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Assays for Oxidative Responses of *Fusarium graminearum* Strains to Superoxide Radicals

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[Abstract] The ascomycete fungus *Fusarium graminearum* is a major causal agent of *Fusarium* head blight (FHB), a devastating disease affecting small grains cereals worldwide. To better understand the pathogenesis of this fungus, we provide here an easy-to-use protocol to examine the sensitivity of the wild-type and mutant strains of *F. graminearum* to oxidative stress from superoxide anions ($O_2^{\bullet-}$) generated by menadione. Similarly, this assay can also be used to detect other stress responses of different fungal strains to various stress agents. The change in stress response of a mutant can offer a clue for the biological function of mutated genes.

Keywords: *Fusarium graminearum*, Mutant, Mycelial disc, Sensitivity, Oxidative stress

[Background] The ascomycete fungus *Fusarium graminearum* (previously also called *Gibberella zeae* for its sexual state) is not only the major causal agent of *Fusarium* head blight and seedling blight on wheat and barley, but also one of the important causal agents of Gibberella stalk rot on maize (Dal Bello *et al.*, 2002; Bai and Shaner, 2004; Kazan *et al.*, 2012). Apart from causing a huge yield loss of cereals, this fungus also produces mycotoxins which affect human and animal health. Therefore, this fungus has received extensive attention, and ranked the fourth among all investigated plant pathogenic fungi (Dean *et al.,* 2012).

F. graminearum overwinters on dead organic matter, particularly on infected crop residues of small grains and corn. To survive such a wide range of environment, *F. graminearum* has evolved the capacity to confront various stresses. Numerous genes in *F. graminearum* have been explored for their roles in counteracting stress treatment, and the resulting mutants exhibited diverse responses to the stresses, which indicated the association of the stress responses and pathogenicity (Son *et al.,* 2011). Some of the frequently used stress agents that act on cell wall or cell membrane of fungi include oxidative stress agents (*e.g.,* menadione and H_2O_2), cell wall-perturbing agents (*e.g.,* Congo red), and membrane stress agents (*e.g.,* SDS). Detection of the stress responses of a fungal mutant strain could provide a clue for further investigating pathogenetic function. Thus, we take oxidative stress treatment as an example to describe a reliable protocol to assess stress response of *F. graminearum* to a superoxide radical generating agent menadione (Kawamura *et al.,* 2006). This protocol can be used to detect other stress responses of a fungal strain in an analogous procedure.

Materials and Reagents

1. Pipette tips (Corning, Axygen®, catalog number: T-300-R-S)
2. 9 cm Petri plates
3. Sterile toothpick
4. Parafilm (Bemis, catalog number: PM996)
5. Fungal strains: *F. graminearum* wild-type strain PH-1 (NRRL 31084), and its deletion mutant △*sod1* (Yao *et al.,* 2016)
6. 75% alcohol (Sinopharm Chemical Reagent, catalog number: 80176961)
7. Menadione (also named Vitamin K3, Sangon Biotech, catalog number: A502486-100g)
8. V8 vegetable juice (CAMPBELL, V8® ORIGINAL)
9. Absolute ethanol (Sinopharm Chemical Reagent, catalog number: 10009259)
10. $CaCO_3$ (Acros Organics, catalog number: 403811000)
11. Double distilled water
12. Agar powder (Oxoid, catalog number: LP0011)
13. $NaNO_3$ (Sinopharm Chemical Reagent, catalog number: 10019918)
14. KH_2PO_4 (Sinopharm Chemical Reagent, catalog number: 10017618)
15. $MgSO_4·7H_2O$ (Sinopharm Chemical Reagent, catalog number: 10013018)
16. KCl (Sinopharm Chemical Reagent, catalog number: 10016318)
17. Sucrose (Sinopharm Chemical Reagent, catalog number: 10021418)
18. N-Z Amine (Casein acid hydrolysate) (Macklin, catalog number: C822594)
19. Yeast extract (Oxoid, catalog number: LP0021)
20. Inositol (Sinopharm Chemical Reagent, catalog number: 63007734)
21. Ca pantothenate (TCI Shanghai, catalog number: P0012)
22. Choline·Cl (Sinopharm Chemical Reagent, catalog number: 69008560）
23. Thiamine (Shanghai Bo'ao Biological Technology, catalog number: H1230)
24. Pyridoxine (Shanghai Bo'ao Biological Technology, catalog number: H0970)
25. Nicotinamide (Shanghai Bo'ao Biological Technology, catalog number: H0850)
26. Ascorbic acid (Sinopharm Chemical Reagent, catalog number: 10004014)
27. Riboflavin (Sinopharm Chemical Reagent, catalog number: 67001734)
28. *p*-aminobenzoic acid (Sinopharm Chemical Reagent, catalog number: 31000116)
29. Folic acid (Shanghai Bo'ao Biological Technology, catalog number: H0550)
30. Biotin (Shanghai Bo'ao Biological Technology, catalog number: H50)
31. Citric acid (TCI Shanghai, catalog number: C1949)
32. $ZnSO_4·7H_2O$ (Sinopharm Chemical Reagent, catalog number: 10024018)
33. $CuSO_4·5H_2O$ (Sinopharm Chemical Reagent, catalog number: 10008218)
34. $Fe(NH_4)_2(SO_4)_2·6H_2O$ (Beijing Ouhe Technology, catalog number: 01000313)
35. $MnSO_4$ (Sinopharm Chemical Reagent, catalog number: 10013418)
36. H_3BO_3 (Sinopharm Chemical Reagent, catalog number: 10004808)

37. $Na_2MoO_4 \cdot 2H_2O$ (Sinopharm Chemical Reagent, catalog number: 10019818)
38. 30 mM menadione stock solution (see Recipes)
39. V8 juice agar medium (Yao *et al.*, 2016; see Recipes)
40. CM medium (Yao *et al.*, 2016; see Recipes)
41. Vitamin stock solution (see Recipes)
42. Trace element solution (see Recipes)

Equipment

1. 500 ml flask
2. Pipettes (Eppendorf)
3. Incubator (Yiheng, model: MJ-150I)
4. Biological safety cabinet (ESCO Micro, model: FHC1200A)
5. Camera (Canon, model: EOS 7D)
6. Autoclave (Zealway Instrument, model: GI54DWS)
7. Ruler
8. Microwave oven (Galanz, model: G70F23N1P-M8(SO))

Software

1. ImageJ software (http://rsbweb.nih.gov/ij/index.html)
2. Microsoft Excel

Procedure

Note: This procedure should be carried out following aseptic techniques, working in a biological safety cabinet.

1. Preparation of *F. graminearum* mycelial discs
   Pick up a bit mycelia of the wild-type strain PH-1 and the mutant △*sod1* and deposit into the middle of a V8 juice agar plate, respectively. Seal the plates with Parafilm and incubate in a 25 °C incubator for 3 days.
   Note: Operations in Step 1 should be carried out in a biological safety cabinet that has been sterilized under UV-light for at least 20 min.
2. Preparation of 30 mM menadione stock solution (see Recipe 1).
3. Prepare the complete medium (CM) beforehand as the recipe provided below, and store at room temperature for further use. Thaw CM agar medium in flask completely in microwave oven, and cool to 50 °C. Then add the menadione to a final concentration of 30 μM into the flask and shake to make menadione well-distributed in the medium. Subsequently pour plates. At the same time, pour the control CM plate without adding menadione as the control.

Note: To keep the activity of menadione, make sure that CM agar medium cool to 50 °C before adding it. Alternatively, consider to examine oxidative stress responses at different concentrations of menadione.

4. Use a yellow 200-µl pipette tip as a cork borer, then hold the tip of a sterile 200-µl pipette tip with fingers to cut mycelial discs (0.5 cm diameter) from the edge of an actively growing colony of PH-1 or △*sod1* from V8 agar plates (Figures 1A-1C). Place one mycelial disc in the center of a CM agar plate amended with 30 µM menadione as the experimental treatment (Figure 2A), and without the addition of menadione as the control. And make sure that the mycelial side of the disc should be in contact with the surface of the medium (Figure 2B). Lastly, incubate in a 25 °C incubator for 4 days. At least three CM agar plates in the experiment treatment or the control are performed for each strain.

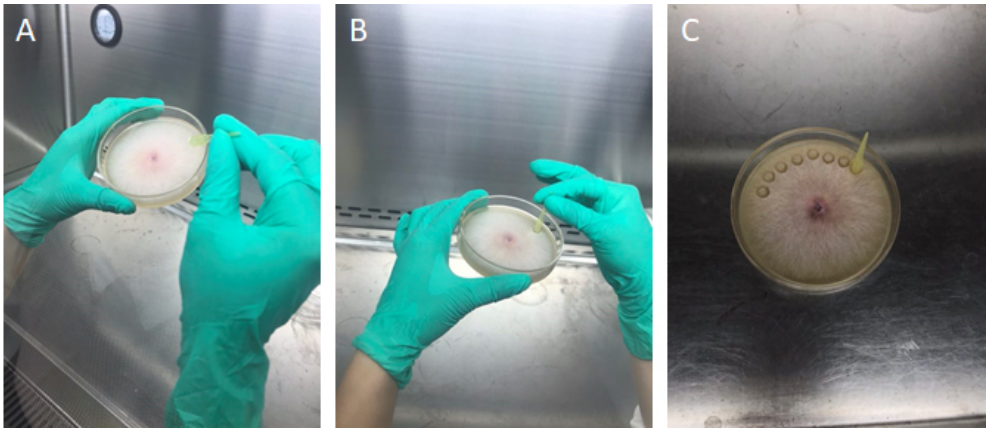

Figure 1. Preparation of *F. graminearum* mycelial discs. Cut mycelial discs (0.5 cm) aseptically using a sterile 200-µl pipette tip as a cork borer from the periphery of a 3-day-old colony grown in V8 agar plates (A, B and C).

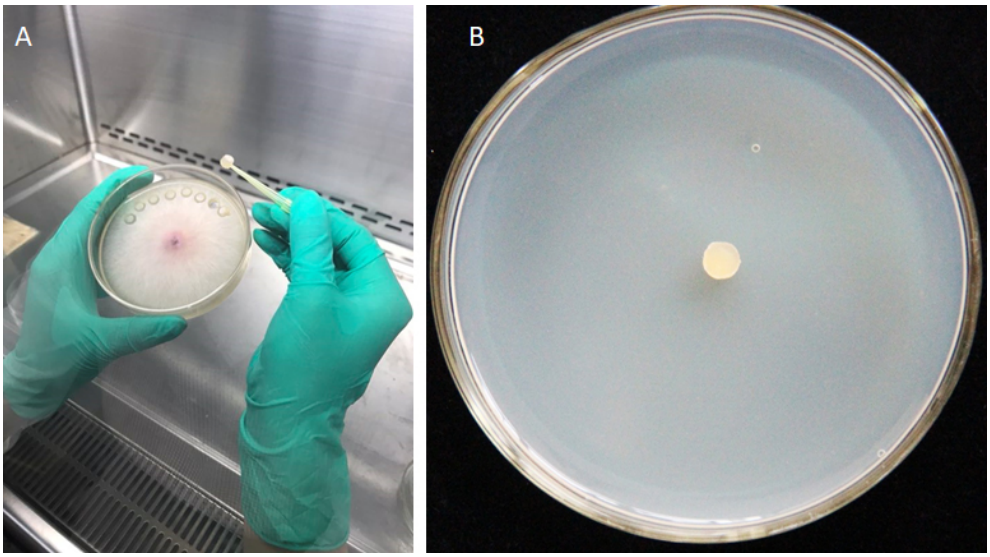

Figure 2. Transfer a mycelial disc into the center of a CM agar plate. A. Pick up a mycelial disc with a pipette tip. B. Place a mycelial disc in the center of the plate and make the mycelial side of the disc attached to the surface of the medium.

5. The cultures should be checked daily. When the growth of the colony on the control approaches the edge of the plate, photograph these plates with a ruler as a reference, as shown in Figure 3.

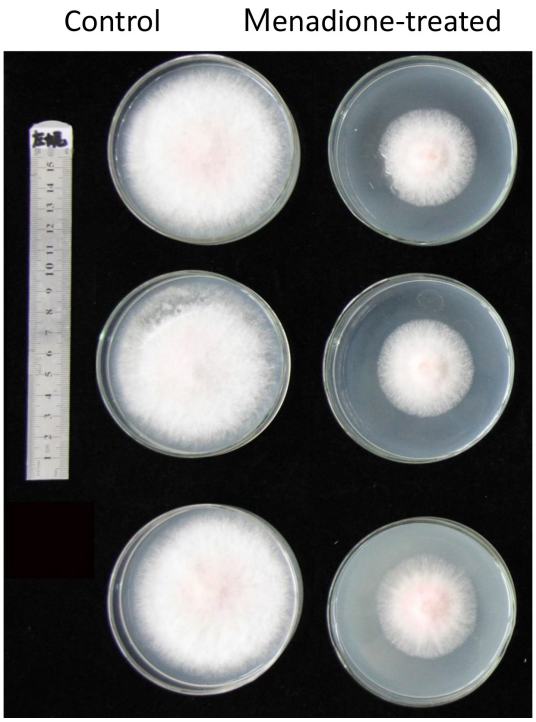

Figure 3. Images of the colony of wild type (WT) grown for 4 days on the control and menadione-treated plates. When the growth of the colony on the control nears the edge of the plate, photograph plates with a ruler as a reference.

Data analysis

1. The colony diameter in each plate was measured using ImageJ software (http://rsbweb.nih.gov/ij/index.html). If a colony is not perfectly round, measure its diameter at several points and use the mean in subsequent calculation. Open the photo in ImageJ (Figure 4A) and choose straight line option to measure 1 cm of the ruler in the photo as scale, then set the length as 1 cm (Analyze → Set Scale → known distance and unit) (Figure 4B). Lastly, measure colony diameter of wild type and mutant successively (Figure 4C) and export the data to Excel sheet (Figure 5).

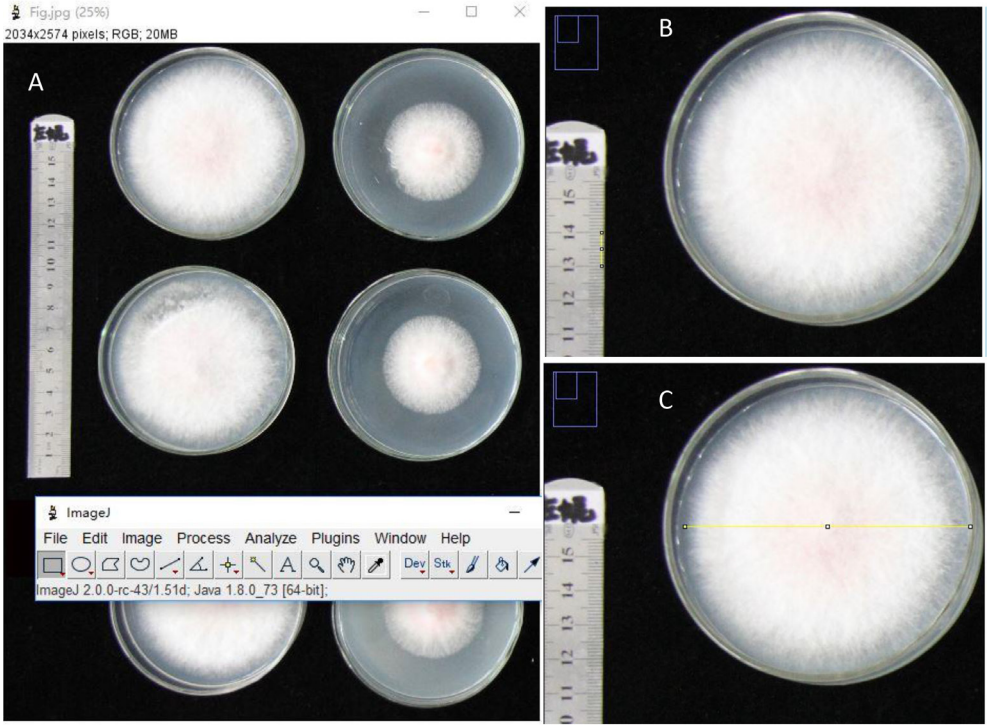

Figure 4. Measurement of colony diameter using ImageJ software. A. ImageJ software window shows the image of the colony of wild type (WT) grown for 4 days on the control and menadione-treated plates. B. Set scale using the ruler in the image. C. Measurement of the colony diameter.

	F	G	H	I	J
		Exp. 1 Colony diameter(cm)			
		PH-1 control	PH-1 treated	△*sod1*	△*sod1 treated*
		8.8	8	8.8	4.6
		8.8	7.9	8.8	3.2
		8.8	7.8	8.8	4.7
	Means	8.8	7.9	8.8	4.17
		Exp. 2 Colony diameter(cm)			
		PH-1 control	PH-1 treated	△*sod1*	△*sod1 treated*
		8.8	6.2	8.5	4.2
		8.8	6.2	8.6	4.8
		8.8	6.3	8.2	4.8
	Means	8.8	6.23	8.43	4.6
		Exp. 3 Colony diameter(cm)			
		PH-1 control	PH-1 treated	△*sod1*	△*sod1 treated*
		8.8	6.8	7.9	2.8
		8.8	6.4	8.8	3.4
		8.8	6.7	8	3.4
	Means	8.8	6.63	8.23	3.2

Figure 5. Data of colony diameter of wild type (PH-1) and mutant (△*sod1*) grown on the control and menadione-treated plates

2. Assess the sensitivity of the wild type and mutant to 30 μM menadione by analyzing the percentage of mycelial radial relative growth inhibition (RGI) of treated colony, as of control. For the relative growth inhibition is calculated using the following equation:

$$RGI = [(Dc - Dt)/(Dc - 0.5)] \times 100$$

where Dc is the mean colony diameter for the control and Dt is the mean colony diameter for the experimental treatment. 0.5 cm should be subtracted from the diameter of each colony because this was the original mycelial disc diameter at the beginning of the procedure. Student's *t*-test was used to analyze the difference in relative growth inhibition of wild type and mutant with Microsoft Excel (Figure 6). Statistical analysis showed that there is significant difference in RGI of PH-1 and △*sod1* at $P < 0.05$ level, suggesting that the mutant is more sensitive than wild type to superoxide anions (Figure 7).

	L	M	N	O	P
		RGI calculation and *t*-test			
		PH-1 RGI	△*sod1* RGI		
	Exp.1	10.84%	56.63%		
	Exp.2	30.96%	48.30%		
	Exp.3	26.14%	65.07%		
	Means	22.65%	56.67%		
	SE	6.07%	5.43%		
	t-test	0.013165 (Significant difference)			

Figure 6. Calculation of relative growth inhibition (RGI) of wild type (PH-1) and mutant (△*sod1*) by 30 μM menadione

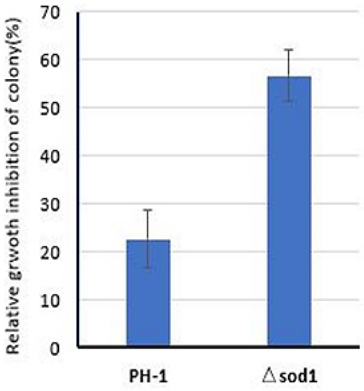

Figure 7. Relative growth inhibition of wild type (PH-1) and mutant (△*sod1*) was shown by Excel bar graph

Notes

To obtain enough discs of the same size, prepare at least two V8 agar plates for culturing each strain (Step 1).

Recipes

1. 30 mM menadione stock solution
   Dissolve 516.54 mg of menadione in a total volume of 100 ml absolute ethanol, and store this stock at 4 °C away from light
2. V8 juice agar medium
   168 ml V8 vegetable juice
   1 g $CaCO_3$
   Add up to 1 L with double distilled water
   Divide it into small aliquots, and add 15 g agar powder
   Autoclave at 121 °C for 20 min
3. Complete medium (CM)
   2 g $NaNO_3$
   1 g KH_2PO_4
   0.5 g $MgSO_4 \cdot 7H_2O$
   0.5 g KCl
   30 g Sucrose
   2.5 g N-Z Amine (Casein acid hydrolysate)
   1 g Yeast extract
   10 ml Vitamin stock solution
   0.2 ml Trace element solution

Add up to 1 L with double distilled water
Divide it into small aliquots, and add 20 g agar powder
Autoclave at 121 °C for 20 min

4. Vitamin stock solution
   4 g Inositol
   200 mg Ca pantothenate
   200 mg Choline·Cl
   100 mg Thiamine
   75 mg Pyridoxine
   75 mg Nicotinamide
   50 mg Ascorbic acid
   30 mg Riboflavin
   5 mg *p*-aminobenzoic acid
   5 mg Folic acid
   5 mg Biotin
   Add up to 1 L with 50:50 ethanol:H_2O, and store at 4 °C in the dark
5. Trace element solution
   5 g Citric acid (dissolve into water first)
   5 g $ZnSO_4 \cdot 7H_2O$
   1 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$
   250 mg $CuSO_4 \cdot 5H_2O$
   50 mg $MnSO_4$
   50 mg H_3BO_3
   50 mg $Na_2MoO_4 \cdot 2H_2O$
   Add up to 1 L with double distilled water, and store at 4 °C in the dark

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