



Classification of *Colletotrichum coccodes* isolates into vegetative compatibility groups using infrared attenuated total reflectance spectroscopy and multivariate analysis



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ABSTRACT

In this study the potential of infrared (IR) spectroscopy for the classification of *Colletotrichum coccodes* (*C. coccodes*) isolates into vegetative compatibility groups (VCGs) was evaluated. Isolates which belong to the same VCG may have similar pathological and physiological traits that differ from those that are not assigned to the same VCG. Early classification of isolates into VCGs is of a great importance for a better understanding of the epidemiology of the disease and improves its control.

The main goal of the present study was to classify 14 isolates of *C. coccodes* into VCGs and differentiate between them, based on their IR absorption spectra as obtained by the FTIR-ATR sampling technique. Advanced statistical and mathematical methods, including Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA), were applied to the spectra after manipulation. The results show that it is possible to assign the isolates into VCGs with more than 90% success based on the wavenumber low region (1800–800 cm⁻¹) and using 15 PCs. However, on the isolate level, the best differentiation results were obtained using PCA (15 PCs) and LDA for the combined regions (2990–2800 cm⁻¹, 1800–800 cm⁻¹), with identification success rates of 87.2%.

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1. Introduction

Colletotrichum coccodes (*C. coccodes*) is a fungal pathogen that attacks different crops, including potato, resulting in large economic losses [1,2]. All parts of the plant, such as potato tubers, could be infected [3]. Infection of potato with *C. coccodes* can cause premature death of the plants and severe damage to tubers, reducing their marketability [4,5]. The growing market for fresh pre-packed potatoes in recent years makes the black dot on potato caused by *C. coccodes* a serious problem [6]. Experimentally,

studies in the U.S.A. [7], Israel [8], the U.K., and Malaysia showed that significant yield losses could be caused by *C. coccodes* (up to 50% of crop). Furthermore, there are additional losses to the seed industry, particularly to the export market [9]. In addition, it is difficult to control *C. coccodes* as it is a pathogen considered as air, seed tuber and soil-borne.

Fungal pathogens are currently detected and characterized mainly by classic microbiological, biochemical, immunological, and molecular methods. These methods are briefly described below:

1. Classic microbiological methods: These methods are based on cultivation of the fungi in selective media, and their identification by visual and microscopic observations. The identification is based on morphological characteristics of the fungus, which is limited [10]. These methods are time-consuming (usually taking a number of weeks) [6], not highly specific [11], and it is impossible to differentiate among different fungi at the species or isolates levels [12,13].

Abbreviations: IR, infrared; *C. coccodes*, *Colletotrichum coccodes*; VCGs, vegetative compatibility groups; nit, nitrate non-utilizing; FTIR-ATR, Fourier transform infrared attenuated total reflectance; PCA, Principal Component Analysis; LDA, Linear Discriminant Analysis; PC, Principal Component; PDA, potato dextrose agar; SA, streptomycin sulphate; CDA, czapek dox agar; DTGS, deuterated triglycine sulfate; LOO, leave-one-out.

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2. Biochemical methods: The use of biochemical techniques enables to characterize interspecific and intraspecific variation at the species level; but these methods are limited to the isolates level due to the poor understanding of specific biochemical variations between different isolates in the same species [14].
3. Immunological methods: These assays rely on the interactions between one of the pathogen proteins with a specific antibody [15,16] and depend on the availability of specific monoclonal antibodies appropriate to the tested fungi.
4. Molecular methods: Molecular techniques are based on the Polymerase Chain Reaction (PCR). Specific DNA fragments are amplified depending on finding two specific DNA primers for each tested fungi [17]. For instance, PCR and real-time PCR tests have been developed for sensitive, specific, and quantitative detection of *C. coccodes* [2,18]. Although the serological and (especially) the molecular techniques are very specific, they are expensive and available only for a small number of fungi, mainly at the species level, but not for different isolates [19–22].

A vegetative compatible group (VCG) consists of isolates that can transfer genetic material by contact [23], producing new stable heterokaryons (cells that contain multiple, genetically different nuclei) and thereby forming subpopulations that tend to be similar due to a common genetic pool. These isolates, which constitute the VCG, have similar pathological and physiological traits compared to isolates that are not assigned to the same VCG [23]. Classification of VCGs has been used to study the genetic structure of populations of plant pathogenic fungi, including *Fusarium oxysporum* [24–26], *Verticillium* [27], and *Colletotrichum* [28,29].

Classification of a new isolate to a VCG is very important because all isolates that are related to the same VCG have similar pathogenic aggressiveness. Therefore, classification of any isolate to a specific VCG improves understanding the epidemiology of the disease and enables its control [30], thus enhances the ability to prevent large economic losses.

Analysis of VCGs has been mainly based on pairings between complementary nitrate non-utilizing (nit) mutants generated from different isolates [29,31,32]. These mutants are selected using potassium chlorate-containing medium. Compatible isolates, whose complementary nit mutants can form stable heterokaryons, are assigned to the same VCG [23,32]. It takes about one month to classify an isolate to a specific VCG using the nit method.

Infrared (IR) spectroscopy has been used widely in applications requiring qualitative and quantitative analysis of biological samples using the molecular fingerprint of their vibrational spectrum. IR spectroscopy has demonstrated encouraging trends in detection and characterization of various types of phyto-pathogens [33–44].

Among various spectroscopic methods, techniques based on evanescent wave absorption (FTIR-ATR spectroscopy), arouse special interest due to their ability to identify specific spectroscopic changes *in situ* or/and *in vivo* [45–47].

Researchers worldwide have used FTIR-ATR in tandem with multivariate analysis and advanced statistical methods, to maximize its potential. For example, FTIR-ATR spectroscopy followed by Principal Components Analysis (PCA) was used to classify fungi at the level of species [48]. Artificial Neural Networks (ANN) [40,49] and Linear Discriminant Analysis (LDA) [36] were employed to differentiate among small numbers of fungi isolates.

As mentioned in our previous studies [34–37], early detection and identification of phyto-pathogens is essential for successful control and effective treatment. The main achievement of this study was to show that by using the FTIR-ATR technique it is possible to differentiate between the examined phyto-pathogens, even at the level of isolates, and to simultaneously classify them into different VCGs.

2. Materials and methods

2.1. Fungal isolates

Fourteen isolates of *C. coccodes* that belong to five different VCGs were examined in this study (Table 1). The samples were obtained from the Department of Plant Pathology, the Institute of Plant Protection, Agricultural Research Organization, at the Gilat Research Center, Israel. The isolates were originally isolated from infected potato plants and tubers sampled from different plots in Southwest Negev, Israel. *C. coccodes* was isolated from surface-sterilized (1% NaClO for 10 min) infected stems or tubers. Sclerotia taken from tuber and stem surfaces were placed on potato dextrose agar (PDA). Plates were incubated in the dark at 27 °C for 7 days and allowed to sporulate. Conidia transferred to medium containing 0.2% sorbose, 15% agar and 100 ppm streptomycin sulphate (SA) were incubated for 24 h at 27 °C in the dark. Monoconidial cultures were obtained from each isolate (by micromanipulation) and maintained on czapek dox agar (CDA) at 6 °C. Assignment of these isolates to VCGs was carried out as previously reported [32,50].

Five replicates of pure cultures of each isolate were cultivated in different batches and grown for 3–10 days at 27 °C with continuous shaking.

For spectroscopic examinations, samples of the fungi were separated and purified by spinning 1.5 ml of the fungal suspension at 13200 rpm for 4 min, rinsing and washing the pellet 4 times with distilled water, and suspending the pellet in an appropriate volume of distilled water (about 1 ml). Pure samples enable us to control as many experimental parameters as possible (including growth conditions, amount of sample examined, and duration of growth), and to verify that each absorption band in the IR spectrum is due to the specific sample.

2.2. Sample preparation

Because of their complicated structures, we encountered some difficulties in preparing a homogeneous suspension of the fungi in water and spreading them on the ZnSe crystal surface of the ATR accessory. Therefore, the fungi were first crushed into small pieces by adding 0.5 ml distilled water to the fungi. We then cut and smashed them with the pipettor tip. By several pipetting actions, a semi-homogeneous suspension was obtained. About 500 µl of each fungal suspension sample was spread as homogeneously as possible on the surface of the ATR ZnSe crystal to cover

Table 1

The details are of the VCGs, isolates, and the number of measurements in this study.

VCG No.	Isolates No.	No. of measurements
3	14	12
	15	21
	72	11
4	4	19
	69	14
	192	20
5	124	15
	145	21
	166	10
7	2	18
	5	25
	30	10
8	28	16
	121	14

The numbers that appear in the “isolates No.” column are serial numbers that were chosen for the different *Colletotrichum coccodes* isolates.

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