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A Spectral-based mathematical approach for identification of *Botrytis* and *Sclerotinia* species

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Botrytis spp. and *Sclerotinia* spp. are related necrotrophic fungi with broad host ranges. The close relationship between them was studied using a spectral-based mathematical approach based on the spectral signals reflected from their cultural growth. A range of seven values corresponding to each spectral measurement was calculated using a polynomial model which significantly distinguished the fungal isolates under study. In parallel, ITS1 sequence similarity of such isolates was compared to that retrieved from the GenBank NR database. The phylogenetic distance was statistically compared to their spectral-based mathematical values. Interestingly, the dataset generated in this study could be a new method for pathogen identification without needing other laborious techniques. Thus, the current dataset library provided a pilot approach to ease fungal identification at genus, species and isolate levels.

Keywords: Diversity; Genetic variation; Molecular phylogeny; Necrotrophic fungi; Pathogen detection; Polynomial model; Spectral reflectance

INTRODUCTION

The necrotrophic fungal pathogens, *Botrytis cinerea* Pers.: Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary, infect hundreds of plant species and produce gray and white molds, respectively. Many plant pathogens had previously been molecularly characterized (Dufresne et al. 2007; Lopez et al. 2009; Abdel Wahab and Balabel, 2011; Abdel Wahab, 2015). In addition, the diversity of *B. cinerea* isolates was studies using both genetic markers and characteristics associated with the pathogenicity process (Srivastava et al. 2018).

Differences between *Botrytis* and *Sclerotinia* at species levels had been intensively investigated using microscopy- and molecular-based methods that were time-consuming and need labor intensive techniques (Reich et al. 2016). Therefore, more rapid and reliable identification methods are urgently needed for pathogen detection and disease management. Recently, spectroscopy-based technology was used to speed fungal identification (Aboelghar and Abdel Wahab, 2013) and early detection of fungal pathogen (Abdel Wahab et al. 2017). Moreover, the polynomial method was used based on hyperspectral data in pathogen identification and the index of some plant disease like wheat stripe and leaf rusts (Hui et al. 2015). Here, we have established a spectral-based mathematical approach to differentiate *Botrytis* spp. from *Sclerotinia* spp. isolates based on the spectral signals reflected from their cultural growth. By applying a mathematical polynomial model, we should calculate a range of seven values corresponding to each spectral measurement of

tested isolate in order to easily identify fungal species.

MATERIALS AND METHODS

Collection of fungal pathogens and culture preparation

This study was conducted during 2017-2019 on five isolates of *Botrytis* spp. and four isolates of *Sclerotinia* spp., which were collected from different host plants and locations (Table 1). One single isolate of the fungal species *Alternaria alternata* was included in the analysis as outgroup on the basis of the phylogenetic analysis. All tested isolates were cultured on potato dextrose agar (PDA; Difco Laboratories, Dickinson and Company) and incubated at 23°C for two weeks. Culture morphology and mycelial growth length (MGL) were characterized according to Aboelghar et al. (2019).

Table 1: Collection of fungal pathogens under study.

Isolate code	Fungal pathogen	Host plant	Location
BCL	*Botrytis cinerea*	Lettuce	Giza
BCS	*Botrytis cinerea*	Strawberry	Giza
BCC	*Botrytis cinerea*	Cucumber	Giza
BF	*Botrytis fabae*	Broad bean	Beheira
BO	*Botrytis* spp.	Onion	Beheira
SSP	*Sclerotinia sclerotiorum*	Pea	Giza
SSM	*Sclerotinia sclerotiorum*	Melon	Sinai
SSPo	*Sclerotinia sclerotiorum*	Potato	Monufia
ST	*Sclerotinia trifoliorum*	Alfalfa	MD*
AA	*Alternaria alternata*	eggplent	Giza

*MD: missing data

Hyperspectral measurements of fungal cultures

Spectral reflectance of fungal cultures was measured using Apogee Portable Spectro-radiometer (Boulder, CO, USA) according to ZHOU et al. (2010) and Zhang et al. (2013) within a wavelength range of 400-1000 nm (visible and near infrared). All hyperspectral measurements have been carried out at the Laboratory of Molecular Diagnostic of Plant Diseases, Department of Plant Pathology, Faculty of Agriculture, Ain Shams University. A halogen light (50 W) was used as the light source for such indoor measurement. A white reference (spectralon) was used every 2 min for the calibration of the spectral measurements with a spectral resolution of 1 nm. For each sample, the spectral data was recorded based on the average of five individual spectral measurements.

Fungal genomic DNA extraction

Total genomic DNA was isolated from fungal mycelia of ten isolates [*B. cinerea* (BCL, BCS, BCC), *B. fabae* (BF), *Botrytis* spp. (BO), *Sclerotinia sclerotiorum* (SSP, SSPo, SSM), *Sclerotinia trifoliorum* (ST), and *A. alternata* (AA)]. All mycelia were harvested at 7-day-old cultures, then frozen in liquid nitrogen. The genomic DNA was extracted using a miniprep procedure according to Möller et al. (1992), quantified, then stored at 4°C.

PCR amplification of fungal ITS

A couple of primers for ITS1 and ITS4 was selected for amplifying fungal ITS region according to White et al. (1990). Each 25 µL PCR reaction contained 0.5 µl of each primer (10 µM), 2 µl (50 ng/µl) of genomic DNA sample, 12.5 µl of Red PCR master mix (Bio-line) and 9.5 µl H_2O. The PCR amplification was performed in a thermocycler (Techne-Progene) with a run program as follows: denaturation for 5 min at 94°C; 27 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min; and final extension at 72°C for 10 min. All PCR products were purified from agarose gels, cloned into *E. coli* DH5α and adapted for the subsequent analysis.

ITS sequencing and Phylogenetic analysis

The PCR amplicons were sequenced using Applied Biosystems (Sigma, Egypt), and identified by BLASTN to the most similar ITS sequences of the GenBank NR database.

Phylogenetic analyses were done by the software of MEGA6, version 6.06 (Tamura et al. 2013). The missing data and nucleotide gaps in the DNA sequences were deleted. The phylogeny tree was constructed using the Neighbour joining method and individually tested with a bootstrap of 1000 replicates to achieve the reliability of any given branch pattern in each Neighbour joining tree.

Mathematical approach

A mathematical approach was established to differentiate between different fungal pathogens. A polynomial equation from the sixth order (i.e. with seven different parameters) was found for

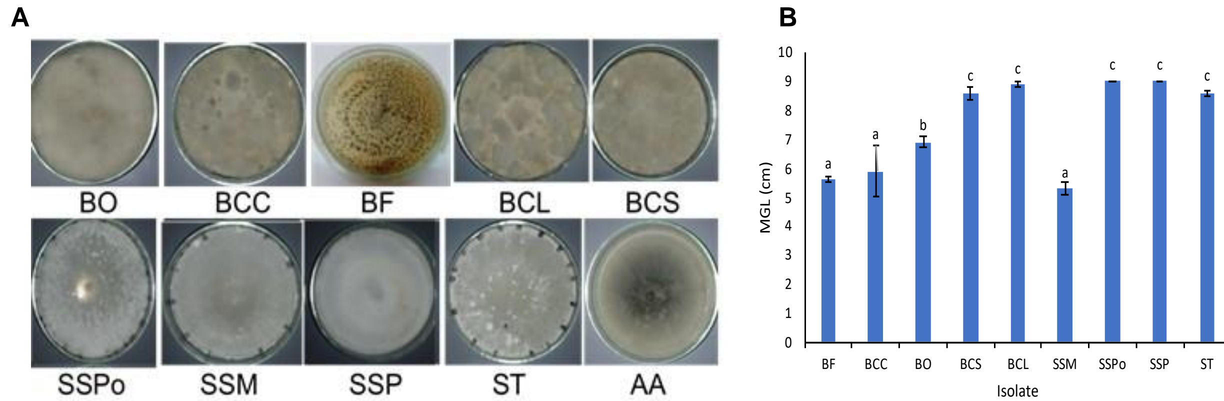


Fig.1: Culture characteristic (A) and MGL (B) of *Botrytis* spp. & *Sclerotinia* spp. isolates on PDA, collected from different locations and host plants. AA was included as an outgroup isolate.

each measurement curves. These seven parameters/values were unique for each pathogen and could enable to identification of the pathogen being tested following the spectral measurements *in vitro*.

The equation of each pathogen's pattern is on the form:

$$y = a_n x^n + a_{n-1} x^{n-1} + \dots + a_2 x^2 + a_1 x + a_0 \quad (1)$$

The coefficients a_0 to a_n are calculated according to Vandermonde's matrix (Gautschi, 1975)

$$\begin{pmatrix} y_0 \\ y_1 \\ \dots \\ y_n \end{pmatrix} = \begin{pmatrix} x_0^n & x_0^{n-1} & \dots & x_0 & 1 \\ x_1^n & x_1^{n-1} & \dots & x_1 & 1 \\ \vdots & \vdots & \vdots & \vdots & \vdots \\ x_n^n & x_n^{n-1} & \dots & x_n & 1 \end{pmatrix} \begin{pmatrix} a_0 \\ a_1 \\ \vdots \\ a_n \end{pmatrix} \quad (2)$$

$$Standard\ Error = \sum (y_p - y_t)^2 \quad (3)$$

Where y_p is the measured y axis of the sample, and y_t is the calculated y axis for each pathogen.

Statistical analysis

All data was statistically analyzed using IBM® SPSS® Statistics (2011) v.20 based on least significance difference (LSD) pairwise comparison test and Duncan's Multiple Range Test (Duncan, 1955) at the level of 5%.

RESULTS AND DISCUSSION

Characterization of *Botrytis* spp. & *Sclerotinia* spp. isolates

Cultural divergence was observed among *Botrytis* spp., *Sclerotinia* spp. isolates (Fig. 1A). Moreover, mycelial growth length (MGL) was significantly different among *Botrytis* isolates that ranged from 5.6 - 8.9 cm. Similarly, a significant variation of MGL was also shown among *Sclerotinia* spp. isolates that ranged from 5.3 - 9 cm, after three days of incubation (Fig. 1B). Such isolates also showed virulence divergence according to another study which was conducted on the detached leaves of both lettuce and broad bean (Abdel Wahab et al. 2020a).

Spectral Patterns of fungal cultures

The spectral measurements of fungi under study investigated a specific spectral pattern for each fungal pathogen at VNIR spectral zone: 400-1000nm on PDA culture (Fig. 2). Interestingly, such spectral signature showed close relation among different species of the same fungal genus and high similarity among isolates of the same species, while varied among different fungal genera (Fig. 2). Such variation could be due to a strong absorption by photoactive pigments (West et al. 2003).

Spectral-based mathematical analysis

The coefficients a_0 to a_n differentiate between different fungal patterns. The more coefficient numbers used in equations, the more precise values will differentiate between patterns. Thus, the polynomial equations were solved to the sixth order, leading to produce 7 coefficients. The choice of the sixth order was not arbitrary, but was the best choice for the current spectral data set. Thus, the polynomial equation including the seven coefficients could be used to identify any unknown fungal species *in vitro* following the spectral measurement. The accuracy of the coefficients ranges (between min and max values) is strongly

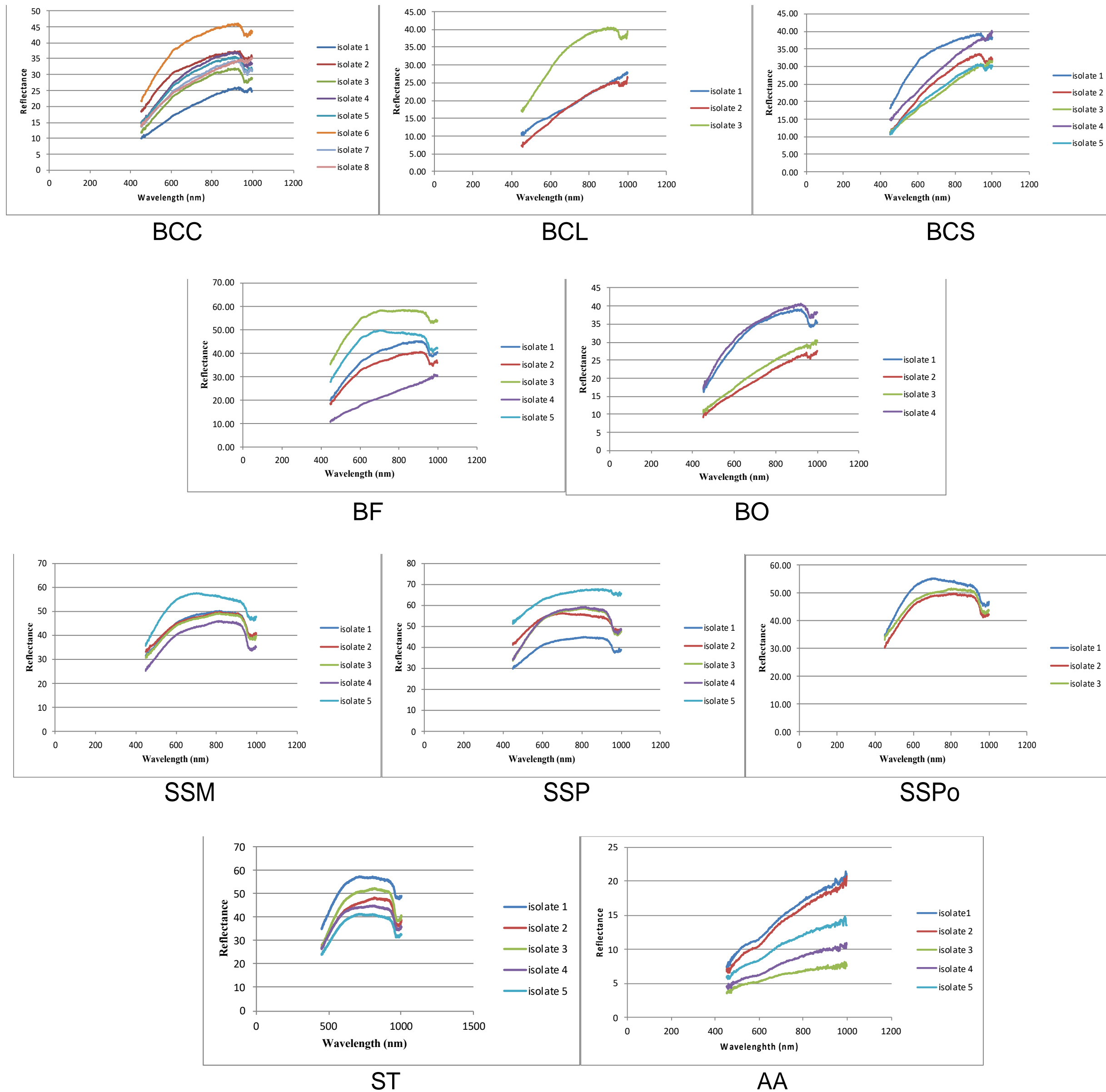


Fig. 2: Spectral signatures of fungal pathogens under study at VNIR spectral region.

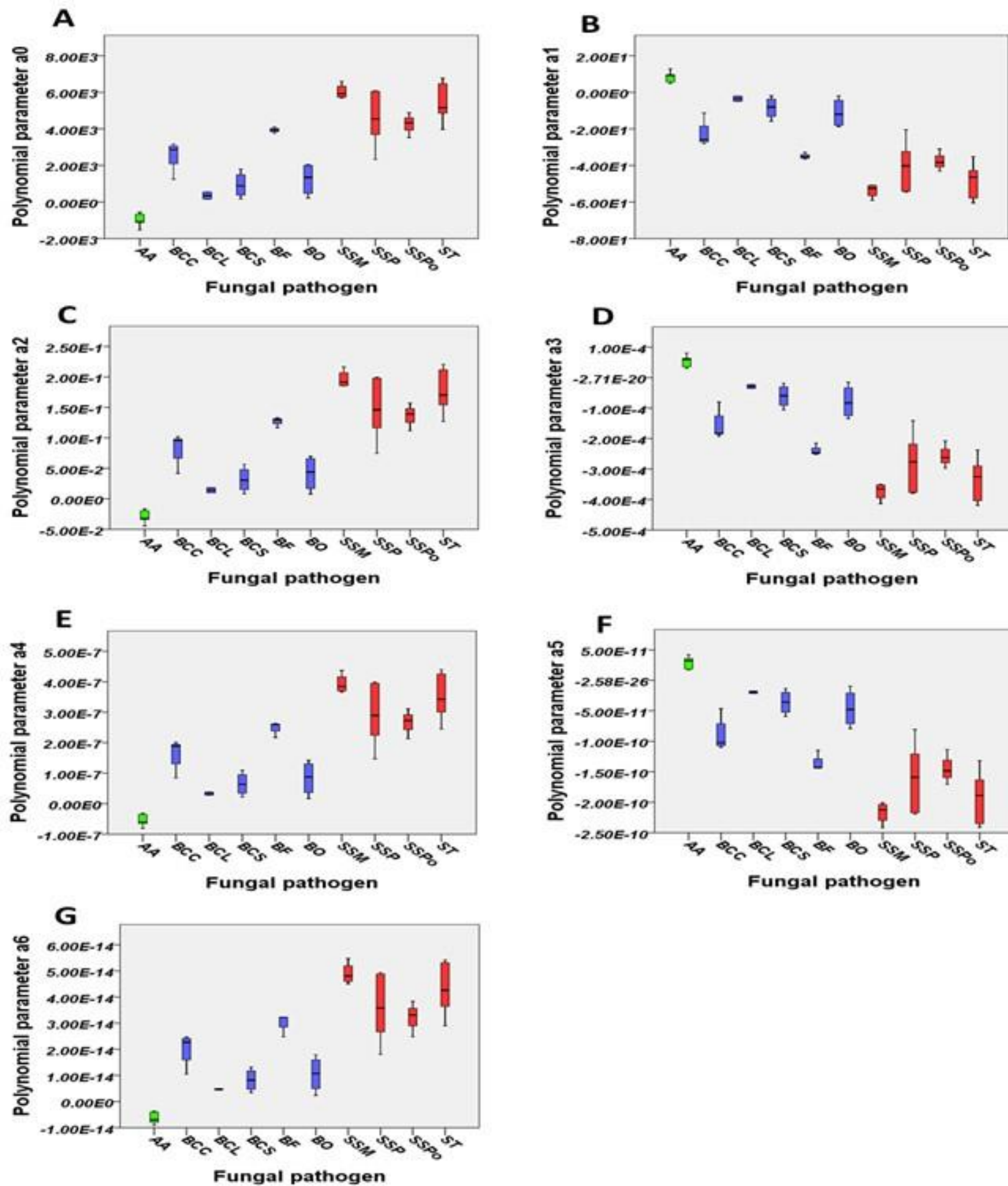
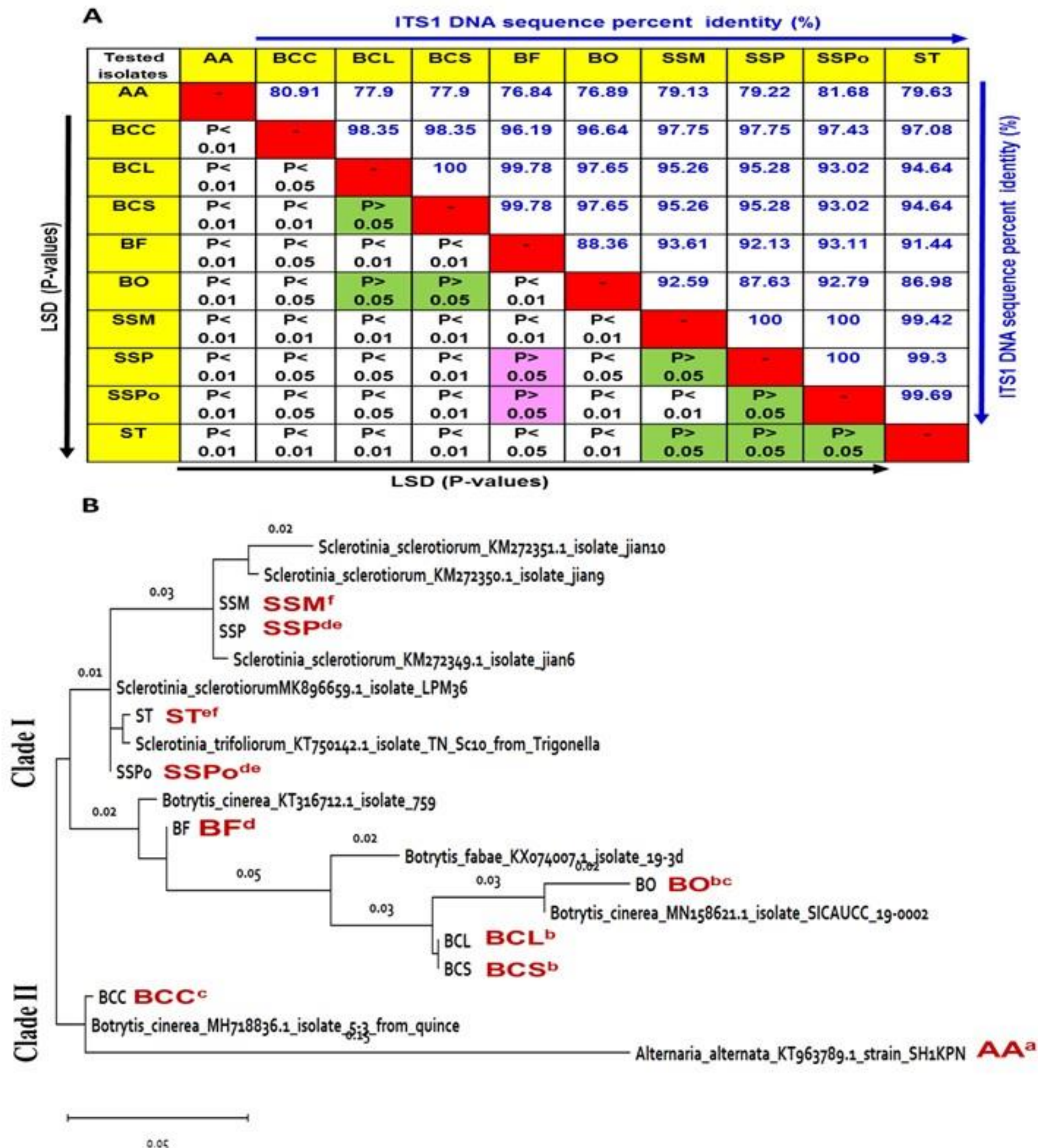


Fig. 3: Polynomial-based model to differentiate isolates of *Botrytis* from *Sclerotinia* represented by seven coefficients (a_0 to a_6).

Polynomial equation 1 was employed to calculate the seven coefficients (a_0 to a_6) that represent the spectral curve of each tested isolate. A to G graphs: Bars reveal the spectral-based mathematical range values of each isolate (a_0 to a_6). The X axis shows the tested fungal isolates collected from different host plants. The Y axis represents the spectral-based mathematical values ranged by a_0, a_1, a_2, a_3, a_4, a_5, and a_6.


A

ITS1 DNA sequence percent identity (%)

Tested isolates	AA	BCC	BCL	BCS	BF	BO	SSM	SSP	SSPo	ST
AA	-	80.91	77.9	77.9	76.84	76.89	79.13	79.22	81.68	79.63
BCC	P< 0.01	-	98.35	98.35	96.19	96.64	97.75	97.75	97.43	97.08
BCL	P< 0.01	P< 0.05	-	100	99.78	97.65	95.26	95.28	93.02	94.64
BCS	P< 0.01	P< 0.01	P> 0.05	-	99.78	97.65	95.26	95.28	93.02	94.64
BF	P< 0.01	P< 0.05	P< 0.01	P< 0.01	-	88.36	93.61	92.13	93.11	91.44
BO	P< 0.01	P< 0.05	P> 0.05	P> 0.05	P< 0.01	-	92.59	87.63	92.79	86.98
SSM	P< 0.01	P< 0.01	P< 0.01	P< 0.01	P< 0.01	P< 0.01	-	100	100	99.42
SSP	P< 0.01	P< 0.05	P< 0.05	P< 0.01	P> 0.05	P< 0.05	P> 0.05	-	100	99.3
SSPo	P< 0.01	P< 0.05	P< 0.05	P< 0.01	P> 0.05	P< 0.01	P< 0.01	P> 0.05	-	99.69
ST	P< 0.01	P< 0.01	P< 0.01	P< 0.01	P< 0.05	P< 0.01	P> 0.05	P> 0.05	P> 0.05	-

LSD (P-values)

Fig. 4: Side by side comparison of the distance between ten fungal isolates using spectral-based mathematical and phylogenetic analysis.

A. LSD pairwise comparison test was used to determine the differences (P<0.05) between each pair of isolates (lower part on the left). The pairwise identity percent measured the genetic relationship of each pair of tested fungal isolates based of their ITS1 sequences (upper part on the right); B. The phylogenetic distance of the fungal isolates constructed by the DNA sequence of ITS1 along with categorized spectral-based mathematical values of the fungal isolates based on Duncan test. Values highlighted in green or purple were not significantly differentiated, belonging to different fungal species or genera, respectively.

depended on the number of samples measured *in vitro*. In the current work, the correlation coefficient was 0.99 for each record. The average value of the measured fungal pathogen depended on the standard error which was minimal and tends to zero. In fact, few models had been

constructed using different data like temperature response equations or wet degree-hours (Butler and Jadhav, 1991; Butler et al. 1994; Erincik et al. 2003; Pfender, 2003). Moreover, some infection models had previously used regression equations based on polynomials (Biggs and Northover, 1988; Lalancette et al. 1988; Schuh, 1991; Evans et al. 1992; Jacome and Schuh, 1992; Trapero and Kaiser, 1992). Such infection models were based on either laboratory or field observations of resulted disease severity at multiple combinations of temperature and wetness (Madden and Ellis, 1988).

Potential identification of the fungal pathogen based on calculated spectral value versus molecular phylogeny

We applied a polynomial model (Equation 1) to identify signatures of the spectral curve readings collected from tested fungal isolates. Seven coefficient parameters (a_0 to a_6) of each spectral curve were calculated (Equation 2) according to Vandermonde's matrix (Gautschi, 1975). Each parameter was able to demonstrate the spectral reflectance reads, and represented a spectral-based mathematical fingerprint of each fungal isolate (Fig. 3A to 3G).

The spectral-based mathematical values significantly distinguished tested genera, except BF isolate from *Botrytis* as it was statistically close to SSP and SSPo isolates from *Sclerotinia* when the least significant difference (LSD) pairwise comparison test was performed to determine the significant differences ($P<0.05$) between each pair of the fungal isolates (Fig. 4A). Meanwhile, AA isolate was highly different ($P<0.01$) from other tested genera accordingly to the phylogenetic analysis (Fig. 4B). The sequenced amplicons were identified by BLASTN to the most similar ITS1 sequences of the GenBank NR database.

The phylogenetic tree generated from the ITS1 sequences divided the tested fungal isolates into two clades: clade I for all *Sclerotinia* & many *Botrytis* isolates and clade II for AA and BCC isolates which showed a similarity to an external isolate of *Botrytis* retrieved from the GenBank. While, BF isolate was the most *Botrytis* species closely related to two isolates of *S. sclerotiorum,* SSP and SSPo (in purple highlight). Moreover, apart from the extremely different value for AA isolate (Fig. 4B[a]), the spectral-based mathematical values of the fungal isolates were sorted and categorized using Duncan test, and they were divided into three groups: values with nonsignificant differences, either among *Botrytis* isolates (Fig. 4B[b,bc,c]), *Sclerotinia* isolates (Fig. 4B[de,ef,f]), or between *Botrytis* and *Sclerotinia* isolates (Fig. 4B[d,de]). The isolates BCC and SSPo were significantly differentiated from BCL/BCS/BO/BF and SSM, respectively. Whereas, the isolates highlighted in green (Fig. 4A) such as BCL, BCS and BO were found to be closer to each other in the ITS1 phylogenetic tree (Fig. 4B[b,**b,bc**], respectively).

This study was conducted to analyze the relationship between the spectral data and molecular phylogeny of *Botrytis* and *Sclerotinia* isolates, as these pathogens are highly related and share amino acid homology for more than 80% (Amselem et al. 2011). It is well known that phenotypic variation of the fungal pathogen depends on the biochemical content, cellular surface, and structure resulting in a clear impact on the spectral pattern (Gamon and Surfus, 1999). This helped us to measure cellular changes in the isolate which may be visibly varied allowing identification of the causal agent of plant disease. Since fungal color and structure affect the reflectance in the wavelengths, and could be related to some genetic changes, the current spectral/molecular analyses of pathogen culture implement useful physiological/genetic information. In addition, the mathematical approach was used to find a value range corresponding to the spectral data of each fungal pathogen which could be directly identified following the spectroradiometer measurements *in vitro*.

Although BCC, BCS and BCL isolates were collected from the same geographic region and showed the same virulence level (Abdel Wahab et al., 2020a), BCC isolate diverged from such two isolates contrarily to previous reported relationship between isolate location and genetic differentiation (Isenegger et al., 2008). The results demonstrated deletions and substitutions occurred in many nucleotide sites of the amplified ITS sequence of BCC isolate, making a questionable cryptic speciation of *Botrytis* spp. Similar polymorphism observation had previously been reported (Staats et al., 2005). Our global phylogeny analyses of Egyptian isolates have been studied using either the amplified ITS sequences (Abdel Wahab et al., 2020b) or the amplified LTR sequences of the retrotransposons *boty* (Abdel Wahab, 2015), being shown high levels of sequence polymorphism within Egyptian *Botrytis* isolates compared to other GenBank accessions. The fact of the occurrence of

nucleotide variation in isolates derived from the same species was previously documented as a status of new adaptations against biotic or abiotic stress (Mousavi-Derazmahalleh et al., 2019). Previously, the genome of different clones from one single *B. cinerea* isolate had been re-sequenced in order to detect point mutations within their sequences (Atwell et al., 2015). Thus, additional sequencing of the current divergent isolates should be done in the future studies to confirm such case as separate species. This would help us to determine whether the tested *Botrytis* isolate with high sequence divergence matches a subpopulation as a sympatric new species submitted in *Botrytis* populations.

CONCLUSION

In this investigation, the generated data showed incredible capabilities in distinguishing highly similar fungal species. The spectral-based mathematical library could be a pilot approach to ease fungal identification at genus, species, and isolate levels. The current study suggests scaling this approach on other fungi to speed fungal identification in the future.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Hala Abdel Wahab proposed the research idea, designed the experiments and conducted the pathological, spectral, and molecular analyses. Walid Abdel-Sattar and Ramy Mawad performed the mathematical analysis of the spectral data. Hala Badr Khalil constructed the phylogenetic tree and sequence similarity analysis. All the authors contributed equally in writing and reviewing the manuscript.

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