



**CSIRO Plant Industry
Cotton Research Unit**

FINAL REPORT

**Project title: Identification and management of
Bunchy Top syndrome in cotton.**

Project code: CSP143C

A final report prepared for the Cotton Research and Development Corporation

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Annual, Progress and Final Reports

REPORTS

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Part 4 – Final Report Executive Summary

Cotton Bunchy Top is a disease of cotton that was first reported in the 1998-99 cotton season. Symptoms of the disease include shortening of internodes and petioles, small leaves and fruit. CBT affected plants also have a reduced photosynthetic rate, and reduced cotton lint and seed yields. Research in this project aimed to (i) identify the causal agent of CBT and develop a probe to detect it (ii) identify the vector(s) of the disease and investigate its transmission characteristics and alternative hosts and (iii) Assist in selection of resistance to CBT in elite cotton germplasm. These are discussed below;

- Identification of the causal agent. Despite exhaustive efforts the agent causing CBT remains unidentified. Tests looked for viruses (common RNA virus, circular DNA Geminivirus and nanovirus, double stranded RNA viruses and a range of virus specific ELISA test including Barley Yellow Dwarf Virus), viroids, phytoplasmas and endoparasitic fungus hyphae. Testing also employed subtractive hybridisation, which did not reveal any non-cotton genes, and cDNA microarrays, which did reveal three clones (genes) that did not match the cotton genome. Unfortunately the project ended before these genes could be characterised.
- The disease is vectored by *Aphis gossypii* (cotton aphid) and attempts to transmit it using *Myzus persicae* (green peach aphid) or *Aphis craccivora* (cowpea aphid) failed. Cotton aphid transmits CBT semi-persistently, meaning they can transmit the disease to multiple plants over several days. Aphids can acquire CBT after feeding on CBT affected plants for five minutes and can inoculate a healthy plant after feeding for one hour. As few as one aphid can transmit the disease though more aphids do it more effectively. Cotton plants infected with CBT are capable of infecting aphids with the disease after a period of about 16-17 days, about 2 weeks before symptoms were obvious in the plants.
- *Gossypium barbadense* cv Pima S7 is a symptomless host of CBT, while the resistant variety DeltaOpal did not host the disease.
- Two alternative hosts of the disease have been identified, both from the family Malvaceae. These are *Malva parviflora* and *Malvastrum americanum*.
- Over the past four seasons we have screened cotton varieties in the field and glasshouse for resistance to CBT. This has led to selection of CBT resistance in several elite lines for commercial release of the first variety in 2005-06.

This project has provided the basic information to begin to understand the field epidemiology of this disease. It is likely that it is acquired by aphids feeding on an alternative host. Aphids transmit the disease semi-persistently, so they are infective for days after leaving alternative hosts and entering cotton crops. Cotton plants can become infected by the feeding of a single aphid, though more aphids results in higher transmission rates. Cotton plants colonised by aphids become new CBT sources after about 17 days and show clear symptoms after 3 – 5 weeks. Initial spread of the disease is slow as aphids are in the apterous form but as populations build alates begin to appear and secondary transmission to other parts of the field can occur.

CBT continues to threaten the industry both due to yield reductions and due changes in growers and consultants perceptions of aphids – resulting in lower thresholds, increase spraying and selection for insecticide resistance in aphids. Potential problems due to CBT have been hidden in recent years due to drought reducing over winter survival of aphids. There is a need for continued research to select for CBT resistance in elite germ plasm, to identify the agent so that a probe can be developed to facilitate understanding field epidemiology and breeding and there is a need to further study the field epidemiology so that outbreaks of CBT can be predicted and managed.

Detailed report

1. Background to the project.

In the 1998-1999 season, a disease-like syndrome commonly termed “Cotton Bunchy Top” (CBT) occurred in cotton crops. The syndrome was reported from crops across NSW and Queensland from the Macquarie Valley in the south to the Emerald region in the north, with the Darling Downs region worst affected. Affected plants consistently show shortened internodes, smaller leaves, and small (pigeon-egg) bolls. These disorders result in an overall compact growth in the affected part of plants and hence the name bunchy top. In addition, mottling of leaves, reddish pigmentation of young growth and occasionally, swelling of the nodes have been observed in some of the affected plants. Bolls from affected parts of plants contain less fibre but the same plants can carry normal bolls on the unaffected lower branches depending on the crop stage and plant position at which the infection occurred. Crop loss estimated from a detailed CRC survey, suggest the value of loss to be about \$70m in that year.

The syndrome recurred in the 1999-2000 growing season but in a limited scale. Nevertheless, it has raised two important issues: a) that CBT may continue to be a limiting factor to cotton yield with variation in severity and spread from year to year, and b) preliminary evidence suggests that the cause may be a disease spread by cotton aphids. This project aimed to identify the agent of CBT, investigate its vectors, transmission characteristics and epidemiology and assist in procedures for breeding of varieties resistant to CBT.

This project had three core objectives;

- i. To identify the causal agent of CBT and develop a probe that could be used to test for the presence of the disease in plants and insects.
- ii. To identify the vector(s), transmission characteristics and alternative hosts of the disease, to help understand its field epidemiology
- iii. To assist the CSIRO plant breeding group in selection and screening of cotton varieties resistant to CBT.

The first component of work was undertaken at the CSIRO Plant Industry, Horticulture Research Unit, at Adelaide, while the second two objectives were largely undertaken at the CSIRO Plant Industry, Cotton Research Unit, at Narrabri. Below a summary of results is provided against these objectives. Further detail can be found in the two manuscripts included as appendices to this report.

2. Achievements against objectives

a. Identification of the causal agent

Despite exhaustive efforts for the duration of the project the causal agent of CBT remains unidentified. Significant progress was made in the final month of the project but unfortunately the termination of the project and subsequent departure of the researcher, Dr Akhtar Ali precluded finalization of the last stages of testing. The tests applied and outcomes are given below;

- i) *Test for common RNA viruses*

Claims of luteovirus infection of cotton have been made overseas. Serological tests on CBT were done with the antisera of 3 luteoviruses and 2 seedborne viruses but the results showed that CBT infected samples did not react with any of them.

ii) *Test for nano viruses*

An aphid-transmitted disease of cotton was reported in Vietnam recently. As CBT is also aphid-transmitted, a suspected DNA virus (Nano-virus) test was carried out. Total DNA from both infected and healthy cotton was subjected to PCR using the primers designed from conserved regions of replication gene of the three nanoviruses. DNA of the replication gene of from nanovirus was used as positive control. The PCR results did not show the expected band from the CBT infected DNA as was observed from that of positive control.

iii) *Double stranded RNA isolation*

Double stranded RNA is a hallmark of many RNA virus infections. A rigorous attempt to isolate viral replicative form dsRNA from different tissues of CBT-infected cotton was unsuccessful while control tissues produced such RNA.

iv) *Viroid test*

Attempts to detect circular viroid-like RNA in infected CBT tissue by 2-D gel electrophoresis were unsuccessful. Viroids were isolated from grapevine tissues used as a control.

v) *Circular DNA pathogen like Geminivirus*

Detection of circular DNA from CBT infected tissues using the bacteriophage ϕ 29 DNA polymerase that amplifies circular DNA in a rolling circle amplification mechanism also failed. A tomato geminivirus was used successfully as a control.

vi) *Test for endoparasitic fungus hyphae*

As a quick test for endoparasitic fungi, different parts of the roots were stained with a fungus stain. However, no mycelium or hyphae growth was observed in CBT-infected roots.

vii) *Phytoplasma*

Using primers from the phytoplasma genome, total nucleic acid from leaf, petiole, stem bark and roots of CBT-infected and healthy plants were tested by PCR. However, the PCR results did not show the expected band from the CBT infected tissues as observed from the positive control.

viii) *Electron microscopy*

A number of tissue samples from infected plants including leaf, stem, main veins, petiole, bark, roots were thoroughly examined by transmission electron microscopy (TEM). However, no virus-like particles or inclusion bodies were observed. Infected cotton aphids, which transmit CBT, were also examined by TEM but so no virus like particles were observed

ix) *Purification*

Different purification protocols for virus extraction were used to purify CBT. Purified protein was examined by TEM and also by electrophoresis. No difference was observed between the CBT infected and healthy cotton tissues suggesting that the agent cannot be purified easily or the concentration is very low.

x) *Subtractive hybridization*

Subtractive cDNA libraries were prepared from CBT infected leaves, petiole and bark tissues and a total of 10,000 clones were screened. Many differentially expressed genes were isolated. However, when the sequence of all these were compared in sequence databases, all of them matched with cotton and other plant genes.

xi) *cDNA microarray*

An EST cDNA library was prepared from CBT infected leaves and DNA chip of 6000 clones were prepared. The chip was hybridized with the probes prepared from messenger RNA of CBT infected and healthy leaves. Of the 20 differentially expressed clones sequenced, 17 of them matched with cotton genes in the sequence database while three of them did not match to any known sequence. Further analysis of these genes is required to examine an association with the CBT pathogen genome.

b. Identification of vector(s), transmission characteristics and field epidemiology

i) *Symptoms*

Leaf symptoms

The most common symptoms of CBT in the field and in glasshouse were a pale yellow, angular mosaic pattern around the edge of the leaves with the remainder of the leaf blade usually dark green in colour, glossy appearance and leathery texture and a distinct stunting of the plants.

Root symptoms

The symptoms included the formation of a mass of hairy structures and small knots on the root branches. The colour of the roots from CBT infected plants were consistently dark brown compared to yellow light brown colour of the roots from healthy cotton plants.

Leaf surface morphology

Stomata of CBT infected leaves on both upper or lower surface were severely affected. They were found to be closed consistently, and to be small, oval or square shaped and in some cases misshapen. The epidermis was rough and ruptured. In contrast, the stomata of healthy leaves were open and long, cylindrical and uniform in shape and the epidermis was smooth and compact.

Effect on cotton growth, photosynthesis and yield

Leaf area, plant height, dry weight of bolls, roots, stems and photosynthetic rate of CBT infected cotton in the field was reduced compared with healthy plants. Specific leaf weight was increased in CBT infected plants but the number of flowers, nodes, squares and the dry weight of flowers and squares were unaffected. The higher specific leaf weight of CBT affected leaves indicates that they were thicker than the controls. The yield of cotton (g lint/metre) was significantly reduced at both sites in CBT affected sections of the crop.

ii) Transmission

Mechanical transmission

Seedlings of cotton varieties (DP90 and Sicot 70) and *Nicotiana benthamiana* that were inoculated mechanically did not show any symptoms 8 weeks post inoculation. These results indicate that CBT is not transmissible mechanically to the above tested species and the extracted sap was non-infectious.

Seed transmission

None of the 3930 plants from three CBT susceptible cotton varieties developed any CBT symptoms over a period of 20 weeks from the time of germination. These results suggest that transmission of CBT through seed is unlikely, or is less than 0.15%.

Graft transmission

Plants of cotton varieties Deltapine 90 and Sicala 40 grafted with CBT infected scions developed typical CBT symptoms 25-35 days after grafting. Symptoms of CBT also appeared on the rootstock and were not affected by the removal of infected scions after the establishment of the graft. None of the plants grafted with healthy scions showed any symptoms.

Aphid transmission

Experiments confirmed that *Aphis gossypii* (cotton aphid) was a vector for CBT. Further experiments aimed to characterise transmission to help understand the patterns of infestation and spread observed in the field. A single aphid per plant was capable of transmitting CBT, though to only 1 in 20 plants. Increasing the number of aphids per plant to three increased transmission to 50% of the cotton seedlings and 20 aphids per plant transmitted the disease agent to nearly 80% of the cotton seedlings. *A. gossypii* acquired CBT after a minimum acquisition access period (feeding time on an infected host) of 5 min. An aphid carrying CBT could transmit the disease to a healthy host after a minimum inoculation access period (feeding time on the healthy host) of 1 h.

Both alate (winged) and apterous (wingless) aphids and nymphal instars 2, 3 and 4 of *A. gossypii* transmitted CBT. Instar 1 did not transmit the disease suggesting that trans-ovariole transmission of the disease (from mother to daughter) is unlikely although the possibility that these stages had not fed before being placed moved from infected to healthy hosts cannot be discounted. *A. gossypii* grown on CBT infected plants could retain and transmit the disease for up to 7 days. These data suggest that *A. gossypii* transmits CBT in a semi persistent manner.

Studies were done to establish the time period for a healthy host to become capable of infecting aphids with the disease. This was done by placing CBT carrying aphids on a healthy plant (source plant), removing them after one day, then placing 'clean' aphids on the plant and at intervals transferring some of these aphids onto healthy hosts (test plants) that were then monitored for symptoms of CBT. Many problems were experienced with these experiments but the results indicate that plants are 'infective' after about 13-15 days after aphid feeding (20 aphids). Significantly this is about 2 weeks before the source plants showed symptoms of the disease.

iii. Alternative Vectors

Cultures of *Aphis craccivora* (cowpea aphid) and *Myzus persicae* (green peach aphid) were established on healthy cotton plants. Groups of 20 aphids were placed on CBT affected plants for at least two days then transferred onto healthy cotton plants of a susceptible variety (Sicot 70), and these plants monitored for symptoms of CBT. This was replicated x time and five healthy plants that did not receive aphids were controls. No symptoms were found in the healthy plants that received either of the aphid species suggesting that they are not vectors, or at best poor vectors.

iv. Alternative hosts and symptomless hosts

Studies were conducted to test if some of the common Malvaceous weeds in the cotton regions were hosts for CBT. Plants of a range of Malvacea were germinated from seed and grown in a glasshouse. These included Sida (*Sida rhombifolia*), Marshmallow (*Malva parviflora*), Malvastrum (*Malvastrum americanum*), Narrow-leaf bladder ketmia (*Hibiscus trionum* var. *trionum*), Wide-leaf bladder ketmia (*Hibiscus trionum* var. *vesicarius*), Velvetleaf (*Abutilon theophrasti*), Anoda weed (*Anoda cristata*), and Native rosella (*Abelmoschus ficulneus*). These plants were infested with CBT carrying aphids, with 2-3 plants left not infested as controls. The aphids were controlled after several days. Plants were then left for 4-5 weeks to grow to see if they developed CBT symptoms. Healthy aphids were then placed on the plants and allowed to feed for several days, before being transferred to susceptible cotton plants. Results were;

Positive results

Marshmallow (*Malva parviflora*): Aphids cultured on bunchy top infected cotton were transferred to four Marshmallow plants. All four plants developed angular mottling on some leaves. Also, when they were young they were considerably smaller than the control plants. Healthy aphids put on infected Marshmallow survived on all four plants. The aphids from three of these plants transmitted CBT when transferred to Sicot 70. One of the *M. parviflora* was infested with healthy aphids twice, and both times these aphids transmitted CBT to Sicot 70.

Malvastrum (*Malvastrum americanum*) : (common around Gwydir valley). Bunchy top aphids survived on three plants. Healthy aphids put on later survived on two plants. The aphids from one of these plants transmitted CBT to Sicot 70.

Negative results

Narrow-leaf bladder ketmia (*Hibiscus trionum* var. *trionum*) : Bunchy top aphids did not survive.

Wide-leaf bladder ketmia (*Hibiscus trionum* var. *vesicarius*) : Bunchy top aphids did not survive.

Velvetleaf (*Abutilon theophrasti*) : Bunchy top aphids did not survive.

Anoda weed (*Anoda cristata*) : Bunchy top aphids survived on two plants, but the healthy aphids put on later did not survive.

Native rosella (*Abelmoschus ficulneus*) : Bunchy top aphids survived on two plants. Healthy aphids put on later survived on one plant, but did not cause CBT when transferred to Sicot 70.

Currently in the glasshouse are *Sida rhombifolia*, *Hibiscus panduriformis*, *Malvastrum americanum* (again), and *Bidens pilosa*. Work in this area needs to continue to understand the range of possible alternative hosts of CBT

Experiments were also done to determine if another cotton species was a host for CBT. Cotton plants of *Gossypium barbadense* (Pima) were grown in pots in a glasshouse. Scions from Sicot 70 cotton showing clear CBT symptoms were grafted onto five healthy Pima

cotton stems, with a further five Pima plants receiving grafts from healthy Sicot 70 as controls. After 9 weeks, the Pima cotton regrowth below the graft showed no symptoms of CBT, compared to control plants. Scions from the Pima cotton regrowth from both treatment and control plants was then grafted onto a healthy Sicot 70 plants. Sicot 70 plants receiving grafts from the controls did not develop CBT but those receiving grafts from the Pima plants that received CBT affected scions did, indicating that Pima was a symptomless host for this disease.

As similar experiment was done to test if a CBT resistant variety did not host the disease or was a symptomless host. This experiment showed that DeltaOpal did not show symptoms nor did it carry the disease.

c. Resistance screening

The CSIRO Cotton Breeding Team, led by Dr Greg Constable, has been breeding for resistance to CBT in Australia varieties, using varieties with established resistance to the disease. This project has facilitated the breeding process by screening new lines for resistance to CBT, both in the glasshouse in winter and in the field through summer.

The fields screening used ratoon rows infected with CBT as a source of infection for aphids that were then transferred to the test lines. A CBT nursery was established in 2000-01 in which designated rows were sown with a CBT susceptible variety. The plants in these rows were infested with aphids carrying CBT. The aphids were obtained from a glasshouse culture maintained on CBT affected plants. The plants were ratooned through the winter and allowed to grow again the following spring. This provided a large number of plants with CBT. Large cages were erected over sections of these ratoon rows and aphids from a glasshouse culture introduced. Other aphids were also placed on plants outside the cages. The cages serve as a predator free environment to mass increase the aphids on CBT affected ratoon growth. These plants in the cages, and those on plants outside the cages with sufficient aphids, were cut into small sections and distributed across the plantings of lines to be screened. The aphid populations on these test lines were allowed to build until all plants were infested, after which they were controlled and CBT symptoms assessed in each line. Using this technique over a thousand lines were screened each year.

In the glasshouse lines to be tested were planted in pots at a density of 5 plants per pot with four replicate pots per line. A known susceptible and resistant line of CBT were included as controls. Pots were arranged in a randomised block design. When plants had several true leaves each plant was infested with about 20 CBT infected aphids. The aphids were allowed to feed and reproduce for about a week after which they were controlled. The plants were monitored for symptoms of CBT for the next 4-6 weeks.

The screening process has allowed Cotton Breeding Team to select several lines which have been to seed nursery and candidates for commercial release in 2005. Further screening will ensure that CBT resistance is included in more elite germplasm.

3. Conclusions (“take home messages”)

- i. CBT was recorded at high levels throughout the industry in the 1998-99 cotton season, and has occurred in each season since at ever diminishing levels. This is probably largely related to drought conditions which have meant that the over winter abundance of hosts for aphids has been low, probably also reducing the abundance of alternative hosts

affected with CBT. Nevertheless, CBT remains a constant threat to the cotton industry for two reasons. Firstly the disease itself can cause significant losses of yield. The earlier that crops are infected the greater the potential yield loss. If we have a wet winter then there is the risk that this disease will occur at levels significant to reduce yields on a widespread basis. Secondly, the desire of growers and consultant not to lose yield to CBT has changed attitudes toward aphids. Where previously growers and consultants were prepared to tolerate aphids at up to the threshold of 90% of plants infested, the fear of CBT has caused a lowering of thresholds. This is because once affected with CBT plants do not recover. This has led in some years to early and more frequent use of insecticides against aphids contributing significantly to widespread selection of carbamate and organophosphate resistant aphid strains.

- ii. The agent that causes CBT remains unidentified, despite a determined effort to isolate it, as does that of similar diseases vectored by cotton aphid elsewhere in the world. Successful identification of the agent of CBT would probably provide the methodology to also identify the various forms of Blue Disease in Africa, South America and South-East Asia.
- iii. CBT is vectored by the cotton aphid. It appears to be at least semi-persistent in the aphids, meaning they can transmit the disease to multiple plants over several days. Aphids can acquire CBT after feeding on CBT affected plants for five minutes and can inoculate a healthy plant after feeding for one hour. As few as one aphid can transmit the disease though more aphids do it more effectively.
- iv. *Malva parviflora* and *Malvastrum americanum* are alternative hosts for CBT and may be important in the field epidemiology of the disease. The outbreaks of CBT in the 1998-99 cotton season followed a wet winter with abundant growth of *M. parviflora*.
- v. The CSIRO breeding team has successfully bred CBT resistance into several elite germplasm lines which will result in commercial varieties in 2005-06.

4. Detail how your research has addressed the Corporation's three Outputs - Economic, Environmental and Social?

This research has identified the vector, two alternative hosts and characterised the transmission characteristics of CBT, a disease capable of significantly reducing cotton yields. The project has also contributed significantly to the selection of new cultivars with resistance to the disease. These outcomes will help to ensure that cotton production can remain viable should seasonal conditions again favour outbreaks of CBT. Understanding of the epidemiology of the diseases provides growers with a realistic understanding of disease progress and the risks associated with the presence of aphids in fields. Combined with the use of resistant varieties this can reduce the fear of CBT, which drives excessive early season control of aphids. It also therefore reduces selection for insecticide resistance in aphids and the risk of environmental contamination due to off-farm movement of insecticides. Ensuring the continued viability for cotton against the threat of a new disease will ensure that communities and support industries dependent on the cotton industry remain healthy.

5. Future research opportunities:

Though significant progress has been made in this project there are still a number of areas that need further research.

- a. The identification of the agent remains a priority. Aphid borne cotton diseases are present in Africa, South America and South East-Asia. Though in some of these

regions the threat from these diseases determines the varieties grown (e.g. Blue Disease in Argentina) or the strategies taken to manage aphids (e.g. Blue Disease in Vietnam). Successful identification of the agent could lead to development of a probe that would enable the disease to be identified in plants and insects, thereby making elucidation of the field dynamics of the disease much more achievable. It would also facilitate the breeding program by allowing plants to be tested for the presence of CBT, thereby reducing the chances of false negatives.

- b. The field epidemiology needs further work to understand how aphids acquire the disease, which alternative hosts are important, how the cycle of infection develops through the year, the relative level of infection of aphids with the disease and the rate of spread of the disease within cotton fields. This would enable simple modelling of disease outbreaks and perhaps of forecasting of season with a high risk. This would enable growers and consultants to alter management strategies or varietal choices to minimise risks in potentially 'bad' CBT years.
- c. The development of molecular markers would greatly assist the breeding process by eliminating the need for the complex field and glasshouse bioassay experiments.

6. Publications.

Industry publications

Reddall, A., Able, A., Stonor, J., Heimoana, S., Tesoriero, L., Rezaian, A. and Wilson, L. (2001) Hunting down the cause of Cotton Bunchy Top
Australian Cottongrower 22(4): 8-10

Wilson, L., Rezaian, A., Forrester, N., Franzmann, B., Wright, P., Heimoana, S and Thomas J (2000) Bunchy Top Research. In, *Proceedings of the Farming Systems Forum*, December 5th and 6th, Dalby, 2001.

Reddall, A. A., Ali, A., Able J., A., Stonor, J., Tesoriero, L., Wright, P., Rezaian, A. M. and Wilson, L. (2002) Cotton Bunchy Top (CBT) characteristics and modes of transmission. Proceedings of the 11th Australian Cotton Conference. Brisbane, Australia.

Referred Publications

Reddall, A. A., Ali, A., Able, J. A., Stonor, J., Tesoriero, L. Wright, P. R., Rezaian, M. A. and Wilson, L. J. (2004). Cotton bunchy top (CBT): an aphid and graft transmitted cotton syndrom. *Australian Plant Pathology* 33(2) 197-202.

Papers in preparation:

Ali, A., Reddall, A. A., J., Tesoriero, L. Wright, P. R., Rezaian , M. A. and Wilson, L. J. (2004). Cytopathology, modes of transmission by aphids and search for the casual agent of cotton bunchy top disease by various techniques. (in internal review CSIRO)

Ali, A. and Rezaian , M. A. and Wilson, L. J. (2004). Identification of differentially expressed genes in cotton by CBT infection using subtractive hybridisation and cDNA microarray analysis.

Appendix 1

Cotton bunchy top: an aphid and graft transmitted cotton disease

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Abstract

A new disease, termed cotton bunchy top (CBT), has been observed in Australian cotton fields since the 1998-99 cotton-growing season. Symptoms include short petioles and internodes, pale light green angular patterns on the leaf margins, and a leathery texture of mature leaves. Affected plants have a reduced photosynthetic rate, leaf area, plant height, number of bolls, dry weight of bolls, roots and stem and ultimately yield. CBT was demonstrated to be graft-transmissible in glasshouse experiments. In the field, CBT hotspots appeared to correlate with cotton aphid (*Aphis gossypii*) density and this species was identified as a CBT vector in controlled transmission tests. CBT symptoms and plant responses recorded in graft and aphid-inoculated plants were similar to those seen in the field. Seed transmission of CBT

appears unlikely as none of 3930 plants grown from seed of CBT infected plants developed symptoms.

Introduction

Cotton (*Gossypium hirsutum*) is an economically important crop to Australia worth approximately \$AU 1.6 billion in exports annually. Several viral diseases of cotton are of economic significance worldwide, including cotton leaf crumple and cotton leaf curl but neither have been observed in Australia (Briddon & Markham 2000; Rahman *et al.* 2001). A new syndrome, termed cotton bunchy top (CBT) after the bunched appearance of affected plants, has recently become a problem in major cotton growing areas in Australia. Growers first reported the disease in the 1998-99 cotton-growing season, from several regions including the Darling Downs and McIntyre Valleys in Queensland and the Namoi and Gwydir Valleys in New South Wales. It has since been reported from the Macquarie Valley in the south to the Emerald region in the north. In many instances, growers or their pest management consultants noted that CBT usually occurred in portions of fields that had been infested with cotton aphids (*Aphis gossypii*). This paper reports the first studies of CBT including characterisation of the symptoms and evidence that it is transmissible by cotton aphid and by grafting.

Materials and methods

Plant culture

Cotton seeds were sown in individual 10 L pots containing a grey vertisol soil typical of cotton growing regions (Constable *et al.* 1992). Seedlings were grown either in a glasshouse at 20-35°C under natural light or in a growth room at 25-30°C and a 16 h photoperiod. Thrive[®] fertiliser (16 g dissolved in 1.5 L water, 27% N; 5.5% P; 9% K plus trace elements) was applied to the plants once every three weeks. Magnesium

sulphate was also added twice during plant growth at a rate of 20 g per pot as the soil is known to be magnesium deficient.

Characterisation of plant responses to cotton bunchy top (CBT) in the field.

Twenty plants (ten with CBT symptoms and ten healthy) were selected from each of two commercial cotton fields, one at 'Hazeldene' cv Sicot 40, near Boomi, NSW, the other at 'Pindara' cv NuPearl, near Wee Waa, NSW, in mid-February 2000 when the plants were at the boll maturation phase. Symptomatic and healthy plants were collected in pairs within 1 m of each other to minimise any differences in soil or other environmental conditions. Plant characteristics including plant height, number of nodes, flowers, squares, bolls and dry weight of bolls, flowers, roots, squares and stems were recorded. Leaf area was measured at every third node upwards from node six, to the plant terminal using a leaf area metre (LI-3100, Lincoln, NE USA). Specific leaf weight was also determined for each of these leaves by dividing the leaf dry weight by the leaf area. Photosynthetic rate was measured on the youngest fully expanded leaf of the mainstem (3-4 nodes below the terminal) using a Licor, LI 6400 portable photosynthesis system (Lincoln, NE, USA). Data from CBT and healthy plants were compared using a paired t test.

The effect of CBT on cotton lint yield was assessed at Hazeldene and 'Whynot' cv Sicot 189, near Goondiwindi, Qld. When the plants had matured, in late March, the opened bolls were harvested from 1 m² areas of cotton in CBT affected and unaffected parts of the field. In each field, each 1 m² area of cotton affected by CBT was matched with an unaffected 1 m² area of cotton located within a distance of 5 m. These served as paired comparisons. Ten such pairs were harvested, 5 m² of CBT affected cotton and 5 m² of healthy cotton in each field. The harvested cotton for

each 1 m² was ginned to separate lint from seed and the lint weighed. Data for the two sites were combined for analysis using ANOVA.

Graft transmission

Sixty healthy plants each of cotton cvs Deltapine 90 and Sicala 40 received grafts, 45 from CBT infected scions and 15 from healthy scions. The grafts were from the same varieties and grafting occurred 40 days after sowing. A 'wedge' graft was carried out at the 3rd to 5th node from the base of the plant. After grafting, all branches and leaves were removed from the recipient rootstock and the subsequent regrowth was monitored weekly for the development of CBT symptoms. After 9 weeks, measurements were taken of the first main branch beneath the graft for petiole length, internode length, branch height, leaf area and specific leaf weight. Data from CBT affected and healthy plants were compared by one-way analysis of variance (ANOVA).

Aphid transmission

Cultures of the cotton aphid (*Aphis gossypii*) were reared on CBT-affected and on healthy cotton plants in insect-proof cages in separate glasshouses for at least 4 weeks. From each colony, aphids were transferred to 20 cotton plants cv. Sicot 70 at the 12 node stage at a rate of 100 aphids/plant. A similar number of uninoculated plants were kept as controls. Each plant was kept individually in an insect-proof nylon mesh cage. Ten days later the aphids were killed using imidacloprid followed by diafenthiuron. Plants were monitored for CBT symptoms over a 5-week period.

Six plants from each of the above three treatments were selected randomly 9 weeks after aphid inoculation and used for growth comparison. Petiole length, internode length and specific leaf weight (g/cm²) were measured from node 10 to the

terminal node. Leaf chlorophyll content was measured in the pale marginal mosaic areas and the darker green central areas of symptomatic plants and from the corresponding leaf of healthy plants using a SPAD 501 chlorophyll meter (Minolta, Osaka, Japan). At each position, five measurements were taken, and this data analysed using a one-way ANOVA

Seed transmission

Seed transmission of CBT was investigated in four cotton cultivars (Delta pearl, Nucleon 37, Siokra V16 and Sicot 70) using seed collected from glasshouse plants showing CBT symptoms. In October 2001, a total of 1930 seeds, and in October 2002, a further 2000 seeds were germinated in the glasshouse or in the field. These seeds were not delinted or treated with insecticide or fungicide to avoid possible effects on the causal agent. Insect pests were controlled as required and the plants were monitored regularly for symptoms until they were 5 months old.

Mechanical transmission

Ten seedlings each of cotton cvs DP90 and Sicot 70 and *Nicotiana benthamiana* were mechanically inoculated according to the procedure in Ali & Randles (1997). Sap extracts in 50 mM sodium borate buffer, pH 8.1, from CBT-affected plants were rubbed onto carborundum-dusted leaves of seedlings at the two-leaf stage. These seedlings and a similar number of uninoculated seedlings were kept in a growth room at 25-30°C and a 16 h photoperiod and monitored for symptoms over an 8-week period.

Results

Symptoms of CBT

The most common symptoms of CBT in the field were a pale yellow, angular mosaic on the margin of the leaf blade (Fig. 1A) and a distinct stunting of the plants. In the field, the pale angular pattern turned red as the leaves matured (Fig. 1B). The leaves of CBT infected plants were also thicker, glossier and had a leathery texture when compared to healthy plants. The affected plants had a compact, stunted and bunched appearance due to the shortening of the internodes and petioles.

Effect of CBT on plant growth and cotton yield

CBT-affected plants were shorter (cm, mean \pm SE, CBT; 64.5 ± 2.7 , Control; 83.9 ± 4.4), had a lower photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, CBT; 28.6 ± 1.6 , Control; 36.9 ± 0.8), smaller leaf area (Fig 2A) and lower boll, root and stem dry weight (Fig. 2D) when compared with the healthy plants. The numbers of flowers, nodes, squares (flower buds) (Fig. 2C) and the dry weight of flowers and squares (Fig. 2D) were unaffected. Specific leaf weight (Fig. 2B) was increased in CBT affected plants indicating that they were thicker than the controls. The yield of cotton was significantly reduced at both sites in CBT affected sections of the crop (g lint m^{-1} , CBT; 95.4 ± 7.7 , Control; 221.2 ± 20.8).

Graft transmission

Cotton cvs Deltapine 90 and Sicala 40 grafted with CBT- affected scions all developed typical CBT symptoms 35-55 days later, similar to those observed in the field. Symptoms of CBT also appeared on the rootstock and were not affected by the removal of the scions after the establishment of the graft. Graft transmission was reproducible in all 43 replicate plants. None of the plants grafted with healthy scions showed any symptoms.

Plants affected with CBT by grafting showed reductions in leaf area, petiole length, internode length and branch height, and increase in specific leaf weight compared to healthy plants (results not shown).

Aphid transmission

All cotton plants inoculated using cotton aphids that were reared on CBT-affected plants developed typical CBT symptoms 25 - 40 days after transfer of the aphids (Fig. 1 C-E), showing a 100% transmission rate. Mild CBT symptoms were observed after 3 weeks and became more conspicuous after 6 weeks. Plants that were inoculated with aphids reared on healthy plants did not show any symptoms and were similar to control plants that did not receive any aphids. Aphid inoculated CBT plants showed a decrease in leaf area (Fig. 3A), length of petiole (Fig. 3C) and internode (Fig. 3D), and increase in specific leaf weight (Fig. 3B). The chlorophyll content of leaf margins did not differ between treatments. In the centre of leaves it was higher ($P < 0.01$) for aphids plus CBT leaves than for the other treatments (Spad units, control; 44.0 ± 1.1 , aphids; 46.2 ± 1.6 , aphids plus CBT; 51.0 ± 1.5).

Seed and mechanical transmission

None of the 3930 plants germinated from seed from three CBT susceptible cotton cvs developed any CBT symptoms over a period of 20 weeks. Similarly, seedlings of cotton cvs DP90 and Sicot 70 and *Nicotiana benthamiana* that were mechanically inoculated did not show any symptoms after 8 weeks.

Discussion

While the cause of CBT has not been identified we have provided strong evidence that the aetiological agent is biotic and can be transmitted by *A. gossypii*. The most

characteristic symptom, a pale, angular mosaic pattern on the leaf margins appears 3 – 5 weeks after transfer of aphids from CBT-affected plants. CBT symptoms were more prominent in plants grown in the glasshouse than in field plants. This may be due to protection from stresses occurring in the field, such as insect damage, UV irradiation and water stress, which could mask symptoms. Other factors, such as plant age at the time of inoculation and the restricted growth period in the field may explain variation in disease symptoms among the CBT-affected plants in the field and glasshouse (Gray *et al.* 1991). The plants grafted with CBT infected scions showed strong responses in the branches that regrew beneath the graft point indicating that the agent moved downward into the rootstock possibly via the phloem tissues. Aphids feed predominantly on phloem cells (Miles, 1987), further indicating that the causal agent may reside in the phloem cells.

Some nutrient deficiency symptoms are similar to CBT symptoms. Magnesium deficiency causes foliar reddening in the field with the main veins remaining green (Hodges, 1992). In the early phase of magnesium deficiency, the leaves have yellow patches, with the veins remaining green (Rochester, 2001), similar to that of CBT. Interveinal chlorosis was noticeable in some of the glasshouse plants inoculated with CBT via graft and aphid inoculation. However, all the plants were regularly fertilised with magnesium sulfate and no magnesium deficiency symptoms were evident in the control plants.

Our results indicate that seed transmission of CBT is unlikely. Seed transmission rates may be related to plant age and symptom severity and the rate of seed transmission of plant diseases may be less than 1% (Pathipanawat *et al.*, 1997; Njeru *et al.*, 1997; Latham & Jones, 2001). While seed transmission of CBT can not be ruled out entirely, lack of symptom development in the 3930 plants tested suggest that seed transmission alone is unlikely to result in CBT epidemics. Similarly, CBT was

not transmitted via sap inoculation to susceptible cotton varieties and *Nicotiana benthamiana*.

A disease similar to CBT, cotton terminal stunt, was first reported in America during the 1960's (Sleeth *et al.*, 1963). While no casual agent was determined for terminal stunt, several similarities with CBT exist including mottled leaves and reduced internode length. Even so, clear differences are also evident between these two diseases. Discoloration (tan to dark brown streaks) of the xylem is the most consistent symptom of terminal stunt, but this was not observed in any CBT-affected plants. Other diseases that have some similarities to CBT include cotton blue disease in Africa and South America, cotton anthocyanosis in Brazil, and cotton leaf roll in Thailand, all of which are transmitted by *A. gossypii* (Cauquil & Follin, 1983; Brown, 1992). However, symptoms of CBT are not completely matched by any of these diseases. In each case, a virus is suspected as the causal agent but so far their aetiology is unclear and the causal agent(s) remain unknown.

The transmission of CBT by aphids poses a serious management problem for the cotton industry in Australia. Historically, this pest only occurred late in the cotton season, and was controlled with insecticides to prevent honeydew contamination of lint. However, in the late 1990's aphids at economic levels occurred earlier in the cotton season, and more alarmingly, many populations were resistant to organophosphate and carbamate insecticides, rendering control difficult and increasing selection for resistance to the remaining effective chemicals. This situation was exacerbated by the concerns of growers that aphids needed to be controlled at sub-threshold levels to prevent spread of CBT. This increased selection for insecticide resistance and threatened integrated pest management systems as most insecticides applied against aphids are non-specific and also affect beneficial insects.

In the 1998-99 cotton season, CBT was widespread across the Darling Downs region of Queensland. CBT was also reported from most other cotton regions, though

at a much lower incidence. Estimates based on grower surveys across the industry (data for 145 fields) suggest that up to 21% of fields across the industry could have been affected by CBT (Anonymous, 1999). More rigorous surveys of plant diseases across the cotton industry have shown that the incidence of CBT has declined significantly over the last four cotton seasons (1999-00, 2000-01, 2001-02, 2002-03) with less than 1% of fields having any affected plants in 2002-03 (D. Nehl, NSW Agriculture and S. Allen, CSIRO, pers comm.). This decline is possibly related to drought conditions which have resulted in (i) a lack of overwintering hosts for the cotton aphid or other potential vectors, and hence low populations in the following spring and summer, and (ii) a low abundance of alternative hosts of the disease, resulting in low inoculum levels. A change to more normal rainfall patterns may lead to conditions favouring survival of aphids and alternative hosts of CBT between cotton seasons and lead to further outbreaks of the disease. Understanding the cause and aetiology of CBT therefore remains a priority.

These initial studies have established CBT as a new cotton disease in Australia. Our efforts to isolate a virus in CBT affected tissue have thus far been unsuccessful (Ali *et al.*, unpublished results). Research is currently continuing on the determination of the casual agent and the development of a molecular probe to test for CBT as well as epidemiological studies. Experiments are also underway to determine acquisition period, the persistent or non-persistent nature of the disease and whether particular aphid instars are linked to transmission. A number of other aphid species including *Myzus persicae* Sulzer, *A. craccivora* Koch, *A. fabae* Scopoli and *Macrosiphum euphorbiae* Thomas also feed on cotton (Blackman & Eastop, 2000) and need to be tested for CBT transmission.

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Figure 1 Leaves of CBT affected cotton showing development of symptoms in the field for cv Sicot 189 showing pale yellow angular mosaic pattern at (A) early stage of development and (B) late stages of (note reddening around the edge of the leaf) and the development of CBT symptoms on leaves of glasshouse-grown cv Sicot 70 following inoculation using *A. gossypii*. C, D and E are symptoms 1, 3, 6 weeks after inoculation, respectively.

Figure 2 The effect of CBT on growth and yield of field-grown cotton plants cvs Sicot 189 and NuPearl combined. (A) Leaf area (B) specific leaf weight and (C) numbers and (D) weight of various plants parts. Vertical bars represented by symbols (*, **, ***) differ at $P = 0.05$, 0.01 and 0.001, respectively. Bars are means plus standard errors.

Figure 3 The effect of CBT on growth of glasshouse-grown cotton cv Sicot 70 plants inoculated using *A. gossypii*. (A) Leaf area, (B) specific leaf weight, (C) petiole length and (D) internode length. Vertical bars represented by symbols (*, **, ***) differ at $P = 0.05$, 0.01 and 0.001 respectively. Bars are means plus standard errors.

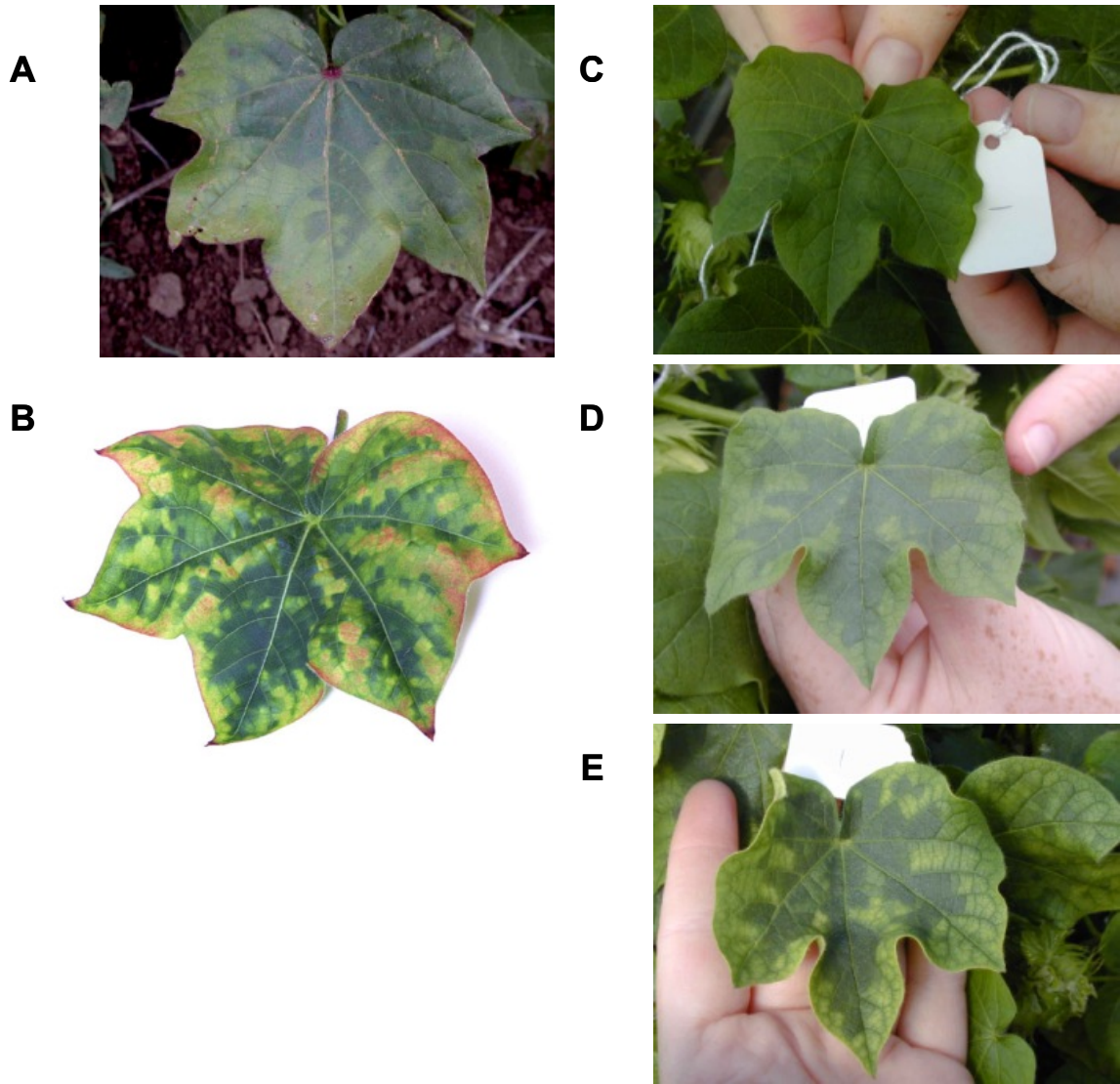


Fig. 1