DEPARTMENT for ENVIRONMENT, FOOD and RURAL AFFAIRS **CSG 15**

Research and Development

Final Project Report

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Project title	Low Sugar Disease of sugar beet - An investigation of the causal agent and vectors of the disease and its likely impact on the UK sugar industry
DEFRA project code	**PH0136**
Contractor organisation and location	Central Science Laboratory Sand Hutton York, YO41 1LZ
Total DEFRA project costs	£ 20,951
Project start date	01/11/99
Project end date	30/04/01

Executive summary (maximum 2 sides A4)

A new disease of sugar beet appeared in 1991 in the Burgundy region of France. The disease was called 'Syndrome des Basses Richesse' or Low Sugar Disease because of its effect on the sugar content of the beet - a reduction to about 14-15% from an average of 17%. After an initial epidemic phase of two years the disease settled down to a low level of sporadic occurrence. The disease reappeared in France in 1997 with about 250 hectares of infected beet in the same region. The French sugar industry is worried about the likely effects of this disease on sugar production.

Research being carried out at INRA Dijon by Dr Boudon-Padieu has identified a stolbur phytoplasma vectored by a cicadellid, (leafhopper) tentatively identified as *Pentastiridius beieri*, as the probable causal agent for the French disease. High populations of this insect were observed in sugar beet plots in the summer. She has transmitted the phytoplasma into indicator hosts and sugar beet experimentally and is working on further characterisation of it as the disease agent. Dr Boudon-Padieu has in a recent publication noted that the presence of detectable phytoplasma DNA in the sugar beet samples analysed could not reliably be related to the expression of symptoms (1) (Gatineau *et al*, 2001)

In 1997 a similar disease was reported in beet crops in Norfolk but infection by phytoplasmas was never confirmed, possibly due to a lack of good diagnostic tests. Communications have been received that some Lavender crops in France may also be affected by phytoplasma. In 1999 sugar beet from SE Hungary with similar symptoms to the French disease were diagnosed as having phytoplasma of the aster yellows group, (first definitive record) by CSL (2) (Mumford *et al*, 2000). The French researchers have suggested that the beet phytoplasma they are investigating is a leafhopper-transmitted member of the stolbur group, (3) (Munchembled *et al*, 1999).

Phytoplasmas, (also known mycoplasma-like-organisms (MLOs) in older literature) are infectious organisms containing DNA that are confined to the phloem and xylem of plants. They are just visible using a light microscope. They resemble bacteria, are irregular in shape but have no rigid cell-walls. Phytoplasmas cause a large and varied group of diseases that affect crops, flowers and trees. Transmission of phytoplasmas is primarily by cicadellids (leafhoppers) and other sap-sucking insects.

Symptoms of Low Sugar Disease (LSD) include beet fields that may be completely yellow or that may develop a brown appearance in late summer. The sugar beet may have a pineapple-shaped crown, stunted growth and chlorotic and necrotic leaves and petioles. Leaves can also have small dots or angular spots with recent regrowth being small or deformed. The internal tap roots may show vascular browning.

The milestones for the project were to:

1. To develop methods for the propagation of phytoplasma obtained from France.

2. To develop a methodology for the diagnosis of the phytoplasma involved in LSD.

In conclusion, control cultures of phytoplasmas have been established and the use of extraction techniques, PCR, nested PCR for detection of the phytoplasmas investigated. We have also established a working collaboration with the beet institute in Sopronhorpacs, Hungary, to investigate the biology of beet phytoplasmas present in that country and identify possible vectors. This has helped to ensure a supply of suspect Low Sugar Disease beet and potential vector cicadellids which have been used to develop disease testing regimes. A publication arising from the project and written by CSL scientists has recorded the first definitive record of a phytoplasma infecting sugar beet in Hungary (2) (Mumford *et al*, 2000) .

The results obtained from this project have allowed us to validate techniques that can now be applied to future investigations. Using the extraction techniques and nested PCR methods we are able to detect phytoplasmas in sugar beet. There still appears to be some slight lack of sensitivity in the detection method when testing beets that are only weakly infected by phytoplasma but are showing symptoms of the disease.

Further research to provide an alternative, even more sensitive technique for phytoplasma detection is clearly needed. The feasibility of developing a TaqMan PCR with a view to improving the reliability of beet phytoplasma testing would merit future investigation. Sample extraction throughput is limited at present and methods to allow greater throughput need to be developed, should large surveys for the disease prove necessary in this country. We need to further establish the significance of this disease for the UK sugar beet industry and develop more fully the tools needed for accurate diagnosis and control of this disease if outbreaks are found.

Scientific report (maximum 20 sides A4)

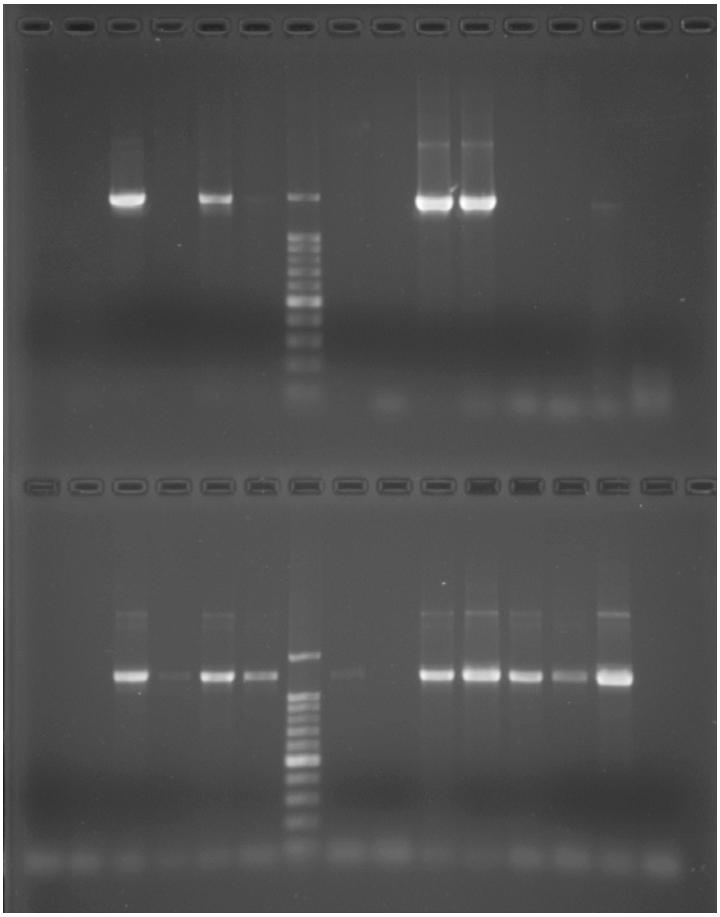
1. Phytoplasma isolates of faba bean phyllody, sweet potato little leaf, Australian tomato big bud and aster yellows have been acquired and maintained on *Catharanthus rosea*, Madagascar periwinkle plants (source Dr D Davies, HRI, E. Malling). Dodder is being maintained to enable transmission from infected beet to periwinkle (maintenance plant). Expertise has been established in grafting from infected periwinkle plants in order to maintain the control phytoplasma isolates. Plant material was collected from Low Sugar Disease outbreak sites in France and preserved at -20^{o}C at CSL until required.

2. A literature search was carried out to identify potential enrichment, extraction and PCR methods. Suitable protocols were tried. Initial extraction methods using the French low sugar disease phytoplasma infected material did not prove successful in PCR tests. Plant material was frozen for development of appropriate PCR test but subsequent experiments showed that fresh material gave much better results in this case. Due to limited supplies of French phytoplasma material continued collaboration has been maintained with our Hungarian contacts. Additional sugar beet material exhibiting unusual symptoms was collected in Hungary and used for the development of suitable PCR DNA extraction methods. In 1999, beets were collected from Hungarian fields suspected of having LSD and other areas of the country where similar symptoms were observed and were brought to CSL together with some leafhopper-like insects found on the crop. This was process was repeated during the 2000 beet season.

An enrichment procedure for DNA extraction, Ahrens and Seemuller 1992 (4) was found to give good results with all isolates. This involved extracting the sample in a specific buffer, then using differential centrifugation to remove much of the plant leaf debris and concentrating the phytoplasma- infected fraction. This fraction was then processed using a CTAB DNA extraction method with a chloroform/isoamyl alcohol step. It became apparent after testing a number of sugar beet samples that it was very important to preferentially extract the phytoplasma from the mid ribs and petioles of the infected beet leaves.

The most appropriate PCR test was found to be that of Gunderson & Lee 1996 (5) using universal nested 16S rRNA primers. The primer pair used for PCR was R16 MR1 -sequence 5' CTTAACCCCAATCATCGAC 3' (19 mer) and R16 MF2- sequence 5' CATGCAAGTCGAACGGA 3' (17 mer). It was found that although some of the other phytoplasma isolates could be detected by ordinary PCR with the 16S primers, the sugar beet phytoplasmas tested were only evident after a nested PCR (a second amplification step) was performed. A 1/40 dilution of the first PCR product was used as the template to perform the second, nested PCR. The primer pair used for the nested PCR was R16 F2n - sequence 5' GAAACGACTGCTAAGACTGG (20 mer) and R16 R2n - sequence 5' TGACGGGCGGTGTGTACAAACCCCG 3' (25 mer).

Detection of phytoplasmas by PCR (top gel), and the more sensitive, Nested PCR (bottom gel)
Samples in the same order on both gels



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Phytoplasma PCR product 1400bp
Phytoplasma Nested PCR product 1200bp

1 Non- infected Catharanthus rosea, (healthy control)
2 Non- infected sugar beet, (healthy control)
3 Faba bean phyllody phytoplasma, maintained in *C. rosea*, (infected control)
4 Aster Yellows phytoplasma, maintained in *C. rosea*, (infected control)
5 Sweet potato little leaf phytoplasma, maintained in *C. rosea*, (infected control)
6 Australian big bud phytoplasma, maintained in *C. rosea*, (infected control)
7 DNA Ladder 100 bp
8 Sweet potato little leaf phytoplasma, maintained in *C. rosea*, (infected control) (frozen -80(C),
9 Hungarian beet sample with symptoms of Low Sugar Disease
10 Sweet potato little leaf phytoplasma, maintained in *C. rosea*, (infected control)
11 Aster Yellows phytoplasma, maintained in *C. rosea*, (infected control)
12 Hungarian beet sample AC-1 with symptoms of Low Sugar Disease
13 Hungarian beet sample CA-6 with symptoms of Low Sugar Disease
14 Pear decline phytoplasma, infected control
15 Water control

Sugar beet infected with phytoplasma were not detected using the PCR test from the 2000 harvest in Hungary but plants were kept growing in the glasshouse and retested at intervals. On retesting in February 2001, 3 very weak positives were detected when testing by PCR. These weak positive samples were tested by 2 alternative PCR methods, Expand^{TM} PCR (Boehringer Mannheim) and PCR beads (Pharmacia). Neither method produced a satisfactory result. The beets from which the leaf petiole samples were originally taken are being maintained for further testing at a later date. The weak positive plants originated from the Hajduszouvat area, one was noted to have had leafhopper-like insects feeding on it in the field, the third was from a different area, Szilsarcony. Designing a TaqMan PCR assay could provide a more sensitive testing method for very weakly infected samples in future projects.

The insect samples were tested using the DNA extraction method of Cenis *et al.* 1993, (6), and the phytoplasma nested PCR method as above. Infected insects were not detected. Insect samples were identified by experts at CSL as being adult *Cicadellidae*, many species of which are important vectors of plant pathogens. Suspect beet samples sent from the Netherlands with similar symptoms to Low Sugar Disease were tested and found negative but were subsequently found to be infected with *Beet soil borne virus.*

As previously mentioned, a collaboration has been established with the beet institute in Sopronhorpacs, Hungary, to investigate the biology of beet phytoplasmas and identify possible vectors. This has helped to ensure a supply of suspect Low Sugar Disease beet and potential vector cicadellids which have been used to develop disease testing regimes at CSL. Sugar beet material exhibiting unusual symptoms was collected in Hungary in 1999. Symptoms observed in the field were characteristic of the French Low Sugar Disease, including pineapple-shaped crown, stunting, chlorotic and necrotic leaves and petioles with recent regrowth being small and deformed. Testing using the methods researched as described above produced a single PCR product (1,247 bp). This was cloned and sequenced twice. The phytoplasma was closest in homology (99.7%) to a phytoplasma belonging to the Aster Yellows (16SrI) group isolated from winter oilseed rape in the Czech Republic (rape green petal or phyllody). This work from the project was published as a new disease report, "The identification of a phytoplasma from the aster yellows group infected sugar beet in Hungary" in 2000 *Plant Pathology*. This is the first definitive record of a phytoplasma infecting sugar beet in Hungary. A poster was also prepared for the PHSI Conference 2001 outlining the disease, its symptoms and the results of this project.

The development of a suitable molecular test for phytoplasmas in sugar beet has enabled a reliable diagnosis to be made. This will enable not only diagnosticians to provide an accurate test result but also better advice can be given to sugar beet growers and the sugar beet industry. These technologies can now be applied to future investigations which may provide even better techniques of phytoplasma detection and diagnosis and add to our knowledge. This will help to provide the essential information needed to contribute to risk assessment of this disease, in order that control/eradication strategies may be devised should the disease be found in the UK.

References

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(2) Mumford, R.A., Potyondi, L., Harju, V.A., Henry, C.M (2000) The identification of a phytoplasma from the Aster Yellows group infecting sugar beet in Hungary. Plant Pathology (2000) **49**, 806.

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(6) Cenis, J. L; Perez, P. and Ferrers, A. 1993. Identification of Aphid (*Homoptera:Aphididae*) species and clones by Random Amplified Polymorphic DNA. *Ann. Entomol. Soc. Am*. **86,** (5), 545-550.

Poster

Mumford, R.A., Potyondi, L., Harju, V.A. & Henry, C.M. New diseases of Sugar beet in Europe 30/1/2001. PHSI Conference (Poster) and displayed at CSL.

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