POMOLOGIA CROATICA
Vol. 25 - 2021., br. 1-4

Original scientific paper
Izvorni znanstveni članak

Etiology of *Colletotrichum* diseases on Sastuma mandarin in Croatia

Etiologija bolesti uzrokovanih *Colletotrichum* vrstama na mandarini Unshiu u Hrvatskoj

D. Ivić, L. Popović, Ivana Križanac, M. Bjeliš

ABSTRACT

During the last decade anthracnose has become a major disease of Satsuma mandarin, the most important citrus crop in Croatia. The aim of this study was to determine *Colletotrichum* species associated with different symptoms and to identify the origin of inoculum. From 2013 to 2016, 437 samples of plant material were collected. *Colletotrichum* spp. was isolated from 93% of dried twigs, 35% of dropped flowers, 89% of leaf spots, all fruit (100%) with anthracnose or calyx-end rot symptoms, 12% of fruit with post-harvest soft rot and from 40% of fruit showing spots remaining on trees after harvest. Out of 258 *Colletotrichum* isolates, 253 has been morphologically identified as *C. gloeosporioides* (Penz.) Penz. & Sacc. species complex. Twenty-seven representative isolates were selected for phylogenetic analysis. Sequencing the inter-spacer gene region of ribosomal DNA confirmed the identity of the species. Artificial inoculation of flowers led to more than 2-fold higher young fruit drop compared to control. Pathogenicity tests on green fruit induced typical anthracnose symptoms on 82% of inoculated fruit two months after inoculation. Inoculation of mature fruit caused the appearance of typical anthracnose symptoms on 87% of inoculated fruit. These results showed that *C. gloeosporioides* species complex is responsible for different disease types on Satsuma mandarin, and that the fungus is present throughout the year on different plant organs.

Key words: anthracnose, *Citrus unshiu*, *Colletotrichum gloeosporioides* species complex, fruit drop, inoculum

SAŽETAK

Antraknoza je tijekom prošlog desetljeća postala gospodarski štetna bolest mandarine Unshiu, najvažnije vrste agruma u Hrvatskoj. Cilj rada bio je istražiti

Colletotrichum vrste povezane s različitim simptomima na mandarini te utvrditi izvor zaraze. Od 2013. do 2016. sakupljena su 437 uzorka biljnog materijala. *Colletotrichum* vrste izolirane su iz 93 % osušenih izbojaka, 35 % otpalih cvjetova, 89 % lisnih pjega, iz svih plodova (100 %) sa simptomima antraknoze ili truleži čaške, 12 % plodova s mekom truleži u skladištu te iz 40 % plodova s pjegama koji su ostali na stablu nakon zime. Od ukupno 258 *Colletotrichum* izolata, 253 je morfološki determinirano kao *C. gloeosporioides* (Penz.) Penz. & Sacc. kompleks. Dvadeset sedam izolata odabrano je za filogenetsku analizu. Sekvenciranjem ITS regije gena ribosomske DNA potvrđena je identifikacija vrste. Umjetna zaraza cvjetova uzrokovala je više nego dvostruko veće otpadanje mladih plodića mandarine u usporedbi s kontrolom. Testovi patogenosti na nezrelim zelenim plodovima doveli su do pojave tipičnih simptoma antraknoze na 82 % inokuliranih plodova dva mjeseca nakon umjetnih zaraza. Inokulacija zrelih ubranih plodova uzrokovala je pojavu tipičnih simptoma antraknoze na 87 % inokuliranih plodova. Rezultati istraživanja pokazali su da je *C. gloeosporioides* kompleks vrsta uzročnik različitih tipova bolesti na mandarini Unshiu, kao i da je patogen prisutan na različitim biljnim organima tijekom cijele godine.

Ključne riječi: antraknoza, *Citrus unshiu*, *Colletotrichum gloeosporioides* kompleks vrsta, otpadanje plodova, inokul

INTRODUCTION

Various *Colletotrichum* species are economically important pathogens of numerous fruit crops worldwide (Freeman et al., 1998; Peres et al., 2005). On citrus crops, these fungi are generally reported as causal agents of post-bloom fruit drop and fruit anthracnose (Agostini et al., 1992; Peres et al., 2005), two diseases frequently occurring in different parts of the world and on different *Citrus* species (Brown and Eckert, 2000; Timmer, 2000; Peres et al., 2005). Both diseases can cause substantial losses in citrus production (Brown and Eckert, 2000; Timmer, 2000). In older literature, *Colletotrichum acutatum* J. H. Simmonds was considered to be the only causal agent of citrus post-bloom fruit drop (Peres et al., 2005), while *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. was commonly regarded as the pathogen causing citrus anthracnose (Agostini et al., 1992). In recent years, many newly described *Colletotrichum* species were reported to occur on citrus crops (Huang et al., 2013; Guarnaccia et al., 2017). Isolated from various plant organs, the ability of these species to cause different disease symptoms on citrus crops has been proven (Huang et al., 2013; Aiello et al., 2015; Ramos et al., 2016). Among

other, recent investigations have shown that both *C. acutatum* and *C. gloeosporioides* can cause citrus post-bloom fruit drop (Lima et al., 2011; McGovern et al., 2012). In Europe, *C. gloeosporioides* was also shown to cause leaf spots, branch dieback and different types of anthracnose or anthracnose-like symptoms on citrus fruit (Aiello et al., 2015; Ramos et al., 2016; Guarnaccia et al., 2017).

Satsuma mandarin (*Citrus unshiu* Marc.) is the most important citrus crop in Croatia, comprising the majority of about 20 000 ha of citrus cultivation area in the country. Satsuma mandarin was introduced from Japan in a period from 1930s to 1960s and was shown to be well-adapted and the most suitable citrus crop for Croatian agro-climatic conditions (Bakarić, 1983). Anthracnose of mandarin in this region was described more than 50 years ago in Montenegro, with *C. gloeosporioides* identified as the causal agent of the disease (Mijušković, 1966). Despite being known for a long time in the East Adriatic region, *C. gloeosporioides* was not considered as an economically important citrus pathogen in Croatia until recently. The first epiphytotics of fruit anthracnose on Satsuma mandarin during the last decade was recorded on several locations in Neretva River valley during 2012. In 2014, a high incidence of calyx-end fruit rot during actively growing period devastated yields in some orchards, while in 2016 typical fruit anthracnose on more than 50% of fruit at certain locations was observed. Fruit anthracnose is becoming a more and more severe disease on Satsuma mandarin in the Neretva River valley, the main citrus-growing area in Croatia (Ivić et al., 2017). Control measures are now needed but effective disease management is difficult to implement without basic knowledge on the etiology and epidemiology of a disease.

The main objectives of this research were to determine the causal agent of Satsuma mandarin anthracnose in Croatia, to assess which disease symptoms can be caused by *Colletotrichum* species, and to investigate the presence of *Colletotrichum* species on different plant parts and organs throughout the year.

MATERIALS AND METHODS

The presence of *Colletotrichum* spp. on different organs of Satsuma mandarin plants was investigated from 2013 to 2016. Partially dried twigs, dropped flowers, leaves with spots and maturing fruit with anthracnose or calyx-end rot symptoms were collected during the actively growing period, from April to November. Fruit from the previous season, showing various spots or rotting symptoms, which remained on trees after the harvest were sampled from February to April in 2015 and 2016. Fruit with post-harvest soft rot were

collected during the storage in 2014 and 2015. In total, 74 dried twigs, 128 dropped flowers and 27 leaves with spots were collected (Table 1). All samples were collected in the area of Opuzen and Metković in the Neretva River valley. Most of the samples were collected in intensive commercial orchards (≥ 0.5 ha in size) and smaller semi-professional orchards (less than 0.5 ha in size). Small number of samples was taken in private gardens, having only several mandarin trees. Twenty-two fruit with calyx-end rot, 54 fruit with anthracnose symptoms and 33 fruit with post-harvest soft rot were sampled, as well as 99 non-harvested fruit remaining on trees from the previous season (Table 1). Only fruit with post-harvest soft rot not showing signs or visible presence of blue or green mould (*Penicillium* spp.) were selected. Among fruit which remained on trees after the harvest, only those showing spots, surface necrosis or anthracnose-like symptoms were collected.

Table 1 Incidence and frequency of *Colletotrichum* spp. and *C. gloeosporioides* species complex isolated from different plant parts of Satsuma mandarin.

Tablica 1. Zastupljenost i učestalost *Colletotrichum* spp. i *C. gloeosporioides* kompleksa vrsta izoliranih iz različitih organa mandarine Unshiu.

Plant organs	No. of samples	No. of samples where *Colletotrichum* spp. were isolated	No. of samples where *C. gloeosporioides* was identified
Shoots	74	69 (93%)	67
Flowers	128	45 (35%)	45
Leaves (leaf spots)	27	24 (88%)	24
Fruits (caylx-end rot)	22	22 (100%)	22
Fruits (anthracnose)	54	54 (100%)	54
Fruits (post-harvest rot)	33	4 (12%)	4
Fruits (remained on trees after the harvest)	99	40 (40%)	37
Total	437	258	253

Colletotrichum spp. presence on dried twigs was analysed by cutting and placing cut fragments of twigs on potato-dextrose agar (PDA). From each twig, 10 to 15 fragments were inoculated on PDA plates. Prior to inoculation, fragments were surface-sterilised with 1% sodium hypochlorite solution for 5 min, rinsed with sterile water and dried. Colonies developed after one week to 10 days of incubation at 22 °C in darkness were examined visually and microscopically. Colony colour, appearance, conidiomata, appressoria and conidia were assessed, following descriptions of Weir et al. (2012) and Damm

et al. (2012). If all colonies developed from a single branch and identified as *Colletotrichum* sp. were similar, only one colony was transferred to new PDA plates for further characterization. If morphologically different colonies were observed, one out of each colony type was sub-cultured. Colonies were sub-cultured from a single conidioma and were considered as isolates.

Dropped flowers were incubated in moist chamber at 24 °C for four days. Fungi developed were examined by stereomicroscope and microscope. If acervuli or mycelia of *Colletotrichum* spp. were observed, they were transferred to PDA and incubated as described above. Isolates collected were identified according to the morphology as described above.

Leaves with leaf spots were incubated in moist chamber at 24 °C until sporulation occurred. Sporulating structures were examined microscopically. If *Colletotrichum* spp. conidia in acervuli developed, conidia from a single acervulus were transferred to PDA, grown to an isolate, incubated and identified as described above.

Fruit with calyx-end rot, fruit with anthracnose symptoms and those showing post-harvest soft rot were rinsed in tap water, briefly surface-sterilised for half a minute in 70% ethanol, rinsed with sterile water, dried and incubated in moist chamber at 24 °C until sporulation. Conidia were examined microscopically and transferred to PDA from a single acervulus, incubated and identified. Fruit collected from the previous season were treated similarly, but rinsing and surface-sterilization time were extended to two minutes.

Colonies developed on PDA from a single conidioma or acervulus were incubated for 14 days at 22 °C under 12 h light/12 h dark regime. Colony appearance, colour, conidiomata and conidia were examined. Twenty conidia of each isolate were measured. Isolates were identified to a species level according to their morphology, following descriptions of Damm et al. (2012), Weir et al. (2012), and Cannon et al. (2008). For molecular confirmation of species identity, 27 isolates were selected for molecular identification by PCR – sequencing of ribosomal internal transcribed spacer (ITS). Six isolates from shoots (CG-M3, CG-M6, CG-M12, CG-M75, CG-M136, GC-M201), three from leaves (CG-M81, CG-M90, CG-M92), five from flowers (CG-M22, CG-M25, CG-M28, CG-M37, CG-M38), four from fruit with anthracnose symptoms (CG-M44, CG-M46, CG-M47, CG-M268), two from fruit with calyx-end rot (CG-M59, CG-M64), one from post-harvest soft rot (CG-M66) and six isolates from previous season fruit (CG-M111, CG-M124, CG-M127, CG-M198, CG-M243, CG-M239) were selected for sequencing. Isolates were

grown on PDA covered with cellophane layer for a week, mycelium was harvested and grinded, and total DNA was extracted using DNeasy® Plant Mini Kit (Quiagen Inc., USA). Approximately 5 µl of diluted DNA extract was used in 50 µl PCR reactions containing 5 µl of 10x PCR buffer, 5 µl of 2.5 mM dNTPs, 2 µl of 2.5 mM $MgCl_2$, 1 U of Taq polymerase and 1 µl of ITS1/ITS4 primer pair (White et al., 1990). PCR conditions were the following: 94 °C for 2 min, 35 cycles of 94 °C for 1 min, 59 °C for 2 min, and 72 °C for 2 min, with a final extension at 72 °C for 5 min. PCR products were purified and sequenced by Macrogen Europe (Amsterdam, The Netherlands). Sequences were edited using Sequencher® software (Gene Codes Corp., USA) and aligned with five sequences obtained from GenBank (*C. gloeosporioides* Acc. No. JX010152, MH156760 and MG543717, *Colletotrichum karstii* Acc. No. KY856462, *C. acutatum* Acc. No. KY856397 and *Diplocarpon rosae* Acc. No. MH855164 as an outgroup). Clustal X (Thompson et al., 1997; Larkin et al., 2007) implemented in MEGA7 software (Kumar et al., 2016) was used for alignment. ITS-based UPGMA phylogenetic analyses (Sneath and Sokal, 1973) was performed, with nodal support evaluated using bootstrap analysis from 1000 replicates (Felsenstein, 1985).

Pathogenicity tests were carried out on mandarin flowers, green fruit and mature fruit. To investigate the possibility of *C. gloeosporioides* isolates from shoots, leaves, flowers or fruit to cause symptoms on other plant organs, several isolates were selected for pathogenicity tests. Isolates CG-M3 (from twig), CG-M19 (flowers) and CG-M54 (fruit, anthracnose) were used for inoculations of flowers. Isolates CG-M21 (from flowers), CG-M51 (fruit, calyx-end rot) and CG-M65 (fruit, post-harvest rot) were used for inoculations of green fruit. Isolates CG-M5 (from twig), CG-M24 (flowers) and CG-M45 (fruit, anthracnose) were used for inoculations of mature fruit (Table 2). Inoculum for tests on flowers and on mature fruit was conidial suspension, while PDA plugs with developed colonies were used for inoculation of green fruit. Suspensions were prepared by harvesting conidia from 21-days old colonies on PDA with a rod and suspending them in sterile water. Suspensions were adjusted to 10^5 conidia/ml with a haemocytometer.

Table 2 Pathogenicity and incubation of *Colletotrichum gloeosporioides* isolates to Satsuma mandarin green fruit inoculated during the vegetation and to mature harvested fruit.

Tablica 2. Patogenost i inkubacija izolata *Colletotrichum gloeosporioides* na zelenim plodovima inokuliranima tijekom vegetacije te na zrelim ubranim plodovima mandarine Unshiu.

Maturity of fruit inoculated	Isolate (origin)	Frequency of symptoms induced	First symptoms appearance (days post inoculation)
Green fruit (field)	CG-M3 (twig)	15/20 (75%)	61±4.7 dpi
	CG-M51 (fruit)	16/20 (80%)	57±8.1 dpi
	CG-M65 (fruit)	18/20 (90%)	64±5.2 dpi
Mature fruit (harvested)	CG-M5 (twig)	9/10 (90%)	3±0.7 dpi
	CG-M24 (flowers)	9/10 (90%)	3±0.7 dpi
	CG-M45 (fruit)	8/10 (80%)	4±0.7 dpi

Inoculation of flowers was performed in an orchard in Jasenska, Opuzen (E 584916, N 4765353) on Satsuma mandarin cv. Okitsu during the inflorescence emergence principal growth stage 5 (BBCH code 56 – flower petals elongating, sepals covering half corolla, "white bud") (Agusti et al., 1995). Flowers on five branches of two trees were sprayed with conidial suspensions with each isolate until runoff, and the same number of branches was sprayed with sterile water. To prevent the potential contamination of control branches, distant trees in an orchard were selected for inoculation. Plastic foil was laid below the trees to collect the eventual dropped fruit. Both control and inoculated branches were closed with plastic bags for approximately 48 h to maintain moisture. Young fruit drop was the symptom monitored and recorded. Senescent petals and dropped fruit were collected for the re-isolation of the fungus. Isolation and identification of re-isolated *Colletotrichum* sp. was performed as described previously.

Inoculation of green fruit was performed in an orchard in an orchard in Buk Vlaka, Opuzen (E 583392, N 4763921) on Satsuma mandarin cv. Kawano Wase during the development of fruit principal growth stage 7 (BBCH code 79 – fruit about 90% of final size) (Agusti et al., 1995). Circular 5-mm cuts of 10-days old sporulating colonies of selected isolates were placed on a surface of wet fruit and sealed with parafilm. Five fruit on four different trees were randomly inoculated. Control fruit were inoculated with sterile PDA cuts. Parafilm was

removed from the fruit after 30 days. Development of symptoms was monitored and recorded until the harvest, when fruit were picked and continued to be monitored in biological chamber at 20 °C/15 °C – 12 h/12 h regime in darkness. *Colletotrichum* sp. was re-isolated from fruit on which symptoms have appeared by transferring mycelia or conidia from acervuli on PDA, following the same procedure as described before. Identification of species also followed the same methodology as previously described.

Inoculation of mature fruit was performed in the laboratory on Satsuma mandarin cv. Chahara collected in a field during the harvest period. Ten fruit were rinsed shortly in 70 % ethanol and water, and were inoculated with isolates CGM-57 and CGM-60. Five µl of conidial suspension was injected under the peel using pipette tip and points of injections were marked and sealed with parafilm. Control fruit were inoculated with sterile water. Fruit were incubated at 25 °C in high humidity (80%) and symptoms development was monitored. Identification of *Colletotrichum* sp. developed on inoculated fruit was performed the same as in previously described experiment on green fruit.

RESULTS AND DISCUSSION

Colletotrichum spp. were detected in 258 out of 437 samples (59%) of Satsuma mandarin (Table 1). It was found on all fruit with typical anthracnose symptoms, as well as on all fruit showing calyx-end rot and it was identified as *C. gloeosporioides* species complex. Acervuli with abundant conidia were present on most of the fruit at the moment of sampling and were developed after several days of incubation. On dried twigs, only two out of 69 *Colletotrichum* isolates were not in line with the descriptions of *C. gloeosporioides* (Cannon et al., 2008; Damm et al., 2012; Weir et al., 2012), and were identified only as *Colletotrichum* sp. On 66 samples of twigs where *C. gloeosporioides* species complex was determined, it sporulated regularly on all samples after incubation. On three leaf samples with grey spots and black dots visible inside the spot pycnidia and *Septoria*-type conidia were detected. Thus, the fungus identified was *Septoria citri* Pass. All colonies developed from flowers were identified as *C. gloeosporioides* species complex. In 2015, *C. gloeosporioides* species complex was detected on 41 out of 66 flowers analysed (62%), while in 2016 only on four out of 62 flowers (7%). Fruit on which *C. gloeosporioides* species complex was found were brown, soft and mostly without any visible sporulation. Scarce acervuli developed randomly on the surface of certain fruit.

The remaining 29 fruit were found affected with *Botrytis cinerea* Pers. or *Alternaria alternata* (Fr.) Keissl., although symptoms were relatively similar. *Colletotrichum* sp. was isolated from three samples. On overwintered fruit, *C. gloeosporioides* species complex was found in lesions, sunken spots or often in necrosis developing around calyx. *C. gloeosporioides* species complex isolates collected within the study were all morphologically similar (grey aerial mycelium with cottony colony surface mainly grey in reverse). Conidiomata developed in all isolates was orange, but varying in abundance and distribution. Certain instability was noted in some isolates after sub-culturing, developing more or less conidiomata, or becoming more dark or bright in reverse. Conidia of all isolates identified as *C. gloeosporioides* species complex were hyaline, smooth, cylindrical, rounded at the ends, 14.4 – 20.3 (mean 18.9) x 3.8 – 5.7 (mean 5.2) μm. Sequencing and subsequent phylogenetic analysis of 27 representative isolates showed 99 % or more similarity with *C. gloeosporioides* species complex sequences available in GenBank. UPGMA phylogenetic tree based on ITS1-5.8S-ITS2 ribosomal DNA sequences from this study showed clear grouping of all isolates within one cluster, together with sequences of isolates IMI 356878 from orange, Italy, isolate PHC 161774 from mandarin, Turkey, and isolate LrLF15 from *Lycoris radiata*, China (Figure 1). *C. gloeosporioides* species complex isolates were also separated from representative sequences of *C. karstii* (isolate CPC 27979 from mandarin, Italy) and *C. acutatum* (isolate CPC 27005 from orange, Italy).

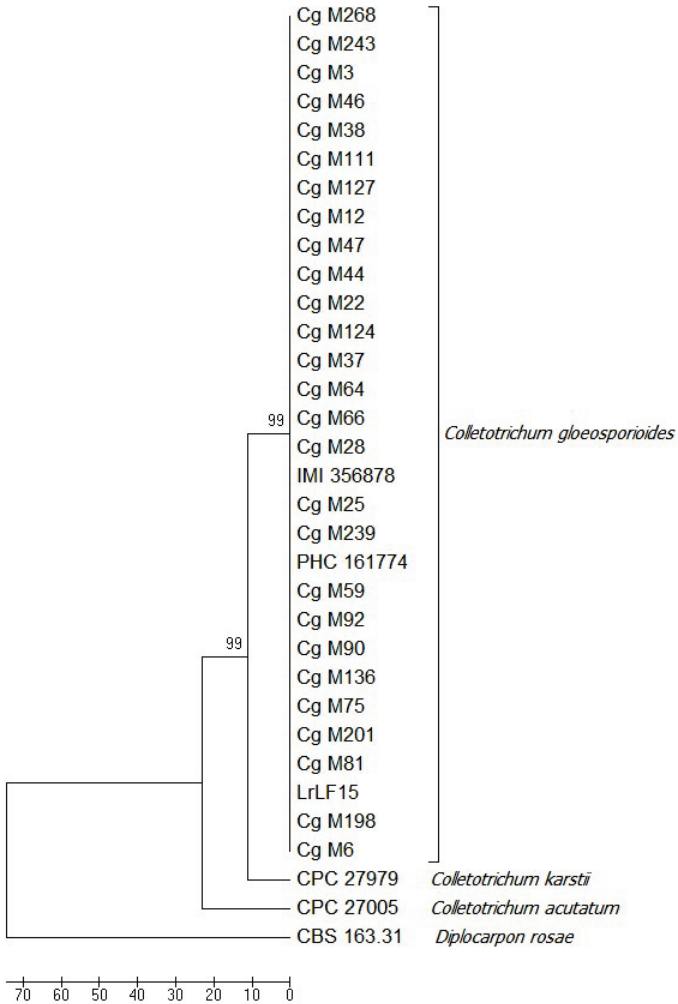

Figure 1 - UPGMA phylogenetic tree derived from sequences of ITS1-5.8S-ITS2 ribosomal DNA of Croatian *Colletotrichum gloeosporioides* isolates from Satsuma mandarin and reference *Colletotrichum* isolates retrieved from GenBank. Bootstrap interior-branch values are based on 1000 replicates.

Slika 1. UPGMA filogenetsko stablo izvedeno iz sekvenci ITS1-5.8S-ITS2 ribosomske DNA hrvatskih izolata *Colletotrichum gloeosporioides* s mandarine Unshiu i referentnih *Colletotrichum* izolata preuzetih iz GenBank-a. Bootstrap vrijednosti grananja dobivene su kroz 1000 ponavljanja.

Selected C. gloeosporioides species complex isolates were pathogenic to Satsuma mandarin in all three types of pathogenicity tests (Table 2 and Table 3). On mature fruit, fruit rot developed on 26 out of 30 inoculated fruit (87%) and in control fruit (Table 2). Lesions developed were dark, sunken and filled with orange acervuli, resembling symptoms of typical anthracnose. However, unlike anthracnose lesions (defined border and demarcation from healthy tissue), lesions induced on inoculated fruit proceeded diffusely into soft rot. On green fruit, the dynamics of symptoms development was different. No signs of infection were visible for about two months. Initial symptoms has become evident from the beginning of September, when bleaching and yellowing of inoculation site on fruit surface started to occur. One to three weeks after, necrotic spots started to appear on bleaches. By the moment of harvest, necrotic areas varied in size from 1 mm spots to 10 mm-diameter sunken lesions. Until the harvest acervuli appeared on seven out of 38 symptomatic fruit. When inoculated fruit were harvested and incubated, lesions advanced and developed on 49/60 inoculated fruit (82%). On a site where mycelial plugs were placed onto fruit surface, mycelium with scattered C. gloeosporioides species complex acervuli developed and progressed into sunken rot. Abundant sporulation occurred at the edge of the lesions and C. gloeosporioides species complex was easily re-isolated. Inoculation of flowers showed the ability of all three isolates to colonize flowers and to cause fruit drop (Table 3). A consequence of flower inoculation was more intensive young fruit drop. Different isolates of C. gloeosporioides species complex originated and isolated from different plant parts shown almost equal ability to cause symptoms on fruit and to colonize flowers. Isolates from flowers were able to cause symptoms on fruit and vice versa. Isolates from twigs caused symptoms on mature fruit and had colonized flowers approximately at the same level as isolates originally obtained from fruit or flowers.

Table 3 Fruit drop incidence and colonisation of *Colletotrichum gloeosporioides* isolates on Satsuma mandarin flowers and young fruit following artificial inoculation.

Tablica 3. Učestalost otpadanja plodova i kolonizacija *Colletotrichum gloeosporioides* izolata na cvjetovima i mladim plodovima mandarine Unshiu nakon umjetne inokulacije.

Symptoms and re-isolation (%)	Isolate (origin)			
	CG-M3 (twig)	CG-M19 (flowers)	CG-M54 (fruit)	Control
Petal re-isolation	77%	69%	70%	6%
Fruit drop	75%	80%	66%	32%
Fruit re-isolation	85%	91%	54%	3%

Citrus diseases caused by *Colletotrichum* species are well-described and known (Brown and Eckert, 2000); Timmer, 2000). However, the taxonomy of *Colletotrichum* species has undergone major revisions during the last ten years (Hyde et al., 2009; Damm et al., 2012; Weir et al., 2012). Studies on genetic level have split *C. acutatum* and *C. gloeosporioides* into a number of closely related species (Damm et al., 2012; Weir et al., 2012). Several described *Colletotrichum* species, previously considered as *C. acutatum* or *C. gloeosporioides*, co-exist on the same host causing similar symptoms (Faedda et al., 2011; Huang et al., 2013; Guarnaccia et al., 2017), without host specificity. *Colletotrichum gloeosporioides* is among the most common and frequent species associated with citrus diseases (Huang et al., 2013; Aiello et al., 2015; Ramos et al., 2016; Guarnaccia et al., 2017). In the present study, only two isolates from twigs and three isolates from previous season fruit were not identified as *C. gloeosporioides* species complex. Thus, this species can be regarded as the causal agent of *Colletotrichum* diseases on Satsuma mandarin in Croatia.

Isolates of *C. gloeosporioides* species complex from twigs, flowers and fruit have shown the ability to cause flower necrosis, typical anthracnose symptoms on fruit and post-harvest rot. These results confirmed that *C. gloeosporioides* species complex, is responsible for different disease types on Satsuma mandarin in Croatia. Observations for several years have shown that fruit anthracnose is the most important type of *Colleototrichum* disease. Post-harvest rot is of minor importance, occurring at very low incidence. The significance of *C. gloeosporioides* species complex flower infections still remained relatively unclear and direct negative effects are hard to assess. Artificial infections of flowers demonstrated that *C. gloeosporioides* species complex can cause flower blight and post-bloom fruit drop. This type of disease was attributed to *C. acutatum* in older literature (Peres et al., 2005), but recent investigations have shown that species from *C. gloeosporioides* complex can also cause post-bloom fruit drop (Lima et al., 2011; McGovern et al., 2012). It seems that *C. gloeosporioides* species complex colonization of flowers may be more important in development of fruit rot later in the season. So, calyx-end rot symptoms on fruit, especially severe in 2014 (Ivić et al., 2017), may originate from flower infections. Observations from 2014 season showed that necrosis of symptomatic fruit started to develop around the calyx, and *C. gloeosporioides* species complex was readily isolated from all fruit with this symptom. Despite that, calyx-end rot was the only disease type on fruit which could not be reproduced artificially. Calyx-end rot might be very complex to induce in artificial infections, since it is not known exactly when the infection occurs, how it is initiated, and what would be the inoculum needed.

Results are showed that fruit which remain on trees could serve as a source of inoculum for the infection of flowers or young fruit. The fungus was also found on leaves, in leaf spots, where it can probably persist all-year long. However, it seems that dry twigs, densely covered with acervuli producing abundant conidia, may represent the most important source of *C. gloeosporioides* species complex inoculum. This was also observed in a study of Guarnaccia et al. (2017). *Colletotrichum gloeosporioides* is mentioned as a common invader of dried twigs (EPPO, 2005). Therefore, plant hygiene can play an important role in reducing *Colletotrichum* inoculum and it can be an important non-chemical measure in disease management.

Pathogenicity tests on fruit showed that latency is an important aspect of anthracnose symptoms development. The significance of latent infections by *C. gloeosporioides* was proven on citrus (Timmer et al., 1998), papaya (Dickman and Alvarez, 1983), peach (Zaitlin et al., 2000) or avocado (Binyamini and Schiffmann-Nadel, 1972). Results of artificial inoculations demonstrated that symptoms coincide with the beginning of fruit maturation (colour change), and this is in line with observations from the field. First signs of anthracnose symptoms are usually observed from the early September and advanced rapidly, appearing on numerous fruit. Croatian growers have often tried to supress disease development with late fungicide treatment after the appearance of symptoms, but this measure was not effective. Confirmation of *C. gloeosporioides* on citrus flowers in this and other studies (Agostini et al., 1992; Lima et al., 2011; Ramos et al., 2016) indicate that the first fungicide treatment might be targeted in bloom or early post-bloom period, followed by another treatment to protect the developing fruit.

REFERENCES

AGOSTINI, J.P, TIMMER, L.W., MITCHELL, D.J. (1992): Morphological and pathological characteristics of strains of *Colletotrichum gloeosporioides* from citrus. Phytopathology, 82(11): 1377-1382.

AIELLO, D., CARRIERI, R., GUARNACCIA, V., VITALE, A., LAHOZ, E., POLIZZI, G. (2014): Characterization and pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* causing preharvest disease on *Citrus sinensis* in Italy. Journal of Phytopathology, 163(3): 168-177.

AGUSTI, M., ZARAGOZA, S., BLEIHOLDER, H., BUHR, L., HACK, H., KLOSE, R., STAUSS, R. (1995): Escala BBCH para la descripción de los estadios fenológicos del desarrollo de los agrios (Gén. Citrus). Levante Agrícola: Revista internacional de cítricos, 332: 189-199.

BAKARIĆ, P. (1983). Uzgoj mandarine Unšiu. Stanica za južne kulture, Dubrovnik.

BINYAMINI, N., SCHIFFMANN-NADEL, M. (1972): Latent infection in avocado fruit due to *Colletotrichum gloeosporioides*. Phytopathology, 62(6): 592-594.

BROWN, G.E., ECKERT, J.W. (2000): Anthracnose. In: Compendium of Citrus Diseases (Timmer, L.W., Garnsey, S.M., Graham, J.H., eds.), APS Press, St. Paul, SAD, pp. 37-38.

CANNON, P.F., BUDDIE, A.G., BRIDGE, P.D. (2008): The typification of *Colletotrichum gloeosporioides*. Mycotaxon, 104: 189-204.

DAMM, U., CANNON, P.F., WOUDENBERG, J.H.C., CROUS, P.W. (2012): The *Colletotrichum acutatum* species complex. Studies in Mycology, 73: 37-113.

DICKMAN, M.B., ALVAREZ, A.M. (1983): Latent infections of papaya caused by *Colletotrichum gloeosporioides*. Plant Disease, 67(7): 748-750.

EPPO (2005): *Phoma tracheiphila*. Diagnostics PM 7/48 (1). Bulletin OEPP/EPPO Bulletin, 35: 307-311.

FAEDDA, R., AGOSTEO, G.E., SCHENA, L., MOSCA, S., FRISULLO, S., MAGNANO DI SAN LIO, G., CACCIOLA, S.O. (2011): *Colletotrichum clavatum* sp. nov. identified as the causal agent of olive anthracnose in Italy. Phytopathologia Mediterranea, 50(2): 283-302.

FELSENSTEIN, J. (1985): Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39(4): 783-791.

FREEMAN, S., KATAN, T., SHABI, E. (1998): Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruit. Plant Disease, 82(6): 596-605.

GUARNACCIA, V., GROENEWALD, J.Z., POLIZZI, G., CROUS, P.W. (2017): High species diversity in *Colletotrichum* associated with citrus diseases in Europe. Persoonia, 39: 32-50.

HUANG, F., CHEN, G.Q., HOU, X., FU, Y.S., CAI, L., HYDE, K.D., LI, H.Y. (2013): *Colletotrichum* species associated with cultivated citrus in China. Fungal Diversity, 61(1): 61-74.

HYDE, K.D., CAI, L., MCKENZIE, E.H.C., YANG, Y.L., ZHANG, J.Z., PRIHASTUTI, H. (2009): *Colletotrichum*: a catalogue of confusion. Fungal Diversity, 39: 1-17.

IVIĆ, D., POPOVIĆ, L., ARNAUT, P., DEAK, S., BJELIŠ, M. (2017): Etiologija i epidemiologija antraknoze agruma u Hrvatskoj. Sažeci 61. Seminara biljne zaštite, 7.-10. veljače 2017., Opatija. 21-22.

KUMAR, S., STECHER, G., TAMURA, K. (2016): MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. Molecular Biology and Evolution, 33(7): 1870-1874.

LARKIN, M.A., BLACKSHIELDS, G., BROWN, N.P., CHENNA, R., MCGETTIGAN, P.A., MCWILLIAM, H., VALENTIN, F., WALLACE, I.M., WILM, A., LOPEZ, R., THOMPSON, J.D., GIBSON, T.J., HIGGINS, D.G. (2007): Clustal W and Clustal X version 2.0. Bioinformatics, 23(21): 2947-2948.

LIMA, W.G., SPÓSITO, M.B., AMORIM, L., GONÇALVES, F.P., MELO DE FILHO, P.A. (2011): *Colletotrichum gloeosporioides*, a new causal agent of post-bloom fruit drop. European Journal of Plant Pathology, 131(1): 157-165.

MCGOVERN, R.J., SEIJO, T.E., HENDRICKS, K., ROBERTS, P.D. (2012): New report of *Colletotrichum gloeosporioides* causing postbloom fruit on citrus in Bermuda. Canadian Journal of Plant Pathology, 34(2): 187-194.

MIJUŠKOVIĆ, M. (1966): Prilog proučavanju *Colletotrichum gloeosporioides* Penz., uzročnika antraknoze agruma. Poljoprivreda i šumarstvo, 12: 1-32.

PERES, N.A., TIMMER, L.W., ADASKAVEG, J.E., CORRELL, J. C. (2005): Lifestyles of *Colletotrichum acutatum*. Plant Disease, 89(8): 784-796.

RAMOS, A.P., TALHINHAS, P., SREENIVASAPRASAD, S., OLIVEIRA, H. (2016): Characterization of *Colletotrichum gloeosporioides*, as the main causal agent of citrus anthracnose, and *C. karstii* as species preferentially associated with lemon twig dieback in Portugal. Phytoparasitica, 44(4): 549-561.

SNEATH, P.H.A., SOKAL, R.R. (1973): Numerical taxonomy - the principle and practice of numerical classification. W. H. Freeman and Company, San Francisco, SAD.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F., HIGGINS, D.G. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 25(24): 4876-4882.

TIMMER, L.W., BROWN, G.E., ZITKO, S.E. (1998): The role of *Colletotrichum* spp. in postharvest anthracnose of citrus and survival of *C. acutatum* on fruit. Plant Disease; 82(4): 415-418.

TIMMER, L.W. (2000): Anthracnose diseases. In: Compendium of Citrus Diseases (Timmer, L.W., Garnsey, S.M., Graham, J.H., eds.), APS Press, St. Paul, SAD, pp. 21-23.

WEIR, B.S., JOHNSTON, P.R., DAMM, U. (2012): The *Colletotrichum gloeosporioides* species complex. Studies in Mycology; 73: 115-180.

WHITE, T.J., BRUNS, T., LEE, S., TAYLOR, J.W. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: A guide to methods and applications (Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., eds.). Academic Press Inc, New York, SAD, pp. 315-322.

ZAITLIN, B., ZEHR, E.I., DEAN, R.A. (2000): Latent infection of peach caused by *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*. Canadian Journal of Plant Pathology, 22(3): 224-228.

Authors address - Adresa autora:
Dario Ivić, e-mail: dario.ivic@hapih.hr
Luka Popović,
Ivana Križanac,
Centre for Plant Protection, Croatian Agency for Agriculture and Food,
Gorice 68b, 10000 Zagreb, Croatia

Mario Bjeliš
University of Split,
Poljička cesta 35, 21210 Split, Croatia