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Food Control 17 (2006) 741–744
FOOD CONTROL
www.elsevier.com/locate/foodcont

Primers from the isoepoxydon dehydrogenase gene of the patulin biosynthetic pathway to indicate critical control points for patulin contamination of apples

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Received 11 November 2004; accepted 27 April 2005

Abstract

The mycotoxin patulin is subjected to European Union statutory limits, and has action levels imposed in the United States of America in fruit products. Samples from soil and an orchard were analysed by primers of the isoepoxydon dehydrogenase gene of the patulin biosynthetic pathway as an HACCP procedure in an initial assessment. A non-orchard soil appeared to inhibit the reaction. However, the reaction was positive from orchard soils. Negative results from orchard samples appeared to be because of target DNA being absent. Bramley samples contained the gene frequently. The method may be useful in the determination of critical control points.

Keywords: Patulin; Apples; Control; IDH gene

1. Introduction

Patulin is an important mycotoxin. Mycotoxins are fungal secondary metabolites which contaminate food, feed and drink (CAST, 2003). Furthermore, the compound has strict regulatory limits in fruit products imposed by the European Union (Byrne, 2004). An HACCP protocol to control patulin in apple juice is available (FAO, 2003), although it does not unfortunately include patulin analytical steps so it is difficult to conclude that the procedures improve the situation.

Patulin is associated with *Penicillium expansum*, the fungus responsible for the blue mould rot of, for example, apples and grapes. However, many other fungi can produce patulin and possess the isoepoxydon dehydrogenase (IDH) gene of the patulin biosynthetic pathway. Specific primers for the IDH gene can amplify the 600bp fragment in various fungi in which a correlation was observed between (a) possession of the gene and patulin detection and (b) non-possession and non-detection (Paterson, 2004).

PCR procedures are being developed increasingly to identify fungi (Haughland, Varma, Wymer, & Vesper, 2004; Marek, Annamalai, & Venkitanarayanan, 2003). These methods are intended ultimately to be used on "virgin" samples. There is a small number of mycotoxins about which the metabolic pathways have been determined sufficiently to enable the development of gene probes of the pathway (Edwards, O'Callaghan, & Dobson, 2002; O'Callaghan, Caddick, & Dobson, 2003). Patulin is such a mycotoxin (Paterson, 2004; Paterson, Kozakiewicz, Locke, Brayford, & Jones, 2003). It is of considerable interest to investigate what occurs when primers are applied to environmental samples where patulin-producing fungi may occur, and will assist in determining "weak spots" in commodity systems (Paterson, Venâncio, & Lima, 2004) with a view to

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doi:10.1016/j.foodcont.2005.04.016

control via HACCP procedures. Towards these ends, the current work provides data on the analysis by the IDH primers of "virgin" soil and orchard samples.

2. Materials and methods

Earth from Egham, UK was sterilised twice at 121 °C for 20 min. Approximately 25 g was mixed with a colony (ca. 1 g) of *P. expansum* (IMI 232297) which was grown on yeast extract sucrose agar for 7 days at 25 °C. It was demonstrated previously to possess the IDH gene and patulin was detected from the strain (see Paterson et al., 2003). Also, the DNA of the fungus was used as a control in electrophoretic gels. Uninoculated sterile and unsterile soil were used as samples for testing the IDH primers.

Samples were from an organic orchard in Ascot, UK of (a) living and dead twigs, (b) soil, (c) fallen apples, and (d) bark from upper and/or lower branches of trees. The varieties were Annie Elizabeth (AE), Cellini, Bramley, and Winter Hawthornden (WH). Samples (ca. 2 g) were ground using a mortar and pestle and stored at −20 °C. DNA extraction was by direct lysis using SDS, Na_2HPO_4 and polyethylene glycol (Saano & Lindström, 1995). The pellet was washed (70% ethanol), dried, re-suspended in 50 µl TE buffer, and purified (Wizard Clean Up System (Promega)). A standard PCR reaction was used as reported in Paterson (2004) and which is not repeated here. MJ Research or Hybaid Thermal Cyclers were employed. Two µl DNA of ×400 DNA from *P. expansum* (IMI 232297) extracted by the Ceris method (Paterson et al., 2003) was added to a soil sample (ca. 2 g) by simple pipetting and mixing. Reamplification involved applying the above procedures to an aliquot of the initially amplified sample of the same size as used in the original analysis. Appropriate positive and negative controls were employed in all cases.

3. Results

The gene was not detected in uninoculated soil from the non-orchard Egham site (Tables 1 and 2). A ×10 dilution of unpurified DNA did not exhibit the gene (Table 1). The 600bp fragment was observed from the sterilised and unsterilised soil which had been mixed with *P. expansum* (i.e. inoculated) at ×100 dilution (Table 1). Similarly, a positive result was obtained at ×100 and ×400 dilution in Table 2, although the band appeared fainter at ×400. The gene fragment was also observed only at the ×100 and 400 dilutions for samples to which was added DNA for the inoculated and uninoculated soils (Table 2; Fig. 1).

Table 1
Presence of the 600bp fragment from non-orchard soil samples

Sample	Dilution				
	0	10U	10	50	100
Sterilised inoculated	−	−	−	−	+
Uninoculated	−	−	−	−	−
Unsterilised inoculated	−	−	−	−	+
Uninoculated	−	−	−	−	−

U = unpurified.

Table 2
Presence of the 600bp fragment in non-orchard soil samples

Sample	Dilution		
	0, 10, 50	100	400
Pure DNA not added			
Sterilised inoculated	−	+	+f
Sterilised uninoculated	−	−	−
Unsterilised inoculated	−	+	+f
Unsterilised uninoculated	−	−	−
Added DNA			
Sterilised inoculated	−	+	+
Sterilised uninoculated	−	+	+
Unsterilised inoculated	−	+	+
Unsterilised uninoculated	−	+	+

DNA from *P. expansum* was added to the indicated samples. f = faint band.

Results were positive for the (a) living twig, (b) bark from an upper branch, and (c) both soil samples from below the Bramley tree (Table 3) at all dilutions. Reamplification of the dead Bramley twigs and lower bark samples remained negative. However, the positive results from the other samples were confirmed.

Few positive results were obtained from the samples of the other trees (Table 4). Only, Cellini twigs and a

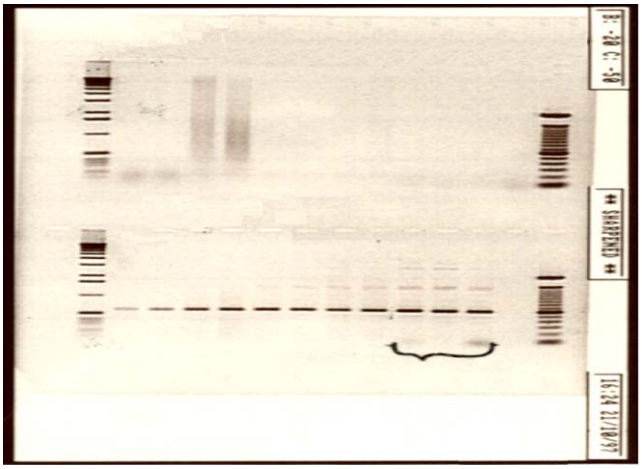

Fig. 1. Gel of the IDH gene fragment from soil samples with *P. expansum* DNA added. Top half of gel and tracks from left to right: DNA marker; four undiluted samples; four ×10 dilution; four ×50 dilution; DNA marker. Lower half of gel and tracks from left to right: four ×100 dilution; four ×400 dilution; three positive controls; one negative control; DNA marker. The gene fragment is the obvious band in the lower gel.

Table 3
Bramley apple related samples tested for the IDH gene at various dilutions

Sample	Dilutions			
	10	200	400	1000
A. Initial amplification				
Dead twig	−	−	−[a]	−[a]
Living twig	+	+	+[a]	+
Soil from 5 cm	+	+	+[a]	+[a]
Soil from 10 cm	+	+	+	+
Upper bark	+[a]	+[a]	+	+
Lower bark	−[a]	−[a]	−	−
B. Reamplification samples including control were tested at three dilution ×(100, 500, 1000) therefore each datum below is from three analyses. Results are of two separate analyses of the same sample				
Dead Bramley twigs			−	−
Living Bramley twigs			+	
Soil from 5 cm depth			+	+
Upper bark from tree	+	−		
Lower bark from tree	−	−		

[a] Samples for reamplification (see Panel B).

Table 4
Amplification of the gene fragment in various apple-related samples

Sample	Dilutions				
	0–10	10	200	400	1000
A. Initial amplification					
Cellini bark	−	−[a]	−	−[a]	−
Cellini twigs	+	+	+[a]	+	−[a]
Fallen Cellini apple	+[a]	−	−	−	−[a]
Top soil Cellini	−	−[a]	−[a]	−	−
Fallen WH apple	−	−[a]	−	−[a]	nd
AE bark	−	−[a]	−	−[a]	−

B. Reamplification. Each sample analysed at ×10, 100, 500 so each datum is from three analyses

	10	200	400	1000
Cellini bark	−		−	
Cellini twig		−		+
Fallen Cellini apple	−			−
Top soil Cellini	+	−		
Fallen WH apple	+			+
AE bark	−			−

[a] Samples for reamplification (see Panel B).

fallen Cellini apple were positive although a dilution effect was observed. Reamplification confirmed the negative result for Cellini and AE barks. However, the positive result for the Cellini apple was not confirmed and the ×200 dilution for the initially positive Celinni twig extract was negative, although the ×1000 reamplification was positive. The initially negative results for Cellini top soil and the WH apple were demonstrated to be positive by reamplification.

4. Discussion

The bands observed at 600bp represent fragments of the IDH gene. The positive results from the initial amplifications could be artefacts; however, the reamplification experiments indicate this is not the case. Obviously, the diversity of organisms in environmental samples could be large. The combined genomes could conceivably yield a 600bp fragment which was not from the IDH gene which may be more likely to occur than from a pure fungus culture. However, this applies to any proposed PCR methods for fungi, etc. of environmental samples. The results of samples dilutions indicted that inhibitors were responsible for the negative results from soil (Tables 1 and 2) (see Paterson, 2004).

On the other hand, dilution did not appear to affect the more frequent negative results from the other orchard samples to a large extent. This indicated that negative results were because the IDH gene was not present. Bramley trees and soil had more positive results than for the other samples in Tables 3 and 4. Bramley apples are known to be particularly susceptible to patulin contamination (Moake, Padilla-Zakour, & Worobo, 2005). It was interesting that the Bramley soil samples were positive, and the Cellini soil was also, although only after reamplification. In contrast, the Egham soil was negative and was not close to apple trees. This may indicate a connection between patulin fungi in soil and the presence of apples trees.

Difficulty remains in interpreting the results in terms of the patulin contamination of apples. It is probable that patulin concentrations in the samples described here would be too low for detection without the use of the most sensitive equipment. The results do indicate the potential for patulin production. More orchards could usefully be investigated in future work. In fact, work was intended on assessing whether the gene could be detected more frequently from orchards which produced juice with high patulin concentrations. However, locating orchards that produced juice with significantly different levels proved impossible and so the experiment had to be abandoned (Paterson, unpublished). Experiments to determine precisely how far from the sites the gene can be detected and how long after cultivation it existed would be illuminating. The primers are for a gene in fungi so if similar fungi are isolated from other orchards such as pears then the probe would also probably be effective.

In conclusion, DNA fragments at the appropriate position of 600bp were observed selectively from orchard and soil samples some of which were liable to reversible inhibition. These indicate critical points for future control of patulin contamination.

Acknowledgements

1. RRMP is grateful for grant SFRH/BPD/14923/2004 from Fundação para a Ciência e a Tecnologia (FCT), Portugal. 2. Some work undertaken as part of

the UK Food Standards Agency project: Reducing patulin in apple juice through manipulation of cultural practices in orchards (contract no. CO30005) which was greatly appreciated. 3. CABI Bioscience, UK is acknowledged for use of its laboratory facilities.

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