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Research Article

# UPLC-Q-TOF-MS-based untargeted studies of the secondary metabolites secreted by *Sclerotinia sclerotiorum* under the axenic condition

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## Abstract

The stem rot disease has emerged globally as a major threat to oilseed Brassica's productivity and seed quality. The generalist causal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary shows large variability in their aggressiveness and pathogenicity. Revealing the pathogen's metabolic profile and signaling components in host-pathogen interaction is fundamental in understanding host resistance to the disease. In this study, the metabolites released by the pathogenic strains of *S. sclerotiorum* under the axenic culture have been identified using the untargeted high-resolution UPLC-QTOF-ESI-MS/MS. The analysis of the ethyl acetate extracts of the *S. sclerotiorum* culture revealed ten major secondary metabolites namely, sclerin, sclerotinin-B, sclerone, melanin, bostrycoidin, botcinin-D, botcinin-A, gliovirin, scleramide, and botcinic acid. The later six metabolites are being reported for the first time in the culture extract of the *S. sclerotiorum* pathogen. Based on the overlapping and unique informative peaks in the chromatograms, the six *S. sclerotiorum* strains were grouped into three major clades in the phylogenetic analysis. The clustering based on metabolic profiles does not substantiate the diversity based on morphology or virulence differences over the host. The findings of the study signified the metabolites secreted under the axenic conditions are varies based on their growth and developmental stages and may not necessarily be the determining factors for their differential aggressiveness and virulence to their host.

**More Information**

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**Abbreviations:** UPLC: Ultra Performance Liquid Chromatography; Q-TOF: Quadruple-Time of Flight; ESI: Electrospray Ionization; MS: Mass Spectrometry; CAZYmes: Carbohydrate Activating enzymes; CWDE: Cell Wall Degrading Enzymes; DHN: Di Hydroxy Naphthalene; HV: Highly Virulent; V: Virulent; MV: Moderately Virulent; ESR: Extramural Stem Rot; UPGMA: Unweighted Pair Group Method with Arithmetic mean; NTSYS: Numerical Taxonomy System; SM: Secondary Metabolites



## Introduction

The phytopathogen *Sclerotinia sclerotiorum* (Lib.) de Bary also known as white mold, causing stem rot disease in oilseed *Brassica* is a broad host range and ubiquitously distributed fungal pathogen [1]. The damage to crops by *S. sclerotiorum* leads to substantial yield losses in susceptible species. It is known to infect more than 400 plant species belonging to 278 genera of 75 families, including various economically important crops including rapeseed-mustard [2,3]. In *Brassica,* 10% - 80% yield reduction and deterioration of seed quality have been observed due to *S. sclerotiorum* infestation [4,5]. In Rajasthan, alone, which is a major *Brassica-growing* state in India, an estimated yield loss of 37%-92% has been reported due to this disease [6].

The life cycle of *S. sclerotiorum* has a very short biotrophic phase followed by a long necrotrophic phase that makes it hemibiotrophic in nature [7,8]. The life cycle of *S. sclerotiorum* involves mycelial growth, appressorium establishment, sclerotia formation, and apothecial and ascospore development followed by virulence [9,10]. Despite sporadic attempts have been made to study the key secondary metabolites produced by the pathogen and their role in virulence on host-pathogen interactions, the molecular mechanism of virulence for *S. sclerotiorum* is poorly understood and necessitates much research on the host and pathogen behaviors. Variation in pathogen virulence and host resistance determines the severity of the disease [7]. Virulence of the pathogen is mediated by various pathogenicity factors which include secretory proteins, effector candidates, secondary metabolites,

and carbohydrate activating enzymes (CAZYmes) that include mostly the cell wall degrading enzymes (CWDEs) [11]. During the disease progression, host defense is usually suppressed by the fungal pathogen either by metabolizing the host secreters or promoting the transporter-based efflux system within the host [12,13]. The necrotrophic mode of infection involves suppression of host defense mediated by the pathogen secretory proteins and induction of cellular death in the host [14]. Oxalic acid, cell wall degrading enzymes like pectinase, cellulase, and xylanase have been found as the major disease-establishing factors which enable the *S. sclerotiorum* entry inside the host. Initiation of the infection is immediately followed by changing the cellular ion efflux of the host cells and degradation of the cell wall components at the site of infection [15-17].

The secondary metabolites secreted by the pathogen at different stages of their interaction with the host serve diverse functions that include facilitation of the infection, escape from the host defense, signaling for cascading the infection, and nutrient uptake for survival and proliferation of the pathogen inside the host. Among them, some of the secondary metabolites secreted by the necrotrophs are broad-spectrum in action whereas many of them are host-specific [18,19]. The botrydial and botcinic acid secreted by *Botrytis cinerea* during pathogenesis have been found to induce tissue necrosis in the host [20,21]. The knock-out of transcriptional regulator *BcReg1* in *B. cinerea* makes the pathogen ineffective to form lesions which was due to the lack of virulence factors botrydial and botcinic acid in the knock-outs [22]. *In-silico* analysis revealed various homologous clusters of *B. cinerea* in *S. sclerotiorum* to produce secondary metabolites such as carotenoids, dihydroxy napthalene (DHN) melanin, siderophores, and botcinic acid. Putative clusters of polyketides sclerin were also identified in *S. sclerotiorum* along with the ABC transporter which aided the suppression of the host defense response through efflux [23]. DHN melanin biosynthesis, reported in *S. sclerotiorum* [24] has been found to play an important role in increasing the longevity of the sclerotia [25]. Another metabolite scleramide was isolated from the sclerotial extracts of *Aspergillus sclerotiorum* [26] whereas sclerin was isolated from *S. sclerotiorum*. Both metabolites were found to play an important role in regulating the maturation and pigmentation of sclerotia [27]. Oxalic acid has been considered essential for the initiation of infection in the host as the oxalic acid-deficient strain of *S. sclerotiorum* showed reduced virulence in several host species [28].

The successful disease establishment seems to be an outcome of the cumulative effects of several downstream processes occurring within the host as well as in the pathogen following the interaction. Although a vast array of information related to different aspects of *S. sclerotiorum* infection has emanated from the recent transcriptomic, proteomic, and secretome studies, however, the wide diversity in *S. sclerotiorum* population remained a major hurdle. This also reflects a likely variation in the mechanism of their pathogenesis which permits further study. In oilseed *Brassica*, the molecular and metabolic processes that regulate the host resistance to *S. sclerotiorum* are largely unknown. Identifying the key metabolites which determine the aggressiveness of the pathogen is important to postulate novel targets for engineered host resistance. To date, a limited number of metabolites *viz.* DHN melanin, sclerin, sclerone, isosclerone, sclerolide, and sclerotinin A and B have been isolated and characterized in *S. sclerotiorum* [24,29]. Earlier, we categorized diverse isolates of the *S. sclerotiorum* into different pathogenicity groups [30]. Here, we have carried out comprehensive metabolite profiling of the six isolates of *S. sclerotiorum,* belonging to three different pathogenicity groups. The metabolites with their diversity under the axenic condition would add to the existing knowledge of the secondary metabolites secreted by *S. sclerotiorum* and their functions in pathogenesis.

## Materials and methods

### *Sclerotinia sclerotiorum* isolates and its culture

From the pathogenically distinct 65 *S. sclerotiorum* isolates [30], two each from the highly virulent (HV; ESR-18, ESR-25), virulent (V; ESR-01, ESR-05), and moderately virulent isolates (MV; ESR-04, ESR-42), were randomly selected for metabolite profiling. For growing the inoculum, all the six isolates were inoculated on potato dextrose agar (PDA) medium and incubated at 22 °C ± 2 °C for 5 days. Mycelial mat from the 5 days old actively growing mycelial culture was scrapped and re-inoculated in 500 mL of potato dextrose broth (PDB) in 1 L flask and grown in the dark at 22° ± 2 °C for 10-days with continuous shaking at 120 rpm. The 10-day-old broth culture was filtered with a double layer of muslin cloth in a sterile 500 mL flask for further processing.

### Extraction of fungal culture filtrate

The cell-free culture filtrates (250 mL) were extracted thrice with ethyl acetate (100 mL × 3). For distinct and clear solvent layer separation, 10 mL saturated sodium hydrochloride solution was added to each extract and shaken vigorously. The upper ethyl acetate layer was filtered out and passed through anhydrous sodium sulfate (20 g) to remove any traces of water. Liquid-liquid partitioning was followed to extract maximum secondary metabolites as described by Kundu, et al. [31]. Extracted ethyl acetate from the culture filtrates was evaporated under reduced pressure below 40 °C in a rotary evaporator (Heidolph, Germany) for obtaining the different amounts of the semisolid residues *viz*. 56 mg (ESR-01), 32 mg (ESR-04), 42 mg (ESR-05), 13 mg (ESR-25) and 67 mg (ESR-42). These extracts were subjected to six UPLC-QTOF-ESI-MS analyses for the separation and identification of the major secondary metabolites.

### UPLC-QTOF-ESI-MS/MS analysis

UPLC-QTOF-ESI-MS/MS analysis was performed on an Acquity Ultra Performance Liquid Chromatography (UPLC) directly attached to a Quadrupole-Time of Flight mass spectrometer (Waters Corporation, Manchester, UK). The data acquisition was done with the $MS^E$ function in continuum mode in the range of m/z 50-1200. The $MS^E$ mode provides the full scan of MS data (low energy, 4V) and MS/MS data (high energy, 15-60V ramping), simultaneously. The ion source parameters were set as default which was fixed with the capillary 4.5kV, sampling cone 25V, extraction cone 5V, source temperature 250 °C, desolvation temperature 400 °C, desolvation gas flow 1000 L/h, and cone gas flow 30 L/h. The chromatographic separation was performed on an ACQUITY UPLC BEH $C_{18}$ column (2.5 × 100 mm, 1.5 µm) at 30 °C. The mobile phase consisted of phase 'A' methanol: water (5:95) and phase 'B' methanol: water (95:5) with 0.1% formic acid in both phases. A gradient program was used with 0.5 mL $min^{-1}$ flow rate, with 0-5 min 100% A, 5-10 min 60% A, 10-15 min 40% A, 15-18 min 60% A, 18-25 min 100% A. The injection volume was 3 µL and the samples were maintained at 25 °C throughout the analysis.

### Molecular ion peak analysis and metabolite prediction

The molecular ion and fragment ion peaks of mass analysis were processed using the UNIFI software package version 1.7. Version (Waters Corporation, Manchester, UK). The database for the secondary metabolites was prepared in the UNIFI software [DG SANTE guideline]. An in-house database for the known species-specific fungal compounds with molecular masses and their elemental composition known in the public domain was constructed. Wherever the species-specific compounds were not available the genus-specific fungal metabolites were included. All the metabolites were identified based on their exact molecular and adduct ion peaks within the acceptable error mass (δ) value of 3 ppm. Tentative compounds were manually further inspected by comparing their mass fragmentation pattern. Chem Draw Ultra, version 15.0 (Bedford, MA, USA) was used for drawing the 2D structure of the compounds and their fragmentation patterns.

### Phylogenetic analysis

Cluster analysis of UPLC-QTOF-MS peaks data was carried out using NTSYS (Numerical Taxonomy and multivariate analysis SYStem). The phenetic classification of NTSYS is based on similarity among the multivariate data generated from the patterns of biological diversity. An input file with scoring into 0, 1 binary matrix for the presence and absence of each peak was prepared. The UPGMA of the SHAN module of NTSYS-pc version 2.2 packages was applied to determine the similarity among the isolates using Jaccard's coefficient between each pair and the isolates were clustered together in the dendrogram.

## Results

### *S. sclerotiorum* isolates and their pathogenicity variance

The pathogenicity grouping of *S. sclerotiorum* isolates includes ESR-01, ESR-04, ESR-05, ESR-18, ESR-25, and ESR-42 (Figure 1) [30]. The virulence grading was done for the individual isolates in terms of mean lesion length which is the function of individual isolates i.e., how fast the pathogen proliferates and establishes infection to develop lesion at the inoculation sites on the host. The virulence evaluation of the selected isolates has grouped them into highly virulent (HV; ESR-18 & ESR-25), virulent (V; ESR-05 & ESR-01), and moderately virulent (MV; ESR-04 & ESR-42) (Table 1). The longest stem lesion was observed in *B. rapa* var yellow sarson (NRCYS 5-2) with ESR-18 isolate whereas the smallest was observed in *B. juncea* (NRCDR-2) with ESR 42. The variability in the severity of infection with different isolates indicates the influence of both the virulence properties of the isolates as well as the defense characteristics of the host.

### UPLC-QTOF-ESI-MS peak simulation and tentative metabolite profiling

UPLC-QTOF-ESI-MS analysis of ethyl acetate extracts of the six different *S. sclerotiorum* isolates revealed various characteristic peaks (S1-S6) corresponding to their molecular and adduct ions (S7). With the manual simulation of the UPLC-MS peaks, ten different metabolites namely, (i) sclerin, (ii) sclerotinin B, (iii) sclerone, (iv) bostrycoidin, (v) botcinin D, (vi) botcinin A, (vii) gliovirin, (viii) melanin, (ix) scleramide, and (x) botcinic acid were putatively identified (Figure 2). The metabolites along with their neutral, observed molecular mass and adducts are listed in Table 2. Reported sources of all the metabolites are also mentioned in Table 2 wherein, sclerin, sclerone, melanin, and sclerotinin B have already been reported in *S. sclerotiorum* whereas the six other metabolites from different fungal species. Botcinic acid, botcinin A, and botcinin D are reported in *B. cinerea* whereas bostrycoidin,

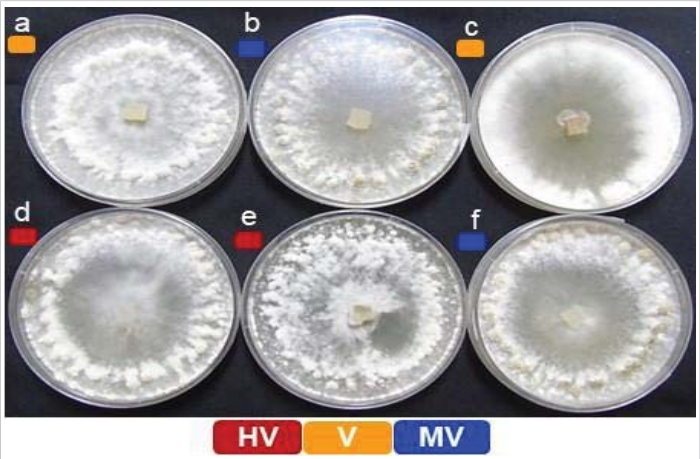


**Figure 1:** Morphological variability and pathogenic diversity among the six different isolates of *Sclerotinia sclerotiorum*. (a) ESR-01, (b) ESR-04, (c) ESR-05, (d) ESR-18, (e) ESR-25, and (f) ESR-42. Colored blocks depicting their pathogenicity grouping.

**Table 1:** The *S. sclerotiorum* isolates with their geographical distribution and virulence grading assessed on *Brassica* cultivars based on the disease severity, measured in lesion length (cm).

| Isolates | Source host | Provinces | Geographical locations | Mycelium growth on PDA (mm) at 72 h | Sclerotia/plate | Mean Lesion length (cm) over the four *Brassica* cultivars | Virulence |
|---|---|---|---|---|---|---|---|
| ESR-01 | *B. juncea* | Rajasthan | 27°15'N; 77°30' E | 85 | 40 | 17.7 | Virulent |
| ESR-04 | -do- | UP | 26°28'N; 80°24' E | 34 | 49 | 7 .7 | Moderately Virulent |
| ESR-05 | *B. rapa* | UP | 25°28'N; 81°54' E | 90 | 33 | 14.5 | Virulent |
| ESR-18 | -do- | Punjab | 30°11'N; 75°00' E | 80 | 38 | 25.8 | Highly Virulent |
| ESR-25 | -do- | UP | 27°28'N; 77°41' E | 90 | 39 | 24.8 | Highly Virulent |
| ESR-42 | -do- | Rajasthan | 27°34'N; 77°37' E | 77 | 38 | 8.8 | Moderately Virulent |

**Table 2:** List of putative secondary metabolites identified with their retention time, neutral mass, observed molecular mass and adducts along with the source from which they were first reported

| Tentative metabolites | Molecular formula | $R_t$ (min) | Neutral Mass (Da) | Observed m/z | Adduct |
|---|---|---|---|---|---|
| Sclerone | $C_{10}H_{10}O_3$ | 3.02 | 178.0630 | 179.0706, 197.1319 | $[H]^+$, $[H+NH_4]^+$ |
| Sclerin | $C_{13}H_{14}O_4$ | 5.29 | 234.0892 | 235.1713 | $[H]^+$ |
| Botcinic acid | $C_{20}H_{34}O_8$ | 5.88 | 402.2254 | 403.1438 | $[H]^+$ |
| Melanin | $C_{18}H_{10}N_2O_4$ | 7.34 | 318.0640 | 319.3947, 336.1815 | $[H]^+$, $[NH_4]^+$ |
| Botcinin D | $C_{20}H_{30}O_6$ | 7.69 | 366.2042 | 367.1489 | $[H]^+$ |
| Gliovirin | $C_{20}H_{20}N_2O_8S_2$ | 8.84 | 480.5110 | 481.6326, 503.3604 | $[H]^+$, $[Na]^+$ |
| Botcinin A | $C_{22}H_{34}O_8$ | 9.15 | 426.2254 | 426.2267 | $[M]^+$ |
| Sclerotinin B | $C_{12}H_{14}O_5$ | 9.05 | 238.0841 | 238.2365, 256.2719 | $[M]^+$, $[NH_4]^+$ |
| Bostrycoidin | $C_{15}H_{11}NO_5$ | 9.64 | 285.0637 | 285.3492 | $[M]^+$ |
| Scleramide | $C_{38}H_{45}N_7O_7$ | 11.81 | 712.3380 | 713.5579 | $[H]^+$ |

**Table 3:** Comparative metabolite profiling based on differential analysis of fungal metabolites in six different isolates of *S. sclerotiorum* using UPLC-QTOF-MS.

| S No. | Metabolites | ESR1 | ESR4 | ESR5 | ESR18 | ESR-25 | ESR42 |
|---|---|---|---|---|---|---|---|
| 1 | Sclerone | 197.1319 | 197.1319 | 197.1319 | 197.1319 | - | 197.1319 |
| 2 | Sclerin | - | 235.1713 | - | 235.1713 | 235.1713 | 235.1713 |
| 3 | Sclerotinin B | 256.2719 | 256.2719 | - | 256.2719 | - | 256.2719 |
| 4 | Bostrycoidin | 285.3492 | - | - | - | - | - |
| 5 | Melanin | 336.1815 | - | 336.1815 | - | - | 336.1815 |
| 6 | Botcinin D | - | 367.1489 | 367.1489 | 367.1489 | - | 367.1489 |
| 7 | Botcinic acid | - | 403.1438 | - | 403.1438 | - | 403.1438 |
| 8 | Botcinin A | - | 426.2267 | 426.2267 | - | 426.2267 | 426.2267 |
| 9 | Gliovirin | - | 503.3604 | 503.3604 | 503.3604 | - | 503.3604 |
| 10 | Scleramide | - | - | 713.5579 | 713.5579 | - | 713.5579 |

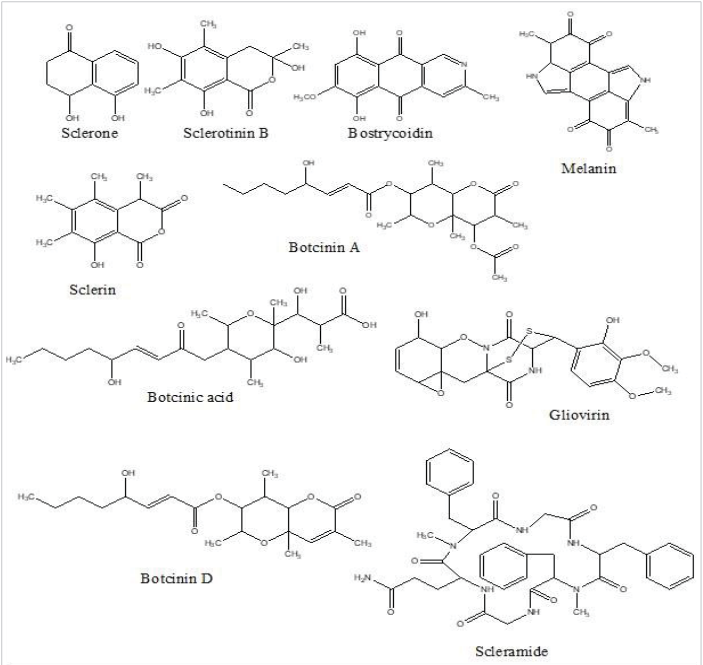


**Figure 2:** Tentative identification of the secondary metabolites in six different isolates of *S. sclerotiorum* using UPLC-Q-TOF-ESI-MS.

gliovirin, and scleramide in *Fusarium bostrycoides*, *Gliocladium virens,* and *Aspergillus sclerotiorum,* respectively. Further, the comparative analysis of the identified secondary metabolites in six different isolates was delineated as shown in Table 3.

The total ion chromatogram (TIC) of the ethyl acetate extract of ESR-1 showed various characteristic peaks, leading to the identification of four major secondary metabolites namely, sclerone, sclerotinin B, bostrycoidin, and melanin (Figure 2). The first tentatively identified compound of ESR-01 was sclerone, eluted from the column at $R_t$ 2.86 min interval, and exhibited distinct protonated molecular ion peak at m/z 179.0706 and ammoniated adduct ion peak at m/z 197.1319. Further, the fragmentation pattern in MS/MS analysis showed daughter ion peaks at m/z 169, formed due to the removal of carbonyl moiety (-CO, 28 amu) (Figure 3i). Similarly, sclerotinin B showed the characteristic molecular ion peak $[M]^+$ at m/z 238.2365 and ammoniated adduct ion peak at m/z 256.2719. Furthermore, fragmentation of sclerotinin B resulted in daughter ion peaks at m/z 149, which originated due to the sequential removal of methyl (-$CH_3$, 15 amu), carbonyl (-CO, 28 amu), and hydroxyl (-OH, -17 amu)

moieties (Figure 3ii). On the other hand, bostrycoidin was characterized by its $[M]^+$ peak at m/z 285.3492, further, fragmentation resulted in fragment ion peaks at m/z 239 after losing both the methyl ($-CH_3$, 15 amu) and methoxy ($-OCH_3$, 31 amu) moieties (Figure 3iii). The fourth metabolite of ESR-01 was identified as melanin which showed $R_t$ 7.34 min with the characteristic protonated adduct ion peak at m/z 319.3947 and ammoniated adduct ion peak at m/z 336.1815 (Figure 3iv).

Similarly, the total ion chromatogram (TIC) of ESR-4 led to the identification of seven secondary metabolites sclerone, sclerin, sclerotinin B, botcinin A, D, botcinic acid, and scleramide (Figure 2). Sclerin was identified from its accurate protonated adduct ion peak at m/z 235.1713, which on the exposure of energy fragmented to form daughter ion peaks at m/z 178 and m/z 149 after subsequent elimination of two carbonyl and methyl groups, respectively (Figure 4i). Another two congeners of botcinin such as botcinin A and D were characterized by their accurate molecular ion peak at m/z 426.2267 and protonated adduct ion peak at m/z 367.1489, respectively. Further botcinin A possessed protonated adduct ion peak at m/z 426.2267, which was cleaved to remove the acetyl group followed by an alkyl ester side chain to get a daughter ion peak at m/z 383 and 243, respectively (Figure 4ii). Similarly, botcinin D showed protonated adduct ion peak at m/z 367.1489, which on fragmentation gave a major fragment ion peak at m/z 227 after the removal of side-chain alkyl ester (Figure 4iii). Similarly, scleramide exhibited protonated adduct ion peak at m/z 713.5579, which on fragmentation produced a daughter ion at m/z 149 (Figure 4iv).

The TIC of ESR-5 showed six similar secondary metabolites such as sclerone, melanin, gliovirin, scleramide, botcinin A, and D (Figure 2). Except for gliovirin, the other five metabolites resembled the previously identified compounds. Thus, gliovirin was characterized by its protonated adduct ion peak at m/z 481.6326 along with the sodiated adduct ion peak at m/z 503.3604 (Figure 5i). Another secondary metabolite, botcinic acid exhibited protonated adduct ion peak at m/z 403.1438. Further fragmentation of parent ion on higher energy produced major daughter ion at m/z 344 after losing acidic moiety (Figure 5ii).

While the TIC of ESR-18 possessed characteristic $[M]^+$ peaks, corresponding to seven secondary metabolites such as sclerone, sclerin, sclerotinin B, botcinin D, botcinic acid, gliovirin, and scleramide (Figure 2). However, the mass spectra of ethyl acetate fraction of ESR-25, unfortunately, showed very few peaks which are matching only with sclerin and botcinin A (Figure 2). Mass spectral analysis of ESR-42 comprised of various peaks corresponding to seven secondary metabolites such as sclerone, sclerin, sclerotinin B, melanin, botcinic acid, botcinin A, and scleramide.

## Comparative metabolite profiling

The differential analysis of secondary metabolites in

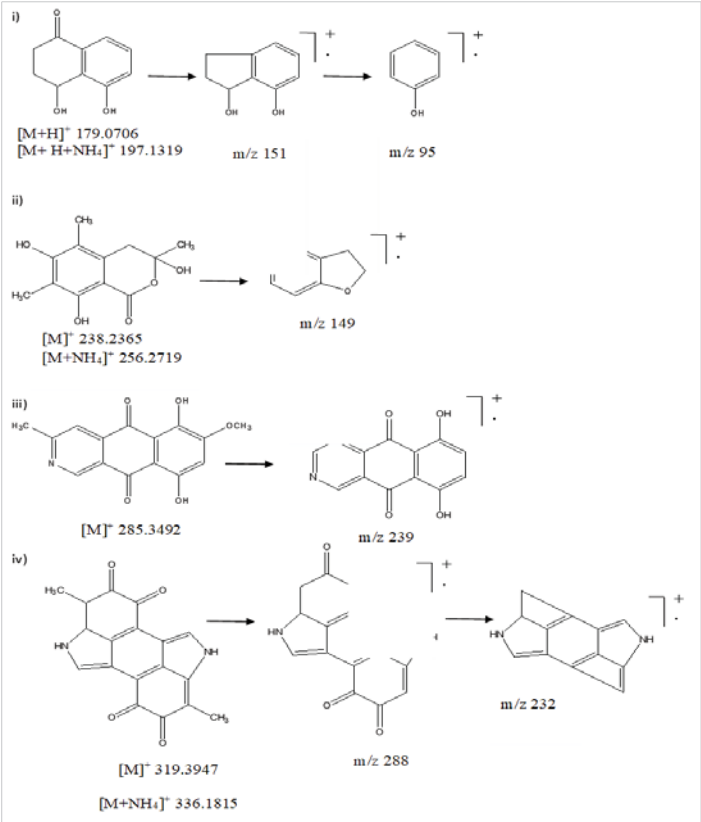


**Figure 3:** Mass fragmentation pattern of secondary metabolites. i) Sclerone, ii) Sclerotinin B, iii) Bostrycoidin and iv) Melanin of S. sclerotiorum using UPLC-Q-TOF-ESI-MS.

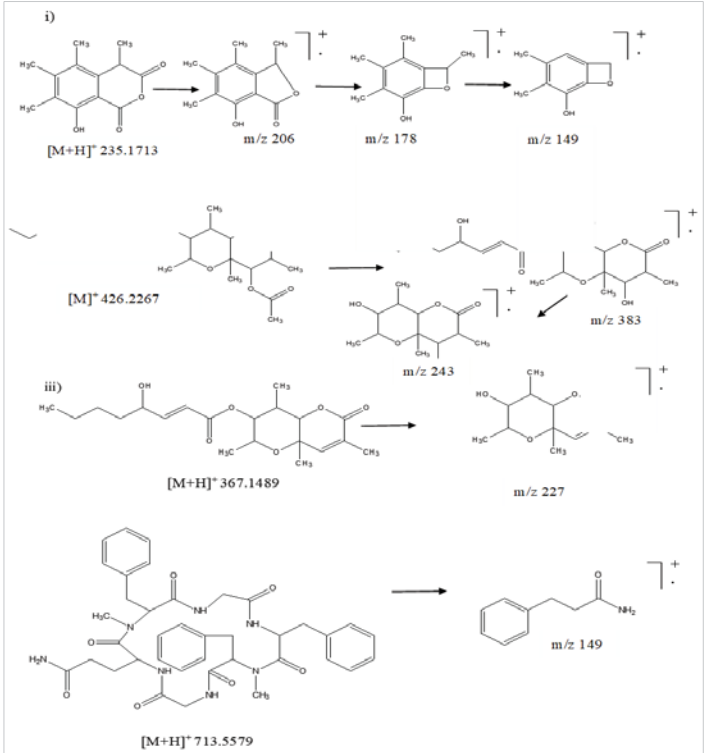


**Figure 4:** Mass fragmentation pattern of secondary metabolites. i) Sclerin, ii) Botcinin A, iii) Botcinic acid and iv) Scleramide of S. sclerotiorum using UPLC-Q-TOF-ESI-MS/MS.

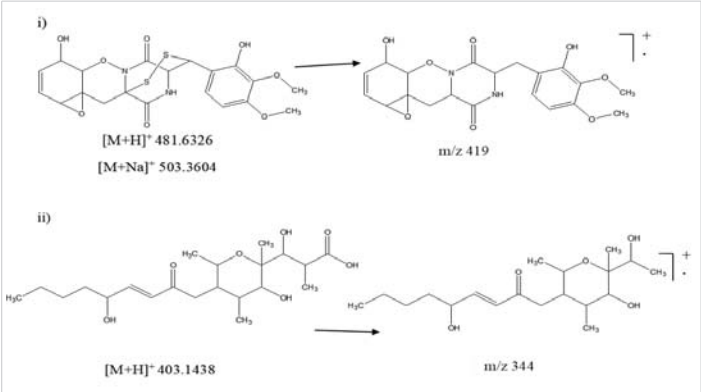


**Figure 5:** Mass fragmentation pattern of secondary metabolites. i) Gliovirin and ii) Botcinin D of *S. sclerotiorum* using UPLC-Q-TOF-ESI-MS/MS.

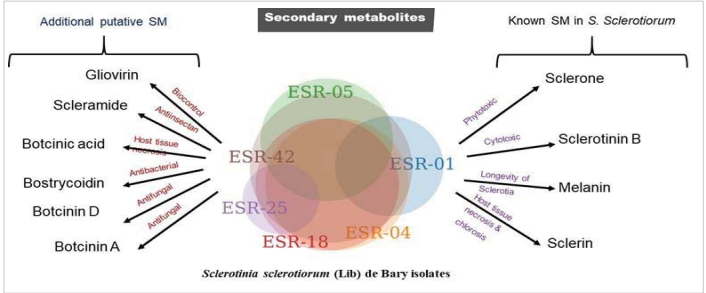


**Figure 6:** Schematic representation of the secondary metabolites identified by LCMS analysis of the liquid culture of *S. sclerotiorum* pathogen. The left panel enlists the six additional putative SM and the right panel shows the known SM identified under the axenic condition.

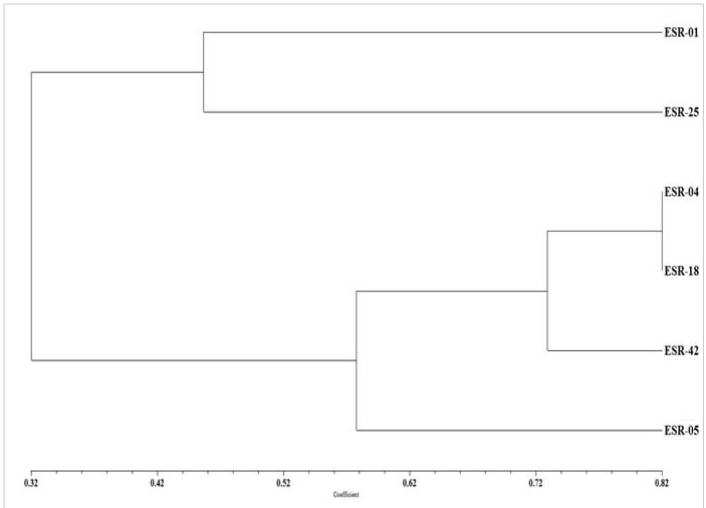


**Figure 7:** Cluster dendrogram of the *S. sclerotiorum* isolates based on comparative metabolite profiling obtained in UPLC-QTOF-ESI-MS/MS analysis.

pathogenically diverse isolates of *S. sclerotiorum* (Table 3) revealed the release of the secondary metabolites under axenic conditions is independent of their virulence and that varies based on the isolate's divergence. The observation has further been supported by the metabolite-based phylogeny that revealed the secondary metabolites secreted under the natural growth condition are not truly depicting the virulence of the pathogen and hence the virulence-based grouping of isolates do not corroborate the metabolite-based grouping (Figure 6). In UPLC-MS, ESR-42 has a maximum number of identified peaks including tentatively identified compounds such as sclerin, sclerotinin B, sclerone, botcinin D, and botcinin A, gliovirin, melanin, scleramide, botcinic acid except for bostrycoidin (Table 3). ESR-04 and ESR-18 have similar compounds identified except for botcinin A and scleramide which include sclerin, sclerotinin B, botcinin D, botcinin A, gliovirin, and botcinic acid. ESR-04 and ESR-18 differ from ESR-42 in the absence of botcinic acid, scleramide, and melanin. ESR-01 includes sclerotinin B, sclerone, bostrycoidin, and melanin whereas ESR-05 includes melanin, sclerone, botcinin D, scleramide, gliovirin, and botcinin A (Table 3). In the isolate ESR-25, only sclerin and botcinin A was detected. Among the ten prominent secretory metabolites identified in the axenic cultures of *S. sclerotiorum* isolates, six metabolites gliovirin, scleramide, botcinic acid, bostrycoidin, botcinin A & D were revealed for the first time in the present studies along with the four other metabolites sclerin, sclerone, sclerotinin-B, and melanin were known earlier (Figure 6).

### Metabolite-based phylogeny of the *S. sclerotiorum* isolates

Phenetic studies were carried out based on identified ten major UPLC-QTOF-MS peaks scored as 1 and 0 for presence and absence, respectively in all the six isolates of the *S. sclerotiorum*. The data was analyzed by similarity-based UPGMA cluster analysis method using NTSYS software. The six isolates of the *S. sclerotiorum* belonging to three different Indian provinces Rajasthan, U.P., and Punjab were grouped into three major clades based on the number of informative characteristic peaks. Clade I carries the ESR-01 (V) and ESR-25 (HV) isolates and shared 45% similarity with the remaining isolates; whereas the three isolates ESR-04, -18, and -42 were present in clade II and solitary ESR-05 isolate was present in clade III with 58% similarity (Figure 7). Clade II with three isolates has further divided into two subgroups with 73% similarity in which subgroup I carry the ESR-04 (MV) and ESR-18 (HV) isolates whereas ESR-42, an MV isolates remained solitary in subgroup II (Figure 7).

## Discussion

*Sclerotinia* stem rot disease causes yield losses in many crops throughout the world [2]. The use of chemical fungicides as the most prevalent control measure has never been effective due to uncertainty in doses and time of application in the field in addition to the incurred high cost [32,33]. Though the natural variation in host resistance has been identified in a few instances, the convergence of resistance to the cultivated varieties has not been achieved much. In addition to breeding efforts, parallel basic studies on the pathogen virulence and determinant of differential pathogenicity in *S. sclerotiorum* isolates are pivotal for the durability of resistance [34]. In the present study aggressiveness of the isolates belonging to pathogenically three different classes namely highly virulent (HV), virulent (V) and moderately virulent (MV) were compared based on their secreted secondary metabolites in growth culture. The assessment clustered ESR-01, a virulent

isolate from Rajasthan, and ESR-25, a highly virulent isolate from UP together in the major clade I. ESR-04, an MV isolates from UP clustered together with HV isolate ESR-18, collected from Punjab, in subgroup I of the major clade II. The subgroup II of the major clad II consisted of a single MV isolate ESR-42 of Rajasthan which shared 73% similarity with the other two isolates present in subgroup I. Clade III represented the isolate ESR-05 from UP though it shared 58% similarity with the other isolates based on secreted metabolites. This signified the role of the metabolites in determining the virulence and pathogenicity of the isolates over the susceptible hosts. No influence of geographical location, as well as the source of origin of the isolates on their aggressiveness, has also been evident from earlier studies [30,35].

The disease severity of *S. sclerotiorum* involves a consequence of complex interactions between several factors including aggressiveness of the isolates and host resistance mechanisms [36]. The differential response of the cultivars to *S. sclerotiorum* isolates can be attributed to variable lignification of stem cortical cells and the number of cell layers in the cortex [36]. A study of different *S. sclerotiorum* isolates revealed that the hyphae in infection cushions of the highly virulent isolates were arranged together in dense parallel bundles whereas hyphae in infection cushions of a less virulent isolate were more diffused independently of the host genotype [36-38]. Moderately virulent isolates ESR-04 and -42 demonstrated a slow growth rate as compared to the other isolates whereas ESR-05 (V) has a high growth rate when the diameter of the lesions is at 72 hours post inoculation (hpi) was compared (Table 1). The growth rate of the pathogen was found to be positively related to the virulence of the isolates. The differential virulence of the isolates was also attributed to effector proteins, secondary metabolites, and carbohydrate activating enzymes [14,15]. Among the major pathogenicity factors such as proteinaceous effectors, secondary metabolites, and cell wall degrading enzymes , secondary metabolites are the less investigated aspect in infection by *S. sclerotiorum* [39]. Many necrotrophs are known to secrete broad-spectrum phytotoxic secondary metabolites to induce host tissue necrosis [40,41].

Many fungal secondary metabolite biosynthetic gene clusters remain silent under axenic conditions [42,43]. The transcriptional regulation of such genes is usually controlled by several external stimuli including environmental and host interacting factors [44]. The present study has also emphasized that the secreted secondary metabolites under axenic conditions may not be solely determining the virulence of the pathogen but it depends on the outcome of their firm interaction with the hosts. Hence, the role of secondary metabolites in standard cultivation condition and their relevance in fungal biology could not be neglected.

The UPLC-QTOF-ESI-MS/MS has been exploited extensively for non-target analysis of a wide range of secondary metabolites [45,46]. The *S. sclerotiorum* isolates under study have 99% conserved ITS DNA sequences yet differ in their metabolome profiles and virulence over the *Brassica* cultivars. Out of the ten predicted secretory compounds with their neutral, observed molecular mass and adducts (Table 2), four of them *viz.* sclerin, sclerone, melanin, and sclerotinin B were reported in earlier studies from *S. sclerotiorum* [24,47-51]. The remaining six secondary metabolites bostrycoidin, botcinic acid, botcinin A and D, gliovirin, and scleramide were detected for the first instance in *S. sclerotiorum* culture.

Fungal secondary metabolites have also been shown to bear medicinal and industrial benefits [52]. Bostrycoidin isolated from *Fusarium bostrycoides* extract was found to bear antibiotic properties [53], whereas bostrycoidin isolated from *Fusarium* spp. was found phytotoxic and possessed antifungal and bacteriostatic properties [54,55]. Botcinic acid is known to be the phytotoxic compound involved in the virulence of *B. cinerea* [20,56]. The *bcreg1* knock-out mutants provided proof of concept for botcinic acid and botrydial being the virulence factors in *B. cinerea* [22]. Botcinins and botrydial in *B. cinerea* are important for virulence as the lack of both toxins leads to reduced disease severity [21,57]. Gliovirin, a diketopiperazine isolated from soil-borne mycoparasite *Gliocladium virens* showed strong inhibitory activity against *Pythium ultimum* [58,59]. DHN Melanin is required for making sclerotia a long-lasting persistent resting body synthesized from the compact mycelial mass of the *S. sclerotiorum* by melanogenesis [24,47]. Melanization protects sclerotia from destruction due to reactive oxygen species (ROS), enzymatic action, and environmental effects like UV exposure [24]. Disruption of genes required for melanogenesis in *S. sclerotiorum* displayed impaired sclerotia formation and resistance to UV rays [7]. Sclerone, extracted from *S. sclerotiorum* has also been observed as phytotoxic [51]. Scleramide, a potent antiinsectan, classified as paraherquamide [26] was isolated from the sclerotial extract of *Aspergillus sclerotiorum*. Sclerin was extracted from *S. sclerotiorum* which has displayed hormone-like activity in some plants [49]. Sclerin plays a role in regulating enzyme activity in the maturation, and pigmentation of fungal sclerotia, and induces melanogenesis [27,50,60]. Co-metabolites such as sclerotinin A and B, sclerolide, and sclerone were also reported along with sclerin [61]. Sclerotinin A and B are said to be biosynthetic intermediates of sclerin isolated from *S. sclerotiorum* and showed a growth-promoting effect on rice seedlings [48]. Botcinin A and botcinin D in *B. cinerea* extracts showed antifungal activity against *Magnaporthe grisea.*

The variation in the number and types of secondary metabolites released by different *S. sclerotiorum* isolates under *in vitro* culture conditions are largely due to the differences in their growth and developmental stages and their correlation with the virulence over the host can be delineated with the metabolic profiling from the infected tissue samples. The possible reason for the variation in the range of secondary metabolites could be due to the factors involved in the growth

culture microenvironment, the developmental stage of the pathogen, and the host-pathogen interaction mechanics. Moreover, the putative secondary metabolites identified in this study are secreted naturally in the growth medium. It has been observed that the profile of the secretory compounds is preferentially governed by the developmental stage of the pathogen; whereas the pathogenicity of the isolates depends on the interacting environments, host architecture, and integral defense mechanisms.

Comparative analysis of the secondary metabolites of *S. sclerotiorum* isolates from rapeseed mustard has been reported for the first time. The study identified the correlation between the differences in the metabolic profile of *S. sclerotiorum* isolates with their differential pathogenicity. ESR-04 and ESR-18 had similar metabolites except botcinin A and scleramide but in the cluster dendrogram, both were present in different clades even though they varied in virulence and geographical location. ESR-42 differed from ESR-18 in virulence, but metabolite-based clustering brought ESR-18 and ESR-42 together in a single clade. The analysis showed that many factors come into play at once in deciding pathogen aggression. The type and abundance of the secreted phytotoxic secondary metabolites are an important factor along with the facets of host defense response.

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## Statements and declarations

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## Data, material and/or code availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Author's contribution

Navin C Gupta conceptualized the project and obtained funding from DBT, India; Navin C Gupta and Aditi Kundu performed the experiments; Shaweta Arora and Aditi Kundu analyzed the data; Navin C Gupta, Mahesh Rao, and Shaweta Arora wrote the manuscript; Pankaj Sharma and Ramcharan Bhattacharya further edited the manuscript.

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