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An effective medium for isolating *Sphaeropsis sapinea* from asymptomatic pines

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Summary

The pathogen *Sphaeropsis sapinea* can persist in stems and branches of asymptomatic pines and can later induce disease when triggered by host stress. Several experiments were conducted to test if: (i) medium amended with tannic acid (TA) can increase the frequency of cultural detection of this shoot blight and canker pathogen from asymptomatic red pine (*Pinus resinosa*) stems, and (ii) *S. sapinea* can persist in asymptomatic red pine in the field following artificial inoculation. TA (0.5% w/v) in 2% (w/v) water agar proved to be the best medium for isolation of *S. sapinea* among a larger number of tested media. The addition of TA had little or no effect on the growth of two group A and two group B isolates of *S. sapinea*. However, when TA was added, 11 other fast-growing fungal isolates from stems/branches of red or jack pines (*P. banksiana*) were inhibited and grew more slowly ($p < 0.05$) than both *S. sapinea* groups. The TA-amended medium improved cultural detection of *S. sapinea* from 2-year-old, asymptomatic red pine nursery seedlings compared with two other methods used for the cultural detection of *S. sapinea* (32% *vs*. 8.5% and 18% recovery; $p < 0.001$ and $p = 0.031$, respectively). A field test using the TA-amended media established that *S. sapinea* can persist asymptomatically in red pine trees for at least 1 year. This medium significantly reduces the frequency of false-negatives from asymptomatic field material.

1 Introduction

Sphaeropsis sapinea (Fr.:Fr.) Dyko and Sutton [syn. *Diplodia pinea* (Desmaz.) J. Kickx fil.] causes shoot blight, cankers, crown wilt, collar rot and root diseases of at least eight coniferous genera throughout the world (Gibson 1979; Farr et al. 1989; Nicholls and Ostry 1990; Swart and Wingfield 1991a; Stanosz and Cummings Carlson 1996). Diseases caused by *S. sapinea* have resulted in extensive losses in nurseries, Christmas tree and ornamental plantings, and forest stands (Gibson 1979; Palmer and Nicholls 1985; Nicholls and Ostry 1990; Swart and Wingfield 1991a). In the Great Lakes region of the United States, some of the greatest losses caused by *S. sapinea* have occurred on red pine (*Pinus resinosa* Aiton) (Nicholls et al. 1977; Palmer and Nicholls 1985; Nicholls and Ostry 1990).

Two distinct forms of *S. sapinea* (A and B) are known and they were originally differentiated by their appearance in culture (Palmer et al. 1987). However, these two groups can be differentiated more clearly using molecular techniques (Stanosz et al. 1999; Zhou and Stanosz 2001; Zhou et al. 2001). On red pine, inoculation of wounded seedlings with isolates of the A group resulted in higher incidences of symptoms, larger cankers and recovery of *S. sapinea* farther from the inoculation site than inoculation with group B isolates (Blodgett and Stanosz 1997a, 1999). However, some group B isolates are more aggressive than some group A isolates on jack pine (*P. banksiana* Lamb.) and

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Colorado blue spruce (*Picea pungens* Engelm.) seedlings (Blodgett and Stanosz 1997a, 1999).

Field observations and experiments have associated stress caused by water deficits (Bachi and Peterson 1985; Chou 1987; Madar et al. 1989; Blodgett et al. 1997a), competing vegetation (Blodgett et al. 1997a), high soil nutrition (Van Dijk et al. 1992), hail damage (Swart et al. 1987a; Zwolinski et al. 1990), seasonal variations (Swart and Wingfield 1991b) and climate differences (Lundquist 1987) to differences in the incidence and severity of disease caused by *S. sapinea*. Although considerable losses caused by *S. sapinea* have been associated with stress, the means by which infection occurred in these situations were not explained, or were attributed to infection through recent wounds.

More recently, it has been shown that *S. sapinea* can persist in stems of asymptomatic red pine seedlings (Stanosz and Cummings Carlson 1996; Stanosz et al. 2001), Austrian and Scots pine branches (Flowers et al. 2001) and in needles of Austrian pines (Jurc et al. 1999). Under these circumstances the fungus can rapidly become pathogenic after planting or water stress (Stanosz and Cummings Carlson 1996; Stanosz et al. 2001). Therefore, infection and asymptomatic colonization can occur prior to the stress-induced disease phase. However, it is not known how long this pathogen might persist in asymptomatic pines.

Potato dextrose agar (PDA), and PDA amended with streptomycin sulphate (PDAS) are often cited as media used for the cultural detection of *S. sapinea* (Palmer 1991; Palmer et al. 1987). An alternative method involved the incubation of surface-disinfested stems segments on 2% w/v water agar (WA) in slants for 3 months (Blodgett and Stanosz 1997a). Recovery was improved using WA in slants compared with PDA and PDAS, with recoveries of 100% from red pine inoculated with several isolates of the A and B groups in greenhouse and growth chamber studies (Blodgett and Stanosz 1997a; Blodgett et al. 1997b). However, the method is time-consuming and still might underestimate the occurrence of *S. sapinea*. Percentage recovery from wound-inoculated red pine in a field study was lower than in the greenhouse and growth chamber studies (82% for A isolates; 63% for B isolates) (Blodgett et al. 1997a). Previous studies demonstrating the recovery of *S. sapinea* from asymptomatic field samples might also have underestimated the occurrence of this pathogen. Given the large number of potentially competing nonpathogenic fungi that live in pines (Vaartaja 1968; Petrini and Fisher 1988; Kowalski and Kehr 1992), there is a need for an effective medium for isolating this pathogen.

Tannic acid (TA) has little or no effect on germination and mycelial growth of the *S. sapinea* groups (Blodgett and Stanosz 1997b). Therefore, the potential to improve the detection of *S. sapinea* from pine tissues was tested using media amended with TA. The objectives of this study were to: (i) test whether media amended with TA can increase the frequency of detection of *S. sapinea* from asymptomatic red pines, and (ii) using the TA media, determine if *S. sapinea* can persist for at least 1 year in asymptomatic red pines in the field following artificial inoculation.

2 Materials and methods

2.1 Media preparation

Tannic acid agar (TAA) was made by autoclaving 10 g reagent-grade TA (Aldrich Chemical Company, Milwaukee, WI, USA) per litre distilled water, and in a separate flask 40 g agar (Difco Laboratories, Detroit, MI, USA) per litre distilled water (4% WA). Flasks containing the two solutions were placed in a water bath, mixed thoroughly when the temperature reached 55°C and were immediately poured into Petri plates. Thus, the final concentration of TA is 0.5% (w/v) in 2.0% (w/v) WA.

Pine needle agar (PNA) was prepared using asymptomatic red pine needles cut into 3 cm segments and autoclaved twice over 2 days, each time for 25 min. Autoclaved 1.5% WA was then poured into small Petri plates (5-cm diameter). After the agar solidified, two pine needle segments were applied to the surface of the medium at the centre of each plate.

Numerous media were tested during growth rate and isolation studies. The main media used were TAA and PNA. Other media used included: 1.5% (w/v) WA; 2% WA; 2% WA amended with various concentrations of TA; PDA (Difco) amended with 0.015% (w/v) streptomycin sulphate (PDAS); and PDAS amended with 0.5% (w/v) TA (PDTAAS).

2.2 Growth rate experiments

Radial growth rates of 17 rapidly growing monoconidial fungal isolates (Table 1) including two isolates of the A group and two isolates of the B group of *S. sapinea* were compared. The fungal species examined were isolated from red and jack pine shoots collected from the field and are commonly isolated from branches of these hosts.

In the first experiment, 2% WA and 2% WA amended with various concentrations of TA (0.01, 0.1, 0.5, 0.7, 0.8 and 1.0% w/v) were used. For concentrations with 0.7, 0.8 and 1.0% TA the media were adjusted to pH 4.8 with NaOH. The first 10 isolates in Table 1 were grown on 2% WA. Plugs (4 mm in diameter) cut from the margin of the actively growing cultures were placed mycelium-side-down on each of the seven media. Five plates per TA concentration were used for each of the isolates. Plates were incubated at 24°C in the dark. Radial growth was measured at 7 days. Growth rates were compared by two-factor analysis of variance with interactions. Factors used as main effects were TA concentration and isolate. Mean values were separated using Fisher's least significant differences (LSD) at $p = 0.05$.

In the second experiment, PDAS, PDTAAS, 2% WA and TAA were used. The first 15 isolates in Table 1 were grown on 2% WA. Plugs (4 mm in diameter) cut from the margin of the actively growing cultures were placed mycelium-side-down on each of the four media.

Table 1. **Origin of isolates used in growth rate studies**

Species/genus	Isolate[1]	Host[2]	Collector	County	State
Sphaeropsis sapinea (A1)[3]	411	RP	M. A. Palmer	Becker	MN
S. sapinea (A2)	128	RP	M. A. Palmer	Grant	WI
S. sapinea (B1)	124	JP	M. A. Palmer	Jackson	WI
S. sapinea (B2)	215	RP	M. A. Palmer	Douglas	WI
Pestalotiopsis sp. 1	95-8A	RP	G. R. Stanosz	Wood	WI
Alternaria sp.	95-26A	RP	G. R. Stanosz	Grant	WI
Fusarium tricinctum	96-145	JP	D. R. Smith	Monroe	WI
Fusarium sp.	95-1A	RP	G. R. Stanosz	Wood	WI
Chaetomium sp.	96-147	RP	D. R. Smith	Monroe	WI
Epicoccum sp.	96-155	JP	D. R. Smith	Monroe	WI
Trichoderma sp.	00-1	RP	J. T. Blodgett	Grant	WI
Pestalotiopsis sp. 2	00-2	RP	J. T. Blodgett	Grant	WI
Rhizoctonia sp. 1	00-4	RP	J. T. Blodgett	Grant	WI
Rhizoctonia sp. 2	00-5	RP	J. T. Blodgett	Grant	WI
Phomopsis sp. 2	00-7	RP	J. T. Blodgett	Grant	WI
Sirococcus conigenus	00-45	RP	J. T. Blodgett	Vilas	WI
S. conigenus	00-48	RP	J. T. Blodgett	Vilas	WI

[1]Culture collection numbers of M. A. Palmer (three-digit number) or G. R. Stanosz (xx-xx).
[2]Host collected from, red (*Pinus resinosa*) and jack (*P. banksiana*) pines.
[3]Representative A and B group isolates used in this study were deposited at the Canadian Collection of Fungal Cultures (CCFC/DAOM), Ottawa, Ontario, Canada. The accession numbers for isolates 128 and 124 are DAOM 222530 and DAOM 222531, respectively.

This experiment was repeated with PDAS, 2% WA and TAA and with two isolates of *Sirococcus conigenus*: another commonly encountered and important pathogen of red pine shoots (Table 1). Five plates per medium were used for each of the isolates in each trial. Plates were incubated at 24°C in the dark. Radial growth was measured at 5 days on PDAS and WA (as some isolates outgrew the plates at 6 days), and at 8 days for PDTAAS and TAA. Growth rates were compared by one-way analysis of variance with the isolate as the factor for each medium in each trial. Means were separated using Fisher's LSD at $p = 0.05$.

2.3 Nursery seedling isolation experiment

The efficacy of TAA was compared with other methods of cultural detection of *S. sapinea*. Dormant, asymptomatic, 2-year-old bare-root red pine nursery seedlings were obtained in late February 2000 from the Wisconsin Department of Natural Resources Griffith Nursery (Wood Co., WI, USA). Asymptomatic persistence of *S. sapinea* in seedlings at Griffith Nursery has been confirmed previously (Stanosz et al. 1997). Seedlings had not been artificially inoculated.

After needle fascicles were removed, the basal 5-cm-long shoot segments were surface-disinfested for 10 s in 95% ethanol followed by 4 min in 1.05% NaOCl solution with two drops per litre of Tween 80 (Fisher Scientific Co., Toronto, Ontario, Canada). Individual segments were placed randomly onto PDAS, 2% WA, or TAA at plate edges, or into 2.0% WA slants. Plates were incubated at 24°C in the dark. Slants were incubated at ambient laboratory temperature (approx. 24°C) and light for 90 days. When the most rapidly growing fungus in each plate had grown at least 33 mm, it was transferred (from the colony margin) to PNA. If there was no growth of at least 33 mm after 26 days, plates were considered negative. PNA plates were incubated at ambient laboratory temperature (approx. 23°C) and light for up to 3 weeks. Identification of *S. sapinea* was based on examination of pycnidia and conidia on pine needles or stem sections for the slants. Other common fungi recovered from plates were identified to genus. One hundred seedlings per medium/method were used in each of two trials (800 seedlings total). Individual chi-square analyses were used to compare the numbers of seedlings from which *S. sapinea* was detected among the different media/methods in each trial. Chi-square two-way cross-tabulation analyses were used to compare the numbers of seedlings from which the different genera were detected among the different media/methods in each trial.

2.4 Field inoculation experiment

A field study was conducted from July 2000 to July 2001 at Wooster, Ohio. The study was conducted in an experimental plot of 24, 10-year-old asymptomatic red pine trees. Three elongating shoot tips on each of the 24 trees were wounded by removing a single needle fascicle (by a sterile scalpel cut flush to the branch) approx. 3 cm below the shoot apex. Two A group monoconidial isolates originating from Scots and Austrian pines (1SP and 3AP, respectively) from central Ohio were used in this experiment. These isolates previously were confirmed as virulent by wound-inoculating both red pine and Austrian pine (*P. nigra* Arnold) seedling (authors, unpublished data). Only the *S. sapinea* A group was used as this is the only group found so far in Ohio (authors, unpublished data), and the *S. sapinea* B group is not aggressive on red pine (Blodgett and Stanosz 1997a). A 4-mm diameter 1.5% WA plug colonized with either of these two *S. sapinea* isolates was placed mycelium-side-down on the wounds; non-colonized 1.5% WA plugs were applied to wounded controls. Plugs were cut from margins of actively growing cultures incubated for 6 days in the dark at 23°C. Parafilm (American National Can Co., Chicago, IL, USA) was wrapped around the shoots at the inoculation site and removed after 7 days. The three branch treatments were assigned randomly to individual branches on the same whorl of each of the 24 trees.

Branches were examined for symptoms and recovery of *S. sapinea* was attempted after 1 year. After needle fascicles were removed, 1-cm-long shoot segments taken at the inoculation site and 3 cm below the inoculation site were surface-disinfested for 10 s in 95% ethanol, followed by 4 min in 1.05% NaOCl solution with two drops per litre of Tween 80. Individual segments were placed into TAA plates at plate edges and incubated at 24°C in the dark. When the most rapidly growing fungus in each plate had grown at least 33 mm, it was transferred (from the colony margin) to PNA. If there was no growth of at least 33 mm after 26 days, plates were considered negative. PNA plates were incubated at ambient laboratory temperature (approx. 23°C) and light. Identification of *S. sapinea* was based on examination of resulting pycnidia and conidia on the pine needles. Chi-square two-way cross-tabulation analyses were used to compare the numbers of seedlings from which *S. sapinea* was detected in relation to the three inoculation treatments.

3 Results

3.1 Growth rate experiments

In the first experiment, the addition of TA at lower concentrations had little or no effect on the growth of group A and B isolates of *S. sapinea* (Table 2). However, the other rapidly growing fungal isolates were inhibited ($p < 0.05$) when TA was added. Two-factor analysis of variance of growth rates at the various concentrations of TA indicated significant effects of the isolate used ($p < 0.001$) and the concentration of TA ($p < 0.001$). There was an interaction between isolate used and TA concentration ($p < 0.001$), indicating that the isolates responded differently to the different TA concentrations. Separation of the *S. sapinea* isolates from all other isolates occurred at concentrations ≥0.10% TA. However, *S. sapinea* was inhibited more at concentrations of 0.7% and higher, and there was difficulty with media solidification above 0.5%.

In the second experiment, the growth rates on the various media differed among isolates (Table 3). TAA was the only medium tested in which both *S. sapinea* groups grew faster than all other fungal species ($p < 0.05$). There were also differences among the isolates in colony colour, morphology and pigment production (colour change of the medium) (Table 4), which can be used to differentiate *S. sapinea* from the other species. *Sphaeropsis*

Table 2. **Colony radius (mm) at 7 days on 2% water agar (WA) and on WA supplemented with various concentrations of tannic acid (TA)**

	Concentrations of TA (%)						
Isolate	0	0.01	0.1	0.5	0.7	0.8	1
411 (A1)	33[1]	29	31	32	29	28	19
128 (A2)	31	30	32	32	30	29	21
124 (B1)	31	29	28	27	25	23	17
215 (B2)	32	32	28	28	24	24	17
95-8A	27	26	24	17	16	16	11
95-26A	38	33	22	7	5	4	2
95-145	52	49	25	8	8	7	3
95-1A	29	24	22	8	6	5	2
96-147	22	20	11	0	0	0	0
96-155	35	34	26	2	1	0	0

[1]Values represent mean values for five plates (replications). Differences among isolates ($p < 0.001$) and among media ($p < 0.001$) are significant based on ANOVA. Fisher's least significant difference = 1.9 at $p = 0.05$.

Table 3. **Colony radius (mm) at 5 or 8 days on potato dextrose agar amended with 0.015% streptomycin sulphate (PDAS), PDAS amended with 0.5% tannic acid (PDTAAS), 2% water agar (WA) and WA amended with 0.5% tannic acid (TAA)**

	Trial 1				Trial 2		
Isolate	PDAS (5 days)	PDTAAS (8 days)	WA (5 days)	TAA (8 days)	PDAS (5 days)	WA (5 days)	TAA (8 days)
411 (A1)	50[1]	69	24	35	47	26	37
128 (A2)	51	74	24	36	48	25	38
124 (B1)	40	47	21	30	39	22	31
215 (B2)	43	48	25	28	40	26	27
95-8A	28	36	24	23	27	24	24
95-26A	25	12	27	4	23	25	2
95-145	48	40	36	5	47	38	7
95-1A	25	10	23	1	25	23	0
96-147	27	1	18	0	26	16	0
96-155	28	16	25	0	27	26	0
00-1	75	34	66	0	71	67	0
00-2	29	41	20	20	29	19	17
00-4	59	66	67	16	59	67	17
00-5	61	59	67	8	65	65	8
00-7	35	43	31	18	39	30	21
00-45					17	3	19
00-48					13	3	19
LSD[2] =	2.3	4.4	2.0	1.6	2.9	1.7	2.5

[1] Values are mean for five plates (replications). Differences among isolates are significant ($p < 0.001$) for all comparisons based on ANOVA.
[2] Fisher's least significant difference among isolates for a given medium at $p = 0.05$.

sapinea is the only species tested that produces dark grey mycelia, a light brown pigment and small black spots in TAA, which can aid in identification.

3.2 Nursery seedling isolation experiment

Sphaeropsis sapinea was recovered from asymptomatic seedlings using all media/methods. However, TAA gave the best detection of this pathogen in both trials ($p < 0.001$ in both trials). Frequency of detection did differ between trials; however, the relative results remained the same among treatments. Therefore, results of the two trials are combined (Table 5).

Fungal species recovered also differed among the media tested ($p < 0.001$). Several rapidly growing isolates of various species were recovered on all media (Table 5). On the original isolation plates, the greatest number of distinct fungal colonies growing from shoot sections occurred on PDAS; the least occurred on TAA. As many as 16 different fungal colonies were observed growing from a single shoot section on PDAS.

3.3 Field inoculation experiment

Symptoms were only observed on one branch inoculated with the 1SP isolate and on one branch inoculated with the 3AP isolate, and consisted of only one dead needle fascicle per branch at the wound site. However, a single dead needle fascicle may have resulted from unintentional wounding during inoculation. Symptoms were never observed on control branches. However, *S. sapinea* was recovered from 63% of the asymptomatic inoculated branches 1 year after inoculation, with the same percentage recovery from branches inoculated with both *S. sapinea* isolates. The frequency of *S. sapinea* recovery was

Table 4. **Growth form and colour on potato dextrose agar amended with 0.015% streptomycin sulphate (PDAS), 2% water agar (WA) and WA amended with 0.5% tannic acid (TAA) at 20 days, for the isolates used in growth rate studies**

	PDAS			2% WA			TAA		
Isolate	Form[1]	Colour[2]	Other[3]	Form	Colour	Other	Form	Colour	Other
411 (A1)	F	DG	–	M	H-DG	BS	M	DG	BS-LP
128 (A2)	F	DG	–	M	H-DG	BS	M	DG	BS-LP
124 (B1)	M	DG	BS	A	H-B	BS-DM	A	DG	BS-LP
215 (B2)	M	DG	BS	A	H-B	BS-DM	A	DG	BS-LP
95-8A	F	W-B-LY	–	A	H	–	A	H-Y	LP
95-26A	F	DG	–	A	H	–	A	DB	BP
96-145	F	P-LO-W	–	A	H	–	A	DB	BP
95-1A	A	W-B-P	–	A	H-P	–	A	H	–
96-147	F	GR	–	A	H	GM	NG	NG	–
96-155	F	O-W-P-R-Y	Z-DR	A	H	DM	NG	NG	–
00-1	F	W	GM	A	H	GM	NG	NG	–
00-2	F	W	BM	A	H	–	A	H	BP
00-4	F	LB	Z-TW	A	H	TM	A	H	–
00-5	F	LB	R-TW	A	H	TC	A	H	–
00-7	F	G-W	–	A	H	BS	A	H	BP
00-45	M	W-LB-DB-B-P	Z	A	H	–	A	H	BS-LP
00-48	M	W-LB-DB-B-P	Z	A	H	–	A	H	BS-LP

[1]Growth form: A, appressed; M, moderately fluffy; F, fluffy; NG, no significant growth.
[2]Culture colour: H, hyaline; G, grey; DG, dark grey; W, white; B, beige; LB, light brown; DB, dark brown; LY, light yellow; Y, yellow; P, pink; R, red; LO, light orange; O, orange; GR, dark green; NG, no significant growth.
[3]Other distinguishing cultural features: Z, zonate growth pattern; R, radiating growth pattern; BS, small black spots in media; TM, tan mass in media; TC, tan chains in media; BM, black tar-like mass on culture; DM, dark mass on culture; GM, green mass on culture; TW, tan to white mass on culture; DR, dark red pigment in media; BP, brown pigment in media; LP, light brown pigment in media.

significantly lower ($p < 0.001$) from control branches (8%) compared with the recovery from inoculated branches. Differences in the frequency of recovery of *S. sapinea* were not significant ($p = 0.581$) at 3 cm from the wound site among the three treatments, with an average *S. sapinea* recovery of 8%.

4 Discussion

Tannic acid agar is an effective selective medium that can significantly reduce false-negatives compared with other cultural methods used to detect *S. sapinea* in asymptomatic red pine stems. Although the TAA method reduces false-negatives compared with the other methods, it still might underestimate the occurrence of this pathogen. Needle fascicle removal, surface-disinfestation and TAA together may reduce competition by other fungal organisms in red pine to improve *S. sapinea* recovery. The TAA method is also faster than the WA in slants method (4–6 weeks *vs*. 12–13 weeks). Another advantage of the TAA method is that TA is relatively inexpensive and may be safer than the chemicals used in other selective media.

Swart et al. (1987b) developed a selective medium for isolating *S. sapinea* from diseased *P. radiata* D. Don. Their medium is effective in inhibiting other fungi found in diseased *P. radiata* in South Africa. However, not all of the ingredients are obtainable in the USA, and the medium is ineffective for isolating *S. sapinea* from diseased red and jack pine

Table 5. **Frequency of isolation of various fungi from asymptomatic, surface-disinfested red pine seedling stems using potato dextrose agar amended with 0.015% streptomycin sulphate (PDAS), 2% water agar (WA), WA amended with 0.5% tannic acid (TAA) and 2.0% WA slants**

	Isolation media/method			
Fungal species/genera	PDAS	WA	TAA	Slants
Sphaeropsis sapinea	17 a[1]	8 a	64 c	36 b
Rhizoctonia sp. 1	62	67	10	–[2]
Trichoderma spp.	48	43	30	–
Pestalotiopsis sp.	9	3	46	–
Phomopsis sp. 2	21	14	3	–
Rhizoctonia sp. 2	9	12	4	–
Phomopsis sp. 1	10	10	4	–
Alternaria sp. 1	4	11	0	–
Other spp.[3]	20	20	8	–
Unidentified isolates[4]	0	11	5	164
No isolate[5]	0	1	26	–
n[6]	200	200	200	200

[1]Differences among media are significant ($p < 0.001$ for both trials) based on chi-square tests. For *S. sapinea*, different letters represent significant differences ($p < 0.05$) in recovery among media/methods based on individual chi-square tests.
[2]Not recorded for the slant method.
[3]Include, from most to least common, four different *Fusarium* spp., a *Phoma* sp., *Alternaria alternata*, an *Alternaria* sp., basidiomycete spp., a *Sordaria* sp., a *Penicillium* sp., a *Didymostilbe* sp., a *Phomopsis* sp. and a *Nigrospora* sp.
[4]Include non-sporulating mycelial fungi. Species identification was not attempted with the slant method other than for *S. sapinea*.
[5]No isolate grew at least 3 cm in 26 days.
[6]Total number of isolation attempts for each media/method.

branches, when benodanil is missing (authors, unpublished data). Swart et al. (1987b) found malt extract agar (MEA) and MEA amended with TA to be ineffective for isolating *S. sapinea*. This is consistent with the poor recovery observed in our study for PDAS and PDTAAS, other high nutrient media.

The medium used in the present study should be useful in further studies involving *S. sapinea* and potentially other pine pathogens. The methods used in the present study also work well for recovering *S. sapinea* from symptomatic Austrian and red pines (authors, unpublished data), and might work well for recovering this pathogen from both symptomatic and asymptomatic hosts of other coniferous genera. In a preliminary test, Bonello and Nielsen (unpublished data) recovered *S. sapinea* from four of 10 adult bark beetles (*Ips pini* Say) collected in baited funnel traps near Austrian pines in an urban location in Ohio. TAA might also be used in the isolation of *S. conigenus* when *S. sapinea* is not present. Although *S. conigenus* grows more slowly than *S. sapinea* on TAA, *S. conigenus* did outgrow many of the common pine fungi on TAA in this study, and the growth rate of *S. conigenus* was actually stimulated by TA.

This field inoculation experiment demonstrates that virulent *S. sapinea* isolates can persist asymptomatically in red pine trees for at least 1 year, and the nursery seedling isolation experiment confirms the asymptomatic persistence of *S. sapinea* in red pine nursery seedlings. This study also indicates that previous estimates of asymptomatic persistence of this pathogen in this host might have been low. The asymptomatic persistence of *S. sapinea* can explain the rapid onset of disease from initially asymptomatic pines when plants are exposed to stress, complicating disease management. This study provides an improved method for screening asymptomatic pines for the presence of *S. sapinea.*

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Résumé

Un milieu efficace pour isoler Sphaeropsis sapinea à partir de pins asymptomatiques

Le pathogène *Sphaeropsis sapinea* peut se maintenir dans les troncs et branches de pins de façon asymptomatique puis induire la maladie en réponse à un stress de l'hôte. Nous avons conduit plusieurs expérimentations pour tester: (1) l'efficacité d'un milieu additionné d'acide tannique (TA) pour augmenter la détection en culture de cet agent de chancre et de dessèchement de pousses à partir de troncs de pins rouges (*Pinus resinosa*) asymptomatiques; (2) la persistance de *S. sapinea* dans les pins rouges asypmtomatiques au champ après inoculation artificielle. Parmi un grand nombre de milieux testés, un milieu gélosé à 2% (p/v) contenant 0.5% (p/v) d'acide tannique s'est révélé le plus efficace pour l'isolement de *S. sapinea*. L'addition de TA a montré peu ou pas d'effet sur la croissance de deux isolats de groupe A et deux isolats de groupe B de *S. sapinea*. Par contre, l'addition de TA a inhibé 11 autres isolats fongiques à croissance rapide provenant de troncs / branches de pins rouges ou pins gris (*Pinus banksiana*) provoquant une croissance plus lente ($p < 0.05$) que pour les deux groupes de *S. sapinea*. La détection en culture de *S. sapinea* à partir de semis de 2 ans asymptomatiques de pins rouges en pépinière a été améliorée par le milieu additionné de TA par rapport à deux autres méthodes utilisées pour la détection en culture de *S. sapinea* (32% contre 8,5% et 18% d'isolements positifs; $p < 0.001$ et $p = 0.031$, respectivement). Un test de terrain utilisant le milieu additionné de TA a montré que *S. sapinea* peut se maintenir de façon asymptomatique dans les pins rouges pendant au moins un an. Ce milieu réduit significativement la fréquence de faux négatifs à partir de matériel asymptomatique de terrain.

Zusammenfassung

Ein wirksames Medium zur Isolierung von Sphaeropsis sapinea aus symtomlosen Kiefern

Der Erreger *Sphaeropsis sapinea* kann in Trieben und Ästen symptomfreier Kiefern überdauern und später, ausgelöst durch Stresseinflüsse auf die Wirtspflanze, zum Krankheitsausbruch führen. Es wurden mehrere Experimente durchgeführt, um zu prüfen, ob (i) mit Tanninsäure (TA) angereichertes Medium die Nachweishäufigkeit dieses Triebsterbenerregers in symptomlosen Trieben von *Pinus resinosa* erhöht und ob (ii) *S. sapinea* in symptomlosen *Pinus resinosa* im Freiland nach künstlicher Inokulation überdauern kann. Unter einer grösseren Anzahl getesteter Medien erwies sich TA (0,5% w/v) in 2% (w/v) Wasseragar als bestes Medium für die Isolierung von *S. sapinea*. Die Zugabe von TA hatte nur wenig oder keinen Einfluss auf das Wachstum von je zwei Isolaten der Gruppe A und B von *S. sapinea*. Die Zugabe von TA hemmte jedoch das Wachstum von 11 anderen, schnell wachsenden Pilzisolaten aus Stämmen und Ästen von *P. resinosa* oder *P. banksiana*. Diese Pilze wuchsen signifikant langsamer ($p < 0,05$) als beide *S. sapinea*-Gruppen.

Das TA-Medium verbesserte den Nachweis von *S. sapinea* aus zwei Jahre alten, symptomfreien *P. resinosa*-Sämlingen im Vergleich mit zwei anderen Medien (32% *vs*. 8,5% und 18% Isolierungshäufigkeit; $p < 0,001$ bzw. 0,031). *S. sapinea* wurde auch ein Jahr nach künstlicher Inokulation an zehnjährigen Bäumen im Freiland auf TA nachgewiesen und kann somit mindestens ein Jahr überdauern, ohne Symptome zu verursachen. Mit Hilfe des mit TA angereicherten Mediums kann der Umfang von falsch negativen Nachweisen aus symptomlosem Freilandmaterial signifikant verringert werden.

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