DBT Ref. No. RR-333

DEVELOPMENT OF TRANSGENIC TEAK RESISTANT TO LEPIDOPTERAN DEFOLIATORS

REPORT OF KFRI-425/2006

PROJECT COMPLETION REPORT

E.M.Muralidharan (PI)
V.V. Sudheedrakumar (Co-PI)
T. Sajeev (Co-I)

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2007

1. Title of the Project : **Development of Transgenic Teak Resistant to Lepidopteran Defoliators**

2. Principal Investigators and Co-Investigators: Dr. E.M.Muralidharan
   Dr. V.V. Sudheedrakumar
   Dr. T. Sajeev

3. Implementing Institutions and other collaborating Institutions : Kerala Forest Research Institute, Peechi, Thrissur

4. Date of commencement : February 2004

5. Planned date of completion : January 2007

6. Actual date of completion : January 2007

7. Objectives as stated in the proposal:

   i. To screen *Bt* strains and endotoxins against *H. puera* and other major leaf feeding pests of teak to select suitable genes for genetic transformation.

   ii. Development of efficient protocols for genetic transformation and regeneration of transgenic plantlets from cells and tissue cultures of teak.

   iii. To carry out assays with the transgenic plants *in vitro* or in lab and controlled green house experiments.

8. Deviation made from original objectives if any, while implementing the project and reasons thereof :

   i. It was envisaged to evaluate the Bt toxins against four insect pests of teak including two defoliators and two borers. As maintenance of the cultures of the borer insects in the laboratory was not successful, further study was not feasible.

   ii. The third objective of assay with transgenic plants against the pests was also not feasible since regeneration of complete plantlets was not achieved except at low frequencies.

9. Experimental work giving full details of experimental set up, methods adopted, data collected, supported by necessary tables, charts, diagrams and photographs

9.1. . Screening of Bt toxins against teak pests:

In the current study, three Bt toxins- Cry1 AC,EC and C were tested for efficiency against the two important pests of teak namely, the teak defoliator, *Hyblaea puera* and the teak skeletonizer, *Eutectona machaeralis*. The toxins were evaluated against the early instars of the pests.

Three Bt toxin genes cloned in plasmid and transformed in *E. coli* strain BL21-DE3 were used. The details of the constructs are as follows.

1. Cry1Ac - Cloned in plasmid pUC19, Resistance Marker Ampicillin, Native Promoter for C*ry1AC*
2. Cry1C - Cloned in plasmid pUC19, Resistance Marker Ampicillin, Native Promoter for C*ry1C*
3. Cry1EC - Cloned in plasmid pET19b, Resistance Marker Ampicillin, T_7 promoter for *Cry1EC* is.

The three plasmids were transformed in *E. coli* strain BL21-DE3. These genes were designed and chemically synthesized in the National Botanical Research Institute, Lucknow and provided for this study.

The *E. coli* strains were cultured on LBA plates and proteins were extracted for evaluation against the defoliator pests. *E. coli* constructs containing Cry1Ac and Cry1C produced protoxin which was transformed to toxin by trypsinisation, while that containing Cry 1 EC produced toxins. The toxins thus obtained were quantified using Lowry's method.

i. Bt cultures

Part of the primary inoculum of Bt was transferred in to 17% final concentration in glycerol and stored at -20^{0}C. For conducting bioassays these were initially sub cultured on Luria Broth Agar (LBA) plates. The LBA plates were prepared by mixing 10gm Luria Broth And 10 gm Agar powder in 500 ml of distilled water and autoclaved. When the medium held skin bearable temperature, 0.1gm of ampicilin was added and poured in to Petri plates. These plates were kept over night in the incubator at 36^{0}C. The protoxins Cry1AC and Cry1C and the toxin EC were streaked on clean Petri plates and incubated overnight at 36^{0}C. The appearance of these strains on the plates were different as Cry1AC grow as pinpointed colonies. Cry1C is difficult to grow on plates and it is observed only in the site of primary

inoculum, it was creamy in color and slimy in appearance. Cry1EC grow faster than the other colonies. It do not produce pinpointed colonies and it is slightly creamy in color.

For multiplication of the cells single colony from the plates were selected and transferred to 5ml of Luria Broth media containing 0.01g of ampicillin and allowed to grow overnight at room temperature in a shaker at 150 rpm. From the cultures of AC and C, 0.5 ml were transferred in to 500 ml of LB medium containing 0.1 g of ampicillin and shaken at 120 rpm at room temperature for 72 hours. 0.1 g of ampicillin was added to this after every 16 hours. After 72 hours of incubation the cells were collected by centrifugation (8500 rpm at 4°C for 10 minutes).

For Cry1EC, cells were collected from 5ml of LB medium by centrifuging at 2000 rpm at 4^0C and washed with ice cold sterile STE buffer prepared by adding 0.1576 g Tris HCl, 0.0372g EDTA and. 0.5844g NaCl in 100 ml of distilled water. After washing in sterile STE buffer it was resuspended in LB medium (20g of LB powder dissolved in 1000 ml of distilled water) and was transferred in to 500 ml of LB medium added with 0.1g of ampicillin. It was allowed to grow at room temperature till the OD is 0.6 (4 to 5 h). Then the temperature was reduced to 15°C . 0.023 g of freshly prepared IPTG was added and allowed to grow for 6 h. Cells were harvested by centrifuging at 8500 rpm at 4°C. The retrieved cells were washed with ice cold sterile STE buffer.

ii. Protein harvesting

The collected cells of Cry1AC and Cry1C were suspended in 100 ml of STE buffer and that of Cry1EC in ice cold STE buffer prepared by dissolving 0.1576 G Tris Hcl, 0.0372g EDTA, and 0.5844g NaCl in 100 ml of distilled water. The cells were recollected by centrifuging at 8500 rpm at 4^0C for 10 minutes and suspended in TE buffer prepared by mixing 0.788g Tris HCl and 0.0372g EDTA in 100 ml distilled water. Then 0.01 g of lysozyme was added for lyses of the cell and this was incubated at room temperature. After 1 hour the lysed cells were centrifuged at 8500 rpm at 4^0C for 20 minutes and decanted the supernatant. To the pellet and 2.922g NaCl was dissolved in 100 ml of distilled water and 2 ml of Triton x-100 dissolved in 100 ml of distilled water was added and centrifuged this at 8500 rpm at 4^0C for 20 minutes.

The procedure was repeated and the pellets of inclusion bodies which contains most of δ –endotoxin protein was obtained. The pellets were washed thrice with 2.922 g of NaCl dissolved in 100 ml of distilled water and washed twice with distilled water. At each washing, the pellets were suspended completely. The inclusion bodies were then dissolved in 0.352 g of Sodium bi carbonate buffer (pH 9.5). This was then stirred for 2 hours continuously at room temperature .This yielded the protoxin of size ~135 kDalton from strain Cry1AC and Cry1C. For the conversion of protoxin to toxin trypsin was added and incubated overnight at room temperature. The trypsin digest was centrifuged at 13500 rpm at 4^0C for 20 minutes. The supernatant which contains toxins ~ 66 k Dalton was collected, the

protein concentration estimated and used for bioassays. In the case of Cry1EC the toxin was solubilised with distilled water, toxin estimated by Lowry's Method using spectrophotometer and used in insect toxicity assay. As per the estimation the amount of protein present in 3.5 ml of Cry1AC, Cry1C and Cry1EC were 3 mg , 3.16 mg and 2.25 mg respectively.

iii.Bioassay Experiment Designs

The insect cultures of *H. puera* and E. *machaeralis* were maintained in the laboratory. Healthy larvae of *H. puera* (First instar) and six day old *E. machaeralis* were challenged using two concentrations (1 & 2 mg/ml) of the three toxins isolated. A total of thirty larvae were used for each toxin/dose/replicate. An untreated control was also observed for natural mortality level.The toxins were presented on tender teak leaf disks.

Mortality of the test larvae was observed at specific time intervals and the percentage mortality data was used to compute LC90 and LT90 values using POLO software

9.2. Development of efficient protocols for genetic transformation and regeneration of transgenic plantlets from cells and tissue cultures of teak.

9.2.1 Explants :

i. Immature embryos : Immature fruits were collected from selected trees in the KFRI campus at Peechi and other localities in Thrissur district, Kerala. The fruits at different stages of development were used but all stages retained the calyx and the downy mesocarp was still light green in colour and still capable of being cut through with a sharp scalpel. The whole fruit with the calyx and downy outer surface scraped off were surface sterilized before excising the immature embryos under sterile conditions. The immature zygotic embryos were carefully excised from the developing seed after slicing through the fruit wall in cross section. The embryos were pearly white globular structures that could be removed with a needle or scalpel and placed on the induction media.

ii. Mature embryos: Mature seeds obtained from stored seeds (less than one year old) were used as explants. The hard endocarp was broken open with a sharp knife and the seeds collected for culture.

iii. Explants of mature tree origin: Leaf and internode explants excised from *in vitro* shoot cultures of teak were used as explants for experiments to induce direct or indirect caulogenesis. The shoots were induced from shoot tips of mature trees of teak collected from the hedge garden maintained at KFRI, Peechi. Shoot were multiplied on MS basal media (Murashige and Skoog , 1962) supplemented with 0.15 mg/l each of Benzyl aminopurine (BAP) and Kinetin (Kn) and 2 % (w/v) of sucrose and solidified with 0.7 % of agar. Leaf explants consisted of the entire leaf

lamina of about 1 cm in length and devoid of petiole and the internode of 1- 1.5 cm long sections devoid of the nodal region.

9.2.2 Surface sterilization

Aqueous mercuric chloride solution was used to surface sterilize the explants used in the study. Immature fruits were sterilized after removing the calyx and scraping off the outer downy layer of the mesocarp and washing with distilled water. The surface sterilization was done under sterile conditions by treating with a 0.1 % (w/v) aqueous solution for 10 min and rinsing three times with sterile distilled water.

Mature seeds removed from stored mature seeds were treated with 0.1 % $HgCl_2$ solution for 6 minutes under sterile conditions and given three washes with sterile distilled water before placing on solid induction medium for induction of somatic embryogeneis.

9.2.3 Culture media

The various explants were inoculated on a range of media in a experiment to induce morphogenesis and plantlet regeneration directly from explants tissues or indirectly through callus. The MS and WPM basal medium consisting of the minerals and vitamins of the media according to Murashige and Skoog (1962) and Lloyd and McCown (1981) respectively were used in the studies. Supplements of plant growth regulators and other adjuvants were made as and found necessary for different stages of culture. Sucrose (2 % w/v) was used as the standard carbon source and the media solidified with 0.7 % (w/v) of agar agar (Hi-Media, Laboratories, Mumbai) and dispensed into test tubes or flasks and autoclaved for 15 minutes. Media were dispensed into petridishes (10 cm X 2 cm) after autoclaving for some of the experiments.

The nodal explants of teak were placed on a medium that was standardized for shoot multiplication consisting of minerals and vitamins of the WPM medium supplemented with 1.5 mg/l of kinetin and BAP and 2 % sucrose and solidified with 0.72 % of agar.

9.2.4 . Experiments to induce *in vitro* regeneration from different explants

The immature and mature zygotic embryos, explants of leaves and internodes and micro cuttings of nodes taken from *in vitro* shoots of mature tree origin were placed on the WPM basal media supplemented with auxins or cytokinins or a combination of the two. WPM was preferred as the basal media since in preliminary experiments it gave a better response than MS or Gamborg's B5 medium. The composition of the media used in the study is given in Table 2.

With immature embryos the objective was to induce somatic embryogenesis as has been reported in several hardwood species like Eucalyptus, walnut, mango, prunus etc.. This was mainly to take advantage of the single cell origin of somatic embryos that is ideally suitable for genetic transformation. The use of leaf and internode explants was aimed at developing either a direct or callus mediated regeneration system through organogenesis or somatic embryogenesis. As a alternative the feasibility of using meristems as a tissue for transformation was also tested.

The basic strategy for obtaining regeneration form tissues was to culture explants on media containing auxins and cytokinins and transfer to a hormone free or lower levels of PGRs so as to permit induction and expression of the morphogenesis.

Since a breakthrough was obtained in induction of somatic embryogenesis in immature embryos the emphasis was on maintaining the embryogenic cultures and obtaining plantlet regeneration with a high efficiency.

9.2.5. Repetitive embryogenesis and conversion of somatic embryos

Somatic embryo were obtained on media containing picloram and picloram + BAP at a low frequency but since secondary embryogenesis was observed to be occurring all cultures of immature embryos were subcultured in the induction medium repeatedly to encourage further increase in number of embryos.

Experiments to convert somatic embryos into plantlets were carried out using two strategies. Mature embryos were transferred to a hormone free basal medium, basal medium at half strength of mineral salts or media containing low levels of cytokinins (BAP or Kin at 0.1 or 0.5 mg/l). In other experiments the following treatments were carried out for maturation of embryos transfer of embryos to hormone free basal medium for conversion.

i. WPM + Sucrose 2 %+ abscisic acid (ABA) (0.01, 0.05, 0.1, 0.5, 1.00 mg/l)
ii. WPM + sucrose 2 % (4%, 6 %)
iii. WPM + Maltose (2 %, 4 %, 6 %)
iv. WPM + Sucrose 2 % + Mannitol (2 %, 4 %, 6 %)
v. WPM + Sucrose 2 % + 0.1 mM Putrescine
vi. Desiccation under sterile air flow or $CaCl_2$ for 5 min

9.3. Establishment of lethal concentration of hygromycin for screeing of transgenics.

Since the Agrobacterium strains received from Dr. Rakesh Tuli, NBRI, Lucknow harboured the *hptII* plant selection marker the lethal concentration of the antibiotic for teak leaf and somatic embryos was established first.

Killing of the leaf and somatic embryo was observed at 35 mg/l. and hence after

The co-cultivation with *Agrobacterium* the explants were screened on media with 35 – 50 mg/l of hygromycin B.

9.4. Genetic transformation of teak using *Agrobactrium tumefaciens*

A. tumefaciens strains (LB4404 with plasmids KSR 1501 or PpK229) obtained form Dr. Rakesh Tuli, NBRI, Lucknow were used for the transformation. Co-cultivation of somatic embryos taken from freshly growing cultures (two weeks after subculture) was done. The cultures on LB medium grown at 28C on shaker (100rpm) in dark was centrifuged at 500 rpm fo r10 min. and pellets resuspended in WPM medium containing Picloram (4 mg/l) + BAP (1 mg/l) + CH (75 mg/l) and Sucrose 2 %. 20uM of acetosyringone was added and media incubated for 3 hrs.. OD was adjusted to 0.9 at 600nm and cocultivation of embryos done for 20 min in dark. The explants were blott dried and washed with 4 % solution of Plant preservative mixture to control the Agrobacterium proliferation. The explants were transferred to ampicillin (700 mg/l) containing medium and maintained for 3 days followed by transfer to WPM media with the PGRs for 1 week and then to media containing hygromycin at 40 mg/l for selection.

10. Detail analysis of results indicating contributions made towards increasing the state and knowledge in the subject.

10.1. Screening of Bt toxins against teak pest larvae:

The dosage mortality relationship between the three Bt toxins and first instar *Hyblaea puera* is shown in Fig.1. It can be seen that in both the doses tested, only Cry1AC could cause cent percent mortality.

Fig.1. Dosage mortality relationship between Bt toxins and first instar *Hyblaea puera*

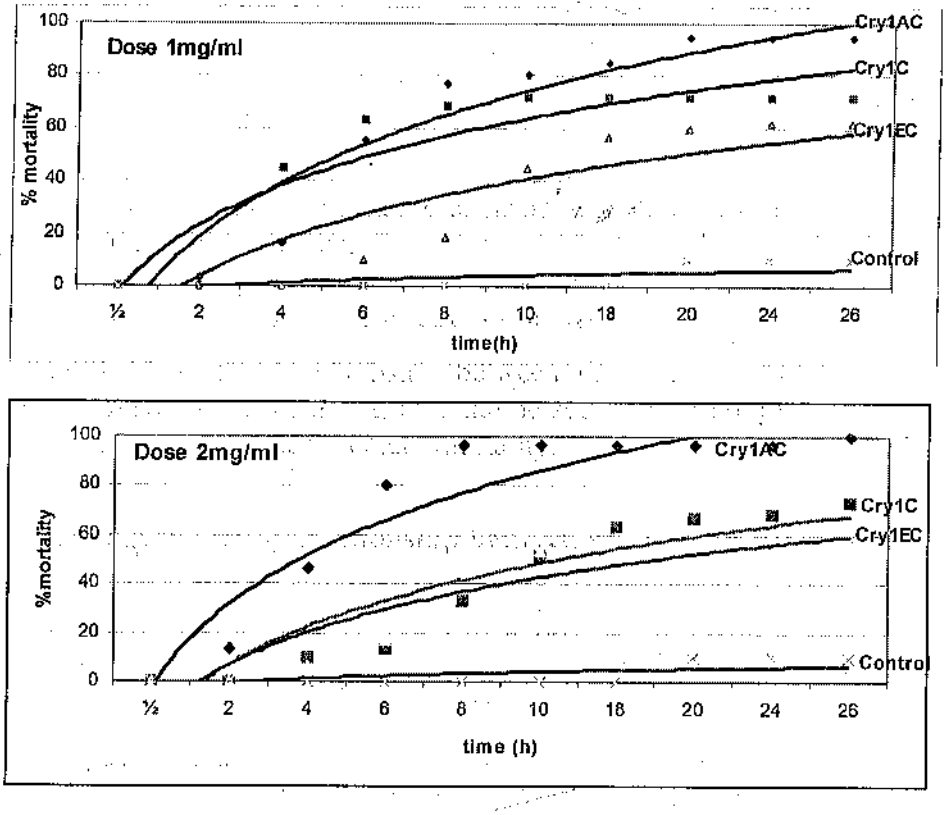


Fig 2 presents the dosage mortality relationship of Bt toxins against 6 day old larvae of *Eutechtona macheralis*. In the lower dose very little mortality was observed. However, in the higher dose, Cry1AC could cause higher mortality than Cry1C and Cry1EC.

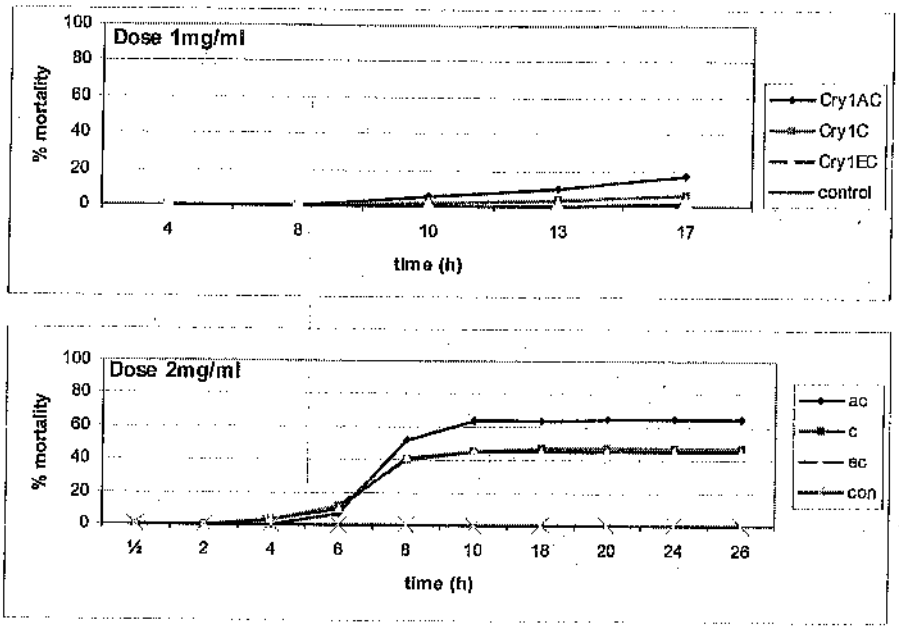


Fig.2. Dosage mortality relationship between Bt toxins and 6 day old *Eutechtona macheralis*

The amount of Bt toxin required for killing 90% of the pest population is given in Table1 along with the time it takes to kill the same proportion of insects. It can be see that Cry1AC requires only 0.9 mg/ml and 2.05 mg/ml of the active ingredient to kill 90% of the test insect population. Among the three toxins tested, Cry1AC recorded the least time to effect the mortality as indicated by the lower LT_{90} values.

Table 1. LC_{90} & LT_{90} values of Bt toxins against *Hyblaea* and *Eutectona* larvae.

Insect	Toxin	LC_{90}	LT_{90}
Hyblaea	Cry1AC	0.90982	8.919
	Cry1C	229.57715	44.74203
	Cry1EC	6.17735	64.17039
Eutectona	Cry1AC	2.05087	18.87690
	Cry1C	5420.8489	24.46361
	Cry1EC	-----	104.42380

10.2. Development of efficient protocols for in *vitro* regeneration of transgenic plantlets

10.2. 1. Leaf and internode explants:

Leaf and internode explants cultured on MS and WPM media containing a wide range of cytokinins and auxins gave rise to calli. The morphology of the calli ranged from soft friable (wet) type to compact hard and nodular (Fig, 3 a-d).

Callus induction was relatively slower in internode explants when compared to leaf explants. Callus initiation was mostly restricted to the cut edges of the explants and was more near the veins. The callus growth could be continued by subculture on fresh medium at 4 -5 week intervals.

Calli derived from the different explants were subcultured on regeneration media without hormones or with low levels of cytokinins to induce morphogenesis. However no sign of morphogenesis was obtained in any of the media except occasional rhizogenesis.

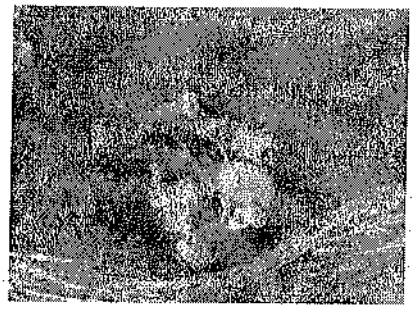
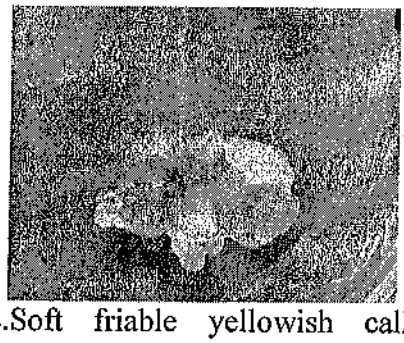


Fig. 3.a. Compact nodular callus from leaf explants growing on 2,4 – D

3.b. Callus obtained on internode explants on 2,4-D containing media.



3.c.Soft friable yellowish callus obtained on NAA containing media

3.d. Brownish yellow callus

10.2.2. Zygotic embryo explants

Mature zygotic embryo explants also failed to show any signs of morphogenesis but for the occasional development of the radicle and greening of the cotyledons.

Immature embryos cultured in the dark on the all the media excepting those with picloram at 4 and 6 mg/l also did not give any positive response. Embryos on 2,4 D containing media gave rise to a brownish white callus which however did not show any morhogenesis on transferring to regeneration media. On media with picloram (4 and 6 mg/l) about 50 % of the immature embryos gave rise to somatic embryogenesis in about 2 weeks after culture (Fig. 4. a & b). On further subcultures to the same media, secondary embryogenesis was observed (Fig 4 c&d). When maintained by subculture on the same media the embryos eventually turned senescent and slao developed a brown callus. Subculture on media supplemented with picloram (4 mg/l) and BAP (0.5 or 1mg/l) permitted better growth and also supported secondary embryogenesis.

The embryogenic cultures could be maintained in this medium in the dark by subculture every 3 weeks. Clusters of 3-5 somatic embryos were used for subculture and cultures were maintained in test tubes. At the end of 3 weeks the explants developed an average of 24.66 embryos. There was lack of synchrony in the stages of embryos. As the embryos matured bright red pigmentation was observed to form on the cotyledonary region . On examination under the microscope the pigmented areas were seen to be protruberances consisting of single or few cells. Similar structures are seen in young leaves of teak forming in in vitro as well as in seedlings. Therefore the pigmentation could be considered as sign of maturation of somatic embryos.

10.2.3. Conversion of somatic embryos:

Somatic embryos turned green when transferred from the embryogenic medium to the embryo conversion medium and to light. On a hormone free basal medium radicle emergence and greening of the plumule portion of the embryos was observed within a week of culture (Fig. 5 a& b.). Abnormality of the plumule development was very common and emergence of the shoot was inhibited in most of the embryos. Growing of embryos in various concentrations of ABA or mannitol or maltose was not effective as has been reported for several other species. Desiccation of the somatic embryos appeared to have a deleterious effect since further survival was effected. Only a few plantlets could therefore we regenerated from the different media attempted (Fig 5.c).

Table 2. Response of different teak explants on different PGRs media
(Basal media: WPM and Sucrose 2 %)

No.	Media composition	Explants	Response
1.	BAP (0.1, 0.5, 1.0, 1.5, 2.0, 5.0)	Leaf, internode Embryos	Friable greenish yellow callus No response
2.	TDZ (0.5, 1.0)	Embryos	No response
3	2,4-D (0.5,1.0,3.0 mg/l)	Leaf, internode Embryos	Soft to compact yellow callus Brownish white callus turning brownish yellow
4.	2,4-D (1) + Kin/ BAP(0.5)	Leaf , internode Embryos	Soft yellow callus Yellowish compact callus
5..	NAA(0.5,1.0, 3.0)	Leaf, internode Embryos	Nodular yellow callus No response
6.	NAA(1)+Kin/BAP/2iP (0.1, 0.5, 2.0 mg/l)	Leaf, internode Embryos	Soft friable pale yellow callus No response
7.	Picloram (2, 4, 8, 6 mg/l)	Leaf, internode Embryos	Pale yellow callus Induction of somatic embryos, maintenance of repetitive embryogenesis. Picloram (4 mg/l) gave best results
8	Dicamba (2, 4, 8,6 mg/l)	Embryos	No response
9	Picloram (4 mg/l) + BAP/Kin (0.5 , 1.0 mg/l)	Somatic embryos	Good multiplication of somatic embryos through repetitive embryogenesis on Picloram(4) + BAP (1).
10	Picloram (4 mg/l) + TDZ (0.5 , 1.0 mg/l)	Somatic embryos	Repetitive embryogenesis

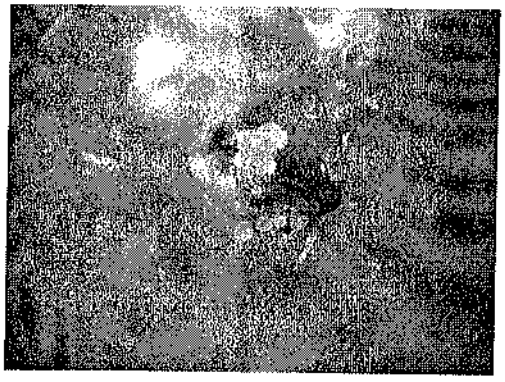


Fig.. 4a. Early stages of somatic embryogenesis on immature seed explants of teak on WPM + picloram

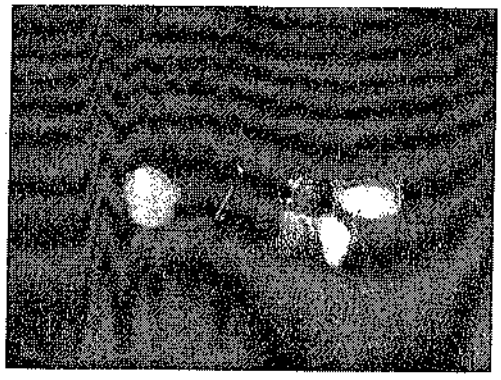


Fig 4b.Torpedo stage somatic embryos

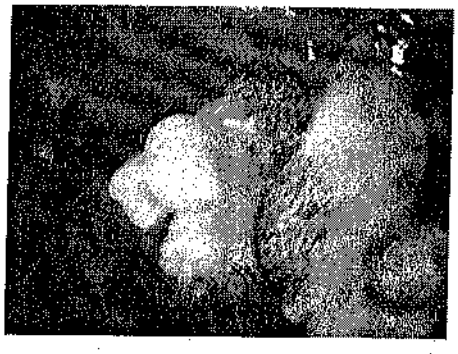


Fig.4 c. Repetitive embryogenesis

Fig.4.d Mature embryo showing secondary embryo formation

Fig.5a. Conversion of somatic embryo on hormone free medium

Fig 5 b. Development of cotyledons of somatic embryo

Fig. 5c. Plantlet derived from somatic embryo

10.2.4. Genetic transformation experiments

Somatic embryos after co-cultivation with two strains of A.tumefaciens was transferred to the embryogenic medium with Picloram and BAP with hygromycin B added at 40-50 mg/l in an effort to induce further cycles of secondary embryos from transformed tissues that survived . Tests with leaf and embryos cultured at different levels of hygromycin B had established that tissues were killed above 35 mg/l (Fig 6a.).

Somatic embryos after cocultivation with both the strains survived on the screening media with upto 50 mg/l of hygromycin but further growth was inhibited and the development of embryos was drastically reduced. No conversion of the embryos could therefore be attempted.

Further experimentation is therefore required to establish if the surviving embryos are indeed transgenic and if so the how these could be recovered and converted to plantlets. Improving the efficiency of the somatic embryo formation and conversion to plantlets is also to be improved to high level before routine transformation could be attempted. Alternatively a regeneration method involving leaves or internodes could also be developed.

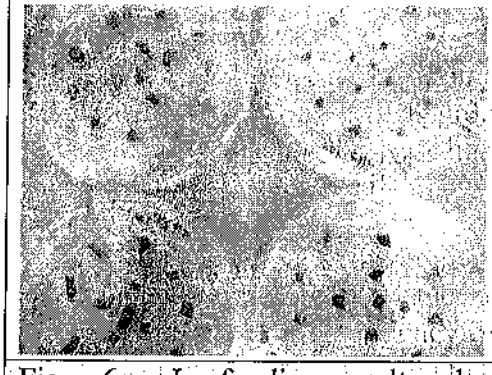


Fig. 6a. Leaf discs cultured on hygromycin B at various concentrations (20, 30 35, 40 mg/)

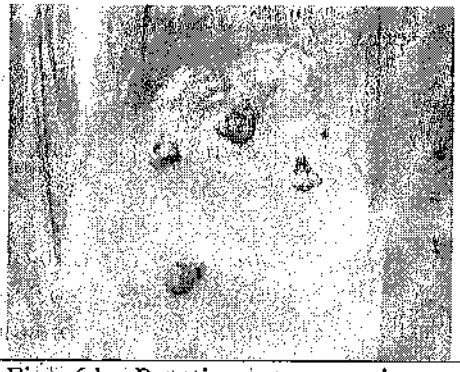


Fig. 6.b. Putative transgenic somatic embryos growing on screening medium with hygromycin B at 40 mg/l

11. Conclusions summarizing the achievements and indicating scope of future work

i. Cry1AC appears to be the best candidate for developing transgenic teak resistant to the major defoliators of teak.
ii. Immature zygotic embryos are suitable explants for induction of somatic embryogenesis in teak.
iii. Picloram is the suitable auxin to induce somatic embryogenesis and maintain embryogenic state through repetitive embryogenesis.
iv. Further experimentation is required to identify conditions leading to normal conversion of somatic embryos and regeneration of whole plantlets
v. Hygromycin B at 35 mg/l in the media results in mortality of teak tissues and somatic embryos.
vi. Putative transformed embryos transformed with Agrobactorium strain LBA4404 with Cry1AC cloned in pCambia (kSR 1501 and PpK 229) survived on selection medium but growth appears to be inhibited and no conversion to plantlets was possible.
vii. Further improvement in quality of teak somatic embryos and further refinement in conditions for conversion of embryos required.

12. S&T benefits accrued:

i. List of Research Publications:
ii.

1. Krishnadas, K.R and E.M. Muralidharan (2006) Repetitive somatic embryogenesis from zygotic embryos of teak (*Tectona grandis* L.) cultured *in vitro*. Paper presented at the National Conference on Tree Biotechnology : Indian Scenario, organised at TFRI, Jabalpur, February 9-10, 2006.

iii. Manpower trained : Three
iv. No. of PhD's trained : One
v. No. of Ph.D's produced : Nil
vi. Others trained : Nil
vii. Patents taken , if any : Nil

13. a. Project costs
b. Actual expenditure Rs. (In Lakhs) Percentage of total cost

i.	Salaries	:	5.79	21.65
ii.	Equipment	:	11.00	41.10
iii.	Consumables	:	5.31	19.86
iv.	Contingency	:	0.56	2.11
v.	Travel	:	1.09	4.07
vi.	Others, if any	:	3.00	11.20
	Total :		26.76	100 %

Name and Signature and Date.

a. Principal investigator:
b. Co-investigator :