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Techniques for Inoculation of *Sclerotium rolfsii* on *Neomarica longifolia* and *Evolvulus pusillus* in Brazil

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Abstract

The causal agent of stem rot was identified on plants of *Neomarica longifolia* and *Evolvulus pusillus*, in the State of Rio de Janeiro and the most efficient inoculation method is proposed. Symptomatic plants collected and fungal structures were transferred to PDA for identification. Two isolates were obtained, H1 (*N. longifolia*) and H2 (*E. pusillus*), and identified as *Sclerotium rolfsii*. *In vitro* culture development at 25ºC/dark was observed daily. Both isolates showed a linear development, where H1 showed faster, however the sclerotia formation was first observed in H2. Six essays were performed with the following inoculation methods: I) 25 mL of mycelium solution in soil; II) Mycelial disc at basal portion of plant, without injury; III) Mycelial disc at basal portion of plant with injury; IV) Toothpicks wrapped by mycelia inserted at basal portion of plant; V) Clothespins wrapped by mycelia inserted at basal portion of plant; VI) Leaf host with mycelium placed on soil pots, close to roots and VII) Leaf host with mycelium placed in the soil pots. The most efficient methods were IV and V.

Keywords: *Basidiomycetes; Etiology Inoculation Methods; Ornamental Plant Diseases*

Introduction

The species *Neomarica longifolia* (Link and Otto) Sprague (Iridaceae) (as Yellow Iris) and *Evolvulus pusillus* Choisy (Convolvulaceae) (Dew drop) are herbaceous ornamental plants native of Brazilian flora and used in landscape gardens in the State of Rio de Janeiro, that is considered one of the main representative examples of national floriculture, which continues to grow [1]. The cultivated ornamental plants are susceptible to a large number of fungal diseases that can be a limiting factor for this sector of the economy [2,3].

The diseases can be observed at several parts of plants, which can be leaves, stems, flowers, fruits and root. Among the plant pathogens that cause root rot and stem rot are *Sclerotium rolfsii* Sacc. [4-7], which is responsible by stem lap rot of more than 500 plant species including vegetables, ornamentals, grasses and forage plants [8]. There are reports of Iridaceaee (*Iris* sp.) and Convolvulaceae (*Dichontra repens* J.R. Forst. and G. Forst) [9-11] as hosts of *S. rolfsii* and it was previously reported in Brazil by Rezende., *et al* [12].

This work aimed show this fungus species associated to stem rot in these ornamental plants found in the gardens of Barra da Tijuca, Rio de Janeiro, Brazil and to evaluate distinct inoculation techniques to obtain a proper protocol for pathogenicity tests.

Material and Methods

On culture

From October 2012 to February 2013, plants of "Yellow Iris" and "Dew drop", showing symptoms of stem lap rot, were collected from the urban gardens of Barra da Tijuca (23º0'00" N, 43º21'00" E) in the city of Rio de Janeiro. Fungal structures (sclerotia and hyphae) were

isolated from culture media of Potato-Dextrose-Agar (PDA), supplemented with antibiotic SEPTIPEN PLUS (Benzylpenicillin, Streptomycin) and kept at incubation chamber and at B.O.D (biochemical oxygen demand), both with regulated temperature to 25°C, in the dark. The colony development was observed daily.

The isolates where labeled as H1 and H2, for the plants of *N. longifolia* and *E. pusillus*, respectively. After viewing its morphological and cultural characters under microscope (optical and dissecting), the identification of isolates was performed and in the sequence outlined the pathogenicity essays.

Pathogenicity Test

The pathogenicity tests were performed at semi-opened greenhouse at different periods, using inoculation methods based on techniques established by Shokes., *et al*. [13]: I) Mycelial solution - 25 mL of mycelial solution (two plates with fungi colonies into 150 mL of distilled sterile water) applied to the soil near the stem; II) Mycelial disc adhered to the basal portion of the plant, without injury (WOI); III) Mycelial disc adhered to the basal portion of the plant, with injury (WII); IV) toothpicks wrapped by mycelia inserted at basal portion of plant; V) Clothespins wrapped by mycelia basal portion of plant; VI) Leaf host (*N. longifolia*) previously colonized by mycelia placed on soil pots, close to roots; and VII) leaf host (*N. longifolia*) previously colonized with mycelia placed in the soil pots.

The seedlings was individually cultivated in plastic pots (5L), with sterilized soil, sand texture, well drained and supplemented with cattle manure (1:1:1). The seedlings were irrigated manually once a day or more often in warmer weather. For inoculum production, fungal colonies were incubated in B.O.D during 30 days for development of mycelia and sclerotia. The inoculation occurred in 60 days after planting and the plants were kept for 48 hours in wet chamber; 3 plants were used for inoculation, plus the plant control.

The disease severity was evaluated using a scale note as following: 0 (without symptoms); 1 (plants with leaves beginning of yellowing); 2 (yellowing and slightly rot of the plant´s lap); 3 (yellowing and drying of some leaves, with sclerotia in the pot); 4 (evident rot, with damping-off and several sclerotia) and 5 (all of the symptoms described and damping-off, almost died). The severity was multiplied by the number of plants showing symptoms of disease, forming a new variable, which was integrated in time to calculate the Area Under Disease Progress Curve (AUDPC) [14].

During March 2013 to January 2014, six assays were performed: 1 – Isolate H1 x method I (evaluated at 2; 5; 10 and 15 days after inoculation - DAI, from March 27th to April 10th); 2 - H1 x Method II, III and IV (evaluated at 2; 5; 10 e 15 DAI, from April 18th to May 3rd); 3 - H2 x Method I, II, III and IV (evaluated at 2; 5; 10 and 15 DAI, from June 12th to June 27th); 4 - H1 and H2 x method I; II; III; IV and V (evaluated at 2; 5; 10, 15 and 25 DAI, from July 1st to July 26th); 5 - H2 x method II and III (evaluated at 2, 20 and 60 DAI, from July 19th to September 17th) and 6 - H1 and H2 x Method II; III; IV; V; VI e VII (evaluated at 2; 5; 10, 15 and 30 DAI, from December 12th to January 11th).

Environmental conditions (temperature and relative humidity-RH) during the experiment were obtained. For essay 5 were used seedlings of *E. pusillus* and for the others *N. longifolia*. The design was completely randomized with three repetitions. The t test was used for comparison among two treatments and Tukey test for comparison of three treatments or more ($p < 0.05$) (Sisvar version 5.3).

Results and Discussion

The plant samples collected from gardens showed dark lesions at lower portion of plants, showing whitish mycelia with several brown, globose sclerotia. Under optical microscope it was observed hyaline hyphae with thin cell walls and transversal cells. The main hyphae presented clamp connection. After seen fungal structures from plants of “Yellow Iris” and “Dew Drop” and those cultured in Petri Dish both isolates H1 and H2 were identified as *S. rolfsii* [4-7].

On Culture

Colonies cultivated in growth chamber presented after 24h of incubation, 6 and 3 mm of diameter for the isolates H1 and H2 respectively. Both isolates showed a linear mycelial growth and formed sclerotia at 5 DAI for H2 and 16 DAI for H1. The isolates incubated in

B.O.D. showed slower development. After 24 hours no mycelial growth was observed for H1 and the colonies of H2 showed nearly 2 mm diameter. The sclerotia formation occurred after 16 and 26 DAI for H2 and H1, respectively. The faster growth in the growth chamber was due to temperature control more constant because the equipment was smaller and less used.

Pathogenicity Test

In the first essay, no infection was observed on inoculated plants using mycelial solution (method I) (Table 1). At essay 2, the plants inoculated with H1 had more disease development without hurting (method II). While in the essay 3, the infection of H2 was favored by the presence of wounds (methods III and IV). In the essay 4, plants inoculated with H1, the method III of inoculation was more effective when compared with I and IV. No statistical differences among the inoculation methods for isolate H2.

At essay 4, plants inoculated with H1 showed higher AUDPC, when compared with H2, with inoculation methods II, III and V (Table 1). No difference among isolates was observed using the methods I and IV. The difference among isolates of *S. rolfsii*, using vegetative compatibility has been tested by researches as Serra and Silva [15] which used PDA to evaluate the genetic variability while Remesal and Navas-Cortes [16] used the Patterson Modified Media increased with 25% glucose in relation to original medium at rate of 23.4 g/L with addition with red dye (180 µL/L).

Inoculation Methods[1]	Area under disease progress curve (AUDPC)						
	Essay 2	Essay 3	Essay 4[3]		Essay 5	Essay 6	
	H1[x]	H2[x]	H1[x]	H2[x]	H2[y]	H1[x]	H2[x]
I. Mycelial Solution	--	0.00 c	1.67 A c	3.33 A a	--	--	--
II. Mycelial Disc WOI	26.00 a	17.33 b	21.67 A abc	0.00 B a	63.00 b	49.00 A b	65.50 A bc
III. Mycelial Disc WII	22.00 b	43.00 a	42.67 A a	2.50 B a	187.00 a	65.50 B b	86.33 A b
IV. Toothpicks inserction	20.17 b	34.00 a	11.50 A bc	8.00 A a	--	110.50 A a	122.50 A a
V. Clothespins inserction	--	--	29.00 A ab	11.33 B a	--	110.50 A a	112.50 A a
VI. Colonized leaves on soil	--	--	--	--	--	49.16 A b	64.33 A bc
VII. Colonized leaves in soil	--	--	--	--	--	23.33 B c	49.17 A c
Variation source							
$QM_{isolate}$ (GL)	--	--	39.91 (1)		--	2131.36 (1)	
$QM_{inoculation}$ (GL)	26.69 (2)	1080.75 (3)	6.56 (4)		23064.00 (1)	6242.56 (5)	
$QM_{interation}$	--	--	6.79 (4)		--	99.23 (5)	
QM_{error} (GL)	2.27 (6)	18.64 (8)	1.75 (20)		1593.50 (4)	102.07 (24)	
CV (%)	6.64	18.31	42.13		31.93	13.35	
p-valor $_{isolate}$	--	--	0.0001		--	0.0001	
p-valor $_{inoculation}$	0.0085	0.0000	0.0199		0.0190	0.0000	
p-valor $_{interation}$	--	--	0.0174		--	0.4546	

Table 1: *Plants of Neomarica longifolia[x] and Evolvulus pusillus[y] inoculated with isolates H1 and H2 of Sclerotium rolfsii under greenhouse condition. Seropédica-RJ Brazil 2015*

[1]I: 25 mL mycelial solution (2 plates containing fungi colonies / 150 mL distilled sterile water) applied to the soil near the stem; II: mycelial disc adhered to the basal portion of the plant, without injury (WOI); III: mycelial disc adhered to the basal portion of the plant, with injury (WII); IV: toothpicks wrapped by mycelia inserted at basal portion of plant; V: clothespins wrapped by mycelia basal portion of plant; VI: leaf host previously colonized by mycelia placed on soil pots, close to roots; and VII: leaf host previously colonized with mycelia placed in the soil pots, near roots.

[2]Means followed different letters differ among themselves by Tukey test ($p < 0.05$).

[3]Data transformed by $\sqrt{(x+1)}$. – No data.

The stem rot development at essays 1, 3 and 4 was lower on plants inoculated with mycelial solution (method I), especially for isolate H1 (note: in addition to inefficiency of this inoculation method, this observation may be related to environmental conditions). According to Pinheiro., *et al*. [17], the development of this fungus on soybean, corn and wheat straw is favored by high humidity of soil (70%) and temperatures between 25 to 30ºC. During the essay 1, the environmental condition of high temperature and high relative humidity are considered favorable to disease development. Thus, an absence of infection can be attributed to two factors, the ineffectiveness of the inoculation method and a high RH has avoided the wilting of the plant (RH average ~80%, table 2).

Essay – isolate x inoculation method (period)	Temperature (ºC)			Relative Humity (%)		
	Maximum	Minimum	Average	Maximum	Minimum	Average
1 - H1 x method I (27/03 to 08/04/2013)[x]	32.00	25.00	25.85	85.00	71.00	80.60
2 - H1 x method II, III and V (18/04 to 03/05/2013)[x]	31.00	20.75	24.00	76.00	68.00	71.50
3 - H2 x method I, II, III and IV (12/06 to 27/06/2013)[x]	29.00	20.00	23.26	76.75	56.75	71.71
4 - H1 and H2 x method I; II; III; IV and V (01/07 to 26/07/2013)[x]	26.55	19.25	21.83	85.00	64.00	72.00
5 - H2 x method II and III (19/07 to 17/09/2013)[y]	27.00	18.00	21.00	85.00	53.00	67.07
6 - H1 and H2 x method II; III; IV; V; VI and VII (12/12/2013 to 11/01/2014)[x]	33.50	23.50	25.00	80.00	63.00	72.17

Table 2: *Environmental conditions during the essays with plants of Neomarica longifolia[x] and Evolvulus pusillus[y] inoculated with isolates H1 and H2 of Sclerotium rolfsii under greenhouse condition. Seropédica-RJ Brazil 2015*

[1]I: 25 mL mycelial solution (2 plates containing fungi colonies / 150 mL distilled sterile water) applied to the soil near the stem; II) Mycelial disc adhered to the basal portion of the plant, without injury (WOI); III) Mycelial disc adhered to the basal portion of the plant, with injury (WII); IV) toothpicks wrapped by mycelia inserted at basal portion of plant; V) Clothespins wrapped by mycelia basal portion of plant; VI) Leaf host previously colonized by mycelia placed on soil pots, close to roots; and VII) leaf host previously colonized with mycelia placed in the soil pots, near roots.

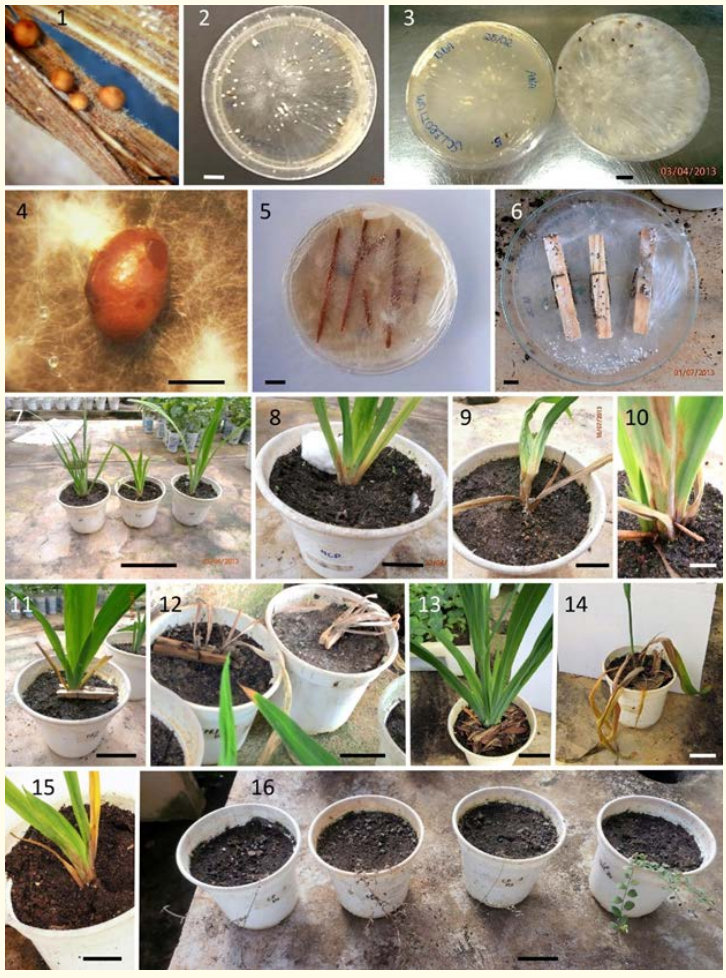
The inefficiency of method I should be due to concentration of solution used. Barbosa., *et al.* [18] when inoculating this fungus in plants of tomato, used soil with sclerotia or mycelial discs, deposited at surface of substrate at 1 cm from plant, with 2, 4, 6 or 8 propagules/treatment; the authors also used rice grain colonized and incorporated into soil before the seedlings transplant at concentrations of 2, 4, 8 or 16 g L^{-1} of soil/treatment. They observed that the deposition of mycelial discs and sclerotia did not cause disease incidence. In contrast, the concentration of 8 g L-1 of soil was considered ideal for inoculation of this pathogen on tomatoes.

At essay 5, the presence of wounds (III) promoted the establishment of do *S. rolfsii* (Table 1). AUDPC was higher in plants inoculated with isolate H2 than H1, with methods III and VII (essay 6). For both isolates, the best inoculation methods were IV and V, these being indicated for this pathosystem. The higher values of AUDPC in this essay can be related to environmental condition. Higher temperatures and RH around 60% may have contributed for the symptoms development. According to Punja [19], the optimal temperature for mycelial growth and sclerotia formation of *S. rolfsii* is between 27 - 30ºC and the high humidity is important only during some stages of life cycle of pathogen, being unfavorable for other stages, since that the pathogen is aerobic.

During the study, the maximum temperature ranged of 26.55 until 33.5ºC, minimum temperature from 18 until 23.50ºC and the average 21 to 25.85ºC (table 2). The RH ranged from 76 to 85% (maximum), 53 to 71% (minimum) and 67.07 to 80.60% (average). In this case, noted that a large variation between a maximum and a minimum relative may have favored the disease development. At essay 4 was found infection at plants inoculated with mycelial solution, which temperatures were milder (26.55; 19.25 and 25.85ºC for maximum, minimum and average, respectively) and RH lower (64 and 72% for minimum and average), when compared to essay 1 (temperature: 32;

25 and 25.85ºC for maximum, minimum and average, respectively/RH: 71 and 80.60% for minimum and average). In addition, the variation between RH maximum and RH minimum was higher in essay 4 (21%) than in essay 1 (14%).

According the results of this study, for inoculation of *S. rolfsii* on plants of *N. longifolia* and *E. pusillus* it is recommended to insert sticks and preachers colonized with mycelium at plant base; and the installation of essays during periods of temperatures between 25- (~33ºC), RH of 63 to 80% and variation between the RH maximum and minimum higher than 15%.



Figures 1-16: *Sclerotium rolfsii em N. longifolia e E. pusillus. 1. Symptomatic leaves of N. longifolia with sclerotia (Bar = 1 mm). 2. Colonies of isolate 1 (Neomarica longifolia) after 12 days after incubation (Bar = 1 cm). 3. Colony after 37 incubation days on PDA (Bar = 1 cm). 4. Detail of sclerotium (Bar = 1 mm). 5. Toothpicks incubated with S. rolfsii on PDA (Bar = 1 cm). 6. Clothespins incubated with S. rolfsii on PDA (Bar = 1 cm). 7. Plants inoculated with mycelial disc at basal portion (Bar = 15 cm). 8. Symptoms after 12 days with mycelial disc (Bar = 3 cm). 9. Symptoms after 20 days with mycelial disc (Bar = 3 cm). 10. Symptoms after 10 days inoculation using toothpicks (Bar = 1 cm). 11. Plants inoculated with clothespin colonized by S. rolfsii (Bar= 5 cm). 12. Symptoms after 15 days inoculated with clothespins (Bar = 5 cm). 13. Colonized leaf host on soil pot around Neomarica seedling (Bar = 5 cm). 14. Symptoms after inoculated with colonized leaves on soil pot (Method VI) (Bar = 5 cm). 15. Symptom of Neomarica inoculated with mycelial disc (Bar = 5 cm). 16. Plants of Evolvulus pulsillus after inoculated with S. rolfsii with inoculation method III (Bar = 5 cm).*

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