SZENT ISTVÁN EGYETEM

SOUR CHERRY ANTHRACNOSE: characterization of the pathogen, genetic diversity and the plant protection technology elaboration

Thesis of PhD dissertation

ANNAMÁRIA TÓTH

GÖDÖLLŐ

2017.

PhD School

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1. INTRODUCTION

Hungary is one of the most important sour cherry producers of Europe, with a unique assortment of varieties. 14 thousand hectares of plantations yield a total of 40-70 thousand tons a year (KSH, 2015). To be able to provide high quality fruits in an adequate amount, certain measures of plant protection against pests and physiological effects are necessary. Producers nowadays are facing a pathogen, which has not been considered a significant problem in the past few decades. The pathogen of sour cherry anthracnose was firstly described by János Lehoczky in 1957 in Hungary as *Gloeosporium fructigenum* Berk. The fungus attacks the fruits, causing mat, enlarging brownish spots. In high humidity sticky orange conidial mass develops on the infected tissues. The infected fruits are not suited for marketing. In recent years the fungus did not cause severe infection, but since 2006 epidemics have been occurred in the orchards of some areas in Hungary. The pathogen caused unexpected losses in fruit production, as the conventional plant protection methods did not include treatments against sour cherry anthracnose; furthermore, not many fungicides were available against this pathogen. Many researchers have tried to find the cause of the sudden reappearance of sour cherry anthracnose. One of the reasons may be the change in variety assortment. Since there was no reason to take anthracnose resistance into account during selection in the breeding process, the new varieties might not be resistant. Another reason for the epidemic could be the development of a new, more aggressive pathogen type and the combination of optimal climatic conditions and the accumulation of inoculums.

The genus *Colletotrichum* contains large number of plant pathogens causing significant damages in cultivation. Simmonds separated *Colletotrichum acutatum* J. H. from *C. gloeosporioides* (Penz.) Penz. & Sacc. in 1965. According to recent studies, the pathogen is more likely to be a species complex, which includes a number of different, phylogenetically closely related *Colletotrichum* species. Since the members of the species complex have very similar morphology, it is crucial to conduct a nucleic acid based assessment, by using multiple genes or regions (so-called multilocus assessment).

Experiments with sour cherry anthracnose are relevant for multiple reasons. From the producer point of view it is essential to provide effective fungicides and plant protection recommendations. To achieve this, the biology of the pathogen, the types of host plants and the susceptibility of the varieties should be described. It is also important to see the diversity, the morphological and molecular variability of the pathogen, and its situation within the species complex.

The aims of this study were the following:

- Collecting infected fruits of different host plants from multiple locations;
- Isolating and describing pathogens based on morphological and other features;
- Identifying and describing pathogens based on nucleic sequence (ITS region, histone 3 gene, calmodulin gene);
- Comparison of pathogens described in 1956 with those causing the sour cherry anthracnose epidemics of the past years;
- Studying the biology of the pathogen (overwintering, spreading during the vegetation period);
- Testing the susceptibility of cultivated sour cherry varieties under *in vitro* conditions;
- Examining the effect of plant protection products and foliar fertilizers on mycelial growth and on conidial germination under *in vitro* conditions;
- Testing and evaluating plant protection practices on the field in small and big scale production.

2. MATERIALS AND METHODS

Collection and storage of plant parts

Infected fruits were collected from different parts of the country. They were then placed into paper bags and labeled accordingly, then stored refrigerated on 4 °C. The evaluation and isolation of the samples were done within 4 days.

Asymptomatic sour cherry leaves were collected from three locations. Leaf samples were taken three times on a former plantation in Lajosmizse, both from the inside and from the outside of the tree crowns. Samples were taken once in Sóskút and in Soponya as well. The leaves were placed into paper bags and kept on 4 °C for maximum one day, when they were processed.

Testing the presence of the pathogen on the leaves

The test was conducted following the method of Børve *et al.* (2010). 10 piece groups of asymptomatic leaves were made. The surfaces of the leaves were disinfected by soaking them in 0.5 % hypochlorite solution for 30 seconds, then in 70 % ethanol for 2 minutes. Following disinfestation, the leaves were washed in sterile water for 2 minutes, and then dried in the laminar flow box. Finally the leaf samples were placed into sterile plastic boxes, and then put in the freezer on -18 °C. The theory is that the fungus sporulation on the leaves is induced by freezing (Børve *et*

al., 2010). To determine the optimal freezing period, different treatments (1, 2, 3, 4 and 5 hours) were tested.

Following freezing, the leaves were incubated in a phytotron on 26 °C, under artificial light. The presence of the pathogen on the leaf surfaces was monitored daily for 2 weeks.

Isolation of the pathogens and maintaining on media

In a laminar flow box, conidia were taken from infected fruits and leaves that underwent artificially induced sporulation. The conidia were then placed on PDA media and incubated on 24 °C, in the dark. After a week 7 mm diam. plugs were taken from the edge of the culture and placed again on sterile PDA. After the cultures have completely colonized the Petri dishes, they were placed on 4 °C for storing. The isolates were transferred every 8-12 weeks for maintenance for the duration of the study. The cultures were used for pathogen description, molecular identification, pathogenicity tests and for *in vitro* fungicide tests.

Morphological features and evaluation

Acervuli and conidia were found on the infected fruits. Conidia were removed from the infected parts. Diameter and length of 100 conidia were then measured by cytoplasm microscope.

Colonies were cultured in complete darkness, on 24 °C during the study. The diameters of the cultures were measured (two measurements, respectively) every fourth day. Based on the data a growth rate was determined (mm/24 h). The color, pattern, shape and the edge of the cultures were also characterized.

Molecular biology methods

The DNA was extracted by CTAB (cetyltrimethyl ammonium bromide) method (Maniatis *et al.*, 1989) followed by chloroform and isoamyl alcohol (24:1) extraction (Gell *et al.* 2007).

ITS5 and NL4 (White *et al.*, 1990; O'Donnell, 1993) primers were used for amplification of ITS region. For the histone 3 gene C.A.Histone3.for and C.A.Histone3.rev primers (Crous *et al.*, 2004), and for the calmodulin gene CA_CAL1 - CA_CAL2 primers were applied (O'Donnell *et al.*, 2000).

The products of the PRC were checked in 1% agarose gel and purified with PCR High Purification Kit (Roche, Germany) according to the manufacturer's protocol. In some cases the purified PCR product was ligated into a pGEM-T Easy vector. *Escherichia coli* DH 5-α and JM 109 strains were used for transformation. After performing the mini prep and checking that the insert is present, the products were purified with Quantum Prep Plasmid Miniprep Kit (BIO-RAD) by following the manufacturer's protocol. 10 µl purified recombinant plasmid were sent to the

BAY-GEN Növénygenomikai, Humán Biotechnológiai és Bioenergiai Intézet (Szeged, Hungary) for sequencing. The NCBI database and its BLAST program was used for sequence identification. Phylogenetical analysis was done by using the BEAST v2.3.2. (Bayesian Evolutionary Analysis Sampling Trees) program (Drummond *et al.*, 2012).

In vitro fungicides and foliar fertilizers assay

Fungicides and foliar fertilizers were tested in *in vitro* experiments in practical dose and in 10x dilution. Mycelial discs of the pathogen were used. The agents were mixed into sterile, cooled PDA media, then poured into Petri dishes. The pathogen disks were placed in the center of the dishes after solidifying. In case of each treatment an untreated control was prepared as well. The test was done in 4 repeats. The Petri dishes were incubated on 24 ºC. The cultures were evaluated when the control samples have colonized the entire surface of the media.

A conidial suspension was prepared from the orange colored conidial mass by suspension in sterile distilled water. The 6 x 10^2 pc/ml concentration was set by Bürker-chamber. 500 µl of suspension was spread on the center of the poisoned agar plate. In case of untreated control plates, the conidia were spread on PDA media without any agent added. The test was done in 4 repeats. The Petri dishes were incubated on 24 ºC, in darkness. The evaluation was done by counting the colonies after 48-72 hours, when the conidia started to germinate on the control plates.

3. RESULTS AND DISCUSSION

Symptoms and host plants

The causal agent of anthracnose disease was isolated 55 times following sample collection. The majority of the isolated were collected from sour cherry (35 from fruits, 7 from leaves), but other host plants (apple, blueberry, banana, cherry, fig, cornelian cherry, tomato, strawberry, grape) were also included in the study.

The fungus cause mat, enlarging brownish spots on the epidermis of the sour cherry and cherry fruits. The spots reach 1-2 cm diameter within a couple of days. The symptoms are the same as described by Lehoczky (1957). The symptoms observed on strawberry correspond with those found on cherry and sour cherry, as described by Leandro *et al.* (2001). Arzanlou and Torbati (2013) found the disease on ripening cornelian cherry fruits (mostly uneven, mat, brown spots, closer to the stem), just like we have observed. The pathogen caused slightly watery, sunken, brown spots on blueberry, although according to Talgø *et al.* (2007) anthracnose spots are dry. Brown, sunken, dry spots appeared on fig. El-Gholl and Alfieri (1994), and Choi *et al.* (2013)

found brown, uneven lesions on fig fruits. The pathogen not only causes symptoms in the orchards, but significant damage can develop during storage as well. The symptoms observed on stored apple correlates with those described by Mari *et al.* (2012).

Molecular identification

Based on the sequences of the ITS region, the histone 3 gene and the calmodulin gene, our isolates (with the exception of two) belong to the *Colletotrichum acutatum* species complex. The Hungarian isolates (except the isolates A37 (tomato) and the A39 (banana)) belong to three species of the species complex. Isolates collected from sour cherry, cherry, strawberry, cornelian cherry (from Bársonyos, Hungary), apple and grape belong to *C. godetiae*. Isolates collected from blueberry, fig and cornelian cherry (from Kecskemét, Hungary) showed closest relations with *C. fioriniae*. These isolates can be differentiated easily, as they produce purple coloration on the media. Three isolates from sour cherry and three from strawberry showed similarity to *C. nymphaeae*. Based on the analysis of the ITS region, isolate A0 (from 1956) showed relations with *C. nymphaeae* and *C. chrysanthemi*. In the meantime histone 3 and calmodulin gene sequences suggest closest relations with *C. godetiae*, which is the case of the other sour cherry isolates.

Biology of the sour cherry anthracnose pathogen

The study has proven that the pathogen overwinters on the bud scales as well as on mummified fruits and fruit stems. Firstly Børve and Stensvand (2006) and Burak and Eris (2008) have detected the overwintering of the fungus on the bud scales of cherry.

The pathogen of sour cherry anthracnose was detected on asymptomatic leaves collected from the Lajosmizse and Soponya sour cherry plantations. The distinctive orange colored conidial mass appeared on both sides of the leaf blades following 9-12 (average 10) days of incubation. Other research groups have also confirmed latent leaf infection in case of lemon (Zulfiqar *et al.*, 1996), strawberry (Leandro *et al.*, 2001; Mertely and Legard, 2004), blackberry (Yoshida and Shirata, 1999), apple (Crusius *et al.*, 2002), and cherry (Børve *et al.*, 2010).

Fungicides and foliar fertilizers test

The poisoned agar plate method proved to be effective in case of testing fungicides and foliar fertilizers *in vitro*. Based on the effect of these products on mycelial growth and conidial germination, triazole chemicals gave the bests *in vitro* results against the pathogen of sour cherry

anthracnose. In their respective studies Freemann *et al.* (1997) found propiconazole and difenoconazole; Adaskaveg and Förster (2000) and Schilder (2002) found fenbuconazole; while Paredes and Muñoz (2002) found propiconazole and hexaconazole to be effective from this group of active agents. In the laboratory experiments of Freemann *et al.* (1997) prochloraz proved to be most effective against *C. acutatum sensu lato*, which has also been confirmed by our experiment: the product Mirage 45EC with prochloraz has inhibited both mycelial growth and conidial germination effectively. Adaskaveg and Förster (2000) and Schilder (2002) achieved best results with active agents fosetyl-Al (Aliette), captan (Captan), benomyl (Benlate), chlortalonil (Bravo), ziram (Zirám), fenbuconazole (Indar 75WP), myclobutanil (Rally 40WP), thiophanate-methyl (Topsin 75WP), azoxystrobin (Abound) and piraclostrobin (Cabrio). We have also tested the active agents captan, chlortalonil, myclobutanil, thiophanate-methyl and azoxystrobin it the laboratory, but not all of them proved to be an effective fungicide in case of all isolates. According to Schilder *et al.* (2001) strobilurins can be used against fruit infesting *Colletotrichum* species. Our studies have not confirmed this statement, as in case of both azoxystrobin and trifloxystrobin containing agar plates, most isolates started growing. According to Glits (2000) mancozeb can also be used against the pathogen. Our results support this finding as well. The data shows that combinations of active agents, such as trifloxystrobin+tebuconazole and fluopyram+tebuconazole are also effective. From the group of copper containing active agents, tribasic copper sulfate and copper oxychloride can be used effectively. These fungicides are available abroad as well (Waller, 1992). The efficiency of contact products decrease significantly when 10 times diluted, thus it is crucial to apply the recommended dose to achieve total protection.

The foliar fertilizer Sergomil applied in a practical dose inhibited mycelial growth by 89%. This product can be used even in case of rainy weather before harvest, as it does not have any withholding period.

4. NEW RESULTS

1. We were the first to find members of the *Colletotrichum acutatum* species complex, *C. fioriniae* on blueberry and on fig, and *C. godetiae* on grape fruits.
2. We have identified cornelian cherry as a new host of *C. acutatum sensu lato* in Europe. We have also classified its place within the species complex (*C. godetiae*).
3. In our research the place of *C. acutatum* (that causes anthracnose in case of many fruit species) within the species complex have been identified for the first time in Hungary: sour cherry, cherry, apple, cornelian cherry, grape - *C. godetiae*; sour cherry, strawberry - *C.*

nymphaeae; fig, blueberry, cornelian cherry - *C. fioriniae*. By molecular methods we have also identified the pathogen that caused the sour cherry anthracnose epidemic in the 1950's.

4. As a result of this study the sequence data of the calmodulin gene from a member of the *C. acutatum* species complex have been published for the first time in the World. Sequence data of the histone 3 gene from a member of the *C. acutatum* species complex have been published for the first time in Hungary.
5. We were the first to prove that the pathogen overwinters on bud scales of sour cherry hosts. We have proven the latent presence of the pathogen during the vegetation period, in the tissue of sour cherry leaves for the first time as well.

Although it is not a scientific result, but still is important from the plant protection practice point of view, that we have developed an effective plant protection technology that can be used against the pathogen in Hungary.

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List of non-related publications

Article published in journals with impact factor

Végh A,, **Tóth A.**, Zámbó Á., Borsos G. és Palkovics L. (2014): First Report of Bacterial Bark Canker of Walnut Caused by *Brenneria nigrifluens* in Hungary. Plant Disease 98 (7), 988. IF: 3,02

Article published in scientific journals

Végh A,, **Tóth A.**, Zámbó Á., Borsos G. és Palkovics L. (2013): A dió (*Juglans regia* L.) kéregrepedése, feketefolyása: új baktériumos betegség Magyarországon. Növényvédelem 49 (9), 397- 401.