Ryegrass Grow out Test
A supplement to the fluorescence test
Sabry Elias, Adriel Garay and Dale Brown
Background

- Fluorescence test sometimes overestimates annual ryegrass contamination in perennial ryegrass seed lots resulting in false labeling and financial loss to ryegrass growers.

- Genetically, fluorescence trait is not always associated with the annual form of ryegrass.

- Environmental conditions affect the level of fluorescence from year to year and from location to location.
Mean % annual ryegrass seeds in 45 perennial seed lots tested at the OSU Seed Lab using the fluorescence test and the grow-out test

% annual type based on

<table>
<thead>
<tr>
<th>FL Test</th>
<th>Grow-out Test</th>
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</thead>
<tbody>
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<td>10.07</td>
<td>3.11</td>
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</table>
As the concern regarding the fluorescence test increased, many ryegrass growers asked to allow the use of grow-out test to supplement the fluorescence test.

The grow out test has proven to give more realistic results on the level of annual ryegrass contamination in perennial seed lots.
The grow-out test in the Federal Seed Act Regulations

- Sec. 201.58a of the FSA Regulations indicates that ryegrass samples can be grown out and plants examined to determine if they are annual or perennial.
Test Procedure

**Sample size for transplanting:**

- All fluorescent seedlings (from the fluorescence test),
- A minimum of 20 random non-fluorescing perennial seedlings from the sample being tested (perennial checks), and
- a minimum of 20 annual seedlings (annual checks)

**Test period and final evaluation:**

Plants should be evaluated after about 42 days of transplanting the fluoresced seedlings, or when full heading of the annual check sample is achieved.
A National Referee Study

- A national referee study was coordinated by the OSU Seed Lab to validate and standardize the ryegrass grow-out test procedure.

- Based on the study, a full protocol including procedure, calculations and an appropriate tolerance table will be published in the Cultivar Purity Handbook by Oct. 2002.
Transplanting in cell trays
Grow-out Test

In Walk-in Germinator

≈ 125 f.c.

(low light intensity)
Ryegrass grow-out test in green house
Plants should be classified as follows:

a. **Perennial types**: Plants that have similar characteristics as the perennial checks and have not headed.

b. **Annual types**: Plants that have headed and/or do not resemble the perennial check characteristics (e.g., have wide blades, light color, elongated stems).
Control perennial ryegrass – No heads, narrow dark blades
Annual type

Perennial type

Ryeegrass

CH-10
35 days after transplanting
**Formulas:** formulas were developed to calculated the % of annual and perennial ryegrass types.

\[
\% \text{ Annual ryegrass} = \frac{\text{Number of fluorescent t plants that have headed or resemble annual checks}}{\text{Total number of normal seedlings} \times \text{Survival factor}}
\]

\[
\text{Survival factor} = \frac{\text{No. of fluorescent seedlings} - \text{No. of plants that died during the grou out test}}{\text{No. of fluorescent t seedlings}}
\]

\[
\% \text{ Perennial ryegrass} = \% \text{ Pure ryegrass} - \% \text{ Annual ryegrass}
\]
Example

% of pure ryegrass (obtained in the purity analysis) = 99.12

No. of normal seedlings (out of 400 seeds planted) = 380

No. of fluorescent seedlings = 12

No. of plants that died during or after transplanting = 3

Number of annual type plants at the end of the grow-out test = 4

\[
\text{Survival factor} = \frac{12 - 3}{12} = 0.75
\]

\[
\text{Annual type} = \frac{4}{380 \times 0.75} \times 99.12 = 1.39
\]

% Perennial Ryegrass = 99.12 – 1.39 = 97.73
Tolerances for grow-out tests:

- Table 4 in Sec. 202.62 of the FSA Regulations contains tolerances appropriate for use when comparing results of grow-out tests.

- Table 4 is adopted from Table V1 'Trueness to variety 2 estimates, 5% 1-way test' in the Handbook of tolerances, Miles, 1963 page 667.

- We used this table in the Grow-out test considering the label as an initial grow-out test (or first estimate) and a subsequent test as a second estimate.
Table V2 "Trueness to variety, 1 specification and 1 estimate, 5% 1-way test; Miles. 1963 page 668.

- The specification is the % of varietal fluorescence (VFL) that is established by the breeder of each variety and is listed in the AOSCA National Grass Variety Review Board. This VFL value is assumed to have only one source of variation which is the variation in the seed or sample variation. In this case Table V2 is appropriate for use.
201.62 Tests for determination of percentages of kind, variety, type, hybrid, or off-type.--Tolerances for tests for determination of percentages of kind, variety, type, hybrid, or off-type shall be those set forth in the following table, added to one-half the required pure seed tolerances determined in accordance with section 201.60, except that one-half the pure seed tolerance will not be applied in determining tolerances for hybrids labeled on the basis of the percentage of pure seed which is hybrid.

Table 4—Tolerances for Purity Tests, When Results Are Based on 10 to 1,000 Seeds, Seedlings, or Plants Used in a Test

<table>
<thead>
<tr>
<th>Seed, seedling, or plant count percent</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>50</th>
<th>75</th>
<th>100</th>
<th>150</th>
<th>200</th>
<th>400</th>
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</table>
**Tolerance Example**

An initial grow-out test reported 98.0% perennial ryegrass, but a subsequent test showed only 94.4%. In the second test 380 normal seedlings were obtained out of the 400 seeds planted for the fluorescence test. Are these two samples within tolerance?

- Apparent test discrepancy = 98 – 94.4 = 3.6%
- Average of the two tests is \((98 + 94.4)/2 = 96.2\) (96%).
- In Table 4, 380 is between column 200 and 400 at row 96/4
- so the tolerance is between 3.2 and 2.3 and must be computed by interpolation.
• Since 380 plants is $\frac{180}{200}$ of the way between 200 and 400, then the allowable tolerance is $\frac{180}{200}$ of the way between 3.2 to 2.3.

• Therefore, the interpolated tolerance is: $3.2 - [(3.2 - 2.3) \times \frac{180}{200}] = 2.4$.

• The test discrepancy of 3.6 exceeds the tolerance 2.4, so the tests are out of tolerance.
Reporting and calculations:

- A grow out test report would include the percentages of perennial, and annual types according to the grow out formulas.
- Purity results based on the grow-out test may differ from those based on the fluorescence test in both the ‘pure seed’ and ‘other crop’ components.
- When grow-out test is used to determine the percentage of annual and perennial ryegrass in a sample, purity report should include the following statement: “Pure seed is based on grow-out test”.
AASCO, AOSCA, and ASTA

Resolutions

- **AASCO** passed a resolution in Feb, 2001 stating that whenever a lot is tested for purity based on a grow-out test, it shall be stated on the label as such. This allows to label and sell PRG based on grow-out test.

- **AOSCA** passed a motion on July 3, 2001 supporting use of the grow-out test as an option to the fluorescence testing until a more accurate, faster test is approved.

- **ASTA**, also supports the grow-out test.
Research Activities

The OSU Seed Lab has conducted the following studies:

1) determination of light intensity and time of exposure for optimum rate and speed of growth and heading of PRG.

2) determination of optimum plant spacing/containers for the grow-out of RG.

3) comparison of two methods for the grow-out of RG: transplanting fluorescent seedlings vs. stand alone test (direct planting of seeds).

- The objective of the above studies was to optimize grow-out test conditions for fast, accurate results.

- The primary results emphasized the importance of light intensity, continuous light exposure (24 h) during the course of the test, and using cell containers for planting.
Reed Barker & Scott Warnke, USDA. Conducting genetic mapping studies to examine annual-perennial inheritance, and located genomic regions suitable for test development.

Jim Dombrowski, USDA. Investigating the possibility of identifying differentially expressed genes by subtractive hybridization of cDNA libraries.

Hiro Nonogaki, OSU. Plans to identify the specific protein or mRNA using subtraction PCR to identify the gene(s) specifically expressed in annual or perennial ryegrass, then isolate the cDNA and develop an ELISA test.

Sabry Elias, OSU Seed Lab. Preliminary exploration shows promise for IEF tests based on phosphohexose isomerase and esterase enzymes. Proposing to develop and verify useable lab tests.
Current/potential Research Activities

Nicholas Hill, U of GA. Initial work with protein monoclonal antibodies and ELISA tests has been done. No data are available.

Glen Freeman, Rutgers Univ. Preliminary work with HPLC (liquid chromatography) shows potential. Proposing to develop ELISA assay test.
For comments or questions contact

Sabry.elias@oregonstate.edu