Determining risks to soil organisms associated with a genetically modified (GM) crop expressing a biopesticide in its roots

Centre for Plant Science
Leeds Institute for Biotechnology & Agriculture
University of Leeds

Executive Summary

This work compares a number of methods that enable both the direct and indirect impact of growing transgenic plants on soil organisms and communities to be evaluated. One aim was to identify reliable and sensitive procedures applicable to any transgenic plants that may be developed in the future. It uses prototype GM nematode resistant (GMNR) plants that express a cysteine proteinase inhibitor (cystatin) based on that from rice seed OcIΔD86) as a specific case around which to develop approaches.

The GMNR plants used provide control of *Globodera rostochiensis* and *G. pallida* (potato cyst nematodes, PCN), growers currently depend on integrated control based on crop rotation (typically 1 in 5-7 years) and chemical control (nematicides) plus resistant cultivars to *G. rostochiensis* where of value. No cultivars combine good levels of resistance to *G. pallida* with other agronomically required traits. Nematicides are one of the most environmentally detrimental compounds used in UK agriculture. They also represent the largest variable cost for many UK potato growers. Their continued use is incompatible with consumer demand for reduced pesticide use. The potential of GMNR crops used in this work to reduce pesticide is recognised by both the GM Science Review and the Prime Minister's Strategy group report on the economic issues surrounding GM crops. Our previous work reported no adverse effects of GMNR-cystatin potato plants on non-target associate aphids and leafhoppers of this crop that feed on potato leaves or on parasitoid natural enemies of the aphids. There is clear evidence that the cystatin poses no toxic or allergenic risk to humans.

The main risk posed by a GM plant relates to the toxicity of any novel protein it expresses and the likelihood of exposure of an organism to harmful levels. Toxicity is usually measured in containment but exposure of soil organisms is influenced by many ecological factors. If a GM plant is developed for pest control, the mode of action of the novel protein it expresses will be known. Therefore, it is possible to devise assays based on the known mode of action of the novel protein to evaluate which non-target invertebrates may be vulnerable. The GMNR plants used in this work express a protein (OcIΔD86) that inhibits cysteine proteinases (a cystatin) which is an important digestive enzyme of many invertebrates but not mammals. A simple histochemical assay was used to establish that several soil invertebrates had digestive cysteine proteinases. Therefore, the central issue is whether or not they are exposed to significant levels of cystatin. This work concentrated on that possibility for soil organisms.

Mites and Collembola were the most abundant micro arthropods at the field site. A collembolan was one of several invertebrates found to have cysteine proteinase activity in its intestine. However, our previous work established that GMNR-cystatin potato imposed no change in abundance of either soil Collembola or mites. Therefore they were not studied further in this work. Instead nematodes were chosen for further study for several reasons: a) the GMNR-cystatin potato is targeted against a nematode (*Globodera* spp), b) they are considered to be reliable bioindicators; c) they are readily sub-divided into species that feed on different food sources and d) previous work has established that many use digestive cysteine proteinases. No effect was detected of growing GMNR-cystatin plants on total nematodes, members of different feeding types or when species diversity was considered. This suggests no adverse effect on sufficient individuals of any that ingest cystatin to affect their number or diversity in soil. Any effect of application of aldicarb prior to planting on nematode numbers was not evident in the following August. In contrast, this soil treatment suppressed earthworm numbers whereas the growing of GMNR-cystatin expressing plants had no adverse effect on them.

The soil microbial community was studied in detail because there are previous reports of an impact on them of GM plants expressing the biopesticide Bt. There was no effect of GMNR plants on soil basal respiration, soil concentrations of nitrate, ammonium or net nitrogen mineralisation. Phospholipid fatty acids (PLFA) were studied as their total amount is a general measure of the active microbial biomass

and fungal and bacterial biomass can be distinguished within total PLFA by their characteristic fatty acids. The PLFA method is sensitive for detecting shifts in microbial community structure caused by crop types. Combined with our earlier work, three years of data at the same site indicated that soil microbes were not affected significantly or deleteriously by any of the GMNR lines used. The changes that occurred reflected both spatial and temporal variations in soil abiotic conditions.

Other approaches were used to detect evidence of impact of GMNR-cystatin plants on soil microbes. No adverse effects were detected for these plants on colony forming units in soil using three media that selected for bacteria, fungi and proteolytic microbes. The methods were sensitive and did detect change due abiotic factors. Community physiological profiles were also studied by providing 31 different carbon substrates for utilisation by microbes (BIOLOG plates). This proved a sensitive method and detected minor differences in growth and attainment of maturity for non-transgenic plants derived from factors such as the size and physiological age of tubers at planting. In spite of this inherent sensitivity, there were no consistent differences in microbial communities that were correlated with the GMNR plants. As with other approaches, the results with BIOLOG plates suggest that GMNR plants did not have a deleterious impact on the soil community. The work suggests both PLFA analysis and BIOLOG plates are sensitive indicators for future studies of factors that may perturb soil microbial communities. The former requires more specialist equipment (GC/MS) than the microplate reader needed for community physiological profiles.

There is value in being able to detect the levels of expression of transgenic protein in plant parts to help define the extent of exposure of organism for instance those browsing on roots. An antibody was raised to OclΔD86 and an ELISA optimised. It measured OclΔD86 reliably for plants expressing that cystatin under control of the constitutive promoter (CaMV35S). RT-PCR detected the corresponding mRNA. RT-PCR would be valuable to define when message is still being produced but ELISA had the sensitivity required to detect OclΔD86 protein to 1% of the maximum expression level recorded in this work. The equipment required for this method is generally available and so it is the approach of choice.

The second type of GMNR-cystatin plant used in this work expressed OclΔD86 under control of a highly specific promoter (ARSK). They produced so little cystatin that detection of expression was close to the minimum possible with both techniques. Previous work has established that this promoter provides expression in roots and particularly in the modified plants cells from which *Globodera* feeds. As a result similar resistance is achieved to that delivered by CaMV35S/OclΔD86 plants. The targeted expression ARSK/OclΔD86 plants provide is beneficial. However, other parts of this work establish that CaMV35S/OclΔD86 plants do not pose a risk to those soil organisms studied. This places the ARSK/OclΔD86 plants at a very high level of environmental safety.

Minor differences in CFU for bacteria were detected for soil that had previously supported CaMV35S/OclΔD86 but not ARSK/OclΔD86 potato plants relative to other soil samples for the mature follow-on winter wheat crop in the subsequent April. However Physiological Community Analysis did not detect any effect of CaMV35S/OclΔD86 and no difference was detected for the more reliable guild rather than individual substrate analysis. Given the sensitivity of analysis there is no evidence that growing GMNR potato plants had an impact on the ability of the soil to support a subsequent wheat crop.

1: Scientific context of this report

The GM science review: (http://www.gmsciencedebate.org.uk; 17/07/03, pdf file) argues for a case-by-case assessment of GM crops and emphasises the benefits of reducing pesticide use. It also sees a need to continue development of safety assessment technologies. It provides few examples of beneficial opportunities for the UK but cites work on GM nematode resistant (GMNR) potato as "*a very strong example of the clear benefits of a GM technology for the UK*" (see Box 6.4; p172).

The report of Prime Minister's strategy group *(*http://www.number10.gov.uk/output/Page3673.asp) provides an assessment of the costs and benefits of GM crops. It also identifies reduced pesticide use as a benefit of interest to consumers. It suggests that this would produce some farm-level savings and convenience of farm management and sometimes provide increased yields. It provides only two detailed examples of such potential impacts of future GM developments and one is GMNR potato (see Box 3.2. page 66). It suggests there are often ways to improve pest control without using pesticides such as crop rotations. The limitations of this approach for nematode control are considerable because the two potato cyst nematode species are highly specific and persistent pests (see section 2).

1.1: The environment and GMNR potato*:*

DEFRA-funded large Farm Scale evaluations (FSEs) of genetically modified herbicide-tolerant crops (GMHT). Key points from the reports are i) effects on herbivores, predators and parasitoid populations associated with weeds at the field scale, ii) changes in detritivore abundance in response to more dead plant material available in GMHT fields and iii) pollinator responses in their foraging behaviour (Hawes *et al.,* 2003). None of these concerns are relevant to GMNR potato. Furthermore the FSEs did not look at soil organisms and in reality compare the consequences of different herbicide management regimes and not the consequences of genetic modification itself (ACRE, 2004).

Potato is an important and safe crop on which to develop early examples of GM technology that have benefits for UK agriculture. It has only two wild relatives in the UK and can only be forced to hybridise with one of them, *Solanum nigrum* (black nightshade) by hand-crossing. It is not naturally invasive of natural or semi-natural habitats. PCN is also an alien species to UK and its distribution in UK is restricted to potato fields. This ensures no ecological advantage to any GM potato plants establishing in field margins. Volunteer (weed potatoes) within field are already controlled as a matter of routine farm management. Potato does not provide nectar and so is not visited by butterflies or hive bees to any great extent. It is pollinated by bumble bees but there is no route from their foraging to human food (they do not produce honey). Our use of root specific promoters limits expression of anti-nematode proteins to parts of the root system (see below). Furthermore potato is vegetatively propagated and so there is no risk of cross-fertilisation resulting in GM presence in the harvest from planted tubers of currently small (c1000 ha) UK organic potato crop.

1.2: Generic methods for testing impact of GM crops and other agronomic practices on soil organisms and communities

Risk is the product of hazard and exposure. For example the direct effect of a transgenic plant on an invertebrate may be the toxicity of the novel protein (e.g. Bt). This value is often expressed in relation to unit body weight of the animal ingesting the protein and is usually measured in the lab. Exposure relates to the quantity the invertebrate is likely to ingest and this is a complex issue that has many ecological aspects. There are a large range of very different non-target soil organisms including the rhizosphere microbial community as well as protozoa and invertebrates, such as soil arthropods and free-living nematodes. Their interactions are highly complex. Non-target organisms may be directly affected if they feed on the roots of transgenic plants or ingest novel proteins released from transgenic plants. Such proteins are released into the soil in plant root exudates (Saxena & Stotzky 2000) and by incorporation of plant residues. In the case of the δ-endotoxin of *Bacillus thuringiensis* (Bt) the toxin is only briefly available in the free state and is readily adsorbed onto surface-active particles. Once bound, the toxin retains its insecticidal properties (Saxena & Stotzky 2001a).

Non-target soil invertebrates may also be indirectly affected if transgenic plants reduce the size of the soil microbial biomass or affect its community structure. Transgenic plants expressing T4-lysozyme and residues of Bt cotton both affected the species composition of rhizosphere bacteria (Donegan *et al*, 1995; Lottmann *et al.,* 1999), while substrate utilization patterns were different between rhizosphere bacterial communities isolated from transgenic and parental alfalfa (Di Giovanni *et al.,* 1999) and transgenic and control potato (Griffiths *et al.* 2000). However Bt maize had no effect on the number of culturable bacteria when the insect toxin was released from root exudates or after incorporation of maize residues into soil (Saxena & Stotzky 2001b).

The impact of GM crops on soil organisms requires examination on a case-by-case basis according to the trait and crop considered. This work compares a number of methods that enable both the direct and indirect impact of growing transgenic plants on soil organisms and communities. The aim is to identify reliable and sensitive procedures applicable to any transgenic plants that may be deployed in the future.

2: Specific scientific background to the project

2.1: Economic importance of Potato cyst nematodes

Globodera rostochiensis and *G. pallida* (potato cyst nematodes, PCN) are key pests of the U.K. potato crop and occur in at least 64% of potato fields in England and Wales (Minnis *et al*., 2002). They are estimated to cause annual yield losses of approximately £43 million based on the mean crop value from 1990-1995 (Haydock and Evans, 1998). The widely planted cv Maris Piper provides resistance to *G. rostochiensis* (Ro1; Dale and De Scurrah 1998) but its frequent use has increased the prevalence of *G. pallida,* to which it is fully susceptible. As a consequence, this latter species is now the predominate form of PCN in UK potato fields (Minnis *et al*., 2002). Conventional breeding for resistance to *G. pallida* has continued for over 50 years but it has proved difficult to combine good levels of resistance with other agronomically acceptable traits. The cv. Sante provides partial resistance to *G. pallida* but populations vary in their virulence to the cultivar (Dale and De Scurrah 1998). It also lacks desired agronomic qualities and is therefore infrequently planted. Such cultivars do not avoid the need for other control measures. Therefore, growers depend on integrated control based on rotation (typically 1 in 5-7 years), chemical control plus resistant cultivars to *G. rostochiensis* where they are effective. The market demand for particular tuber characteristics overrides the value of nematode resistance in indifferent cultivars. PCN may make potato cropping uneconomic for some UK growers and ensure many more will remain nematicide-dependent. The way forward is cost-effective control that is centred on the cultivars that the market demands. Current, nematicide-dependent, control practices are inconsistent with a sustainable approach to potato cropping.

2.2: Cystatins and GMNR-cystatin expressing potato

Cysteine proteinases are important digestive enzymes in many nematodes, but not humans. Therefore inhibitors of this class of proteinases (cystatins) are of particular interest for plant nematode control because of their efficacy and inherent food safety. Cysteine proteinase activity occurs in potato cyst nematodes (Koritsas & Atkinson, 1994). A gene encoding a rice seed protein, OcI, was engineered using crystallographic data to have an enhanced inhibitory activity. Expression of the engineered variant (OcIDΔ86) in root cultures conferred higher levels of resistance against nematodes than the unaltered molecule (Urwin *et al*., 1995). This work has culminated in successful field and containment trials of transgenic potatoes expressing OcIΔD86. The best transgenic lines of the fully susceptible cv. Desiree were shown to have a degree of resistance bordering on commercial value (Urwin *et al.,* 2001). Full resistance to *G. pallida* was observed under UK field conditions by stacking natural and transgenic resistance (Urwin *et al.,* 2003).

Food safety of the cystatin OcIΔD86:

A *prima facie* case has been established for the food safety of GMNR-cystatin potato plants. They are not toxic, nor stable in simulated gastric fluid. It can provide margin of exposure (MOE) many fold greater than generally accepted safety margin of 100x (Atkinson *et al*., 2004) when used with root specific promoters (see section 2.4). In contrast, natural glycoalkaloids may be consumed in potato with a MOE as little as 2-6 (Kuiper *et al*., 2001).

Others have confirmed that the cystatin used is not an allergen (BIBRA FSA [awarded by MAFF] grant F 3023, end date 30/04/2000).

2.4: Promoters that enable targeted expression of cystatins in GMNR potato

The CaMV35S promoter provides sufficient expression of a cystatin for partial resistance to *G. pallida* in the field (Urwin *et al*., 2001) but it is progressively down regulated in the syncytial feeding cells of cyst nematodes (Goddijn *et al*., 1993; Urwin *et al*., 1997b). It is often more active in younger than older roots, resulting in patchy protection of the root system (Atkinson *et al*., 1998).

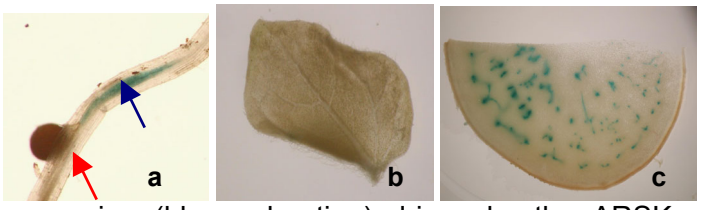

Fig. 1: GUS reporter expression (blue coloration) driven by the ARSK promoter. Expression is precisely driven in the feeding cell (a; blue arrow) of *G. pallida* female (red arrow; diameter c 0.7mm). Expression is absent in the leaf (b) and very low in the tuber (c).

Promoters that direct expression preferentially in root tissue play an important role in addressing public concerns over transgenic nematode-resistant crops. They offer a clear food safety benefit of little expression in tubers and they reduce exposure of non-target organisms feeding on aerial tissues to transgenic products. Several root-specific promoters including two from *Arabidopsis* genes of a root specific tubulin (*TUB-1*) and particularly a serine kinase (*ARSK*; Fig. 1) are used in this work and shown in parallel work to provide similar levels of resistance due to OcIΔD86 as achieved using CaMV35S to control expression (Lilley, *et al.,* 2004).

2.5: GMNR potato and non-target organisms

The growth and survival of a root feeding insect, *Diabrotica undecimpunctata* (Edmonds *et al,* 1996) was adversely affected on artificial diets containing a cystatin. The survival of the leaf-feeding insect *Chrysomela tremulae* was also reduced on plants expressing oryzacystatin-I (Leple *et al.* 1995). However, *Eupteryx aurata* (a leafhopper) and *Myzus persicae* were not adversely affected in the field for potato plants that provided control of nematodes (Cowgill, *et al.,* 2002, 2003) even when the cystatin was expressed in potato leaves. GMNR cystatin potato also has no adverse effects on the parasitoid *Aphidius ervi* that attacks *M. persicae* (Cowgill *et al*., 2004). In contrast to aldicarb, transgenic nematode resistant potato plants are fully compatible with biocontrol approaches to insect pest control.

We had initiated some work on non-target organisms in soil and GMNR-cystatin potato plants prior to the current research. Microbial community structure, soil microarthropods and litter decomposition were studied during two growing seasons of GMNR potato. In the first year, the transgenic lines had no effect on the abundance, evenness or metabolic activity of the soil microbial community as determined by ester-linked phospholipid fatty acid analysis (PLFA). However, one transgenic line (CaMV35S/chicken egg white cystatin) had some effect on the structure of the soil microbial community. PLFA suggested it favoured fungal growth relative to bacterial growth during the latter parts of the growing season. A second transgenic line expressing the cystatin was more effective against PCN. It reduced the abundance of the fungal fatty acid 18:2 ω6 in late season, suggesting a suppression of fungal growth. However, there were no changes in the rate of leaf litter decomposition. The transgenic lines had no significant effect on the abundance of soil microarthropods or free-living nematodes (Cowgill *et al.,* 2002).

2.6: GMNR potato: an opportunity to reduce pesticide use:

Potato receives more pesticide applications than any other broad acre crop and ranks first in the proportion of the national crop receiving fungicides, nematicides and molluscicides (DEFRA and SEERAD pesticide usage survey, 2002). Therefore there is a strong case for developing technologies that reduce this dependence.

Nematicides are a suitable starting point because prototype GMNR plants are available and these pesticides are probably the most environmentally detrimental compounds used on any UK crop. They also represent the largest variable cost for many UK potato growers. Two main types of nematicide are used. In 1999, 17k ha received the oxime carbamates aldicarb and a further 13k ha the carbamate oxamyl. Together they represent 79% of the 37k hectares of land treated with nematicides before potato cropping. Both are registered as poisons under the UK poison's act. Failure to incorporate granules is a cause of farmland bird mortality and the compound is used illegally to poison raptors. The EU plans to withdraw aldicarb from use on potato crops in 2007 but the future of oxamyl has yet to be resolved by the EU review process. One possibility is that other nematicides with the same mode of action will increase in importance. This will do little to reduce environmental concern from nematicide use. A second possibility is that an older type of nematicides will be increasingly used. They are soil fumigants and that currently available (1,3–dichloropropene) is increasing from a very low level (DEFRA). Switching to such compounds offers little prospect of reduced environmental harm from nematicides.

This work uses GMNR-cystatin potato as a specific case with potential for UK agriculture around which to develop and compare procedures that can be used to assess the impact of GM crops on soil organisms and communities.

3: Evaluation of risk to meso and micro soil fauna from root expression by GMNR cystatin expressing plants (*D.T. Kiezebrink and H. J. Atkinson, University of Leeds.*)

Initial soil samples were analysed using a standard method (Tullgren funnel) to determine the key invertebrate groups present at the field site (Fig. 1) at the University of Leeds farm. Nematodes and earthworms were extracted using different procedures (see later). Mites (cryptostigmata, astigmata, other acari) followed by springtails (Collembola) and potworms (enchytraeids) were the most numerous mesofauna in the sample.

In our previous work (Cowgill *et al*, 2002b), mites and Collembola were found to be numerically the most abundant soil fauna group at the field site to be used in the current research. In two years of study, they showed no significant variation in density that could be correlated with the growth of GMNR cystatin-expressing potato although they did show changes in seasonal abundance. In one of the two years densities of Collembola were too low for quantitative analysis. In both years, the number of other soil microarthropods was too low for quantitative analysis.

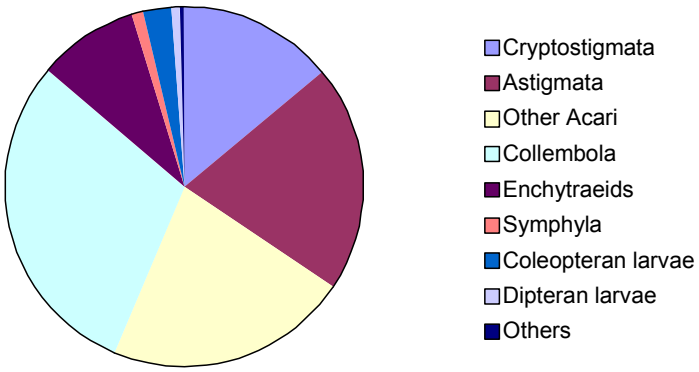

Fig. 1: Pooled data for the proportion of different soil invertebrate groups in several potato fields down to potato in Yorkshire. Macrofauna and nematodes are not included.

3.1: Laboratory-based definition of impact of GM plants on soil fauna

Cystatins are selective inhibitors of cysteine proteinases hence non-target invertebrates utilising this class of proteinases in their digestive tract are potentially at risk from browsing on GMNR plants or debris from such plants. Key members of the fauna were examined for cysteine proteinases using the aphid *Myzus persicae* and leaf hopper (*E. aurata)* as positive controls and methods defined in previous work (Cowgill *et al*., 2002a, 2003). This activity could be assigned to one or more of three sub-groups by the synthetic protein substrate required for positive detection (Table 1).

The cysteine proteinase activity for the springtail (*Folsomia candida)* is shown in Fig. 2 as an example of positive detection. Cysteine proteinases activity was not detected in the digestive tract of potworms.

Invertebrate, common name	Species	Synthetic substrate	Cysteine proteinase subgroup detected	Activity observed
Springtail;	*F. candida*	Z-Ala-Arg-Arg-4MNA	B, L	+
	F. candida	L-Arg-NA	B, H	+
Springtail;	*H. nitidus*	Z-Ala-Arg-Arg-4MNA	B, L	+
	H. nitidus	L-Arg-NA	B, H	+
Leafhopper;	*E. aurata*	Z-Ala-Arg-Arg-4MNA	B, L	+
	E. aurata	L-Arg-NA	B, H	+
Symphyllid;	*S. immaculata*	Z-Ala-Arg-Arg-4MNA	B, L	+
	S. immaculata	L-Arg-NA	B, H	?
Potworm;.	*Enchytraeus* spp	Z-Ala-Arg-Arg-4MNA	B, L	-
	Enchytraeus spp	Z-Arg-Arg-4MNA	B	-
Peach-Potato Aphid	*M. persicae*	Z-Ala-Arg-Arg-4MNA	B, L	+
Leafhopper;	*E. aurata*	Z-Ala-Arg-Arg-4MNA	B, L	+
	E. aurata	L-Arg-NA	B, H	+

Table 1: Cysteine proteinase activity in cryosections of soil invertebrates plus *M. persicae* and *E. aurata*. Cysteine proteinase activity was categorised into 3 levels; frequently detected (+), low activity in some sections (?) and no activity detected (-).*M. persicae* and *E. aurata* occur on potato leaves and are included as positive controls from previous work (Cowgill *et al*, 2002a, 2003).

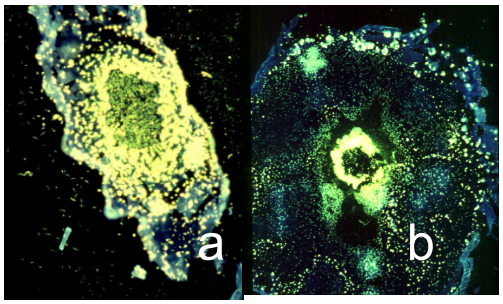

Fig. 2: Detection of cysteine proteinase activity in frozen sections of springtail *Folsomia candida*. **a**, longitudinal and **b**, cross-section showing high activity in the digestive tract. This proteinase is indicated by the histochemical assay as fluorescent, yellow crystals.

3:2 Conclusions

3.1. If a GM plant is developed for pest control, the mode of action of the effector protein against targeted pest will be known. It is possible to devise assays that evaluate risk to non-target invertebrates.

3.2. In this work, GMNR plants express a cystatin. This work establishes several invertebrates (but not an enchytraeid) have digestive cysteine proteinases and so may be at risk.

3.3. Mites and Collembola were the most abundant microarthropods at the field site.

3.4. A Collembolan was found to have cysteine proteinase activity in its intestine but we have already established that GMNR plants express a cystatin imposed no change in abundance on either soil Collembola or mites. Therefore, they were not studied further in this work.

3.5. Nematodes were chosen for further work for several reasons: a) the GMNR is targeted against nematodes, b) are considered to be reliable bioindicators; c) They are readily sub-divided into species that feed on different food sources (see later) and d) previous work has established that many use digestive cysteine proteinases.

4: Impact of GM plants on soil fauna in the field *D.T. Kiezebrink and H. J. Atkinson, University of Leeds*

4.1: Field trial in 2001 : The first field trial was carried out in 2001 at University of Leeds farm between 18/05/01 and 19/09/01 under the terms of DTER consent 98/R31/01. The trial was on a heavy clay soil and involved a much-randomised block design with four treatments, three replicates and 36 plants per treatment. Two GMNR lines of cv Desiree were used and taken directly from tissue culture, grown in glasshouse to establish them and then transplanted to the field. Both expressed the rice cystatin OcIΔD86 under control of the constitutive promoter CaMV35S or the promoter of a serine kinase of *Arabidopsis* (ARSK; Lilley *et al*., 2004). In parallel work at the same field site, they provided 73 ± 9% and 70 ± 4 % resistance to *Globodera* spp respectively (Urwin *et al*., 2001, Lilley *et al*., 2004). The other two treatments in the trial were aldicarb (Temik) at 33.6 kg/ha (10% active ingredient), which is the most commonly applied nematicide against PCN in UK potato production and untransformed cv Desiree. Further details of methods are given below.

4.1: Field trial in 2002

Potato plantlets were transplanted directly from tissue culture for the 2001 field trial. Possibly the rhizosphere microbial flora and fauna composition were influenced by compost adhering to roots at planting. Therefore, for the 2002 trial, only tubers were used as planting material. Some were produced in early 2002 and so plants were treated with gibberelic acid prior to harvest to break the dormancy of their tubers. The two GMNR lines used in 2001 were again used plus CaMV35S/sunflower cystatin line (Urwin *et al.,* 2001). Tubers of untransformed line and CaMV35S/OcIΔD86 produced in 2001 were used as controls. This was for the effect of tuber size (those from 2001 were larger than 2002 tubers) and for any effect of breaking tuber dormancy or physiological ages at planting. The trial consisted of a randomised block design with three replicates for each treatment.

The trial was planted on the 18th of June 2002. Very wet conditions delayed planting in common with commercial plantings at the University farm that year. Soil samples were taken using a 3cm diameter core (10 cm depth), on three sample dates in July, August and September. Soils were sampled by coring into the root zone of one randomly chosen plant within each of the four rows of each treatment replicate plot. These soils were bulked to give one composite sample for each treatment replicate plot. Soil moisture contents were determined by drying soil at 105^{o}C for 24 hours (dry weight basis). At the same time, one randomly chosen plant from each row was harvested, to give an assessment of plant productivity at each sample date. In addition, four plants were selected randomly on three occasions, 02 August, 10 September and 12 October, divided into shoot, root and tuber and oven dried at 105°C. On the October sampling dates, the plants were separated into shoot plus root and tuber as it was difficult to separate the roots from the shoots and senescence had already started in some of the plants. Dry weights were recorded.

Soil dilutions were prepared for all soil samples and plated out on fungi selective media (Martin's Rose Bengal agar), bacteria specific medium (Tryptone soy agar) and proteolytic organism selective media. Colony forming units (CFU) were counted after 3 days incubation at 25°C. Soil samples were also passed through a 6 mm sieve and analysed for shifts in Community Physiological Profiles between the different treatments using BIOLOG plates. The approach is widely used in soil ecology to look at the effect of disturbances and at differences between different ecosystems. BIOLOG plates (Oxoid) are 96 well plates containing different substrates. BIOLOG Eco plates contain 31 different carbon substrates in triplicate per plate. Each sample occupied one plate and each well received 150 µl of a soil suspension. This provides three replicates per substrate. The plates were read with a microplate reader at 590 nm at 12 hourly intervals for 7 days.

Nematodes were extracted from the soil samples using the standard tray method (Whitehead & Hemming, 1965) and fixed in double strength TAF solution. The number of nematodes in each sample was counted and 100 individuals from each sample were identified down to species or genus level in the few cases when such identification required a taxonomic specialist.

4.2: Plant growth in 2002 trial

In August, growth was more rapid for the 2001 than 2002 tubers (SNK, $P< 0.05$; Fig. 3). Only differences in dry weight occurred and this difference was not evident by October. The effect is probably due to differences in tuber size at planting. In August, the dry weight of shoot and tuber was significantly higher for CaMV35S/OcIΔD86 2001 tubers than for this line from 2002 ($P<0.05$, Fig. 3) but not for either the September

and October sampling date. There were no significant differences in yield among the treatments except for poor growth and low yield for the sunflower cystatin expressing line. This may be related to the particular transformation event as there was no significant difference between the wild type and the other lines expressing the other cystatin, OcIΔD86.

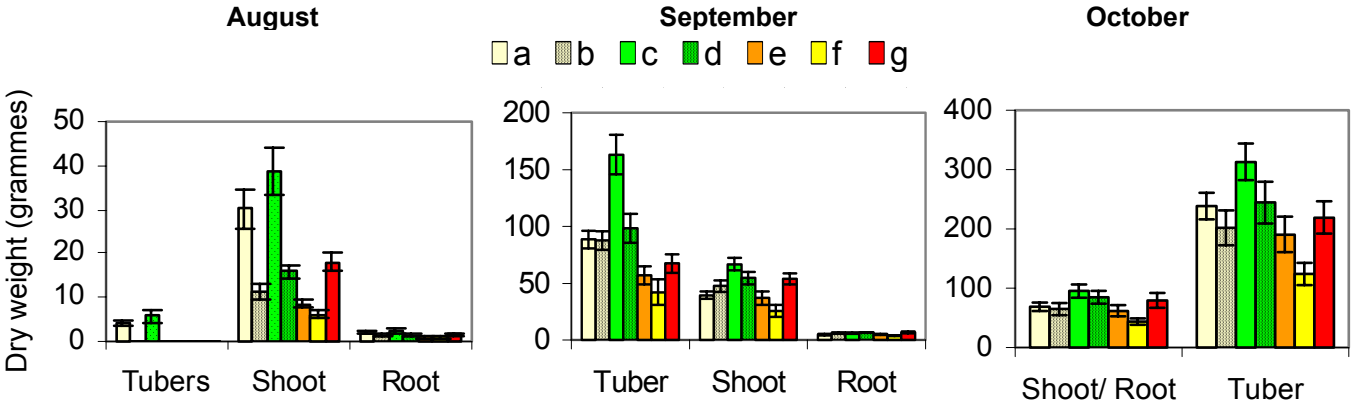

Fig. 3: Dry weights of plant parts during the 2002 growing season for different 7 different lines of cv Desiree. Lines from left to right are: transgenic lines CaMV35S/OcIΔD86 from 2001 (**a**) and 2002 (**b**) tubers, untransformed plants from 2001 (**c**) and 2002 tubers (**d**), transgenic lines ARSK/OcIΔD86 (**e**) CaMV35S/sunflower cystatin (**f**) and untransformed with nematicide (aldicarb) treated soil (**g**; d-g all from 2002 tubers).

4.3: Earthworm density in the 2001 trial

Earthworm numbers were recovered using standard methods (Edwards and Bohlen, 1996). The number recovered from the plot treated with aldicarb were significantly less than for all three plots (Fig. 4; P= 0.01, one-way ANOVA *a priori* contrast). The greater number recovered from the plots supporting growth of the two transgenic potato plants was not significantly greater than for the untransformed control. We choose not to study earthworms further as there was no evidence that GMNR cystatin potato had an adverse effect on them. In addition, they are severely and adversely affected by other soil cultivation associated with stone removal and harvesting of potatoes (Curry *et al*., 2003). This complicates evaluation of the impact of other minor impacts on these beneficial organisms.

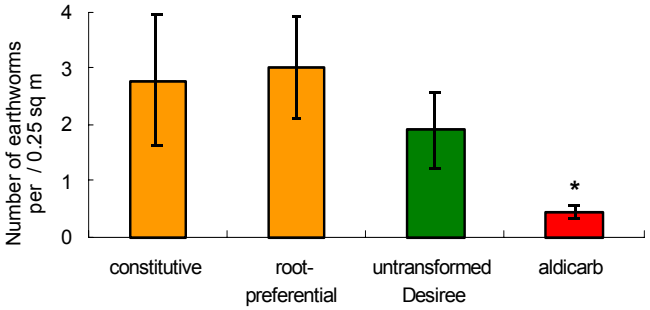

Fig. 4: Total number of earthworms per 0.25 m^2 from soil samples for the four treatments. The transgenic lines expressed OcIΔD86 under control of CaMV35S (constitutive) or ARSK (root-preferential) promoters.

4.4: Soil nematode density and biodiversity (2002 trial)

Nematodes were divided into categories (trophic forms) depending on their primary food source viz: bacteria, plants, fungal hyphae, omnivores or predators on other small animals particularly nematodes. Nematodes are considered to be the most promising faunal indicators for indicating soil quality (Schloter, *et al*., 2003). They form a dominant group, occur in all soil types, have recognisable trophic forms and have both high abundance

and biodiversity. They also play an important role in soil functioning. It is particularly pertinent to examine them given that GMNR potato is being used targeted at one nematode genus *Globodera* and nematicides with potency against all nematodes are frequently applied to soils. In previous work in Canada, aldicarb did favour opportunistic nematodes with rapid life cycles for a potato crop treated with either aldicarb or fosthiazate nematicides (Sturza and Kimpinski, 1999).

The results provide typical proportions with bacterial feeders and plant nematodes representing 61.3 ± 1.67 and 27.6 ± 2.18 % respectively of the population (Fig. 5). There were no significant differences in proportions (after arcsin transformation) of the different trophic forms found in soil for GMNR plants compared with the control. Similarly at the time of sampling, there was no significant residual effect of previous soil treatment with aldicarb before planting relative to controls. No significant differences occurred in the number of species recorded (Fig. 5). A range of diversity indices did not detect significant differences between the samples. Maturity index is widely used to detect changes in species composition. The values correlate positively with diversity of species. No significant effect on maturity index occurred for all nematodes, plant parasite nematode considered alone or when the two indexes were expressed as a ratio. In each case the plots supporting GMNR plants returned the highest values. In contrast aldicarb provided the lowest value for the plant parasitic nematode index or the ratio of the two indices.

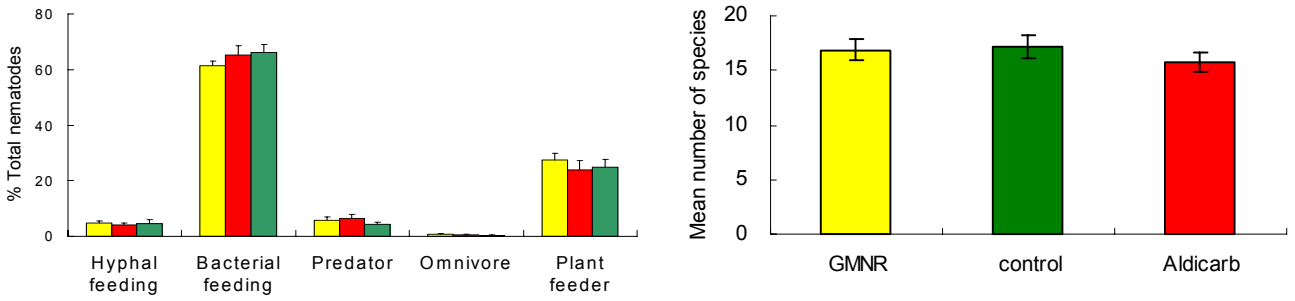

Fig. 5: Percentage of nematode of different trophic forms (left) and total number of species (right) associated with plots of GMNR plants (yellow), or wildtype cv Desiree plants receiving no nematode control (green) or aldicarb (red).

Overall there is no evidence that GMNR plants grown in the field used for this trial has any impact on soil nematodes. The lack of effect on the plant parasitic nematodes included in the analysis may arise as many of the species are ectoparasites and the level expression of the cystatin in the cells from which they feed is not known. Some individuals may feed extensively on potato roots but others may depend on roots of weeds plus crops in other years. The effect of aldicarb soil treatment was not evident by the time of soil sampling. It is normally only present in soil at effective levels for a few weeks after application and so nematode populations could have recovered by the sampling point in August. This effect has been reported in previous work (Griffith *et al*., 2000). The August sample date was chosen for this labour-intensive work because GMNR plants produce cystatin most abundantly from the large root system of mature plants (see section 7).

4.5: Conclusions

4.1. In contrast to soil treatment with aldicarb, the growing of GMNR cystatin expressing plants did not have an adverse effect on earthworm numbers

4.2. Nematodes were chosen for study as they are an important bioindicator group in soil and the GMNR plants are targeted against a plant parasitic nematode (*Globodera* spp).

4.3. No effect was detected on total nematodes, members of different trophic forms or when species diversity was considered.

4.4. This suggests too few individuals ingested sufficient cystatin, if any was available, to affect their number.

4.5. The conclusion is reached that GMNR plants expressing cystatin had no adverse effects on soil nematodes at this site.

5: Impacts on microbial community structure Part I (*a sub-contract, Prof. R. Bardgett, U. Lancaster*)

Changes in the rhizosphere microbial community structure were assessed by analysing the ester-linked phospholipid fatty acids (PLFA) composition of the soil. This method relies on the fact that certain groups of microorganisms have different 'signature' fatty acids which act as markers for microbial groups (Tunlid and White 1992). In addition, phospholipids released upon cell death are used as substrates by living micoorganisms and within minutes to hours are metabolised to diglyceride and PO_4^{3-} (White *et al.,* 1979). Because of this rapid turnover, the total amount of PLFA in soil serves as a general measure of the active microbial biomass (Zak *et al.,* 1996). Specifically, the technique was be used to measure the relative abundance of active fungi and bacteria (Bardgett *et al*. 1996), which constitute some 90-95% of total heterotrophic metabolism in most soils. The fatty acids i15:0, a15:0, 15:0, i16:0, 17:0, i17:0, cy17:0, cis18:1ω7 and cy19:0 represent bacterial PLFAs (bactPLFAs), and 18:2ω6 and 10Me:18:0 can be used as indicators of fungal biomass and actinomycetes. The ratio of 18:2ω6:bactPLFAs can be taken to represent the ratio of fungal: bacterial biomass in soil (Bardgett *et al.* 1996). To assess changes in the dominance, or evenness, of the soil microbial community, a Shannon-Weiner evenness index was calculated using the above PLFAs known to be signatures of different groups of microorganisms. Lipids were extracted from soil, fractionated and quantified using the procedure described by Bardgett *et al*. (1996), which is based on the method of Bligh & Dyer (1959) as modified by White *et al*. (1979). Separated fatty acid methyl-esters were identified by chromatographic retention time and mass spectral comparison using standard qualitative bacterial acid methyl ester mix (Supelco) that ranged from C11 to C20. For each sample, the abundance of individual fatty acid methyl-esters was expressed on a dry weight basis per unit area (m^{-2}). Fatty acid nomenclature was used as described by Frostegård *et al*. (1993).

5.1: Microbial activity: Microbial activity, measured as basal respiration, did not differ significantly between the crop types, but did vary with sampling date being lowest at the end of the growing season in September (Fig. 1). This difference, however, was not significant ($F_{2,18} = 3.49$, $P = 0.053$).

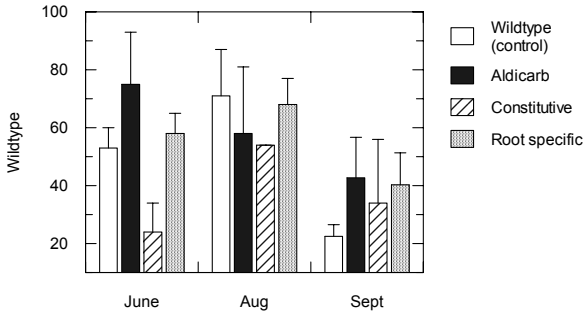

Fig. 6: Effects of crop type on microbial activity, measured as basal respiration.

Soil concentrations of plant available, inorganic N forms (NO_3^--N and NH_4^+-N) also did not differ between crop types, and only nitrate-N varied significantly ($F_{2,24} = 9.70$, $P < 0.001$) between sampling dates, being greatest at the start of the growing season and lowest in mid season in all crops types (Fig. 2a and b). Similarly, net N mineralisation, a measure of the net rate of inorganic N production in soil, varied only with sampling date ($F_{2,24} = 18.54$, $P < 0.0001$), with net immobilisation occurring at the start of the growing season in soils growing all crop types (June) (Fig. 2c). Decomposition of standard substrates, measured as % weight loss of litter material, did not differ significantly between soil types (data not shown).

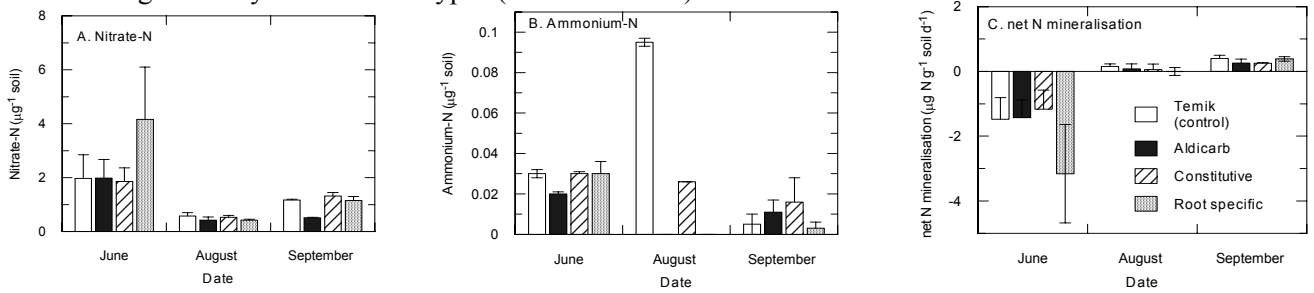

Fig. 7: Effects of crop types on soil concentrations of nitrate (a) and ammonium (b), and on net nitrogen mineralisation (c) at three sampling dates over the growing season

5.2: Microbial biomass and community structure: Using the phospholipid fatty acid approach we detected no measurable effect of the GM lines, in comparison to a control line and aldicarb treatment, on the abundance of 'active' microorganisms in soil over the whole growing season, as measured as total PLFA (Table 2 and Fig. 8a). Similarly, the total abundance of PLFAs synthesised by bacteria was not affected by crop type, but did differ significantly between sampling dates (Table 2, Fig. 8b). None of the individual marker PLFAs of gram positive or negative bacteria were affected by the crop type, indicating that certain individual groupings of the bacterial community were not sensitive to the GM lines (Table 3). Likewise, the evenness of PLFA was not affected significantly by crop types, again indicating that there was no change in the relative abundance, or diversity, of bacterial groups within the microbial community (Table 2; Fig. 8c). In general, concentrations of the fungal PLFA 18:2ω6 were too low to be detectable in several samples; therefore, calculation of fungal: bacterial PLFA ratios, an indicator of shifts in the relative abundance of fungi and bacteria, were not possible at this site (Table 2).

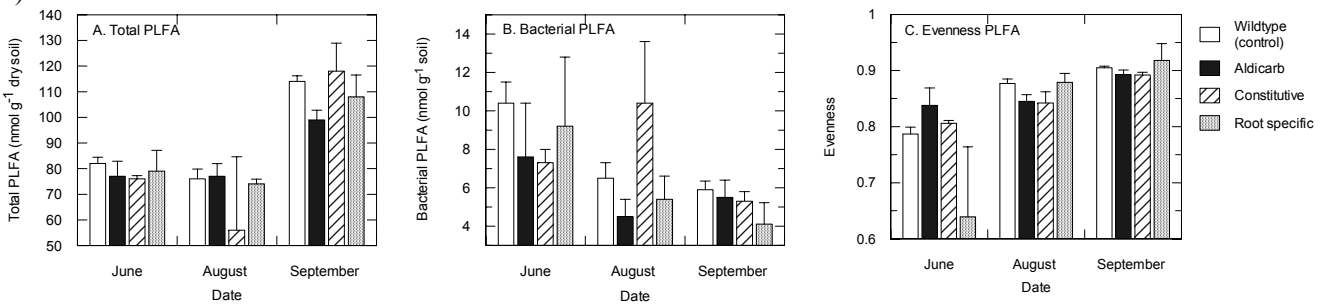

Fig. 8: Effects of crop type on total PLFA (a), bacterial PLFA (b), and (c) the evenness of PLFA at three sampling dates over the growing season.

		Total PLFA		BactPLFA		Fungal PLFA		Evenness	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Main effects									
Crop	3	0.04	0.770	0.49	0.693	nd	nd	0.89	0.460
Time	2	17.48	0.001	4.45	0.023	nd	nd	11.95	0.001
Interactions									
Crop x time	6	0.71	0.643	0.31	0.927	nd	nd	2.16	0.086

Table 2: Summary statistics for analysis of variance of measures of total PLFA, bacterial PLFA, fungal PLFA and the evenness of microbial PLFA.

The data show that the growth of constitutive and root specific crop lines, or the use of aldicarb, had no deleterious effect on either the abundance, structure, or metabolic activity of the soil microbial community. Likewise, these crops had no effect on the availability of plant available inorganic N forms, measured as the concentration of nitrate-N and ammonium-N, or as the rate of mineral N production in soil. As in previous studies, the main factor influencing these soil biological properties was temporal variations, presumably in soil abiotic factors such as temperature and moisture availability. It is of note that the fungal PLFA was not detected in these soils, unlike in previous studies of this site (Cowgill *et al.* 2002). This does not mean that fungi were not present in the soil, but rather that fungal growth was very low during this growing season as compared to previous years, again presumably due to abiotic factors such as moisture availability in soils.

PLFA	Crop line	Time of sampling	Crop x time
i15:0	NS	0.0002	NS
a15:0	NS	0.0004	NS
15:0	NS	NS	NS
i16:0	NS	0.0006	NS
i17:0	NS	0.03	NS
cy17:0	NS	NS	NS
cis18:1ω7	NS	NS	NS

10Me:18:0	NS	NS	NS
cy19:0	NS	NS	NS
18:2ω6	NS	NS	NS

Table 3: Summary for Analysis of Variance (*P* values) of individual fatty acids. Degrees of freedom (df) are as for Table 2.

5.3: Conclusions

5.1. Overall, this study and that already completed (Cowgill *et al.* 2002b), indicate that the PLFA method is sensitive for detecting shifts in microbial community structure caused by crop types

5.2. However soil microbes were not affected significantly or deleteriously by any of the GMNR lines used.

5.3. Further, consistent with previous studies of cropping systems (Cowgill *et al.* 2002b), we show that temporal variations in soil abiotic conditions are the main determinant of microbial properties of these soils.

6: Impacts on microbial community structure Part II (*D.T. Kiezebrink and H. J. Atkinson, University of Leeds*)

6.1: Soil flora: fungi and bacteria (Colony forming units; 2001 trial)

The work with PLFA and other methods listed above were complemented by measurements of colony forming units using standard methods for media that provided a selective medium for fungi, bacteria and proteolytic organisms. The counts were ranked for each culture medium with rank 1 assigned to the highest and 4 to the lowest number of CFUs in October. Kendall W test was not significant (P = 0.44) suggesting no significant differences in ranking were obtained in this preliminary experiment.

Desiree line	Mean rank
Untransformed lines	
No nematode control	2.8
Nematicide (aldicarb) treated soil	3.0
GMNR lines	
CaMV35S/OclΔD86	1.5
ARSK/OclΔD86	2.7

Table 4: Mean rank of soils supporting different potato cv Desiree lines field planted in 2001

6.2: Soil flora: fungi and bacteria (2002 trial)

The number of CFU was obtained as in the 2001 trial.

Fungi selective medium: There was no significant difference in the number of CFU between plants raised from 2001 or 2002 tubers. There was no significant difference among the treatments in the number of CFU on fungi selective media on any sampling date (Fig.9). However there was a significant date effect demonstrating that the approach has the sensitivity to detect differences.

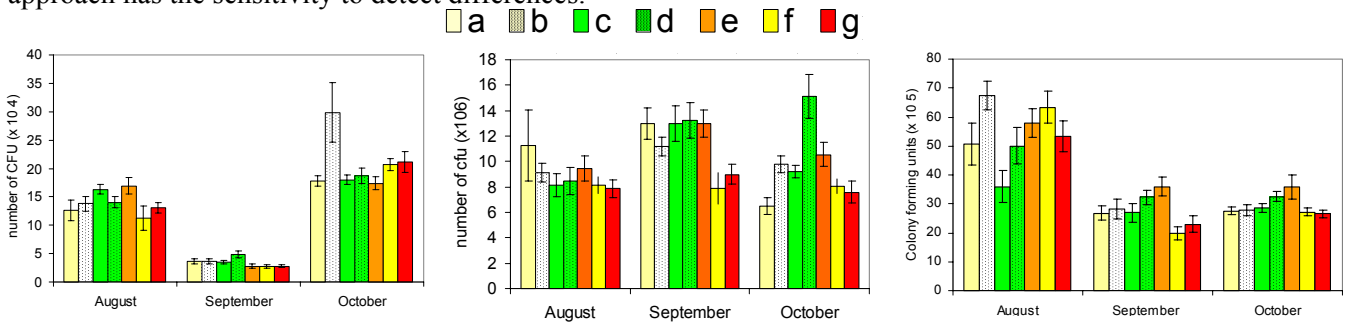

Fig. 9: Number of colony forming units (CFU) from 10 gram fresh soil on **left**, fungi selective media; **centre**, on bacteria selective media, **right** on proteolytic organism selective media in August, September and October. Each block of histograms shows data for soil from the around the roots of

different plants. They are from left to right are: transgenic lines CaMV35S/OcIΔD86 from 2001 (**a**) and 2002 (**b**) tubers, untransformed plants from 2001 (**c**) and 2002 tubers (**d**), transgenic lines ARSK/OcIΔD86 (**e**) CaMV35S/sunflower cystatin (**f**) and untransformed with nematicide (aldicarb) treated soil (**g**; d-g all from 2002 tubers).

Bacterial selective media: there was no significant difference in the number of CFU for the August sampling but in September this value was significantly less in the sunflower treatment that the other three treatments (Fig. 9). This may be explained by the slow growth of this line. In the October sampling, the number of CFU was significantly higher in soil samples taken from the rhizosphere of wildtype and CaMV35S/OcIΔD86 plants grown from 2001 tubers than for these lines with the 2002 tubers. By October most of the plants from 2001 but not 2002 tubers had started to senesce. Presumably, the decaying root material of the 2001 tuber plants provided an accessible substrate for bacteria that normally respond quickly to changes in nutrient availability. The number of CFU on bacteria specific media was significantly lower in the CaMV35S/OcIΔD86 plants with a higher cystatin expression than the ARSK/OcIΔD86 plants. However the differences are probably not due to cystatin expression as there was no significant difference between transgenic and the untransformed Desiree lines (Fig 9).

Proteolytic organism media: there were no significant differences between sample dates in the number of CFU between plants grown from 2001 or 2002 tubers (Fig. 9). However there were significant differences between treatments for the 2002 tubers by sampling date ($P<0.05$). For both the September and October sampling dates, the number of CFU was significantly higher in the ARSK/OcIΔD86 plots than either the aldicarb treated soil or that in which CaMV35S/sunflower cystatin grew. The CFU significantly different compared from the wild type treatment only in providing higher values for CaMV35S/sunflower and CaMV35S/OcIΔD86 lines in August. The transient effect recorded is unlikely to influence the general functioning of the soil ecosystem. The date effects were greater than those due to comparison between lines.

Desiree line	Tuber production year	Mean rank
Untransformed lines		
No nematode control	2001	2.9
No nematode control	2002	4.3
Nematicide (aldicarb) treated soil	2002	5.7
GMNR lines		
CaMV35S/OcIΔD86	2001	3.0
CaMV35S/OcIΔD86	2002	4.1
CaMV35S/suncystatin	2002	5.3
ARSK/OcIΔD86	2002	2.7

Table 5: Mean rank of soils supporting different potato cv Desiree lines field planted in 2002 from using tubers produced in either 2001 or 2002.

The three media and three sampling dates provide a total of nine occasions on which the number of CFUs were judged in different soils. If the cystatin had an overall suppressive effect on the microorganisms, they would rank low overall for the nine different samples (i.e. 1 is assigned to the highest value in the ranking; Table 5). On this basis, Kendall W test for ranking difference is significant ($P<0.05$) but only when the lowest rank (aldicarb treated soils) was included. There are no significant differences when this treatment is omitted. Furthermore ARSK/OcIΔD86 plants grow in the soil samples ranked highest overall.

6.4: Community-Level Physiological Profiles (BIOLOG; 2002 trial)

Soil samples as collected above were analysed for shifts in community-level physiological profiles using BIOLOG plates. The approach is widely used in soil ecology to look at the effect of disturbances and at differences between different ecosystems (Zak *et al*., 1994; Grayston *et al*., 2004). They are 96 well plastic plates containing different substrates. The absorbance per substrate per triplicate were used for Canonical Discriminant Analysis (CDA ; Fig. 11). The CDA was performed on the absorbance data using the time point when the average absorbance for all 31 substrates on a plate had an OD of 0.5. The CDA was carried out for all substrates separately and for the substrates grouped in guilds of substrates that indicate functional differences between bacterial communities in substrate utilisation. The guilds were carbohydrates, carboxylic acids, polymers, polyphenols, amino acids and amines plus amides.

The soil that supported growth of ARSK/OcIΔD86 plants could be separated from CaMV35S/OcIΔD86 on function one for the August samples using all substrates (Fig. 11). The largest correlation was between function one and the substrates D- Glucosaminic acid (Carboxylic acid) and Putrescine (Amines/ amides) with more utilisation of them by the CaMv35S/OcIΔD86 line. When the August sampling data were analysed grouping the substrates into guilds, the microbial communities of the aldicarb treated soil could be separated from that in which ARSK/OcIΔD86 plants grew (Fig. 11). This separation could be attributed to differences in substrate utilisation of the polymer and amino acids guilds. The soil supporting the wild type plants could be separated from that associated with the three transgenic lines on function 1. The substrate with largest absolute contribution to this function was itaconic acid (carboxylic acid). This substrate was used more by the microbial community in the rhizosphere of the untransformed than transformed Desiree.

None of treatments differed in utilisation of particular substrates or substrate guilds for the September data (Fig. 11). In the October samples, only the sunflower treatment separated from all other treatments on function one. The largest correlation was between function one and D- mannitol (Carbohydrate) and Glucose-1-Phosphate (Miscellaneous). These substrates were used less by the microbial communities of soil growing the plants expressing sunflower cystatin than those associated with other treatments. The soil from untransformed Desiree plants grown from 2001 tubers separated from those produced in 2002 function one (Fig. 11). The microbial community in the sample for soil supporting the 2001 tubers utilized more D- mannitol and Glucose-1-Phosphate than that of the 2002 tubers. This might have been be due to senesce of plants from the 2001 tubers by this sampling date. The effect detected of more CFU on bacteria selective media for plants from 2001 tubers than 2002 tubers for both the wild type and CaMV35S/OcIΔD86 plants in section 6.2 was not evident on function 1 (Fig. 11). None of the treatments separated on either function 1 or 2 when all substrates were grouped into guilds for the October sampling date before Canonical Discriminant Analysis. Therefore the separation found when using all substrates is due to differences in utilisation of individual substrates and not substrate guilds.

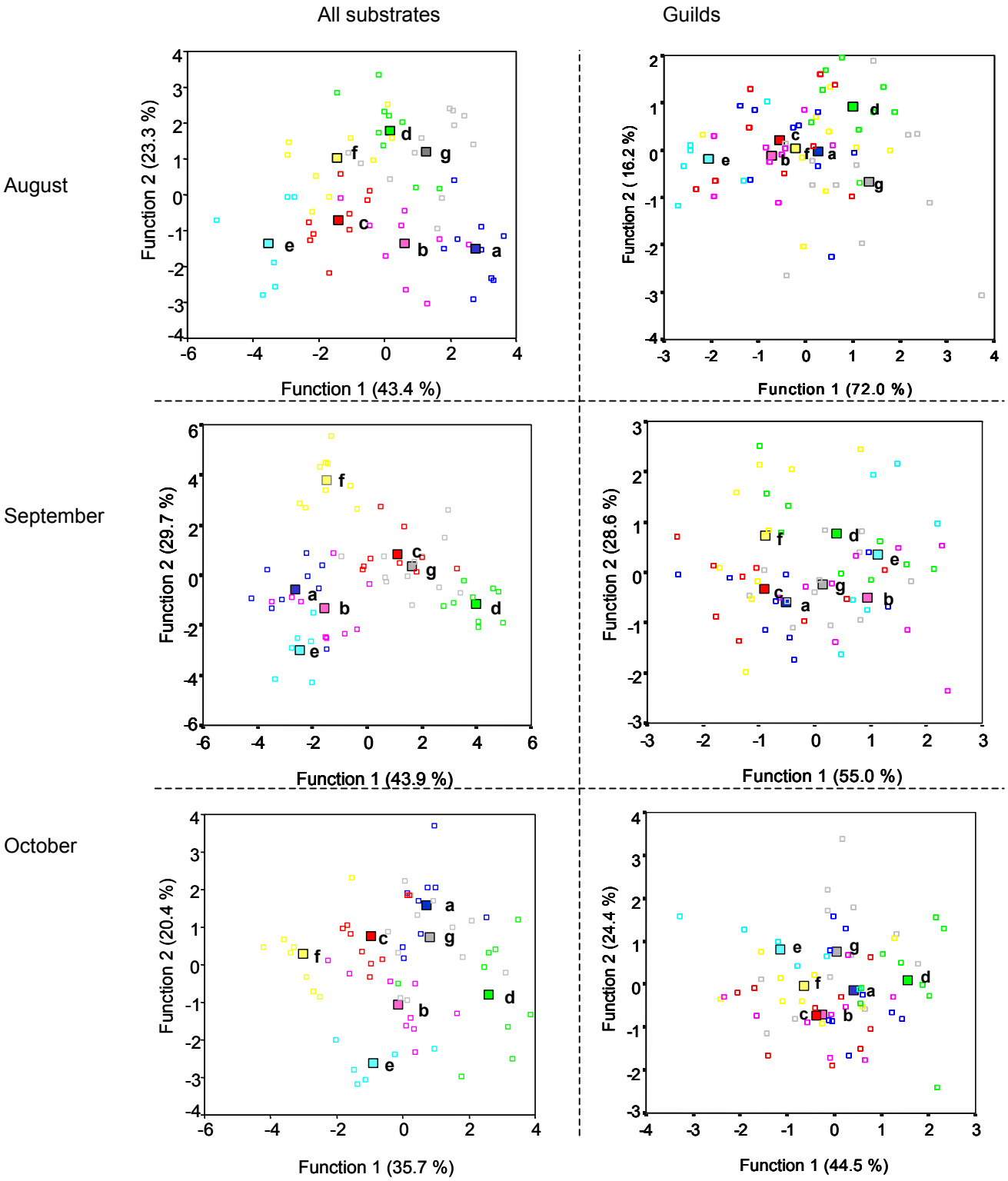

Fig. 11: Canonical Discriminant Analysis using all substrates and guilds to provide community-level physiological profiles for the three monthly samples. Closed, colour squares are group centroids. Lines are: transgenic lines CaMV35S/OclΔD86 from 2001 (**a**) and 2002 (**b**) tubers, untransformed plants from 2001 (**c**) and 2002 tubers (**d**), transgenic lines ARSK/OclΔD86 (**e**) CaMV35S/sunflower cystatin (**f**) and untransformed with nematicide (aldicarb) treated soil (**g**; d-g all from 2002 tubers). The Y-axis is to an expanded scale relative to the X-axis to separate data points for visualisation.

6.5: Conclusions

6.1. The results establish that neither the fungal nor bacterial component of the soil microflora are influenced by plants expressing a cysteine proteinase inhibitor (OcIΔD86).

6.2. No adverse effects were detected for the GMNR plants. The ARSK/OcIΔD86 plants were not distinguishable from the non-transformed plants in their effects on CFU of bacteria, fungi or organisms favoured by proteolytic organisms.

6.3. The methods were sensitive and they did detect change. Overall the results suggest abiotic factors such as temperature and moisture play a greater role in influencing microbial organism numbers than any of the transgenic potato lines used in this study.

6.4. The results are consistent with the lack of effect shown earlier for the GMNR plants by PFLA analysis.

6.5. Community-level physiological profiles proved a sensitive method and it detected differences in growth and attainment of maturity for non-transgenic plants derived from 2001 and 2002 tubers that differed in size and physiological age at planting.

6.6. In spite of this inherent sensitivity, there were no consistent differences in microbial communities that were correlated with GMNR plants.

6.7. As with other approaches, the results suggest GMNR plants did not perturb the soil community.

6.8. Any differences indicated are due to seasonal abiotic differences and the consequences of plant maturity and senescence on the availability of substrates to soil micro-organisms.

7: Comparisons of RT-PCR relative to ELISA for detection of low expression in plant tissues during growth and decomposition

a sub-contract , Dr Neil Boonham and Dr Chris Danks, Central Science Laboratory

Transgenic potato plants with OcIΔD86 expressed under control of either a constitutive promoter (CaMV35S) or a root-preferential promoter (ARSK) plus untransformed Desiree were grown in containment. These plants were used for parallel measurement of the cystatin by ELISA and corresponding mRNA expression. Measurements were made from samples taken from the plant apex, leaves, stems, roots and tubers when the latter formed i.e. for the third to fifth sample occasions. Samples were taken at 14, 32, 50, 60,128 and 152 days post tuber planting to span the progress of the plant from early growth to senescence.

7.1: Protein expression

A rabbit polyclonal raised to OcIΔD86 was coated overnight at 4^{o}C diluted 1/5000 in coating buffer. The plate was washed and 100µl of samples/standards was applied in PBST containing 2%PVP and 0.5% Casein, and incubated at 33^{o}C for 2 hours. Following a subsequent wash 100 µl of monoclonal was applied diluted 1/1000 in buffer, and incubated at 33^{o}C for 1hr. Another wash was performed followed by addition of 100µl of anti-mouse AP conjugate diluted at 1/4000 in buffer before further incubation at 33^{o}C for 1hr. After a final wash 100µl of pNPP substrate was added and the plate read at 405nm after 1hr. CaMV35S/OcIΔD86 plants expressed cystatin in all samples taken for each sample data with little variation between the replicate five plants. There was no significant differences between sample occasions so the data for different sample dates has been pooled in Fig. 12. Differences between expression levels between tissues was also not evident. However, the assays were not optimised to detect such an effect. The planned comparison was between expressions levels provide by the two promoters and so assays were optimised to detect expression levels provided by the less active ARSK promoter. Although the level OcIΔD86 provided in bulk tissue by the ARSK promoter was extremely low, values were significantly different from those for the untransformed control ($P<0.05$). The detected very low expression levels were significant for leaves, stems and roots but neither tubers nor plant apex.

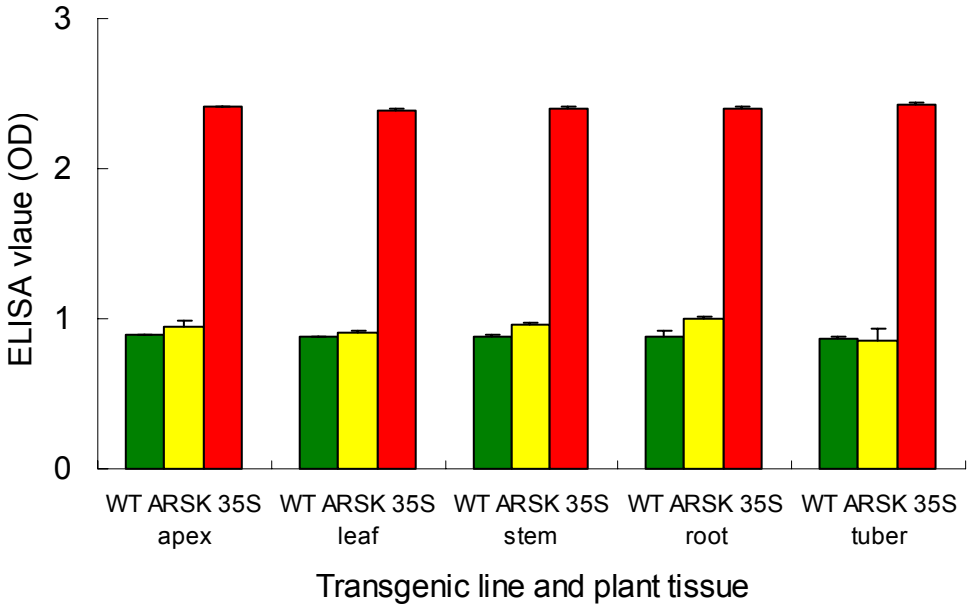

Fig. 12: Relative expression of OcIΔD86 when expression is under the control of either a constitutive (CaMV35S) or root-preferential promoter (ARSK). Data are the grand meand for five sample periods during potato growth

7.2: RNA message abundance

Real time PCR using standard equipment (TaqMan) was directed at detection of the Oc-IΔD86 coding sequence (Table 6).

Primer and probe	Sequence
Oc-IΔ D86-22F	GTT TTG GGA GGC GTC GAA
Oc-IΔ D86-98R	TCA GTA ACA GCA AAC CTA GCC AAA T
Oc-IΔ D86-41T (probe)	CAG TTG GAA ATG AAA ATG ATT TGC ATT TGG TT

Table 6 : Sequences for the primers and probe used to detect rice cystatin.

Primer optimisations were carried out for both the cystatin and the 18S ribosomal assay (normaliser). The difference in slope of the standard curve between these assays is greater than 0.1 and so a standard curve was incorporated onto each reaction plate. mRNA was extracted using magnetic silica and a magnetic bead particle separator (Kingfisher total RNA purification kit cat# 6300051 and Kingfisher ml: Thermo Labsystems). DNA was removed using DNase (Promega). Nucleic acid extractions were incubated in the presence of DNase in a buffer containing 10mM $MgCl_2$, for 30min at 37°C, the DNase was inactivated by incubating for 10min at 94°C, the treated RNA was stored at -80°C prior to use; 10µl of sample was used per reaction. Real time PCR reactions were carried out using reagents and protocols supplied (Applied Biosystems) with the exception of a reduced $MgCl_2$ concentration (1.5mM final concentration – from the TaqMan kit) and also 0.5u of MMLV per reaction.

RT-PCR detected significant message for OcIΔD86 under control of CaMV35S for all tissue types. There was no significant differences between sample occasions so the data has been pooled in Fig. 13. The low level of OcIΔD86 message provided overall for all tissues by the ARSK promoter was significantly greater than the no message level in untransformed control ($P<0.05$). However the effect was too small to be significant when individual tissues of the transgenic and control plants were compared.

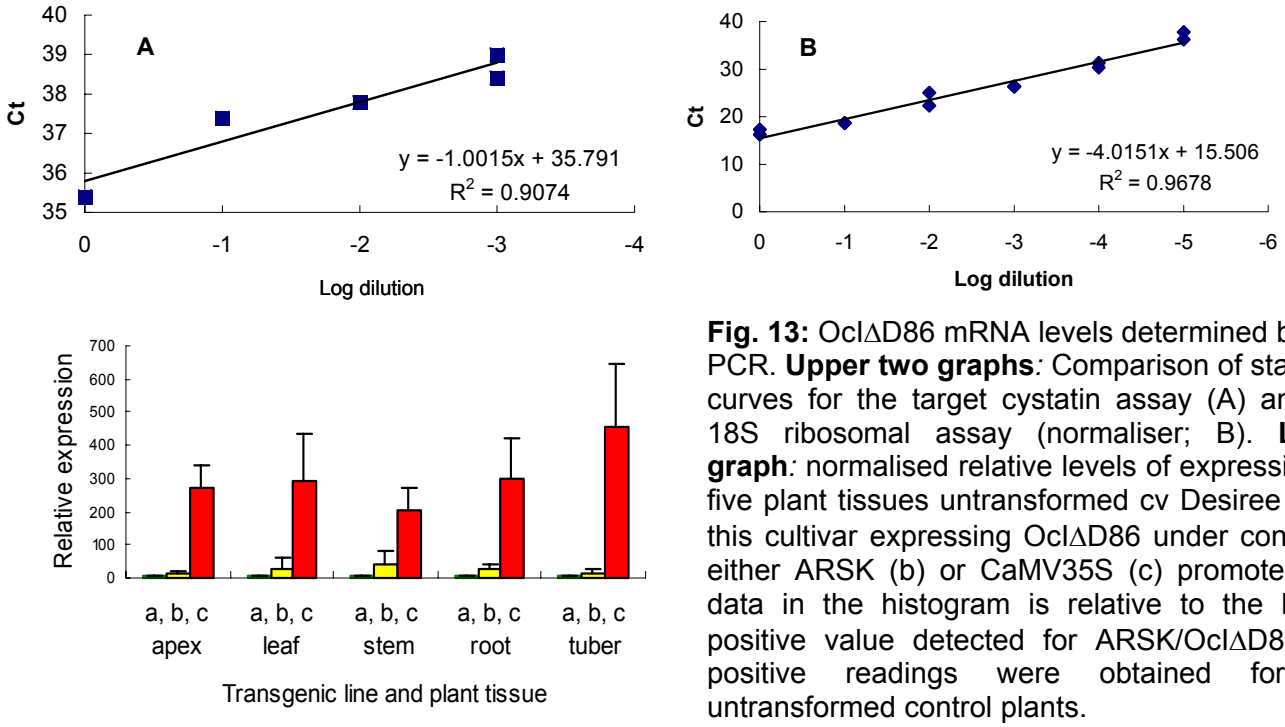

Fig. 13: OclΔD86 mRNA levels determined by RT-PCR. **Upper two graphs***:* Comparison of standard curves for the target cystatin assay (A) and the 18S ribosomal assay (normaliser; B). **Lower graph***:* normalised relative levels of expression for five plant tissues untransformed cv Desiree (a) or this cultivar expressing OclΔD86 under control of either ARSK (b) or CaMV35S (c) promoters. All data in the histogram is relative to the lowest positive value detected for ARSK/OclΔD86. No positive readings were obtained for the untransformed control plants.

Both approaches readily detected expression of OclΔD86 under control of CaMV35S. Both techniques demonstrate the large different in expression in all tissue samples provided by this promoter in comparison with ARSK. The difference was larger than expected at the onset of the work but is fully consistent with parallel work using ARSK/GUS reporter lines. That work established that ASRK gave very limited expression. However, it was sufficiently active in the feeding cells of *Globodera* to provide partial resistance to cv Desiree (Lilley *et al*., 2004). Clearly ASRK localises expression to an extent in roots that explains the lack of negative environment impact in other sections of this work. ELISA-based detection of a novel protein is an approach that could be adopted in many laboratories. In contrast, RT-PCR depends on both higher capital and running costs to establish such assays. There is no evidence in this work that it is the approach of choice for detecting low levels of transgene activity. It may be of particular value for detecting any message either made or persisting in roots after harvest. Such work was not attempted in this study as use of the ARSK promoter effectively limits any concern about transgene expression associated with the nematode resistance potato plants.

7: Conclusions

7.1. The ELISA detected OclΔD86 reliably for plants expressing that cystatin under control of CaMV35S promoter. Likewise RT-PCR detected the corresponding mRNA.

7.2. The high correlation between the two measurements indicates that protein was being continually translated in the plants throughout growth with evidence for more message and protein as the roots matured.

7.3. The ARSK/OclΔD86 plants produced too little cystatin for reliable detection using whole roots. **This is an important result as other work demonstrates these plants do control *Globodera* as effectively as provided by constitutive expression of the cystatin.** Therefore targeted expression is clearly beneficial. It limits expression in the plant without reducing efficacy of the defence. Other parts of this work establish that CaMV35S/OclΔD86 do not pose a risk to those soil organisms studied.

7.4. ARSK promoter prevented the expression in tubers provided by the CaMV35S promoter.

7.5. RT-PCR would be valuable to define when message is still being produced

7.6. In this case, ELISA does have the sensitivity to detect OclΔD86 to 1% of the maximum expression level recorded in this work.

8: Determine safe expression levels in plants and rhizosphere during cropping and post-harvest

(*D.T. Kiezebrink and H. J. Atkinson, University of Leeds*)

Data from previous work (Cowgill *et al.,* 2002, 2003) suggested that expression levels of cystatin under control of CaMV35S was safe for non-target invertebrates throughout plant growth. The ARSK/OcIΔD86 proved not to express in most potato tissues throughout plant growth (section 7). Atkinson *et al*., (2004) suggest that even CaMV35S/OcIΔD86 plants provide a margin of exposure of > 200 for a mammal highly dependent on potato as a food source. The above work and results earlier in this report suggest neither CaMV35S/OcIΔD86 nor ARSK/OcIΔD86 plants have a measurable impact on soil fauna. However there is a possibility that such plants and root litter left after harvest may affect soil organisms and communities in a subsequent crop. Our DTER consent required a non-root crop to follow transgenic potatoes. A winter wheat crop was sown only one month after potato harvest in 2001 and the plot boundaries of the potato trial were marked. Soil samples were taken from the various plots at potato harvest and on the 14th of April 2002 when the wheat was growing i.e. about 7 months after the potato harvest. The samples were used to determine microbial abundance for microbial community analysis. On 28 June 2002 the wheat crop was sampled using a 1 m^2 quadrat with three replicates for each treatment All plants in within this area were dug from the ground, cleaned of adherent soil and separated into shoot plus root and ears. The plant material was dried at 105 °C for 48 hours.
The data were analysed using ANOVA. There was no significant difference in crop yield between the different treatments (Fig. 14).

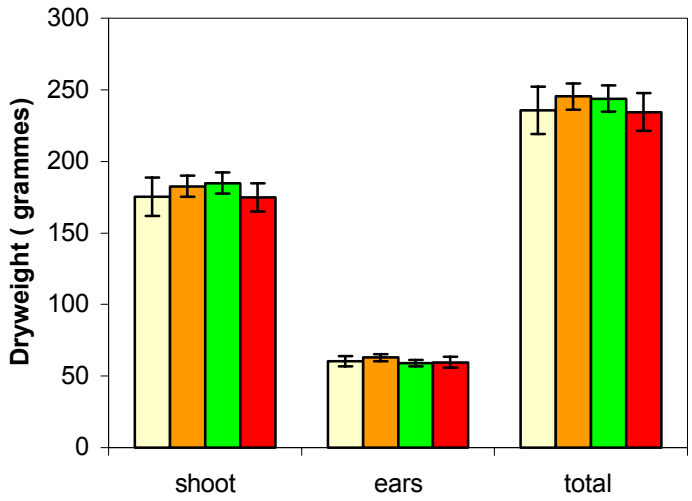

Fig. 14: Dry weight of winter wheat plant shoots plus roots and ears in a 1 m^2 quadrat for different treatments in follow up experiment from a winter wheat crop growing on soil plots previously associated with different potato treatments. The latter were (from left to right) CaMV35S/OcIΔD86, ARSK/OcIΔD86, untransformed with and without and aldicarb treatment of the soil.

8.1: Soil fungi and bacteria (Colony forming Units)

A soil serial dilution was made of these soil samples. The 10^{-6} dilution was plated out on to three different media. In addition, the 10^{-5} dilution was plated out on the fungi selective media. The number of colony forming units (CFU) was transformed to a log10 scale to achieve the homogeneity of variance before being analysed with ANOVA and *post hoc* tests (SNK).

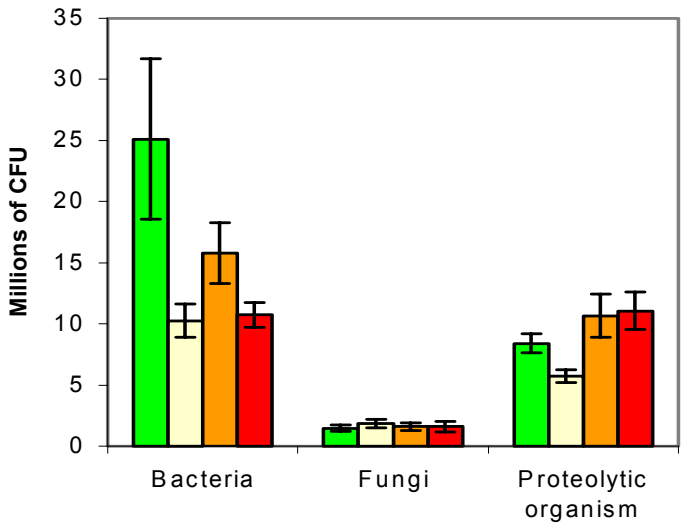

Fig. 15: Number of colony forming units per 10 gram wet weight of soil from a winter wheat crop growing on soil plots previously associated with different potato treatments from the left are: untransformed, CaMV35S/OclΔD86, ARSK/OclΔD8 and untransformed with aldicarb treatment of the soil.

There was no difference for the number of CFU on fungi selective media between the treatments (Fig. 15). The number of CFU on bacteria selective media just after harvest of 2001 potato field trial was significantly higher in the aldicarb treatment than in the other treatments. In the following April under wheat, the number of CFU on bacteria selective media were significantly lower in the ex-CaMV35S/OclΔD86 potato and ex-aldicarb treated soils than the ex-wild type potato plot. (Fig. 15; $P<0.05$, SNK test) There was no corresponding, significant difference between the wild type and the ex-ARSK/OclΔD86 plot. The number of CFU on proteolytic organism media was significantly lower in April 2002 for the soil previously supporting constitutively expressing CaMV35S/OclΔD86 potato compared to the other treatments (see Fig. 15). These differences did not have any impact on the performance of the wheat crop planted after the GM potato.

8.2: Community-level physiological profiles (BIOLOG)

The results from the Canonical Discriminant Analysis using absorbance values for all substrates separated the different treatments on both function 1 and function 2 (Fig. 16).

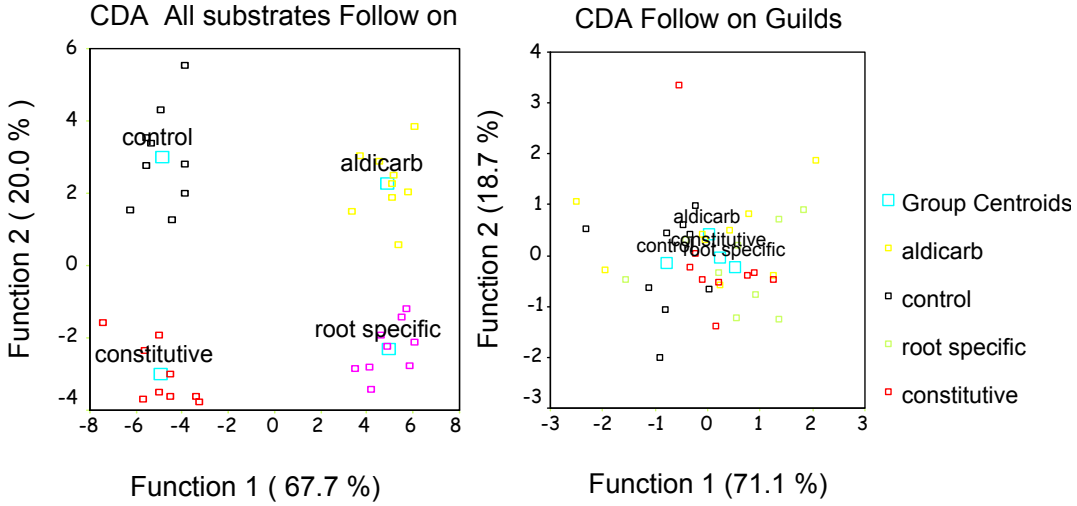

Fig. 16: Canonical Discriminant analysis of all 31 substrates (left) and guilds (right) for BIOLOG ECO plates from soil in a follow-on winter wheat crop growing on soil plots previously associated with different potato lines and soil treatments. Open squares (□) are group centroids. Untransformed

Desiree control), CaMV35S/OcIΔD86 2002 tubers (constitutive), ARSK/OcIΔD86 (root specific), and finally untransformed plus aldicarb treatment (aldicarb). The Y-axis is on an expanded scale relative to the X-axis to separate data points for visualisation.

On function 1 for all substrates, (67.7% variation), the soils formerly receiving aldicarb or growing ARSK/OcIΔD86 potato in the previous season separated from the other two treatments. CaMV35S/OcID86 did not separate on function 1 with ARSK/OcIΔD86. Furthermore grouping the substrates into guilds did not lead to any separation of the different treatments (Fig. 16). Therefore, it is unlikely that the functioning of the soil ecosystem is affected by any of the treatments relative to the untransformed Desiree. Separate ANOVA of the absorbance for each of the different treatments did not show significant differences for any of the substrates on its own. Therefore, the separation of the different treatments is due to minor variations over a set of substrates.

8.3: Conclusions

8.1. There was no effect of the GMNR potato plants on the soil that influenced the dry weight of shoot, root or grain obtained from the subsequent wheat crop.

8.2. Minor differences were detected in CFU for bacteria for CaMV35S/OcIΔD86 but not ARSK/OcIΔD86 plants relative to other soil samples

8.3. Community-level physiological profile analysis did not detect any effect of CaMV35S/OcIΔD86 and no difference was detected for the more reliable guild rather than individual substrate analysis.

8.4. Given the sensitivity of the physiological-level community analysis, there seems no evidence that growing GMNR potato plants had an impact on the ability of the soil to support a subsequent wheat crop.

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