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INTERRELATION OF OXALIC ACID FORMATION WITH PATHOGENICITY OF *SCLEROTINIA SCLEROTIORUM* (LIB.) DE BARY CAUSING WHITE MOLD DISEASE OF MUSTARD

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Abstract

Sclerotinia sclerotiorum (Lib.) de Bary, the causal agent of white mold disease of mustard is one of the most devastating plant pathogens. Production of oxalic acid, the key pathogenicity factor of *S. sclerotiorum* was studied with 15 isolates collected from different districts of Bangladesh. Potato dextrose agar and potato dextrose broth adjusted to pH 7.0 with bromophenol blue (Bb) was used to assess the acid production by *S. sclerotiorum* at 25 ± 1ºC in dark. Oxalic acid production is an evidence of changing color of the medium from blue to yellow. Isolates of *S. sclerotiorum* form Joypurhat *viz*., Joy1, Joy2 and Joy3 produced higher quantity of oxalic acid where the pH levels were measured by 3.10, 3.20 and 3.35, respectively and the color changed into blue to deep yellow while the isolates of Hobiganj *viz*., H1, H2 and H3 produced lower quantity of oxalic acid where the pH level were 4.30, 4.45 and 4.30, respectively and color changed from blue to purple. Oxalic acid productions by Kustia (K1, K2 and K3) and Jamalpur (J1, J2 and J3) isolates were intermediate and the color changed from blue to light yellow. The highly virulent isolates of *S. sclerotiorum* from Joypurhat (Joy1, Joy2 and Joy3) produced higher amount of oxalic acid (4.46, 4.50 and 4.24 mg/l). Isolates of Kustia *viz*., K1, K2 and K3 produced 3.88, 3.47 and 3.33 mg/l oxalic acid while the isolates of Jamalpur *viz*., J1, J2, J3 produced 3.45, 3.18 and 3.42 mg/l oxalic acid, respectively. Oxalic acid was poorly produced (2.38, 2.41, 2.46 mg/l) by the most weakly virulent isolates of Hobiganj (H1, H2 and H3). The moderately virulent isolates of Bogra (B1, B2 and B3) produced modest amount of oxalic acid (3.23, 3.35 and 3.07 mg/l).

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a devastating fungal pathogen that causes disease on many economically important vegetables, oilseed and pulse crops, infects over 500 species worldwide (Boland and Hall 1994, Saharan and Mehta 2008). In Bangladesh, *S. sclerotiorum* was first recorded on mustard in 2008 (Hossain *et al*. 2008), then on chilli, auber-gine and cabbage (Dey *et al*. 2008). Recently the disease is found to attack almost all the vegetables (Hyacinth bean), flowers (Mary-gold, gerbera) and fruit crops (jackfruit) and pulse croup (Lentil) (Prova *et al*. 2014, Rahman *et al*. 2015a, Rahman *et al*. 2015b and Ahmed and Akhond 2015). Stem rot is the most significant Sclerotinia disease in soybean and canola (*Brassica napus*). Sclerotinia disease can cause serious yield losses of crops including sunflower, canola and soybean. The grain yield losses can be 100% (Purdy 1979). *S. sclerotiorum*, *S. trifoliorum*, and *S. minor* all can produce oxalic acid into their surrounding media (Cessna *et al*. 2000, Livingstone *et al*. 2005). The role of oxalic acid as an essential determinant of pathogencity of *S. sclerotiorum* is well documented (Li *et al*. 2008, Williams *et al*. 2011). *Sclerotinia sclerotiorum* mutants unable to produce oxalic acid proved to be non-pathogenic on bean plants, while the oxalic acid producing wild type was pathogenic (Godoy *et al*. 1990). It has been focused on oxalic acid production by

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this pathogen and the following mechanisms of action have been proposed to explain its involvement in pathogenesis: (a) lowering infected tissues pH that enhances the activity of extracellular enzymes produced by the pathogen, (b) chelation of cell wall Ca^{++} by the oxalate anion, that softens plant cell wall and compromises the function of Ca^{++}dependent defense responses, (c) direct toxicity to host plants that weakens the plant and facilitates invasion and (d) suppression of the host plant oxidative burst (Noyes and Hancock 1981, Cessna *et al*. 2000).

The present study was undertaken to evaluate the oxalic acid production by different *Sclerotinia sclerotiorum* isolates from economically important mustard plant and to determine the relationship between oxalic acid production and pathogenicity of *S. sclerotiorum* isolates.

Materials and Methods

Fifteen isolates of *Sclerotinia sclerotiorum* were isolated from infected mustard plant samples collected from five districts of Bangladesh during winter 2014-15. All the collected isolates were purified and maintained on potato dextrose media (PDA) for testing its pathogenic potentiality on mustard and to examine its ability to produce oxalic acid in culture media.

Production of oxalic acid was determined using PDA amended with bromophenol blue (50 mg/l). Petri plates containing PDA inoculated with 15 isolates of *S. sclerotiorum* were incubated at 25 ± 1ºC for 5 days in dark. Oxalic acid production was confirmed by the change of color of the medium. Since bromophenol blue is a pH indicator that turns from blue to yellow when pH is 3 to 4.6. Presence of oxalic acid blue color medium turn into yellow color was taken as an indication for oxalic acid production. Oxalic acid production was ranked on a scale from no production (–) to maximum production (++++) based on the visual degree of color change observed on the bromophenol blue amended PDA plates according to Steadman *et al*. (1994). The medium color in Petri plates were measured by using chromameter CR- 400 according to Hunter and Harold (1987). Color measurements were recorded using Hunter L*, a* and b* scale. The b* scale ranges from negative values for blue to positive values for yellow.

For acid identification in PDB with bromophenol blue was adjusted to pH 7.0 by using 1 mM NaOH. Isolates of *S. sclerotiorum* were inoculated in 100 ml conical flasks containing 50 ml of PDB + Bb and incubated at 25 ± 1ºC for 7 days. The pH was recorded every three days after inoculation. After 7 days of inoculation, pH of the spent broth was measured for the presence of oxalic acid. Oxalic acid production confirmed as evident by the change of color in the medium from purple to yellow was taken as an indication for oxalic acid.

For oxalic acid determination, isolates were inoculated in 100 ml conical flasks containing 50 ml of PDB with four replications of each. Flasks were incubated for 7 days at 25 ± 1°C. All of the cultures were vacuum filtered and oxalic acid was determinate in the supernatant of culture of each isolate according to Xu and Zhang (2000). This was conducted by preparing a mix reaction contained 0.2 ml of sample (or standard oxalic acid solution), 0.11 ml of bromophenol blue (BPB 1 mM), 0.198 ml of sulfuric acid (1 M), 0.176 ml of potassium dichromate (100 mM) and 4.8 ml of distilled water. Then the reaction mixtures were placed in a water bath at 60°C and quenched after 10 min by adding 0.5 ml sodium hydroxide solution (0.75 M). The absorbance was measured at 600 nm by means of a spectrophotometer and PDB was used as the blank control. Oxalic acid concentration was calculated comparing with a standard curve and was expressed as mg oxalic acid/liter PDB medium. The data were statistically analyzed using the American SAS/STAT Software version 6 and means were compared by the least significant difference test (LSD).

Results and Discussion

The results of mycelial growth and detection of oxalic acid production are summarized in Table 1. Indication of oxalic acid production in culture medium showed that all the *S. sclerotiorum* isolates produced oxalic acid after one day of inoculation as evident by the change of color of bromophenol blue amended PDA medium (Fig. 1). Isolates of Joypurhat (Joy1, Joy2 and Joy3) district showed deep bright yellow (++++) color whose luminosity value was (+b*) 20.44, 20.11 and 19.39, respectively. Bright yellow (+++) color produced by the isolates of Kustia (K1, K2 and K3) and Jamalpur (J1, J2 and J3) districts and luminosity value were recorded 16.87, 15.93 and 15.74 for Kustia, and 14.80, 14.18 and 13.91 for Jamalpur, respectively. The isolates of Bogra (B1, B2 and B3) showed intermediate yellow color (++) and luminosity values were 12.09, 11.98 and 11.34. Lower luminosity value (+b*) 08.9, 08.77 and 08.65 were recorded in Hobiganj isolates (H1, H2 and H3) that showed faint yellow color (+).

Production of oxalic acid is shown in Table 2. It was observed that the isolates of five districts produced oxalic acid after seven days of inoculation giving evident by changing the color of bromophenol blue from purple into yellow in PDB.

Sclerotinia sclerotiorum isolates of Joypurhat *viz*., Joy1, Joy2 and Joy3 produced higher oxalic acid where the pH levels were 3.10, 3.20 and 3.35, respectively and color changing recorded into blue to deep yellow. While the isolates of Hobiganj *viz*., H1, H2 and H3 produced lower percentage of oxalic acid where the level were calculated by pH 4.30, 4.45 and 4.30, respectively and color changed into blue to purple. Oxalic acid production by Kustia (K1, K2 and K3) and Jamalpur (J1, J2 and J3) isolates was medium and the color changed from blue to yellow.

Table 1. Oxalic acid production of *Sclerotinia sclerotiorum* isolates on PDA with bromophenol blue medium.

Isolates	Source of Isolates	Medium color before inoculation	Mycelial growth of 3 dpi (mm)	Mycelial growth of 5 dpi (mm)	Medium color of 5 dpi	brightness /luminosity value (+ b*)	Rank of color bright-ness
Joy 1	Joypurhat	Blue	51.5	87.0	Deep bright yellow	20.44	++++
Joy2	"	"	56.0	89.0	Deep bright yellow	20.11	++++
Joy3	"	"	53.5	85.5	Deep bright yellow	19.39	++++
K1	Kustia	"	66.5	90.0	Bright yellow	16.87	+++
K2	"	"	62.0	90.0	Bright yellow	15.93	+++
K3	"	"	65.5	90.0	Bright yellow	15.74	+++
J1	Jamalpur	"	25.0	83.5	Bright yellow	14.80	+++
J2	"	"	30.5	80.0	Bright yellow	14.18	+++
J3	"	"	29.0	84.0	Bright yellow	13.91	+++
B1	Bogra	"	60.5	90.0	Intermediate yellow	12.09	++
B2	"	"	66.5	90.0	Intermediate yellow	11.98	++
B3	"	"	62.5	90.0	Intermediate yellow	11.34	++
H1	Hobiganj	"	61.5	90.0	Faint yellow	08.91	+
H2	"	"	63.5	87.5	Faint yellow	08.77	+
H3	"	"	57.0	90.0	Faint yellow	08.65	+

dpi = Days post- inoculation, + and ++++ indicate minimum and maximum brightness.

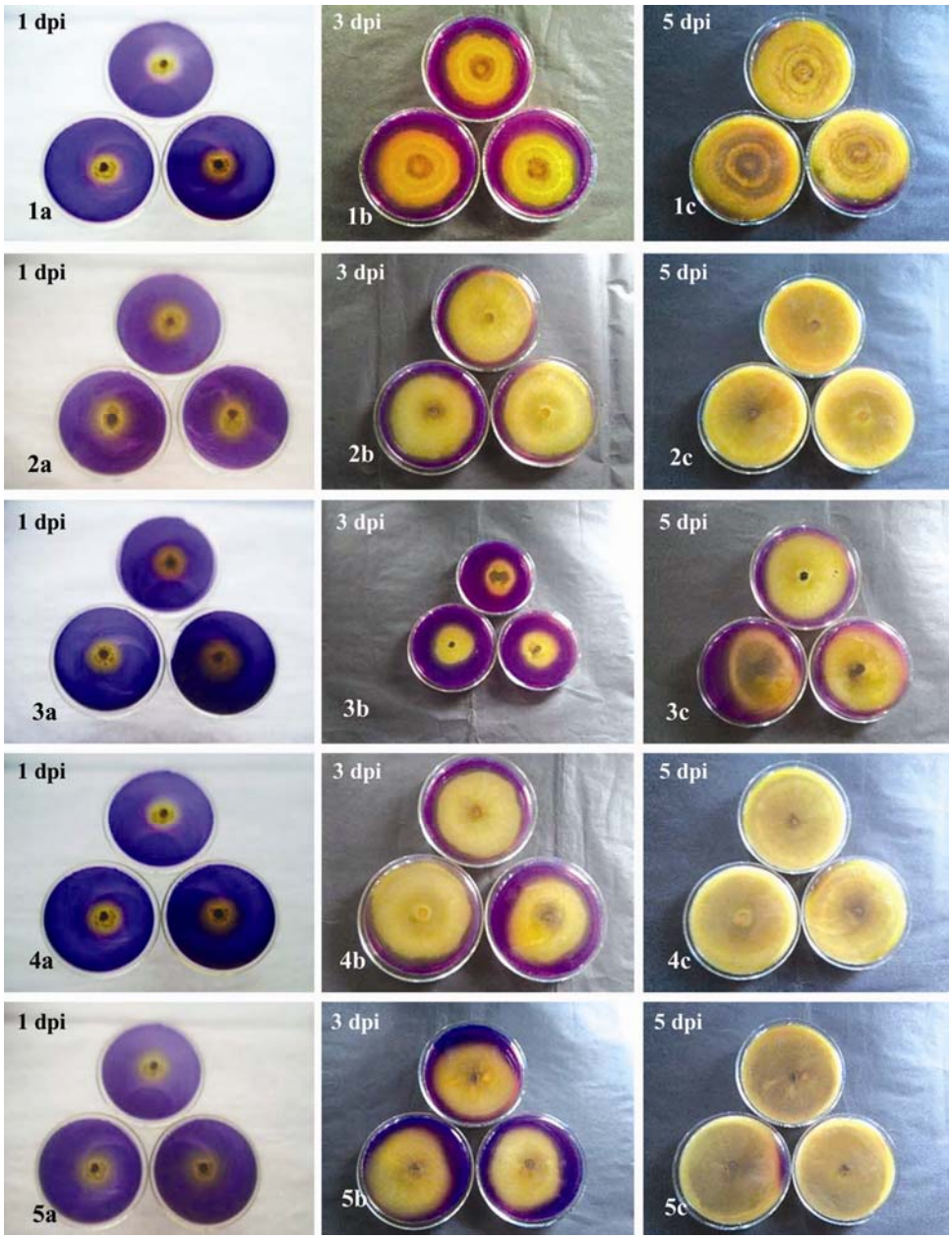

Fig.1. Oxalic acid production of three isolates from five districts on PDA medium amended with bromophenol blue: 1a-c. Joypurhat; 2a-c. Kustia; 3a-c. Jamalpur; 4a-c. Bogra and 5a-c. Hobiganj. (dpi = Days post- inoculation).

However, the pH altered into 3.15, 3.16 and 3.10 for K1, K2 and K3 isolates of Kustia, respectively and 3.26, 3.30 and 3.15 for J1, J2 and J3 isolates of Jamalpur, respectively. Isolates of Bogra viz., B1, B2 and B3 showed orange color and the pH levels were 4.30, 4.45 and 4.30,

respectively. However, the un-inoculated PDB amended with bromophenol blue (control) remained deep blue in color.

Bromophenol blue is a pH-indicator that turns from blue to yellow when pH is 3 - 4.6. Meantime, oxalic acid production increased by the following days up to seven days as indicated by the visual degree of color change was considered an evidenced of acid releasing by the fungus (Durman *et al*. 2005).

Table 2. Oxalic acid production of *Sclerotinia sclerotiorum* isolates on PDB at pH 7.0 with bromophenol blue medium.

Isolates	Medium color (7 days after inoculation)	Medium pH (7 days after inoculation)
Joy 1	Bright yellow	3.10
Joy2	Bright yellow	3.20
Joy3	Bright yellow	3.35
K1	Yellow	3.15
K2	Yellow	3.16
K3	Yellow	3.10
J1	Yellow	3.26
J2	Yellow	3.30
J3	Yellow	3.15
B1	Orange	3.35
B2	Orange	3.32
B3	Orange	3.35
H1	Purple	4.30
H2	Purple	4.45
H3	Purple	4.30

Quantification of oxalic acid production data tabulated in Table 3 showed that oxalic production by the tested *S. sclerotiorum* isolates was obviously correlated with their pathogenicity degree.

The extremely high virulent *S. sclerotiorum* isolates of Joypurhat (Joy1, Joy2 and Joy3) produced high amount of oxalic acid (4.46, 4.50 and 4.24 mg/l). Isolates of Kustia *viz.,* K1, K2 and K3 produced 3.88, 3.47 and 3.33 mg/l oxalic acid while the isolates of Jamalpur *viz.,* J1, J2, J3 produced 3.45, 3.18 and 3.42 mg/l oxalic acid, respectively. Oxalic acid was poorly produced (2.38, 2.41, 2.46 mg/l) by the most weak isolates of Hobiganj (H1, H2 and H3). The moderately virulent isolates of Bogra (B1, B2 and B3) produced modest amount of oxalic acid (3.23, 3.35 and 3.07 mg/l).

Oxalic acid was associated with pathogenesis. Effective pathogenesis by *S. sclerotiorum* requires the secretion of pathogenicity factors like oxalic acid (Cessna *et al.* 2000). Oxalic acid can degrade or weaken the plant cell wall via acidity or chelation of cell wall Ca^{++} (Bateman and Beer 1965). Marciano *et al*. (1989) found that highly aggressive and weakly aggressive isolates could equally utilize several components of host cell wall as nutrients for mycelial, but differed in their ability to utilize them for oxalate production. The poor ability to produce oxalic acid by weakly aggressive isolates seems to be due to a lower efficiency in the synthetic pathway. Godoy *et al*. (1990) showed that mutants of *S. sclerotiorum* deficient to synthesize oxalate were non

pathogenic, whereas relevant strains that regain their oxalate biosynthetic capacity exhibited normal virulence. These data clearly demonstrate that oxalic acid is a pathogenicity factor for *S. sclerotiorum*. This factor suppresses the hypersensitive response of the host plants (Cessna *et al*. 2000). The results of the experiments showed that the highly virulent isolates (Joy1, Joy2 and Joy3) of *S. sclerotiorum* produced high amount of oxalic acid while the weak isolates (H1, H2 and H3) were poor oxalic acid producer. The findings are in agreement with the findings of

Table 3. Oxalic acid production on PDB medium by *Sclerotinia sclerotiorum* isolates of different degrees of pathogenicity.

Isolates	Pathogenicity	Oxalic acid (mg/l) of medium
Joy 1	Extremely high	4.46 [a]
Joy2	Extremely high	4.50 [a]
Joy3	Extremely high	4.24 [a]
K1	High	3.88 [b]
K2	High	3.47 [b]
K3	High	3.33 [b]
J1	High	3.45 [b]
J2	High	3.18 [b]
J3	High	3.42 [b]
B1	Moderate	3.23[b]
B2	Moderate	3.35[b]
B3	Moderate	3.07 [b]
H1	Weak	2.38[c]
H2	Weak	2.41[c]
H3	Weak	2.46 [c]

Data are means of four replicates. Data with the same letters are not significant at p = 0.05

several other reports worldwide (Harel *et al*. 2006, Li *et al*. 2008). pH is another important factor in *Sclerotinia* spp. pathogenesis (Chen *et al*. 2004, Dickman 2007). It considered influencing the oxalate secretion by *S. sclerotiorum*, with decreasing culture pH being the result of increasing oxalate accumulation (Hegedus and Rimmer 2005, Bolton *et al*. 2006). During the course of the present study pH progressively decreased from 3.5 to 2.8 due to oxalic acid production in unbuffered medium, (Bueno *et al*. 2012). If the pH is not reduced, pathogenic development does not occur (Chen *et al*. 2004, Rollins and Dickman 2001). In the present study it was found that the extremely high virulent *S. sclerotiorum* isolates of Joypurhat (Joy1, Joy2 and Joy3) reduced the maximum pH level in culture media (3.10, 3.20 and 3.35) and was high oxalic acid producer (4.46, 4.50 and 4.24 mg/l).

Based on the findings of the study it may be concluded that there are closely relationship between oxalic acid production and pathogenicity. Oxalic acid may aid in infection through a number of proposed routes, including acidification to facilitate cell wall degrading enzyme activity, through pH mediated tissue damage or via sequestration of Ca^{++} ions (Bateman and Beer 1965). The low pH resulting from oxalic acid production may weaken plants to improve access to fungal infection. Oxalic acid has been reported to disturb guard cell function during infection by *S. sclerotiorum* by including stomatal opening and inhibiting absicicic acid induced stomatal closure (Guimaraẽs and Stotz 2004).

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