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USE OF THE NEMATODE *DELADENUS SIRICIDICOLA* IN THE BIOLOGICAL CONTROL OF *SIREX NOCTILIO* IN AUSTRALIA

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Abstract

A knowledge of the life cycles of free-living, mycetophagous nematodes has been utilized in developing methods for the monoxenic mass rearing of hundreds of millions of *Deladenus siricidicola* Bedding. This tylenchid nematode causes sterility in female *Sirex noctilio* F., a serious pest of pine trees in south-eastern Australia; it has been liberated over many of the areas affected by *Sirex* using the simple but highly effective techniques described.

Introduction

There are in Australia over $\frac{1}{2}$ million hectares of coniferous forest of which more than $\frac{3}{4}$ is the introduced pine species *Pinus radiata* D. Don; the estimated annual return from the timber harvested is now approaching 250 million dollars and the industry continues to expand rapidly.

The woodwasp *Sirex noctilio* F., a serious pest of radiata pine trees, was accidentally introduced, probably from New Zealand and was found in southern Tasmania in 1952. As a result of spectacular damage by this insect in one Tasmanian forest, where at least 40% of all trees were killed over several years, and the finding of *Sirex* in Victoria in 1961, the National Sirex Committee was set up in 1962 to examine means for eradication or control. When initial attempts at eradication proved unsuccessful, an extensive programme of "Search and Destroy" was carried out in Victoria in an effort to contain the spread of *Sirex* while research into various other aspects of control including attractants, selective breeding of resistant trees, and the introduction of insect and nematode parasites was carried out. Despite all efforts to prevent its spread, infestations of *Sirex* have spread steadily from the initial outbreak area in Melbourne over many thousands of hectares and are now only about 120 km from the vast coniferous forests of Mt. Gambier 80,000 ha). Following the discovery of nematodes infecting *S. noctilio* in New Zealand (Zondag 1962), CSIRO commenced investigations into nematode parasites of various siricids and their parasitoids from world wide sources in 1965. The life history of these nematodes was shown by Bedding (1967, 1972a, 1972b) to be extraordinary, involving profound female dimorphism associated with free-living mycetophagous and parasitic life cycles, and seven species of *Deladenus* (Neotylenchidae) found parasitizing siricids were described by Bedding (1968, in press). The present paper is concerned mainly with techniques used in the manipulation of *D. siricidicola* for the control of *Sirex* in Australia.

Biology of *Sirex*

Female *S. noctilio* drill into the wood of living pine trees to oviposit and implant toxic mucus and a symbiotic fungus (*Amylostereum areolatum* (Fr.) Boidin). The mucus and fungus kill susceptible trees (Coutts 1969) and *S. noctilio* larvae bore through, and feed on, the fungus infected wood. Adult *S. noctilio* emerge 1, 2, or 3 years after oviposition. Immature stages of *Sirex* are subject to attack from various ichneumonid, cynipid and nematode parasites and adult *Sirex* to predation by birds.

Biology of *D. siricidicola*

Parasitized *Sirex* larvae contain 1 to 100 or more adult female nematodes dispersed in the haemocoel. These nematodes vary from 5 to 25 mm in length, are cylindrical and often bright green. Their reproductive system remains undeveloped (about 0.5 mm in length) until the onset of host pupation and then expands rapidly throughout the length of the nematode, soon producing several thousand eggs,

which hatch within the parent. Juvenile nematodes escape into the haemocoel of the host pupa and migrate into its reproductive organs. In a female host, juveniles penetrate all developing eggs and tend to suppress the developing ovaries, while in a male host the testes become a solid mass of many thousands of juveniles. The female *Sirex* is sterilized, with each of its eggs containing 50 to 200 juveniles, but the male *Sirex* remains fertile because early in pupation, before juvenile nematodes penetrate the testes, most spermatozoa pass into the vesiculae seminales which are not penetrated by the nematodes.

During oviposition by parasitized female *Sirex*, eggs containing juvenile nematodes are implanted into pine trees together with symbiotic fungus. Juvenile nematodes migrate from the host's eggs, feed on the developing fungus and grow into adult free-living nematodes which lay many eggs within the tracheids around *Sirex* oviposition holes. Juveniles hatching from these eggs also feed on fungus and can develop into free-living mycetophagous adults. This free-living cycle can be repeated indefinitely.

As the tree dries out, the symbiotic fungus spreads throughout it and nematodes breed within tracheids, resin canals and even beneath the bark. In these relatively aseptic areas where fungus is sparse, juveniles develop only into mycetophagous forms, but in the vicinity of *Sirex* larvae (several *Sirex* usually attack a single tree), juvenile nematodes may develop into adult infective females which are quite unlike the mycetophagous females. After insemination, infectives penetrate *Sirex* larvae and grow as much as a thousand fold in volume within a few weeks and commence reproduction after host pupation.

Aspects of greatest importance in the use of this nematode for biological control are its ability to sterilize female *Sirex*, the free living cycle which can be utilized for maintaining cultures and mass rearing, and (Bedding, in preparation) its specificity to the *Sirex* symbiotic fungus (which restricts it to the environment of *Sirex*) and inability to affect important insect parasitoids.

Culturing techniques

Culture on agar plates

To obtain uncontaminated cultures of the symbiotic fungus *Amylostereum areolatum*, adult female *Sirex* are dipped in ethanol, ignited, and plunged into and then dissected under sterile water to remove the ooidial glands (situated internally at base of ovipositor). Under sterile conditions the burst glands are streaked on plates of potato-dextrose agar (P.D.A.) and the growing fungal front is sub-cultured after incubation at 24°C. Testes containing juvenile *D. siricidicola* similarly removed from male *Sirex* are placed in the centre of fungal cultures that have grown over about one third of the plate. After 1 day, sub-cultures (about 1 cm square) of several hundred nematodes together with fungus are cut from the fungus front and placed at the edge of P.D.A. plates to establish monoxenic cultures. At 24°C larvae mature into adults about 5 days after removal from a host insect and sub-cultures are made about 1 week later when many eggs have hatched. Further sub-cultures are made at about 2 weekly intervals just before the agar is completely covered with fungus. In sub-culturing, a balance must be maintained between nematodes and fungus since the nematodes can breed successfully only on the growing edge of the fungus; with too few nematodes the fungus covers the plate before much reproduction occurs, and with too many, fungal growth may be completely stunted. A noticeable improvement in rate of reproduction occurs during the first few months of culturing.

Use of P.D.A. produces cultures far superior to those on malt, corn meal, Czapek-Dox, nutrient, or water agars although corn meal agar is sometimes useful for storing cultures.

Cultures can be stored at 5°C in the dark for 6 to 12 months on P.D.A. or corn meal agar slopes in test tubes without sub-culturing. A 1cm square inoculum of a high density of nematodes on fungus is placed at the bottom of the tube which is closed with a tight-fitting non-absorbent cotton wool bung covered with aluminium foil. At 5°C the fungus grows and the nematodes can still feed and reproduce slowly.

Mass culture

Although P.D.A. plates each yield several thousand nematodes, this method of obtaining *D. siricidicola* in large numbers is expensive and time consuming and the nematodes are difficult to harvest. Evans (1970) developed an excellent method for mass rearing the nematode *Aphelenchus avenae* which fed on the fungus *Rhizoctonia solani* growing on autoclaved wheat in jars, but he had some contamination problems. The method used for mass rearing *D. siricidicola* is similar but utilizes Erlenmeyer flasks with cotton wool bungs and foil caps to eliminate contamination; the proportion of wheat to water is very different (1.5 cc tap water to 1 g of wheat), and the fungus used is *A. areolatum*.

Flasks of 500 ml capacity are most frequently used; 100 g wheat and 150 ml water are added to each flask and after plugging and capping they are autoclaved at 1.05 kg/cm^2 for 20 min; while still warm, the flasks are strongly agitated to separate wheat grains and aid aeration. They are inoculated from vigorous P.D.A. plate cultures of *D. siricidicola*, using sub-cultures of about 2 cm^2 of fungus and 1000 to 1500 nematodes. Incubation is at 24°C, preferably in the dark, and nematodes are ready for harvesting 4 to 6 weeks later when most of the fungus has become brown and nematodes swarm up the sides of the flask (Fig. 2).

To harvest, cold tap water (5-10°C) is added to cover the wheat and left a few minutes prior to agitation. The resulting suspension of nematodes is poured off (through a sieve to remove debris) and the nematodes allowed to settle, or centrifuged prior to washing 2 or 3 times in cold tap water. Flasks are washed out 3 or 4 times over an hour or more, but should not be left full of water for more than 5 to 10 min because of possible nematode asphyxiation. Yields vary from 3 to 10 million nematodes per 500 ml flask. Since the contents of a single flask are sufficient to inoculate about an hundred metres of timber, attempts were not made at larger scale mass rearing.

With some strains of *D. siricidicola*, infective nematodes are formed readily in such cultures so that at the usual harvest time, most nematodes are either infectives or associated males. These cannot reproduce in *Sirex* infested timber and so are of little use for inoculation. To reduce the proportion of infectives produced, flasks can be either harvested early (after 3-4 weeks) resulting in rather low yields, or infective production can be greatly reduced by blowing moist, sterile air through cultures.

Storing and transporting

Unlike many other insect parasitic nematodes, *Deladenus* has no special resistant stage adapted for prolonged survival. Generally, the nematodes are cultured as and when they are needed but can be stored for several weeks in shallow tap water with an atmosphere of almost pure oxygen at 5 to 10°C (Fig. 1).

Use of dilute formaldehyde solution, although successful for storing *Neoaplectana* spp. (Dutky *et al.* 1964), kills *Deladenus* spp. which are usually transported and stored in 150 ml Erlenmeyer flasks with up to 1 million nematodes in 20 ml tap water per flask. Each flask is flushed out with pure oxygen for several seconds before being closed by a tight fitting rubber bung.

Inoculation of timber

In order to introduce *Deladenus* into wild populations, either *Sirex* infested timber is inoculated with nematodes and distributed in *Sirex* affected areas, or trees scattered throughout affected areas are felled and inoculated on the spot. Timber of a wide range of moisture content, from freshly killed down to 50% oven dry weight can be inoculated with resulting successful establishment. Most female *Sirex* emerging from correctly inoculated timber are parasitized and oviposit on trees usually also attacked by wild unparasitized *Sirex*. The techniques used for inoculation are important; early methods of inoculating timber resulted in low, variable or no parasitism, but methods described below regularly produce over 99% parasitism.

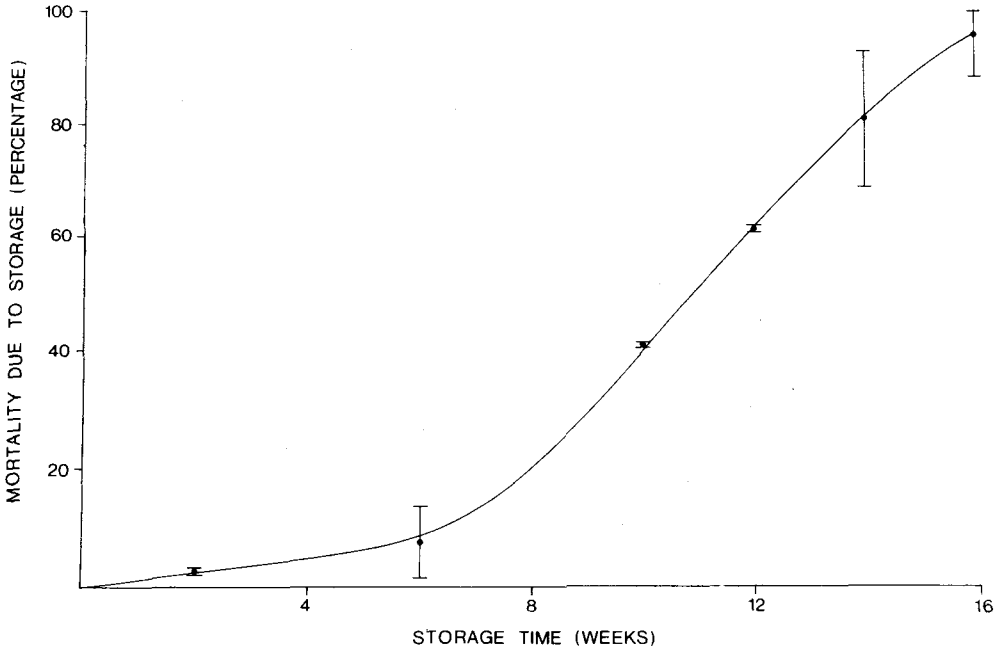

FIG. 1.—Graph of the mortality of *Deladenus siricidicola* stored at 5°C in 150 ml Erlenmeyer flasks each with 10^6 nematodes in 20 ml of tap water and an atmosphere enriched with oxygen.

Inoculation medium

Water suspensions were found to be unsatisfactory for introduction of nematodes into timber because water is rapidly absorbed into the wood leaving nematodes stranded and soon desiccated in inoculation holes, and resulting parasitism of the *Sirex* is low and variable. Most nematodes tend to remain in agar inoculum even after it dries out in inoculation holes, but gelatin based inoculum appears to be ideal.

A 12% solution of gelatin in hot tap water is cooled in a small bucket until beginning to set and is then easily made into a foam with an egg beater. A concentrated suspension of nematodes is thoroughly mixed with the foam (usually 250,000 juveniles per 100 ml gelatin) and the medium is ready for use or can be stored for several hours at 5 to 10°C. Nematode survival in the foam is high because of adequate aeration; the medium is not readily absorbed into wood, and the nematodes rapidly migrate from it into the tracheids.

Preparation of inoculation holes

Drilling holes with a wide variety of bits and speeds proved unsatisfactory. Staining in cotton blue/lactophenol (Goodey 1963) sections taken across such holes revealed that insignificant nematode penetration into the tracheids occurred because tracheal ends were damaged and blocked after drilling. Early mass inoculation of timber was made into cuts across the grain, made by a sharp 2.5 cm chisel. This gave good parasitism but required 2 operators for efficiency, used more inoculum and was more time consuming than the present method.

Mr Victor Gould of Forests Commission, Victoria, designed and manufactures a special tool for nematode inoculation (Figs. 3, 4). This consists of a wad-punch with one side cut away to release wood cores, and a heavy head welded on top. The punch is mounted in a handle and can be used like a hammer to produce inoculation holes with great rapidity. The tool is self sharpening and cuts sufficient tracheal ends cleanly enough to ensure ready access by the nematodes.

Inoculation

Inoculum (averaging 0.8 ml) is introduced into each punch hole using a 50 ml plastic syringe fitted with a 1 cm long narrow polythene tube in place of a hypodermic needle. For maximum efficiency, an operator holds the punch in one hand and syringe and a reserve of inoculum in the other (Fig. 3). After being driven in, the punch is pulled out by the head and inoculum introduced with the other hand immediately afterwards. With practice one operator can inoculate about an hundred m of timber per hour using this technique. The reserve of inoculum consisting of 200 ml in a 15 cm × 20 cm polythene bag is squeezed into the syringe via a small hole cut in one corner. During inoculation, the bag is suspended from the 2 smallest fingers while the syringe is operated by thumb and first 2 fingers.

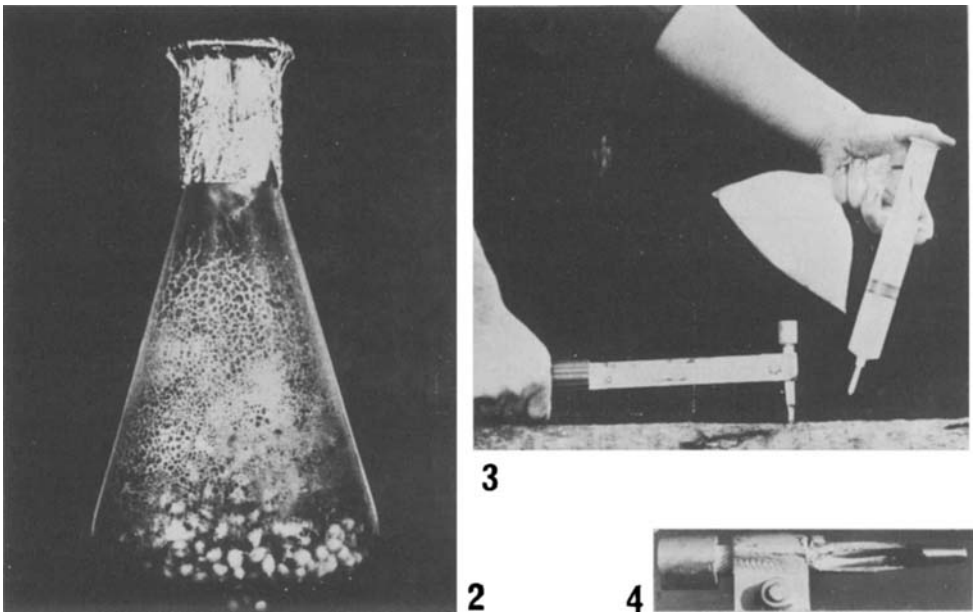

FIG. 2-4.—(2) Culture flask of *Deladenus siricidicola* ready for harvesting. (3) Inoculation of timber with *Deladenus siricidicola* by one operator using a Gould punch and syringe. (4) Head of Gould punch.

Spacing and strength of inoculations and resulting parasitism

Initially inoculations were made at 10 cm intervals on both sides of *Sirex* infested timber, but the following experiments show this density of inoculation to be unnecessarily high; the level of inoculation was in fact detrimental in that smaller parasitized *Sirex* (which fly less far and produce less nematode infected eggs) resulted, probably because of early competition for fungal food between *Sirex* larvae and nematodes (Bedding & Akhurst, in preparation).

In the following experiments (Table 1), 10 randomised, 1 m long, *Sirex* infested billets of similar source, age and moisture content were used for each treatment. The first 2 treatments were made a year later than the others.

It can be seen that excellent and almost identical results were obtained from most treatments but that 1 inoculation per m is inadequate to produce very high levels of parasitism. It is recommended that inoculations of 2000 nematodes (250,000 per 100 ml of inoculum) be made at 30 cm (1 ft) intervals along one side of *Sirex* infested logs or trees.

Field liberations

In 1971, over 1000 1 m *Sirex* infested billets were inoculated with nematodes at Hobart and sent to the Forests Commission, Victoria for distribution in areas of known *Sirex* infestation. Since then, several hundred million nematodes of 5 strains

TABLE 1

EFFECT OF INOCULATION DENSITY ON PARASITISM OF *S. NOCTILIO* BY *D. SIRICIDICOLA* IN BILLETS OF *P. RADIATA*

No. nematodes per inoculation	No. inoculations per billet	No. *Sirex* dissected	No. *Sirex* parasitized	% parasitism
1000	1	529	221	41.8
	1	844	349	41.4
	4	208	206	99.0
	16	337	335	99.4
2000	4	252	251	99.6
	16	287	280	97.6
4000	1	287	226	78.7
	2	165	162	98.2
	4 (2 rows)	335	324	96.7
	8 („)	210	209	99.5
	8 („)	368	366	99.5
	8 („)	144	143	99.3
	16 (4 rows)	275	273	99.3
	32 („)	184	183	99.5
8000	4 (2 rows)	368	366	99.5
	16 („)	144	143	99.3

have been sent to the Commission, which in a major operation, has searched for *Sirex* infested areas and cut down and inoculated selected trees over several thousand hectares of Victoria. Early results from the Commission's investigations indicate establishment of nematodes in most areas treated, with parasitized *Sirex* from original inoculations carrying nematode infection up to 19 km in a single year. Optimal levels of parasitism are not to be expected for several years.

Results from the first experimental liberation of *D. siricidicola,* made in 1970, in a 400 ha pine forest in northern Tasmania, indicate that the nematode may have great potential to control *Sirex*. An estimated 50 parasitized female *Sirex* emerged from inoculated logs at a single point in one corner of the forest and by 1972, 37% of the *Sirex* infested trees in the whole forest, and 92% in the 12 ha compartment of liberation contained nematodes (Bedding and Akhurst, in preparation). Usually over 70% of the *Sirex* emerging from a nematode infested tree are parasitized. In normal control operations, *Sirex* infested timber, inoculated with nematodes, is scattered throughout a forest, and in similar forests results comparable with those in the compartment of liberation might be expected. Forests treated in this manner are being assessed annually for development of nematode parasitism and the *Sirex* population is being monitored by ground and aerial surveys.

Discussion

This is a good example of a full study of the life history and biology of a potential biological control agent leading to developments that are of great practical importance: without knowledge of the mycetophagous phase of the life cycle of *Deladenus*, the mass manipulation of this organism for the biological control of *Sirex* would have been almost impossible. With the exception of *Neoaplectana* spp, no other nematode has been manipulated on a large scale for biological control but, since many other sphaerulariids may have comparable life cycles to *Deladenus* (Bedding 1972b), the possibility exists of similarly manipulating these.

Developments in techniques of inoculation have made it possible to produce readily large supplies of logs in which almost 100% of the *Sirex* are parasitized, an important factor when nematodes have to be released over the very long and rapidly advancing front of *Sirex* infestations. In order to obtain maximum nematode parasitism as early as possible, and hopefully to control new infestations, the greatest possible number of *Sirex* infested trees should be inoculated with nematodes; as about an hundred metres of timber can be inoculated per hour the main limiting factor is now the finding and felling of *Sirex* infested trees.

Almost inevitably *Sirex* infestations will eventually spread to all forests of *Pinus radiata*, at least in eastern Australia, but by the time this occurs, nematode and

insect parasitoids should be well distributed throughout these regions and continued inoculation of *Sirex* infested timber with nematodes and release of parasitoids should not be necessary. However, it could well become normal forestry practice to transfer *Sirex* infested timber from areas known to have high nematode parasitism to fresh outbreaks as these occur since one of the main weaknesses in nematodes as biological control agents is their inability to disperse from one host population to another.

At this stage, the techniques of liberation have been more than adequate, but it remains to be seen whether *D. siricidicola* together with insect parasitoids and changing forestry practices will be able to control *Sirex* sufficiently. In the meantime, 5 new species of *Deladenus* are being screened and selectively reared for biological control of *Sirex*, and there also exists the possibility of releasing the harmless siricid, *Xeris spectrum* (L.) as an alternative host for both nematodes and the insect parasitoids.

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