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A Comparative Analysis of Extraction Methods for the Recovery of *Anguina* sp. from Grass Seed Samples

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Abstract: Four procedures were compared in their efficacy to extract juveniles of *Anguina agrostis* from commercial grass seed. The procedures included those currently used by the state regulatory laboratories of Oregon and California, as well as new tests developed to determine juvenile viability for the phytosanitary certification of fumigated grass seed. Eleven seed lots of *Agrostis tenuis* (bentgrass) and *Dactylis glomerata* (orchardgrass) naturally infested with varying levels of juveniles of *Anguina* were individually analyzed. Only one procedure, a new live recovery test, yielded nematodes in all 11 samples and is recommended as the best method for use by regulatory agencies. In comparison, although the other three extraction procedures resulted in greater numbers of *Anguina agrostis* juveniles per gram of seed, they failed to yield any nematodes in as many as four seed lots with low infection levels.

Key words: Anguina agrostis, assay, bentgrass, extraction procedures, nematode, orchardgrass, phytosanitary certification, regulatory nematology, seed-borne nematodes, technique.

Anguina agrostis (Steinbuch, 1799) Filipjev, 1936, the bentgrass nematode, infects many economically important species of the Poaceae (Krall, 1991; Southey, 1969, 1973). *Anguina agrostis* is a seed-borne plant-parasitic nematode with a cosmopolitan distribution, due in part to the wide dispersal of infested host seed material. This species of nematode has been reported to occur on bentgrass (*Agrostis* spp.) and has tentatively been identified on other grasses in the cool-season grass seed production areas of western Oregon (Courtney and Howell, 1952; Jensen, 1961). In 1997, the Oregon Department of Agriculture (ODA) conducted 850 official detection tests for *A. agrostis* in commercial seed lots. While nearly all of these tests failed to extract *Anguina* spp., some lots of *Agrostis tenuis* Sibth. (bentgrass) and *Dactylis glomerata* L. (orchardgrass) were infected.

Western Oregon is one of the world's leading cool-season grass seed production areas, producing approximately 273,000 metric tons of seed on about 170,000 ha, with a value of US $295.4 million in 1996 (Rowel and Kreissel, 1997). Much of the grass seed produced is exported to a number of countries, many of which have strict phytosanitary requirements against certain nematode pathogens. Official tests for pathogens including *A. agrostis* are needed and often required by the governments of importing countries. Presence of any *A. agrostis* individuals in a sample can result in the non-issuance of federal phytosanitary certificates and preclusion of commodity shipments to foreign markets.

In certain cases, seed commodity shipments found infested with *A. agrostis* will require treatment to qualify for export. Evaluation of fumigants for seed treatment are currently under way, and several schedules look promising (Alderman, pers. comm.). However, to demonstrate the efficacy of the treatment, a live nematode extraction retest may be required by some foreign governments. This retest must allow for the extraction of live nematodes in order to differentiate from those nematodes killed by fumigation or other treatments.

The goals set for the current study were to develop (i) a standard test that could be used in official certification laboratories to meet proposed federal standardization and accreditation rules (7 CFR 353), (ii) an assay for live nematodes that could be used to demonstrate seed treatment efficacy, and (iii) a test that produced a clean and readily

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viewed sample in a timely and efficient manner.

Materials and Methods

Seeds from 10 orchardgrass seed lots and one bentgrass seed lot, predetermined to be infested with *A. agrostis* by routine ODA laboratory testing, were obtained from commercial seed companies in Oregon (Table 1). All seeds were produced in the Willamette Valley during the 1996 growing season. Seeds were commercially harvested and mechanically cleansed of inert debris and contaminants. All seeds were stored in commercial bins prior to sampling. Samples from seed lots 1–4, 6, 7, and 11 had low levels of infestation based on previous tests and were included to examine experimental precision. Samples from each lot were equally divided on a gravity seed splitter and were shared by the testing laboratories of the Oregon Department of Agriculture (ODA) Plant Division and the California Department of Agriculture (CDFA) Nematology Laboratory.

Four extraction methods were conducted at CDFA-Nematology and ODA Laboratories and compared for total numbers of collected juveniles and ability of a method to extract any nematodes (presence or absence in a given repetition). In addition, at ODA, a test was conducted to determine the effect of aeration and host plant foliage on nematode extraction. At each laboratory, treatments for each seed lot were replicated 5 times unless otherwise noted, and a total of 220 samples were processed for the comparison of the four main methods.

Method 1, water agar blend: To determine an average number of *A. agrostis* juveniles per gram of seed, 5 g from each lot were soaked in distilled, aerated water for 24 hours. The soak water was then passed through 60- and 500-mesh sieves of 250-µm and 25-µm pore size, respectively. The effluent water was examined, and counts were added to the final totals. The seeds were then placed on petri dishes containing 10–12 ml of 0.9% water agar and sealed with Parafilm® (American Can Company, Greenwich, CT). These dishes were incubated at 15 °C in darkness for 16 hours and 25 °C in fluorescent light for 8 hours to optimize germination of orchardgrass seed. After 7 days of incubation, the contents of each plate, including germinated seeds, media, and ungerminated seeds, were blended in 200 ml distilled water for 30 seconds at high speed in a Waring blender (Waring Products Division, New Hartford, CT). The homogenate was passed through a 25-µm pore-size sieve, and nematodes were extracted from the back-washed sieve residue, with sugar flotation-centrifugation (Jenkins, 1964) then counted.

Method 2, sieve-blend: Five grams of seed from each lot were soaked in distilled water

Table 1. Number of juveniles of *Anguina agrostis* collected per gram seed from 11 naturally infested commercial seed lots with four extraction methods.

Lot	Grass	Cultivar	Method 1	Method 2	Method 3	Method 4
1	Bentgrass	Highland	3.9[a]	0.7	4.5	0.8
2	Orchardgrass	Hallmark	0.0	3.5	0.0	0.1
3	Orchardgrass	Hallmark	0.0	0.0	0.5	0.2
4	Orchardgrass	Hallmark	1.4	56.8	0.0	1.3
5	Orchardgrass	Frode	178.5	258.2	0.1	3.2
6	Orchardgrass	—	0.0	0.3	0.0	0.1
7	Orchardgrass	—	14.9	14.3	0.1	1.0
8	Orchardgrass	Juno	40.5	224.0	0.1	2.0
9	Orchardgrass	Crown	223.7	826.0	0.7	1.0
10	Orchardgrass	Potomac	149.5	154.0	0.1	0.6
11	Orchardgrass	Crown	6.8	14.0	0.0	0.2
	Mean		56.3	141.0	0.6	1.0
	Standard deviation		95.7	304.3	0.8	0.3

[a] Means of five replicates per lot per method, divided by total weight in grams of seed processed per method.

and aerated with an air stone and aquarium pump for 24 hours. The seeds were then collected on a 25-µm-pore-size sieve and back-washed into a blender with 400 ml distilled water. The mixture was homogenized for 30 seconds. One liter of tap water was added progressively as the mixture was passed through 850-µm-pore-size (20 mesh) and 25-µm-pore-size sieves. Residue from the 25-µm sieve was back-washed into counting dishes, and nematodes were counted.

Method 3, misting: Fifty grams of seed from each lot were soaked in distilled water and aerated for 24 hours. The seeds were then collected on a 25-µm-pore-size sieve and transferred with a spoon to a tissue-lined stainless-steel wire basket, which was placed over a large funnel and tube apparatus in an intermittent mist chamber. After 2 to 7 days, until seed germination, the suspension in the tube was microscopically examined and the nematodes were counted.

Method 4, blender-funnel-host stimulant: Fifty grams of seed from each lot were placed in a blender with 300 ml of tap water and blended for 15 seconds, shaken, and blended again for 15 seconds. The mixture was then placed on a single tissue draped on an 850-µm-pore-size sieve supported over a large funnel containing tap water. Approximately 0.1 g of orchardgrass leaves was put in the funnel as a stimulant, and the water column was aerated. Check valves at the funnel base enabled the suspension to be drawn off after 24 and 48 hours. The suspension was passed through a 25-µm-pore-size sieve and microscopically examined.

In addition to these four methods, trials were conducted to further examine the effects of aeration and presence of host material on extraction efficiency. While aeration may routinely be applied to seed samples at CDFA and ODA, host material other than seeds is not added. The protocol was similar to method 4, except that 25 g of seeds were used instead of 50 g. Three treatments included aeration with an aquarium air stone, aeration plus 0.2 g orchardgrass leaves added to the collection funnel, and 0.2 g orchardgrass leaves alone added to the collection funnel. Each treatment was replicated twice and nematode counts from four seed lots were compared.

Data obtained in all tests were subjected to ANOVA with Systat software (SPSS, Chicago, IL). All juvenile counts were transformed with log $(n + 1)$ for statistical analysis. The effects of aeration and host tissue alone and in combination also were subjected to ANOVA after $\log(n + 1)$ transformation. In all analyses, Tukey's test was used for mean separation.

Results

Extraction of juveniles: Numbers of nematodes obtained in the four methods are shown in Table 1. Methods 1, 2, 3, and 4 averaged 56.3, 141.0, 0.6, and 1.0 juveniles/gram seed, respectively, for all seed lots. Although all 11 seed lots contained *A. agrostis* juveniles in varying numbers, no single method recovered juveniles consistently in every replication. Additionally, method 1 failed to yield any nematodes from all replicates of seed lot numbers 2, 3, and 6. Method 2 also failed to detect nematodes in lot 3, and method 3 similarly failed in lots 2, 4, 6, and 11. A few nematodes were extracted from all sample seed lots in at least one replicate of method 4. For all sample seed lots, the percentages of replicates with at least one juvenile (or absolute recovery) found with methods 1, 2, 3, and 4 were 20.0, 58.8, 50.1, and 85.5%, respectively.

Total numbers of nematodes in the four methods: The highest number of nematodes was collected with method 2 ($P < .001$, Table 2).

Table 2. Comparison of four methods for the recovery of *Anguina agrostis* juveniles per gram of seed from 11 commercial seed lots collectively.

Method	Total samples[a]	Juveniles per gram of root[b]	Standard deviation
1	55	0.341 c	0.859
2	55	1.481 a	1.417
3	55	0.554 c	0.716
4	55	0.761 bc	0.484

[a] Total numbers of samples for all 11 seed lots.
[b] Numbers given are log $(n + 1)$ transformed values of actual mean values. Means followed by a common letter are not significantly different according to Tukey's test ($P < 0.05$).

The other methods did not differ among themselves (Table 2).

For method 4, counts were taken at 24 and 48 hours after initiation of the experiment. Of the 55 replicates, 44 replicates had at least one nematode collected at 24 hours (80%), but, at 48 hours, all replicates had at least one nematode collected (100%). Additionally, counts were significantly higher at 48 hours compared to 24 hours ($P = 0.0137$) (Fig. 1).

The combination of aeration and orchardgrass leaf tissue provided significantly higher counts than aeration alone ($P = 0.038$); however, this combination was not significantly better than orchardgrass alone, nor was aeration alone significantly different from orchardgrass tissue alone.

Discussion

The level of precision necessary for regulatory nematology requires any extraction method to be carefully optimized so as to enable the detection of even a single plant-parasitic nematode that might be present within a sample. Quarantine action against a commodity is often necessary regardless of the number of regulated nematodes present. A sample containing one *A. agrostis* individual will receive the same regulatory action as that of a sample containing 1,000 individuals. Hence, the need to assess an extraction method for total nematode extraction (absence or presence), and not just quantitative estimates, must not be underrated.

In assessing the different methods for assaying seed-borne nematodes, one problem faced was in determining the actual number of nematodes already present within seeds. Variations in numbers of nematodes within a variety are expected in naturally infested, field-grown seeds. It is probable that even if known nematode inoculum were added experimentally to growing plants, similar variations in final numbers of nematodes would result. In this study, seeds from lots that were predetermined to be infected with low levels of *A. agrostis* were included with those

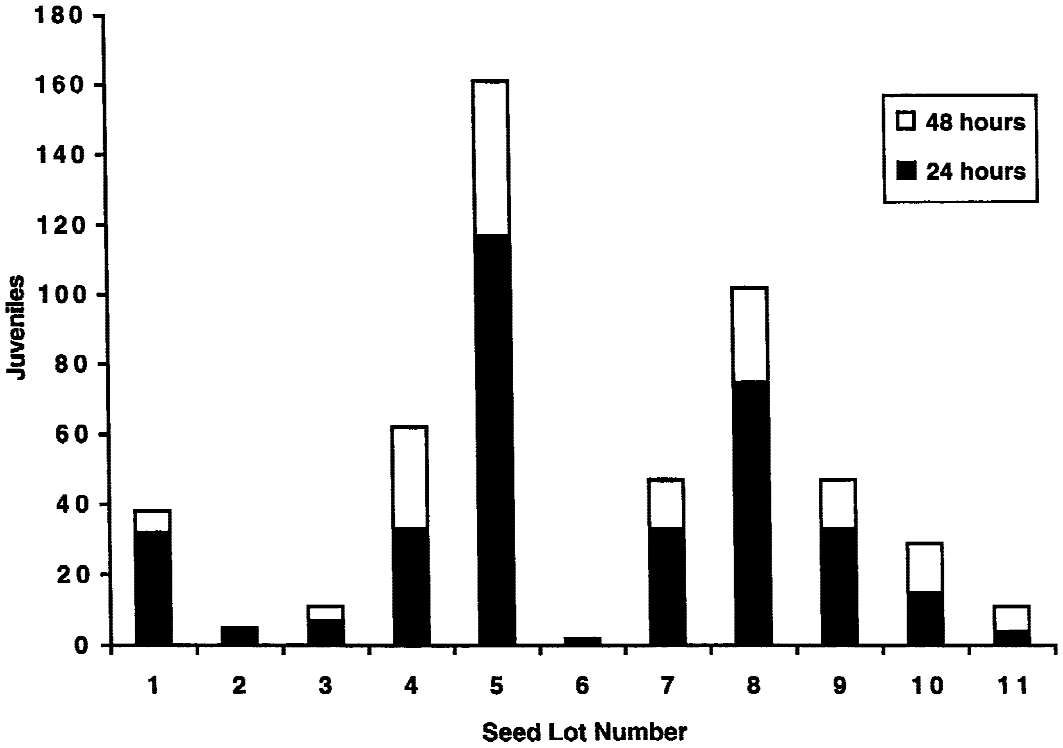

Fig. 1. Numbers of *Anguina agrostis* juveniles extracted from bentgrass and orchardgrass seed lots over 24 and 48 hours.

from heavily infested fields. Samples from these lots were representative of exported seed commodities, which usually require state certification. The comparison of replicated trials provided a sound basis for estimating total numbers of nematodes per gram seed for each variety.

Method 2, which comprised a three-step soak-blend-sieve process prior to direct microscopic examination, produced the highest number of nematodes. While this test resulted in the greatest number of juveniles, it also involved difficulty in examination due to large amounts of seed debris present in the blended suspension. Other contaminants often present in the suspension included free-living Rhabditidae and *Aphelenchoides* spp. This method is a passive process not suitable as a bioassay for seed fumigation assessments as it does not depend on the viability of nematodes.

Method 1 was devised to accurately quantify the number of juveniles present by culturing nematodes in a growth chamber in the presence of germinating seed. Unfortunately, this method gave only the second-highest counts per gram seed and also had the highest number of replicates with no nematodes extracted (80%). While many plant-parasitic nematodes can be successfully cultured on sterile agar in the presence of host tissue (Hooper, 1986), this method produced only limited success. Compared with total nematode counts obtained in method 2 (also with 5-g samples), it is obvious that many nematodes were lost in the extraction process. It is likely that the nematodes were not successfully separated from plant tissue and media by the blending-and-sucrose-centrifugation methods and, therefore, were not quantified.

Methods 3 and 4 required nematodes to actively move through the sample mass and tissue barrier. This migration depended on a high degree of viability compared to other tests for viability, such as the direct observation of active larval movement or passive movement when probed by the tip of a pick. If a high degree of mobility is required for *Anguina* sp. juveniles to move from a seed gall on the soil surface or buried in field litter to a new host's inflorescence, then a robust test of nematode viability, such as barrier migration, may be a more appropriate evaluation.

Methods 3 and 4 both used 50-g seed samples and required 2 to 7 days to complete the tests. Thus, these methods would seem to be more likely to detect low-level infestations of nematodes, but this was not the case for method 3. Method 3, the mist chamber extraction, failed to detect *A. agrostis* juveniles in nearly all of the orchardgrass lots with low populations (samples 2, 4, 6, 11). It also failed to find any juveniles in more than half of the orchardgrass replicates, making it a poor choice for either live extraction or standard certification testing. However, method 3 extracted the highest counts of any method on the single bentgrass sample and also extracted juveniles in each of the five bentgrass replicates. While this observation is limited to a single sample, it may indicate either an important effect of seed or juvenile population on a given method of extraction.

Under the conditions tested, method 4 appeared to be the best procedure for live extraction. Significant effects of both aeration in combination with host material and a 48-hour extraction period appear to be critical factors in allowing this test to detect nematodes in every sample. Along with the high extraction rate of at least a few nematodes per replication, the resulting suspensions were clear and easy to observe and quantify.

With the dramatic increase in world trade of seed comes an increased need for biologically based phytosanitary standards and corresponding certification tests for pests and pathogens of concern. To assist this trade, official certification tests ideally should be cost-effective, timely, accurate, and reproducible. This report compares and contrasts current and newly devised tests for both the standard detection and viability of *Anguina* larvae in seed samples and can be used as a basis for developing universal testing procedures. Based on the results of this study, we recommend method 4 as the best method for extracting nematodes from seed and, therefore, suitable for adoption by regulatory agencies.

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