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In vitro evaluation of fungal isolates for their ability to influence leaf rheology, production of pseudothecia, and ascospores of *Venturia inaequalis*

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Abstract

As part of an ongoing research project on biological control of apple scab, this study presents a novel approach for the *in vitro* selection of potential antagonists of the saprophytic phase of *Venturia inaequalis*. A collection of forty-two fungal isolates were tested for their *in vitro* ability to degrade apple leaf tissue, inhibit pseudothecia, and ascospore production. The inhibition of ascospore production cannot always be linked reliably with leaf degradation or the evaluation of pseudothecia production. Consequently, ascospore production was retained as the most useful screening parameter. Six isolates proved to significantly reduce the ascospore production of *Venturia inaequalis*. Two were as effective as *Athelia bombacina*, a previously reported antagonist of pseudothecia formation and inhibited over 98% of the ascospore production. These new organisms are now available for future field tests. Future selections from a large collection of fungal and bacterial saprophytes can now be based on a reliable and simple *in vitro* screening method.

Abbreviations: CRBD – completely randomized block design; CRD – completely randomized design; CRDA – centre de recherche et de développement agro-alimentaire; CRDH – centre de recherche et de développement horticole; DMI – sterol demethylation inhibitors.

Introduction

After years of research and treatment, apple scab, caused by *Venturia inaequalis* (CKE.) Wint., is still by far the most important disease of apple trees (*Malus domesticus* L.) worldwide (MacHardy, 1996). The consequences of this disease are: the loss of leaf photosynthetic area, which is due to both lesions and leaf fall (in the more serious cases), the unsightly lesions on fruits that render them unacceptable to the average consumer, and eventually can also lead to reduced size and premature windfall. Thus the pathogen affects the tree and the fruit in terms of vigor, yield and quality.

Since it is very difficult and costly to control the secondary cycles of the disease, the strategies for controlling this pathogen are mostly concerned with the destruction of the primary inoculum. Scab control is mostly achieved with systemic and protective fungicides, despite reports that tolerance to systemic chemical fungicides has been observed for a number of years (Jones and Walker, 1976), that resistance to two of the major systemic chemical fungicide families, namely: benomyl and dodine, is increasing (Jones, 1981) and more importantly, that resistance is already of concern in the most recent line of chemical defense: the sterol demethylation inhibitors (or DMI) (Hildebrand et al., 1989; Smith et al., 1991; Carisse and Pelletier, 1994).

Much research has been devoted to the control of primary infections, and it has yielded very costly and ecologically questionable spraying schedules (Funt, 1990). A change in practice is advocated by recent reports which recommend a considerable reduction in

overall pesticide use in agricultural systems (Anonymous, 1992). The whole controversy surrounding the use of synthetic chemical pesticides has fueled an interest in alternative solutions. The biological control approach has been tested with little success in the past to reduce the summer scab (Andrews et al., 1983; Boudreau and Andrews, 1987, Cullen et al., 1984) but with great success to reduce the overwintering inoculum (Young and Andrews, 1990).

Several studies suggest methods to reduce the overwintering inoculum to reduce disease pressure the following spring (MacHardy, 1996). Until recently, (MacHardy et al., 1993) this discovery had very little impact on scab management in the field since no tool was available to scientifically integrate the impact of reduced inoculum levels on the number of fungicide applications the following spring. Under the Potential Ascospore Dose (PAD) system, orchards with a low inoculum density (< 700 ascospores per m^2, as determined with a late fall assessment of scab) can delay the first fungicide applications up to pink stage. Such practical methods are likely to fuel a renewed interest in inoculum reduction techniques including the use of biological control.

Heye (1982) screened 57 organisms for their ability to inhibit pseudothecia formation. The results showed that a basidiomycete, *Athelia bombacina* Pers. proved to completely inhibit pseudothecial formation in both controlled laboratory experiments and in the field. Moreover, subsequent reports (Young and Andrews, 1990) showed that this approach is encouraging, even more so if the potential synergism of other compatible methods are considered alongside of it. These include fall urea applications (Burchill and Cook, 1970), certain fall chemical applications of etephon or Ethrel® (2-chloroethylphosphonic acid) to promote defoliation (Heye, 1982), and/or chemical fungicides to assist the potential mycological antagonist (Miedtke and Kennel, 1990).

The purpose of our experiment was to test the differential ability of fungal isolates to inhibit the ascospore production and evaluate the reliability of other parameters used to measure antagonism. Since the number of pseudothecia per unit area was reported in the past as the main parameter to demonstrate fungal inhibition (Heye, 1982), this study compared the evaluation of ascospore production and pseudothecia production for the ease of use and reliability. Leaf rheology measurements were included to determine if leaf strength and the relative competition for the substrate are linked. A collection of approximately forty orchard saprophytes and a known antagonist, *Athelia bombacina*, were used to test the *in vitro* screening methodology and were compared to previous methods.

Materials and methods

Leaf material

Non infected apple leaves were collected randomly from McIntosh trees in an orchard located in Frelighsburgh, Quebec on 9 June, 1993. Disks were cut using a home-made stainless steel cork borer of 2.7 cm diameter. Major veins were avoided in order to facilitate colonization by both the pathogen and the potential antagonists and to facilitate readings. The leaf disks were then pooled and sterilized by irradiation in a glass jar. The jar was placed 35 cm from the radiation source on a rotating platform with four dosimeters. The irradiation process lasted 10 hours. The jar and its contents were submitted to 40 kGy (4 Mrad) of gamma radiation.

Incubation chamber

Standard, universal short cylinder, screw cap, transparent glass jars of about 500 ml (8 cm diameter $\times$ 10 cm high) (Consumer Glass #B6336) were used to produce pseudothecia and observe the effects of antagonists. Jars were filled with 100 ml of Perlite™ (Whittemore Perlite Co. Ltd., Lawrence, Ma.) and 50 ml of distilled water and were then autoclaved for 15 min at 121 °C. After the jars were slowly cooled to laboratory temperature, the leaf disks were placed in them under sterile conditions, abaxial surface up, at a rate of 4 disks per jar. The jars were used as sampling units.

Inoculum of Venturia inaequalis

A conidial suspension of *Venturia inaequalis* was made from a equi-proportional mixture of conidia originating from 5 different isolates (1An1.3, 9A1.1, 15A1.1, 18b2.2, and 13c1.1) chosen randomly from a vast collection of single spore isolates maintained at the Agriculture and Agri-food Canada Horticultural Research and Developent Center (CRDH) at St-Jean-sur-Richelieu, Quebec (Canada). The conidia of *V. inaequalis* were produced on a large scale using very

slight modifications of previously published methods (Keitt and Palmiter, 1938; Williams, 1976). A second *V. inaequalis* inoculation method was tested alongside conidia. A mycelial suspension of *V. inaequalis* was made from 7 isolates (1An1.3, 9A1.1, 15A1.1, 18b2.2, 4b1.1, 26a3.3, and 15d2.1) obtained from the same collection. One-month-old cultures grown on potato dextrose agar were cut and inserted in a sterile plastic bag, along with 100 ml of sterile water. The bag contents were homogenized in a Stomacher™ laboratory blender (Seward medical, London, U.K.) for 30 seconds at normal speed or longer depending on the homogeneity of the contents of the bag after the initial treatment. Sterile repeater type syringes (Becton Dickinson, Canada Inc.) were used to inoculate the disks for both inoculation methods. For the mycelia inoculation (M), 50 μL of the mycelial suspension was drawn directly from the bottom of the bag and then inoculated onto the disk surfaces. Since the petri cultures produced conidia, the *V. inaequalis* mycelial suspension contained both conidia and mycelial fragments. Although the inoculum concentration was not measured, the contents of the Stomacher™ bag were thoroughly homogenized and the same volume of inoculum was used on all disks. Consequently, it was assumed that the same number of propagules or colony forming units was inoculated on each disks. For the conidia inoculation (C) each disk received 50 μL of the conidial suspension, which corresponded to approximately 5000 spores per disk. The disks were then incubated at room temperature in full darkness until inoculation with the fungal isolates.

Inoculum of fungal isolates

Forty-two partially identified fungal isolates in the conidia experiment (C) and thirty-seven in the mycelium experiment (M) were chosen from a collection of fungi isolated from different orchards in Quebec (Bernier, 1995). The fungal isolates were chosen according to the temperature at which they grew. The most cold tolerant were tested in this study. Two others, *Epicoccum purpurascens* Ehrenb. and *Trichothecium roseum* Link. were included in this study, following earlier work by Pelletier (unpubl.). An isolate of *A. bombacina* found antagonistic in Heye's study (1982) was kindly provided by Dr. Andrews (University of Wisconsin, Madison) and was included as a positive control to determine if our test would select it. *A. bombacina* served as a means of comparing the relative efficiency of the other candidate antagonists. The fungal isolates were all grown on V8 agar for approximately two weeks or longer for certain isolates in order to obtain colonies which covered approximately half the surface of ordinary petri plates. Approximately three to four weeks after the artificial apple scab inoculation, a mycelial suspension of each fungal isolate was made and applied directly on the leaf disks of 5 jars. For both the conidial and the mycelial experiment, a total of 20 disks were inoculated for each candidate, 5 replicates (blocks) of 4 disks each in a completely randomized block design. Two controls per block were used: sterile disks and disks inoculated with *V. inaequalis* alone. Each mycelial suspension was made by homogenizing cultures the same way as for the *V. inaequalis* (M) inoculum. Each disk received 50 μL of the mycelial suspension. The jars were kept at 25 °C in full darkness for approximately one week to favor fungal colonization, incubated at 4 °C for about a month and then were transferred to 10 °C for an extra 3 months. Observations were made by randomly squash mounting pseudothecia (Gadoury and MacHardy, 1982) found on disks inoculated with *V. inaequalis* alone to insure that pseudothecia had matured during this simulated winter.

Leaf rheology

All the disks were submitted to penetrometer rheology tests. Leaf disk thickness was measured with a micrometer and then individual disks were firmly mounted between two plexiglas plates with heavy paper clips. The plates were pierced in their center with a hole (1 cm diameter) large enough for the penetrometer anvil (6.35 mm diameter) but small enough to hold the disk in place. Care was taken to insure that the disk was placed over the hole in such a way that the anvil was aligned exactly where the thickness reading was taken. Major veins were avoided. The plexiglas plates were designed to be inserted in a slot of the Instron® penetrometer (Instron Corp. model 4201, Mass. USA) available at the Centre de recherche et de développement agro-alimentaire (CRDA) facility in St. Hyacinthe. A 50-Newton cell was used to take readings. The penetrometer crosshead speed was set at 100 mm/min. The maximum rupture force (peak load) (N), per leaf thickness (mm) (firmness) was recorded for each disk. All disks were recovered for the following tests.

Ascospore production

The ascospore production by the pseudothecia of each treatment was measured after the simulated winter on two disks per jar. Three different readings at weekly intervals were taken to observe any possible lag in spore maturation. A specially designed bubbler type apparatus, which was described in detail by Philion (1995), was used to force ascospore ejection. The results of the three extractions are reported for each treatment as the ascospore production per cm^2. Only the total productivity was considered in the analysis.

Pseudothecia production

The two remaining disks of each jar were cleared by autoclaving them in glass petri dishes with approximately 25 ml of a KOH solution 0.4 m (20 g/L). After careful decantation of the caustic solution, a few drops of lactophenol were added to each disk. The pseudothecia were counted under a binocular at a low magnification, and expressed as the number of pseudothecia per square centimeter.

Statistical analysis

For both inoculation methods (mycelium (M) and conidia (C)) of the the leaf rheology experiment, the variances of the populations of the two trials were homogeneous based on a F-test and were pooled for the analysis. However, the variance of the two trials of each method (M and C) for pseudothecia, and ascospore production were not homogeneous so the data was transformed using Y=Log(X+1) to stabilize the variance. The resulting transformed data could be compared and the two trials of each experiment were pooled. Simple analysis of variance models were analyzed with the pooled data using either leaf strength, log(ascospore+1), or log(pseudothecia+1) production as the dependent variables and the main effects, treatments and blocks, as sources of variation. The use of a completely randomized block design (CRBD) over a simple completely randomized design (CRD) increased the resolution of the experiment for each parameter analysed, since blocking had a significant effect on the ANOVA in all experiments. The effects of the treatments on rheology, ascospore, and pseudothecial production were compared to a control with a Dunnett one-tail test at the 0.05 confidence level. The control treatment was disks inoculated only with *V. inaequalis* for all rheology, pseudothecia and ascospore experiments. This test is not designed to compare differences among treatments but only to determine if they are better than the standard. As such, the critical value is smaller than a standard Tukey test (Steel and Torrie, 1980). Nonetheless, a Tukey test was used to compare successful antagonists to one another by grouping them according to significant differences and also to rate their relative efficiencies relative to a positive control *Athelia bombacina*. Tukey families were reported for treatments significantly different from the control according to Dunnet only, and would otherwise normally extend beyond. Pearson correlation moment products (Steel and Torrie, 1980) were computed between all measured variables. No direct comparisons between the two inoculation methods were possible.

Results

Effect of fungal isolates on leaf rheology

The *V. inaequalis* control significantly affected leaf firmness as compared to the sterile disk, consequently it served as the reference for the other treatments instead of the sterile disks. Aside from the positive control (*A. bombacina*), a total of ten isolates in the C and twenty-one in the M experiment significantly reduced the leaf strength *in vitro* at the 95% confidence level (Table 1). Of these, five of the six best are common to both inoculation methods. These are isolates *Rhizoctonia* sp., *Coniothyrium* sp., *Phoma* sp. (6J-2), *Diplodia* sp., and *E. purpurascens*. Except for the difference between the sterile control and all the treatments, the leaf strengths showed no obvious groupings and vary smoothly with the different treatments. This is reflected by the multiple Tukey family groupings that overlap one another. The nine best treatments in the M experiment are not statistically different from one another, and similarly for the nineteen best treatments of the C method. Certain treatments rate differently in the two methods. Treatments with isolates *Pyrenochaeta* sp., *Cladosporium* sp. (2F10), *Cephalosporium* sp. (6J0), *Alternaria* sp.(1H4), *Chaetomium* sp. and *Penicillium* sp. (1F2) show very large differences in firmness when the two methods are compared. All of these show a larger leaf strength in the C experiment than in the M experiment.

Table 1. The effect of fungal isolates on the leaf rheology of leaf disks inoculated with *Venturia inaequalis* using two inoculation methods

Mycelium inoculation				Conidial inoculation			
Isolates	Firmness[a] (N/mm)		Reduction[b] (%)	Isolates	Firmness[a] (N/mm)		Reduction[b] (%)
Rhizoctonia sp.	2.73	∗a[cd]	55	*Rhizoctonia* sp.	3.21	∗a[cd]	49
Athelia bombacina	2.78	∗ab	55	*Athelia bombacina*	3.56	∗ab	44
Coniothyrium sp.	3.51	∗abc	43	*Phoma* sp. (6J-2)	3.88	∗abc	39
Phoma sp. (6J-2)	3.55	∗abcd	42	*Diplodia* sp.	4.09	∗abcd	36
Diplodia sp.	3.59	∗abcde	41	*Epicoccum purpurascens*	4.17	∗abcde	34
Epicoccum purpurascens	3.60	∗abcdef	41	*Phoma* sp. (J2C)	4.27	∗abcdef	33
Pyrenochaeta sp.	3.72	∗abcdefg	39	*Coniothyrium* sp.	4.27	∗abcdefg	33
Phoma sp. (010B)	3.96	∗abcdefgh	35	*Phoma* sp. (010B)	4.56	∗abcdefgh	28
Botrytis sp. (F4B)	4.27	∗abcdefghi	30	*Coniothyrium* sp. (6D0)	4.63	∗abcdefghi	27
Fusarium sp. (*306*)	4.37	∗bcdefghij	29	D-2B	4.65	∗abcdefghi	27
Coniothyrium sp. (6D0)	4.45	∗bcdefghijk	27	6D-2	4.68	∗abcdefghi	26
Coniothyrium sp. (2J4)	4.49	∗cdefghijkl	27	*Alternaria* sp. (D6A)	4.91		23
Cladosporium sp. (2F10)	4.51	∗cdefghijkl	26	*Fusarium* sp. (*306*)	4.94		22
D-2B	4.51	∗cdefghijkl	26	C2A	4.97		22
Phoma sp. (J2C)	4.56	∗cdefghijkl	25	*Botrytis* sp. (F4B)	5.06		20
Cephalosporium sp. (6J0)	4.56	∗cdefghijklm	25	*Trichoderma* sp. (*4J2*)	5.14		19
6D-2	4.58	∗cdefghijklm	25	*Coniothyrium* sp. (2J4)	5.15		19
Alternaria sp.(1H4)	4.77	∗cdefghijklm	22	5D10	5.17		19
Alternaria sp. (D6A)	4.79	∗cdefghijklm	22	*Ophiostoma* sp.	5.30		17
Chaetomium sp.	4.85	∗cdefghijklm	21	*Aureobasidium* sp. (20J22)	5.40		15
Fusarium sp. (*D2B*)	4.87	∗cdefghijklm	20	*Fusarium* sp. (*D2B*)	5.40		15
Penicillium sp. (*1F2*)	4.94	∗cdefghijklm	19	*Pyrenochaeta* sp.	5.41		15
Trichoderma sp. (*4J2*)	5.02		18	*Cladosporium* sp. (2F10)	5.45		14
6D2	5.05		17	*Trichoderma* sp. (1H22)	5.46		14
Chaetomium sp.	5.07		17	*Penicillium* sp. (*F0A*)	5.49		14
C2A	5.11		16	*Cephalosporium* sp. (9H22)	5.51		13
Penicillium sp. (*F0A*)	5.15		16	*Arthrobotrys* sp.	5.55		13
5D10	5.16		16	*Cephalosporium* sp. (6J0)	5.78		9
3H18	5.18		15	12J18	5.83		8
010A	5.22		15	*Chaetomium* sp.	5.84		8
1H10	5.26		14	*Phoma* sp. (3D0)	5.88		7
Trichoderma sp. (1H22)	5.26		14	3H18	5.91		7
Cladosporium sp. (7D10)	5.31		13	010A	5.98		6
Cephalosporium sp. (9H22)	5.37		12	*Trichothecium roseum*	6.02		5
F18A	5.43		11	1H10	6.1		4
7D18	5.43		11	F18A	6.13		3
Aureobasidium sp. (20J22)	5.58		9	*Penicillium* sp. (*3H22*)	6.27		1
Phoma sp. (3D0)	5.64		8	*Cladosporium* sp. (7D10)	6.29		1
Ophiostoma sp.	5.69		7	7D18	6.32		1
Unidentified yeast (502)	5.86		4	*Venturia inaequalis*	6.35		0
Penicillium sp. (*3H22*)	5.89		4	*Penicillium* sp. (*1F2*)	6.37		0
Aureobasidium sp. (*1H2*)	5.96		2	6D2	6.41		–1
Verticillium sp.	6.00		2	Unidentified yeast (502)	6.51		–3
Trichothecium roseum	6.05		1	*Alternaria* sp. (1H4)	6.58		–4
12J18	6.07		1	*Aureobasidium* sp. (*1H2*)	6.78		–7
Venturia inaequalis	6.11		0	*Verticillium* sp.	7.16		–13
sterile	7.90	∗	–29	sterile	7.87	∗	–24

[a] Leaf firmness measured in N/mm. Mean of 8 to 10 measurements.
[b] Percent variation from the sterile disks.
[c] Values followed by a ∗ are significantly different from *V. inaequalis* according to Dunnett (p=0.05). The critical values for the mycelial and conidial inoculation were 1.11 and 1.49, respectively.
[d] Values followed by the same letter are not significantly different according to Tukey test (p=0.05). Tukey groupings can extend beyond treatments significantly different from the control.

Effect of fungal isolates on ascospore production

Aside from the control, three isolates (*Coniothyrium* sp., *Phoma* sp. (6J-2), and *Trichoderma* sp. (1H22)) significantly reduced the ascopore production in the C experiment while six did the same in the M experiment (Table 2). The three first isolates are common to both sets. Treatment with fungal isolate *Coniothyrium* sp. was reported by both methods to be as efficient as *A. bombacina*, while *Phoma* sp. (6J-2) is rated similarly only in the M experiment. More than 98% of the ascospore production ($>$1.84 log reduction) was inhibited by the fungi classed alongside *A. bombacina*. Similar to rheology, certain isolates rated differently with the C and the M methodologies. These were isolates *Cladosporium* sp. (2F10), *Pyrenochaeta* sp., *Chaetomium* sp., *Diplodia* sp., and *Ophiostoma* sp. Contrary to rheology, a clear demarcation could be found between the very good inhibitors and the non inhibitory isolates.

Effect of fungal isolates on pseudothecia production

In the C experiment, 5 isolates aside from the positive control (*A. bombacina*) significantly reduced the pseudothecial production (Table 3). These were isolates *Coniothyrium* sp., *Trichoderma* sp. (1H22), *Phoma* sp. (6J-2), *Trichoderma* sp. (*4J2*), and *Fusarium* sp. (*306*). Of these, the efficiency of the first three was not significantly different from *A. bombacina*, similarly to the two first isolates of the M experiment. A total of nine isolates significantly reduced pseudothecial production in the M experiment. The isolates in the best Tukey family of both inoculation techniques inhibited more than 96% of the pseudothecial production ($>$1.1 log reduction).

Correlation between the variables

No strong correlation could be found among any of the variables for the two inoculation methods. The correlation between the log of pseudothecia production and the log of ascospore production was the only one significantly different from zero (r=0.463) (Table 4). The three best candidates (*A. bombacina*, *Coniothyrium* sp., and *Phoma* sp. (6J-2)) appeared in the top ten of all tests of rheology, ascospore and pseudothecia production. However, fungal isolates ineffective against either the ascospore or the pseudothecia production such as *Rhizoctonia* sp., *E. purpurascens*, and *Phoma* sp. (J2C) are equivalent or better degraders than the ascospore inhibitors. Conversely, *Trichoderma* sp. (1H22), a strong ascospore inhibitor, does not affect leaf firmness. Isolates *Cladosporium* sp. (2F10), *Pyrenochaeta* sp. and *Chaetomium* sp. rated differently in the C and M experiment for both ascospore and rheology. These three isolates degraded the leaf tissue and inhibited the ascospore productivity more in the M experiment than in the C experiment.

Discussion

Two different types of *Venturia inaequalis* inoculum were used and both methods included several strains of *V. inaequalis* to avoid reproduction failure. In the first, conidia of the pathogen were produced (Keitt and Palmiter, 1938; Williams, 1976). The whole process took approximately 6 weeks. The second method, which made use of a mixed suspension of *V. inaequalis* mycelia and conidia was easier and faster since no intermediate steps were necessary. The working hypothesis was that the efficiency of mycelia to infect a leaf disk *in vitro* and eventually lead to sexual reproduction is similar to that of conidia. With both inoculation methods, a mycelial mat grew on the surface of the disks and caused the production of a certain number of 'aerial' pseudothecia which were not imbedded in the leaf tissue. A certain number of these 'aerial' pseudothecia were observed and squash mounted (Gadoury and MacHardy, 1982). They appeared no different from regular imbedded pseudothecia. This erratic growth pattern is likely to be more a side effect of using a sterilized environment than a different inoculum source. This is of no real concern in this study since the vast majority of pseudothecia grew normally. However, Young and Andrews (1990) reported a similar observation which prevented them from observing mycoparasitic associations under microscope. This could preclude future use of the current methodology for this type of observation.

Since preliminary tests showed that similar to the *V. inaequalis* inoculum, all candidate antagonists could grow from mycelial fragments, all candidate antagonists were inoculated as a suspension containing mostly mycelia obtained with the Stomacher®. Candidate antagonist colonization of leaf disks was not assessed directly. Heye (1982) reported that observation showed that the extent of fungal colonization is difficult to quantify especially since fungi with inherently different growth patterns are compared. Furthermore, *in vitro* colonization would not be indicative

Table 2. The effect of fungal isolates on the ascospore production of *Venturia inaequalis* using two inoculation methods

Mycelium inoculation				Conidial inoculation			
Isolates	Ascospore[a] (Log)		Reduction[b] (%)	Isolates	Ascospore[a] (Log)		Reduction[b] (%)
sterile	0.00	∗a[cd]	100.0	sterile	0	∗a[cd]	100.0
Phoma sp. (6J-2)	1.30	∗b	99.7	*A. bombacina*	1.76	∗b	99.3
A. bombacina	1.31	∗b	99.7	*Coniothyrium* sp.	1.79	∗b	99.2
Coniothyrium sp.	1.91	∗bc	98.6	*Trichoderma* sp. (1H22)	2.47	∗c	96.3
Trichoderma sp. (1H22)	2.59	∗cd	93.2	*Phoma* sp. (6J-2)	2.82	∗cd	91.6
Ophiostoma sp.	2.81	∗cde	88.7	*Cephalosporium* sp. (9H22)	3.43		66.2
Diplodia sp.	2.82	∗cde	88.3	F18A	3.53		57.1
Trichoderma sp. (*4J2*)	2.96	∗cde	83.8	Unidentified yeast (502)	3.58		51.5
Chaetomium sp.	3.11		77.2	*Trichoderma* sp. (*4J2*)	3.59		50.4
F18A	3.12		76.9	*Fusarium* sp. (*D2B*)	3.60		49.1
Cephalosporium sp. (9H22)	3.18		73.0	*Rhizoctonia* sp.	3.65		43.9
Rhizoctonia sp.	3.24		69.5	*Botrytis* sp. (F4B)	3.69		38.2
12J18	3.41		54.6	*Chaetomium* sp.	3.71		35.2
Fusarium sp. (*D2B*)	3.47		47.8	*Diplodia* sp.	3.72		34.0
Trichothecium roseum	3.47		47.4	010A	3.74		30.7
Aureobasidium sp. (*1H2*)	3.49		44.9	*Aureobasidium* sp. (20J22)	3.74		30.7
Aureobasidium sp. (20J22)	3.53		39.8	D2B	3.76		27.7
3H18	3.54		39.0	*Penicillium* sp. (*1F2*)	3.77		25.1
1H10	3.54		38.5	*Coniothyrium* sp. (6D0)	3.78		23.7
Pyrenochaeta sp.	3.55		38.0	3H18	3.78		22.8
D-2B	3.56		36.1	5D10	3.79		22.6
Phoma sp. (J2C)	3.60		30.1	*Epicoccum purpurascens*	3.79		21.3
Unidentified yeast (502)	3.61		27.4	*Penicillium* sp. (*3H22*)	3.80		19.9
Fusarium sp. (*306*)	3.66		19.1	6D-2	3.81		18.1
Penicillium sp. (*3H22*)	3.66		18.3	*Verticillium* sp.	3.82		16.7
Verticillium sp.	3.67		17.6	*Trichothecium roseum*	3.83		15.0
Cladosporium sp. (2F10)	3.68		15.1	6D2	3.83		14.9
Botrytis sp. (F4B)	3.69		12.5	*Arthrobotrys* sp.	3.84		13.1
010A	3.70		11.4	*Fusarium* sp. (*306*)	3.84		11.3
7D18	3.72		6.4	1H10	3.85		10.9
5D10	3.73		5.4	*Ophiostoma* sp.	3.86		8.2
Arthrobotrys sp.	3.73		4.8	7D18	3.87		6.9
Penicillium sp. (*1F2*)	3.74		2.8	*Aureobasidium* sp. (*1H2*)	3.87		5.8
Coniothyrium sp. (2J4)	3.75		1.6	*Cladosporium* sp. (2F10)	3.89		1.6
6D-2	3.75		0.8	*Venturia inaequalis*	3.90		0.0
Venturia inaequalis	3.75		0.0	*Penicillium* sp. (*F0A*)	3.90		–0.7
6D2	3.76		–2.1	*Cladosporium* sp. (7D10)	3.91		–3.9
Coniothyrium sp. (6D0)	3.77		–3.0	C2A	3.92		–4.3
Alternaria sp. (1H4)	3.77		–3.3	*Pyrenochaeta* sp.	3.92		–5.7
Penicillium sp. (*F0A*)	3.77		–3.8	*Cephalosporium* sp. (6J0)	3.93		–8.7
Phoma sp. (010B)	3.78		–6.3	12J18	3.95		–12.5
Epicoccum purpurascens	3.88		–33.2	*Phoma* sp. (3D0)	3.98		–19.9
				Alternaria sp. (D6A)	3.98		–20.9
				Phoma sp. (J2C)	3.98		–21.5
				Coniothyrium sp. (2J4)	4.03		–36.1
				Phoma sp. (010B)	4.05		–43.5

[a] Log (X+1) of the ascospore production of *V. inaequalis* per square cm. Mean of 5 to 10 measurements.
[b] Percent reduction in ascospore productivity compared to *V. inaequalis* inoculated alone.
[c] Values followed by a ∗ are significantly different from *V. inaequalis* according to Dunnett (p=0.05). The critical values for the mycelial and conidial inoculation were 0.75 and 0.53, respectively.
[d] Values followed by the same letter are not significantly different according to Tukey test (p=0.05). Tukey groupings can extend beyond treatments significantly different from the control.

Table 3. The effect of fungal isolates on the pseudothecia production of *Venturia inaequalis* using two inoculation methods

Mycelium inoculation				Conidial inoculation			
Isolates	Pseudothecia[a] (Log)		Reduction[b] (%)	Isolates	Pseudothecia[a] (Log)		Reduction[b] (%)
sterile	0.01	∗a	100.0	sterile	0.00	∗a	100.0
A. bombacina	0.01	∗a	99.9	*A. bombacina*	0.01	∗a	99.9
Trichoderma sp. (1H22)	0.08	∗a	99.3	*Coniothyrium* sp.	0.13	∗a	98.6
Coniothyrium sp.	0.29	∗ab	96.7	*Trichoderma* sp. (1H22)	0.22	∗a	97.3
Phoma sp. (6J-2)	0.49	∗bc	92.7	*Phoma* sp. (6J-2)	0.29	∗a	96.1
Trichoderma sp. (*4J2*)	0.73	∗c	84.8	*Trichoderma* sp. (*4J2*)	0.84	∗b	75.4
Chaetomium	1.12	∗d	57.5	*Fusarium* sp. (*306*)	1.01	∗bc	62.5
Aureobasidium sp. (*1H2*)	1.16	∗de	53.6	*Botrytis* sp. (F4B)	1.15		46.5
Fusarium sp. (*D2B*)	1.20	∗def	48.8	F18A	1.18		42.1
Aureobasidium sp. (20J22)	1.20	∗def	47.8	*Fusarium* sp. (D2B)	1.19		40.7
Rhizoctonia sp.	1.22	∗def	45.6	*Aureobasidium* sp. (1H2)	1.19		40.5
Unidentified yeast (502)	1.28		37.0	*Penicillium* sp. (3H22)	1.19		40.4
D-2B	1.29		35.6	Unidentified yeast (502)	1.19		40.2
F18A	1.30		34.6	010A	1.21		37.2
Ophiostoma sp.	1.30		34.2	*Penicillium* sp. (F0A)	1.22		36.4
Fusarium sp. (*306*)	1.30		33.8	3H18	1.23		35.0
Pyrenochaeta sp.	1.30		33.5	6D2	1.23		33.6
010A	1.31		33.2	12J18	1.24		33.1
12J18	1.31		31.7	*Penicillium* sp. (1F2)	1.26		29.6
7D18	1.32		30.9	*Pyrenochaeta* sp.	1.27		28.4
Coniothyrium sp. (6D0)	1.35		26.4	1H10	1.28		25.3
6D-2	1.35		26.1	5D10	1.28		25.1
3H18	1.36		24.1	*Ophiostoma* sp.	1.29		24.8
Penicillium sp. (*3H22*)	1.36		23.7	D-2B	1.29		24.8
Cephalosporium sp. (6J0)	1.37		22.0	*Trichothecium roseum*	1.29		24.2
6D2	1.38		20.1	C2A	1.31		19.7
Trichothecium roseum	1.38		19.8	*Epicoccum purpurascens*	1.32		18.7
Epicoccum purpurascens	1.39		18.6	*Arthrobotrys* sp.	1.32		17.3
1H10	1.39		18.4	7D18	1.33		16.7
Phoma sp. (J2C)	1.40		17.0	*Chaetomium* sp.	1.33		16.1
Arthrobotrys sp.	1.41		13.4	*Rhizoctonia* sp.	1.34		15.1
Cladosporium sp. (2F10)	1.41		13.4	*Cladosporium* sp. (7D10)	1.34		14.9
Botrytis sp. (F4B)	1.42		12.3	*Coniothyrium* sp. (2J4)	1.34		13.9
Diplodia sp.	1.42		11.9	*Aureobasidium* sp. (20J22)	1.35		13.0
Penicillium sp. (*1F2*)	1.44		6.7	*Cladosporium* sp. (2F10)	1.35		12.8
5D10	1.45		5.1	*Alternaria* sp. (1H4)	1.35		11.3
Penicillium sp. (*F0A*)	1.45		4.7	*Coniothyrium* sp. (6D0)	1.36		10.3
Verticillium sp.	1.47		1.7	*Phoma* sp. (3D0)	1.37		8.5
Coniothyrium sp. (2J4)	1.47		1.4	*Cephalosporium* sp. (6J0)	1.37		7.4
Venturia inaequalis	1.47		0.0	*Verticillium* sp.	1.38		5.6
Phoma sp. (010B)	1.53		–14.8	*Cephalosporium* sp. (9H22)	1.39		2.8
				Alternaria sp. (D6A)	1.40		1.7
				Venturia inaequalis	1.40		0.0
				6D-2	1.43		–6.4
				Phoma sp. (J2C)	1.46		–13.2
				Diplodia sp.	1.47		–16.8
				Phoma sp. (010B)	1.57		–16.8

[a] Log (X+1) of the pseudothecia production of *V. inaequalis* per square cm. Mean of 5 to 10 measurements.
[b] Percent reduction in pseudothecia productivity compared to *V. inaequalis* inoculated alone.
[c] Values followed by a ∗ are significantly different from *V. inaequalis* according to Dunnett (p=0.05). The critical values for the mycelial and conidial inoculation were 0.2 and 0.3, respectively.
[d] Values followed by the same letter are not significantly different according to Tukey test (p=0.05). Tukey groupings can extend beyond treatments significantly different from the control.

Table 4. Correlation among variables used to select the fungal isolates

Variables	Mycelium inoculation		Conidial inoculation	
	Coefficient	P-value	Coefficient	P-value
Firmness and Pseudothecia	–0.028	0.5836	–0.18	0.5560
Firmness and Ascospores	0.038	0.4664	0.023	0.4300
Pseudothecia and Ascospores	0.463	0.0001	0.429	0.0001

of natural conditions since the use of large amounts of inoculum in sterile conditions excludes competition with other fungi.

The results of the two inoculation methods for all three variables were very similar, although certain notable exceptions raise questions. The mycelial inoculation experiment (M) selected more organisms for all parameters than its counterpart. Since the variability of the mycelia experiment was lower than the conidia experiment, it follows that observable statistical differences were smaller and thus more organisms end up statistically different from the control. However, isolates such as *Pyrenochaeta* sp. in the mycelia rheology and ascospore experiment rates entirely differently with the conidia procedure. Since the five blocks of each repeated trial were inoculated at the same time from the same inoculum source, a fungus which would fail to grow entirely at this point would be strongly biased against in the pooled analysis. This example is true for the measurement of any parameter. In the future, reisolating the candidate antagonist from the disks at the end of the experiment would eliminate this problem. Similarly, Potato dextrose agar plates were inoculated periodically when inoculating *V. inaequalis* on the disks to insure that it actually grew. Unfortunately, this step was omitted for the fungal isolates inoculation. More importantly, the same organisms mostly gave high ratings with both methods. Furthermore, the procedure presented here could not have selected for ineffective isolates it can only exceptionally omit a good candidate. Since the mycelial inoculation is easier, one can safely choose it instead of conidial inoculation.

Following the pioneering work of Ross and Burchill (1968), who measured the relative difficulty of manually tearing up disks submitted to certain bacterial treatments, our experiment used leaf firmness to demonstrate relative disk decomposition. Heye (1982) used a hand-held penetrometer to observe differences between treatments but similarly to Ciotola (1987) we used an Instron® device. This very sophisticated equipment is generally more precise and permits finer studies of leaf degradation. Since maximum force (N) was used in the past (Heye, 1982; Ciotola, 1987) as a measure of leaf degradation, it was adopted in this experiment as well. However, since the peak load at the rupture point is proportional to the leaf thickness (G. Doyon, pers. comm.), the force per unit thickness (N/mm) was reported instead. The use of a very precise penetrometer resulted in a reduced variance and contrary to Heye (1982), data could be analyzed directly without transformation. Values of the first Tukey family extended beyond the significance level of Dunnett's comparison to the control in the conidia experiment because it is by design larger than Dunnett's (Steel and Torrie, 1980). Again because of the variability of the conidia experiment, contrary to the mycelia experiment we cannot determine the relative efficacy of these degraders. A very small difference can be found between our results and that of Heye (1982) on the degrading ability of *A. bombacina*. They observed a 72% loss of leaf firmness of that of sterile disks as compared to our results of 55% in the conidia experiment and 65% in the mycelia experiment, when similarly compared to the sterile control. However, any observed difference can be related to a number of factors including the speed of penetration, the temperature and relative humidity of the material being tested. This is a serious limit of the rheology technology which can only be overcome by testing the material exactly in the same manner. (G. Doyon, pers. comm.)

We observed a significant degradation of the leaf material by *V. inaequalis*, unlike Heye (1982). However, this result is not surprising, since the leaf disk is the sole source of carbon for *V. inaequalis* as well as the fungal treatments and a certain level of degradation is expected as the pathogen feeds itself. The corollary of this, is that the other fungi also feed on the same substrate and many treatments further degraded the disks and were found to be significantly different even from *V. inaequalis*. The multiple Tukey family overlapping is indicative that the degrading ability is shared by most isolates and no clear demarcation can be found between degraders and non degraders. This high success rate for degrading ability forces us to reconsider the usefulness of this parameter. Except for both *Trichoderma* sp. (1H22 and 4J2) tested, all

isolates antagonistic to pseudothecia or ascospore production were also leaf degraders. However, some good leaf degraders such as *Epicoccum* sp., were not antagonistic. This does not rule out a possible role of leaf degradation in spore reduction in conjunction with other mechanisms for certain isolates but it confirms that rheology cannot be used as a main screening parameter.

Irrespective of an earlier report (Heye, 1982) that leaf degradation is not a true measure of antagonism, our *a priori* assumption was that leaf rheology could be used to evaluate the contribution of one of the main modes of antagonism, the relative competition for the substrate. However, since certain non antagonists degraded the leaves better than the best antagonist (for example, *Rhizoctonia* sp.) rheology results become impossible to interpret. In essence, the precise measure of leaf firmness is more an indication of successful establishment of the agent than a measure of competition. The rheology procedure can thus be discarded to measure competition. However, it is still useful in the quest for leaf decomposers. Non-antagonistic fungal leaf degraders could act synergistically with earth worms, other naturally occurring scavengers and physical methods such as mowing or tillage to completely eliminate the substrate. Such a use should be further investigated.

For the ascospore and pseudothecia production, measures taken to stabilize the variance following earlier reports (Heye, 1982) such as incubating multiple disks in a chamber, proved insufficient and a log (X+1) transformation was necessary. Simply rating pseudothecia production would not be advisable since treatments with *Diplodia* sp. and *Ophiostoma* sp., which inhibited ascospore production were not selected with this parameter. Perithecia and pycnidia of other fungi were previously reported as a complication in assessing pseudothecial production on naturally infected disks (Heye, 1982) but these can also affect counting in artificial inoculation conditions. The fruiting bodies of certain fungal treatments inoculated alongside the apple scab pathogen are not always easily differentiated from *V. inaequalis* pseudothecia and this could lead to a bias. This problem cannot arise while assessing ascospore production, since these are uniquely shaped. Moreover, it is possible to automate ascospore counting (V. Philion, unpubl.). In the light of this information, one could consider eliminating pseudothecia counting altogether in a primary screening and concentrate on ascospore production.

The ascospore production parameter alone can confidently and rapidly select promising candidates for future outdoor trials. In this first batch tested, a total of six new organisms were found to be antagonistic to ascospore production. The last significant Tukey family (*Trichoderma* sp. (*4J2*), *Diplodia* sp., *Ophiostoma* sp., and *Trichoderma* sp. (1H22) inhibited over 83.8% of the ascospore production ($>$0.79 log reduction). Three isolates appear to be very interesting for future outdoor trials. Of these, at least 98% of the ascospore production ($>$1.84 log reduction) was inhibited by isolates *Coniothyrium* sp. and *Phoma* sp. (6J-2) which were as inhibitory to ascospore production as *Athelia bombacina*. The third, *Trichoderma* sp. (1H22), inhibited more than 93% ($>$1.16 log reduction). None were better than *Athelia bombacina*.

Despite an initial report that they were ineffective against the saprophytic phase of *V. inaequalis* (Heye, 1982), both *E. purpurascens* and *T. roseum* were initially included in the screening following reports of considerable antagonism in other plant pathogen systems and because these two fungi are almost always reported in the natural fungal community of apple and pear trees (Andrews and Kenerly, 1979; Bernier, 1995; Burchill and Cook, 1970; Hislop and Cox, 1969; Picco, 1985). Moreover, the two fungi are reported effective against several pathogens (Brown et al., 1987; Manandhar et al., 1987; Peng and Sutton, 1991; Singh and Sekhon, 1988; Upadhyay, 1980; Wu, 1976; Zhou and Reeleder, 1990) including fruit tree pathogens (Martinovic, 1967; Schulz, 1981). This study confirms the earlier report that the two showed no antagonistic ability against scab. Antagonists are often reported to be effective against more than one pathogen and we had high expectations for both the hyperparasitic ability and antibiotic ability of *T. roseum* (Bawden and Freeman, 1952) and of *E. purpurascens*, (Singh, 1985; Urbasch, 1989; Wu, 1977) but evidently there are limits to the scope of their activity.

The screening methodology was not designed to determine with precision the mode of action of the selected isolates. Heye's (1982) hypothesis that basidiomycetes are likely to be found as antagonists when decomposition of the substrate is the key to antagonism could not be confirmed here since some of the best degraders in this study (*Rhizoctonia* sp., *E. purpurascens, Pyrenochaeta* sp., *Phoma* sp. (010B), and *Phoma* sp. (J2C)), some of which as efficient as *A. bombacina*, were not basidiomycetes and were not inhibitory to *V. inaequalis*. Consequently, the leaf degrading ability of *A. bombacina, Coniothyrium* sp., and *Phoma* sp.

(6J-2) may or may not be involved in their inhibitory effect. Isolate *Trichoderma* sp. (1H22) is interesting because it is the only fungus that significantly reduced ascospore production without affecting leaf firmness. It is possible that only one or all modes of antagonism are used in conjunction. Specific tests would be required to answer this.

When pseudothecial production results of Heye (1982) are converted to a comparable scale, notwithstanding that our *V. inaequalis* control was slightly less productive, one finds that their least significant treatment had 1.046 (log pseudothecia cm^{-2}) while in our mycelia experiment, the least significant treatment produced 1.22 pseudothecia on the same scale. Our experimental setup could detect smaller differences in pseudothecia inhibition. This lower variation would select more treatments unless a further criterium is included. One could eliminate organisms unlikely to reach commercial development. For instance: potential pathogens, fungi which could do not grow at low temperatures or not well adapted to the orchard environment, and last but not least, organisms ill suited for mass production. The prior selection of cold tolerant fungi which can survive during the winter, organisms previously reported as antagonists, and members of genera previously unreported, or rare, presumably resulted in an artificially high success rate. This is questionable since known antagonists such as *E. purpurascens* and *T. roseum* were not selected. The *a priori* assumption that antagonism screening tests directed towards the stage of interest of a pathogen are superior to tests of mycelial inhibition was substantiated by our study. Conidia of *V. inaequalis* were found in all treatments including the best ascospore inhibitors. This last finding would indicate that the antagonists effective against ascospore production were not necessarily effective against conidia. Inversely, tests directed against mycelial growth or conidia production would probably not be suitable.

New organisms are now available to develop as biocontrol agents of apple scab and offer promise for the next step, field tests. Eventually, determining the mode of action of the organisms effective against scab would be useful to improve the antagonistic activity of the isolates. The proposed screening method based on ascospore production proved successful in selecting antagonists out of a collection of fungal isolates. Moreover, the modifications suggested in earlier work, introduced here, and proposed for future work will simplify, hasten and improve the screening methodology. Eliminating rheology and direct pseudothecia counts would contribute to reduce the time and cost associated with selecting organisms.

One has to keep in mind that no unique solution will solve the scab problem. In the foreseeable future, environmentally benign solutions reside in finding a multiple approach system. For instance, improved IPM strategies including methods aimed at managing the inoculum (PAD) which may include microbial antagonism (MacHardy, 1996). Moreover, any fruitful solution to any pest or disease including scab must also be compatible with the other parts of the production puzzle. Thus the combination of solutions will also be an integration of solutions.

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