VG324
Microbial products for biological control of root-knot nematodes

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Know-how for Horticulture™

VG324

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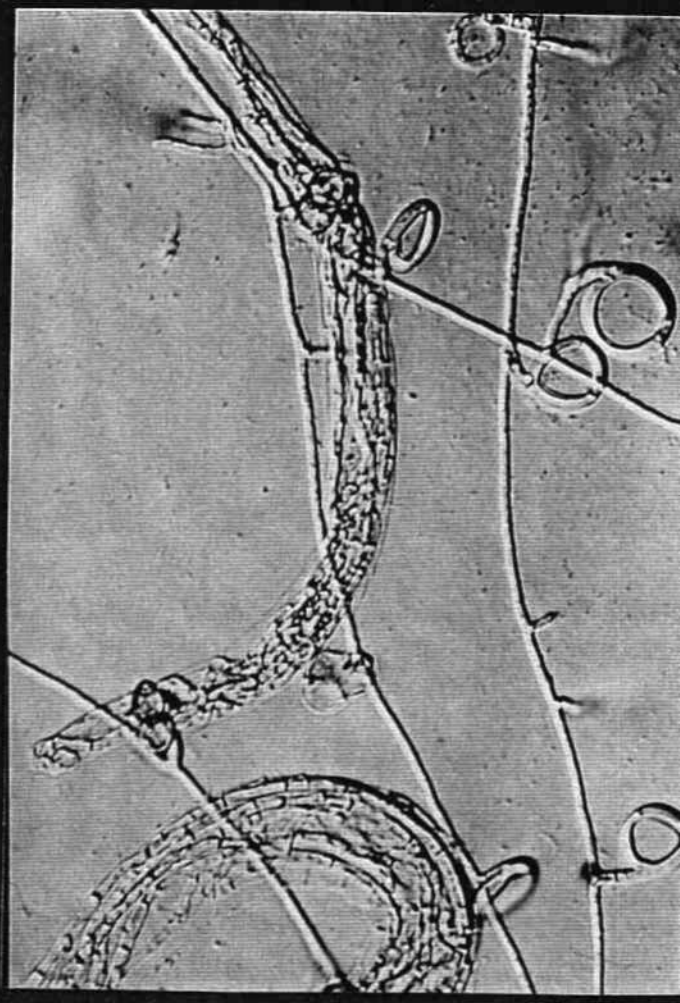
MICROBIAL PRODUCTS FOR BIOLOGICAL CONTROL OF ROOT-KNOT NEMATODE



REPORT FOR THE HORTICULTURAL RESEARCH AND DEVELOPMENT CORPORATION

FINAL REPORT OF PROJECT

PROJECT TITLE: Microbial products for biological control of root-knot nematode

HRDC REFERENCE NO.: VG324

ORGANISATION: Queensland Department of Primary Industries
GPO Box 46, Brisbane Qld 4001

PROJECT CHIEF INVESTIGATOR: Dr G R Stirling

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HORTICULTURAL RESEARCH & DEVELOPMENT CORPORATION
The Research Arm of the Australian Horticultural Industries

DPI QUEENSLAND DEPARTMENT OF PRIMARY INDUSTRIES AGRICULTURAL PRODUCTION

INDUSTRY SUMMARY

Root-knot nematodes cause losses to Australian agriculture that are estimated at more than $100 million per annum. Vegetables are some of the most susceptible crops and soil fumigants and nematicides such as methyl bromide and fenamiphos are widely used, particularly on crops such as tomatoes, potatoes, carrots and cucurbits. Since these chemical control strategies are coming under close scrutiny from health authorities and environmental protection agencies, there is an urgent need to develop alternative nematode control practices. The objective of this project was to test new biological control products for nematodes that had been developed jointly by Queensland Department of Primary Industries and Crop Care Australasia Ltd. These products contained one of two fungi: *Verticillium chlamydosporium*, a parasite which grows into egg masses of root-knot nematode and kills a large percentage of the eggs, and *Arthrobotrys dactyloides*, a fungus which captures nematodes with lasso-like trapping structures.

Granular formulations of the two fungi were tested on tomato in seven field trials at Bundaberg and Stanthorpe, Queensland. Granules were incorporated into soil prior to planting and their effectiveness in reducing damage caused by root-knot nematodes was assessed. The egg-parasitic fungus was ineffective, as egg mortality was not high enough to reduce nematode damage to the current crop. However, results showed that the nematode-trapping fungus gave some nematode control at application rates of 220-440 kg/ha. Control was not as good as with chemicals, because the number of nematodes entering roots was reduced by only 75-80%, compared with 95-99% for fenamiphos, a widely used nematicide.

This research has produced encouraging results, as activity against root-knot nematodes was achieved at application rates far lower than has been achieved elsewhere with other biological products. Although the level of activity is not good enough to commercialise, the results suggest that biological control of root-knot nematode may eventually be feasible. Further improvements in formulations are needed so that levels of activity can be increased and application rates reduced.

TECHNICAL SUMMARY

Research on nematophagous fungi for biological control of root-knot nematode (*Meloidogyne* spp.) commenced in Brisbane in 1991. Initial work funded by Queensland Department of Primary Industries and Crop Care Australasia Ltd aimed to mass produce fungi by liquid fermentation and formulate this biomass into a granular product suitable for use as a biological nematicide. In 1994 this HRDC-funded project commenced with two main aims: 1) to test the efficacy of these products in controlling root-knot nematodes on tomato in the field and 2) to develop molecular technologies for monitoring fungal biocontrol agents following their introduction into soil.

Granular formulations containing *Verticillium chlamydosporium* (a parasite of nematode eggs) and *Arthrobotrys dactyloides* (a fungus which snares nematodes with lasso-like appendages) were applied at 55-880 kg/ha in seven field trials. Granules were incorporated into soil prior to planting tomatoes and their effectiveness in reducing damage caused by root-knot nematodes was compared to a granular formulation of fenamiphos. *V. chlamydosporium* was ineffective but *A. dactyloides* consistently reduced galling during the first two months after planting. At application rates of 220-440 kg/ha, the fungus reduced the number of nematodes invading roots by about 75-80% whereas the chemical nematicide reduced nematodes by 95-99% at rates of 100 kg/ha. These data suggest that biological control of root-knot nematodes is a feasible proposition but that further formulation work will be needed to improve the level of control.

Molecular studies using RAPD and ITS sequence analysis have shown that *A. dactyloides* is genetically diverse. Work is in progress to develop PCR primers that will amplify only specific isolates of *A. dactyloides*. The ITS sequence is also showing that there is potential to develop primers that will differentiate various species of nematode trapping fungi. It will then be possible to use these techniques to confirm the identify of biological control agents that have been recovered from soil using traditional cultural methods. Assays of soil without isolating the nematode-trapping fungi may also be feasible.

PUBLICATION SCHEDULE

Publications arising from initial work on formulation development:

Stirling, G.R. and West, L.M. (1991). Fungal parasites of root-knot nematode eggs from tropical and sub-tropical regions of Australia. *Australasian Plant Pathology* **20:** 149-154.

Galper, S., Eden, L.M., Stirling, G.R. and Smith, L.J. (1995). Simple methods for assessing the predacious activity of nematode-trapping fungi. *Nematologica* **41:** 131-140.

Stirling, G.R., Licastro, K.A., West, L.M. and Smith, L.J. (1997). Progress towards the development of commercial biocontrol formulations containing nematophagous fungi. 1. *Verticillium chlamydosporium. Biological Control* (to be submitted in 1996).

Stirling, G.R., Smith, L.J., Licastro, K.A. and Eden, L.M. (1997). Progress towards the development of commercial biocontrol formulations containing nematophagous fungi. 2. *Arthrobotrys dactyloides* and *Dactylella candida. Biological Control* (to be submitted in 1996).

Eden, L.M., Aitken, E.A.B. and Stirling, G.R. (1997). Morphological measurements and RAPD analysis differentiate *Dactylella nullanulla* sp. nov. from other nematode-trapping species of Dactylella. *Mycological Research* (to be submitted in 1996).

Publications arising from HRDC project VG324.

Stirling, G.R. and Smith, L.J. (1997). Progress towards the development of commercial biocontrol formulations containing nematophagous fungi. 3. Field tests with formulated products containing *Verticillium chlamydosporium* and *Arthrobotrys dactyloides. Biological Control* (to be submitted in 1996).

Eden, L.M. (1994). Diversity within species of the nematode-trapping fungi *Dactylella candida* and *Arthrobotrys dactyloides.* B.Sc. (Honours) thesis, University of Queensland, 67 pp.

Eden, L.M. (1998). Molecular methods for detecting and differentiating nematode-trapping fungi. Ph.D. Thesis, University of Queensland (to be submitted in 1998).

BENEFITS OF PROJECT AND FUTURE DIRECTIONS

Extension/adoption by industry

Results of this work were publicised at a vegetable industry field day at Bundaberg (November, 1995), in a poster at the 1996 National Vegetable and Potato Industry Conference and in articles on integrated nematode management in Good Fruit and Vegetables. Since a commercial product has not been obtained at this stage, further extension work is not yet warranted.

Directions for future research

Although claims of successful biological control of nematodes have been made by some researchers in recent years, there is not yet any documented example anywhere in the world of the commercial use of a biological agent for nematode control. This study was one of the first attempts to formulate nematophagous fungi for biological control purposes and the results were promising enough to warrant research in two areas:

(1) Formulation

The formulations used in this study were relatively crude. Preliminary results with other formulations (e.g. alginate) suggest that substantial improvements in activity could be achieved and this should result in better control being obtained at lower application rates. Research should focus on formulation ingredients that improve viability, procedures for eliminating contamination problems and options for maintenance of fungal viability during the mixing and drying process.

(2) Utilisation of other fungi

The search for potentially useful fungi in this study was not exhaustive and it is likely that other isolates could also be useful. The success obtained with *A. dactyloides* suggests that research should concentrate on relatively slow growing predacious species that produce trapping structures other than three-dimensional networks.

Financial/commercial benefits

The potential market for a good biological nematicide involves millions of dollars, as root-knot nematode is a universal problem on vegetable crops in Australia and overseas. The deregistration of some soil fumigants (e.g. EDB, methyl bromide) will create opportunities for biological products, but several chemical nematicides are also likely to be available. These organophosphate and carbamate nematicides are highly toxic, they are potential groundwater contaminants and they are subject to microbial degradation problems, but they are likely to remain competitors to any biological product for the foreseeable future.

Crop Care Australasia Ltd, the commercial partner in this project, made a decision not to proceed to commercialisation with the biological formulations that were developed in this project. The decision was based mainly on the observation that the level of nematode control

was not as good as could be obtained with fenamiphos. Additional investment in formulation research and on a pilot production plant would also have been needed.

TECHNICAL REPORT

COMPONENT A. FIELD TESTS FOR EFFICACY OF BIOLOGICAL CONTROL PRODUCTS

INTRODUCTION

For many years there has been interest in the use of nematophagous fungi for biological control purposes. However, there have been relatively few attempts to develop formulations of these fungi that are suitable for commercial use. Most mass production systems have involved solid substrate fermentation on materials such as cereal grains or bran (Stirling, 1991) but the low bulk density of the final product means that it is difficult to store and transport and cannot be readily applied using conventional farm machinery. Fermentations based on alginate and other materials have been tested only on a limited scale (Rhoades 1985, Kerry 1988, Cabanillas, 1989).

In two companion papers (Stirling *et al.* 1996a, b) we reported on our attempts to mass produce both egg-parasitic and nematode-trapping fungi by liquid fermentation and convert the fungal biomass into a granular product suitable for commercial use. Experimental evidence was also provided which showed that formulations containing *Verticillium chlamydosporium* and *Arthrobotrys dactyloides* could be produced that were biologically active, the latter fungus consistently reducing the number of eggs and juveniles respectively of *Meloidogyne javanica* Treub (Chitwood) in laboratory microcosms and in glasshouse tests. This paper reports the results of field experiments with these formulations against root-knot nematodes on tomato.

MATERIALS AND METHODS

Assessment of Formulations

Formulations containing *V. chlamydosporium* LS53 and *A. dactyloides* A4 were prepared using standard procedures described elsewhere (Stirling *et al.* 1996) and stored in vacuum sealed plastic bags at 4°C. At the time field trials were established, a sample of the formulation used in each experiment was retained for evaluation in a series of standard laboratory and glasshouse tests. Viability was assessed by placing 30 granules on water agar and scoring for the presence or absence of the fungus after 4 days. At the same time, viable granules were rated for vigour on a 1-5 scale, where 1 = growth only on agar, no hyphae visible on granule; 2 = few hyphae growing from granule, 3 = sparse but even coverage of granule by hyphae; 4 = moderate coverage of granule by hyphae; 5 = profuse mass of hyphae on granule.

The capacity of *V. chlamydosporium* to grow from granules into the soil to which granules were to be added was assessed in a laboratory microcosm. Six granules were placed at marked positions on glass slides sitting in petri dishes. A piece of 100 µm nylon fabric cut to the same size as the slide was placed over the beads and 60g soil was added. The soil was gently compacted, moisture content was adjusted to 2/3 field capacity and then dishes were incubated in a humid chamber at 27°C. After 5 and 10 days, soil and mesh was removed and the amount of hyphal growth from granules was rated according to the number of hyphal strands per granule: 0 = none; 1 = <10; 2 = 11-20; 3 = 21-50; 4 = 51-100; 5 = >100. Parasitic activity was assessed in the glasshouse by incorporating granules into the test soil at 1% w/v, planting a tomato seedling and inoculating it with *M. javanica* eggs and observing egg masses for parasitism 8-11 weeks after inoculation (Stirling *et al.*, 1996a). The effects of parasitism due to any egg parasites occurring naturally in the test soil were removed by subtracting the proportion of parasitised egg masses observed in soil to which no formulation had been added.

Production of traps by *A. dactyloides* in the test soils was confirmed by placing granules at marked positions on a glass slide, covering them with 100 µm mesh and soil, and counting the number of ring traps surrounding each granule after 5 days (Stirling and Mani, 1995; Stirling *et al.* 1996b). Activity of formulations was further assessed by incorporating granules into test soils at 1% w/v and comparing the number of *M. javanica* juveniles recovered from soil microcosms or the number of galls produced on tomato plants in pots with numbers in untreated soil (Stirling *et al.* 1996b).

Details of Field Experimentation

Field experiments were carried out in two vegetable-growing areas in the south-east region of Queensland. Experiments at Bundaberg, a coastal area with a sub-tropical climate, were carried out at all times of the year except for mid summer, while in the cooler, more temperate climate at Stanthorpe, crops were grown only from October to March. Six locations were used but only three soil types were involved: a well aggregated, clay loam soil of volcanic origin (14% sand, 16% silt and 70% clay); a fine sandy loam (85% sand, 7% silt and 10% clay) and a coarse granitic sand (approximately 60% coarse and 30% fine sand). A number of varieties were used, including Floradade, Mutiny, Bermuda and Tornado, and agronomic practices were typical of the Queensland fresh market tomato industry. Transplants were planted 50 cm apart in raised beds approximately 1.8 m apart, beds were mulched with plastic, irrigation water was supplied through trickle tape and the crop was grown on trellises.

In most cases, fields naturally infested with root-knot nematodes were used and initial nematode density (Pi) was determined by extracting nematodes from at least five 200 ml soil samples using a Baermann tray or by counting galls on tomato bioassay plants that had grown in 1 L soil samples for 3-4 weeks. In sites where few nematodes were present, the soil was inoculated with *M. javanica.* Inoculum was prepared by chopping infested tomato roots into pieces and mixing the root material with sand. The inoculum was then sprinkled onto the surface of beds and incorporated to a depth of about 10 cm with a rotary hoe. The number of eggs present in an aliquot of root material was determined by extracting eggs in 1% NaOCl and Pi was estimated by calculating the number of eggs incorporated into a known volume of soil in the bed.

Experimental formulations were generally applied in the field by sprinkling the appropriate amount of granules in a 50 cm band on the surface of the bed and immediately incorporating the product with a rotary hoe to a depth of 10cm. Plastic mulch and irrigation tubing were then laid and beds were irrigated before planting. Some experiments involved applying granules to the planting hole and in these cases, 1-4L soil was removed with a spade, mixed with the appropriate quantity of granules and then returned to the planting hole.

Most experiments involved 5-10 treatments set out in a randomised block design with five replications. Plots generally consisted of 5-10 m row and contained 10-20 plants. Each experiment included a standard nematicide treatment (5g Nemacur 100G (100g/kg fenamiphos)/m row applied in a 50 cm band to the soil surface and incorporated by rotary hoeing) and an untreated control. In experiments where formulations of *A. dactyloides* A4 were evaluated, several of the outer plants in each plot were dug up 4-8 weeks after planting to observe the effects of treatments on early infection. Roots were rated for galling on the 0-10 scale of Zeck (1971). The remaining plants in each plot grew to maturity so that yield data

could be collected (generally 3-5 harvests at weekly intervals). Roots of at least 5 plants in each plot were then rated for galling and in cases where *V. chlamydosporium* treatments were included, a sample of at least ten egg masses from each plot were examined for parasitism and the presence of chlamydospores. Details of each experiment are detailed in Table 1.

RESULTS

Assessment of Formulations

The formulations of *V. chlamydosporium* that were used in field experiments were all 100% viable and granules produced a profuse mass of hyphae when they were plated on water agar. However, relatively few hyphae grew from granules into soil contained within laboratory microcosms and when nematode infested tomatoes were grown in potted soil into which granules had been incorporated, only 11-49% more egg masses were parasitised than in untreated soil (Table 2). Granules of *A. dactyloides* always showed less vigorous growth on agar than those containing V. *chlamydosporium* but were generally 100% viable and showed acceptable vigour. Traps were invariably produced when hyphae grew from granules into the test soil but the number of traps was never very high. However, a high level of nematode control was generally achieved in laboratory and glasshouse tests. Numbers of *M. javanica* juveniles in microcosms were always reduced by more than 90% and three of five batches substantially reduced root galling on potted tomato plants (Table 3).

Results of field experiments

Root-knot nematodes increased to high populations in untreated plots in all trials and at harvest, gall indexes on a 0-10 scale were generally greater than 7 (Tables 4-10). Fenamiphos, the standard nematicide treatment, generally reduced nematode numbers and galling at 4-8 weeks and at harvest but a significant increase in yield from nematicide application was never obtained. Biological treatments also did not affect yield.

Formulations containing *V. chlamydosporium* were applied in experiments 1,2,4 and 7 but treatments in which the fungus was used alone did not reduce either galling or nematode numbers (Tables 4,5,7 and 10). Reductions in root galling at 4-8 weeks were observed with at least one treatment containing *A. dactyloides* in 4 of the 5 experiments in which data was collected at this time (Tables 4,5,6,8 and 9). However, these effects generally disappeared by harvest as significant reductions in galling at this time were only observed in one experiment (Table 9). Populations of root-knot nematode were never reduced by treatment with formulations containing *A. dactyloides*.

In experiment 7, where the effect of organic matter was examined, samples taken 18 days after a mixture of poultry manure and sawdust was applied showed that populations of free living nematodes had increased from 420 to 673 nematodes/200 ml soil in manured plots. This increase was significant ($P = 0.05$). However, the organic treatment did not affect galling, nematode populations or yield when used alone or in combination with formulations containing *V. chlamydosporium* or *A. dactyloides* (Table 10).

In most experiments, a sample of granules was retrieved 10-25 days after application and plated onto water agar to check for the presence of *V. chlamydosporium* or *A. dactyloides*. The appropriate fungus generally grew out of 10-40% of granules. However, in experiment 1, *A. dactyloides* was not detected after 25 days, while in experiment 5, an additional sample at 57 days showed that *A. dactyloides* was present in 44% of granules.

Observations of egg masses collected at harvest from the four experiments in which formulations containing *V. chlamydosporium* were applied showed that this fungus was frequently present in untreated plots and that fungal parasitism was common (Table 11). At these sites, there was no suggestion that pre-plant application of *V. chlamydosporium* increased levels of fungal parasitism. In experiment 4, when natural levels of fungal parasitism were low, virtually no parasitism of eggs was observed in plots receiving *V. chlamydosporium*. In some experiments, egg masses were examined from plants that were removed 4-11 weeks after planting, but the number of egg masses with some parasitised eggs was never greater than 47%.

DISCUSSION

The formulations of *V. chlamydosporium* used in this study showed good viability and vigour on agar but parasitised less than 50% of *Meloidogyne* eggs in glasshouse tests. These relatively low levels of paratisism were sometimes observed during developmental work with these formulations (Stirling et al. 1997) and were commonly seen in samples of first generation egg masses from our field trials. *V. chlamydosporium* therefore did not provide the high levels of egg mortality that are needed 4-8 weeks after planting if nematode multiplication and nematode-induced galling are to be substantially reduced.

Although *V. chlamydosporium* proved to be inadequate as a biological control agent in our experiments, improved activity could perhaps be expected in other situations. Soil temperatures in our glasshouse and field tests generally ranged from 25-35°C, which is generally above the optimum temperature for parasitic activity of other isolates of the fungus (De Leij, 1992). Also, the target nematodes were *Meloidogyne* species that tend to produce egg masses within gall tissue on crops such as tomato. Such egg masses are protected to some extent, from attack from soil and rhizosphere-inhabiting egg parasitic fungi. It is also possible that the absence of chlamydospores in our formulations contributed to their poor activity. Inocula containing *V. chlamydosporium* have been used elsewhere with some success (Kerry et al. 1993), perhaps because of improved survival during the period from planting until the first generations of egg masses are produced.

The relatively poor performance of *V. chlamydosporium* in an environment and cropping system where root-knot nematode is an intractable problem raises questions about whether it has real potential on annual crops. One important consideration is our observation that, whether soil was treated with fungus or not, egg masses collected at harvest were always much more frequently parasitised than those collected 4-11 weeks after planting. Results of glasshouse tests (unpublished data) also showed that levels of parasitism increased as inoculum density increased. *V. chlamydosporium* may therefore be adapted to take advantage of the exponential increase in food supply that becomes available with second and later generations of root-knot nematode. If this hypothesis is true, it is doubtful whether the fungus could ever produce the

levels of egg mortality that would be required to reduce damage from root-knot nematodes on susceptible annual crops.

Laboratory tests with the formulations of *A. dactyloides* used in this study confirmed that they were viable and that the fungus grew from them with moderate vigour when granules were plated on agar. Each formulation also reduced the number of nematodes in laboratory microcosms and reduced galling when incorporated into field soil in the glasshouse. Levels of activity were generally consistent with that obtained in previous studies with similar formulations (Stirling et al. 1997_a).

In the field, formulations containing *A. dactyloides* consistently reduced galling when roots were assessed 4-11 weeks after planting. These reductions were sometimes significant (P = 0.05) and in four out of five experiments involving various application rates there was a trend towards lower galling as application rate increased (Tables 4, 5, 6, 8 and 9). We thereforé believe that formulations had an effect when applied at 40 and 80 g/m row. At a row spacing of 1.8m, this equates to application rates of 220 and 440 kg/ha, which is 2-4 times the registered application rate for granular formulations of fenamiphos. Since most experiments on biological control with the nematode - trapping fungi have been done by applying formulations at more than 2t/ha (Stirling 1991), our results are a significant advance on what has been achieved previously. The results are even more significant when it is considered that most of the weight of the granules is made up of kaolin. Since approximately 10g of dry mycelium is harvested from 1L of fermented biomass, and this biomass is used to produce about 1kg of granules, it is probably more useful to think of application rates in terms of amounts of fungal biomass or as fermentor volumes. Thus, an application rate of 220 kg/ha is equivalent to about 2.2 kg of dried fungal material/ha, and this biomass can be produced in a fermentation volume of about 220L.

A superficial examination of our data might suggest a relatively low level of nematode control, as gall ratings early in the season were only reduced by about one unit on the 0-10 scale of Zeck (1971). However, we have used cellulolytic and pectolytic enzymes to extract nematodes from roots with various gall indexes and our data suggests that this rating scale approximates a geometric series. Thus, indexes of 1, 2, 3, 4 and 5 typically observed 4-8 weeks after planting are approximately equivalent to about 4, 20, 80, 300 and 1200 nematodes/plant respectively. Thus, a reduction in galling of one rating unit is equivalent to about a 75-80% reduction in the number of nematodes entering roots in the period shortly after planting. Although significantly less than the 95-99% reduction achieved with fenamiphos, such data suggest that formulations containing *A. dactyloides* have considerable potential.

Although the performance of *A. dactyloides* in these field studies might appear to be poorer than in the laboratory and glasshouse, differences in application rate must be taken into account. Assuming the formulation is broadcast in a band 50cm wide and is incorporated evenly to a depth of 10cm, application rates of 40, 80 and 160 g/m row are equivalent to 0.8, 1.6 and 3.2g formulation/L soil, whereas standard glasshouse tests were done with application rates of 10g/L soil. When rates of 0.8 and 1.6 g/L were applied to two soils two weeks before tomatoes were transplanted, the number of galls were reduced by 66-85% (Stirling et al. 1997b). This reduction is of the same order of magnitude as that achieved in our field trials.

Our work with *A. dactyloides* was based on the premise that granules containing the fungus could be located around and distal to the roots which grow from transplanted tomato seedlings

and that a protective network of traps could therefore be interposed between the roots and the second-stage juveniles migrating towards them from afar. In such a scenario, application rate and placement of granules could be expected to impact on efficacy and this was observed in some of our experiments. Improved control was obtained as application rate increased, while treatments in which a broadcast application was supplemented by mixing granules with soil immediately surrounding the transplant also had some effect (Tables 5, 8). Assuming formulations contain 300 granules/g and that granules are incorporated evenly in soil, an application rate of 40g/m row is equivalent to 1 granule/4 ml soil and treatments of 10g/L in the transplant holes (ie. 110 kg/ha) involved 3 granules/ml soil. Since hyphae of *A. dactyloides* can radiate at least 2mm from granules in 5 days, the fungus in each granule has the potential to occupy a soil volume of about 0.1ml. It is therefore conceivable that application rates of this magnitude could produce the desired level of nematode control, providing adequate numbers of traps were produced and they remained in place for a reasonable period.

Since cadavers of trapped nematodes provide an energy source from which further growth and trap production can occur (Jaffee et al. 1992), we attempted to extend the length of the predacious phase by increasing populations of free-living nematodes in soil. Addition of organic matter did increase populations of free-living nematodes in the one experiment in which it was used, but control of root-knot nematode was not improved. The relationship between organic matter, free-living nematodes and trapping activity of *A. dactyloides* therefore requires more detailed examination.

Formulation development is one obvious area where biological control activity could be improved. Elimination of contamination problems and maintenance of fungal viability during the mixing and drying process are two areas requiring further research. Our results with alginate granules would also suggest that such formulation work would lead to substantial improvements in activity. The kaolin/gum arabic formulations used in this study produce 1-6 traps per granule whereas more than 100 traps are usually associated with alginate granules (Sudirman, unpublished data). Differences of this magnitude would be expected to have a major impact on efficacy and could result in better control being obtained at lower application rates.

One interesting observation arising from this work was the absence of a yield response to fenamiphos in any of our field experiments, despite the fact that the nematicide consistently reduced galling. This phenomenon is related to the fact that well-managed crops with adequate water and nutrients can cope with severe root damage with little loss in yield. This is particularly true in situations where the crop matures during the coolest months of the year, where trickle irrigated crops are rarely under moisture stress. Such observations suggest that nematicides are often used excessively and provide cause for optimism that the production levels now achieved with nematicides will eventually be achieved with a combination of cultural and biological controls.

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TABLE 1

General details of field experiments with granular formulations of *Arthrobotrys dactyloides* (Ad) and *Verticillium chlamydosporium* (Vc) against *Meloidogyne* spp on tomato

Experiment No.	Location	Pi*	Soil Texture	Date	Soil Temp. (°C) †	Biological Treatments Tested	Comments
1	Stanthorpe	5	Granitic sand	Nov 1994 - Feb 1995	17 - 24	Ad and Vc applied in band, alone or in combination, at two rates	—
2	Stanthorpe	32	Granitic sand	Nov 1994 - Mar 1995	19 - 26	As above, but with additional treatments of Ad in planting hole	—
3	Bundaberg	6	Clay loam	March - May 1995	22 -33	Three rates of Ad applied as a band	Experiment terminated at 8 weeks
4	Bundaberg	6	Clay loam	March - July 1995	22 - 33	Ad and Vc applied as a band alone or in combination	—
5	Bundaberg	9	Clay loam	Aug - Nov 1995	20 - 28	Ad at three application rates, applied in band or in band + planting hole	—
6	Bundaberg	23	Fine sand	Sept - Dec 1995	25 - 33	Ad in band at three application rates	Yield reduced by leaf miner damage
7	Stanthorpe	8	Granitic sand	Nov 1995 - Feb 1996	16 - 24	Ad and Vc alone or in combination with or without organic matter	Yield reduced by hail damage

* *Meloidogyne*/200 ml soil immediately prior to planting.

† Soil temperatures at 7.5 cm depth in the first month after planting.

TABLE 2

Laboratory evaluation of *Verticillium chlamydosporium* formulations used in field experiments.

Formulation No.	Field Expt. No.	Viability (%)	Vigour Rating	Rating of growth from granules into soil	% egg masses parasitised in glasshouse test *
H18	1, 2	100	5.0	1	32
H23	4	100	5.0	2	11
H29	7	100	4.0	-	49

* Difference between levels of parasitism in untreated soil and soil to which a formulation was applied.

TABLE 3

Laboratory evaluation of *Arthrobotrys dactyloides* formulations used in field experiments.

Formulation No.	Field Expt. No.	Viability (%)	Vigour Rating	No. Rings/ Granule	% reduction in nematodes in microcosms	% reduction in galling
H17	1	100	3.0	2.5	91	57
H19	2	97	2.9	6.0	98	98
H21/22	3, 4	100	2.5	1.0	94	89
H26	5,6	100	2.4	6.0	93	89
H28	7	87	2.5	-	-	83

TABLE 4

Effect of granular formulations of *Verticillium chlamydosporium* (Vc) and *Arthrobotrys dactyloides* (Ad) on root-knot nematode on tomato when granules were applied in a 50cm band immediately prior to planting (Experiment 1).

Treatment	Application Rate (g/m row)	Gall Index 6 wk	Gall Index Harvest	Nematodes/200ml soil at harvest*	Yield (kg/plant)
Ad	10	4.4	8.1	1104 (3.04)	4.1
Ad	40	3.7	8.0	1424 (3.15)	4.5
Vc	10	4.8	8.0	1105 (3.04)	5.0
Vc	40	5.0	7.6	1195 (3.08)	4.9
Ad + Vc	10 + 10	4.7	7.0	1195 (3.08)	6.4
Ad + Vc	40 + 40	5.0	7.3	1658 (3.22)	5.1
Fenamiphos		2.2	6.7	1017 (3.01)	6.8
Control	—	5.7	7.6	987 (2.99)	5.4
LSD (P = 0.05)		1.2	0.8	n.s.	n.s.

TABLE 5

Effect of granular formulations of *Verticillium chlamydosporium* (Vc) and *Arthrobotrys dactyloides* (Ad) on root-knot nematode on tomato when granules were applied to the planting hole or in a 40cm band immediately prior to planting (Experiment 2).

Treatment	Application Rate	Gall index			Nematodes/200ml	Yield
	(g/m row)	4 wk	6 wk	Harvest	soil at harvest*	(kg/plant)
Ad	10	7.3	7.5	7.2	3013 (3.48)	3.0
Ad	40	5.8	6.8	7.3	1902 (3.28)	3.5
Ad	10†	6.6	6.8	7.4	2294 (3.36)	3.2
Ad	40†	5.9	6.87	7.0	2161 (3.33)	3.4
Vc	10	7.3	7.2	7.3	3893 (3.59)	3.1
Vc	40	7.5	7.1	7.4	4183 (3.62)	2.9
Ad + Vc	10 + 10	6.9	6.8	7.4	3014 (3.48)	3.0
Ad + Vc	40 + 40	6.2	7.9	7.2	2296 (3.36)	2.9
Fenamiphos		5.6	6.6	7.2	3351 (3.52)	3.3
Control		7.4	7.1	7.5	2768 (3.44)	2.7
LSD (P = 0.05)		0.97	n.s.	n.s.	n.s.	n.s.

* Data presented as equivalent means with transformed means (log x + 1) in parentheses.

† Either 4.5 or 18g granules were mixed with 4L soil from the planting hole. Since plants were 0.45m apart, this was equivalent to 10 and 40g/m row respectively. All other treatments were applied as a band.

TABLE 6

Effect of granular formulations of *Arthrobotrys dactyloides* (Ad) on root-knot nematode populations and galling of tomato when granules were applied in a 50cm band one week prior to planting (Experiment 3)

Treatment	Application rate (g/m row)	Gall index at 8 weeks	Nematodes/200ml soil at 8 weeks*
Ad	20	2.0	48 (1.68)
Ad	40	1.8	125 (2.09)
Ad	80	1.4	182 (2.26)
Ad	160	1.3	18 (1.26)
Fenamiphos		1.0	23 (1.36)
Control		2.5	97 (1.99)
LSD (P = 0.05)		0.95	n.s.

* Data presented as equivalent means with transformed means (log x + 1) in parentheses.

TABLE 7

Effect of granular formulations of *Verticillium chlamydosporium* (Vc) and *Arthrobotrys dactyloides* (Ad) on root-knot nematode on tomato when granules were applied in a 50cm band immediately prior to planting (Experiment 4)

Treatment	Application rate (g/m row)	Gall index at harvest	Nematodes/200ml soil at harvest*	Yield (kg/plant)
Ad	40	7.4	1394 (3.14)	6.8
Vc	40	7.4	1640 (3.21)	6.2
Ad + Vc	40 + 40	7.1	842 (2.92)	6.6
Fenamiphos		5.7	450 (2.70)	7.0
Control		7.8	1493 (3.17)	6.5
LSD (P = 0.05)		1.06	(0.37)	n.s.

* Data presented as equivalent means with transformed means (log x + 1) in parentheses.

TABLE 8

Effect of granular formulations of *Arthrobotrys dactyloides* on root-knot nematode on tomato when granules were applied in a 50cm band or the planting hole immediately prior to planting (Experiment 5)

Treatment		Gall Index		Meloidogyne/200ml soil		Yield
Application rate (g/m row)	Method	7 wk	Harvest	7 wk	Harvest	(kg/plant)
20	Band	2.8	6.6	247 (2.39)	2572 (3.41)	5.7
40	Band	2.6	6.5	191 (2.28)	3488 (3.54)	6.4
80	Band	2.6	6.0	138 (2.14)	1813 (3.26)	5.8
20	Planting hole +	2.3	6.2	139 (2.14)	3626 (3.56)	5.2
20 + 20	Band and planting hole +	2.2	7.0	227 (2.35)	2454 (3.39)	5.6
40 + 20	Band and planting hole +	2.8	6.6	274 (2.44)	2261 (3.35)	6.0
80 + 20	Band and planting hole +	2.2	5.8	170 (2.23)	3595 (3.55)	6.2
Nemacur		0.8	3.3	3 (0.49)	730 (2.86)	5.9
Nil		3.3	6.6	394 (2.59)	3131 (3.49)	5.9
Nil (planting hole)		3.1	5.5	294 (2.47)	3372 (3.53)	7.0
LSD (P = 0.05)		0.78	0.90	(0.56)	(0.33)	n.s.

\+ This treatment consisted of 10g granules applied to 1L soil in the planting hole. Since plants were 0.5m apart, this was equivalent to 20g/m row.

* Data presented as equivalent means with transformed means (log x + 1) in parentheses.

TABLE 9

Effect of granular formulations of *Arthrobotrys dactyloides* (Ad) on root-knot nematode on tomato when granules were applied in a 50cm band 3 weeks prior to planting (Experiment 6)

Treatment	Application Rate (g/m row)	Gall Index		Nematodes/200ml soil		Yield
		7 wk	Harvest	7 wk	Harvest	(kg/plant)
Ad	10	3.2	8.2	440 (2.64)	247 (2.39)	2.3
Ad	20	3.3	8.0	635 (2.80)	326 (2.51)	2.2
Ad	4.0	2.7	7.5	486 (2.68)	186 (2.27)	1.7
Nemacur		1.8	6.2	156 (2.19)	226 (2.35)	2.3
Nil		3.2	8.5	579 (2.76)	156 (2.19)	2.1
LSD (P = 0.05)		0.8	0.61	(0.33)	(n.s)	(n.s.)

* Data presented as equivalent means with transformed means (log x + 1) in parentheses.

TABLE 10

Effect of granular formulations of *Arthrobotrys dactyloides* (Ad), *Verticillium dilanydosporium* (Vc) and poultry manure on root-knot nematode on tomato when granules and manure were applied in a 50cm band 4 days prior to planting (Experiment 7)

Treatment	Application rate (g/m row)	Poultry manure (g/m row)	Gall index		Meloidogyne/200ml soil		Yield (kg/plant)
			11 wks	Harvest	11 wks	Harvest	
Ad	80		5.1	7.0	11 (1.07)	3508 (3.54)	4.5
Ad	80	240	5.5	6.1	26 (1.43)	6293 (3.80)	5.9
Vc	80		5.4	7.2	26 (1.43)	3766 (3.57)	5.0
Vc	80	240	5.8	6.6	19 (1.30)	3149 (3.50)	4.5
Ad + Vc	80+80		5.7	6.2	41 (1.62)	11796 (4.07)	5.7
Ad + Vc	80+80	240	4.3	6.2	20 (1.32)	2063 (3.31)	6.2
Nemacur			4.2	6.6	6 (0.86)	2616 (3.42)	4.1
Control	-		6.4	7.7	30 (1.49)	3370 (3.57)	3.6
Control	-	240	6.1	7.0	15 (1.21)	3904 (3.59)	5.0
LSD (P + 0.05)			n.s	n.s.	(0.39)	(n.s.)	n.s.

* Data presented as equivalent means with transformed means (log x + 1) in parentheses.

TABLE 11

Presence of *Verticillium chlamydosporium* and levels of fungal egg parasitium in *Meloidogyne* egg masses at four field sites in plots either treated with formulation containing *V. chlamydosporium* (Vc) or left untreated (C)

Experiment No.	% egg masses containing chlamydospores		% egg masses parasitised		% egg masses with >75% parasitised eggs	
	C	Vc	C	Vc	C	Vc
1	17	17	77	83	39	37
2	23	27	78	80	47	42
4	0	0	3	5	0	2
7 (11 weeks)	13	14	23	31	23	31
7 (16 weeks)	48	23	65	77	26	35

COMPONENT B. MONITORING OF BIOLOGICAL CONTROL AGENTS

INTRODUCTION

Ecological studies of the nematode-trapping fungi are hampered by the absence of techniques for monitoring these fungi in soil. Thus, it is difficult to determine whether an introduced fungus has established and whether trapping activity proceeds for long enough to achieve nematode control. This work therefore aimed to develop methods for differentiating specific isolates of nematode trapping fungi from the background population that may already exist in soil. Research concentrated on *Arthrobotrys dactyloides*, a fungus which had shown biocontrol potential in previous studies (Section 4).

Initial morphological studies showed that there was little variation within *A. dactyloides*, so that data based on morphology could not be used to distinguish isolates. However, evidence from random amplified polymorphic DNA (RAPD) analyses suggested that the fungus was genetically polymorphic, which meant that there was a good basis for developing molecular diagnostic systems. This work therefore had the following specific objectives :

1. To determine the intra-specific morphological and genetic variation in *D. candida* and *A. dactyloides*;

2. To identify unique genetic regions that differentiate: (*i*) individual isolates within the species *A. dactyloides*, (*ii*) *A. dactyloides* from other species of nematode-trapping fungi, and *(iii)* nematode-trapping fungi from other soil inhabiting species;

3. To develop a polymerase chain reaction (PCR) -based system for the rapid detection identification of: (*i*) individual isolates within the species *A. dactyloides*, and (*ii*) *A. dactyloides* as distinct from other species of nematode-trapping fungi, and (iii) nematode-trapping fungi from other soil inhabiting species;

4. To develop a standard system for assessing the level of intraspecific morphological and genetic diversity within the species *A. dactyloides* and to add all new species to this data base.

MATERIALS AND METHODS

DNA isolation

Isolates were grown in 250 ml flasks containing 100 ml of glucose peptone yeast broth (glucose 15 g l^{-1}, Difco peptone 2 g l^{-1}, Difco yeast 5 g l^{-1}, asparagine 1 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, $MgSO_4.7H_2O$ 0.25 g l^{-1}). Flasks were incubated on an orbital shaker (150 rpm) at 25°C in natural daylight for 5 to 7 days. Mycelia was then recovered by vacuum filtration through Miracloth (Calbiochem Inc.), frozen with liquid nitrogen and stored at -70°C. For the initial variability studies DNA was extracted using a modified method of Yoon *et al.*, 1991, while all subsequent DNA was extracted using a modified method of Raeder and Broda, 1985.

RAPD analysis

DNA was amplified by the PCR procedure in 25 µl reaction mixtures prepared in sterile deionised water. For the initial variability studies, this mixture contained: 4 mM $MgCl_2$; 200

μM each of dATP, dCTP, dGTP, and dTTP (Biotech International); 67 mM Tris-HCl (pH 8.8); 16 mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 5 μg gelatin; 0.3 μM decamer primer (Operon); 1.6 units thermostable *T.th* plus polymerase (Biotech International) and 24 *ng* DNA. This mixture was subsequently modified so that it contained 0.4 μM primer (Operon).

PCR reactions were carried out using a Programmable Thermal Controller PTC-100-96V (M.J. Research Inc.) which performed forty cycles after an initial DNA denaturing step at 94°C for 5 minutes. Each cycle consisted of: 1 minute at 94°C to denature the DNA, 1 minute at 37°C to anneal the primers to the single stranded DNA template and 2 minutes at 72°C to allow the DNA polymerase to extend the new DNA strands. The forty cycles were followed by a final extension step of 72°C for 5 minutes. After the initial variability studies the annealing temperature was altered to 45°C.

Amplification products were loaded into individual wells of 1.5% agarose gels and electrophoresis with 1BE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA) was conducted at 120 V or 100 V for 5 to 6 hours. Following staining in ethidium bromide (1 μg ml^{-1}), the bands of amplified DNA were visualised on a transluminator (UVP hama Repro) and photographed using a polaroid camera.

PCR of rDNA ITS region

DNA was amplified by the PCR procedure in 12.5, 25 or 50 μl reaction mixtures prepared in sterile deionised water. This mixture contained: 4 mM $MgCl_2$; 200 μM each of dATP, dCTP, dGTP, and dTTP (Biotech International); 67 mM Tris-HCl (pH 8.8); 16 mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 2.5, 5 or 10 μg gelatin; 0.3 μM each of primers AB28 and TW81; 0.8, 1.6 or 3.2 units thermostable *T.th* plus polymerase (Biotech International) and 12, 24 or 48 *ng* DNA.

PCR reactions were carried out using a Programmable Thermal Controller PTC-100-96V (M.J. Research Inc.) which performed thirty cycles after an initial DNA denaturing step at 94°C for 5 minutes. Each cycle consisted of: 1 minute at 94°C to denature the DNA, 1 minute at 72°C to anneal the primers to the single stranded DNA template and 2 minutes at 72°C to allow the DNA polymerase to extend the new DNA strands. The thirty cycles were followed by a final extension step of 72°C for 5 minutes. To determine the size and purity of the ITS fragments, 3 μl of amplification products were loaded into individual wells of 0.9% agarose gels and electrophoresis with 0.5 x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA) was conducted at 90 V for 3 - 4 hours. To determine the size of fragments, a 100 bp marker (Pharmacia Biotech) and a pUC 19 */Hpa*II marker (Biotech) were also run on the gels. Following staining in ethidium bromide (1 μg ml^{-1}), the bands of amplified DNA were visualised on a transluminator (UVP hama Repro) and photographed using a polaroid camera.

Restriction analysis of ITS fragments

Restriction analysis was conducted in deionised water in 20 μl reaction volumes containing 10 μl PCR product, BSA (1 mg ml^{-1}), 2 μl buffer specific to the restriction enzyme, and 1μl restriction enzyme. Reactions were incubated overnight at 37°C and both cut (6 μl) and uncut (3μl) products were run on an agarose gel (1.5% agar) in 0.5 x TBE for 4 - 5 hours at

90 V. To determine the size of fragments, a 100 bp marker (Pharmacia Biotech) and a pUC 19 /*Hpa*II marker (Biotech) were also run on the gels.

Sequencing DNA

DNA was sequenced using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems). Reaction mixes, made up to 20 µl with deionised water, contained 300 - 500 *n*g DNA purified in QIAquick (Qiagen) columns, 3.2 *p*mole primer and 8 µl Terminator Premix. PCR reactions were carried out using a Programmable Thermal Controller PTC-100-96V (M.J. Research Inc.) which performed twenty-five cycles after an initial DNA denaturing step at 94°C for 5 minutes. Each cycle consisted of: 1 minute at 94°C to denature the DNA, 1 minute at 55°C to anneal the primers to the single stranded DNA template and 2 minutes at 72°C to allow the DNA polymerase to extend the new DNA strands until their extension is terminated arbitrarily by one of the dye-labelled dideoxy nucleotides.. The twenty-five cycles were followed by a final extension step of 72°C for 5 minutes.

The PCR product was precipitated in 2 µl 3 M Na acetate (pH 4.8) and 50 µl ethanol, centrifuged at 13200 rpm for 30 minutes and the pellet dried under vacuum. Samples were then loaded onto an Applied Biosystems 373A DNA sequencer (Applied Biosystems) and run for 12 hours. The sequencer is able to determine the sequence by analysing the different fluorescence produced by each of the four dye-labelled dideoxy nucleotides which terminate the extended DNA strands.

RESULTS AND DISCUSSION

RAPD analysis to investigate isolate specific sequences

RAPD analysis, based on the PCR, using small primers of random sequence, is a fast, relatively inexpensive, highly sensitive method to locate intraspecific polymorphisms. The strategy employed with RAPD analysis was to screen isolates of *A. dactyloides* with a large number of primers to locate polymorphisms unique to isolate A4. Polymorphic DNA, when sequenced would allow the design of PCR primers capable of amplifying a section of the genome present only in isolate A4.

RAPD conditions were optimised to obtain optimum reproducibility and clarity. Two conditions were altered: 1. the annealing temperature was raised from 37°C to 45°C, thus increasing stringency, and 2. the concentration of the 10 base primer was increased from 1.5 µl to 2.0 µl per 25 µl reaction mix. These changes resulted in agarose gels which showed a greater number of bands of increased sharpness.

Using these conditions, a range of decamer primers of random design were used to screen isolate A4 against isolates D2 and CBS 264.83. These two isolates were chosen because RAPD analysis conducted during the initial variability studies indicated that they were the closest genetically to isolate A4. A band present in isolate A4, but absent in both these isolates would be less likely to be present in other, less genetically similar isolates.

Thirty primers were screened and three of these produced bands unique to isolate A4. However, when the screening was extended to include all the isolates from the initial study and new isolates from Queensland soil, only primer OPH-07 produced a band unique to isolate A4. This band comprised DNA of 1700 bp length. DNA from this unique band was extracted, purified using QIAquick Spin columns (Qiagen Inc.) and re-amplified in further rounds of PCR using primer OPH-07. The aim was to increase the quantity and purity of the target DNA by further PCR amplifications and agarose gel separations. However, while this procedure resulted in the re-amplification of the 1700 bp DNA fragment, it also amplified many smaller fragments. This suggested the presence of internal priming sites or smaller fragments of DNA trapped in the gel matrix by the larger fragments.

Further studies were conducted altering gel consistency and gel running voltage to optimise conditions for the efficient separation of smaller fragments of DNA. PCR amplification conditions were altered, further increasing stringency, to prevent non-specific primer binding to the 1700 bp DNA template. However, these procedures were unsuccessful, suggesting even more convincingly the presence of internal priming sites.

Due to the presence of internal priming sites it was not possible to sequence the fragment directly using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit with OPH-07 as the primer. Therefore, the insert will be ligated into the pGEM®-T vector (Promega Corp) and transformed into *Eschericheria coli* JM109 competent cells (Promega Corp). Sequencing can then be conducted using the pUC/M13 sequencing primers which bind to the pGEM®-T vector on either side of the 1700 bp insert.

ITS analysis

Ribosomal DNA (rDNA) contains both highly conserved and variable regions. The highly conserved regions have evolved slowly and have been well characterised. Therefore "universal" PCR primers have been designed which will amplify DNA from a wide range of organisms. At the same time, the internal transcribed spacer (ITS) region of these genes are non-coding sections and therefore have been able to evolve at a faster rate. This may result in sequence variations between related species and within a population of a species.

Primers AB28 and TW81 successfully amplified a section of the rDNA of *Phytophthora* spp. which contained ITS1 and ITS2 flanking the 5.8S coding section. Using these primers, a 600 bp fragment was produced for the QDPI isolate *A. dactyloides*. A "ghost" band at 1200 bp was present on the agarose gels used to visualise the ITS fragment, suggesting self-priming of the 600 bp fragments due either to depletion of the primers or inadequate stringency. Reducing the number of cycles from 35 to 30 and increasing the annealing temperature from 65°C to 72°C eliminated the "ghost" band.

The 600 bp fragment was amplified for all isolates of *A. dactyloides*, an isolate of *A. brochopaga* (which also produces constricting rings to trap nematodes), four isolates of *Dactylella candida* and four isolates of another *Dactylella* species. However, the ITS fragment amplified for other *Arthrobotrys* species and three isolates of *Monacrosporium cionopagum* varied in size between 600 bp and 700 bp.

Restriction analysis on the ITS fragment was conducted to determine the degree of variation within isolates of *A. dactyloides* and between *A. dactyloides* and other species of nematode-

trapping fungi. Using two restriction enzymes, *Hae* III and *Hpa* II, that recognise and cut DNA at particular 4 bp sequences, inter-specific and intra-specific polymorphisms were found. Subsequent sequence analysis was conducted with the aim of designing PCR primers able to distinguish the QDPI isolate of *A. dactyloides* from other isolates of *A. dactyloides* and *A. dactyloides* from other species of nematode trapping fungi. Initially, sequencing reactions used primers specific to *Phytophthora* species rDNA conserved regions: S1, S2, S4 and S6. However, although some results were obtained, the primers were not specific enough to give reliable results. For all primers except S2, results often gave a weak signal, had overlapping sequences or gave a low signal/noise ratio, indicating that the primer sequences were not specific enough.

New primers NTF-S1, NTF-S6 and NTF2 were designed using sequence information obtained from this initial sequencing. NTF-S1 and *Phytophthora* S2 were used to sequence one strand while NTF2 and NTF-S6 were used for the opposite strand. Sequence information corresponding in both directions was required for the sequence to be considered reliably confirmed. The ITS regions of four isolates were sequenced: isolate A4, isolate LE4, a Queensland isolate which displayed RAPD patterns similar to A4, isolate LS71, a Queensland isolate which displayed RAPD patterns dissimilar to A4 and CBS 218.61 (1887), an isolate of *A. brochopaga* which also produces constricting rings. The results (Figure 1) indicated that regions with enough variability are present to allow primers to be designed that will differentiate between *A. dactyloides* and *A. brochopaga*. However, since A4, LE4 and LS71 are all Queensland isolates, other isolates of *A. dactyloides* from international culture collections, CBS 264.83, ATCC 15595 (the type species) and ARS 3733, will also be sequenced to determine whether the variation observed is due to geographical distance.

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Figure 1: Sequenced *A. dactyloides* ITS Strands with bases differing from isolate A4 marked in bold. Isolates LE4 and LS71 are isolates of *A. dactyloides*. Isolate CBS 218.61 is an isolate of *A. brochopaga*

1	**A4**	CGTTTCCGTA	GGTGAACCTG	CGGAAGGATC	ATTACCAAAA
	LE4		GGTGAACCTG	CGGAAGGATC	ATTACCAAAA
	LS71	CGTA	GGTGAACCTG	CGGAAGGATC	ATTACCAAAA
	LBS 218.61	CGTTTCCGTA	GGTGAACCTG	CGGAAGGATC	ATTACCAAAA
41	**A4**	CAAAGCCAAC	TTGGCCTTCG	GGTCTTGAGA	GCTTCAACCT
	LE4	CAAAGC**G**AAC	TTGGCC**CTAT**	GG**C**CTTGAGA	GCTTCAACCT
	LS71	CAAAGC**G**AAC	TTGGCCTCC**A**	GGTCTTGAGA	GCTTCAACCT
	LBS 218.61	CA**G**AGCA**A**TC	TCGGC-TTCG	G-CC**C**TGAAA	GCTTCAACCT
81	**A4**	TTTGTGAACC	AAACCTTTAT	TTTCGCTTCG	GCAGCAATGG
	LE4	TTTGTGAACC	AAACCTTTAT	TTTCGCTTCG	GCAGCAATGG
	LS71	C**C**TGTGAACC	AAACCTTTAT	TTTCGCTTCG	GCAGCA**G**TGG
	LBS 218.61	TTTGTGAACC	AAACCTTT**C**T	TTTCGCTTCG	GCAGCA**GC**GG
121	**A4**	C—TTCCGCCAT	GTCAGCCTGC	CGTTAGCACC	CAACCAAAAC
	LE4	C—TTCTGCCAT	GTCAGCCTGC	CGTTAGCACC	CAACCAAAAC
	LS71	C—TTC**T**G**T**CAT	GTCAGCCTGC	CGTTAGCACC	**A**AACCAAAC
	LBS.61	C**GATCCCGCCGC**	GTCAGCCTGC	CG**C**TAGCACC	C**TTG**-AAAAC
161	**A4**	TTGCAGTATC	TCATGTCTGA	AATCAAATTT	T-TGAATTAAA
	LE4	TTGCAGTATC	TCATGTCTGA	AATCAAATTT	T-TGAATTAAA
	LS71	TTGCAGTATC	TCATGTCTGA	AATCAAATTT	T-TGAAT**C**AAA
	LBS.61	TTGC**T**GTATC	TCATGTCTGA	A**C**A**C**GAATA**T**	**TTT**GAATT**C**AA
201	**A4**	TCAAAACTTT	CAACAACGGA	TCTCTTGGTT	CCCGCATCGA
	LE4	TCAAAACTTT	CAACAACGGA	TCTCTTGGTT	CCCGCATCGA
	LS71	TCAAAACTTT	CAACAACGGA	TCTCTTGGTT	CCCGCATCGA
	LBS.61	TCAAAACTTT	CAACAACGGA	TCTCTTGGTT	CCCGCATCGA
241	**A4**	TGAAGAACGC	AGCGAAACGC	GATAGTTAAT	GTGAATTGCA
	LE4	TGAAGAACGC	AGCGAAACGC	GATAGTTAAT	GTGAATTGCA
	LS71	TGAAGAACGC	AGCGAAACGC	GATAGTTAAT	GTGAATTGCA
	LBS 218.61	TGAAGAACGC	AGCGAAACGC	GATAGTTAAT	GTGAATTGCA
281	**A4**	GAATTCAGTG	AATCATCGAG	TCTTTGAACG	CATATTGCGC
	LE4	GAATTCAGTG	AATCATCGAG	TCTTTGAACG	CATATTGCGC
	LS71	GAATTCAGTG	AATCATCGAG	TCTTTGAACG	CATATTGCGC
	LBS 218.61	GAATTCAGTG	AATCATCGAG	TCTTTGAACG	CATATTGCGC
321	**A4**	CCATTGGTAT	TCCATTGGGC	ATGTCTGTTT	GAGCGTCATT
	LE4	CCATTGGTAT	TCCATTGGGC	ATGTCTGTTT	GAGCGTCATT
	LS71	CCATTGGTAT	TCCATTGGGC	ATGTCTGTTT	GAGCGTCATT
	LBS 218.61	CCATTGGTAT	TCCATTGGGC	ATGTCTGTTT	GAGCGTCATT

361	**A4**	TCAAACCCTC	GACTTAGGTC	GGTTTTGAGC	TGGCTTAACG
	LE4	TCAAACCCTC	GACTTAGGTC	GGTTTTGAGC	TGGCTTAACCG
	LS71	ACAAACCTC	GACCCAGGTC	GGTTTTGAGC	TGGCTTAACG
	LBS 218.61	TCAAACCCTC	GACTTTGGTC	GGTATTGAGC	TGGCTTTGCG
401	**A4**	GGTGCAAACC	CAAGCCGGTT	TTAAAGTTGT	AGGCTTTGCT
	LE4	GGTGCAAACC	CAAGCCGGTT	TTAAAGTTGT	AGGCTTTGCT
	LS71	GGTGCAAACC	CAAGCCGGTT	TTAAAGTTGT	AGGCTTTGCT
	LBS 218.61	GGTGCGAACC	CAGGCCGGTT	TTAAAGTGGT	AGGCTTTGCT
441	**A4**	GACAGCTGCT	CCAAACCAAA	AACATAGTAA	AACATACTTG
	LE4	GACAGCTGCT	CCAAACCAAA	AACATAGTAA	AACATACTTG
	LS71	GACAGCTGCT	CCAAACCAAA	AACATAGTAA	AACATACTTG
	LBS 218.61	GTCTGCTGCT	CCAAACCAAA	AACATAGTAA	ACTGTACTTG
481	**A4**	TTCTAGGTGG	GGTGGTTCCA	GCCTTGAAAA	TGACAC-TTGT
	LE4	TTCTAGGTGG	GGTGGTTCCA	GCCTTGAAAA	TGACAC-TTGT
	LS71	TTCTAGGTGG	GGTGGTTCCA	GCCTTGAAAA	TGACAC-TTGT
	LBS 218.61	TTGATGGTGG	GGTGGCTCCA	GCCTTGAAAA	TGACACTTTGT
521	**A4**	GGTTTGACCT	CAGATCAGAT	AAGGATACCC	GCTGAACTTA
	LE4	GGTTTGACCT	CAGATCAGAT	AAGGATACCC	GCTGAACTTA
	LS71	GGTTTGACCT	CAGATCAGAT	AAGGATACCC	GCTGAACTTA
	LBS 218.61	GGTTTGACCT	CAGATCAGAC	AAGGATACCC	GCTGAACTTA
561	**A4**	AGCAT			
	LE4	AGCATATG			
	LS71	AGCATATGAT	C		
	LBS 218.61	AGCATATGAT	C		