationship between weight loss after being dried and their viability; assessing the survival of dried sclerotia and then they subjected to the following stress treatments: none, buried in sand flooded with glucose added, immersed in a solution of sodium hypochlorite, buried in moist sand.

Material and methods

Reproduction and management of the sclerotia

Sclerotia were reproduced, harvested and management as indicated in Samaniego-Gaxiola (2008a). Harvest sclerotia were separated in vials and stored until were used. The experiments began 10 days after of obtain the first vials (25 sclerotia per vial). Each vial was treated as a replication and each experiment had four replicates.

Selection of sclerotia

Visually four types of individual sclerotia were selected by size and pigmentation. By size and pigmented the sclerotia were large, medium and small, and of medium size the albino that corresponding to > 6, < 6 >3, < 3 and < 6 >3 mg per sclerotia, respectively. Pigmented sclerotia were clear brown color and albino cream color.

Preliminary tests drying of the sclerotia

In this test and subsequent experiments, all sclerotia extracted from the vials were dried using absorbent paper, until water was not visible around them. Then, medium size sclerotia were subjected to drying, in a flow air camera, at constant temperature at 28 °C. Periods of drying the sclerotia were of 1 to 7 days: 1, 3, 6 and 24 hours; and 5, 15, 30 and 60 minutes. Immediately after the period of drying, the sclerotia were placed over moist sand in Petri dishes (saturated with water) and incubated up to 10 days at 28 °C. During incubation time the sclerotia were reviewed to determine their survival. The criterion for considering viable the sclerotia was the development of at least a strand. In contrast, when sclerotia did not form strand, they were invaded by other soil fungi (Samaniego-Gaxiola, 2008a).

Experiment of drying time

The weight of sclerotia was recorded using analytical balance before and after beginning the period of drying. The four types of sclerotia (large, medium, small, and albino) were dried at periods of 0, 5, 15, 30 and 60 minutes at a temperature of 28 °C; then the sclerotia were placed in Petri plates to determine their survival. The sclerotia used in this experiment had no more than two months after being harvested.

Experiment of drying and subsequent stress treatments

Here were used sclerotia between two and three months after being harvested. This sclerotia were dried by the same time of the previous experiment, and subsequent were applied following treatments: a) neither, sclerotia placed in plates with sand to assess their survival; b) sclerotia buried in 400 g of sandy soil in 1 L glass jars, the moisture content of the sand was 25% w/w, after, the glass jars with the sclerotia were incubated two weeks at 28 °C; c) the sclerotia were immersed in 10 mL of 0.3% sodium hypochlorite commercial solution (Cloralex) for 4 h at 28 °C; d) sclerotia treated as b), except because the water added to soil contained glucose a rate of 1 mg glucose/g soil. After each treatments the sclerotia was placed in plates with sand to assess their survival, except, the sclerotia immersed in hypochlorite solution, which were planted on Petri dishes containing water agar, and were incubated at 28 °C for one week to evaluate their survival (mycelium growth).

Data analysis

The experiments were repeated twice. Each experiment was carried out in a completely random design with factorial arrangement and an analysis of variance with repeated means over time was applied. The survival of the sclerotia was expressed as a percentage, and these values (before be analyzed) were transformed as a sine arc. Least Significant Difference (Tukey) was used to compare means of proof. The relation between loss weight and loss survival of sclerotia were analyzed by correlation. All the statistical analyses were carried out using SAS program SAS (1999).

Results

Drying the sclerotia for two hours or more resulted in null survival of the fungus and with a weight loss of ~ 50%. The analysis of variance in both experiment, indicates no statistical difference for initial weight of sclerotia and for time of drying; but if there were differences between each type of sclerotia according to initial weight average ($p = 0.001$); also were detected differences to interactions of experiments x type, and type x time ($p = 0.001$).

Each experiment of stress treatment applied to sclerotia (after drying) was analyzed separately, in each case no statistical difference was found between the two repetitions between experiments ($p = 0.001$), but were detected differences according time of drying, type of sclerotia, and for interaction of type x time ($p = 0,001$).

The greater initial weight and the greater weight loss of the sclerotia were in the following order: large, medium, albino and small (Tables 1-3). In contrast, the lower weight loss of sclerotia (as percentage of initial weight) was for large sclerotia continued by medium and very similar between albino and small (Figure 1, Table 4). In the first and the second experiment, survival in all type of sclerotia of *P. omnivora* decreased with the drying time; however, albino sclerotia survival was higher than anyone (Figure 2). The large sclerotia survived more than medium size, and the small sclerotia had lowest survival, although in the second experiment, in general was observed that the survival of the sclerotia was lower than in the first experiment (Figure 2). So, large, medium, small and albino sclerotia after being dried for 60 minutes had an average of survival (two experiments) of 57, 43, 14 and 76 %, respectively.

As increasing the drying time of sclerotia and then stress treatments were applied, the survival of them declined, although small sclerotia were most likely to lose survival (Figure 3). All type of sclerotia (large, medium, small, and albino) with two or more months after harvest, and subsequently dried had similar survival (Figures 2 and 3A); except large sclerotia (dried 60 min) with over two months, which survived more than their counterparts with less than two months (Figures 2 and 3A). The treatment of sclerotia

Table 1. Initial weight (mg) of sclerotia before being dried

Minutes of drying	Type of sclerotia			
	Large	Medium	Small	Albino
5	†269.5 (35.7)	89.9 (16.9)	44.1 (9.8)	80.4 (14.7)
15	243.4 (36.9)	99.6 (9.9)	42.1 (6.5)	65.0 (6.9)
30	237.7 (26.5)	98.2 (9.5)	42.8 (6.5)	67.7 (10.7)
60	206.2 (32.3)	99.9 (12.4)	37.9 (3.8)	78.6 (12.3)
Average	239.2	96.9	41.7	72.9

†Data average of two experiments, n = 8.
The numbers in parentheses indicate the standard deviation.

Table 2. Weight loss (mg) of sclerotia after being dried at 28 °C

Minutes of drying	Type of sclerotia			
	Large	Medium	Small	Albino
5	†10.2 (2.8)	6.7 (1.9)	4.7 (1.3)	6.1 (2.1)
15	21.7 (3.8)	12.9 (3.8)	8.8 (1.6)	11.7 (3.4)
30	32.2 (1.9)	21.3 (4.1)	13.5 (2.3)	15.4 (4.3)
60	53.1 (9.1)	31.5 (3.0)	14.8 (3.6)	27.4 (3.7)
Average	29.3	18.1	10.4	15.2

†Data average of two experiments, n = 8.
The numbers in parentheses indicate the standard deviation.

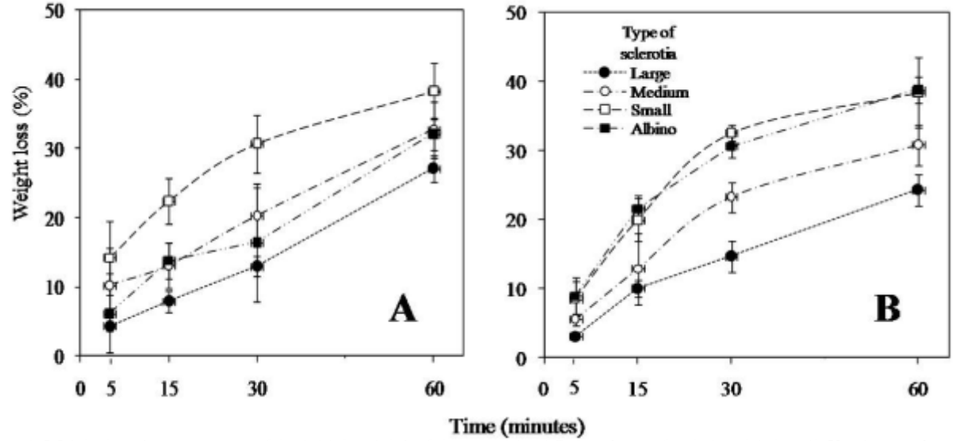

Figure 1. Weight loss (as percentage of the initial weight) of the sclerotia of *P. omnivora* after being dried until 60 minutes. A and B, data of the experiments one and two, respectively. The bars mean standard deviation, n = 4.

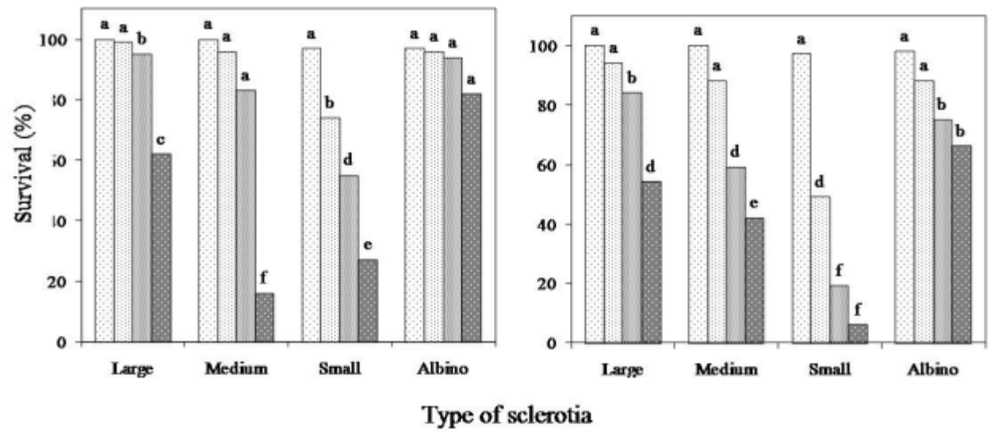

Figure 2. Survival of sclerotia of *P. omnivora* after being dried by 5□, 15□, 30■, and 60■ minutes at 28 °C. A and B, figures for first and second experiment, respectively. Bars represent the mean of two experiments (n = 8). Different letters, indicate statistical difference according to means separation test Tukey p = 0.05.

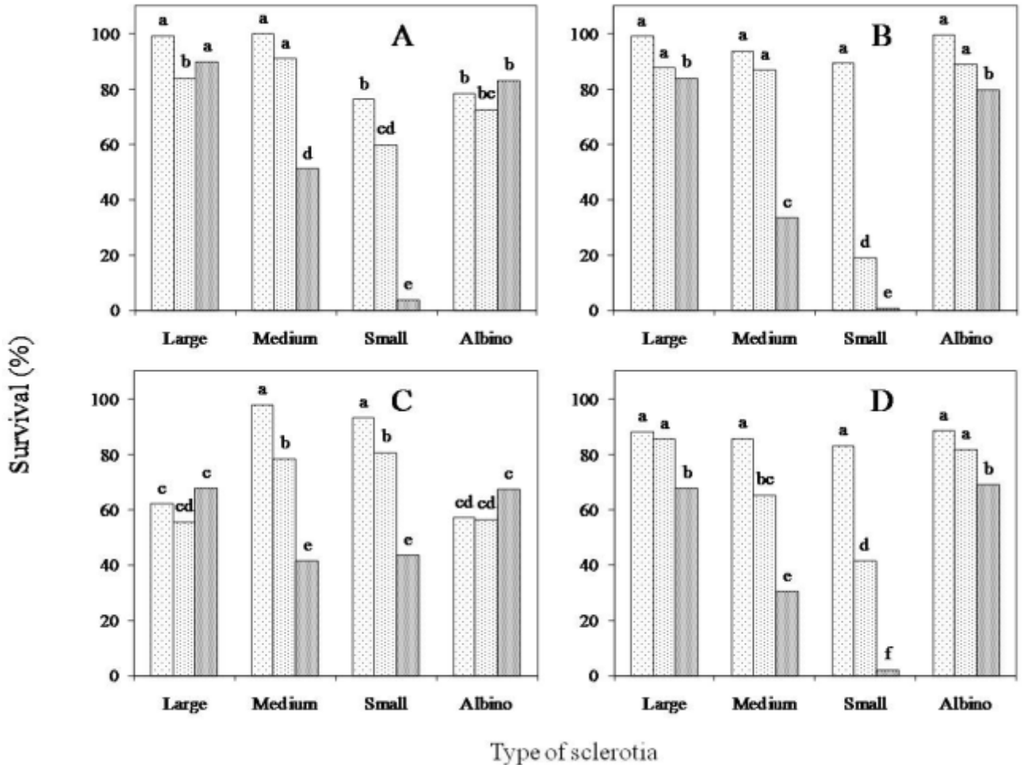

Figure 3. Survival of sclerotia of *P. omnivora* after being dried by 15□, 30□, and 60□ minutes at 28 °C; and then to apply treatments to them. A, neither, sclerotia placed in plates of sand. B, sclerotia remained in moist sand during two weeks. C, sclerotia remained in solution of NaOCl at 0.3% during four hours. D, sclerotia remained during two weeks in sand flooded that contained 1 mg g^{-1} glucose. Bars represent the mean of two experiments (n = 8). Different letters, indicate statistical difference according to means separation test Tukey p = 0.05.

immersed in sodium hypochlorite solution, resulted in lower survival of large and albino sclerotia (Figure 3C). Albinos and large sclerotia survived similarly after drying and apply those stress treatments (Figure 3B, C and D).

At increasing the loss weight of the sclerotia (as a proportion of its initial weight) their survival decreased, when the small sclerotia lost ~ 40% of their weight, they survived with almost zero percent (Figure 4C and G). In contrast albino sclerotia weight loss almost as small sclerotia, but the survival of albino sclerotia was the highest of all, which fluctuated between 60 at 80% (Figure 4D and H). The medium sclerotia had an intermediate survival of 20-40% with a weight loss for about 40% (Figure 4B and F). Large sclerotia had the lowest loss weight although its survival was only of 50-60% (Figure 4A and E).

Discussion

Initial weight of large sclerotia had the highest variation inside and between the periods of drying (Table 1). This variation may partially explain why there were difference statistics in the interactions type (sclerotia) x time or type x experiment. This means, that large sclerotia were not homogenously selected for both, drying time and experiments. However large sclerotia had the same behavior

Table 3. Initial weight of sclerotia (mg) after being [§]dried at 28 °C, and subsequently apply treatments

Type of sclerotia	Treatments, sclerotia dried and then: Placed in Sand		Buried in moist sands		Immersed in solution of NaClO		Buried in sand floded with glucose	
Large	[†]256.9	(51.1)	252.1	(44.6)	247.8	(62.6)	231.5	(48.9)
Medium	128.1	(33.9)	100.1	(25.2)	113.8	(29.7)	105.4	(15.0)
Small	45.0	(11.7)	44.0	(12.9)	45.0	(14.4)	43.8	(10.3)
Albino	63.9	(24.8)	66.4	(22.6)	58.8	(24.2)	69.0	(22.2)

[§]Each type of sclerotia was dried by 15, 30 and 60 minutes.
[¶]To see details in text.
[†]Data average of two experiments, n = 8.
The numbers in parentheses indicate the standard deviation

Table 4. Weight loss of sclerotia in mg after being [§] dried at 28 °C, and subsequently apply treatments

Type of sclerotia	[¶]Treatments, sclerotia dried and then: Placed in sand		Buried in moist sand		Immersed in solution of NaClO		Buried in sand flooded with glucose	
Large	13.8	(6.3)	16.5	(7.0)	15.5	(5.6)	13.4	(4.6)
Medium	18.9	(4.9)	22.0	(7.5)	20.0	(6.2)	20.9	(11.3)
Samll	29.2	(7.1)	29.9	(11.6)	32.3	(9.7)	28.4	(10.6)
Albino	26.7	(14.7)	27.1	(16.6)	28.2	(16.7)	23.3	(11.4)

[†]Data average of two experiments, n = 8.
[§]Each type of sclerotia was dried by 15, 30 and 60 minutes.
[¶]To see details in text.
The numbers in parentheses indicate the standard deviation

than other type of sclerotia, respect to weight loss when increasing drying time.

Particularly weight loss of large sclerotia dried for 60 minutes had the highest standard deviation (Table 2) which suggests a strong variation in initial weight of sclerotia as this was indicated. The weight loss of sclerotia may mainly be attributed to their loss of humidity, due that one hour is a very little time for to release significant quantities of gases, liquid exudates, and product of their decay (necrosis). Although is not excluded the loss of small amounts of exudates such as nutrients. Then we may think that the sclerotia are analogue to deposits of different sizes with their respectively water content. Experiment conduced with maize and soybean in plots of different size, where the loss of humidity (only as transpiration of these plants) was measured (Ray and Sinclair, 1998) had a similar behavior as the way the sclerotia lost weight in this study.

Larger sclerotia contained more water than the smaller sclerotia, and therefore, the first lost more weight which is observed in the tables 2 and 4. A possible loss of nutrients to the *P. omnivora* sclerotia could explain the why the fungus (after being dried) was invaded by Mucoraceae molds (dates did not show). According to the size versus initial weight of sclerotia, they lose weight e.g. large sclerotia

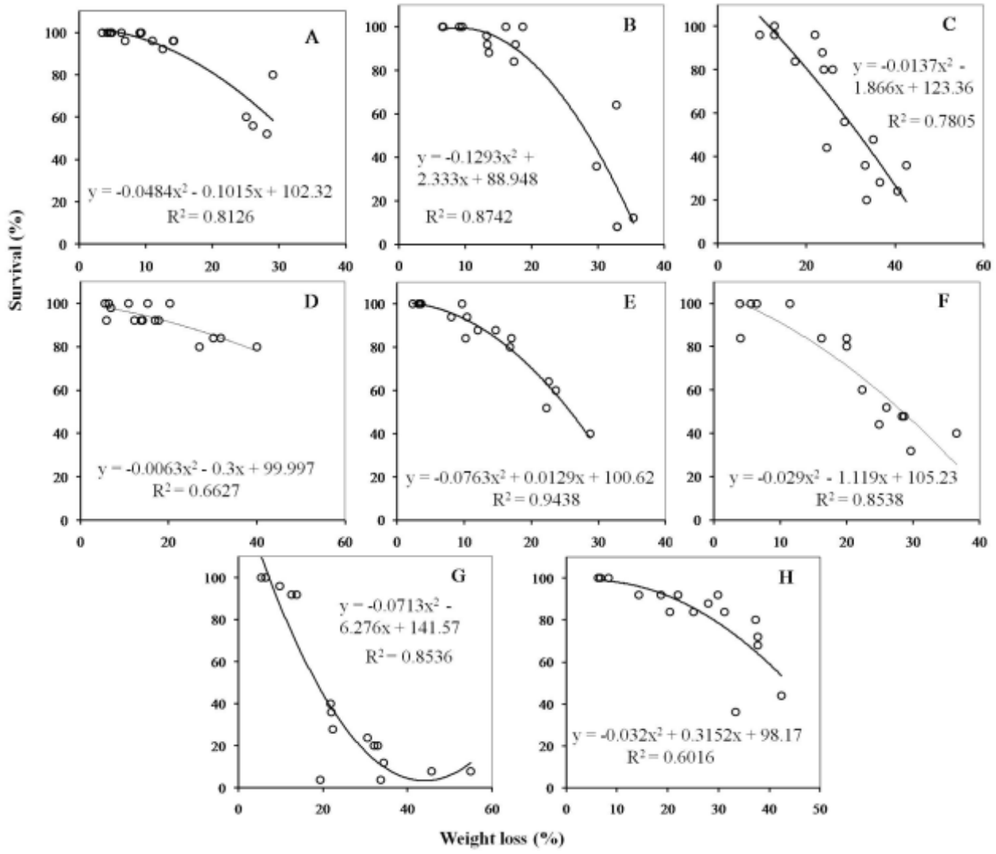

Figure 4. Survival of sclerotia of *P. omnivora* with respect to their weight loss (as percentage of the initial weight). A, B, C and D, large, medium, small and albino sclerotia, respectively to the first experiment. E, F, G and H, same type of the sclerotia to the second experiment.

lost an average of 29.3 mg, and small sclerotia only 10.4 mg (Table 1); meanwhile initial average weight of large and small sclerotia were of 239.2 and 41.7 mg, respectively (Table 2). So, the effect of drying in the initial weight loss of sclerotia may be proportionally minor in the larger sclerotia than in the smaller sclerotia. This behavior may explain the results of the Figure 1.

In this study was observed that sclerotia of *P. omnivora* began to decrease their survival since 15 minutes of drying and all sclerotia died after two hours of drying, this result differ from the reported by King and Eaton (1934). A probable difference between studies may be the type of sclerotia used; sclerotia of natural origin could have differences in melanin (more melanin) with respect on sclerotia artificially reproduced. The melanins in fungi allowed tolerate drying (Bell and Wheeler, 1986).

Drying time affected survival sclerotia, but each type of sclerotia was affected in a different way. Sclerotia of larger size (initial weight) were less affected in their survival; except albino sclerotia, which had a weight loss similar to medium sclerotia, and the highest survival of all type of sclerotia. We observed that albino sclerotia acquired pigmentation few minutes after putting them over sand where their survival was evaluated. The major capacity of survival albino sclerotia (after drying) could be due to the formation of melanin. This idea is supports by Abo Ellil (1999) who found that melanins are simultaneously formed when mycelium and sclerotia of *S. rolfsii* mature. The melanins are compounds that protect the fungi of the drying, uv radiation, attack for microorganisms, among others (Bell and Wheeler, 1986). Soluble pigment of *P. omnivora* sclerotia can be excreted to the media; this has been observed by us in sclerotia maintained in distilled water. Rush and Lyda, (1982) also mentioned the excretion of soluble pigment of exposed sclerotia to anhydrous ammonia. Also is probable that drying sclerotia may suffer some change in their melanins, doing them susceptible to antagonistic microbiota of the soil.

Excreted pigment by sclerotia fungi has been recorded (Bell and Wheeler, 1986). Some pigments excreted by *Sclerotinia sclerotiorum* (Lib.) de Bary are precursors in the synthesis of melanins or indicate an inhibition of this one (Butler *et al*., 2009). *S. sclerotiorum* produces a dark pigment presumably melanin (Sanogo and Puppala, 2007). The melanin plays an important function as defense of sclerotia of *P. omnivore*, however, there are not many studies in this topic. Differences in production, excretion or structure in melanin of sclerotia of *P. omnivora* isolated from soil or produced in the laboratory could explain the difference in survival of both type of sclerotia.

Our results indicate that stress treatments compared to drying did not decrease significantly the survival of sclerotia. In previous reports, Samaniego-Gaxiola (1994, 2008b), flooded soils amended with glucose, sodium hypochlorite solution, and moist soil decreases the survival of sclerotia of *P. omnivora* of way similar to that observed in this study. This suggests that the sclerotia primarily die before applying stress treatments.

The effect of the reproduction and management of the sclerotia of *P. omnivora* obtained in laboratory may be associated with its response to drying and other stresses, although, there are not studies on the subject. Sclerotia of *Corticium rolfsii* syno. *Athelia rolfsii* Tu & Kimbr. and mycelium of *Sporothrix schenckii* reproduced in different conditions of light and culture media, showed different sizes of cells and accumulated melanins (Romero-Martínez *et al*., 2000; Motomura *et al*., 1992).

The correlation between weight loss of sclerotia and their survival suggest a possible loss of nutriments the sclerotia to their surrounding, which may attract to soil microbiota that subsequently could invade the sclerotia. Excretions of sclerotia of *S. rolfsii* and *P. omnivora* were related with their susceptibility to be attacked by *Trichoderma harzianum* Rifai and soil mycobiota (Henis and Papavizas, 1983; Samaniego-Gaxiola, 1994; Samaniego-Gaxiola and Rivera-González, 1992). In this study, we observed that the surface of sclerotia of *P. omnivora* (that did not survive after being dried) was invaded by mold (Mucoraceae), this support the idea of nutriment loss by the sclerotia; because is know that the Mucoracae invades easily substrates containing simple carbohydrates (Dighton, 2003).

The results obtained here explain partly why deep-chiseled was a more efficient method than the fumigation with anhydrous ammonia to control *P. omnivora* in cotton (Rush, 1984), this may due to the exposure of sclerotia and strand to drying. The effect of the humidity over strand and sclerotia growth or germination has been studied (King and Eaton, 1934; Wheeler and Hine, 1972; Rush, 1984; Stapper *et al*., 1984). These survey and the results obtained here, suggest that the sclerotia and strand are very susceptible to drying, and that this could be implemented to diminish the inoculum of the fungus in the soil, and consequently, decrease the Phymatotrichopsis root rot.

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