AR1 quality assurance by ELISA

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Abstract

Tests are required to monitor contamination of AR1-infected seed with ryegrass seed infected with the common toxic endophyte to ensure contamination is maintained below 5% for First Generation seed and below 2% for Breeders and Basic seed. To achieve this AR1 seed is tested for the presence of lolitrem toxins that are produced by the endophytic fungus in toxic seeds. This was done by HPLC analysis until 2006 when an ELISA method, more suited to processing large sample numbers, was successfully established as a replacement. The ELISA is specific for lolitrems and lolitriol, provides rapid sample turnaround and analytical costs have been reduced. It is anticipated that in the future the test will be transferred to a commercial testing laboratory and will be extended to monitor contamination of AR37 seed with common toxic ryegrass seed.

Keywords: AR1 ryegrass, wild-type endophyte, lolitrem, ELISA, quality assurance

Introduction

Ryegrass pastures sown to seed lines infected with newly developed endophyte strains such as AR1 retain the beneficial properties of the endophyte but unlike ryegrass infected with common toxic endophyte, have nil or low toxicity. Common toxic (sometimes called "wild-type") endophyte produces lolitrem toxins that cause ryegrass staggers in animals. In establishing low toxin pastures it is necessary to have quality assurance seed tests to limit contamination from common toxic seed and therefore reduce risk of animal neurotoxicoses. Effective quality assurance programmes also provide added value to commercial seed lines. The specification for AR1 is that contamination is below 5% for First Generation seed and below 2% for Breeders and Basic seed. To achieve this, AR1 seed was tested for the presence of lolitrem toxins by HPLC analysis until sample volume was such that alternative methods more suited to processing large sample numbers were investigated. ELISA methodology was selected as immunoassays often require minimal sample clean-up and provide high sensitivity and rapid throughput. Lolitriol-protein conjugates were synthesised for use as immunogens and coating antigens (Miles *et al*. 1992) and an ELISA for the lolitrem toxins was developed (Miles *et al*. 1992; Garthwaite *et al.* 1993). The assay detects 'lolitrem-like' compounds and sample concentrations measured are expressed in lolitriol immunoreactive equivalents (IRE) as lolitriol is the reference toxin used to generate standard curves for the assay. Sprosen *et al.* (2001) described the first application of this assay to determine contamination of lolitrem-containing common toxic seed in seed lots. Hydrolysis of seed extracts was included in sample preparation so that lolitrem was converted to lolitriol which was better recognised by the toxin-specific antibodies and assay sensitivity maximised. In this format, results indicated that assay sensitivity was sufficient to detect a minimum of two contaminating seeds out of 10. This would not be sensitive enough, however, to detect less than 2% contamination which is now required for the quality assurance test. Here we describe modification to the assay conditions and optimisation of seed extraction procedures to achieve the sensitivity required for the successful establishment of the ELISA method as a replacement for quality assurance testing by HPLC.

Methods

Sample preparation

i) Bulk extraction

Seeds (10 g) were ground in a modified (reduced milling volume) domestic coffee mill by three pulses, each for 4 sec. Ground material (150 mg) was weighed into a capped polypropylene microcentrifuge tube (2 mL) and extracted with 90% methanol in water (1 mL) for 1 h by end-over-end rotation. Extracts were centrifuged and an aliquot (200 µL) was reacted with HCl (0.1M, 100 µL) for 15 min at 37°C. In a separate tube the hydrolysed extracts, were diluted 1 in 6 in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) to give 10% methanol in PBST and then further diluted 1 in 10 in 10% methanol in PBST before analysis by ELISA. Each extract was analysed at 1 in 90 dilution in duplicate wells.

ii) Multiplex extraction

When more accurate testing was required, it was possible to estimate contamination rates to specified probability levels, by extraction and analysis of multiple pools of seeds. Eighteen lots of 30 seeds from a single seed line were counted into 2 mL

Figure 1 Concentration ranges of lolitrem toxins determined by ELISA for AR1 seed contaminated with seed from various common toxic ryegrass cultivars. Bulk extraction of contaminated seeds was used. LOQ indicates assay limit of quantitation.

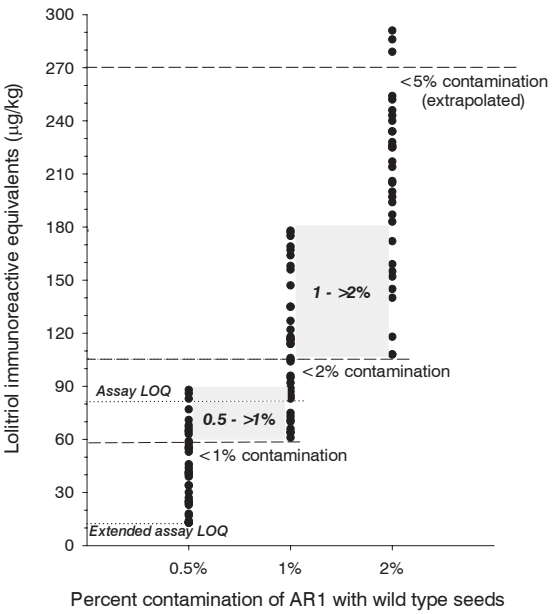

Table 1 Quality assurance grades based on lolitrem ELISA results. Breeders and Basic contamination limit is set at 2% and the First Generation limit is set at 5%.

	Grade	Lolitrems ELISA immunoreactivity (µg/kg)
1	No contamination detected, pass	<13
2	Trace contamination, likely to be less than 1%, pass without further testing	<50
3	Trace contamination, likely to be less than 2%, pass without further testing	<100
4	Some contamination, <2 or >2% Breeders seed - should be further tested	100-270
5	<5%, pass without further testing for First Generation seed	<270
6	Contamination, <5 or >5%, further testing possible for First Generation seeds	270-725

plastic vials (QSP 520) with 2 grinding beads (tungsten/carbide, 6.0 mm) and 90% methanol in water added (600 µL). The vials were secured onto a tissue disrupter (Mikro-Dismembrator U) and shaken for 90 sec at 2000 rpm before extraction end-over-end for 30 min. The extracts were left to settle before an aliquot (200 µL) was taken and hydrolysed as above. Analysis of results was performed using an exact upper confidence limit statistical test for tests in pools to predict the contamination range for any given number of positive tests, for the number of pooled samples taken, and at the significance required (95%). The upper limit was taken as an indication of a plausible level of contamination and this was required to be less than 2 or 5% depending on the seed type.

Competitive indirect ELISA

Extracts were analysed for lolitrem analogues by ELISA using an indirect competitive ELISA described by Garthwaite *et al.* (1993) with modifications. In the competitive assay the analyte competes with coating conjugate for binding to anti-analyte antibody. Inhibition of colour development in the ELISA indicates presence of analyte in samples.

Microtiter plates were coated with bovine serum albumin (BSA)-lolitriol conjugate and incubated overnight. ELISA washing buffer consisted of PBST and plates were blocked for 1 h with blocking buffer consisting of 1% BSA in PBST. Lolitriol standard was diluted in 10% methanol (v/v) in PBST to give standards ranging from 0.005 to 20 ng/mL. To each well was added 50 µL of standard or hydrolysed seed extract, diluted 1 in 90 to give a final concentration of 10% methanol in PBST. This was followed by 50 µL of sheep anti-lolitriol antibody diluted in blocking buffer so that the maximum absorbance in the assay (in the absence of analyte) was approximately 1.0 absorbance. All samples were analysed in duplicate and incubated for 1 h. Binding of sheep antibodies to coating conjugate was determined by the addition of rabbit anti-sheep immunoglobulin–horseradish peroxidase conjugate. A commercial (3,3',5,5' tetramethylbenzidine) substrate for horseradish peroxidase was used and the enzyme reaction was stopped after 15 min by addition of sulfuric acid (0.3 M).

The absorbance of wells was determined at 450 nm using a $Versa_{max}$ microplate reader (Molecular Devices Corporation, California, USA) and data analysis was performed using SOFTmax PRO data analysis software (Molecular Devices Corporation). Curve fits of mean absorbance versus the logarithm of the analyte concentration were performed by 4-parameter curve fit.

Optimisation of extraction

Common toxic ryegrass seeds known to contain high levels of lolitrem toxins, were extracted in a range of solvents to determine which system gave the most efficient extraction of immunoreactivity. The solvents investigated were 70, 80 and 90% methanol in water, 90% dichloroethane in methanol and isopropanol. Milled seed to solvent ratios were varied to determine the maximum ratio possible before mixing and extraction became compromised. Extraction times were varied from 0.25 to 1.5 h to determine optimum extraction time.

Establishment of an ELISA grading system

AR1 seed (15 g/preparation), known to be free of lolitrem toxins, was mixed with 40 seed lines containing 12 common toxic ryegrass cultivars that had a range of endophyte infection rates. Each seed line was mixed (w/w) to give three contamination rates, i.e. 0.5, 1.0 and 2.0% to give 120 samples in total. A range of cultivars and infection rates were selected to obtain a good estimate of variation in possible toxin concentrations and to establish whether assay sensitivity would be sufficient in all cases. Each sample was analysed using the bulk extraction procedure and contamination rates were related to the concentration of immunoreactivity measured. Cut-off concentrations for the ELISA results were established for each contamination level.

Results and Discussion

Optimisation of assay and extraction

The ELISA was modified to achieve an increase in assay sensitivity. Assay and washing buffers were replaced by PBST, incubation with specific antibody was reduced to 1 h and a commercial TMB substrate for HRP was utilised. Inclusion of the hydrolysis step was found to provide an increase of 70% in immunoreactivity compared with extracts analysed without hydrolysis. A 3-fold increase in assay sensitivity was achieved with the assay limit of quantitation (I_{20}) reduced to 150 pg/mL. Methanol (90%) in water was found to provide the most efficient extraction and the bulk extraction procedure was optimal after extraction for 1 h. Standard curves prepared in toxin-free AR1 seed extracts diluted 1 in 90 were coincidental with those prepared in sample buffer (data not shown). This demonstrated that assay matrix effects caused by AR1 seed were removed by dilution and 1 in 90 was therefore the minimum dilution used for test extracts.

ELISA quality assurance grading system

The ranges of immunoreactivity concentrations determined by ELISA for each contamination rate were determined (Fig. 1). A conservative cut-off limit was set for each rate at the lowest concentration measured for each range. The cut-off point for 5%

contamination was extrapolated using the limits determined for 0.5, 1.0 and 2.0% contamination. With the cut-off points defined, it was possible to establish a seed grading system (Table 1).

Application of the quality assurance method

The assay was applied to samples supplied by the AgriQuality National Seed Testing Laboratory. Samples had been provided for testing by seed producers during the 2006 season. Analysis of results indicated that only six out of 560 samples failed to meet specifications, four of the Breeders and Basic seed lines were recommended for further multiplex testing and five of the First Generation seed lines were also recommended for further testing. During the season, the ELISA analysis time has provided quick sample turnaround and analysis costs have been reduced. The assay is suitably formatted to be transferred to a commercial testing laboratory and it is anticipated that the assay will also be applied in determining contamination of seed lines infected with other selected endophyte strains, such as AR37, with common toxic ryegrass.

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