A STUDY OF ELECTROPHORESIS TESTING OF KENTUCKY 31 TALL FESCUE

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Introduction

The Federal Seed Act (FSA; 7 CFR §§ 201.1 to 201.78) requires that seed traded across state lines be labeled to accurately identify the species and variety. The United States Department of Agriculture Agricultural Marketing Service Seed Regulatory and Testing Division (SRTD) is responsible for FSA enforcement. State departments of agriculture may collect samples from seed lots suspected of being incorrectly labeled and submit them to SRTD for testing to evaluate germination, purity, and trueness to variety.

The SRTD determines the appropriate testing methods to verify trueness to variety (7 U.S.C. 1593). The traditional method used to evaluate trueness to variety is a grow-out test: the seed in question is planted, and the morphological characteristics of the resulting plants are compared to the characteristics established by the original breeder of that particular variety. Grow-out tests for tall fescue take several months because seed head characteristics are used to distinguish varieties.

Electrophoresis testing is an alternative molecular approach used to differentiate varieties of many crop species (Cooke, 1995). Proteins are extracted from plant tissue and forced through a gel medium that causes them to separate by size. The proteins create a pattern of bands in the gel, and differences in banding pattern can be used to differentiate among varieties. Testing protocols are crop-specific and target groups of proteins that are known to differ among varieties of that crop species. Electrophoresis methods are becoming increasingly common because results can be generated in weeks instead of months. Electrophoresis and grow-out tests are the only methods commonly used to differentiate among tall fescue varieties.

The SRTD has developed and adopted an electrophoresis test to differentiate between the tall fescue variety 'Kentucky 31' (K31) and other varieties (Wu and Payne, 2018). For the K31 electrophoresis test, proteins are extracted from the stem tissue of 4-week-old seedlings. Kentucky 31 produces a characteristic banding pattern. If a test sample produces a banding pattern that is not consistent with the expected K31 banding pattern, the SRTD determines that there is a "significant presence of a variety other than K31." (The term "off-type" will be used here.)

In 2018, seed from a pair of Oregon commercial tall fescue seed production fields of K31 (treated as a single lot) was tested using electrophoresis and was determined to be off-type. However, a follow-up electrophoresis test was conducted on seed harvested from the same fields in 2020, and the test returned the opposite result, identifying the seed as K31. The seed used to plant the fields was purchased from a supplier of K31 seed stock, and seed from the source fields was tested and found to be K31 in 2019. This incident raised questions about the K31 electrophoresis test. Thus, this study was initiated to better understand how the conflicting electrophoresis test results came about.

Conflicting test results could occur if the test did not produce consistent results, or they could be a result of actual differences among samples. The repeatability of the electrophoresis test must be evaluated before it is possible to investigate differences among samples. Wind-blown pollen from a neighboring field of another variety might cause seed from part of a K31 field to test as off-type. This effect was expected to be most detectable as heterogeneity in the seed lot. Seed from multiple fields of the same variety are often combined in a single lot, so differences among fields could also result in one portion of a lot testing differently from another portion.

This project addressed two research questions:

1. Does the electrophoresis test produce consistent results for identical samples?

2. Is there heterogeneity in seed lots that leads to inconsistent electrophoresis test results?

Materials and Methods

Seed lots

Samples from nine tall fescue seed lots were included in this study (Table 1). These included five lots that were expected to be K31 and four lots of other varieties. The K31 lots included K31 seed stock, the 2020 lot that had

Table 1. Summary of tall fescue seed lots and number of samples submitted for electrophoresis testing.

Seed lot	Description	-- Round 1 --		-- Round 2 --	
		WL[1]	PL[2]	WL[1]	PL[2]
		----- (Number of samples) -----			
K31-1 2020	Seed harvested from this pair of fields produced conflicting test results (2018, 2020). Uncertified.	3	3	1	3
K31-2 2020	Uncertified seed harvested from a group of three	3	3	1	3
K31-2 2019	commercial fields in three crop years	3	—	1	—
K31-2 2018		3	—	1	—
K31-SS	MO-certified K31 seed stock, grown in Missouri	3	—	1	—
Honky Tonk II 2020	OR-certified turf-type tall fescue, field adjacent to K31-1	3	3	1	3
Fawn 2020	Uncertified forage type	3	3	1	3
Rustler 2019	Uncertified forage type	—	—	1	—
Brutus 2020	Uncertified forage type	—	—	1	—

[1]Samples that are representative of the whole lot. A single sample representative of the whole lot was collected, mixed, and split into replicate subsamples.
[2]Partial-lot samples collected from three individual pallets of each seed lot. Three samples were collected, and each was tested twice, once in each round of testing.

previous inconsistent test results (K31-1), and seed lots from the 2018–2020 crop years from another grower (K31-2). Other varieties included a turf-type ('Honky Tonk II') grown adjacent to K31-1 and three forage varieties: 'Fawn', 'Rustler', and 'Brutus'.

Experimental design

Two different approaches were used in this study to address the two research questions. The number of samples tested for each lot is shown in Table 1.

To address question #1, a representative sample was collected from each lot, mixed thoroughly, and split into replicate subsamples. This approach was designed to produce replicate subsamples that were as identical as possible. Four replicates were tested for each "whole-lot" (WL) sample: three replicate samples from each lot in the first round of tests, and one from each lot in the second round of tests (Table 1, see below for explanation of test rounds).

Sample collection to address question #2 was designed to capture heterogeneity within lots. Four lots (Table 1) were included in this portion of the study. Three partial lots, each consisting of a single pallet (50 or more 50-lb bags), were randomly selected from each lot, and a representative sample was collected from each pallet. These samples are referred to as partial-lot samples (PL). If there is heterogeneity in the lot, PL samples from that lot have the potential to differ from one other. Each PL sample was mixed thoroughly and split to form replicates. A total of six tests from each lot were completed: three potentially unique PL samples, each tested in the first round of testing and again in the second round of testing (two replicates).

The seed lots Rustler 2019 and Brutus 2020 were added at a later stage of the project to increase the number of non-K31 varieties tested. A single representative sample was collected from each lot and tested once. Partial-lot samples were not collected from these lots.

Sample collection and submission

Seed samples were collected by an Oregon Department of Agriculture seed regulatory specialist in February 2021, except for the Rustler 2019 and Brutus 2020 samples, which were collected in June 2021. Seed lots K31-2 2018 and K31-2 2019 were sampled at the time of cleaning by an in-process auto-sampler in 2018 and 2019, respectively. The grower saved seed samples (one 1-gal bag each), and a representative portion of these samples was collected by the seed regulatory specialist for this study. The K31-2 2018 and K31-2 2019 samples are considered WL samples because they are representative of the whole lot. All other samples were collected using the standard AASCO sampling protocol (Guerke, 2006). All samples were collected once, and a portion of each sample was retained for future testing.

Samples were submitted to the SRTD for K31 electrophoresis testing by the ODA on two separate dates. A second round of testing was done because identifying information had been included with the first sample submission, so the samples were not blind. All potentially unique samples were submitted for testing in the second round, as well as two samples from lots that were not included in the first round (Table 1). The second batch of samples was blind; only randomly generated sample numbers were used to identify samples.

Results and Discussion

The results from SRTD included images of gel banding patterns in addition to the determination of whether each sample was K31 or off-type. Each gel included a K31 control sample. Four bands are used to distinguish K31 from other varieties, and these bands were noted on the control sample with labels "a" through "d." Figure 1 shows one of the gels from the second round of tests.

The SRTD had information about the identities of the samples in the first batch of tests, which could bias the results. However, we have several reasons to believe that the first round of testing was valid and worth including here. Samples submitted to the SRTD as part of routine regulatory activities are provided with all label information and are collected from lots that are believed to have inaccurate labeling. Therefore, as a matter of course, all samples submitted to SRTD are not submitted blindly, and SRTD considers the label information to be potentially inaccurate.

Knowledge of sample identity could not alter the banding pattern produced by the sample, but it could affect how a human interprets that banding pattern. Since images of the gels were provided by the lab we were able to independently interpret the gels (without knowing sample identities) and compare our interpretation to that of the lab. Comparisons of round one (not-blind) and round two (blind) test results from the same lots confirmed that the gel banding patterns were the same. There were no discrepancies between the results obtained in the first and second batches of tests.

In regards to question #1, all replicate WL samples collected from the same lot returned the same test result and showed the same banding pattern. These results include seven lots replicated four times each. Additionally, 2 replicate tests were conducted for each PL sample, for another 12 pairs of replicate tests. These results show that the K31 electrophoresis test returns repeatable results when identical samples are tested.

In regards to question #2, no evidence of heterogeneity was detected in this study. Three PL samples were tested each for four lots. All PL samples from the same parent lot returned the same result and banding pattern. Although heterogeneity was not detected, this study tested only four lots. More research would be needed to rule out the possibility of heterogeneity as a cause of inconsistent test results.

All samples that were believed to be K31 (K31-1, K31-2, and K31-SS) produced a banding pattern that matched that of the laboratory check samples and were determined to be K31 by SRTD. Samples from other varieties produced different distinctive banding patterns. For example, the turf-type variety 'Honky Tonk II'

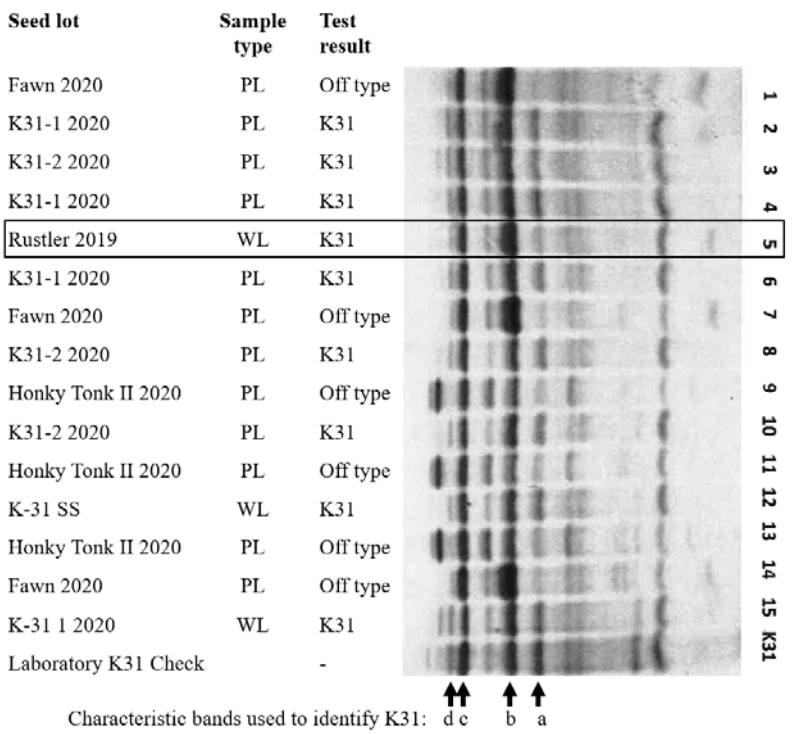

Figure 1. Image of electrophoresis test results from the second round of testing annotated with seed lot, sample collection method (WL = whole-lot replicate; PL = partial-lot), and the test result determined by the SRTD laboratory. A box highlights the sample that was incorrectly determined to be K31 by the testing laboratory. The gel is shown sideways to make text easier to read.

showed evenly spaced dark bands (Figure 2). Combining replicate and PL samples, four seed lots were tested ten times each, and three seed lots were tested four times each. All tests of the same lot produced the same banding pattern and test result.

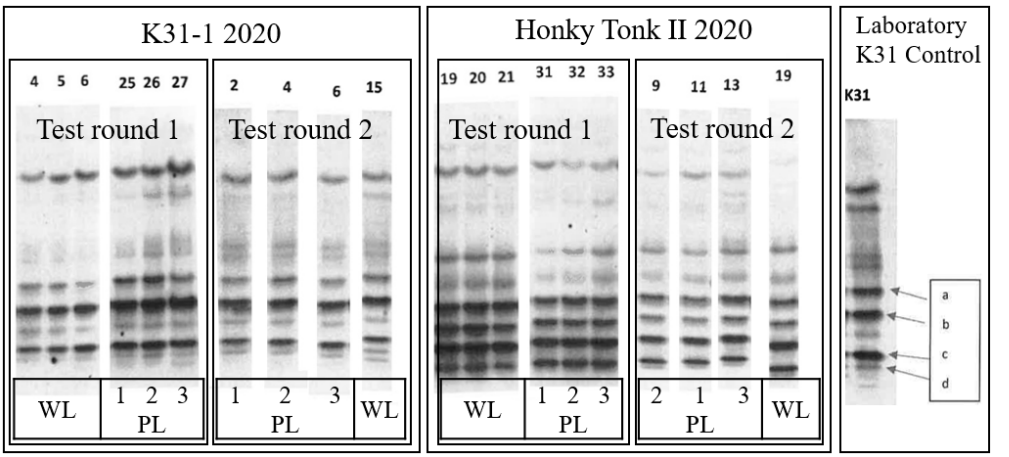

Figure 2. Electrophoresis test results for the lots K31-1 2020 and Honky Tonk II 2020 in test rounds 1 and 2. Samples are identified as whole-lot (WL) or partial-lot (PL). PL samples with the same number and variety are replicates of the same sublot.

Of the 54 samples submitted for testing, 1 sample was incorrectly identified. This sample was a non-K31 forage type (seed lot Rustler 2019), but the test results identified it as being K31 (a false positive). Unlike the other seed lots, which were tested in both rounds, only a single sample each of Rustler 2019 and Brutus 2020 was included in the second (blind) round of testing.

The banding pattern of the Rustler 2019 sample (Figure 1, row 5) has a faint "a" band and a dark "b" band compared to the K31 check sample. The Rustler 2019 banding pattern shows some similarities to the banding pattern of Fawn 2020 samples (Figure 1, rows 7 and 14). The determination by SRTD for this sample should be considered in context: The electrophoresis test is used on samples that are labeled as K31 but suspected of being mislabeled (a violation of the FSA). It would be reasonable to consider the banding pattern of the Rustler 2019 sample to be insufficiently different from the K31 banding pattern to determine that it was not K31.

These results indicate that the K31 electrophoresis test can produce consistently repeatable results, producing similar banding patterns. One false positive result was obtained when a forage-type tall fescue lot was incorrectly determined to be K31, likely due to human interpretation of the banding pattern.

References

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