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Microbial Antagonists: New Biocontrol Approach to Control Patulin-producing fungi in fruits during Postharvest storage

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Abstract

Patulin is a mycotoxin produced by Penicillium expansum, which mostly contaminates stored pome fruit and their-derived products. The excessive exposure to this mycotoxin through consumption of polluted foods poses a serious health hazard. Due to the ethical, technical and health issues in the use of conventional chemicals, various strategies are being explored to control postharvest fungal diseases, which present the use of biocontrol agents as promising alternative method. Several factors (such as pH, temperature and cultivar) have been cited to contribute to contribute to patulin accumulation; whiles other factors inducing or modulating their synthesis are not entirely understood. However, antagonistic biocontrol agents have been found capable of preventing postharvest fungal infections, resisting patulin and degrading it into lesser toxic compounds via different pathways. Apparently, the complex mechanisms of biocontrol are made more effective in antagonistic yeast through the addition of enhancing exogenous compounds. This review discusses the occurrence of patulin in fruit and derived products, possible factors influencing the initiation and accumulation of patulin in fruit, enhancement of biocontrol efficacy of antagonist yeasts and the mechanisms of action in patulin degradation.

Keywords: *Patulin, mycotoxins, antagonistic microbes, Penicillium expansum, biocontrol agents.*

INTRODUCTION

The prevalence of mycotoxins in agricultural raw materials has become an increasingly important issue worldwide. Growing demand for food in accordance with trends in global climate change accentuates further, the importance of discussing the role of mycotoxins in the changing environment. According to the Food and Agriculture Organization of the United Nations (FAO), approximately 25% of the global food and feed crops output are affected by mycotoxins including aflatoxins (AF), ochratoxins (OT), trichothecenes, zearalenone (ZEN), fumonisins (F), tremorgenic toxins, patulin and ergot alkaloids (Lawlor and Lynch, 2005). The annual losses worldwide in terms of human health, animal health, and condemned agricultural products caused by these mycotoxins amount to millions of dollars (Zain, 2011). Mycotoxins occur naturally and

the most relevant of them occur in food (Mahunu *et al*., 2015). In addition the same infected crop/food during production or harvest and storage can contain more than one mycotoxin at the same time (Speijers and Speijers, 2004). The intake of such different types of combined mycotoxins can lead to a potential higher risk for adverse health effects than the intake of one of these mycotoxins alone.

In general, mycotoxins comprise of highly diverse organic structures characterized by a variety of heteroatom-containing functional groups. Most significantly, mycotoxins consist of low molecular weight fungal metabolites that may be injurious if consumed over an extended period of time (Marroquín-Cardona *et al*., 2014). In the case of their simultaneous occurrence in the same substrate, OTA can intensify the mutagenicity of AFB1 (Sedmikova *et al.*, 2001). However, globally there are few reported studies on the incidence of two or more mycotoxins in host (Ozbey and Kabak, 2012).

Fungi, the main cause of some mycotoxins play a considerable role in the damage of fruit and vegetable, due to their pathogenicity to the products after harvest. At various stages in the food chain the different mycotoxins generated by some fungi are toxic to humans and animals that consume them (Beretta *et al.*, 2000). The core mycotoxigenic fungi that attack fruit and vegetable particularly harvested include *Aspergillus, Penicillium, Alternaria, Fusarium, and Claviceps* species (Marin *et al*., 2013). The specific mycotoxins produced by these fungal genera during pathogenesis, can survive the effects of several food processing methods. It is obvious that consumers will reject a visibly rotten fruit, but the processed fruit products may still constitute a significant source of these mycotoxins, because some of them are highly stable during processing (Mahunu *et al.*, 2015, Paster and Barkai-Golan, 2008). For instance, some of the compounds are known to tolerate very high melting point (González-Osnaya *et al*., 2007).

The control of mycotoxins has depended heavily on the use of conventional chemical. Approximately, 26% of the plant protection market consist of these chemical products in Europe and Asia and 6% in the US (Jutsum *et al.*, 1988). Estimates of 23 million kg of fungicides are applied annually to reduce postharvest diseases of fruit and vegetables on the global scale (Ragsdale and Sisler, 1994, Karabulut *et al*., 2005a). Yet, the significant contributions of these chemicals do not negate the need for stringent measures to reduce their impact on food safety and security. Over the years the use of conventional chemicals against mycotoxins in foods and feeds has been the practice. To overcome their limitations, various countries remain focused on the use of pragmatic control strategies. Also, notwithstanding the direct health risk from mycotoxicoses, several developing countries have recognized the significance of reducing mycotoxins levels in foods to reduce financial burden on health care and also take advantage of international trade exports to the attractive European markets (Zain, 2011). Therefore, special attention has been given to biological methods for their ability to suppress the growth of mycotoxigenic molds, as well as directly decontaminate and detoxify fruit. It has also been reported that interactions of mycotoxins (combined toxicity) are very difficult to predict because of the influence of several factors. While, the diversity of these factors affecting the production or presence of mycotoxins in foods or feeds makes the assessment of mycotoxins difficult (Šegvić Klarić, 2012). Therefore, the best approach is to establish how individual mycotoxins act on their hosts, which is the focus of recent investigations.

This review discusses the occurrence of patulin in fruit and derived products, possible factors influencing the initiation and accumulation of patulin in fruit, enhancing

biocontrol efficacy of antagonist yeasts using exogenous compounds and the mechanisms of action in patulin degrading.

Occurrence of patulin in fruit and derived products

PAT is a secondary metabolite with a molar mass of 154.12 g mol^{-1}, with a molecular formula of 4-hydroxy-4Hfuro [3, 2-c] pyran-2(6H)-one (Fig. 1). As early as 1943, Birkinshaw carried out the first isolation of PAT from *P. expansum* in search for new fungal molecules featuring antibiotic properties after Fleming discovered penicillin. Consequently, the attention in PAT as a potential antibiotic dwindled when it was discovered that PAT was toxic to humans and animals. PAT is produced by several species belonging to *Penicillium*, *Aspergillus*, *Paecilomyces* and *Byssochlamys* (Piqué *et al.*, 2013). Only 3 *Aspergillus* species of the Clavati group (*A. clavatus*, *A. giganteus*, and *A. longivesica*) are known to produce PAT. With regards to *Penicillium*, 13 PAT-producing species; *P. carneum*, *P. clavigerum*, *P. concentricum*, *P. coprobium*, *P. dipodomyicola*, *P. expansum*, *P. gladioli*, *P. glandicola*, *P. griseofulvum*, *P. marinum*, *P. paneum*, *P. sclerotigenum*, and *P. vulpinum* were identified (Andersen *et al*., 2004). *P. expansum*is responsible for the decay in stored pome fruit, particularly apple fruit; characterized by rapid soft rot and finally form blue mold (Fig. 2). *P. expansum* is the main source of PAT in susceptible fruit and their derived products (González-Osnaya *et al*., 2007).

In recent years PAT is seen as an important chemical contaminant among the list of important mycotoxin(Puel *et al.*, 2010) base on its toxicity and extent of spread (Spring and Fegan, 2005). An assessment of global occurrence of mycotoxins has shown that the threat of PAT contamination to the apple processing industry is rising. Sample of apples and apple products from some countries have revealed various levels of PAT accumulations in table 1 (Zhu *et al.*, 2015).

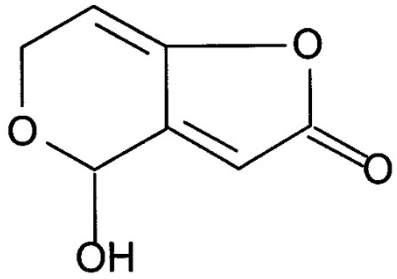

Fig. 1 Structure of patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one)

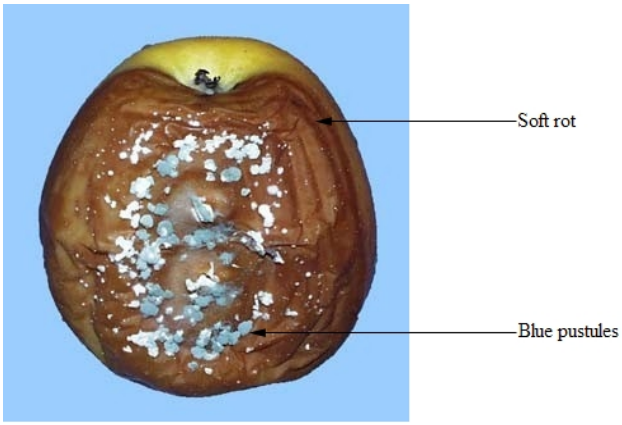

Fig. 2 Blue mold on apple fruit (Janisiewicz, 1999)

Table 1. Samples of apple and their derivative products containing PAT from some countries

Country	Proportions of PAT contaminated Products	References
Portugal	69% of rotted apples	Martins *et al*. (2002)
	23% of apple-based products	Barreira *et al.* (2010)
Belgium	12% of 177 apple juice samples	Baert *et al*. (2006)
India	24% of apple juice samples; 16% of samples contain more than 100 µg/l patulin	
	Note: higher than the maximum amount of patulin permitted in fruit juice (50 µg/l) in many regions, including the EU and USA	Saxena *et al*. (2008)
China	16% of 95 samples of apple products in Northeast China exceeded the maximum permitted patulin limit, while just 12.6% of the samples were below the detectable level for patulin (1.2 µg/kg).	Yuan *et al*. (2010)

Note: content of table adopted with modification (Zhu *et al*., 2015)

Different organizations and countries have established their maximum tolerable daily intake for PAT, in order to reduce the risk of contaminated fruit and fruit products. The following are some major maximum levels that have been set: 50 mg/L for fruit juice and derived products, 25 mg/L (solid apple products) and 10 mg/L for juices and foods as raw materials for babies and young infants. The US Food and Drug Administration (FDA) to adopt similar PAT level (50 mg/L) in fruit and their products (Van Egmond *et al*., 2008). The Gulf Cooperation Council (GCC) in China also adopts similar regulations in apple fruit and their products (FAO and FOODS, 2004). Table 2 shows patulin limits in products adopted by different countries. The World Health Organization's Global Environment Monitoring System/Food Contamination Monitoring and Assessment Program (GEMS/Food) also reported 1.04 ng kg^{-1} body weight (bw)/day to be the highest PAT intake based on apple-juice consumption (Organization, 2009). The PAT intakes among adults, children and babies who consume apple juice were estimated to be 28.1, 67.5 and 110 ng kg^{-1}bw/day, respectively (Van Egmond *et al*., 2008). PAT is known as a food pollutant that can cause oxidative damage to cells (Speijers and Speijers, 2004). They also indicated that PAT to react with sulfhydryl groups is emerging as a key explanation for the cytotoxic and certain genotoxic effects. The interaction of PAT with hormone-production systems negatively alters the immune system (Marin *et al*., 2013). In view of the potential health impact, PAT contamination is considered a corporate global issue (Morales *et al*., 2010).

Table 2. Patulin limits in products (FAO and FOODS, 2004).

Country	Products	PAT μg kg^{-1}
China	Fruit products containing apple or hawthorn (excluding Guo Dan Pi, a Chinese-style fruit snack)	50
	Fruit or vegetable juice containing apple or hawthorn juice	50
	Alcoholic beverages containing apple or hawthorn	50
Codex, GCC, Kenya, Nigeria	Apple juice	50
South Africa	Apple juice, apple-juice ingredients in other juices	50
USA	Apple juice, apple-juice concentrates and products	50
India	Apple juice, apple-juice ingredients in other beverages	50
Japan	Apple juice, food made using only apple juice as raw material	50

Factors that affect the initiation and accumulation of patulin in fruit

Various factors including the amount of free water (a_w), temperature, the presence of oxygen, the nature of the substrate and pH control PAT production (Yiannikouris *etal*., 2007). Relatively, the interactions among these factors than any single factor influence PAT production. At present there is no approach globally that has been started to investigate the multiple physicochemical parameters concurrently, which might be helpful to control the fungal growth and subsequent biosynthesis of PAT (Tannous *et al*., 2015). Most *Penicillium* species are recognized to be saprophytic, thus they can survive on dead, decaying matter or soil (Pitt, 2000) but *P. expansum* in particular is a psychrophilic fungus that grows at low temperatures (Deming, 2002). The ecological adaptation makes it possible to survive cold treatment of fruit during storage.

The PAT distribution during contamination reveals the presence of high concentration within decayed portions of fruit toward the inner cortex (where probably no visible decay) (Bandoh *et al*., 2009). In fig. 3 the outer part of apple pulp showed complete decay with PAT concentration of 40 mg kg^{-1}; section b is next to the completely decay area and extending up to 5 mm was 0.14 mg kg^{-1}; and section c is the section within 5–10 mm, showed only 0.003 mg kg^{-1}. Other researchers indicated that in apple fruit, PAT can penetrate to a depth of 10 mm (Welke *et al*., 2009), whiles its distribution in pear fruit can extend further into the surrounding healthy tissue (10–20 mm) (Laidou *et al*., 2001). These authors further indicated that PAT gradient in pear fruit\ within the first three sections showed 288, 144 and 144 ppm (outside to inside); but these concentrations were 115, 58 and 58 ppm, respectively, above the maximum residual limit of 50 ppm. Surprisingly, the third section of the fruit which appeared healthy was contaminated, but beyond that PAT was not detected. The conclusion of the various findings revealed that PAT contamination was not confined to the decayed tissues and the concentrations declined with increasing distance from the rotten area towards the supposedly healthy cortex (Mahunu *et al*., 2015). Again, it was found that PAT concentrations did not

correlate with lesion diameter, which explains the detection of PAT in small affected spots on fruit (Beretta *et al.*, 2000). PAT could be absent in apple fruit yet infected with *P. expansum* (Neri *et al*., 2010). Some factors associated with occurrence of PAT have been outlined; fruit variety and degree of ripening, the fungal strain, the presence of additional microbes or the postharvest storage conditions (Ballester *et al*., 2015). It was also reported that the inability of the fungus to produce PAT could be responsible for its absence (Barad *et al*., 2016). On the other hand, the pathogenicity exhibited by *P. expansum* has a direct function in patulin level, using either gene-disruption or RNAi mutants. It was also indicated that since the mutants still produced some level of patulin, there was no correlation between the level of pathogenicity of the *Penicillium* strain and ability to produce patulin (Ballester *et al*., 2015).

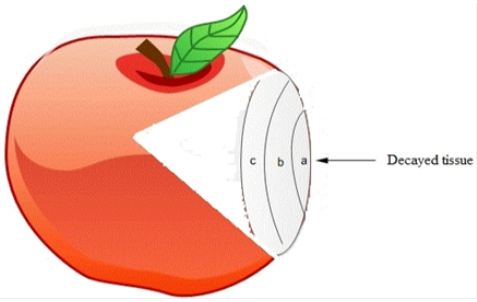

Fig. 3 An illustration of how of patulin is diffused in decayed apple fruit. PAT concentration decreases from the visibly decayed outer cortex "a" toward the inner area "c."(Mahunu *et al*., 2015)

Infection of apple fruit by *P. expansum* occurs before or during harvest through wounds on the fruit. The implications associated decay and patulin accumulation) are evidential later during storage. Some reports suggested that *P. expansum* pathogenicity is controlled by the changes in pH intermediated by secretion of organic acids such as gluconic acid (GLA) accumulation, which influence activation of polygalacturonase under acidic conditions (Prusky *et al*., 2014). Prusky *et al*. (2014) also reported that the virulence of pathogens is based on modulation of pH, which is response to host signals including (1) alkalization by ammonification of the host tissue in *Colletotrichum* and *Alternaria* or (2) acidification by secretion of organic acids in *Penicillium*, *Botrytis* and *Sclerotinia*. The same author mentioned that patulin in tissues of apple fruit contributes to fungal colonization by enhancing cell death. This implies that the detection of patulin accumulation probably could be an independent factor moderating *P. expansum* pathogenicity or virulence. Although, to date the general biological role of fungal secondary metabolites has not been completely exposed, there are some toxins (such as fungal phytotoxins) found to act as mediators of pathogenicity or virulence in several mycotoxigenic genera that cause significant disease (Scharf *et al*., 2014).

Control of Patulin by enhancing antagonist yeasts

The control of postharvest decay caused by *P. expansum* is the primary method to prevent PAT accumulation in stored fruit. The combination of cold storage and chemical fungicides treatments has been relied upon to control postharvest decay over the years. It was estimated that annually 23 million kg of fungicides are used globally to control postharvest diseases of fruit and vegetable (Karabulut *et al*., 2005b). Whereas less than 1% of the annual market share is represented by biological control agents (BCAs) (Mahunu *et al*., 2015). Nonetheless, ethical, technical and health issues related to the use of most chemicals rather favour the promotion of BCAs as promising alternative methods for the industry (Morales *etal.*, 2010). The prevention of *P. expansum* attack on stored fruit and implementation of viable

approaches to detoxify PAT can provide safer fresh fruit and juices for consumption (Ianiri *et al*., 2016). In this regard, various microbes that are naturally resistant to PAT and others with the ability to degrade it have been investigated. For example, *Rhodotorula glutinis* LS11 cells were found to resist concentration of PAT and degraded it *in vitro*; consequently it was able to metabolize PAT accumulation (Castoria *et al.*, 2005). It was also observed that initial PAT of 223 mg decreased (83%) following incubation with *Pichia ohmeri* 158 for 2 days at 25 ºC, then after 5 days it decreased by and finally become undetectable after 15 days (Coelho *et al*., 2007). Similarly, incubation of *P. caribbica* yeast for 15 days at 20 ºC, reduced PAT contamination in apples than the untreated the control samples (Cao *et al.*, 2013). Also, reduction of PAT accumulation was achieved by two ascomycete yeast species (*P. ohmeri* and *C. sake*), and a bacterium (*Pantoea agglomerans*) (Ianiri *et al*., 2016).

Apparently, it was reported that patulin reduction was due to fruit protection against infection caused by PAT-producing *P. expansum* or by PAT absorption through the cell wall and/or into the cells but not direct PAT metabolization (Morales *et al.*, 2010). Recent reports of an *in vitro* experiment indicated that BCAs in the Pucciniomycotina, including the yeasts *Sporobolomyces* sp. strain IAM 13481 and *Rhodosporidium kratochvilovae* strain LS11 have competence to resist patulin and degrade it into the less toxic compounds (desoxypatulinic acid and ascladiol) (Ianiri *et al.*, 2016). In addition, the results indicated that *Sporobolomyces* sp. converts PAT to desoxypatulinic acid (DPA) and ascladiols, but then (E) ascladiol was found as a transient metabolite, while DPA and (Z) ascladiol were the two final breakdown products (Fig. 4) (Ianiri *et al.*, 2013). Another reported also indicated that the isomers (E) and (Z) of ascladiol, intermediate in PAT biosynthesis, which were found as transient products, while desoxypatulinic acid (DPA) was recognized as the final product of PAT degradation (Castoria *et al*., 2005, Castoria *et al*., 2011). PAT degradation involves different and complex mechanisms but particularly pH and storage temperature and the species ripening have preeminence on the patulin accumulation in fruit as well as expression of the degradation pathways.

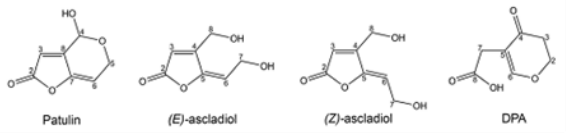

Fig. 4 Chemical structures of the three breakdown products (*E*)-ascladiol, (*Z*)-ascladiol and desoxypatulinic (*DPA*)

Reports on joint activity of BCAs in fruit or artificial medium, corroborate their contribution as additively or synergistically to enhance the biocontrol efficacy of antagonistic microbial agents (AMA) against *P. expansum* pathogenicity. According to Castoria *et al*. (2008), the improvement of the biocontrol efficacy of BCAs depends on their colonization, persistence and stabilization. Probably, the slow degradation of the exogenous compound might have supported the stability and efficacy of the biocontrol mixture (Mahunu *et al*., 2016). It was notice that addition of nitrogenous compounds (L-serine and L-aspartic) to *Candida sake* enhanced significantly its bioefficacy against *P. expansum* on apples. Also, the biocontrol efficacy of *C. sake* was greatly enhanced in cold storage by adding ammonium molybdate, which completely eradicated the incidence of blue mold on pears and reduced the severity and incidence of the disease by more than 80% on apples (Nunes *et al.*, 2001). Similarly, by the adding

nitrogenous compounds (L-asparagine and L-proline), the control efficacy of *Pseudomonas syringae* bacterium was improved tremendously, which reduced blue mold completely to undetected levels as against 50% decay in control (Janisiewicz *et al.*, 1992). During the process of biocontrol activity, important mechanisms of action of antagonistic yeasts include competition for space and nutrients, activation of host defenses, and production of extracellular depolymerases which apparently act on pathogen cell walls (Castoria *et al*., 2003). In addition, increased population density of antagonistic yeasts, as a result of the presence of exogenous compound plays a positive role in its competing abilities for space and nutrients against postharvest fungal pathogens (Li *et al*., 2008). For successful competition against pathogens, a timely colonization of wounds or surfaces of fruit by biocontrol agents is vital.

Some authors agreed that enzymes could be the basis for detoxification processes of products and juices derived from pome fruit (Ianiri *et al.*, 2016). Further, antioxidant enzymes and other related enzymes play a crucial role in eliminating reactive oxygen species (Castoria *et al.*, 2011). This also can elicit and regulate the biosynthesis of mycotoxins produced by other fungi (*Aspergillus flavus*, *A. parasiticus* and *A. ochraceus*) (Reverberi *et al*., 2008). Natural antioxidants from compounds in this regard have shown considerable usefulness in postharvest fungal control and inhibition of PAT (Ricelli *et al*., 2007) as well as other mycotoxins including aflatoxin, ochratoxin (Reverberi *et al*., 2005). Some reports indicated that there is a correlation between bioefficacy of antagonistic yeasts and related enzymes activities and eventual effect on yeast's resistance to oxidative stress (Castoria *et al*., 2005). Nevertheless, co-inoculation treatments of BCAs have proved to provide effective protection for AMAs against reactive stress conditions in biocontrol activities. Although, there are few reports that show evidence of PAT degradation in apple fruit, but co-treatment of antagonistic yeast cells with other BCAs control or inhibit fungi infection thereby preventing PAT production as well. However, it is not entirely possible to limit PAT diffusion after contamination has occurred.

Presently, there is scarceness of data on actual degradation of PAT in raw contaminated pome fruit according to several investigative reports and even more importantly, data on year-to-year dietary exposure through assessment is inadequate. Apart from the few countries such as South Africa, Morocco, Kenya and Nigeria, data on PAT contaminated products from other African countries are non-existent. It could also be stated that perhaps the results are available but not published in literature, since they export commodities to nations such the EU and USA.

In conclusion, this review paper shows that environmental factors controlling patulin biosynthesis are diverse and probably act together rather than one factor act alone. Also, the findings of the review suggest that ultimate factors controlling patulin synthesis are reliant on host. For this reason, further studies must focus on specific host component(s) that have the abilities to control colonization by fungal strain. In addition, once inoculation of PAT commences it cannot be reversed. Therefore, prevent of its occurrence in raw fruit is cardinal. The introduction of biological control microbes that affect *P. expansum* colonization by or patulin metabolism is a major technique of controlling patulin contamination during the extended storage and shelf-life of fruit especially apples in cold rooms. This paper also gathered that antagonistic microbial activities transformed PAT through intracellular activity to transformed products that are significantly

less toxic than PAT itself. Furthermore, the use of antagonistic microbes has potential to detoxify PAT but resistance to oxidative stress could characterize a principal mechanism of action involved in wound competence of biocontrol antagonistic yeasts.

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