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Physiological studies on *Stemphylium vesicarium* causing Stemphylium blight of onion

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Abstract

Stemphylium vesicarium **the causal agent of Stemphylium blight of onion had incubation period of 5 and 3 days whereas, 7 and 5 days of latent period on potted plants and detached leaves, respectively. The inoculum level i.e., 3x10^4 conidia/ml, required minimum incubation period for disease expression and development whereas at lower level i.e., 1x10^4 conidia/ml, the incubation period increased resulting in less disease severity. The effect of different culture media and temperatures on mycelial growth and sporulation of *S. vesicarium* of onion was investigated. Maximum colony growth of *S. vesicarium* was recorded on Potato dextrose agar (PDA) and Oatmeal agar while the highest sporulation was observed on Oatmeal agar and V8 agar media. Similarly, maximum colony growth of *S. vesicarium* was recorded between 20°C and 25°C temperature. Whereas, high sporulation was recorded at 15°C while at 20°C and 25°C a good amount of spore production was observed.**

Key words: Media, temperature, colony growth, inoculum and *Stemphylium vesicarium*

Onion (*Allium cepa* L.) is one of the important vegetable crops grown throughout the world. Stemphylium leaf blight (SLB) caused by *Stemphylium vesicarium* (Wallr.) Simmons is among the major diseases of onion severely affecting the foliage, thus resulting in crop losses ranging from 30 to 100 per cent both in seed and bulb crop from year to year (Aveling et al. 1994; Chaput 1995; Cramer 2000). The disease is prevalent in warm and humid environment (Suheri and Price 2000). This disease is one of the most important foliar diseases in the northern parts of India (Gupta and Pandey, 1986) and was first reported by Rao and Pavgi in 1975 from Uttar Pradesh. The disease is, however, more severe on seed crop as compared to bulb crop (Gupta and Pathak 1988; Tomaz and Lima 1988) causing 100 per cent loss of seed production sometimes (Singh *et al*. 1992). In the present study, the physiological conditions required for the growth and sporulation of the pathogen were studied. The identification of suitable culture medium, optimum temperature, sporulation of the pathogens and different inoculum concentrations would help and aid in inoculum preparation required for creating artificial epiphytotic conditions and thus, would contribute to disease resistance breeding as well as fungicides evaluation. The study would be useful in formulating promising strategies for integrated disease management.

Materials and Methods

Isolation and purification

Isolations of the pathogen were made from the diseased leaf tissue collected from different locations. Typical diseased spots on the leaves were selected and cut into bits of about 1 to 1.5 mm with the help of a sterilized scalpel, washed with sterilized distilled water and disinfected with 1 per cent sodium hypochlorite solution for 10-15 seconds. These disinfected bits were immediately rinsed in double sterilized distilled water repeatedly to remove the traces of sodium hypochlorite and towelled on sterilized filter paper, prior to their being aseptically transferred to Petri plates containing 20 ml of autoclaved Potato dextrose agar (PDA) in a laminar flow and incubated at 25±1°C in BOD incubator for 10 days.

Incubation and latent period

To study the incubation and latent period, a conidial suspension was prepared from 15 days old fungal culture. Conidia harvesting was done by flooding the culture plate with 10 ml of sterilized distilled water and gently scraping the surface with a spatula to form a conidial suspension. This conidial suspension is then filtered through muslin cloth to remove mycelial bits and remains of media. A conidial suspension of $3x10^4$ standardized with a haemocytometer was used in the experiment. Later two months old healthy seedlings (var. Agri Found Light Red) raised in plastic pots (15 cm dia) filled with a mixture of sterilized soil and FYM were inoculated by spray method using above prepared homogenized conidial suspension of the pathogen. The inoculated plants were placed in net-house by maintaining RH > 90% for 48 hours at temperature 25-30^{0}C and thereafter the pots were watered daily until the development of disease symptoms. The data on the incubation and latent period were recorded. The pathogen was re-isolated from the diseased plants for confirmation of Koch's postulates.

The incubation and latent period were also studied on the detached leaves. For this leaves were obtained from 3-4 leaf stages of onion plants of susceptible variety AFLR for incubation. These leaves were sterilized with 1 per cent sodium hypochlorite solution followed by three times washing with distilled water and were dried with sterilized blotting sheets. Then leaves were kept over a clean sterilized glass slide placed in Petri dishes lined on both sides with double layered moist filter paper. The surface of the leaves was then punctured with a sterilized needle and inoculated with conidial suspension with the help of atomizer and were kept for incubation under different temperature conditions i.e., 10, 15, 20, 25 and 30^{0}C in BOD incubator. The required relative humidity conditions were also maintained for pathogen initiation. The leaves were then observed for incubation and latent period after every 24 hours.

Effect of different media on growth and sporulation of *Stemphylium vesicarium*

Five different solid media, viz., Potato dextrose agar (PDA), V-8 juice agar, Oatmeal agar, Mathur's media and Malt extract agar media were used to study growth and sporulation of *S. vesicarium*. Twenty ml of each sterilized medium was aseptically poured into the sterilized Petri plates in a laminar flow. The actively growing 5 mm mycelial disc was cut with the help of sterilized cork borer from 7 days old culture of *S. vesicarium* raised on PDA and the Petri plate was inoculated with 5 mm mycelial disc of the fungus with the help of sterilized inoculating needle. Four replications were kept in each treatment under completely randomized design. The Petri plates were incubated at 25±1°C and observations on colony diameter of the fungus was observed. The linear growth of the respective fungus was measured in millimetre at 48 h interval for 10 consecutive days. Sporulation was also checked out time to time after 48 h and different cultural characteristics and pigmentation were also observed on the growth media.

Effect of different temperature levels on growth and sporulation of *Stemphylium vesicarium*

To determine the optimum temperature for growth of *S. vesicarium* different temperature levels, *viz*., 10, 15, 20, 25 and 30ºC were studied to observe mycelial growth and sporulation of the pathogen. The culture of pathogen was grown on PDA for 7 days and mycelial disc of 5 mm diameter from actively growing colony margins were cut with sterilized cork borer. Each mycelial disc was transferred on to 90 mm Petri plates containing 20 ml of PDA medium and then incubated at 10, 15, 20, 25 and 30ºC. The colony diameter measurements were taken after 48 h till 240 h i.e., up to 10 days of incubation. Sporulation was also checked out time to time after 48 h and different cultural characteristics were also observed during the growth period at all temperature levels.

Effect of different inoculum concentrations of pathogen on disease development

Different inoculum levels were evaluated to find out the optimum inoculum required for the disease development. For this seedlings of susceptible onion variety (AFLR) were raised in surface sterilized trays filled with mixture of sterilized soil and FYM (3:1). After two months, seedlings were transplanted to pots (15 cm dia) and allowed to grow by following standard agronomic practices. These potted onion plants were then inoculated with different conidial concentrations, *viz*; $1x10^4$, $2x10^4$, $3x10^4$ and $4x10^4$ conidia/ml. Simultaneously, control treatment by spraying sterilized water was also maintained. The inoculated plants were

placed in net-house by maintaining RH > 90% for 48 hours at temperature 25-30^{0}C and thereafter the pots were watered daily until the development of the disease symptoms. Ten plants were taken per replication and the treatments were replicated thrice. The incubation period for inoculated leaves at different inoculum concentrations were recorded and per cent disease severity was recorded as 0-9 disease scale modified by Zheng *et al*. (2010) and area under disease progress curve was also calculated. Progression of the disease on different inoculum concentrations was measured by calculating apparent rate of infection (r) based on area covered by lesion as per Vanderplank (1963) using logistic equation as given below:

$$r = \frac{2.303}{t_2 - t_1} \log 10 \frac{X_2(1 - X_1)}{X_1(1 - X_2)}$$

Where,

r= apparent infection rate per unit per hour

t_2-t_1= time interval between first and last observation

X_1&X_2= proportion of leaf area covered by lesion at t_1 and t_2 time intervals, respectively

(1-X_1) & (1-X_2) = proportion of healthy leaf area at t_1 and t_2 time intervals, respectively

Studies on the host range of pathogen

The studies on the host range were carried with different plants *i.e.*, tomato, garlic, lentil, pea and coriander. The seeds of the respective plant species were procured, and their seedlings were raised in surface sterilized trays filled with mixture of sterilized soil and FYM (3:1). Then after two months seedlings were transplanted in pots and at 3-4 leaf stage were inoculated by spray method using homogenized conidial suspension of pathogen. Inoculated plants were irrigated with sterilized water to maintain moisture for plant growth as well as disease development. Observations on the disease appearance were recorded periodically to find out the host range of the pathogen.

Results and Discussion

Incubation and latent period

In vitro

The detached leaves incubated at different temperature and observed for the symptom development every 24 hours up to 10 days and it was observed that no symptoms were developed at 10, 15 and 20^{o}C. The symptoms appeared after 3 days at 25^{o}C and also sporulation after 5 days. However, there was appearance of symptoms at 35^{o}C but no sporulation was observed at this temperature. The results thus revealed that 25^{o}C as the optimum temperature for the development of the disease as at less than 25^{o}C there was no initiation of disease and above 25^{o}C symptoms appeared but disease will not spread as there was no sporulation was observed above 25^{o}C (Table 1).

Table 1. Studies on incubation and latent period under in *vitro* and in *vivo* conditions

In vitro **Temperature (^{o}C)**	**Symptoms appearance**	**Sporulation**	**Incubation period (days)**	**Latent period (days)**
10	-	-	-	-
15	-	-	-	-
20	-	-	-	-
25	+	+	3	5
30	+	-	3	-
In vivo				
Apparent temperature	+	+	5	7

(+ = Yes) (- = No)

In vivo

To study the incubation and latent period symptom development and conidia formation has been recorded regularly. The observations indicated that initial symptoms appeared as small, irregular to oval, white flecks surrounded by bright yellow margin developed after 5 of inoculation on the onion plants potted in pot culture. However, conidia formation was observed after 7 days of inoculation on the plants. Thus, incubation and latent period of 5 and 7 days had been observed on the plant under net house in pots.

Basallote *et al*. (1999) while studying the pathogenicity of *S. vesicarium* on garlic observed initial disease symptoms after 4-6 days of inoculation in pot plants as minute, numerous white flecks on the leaves under in vivo conditions. Devi (2014) observed incubation period of 2 and 4 days with latent period of 6 and 8 days in young and old tomato seedlings, respectively in Stemphylium leaf spot of tomato. They also observed that on detached leaves of tomato symptoms appeared at 25 and 30°C and sporulation at 25°C only whereas no symptoms were observed at other temperatures (15 and 20°C). Shabnam (2015) also observed incubation period of 4-5 days with 6-7 days period for sporulation while studying pathogenicity of *S. vesicarium* on garlic plants.

Effect of different media on growth, sporulation and colony characteristics of *Stemphylium vesicarium*

The data presented in the Table 2 revealed that the fungus showed considerable growth on all the tested media. Also, the colony characteristics of the fungus

Table 2. Evaluation of different media on growth, sporulation and colony characteristics of *Stemphylium vesicarium*

Media	Mycelial growth after hrs. of inoculation (mm)							Cultural characteristics	
	48 h	96 h	144 h	192 h	240 h	Mean	Sporulation	Mycelial growth	Pigmentation
Potato Dextrose Agar	16.50	35.67	60.33	80.33	85.00	55.57	Good	Growth fast, cottony, olive grey colony, raised centre	Orange
Oatmeal agar media	16.23	34.67	51.33	74.00	81.67	51.58	High	Growth fast, greenish grey cottony colony	No
Mathur media	10.17	28.33	41.83	59.33	79.00	43.73	Nil	Growth fast, whitish fluffy, greyish at centre, less dense	No
Maltose media	9.50	24.50	40.83	60.67	77.17	42.53	Nil	Growth fast, whitish less dense growth	No
V8 agar media	15.77	20.33	23.67	30.50	35.33	25.12	High	Slow growth, adpressed uniform whitish growth	No
Media						0.958			
Interval						0.958			
Media x Interval						2.141			

varied significantly among the different media. Mycelial growth was fast on all the tested media except V8 agar media. On PDA media mycelia was cottony with olive grey colony and raised centre whereas, on Oatmeal agar there observed greenish grey cottony colony. White fluffy grey centred colony, whitish less dense colony and adpressed uniform white colony growth were observed on Mathur media, Maltose media and V8 agar media, respectively. Orange colour pigmentation was also observed on lower side of PDA plate whereas, there was no pigmentation on other media plates. The mean radial growth varied from 25.12 to 55.57 mm on all the tested media. The maximum radial growth (85.00 mm) on 10th day of incubation was recorded on Potato dextrose agar followed by Oatmeal agar (81.67mm) and Mathur media (79.00 mm) while the least radial growth (35.33 mm) was recorded on V8 agar media. Among all the tested media V8 agar media, Oatmeal agar media and PDA supported the production of conidia of *S. vesicarium*. Whereas, no sporulation was observed in Mathur media and Maltose media.

Kim *et al*. (2004) reported higher sporulation of *S. solani* and *S. lycopersici* on V8 juice agar medium followed by Potato carrot agar and Potato dextrose agar, respectively. *S. botryosum* colonies grow rapidly at 25°C on PDA (Mathur and Kongsdal 2002; Hashemi *et al*. 2005). Kumar 2007 reported the highest sporulation of *S. botryosum* on V8 juice Potato dextrose agar medium (V8P) followed by V8 juice agar, V8P+2 per cent tamarind juice medium and V8P+4 per cent tamarind juice medium, respectively. Chowdhury *et al*. (2015) reported V-7 juice agar and V-7 juice mixed with Potato dextrose agar the most suitable culture media for mycelial growth and sporulation of *S. vesicarium*. Yadav *et al*. (2017) perceived maximum radial growth (65.30 mm) on 10th day of incubation on Richard's agar followed by Potato dextrose agar (60.95 mm) and Czapek's Dox agar (56.90 mm) while the least radial growth (53.35 mm) was recorded on V8 juice agar followed by Rye agar B (55.58 mm). The production of conidia of *S. vesicarium* was supported fairly by all the tested media.

Effect of different temperatures on growth, sporulation and colony characteristics of *Stemphylium vesicarium*

The data presented in Table 3 revealed significant differences among temperature levels in terms of mean radial growth of *S. vesicarium*. The mean radial growth varied from 29.20 to 50.30 mm on all the tested temperature levels. The fungus grew well at temperature 20 and 25ºC. The maximum radial growth (87.67 mm) was recorded at 25ºC followed by 20ºC (81.67 mm) and 30ºC (64.67 mm) after 10 days of incubation while at 10ºC there was least growth (33.33 mm) of fungus was recorded. At 15ºC highest sporulation was recorded followed by temperature 20 and 25ºC with good amount of sporulation while at 10 and 30ºC there was no sporulation. Mycelial growth also varied at different temperatures as cottony olive growth at 10ºC, grey white at 15 and 20ºC and dull

Table 3. Evaluation of different temperature levels on growth, sporulation and colony characteristics of *Stemphylium vesicarium*

Temperature (ºC)	Mycelial growth after hrs. of inoculation (mm)						Cultural characteristics		
	48 h	96 h	144 h	192h	240 h	Mean	Sporulation	Mycelial growth	Pigmentation
10	11.67	17.67	24.00	28.33	33.33	23.00	Nil	Cottony olive grey	Nil
15	14.67	24.33	29.33	35.33	42.33	29.20	High	Grey white	Nil
20	18.67	30.33	46.33	60.33	81.67	47.47	Good	Grey white	Nil
25	20.00	31.67	47.67	64.50	87.67	50.30	Good	Dull white	Orange
30	11.67	26.67	32.67	47.67	64.67	36.67	Nil	Dull white	Orange
Temperature						0.759			
Interval						0.759			
Temperature x Interval						1.696			

white at 25 and 30ºC has been observed. Only orange colour pigmentation has been observed at temperature 25 and 30ºC whereas, no pigmentation was recorded at other temperature ranges.

Montesinos *et al*. (1995) found that the optimal temperature for mycelial growth of *S. vesicarium* was 15-25°C. Kim *et al*. (2004) observed 25°C as optimum temperature for the growth of *S. solani* and *S. lycopersici*. Bhat *et al*. (2008) reported that *S. vesicarium* infecting onion, isolated on PDA produced greenish grey to black colonies within 8-10 days at 24±1°C as fluffy, lanose to loose cottony growth. Rahman *et al*. (2010) mentioned that radial growth of *S. botryosum* was highest at temperature 25°C followed by 20°C. In addition to this they also reported that with the increase in temperature the radial growth increased up to 25°C and decreased subsequently.

Hosen (2011) found 25°C as the optimal temperature for the growth of *S. botryosum*. Tomioka and Sato (2011) studied the effect of temperature (5-35°C) on mycelia growth of *Stemphylium* spp. and thus, observed that 25°C temperature was optimum for mycelial growth and the grey mycelial colonies at this temperature secreted yellow to reddish brown pigment. Subash and Saraswati (2016) observed that the *S. vesicarium* isolates had higher colony diameter of 7.66 cm and conidial dimension of 29.42×18.12 µm (L/B ratio-1.62) with abundant sporulation intensity (46.67 conidia /0.01 ml) was observed at 25°Cafter 15 days of incubation on PDA.

Effect of different inoculum levels of pathogen on the disease development

The data in Table 4 & Fig.1 showed considerable variation among different inoculum levels for incubation period. Per cent disease severity was recorded at 3, 6 and 9 days after incubation. Inoculum level of $3x10^4$ conidia/ml gave maximum disease severity (44.62%) and was found best with minimum incubation period of 4 days followed by $2x10^4$ (38.68%) and $4x10^4$ (30.32%) conidia/ml with 5 and 7 days of incubation period, respectively. However, minimum disease severity (18.87%) was observed at inoculum level $1x10^4$ having 8 days of incubation period. The progress of disease was maximum at $3x10^4$ conidial concentration and which was reflected from disease severity depicting highest AUDPC (258.54) and apparent infection rate (0.85/day). This was followed by $2x10^4$ and $4x10^4$ conidial concentration with 38.68 & 30.32 AUDPC and 0.75 & 0.69 rate of infection, respectively. Considerably lowest disease severity was observed at $1x10^4$ with minimum AUDPC (108.01) and apparent infection rate of 0.52 /day.

Gupta and Pathak (1988) at inoculum concentration of 3.28 $x10^5$ mycelial propagules/cm^3 observed maximum disease incidence (100%) and severity (68.8%) and short incubation period. As the density of inoculum decreased there observed late appearance and poor purple blotch disease development. Kareem *et al*. (2012) reported that with

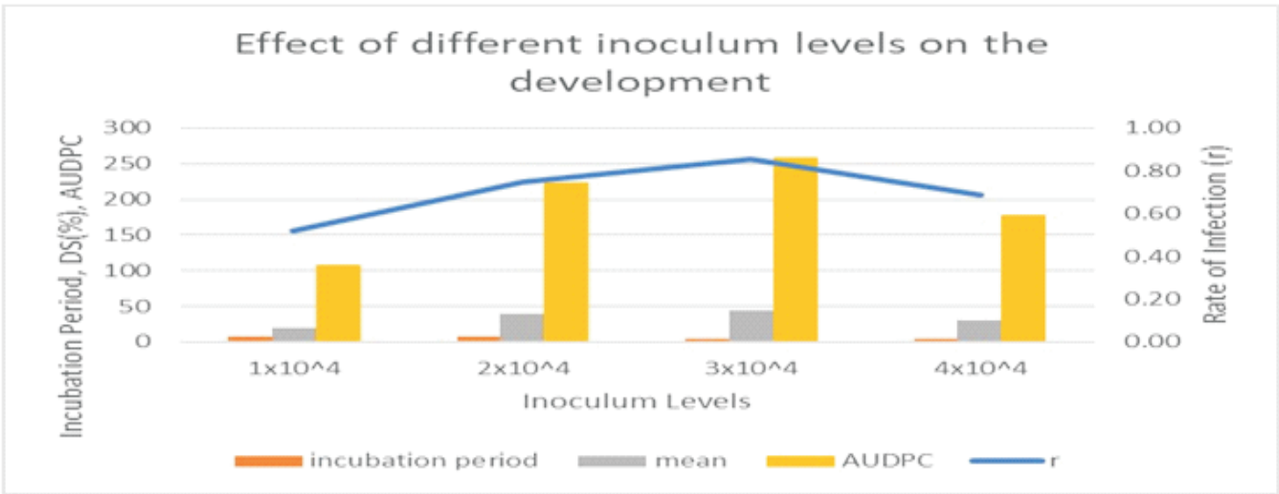

Figure 1. Effect of different inoculum levels on the development of Stemphylium blight of onion in pot conditions

Table 4. Evaluation of different inoculum levels on the disease development by *Stemphylium vesicarium*

Inoculum levels	Incubation period	Disease severity (%) after days of incubation period				AUDPC	Apparent rate of infection(r)
		3	6	9	Mean		
$1x10^4$	8	7.82	15.40	33.38	18.87	108.01	0.52
$2x10^4$	5	14.22	32.93	68.89	38.68	223.48	0.75
$3x10^4$	4	18.39	38.48	77.00	44.62	258.54	0.85
$4x10^4$	7	9.89	27.62	53.46	30.32	177.89	0.69
Inoculum level					1.539		
Interval					1.333		
Inoculum level x Interval					2.666		

the increase in the inoculum level there was significant increase in the disease development of *Alternaria porri* causing purple blotch of onion. Highest per cent disease index (57.03) was recorded with 10^8 spores/ml inoculum concentration followed by 10^6 and 10^4 spores/ml with 52.00 and 43.75 disease index, respectively. Lowest per cent disease index was observed with 10^2 conidial concentration. Bhardwaj (2018) observed $6x10^4$ as the ideal inoculum concentration for rapid advancement of Stemphylium blight disease of garlic. While with inoculum concentration of $2x10^4$ the infection process was noticeably delayed.

Studies on the host range of the pathogen

To determine host range of the pathogen, plant species belonging to same or different botanical families were inoculated artificially with *S. vesicarium inoculum*. Tomato, garlic and lentil plant species showed prominent symptoms whereas, pea and coriander did not show such prominent symptoms. The initial symptoms of the disease appeared within 4-5 days. Tomato plants developed brown to black specks on leaves. Later, angular to oval brown spots developed with grey centre surrounded by yellow halo. On lentil plants initially symptoms appeared as small, light beige lesions on leaves. Later these small lesions coalesce to produce large, irregular shaped lesions that killed entire branches. On garlic plants initially white oval lesions appeared which later become sunken with purple colour surrounded by white margin and ultimately extensive necrosis followed by pre-mature plant desiccation. Other plant species, i.e., pea and coriander did not show any prominent symptoms except some minute leaf spots.

Various workers reported *S. vesicarium* as host of different plant species like garlic (Basallote 1993), leek (Suheri and Price 2001), asparagus (Falloon 1987) and European pear (Llorente and Montesinos 2006). Barnwal (2003) also reported Lentil and tomato as the host plant of *S. vesicarium* while studying Stemphylium blight of onion.

Conclusion

From present work, it is concluded that the knowledge of incubation and latent period will be helpful in understanding the infection cycle of the pathogen to manage the disease. All tested media, temperature levels and different inoculum levels showed variations in the terms of cultural, morphological aspects and in disease development process of *S. vesicarium*. PDA and oatmeal agar were the best culture medium for *S. vesicarium*. Different culture media had profound effect on radial mycelial growth of pathogen. Oatmeal agar and V8 agar media appeared to be the best for the sporulation of *S. vesicarium* whereas, best fungal growth with sufficient sporulation was observed at 20 and 25ºC temperature. Standardization of inoculum load also helped to know the amount of inoculum that can initiate the infection for successful disease development with minimum incubation and latent period.

Conflict of interest: The authors declare that they have no conflict of interest.

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