Preliminary investigations on pathogenicity of European *Bursaphelenchus* species in comparison to *Bursaphelenchus xylophilus* from Japan

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SUMMARY

Laboratory studies were conducted to investigate pathogenicity of different European *Bursaphelenchus* species in comparison to *Bursaphelenchus xylophilus* from Japan. Inoculation tests revealed that *B. mucronatus* from Norway can cause death to young *Pinus sylvestris*, if test plants were inoculated with 12 500 nematodes each and the chosen day/night temperatures reached 23 ± 2 °C/21 ± 2 °C, respectively. Under these conditions disease development and tree damage due to *B. mucronatus* from Norway were similar to those caused by *B. xylophilus*. *B. fraudulentus* from Germany did not cause any damage to *P. sylvestris*. In an experiment with reduced inoculum and lowered temperature, distinct differences in pathogenicity between *B. mucronatus* from Norway and *B. xylophilus* became visible. All *B. xylophilus* inoculated plants died, whereas in *B. mucronatus* (Norway) inoculations only minor wilting symptoms occurred. The same was the case for the French *B. mucronatus* strain. *B. fraudulentus* (Germany) and *B. mucronatus* (Japan) dit not cause any disease symptoms in *P. sylvestris*. The pathogenicity shown on pine seedlings in laboratory tests does not imply any damage on adult trees under natural conditions by European *Bursaphelenchus* species.

RÉSUMÉ

Recherches préliminaires sur le pouvoir pathogène d'espèces européennes de Bursaphelenchus *en comparaison avec* Bursaphelenchus xylophilus *du Japon*

Des études ont été conduites au laboratoire pour évaluer le pouvoir pathogène de différentes espèces européennes de *Bursaphelenchus*, en comparaison avec une souche de *Bursaphelenchus xylophilus* du Japon. Des inoculations artificielles ont montré qu'une souche de *B. mucronatus* de Norvège peut gravement endommager de jeunes *Pinus sylvestris* lorsque les plants sont inoculés avec 12 500 nématodes chacun et maintenus à une température de 23 ± 2 °C pendant le jour et 21 ± 2 °C pendant la nuit. Dans ces conditions, l'évolution des symptômes et les dommages causés aux plantes par *B. mucronatus* de Norvège sont semblables à ceux causés par *B. xylophilus*. Une souche de *B. fraudulentus* d'Allemagne ne cause aucun dommage à *P. sylvestris*. La réduction de l'inoculum à 10 000 nématodes et l'abaissement de la température à 21 ± 1 °C entraînent de nettes différences dans le pouvoir pathogène des différentes souches : tous les plants inoculés avec *B. xylophilus* sont morts tandis que ceux inoculés avec *B. mucronatus*, de Norvège ou de France, ne montrent que de légers symptômes de flétrissement. Dans ces mêmes conditions, *B. fraudulentus* d'Allemagne et *B. mucronatus* du Japon ne provoquent aucun symptôme sur *P. sylvestris*. L'effet pathogène constaté au laboratoire sur de jeunes plants de pins n'implique cependant pas que les *Bursaphelenchus* européens causent des dommages aux arbres adultes en milieu naturel.

Bursaphelenchus xylophilus (Steiner & Buhrer, 1934) Nickle, 1970 (= *B. lignicolus* Mamiya & Kiyohara, 1972) is the causal agent of the pinewilt disease in Japan. This nematode is also distributed in other East Asian countries (Li *et al.*, 1983; Tzean, 1985; Yang, 1985), in the USA (Dropkin & Foundin, 1979; Dropkin *et al.*, 1981), and Canada (Knowles *et al.*, 1983; Anon., 1986*a*). On the North American continent tree damage occurs to a much lesser extent and is often restricted to tree species not native to this area (Blakeslee *et al.*, 1987; Bergdahl & Halik, 1987). To our present knowledge *B. xylophilus* does not exist in Europe, but it is known that many pine species common in Europe can be seriously damaged by *B. xylophilus* (Futai & Furuno, 1979; Linit & Tamura, 1987). European countries have therefore been keen to prevent the accidental introduction of *B. xylophilus* with imported wood products from its infested area and, as a consequence the European

Plant Protection Organisation (EPPO) has declared *B. xylophilus* as an A1 quarantine pest in 1986 (Anon., 1986*b*). The A1 list contains quarantine organisms which are not considered to occur in countries belonging to the EPPO-region.

In recent years some surveys have been carried out to check if *B. xylophilus* or closely related species occur in European countries. In 1979 Baujard *et al.* reported the finding of *B. xylophilus* (under the name of *B. lignicolus*) in *Pinus pinaster* in the south-west of France, but these nematodes are today regarded (de Guiran & Boulbria, 1986) to be a non-pathogenic form, morpho-biometrically similar to *B. mucronatus* Mamiya & Enda, 1979.

Weischer (1983) surveyed several hundred samples of conifers in the Federal Republic of Germany for pinewood nematodes, but did not find *B. xylophilus* or related species. Schauer-Blume (1989) and Balder (1987, 1988) reported the existence of " *B. mucronatus*–like " and *B. mucronatus*, respectively, in heavily damaged deciduous trees in West-Germany. By crossing experiments (unpubl.) the author identified these nematodes later as *B. fraudulentus* (Rühm, 1956) Goodey, 1960, which is morphologically similar to *B. mucronatus*. *B. fraudulentus* was also found in the wood in heavily damaged oak trees in Hungary (Schauer-Blume & Sturhan, 1989). Tomiczek (1988) mentioned the presence of *Bursaphelenchus* sp. in dying oak trees in Austria.

Surveys conducted by McNamara and Stoen (1988) in Norway and by Magnusson and Schroeder (1989) in Sweden revealed the presence of *B. mucronatus* in *P. sylvestris*. None of these reports of *Bursaphelenchus* species in Europe recorded damage to trees that could be directly attributed to the nematodes.

The aim of the present research was, therefore, to investigate the possible pathogenicity of European *Bursaphelenchus* isolates in comparison to the pathogenic *B. xylophilus* from Japan.

Material and methods

Nematodes

The following nematode species and/or populations were used for the inoculation tests :

Bursaphelenchus xylophilus from *Pinus densiflora*, Mito, Ibaraki Prefecture, Japan (hereafter referred to as BxJP)*

Bursaphelenchus mucronatus from *Pinus densiflora*, Yachiyo, Chiba Prefecture, Japan (BmJP)*

Bursaphelenchus mucronatus from *Pinus pinaster*, Casteljaloux, France (BmFR)**

Bursaphelenchus mucronatus from *Pinus sylvestris*, Hanestad, Norway (BmNo)***

Bursaphelenchus fraudulentus from *Prunus avium*, Erlangen, West-Germany (BfDE)

All nematode species were reared at 21 ± 2 °C on *Botrytis cinerea* on malt agar (4 %) and were subcultured monthly.

Test method

Starting two days before tree inoculation, the nematodes were isolated from the agar-plates by the Baermann-funnel technique and rinsed twice in clean tap water. At the day of inoculation the inoculation density of living specimens was established and then concentrated in a maximum water volume of 400 µl.

Test plants for the pathogenicity tests were young trees of *Pinus sylvestris*, *Quercus robur* and *Fagus sylvatica*, all aged 3-4 years. A small slit (1-2 cm) was cut into the upper part of the main stem of each plant, into which a small piece of cotton wool was placed. A few droplets of water with the required number of nematodes as inoculum were applied into the cotton wool. Then the inoculation site was sealed with a plastic band to prevent desiccation. The controls were injured in the same way as described above but were inoculated with water only. After inoculation the trees were maintained either in a phytotron or in a growth chamber under a 12 h photoperiod (light intensity 6 000 lux). The test plants were watered every third day if necessary and were checked for wilting symptoms weekly. First wilting symptoms became apparent in single needle chloroses at the inoculated branch. These disease symptoms could spread and complete chloroses could finally lead to the death of the plant.

In order to reisolate nematodes from the test plants, stem and/or branches were cut off, weighed, chopped into small pieces (maximum length 1 cm) and placed on a Baermann-funnel for three days. The total amount of reisolated nematodes was then calculated per gram of fresh wood.

Three inoculation experiments were performed :

Inoculation test 1 : Pine, oak and birch trees were inoculated with 12 500 of BxJP each and the test plants were then kept at day/night temperatures of 23 ± 2 °C/21 ± 2 °C. This test was conducted to find out if *B. xylophilus* is pathogenic also for deciduous tree species.

Inoculation test 2 : It was conducted to investigate possible pathogenicity of the *B. mucronatus* isolate from Norway and of the *B. fraudulentus* isolate from Germany against *P. sylvestris*. For this experiment the same

* Nematode cultures kindly provided by : Dr. Y. Mamiya, Forestry and Forest Products Research Institute, Ibaraki, Japan; ** Dr. G. de Guiran, INRA, Station de Recherches de Nématologie et de Génétique Moléculaire des Invertébrés, Antibes, France; *** S. Haukeland, Department of Agriculture, Reading, England.

test conditions (inoculation density : 12 500 nematodes/tree, temperature day/night : 23 ± 2 °C/21 ± 2 °C) were chosen as in the first inoculation test with *B. xylophilus*.

Inoculation test 3 : The temperature was lowered to 21 ± 1 °C and the inoculum density was reduced to 10 000 nematodes per tree. *P. sylvestris* trees were inoculated with BmNO, BmFR, BfDE, BmJP and BxJP to find out, if under conditions of lower temperatures and inoculum density, isolates other than BxJP are pathogenic to *P. sylvestris*.

Results

Inoculation test 1

Results are demonstrated in Table 1. Only *P. sylvestris* developed wilting symptoms. The first distinct needle chloroses near the inoculation site were visible in the third week after inoculation and these symptoms extended continuously thereafter. After five weeks the soil of inoculated *P. sylvestris* remained moist, indicating that the plants had reduced or stopped water uptake from the soil. This was not the case for the deciduous tree species. Nine weeks after the beginning of the test all *P. sylvestris* had died. During the whole test period none of the inoculated *Q. robur* or *F. sylvatica* and none of the control plants developed any disease symptoms.

This test was terminated after three months. The wood of *P. sylvestris, F. sylvatica* and *Q. robur* was then investigated for the presence of *B. xylophilus*. No nematodes could be reisolated out of stem and branches of any *F. sylvatica* tree. Only in three of the five inoculated *Q. robur* an average of 30 viable *B. xylophilus* was discovered. These nematodes were found in the stem sector 5 cm above and below the inoculation site. All other stem and branch parts were free of nematodes. In all inoculated *P. sylvestris, B. xylophilus* had spread within the main stem and branches.

Table 1

Development of disease symptoms in 3 year-old *Pinus sylvestris, Quercus robur* and *Fagus sylvatica* after inoculation with *Bursaphelenchus xylophilus* (inoculum density : 12 500 nematodes/tree; temperature day/night : 23 ± 2 °C/21 ± 2 °C).

Trees	*Weeks after inoculation*				
	3	*5*	*7*	*9*	*12*
P. sylvestris :					
inoculated (n = 5)	2N/3W	5W	1D/4W	5D	5D
control (n = 5)	5N	5N	5N	5N	5N
Q. robur :					
inoculated (n = 5)	5N	5N	5N	5N	5N
control (n = 3)	3N	3N	3N	3N	3N
F. sylvatica :					
inoculated (n = 5)	5N	5N	5N	5N	5N
control (n = 3)	3N	3N	3N	3N	3N

(N = no disease symptoms; W = wilt symptoms; D = plant has died.)

Inoculation test 2

Results are demonstrated in Table 2. Again three weeks after inoculation first wilting symptoms were noticed in pine trees inoculated with BmNO. As time passed the wilting of the trees became more pronounced and, after nine weeks, nine *P. sylvestris* had died, two trees showed chlorotic needles at the stem and on one branch, and only one tree showed no disease symptoms. The three pine trees inoculated with BmNO continued living after three months, did not develop any further symptoms and developed new shoots after seven months. The wilting symptoms and disease development in trees inoculated with BmNO under these test conditions were similar to those in the first test which were inoculated with *B. xylophilus* from Japan. *B. fraudulentus* did not cause any disease symptoms in the pine trees. These plants, as well as the controls, stayed healthy during the whole test period and beyond.

Seven weeks after the beginning of this test, single branches were cut off and classified for degree of

Table 2

Development of disease symptoms in 3 year-old *Pinus sylvestris* after inoculation with *Bursaphelenchus mucronatus* (Norway) and *Bursaphelenchus fraudulentus* (Germany) (inoculum density : 12 500 nematodes/tree; temperature day/night : 23 ± 2 °C/21 ± 2 °C).

Nematodes	*Weeks after inoculation*				
	3	*5*	*7*	*9*	*12*
B. mucronatus (n = 12)	5N/7W	2N/10W	1N/8W/3D	1N/2W/9D	1N/2W/9D
B. fraudulentus (n = 12)	12N	12N	12N	12N	12N
Control (n = 6)	6N	6N	6N	6N	6N

(N = no disease symptoms; W = wilt symptoms; D = plant has died.)

damage. In BmNO-inoculated trees an average of 337 nematodes/g wood were isolated from branches (n = 6) with totally chlorotic needles, 232 nematodes/g wood out of branches (n = 6) with some chlorotic needles, whereas branches with no wilting symptoms contained only 0.4 nematodes/g wood. Healthy looking branches (n = 6) of BfDE-inoculated pine trees contained 0.2 nematodes/g wood and the controls were free of nematodes.

INOCULATION TEST 3

Results are demonstrated in Table 3. Distinct differences in the pathogenic potential between BxJP and BmNO appeared. The first wilting symptoms occurred in BxJP-, BmNO- and BmFR-trees three weeks after the beginning of the test. After nine weeks in BxJP-inoculated plants three of six trees had already died and two other ones were severely damaged, whereas in the BmNO- and BmFR-inoculations only one plant had developed chlorotic needles above the inoculation site. The chlorosis was restricted to the upper part of the main shoot and did not affect the entire plant. In none of the trees inoculated with BfDE and BmJP wilting occurred. Five months after the beginning of the test also the last BxJP-inoculated plant had died. All plants in the BmNO-, BmFR-, BmJP- and BfDE-inoculations were living without any further development of disease symptoms.

Table 3

Development of disease symptoms in 3 year-old *Pinus sylvestris* after inoculation with *Bursaphelenchus xylophilus* (Japan), *Bursaphelenchus mucronatus* (Norway), *Bursaphelenchus mucronatus* (France), *Bursaphelenchus mucronatus* (Japan) and *Bursaphelenchus fraudulentus* (FRG). (Inoculum density : 10 000 nematodes/tree; temperature : 21 ± 1 °C.)

Nematodes	*Weeks after inoculation*				
	3	*5*	*7*	*9 & 12*	*20*
B. xylophilus (JP) (n = 6)	3N/3W	3N/3W	2N/2W/2D	1N/2W/3D	6D
B. mucronatus (NO) (n = 6)	5N/1W	5N/1W	5N/1W	5N/1W	5N/1W
B. mucronatus (FR) (n = 6)	5N/1W	5N/1W	5N/1W	5N/1W	5N/1W
B. mucronatus (JP) (n = 4)	4N	4N	4N	4N	4N
B. fraudulentus (DE) (n = 6)	6N	6N	6N	6N	6N
Control (n = 6)	6N	6N	6N	6N	6N

(N = no disease symptoms, W = wilt symptoms, D = plant has died.)

Discussion

The inoculation tests have shown that *B. xylophilus* does not damage either young birch nor oak trees. This nematode did not survive in birch trees and in oak trees in only three out of five seedlings a few *B. xylophilus* (0.2 % of the inoculum) survived more than three months. Under certain test conditions the Norwegian isolate of *B. mucronatus* possesses the ability to kill young pine trees. Expression of the first wilting symptoms and disease development under these conditions were similar to those in the *B. xylophilus* inoculations. Under lower temperature- and inoculation density conditions, distinct differences in pathogenicity between BxJP and BmNO were evident. In contrast to the *B. xylophilus* inoculations no plant died when inoculated with the Norwegian *B. mucronatus* isolate. BmNO and BmFR caused only minor damage, locally restricted to the inoculated tree branch.

Although the laboratory tests reported here indicate a potential pathogenicity in *B. mucronatus* from Norway it is very questionable if these nematodes can do any harm to pine trees under the natural climate in northern Europe. Similar results were described by de Guiran and Boulbria (1985). They reported, that the French *B. mucronatus* strain possessed a certain pathogenicity against four year-old *P. pinaster* in laboratory tests (temperature : 20-28 °C, inoculation density : 10 000 nematodes/tree), but when nine-year old trees were inoculated under the natural climate in south-western France, no disease symptoms or damage could be evoked.

B. fraudulentus was not pathogenic under any of the test conditions. Further tests are needed in order to clarify wether European *B. mucronatus* isolates could contribute to tree damage in the field under natural and sometimes stressful climatic conditions.

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