Multivariate Evaluation of Isolation Techniques for Fungi Associated With Stored Rapeseed

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ABSTRACT

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A multivariate statistical method was used to evaluate six techniques for isolating seed-borne fungi. The objective was to determine the most reliable mycological isolation technique for fungi known to be actively involved in the deterioration of stored rapeseeds. Analyses were based on examination of 106 samples of *Brassica napus* 'Midas' rapeseed. The seeds were either sound, spoiled, or heated and were obtained from primary elevators across western Canada. Isolation techniques were plain (PA), malt salt (MSA), and Czapek solution (CZA) agars and filter paper soaked with either water (FP), 7.5% salt (SFP), or Czapek (CZFP) solutions. The results of principal component analyses within individual techniques indicated that the FP and SFP techniques provided information on most of the major fungal variables associated with seed deterioration. Because of their simplicity these two techniques together were preferred. The FP-SFP combination was considered superior after interpretation of the results of the principal component analyses of pooled data of the seven combinations of techniques (PA-MSA-CZA-FP-SFP-CZFP, PA-FP, MSA-SFP, CZA-CZFP, FP-SFP-CZFP, PA-MSA-CZA, and FP-SFP). In the PCA of the FP-SFP combination, 52.7% of the variability was accounted by the first, and 13.3% by the second of the principal components. The key fungal variables associated with the first two components were: *Aspergillus glaucus*, *Penicillium* spp., and *Cladosporium* spp. Data on seed germination were obtained from FP but not SFP necessitating the use of both techniques. In the absence of a single technique, the FP-SFP combination appeared to be the most desirable for the mycological study of rapeseeds.

Additional key words: stored rapeseed, deterioration, fungal pattern, principal component analysis.

Techniques to isolate saprophytes from plant material can be broadly divided into (i) techniques for isolation of the maximum number of organisms or species, and (ii) techniques for specific species. The techniques presently available exist in the original published form and also as numerous modifications (12). The choice of techniques will depend on the objective of the study, the facilities available, and the prior training and personal idiosyncracies of individuals. Many techniques, however, are not designed for solving ecological problems in grain storage. The techniques that could be used for solving such problems should be evaluated through isolation of the most relevant fungal species. In particular, an objective evaluation of mycological techniques used in seed storage problems is urgently needed.

The objective of this research was to determine whether one single isolation technique is adequate for ecological studies of the mycological flora of stored rapeseed, and if not, what combination of techniques should be used for this purpose. Our objective was carried out in four steps: (i) to record all fungal variables isolated by each of six mycological isolation techniques; (ii) to eliminate from further analyses those variables that are not represented in more than 10% of the samples; (iii) to summarize and determine the natural pattern of relationships among groups of the remaining fungal variables by using a multivariate statistical method, principal component analysis (PCA); and (iv) to choose one or more mycological techniques to obtain the most representative natural combination of fungal species that cause decay of stored rapeseed. Such combinations were evaluated through practical experience with grain storage fungi.

We minimized the bias in the choice of techniques by including as many relevant techniques as possible. Data obtained from the application of the selected set of techniques to seeds of the same storage history were subjected to PCA to determine patterns of occurrence of fungi. The term "fungal pattern" was used to indicate the natural grouping of different fungi that could be isolated with the techniques. The relation of fungal population patterns to grain spoilage was tested for significance. If the selected patterns were relevant, then the technique combinations involved could be considered by seed mycologists for diagnosing grain spoilage problems.

MATERIALS AND METHODS

Seed samples.—During November-December 1975, 106 samples of sound, heated, or spoiled rapeseed

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(*Brassica napus* L. cv. 'Midas') were received from grain elevators in all major rapeseed growing areas of Manitoba, Saskatchewan, and Alberta. Each sample was thoroughly mixed and a 20-g subsample hand-cleaned to remove dockage, then stored in a screw-capped glass bottle at 4 C.

Mycological isolation techniques.—Preharvest, harvest, and postharvest fungi occur on seeds in storage. Preharvest fungi attack seeds on plants in the field before they are harvested; harvest fungi contaminate seed during cutting, swathing, combining, and trucking; and postharvest fungi multiply and infect during storage and transportation until the grain is processed for consumption. Particular isolation techniques favor the isolation of one or more of these groups. The six isolation procedures used were designated as "isolation techniques" for convenience in discussion and to avoid confusion with statistical methodology even though some of these techniques merely involve usage of different agars. Agar media used were: plain agar (PA) (8), malt salt agar (MSA) (3), and Czapek solution agar (CZA) (12). Filter-paper-based media were: filter paper soaked with water (FP) (13), with 7.5% NaCl solution (SFP), and with Czapek solution (CZFP). Czapek solution contained the same constituents as Czapek solution agar (12) but without agar, and PA contained 15 g Difco Bacto® agar per liter. The SFP technique was developed by H. A. H. Wallace (*unpublished*) when he was investigating the effect of sea water on flooded grain cargoes. We selected FP for isolation of preharvest and harvest fungi, PA as a minimum nutrient medium for isolating preharvest and harvest fungi and actinomycetes, MSA and SFP for xerophilic postharvest fungi and some preharvest fungi, and CZA and CZFP for preharvest fungi, some postharvest fungi, actinomycetes, and bacteria. All solutions were prepared with distilled water and then sterilized. Media with agar contained 12 ml agar per 90-mm diameter plastic petri dish and those with filter paper 4.5 ml of solution per dish. Each of the 106 seed lots was plated on the six media. For each agar medium, 10 seeds were placed in each of five 90-mm-diameter petri dishes using sterile forceps. For each filter paper medium, 25 seeds were placed in each of two petri dishes. The rapeseeds were not surface-sterilized as we wished to include superficial fungi as well as fungi in the seed coat and embryo. All dishes were incubated for 4 days in plastic bags at 25 C in darkness; dishes were read 3 days later. Fungi on the seeds were identified using a stereo microscope with magnifications up to ×100, and the frequency of occurrence was recorded.

Description of variables.—Each variable is defined as the number of seeds in a sample exhibiting a particular fungus. Variables other than those described below were measured but were omitted from the statistical analyses when zero values occurred in 90% or more of the total samples.

The variables were:

1. *Absidia* spp. (Phycomycetes) (ABSI), included the postharvest fungi *A. corymbifera* (Cohn) Sacc. & Trott. and *A. ramosa* (Lindt) Lendner.

2. *Alternaria alternata* (Fr.) Keissler (Fungi Imperfecti) (ALTE) (preharvest).

3. *Aspergillus glaucus* series group (AGLA), included the postharvest fungi *Eurotium amstelodami* Mang., *E. repens* de Bary, *E. rubrum* König et al. and *E. chevalieri* Mang.

4. *Aspergillus candidus* Link ex. Fr. (ACAN), (postharvest).

5. *Aspergillus flavus* Link ex. Fr. (AFLA), (postharvest).

6. *Aspergillus fumigatus* Fres. (AFUM), (postharvest).

7. *Aspergillus nidulans* (Eidam) Wint. (ANID), (postharvest).

8. *Aspergillus versicolor* (Vuill.) Tiraboschi (AVER), (postharvest).

9. *Cladosporium* (CLAD) (Fungi Imperfecti), included two species *Cladosporium cladosporioides* (Fres.) de Vries and *C. herbarum* (Pers.) Link ex. Fr., both usually regarded as preharvest fungi.

10. *Gonatobotrys simplex* Corda (Fungi Imperfecti) (GONA) (harvest).

11. *Mucor* (Phycomycetes) (MUCO), included several species (postharvest).

12. *Papularia arundinis* (Corda) Fr. (Fungi Imperfecti) (PAPU) (harvest).

13. *Penicillium* (Fungi Imperfecti) (PENI), included several species of which the most common were *Penicillium cyclopium* Westling, *P. brevicompactum* Dierckx, *P. patulum* Bain, and *P. chrysogenum* Thom. (postharvest).

14. *Rhizopus oryzae* Went & Geerligs (Phycomycetes) (RHIZ), was a postharvest fungus which usually invaded damp seeds with broken seed coats.

15. *Streptomyces griseus* (Krainsky) Waksman and Henrici (Actinomycete) (ACTI); it was the most common seed-borne storage actinomycete in western Canada. For statistical purposes it was treated as a postharvest fungus.

16. Bacteria (BACT) included several species common on grain. For statistical purposes they were treated as fungi.

Statistical procedures.—Statistical analyses of up to 16 variables, which were used to describe the fungi on the seed, were carried out with computer programs developed by Lee (7). Principal component analysis (PCA) was carried out on the results from all samples plated on the 6 isolation media. All variables were subjected to log 10 (x + 1) transformation because their frequency distribution was J- or U-shaped. The statistical methodology used in the analyses was given in Anderson (1), Kendall (6) and Seal (10).

Principal component analysis (PCA).—Data can be screened by PCA to determine the main constituents, the underlying structure (if any), and order in the system. One also can determine whether most of the information contained in several subjectively chosen variables can be expressed by a few transformed variables or vectors, called principal components; underlying structures are not assumed but variables are measured on random samples (11). We used PCA to determine the patterns of fungal variables yielded both within each technique (1st set of PCA's) and within several technique combinations (2nd set of PCA's). The number of variables to be used in an analysis was decided by including only those variables which occurred in at least 10% of the total number of samples.

PCA within an isolation technique (1st set).—These analyses were applied to explore the pattern existing

among fungal variables in each of the six isolation techniques. The aim was to determine the best pattern of fungi that can be obtained by using that particular technique, compatible with existing knowledge of the grain storage microflora.

PCA on pooled data of isolation technique combinations (2nd set).—The technique combinations contained: I. all agar and filter paper techniques used in this study (PA, FP, MSA, SFP, CZA, CZFP); II. techniques with no artificial nutrient (PA, FP); III. saline solution (MSA, SFP); IV. Czapek solution (CZA, CZFP); V. filter paper base (FP, SFP, CZFP); VI. agar base (PA, MSA, CZA);and VII. a simple comprehensive combination (FP, SFP).

RESULTS

Interpretation of data.—The component loadings (= principal component loadings) were examined to interpret the contribution of each variable to a particular component. An arbitrary cut-off point of 0.30 was set for all loadings. Generally opposite signs before the loadings of two variables in one component was interpreted as an inverse relationship for that component. Further, if two variables had the same signs in one component, and opposite signs in another, we interpreted this to mean that the variables behaved in both similar and opposing ways at different times. Often it was difficult to interpret C_3 and subsequent components, having already interpreted C_1 and C_2 (6). The difficulty lay in reconciling the apparently contradictory behavior pattern of a single variable as indicated by a change of sign. A change of sign may not necessarily have meant a biological contradiction or a statistical error. This was because several fungal species infect the same seed and behavior and ecological requirements often overlap. The behavior of some variables; e.g., *Penicillium* and *Cladosporium,* radically changed in time, in space, in association with other organisms or in nutrient media. Such changes resulted in expression of diametrically opposite relationships at one point of time to another, one point in space to another, one association of organisms to another or from substrate to another.

PCA within a technique (1st set).—The number of fungal variables occurring in 10% or more of seed samples ranged from 14 with CZA to 7 with MSA (Table 1). Principal component matrices of six PCA's based on individual correlation matrices of data from the six isolation techniques are given in Table 1. Principal components C_1 to C_3, added together, accounted for 64.4, 68.3, 77.2, 60.1, 58.6, and 72.5% of variability for techniques PA, FP, MSA, SFP, CZA, and CZFP, respectively. Following general practice (6), the first three principal components are listed, but only C_1 and C_2 accounting together for about half, or more than half of the variability were used for interpretation.

PCA on pooled data of technique combinations (2nd set).—Principal component matrices of six PCA's based on individual correlation matrices of data from the seven isolation technique combinations (analyses I-VII) are given in Table 2. When the first three components (C_1 - C_3), with most interpretative value, were added together in each of the analyses they accounted for: I - 90.3, II - 63.7, III - 69.5, IV - 69.8, V - 70.7, VI - 76.1,and VII - 76.5% of total variability. However, as in the first set, only C_1 and C_2 accounting together for about half or more than half of the variability were used for interpretation.

Fungal patterns revealed by individual techniques (1st set).—No single technique represented all the major variables. *Alternaria, Aspergillus glaucus, A. candidus, A. versicolor, Cladosporium, Penicillium,* and actinomycetes were considered to be the major variables associated with seed deterioration. We interpreted the principal component matrices (Table 1) to mean that *Aspergillus flavus* and *A. fumigatus,* among others, were negligible in importance.

The FP technique represented the most common fungal variables associated with western Canadian grains and oilseeds; i.e., *Alternaria, Cladosporium, Penicillium,* and actinomycetes; and the variable, seed germination, was also associated. Data on fungal variables most frequently associated with deteriorating grain; i.e., *Aspergillus candidus, A. glaucus,* and *A. versicolor,* were represented by SFP, but not by FP. Fungi developing from seeds plated on SFP were discrete, well-developed and did not usually overgrow onto the filter paper. Data on seed germination and those on most of the major fungal variables associated with seed deterioration were represented by FP and SFP together. The technique combination FP-SFP, was simple and easy to use. Thus, we considered these as the preferred techniques; the other techniques were less satisfactory.

The CZFP technique, which was only moderately convenient as more chemicals had to be weighed and mixed, did not yield isolates of *Aspergillus candidus, A. glaucus, A. versicolor,* and *Penicillium.* The PA technique required agar preparation, yielded actinomycetes (8) but not *Penicillium.*

More time was needed to prepare MSA and CZA than PA. *Aspergillus candidus, A. versicolor,* and actinomycetes were not obtained in appreciable amounts with MSA and CZA. Data on seed germination were not obtained with MSA.

Fungal patterns revealed by technique combinations (2nd set).—The PCA was applied on an exploratory basis to determine whether various combinations yielded an interpretable fungal pattern. These isolation techniques were applied separately but pooled together for statistical analysis.

The PCA's of pooled data of the seven combinations of techniques were used to demonstrate the capability of each combination to yield useful information (Table 2). The seven combinations were further evaluated using several criteria. These were the variability accounted for in C_1, the ability to reveal the key fungal variables associated with seed deterioration in C_1 and C_2, the simplicity of preparation, (i.e., the number of techniques and total time involved), whether agar preparation was required, and the germination data given. Combination VII (FP-SFP) was considered to be more useful than the other combinations as the maximum variability of fungal infection (52.7%) was brought out in C_1. Also the key variables–*A. glaucus, Penicillium* spp., and *Cladosporium* spp.–were revealed in C_1 and C_2; only two techniques were involved requiring minimum preparation with no agar, and germination data was given. Combinations V (FP-SFP-CZFP) and VI (PA-MSA-CZA) were next in merit; of these combination V

TABLE 1. First three principal components of six principal component analyses based on individual correlation matrices of data from six mycological isolation techniques[a]

	Mycological isolation techniques[b]																	
	PA			FP			MSA			SFP			CZA			CZFP		
Variable[c]	C_1[d]	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3
ABSI	−33	24	17	23	18	−12	−04	−03	09	23	−04	08	−26	−10	−13			
ALTE	46	49	02	−37	72	29	81	−34	25	−36	29	02	55	28	−68	−25	73	05
AGLA	−42	21	−48	12	15	−04	−47	−09	22	65	−34	−36	−45	−49	−35	23	−10	−04
ACAN	−34	20	01	11	09	−03	−10	−02	87	48	19	77	−21	−14	−06			
AFLA	−15	13	11	07	15	−12				01	12	10	−05	08	−05	−03	12	63
AFUM	−05	01	02	08	03	−07												
ANID													−04	−01	02			
AVER	−05	06	08				−12	−13	30	28	48	−09	−08	−03	−03	05	01	04
CLAD	48	41	12	−44	05	−87	18	−06	−10	−16	06	−01	44	−26	55	−13	48	−23
GONA				−09	12	11												
MUCO													−03	09	−05			
PAPU	09	10	−03	−07	07	02							08	01	−03			
PENI	−01	−25	48	67	02	−23	25	92	17	−09	26	24	−31	58	29	−25	−23	64
RHIZ	−28	16	68	10	20	−19				02	−01	01	−12	10	−01			
ACTI	−22	58	−01	32	58	−15				22	66	−44	−04	−09	−01	31	39	35
BACT	−08	02	−09	05	06	05				−01	09	−10	26	−46	06	84	11	08
Variability explained (%)	28.3	19.6	16.5	38.2	17.9	12.2	37.6	27.7	11.9	30.9	16.7	12.5	30.5	16.2	11.9	38.4	22.3	11.8

[a]Variables used in the analyses for each technique are represented by a vector loading in each column. Decimal points are omitted, except in the bottom row.

[b]Abbreviations for techniques: PA = plain agar, FP = filter paper with water, MSA = malt salt agar, SFP = filter paper saturated with 7.5% NaCl solution, CZA = Czapek solution agar, CZFP = filter paper with Czapek solution.

[c]Abbreviations for variables: ABSI = *Absidia* spp.; ALTE = *Alternaria alternata*; AGLA = *Aspergillus glaucus* series group; ACAN = *A. candidus*; AFLA = *A. flavus*; AFUM = *A. fumigatus*; ANID = *A. nidulans*; AVER = *A. versicolor*; CLAD = *Cladosporium* spp.; GONA = *Gonatobotrys simplex*; MUCO = *Mucor* spp. PAPU = *Papularia arundinis*; PENI = *Penicillium* spp.; RHIZ = *Rhizopus oryzae*; ACTI = *Streptomyces griseus*; BACT = bacteria.

[d]The letters C_1, C_2, and C_3 = first, second, and third principal components.

TABLE 2. First three principal components of six principal component analyses based on individual correlation matrices of data from six combinations of mycological isolation techniques[a]

	Mycological isolation technique combinations[b]																				
	I			II			III			IV			V			VI			VII		
Variable[c]	C_1[d]	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3
ABSI				−22	33	−03	−15	10	16							03	−09	−03	12	09	−07
ALTE	−34	54	72	41	36	47	62	−33	53	39	66	05	−29	−17	−17	−37	67	62	−24	−26	92
AGLA	84	48	12	−18	27	−34	−62	−31	−01	10	−49	22	79	−32	−48	83	39	14	75	−57	07
ACAN				−17	23	−08	−32	28	72							13	−05	03	23	−13	−08
AFLA				−06	19	01				−01	03	09	−01	05	07				01	12	05
AFUM				−05	04	−01															
AVER							−19	17	38	02	−06	12				05	02	−02			
CLAD	−38	27	−05	50	11	37	18	−03	05	37	32	−05	−36	−27	−23	−39	20	−32	−36	−25	−11
PAPU				08	05	05															
PENI	18	−64	68	−63	−19	66	19	82	−13	−28	09	77	40	26	65	−02	−59	70	43	70	35
RHIZ				−19	26	27													−04	09	03
ACTI				−15	69	−05				−19	−12	−57	06	49	−37						
BACT				−03	05	−08				77	−43	11	−04	69	−34						
Variability explained (%)	50.0	23.5	16.8	32.8	17.1	13.8	33.3	20.4	15.8	39.0	20.0	10.8	37.7	19.2	13.8	42.9	21.2	12.0	52.7	13.3	10.5

[a]Variables used in the analyses for each combination of techiques are represented by a vector loading in each column. Decimal points are omitted, except in the bottom row.

[b]Technique combinations with: I. all and no modifications; II. no artifical nutrient; III. saline solution; IV. Czapek solution; V. filter paper base; VI. agar base; VII. a simple comprehensive combination.

[c]Abbreviations for variables: ABSI = *Absidia* spp.; ALTE = *Alternaria alternata*; AGLA = *Aspergillus glaucus* series group; ACAN = *A. candidus*; AFLA = *A. flavus*; AFUM = *A. fumigatus*; AVER = *A. versicolor*; CLAD = *Cladosporium* spp.; PAPU = *Papularia arundinis*; PENI = *Penicillium* spp.; RHIZ = *Rhizopus oryzae*; ACTI = *Streptomyces griseus*; BACT = bacteria.

[d]The capital letters C_1, C_2, and C_3 = first, second, and third principal components.

was superior as it involved less time.

Mycological significance of principal component analyses of the data.—The PCA has provided a multivariate summarization of the mycological data to determine the main fungal constituents; i.e., identified fungi characteristic of each isolation technique or combination of techniques. The PCA has also generated an hypothesis from the data that a combination of filter paper and salt filter paper techniques brings out the most representative types of seed-borne fungi from elevator samples of rapeseed.

When we look for the common pattern of a fungal species represented in all six techniques through visual comparisons, and the use of a 0.30 loading cut-off point, we find only *Alternaria* common to all techniques (Table 1). When the same samples are pooled together and analyzed by single PCA combination I (Table 2) we obtain a wider range of fungal species. In combination I based on pooled samples of all techniques, four variables, *Alternaria*, *Aspergillus glaucus*, *Cladosporium*, and *Penicillium* were represented. This wider range of fungi demonstrated that when one seeks a common fungal pattern, a visual comparison of PCA data is less efficient than PCA of the pooled samples of results from various techniques. Nevertheless, mycologists often have little choice but to use the visual comparison method instead of a statistical comparison of pooled samples.

DISCUSSION

In the absence of a single technique, the FP-SFP combination appeared to be most desirable for the mycological study of stored rapeseeds. This combination had some advantage over use of agar-based techniques. The SFP isolation technique yielded more recognizable fungal types on seeds that had less overgrowth and probably less exaggerated antagonistic effects (2). A major disadvantage of the many agar media (14) presently used for fungal isolation from seeds is that the fungi often differ in appearance with the medium used. On agar media some fungi fail to sporulate, and require time-consuming UV light application and/or other refinements. Also, media are often modified by individuals thus increasing the variability of media available for use. The FP-SFP combination, however, provided a means of obtaining standardized morphological descriptions of species confined to and sporulating on seeds on minimum nutrient media. At present, the mycologist concerned with stored seed often evaluates hundreds of grain samples and lacks morphological descriptions of the range of forms of individual microbial species occurring on seeds. Such descriptions now available are mainly those on standardized agar media. Disadvantages of the FP and SFP isolation techniques are a need for careful moisture monitoring in dishes because of the wet-blotter effect (5) and salt crystallization when SFP dishes dry out.

Seed pathologists and mycologists in general usually isolate fungi from seeds for either solving seed health, taxonomic, or ecological problems. The seed health approach, used by pathologists in seed testing stations, utilizes different techniques; e.g., filter paper and potato-dextrose-agar (PDA) (4) for determining the presence of specific pathogens in seed samples. In the taxonomic approach PDA (9) and less often CZA have been used for identifying as many species and strains of fungi as possible in a seed sample. The ecological approach is used by mycologists (13) for determing what ecologically relevent fungi occur naturally before, during, and after a problem arises in stored crops. In the ecological approach, we are mainly concerned with those fungi that are common and/or abundant in deteriorating grain, but not with those that are rare. This limitation is imposed by the requirements of the statistical procedures used in ecological studies. When the primary objective is to obtain an insight into the problem the way it occurs in situ, isolation techniques with an ecological goal in mind, rather than taxonomic techniques, are desirable.

Prior to this study, few attempts have been made to evaluate and recommend isolation techniques suitable for ecological purposes. Consequently, many seed storage studies showed more taxonomically represented forms rather than ecologically relevant forms of species simply because only taxonomic techniques were employed. If the present results for rapeseed also hold true for other crops, then earlier ecological seed storage studies, primarily using agar media, may need to be carefully re-evaluated.

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