H

January, August & Final Reports

Part 1 - Summary Project Details **REPORTS**

Please use your TAB key to complete parts1,2,4 & 5

CRDC Project Number: **CSP111C**

January Report:	☐	Due 29-Jan-01
August Report:	☐	Due 03-Aug-01
Final Report:	☒	Due within 3 months of project completion
Project Title:		New methods to enhance regeneration of cotton plants from tissue cultures to aid crop improvement

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The points below are to be used as a guideline when completing your final report.

1. Outline the background to the project.

Cotton is currently the only crop whose transgenic varieties are used in large-scale production in Australia. Transformation of cotton is therefore of special importance for the healthy development of plant biotechnology in Australia.

Unfortunately, cotton is also one of the most difficult plants to transform and regenerate from tissue culture. Currently, transformation of elite Australian cottons is through backcross to Coker varieties, which are no longer used in production. The main obstacle for direct transformation of Australian varieties, as well as most other non-Coker varieties around the world, is the lack of a reliable plant regeneration system for these varieties. CSIRO Plant Industry has had some success with one Australian variety, Siokra 1-4, and a breeding line, Siokra 1-3, but only at low frequency in both cases. There are no published reports about embryogenesis or regeneration of other Australian cotton varieties.

Even in the Coker varieties, which have been used as the model varieties in cotton transformation studies, transformation efficiency is low. To make things worse, this process is very time consuming, requiring more than 9 months to obtain transgenic plantlets from Coker varieties. In addition, it has been difficult to regenerate normal plants from cotton embryogenic callus, so the frequency of healthy transformed plants is very low even in the best available system.

One way to speed up the genetic transformation of cotton is to substantially improve the regeneration of plants from tissue culture. The initial phase of our research focussed on applying techniques that have proven successful in lower plants and in carrot tissue cultures to induce elite lines of cotton to regenerate from tissue culture.

In the course of this project, we concluded that a systematic study of the response of cotton to tissue culture conditions was necessary for development of reliable regeneration systems for cotton transformation. Most published reports are about success with specific genotypes, and the protocols outlined are essentially identical to earlier published protocols with small modifications. The paucity of information about culture conditions favouring cotton regeneration may be due to difficulties in collecting sufficient solid data for publication, as cotton tissue culture requires much more time and space than other commercial crops because the success rate is so low.

From 2000, we shifted the emphasis of this study to induction of embryogenic callus, hoping to trial plasmolysis when embryogenic callus became available. For this purpose, we started with a systematic test of published protocols for cotton embryogenesis, and also examined the response of cotton to plant hormones.

This approach proved productive. Within 12 months, we achieved embryogenesis in three Australian varieties (Siokra S101, Siokra 1-4 and V16). More importantly, we established a hormone-free culture system, which makes it possible to test the various factors affecting cotton regeneration and would be a very useful tool for future studies.

2. List the project objectives and the extent to which these have been achieved.

The specific objectives and achievements for each year of the project are listed below. Detailed results are given under point 4 below.

Year 1: **(i)** Establish tissue cultures of cultivars important to Australia
(ii) Multiply callus and suspension cultures for experiments
(iii) Induce embryogenesis from amenable cultures
(iv) Determine type, strength and duration of plasmolytica required to plasmolyse cells in culture, assess viability after recovery

(i) Both callus and suspension cultures of three cultivars important in Australia, namely, Siokra V-16, Sicala V-2 and Sicot 189, and cultures of the embryogenic line Coker 315, were established at Monash University. We also established carrot callus and suspension cultures since they are highly embryogenic, very fast to regenerate and have been very well characterised. This system was then used to carry out trial experiments before testing treatments in the much slower-growing cotton cultures.

(ii) Callus and suspension cultures were multiplied up in sufficient quantities for experiments.

(iii) We established cultures of the embryogenic Coker 315 line as a baseline against which to assess how well the treatments worked. However, the Coker callus grew more slowly than anticipated and we used a longer callus proliferation stage than the minimum time reported in the literature.

(iv) We carried out preliminary trials using three different solutions, glucose, calcium chloride and mannitol, to plasmolyse the tissue cultures. The optimum concentrations for the plasmolysis solutions were 1 M glucose, 0.6 M calcium chloride or 1 M mannitol, applied for 45 min, and best results were obtained using glucose as the plasmolyticum.

Embryoids were found, but they did not look like typical somatic embryos formed in suspension culture. The cotton embryoids we examined appeared to be encased in a layer of cells, similar to the layer found around zygotic embryos. The embryoids appear to burst through this layer of cells when maturing from the globular to the torpedo stage of development. Interestingly, this unusual type of regeneration is also seen in *Eucalyptus* suspension cultures.

Year 2: **(i)** Continue determination of appropriate plasmolytica
(ii) Preliminary trials of plasmolytica on amenable and recalcitrant tissues
(iii) Assess methods to determine embryogenic potential of cultures
(iv) Trial combinations of plasmolytica and cell polarity disrupters

Objectives (i), (ii), and (iii) were achieved. Note that in our standard protocol, seedlings were harvested 12 days after germination, then seedling tissue was placed onto agar containing hormones for 8 weeks (subcultured once at 4 weeks), then 1 g aliquots of the resulting callus were transferred to 250 ml flasks containing liquid medium without hormones. Embryos appeared after 4-6 weeks in suspension culture (subcultured every 2 weeks).

(i) We established that plasmolysis of the 8-week callus for 45 min in 1 M glucose, followed by a two-step deplasmolysis treatment prior to transfer into liquid medium, produced suspension cultures with the highest, most consistent embryogenesis. Treatments with other plasmolytica produced less suspension cell growth and fewer embryos.

(ii) Plasmolysis of callus from Sicala V-2, Siokra V-16 and Coker 315 prior to transfer into suspension culture did promote embryogenesis, but this promotion was not as great as we had hoped.

(iii) In our hands, the callus giving greatest embryogenic suspension cultures was wet, dark and very loose. Under the microscope, this callus had a diversity of cell sizes, and we suspect that the small clumps of small cells give rise to embryos in suspension culture. Light, friable, "fluffy" callus was much less embryogenic, and contained larger cells which often displayed irregular shapes. These cells were also very easily separated from each other.

Globular stage embryos were readily detected by flitering the suspension culture through a tea strainer. They appeared as small, dense globules, which were white in cultures from the Australian varieties of cotton, and green in cultures from the Coker variety.

Objective (iv) was not achieved in Year 2 since we planned to start this part of the project in early 2000. This objective was modified since the principal researcher, Dr. Janine Radford, ceased work on the project in December 1999. The project then moved from Monash University to CSIRO Plant Industry and a new principal researcher, Dr. Ding He, was appointed to the project and commenced work in April, 2000.

We also investigated several other treatments known to promote regeneration and embryogenesis in other species, and these are detailed in the Results (point 4 below).

Year 3: **(i)** Continue combinations of plasmolytica and cell polarity disrupters
(ii) Assess uniformity and synchronicity of embryogenesis from combinations of plasmolysis and herbicide treatments
(iii) Repeat and refine treatments that significantly promote embryogenesis
(iv) Trial new treatments that may promote embryogenesis and, in particular, trial treatments that may overcome the arrested state of somatic globular embryos formed in tissue culture.

After discussion with Dr. Ding He, these objectives were revised, and in effect, we jumped ahead to Objective (iv) of Year 3 (see below).

(i) Compare the responsiveness of Coker and elite Australian varieties to hormones and other factors, aiming to promote embryogenesis;
(ii) Establish a method to reduce the formation of non-embryogenic "fluffy" callus cells on callus induction medium, which may be detrimental to cotton embryogenesis;
(iii) Assess explants other than leaf and hypocotyl, for example immature embryos and flower organs, for their embryogenic potential;
(iv) Test the responsiveness of Coker and elite Australian varieties to different regeneration regimes that promote embryogenesis in other labs or in other species, including media containing a) 2,4-D and kinetin (CSIRO's current protocol); b) no hormones (to replicate a successful protocol established in Belgium); c) NAA and 2iP; d) zeatin; e) other combinations of hormones if time permits.

(i),(iii) These two objectives were combined and a range of hormone regimes was tested on different tissues from 10 lines of cotton. The three lines forming embryogenenic callus in other experiments, Coker 315, Siokra 101 and Siokra 1-4, showed fewer side-effects on BA-containing media. We concluded that to reduce the severe vitrification seen in shoot meristem tissue, the strong cytokinin BA should be omitted from induction media.

Our overall conclusion is that embryogenic callus can probably be obtained from many types of source tissue, but that hypocotyl tissue forms regenerative callus most readily.

(ii) From early results from experiments conducted at Monash, or outlined in Objective (i) above, and from subsequent experiments, we conclude that the formation of fluffy cells and the severe vitrification of explants on callus induction medium is a major obstacle for direct regeneration and is a cause of delayed embryogenesis in cotton. However, we cannot yet identify the main cause of these adverse responses, and therefore cannot yet suggest a method for overcoming them, but we suspect that a wound response to cutting of the tissue and placing on growth medium may be involved.

(iv) In conjunction with the experiments outlined above, we also tested other published protocols for inducing regenerative cotton callus. We concluded that either hormone-free induction medium, or medium with weaker hormones, produced greater quantities of embryogenic callus than media with high hormone levels. Once embryogenic callus has formed, it appears to be much less sensitive to hormones, particularly to cytokinins.

3. **How has your research addressed the Corporations three outputs: Sustainability of natural resources, profitability and competitiveness, and/or people and communities?**

The research addressed one of the three outputs: profitability and competitiveness. Our work aimed to improve profitability and competitiveness by shortening the time needed to generate new transgenic lines of cotton. We aimed to achieve this by developing new techniques to accelerate the regeneration of whole plants from transformed cells and tissues of elite lines of cotton. To date, we have demonstrated an improvement in the production of cotton tissue cultures that will go on to form embryo-like structures (embryoids) that are the precursors to new plants. However, before recommending general adoption of a new protocol, we need to demonstrate a much greater improvement in regeneration from tissue culture, and we need to demonstrate that the new protocol gives repeatable results.

Because the project started late (in October rather than July, 1999), then was interrupted during the shift from Monash University to CSIRO Plant Industry (December 2000 – April 2001) our observations are restricted to identification of embryoids in callus and suspension cultures. In order to establish the usefulness of any new protocol, we would need to demonstrate that these embryoids will go on to form healthy new plants that can be propagated for use on-farm. Even in Coker 315, which shows the highest rate of regeneration from culture, plantlets may become arrested or malformed early in development, so it will be essential to confirm the regeneration potential of these embryoids from other cotton lines.

4. Detail the methodology and justify the methodology used.

Methods used by Dr. Janine Radford, Monash University, October 1998 to December 1999

Adapted from protocols provided by Dr. Danny Llewellyn, CSIRO Plant Industry.

Sterilise seeds

- Sterilise cotton seeds in 70% ethanol for 30 seconds, followed by 20 min in 30% bleach with a drop of Tween 20 per 100 ml.
- Rinse at least 6 times with sterile distilled water.

Germination

- Place 2 seeds per tall flask (~40 ml of Germination Medium, Flask: 75.9922410 container 120 ml Natural cap. from Proscience). The seeds have a high germination rate ~90%.
- Grow at 28°C for 6 to 12 days under constant light using Gro-Lux tubes.

Callus induction

- The seedlings are harvested once they have secondary roots. (Wait until they reach the top of the flask or until the first true leaves have emerged.)
- Harvest cotyledons or hypocotyls and cut each one into four pieces.
- Place 2 pieces per dish on 90 mm petri dishes with MST medium (no more than 2 per dish or growth will be retarded).
- Seal plates with two layers of Husky masking tape and grow at same temp and lights as above. (Note: parafilm tends to retard growth rate).
- Subculture at 4 week intervals.

Plasmolysis (optional)

- Place 1 g of dark loose callus into a 10 ml centrifuge tube and add up to 10 ml of plasmolyticum (1M glucose, 1M mannitol, or 0.6M $CaCl_2$; 1M glucose works best).
- Shake to disperse callus and put on rotary shaker at 60 rpm for 45 min.
- Centrifuge for 1 minute at 1000 rpm, take off half of the supernatant and replace with Embryo Suspension Medium.
- Shake to disperse and leave for 5 min.
- Repeat twice but on the last rinse remove all of the supernatant and replace with embryo suspension medium.
- Alternative method: put 1g [~ 1.5 ml mark on tube] of callus in tube and top up to the 3.5 ml mark with plasmolyticum. Shake for 45 min, top up to 5.5 ml with ESM, shake for 5 min, top up to 9.5 ml with more ESM, shake for 5 min then spin, discard supernatant and replace with ESM as above).

Embryo regeneration

- Add 1 g dark, loose callus to 100 ml embryo suspension medium in 250 ml flask.
- Place on platform shaker at 100 rpm at 28°C.
- Subculture every 2 weeks with filtering.
- After 4 - 6 weeks separate out the embryos (by filtering through a tea strainer) and plate onto MSK agar plates.
- After another 4 weeks plant embryos on SH agar plates until roots form.
- Transfer into short fat pots with SH medium and grow until a proper root system is established and true leaves form.
- Transfer into potting mix and harden off.

To Plasmolyse:

Method adapted from Wetherell DF (1984) Enhanced adventive embryogenesis resulting from plasmolysis of cultured wild carrot cells. Plant Cell Tissue Organ Culture 3: 221-227

Autoclave:

2 autoclavable wash bottles
16 centrifuge tubes (10 ml) in a 400 ml beaker
5 ml pipette tips
16 x 250 ml flasks with sun caps (Sun caps: E6670 from Sigma)
300 ml distilled water
2 x 1 l Embryo Suspension Medium (ESM)
Glucose plasmolysing solution: remove 100 ml of the ESM and put into 250 ml Schott bottle with 18.016 g glucose (to give 1 M glucose)

Put 16 centrifuge tube lids in 30% bleach with a drop of Tween 20 into a 150 ml beaker and leave for 1 hour (they can't be autoclaved - they melt).

Procedure: All in laminar flow cabinet

- Put centrifuge tubes into blue rack.
- Pour distilled sterile water into the 400 ml beaker.
- Transfer (1 at a time, shaking the liquid) lids to 400 ml beaker and stir (to rinse off the bleach).
- Fill one wash bottle with glucose plasmolysing solution and one with embryo suspension medium.
- Place ~1 gram (~1 ml volume) of callus tissue into each centrifuge tube*. Each tube is from a new hypocotyl segment. Put lid on loosely.
- When all tubes are filled use the glucose plasmolysing solution to bring total volume in tube to 3.5 ml.
- Place lids on tightly, put on shaker at 60 rpm and leave for 45 min.
- Take tubes back to laminar flow cabinet and top volume up to 5.5 ml with the embryo suspension medium.
- Put on shaker for 5 min.
- Take tubes back to laminar flow cabinet and top volume up to 9.5 ml with embryo suspension medium.
- Put on shaker for 5 min.
- Spin tubes for 1 minute at 1000 rpm.
- In laminar flow, remove solution using 5 ml pipette.
- Rinse the tissue into sterile flasks twice using embryo suspension medium and top it up to 100 ml.
- Label with cotton species, medium type, plasmolysis treatment, date.

- Put on platform shaker at 28°C for 2 weeks then filter.

Note: You must gradually deplasmolyse your cells as per this procedure or you get explosive expansion and cell death.

*There are 2 different types of callus. White and prolific fluffy callus that does not form embryos, or grey-green wet shining callus. You want the grey-green wet callus.

To filter embryo suspensions:

Autoclave:

2 autoclavable wash bottle
2 x 1l embryo suspension medium
1 l conical flask
Large glass funnel
2 mm metal mesh
Special filter (short fat pot with end cut off and hole in lid with 149 μm hole (104 mesh) nylon cloth over the end).
600 ml distilled water
250 ml beaker

Procedure:

- Fill one autoclaved bottle with suspension medium and the other with sterile distilled water.
- Put funnel in 1 l conical flask. Put special filter in funnel, then put metal mesh on top of filter.
- Take sun cap off first 250 ml flask to be subbed.
- Pour in through metal mesh/filter/funnel.
- Take off metal mesh and place on top of 250 ml beaker and rinse with the sterile water.
- Transfer funnel and filter back to the 250 ml flask.
- Turn filter upside down and rinse into flask with embryo suspension medium.
- Remove funnel and filter and place back on 1 l conical flask.
- Top up 250 ml flask liquid to 100 ml using fresh embryo suspension medium.
- Replace suncap, put sub date on flask.
- Repeat with each flask.
- If doing multiple filtrations at 2 weeks apart you can omit the 2 mm metal mesh step after the first filtration.

For dry weight:

- Place a small petri dish into a normal petri dish and add dry silica gel between the two.
- Place a small filter disk into the small petri dish, cover with a large petri lid and dry the whole lot in oven at 70°C overnight. Weight the filter disk.
- Place disk in ceramic filter funnel and filter suspension onto filter using a vacuum.
- Wash the disk and dry in the petri dishes at 70°C for 24 h.
- Weigh again and subtract original disk weight to get the sample dry weight.

Germination medium (per litre)

Macronutrients	50 ml	pH to 5.8
Micronutrients	1 ml	
EDTA	5 ml	
$FeCl_3$	5 ml	
Vitamins	10 ml	
Glucose	30 g	
$MgCl_2$	0.94 g	
Phytogel	2 g	

Autoclave

Macronutrients (1 L)

NH_4NO_3	33.0 g
$CaCl_2$	8.8 g
KNO_3	38.0 g
$MgSO_4$	7.4 g
KH_2PO_4	3.4 g

Micronutrients (500 ml)

H_3BO_3	3.11 g
$MnSO_4$	11.15 g
$ZnSO_4$	4.3 g
KI	0.415 g
Na_2MoO_4	0.125 g
$CuSO_4$	0.0125 g
CoCl	0.0125 g

EDTA (500 ml)

Na_2EDTA	3.35 g

$FeCl_3$ (500 ml)

$FeCl_3$	2.70 g

Vitamins (100 ml - freeze in 10 ml lots)

Nicotinic acid	5 mg
Pyridoxine HCl	5 mg
Thiamine HCl	1 mg
Glycine	20 mg

MST (per litre)

Frozen basal stock	100 ml	pH to 5.8
Glucose	30 g	
$MgCl_2$	0.94 g	
Phytogel	2 g	

Autoclave. Leave about 50 min or until cool to touch.

2,4-D stock	100 µl
Kinetin stock	1 ml

(Do not autoclave the hormones but filter sterilise with a 0.22 µm filter and add to cool medium after autoclaving with phytogel.)

1 litre of medium fills ~40-50 petri dishes depending on the volume in each plate.

Basal Stock (2 L - freeze in 100 ml lots)

NH_4NO_3	33 g
KNO_3	38 g
$CaCl_2$	8.8 g
$MgSO_4$	7.4 g
KH_2PO_4	3.4 g
micronutrients	20 ml
$FeCl_3$	0.54 g
EDTA	0.67 g
Nicotinic acid	20 mg
Pyridoxine-HCl	20 mg
Thiamine-HCl	200 mg
Myo-Inositol	2 g

2,4-D stock
Dissolve 100 mg 2,4-D in 1 ml absolute alcohol.
Add 3 ml 1 M KOH.
Quickly add 80 ml dH_2O while stirring.
Adjust pH to 6 with 1 M HCl.
Adjust volume to 100 ml and store in fridge.

Kinetin stock
Dissolve 10 mg in 2 ml of 0.2 M HCl.
Slowly adjust volume to 100 ml with dH_20 and store in fridge.

Embryo Suspension Medium (per litre)

Frozen basal stock	100 ml	pH to 5.8
Glucose	30 g	
(Glutamine stock	40 ml) optional	

Autoclave

Glutamine (final sol. conc. = 10 mM)
Dissolve 14.6 g glutamine in 300 ml dH_20.
Heat gently and make up to 400 ml.

MSK medium (per litre)

Frozen basal stock	100 ml	pH to 5.8
Glucose	30 g	
KNO_3	1.9 g	
$MgCl_2$	0.94 g	

Adjust pH to 5.8

Phytogel 2 g

Autoclave

SH medium (1 L)

Frozen SH stock	100 ml	pH to 6.8
Sucrose	20 g	
$MgCl_2$	0.94 g	

Phytogel 2 g

Autoclave

SH stock (2 L - freeze in 100 ml lots)

KNO_3	10.1 g
NH_4NO_3	4.8 g
$MgSO_4$	9.86 g
$CaCl_2$	3.54 g
KH_2PO_4	0.54 g
Micronutrients (from stock)	40 ml
$FeCl_3$ (from stock)	15 ml
Na_2EDTA (from stock)	15 ml
Nicotinic acid	9.8 mg
Pyridoxine-HCl	16.4 mg
Thiamine-HCl	2.7 mg

Methods used by Dr. Ding He, CSIRO Plant Industry, April 2000 to July 2001

Tissue culture medium

In this study, MS medium was used as the basal medium (see pp. 14-15 for preparation of the medium), and the suspension culture stage was omitted.

The medium was supplemented with 3% glucose and 0.25% Phytagel in most experiments. These were changed in some experiments where carbohydrate type and gelling agents were tested.

All hormones were filter sterilised (except the few dissolved in DMSO) and added to the culture medium after autoclaving.

Sterilisation of seeds

1. Discard broken seeds and dirt.
2. Wash seeds with 30% swimming pool bleach (20 µl of Tween 20 added to 50 ml of bleach) to get rid of the dye and fungicide.
3. Sterilise seeds in 30% bleach for 30 minutes.
4. Wash at least 5 times with sterile water.
5. Blot dry the seeds with sterile filter paper.
6. Transfer the seeds into petri dishes and leave them open for several hours in the hood to dry the seeds.
7. The dry seeds can be stored at RT for at least a couple of months.

Germination of seeds

Place 20 seeds in a 9 cm petri dish with 40 ml of hormone free MS medium.

Explants

As the experiments were for research purposes, not for bulk production of regenerated plants, only the first two hypocotyl segments and the two pieces of cotyledon near the petiole were used. Explants from a single seedling were distributed into different treatments to reduce any artefactual responses due to variation between individual donor plants. The hypocotyls were cut into 3-4 mm long segments and cotyledons cut into 2-3 mm wide pieces. In most experiments, 6 explant pieces were placed in each petri dish containing 30-40 ml medium.

Treatments

A treatment consisted of 1-6 replicate petri dishes; most treatments contained 3 replicate dishes. As the experiments were mainly for observing cotton's response to a very wide range of treatments, rather than for collecting replicate data from a few treatments, no statistical analysis was carred out at this stage.

Culture conditions

Cultures were maintained at 28°C under dim light, except in the experiments testing effects of light intensity.

Formation of embryogenic callus

Formation of embryogenic callus was determined by microscopic examination of callus. Callus that contained distinct embryoids was defined as embryogenic. Shining friable (not fluffy) callus was termed pre-embryogenic.

Growth media

MS medium
Murashige and Skoog 1962

Chemical	MW	mg/l	mM
KNO_3	101.11	1900	18.79141
NH_4NO_3	80.05	1650	20.61212
$CaCl_2$ $2H_2O$	147.03	440	2.99259
$MgSO_4$ $7H_2O$	246.5	370	1.50101
KH_2PO_4	136.09	170	1.24917
$FeSO_4$ $7H_2O$	278.03	27.8	0.09999
Na_2EDTA $2H_2O$	372.24	37.3	0.10020
$MnSO_4$ $4H_2O$	223.06	22.3	0.09997
$ZnSO_4$ $7H_2O$	287.56	8.600001	0.02991
KI	166.02	0.83	0.00500
H_3BO_3	61.84	6.2	0.10026
$CuSO_4$ $5H_2O$	249.69	0.025	0.00010
$CoCl_2$ $6H_2O$	237.95	0.025	0.00011
Na_2MoO_4 $2H_2O$	241.98	0.25	0.00103
Inositol	180.16	100	0.55506
Thiamine HCl (vitamin B1)	337.27	0.1	0.00030
Nicotinic acid (vitamin B3; Niacin)	123.11	0.5	0.00406
Pyridoxine HCl (vitamin B6)	205.64	0.5	0.00243
Glycine	75.07	2	0.02664
pH	5.8		

Ion concentrations in the medium (mM)

NH_4	NO_3	(N)	K	Na
20.61212	39.40353	60.01565	20.04559	0.2024746
Ca	Mg	PO_4	SO_4	Cl
2.992587	1.501014	1.249173	1.730983	5.985384
Total cations	Total anions	Total ions	Amino acid N	Total N
45.58386	48.47036	94.05421	0.0266418	60.04229

MS Stock solutions

Chemical	mg per 100 ml	250 ml	500 ml	1000 ml	2000 ml
Macronutrients					
10 X Stock					
KNO_3	1,900.00	3,800.00	9,500.00	19,000.00	38,000.00
NH_4NO_3	1,650.00	3,300.00	8,250.00	16,500.00	33,000.00
$CaCl_2\ 2H_2O$	440.00	880.00	2,200.00	4,400.00	8,800.00
$MgSO_4\ 7H_2O$	370.00	740.00	1,850.00	3,700.00	7,400.00
KH_2PO_4	170.00	340.00	850.00	1,700.00	3,400.00
Iron					
100 X Stock					
$FeSO_4\ 7H_2O$	278.00	556.00	1,390.00	2,780.00	5,560.00
$Na_2EDTA\ 2H_2O$	373.00	746.00	1,865.00	3,730.00	7,460.00
Micronutrients					
100 X Stock					
$MnSO_4\ 4H_2O$	223.00	446.00	1,115.00	2,230.00	4,460.00
$ZnSO_4\ 7H_2O$	86.00	172.00	430.00	860.00	1,720.00
KI	8.30	16.60	41.50	83.00	166.00
H_3BO_3	62.00	124.00	310.00	620.00	1,240.00
$CuSO_4\ 5H_2O$	0.25	0.50	1.25	2.50	5.00
$CoCl_2\ 6H_2O$	0.25	0.50	1.25	2.50	5.00
$Na_2MoO_4\ 2H_2O$	2.50	5.00	12.50	25.00	50.00
Vitamins					
100 X Stock					
Inositol	1,000.00	2,000.00	5,000.00	10,000.00	20,000.00
Thiamine HCl	1.00	2.00	5.00	10.00	20.00
Nicotinic acid	5.00	10.00	25.00	50.00	100.00
Pyridoxine HCl	5.00	10.00	25.00	50.00	100.00
Glycine	20.00	40.00	100.00	200.00	400.00

4. Detail results including the statistical analysis of results.

The specific objectives and results achieved in each year of the project are detailed below.

Year 1: **(i)** Establish tissue cultures of cultivars important to Australia
(ii) Multiply callus and suspension cultures for experiments
(iii) Induce embryogenesis from amenable cultures
(iv) Determine type, strength and duration of plasmolytica required to plasmolyse cells in culture, assess viability after recovery

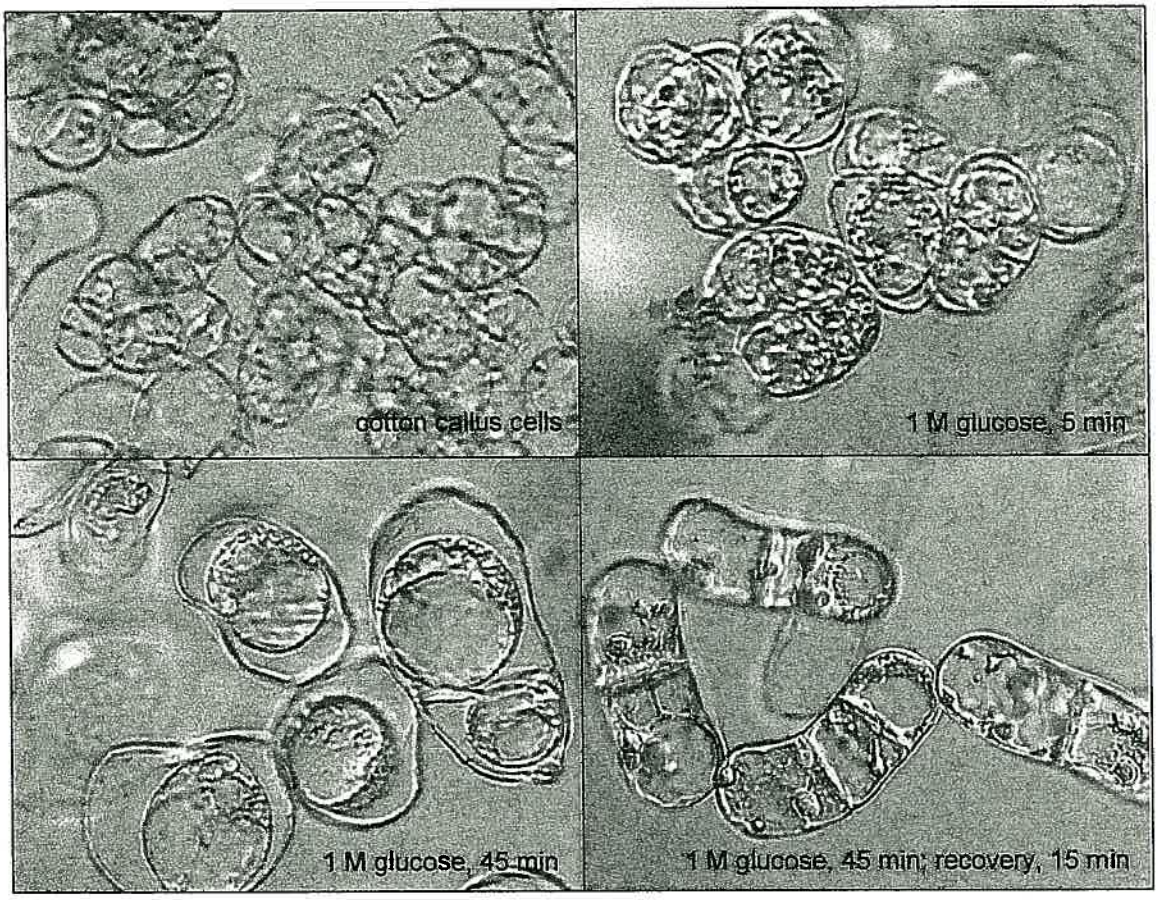
(i) Both callus and suspension cultures of three cultivars important in Australia, namely, Siokra V-16, Sicala V-2 and Sicot 189, and cultures of the embryogenic line Coker 315, were established at Monash University. We also established carrot callus and suspension cultures since they are highly embryogenic, very fast to regenerate and have been very well characterised. This system was then used to carry out trial experiments before testing treatments in the much slower-growing cotton system.

Initially, we followed the protocol developed at the CSIRO by Cousins, Lyon and Llewellyn (1991, Aust J Plant Physiol 18: 481-494) for Siokra I-3 tissue cultures, which gave low regeneration rates in this variety. Under this protocol, the time allowed for initial callus proliferation is 6 weeks. However, for the varieties we used, we found that this time was too short for adequate proliferation of embryogenic callus. We note that in the literature, the time required for generation of embryogenic callus ranges from 6 weeks to 7 months, depending on the variety used, with 2-4 months commonly used for many varieties. We determined that growing the callus cultures for a minimum of 3 months, with a transfer onto fresh medium each month, maintained rapid growth. This appeared to produce good quantities of callus that gave rise to vigorous suspension cultures.

(ii) Callus and suspension cultures were multiplied up in sufficient quantities for experiments, with preliminary results reported in (iv) below.

(iii) We established cultures of the embryogenic Coker 315 line as a baseline against which to assess how well the treatments worked. However, the Coker callus grew more slowly than anticipated and we used a longer callus proliferation stage than the minimum time reported in the literature. Embryoids formed and were transferred to solid medium then to soil as outlined in Embryo regeneration (p. 7).

(iv) We carried out preliminary trials using three different solutions, glucose, calcium chloride and mannitol, to plasmolyse the tissue cultures. Callus cells were monitored while they were undergoing plasmolysis in the treatment solutions and again during their recovery and deplasmolysis in nutrient solution without the plasmolytica. The optimum concentrations for the plasmolysis solutions were 1 M glucose, 0.6 M calcium chloride or 1 M mannitol, applied for 45 min. The cells survived best if allowed to recover slowly after plasmolysis. The plasmolyticum was diluted by half with the nutrient solution and the callus was allowed to equilibrate in this solution for 5 minutes. This process was repeated twice more, followed by a final 5 minute rinse in the suspension culture nutrient solution.

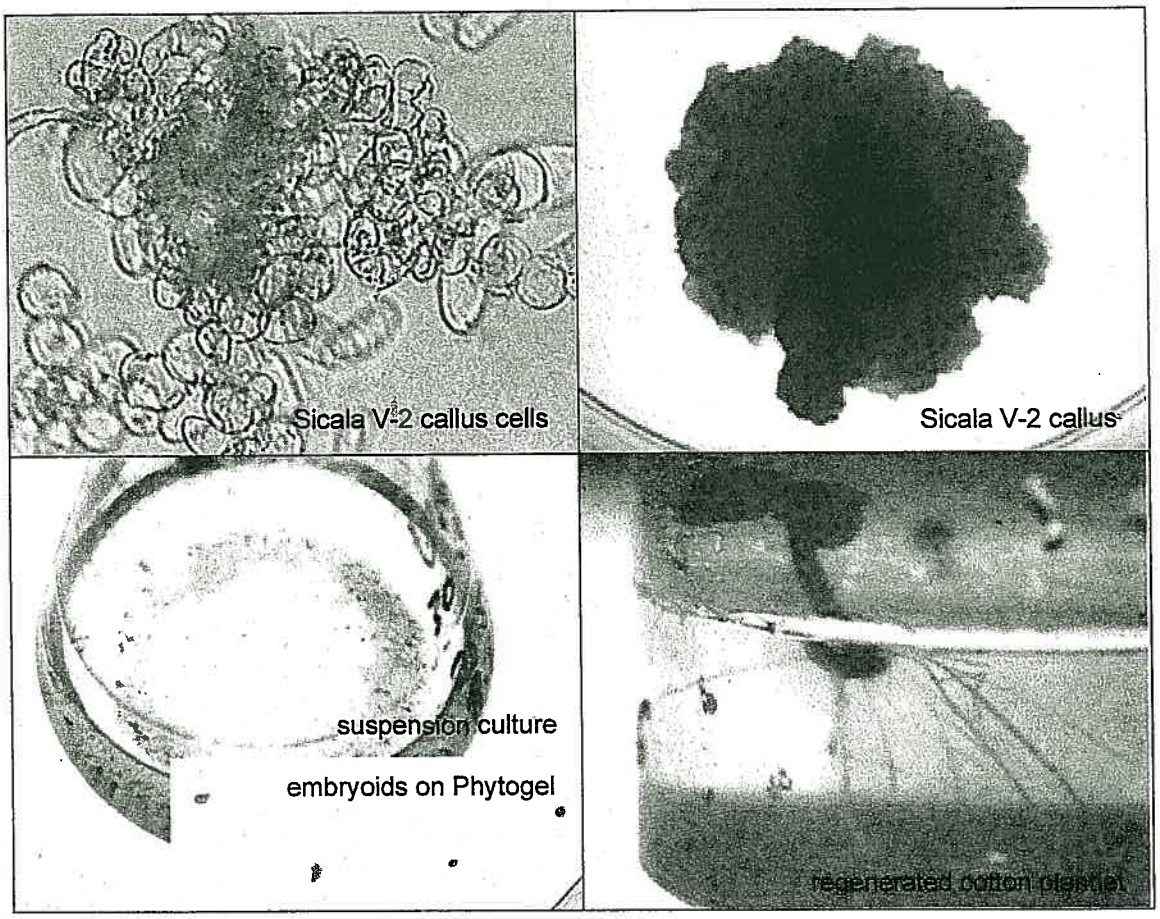


Callus cultures from all three Australian varieties that had been grown on the usual solid (Phytogel) medium with hormones to promote growth, were treated just before transfer to the liquid suspension solution, which lacks hormones.

The callus samples survived the treaments, and they were then grown in suspension culture for 4 weeks. They were harvested and examined much earlier than planned because they became contaminated. The autoclave we use to sterilise all media and glassware was not reaching a sufficiently high temperature and pressure for long enough for proper sterilisation. This meant that the suspension cultures all became contaminated with bacteria following their most recent transfer into fresh medium. In some lines, embryos can be isolated from suspension cultures at 2 weeks after transfer, but it is more usual to allow 2-4 months growth after transfer from the solid medium.

Nevertheless, there were a few young embryoids in the cultures. The table below gives the average number of embryoids found per 250 ml culture flask (total of 25 flasks examined).

cotton variety	plasmolysis treatment			
	no treatment	1 M glucose	1 M mannitol	0.6 M calcium chloride
Sicala V-2	1.0	4.2	3.3	2.5
Sicot 189	0.6	5.0	1.0	1.0
Siokra V-16	0.5	4.0	2.5	2.0



The embryoids did not look like typical somatic embryos formed in suspension culture. Somatic embryos are usually recognised first as round clumps of small, dense cells, which are the globular stage embryos. The later stages, torpedo and heart-shaped embryos, are usually also found naked in older suspension cultures. In contrast, the cotton embryoids we examined appeared to be encased in a layer of cells, similar to the layer found around zygotic embryos. The embryoids appear to burst through this layer of cells when maturing from the globular to the torpedo stage of development. Interestingly, this unusual type of regeneration is also seen in *Eucalyptus* suspension cultures.

Year 2: **(i)** Continue determination of appropriate plasmolytica
(ii) Preliminary trials of plasmolytica on amenable and recalcitrant tissues
(iii) Assess methods to determine embryogenic potential of cultures
(iv) Trial combinations of plasmolytica and cell polarity disrupters

Objectives (i), (ii), and (iii) were achieved. Note that in our standard protocol, seedlings were harvested 12 days after germination, then seedling tissue was placed onto agar containing hormones for 8 weeks (subcultured once at 4 weeks), then 1 g aliquots of the resulting callus were transferred to 250 ml flasks containing liquid medium without hormones. Embryos appeared after 4-6 weeks in suspension culture (subcultured every 2 weeks).

(i) We established that plasmolysis of the 8-week callus for 45 min in 1 M glucose, followed by a two-step deplasmolysis treatment prior to transfer into liquid medium, produced suspension cultures with the highest, most consistent embryogenesis. Treatments with other plasmolytica produced less suspension cell growth and fewer embryos.

(ii) The percentage of suspension flasks (10-26 250 ml flasks per treatment) containing embryos for three varieties was:

	Sicala V-2	Siokra V-16	Coker 315
non-plasmolysed callus	23.5	54.5	57.1
plasmolysed callus	35.1	88.9	77.6

We concluded that plasmolysis of the callus prior to transfer into suspension culture did promote embryogenesis, but this promotion was not as great as we had hoped.

Further treatments were investigated and assessed for the variety Sicala V-2:

	Percentage of flasks containing embryos	
	non-plasmolysed callus	plasmolysed callus
3% glucose in medium	23.5	35.1
1.5% glucose in medium	21.7	36.6
3% gluc. + activated charcoal	25.0	25.0
3% gluc. + activated c. in agar	8.3	6.7

	Number of embryos per gram fresh weight of suspension culture	
	non-plasmolysed callus	plasmolysed callus
3% glucose in medium	24.7	34.5
1.5% glucose in medium	12.7	43.0
3% gluc. + activated charcoal	-	-
3% gluc. + activated c. in agar	8.6	8.7

Key to treatments in tables above:

3% glucose in medium = standard sugar level in callus and suspension media

1.5% glucose in medium = reduced sugar level in callus and suspension media, tested because some species/varieties grow better with reduced sugar - this promoted embryogenesis somewhat in plasmolysed callus

3% gluc. + activated charcoal = the callus was placed in suspension medium containing activated charcoal in case exudates from the cells inhibited embryogenesis - this had no apparent effect on embryogenesis

3% gluc. + activated c. in agar = callus was transferred to agar containing activated charcoal and maintained on this medium for 3 days prior to transfer to suspension culture - this treatment was surprisingly inhibitory to cell growth and embryogenesis

(iii) In our hands, the callus giving greatest embryogenic suspension cultures was wet, dark and very loose. Under the microscope, this callus had a diversity of cell sizes, and we suspect that the small clumps of small cells give rise to embryos in suspension culture. Light, fluffy callus was much less embryogenic, and contained larger cells which often displayed irregular shapes. These cells were also very easily separated from each other.

Globular stage embryos were readily detected by flitering the suspension culture through a tea strainer. They appeared as small, dense globules, which were white in cultures from the Australian varieties of cotton, and green in cultures from the Coker variety.

We also tested a number of other treatments not outlined in the objectives. Some of these treatments had been used in other laboratories for inducing cotton embryogenesis. The remaining treatments did not appear to have been tested on cotton tissue cultures, or, if they had been tested this was not reported in the literature, but they were reported to promote embryogenesis in other species.

a) The 2,4-D concentration was increased from the usual 0.1 mg/l to 5 mg/l after one month of callus culture. This had no effect on callus growth or embryogenesis.

b) Sucrose was used instead of glucose as the energy source in callus initiation medium, but gave very poor growth with dark, necrotic callus.

c) Hormones were added to the suspension culture medium, which is normally hormone-free. This did not give any detectable increase in embryo initiation. The treatment was discontinued as it was very labour-intensive.

d) The callus was transferred to solid, hormone-free medium rather than into liquid, hormone-free medium, with or without glutamine, which some groups have found to promote embryogenesis. None of the Australian varieties showed any embryogenesis on solid medium. There was some promotion of embryogenesis in Coker callus on agar with glutamine, but not on agar without glutamine.

e) Zygotic embryos from imbibed seeds were used as the source material for the callus. No callus was produced from embryos.

f) Seedling hypocotyls were used as the source material for callus, rather than cotyledons. This gave less callus, but more of it was the embryogenic type. Hypocotyls were used as the source material for all subsequent experiments.

g) The glucose level was reduced from 3% to 1.5% in callus and suspension media. Embryogenesis appeared unaffected in untreated callus, but was promoted somewhat in plasmolysed callus. However, this difference was not statistically significant.

h) Light levels were varied, but did not appear to affect callus or suspension culture growth, or embryogenesis.

i) Other additives that might promote embryogenesis were added to the suspension medium, including extract of ground whole seeds, and the filtered suspension medium in which embryogenic Coker cultures had been growing for 2 weeks. The ground seed extract had no effect, but this was tested only once. The filtered medium promoted embryogenesis in the Australian suspension cultures, and most excitingly, it induced the embryos to develop to the torpedo stage, rather than remain arrested at the globular-heart transition. This treatment was also tested only once.

Objective (iv) was not achieved in Year 2 since we planned to start this part of the project in 2000. This objective was modified since the principal researcher, Dr. Janine Radford, ceased work on the project in December 1999. The project then moved from Monash University to CSIRO Plant Industry and a new principal researcher, Dr. Ding He, was appointed to the project and commenced work in April, 2000.

As noted in our previous report, we were successful in promoting embryo formation in tissue culture. However, in the Australian varieties of cotton, the embryos that formed remained white and appeared to be arrested, and at Monash, we did not succeed in developing a technique to release them from this arrested state so that they developed into viable new plants. (Embryos from Coker callus are green, and grow on to form new plants.) Over the final 15 months of the project, we planned to focus on overcoming this problem, as well as completing the work outlined in our initial objectives.

Year 3: **(i)** Continue combinations of plasmolytica and cell polarity disrupters
(ii) Assess uniformity and synchronicity of embryogenesis from combinations of plasmolysis and herbicide treatments
(iii) Repeat and refine treatments that significantly promote embryogenesis
(iv) Trial new treatments that may promote embryogenesis and, in particular, trial treatments that may overcome the arrested state of somatic globular embryos formed in tissue culture.

After discussion with Dr. Ding He, these objectives were revised, and in effect we jumped ahead to Objective (iv) of Year 3 (see below).

(i) Compare the responsiveness of Coker and elite Australian varieties to hormones and other factors, aiming to promote embryogenesis;
(ii) Establish a method to reduce the formation of non-embryogenic "fluffy" callus cells on callus induction medium, which may be detrimental to cotton embryogenesis;
(iii) Assess explants other than leaf and hypocotyl, for example immature embryos and flower organs, for their embryogenic potential;
(iv) Test the responsiveness of Coker and elite Australian varieties to different regeneration regimes that promote embryogenesis in other labs or in other species, including media containing a) 2,4-D and kinetin (CSIRO's current protocol); b) no hormones (to replicate a successful protocol established in Belgium); c) NAA and 2iP; d) zeatin; e) other combinations of hormones if time permits.

Objectives (i) and (iii) were mostly achieved and we made progress towards Objectives (ii) and (iv).

(i),(iii) These two objectives were combined. Shoot tips from 10 cotton lines were cultured on media containing a range of concentrations of either the cytokinin BA or the auxin 2,4-D or a combination of the two hormones. On media containing BA, multiple shoots and occasionally, enlarged meristems, were formed at 0.3 mg/l BA, and this response is similar to that in many other dicot species. However there were severe side-effects of BA, including swelling of stems and leaves, development of abscission layers, browning, vitrification and formation of large amounts of non-regenerative "fluffy" callus cells. Some of these side-effects were long-term, in that they were still expressed by the tissue long after subculture to hormone-free medium. The three lines forming embryogenenic callus in other experiments, Coker 315, Siokra 101 and Siokra 1-4, showed fewer side-effects on BA-containing media. Similar side-effects were seen in tissue grown on 2,4-D-containing media, but these

gradually disappeared after transfer to hormone-free medium. We concluded that to reduce the severe vitrification seen in shoot meristem tissue, the strong cytokinin BA should be omitted from the callus induction medium.

Hypocotyls and cotyledons from mature seeds were grown on media containing one of the cytokinins BA, kinetin, 2iP or zeatin. Unfortunately, most of these experiments were lost in a growth room fire. On zeatin, the tissues produced much non-embryogenic fluffy callus. After 6 months, Siokra 101 and 1-4 produced embryogenic callus on hormone-free medium, and Siokra V16 produced embryogenic callus on medium with 0.1 mg/l zeatin. Friable, pre-embryogenic callus was also obtained from varieties Siokra L23 and Sicot 189. It is interesting that the embryogenic line Coker 315 did not produce embryogenic callus under these conditions, suggesting that the elite Australian lines may have quite different hormone requirements for embryogenesis.

Our overall conclusion is that embryogenic callus can probably be obtained from many types of source tissue, but that hypocotyl tissue forms regenerative callus most readily.

These experiments were extended by testing the response of embryogenic Coker 315 callus to different hormone regimes. (Normally, callus is transferred from hormone-containing induction medium to hormone-free medium.) Calluses were transferred to media containing a range of kinetin and 2,4-D concentrations. Prolonged maintenance on 2,4-D caused browning of callus, but there were no side-effects from kinetin. Neither hormone caused formation of non-embryogenic fluffy callus. This result suggests that once embryogenic callus has formed, the tissue is much less sensitive to hormones, particularly to cytokinins.

(ii) From early results from the experiments outlined in Objective (i) above, our hypothesis was that the formation of fluffy cells and the severe vitrification of explants on callus induction medium may be a major obstacle for direct regeneration and could be a cause of poor or delayed embryogenesis in cotton. We speculate that as part of a wound response to cutting the tissue before placing on callus induction medium, cotton tissue may undergo a burst of hormone synthesis that promotes the formation of non-regenerative callus. Furthermore, previous work in other species has shown that fluffy callus can be caused by gibberellin in the growth medium or by high internal auxin levels in the callus. Hence, we tested a number of hormone antagonists on the embryogenic lines Coker 315 and Siokra 1-4 to see if they would inhibit the formation of fluffy cells. There was no effect of the gibberellin antagonists paclobutrazol or ancymidol, the auxin antagonist NPA or the ethylene antagonist silver thiosulphate (we tested this because high auxin levels are known to induce ethylene synthesis in many plant tissues). However, the induction medium in these experiments contained a low level (0.1 mg/l) of the cytokinin BA, which we now suspect may induce the fluffy callus. These experiments were repeated (only once) using media either without hormones or containing the weaker cytokinin, zeatin, and we obtained embryogenic or pre-embryogenic callus from 5 Australian lines, as noted in (i) above.

In addition, we tested agar-based media against gelrite. Gelrite is the standard gelling agent currently used for cotton tissue culture. We found that agar media reduced vitrification but also gave much slower callus growth.

Our overall conclusion here is that prevention of fluffy cell formation and callus vitrification is crucial to development of a more efficient regeneration system in the Australian cotton lines. We cannot yet identify the main cause of these adverse

responses, and therefore cannot yet recommend a reliable method for overcoming them, but we suspect that a wound response to cutting of the tissue and placing on growth medium may be involved. In addition, inclusion of hormones in the callus initiation medium appears to enhance the formation of non-embryogenic callus, so we would recommend using hormone-free medium for callus initiation (see also (iv) below).

(iv) In conjunction with the experiments outlined above, we also tested other published protocols for inducing regenerative cotton callus.

The first protocol tested included 0.1 mg/l 2,4-D and 0.1 mg/l kinetin in the callus induction medium. This is the most commonly used system for cotton. Ten lines of cotton (Coker 315 plus 9 Australian lines – CS 85, Sicot 189, Sicala V2 and 40, Siokra V16, 101, 1-4, L23 and V17) were tested. By the conclusion of the project, Coker 315, Siokra 1-4 and Siokra 101 produced embryogenic callus. Calluses from all experiments were subcultured on hormone-free medium to encourage formation of embryoids, but no other lines showed regeneration by June 2001.

The second protocol tested included no hormones in the induction medium. In general, regeneration was better on media without hormones and 5 lines produced embryogenic callus. By June 2001, 30% regeneration was obtained from Siokra 1-4 callus and 50% regeneration from Siokra 101 callus. Friable pre-embryogenic callus was obtained from Siokra L23 and V16 and from Sicot 189 tissues.

The third protocol tested used weaker hormones; 5 mg/l 2iP and 0.1 mg/l NAA, in the induction medium. Explants from all 9 lines tested formed watery green callus, but no embryoids were observed by June 2001.

The fourth protocol tested avoided auxin and used only the weaker cytokinin, zeatin, at 0.1 mg/l, in the induction medium. Very little non-embryogenic fluffy callus was formed on this medium. Calluses from the eight lines tested were transferred either to hormone-free medium or to fresh induction medium, and embryoids were observed in Siokra V16 callus.

5. Discuss the results, and include an analysis of research outcomes compared with objectives.

Our primary objective was to improve regeneration of Australian cotton lines from tissue culture. In order to achieve this objective, we aimed to test a number of protocols known to promote embryogenesis in other plant species.

The main new protocol we tested was the plasmolysis of callus to promote regeneration. As noted in 4. above, this did promote embryogenesis, but not sufficiently to warrant the inclusion of a plasmolysis step during routine initiation of cotton callus from seedling tissue.

Since the cotton callus was so recalcitrant with regard to regeneration, we changed focus somewhat. Rather than testing additional new protocols on the callus, we decided to test some more basic modifications of the callus initiation and regeneration media. As noted above, the most promising results were obtained by initiating callus on MS medium without additional hormones, or with much lower levels of hormone. We obtained regeneration in at least 5 different Australian varieties. Four of these showed regeneration on hormone-free medium, and one on medium with a low level of zeatin. The most regenerant line, Coker 315, did not show regeneration on either of these media.

We conclude that reduced hormone levels may promote regeneration in the Australian varieties. Our second main conclusion is that methods for optimum regeneration may differ between the different lines, even those that are closely related. Thus it may be useful, in future, to test a range of regeneration protocols on new cotton lines as they are developed.

The main limitation to these conclusions is that we did not have time, either at Monash University or CSIRO Plant Industry, to propagate sufficient numbers of the embryoids formed in the callus to confirm that they would normally grow on into healthy plants. This step is known to be a major bottleneck even in highly regenerative lines of cotton.

6. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian cotton industry and future research needs.

Further development of these methods could shorten the time required to generate transgenic cotton lines and could broaden the number of Australian lines amenable to genetic transformation.

7. Describe the project technology (eg. commercially significant developments, patents applied for or granted licenses etc).

N/A

8. Provide a technical summary of any other information developed as part of the research project. Include discoveries in methodology, equipment design, etc.

As noted above, the use of a callus initiation medium containing no hormones, or only a low level of a weak cytokinin such as zeatin, may substantially enhance production of regenerant callus. A suspension culture step during initial callus induction can also promote regeneration. However, because the different cotton lines respond somewhat differently to culture conditions, these treatments need to be tested with new lines as they are developed.

9. State the recommendations on the activities or other steps that may be taken to further develop, disseminate, or to exploit the project technology.

The hormone-free system will be a good tool for further study. It makes it time and space economic to study the culture conditions for cotton.

It should be not too difficult to establish a regeneration system of S101 and 1-4 with 50% of regeneration frequencies in 5-6 months. Further development of this system could provide a very powerful tool for studies on cotton embryogenesis/ regeneration.

Regeneration of normal plants from embryogenic callus is a serious problem even in the commonly used variety Coker 315. We suspect that the same physiological responses may underlie the production of both abnormal regenerants from embryoids and non-regenerant fluffy callus from cotton tissues.

10. List the publications arising from the research project.

N/A

Part 4 – Final Report Plain English Summary

You must submit a half to one page Plain English Summary of your research proposal that is not commercial in confidence, and that can be published on the World Wide Web. An electronic copy of the Plain English Summary must also be forwarded by e-mail (angela@crdc.org.au).

A major limitation to the development of new varieties of cotton by introduction of foreign genes, or amplifying or suppressing native genes, is that fertile plants cannot be regenerated from genetically modified cells and tissues of most of the important cotton cultivars. This project tested a variety of techniques that promote regeneration from tissue culture in other plant species to determine whether they could also promote regeneration from cotton tissue cultures.

We used tissue cultures from a cultivar of cotton that will regenerate from culture, and from nine commercially important Australian cultivars that are difficult to regenerate from culture. The first treatment we tested was incubation of the cultures in a variety of sugar and salt solutions that cause the cell membrane to detach from the surrounding cell wall (= plasmolysis). The tissue was then allowed to recover in normal growth solution. This treatment promoted regeneration in the four cotton lines tested but this promotion was not sufficient to warrant the inclusion of a plasmolysis step in the normal protocol for production of cotton tissue cultures.

We also tested a large number of other variations on the standard protocol for initiating cotton tissue cultures. The most promising results were obtained by initiating tissue cultre on the normal growth medium but without additional plant hormones, or with much lower levels of hormone than normal. We obtained regeneration in at least 5 different Australian varieties. Four of these showed regeneration on hormone-free medium, and one on medium with a low level of the hormone zeatin.

We conclude that reduced hormone levels may promote regeneration in the Australian varieties of cotton. Our second main conclusion is that methods for optimum regeneration may differ between the different cotton lines, even those that are closely related. Thus it may be useful, in future, to test a range of regeneration protocols on new cotton lines as they are developed.

The main limitation to these conclusions is that we did not have time to propagate enough plants from tissue culture to confirm that they would normally produce healthy, fertile plants. This step is known to be a major bottleneck even in highly regenerative lines of cotton.