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METODE DE IDENTIFICARE TIMPURIE A CIUPERCILOR FITOPATOGENE CARE AFECTEAZĂ MERELE ÎN TIMPUL DEPOZITĂRII

EARLY DETECTION METHODS FOR APPLE FUNGAL PATHOGENS DURING POSTHARVEST PERIOD

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Abstract

Postharvest diseases caused by fungal pathogens are one of the major causes of economic losses of horticultural fresh products during the supply chain. Undetected fungal pathogens and mycotoxins contamination caused by some of these fungi are already a concern for food safety scientists. Apple fruits decay caused by fungal infections results in considerable losses during post-harvest storage. The detection of the decay and infection type in the early stage is necessary and helpful for reducing the losses; therefore, rapid and accurate methods are needed for early detection. The polymerase chain reaction and the real-time PCR (qPCR), using specific primers, have been used for the presence and dynamics of pathogen populations during the storage. Apples are suitable substrates for fungal colonization, in storage conditions, mostly caused by *Penicillium sp.*, *Botrytis sp., Gloeosporium sp., Neofabrea sp., Monilinia sp.* etc.LAMP assays, which can confirm the presence or absence of a specific pathogen in less than half an hour have been also developed for many apple pathogens, as *Monilinia laxa*, *M. fructicola*, *M. fructigena* and *Venturia inaequalis*.

Therefore, this review summarizes the use of early detection by molecular biology technologies, for high sensitivity and specificity, as real time PCR (qPCR) assay and loop-mediated isothermal amplification (LAMP) of DNA, in detecting and identifying apples fungal pathogens and mycotoxin producing food fungi.

Cuvinte cheie: identificare timpurie, mere, bolimicotice, micotoxine, qPCR
Keywords: early detection, apple, fungal diseases, mycotoxins, qPCR

1. Introduction

Apple is one of the few fruit species that allow long time storage without quantitative losses and qualitative depreciations, being offered on the market almost all year round for fresh consumption (Sumedrea et al., 2017). Regardless of their storage capacity (low variation of major physical, chemical or biological characteristics during storage), apple fruits become particularly susceptible to postharvest disease caused by fungal pathogens during storage, packing and transportation. Special attention should be given during harvest, handling and transportation to the storage facilities, to diminish the so called ”wound” pathogens (Wenneker and Köhl, 2014).Scientific data showed that the most important apples pathogens, as *Neofabraea* spp., *Botrytis* spp., *Pencillium* spp., *Fusarium* spp., *Alternaria* spp., or *Cladosporium* spp., are identified as causal agents of postharvest decay, also associated with the formation of mycotoxins or different volatile compounds as secondary metabolites (Kim et al., 2018).

To date over 90 fungal species have been identified as causal agents of postharvest decay of apples during storage (Li et al., 2011), some of them, as the blue mold, caused by *Penicillium expansum*, causing losses of up to 50% (Quaglia et al., 2011; Vilanova et al., 2014) and produces patul in, a mycotoxin which can have acute and chronic effects on human health (Etebarian et al., 2005; Quaglia et al., 2011, Rhiannon L. Wallace, et al., 2017). The food sector has integrated the use of real time PCR (qPCR) analysis, for the quantification of the fungal contamination level in a wide variety of food products and lately, the LAMP analysis, as new useful tools, for very fast confirmation of the presence/absence of a specific pathogen (Hayat et al., 2012; Konietzny and Greiner, 2003; Rodríguez et al., 2012; Sardinas et al., 2011; Selma et al., 2008; Vegi and Wolf-Hall, 2013).

Our work summarizes the use of real time PCR (qPCR) assay and loop-mediated isothermal amplification (LAMP) in detecting apples fungal pathogens and identifying the mycotoxins these fungi produce.

2. Material and methods

For the present paper the available online and offline bibliographical references were used, mostly thethe following international databases: Web of Science - Core Collection, Springer Link Journals (Springer), Scopus (Elsevier), Science Direct Freedom Collection (Elsevier), PROQUEST Central Oxford Journals, CAB Abstracts, Google Scholar, Agris FAO, simple Google research.

The present article integrates the review of recent literature with the preliminary research on apple postharvest fungal pathogens done at University of Agronomic Sciences and Veterinary Medicine of Bucharest (USAMVB), aiming at screening the apple storage diseases from our area. Decayed apple fruits were sampled from USAMV B orchard, kept in cold storage and controlled atmosphere chambers and the cold storage of a private producer in Arad County. Approximately 10 representative decaying fruits in different periods (once a month) were collected. As no results were made available yet, some unpublished pictures and data were used here, as they were considered necessary to demonstrate the actual situation in Romania.

3. Results and discussions

Postharvest diseases are mainly caused by fungal pathogens (Kim et al., 2018) and they produce important economic losses of horticultural fresh products during the supply chain. Undetected fungal pathogens and mycotoxins contamination caused by some of these fungi are already a concern for food safety scientists. Apples fruit decay caused by fungal infection results in considerable losses during post-harvest storage. Detection of the decay cause and infection type in the early stage is necessary and helpful for reducing the losses, therefore rapid and accurate methods are needed for early detection.

3.1. The main apple fungal pathogens

More than 90 fungal species have been presented in the Compendium of Apple and Pear Diseases, by the American Phytopathological Society in 1991 (Leibinger et al, 1997). Since then, many other new fungal pathogens were identified / renamed around the world, especially due to the new molecular identification techniques.

Apple fungal pathogens were categorized during time by different criteria. Some authors refer to two main disease groups: “latent infection” as *Neofabraea* spp. and “wound” pathogens, as *Botrytis* spp., *Penicillium* spp. or *Monilia* spp. (Wenneker and Köhl, 2014). Other researchers refer tothree types of pathogens that occur during storage: pathogens that infect and occur in vegetation and storage period like *Venturia inaequalis* and *Glomerella cingulata*; pathogens that infect in vegetation and occur only in storage, as *Neofabraea* spp. and pathogens that infect and occur only in storage as *Penicillium* spp. or *Fusarium* spp. (Florian et al, 2018). Another way of categories is by symptoms: some pathogens have obvious symptoms, as *Fusarium* spp., *Penicillium* spp, *Botrytis* spp., *Fusicladium* spp., *Alternaria* spp. while some fruits are rotting unobserved, with symptoms without fructifications, as *Phomopsis* mali or *Trichothecium roseum* (Florian et al, 2018).

The taxonomy of postharvest fungi is continuously changing and sometimes difficult to follow, also because of the dual nomenclature of pleomorphic fungi, which, although discontinued since 2011 (Hawksworth, 2011), still raise many problems. Giraud et Bompeix, (2012) remind about the lenticels spot diseases (“*Gloeosporium*” rots group), the most common European storage disease of apples, that it can be actually produced by *Neofabraea* (previously *Pezicula*) *alba*, *N. perennans*, *N. malicorticis*, and to a lesser extent, by *Cylindrocarpon mali* and *Colletotrichum acutatum*. Actually, the anamorphs of these species were formerly known as *Gloeosporium* spp., so different reports about this fungus may actually hide the presence of other species in different territories.

In Romania, the most throughout study on apple decay identifies not less than 16 fungal agents: *Venturia inaequalis, Penicillium* spp., *Glomerella cingulata, Giberella* spp., *Neofabrea* spp., *Monilinia fructigena, Neonectria ditissima, Botryosphaeria obtusa, Diaporthe eres, Alternaria* spp., *Botryotinia fuckeliana, Aspergillus niger, Phytophtora cactorum, Rhizopus stolonifer, Trichotecium roseum and Leucostoma* spp., which appear with a frequency between less than 5% and over 50%. (Florian et al. 2018). A country level screening of postharvest diseases of apples is definitely required, as many private farmers that hold storage facilities, regardless of their degree of technological advanced techniques, are yearly challenged by losses caused by different fungal pathogens. Some of them are forced to make monthly manual sorting of all apples in their storage, but this method is very costly and has a low impact on fungal storage diseases development (Figure 1).

3.2. The most important secondary metabolites – mycotoxins and volatile compounds

The presence of mycotoxins in the food chain is of high concern for human health due to their properties to induce severe toxicity effects at low dose levels. The contamination of fruits with mycotoxins has not only caused health hazards but also resulted in economic losses, especially for exporting countries. Patulin is a mycotoxin produced by a variety of molds, particularly *Aspergillus*, *Penicillium* and *Byssochlamys*. Most secondary metabolites are produced by organisms (fungi) that exhibit filamentous growth and have a relatively complex morphology. Fungi growth can occur either before harvest or after harvest, during storage, in the food itself often under warm, damp and humid conditions. Mycotoxins are toxic compounds that are naturally produced by certain types of fungi, chemically stable and survive food processing. Several hundred different mycotoxins have been identified, but the most commonly observed mycotoxins that present a concern to human health and livestock include aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and nivalenol / deoxynivalenol. (Fernández-Cruz, et. al., 2010).

Although the presence of patulin is reported, in various moldy fruits, grains and other foods, its occurrence especially in rotting apples and apple products, remains the major concern (Baert et al., 2012; Salomao et al., 2009). Patulin has been revealed as an appreciably stable compound and chronically toxic causing genotoxic, cytotoxic, mutagenic as well as immunotoxic health effects. The acute symptoms in animals include liver, spleen and kidney damage and toxicity to the immune system. For humans, nausea, gastrointestinal disturbances and vomiting have been reported (Puel et al., 2010). Major human dietary sources of patulin are apples and apple juice made from affected fruit. Due to its related hazards, the European Union has set maximum acceptable levels of 50, 25 and 10 mg patulin/kg, respectively for fruit juices, nectars and fermented apple beverages, solid apple products and apple-based products for infants and young children (European Commission Regulation No 1425/2003 and No 1881/2006, Tannous et. al, 2015).Therefore, it is important to prevent patulin presence in the food chain by ensuring a fast and specific method to early detect the potential producing fungi before reaching the toxin's unacceptable level or even prior its synthesis. These tolerable daily intakes are used by governments and international risk managers, such as the Codex Alimentarius Commission, to establish maximum levels for mycotoxins in food. The maximum levels for mycotoxins in food are very low due to their severe toxicity. For example, the maximum levels for aflatoxins set by the Codex in various nuts, grains, dried figs and milk are in the range of 0.5 to 15 µg/kg. The maximum limit for patulin in apple juice is 50 µg/L (Ioi et al., 2017).

3.3. The most frequently used early detection methods for apple fungal storage diseases and their secondary metabolites

Generally, for the early detection of apple fungal storage diseases two distinct approaches were considered, the optical sensors and the molecular analysis. In 2018, Pieczywek and his team published a research regarding the early detection of fungal infection of stored apple fruit using optical sensors. They compared biospeckle, hyperspectral imaging and chlorophyll fluorescence, reaching to the conclusion that biospeckle activity and hyperspectral imaging enable statistically significant detection of infected areas as early as 2 days after *Pezicula malicorticis* inoculation (Pieczywek et al., 2018).

The polymerase chain reaction by real-time PCR (qPCR) analysis ensures the fastest detection of fungal agents and fungal metabolites in comparison with the conventional, time consuming, microbiological analysis. Several publications described the practical use of qPCR in detecting mycotoxins in food samples (Luque et al., 2011; Manonmani et al., 2005; Noorbakhsh et al., 2009; Shapira et al., 1996; Spadaro et al., 2011a, Spadaro et al., 2011b).

Regarding patulin, some conventional PCR assays have been reported for the detection of patulin producing molds (Dombrink-Kurtzman, 2007; Paterson, 2004; Paterson et al., 2000).Recently, Tannous et al. (2015) reported a quantitative real-time PCR assay to quantify the DNA amounts of the main patulin producer *P. expansum* in apple samples and to correlate *P. expansum* DNA with patulin content in apples in order to have an approximately estimation of the patulin contamination level (Tannouset al., 2015).

In literature, the use of the qPCR technique was not only restricted to the quantification of the fungal contamination level in food stuffs but a direct application of this method was also described to estimate the content of the associated mycotoxin level. In this regard, a number of papers have demonstrated the correlation between the amount of toxin and fungal DNA content (Atoui et al., 2007, 2012; Fredlund et al., 2008; Mule et al., 2006; Schnerr et al., 2002).

Loop-mediated isothermal amplification (LAMP) is known as the best on-site diagnostic assay, because it is fast, highly specific to a target, and less sensitive to PCR inhibitors in samples (Kiddle et al., 2012). Development of portable apparatus, such as Genie III (OptiGene Ltd, West Sussex, UK), that can detect DNA amplification in LAMP in real time makes the LAMP assay more accessible for on-site diagnosis (Bühlmann et al., 2013).

4. Conclusion

Identify the storage disease might be a difficult task, but recent molecular techniques have been developed. In practice, these methods may save human lives, by detecting small amounts of mycotoxins in food commodities, but they can contribute to economic and sustainable growth of farmers or storage owners and managers by reducing the quantitative and qualitative losses.

Developing early detection methods, as real-time PCR, LAMP or other methods, for the most important pathogens in a given area, represent the key for a safer and better apple growing activity.

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Figures

Fig.1. Different postharvest storage diseases found during manual monthly sorting of apples, in private storage facilities in Arad county and in the research climatic chambers of the USAMV Bucharest.