Food Control 84 (2018) 246–254

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Detection and identification of five common internal grain insect pests by multiplex PCR

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ARTICLE INFO

Article history:
Received 9 June 2017
Received in revised form
28 July 2017
Accepted 5 August 2017
Available online 7 August 2017

Keywords:
Insect pests
Internal feeders
Grain cereals
Detection
Identification
Multiplex PCR

ABSTRACT

Consumer demands for better quality food have led to research on new tools aimed at early detection of insect pests in agro food industries. In these industries, internal grain feeders are the most concerning pests because of being the first colonizers of stored grain and transmitting harmful micro-organisms, such as fungi and bacteria, which affect both food quality and human health. The immature stages of these cosmopolitan pests develop and feed inside the grain kernels, easily evading visual analysis in food industries. To avoid the consequent underestimation of contamination by internal pest species, a multiplex PCR approach for the detection and identification of the five most concerning primary pests that develop and feed hidden inside the grain kernels (*Rhyzopertha dominica*, *Sitophilus granarius*, *S. oryzae*, *S. zeamais* and *Sitotroga cerealella*) has been developed. Results have demonstrated that the designed protocol can be used for the diagnosis of grain contamination with high sensitivity (0.1 pupa/kilo of rice, except for *R. dominica* 10 pupae/kilo). This tool proved to be specific when 46 other species potentially present in grain commodities were tested, and to detect all developmental stages of *S. zeamais* in different kinds of grain (barley, maize, oat, spelt, rice and wheat) and pasta (macaroni). Detection was even possible when grain was treated with CO_2. Finally, in order to confirm its applicability in food industries, this method has also been tested in real commercial grain samples from a pasta mill. The multiplex PCR method presented here could be of great help when making commercial decisions aimed at satisfying the current market demands.

1. Introduction

Cereal grain, either as raw or processed material, constitutes 80% of consumed food (Pimentel et al., 1997). Unfortunately, since the routine procedures before food consumption harbor several pest species, the safety and security of this food are susceptible to being affected when grain is stored, transported and processed (Hagstrum, Reed, & Kenkel, 1999; Nopsa et al., 2015; Stejskal, Hubert, Aulicky, & Kucerova, 2015). Phillips and Throne (2010) estimated post-harvest losses due to stored-product insects of between 9% and 20% or more in developed and developing countries, respectively.

Among insect pests, internal feeders, which are primary pest species that develop and feed inside the grain kernels, have generally been regarded as the most damaging pests of stored cereals (Toews, Campbell, Arthur, & Ramaswamy, 2006). These species not only consume large quantities of grain, but are hidden inside the grain kernels during their preimaginal development. Furthermore, these insects facilitate grain contamination by secondary pests, which might increase the damage to the food by depositing faeces and cast skins. This all causes localized increases in heat and moisture that might lead to accelerated mold growth and mycotoxin production threatening the grain quality and human health (Beti, Phillips, & Smalley, 1995; Phillips & Throne, 2010; Shah & Khan, 2014).

Because these internal feeders are not easily detected and removed during routine cleaning or processing practices, a situation where contamination is underestimated can often occur (Perez-Mendoza, Throne, Maghirang, Dowell, & Baker, 2005; Toews et al., 2006). Hence, Storey, Sauer, Ecker, and Fulk (1982) reported that 12% of wheat samples from export loads contained hidden internal insects in the United States. Consequently, it is not surprising that primary pests are mainly present in filth contamination of finished cereal products (Trematerra, Stejskal, & Hubert, 2011). The most concerning internal feeders in grain worldwide are the

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http://dx.doi.org/10.1016/j.foodcont.2017.08.002
0956-7135/

following five species: *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae); three species of the genus *Sitophilus* (*S. granarius* (L.), *S. oryzae* (L.) and *S. zeamais* (Motschulsky) (Coleoptera: Curculionidae)) and *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) (Castañé & Riudavets, 2015; Toews et al., 2007; Trematerra, Ianiro, Athanassiou, & Kavallieratos, 2015). Also, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), which is an important internal feeder of stored maize and cassava, has also become a serious pest in tropical and subtropical areas (CABI, 2017).

The increased consumer concerns about food safety and wholesomeness have produced a general trend toward a decrease in tolerance of live insects in food (Hagstrum et al., 1999; Trematerra, 2013). This situation has brought changes in grain standards in terms of food quality, which has emphasized the need for regulative approaches in the commercial sequence from the growers to consumers, driving market changes, politically and industrially (FDA, 1997; Stejskal, Aulicky, & Kucerova, 2014). For example, domestic flour millers generally report zero tolerance for live insects, while the national agency in charge of food safety in the US, the Food & Drug Administration (FDA), has produced administrative guidelines that set maximum levels for natural or unavoidable defects in food for humans (FDA, 1997). Because failure to control insect infestations when they initially occur in storage (or in the field) can lead to extensive contamination of the stored grain that could affect food security (Nopsa et al., 2015), the importance of establishing strategies for early diagnosis of insect contamination is evident.

With the purpose of detecting insect contamination, hazard analyses are routinely conducted in grain industries. At the moment, grain is inspected with sieves and all sorts of methods to crack kernels for the identification of insect adults, damaged kernels or insect fragments. However, when those visual methods are used alone, internal infestations are not evident (Brader et al., 2002; Hubert, Nesvorna, & Stejskal, 2009). Additionally, insect fragments produced are not equivalent at each development stage of the pest (immature stages and eggs have low to no chitin content, respectively), highlighting the need for other analysis approaches (Brabec, Pearson, Flinn, & Katzke, 2010; Perez-Mendoza et al., 2005).

Nowadays, there is a panoply of techniques available for insect detection (Hagstrum & Subramanyam, 2014; Neethirajan, Jayas, & White, 2007; Parkin, 1956; Phillips & Throne, 2010; Trematerra, 2013). Unfortunately, although acoustic emissions, ELISA, NIR and X-ray are diagnostic techniques that are capable of detecting hidden infestations (Chen & Kitto, 1993; Fleurat-Lessard, Tomasini, Kostine, & Fuzeau, 2006; Fornal et al., 2007; Maghirang, Dowell, Baker, & Throne, 2003; Perez-Mendoza et al., 2005), they also present some limitations. Among their main drawbacks, some of these approaches do not accomplish the cost-time compromise, while others are less sensitive to low population densities (Neethirajan et al., 2007; Nowaczyk et al., 2009).

In recent years, the application of molecular techniques has gained importance in food diagnostics because of their simplicity, speediness and specificity (Obrepalska-Steplowska, Nowaczyk, Holysz, Gawlak, & Nawrot, 2008; Solà, Lundgren, Agusti, & Riudavets, 2017). DNA-based approaches such as PCR have become relevant for the analysis of genetically modified organisms (GMOs) in food (Ciabatti, Froiio, Gatto, Amaddeo, & Marchesi, 2006; Datukishvili, Kutateladze, Gabriadze, Bitskinashvili, & Vishnepolsky, 2015), as well as for identifying insect species (Barcenas, Unruh, & Neven, 2005; Zhang et al., 2016), providing an excellent method for both adult and immature forms even for sibling species (Correa, de Oliveira, Braga, & Guedes, 2013; Hidayat, Phillips, & FrenchConstant, 1996; Peng, Lin, Chen, & Wang, 2002). Among PCR approaches, the multiplex is the most suitable technique for screening multiple species because it is able to simultaneously identify all species present in a sample within a single PCR reaction (King et al., 2011; Solà, Agusti, & Riudavets, 2015). It also offers simplicity of execution, a reduction of carry-over errors and time saving, compared to the traditional singleplex PCR (Bai et al., 2009).

A multiplex PCR approach was here developed and described as a reliable molecular method for routine detection and identification of the five main internal feeders in grain samples, namely: the lesser grain borer (*R. dominica*), the three grain weevils species (*S. granarius*, *S. oryzae*, and *S. zeamais*) and the Angoumois grain moth (*S. cerealella*). One major consideration was to perform a large specificity test covering a wide range of species potentially present in stored grain facilities. The sensitivity of this protocol has been determined taking into consideration all developmental stages of the insect pests (egg to adult), the post-mortem time, different grain types and the potential of a grain treatment with modified atmospheres. Finally, some real commercial samples have been analyzed using the developed method.

2. Material and methods

2.1. Biological material

Five target pest species (*R. dominica*, *S. granarius*, *S. oryzae*, *S. zeamais* and *S. cerealella*) were maintained in laboratory cultures at IRTA (Barcelona, Spain). Coleopteran species were grown on organic rice (Eco-Salim, Maquefa, Spain), while the lepidoptera species was reared on maize (Crit d'or, Granollers, Spain). All insect cultures were maintained in climatic chambers at 28 °C, 70% RH, and 16L: 8D.

Forty-six species were tested in the specificity test of the designed primers. The specimens of these non-target species were found in alimentary factory surveys since 1997 or came from laboratory colonies (Table 1). Identification of all species was performed using morphological keys before storing the specimens in alcohol 96° or frozen at −20 °C until DNA extraction.

The following insect-free grain and pasta were also tested for the characterization of the protocol: brown rice and wheat (Eco-Salim, Maquefa, Spain), maize (Crit d'or, Granollers, Spain), spelt (Biogrà, Polinyà, Spain), barley and oat (Celnat, Saint-Germain-Laprade, France) and macaroni pasta (Castagno Bruno, Giaveno TO, Italy). In order to ensure that the food samples used in the analyses were insect-free, a sample of 125 g of each grain and pasta was maintained at 28 °C, and 70% RH for three months and checked for insect adult presence by sieving it with a 2 mm mesh. Also, for the same purpose, three samples of 5 g of each grain type and pasta were first ground with a laboratory grinder (Laboratory Mill 3303, Perten Instruments, Hägersten, Sweden) to be then analyzed for insect presence with the multiplex PCR described below.

2.2. DNA extraction and multiplex PCR

Two different DNA extraction protocols were performed: one for the insect DNA extraction and another for the grain (infested or not). Insect DNA was extracted from whole individuals using a SpeedTools Tissue DNA extraction kit (Biotools, Madrid, Spain) and eluted in 100 μl of AE buffer. In addition, 5 g (or 10 g in the case of the sensitivity test) of homogenized infested grain and pasta DNA was extracted with the Extragen Alimentos extraction kit (Sistemas Genómicos, Valencia, Spain) following the manufacturer's instructions and eluted in 1 ml of purified water. One negative control was included in each DNA extraction group. DNA was stored at −20 °C until PCR.