ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Hortorum Cultus, 20(5) 2021, 97–106
https://czasopisma.up.lublin.pl/index.php/asphc ISSN 1644-0692 e-ISSN 2545-1405 https://doi.org/10.24326/asphc.2021.5.9
ORIGINAL PAPER
Accepted: 26.11.2020

THE INFECTION PROCESS OF *Colletotrichum fuscum* ON OREGANO LEAVES AND STEMS

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ABSTRACT

Anthracnose, caused by *Colletotrichum fuscum*, produces regular necrotic spots on oregano leaves and stems, causing severe crop losses. In this study, Koch's postulates were fulfilled and infection process was investigated using scanning electron microscopy. Leaves and stems of *Origanum vulgare* were inoculated and incubated at 24 C in wet chambers under high relative humidity. Pathogenicity experiments demonstrated that all tested *C. fuscum* isolates had infected stems and leaves of oregano. Of all inoculation methods, direct placement of colonized agar plugs on injured epidermis and soaking plant organs in conidial suspension were the most effective. The behavior of the conidia deposited on the oregano leaves was investigated at different time intervals after inoculation: at 12, 18, 32, 48, 67 and 98 h. Conidia produced an appressoria of varying shapes which has been formed at the end of germ tubes of different lengths. Penetration to host tissue through stomata was observed. Acervuli formed on the leaves surface after 98 h after inoculation, typically with sharp pointed setoses.

Key words: *Origanum vulgare*, anthracnose, SEM, ultrastructure

INTRODUCTION

Colletotrichum (Ascomycota, Sordariomycetes) is one of the most important genera of plant pathogenic fungi worldwide, having been voted as one of the top 10 fungal pathogens by 495 scientists associated with the journal Molecular Plant Pathology [Dean et al. 2012, Joshi 2018]. Species of *Colletotrichum* affect a wide range of woody and herbaceous plants, including medicinal and aromatic plants (MAPs), causing anthracnose diseases [Cannon et. al 2012, da Silva et al. 2020]. *Colletotrichum gloeosporioides* is one of the most important pathogens St. John's wort (*Hypericum perforatum* L.) in Germany, Switzerland and Poland [Debrunner et al. 2000, Gärber and Schrenk 2002, Zimowska and Machowicz-Stefaniak 2004] and basil (*Ocimum basilicum* L.) grown in Italy [Garibaldi et al. 1995]. *Colletotrichum dematium* is a pathogen of caraway (*Carum carvi* L.) [Machowicz-Stefaniak 2010]. *Colletotrichum malvarum* causes the anthracnose of common mallow (*Malvae sylvestris* L.) [Máthé 2015].

Colletotrichum fuscum has been isolated for the first time in Poland in 2012 from symptomatic oregano (*Origanum vulgare* L.) leaves [Zimowska 2015]. The pathogen was previously recorded in Japan as the causal agent of anthracnose of nemesia (*Nemesia strumosa* L.), and in the United States as the pathogen of foxglove (*Digitalis purpurea* L., *Digitalis lanata* Ehrh.) [Goodman 1960, Tomioka et al. 2001]. Identification of *C. fuscum* isolates obtained from diseased oregano leaves was performed through morphological approach as well as rDNA-ITS (internal transcribed spacer) sequencing [Zimowska and Abramczyk 2014]. According to Taylor et al. [2000], the three most com-

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mon ways to recognize species are the morphological, biological, and phylogenetic species concepts. Whereas the identification and classification of species of *Colletotrichum* has undergone a taxonomic revolution in the last decade through the application of molecular phylogenetic methods very little is known about the biology, pathogenicity, host range and geographical distribution many of the recently recognized *Colletotrichum* species [Cai et al. 2009, Crouch et al. 2009, Hyde et al. 2009a, 2009b, da Silva et al. 2020]. Moreover, understanding the modes of infection shown by *Colletotrichum* may be very important in preventing and controlling disease development and spread. The mechanisms by which species of *Colletotrichum* penetrate their hosts have been discussed for many years. Several modes are possible: by direct penetration of the cuticle, through natural openings, e.g. stomata, and through wounds [Bailey et al. 1992, Marques et al. 2016].

Considering the immense importance of *O. vulgare* as the medicinal plant in Poland, the present work has been carried out to to examine the infection strategy of *C. fuscum* isolates. To the best of our knowledge this is the first report considering ultrastructural aspect of the infection of oregano by *C. fuscum* aim of explaining the relation between pathogen and the host plant.

MATERIAL AND METHODS

Plant material. The plant material consisted the symptomatic leaves and stems of 2-year-old plants of oregano grown on three plantations in the Lublin province in 2012–2014 [Zimowska 2015, Zimowska et al. 2016]. The fore crops on those plantations were usually other herbs, e.g. lemon balm, common thyme and sage. For mycological analysis 10 symptomatic leaves and stems were taken from. The plan material were sterilized by soaking in a 10% blench (0.525% sodium hypochlorite) solution for 3 min and then rinsing 3 times with sterile distilled water. Small (approximately 3 × 3 mm) section of tissue were aseptically excised and placed into 90 mm diameter petri plates (10 pieces/plate) containing the mineral medium (0.7 g NH_4NO_3, 0.3 g KH_2PO_4, 0.3 g $MgSO_4 \times 7\ H_2O$, 0.01 g $FeCL_3 \times 6\ H_2O$, 0.01 g $ZnSO_4 \times 7\ H_2O$, 0.01 g $CuSO_4 \times 7\ H_2O$, 0.01 g $MnSO_4 \times 5\ H_2O$ + 38 g saccharose + 20 g agar + 1000 ml H_2O). Within 4 days of incubation in the dark at 24°C, small parts of colonies growing around the inocula were transferred into PDA (potato dextrose agar) medium (Difco™ Potato Dextrose Agar, USA) slants.

Fungal isolates. The studies used one-spore cultures of three isolates of *C. fuscum* obtained from the naturally infected leaves and stems of oregano with anthracnose symptoms and isolate CBS 102189 obtained from CBS-KNAW Fungal Biodiversity Centre (actually: Westerdijk Fungal Biodiversity Institute), Utrecht, Netherlands.

Inoculation techniques. Isolates of *C. fuscum* were incubated on a potato-dextrose agar (PDA, Difco) at the temperature of 24°C without any light access, throughout 2 weeks. Three methods of inoculation were used to prove the pathogenicity. The first method used plugs of the colonized agar (5 mm diameter) cut out from 2-week-old cultures of each isolate. Those plugs were placed at the leaves and stems fragments which were disinfected on the surface by being immersed for 60 s in 10% sodium hypochlorite. In the second method, colonized plugs were placed at leaves and stems, together with the epidermis injured with a needle puncture [Zimowska, 2004]. The third method used a suspension of conidia with the density of 10^5 conidia per 1 ml (as measured with a hemacytometer). It was obtained by rinsing the surface of the cultures of particular isolates with sterile distilled water. The disinfected fragments of stems and leaves were soaked in the suspension for 5 min [Zimowska 2012]. Each method was tested in humidity chambers. Those were Petri dishes with the diameter of 9 cm, containing 3 sheets of cellulose tissue and one layer of filter paper moistened with 4 ml of distilled sterile water. For each method 120 fragments of stems and leaves were used. Control stems and leaves were inoculated with mycelium-free agar plugs (methods I and II) or sterile water (method III). The experiment was conducted twice. Humidity chambers were kept in a thermostat at the temperature of 24°C for 12 days. During that time, observations were made every 3 days on the development of disease symptoms. After 9 days, the infection index was calculated on the basis of the disease scale. Next, all inoculated organs were analyzed for the presence of fungus according to Koch's postulates. Moreover, the leaf samples were taken at 12, 18, 32, 48, 67 and 98 h to be used to study the infection and

colonization process using scanning (SEM) electron microscopy. The results obtained from the experiment were statistically analyzed using a two-factor variance analysis (Anova) according to SAS program [Snedecor and Cochran 1982].

Sample preparation for scanning electron microscope (SEM). The leaf samples inoculated by conidial suspension were cut into 2–3 mm section. Next, the specimens were fixed with 4% glutaraldehyde for 3 h at room temperature and then, for 24 h, at 5°C. After that time, the specimens were placed in 1% cacodylate buffer for 2 h at room temperature [Bozzola and Russell, 1998]. Next, they were dehydrated in an alcohol series (30, 50, 70, 95 and 100%), for 15 min at each concentration. The specimens were then dried in liquid CO_2 by using a BAL-TEC CPD 030 Critical Point Dryer, and finally gold sputter-coated. Observations of six samples were carried out at different time intervals after inoculations: 12, 18, 32, 48, 67 and 98 h. Micrographs were obtained using a Vega 2, Tescan scanning electron microscope.

RESULTS

Pathogenicity tests and inoculation techniques. All studied isolates of *C. fuscum* caused symptoms on inoculated fragments of stems and leaves as small round to oval brown spots, which subsequently enlarged. As early as already 3 days a.i. (after inoculation), symptoms were observed on oregano parts inoculated according to method II. Those were necrotic spots, around the infection site. After 6 days, the necrosis enlarged and covered from 25% to 30% of the area of the inoculated parts. After 9 days, the necrosis covered from 31% to 40% of the surface of the stems and leaves, and after 12 days – from 41% to 50% of the surface of the inoculated parts of oregano showed necrosis symptoms. The development of disease symptoms on oregano stems and leaves inoculated according to method III was similar. In the combination with inoculation through the undamaged epidermis (method I), a trace of necrosis was seen after 6 days around the inoculation site. After 6 days, necrosis covered from 15% to 25% of the surface of the inoculated parts, while after 12 days, from 30% to 50%. Those symptoms were similar to those observed on the plants in conditions of field cultivation. The most effective methods of inoculation proved to be methods II and III. Values of the infection index were, respectively, 86.6% and 99.4% for the leaves and 88.3% and 93.5% for the stems and they were significantly different from values of the index obtained for method I (Tab. 1). The highest values of the infection index in all inoculation methods among the tested isolates were for isolate O 1001/2013 and isolate O 886/2012 (Tabs 2, 3, 4). Those values were significantly differ from the values of the index obtained for the other isolates, except isolate O 987/2012 tested according

Table. 1. Pathogenicity of *Colletotrichum fuscum* to stems and leaves of oregano using 3 different methods of inoculation (mean for 4 isolates)

Inoculation methods	Infection index (%) after 12 days[x]	
	stems	leaves
Colonized plugs placed at injured epidermis of stems and leaves	88.3a*	86.6a
Untreated	0c	0c
Colonized plugs placed at not injured epidermis of stems and leaves	62.2b	64.8b
Untreated	0c	0c
Stems and leaves soaked in conidial suspension (1×10^6 conidia/ml)	93.8a	95.4a
Untreated	0c	0c
HSD	22.0659	18.2701

[x] Infection index evaluated on the basis of scale: 0° – lack of disease symptoms; 1° – signs of necrosis visible only around of the inoculation points; 2° – up to 25% surface of inoculated organs showed disease symptoms; 3° – from 25% to 50% surface of inoculated organs showed disease symptoms; 4° – more than 50% surface of inoculated organs showed disease symptoms.
* Values marked with the same letter do not differ significantly
HSD – honest significant difference

Table. 2. Effect of infection *Colletotrichum fuscum* isolates at injured epidermis on occurrence of necrosis stems and leaves of oregano – method I

Isolate	Infection index (%) after 7 days[x]		Reisolation (%)[y]	
	stems	leaves	stems	leaves
O 886/2012	89.0b*	92.5ab	100	100
O 987/2012	85.5b	90.0b	100	100
O 1001/2013	99.0a	95.3a	100	100
CBS 102189	79.7c	68.6c	100	100
Control	0d	0d	0	0
HSD	5.7023	4.8077	–	–

[x] Note: see Table 1
[y] For leaves and stems showing anthracnose symptoms, percent isolations that resulted in *C. fuscum* colonies
* Values marked with the same letter do not differ significantly
HSD – honestly significant difference

Table. 3. Effect of infection *Colletotrichum fuscum* isolates at not-injured epidermis on occurrence of necrosis stems and leaves of oregano – method II

Isolate	Infection index (%) after 7 days[x]		Reisolation (%)[y]	
	stems	leaves	stems	leaves
O 886/2012	67.6a*	71.0a	100	100
O 987/2012	59.0b	68.8a	100	100
O 1001/2013	68.3a	70.5a	100	100
CBS 102189	54.0b	49.0b	100	100
Control	0c	0c	0	0
HSD	7.0532	8.2055	–	–

[x] Note: see Table 1
[y] For leaves and stems showing anthracnose symptoms, percent isolations that resulted in *C. fuscum* colonies
* Values marked with the same letter do not differ significantly
HSD – honestly significant difference

Table. 4. Effect of infection *Colletotrichum fuscum* isolates by conidial suspension on necrosis of stems and leaves of oregano – method III

Isolate	Infection index (%) after 7 days[x]		Reisolation (%)[y]	
	stems	leaves	stems	leaves
O 886/2012	98.3a*	100.0a	100	100
O 987/2012	88.5b	92.6ab	100	100
O 1001/2013	100.0a	100.0a	100	100
CBS 102189	88.5b	89.0b	100	100
Control	0 c	0c	0	0
HSD	7.5203	10.0352	–	–

[x] Note: see Table 1
[y] For leaves and stems showing anthracnose symptoms, percent isolations that resulted in *C. fuscum* colonies
* Values marked with the same letter do not differ significantly
HSD – honestly significant difference

to method II (Tab. 3). *Colletotrichum fuscum* cultures were reisolated from all inoculated organs, for all methods (Tabs 2, 3, 4). Morphological features of reisolated cultures corresponded to the features of cultures considered in the research. Uninoculated controls remained symptomless, and results from the two experiments were similar.

Scanning electron microscope. Twelve hours after the inoculation, conidia of *C. fuscum* were visible on the surface of oregano leaves (Fig. 1a). Conidia started to germinate 18 h a.i. Appressoria were first observed at 32 h. They were produced either from the tips of the short germ tubes or directly from conidia (Fig. 1b, c, d). The growth of the germ tubes and formations of appressoria were apparently not influenced by the proximity of stomata. Appressoria varied in shape, from round, ovate to lobed (Fig. 1b, c, d). Conidia produced a single germ tube with moderate length (Fig. 1b, e). At the time of germination, a septum (Fig. 1b, c, d) formed in the center of the germinating spore. Penetration of the pathogen through stomata was observed 48 h after the inoculation (Fig. 1f). Extensive networks of runner hyphae were observed 67 h after the inoculation (Fig. 1g). By 98 h acervular stroma was formed. The stroma erupted through the cuticle and began to produce acervuli with setae (Fig. 1h).

DISCUSSION

Pathogenicity studies showed that all tested isolates of *C. fuscum* caused infection on the inoculated organs. This has been testified according high value of infection index and fulfillment of Koch's postulates. Of all inoculation methods, the most effective proved to be the one including putting plugs of colonized agar on the injured tissue, and the method considering soaking the plant organs in the conidial suspension, which is typical in case of facultative pathogens [Cannon et. al 2012]. Moreover, all re-isolated cultures of *C. fuscum* showed the same morphological features, corespondented with strains used in the studies. Numerous acervuli were observed, which were formed on the entire surface of the colony. They were black, oval or almost round and slightly immersed in the medium. The diameter of acervuli was 350.68 × 251.45 µm. On the surface, numerous dark, sharp pointed setoses were developed, with size ranging from 17.81 µm to 85.59 µm in length and at the base 4.20 µm to 5.85 µm in width. Salmon-cream drops of conidial exudate emerged from the fruting bodies. *C. fuscum* formed hyaline, aseptate, smooth conidia. They are cylindrical to ellipsoid, straight or slightly bent. The top of the conidia is rounded, the base is slightly cut. The size of the conidia was 14.07–21.98 µm by 3.58–4.89 µm. At the end or in the middle of hyphae abundant brown, ellipsoidal and lobe appressoria were formed.

Due to its economic importance as a plant pathogen, *Colletotrichum* has received a lot of attention from great number of researches all over the world, who try to determine molecular methods useful to detect species and figure out phylogenetic relationships, they also put the effort to establish clear taxonomical borders within *Colletotrichum* [Cano et al. 2004]. On the other hand, the detailed studies are still needed to provide knowledge on the infection process of *Colletotrichum,* knowledge of specialized infection structures, e.g., germ tubes, appressoria, primary and secondary hyphae to better understand the biology of this pathogenic fungi [O'Connel et al. 2000, Hiruma 2019].

The early stages of fungal development during the infection process is a critical stage in any plant-pathogen interaction [Howard and Valent, 1996, Nordzieke et al. 2019]. In *Colletotrichum*, this process can be separated into stages including: the deposition of conidia on plant surfaces, attachment of conidia to those surfaces, germination of conidia, production of appressoria, penetration of the plant epidermis, growth and colonization of plant tissues, production of acervuli and sporulation [Jeffries et al. 1990, Prusky et al. 2000]. It was observed in udertaken studies germinated conidia, germ tubes and appressoria of *Colletotrichum fuscum* adhered to the surface of the inoculated organs. Conidial germination of *Colletotrichum* is highly variable, between 3 and 48 after inoculation [Lopez 2001]. In this study, conidial germination was observed 18 h a.i. During this process, a septum formed in the center of the germinating spores, however *C. fuscum* conidia are normally aseptate [Zimowska et al. 2016]. The unicellular conidium divides, due to mitosis, and then only one of the resulting cells germinates. Nuclear division in the other cell is arrested, and the cell remains inactive but can resume growth at a later stage [Nesher et al. 2008]. In *Magnaporthe grise*, blockage of mitosis prevents completion of germination and ap-

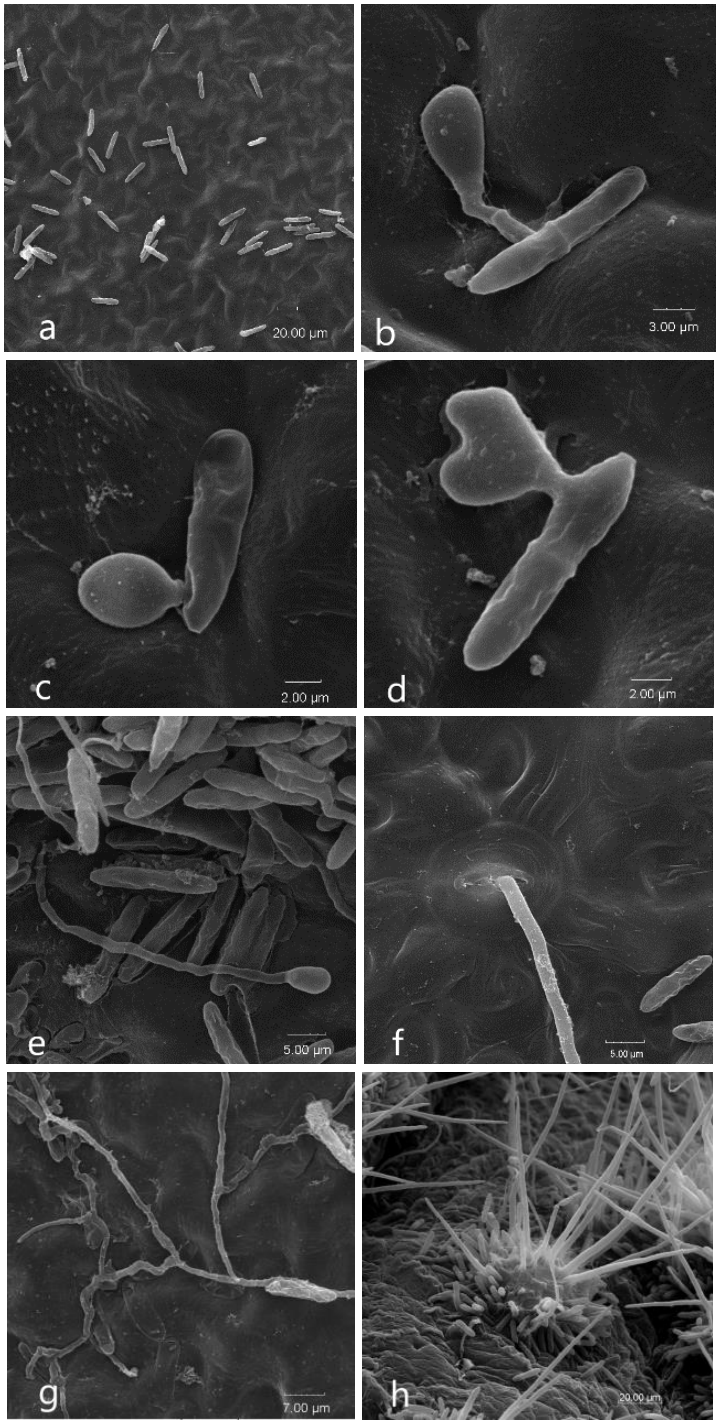

Fig. 1. Scanning electron micrographs of *Colletotrichum fuscum* infection on oregano leaves: **a.** Conidia 12 h a.i. (after inoculation); **b.** Germinated conidia 32 h a.i., with formation of the septum and oval appressorium at the end of the germinative tube; **c.** Germinated conidia 32 h a.i., developing rounded appressorium; **d.** Germinated conidia 32 h a.i., with formation of septum and lobed appressorium; **e.** Appressorium developed at the end of the long germ tube; **f.** Penetration through stomata 48 h a.i.; **g.** Network of runner hyphae on the leaf surface 67 h a.i.; **h.** Acervular stroma with setae, 98 h a.i.

pressorium formation [Veneault-Fourrey et al. 2006]. Thus, genetic intervention during mitosis prevent both appressorium development and conidium death, and in the same time makes infection unsatisfactory. Impairment of autophagy, by the targeted mutation of the certain gene responsible for nuclear mitosis can makes the fungus nonpathogenic, whitch also has been proved by Nesher et al. [2011], in case of *Colletotrichum gloeosporioides.*

In *Colletotrichum* appressoria play the crucial role, allowing the fungus to penetrate host cuticle and epidermal cell wall directly by means of a narrow penetration peg that emerges from the base of the appressorium [Perfect et al. 1999, Fukada et al. 2019]. Although rare in *Colletotrichum,* there are reports of indirect penetration of tissue through stomata [Latunde--Dada et al. 1999], or wounds without the formation of appressoria [Sénéchal et al. 1987, Van der Bruggen and Maraite 1987, Zulfiqar et al. 1996]. In undertaken studies *C. fuscum* penetrated oregano leaves through stomata with appressoria formation. The same mode of penetration has been observed in case of *C. gloeosporioides* on mullberry leaf [Kumar et al. 2001]. *Colletotrichum fuscum* appressoria developed in different position, both on the end of the germ tube and different positions on the conidia and presented distinct shapes, including round, ovate to lobed. Thus, due to the great diversity, appressoria morphology should not be used for species identification. Appressorium formation represents a distinct morphogenetic stage and is associated with a switch from polar to isotropic growth and triggered by perception of physical and/or chemical signals. In the rice blast fungus *M. grisea,* surface hydrophobicity and wax components stimulate appressorium formation [Lee and Dean 1994, Uchiyama and Okuyama 1990, Gilbert et al. 1996]. Also, the avocado pathogen *C. gloeosporioides* requires both, hard surface contact and host surface wax for germination and appressorium formation [Podila et al. 1993, Flaishman et al. 1995]. Pharmacological analysis suggests that the perceptionof environmental signals initiates intracellular signal trans-duction pathways in these fungi, then promotes infection-related morphogenesis [Lee and Dean 1993, Flaishman et al. 1995, Kim et al. 1998]. The importance of appressoria in the infection process has been showed in several studies with melanin deficient mutants and the inhibitors of melanin biosynthesis [Kubo and Furusawa 1991, Mendgen and Deising 1993].

The next stage observed in this study was the network of runner hyphae which grew on the leaf surface and developing acervuli with seta. Such process of *C. fuscum* conidiogenesis is typical for many previously described *Colletotrichum* species. *Colletotrichum gloeosporioides* developed clusters of conidia in acervuli on the adaxial surface of mulberry leaves [Kumar et al. 2001]. Conidia of *C. destructivum* were also released through acervuli in tobacco leaves [Shen et al. 2001]. Although, there are species which form conidia mostly along runner hyphae or secondary hyphae at short intervals [Babu et al. 2008].

The present studies showed different mode of penetration by *C. fuscum* on oregano leaves, when compered to majority of known *Colletotrichum* spp., including appressoria formation and infection through stomata. Now the detailed studies on post-infection events (including the quiescent phase) should be carry on to distinguished which type of colonization strategy plays role in case of *C. fuscum.*

CONCLUSIONS

There have been many studies on infection process of *Colletotrichum* spp., but none of them considered *C. fuscum.* The present study showed that the infection is an independent and specific mechanism, which can be different according to the host-pathogen interaction and additional factors which should be fulfiled to follow the successful infection.

Explaining the mechanism of *C. fuscum* infection provides the new knowledge to the literature and may be helpful in planning plants protections treatments against this pathogen.

SOURCE OF FUNDING

The research was funded by subvention no. OK-K/s/44/2021.

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