e-ISSN 2795-4362
p-ISSN 2795-4340

10.5281/zenodo.12665885 Vol. 07 Issue 05 May - 2024 Manuscript ID: #01453

FIRST REPORT: MOLECULAR IDENTIFICATION OF THE CAUSES OF ANTHRACNOSE DISEASE IN RAMBUTAN FRUITS IN BALI

I Made Sudarma* and Ni Nengah Darmiati*
Lecturer staff at the Agroecotechnology Study Program, Faculty of Agriculture, Udayana University, Jl. PB. Sudirman Denasar-Bali.

Corresponding email: madesudarma@unud.ac.id

Abstract

Anthracnose disease on post-harvest rambutan fruit is often found in traditional markets as well as freshly picked and post-harvest fruit. The disease really interferes with the appearance of the fruit, the skin color of the fruit is black and is not good for use as offerings or consumption. Molecular search results found that a 650 bp DNA fragment was successfully amplified from 5 fungal samples using universal primers ITS1/ITS4. The DNA samples resulting from the amplification are then used for the sequencing stage to determine the fungal species. Sequencing analysis confirmed that the identity of the fungus in the sample was *Colletotrichum fructicola* with 68-100% homology to several *C. fructicola* isolates and out groups in the genebank. Further phylogenetic analysis showed that *C. fructicola* isolates formed three groups. The *C. fructicola*_Bali isolate formed a group with isolates from Taiwan. The second group consists of isolates from China, while the third group consists of isolates from Japan. Meanwhile, outside the cluster there are 3 species of Colletotrichum including C. siamense, *C. musae*, and *C. gloeosporioides*. As an outgroup isolate, the *Lasiodiplodiatheobromae* isolate originating from Indonesia was used.

Keywords

Anthracnose, rambutan, DNA, *Colletotrichum fructicola*, and phelogenetics

INTRODUCTION

Colletotrichum fructicola Prihast., a well-defined polyphagous fungus of the *C. gloeosporioides* complex that has been reported from five continents to cause anthracnose, bitter rot, and leaf spot on more than 90 cultivated plants and non-cultivated woody or herbaceous plant species [1].

Fruit rot (anthracnose) in rambutan fruit is a disease that is quite serious in affecting rambutan fruit, both those that have just been harvested and those that are in storage (post-harvest). During the early stages, symptoms develop similar to brown spot disease. Furthermore, the infected area becomes extensively overgrown with air mycelia which cannot be observed at later stages of infection. The spots appear as black, circular lesions that increase in size and become large, sedimentary spots, with masses of orange to pink colored conidia observed in infected pericarp under moist conditions after 5-6 days of age [2].

Rambutan fruit rot disease was first reported in 2017 attacking rambutan fruit in Thailand and Sri Lanka. There are two reported pathogens, namely *C. fructicola* and *C. queenslandicum*[3]. In tropical fruit plants, anthracnose is mainly caused by species belonging to the fungal genus, *Colletotrichum*. This phytopathogen can infect several parts of fruit plants; however, infection during postharvest or ripening stages is responsible for major economic losses. As a result of the formation of black to dark brown concave lesions on the surface of the fruit, anthracnose reduces fruit quality and marketability. Among the tropical fruit crops most commonly susceptible to anthracnose are mango, papaya, banana, avocado, guava, and dragon fruit; This is an economically relevant product in many developing countries. It is important to document the newly recorded *Colletotrichum* spp. Fruit-associated anthracnose can infect many hosts, but some species can be host-specific. Through the use of multiple markers, many phylogenetic species of Colletotrichum have been found to be reported as pathogens that cause anthracnose. Considering that disease management strategies rely heavily on adequate knowledge of the causative agents, up-to-date information on *Colletotrichum* species and the dangers posed by the most recently identified species in tropical fruit plantations and harvested fruit becomes vital. In addition, the newly recorded species may be important for biosecurity and should be listed as quarantine pathogens, considering that tropical fruits are traded worldwide [4].

Colletotrichum fructicola also causes fruit rot and tip dieback on nes plants in Thailand [5], causing anthracnose disease on Hylocereus plants in the Philippines [6]. Anthracnose is a disease caused by the *Colletotrichum* species complex, an intracellular filamentous Ascomycota characterized by the formation of sunken spots and cankers on stems, twigs, branches and fruit [7]. This pathogen attacks many hosts throughout the world such as avocado (*Persea americana*), annona (*Annona cherimola*), coffee (*Coffea arabica*), apple (*Malus domestica*), peach (*Prunus persica*), mango (*Mangifera indica*), and, most recently, tea oil (*Camellia oleifera*), among others [8-12].

MATERIALS AND METHODS

Place and time of research

The research was carried out in two places: 1) looking for sick and healthy panicle specimens from farmer's plantations in Paku Dui Village, Tegalalang, Gianyar. 2) Plant Disease Laboratory and Agricultural Biotechnology Laboratory. The research was carried out from April to August 2023.

Macroscopic Identification

Symptoms of the disease were observed to determine macroscopically, then continued with isolating the pathogen by growing it in a Petri dish which was first given PDA media, after growing for 5 days then inoculating, and then viewing the pathogen under a microscope. The Optilab tool is used to help see under a microscope with magnifications of 100 x and 400 x.

Molecular Identification

DNA extraction followed the procedure from [19]. A 0.2 g sample of pathogenic fungal mycelium was ground with liquid nitrogen and the pathogenic fungal powder was put into an Eppendorf tube. Next, 500 µL of CTAB buffer and 50 µL of β-mercaptoethanol were added, then mixed until homogeneous with a vortex. To lyse the cell walls, heating is carried out at a temperature of 70ºC for 60 minutes, where every 10 minutes it is turned back and forth to speed up the lysis process. Then cooled until it reaches room temperature. Next, 500 µL of isoamylalcohol chloroform (24:1) was added to the tube, then mixed until homogeneous using a vortex and centrifuged at 12,000 rpm for 15 minutes. The supernatant obtained was transferred to a new Eppendorf tube by adding 500 µL sodium acetate, mixed until homogeneous by vortexing and centrifuged again at 12,000 rpm for 10 minutes. The supernatant was transferred to an Eppendorf tube then 500 µL of sodium acetate and isopropanol were added each, mixed until homogeneous by vortexing and centrifuged again at 12,000 rpm for 10 minutes. The tube was shaken gently to bind DNA and incubated at -20 ºC for 30 minutes. The DNA strands obtained were precipitated by centrifugation for 10 minutes. The supernatant was discarded, the pellet was washed with ethanol (70%) then centrifuged at 8,000 rpm for 5 minutes. The ethanol is discarded and the pellet is dried. The pellet was resuspended with 50 µL of TE buffer and stored at -20ºC for further use in the DNA amplification process.

DNA Amplification

DNA amplification was carried out on a Thermo Cycle PCR machine. Amplification was carried out using universal primers to detect the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA), namely forward primer ITS1 (5'- CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4 (5'- TCCTCCGCTTATTGATATGC-3') with a target yield size amplification was 490 bp (Doyle & Doyle, 1987). The DNA amplification reaction was carried out with a total volume of 25 µL consisting of 1 µL DNA, 2.5 µL buffer 10 x and Mg2+, 0.5 µL dNTP 10 mM, 1 µL each primer, 12.5 µL Taq DNA (10 units/ µL), and 9.5 µL H2O. Amplification conditions are divided into several stages, namely pre-denaturation 94 ºC for 3 minutes, followed by 30 cycles of amplification, each cycle consisting of DNA strand separation / denaturation 94 ºC for 1 minute, primer attachment / annealing 45 ºC for 1 minute, DNA synthesis 72 ºC for 2 minutes. Especially for the last cycle, the synthesis stage is added for 10 minutes, then the cycle will end with a temperature of 4 ºC.

DNA electrophoresis

The amplification products were analyzed using Blued electrophoresis with 1% agarose gel (0.5xTris-Borate EDTA/TBE). Electrophoresis was carried out at 100 volts for 28 minutes and then the agarose gel was incubated in a dye containing ethidium bromide (1%) for 15 minutes, then washed with H2O for 10 minutes. The electrophoresis results were visualized with an ultraviolet transilluminator. The DNA bands formed as a result of electrophoresis were documented with a digital camera.

DNA Sequence Analysis

The amplification product was sent to 1st Base (Malaysia) for nucleotide sequencing. The tracing results were then analyzed using the basic local alignment search tool (BLAST) program with an optimization program to obtain DNA base sequences that have homology to the DNA sequences found on the National Center for Biotechnology Information (NCBI) website. The nucleotide sequences obtained were then analyzed using ClustalW multiple alignment on the Bioedit sequence alignment editor version 7.0.5 software. Homology results approaching 100% similarity are categorized as the same species as the sample species.

RESULTS AND DISCUSSION

Disease Studies

Anthracnose disease on rambutan fruit shows black spots on the skin of the fruit which over time can enlarge, damaging the skin of the fruit and resulting in damage to the fruit seeds inside (Figure 1A), which is very different from healthy rambutan fruit (Figure 1B). After being isolated from the skin of a diseased fruit and growing it in a Petri dish, mycelium was seen growing around pieces of white fruit skin (Figure 1C). Next, when the pathogen was viewed under a microscope, the conidia were oval-shaped with a length of 8-10 µ and a width of 2-5 µm (Figure 1D).

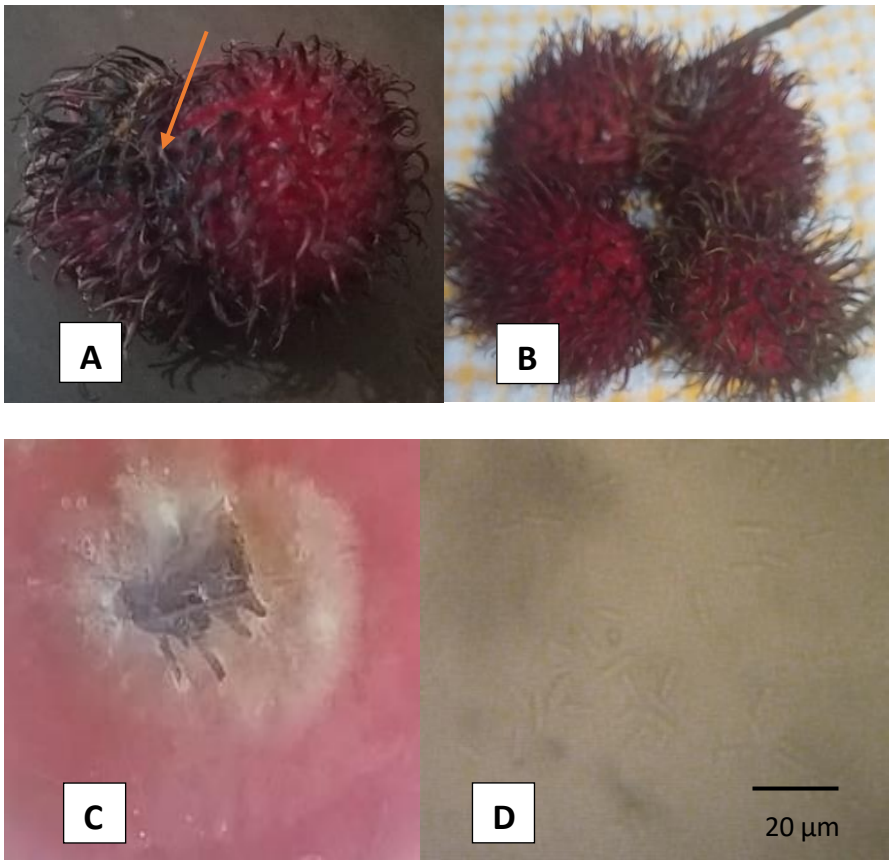

Figure 1. Study of anthracnose disease on rambutan fruit, (A) diseased fruit, (B) healthy fruit, (C) mycelium growing around the skin of the fruit and (D) pathogenic conidia (direction of anthracnose symptom arrows) (private collection)

Anthracnose, caused by *Colletotrichum gloeosporioides*, is a serious disease of rambutan that attacks the leaves, flowers, and fruit harvested in areas with high rainfall including the Philippines, Sri

Lanka, and Thailand. Fruit infection occurs in the field but usually does not occur until the fruit is ripe. Four fungi consistently isolated from symptomatic fruit include Lasmenia, Pestalotiopsis, Phomopsis, and *Colletotrichum* spp. Over a 2-year sampling period, the disease incidence of more than 300 fruits sampled was 84.6% [13]. Pathogens attack rambutan fruit from the field and then develop after harvest. Meanwhile, the fruit rot contamination phase is caused by *C. gloeosporioides* [14].

Molecular Identification

DNA fragments measuring 650 bp were successfully amplified from 5 fungal samples using universal primers ITS1/ITS4 (Figure 2). The DNA samples resulting from the amplification are then used for the sequencing stage to determine the fungal species. Sequencing analysis confirmed that the identity of the fungus in the sample was *C.fructicola* with 68-100% homology to several *C.fructicola* isolates and out groups in the genebank (Table 1).

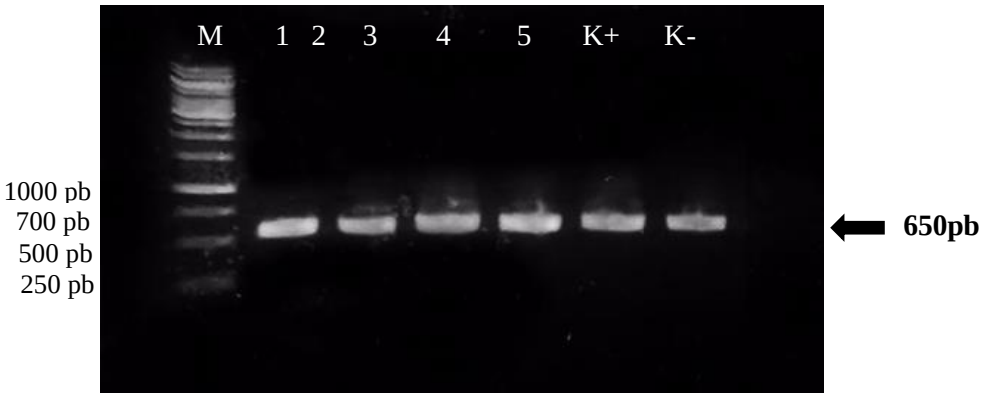

Figure 2. Visualization of *Colletotrichum fructicola* DNA amplified using universal primers ITS1/ITS4 on a 1% agarose gel. M: DNA marker (1kb ladder); Sample no. 1-5 (Bali Isolate); K+: Positive Control; K-: Negative control

Further phylogenetic analysis showed that C.fructicola isolates formed three groups. *C.fructicola*_Bali isolates formed a group with isolates from Taiwan. The second group consists of isolates from China, while the third group consists of isolates from Japan. Meanwhile, outside the cluster there are 3 species of Colletotrichum, including *C.siamense*, *C.musae*, and *C. gloeosporioides*. As an out group isolate, *Lasiodiplodiatheobromae* isolate originating from Indonesia was used (Figure 3).

Table 1. Homology (%) of nucleotide sequences of Colletotrichum fructicola isolates with several isolates that have been reported in GenBank

Seque-nce	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	G (%)	H (%)	I (%)	J (%)	Clustal Consensus (%)
A (%)	ID	100	100	100	100	100	99	99	99	68	0
B (%)	100	ID	100	100	100	100	99	99	99	68	0
C (%)	100	100	ID	100	100	100	99	99	99	68	0
D (%)	100	100	100	ID	100	100	99	99	99	68	0
E (%)	100	100	100	100	ID	100	99	99	99	68	0
F (%)	100	100	100	100	100	ID	99	99	99	68	0

G (%)	99	99	99	99	99	99	ID	99	99	68	0
H (%)	99	99	99	99	99	99	99	ID	99	69	0
I (%)	99	99	99	99	99	99	99	99	ID	68	0
J (%)	68	68	68	68	68	68	68	69	68	ID	0
Clustal Consensus	0	0	0	0	0	0	0	0	0	0	ID

Where: A = *Colletotrichum fructicola* Bali, B = KU642470 *C. fructicola*Jepang, C = MF540883 *C.fructicola* China, D = ON763369 *C. fructicola* China, E = OK145563 *C.fructicola* China, F = MK326868 *C. fructicola* Taiwan, G = ON763399 *C. siamense* China, H = AI301988 *C. gloeosporioides* Jerman, I = MT351114 *C. musae* China, J = LC490867 *Lasiodiplodiatheobromae* Indonesia

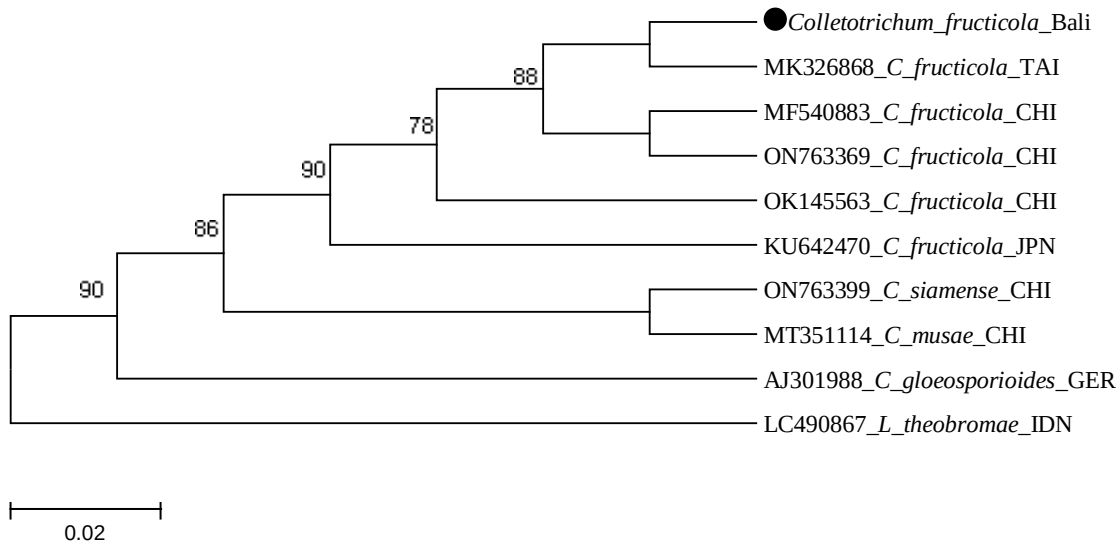

Figure 3. Phylogenetic analysis of Colletotrichum fructicola isolates based on partial nucleotide sequence alignment of the internal transcribed spacer 1, 5.8S ribosomal RNA gene using Mega 6.06 (Neighbor Joining Algorithm with 1,000 bootstraps replicates)

Colletotrichum is one of the most economically important fungal genera that causes anthracnose. This disease affects many host plants, especially tropical and subtropical plants, thereby reducing crop yields and the quality of plant products [15]. In rambutan production, fruit rot is the main disease before and after harvest. In the 2008–2013 fruit disease survey in Puerto Rico, during August and September, fruit rot was observed in eight orchards. Infected fruit were collected and 1 mm2 tissue sections were surface disinfected with 70% ethanol followed by 0.5% sodium hypochlorite, rinsed with sterile deionized distilled water, and transferred to acidified potato dextrose agar (APDA). *C. fructicola* (Cof) and *C. queenslandicum* (Coq) were identified morphologically using a taxonomic key [16].

Colletotrichum, a genus in the phylum Ascomycota (Fungi) and family Glomerellaceae is a globally important plant pathogen. In this paper, we detail four Colletotrichum species found in mangrove ecosystems. Two new species, Colletotrichum rhizophorae and C. thailandica, and a record new host *C. fructicola* were identified in Thailand [17]. *C.fructicola*Prihast., a complex polyphagous fungus *C. gloeosporioides* that has been reported from five continents to cause anthracnose, bitter rot, and leaf spot on more than 90 cultivated plants. and uncultivated woody or herbaceous plant species [18].

CONCLUSION

Sequencing analysis confirmed that the identity of the fungus in the sample was Colletotrichum *fructicola* with 68-100% homology to several *C. fructicola* isolates and out groups in the genebank. Further phylogenetic analysis showed that C. fructicola isolates formed three groups. The *C. fructicola*_Bali isolate formed a group with isolates from Taiwan. The second group consists of isolates from China, while the third group consists of isolates from Japan. Meanwhile, outside the cluster there are 3 species of Colletotrichum including *C. siamense*, *C. musae*, and *C. gloeosporioides*. As an out group isolate, the *Lasiodiplodiatheobromae* isolate originating from Indonesia was used.

Acknowledgements

Authors wish to thank to the Rector of Udayana University for their assistance and the opportunity given so that research can be resolved, Dean of the Faculty of Agriculture, Udayana University, and Chairman of the Institute for Research and Community Service Udayana University, for their help and cooperation so that research can be funded to completion.

REFERENCES

1. Bragard C. *et al*., 2021. Pest categorisation of Colletotrichum frukticola. Scientific Opinion. Ej, efsa Journal. doi: 10.2903/j.efsa.2021.6803.
2. Sivakumar D., R.S.W. Wijeratnam, R.L.C. Wijesundera and M. Abeysekera. 1997. Postharvest diseases of rambutan (Nephelium lappaceum) in the western province. J. Natn. Sci. Coun. Sri Lanka 25(4): 225-229.
3. Serrato-Diaz, L.M., L. I. Rivera-Vargas, R. Goenaga, E. D. Navarro, and R. D. French-Monar. 2017. First Report of Colletotrichum fructicola and C. queenslandicum Causing Fruit Rot of Rambutan (Nephelium lappaceum). Plant Disease 101(6).
4. Zakaria L. 2022. Fungal and oomycetes diseases of minor tropical fruit crops. Horticulture 8(323): 1-31.
5. Armand A., K. D. Hyde and R. S. Jayawardena. 2023. First Report of Colletotrichum fructicola Causing Fruit Rot and Leaf-Tip Dieback on Pineapple in Northern Thailand. Plants 12(971): 1-14.
6. Evallo E., Taguiam J.D., Bengoa J., Maghirang R., Balendres M.A. 2021. First report of *Colletotrichum fructicola*, causing anthracnose of Hylocereus plants, in the Philippines. – Czech Mycol. 73(1): 79–90.
7. Freeman S, Katan T, Shabi E.1998. Characterization of Colletotrichum species responsible for anthracnose diseases of various fruits. Plant Dis. 82:596–605.
8. Silva DN, Talhinhas P, Várzea V, *et al*..2012. Application of the Apn2/MAT locus to improve the systematics of the Colletotrichum gloeosporioides complex: an example from coffee (Coffea spp.) hosts. Mycologia.104:396–409.
9. Lima NB, Batista MV de A, De Morais MA Jr, *et al*..2013. Five Colletotrichum species are responsible for mango anthracnose in north-eastern Brazil. Fungal Divers.61:75–88.
10. Cristobal-Martínez AL, Yañez-Morales MJ, Solano-Vidal R, *et al*.. 2017. Diversity of Colletotrichum species in coffee (*Coffea arabica*) plantantions in Mexico. Eur J Plant Pathol.147:605–614.
11. Li H, Zhou G-Y, Liu J-A, *et al*..2016. Population genetic analyses of the fungal pathogen *Colletotrichum fructicola* on tea-oil trees in China. PLoS One.
12. Farr DF, Rossman AY. 2017. Fungal database: fungus–host distributions, U.S. National Fungus Collections, ARS, USDA [Internet].

13. Keith L., T. Matsumoto, K. Nishijima, M. Wall and M. Nagao. 2011. Field Survey and Fungicide Screening of Fungal Pathogens of Rambutan (Nephelium lappaceum) Fruit Rot in Hawaii. American Society for Horticultural Science 46(5): 730-735. Bellec, 2014
14. Phoulivong S. 2011. Colletotrichum, naming, control, resistance, biocontrol of weeds and current challenges. Current Research in Environmental & Applied Mycology p: 53-73.Serrato-Diaz *et al.*, 2017
15. Norphanphoun C. and K. D. Hyde. 2023. First Report of Colletotrichum fructicola, C. rhizophorae sp. nov. and C. thailandica sp. nov. on Mangrove in Thailand. Pathogen 12: 1436.
16. Serrato-Diaz, L.M., L. I. Rivera-Vargas, R. Goenaga, E. D. Navarro, and R. D. French-Monar. 2017. First Report of Colletotrichum fructicola and C. queenslandicum Causing Fruit Rot of Rambutan (Nephelium lappaceum). The American Phytophathological Society (APS) Publication.
17. Norphanphoun C. and K. D. Hyde. 2023. First Report of Colletotrichum fructicola, C. rhizophorae sp. nov. and C. thailandica sp. nov. on Mangrove in Thailand. Pathogen 12: 1436.
18. Bragard C., K. Dehnen-Schmutz, F. Di Serio, P. Gonthier, M.-A. Jacques, J. A. J. Miret, A.F., A. MacLeod, C.S. Magnusson, P. Milonas, J. A Navas-Cortes, S. Parnell, R. Potting, Hans-Hermann Thulke, W. Van der Werf, A. V. Civera, J. Yuen, L. Zappala, Q. Migheli, I. Vloutoglou, C. Campese, A.Maiorano, F. Streissl and P. L. Reignault. 2021. Pest categorisation of Colletotrichum fructicola. EFSA Journal 19(8): 6803.
19. Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin, 19: 11-15.