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Development of fast methods for the detection of patulin producing fungi

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"Recomeça...

Se puderes
Sem angústia
E sem pressa.
E os passos que deres,
Nesse caminho duro
Do futuro
Dá-os em liberdade.
Enquanto não alcances
Não descanses.
De nenhum fruto queiras só metade.

E, nunca saciado,
Vai colhendo ilusões sucessivas no pomar.
Sempre a sonhar e vendo
O logro da aventura.
És homem, não te esqueças!
Só é tua a loucura
Onde, com lucidez, te reconheças..."

"Sísifo", Miguel Torga

Obrigada aos meus pais, à minha irmã, à minha família, aos amigos de sempre, aos amigos que Braga me deu e aos que fui fazendo pelo caminho. Obrigada à Fay, ao Alex, à Marta, à Sarah, ao professor Venâncio e à Célia. Por todos os começos e recomeços, por todas as oportunidades. Pela liberdade de tentar, de errar e de aprender. Obrigada por toda a generosidade e obrigada por tudo o que me ensinaram. A experiência que vivi vai além do tempo e do espaço, e levo-a comigo para o resto da Aventura.

RESUMO

Micotoxinas são metabolitos fúngicos secundários que podem ser tóxicos para humanos, animais e plantas. Algumas das micotoxinas mais observadas são ocratoxina A (OTA), aflatoxinas, fumonisinas e **patulina** (**PAT**), que representam uma preocupação para a saúde humana e pecuária. Membros dos três géneros de fungos: *Aspergillus*, *Fusarium* e *Penicillium* são os principais produtores de micotoxinas, sendo *Penicillium expansum* o principal produtor de **PAT**. As culturas podem ser infetadas antes ou depois da colheita, resultando na contaminação da cadeia alimentar. Existem alguns efeitos adversos das micotoxinas para a saúde, como a toxicidade aguda, resultando na deterioração das funções hepáticas ou renais e imunodeficiência. Deste modo, o potencial das micotoxinas em prejudicar a saúde através da exposição às mesmas, conduziu ao desenvolvimento de métodos que detetam fungos produtores de micotoxinas. Os métodos tradicionais são baseados em técnicas de cultura que consomem muito tempo e que requerem grandes quantidades de meios e reagentes. Devido a estas limitações, há um interesse crescente em métodos mais rápidos e sensíveis para a deteção dos principais contaminantes dos alimentos. Neste sentido, técnicas moleculares de DNA têm sido utilizadas como um método alternativo devido ao menor tempo de análise e maior sensibilidade. Alguns exemplos são a Reação em Cadeia da Polimerase (PCR), o PCR em Tempo Real (qPCR) e a **Amplificação da Polimerase de Recombinação** (**RPA**). A **RPA** é uma técnica de amplificação isotérmica que apresenta grandes vantagens como a sensibilidade, o baixo custo de operação, temperatura baixa constante e não requer instrumentos complexos. Uma forma de detetar os produtos de amplificação da reação de RPA são as **Fitas de Fluxo Lateral** (**LFS**) – dispositivos simples para a deteção de analitos onde os resultados podem ser observados diretamente a **olho nu** dentro de 5 a 15 minutos. O analito de interesse é capaz de se mover por ação capilar através das várias secções da fita, onde estão ligadas moléculas que podem interagir com o analito. Na presente tese foi desenvolvida uma combinação do método **RPA** com o **LFS** para a deteção a **olho nu** de fungos produtores de **PAT**, utilizando um conjunto de primers e uma sonda que têm como alvo o **gene isoepoxydon dehydrogenase** (***idh***). Esta combinação foi bem-sucedida para todas as estirpes de *P. expansum*.

Palavras-Chave: Patulina, Olho nu, Gene *idh*, Amplificação da Polimerase de Recombinação, Fitas de Fluxo Lateral.

ABSTRACT

Mycotoxins are secondary fungal metabolites that can be toxic to humans, animals and plants. Some of the most commonly observed mycotoxins are ochratoxin A (OTA), aflatoxins, fumonisins and **patulin** (**PAT**), which present a concern to human health and livestock. Members of three fungal genera: *Aspergillus*, *Fusarium* and *Penicillium* are the mainly producers of mycotoxins, where *Penicillium expansum* is the main producer of **PAT**. Crops can be infected before or after harvesting, resulting in contamination in the food chain. There are some adverse health effects of mycotoxins, such as acute toxicity, resulting in deterioration of the liver or kidney function and immunodeficiency. Thereby, the potential of mycotoxins to cause harm health through exposure has led to the development of methods that detect mycotoxins producing fungi. Traditional methods are culture-based techniques which are time consuming and require huge amounts of media and reagents. Because of these limitations, there is an increasing interest in faster and more sensitive methods for the detection of major food contaminants. In these sense, DNA molecular techniques have been used as an alternative method owing to their lower analysis time and higher sensitivity. Some examples are Polymerase Chain Reaction (PCR), Real-time PCR (qPCR) and **Recombinase Polymerase Amplification** (**RPA**). **RPA** is an isothermal amplification technique that has great advantages such as its sensitivity, its operation low cost, constant low temperature and it does not require complex instruments. A way to detect the amplification products of the **RPA** reactions is the **Lateral Flow Strips** (**LFS**) – simple devices for the detection of analytes where the results can be directly observable by **naked-eye** within 5 to 15 minutes. The analyte of interest is able to move by capillary action through the various sections of the strip, where attached molecules can interact with the analyte. In the present thesis it was developed a combination of the **RPA** method with the **LFS** for the **naked-eye** detection of **PAT** producing molds, using a set of primers and probe that target the **isoepoxydon dehydrogenase** (***idh***) **gene**. This combination was successful for all the *P. expansum* strains.

Keywords: Patulin, Naked-eye, *Idh* gene, Recombinase Polymerase Amplification, Lateral Flow Strips.

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List of Abbreviations and Acronyms

AMC – *Cooperativa de Productores Mejilloneros de Cabo de Cruz*

a_w – Water Activity

BLAST – Basic Local Alignment Search Tool

CECT – Spanish Type Culture Collection

Cq – Quantification Cycle

DAD – Diode Array

DNA – Deoxyribonucleic Acid

ECD – Electron Capture Detector

FAO – Food and Agriculture Organization

FID – Flame Ionization Detector

FLD – Fluorescence Detector

GS – Gas Chromatography

HPLC – High performance liquid chromatography

IARC – International Agency for Research on Cancer

Idh – Isoepoxydon dehydrogenase

INL – International Iberian Nanotechnology Laboratory

LFS – Lateral Flow Strip

MS – Mass Spectrometry

MUM – *Micoteca da Universidade do Minho*

N.C. – Negative Control

NTC – No Template Control

OTA – Ochratoxin A

PAT – Patulin

P.C. – Positive Control

PCR – Polymerase Chain Reaction

PDA – Potato Dextrose Agar

qPCR – Real-time PCR

RASFF – European Union Rapid Alert System for Food and Feed

RPA – Recombinase Polymerase Amplification

TLC – Thin Layer Chromatography

Tm – Melting Temperature

UCP – *Universidade Católica do Porto*

UHPLC – Ultrahigh performance liquid chromatography

UPLC – Ultra-performance liquid chromatography

UV – Ultraviolet

1. INTRODUCTION

This dissertation was developed in the context of the Masters in Biological Engineering, and the internship was carried out at the International Iberian Nanotechnology Laboratory (INL).

1.1 Theme framework

The growth of the population is the primary driver for global food request: more people demand more food (Nonhebel and Kastner, 2011), what is leading to a massive food production where food safety risks may be increasing. These risks result not only from uncontrolled endemic diseases but also from those that arise in more developed productions systems where foods and feeds contain contaminants, or are not handled properly (Delgado *et al.*, 1999). In addition to this, quality and safety are extremely important elements in the consumers' food choices (Rijswijk and Frewer, 2008).

During food and feed storage, fungi can cause serious problems because of mycotoxins production and spoilage, leading to health problems and great economic losses. In this sense, it is urgently needed to improve detection methods, and to evaluate the level of fungal contamination in the products (Kabal, 2009; Kocić-Tanackov and Dimić, 2013; Schnurer, 1993).

1.2 Mycotoxins

Mycotoxins are toxic secondary metabolites with low molecular weight produced by filamentous fungal species, commonly known as molds that grow naturally in animal feed and in various food products intended for human consumption. These secondary metabolites are not fundamental for the growth of the microorganisms that produce them, mycotoxins appear to be the result of the accumulation of precursor primary metabolites, for example, amino acids, pyruvates and acetates (Pimenta, 2002).

Since they are natural contaminants, it may not be possible to eliminate mycotoxins from food. However, their levels should be reduced as much as possible allowed by technology, so that they do not pose a risk to public health (Soares *et al.*, 2013).

Such small molecules induce no response in the human immune system, so our inability to detect them biologically makes mycotoxins a major potential danger in the human diet (Seo Jeong Ah and Yu Jae Hyuk, 2004).

Mycotoxicosis is the name given to human and animal intoxications associated with eating contaminated food or feed. Contaminated foods can be endowed with acute or chronic toxicity. Acute toxicity usually results from exposure to high levels of mycotoxins and its effects are visible in a short period of time (Pimenta, 2002). The most commonly described effect of acute mycotoxin poisoning is deterioration of liver or kidney function, which in extreme cases lead to death. Moreover, some mycotoxins act mainly by interfering with protein synthesis, and their effects ranges from skin sensitivity or necrosis to extreme immunodeficiency. Other mycotoxins in low doses might cause sustained trembling in animals, but at only slightly higher doses cause brain damage or even death (Seo Jeong Ah and Yu Jae Hyuk, 2004). On the contrary, chronic toxicity is originated by the ingestion of low levels over a long period of time and its chronic effects become visible later (Pimenta, 2002). Long-term effects of low levels of mycotoxin ingestion can be diverse. The main chronic effect of many mycotoxins is cancer, especially liver cancer. Some toxins affect DNA replication, and hence may produce mutagenic or teratogenic effects (Seo Jeong Ah and Yu Jae Hyuk, 2004). Thus, the effects of mycotoxicosis depend not only on the amount and duration of exposure, but also on the type of mycotoxin (Pimenta, 2002).

1.3 Mycotoxin-producing molds

Some mycotoxins are produced by a limited number of species and others might be produced by a relatively large range of species from several genera. Fungal genera producing mycotoxins include *Alternaria* spp., *Cladosporium* spp., *Botrytis* spp., *Claviceps* spp., *Stachybotrys* spp., *Mucor* spp., *Rhyzopus* spp., however *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. are the most important ones regarding to food safety (Sánchez-Hervás *et al.*, 2008; Venâncio, 2019). It is estimated that 25 to 50 % of crops harvested worldwide are contaminated with mycotoxins (Konietzny and Greiner, 2003).

The genus *Aspergillus* is inside a large, diverse family of fungi that primarily occupy subtropical and warm temperature climates. They grow at high temperatures and low water activity (a_w), for example, *A. flavus* growth temperatures vary with a minimum of 10 °C to a maximum of 48.8 °C, with an optimal of 33.8 °C and the a_w range from 0.80 to 0.82, contributing for their involvement in the colonization of a variety of crops (Venâncio, 2019). Strains belonging to *Aspergillus* section *Flavi* – *A. flavus*, *A. parasiticus* and *A. nomius* are aflatoxins producers and they grow essentially, as mentioned above, in high temperature and high humidity. Thus, aflatoxins accumulate in post-harvest when food is stored under conditions that contribute to fungal growth (Soares, 2019).

Fusarium is a large and complex genus containing species adapted to a large range of habitats: a few significant mycotoxin-producer species are present at pre-harvest in contaminated grains, and other plants, in regions like Australia, United States and Europe. *Fusarium* populations in agricultural field soils include saprophytes that decompose plant residues in the soil as well as pathogens that can cause rots, wilts, and other diseases (Venâncio, 2019). These fungi require slightly lower temperatures for growth and mycotoxin producing –, ranging from 4 °C to 45 °C, – compared to the aflatoxigenic *Aspergillus* species. *Fusarium* species synthesize a wide range of mycotoxins, and it is believed that the most important from the point of view of animal health are the trichothecenes, zearalenone, moniliformin and fumonisins (Placinta *et al.*, 1999). In Figure 1 it is possible to see macroscopic and microscopic appearance of the fungi mentioned above.

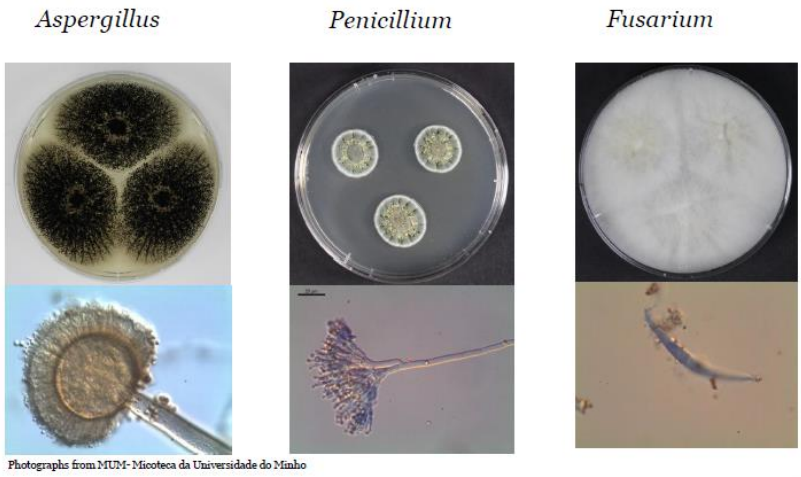

Figure 1 – Fungal genera producing mycotoxins (Soares, 2019).

1.4 *Penicillium* spp.

Penicillium spp. can be found in natural environment and also in food and drug production, having a worldwide distribution and a considerable economic impact on human life. Its main function in nature is decomposing organic materials and producing a large range of mycotoxins (Yadav *et al.*, 2017). The genus *Penicillium* contains many toxigenic species, and the range of mycotoxins produced is much extensive than that of any other genus (Cheung Chun Tung *et al.*, 2004). For instance, the most common mycotoxin produced by *Penicillium expansum* is patulin (PAT), a relatively small molecule that may also be produced by other *Penicillium* species (Jackson Lauren S. and Al-Taher Fadwa, 2008). However, some members produce penicillin that is used as an antibiotic that stops the growth and kill bacteria (Yadav *et al.*, 2017).

Most of *Penicillium* species are opportunistic saprophytes – they absorb organic substances normally from decomposing organic matter. The asexual spores produce a typical structure of the genus called penicillus that is supported by the stipe. The set of the penicillus and the stipe is denominated conidiophore, that ranges from being simple, to have multiple levels of branching. On the stipe there are cells named metulae and rami that support the phialides, even though they could sit directly on the stipe. Phialides produce chains where conidia are born. This morphology is represented in Figure 2. The wall texture, ornamentation and dimensions of stipes and conidia, and the colours of all elements of the conidiophore are other important microscopic characteristics (Serra, 2005; Visagie *et al.*, 2014).

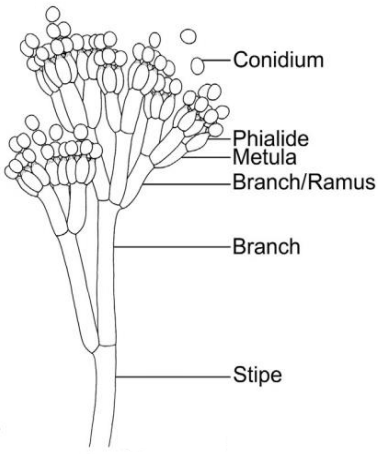

Figure 2 – Morphology of *Penicillium* spp. Adapted from (Visagie *et al.*, 2014).

Members of the genus *Penicillium* generally grow over a wider range of temperatures than those of the genus *Aspergillus*, for example, *P. expansum* can occur between 0 °C and 24 °C. The abundance of *Penicillium* species is higher in temperate climates and members of the genus are far more associated with storage than with pre-harvest contamination of grains (Venâncio, 2019).

1.5 Most important mycotoxins

Over 300 mycotoxins have been identified, but most of them have been produced under laboratory conditions. Only a relatively small number, of about 20, have been so far shown to occur naturally in foods and animal feeds at a significant level, and with enough frequency to be a safety concern (Konietzny and Greiner, 2003). Some of the most important ones are ochratoxin A (OTA), aflatoxins, fumonisins and PAT, that is the mycotoxin of interest for this project.

OTA is produced by several species of *Aspergillus* fungi and by a single species of the genus *Penicillium*, *P. verrucosum* (Pimenta, 2002). Humans might be exposed to this mycotoxin through the consumption of contaminated grains, and it can also appear in oilseeds, coffee, cocoa, beer and wine. Chickens, turkeys and ducklings are affected by ochratoxicosis, leading to poor weight gain and poor shell quality (Soares, 2019). Despite reported to appear in foods around the world, the main regions of concern are Europe and, for some foods, Africa (Murphy *et al.*, 2006) and the International Agency for Research on Cancer (IARC) classified in 1993 OTA as a potential human carcinogen (Group 2B) (Ostry *et al.*, 2017).

The main naturally produced aflatoxins are known as aflatoxins B_1, B_2, G_1, and G_2 where "B" and "G" refer to the blue and green fluorescent colours produced by these compounds under UV light on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds respectively. When aflatoxin B_1 and B_2 are ingested by lactating cows, about 1.5 % is hydroxylated and excreted in the milk as aflatoxins M_1 and M_2, compounds of lower toxicity than the parent molecules, but significant considering the widespread consumption of cows' milk by children. Aflatoxins can contaminate many crops including peanuts, corn, Brazil nuts, pistachios and figs with widespread contamination in hot and humid regions of the world (Murphy *et al.*, 2006) and are both acutely and chronically toxic to animals and human beings, causing acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects. Among the four major aflatoxins, B_1 has the greatest acute toxicity to animals and was acknowledged as the most carcinogenic type since it causes malignant tumours in a lot of animals, being the liver the major target organ. It was considered as a group I carcinogen by the IARC, owing to be the cause of human primary hepatocellular carcinoma.

Fumonisins are produced mainly by *Fusarium verticilloides* and *F. proliferatum* and the predominant group of fumonisin is B (FB) (Murphy *et al.*, 2006). Fumonisins are most associated with maize, however, can also be found in rice, hazelnuts and cheeses. Some diseases are associated with this mycotoxin, for example, "crazy horse disease" in horses, pulmonary edema in pigs and liver cancer in rats. In addition, fumonisins are associated with esophageal cancer in humans (Soares, 2019). IARC classified fumonisins as a potential human carcinogen (Group 2B) (Ostry *et al.*, 2017).

1.6 Patulin

Within the species that produce PAT, some of the most common ones are *Penicillium expansum*, *P. patulum*, *P. crustosum* and *P. roqueforti*, where *P. expansum* is the main producer (Soares, 2019).

PAT is an extremely common contaminant of fruits and fruit juices, such as apple, pear, grapes and strawberries, but it can appear in cereals and vegetables and it has also been isolated from cheddar cheese. Normally, within the food industry, apple and apple-based products have been products of high concern with regard to PAT contamination. Although the presence of these fungi does not guarantee the production of PAT, damages on the surface of fruit causes vulnerability to *Penicillium* infections, so PAT is not found in intact fruit (Murphy *et al.*, 2006; Moake *et al.*, 2015).

Fruit contamination may occur before the harvesting when microorganisms infect fruits by the calyx or through the stem or after, during the storage, since *Penicillium* species have the capability to grow in low temperatures, and during packaging, transportation and processing of fruits. Besides, as it was mentioned previously that damages on the skin of fruits induce vulnerability to *Penicillium* infections, if the surface of the fruit is damaged by insects, animal bites or accidentally in any post-harvesting steps, the fungi can access the soft tissue of the fruit. Another way of contaminating the fruit is by cross contamination in the markets as a result, for example, of an inappropriate display of fruit (Saleh and Goktepe, 2019). To reduce the incidence of *P. expansum* infections in order to avoid patulin contaminations of fruits, some practices can be applied such as the application of fungicides, the removal of infected tissue on the fruit and thermal processes. Regarding fruit-based products, in addition to what was mentioned, adding ascorbic acid and ascorbate to the product can reduce PAT levels (Ritieni, 2003; Wirght, 2015).

The acute symptoms of the consumption of contaminated products include convulsion, edemas, vomiting and intestinal ulceration (Pereira *et al.*, 2012) and the chronic health effects include immunotoxicity, genotoxicity and neurotoxicity in rodents (Barreira *et al.*, 2010). Although IARC has expressed much concern about the possible carcinogenicity of PAT, PAT is placed in carcinogenicity Group 3 (Alshannaq Ahmad and Yu Jae Hyuk, 2017), meaning that there are not enough studies that support PAT's carcinogenesis (Saleh and Goktepe, 2019).

Biosynthesis pathways for mycotoxins are consecutive enzymatic reactions where enzymes are successively activated as the newly synthesised product is being metabolised. The biosynthetic pathway of PAT consist of about 10 steps and a better understanding of its mechanisms can help to define strategies to detect this secondary metabolite (Barad *et al.*, 2016; Tannous *et al.*, 2014). In this sense, the present thesis will focus on the detection of the isoepoxydon dehydrogenase (*idh*) gene, which encodes the production of patulin that, as mentioned before, is the most common mycotoxin produced by *P. expansum*. This gene is involved in the mycotoxin biosynthetic pathway, as it can be seen in Figure 3. Genes involved in mycotoxin biosynthetic pathways are supposed to be only present in

potentially producing fungi. This is the reason why the *idh* gene was the selected gene for the current project/thesis (Konietzny and Greiner, 2003).

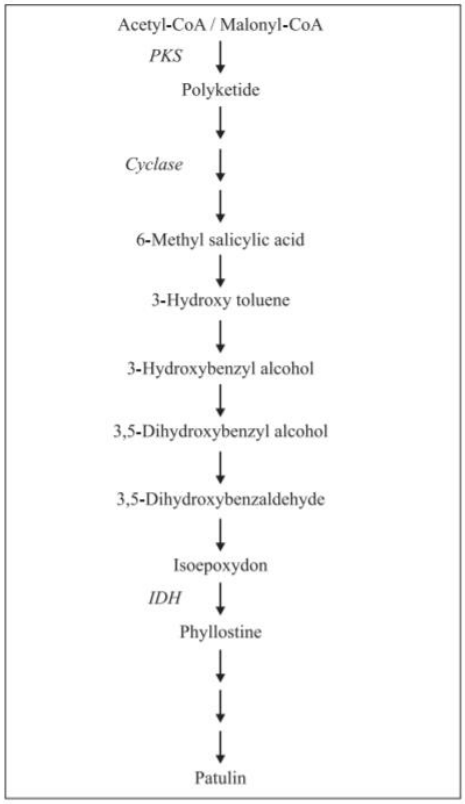

Figure 3 – Biosynthetic pathway of PAT (Konietzny and Greiner, 2003).

1.7 Regulation relating to mycotoxins in food

In Europe, the occurrence of mycotoxins in food products, as well as other contaminants that pose a risk to public health, is the subject of extensive monitoring by all countries, the results of which can be consulted on the European Union Rapid Alert System for Food and Feed (RASFF) website. According to the RASFF Annual Report of 2019, mycotoxins were the most reported type of hazard in products from non-member countries, with 534 notifications in which aflatoxins and OTA were the most frequently reported mycotoxins in food (Publications Office of European Union, 2020).

Most countries have adopted legislation that sets maximum limits for the presence of certain mycotoxins in food and, in Table 1, it is presented the maximum levels of PAT in food. In developed countries, the legislation in force is very restrictive and the most common health problems associated with mycotoxins are related to the appearance of tumours and the weakening of the immune system of

individuals, which reduces their resistance to infectious diseases. According to the Food and Agriculture Organization (FAO), 39 European countries were known to have mycotoxins regulation. In underdeveloped countries, exposure to mycotoxins occurs more easily since agricultural practices, storage methods and legislation are not the most suitable. In some of these countries, high morbidity and premature deaths continue to occur among the human population due to exposure to mycotoxins (Soares *et al.*, 2013).

Table 1 – Maximum levels of PAT in food according to the Commission Regulation (EC) N° 1881/2006

Foodstuffs	Maximum levels (µg/kg)
Fruit juices, concentrated fruit juices and fruit nectars	50
Spirit drinks, cider and other fermented drinks derived from apples or containing apple juice	50
Solid apple products, apple compote and apple puree for direct consumption	25
Apple juice and solid apple products, apple compote and apple puree for infants and young children	10
Baby foods except processed cereal-based foods for infants and young children	10

A variety of factors might affect the promulgation of mycotoxin limits and regulations. These include the availability of toxicological data of mycotoxins and exposure data of mycotoxins, the knowledge of the distribution of mycotoxins concentrations within commodity or product lots, the availability of analytical methods, legislation in other countries with which trade contacts exist and, for last, the need for sufficient food supply (Van Egmond *et al.*, 2007).

1.8 Methods for the detection and analysis of mycotoxins and mycotoxin-producing fungi

Determination of mycotoxin levels in food sample is normally accomplished by methods that combine common steps: sampling, homogenization, extraction followed by a clean-up and, lastly, the detection and quantification (Alshannaq Ahmad and Yu Jae Hyuk, 2017), as there are several methods that have been validated and used for the analysis and detection of mycotoxins (Turner *et al.*, 2009).

1.8.1 Chromatography Techniques

Chromatography techniques are undoubtedly the most used method for mycotoxins analysis in food and feed. Thin layer chromatography (TLC) is presently used as a rapid screening method for certain mycotoxins by visual assessment or instrumental densitometry. Additionally, HPLC coupled with

ultraviolet (UV), diode array (DAD), fluorescence (FLD), or mass spectrometry (MS) detectors and ultra-high-performance liquid chromatography (UHPLC) or ultra-performance liquid chromatography (UPLC) can detect and quantify various mycotoxins. Besides that, gas chromatography (GC) coupled with electron capture detector (ECD), flame ionization (FID), or MS detectors have been used to identify and quantitate volatile mycotoxins such as PAT (Alshannaq Ahmad and Yu Jae Hyuk, 2017).

In the case of PAT, TLC was the first method used for the identification and quantification of this mycotoxin in apple juice, however the most commonly chromatographic method used for the analysis of this secondary metabolite is HPLC coupled with UV detection (Barad *et al.*, 2016; Moake *et al.*, 2015).

1.8.2 Molecular Techniques

There is an increasing interest in developing rapid tests for detection of major food contaminants like mycotoxins, and results are expected to be obtained within a short time, with the help of simple devices (Alshannaq Ahmad and Yu Jae Hyuk, 2017).

Polymerase Chain Reaction (PCR)

The origins of PCR emerged from researches performed in the early 1980s at Cetus Corporation in California. The innovation in Mullis's concept was using the juxtaposition of two oligonucleotides, complementary to opposite strands of the DNA, to amplify the specific region between them and to achieve this in a repetitive manner so the product of one round of polymerase activity was added to the pool of template for the following round, from here arises the chain reaction (Sgaramell, 1996).

PCR has been used as an alternative technique for microbiological and chemical methods in the detection and identification of some toxigenic molds in food (Luque *et al.*, 2011) and it is based on genes presented in mycotoxin biosynthesis (Paterson, 2006).

The fundamental process involves 3 steps: double-stranded DNA denaturation at a temperature higher than 90 °C, primer annealing at 50 °C - 75 °C, and extension at 72 °C - 78 °C (Mackay *et al.*, 2002). The DNA denaturation consists in separating the individual strands by heat during specific lengths of time then, a short chain of specifically ordered nucleotide bases – the primers – bind to a small segment of DNA of interest – the target. The target DNA is exponentially amplified by a heat-stable polymerase – DNA polymerase. The process is cycled approximately 40 times to provide the necessary quantity of DNA product (Mullis, 1990; Paterson, 2006; Schrader *et al.*, 2012). This process is represented in Figure 4 below.

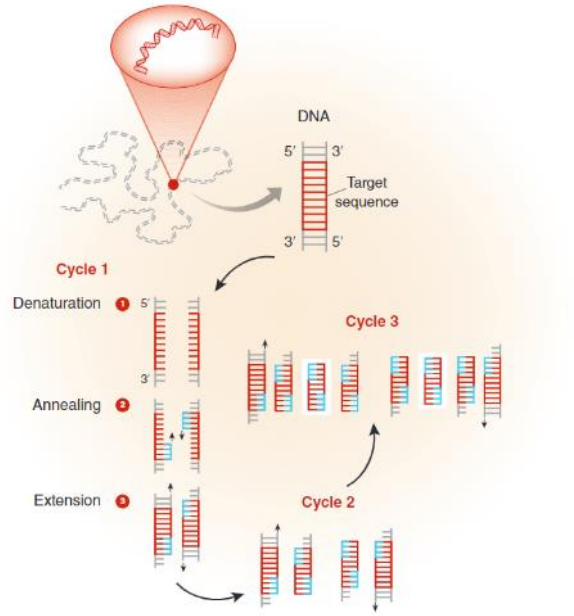

Figure 4 – Schematic representation of PCR (Garibyan and Avashia, 2013).

In other words, PCR allows the amplification of certain fragments of DNA from complex DNA samples where the resulting PCR product can be seen after gel electrophoresis and staining with a DNA binding fluorescent dye, for instance, Green Safe (Edwards *et al.*, 2002). The efficiency of a PCR is defined as the fraction of the target DNA that is copied in one cycle and it should be, at least, 90 % (Svec *et al.*, 2015).

Real-time PCR (qPCR)

Even though conventional PCR techniques are faster than microbiological techniques, qPCR techniques allow the elimination of time-consuming techniques to analyse the reaction product, for example, agarose gel electrophoresis (Suanthie *et al.*, 2009). The qPCR enables the detection and measurement of products generated during the PCR cycles, which are directly proportional to the amount of template in the beginning of the PCR process, by using dual-labelled oligonucleotide fluorescent probes (Arya *et al.*, 2005) or intercalating dyes (Ginzinger, 2002).

The correlation between the amount of target sequence at the beginning of the PCR reaction and the amount of amplified PCR product at any given cycle follows an exponential rate that causes an exact doubling of product that is accumulated at any cycle – this is known as the "exponential phase" (Raso and Biassoni, 2014). However, due to inhibitors of the polymerase reaction found within the template, reagents consumption or accumulation of pyrophosphate molecules, at some point the PCR reaction is not generating template at an exponential rate anymore. This is known as the "plateau

phase" (Ginzinger, 2002). The difference in the initial amounts of template molecules can be quantified by comparing the number of amplification cycles required for the response curve of the samples to reach a threshold fluorescence signal level. This number of cycles required is called Cq value (Kubista *et al.*, 2006; Bustin *et al.*, 2009) and the threshold is calculated as a function of the amount of background fluorescence and is traced at a point where the signal generated from a sample is substantially higher than background fluorescence (Ginzinger, 2002). So, as it can be seen in Figure 5, the reaction is going to be exponential in the beginning, then will enter an almost linear phase and finally, in later qPCR cycles, it reaches a plateau (Kainz, 2000).

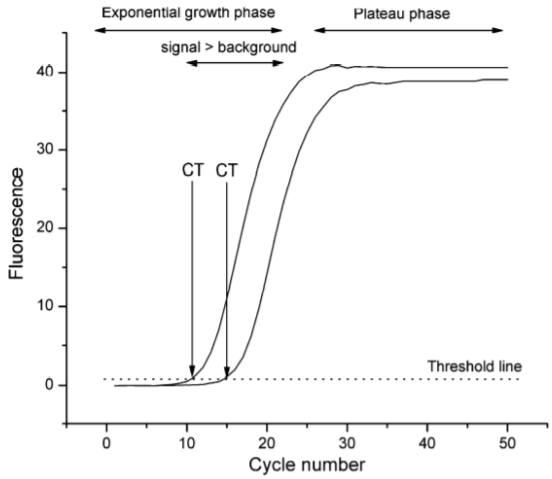

Figure 5 – qPCR response curve (Kubista *et al.*, 2006).

For the detection and quantification of mycotoxigenic fungi, intercalating dye and hydrolysis probes are largely used (Konietzny and Greiner, 2003). In the first method mentioned, the fluorescence technique is based on the SYBR Green dye. The dye has selective affinity to double stranded DNA and binding highly enhances fluorescence emission of the molecule at 530 nm and does not react with single stranded DNA present in the sample. It intercalates into double stranded DNA to monitor the amplification of the target gene (Cao and Shockey, 2012).

SYBR Green is widely used for qPCR due to cost efficiency, universal detection of amplified DNA and its capacity to differentiate PCR products by melting curve analysis (Gudnason *et al.*, 2007). The use of melt curve analysis eradicates the need for agarose gel electrophoresis since the melting temperature – Tm – of the specific amplicon is analogous to the detection of an electrophoretic band (Giglio *et al.*, 2003). One drawback of this method is that the dye is non-specific and might generate false positive signals if non-specific products or primer-dimers are present in the assay. This problem

can be minimized by designing the primers carefully and validating the PCR products with dissociation curve analysis. Likewise, some approaches have been developed to further increase the specificity of SYBR Green detection, for example, a "hot start" strategy that uses a DNA polymerase that needs heat activation (Arikawa, 2008).

The second method, the hydrolysis probe, uses dual-labelled probes (Heid *et al.*, 2001). This probe is a short oligonucleotide containing a fluorescent dye as a reporter at 5' end and a quencher dye at 3' end, designed to hybridize with the target DNA at the annealing step (Espy *et al.*, 2006; Tsai *et al.*, 2012). At the extension step, the DNA polymerase separates the reporter from the quencher dye, which results in the emission of fluorescent signals from the reporter dye (Tsai *et al.*, 2012), as it can be seen in Figure 6. Since hydrolysis probes can specifically bind to each target, non-specific products are not detected. However, one drawback of these probes is their cost, because for each target, a specific probe must be synthesized (Fattahi *et al.*, 2018).

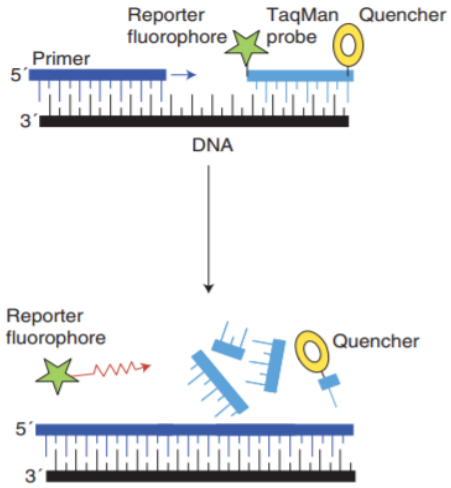

Figure 6 – Schematic representation of the hydrolysis probe action. Adapted from (Arya, 2005).

Recombinase Polymerase Amplification (RPA)

An alternative DNA amplification technique is the RPA – which is an isothermal amplification technique (Piepenburg *et al.*, 2006). The RPA reaction starts with the binding of the recombinase to the primers with the help of a loading factor, forming a nucleoprotein filament. This filament searches the homologous sequence in the double-stranded DNA and, once it is located, the complex invades the double-stranded DNA and a D-loop structure is formed to initiate a strand exchange reaction. When this strand exchange is performed, the recombinase disassembles from the nucleoprotein filament in order

to be available for the next pair of primers. Then, the DNA polymerase extends from the 3' end of the primer (Li *et al.*, 2019).

RPA has great advantages such as its sensitivity, ability for multiplexing, its operation at a low cost and constant low temperature (25 °C - 42 °C), it does not need an initial denaturation step, high speed and it does not require complex instruments (Ma *et al.*, 2019; Lobato and O'Sullivan, 2018). The amplification products of the RPA reactions are detected by gel electrophoresis or through lateral flow strips (LFS) (Liu *et al.*, 2017), through fluorescent probes for a real time detection (Lobato and O'Sullivan, 2018).

The LFS are simple devices for the detection of analytes in complex samples, where the results are directly observable by naked eye within 5 to 15 minutes (Koczula and Gallota, 2016; Li *et al.*, 2019). The principle is simple, a sample containing the analyte of interest moves by capillary action through the various sections of the strip, where molecules that can interact with the analyte are attached (Koczula and Gallota, 2016).

The four main sections of the LFS are: sample pad, conjugate pad, detection pad and lastly, absorption pad, as it can be seen in Figure 7 (A). The sample pad, made of cellulose, on which the sample is dropped, ensures that the analyte on the sample will be able to bind to the capture reagents and on the membrane. Then, the sample migrates through the conjugate pad, made of glass fiber containing antibodies that are specific to the target analyte and are conjugated, most commonly, to colloidal gold particles. The sample with the conjugated antibody bound to the target analyte, run through the strip into the detection pad. This section is made of a nitrocellulose sheet with specific biological components – antibodies or antigens – immobilized in lines. The biological components will react with the analyte bound to the conjugated antibody, which forms the test line and the control line. Finally, the absorption pad made of cellulose is attached to the end of the strip where the excess of reagents is wicked, preventing backflow of the liquid (Quesada-González and Merkoçi, 2015; Koczula and Gallota, 2016).

If the amplification is successful, a red line is produced on the test line owning to the accumulation of colloidal gold. On the contrary, in the absence of the target DNA, no red line is showed in the test line – Figure 7 (B). The gold particles that are not captured, flow through and are immobilized on the control line (Li *et al.*, 2019).

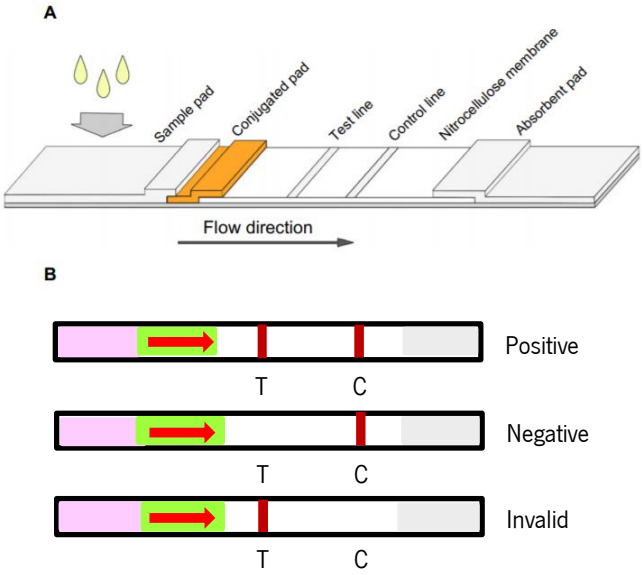

Figure 7 – **(A)** Schematic representation of LFS Adapted from (Wang *et al.*, 2014). **(B)** Schematic representation of possible results. T, test line; C, control line.

Some advantages of LFS are increased specificity, and the fact of being very stable under different set of environmental conditions. They are extremely easy to use – simple instrumentation and one step analysis. Besides, LFS have relatively low operational cost (Amerongen *et al.*, 2018; Sadjid *et al.*, 2015). Nevertheless, the main limitation are low sensitivity and lack of quantification capacity (Liu *et al.*, 2017a; Rivas *et al.*, 2014).

The kit used for the lateral flow, TwistAmp® nfo kit (TwistDx), requires a specific probe – the TwisAmp® nfo probe – that is intended to be used in lateral flow strips. This probe is an oligonucleotide with a 5'-antigenic label (FAM), an internal abasic nucleotide analogue (THF residue) and a blocking group at the 3' end (C3 spacer). A schematic representation of the probe is present in Figure 8 (TwistDx, 2020).

Figure 8 – Schematic representation of the TwistAmp® nfo probe.

The probe is used with a reverse primer that is also labelled on the 5' with another antigenic label (Biotin), and a conventional forward primer. The TwistAmp® nfo kit (TwistDx) has an

endonuclease IV (nfo) that will cut the probe on the THF residue site then, the processed probe and the reverse primer will generate amplification products that co-join the two antigenic labels – the FAM and the biotin. This is schematized in Figure 9. On the lateral flow strip, the amplification products will be captured by the anti-biotin test line (TwistDx, 2020).

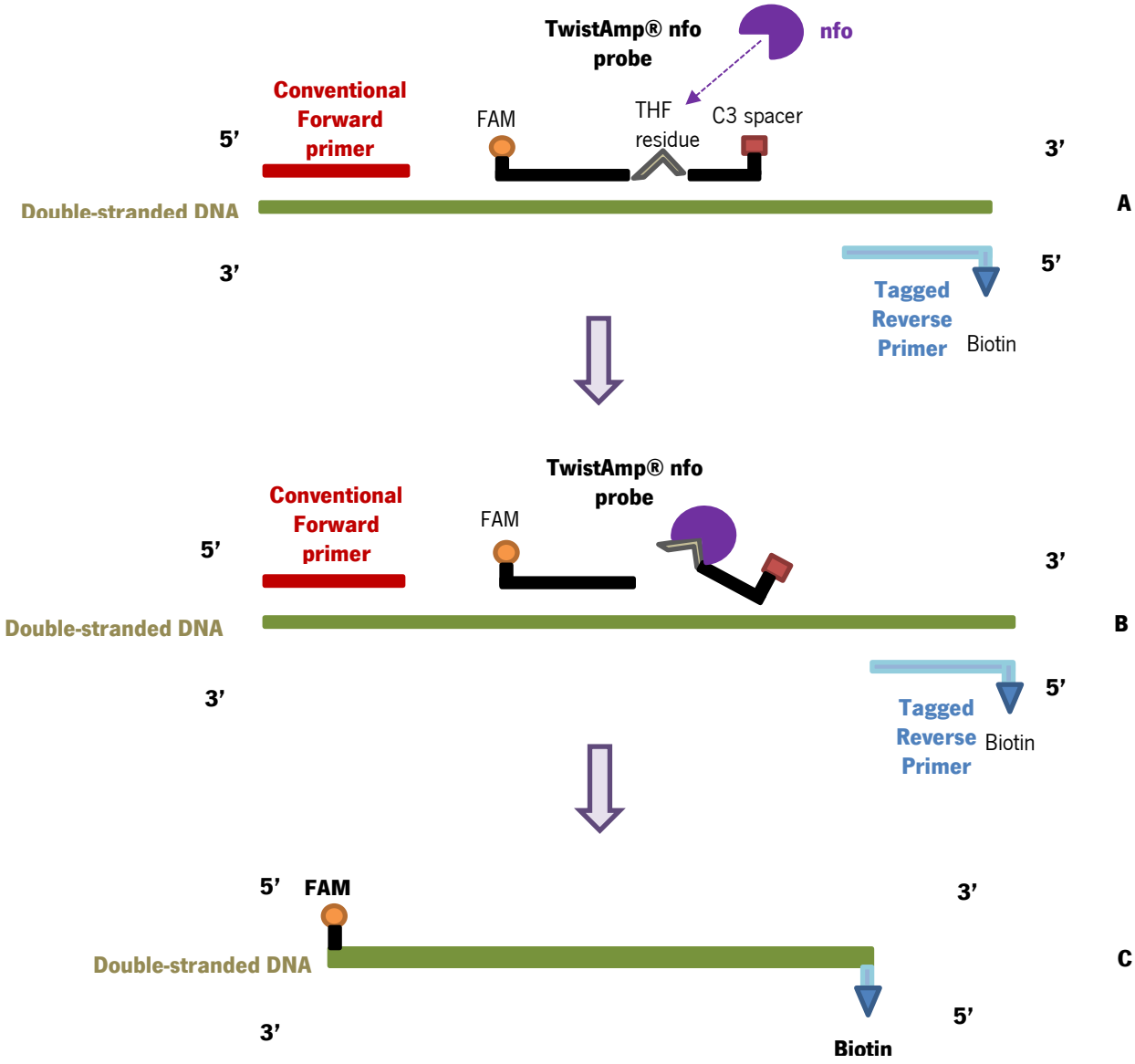

Figure 9 – Schematic representation of the arrangements of the conventional forward primer, the tagged reverse primer and the TwistAmp® nfo probe **(A)**, schematic of the endonuclease IV (nfo) **(B)** and generated amplification products with FAM and biotin **(C)**.

1.9 Objetives

The main objective of this project was to develop an isothermal DNA amplification method combined with lateral flow strips (LFS) for naked-eye detection of patulin producing molds. To accomplish this, the following specific objectives were addressed:

- ✓ Development and optimization of a qPCR method targeting the *idh* gene;
- ✓ Development and optimization of a RPA method targeting the *idh* gene;
- ✓ Combination of the optimized RPA protocol with LFS;
- ✓ Evaluation of the analytical methods developed.

2. MATERIALS AND METHODS

2.1 Strains used for the development of the assay

The target gene for this project was the *idh* gene since this gene is involved in the biosynthetic pathway of PAT. In order to develop and evaluate the assay for the naked-eye detection of PAT producing molds, some mold, fungal and bacterial strains were obtained from the *Micoteca da Universidade do Minho* (MUM) culture collection, the Spanish Type Culture Collection (CECT), the *Universidade Católica do Porto* (UCP), from *Cooperativa de Productores Mejilloneros de Cabo de Cruz* (AMC) and from a partner company. The information regarding the species name, culture collection they are from, the number of the strains and their source, as well if the species have the *idh* gene and if they are PAT producers are presented in Table 2.

For developing the qPCR assay, the strains used as reference strains were *Penicillium expansum* (MUM 17.69), used as the positive control (P.C.), and *P. tunisiense* (MUM 17.62) as the negative control (N.C.).

Table 2 – Strains used for the development and evaluation of the assay

Species name	Culture collection	Number of the strain	*Idh*	Patulin	Source
Penicillium expansum	MUM	17.41	+	+	Apple
Penicillium tunisiense	MUM	17.62	-	-	Apple
Penicillium expansum	MUM	17.69	+	-	Apple
Aspergillus flavus	MUM	18.41	-	-	-
Aspergillus niger	MUM	19.133	-	-	Pepper
Penicillium brevicompactum	MUM	17.45	+	-	Apple
Penicillium crustosum	MUM	17.31	+	-	Apple
Penicillium polinicum	MUM	17.37	+[a]	-	Apple
Penicillium solitum	MUM	17.33	+	-	Apple
Penicillium expansum	MUM	17.67	+	-	Apple
Penicillium expansum	MUM	00.02	+	+	Grapes
Penicillium expansum	MUM	17.38	+	+	Apple
Penicillium expansum	MUM	17.44	+	+	Apple
Penicillium expansum	CECT	2278	+	+	Mold-fermented sausage
Penicillium griseofulvum	CECT	2919	+	+	Corn
Salmonella Typhimurium	CECT	4594	-	-	-
Escherichia coli	CECT	434	-	-	-
Escherichia coli	CECT	4972	-	-	-
Staphylococcus aureus	CECT	240	-	-	-
Campylobacter coli	INL	2	-	-	-
Listeria innocua	UCP	2110	-	-	-
Salmonella Enteritidis	AMC	82	-	-	Proficiency test
Penicillium spp.	Partner Company	Fungus 2	ND[b]	+	Chestnuts
Meyerozyma spp.	Partner Company	-	ND	ND	Fruit preparations
Pichia fermentans	Partner Company	-	ND	ND	Fruit preparations
Neosartorya fischeri	Partner Company	-	ND	ND	Fruit preparations
Penicillium spp.	Partner Company	-	ND	ND	Fruit preparations
Penicillium spp.	Partner Company	Fungus 1	ND	ND	Chestnuts
Mucor spp.	Partner Company	Fungus 3 and 5	ND	ND	Chestnuts

MUM – *Micoteca da Universidade do Minho*
CECT – Spanish Type Culture Collection
INL – International Iberian Nanotechnology Laboratory
UCP – *Universidade Católica do Porto*
AMC – *Cooperativa de Productores Mejilloneros de Cabo de Cruz*
ND – Non-defined

[a] – Due to a discrepancy of the results with this strain in this project, MUM confirmed a possible misidentification of this strain

[b] – With the qPCR developed in this project, the Fungus 2 was identified as *idh* +

2.2 Culture conditions

Stock cultures were maintained at - 80 °C in cryogenic vials that were prepared with glycerol and spores resulted from the harvest of the grown mycelium of the molds, as it is explained below. For

inoculum preparation, stock cultures were sub-cultured on Potato Dextrose Agar (PDA) (Biokar diagnostics) and grown at room temperature for 7 days in the dark.

2.3 DNA Extraction

For the DNA extraction from pure cultures, 10 mL of distilled water were added to each plate, spores were harvested from the surface of the plate and a syringe with cotton was used to retain the mycelium. Then, 1 mL of the filtered solution was centrifuged at 9000 × g for 5 minutes and the supernatant was discarded. The pellet was used for DNA extraction with the DNeasy® PowerSoil® Pro Kit (QIAGEN) following the supplier's specifications. Since this kit is made for soil, the pellet was first dissolved with solution CD1 and then transferred for the PowerBead Pro Tube (both supplied with the DNeasy® PowerSoil® Pro Kit). DNA concentrations were quantified using the QubitTM 1X dsDNA HS Assay Kit (Thermo Fisher Scientific).

2.4 Real-time PCR (qPCR)

2.4.1 Selection of the primers

From previous studies, primer sequences – presented in Table 3 – were selected and with the CLC Sequencer Viewer it was possible to see if those sequences could be found in the strains of interest. Then, the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to ensure that those primer sequences could not be found in other strains.

Table 3 – Primers selected from previous studies

Primers	Nucleotide Sequence (5'-3')	Reference
FC2	CGATGTTGCTAGCAAAGACG	Luque *et al.*, 2011
IDH2	ACCTTCAGTCGCTGTTCCTC	
F-idhtrb	GGCATCCATCATCG	Rodrígez *et al.*, 2012
R-idhtrb	CTGTTCCTCCACCCA	
idh2444	ATGCACATGGAAGGCGAGAC	
idh2887	CAAVGTGAATTCCGCCATCAACCAAC	Hosoya *et al.*, 2013
idh2444	ATGCACATGGAAGGCGAGAC	
idhBP1R	CTGCGCTGCCTTGCAGGGCCC	Hosoya *et al.*, 2012

2.4.2 Primers Evaluation

The qPCRs done to test the primers were performed for a total volume of 20 µL, with 5 µL of DNA template, using the PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) and the DNA from the strains obtained from MUM was used as the template. The primers concentrations and thermal cycling conditions are detailed in Table 4.

Table 4 – Primers concentration and thermal cycling conditions

Set of Primers	Concentration	Thermal cycling conditions			
		Cycling Stage			
		Denaturation	Annealing	Extension	
FC2/IDH2 [a]	400 nM	95 °C, 15 s	52 °C, 1 min	72 °C, 1 min	72 °C, 5 min
		40 cycles			
F-idhtrb/R-idhtrb [b]	720 nM	95 °C, 15 s	58 °C, 1 min		
		40 cycles			
idh2444/idh2887 [c]	200 nM	95 °C, 15 s	59 °C, 1 min	72 °C, 1 min	
		40 cycles			
idh2444/idhBP1R [d]	200 nM	95 °C, 15 s	59 °C, 1 min	72 °C, 1 min	
		40 cycles			

[a] – Set of primers from (Luque *et al.*, 2011)

[b] – Set of primers from (Rodríguez *et al.*, 2012)

[c] – Set of primers from (Hosoya *et al.*, 2013)

[d] – The primer idh2444 if from (Hosoya *et al.*, 2013) and the primer idhBP1R is from (Hosoya *et al.*, 2012)

2.5 qPCR Optimization

The premier set providing the best preliminary results were selected for further optimization. This sense, the concentration of the selected set of primers, the temperature and time of the annealing step of the thermal cycling profile of the qPCR were changed and tested. The qPCR optimization was performed with 3 µL of DNA template for a total volume of 20 µL, using the PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific).

2.5.1 Primers concentration optimization

For both forward and reverse primers, 5 different concentrations were tested: 500 nM, 600 nM, 700 nM, 720 nM – that is the concentration used in (Rodrígez *et al.*, 2012) –, and 800 nM.

2.5.2 Temperature optimization

The concentration of the forward and reverse primers was 720 nM and 6 different annealing temperatures were tested for a time of 1 minute: 55 °C, 56 °C, 57 °C, 58 °C – that is the temperature used in (Rodrígez *et al.*, 2012) –, 59 °C and 60 °C.

2.5.3 Annealing time optimization

From the 6 temperatures tested previously, with the temperature that got the better amplification plot and lower Cq values, 2 annealing times were tested: 45 seconds and 30 seconds.

2.6 Specificity Assay

With the best parameters regarding the concentration of the primers, temperature and time of the annealing step of the thermal cycling profile, the qPCR assay was tested for its specificity to confirm if it was detecting the right sequence. In this sense, to evaluate the specificity of the qPCR assay, an inclusivity/exclusivity assay was performed using mold strains that are *idh* +, fungal strains that are *idh* - and bacterial strains which information is presented in Table 2.

2.7 Sensitivity Assay and qPCR Efficiency

With the best conditions of the concentration of the primers, temperature and time, the qPCR assay was tested for its sensitivity to know until which DNA concentration of the template it was possible to get amplification. For the sensitivity assay, with the *Penicillium expansum* (MUM 17.69), ten-fold serial dilutions were prepared, as it is shown in Figure 10. For this assay, three independent experiments were performed with three technical replicated per dilution.

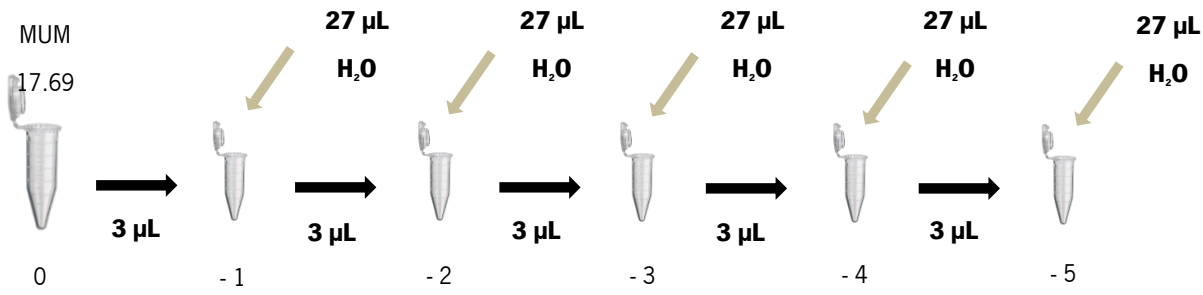

Figure 10 – Schematic representation of the ten-fold dilutions of *Penicillium expansum* (MUM 17.69).

In order to assess the sensitivity of the qPCR, the efficiency of the assay was calculated using the following formula (Kawasaki *et al.*, 2010):

$$E = 10^{\frac{-1}{b}} - 1 \quad (1)$$

Where, E is the PCR efficiency and b represents the slope. For each dilution, an average of the Cq values was calculated and the DNA concentration was quantified. Then, a plot of the Cq values versus the logarithm of the sample's concentration was constructed. With the regression line, the slope was determined and, using the formula (1), the qPCR efficiency was finally calculated.

2.8 RPA Assay

2.8.1 Selection of the RPA primers

The primers used for the RPA were designed using the PrimedRPA software (Higgins *et al.*, 2019) for which a consensus file of the sequences was inputted. Then, filtering parameters were tested such as, primer, probe and amplicon lengths, GC content and capacity to form secondary structures and with these parameters, the following primers and probes, presented in Table 5, were generated.

Table 5 – Primers and probes generated with PrimedRPA Software

	Nucleotide Sequence (5'-3')	
Forward Primer	AACGCGGGAACTAGGAGGCACGCGGGGTAT	
Probe	TACTGAGTATGATGACTTCTATCCGACGCTCGCTGAGGGAAATACC	1st set
Reverse Primer	GATAATCACGTCAATTCGTCCGAGTCGCTC	
Forward Primer	ACGCGGGAACTAGGAGGCACGCGGGGTATG	
Probe	ACTGAGTATGATGACTTCTATCCGACGCTCGCTGAGGGAAATACCG	2nd set
Reverse Primer	CGATAATCACGTCAATTCGTCCGAGTCGCT	
Forward Primer	AGTCCCCCCAGGCATCCATCATCGTGCAGA	
Probe	AGCATGTCCAGGAATTGTCCCACTTTTTGGGGCCGAAGGGCATCCG	3rd set
Reverse Primer	ATCTTCAGTCGCTGTTCCTCCACCCATTCC	
Forward Primer	GTCCCCCCAGGCATCCATCATCGTGCAGAG	
Probe	GCATGTCCAGGAATTGTCCCACTTTTTGGGGCCGAAGGGCATCCGG	4th set
Reverse Primer	AATCTTCAGTCGCTGTTCCTCCACCCATTC	
Forward Primer	TCCCCCCAGGCATCCATCATCGTGCAGAGC	
Probe	CATGTCCAGGAATTGTCCCACTTTTTGGGGCCGAAGGGCATCCGGG	5th set
Reverse Primer	GAATCTTCAGTCGCTGTTCCTCCACCCATT	
Forward Primer	GAATCTTCAGTCGCTGTTCCTCCACCCATT	
Probe	ATGTCCAGGAATTGTCCCACTTTTTGGGGCCGAAGGGCATCCGGGT	6th set
Reverse Primer	GGAATCTTCAGTCGCTGTTCCTCCACCCAT	

Similarly to what was done with the qPCR primers, with CLC Sequencer Viewer it was possible to see if the primers and probes generated by PrimedRPA were present in different sequences in conserved regions or not and, with the BLAST, the sequences of the selected primers and probes were verified in the target species. Beyond this, with the OligoAnalyzer (https://eu.idtdna.com/pages/tools/oligoanalyzer), the general structure of the primers and probes and the formation of secondary structures were analysed. Then, the set with the best results was selected to be tested.

2.8.2 Specificity Assay

The conditions selected and the performance of the assay under those conditions were confirmed by the evaluation of the specificity. To test the specificity of the RPA, an assay was performed with the set selected at a concentration of 780 nM, for 40 minutes at 37 °C with the TwistAmp® Basic Kit (TwistDx). For this assay it was used 2 µL of DNA template for a total volume of 25 µL, were the DNA from mold strains that are *idh* +, fungal strains that are *idh* - and bacterial strains were used as template. The information about the strains is presented in Table 2.

2.9 RPA combined with Lateral Flow

2.9.1 nfo probe

Milenia Genline HybriDetect (TwistDx) strips were used for the lateral flow tests and the RPA reaction was performed using TwistAmp® nfo kit (TwistDx). The nfo probe used was the idh-nfo-probe, the conventional primer used was the F-idhRPA and the tagged reverse primer was the idhR-bio.

For the RPA reaction it was used, as mentioned above, the TwistAmp® nfo kit (TwistDx) with a concentration of the probe of 120 nM, that is the recommended concentration from the supplier, and 780 nM for the forward and reverse primers, for a total volume of 25 µL with 2 µL of DNA template, where the strains from MUM and CECT were used as template, for 40 minutes at 37 °C. Afterwards, the RPA reactions were diluted 1:50 and 1:100 with the running buffer (supplied in the Milenia Genline HybriDetect) and 2 µL were loaded on the lateral flow strips and, besides this, it was also tested the loading of 2 µL of the RPA reaction directly on the lateral flow, as it is schematized in Figure 11. After loading the samples in the lateral flow strips, the strips were placed on Eppendorf tubes containing 100 µL of running buffer to help the reagents to move along the strip.

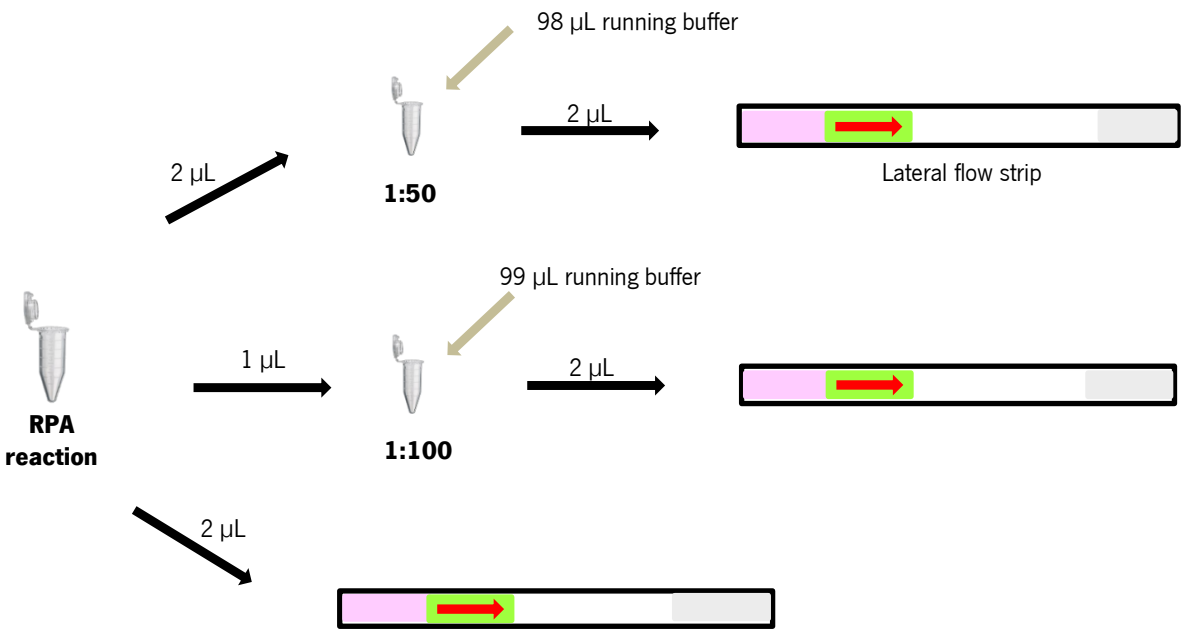

Figure 11 – Schematic representation of the steps done before loading the samples on the lateral flow strips.

2.9.2 Modified primers

Another approach tested in lateral flow, as it can be seen in Figure 12, was to use the labelled reverse primer (idhR-bio) but, instead of using the nfo probe, to label the forward primer with FAM on the 5' – F-idhRPA-FAM – and use the TwistAmp® Basic Kit (TwisDx).

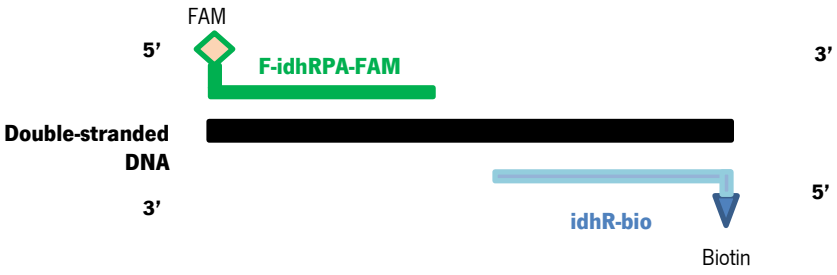

Figure 12 – Schematic representation of the F-idhRPA-FAM and idhR-bio.

For the RPA reactions, 3 different primers concentrations were tested: 480 nM that is the recommended concentration from the supplier, 780 nM which was the concentration used in all the previous RPA reactions and 630 nM, a concentration between 480 nM and 780 nM, in order to optimize the method. The reaction was done for a total volume of 25 µL with 2 µL of DNA template – the strains from MUM and CECT were used as template –, at 37 °C for 40 minutes. Then, 1:50 and 1:100 dilutions were done with the running buffer before loading 2 µL of the RPA reaction on the lateral

flow strips. Besides these dilutions, it was also done a cleaning step following the NucleoSpin® gDNA Clean-up protocol (Macherey-Nagel) and a 1:10 dilutions and only after that, 2 µL of the RPA reaction was loaded on the lateral flow strip. Those dilutions were made to avoid false positive results from reaction components, as the running buffer reduces non-specific binding on the membrane. After loading the samples in the lateral flow strips, the strips were placed on Eppendorf tubes containing 100 µL of running buffer to help the reagents to move along the strip.

2.9.3 Specificity Assay

To test the RPA assay for its specificity to confirm if it was amplifying the right sequence, the RPA reaction was performed with the TwistAmp® nfo kit (TwistDx) with 120 nM of the idh-nfo-probe and 780 nM for both primers, F-idhRPA and idhR-bio, for a total volume of 25 µL with 2 µL of DNA template. Then, 2 µL of the RPA reaction were loaded directly in the lateral flow strip since with the tests done before it was possible to see that no false positive result was appearing when the RPA reactions were being loaded without dilution. The mold strains that are *idh* +, fungal strains that are *idh* - and bacterial strains used in this assay are presented in Table 2.

2.9.4 Sensitivity Assay

To determine the lowest detectable DNA concentration, ten-fold dilutions were prepared with the same strain used for the qPCR sensitivity assay. For this assay it was used the TwistAmp® nfo kit (TwistDx) and the RPA reaction was performed with the same conditions mentioned above. Then, 2 µL of the RPA reactions were loaded with no dilution on the lateral flow strip.

3. RESULTS AND DISCUSSION

3.1 qPCR

3.1.1 Primers Evaluation

In Table 6, it is presented the Cq and the Tm of the qPCRs performed in order to evaluate the better set of primers to detect the *idh* gene.

Table 6 – Quantification cycles and melting temperatures of the qPCR primers evaluation

Set of primers	Concentration	Species name	Number of the strain	*Idh*	Cq	Tm /°C
FC2/IDH2	400 nM	*Penicillium expansum*	17.41	+	Undetermined	-
		Penicillium tunisiense	17.62	-	Undetermined	-
		Penicillium expansum	17.69	+	Undetermined	-
		NTC			Undetermined	-
F-idhtrb/R-idhtrb	720 nM	*Penicillium expansum*	17.41	+	22.66	88.08
		Penicillium tunisiense	17.62	-	35.98	87.93
		Penicillium expansum	17.69	+	23.96	88.23
		NTC			Undetermined	-
idh2444/idh2887	200 nM	*Penicillium expansum*	17.41	+	16.95	87.93
		Penicillium tunisiense	17.62	-	23.55	87.93
		Penicillium expansum	17.69	+	16.57	88.07
		NTC			Undetermined	-
idh2444/idhBP1R	200 nM	*Penicillium expansum*	17.41	+	11.21	86.88
		Penicillium tunisiense	17.62	-	18.15	86.59
		Penicillium expansum	17.69	+	12.82	86.74
		NTC			Undetermined	-

All the strains are from MUM (*Micoteca da Universdade do Minho*) culture collection

Cq – Quantification cycle

Tm – Melting temperature

With the set of primers FC2/IDH2, no amplification product was detected. Since the *Penicillium tunisiense* (MUM 17.62) does not have the *idh* gene, it was expected to have a negative result. However, the other 2 strains used in the experiment have the gene, so it should have been an amplification. In this way, since there was no amplification of these strains, this set was excluded to be part of the rest of the project.

Comparing the other 3 sets of primers, there was non-specific amplification of the *Penicillium tunisiense* (MUM 17.62) in all the experiments. As the recorded Tm's of this strain were 87.58 ± 0.69 °C, this unspecific amplification was not a result of primers dimers since they have relatively lower Tm

compared to the qPCR products. However, with the set F-idhtrb/R-idhtrb, it got a higher Cq value (Cq= 35.98). For this reason, this was the selected set to continue the rest of the project. In Figure 13, where the amplification plots of the experiments are represented, it is possible to see that the *Penicillium tunisiense* (MUM 17.62) was amplifying later than the *Penicillium expansum* (MUM 17.41 and MUM 17.69) in all the experiments shown in Figure 13a, 13b and 13c and in the qPCR performed with the set F-idhtrb/R-idhtrb, it amplified even later.

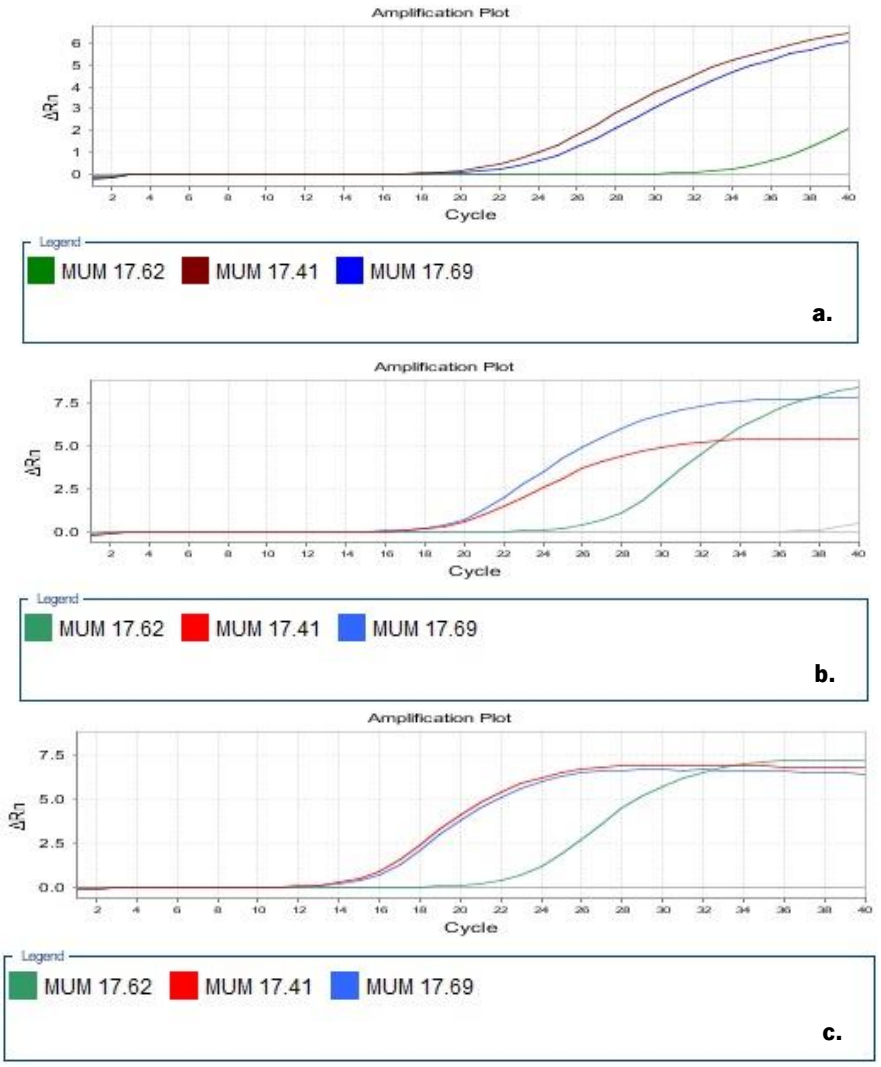

Figure 13 – Comparison of the amplification plots of the qPCR performed with the 3 different set of primers: **a.** It was used the set of primers F-idhtrb/R-idhtrb; **b.** The set idh2444/idh2887; **c.** The qPCR was performed with the set of primers idh2444/idhBP1R. The strains are from *Micoteca da Universidade do Minho* (MUM) culture collection, where the strain number 17.62 is a *Penicillium tunisiense* (*idh* -) and the strains number 17.41 and 17.69 are *Penicillium expansum* (both species are *idh* +).

3.2 qPCR Optimization

3.2.1 Primers concentration optimization

In Table 7, it is possible to see the Cq values and the Tm of the qPCRs performed with different primers concentration in order to optimize the assay.

Table 7 – Quantification cycles and melting temperatures of the annealing temperature optimization

Specie name	Number of the strain	*Idh*	Concentration of the primers	Average Cq ± SD	Average Tm/°C ± SD
Penicillium expansum	17.69	+	500 nM	23.52 ± 0.43	88.25 ± 0.00
			600 nM	21.78 ± 0.12	88.17 ± 0.08
			700 nM	21.03 ± 0.05	88.10 ± 0.00
			720 nM	21.50 ± 0.13	88.32 ± 0.08
			800 nM	20.98 ± 0.00	88.25 ± 0.00

The strain is from MUM (*Micoteca da Universidade do Minho*) culture collection
Cq – Quantification cycle
Tm – Melting temperature
SD – Standard Deviation

As it was expected, there was amplification of the *Penicillium expansum* (MUM 17.69), that is *idh* +, in all the experiments as it can also be seen in Figure 14. From all the experiments, the qPCR performed with a primer's concentration at 700 nM and 800 nM got the lowest Cq values, Cq = 21.03 and Cq = 20.98 respectively. Between these 2 concentrations, the lowest one was selected in order to avoid primer dimers which are more likely to be formed with higher primer concentrations. Besides, the Tm of all experiments was 88.22 ± 0.08 °C, which means that the primers were amplifying the same sequence.

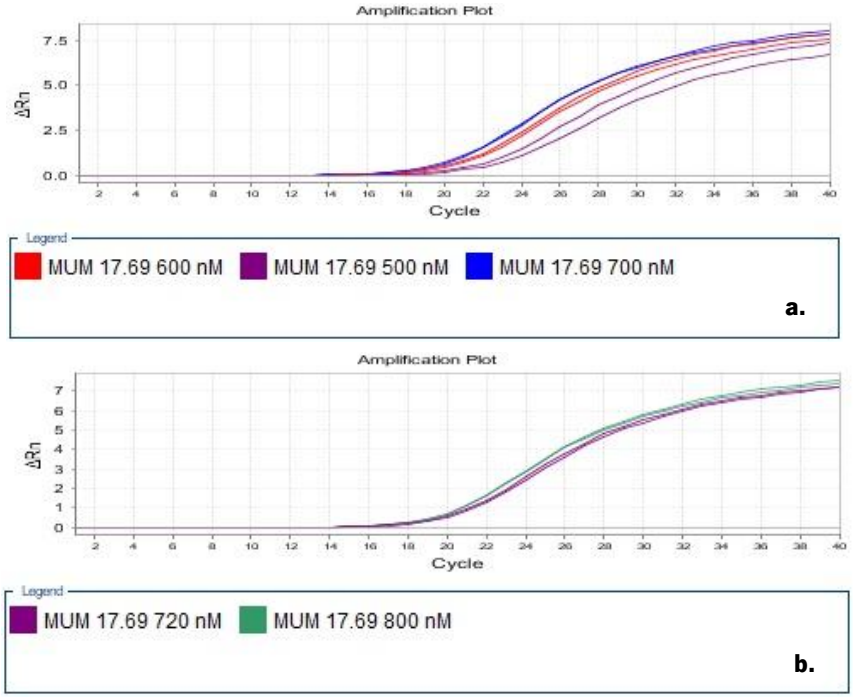

Figure 14 – Amplification plots of the qPCRs performed with the set of primers F-idhtrb/R-idhtrb, with 5 different primers concentrations: **a.** Primers concentration at 500 nM, 600 nM and 700 nM; **b.** Primers concentration at 720 nM and 800 nM. The strain number 17.69 is a *Penicillium expansum* and it is from the *Micoteca da Universidade do Minho* (MUM) culture collection.

3.2.2 Annealing temperature optimization

As it can be seen in Figure 15, there was only amplification of the *Penicillium expansum* (MUM 17.69) at temperatures of 58 °C, 59 °C and 60 °C and in any experiment the *Penicillium tunisiense* (MUM 17.62), that does not have the *idh* gene, amplified. Since this strain had amplified on the evaluation of the primers, it was used another stock culture of the *Penicillium tunisiense* (MUM 17.62) and, this time, it showed a negative result as it was expected. From the 3 temperatures mentioned before, with at 58 °C it was possible to obtain the lower Cq value of 23.98. In this sense, 58 °C was the temperature selected as the optimal temperature of the qPCR.

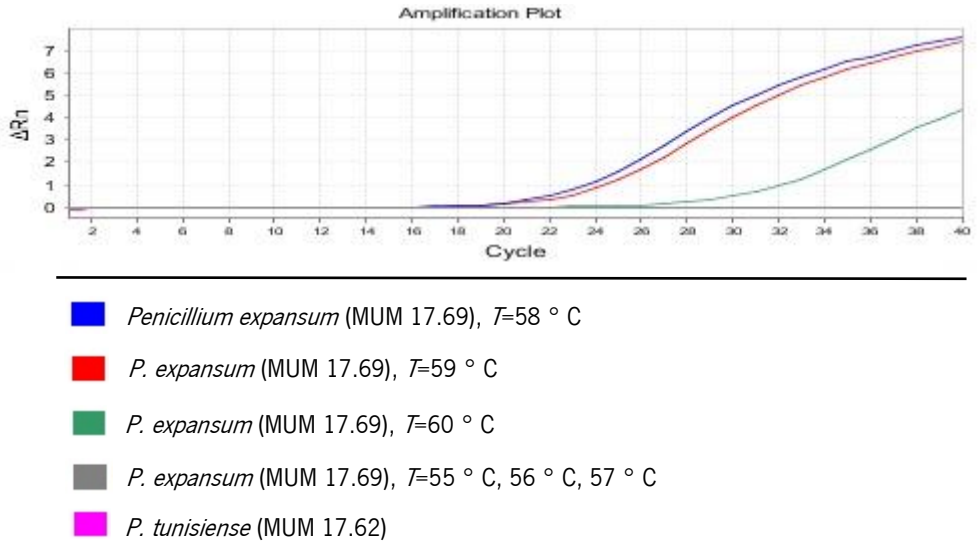

Figure 15 – Amplification plot of the qPCR performed to optimize the annealing temperature. It was used the *Penicillium expansum*, strain number 17.69 (*idh* +), and the *Penicillium tunisiense*, strain number 17.62 (*idh* -), both from the *Micoteca da Universidade do Minho* (MUM) culture collection.

3.2.3 Annealing time optimization

As it can be observed in Table 8, when compared the Cq values of the qPCR performed with an annealing time of 45 seconds to a time of 1 minute, that is the time used in all the previous qPCR performances, it is possible to see that the Cq value increased from 23.98 to 25.89. For this reason, the annealing time of 30 seconds was not tested and 1 minute was chosen as the optimal time of this assay.

Table 8 – Quantification cycles and melting temperatures of the annealing time optimization

Annealing Time	Specie name	Number of the strain	*Idh*	Average Cq ± SD	Average Tm/°C ± SD
45 s	*Penicillium expansum*	17.69	+	25.89 ± 0.10	88.17 ± 0.08
	NTC			Undetermined	-
1 min	*Penicillium expansum*	17.69	+	23.98	88.10

The strain is from MUM (*Micoteca da Universidade do Minho*) culture collection
Cq – Quantification cycle
Tm – Melting temperature
SD – Standard Deviation

3.3 qPCR Specificity Assay

In Table 9 the Cq values and the Tm's of the mold strains selected for the evaluation of the inclusivity of the qPCR Assay are presented.

Table 9 – Results of the qPCR assay inclusivity evaluation

Species name	Culture collection	Number of the strain	*Idh*	Average Cq ± SD	Average Tm/°C ± SD
Penicillium expansum	MUM	17.41	+	18.81 ± 0.02	88.25 ± 0.00
Penicillium expansum	MUM	17.69	+	21.18 ± 0.07	88.18 ± 0.08
Penicillium expansum	CECT	2919	+	19.88 ± 0.03	85.79 ± 0.08
Penicillium griseofulvum	CECT	2278	+	18.94 ± 0.03	87.88 ± 0.07
Penicillium spp.	Partner Company	Fungus 2	ND	28.20 ± 0.22	87.50 ± 0.00

MUM – *Micoteca da Universidade do Minho*

CECT – Spanish Type Culture Collection

Cq – Quantification cycle

Tm – Melting temperature

SD – Standard Deviation

As it can be seen, all the strains amplified which was expected since they are *idh* +. The Tm's vary from 86 °C to 88 °C probably due to some variations in the sequence that the primers are amplifying. In (Rodríguez *et al.*, 2011) the patulin-producing strains used as template showed also Tm values ranging from 86.90 °C to 88.50 °C. Regarding the species that are *idh* -, there was no amplification which prove that the set of primers selected are amplifying the *idh* sequence (Figure 16).

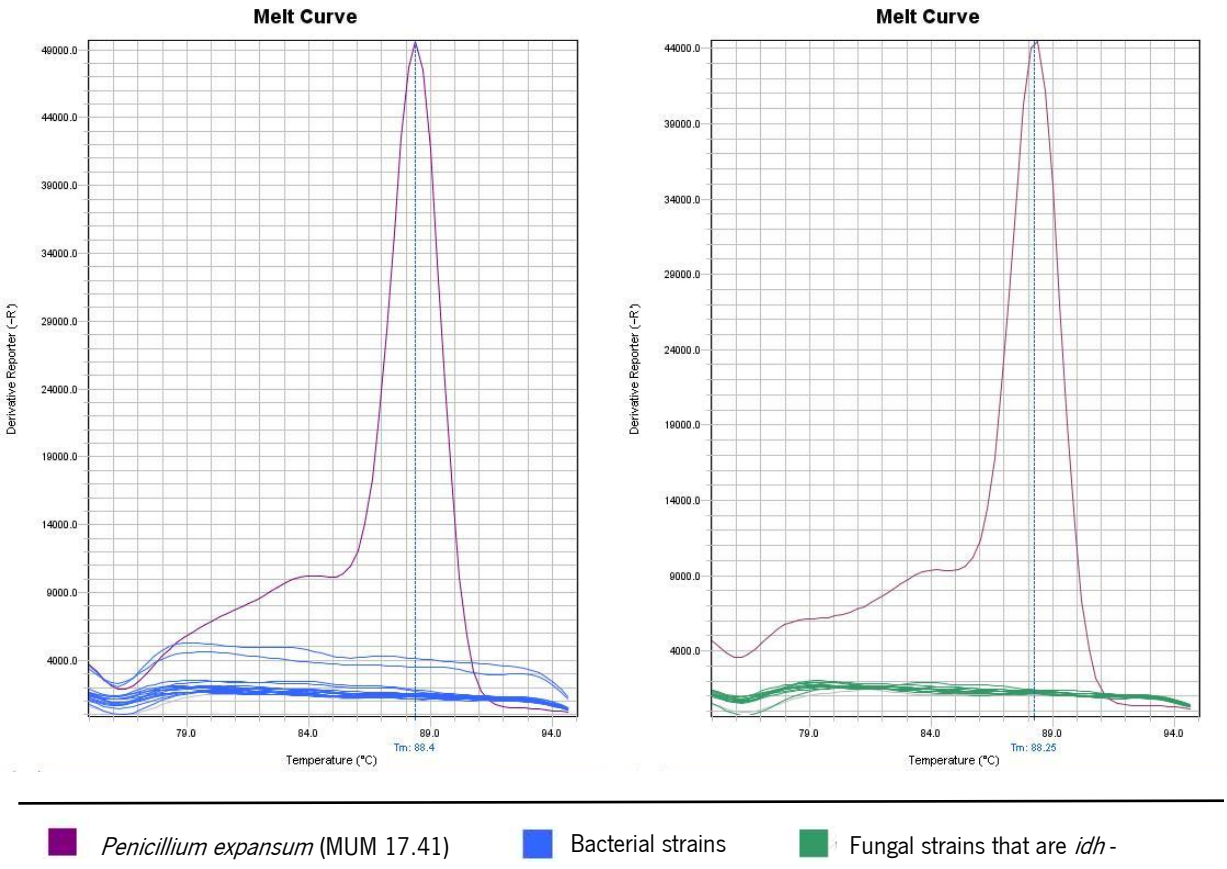

Figure 16 – Melt curves of the qPCR assay exclusivity evaluation.

3.4 qPCR Sensitivity Assay

In Table 10 can be seen the Cq values and the Tm's of the qPCRs performed with ten-fold serial dilutions of the *Penicillium expansum* (MUM 17.69).

Table 10 – Cq values and Tm's of the qPCRs of the ten-fold dilutions

Sample	Average Cq ± SD	Average Tm/°C ± SD
MUM 17.69 0	17.10 ± 0.15	88.40 ± 0.00
MUM 17.69 -1	19.38 ± 0.16	88.35 ± 0.07
MUM 17.69 -2	23.08 ± 0.13	88.35 ± 0.07
MUM 17.69 -3	27.35 ± 0.20	88.30 ± 0.07
MUM 17.69 -4	31.47 ± 0.11	88.25 ± 0.00
MUM 17.69 -5	36.12 ± 0.71	88.05 ± 0.07
MUM 17.69 0 2nd test	18.39 ± 0.05	88.25 ± 0.00
MUM 17.69 -1 2nd test	20.85 ± 0.06	88.20 ± 0.07
MUM 17.69 -2 2nd test	24.60 ± 0.12	88.20 ± 0.07
MUM 17.69 -3 2nd test	28.93 ± 0.25	88.15 ± 0.07
MUM 17.69 -4 2nd test	33.22 ± 0.17	88.25 ± 0.00
MUM 17.69 -5 2nd test	36.94 ± 0.05	88.05 ± 0.07
MUM 17.69 0 3rd test	15.20 ± 0.02	88.25 ± 0.00
MUM 17.69 -1 3rd test	17.79 ± 0.17	88.20 ± 0.07
MUM 17.69 -2 3rd test	21.68 ± 0.12	88.20 ± 0.07
MUM 17.69 -3 3rd test	26.23 ± 0.21	88.20 ± 0.14
MUM 17.69 -4 3rd test	30.73 ± 0.44	88.30 ± 0.07
MUM 17.69 -5 3rd test	35.60 ± 1.03	88.10 ± 0.12

MUM – *Micoteca da Universidade do Minho*

Number of the strain – 17.69

Specie name – *Penicillium expansum*

idh +

Cq – Quantification cycle

Tm – Melting temperature

SD – Standard Deviation

Between ten-fold dilutions the difference in the Cq values should be of 3.3 and, in this case, the dilutions did not match to this trend. This poor linearity of the Cq values across the serial dilution can be due to pipetting errors. It is possible to observe that the Tm's, for all samples, it was 88 ± 0.10 °C which indicates that the same sequence was amplified in all tests. Besides, there were amplifications down to a DNA concentration of 0.13 pg/µL. In (Tannous *et al.*, 2015) with the developed qPCR in the mentioned study, was possible to have amplifications down to 40 pg/µL, which shows that the developed qPCR in this project is more sensitive.

3.5 qPCR Efficiency

With the Log_{10} of quantified DNA concentration of the *Penicillium expansum* (MUM 17.69) and the Cq average of the three independent experiments performed, presented in Table 11, the plot in Figure 17 was constructed.

Table 11 – Log_{10} of the DNA concentration of the MUM 17.69 and the Cq values

Sample	Log_{10} concentration (pg/µL)	Average Cq ± SD
MUM 17.69	1110	16.90 ± 1.39
MUM 17.69 -1	110	19.34 ± 1.34
MUM 17.69 -2	- 890	23.13 ± 1.28
MUM 17.69 -3	- 1890	27.50 ± 1.20

The strain number 17.69 is a *Penicillium expansum*

MUM – *Micoteca da Universidade do Minho*

idh +

-1, -2, -3 – Dilutions

SD – Standard Deviation

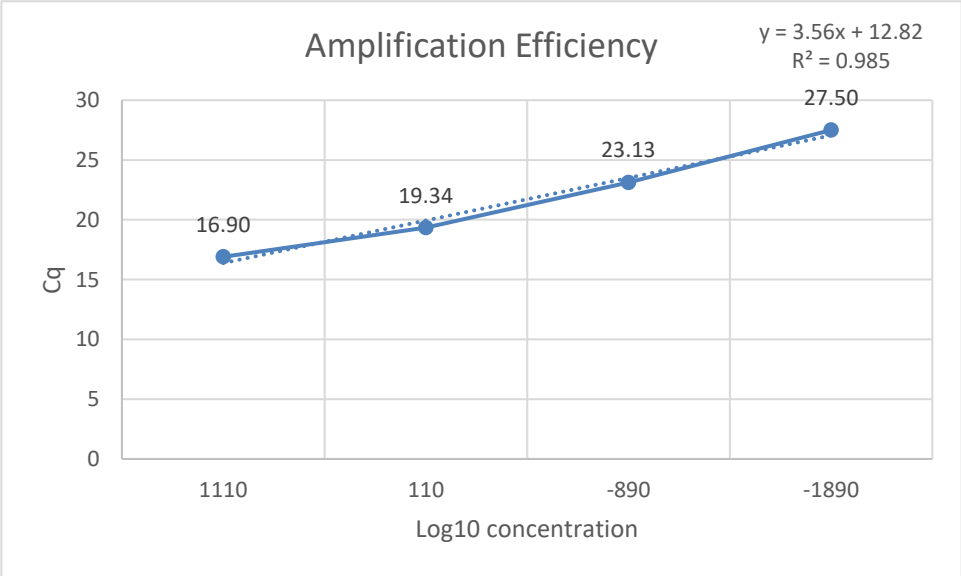

Figure 17 – Plot of the Amplification Efficiency.

With a slope of -3.56, the Amplification Efficiency is of 90.94 %, so the reaction is efficient down to a DNA concentration of 13 pg/µL, however it is also possible to detect less than that and reach a concentration of 0.13 pg/µL like it was shown on the Sensitivity Assay.

As it is mention in the chapter of the introduction, the efficiency should be, at least, of 90 %. There are some factors that can influence the efficiency, such as the length of the amplicon, since the

larger the amplicon, the more difficult it is to amplify, as well the reaction dynamics and enzyme quality. In (Rodríguez *et al.*, 2011), that used the same set of primers, got an efficiency of 100.9 %. This difference may be due to pipetting errors, for the reason that any reaction component in other concentration other than the ideal one, as well as poor homogenization, can decrease the amplification efficiency.

3.6 RPA Reaction

3.6.1 RPA Specificity Assay

All the mold strains presented that are *idh* + were confirmed by the agarose gel electrophoresis through the detection of a 250 bp amplicon, as it is shown in Figure 18.

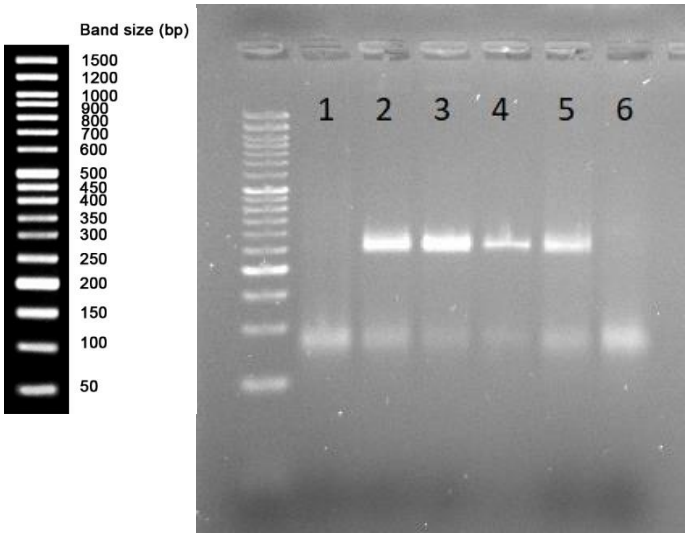

Figure 18 – Agarose gel electrophoresis of RPA inclusivity assay performed with the set of primers F-idhRPA/R-idhRPA: **1** – *Penicillium tunisiense* (MUM 17.62) [*idh* -]; **2** – *P. expansum* (MUM 17.41); **3** – *P. expansum* (MUM 17.69); **4** – *P. expansum* (CECT 2278); **5** – *P. griseofulvum* (CECT 2919); **6** – NTC.

In Figure 19, can be seen the gel electrophoresis of the RPA exclusivity assay in which a 250 bp amplicon was detected in the strains used as P.C., that was the case of the *Penicillium expansum* (MUM 17.41). On the other fungal and bacterial strains that are *idh* - nothing was detected.

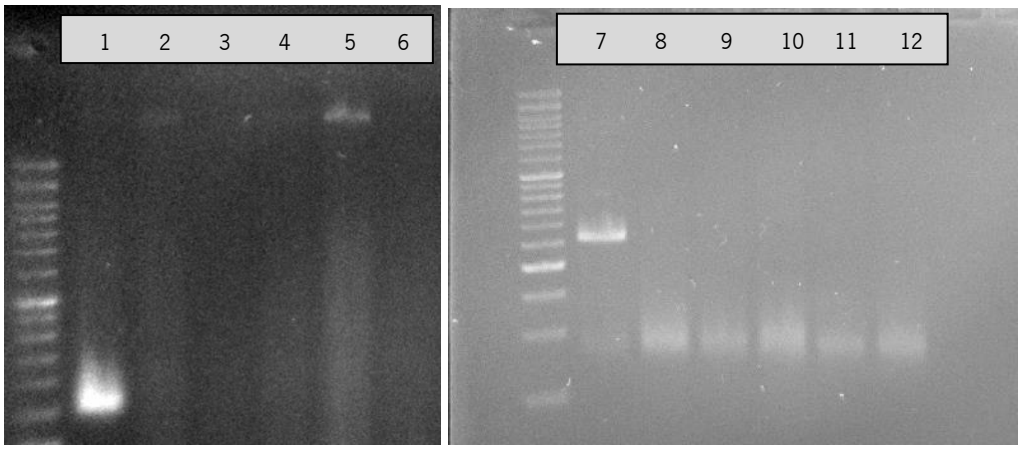

Figure 19 – Agarose gel electrophoresis of RPA exclusivity assay performed with the set of primers F-idhRPA/R-idhRPA: **1** – *Penicillium expansum* (MUM 17.41); **2** – *Mucor* spp. (Fungi 3); **3** – *Meyerozyma* spp.; **4** – *Penicillium* spp. (Fungi 1); **5** – *Mucor* spp. (Fungi 5); **6** – NTC.

3.7 RPA combined with Lateral Flow Strips

3.7.1 Modified primers approach: F-idhRPA-FAM/idhR-bio

As presented in Figure 20, there were false positive results when the RPA reactions were diluted 1:50 before the load on the lateral flow strip, with the 3 primer's concentrations, since both the NTC and the *P. tunisiense* (MUM 17.62), which is *idh* -, shown a test line on the lateral flow strip.

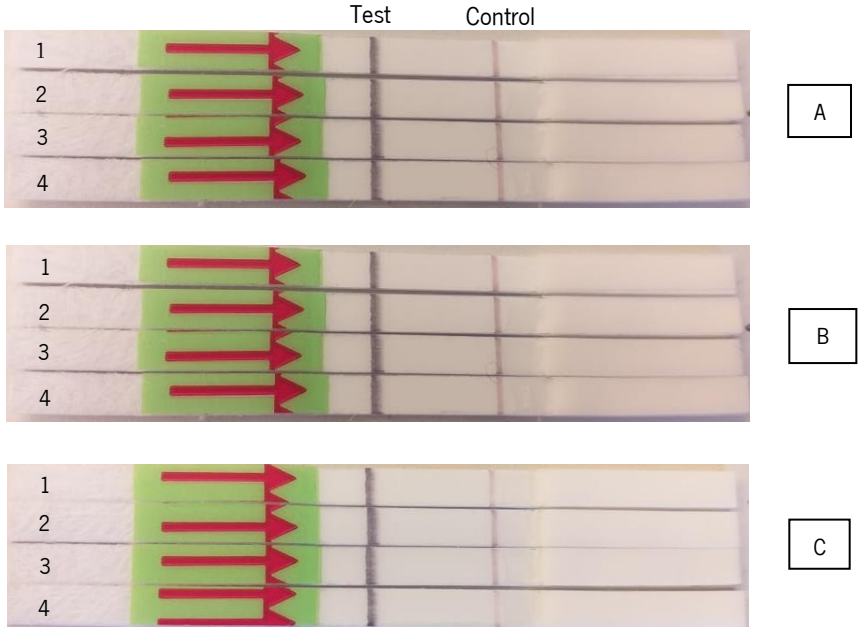

Figure 20 – Test of 1:50 dilution of the samples before being loaded on the lateral flow strips with three different primer concentrations on lateral flow strips: **A** – 480 nM; **B** – 630 nM; **C** – 780 nM, where **1** – *Penicillium expansum* (CECT 2278); **2** – *P. griseofulvum* (CECT 2919); **3** – *P. tunisiense* (MUM 17.62); **4** – NTC. The set of primers used was F-idhRPA-FAM/idhR-bio.

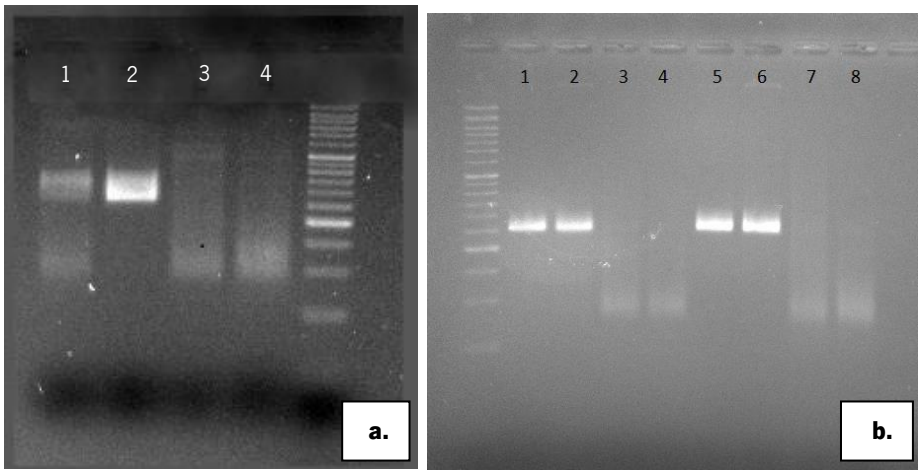

Figure 21 – Agarose gel electrophoresis of the RPA performed with the set of primers F-idhRPA-FAM/idhR-bio **a**. at 480 nM: **1** – *Penicillium expansum* (CECT 2278); **2** – *P. griseofulvum* (CECT 2919); **3** – *P. tunisiense* (MUM 17.62); **4** – NTC; **b.** at 630 from 1 to 4: **1** – *Penicillium expansum* (CECT 2278); **2** – *P. griseofulvum* (CECT 2919); **3** – *P. tunisiense* (MUM 17.62); **4** – NTC and at 780 nM from 5 to 8: **5** – *Penicillium expansum* (CECT 2278); **6** – *P. griseofulvum* (CECT 2919); **7** – *P. tunisiense* (MUM 17.62); **8** – NTC.

In the gels, presented in Figure 21, it is possible to see that both the *Penicillium tunisiense* (MUM 17.62) and the NTC, did not present any band, which indicates that there were no contaminations or unspecific amplification in the RPA reaction. For this, further tests were performed at a 1:100 dilution before loading the RPA reaction on the lateral flow strips. As it is showed below in Figure 22, the false positive results happened once again.

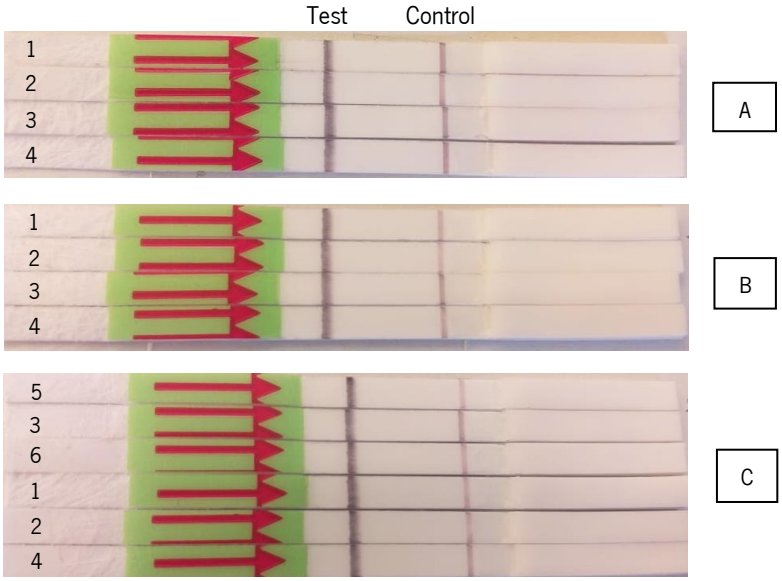

Figure 22 – Test of 1:100 dilution of the samples before being loaded on the lateral flow strips with three different primer concentrations on lateral flow strips: **A** – 480 nM; **B** – 630 nM; **C** – 780 nM, where **1** – *Penicillium expansum* (CECT 2278); **2** – *P. griseofulvum* (CECT 2919); **3** – *P. tunisiense* (MUM 17.62); **4** – NTC; **5** – *P. expansum* (MUM 17.41); **6** – *P. expansum* (MUM 17.69). The set of primers used was F-idhRPA-FAM/idhR-bio.

A purification of the RPA reaction was done with the purification kit and then a 1:10 dilution before loading the samples on the lateral flow strip and even then, the *Penicillium tunisiense* (MUM 17.62) and the NTC showed a positive result, as it can be seen in Figure 23.

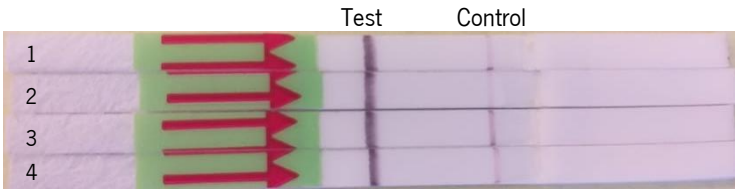

Figure 23 – Test of 1:10 dilution of the samples before being loaded on the lateral flow strip with a concentration of the primers at 780 nM, where **1** – *Penicillium expansum* (CECT 2278); **2** – *P. griseofulvum* (CECT 2919); **3** – *P. tunisiense* (MUM 17.62); **4** – NTC.

The appearance of false positive results has been pointed out in several studies as a disadvantage of the RPA-LFS (Du *et al.*, 2018; Liu *et al.*, 2017b) and some points are suggested to avoid these results. An example is a dilution step for the amplified products because crowing reagents used in the buffer can cause false positive results. Since in all the tests there were false positive results that were not solved with the dilutions of the samples, a possible explanation for the false positive results can be due to non-specific binding from a non-specific interaction between the gold nanoparticles and the streptadavin on the test line, regardless of the presence or not of the target in the sample.

3.7.2 nfo probe approach: idh-nfo-probe/F-idhRPA/idhR-bio

As the clearest bands obtained in the gel when the three different primer concentrations were tested previously, were achieved with the concentration of 780 nM, this was the concentration chosen to continue the lateral flow tests with the probe.

As it can be seen in Figure 24b, the samples that have the *idh* gene showed a test line on the lateral flow strip, except for the *Penicillium griseofulvum* (CECT 2919), although a band appeared on the gel (Figure 24a). So, the test was repeated, and it was possible to observe an extremely faint test line, presented in Figure 25.

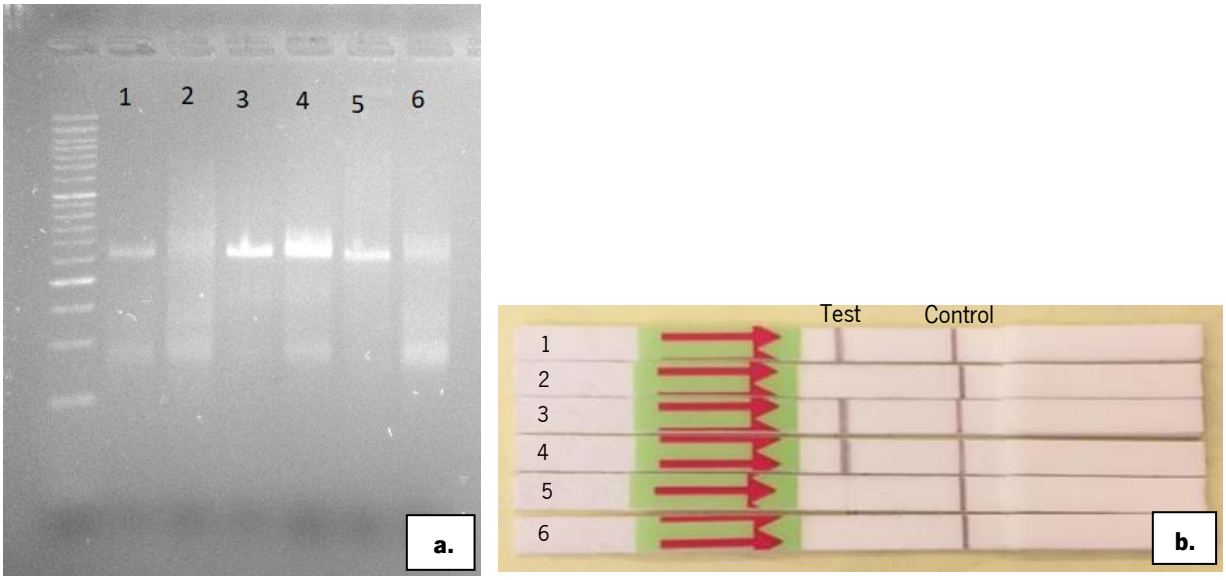

Figure 24 – **a.** Agarose gel electrophoresis of the RPA performed with the set of primers F-idhRPA/idhR-bio at 780 nM and the idh-nfo-probe at 120 nM; **b.** Test of 1:50 dilution of the samples before being loaded on the lateral flow strip. **1** – *Penicillium expansum* (MUM 17.41); **2** – *P. tunisiense* (MUM 17.62); **3** – *P. expansum* (MUM 17.69); **4** – *P. expansum* (CECT 2278); **5** – *P. griseofulvum* (CECT 2919); **6** – NTC.

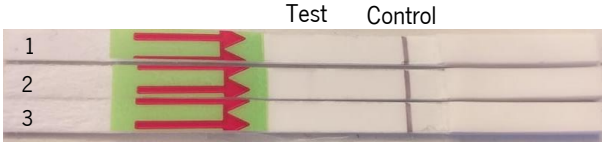

Figure 25 – Second test of 1:50 dilution of the samples before being loaded on the lateral flow strip with a concentration of the primers at 780 nM and 120 nM for the probe, where **1** – *Penicillium tunisiense* (MUM 17.62); **2** – *P. griseofulvum* (CECT 2919); **3** – NTC. It was used the primers F-idhRPA/idhR-bio and the idh-nfo-probe.

Since the extremely faint test line appeared, another test was done without dilution and with a dilution of 1:100 before loading the sample on the lateral flow strip and it was possible to verify that, without dilution, a test line appeared which, although clear, was stronger than the ones formed when the RPA reaction was diluted – Figure 26.

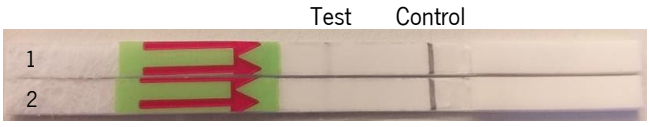

Figure 26 – Test with the sample *Penicillium griseofulvum* (CECT 2919) without dilution **(1)** and with a 1:100 dilution **(2)** before loading the sample on the lateral flow strip. It was used the primers F-idhRPA/idhR-bio and the idh-nfo-probe.

Thereby, it was decided to continue the tests without diluting the RPA reactions before loading them on the lateral flow strip. To ensure that there were no false positive results by not diluting the reactions, *Penicillium expansum* (MUM 17.41), *P. expansum* (MUM 17.69), *P. expansum* (CECT

2278), *P. griseofulvum* (CECT 2919) that are *idh* + and *P. tunisiense* (MUM 17.62) that is *idh* - were tested. As the results in Figure 27 show, there were no false positive results.

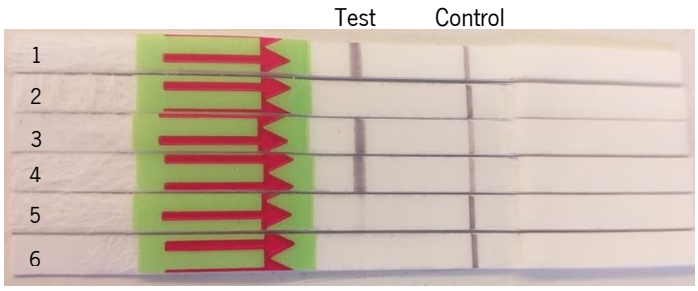

Figure 27 – Test without dilution of the samples before being loaded on the lateral flow strip with a concentration of the primers at 780 nM and 120 nM for the probe, where **1** – *Penicillium expansum* (MUM 17.41); **2** – *P. tunisiense* (MUM 17.62); **3** – *P. expansum* (MUM 17.69); **4** – *P. expansum* (CECT 2278); **5** – *P. griseofulvum* (CECT 2919); **6** – NTC. It was used the primers F-idhRPA/idhR-bio and the idh-nfo-probe.

Since in the gel for the *Penicillium griseofulvum* (CECT 2919) a 250 bp amplicon appear and in the lateral flow the test line does not, a possible reason for this may be that the primers are annealing but the probe cannot bind.

3.7.3 Lateral Flow Specificity Assay

As there were no false positives results, a specificity assay was performed, and the results can be seen in Figure 28b. All *idh* - samples only registered the control line on the lateral flow strip and, regarding the samples that have the gene, besides the control line, a test line was shown, which was consistent with the result of the gel, presented in Figure 28a.

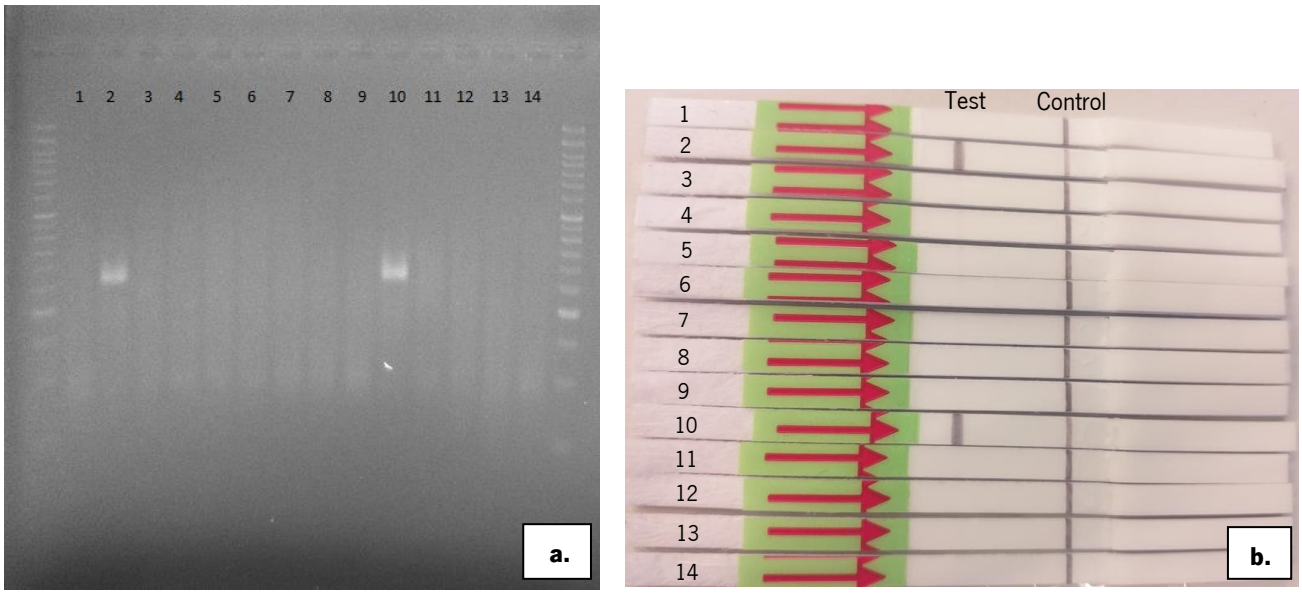

Figure 28 – **a.** Agarose gel electrophoresis of the RPA Specificity Assay performed with the set of primers F-idhRPA/idhR-bio at 780 nM and the idh-nfo-probe at 120 nM; **b.** Selectivity assay of the lateral flow strips with no dilution of the samples before being loaded on the strip. **1** – NTC; **2** – *Penicillium expansum* (MUM 17.69); **3** – *Penicillium* spp.; **4** – *Meyerozyma* spp.; **5** – *Mucor* spp. (Fungi 3); **6** – *Penicillium* spp. (Fungi 1); **7** – *Neosartorya fischeri*; **8** – *Pichia fermentans*; **9** – NTC; **10** – *P. expansum* (MUM 17.69); **11** – *Salmonella* Typhimurium (4595); **12** – *Escherichia coli* (434); **13** – *Salmonella* Enteridis (82); **14** – *Listeria innocua* (2110).

3.7.4 Lateral Flow Sensitivity Assay

Regarding the sensitivity assay of the lateral flow, it was possible to register a test line until the DNA concentration of the *Penicillium expansum* (MUM 17.69) of 13 pg/µL, revealing a strong positive sign while the gel gave an extremely faint band, as it can be seen in Figure 29b and the gel in Figure 29a. This sensitivity was also possible to achieve in other studies, such as in (Yin *et al.*, 2017).

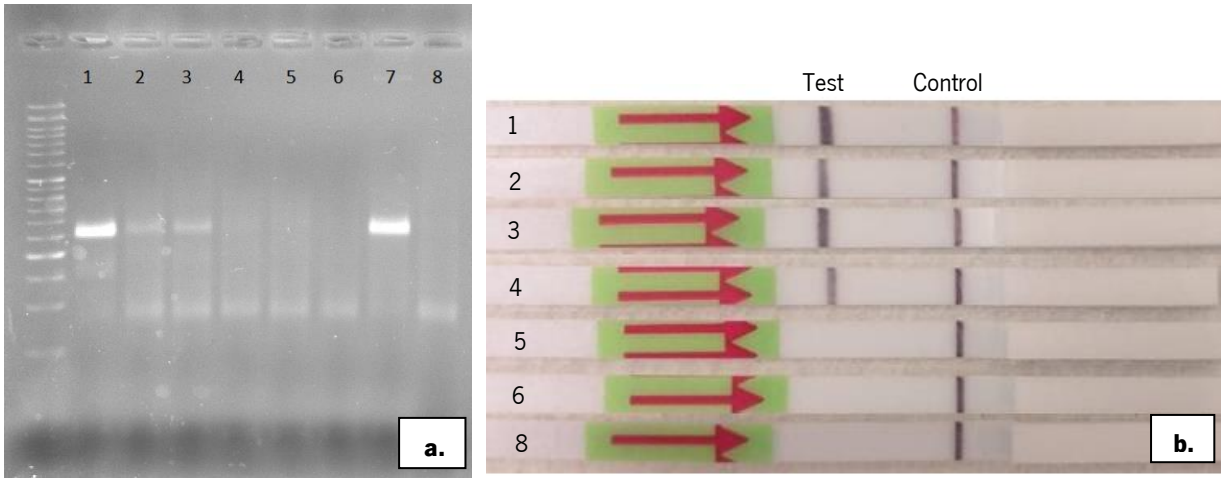

Figure 29 – **a.** Agarose gel electrophoresis of the RPA Sensitivity Assay performed with the set of primers F-idhRPA/idhR-bio at 780 nM and the idh-nfo-probe at 120 nM; **b.** Sensitivity assay of the lateral flow strips with no dilution of the samples before being loaded on the strip. **1** – *Penicillium expansum* (MUM 17.69) (DNA concentration of 13000 pg/µl); **2** – Dilution -1 of MUM 17.69 (DNA concentration of 1300 pg/µl); **3** – Dilution -2 of MUM 17.69 (DNA concentration of 130 pg/µl); **4** – Dilution -3 of MUM 17.69 (DNA concentration of 13 pg/µl); **5** – Dilution -4 of MUM 17.69 (DNA concentration of 1.3 pg/µl); **6** – Dilution -5 of MUM 17.69 (DNA concentration of 0.13 pg/µl); **7** – *P. griseofulvum* (CECT 2919) *; **8** – NTC.

* Does not make part of the Sensitivity Assay.

3.8 Evaluation of the developed methods

3.8.1 qPCR

The results of the evaluation assay of the developed qPCR method are presented in Table 12 below.

The *Penicillium polonicum* (MUM 17.37), which is *idh* +, obtained a negative result even though it exhibited amplification, it did not present the expected Tm. The remaining samples showed the results that were expected, and the Tm varied from 87 °C to 88 °C that are the common temperatures recorded in the qPCRs that were performed throughout the project.

Table 12 – Evaluation assay of the developed qPCR method

Species name	Number of the strain	*Idh*	Average Cq ± SD	Average Tm/°C ± SD
Penicillium expansum	00.02	+	15.36 ± 0.32	88.02 ± 0.11
Penicillium crustosum	17.31	+	23.57 ± 0.21	87.36 ± 0.00
Penicillium solitum	17.33	+	18.07 ± 0.10	87.81 ± 0.00
Penicillium polonicum	17.37	+	35.40 ± 0.43	-
Penicillium expansum	17.38	+	15.27 ± 0.02	88.25 ± 0.00
Penicillium expansum	17.44	+	14.73 ± 0.07	88.40 ± 0.00
Penicillium brevicompactum	17.45	+	20.28 ± 0.08	87.21 ± 0.00
Penicillium expansum	17.67	+	14.70 ± 0.60	88.10 ± 0.00
Aspergillus flavus	18.41	-	Undetermined	-
Aspergillus niger	19.133	-	35.91 ± 1.73	-
Penicillium expansum	17.41	+	13.18	88.40

All the strains are from MUM (*Micoteca da Universidade do Minho*) culture collection

Cq – Quantification cycle

Tm – Melting temperature

SD – Standard Deviation

The qPCR was repeated with the *Penicillium polonicum* (MUM 17.37) to verify that there was no error in the qPCR previously performed and, as can be seen in Table 13, there was no peak in the melt curve again.

Table 13 – Repetition of the qPCR performed with the *Penicillium polonicum* (MUM 17.37)

Species name	Number of the strain	*Idh*	Average Cq ± SD	Average Tm/°C ± SD
Penicillium solitum	17.33	+	18.07 ± 0.10	87.81 ± 0.00
Penicillium polonicum	17.37	+	36.40 ± 0.98	-
Penicillium expansum	17.41	+	13.42	88.40

All the strains are from MUM (Micoteca da Universidade do Minho) culture collection

Cq – Quantification cycle

Tm – Melting temperature

SD – Standard Deviation

One possibility for the negative result of this sample may be the fact that it requires a greater number of cycles to amplify. In this way, a qPCR was performed with 50 cycles, instead of 40 cycles. In this qPCR, fungal strains that do not have the *idh* gene were also tested to exclude non-specific results.

As it is presented in Figure 30, there was no amplification of the samples that do not have the *idh* gene and, in one of the replicas of *Penicillium polonicum* (MUM 17.37), a small peak of the melt curve was recorded at a temperature of 87.51 °C.

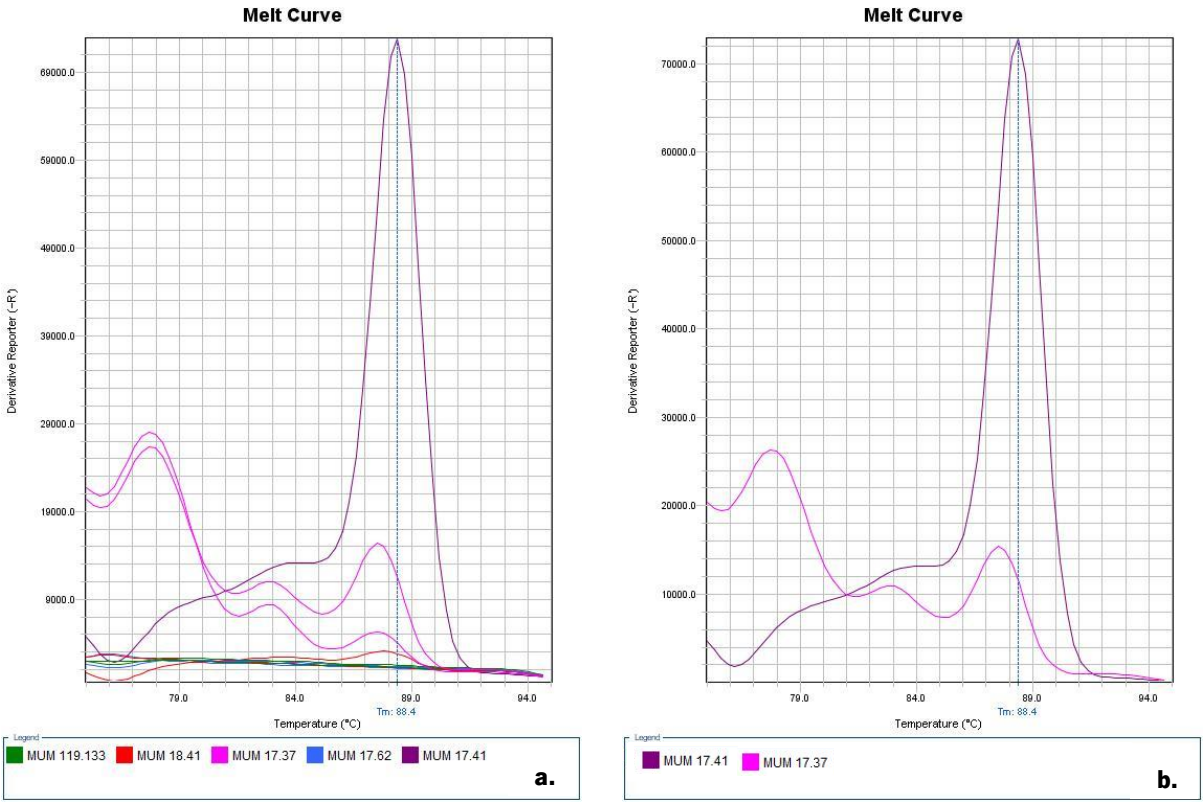

Figure 30 – Melt curves of the qPCR performed with 50 cycles. **a.** The melt curve of all the samples; **b.** The melt curve of one of the replicates of the *Penicillium polonicum* (MUM 17.37) and of the *Penicillium expansum* (MUM 17.41) used as P.C. The *idh* - fungal strains used were *P. tunisiense* (MUM 17.62), *Aspergillus flavus* (MUM 18.41) and *A. niger* (MUM 19.133).

3.8.2 RPA Reaction

Regarding to the RPA reactions, as can be seen in Figure 31a and 31b, a 250 bp amplicon was obtained on the gel in the samples that are *idh* +, except for the *Penicillium brevicompactum* (MUM 17.45), which has the gene, and has not shown any band. In addition to this, the sample of the *Penicillium crustosum* (MUM 17.31), despite showing a band, was very clear.

A quantification of the DNA of these two samples was made to verify if they had enough DNA. The *Penicillium crustosum* (MUM 17.31) had a DNA concentration of 13.1 ng/µL and *Penicillium brevicompactum* (MUM 17.45) had 32.4 ng/µL. Thus, both samples were purified with the purification kit and, in addition, the *Penicillium brevicompactum* (MUM 17.45) was also diluted to a 1:2 dilution. With these samples, a qPCR was performed to see how they would react because, if the Cq value decreased, in the case of the *Penicillium crustosum* (MUM 17.31), it might mean that there were contaminants in the reaction and, in the case of the *Penicillium brevicompactum* (MUM 17.45), if the Cq value decreased, it could mean that there was too much DNA concentration which could be inhibiting the amplification. However, as it is presented in Table 14, the Cq values increased.

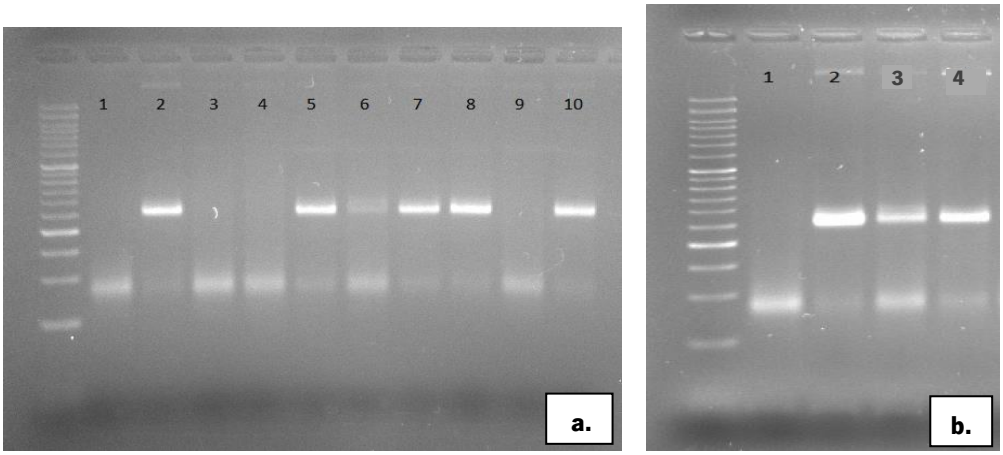

Figure 31 – Agarose gel electrophoresis of the RPA Specificity Assay performed with the set of primers F-idhRPA/R-idhRPA at 780 nM. **a. 1** – NTC; **2** – *Penicillium expansum* (MUM 17.41); **3** – *Aspergillus niger* (MUM 19.133); **4** – *A. flavus* (MUM 18.41); **5** – *P. expansum* (MUM 00.02); **6** – *P. crustosum* (MUM 17.31); **7** – *P. expansum* (MUM 17.38); **8** – *P. expansum* (MUM 17.44); **9** – *P. brevicompactum* (MUM 17.45); **10** – *P. expansum* (MUM 17.67); **b. 1** – NTC; **2** – P. expansum (MUM 17.41); **3** – *P. solitum* (MUM 17.33); **4** – *P. polonicum* (MUM 17.37).

Table 14 – Cq values and Tm's of the qPCR performed with the purified samples

Species name	Number of the strain	*Idh*	Average Cq ± SD	Average Tm/°C ± SD
Penicillium crustosum	17.31	+	23.57 ± 0.21 [a]	87.36 ± 0.00 [a]
			33.10 ± 23.40 [b]	86.91 ± 0.00 [b]
Penicillium brevicompactum	17.45	+	20.28 ± 0.08 [a]	87.21 ± 0.00 [a]
			28.81 ± 0.22 [c]	86.24 ± 0.11 [c]
			26.44 ± 0.30 [d]	87.06 ± 0.00 [d]
Penicillium expansum	17.41	+	19.54	88.40

All the strains are from MUM (*Micoteca da Universidade do Minho*) culture collection

[a] – Values from the previous qPCR performance

[b] – The sample was purified with the purification kit

[c] – The sample was purified with the purification kit and diluted 1:2

[d] – The sample was diluted 1:2

SD – Standard Deviation

A new extraction of the *Penicillium brevicompactum* (MUM 17.45) was made and the qPCR was repeated with the samples from this extraction and with the samples that were being used previously to ensure that the mismatch of the results with the RPA and with the qPCR were not due to any problem in the DNA extraction. Again, the samples amplified, as shown in Table 15.

Table 15 – Cq values and Tm of the qPCR performed with the new DNA extraction of the MUM 17.45

Species name	Number of the strain	*Idh*	Average Cq ± SD	Average Tm/°C ± SD
Penicillium brevicompactum	17.45	+	21.56 ± 0.13 [a]	87.21 ± 0.00 [a]
			21.40 ± 0.00 [b]	86.91 ± 0.00 [b]
Penicillium expansum	17.41	+	14.24	88.25

The strains are from MUM (*Micoteca da Universidade do Minho*) culture collection

[a] – Values from the previous qPCR performance

[b] – Sample from the new DNA extraction

SD – Standard Deviation

Given these results, although it was not possible to find the sequence of the *idh* gene in this species, a possible reason for this sample to be positive in the qPCR and negative with RPA is the fact that the primers of the qPCR are able to bind with the sequence and those of the RPA are not.

3.8.3 RPA combined with Lateral Flow Strips

In relation to the lateral flow, in Figure 32b is possible to see the result of the evaluation tests. All *idh* - samples only registered the control line on the lateral flow strip. Regarding the samples that have the gene, only *Penicillium brevicompactum* (MUM 17.45) did not register a test line on the lateral flow strip, which was consistent with the result of the gel (Figure 32a). It was not possible to find the sequence of the *idh* gene of this specie, however, a possible reason for this negative result may the fact that the probe is not binding to the target DNA.

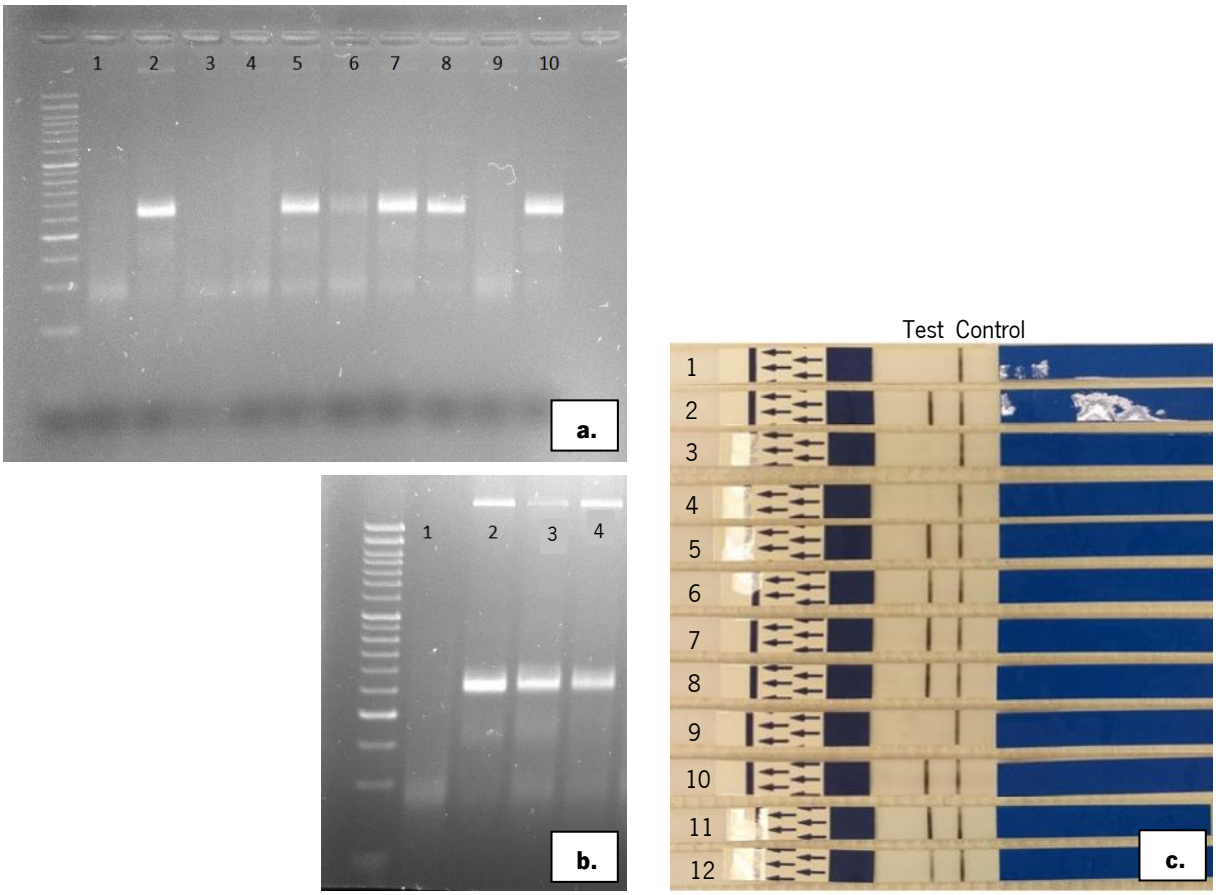

Figure 32 – **a.** Agarose gel electrophoresis of the RPA Specificity Assay performed with the set of primers F-idhRPA/idhR-bio at 780 nM and the idh-nfo-probe at 120 nM: **1** – NTC; **2** – *Penicillium expansum* (MUM 17.41); **3** – *Aspergillus niger* (MUM 19.133); **4** – *A. flavus* (MUM 18.41); **5** – *P. expansum* (MUM 00.02); **6** – *P. crustosum* (MUM 17.31); **7** – *P. expansum* (MUM 17.38); **8** – *P. expansum* (MUM 17.44); **9** – *P. brevicompactum* (MUM 17.45); **10** – *P. expansum* (MUM 17.67); **b.** Agarose gel electrophoresis of the RPA Specificity Assay performed with the set of primers F-idhRPA/idhR-bio at 780 nM and the idh-nfo-probe at 120 nM: **1** – NTC; **2** – *Penicillium expansum* (MUM 17.41); **3** – *P. solitum* (MUM 17.33); **4** – *P. polonicum* (MUM 17.37); **c.** Specificity assay of the lateral flow strips with no dilution of the samples before being loaded on the strip. **1** – NTC; **2** – *Penicillium expansum* (MUM 17.41); **3** – *Aspergillus niger* (MUM 19.133); **4** – *A. flavus* (MUM 18.41); **5** – *P. expansum* (MUM 00.02); **6** – *P. crustosum* (MUM 17.31); **7** – *P. expansum* (MUM 17.38); **8** – *P. expansum* (MUM 17.44); **9** – *P. brevicompactum* (MUM 17.45); **10** – *P. expansum* (MUM 17.67); **11** – *P. solitum* (MUM 17.33); **12** – *P. polonicum* (MUM 17.37).

4. CONCLUSION

The combination of the RPA method with the LFS for the naked-eye detection of PAT producing molds worked for all the *Penicillium expansum* strains, however with two other species some problems occurred. The *P. griseofulvum* (CECT 2919) (*idh* +) registered a test line extremely light and with the *P. brevicompactum* (MUM 17.45) (*idh* +) did not register a test line in the LFS. Thus, the method developed proved to be specific for the specie *P. expansum*, the main producer of PAT.

In order to improve this combination for the detection of PAT producing fungi, further studies will have to be performed to elucidate the reasons why the *idh* was not detected in the *P. griseolfulvum* (CECT 2919) and *P. brevicompactum* (MUM 17.45). Nevertheless, RPA combined with LFS for rapid detection of PAT producing fungi has proved to be a promising technique for its fastness and simplicity of handling.

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