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BEATRICE CARLETTI (*) - ALBA COTRONEO (**) - PIO FEDERICO ROVERSI (*)

INOCULATION EXPERIMENTS OF *BURSAPHELENCHUS EREMUS* RÜHM (GOODEY) (NEMATODA PARASITAPHELENCHIDAE) ON *QUERCUS ROBUR* L. [1]

(*) *CRA - Agricultural Research Council, Research Centre for Agrobiology and Pedology, Laboratory of Nematology, Via Lanciola 12/A, Cascine del Riccio - 50125 Firenze, Italy;* e-mail: beatrice.carletti@isza.it
(**) *Servizio Fitosanitario Regione Piemonte c/o Environment Park, Palazzina A2, Via Livorno 60 - 10144 Torino; Italy.*

Carletti B., Cotroneo A., Roversi P.F. – Inoculation experiments of *Bursaphelenchus eremus* Rühm (Goodey) (Nematoda Parasitaphelenchidae) on *Quercus robur* L.

Bursaphelenchus eremus Rühm (Goodey) occurs in declining oak forests of some European countries, although data are lacking on the possibility of the nematode to colonize healthy trees. To evaluate the pathogenicity of the nematode, we carried out an inoculation test in Tuscany (central Italy) in 2007-2008: on May 2007, 32 *Quercus robur* L. plants (7 years old) were inoculated with an Italian isolate of *B. eremus* (IT37w) and 16 plants were maintained as controls. There were two inoculation sites on each plant: one immediately below the fresh top shoot and another 30 cm below the first site. All plants were kept outdoors under a zinc-coated cage covered with a cloth. After four months, 16 inoculated plants and eight controls were randomly selected and each stem was cut into three parts, weighed, crushed in a grinder and subjected to Baermann funnel extraction. After 16 months, the remaining 18 inoculated and 6 control plants were chosen and examined with the same procedure.

Bursaphelenchus eremus was obtained from the stems of all infected oak trees. The difference was in the number of nematodes/g of fresh wood in the different plant portions both after four ($F_{2,16}$=86.94) and 16 months ($F_{2,18}$=400.71). The highest density was always recorded in the fresh top shoot tissue, while no nematode was obtained from the lower stem portion. The RHS (relative host suitability) index value increased from 0.6 (4 months) to 2.1 (16 months) but it was still too low to be accompanied by signs of wilting. Because of the resemblance between *B. eremus* and the Pine Wood Nematode, we suggest that further experimental studies are needed to evaluate the potential risk of this nematode for different species of oaks common to the Mediterranean area, where the maximum summer temperatures reach the optimal temperature range for *B. eremus* development.

KEY WORDS: Aphelench nematode, broadleaf plants, oak decline, pathogenicity.

INTRODUCTION

Bursaphelenchus eremus Rühm (Goodey) was first described in Germany based on specimens isolated from wood of *Quercus* spp. and from the bark beetle *Scolytus intricatus* Ratzeburg (RÜHM, 1956). It was subsequently recorded in other European countries, both from *S. intricatus* adults and from wilted, weakened or dead broadleaf trees (KURASHVILI *et al.,* 1980; KUBÀTOVÁ *et al.,* 2000; CARLETTI *et al.*, 2004; BRAASCH *et al.*, 2006). In Italy, the nematode occurs in declining oak forests of Lombardy, Piedmont and Tuscany, generally associated with individuals of *Quercus* spp. that are recently dead or showing wilting of unclear aetiology and whose crowns are frequently colonized by *S. intricatus* (CARLETTI *et al.,* 2007).

The biology of this phytoparasitic nematode shows a strong affinity with that of the Pine Wood Nematode *Bursaphelenchus xylophilus* (Steiner *et* Bührer) Nickle (BRAASCH *et al.*, 2006); high numbers of dauer juveniles are found beneath the elytra or the wings of *S. intricatus* when the scolytid emerges from declining trees and moves to other *Quercus* plants to feed on young shoots for sexual maturation (CARLETTI *et al.*, 2005). The virulence of *B. xylophilus* has been investigated under natural and controlled conditions and has proved to be an important quarantine pest causing high mortality to pines in a very short time (MAMIYA, 1983; RIGA *et al.,* 1991; BAKKE *et al.,* 1991; KISHI, 1999). In contrast, the pathogenic potential of *B. eremus* against broadleaf trees is unknown and only some preliminary data, obtained recently in Italy, are available (CARLETTI *et al.,* 2010). Therefore, to evaluate the potential risk of this species in the Mediterranean area, we carried out experimental trials to verify the possibility of *B. eremus* to reproduce in healthy pedunculate oak trees (*Quercus robur* L.), one of the most important trees in broadleaf forests in plains and hills.

MATERIALS AND METHODS

NEMATODE ISOLATE

The nematode population (IT37 w) used in this experiment was extracted from wood of diseased *Q. robur* trees in the Ticino Park Forest (Piedmont). The population had been cultured *in vitro* for two months at 26°C on Petri dishes (9 cm ø) with *Botrytis cinerea* Pers. ex Fr. containing malt extract agar added to 5% glycerol. In order to utilize biologically active and clean material, just before the inoculation we collected only individuals gathered on droplets of water moisture on the Petri dish covers with a wash-bottle containing sterilized bidistilled water. This suspension was concentrated by gravity and the excess was poured out. The remaining material was homoge-

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nized and the nematodes were counted by considering 1 cc of water to apply to each plant. The 1 cc of water, containing about 25,000 individuals of all the nematode stages, was divided into two 0.5 cc inocula per plant.

Inoculation test

In spring 2006, 48 *Q. robur* plants from nursery beds in Piedmont (Italy) were transferred to the Centre for Agrobiology and Pedology in Florence (Tuscany – central Italy). The 7-year-old plants were potted in plastic pots (50 cm ø) containing the same substrate as used in the nursery. The plants were kept until 2007 in a hilly area near Florence (68148384 E, 4844551 N), during which time they were subjected to pollarding and treatments against defoliators.

Inoculation was carried out in May 2007: 32 *Q. robur* plants were inoculated with almost 25,000 nematodes/plant. Sixteen plants were used as controls. Two inoculation sites were selected on the plants, one immediately below the fresh top shoot and another 30 cm below the first. At both sites, the bark was cut ca. 1 cm lengthwise with a knife. A strip of previously sterilized thin cotton cloth, 1 cm wide and 7 cm long, was inserted into the slit slightly underneath the bark and folded over the slit. A strip of polyethylene was wrapped around the stem at the inoculation site and fixed at the bottom with a small strip of adhesive tape.

The nematode suspension (0.5 cc for each site) was then carefully pipetted from the top of the polyethylene strip onto the folded cotton cloth strip. Finally, to prevent water loss, the top of this strip was closed with adhesive tape. The control plants were treated in the same way but inoculated with 1 cc/plant of sterilized bidistilled water. To assess the inoculum viability, we poured the remaining part of the solution with nematodes into five Petri dishes containing *B. cinerea* on malt extract agar. After one week, the *B. eremus* isolate was increased on each Petri dish.

All inoculated plants were labelled and put under a zinc-coated cage with rectangular base (4x10 m) and 4 m high in the mid point of the area centre, which was covered with a fine-mesh anti-insect cloth. A barrier with the same cloth divided the inoculated plants from those used as controls. Inside the cage, the daily temperature was recorded with a data logger (Escort Junior). An electronic watering system was set up to provide 8-10 litres of water/pot twice a week when no rainfall occurred (Caroppo *et al.,* 2000).

Nematode extraction

In September 2007, i.e. four months after the inoculation procedure, 16 plants inoculated with *B. eremus* and eight control plants (all randomly selected) were cut 2 cm above the soil; after the leaves were removed, the stem was divided into three parts by cutting a 15 cm portion below the first inoculation site and 15 cm below the second inoculation site. The stem parts were crushed in a grinder. The resulting material was collected separately in tightly sealed plastic bags, which were labelled and stored in a refrigerator (at +4°C) until the Baermann funnel extraction. This method makes it possible to recover most living nematodes even after 24-48 hours. The re-isolated nematodes were counted under a Nikon SMZ-1500 stereomicroscope.

In September 2008, 16 months after inoculation, the remaining 24 plants maintained under the cage (18 inoculated with *B. eremus* and 6 controls) were subjected to the same procedure described above.

Relative Host Suitability (RHS)

In view of the lack of published data on pathogenicity tests of *B. eremus* on broadleaf seedlings or plants, the *Quercus robur* plants were screened for suitability for the nematode by means of the Relative Host Suitability (RHS) index (see Braasch, 1997):

RHS = (percentage of successfully inoculated plants x mean number of re-isolated nematodes/g of fresh wood)/1000.

Symptom observation

Partial wilting, discolouration and leaf loss were categorized and assessed every two weeks during the summer and monthly during the other seasons.

Statistical analysis

The effect of treatment/inoculation was evaluated by recording the number of nematodes per gram of fresh wood. Data were subjected to two-way analysis of variance and the differences were compared with the Tukey HSD test ($P<0.05$).

RESULTS

Nematode density after four months

No nematode was recovered from the control plants at the end of the first stage of the experiment. In the 16 plants treated with the nematode suspension, the nematode population developed and increased, with strong differences from one plant to another. The number of nematodes/plant showed strong variation among plants, from a maximum of 9510 to a minimum of 542 individuals/plant. On fresh wood, 5.28±3.28 (mean±SD) individuals per gram were recorded (fig. I). Nematodes were reisolated only from the top shoot, above the first inoculation site, and from the middle part of stem, around the second inoculation site; no specimen was collected from the basal portion. Furthermore, the number of nematodes recovered after four months was lower in the middle portion of the stem than in the top shoot ($F_{2,16}= 86.94$, $P < 0.05$) (fig. II).

After four months, no external disease symptoms appeared on the inoculated plants. The RHS value was 0.6.

Nematode density after 16 months

Table 1 reports the mean monthly temperatures recorded under the cage and the rainfall in the area from May 2007 to September 2008. After the 16th month, no wilting was observed on the remaining 24 plants.

All inoculated plants maintained for 16 months showed nematode infestation. The population density of the reisolated nematodes varied between 16,986 and 28,510 individuals/plant (22,575.28±3450.01, mean±SD), with a mean number of specimens/g of fresh wood of 20.35±0.13 (fig. I).

The highest density was always recorded from the fresh wood of the top shoot, with a mean of 41.69 nematodes/g (±4.91 SD); this density was higher than that recorded in the middle stem portion ($F_{2,16}= 86.94$, $P < 0.05$) (fig. II). At 16 months, the RHS value was 2.1.

DISCUSSION

Our results suggest that the *B. eremus* strain tested in this study was able to develop successfully on healthy young *Q. robur* plants. During the trials, *B. eremus* multi-

Table 1 – Temperatures (°C) and rainfall (mm) registered over the period May 2007 - September 2008.

Year	Month	Average temperature	Maximum	Minimum	Rainfall
2007	May	15.9	33.6	3.4	74.4
	June	20.6	37.0	8.2	25.4
	July	23.1	41.4	7.9	n.d.
	August	20.9	36.1	8.5	116.6
	September	19.7	26.3	13.6	54.6
	October	16.1	22.0	11.3	49.3
	November	10.4	15.3	5.8	27.4
	December	6.7	11.6	2.2	87.9
2008	January	9.0	13.1	4.9	98.3
	February	9.1	14.1	4.8	33.0
	March	10.6	15.4	6.0	72.1
	Avril	13.6	19.2	8.3	113.3
	May	18.7	25.1	12.0	76.9
	June	22.4	28.4	16.6	45.7
	July	24.5	31.5	18.0	25.9
	August	25.4	32.9	18.0	9.1
	Septemebr	19.8	25.9	14.2	24.1

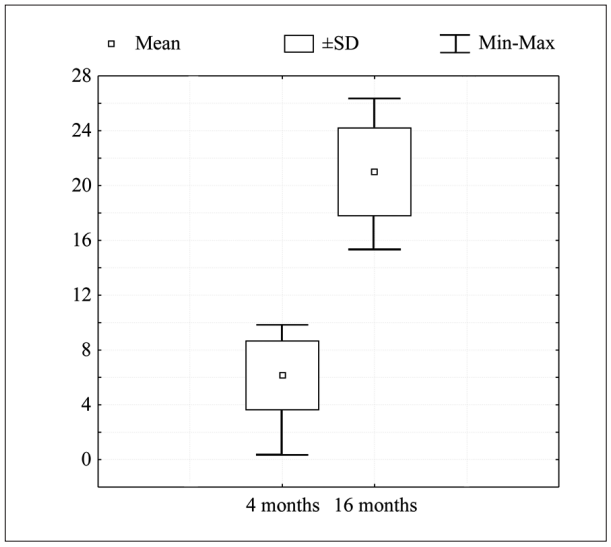

Fig. I – Number of nematodes/g of fresh wood (mean±SD) after 4 and 16 months.

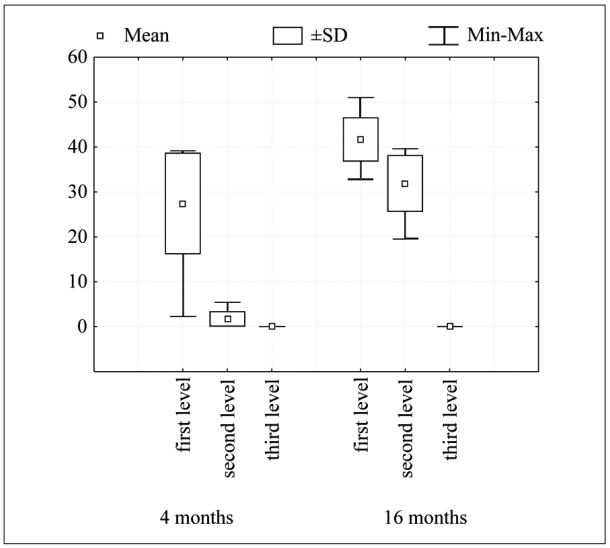

Fig. II – Number of nematodes/g of fresh wood in the three different stem parts (mean±SD) after 4 and 16 months.

plied and spread in the middle and the top of the plant stems. The number of nematodes extracted from the top shoot, the most intensively colonized part, was 20 times higher after 16 months than after four months, and the RHS index was nearly four times higher. Similar results were reported by BRAASCH *et al.* (2000) with 11-year-old coniferous plants inoculated with *B. mucronatus* Mamiya *et* Enda; after inoculation, nematodes were only found close to the inoculation site.

During summer, the mean monthly temperature fluctuated between 20.6°C and 23.1°C in 2007 and between 22.4°C and 25.4°C in 2008 (Table 1). These values are too low for optimal development of *B. eremus*, as demonstrated by recent tests performed *in vitro* with 3 Italian isolates of this nematode, showing that the reproduction rate reached the highest value at 26°C (CARLETTI *et al*., 2008). Despite the success of the inoculation, none of our infested plants showed wilting, probably due to the low RSH values with respect to those recorded by BRAASCH *et al.* (2000). In previous pathogenic tests conducted with *Bursaphelenchus* species on different 3-year-old coniferous seedlings, only RHS index values higher than 10 indicated a pathogenic potential (BRAASCH *et al.*, 1998; 2000).

Since the present study has demonstrated the possibility of *B. eremus* to develop on healthy plants, further research is needed to clarify the role of this nematode in the oak decline observed in an increasing number of southern European deciduous oak woods.

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RIASSUNTO

INOCULAZIONE DI BURSAPHELENCHUS EREMUS *RÜHM (GOODEY) IN* QUERCUS ROBUR *L.*

Bursaphelenchus eremus Rühm (Goodey) è un nematode segnalato in alcuni paesi europei in boschi di latifoglie interessati da fenomeni di deperimento di non facile interpretazione. Per acquisire dati sulla patogenicità di questo nematode è stata effettuata una prova all'aperto, utilizzando piante di *Quercus robur* L. di sette anni. Per la prova sono state utilizzate 48 piante: 34 piante inoculate con un isolato italiano di *B. eremus* (25000 individui per pianta) e 14, da utilizzare come testimone, trattate con acqua sterile bidistillata. Le inoculazioni sono state effettuate nel fusto di ciascuna pianta in due punti distinti, immediatamente sotto il getto apicale e più in basso a distanza di 30 cm. Tutte le piante sono state messe a dimora sotto una struttura zincata rivestita da tela antiinsetto, al cui interno è stata inserita una sonda Dataloger per registrare le variazioni giornaliere di temperatura. La prova è durata 16 mesi. A distanza di 4 mesi dall'inoculazione sono state scelte a caso 16 piante inoculate con *B. eremus* e 8 di controllo. Il fusto di tutte le piante è stato tagliato in 3 parti e ciascuna porzione è stata pesata, triturata e sottoposta a estrazione mediante imbuto di Baermann, con successivo conteggio dei nematodi vivi reisolati. Le piante rimaste, sono state mantenute nel medesimo ambiente per ulteriori 12 mesi, terminati i quali sono state sottoposte allo stesso trattamento adottato in precedenza. Nel corso dell'intera ricerca tutte le piante non hanno evidenziato alcun sintomo di deperimento. Tutte le piante inoculate hanno fatto registrare in seguito popolazioni insediate di *B. eremus* con colonizzazione delle sole porzioni di fusto nelle quali erano state effettuate le inoculazioni. Considerando la distribuzione verticale del nematode si evidenzia inoltre che la massima densità di nematodi per grammo di legno fresco è stata registrata sia dopo 4 mesi ($F_{2,16}$= 86.94, $P < 0.05$) che dopo 16 mesi ($F_{2,16}$= 86.94, $P < 0.05$) nella parte della pianta comprendente il getto apicale. Dopo 16 mesi dall'inoculazione il numero di nematodi reisolati è risultato quasi quadruplicato rispetto a quello rilevato dopo 4 mesi. Di conseguenza anche l'indice RHS ha subito nel tempo un marcato aumento, ma i valori raggiunti non sono stati accompagnati dalla manifestazione di fenomeni di deperimento.

Da rilevare che nel biennio le temperature medie estive sono rimaste al di sotto dei valori considerati ottimali per lo sviluppo di *B. eremus* nel corso di precedenti ricerche svolte con 3 isolati italiani. Gli Autori evidenziano infine l'importanza di condurre ulteriori studi per definire il ruolo di questo nematode nel progressivo intensificarsi e diffondersi dei fenomeni di deperimento che interessano vari querceti caducifogli del sud Europa.

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