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CSIRO REPORT - 1993/4

NEMATODES TO CONTROL SIREX

Research has centred mainly around problems associated with use of the Kamona strain to replace the "Old" or "Defective" strain of *Deladenus siricidicola*, it already having been demonstrated that the Kamona strain gives much higher levels of parasitism at least in inoculated trap trees.

Nematode culture

It was the continual sub-culturing of *D. siricidicola* over many years in the fungal feeding cycle without intervention of the cycle parasitic in sirex that led to the nematode's decline in infectivity. Likewise this continual sub-culturing led to much improved yields in artificial fungal cultures so that Bedding and Akhurst in 1974 were able to report average yields of 15 million nematodes (198 strain) per 500ml wheat culture flasks. However, in re-isolating this strain (now called the Kamona strain) from where it was originally liberated in 1971, it was found to be, just like the wild type, much more difficult to rear and in fact gave average yields of fewer than 1 million per 500ml wheat flask. Artificial selection for better yields would doubtless result, once again, in decline of infectivity so that other means of improving yields from mass rearing the Kamona strain had to be investigated.

Nematode/fungal balance in plate culture

In artificial culture, *D. siricidicola* will feed and reproduce readily only on the growing front of the symbiotic fungus *Amylostereum chailletii*. If this fungal front advances too rapidly through the culture, nematode breeding cannot keep up with it and yields are negligible. The nematode population can by itself hold back the advancing front if there are adequate nematode numbers. With some strains of *D. siricidicola* such as the defective strain, too many nematodes can completely stop the fungal advance but this never occurs with the Kamona strain.

One problem with the Kamona strain is that reproduction and feeding is so slow that it is not usually possible for there to be more than one generation on the starter plates before the plate is covered with fungus particularly when the nematodes have just been established from liquid nitrogen storage. This problem has been attacked in a number of ways but none have proved entirely satisfactory.

- Fungal nutrient was reduced by using ½ strength potato dextrose agar (now adopted) and various lesser strengths were tested but found inadequate except for establishing cultures directly from liquid nitrogen

- Various other agars were tested but proved unsatisfactory. Low levels of lactic and citric acid are currently being tested since they may suppress fungal growth without affecting nematode reproduction

- Various methods of narrowing the fungal front by sterile removal of areas of agar to channel fungal growth through narrow necks were tested and found initially successful but subsequent culturing saw nematode densities decrease to previous levels

- A stainless steel ring was inserted into the agar surface of each plate and inoculum of nematodes + fungus placed within the ring. As each ring filled with fungus it was replaced by a sequence of larger rings. This method held back the fungal front sufficiently to allow increase of nematode density but again subsequent culturing saw nematode densities decrease to previous levels. Adding surface sterilised nematodes eggs to fungus growing within the ring produced no improvement.

- The strain (198) from which Kamona was derived , came from Sopron in Hungary so climatic data were sought for this region to ascertain average and ranges of temperatures. These were a little below the 23°C that Kamona has routinely been cultured. Therefore, nematodes were test-cultured at various temperatures (12°C, 15°C, 18°C, 21°C, 23°C) in the hope that one temperature would favour nematode reproduction more than fungal growth. However, this produced no significant improvement since fungal and nematode growth appeared to be equally affected.

- The simple expedient of reversing the inoculum so that the youngest region of the inoculum faced towards the petridish wall unexpectedly resulted in some improvement

- Current trials involve sterilely extracting nematodes from plates and wheat flasks and concentration by filtration before adding to culture plates to supplement the original nematode population. This process is particularly laborious but may be useful to initiate dense populations which can then be sub-cultured in the usual manner

Mass rearing

Attempts to ameliorate the very low yields achieved with the Kamona strain of *D. siricidicola* have involved changing conditions within 500 ml flask cultures and increasing the size of culture containers and hence quantity of medium.

Within flasks the following have been tested:

- Size of inoculum had little effect when in excess of a minimal level

- Oxygen permeability of flasks (normal narrow necked flasks vs wide necked) resulted in negligible reduction of O_2 and accumulation of CO_2 in both types of flask and yields were therefore not significantly different.

- Testing various grain and legume seeds and combinations of these to replace wheat showed that none of these except rice was as good as the standard wheat flask. However, a combination of 50% wheat and 50% brown rice resulted in a more than doubling of yields. Thus :

  600 flasks of standard wheat had an average yield of 718,000 (0.54 - 1 million)
  120 flasks of 50:50 rice/wheat had an average yield of 1.9 million (1.6 - 2.6 m)

Larger culture vessels

In an effort to reduce labour input/yield, two types of container have been tested Firstly, work was continued on the use of CSIRO patented self-aerating, sterility-maintaining trays which work well for the different entomopathogenic nematodes. For *D. siricidicola* culture, each tray contained 5 kilo of autoclaved wheat (as compared to 250 g in a flask) and a wide range of methods for sterilely inoculating this were explored. However, a major problem remained that most trays eventually succumbed to infections of a sporulating *Bacillus* species which had obviously survived autoclaving because of the larger mass involved. Longer autoclave times were tested, but this resulted in the grain losing its integrity becoming fused so that oxygen penetration was minimal. Recently, it has been found that if the grain is autoclaved dry for 90 minutes and then water is added while the grain is still hot and then the whole autoclaved again for a further 45 minutes, there is no survival of sporulating bacteria and the grain maintains its integrity.

Nevertheless, these trays are still cumbersome to sterilely inoculate so that 5 litre polypropylene Nalgene bottles with surgical cloth filters inserted into the lids are now being tested. These hold only 2 kilo of autoclaved grain but are relatively easy to inoculate within a laminar flow cabinet and are held horizontally during incubation. To date, monoxenic cultures have been successfully established in these bottles.

Factors affecting infectivity

It is important to discover the exact mechanisms involved in determining what causes juveniles to become either infective or fungal feeding nematodes because it is believed that this plays a large part in influencing levels of parasitism of sirex in the field. In order to test nematodes from the field to determine whether they are adequately infective it is important to determine whether plate tests accurately mirror what happens in the field. A combination of 0.2% lactic acid and high levels of CO_2 had earlier been shown to be important and results shown in "acids.xls" show that it is the pH that is important rather than any other attribute of lactic acid.

Further to this, it was expected that frass from within 10mm of sirex larvae would produce these low levels of pH, but incorporating frass into cultures did not induce infective female production (frass.xls). This is possibly because not all the frass used was fresh and the high water level in the agar plates, compared to that in the frass diluted out any acids to be found there. Uric acid, which is a common component of insect frass did not induce infective production in the absence of high levels of CO_2 but has not yet been tested in combination with CO_2.

Cross-breeding Defective with Kamona strains

In introducing the Kamona strain into the field where the defective strain is already present, populations of cross breeds between these two strains are to be expected. Early results indicated that crosses were more like the Defective than the Kamona strain.

Large numbers of single pair matings between Kamona females and Defective males, and between Kamona males and Defective females were set up and then allowed to breed through many generations. Because of the difficulties of starting from single parents, many of these crosses were lost. Others were well established and some of these tested as shown in "Crosses.xls". These tests indicated that at least on plates, crosses were inevitably much more similar to the Defective than Kamona strain and if the plate results are born out in the field this result is worrying indeed.

Inoculation of timber

Billets exposed to nematode free sirex provided by KTRI which had been injected with various nematode strains, crosses and methods were appropriately caged and periodically checked. Unfortunately only a few sirex emerged from these billets rendering the extensive experiments useless.

During late July 1994, Woods and Forests kindly provided 100 logs from Kangaroo Island which chipping indicated to be nematode free. These have been divided into 300 billets of 700 ml length, end coated with wax and using random numbers allotted each treatment. One billet from each of the 100 original logs was kept as a control. Each of the strains indicated below were bred up in wheat flasks over the same time period and typical flasks of each were selected for the experiment.

The treatments were as follows:

Kamona
Defective
Kamona male X Defective female 1
Kamona male X Defective female 2

Infectivity experiments - Kamona strain

12% CO2+ various acids — All acids 0.2% pH 4

Acid	Fungal F. Females	Infective females	%infectives	Av.%infs.
Malic+CO2	100	56	35	
	88	112	56	43
	98	62	38	
Malic Control	100	0	0	
	100	0	0	0
	100	0	0	
Citric+CO2	50	48	49	
	60	100	62	56
	70	93	57	
Citric Control	100	2	2	
	97	2	2	2
	100	1	1	
Lactic+CO2	100	82	45	
	40	100	71	54
	96	86	47	
Lactic Control	100	2	2	
	100	8	7	4
	100	3	3	
0.5 PDA+CO2	100	1	1	
	100	52	34	26 (35)
	100	46	32	
	100	61	38	
0.5PDA Control	100	0	0	
	100	0	0	0
	100	0	0	
	100	0	0	

Infectivity test using frass/agar and 12%co2.

Frass collected from sirex infested logs,all frass samples gathered a d approx. 10mm from insect.
Frass was ground into a fine powder by freezing in liquid nitrogen then grinding with a pestle and mortar.This powder then incorporated with 0.5 strength PDA and autoclaved.

	F.F.F.	Inf.fems.
1	100	28
2	100	30
3	100	24
4	100	18
5	100	52
	Control plates,0.5 PD	A and 12% co2.
1	100	51
2	100	63
3	100	60
4	100	48
5	100	39

* pH of agar and frass measured at 4.5

Experiment to examine individual crosses of Kamona strain and Old/Defective strain using 0.2% lactic acid and 12%CO2 and any differences in rates of infectivity.

28.2.94

Cross		E.L.F.	Infectives	%Infective
K(f) O(m)	E	100	0	0
		100	7	6.5
		100	20	16.6
		100	18	15.2
		100	11	9.9
K(f) O(m)	D	100	14	12.2
		100	24	19.4
		100	12	10.7
		100	30	23
		100	28	21.9
O(f) K(m)	D	100	1	0.99
		100	1	0.99
		100	4	3.8
		100	2	1.9
		100	6	5.6
O(f) K(m)	K	100	11	9.9
		100	8	7.4
		100	16	13.8
		100	11	9.9
		100	19	15.9
Control Plates				
Kamona (K94)		32	100	75.8
		42	100	70.4
		10	100	90.9
		48	100	67.6
		51	100	66.2
Old/Defective		100	13	11.5
		100	2	1.9
		100	12	10.7
		100	2	1.9
		100	1	0.99

Kamona female X Defective male 1
Kamona female X Defective male 2
K X D backcross to Kamona
Lady Barron
Wingello
Colac
APM
Kamona in polyacrylamide gel

With the strain inoculations it will be important to determine whether these mirror results on plates particularly with regard to Kamona X Defective resulting in poor infectivity.

The polyacrylamide gel is obviously more simple to use than aerated gelatine and should this prove to give levels of parasitism at least equal to the normal inoculation method, its use will be recommended in the field.

Know by Feb/Mar if this works

Mass Rearing of *D. siricidicola* for liberation

One vial of Kamona was thawed from liquid nitrogen on 30 November 1993. The nematodes were placed onto agar plates to initiate the culture for the 1994 mass rearing season. The nematodes have been subcultured on ½ strength PDA fortnightly since and then into wheat flasks as required by advance orders. Orders were received as follows:

Forestry Commission of NSW

		Delivery Dates
Bathurst	305million	26/4-7/6
Oberon	340	12/4-31/5
Bombala	90	3/5-17/5
Queanbeyan	31.5	3/5-17/5
Moss Vale	20	26/4
Tumbarumba	25	3/5-10/5
Tumut	39	19/4-26/4

Total for these areas **850 million.**

Primary Industry Forestry (SA)

Mt.Gambier	39 million	14/6-8/8
Kuitpo	80	31/5-7/6

Total for these areas **119 million.**

ACT Forests

Total for this area **110 million** 17/5-1/8

Private orders

Timberland Oberon	90	12/4-17/5
FJ Hanrahan Oberon	20	26/4
Timberland F. Baker	5	27/6
Treecorp Kangaroo Island	30	11/7
Anon Oberon	35	4/7
Smorgen Colac	50	25/7
Seas Sapfor Mt Gambier	10	8/8
Furneaux Forests Flinders Is.	10	22/8
Total	**250 million**	

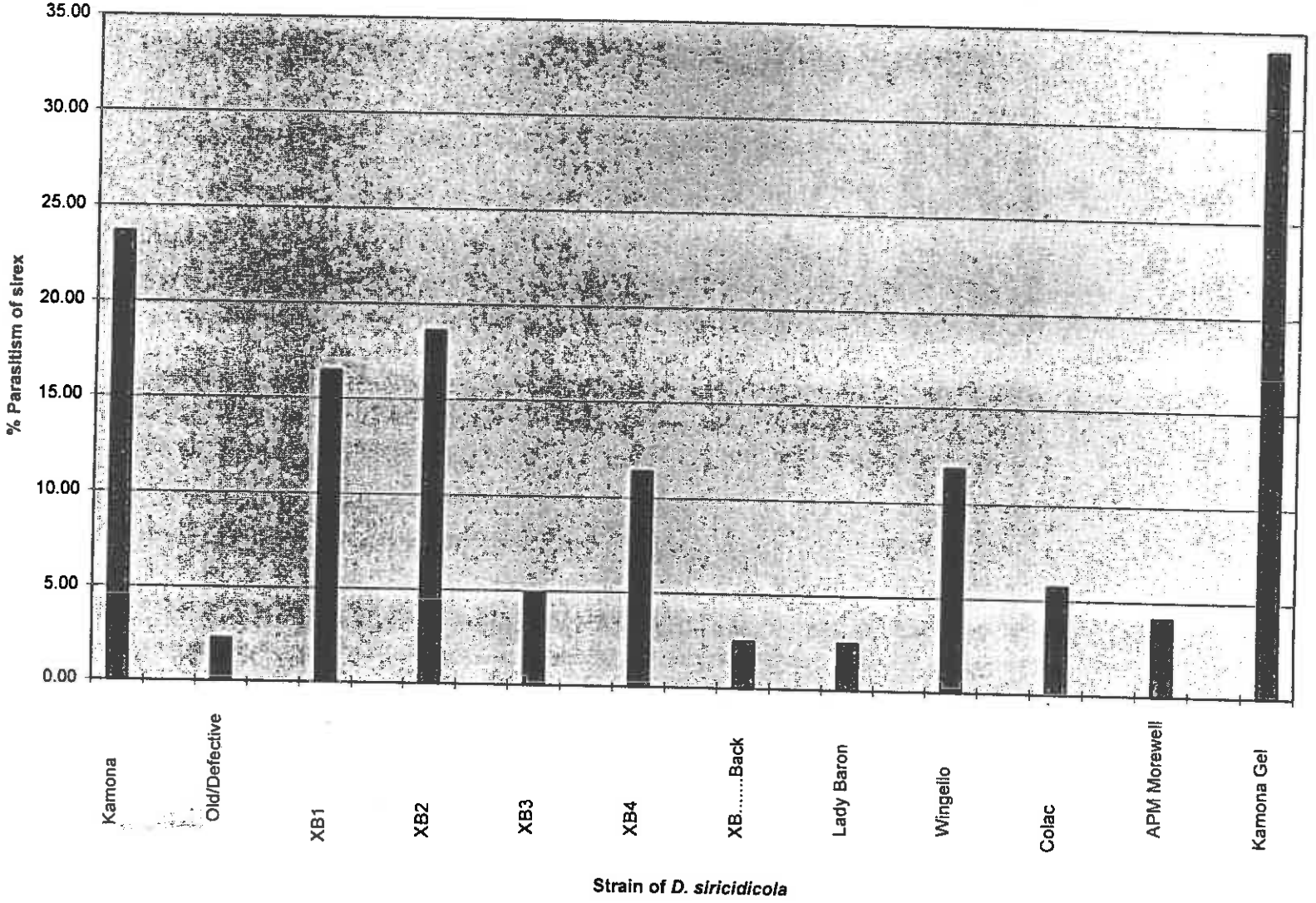
Total number for season (all areas) 1329 million

Initially, 1,000 flasks were set up using the normal wheat rearing medium. As the harvesting phase progressed yields were consistently below 1 million per flask and it proved necessary to set up 800 extra flasks to provide enough nematodes to fulfil all orders.

Nematodes were dispatched in heat sealed plastic sachets in quantities convenient to each customer.

All shipping was carried out by *TNT Air Cargo,* with only one late delivery in 34 shipments.

Comparison of *D. siricidicola* strains inoculated into sirex infested billets



Comparison of *Deladenus siricidicola* strains for parasitism of sirex

	Pos		Neg		% Para	Total	Adults		Av No/Par. in		Av /Tot.ins.	
Strain ID	m	f	m	f		% Para	m	f	m	f	m	f
Kamona	20	10	31	19	37.50	23.70	275	82	13.75	8.20	5.39	2.83
	1	1	40	13	3.64		29	4	29.00	4.00	0.71	0.29
Old/Defective	1	1	81	33	1.72	2.29	3	4	3.00	4.00	0.04	0.12
	3	0	69	30	2.94		15	0	5.00	#####	0.21	0.00
XB1	8	4	77	29	10.17	16.48	81	51	10.13	12.75	0.95	1.55
	9	9	29	17	28.13		88	51	9.78	5.67	2.32	1.96
XB2	6	0	29	3	15.79	18.67	130	0	21.67	#####	3.71	0.00
	13	12	58	45	19.53		40	27	3.08	2.25	0.56	0.47
XB3	3	1	57	33	4.26	4.96	82	3	27.33	3.00	1.37	0.09
	2	0	15	10	7.41		66	0	33.00	#####	3.88	0.00
XB4	0	2	24	16	4.76	11.43	0	5	#####	2.50	0.00	0.28
	4	6	35	18	15.87		36	15	9.00	2.50	0.92	0.63
XB.....Back	1	1	17	16	5.71	2.53	2	23	2.00	23.00	0.11	1.35
	0	0	29	15	0.00		0	0	#####	#####	0.00	0.00
Lady Baron	1	1	36	19	3.51	2.53	2	2	2.00	2.00	0.05	0.10
	0	0	19	3	0.00		0	0	#####	#####	0.00	0.00
Wingello	2	1	9	2	21.43	11.90	28	5	14.00	5.00	2.55	1.67
	2	0	20	6	7.14		11	0	5.50	#####	0.50	0.00
Colac	2	0	94	23	1.68	5.76	20	0	10.00	#####	0.21	0.00
	7	2	39	24	12.50		47	4	6.71	2.00	1.02	0.15
APM Morewell	0	2	44	17	3.17	4.17	0	2	#####	1.00	0.00	0.11
	1	2	39	15	5.26		6	6	6.00	3.00	0.15	0.35
Kamona Gel	5	8	3	5	61.90	34.04	30	8	6.00	1.00	3.75	0.62
	17	2	47	7	26.03		166	25	9.76	12.50	2.59	2.78