Indian Journal of Agricultural Sciences **83** (7): 717–20, July 2013/Article

Development of artificial inoculation technique and cross infectivity test of *Sclerotinia* species on different hosts

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Received: 31 March 2012; Revised accepted: 18 March 2013

ABSTRACT

Out of five inoculation techniques, the colonized petal method was best for quick and reproducible infections which can be successfully used for screening large number of germplasms at nursery stage, host pathogen interaction study, virulence test and race characterization. There was no significant difference in colony characters, sclerotial initiation period, arrangement and size of sclerotia in culture of different *Sclerotinia* species after inoculation on berseem and subsequent re-isolation. Initially sclerotial size was small and abundant in numbers but subsequently size increased and number decreased in berseem isolate. There was no significant difference in sclerotial weight of all the seven isolates when it was isolated from the cross inoculated hosts. It indicates that large sclerotia formation character of *S. sclerotiorum* from other hosts, changed in smaller sclerotia when inoculated on berseem. Indiscriminate colonization by *Sclerotinia* species on these hosts without any selective host specificity and no variation in cultural condition indicates that all may be same species of *Sclerotinia*. Confirmation of cross infectivity test on different hosts confirms that *S. trifoliorum* and *S. sclerotiorum* may be the same species to be known as *S. sclerotiorum* like *Sclerotium rolfsii* and *Rhizoctonia solani*.

Key words: Berseem, Cross-infectivity, Inoculation technique, *Sclerotinia* spp

Sclerotinia is an ubiquitous, necrotropic, omnivorous and sclerotial fungal pathogen causing disease on almost all agricultural crops including forage crops. Berseem (*Trifolium alexandrinum*) is an important forage crop of India. The crop is severely infected by sclerotinia stem rot. The associated pathogen is reported as *Sclerotinia trifoliorum* Errk. in 1880. It becomes a devastating pathogen during in rainy period in winter season and the damage was recorded as high as 70% in Northern India. The pathogen is rapidly killing all above ground part of plants. Effective artificial inoculation technique is prerequisite for disease resistance screening and host pathogen interaction study. The inoculation technique for stem rot disease in berseem is not properly standardized to create artificial epiphytotics. Stem inoculation technique in alfalfa by applying saturated pads in 20% V-8 juice infested with sclerotinia pathogen (*Sclerotinia sclerotiorum* or *S. trifoliorum*), around tips and enclosing with tape for three days in saturated atmosphere followed by 11 days in ambient air was reported by Pratt and Rowe (1991).

The basic type species of this necrotrophic pathogen is universally described and well established as *S. sclerotiorum* (Lib.) de Bary. *S. trifoliorum* and *S. sclerotiorum* are reported on different clovers (*Trifolium* spp.) and *Medicago sativa*. *Sclerotinia sclerotiorum* reported to cause crown and stem rot of crimson clover (*Trifolium incarnatum*), Berseem clover (*T. alexandrinum*) and allfalfa (*Medicago sativa*) in temperate climates (Rowe 1993). *S. sclerotiorum* is reported on *T. repense* (white clover) the closely related to Egyptian clover (Berseem). Crown and stem rot caused by *S. sclerotiorum* on lucerne was confirmed by Gilbert (1987) on the basis of morphology of ascospore and sclerotial size with 0.85 to 3.35 mm diameter. *Sclerotinia* stem rot is also reported on faba bean (*Vicia faba)* caused by *Sclerotinia trifoliorum* and is often a serious problem in Greece (Lithourgidis *et al.* 2003). Scott (1981) reported that no evidence of other species other than *Sclerotinia trifoliorum* on white clover to cause clover rot. The highest variability was detected among isolates of *S. trifoliourum* from *Trifolium pratense* (red clover) and also observed that *S. trifoliourum* and *S. sclerotiorum* being more pathogenic on diploid than tetraploid red clover (Arseniuk 1990). Pratt and Rowe (1995) reported that host origin is not an important determinant for the virulence of *Sclerotinia* isolates on lucerne and no evidence for different pathogenic races among the isolates of *Sclerotinia trifoliorum* and *S. sclerotiorum* originated from different forage legumes in USA. The pathogen has a number of morphological strains such as *S. minor*, *S. intermedia*, *S. trifoliorum* and *S. sativa*

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that are now considered synonymous with *S. sclerotiorum* (Singh 1982).

Considering the above facts, the present study was carried out with the objective to develop effective inoculation technique for creating successful infection on berseem in artificial conditions and to characterize different isolates of *Sclerotinia* spp on the basis of cross inoculation test as well as colony characters**.**

MATERIALS AND METHODS

In the present study, five different inoculation techniques, i e petal colonization method, sclerotia and mycelial mat inoculation, colonized grain placement, mycelial suspension spray and sick nursery bed were tested on berseem. Ten plants per pot of berseem variety Wardan was grown in sterilized soil in earthen pots under glass house conditions and inoculated after 25 days of germination. In colonized petal inoculation technique, dry petals of cruciferous flowers were aseptically placed in the growing culture of sclerotinia and incubated till growing mycelium come in contact with the petals. The colonized petals were inoculated on berseem plant with the help of one drop sterilized water. Inoculated pots were kept in indigenously designed moisture chamber by maintaining 12-15°C temperature and 95-100% relative humidity. Observations were recorded after five days of inoculation on ten randomly selected inoculation sites on berseem. Five berseem isolates of *S. trifoliorum* belongs to Jhansi (1), Hisar (2 & 3), Ludhiana (4S & 4L) while two isolate of *S. sclerotiorum* from brinjal (7) and *Chenopodium* (8) belongs to Deoria were collected from their natural hosts. Isolate 4S represents small sclerotia formed on the berseem host while isolate 4L forming large sclerotia on berseem in Ludhiana region. All the isolates were cultured on PDA and colony characters, growth rate and sclerotial size were recorded. Petal colonization method, the most effective technique among five inoculation technique was used for inoculation on different host. These isolates were cross inoculated artificially on brinjal, tomato and berseem under controlled conditions. Cross inoculation of berseem isolate (*S. trifoliorum*) on brinjal and tomato while brinjal, *Chenopodium* isolate (*S. sclerotiorum*) inoculated on berseem. Pathogen was isolated from these inoculated hosts after complete rotting of the tissues. The colony characters, growth rate, sclerotial size and its developmental pattern were compared with the original native culture.

RESULTS AND DISCUSSION

Periodical observations revealed that no infection was recorded in any of the inoculation method after 24 hours of inoculation except colonized petal inoculation technique. Out of ten inoculation site, none was infected even after 48 hours of inoculation. Cent percent infection was initiated on all inoculated site after 48 hours of inoculation in colonized petal method. The rotting of leaves, pedicel and stem progressed as the incubation period increased. Only one infection site was observed in sclerotia + mycelial mat placement method even after heavy inoculum load. Out of five inoculation technique, colonized petal inoculation method was highly effective, simple, quick and reproducible (Table 1)**.** *Vicia faba* inoculated at different internodes using carrot root pieces colonized by *Sclerotinia trifoliorum* clearly results the differentiable symptoms after 48 to 72 hr after inoculation (Lithourgidis *et al.* 1991). Inoculation on excised lucerne leaf tissues by putting leaf on water agar in Petri plates with mycelium of *Sclerotinia trifoliorum* and incubation at 17°C was used for disease resistance screening (Pratt 1996). Nelson *et al.* (1989) have also reported that an inoculation density of less than 0.5 sclerotia/800 cm^3 of soil can result in significant incidence of *Sclerotinia* disease in sunflower. Inoculation with mycelium of *S. sclerotiorum* on alfalfa (*Medicago sativa*) and incubated at 22°C for 72 hr followed by 14 days incubation showed significant difference in stem necrosis (Elgin 1988). However, Mycelium-on-wheat formulation of *S. sclerotiorum* inoculated at 500 kg/ha in pasture to as mycoherbicide, but it had no any effect on either the grass or the clover (Hurrell and Bourdot 1993).

Cultural characterization of Sclerotinia *species*

The colony l growth on potato dextrose agar medium was varied between 73.5 to 85.2 mm after five days of incubation. Lowest growth as colony diameter was 73.5 mm in *Chenopodium* isolate (8) while maximum radial growth 85.2 mm was in berseem isolate of Hisar (3). Statistically isolates 1, 2, 4S,7 and 8 were at par while 2, 3 and 4L were at par (Table 2). Interestingly the original berseem isolate native to Ludhiana changed distinctly its colony characters from creamy white, sparse, numerous small sclerotia (4S) to white, compact, few, large sclerotia (4L) in sub-culturing. There was no significant difference in colony clours and sclerotial initiation period. The colony colour was white, compact regular in most of the isolates (2, 3, 4L, 7, 8) while creamy white, sparse, regular in 1,4S. The several sets of experimentations revealed that sclerotial formation was in the center or outer periphery of colony close to plate, in a circular ring or scattered with varying size. Usually large

Table 1 Different inoculation methods of *Sclerotinia* on berseem

Test methods	Inoculation sites	Infection site (mean)	Infection (%)
Colonized petal inoculation	10	9.66	96.6
Sclerotia + mycelial bits placement	10	0.33	3.33
Colonized grain placement	10	0.0	0.0
Mycelial suspension + spray + drench	10	0.0	0.0
Sick nursery bed	10	0.0	0.0

Table 2 Colony characters of different *Sclerotinia* spp on subsequent culturing

Isolate No.	Native Host (A)	Sclerotial size (mm)	Radial growth (mm) 5 DAI	Sclerotial weight (mg)		
				Isolation from A (B)	Re-cultured from B plate (C)	Isolation from inoculated berseem (D)
1	Berseem (Jhansi)	3.7 × 2.9	73.8	7.4	13.7	15.4
2	Berseem (Hisar)	3.5 × 3.0	79.7	9.4	13.0	15.4
3	Berseem (Hisar)	4.3 × 3.1	85.2	9.7	13.1	9.7
4S	Berseem (Ludhiana)	3.4 × 3.0	76.8	8.7	15.0	7.9
4L	Berseem (Ludhiana)	5.0 × 3.6	85.1	21.2	21.1	14.9
7	Brinjal (Deoria)	4.5 × 4.1	74.9	15.3	24.5	8.2
8	Chenopodium (Deoria)	4.5 × 3.7	73.5	16.7	18.8	10.5
	CD (P=0.05)		6.2	3.2	5.1	4.5
	CV (%)		13.4	9.7	7.2	9.2

sclerotia formed in the central periphery of colony in a circular fashion with few in numbers (4L, 7, 8) while small sclerotia were scattered and numerous in isolates of 1, 2, 3, 4S. It was observed that initially the sclerotial size was small and abundant in numbers when first time isolated from its native host particularly in berseem (1, 2, 3, 4) isolates but subsequently sclerotial size increased and its number decreased. There was not a definite pattern of arrangement and size of sclerotia. Arseniuk and Macewicz (1991) reported that of the SEM level, the structural organization of sclerotial stomata of *Sclerotinia trifoliorum* was identical to that of *Sclerotinia sclerotiorum* and *S. minor*.

Apparently there was no difference in sclerotial size of all the seven isolates (1-8) and it varied from 3.6 × 3.0 mm to 5.6 × 3.6 mm (Table 2 B). The weight of sclerotia was less and at par in isolate 1, 2, 3, 4S but it increased significantly after subsequent re-culturing (Table 2 C). The weight of sclerotia was more and at par in isolates 4L, 7, 8 in both the culturing conditions. Interestingly the sclerotial weight becomes almost similar in all the seven isolates when it was isolated from the cross infected berseem host (Table 2 D). It indicates that large sclerotia forming *Sclerotinia sclerotiorum* isolate from other hosts, formed smaller sclerotia after inoculation on berseem. Cross inoculation revealed that infection was clearly recorded cent percent on all the test hosts by all the isolates irrespective of pathogen and host species (Table 3). Indiscriminate colonization by the *Sclerotinia* species on these hosts without any selective host specificity clearly indicates that all may be the same species of *Sclerotinia* (Table 3). The number of sclerotia was statistically at par in 4S, 8 when natural berseem isolate (1,4S) was inoculated on berseem and brinjal while *Chenopodium* (8) inoculated on berseem. Similarly berseem native isolate (2,4L) and brinjal isolate 7 was inoculated on berseem was at par to each other. Berseem and brinjal isolate inoculated on berseem and tomato respectively resulted similar result (Table 4). There was no significant difference

Table 3 Cross infectivity test of different *Sclerotinia* spp on different hosts under controlled conditions

Isolate No.	Native Host	A-Inoculated host (Infection %)	Re-inoculated host from-A (Infection %)
1	Berseem	Tomato (100%)	Berseem (100%)
2	Berseem	Tomato (100%)	Berseem (100%)
3	Berseem	Brinjal (100%)	Berseem (100%)
4S	Berseem	Brinjal (100%)	Berseem (100%)
4L	Berseem	Brinjal, Tomato (100%)	Berseem (100%)
7	Brinjal	Berseem, Tomato (100%)	Berseem (100%)
8	Chenopodium	Brinjal, Tomato (100%)	Berseem (100%)

Table 4 Sclerotial number and size of different *Sclerotinia* spp isolated from cross inoculated host

Isolate No.	Native isolate	Inoculated host	Number/ plate	Sclerotial size (mm)
1	Berseem	Berseem	18.0	4.6 × 2.5
2	Berseem	Berseem	29.0	4.8 × 2.7
3	Berseem	Berseem	34.0	4.7 × 2.9
4S	Berseem	Tomato	14.5	6.3 × 4.4
4L	Berseem	Berseem	28.5	4.3 × 2.7
7	Brinjal	Berseem	24.0	5.2 × 3.6
8	Chenopodium	Berseem	22.0	5.3 × 4.4
4S	Berseem	Brinjal	17.0	5.1 × 3.6
7	Brinjal	Tomato	37.0	3.8 × 2.7
	CD (P=0.05)		8.9	
	CV (%)		11.3	

in sclerotial size in most of the inoculated isolates except 4S where size increased (6.3 mm × 4.4 mm) and number decreased to 14.5 unexpectedly. Confirmation of cross infectivity test and gradual change of colony characters of *S. trifoliorum* to *S. sclerotiorum* after subsequent sub-culturing may be considered as reasonable criteria to classify it under *S. sclerotiorum.* Taxonomically most of the *Sclerotinia* spp infecting several hosts has classified under *S. sclerotiorum.* Lack of host specificity and necrotrophic nature of *Sclerotinia* may be considered another reason for a single species like *Sclerotium rolfsii* and *Rhizoctonia solani*. Double diffusion tests showed that *Sclerotinia trifoliorum*, *Sclerotinia sclerotiorum* and *S. minor* are serologically related, but the relationship between *Sclerotinia sclerotiorum* and *S. minor* is closer than that between either of these and *Sclerotinia trifoliorum* (Scott 1981). Nelson *et al.* (1989) reported that *Sclerotinia trifoliorum* is similar in biology and morphology to *Sclerotinia sclerotiorum*, but *S. minor* produces much smaller sclerotia and generally does not produce apothecia in nature. Singh (1982) reported that the pathogen has a number of morphological strains such as *S. minor*, *S. intermedia*, *S. trifoliorum* and *S. sativa* that are now considered synonymous with *S. sclerotiorum*. However, Arseniuk (1996) reported that *S. minor*, *Sclerotinia sclerotiorum* and *Sclerotinia trifoliorum* are distant species based on size of ascospores, mycelial interaction, colony growth rate at various temperature and colony morphology and PDA.

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