

The effect of fungal species on the fluorescent lectin test

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Abstract

Fungal (mold) contamination is an important indicator of low-quality raw product used in food processing operations. Fluorescent-labeled lectins, specific for chitin, have been shown to be valuable for quantitative detection of mold in raw tomatoes.

In this research, the response of individual fungal species to a rapid fluorescent lectin assay was investigated. Ten of the most common mold species were grown on two types of artificial broth media, and added to blended field tomatoes. The assay was conducted on each species, and linear regressions were developed, comparing the fluorescent lectin assay score with the fungal dry weight. The assay was able to detect all molds at sensitivities required for the tomato industry, and had high linearity (r^2 ranging from 0.72 to 0.99) and low variability (standard error of calibration ranging from 20 to 116 μg of fungal biomass/ml of tomato juice) for individual species grown on V-8 juice broth. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

A major challenge in measuring the fungal contamination in food products is the different response level of the measurement method to various species. This is an important issue because many different fungal organisms are responsible for decay in food products. More than 10 different species have been identified in California processing tomato fields (Jones et al., 1991; Mislivec et al., 1987; Butler, 1959). Previous work to detect mold contamination in processed product have shown variation in several

assays' response to differing mold species: Howard mold count (HMC; Eisenburg, 1952; Potts et al., 2000), ergosterol concentration (Battilani et al., 1996), chitin concentration (Ride and Drysdale, 1971; Lin and Cousin, 1985) and antibody response (Lin et al., 1985). Variation in assay sensitivity to differing species may allow varying amounts of contaminated raw material to be used in processed product.

The varying species response of many assays may be due to varying levels of chitin in the fungal cell walls of the organisms. Chitin content differs between fungal species because of growth environment (nutrient media and dissolved oxygen), and age of the infection (Cousin et al., 1984; Sharma et al., 1977). The organisms *Phytophthora parasitica*, *Pythium ultimum* and *Pyt. aniderphermatum* are not true fungi but are considered to be mold by the

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tomato processing industry. These organisms have been considered for many years to have little or no chitin in their cell walls (Bartnicki-Garcia, 1968). However, Cherif et al. (1993) found that *Pyt. ultimum* had measurable levels of chitin, but confirmed the near-absence of chitin in *P. parasitica*.

Potts et al. (2000) developed an assay based on binding a fluorescent-labeled lectin to chitin. The assay was shown to be a good method for the detection of mold in field tomatoes destined for processing (Potts et al., 2000; Patel et al., 1993). It is simple and fast to run and can detect molds at levels of interest to the tomato industry. Potts et al. reported some variability in the response to their fluorescent lectin assay among four fungal species grown on field tomatoes. However, this result was based on a dilution series using spoiled tomato volume not a measured fungal mass.

The goals of this research were to (1) measure differences in lectin assay response associated with the various fungal species expected in tomato processing fields and (2) determine if the assay response level of the fungus with the weakest response is adequate to allow the commercial use of the assay.

2. Materials and methods

2.1. Mold culturing

Tomato juice and potato dextrose broths were prepared according to the manufacturer's instructions (Sigma). Both solutions were filtered through a 0.45- μm nylon filter to remove large particulate matter. Broth (100 ml) was poured into 250-ml Erlenmeyer flasks, and autoclaved for 15 min at 121°C. Ten fungal cultures of the species: *Alternaria alternata*, *Stemphylium botryosum*, *Cladosporium herbarum*, *Geotrichum candidum*, *P. parasitica*, *Pyt. anidiphematum*, *Pyt. ultimum*, *Fusarium oxysporum*, *Rhizopus stolonifer*, and *Botrytis cinerea*, were each grown on both V-8 juice agar and potato dextrose agar (Sigma), until they reached the exponential growth phase. Cultures were obtained from Mike Davis, Department of Plant Pathology, University of California, Davis. A set of nine replicate flasks was inoculated with one of the 10 pathogens, using a sterilized inoculating loop. Fungi grown on V-8 agar

were used to inoculate tomato juice broth and fungi grown on potato dextrose agar were used to inoculate potato dextrose broth. The flasks were sealed with a sterile foam stopper, covered loosely with aluminum foil, and placed on a horizontal shaker at room temperature (22°C). The cultures were allowed to grow until they reached approximately 2-cm-diameter circular mycelial balls: 3–10 days depending on species grown on V-8 juice and 5–15 days for the same species grown on potato dextrose agar.

Each culture was harvested by combining contents of all nine flasks of a culture into a blender, and blending on high speed for 1 min. This mold solution was then poured into 15-ml centrifuge tubes (Falcon), autoclaved for 15 min at 121°C, and then stored at 6°C. Fungal dry mass concentration was determined by pouring 10 ml of each mold solution into a filtration unit, and filtering it through a 1- μm preweighed polycarbonate filter (Osmonics K10CP04700, 47 mm) using a 700-mm Hg vacuum. The cells on the filter were washed with 10 ml of distilled water, and dried at 90°C for 24 h. The amount of fungal dry mass per volume mold solution was calculated by taking the dry weight of the filtered material divided by the weight of the 10-ml mold solution.

2.2. Lectin assay procedure on chitin spiked tomato juice

Purified crab shell chitin (Sigma) was finely ground using a mortar and pestle. One hundred milliliters of distilled water was added to 60 mg of the ground chitin. Defect-free processing tomatoes were handpicked from California tomato fields, and comminuted for 40 s in an industrial blender. Various amounts of chitin solution were added to tomato juice, to obtain 10 levels of chitin concentration, ranging from 0 to 400 μg dry chitin/ml tomato juice. Six milliliters of each solution was pipetted into clean 50-ml centrifuge tubes (Falcon), to obtain four replicates for each solution (4 replicates \times 10 chitin levels + 8 controls = 48). Because the centrifuge held only four tubes, the analysis was conducted in batches of four randomly selected samples. Five milliliters of a 10- μg /ml fluorescein isothiocyanate-labeled wheat germ agglutinin (WGA) solution (EY Labs, San Mateo, CA) in a 0.5-M Tris/HCl

(pH 10) solution was added to each sample. The tubes were capped and placed on a shaker for 3 min. After this binding time, 40 ml of distilled water was added to each tube and they were centrifuged at 3800 rpm ($2250 \times g$) for 2 min. The supernatant was gently poured off, leaving the pelleted cells at the bottom. An additional 40 ml of distilled water was added, and the tubes were again centrifuged for 2 min at the same speed. Fifteen milliliters of Tris/HCl solution was added to the sample and the liquid was poured into a borosilicate glass cuvette. The average fluorescence level was determined from three fluorometer readings (Turner 450, Barnstead-Thermolyne). The fluorometer was calibrated and checked for drift using a fluorescent standard of Oregon Green (Molecular Probes, OR). Four controls (without lectin) each at the highest chitin concentration (400 μg chitin/ml tomato juice) and the lowest chitin concentration (0 μg chitin/ml tomato juice) were also measured using this procedure.

2.3. Lectin assay on mold spiked tomato juice

Six concentrations of mold (0, 50, 100, 200, 400, and 600 μg dry mass mold/ml tomato juice) were used to obtain linear relationships between fluorescent score and mold concentration for each mold species. For each mold species and each concentration, the appropriate amount of mold was added to enough distilled water to obtain 15 ml of a diluted mold solution (5 levels \times 10 molds = 50 diluted mold solutions). To each of these was added 15 ml of blended clean field tomatoes that had been previously filtered through a 650- μm filter and autoclaved. Six milliliters of these solutions was pipetted into clean 50-ml centrifuge tubes (Falcon), to obtain four replicates for each solution (5 levels \times 10 molds \times 4 replicates + 8 controls tubes with only distilled water and tomato juice = 208 tubes). These 208 tubes were chosen at random, four tubes at a time, and the fluorescent lectin test was conducted as described for the crab shell chitin test.

2.4. Data analysis

A linear regression between fluorescent score and mold concentration was conducted for each fungal species and each broth media. Slopes and standard

errors were calculated. A linear regression using indicator variables for each species was used to determine whether the slopes between species were significantly different (Neter et al., 1996).

3. Results and discussion

3.1. Lectin assay of crab shell chitin spiked tomato juice

The linear regression of fluorescence reading versus crab shell chitin concentration had a high linearity as measured by an $r^2 = 0.994$ and a high level of precision as measured by a standard error of calibration of 11 μg dry chitin/ml tomato juice. Any differences in response of the lectin assay to different species are likely caused by differing amounts of chitin in the mold mass or by the chitin being less accessible to the lectin.

3.2. Effect of mold species on the lectin assay

The lectin assay response was quite different for the 10 species that were tested (Table 1). Grown on V-8 juice broth, *A. alternata* and *S. botryosum* had much higher responses than the other eight species. The high response of *A. alternata* agrees with Lin and Cousin's (1985) measurements of high amounts of chitin in this organism. They also found large amounts of chitin in *R. stolonifer* and *F. oxysporum*. *R. stolonifer* had the third highest lectin assay response on V-8 juice and the highest response on potato dextrose broth, while *F. oxysporum* gave some of the lowest lectin assay responses, for fungi grown on both V-8 juice and potato dextrose broths. In fungal cell walls, chitin is located on the inner portion of the wall, covered usually by a layer of beta glucans. The outer layer may slow lectin binding in some species, causing a lower lectin assay score.

The growth media also influences the lectin assay results. All molds except *F. oxysporum* showed a significant difference ($\alpha = 0.01$) in the level of response between the two broth types. Grown in V-8 broth *A. alternata* and *S. botryosum* had the highest responses; however, when grown on potato dextrose,

Table 1
Linear regression analysis of lectin assay response to differing levels of mold spiked tomato juice

Mold species	Slope* (relative fluorescence/ μg dry fungal biomass/ml tomato juice)	r^2 Value	Standard error of calibration (μg dry fungal biomass/ml tomato juice)
<i>Tomato juice broth</i>			
<i>A. alternata</i>	1.50a	0.99	20
<i>S. botryosum</i>	1.12b	0.98	31
<i>R. stolonifer</i>	0.32c	0.97	36
<i>C. herbarum</i>	0.23d	0.99	25
<i>B. cinerea</i>	0.20e	0.99	27
<i>P. parasitica</i>	0.20e	0.96	40
<i>G. candidum</i>	0.16f	0.88	76
<i>Pyt. aniderphermatum</i>	0.15f	0.80	99
<i>F. oxysporum</i>	0.10g	0.95	48
<i>Pyt. ultimum</i>	0.10g	0.72	116
<i>Potato dextrose broth</i>			
<i>R. stolonifer</i>	2.52a	0.99	16
<i>S. botryosum</i>	0.90b	0.99	21
<i>Pyt. aniderphermatum</i>	0.56c	0.99	20
<i>P. parasitica</i>	0.38d	0.90	70
<i>B. cinerea</i>	0.31d	0.86	82
<i>A. alternata</i>	0.22e	0.97	41
<i>Pyt. ultimum</i>	0.20e	0.83	92
<i>G. candidum</i>	0.10f	0.96	46
<i>F. oxysporum</i>	0.08f	0.77	105
<i>C. herbarum</i>	0.06f	0.36	177

* Slopes with the same grouping letter are not significantly different at the $\alpha = 0.05$ level.

A. alternata had a much lower response. *R. stolonifer* had a higher lectin response when grown on potato dextrose broth than when grown on V-8 broth. Growth media clearly had a great influence on lectin assay response and supports the observations particularly by Sharma et al. (1977) that environment influences chitin content of fungi.

The results from the tomato juice broth cultures show that the lectin assay is able to detect *Pythium* species and *P. parasitica* despite the contradictory evidence in the literature documenting chitin in their cell walls. The ability to detect *Pythium* spp. is an important benefit, since these mold species are common in California tomato fields.

3.3. Sensitivity required in the processing tomato industry

In order for the lectin assay to be used as a measure of fungal contamination of raw product, it must be able to detect mold at levels of importance

to the tomato industry. The California industry currently uses a rapid visual estimate of raw product contamination (PTAB, 1996) to predict the Howard mold count of the processed product. If HMC were used for raw product, we would expect that the raw product should have a HMC score less than the limit for processed product. For many years, the United States standard for mold in tomato products has been 40 (USDA, 1978; AOAC, 1984). Jarvis (1977) observed that an HMC of 50% would be approximately equal to 4.2–4.9 mg fungal dry mass/ml juice. The standard error of calibration for *P. ultimum*, the species with the lowest lectin assay response, was about 0.116 mg/ml. Therefore, a 95% confidence limit would be ± 0.232 mg/ml and would correspond to a 95% confidence limit of about ± 2 in HMC units. A previous study (Potts et al., 2000) demonstrated the HMC determinations of fungal contamination of raw product have a CV ranging from 20% to 50% depending upon the inspector, so the lectin assay was far more precise than the HMC.

The response difference between *A. alternata* and *S. botryosum* and the other eight species would probably cause the industry to set standards to detect the lower responding species. The practical effect of this would be to allow the use of very little product contaminated with *A. alternata* and *S. botryosum*.

This study was based on individual species evaluation. Natural infections are likely to be a variable mix of organisms, with the specific mix depending on cultural and environmental conditions. In order for this method to be utilized as a replacement for the current raw product mold contamination method, more work is required on naturally infected samples taken from California tomato fields.

4. Conclusion

The lectin assay proved to be linear with chitin concentration ($r^2 = 0.994$) and to have a low variability when used with pure chitin, but a range of responses were observed when different species of fungal organisms were tested. The lectin assay was able to detect *Pythium* spp. and *P. parasitica* that are considered by some to have little or no chitin content. The assay appears suitable for use in detecting mold-contaminated processing tomatoes, if threshold levels are set to detect the species with lower lectin assay response.

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