Interactive key (Lucid) for identification of fungi in vegetable seeds

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Data de chegada: 21/06/2018. Aceito para publicação em: 19/12/2019.

10.1590/0100-5405/204669

ABSTRACT

Pierozzi, C.G; Fujihara, R.T.; Souza, E.S.; Pizetta, M.; Sartori, M.M.P.; Kronka, A.Z. Interactive key (Lucid) for identification of fungi in vegetable seeds. ***Summa Phytopathologica,*** v.46, n.1, p.14-19, 2020.

Interactive keys are tools that aid research and technical work since identification of organisms has become increasingly present in the scientific and academic context. An interactive key was developed with the software Lucid v. 3.3 for the identification of eleven fungal species associated with onion, carrot, pepper and tomato seeds. It was based on a matrix composed of six features: crop, conidium, conidiophore, color of long conidiophore, color of mycelium and presence of setae, besides 21 character states. In addition, descriptions, illustrations and high-resolution photographs of the morphological characters and states were made available to aid in the correct identification of fungal species. Validation of the interactive key was performed by distinct groups of volunteers: (i) graduate students with prior knowledge and using the interactive key; (ii) undergraduate students with little prior knowledge and using the interactive key, and (iii) undergraduate students with little prior knowledge and using the conventional identification system such as the printed manuals used in seed pathology laboratories. We analyzed the time spent by each volunteer to evaluate 25 seeds infected with the fungal species in the key, as well as the percentage of success and the difficulty level for each participant. The high percentage of correct answers with the use of the interactive key and the ease of use by the volunteers confirmed its efficiency because there was an increase in the identification accuracy when compared to the conventional system. Furthermore, the rate of success and the difficulty level presented low variability within groups (i) and (ii). These results are a consequence of the interaction of the user with characteristics of the developed tool, such as high-resolution photographs, which faithfully reproduce the fungal characteristics observed in the seeds under a stereomicroscope. Thus, the interactive key presented here can aid in teaching, institutional and commercial research, inspection and certification of seeds, making diagnosis safer and more accurate. The key is available for free at https://keys.lucidcentral.org/keys/v3/seed_fungi/.

Keywords: ID key; seed pathology; teaching; fungal characteristics; photographs.

RESUMO

Pierozzi, C.G; Fujihara, R.T.; Souza, E.S.; Pizetta, M.; Sartori, M.M.P.; Kronka, A.Z. Chave interativa (Lucid) para identificação de fungos em sementes de hortaliças. ***Summa Phytopathologica,*** v.46, n.1, p.14-19, 2020.

Chaves interativas são ferramentas que auxiliam a pesquisa e trabalhos técnicos, de forma que a identificação de organismos tem se tornado cada vez mais presente no meio científico e acadêmico. Foi desenvolvida uma chave interativa através do software Lucid v. 3.3 para a identificação de onze espécies fúngicas associadas às sementes de cebola, cenoura, pimentão e tomate. Esta foi baseada em uma matriz composta por seis caracteres: cultura, conídio, conidióforo, coloração do conidióforo longo, coloração do micélio e presença de setas, e 21 estados de caráter. Além disso, descrições, ilustrações e fotografias de alta resolução dos caracteres e estados foram disponibilizados para auxiliar na correta identificação das espécies fúngicas. A validação da chave interativa foi realizada por grupos distintos de voluntários: (i) acadêmicos de pós-graduação com conhecimento prévio e utilizando a chave interativa; (ii) alunos de graduação com pouco conhecimento prévio utilizando a chave interativa; e (iii) alunos de graduação com pouco conhecimento prévio e utilizando sistema convencional de identificação como os manuais impressos utilizados em laboratórios de patologia de sementes. Analisou-se o tempo despendido por cada voluntário para avaliar 25 sementes infectadas com as espécies fúngicas da chave e a porcentagem de acerto e o grau de dificuldade de cada participante. A elevada porcentagem de acertos na diagnose com o uso da chave interativa e a facilidade de uso pelos usuários confirmaram sua eficiência, pois houve um aumento da acurácia de identificação quando comparado ao sistema convencional. Além disso, a porcentagem de acertos e o grau de dificuldade apresentaram baixa variabilidade dentro dos grupos (i) e (ii). Estes resultados são consequência da interação do usuário com características inerentes ao material desenvolvido, como fotografias de alta resolução, que reproduzem fielmente as características fúngicas observadas nas sementes por meio do estereoscópio. Portanto, a chave interativa desenvolvida pode auxiliar no ensino, pesquisa institucional e comercial, fiscalização e certificação de sementes, tornando as diagnoses mais seguras e precisas. A chave encontra-se disponível gratuitamente no endereço https://keys.lucidcentral.org/keys/v3/seed_fungi/.

Palavras-chave: Chave de identificação, Patologia de sementes, Ensino, Características fúngicas, Fotografias.

Seeds are the main propagation technique of most farm crops (13), including onion, carrot, pepper and tomato. On the other hand, they are also efficient disseminators of phytopathogenic microorganisms that can cause production losses (7, 14). Therefore, the use of healthy seeds is a basic premise for good agricultural practices.

The biological association between seeds and fungi is even more

relevant when it comes to soilborne phytopathogenic fungi, such as *Fusarium*, which is disseminated by seeds to previously non-infested cultivable areas. Fungi of this type can survive in the soil for several years, limiting agricultural production (20).

Seed Pathology is a branch of Phytopathology which has been involved in the identification of pathogens associated with seeds since 1923 (2). Since then, detection of fungi in seeds has been gaining visibility and importance in research and certification of seeds worldwide (32).

Traditionally, identification of fungi in seeds is done by methods such as Blotter test and plating in culture media (11, 19), based on the morphological characteristics of the pathogen. Such tests are often difficult for non-specialists or beginners in the field.

Illustrated identification guides or catalogs for seed fungi are scarce (11, 35) or outdated and, in some cases, present poor illustrations, figures and/or photographs. Advances in information technology and hand-held technology have enabled the development of interactive or multi-access identification keys (10, 15, 21, 25). However, there are still very few interactive keys developed for the identification of fungi in seeds (5).

Our goal was to develop and validate an easy-to-use interactive identification key with Lucid 3.3® software for the identification of fungal species associated with seeds of onion, carrot, pepper and tomato. This tool aims to assist researchers and the regulatory community that works to mitigate fungal diseases transmitted via seeds. This key can also be helpful to the academic community, especially students, to perform seed health testing with greater reliability.

Table 1. Crops and respective fungal isolates used in the interactive key.

Crop	Fungal isolates
Onion	*Aspergillus flavus* Link
	Aspergillus niger Tiegh.
	Cladosporium spp. Link
	Colletotrichum gloeosporioides f. sp. *cepae* (Penz) Penz & Sacc
	Fusarium oxysporum f. sp. *cepae* (Hanzawa) W.C. Snyder & H.N. Hansen
Carrot	*Alternaria alternata* (Fr.) Keissl
	Alternaria dauci (J.G. Kuhn) J.W. Groves & Skolko
	Cladosporium spp.
	Fusarium oxysporum Schltdl.
Pepper	*Alternaria solani* (Ellis & G. Martin) L.R. Jones, Bull
	A. flavus
	A. niger
	Cladosporium spp.
	C. gloeosporioides (Penz.) Penz. & Sacc.
Tomato	*A. solani*
	A. flavus
	Cladosporium spp.
	Fusarium oxysporum f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen

MATERIAL AND METHODS

Selected fungal isolates and seeds

Fungal isolates were obtained from the culture collection of "Instituto Biológico de São Paulo", São Paulo, Brazil (Table 1). Chemical treatment-free seeds of onion, carrot, pepper and tomato were purchased from ISLA Sementes®.

Most of the selected fungi affect these crops (20), which stand out in production and commercialization in Brazil among the other vegetables (3).

Seed inoculation

Initially, fungal isolates were grown in a Petri plate with PDA (Potato Dextrose Agar) as culture medium and incubated for seven days in a BOD (Biochemical Oxygen Demand) incubator at 22°C and 12h photoperiod, as described by Lucca Filho (21) and Brasil (11).

Culture medium discs of 0.5 cm diameter presenting fungal growth were transferred to Petri plates containing PDA supplemented with mannitol (PDA + mannitol), and the water potential was adjusted to -1.0 MPa (PDA plus 73.77 g mannitol, 1000 mL sterile water). The concentration of the solute (mannitol) was obtained by the Van't Hoff formula (31): Po = -CiRT, where: Po = Osmotic potential (MPa); i = Ionization constant; R = General gas constant (0.00831 x Kg x MPa x mol^{-1} x K^{-1}); T = Absolute temperature (T°C + 273); C = Concentration (moles Kg^{-1} water). The water potential was adjusted to -1.0 MPa, as it provides higher infection rates in seeds without hindering them for subsequent use (22, 23, 24).

Prior to inoculation, seeds were disinfested with 2% sodium hypochlorite for one minute, washed with sterile water and dried on sterile filter paper sheets in ambient condition for 24 hours. Then, they were distributed in a single layer on the inoculum grown in the osmotically modified medium (PDA + mannitol), allowing their contact with the pathogen for 24 hours. Finally, the seeds were again disinfested as previously described.

Seed health test

To obtain the fungal structures in the seeds, the seed health test Blotter test was performed according to the International Seed Testing Association (18). Twenty-five seeds were equidistantly distributed per plate lined with three filter paper sheets previously moistened with sterile water. Afterwards, seeds were incubated at 20 ± 2°C and photoperiod of 12 hours light and 12 hours darkness, for seven days.

Imaging

Seeds were individually examined under a stereoscope, and those presenting well-defined fruiting bodies were selected for image capturing. The images were obtained from the Department of Animal Biology of the Institute of Biology of University of Campinas, Campinas, São Paulo, Brazil, under a stereoscope coupled to a digital camera (Zeiss® - Axio Zoom.V16 with AxioCam MRc camera) and exclusive software. Important structures, such as spores and hyphae, which could not be obtained under the stereoscope, were captured under a light microscope (Zeiss® - Axio Imager M2).

Lucid Key

Lucid software (LucidCentral.org, Queensland, Australia) is a versatile tool that facilitates the development of interactive keys to aid in identification and diagnosis (33).

A set of six features and 21 character states (Table 2) were selected based on fungal characteristics visible under a stereomicroscope (6, 11, 15) and scored through a matrix, where the features and their respective states were associated with the fungal species (Figure 1).

Table 2. Features and character states used in the interactive key.

Features	Character states
Crop	Onion
	Carrot
	Pepper
	Tomato
Conidia	Branched chains
	Single
	Not visible
Conidiophore	Short
	Long
	Short (not visible)
Color of long conidiophore	Dark (black)
	Hyaline (transparent)
Color of mycelium	Brown
	Grey
	White
	Roseate (salmon)
	Black
	Green
	Yellow
Presence of setae	Presence
	Absence

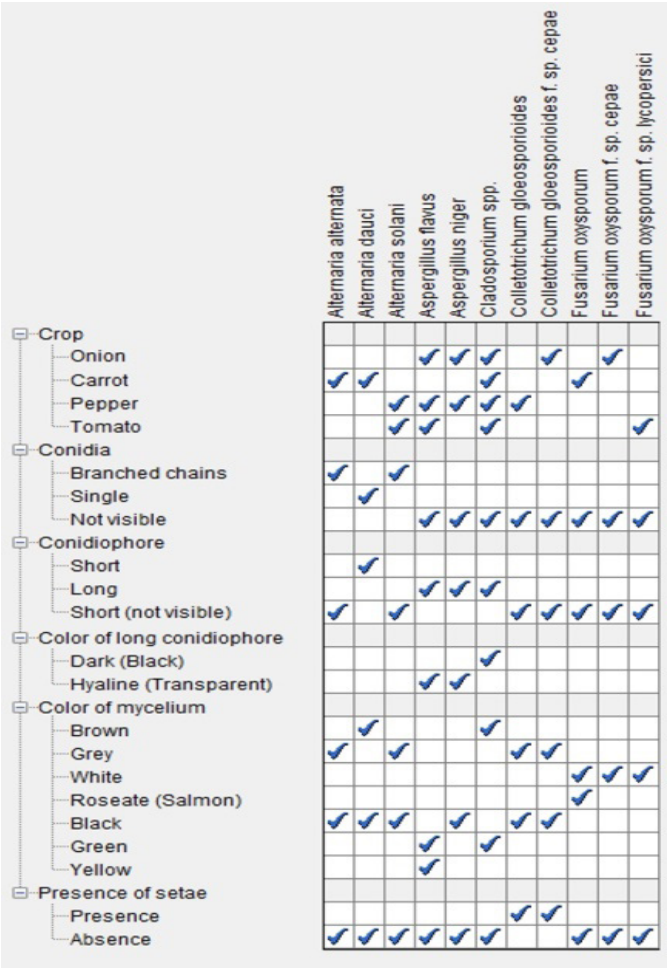

Figure 1. Matrix spreadsheet used to score character states for a given fungal species in Lucid Builder.

Key evaluation

To test the efficiency of the interactive key, the fungi obtained from a seed health test were provided to potential users, and the percentage of success (=correct identification), time spent and difficulty level of users were tallied.

Healthy and untreated carrot and pepper seeds ISLA Sementes® were used. For each crop, samples were prepared with 25 seeds associated with fungal species, following the Blotter test protocol (11, 18).

Three groups of volunteer evaluators (n = 30), from the School of Agriculture (FCA) of São Paulo State University (Unesp), Botucatu, São Paulo, Brazil, were classified according to their previous knowledge of seed pathology: (i) graduate students of Plant Protection and Forest Science, with prior knowledge and using the interactive key; (ii) undergraduate students of Agricultural Engineering, with little or no prior knowledge and using the interactive key, and (iii) undergraduate students of Agricultural Engineering, with little prior knowledge but using conventional literature (6, 11) for identification.

Each evaluator had access to a plate containing 25 seeds, a stereomicroscope and a computer with the interactive key or the conventional material. Based on the volunteers' answers, the percentage of success was analyzed, as well as the time spent to analyze a plate (25 seeds) and the difficulty level, classified as: 1.0 - Very easy; 2.0 - Easy; 3.0 – Difficult, and 4.0 - Very difficult. All participants signed a Free and Informed Consent Term (TCLE) of the Research Ethics Committee (CEP) of Unesp – Botucatu Medical School (Process No. 62179116.2.0000.5411).

Statistical analysis

A Kruskal-Wallis ranks test was used to compare the response groups. The result was considered significant when $p<0.05$. The used software was Minitab 16 Statistical Software (26). Data were represented as a boxplot to allow visualization of the variability of groups.

RESULTS AND DISCUSSION

Presentation of the interactive key developed in Lucid Player

Keys to identify pathogens are extremely useful tools and, when existent, they should be widely available to those involved in the agricultural chain, such as researchers, students, companies and producers. However, the use of identification keys is not such a common practice in Phytopathology.

The developed "Interactive key for identification of fungi in vegetable seeds" consists of a matrix based on the compilation of important morphological characters (6) for the identification of the 11 fungal species contained in the key.

To use the key, which is displayed in the Lucid Player, the user must have the results of a seed health test, like the Blotter test, as well as seeds with fruiting bodies present on their surface and a stereomicroscope for observation of these structures.

An initial screen for accessing seed health testing protocols, useful for the acquisition of fungal structures, is available to users. When the key is started, four windows can be observed: the upper left window (Figure 2-A) contains the features and their respective character states. The states can be visualized by clicking on the symbol (+) and can be selected by clicking on the thumbnail image or by checking the box when thumbnails are not showing on the left side of the feature text. In addition, descriptions, illustrations and photographs of the

morphological characters and states can be observed by clicking on the icons that represent each of them. The list of features can be closed by clicking on the symbol (-). In the lower left window (Figure 2-B), the selected characteristics are displayed, and the lower right window shows the fungal species that were discarded (Figure 2-D). At the end of the process, only one fungal species should be presented in the upper right window (Figure 2-C). It is not necessary to click on all character states to reach a result.

After identifying the species, the user has access to a fact sheet containing a species description page in HTML (Figure 3) and images of each species (Figure 4). The fact sheet is represented by the icon next to the name of each species, so that the user can find additional information such as the type and dimensions of spores, the colony coloration in culture medium and other crops it may affect.

Similarly to the present interactive key, to optimize the process of diagnosing diseases in seeds, some tools such as conventional identification keys, fungal databases and even other interactive keys have already been developed (1, 8, 9, 10, 12, 16, 17, 28, 29, 30, 34); however, those designed for the identification of pathogens associated with seeds are rare, or even nonexistent, especially relative to the availability of high-resolution images and structural details. One of the few tools available is the "Doctor Seed Fungi" system, which is directed

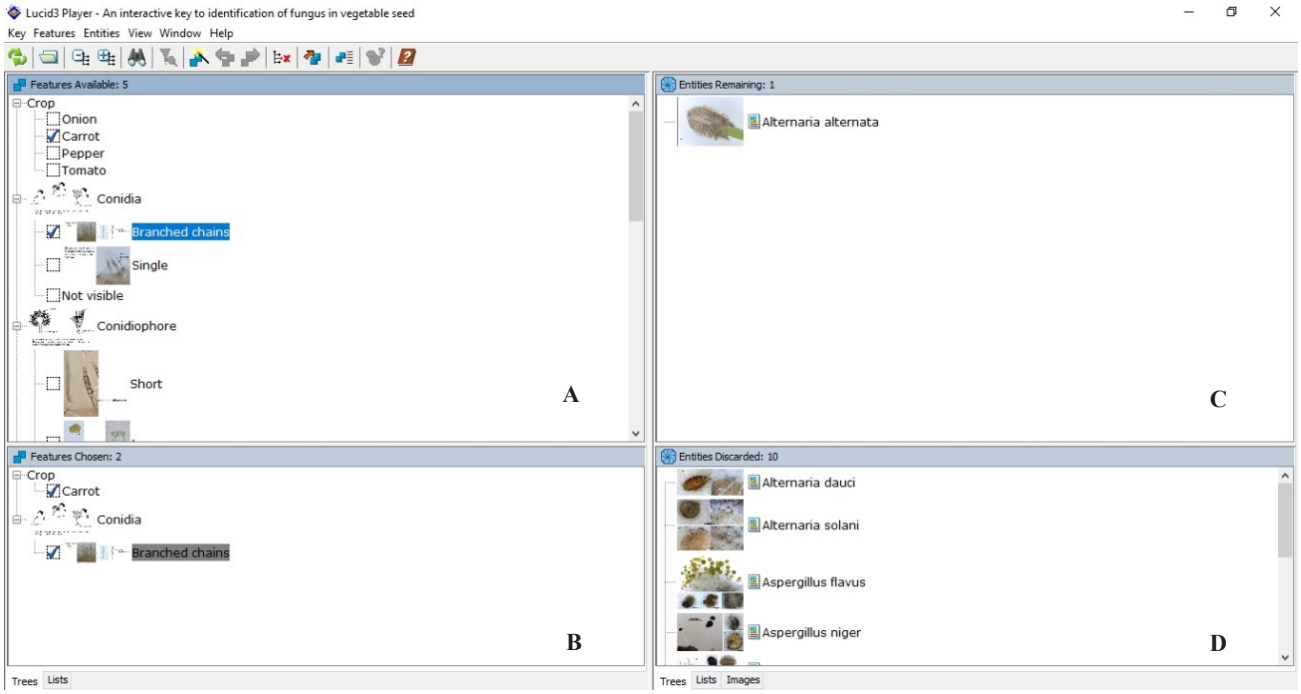

Figure 2. Interactive key developed with Lucid software (v 3.3). A) Features of the specimen are examined, and known character states of a feature are entered into the key; B) By a process of elimination, species with character states of interest are selected; C) Selected fungal species, and D) Discarded fungal species.

Alternaria alternata

The fungus *Alternaria alternata* target host is carrot, but can also be found in cotton, rice, oats, barley, beans, apple, papaya, corn, sorghum, wheat, among others. In some cases, it is considered a contaminating species in seeds. In carrot, it is the causal agent of the alternating spot. Conidia are produced in **long, usually simple, chains of numbers 2 to 11** (Figure 1.1). Their coloration may vary from dark gray to black and their **shape may be oval or cylindrical** (Figure 1.2). As for the number of septa, they may have up to **8 transverse and usually several longitudinal or oblique** (Figure 1.3). The size of the conidium varies from 20-63 (37) µm x 9-18 (13) µm in the widest part, the tip thickness (nozzle) being 2-5 µm. The colony in culture medium has a **grayish-green coloration** (Figure 1.4).

Figure 1.1. Conidia produced in long, simple chains and in numbers from 2 to 11.

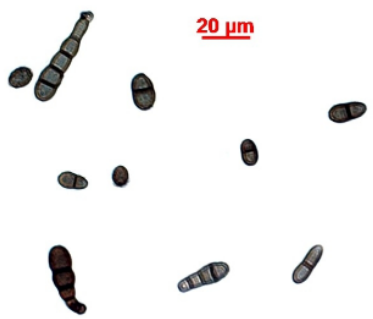

Figure 1.2. Conidia with oval or cylindrical shape.

Figure 3. Example of fact sheet (html) containing the description of the fungal isolates.

Figure 4. Images of *Alternaria alternata* in carrot seeds.

to the identification of fungi in seeds of large crops (5). However, this system is not available for public access.

In addition, guides for the identification of fungi in seeds have also been published (4, 11, 27, 35), which compile the main phytopathogenic fungi of some crops. Nevertheless, most images do not have a good definition of colors and fungal structures, which compromises the comparison of the characteristics observed in the seed under the stereomicroscope with those on the printed key, leading to unreliable and/or erroneous identification. Another disadvantage of printed guides is the difficulty to update the information, which would require preparation and printing of a new publication.

Evaluation

Regarding the percentage of success, group (i) reached 82.38% correct answers, while group (ii) obtained an average success of 84.71% (Table 3). Group (iii) obtained a lower average, only 58.62%. As expected, significant differences among groups were found for this criterion ($p < 0.05$). However, when analyzing the time spent by each evaluator, there was no significant difference among groups, but group (iii) spent more time (Table 3). Regarding the difficulty level for the use of the interactive key, groups (i) and (ii) considered the system easy to use, while group (iii) had greater difficulties and classified it as difficult, statistically differing from the others (Table 3).

Group (iii) presented greater variability, both regarding the percentage of success and the difficulty level, proving that different results were obtained depending on the evaluator and not on specific knowledge. However, the percentage of success and the difficulty level did not vary significantly within groups (i) and (ii).

The wide use in Brazil of diagnosis based on morphological characteristics, such as Blotter test, and the analysis of the results obtained after evaluation of the present tool lead us to infer that the use of this type of key in seed health tests increases the accuracy and the precision of results, besides facilitating the work of the researcher. Similar results were obtained with other systems for diagnosis of plant diseases (1, 5, 16, 17, 30), highlighting the importance of this tool in the area.

The present key provides the user with high-resolution images to faithfully reproduce the fungal characteristics present in the seeds observed under a stereoscope and a light microscope. Thus, this key is differentiated from the other currently available tools since it allows more accurate identification, simplifying the diagnostic work in Phytopathology, such as sanitary certification, as well as in quarantine stations and research agencies. In addition, this tool can be used in the academic environment, helping teach and train students.

As important as the effectiveness of this key is the public availability of this tool. The interactive key that gathered the fungal species most frequent in the evaluated crops is available for free at https://keys.lucidcentral.org/keys/v3/seed_fungi/ and species common to other crops, such as soybean and corn, are planned to be included in the future.

Table 3. Average percentage of success and error, average time spent and difficulty level for each group when analyzing a plate containing 25 infected seeds.

Groups	% Success	Average	% Error	Average	Time (min)	Average	Difficulty level*	Average
(i)	82.38 a	80.38	17.62 b	19.08	25 a	28.97	2.0 b	2.05
(ii)	84.71 a	82.35	15.29 b	17.64	29 a	28.33	2.0 c	1.66
(iii)	58.62 b	45.55	41.38 a	54.45	35 a	34.60	3.0 a	3.20
P	0.005		0.005		0.195		0.0001	

(i) Graduate students with prior knowledge and using the interactive key; (ii) undergraduate students with little prior knowledge and using the interactive key; (iii) undergraduate students with little prior knowledge and using the conventional identification system of printed manuals used in seed pathology laboratories. *Difficulty level: 1.0 – Very easy; 2.0 – Easy; 3.0 – Hard, and 4.0 – Very hard. Median followed by different letters are significantly different according to Kruskal-Wallis ranks test ($p<0.05$).

ACKNOWLEDGMENTS

The first author would like to thank National Council for Scientific and Technological Development (CNPq) for a granted scholarship. We are thankful to Antonia Cecilia Amaral and Camila Fernanda da Silva (University of Campinas) for the assignment and guidance in the use of the equipment to produce the images. We thank Christiane Ceriani ("Instituto Biológico de São Paulo") for providing the fungal isolates used in this study.

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