Karstenia 39: 69–75, 1999

The effect of storage temperature, time and spore source on the germination of Cronartium flaccidum and Peridermium pini aeciospores in vitro

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KAITERA, J. 1999: The effect of storage temperature, time and spore source on the germination of Cronartium flaccidum and Peridermium pini aeciospores in vitro. – Karstenia 39: 69–75. Helsinki. ISSN 0435-3402

The effect of temperature and time of storage on the germination of aeciospores of *Cronartium flaccidum* (one source) and *Peridermium pini* (5 sources) were studied on malt agar with added pine needle extracts, and water agar. The storage temperatures ranged from –160 °C to +25 °C, and the time of storage from 7 days to 2 years. The rate of aeciospore germination remained higher after storage at low temperatures than at high temperatures, as spores stored at high temperatures lost their viability within a few months after storage. The two-year period of storage, however, reduced the germinability of the aeciospores significantly even at low temperatures. Aeciospores of *C. flaccidum* lost their viability more rapidly than those of *P. pini*.

Storage strongly reduced the subsequent ability of germ tubes to form vesicles. No vesicles were formed on a water agar substrate after 3–6 month-storage at +4 – +25 °C. At lower temperatures, the ability to form vesicles decreased more slowly, but vesicles were formed in individual spore samples as abundantly as at the beginning of the experiment when these were stored at between –22 °C or –70 °C for 2 years. The results suggest that aeciospores should be stored at low temperatures when needed for future inoculations in order to retain the highest possible viability.

Key words: *Cronartium flaccidum*, germination, *Peridermium pini*, pine stem rust, *Pinus sylvestris*, storage, temperature

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Introduction

The pine stem rusts *Peridermium pini* (Pers.) Lév. and *Cronartium flaccidum* (Alb. & Schwein.) G. Winter are among the most destructive pathogens damaging Scots pine (*Pinus sylvestris* L.) in Scandinavia (Jørstad 1928, Rennerfelt 1943). In Finland, *P. pini* is the dominating species, but *C. flaccidum* occurs also sporadically across the country (Liro 1908, Hantula et al. 1998, Kaitera & Hantula 1998, Kaitera et al. 1999).

Cronartium spp. and *Peridermium* spp. are also among those rusts that have been successfully cultured on specific media (e.g. Harvey & Grasham 1974, Allen et al. 1988, Pei & Pawsey 1990, Moricca & Ragazzi 1994). *Peridermium pini* aeciospores germinate at temperatures between 5 °C and 30 °C, the optimum being 20–25 °C (Klingström 1963, Olembo 1971a). The frequency of germ tubes differentiating to form vesicles (Klingström 1963, Gibbs et al. 1988), however, varies greatly with different incubation temperatures (Olembo 1971a). Germination is reduced when spores are seeded at high densities with pH between 2.0–7.5 (Olembo 1971a). The optimum pH for maximum germination is 5.5 (Olembo 1971a). In addition, the prevailing relative humidity of the air must be over 80% to promote germination (Olembo 1971a). According to Klingström (1963) *P. pini* aeciospores maintain their viability unaltered for at least one year after of being stored dry at 0 °C.

The aim of this study was to investigate the effect of storage temperature and storage time on the germination of pine stem rust aeciospores from different sources on two agar media *in vitro*. The information thus obtainable is important, as aeciospores must, in most cases, be stored for some time before final usage, e.g. in inoculations, and therefore, the viability of the spores needs to be maintained high for as long as possible after spore collection.

Material and methods

Rust aeciospores were collected from unruptured aecia from a number of lesions (a mixed sample from several lesions per location) during dry weather in late June 1995. The aeciospores represented four geographic *P. pini* populations (Jokela 1, Kesälahti 2, Inari, Pudasjärvi) and one *C. flaccidum* population (Kolari 1) in Finland (for identification of the rust populations, see Hantula et al. 1998 and Kaitera et al. 1999). Shortly after collection, the aeciospores were air-dried and sieved through a fine mesh onto petri dishes just before storage. Initial viability was measured by dusting spores onto water agar followed by incubation for 24 h at 25 °C. The germination rates of the spore sources (Jokela, Kesälahti, Inari, Pudasjärvi and Kolari) were 95%, 80%, 100% 98% and 96% on water agar at the beginning of the experiment. Aeciospores were stored dry in Eppendorf vials in the dark at 25 °C on a laboratory worktop, at 15 °C in a warming cupboard, at 4 °C in a cold room, at –7 °C in a refrigerator, at –22 °C in a cold room, at –70 °C in a freezer or at –160 °C in a tank containing liquid nitrogen. Spores from a vial of each aeciospore source were dusted on both water agar or 1.5% malt agar with added pine needle extract (Kurkela 1979) after storage for 7 days, 1 month (35 days), 3 months, 6 months, 1 year and 2 years. The frequency of germ tube formation and their subsequent ability to form vesicles (Klingström 1963, Gibbs et al. 1988) was estimated for each sample (vial) using the following classes: Abundant vesicles (>50% of the germinated spores bearing vesicles), moderate number of vesicles (10–50%), low number of vesicles (1–9%) and no vesicles (0%). Aeciospore germination was determined from ten randomly sampled microscope fields under a light microscope after a 24 h incubation period at 25 °C on the agar plates. Before statistical analysis, the percent figures were arcsine-transformed. The germination of the spores was compared between spore sources, storage temperatures, storage times, and media by analysis of variance using the GLM procedure of SAS (Anonymous 1989). The germination rates of individual spore sources were compared using Tukey's test (Anonymous 1989).

Results

Analysis of variance revealed that storage time was the most significant explaining variable, when modelling the variation in germination on both media (Table 1). Also temperature, spore source, and their interactions, were statistically significant in modelling the germination of the aeciospores, regardless of the basal media (Table 1). These variables and their interactions explained 80% of the total variation in spore germination. When modelling the variation in germination separately on the different media, the former variables and their interactions were statistically significant at 0.001 p-level (ANOVA, data not shown), The respective models explained 86% and 77% of the total variation on water agar and on malt agar with added pine needle extract.

Table 1. ANOVA table of the effect of storage time (A), storage temperature (inoculum C), media (D), and their first-degree interactions on the germination of *Peridermium pini* and *Cronartium flaccidum* aeciospores after a 24 h incubation period.

Source	df	F	P > F
Time (A)	6	1076.89	0.0001
Temperature (B)	6	464.67	0.0001
Inoculum (C)	4	204.83	0.0001
Media (D)	1	614.37	0.0001
A X B	30	52.14	0.0001
A X C	24	21.35	0.0001
A X D	5	266.69	0.0001
B X C	24	20.29	0.0001
B X D	6	4.85	0.0001
C X D	4	48.25	0.0001

After 7-day storage at temperatures below 4 °C, the germination of the aeciospores remained almost unaffected on water agar, regardless of the spore source. At higher temperatures spore samples from different sources showed statistically significant differences in germination (e.g. Pudasjärvi > Kesälahti; Tukey's test, data not shown). Thereafter, the germination of aeciospores from individual aeciospore sources fell rapidly at 25 °C to nearly zero after 3 months (Fig. 1). No statistically significant differences occurred among the spore sources after 6 months at 15 °C and 25 °C on water agar. At lower temperatures, the germination rate of the aeciospores diminished slowly with increasing time of storage (Fig. 1). After storage of 2 years, germination of the aeciospores was less than 20% in nearly all the cases. The aeciospores maintained their viability best when stored at –22 °C. Storage at –160 °C broke some of the vials during the experiment, leading to the rejection of some of the

observations due to possible condensation of moisture in the test tubes. Nevertheless, the possibility that extremely low temperatures (below –70 °C) may also have some additional inhibiting (or for some *P. pini* sources even stimulating) effects on the germination of the aeciospores when compared to higher temperatures below 0 °C cannot be excluded. There were statistically significant differences between individual spore sources at different temperatures after for different storage times, but in general *C. flaccidum* aeciospores (Kolari) lost their viability more rapidly than those of *P. pini* (e.g. Pudasjärvi). On malt agar with pine needle extract, the aeciospores germinated less vigorously than on water agar, regardless of the spore source. On the former medium, germination was more variable giving rise to statistically significant differences among aeciospore sources at different temperatures (Tukey's test, data not shown). Nevertheless, *C. flaccidum* germinated poorest among spore sources also on this medium, as was the case on water agar, regardless of the storage time or temperature. Also a general downward trend in germination on water agar was evident after the 1-month storage period, but some additional stimulative effect was observed when compared to germination after the 7-day storage period for all *P. pini* sources at some temperatures.

Vesicles were produced variably by germ tubes originating from individual spore sources during the experiment. *Peridermium pini* spores from Inari and Pudasjärvi produced vesicles most frequently on water agar, whereas *C. flaccidum* spores from Kolari produced only a few individual vesicles during the experiment (Table 2). On water agar, the frequency of vesicles remained unaffected by a 7-day storage period at all temperatures. Thereafter, the frequency of vesicle production by *P. pini* after storage for 1–6 months at 4–25 °C diminished (Table 2). Storage time and temperature did not significantly affect the frequency of *C. flaccidum* vesicles. Vesicles occurred frequently after storage below –7 °C, depending somewhat on the aeciospore source. In most cases, the frequency of vesicle production remained high after storage for 1 year. The formation of vesicles was highest in material stored at the lowest temperatures, although the breaking of some of the vials at –160 °C prevented final conclusions to be drawn. Vesicles were formed most frequently on water agar by aeciospores stored at –22 °C to –70 °C in the case of *P. pini* from Inari during the entire 2-year storage period. On malt agar with pine needle extract, vesicles were formed only occasionally in *P. pini* from Inari, Pudasjärvi and Jokela (one case). No trends in their frequency were observed in relation to either storage temperature or time.

Table 2. Frequency of *Peridermium pini* (1) and *Cronartium flaccidum* (2) aeciospores producing germ tubes with vesicles after storage periods of 7 days to 2 years at temperatures between 25 °C and –160 °C, followed by inoculation on water agar and incubation for 24 h. 1 = No vesicles, 2 = Low number of vesicles (1–9% of the germinated spores producing vesicles), 3 = Moderate number of vesicles (10–50%), 4 = Abundant vesicles (>50 %). – = Missing data.

		Storage time					
Spore source	°T	7 d	1 month	3 months	6 months	1 yr	2 yrs
Inari[1]	25	4	3	1	1	1	1
	15	4	4	3	1	1	1
	4	4	3	4	1	1	1
	–7	4	4	4	3	3	3
	–22	4	4	4	4	4	4
	–70	4	4	4	4	4	4
	–160	4	4	4	4	1	–
Pudasjärvi[1]	25	3	3	1	1	1	1
	15	4	4	3	1	1	1
	4	4	3	3	2	1	1
	–7	3	4	3	3	3	3
	–22	3	4	3	3	2	2
	–70	4	3	3	3	2	1
	–160	3	3	3	4	1	3
Kesälahti[1]	25	3	3	1	1	1	1
	15	1	3	4	1	1	1
	4	3	3	1	1	1	1
	–7	3	3	1	1	2	1
	–22	3	3	3	1	3	2
	–70	3	3	4	4	3	2
	–160	1	3	3	3	1	–
Jokela[1]	25	1	1	1	1	1	1
	15	3	1	1	1	1	1
	4	3	3	1	2	1	1
	–7	3	3	1	3	1	1
	–22	4	3	3	3	3	1
	–70	3	3	3	3	3	2
	–160	1	3	3	2	–	–
Kolari[2]	25	1	1	1	1	1	1
	15	1	1	1	1	1	1
	4	2	1	1	1	1	1
	–7	1	1	1	1	1	1
	–22	1	1	2	1	1	1
	–70	1	1	1	2	1	1
	–160	1	1	1	2	1	–

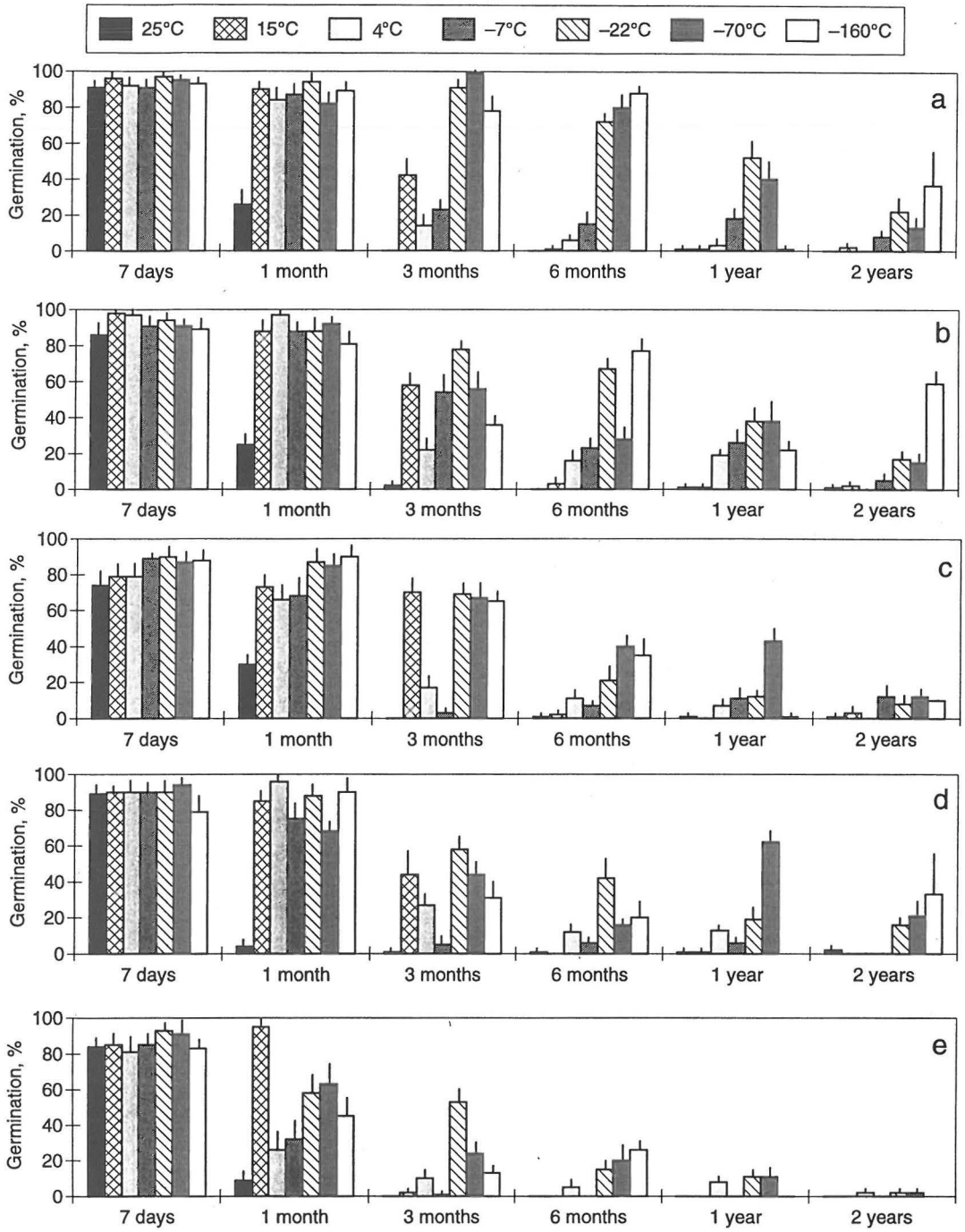
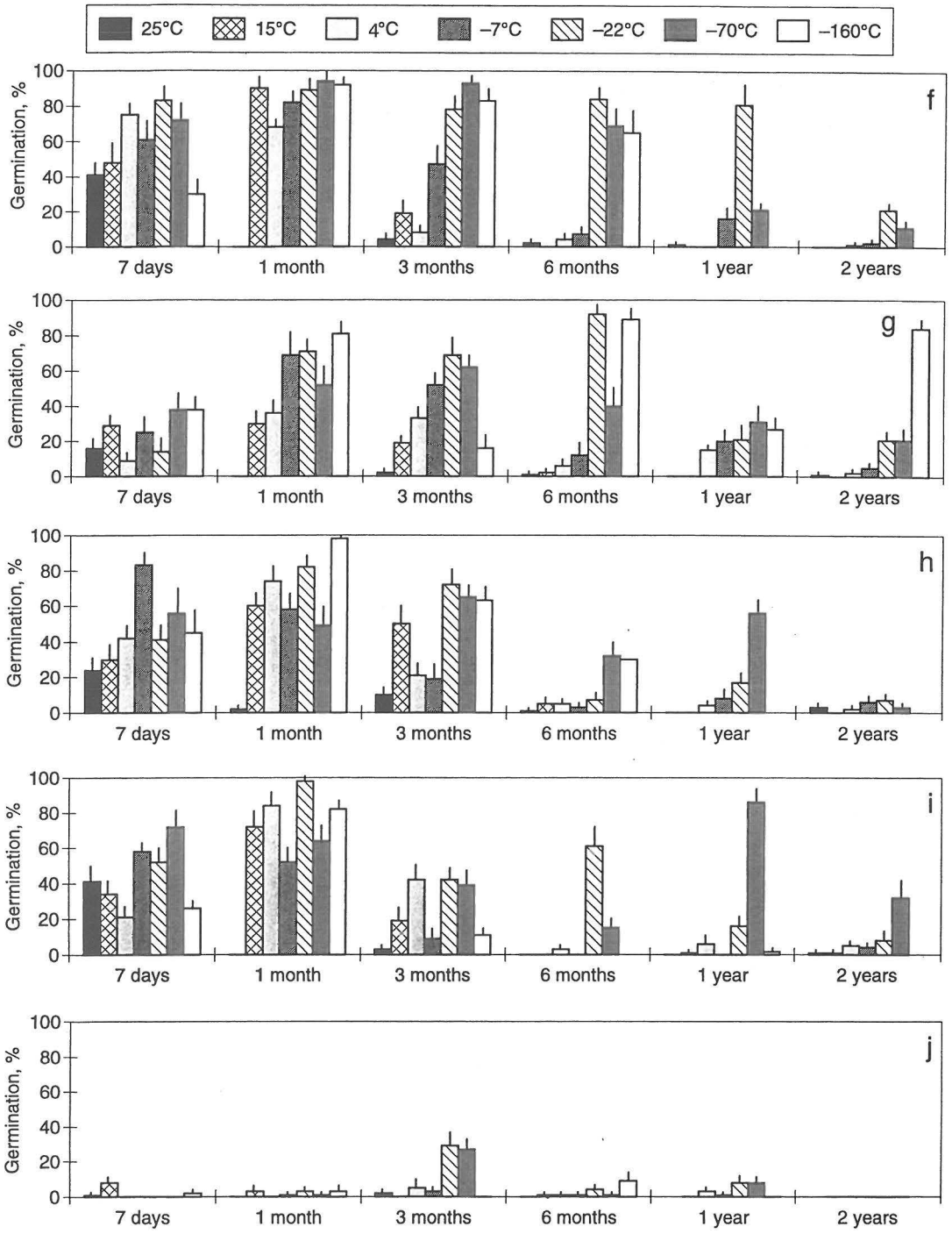


Fig. 1. Germination (%) of *Peridermium pini* aeciospores from Inari (a, f), Pudasjärvi (b, g), Kesälahti (c, h) and Jokela (d, i), and of *Cronartium flaccidum* aeciospores from Kolari (e, j) after storage at 25 °C, 15 °C, 4 °C, –7 °C,



–22 °C, –70 °C and –160 °C for 7 days, 1 month, 3 months, 6 months, 1 year and 2 years, followed by 24 h incubation on water agar (a–e) and malt agar added with pine needle extract (f–j). Vertical bars indicate the SD of the mean.

Discussion

The results of the present study revealed that the germination of aeciospores was rapidly reduced when stored at high temperatures, whereas high viability was maintained for longer at temperatures below 0 °C. Earlier Klingström (1963) had shown that 10 days at –27 °C did not affect the capacity of *P. pini* aeciospores to germinate. Also, aeciospores stored at –25 °C for 1 year were still infective on pine (Klingström 1972). At higher temperatures, Siggers (1947) reported that only a few aeciospores of fusiform rust (*C. quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*) preserved their viability after a 7-month -storage period at below 10 °C. Klingström (1963), however, noted that germination of *P. pini* aeciospores remained unaffected at 4 °C for at least a year. In this study, the germination of *P. pini* aeciospores was greatly reduced after long-term storage at temperatures above 0 °C, the reduction being most rapid at the highest temperatures applied. Also, Lightle (1955) reported that germination of *Endocronartium harknessii* (Moore) Hiratsuka (syn. *P. harknessii* Moore) aeciospores was less than 15% after a 14-month -incubation period at 4 °C. In addition, according to Klingström (1972), the germination of *P. pini* aeciospores declined after a 4-week-storage period at 20 °C, which is in rather good agreement with the results of the present study (at 25 °C after a similar time of storage).

In this study, the unexplained variation in the model describing aeciospore germination was low suggesting that the effect of storage moisture on spore viability was minimal. In some cases, however, condensed moisture in the test tubes at –160 °C, due to breaking of some of the tubes during storage may have affected the germinability of the spores contained within them. Earlier, Klingström (1963) reported that condensation in test tubes and collecting of *P. pini* spores in rainy weather decreased aeciospore germination. Lightle (1955), however, found only small variation in viability between air-dried, desiccated and undried aeciospores of *P. harknessii* after a 20-month-incubation period at 4 °C.

In the present study, viability of *C. flaccidum* aeciospores decreased more steeply than that of *P. pini* aeciospores especially at low temperatures. No similar reports are, however, available to confirm the applicability of the results. In addition, *C. flaccidum* was found to produce vesicles less frequently than *P. pini* regardless of the storage temperature, time or medium used. According to Gibbs et al. (1988) and Kaitera et al. (1999), there is high variation in vesicle formation between *P. pini* and *C. flaccidum*, with *P. pini* producing vesicles more frequently than *C. flaccidum*. In the present study, the formation of vesicles was greatly reduced when storage was arranged at high temperatures, but their formation remained relatively unaltered after storage at lower temperatures. No similar studies are available to confirm these results.

In this study, germination was poorer and more variable on malt agar with pine needle extract than on water agar, regardless of the time or temperature of storage, or the spore source. The general trend on the effect of storage time and temperature in germination was, however, similar to that on water agar. Earlier, Klingström (1963) reported that *P. pini* aeciospores germinated better on agar media with added nutrients than on water agar. Similarly, aeciospores of *C. ribicola* (J. C. Fisch.) Rabenh. germinated better on *Ribes*-leaf-decoction agar than on water agar (Van Arsdel et al. 1956). Olembo (1971b), however, reported that added pine needle extract had a more pronounced inhibiting effect on *P. pini* aeciospore germination as compared to pure water agar. This was also the observation made in the present study.

Acknowledgements: I wish to thank Ms Leena Seitamäki for her assistance in the laboratory, Tarmo Aalto, B.Sc. (For.), for drawing the figures for the publication, and Erkki Pekkinen, MSc. (For.), for checking the English.

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Received on 3 September 1999