DETECTION OF *Drechslera avenae* IN OAT SEEDS

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ABSTRACT

The fungus *Drechslera avenae*, the causal agent of Helminthosporium leaf spot on oats (*Avena sativa*), survives as mycelium in crop residues and in infected seeds. In trials carried out in the laboratory, ten methods were evaluated for their efficiency to detect *D. avenae* in oat seeds. In each experiment, groups of two or three methods were compared to a standard protocol, in which seeds were placed in Petri dishes containing the Reis selective medium and incubated at 25±2 °C for ten days. Data were submitted to analysis of variation and the means of the methods were compared using the Dunnett test at the 5% significance level. Overall, the highest levels of seed infection by *D. avenae* were observed on oat seeds plated in the osmotic, the oat-agar and the Reis media, or on seeds subjected to heat treatment previous to incubation in malt-agar. Therefore, these methods should be recommended for detection of *D. avenae* in oat seed testing.

Additional Key words: Deuteromycetes, *Pyrenophora avenae* and, detection methods.

RESUMO

Detecção de *Drechslera avenae* em sementes de aveia

O fungo *Drechslera avenae*, agente causal da helmintosporiose da aveia (*Avena sativa*), sobrevive como micélio em restos culturais e em sementes infetadas. Em ensaios conduzidos em laboratório, dez métodos foram avaliados considerando-se sua eficiência em detectar *D. avenae* em sementes de aveia. Em cada experimento, grupos de dois ou três métodos foram comparados a um padrão, no qual as sementes foram dispostas em placas de Petri contendo meio seletivo de Reis e incubadas a 25±2 °C por dez dias. Os dados foram submetidos à análise de variância e as médias comparadas pelo teste de Dunnett (5%). Entre os métodos testados, maiores níveis de infecção de *D. avenae* foram observados nas sementes dispostas nos meios ou métodos osmótico, aveia-ágar e seletivo de Reis, ou no qual as sementes foram aquecidas previamente à incubação em malte-ágar. Sugerem-se que, em análise de sanidade de sementes de aveia, sejam utilizados esses métodos para a detecção de *D. avenae*.

INTRODUCTION

The white oat (*Avena sativa* L.), a cereal of the Gramineae family, is mainly cultivated in temperate climate regions. It is grown with the purpose of producing grain, green pasture, and hay, and as a winter cover crop for no-till farming. The grain is used for animal feeding and for human consumption as flakes, flour, and bran (Floss, 1991).

Among the factors that reduce grain production and the quality of oat is the Helminthosporium leaf spot**,** caused by the fungus *Drechslera avenae* (Eidam) Sharif (teleomorph = *Pyrenophora avenae* Ito & Kurib). This disease occurs in most oat producing areas (Reis & Soares, 1995) and the pathogen survives as mycelium in oat crop residues (Shaner, 1981; Kohli & Reis, 1994) and in infected seeds. Seeds are the main source of inoculum, especially in field areas cultivated under crop rotation (Turner & Millard, 1931).

The efficiency of methods for detecting of *D. avenae* in oat seeds can be influenced by the amount and position of the inoculum in the seed, by the presence of antagonists and by environmental factors (Soteros & Sheridan, 1972). The first known detection method for *D. avenae* was the filter-paper, developed by Muskett (1938). In this method, the inconvenient growth of antagonist fungi and bacteria is a major disadvantage that has led many authors to search for more accurate protocols (Soteros & Sheridam 1972; Chidambaram *et al.*, 1974; Elekes, 1983; Mathur, 1983; and Reis, 1983).

Some species of *Bipolaris* and *Drechslera* produce pigments, called anthraquinones, in culture media (Sivanesan, 1987). Brodal (1993), using the method developed by Joelsson (1983), observed production of a fluorescent blue pigment on filter-paper after incubating oat seeds infected by *D. avenae*.

Molecular markers such as RAPD (random amplified polimorphic DNA) and RFLP (restriction fragment length polymorphism), have been developed for genetic studies on the causal agents of fungal diseases in wheat (*Triticum*

aestivum L.) (Faris *et al.*, 1997), barley (*Hordeum vulgare* L.) (Valè *et al.*, 1994; Graner *et al.*, 1996) and oat (*Avena sativa* L.) (Chong *et al.*, 1994). These techniques could be extended to routine seed testing of specific fungal infections, including *D. avenae.*

In eradication tests of seed-borne pathogens**,** when seeds are treated with an efficient fungicide, pathogens are present in very low incidence and cannot be detected by health tests, such as the filter-paper. The objective in this research was to test ten laboratory methods in order to select the most sensible for detection of *D. avenae* in seed eradication tests.

MATERIALS AND METHODS

To select the most efficient method for *D. avenae* detection, ten protocols were compared in four laboratory-conducted experiments. The various protocols were organized in groups of two or three and compared to the same standard method, the Reis selective medium (Reis, 1983). In all tests seeds of the UPF 17 cultivar were used. In each substrate seeds were placed at similar distances from each other, for a total of ten seeds per 9 cm Petri dish. This work was done in a laminar flow hood and forceps were very often flamed. Plates were then sealed and incubated for seven days at 25±2 ºC and a 12 h photoperiod (except for the heating, osmotic, pigment, and Reis methods). In tomato-extract-agar, filter-paper, potato-sucrose-agar (PSA), filter-paper + nylon sponge disc, oat-agar, and V8-agar, 200 ppm of streptomycin sulfate were added to the substrate. In these methods, seeds were previously disinfested in sodium hypochloride at 2% (Fernandez, 1993). After colony development, fungus structures were examined under a stereo microscope for the identification of *D. avenae*. Seeds on which the fungus formed conidiophore and conidium were considered infected. The following methods were tested:

Experiment 1

(a) Tomato-extract-agar (Fernandez, 1993): seeds were plated in tomato-extract-agar (200 ml tomato extract; 3 g $CaCO_3$, 15 g agar and 1000 ml sterile distilled water); (b) filter-paper (Chidambaram *et al*., 1974): seeds were distributed over three sterile filter-paper layers embedded with water; and (c) standard Reis selective medium for isolation of *Cochliobolus sativus* (Ito & Kurib) Drech. ex Dastur (Reis, 1983): seeds were distributed on a PSA medium (15 g potato; 25 g sucrose; 10 g agar; and 700 ml distilled water) amended with 50 ppm of benomyl, 500 ppm of streptomycin sulfate, 300 ppm of neomycin sulfate, 3 ml of captan (stock solution = 133,33 mg/100 ml of sterile distilled water), and 5 ml of botran (dicloran) (stock solution = 200 mg/100 ml of sterile water).

Experiment 2

(a) PSA (200 g potato, 20 g sucrose, and 15 g agar in 1000 ml sterile distilled water) (Fernandez, 1993); (b) filter-paper + nylon sponge disc (5 mm in thickness) was placed below three filter-paper layers to maintain moisture; and (c) the standard protocol as described previously.

Experiment 3

(a) Heat treatment (Malone, 1962): seeds were placed on sterile glass Petri dishes and heated in a circulating air oven at 100 ºC, for 1 h. Afterwards, seeds were cooled to ambient temperature and then plated in malt-agar medium (Soteros & Sheridan, 1972); (b) oat-agar (100 g oat flour and 15 g agar in 1000 ml distilled water) (Fernandez, 1993); and (c) the standard described previously.

Experiment 4

(a) Osmotic (Brodal, 1993, adapted from Joelsson, 1983): seeds were placed on glass Petri dishes, heated to 90 ºC for 2 h, and cooled to ambient temperature. The seeds were then placed over three filter-paper layers and dipped in a 0,5 M sucrose solution (170g of cane sugar per litre of distilled water) for a few seconds. After seven days, in alternating 16 h- periods at 27±1 ºC, under white light (5.000 to 6.000 Lux), and 8 h at 21±1 ºC in the dark, seeds were removed and the filter-paper was sprayed with an 1% NaOH solution; (b) V8-agar (200 ml V8 juice, 3 g $CaCO_3$, and 15 g agar in 1.000 ml distilled water) (Fernandez, 1993); (c) pigments (Knudsen, 1982, for *Pyrenophora graminea* (Rabh.) Ito & Kurib and *P. teres* (Died.) Drech. in barley): seeds were disinfested by immersion in sodium hypochloride for 10 min and then distributed in Petri dishes over three layers of filter-paper moistened with 12 ml of 0,04% acetic acid; were incubated at 18–23 ºC, for 48 h, followed by 25 h at -20 ºC, and 12 days at 12-16 ºC in a 12×12 dark-ultraviolet light (400 lux) cycle; after incubation, the filter-paper was examined for presence of pigments and *D. avenae* spores; and (d) standard as described before.

Experiment 5

This experiment was carried out to examine the selectivity of the osmotic method and to differentiate the colour pigment produced by *D. avenae* from those of other *Drechslera* and *Bipolaris* species. Wheat (Emprapa 40 cultivar), barley (MN 697 and BR 2 cultivars), and oat seeds (UPF 17 cultivar), were tested by the osmotic method as compared to the BSA and Reis methods; a total of 12 treatments were tested. Two hundred seeds were used for each treatment (five plates × four replicates) and incubated under conditions previously described.

Statistical procedures

In all experiments a completely randomised design was used. Means for methods were submitted to analysis of variation and the incidence of *Bipolaris sorokiniana* (Sacc. in Sorok) in wheat, of *Drechslera teres* (Sacc.) Shoem., and *Bipolaris sorokiniana* in barley, and of *D. avenae* in oat were compared by the Dunnett test ($p < 0,05$) (Dunnett, 1964).

RESULTS AND DISCUSSION

The efficiency of the various methods (Experiments 1, 2, 3 e 4) in detecting *D. avenae* in oat seeds is presented in Table 1. In the Reis selective medium, used as the standard protocol, *D. avenae* formed conidiophore and conidia after ten days of seed incubation. There was no need for previous seed disinfestation and plating in laminar flow hoods. However, this medium did not avoid colony formation for *B. sorokiniana, Alternaria* spp., and other *Drechslera* species.

In experiment 1 (Table 1), sporulation of *D. avenae* initiated after ten days of incubation in tomato-extract-agar and after seven days in filter-paper. Although Petri dishes were sealed with plastic film, the filter-paper did not keep enough moisture throughout the incubation period.

In PSA, experiment 2 (Table 1), *Fusarium* spp., *Alternaria* spp., and storage fungi as *Penicillium* spp. and *Aspergillus* spp. developed. Observation of *D. avenae* conidia was possible from nine days of incubation. The presence of nylon sponge disc in the filter-paper method, kept enough moisture throughout the incubation period, not needing water reposition. In this method, observation of *D. avenae* sporulation was possible from the 7th day of incubation.

Heating the oat seeds to 100 ºC allowed for the recuperation of *D. avenae*. According to Malone (1962), heating eliminates most contaminating microorganisms, with little or no effect over *D. avenae*. After seven days of incubation, heating made seed examination easy due to the low production of fungus mycelium, and strong, uniform, and typical conidia and conidiophores could been seen. Seeds tested in this method showed only *D. avenae* colonies. From such results, we conclude that *D. avenae* was the only fungus to survive the tested temperature (100 °C). In oat-agar, evaluations were possible after ten days.

In the osmotic method (Table 1), infected oat seeds did not produce fungal colonies. After incubation, a rose coloured ring was observed on filter-paper around seeds. After removing seeds and spraying the filter-paper with a NaOH solution, blue pigments formed in 10-30 min. Such results were similar to those described by Brodal (1993). Among all protocols tested in experiments 1, 2, 3, and 4, the highest incidence of the target pathogen was detected by the osmotic method.

In V8-agar (Table 1), the assessment was possible after nine days of seed incubation. The method based on pigment production was laborious and required a longer incubation period. Pigments formed in filter-paper were not clearly seen. Also *D. avenae* grew weakly and did not sporulate in this substrate. Knudsen (1982), in tests with *Pyrenophora graminea* and *P. teres*, reported the pigment-based method provided a lower index (about 16%) of detection than the filter-paper + freezing.

In Experiment 5 (Table 2), *B. sorokiniana* formed a fluorescent lilac pigment when wheat seeds were analysed through the osmotic method and sprayed with NaOH solution. Blue pigments were also seen in 3% of the analysed seeds. According to Knudsen (1982) and Brodal (1993), pigments of different colours may form on filter-paper when seeds are infected by more than one species of *Drechslera*. For barley seeds (cultivar MN 697) plated in the osmotic method, a pale orange pigment formed on filter-paper. Such pigment turned purple after being sprayed with NaOH. Brodal (1993), evaluating *Drechslera graminea* (Rabh.) Shoem. and *D. teres* in barley, observed reddish pigments that turned lilac-rose after treatment with NaOH. According to the author, such pigments did not allow for differentiating such *Drechslera* species. On filter-paper, BR 2 barley seeds formed a light orange pigment, which did not change colour after treatment with NaOH. For oat seeds, a fluorescent blue pigment was again observed on filter-paper, which confirms previously obtained results (Experiment 4a). Under these experiment conditions, the osmotic method was selective to *D. avenae* because only seeds infected by this fungus formed blue pigments on filter-paper.

TABLE 1 - Incidence of *Drechslera avenae* in oat (*Avena sativa*) seeds as detected by ten seed-testing methods based on four experiments

Method	Experiments (means)*			
	1	2	3	4
Reis (standard)	46,00 a	49,25 a	44,7 a	49,25 b
Tomato-extract-agar	21,50 b	-	-	-
Filter-paper	11,00 b	-	-	-
PSA	-	27,25 b	-	-
Filter-paper-sponge**	-	18,00 c	-	-
Heat treatment	-	-	41,75 a	-
Oat-agar	-	-	36,50 a	-
Osmotic	-	-	-	61,50 a
V8-agar	-	-	-	39,75 c
Pigment	-	-	-	25,00 d
C. V. (%)	24,40	20,44	22,83	13,29
L. S. D. (%)	16,30	7,64	11,11	7,24

*Means followed by the same letter are not different from each other by the Dunnett test at 5% significance.
** Filter-paper + nylon sponge disc
- Treatment not tested in this experiment.

TABLE 2 - Incidence of *Bipolaris sorokiniana* on wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), *Drechslera teres* on barley, and *Drechslera avenae* on oat (*Avena* sativa) seeds as detected by three seed-testing methods (experiment 5)

Method	Pathogen incidence*			
	B. sorokiniana [1]	*D. teres* [2]	*B. sorokiniana* [3]	*D. avenae* [4]
Osmotic	17,50 a	38,00 a	16,50 b	18,00 a
PSA	23,75 a	43,75 a	92,75 a	12,00 b
Reis	22,75 a	47,50 a	88,50 a	15,25 a
C. V. (%)	17,71	10,60	7,06	14,11
L. S. D. (%)	9,64	11,66	11,87	5,43

* Means followed by the same letter are not different from each other by the Dunnett test at 5% significance.
[1] From cultivar Embrapa 40, wheat; [2] MN 697, barley; [3] BR 2, barley; and [4] UPF 17, oat.

Compared to the standard Reis medium, the osmotic method was the most sensitive for the detection of *D. avenae.* Seed tests such as the heat treatment and the oat-agar did not differ from the standard. Potato-sucrose-agar, tomato-agar extract, filter-paper, filter-paper + nylon sponge disc, pigments and V8-agar were not as good as the standard. Therefore, the osmotic, Reis, oat-agar, and heat treatment methods should be used in oat seed pathology tests.

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00138