FINAL PROJECT REPORT
WTFRC Project Number: PH-02-240 (Amendment for 2005 project)

Project Title: Molecular techniques to study apple and pear pathogens in CA storage

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University of British Columbia – Okanagan, Kandis Pohl and Dan Durall

Budget History:

Item	**Year 1:**	**Year 2:** N/A	**Year 3: N/A**
Salaries	20,000		
Benefits			
Wages	3,522		
Benefits			
Equipment			
Supplies	3,478		
Travel			
Miscellaneous			
Total	27,000		

Objectives:

1. Study epidemiology of fungal pathogens on storage room walls using molecular techniques for identification. Additionally determine if these fungi are pathogenic on apple and resistant to fungicides such as thiabendazole.

2. Develop a PCR assay for two new postharvest diseases (Phacidiopycnis and Sphaeropsis rot) known to occur in Washington and British Columbia.

Significant findings:

- Thirty nine *Penicillium* isolates were identified and sequenced from a survey conducted of different Controlled Atmosphere (CA) rooms in the Okanagan Valley

- Eleven of the 20 *Penicillium* spp. isolated from the CA walls were capable of decaying apples and seven of the 20 isolates were found to be resistant to thiabendazole (TBZ).

- Twelve *Phacidiopycnis* and 27 *Sphaeropsis* isolates were identified using molecular techniques that relied on unique DNA sequences from samples of a packinghouse survey of diseased winter pears conducted in January 2006.

- Polymerase chain reaction (PCR) primers were developed for both *Phacidiopycnis* and *Sphaeropsis*.

- *Phacidiopycnis* was identified in the orchard from canker-like areas and diseased branches of pear fruit trees.

Objective 1. Identification of the *Penicillium* pathogens using molecular techniques.

Methods:

1.1 Initial collection

Wall swab samples were taken from CA rooms in the seven major packinghouses in the Okanagan Valley between March and June 2005. The packinghouses had walls that were made of a number of different materials ranging from metal, painted plywood, styrofoam, concrete foam, white fire retardant mineral fiber and a dark fiber material. Samples were taken by dipping a sterile cotton swab or Q-tip into a sterile tube containing 10 ml of sterile distilled water (SDW) and swabbing over an area of 10 cm^2 by using a template. For one wall, five swabs were taken across the wall and placed into the same tube. For the walls covered with the fiber material five sample areas were collected across the wall, with the material being removed from a 2 cm^2 area and placed into one tube. To plate out the samples each tube containing the swabs were vortexed for 30 seconds before plating. A 100 µl aliquot was taken from each sample and then pipetted onto 100 ml Petri plates containing acidified potato dextrose agar (APDA) in triplicate. These were then incubated at 20ºC for five days or until the fungal colonies could be identified and counted. For those samples taken from the fiber material, 10 ml of SDW was added to each tube, which was then vortexed, plated and incubated as above. An initial selection of 100 *Penicillium* isolates was made from these plates taking into account different locations, wall materials and *Penicillium* morphology. Thirty-nine of the 100 isolates were further purified by single-sporing them for use in the molecular identification studies.

1.2 Molecular identification

The isolates were grown on 60 mm plates containing Potato dextrose agar (PDA) at 20^{o}C, for one to two weeks. Approximately 0.5 ml of wet fungal tissue was placed in a 2 ml extraction tube. Extraction of total DNA was accomplished using a FastDNA kit (BIO 101 Inc., Vista, CA) and eluted in 100 µl volumes. PCR amplification was carried out using the parameters in Sholberg et al. (2005) with a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA). The modifications made to the method of Sholberg et al. (2005) were the use of forward primers Bt-LEV-Up4 and Pex-ITS-35f in conjunction with reverse primers Un28S22, Pex-Bt-384r and Pen_other-233r. Three separate PCR reaction mixtures including known samples of *P. expansum*, *P. commune* and *P. solitum* were included as positive controls. A negative control of the PCR reaction mixture lacking DNA was also included. Purification using a Wiaquick purification kit (Qiagen, Mississauga, Ontario) and electrophoresis using a Mass ladder (Gibco-BRL, Burlington, Ontario) was carried out using the method in Sholberg et al. (2005). The primers Bt-LEV-Up4 and Bt-LEV-Lo1 (De Jong et al., 2001) along with the Big Dye Terminator sequencing mix (Applied BioSystems, Foster City, CA) were used to sequence the β-tubulin region of the *Penicillium* spp. in separate reactions. The sequences were then imported into SeqMan Pro sequence analysis software Lasergene 7.1 (DNSTAR Inc., Madison, WI) for contig assembly and visual editing. Ambiguities identified in the sequence data were resolved by comparison with reverse complimentary sequences.

1.4 Pathogenicity test.

Fifteen Gala apples were used per isolate (5 apples replicated 3 times); each apple was wounded in triplicate with a sterile nail (3.0 mm diameter). Fruit was then inoculated by pipetting 20 µl of a 1 x 10^{4} (conidia/ml) spore suspension of each isolate into each wound. The fruit was then incubated at 20^{o}C for 6 and 15 days, decay area was then measured and recorded using electronic calipers. Each of the decay areas were measured twice and the readings averaged. Only those isolates which caused decay on apples were tested for fungicide resistance.

1.3 Fungicide Resistance

Potato dextrose agar (PDA) amended with thiabendazole (TBZ) was used to test the sensitivity of the *Penicillium* isolates. Stock solutions of commercial grade thiabendazole (Mertect SC; Syngenta Crop Protection Canada Inc., Guelph, Ontario) were made by suspension in sterile distilled water (SDW) with different concentrations of active ingredient (µg ml = ppm) (Table 1). The stock solutions were then added to the PDA which had been autoclaved and cooled to 50°C to obtain the test concentrations. The media was then poured into 60 mm plates.

The stock solutions of the commercial grade thiabendazole at 500 g active ingredient /l were made as follows:

Stock solution A: 10 ml of TBZ (500,000 ppm)
Stock solution B: 1/10 dilution of stock A (50,000 ppm)
Stock solution C: 1/10 dilution of stock B (5000 ppm)
Stock solution D: 1/10 dilution of stock C (500 ppm)

Table 1: Amount of thiabendazole stock solution added to 200 ml of autoclaved PDA agar.

Required concentrations (ppm)	Amount added to agar (ml)	Stock solution
0	-	-
1	0.4	D
5	0.2	C
10	0.4	C
50	0.2	B
100	0.4	B
500	0.2	A
1000	0.4	A

A 7 mm diameter plug was then taken from the margin of a 10-14 day old pure culture and placed in the center of each plate. Each concentration was replicated twice. Plates were incubated at 20^{o}C for 4 days; mean colony diameters were measured and calculated to determine resistance.

Results and Discussion:

Penicillium expansum and *P. solitum* are important postharvest pathogens of apple, in this study they were found to occur on all the wall materials in the CA rooms as well as in the packing line and defrost water. The *P. cyclopium* was predominantly found on the fire retardant fiber material and occasionally on the other materials. Using molecular techniques the isolates were identified as 8 *P. expansum*, 11 *P. solitum*, 2 *P. commune*, 15 *P. cyclopium*, 1 *P. cyclopium-like*, 1 *P. melanoconidium* and 1 *P. polonicum* (Table 2).

The pathogenicity tests showed that of the 6 *P. expansum* isolates tested all were able to decay fruit at 6 and 15 days when compared with the positive control and *P. melanoconidium* which is not usually associated with fruit decay was also comparable with the control. One out of the 9 *P. solitum* isolates tested was found to cause decay at only 15 days while the others caused only mild decay at 15 days. One out of the 3 *P. cyclopium* isolates caused decay to the same level as the control (Fig.1 and Table 3).

Of the 20 isolates tested 4 were found to be resistant and 3 moderately resistant (50% growth reduction at 1000 ppm) to TBZ. These isolates were shown only to cause mild decay at the 15 day rating. All the *P. expansum* isolates tested were found to be sensitive to TBZ. Of the 9 *P. solitum* isolates tested only 1 was sensitive and the rest were found to be either resistant, moderately resistant or sensitive at the 500 ppm level (Table 3).

Table 2: *Penicillium* isolates with their locations and identifications.

Isolate	Location	Source	Material[1]	sequence ID
P-2	Kelowna	Room 82	dark fiber	*P. solitum*
P-3	Kelowna	Room 61	fire retardant fiber	*P. commune*
P-5	Kelowna	Room 72	metal/steel	*P. expansum*
P-8	Summerland	Dump tank	water	*P. expansum*
P-9	Summerland	Dump tank	water	*P. cyclopium*
P-10	Summerland	Packing line	water	*P. expansum*
P-11	Summerland	Packing line	water	*P. solitum*
P-12	Kelowna	Room 63	fire retardant fiber	*P. cyclopium*
P-13	Kelowna	Room 63	fire retardant fiber	*P. expansum*
P-17	Rutland	Room 57	fire retardant fiber	*P. solitum*
P-22	Kelowna	Room 61	fire retardant fiber	*P. cyclopium*
P-23	Kelowna	Room 83	fire retardant fiber	*P. cyclopium*
P-24	Kelowna	Room 61	dark fiber	*P. cyclopium*
P-26	Kelowna	Room 63	fire retardant fiber	*P. cyclopium*
P-28	Kelowna	Room 61	dark fiber	*P. cyclopium*
P-31	Kelowna	Room 64	metal/steel	*P. commune*
P-33	Kelowna	Defrost tank	water	*P. expansum*
P-35	Kelowna	Dump tank	water	*P. melanoconidium*
P-37	Kelowna	Room 63	pipes	*P. cyclopium*
P-39	Rutland	Room 54	fire retardant fiber	*P. expansum*
P-41	Winfield	Room 525	fire retardant fiber	*P. solitum*
P-43	Winfield	Room 526	fire retardant fiber	*P. solitum*
P-45	Winfield	Room 510	plywood	*P. cyclopium*
P-48	Winfield	Room 611	concrete foam	*P. cyclopium*
P-51	Winfield	Room 508	plywood	*P. solitum*
P-55	Naramata	Room 253	plywood	*P. solitum*
P-56	Summerland	Room 232	plywood	*P. cyclopium-like*
P-58	Naramata	Room 258	concrete foam	*P. cyclopium*
P-61	Oliver	Room 107	plywood	*P. cyclopium*
P-63	Oliver	Room 104	metal/steel	*P. cyclopium*
P-64	Oliver	Room 115	plywood	*P. solitum*
P-72	Osoyoos	Room 144	fire retardant fiber	*P. polonicum*
P-76	Osoyoos	Defrost tank	water	*P. solitum*
P-84	Winfield	Room 511	spore trap	*P. solitum*
P-87	Kelowna	Room 61	spore trap	*P. cyclopium*
P-91	Kelowna	Room 82	spore trap	*P. solitum*
P-96	Kelowna	Room 83	spore trap	*P. cyclopium*
P-97	Kelowna	Room 83	spore trap	*P. expansum*
P-100	Kelowna	Room 60	plywood	*P. expansum*

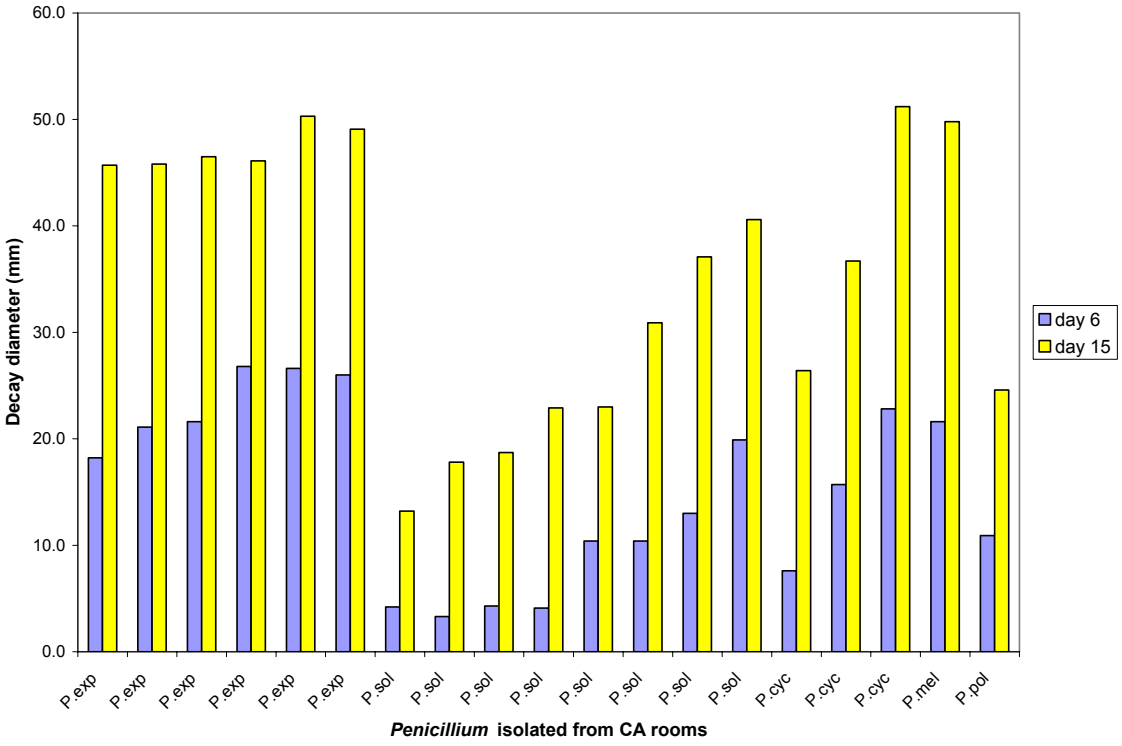
[1]Samples were taken by dipping a sterile cotton swab or Q-tip into a sterile tube containing 10 ml of sterile distilled water (SDW) and swabbing over an area of 10 cm^2 by using a template. For plywood and metal walls 5 swabs (10 cm^2)/ wall into 1 tube. For fiber walls 5 samples (2 cm^2) into 1 tube.

Table 3: *Penicillium* isolates screened for decay on 'Gala' apples and tested for thiabendazole fungicide resistance.

Isolate	Sequence ID	Pathogenicity		Level of fungicide resistance (ppm)
		Day 6	Day 15	
Group 1[1]				
Water	-	3.0 d[3]	5.5 h	-
1790 (positive control)[2]	*P. expansum*	21.3 b	50.6 ab	1000 (R)[4]
P-8	*P. expansum*	18.2 c	45.7 b	5
P-33	*P. expansum*	21.6 ab	46.5 ab	5
P-10	*P. expansum*	21.0 b	45.8 b	1
P-2	*P. solitum*	4.2 d	13.2 ef	1000 (R)
P-51	*P. solitum*	4.3 d	18.7 cd	1000 (MR)[5]
P-64	*P. solitum*	4.1 d	22.9 c	500
P-17	*P. solitum*	4.1 d	11.42 f	500
P-11	*P. solitum*	3.3 d	17.8 de	1
P-9	*P. cyclopium*	22.8 a	51.2 a	5
P-35	*P. melanoconidium*	21.6 ab	49.8 ab	1
Group 2[1]				
Water	-	15.9 c	35.2 cd	-
1790 (positive control)	*P. expansum*	27.0 a	47.7 a	1000 (R)
P-39	*P. expansum*	26.8 a	46.1 ab	5
P-97	*P. expansum*	26.6 a	50.3 a	1
P-100	*P. expansum*	26.0 a	49.1 a	1
P-55	*P. solitum*	10.4 def	23.0 f	1000 (R)
P-43	*P. solitum*	19.9 b	40.6 bc	1000 (R)
P-41	*P. solitum*	13.0 d	37.1 cd	1000 (MR)
P-91	*P. solitum*	10.4 def	30.9 de	1000 (MR)
P-48	*P. cyclopium*	7.6 fgh	26.4 ef	50
P-45	*P. cyclopium*	15.8 c	36.7 cd	1
P-72	*P. polonicum*	10.9 de	24.6 ef	1000 (R)

[1] Group 1 isolates were tested in January 2006 and group 2 isolates were tested in March 2006. The groups were analyzed separately to account for any differences in storage time of the fruit.
[2] Isolate 1790 is *P. expansum* previously isolated from decayed fruit and identified as TBZ resistant.
[3]Numbers followed by the same latter in each column are not statistically different at the p=0.05 level.
[4] R = resistant where colony growth reduction at 1000 ppm is not more than 25% compared to growth at 0 ppm.
[5] MR = moderately resistant where colony growth reduction at 1000 ppm is between 50-60% compared to growth at 0 ppm.

Fig.1 Screening of the *Penicillium* isolates on 'Gala' fruit incubated at 20°C and rated at 6 and 15 days. Decay diameter was the mean of 15 fruit (3 wounds/fruit).



Objective 2. Develop a PCR assay for two new postharvest diseases (Phacidiopycnis and Sphaeropsis rot) known to occur in Washington and British Columbia.

Methods:

2.1 Packinghouse survey

Winter pears were surveyed in British Columbia in January 2006 for *Phacidiopycnis* and *Sphaeropsis* rot. Two CA rooms of 'Anjou' pears at a Kelowna packinghouse were examined during this study. Room 67 contained 841 bins (366,564 kg) from 14 different growers and room 73 held 912 bins (398,876 kg) from 7 different growers. During pear packing only fruit with stem or calyx end rot were sampled from the cull bins. The decayed area was wiped with 95% alcohol and a small piece of the infected tissue from each pear was removed and placed on acidified potato dextrose agar (APDA) plates. These plates were then incubated at 20°C for 5-7 days and the resulting fungus identified to genus using a dissecting microscope.

2.2 Field isolations

Infection of the fruit by these organisms is known to occur in the field so two orchards were examined for any signs of these fungi on dead bark, twigs and canker-like areas. Small sections of the canker-like areas and diseased branches from the orchard were surface sterilized, placed on to APDA plates and incubated at 20°C for 5-7 days.

2.3 Single spore cultures

From the microscope identifications a selection of *Phacidiopycnis* and *Sphaeropsis* cultures were single spored before DNA extraction to remove any possibility of cross contamination. From previous work by Liu and Xiao (2005) for the *Phacidiopycnis* spp., and Xiao and Rogers (2004) for the *Sphaeropsis* spp. it was determined that in order for both species to produce pycnidia 12 hour cycles of alternating light and dark at 20°C were needed. Pycnidia were collected by gently scraping them off the surface of the cultures and put into a 1.5 ml eppendorf tube with a few drops of 10% pear juice for *Phacidiopycnis* or sterile distilled water for the *Sphaeropsis*. The pycnidia were then crushed using a 1.5 pellet pestle (VWR International Ltd). Spores released from the pycnidia were streaked onto 60 mm PDA plates, incubated at 20°C for 12-24 hours from which the single spore cultures were made.

2.4 DNA and sequence identification

DNA was extracted using a Fast DNA extraction kit (BIO 101 Inc., Vista, CA) for all samples. Polymerase chain reaction (PCR) amplification of the internal transcribed spacer region (ITS) was made by using the universal primers UN18S-42 and UN28S-22. Samples were amplified using low or limiting levels of primers so that no clean up of PCR product was needed prior to using it as a sequencing template. For all samples the primers UN18S-42 and UN28S-22 along with the Big Dye Terminator sequencing mix (Applied BioSystems, Foster City, CA) were used to sequence the ITS region in separate reactions. This method was repeated but using the Bt-LEV-Up4 and Bt-LEV-Lo1 (De Jong et al., 2001) primers to all the *Phacidiopycnis* and some of the *Sphaeropsis* samples for the initial PCR and sequencing mix to sequence the β-tubulin region. The sequences were then imported into SeqMan Pro sequence analysis software Lasergene 7.1 (DNSTAR Inc., Madison, WI) for contig assembly and visual editing. Ambiguities identified in the sequence data were resolved by comparison with reverse complimentary sequences.

DNA obtained from Washington State isolates were also sequenced as described above and compared to our sequences.

2.5 Primer design

Using the Primer 3 analysis program a number of primers were designed and made up by Invitrogen Canada Inc, (Burlington, Ontario). These primers are able to target specific differences in the β-tubulin region between the *Phacidiopycnis* and *Sphaeropsis* so when DNA is extracted from decayed fruit and used in a PCR reaction with these primers it should be possible to identify the causal agent without additional PCR and sequencing.

Results and Discussion:

Packinghouse survey:

From room 67, 41% of the rots were caused by *Phacidiopycnis* and 35% by *Botrytis* and from room 73, 37% were caused by *Phacidiopycnis* and 41% by *Botrytis*, consequently a significant amount of the stem and calyx end rot previously thought to be caused by *Botrytis* was found to be *Phacidiopycnis* spp. *Sphaeropsis* spp. was also isolated from rooms 67 and 73 at 2.4% and 1.1% respectively (Fig.2).

Phacidiopycnis spp. was isolated from the orchard from small canker-like areas and diseased branches from pear trees. The field isolations were made in May, June and October 2006 and it was

observed that the *Phacidiopycnis* was re-isolated in conjunction with *Botrytis* showing that it is active in the orchard for a number of months. There may be some connection with the *Botrytis* however; a more extensive field survey would be required to ascertain the level and length of field infections. Effective control measures such as good orchard sanitation to remove over wintering inoculum and the use of effective fungicides should be considered in orchards that harbor the disease.

Sequence identification:

Both *Phacidiopycnis* and the *Sphaeropsis* sequences matched 99-100% with the isolates from Washington State showing that they are *Phacidiopycnis pyri* and *Sphaeropsis pyriputrescens* (Fig 3). Six primers were designed for the *Phacidiopycnis* and four for *Sphaeropsis*. These primers will be used in future studies for accurate identification of these fungi.

Fig.2 Survey results showing the identity of fungi and percent rot isolated from calyx and stem end rots of 'Anjou' pears in 2006.

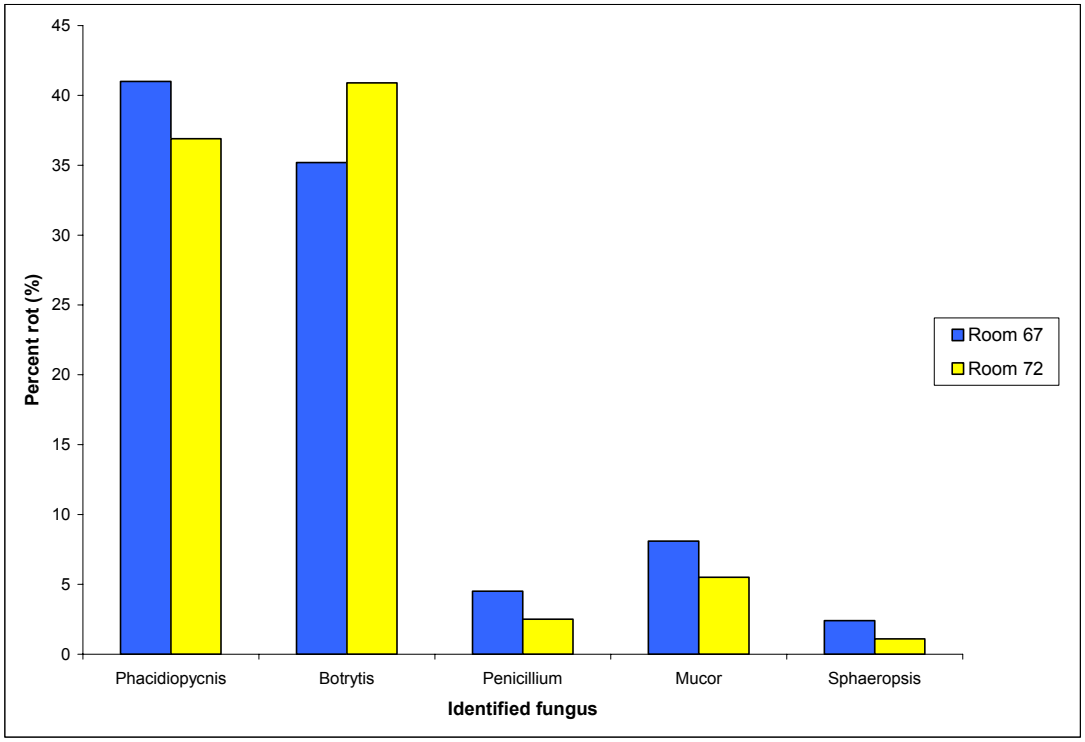


Fig.3 *Phacidiopycnis* and *Sphaeropsis* β-tubulin DNA gene sequences compared to one another showing differences between bases. WI - Washington isolates; Pha - *Phacidiopycnis* isolates; SRS - *Phacidiopycnis* isolated from the orchard; Sph - Sphaeropsis isolates; Major differences between the sequences are in bold type.

WI 2271: CCCACCCTCTGT**T**TACAA**T**ACC**AT**TGTTGCTTTGGCGGCCCCGTCGCAA**G**ACAACCGGC**T**CCGGCTGGTCAGCGGCCGCCAGAGGACTCCAAAAC**T**CATAT**TG**TCA**T**TGTCGTCTGAGTA

Pha 2300: CCCACCCTCTGT**T**TACAA**T**ACC**AT**TGTTGCTTTGGCGGCCCCGTCGCAA**G**ACAACCGGC**T**CCGGCTGGTCAGCGGCCGCCAGAGGACTCCAAAAC**T**CATAT**TG**TCA**T**TGTCGTCTGAGTA

SRS 2383: CCCACCCTCTGT**T**TACAA**T**ACC**AT**TGTTGCTTTGGCGGCCCCGTCGCAA**G**ACAACCGGC**T**CCGGCTGGTCAGCGGCCGCCAGAGGACTCCAAAAC**T**CATAT**TG**TCA**T**TGTCGTCTGAGTA

WI 2373: CCCACCCTCTGT**C**TACA**G**TACC**TC**TGTTGCTTTGGCGGCCCCGTCGCA**GG**ACAACCGGC**C**CCGGCTGGTCAGCGGCCGCCAGAGGACTCCAAAAC**C**CATAT**CA**TCA**G**TGTCGTCTGAGTA

Sph 1206: CCCACCCTCTGT**C**TACA**G**TACC**TC**TGTTGCTTTGGCGGCCCCGTCGCA**GG**ACAACCGGC**C**CCGGCTGGTCAGCGGCCGCCAGAGGACTCCAAAAC**C**CATAT**CA**TCA**G**TGTCGTCTGAGTA

Sph 2381: CCCACCCTCTGT**C**TACA**G**TACC**TC**TGTTGCTTTGGCGGCCCCGTCGCA**GG**ACAACCGGC**C**CCGGCTGGTCAGCGGCCGCCAGAGGACTCCAAAAC**C**CATAT**CA**TCA**G**TGTCGTCTGAGTA

Sph 3362: CCCACCCTCTGT**C**TACA**G**TACC**TC**TGTTGCTTTGGCGGCCCCGTCGCA**GG**ACAACCGGC**C**CCGGCTGGTCAGCGGCCGCCAGAGGACTCCAAAAC**C**CATAT**CA**TCA**G**TGTCGTCTGAGTA

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References:

De Jong S.N., Lévesque C.A., Verkley G.J.M., Abeln E.C.A., Rhae J.E. and Braun P.G., 2001. Phylogenetic relationships among *Neofabraea* species causing tree cankers based on DNA sequencing of the ITS nuclear rDNA, mitochondrial DNA and β-tubulin gene. Mycol. Res. 105, 658-669.

Liu Q. and Xiao C.L., 2005. Influence of nutrient and environmental factors on conidial growth of *Potebniamyces pyri*. Phytopathology 95:572-580.

Sholberg P.L., Harlton C., Haag P., Lévesque C.A., O'Gorman D. and Seifert K., 2005. Benzimidazole and diphenylamine sensitivity and identity of *Penicillium* spp. that cause postharvest blue mold of apples using β-tubulin gene sequences. Postharvest Biology and Technology. 36:41-49

Xiao C.L. and Rogers J.D., 2004. A postharvest fruit rot in d'Anjou pears caused by *Sphaeropsis pyriputrescens* sp. nov. Plant Disease 88:114-118.