Achievements in Genetic Transformation of a Tree Crop and Obstacles for Field Trials: The case of natural rubber (*Hevea brasiliensis*) from India

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Natural rubber is a strategic engineering/industrial raw material, like steel or coal.

More than 55,000 products are made from rubber; key driver of economy.

Renewable, eco-friendly.

*Natural rubber: Hevea brasiliensis*

Centre of origin: Amazon forests.

Short history of domestication (just over a century old).
Main Rubber Producing Countries

Brazil
Liberia
Cote D'Ivoire
Nigeria
Cameroon
D. R. of Congo
India
Sri Lanka
China
Indonesia
Malaysia
Philippines
Vietnam
Cambodia
Myanmar
Thailand

Map showing the main rubber producing countries around the world.
RUBBER RESEARCH INSTITUTE OF INDIA

(R&D Department of Rubber Board)

(Established 1956)
RRII: Started in 1955 as the R&D Department of Rubber Board of India

117 Scientists and over 300 supporting staff
11 Regional research stations across India
Annual maximum temperature anomaly with 1961-1990 mean - RRII, Kottayam

\[ y = 0.044x - 0.848 \]
\[ R^2 = 0.683 \]
Young plants without irrigation during summer
Limitations for Crop Improvement by Conventional Methods

1. Narrow genetic base
2. Long breeding cycle
3. Long juvenile period
4. Highly heterozygous nature
5. Poor seed set
THE HEVEA BREEDING CYCLE
### Modified Breeding and Selection Cycle

<table>
<thead>
<tr>
<th>Year</th>
<th>Process</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hybridization/ Ortet selection/ Polycross Progeny - nursery establishment</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Nursery selection – Medium to high yielders (Certain vigorous medium yielders may yield high and less vigorous high yielders can give medium yield in the juvenile phase) with reference to that of high yielding clones like RRII 105.</td>
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</tr>
<tr>
<td>4</td>
<td>Multiplication of selections</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Clonal Nursery (2.5 m x 2.5 m; RBD/S. Lattice; 6x4) Include 2-3 high yielding clones as controls</td>
<td></td>
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<tr>
<td>8</td>
<td>Test Tapping (April- May and October - November)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Test Tapping (April- May and October - November)</td>
<td></td>
</tr>
<tr>
<td>10-11</td>
<td>Selection, establishment of Source Bush Nurseries and multiplication for Participatory Clone Evaluation Selection in comparison with the controls</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>LST (on station) OFT (participatory, multi location) Regular yield &amp; sec. characters Regular yield &amp; sec. characters</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Selection based on mature yield for three years Selection based on mature yield for six years Recommendation in CATEGORY II Recommendation in CATEGORY I</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
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<tr>
<td>24</td>
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</table>

*Evaluation to be continued for further data collection.*

As per the conventional procedure, breeding cycle takes > 30 years; reduced to 24 years according to this modified scheme.
BREEDING ORCHARD FOR HYBRIDIZATION PROGRAMMES
Fig. 3a. Longitudinal section of a male flower

Fig. 3b. Longitudinal section of a female flower
Reproductive Biology

1. Cross pollinated by insects
2. Pollen viability average 50%
3. Pollen trap experiments show that pollen cannot travel more than 15 metres
4. Suggested isolation distance 100 metre in field trials
5. Seeds are large weighing 3.5 g to 6g
HYBRIDIZATION PROCEDURE

HAND POLLINATION

POLLINATED PANICLE

BAGGED IMMATURE HP FRUIT
Hybrid seedlings under test tapping in the nursery
Constraints in genetic engineering of rubber

- Explants highly recalcitrant to *in vitro* manipulations
- Short flowering season
- Low frequency of transformation (3-4%)
- Poor repeatability
- Long time for conversion of the transgenic cell lines to embryogenic callus (about one year if transgenic, but slightly less if not transformed)
Constraints …..

- Low regeneration potential of the transgenic tissues (1-1.5 years)
- Hard to harden (6 months; high rate of mortality as high as 90% if transformed; if not transformed 20-30%)
- Regulatory issues
Development of GM rubber plants

Methods – *Agrobacterium tumefaciens* mediated genetic transformation

- Standardized somatic embryogenesis and plant regeneration protocols for *Hevea brasiliensis* using different explants (immature anther, inflorescence and leaf)
Genetic transformation in Hevea brasiliensis: Opportunities

– Tolerance to abiotic stresses (drought, high temperature, cold etc.)
– Production of valuable recombinant proteins and other molecules
– Increased rubber yield
– Tolerance to diseases
# Development of transgenic plants in *Hevea*

<table>
<thead>
<tr>
<th>Objectives</th>
<th>Genes Selected</th>
<th>Status</th>
<th>Molecular/Biochemical studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPD &amp; abiotic stress tolerance</td>
<td>Superoxide dismutase (CaMV 35S &amp; FMV 34S promoter)</td>
<td>Plants developed and hardened</td>
<td>PCR, Southern, Northern, Enzyme analysis, Physiological studies.</td>
</tr>
<tr>
<td></td>
<td>Isopentenyl transferase</td>
<td>plantlets developed <em>in vitro</em> and hardened</td>
<td>PCR</td>
</tr>
<tr>
<td>Drought tolerance</td>
<td>Sorbitol-6-phosphate dehydrogenase</td>
<td>Plantlets developed <em>in vitro</em> and hardened</td>
<td>PCR</td>
</tr>
<tr>
<td>Biotic and abiotic stress tolerance</td>
<td>Osmotin</td>
<td>Plantlets developed and hardened</td>
<td>PCR, Southern, RT-PCR, Biochemical studies</td>
</tr>
<tr>
<td>Enhanced Latex Biosynthesis</td>
<td><em>hmgr 1</em></td>
<td>Plantlets developed and hardened</td>
<td>PCR, Southern, ELISA, Northern</td>
</tr>
<tr>
<td></td>
<td>FDP</td>
<td>Plantlets developed and hardened</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TB Antigen</td>
<td>Embryos developed</td>
<td>PCR</td>
</tr>
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</table>
Characterization of MnSOD gene and development of functional binary vector

- Isolation of total RNA from healthy bark of *Hevea basiliensis* (clone RRII 105)
- Preparation of cDNA by reverse transcription
- PCR amplification of a 702 bp MnSOD gene using restriction site tagged primers
- Insertion of the MnSD gene into a binary vector between the promoter and termination sequences
- Amplification of the vector in *E. coli*
- Transformation of *Agrobacterium tumefaciens*
• **Binary Plasmid vector:**
  - MnSOD cDNA with CaMV 35S promoter
  - Source of MnSOD gene – *Hevea brasiliensis*
  - *npt II* gene for Kanamycin resistance
  - GUS gene as reporter
Transgenic callus
Transgenic embryogenic callus
Confirmation of Transgene – PCR analysis of transgenic plants

- The integrated *HbSOD* gene was amplified with gene specific primers.

- 3.2 kb native SOD gene and 702 bp SOD transgene was amplified from transgenic plants.

- Only the 3.2 kb native SOD gene was amplified from control plants.
Matured embryos

Transgenic plant
Transgenic plant regeneration …

• Regeneration through somatic embryogenesis and hardening
PCR analysis of Transgenic Plants

uidA gene

nptII gene

650

800
Southern Analysis of Transgenic Plants

Probed with GUS and nptII genes

Lanes 3-HindIII
4-BamHI
5-EcoRV
6-XbaI

Lanes 3-BamHI
4-HindII
5-EcoRV
6-XbaI
Budgrafted transgenic plants maintained in net house

Two transgenic lines (L1 & L2) were developed, acclimatized, multiplied by budgrafting and maintained in net house.
Physiological and molecular analysis of over expression of MnSOD and other drought tolerant traits

Budgrafted plants – six month old:
- Transgenic plants (clone RRII 105), L1 & L2
- RRII 105 (control)
- Untransformed somatic plants (control)

In summer, the plants were divided into two sets of six plants each.

One set of plants was irrigated on alternate days up to field capacity and the other set was kept un irrigated for 2 weeks to induce drought.
Physiological & biochemical parameters tested

- Leaf water potential
- Photosynthetic oxygen evolution and respiratory oxygen uptake
- Photosystem II activity
- Total SOD activity
- Peroxidase activity
- $\text{H}_2\text{O}_2$ content
Table. 1. Mid – day leaf water potential (recorded at 12 noon)

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Plant material</th>
<th>Leaf water potential (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Irrigated</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>-1.2 ± 0.52</td>
</tr>
<tr>
<td>2</td>
<td>Somatic plant</td>
<td>-1.1 ± 0.46</td>
</tr>
<tr>
<td>3</td>
<td>L1</td>
<td>-1.2 ± 0.18</td>
</tr>
<tr>
<td>4</td>
<td>L2</td>
<td>-1.1 ± 0.52</td>
</tr>
</tbody>
</table>
Table 2. Chlorophyll \(a\) fluorescence in SOD transgenic and non-transgenic *Hevea* plants after imposing drought (withholding water for two weeks)

<table>
<thead>
<tr>
<th></th>
<th>Plant material</th>
<th>Max. photochemical efficiency (Dark (F_{v}/F_{m}))</th>
<th></th>
<th>PS II quantum yield ((\Phi_{PSII}))</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Irrigated</td>
<td>Drought</td>
<td>Irrigated</td>
<td>Drought</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>0.81 ± .002</td>
<td>0.78 ± .007</td>
<td>0.36 ± .010</td>
<td>0.25 ± .013</td>
</tr>
<tr>
<td>2</td>
<td>Somatic plant</td>
<td>0.80 ± .003</td>
<td>0.70 ± .023</td>
<td>0.33 ± .008</td>
<td>0.20 ± .010</td>
</tr>
<tr>
<td>3</td>
<td>SOD-L1</td>
<td>0.81 ± .005</td>
<td>0.78 ± .005</td>
<td>0.38 ± .016</td>
<td>0.33 ± .015</td>
</tr>
<tr>
<td>4</td>
<td>SOD-L2</td>
<td>0.81 ± .003</td>
<td>0.76 ± .008</td>
<td>0.39 ± .013</td>
<td>0.28 ± .007</td>
</tr>
</tbody>
</table>
**Table 3. Photosynthetic oxygen evolution and dark respiration rates.** Figures in the parenthesis indicate percentage of reduction/increase from the respective irrigated and drought induced plants.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Plant material</th>
<th>Photosynthetic O$_2$ evolution rate (µ mole/m$^2$/sec)</th>
<th>Respiratory O$_2$ uptake (µ mole/m$^2$/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Irrigated</td>
<td>Drought</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>7.2 ± 0.5</td>
<td>3.2 ± 0.6 (-56)</td>
</tr>
<tr>
<td>2</td>
<td>Somatic plant</td>
<td>6.43 ± 0.5</td>
<td>2.2 ± 0.4 (-66)</td>
</tr>
<tr>
<td>3</td>
<td>L1</td>
<td>7.9 ± 0.3</td>
<td>4.6 ± 0.3 (-42)</td>
</tr>
<tr>
<td>4</td>
<td>L2</td>
<td>6.9 ± 0.5</td>
<td>2.9 ± 0.4 (-58)</td>
</tr>
</tbody>
</table>
Physiological and molecular analysis under drought condition showed enhanced SOD expression and significant reduction in oxidative stress in the transgenic plant L1 compared with the control.

The recovery of the transgenic plants on re-watering after induction of drought was also better with transgenic plants.

Developing transgenic plants for abiotic stress tolerance is a novel approach towards crop improvement in *Hevea*. 
Field evaluation of transgenic *Hevea brasiliensis*

**Purpose**

- To evaluate the potential of transgenic *Hevea brasiliensis* plants integrated with MnSOD gene for enhanced tolerance to abiotic stress and tapping panel dryness syndrome, under field conditions.

- To assess the capability of the transgenic plants to grow better with higher yield under adverse environmental conditions.
Experimental Design

- The field experiment will be carried out with the two transgenic events (Hb. Mn.SOD- \( L_1 \) & HbMn.SOD-\( L_2 \)).
- The two plants (\( L_1 \) & \( L_2 \)) will be multiplied by bud grafting.
- The non-transgenic control plants are bud grafted plants of the clone RRII 105 as well as the bud grafted plants of the same clone developed through somatic embryogenesis.
- The proposed design is RBD with four treatments, five replicates and a plot size of six.
Type of Data to be Collected

A. Growth data (every three months)
   1. Girth of the stem
   2. Height of the plant
   3. Number of leaf whorls \[\text{upto three years only}\]
   4. Number of leaves

B. Physiological parameter (once in every year in the summer months)
   1. Leaf water potential
   2. Photosynthetic net assimilation (‘A’)
   3. Fluorescence parameters
      a) Dark FV/FM
      b) $\Phi$. PS II
   4. Antioxidant enzyme assay
      a) Superoxide dismutase
      b) Peroxidase
      c) $\text{H}_2\text{O}_2$ content

C. Latex yield

D. Rate of tapping panel dryness incidence.
Seed Set

- The transgenic *Hevea brasiliensis* plants are expected to start flowering by the 5th year. The seed set ratio is also expected to be very low. The seeds, after dispersal, will be collected manually and destroyed by burning.
On the positive side…

• Once a agronomically useful GM event has been obtained, no need for any back crossing
• Propagated through bud-grafting
• Exotic species; no sexual compatibility with any species in India
• Does not exist under natural (unmanaged, free living) conditions
• Does not flower for about four or five years
• Fruits and seeds are not consumed by birds or animals
• Final produce (economic yield) has no GM components
• Has a “safe biology” as far as GM research, field trials and commercial cultivation are concerned
• Approved by RCGM and GEAC
• However, unable to get state NOC for conducting field trials
THANK YOU FOR YOUR ATTENTION