

Rules Change Review Proposal – Identification of ryegrass types

For Review Only

PURPOSE OF PROPOSAL: To add additional or optional test methods to further identify ryegrass growth types.

PRESENT RULE: This is a new Rule.

PROPOSED RULE:

5.2 Identification and cultivar determination

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c. Grow-out tests

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d. PCR tests to predict growth-type in ryegrass.

- (1) **Allelic discrimination** is a method that can be used to predict growth types of ryegrasses based on multiple genetic markers of genes involved in flowering control of grasses. Testing protocols are included in the AOSA Cultivar Purity Testing Handbook, Contribution No. 33 to the Handbook on Seed Testing, AOSA, 2008, and subsequent updates.

HARMONIZATION AND IMPACT STATEMENT: Canada Methods and Procedures and ISTA rules do not require fluorescence testing on ryegrass; therefore there is no need for further clarifications to the false positives provided by Allelic Discrimination testing. The FSA does not address genetic testing. The AASCO in their 2009 meeting requested that AOSA investigate solutions to the SRF and GOT tests.

SUPPORTING EVIDENCE:

Seed analysts have known for years that the Seedling Root Fluorescence Test (SRF) in ryegrass has been a problem. Generally, germinating Italian (or annual) ryegrass seedlings fluoresce under ultraviolet light and perennial ryegrass does not. False positives occur due to lack of complete test standardization, age of seed, seed analyst subjectivity in scoring results, and plant biology (Floyd and Barker, 2002). This false-positive bias has been costly to grass seed growers who receive lowered seed payments and costly to seed companies because of inappropriately labeled seed being sold to the consumer.

An accurate test that detects both physical (mixtures) and genetic (pollen flow) contamination of Italian ryegrass in perennial ryegrass cultivars is necessary, especially if the perennial ryegrass seed is for permanent turf. Contamination of perennial turf with annual type ryegrasses detracts from the otherwise aesthetically pleasing appearance of perennial ryegrass with dark-green color and fine turf texture. Other than a physical plant grow-out test for each seed lot (Barker et al., 2002, Nittler and Kenny, 1972), SRF is the only accepted test for detecting the presence of Italian ryegrass in seed lots of perennial ryegrass. Additionally, tolerances are not applied to test results from year to year, or field to field.

To alleviate the income loss to seed growers, a maturity grow-out test (GOT) was developed and implemented for labeling seed in 2001. After several years experience with the GOT, we found that the GOT is causing problems in the grass seed industry as well. Research showed that it consistently underestimated annual-type plants in the SRF seedlings. This is because the implemented GOT is conducted for too short a time and artificial lighting conditions are not standardized at high enough intensity levels.

A new genetic test, (referred to as Allelic Discrimination (A/D)) is an optional test used as a supplement to the SRF. It is a replacement to the ryegrass grow-out test and can be used in VFL determinations. This test is a method to predict growth types of individual ryegrass plants based on multiple genetic markers of genes involved in flowering control of grasses. The two genes are the indeterminate (*LpID₁*) and one of the vernalization (*LpVrn-1*) genes. To reduce costs involved in conducting this TaqMan[®] assay, it is conducted on individual seedlings and is done on all individual seedlings that have fluorescent root traces as done in the GOT. Up to 25 non-fluorescent seedlings and 25 seedlings from an Italian (or annual) ryegrass cultivar are included in the test as controls. Once allelic determination for each seedling has been made, results are reported the same as with the GOT.

For the past 13 to 14 years, Barker from USDA/ARS in cooperation with Oregon State University, Agri Seed Testing and others have been working on a PCR test that is faster and more accurate than either the SRF test or the GOT. An Allelic Discrimination (A/D) test was developed based on single nucleotide polymorphisms (SNPs) to two major genes involved in the flowering control process in grasses. A SNP is an allele, which is an alternate form of a gene. SNPs of the indeterminate gene (*LpID₁*) and one of the vernalization genes (*LpVrn-1*) are used in the A/D test. Implementation of this rule will be more accurate than GOT for ryegrass because it is based on actual flowering control genes, it will remove the bias to SRF tests, lessen the burden placed on seed growers, and provide answers six weeks earlier than a GOT and at similar costs for low contamination seed lots.

- A. Current data:** Twenty-two seed lots chosen for their wide range of SRF values were examined to verify that the A/D tests could be conducted on a commercial scale. Agri Seed Testing provided the seed test data and GGT conducted the A/D analyses. Seed for each seed lot were germinated, a SRF conducted, and a grow-out test started according to AOSA protocols. During the grow-out, a leaf from each plant was harvested, DNA extracted, and an A/D conducted for both *LpVrn-1* and *LpID₁*. Annual-like and perennial-like using the markers was determined using a multi-point decision.

From the A/D test, individual plants were determined to be “annual-like” or “perennial-like.” This determination was made using a multi-point decision process. Plants that had roots that were fluorescing (FL+) were declared annual-like if either *LpVrn-1* or *LpID₁* had an “annual” allele. For non-fluorescing plants (FL-), two “annual” alleles were needed to declare it annual-like. This is a “two of three” marker determination (called GGT M*3 AR/PR test). Multiple markers are more reliable than one marker alone, unless the marker is the actual “annuality” gene, if there is one.

Values were used as described in the AOSA Cultivar Purity Testing Handbook “Grow-Out of Fluorescent Ryegrass Seedling to Differentiate Between Annual and Perennial Types” to calculate actual “annual-type” contamination (%) (Table 1).

Table 1. Annual-type contamination in perennial ryegrass seed lots (%) as determined by the GOT formulae for both the GOT and molecular markers.

Test#	TFL	From grow-out	Using molecular markers
----- % -----			
59216	5.39	0.60	0.90
59337	5.29	0.28	1.11
59669	6.50	0.00	0.89
60079	8.84	1.26	4.42
60080	9.83	2.53	6.65
60660	11.65	1.36	6.78
61248	4.01	0.27	1.07
61249	7.22	1.67	4.16
61490	0.75	0.00	0.00
61491	1.16	0.00	0.00
61492	0.83	0.00	0.09
61493	1.24	0.00	0.31

(Table 1. Continued)

Test#	TFL	From grow-out	Using molecular markers
----- % -----			
61494	0.94	0.00	0.00
61495	2.56	0.09	0.28
61496	1.10	0.00	0.00
61497	2.90	0.00	0.68
61761	22.04	3.54	14.59
62408	8.99	0.00	4.17
62409	6.29	0.00	0.39
62411	4.31	0.00	2.16
62476	13.67	1.09	6.27
62477	10.93	0.00	0.33
Mean	6.20	.58	2.51

The Variety Fluorescent Level (VFL) determines the number of FL+ plants that should actually be classed as “perennial-like.” Table 2 uses numbers to calculate results as if each lot was a “VFL.”

Table 2. Annual-type contamination (as VFL*) in perennial ryegrass as determined by the GOT formulae for both the GOT and molecular markers.

Test#	TFL	From grow-out	Using molecular markers
----- % -----			
59216	5.39	4.79	4.49
59337	5.29	5.01	4.18
59669	6.50	6.50	5.62
60079	8.84	7.58	4.42
60080	9.83	7.30	3.18
60660	11.65	10.30	4.88
61248	4.01	3.74	2.94
61249	7.22	5.55	3.05
61490	0.75	0.75	0.75
61491	1.16	1.16	1.16
61492	0.83	0.83	0.73
61493	1.24	1.24	0.93

(Table 2. Continued)

Test#	TFL	From grow-out	Using molecular markers
----- % -----			
61494	0.94	0.94	0.94
61495	2.56	2.47	2.28
61496	1.10	1.10	1.10
61497	2.90	2.90	2.12
61761	22.04	18.51	7.45
62408	8.99	8.99	4.82
62409	6.29	6.29	5.89
62411	4.31	4.31	2.16
62476	13.67	12.58	7.41
62477	10.93	10.93	10.60
Mean	6.20	5.63	3.69

*Assumes the Variety Fluorescence Level (VFL) of the perennial seed lot was 0%, the annual check was 100%, and purity at 100% ryegrass.

Eight of the seed lots (in bold) were actually being tested for VFL. Lots 61490 through 61493 were from the cultivar Prospert 2 and 61494 through 61497 were from Pacesetter II. The VFL using GOT values were 1.87 for Prospert 2 and 0.99 for Pacesetter II. Using DNA marker values were 1.64 for Prospert 2, and 0.90 for Pacesetter II. Results for actual VFL are similar between GOT and molecular markers, but are obtained six weeks earlier when using molecular markers.

B. References

Barker, R.E., S.E. Warnke, S.G. Elias, A.E. Garay, and R.L. Cook. 2002. Ryegrass grow-out tests in relation to seedling root fluorescence. p. 90-95. *In* W.C. Young III (ed.). 2001 Seed Production Research. Dept. Crop and Soil Sci. Ext/CrS 121, 4/02.

Floyd, D.J., and R.E. Barker. 2002. Change of ryegrass seedling root fluorescence expression during three generations of seed increase. *Crop Sci.* 42:905-911.

Nittler, L.W., and T.J. Kenny. 1972. Distinguishing annual from perennial ryegrass. *Agron. J.* 64:767–768.

Complete research details and additional data are available from the authors.

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