BUREAU OF SUGAR EXPERIMENT STATIONS
QUEENSLAND, AUSTRALIA

FINAL REPORT – SRDC PROJECT BS99S
RSD HOST-PATHOGEN RELATIONSHIPS IN SUGARCANE

by

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SD97014

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This project was funded by the Sugar Research and Development Corporation, The Cooperative Research Centre for Tropical Plant Pathology, and by BSES during the 1993/94, 1994/95 and 1995/96 financial years.

BSES Publication
SRDC Report
SD97014 **July 1997**

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1.0 SUMMARY

Clavibacter xyli subsp. *xyli* (Cxx) is a gram +, fastidious, slow growing bacterium that causes ratoon stunting disease (RSD) of sugarcane. Because of its fastidious nature, slow growth, and poor symptom expression, it has traditionally been put into the 'too hard basket' by molecular plant pathologists. Systems for transformation and transposon-based mutagenesis of Cxx have been successfully established. In addition, a very sensitive polymerase chain reaction (PCR) based detection system was developed for Cxx so that as few as 100 cells per reaction can be detected in the xylem extracts from Cxx infected plants.

It was demonstrated that a variety of plasmids and transposons can be shot into Cxx by electroporation. These include shuttle vectors designed to move DNA back and forth between *Escherichia coli* and *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) or *Clavibacter xyli* subsp. *cynodontis* (Cxc), cosmid cloning vectors for generating DNA libraries, and vectors carrying various transposons (jumping genes).

It was shown that the transposon Tn4431 jumps from the plasmids it is carried on into the Cxx genome to generate random mutants. Using this mutagenesis system, approximately 800 independent transposon mutants were generated. The transposon carries the complete operon encoding light production from *Vibrio fischeri,* lacking only a promoter. Most of the transposon mutants produce light from this operon, suggesting that the majority of the insertions are into functional genes within the Cxx genome.

Two PCR primers, OPC-2 and OPC-11 (Operon Technologies, USA) were used. These primers were previously shown to amplify products from Cxx (van de Velde and Smith, unpublished), to generate Cxx specific PCR primers and DNA probes. The PCR-amplified products from Cxx were cloned and sequenced. Internal PCR primers to these sequences were generated and the clones were used directly as probes for Southern blots of restriction-digested Cxx genomic DNA. Both the internal PCR primers and the probe generated from the OPC-2 PCR amplified product proved to be very sensitive and highly specific for Cxx. A provisional patent has been filed on these primers. Unfortunately, inhibitors found in the xylem extracts from sugarcane plants make this technique unreliable as a routine method for screening for Cxx. The Cooperative Research Centre for Tropical Plant Pathology (CRCTPP) has a project running in Program 1 to try to solve the problem of these PCR inhibitory compounds.

An assay for screening for 'loss of pathogenicity' of these mutants *in planta* was developed. Symptom expression is not a reliable assay for ratoon stunting disease. Therefore, an assay based on the presence or absence of Cxx in the xylem extracts of plants is being used. This allows screening of the assays ten weeks after inoculation rather than six to eighteen months as previously reported in the literature.

One of several biochemical mechanisms involved in plant cell recognition of Cxx was studied by measuring endogenous peroxidase activity produced by susceptible and resistant cultivars of sugarcane after infection. Preliminary results indicated that peroxidase activity may not be an important host defence response to infection by *Cxx,* although a small increase in activity occurred in the resistant cultivar seven days after inoculation. This avenue of research had to be discontinued when the project leader resigned.

2.0 BACKGROUND

Clavibacter xyli subsp. *xyli* (Cxx), the organism responsible for ratoon stunting disease (RSD) of sugarcane, causes losses to the Australian sugar industry in excess of $15m annually despite extensive control programs. Existing controls for RSD are becoming more difficult to implement, and loss of effective controls could result in crop losses exceeding $50m per year in Australia alone. Steindl (1961) first reported the use of Integrated Pest Management (IPM) programs to control RSD in Australia. However, RSD is still the most economically important disease of sugarcane in Australia (Croft *et al.* 1995, 1996) and one of the most economically important diseases of sugarcane worldwide (Gillaspie and Teakle 1989). As important as this disease is, very little is known about the basic mechanisms by which this pathogen infects, colonises and causes disease on sugarcane. An understanding of these basic mechanisms is essential for the development of a comprehensive control strategy.

RSD is not controlled adequately in a number of areas, eg in one mill area, over 25% of the commercial cane is infected. Control procedures currently need high levels of management, which is difficult on larger farms. Even with hot water treatment and a planting material assay, Cxx infected cane occasionally gets distributed, sometimes into areas where RSD did not previously exist. In addition, in both the Bundaberg and the Mackay regions, there have been unexplained outbreaks of RSD. In one trial in Bundaberg, a field where sugarcane had never been grown was planted with long hot water treated setts originally grown in clean seed plots. Within the first year, three RSD infected stools were discovered. The other farms in the region that received cane from the same clean seed plot were all found to be free of RSD.

RSD infection occurs on farms throughout sections of most mill areas of the Australian sugar industry. In the future, RSD may be even more important as changes in cane production practices are likely to reduce the efficacy of current controls. These changes include longer ratoon cycles, reduced fallowing, increased ploughout and replant, larger harvesting groups and more use of billet planters.

Resistant varieties would offer an attractive alternative to current RSD controls but these are expensive to produce by conventional breeding. Resistant varieties are difficult to identify at times because some varieties can be infected with high numbers of bacteria but show no symptoms of the disease. This is true of H60-6909 that was previously reported to be immune but two years ago was found to contain high numbers of Cxx. Determining the infection and colonisation process for the RSD bacteria in sugarcane will be useful for improving control strategies and for investigating resistance in varieties. Determining the mechanisms of pathogenicity and host resistance will allow the development of novel resistance genes for producing transgenic plants resistant to RSD.

3.0 OBJECTIVES

- Develop a stable transformation system for Cxx to incorporate the luciferase gene.
- Develop an assay for plant infection and colonisation by the tagged RSD bacteria.
- Test RSD-specific RAPDs developed by CRC-TPP for detection and tracking Cxx during plant infection.
- Screen for RSD mutants and investigate pathogenicity genes by cloning and complementation.
- Establish *in vitro* cell cultures to identify biochemical mechanisms involved in plant cell recognition by Cxx.

The last of the objectives was dropped when the project leader resigned to take up a position at Melbourne University.

4.0 OUTCOMES

- Single cell cultures of Cxx.
- A transformation system for Cxx with a variety of plasmids including *Clavibacter*/*E. coli* shuttle vectors, cosmid cloning vectors, reporter vectors and suicide vectors containing transposable elements.
- A mutagenesis system for Cxx based on transposable elements that act as discrete tags in the genes whose function they disrupt allowing for easy cloning and characterisation.
- Light producing Cxx transposon mutants.
    1. The transposon Tn4431 containing a promoterless *lux* operon from *Vibrio fischeri* and a tetracycline resistance gene.
    2. All but 1% of the tetracycline resistant transformants involving this transposon produce light at varying levels indicating transposition into functional Cxx genes.
- Cxx specific PCR primers designed from internal sequences of the cloned OPC-2 PCR product of Cxx that are very sensitive (>100 cells per reaction) [Preliminary Patent].
- A pathogenicity assay that can be run in 10 weeks rather than the six to eighteen months previously reported.
- A DNA probe that is specific for Cxx and could be used for *in situ* hybridisation studies.
- A transposon mutant that is no longer able to colonise sugarcane.

In conclusion, it has been shown that some of the standard tools that have been used to unravel the molecular basis of pathogenicity and virulence in the gram negative bacterial plant pathogens will work on Cxx, a gram positive, fastidious, and very slow growing sugarcane pathogen.

5.0 RESEARCH METHODOLOGY, RESULTS AND DISCUSSION

5.1 Growth of Cxx as single colonies on plates

In Australia, it was thought that Cxx could not be grown as single colonies. Therefore, Cxx was cultured by scraping up large spots of cells off plates, resuspending these into sterilised dH_20 or buffer, and then putting several 50 to 100 µl drops of the suspension onto fresh MSC plates. A simple modification of the culture media, the addition of 4 g/l bacto-agar, resulted in media firm enough to streak onto with resultant growth of Cxx as single colonies. This technique worked with all the isolates in our collection, demonstrating that it was not a chance mutation but a fault in the media composition. Achieving single colony growth was an essential first step for doing any form of genetics on Cxx. For the composition of the media used to culture Cxx see, Appendix 1. In addition, obtaining growth of Cxx in broth was not successful. A modification of the S8 broth sent from Crop Genetics International (unpublished data) solved that problem and resulted in faster growth of Cxx (four weeks on plates to < two weeks in broth).

5.2 Transformation of Cxx

There were no reports in the literature of Cxx transformation. However, there were reports of successful transformations of other *Clavibacters* (Meletzus and Eichenlaub 1991; Meletzus *et al.* 1993), including Cxc (Taylor *et al.* 1993), a close relative of Cxx that infects grasses. Initially, both bi- and tri-parental matings were attempted using *Escherichia coli* as the donor and helper strains respectively. These attempts were unsuccessful probably because Cxx is not much bigger than the pillae used to transfer the plasmid DNA from one bacterial cell to the other by the *E. coli* donor strain. Using electroporation, the first ever transformants of Cxx were generated. Initially, three *Clavibacter/E.coli* shuttle vectors (Table 2) were used which were originally created for Cxc and Cmm. Although there were very few transformants in these early trials, the positive results were encouraging.

Using a modification of the electroporation protocols developed by Crop Genetics International (CGI) for Cxc, successful and routine transformation of Cxx was achieved. The most critical factor was the addition of glycine to the growth medium at sublethal concentrations (Figure 1). Glycine interrupts cell wall formation and therefore makes it easier to shoot the DNA through the very tough cell wall of Cxx. A variety of plasmids, including the *Clavibacter*/*E. coli* shuttle vectors mentioned above, cosmid cloning, reporter, and suicide vectors containing transposable elements were electroporated into Cxx (Table 2). Transformation frequencies varied from as low as 20 up to 10^3 colony forming units (cfu) per µg of DNA depending on the size of the plasmid. It was necessary to amplify the largest plasmids, such as pUCD623 (43 kb) or pLAFR3 (20 kb), in a *dam*$^-$ /*dcm*$^-$ *E. coli* strain, such as Stratagene's GM2163 because it appears that Cxx has a unique DNA restriction/methylation system (Table 3). In addition, it was necessary to purify the plasmids using Qiagen columns in order to get plasmid DNA of a high enough quality so that it was not recognised by Cxx as foreign DNA and destroyed by the bacterial defence mechanisms.

5.3 Transposon mutagenesis

The transposon Tn*4431*, on the suicide vector pUCD623 (Figure 2), contains a promoterless *lux* operon from *Vibrio fischeri* and a tetracycline resistance gene which are transcribed in opposite directions. All but 1% of the tetracycline resistant transformants involving this transposon produced light at varying levels indicating transposition into functional Cxx genes (Figures 3 & 4). Southern blot analysis of a random selection of these transposon mutants indicates that most insertions were at single sites with only a few multiple site insertions occurring (Figure 5). This shows that it will be easy to clone the gene that is tagged by the transposon insertion. In addition, the insertions were at unique sites throughout the Cxx genome (Figure 5), indicating an increased likelihood of tagging the gene(s) of interest. The development of a transformation and transposon mutagenesis system opens the way for molecular analysis of pathogenicity determinants in Cxx.

Initially, it was anticipated that the light production from the Cxx mutants would serve as a distinct marker to follow the processes of infection and colonisation of the sugarcane plants. The light production from mutants of *Xanthomonas albilineans,* generated using this same transposon, could be detected in diffusate from leaves or in the intact leaves (Plate 1) of infected sugarcane plants using a luminometer or ultra low light sensitive camera respectively (Homer, Honors project). Unfortunately, very soon after inoculation of the Cxx light producing mutants onto sugarcane plants, light production was turned off. This may be because there is insufficient energy sources in the xylem to drive the luciferase enzyme, or it may be because special regulatory systems are turned on in Cxx during the interaction with sugarcane which regulate expression from the lux operon. Another possibility is that the genes encoding the light production are lost once the antibiotic selection pressure is removed. This last option is unlikely because the transposition appears stable under laboratory conditions (data not shown).

5.4 Randomly amplified polymorphic DNA primers for sensitive detection of Cxx

RSD is very difficult to diagnose in the field. In side by side, diseased and healthy, demonstration plots it is easy to see how much yield loss can be caused by this pathogen. However, in the field, diagnosis is difficult because there are no reliable external symptoms. Direct serological techniques such as microagglutination (Gillaspie 1978) and immune electron microscopy (Damann *et al.* 1978) or indirect serological techniques such as the fluorescent-antibody method (Harris and Gillaspie 1978) have been used in the diagnosis of RSD. Enzyme-linked immunosorbent assays (ELISA) were developed in 1979 (Gillaspie and Harris 1979) and were capable of detecting Cxx at 1 x 10^5 cells/ml. Davis and Dean (1984) developed a more sensitive, direct fluorescent-antibody technique which detects 1 x 10^4 cells/ml. However, this technique is limited to research use since it is very slow to operate and requires fluorescence microscopy. Serological diagnostic techniques based on polyclonal antiserum are not specific to Cxx because the closely related bacterium Cxc is also detected. Cxc and Cxc-like bacteria do not naturally infect sugarcane but are found in grass species, which grow near sugarcane. Nucleic acid probes increase the sensitivity of detection of Cxx to 1 x 10^4 cells/ml (Chung *et al*. 1994) but are slow and tedious, thus limiting their usefulness as a routine test. Several sugarcane growing regions of the world, including South Africa, rely upon phase contrast microscopy (PCM) to accurately diagnose RSD.

In 1993, van de Velde and Smith (unpublished data) used polymerase chain reaction (PCR) to screen several libraries of primers (Operon Technologies, USA) for amplification of genomic DNA from Cxx. Two ten-mer primers, OPC-2 and OPC-11 (Table 4), each amplified a single band from Cxx (Figure 6). Both primers amplify unique bands in Cxx which are easily differentiated from bands amplified from either sugarcane or other *Clavibacters* (Figure 7). These primers were able to detect Cxx in xylem extracted from a number of different Cxx infected sugarcane cultivars (data not shown). In addition, as few as 100 Cxx cells per PCR reaction could be detected with these primers, showing that these primers can be used as a sensitive diagnostic tool for RSD (Figures 8a & 8b).

To make the test more reliable, the DNA bands amplified from Cxx by OPC-2 and OPC-11 were cloned, partially sequenced, and new primers designed based on the internal sequence data (Table 5). These primers were tested for specificity (Figure 9; Table 6) and sensitivity to Cxx (Figure 10). In addition, Dr Fegan in the University of Queensland Microbiology Department completely sequenced both clones (Figures 11 & 12) and designed his own internal primers (Table 7). These also were tested for specificity to Cxx (Figures 13 & 14; Table 8). The PCR primers designed by Dr Fegan were tested against our internal primers, the evaporative binding enzyme immunosorbent assay (EB-EIA), and phase contrast microscopy (Tables 9a & 9b). Unfortunately, the PCR-based tests are unreliable due to inhibitory compounds found in the xylem extract from sugarcane that interfere with the Taq polymerase. In Program 1 of the CRCTPP there is a project to try and solve the problem of these inhibitory compounds. Once solved, these primers will be powerful diagnostic tools for the sugar industry, especially for screening clean seed plots.

5.4.1 Genetic diversity of Cxx

To do a preliminary study of the genetic diversity of the various Cxx isolates in our collection, total genomic DNA from these isolates was digested to completion with the restriction enzyme PstI and run out on an agarose gel. This gel was blotted onto Hybond N+ membrane (Amersham) and was probed with α^{32}P dCTP labelled cloned PCR products from either OPC-2 or OPC-11. The results of these gels show that, based on these two probes, there is no diversity in the Cxx isolates tested (Figures 15 & 16). In addition, these Southern blots showed that the product of OPC-2 is highly specific for Cxx whereas the OPC-11 probe hybridises to bands of some but not all of the Cxc and Cxc-like bacteria. The OPC-11 probe also shows that unlike the Cxx there are distinct polymorphisms in Cxc (Figure 16). Dr Fegan has also found no diversity in Cxx isolates from Australia (ACM2271) and overseas (B1B, J1, L1A, F1, & S1A) when he sequenced through the intergenic nontranscribed spacer region between the 16s and 23s ribosomal RNA genes of these six isolates (personal communication).

5.5 A pathogenicity assay for screening Cxx transposon mutants

Like many plant pathogens, Cxx does not kill its host plant outright. In addition, Cxx produces no external symptoms that can be reliably assayed for. This makes the pathogenicity assay one of the more difficult aspects of this research project. Because nothing is known about the mechanisms by which Cxx causes RSD, it is not known what exactly to assay for, (ie an enzyme, polysaccharide or some other pathogenicity or virulence factor). In addition, Cxx is fastidious and will only grow on a very rich culture

medium. This means that the Cxx mutants generated cannot be screened for auxotrophy (mutants in one of the essential biosynthetic pathways such as amino acids or vitamins). If one of the Cxx mutants is auxotrophic, and the sugarcane plant does not supply the missing component, these mutants will be unable to colonise sugarcane. These types of mutants will indicate something important about the interaction between Cxx and sugarcane but they are not the purpose of this study. Therefore, each of the mutants that cannot colonise sugarcane will have the flanking DNA around the site of the mutation cloned and partially sequenced to determine if it is an auxotropic mutant or not. This means that doing these types of studies on a bacterium like Cxx is much more time consuming and tedious. However, for a disease such as RSD, that is so economically important to the majority of the sugarcane growing regions of the world, a molecular-based control strategy could provide a very powerful bargaining chip for the sugar industry that owns it.

Prior to this study, infectivity studies of Cxx on sugarcane took from six to eighteen months. To screen several hundred Cxx mutants with enough replications to be confident of the result demands a quicker assay. Both glasshouse and tissue culture techniques were tested in an attempt to devise a rapid and reliable ‘pathogenicity’ assay for Cxx (Homer 1993). In the glasshouse, two inoculation methods were tested. One-eye setts were dipped into Cxx cells suspended in H_2O (Table 10). Alternatively, sugarcane plantlets were decapitated and a drop of the Cxx cell suspension was placed directly onto the cut surface (Table 11). To determine which method, sett or stem inoculations, gives the best results, Homer (1993) did a side by side comparison. At two, four and six weeks the sett inoculations consistently gave higher bacterial counts (Figure 17). In these studies, the only determinant for pathogenicity is the presence or absence of Cxx. As a positive control, juice from a Cxx-infected sugarcane plant was used following the same inoculation protocols described above. In preliminary inoculation studies with the transposon mutants, bacteria were reisolated only from stem-inoculated plants, none from sett inoculated sugarcane (data not shown). Therefore, we chose the cut stem inoculation method as the method for screening the transposon mutants.

The inoculation of tissue culture plants turned out to be quite tedious. Homer (1993) did one study to see if tissue culture plants could be used to assay for pathogenicity. She demonstrated that both an older culture and fresh culture of Cxx could be used as the inoculum source and that within a very short time, one week for the fresh and two weeks for the old Cxx culture, high concentrations of Cxx could be recovered (Table 12). In addition, a wide range of varieties was grown on ½ strength MS medium using the bud and apical meristem techniques (Taylor & Dukic 1993). For the inoculation with Cxx, the plantlets were handled as for a normal subculturing. The individual plantlets for the varietal effect were decapitated and inoculated with Cxx isolate QPF110-45 scraped from a plate culture with an inoculation loop. These plantlets were then incubated for three weeks in the tissue culture room with continuous lighting at 28°C. The plantlets were then chopped into 1 to 2 mm segments that were put into 5 ml sterile distilled water for approximately one hour with gentle agitation. The liquid was removed by pipetting to a sterile 1.5 ml eppendorf tube and this liquid was microfuged for 10 minutes at 13,000 rpm. The supernatant was discarded and the pellet resuspended in 100 µl sterile distilled water. This suspension was assayed for the presence of Cxx using three separate techniques.

1. A 10 µl sample of the juice was examined under phase contrast microscopy (Table 13).
2. A sample was PCR-amplified using the OPC-2/OPC-11 primers (data not shown).
3. The samples were plated onto MSCm and were incubated at 28°C for approximately four weeks.

The results in Table 13 were confirmed by both the PCR (data not shown) and reculturing of the Cxx. Because PCR is so sensitive, the biggest concern is whether there were enough residual Cxx cells still present from the inoculation to give the positive result. Also, the PCR amplification of Cxx from plants has never worked consistently because of plant produced inhibitory compounds. A similar problem may exist in terms of culturing the bacterium from the plant exudates, because the cells that grew could be residual inoculum still viable, but on the outside not the inside of the plantlets. However, the high numbers of cells observed in many of the varieties indicate that the Cxx bacteria were multiplying, and there is no evidence that these fastidious pathogens can grow anywhere but inside the xylem of plants or on the very rich culture media. Cxx will not grow on the ½ strength MS sugarcane tissue culture media unless this media is supplemented with 10% or more S8 (Brumbley & Pillay Unpublished).

For the effect of inoculum concentration, a 1 µl drop of the various dilutions of Cxx was placed on the cut surface of the three varieties tested. These plantlets were treated in the same way as described above. However, for this experiment, the diffusate from the chopped up sugarcane plantlets was assayed for the presence of Cxx by phase contrast microscopy only (Table 14).

Once again Cxx proved to be a difficult organism to work with. Within one week for the Cxx inoculated tissue culture plants and two weeks for the glasshouse plants, Cxx could be reisolated from the vascular tissue if juice from an infected plant was used for the inoculum. However, for Cxx isolates that had been in culture for a period of time it took considerably longer to be able to detect the bacterium in the vascular tissue of the inoculated sugarcane plants. During the warmer months it took from five to 10 weeks after inoculation before sufficiently high concentrations of Cxx built up in the vascular tissue to be detected by phase contrast or EB-EIA. During the colder months only an occasional bacterium from culture would successfully grow in the vascular tissue to detectable concentrations whereas all of the plants inoculated with infected sugarcane xylem extract had high levels of Cxx in the vascular bundles. It could be argued that this is an indication that Cxx loses its pathogenicity in culture but the evidence points to a difficulty in making transmission. Once inoculated into plants the bacteria simply go into a lag or dormant phase for a period of time. However, it was demonstrated that several hundred of the Cxx mutants, some of which had not been back into sugarcane for several years, were able to colonise sugarcane xylem to very high numbers in 10 weeks.

5.6 Enhanced growth of Cxc using extracts from the xylem of sugarcane, and corn and a root extract of Bermuda Grass

One of the complications in the pathogenicity assay is the inconsistency of getting the Cxx isolates to shift from growing in cultures to growing in sugarcane (Figure 18 & Tables 10, 11, & 12). However, many of the transposon mutants, some of which have been in culture for over three years, have been able to successfully recolonise sugarcane. Unfortunately,

it appears that there is some specific set of conditions that need to be met for colonisation to be successful. One of the experiments to try and understand this phenomenon was to look at xylem or root extracts as supplements to the growth media. Because Cxc grows considerably faster than Cxx, this initial study was carried out with a Cxc. This work was done in Dr Metzler's lab in Turku Finland during a CRCTPP funded study tour in June of 1996. Dr Metzler's group had reported that they could enhance the growth of Cxc in broth culture by the addition of xylem extracts from corn or root exudates from Bermuda Grass (personal communication). Their experiments were repeated with the addition of xylem extract from healthy and Cxx-infected Q110 sugarcane plants. The xylem extract from the Cxx-infected sugarcane had the greatest effect on growth of Cxc followed by Bermuda Grass Root extract and then both the healthy sugarcane and the corn (Figure 17). It is interesting to note that the extract from the corn xylem took considerably longer to influence the growth of the Cxc but, when it did, the growth was very rapid. This suggests that there may be different compounds (sugarcane –vs- corn) that are responsible for this growth enhancement. These results indicate that there is something in the xylem and roots of these plants that encourages the growth of Cxc and more importantly, either there is more of this compound in the Cxx infected xylem or the Cxx itself is producing something that also enhances growth of Cxc (Kaprelyants & Kell 1996). These results have not been repeated with Cxx because of the lack of manpower.

5.7 Recommendations for future research

The initial work at developing molecular tools for studying the basis of ratoon stunting disease caused by Cxx is well underway. This work should continue by further characterisation of the transposon tagged gene from the Cxx mutant that cannot colonise sugarcane to determine what role, if any, it plays in pathogenicity. This includes searching the databases for genes with similar homology to determine what the cloned genes encode. However, should no homology be found there are other approaches to try to understand the function of this gene. Bacteria organise their genes into operons, several genes with related or complementary functions whose expression is jointly controlled. Therefore, the Cxx genes adjacent to the one tagged by Tn4431 should be cloned and characterised to determine their role in pathogenicity.

The cloned gene from the wild type genome should be used to try and restore the transposon mutant to a wild type phenotype. This involves inserting a functional copy of the gene into the Cxx mutant and testing to see whether the cells containing the functional copy can now colonise sugarcane. Again, this may be difficult because no Cxx vectors, plasmids that are stable and replicate in Cxx, exist. We do have some shuttle and integration vectors from Cxc. An alternative would be to marker exchange the transposon back into a wild type Cxx. This is done by cloning the transposon and flanking DNA from the noncolonising mutant into a plasmid that cannot replicate in Cxx. This construct is then put into a wild type Cxx which is then screened for isolates that have the antibiotic resistance of the transposon but lack the antibiotic resistance encoded by the plasmid. These isolates can then be screened to see if the transposon jumped or if there was homologous recombination between the DNA flanking the transposon insertion and the identical sequences in the Cxx chromosome. The isolates where homologous recombination occurred are then tested for loss of pathogenicity.

Once the functions of the various genes involved in pathogenicity are determined, easily identifiable markers can be incorporated into them to study patterns of expressions. This

work is important to determine the specific signals that control the expression of these genes. To accomplish this, gene fusions are generated by inserting a reporter gene such as β-galactosidase or GUS into, and in frame with, the genes involved in pathogenicity. Then the expression of the reporter gene can be measured under various conditions to determine when the particular gene is activated and what levels of gene expression occur.

Long term, the objective of this research is to design a gene construct that can be used to transform sugarcane plants with RSD resistance. The transposon tagging work is currently the best approach for identification of genes involved in pathogenicity. It is also the best way to identify something new and unique in terms of bacterial pathogenesis. It is specificly this unique pathway for causing disease that is being researched so that a control strategy for RSD can be designed.

5.8 Conclusions

Some major advances have occurred in the study of the molecular basis of disease caused by Cxx. The first transformation and mutagenesis systems for Cxx have been developed. A pathogenicity assay for screening Cxx mutants has been established and the first Cxx mutant that cannot colonise sugarcane identified. The progress in the area of Cxx molecular biology has been admittedly slow, primarily because Cxx is so difficult to work with. However, this approach to studying Cxx has several advantages over other methods of controlling RSD. The breeders do not want to have another selection criterion put on their breeding lines. However, they would welcome transgenic plants resistant to Cxx. The current control practices are costly and difficult to implement. In addition, because Cxx produces no reliable external symptoms it is easy for escapes to occur, as is evidenced by the recent infection of clean seed plots. By tagging genes and studying their specific function, there is potential to find something new and unique which is, therefore, patentable. Any genes that develop from this project will be owned by the industry, and can be traded or sold to all the sugarcane growing regions of the world.

An alternative approach would be to sequence the genome of Cxx. There are two disadvantages to this. One is that it is expensive and the second is that unique pathogenicity loci cannot be identified this way. However, the advantages far outstrip the disadvantages. Genome sequencing is one of the fastest growing areas of biological research. The genomes of a dozen microbes have already been sequenced. This year the complete genomes of at least nine additional bacteria will be completed and next year twice that many. If the genome of Cxx were sequenced, it could be compared to all the other genomes and DNA databases. All of the Cxx regions with homology to the well characterised genes and regions from other bacterial pathogens (ie pathogenicity islands) can be identified from the databases. This information will be invaluable for indicating how this pathogen causes disease on sugarcane. In addition, the rest of the Cxx genome would be available for analysis.

The most powerful aspect of this type of research is that the genome of Cxx could be sequenced in less that three years based on current technologies. This is the same amount of time it will take to clone, sequence and characterise the other genes flanking the one tagged by the transposon insertion in the Cxx mutant that cannot colonise sugarcane. Both techniques are very powerful tools for unravelling the basis of pathogenicity in Cxx.

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Table 1 Bacterial strains used in this study

Bacterium	Abreviation	Strain	Source
Clavivacter xyli subsp *xyli*	Cxx	ACM2272	Type Culture Strain
		ACM2271	UQ Microbiology
		QPF110-45	This study
		QPF110-108	This study
		QPF110.1	This study
		QPF83S	This study
		QPF115	This study
		QPF122	This study
		QPF124	This study
		QPF130	This study
		QPF138	This study
		QPF153	This study
		B1B	Mike Davis
		SP70-3370	Eugenio Ulian
		NA56-79	Eugenio Ulian
		F1	Mike Davis
		L1A	Mike Davis
		J1	Mike Davis
		S1A	Mike Davis
Clavibacter xyli subsp. *cynodontis*	Cxc	MDE1	Crop Genetics International
		FB2	Mike Davis
		TB1A	Mike Davis
		TB2A	Mike Davis
	Cxc-like	ACM3	Trevor Leaman
		ACM63	Trevor Leaman
		ACM4963	Trevor Leaman
		ACM4964	Trevor Leaman
		ACM4966	Trevor Leaman
		ACM4968	Trevor Leaman
Clavibacter michiganensis subsp. *insidiosum*	Cmi	ACM4951	UQ Microbiology
Clavibacter michiganensis subsp. *michiganensis*	Cmm	ACM932	UQ Microbiology
		ACM1745	UQ Microbiology
		ACM4950	UQ Microbiology
Clavibacter michiganensis subsp. *nebraskensis*	Cmn	ACM4952	UQ Microbiology
Clavibacter michiganensis subsp. *sepedonicus*	Cms	BSES155	Salke DeBoer
Clavibacter michiganensis subsp. *tesselarius*	Cmt	BSES154	Salke DeBoer
Clavibacter tritici	Ct	ACM3998	UQ Microbiology
Clavibacter iranicus	Ci	ACM3997	UQ Microbiology
Clavibacter rathayi	Cr	ACM4954	
Xanthomonas albilineans	Xa	BSES868	This Study
Pseudomonas rubrilineans	Prub	BSES	This Study
Pseudomonas syringae	Psyr	BSES	Chris Hayward
Pseudomonas solanacearum	Psol	BSES	Chris Hayward

Table 2 Plasmids used to transform Cxx

Plasmid	Relevant Characteristics	Source or Reference
PDM302	Cmm/*E. coli* shuttle vector Km^R, Cm^R	Meletzus *et al.* 1993
PDM306	Cmm/*E.coli* shuttle vector Km^R, Gn^R	Meletzus *et al.* 1993
PCG188	Cxc/*E. coli* shuttle vector Ap^R, Cm^R	Taylor *et al.* 1993
PUCD623	Tn4431 containing promoterless lux operon from *Vibrio fischeri* on pSa325 suicide vector Amp^R, Cm^R, Tc^R	Shaw *et al.* 1988
PSUP1021	Suicide vector containing Tn5 Ap^R, Cm^R, Km^R	Berg & Berg 1983
PLAFR3	Broad host range cosmid cloning vector Tet^R	Staskawicz *et al.* 1987
PLAFR5	Improved broad host range cosmid cloning vector Tet^R	Keen *et al.* 1988
PΔLUX19XK	*Vibrio harveyi* lucuferase (*lux*AB) fusion driven by *lac* promoter, in pBIN19 Km^R	Mudge *et al.* 1996

Table 3 *Clavibacter xyli* subsp. *xyli* appears to have a *dam* and possibly some other type of DNA methylation system

Restriction Enzyme	Cxx DNA Digested	Blocked by *dcm* †	Not Blocked by *dcm*	Blocked by *dam* ‡	Not Blocked by *dam*
*Alu*I	+				
*Apa*I	+	+	-		
*Bam*HI	-				
*Bst*YI	+				
*Eco*RI	-				
*Eco*RV	-				
*Hha*II	+				
*Kpn*I	-	-	+		
*Mbo*I	-			+	-
*Mse*I	-				
*Not*I	+				
*Nsi*I	-				
*Pst*I	+				
*Pvu*I	+				
*Sac*I	+				
*Sac*II	+				
*Sau*3A	+			-	+
*Sph*I	+				
*Ssp*I	+			-	+
*Xba*I	-				

† *dcm* – A gene encoding a DNA methylation enzyme that methylates cytosines bases on the DNA strand; ‡ *dam* – A gene encoding a DNA methylation enzyme that methylates adenosine bases on the DNA strand

Table 4 Sequence of Operon Technology (USA) Primers that amplify bands from Cxx

Oligonucleotide Primer	Oligonucleotide Sequence (5'-3')	Size Band Amplified
OPC-2	GTGAGGCGTC	797 bp
OPC-11	AAAGCTGCGG	1127 bp

Table 5 Oligonucleotide primer pairs selected from Cxx-specific, partially sequenced, SCAR regions

Oligonucleotide Primer	Oligonucleotide Sequence (5'-3')	Plasmid from which Oligonucleotide Primer Designed
2T3	AGGTGGCAGCGACAACGGTGC	PSKC-2
2T7	CGAGTTTGGGTTTCTGCCTGC	PSKC-2
11T3	GTGATTCGCACCATGAAGAGC	PSKC-11
11T7	TTCGAGGATAAAGTTAGGTGC	PSKC-11

Table 6 Samples analysed in Figure 9

Lane # Top Gel	Species	Strain Designation	Lane # Bottom Gel	Species	Strain Designation
1	Cxx	QPF83S	20	Cxc like	ACM4963
2	Cxx	B1B	21	Cxc like	ACM4964
3	Cxx	SP70-3370	22	Ct	ACM3998
4	Cxx	L1A	23	Cmm	ACM4950
5	Cxx	NA56-79	24	Cmn	ACM4952
6	Cxx	QPF110-1	25	Ci	ACM3997
7	Cxx	QPF138	26	Cmi	ACM4951
8	Cxx	QPF122	27	Cr	ACM4954
9	Cxx	QPF115	28	Cxc	TB2A
10	Cxx	QPF110-108	29	Cxc	FB2
11	Cxx	J1	30	Psol	
12	Cxx	S1A	31	Psyr	
13	Cxx	QPF153	32	Cxc	MDE1
14	Cxx	QPF130	33	Prub	BSES127
15	Cxx	F1	34	Cmt	BSES154
16	Cxx	QPF124	35	Cms	BSES155
17	Cxx	ACM2271	36	Xa	BSES868
18	Cxx	QPF110-45	37	Cxx	QPFUNK
19	Negative control		38	Cxx	ROY1

Table 7 Oligonucleotide primer pairs selected from Cxx-specific SCAR regions

Oligonucleotide Primer	Oligonucleotide Sequence (5'-3')	Plasmid from which Oligonucleotide Primer Designed
CL-2F	GAACGCGGTTTGGAATCAGAG	pSKC-2
CL-2R	ACCGAGAACGTCAGGGCTATC	pSKC-2
CL-11F	CGCGAATCCACCATCACACACA	pSKC-11
CL-11R	TCCACAGCCGAAGCGTCTCAG	pSKC-11

Table 8 Samples analysed in Figures 13 and 14

Lane	Species
1	*Clavibacter xyli* subsp. *xyli*
2	*Clavibacter xyli* subsp. *cynodontis*
3	*Clavibacter michiganensis* subsp. *michiganensis*
4	*Clavibacter michiganensis* subsp. *nebraskensis*
5	*Clavibacter michiganensis* subsp. *insidiosum*
6	*Clavibacter tritici*
7	*Clavibacter iranicus*
8	*Clavibacter tritici*
9	*Agromyces ramosus*
10	*Curtobacterium lutem*
11	Negative control

Table 9a Analysis of xylem extracts for Cxx using 4 different techniques

Variety	Plot	PCM	EIA Rating[1]	PCR-UQ	PCR-BSES
Q138	RSD	+	++	+	+
Q138	HEALTHY	-	-	-	-
Q122	HEALTHY	-	-	+	-
Q107	RSD	-	-	+	-
Q122	RSD	+	+	+	+
Q107	HEALTHY	-	-	-	-
Q107	HEALTHY	-	-	-	-
Q138	RSD	+	++	+	+
Q122	RSD	+	+	+	+
Q107	RSD	-	-	+	-
Q117	HEALTHY	-	-	-	-
Q117	HEALTHY	-	-	-	-
Q138	HEALTHY	-	-	-	-
Q107	RSD	-	-	-	-
Q117	RSD	+	+	+	+
Q107	HEALTHY	-	-	+	-
Q107	HEALTHY	-	-	-	-
Q117	RSD	+	+?	+	+
Q122	RSD	+	+	+	-
Q117	RSD	+	+?	+	-
Q117	HEALTHY	-	-	-	-
Q138	RSD	+	++	+	+
Q122	HEALTHY	+	+	+	+
Q122	HEALTHY	+	+?	+	-
Q117	RSD	+	+	+	+
Q117	HEALTHY	-	-	-	-
Q107	RSD	+	+?	+	+
Q122	HEALTHY	-	-	-	-
Q138	HEALTHY	-	-	-	-
Q122	RSD	+	+	+	+
Q138	HEALTHY	-	-	+	-
Q138	RSD	+	++	+	+

[1] ? – The optical density readings were borderline positive from the EB-EIA.

Table 9b Analysis of xylem extracts for Cxx using 4 different techniques

Variety	Plot	PCM	EIA Rating[1]	PCR-UQ	PCR-BSES
Q107	HEALTHY	-	-	-	-
Q107	HEALTHY	-	-	-	-
Q107	HEALTHY	-	-	+	-
Q107	HEALTHY	-	-	-	-
Q117	HEALTHY	-	-	-	-
Q117	HEALTHY	-	-	-	-
Q117	HEALTHY	-	-	-	-
Q117	HEALTHY	-	-	-	-
Q122	HEALTHY	-	-	+	-
Q122	HEALTHY	+	+	+	+
Q122	HEALTHY	+	+?	+	-
Q122	HEALTHY	-	-	-	-
Q138	HEALTHY	-	-	-	-
Q138	HEALTHY	-	-	-	-
Q138	HEALTHY	-	-	-	-
Q138	HEALTHY	-	-	+	-
Q107	RSD	-	-	+	-
Q107	RSD	-	-	+	-
Q107	RSD	-	-	-	-
Q107	RSD	+	+?	+	+
Q117	RSD	+	+	+	+
Q117	RSD	+	+?	+	+
Q117	RSD	+	+	+	-
Q117	RSD	+	+	+	+
Q122	RSD	+	+	+	+
Q122	RSD	+	+	+	+
Q122	RSD	+	+	+	-
Q122	RSD	+	++	+	+
Q138	RSD	+	++	+	+
Q138	RSD	+	++	+	+
Q138	RSD	+	++	+	+
Q138	RSD	+	++	+	+

[1] ? – The optical density readings were borderline positive from the EB-EIA

Table 10 Detection of *Clavibacter xyli* spp. *xyli* in sett-inoculated glasshouse plants

Treatment	Plant	Cxx cells x 10^3 per sample at intervals after inoculation		
		Week 2	Week 4	Week6
SDW	1	0	0	0
	2	0	0	0
	Average	0	0	0
Pure Culture (RSD 1)	1	0	0	0
	2	0	0	0
	3	0	0	540
	4	0	0	38
	5	0	0	84
	Average ± Std. Error	0	0	130 ± 100
Infective xylem exudate	1	35	230	7 700
	2	130	390	5 800
	3	180	27	4 400
	4	8.1	88	6 900
	5	14	58	63
	Average ± Std. Error	73 ± 34	160 ± 67	5 000 ± 1 400

Table 11 Detection of Cxx cells in shoot inoculated glasshouse plants

Treatment	Plant	Cxx cells x 10^3 per sample at intervals after inoculation		
		Week 2	Week 4	Week6
SDW	1	0	0	0
	2	0	0	0
	Average	0	0	0
Pure Culture (RSD 1)	1	0	0	0
	2	0	0	0
	3	150	0	0
	4	55	0	0
	5	0	0	0
	Average ± Std. Error	41 ± 29	0	0
Infective xylem exudate	1	1 400	45 000	170 000
	2	0	4 300	180 000
	3	1 500	19 000	160 000
	4	0	21 000	310 000
	5	1 400	4 600	210 000
	Average ± Std. Error	860 ± 350	19 000 ± 7 400	210 000 ± 27 000

Table 12 Detection of Cxx cells in shoot inoculated tissue culture plants

Treatment	Plant	Cxx cells x 10^3 per sample at intervals after inoculation		
		Week 1	Week2	Week3
SDW	1	0	0	0
	2	0	0	0
	Average	0	0	0
Pure Culture (RSD 1)	1	0	0	19 000
	2	0	5 800	43 000
	3	8 800	57 000	25 000
	4	0	0	17 000
	5	0	37 000	31 000
	Average ± Std. Error	1 800 ± 1 800	20 000 ± 12 000	27 000 ± 4 700
Pure Culture (RSD 2)	1	TMTC[1]	25 000	38 000
	2	TMTC[1]	72 000	61 000
	3	9 400	39 000	39 000
	4	TMTC[1]	17 000	37 000
	5	TMTC[1]	12 000	50 000
	Average ± Std. Error	9 400	33 000 ± 11 000	45 000 ± 4 600

[1]TMTC – Too many to count

Table 13 Effect of varieties on the colonisation of sugarcane plants in tissue culture by Cxx

	Inoculum[1]	Cultivars Tested							
		Q90	Q110	Q124	Q136	Q137	Q145	Q155	Peloris
Cxx Isolates per field of view[2]	None	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)
	QPF110-45	+ (4 – 5)	++ (>20)	+++ (>50)	+++ (>50)	+ (<10)	+++ (>100)	+ (<10)	++ (<20)

[1]Cells were scooped up off a plate culture of Cxx using a flame-sterilized metal loop and were smeared onto the cut surface of each of the different varieties.
[2]Samples were examined using oil immersion, 100x plan objective, phase contrast microscopy.
– no Cxx seen in any field of view; + < 10 Cxx seen per field of view; ++ >10 but <50 Cxx seen per field of view; +++ >50 Cxx seen per field of view; numbers in parenthesis represent the approximate number of Cxx seen per field of vision.

Table 14 Tissue culture pathogenicity assay for Cxx

Inoculum Concentration[1]	Varieties Tested		
	Q110[2]	Q136	H60-6909[3]
10^7 CFU/µl	3/3	3/3	2/3
10^5 CFU/µl	2/3	2/3	2/3
10^3 CFU/µl	1/3	1/3	0/2
0 CFU/µl	0/3	0/3	0/3

[1] CFU – colony forming units
There were 3 reps of each and data are reported as the number of plants that were positive for Cxx / the total number of plants in the rep. In one of the experiments one of the reps became contaminated and was discarded.

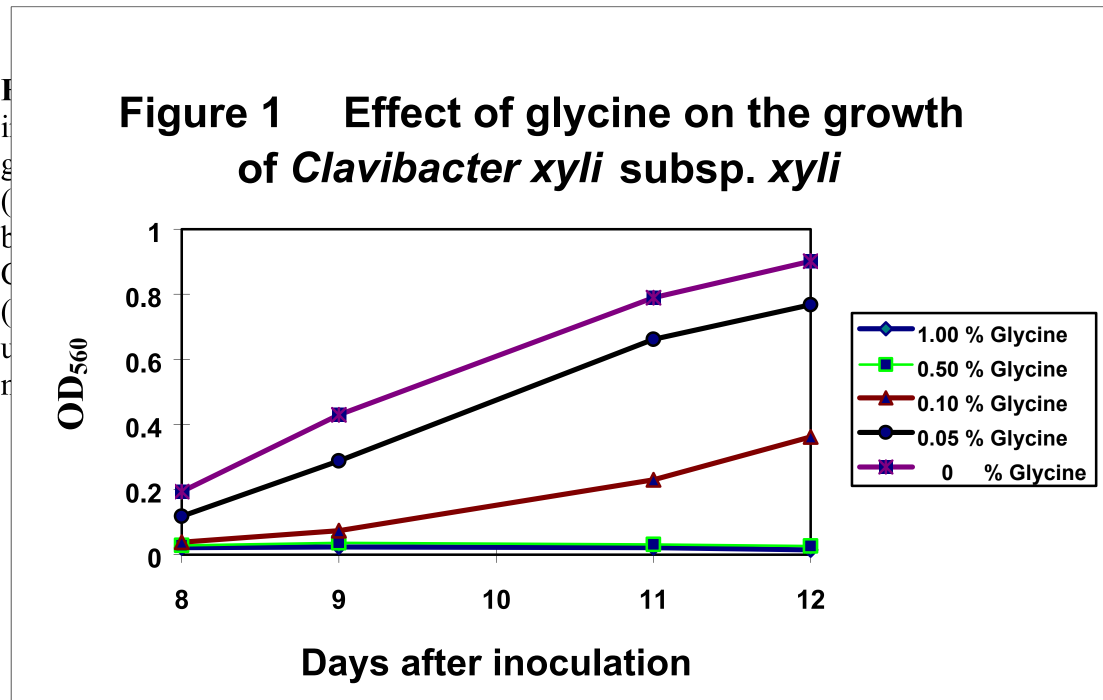

Figure 1 Effect of glycine on the growth of Clavibacter xyli subsp. xyli
OD560
1
0.8
0.6
0.4
0.2
0
8
9
10
11
12
Days after inoculation
1.00 % Glycine
0.50 % Glycine
0.10 % Glycine
0.05 % Glycine
0 % Glycine

Figur ... ure and placing
500 µ ... a scintillation
count ... nter and 0.5 µl
of n-c ... cap and were
reassa ... enhanced with
additi ... ate was added,
light p ... three different
cultur

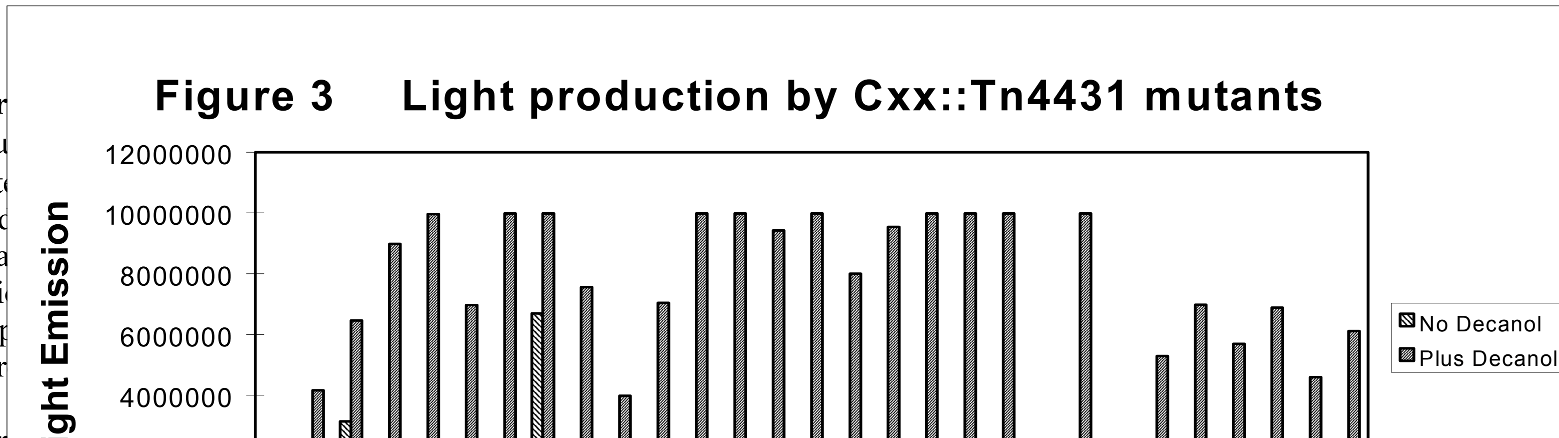

Figure 4 Light production by Cxx::Tn4431 mutants

Figure 17: A comparison of two different inoculation techniques, sett or inoculation onto the cut surface of decapitated plantlets. This histogram shows that Cxx is able to colonise decapitated sugarcane plants faster than setts. In studies with the transposon mutants, we were unable to get any colonisation into setts except when xylem extract from a Cxx infected sugarcane plant was used, the positive control. However, all of the mutants that were inoculated onto decapitated 5 week old plantlets colonised and were reisolated (data not shown). Bars show means with standard errors from five replicates.

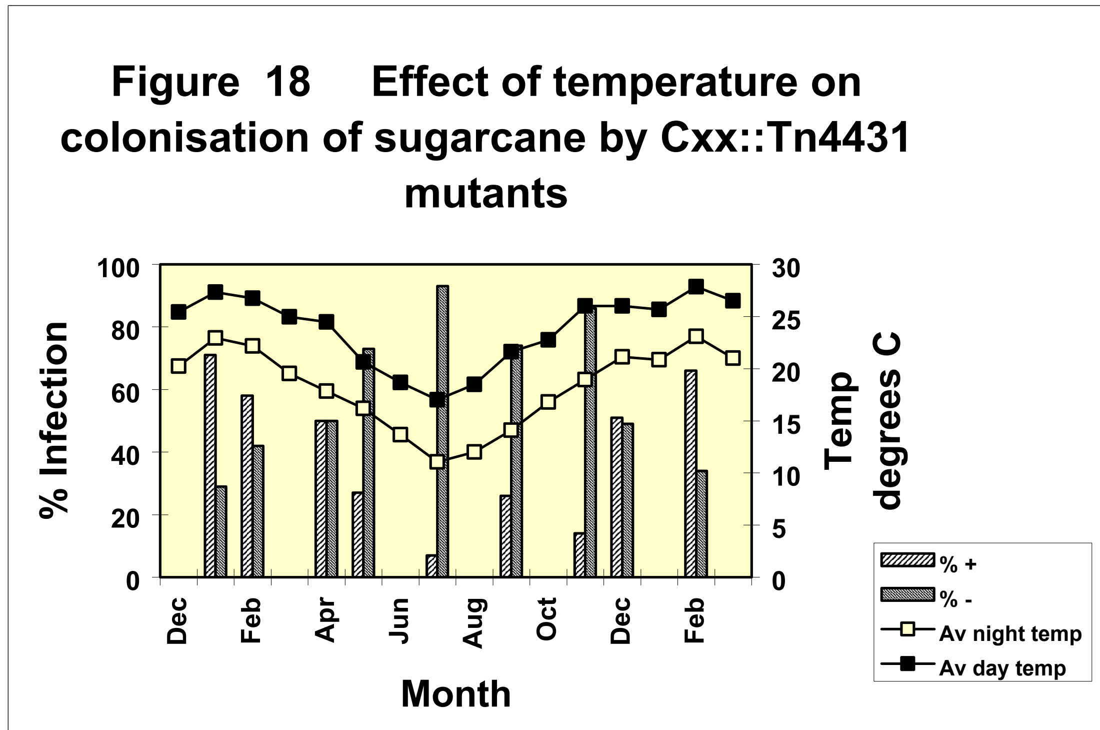
Figure 18 Effect of temperature on colonisation of sugarcane by Cxx::Tn4431 mutants
% Infection
100
80
60
40
20
0
30
25
20
15
10
5
0
Temp degrees C
Dec
Feb
Apr
Jun
Aug
Oct
Dec
Feb
Month
% +
% -
Av night temp
Av day temp

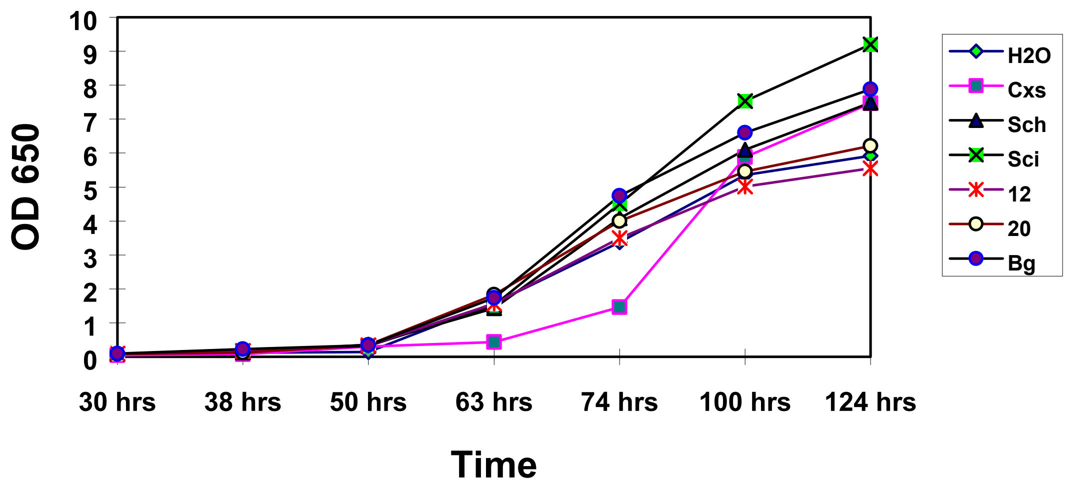
Figure 19 Growth induction of Cxc by plant extracts
OD 650
10
9
8
7
6
5
4
3
2
1
0
30 hrs
38 hrs
50 hrs
63 hrs
74 hrs
100 hrs
124 hrs
Time
H2O
Cxs
Sch
Sci
12
20
Bg