FINAL TECHNICAL REPORT

R6743 The Development and Testing of Transgenic Cultivars of Banana Resistant to Nematodes

Executive Summary

This project built on previous work in which a generic defence strategy against nematodes was developed. Appropriate methods, both in the field and in the laboratory, for challenging banana plants with nematodes were developed after monitoring the population dynamics of endemic species that parasitize bananas in the field in the Windward Islands. A gene coding for the production of a cysteine proteinase inhibitor was linked to a root-specific promoter and used to transform rice cultivars to be resistant to nematodes. The gene product, normally expressed in rice grains, is harmless to humans.

This transformation exercise was achieved within a commercial collaboration between the University of Leeds and Zeneca (now Syngenta). Negotiations between these two parties and PSP resulted in: 1) significant price reductions in the event of the deployment of commercial transgenic cultivars for the WI banana industry in return for testing; 2) permission from Syngenta for PSP to use, royalty free, the nematode defence in non-commercial bananas and plantains. A new PSP-funded project is exploiting this concession to improve nematode resistance of cooking bananas for Uganda.

Delays in the provision by Syngenta of test materials and a failure to develop and adopt biosafety regulations by the Government of St. Lucia prevented all objectives of this project being met. Nevertheless, much valuable information was generated on testing procedures that will be of great benefit for other ongoing work on improving nematode resistance in bananas and plantains.

Background

More than 22,000 farmers are estimated to produce bananas for export from the islands of St. Lucia, St. Vincent, Dominica and Grenada out of a total population of 445,000 and their combined efforts produced 280,000 tonnes of fruit in 1992, generating revenues for the islands of US$140.8 million. Banana production is the main single source of foreign exchange, is the mainstay of the economies and will remain so for many years to come. Yields and profitability are low relative to larger-scale producers in Central America for a number of reasons, one of which is the damage done to the crop by nematodes parasitic on banana roots.

The four most common nematodes found parasitizing bananas in the Windward Islands are:

Radopholus similis
Rotylenchulus reniformis
Helicotylenchus multicinctus
Meloidogyne incognita

All are widespread on banana roots in fields in St. Lucia, St. Vincent and Dominica.

The importance of nematodes was recognised early in the Windward Islands, with the secondment by ODM (ODA now DFID) of nematologists to the fledgling banana industry. Early control was by the use of fumigants e.g. DBCP (dibromochloropropane) a volatile compound with relatively “benign” LD50 values (see Table 1) but this was discontinued after it was found to be carcinogenic. Fumigants

were replaced during the mid-1970s by granular nematicides that afforded similar levels of control but were easier to apply.

The yield losses attributable to nematode infestation have only ever been estimated indirectly by comparing production with and without the use of nematicides on infected soils. Early reports suggest yield increases following the use of nematicides from 17% to as much as 350% where high winds blow over infected plants with extensively damaged root systems.

The nematicides currently in use in the Windward Islands are potentially very effective in reducing yield losses and a recent survey of production practices and costs suggests that they are also cost-effective (Alexander-Louis, 1993a). Nevertheless this, and a similar survey (Alexander-Louis, 1993b) report that many farmers use no nematicides at all and those who do manage them poorly. Even so, St. Vincent paid, on average, over US$584,000 per year for nematicides in the four years 1988-1991. The costs to St. Lucia farmers during the period 1992-1994 were similar at over US$582,000 per year (BGA Statistics). Despite this heavy expenditure the average yield of farmers, reported by Alexander-Louis (1993a), of 15 tonnes/hectare/year is only about half of the potential yields reported from WINBAN field trials in farmers' fields. Hence, it is clear that:

1. control of nematode damage is very expensive but
2. it is not very effective at the industry level since there is inadequate adoption of recommended practices.

The real cost to the WI of not controlling nematodes may be illustrated by a simple example. In 1994, St. Lucia imported 155 tonnes of Mocap and 88 tonnes of Miral. This is approximately 30% of the nematicide required for full control using recommended rates of application over all 12,000 acres (WINBAN, 1993). During the same year St. Lucia exported 90,119 t bananas with revenues of US$42.85 million. If we make the simplifying assumptions that a doubling of yields occurs when nematodes are controlled (see references above) and that 30% of all fields were treated then 60% of all production was "nematode-free". It follows then that the other 40% of production can be seen as limited by nematode damage. If this is so then potential production in 1994 in the absence of nematode damage would have been 126,166 tonnes, a difference of 36,047 tonnes and equivalent to a net benefit of US$17.03 million after the costs of additional nematicides. This, together with the cost of the nematicide already imported (see above) and the labour cost of applying the chemicals (unknown, but not insignificant) could potentially all be saved if nematode-resistant bananas were available to all farmers. The possible savings are of a similar order of magnitude in the other islands.

The control of nematodes with chemical nematicides also has social and environmental costs. The case, cited above, of DBCP illustrates this well. This chemical was the mainstay of the industry in the WI and elsewhere for many years and its toxicity, based on the LD50 approach was no worse than many of the compounds in use today. Over the long term, however, it was found to be highly carcinogenic and was withdrawn completely, although not before many hundreds of banana workers were affected by it. The removal of these toxins from the environment will have highly beneficial effects.

Overview of strategy for generic resistance

Nematodes are important parasites with complex host-parasite relationships (Sijmons, Atkinson and Wyss, 1994) and several plant defences directed against them (Atkinson, 1995). Unfortunately natural resistance does not occur in important crop plants or provides an unsuitable basis from which to develop new varieties by conventional plant breeding (Roberts, 1992). CPBB has pioneered two

approaches for nematode control that overcome such limitations (Atkinson *et al* 1994, 1995). One is based on limiting the feeding cells that cyst and root-knot nematodes must form in roots. The second approach is based on an anti-feedant strategy. The University of Leeds arranged for this latter technology to be licensed from the patent application holder (originally Nickerson BIOCEM) to DFID (then ODA) on a royalty-free basis for rice and several other crops. The anti-feedant approach offers major practical benefits for use in the developing world. It offers an ability to control several nematodes simultaneously attacking a crop such as rice or to control nematodes at many different locations without any need for awareness of nematode problems at the grower level. Furthermore, progressive enhancement of the approach is possible and this will ensure durability of resistance. The well established scientific basis of resistance enables any incidents of resistance-breaking to be countered by examining changes in the nematode proteinases.

Table 1. Comparative Toxicologies of Nematicides Available in the Windward Islands

			LD 50 Values (mg/kg)							
			Formulation				Technical			
Chemical	**Type**	**Solubility (ppm)**	**Dermal**		**Oral**		**Dermal**		**Oral**	
In current use										
Rugby 10G (Cadusofos)	OP	248	150	(mt)	679	(st)	24	(mt)	37	(ht)
Nemacur 10G (Phenamiphos)	OP	700	500	(st)	42	(ht)	84	(mt)	15	(ht)
Mocap 10G (Ethoprop)	OP	750	510	(st)	355	(mt)	226	(st)	62	(mt)
Miral 10G (Isazofos)	OP	150	2200	(nt)	327	(mt)	500	(st)	50	(ht)
Vydate 24L (Oxamyl)	Carb.	280,000	2960	(nt)	37	(ht)	710	(st)	5	(ht)
Furadan 10G	Carb.	700	10,200	(nt)	132	(mt)	2240	(nt)	11	(ht)
Withdrawn										
Nemagon (DBCP)			1,420	(st)	172	(mt)				

Note:
(et) extremely toxic
(ht) highly toxic
(mt) moderately toxic
(st) slightly toxic
(nt) practically non-toxic

Proteinase inhibitor (PI) proteins are natural, defence-related, proteins induced in aerial parts of plants and certain other tissues by wounding and herbivory. While they are induced systemically in the aerial parts of plants by nematode parasitism of roots they are, surprisingly, not present in roots. A serine proteinase inhibitor (Cowpea trypsin inhibitor, CPTI) has some potential against insects when expressed as a transgene (Hilder et al., 1987). For those advocating their use in transformed plants, PIs have the particular advantage of already being consumed by humans in many plant foods. The work by CPBB to-date for DFID has been based on a rice cystatin that, because it is expressed in seed of the plant, is widely consumed by people and livestock.

Proteinases occur in all nematodes so far examined, including plant parasitic forms (Koritsas and Atkinson 1994a, Urwin et Al., 1995), animal parasites (Cox et al., 1990) and a free-living species (Ray and McKerrow, 1992). We have shown that inhibitors of both serine (Hepher and Atkinson, 1992) and cysteine proteinases (Urwin et al., 1995) have effects against plant parasitic nematodes. Interest in the approach is heightened by the lack of expression of PIs in roots and so a lack of co-evolution between the nematode and this form of plant defence.

Project Purpose

'Plant genes conferring resistance to pests identified and incorporated into adapted genetic backgrounds of target crops'. Specifically, this project attempted to provide an alternative way for banana farmers to control economically damaging nematodes in their banana crops. Current practice involves the use of chemical nematicides that have serious health, safety and environmental consequences.

Research Activities

From the logical framework, proposed activities were:

1. Gene constructs optimised for banana transformation.
2. Banana cultivars transformed.
3. Transgenics tested against nematodes under laboratory conditions.
4. Population dynamics of nematodes parasitic on bananas in the Windward Islands characterised.
5. Transgenic cultivars tested under field conditions in the Windward Islands.

Gene constructs optimised for banana transformation.

This was achieved later than anticipated using non-PSP funding but details are protected by IPR and hence are not available.

Banana cultivars transformed.

This was achieved later than anticipated using non-PSP funding but details are protected by IPR and hence are not available.

Transgenics tested against nematodes under laboratory conditions.

This is continuing using non-PSP funding. Because of IPR, precise details are not available. It is possible to report that there is evidence that some of the transgenic banana lines exhibit resistance to nematodes.

Population dynamics of nematodes parasitic on bananas in the Windward Islands characterised.

The population dynamics of endemic nematodes were studied and damage to bananas in the field throughout the crop cycle were quantified. These results were used to develop *in vitro* and *in vivo* laboratory sytems for challenging banana plantlets with the appropriate nematode spp endemic to the Windward Islands. A method for minimising variability between inoculated plants in the field was also developed.

Transgenic cultivars tested under field conditions in the Windward Islands.

At no time during this project were transgenic plants tested in St. Lucia. The provision of transgenic lines for testing was delayed but, in any case, the Government of St. Lucia was unable to develop, adopt and implement biosafety legislation to allow the testing of any transgenics in the country. Nevertheless, the project provided secure facilities for hardening transgenic plantlets and testing them

in the field. Operational protocols were also developed that would be useful if ever testing of transgenics were to be considered there in the future.

Results

Field studies

A field experiment was established in The Roseau valley near the WIBDECO research station on a small farmer's holding. The site was sampled at monthly intervals following initial banana plant treatment with nematicide. Chemical treatment was repeated when the mean sample exceeds 1000 nematodes/25 g roots. The trial had four replicates and treatment with either Vydate or the more efficient nematicide Butylfos. Nematodes were counted in 25 g root samples and in 100g soil samples. Nematodes were counted as *Radopholus*, *Helicotylenchus*, *Rotylenchulus* and *Meloidogyne* and saprophytic species. The total numbers of plant parasitic species are shown in Fig 1. The increase in numbers fits a growth curve. This model has been used to predict the build up expected if transgenic plants cause a 70% or 90% reduction in population build-up in the roots.

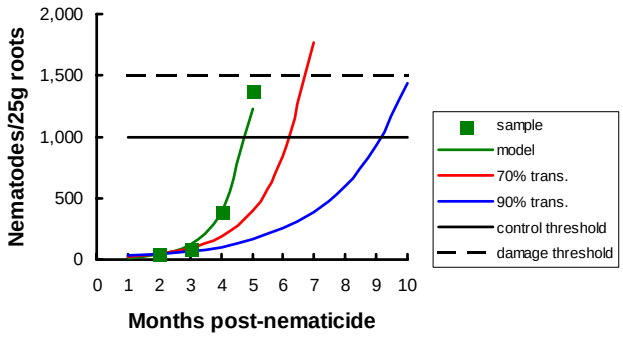

Fig.1 Changes in plant parasitic nematodes/25g roots. Curves are fitted using a growth model based on the data (nematodes/25 g roots $=e^{(b0+b1t)}$ where b_0= 1.493; b_1= 1.1381; t= months post-nematicide treatment). The control threshold and damage threshold are those commonly used in the industry for making decisions in commercial situations.

There was considerable variation between the levels of *R. similis* infection among banana plants at this locality. Therefore a field trial was established at Union, St Lucia (Fig. 2) in which the soil was treated with the nematicide Oxamyl 6 weeks before planting. The objective was to reduce the natural soil infestation by nematodes to a low level before planting. Then different numbers of *R. similis* were added to the banana plant rhizosphere at planting. The subsequent variation in nematode density among the plants was analyzed. Adding *R. similis* to the rhizosphere of banana at planting following nematicide treatment of the soil 6 weeks earlier did influence the levels established relative to that achieved by nematodes surviving the chemical treatment of the soil (Fig. 2 and 3).

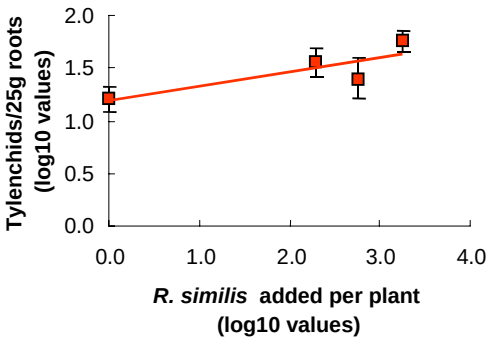

Fig.2: Increase in number of nematodes recovered from roots of banana with increasing inoculum levels of *R. similis* added 2 months earlier.

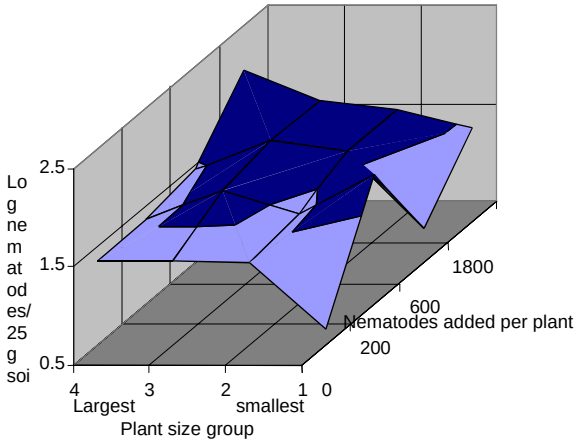

Fig 3: Number of nematodes recovered from banana plants in the field trial of 4 size classes at planting 2 months after adding different levels of nematodes to the banana rhizosphere. See Table 2 for further details.

The results establish that adding nematodes did increase the level of infestation of roots at 2 months post-planting (Table 2; $P<0.02$). The coefficient of variance was less when the highest number of nematodes were added relative to the two lower values. It is possible from these values to estimate the minimal detectable lower value due to resistance that would be detectable with that number of plants per treatment (Table 2). Therefore it seems that adding c 1500-2000 nematodes per banana provides the most certain basis for establishing a reliable infection level for each plant in the field.

Table 2: Mean nematodes/25g banana root at 2 months post-planting when different numbers of *Radopholus similis* were added at planting to soil treated with Oxamyl at 6 weeks pre-planting. Analysis was based on (Log_{10} +1) values but results are shown after re-transformation to linear values. [a] Linear contrast establishes a lower mean when no nematodes added than for other values P<0.02.

R. similis added	Number of plants	Nematodes / 25 g root ± SEM	95% Confidence lower limit	Minimum % resistance detectable
0	11	21.6 ± 26.2[a]	13.8	36%
200	11	29.5 ± 39.1	15.6	47%
600	10	40.3 ± 65.1	13.3	67%
1800	10	68.5 ± 88.2	38.6	44%
All plants	42	35.9 ± 42.3	25.6	29%

Assuming the subsequent results provide similar variance, it is possible to determine the levels of precision likely in the field trials depending on the number of replicate plants per treatment. This estimate is provided in Fig 4. It suggests that establishing c10-20 plants per replicate provides the optimal compromise between accuracy and the feasibility of providing sufficient plants for the trials.

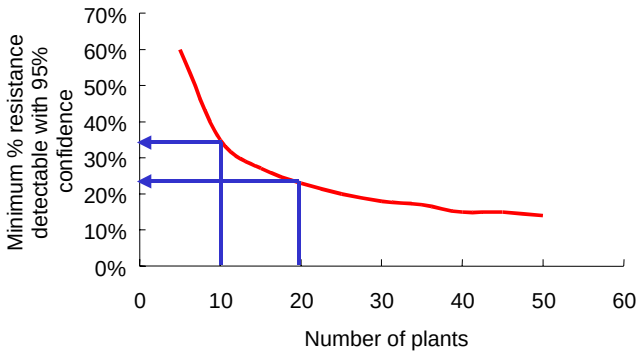

Fig 4: Preliminary estimate of the relationship between minimal detectable level of resistance detectable with 95% confidence limit and number of plants used per treatment.

In vitro studies

Further improvements were made to the method of inoculation of *R. similis* to the rhizosphere of banana roots and in extractions. As result quite a low variation between nematode infections/ root system were obtained in containment (Table 3). Analysis suggests that the precision obtained with 10 plants is more than sufficient to detect levels of resistance of value (see Fig 4).

Table 3: Variation about mean for *Radopholus* in challenging banana plants. The method of extraction is by mistifier using hot (25^{o}C) or cold (direct from the mains water in UK) for extraction. The variance allows the critical difference in means between two treatments that can be detected at P=0.05 to be estimated.

Inoculum Nematodes /plant	Water Temp. used in mistifier	Mean	Minimal detectable Reduction
100	coldmist	3.517 ± 0.16	32%
	hotmist	3.487 ± 0.24	17%
200	coldmist	3.504 ± 0.18	28%
	hotmist	3.796 ± 0.26	17%

WIBDECO has all the essential scientific and technical infrastructure to conduct field trials. It lacked the facilities to conduct measurements more complex than simple nematode sampling. An ELISA plate reader and associated equipment was supplied by the project to allow local measurement of expression levels at the protein level for plants in any field experiments. Dr Henry Fagan visited the University of Leeds for training in the use of this equipment.

Biosafety

Any activities involving transgenic bananas in St. Lucia would be appraised by a committee comprised of appropriate scientists and GoSL officials. Since, during the life of the project, the regulation of genetically modified organisms was not well developed in St. Lucia, the project followed guidelines for the UK as outlined in 'Guidance to the Genetically Modified Organisms (Deliberate Release) Regulations 1995' by the Department of the Environment.

Several meetings were held with senior government officials in St. Lucia. Despite considerable enthusiasm and verbal support for the transgenic approach advocated by this project, the government of St Lucia has yet to establish national regulations for use and field trials of transgenic plants.

The need for secure housing for tissue culture plants after transit from UK before field trial was considered. A screen-house was identified that could be subdivided at low cost to provide a secure area of sufficient size. It is on the WIBDECO station within 10 yards of the main building. This facility was upgraded (Figure 5).

Fig. 5. Screenhouse modified to hold transgenic banana plants after shipment from UK until of sufficient size for field planting. The capacity of the house is one limitation to the number of plants that can be field trialled on one occasion.

A potential site was identified for possible future transgenic field trials. It is in the Roseau valley on alluvial soil about 4 miles south of the capital Castries. The site is within the holding of WIBDECO and within 100 metres of the main building on an area not managed since tropical storm Debbie caused major flooding in the area in 1994. It is surrounded by wild cane that screens the potential site from view. The site was fenced with link fencing to eliminate potential pilfering. The site has the potential for increasing the trial area if necessary.

Standard operating procedures were developed for adoption under the auspices of any national biosafety regulations. Any additional risk not considered below and defined by a future national biosafety committee must be covered by modified SOPs. The guidelines to be established do not apply to any future commercial use which will be covered by different aspects of any national regulations. The SOPs (Table 4) consider: I) the movement of transgenic banana from the UK to a screen house in St Lucia (Fig. 5) and II) conduct of a field trial of such plants on St Lucia. A mock GM trial was conducted in St Lucia at the fenced site in order to practice, refine and develop these SOPs.

Table 4. Summary of areas defined by standard operating procedures for any experimentation with transgenic banana.

I Establishment of transgenic banana plants in a screenhouse
1. Shipment and handling of plants from UK to St Lucia
2. Security and use of the Screen-house
3. Disposal of Plants
4. Completion of experiments
II Deliberate release of transgenic banana plants for nematode resistance
1. Transfer and release in the Field
2. Removal and destruction of plant material
3. Disposal of parts of plants, fruit and whole plants
4. Post-trial monitoring

Contribution of Outputs

Of the five original project Activities, four had been addressed by the end of the project. Gene constructs had been optimised for banana transformation (University of Leeds) and banana cultivars had been transformed (Syngenta) although the latter had taken much longer than expected. Transgenics had begun to be screened in the laboratory (University of Leeds) during 2000 and is still ongoing with other, non –DFID, funding. The population dynamics of nematodes parasitic on bananas in the Windward Islands were quantified and effective *in vitro* test systems were developed. In anticipation of field testing, biosafety-conscious operational protocols were developed and rehearsed using upgraded containment facilities (a secure shadehouse and a fenced enclosure) supplied by the project.

Successful implementation of most of the project activities did not lead to fulfilment of the project Output because of the failure of one important assumption, that 'National regulations for testing transgenic materials are in place'. Despite repeated lobbying and technical support from the project, the Government of St. Lucia was unable to draft and adopt biosafety legislation during this project and there is little likelihood of it so doing in the near future. Nevertheless, field testing of materials remains an option for the future if circumstances change.

Collaboration between University of Leeds, WIBDECO and Syngenta for this project required that Syngenta agree to allow the royalty-free use of the nematode defence in non-commercial bananas and plantains in Africa. This has allowed PSP to plan future research on banana transgenics in collaboration with scientists in Uganda.

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