COMPARISON OF DIFFERENT METHODS TO DETECT *PHYTOPHTHORA* SPP. IN RECYCLING WATER FROM NURSERIES

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SUMMARY

Five methods for the detection of *Phytophthora* spp. in water recirculation systems of four commercial nurseries were compared: direct plating, three bait tests (apple, lupin seedling, Rhododendron leaf test) and DAS-ELISA with two *Phytophthora* specific polyclonal antisera. Water and sediment samples were taken from different places in the recirculation systems over one year. With all methods except direct plating *Phytophthora* spp. could be detected in the four nurseries. In most cases the detection rates were influenced more by the method itself than by different conditions on particular nurseries. The Rhododendron leaf test trapped the widest range of *Phytophthora* species and was the most successful method followed by ELISA with antiserum against *P. cinnamomi*. Although the chemical analysis of the water samples showed a good water quality for nursery cultivation, there was a close relationship between the detection rates obtained with ELISA and the total salt content of the water. The results are compared with those of *in vitro* experiments and their relevance for commercial nurseries is discussed.

Key words: detection methods, *Phytophthora* spp., recycling water, commercial nurseries.

INTRODUCTION

Container production of woody ornamentals on large outdoor stands is of great economic importance. To reduce waste after irrigation surplus water is collected by different drainage systems and stored in special basins. In German nurseries these basins are mostly ponds with water storage capacities of up to 6000 m^3. From these ponds the water is taken again for irrigation. Water from wells is pumped into the ponds to refill them during periods of low rainfall and high irrigation (*e.g.* during summer).

Water recycling is a risk to plant health as it is an infection source of plant pathogens par excellence. One of the most important soilborne pathogens in nurseries are the fungal-like *Phytophthora* species. Many of these species can attack a wide range of woody plants causing mainly root, collar and stem rots but also twig blight and fruit rots (Erwin and Ribeiro, 1996). *Phytophthora* species are well adapted to live in water which they need to produce sporangia and motile zoospores, the most important propagules for new infections. Studies have shown that surface irrigation water, rivers and recirculation systems of nurseries can be contaminated with *Phytophthora* species (Klotz *et al.*, 1959; McIntosh, 1966; Whiteside and Oswalt, 1973; Thomson and Allen, 1974; Taylor, 1977; Shokes and McCarter, 1979; Hallett and Dick, 1981; von Broembsen, 1984a, b; Ali-Sthayeh and MacDonald, 1991; Lutz and Menge, 1991; MacDonald *et al.*, 1994).

Therefore, for the plant protection service and for nurserymen it is very important to have a reliable diagnostic tool for screening the irrigation water routinely for *Phytophthora* species before reuse. There are many publications concerning detection methods for *Phytophthora* (Erwin and Ribeiro, 1996), but only a few concerning water samples from recirculation systems in hardy ornamental nurseries (Klotz *et al.*, 1959; McIntosh, 1966; Thomson and Allen, 1974; Taylor, 1977; Shokes and McCarter, 1979; Ali-Sthayeh and MacDonald, 1991; Ali-Sthayeh *et al.*, 1991; MacDonald *et al.*, 1994). Furthermore there is a lack of information about whether factors characteristic of particular nurseries, like the pH-value of the water, can influence the detection rate. The following study compares different methods for the routine screening of water recirculation systems for *Phytophthora* spp. in commercial tree nurseries. These detection methods had been previously evaluated for water samples only under controlled conditions (Themann and Werres, 1997, 1998a, b; Hahn *et al.*, 2000).

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MATERIALS AND METHODS

Sampling sites and procedures. Four different nurseries, that collect surplus water from container stands and reuse it for irrigation were chosen. All four produced a wide range of containerised woody ornamentals, *e.g. Taxus* spp., *Chamaecyparis* spp. and *Rhododendron* spp. The recirculation system in nursery 1 was about three years old. Although the container stands of nursery 2 were three to twelve, of nursery 3 eight to ten and of nursery 4 two to seven years old. The ponds for storing surplus water in these latter three nurseries were about 20 years old. Water and sediment samples were taken every seven weeks from August 1994 to July 1995 from different sites in the recirculation systems. Water samples were taken from different drains, from the ponds and from the wells. Sediment samples were taken from the drains if no water was in them and from the bases of the ponds. In nursery 1 there was as yet no sediment in the very young pond.

Water samples (3 l) and sediment samples (3 l) were taken from each sampling site, transported in 3 l plastic containers and stored overnight at +4°C.

Chemical analysis of the water. All chemical data for the water samples were determined with the techniques and methods specified in VDLUFA-Methodenbuch (1991) for soil samples. These leaflets contain summarized methods that are used routinely for chemical analysis of soil and substrates in horticulture by all Institutes for Agriculture Analysis and Research (LUFA) in Germany.

Detection methods and media. All methods used in the present study have been previously tested under controlled conditions (Themann and Werres, 1997; 1998a, b; Hahn *et al*., 2000). These methods were used in the present study with only slight modifications as described below.

Media. Two media were used: the first was carrot piece agar, medium as described by Kröber (1985). It was used for the growth of the *Phytophthora* species used as positive controls (*P. cactorum* and *P. cinnamomi*), for the isolation of *Phytophthora* spp. from baits and for the identification procedure. The second medium (NVP) a selective medium, was used for direct plating. It was based on a vegetable-oatmeal agar [15 g oatmael (Holo® Hafergold Neuform®), 100 ml Rabenhorster® vegetable juice without salt, 2 g $CaCO_3$ (Merck no. 2063) and 15 g agar-agar (Difco no. 0140-01)] with 50 ppm Nystatin (Sigma N-3503), 100 ppm Vancomycin (Sigma V-2002) and 10 ppm Pentachloronitrobenzene (Sigma P-3395) as described by McCain *et al.* (1967).

Direct plating. 500 ml water per sample was filtered through filter pads (Schleicher and Schuell no. 400 114, AE 99, 5 cm) using an apparatus for sterile filtration (Satorius, Göttingen) and a vacuum pump. The filter pads were then placed on the surface of NVP-Agar in Petri dishes. After an incubation period of three days, the filter pads were removed and the incubation continued. Organic material such as leaves or needles were removed from the sediment samples, surface sterilized and placed on the selective medium. One Petri dish with a filter pad and two further Petri dishes each with five pieces of plant debris were prepared for every sample. All agar dishes were incubated at +20°C in the dark until *Phytophthora* grew out and could be detected by microscopic examination.

Baiting methods. For the apple test, 500 ml from each water sample was filtered through a pad (see 'direct plating'). The filters were then cut into twelve pieces and each piece put into a hole cut out of apple fruit. From each sediment sample 1.5 ml was inserted into a hole. Four apples with three holes each were prepared per sample.

For the lupin seedling test, 250 ml of each sediment sample was diluted with 250 *aqua deion*. 500 ml of each water sample and 500 ml of each sediment suspension were baited with 20 lupin seedlings of *Lupinus angustifolius* cv. 'Blue Stevens'.

For the Rhododendron leaf test the same quantities of each water sample and each sediment suspension were baited with ten leaves of the *Rhododendron* hybrid 'Cunningham's White', the leaves being placed on the surface of the water or the sediment suspension.

Every bait test contained two negative controls consisting of non contaminated soil extract and non contaminated *aqua deion.* and a positive control consisting of a *P. cinnamomi* culture (Themann and Werres, 1997).

DAS-ELISA. For the DAS-ELISA (double antibody sandwich *enzyme-linked immunosorbent assay*) procedure, polyclonal antisera produced against *P. cinnamomi* 8/88/92 and *P. cactorum* 9/88/92 were used. The standardization of the ELISA (Werres, 1988) was slightly modified as follows. All samples were applied in microtiter plates with high binding capacities (Greiner no. 655061). Incubation for γ-globuline and conjugate was at +37°C in a shaker (Wellwarm 1, Denley Instruments Limited) for one hour and for a further three hours at room temperature without shaking. The washing steps were done by a washer (Easy Washer Eaw 812

Sw 1 Slt Labinstruments Deutschland GmbH). The optical density (ELISA value) was determined at 405 nm (monochromatic) with a photometer (Titertek Multiscan Plus Mcc/340, Flow Laboratories). In every ELISA the test samples were measured, when positive controls (mycelium and propagules of *P. cinnamomi* 8/88/92 and *P. cactorum* 9/88/92 from liquid cultures diluted 1: 20,000) reached a value between 0.7-1.1 ($E_{405\ nm}$). For negative controls buffer and nutrient solutions diluted 1:100 were applied to every microtiter plate.

From each water sample 500 ml was filtered, (see 'direct plating') and then frozen at –60°C, until further processing as described by Ali-Shtayeh *et al.* (1991). From each sediment sample 250 ml was mixed with 250 ml *aqua deion*. The sediment-water mixture was filtered (Schleicher and Schuell no. 331 412) and prepared as described above for water samples. Every sample was replicated three wells on a microtiter plate.

Tap water, non filtrated and filtrated soil extract were taken as negative control. Their mean ELISA values using both antisera were 0.026, 0.081 and 0.079 respectively. Therefore two thresholds were fixed for the samples: if the ELISA value ($E_{405\ nm}$) was below 0.100, detection was said to be negative. ELISA values between 0.100 and 0.150 were considered uncertain and not interpreted. Values above 0.150 were said to be positive ('*Phytophthora* detection positive'). These thresholds were fixed according to recent experience with other sample material (Werres, 1988; Werres and Themann, 1993). Therefore in the following interpretation of factors influencing detection rates, the so called 'uncertain values' were not included.

Identification procedure. The very time consuming identification procedure was done only with *Phytophthora* isolates from water and sediment from the ponds. Preparation of the cultures, induction of the propagules and the determination was done according to Kröber (1985) and Werres *et al.* (2001).

RESULTS

Chemical data for the water. In the ponds the pH-value of the water varied between 4.6 and 7.9 (Table 1a). On average it was lowest in nursery 1 (mean 5.7) and highest in nursery 3 (pond a, mean 7.5). The variability throughout the year was highest in nursery 1 where the pH-value decreased from early autumn (7.3 at the end of August) to spring (4.6 at the end of March) and then increased again up to 6.6 in July. A moderate decrease during winter occurred in nursery 2 but not in nurseries 3 and 4. In the wells the pH-values of the water samples varied from 5.3 to 7.2 (Table 1b). As with the samples from the ponds the lowest pH-values in the wells were detected in nursery 1 and the highest in nurseries 2 and 3. pH-values in the wells were very uniform throughout the year.

The irrigation water of all four nurseries had total salt contents and nutrient values below those recommended for outdoor recirculation systems (Krüssmann, 1997). Nursery 2 had the highest levels of nutrients in the ponds (Table 1a). Nursery 4 had the highest level of NH_4-N, but otherwise had very low levels of nutrients. The total salt content of the ponds varied from 54 to 317 mg l^{-1}. On average it was lowest in nursery 4 (54-72 mg l^{-1}) and highest in nursery 2 (141-317 mg l^{-1}). In all four nurseries there was a decrease in the total salt content in the ponds from the end of August '94 up to the end of March '95. The values increased again in the spring. There was less variability in total salt content and nutrient levels in the wells compared to the ponds. As with the ponds nutrient levels in wells were highest in nursery 2 and lowest in nursery 4.

Specifity of the antisera. Both antisera used in the DAS-ELISA detected a wide range of *Phytophthora* species *in vitro* but not other microorganisms like the closely related *Pythium* spp. (Themann and Werres, 1997; 2000a, b). That means they were both genus specific, but not specific for a single *Phytophthora* species.

Detection rates depending on the nursery. *Phytophthora* spp. could be detected in the four nurseries with all methods except direct plating (Fig. 1a), that was unsuccessful in nursery 1. Detection rates varied between 0% and 71%. The baiting techniques as well as ELISA showed a similar range for the four nurseries: most contaminated samples were found in nurseries 2 and 4 and the fewest in nursery 1. But within a single nursery the detection rates varied greatly with the method. For nurseries 2, 3 and 4 the highest detection rates were obtained with the Rhododendron leaf test (71.0%, 43.1%, 68.1%) followed by the ELISA using antiserum against *P. cinnamomi* (38.7%, 25.4%, 40.9%). ELISA with the antiserum against *P. cactorum* gave the lowest numbers with positive results for nursery 2 (16.1%), 3 (9.8%) and 4 (13.6%). For nursery 1 the ELISA using antiserum against *P. cinnamomi* was the most successful detection method (20.8%) followed by the Rhododendron leaf test (8.3%).

Table 1a. Chemical data of the water - from the ponds.

Nursery/ pond	Sampling date	pH-value	Total salt content (mg l^{-1})	NO_3-N (mg l^{-1})	NH_4-N (mg l^{-1})	K (mg l^{-1})	Mg (mg l^{-1})	P_2O_5 (mg l^{-1})
1	29 Aug	7.3	127	2.0	1.5	4.0	3.0	0.05
	24 Oct	5.1	118	1.5	1.5	6.0	3.0	0.08
	06 Feb	5.0	87	1.3	1.8	3.0	2.0	0.03
	27 March	4.6	86	2.1	0.9	2.0	3.0	0.03
	15 May	5.5	99	1.4	1.2	2.0	3.0	0.03
	11 Jul	6.6	104	0.0	1.9	1.0	3.0	0.03
	Mean value	5.7	103	1.4	1.5	3.0	2.8	0.04
2	29 Aug	7.1	247	4.0	1.5	8.0	8.0	0.09
	24 Oct	6.8	188	2.0	1.5	11.0	7.0	0.15
	06 Feb	6.9	184	1.0	1.0	4.0	7.0	0.14
	27 March	6.9	141	1.7	0.6	2.0	5.0	0.60
	15 May	7.2	317	6.9	4.3	7.0	10.0	0.60
	11 Jul	7.4	277	5.5	2.4	5.0	9.0	0.60
	Mean value	7.0	226	3.5	1.9	6.2	7.7	0.36
3a	29 Aug	7.4	200	1.0	0.5	4.0	7.0	0.11
	24 Oct	7.1	158	0.5	0.5	6.0	5.0	0.08
	06 Feb	7.4	139	1.3	0.8	7.0	5.0	0.18
	27 March	7.7	153	0.4	0.2	4.0	5.0	0.03
	15 May	7.5	193	0.2	0.9	3.0	6.0	0.10
	11 Jul	7.9	201	2.7	0.6	7.0	8.0	0.92
	Mean value	7.5	174	1.0	0.6	5.2	6.0	0.24
3b	24 Oct	7.6	186	0.5	0.5	5.0	6.0	0.03
4	29 Aug	6.6	71	0.5	1.5	2.0	1.0	0.14
	24 Oct	6.6	69	0.5	1.5	5.0	1.0	0.13
	06 Feb	6.4	61	1.8	1.0	3.0	2.0	0.55
	27 March	6.9	54	2.3	0.4	2.0	2.0	0.11
	15 May	6.9	72	0.4	3.4	1.0	1.0	0.03
	11 Jul	6.3	69	0.4	4.5	1.0	1.0	0.03
	Mean value	6.6	66	0.9	2.0	2.3	1.3	0.16

The sampling site. None of the methods detected *Phytophthora* spp. in the wells. But with all methods they could be detected in the drains and in the ponds (Fig. 1b). ELISA and the Rhododendron leaf test detected more *Phytophthora* in the drains than in the ponds. But with the apple test and with direct plating, detection rates for water and sediment samples were nearly identical. Again the detection rate at a particular site varied greatly with the method used. For the drains it was highest with the Rhododendron leaf test (61.0%), followed by the DAS-ELISA with the antiserum against *P. cinnamomi* (40.2%). It was lowest with the apple test (20.6%). Similar results were obtained for the ponds: again the Rhododendron leaf test was the best method (49.5%), followed by DAS-ELISA with the antiserum against *P. cinnamomi* (30.7%). The lowest detection rate for the ponds was with the DAS-ELISA using the antiserum against *P. cactorum* (8.7%).

The sampling/sample material. All methods could detect *Phytophthora* spp. in pond water and in pond sediment (Fig. 1c). Also all methods detected more contamination in sediment than in water, except the DAS-ELISA using antiserum against *P. cinnamomi*. With this antiserum the detection rates were higher with the water samples. For water the detection rate of the different methods varied from 1.5% to 37.9%. It was highest with ELISA using antiserum against *P. cinnamomi* (37.9%) followed by the Rhododendron leaf test (27.3%) and was lowest with direct plating (1.5%). In sediments the detection rate was highest with the

Table 1b. Chemical data of the water - from the wells.

Nursery	Sampling date	pH-value	Total salt content (mg l^{-1})	NO_3-N (mg l^{-1})	NH_4-N (mg l^{-1})	K (mg l^{-1})	Mg (mg l^{-1})	P_2O_5 (mg l^{-1})
1	24 Oct	5.4	96	0.5	1.0	4.0	2.0	0.05
	15 May	5.5	114	0.2	1.2	1.0	3.0	0.10
	11 July	5.3	96	0.2	1.5	1.0	3.0	0.07
2	24 Oct	7.2	218	0.5	0.5	4.0	7.0	0.03
	15 May	6.9	258	0.2	0.5	1.0	8.0	0.64
	11 July	7.1	248	0.4	0.2	1.0	8.0	0.07
3	24 Oct	6.9	199	0.5	0.5	5.0	7.0	0.06
	15 May	6.9	224	0.2	0.3	1.0	7.0	0.32
	11 July	7.2	197	0.5	0.4	1.0	7.0	0.32
4	24 Oct	5.7	86	0.5	2.0	5.0	1.0	0.11
	15 May	6.1	106	0.2	4.6	2.0	2.0	0.14
	11 July	6.0	80	0.5	5.1	1.0	2.0	0.14

NO_3-N: nitrate nitrogen; NH_4-N: ammonium nitrogen; K: potassium; Mg: magnesium; P_2O_5: phosphate.

Rhododendron leaf test (69.4%) and lowest with the ELISA using antiserum against *P. cactorum* (7.5%). But a successful detection depended not only on the method. It was also influenced by the combination of sample type and nursery. For example, in the pond water from nursery 1 and in the sediments from nursery 3 (pond b) *Phytophthora* spp. could only be detected with the ELISA using antiserum against *P. cinnamomi* (Table 2). However with pond sediments from nursery 2, direct plating was the most successful method.

The sampling date. Not all methods could detect *Phytophthora* spp. at every sampling date (Table 3). Direct plating and the apple test gave negative results in August and October. With ELISA using antiserum against *P. cactorum*, none of the pathogens could be detected in July. But with the lupin seedling test, with the Rhododendron leaf test and with ELISA using antiserum against *P. cinnamomi* detection was possible at any sampling date. From August '94 to May '95 the detection rates were highest with the Rhododendron leaves. In March the results with ELISA using antiserum against *P. cinnamomi* were identical to those obtained with the Rhododendron leaf test. In July the apple test was the most successful method followed by the Rhododendron leaf test.

ELISA showed a close relationship between the sampling date and the detection rate (Table 3). With the antiserum against *P. cinnamomi* the detection rates increased from 9.1% at the end of August '94 to 58.8% at the end of March '95. With the antiserum against *P. cactorum* they increased from 9.1% to 43.8% between August '94 and February '95.

There was no clear relationship between the maximum and minimum detection rate and the sampling date (Table 3). Direct plating, the lupin seedling test and the ELISA using antiserum against *P. cactorum*, gave the highest detection rates in February but the apple test gave the highest detection rate in July, the Rhododendron leaf test in August and the ELISA using antiserum against *P. cinnamomi* in March. On the other hand the ELISA using both antisera gave the lowest detection rates in July, the Rhododendron leaf test the lowest in May and July, the lupin seedling test in November and May, direct plating and the apple test in August and October.

The water quality. There was no clear correlation between either the pH-values, or the nutrients of pond water (Table 1a) and the detection rates of the different methods in different months (Table 3). But there was a negative correlation between the total salt content and the detection rates obtained with ELISA: from the end of August '94 to the end of March '95 a decreasing total salt content in the pond water corresponded with increasing detection rates obtained with ELISA, the very low detection rate with the antiserum against *P. cactorum* in March being the only exception.

Range of *Phytophthora* species detected with the methods. Although both antisera detected a wide range of *Phytophthora* species *in vitro* they were specific for the genus *Phytophthora* but not for a single species. Therefore single *Phytophthora* species in samples from commercial nurseries could only be surveyed using direct plating and the three bait tests.

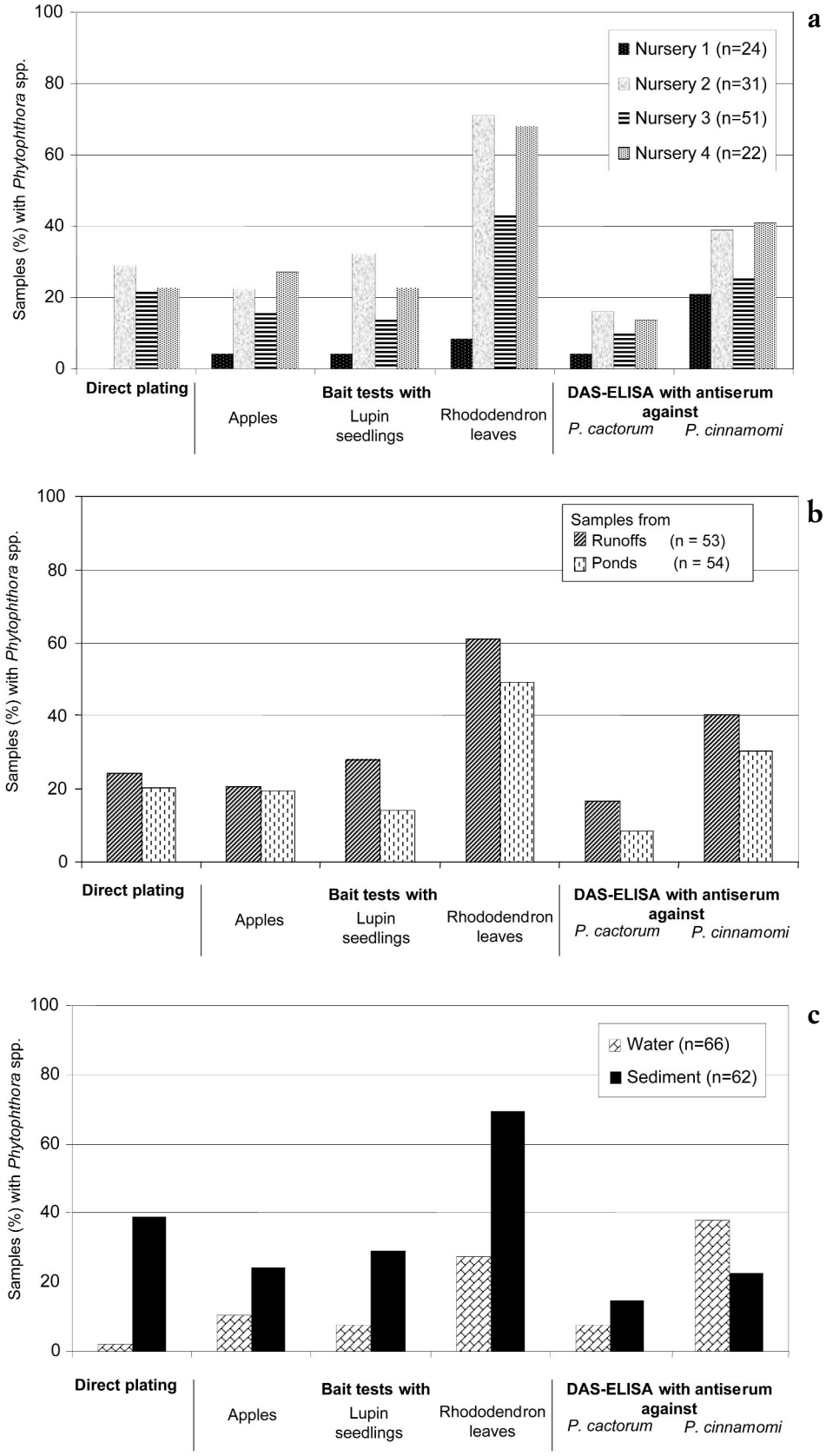

Fig. 1. Detection rates depending on:
a: - the nurseries;
b: - the sampling site;
c: - the sample material.

Table 2. Comparison of different methods for detecting *Phytophthora* spp. in ponds.

Nursery	Pond	Sample material	no. of samples	Samples (%) with *Phytophthora* spp. in the test with					
				Direct plating	Apples	Lupin seedlings	Rhododendron leaves	DAS-ELISA with antiserum against[1]	
								P. cactorum	*P. cinnamomi*
1		water	7	0	0	0	0	0	28.6
2		water	7	0	28.6	14.3	85.7	28.6	42.9
		sediment	7	57.1	14.3	14.3	42.9	0	14.3
3	a	water	7	0	14.3	14.3	42.9	0	28.6
	a	sediment	6	42.9	0	0	57.1	0	28.6
	b	water	6	16.7	33.3	16.7	50.0	0	0
	b	sediment	6	0	0	0	0	0	16.7
4		water	6	0	16.7	16.7	66.7	33.3	66.7
		sediment	6	66.7	66.7	50.0	100.0	16.7	50.0

Maximum detection rates are underlined.
[1] Both antisera were specific for the genus *Phytophthora*, but not species specific.

Table 3. Comparison of different methods for detecting *Phytophthora* spp. according to sampling date.

Date of sampling	no. of samples	Samples (%) with *Phytophthora* spp. in the test with					
		Direct plating	Apples	Lupin seedlings	Rhododendron leaves	DAS-ELISA with antiserum against[1]	
						P. cactorum	*P. cinnamomi*
29 August '94	11	0.0	0.0	27.3	63.6	9.1	9.1
24 October '94	21	0.0	0.0	19.0	52.3	4.8	42.8
28 November ' 94	21	23.8	28.6	9.5	47.6	14.2	33.3
06 February '95	16	43.7	12.5	31.3	56.3	43.8	50.0
27 March '95	17	17.6	29.4	23.5	58.8	5.9	58.8
15 May '95	21	19.0	4.8	9.5	33.3	4.8	14.2
11 July '95	21	28.6	38.0	14.2	33.3	0.0	4.8

Maximum detection rates are underlined.
[1] Both antisera were specific for the genus *Phytophthora*, but not species specific.

Thus using only non serological methods, different *Phytophthora* species were identified in the pond samples (Table 4). Species isolated could be attuned to a well known species or classified as confer (cf.), meaning it was similar to a known species. There were also isolates of the new species *P. ramorum* (Werres *et al.*, 2001) and of unknown *Phytophthora* species.

The widest range of *Phytophthora* species was isolated with the Rhododendron leaf test, the lowest range with the apple and the lupin seedling tests (Table 4). Not all *Phytophthora* species were detected by all methods.

For example, *P. cryptogea*/cf. *P. cryptogea* and *P. drechsleri*/cf. *P. drechsleri* were isolated by direct plating, by the lupin seedling and the Rhododendron leaf test but not by the apple test. *P. citrophthora* was isolated with the apple test and the Rhododendron leaf test but not with the other two methods. Certain *Phytophthora* species could only be trapped with one method: *P. cinnamomi* only by direct plating, *P. cactorum* only with the apple test, *P. cambivora*/cf. *P. cambivora* and *P. syringae* only with the Rhododendron leaf test. This last method was particularly favourable for detection *P. cryptogea*/cf. *P. cryptogea*, *P. drechsleri*/cf. *P. drechsleri*, cf. *P. gonapodyides*, *P. ramorum* spec. nov. and unknown *Phytophthora* species.

Table 4. The different species of *Phytophthora* detected in pond water and number of isolates obtained with different methods of detection.

Detection method	*Phytophthora* species	no. of isolates
Direct plating	*P. cinnamomi*	3
	P. cryptogea and cf. *P. cryptogea*	11
	P. drechsleri and cf. *P. drechsleri*	4
	cf. *P. gonapodyides*	12
	cf. *P. richardiae*	1
	P. ramorum spec. nov.	1
	unknown *Phytophthora* species	1
Apple test	*P. cactorum*	6
	P. citricola	4
	P. citrophthora	6
	cf. *P. gonapodyides*	17
	P. ramorum spec. nov.	2
Lupin seedling test	*P. citricola*	1
	P. cryptogea and cf. *P. cryptogea*	2
	P. drechsleri and cf. *P. drechsleri*	1
	cf. *P. gonapodyides*	8
	cf. *P. undulata*	3
Rhododendron leaf test	*P. cambivora* and cf. *P. cambivora*	5
	P. citricola	5
	P. citrophthora	1
	P. cryptogea and cf. *P. cryptogea*	28
	P. drechsleri and cf. *P. drechsleri*	26
	cf. *P. gonapodyides*	63
	cf. *P. richardiae*	4
	P. syringae	2
	cf. *P. undulata*	4
	P. ramorum spec. nov.	6
	unknown *Phytophthora* species	21

DISCUSSION

Data obtained with samples from water recirculation systems of commercial nurseries showed great variations in the detection rates of the different methods. The results were not always identical to those of obtained *in vitro* (Themann and Werres, 1997).

One important factor for successful *Phytophthora* detection is the sensitivity of a method towards different species of these pathogens. In commercial water recirculation systems the range of these pathogens is usually not known and can change from season to season and from year to year. That means the method should detect the widest range possible if the interest is not focused on a single species (*e.g.* a quarantine organism). Detection of *Phytophthora* species, for example by direct plating on selective media, is greatly influenced by the supplements added to the medium. Many antibiotics and fungicides can suppress development of different *Phytophthora* species (Jeffers and Martin, 1986; Kato *et al.*, 1990; Erwin and Ribeiro, 1996). In a bait test the attractiveness of the bait for a particular *Phytophthora* species determines the detection rate (Erwin and Ribeiro, 1996). With nursery samples, trapping rates of the apple and lupin seedling tests for*, P. cryptogea*/cf. *P. cryptogea* and *P. drechsleri*/cf. *P. drechsleri* were much lower than those of the Rhododendron leaf test. Of great interest for the nurserymen is the sensitivity of a test towards unknown or new *Phytophthora* species. If new species are imported onto container stands by, *e.g.*, latently infected plants, like the new species *P. ramorum* (Werres *et al.*, 2001), it is important to detected them in the water recirculation system as soon as possible. The Rhododendron leaf test was the best baiting method for this purpose.

A further uncertainty regarding methods for detection of *Phytophthora* is insensitivity towards different propagules. At a particular site and in a particular season there is uncertainty regarding type of propagule and species of *Phytophthora* present. Zoospores are the propagules expected to predominate in water. However in the organic material of the sediment other propagules can be present, and they can enter the water when the sediment is churned up by water being pumped in at high pressure. They can also contaminate directly water and sediment *via* infected plant debris. *In vitro* experiments had shown that the methods similar to those used in this study differed in their sensitivity towards different propagules (Themann and Werres, 1997).

A good detection method presupposes high sensitivity. *In vitro* experiments have shown that sensitivity depended on the *Phytophthora* species, on the propagule and on the method itself (Ali-Shtayeh *et al.*, 1991; Werres and Steffens, 1994; Themann and Werres, 1997). However, the results obtained with nursery samples in this study do not confirm the *in vitro* results (Themann and Werres, 1997). With ELISA the serological and enzymatic reactions may also have been negatively influenced by organic material in the samples. MacDonald *et al.* (1994) held them responsible, among other things, for decreasing ELISA values in recycling water from nurseries using a commercial ELISA test kit.

Indeed, when using water samples from nurseries, rivers or streams, it is helpful to know the possible influence of water quality on different detection methods. Under laboratory conditions, the chemical nature of the water can be standardized. Under commercial conditions this varies from nursery to nursery, and within a single nursery it can change with the season, as this study shows. However pH and nutrient levels had no influence on detection rate of any method used. There was only a negative correlation of total salt content of water and detection rate with ELISA, even though the highest salt content was that recommended for woody plants (Krüssmann, 1997). The ions present may have negatively influenced the epitopes of the antigens of *Phytophthora*, or indirectly influenced results by decreasing propagation of *Phytophthora* (Gisi *et al.*, 1977;

von Broembsen and Deacon, 1997; Hill *et al*., 1998; Toppe and Thinggaard, 1998). Thus the increase in detection rates with ELISA at lower salt concentration could have been caused by an increase of *Phytophthora* propagules. This is much more probable because the number of *Phytophthora* isolates in the water recirculation systems actually increased during the period when the total salt contents decreased (Themann, unpublished data). But due to the variable nature of the water from commercial nurseries, high background reactions or interference in the antibody reaction could not be excluded. For this reason ELISA is best used as a qualitative and not as a quantitative tool (MacDonald *et al*., 1990; Benson, 1991; Timmer *et al*., 1993; MacDonald *et al*., 1994; Werres and Steffens, 1994; Cacciola *et al*., 1995).

Last but not least, the volume of the sample greatly affects the probability of detecting low amounts of a pathogen (Werres *et al*., 1997). In ponds up to 6000 m^3, the probability of trapping low amounts of propagules seems to be low. Filtration of the water samples before direct plating, as done for the apple test and for ELISA is a possible way of concentrating propagules. Unfortunately this procedure also loses propagules (MacDonald *et al*., 1994). So, the trapping rate is best optimized by screening large sample volumes without further treatment, as is possible with Rhododendron leaf and lupin seedling tests.

The results clearly show that the quality of a detection method developed *in vitro* must be verified *in vivo* with samples from commercial nurseries before it can be used for routine screening. For water recirculation systems of commercial nurseries in Central Europe, a combination of the Rhododendron leaf test and the DAS-ELISA with antiserum against *P. cinnamomi* is excellent for the detection of *Phytophthora* spp. Furthermore, ELISA gives results within two days; a helpful first indication of potential contamination of a water recirculation system with *Phytophthora* spp.

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