Chapter 7: *Agrobacterium tumefaciens* mediated transformation in *Withania somnifera*

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7.5. Summary
7.1. Introduction

The Ti plasmid in *Agrobacterium tumefaciens* causes crown gall disease, which forms unorganised proliferated plant tumors at the site of infection by the bacteria (Hooykaas and Schilperoot, 1992). This is due to the integration of a T-DNA fragment of approximately 25Kb from the Ti-plasmid into the chromosomal DNA of plant nuclei and its subsequent expression (Chilton *et al.*, 1980, Citovsky *et al.*, 1992, Hooykaas and Beijersbegen, 1994). The T-DNA of wild type strains of *Agrobacterium tumefaciens* contains gene for auxin and cytokinin biosynthesis in plants. The crown gall and gall derived cell suspension cultures, incited with wild type Ti-plasmid have been used for production of some specific secondary metabolites (Di Cosmo, 1990, Saito *et al.*, 1992, Verpoorte *et al.*, 1993, Mukherjee *et al.*, 2000a, 2000b). The plasmid mutants in gene 1 and/or gens2 (shoot inhibition) or in gene 4 (root inhibition) induced shooty teratomas or rooty teratomas respectively on host plants (Leemans *et al.*, 1982, Inze *et al.*, 1984). There are several lines of evidence in literature supporting the close relationships between cell differentiation and the various features of secondary metabolism i.e. biosynthesis, transport, accumulation, transformation, degradation and release (Luckner, 1984) e.g. Lupin alkaloids in Leguminosae are synthesized in the green parts of the plants and stored in other parts such as roots (Wink, 1987). Tropane alkaloids in Solanaceae are produced in roots and transported in leaves (Liebisch and Schutte, 1985, Parr *et al.*, 1990). In this context transformed shooty and rooty teratomas (Rhodes *et al.*, 1987, Saito *et al.*, 1992, Jha, 1999) are interesting for the study of relationship between cell differentiation and secondary metabolites.

A limitation associated with hairy roots, formed following transformation with *A. rhizogenes*, is that they normally produce only those chemicals synthesized in roots of intact plants. The hormone autotrophic shooty teratomas produced by genetic transformation of plants with nopaline or genetically modified strain of *A. tumefaciens* have been used for producing secondary metabolites normally produced in leaves of plants and for biotransformation of some specific secondary metabolites (Table 7.1).
### Table 7.1: Production of secondary metabolites by shooty teratomas and transgenic plants

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Agrobacterium strain / Ti plasmid</th>
<th>Type of culture</th>
<th>Secondary metabolites</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arachis hypogaea</em></td>
<td>EHA101, C58</td>
<td>Shooty teratoma</td>
<td>-</td>
<td>Cheng et al., 1996, Egnin et al., 1998</td>
</tr>
<tr>
<td><em>Artemisia annua</em></td>
<td>CS8, N2/73</td>
<td>Shooty teratoma</td>
<td>Artemisinin</td>
<td>Paniego and Giulietti, 1996, Ghosh et al., 1997, Vergauwe et al., 1998</td>
</tr>
<tr>
<td><em>Atropa belladonna</em></td>
<td>pGV2215</td>
<td>Shooty teratoma</td>
<td>Biotransformation of hyoscyamine to scopolamine</td>
<td>Saito et al., 1991</td>
</tr>
<tr>
<td></td>
<td>Ti binary</td>
<td>Transgenic plants</td>
<td>Scopolamine</td>
<td>Yun et al., 1992</td>
</tr>
<tr>
<td><em>Coleus forskohlii</em></td>
<td>C58</td>
<td>Shooty teratoma</td>
<td>-</td>
<td>Mukherjee et al., 1996</td>
</tr>
<tr>
<td><em>Digitalis lanata</em></td>
<td>-</td>
<td>Transformed plant</td>
<td>Cardenolides</td>
<td>Lehman et al., 1995</td>
</tr>
<tr>
<td><em>Mentha citrata</em></td>
<td>pTi T37</td>
<td>Shooty teratoma</td>
<td>Mint oil terpenes</td>
<td>Spencer et al., 1990a</td>
</tr>
<tr>
<td><em>Mentha piperita</em></td>
<td>pTi T37</td>
<td>Shooty teratoma</td>
<td>Mint oil terpenes</td>
<td>Spencer et al., 1990b, Niu et al., 1998, Diemer et al., 1998</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>pGV3845(aux-)</td>
<td>Transformed shoot</td>
<td>Biotransformation of nicotine to normicotine</td>
<td>Saito et al., 1989</td>
</tr>
<tr>
<td><em>Rubus arcticus L.</em></td>
<td>EHA 101</td>
<td>Transformed shoot</td>
<td>-</td>
<td>Kokko et al., 1998</td>
</tr>
<tr>
<td><em>Solanum eleagnifolium</em></td>
<td>T37</td>
<td>Transformed shoot</td>
<td>Solasodine</td>
<td>Alvarez et al, 1994</td>
</tr>
<tr>
<td><em>Spinacia olracea L.</em></td>
<td>-</td>
<td>Transformed shoot</td>
<td>-</td>
<td>Zhang et al., 1999</td>
</tr>
</tbody>
</table>

Whilst there is no species of *A. tumefaciens* that can normally produce transformed shoots following plant infection, the formation of shooty teratomas from galls has been obtained...
following infection of a limited range of plants with certain nopaline strains of *A. tumefaciens* (Ooms *et al.*, 1981). *Bidens alba*, *Solanum tuberosum cv Maris bard*, *Brassica napus* and a populus hybrid have also been reported to develop shooty teratomas from transformation with pTi T-37 (Norton and Towers, 1983, 1985, Ooms *et al.*, 1983, 1985 and Filatti *et al.*, 1987). Shooty teratoma development has also been reported from transformation of *Nicotiana langsdorffii* by the nopaline strains pTi C58. However, transformation of *N. silvestris* with pTi C58 induced rooty teratoma development and undifferentiated galls were reported to have developed in four *N. tabacum* varieties infected with pTi C58 (Willmitzer *et al.*, 1983). Rooty teratoma also developed in a large proportion of the transformation of *B. napus* with pTi T-37 (Ooms *et al.*, 1985). The loss of apical dominance with the production of shoots from axillary buds afore the sites of gall development has been reported in *Kalanchoe* stems transformed with pTi T 37 (Ooms *et al.*, 1981). This result is similar to that observed on *Kalanchoe* in galls induced by the auxin mutant strains, LBA 4060 and LBA 1501 (Ooms *et al.*, 1981).

There is a limited number of reports in the literature about the development and application of shooty teratomas (Table 7.1). Saito *et al.*, (1989) used strains of *A. tumefaciens* with mutations in the auxin loci to develop shoot cultures of *Nicotiana tabacum* for nicotin biotransformation. Wild type nopaline strains of *A. tumefaciens* and disarmed strains carrying the ipt gene controlled by the CaMV 35S promoter have been used by Spencer *et al.*, (1990a) to produce shooty teratoma of *Mentha citrata*. Oil glands were found on the leaves of these shoots, chromatographic analysis confirmed presence of significant quantities of terpenes characteristic of mint oil, from native plant. In other work, *Atropa belladonna*, *N. tabacum* and *Solanum tuberosum* teratomas were examined for synthesis of tropane, nicotine and steroidal alkaloids, respectively (Saito *et al.*, 1991). Solasodine has been reported in shooty teratomas of *Solanum eleagnifolium* initiated using a nopaline strain of *A. tumefaciens* (Alvarez *et al.*, 1994). Recently transformed shoot cultures of *Pimpinella anisum* initiated using nopaline strain T-37, have been reported to accumulate essential oils at a lower level than normal shoot cultures (Khaled Salem *et al.*, 1995). On the other hand transformed shoot culture of *Artemisia annua* obtained using nopaline strain C58 have recently been reported to synthesize artemisinin at a higher level than untransformed shoot cultures (Ghosh *et al.*, 1997).

*Withania somnifera* Dunal (Solanaceae) is held with high reputation in traditional Indian medicine (Chopra *et al.*, 1958, Sharma and Dandiya, 1992, Ray and Gupta, 1994). The active pharmacological components of *W. somnifera* are steriodal lactones of withanolide type. Several
chemotypes exist differing in their withanolide content (Glotter et al., 1973). The principle withanolides in Indian *W. somnifera* are withaferin A and withanolide D (Kirson et al., 1971). Both leaves and roots of the plant are used as drug and steroidal lactones occur in both parts (Ray and Gupta 1994). The purpose of the present study was to investigate the transformation of *W. somnifera* through *A. tumefaciens* and the synthesis of withanolides in the transformed tissues.

7.2. Materials and methods

7.2.1. Plant material

Plants of two populations of *W. somnifera* viz. population I obtained from Agrihorticultural horticultural society, Alipore, Calcutta, West Bengal and population II obtained from Botanical Survey of India, Southern Circle, Tamil Nadu were grown in the Experimental Garden of Department of Botany, University of Calcutta. As seed germination frequency was very low (10%) in population II, only one experiment on virulence of *A. tumefaciens* strains could be carried out using leaf explants from axenic cultures of population II.

7.2.2. Production of plantlets as source of explants

Seeds of population I and II were presoaked in sterile water for 24hrs. They were then surface sterilized in 0.1% mercuric chloride for half an hour, washed repeatedly in sterile double distilled water and cultured on nutrient medium (MS) with 0.32% agar. Germination started within 21-25 days. A single shoot tip from *in vitro* germinated seedling of each population was used to raise multiple shoot cultures in MS medium containing BA (1mgl\(^{-1}\)) as described in Chapter three. These shoots were regularly subcultured to MSO medium to obtain explants for transformation experiments. Leaves from 4-6 week old plants were used as explant. For each experiment 150 medium sized, healthy leaves of population I were used. Parallely 50 noninoculated explants were plated as controls. The pH was adjusted to 5.6 prior to autoclaving at 121°C, 1.05 kg/cm\(^2\), for 15 min. The cultures were kept under 16/8h light/dark cycles of cool white fluorescent 40W tubes providing 37.5μmol m\(^{-2}\)s\(^{-1}\) light intensity and at 25°C±1°C. The shoots multiplied and proliferated within 4-6 weeks. Individual shoots were excised and rooted in MS medium without growth regulators (MSO) within 3-4 weeks. 8- week-old plantlets were used for *A. tumefaciens* mediated tumor production.
7.2.3 **Bacterial strains**

Agrobacterium tumefaciens wild type nopaline strains C58 (Depicker *et al.*, 1980), N2/73 (Anderson and Moore 1979), T37 (Sciaky *et al.*, 1978), L-L-succinamopine strain A281 (Guyon *et al.*, 1980), octopine strain Ach5 (Hoekema *et al.*, 1983) and disarmed strain LBA 4404 (Hoekema *et al.*, 1983) were used for transformation experiments. Strains were maintained on TY agar slants containing tryptone (10g l⁻¹), yeast extract (5g l⁻¹), NaCl (10g l⁻¹), Difco bacto agar (15g l⁻¹) at 25°C for 48 hr. Petri dishes were incubated upside down at 28°C for 48 hrs and stored at 4°C after sealing with Parafilm up to one month. Before infection bacterial strains were cultured in liquid TY medium (under shake condition of 140 rpm, at 28°C for 48 hrs).

7.2.4 **Tumor formation by *A. tumefaciens***

Leaves of *in vitro* grown plants were pricked with a needle and immersed for 1-2 min. in bacterial suspension to which aceto-syringone (100μM) had been added. The leaves were then placed on sterile Petri dishes containing filter paper soaked in MSO liquid medium for 24 hr. Co-cultivation occurred under controlled environmental conditions viz., 16/8 hr light/dark cycles ca. 3000 Lux intensity and 25°C ±1°C. For each experiment, to produce tumorous tissues, 30 explants were inoculated, five per plate. Parallely, 30 non-inoculated explants were plated as controls. All the experiments were performed with three replicates.

7.2.5 **Culture of tumorous tissues**

After co-cultivation leaves were transferred to solid MSO medium containing 500mg l⁻¹ cefotaxime and cultures were kept under a 16/8 hr light/dark cycle at 25°C. Approximately 3-4 weeks after infection the tumors that appeared at points of infection were excised and transferred to fresh media and maintained in MSO with cefotaxime (250 mg l⁻¹).

7.2.6 **Pre-culture and post-culture of explants in hormone supplemented MS media**

To study the effect of pre-culture of explants on infectivity and gall morphology incited with strain T-37 and N2/73, experiments were performed with leaves (population I) grown on MS medium supplemented with BA (1mg l⁻¹) for 8 weeks. In one experiment, (set A) leaves after
infection were post-cultured in MS medium supplemented with BA (1mg/l) and cefotaxime for another 8 weeks and then subcultured to unsupplemented MS medium containing cefotaxime (250mg/l). In another experiment (set B) the leaves were excised from plants growing on MS media containing BA (1mg/l), the leaves were pre-cultured for 7 days (set BI) and 15 days (set BI) in MS + BA (1mg/l) media, then infected with strain T-37 and N2/73, and placed in MSO media containing cefotaxime 250mg/l.

7.2.7. Verification of asepsis

The possible presence of contamination of Agrobacterium in the tumorous cultures was investigated by crushing samples of approximately 500mg of tumorous tissue under sterile conditions, and plating the crushed tissue onto TY medium. Initially the presence of bacteria was detected by this method. The initial washing after co-cultivation with higher level of cefotaxime (1g/l) for 10 min was followed by culture for one week in MS + cefotaxime (1g/l), to remove any contaminating Agrobacteria. After about 48-60 weeks of culture in presence of cefotaxime (250mg/l) axenic transformed tissues could be established.

7.2.8. Confirmation of transformation by opine assay

To confirm the transgenicity of tumcur tissue, opine assays were performed in samples of 50-70mg tissues each, using the method described by Otten and Schilperoort (1978). In nopaline type of crown galls transcript 3 in the T-DNA codes for the opine synthase. The enzyme nopaline synthase, catalyses the synthesis of nopaline from arginine and a ketoglutaric acid, similarly the enzyme octopine synthase catalyses condensation between several aminoacids and pyruvate. The activity of this gene can readily be used as a screenable marker in transformation experiments by screening for the presence of nopaline or octopine using a simple paper electrophoresis assay. The transformed tissues were initially cultured in nopaline test incubation medium (NIM) containing arginine and a ketoglutlaric acid. Standard nopaline was loaded onto a paper electropherogram and electrophoresed to separate the aminoacids (Draper and Scott, 1988). Arginine, octopine and nopaline contain a guanidium group that can be visualized from fluorescent staining with phenenthrenequinone.

Solutions:

1) Nopaline test incubation medium (NIM): 10mM arginine, 10mM a ketoglutaric acid, MSO media.
2) Standard arginine, octopine and nopaline (Sigma) solutions at 1mg ml\(^{-1}\) in double distilled water.


4) Staining solutions:
   - Solution A: 2mg phenanthrenequinone (BDH) in 10ml of 100% ethanol.
   - Solution B: 10% (w/v) NaOH in 60% ethanol.

One volume of solution A was mixed with one volume of solution B, immediately prior to use.

**Procedure:**

200mg of the tissue were incubated in an Eppendorf tube in 500\(\mu\)L of NIM at 25°C overnight. The tissue was rinsed with distilled water, plotted dry and homogenised in a clean Eppendorf tube with a sterile glass rod. The homogenate was centrifuged at 14,000 rpm for 2 minutes in an Eppendorf tube, and 5\(\mu\)L of the supernatant was loaded onto a 20cm x 15cm piece of 3MM Whatman paper. 1\(\mu\)L each of nopaline and arginine (1mg ml\(^{-1}\)) were also loaded as standards. The paper was equilibrated in electrophoresis buffer and the reaction products were separated by electrophoresis in a flat bed electrophoresis tank at 500V for 45 min. The origin was at anode and the reaction products separated towards the cathode. After electrophoresis, the electropherogram was blotted on a paper towel to remove surplus moisture and dried origin up, in air for several hours in a fume hood. The dried electropherogram was stained with the guanidium staining solutions, dried in the fume hood, and viewed on an UV-transilluminator. The yellow green fluorescence of nopaline and arginine was photographed using a Cannon SLR Camera fitted with red filter.

7.2.9. **Extraction and analysis of withanolides**

The extraction and quantification of withanolide D and withaferin A was done following published methods (Roja *et al.*, 1991, Ray *et al.*, 1996). The methanol extracts were spotted with reference compounds on Merck Silica gel TLC plates and analysed in solvent system CHCl\(_3\): EtOAc: CH\(_3\)OH: C\(_6\)H\(_6\) (72:4:8:16) and sprayed with Liebermann-Burchard reagent. Standard withaferin A and withanolide D have Rf values of 0.34 and 0.51 respectively. For quantitation these extracts were evaporated to dryness dissolved in 1ml of methanol, clarified using milipore filters (0.22\(\mu\)) and subjected to HPLC analysis. HPLC was done by the method of Roja *et al.*, (1991) but at a higher flow rate (1.5ml/min) with detection at 254 nm using a Shimadzu liquid chromatography (LC10AD) employing a reverse phase ODS-supelcosil C18 column (2.5cm x 4.6mm id) and isocratic elution with CH\(_3\)OH : H\(_2\)O (57:43). The system was calibrated with
standard sample of withaferin A and withanolide D. Withaferin A and withanolide D were isolated by preparative HPLC and identified by its m.p., retention time, spiking with standard, superimposable IR spectra and mass spectrometry (Lavie et al., 1968). For qualitative analysis, peak areas were used to calculate the amount of withanolide present in cultured tissues as compared to the standard. For analysis at least 1g of cultured tissue was used and the method was quantitative and reproducible. The detection limit was 50ng. The samples for which data have been presented here were initially extracted and analysed in triplicate with an average variation of ± 0.006 in the % of withaferin A and ± 0.003 in the % of withanolide D obtained. Reproducibility of injections, expressed as the variation (%) of injection was 2%.

7.3. Results

7.3.1. Tumor formation mediated by *A. tumefaciens*

Virulence of five oncogenic strains of *A. tumefaciens* on leaf explants of two populations of *W. somnifera* were studied and expressed as the proportion of explants forming tumors after 30 days. No tumor formation was observed in uninoculated leaf explants or in those leaf explants infected with disarmed strain LBA 4404. The oncogenic stains had different levels of virulence on two populations (Fig: 7.1), the main difference was found in the nature and growth of galls formed and in their subsequent morphological competence. The effect of strain types and genotype on the frequency of tumor induction as determined by ANOVA was significant at 1% level.

Table 7.2: Analysis of variance for effect of strain type and genotype on the frequency of tumor formation.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>Table value of F at 1% level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>4</td>
<td>10875.0</td>
<td>2718.7</td>
<td>21.75</td>
<td>4.43</td>
</tr>
<tr>
<td>Populations</td>
<td>1</td>
<td>1080.0</td>
<td>1080.0</td>
<td>8.64</td>
<td>8.10</td>
</tr>
<tr>
<td>Strain*population</td>
<td>4</td>
<td>795.0</td>
<td>198.8</td>
<td>1.59</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>2500.0</td>
<td>125.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>15250.0</td>
<td>525.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| S.E.             | 4.56|        |       |     |                             |
| C.D.             | 17.77|        |       |     |                             |

(At 1% level)

The supervirulent broad host range strain A281 showed 61-71% tumorigenesis within 20-30 days of infection where as strain Ach5 was more virulent and induced tumors on 80-90% of explants
with galls developing at all sites of infection. Growth of the galls was slow initially up to three months after which they grew faster in MSO medium supplemented with cefotaxime (250mg/l). Among the three nopaline type strains tested, although tumor induction was noted with all the three strains, the frequency of tumorigenesis and primary response of leaf explants differed among the two populations with the strain used. Infectivity was maximum (85-90%) with strain T-37 in both populations (Fig7.1). Galls appeared earliest with strain T-37 (16-18days) in both populations and later (30-35days) with strain C-58 (in population II) and strain N2/73 (in population I).

Fig 7.1 : Virulence of five oncogenic strains of *A. tumefaciens* on two populations of *W. somnifera* (% of tumor induction after 30 days)

One noticeable change in leaf explants following infection with nopaline strains was enlargement in the size of leaf explants with 7-8 days of infection in population I. Maximum enlargement (3-4 times) was observed with strain C-58 (Fig 7.2a). Leaves of population II did not show any enlargement in size after infection with any strain used. With strain C-58, galls appeared in the midrib region and on lamina towards the petiolar end. Tumors obtained following infection with strain C-58 were morphologically distinguishable as two types: 12-15% of the galls (Type I gall, Fig 7.3.a) were friable and greenish and the rest (85-87%) were compact, white and large (Type II
Fig 7.2. Tumor induction following infection with different strains of *A tumefaciens* in *W. somnifera* after 30 days of culture in MSO + cefotaxime 500 mg

a. Leaf showing tumor induction at petiolar end following infection with strain C58 (x7).

b. Leaf showing tumor induction all over the surface including margins, abaxial and adaxial surface of lamina and cut edges of explant following infection with strain T37 (x6).

c. Leaf showing tumor induction all over the surface following infection with strain N2/73 in population II of *W. somnifera* (x2).

d. Induction of gall callus from petiolar end, leaf margin and midrib region with strain N2/73 in population I of *W. somnifera* (x6)
gall, Fig 7.3.b). While the Type I gall grew rapidly (5-8 fold every 3 weeks) in MSO medium, Type II galls grew slowly up to six months after which their growth rate improved (Table 7.3).

With strain T-37, tumors appeared at all infection sites on the leaf i.e. in the leaf apex, leaf margin, midrib region and lamina-petiolar end in population I (Fig 7.2 b). Three types of morphologically distinguishable tumors (Table 7.3) were obtained; in addition to Type I and Type II galls, another type of gall (Type III) appeared in the apical and midrib regions of leaves of genotypes I, which were minute (microgall’s ~1mm in diameter), white and compact.

With strain N2/73, galls did not develop from all infection sites. They developed mainly from leaf apex, midrib and lamina-petiolar end (Fig 7.2 c, d). Three types of morphologically distinguishable tumors (Table 7.3) developed: greenish or globular microgalls (Type IV galls); greenish white compact large galls (Type V gall) and unorganised brownish galls, which developed friable calli (Type VI galls). Galls developed on both surfaces of lamina with strain N2/73. While all the three types of galls developed in leaves of population II. All the gall lines were maintained in MSO medium supplemented with 500mg/l.

Table 7.3: Type of galls obtained following transformation of \emph{W. somnifera} leaf explants (population I) with three nopaline strains of \emph{A. tumefaciens}

<table>
<thead>
<tr>
<th>Gall type</th>
<th>Incited by strain</th>
<th>Phenotype of galls observed after 120 days of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>type</td>
<td>u*</td>
</tr>
<tr>
<td>Type I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Friable, greenish, fast growing</td>
<td>C58</td>
<td>+</td>
</tr>
<tr>
<td>T37</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compact, large, white slow growing</td>
<td>C58</td>
<td>+</td>
</tr>
<tr>
<td>T37</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Type III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compact, minute (micro galls) white</td>
<td>T37</td>
<td>+</td>
</tr>
<tr>
<td>Type IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Globular (micro galls) greenish</td>
<td>N2/73</td>
<td>+</td>
</tr>
<tr>
<td>Type V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compact, large greenish white</td>
<td>N2/73</td>
<td>+</td>
</tr>
<tr>
<td>Type VI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Friable, brownish</td>
<td>N2/73</td>
<td>+</td>
</tr>
</tbody>
</table>

u* = unorganised callus. s* = shoot developed on galls, r* = root developed on galls.
Fig. 7.3 Development of different gall lines following infection with *A. tumefaciens* in MSO + cefotaxime 500 mg/l.

a. Type I friable, greenish, fast growing gall developed following infection with T37 (x7)
b. Type II compact, white slow growing gall developed following infection with strain C58 (x6)
7.3.2. Effects of pre-culture and post-culture of explants with BA (1mg/l) in population I

In experiment set A leaves from plants growing in MS basal medium containing BA (1mg/l) after infection were post-cultured in MS basal medium + BA (1mg/l). With both strains T-37 and N2/73, 60% of the explants formed tumors within 15 days. The tumors appeared initially at the leaf petiolar end and later developed localised callus at wound points and along mid vein in both types. With strain N2/73, leaf enlargement was observed in 40% of the explants and both the Type V and Type IV gall morphology was obtained. With strain T-37 unorganised brownish Type VI galls were detected. All the gall lines were maintained in MSO medium supplemented with cefotaxime (250mg/l) but they failed to induce any shooty or rooty structure.

In a second experimental set B, isolated leaves from plants grown in MS basal media containing BA (1mg/l) were pre-cultured in MS+BA (1mg/l) for 7 days (set BI) and 15 days (set BII). They were then infected and placed in MSO medium supplemented with cefotaxime (500mg/l). With strain N2/73 the frequency of tumor induction reached 100% in both set BI and set BII pre-culture conditions. Within 15-16 days tumors developed at the lamina-petiolar end and then the tumors extended along mid-rib as well as on laminar region. The morphology of galls was distinguished as Type V and Type VI. Two rooty lines were obtained from the petiolar end tumor induced in set BII which could be maintained with subculture in MS + cefotaxime (250mg/l).

With strain T-37, frequency of development of galls in both set BI and BII decreased (37% and 27% respectively). Galls developed at lamina-petiolar end within 15-19 days and few along mid vein. The galls were compact, brown and large, with subculture on MSO medium containing cefotaxime (500mg/l) the growth slowed and finally got checked. Thus although pre-cultured enhanced tumorigenesis with strain N2/73, tumor induction was decreased in T-37 and no shoot morphogenesis was noted under these conditions in either of the two strains studied.

7.3.3. Development of rooty teratomas and transformed root cultures

Rooty teratomas developed from gall callus spontaneously in MSO medium following infection with all strains except strain T-37. However, while strains A281 and Aeh incited rooty galls in both genotypes, galls derived following infection with nopaline strains C-58 and N2/73 developed rooty teratomas only in population I.
Fig 7.4 Development of rooty teratomas following infection with *A. tumefaciens* strain TrACh5 in MSO + cefotaxime 500 mg/l.

- a. Initiation of roots at point of infection (x3)
- b. Greenish, thicker roots with fewer laterals in presence of light conditions (x3)
- c. Brownish, thinner roots with more laterals in presence of dark conditions (x3)
fig 7.4.
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With strain C-5S, 15-20% of the Type II galls developed profuse roots. Roots were thick and could be excised and cultured as root organ cultures up to 4-5 months in MSO medium after which the root growth ceased. These roots did not show presence of nopaline and were probably not transformed.

With strain N2/73, 10% of the Type V galls developed roots. The roots were pale brown and grew as clusters. The roots did not elongate beyond 1.5-2.0 cm in length and developed callus, growing as a rhizogenic mass (line N2/73/EI/R2) in MSO medium (Table 7.3).

Strain Ach5 induced maximum (20-30%) rooty galls in both populations. Greenish organised galls (5-10 galls/leaf) gave rise to roots (1-2 rooty galls/leaf). It was, however, very difficult to free the developing roots from contamination Agrobacteria. One axenic rooty line (derived from a single root tip) was developed following infection with strain Ach5 in leaves of populations II (Fig 7.4.a). Root cultures grew both in the light and the dark. Roots grown under a 16hr photoperiod were greenish, thicker with few laterals (4-5 laterals per 25mm long root, Fig 7.4.b) as compared to those grown in the dark, which were thinner with more laterals (12 laterals per 25mm long root, Fig 7.4.c). This rooty line Ach5 /Sl/R1 was maintained in MSO medium supplemented with 250mg/l cefotaxime. Rooty teratomas contained octopine confirming insertion and expression of the ocs gene. No activity was found in control culture lines (Fig 7.6a). Strain A281 induced 10-40% rooty galls, which developed laterals, were hairy and grew 2-5 fold within 6-8 weeks. But these roots could not be freed from contamination Agrobacteria and hence could not be maintained.

7.3.4. Development of shooty teratomas

Shoot differentiating gall callus or 'shooty galls' were obtained with two nopaline strains (T-37 and N2/73) in population I only. With strain T-37 the Type I greenish galls developing from the base of lamina regenerated shoot buds after six months of culture in MSO medium supplemented with cefotaxime (250mg/l). The shoot buds could be excised and cultured in MSO medium, but even after 1 year of culture they did not proliferate. The Type IV microgalls developing from the leaf apex, midrib and laminar petiolar end were excised after 6-8 weeks of infection and cultured in MSO medium supplemented with 500mg/l cefotaxime. Shoot buds (2-6) differentiated from such green calli after 4-5 months of infection (Fig 7.5.a). Clusters of developing shoot buds (1-1.5cm long and 14-15 shoot buds per cluster) were grown in MSO solid and liquid medium.
Fig. 7.5 Development of shooty teratomas from gall callus following infection *A. tumefaciens* strain N2/73 in MSO + cefotaxime 500 mg l⁻¹.

a. Induction of shoot buds from gall callus (Type IV) after 4-5 months (x10).
b. Development of shoot buds from gall callus (x7)
c. Multiplication of shoot buds in MSO and establishment of a shoot line after 1 year of inoculation (x3)
d. Proliferation of shoots with altered morphology, thicker stem and narrower leaves (x1)
supplemented with 250mg l\(^{-1}\) cefotaxime. The shoot grew better in solid medium as compared to liquid medium. The regenerating gall calli continued to produce new shoot buds when the first crop of shoots was excised at 2-4 weeks, after which the gall calli lost their potential for regeneration (Fig 7.5. b, c).

The shooty teratomas differed phenotypically from normal untransformed shoots (Fig 7.5.d). The stem was thicker and leaves were narrower in untransformed shoots as compared to untransformed shoots. The shooty teratomas grew 2-3 fold every four weeks. While untransformed shoots cultures can be easily rooted (90%) in MSO medium, roots developed form shooty teratomas in unsupplemented basal medium at a very low frequency (2-5%). Shooty teratomas contained nopaline confirming the expression of nos gene (Fig 7.6.b). No nos activity was found in control culture lines.

7.3.5. **Withanolide synthesis in transformed cultures**

The two principle withanolides of Indian *W.somnifera* – withanolide D and withaferin A was present in shooty teratoma cultures, while withanolide D alone was detected in rooty teratomas (Table 7.4). In the present study gall callus or untransformed callus did not show withanolides. The rooty teratoma lines differed in their ability to synthesize withanolide D. Line Achy/SI/R\(_1\) showed a higher level of withanolide D (0.015%, Fig 7.6.c) as compared to line N2/73/EI/R\(_2\) (0.006%). The shooty teratoma lines showed maximum synthesis of withaferin A (0.07-0.1%) and withanolide D (0.025-0.085%, Fig 7.6.d). The shooty teratomas synthesized both the withanolides at higher levels when compared with the roots and shoots of 8-week-old untransformed plants of both populations (Table 7.4). Withanolide content in untransformed shoot cultures growing in presence of BA (1mg l\(^{-1}\)) was also lower than the withanolide content of shooty teratoma cultures (Table 7.4). Thus shoot differentiation seems to be a prerequisite for the synthesis of withaferin A.
Fig. 7.6 Electropherograms showing the presence of octopine and nopaline in rooty and shooty teratomas respectively.

- b. Lane 1: Nopaline + arginine, Lane 2: Arginine, Lane 3: shooty teratoma Lpt1 N2/73, Lane 4: T-37 shoot line, Lane 5: Untransformed shoot, Lane 6: Arginine, Lane 7: Nopaline.

Withanolide detection in different shooty teratoma lines.

- c. Detection of withafarin A (rt 15.1) and withanolide D (rt 20.4) in shooty teratoma LpE3 obtained following infection with A. tumefaciens strain N2/73 by HPLC.
- d. Detection of withafarin A (rt 15.3) and withanolide D (rt 20.5) in shooty teratoma LpE1 N2/73 obtained following infection with A. tumefaciens strain N2/73 by HPLC.
Table 7.4: Withanolide contents in different transformed and untransformed culture lines of *W. somnifera* (values are average of 3 determinations; S.E. < 10%)

<table>
<thead>
<tr>
<th>Culture lines analysed</th>
<th>Opine present</th>
<th>Age</th>
<th>Withanolides present (mg/100g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Witherferin A</td>
</tr>
<tr>
<td><strong>Gall callus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II (C-58)</td>
<td>nopaline</td>
<td>4week</td>
<td>nd</td>
</tr>
<tr>
<td>Type V (N2/73)</td>
<td>nopaline</td>
<td>4week</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Rooty teratoma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Achs/Si/R1</td>
<td>octopine</td>
<td>4week</td>
<td>nd</td>
</tr>
<tr>
<td>N2/73/Ei/R2</td>
<td>nopaline</td>
<td>4week</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Shooty teratoma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lpt1 N2/73</td>
<td>nopaline</td>
<td>4week</td>
<td>100.0</td>
</tr>
<tr>
<td>Lpt2 N2/73</td>
<td>nopaline</td>
<td>4week</td>
<td>70.0</td>
</tr>
<tr>
<td><strong>Untransformed shoot culture</strong> (population I)</td>
<td>-</td>
<td>4week</td>
<td>40.0</td>
</tr>
<tr>
<td><strong>Untransformed plants</strong> (population I)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>shoot</td>
<td>-</td>
<td>8week</td>
<td>50.0</td>
</tr>
<tr>
<td>root</td>
<td>-</td>
<td>8week</td>
<td>18.0</td>
</tr>
<tr>
<td><strong>Untransformed plants</strong> (population II)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>shoot</td>
<td>-</td>
<td>8week</td>
<td>40.0</td>
</tr>
<tr>
<td>root</td>
<td>-</td>
<td>8week</td>
<td>19.0</td>
</tr>
</tbody>
</table>

* Media: MS basal media + BA (1mg/l).

** Media: MS basal media + IBA (0.5mg/l).

### 7.4. Discussion

Transformation of *W. somnifera* with wild type strains led to the induction of morphologically distinct tumors with different responses. Similar difference in tumor phenotypes have been reported for *Nicotiana tabacum* (Saito *et al.*, 1989), *Mentha citrata* (Spencer *et al.*, 1990) and *Coleus forskohlii* (Mukherjee *et al.*, 1996). The *A. tumefaciens* wild type strains have been reported to induce rooty teratomas and shooty teratomas in a number of herbaceous and woody species (Saito *et al.*, 1992, Caissard *et al.*, 1996, Li and Wolyn, 1998). Rooty teratomas of *N. tabacum* and *Coleus forskohlii* were reported to
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synthesize secondary metabolites characteristic of the roots of the parent plant. In the present study, rooty teratoma lines obtained following infection with Ach5 (octopine strain) and N2/73 (nopaline strain) grew in MSO medium in absence of auxins. Withanolide D was detected in both rooty lines and the content in line Ach5/SI/R1 was higher (0.0% 15) than in line N2/73/El/R2 (Table 7.4). Withaferin A was not detected in such root cultures. It is noteworthy that hairy root cultures obtained following infection with A. rhizogenes as well as untransformed root cultures of W. somnifera have been reported to synthesize withanolide D but withaferin A was not detected in such cultures. This suggests that withaferin A is synthesized in the aerial parts of the plant and translocated to roots in rooted plants. Withanolide D and withaferin A was not detected in gall callus and root differentiation stimulated synthesis of withanolide D even in absence of growth hormones.

Nopaline strains of A. tumefaciens have been reported to induce shooty galls in a number of plant species like, Mentha citrata (Spencer et al., 1990 and Caissard et al., 1996 Diemer et al., 1998), Solanum eleagnifolium (Alvarez et al., 1994), Pimpinella anisum (Khaled et al., 1995), Coleus forskohlii (Mukherjee et al., 1996) and Artemisia annua (Ghosh et al., 1997). The reason for shooty and rooty teratoma formation by nopaline strains is unclear (Turgeon, 1982). The expression of bacterial genes affecting the response of plants cells to auxin and cytokinin may need to be manipulated before shooty teratomas can be produced for a wide range of plant species (Hamill and Rhodes 1993, Hamill, 1993). The successful development of A. tumefaciens transformed shoot culture may be of considerable value as a model system in which the effects of gene transfer on the synthesis, transport and storage of secondary metabolites may be investigated (Khaled et al., 1995). The induction of shooty teratomas from a particular gall callus line may be due to the altered endogenous ratio of auxin and cytokinin favouring shoot induction (Ghosh et al., 1997). Although the molecular mechanism of shooty teratoma formation is not yet completely clear, shooty teratomas form after integration of part of the A. tumefaciens Ti plasmid into the plant genome in an analogous way to hairy root induction by A. rhizogenes.

Pre conditioning and post conditioning of explants used in the transformation studies in an appropriate medium enhances the regenerative capacity of leaf explants derived from the shoots, so that adventitious buds form in high frequency. The use of conditioning enables the transformation and rapid recovery of plants form otherwise recalcitrant cultivars without the need for an extended callus phase as reported in apple by Sriskandarajah and Goodwin (1998). Conditioning of apple shoots promoted the recovery of transformed plants from leaf explants by
two processes: increasing the number of cells containing and expressing the introduced genes, and by increasing the probability that cells will regenerate directly to shoots. In the present study W.somnifera plants from which leaf explant were obtained for transformation studies were preconditioned in BA containing media as well as the isolated leaves were preconditioned in BA containing media. Shoot organogenesis from leaf explants was obtained with the same media in the same species (Chapter 3). But preconditioning and post conditioning failed to enhance the regenerative capacity of the leaf explants infected with A.tumefaciens in W.somnifera.

The withanolide synthesis in shooty teratomas was much higher than in untransformed shoot cultures. On the basis of results obtained with transformed organ cultures in W.somnifera, it can be inferred that withanolide synthesis is related to differentiation and transformed shoot differentiating cultures growing in MSO medium are able to synthesize both the major withanolides of Indian W.somnifera. Thus a useful system has been developed for studying withanolide synthesis and production in vitro.

7.5. Summary

Transformed organ cultures of W. somnifera were established following infection with wild type nopaline and octopine strains of Agrobacterium tumefaciens. The oncogenic strains had different levels of virulence on two populations studied, but the main difference was found in the nature of galls formed and in their subsequent morphological competence. Ten percent of the galls obtained following infection with nopaline strain N2/73 spontaneously developed shooty teratomas of altered phenotype. Pre-conditioning and post-conditioning of isolated leaves in BA (1mg/l) failed to enhance the regenerative capacity of the leaf explants infected with A.tumefaciens. The shooty teratomas grew in unsupplemented basal medium and were able to synthesize both the major withanolides of the parent plants. Withanolide synthesis in shooty teratomas was much higher (0.07-0.1% withaferin A and 0.085%-0.025% withanolide D) than in non-transformed shoot cultures.