GENETIC TRANSFORMATION AND MICROPROPAGATION OF *THAPSIA GARGANICA* L.- A MEDICINAL PLANT

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DECLARATION

The experimental work described in this thesis was carried out in the Centre of Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaritzburg from June 1997 to October 2003 under the supervision of Professor J. van Staden and Doctor A.K. Jäger.

These studies represent work done by the author and have not been submitted in any form for any degree or diploma to any other University. Where the work of others has been used , it is duly acknowledged.

I declare that the above statement is correct.

Nokwanda. P. Makunga

Professor J. van Staden
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Problems worthy of attack prove their worth by hitting back.

P. Hein (1966)

Just as physics and chemistry discover the mineral component of compound bodies of experimental investigation, so to comprehend the phenomena of life, that are so complex, it is necessary to go deep into the organism and to analyze the organs and tissues in order to reach the organic components

C. Bernard (1872)

SCIENTIFIC OUTPUTS

(i) PUBLICATIONS IN PEER-REVIEWED JOURNALS

MAKUNGA NP, JÄGER AK, VAN STADEN J (2003) Micropropagation of *Thapsia garganica* – a medicinal plant. *Plant Cell Reports 21: 967-973.*

NIGRO SA, MAKUNGA NP, GRACE O Medicinal plants at the ethnobotany-biotechnology interface in Africa. *South African Journal of Botany 70(1): 89-96*

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(ii) CONFERENCE CONTRIBUTIONS

MAKUNGA NP, VAN STADEN J, JÄGER AK, CRESS WA (1998) Establishment of *in vitro* cultures of *Thapsia garganica* for infection with *Agrobacterium rhizogenes.* South African Association for Botanists Conference (University of Cape Town, Cape Town)

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MAKUNGA NP, VAN STADEN J, JÄGER AK (2002) Transfer of the root inducing genes of *Agrobacterium rhizogenes* into *Thapsia garganica*, a medicinal plant. South African Association for Botanists Conference (Rhodes University, Grahamstown)

MAKUNGA NP, JÄGER AK, VAN STADEN J (2002) Micropropagation and genetic transformation of *T. garganica*: Progress and Perspectives. 8th International Pacific Rim Biotechnology Conference (Auckland, New Zealand [Poster])

MAKUNGA NP, JÄGER AK, VAN STADEN J (2003) Micropropagation and genetic transformation of *T. garganica*: Progress and Perspectives. South African Association for Botanists Conference (University of Pretoria, Pretoria)

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Mama no Tata, ndiyayibulela ngentliziyo yam yonke inxaso yenu ukusukela ku-A kude kubelelixa. Niye nandixhasa ixesha elide. Enkosi kakhulu for your unselfish sacrifices. Ngaphandle kwenu, bendingazukwazi ndifunde ndide ndifikelele apha. "Ndinje nje nje nguwe". Enkosi – ningadinwa.

ABSTRACT

The roots and fruits of the umbelliferous plant, *Thapsia garganica* (Apiaceae), contain sesquiterpene lactones – thapsigargins, and presently these compounds are being developed as chemotherapeutic agents for prostate cancers due to their ability to induce apoptosis of TSU-Pr1 prostatic cancer lines. The most prominent of these compounds, thapsigargin, is an important pharmacological tool used extensively for investigating calcium homeostasis. Increasing economic pressure for thapsigargin combined with diminishing *T. garganica* plants in the wild led to the biotechnological approach used in this study in pursuit of a conservation-through-cultivation strategy for this important medicinal species. Therefore, plant tissue culture methodologies and gene transfer techniques were examined as they offer a solution, in such instances. Introduction of the *rol* genes of the Ri TL-DNA for the production of hairy root cultures provides a genetically improved source of secondary metabolites and mass propagation *in vitro* of medicinally important is beneficial and complements field cultivation.

This research effort has culminated in an efficient *in vitro* regeneration protocol with a high-rate microplant multiplication for propagation of *T. garganica* as a prerequisite for gene transfer with the leaf material proving most useful for induction of culture *in vitro* on a MS medium (MURASHIGE & SKOOG, 1962) supplemented with 0.5: 1.5 or 1:3 NAA: BA combination. Regeneration on this medium followed an indirect organogenesis route. The 1:3 NAA: BA medium promoted callugenesis and unprecedented hyperhydrification of *in vitro* propagules. In an attempt to minimise the onset of aberrant phenotypes and circumvent the callus phase, often associated with somaclonal variation, an optimal medium for regeneration of plantlets was investigated using factorial experimentation procedures. Results from those experiments indicated that inclusion of 2,4-D in the growth medium strongly encouraged development of somatic embryos and thus verified the findings of JÄGER *et al.* (1993) and other researchers as somatic embryogenesis occurred with spontaneity in *T. garganica*.

Induction of roots through popular-used application of auxins was not sufficient in *T. garganica* as this species did not readily root. Utilisation of a two-step liquid rooting procedure with filter paper bridges as supports for microplants in the presence of high auxins (higher than 5 mg l^{-1}) improved rooting significantly. Rooting on solid medium in the presence of charcoal initiated roots on microplants to a similar extent as the liquid medium. Polyethylene glycol (PEG) and use of better ventilated culture jars prior to re-introduction of cultures from vitreous conditions to the natural environmental allowed for better quality management and those plants treated in this manner were healthier as compared to control plants. The stage of acclimatisation presented in this thesis was disappointing due to susceptibility of *T. garganica* to damping-off diseases. This is not a surprising result as *ex vitro* survival is generally low in cultured herbaceous umbelliferous plants. Intensive evaluation of acclimatisation procedures preventing or reducing attack by fungal pathogens was the most efficient way of eliminating the problem of wilt-diseases. Pre-treatment and post-treatment with a variety of antifungal agents significantly improved the frequency of survival post-*vitrum* from 0 % to 52 %. It is advised that antifungal cocktails become an integral part of the acclimatisation of the genus *Thapsia*. The fungicidal cocktail targeting a variety of fungi, - *Pythium*, *Phytophtora*, *Rhizotonia*, *Fusarium*, *Thielaviopsis* and *Verticillium*, - commonly associated with causing "damping-off" was used as a soil drench and a spray. This cocktail composed of Previcur N, Rovral Flo and Kelpak (a seaweed concentrate), quadrupled the number of hardened off plantlets to 52%. Acclimation after 8 months was achieved, with these plants becoming phenotypically similar to mother plants, dying back for four months of the year.

Several transformation protocols were tested against a variety of explants (*in vitro* and *ex vitro* grown material) throughout the course of the study. Standard transformation procedures with a variety of wildtype and recombinant strains of *Agrobacterium* were inefficient in transforming a variety of explants as target material. The effect of combining *A. rhizogenes* infection and biolistic transformation on the transformation of *T. garganica* shoot bases was compared to alternative methods of genetic transformation. This novel approach of biolistics-

assisted *Agrobacterium*-mediated transformation using DNA-coated tungsten (BAAT 2) in *T. garganica*, showed its usefulness by transforming 35.3 and 53 % of explants using *A. rhizogenes* strains A4T and C58C1, respectively. Transgenic tissues were selected on the basis of kanamycin selection with a survival ranging from 0 to 53 %. Transformation of lines was confirmed by PCR-mediated detection of *rol* transgene fragments with a TL-rol A_1 and TL-rol C_2 primer combination. Furthermore, resolution of positive amplification products served as support for data obtained with the GUS (β-glucuronidase) histochemical assay where 64 % of the explants tested positive, thus validating *T. garganica* as a species that is amenable to transformation upon employment of BAAT. Of the gene transfer methodologies presented here, BAAT-treatment may further be employed to other species of *Thapsia* and the Apiaceae that do not respond favourably to conventional application.

In spite of successful gene transfer, true-to-type hairy root production was not realised in the media tested for establishment of a liquid-shake system. Such findings draw into question whether mechanisms of transgene silencing are acting to hinder phenotypically characteristic hairy roots. This was evidenced by putative transgenic roots behaving unexpectedly in culture with a growth rate similar to untransformed roots. Even though such ideas require further research to be conducted, comprehensive investigation into the transformation of *T. garganica* as a first time attempt has validated that this plant does indeed lend itself to gene transfer.

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CHAPTER 1
GENERAL INTRODUCTION

Thapsia garganica L. (Apiaceae [Umbelliferae]) has generated considerable attention because the thapsigargins isolated from the fruits and roots of this umbelliferous plant have recently been shown to induce apoptosis (programmed cell death) of TSU-Pr1 human prostatic cancer lines (FURUYA *et al.*, 1994; XIAOHUI *et al.*, 1997; JAKOBSEN *et al.*, 2001) provoking interest anew in this ancient species in Europe. These sesquiterpene lactones are currently being developed as therapeutic agents against prostate cancer. The resin of this ancient plant has been known to provoke a vigorous contact dermatitis associated with inflammation (JÄGER, 1993), and intolerable itching and often leads to scarring of tissue (GRIEVE, 1931). The plant was named after the Isle of Thapsus where it is believed to have originated according to folklore and Algerians, in particular, consider every part of the plant to be efficacious with the root being a strong purgative and emesis (GRIEVE, 1931). Its use as a medicinal plant to relieve rheumatism by the Arabs of Northern African is well-established (JÄGER *et al.*, 1993). *Thapsia villosa* also contains a resin in the roots and this resin is milder in its actions than that of *T. garganica* (GRIEVE, 1931).

According to the recent review by EKEIRT (2000), the species of the Apiaceae are rapidly becoming an example of medicinal plant biotechnology as European pharmocopoeias carry a wide variety of herbal medicinal products derived from this family. These herbal medicinal products act via a variety of secondary metabolites such as coumarins, polyacetylenes, triterpenes, furanochromes, essential oils, amongst others. In addition, these plants are commonly utilised as spices and vegetables (HEYWOOD, 1971; RASMUSSEN and AVATO, 1998; EKIERT, 2000). Many of these species are endemic to the Mediterranean area and Asia minor where temperate climates prevail (EKIERT, 2000). According to Drude's classification (DRUDE, 1898), the Apiaceae family is divided into three subfamilies

and the genus *Thapsia* belongs to the largest subfamily. These plants are further characterised in the tribe Laserpitieae and subtribe Thapsiineae. In Flora Europaea (TUTIN *et al.*, 1968), three species are recognised, namely, *T. garganica* (L.), *T. maxima* Miller and *T. villosa* L and these are distinguished according to leaf morphology. A combination of phytochemical studies, morphological observations and chromosome counting has elicitated doubt in this taxonomic classification of the genus. Geographically, *T. garganica* is distributed over the Southern part of the Mediterranean through to Portugal (Figure 1.1). *T. maxima* is found in Eastern, Central and Southern Spain and *T. villosa* may be found in Portugal to Southern parts of France (JÄGER, 1993).

Figure 1.1: Distribution of *Thapsia* species. Locations where plant material may be collected (JÄGER, 1993)

Thapsigargin is the most prominent of the secondary compounds isolated from *Thapsia* plants and since its commercialisation it has been extensively used as a pharmacological tool for studying calcium homeostasis (JÄGER *et al.* 1993, CHRISTENSEN *et al.*, 1997). The increasing economic demand for thapsigargins as a pharmacological tool is endangering the wild and sparsely distributed *Thapsia* populations in the Mediterranean region (JÄGER, 1993). Thapsigargins are solely accessible from the roots and fruits of naturally growing plants, at present. There

is a pressing need to develop a genetically modified root culture system for the enhanced production of thapsigargins to alleviate this problem. Transgenic hairy root cultures of *T. garganica* would provide an alternative source for thapsigargin and its analogues and thereby reduce harvesting of the plants. The use of classical breeding methodology for the improvement of the Apiaceae may not be a feasible option as it is said to be difficult, laborious, time consuming and extremely slow (HUNAULT *et al.*, 1989, TAWFIK and NOGA, 2002). Plant biotechnology offers a more efficient approach to improve the accumulation of secondary metabolites for medicinal purposes (BAJAJ and ISHIMARU, 1999; TAWFIK and NOGA, 2002) and for germplasm conservation and multiplication of rare, threatened or endangered plant species (BAJAJ and ISHIMARU, 1999). This approach usually guarantees the preservation of the same genotype and phenotype of the plants resulting from *in vitro* propagation (EKIERT, 2000). The production of thapsigargins is not spontaneous in culture and has only been reported in embryogenic cultures of *T. garganica* (JÄGER *et al.*, 1993), suggesting that differentiation in an *in vitro* culture system is concomitant with thapsigargin biosynthesis. This requirement for differentiated cell cultures for the production of thapsigargins *in vitro* further intensifies the interest in developing an efficient plant transformation regime to be able to exploit the hairy root culture system. Such cultures provide a more efficient alternative for the production of root derived bioactives as they have been proven to be stable, biochemically and genetically superior as compared to a non-transgenic root culture system, and often lead to higher levels of extractable secondary metabolites (TEPFER, 1989; FLORES *et al.*, 1999; VERMA *et al.*, 2002). These properties have made hairy root cultures more desirable to industry than callus cultures. Crucial for exploiting the potential of these biotechnological approaches has been the development of more efficient gene transfer methods which have culminated in 200 plant species being manipulated. Of these, about 70 species belong to medicinal plants (BAJAJ and ISHIMARU, 1999) with studies in the Apiaceae focussing mainly on two species, namely, *Pimpenella anisum* (CHARLWOOD and SALEM, 1999; EKIERT, 2000) and *Anethum graveolens* (SANTOS *et al.*, 2002). *Agrobacterium*-mediated transformation has been favoured over direct gene delivery methods like particle bombardment as it offers several advantages: such as, defined transgene

integration, potentially low copy number insertion; and, preferential integration into transcriptionally active regions of chromosomes (KONCZ *et al.*, 1989; BIRCH, 1997). However, some plants are recalcitrant to *Agrobacterium* infection and in those species amenable to manipulation, transformation efficiencies and subsequent regeneration may be low (TRICK and FINER, 1997). These factors have been attributed to *Agrobacterium* exhibiting host specificity as well as the failure of T-DNA transfer to some plant species and transformation via *Agrobacterium* is also cultivar and target tissue dependent (HANSEN and CHILTON, 1999). A tissue culture phase is often essential to facilitate molecular manipulations in plants (BIRCH, 1997; HANSEN and WRIGHT, 1999). In this instance, the application of successful plant gene transfer technology is largely dependent on a reliable and efficient regeneration system for a particular plant species (HANSEN and WRIGHT, 1999) The infection of plants with *Agrobacterium rhizogenes* to induce the hairy root syndrome followed by the excision of the transgenic roots to initiate root cultures is preferred for the synthesis of secondary metabolites with intent to industrialise.

This study was undertaken as there were no successful reports on genetically enhanced culture systems of *Thapsia* species at the time of initiation. In this investigation, genetic transformation with the aid of tissue culture was applied and this thesis describes the response of *T. garganica* to gene transfer combined with the search for a suitable *in vitro* propagation for this medicinally important plant.

CHAPTER 2
LITERATURE REVIEW

2.1 *Thapsia garganica* L. – A MEDICINAL PLANT

The genus *Thapsia* is one of approximately 300 genera comprising the Family Apiaceae. The members of the Apiaceae (3 000 species) are mostly temperate herbs characterised by the presence of hollow stems, sheathing petioles and almost always an umbellate inflorescence (SMITT *et al.*, 1995). Flowers of the Apiaceae are typically small, mostly bisexual and actinomorphic and the fruits are schizocarps (WATSON and DALLWITZ, 1992). Inspection of the genus *Thapsia* based on morphological characters (SMITT *et al.*, 1995), phytochemical analysis (AVATO *et al.*, 1993, CHRISTENSEN *et al.*, 1997), and molecular characterisation of chromosomes and genome organization of *T. garganica* L. (RASMUSSEN and AVATO, 1998) provided evidence suggesting that four species belong to this genus and *T. garganica* should be considered as a separate species from *T. transtagana*.

According to SMITT *et al.* (1995), *T. garganica* is a perennial herb that is 80-200 cm tall and has a stem that is cylindrical (1-3 cm in diameter) and the pinnate glabrous leaves (1-3) with entire linear oblong lobes (Figure 2.1). The petals are yellow without bracts and bracteoles. The fruits (15-25 mm) are elliptical to oblong with very broad lateral wings (3-6 mm) that have deeply emarginated bases and apexes. Although it is difficult to distinguish *T. garganica* from *T. transtagana* when comparing macromorphological characters, these two species are easily identified from the other *Thapsia* species on the basis of fruit size and leaf morphology. With regard to micromorphological characters, the placing and the number of secretory canals in the pericarp is of great value taxonomically. Both species contain companion cells and true vittae with the vascular bundles of *T. garganica* accompanied by one secretory canal situated at the outer side of the bundle. On

the other hand, *T. transtagana* has two secretory canals accompanying each vascular bundle, one situated at the inner side of the vascular bundle and the other at the outer side. *Thapsia* plants have a single tap root which accumulates thapsigargins at highest levels and similarly to fruits. Two thapsigargins are the major constituent of the *T. garganica* plant resin, thapsigargin and thapsigargacin (Figure 2.2). Nortrilobolid, thapsivillosin J and thapsivillosion I are the three minor constituents that were isolated and identified from the resin of *Thapsia* plants by SMITT *et al.* (1995).

2.2 CHEMICAL STRUCTURE OF THAPSIGARGINS

Sesquiterpene lactones are one group of secondary compounds which have been briefly studied even though they are important for the pharmaceutical industry (LUCZKIEWICZ *et al.*, 2002). Thapsigargins are a group of highly oxygenated sesquiterpene lactones and possess a unique configuration as they have a *cis* annulation of the lactone ring and two non-esterified hydroxy groups at C_7 and C_{11} (Figure 2.2) (CHRISTENSEN, *et al.* 1997). All the thapsigargins are esterified with angelic acid and acetic acid at C_2 and C_{10}, respectively (CHRISTENSEN *et al.*, 1997). Different acids may be esterified to the C_1 and C_8 hydroxy groups, although in some thapsigargins, the ester group is lacking (SMITT *et al.*, 1995). The chemotypes of thapsigargins known to date are shown in Figure 2.2.

2.3 TERPENOID BIOSYNTHESIS

Terpenoids are the largest group of plant chemicals comprising of over 15 000 fully characterised compounds with terpenoids being amongst the most expensive secondary compounds to synthesise per gram in leaves (LANGENHIEM, 1994). At times, terpenoids may accumulate to concentrations of at least 5 % of the dry weight of the plant (GERSHENZON, 1994).

Figure 2.1: A flowering *T. garganica* plant growing in the wild

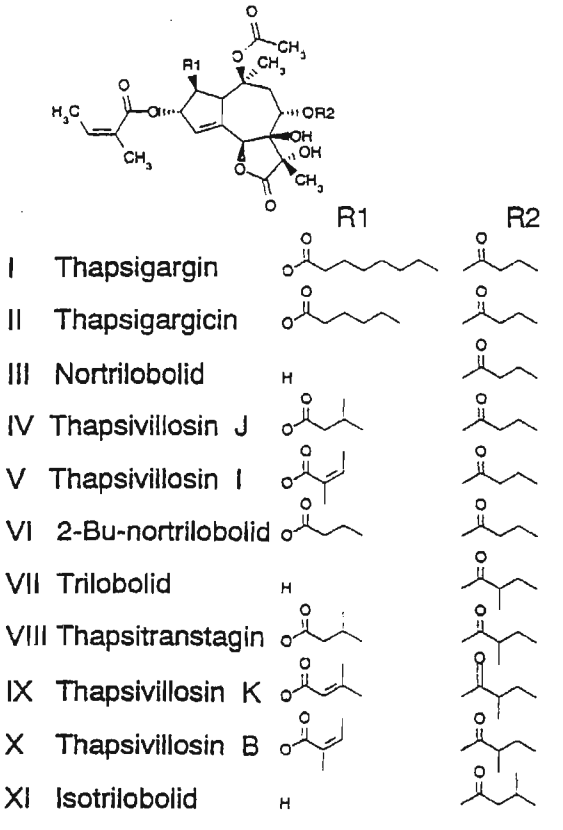

Figure 2.2: Chemotypes of thapsigargins (JÄGER, 1993)

Terpenoid compounds are synthesised from isoprene, a precursor found rarely in nature, via the acetate-mevalonate pathway (GERSHENZON, 1994) (Figure 2.3). They are synthesised in various cellular organelles and are then generally stored in a specialised secretory structures for the protection of the plant's primary metabolic processes from their toxic effect (GERSHENZON, 1994). Mevalonate is the accepted universal primary precursor of all isoprenoids and is derived from acetyl-CoA through the intermediate formation of acetoacetyl-CoA. These reactions are catalysed by acetyl-CoA transferase and hydroxy-3-methlyglutaryl-CoA (HMG-CoA) synthase, respectively. A reduction of HMG-CoA performed by hydroxy-3-methlyglutaryl reductase (HMGR) produces mevalonate. This enzyme requires NADPH as a co-substrate as well as for protection against oxidative inactivation of thiol groups. The mevalonate pathway utilizes three molecules of ATP and two molecules of NADPH (GERSHENZON, 1994) (Figure 2.3).

The terpenoid biosynthetic pathway is well-documented and understood (see LANGENHEIM, 1994 for review). Once produced, mevalonate is phosphorylated by mevalonate kinase in the presence of ATP producing mevalonate phosphate. This phosphorylation of mevalonate is the control point of terpenoid biosynthesis and a second phosphorylation carried out by phosphomevalonate kinase to yield mevalonate pyrophosphate occurs. Isopentenyl pyrophosphate (IPP), the biogenic isoprene unit, is then produced by the decarboxylation-dehydration reactions of mevalonate pyrophosphate by the actions of pyrophosphomevalonate decarboxylase. Isomerisation of IPP to dimethylallyl phosphate involves IPP-isomerase and produces the precursor of the single isoprene units in a number of plant compounds. These units are subsequently converted to individual isoprenoid compounds. Sesquiterpenes are a result of the condensation of dimethylally pyrophosphate and IPP to yield a C_{10} monoterpenoid, geranylpyrophosphate farnesylpyrophosphate. Formation of 2-trans-6-farnesylpyrophosphate then occurs and leads to a variety of sesquiterpenoids which may be hydroxylated, dehydrogenated, carboxylated and acquire lactone groups. These reactions occur later in the biosynthesis (LANGENHEIM, 1994) (Figure 2.4).

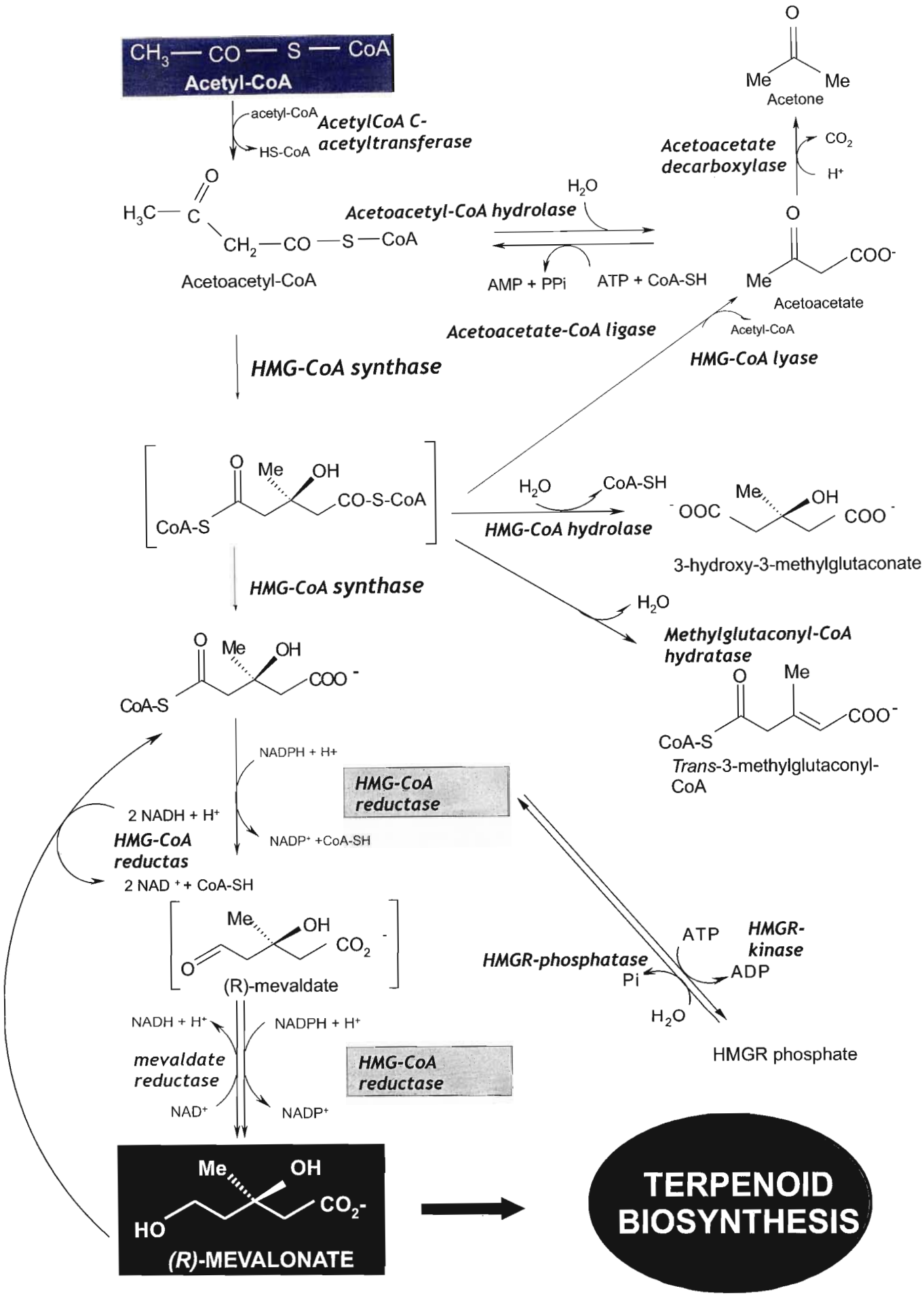

Figure 2.3: Acetate-mevalonate biosynthetic pathway (Adapted from http://www.chem.qmul.ac.uk.iubmb/enzyme/reaction/terp/mva/htm using the ISIS DRAW version 2.4 computer package)

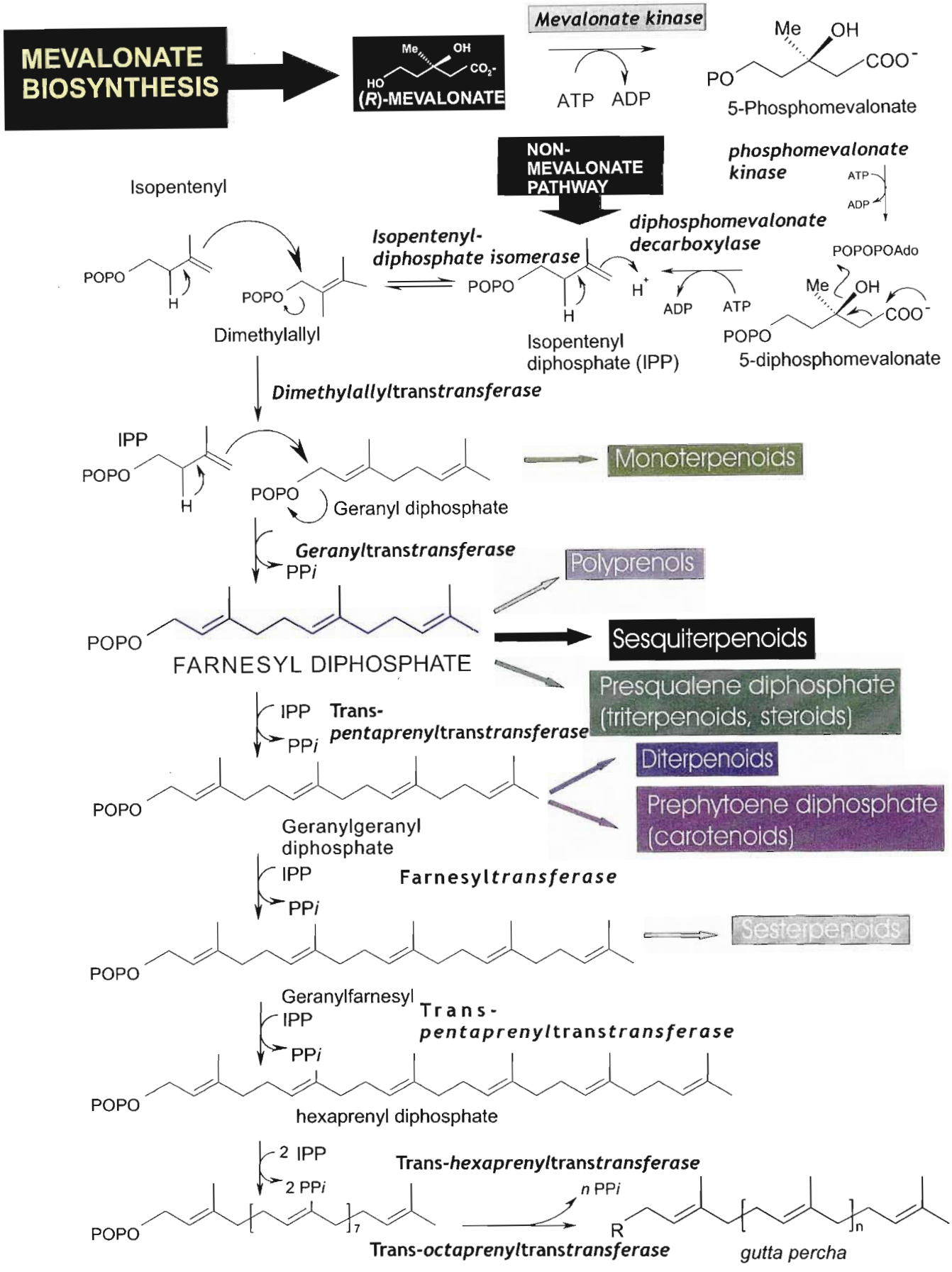

Figure 2.4: Terpenoid biosynthesis
(Adapted from http://www.chem.qmul.ac.uk.iubmb/enzyme/reaction/terp/terp.html using the ISIS DRAW version 2.4 computer package

2.4 MECHANISM OF THAPSIGARGINS ACTION IN THE CELL

Calcium (Ca^{2+}) is known to play a pivotal role in many cellular reactions of eukaryotes and is also implicated in the regulation of the release of compounds involved in signal transduction pathways and developmental processes (BUSH, 1995; CLAPHAM, 1995; LIANG and SZE, 1998). However, in plant systems the regulation of cytosolic and organellar Ca^{2+} is poorly understood (LIANG and SZE, 1998) whereas in animal cells the role of Ca^{2+} is well-studied and characterised. According to studies performed in animal cells (DENMEADE *et al.*, 2003), high affinity Ca^{2+}-ATPases are ATP-coupled pumps located in a variety of cell and organ membranes that regulate the Ca^{2+} concentration inside the cell by removing Ca^{2+} from the cytosol to re-establish the basic concentration of Ca^{2+} after a signalling event has passed (Figure 2.5A). Na^{+}/Ca^{2+}-exchange proteins in the plasma membrane are also necessary for channelling Ca^{2+} from the cytosol (RASMUSSEN and AVATO, 1998; DENMEADE *et al.*, 2003).

Thapsigargins have been utilised extensively as a tool for the study on calcium homeostasis and the elucidation of Ca^{2+}-transport mechanisms (RASMUSSEN and AVATO, 1998). Biochemical analyses (CHRISTENSEN *et al.*, 1997; JAKOBSEN *et al.*, 2001) have shown that thapsigargin selectively inhibits the function of the ubiquitous sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCA's), thereby blocking intracellular sequestration of Ca^{2+} leading to the depletion of the endoplasmic reticulum (ER) Ca^{2+} accumulation of increased levels of Ca^{2+} within the cell (Figure 2.5B). The rise in the Ca^{2+} concentration due to thapsigargin is followed by a sustained influx of Ca^{2+} from the extracellular medium. Thapsigargin thus induces a sustained increase in intracellular Ca^{2+} levels as movement of Ca^{2+} from the cell is inhibited when thapsigargin forms a "dead end " complex with ATPase. Elevated Ca^{2+} may cause the release of signal compounds such as histamine which in turn can cause blisters and skin irritation. Sustainment of these high levels of Ca^{2+} subsequently leads to DNA fragmentation and apoptosis (programmed cell death) (FURUYA *et al.* 1994, XIAOHUI *et al.* 1997).

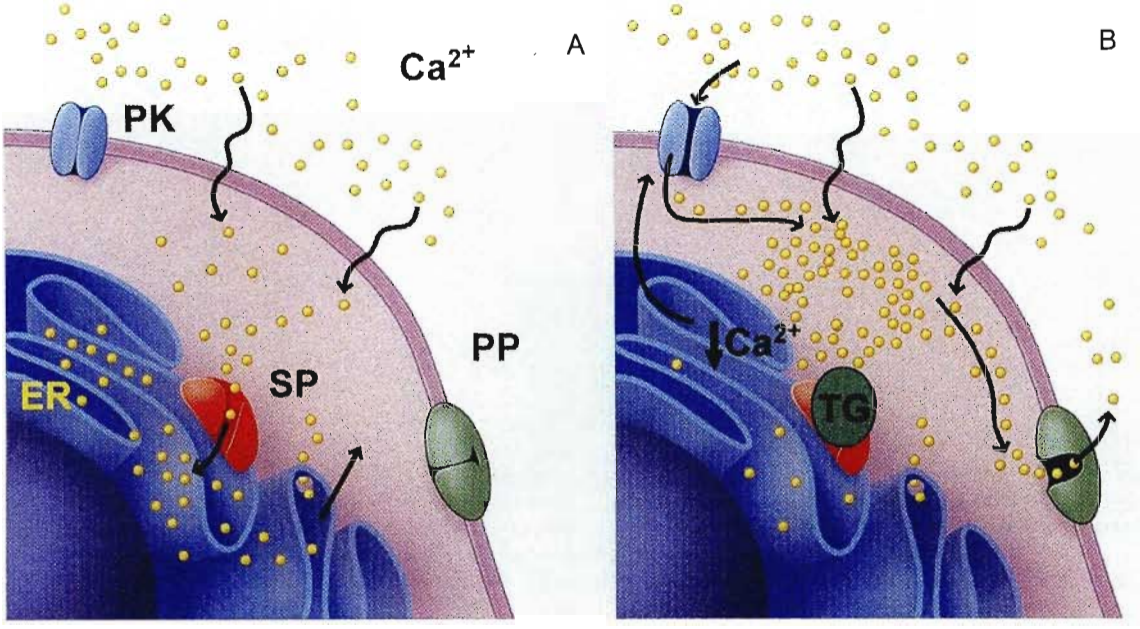

Figure 2.5: A schematic diagram of a cell showing different calcium pools and calcium transport proteins (A) Normally the SERCA-pump **(SP)** keeps the concentration of calcium ions (yellow dots) low in the cytoplasm by pumping the ions into intracellular calcium stores **(ER)**. **(B)** If the pump is blocked by thapsigargin **(TG)**, the calcium ions are slowly released from the stores and simultaneously, a channel in the cell membrane **(PK)** is opened allowing inflow of calcium ions from the extracellular space elevating calcium concentrations to abnormally high levels. Due to the efforts of a membrane located pump **(PP)** trying to remove the excess calcium ions, a series of uncontrolled reactions occurs within the cell. Finally, these reactions lead the cell to self-destruct.

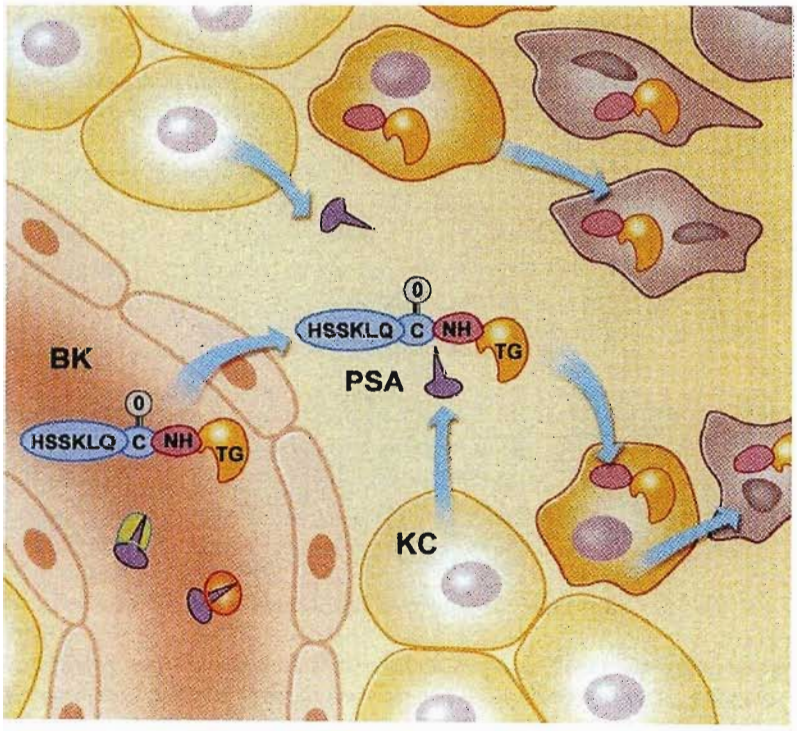

Figure 2.6: A schematic diagram of the action of a thapsigargin derivate against prostrate cancer cells. The anti-prostrate cancer prodrug is a protein which is bound to a compound derived from thapsigargin **(HSSKLQC=ONHTG)**. The compound is cleaved by the enzyme PSA in close proximity to the PSA-releasing prostate cancer cells **(KC)**, whereafter thapsigargin **(TG)** enters the cells and leads to apoptosis. In the vascular system **(BK)**, as indicated on the left, PSA is inactivated by blood proteins and the prodrug remains uncleaved preventing the release of thapsigargin into the blood stream.

JAKOBSEN *et al.* (2001) recognised that this response is independent of the rate of cellular division and consequently, thapsigargin induces apoptosis of slow-growing prostate cancer cells. However, this action is not selective against cancerous cells of the prostate and thus would render thapsigargin cytotoxic to the host if administered as a therapeutic agent in its present state. This has been the most limiting factor with respect to the use of thapsigargin as a chemotherapeutic agent against cancer as the compound eventually kills all cell types (Figure 2.5B).

2.5 THAPSIGARGIN ACTION AGAINST PROSTRATE CANCER

Currently, thapsigargin is being targeted to act solely on prostate cancer cells. JAKOBSEN *et al.* (2001) converted thapsigargin to debutanolythapsigargin and thereafter, coupled this analogue to a protein which can only be cleaved by a prostate-specific antigen (PSA), a serine protease uniquely secreted by prostate cancer cells (Figure 2.6). PSA regulates the viscosity of sperm fluid and is only active enzymatically in prostate extracellular fluid. Moreover, PSA is inactivated by blood proteins and thus, active in the immediate proximity of the cells secreting it. Binding a thapsigargin analogue to a highly specific and efficient PSA substrate specifically targets the cytotoxicity to prostate cancer cells (Figure 2.6). The thapsigargin analogue is brought into closer proximity to PSA and will then only enter and kill those prostate cells producing PSA. Until recently, the structure of thapsigargin made it difficult to bind it to a protein. Thus far, tests performed on mice implanted with prostate cancer treated with a variety of thapsigargin analogues have shown a marked reduction in the proliferation of the cancer. This was due to the maintenance of elevated intracellular Ca^{2+} (Figure 2.5B) in TSU-Pr1 cells which culminated in apoptosis.

2.6 *IN VITRO* PRODUCTION OF SECONDARY METABOLITES

Secondary metabolism was once regarded as the non-essential process that was responsible for the production of by-products or waste products in plants (LIU *et al.*, 1998). It is now apparent that secondary metabolites such as alkaloids, phenolics and terpenoids contribute significantly to the defence mechanisms deployed by against herbivory and microbial attack; and these secondary

metabolites are medicinally important due to their cytotoxicity (LANGENHEIM, 1994; LIU *et al.*, 1998). Although much progress and many developments in the synthesis of chemical compounds have occurred, plants are still the major source of indispensable industrially important secondary metabolites such as pharmaceuticals (steroids, alkaloids, glucosides amongst others), dyes, pesticides, natural flavours, fragrances, colourants, dyes (BALANDRIN *et al.*, 1985; BAJAJ and ISHIMARU, 1999) and in recent years nutraceutics (BOURGAUD *et al.*, 2001). Many compounds of popular use are remarkably complex in structure rendering chemical synthesis difficult and economically non-viable (HAMILL *et al.*, 1987).

Cultivation of plants for medicinal purposes is a common practice, however, this is limited to plants that establish well in cultivation outside their traditional ecosystem (BOURGARD *et al.*, 2001). For pharmaceutical purposes, plant cell and callus cultures have become a means of producing secondary metabolites and are exploited particularly for biologically active secondary metabolites which have found medicinal application as drug entities or model compounds for drug synthesis (HAMILL *et al.*, 1987). Application of plant cell cultures to produce secondary metabolites *in vitro* has been utilised in the past for plant species which are rare, endangered or threatened due to diminishing natural habitats, and the use of this technology continues (BAJAJ and ISHIMARU, 1999). Plant cell biomass can be produced in large bioreactors for commercially interesting secondary compounds, similar to those in use for the production of microbial drugs (BAJAJ and ISHIMARU, 1999). Biotechnological approaches are being applied for plants whose germplasm needs to be conserved, multiplied and improved for various traits, more especially for increased secondary metabolite accumulation (NESSLER, 1994). Particularly plant metabolic engineering involving the redirection of one or more enzymatic reactions has the ability to provide a better understanding and subsequent manipulation of secondary pathways (DELLAPENNA, 2001). Biotechnology is also being utilised for generating and incorporating genetic variability into medicinal plants and recently for metabolic engineering of pathways for the production of these biologically active compounds of high-value (NESSLER, 1994; DIXON *et al.*, 1996).

A large amount of studies have concentrated on *Atropa belladonna* and *Nicotinia tabacum* (BAJAJ and ISHIMARU, 1999) and the main objective of research performed on medicinal plants focuses on enhancing or increasing the production of pharmaceutics, drugs, flavourants, colourants and medicinal compounds *in vitro* (BAJAJ and ISHIMARU, 1999) by manipulating plant cells in culture to increase metabolic flux into specific pathways (DIXON and BOLWELL, 1986). Additionally, DIXON and BOLWELL (1986) recognised that tissue culture systems offer alternative means to produce medicinal compounds from endangered plants. Cell suspension cultures are favoured for large-scale production of secondary metabolites from such plants as culture conditions are optimised with ease. The use of a controlled environment that may be manipulated is preferred as environmental and geographical instabilities can make it difficult to acquire certain plant-derived chemicals from plants growing in the wild (DIXON and BOLWELL, 1986; BALANDRIN *et al.*, 1985; BALANDRIN and KLOCKE, 1988). Biotechnology has the potential to provide a continuous and reliable source of natural products, as well (BALANDRIN and KLOCKE, 1988). Extraction of secondary compounds from intact plants may have problems associated with it as isolation from *in vivo* plant matter is often limited by season in which plants can be collected; the age of the plant; and, other environmental and physiological factors. *In vitro* cultures, on the other hand, tend to accumulate secondary products when subjected to chemical stress, specific stimuli (such as fungal elicitors) and under specific culture conditions (TOIVONEN and ROSENQVIST, 1995; DIXON and STEELE, 1999; KOVALENKO *et al.*, 2002). The manipulation of the environment and media often affects the growth rate and accumulation of a secondary metabolite (BALANDRIN and KLOCKE, 1988). At times, during this process *de novo* synthesis of sometimes new compounds not occurring normally in the intact plant occurs (BAJAJ and ISHIMARU, 1999).

The technology was first described by NICKELL (1962) for large-scale production of secondary metabolites by plant cell cultures and since inception a variety of metabolites ranging from allergens, diosgenin, L-dopa, saponin glycosides and glycyrrhizin have been produced and patented (DODDS and ROBERTS, 1985). The anti-inflammatory drug, shikonin, was the first product derived from *in vitro* cell

cultures of *Lithospermium erythrorhizon* (TABATA and FUJITA, 1985) to become commercially available in Japan and since then a limited amount of cell culture derived products have been successfully industrialised in spite of the large amount of research efforts focussing in this particular area (BUITELAAR and TRAMPER, 1992). In 1990, cultured ginseng products derived from cell suspension cultures of *Panax ginseng* were commercialised by Nitto Denko (Japan) with a nett sale of $ 3 million in 1995 (FU, 1998). In the past, the major limitations which have led to fewer than anticipated products being produced and available in the market include: the lack of basic knowledge of biosynthetic pathway and the mechanisms involved in secondary metabolite synthesis (BUITELAAR and TRAMPER, 1992) and the production rate of plant cell cultures of desired products is frequently poor (HAMILL *et al.*, 1987). A greater knowledge of the biochemical and genetic regulation of plant secondary metabolism has since the commercialisation of the first *in vitro* cell culture derived products been acquired. Recent advances in the fields of cell, developmental and molecular biology of secondary metabolism have heightened the appreciation for the complexity of biosynthetic pathways. It is now clear that these pathways are under strict developmental regulation in plants and this is one reason that has severely restricted the exploitation of cultured plant cells for the production of valuable phytochemicals (FACCHINI, 2001).

According to FU (1998), efforts focussing on the study of metabolics for the analysis and the optimisation of biochemical pathway have given major impetus to the use of phytotechnology for the exploration and utilisation of plants in medicine. Identification and cloning of enzymes involved in the synthesis of bioactive compounds as well as the understanding of the regulation of complex metabolic pathways is essential in the control and optimisation of secondary metabolite production. More advances in the development of strategies for yield improvement and design of large-scale bioreactors to meet industrial demands have the potential to aid the commercial development of plant cell culture derived products. Moreover, selection for clones arising from individual cells with high producing capabilities and maintenance of elevated productivity through repeated screening has the ability to alleviate the problems associated with poor production rates. Disorganised cell cultures have a tendency to revert to lower productive capacity

(HAMILL and LIDGETT, 1997) and this inherent instability is due to altered gene expression (FACCHINI, 2001). The widespread application of molecular technology in the 1990's has facilitated the isolation of several genes involved in secondary metabolite biosynthesis. This is coupled with the identification of the early events of signal perception, investigations into signal transduction pathways and the function of gene promoters which regulate the secondary metabolic pathways and enormous emphasis has been placed in particular on alkaloid biosynthesis in the past decade. This research, recently reviewed by FACCHINI (2001) has shown that secondary biosynthetic pathways are strictly developmentally regulated and this fact has resulted in major limitations to the exploitation of cultured plant cells for the production of valuable phytochemicals, as previously mentioned. Dedifferentiated cells often fail to display the same range of compounds as intact plants suggesting that differentiation of specific cell types is essential for the synthesis as many of the key enzymes involved in biosynthesis are developmentally regulated and furthermore often regulated environmentally, for example by light. This was shown in *Catharanthus roseus* cell cultures that were able to accumulate tabersonine and catharanthine but failed to synthesise vindoline or vinblastine (MEIJER *et al.*, 1993). Current research has begun to reveal the *cis*-elements and *trans*-acting transcription factors implicated in genetic mechanisms of developmental and inducible regulation of secondary metabolic pathways (FACCHINI, 2001). A rising number of genes and their regulatory sequences, which are involved in biosynthesis of secondary metabolic pathways, being identified and cloned greatly improves the opportunity to affect complex pathways by metabolic engineering (HERMINGHAUS *et al.*, 1995). This infantile technology, with respect to its use in the alteration of secondary metabolism, encompasses the improvement of cellular activities by manipulating enzymatic activities, transport and or regulatory functions by applying recombinant DNA technology (FACCHINI, 2001). It has been suggested that a focus on regulatory controls and rate-limiting steps of pathways should be the starting point for alteration.

The types of cultures that are favoured for the production of secondary metabolites include cell suspension, organised tissues and transformed shoot and root cultures (LUCZKIEWICZ *et al.*, 2002). For production of secondary metabolites on a large-scale, cell suspension cultures are preferred due to the similarities that exist when compared to microbial cultures as both systems exhibit rapid growth cycles and plant cell cultures are totipotent (FU, 1998). For the production of compounds requiring a differentiated system; shoot, root and other plant organs are utilised and the profiles of products generated by such organised or specialised cultures are similar to intact plants growing in the wild (FU, 1998; LUCZKIEWICZ *et al.*, 2002). The genetic stability in these cultures surpasses cell and callus cultures and stable growth accompanied by consistency in the production of secondary metabolite has been observed. However, product profiles may sometimes differ in organised cultures when compared to plants growing *in vivo* (FU, 1998).

There are several techniques which exist to facilitate growth, production and excretion of secondary metabolites in a continuous production process including immobilisation of plant cells, permeabilisation, excretion of secondary metabolites using two-phase technology, use of bioreactors, use of elicitors, cell selection or genetic manipulation (BUITELAAR and TRAMPER, 1992; CHOI *et al.*, 1994). These methods are able to overcome the limitations associated with plant cell cultures such as low metabolic rate, low product yield, and sensitivity to shearing (CHOI *et al.*, 1994). In addition, plant cell cultures provide tools for the standardization and optimization of the production of bioactives isolated from traditionally used plants have shown clinical efficacy but whose composition and production may be highly variable *in vivo* (CHOI *et al.*, 1994).

The immobilisation of plant cells has been shown by many researchers to result in increased yield of extracellular secondary metabolites and this system is said to facilitate the continuous flow process (BUITELAAR and TRAMPER, 1992; FU, 1998). Immobilised cell systems, reviewed by FU (1998), have a positive effect on cultured cells and show prolonged cell viability and stable synthesis of important secondary metabolites. Active cell immobilisation involves the enclosure of cells into polymers, which polymerate after adding of monomers (mainly sodium alginate, agar, agarose and polyacrylamide), whereas, passively immobilised cells

are placed or sedimented into spongy material, such as polyurethane froth. It has been suggested in the review that the immobilisation of cells affects cellular physiology and enhances cell-to-cell contact resulting in higher levels of certain bioactives produced in comparison to freely suspended cells. However, this system does have its disadvantages. Firstly, few studies have compared the level of compound when applying cell immobilisation methods versus free cell suspension methods. It is speculated that immobilisation creates a matrix effect around cells which possibly mimics tissue organization between them and may give rise to 'biochemical differentiation' which is known to favour the accumulation of secondary bioactives (BRODELIUS, 1984; BOURGAUD *et al.*, 2001).

The use of elicitors is a widespread and favoured method for increasing secondary metabolite production *in vitro* (reviewed by BOURGAUD *et al.*, 2001). The application of chemical or physical stresses, namely fungal cell wall materials; plant and microbial polysaccharides; and, specific chemical stimulants for secondary metabolism triggers the production of secondary metabolites at times not normally associated with a specific cell culture. Presently, the favoured biotic elicitors are fungal cell wall materials, chitosan, plant and microbial polysaccharides; and specific protein extrascts. Abiotic factors are also utilised to elicit a response in cell cultures such as temperature, UV light, heavy metal salts, and pH. Transfer of cells to a new production medium is not a requirement and the elicitation treatment is usually awarded with a rapid response resulting in the production of increased yield of bioactive compounds. This has led to increasing popularity of this technology. Elicitation has been found not only to activate the expression of several enzymes but also to increase activity of already existing enzymes involved in the biosynthesis of terpenoid indole alkoloids, phenylpropanoids and terpenoids. However, sometimes the activity of other enzymes can be reduced or remain unchanged. This type of treatment is also capable of activating novel genes which are not similar to genes already known. It is generally accepted that elicitation ultimately has an effect at the transcriptional level by increasing the rate of gene transcription of those genes involved in plant defense mechanisms. Coordination of the activation and inactivation of such genes is tightly regulated but a detailed understanding of these regulatory controls is still amiss (FU, 1998). A more detailed understanding of secondary metabolism is said to be essential as these

studies will not only provide for better understanding of primary metabolism but will also affect the practical implications for the production of secondary metabolites (LIU *et al.*, 1998).

Plant genetic manipulation has gained considerable attention for the production of transgenic root and shooty teratoma cultures to produce plant metabolites, as many characteristics of transformed cultures are advantageous for the production of secondary plant metabolite production (FU, 1998). Stably transformed hairy root cultures are rapidly growing and highly productive. It is such features that have made them attractive for exploitation in order to produce secondary metabolites (HAMILL *et al.*, 1987).

2.7 HAIRY ROOT CULTURES

Hairy root cultures represent differentiated genetically transformed organ cultures that may frequently produce higher contents of the secondary metabolite compared to the intact non-transgenic plant (HAMILL *et al.*, 1987). These cultures show generally a high biosynthetic ability concomitant with genetic and biochemical stability (TOIVONEN and ROSENQVIST, 1995). Due to their inherent genetic stability, they show stable production of secondary compound in contrast to cell suspension cultures and remain fairly active and prolific in medium devoid of plant growth regulators (FLORES *et al.*, 1999). Consequently, they are considered to offer better prospects for the commercial production of secondary metabolites than undifferentiated cell cultures (SIM *et al.*, 1994). RHODES *et al.* (1989) reviewed the characteristics of hairy root cultures in comparison to normal cultures. Callus and/or suspension cultures have the tendency to lose their abilities to produce specific secondary compounds, a characteristic of these type of cultures noted by several researchers. On the other hand, hairy root cultures represent a differentiated *in vitro* system for the production of secondary products that often matches or even surpasses the concentration of compound in the intact plant. However, these cultures are not as easy to manipulate as cell suspension cultures by the simple alteration of medium components, whereas the addition of fungal elicitors has sometimes increased the secondary product content of those cultures not responding to changes in medium composition (OKSMAN-CALDENTY *et al.*,

1994). Hairy root cultures since the 1980's have also allowed for studying previously unidentified and unique secondary metabolites and the development and exploitation of this technology has revolutionised the role played by *in vitro* cultures in fine chemical synthesis (HAMILL *et al.*, 1987).

All root-derived secondary products from dicotyledonous were regarded as the main potential targets for the induction of hairy root cultures in the 1980's as monocotyledonous plants were more difficult to transform with *Agrobacterium* due to its host specificity. The first report on the intitiation of hairy root cultures for production of secondary metabolites was in 1985 by FLORES and FILNER. These authors were able to induce the hairy root cultures of *Hyoscyamus spp* which accumulated tropane alkaloids. This study has since then been followed by an ever growing number of reports recording hairy root cultures being applicable to many more plant species including monocotyledons and gymnosperms. Thus, this literature review cannot cover all the productive hairy root culture systems which have been described in recent years. For recent reviews consult DORAN (1997) and BAJAJ (1999) and secondary products which have been isolated from hairy root cultures were summarised by BAJAJ (1999). Approximately 200 species of higher plants representing a minimum of 30 plant families have been transformed (BAJAJ and ISHIMARU, 1999). Therefore, this makes hairy root culture a well-established experimental system. Since its inception, it has generated many insights into root metabolism and synthesis of phytochemicals, and thus becoming a system for acquiring a better understanding of the coordination existing between primary and secondary metabolism (FLORES *et al.*, 1999). Hairy roots have also been applied as a tool in rhizosphere interaction studies. These cultures are of great value as the undergrowth habit of roots poses enormous technical difficulty for their study and has hampered research in the biology of root specific metabolism (FLORES *et al.*, 1999).

Despite transformed tissue cultures showing higher stability in comparison to non-transgenic cultures, clone-to-clone variation still exists and several researchers have recorded variations with regard to the growth rate and production of compounds (FU, 1998). This variation has been attributed to differences in the

length and the copy number as well as the chromosomal position of the Ri T-DNA transgene upon intergration into the plant genome.

2.8 GENE TRANSFER TO PLANTS

The *Agrobacterium* system is technically more attractive for the introduction of foreign DNA into plant genomes because of its precision and simplicity; furthermore steps followed the mimic closely course of the infection process that occurs in nature (HANSEN and CHILTON, 1999). Gene transfer is a well-established and a routine technique in many laboratories around the world and it has contributed to rapid progress in basic and applied plant sciences (LINDSEY, 1992; NESSLER, 1994; POTRYKUS, 1995).

Genetic transformation is a naturally occurring process (HOOYKAAS and SCHILPEROORT, 1992; BENT, 2000) and scientists have been able to perform controlled transformation of plants with specific genes since the mid 1970's (BENT, 2000). Tremendous progress in plant gene transfer technology has been achieved since the recovery of the first stably transformed plants in the 1980's. Pioneer experiments involved gene transfer via *Agrobacterium tumefaciens* for the introduction of antibiotic resistance genes. In 1983, the gene encoding for kanamycin resistance was introduced into tobacco plants by HERRERA-ESTRELLA *et al.* The host specificity of *A. tumefaciens* limited its use to mainly solanaceous species and other dicotyledonous plants. The development of other sophisticated methods of transformation has since led to the rapid progress in this field of study and this technology has now been applied to numerous species including monocotyledonous species which previously were recalcitrant to *Agrobacterium* infection.

In the mid 1980's reporter gene systems, still currently in use, enabled for rapid selection of transgenic tissues. These systems also allowed for the investigation of regulatory factors, *cis*-acting and *trans*-acting factors in stable transformants in deletion assays and have thus led to a greater understanding of the regulatory mechanisms controlling the expression of plant genes. The *gusA* gene, isolated

from *Escherichia coli* strain K12 by JEFFERSON, was developed for use as a reporter system in the mid-1980's, with its use being wide and extensive for the selection of putative transformants (JEFFERSON, 1987; JEFFERSON, 1989; JEFFERSON *et al.*, 1987). The introduction of the first commercial genetically modified plant product to be readily available to consumers in the market was the Flavr Savr or Endless Summer™ tomato. These transgenic tomatoes, first released in America, were modified to be tastier, less prone to spoilage and richer in colour. The approval for sales of Bollgard™ - a transgenic cotton containing resistance to bollworm and budworm (*Heliothis* spp) occurred in 1995 and this commercial product, sold as Yieldgard, is also available in South Africa (BRINK *et al.*, 1999).

The list of plants (vegetables, ornamentals, medicinal plants, fruits, trees and pasture plants) with a variety of novel traits acquired through genetic modification is on the increase. This list includes plants modified for the production of biodegradable plastic; edible vaccines as a source of oral immunization; speciality oils; and, hypo-allegernic products including rice. The use of non-destructive reporter gene assays since the mid 1990's has led to the development of green fluorescent protein (GFP) as a means of selection of putative transgenic tissues (CHALFIE *et al.*, 1994). Advancements in transgenic technology has had an enormous impact on the pharmaceutical industry as well with hairy root cultures being employed to manufacture phytochemicals.

BIRCH (1997) alleged that a threshold for plant transformation was being reached with over 3 000 field trials in progress or completed in over 30 countries. Plant transformation research was fast emerging from an era dominated by development of proven and effective gene transfer methodologies for species of economical and experimental purposes into an age of the application of this technology as a core tool for research for the better understanding of plant biology. The rapid development of transformation technology concurring with flourishing progress in information technology makes the tabulations of transformed species to become quickly outdated. Bearing this in mind, BIRCH (1997) advised for the use of computer-based searches to access species of interest currently being

transformed. Refinement and diversification of transfomation techniques in order to achieve higher efficiency and convenience; a wider genotype range; and, more desired molecular traits will continue into the future. Future prospects are thus likely to concentrate on the improved patterned of expression and dealing with economic constraints as gene transfer and subsequent regeneration of transformed plants is fast ceasing to be an issue of concern.

2.8.1 Methods of transformation

Genetic transformation of medicinal plants is commonly brought about by two methods, ie, direct gene transfer or uptake of DNA, and using the *Agrobacterium* transgene delivery system (BAJAJ and ISHIMARU, 1999). However, the use of *A. rhizogenes* is preferred over the *A. tumefaciens* as the former mentioned bacterium rapidly induces hairy roots which easily lend themselves to industrialisation for large-scale production. For successful gene transfer to plants several biological requirements need to be met (HANSEN and WRIGHT, 1999):

(i) the availability of suitable target tissue with regenerable cells;

(ii) an appropriate method for targeting the gene(s) of interest efficiently into those cells competent for regeneration;

(iii) selection procedures for transgenic tissues including protocols allowing for ease of regeneration of transgenic plants at reasonable frequency;

(iv) a simple efficient reproducible genotype independent and cost effective process; and,

(v) avoidance of somaclonal variation and possible sterility by limiting time in culture.

Protoplast transformation, biolistics and *Agrobacterium* transformation fulfil these criteria (HANSEN and WRIGHT, 1999). The protoplast method however requires the most finesse, as a result the other two methods have been more widely used.

2.8.2 *Agrobacterium:* nature's 'genetic engineer'

The genus *Agrobacterium* contains Gram-negative saprophytic or parasitic soil-borne bacteria belonging to the Family Rhizobiaceae (NILSSON and OLSSON, 1997). *A. tumefaciens* and *A. rhizogenes*, incite crown gall disease and the hairy root syndrome, respectively, on a wide range of plants. SMITH and TOWNSEND (1907) identified *A. tumefaciens* as responsible for the neoplastic effect of crown gall tumours at the turn of 20th century and later in the thirties, the causative agent of hairy root disease, characterised by extensive rhizogenesis, was discovered and the bacterium denominated for its effect *A. rhizogenes* (RICKER, 1930). It was only in the seventies that the unprecedented event which caused tumours associated with crown gall in plants was revealed to be an example of unique interkingdom gene transfer and integration of genetic material from the soil bacterium to the infected plant (SCHEIFFELE *et al.*, 1995). Since then the scientific community has gone through great lengths to comprehend the complex processes of plant transformation by *Agrobacterium* (CONSTANTINO *et al.*, 1994). Although, other bacteria have been shown to transport proteins into eukaryotic cells such as bacteria with type III secretion capabilities (DUMAS *et al.*, 2001), is the only bacterium known to possess the ability to transfer DNA into a eukaryotic target. This bacterium had therefore evolved unique and specialised mechanisms for interkingdom transfer of DNA. To date, transformation via *Agrobacterium* is still not fully understood and specifically, fundamental biological events comprising of DNA transfer from bacterium to plant still remain elusive (DUMAS *et al.*, 2001). However, the use of molecular biology to elucidate these mechanisms is slowly providing answers and the interaction between the pathogen (*Agrobacteria*: *A. tumefaciens*, *A. rhizogenes, A. rubi* and *A. vitis*) and host is now viewed as a complex series of chemical signals communicated between the two organism. In recent years, this topic has been the subject of much research which has been synthesised or summarised in a number of review articles (WINANS, 1992; ZAMBRYSKI, 1992; ZUPAN and ZAMBRYSKI, 1995; TZIFIRA and CITOVSKY, 2000; GELVIN, 2000; GELVIN, 2003).

GELVIN (2000) suggested that the current understanding of the functional role played by the plant genes and proteins that lead to transformation is relatively poor when compared to the knowledge acquired at the genetic and molecular levels with regard to the mechanisms at play within *Agrobacterium* involved in the process. It is a growing belief that a comprehensive understanding of the role of plant genes in the transformation process and the manner in which plant-encoded gene products interact with the incoming DNA transferred from the *Agrobacterium* will extend the host range for genetic manipulation of plants as well as benefit basic plant cell biology. A limit of extending the host range via the manipulation of the bacterium may fast be approaching and the manipulation of plant genes to increase the efficiency of transformation will probably influence not the host range but also the frequency of gene transfer via *Agrobacterium*. At present, for a large number of plant species and elite cultivars recalcitrant to transformation mediated by *Agrobacterium*, this recalcitrance seems not to be a consequence of T-DNA transfer failure or lack of directed T-DNA targeting into the nucleus as in many cases transient transformation has shown relative efficiency. Rather, stable integration into the genome of cells that regenerate efficiently poses a restraint in mitigating the host specificity of *Agrobacterium*. Moreover, proficient transformation due to a better control of the T-DNA transfer process and less random T-DNA integration associated with multicopy, inverted repeats T-DNA resulting in homology dependent gene silencing may be averted with a greater understanding of *Agrobacterium*-plant interactions. Thus far, the bacterial factors involved in the infection process are relatively well-studied and identified. This knowledge is accompanied by complete sequences of the T-DNA region of nopaline type *Agrobacterium* strains and the T-DNA regions has been fully characterised. However, far less is known with regards to the host plant factors involved in the transformation process.

2.8.3 Biology of the *Agrobacterium*-plant Interaction

Virulent strains of *A. tumefaciens* and *A. rhizogenes* both carry the tumour inducing (Ti) plasmid and root inducing (Ri) plasmid, respectively. These megaplasmids are very large (200 to greater than 800 kb) and the transcription of a DNA fragment

located in the Ti/Ri plasmid but transcribed in the plant genome, termed the transferred DNA (T-DNA) (20-30 kb) leads to the disease characteristics of crown gall and hairy roots, respectively (SCHEIFFELE *et al.*, 1995; DICOLA *et al.*, 1997). The transfer of the mobile T-DNA segment occurs through the cooperative action of gene products of the Ti plasmid virulence operon (*vir* genes) and the chromosomal genes of the bacterium (DE LA RIVA *et al.*, 1998). The megaplasmids also contain genes that control the conjugative transfer, mobilisation or transfer and stable integration. The T-DNA once stably integrated directs *de novo* synthesis of special compounds, opines (amino acid and sugar phosphate derivates) which the bacteria exclusively catabolises for a carbon/nitrogen source (TZIFIRA and CITOVSKY, 2000; AKAKURA and WINANS, 2002). *In planta* T-DNA expression includes the expression of oncogenic (*onc*) genes producing enzymes for the synthesis of plant hormones causing tumourogenesis or rhizogenesis. The virulence region (*vir*) is a 30 kb regulon organized as six operons with *vir*A, *vir*B, *vir*D and v*ir*G determining T-DNA excision and transfer and *vir*C and *vir*E function to increase the efficiency of T-DNA (ZUPAN and ZAMBRYSKI, 1995; DE LA RIVA *et al.*, 1998; JEON *et al.*, 1998). The T-DNA is flanked by 25 bp border repeats which are essential for transfer and recognised by two vir proteins, VirD1 (49,6 kDa) and VirD2 (16.1 kDa) (SCHEIFFELE *et al.*, 1995; ZUPAN *et al.*, 1996; RELIĆ *et al.*, 1998). A cascade of events is triggered when a plant cell susceptible to infection by *Agrobacterium* is wounded as the wounding releases phenolic compounds from the plant cell. Acetosyringone and hydroxyacetosyringone induce the expression of the *vir*A gene product encoding for the transmembrane sensing protein which senses the phenolic compounds (WALDEN, 1993). The phenolic compounds not only result in the activation of the *vir* operon which causes several changes to the T-DNA segment but also chemotaxis of the bacterium to the plant cell. The binding of *Agrobacterium* is a prerequisite for successful transfer of the T-DNA and the process occurs in a polar manner in a two-step process. The genes necessary for attachment are located in a 20 kb segment in the chromosomal DNA of the bacterium (HANSEN and CHILTON, 1999) and are referred to as the chromosomal virulence genes (*chv* genes) partaking in the early stages of bacterial chemotaxis and attachment to plant cells (TZIFIRA and CITOVSKY, 2000). These chromosomally determined

genetic elements display their functional role in bacterial colonisation via the *chv*A and *chv*B loci synthesising the excreted β-1,2-glucan (CANGELOSI *et al.,* 1990; DE LA RIVA *et al.*, 1998). The attachment of *Agrobacterium* produces a mass of cellular fibrils organised in a network tightly binding the bacterium anchoring the bacterium firmly on the surface of the plant cell forming a trap for other *Agrobacteria* that are not bound as yet (MATTHYSE, 1983; HANSEN and CHILTON, 1999). The binding of *Agrobacterium* is thought to occur at specific sites on the plant cell and the expression of *chv*A, *chv*B and *pscA* loci mediates this process (GRIERSON and COVEY, 1988; WALDEN, 1993; HANSEN and CHILTON, 1999). Even though the genes encoding for this process have been studied, their mechanism of action still remains poorly understood. The other chromosomal virulence genes are *chv*E required for the sugar enhancement of the induction of the *vir* operons; *pscA* (*exoC*) responsible for synthesis of cyclic glucan and acid succinoglycan; *cel* locus functions in the synthesis cellulose fibrils and the *att* locus plays a role in the cell surface proteins (HANSEN and CHILTON, 1999). ChvB is a 235 kDa protein which is directly involved in the formation β-1,2-glucans, whereas the *chv*A locus determines the synthesis of an inner membrane located transport protein and mediates the export of the β-1,2-glucans into the periplasm (HANSEN and CHILTON, 1999). Therefore, the export of cyclic β-1,2-glucans and other sugars is under the control of *chv*A, *chv*B and *pscA*(*exoC*) loci and these sugars are essential for bacterial attachment indirectly as *Agrobacterium* strains with mutations in these genes function poorly and exhibit highly attenuated virulence or avirulence under normal inoculation conditions (DOUGLAS *et al.*, 1982; DOUGLAS *et al.*, 1985; GELVIN, 2000). Direct microinjection into the cytoplasm does allow for T-DNA delivery from non-attaching mutants of *Agrobacterium* suggesting that T-DNA export from the bacterium is independent of attachment. Other pleiotropism is displayed by the *chvB* locus including the loss of rhicadhesin activity, an adhesive protein implicated in bacterial attachment. The loss of rhicadhesin activity may however be restored under condition of high osmoticum. Mutation in *psc*A(*exo*C) also results in pleitrophic effects associated with the loss of β-1,2-glucans (HANSEN and CHILTON, 1999).

Although, the attachment process has been studied extensively and the subject of several reviews by ZAMBRYSKI *et al.*, 1989; CITOVSKY *et al.* 1992a; CITOVOSKY *et al.*, 1992b; ZAMBRYSKI, 1992; HOOYKAAS and BEIJERSBERGEN, 1994; SHENG and CITOVSKY, 1996), plant molecules recognised by *Agrobacterium* in this process are poorly identified. Thus far, apart from a rhicadhesin-binding protein (SWART *et al*, 1994), a vitronectin-like protein (WAGNER and MATTYHSEE, 1992) on the cell wall of the plant is also implicated as surface receptor(s). In animal cells, the role of vitronectin as an extracellular component utilised as a specific receptor by some pathogenic bacteria is well-documented (TZIFIRA and CITOVSKY, 2000). In plants, the exact role of such proteins has not yet been clearly resolved by genetic analysis (GELVIN, 2000). Studies that have recently explored the function of plant proteins in the binding process using mutant plant ecotypes (resistant to *Agrobacterium* transformation, termed *rat* mutants) have shown that *Agrobacterium* is unable to attach to the *rat* mutants. This indicated a block at an early stage of the transfomation process (reviewed recently by GELVIN, 2000). However, transformation mediated by attachment deficient *Agrobacterium* using flower vacuum infiltration in *Arabidopsis* proceeds with efficiency comparable to implementation by wild type bacterial strains. These results suggest that vacuum infiltration of female gametophytes bypasses the requirement of the plant cell surface proteins for successful transformation (MYSORE *et al.*, 2000; GELVIN, 2000).

According to GELVIN (2000), inefficient *Agrobacterium* infection has been shown to occur in the absence of wounding and this has been speculated to take place through stomatal entry. The process of *Agrobacterium* infection to be efficient, sufficient wounding and/or rapidly dividing cells cultures in suspension is a requirement. Bacterial attachment is thought to require the presence of a plant cell surface and is speculated to be mediated by a protease-sensitive molecule located on the plant cell surface. This attachment process can reach saturation. Once the bacteria are firmly enmeshed on the plant cell wall, T-DNA transfer follows rapidly thereafter. The process can occur within two hours of co-cultivation as shown by several reports.

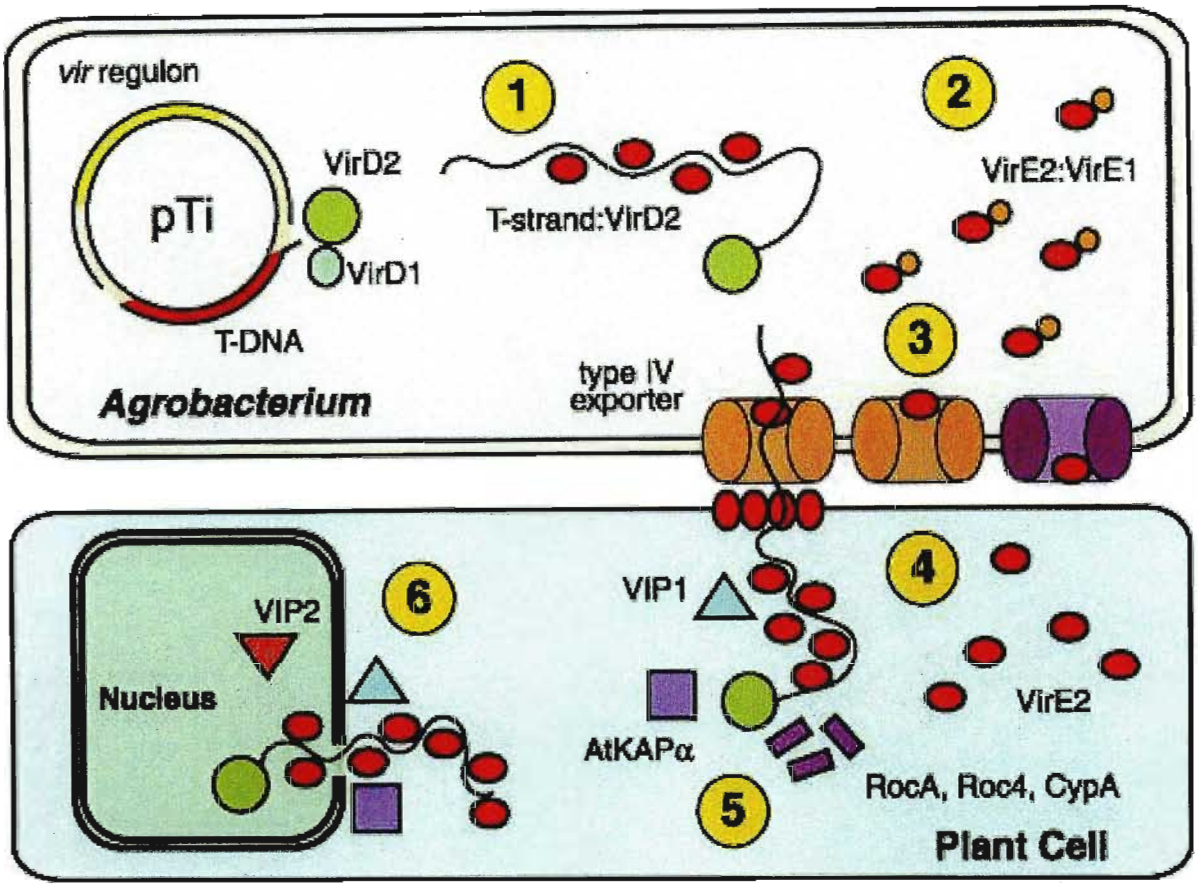

Figure 2.7: Schematic representation of the biology of the *Agrobacterium*-plant interaction. The *vir* regulon, encoding the major loci *vir*A-E and *vir*G-H, is expressed upon chemotactic detection of plant phenolic signals released due to wounding. VirD2 and VirD1 proteins excise the T-strand and VirD2 remains covalently bound to the 5' end. **(1)** VirE2 coats the T-strand, protects it from nucleolytic degradation, and maintains it in a transportable conformation. **(2)** VirE2 interacts with VirE1 prior to VirE2 export from *Agrobacterium* to the plant cell. **(3)** VirE2 exits *Agrobacterium* cell via the type IV exporter independently, or as part of the T-complex. Alternatively, VirE2 may exit by an alternate pathway (pink). **(4)** VirE2 forms a pore in the plant plasma membrane allowing passage of the T-complex and coats the T-strand in the plant cytoplasm. **(5)** VirD2 and VirE2 interact with plant cytoplasmic. Alternatively, VirE2 is transported through the VirB-VirD4 channel (DUMAS *et al.*, 2001) and subsequently inserts into the plant plasma membrane, allowing the transport of the ssDNA-VirD2 complex. The way in which the VirE2 molecules enter the cytoplasm is not clearly understood. The plant cytoplasmic chaperones (RocA, Roc4, and CypA) interact with VirD2 and other factors, such as AtKAP and VIP, may be involved in targeting the T-complex to the nucleus. **(6)** VirE2 associates with nuclear factors (VIP2) that facilitate integration of the T-strand into the plant DNA (WARD and ZAMBRYSKI, 2001).

It is well-documented that the regulation of the *vir* operon is tightly controlled (HERRERA-ESTRELLA *et al.*, 1988; HERRERA-ESTRELLA *et al.*, 1990) in the presence of phenolic and sugar compounds, usually involved in phytoalexin and lignin biosynthesis, are excreted by the plant and then synergistically perceived by the bacterium resulting in the induction of *vir* gene expression. Thus, *Agrobacterium* sabotages part of the plant's defence mechanism for its autonomous growth (GELVIN, 2000). The *vir* region is under stringent regulatory control and operates through *virA* and *virG* genes which are transcribed in a monocistronic fashion (reported in a review by HOOYKAAS and SCHILEPEROORT, 1992; HOOYKAAS and BEIJERSBERGEN, 1994; HANSEN and CHILTON, 1999). The dimeric transmembrane VirA sensor protein perceives the phenolic signal (such as acetosyringone) including monosaccharides which serve to augment the low level phenolic signal (PAN *et al.*, 1993) and VirA undergoes autophosphorylation which subsequently leads to transphosphorylation of the cytoplasmic DNA binding VirG protein (CHANG and WINANS, 1992). This in turn activates the expression of the rest of the vir operons (*virB*, *virC*, *virE*, *virF*). These *vir* gene products are then directly involved in the processing of the T-DNA in preparation for transport from the bacterial cell into the plant nucleus. The number of genes per operon differs as virA, virG and the non-essential virF have one gene only whereas virE, virC, virH have two genes each. Four and eleven genes have been assigned for virD1 and virD2, respectively. The expression of virA and virG operons is constitutive, and has been said to encode for a two component VirA-VirG system (DOTY *et al.*, 1996).

CHANG and WINANS (1992) reviewed the induction of the *vir* operon and reported that structurally, the virA protein has three domains. One of these being the periplasmic or input domain, which is essential for monosaccharide detection; and within this domain is an amphipathic helix that has strong hydrophilic and hydrophobic regions. These regions allow for protein folding and simultaneous alignment with the inner membrane and thus, anchoring the virA protein in the membrane. The other two transmembrane domains, TM1 and TM2, serve as the signal transmitter and the signal sensor/receiver, respectively. The TM2 is a kinase region that has a crucial role in the autophosphorylation of the VirA protein on a conserved Histidine-474 residue in response to phenolic and sugar

compounds upon plant wounding. The TM2 domain acts by activating the VirA protein. This is then followed by the activated VirA protein activating the VirG protein. The detection of monosaccharides by VirA functions as an amplification system because the periplasmic sugar binding protein, ChvE, complexes with VirA exposing the amphipathic helix of VirA to small phenolics. Once activated, VirA donates a phosphate to a conserved aspartate residue at the N-terminal region of VirG protein and subsequently, the phosphorylated VirG then acts as a transcription factor regulating the expression of the *vir* genes including its own. The C-terminus of VirG enables for the DNA binding activity allowing the protein to function as a trans-acting factor whereas the N-terminus shares homology with the VirA sensor domain. Expression of the *vir* operons is also under environmental regulation by external factors such as temperature and pH. The complexity of the structure of the virA protein enables its response to subtle changes in environmental conditions and temperatures higher than 32 °C inactivate VirA as it undergoes conformational change in the manner in which it folds (HANSEN and CHILTON, 1999). It has been postulated that other proteins involved in T-DNA transfer may be affected and inactivated at higher temperatures (FULLNER *et al.*, 1996). The *virH* (or *pinF*) locus is thought detoxify some of the plant-secreted phenolics which are potentially deleterious to *Agrobacterium* (HANSEN and CHILTON, 1999).

It is generally accepted that activation and expression of the *vir* genes ultimately generates a linear copy of the bottom strand of the T-DNA segment (designated the T-strand) and this single stranded (ss) T-strand is then exported from the bacterium and transferred to the plant cell (Figure 2.7). All T-DNA elements are flanked by 25 base pair (bp) imperfect repeat T-DNA borders where the nicking of the T-strand prior to transfer occurs. The right border at the 5' end is essential for excision and mutations in this regions result in loss of tumorigenecity. On the other hand, the left border is less important and is not essential for pathogenecity. T-strand production occurs in a 5' to 3' direction with the nick site located on the 5' end at the right border. Excision of the T-strand terminates at the left border. The VirD1 and VirD2 polypeptides are essential for the processing of the ss T-strand as the VirD1 protein aids the VirD2 protein in recognising and subsequently cleaving within the two border sequences (DUMAS *et al.*, 2001). This process involves the

two proteins interacting in order to function as a site- and strand-specific endonuclease which forms a bond with the supercoiled Ti plasmid at the T-DNA borders. This action relaxes and unwinds the T-DNA and the antisense strand between the third and the fourth base of the borders is nicked. After endonucleotidic cleavage, VirD2 remains strongly covalently attached to the resulting 5'-end of the ss-T-strand via tyrosine residue 29 (ZUPAN and ZAMBRYSKI, 1995). This distinguishes the 5' end as the leading end of the T-DNA transfer complex and the association of the VirD2 protein prevents exonuclease degradation of the T-strand at the 5' end. The gap between the right and the left borders is repaired using the bacterial DNA synthesis machinery. Thus far, it remains unknown whether the release of the T-strand occurs at the same time as gap repair or immediately follows thereafter. The left border and right borders of the T-DNA have been compared to sequences found at oriT (origins of transfer) sites of some bacterial conjugative plasmids (COOK and FARRAND, 1992; LLOSA *et al.*, 2002).

At present, there are two models that have been proposed for the translocation of the T-DNA strand. The most accepted model proposes that T-DNA is transferred to the plant as a protein/nucleic acid complex composed of a single VirD2 protein attached to a ss T-strand with the VirE2 protein (69 kDa) coating the T-strand/VirD2 complex forming a semi-rigid hollow filament (ZUPAN and ZAMBRYSKI, 1995). Thus, VirD2 is thought to act as the guiding protein for the T-strand from the bacterium into the plant and the VirE2 coat not only prevents the action of nucleases but also extends the ss T-DNA strand reducing the complex diameter to approximately 2 nm, making easier the translocation through membrane channels. Therefore, VirE2 and VirD1 are the only essential virulence components that are finally mobilized out of the bacterial cell and translocated into the plant cell (DUMAS *et al.*, 2001). The VirE1 protein stabilizes the VirE2 coat protein thereby preventing self-aggregation and maintains VirE2 in a competent state for export. Thus, the small VirE1 protein acts as a chaperone of the VirE2 and the requirement for a chaperoning protein has been likened to effector proteins of Type III secretion systems functioning in eukaryotic cells (BINNS *et al.*, 1995; DUMAS *et al.*, 2001). ZUPAN and ZAMBRYSKI (1995) and CHRISTIE *et al.* (1997) also suggested that transfer occurs through a pilus composed of several

protein products of the *virB* and *virD4* loci. The structure and assembly of this pilus still remains to be elucidated, and moreover, the export of the VirE2 and VirD2 proteins remains possible without the assembly of the VirB apparatus. Mechanism(s) required for the movement of the T-DNA complex across the plasma membrane of the plant remain elusive. Thus far, T-DNA mobilisation into the nucleus from the plant cytoplasm under the guidance of the VirD2 pilot protein is now known. Biophysical experimentation has recently shown that VirE2 interacts with lipids to form a transmembrane channel that is capable of ss DNA transport (DUMAS *et al.*, 2001). These findings have led to a hypothetical model for T-DNA transfer into the plant cell. The model proposes that VirE2 is transported through the VirB1-VirD4 channel or via an alternative manner and subsequently becomes located in the plasma membrane allowing for movement of the T-DNA complex. The movement of the VirE2 protein then follows in a manner not yet elucidated and these proteins coat the ss DNA facilitating import into the plant.

Even though VirE2 is the most abundant protein that accumulates in *Agrobacterium* due to the presence of acetosyringone, several researchers believe that it performs its primary functions only once in the plant cell. This then discredits the hypothetical model described above. SUNDEBERG *et al.* (1996) were able to show that export of VirE2 can be independent of T-DNA transfer using VirE2 bacterial mutants and also movement of naked T-DNA from the bacterium to the plant can occur without the presence of VirE2 protein (LESSL *et al.*, 1992; BINNS *et al.*, 1995). This experimental data led to a second model being brought about which proposes that the transfer complex is ss T-DNA covalently associated with VirD2 and lacking a VirE2 protein coat. Transfer of VirE2 to the plant cell occurs on its own and it is only when the naked ss T-complex reaches the plant cell that the VirE2 protein molecules interact and covers this strand in preparation for nuclear import (LESSL *et al.*, 1992; BINNS *et al.*, 1995; DE LA RIVA *et al.*, 1998; GELVIN 1998a). Recent experiments conducted in support of this model suggesting T-DNA transfer as an uncoated ssT-DNA/ VirD2 complex are summarized in the review by GELVIN (2000). This author further suggests that this model concurs with current models of conjugal DNA transfer between bacteria where single stranded conjugal intermediates are not produced in the bacterial cytoplasm but rather are generated at the membrane surface of the bacterium

concomitantly with ss DNA export. In the case of T-DNA, there would be no strands present in the cytoplasm to interact with VirE2 protein molecules and perhaps the detection of T-strands by certain researchers may be due to over-stimulation of T-DNA synthesis and the *vir* operon due to high concentrations of acetosyringone used during experimentation. Some laboratories have been unable to detect ss strands in the absence of proteases and ionic detergents.

The debate surrounding the manner in which the T-strand is imported into the plant cell continues, whether be it as a mature or immature T-complex continues. Additionally, the mechanisms of delivery across the bacterial envelope and the plasma membrane of the host plant have also remain largely unknown. T-complex transport has been likened to Type IV secretion systems (ZUPAN *et al.*, 1998) which have up to 12 proteins forming a functional filamentous pilus and transporter complex for the translocation of substrates via cell membranes (TZIFIRA and CITOVSKY, 2000). The products of the *vir*B operon and the virD4 protein are thought to function as the major components in the assembly of the Type IV transport apparatus. DUMAS *et al* (2001) incorporated new data into a hypothetical model based on the research of others as well as their own where they identified VirE2 as the channel mediating T-DNA uptake by the plant cell. DE LA RIVA *et al* (1998) reviewed the function of the vir proteins and VirB proteins are thought to play a major role as membrane spanning proteins forming a channel from the bacterium to the plant cell. The majority of VirB proteins are assembled as a membrane-spanning protein channel linking the two. In structure, the pilus has been accepted to be made of VirB proteins, with the exception of VirB11. These proteins have multiple periplasmic domains, whereas VirB11 has an extracellular. VirB4 and VirB11 are hydrophilic ATPases which are essential for actively transferring DNA transfer. VirB4 intertacts strongly with the cytoplasmic membrane VirB7 protein. VirB9 monomers are exported to the membrane and processed to a heterodimer which has been thought to assume a stabilization function of the other Vir proteins during assembly of the transmembrane channel. The VirB7-VirB9 heterodimmer is then sorted to the outer membrane and this sorting mechanism is still largely unknown. The products of virF and virH are thought to act as accessory proteins as their true function has not yet been fully elucidated. VirF appears to function in the targeting of the T-DNA complex into the

plant cell nucleus and VirH exists as two genes, VirH1 and VirH2. These genes are non-essential although do enhance the efficiency of T-DNA transfer and are thought to detoxify plant compounds that potentially are deleterious to the existence of the bacterium, as afore mentioned. This detoxification process occurs once the phenolics have fulfilled their role as signaling compounds as the expression of *virH* gene is induced by the phosphorylated VirG protein which, in turn, is activated when the phenolic signal has been received. The *virH* locus is also thought to play a role in the host range specificity of the bacterial (TZIFIRA and CITOVSKY, 2000).

2.8.4 T-DNA import into the nucleus

Once inside the plant cell, the ss T-DNA complex needs to cross the nuclear membrane in order to reach the nucleus and VirD2 and VirE2 are important facilitators of this process. VirF has been implicated as a minor contributor to nuclear targeting (DE LA RIVA *et al.*, 1998). The proteins involved in nuclear import contain motifs termed nuclear localization signals or sequences (NLS). These motifs are composed of one or two stretches of basic amino acids that control the import of the T-DNA into the nucleus. The two NLS's of VirE2 are thought to play a major part in nuclear import of the ss T-DNA complex by probably keeping both sides of nuclear pore simultaneously open. Those who argue that the T-complex is imported as an immature strand believe that on its arrival VirE2 proteins binds to the naked strand and this complex then recruits plant proteins existing in the cytoplasm. Thus, plant proteins have been assigned a role as mediators of nuclear targeting and this is under the control of NLS's in the amino acid sequence (see DE LA RIVA *et al.*, 1998 for review).

The NLS's are important as they are recognized by the nuclear import machinery. Nuclear import is a two step process which firstly involves NLS-dependent docking of the protein at the nuclear pore and then secondly channeling through the nuclear pore complex. VirD2 and VirE2 contain NLS's that mediate translocation of the T-DNA complex into the plant nucleus. ZIEMIENOWICZ *et al.*, (2001) accepted this model and additionally proposed that VirD2 has dual roles of firstly, chaperoning the T-complex into the plant cell and secondly, initiating the import

process into the nucleus. These researchers hypothesise that the C-terminal region of VirD2 NLS is essential for recognition by the NLS receptor importin-α of the nuclear import machinery. The T-complex is docked to the nuclear pore channel (NPC) via the importin-β when bound to the importin-α. The 5' end of the T-DNA is directed toward the channel of the nuclear pore where the translocation process commences. The efficiency of this process is increased by the presence of VirE2 cooperatively binding to the ssT-DNA, covering its negative charges and shaping it into a structure revealed by electron microscopy to resemble a telephone cord (CITOVSKY *et al.*, 1997). This form is most competent for translocation through the nuclear pore. The proposal highlights the role of the NLS of the VirE2 protein as they function to allow for specific interaction between the NPC and the T-DNA complex. The VirE2 is also thought to additionally (or alternatively) interact with nuclear import intermediates located on the inside of the nuclear pore in order to facilitate translocation (ZIEMIENOWICZ *et al.*, 2001).

The integration of the T-DNA by illegitimate recombination into plant genome, as generally accepted, is the final step of T-DNA transfer (GHEYSEN *et al.*, 1991; LEHMAN *et al.*, 1994; PUCHTA, 1998; ZUPAN *et al.*, 2000; BRUNAUD *et al.*, 2002). The precise mechanism remains largely uncharacterized, particularly the role played by specific plant proteins (GELVIN, 2000). The amount of T-DNA stably integrated into plants has been shown to be far less than that is transiently inserted indicating that the T-DNA that is transferred does not always all stably integrate and express (GELVIN, 2000). T-DNA strand invasion results in plant DNA denaturation at the site of integration. It remains unclear whether repair synthesis of a new second strand is preceded by T-DNA strand invasion or whether conversion of the T-strand to an extra-chromosomal double stranded form occurs before integration as per suggestion by DE NEVE *et al.* (1997).

BRUNAUD *et al.* (2002) have recently shown that T-DNA integration likely occurs in plant DNA regions rich in AT content. The process of recombination is initiated by the formation of a short DNA duplex between the host and the left end of the T-DNA. The preference for integration in a T-rich region is the main difference between the model presented by BRUNAUD and his associates (BRUNAUD *et al.*,

2002) and the previously accepted model by TINLAND (TINLAND, 1996). Five steps are thought to be involved in T-DNA insertion:

(i) the process of integration is initiated at the 3' end left border of the T-DNA in the vicinity of a poly T-rich area of the plant DNA;

(ii) a duplex is formed with the top strand of the host DNA upstream of the 3' end of the ssT-DNA;

(iii) the 3' end of the T-DNA downstream of the duplex is degraded and this is followed by ligation of the digested bottom strand and the 3' of the T-DNA. This ligation is under the control of the plant enzymes;

(iv) nicking of the upper strand of the host downstream of the duplex allows for the synthesis of a strand complimentary to the invading T-DNA. Detection and repair of imperfect matches in the duplex by the enzymes of the plant host using the sequence of the T-DNA template follows; and finally,

(v) the T-DNA right border is ligated to the bottom strand of the host DNA and this pairing often involves a G and another upstream nucleotide. This is followed by the degradation of the host DNA top strand. Ligation with the newly synthesized T-DNA takes place.

The AT-rich regions for the docking of the T-DNA are thought to be favoured as they have low DNA duplex stability and exhibit strong bending. In retroviral integration and transposable element insertion bending is essential for successful placement. A bended DNA region has been speculated to act as a common recognition site for the integration of foreign DNA segments in the genomes of eukaryotes.

According to the model proposed by TINLAND (1996) model, pairing of a few bases provides just minimum specificity for the recombination process by positioning VirD2 for the ligation. The 3´-end or adjacent sequences of T-DNA find some low homologies with plant DNA resulting in the first contact (synapses) between the T-strand and plant DNA forming a gap in 3'-5' strand of plant DNA. The displaced plant DNA is subsequently cut at the 3'-end position of the gap by endonucleases, and the first nucleotide of the 5' attached to VirD2 pairs with a

nucleotide in the top (5'-3') plant DNA strand. The 3' overhanging part of T-DNA together with displaced plant DNA are digested away, either by endonucleases or by 3'-5' exonucleases. Then, the 5' attached to VirD2 end and other 3'-end of T-strand (paired with plant DNA during the first step of integration process) joins the nicks in the bottom plant DNA strand. Once the introduction of the T-strand in the 3'-5' strand of the plant DNA is completed, a torsion followed by a nick into opposite plant DNA strand is produced. This situation activates the repair mechanism of the plant cell and the complementary strand is synthesised using the early inserted T-DNA strand as a template.

The VirD2 in concert with VirE2 and other plant nuclear factors have a role in the precise integration of the T-strand into the chromosome to form a stable genetically transformed cell. The release of VirD2 protein from the T-complex occurs after intra-nuclear import. Due to the ability of VirD2 to perform ligation *in vitro* (PANSEGRAU *et al.*, 1993), it was speculated that it also functions by ligating the 5' end of the T-DNA to the genomic plant DNA and thereafter, second strand repair synthesis provided by the plant machinery follows (TINLAND *et al.*, 1996). The integration of the T-DNA occurs with precision and usually a few nucleotides at the 5' region are deleted in order for the VirD2 cap to protect this strand from degradation by exonucleases. Besides its involvement in the ligation process, VirD2 is implicated in the integration process as it contains two amino acid sequence motifs that are generally associated with integration, one of these being the C-terminal ω domain and the other an integrase motif similar to the motifs found in integrase motifs of the recombinase family. VirE2 is also thought to have a role in T-DNA insertion as it may be required for the fidelity of the 3' end of the T-DNA strand (reviewed in GELVIN, 2000; TZIFIRA and CITOVSKY, 2000).

It is only now that the role of plant proteins in the integration process are being elucidated. Deficient plants in the mechanisms of DNA repair and recombination have a potential to be deficient in T-DNA integration as this process occurs by illegitimate recombination (GELVIN, 2000). In recent years, the isolation of several *Arabidopsis* mutants resistant to *A. tumefaciens* transformation at different stages of the transformation process has led to mapping of a H2A histone gene which is thought to function in T-DNA integration (GELVIN, 2000; TZIFIRA and CITOVSKY,

2000). Although the role of the H2A histone is currently unclear in the integration process, recent findings may have elucidated to the interaction of this protein with the incoming T-strand to form a putatitve integration complex (GELVIN, 2000). An "open" chromatin conformation of the target site where active transcription is taking place is preferred for T-DNA integration and this is thought to possibly influence the efficiency of T-DNA insertion.

Analysis of T-DNA inserts, once integrated into the plant genome using mapping techniques, has revealed that T-DNA integration occurs at random positions in the plant chromosome. However, activation of expression can occur subsequent to integration if the T-DNA inserts into transcriptionally active regions and this gene activation has a relatively high rate of 30-50%. It is also common for the T-DNA to be integrated in more complex patterns consisting of direct or inverted repeats of two or more T-DNA segments located in the same locus. Moreover, plant DNA flanking the T-DNA inserts has been shown to undergo deletions, rearrangements as well as suffer duplication yielding perfect or imperfect, direct or indirect repeats (see review HANSEN and CHILTON, 1999). Several researchers including BRUNAUD *et al.* (2002) have analysed T-DNA integration in depth and found that the left border of T-DNA sequence often exhibits "microhomology" or "microsimilarity" with short segments of the host DNA during integration. BRUNAUD *et al.* (2000) found that the "microsimilarity" exists with the first 25 nucleotides of the T-DNA at the 3' end.

According to HANSEN and CHILTON (1999), ideally transformants with single copy genes segregating in a Mendelian fashion exhibiting uniform expression from one generation to the other are most desirable. This is not always the case in practice as the success of the transformation depends on the plant material to be transformed as well as to a lesser extent the origin and the complexity of the inserted gene. The tissue specificity of a transgene may be variable from one transformant to another.

Although great progress has been achieved in understanding the *Agrobacterium*-mediated transformation system since the recovery of the first transformed tobacco plant performed by HERRERA-ESTRELLA (1983). The application of this system

is limited to those plants that are natural hosts of *A. tumefaciens* and *A. rhizogenes*. For many economically important plants, including the cereals, which for many years remained inaccessible for genetic manipulation, alternative direct means of gene transfer were traditionally used (TRIFONOVA *et al.*, 2001). Despite this particular disadvantage, *Agrobacterium* transformation remains superior to other methods for a variety of reasons: fewer copies of the transgene are inserted into the host genome producing simpler integration patterns, thus lessening the probability for transgene silencing particularly co-suppression and the instability of the transgene over generations is also reduced compared to the application of direct gene transfer methods. Transgene instability is generally triggered by occurrence of an inverted repeat transgenic locus, by insertion of transgene into heterochromatin regions which become methylated; and even extraordinarily high levels of expression may lead to transgene inactivation. "Position effect variation" is another property that leads to variable expression of introduced genes in plants due to the state of the chromatin at the site of insertion (HANSEN and CHILTON, 1999). These conclusions have mainly been drawn for dicotyledenous plants and for plants that are regarded as recalcitrant to *Agrobacterium* gene transfer. However, more data is required for a better understanding and improvement of the system in order to increase reproducibility and overall efficiency. Furthermore, *Agrobacterium* is a single-cell transformation system and thus the production of mosaic plants often associated with the use of direct gene transfer can be avoided.

2.9 THE Ri PLASMID OF *A. rhizogenes*

The insertion and expression of the T-DNA of the Ri plasmid induces a neoplastic response associated with extensive adventitious root formation (TEPFER, 1984). Whole fertile plants can easily be regenerated from *in vitro* cultured hairy roots and the Ri T-DNA transgene is transmitted in Mendelian fashion (BAJAJ and ISHIMARU, 1999). The *A. rhizogenes* strain and the plant species infected determine the pathogenecity of an infection (PORTER, 1991). The type of opine synthesis genes that are integrated and expressed in the host after a transformation event have been used to classify strains of *A. rhizogenes* and thus *A. rhizogenes* strains have been subdivided into three classes: agropine, mannopine and cucumopine (PETIT *et al.*, 1983).

2.9.1 Rol genes of A. rhizogenes

Due to the clonal nature of hairy roots plants, obtained via this method obtained from single roots are derived from a single T-DNA transformed cell (SLIGHTOM *et al.*, 1986). These plants express a transmittable phenotype defined by the appearance of wrinkled leaves, altered internode length, non-geotropic roots and altered flower morphology and reduced production of seed. These symptoms as a whole define the hairy root syndrome (TEPFER, 1984; CONSTANTINO *et al.*, 1994). This phenotype is controlled by the *r*oot *l*oci or *rol* genes which are encoded by four of the 18 open reading frames (ORFs) described by SLIGHTOM *et al.* (1986) after sequencing a 21 kb fragment of the Ri T-DNA. The four loci involved responsible for the hairy root syndrome (designated *rol A*, *rol B*, *rol C* and *rol D*) correspond to ORF 10, 11, 12 and 15 among 18 ORF'S of the TL-DNA. Each locus is responsible for a specific phenotypic characteristic that is expressed in transgenic regenerated plants (SÉVON *et al.*, 1997). TR-DNA encodes for auxin biosynthesis genes and the *iaaH* and *iaaM* gene products appear to encourage rhizogenesis (CARDARELLI *et al.*, 1987).

According to VAN DER SALM *et al.* (1996), the functioning of *rol B* and *rol C* loci is most well-understood as these two genes have a contribution to the hairy root syndrome and as a result have been extensively studied whereas little is known about *rol A* and *rol D*. *Rol A* transgenic plants usually have wrinkled leaves and the internodes are reduced, whereas *rol* B causes heterostly of flowers accompanied by prolific root production and *rol C* has been shown to reduce apical dominance, alter leaf morphology and decrease seed production. *Rol D* appears to encode for a transportable factor which induces premature flowering. Therefore, each *rol* gene is capable of acting alone and produces its own characteristic effect on plant development and morphogenesis. However, their synergistic effect is more pronounced with expression being tissue specific, moreover, the roots strongly express the *rol* genes as compared to leaves, once transformed. The *rol* B locus is thought to play the most important role in root induction as it has been the only one shown to be capable of initiating root growth on all plant hosts tested, individually. *rol B* is mainly expressed in the root caps, where the cells are actively undergoing division and in the vasculature of mature organs. The roots induced by

the *rol B* are typically characteristic of the hairy root syndrome as they are plagiotropic, highly branched and have an elevated growth rate. *Rol* B has been assigned the role of a morphogen able to stimulate meristematic tissue which then has the potential to differentiate into other organs. Whole plants regenerated from transformed roots exhibit the hairy root syndrome (reviewed by CONSTANTINO *et al.*, 1994). *Rol C* has been described as possessing cytokinin like activity resulting in decreases apical dominance, internode length, and; chlorophyll content. *Rol C* expression occurs mainly in the phloem of roots, stems and leaves. Although both *rol* B and *rol* C are well-understood, their biochemical action at the protein level requires further investigation.

2.10 COMPLICATIONS OF GENE TRANSFER

Agrobacterium-mediated transformation was generally considered out of the host range for monocotyledonous plants and initiatives were taken to develop direct means of gene transfer methods for the transformation of such plants. Even though *Agrobacterium* has been extensively exploited for transformation, highly efficient transformation events still remain elusive (BUER *et al.*, 1998). For the development of this methodology for monocotyledonous plants aspects in the *Agrobacterium*-plant interaction require critical consideration and this impacts on the cellular and tissue culture aspects being developed for a particular species as well as the bacterial strains, binary vectors, reporter genes and promoters being utilised. Ease of identification of transformed plants under selection conditions and application of molecular biology techniques for testing and characterizing the event of stable integration is only possible when the transgene is expressible in the host plant (reviewed by BIRCH, 1997).

Currently, three methods (biolistic DNA delivery; electroporation and polyethylene glycol mediated DNA transfer into protoplasts) are being utilised for the successful introduction of new DNA allowing for the application of transgenic technology to plants which were traditionally difficult to transform via *Agrobacterium*-mediated transformation. Although the host range of *Agrobacterium* has been extended to traditionally recalcitrant plants, none of the popular methods provide a mechanism

for the introduction of foreign genes at a defined locus. There is also no control over the number of copies inserted into the genome. Transformation usually results in the insertion of new DNA at random locations in the host genome. Suppresion of the transgene expression occurs with its insertion into heterochromatin regions which are inactive and present methods of selection are unable to allow for its recovery in the absence of expression. Highly augmented expression may also occur when introduced genes are located in close proximity to an enhancer element. Genes that in the subtelomeric region are highly expressed and insertion of a transgene in such a region results in a positive position effect (BHAT and SRINIVASAN, 2002).

Insertion of multiple copies of the transgene often leads to its silencing. This has been well-documented for direct gene transfer methods such as particle bombardment but reports do exist for *Agrobacterium* transformation as well. The changes in transgene organisation include truncation, inversion, deletion and other complex rearrangements. Transgene rearrangements are particularly common to biolistic delivery and it is speculated that the physical force may attribute to the rearrangements and subsequently to non-expression of the transgene. Over expression has also been shown to decrease or inhibit transgene expression (BIRCH, 1997).

In recent publications by BREGITZER *et al.* (1998); and, BHAT and SRINIVASAN (2002), most transformation methods are said to require the use of tissue culture, somaclonal variation may also influence transgene expression. For the recovery and propagation of transgenic single cells differentiated into whole plants, tissue culture is an integral part of this process and epigenetic variation has also been shown to be induced at this stage. Variation in transgene expression due to the activation of transposable elements, production of point mutations, altered methylation patterns and DNA amplification is well-established in transgenic generated *in vitro*. Successful plant regeneration is often the most limiting step of transformation as many important species are difficult to regenerate and this restricts use of this technology to a narrower range of plants for fundamental and applied research purposes. Recently, evidence to support the cause of

somaclonal variation as being the transformation process itself has become apparent or available. Nowadays, a growing trend is the utilisation of approaches which aid in the reduction of variation among transgenics, such as (i) *in planta* vacuum infiltration transformation which was originally developed for *Arabidopsis thaliana*; inclusion of matrix-associated region sequences in T-DNA and employment of the *cre-lox* system for reverting multicopy insertions into single copies. These approaches need further refinement to make them accessible to a wider range of plant species for their applicability universally (BHAT and SRINIVASAN, 2002).

2.11 ACHIEVEMENTS IN THE GENETIC TRANSFORMATION OF MEDICINAL PLANTS

The number of transformed medicinal plants for the production of secondary metabolites is on the increase as genetic transformation has become an established method for introduction of genes into plants of medicinal value. Some achievements have been the production of pharmaceutics, mainly utilising hairy root cultures for the production of alkaloids, polyphenols, terpenoids, nematodical compounds and novel compounds. Solanaceous species (*Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and others) are favoured as a source of tropane alkaloids and the extensive and intense research performed on these species has culminated in several useful products. Research into other alkaloid, apart from tropane alkaloids, has also resulted in the transformation of various plants such as *Amsonia elliptica* (Apocynaceae) to produce indole alkaloids (SAUERWEIN and SHIMOMURA, 1990) and *Lobelia inflata* (ISHIMARU *et al.*, 1992) for the production of piperidine-type lobeline, an alkaloidal constituent isolated from hairy roots. Polyphenols isolated from transgenic genera including *Geranium*, *Sanguisorba*, *Swertia*, *Pyllanthus*, *Ocimum*, *Fagopyrum* treatment of wide range of medical conditions such as they produce tannins, xanthones, phenylglycosides, flavanols, antioxidant polyphenols amongst others. Cardioactive glycosides (terpenoid compounds) were produced from transformed cultures of *Digitalis purpurea* mediated by *Agrobacterium* (SAITO *et al.*, 1990). Polyacetylenes isolated from hairy root clones of Asteraceae, Umbelliferae, Araliaceae and others

have been utilised as anticancer agents as they exhibit cytotoxic properties and even nematocidal agents have been extracted from transformed *Tagetes* and *Rudbeckia spp*. For a summary of achievements with medicinal plants, the reader is directed to consult the review by BAJAJ and ISHIMARU (1999).

Success has also been achieved with the production of secondary metabolites using shooty teratomas induced by *A. tumefaciens* gene transfer. These differentiated shoots grow in the absence of growth regulators and are capable of producing secondary metabolites (Table 2.4). The use of shooty teratomas is particularly useful in cases where the production of hairy roots is not feasible (BAJAJ and ISHIMARU, 1999). Medicinal plants have also been targeted for the production of edible vaccines as well as the expression of different phenotypical traits, introduction of herbicide, disease and insect resistance genes. These developments and those that will follow in the future have far-reaching implications especially for the pharmaceutical and nutriceutical industries (BAJAJ and ISHIMARU, 1999).

2.12 AIMS AND OBJECTIVES OF STUDY

In view of the importance of thapsigargin as a pharmacological tool and the recent discovery of this compound and its analogues as potential anti-prostate cancer agents necessitated investigation into an alternative system for their production as to date, thapsigargin is still mainly extracted from roots and fruits of wild plants by destructive harvesting. Evidently, this practice cannot meet the increasing demands for thapsigargins from the pharmaceutical industry due to the limited resources and also will lead to considerable reduction of the natural medicinal plant population and possibly environmental damage in terms of soil erosion. *T. garganica* was therefore regarded as an important target for the application of transgenic technology as hairy root cultures can be harnessed *in vitro* for the production of complex bioactives that are difficult to synthesise and which require a differentiated culture system. Production of of thapsigargins is organ specific in nature and previously displayed a requirement for specialised tissue in culture (JÄGER *et al.*, 1993). For this study, plant tissue culture was not only investigated

for use as part of the transformation regime but also as a means of mass propagating *T. garganica* for the conservation of this species in mind. An effective and alternative way towards large-scale production of *T. garganica* plants and compounds synthesised within instead of using wild plants was a major motivating factor for the establishment of *in vitro* cultures and more especially a hairy root system induced by *A. rhizogenes*. Although, JÄGER (1993) attempted genetic transformation of this species, this study was preliminary and a more comprehensive and extensive investigation into the inherent ability of *T. garganica* to undergo genetic transformation was necessary. The study focussed on three main objectives:

(i) Development of an *in vitro* propagation regime, excluding a somatic embryogenesis phase, that would culminate in successful introduction of cultured plants in the wild; and,

(ii) Investigation of gene transfer method(s) for the ultimate recovery of transgenic hairy roots for potential use in a bioreactor system in order to produce thapsigargins at higher or similar levels as those accumulating in intact plants.

CHAPTER 3
MICROPROPAGATION OF *THAPSIA GARGANICA* [1]

3.1 INTRODUCTION

Micropropagation is a valuable method for vegetative plant propagation of the Apiaceae as its application often ensures mass production of plants with clonal properties expressing the same genotypic and phenotypic traits (EKIERT, 2000). This technology exploits the 'totipotent' nature of plant cells as they have whole plant regenerability under controlled environmental conditions in the presence of the correct combination of plant growth regulators and macro and micronutrients. Particularly, this is a useful method for cultivating plants that fail to generate seeds as a result of climatic conditions, as is often the case with members of the Apiaceae when grown out of their natural habitats (EKEIRT, 2000). The problem of plants which originate from particular biotopes being difficult to cultivate outside their local biosystem is not unique to the Apiaceae. Moreover, it is common that plants under large-scale field cultivation are also sensitive to pathogen attack (BOURGAUD *et al.*, 2001) whereas plants derived from tissue culture are disease-free (CASSELLS and CURRY, 2001). It is ongoing practice for plants with medicinal value to be collected from their native ecosystems as opposed to being cultivated for commercial purposes (MÀTHÉ, 1988; TAYLOR and VAN STADEN, 2001). However, domestication of medicinal plants has gained considerable popularity as this ensures the continuous supply of the plants and their products for commercial exploitation (TAYLOR and VAN STADEN, 2001). Continuous micropropagation techniques complement field cultivation as this provides a rapid and bulk supply of plantlets that are ready to plant, thus making plant tissue culture a powerful tool for conservation of germplasm (REDDY *et al.*, 2001).

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In vitro culture of the Apiaceae has focussed on the use of somatic embryogenesis as this process occurs spontaneously in the Apiaceae (HUNAULT *et al.*, 1989; IGNACIMUTHU *et al.*, 1999; EKIERT, 2000), making this a special feature of these plants. Somatic embryogenesis may be induced from a variety of explants including ovaries, hypocotyls, cotyledons, pedicels and parts of the compound leaf, such as petioles and petiolules. As a result, since the first report by STEWARD (1958), *Daucus carota* has for several decades been used extensively as a model to study the mechanisms governing the process of somatic embryogenesis. The type of somatic embryogenesis prevalent in carrot cultures is regarded as originating from 'induced embryogenic cells' where proliferation of callus is a prerequisite due to the presence of auxin in the induction medium (DODDS and ROBERTS, 1985), thereafter callus undergoes developmental processes of embryogenesis on the same medium or that lacking plant growth regulators after transfer. Production of plantlets without somatic embryogenesis and with the intent to transfer plants out of culture has been achieved with a few members of the Apiaceae (EKIERT, 2000).

Organogenesis *in vitro* is a complex phenomenon controlled by synergistic interactions of both physical and chemical factors and requires optimisation for each species (CHAND *et al.*, 1997). Special attention to the balance of plant growth regulators that govern differentiation is often required (BOURGAUD *et al.*, 2001) and the auxin:cytokinin ratio controls the shoot differentiation in cultured tissues where high levels of cytokinin to auxin favour shoot induction (CHAND *et al.*, 1997). Higher levels of auxins or absence of plant growth regulators often result in root production and cytokinin autotrophy has been shown to decrease the potential to differentiate in long term cultures (CHAND *et al.*, 1997). *In vitro* cultures are generally cultivated with 3 % sucrose as a source of carbon and energy in the media as *in vitro* plants rarely exhibit autotrophic growth due to insufficient carbon dioxide for photosynthesis in tightly closed culture vessels (VÍTOVÁ *et al.*, 2002).

Leaf based regeneration protocols are generally favoured for genetic improvement of plants as in the case of *E. foetidum* since nutrients readily penetrate thereby

facilitating antibiotic selection during transformation in order to suppress growth of non-transformed tissues (IGNACIMUTHU *et al.*, 1999). Procedures avoiding regeneration via a callus phase are superior as the undesirable effects of somaclonal variation associated with indirect organogenesis often induce chromosomal aberrations resulting in phenotypic instability (ŠUŠEK *et al.*, 2002). This in turn results in plants that are difficult to acclimatise *ex vitro* and decreases the potential for successful transfer and expression of introduced genes following transformation (CASSELLS and CURRY, 2001). Moreover, the expression of epigenetic and genetic instability in explants utilised for *Agrobacterium* may result in the production of transformants with chimaeras which may lead to transgene silencing (MATZKE and MATZKE, 1998; CASSELLS and CURRY, 2001).

Thapsia plants are difficult to cultivate out of their natural environment. Firstly, germination from seed does not occur with ease and secondly, maintenance under greenhouse conditions is labour intensive because these plants are prone to insect attack, such as aphids. The increasing worldwide demand for thapsigargin is endangering the sparse populations of *T. garganica* in the Mediterranean area and may lead to their extinction. As cultivation of these plants is not a feasible option, *in vitro* propagation often offers a solution in such instances. In 1993, JÄGER and fellow researchers reported for the first time the use of *in vitro* cultures of *T. garganica* for the production of thapsigargins (JÄGER *et al.*, 1993). These authors were successful in isolating two thapsigargins, namely nortrilobolid and trilobolid, which had accumulated in callus-derived somatic embryos of *T. garganica*. However, the ability to convert somatic embryos into plantlets was not investigated, as the purpose of that particular study was to understand the correlation between the state of differentiation and thapsigargin content during transition from callus to somatic embryo. JÄGER *et al.* (1993) also noted a high degree of shape variation indicating abnormal embryo development and this was attributed to the onset of genetic variation as a consequence of the age of the cultures. Pre-empting an intermediate callus stage as a precursor for plantlet regeneration is the preferred strategy for maintenance of genetic stability (GEORGE and SHERRINGTON, 1984, SKIRVIN *et al.*, 1994, CASSELLS and CURRY, 2001, ŠUŠEK *et al.*, 2002). Insomuch that, the risk of producing variable plants deters many laboratories from

practising methods of indirect morphogenesis (GEORGE and SHERRINGTON, 1984).

During the inititiation of this study there were no successful reports on the regeneration of *T. garganica* plantlets *in vitro*. Subsequently, the research conducted for this thesis resulted in the successful development of a tissue culture regime that circumvented a somatic embryo stage (MAKUNGA *et al.*, 2003) in order to avoid genetic disturbances associated with lengthy periods in culture.

The main objective of the experiments described in this Chapter was to apply tissue culture methods for the clonal propagation of *T. garganica* with the intention to introduce and thereafter monitor the development of the *in vitro* derived plantlets out of culture. The focus was on the development of a protocol, even though circumventing somatic embryogenesis as a precursor for plantlet production, would still remain efficient and reliable. *In vitro* propagation of *T. garganica* would not only provide sufficient target tissue for genetic transformation but would also allow for identification of organs most suitable for that particular purpose.

3.2 MATERIALS AND METHODS

3.2.1. Plant material

Fruits of *T. garganica* were a kind donation from Professor N. Villalobos (University of Salamanca, Spain) who collected these in May 1994. They were stored at -20 °C for two months after collection. Before the fruits were germinated (August 1994), they were hand-sorted. Only intact fruits, without visible predation, were used. Fifty fruits were washed in tap water with a few drops of Tween-20® for 15 min. They were then immersed in a fungicidal solution of 1 % Benlate (Benomyl 500 g kg^{-1} active ingredient; Du Pont de Nemours Int., South Africa) for 10 min and rinsed twice in decontaminated water. The fruits were subsequently surface-decontaminated by soaking in 1 % sodium hypochlorite (NaOCl) (w/v) for 15 min, rinsed three times in sterile water and then placed on 1 % water-agar (Agar-agar, Associated Chemical Enterprises c.c., South Africa) in tissue culture tubes in the

dark at 5 °C. Once they had germinated, they were transferred to 6 cm pots containing a sand: compost mixture (1:1; v/v). The seedlings were watered with a modified Hoagland's nutrient solution (HEWITT, 1966) and placed at 26 °C in a Conviron incubation unit with an 8 h light (50 µmol m^{-2} s^{-1})/16 h dark regime. After one month, the plants were transferred to the greenhouse (light intensity 1 020 µmol m^{-2} s^{-1}) and watered twice a week. The germination experiment was repeated three times at monthly intervals and was performed by Dr A.K. JÄGER.

3.2.2. Explant decontamination

Petiole, petiolule and leaflet explants were taken from plants grown under greenhouse conditions in their second growth season (Figure 1A) and used to initiate *in vitro* cultures. The explants were surface-decontaminated by firstly dipping in 70 % ethanol (v/v) for two min, treating with 1 % Benlate (Benomyl 500 g kg^{-1} active ingredient; Du Pont de Nemours Int., South Africa) (w/v) for 10 min, and then immersing in 1 % NaOCl (w/v) for 5, 10, 15, or 20 min, respectively. Thereafter the plant material was rinsed three times in decontaminated distilled water. The explants were cut under aseptic conditions into 10 to 15 mm segments and placed onto decontaminated tissue culture media. Leaflet explants were placed with the abaxial surface in contact with the medium.

3.2.3. Culture methods

(i) Establishment of cultures

A MURASHIGE and SKOOG (MS) medium (1962), supplemented with 3 % sucrose (w/v) and solidified with 8 g l^{-1} agar (Agar-agar powder CP, Associated Chemical Enterprises c.c., South Africa) was used for the establishment of *in vitro* cultures. The pH was adjusted to 5.8 with 1 M KOH prior to the addition of the agar and then autoclaved at 121 °C and 103 kPa (1.1 kg cm^{-2}) for 20 min. A plant growth regulator (PGR) grid combining various concentrations of NAA (0, 0.5, 1, 3 and 5 mg l^{-1}) and BA (0, 0.5, 1, 3 and 5 mg l^{-1}) was tested for spontaneous induction of shoots. The explants were aseptically transferred to initiation media (10 ml) in culture tubes (24 mm x 100 mm). The culture tubes were capped with plastic caps and a 5 cm x 1 cm strip of laboratory film (Parafilm "M"®, American

National Can™, USA) was used to seal the tubes. The cultures were then placed in a growth room with a 16 h light (45 µmol m^{-2} s^{-1})/8 h dark regime under cool-white fluorescent light (L75W/20X Osram, USA; Code number F96T12) at 22 - 25 °C. Resultant callus (approximately 200 mg) produced by explants on MS medium was transferred to growth media supplemented with NAA and BA (0.5:1.5, 1:3, 1:5,or 3:1 mg l^{-1}, respectively). *In vitro* cultures were subcultured every four weeks to multiply the callus and shoots.

(ii) Acclimatisation of shoots

Roots were induced by transferring elongated shoots (5 cm - 10 cm) onto 1 % agar-solidified MS medium supplemented with 30 g l^{-1} sucrose and various concentrations of IBA (0.5, 1, 2 and 5 mg l^{-1}) or PGR-free basal MS medium. Once rooted, the plantlets were carefully extracted from the tissue culture vessels and washed gently in water to remove excess agar-medium and sucrose traces to discourage infection by fungal contaminants. The plantlets were blotted lightly on filter paper and then planted into pots. If plantlets were not rooted, they were dipped in Seradix® B No. 1 powder (IBA, 1g kg^{-1}; Maybaker Agrichem, South Africa) to stimulate *ex vitro* root production prior to transfer into soil. To maximise plantlet survival, three acclimatisation regimes were tested. The first strategy involved planting the plantlets into a sand: soil: vermiculite: seedling bark mix (1:1:1:1 [v/v/v/v]). The potted regenerated shoots were kept covered under polyethylene bags for two weeks in a Conviron incubator at 22 °C. The conditions of high humidity, created by the polyethylene bag covers, were maintained to prevent excessive water loss during the "hardening-off" period. The bags were removed and the plantlets were transferred to a mist house for a further two weeks. After "hardening-off", the *T. garganica* plants were placed under greenhouse conditions (light intensity 1 350 µmol m^{-2} s^{-1}) and watered twice a week. Their success at growing *ex vitro* was monitored.

The second acclimatisation protocol included pre-treating against fungal infection by soaking plantlets in a solution of Dithane® M-45 solution (0.22 % [w/v]) for 30 min. This fungicidal agent contains dithiocarbamate (800 g kg^{-1}) as the active ingredient and reduces the "damping-off" of *T. garganica* under *ex vitro* conditions.

The plantlets were potted in a mixture of soil, seedling bark and vermiculite (1: 0.7: 0.1). The soil mixture was moistened with the Dithane® M-45 solution and sulphur powder was lightly sprinkled on the soil and over the plantlets. The micropropagated plantlets were "hardened-off" in a misthouse with a bottom heat of 30 °C and supplied with intermittent mist irrigation, which was automatically regulated by an 'electronic leaflet' solenoid valve system. After 72 h the plants were removed from misthouse conditions and relocated to a 80 % shadehouse (light intensity 698.83 µmol m^{-2} s^{-1}). The plants were watered automatically by a sprinkler system at 11 am and 2 pm for 1 min and at 5:55 pm for 2 min daily for six weeks. The watering regime was then changed to once daily. The plants were acclimatised in Autumn (Southern Hemisphere) and the plants were subjected to temperatures that ranged from 20 °C to 26 °C. These plants were able to adapt with ease to the Southern Hemisphere climate.

The third protocol involved pre-treating the plantlets in a 0.1 % Benlate (w/v) soaking solution for 30 min prior to planting out. The soil mixture (sand: compost: vermiculite: bark [7:3:0.1:0.1; v/v/v/v]) was autoclaved for 20 min at 121 °C and 103 kPa, transferred to pots when cool and then drenched with a solution of Dithane® M-45 solution containing one Fongarid tablet (62.5 mg Furalaxyl (phenylamide), Syngenta [Pty] Ltd, South Africa). After planting out, the micropropagated plantlets were sprayed with an anti-fungal cocktail containing 1 g l^{-1} Benlate, 2.5 ml l^{-1} Previcur® N (Propamocarb-HCl 722 g l^{-1} active ingredient, FBC Holdings Pty, Germany), 2 ml l^{-1} Rovral Flo (Iprodione [dicarboximide] 250 g l^{-1} active ingredient, Rhonê-Poulenc Agrochimie, France) and 2 ml l^{-1} Kelpak (0.0031 mg l^{-1} cytokinin active ingredient, Kelp Products [Pty] Ltd, South Africa), a seaweed concentrate prepared from *Ecklonia maxima* (KOWALSKI *et al.*, 1999). They were then kept in a misthouse with a bottom heat of 30 °C, as previously described, for five days to accustom the plantlets to conditions of reduced humidity. The plants were then placed in a greenhouse (light intensity 2 380 µmol m^{-2} s^{-1}) and watered by a sprinkler system as previously described. They were also sprayed every four days with a post-acclimatisation cocktail containing 1 g l^{-1} Benlate, 0.22 g l^{-1} Dithane® M-45 and 2 ml l^{-1} Kelpak for six weeks *ex vitro*. The number of plantlets surviving out of culture was recorded.

3.2.4. Data analysis

Twenty-five replicates were used for all experiments. Experiments were repeated three times and data were subjected to a one-way analysis of variance (ANOVA). When the ANOVA indicated statistical significance, a Tukey's multiple comparison test (Minitab Release 12.1 computer package) was used to distinguish differences between treatments.

3.3. RESULTS AND DISCUSSION

T. garganica seeds were difficult to germinate. From the first fruit lot, 98 % of the seeds germinated while only 39 % and 15 % germinated from the second and third germination trials, respectively. This decrease in germination could be due to loss of viability or the induction of secondary dormancy. The fruits of the Apiaceae often fail to ripen fully and are prone to attack by fungal and bacterial pathogens (EKIERT, 2000) and this in turn prevents successful seed germination. In a number of species, the use of NaOCl is common practice to inhibit or reduce invasion of seeds by pathogens and it improves germination (DREW and BROCKLEHURST, 1984; DREW and BROCKLEHURST, 1987). In this study, germination of *T. garganica* seeds was ameliorated by scarification with NaOCl as this was used as the decontamination agent of these seeds. On the other hand, RASMUSSEN and AVATO (1998) imbibed seeds and treated them with gibberellic acid for 18 h in order to initiate germination of *T. garganica* and in this instance, seedling emergence began within four weeks of sowing. In this investigation, a period of approximately two months was required for germination of seeds. Once germinated, the plants established well in soil and the morphology of the primordial leaflet and the first leaflets resembled that of the type as described for *T. garganica* L. by SMITT *et al.* (1995). One plant flowered after the first year and most of the plants after the second year (Figure 3.1A).

Table 3.1: Effect of different combinations of NAA and BA on decontaminated explants of *Thapsia garganica*

PLANT GROWTH REGULATOR (mg l^{-1}) NAA	BA	Type of explant showing response	Explant response	Responding explants (%)
0	0	-	-	0 a
0	0.5	-	-	0 a
0	1	Petiolules	Callus initiation	4 b
0	1.5	Petioles	Explant elongation	10 b
0	3	Petioles	Explant elongation	28 b
0	5	Petioles	Explant elongation	12 b
0.5	0	-	-	0 a
0.5	0.5	-	-	0 a
0.5	1	Petioles	Explant elongation	5.5 a
0.5	1.5	Petioles	Adventitious buds	64 e
		Leaflet midrib	Adventitious buds	60 e
0.5	3	Petioles	Explant elongation	5 b
0.5	5	-	-	0 b
1	0	-	-	0 a
1	0.5	-	-	0 a
1	1	-	-	0 a
1	1.5	Petioles	Callus initiation	40 cd
1	3	Petiolules	Callus initiation	64 e
1	5	Petioles	Callus initiation	40 cd
		Petioles	Adventitious buds	52.6 d
3	0	Petioles	Explant elongation	8 b
3	0.5	Leaflet midrib	Callus initiation	8 b
3	1	Petioles	Callus initiation	30 c
		Petiolules	Callus initiation	30 c
3	1.5	-	-	0 a
3	3	-	-	0 a
3	5	Petioles	Explant elongation	4 b
5	0	-	-	0 a
5	0.5	Leaflets	Callus initiation	58 ed
5	1	Leaflets	Callus initiation	30 c
5	1.5	Petioles	Explant elongation	4 b
5	3	Petioles	Explant elongation	10 b
5	5	-	-	0 a

Treatments denoted by the same letter were not significantly different, $P < 0.05$. At least 25 replicates were used per treatment.

3.3.1 Indirect and direct organogenesis

The explants of *T. garganica* were successfully decontaminated by treating with 1 % NaOCl for 15 min. On average, 66 % of the explants did not manifest symptoms

of bacterial and fungal contamination. The 5 and 10 min decontamination treatments were not effective in eliminating microorganisms from either petiole or leaflet explants. Leaflet explants immersed in the sterilant for 20 min did not show symptoms of contamination. This treatment was not only lethal to microorganisms but also caused extensive explant bleaching which proved lethal. Removal of all the microorganisms with minimum damage to the plant system to be cultured is the major objective of surface-decontamination (DODDS and ROBERTS, 1985). Fifteen min were optimal for decontamination and used for all experiments that followed. Parts of the compound leaf of *T. garganica* were chosen as a suitable source of explants since harvesting is non-destructive and plant material was readily available when compared to seeds. In general, shoot apices, roots and leaf petioles are popular as explant material for the initiation of *in vitro* cultures of plants belonging to the family Apiaceae. These explants often produce embryogenic callus and the embryoids may easily be converted to whole plants (GEORGE and SHERRINGTON, 1984; CHAND *et al.*, 1997). GEORGE and SHERRINGTON (1984) reported that zygotic embryos may be used to initiate embryogenic callus and the induction of embyrogenic cells in suspension cultures that were not morphogenic occurred in several species of the Apiaceae, namely, *Apium graveolens*, *Carum carvi*, *Daucus carota*, *Sium suave* and *Pimpinella anisum*.

Both petiole and leaflet explants had a tendency to brown after a week in culture. These brown explants were not discarded as they were still viable and were capable of callus and shoot initiation (Figure 3.1C). The appearance of white callus from the midrib of leaflet explants on MS supplemented with 1 mg l^{-1} NAA and 3 mg l^{-1} BA (Table 3.1) after two weeks was the first response in culture. After four months, this callus began to produce organised tissue (Figure 3.1B), and shoot initiation quickly became abundant. The shoots were separated from the callus and then prolific shoot formation from the shoot bases was achieved on the same medium (1 mg l^{-1} NAA and 3 mg l^{-1} BA). In comparison, explants on other treatments underwent elongation in the first two weeks in culture and only in some of these did organogenesis occur after a month.

Regeneration of plants from callus is often not desirable as these plantlets may be genetically variable. Numerous reports have described a wide range of species that lack genotypic and phenotypic uniformity amongst plants that are callus-derived. The preservation of genetic stability associated with direct organogenesis is desirable in culture (GEORGE and SHERRINGTON, 1984). Spontaneous induction of adventitious buds from petiole explants was optimal for a 0.5:1.5 (NAA: BA) medium (Table 3.1, Figure 3.1C) with petiole and leaflet explants producing adventitious buds. Although supplementing the induction medium with NAA and BA at a concentration of 1 mg l^{-1} and 5 mg l^{-} promoted direct shoot initiation from petiole explants, subculture onto the same medium high in cytokinin resulted in plantlets prone to hyperhydricity. The hyperhydric shoots had a tendency to brown and their growth was short-lived. Browning of both shoot and callus cultures was observed during maintenance. This effect was not detrimental to plantlet survival but accelerated the death of callus tissue. CHAND *et al.* (1997) eliminated browning of cultures of *Pimpinella anisum* L. (Apiaceae) by shortening the frequency of subculture to 2 weeks. The 5:0.5 (NAA: BA) medium was most effective for the proliferation of callus (Figure 3.1D) from leaflet explants. This medium could not support shoot production due to the elevated auxin levels as compared to cytokinin levels. A combination of NAA and BA (3:1) promoted callus formation from petiole and petiolule explants after 3 weeks (Table 3.1). Subculture of callus onto the same medium promoted shoot organogenesis (Table 3.2). Inclusion of a single phytohormone in the induction medium could not support further *in vitro* development of explants.

Data on the regeneration of plantlets from callus are shown in Table 3.2. The medium optimal for direct organogenesis (0.5 mg l^{-1} NAA: 1.5 mg l^{-1} BA) (Table 3.1) also gave the highest rate of multiplication (Table 3.2) and moreover the shoots were less prone to hyperhydricity. Similarly, extensive shoot proliferation (80%) was also noted when the basal medium contained NAA and BA (1:3). The shoots produced on this medium had the highest mean length (11.5 mm) (Table 3.2). However, the onset of hyperhydricity was often observed on some of these cultures. A concentration of 1 mg l^{-1} NAA and 5 mg l^{-1} had a similar effect on the number of shoots produced per culture tube as the 0.5:1.5 NAA: BA and 1:3 NAA:

BA treatments. Although, the number of shoots recorded ranged from 10–25 plantlets per culture tube for these treatments, increasing the BA concentration did not result in an increase in the length of the shoots produced (Table 3.2). It was difficult to reduce or arrest callus formation at the bases of the plantlets growing on the higher levels of BA (Figure 3.1F). This callus often accumulated anthocyanins especially towards the end of the growth cycle prior to the time of subculture (Figure 3.1F).

Table 3.2: Effect of NAA and BA combinations on shoot production of *Thapsia garganica* from callus

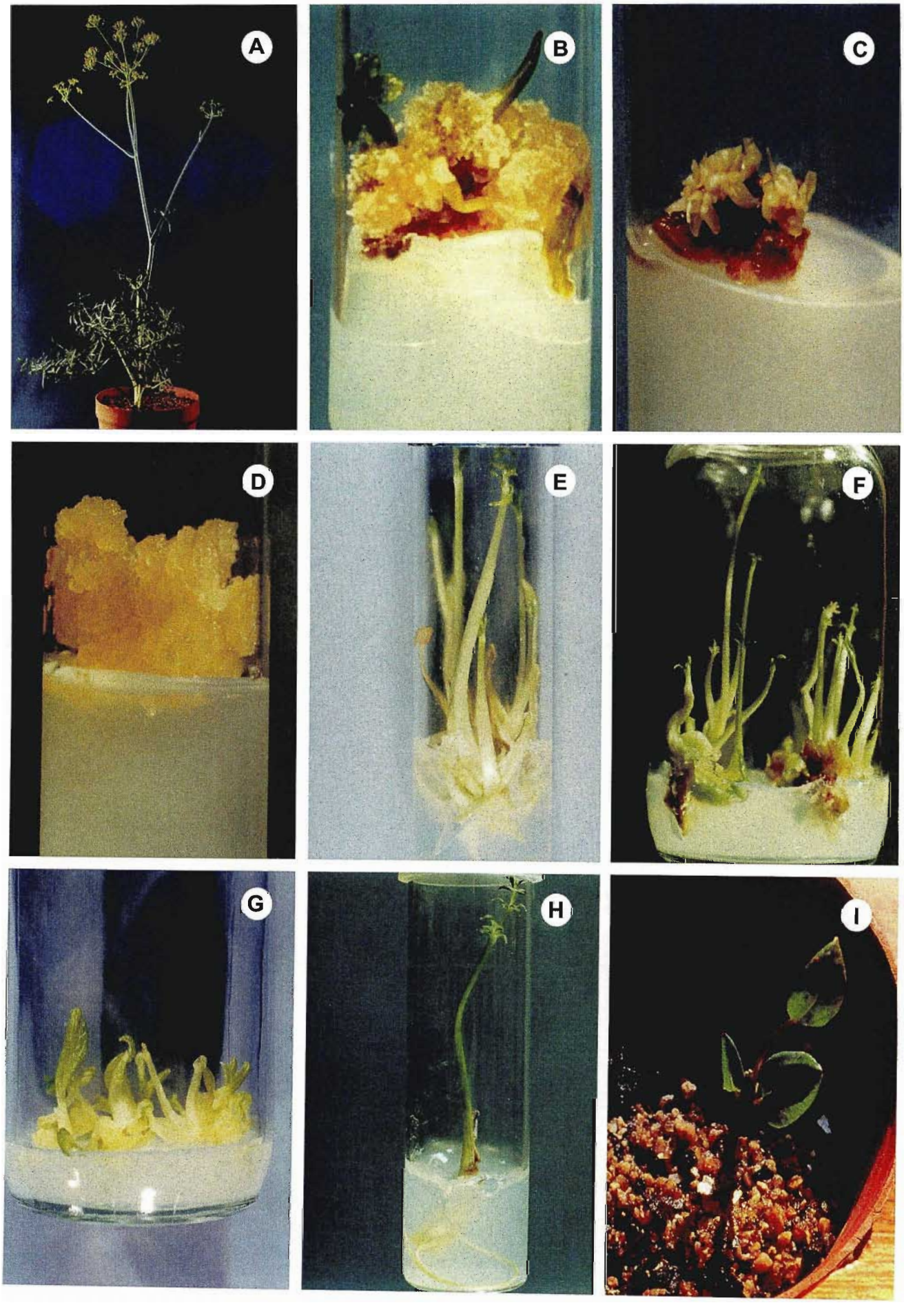
PLANT GROWTH REGULATORS (mg l^{-1}) NAA	BA	Frequency (%)	Shoot length (cm)
0.5	1.5	85 b	10.8 a
1	3	80 b	11.5 a
1	5	73 b	8.7 b
3	1	33 a	8.3 b

Data were collected prior to transfer of shoots to rooting medium or prior to *ex vitro* rooting. Treatments denoted by the same letter were not significantly different, $P < 0.05$. At least 25 replicates were used per treatment.

Table 3.3: Effect of IBA on the rooting of *T. garganica in vitro* shoots

PLANT GROWTH REGULATOR (mg l^{-1}) IBA	Plantlets rooted *in vitro* (%)	Number of roots per plantlet
0	32 c	3 b
0.5	0 a	0 a
1	35 c	3 b
2	25 bc	10 c
5	16b	8 c

Data were collected prior to transfer of plantlets to ex vitro conditions. Treatments denoted by the same letter were not significantly different, $P < 0.05$. At least 25 replicates were used per treatment.


A
B
C
D
E
F
G
H
I

3.3.2 Root initiation and acclimatisation

Rooting and acclimatisation were the most labour intensive stages of this protocol as the plantlets of *T. garganica* did not always respond favourably. In some plant species, rooting *in vitro* is largely genotype-dependent with shoots rooting naturally during propagation, thereby, obviating the inclusion of a separate rooting stage. In other species, a PGR-free medium encourages root formation. However, root formation is often inhibited by cytokinins which are important for shoot multiplication (DEBERGH and MAENE, 1981; GEORGE and SHERRINGTON, 1984). Initial attempts at rooting *T. garganica in vitro* shoots on PGR-free medium were promising as the appearance of roots was observed after a week. On average, three roots were initiated per plantlet from 32 % of the cultures. The low incidence of root formation warrants further investigation. This rooting procedure is far from ideal as a larger percentage of shoots should form roots for the protocol to be financially viable. Comparison of the frequency of rooting in *T. garganica* plantlets (Table 3.3) when treated with IBA (0.5-3 mg l^{-1}) revealed that a root initiation medium with 1 mg l^{-1} IBA had a similar effect on the number of adventitious roots as PGR-free medium (Figure 3.1H). However, these roots appeared after three weeks. Application of IBA levels greater than 1 mg l^{-1} caused extensive root proliferation but the shoots died after a few days. The ability to initiate prolific rhizogenesis may be useful for the establishment of *T. garganica* root cultures as such cultures may prove useful for the production of thapsigargin compounds. With an intention of utilising the *in vitro* culture system for conservation purposes, it is best to maintain in culture the naturally occurring phenotype for successful transplanting. In this case, a single tap root system typically displayed by *T. garganica* plants growing in the wild was most sought after.

Numerous futile attempts were made to acclimatise rooted *T. garganica* plantlets which were transplanted to a sand: soil: vermiculite: seedling bark mixture (1:1:1:1 [v/v/v/v]). A fully functional root system is harder to induce *in vitro* than *ex vitro* (RAYNS, 1993). Frequently, these roots lack root hairs and are prone to damage during transplanting (GEORGE, 1993). Some plantlets were rooted *ex vitro* in the same soil mixture that was used for plantlet acclimatisation (sand: soil: vermiculite:

seedling bark mixture [1:1:1:1; v/v/v/v]). The extent of rooting induced by the application of Seradix®, a rooting powder, was not determined. This was due to the micropropagated plantlets being highly susceptible to a fungal rot once removed from culture. This type of infection accelerated plantlet mortality. This phenomenon of "damping off" has been attributed to thin epicuticular wax deposits and insufficiently developed roots (RAYNS, 1993). Application of Dithane solution and sulphur powder as anti-fungal pre-acclimatisation agents to *T. garganica in vitro*-derived plantlets during transplanting and hardening-off in the misthouse, significantly reduced plantlet mortality in the greenhouse. The acclimatisation frequency was still very low, with only 13 % plantlet survival being recorded when planted in a mixture of soil, seedling bark and vermiculite (1:0.7:0.1; v/v/v) (Figure 3.1I). In comparison, the third acclimatisation regime which had a post–treatment targeting a variety of fungi: *Pythium*, *Phytophthora*, *Rhizoctonia*, *Fusarium*, *Thielaviopsis* and *Verticillium*, commonly associated with causing "damping-off", significantly improved the number of plantlets surviving out of culture to 52%. The inclusion of fungicides can suppress root formation and need to be used sparingly. The seaweed concentrate Kelpak could have contributed considerably to the improved acclimatisation frequency as it has been previously shown to boost plantlet vigour and root development after tissue culture (KOWALSKI *et al.*, 1999).

3.4 CONCLUSION

The low survival percentage of plantlets was the main limitation of this protocol and warranted more comprehensive investigation. The transfer of rooted plantlets or microcuttings to an optimal acclimatisation medium is, however, desirable as it maximises survival during the transitional period from culture vessel to an *ex vitro* environment and ensures minimal losses common to these stages of *in vitro* propagation. The protocol described in this chapter does have its advantages (1) the use of leaf material to initiate cultures proved beneficial in terms of the renewable nature of the plant organ and in the relative ease of decontamination; (2) a leaf based regeneration protocol is particularly useful for use in transgenic technology as nutrients penetrates easily thereby facilitating selection procedures; and, (3) a medium with high rate of plantlet multiplication, increasing the efficiency

of this particular stage, has been identified. Optimisation of the components of the microenvironment would facilitate production of better quality plantlets in order to assist further plantlet acclimatisation. However, the regime presented is the first successful attempt of the micropropagation of *T. garganica* plants without the intervening step of somatic embryogenesis.

CHAPTER 4
INVESTIGATING IMPROVED PLANTLET REGENERATION IN CULTURE

4.1 INTRODUCTION

During *in vitro* propagation, the composition of the culture medium has the greatest influence on the explant response and as a consequence has received the most attention from clonal propagators (COHEN, 1995). Investigations on optimisation of media components are abundant in the literature, thus indicating copious attempts to overcome problems encountered in culture by adjusting media formulation (COHEN, 1995). A large majority of plant species are able to tolerate a wide range of micronutrients in tissue culture and for changes in micronutrient composition to elicit a response on explants, more than one subculture is generally required. Conversely, macronutrients manifest their effect often at the onset of the tissue culture process. Optimisation of PGR types and concentrations is essential for each stage of microprapagation as phytohormones are used to achieve specific responses for the establishment of different types of culture. The medium developed by MURASHIGE and SKOOG in 1962 has been extensively utilised for micropropagation of a broad spectrum of plants species and is regarded as a good starting point for the *in vitro* propagation of herbaceous plants (COHEN, 1995).

Morphogenesis from *T. garganica in vitro*, directly from leaf material, is feasible also on MS (MURASHIGE and SKOOG, 1962) medium when supplemented with 0.5 mg l^{-1}NAA and 1.5 mg l^{-1} BA but not always reliable (MAKUNGA *et al.*, 2003). However, conversion of plantlets from callus occurs with greater frequency and reproducibility compared to direct vegetative budding under these conditions. Adventitious shoots, especially derived from callus, may express epigenetic and genetic instability often associated with somaclonal variation - minimising the organogenetic potential of the plant; and, is often accompanied by hyperhydricity (CASSELLS and CURRY, 2001).

Moreover, suboptimal *in vitro* growth components and environmental factors may exacerbate the occurrence of somaclonal variants and consequently, hamper further microplant development in culture, such as rooting (CASSELS and CURRY, 2001) and even the expression of transgenes (MATZE and MATZE, 1998). The special needs of tissue cultures – high humidity, superfluous nutritional components, high levels of PGRs and low light intensity are the major inducers of shoot abnormalities. The relative water potential and high humidity are key factors in the onset of malformations (ZIV, 1991). Oxidative stresses have been implicated as the causal factor of some problems observed in cultured plants including hyperhydricity, habituation, recalcitrance, poor physiological function, genetic and epigenetic variation. Studies of molecular mechanisms underlying the epigenetic modification induced by tissue cultures have the potential to improve the current understanding and provide better tools for monitoring and controlling somaclonal variation. Thus far, the inherent stresses associated with *in vitro* cultivation are also capable of eliciting retrotransposition in plants and this action is mediated through promoter sequences that share a close resemblance to plant-defence related genes as revealed using molecular techniques (MHIRI *et al.*, 1997; TREGEAR *et al.*, 2002). Abiotic stresses *in vitro* induce aberrant genomic expression and result in altered methylation of DNA; changes in chromosome number and integrity as well as DNA base deletions and substitution (CASSELLS and CURRY, 2001).

According to ZIV (1991), abnormal morphogenesis and physiological events that occur in culture closely resemble events associated with plants under stress; as a result there is often a need for the optimisation of plant tissue culture conditions. Morphologically and physiologically challenged plants are usually unable to cope with environmental stress after transplanting *ex vitro* and are more prone to damping-off diseases. A gradual transition period enabling for development of normal morphology ensures better survival *ex vitro*. However in some herbaceous plants, the continued persistence of some features acquired *in vitro* is of great concern out-of-culture. ZIV (1991) recommended for tissue culture practitioners to encourage normal development *in vitro* by paying special attention to *in vitro* components of growth and conditions to create a microenvironment optimal for development of true-to-type plants. Propagules derived under such conditions are better equipped for survival in the greenhouse and this is controlled by the state of the medium and the container atmosphere.

Rooting can be one of the more demanding stages of the tissue culture process, often requiring optimisation in such situations. Frequently, however, *in vitro* rooting occurs in some species spontaneously during the step used to encourage multiple shoot formation and these plants can be transferred directly to the external environment for acclimatisation. In most cases, exposure to cytokinins during the multiplication stage potentially inhibits root production and a separate root-induction phase has to be implemented (GEORGE and SHERRINGTON, 1984). The dipping of plants in highly concentrated auxin solutions, as a means to encourage rooting for short periods of time, is also well-established. Although, most published micropropagation regimes report on an *in vitro* rooting stage, several reasons exist for preferring *in vivo* rooting over *in vitro* (DEBERGH and READ, 1991) – (1) shoot clusters need to be separated into single shoots making this *in vitro* rooting more labour intensive; (2) production of roots *in vitro* often leads to a poorly-formed and non-functioning root system; (3) *in vitro* roots are susceptible to physical damage during handling while transplanting enhancing the chances for onset of root and stem diseases; and, (4) for difficult to root plants application of good rooting practices *in vivo* is far easier and less expensive as compared to *in vitro*.

Plants expressing stress-induced aberrations may lose competence for development *in vitro* and for instance become recalcitrant to rooting procedures, exhibit flower induction *in vitro* (early flowering) and lowered performance after transplanting, as previously mentioned, further complicating the tissue culture process. Damage induced by *in vitro* stress is genotype-dependent. Remediation strategies include genotype screening for sensitivity to stress; choice of a suitable explant for clonal propagation and transformation as for instance protoplasts and mature tissues are more prone to express greater variability in culture; application of methods that encourage stability during multiplication of shoots; optimisation of the auxin-cytokinin balance regulating adventitious regeneration; and, reducing non-essential steps in culture as the culture age is one of the factors controlling genetic instability (CASSELLS and CURRY, 2001; JOYCE *et al.*, 2003).

In light of these facts, the main objective of this part of the study was to refine the methodology described in the previous chapter in order to optimise the tissue culture regime for *T. garganica* so as to circumvent indirect organogenesis and to address the limiting stages identified previously. To reiterate, these include (i) the elimination of the callus phase for production of propagules; (ii) the use of an optimal medium for multiplication thus ensuring regeneration of better quality plants of *T. garganica* in culture; and, (iii) to improve the rooting protocol in order to facilitate acclimatisation of the *in vitro* derived plants. Such procedures would be superior to the existing one described by MAKUNGA *et al.* (2003), since regeneration through callus can cause undesirable genetic aberrations resulting in phenotypic instability. Moreover, regeneration of plants from explants in a shorter time without a long intermediate callus stage; and with minimal use of PGRs is part of an important step towards the establishment of a genetic transformation system in *T. garganica*. Therefore, this chapter reports on the efforts undertaken so as to develop a reliable and predictable regeneration system that would offer a high rate of microplant multiplication and acclimatisation for *ex vitro* planting and gene transfer. With intention to adhere to these goals, specific focus was placed on aspects of multiplication and rooting. By comparing the use of different PGRs in culture versus to those reported previously (MAKUNGA *et al.*, 2003; Chapter 3) and use of a variety of rooting procedures prior to transplanting, the response of *T. garganica* plantlets to this new set of conditions was monitored.

4.2 MATERIALS AND METHODS

Stock plants were maintained under greenhouse conditions at the University of Natal Botanical garden, University of Natal Pietermaritzburg. The plants growing in a sand: soil (1:1; v/v) mixture were watered twice a week. These plants lost their leaves during the Southern Hemisphere summer and died back from October until appearance of new leaves at the beginning of March. Initiation of cultures was performed only when leaf material was readily available.

4.2.1 Plant material and explant preparation

Parts of the compound leaf of *T. garganica* were collected from plants growing in the greenhouse. The freshly harvested plant material was washed to remove loose dirt and placed in 70% ethanol (v/v) for 5 min, followed by 10 min in 1% Benlate® (w/v). The material was surface decontaminated by soaking in 1% NaOCl for 15 min. To thoroughly remove the surface-decontamination agent, the plant material was washed three times with sterile water in a laminar flow hood. The plant material was used immediately or else stored at 4 °C for not longer than 24 hours.

4.2.2 Initiation medium optimisation

Explants (10 mm - 15 mm) were aseptically transferred to culture tubes (24 mm x 100 mm) containing 10 ml modified MS nutrient medium (MURASHIGE and SKOOG, 1962) supplemented with 30 g l^{-1} sucrose and solidified with 8 g l^{-1} agar (Agar-agar powder CP, Associated Chemical Enterprises c.c., South Africa). The pH of all media was adjusted to 5.8 with 1 M KOH before autoclaving at 121 °C and 103 kPa for 20 min. Where instructed by the manufacturer, PGRs were filter-sterilised. Once the media had cooled to approximately 50 °C, filter-sterilised[1] PGRs were added prior to dispensing 30 ml of the agar medium per culture bottle.

In order to determine a suitable optimal medium and an effective PGR combination for inducing cellular differentiation *in vitro*, PGRs were added to the initiation medium in a factorial grid. As a new set of PGR combinations was being determined in comparison to the results obtained for MS supplemented with NAA and BA (Chapter 3), the response of different explants was once again monitored. The PGR combinations of auxins (0, 0.5, 1, 3 and 5 mg l^{-1}) and cytokinin (0, 0.5, 1, 3 and 5 mg l^{-1}) are listed below:

i) NAA and BA;
ii) IAA[1] and BA;
iii) NAA and Kinetin[1];

[1] IAA (100 mg l^{-1}) and Kinetin (100 mg l^{-1}) stock solutions were filter-sterilised by passing through a 22 µM sterile Millipore® filter and then stored at 4 °C until use.

iv) IAA and Kinetin;
v) 2,4-D and BA; and;
vi) 2,4-D and Kinetin.

Decontaminated petiole and petiolule explants were placed with adaxial surface directly on the growth medium exposing the midrib. Leaflet explants were also placed with the adaxial surface in contact with the medium. The test tubes (24 mm x 100 mm) were sealed with a laboratory film strip (5 cm x 1 cm) (Parafilm "M"®, American National Can™, USA) and thereafter all the explants were incubated in a growth room under similar conditions as previously described in Section 3.2.3. The growth room was fitted with cool white-fluorescent lights automated to provide a photoperiod of 16 h of light (45 µmol m^{-2} s^{-1}) and 8 h of darkness. The growth room temperature was maintained at 22 – 24 °C and these incubation conditions were kept throughout the whole course of the study, unless otherwise stated. Under these growth conditions, successful shoot and/or callus initiation was obtained for combinations of PGRs used.

These experiments were repeated three times to determine reproducibility of the response in culture. Percentage of explants initiating shoots and the number of shoots was noted each week until the end of the primary culture passage (four weeks after explant culture). Thereafter, explants showing a response were transferred to the same fresh medium, which had elicited a response and these were the experiments that were repeated. Therefore, results for the remaining hormone combinations are not presented. Successful culture initiation was obtained for the different combinations of PGRs listed below:

i) 1 mg l^{-1} IAA and 1 mg l^{-1} BA;
ii) 1 mg l^{-1} IAA and 2 mg l^{-1} BA;
iii) 1 mg l^{-1} IAA and 3 mg l^{-1} kinetin;
iv) 2 mg l^{-1} 2,4-D and 1 BA mg l^{-1}; and,
v) 2 mg l^{-1} kinetin

As positive controls, tissue cultures were initiated on medium containing 0.5 mg l^{-1} NAA and 1.5 mg l^{-1} BA or 1 mg l^{-1} NAA and 3 mg l^{-1} BA, as these combinations were previously shown to induce a response in culture (MAKUNGA *et al.*, 2003; Chapter 3).

4.2.3 Shoot multiplication

Sterile shoot bases (2 - 3 cm) were excised from the *in vitro* plantlets and used to reinitiate cultures in a continuous system with six shoot bases placed on growth medium per culture bottle being used. Differences in the number of shoots initiated from each explant base were determined for each of type of medium (Refer to list; Section 4.2.2) after a month. All cultures were maintained by subculturing at four week intervals. Plantlets were allowed to grow and reach at least 5 mm in length. Thereafter, they were removed and transferred to rooting media.

4.2.4 *In vitro* rooting of plantlets

Several rooting experiments (listed below) were performed throughout the course of the study in order to establish an efficient and reliable rooting protocol with the following properties: 1) induction of strong roots within the shortest time frame in order to decrease time in culture so as to minimise possible somaclonal variation; 2) minimum callusing of shoot bases; and, 3) formation of roots with a similar phenotype as those that form on plants growing in a natural environment. The rooting procedures followed are described below:

i) Three different auxins (IAA, NAA, and IBA, all at 1 mg l^{-1}) were compared for root induction. These auxins were included individually in the growth medium (MS medium containing 30 g l^{-1}, 0.1 g l^{-1} myo-inositol, 8 g l^{-1} agar [adjusted to pH 5.8 as previously described Section 4.2.1]);

ii) The effect of charcoal on root induction was first tested by growing the plantlets on media supplemented with 5 mg l^{-1} IBA for four weeks and then transferring to MS agar medium (0.8 % [w/v]) supplemented with 0.5, 1, 2 or 5 % charcoal (w/v) until the appearance of root. The omission of the step exposing the plantlets to IBA would thus shorten the period required for establishment of roots *in vitro*. Therefore, the other treatment involved transferring the plantlets directly from shoot multiplication medium to rooting medium containing charcoal (0.5, 1, or 5 % [w/v]). The rooting medium was 30 ml liquid MS medium (pH 5.8)

containing 8.5 g sterile vermiculite as a supporting agent. These experiments were repeated and the supporting agent was changed to vermiculite (4.25 g) and sand (40 g) per 30 ml medium;

iii) Charcoal (1%, w/v) and NAA, IAA or IBA (all at 1 mg l^{-1}) was added to liquid MS medium. Vermiculite (8.5 g) was added to 30 ml liquid medium per culture bottle; and,

iv) Finally the plantlets were rooted by subjecting to a) half strength liquid MS medium (10 ml) b) full strength MS, both free of PGRs or c) half strength liquid MS with elevated levels of IBA (5, 12.5 or 25 mg l^{-1}) for varying time periods (3 days, 6 days and 9 days). Filter paper bridges were used to support and stand the plants in the liquid medium. The filter paper bridges were prepared from Whatman® number one paper strip (14 cm x 2 cm), wrapped in aluminium foil and thereafter sterilized by autoclaving at 121 °C and 103 kPa for 20 min. One filter paper bridge was then subsequently transferred to a culture tube (24 mm x 100 mm) containing 10 ml of rooting medium, left to soak for 30 min prior to transfer of a single plantlet per tube. All dead tissue and callus (if present) was removed from the bases of the shoots before placing on top of the filter paper supports. These tubes were then incubated in the dark for 3 days at 25 °C. Thereafter, all the plantlets were transferred to solidified half strength MS and then incubated at 25 °C in a growth room with a 16 h light / 8 h dark regime (as per previous description, Section 4.2.1) until the appearance of roots.

The experiments were terminated after six weeks of initiation and the number of roots per plantlet; the number of shoots and the induction of callus and onset of hyperhydricity were recorded. Plantlets, including those failing to root under the above mentioned conditions, were then acclimatised in preparation for their continued growth *ex vitro* (Section 3.2 3. ii).

4.2.5 Statistical evaluation of data

Twenty-five replicates were used per treatment and experiments were repeated at least twice. One-way ANOVA followed by a Tukey's multiple range test, using the Minitab Release 12.1 computer package, was conducted to evaluate statistical differences among treatments.

4.3 RESULTS AND DISCUSSION

4.3.1 Towards improved culture induction

To date most studies performed *in vitro* on the Apiaceae concern the utilization of the somatic embryo induction and development, for example *Ammi spp, Angelica sp*, *Foeniculum spp*, *Carum carvi* L., *Bupleurum falcatum* L., *Levisticum officinale* Koch, *Apium graveolens* L. (reviewed by EKIERT, 2000) with carrot plants being used as a model species to study the regenerative processes of somatic embryos. A limited number of studies have focused on plant regeneration by adventitious bud formation in the species of the Apiaceae (ANZIDEI *et al.*, 2000). Other studies on the Apiaceae have shown that the organogenic response is not only genotype-dependent but also dependent on the PGR(s) used for culture establishment, such as in fennel (ANZIDEI *et al.*, 2000).

Using factorial design, the only plant growth combinations to induce a significant response in culture on the explants of *T. garganica* are presented in Table 4.1. The other PGRs had little or no effect on regeneration in culture and their use was thus discontinued for inspection in further experiments. Although, 66 % percent of explants on medium supplemented with 1 mg l^{-1} IAA and 1 mg l^{-1} BA were the first to show a morphogenic response in culture compared to all the other treatments, this level of BA was not sufficient enough to induce organogenesis.

Table 4.1: Morphogenic response of leaf explants of *T. garganica* on MS media with different auxin/cytokinin combinations as supplements [a]

PLANT GROWTH REGULATORS (mg l^{-1})		Explant regenerating shoots via adventitious buds(%)	Explant regenerating callus (%)	Explants regenerating shoots from callus	Contaminated explants (%)
AUXIN	CYTOKININ				
1 IAA	1 BA	0	66 (7 d)[b]; 83	20	0
1 IAA	2 BA	0	30 (14 d); 35	25	16
1 IAA	3 Kinetin	0	9.1	0	12
0 IAA	2 Kinetin	25 (21 d); 36.4	0	0	12
2 2,4-D	1 BA	0	44	0	0
0.5 NAA	1.5 BA	42	30 (7 d); 55	28 (14 d)	0
1 NAA	3 BA	0	36.4 (14 d); 10	68.1	9.12

[a] Data collected from media that did not induce a response in culture have been omitted since the percentage was 0 in all cases

[b] Brackets indicate the day when the response was first noted if the response occurred before the data collection date

Table 4.2: Shoot regeneration and callus formation in explants from *T. garganica* for different types of shoot multiplication media supplemented with different combinations of auxin and cytokinin

PLANT GROWTH REGULATORS (mg l^{-1})		Mean number of shoots per culture tube	Shoot length (cm)	Callus formation (%)	Occurrence of hyperhydration (%)
AUXIN	CYTOKININ				
1 IAA	1 BA	5 ab	3.8 a	47 c	3.3 a
1 IAA	2 BA	7 a	4.6 a	13 b	27 b
1 IAA	3 Kinetin	8 a	4.1 a	10 b	7.3 a
0 IAA	2 Kinetin	6 ab	4.3 a	3.3 a	2.3 a
2 2,4-D	1 BA	2 b	3.3 a	89.3 d	10.1 ab
0.5 NAA	1.5 BA	8 a	8.7 b	35 bc	25 b
1 NAA	3 BA	6 ab	7.5 b	60 cd	40 c

Treatments denoted by the same letter were not significantly different, $P < 0.05$. At least 25 replicates were used per treatment.

Callus initiation was noticed from the cut surfaces of petiole, petiolule explants and the midrib of leaflet explants on this medium 10 days after explant decontamination (Figure 4.1A). This white, friable callus showed no organogenic potential at that point in time. By means of comparison the 0.5:1.5 NAA: BA combination (positive control) at the same time had resulted in callogenesis in 30 % of cultures, with the callus production being particularly abundant from the midrib of leaflet explants. After four weeks in culture, a total of 55 % of the explants on the 0.5:1.5 NAA: BA combination had differentiated into white callus.

Formation of nodular tissues on swollen explants was a response frequently observed when adventitious bud formation occurred directly from explants cultured on 2 mg l^{-1} kinetin (Figure 4.2B). Twenty four percent of these cultures (Table 4.1) were showing signs of direct organogenesis after a month as the nodular tissues on the explants had subsequently developed into visible shoot initial buds (Figure 4.2B). This developmental pattern was the same for explant tissues on 1:3 IAA: kinetin combination and 2 mg l^{-1} kinetin. This result is similar to the effect noted in cumin by TAWFIK and NOGA (2001) where nodular tissues developed only on areas at the cut end in direct contact with the medium only in the presence of BA. These authors concluded that BA was essential for adventitious meristem organogenesis. It was also suggested that BA triggers two types of responses in cumin simultaneously, with one of these responses being the proliferation of callus-like tissues from cells lacking the competence for adventitious bud formation. The other response was shoot regeneration from initially existing competent cells via adventitious buds. A similar process may govern the conversion of nodular tissues into plantlets in *T. garganica* as nodular tissues on some of the explants failed to develop into shoots even after six weeks in culture. Instead they remained as white buds showing no further signs of development (Figure 4.2C).

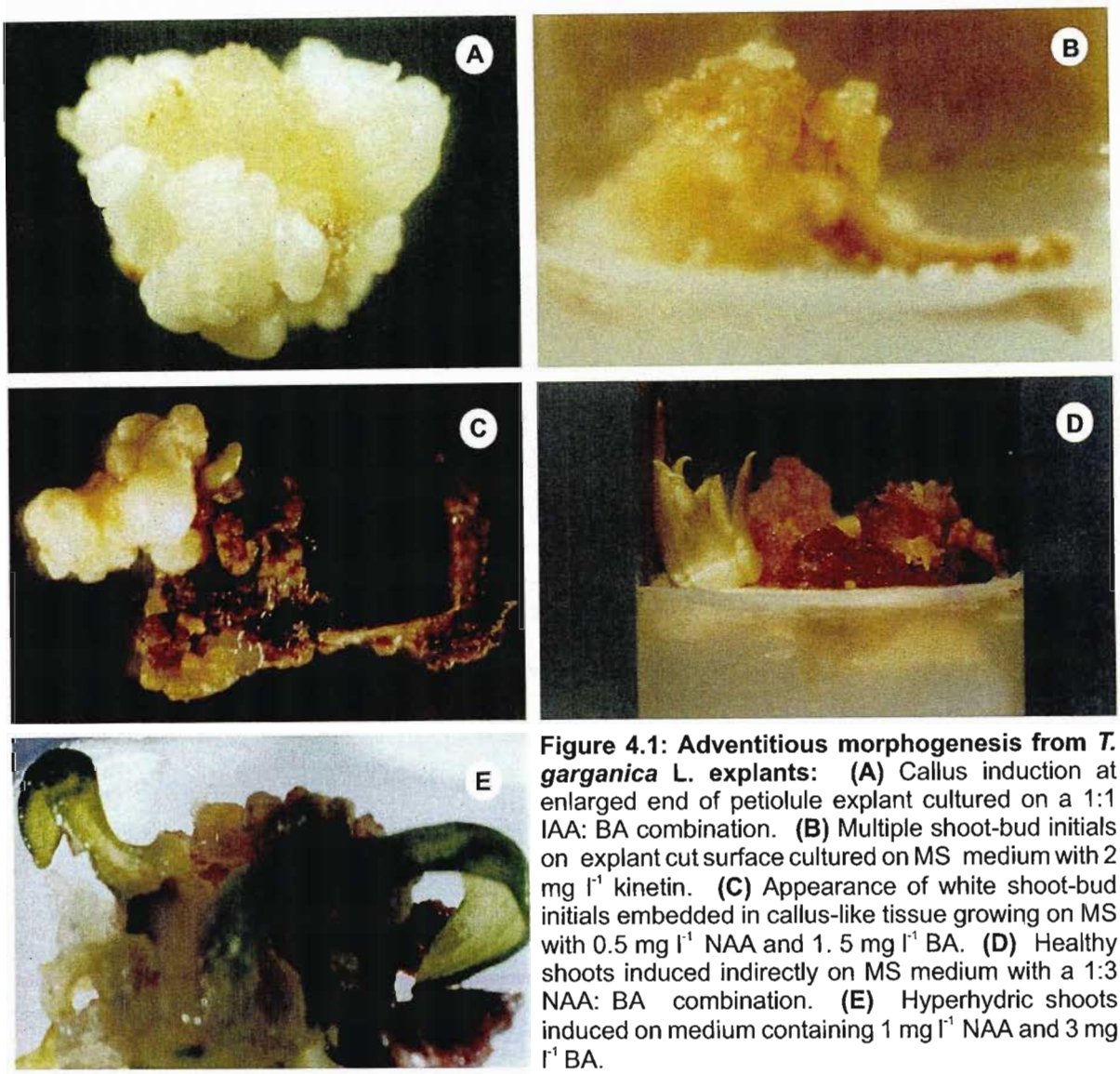


Figure 4.1: Adventitious morphogenesis from *T. garganica* L. explants: **(A)** Callus induction at enlarged end of petiolule explant cultured on a 1:1 IAA: BA combination. **(B)** Multiple shoot-bud initials on explant cut surface cultured on MS medium with 2 mg l^{-1} kinetin. **(C)** Appearance of white shoot-bud initials embedded in callus-like tissue growing on MS with 0.5 mg l^{-1} NAA and 1. 5 mg l^{-1} BA. **(D)** Healthy shoots induced indirectly on MS medium with a 1:3 NAA: BA combination. **(E)** Hyperhydric shoots induced on medium containing 1 mg l^{-1} NAA and 3 mg l^{-1} BA.

This study has shown that the presence of a cytokinin in the induction medium is essential for adventitious shoot formation as an organogenic response was observed only when cytokinins were present (Table 4.1). Furthermore, stimulation of shoot regeneration was only high when the concentration of the cytokinin used was higher than 1 mg l^{-1}. It is noteworthy that the 1: 1 IAA: BA treatment had induced callus production in 83 % of the explants placed in culture. However, no signs of plantlet regeneration were evident from this treatment whereas the medium with a 1:2 IAA: BA combination also resulted in callus production three weeks from the time of culture initiation, with the higher concentration of BA triggering shoot induction. Only 25 % of the explants responded in this manner.

The only medium to result in direct shoot production alone from adventitious buds was MS supplemented with 2 mg l^{-1} kinetin in 36 % of leaflet explants (Table 4.1). This is not only a new result for *T. garganica* but this response is also unique as all other PGR combinations also had the ability to induce callus formation. This result has several advantages. A great number of leaflet explants could be prepared from one or limited number of mother plants material explants and placed on this medium to induce organogenesis only and thus reducing the potential introduction of somaclonal variation. Secondly, the occurrence of adventitious shoot initiation on cut surfaces of *T. garganica* explants, also similarly indicated in *Cuminum cyminum* L. (TAWFIK and NOGA; 2001), or leaflet midrib increases the probability for transformation of a species through *Agrobacterium* infection.

The factorial grid combining 2,4 D and BA did not prove a very effective treatment as most of the explants did not respond except for those on 2 mg l^{-1} 2,4 D and 1 mg l^{-1} BA (Table 4.1). The 1:3 NAA: BA treatment resulted in indirect organogenesis in 75 % of the explants after four weeks, once again confirming that this is a good medium to initiate shoots via a callus phase from *T. garganica* as observed from previous experiments (MAKUNGA *et al.*, 2003; also refer to Chapter 3). However, the ramification of inducing cultures on this particular medium seems unpredictable as the onset of hyperhydricity occurred at random. At times, shoots would emerge from explants with acute or severe morphological defects due to hyperhydration (Figure 4.2D). Whilst at other times, the shoots induced from this PGR would show no anomalous morphological defects and exhibited a healthy appearance. Continued culture of plantlets on the medium with NAA plus BA at 1 mg l^{-1} and 3 mg l^{-1}, respectively, for purposes of shoot multiplication further exacerbated the frequency of hyperhydration (40 % hyperhydric plants; Table 4.2). CASSELS and CURRY (2001) suggested oxidative stress is the one stress often associated with initiation of cultures. The wounding process during explant preparation is one of the causal factors of this type of stress and the presence of oxidative stress induces aberrations in culture such as hyperhydricity. Other factors implied in triggering the hyperhydric state include auxin over-exposure, water stress and mineral deficiency amongst others.

Tissues presenting with a severe hyperhydrated condition such as the translucent shoots induced on the 1:3 NAA: BA combination (Figure 4.1D), have impaired or poor stomatal function which inevitably causes problems in microplant establishment (CASSELS and CURRY, 2001; JOYCE *et al.*, 2003). Mechanisms regulating abnormal morphologies *in vitro*, resulting in aberrant genomic expression, are similar to those involved *in vivo*. Stress responses act via signal pathways that involve Ca^{2+}, PGRs, sucrose and other components in transduction of the signal (JOYCE *et al.*, 2003). The nature of the *in vitro* environment may affect transpiration and consequently, uptake of Ca^{2+} and auxin movement. Media components interacting with compounds in tissues may also lead to breakdown of regulatory processes leading to abnormal phenotypic expression in culture (JOYCE *et al.*, 2003). It can thus be deduced that the 1:3 NAA: BA medium, although reliable for shoot proliferation, is not an optimal medium. It is presumed that this auxin:cytokinin ratio, albeit having the ability to control adventitious regeneration in *T. garganica*, is also responsible for occurrence of hyperhydricity; particularly under the set of growth conditions the plants were exposed to during experimentation. It can also be assumed that the alteration of the other abiotic culture conditions, including the use of ventilated vessels for gas permeability (CASSELLS and WALSH, 1994; CASSELLS and WALSH, 1998), bottom-cooling of culture jars (CASSELLS, 2000) and/or alterations in Ca^{2+} levels would provide better quality management *in vitro* (CASSELLS, 2000) and thus maintain high health of *T. garganica* plants during clonal propagation. Rather than disregard the 1:3 NAA: BA medium, it is proposed that such alterations would reduce the appearance of hyperhydricity and increase plantlet fitness for transplanting out of culture.

Quantitative evaluation of results (Table 4.2) investigating the optimal medium for shoot multiplication showed that 1:2 IAA: BA, 1:3 IAA: kinetin and 0.5:1.5 NAA: BA had a similar effect on the number of shoots obtained per culture tube (7-8 plantlets per tube). The longest shoots differentiated on MS with 0.5 NAA and 1.5 BA. Doubling the concentration of these phytohormones, respectively reduced the number of plantlets obtained but the effect on shoot length was the same. Even though, these treatments had a similar effect on both plantlet regeneration and hyperhydration, their effect on callus production varied. The 0.5: 1.5 NAA: BA PGR combination encouraged callus induction to a lesser extent as compared to the 1: 3 NAA: BA combination.

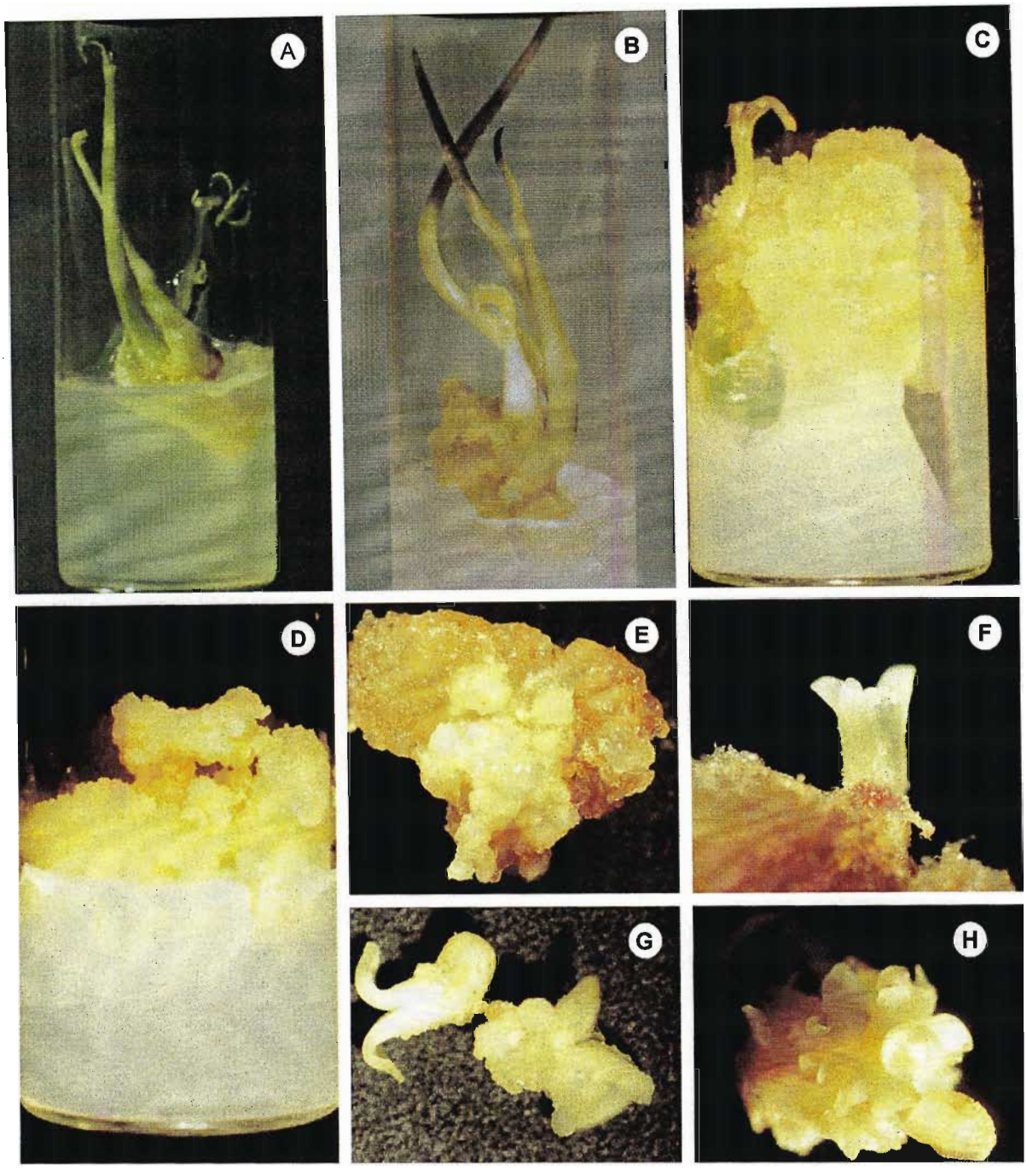


Figure 4.2: Effect of 2.4-D on morphogenesis in *in vitro* cultures of *T. garganica* L.: (A) Enlarged shoot base at the onset of callogenesis **(B)** Prolific callus formation at the shoot base after a month on MS with 2.4-D (2 mg l^{-1}) and BA (1 mg l^{-1}) as adjuvents **(C)** Conversion of shoot tissue into dedifferentiated white friable callus after 8 weeks on MS with a 2: 1 2.4-D: BA combination **(D)** Transfer of callus onto PGR-free medium resulted in callus becoming compact and granular subsequent to somatic embryo production **(E)** Compact embryogenic callus initiated from nodular callus **(F)** Torpedo-shaped embryo emerging from callus. Note anthocyanin production at the base of the embryo. **(G) Somatic embryos seen at the globular** stage and heart-shaped embryo exhibiting more torpedo character. **(H)** Most embryos easily converted spontaneously to form shoots on PGR-free medium.

ANZIDEI *et al.* (2000) found that a ratio of 1:1 auxin:kinetin strongly stimulates shoot regeneration and that kinetin is the more effective cytokinin for plant regeneration from callus through adventitious buds when compared to BA during the micropropagation of fennel. In comparison, the results suggest that BA and kinetin are equally compatible for shoot multiplication for the number of plants produced in each tube and shoot height as they had a similar effect statistically (Table 4.2).

Presence of 2,4-D in the multiplication medium was observed to reduce the organogenetic potential and instead promoted callogenesis (Table 4.2; Figure 4.2A-B). It is noteworthy that the transferal of plantlets from another plant growth combination resulted in the callusing of shoot bases becoming apparent within a week on this medium (Figure 4.2A). This indicated that the inclusion of 2,4 D is necessary for rapid callus proliferation. This auxin has a stronger promotory effect on callus induction than NAA. This system is also potentially useful for expeditious induction of embryogenic tissue with somatic embryos being useful as an explant for transformation. However, problems associated with somaclonal variation would remain a concern, as previously discussed (Chapter 3, Section 3.1). In support of this observation, statistical analysis revealed that the 2 mg l^{-1} 2,4-D and 1 mg l^{-1} BA treatment does significantly encourage callus formation in comparison to other treatments (Table 4.2). Plantlet subculture onto the 2 mg l^{-1} kinetin, lacking in auxins, resulted in callus induction not previously noted at the time of initiation. Once callus was produced, this dedifferentiated tissue proved stubborn to eliminate after transfer of the same plantlets to medium devoid of PGRs during the rooting stage. This may imply that the plantlets produce enough endogenous auxin on their own to support callus production. It was thought that shortening of exposure time of shoots on this multiplication medium or reducing the number of subcultures prior to rooting would eliminate the unprecedented production of callus. However, when the plantlets were treated in this manner, callus proliferation continued in the absence of PGRs. Instead, the callus seems to have a high potential for producing somatic embryos once transferred from 2 mg l^{-1} kinetin to PGR-free medium. This is in accordance with previous observations made by JÄGER *et al.* (1993). A similar effect was noted during the course of this study when the callus obtained on the 2,4 D: BA (2:1) medium was transferred onto PGR-free medium. The callus became more compact after 10 days on medium without PGRs and different stages of embryo

formation were noted after eight weeks (Figure 4.2). SMITH *et al.* (1997) investigated the optimal medium for somatic embryogenesis in carrot cultures and found that the presence or absence of 2,4-D and the medium type had no influence on the number of embryos obtained. In that particular case, embryogenesis was more sensitive to culture age as the young cultures were more vigorous in their morphogenetic response.

4.3.2 Improving the rooting stage

The use of different types of auxins (NAA, IAA and IBA at 1 mgl^{-1}) on the rooting of plantlets in this study had no significant effect as all three different types of auxins used did not promote rooting at all (results not shown). These auxins did not have any further effects on the development of plantlets in culture (Table 4.3). IBA has previously been recorded to promote induction of roots with similar characteristics to those growing *in vivo* at low incidence (32 %, MAKUNGA *et al.*, 2003; Section 3.3.2). However, when IBA was used for comparative purposes against NAA and IAA for root initiation, the results previously obtained were not noted again. Instead, no roots were produced from all three treatments. This indicated that the previous results were not reproducible and perhaps the influence of the culture age was negative on rooting. Young cultures were presumed to respond more positively to these rooting phytohormones. Reports on other species of the Apiaceae, such as cumin (TAWFIK and NOGA, 2001; EBRAHIMIE *et al.*, 2003) and fennel (ANZIDEI *et al.*, 2000), have discussed the successful use of a medium devoid of PGRs for root induction, with rooting occurring within two weeks. In contrast, PGR-free medium does induce rooting but at low incidence in *Thapsia* as previously discussed (Chapter 3, Section 3.3.2) halving the concentration of MS salts had no significant effect on root production in *T. garganica*. In a recent study, a rooting medium of half-strength MS with 0.2 mg l^{-1} IAA as a supplement was highly successful in inducing roots 95 % of *in vitro* shoots tested by EBRAHIMIE *et al.* (2003) during in *Cuminum cyminum* (Umbellifereae) micropropagation using excised embryos as explants. In cumin, only 30 % survival was noted *ex vitro*.

It needs to be emphasized that attempts that were made to improve rooting using various treatments were mostly futile and consequently, several experiments that were conducted are not reported here. Introduction of activated charcoal in the rooting

regime significantly improved the incidence of rooting and the number of roots produced per plantlet, especially when plantlets were exposed to 5 mg l^{-1} IBA and transferred onto charcoal supplemented medium, thereafter (Figure 4.3D-F). This two-step regime resulted in 50 % rooted plantlets with 6 roots per plant, on average.

Table 4.3: Effect of auxins and charcoal as adjuvents on the rooting of *T. garganica in vitro* shoots

ROOTING TREATMENT	Plantlets rooted *in vitro* (%)	Number of roots per plantlet	Callus formation (%)	Presence of hyperhydration (%)
0 mgl^{-1} PGR + MS solid medium	0 a	0 a	12	4
0 mgl^{-1} PGR + ½ MS solid medium	0 a	0 a	24	0
IBA + 0 mgl^{-1} charcoal	0 a	0 a	32	0
IBA + 0.5 mgl^{-1} charcoal	0 a	0 a	8	0
IBA + 1.0 mgl^{-1} charcoal	36 d	4 bc	52	12
IBA + 2 mg l^{-1} charcoal	4 b	5 bc	100	0
IBA + 5 mg l^{-1} charcoal	50	6 c	83	0
IBA + 0 charcoal + vermiculite	0 a	0 a	NQ[c]	0
IBA + 0.5 charcoal + vermiculite	10 b	2 b	NQ	0
IBA + 1.0 charcoal + vermiculite	28 cd	2 b	NQ	0
IBA + 2 charcoal + vermiculite	4 b	1 b	NQ	18
IBA + 5 charcoal + vermiculite + sand	40 d	4 bc	NQ	0
10 min IBA [b] pulse + 2 mg l^{-1} charcoal	8 bc	3 b	32	0
30 min IBA [b] pulse + 2 mg l^{-1} charcoal	32 d	6 c	24	8

[a] IBA was supplemented in the growth medium at a concentration of 5 mg l^{-1}, [b] except when IBA was used as a pulse treatment. In this instance, 5 mg l^{-1} or 10 mg l^{-1} IBA solution were made by dissolving in sterile water and filter sterilised, and then used as a pulse treatment to induce rooting

[c] NQ means that a quantitative analysis was not performed

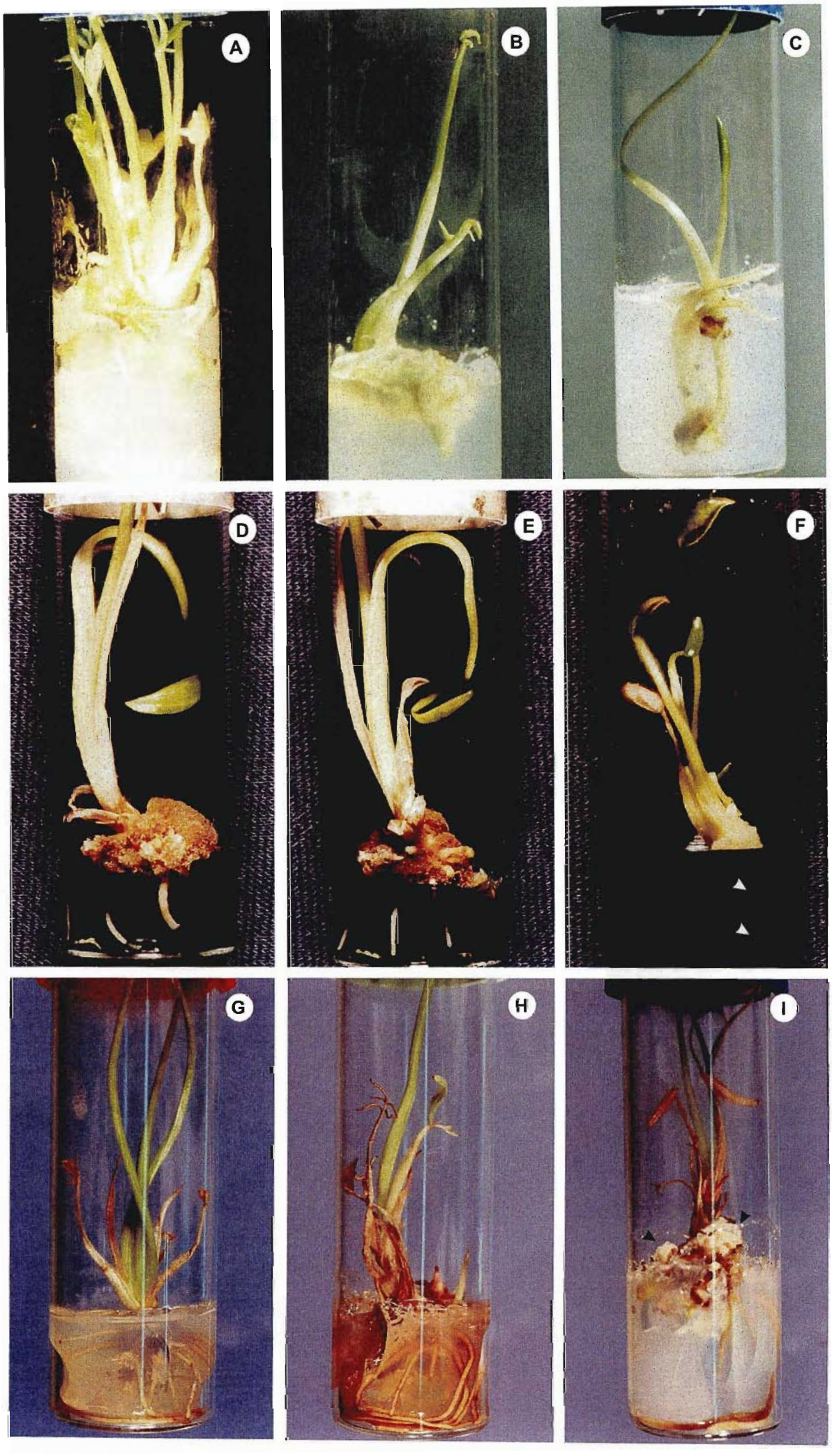
[d] Data were collected prior to transfer of plantlets to *ex vitro* conditions. Treatments denoted by the same letter were not significantly different, $P < 0.05$. At least 25 replicates were used per treatment

Table 4.4: Effect of liquid medium at half-strength MS (½ MS) or full- strength (MS) with filter paper bridges (F) on the rooting of *in vitro*-formed and elongated shoots of *T. garganica* L.

ROOTING PROCEDURE	Plantlets rooted *in vitro* (%)	Mean number of roots per plantlet	Callus formation (%)	Occurrence of hyperhydration (%)
½ MS F[b] 5 IBA[a] (3d)	12 b	3 bc	12	14
½ MS F 5IBA (6d)	0 a	0 a	45.5	17.4
½ MS F10 IBA (3d)	40 e	7d	9.1	0
½ MS F10 IBA (6d)	35.3ed	8 d	24	0
½ MS F25 IBA (3d)	13b	4 bc	8	0
½ MS F25 IBA (6d)	9.5 b	5 cd	20	0
MS F5 IBA (3d)	13.3 b	3 bc	4	16
MS F5 IBA (6d)	30 d	2 b	45	24
MS F10 IBA (3d)	30 d	3 bc	68	18
MS F10 IBA (6d)	20 c	7 d	60	22
MS F25 IBA (3d)	18.1 bc	2 b	78.3	64
MS F25 IBA (6d)	0 a	0 a	76	65.2

[a] The IBA was added to the liquid medium at a concentration of 5, 10 or 25 mg l^{-1}, denoted as 5 IBA, 10 IBA and 25 IBA, respectively
[b] F denotes filter paper bridge
[c] Treatments denoted by the same letter were not significantly different, $P < 0.05$. At least 25 replicates were used per treatment
Brackets indicate the number of days that the shoots were exposed to the rooting phytohormone

It is generally accepted that the rooting process is governed by four phases: (i) induction phase that determines the capacity for root formation; (ii) initiation phase characterized by the occurrence of visible changes at the cytological level; (iii) organization phase involves the histological development of root primoridia; and, (iv) root growth (root elongation) defined by elongation of root primordial into roots (GEORGE, 1996; PAN and VAN STADEN, 2002). This two-step rooting regime most probably resulted in IBA influencing formation of rooting initials and then charcoal subsequently promoted further root development. The inclusion of charcoal benefits the tissue culture process in a variety of ways (for review refer to PAN and VAN STADEN, 1998). These include the adsorption of phytohormones and other artifacts that may hinder rhizogenesis as well as the provision of a darkened environment (PAN and VAN STADEN, 1998; PAN and VAN STADEN, 2002) which simulates *in vivo* conditions for root induction.


A
B
C
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E
F
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I

Although, the roots obtained appeared strong and unlikely to experience transplantation damage, they were accompanied by extensive callusing at the shoot bases (Table 4.3; Figure 4.3D-F) on medium with charcoal. The callusing area forms a point of weakness as it is a site for opportunistic fungal and bacterial infections. This is also a site where the vascular system is disrupted or poorly formed and acclimatisation of such plants is generally difficult. This method of rooting cannot possibly be applied routinely unless this particular step is optimized as CASSELLS and CURRY (2001) highlighted that techniques chosen for *in vitro* regeneration influence subsequent development and performance of plantlets following transplantation to *ex vitro* conditions.

Micropropagators are often reluctant to expand time investigating optimal rooting *in vitro* as often the roots induced under the microenvironment fall-off during transplantation and show limited physiological functioning when in contact with soil (GANGOPADHYAY *et al.*, 2002). Even though this is true, the presence of roots at the time of transplanting increases *ex vitro* survival, thus treatments that encourage rooting *in vitro*, reduce mortality out of culture (WHISH *et al.*, 1992). While these statements are paradoxical, the possibility of better rooting of plantlets whilst at the *in vitro* stage plus the spin-off of increased survival of *T. garganica* microplants after deflasking, was sufficient incentive to encourage the search for a better rooting medium. Apart from this reason, the motivation to study a liquid rooting system was driven by the susceptibility of *T. garganica* to damping-off diseases due to fungal attack. Remains of agar-gelled medium in the roots would thus increase the vulnerability of microplants to unwanted bacterial and fungal diseases such as, root rot. It became evident that rooting in liquid MS particularly when the concentration of salts was reduced to half, with *T. garganica* plantlets on filter paper supports was the best method investigated thus far. These results are tabulated in Table 4.4. Three days (40 % rooting frequency; 7 roots per plant) and six days (35.3 % rooting frequency; 8 roots per plant) of IBA (5 mg l^{-1}) ½ MS treatment prior to transfer onto solid medium for further root development were comparable for rooting *in vitro* (Table 4.4). The roots induced were strong and healthy (Figure 4.3G and Figure 4.3H). However, a small number of plantlets callused at the shoot bases (9.1 %, Figure 4.3I). When root elongation was continued in a liquid medium (data not shown), some of the plantlets became hyperhydric. Precautions to prevent hyperhydricity should be taken because roots of this nature are unable function

properly (JAYANAND *et al.*, 2003). On the other hand, when cultures were rooted on solid medium after the filter paper bridge phase, the adventitious roots induced directly from the shoot bases exhibited normal positive gravitropic behaviour, a highly desired effect. The incidence of callus formation at the shoot bases was also minimal 9.1 % for the 3 day treatment and for the 6 day treatment at 10 mg l^{-1} IBA. The decision to use liquid medium in subsequent rooting procedures versus the 5 mg l^{-1} IBA treatment on solid medium for one month followed by removal of the PGR using charcoal (Table 4.3) was based on the callus being kept in check under such conditions. Adsorption of PGR by the microcutting is far more effective in a liquid system. Therefore, this is presumably the primary reason for increased rooting in *T. garganica*. Additional support can be drawn from results presented on the use of IBA pulse treatment for 30 min as 32 % rooting was achieved but 64 % of the shoots callused.

The use of liquid medium for rooting at concentrations of 25 mg l^{-1} auxin was modified from RATHORE *et al.* (1992) for rooting of tree species, *Maytenus spp.* However, the authors did not discuss the reason for the success of this method. Rooting in liquid medium is popular. JAYANAND *et al.* (2003) established a three-phase system for the rooting of chickpea microplants. This species is also notoriously difficult to root and these authors reported on the use of filter paper bridges immersed in liquid medium subsequently followed by a pulse treatment with IBA at 100 µM. The third stage of the protocol involved transfer of unrooted shoots to a static hydroponic system. The high frequency of rooting that was realized was attributed to the last phase involving the use of hydroponics. The positive response of *T. garganica* plantlets to rooting in liquid medium is possibly an incentive towards the application of a hydroponic system in future studies of *Thapsia* in culture.

Inclusion of cytokinins, alone or in combination, to achieve adventitious plant regeneration by is common practice in tissue culture protocols. However, at times the inhibitory effect of cytokinins on rooting is pronounced especially when the shoots are on the shoot regeneration medium for lengthy time periods prior to rooting (POLANCO and RUIZ, 2001). It seems that the longer the shoot cultures of *T. garganica* were maintained on shoot proliferation medium, the more difficult it became to induce rhizogenesis (data not shown). The rooting was most probably directly proportional to

the number of subcultures on shoot proliferation medium as observed qualitatively. This, however, was not investigated quantitatively in this study but requires further scrutiny in the future for optimization of the rooting regime in *T. garganica*. No significant improvements were made on *ex vitro* survival in this part of the study. The following chapter deals with this particular aspect in greater detail (refer to Chapter 5, Section 5.2.3). In cumin (TAWFIK and NOGA, 2001), the application of IBA increased the number of roots but affected the frequency of rooting negatively as a decrease. In that instance, plantlet survival *post-vitrum* was significantly improved (above 75 %) and these authors contributed this effect to the use of sand in the potting medium as previously it had been much lower. In this particular study, vermiculite and sand was intended to commence the weaning of plantlets *in vitro* whilst rooting simultaneously (Table 4.3), so as to strengthen plantlet survival for the *ex vitro* environment. However, this was not the case as the plantlets rooted in this way did not survive planting out. A large number of losses were still experienced out of culture. Hardening-off of the Apiaceae is difficult (EKIERT, 2000). This statement is in agreement with results based on cumin *in vitro* culture reported by EBRAHIMIE *et al.* (2003) as these authors obtained 32.8 % of the microplants successfully hardened-off. In *T. garganica* application of antifungal agents during planting out improves acclimatization. It can be argued that although the efficiency of hardening is to an extent low, the highly efficient rate of multiplication easily overlaps and thus overcomes this problem in both *C. cyminum* and *T. garganica*.

4.4 CONCLUSION

In conclusion, the comprehensive methods presented in this chapter are superior to those previously reported and indicate anew the relative ease of inducing and regenerating microplants of *T. garganica* through the use of clonal propagation. Highly inductive media have been obtained. For direct organogenesis induction on 2 mg l^{-1} kinetin is best for the avoidance of a callus phase with intention to micropropagate *T. garganica*. The response on this medium is similar to that obtained on a 0.5:1.5 NAA: BA combination except that callus production, more often than not, precedes vegetative budding on the auxin:cytokinin medium. However, the 0.5:1.5 NAA: BA medium is efficient and more importantly, reliable. The initiation response on this medium is similar

or resembles that obtained previously (MAKUNGA *et al.*, 2003). Thus, this chapter has formed a basis to verify the results previously obtained and high multiple shoot regeneration concludes the stability of the method used. By application of a new set of growth media for purposes of shoot multiplication, the production of better quality plantlets and increased rooting has been achieved in this study. This means that two stages of the micropropagation regime of *T. garganica* have been greatly improved in this chapter. The elongation of shoots on 2 mg l^{-1} kinetin medium or 0.5 mg l^{-1} NAA and 1.5 BA supplemented MS medium followed by passage onto 5 mg l^{-1} IBA and 2 % charcoal or use of liquid medium with filter paper bridges to condition *T. garganica* for production of root initials and root elongation was found to be very useful. The preparation of filter paper bridges is time-consuming and requires the inclusion of a new step to elongate roots in culture on ½ MS devoid of PGRs. Even though this increases the time period of *T. garganica* plantlets under vitreous conditions which can precipitate the onset of somaclonal variation, the benefits of rooting microplants in this way outweighs the potential drawbacks or disadvantages. A larger number of plants have the possibility of surviving *ex vitro* as unrooted microshoots are particularly susceptible to damping-off diseases. This protocol should provide an efficient means for future studies on other species of Apiaceae, and in particular those of the genus *Thapsia*, should similar problems *in vitro* be experienced. Furthermore, improved rooting in *T. garganica* at the *in vitro* stage also becomes important while inducing root cultures as a source of thapsigargins.

CHAPTER 5

IMPORTANCE OF *IN VITRO* PRE-TREATMENT FOR *EX VITRO* ACCLIMATISATION

5.1 INTRODUCTION

The phenomenon of hyperhydricity, formerly termed vitrification (DEBERGH *et al.*, 1992) has been described in a large number of plant species (OCHATT *et al.*, 2002) cultivated through *in vitro* propagation. It is evident that *T. garganica* plants produced *in vitro* are not exempt from this anomalous condition. In *T. garganica*, production of hyperhydric shoots in culture is not an exception and its occurrence is not easily predictable as it occurs at all stages of propagation, from Stage 1 of the *in vitro* culture process (as defined by MURASHIGE, 1974; CASSELLS, 2000) through to the rooting stage prior to acclimatization. Its occurrence is characterized by random appearance in culture and the intensity of this condition is variable. The preferential occurrence of hyperhydration on some of the regeneration media is capricious. In some instances, cultures are free of hyperhydric tissues (Figure 5.1A) with their morphological appearance normal, whereas occasionally hyperhydric individuals of *T. garganica* are grossly malformed (Figure 5.1B) with acute hyperhydration. Hyperhydric plants are often characterised by translucent tissues with impaired stomatal function which causes difficulties in the establishment of microplants *ex vitro* (DEBERGH *et al.*, 1992; DEBERGH et *al.*, 2000), as shown in Figure 5.1B. Plant growth regulators implicated in hyperhydricity are cytokinins, auxins, gibberellic acid and ethylene (ZIV, 1991; CASSELLS and CURRY, 2001), whereas oxidative stress caused by presence of hyperactive oxygen species in culture is due to mainly auxins and cytokinins.

Many investigations have focused on the production of better quality plantlets *in vitro*. A subject of several reports has been the photosynthetic ability of *in vitro* microplants. It has been deduced or proven from these studies that photosynthesis *in vitro* is hampered by a low capability for inorganic carbon assimilation, low photochemical quenching, and relatively low value of variable over maximal fluorescence ratio, an indication of reduced Photosystem II photochemistry (PREMAKUMAR *et al.*, 2003). Diminished photosynthetic performance of tissue culture has been correlated with the supplementation of culture medium with sugars as a source of carbon (DESJARDINS, 1995; PREMAKUMAR *et al.*, 2003).

According to JOYCE *et al.* (2003), plant growth is under the control of phytohormones acting synergistically and plant development is tightly regulated by environmental stimuli, in particular light *in vivo*. The interaction of light receptors with phytohormones controls biosynthesis of chlorophyll, elongation of stem tissue and leaf expansion and the transition from vegetative development to reproductive growth. Environmental stress affects progression of development and plants have evolved mechanisms to cope with a wide variety of environmentally induced stress. Responses to stress are elicited through complex signal transduction pathways, not yet fully elucidated. *In vitro* abnormal morphological and unconventional physiological behaviour of microplants has been attributed or hypothesised to be due to *in vitro* stresses as culture conditions are controlled. The primary putative stresses *in vitro* are abiotic and manipulation of growth parameters, culture vessel design and media components are of important consideration for management of stress in plants during propagation. Techniques for improved plant health *in vitro* include bottom-cooling of culture vessels and ventilation of vessels to reduce the signs of hyperhydration. Induction of a transpiration stream in better ventilated culture vessels is thought to lead to increased Ca^{2+} uptake. This is a counter ion for basipetal auxin movement. Auxin accumulation in poorly transpiring microplants may be involved in cell extension leading to symptoms associated with the hyperhydric condition (reviewed recently by JOYCE *et al.*, 2003).

CASSELLS and CURRY (2001) consider oxidative stress as the underlying cause of stresses *in vitro* contributing to poor genetic quality in microshoots. These authors advocate not only the manipulation of media composition but also modification in culture vessel design to facilitate exchange of gases during *in vitro* culture as this increases the resistance to hyperhydricity in cultured plants and leads to improved ontogenic development. Oxidative stress-induced damage is also in part implicated in recalcitrance to genetic transformation, genetic and epigenetic variation associated with somaclonal variation.

Induction of abnormal vitromorphologies due to the gelling agent is often a parameter that is overlooked in micropropagation protocols (LAINE *et al.*, 2000). It is possible for hyperhydration to be induced by the type of gelling agent used to solidify medium (COHEN, 1995; LAINE *et al.*, 2000). The use of agar (extracted from *Laminaria algae*) and gellan gum (popularly referred to as Gelrite or Phytagel) has an influence on the onset of aberrant morphological conditions in culture (LAINE *et al.*, 2000) as the availability of mineral ions is also influenced by the gelling agent used. The two types of gelling agents (agar and gellan gum) that are common in use, are different in their composition and salts. In addition, the quality is a factor that needs to be considered when choosing a gelling agent (CASSELLS and ROCHE, 1994; CASSELLS and WALSH, 1994; CASSELLS and COLLINS, 2000). Hyperhydricity may become a problem with some of the purified grades of agar that are commercially available (COHEN, 1995). However, partial hydrolysis of agar with acid produces compounds that are capable of reducing the hyperhydric condition. In spite of negative influences in tissue culture, a wide range of plants respond favourably to agar-medium. The gelling action of gellan gum requires Ca^{2+} sequestration for its action, thus affecting the availability of Ca^{2+} to the tissues growing on it (LAINE *et al.*, 2000). Ca^{2+} is an important compound implicated in many signal pathways controlling stress responses in plants (CASSELLS and COLLINS, 2001). Moreover, Ca^{2+} concentration is important for the inhibitory effect of amino glycoside antibiotics such as kanamycin, which are a popular choice for the selection of transgenic plants. The effect of gelling agent on selection efficiency is therefore an important consideration.

Interactions in culture leading to aberrant phenotypes are complex. Controlled stress *in vitro* facilitates post-culture establishment. Therefore, *in vitro* weaning may lead to improved performance of microcuttings once out of culture (CASSELLS and CURRY, 2001; JOYCE *et al.*, 2003). Stress responses of genotypes are often unpredictable as each genotype enters the culture environment in a specific physiological and hormonal state (JOYCE *et al.*, 2003). Introduction of plants into the external environment from tissue cultures still poses many problems for many plant species due to rapid foliar water loss leading to dessication (SHORT *et al.*, 1987). Plantlet mortality can increase if microplants are handled poorly and when acclimatization procedures are suboptimal (DAMI and HUGHES, 1997).

According to ZIV (1991), the manifestation of the hyperhydric state is still poorly understood but is believed to be a function of synergistic mechanisms. The defects are of an anatomical and physiological nature due to various disorders in metabolic pathways governing plant growth and development. Changes in protein biosynthesis due to hyperhydration affects a variety of enzymes associated with photosynthesis, for example, Ribulose-1,5-bisphosphate (Rubisco); enzymes of secondary metabolism such as phenylammonia lyase (PAL) and glucan synthase and/or processes of ethylene production. Such changes, in actual fact, are affecting metabolic pathways that are interlinked. Regarding PAL, this enzyme is a key one in phenylpropanoid metabolism and the products from PAL activity are shunted into various secondary metabolic pathways including lignin biosynthesis and flavonoid synthesis resulting in the production of anthocyanins. Ethylene gas, which accumulates in tissue culture vessels, has the ability to affect PAL activity and phenolic levels and this in turn results in hypolignification associated with hyperhydric plants. When in poor physiological and morphological condition, plants are unable to cope with *ex vitro* stress at the time of establishment in the environment because their resilience is lacking.

Findings by KADLEČEK *et al.*, (2001) are in accordance with other researchers (VAN HUYLENBROECK *et al.*, 1998; LAFORGE *et al.*, 1991) in supporting that optimal *in vitro* conditions for growth of plants in culture prior to acclimatisation

have a positive effect on the growth of micropropagated plants once transferred *ex vitro*. During the last stage in culture prior to *ex vitro* transfer, an increase in the water vapour gradient between the culture atmosphere and the leaf *in vitro* significantly improve the leaf structure and function of young and newly formed leaves from existing leaf primordia. Leaves developing under conditions of reduced humidity and elevated irradiance perform better due to their increased photosynthetic ability and metabolic fitness. The adaptation of leaves toward active photosynthesis is one of the major challenges in the development of plantlets once out of culture. It has been suggested that the success in hardening-off propagules is largely dependent on reserve nutrients stored in the leaves during *in vitro* leaf development. As many stresses affect photosynthesis, water deficit and photoinhibition are used as a broad screen for stress and monitored by chlorophyll fluorescence.

According to DAMI and HUGHES (1997), several methods are available to plant biotechnologists to reduce the relative humidity in culture jars in order to encourage *in vitro* hardening. These include the use of polyethylene glycol (PEG), an inert non-ionic long chain polymer that is highly soluble in water. There is a wide range of molecular weights for this compound and when the molecular weight (MW) is higher than 4 000 it induces water stress in plants by decreasing the water potential. The compound itself is not toxic to the plant, nevertheless its dehydration effects on the medium can result in the levels of the minerals of the medium becoming toxic to the plant (WHISH *et al.*, 1992). This inert osmoticum can influence microplant quality as it induces morphological and anatomical *in vitro* changes. Consequently, PEG positively influences post-culture survival (DAMI and HUGHES, 1997; TAWFIK and NOGA, 2001). However it can delay and even hinder some *in vitro* growth process (VÍTOVÁ, 2002) such as rooting, as observed in cumin species (TAWFIK and NOGA, 2001).

Management of *in vitro* plants for better health increases their resilience to out-of-culture stress and pathogens. The fungal diseases that naturally infect the family Apiaceae have been defined in the online publication of the Connecticut

Agricultural Experiment Station's Plant Pest Handbook (DOUGLAS and COWLEY, 2003). This publication is available at the following site:
http://www.caes.state.ct.us/PlantPestHandbookFiles/pphIntroductory/pphintrohtm.htm
The causal agents of rot or wilt diseases infecting umbelliferous plants are listed below:

i) Damping-off diseases are due to *Rhizoctonia solani* and *Pythium spp.* These pathogens cause seedlings to rot and frequently attack the bases of the seedling;

ii) *Rhizoctonia solani, Pythium spp* and *Fusarium spp* are the causal agents of root and crown rot diseases and the symptoms appear as wilting and slow or rapid collapse of the plant. The roots appear brown and watery-soaked. This disease often begins as a water-soaked lesion at the base of the stem;

iii) Fusarium yellows is a disease associated with *F. oxysporum* f. sp. *appii* and the symptoms include stunted, yellow and wilting plants. This disease leads to death if susceptible cultivars are infected; and,

iv) Stem rot in umbelliferous plants is a result of infection with *Sclerotinia sclerotiorum*, a soil-borne fungus that results in collapse and wilting.

Fusarium species, particularly *F. oxysporium*, are notorious for causing disease in a wide range of different plant hosts and this soil-borne fungus can persist in soil for lengthy periods of time. It is also able to survive on weeds and other crop plants without exhibiting disease symptoms. *F. solani* infections in plants are responsible for vascular wilting (or damping-off). For disease control sterile potting media, clean pots and fungicidal treatments are advised (DOUGLAS and COWLEY, 2003). When seedlings are infected, plants should be rogued out and discarded as asymptomatic plants may subsequently become infected also.

The persistence of hyperhydricity in cultures of *T. garganica* (MAKUNGA *et al.*, 2003; refer also to Chapter 4, Section 4.2.1) is an indication of the failure of media manipulation attempts, especially the auxin:cytokinin ratio, to eliminate or reduce this problem in the clonal propagation of this species. It was hoped that optimisation of culture induction would decrease the regeneration of hyperhydric tissues. The changes made to the *in vitro* procedures were not efficient regarding plant health, thus the aims of this particular set of experiments was to circumvent the onset of hyperhydricity by the application of other *in vitro* strategies, including modifications in the gelling agent of culture medium and incorporation of PEG pretreatments prior to *ex vitro* acclimatization. Thus the study was undertaken with several objectives including the regeneration of true-to-type propagules of *T. garganica* L. *in vitro*. The other goal was to minimize *ex vitro* acclimatization or omit this step completely in order to reduce fungal attack, more especially in the misthouse where *T. garganica* plants were particularly susceptible. The contribution of *in vitro* hardening to management of plantlet quality and vigour *post vitrum* was also under examination.

5.2 MATERIALS AND METHODS

5.2.1 Plant material and *in vitro* culture conditions

Sterile shoot bases from *in vitro* plantlets of *T. garganica* (established as described in the previous Chapter [Section 4.2.2]), were used to reinitiate cultures in a continuous culture system. The leaves of the shoots were removed until about 1 cm of the shoots remained and the base was then cultured on solidified MS basal medium (MURASHIGE and SKOOG, 1962) with 0.8 % agar (w/v) (Agar-agar powder CP, Associated Chemical Enterprises c.c., South Africa) with 3 % sucrose (w/v), 0.1 g l^{-1} myo-inositol, and solidified with 8 g l^{-1} agar. The pH of medium was adjusted to 5.8 with 1 M KOH after the addition of phytohormones at a ratio of 0.5:1.5 NAA: BA. The medium was then autoclaved at 121 °C and 103 kPa for 20 min. Thereafter, it was left to cool prior to pouring 30 ml per culture vessel. Once the medium was solidified, shoot bases were then placed on it. Stock plants were subcultured every four weeks onto freshly prepared growth medium. The cultures

were incubated in a growth room at a temperature of 22 – 24 °C under a 16 h light (36 µmol m^{-2} s^{-1})/ 8 h dark regime. These conditions were maintained throughout the course of the study.

5.2.2 Effect of the gelling agent

Gelling agents have previously been shown to affect the occurrence and the frequency of hyperhydricity in cultured plants (ZIV, 1991; CASSELLS and COLLINS, 2000). With respect to the elimination of this problem, an experiment was included to determine the effect of using 2.5 g l^{-1} Gelrite® (Labretoria, South Africa) compared to agar in the vegetative propagation of *T. garganica*. For experiments studying the effect of Gelrite® on shoot proliferation and plantlet health, a combination of 0.5 mg l^{-1} NAA and 1.5 mg l^{-1} BA or 1 mg l^{-1} NAA plus 3 mg l^{-1} BA was added to the MS medium containing, 0.1 g l^{-1} myo-inositol and 30 g l^{-1} sucrose. These combinations were chosen due to their ability to promote both shoot multiplication and to induce hyperhydricity in the cultures. Gelrite® (2.5 g l^{-1}) was added to the medium prior to dissolving the gelling agent completely in a microwave oven. The agar (8 g l^{-1}) medium was treated in the same manner and the same combination of plant growth regulators was added as supplements. All media were then autoclaved (Section 5.2.3) after the pH had been adjusted to 5.8. Single shoot bases were transferred aseptically to culture tubes (24 x 100 mm) containing the different types of medium to be tested. Tubes were sealed around the lids with a 5 cm x 1 cm Parafilm "M"® and transferred to a growth room with cool white-fluorescent lights providing 16 h of light (45 µmol m^{-2} s^{-1}) and 8 h of darkness. The cultures were incubated at 22 – 24 °C and differences in the number of shoots and the percentage of hyperhydric shoots were recorded after a month.

5.2.3 *In vitro* hardening

(i) PEG pre-treatment

Two types of media were tested throughout the experiment. Healthy shoots, without apparent signs of hyperhydricity, that were at least 3 cm long were excised

from multiple shoot clusters growing on maintenance MS culture medium with 0.5 mg l^{-1} NAA and 1.5 mg l^{-1} BA. They were then transferred to half-strength (½) MS medium containing 3 % sucrose (w/v) (unless otherwise stated), 0.1 g l^{-1} myo-inositol and 8 g l^{-1} agar (pH 5.8) as additives; and placed vertically in it. In an attempt to reduce or eliminate the onset of hyperhyricity prior to *ex vitro* transfer, adjustments were made to media composition and then compared for their efficiency in alleviating hyperhydration of shoots after a period of four weeks. The treatments were as follows:

i. PGR-free medium with ½ MS salts , used as a control;
ii. PGR-free ½ MS plus 2% PEG 6000 (w/v) (BDH laboratory supplies, England);
iii. PGR-free ½ MS plus 2% PEG 6000 and 2.5 mg l^{-1} IBA;
iv. PGR-free ½ MS plus 2% PEG 6000 and 5 mg l^{-1} IBA;
v. Cultures were grown on PGR-free ½ MS with 5 mg l^{-1} IBA for six days prior to transfer onto PGR-free ½ MS medium with 2 % PEG (w/v) and 3 g l^{-1} sucrose; and,
vi. Cultures were placed in PGR-free ½ MS medium with 5 mg l^{-1} IBA for six days prior to transfer onto PGR-free ½ MS medium supplemented with 2 % PEG and 0.3% sucrose (w/v)

For each treatment 20 culture vessels were prepared. Out of the twenty, ten flasks were sealed with normal lids but the cultures were kept slightly ajar (i.e. the lids were not tightly screwed on) for all the above-listed experiments. The other ten jars were sealed with lids that had a 1 cm hole. Five microplants were placed per culture vessel. The *in vitro* plants under the various conditions were monitored for four weeks prior to *ex vitro* acclimatisation.

(ii) Effect of culture lids

An experiment was conducted to test the effect of using culture jars sealed with lids that had a hole drilled for purposes of *in vitro* weaning. Unrooted microplants were transferred from MS medium containing 5 mg l^{-1} IBA after they had been growing for a month on this rooting medium, to ½ MS medium supplemented with 30 g l^{-1}

sucrose, 0.1 g l^{-1} myo-inositol and 8 g l^{-1} agar (pH 5.8). Two different sized holes were tested for their effect on *in vitro* hardening. The cultures were placed in culture vessels with screw caps which had (i) a 1 cm diameter hole or (ii) a 2 cm diameter hole, sealed against bacteria and fungal pathogens with a cotton wool bung. The control jars were tightly sealed and had normal screw caps. Plants were placed on the same growth medium as experimental plants. The response of cultures regarding growth and hyperhydricity were monitored for one month.

5.2.5 *Ex vitro* transfer and acclimatization of plantlets

a) Plantlets were removed from the *in vitro* environment and hardened-off. Several regimes were attempted for this process in order to discourage damping-off diseases. Plantlets were washed thoroughly with sterile distilled water to remove all traces of culture medium and sucrose to limit bacterial and fungal growth once planted in soil. The treatments investigated are listed below: Cultures were transferred to glass bottles with caps that had a hole of 1 cm. Each bottle contained sterile vermiculite (28.3 %; w/v or 8.5 g per 30 ml liquid medium); ½ MS liquid medium without sucrose or PGRs; and, the hole of the cap was sealed with a cotton wool bung. These cultures were incubated in a growth chamber with a 16 h light (48 µmol m^{-2} s^{-1})/ 8 h dark photoperiod. The growth room was fitted with cool-white fluorescent lights. The cotton wool plug was slowly removed after a month's incubation until the hole of the cap was completely exposed. The plantlets remained in the growth room for a further two weeks, then they were extracted from the growth medium with care, washed with sterile water to remove residual medium and treated with a 1 % Benlate (Benomyl 500 g kg^{-1} active ingredient; Du Pont de Nemours Int., South Africa) solution for 30 min to eliminate fungal contaminants and placed into a soil mixture of vermiculite: bark: sand: compost at a ratio of 2:1:2:1 (v:v:v:v).

Thereafter, the pots (7.5 cm) were moved directly to a greenhouse;

b) Plants were treated with a solution containing 5.5 ml Dithane (dithiocarbamate, 800 g kg^{-1}), Chemicult commercial nutrient mix (6.5 % nitrogen, 8.4 % phosphorus and 23,5 % potassium active ingredients; Chemicult products, South Africa), 5 ml Kelpak (0.0031 mg l^{-1} cytokinin active ingredient, Kelp Products [Pty] Ltd, South Africa), (KOWALSKI *et al.*, 1999) made up to 2.5 l with sterile distilled water. A soil mixture of potting soil and bark (1:1; v:v) was drenched with the anti-fungal treatment prior to planting of microshoots;

c) The microplants were soaked in 0.1 % Benlate solution for 30 min prior to planting out. The soil mixture (sand: compost: vermiculite: bark [7:3:0.1:0.1; v:v:v:v) was autoclaved for 20 min at 121 °C and 103 kPa, transferred to 10 cm pots when cool and treated with a Dithane M-45 and Fongarid (62.5 mg Furalaxyl (phenylamide) active ingredient) solution. Once the plantlets were transferred, they were then sprayed with an anti-fungal solution containing 2.5 g l^{-1} Benlate, 2 g l^{-1} Rovral Flo (Iprobione [2ml l^{-1}]) and 2.5 g l^{-1} Previcure N (Propamocarb HCl) prior to transfer to the misthouse with bottom heat of 30 °C to accustom the plantlets to conditions of reduced humidity. The plants were placed for three days in a misthouse with bottom heat of 30 °C and supplied with intermittent mist irrigation, which was automatically regulated by an 'electronic leaflet' solenoid valve system. Two time periods were investigated for this process, namely three days and five days of misthouse treatment. The plants were then placed in a greenhouse. This regime proved successful for the hardening-off *T. garganica* plants (MAKUNGA *et al.*, 2003), albeit at low incidence (52 %). However, the time period required in the misthouse was not a parameter previously tested.

d) The plantlets were removed from culture bottles and incubated in 1% Benlate (w/v) for 30 min. A fungicide cocktail was used as a pre-treatment to protect plantlets against fungi associated with damping-off diseases. The anti-fungal combination was prepared by dissolving 2.2 g Dithane M-45 and 1 g Benlate in 1 l sterile distilled water. Kelpak and Folio Gold (500 g l^{-1} chlorothalonil and 37.5 g l^{-1} metalaxyl active ingredients), 2 ml and 4 ml, respectively, were added to the antifungal cocktail and thereafter, the solution was shaken thoroughly to mix. This solution was used to drench the soil mixture, that had previously been autoclaved and allowed to cool. Subsequently, rooted or unrooted microcuttings were planted in the soil. Two types of soil mixtures were tested. The first mixture had potting soil plus vermiculite at a ratio of 3:1 (v:v). The second mixture contained compost: potting soil: vermiculite: bark (3:7:0.1:0.1; v:v:v:v), afterwards the soil was wetted thoroughly after planting with the anti-fungal treatment. Every four days for a six week period, the plants were sprayed with the cocktail. Every alternate spray, Benlate was excluded from the cocktail and replaced with 2 ml l^{-1} Rovral Flo. The other contents were kept the same. During spraying, the cocktail was agitated thoroughly to ensure mixing of anti-fungal compounds. The plantlets were monitored very closely and were watered daily by hand for four weeks. After one month the watering regime was reduced to twice a week.

e) The pre-transplanting treatment was the same as described above (Section 5.2.5d) except that the soil drench and fungicidal cocktail were changed. A soil drench combining Captab (dicarboximide 50% m/m; active ingredient) and Benlate was used instead. The roots (when available) were then dipped in Kelpak (1 %) before planting out, otherwise shoot bases were first treated with Seradix® B No. 1 powder (IBA, 1g kg^{-1}; Maybaker Agrichem,

South Africa) to encourage rooting. Afterwards, the microplants were transferred to the misthouse and then to the greenhouse after three days. While the plantlets were acclimatizing to conditions out of culture, they were given a foliar feed of 1 % Kelpak (v/v), once a week for four weeks. The plants were watered as described in Section 5.2.5.d. Once again, they were monitored closely *ex vitro*.

For all the above treatments, the soil mixtures tested were autoclaved prior to use to destroy pre-existing microorganisms. The potting soil was left to cool at room temperature and then it was transferred to pots and treated as specified in the above list. Control plants were not treated with anti-fungal cocktails. Instead, the fungicidal cocktail was replaced with water. When plants had not rooted, they were treated with Seradix rooting powder to encourage rooting *ex vitro*. The microplants were monitored closely for symptoms of damping-off diseases, once transferred to soil. The different hardening-off regimes were compared. The number of plantlets surviving *ex vitro* was recorded.

5.2.6 Statistical analysis

Significant differences resulting from different treatments were revealed after a one-way ANOVA test followed by a Tukey's multiple range test (Minitab Release 12.1 computer package). In most cases, twenty-five replicates were used per treatment and experiments were repeated at least twice. For exceptional cases, the number of replicates per experiment has been indicated (Section 5.2.3 and 5.2.5). The number of times the experiment was performed has also been described.

5.3. RESULTS AND DISCUSSION

Malformation of shoots due to hyperhydricity posed a problem with *T. garganica in vitro* culture (Figure 5.1). Cytokinikn supplementation of 5 mg l^{-1} in the medium in a factorial combination such as 1:5 NAA: BA resulted in abnormal shoots with typical features of hyperhydricity (Figure 5.1A-B). On occasion, shoots regenerated from the maintenance medium with a 0.5:1.5 NAA: BA combination became hyperhydric and as a result strategies to reduce the occurrence of atypical vitromorphologies were implemented. The apparent inherent ability of cultures to become hyperhydric on media that mostly promoted regeneration of healthy shoots (1:3 NAA: BA and 0.5:1.5 NAA: BA) (Figure 5.1C) became a concern during the study and as a result, necessitated attempts to eliminate the appearance of hyperhydric shoots.

Increasing the agar or Gelrite® concentration is one strategy that allows for elimination of the hyperhydric state, although often lowering the rate of propagation vegetatively *in vitro*. Other strategies involve the use of PEG and decreasing concentrations of MS salts to curb hyperhydricity. In some species PEG reduces the relative humidity and subsquently improves stomatal function (ZIV, 1991) and encourage wax deposition. Lowering MS salts reduces hyperhydration as this induces the plant to change from the heterotrophic state to the autotrophic phase (ZIV, 1991).

Table 5.1: The effect of the gelling agent on shoot multiplication and the morphological appearance of *T. garganica* shoots after subculturing

GELLING AGENT (mg l^{-1})	Frequency of shoot production (%)	Number of shoots/ per tube	Shoot length (cm)	Hyperhydric shoots (%)	Callus formation (%)	Anthocyanin production (%)
AGAR (8)						
0.5: 1.5 NAA: BA	100	8.3 a	8.9 a	15	35	10
1: 3 NAA: BA	100	10.2 a	7.6 a	25	40	24
GELRITE (2.5)						
0.5:1.5 NAA: BA	100	12.5 a	4.6 b	60	55	80
1: 3 NAA: BA	100	10.6 a	3.5 b	50	75	67

Treatments denoted by the same letter were not significantly different, P < 0.05. At least 25 replicates were used per treatment.

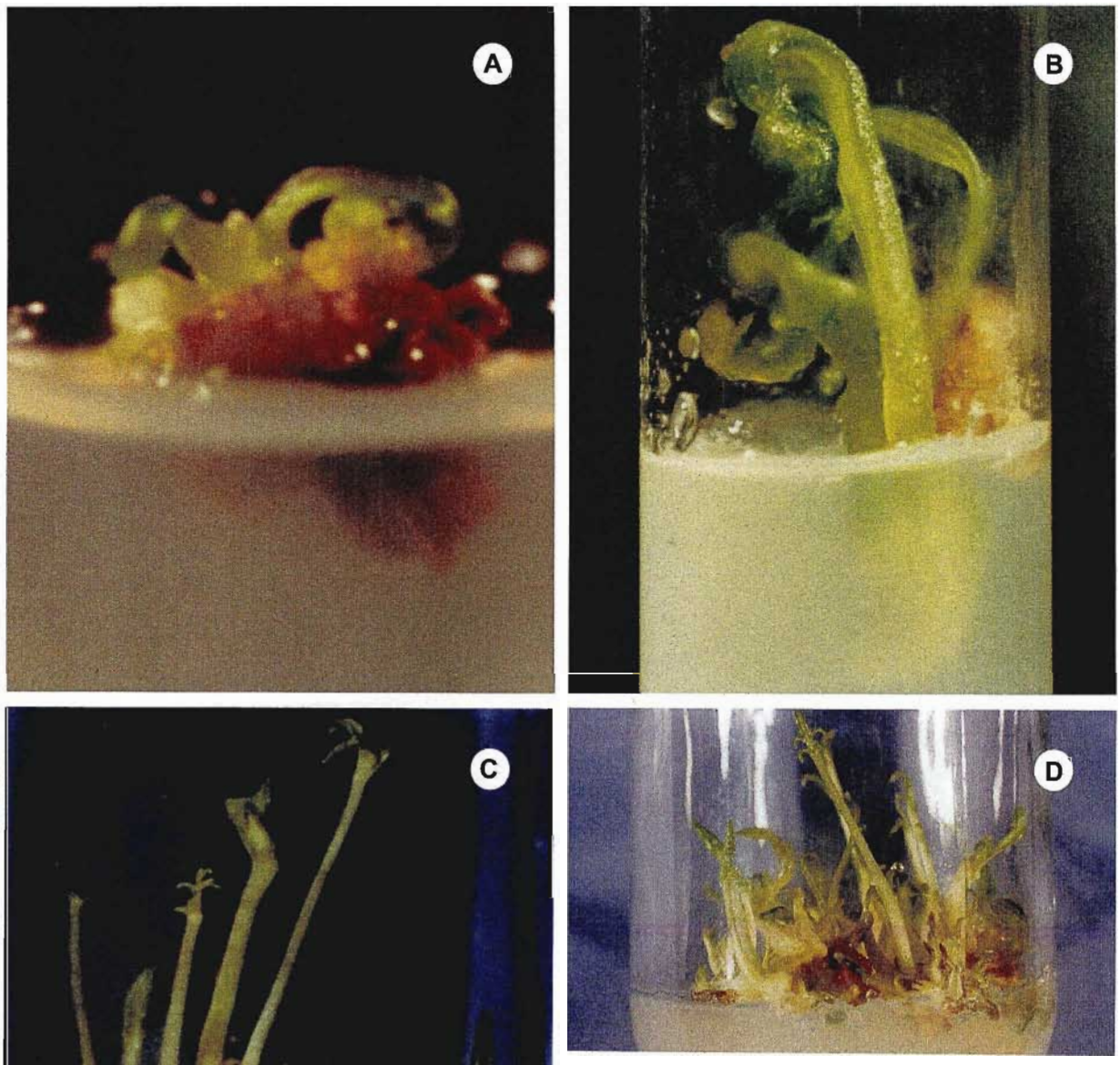


Figure 5.1: Phenotypic morphologies of *in vitro* plants of *T. garganica* L. on MS agar medium (0.8 %; w/v) (A) Induction of hyperhydric shoots (culture initiation, Stage 1). **(B)** Severely hyperhydric shoot on a 1: 5 NAA: BA combination. **(C)** At times, normal and hyperhydrated shoots proliferated on a 1: 3 NAA: BA medium. Red anthocyanin pigment production at plantlet bases accompanying prolific adventitious shoot growth. **(D)** Multiplication of hyperhydric and normal shoots on 0.5: 1.5 NAA: BA MS medium.

Table 5.1 shows the response of *T. garganica* propagules to different gelling agents. The use of agar as a gelling agent proved more beneficial as compared to Gelrite®. For example, the major features of the response, characterised on the 0.5:1.5 NAA: BA medium (solidified with Gelrite®), were prolific shoot induction and occurrence of hyperhydricity. Agar gave stronger gelling compared to Gelrite®, during the investigation. The agar-gelled medium was also less watery whereas accumulation of liquid was observed on Gelrite®-supplemented media. Moreover, towards the end of the growth cycle the medium became liquefied. The strength of the gellan gum requires to be increased for this problem to be solved as excessive water availability to the plantlets presumably increased the incidence of hyperhydricity with this gelling agent. Growth medium containing a combination of 0.5:1.5 NAA: BA solidified with agar had fewer hyperhydric shoots vs the 1:3 NAA: BA treatment. Multiplication was statistically similar on the 1:3 NAA: BA medium. However, five shoots per explant were observed on the agar medium with 0.5 mg l^{-1} NAA and 5 mg l^{-1} BA (Table 5.1). As with other species, agar displayed some protective properties against hyperhydricity and somaclonal variation in this study. This is highly desirable for the micropropagation of *T. garganica*. The production of anthocyanins in plant cells is often an indication of the existence of stress. These flavonoid compounds are induced under the influence of a variety of environmental stimuli including light irradiation, low temperature, UV light, phytohormones, fungal elicitors and low nutrient levels (MOL *et al.*, 1996). Gellan gum was therefore assumed to result in a microenvironment more stressful than agar, and *de novo* anthocyanin synthesis was regarded as an indication of stress experienced by the plantlets *in vitro*. Table 5.1, shows that anthocyanin production was recorded in 80 % (0.5:1.5 NAA: BA medium) and 67 % (0.5:1.5 NAA: BA medium) of the cultures growing on Gelrite®. Such stresses have been implicated in increasing the frequency of somaclonal variation in tissue culture and their avoidance is a prerequisite for successful *ex vitro* transfer (CASSELLS and CURRY, 2001).

Hyperhydration of callus also increased when Gelrite® was utilized as the gelling agent in the culture medium. The callus was also more brittle compared to the callus growing on agar-media and some cell clusters were red in colour due to the synthesis of anthocyanin pigments. Often agar is preferred for keeping

hyperhydricity in check in tissue cultures as Phytagel®/Gelrite® can encourage occurrence of the hyperhydric condition (THOMAS *et al.*, 2000). However, it is often worth determining the better gelling agent for a particular species during *in vitro* propagation as the onset of hyperhydricity resulting from the gelling agent appears to be genotype-dependent. For instance, tissue cultured watermelon became hyperhydric when gellan gum was substituted for agar in the growth medium (THOMAS *et al.*, 2000). Contrastingly, no hyperhydricity was observed with the use of gellan gum as a solidifying agent in grapes (THOMAS, 1997). Even though the use of gellan gum increases the occurrence of hyperhydric plantlets, it does have its advantages. According to THOMAS *et al.* (2000), it may be favoured for use in culture, if there are no other side effects, as it produces a clear solidified medium. The clarity of the medium makes it easier for plant tissue culture practitioners to visualize the roots produced *in vitro*, more especially when roots are being washed prior to transplanting. Less damage of the roots occurs when plantlets are being excised from a gellan gum-solidified medium. Cleaning of culture tubes and jars is also easier when gellan gum is utilized to gel the medium.

The supplementation of medium with PEG 6 000 as well as the effect of culture vessels with better ventilation on growth and quality of plants are shown in Table 5.2. Generally, fewer cultures produced callus when PEG was included in the medium. For instance, all the shoots derived from the 1:3 NAA: BA combination did not produce callus once transferred to medium supplemented with PEG when grown in culture jars that were plugged with a cotton wool bung (Table 5.2). In addition, there were fewer symptoms of hyperhydricity noted for PEG supplemented medium and/ or when culture jars with cotton wool plugs were employed. After one month, the plantlets growing in 2 % PEG and/or better ventilated vessels (Figure 5.2B-E) were healthier and of a better quality in comparison to the control plants (Figure 5.2A). The deeper colouration of plants (Figure 5.2B-D) was an indication of the increased accumulation of chlorophyll pigments. Although not measured, the increase in photosynthetic pigments was taken as a sign of more active photosynthesis in treated plantlets. Expectedly, PEG encouraged deposition of a waxy cuticle in cultured *T. garganica* (Figure 5.2D). This was a new result, not previously observed with the other treatments in

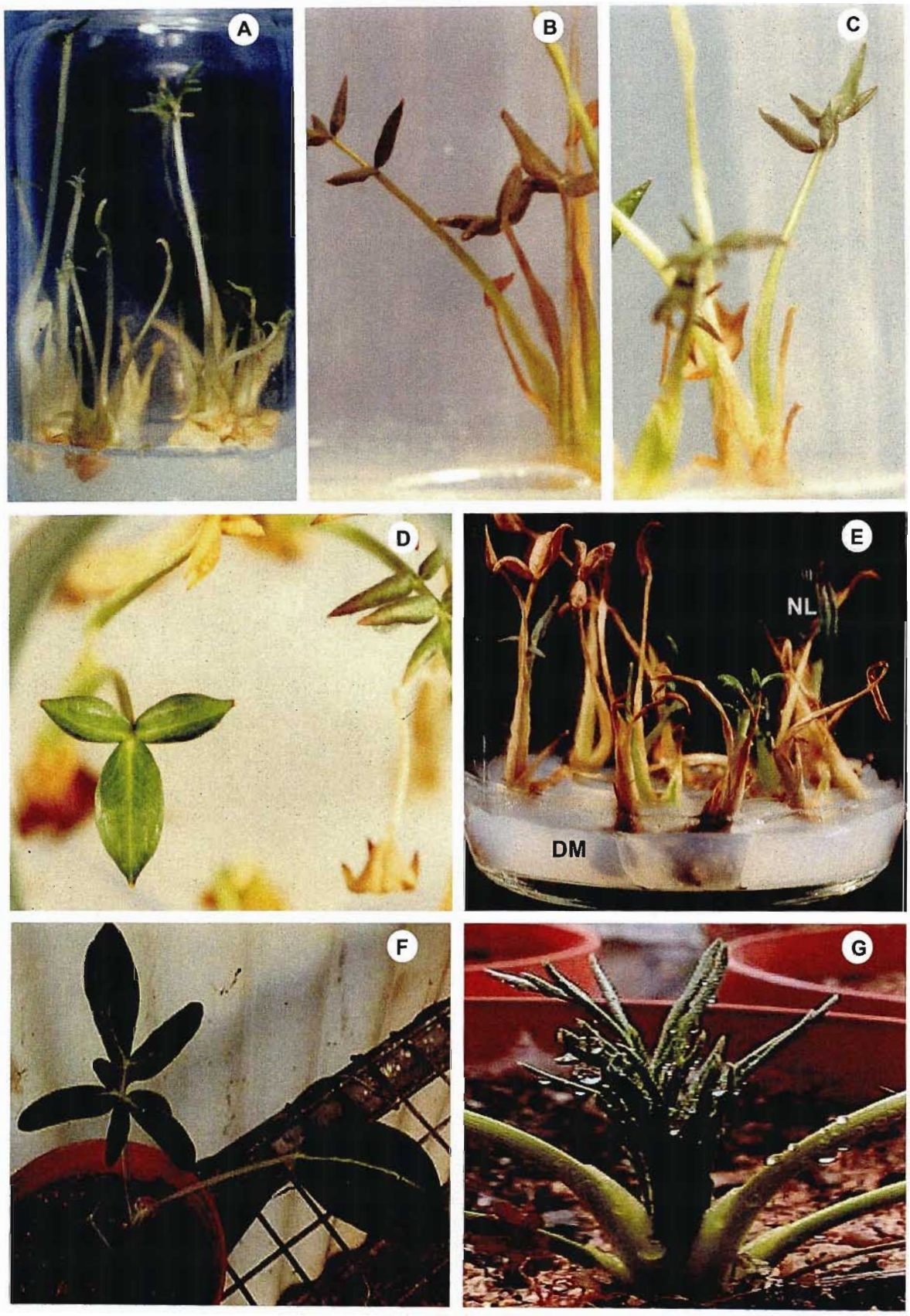
the micropropagation of this plant. The low deposition of surface wax, stomatal abnormalities and non-continuous deposition of a cuticle are disadvantages of the tissue culture of herbaceous species and in chrysanthemum, the addition of PEG increased production of epicuticular surface wax (SHORT *et al.*, 1987). DAMI and HUGHES (1997) reported that mortality rates of tissue culture grapes *ex vitro* were significantly reduced by the application of PEG in a study investigating the use of this polymer as a transplantation pre-treatment.

In spite of *T. garganica* plants being much darker in colour on the PEG-containing growth medium in this study, sometimes the leaf tips were slightly red showing anthocyanin production (Figure 5.2B and D. The use of vented lids plus PEG in the medium promoted production of anthocyanins in 50 % and 70 % when the medium contained 2.5 mg l^{-1} IBA and 5 mg l^{-1} IBA, respectively (Table 5.2). In addition, some of the older leaves had dried and eventually died after a month of treatment when the sucrose concentration was lowered to 0.3 % (Figure 5.2). The stringency of the treatment requires adjustment to discourage excessive desiccation of shoots. The use of PEG although beneficial for reducing the relative humidity in the culture vessel, can result in effects detrimental to further development in culture (SHORT *et al.*, 1987; LAINE *et al.*, 2000). Plants growing in the presence of this osmoticum can be severely restrained regarding shoot, leaf and root growth (SHORT *et al.*, 1987) as excessive dehydration of the medium results in the increased concentration of nutrients and minerals to levels that are, at times, toxic to the plant (MAENE and DEBERGH, 1987; WHISH *et al.*, 1992). It remains a possibility that *T. garganica* plantlets suffered in this way on medium with PEG as an additive (Figure 5.2E). It is thus advisable to exercise caution when applying PEG as an osmoticum to *T. garganica* shoots as cultures showed complete medium dehydration and desiccation of leaves at the end of one month (Figure 5.2E, DM). However, newly formed leaves on this medium appeared healthy(Figure 5.2E, NL). Their physiological condition requires inspection to ascertain whether the morphological appearance is comparable to their physiological state in the future.

Table 5.2: The effect of PEG and type of vessel closure in the presence of 0.5:1.5 NAA: BA or 1:3 NAA: BA in MS medium on plant growth, incidence of hyperhydricity, callus formation and anthocyanin production in *T. garganica* shoots during *in vitro* hardening

TREATMENT	Shoot length (cm)	Number of shoots derived per explant	Hyperhydric shoots (%)	Callus formation (%)	Anthocyanin production (%)
Explant source: 0.5: 1. 5 NAA: BA					
		NORMAL LIDS			
PGR-free medium	4.6	2	32	28	0
½ MS + PEG	5.5	1.6	4	24	0
½ MS + PEG + 2.5 IBA[a]	4.1	3	0	0	0
½ MS + PEG + 5 IBA [b]	2.4	2	0	0	0
½ MS + PEG+5 IBA + 0.3 % sucrose	3.5	2.6	0	0	0
		LIDS WITH A 1 CM HOLE			
PGR-free medium	5.7	1	8	0	0
½ MS + PEG	5	2	0	0	0
½ ms + PEG + 2.5 IBA	3.4	3	8	9	5
½ MS + PEG + 5 IBA	2.4	1.4	0	0	0
½ MS + PEG + 5 IBA + 0.3 % sucrose	3.5	2.6	0	0	10
Explant source: 1: 3 NAA: BA					
		NORMAL LIDS			
PGR-free medium	6.7	3	12	0	0
½ MS + PEG	5.9	1	0	0	0
½ MS + PEG + 2.5 IBA[a]	2.9	2	0	0	0
½ MS + PEG + 5 IBA [b]	6	3.2	20	25	0
½ MS + PEG + 5 IBA +0.3 % sucrose	2.8	1.3	0	0	0
		LIDS WITH A 1 CM HOLE			
PGR-free medium	4.6	3	0	0	0
½ MS + PEG	3.4	2	0	0	0
½ ms + PEG + 2.5 IBA	3.6	2	0	0	50
½ MS + PEG + 5 IBA	3.5	1.7	0	0	70
½ MS + PEG + 5 IBA + 0.3 % sucrose	5.4	1	.0	0	50

[a] Denotes the inclusion of IBA to the medium at a concentration of 2.5 mgl^{-1}
[b] Denotes the inclusion of IBA to the medium at a concentration of 5 mgl^{-1}
Treatments denoted by the same letter were not significantly different, $P < 0.05$


A
B
C
D
E
NL
DM
F
G

The inclusion of IBA to promote rooting on the medium with PEG as an adjuvant was not effective. The rooting was unreliable with only 12 % of plants rooted when a ½ MS medium supplemented with 2 % PEG, 5 mg l^{-1} and 0.3 % sucrose (w/v) was used. On ½ MS medium with 2 % PEG, 20 % of the microplants rooted (data not shown). Those plantlets rooting had strong well-formed roots but extraction of the propagules from the nutrient medium without damaging and pulling off the roots was difficult prior to explantation. Although such mechanisms, namely, PEG and use of better ventilated vessels, are effective in reducing the humidity in culture and allow for plants that are hardier for survival out of the culture environment, their effect on the medium can be detrimental to the plant (WHISH *et al.*, 1992) and such effects can increase stress-related defects. It is highly possible but speculative that some of these effects are then expressed as part of the stress response resulting in production of compounds such as anthocyanins. The production of anthocyanins during *in vitro* hardening was observed in *T. garganica* cultures. However, synthesis of anthocyanins by the Apiaceae (MOL *et al.*, 1996) is one of those processes in culture that appears to prevail with little encouragement or persuasion. It has been noted in carrot cell culture, for instance; and this system has been used as a model to study anthocyanin biosynthesis.

Highly dehydrated agar also inhibits root production *in vitro* (WHISH *et al.*, 1992). The severity of using PEG in the medium plus culture jars with better ventilation had major drawbacks as more visible signs of stress were noted (Table 5.2) such as anthocyanin production in leaf tissue, dehydration and resultant death of older leaves (Figure 5.2E). Therefore, it is advisable to keep the culture vessel closed with a normal lid if PEG is to be included in the medium rather than lids with cotton wool bungs. PEG proved an extremely effective osmoticum when normal screw caps were utilized as non-hyperhydric plantlets were obtained. The use of cotton wool plugged lids together with an osmoticum, in this case PEG, encouraged excessive dehydration of the medium during experimentation. In this study, the level of medium in the jar was rapidly desiccated (Figure 5.2E, DM) and edges of the medium shrunk away from the glass container within four days.In another study where improved aeration significantly reduced water condensation and resulted in

faster medium desiccation but growth was affected, the authors advocated frequent transfers to newly prepared medium (THOMAS *et al.*, 2000). This advice is in agreement with that of ZIV (1991). It is noteworthy that improved aeration facilitates higher transpiration associated with nutrient uptake of Ca^{2+} (THOMAS *et al.*, 2000) as well as encouraging gaseous exchange including the removal of ethylene which may enhance the hyperhydric state in cultured cells (ZIV, 1991). A compromise needs to be reached as ventilation should not sacrifice or hinder plant development.

Active photosynthesis is a major goal in the development of transplantable plants. *In vitro* foliage with poor photosynthetic activity hampers adaptation of leaves towards independence. Lowering sucrose levels in the hardening medium promotes active foliage development even if plants do not become fully autotrophic. CO_2 levels stimulates photoautotrophy due to CO_2 having an antagonistic effect to ethylene. This stabilizes lignin metabolism and contributes significantly to the prevention of hypolignification; abnormal cell wall and aerenchyma formation; and, hyperhydration. These listed events are features of abnormal morphogenesis in culture. Lowering the sucrose level in the medium without the addition of PEG was unfortunately not investigated in this study. Future studies should involve the determination of the activity of photosynthetic (PS) enzymes or photosynthesis using chlorophyll fluorescence when the carbohydrate concentration is being manipulated. This would then verify the extent of the dependency of cultured microplants on the carbohydrate source prior to transplanting. It would be of interest to investigate the anatomical changes and physiological changes encouraged by increased ventilation in micropropagated *T. garganica* as it had allowed for production of better quality plantlets.

It became apparent during the course of the project that the acclimatization of the Apiaceae is not always successful due to attack by pathogens, especially those responsible for fungal rot or wilt diseases. This is in agreement with several findings, as an example the acclimatization of *Ammi visgana* (EKIERT, 2000). These difficulties were also reported by TAWFIK and NOGA (2001) who found that generally the establishment of cumin and other herbaceous plants of the

Umbellifereae is low, an additional complication to the tissue culture of these plants for purposes of conservation. Similarly, fungal infections causing damping-off sabotaged the final stage of the clonal propagation of *T. garganica*.

The type of potting mix becomes of importance in the acclimatization of *in vitro* propagated umbelliferous plants as they are highly sensitive to water saturation (HUNUALT, 1984), such as fennel, cumin and *Thapsia*. A mixture with sand benefited *ex vitro* survival (*C. cyminum*; TAWFIK and NOGA, 2001). In the field cultivation of carrots, the management of fungal diseases involves thorough foliar spray coverage as soon as symptoms are visible and the compounds that are used for prevention and/or as a cure are chlorothalonil, mancozeb and iprodione (DOUGLAS and COWLEY, 2003).

Attempts to harden-off *T. garganica* microplants were mostly futile due to damping -off diseases. One hundred percent plantlet mortality was observed with rooted and non-rooted specimens and several transplanting regimes were tested. Numerous experiments were conducted, most of which were terminated due to the entire population becoming infected as a result of wilt diseases. Microcuttings were thus best hardened-off when the pretreatment included washing in a fungicidal agent prior to hardening-off. The plantlets could best be acclimatized once incubated in the misthouse for a maximum of three days, otherwise longer misthouse exposure resulted in greater losses in the greenhouse. Kelpak is extremely beneficial to their survival and encouraged development in the natural environment after *in vitro* culture (KOWALSKI *et al.*, 1999). The use of seaweed concentrates as foliar sprays or soil drench is becoming increasingly popular as they have a positive effect on plant growth after transplantation.

Larger pot size (10 cm) for root development appears to be essential in *T. garganica* as those plants that were hardened-off more readily acclimated to *ex vitro* conditions and were larger in size as compared to those that were planted in smaller pots (7.5 cm). The plants growing in the 10 cm pots behaved in the same manner as the stock plants in the greenhouse, dying back during the southern

African summer (November until February). Appearance of new leaves occurred from the modified stem on top of the root occurred in April. The morphology of the *in vitro*-derived plants became increasingly characteristic of the stock plants (Figure 5.2G) with the primary leaf splitting into a typically compound leaf of *T. garganica* (Figure 5.2F). The ultimate goal in micropropagation of plants is the successful establishment of not only acclimatized plants but those that acclimate to the external environment without apparent abnormalities resulting from the *in vitro* process.

5.4 CONCLUSION

A study solely dedicated to resolve problems associated with aberrant *in vitro* morphologies in tissue cultures of *T. garg*anica; and, subsequently eliminate such problems would thus plausibly provide a better understanding of the behaviour of this species in culture, especially regarding the occurrence of hyperhydricity. A more comprehensive investigation into the mechanisms governing the induction of this phenomenon is thus proposed and such a study should also assess factors that influence establishment of *in vitro* propagules in the greenhouse. It is envisaged that the use of quantitative markers, for example chlorophyll fluorescence to analyse the effectiveness of the meaning and validity to the results obtained. The parameters that require monitoring include chlorophyll fluorescence (afore mentioned), chlorophyll content and stomatal function. An assessment of the genome using molecular biological techniques would increase the knowledge with respect to the genetic mechanisms governing plantlet quality in *T. garganica* cultures.

It is worthy to mention that, in this Chapter the primary goal was never an attempt to elucidate the factors regulating the establishment of the hyperhydric state but rather to overcome the regeneration of hyperhydric plants for better post-culture performance in *T. garganica*. Therefore, the main purpose of the inquiry has been satisfied as better ventilation of culture vessels contributed to the quality assurance of *in vitro* propagated plants. Acclimatisation of *T. garganica* plants remains a major setback in the micropropagation of this species due to their susceptibility to

fungal rot. This study was able to identify the problem and a regime for weaning was successfully established. Future studies in this regard should focus on treatments recommended to improve plantlet quality in *T. garganica* would add identifying, at species level, the fungi that are pathogens of *Thapsia* plants for better disease management.

CHAPTER 6
DEVELOPING A PROTOCOL FOR EFFICIENT DELIVERY OF Ri TRANSGENES

6.1 INTRODUCTION

Breakthroughs in plant molecular biology and physiology; transformation technologies; and rapid advances in *in vitro* plant propagation have culminated in many transgenic plants and products of commercial value (GELVIN, 1998b). The development of efficient transformation techniques concomitant with improved tissue culture protocols for so-called recalcitrant species, namely, maize, cotton, barley, soybean and gymnosperms have revolutionised the introduction of exogenous DNA into plant cells (BIRCH, 1997). There are two classes of gene transfer methodologies, namely; 1) non-natural methods such as microprojectile transformation; direct DNA uptake into protoplasts using electroporation or chemical means, for example PEG-mediated transformation; microinjection which is not popularly utilised in plant transformation; and, 2) natural technologies including *Agrobacterium*-mediated transformation and transfer mediated via viral vectors (GELVIN, 1998b). The methods primarily used to achieve advances in gene transfer technologies for both dicotyledonous and monocotyledonous plants in the last two decades are *Agrobacterium* and particle bombardment. However, most monocotyledonous and certain species of dicotyledonous are still not receptive to *Agrobacterium*. The many years of scientific endeavour have culminated in improvement of tissue culture methodology, transformation techniques and recombinant DNA technology for the production of more efficient vectors for transformation (HANSEN and WRIGHT, 1999). Recently, success has been achieved with the transformation of monocotyledonous plants utilising the *Agrobacterium* gene transfer system (HIEI *et al.*, 2000).

BIRCH (1997) considered tissue culture a practical necessity as virtually all current transformation systems require it as a tool to achieve gene transfer, selection of transformed cell lines and for the regeneration of transformed plants. When developing a tissue culture regime with transformation in mind, it is most important to consider that a large number of regenerable cells need to be accessible to the gene transfer treatment. Secondly, these cells need to be able to retain their capacity for regeneration throughout preparation of target, as well proliferate once transformed. Appropriate selection procedures for transformed cells require optimisation. The development of micropropagation protocols that have a high multiplication frequency for a particular species can be deceiving as highly prolific tissues are not an indication of accessibility to gene transfer. In addition, if the capacity for efficient regeneration is short-lived it becomes limiting with regard to the recovery of transgenics even if the target tissue is highly regenerable. The choice of explant for transformation is often governed by the ready-availability of the plant material and the minimum time required during the tissue culture phase for successful regeneration without the introduction of random genetic change resulting from somaclonal variation. Tissue culture regimes tending to introduce somaclonal variation need to be avoided as expression of the transgene may be negatively affected. The development of transformation protocols with minimal use of tissue culture or with complete avoidance of an *in vitro* culture phase have become desirable and sought after. It is now possible to dispense with micropropagation for target tissue preparation and genetic transformation without an intervening *in vitro* regeneration stage is the focus for an increasing number of laboratories. Techniques such as *in planta* transformation (BECHTOLD *et al.*, 1993) are applicable to plants without passage of the transformants in tissue culture but their application has mainly been limited to transformation of *Arabidopsis*. In the recent past, *in planta* transformation using vacuum infiltration to introduce transgenes has been extended to other species such as soybean. However, tissue culture does still hold merit as it remains a reliable means of efficiently generating genetically modified plants (BIRCH, 1997) within a short space of time (JAIN *et al.*, 1992). The development of efficient tissue culture protocols for transformation does not guarantee the regeneration of regenerable cells even in the hands of a most competent tissue culturist due to the greater

complexity of *Agrobacterium*–plant interaction during genetic transformation in comparison to direct gene delivery methods (BIRCH, 1997).

Most gene transfers have been accomplished using *Agrobacterium* as a vehicle to deliver transgenes. For historically recalcitrant species, direct methods of transformation were developed for successful transformation (JAIN *et al.*, 1992). Certain criteria need to be met when developing a practical gene transfer system for a particular species and these include 1) genotype-independent transformation; 2) recovery of a large number of transgenic plants for analysis of gene expression; and, 3) minimal time in culture to circumvent potential somaclonal variation. These are general criteria and more specifically for *Agrobacterium* transformation it is considered more efficient to optimise the conditions for gene transfer and thereafter, develop a system for regeneration of the transformed cells. However, with microparticle bombardment as a tool for gene transfer, it is considered best to establish a micropropagation protocol first prior to developing the optimal conditions for direct gene transfer. The essential requirements for efficient regeneration include the determination of regenerable cells and their location as well as the optimisation of the tissue culture protocol in order to increase the number of cells accessible for transformation (JAIN *et al.*, 1992).

6.1.2 Microparticle bombardment

Microprojectile bombardment involves the delivery of biologically active DNA by employing high-velocity metal particles, gold or tungsten, into plant cells and which subsequently can be regenerated into whole plants. Of important consideration for successful gene transfer, are the momentum of the particles, impact distribution and the DNA-carrying capacity (KLEIN, 1995). The amount of vacuum, macrocarrier distance to the stopping plate and the distance between the stopping plate; and the target tissues controls the velocity of the microcarriers for each bombardment. This in turn affects the trauma induced on the target tissue due to the transformation event after bombardment. The biological parameters to consider for particle gene transfer are the choice of target tissue, the exposed cell surface area, the state of the cells regarding active cellular division and the osmotic

conditioning which controls turgor pressure of the cells to be transformed. Growth of cells in conditions of lowered turgor pressure before particle bombardment seemingly increases the survival of cells after the bombardment event. The osmotic pre-treatment usually involves air-drying of tissues using laminar flow or the inclusion of an osmoticum in the medium (HANSEN and WRIGHT, 1999), such as mannitol or sorbitol. Optimisation of these conditions for transformation ensures minimum variation in the transformants and more efficient transformation.

Particle bombardment and other indirect transformation methods have several disadvantages. The achievements accomplished with the employment of biolistic transfer for the modification of plants revealed that this method results in high copy number integration of the transgene (GELVIN, 1998b) and in complex integration patterns revealing catenation and rearrangement of transgenes. This is one of the major disadvantages of this technique as transgene expression is often affected due to homology-dependent suppression, also termed transgene silencing (GELVIN, 1998b; HANSEN and WRIGHT, 1999). The primary advantage of this method of transfer is the delivery of exogenous DNA to plant cells that is genotype and tissue independent (FINER *et al.*, 1999) as compared to *Agrobacterium*-mediated transfer. However, it is inefficient as few cells are stably transformed. One must bear in mind that transformation of recalcitrant species such as rice, has shown that responses to particle transfer are at times varied among genotypes. Some cultivars respond by producing more cell clusters that are potentially transformed than others. The damage to tissues as a result of bombardment is also an issue of concern as this often hinders and limits the regenerability of putatively transformed cells. The severity of tissue damage may also be correlated with the type of instrument utilised as the old-fashioned model resembling the original version - the gun powder biolistics gene gun, are more likely to aggravate cellular injury (CHRISTOU, 1997). Apart from potential cellular damage, ethylene is one of the sides effects of particle transfer and uncontrolled accumulation of this gas decreases the expression of introduced transgenes (KLEIN, 1995). *Agrobacterium* transformation, on the other hand, is more efficient with respect to low copy integration of non-rearranged transgenes that become stably incorporated into the plant genome. Although much of the limitations of

Agrobacterium transfer have been greatly reduced since the recovery of the first group of transgenic plants, host range barriers still exist. The *Agrobacterium* transformation system is further complicated by the requirement for efficient wounding of explants and the elimination of *Agrobacterium* from infected tissues (FINER *et al.*, 1999).

6.1.3 Combining direct and indirect methods for improved gene transfer

The combination of the positive attributes of *Agrobacterium* and biolistic transformation is gaining popularity as a means of transformation (GELVIN, 1998b). The microparticle bombardment of tissues prior to *Agrobacterium* transformation may be employed for the enhancement of the frequency of genetic transformation. Particle bombardment results in microwounds in the target tissue and is thus a more efficient method of wounding the target tissue for *Agrobacterium* infection (JAIN *et al.*, 1992). In this thesis this approach has been termed 'Biolistics-assisted *Agrobacterium* transformation' or BAAT. Another approach which is proving very useful and has a potential to gain popularity is 'Agrolistic' transformation where the T-DNA genes, *vir* D1 and *vir* D2, are bombarded on a separate plasmid from the gene of interest into the plant . The integration of these genes including the transgene is at a low copy number compared to when the gene of interest is bombarded when cloned on a plasmid with just T-DNA borders in the absence of *vir* genes responsible for nicking the T-DNA borders. It seems that this approach also results in the integration of the gene of interest without the incorporation of the plasmid vector backbone – a side effect of the gene transfer process (GELVIN, 1998b).

TRICK and FINER (1997) described a method based on *Agrobacterium* transformation technology called sonication-assisted *Agrobacterium*-mediated transformation (SAAT) that induces fissures and channels throughout the sonicated target tissues. A variety of explants were tested and even the more recalcitrant taxa become more compliant to *Agrobacterium* transformation. The success of this technique was ascribed to micropockets allowing continued bacterial growth within, bringing the pathogen in closer proximity to the target cells

without causing deleterious effects that would impact negatively on the regeneration potential of the plant after the transformation event as a result of wounding. Agro-infiltration, as termed by PAGE and ANGELL (2002) involves the use of vacuum infiltration to draw agrobacteria into plant cells. This method is gaining popularity for transformation of plant cells that are difficult to transform via *Agrobacterium* using conventional procedures.

6.1.4 Typical laboratory methods for transformation using *Agrobacterium* and biolistic transfer

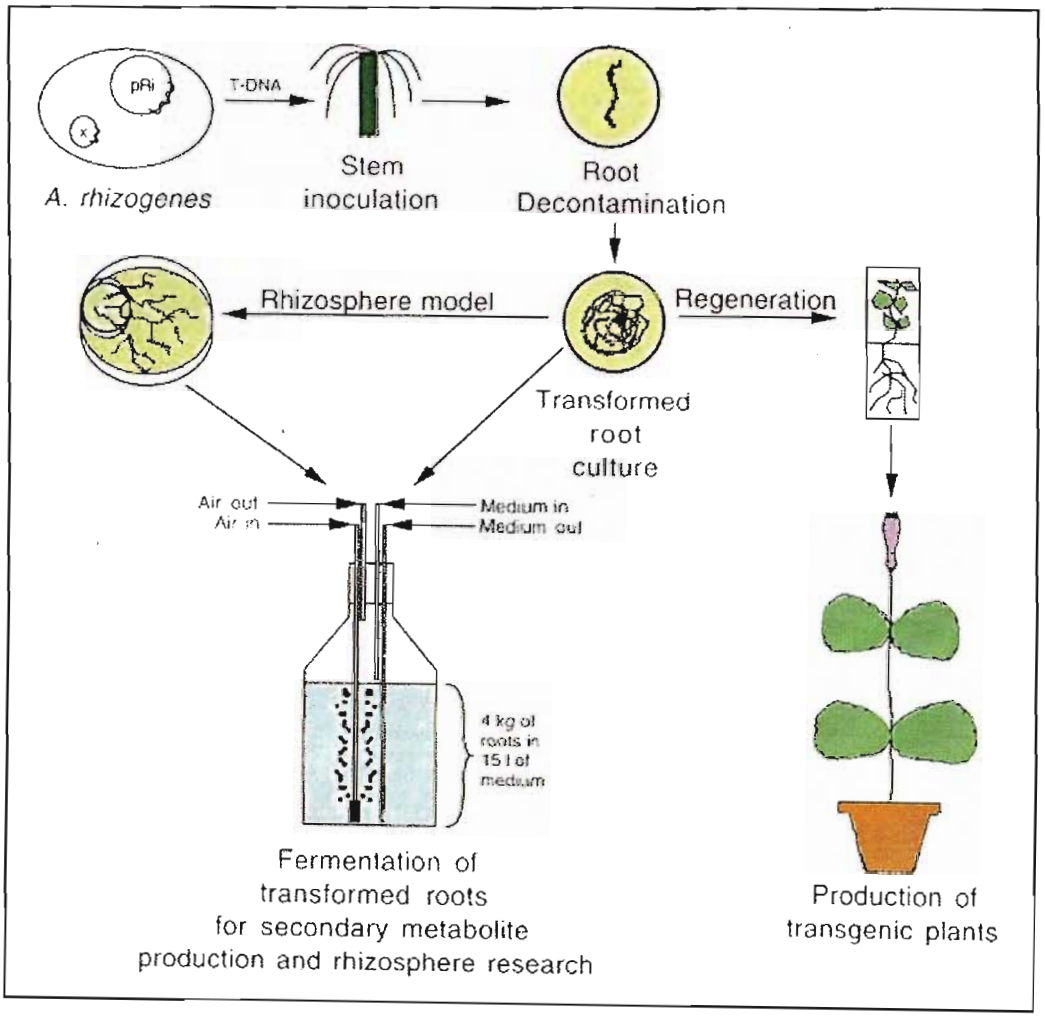


Figure 6.1: Schematic diagram of the transformation of plant cells mediated via *Agrobacterium rhizogenes* with or without the binary plasmid induces formation of plant roots. When these roots are excised and decontaminated of bacteria using antibiotics, they may be utilised for secondary metabolite production. These roots are also useful as models for rhizosphere studies and the regeneration of plants with traits characteristic of the hairy root phenotype.

In nature, *Agrobacterium* infects plants that are susceptible to it through wound sites. In the laboratory, *in vitro* genetic transformation attempts to mimic the phases of transformation that occur naturally as closely as possible, except that in a controlled *in vitro* environment. The basic protocol (Figure 6.1) involves the inoculation of the explants with the *Agrobacterium* donor strain and after a period of *Agrobacterium* and explant co-cultivation, the growth of the bacterium is inhibited by bacteriostatic or bacteriocidal action of an antibiotic such as ampicillin or cefotaxime. The inhibition of bacterial cell wall synthesis resultant from impaired peptidoglycan cross-linking is the main cause of those bacteria that are due to sensitivity to ampicillin dying (AUSUBEL *et al.*, 1988).

After selection procedures, the explants are induced to regenerate in culture and assayed firstly for transient gene expression of the introduced gene followed by analysis for stable integration using molecular techniques such as the polymerase chain reaction (PCR) (HAMILL *et al.*, 1991; BHAT and SRINIVASAN, 2002) and Southern blotting (SOUTHERN, 1975; BHAT and SRINIVASAN, 2002) to detect stable incorporation and as well as the number of copies that are inserted into the genome. Expression of the transgene may be studied using Northern blot analysis or reverse transcriptase polymerase chain reaction (RT-PCR). Transformed cells are often capable of growing in the presence of kanamycin as the gene encoding for kanamycin resistance is often included as part of the gene construct in engineered strains of *Agrobacterium*. Transformation with the root-inducing genes of the Ri plasmid not only results in extensive rhizogenesis but also the formation of hairy roots on transformed tissue is accompanied by active expression of opine synthesising genes. Thereafter, secretion of these opines into the intracellular region of the hairy root rhizosphere serves as a source of nitrogen and carbon for the agrobacteria (GRIERSON and COVEY, 1988). The plant, on the other hand, is unable to catabolise these compounds; indicating that the bacterium colonises the plant solely for its own benefit. The T-DNA encodes for type-specific opine synthesis and the genes for opine catabolism are harboured on another region of the Ri plasmid. Hence, *Agrobacterium* strains and their Ri plasmids are often classified according to the type of opine that is synthesised (Table 6.1) (MACRAE, 1994).

Of the direct gene transfer technologies, particle bombardment has proven the most widely utilised method due to the successful transformation achieved with it in species that are less receptive to gene transfer as mediated by *Agrobacterium*, which are consequently classified as difficult to transform. This method involves the direct delivery of DNA into intact cells and tissues via DNA-coated microparticles. The negatively charged DNA is precipitated onto tungsten or gold particles (0.5 - 5 µm) and subsequently the DNA-coated particles are accelerated in a particle gun with the starting energy of expanding highly compressed gas to velocities that allow for them to penetrate the target tissue without causing excessive damage destroying the cells. Coating procedures usually involve the addition of spermidine and calcium salts to the microparticle/DNA suspension. Significant improvements of DNA precipitation are needed for optimisation of the particle gene transfer technique (HANSEN and WRIGHT, 1999). The features that have enabled for the optimisation or improvements in biolistic transfer also include the proper handling of explant materials with respect to preculture practices and the use of baffling screens at the time of biolistic transfer. The DNA is released within the plant cell and then transiently or actively integrated into the plant genome. Nitrogen gas or helium released by the electric valve and partial vacuum is used to propel the macroprojectile or bullet with loaded DNA microcarriers. The travelling bullet, once shot, is then stopped by the stopping plate after a short acceleration and the DNA coated particles are released from the macrocarrier. These continue to travel towards the target cells and eventually transform them subsequent to penetrating cell walls and cell membranes.

6.1.5 Overcoming host range incompatibilities of *Agrobacterium*

Numerous strains of *Agrobacterium* were isolated from the wild and out of these naturally occurring strains, few have been modified to function as plant transformation vectors. The nopaline strains C58, are one of the few that are widely used for co-cultivation with explants (GELVIN, 2003). *Agrobacterium* host range is affected by several factors with the Ti/Ri plasmid; T-DNA region and the *vir* gene operon in particular, primarily determine specificity for plant genotypes and tissue types. It appears that host range of a limited host range strain may be

extended by transferring the TL-DNA from a wide host range strain or the oncogenes which are encoded on the TR-DNA of the Ri plasmid into it. As the exact interaction of the bacterium and the plant cell remains not clearly understood, it is still necessary to screen a series of *Agrobacterium* strains on a range of plant genotypes and/or tissues for the most efficient virulent strain/genotype combination (BAUER *et al.*, 2002), when the ultimate goal is the production of transgenic plants. The strains that were used in initial transformation experiments were created by inactivation or removal of oncogenes. The co-integration system of transformation (GRIERSON and COVEY, 1988) was then employed and this involved the incorporation of selectable and chimaeric constructs into the Ti plasmid by recombination between homologous regions in a suicide vector and the T-DNA (REMPEL and NELSON, 1995). The binary vector system as an alternative system was established later for use (GRIERSON and COVEY, 1988; for a review also consult GELVIN, 2003). The development of binary vectors such as pBI121 (JEFFERSON *et al.*, 1987) was based on the knowledge that the 25 bp repeat borders flanking the T-DNA are essential for the transfer of any segment of DNA which they enclose, provided that the *vir* genes controlling the transfer capabilities of the T-DNA to the plant are acting in *trans*. The greatest advantage of the application of binary vectors as vehicles of transformation is that they facilitate the cloning of any gene of interest between the 25 bp repeat T-DNA borders. The gene of interest thus replaces the sequences causing oncogenic expression.

Although, particle bombardment is very effective in transforming challenging species, it still remains a primary goal for many practitioners of transgenic technology to use agrobacteria and increase compatibility for a wide range of species as this system of transformation offers several advantages. According to REMPEL and NELSON (1995), the advantages of *Agrobacterium*-mediated gene transfer include low copy insertions and integration into areas that are transcriptionally active in the chromosome and the production of fewer chimaeric insertion patterns. To broaden the host range and increase T-DNA efficiency regarding stable transformation, the oncogenic wild-type strains were reassessed. Modification in the strains utilised include the development of a p35SGUS-INT gene construct which contains a eukaryotic intron which is solely spliced in plant

cells for the expression of GUS. This intron cannot be spliced out of RNA transcripts by prokaryotic cells (REMPEL and NELSON, 1995) and as a result decreases the number of false positives that are obtained when performing GUS analysis.

There are several factors that have been hypothesised to limit the ability of *Agrobacterium* strains in transforming so-called recalcitrant plants and these include 1) inability of agrobacteria to effectively bind to plant cell walls; 2) reduced activity of the T-DNA promoters; 3) incorrect auxin-cytokinin balance in plant tissues that is conducive to transformation; and, 4) a wound response that is not sufficiently pronounced for the highly sensitive chemotaxis system and induction of *vir* activity of *Agrobacterium* (MACRAE, 1994). Successful infection of plants by *Agrobacterium* also requires a compatible reaction between the host and a particular bacterial strain. It is thus still regarded as important to test for strain-cultivar compatibility during the establishment of a transformation regime. The transformation of *T. garganica* to the best of our knowledge, has not been comprehensively studied, except for a study by JÄGER (1993). This preliminary study utilised leaf material as the target for gene transfer. As the study's main focus was the production of an organogenetic system for *in vitro* synthesis of thapsigargins the number of trials was minimal regarding *Agrobacterium* transformation *in vitro*.

Thus, the aim of the present investigation was to devise a regime for the transfer of Ri genes from the T-DNA into *T. garganica* for the production of hairy roots and thereby examine a variety of methods for the delivery of *rol* transgenes. Several factors were evaluated including the establishment of *T. garganica* as a compatible host for *Agrobacterium* and an assessment of the most infective *Agrobacterium* strain(s) for transformation. Upon transformation, we aimed at establishing transgenic *T. garganica*.

6.2 MATERIALS AND METHODS

6.2.1 Chemical reagents and enzymes

All chemicals were of the highest quality available. Generally, inorganic salts were purchased from BDH (Poole, England) and Merck (Darmstadt). Agar for bacterial media, tryptone and yeast extract were purchased from Oxoid Ltd. (Basingstoke, England). Agar used in gelling the tissue culture media was purchased from (Associated Chemical Enterprises, South Africa) and agarose for gel electrophoresis was from Hispanagar D1-LE, Burga, Spain. Unless otherwise specified, all enzymes, deoxynucleotides and antibiotics were bought from Roche Biochemicals and were used according to the instructions of the manufacturer, unless otherwise stated. For all extraction procedures requiring phenol, a molecular biology grade of phenol was purchased from Sigma®, St Louis, USA. The phenol was buffer-saturated with TE buffer (10mM Tris-HCl [pH 8], 1 mM EDTA, pH 10.5). The phenol phase was at a pH of 6.7- 6.9. Ethanol (SeccoSolv®, max 0.025 H_2O) for DNA precipitation procedures was purchased from Merck (Darmstadt). Fungal and bacterial pathogens as well as DNases were destroyed from all molecular biology media, reagents and solutions by autoclaving. Solutions which were not decontaminated by autoclaving were filter-sterilised, and such cases are clearly specified.

6.2.2 Plasmids and bacterial strains

Three types of growth medium were utilised to grow the different strains of *Agrobacterium*, YMA (5 g l^{-1} yeast, 0.5 g l^{-1} casein hydrolysate, 8 g l^{-1} mannitol, 2 g l^{-1} ammonium sulphate, 5 g l^{-1} NaCl; pH 6.6); YMB (5 g l^{-1} yeast extract, 10 g l^{-1} mannitol, 0.1 g l^{-1} $MgSO_4$, KH_2PO_4, pH 7.0) or LB (5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 10 g l^{-1} NaCl, pH 7.0). For solid bacterial cultures, agar was added to the different media at 15 g l^{-1} to solidify the medium. Both wildtype strains and genetically modified *A. rhizogenes* strains were tested for transformation (Table 6.1). The recombinant vectors had the CaMV35S RNA polymerase promoter to drive transcription of the *gus A* gene. This is a constitutively expressed promoter

that directs strong expression of the transgene at high frequency for the selection of transformants (HIEI *et al.*, 1997). In most instances the nopaline synthase (*nos*) promoter drove the expression of the *nptII* gene.

Several strains (LBA9402, R 1600 Nester, R 1601 Nester, TR 8/3 Wits, A4 Tempé) were obtained from Dr S. Macrae in 1994 and were kept in culture by Dr A.K. Jäger until the commencement of this particular project. The other strains used in this investigation were donated by other researchers in the field. The A4 and A4T strains were a kind donation from Dr I. Puddephat (HRI, United Kingdom) and Dr P. Lemaux (University of California, Berkeley) was instrumental in the request and acquisition of these particular vectors. The R1601 strains were kindly provided by Dr K-H Han from Kumho, Korea, upon request. All the bacterial strains were sent as stab cultures and upon arrival were plated on YMA agar medium using three-way dilution streaking (AUSUBEL *et al.*, 1988), except for the *A. rhizogenes* LBA9402 which was best maintained on YMB medium. The growth of this particular strain was very poor after a long time in culture and a replacement strain was donated by Dr D. Tepfer (INRA, Versailles, France), upon request. Dr I. Puddephat also donated a strain of LBA9402 for utilisation in this project and this strain was cryopreserved and kept frozen in case the strain donated by Dr D. Tepfer lost its viability.

All strains were grown at 28 °C in an incubator until the appearance of single colonies on agar-solidified bacterial medium. Afterwards, a single colony from each of the different plates was inoculated in liquid medium (Table 6.1) and grown at 28 °C. The liquid cultures were shaken at approximately 60 rpm in a rotary shaker. Two sets of cultures were prepared for each recombinant strain. The one set of cultures was grown without antibiotics whereas the other set was grown in rifampicin (100 mg l^{-1}) and kanamycin (150 mg l^{-1}), where appropriate. The strains were grown until they reached log phase or a point of saturation and 800 µl of the culture was cryopreserved. The freezer stocks of all bacterial strains were prepared by adding the 800 µl of bacterial suspension into 800 µl cryopreservation glycerol solution (65 % glycerol [v/v], 0.1 M $MgSO_4$, 0.025 M Tris [HCl; pH 8.0], which had been pipetted into a 1 ml cryovial, to make up a 1:1 (v/v) mixture of a

logarithmic-phase culture in *Agrobacterium* growth medium with appropriate antibiotics. The vials were then cryopreserved in a -70 °C freezer until further use.

6.2.3 Pre-induction of *Agrobacterium* strains

Table 6.1: *A. rhizogenes* and *A. tumefaciens* strains tested for their inductive ability pertaining to hairy roots

Strain	Relevant Characteristics	Source	Growth Medium	Opine type synthesised	[b]Ri transformation of tobacco
A. rhizogenes					
LBA9402	Ri plasmid		YMB; rif$^+$	Agropine	+++
TR8/3 [a]	Ri plasmid		YMA		+
R 1600	Ri plasmid	E. Nester	YMA		+
R 1601	Ri plasmid	E. Nester;	YMA		+
A4R	Ri plasmid	D. Tepfer;	YMA	Agropine	++
A4	Ri plasmid	I. Puddephat	YMA	Agropine	++
A4T	Ri plasmid	I. Puddephat			+++
C58C1 pGUS1	Ri plasmid (pRiA4) with binary vector pLTCgus1	J. Berlin	YMA or YMB with rif$^+$, kan$^+$		+++
A. tumefaciens					
	pMRKE15 with ORFS 10,11, 12 encoding rol A, rolB, rol C	D. Tepfer	YMA		ND
	pMRKE15 with ORFS 1a0,11,12	D. Tepfer	YMA		ND

[a] Strains written in blue indicate that were passed on from Dr S. Macrae to Dr A.K. Jäger and maintained in culture by subculture monthly onto YEB () at 10 °C

[b] The strains were tested against tobacco explants to test whether they were infective after long-term culture. Those that were requested from other laboratories were also evaluated for their infectivity against tobacco on arrival. Their effect on tobacco transformation for hairy root induction was rated as +++, ++, + and –, denoting strong, intermediate, weak and no response, respectively

kan$^+$; kanamycin was added to the medium, rif$^+$; rifampicin was added to the medium; ND, not determined

All the *Agrobacterium* strains used for transformation were pre-induced by acetosyringone before co-cultivation with plant tissues. A fresh bacterial colony was picked after growing as a solid culture and grown at 28 °C and fresh liquid medium (YMA or YMB), depending on the strain that was being inoculated, it was pipetted from a stock solution which was previously prepared by dissolving the rifampicin crystals in methanol and was stored at -20 °C, for no longer than one week. After inoculation, the bacterial strains for the transformation were allowed to grow to mid-logarithmic phase in the various growth media (Table 6.1) containing 150 mg l^{-1} rifampicin and 20 µM acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone, Sigma), plus 100 mg l^{-1} kanamycin, where appropriate. The acetosyringone was added to enhance gene expression of the *vir* operon and thereby increase the probability for genetic transformation. Several methods for the transformation of various explants of *T. garganica* were evaluated. These are described in Section 6.2.5.

(i) Optimisation of the acetosyringone concentration

An experiment was included to determine the optimal concentration of acetosyringone to positively influence *Agrobacterium* transformation of *T. garganica.* The concentrations of acetosyringone tested were 20 µM, 50 µM, 100 µM and 200 µM. The transformation experiments were performed as described in Section 6.2.5i on leaf material as the explant, except that the acetosyringone concentration in the bacterial growth medium was manipulated. Control cultures were grown in medium without acetosyringone.

6.2.4 Decontamination of explant after co-cultivation

After co-cultivation, explants were washed by agitation in liquid MS with 500 mg l^{-1} cefotaxime (Claforan, Roussel Pharmaceuticals) to remove the bacteria and subsequently inhibit further bacterial growth. Three to four changes of washing solution were performed or else, the explants were washed until the washing solution was no longer turbid in appearance but clear. Thereafter, the explants were blot-dried and transferred to growth medium with 500 mg l^{-1} cefotaxime and maintained on it for two subcultures to prevent bacterial contamination. Selection

of putative transformants was often performed at this stage on kanamycin-supplemented medium (Section 7.2.1).

6.2.5 *Agrobacterium*-mediated methods of transformation: identifying a suitable explant

(i) Transformation of in vivo *plants*

Plant material was surface decontaminated as previously described in Section 4.2.1 (Chapter 4) and cultured on MS medium with 30 g l^{-1} sucrose, 0.1 mg l^{-1} myo-inositol, 0.5 mg l^{-1} NAA, 1.5 mg l^{-1} BA and 8 g l^{-1} agar. Stem and leaf explants were excised (3 cm) and placed on the plant culture medium, incubated at 22 - 24 °C in a growth room fitted with cool-white fluorescent light providing fluence at 38 µmol m^{-2} s^{-1}. The cultures were left for one week in the growth chamber in order to check the amount of contaminated tissues prior to transformation. Those tissues that were infected by bacterial or fungal pathogens were discarded as well as tissues damaged by the decontamination process using commercial bleach. A fresh culture of *Agrobacterium* was utilised for the transformation. The culture was poured into centrifuge tubes under laminar flow conditions and centrifuged at 5 000 rpm in a Sorvall superspeed centrifuge using a SS-34 rotor for 10 min. The pellet was resuspended in 15 ml growth medium without antibiotics after the supernatant was discarded. The centrifugation was repeated to remove the culture broth containing antibiotics and the *Agrobacterium* pellet was diluted in YMA or YMB (for LBA9402) to an A_{600} of 1 (2 x 10^9 ml). A microfine insulin syringe (Becton-Dickinson and Co., Ireland) (1 ml) was filled with the bacterial suspension and then incisions or pin-pricks were made on the week-old petiole and leaflet explants. Vascular tissues of the petiole explant and the leaflet midrib were generally infected as transformation generally occurs in the vasculature and these cells were previously shown to be regenerative (Chapter 3; Section 3.3.1). At times, the tissue was wounded with a needle free of *Agrobacterium* and thereafter immersed in the diluted agrobacteria for 30 min. The explant was then removed from the agrobacterial suspension and the excess liquid blotted-off

on sterile filter paper to prevent excessive agrobacteria growth during co-culture.

Following the infection process, 10 explants were placed onto solidified MS medium with 30 g l^{-1} sucrose, 0.1 mg l^{-1} myo-inositol, 8 g l^{-1} agar plus 20 µM acetosyringone but without antibiotics. Twenty Petri dishes were prepared for each strain and the experiments were repeated three times. The explants were co-cultivated with the bacteria for 36 h at 22 - 24 °C in the dark. After this time, slight bacterial growth around the edges of the explant was evident.

(ii) Transformation of* in vitro *plantlets

Cells of agrobacteria were grown for one day at 25 °C and transferred to a 19 °C rotary shaker and their growth was continued at the lower temperature again for another day or until mid-log phase in liquid-shake culture. These cells were then utilised to infect *in vitro* derived plantlets of *T. garganica*. The antibiotics were removed from the agrobacterial suspension as described in Section 6.2.3i by centrifugation. Micropropagated plantlets were cut into stems, leaves and shoot bases. Thereafter, they were incubated for 30 min in the agrobacterial suspension. The excess liquid was removed by blotting on filter paper and afterwards the explants were transferred onto acetosyringone-supplemented (20 µM) co-cultivation medium with a 1:3 NAA: BA PGR combination and co-cultivated for three days at 19 °C. After three days of co-cultivation, a bacterial halo was generally noted on the explants, they were washed three times with sterile distilled water containing cefotaxime (250 mg l^{-1}), as previously described (Section 6.2.4). The tissues were placed on growth medium after the excess washing solution was blotted-off and the cultures were incubated at 22 - 24 °C in a growth room with cool-white fluorescent lights providing 24 h light (38 µmol m^{-2} s^{-1}).

(iii) Transformation of in vivo *root explants*

Transformation of the Apiaceae, in particular carrot, has been achieved with root explants and seedling hypocotyl explants from germinating seeds. Experiments conducted by SPANO *et al.* (1989) using root explants were used as a basis for the transformation of *T. garganica* roots as seeds were not readily available. Therefore, hypocotyls were not tested as an explant for transformation in this study.

Tap roots of *T. garganica* were excised from plants growing under greenhouse conditions. Latex gloves were worn throughout the decontamination of roots to prevent thapsigargins from coming into contact with skin as they cause skin dermatitis. After harvesting, the brown epidermal layer of the roots was washed thoroughly with running tap water to remove soil and then it was peeled-off. Thereafter, the tap roots were decontaminated by rinsing with 70% ethanol (v/v) for 10 min and treated with 1 % Benlate (w/v), whilst continually agitating, overnight. Using full strength commercial bleach (3.5 % $NaOCl_2$; w/v), the roots were decontaminated for 15 min and the decontaminating solution was then rinsed-off with four changes of sterile distilled water. The roots were sectioned into discs and then placed on growth medium with different combinations of PGRs. The MS medium was supplemented with various auxin:cytokinin combinations. These were 1:3 NAA: BA, 2:1 2,4-D: BA, 1:1:1 2,4-D: NAA: BA or 0.5; 1.5 NAA: BA 30 g l^{-1}. The medium also contained sucrose, 0.1 mg l^{-1} myo-inositol and 8 g l^{-1} agar (pH 5.8). The excised root discs were placed on the tissue culture medium and transferred to a growth room with cool-white fluorescent lights providing light for 24 h (35 µmol m^{-2} s^{-1}). The growth room temperature was maintained at 22 - 24 °C. The cultures were monitored for fungal and bacterial growth to determine whether the decontamination procedure had been effective. Root explants that had become infested with fungus and/or bacteria were eliminated for use in subsequent transformation experiments. Those that were healthy, without visible signs of infection, were then used. *Agrobacterium* cells grown for 48 h and treated as previously described

(Section 6.2.3i) were utilised to transform the explants. Small incisions were made on the root explants to facilitate wounding for transformation by *Agrobacterium*. These were then transferred to a bacterial suspension for 30 min or the scalpel blade was used to smear a single colony of bacterial growth on the explants while the explants were being scratched. Otherwise pinprick punctures were made with the needle of a syringe and a droplet of bacterial suspension was released from the syringe. These were then placed on MS agar medium (0.8 % [w/v]; pH 5.8) with 20 µM acetosyringone and incubated in the dark at 25 °C for 3 days. The co-cultivation period of three days was sufficient time to encourage agrobacteria to grow as visible agrobacterial growth could be detected on the edges of the explants. The infected explants were then transferred to culture jars with a cefotaxime solution (500 mg l^{-1}), washed by agitation in the antibiotic solution three times to kill agrobacteria, blotted dry on filter paper and then cultured on MS with 500 mg l^{-1} cefotaxime, 1 mg l^{-1} NAA and 3 mg l^{-1} BA, as per previous description Section 6.2.4. They were then transferred to a growth room with a temperature of 22 - 24 °C and monitored for six weeks to assess induction of hairy roots. This experiment was repeated and the growth medium was changed to include 250 mg l^{-1} cefotaxime and 1 mg l^{-1} IAA or 500 mg l^{-1} cefotaxime and 1 mg l^{-1} IAA.

(iv) Transformation of callus tissue

Logarithmic cultures of *Agrobacterium* strains were diluted 10 times in MS liquid medium (pH 5.8) supplemented with 30 g l^{-1} sucrose and 0.1 mg l^{-1} myo-inositol and incubated for 30 min with callus derived from *in vitro* culture of leaf explants on 2 mg l^{-1} 2,4-D and 1 mg l^{-1} BA. After co-cultivation, the callus and the agrobacteria were washed three times with liquid MS three times, blotted-dry and then cultured onto MS solid medium supplemented with 0.5 NAA: 1.5 BA. After 48 h the callus was resuspended in liquid MS and the agrobacterial cells were removed by agitating the callus suspension. This washing step was repeated twice. Afterwards, the callus was dried by blotting on filter paper and then transferred onto solid MS medium for induction of hairy roots. Control experiments were set up and

performed in a similar manner except that agrobacterium inoculation of the transformation solution was omitted. Transformation of callus derived from root explants was also performed as described above.

6.2.6 *Agrobacterium* transformation assisted by enzyme digestion of explant

Shoot bases were excised from *in vitro* grown plantlets and placed in an enzyme solution composed of 0.5 % Onozuka R 10 (cellulose) (w/v) and 0.1 % macerozyme (pectinase) (w/v) in plasmolysing solution, CPW 13M. The CPW salts were 27.2 mg l^{-1} KH_2PO_4, 101 mg l^{-1} KNO_3, 1.48 mg l^{-1} $CaCl_2.2H_20$, 246 mg l^{-1} $MgSO_4.7H_20$, 0.16 mg l^{-1} KI, 0.16 mg l^{-1} $CuSO_4.5H_20$ and 13 % mannitol (w/v). The enzyme solution was filtered through a 0.22 µM filter to remove contaminants. After the digestion (45 min), the plant material was washed twice in a solution of CPW21S. The washing solution contains the same CPW salts as the enzyme with mannitol substituted for 21 % sucrose (w/v). The procedures outlined in Section 6.2.5i and Section 6.2.4 for *Agrobacterium* transformation and decontamination were subsequently followed.

6.2.7 Sonication-assisted *Agrobacterium* transformation (SAAT)

Sonication-assisted *Agrobacterium*-mediated transformation was performed according to the methods described by TRICK and FINER (1997), with modifications. The agrobacterium was grown until mid-logarithmic phase in appropriate medium and antibiotics for each particular strain. An overnight culture was then set up. The following day this culture was centrifuged at 1 500 x *g* for 10 min and resuspended in 10 ml liquid bacterial medium. The centrifugation was repeated and the bacterial cells were subsequently diluted until a reading of 0.5 was obtained at A_{600} nm. Five ml of the suspension were placed in a plastic vial and the explants of *T. garganica* were then sonicated for 30 s, 1 min or 2 min. Thereafter, they were blotted on sterile filter paper and co-cultivated for 3 days with different strains of *Agrobacterium* at 19 °C on medium containing 20 µM acetosyringone. On the third day, the plant material was decontaminated of the

agrobacteria as previously described (Section 6.2.4i) and then transferred onto medium with cefotaxime to kill-off the bacterial cells. The tissue culture medium was changed weekly for three weeks to prevent tissue browning and the explants were observed for hairy root induction up to eight weeks.

Sonication of the tissue prior to addition of agrobacteria was compared to when sonication was performed with the explants incubated in the agrobacterial suspension as described above. All the other experimental variables were kept the same.

6.2.8 Biolistics transformation

(i) Preparation of microparticles

The vector DNA for microparticle bombardment was prepared according to methods outlined in Section 6.2.13 and the DNA was quantified as specified in Section 6.2.14.

The method of precipitating the DNA onto the microparticles is routinely used by Dr B. Jénés and his research group at the Agricultural Biotechnology Centre (ABC), Gödöllö (Hungary) and Dr Jénés is hereby acknowledged for sharing his technical expertise while learning this technique. Fifty mg of 1.1 µm tungsten particles were incubated in 70 % ethanol (v/v) overnight. To wash particles, they were centrifuged at 10 000 rpm in a Sigma-113 desk top centrifuge and the supernatant was removed prior to the particles being resuspended in sterile distilled water by vortexing. They were then washed two more times and then resuspended in 1 ml of sterile 50 % glycerol solution (v/v). While the suspension was being vortexed continually, 500 µl aliquots of this final suspension were stored in sterile microfuge tubes at -20 °C. For the precipitation of the DNA onto the particles, 25 µl of the tungsten suspension were added to a microfuge tube and 5 µg of DNA of each plasmid was also pipetted into the tube and 25 µl of 1 M $CaCl_2$ were added and followed immediately by 10 µl of sterile deinonised water. The components were added in the order described above. The contents of the tube were mixed thoroughly by vigorous hand-vortexing and then left on ice for 10 min to allow for

precipitation and sedimentation. Once 10 min had passed, 40 µl of the supernatant were removed as the DNA-coated particles had formed a pellet at the bottom of the tube. The DNA-coated particles were then resuspended by vigorous hand-vortexing to prevent particle agglomeration prior to loading and 5 µl were used per shot. The target tissue was shot twice.

(ii) Preparation of target tissue

Approximately 4 - 16 h prior to gene delivery, the target tissue was arranged on the MS basal medium in a circle at the centre of the plate. This medium was supplemented with 0.2 M mannitol and 0.2 M sorbitol to provide osmotic pre-bombardment conditioning to the explants. The inclusion of osmotic agents minimises bombardment damage and permits maximum response from the target tissue (DUNDER *et al.*, 1995). Fifteen shoot bases were placed on an agar plate for each bombardment and the experiments were repeated three times.

(iii) Conditions for biolistic gene transfer

All parts of the GENEBOOSTER™ (Agricultural Biotechnology Centre, Hungary) were cleaned thoroughly by spraying with 70 % ethanol (v/v) and the surface of the laminar flow hood also sprayed thoroughly with 70 % ethanol (v/v) 30 min before bombardment of the first plate. The stopping plate mesh was autoclaved prior to use and subsequently fitted onto the stopping plate before the first shot was fired. When a new construct was used, the stopping mesh was sterilised by using dry heat provided by an alcohol flame, cooled and then transferred onto the stopping plate.

The macroprojectiles were always stored in a jar filled with absolute ethanol at least 16 h before use and sufficient macroprojectiles for the biolistics process were removed from the storage solution, placed on a Petri dish and the ethanol left to evaporate under laminar flow air for 30 min before use. Once the macroprojectiles had dried completely, the DNA/tungsten suspension (5 µl) was pipetted onto the centre of the top of the macroprojectile. Thereafter, the macroprojectile was loaded or inserted into the upper end of the acceleration barrel, as per manufacturer's instructions. For each shot, the Petri dish with the target tissue was

opened, then placed on the 4th shelf (from the top) of the GENEBOOSTER vacuum chamber and locked inside. The tissue was transformed by microparticle bombardment with 4 µg DNA mg^{-1} tungsten particles at a time and the biolistic process was repeated for a second shot. Throughout the entire procedure or study, the bombardment conditions were kept the same. The distance of the stopping plate from the target tissue was kept constant (7-8 cm). The bombardment chamber was evacuated to -0.4 bar and target tissue was bombarded twice with a gas pressure of 40 bar.

After the second shot, the plate was removed from the biolistic device, the lid replaced and sealed with sealing film. The bombarded plates were transferred for 16 h to a growth room with a 16 h light and 8 h dark cycle provided by cool-white fluorescent light (37 µmol m^{-2} s^{-1}). The temperature in the growth room was kept between 22 - 24 °C. After 16 h, the explants were transferred to medium without sorbitol and mannitol; and, monitored for subsequent development of hairy roots post-bombardment or analysed for GUS transient expression of introduced genes 48 h after microparticle bombardment (Section 7.2.3).

6.2.9 Biolistic-assisted *Agrobacterium*-mediated transformation (BAAT)

Two systems of BAAT were tested for their efficiency to transform *T. garganica* plantlets, and are schematically outlined in Figure 6.2. The first system involved wounding of the target tissues with tungsten particles that were not coated with DNA binary vectors. Instead, the DNA was substituted with 5 µl of water for each reaction (Table 6.2). This system was denoted BAAT 1. The second system (defined as BAAT 2; Figure 6.2) involved a two-step transformation where the target tissue was subjected to microparticle transfer with plasmid-coated tungsten as described in Section 6.2.6, without amendments. Immediately thereafter, the bombarded plant materials were inoculated in an *Agrobacterium* suspension and, the *in vitro* propagated tissues or root explants were transformed via *Agrobacterium* according to methods previously described in Section 6.2.3ii and Section 6.2.3iii, respectively. The methods were kept the same except that the co-cultivation of *Agrobacterium* and the plant material was extended from 30 min to 16

h or overnight. After the co-cultivation, tissues were decontaminated of agrobacteria by washing three times in sterile water with cefotaxime at a concentration of 500 µg ml^{-1}.

Isolate plasmid DNA

↓

Precipitate DNA onto tungsten particles

↓

Load DNA-coated tungsten particles onto a macroprojectile

↓

Bombard *in vitro* derived tissues

↓

Transfer onto *Agrobacterium* suspension and co-culture

↓

Remove excess suspension prior to 3 day co-cultivation

↓

Kill off agrobacterial cells with antibiotics

↓

Select for transformants and regenerate transgenic tissues

Figure 6.2: An outline of the steps followed for biolistic-assisted *Agrobacterium*-mediated transformation (BAAT 2)

6.2.10 Introduction of *Agrobacterium* by vacuum infiltration methods

Attempts to introduce *rol* genes via *Agrobacterium* transformation with vacuum infiltration to facilitate this process were made on decontaminated leaf material (refer to Section 6.2.2 for decontamination procedure), and shoot bases that had been excised from *in vitro* propagules. The tissues were placed on MS without PGRs and the *Agrobacterium* was grown until mid-logarithmic phase in acetosyringone (Section 6.2.4) and appropriate antibiotics. The *Agrobacterium* culture was centrifuged at 5 000 rpm in a SS-34 rotor (Sorvall R-65 Superspeed centrifuge). The cells were washed once with MS medium plus acetosyringone (200 µM). The centrifugation was repeated to collect the cells and each pellet was then resuspended in 15 ml of the MS liquid medium (pH 5.8) with 3 % sucrose (w/v), 0.1 g l^{-1} myo-inositol. Each explant was infiltrated with 10 µl of the bacterial mixture at 800 mbar of vacuum pressure for 30 min. The vacuum was then released and the plate containing the target tissues was removed and sealed with sealing film (Parafilm®). The plant material and the agrobacteria were left to co-cultivate overnight and the following day, the excess bacteria was removed from the tissue by lightly rubbing the plant material against a piece of sterile filter paper. The plant material was then incubated for 24 h in a growth room with 24 h light at 22 – 24 °C on MS medium solidified with 0.8 % agar (w/v) supplemented with 20 µM acetosyringone. Subsequently, further agrobacterial growth was inhibited by washing the explants with three changes of liquid MS, devoid of PGRs, containing cefotaxime (250 mg l^{-1}). Thereafter, tissues subjected to *Agrobacterium*-infection were grown in MS agar-medium (0.8 %, w/v) on a combination of 0.5:1.5 NAA: BA with kanamycin (100 mg l^{-1}) and cefotaxime (250 mg l^{-1}) for two weeks prior to transfer onto medium lacking PGRs.

6.2.11 Preparation of experimental controls

With all transformation experiments, tobacco plants growing in a continuous culture system on solid ½ MS medium (8 g l^{-1} agar; pH 5.8), supplemented with sucrose at 3 % (w/v), myo-inositol (0.1 g l^{-1}) and without PGRs were used a positive controls as the transformation of this species is well-documented and routine in many

laboratories. Tobacco *in vitro* plantlets were initiated and subsequently donated by Mrs S. Stuart. Throughout the course of the project, they were subcultured on a monthly basis and grown under the same growth room conditions as *T. garganica in vitro* stock plants at 22 – 24 °C under a 16 h light (35 µmol m^{-2} s^{-1})/ 8 h dark cycle. Bacterial strains were tested against tobacco for their efficiency to transform plants except for the strain donated by Dr D. Tepfer with pMRKE15 (Table 6.1) as most of them had been in long-term storage or sent from overseas by surface mail. It was considered a possibility that, during postage conditions were not necessarily conducive to maintaining bacterial infectivity.

As a negative control, sterile distilled water was used as a substitute for the agrobacterial suspension employed to infect *T. garganica*; and, leaf and stem explants of tobacco was substituted with bacterial growth medium that had not been inoculated with agrobacteria. Otherwise, both negative and positive controls were treated in the same manner as the experimental plants.

6.2.12 Selection of putative transformants

After transformation, tissues were placed on selection MS medium containing kanamycin (100 mg l^{-1}). All selection procedures were performed on solid medium after removal of agrobacteria from the cells by washing. The cultures were kept on the selective medium for two to three passages. Thereafter, they were transferred to medium devoid of antibiotics and the re-growth of residual *Agrobacterium* was monitored. Detailed methodology regarding selection of transformants is described in Chapter 7 (Section 7.2.1).

6.2.13 Isolation of plasmid vector DNA from *Agrobacterium*

The method for the isolation of plasmid DNA from *Agrobacterium* was based on the methodology for the preparation of Ti plasmids and binary vectors described by LI *et al.* (1995), with modifications. This method deals with inherent difficulties associated with the preparation of plasmids from *Agrobacterium*. DNA was isolated from mid-logarithmic phase cultures of agrobacteria that had been grown

in appropriate antibiotics. Bacterial cells (0.5 ml) were transferred to 15 ml tubes and centrifuged at 8 287 rpm (Hettich Universal KT2S centrifuge). The pellet was then resuspended in 100 µl of Buffer 1 (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM ethylene diamene tetra-acetic acid [EDTA]). Bacteria were lysed by the addition of 200 µl of lysing solution containing 0.2 M NaOH combined with 1 % sodium dodecyl sulphate (SDS) (w/v). The cells and the lysing solution were mixed well and set on ice for 15 to 30 min and then 1.5 volumes of ice-cold 5 M potassium acetate solution (pH 4.8), pre-chilled by incubating at -20 °C, were added for neutralisation. Thereafter, the tubes were mixed gently by inversion and then transferred to an ice bucket filled with ice for 60 min. Afterwards, the tubes were centrifuged to collect the proteins and cellular debris at 10 000 x *g* for 10 min and the pellet discarded. This centrifugation was repeated until the white cellular debris was no longer visible. Each time the supernatant was transferred to a fresh tube. To digest the RNA, 2 µl of a 500 µg ml^{-1} stock of RNase A per ml were added to the plasmid DNA extract and incubated at room temperature for 20 min. Thereafter, a phenol: chloroform: isoamyl alcohol extraction (25:24:1; v/v/v) was performed in the fumehood to purify the DNA. Whilst extracting, gloves were worn and organic solvents and the DNA extract were mixed well, by careful inversion. The tubes were then centrifuged for 10 min at 10 000 x *g* and in most instances this step was repeated until there were no visible signs of proteinaceous material in the DNA extract at the interface. DNA was precipitated with an equal volume of prechilled isopropanol by placing at -20 °C for at least 1 h prior to centrifugation. The nucleic acid was collected by centrifugation at 10 000 x *g*. The DNA was washed with 70 % ethanol (v/v) to remove residual salts, followed by a 100 % ethanol precipitation step and finally, vacuum-drying or air-drying by exposing open tubes to laminar air for 10 – 20 min. The DNA was resuspended in a small volume (20 - 50 µl) of TE buffer (10 Mm Tris-HCl [pH 8]; 1 mM EDTA) or distilled water. TE buffer was used particularly for long-term storage of DNA and moreover, when the DNA was not intended for amplification via PCR. DNA isolated for PCR analysis was stored in sterile distilled water and the integrity of all DNAs was assessed through electrophoretic analysis in 1.5 % agarose gels, as described below, prior to application of PCR and other molecular techniques such as

Southern blotting (SOUTHERN, 1975; BHAT and SRINIVASAN, 2002) (refer to APPENDIX for Southern hybridisation).

6.2.14 DNA quantification and agarose gel electrophoresis

DNA was quantified by ultraviolet (UV) absorbance spectrophotometrical analysis at A_{260}, A_{280} and A_{320}. Two µl of DNA suspension were diluted by pipetting into a quartz cuvette holding TE or distilled water. The contents of the cuvette were mixed well and the readings were taken using a Varian Cary UV-visible spectrophotometer. Thereafter, the concentration of the DNA (µg μl^{-1}) was calculated according to the formula below:

$$([A_{260} - A_{320}] \times \text{dilution factor} \times \Delta E) \div 1000$$

(ΔE = 50 µg μl^{-1} double stranded DNA)

The value of the A_{260} reading divided by the A_{280} value, was used to determine the purity of the DNA where an answer of 1.8 indicated 100 % ultra-pure DNA, free of protein, residual phenol and other contaminants (AUSUBEL *et al.*, 1988; BROWN, 1996).

The integrity of the DNA was analysed using agarose gel electrophoresis in a 1.5 % agarose gel (w/v) with TAE buffer (0.04 M Tris-acetate, 0.002 M Na_2EDTA, pH 8.0). Loading dye (50 mM NaOH, 1 mM EDTA, 2.5 % glycerol [v/v], 0.0 5 % bromophenol blue [w/v]) (5 µl) was used to track the progress of electrophoresis. The gels were run at 65 volts (V) at room temperature and the DNA was visualised by ethidium bromide (50 µg ml^{-1}) staining. The size of the bands was determined by comparing to Molecular Weight Marker III (MIII) or Molecular Weight Marker (MXIV) (Roche). The gels were photographed using a Pentax ME-Super with an orange filter or utilising the UVItec Gel-Documentation System DOC-008. TFT.

6.3 RESULTS AND DISCUSSION

One of the primary objectives of this part of the study was to determine favourable strains to bring about genetic transformation of *T. garganica*. The strains LBA9402, R 1600 Nester, R 1601 Nester, TR 8/3 Wits and A4 Tempé that were donated by Dr S. Macrae and were kept in long-term culture for approximately three years, prior to the initiation of this particular study. These were easily grown on media containing rifampicin, with the exception of LBA9402. Inclusion of rifampicin as a means of selecting for a particular strain of *Agrobacterium* is routine practice and thus served to verify that indeed the bacterial strains were *Agrobacterium* rather than some other contaminating strains. Rifampicin exerts its bacteriostatic activity by inhibiting RNA synthesis by binding to and inhibiting the β-subunit of RNA polymerase (AUSUBEL *et al.*, 1988). *Agrobacterium* strains used in this study had chromosomally-located rifampicin resistance (HELLENS *et al.*, 2000). This type of resistance is conferred due to a mutation in the β-subunit of RNA polymerase resulting in the inhibition of the antibiotic from complexing at this site (AUSUBEL *et al.*, 1988). The LBA9402 strain did not grow at all and it was thus necessary to obtain another strain as it was unclear whether the strain (donated by Dr Macrae) was still infective. The loss of rifampicin resistance suggested changes in the chromosomal makeup of this *Agrobacterium* as a result of long-term culture and its capability for genetic modification was questionable and untrustworthy. The possibility that another bacterium had contaminated the plates and was able to survive on rifampicin was also considered; and the element of doubt brought about by the lack of growth was disconcerting. LBA9402 was identified as an important strain to test for its efficiency in the transformation of *T. garganica* as it is supposedly supervirulent or rather, less genotype-dependent regarding gene transfer because it has resulted in the transformation of many plants (RHODES *et al.*, 1997). The strains that proved most efficient in transforming tobacco are shown in Table 6.1 and strains that elicited a strong or intermediate response regarding root induction were used for subsequent transformation experiments. Their response on tobacco explants was taken as in indication of virulence as they were still infective after long-term storage.

Therefore, the strains that were evaluated for transformation of *T. garganica* explants were LBA9402, C58C1 (pGUS1), A4R, A4T (pBIN19) and pMRKE15.

Although, a variety of strains are responsible for numerous reported transformations culminating in the successful establishment of hairy root cultures, including mannopine-producing, cucumopine-producing, and agropine-producing strains, the latter such as LBA9402, are generally preferred for their efficiency of transformation (HAMILL and LIDGETT, 1997). Mobilisation of binary vectors containing marker genes such as GUS can be easily achieved via triparental mating to *Agrobacterium* (HAMILL and LIDGETT, 1997), if desired, when wildtype strains are used. For instance the C58C1 strain and A4T strain had binary vectors (pGUS1 and pBIN19) with *gus* A and *nptII* genes under the control of the CaMV35S promoter and nopaline synthase (nos) promoter, as previously mentioned.

Several transformation protocols utilising different or varied explants as subjects for *Agrobacterium* colonisation were examined to assess whether *T. garganica* would yield to transformation. A positive control was included with all transformation experiments using tobacco explants. This plant is often used in experiments as a model species for genetic manipulation as its transformation is technically simple, easy, routine in many laboratories and highly efficient. The transformation of tobacco and petunia, more specifically leaf explants, are often exploited as model systems to study the transformation process pertaining particularly to the biology of the *Agrobacterium*-host interaction (WALDEN, 1988). These plants are favoured positive controls due to the plants being highly susceptible to transformation as they are natural hosts of *Agrobacterium*. In the current investigation, *A. rhizogenes* LBA9402, A4T and C581 were the most virulent strains and induced massive prolific hairy roots on tobacco leaf explants (Table 6.2, Figure 6.3A and C). As a consequence of stable integration of the *rol* genes, a highly branched, plagiotropic, prolific root system (Figures 6.3A and C), typical of hairy roots (TEPFER, 1983), was produced on medium devoid of PGRs on tobacco explants indicating that the strains utilised at the time of experimentation were virulent. The roots produced were induced between 3 days to 10 days after co-cultivation and exhibited

extensive lateral branching and a lack of geotropism. The roots were initiated, at first, from cut surfaces of explants and even undamaged tissues of the explant rapidly became rhizogenic to the point where the entire explant was enmeshed in a network of tangled hairy roots (Figure 6.3C). This is in agreement with other findings where proliferation of hairy roots at the site of infection at the cut surface, normally occurs within two to three weeks. The uptake of the genes of the Ri-plasmid is characterised by the insertion of both TR- and TL-DNA segments. At other times, the TR-DNA or TL-DNA is integrated into the plant genome only when a co-integration vector system is utilised, whereas with binary vectors the sequence on the vector might be inserted with or without the TR- or TL-DNA or both. The TL-DNA carries the *rol* operon responsible for the hairy root syndrome whereas the TR-DNA harbours the other oncogenes promoting the hairy root syndrome; such as those controlling auxin synthesis.

On one hand, tobacco transformation was an excellent example of Ri-transformation and allowed for positive identification of the most virulent strains as well the development of the necessary skills to carry out other transformation experiments on *T. garganica*. On the other hand, *Thapsia* proved stubborn to genetic transformation by the Ri T-DNA. Several of the methods tested failed to successfully genetically alter the explants on trial and symptoms of the hairy root syndrome never became apparent extensively after experimentation. This result was not expected as inoculation of leaf material in other species with *A. rhizogenes* or *A. tumefaciens* with binary vectors containing the *rol* genes generally and readily brings about transformation. Occasionally hairy roots are produced within the first week particularly in species that easily comply to genetic modification. The experiments evaluated for transformation in this particular study, were based on published protocols that were successful for other species in the Apiaceae family and application of these procedures to *T. garganica* were unsuccessful. It must be noted that the expression of *rol* genes is tissue-specific, with expression in root tissues being more prominent compared to expression of genes introduced into leaf material. Carrot slices have proved useful as an explant source for the transformation of *D. carota*. A number of groups have used them since the 1980's as hosts to both phytopathogens

Table 6.2: Effect of various gene transfer methods on root formation on *T. garganica* explants

Transformation Method	Root formation (%)	[a]Degree of rhizogenesis in responding cultures	Other response in culture
Agrobacterium + *in vivo* explants	-	-	-
Agrobacterium + *in vitro* shoots	-	-	Adventitious shoot regeneration
Agrobacterium + callus	-	-	Extensive browning and somatic embryo induction
Enzyme digestion	-	-	-
SAAT	1.2	+	Adventitious shoot regeneration
Biolistics	-	-	Transient GUS expression; shoot regeneration and somatic embryogenesis
BAAT 1	-	-	Transient GUS expression; shoot regeneration
BAAT 2	3	++	Transient GUS expression, shoot regeneration and embryogenesis
Agrobacterium infiltration	-	-	-

[a] The number of roots were counted and the growth rate monitored. The degree of rhizogenesis was rated according to the number of roots observed where +++, ++, + and - refers to 10 or more roots, 5 or more roots, 1 or more and no roots observed on each explant, respectively

A. rhizogenes and *A. tumefaciens*. KĘPCZYŃSKA *et al.* (2003) cited and employed the method of carrot discs infection devised by CANFIELD and MOORE (1983). The establishment of transgenic roots in liquid culture by the former group was not an incentive that was explored. Somatic embryos that have been regenerated from root discs have also been utilised for carrot transformation using C58C1 (pGSGluc1) by PAWLICKI *et al.* (1992) and a comprehensive study of a variety of parameters which were hypothesised to impact on carrot transformation followed. It is worth mentioning that these authors found transformation to be dependent on the plant genotype, the explant age and type as well as the duration of co-cultivation where for most of the parameters tested the transformation

frequency was below 15 % - making the system of transformation rather inefficient. On the other hand, SPANÒ *et al.* (1989) were able to fulfil their research goals by inducing hairy root cultures from carrot discs with introduction of single or groups of *rol* genes harboured by agropine-type Ri plasmid, pRi1855. The inefficiency of employing the carrot disc method for transformation, in that particular study, was not expressed. Presumably, the gene transfer lived up to expectations and these authors identified auxin production by the *aux* genes of ORF 13 and 14 of the Ri plasmid being vital for roots with typical characteristics of the hairy root syndrome. However, roots formed when only constructs of *rol* A + *rol* B + *rol* C were transferred into carrot discs were surprisingly geotropic and the growth rate was slighter faster compared to normal roots, that had been transformed with the entire Ri plasmid with the *aux* genes. Similarly, putative transgenicity of *T. garganica* (Figure 6.3) obtained via BAAT had similar properties of being geotropic and the growth rate was not exceptionally fast in comparison to non-transgenic roots. It is, however, noteworthy to mention that LBA9402 had the entire Ri plasmid while binary vectors (pMRKE15) carried only a complement of the *rol* genes, without the TR-DNA. The lack of formation of secondary roots was also a concern during the course of the present study. This subject is discussed further in the following Chapter which partly deals with the initiation of a transgenic root culture system for *T. garganica*. It is worthy to note that although transformation was achieved using BAAT, it was as low as 3 % (Table 6.2).

In the past, some studies have documented the use of protoplasts for transformation of the Apiaceae. As an example, carrot protoplasts were transformed using electroporation by RATHUS *et al.* (1993) in order to study the effects of promoter, intron and enhancer elements on transient gene expression. These authors did not attempt to regenerate plantlets from the transformed protoplasts. In this particular case, carrot was used as dicotyledonous model to compare transformation to a monocotyledonous species such as sugarcane. Transformation of carrot protoplast was not investigated in the current study as the protoplast system is said to be difficult to handle in the laboratory. Problems associated with the regeneration of protoplasts to whole plants are a discouragement to researchers wishing to explore the use of such a system for

transformation and PEG-mediated protoplast transformation often leads to increased cell death and low transformation frequencies (BALLAS *et al.*, 1987). These types of transformation protocols have added disadvantages of being time-consuming as well as expensive, apart from being technically difficult to establish as routine practices for transformation. However, enzymes utilised in protoplast transformation were employed for partial digestion of plant cell walls of explants to create micropores as sites for T-DNA entry into the cells of *T. garganica* during transformation. It was envisaged that the micropores would facilitate movement of T-DNA from the bacterium through the cell envelope. Further optimisation experiments are needed to verify the usefulness of enzymes as part of the transformation protocol for *T. garganica*.

Genetic transfer, via *Agrobacterium*, of previously untransformed species often fails due to the wounding process being ineffective at the stages of explant infection and consequently little to no production of phenolic signal for the *Agrobacterium* to sense wounded plant cells circumvents successful ss T-DNA processing due to poor *vir* expression (HIEI *et al.*, 1997). Despite the belief by some groups that the inclusion of acetosyringone is not essential for transformation, many laboratories still include the *vir* operon inducer in the growth medium as it appears to be a critical requirement for acceptable transformation frequencies for many species. Acetosyringone is essential for the *vir* operon induction which controls the transfer and integrative capabilities of *Agrobacterium* (GELVIN, 2000). Production of the *vir* proteins facilitated by the phenolic signal is also implicated in the pilus assembly for the attachment of agrobacteria onto the surface of the plant cell walls. In recalcitrant species, such as rice, concentrations of 100 µM are vital for bringing about successful transformation and acetosyrigone-pretreated agrobacteria seemingly have a slight positive influence on the efficiency of transformation despite T-DNA transfer occurring at early stages during co-cultivation (HIEI *et al.*, 1997). For these reasons, the concentration of acetosyringone that was optimal for transformation was tested. In this study, there were no significant differences obtained with respect to hairy root production regardless of the acetosyringone concentration incorporated into the medium. At the highest concentration (200 µM) tested, no deleterious effects were noted in

culture on the regenerative capability. GELVIN (2000) suggested that in most cases the concentration of the acetosyringone used by researchers to induce the *vir* operon *in vitro* far exceeds the level required by the bacterium. This results in over-stimulation of the *vir* genes and in turn, saturation of *vir* proteins within the bacterial cell during experimentation, more especially *vir* E which is thought to protect the ssT-DNA from nucleolytic degradation while in transit to the plant nucleus from the *Agrobacterium* cell.

The elimination of residual agrobacteria after co-cultivation is often one of the problems that arise, even when antibiotics are applied to subsequent *in vitro* regeneration medium during selection. Although occurring infrequently, agrobacteria was at times stubborn to the application of antibiotics with occasional development of bacterial growth during selection or after transfer of explants to medium without antibiotics (Figure 6.3E). The occurrence of bacterial growth was noted when the cultures were presumed decontaminated of agrobacteria. In such instances, the steps taken to eliminate *Agrobacterium* from the explants were repeated (Section 6.2.4) and the explants were transferred back onto the selective medium until they were free of bacterial infection. However, more often than not, the initial methods used were appropriate and effective in eliminating residual *Agrobacterium* infection after co-culture. It must be stressed that this agrobacterial overgrowth was not a major problem as none of the putative transformed lines were lost due to its presence and a repeat treatment with cefotaxime killed the residual cells.

As the host range of *A. rhizogenes* is restricted to dicotyledonous plants in nature, with gymnosperms and monocotyledonous plants showing limited susceptibility to infection (KODAMA *et al.*, 1993). Furthermore, some dicotyledonous plants are presumed or thought not to be compatible hosts for *A. rhizogenes* (KODAMA *et al.*, 1993). In such cases, the possibility of direct gene transfer of the *rol* genes becomes attractive as an option for the production of hairy roots. The successful generation of transgenic hairy roots, which were fast growing and showed extensive lateral branching, after particle bombardment of cucumber cotyledons as reported by KODAMA *et al.* (1993) was motivation to investigate biolistic-mediated

gene transfer as a means to transform *T. garganica*. It was unfortunate that their protocol could not be reproduced in its entirety as seeds of *T. garganica* were a limiting factor for evaluating cotyledons as an explant for transformation. Although, no transformed roots were regenerated using microparticle bombardment in this study, positive transient expression, indicating putative transformation (discussed extensively in Chapter 7), identified this method as having potential for stable integration, more especially, when a suitable explant (for instance seeds) for transformation is readily available. In other plant species, seedling hypocotyls are valuable as an explant for transformation when other tissue organs fail to respond to *A. rhizogenes* infection, for example plaigiotropic hairy roots were only at the site of infection from hypocotyls after LBA9402 inoculation (DREWES and VAN STADEN, 1995) following numerous attempts with leaf explants with *Solanum mauritianum*. The positive response of cells derived from hypocotyls to transformation has been attributed a high organogenetic potential in comparison to callus derived from more mature explants (SENIOR *et al.*, 1995), for instance. Unfortunately, seeds of *T. garganica* were not easy to obtain to test the hypocotyls for their efficiency to undergo genetic transformation due to lack of availability, as aforementioned.

Extensive attempts by SENIOR *et al.* (1995) to produce hairy roots following the co-cultivation of mature tissues of *Antirrhinum majus* and *A. rhizogenes* were futile. Several conditions of transformation were tested including co-cultivation, varying *Agrobacterium* strains, pH and a variety of secondary metabolites as *vir* gene inducers. None of these parameters improved the ability to transform *Antirrhinum majus*. Success was only achieved by those authors with the use of three-week-old hypocotyls after *A. rhizogenes* co-culture, where prolific hairy roots were excised to establish a root culture system. These authors (SENIOR *et al.*, 1995) identified the disadvantages of their method for transformation as being genotype-dependent and time-consuming. It must be emphasised that DREWES and VAN STADEN (1995) experienced difficulties of a similar nature regardless of the method of transformation until hypocotyl tissue was tested for transformation. Furthermore, the former group of authors used similar strains as the current study, most of which did not induce transformation. The utilisation of hypocotyls as

explant material for the transformation of *T. garganica* is a future consideration, should a large number of seeds become more readily available. It would be interesting to test the susceptibility of hypocotyls of *T. garganica* as starting material to infection by LBA9402; and, other strains such as A4T and C58C1 as well as their competence for regeneration of hairy roots upon transformation.

The successes obtained by other research groups in transformation of umbelliferous plants using explants derived from germinating seeds are documented in the literature but are not necessarily prolific in representation. Carrot transformation was achieved by TEPFER (1984) and the following year, a different set *A. rhizogenes* strains was demonstrated to have varying transformation capabilities on the apical surface of carrot discs (CARDARELLI *et al.*, 1985). These experiments generally indicated that strains of *A. rhizogenes* exhibited asymmetrical (polar) infectivity with exception of an agropine strain, 1855, that induced root proliferation on both basal and apical surfaces of carrot discs. The differential rooting ability of the various strains was attributed to the higher level of auxin on the apical tissues compared to the basal surface. The authors further advocated for exogenous auxin application to induce hairy roots, followed by removal of the auxin after induction as Ri-transgenic roots can readily proliferate in the absence of PGRs. *P. anisum* was first transformed by *A. tumefaciens* using a method of co-cultivation reported by KOMARI (1989) and further discussed by CHARLWOOD and SALEM (1999), where hypocotyls were used for callus regeneration prior to subsequent transformation with *A. tumefaciens* A281 harbouring pTOK119 as a binary vector. The bacterial strains tested were also different from the agrobacteria available to conduct this particular investigation. For instance, transformation of *P. anisum* with *A. tumefaciens* strains 337 (pBI121) and LBA4404 (pMON9793) for the production of transgenic shooty teratomas was achieved by CHARLWOOD and SALEM (1999). Fennel transformation by a wild type strain of *A. rhizogenes* was first reported by MUGNIER (1988) (see review article by CHRISTEY, 1997). Transformation with *Agrobacterium* is strain- and explant-dependent, thus further complicating gene transfer to non-compliant or hardy-to-transform species. Such circumstances led to our turning to other innovative transfer techniques including those combining direct and indirect

methods to assist and coerce introduction of transgenes into *T. garganica* plant cells. The application of SAAT (1 min) with explants incubated in the bacterial suspension facilitated transgene introduction compared to vacuum infiltration as indicated by induction of roots on putatively transformed shoot bases. Prolific rhizogenesis occured at the shoot bases in 1.2 % of explants (Figure 6.3D) and this effect was comparable to BAAT application where extensive root production was also observed. Nevertheless, the transfer of these roots to selective medium containing kanamycin (100 mg l^{-1}) (Section 7.2.1) resulted in mortality of transformed roots. Vacuum infiltration of agrobacteria into *T. garganica* explants produced no significant effects on root induction. Agro-infiltration on leaf tissue has recently been utilised to study transient expression of reporter genes by PAGE and ANGELL (2002) using *Nicotiana benthaniana*. The conditions for infiltration were not reported and it is difficult to assess the vacuum requirement for successful application of this technique. During this study, no positive transient expression of reporter genes was noted after agro-infiltration of tissues. The amount of vacuum pressure applied on the tissue presumably plays a significant role in the success of the application of this technique and such parameters require optimisation for *T. garganica* transformation.

Roots are valuable explants for generating transgenic plants for many plant species by *Agrobacterium* co-culture. Recently, LOMBARI *et al.* (2003) showed that root explants were an excellent starting material for use in a highly efficient transformation-regenerating protocol of *Lotus japonicus*. In that particular study, root explants were more beneficial for transformation in comparison to hypocotyl transformation as efficiencies of plantlet regeneration using hypocotyl-derived tissue are low in *L. japonicus*. From our experience, the use of root explants was often hindered by the difficulties experienced with the decontamination of the root discs of *Thapsia* plants prior to transformation. In one decontamination trial, only 6 % of the root explants were free of fungal and bacterial pathogens. The tissues free of contaminants were retained and afterwards employed for transformation. No roots were initiated from root discs that were exposed to agrobacteria, irrespective of the method of inoculation and the strain of *Agrobacterium* used. The inclusion of auxins in the transformation medium is supposed to assist Ri gene

expression (SPANO *et al.*, 1989). Instead, root discs responded by producing a profuse callus with a high embryogenic potential from the tissues of the cambia (Figure 6.3F). Carrot slice utilisation for purposes of generating embryogenic callus is a well-established system. In the present study, the number of experiments was limited by the ready-availability of *T. garganica* plants as only 20 plants were growing under greenhouse conditions for harvesting. Optimisation experiments would have required a larger number of roots. The acquisition of *in vivo* roots with transformation as an objective in *T. garganica* requires the utilisation of a destructive method of harvesting. Hence, root discs were not regarded as an easy-to-renew starter or target material for transformation. Generation of a large number of non-transgenic root clones for transformation also proved a formidable task due to difficulties experienced in inducting rhizogenesis *in vitro* (discussed extensively in Chapter 4). This problem was further exacerbated by root culture initiation in shake flasks being mostly unrewarding (further discussion to follow; Chapter 7).

Upon setting up continuous solid and liquid-shake cultures on 2:1 2,4-D: BA medium for the callus derived from the carrot cambium, transformation experiments were initiated. However, no response to *Agrobacterium* infection, regardless of the method utilised, was obtained. Browning of callus at the time of co-incubation with agrobacterial suspensions whilst handling, was extensive and rapid in its occurrence. Callus browning was presumed to result from phenolic production. Even when care was taken to minimise rough-handling of flasks and tubes, the white callus became brown, once in contact with the agrobacterial suspension. Production of secondary compounds such as phenolics is desirable during *Agrobacterium* transformation. In this particular case, phenolic production was presumed to be excessive to the extent of inhibiting further development in culture after transformation. The callus was difficult to revive as cellular division was minimal and very little growth occurred after transformation; and the colour remained brown. Release of phenolics often adversely affects growth in culture as these compounds inhibit biochemical and other physiological processes by complexing with enzymes and other proteins (GEGENHEIMER, 1990). Phenolics have an affinity to also form insoluble or irreversible complexes with nucleic acids.

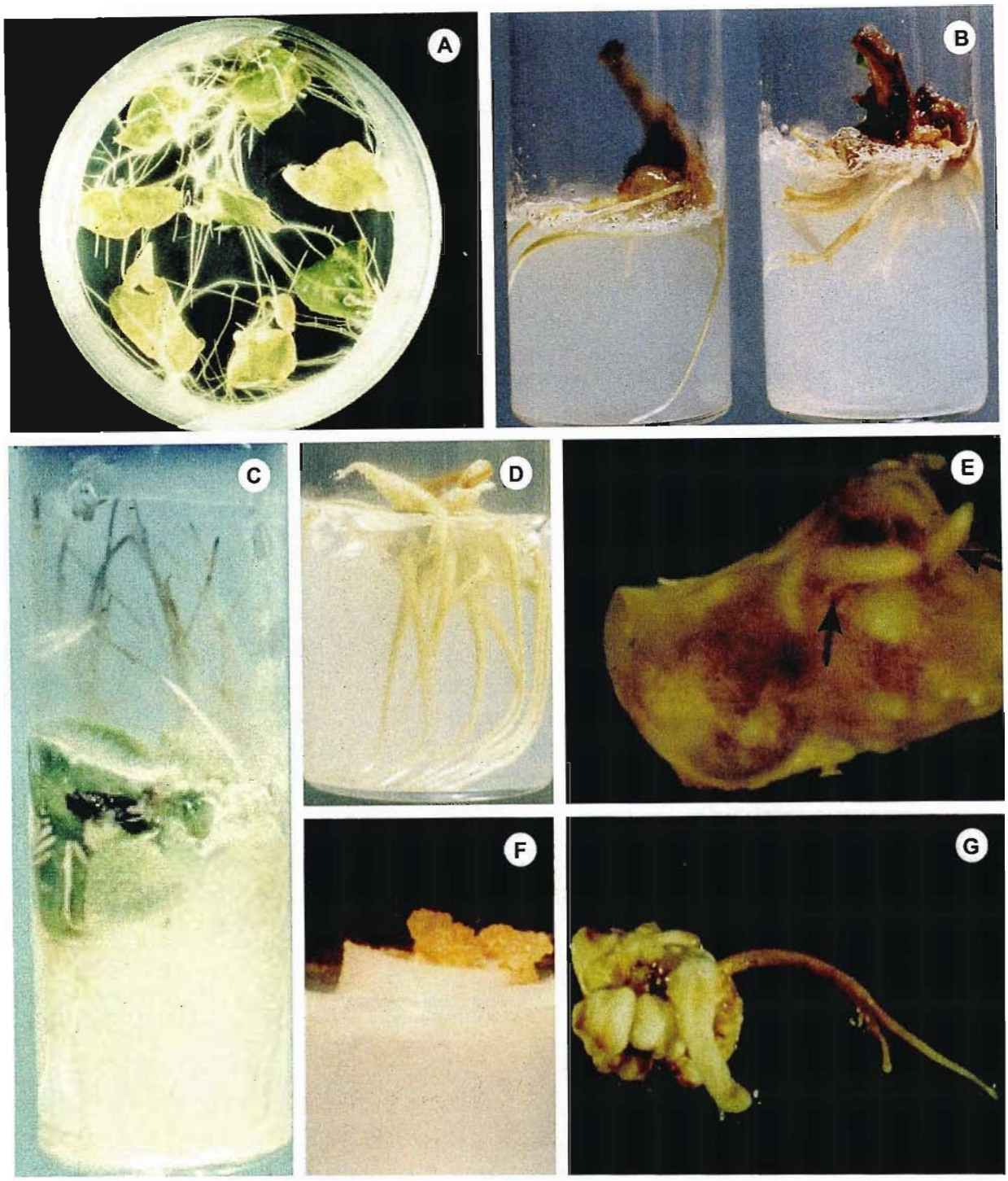


Figure 6.3: Morphology of putative transgenic root cultures obtained after transformation of *T. garganica* L. with the Ri plasmid. **(A)** Proliferation of true hairy roots after transformation of tobacco explants with *A. rhizogenes* LBA9402. **(B)** Abundant root initiation on shoot bases after *T. garganica* explants were transformed with *A. rhizogenes* C58C1 (pGUS1) using BAAT 2. **(C)** Massive induction of non-geotropic hairy roots after transformation of tobacco explants with *A. rhizogenes* C58C1 (pGUS1) using BAAT 2. The inclusion of tobacco as a positive control was routine practice for all transformation experiments. **(D)** Effect of SAAT-treatment on root growth in *T. garganica*. The explant was transformed with A4T after 2 min of SAAT. **(E)** Induction of roots after BAAT-treatment with *A. rhizogenes* LBA9402 to mediate transformation. In this particular instance, a slight bacterial film developed over the explant upon transfer onto cefotaxime-free medium. Roots were induced after transfer to MS with kanamycin, indicating their tolerance for this antibiotic. **(F)** Transformation methods tested on root explants were not successful in inducing the hairy root phenotype instead abundant callus proliferation initiated from the cambium was noted. This callus became embryogenic upon transfer onto selective medium lacking in PGRs. **(G)** Transgenic roots growing into selection medium containing kanamycin (100 mg l^{-1}).

Callus transformation appears to be successful in the hands of other researchers. Different carrot cell culture lines have also been successfully transformed using *Agrobacterium* or particle bombardment and analysis for transient expression was positive (DEROLES *et al.*, 2002). These authors, however, made no attempts to generate stable transformants.

The resilience of *T. garganica* against transformation using standard methods resulted in a double transformation system being tested. Transformation via BAAT 2 proved to be the most effective method for *T. garganica* explants. The shoot base was the most useful explant for transformation with all the methods tested. It was speculated that the cells of the shoot bases were more amenable to Ri-transformation and perhaps these cells have a higher potential for producing root initials. This is where roots naturally originate upon receipt of the correct stimuli for rhizogenesis. In culture, root induction at the base is more reliably stimulated, for instance, by application of high concentrations of auxins (5 mg l^{-1}) and transfer onto ½ MS medium with charcoal as an adjuvant (Chapter 4). The type of rhizogenesis occurring under such conditions was similar to that noted on shoot bases after BAAT-delivery of *rol* genes. Extensive rhizogenesis at the shoot bases was realised on MS medium without PGRs after the utilisation of the two-phase transformation and no rhizogenesis occurred in control explants. This suggested that root proliferation was more likely to be a consequence of BAAT-treatment rather than spontaneous induction.

Thus, the application of BAAT on *T. garganica* tissues was able to induce rhizogenesis at a low frequency (3 %) (Figure 6.3B and G), at the site of infection. Roots did not exhibit characteristic features of the hairy root syndrome, albeit elongating but failing to propagate *en masse* in liquid shake culture. The *rol* genes were assumed not to express their rooting function in *T. garganica* fully (for more comprehensive discussion, refer to Chapter 7). Recently, LUCAS *et al.* (2000) applied similar principles for the transformation of *Helianthus annuus* L., a species that is not very amenable to transformation. Utilising a BAAT 1 approach for transformation of *H. annuus*, these authors were able to devise a method that was reproducible for obtaining transgenic plants with immature embryos.

Recently, there has been some debate regarding the temperature which best promotes *vir* expression; particularly bacterial attachment and T-DNA transfer. Growth of *Agrobacterium* at a lowered temperature of 19 °C as opposed to the traditionally used temperatures, between 25 and 30 °C, increased the transformation efficiency in tobacco explants tested by FULLNER *et al.* (1996). T-DNA transfer has also been shown to be thermo-sensitive with temperatures of 19 - 22 °C being optimal compared to the traditionally used co-cultivation of 25 °C (DILLEN *et al.*, 1997). Similarly, data obtained for transient GUS expression in garlic by KONDO *et al.* (2000) were in good agreement with those of DILLEN *et al.* (1997) and the former group further suggested that the optimal temperature for transformation is dependent on the *Agrobacterium* strain and explant material used. In this particular study, the lowered temperature was assumed to be instrumental in facilitating transformation as the 3 % of explants (Table 6.2) that responded to transformation with BAAT 2 were transformed with cells of *Agrobacterium* grown at 19 °C, a result not previously obtained with the other experiments conducted at higher temperatures. Perhaps the more effective means of wounding through the use of the particle gun, which in actual fact results in microwounds, plus the transformation with the binary vector via biolistic transfer as well as the lowered temperature to facilitate better pili formation for agrobacterial attachment and the stimulatory effect of acetosyringone provided conditions that complemented each other and were altogether more condusive to transformation of *T. garganica*.

It is not common to report the transformation or problems associated with the transformation of difficult or recalcitrant species as pertaining to introduction of Ri genes with the intention to produce hairy root cultures. One of the few reports on the species that were difficult to transform in their laboratories was compiled by HAMILL and LIDGETT (1997). This list included species such as *Amaranthus retroflexus*, *Sedum acris*, *Coleus blumei*, *Capsella bursa-pastoris*, *Thlaspi arvensis*, *Achillea millefolium*, *Matricaria chamomilla*, *Senecio vulgaris*, *Hibiscus esculentus*, *Llysimachia vulgaris*, *Borrago officinalis*, *Silene inflate*, *Rumex obtusifollus*, *R. acetosella* and several species of *Euphorbia*.

Researchers are often reluctant to report on species that have failed to respond favourably to genetic transformation with the Ri genes. It is also not obligatory or mandatory to report in scientific publications the number of attempts or trials performed to obtain successful transformation with the *rol* genes. Even with plants where transformation is successful, transformation efficiencies are not reported at all times in the literature (DREWES and VAN STADEN, 1995), thus making the task of evaluating the number of trials taken to achieve a successful result difficult for the reader. For instance, out of the experiments conducted by DREWES and VAN STADEN (1995) the susceptibility of *S. mauritianum* was low, with only one single inoculation of an explant being successfully transformed. In 1997, HOSOKAWA and others reported that the medicinally important genus, *Gentiana*, is difficult to transform with attempts on this herbaceous species failing with strains of *A. tumefaciens* as indicated by the lack of positive GUS expression. This group of authors only achieved success with *A. rhizogenes* A4. HAMILL and LIDGETT (1997) also emphasised that an account of the number of species that have proven difficult or recalcitrant to transformation after numerous transformation experiments is impossible to put together with accuracy, due to the failure of researchers reporting such results as it is unconventional. In almost all the scientific papers published, only accounts of successful transformation are reported.

Amongst some of the species that were listed by HAMILL and LIDGETT (1997) as being difficult is *Castanospermum australe*, which only induced roots from seedlings after transformation, but these were never established as a hairy root culture due to a lack of response to standard growth media. On occasion, transformation attempts of specific members of a family is possible with slightly altered transformation conditions such as the successful transgenic root culture system developed by MACRAE and VAN STADEN (1993) for *Eucalyptus grandis*, a subtropical species, after utilisation of an agropine strain, LBA9402. On the contrary, transformation of other temperate *Eucalyptus* species using agropine strains failed completely (HAMILL and LIDGETT, 1997).

MUGNIER (1988) reported numerous species whose transformation for hairy root induction was limited. In most cases the transformation was successful to some

extent but the hairy root cultures failed to establish easily or proliferate with ease, if at all. Some families such as Ranunculaceae and Papaveraceae were altogether recalcitrant to transformation with *A. rhizogenes* A4. Other research groups have also expressed their lack of success in transformation, for example, *Papaver* species (reported in HAMILL and LIDGETT, 1997). One report of gene transfer to *Papaver somniferum* with *A. rhizogenes* described the difficulties with establishing and maintaining a stable culture of the transformed roots of this particular species. HAMILL and LIDGETT (1997) advocated the use of different wild isolates or recombinant strains of *Agrobacterium* with altered groupings of Ri genes combined with optimisations of tissue culture practises, pertaining to growth media and culture conditions, as a focus with intention to transform problematic species.

For genotypes that do not respond favourably to Ri transformation, where the efficiency of transformation is low, steps to increase virulence of strains are amongst the modifications often undertaken. These include the utilisation of *vir* regions of supervirulent plasmids in order to enhance transformation in *trans* of *A. rhizogenes* (CHRISTEY, 1997). Improvements on traditional methods and/or invention of novel methodologies, also enhances the probability for genetic transformation in difficult species. Recently, the transformation of a Mediterranean strawberry using *Agrobacterium* cells as a coating for microprojectile bombardment (DE MESA *et al.*, 2000) proved extremely beneficial for improving the efficiency of transformation in this species versus conventional *Agrobacterium*-mediated transformation. For future studies of *T. garganica,* such a novel strategy may hold much promise for competent *rol* gene delivery.

6.4 CONCLUSIONS

Using standard procedures generally applied in other Apiaceae, the transformation of *T. garganica* for the introduction of *rol* genes proved to be challenging. Conventional application of strains of *Agrobacterium* tested failed to induce rhizogenesis in *T. garganica* plants. Nevertheless, the results from this investigation are valuable as they have led to the identification of *T. garganica* as a difficult species to transform, thereby providing an explanation for the negative

results obtained by JÄGER *et al.* (1993) in their attempt to transform this species using leaf explants. Conventional *Agrobacterium*-transformation alone was not able to induce the hairy root syndrome in that preliminary study. We have shown that putative transformation of *in vitro*-derived shoot bases as explants is possible upon application of BAAT 2 – a two-phase regime for transformation. Although meeting with limited success, the experiments presented in this Chapter attest to the merits of combining two powerful methods for genetic transformation - biolistic transformation and *Agrobacterium,* for recalcitrant species. Several modifications to the protocol presented here may optimise gene transfer into *T. garganica* and thus allow for the adoption of such a protocol in other related plant taxa, should similar problems be experienced. Since they easily lend themselves to transformation, seedling hypocotyls are possibly another explant for future consideration. The usefulness of somatic embryogenic cultures in the production of transgenic plants in other genera coupled with the ease of production and development in *T. garganica* may prove a more amenable tissue for transformation and requires further exploration. To extend our understanding of the *Agrobacterium*-plant interaction in *T. garganica*, optimising conditions for BAAT as well as employing *Agrobacterium*-coated microprojectiles is proposed as a focus for future experimentation.

CHAPTER 7
ANALYSIS OF PUTATIVE TRANSGENIC PLANTS

7.1 INTRODUCTION

The advantage of root organ cultures as a tissue culture system apart from being easy to handle, is their genetic stability as regenerated roots carry faithful genetic copies of the mother root and are thus similar to the root shape of the parent (OUZUMI and KOBAYASHI, 1997). The *rol* gene-transformed phenotype is typically represented by extensive lateral branching (TEPFER, 1983; TEPFER, 1984) and if plants are regenerated from the Ri-transformed roots wrinkled leaves and shortened internodes are also symptoms that may be expected from stable integration of the transgenes. However, differential gene expression may control the degree of the representation of the symptoms (TEPFER, 1999). Another advantage of using Ri-transformation is that transgenic plants may be produced without the use of chemical compounds that have the potential to inhibit further growth and development, during selection of transformants. High rates of co-transformation on the second binary vector occur in the absence of selective pressure (CHRISTEY, 1997; TEPFER, 1999). The utilisation of the standard protocols for *A. rhizogenes*-mediated transformation generally ensures the prevention of chimaeric plants being regenerated. Compared to *A. rhizogenes* transformation, *A. tumefaciens* may result in non-transformed cells being regenerated due to the nurse effect of surrounding cells during selection and thus resulting in higher frequencies of escapes and/or chimaeric plants regenerating (CHRISTEY, 1997). On the contrary, stable integration of the *rol* genes may be confirmed by the generation of hairy roots in culture, with root-tip culture ensuring the selection of only stable transformants after several subcultures. Another advantage is that long-term hairy root cultures seem less plagued by the occurrence of somaclonal variation in comparison to callus cell cultures and less incidence of cytological abnormalities are apparent in hairy root cultures. Due to

the inherent genetic stability and incremental growth frequency compared to normal cultures, hairy roots are primarily employed for the synthesis of secondary metabolites even though there are individual instances where the production of the phytochemical is higher in other organs such as leaves as compared to transgenic roots. There are two major biotechnological strategies utilised for obtaining secondary metabolites from transgenic roots and these are 1) a combination of the use of bioreactors and effective control techniques for production of a high density hairy root culture; and, 2) regeneration of transgenic microplants from transformed hairy roots. For studies focussing on root-organism interactions, hairy roots are becoming increasingly important as they offer a simplified and controlled environment *in vitro* as a means of understanding such complicated interactions, as for example the interaction of vesicular-arbuscular mycorrhizae obligate organism interactions with the root systems (MUGNIER, 1997).

7.1.1 Hairy roots in culture

According to YU *et al.* (1997) the culture conditions affect behaviour of transgenic hairy roots whether in a shake-flask or bioreactor system. Several media components such as nutrient level, carbon source, plant growth regulators and osmolality of medium have been extensively investigated and reports are abundant in the literature. The medium pH, incubation temperature and gas phase carbon dioxide concentrations are also important factors that influence the growth rate and behaviour of hairy roots as a whole. Hairy roots are regarded as heterotrophic, respiratory organisms that rely on oxygen for generating energy and other functions; and thus an inadequate supply of oxygen can influence the growth rate plus also directly affecting the synthesis of specific secondary compounds. The most common method of growing hairy roots is in submerged liquid-shake culture or bioreactors. Under such conditions of culture, hairy roots tend to exist as dense root mats or clumps growing to a diameter similar to that of the culture vessel. Submerged liquid culture is considered unnatural as it forces the plant material to be in a waterlogged state and few plant species can naturally tolerate a continuous state of water-logging. Other methods of bioreactor-engineering available that do not impose as much water-logging on tissues include the use of nutrient mist, a

trickle beds and rotating drums. In such systems the roots are not continually submerged but a liquid boundary layer is available to prevent dehydration of the tissues. In liquid-shake culture, several factors are of important consideration for general health of hairy roots and these include the type of closure for the vessel, the shaking speed, the size of the flask and the volume of liquid. Gas-liquid transfer occurs by absorption in the thin liquid film thrown up onto the flask wall by the shaking motion and the presence of hairy roots tends to lessen the motion of the liquid and the efficiency of this process.

CARVALHO *et al.* (1997) extensively reviewed the important factors influencing the establishment of hairy root cultures in bioreactors that are suitable for industrial application. Morphology of hairy roots becomes an important factor for consideration with intention to scale-up as a practical and suitable system for industrial application, more especially for bioreactor design and operation. The thickness of the roots is dependent on the plant species. The selection of transformants for the establishment of root cultures is naturally based on the growth rate. Those with a higher growth are assumed to have the higher biosynthetic potential as the growth rate is directly proportional to the production of secondary metabolites. It has also been noted that the age of the culture has an effect on the morphology where maintenance of transformed root cultures over long periods of time not only changes the morphology but also causes variations in the growth rate. Transformed hairy root cultures are often described as 'highly branched' and the degree of branching is largely dependent on the frequency of branch formation as compared to root elongation. Branching in a soil environment is critically important for determining nutrient and water uptake. Roots thus display considerable plasticity in response to environmental conditions and in an *in vitro* environment alterations in different factors can significantly alter the degree of branching of hairy roots. Higher growth rates also tend to correlate with higher levels of branching in culture and the growth rate is affected by the activity of growth meristems. In root clones where greater dominance is a feature, less branching occurs and the entire root mass has a more uniform availability of nutrients as compared to highly branched forms with higher localised clumped areas. Proliferation in such cases is often hindered by the inner regions of the

clumps suffering from inadequate oxygen availability. One of the typical features of hairy roots is the prolific production of root hairs, as suggested by their name. These root hairs are important for nutrient uptake due to increased surface area. In nature, increased root hair production is associated with limiting nutrient availability and thus root hairs are produced to facilitate more effective uptake of nutrients. However, they can also result in a stagnant boundary layer which is inhibitory to nutrient transfer in culture.

The biosynthetic ability of hairy roots for secondary metabolite production is also affected by various factors, including *Agrobacterium* strains and medium components. Optimal growth conditions are investigated using a random approach which is time-consuming and labour intensive. Apart from media manipulation studies, the metabolite enhancement can thus be manipulated by using fungal elicitors, *in situ* extraction, and manipulation of signal transduction using enzymatic control (SINGH, 1997). At the molecular level, the morphology is affected by the location and number of T-DNAs integrated into the plant genome (CARVALHO *et al.*, 1997). The use of multiple transformations using the same species of *Agrobacterium* may introduce morphological variations and affect the growth rate of the transformed roots. Transgenes are susceptible to silencing in all plant species studied as a result of multiple copy insertion interaction. Additional copies of endogenous genes also become silenced when expressed ectopically due to homology-dependent gene silencing (HDGS). Methylation of the silenced loci is often observed where regions are homologous to each other (SRINIVASA-REDDY *et al.*, 2003). Variation in hairy root morphology is largely controlled by the number of T-DNA integrations. Different chromosomal insertions can affect expression. The number of copies integrated into the host genome is unpredictable as a result of the transformation event and including the position of the insertion event regardless of the method of transformation (VAUCHERET *et al.*, 1998). The current limitations to genetic transformation of plants are a low frequency of transfer and high rate of unpredictable transgene expression as well as uncontrollable genetic change. It is thus necessary to perform transformation treatments and screening procedures on a large-scale in order to produce transformants with the desired traits. The key to transformation of the so-called

recalcitrant species is thought to be the development of methods that allow for many regenerable cells to be exposed to non-destructive transformation methods.

The targeting of genes into specific areas of the genome that are transcriptionally active would thus ensure better or more predictable transgene expression. The constraints posed by transgene silencing need to be considered and dealt with in the future. Already, research is now currently being conducted to target transgenes into transcriptionally active areas of the genome so as to ensure low copy integration and more predictable expression (GELVIN, 1998b).

The major goals of this Chapter were two-fold; firstly, we aimed at introducing the putative transgenic roots into a liquid-shake system and monitoring their growth and subsequently determining the best medium for their regeneration. Secondly, the study analysed the putative transformants using molecular methods and to determine whether *rol* genes were stably integrated.

7.2 MATERIALS AND METHODS

7.2.1 Evaluation of *nptII* gene expression and liquid root culture initiation

Few explants initiated roots as a result of transformation experiments. Selection for the expression of the *nptII* gene involved the growth of all tissues that had been through the transformation process on medium supplemented with 100 mg l^{-1} kanamycin and their growth was monitored. Explants were placed on the medium supplemented with cefotaxime (250 mg l^{-1}) for the first four weeks after transformation, thereafter the concentration was halved and it was included in the medium at a concentration of 125 mg l^{-1} to inhibit the regrowth of *Agrobacterium* for one month prior to transfer of transformants onto solid medium with kanamycin at 100 mg l^{-1}. Root growth was monitored by measuring the length (mm) every 3 days at the same time, upon root induction. Root tips (2 cm in length) that survived the selection process were finally transferred onto liquid medium without antibiotics. They were grown in the dark in 50 ml flasks, plugged with a cotton wool bung covered with a tin foil cap, on an orbital shaker (120 rpm) at 22 - 24 °C. The liquid medium was changed after four weeks in the first cycle of liquid-shake

culture thereafter, every two weeks, the roots were transferred into clean flasks and fresh growth medium was added. The growth response of the putative transgenics was monitored.

7.2.2 Towards an efficient medium for hairy root growth

The growth response of putatively transformed roots in liquid culture was not phenotypically characteristic of hairy root cultures. Thus, different types of media were tested to determine the most suitable medium for encouraging prolific hairy root growth of *T. garganica*.

Two experiments were carried out. Initially MS, ½ MS and ¼ MS (quarter strength MS) were tested for their effect on growth of normal roots in liquid culture. Secondly, Miller's medium (APPENDIX) adapted from MILLER (1965) was compared to MS for its ability to encourage secondary root differentiation. Miller's medium supplemented with IBA (1 mg l^{-1}) was evaluated against MS supplemented with IBA (1 mg l^{-1}). All treatments were placed in the dark at 22 - 24 °C and incubated with shaking (120 rpm) for one month prior to transfer onto fresh medium.

7.2.3. GUS analysis of transformed tissues

For most experiments, β-glucuronidase (GUS) analysis was performed at least 48 h after the transformation event. In some cases, GUS assays were conducted two weeks after transformation and those instances are clearly identified. Tissue selection for the assay was performed on a random basis. The assays were essentially based on that first described by JEFFERSON *et al.*, (1987). Different plant parts were excised for GUS analysis and these were incubated in a solution containing 0.3 % 5-bromo-4-chloro-3-indolyl β-D-glucoronide (w/v) (X-GlcA, Sigma, but for purposes of this study referred to as X-Gluc in order to follow scientific convention), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 100 mM sodium phosphate buffer (pH 7.0) and 0.005 % Triton X-100 (v/v). The principle of this method essentially involves the indolyl cleavage of the substrate through

oxidative dimerisation to yield a highly coloured product, 5'5'-dibromo 4'4'-dichloro-indigo, and this stable blue/purple precipitate allows for histochemical localization of the product of the *uidA* gene. Potassium ferrocyanide and potassium ferricyanide act as oxidation catalysts, enhancing the dimerisation which is stimulated by atmospheric oxygen. The tissue samples were incubated in the staining solution for 16 h at 37 °C in either sealed microfuge tubes or an Elisa plate closed with a lid. When the tissues were assayed in an Elisa plate they were placed under conditions of high humidity in a plastic container to prevent evaporation of water.

After 16 h in the staining solution, they were transferred to room temperature and fixed for 10 min in a fixative (5 % formaldehyde [v/v], 35 % ethanol [v/v] and 5 % acetic acid [v/v]), then transferred to 50 % ethanol for at least 5 min and finally incubated in 95 % ethanol for 3 h to remove the chlorophyll in the tissues as this can camouflage the blue coloured product. The tissues were subsequently rehydrated in a graded ethanol series [v/v] (90 %, 75 %, 60 %, 45 %, 30 % and 15 %) prior to visualization of GUS activity. After they were rehydrated, the last ethanolic solution (15 %) was replaced with distilled water.

Control transformation treatments were also assayed and then compared to putatively transformed tissues. The number of GUS positive tissues was counted and the intensity as well as the extent of GUS activity was recorded. GUS expression was visually scored for each explant assayed according to a scale where GUS activity was rated as +++, ++, + and – denoting strong, intermediate weak and no detectable GUS activity, respectively. The number of blue spots or zones was counted for each explant, where possible.

The localization of the blue precipitate resultant from GUS expression and activity was visualized with a dissecting microscope (WILD HEERBRUGG M400) fitted with a camera for photographical purposes (WILD Photo-automat MPS 55) or a light microscope (Olympus BH-2 light microscope).

7.2.4 Genomic DNA isolation

Genomic DNA for both PCR and Southern hybridization (APPENDIX) was extracted by grinding sample tissues (from putative transgenic tissues) in liquid nitrogen to a fine powder and thereafter the powder was transferred to a 1.5 ml microfuge tube and 500 µl urea extraction buffer (420 g urea crystals, 70 ml 5 M NaCl, 50 ml 1 M Tris-HCl [pH 8.0], 50 ml 20 % sarkosyl [v/v], 40 ml 0.5 M EDTA and made up to 1 l with deionised distilled water). The contents of the tubes were mixed well by vortexing. The sarkosyl was added after the other solutions had been autoclaved and cooled to room temperature. The samples were kept on ice at all times during extraction. After the extraction buffer was added to the samples, phenol extraction was performed by the addition of 500 µl phenol: chloroform (1:1, v/v) and tubes were shaken for 1 h on a table top shaker. The samples were centrifuged to separate the cellular debris from the nuclear extract for 15 min at 15 000 rpm using a desk-top microcentrifuge with a fixed angle rotor (Sigma-113). The top aqueous phase was then transferred to a new tube and the nucleic acids were precipitated with 1/10 volume 4.4 M ammonium acetate (pH 5.2) and an equal volume of pre-chilled isopropanol. The tubes were then inverted gently several times to precipitate the genomic DNA and placed at – 20 °C for 20 min. The nucleic acids were collected by centrifugation at 15 000 rpm for 15 min, rinsed with 70 % ethanol and washed with 100 % ethanol prior to vacuum drying the pellet for 5 to 10 min. Once the ethanol, had evaporated the pelleted DNA was resuspended in 50 µl distilled water and thereafter stored at – 20 °C until analysed via electrophoresis to check its integrity (Section 6.2.14) or amplified via PCR.

7.2.5 PCR analysis of transgenes

Genomic DNA, isolated as described in Section 7.2.4, was used as target DNA. The DNA that was resuspended in distilled water was utilized as the EDTA in the TE buffer can act as a chelating agent, sequestering magnesium ions that are essential in the PCR reaction for the functioning of the *Taq* polymerase. Transformation of putative lines was confirmed by PCR mediated amplification of the *rol* A, *rol* B, *rol* C primers genes. All the primer sets used in this study were

synthesized by Roche Biochemicals (Table 7.1). The primers were delivered as freeze-dried samples and were resuspended in sterile ultra-pure water upon arrival and prepared as 100 µM stock solutions and then stored at – 20 °C until required. The homology of primers was confirmed using the Basic Local Alignment Search Tool (BLAST), for the use of BLAST analysis the reader is directed to visit http://www.ncbi.nlm.nih.gov/BLAST/ .

Table 7.1: Oligonucleotide primer sets tested for PCR-mediated amplification of *rol* genes

Primer Set	Sequence
rol A1	5'-ATGGAATTAGCCGACTAAACG-3'
rol B1	5'-ATGGATCCCAAATTGCTATTCCTTCCACGA-3'
rol B2	5'-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3'
rol C1	5'-ATGGCTGAAGACGACCTGTG-3
rol C2	5'-GCTCCATCTGCTCATTCAGC-3'
vir D1	5'-ATGTCGCAAGGCAGTAAGCCC-3'
vir D2	5'-GGAGTCTTTCAGCATGGAGCAA-3'

Several PCR regimes were tested for their ability to amplify *rol* genes. The thermostable *Taq* (Roche) was supplied with a standard 10X PCR buffer. When reaction conditions were being optimized the concentration of $MgCl_2$ was also manipulated and the PCR buffer used in these experiments was supplied by Perkin-Elmer but it lacked $MgCl_2$. Magnesium ions which are necessary for efficient activity of the enzyme were then added separately.

The regimes tested are listed below:

i) The amplification reaction (50 µl total volume) contained 50 ng of genomic template DNA, 1 µM of each primer (*rol* B1/B2, *rol* C1/C2 or *vir* D1/D2 per reaction; Table 7.1), 20 µM of each dATP, dTTP, dGTP and dCTP, 1X PCR Buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl, 0.01 % gelatin [w/v]) and 2.5 U *Taq* DNA polymerase (Roche) plus DMSO (10 %, v/v) to enhance reaction specificity and efficiency. Samples were overlaid with 50 µl paraffin oil to prevent evaporation

whilst amplifying and subjected to 36 cycles of amplification (PC-960G gradient thermal cycler). The first 35 cycles each comprised of a 94 °C melting temperature (30 s), a 60 °C primer annealing temperature (30 s) and a 72 °C primer extension step (45 s). During the final cycle an extension step of 5 min was used. Upon completion of amplification, reactions were electrophoresed immediately or stored at – 20 °C until electrophoresis on 0.8 % agarose gels (w/v), as previously described (Section 6.2.14).

ii) The reaction mixture had the same contents as described above (Section 7.2.5i) except that 1 µM *rol* B primer set (Table 7.1), 200 µM of each dATP, dTTP, dGTP and dCTP and 0.5 U *Taq* DNA polymerase were used. Thereafter, the reaction was overlaid with mineral oil. Thermal cycling comprised of a initiation cycle (denaturation step for 5 min at 95 °C, a primer annealing step for 2 min at 55 °C and primer elongation for 72 °C min) followed by 30 cycles (denaturation step for 1 min at 95 °C, primer annealing step for 2 min at 55 °C and primer elongation for 72 °C min). An extra cycle with a 10 min extension step at 72 °C was also programmed into a temperature-control system for the PCR (PC-960G gradient thermal cycle) to be carried out.

iii) A PCR method originally devised by WAN and LEMAUX (1994) was also evaluated for its efficiency to detect *rol* genes. Amplifications were performed in a 50 µl reaction with *Taq* DNA polymerase (Roche) using a Hybaid thermal cycler. The reaction components were exactly the same as those described in Section 7.2.5i. In a sample PCR protocol, the regime consisted 1 min denaturation step at 95 °C, 30 s annealing steps (5 cycles at 65 °C, 10 cycles at 60 °C, 10 cycles at 55 °C) and 2 min elongation step at 72 °C. The final cycle was an extension step with a duration of 7 min at 72 °C. The target DNA was amplified for 25 cycles (Hybaid thermal cycler, Figure 7.2) prior to electrophoretic analysis on a 0.8 % agarose gel and examined for products indicating presence of *rol* A, *rol* B and *rol* C gene integration into the *T. garganica* genome. This

protocol was also used to detect the presence of *rol* products amplified via the TL-*rol* A_1 and TL-*rol* C_2 oligonucleotide primer set.

iv) Analysis of the integration of *rol* A was performed according to the methods outlined by SÉVON *et al.* (1997) using a single primer (*rol* A; Table 7.1) to anneal to template DNA. The following amplification regime was utilised: initial denaturation at 94 °C for 2 min for the first cycle, thereafter 24 cycles under identical temperature conditions followed but the time of each step was reduced to 1 min.

7.2.6 Optimisation of the components of PCR reaction

Several measures were taken to optimize and enhance reaction specificity and efficiency (Table 7.2). These included the addition of 0.5 µl dimethyl sulphoxide (DMSO) per 50 µl reaction plus the manipulation of the concentration of magnesium (Table 7.2) to obtain an optimal concentration (RIEDEL *et al.*, 1992) for correct fragment amplification. The Perkin-Elmer PCR kit, with $MgCl_2$ supplied separately, was used in this instance. Magnesium can reduce non-specific priming and enhance specificity of the amplification reaction (RIEDEL *et al.*, 1992). The concentration of DNA template (50, 100 or 250 ng), dNTPS (20, 100 and 200 µM) and the *Taq* polymerase concentrations (0.5, 1.25, 2.5 or 5 U) were tested for their effect on product generation. Each of these parameters was tested individually, i.e. one parameter was manipulated at a time.

In most instances a cocktail with all the components of the reaction mixture was prepared to ensure uniformity of the reaction mixtures and then it was divided as aliquots into separate microfuge tubes prior to amplification via PCR. For all the amplifications, several control reactions for PCR were set up to detect spurious amplification and other errors due to contaminating DNAs. The *vir* D1 and *vir* D2 primer set was used to detect the presence of the virulence region of *A. rhizogenes* plasmid and thereby test for contaminating residual agrobacteria. Untransformed genomic DNA template was also used as a negative control and the absence of PCR products for this particular sample was expected. On the other hand, positive

amplification was anticipated when plasmid DNA was incorporated into the amplification reaction due to primers annealing to genes of the *rol* operon on the TL-DNA. Amplification products of *rol* B and *rol* C were compared to Molecular Weight Marker XIV (Roche) whereas amplification products of the TL-*rol* A_1 and TL-*rol* C_2 were run alongside Molecular Weight Marker III (Roche).

Table 7.2 Components of the PCR (50 µl) manipulated for *rol* analysis

PCR Components	Concentrations evaluated				Optimal reaction
DNA (ng)	25	50	100	-	50
Primers (µM)	N/A	N/A	N/A	N/A	1
Taq DNA polymerase (U)	0.5	1.25	2.5	5	2.5
DNTPs (µM)	20	100	200	-	20
$MgCl_2$[a] (mM)	0.75	1.5	3	-	1.5
DMSO (%; v/v)	1	5	10	-	10
10x PCR BUFFER[b] (µl)	N/A[c]	N/A	N/A	N/A	5

[a] The Perkin-Elmer PCR kit, with $MgCl_2$ supplied separately was used for determining the optimal concentration of Mg^{2+} ions required for *rol* amplification
[b] When the concentration of $MgCl_2$ was manipulated, the buffer without $MgCl_2$ was employed
[c] NA refers to reaction components that were not manipulated and the concentrations were as described for Section 7.2.5i

7.2.7 Nomenclature for putative transformants

Root clones were differentiated according to the transformation method used, the type of explant used as target tissue and the vector used as a vehicle for transformation. In Table 7.3, BAAT 2-transformed tissues have been abbreviated to BT and SAAT transformation has been coined ST. The abbreviation EV refers to use of *ex vitro* material as target tissue for transformation and the letter C is an abbreviation for callus tissue. Thus, putative transgenic clone PB-IVpGUS1 denotes tissues transformed by particle bombardment (PB) using *in vitro*-derived tissues (IV) and the pGUS1 plasmid (Table 6.1, Chapter 6).

7.3 RESULTS AND DISCUSSION

7.3.1 Characterisation of kanamycin resistance and GUS activity

In plant transformation, use of antibiotics and herbicides as selectable markers is wide even though the regeneration rate may be reduced and some plants are insensitive to selection agents (MINLONG *et al.*, 2000). Most vectors, with the exception of LBA9402, used in this study contained the *nptII* gene encoding for kanamycin resistance. Thus transfer of this chimaeric gene can be selected by the ability of cells to grow in doses of kanamycin that are ordinarily lethal to the plant. In contrast, tissues that were not transformed turned brown and died within 30 days on medium with 250 mg l^{-1} kanamycin. Line PB-IV (gus1) (22.9 % of explants) responded on kanamycin supplemented medium by producing a white soft callus. From the callus-regenerating explants 11.7 % produced a more globular callus indicating a high embryogenic potential (Table 7.2). Similarly, those bombarded with the pBIN19 plasmid of the A4T strain survived kanamycin selection (35 %) and cellular differentiation through somatic embryo induction was observed in 18.5 % of the cultures.

Overall, the explants surviving kanamycin selection were those bombarded with C58C1 (53 %) and A4T (25 %). Even though, survival on medium with kanamycin was an indication of stable transgene incorporation, all the bombarded tissues failed to initiate roots. It is possible that the uptake of the *nptII* gene was not always coupled with the co-integration of *rol* genes. Biolistic transfer of pMRKE15 produced a limited response in selection medium with 17 % of the explants remaining green but failing to regenerate. Of the explants transformed via SAAT, 1.2 % produced roots after treatment. However, transfer onto selection medium killed all the putative transgenic roots. Those explants transformed using LBA9402 were transferred directly onto liquid medium for initiation of hairy root liquid cultures and the selection phase was omitted as LBA9402 is a wildtype strain that lacks the *nptII* gene. Explants that were untransformed with the *nptII* gene showed their sensitivity to kanamycin by losing their green pigmentation, becoming more yellow with passage of time on selection medium. This was an indication of the loss or

breakdown of chlorophyll pigments due to exogenously-applied kanamycin. However, the lines that were resistant to kanamycin allowed for confirmation of their transgenic status. Analysis for stable integration through application of molecular methods was not performed on these lines and therefore, it was difficult to determine the number of escapes or false-positives. Nonetheless, the ability of BAAT-treated lines (BT2-IVgus1/LBA9402 and BT2-IVgus1/C58C1) to initiate roots (Figure 7.1) in the presence of kanamycin was an indication of their transgenic status. Moreover, these roots continued to grow into the selection medium, for example the BT2-IVgus1/C58C1 transformed roots grew at a growth rate of 0.75 mm per day. Kanamycin supplemented medium often reduced the growth rate of the putative hairy roots. It was thought that subsequent transfer of *T. garganica* roots onto non-selective liquid medium would encourage abundant root proliferation. On the contrary, however, upon transfer onto liquid medium their growth rate was comparable to non-transgenic roots. The growth character expressed was untypical of hairy roots as they failed to produce secondary roots.

Table 7.3: Analysis of *T. garganica* putatitve transformants for kanamycin and GUS activity in various parts of plant organs

[a]Line surviving selection	Kanamycin resistance assay		[b]GUS activity
	Survival (%)	Regeneration response	
PB-Cpgus1	23.5	Callus regeneration	-
PB-Cpgus1	5.8	Embryo production	-
PB-CpBIN19	18	Callus regeneration	-
PB-IVpGUS1	22.9	Callus regeneration	++
PB-IVpGUS1	11.7	Embryo production	++
PB-IVpBIN19	35	Callus regeneration	++
ST-IVLBA9402	ND	Root production	+
ST-EVLBA9402	ND	Root production	+++
ST-IVC58C1	0	Root production	+
BT2-IVgus1/LBA9402	45	Callus and root production (4.5%)	++
BT2-IVbin19/A4T	35.3	Callus and root production (9.4%)	++
BT2-IVgus1/C58C1	53	Callus and root production (6.7%)	++

[a]Treatments that did not result in survival of explants on selection medium or where the only regenerative response was the elongation of explants, data has been excluded and not shown
[b]GUS activity was assessed visually following histochemical staining (Section 7.2.3) and ratings of GUS activity as +++, ++, + and – denote strong, intermediate, weak and no detectable GUS activity, respectively
[c]ND refers to explants transformed using LBA9402 where sensitivity was not determined as this *Agrobacterium* does not carry the kanamycin resistance gene

The use of BAAT 2 using pBIN19 DNA-coated tungsten and the A4T strain for the double transformation resulted in the production of a single root from the shoot base. The root produced was 3 mm in length and 1 mm in diameter after 10 days. It grew readily into the kanamycin-supplemented selection medium, indicating its ability to tolerate the selective pressure (Table 7.3). The root was growing at a rate of 0.72 mm day, whereas the putative transgenic roots that had been initiated after bombardment with A4T and exposed to the LBA9402 strain had a growth rate of 0.52 mm per day and they grew to 2.5 cm prior to initiation of liquid root cultures. On the other hand, SAAT putative transformants, when cultured in kanamycin-supplemented medium, were sensitive to the antibiotic and 100 % root mortality was observed. Nonetheless, GUS expression of assayed SAAT-tissues was indicative of positive gene delivery and expression (Figure 7.1A). The loss of kanamycin resistance was presumed to show that although SAAT resulted in transient transformation, co-transformation of both the stable *npt II* gene and GUS gene expression did not occur. In other words, only the GUS gene was stably incorporated as the histochemical GUS assay was performed a month after co-cultivation. In a study conducted by OTTAVIANI and HÄNISCH TEN CATE (1990), selection of transformed roots was based on a growth rate of 2 to 3 cm per week. The authors considered such a growth rate prolific for potato hairy roots even though the presence of kanamycin as selection pressure inhibited the growth rate slightly. As a comparison, roots for line BT2-IVgus1/LBA9402 (Table 7.3) obtained for *T. garganica* in this study grew ten-fold slower and the average growth rate of these roots was 0.45 mm per day.

Variable patterns of activity of the glucuronidase enzyme were observed following assessment of GUS in assayed tissues transformed with microparticle bombardment, BAAT 2 and SAAT whereas none of the other explants transformed via conventional *Agrobacterium* application stained positive for GUS. As indicated in Table 7.3, GUS activity was strongly influenced by the method of transformation used. Direct gene transfer via biolistics and use of BAAT were the most influential on transient gene expression of GUS as a larger percentage of the tested tissues assayed positively for the incorporation of the GUS gene construct. Overall, visual assessment of the intensity of the histochemical staining for GUS expression

elucidated the uptake of the GUS reporter in 64 % of the tissues tested. Although the number of GUS positive foci for BAAT-treated tissues (Figure 7.1C-D) was not necessarily high per explant the intensity of the stain was higher in comparison to GUS-stained foci obtained with either SAAT or microparticle bombardment. Presumably, BAAT 2 application increased the efficiency of T-DNA transfer. The use of *Agrobacterium*-mediated transformation, without special measures being undertaken to facilitate the transformation process, resulted in absence of visually detectable GUS expression and this was similar to wildtype explants that were incubated in X-gluc. It became apparent that there was little to no spurious GUS activity visualized in the control explants as a result of endogenous bacterial contaminants. The appearance of false-positives becomes a concern as contaminating bacteria are able to metabolise X-gluc – a disadvantage of using GUS constructs that do not carry an eukaryotic intron. Inclusion of an intron that is soley spliced by the eukaryotic transcription machinery during processing of GUS mRNA is now an accepted method of reducing false-positives in GUS histochemical assays. Constructs lacking an intron can result in an incorrect impression as the accumulation of a blue precipitate, upon inspection resultant from bacterial contaminants catabolising the chromogenic substrate, can be taken as an indication of positive transient expression and thus increase the number of false-positives. The fact that all the negative controls assayed for GUS expression did not at any stage exhibit the activity of this reporter enzyme, increased our confidence in the results that were obtained. Experiments that omit such controls typically show high errors in resulting data and increase the occurrence of false-positives at later stages of the transformation protocol, for example during greenhouse analysis of transgenic plants. The occurrence of escapes is a major limitation of many transformation methods and the use of intron-inserted selectable markers becomes useful for screening of putative transformants. Another concern is the inefficiency of transformation procedures with respect to the survival and subsequent regeneration of non-transgenic or chimaeric tissues under selection (BHAT and SRINIVASAN, 2002).

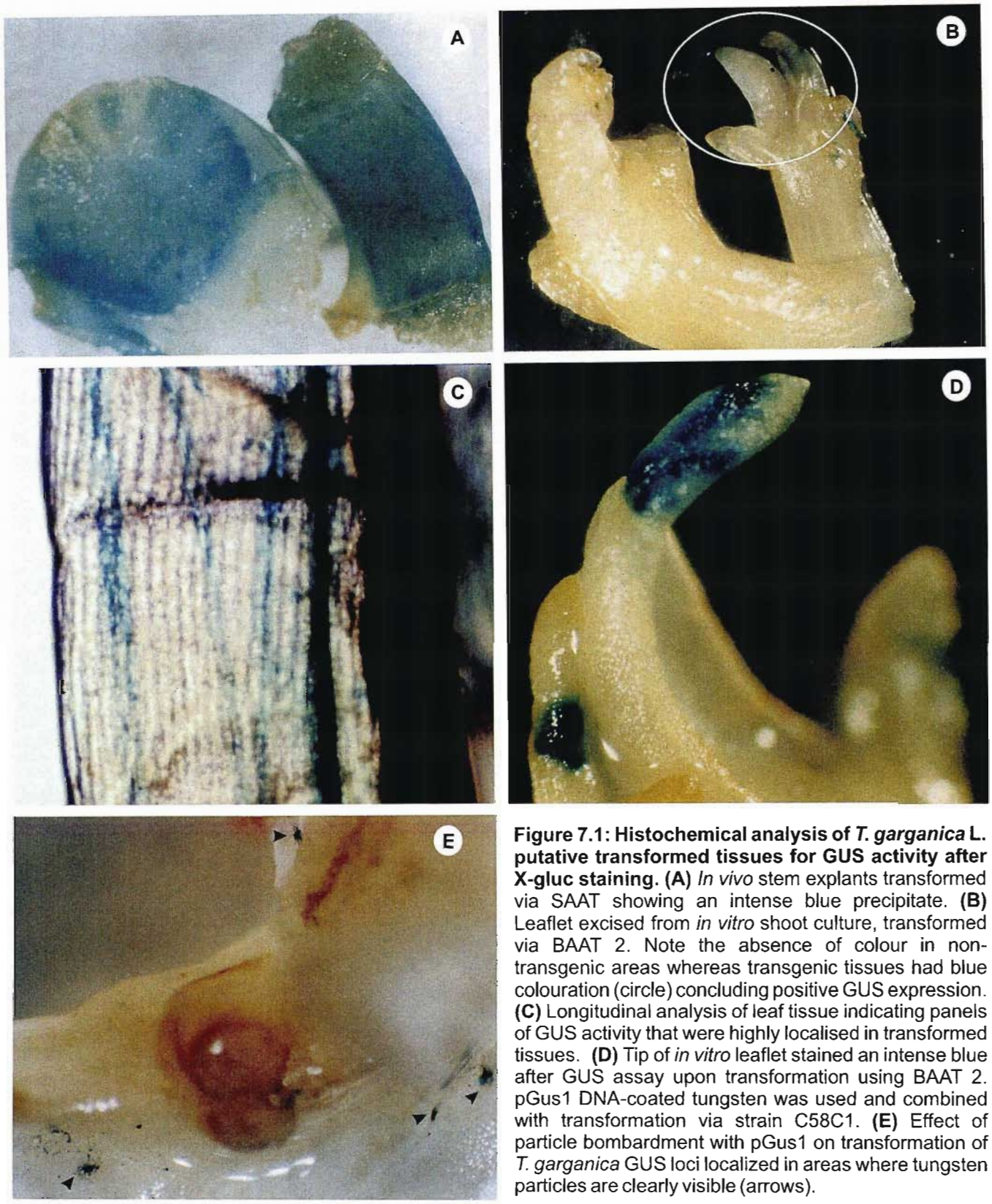


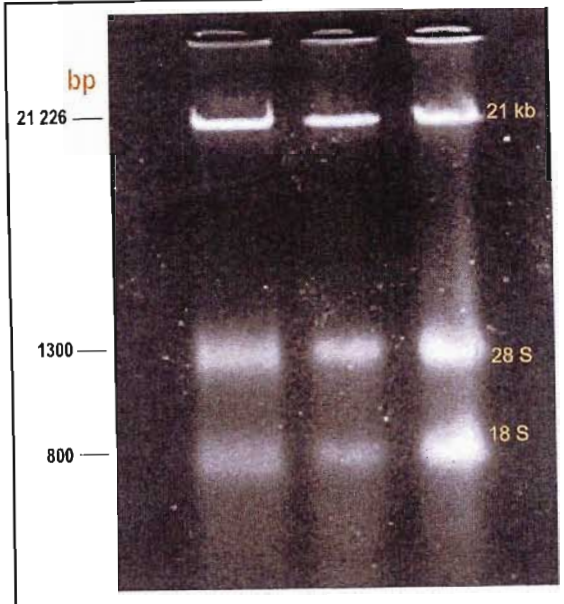
Figure 7.1: Histochemical analysis of *T. garganica* L. putative transformed tissues for GUS activity after X-gluc staining. (A) *In vivo* stem explants transformed via SAAT showing an intense blue precipitate. **(B)** Leaflet excised from *in vitro* shoot culture, transformed via BAAT 2. Note the absence of colour in non-transgenic areas whereas transgenic tissues had blue colouration (circle) concluding positive GUS expression. **(C)** Longitudinal analysis of leaf tissue indicating panels of GUS activity that were highly localised in transformed tissues. **(D)** Tip of *in vitro* leaflet stained an intense blue after GUS assay upon transformation using BAAT 2. pGus1 DNA-coated tungsten was used and combined with transformation via strain C58C1. **(E)** Effect of particle bombardment with pGus1 on transformation of *T. garganica* GUS loci localized in areas where tungsten particles are clearly visible (arrows).

The GUS gene fusion system is popular due to its robustness, versatility and straightforward simplicity (GALLAGHER, 1992). In this study, this system proved highly valuable for the analysis of transient expression and 64% of the tissues assayed throughout the entire study showed positive GUS activity. None of the putatively transformed roots were assayed as the *in vitro* evaluation of GUS activity is a destructive method of analysis (JEFFERSON, 1992) and the number of putative transgenic roots was limited. Assay of transgenic roots would have possibly resulted in sacrifice of those that could have developed more typical symptoms of the hairy root syndrome as the GUS assay is a destructive assay unlike using the Green Flourescent Protein Assay (CHALFIE *et al.*, 1994). However, GUS assays on other explants – such as leaf and shoot bases, were sufficient indication of the efficacy of the transformation methods examined (Figure 7.1) and furthermore, showed that *T. garganica* is transformable using BAAT-treatment, in particular. It was unclear whether tissues that did not express GUS activity were false-negatives or were truly non-transgenic as their molecular analysis was not pursued. Apparently, the lack of detectable GUS activity does not necessarily imply failure of stable transformation because as other researchers have found the *gus* A*luid* A gene to be stably integrated, in spite of plant tissues testing negatively for GUS expression. Results of this nature were reported by OTTAVIANI and HÄNISCH TEN CATE (1990). These authors found that the number of copies of the GUS gene integrated into the host genome had no correlation on gene expression. In this particular study, intense GUS colouration was observed in areas particularly where a high number of microparticles were visible. Therefore, localization of the blue precipitate was in close proximity to the particles (Figure 7.1E) and this indicated particle transfer was responsible for transgene delivery to the cells that had stained blue rather than some other factor. The most *Agrobacterium* effective strains for transformation of *T. garganica* using the diphasic process of BAAT 2 were C58C1, LBA9402 and A4T, in combination with pBIN19 and pGus1. Both large areas and small foci were observed on assayed tissues. Thus, it was presumed unlikely that diffusion of the blue chromogenic precipitate was to blame for larger areas of GUS activity that were visualized after staining. Longitudinal inspection of transformed leaf tissues (Figure 7.1C) also showed highly localized blue-coloured panels with neighbouring

tissues free of colour, providing more evidence to support this presumption. BAAT allowed for localized yet deep microwounding of tissues without apparent damage to occur that facilitated transgene delivery and expression into subepidermal tissue layers. GUS staining can be deceiving as it may give rise to a false impression of a large number of cells being transiently transformed whereas in actual fact the stain had diffused to non-transformed cells.

7.3.2 Molecular analyses for stable transformation

As no other molecular studies on *T. garganica* DNA for *rol* transgenes had previously been conducted, as revealed by the literature, it was necessary to develop an efficient and reproducible PCR protocol for analysis of *T. garganica* plants. An efficient PCR regime would also be of importance to ongoing studies in our laboratories focusing on this genus. PCR is a powerful and sensitive technique (HAMILL *et al.*, 1991) for analysis of transformed tissues for specific integration of introduced genes in plants. It is highly sensitive to contaminating plasmids and other DNAs, and control reactions allow for comparison regarding the efficacy of the reaction as well as being a check for spurious or erroneous amplification. In this study, the modified method of WAN and LEMAUX (1994) was the most suitable method for positive amplification of *rol* genes. The bands resolved were bright (Figure 7.2B) showing positive amplification of plasmid DNA and no bands were observed after EtBr staining in the control wells, as per expectations. In all subsequent PCR, binary plasmid DNA was incorporated as a positive control. The other methods tested were not suitable for amplification of the *rol* genes integrated into *T. garganica* regardless of the amendments made to the basic protocol to enhance the specificity of the reaction. Figure 7.2 illustrates the steps necessary for the amplification of *rol*-specific transgenes in *T. garganica*. All the PCRs conducted also revealed the absence of *vir* genes, again confirming the absence of contaminating agrobacterial cells residual from the transformation process; thus indicating that the efficiency of the decontamination after co-cultivation was effective. This further increased our trust in the positive results revealed upon examination of *T. garganica* DNA when assaying for stable incorporation of the *rol* genes.



A. Isolation of ultra-pure genomic DNA from putative transgenic tissues of *T. garganica* for PCR, using a urea-extraction method. Intact RNA (28S and 18S rRNA) was also isolated.

Steps for optimisation of reaction components for more efficient amplification of *rol* genes

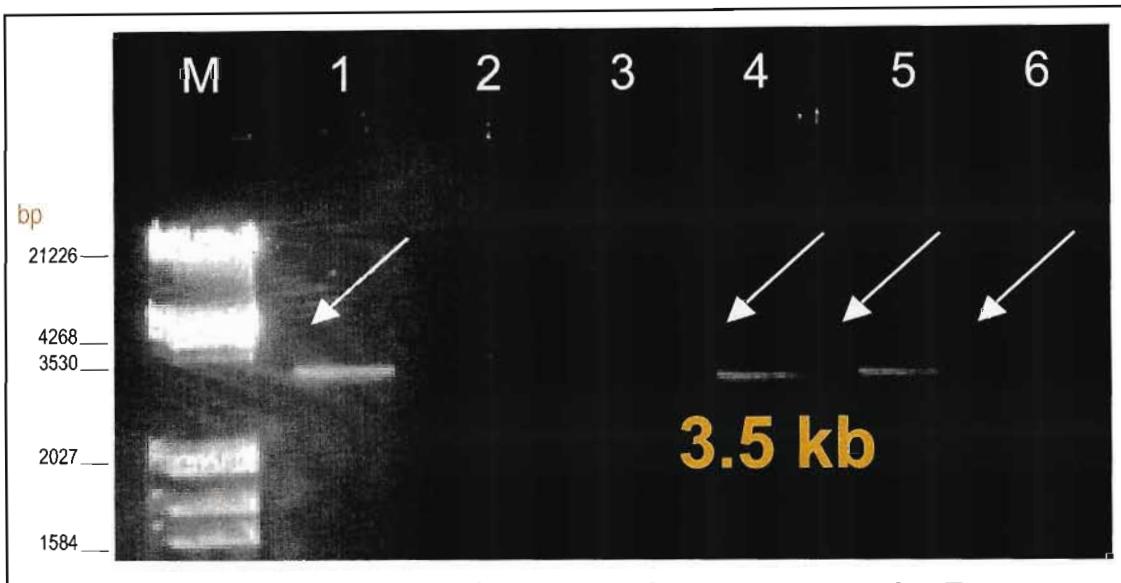
50-200 ng DNA
20-200 µM dNTPs
0.5-5 U *Taq* Polymerase
1 µM Primers
1-10 % DMSO (v/v)

B. Optimisation of the PCR using binary vector DNA isolated from *A. tumefaciens* C58C1 (pGus1) as a template to test the efficacy of the reaction prior to evaluation of stable integration of *rol* genes in putative transformants. The *Rol* B primer pair amplified a 400 bp fragment.

WAN & LEMAUX (1994) MODIFIED PROTOCOL

Cycling regime for PCR detection

PHASE II	PHASE II	PHASE III	PHASE IV
95 °C: 1 min	95 °C: 1 min	95 °C: 1 min	95 °C: 1 min
65 °C: 30 s	55 °C: 30 s	60 °C: 30 s	55 °C: 30 s
72 °C: 2 min	72 °C: 2 min	72 °C: 2 min	72 °C: 7 min
5X	9X	10X	1X



C. Confirmation of the inclusion of *rol* genes into the *T. garganica* genome was obtained through amplification of a 3.5 kb fragment following PCR-mediated detection using the TL-A_1 and TL-*rol* C_2 primer pair in 5 % of the samples tested. Each gel slot was loaded with 25 µl of the 5O µl amplification product and resolved on a 1 % agarose gel (v/v). The amplicons were compared to size markers (M) generated by digesting Lambda DNA with *Hind* III (Roche).

Molecular analysis using PCR-mediated amplification further authenticated the transgenic nature of *T. garganica* tissues assayed obtained from GUS positive and kanamycin resistant tissues. Integration of *rol* B and *rol* C into the genome of the plants assayed became apparent after PCR, illustrated in Figure 7.2B. Uptake of individual TL-*rol* B and TL-*rol* C genes was revealed in 25 % and 20 % of putative transformants after PCR analysis, respectively, with distinct 400 bp amplicons being resolved following electrophoresis in a 1 % agarose gel (v/v). The TL-DNA encodes for *rol* A, *rol* B, *rol* C and *rol* D (ORFs 10, 11, 12 and 15; respectively) and TR-DNA contains auxin biosynthesis genes. Thus primers specific to amplification of the TL -DNA were utilized. Partial analysis of the *rol* operon with the *rol* A_1 and *rol* C_2 primer combination was conducted as to gain some insight into co-transformation patterns of these genes, as *rol* genes act synergistically. These genes are more effective when activating in pairs compared to single genes. When these genes are singly transformed into the plant, phenotypic expression is specific to each gene in regenerated plants (SCHMÜLLING *et al.*, 1988; SÉVON *et al.*, 1997). It is generally accepted that *rol* B is the most vital of all the agropine Ri genes for induction of root initials even though all the other genes also have a role in root proliferation. *Rol* B is powerful enough on its own to initiate the growth of non-geotropic hairy roots (DICOLA *et al.*, 1997; MINLONG *et al.*, 2000). However, its effect is significantly enhanced by the activity of the other genes of the *rol* operon.

Rol operon specific amplification mediated by the PCR yielded clear, bright 3.5 kb fragments (Figure 7.2C) in 5 % of samples tested partially with the TL-*rol* A_1 and TL-*rol* C_2 primer pair. This implied that the three of the four genes of the *rol* operon were at least stably integrated into the plant genome. These results clearly indicate that *T. garganica* is amenable to stable genetic transformation. Expression of co-introduced genes can vary independently irrespective of their position on the T-DNA recombinant construct even if they are on the same or separate strands. The expression of the transgene is not only influenced by the promoter elements and terminator sequences that flank it but also by enhancer elements located outside the T-DNAs on the plant chromosome. These *cis*-acting regulatory sequences also exert their effect on the promoter and terminator

sequences of the transgene (OTTAVIANI and HÄNISCH TEN CATE, 1990). Tissue culture practices influence both the integration and expression of introduced genes; and in some cases have been attributed to deletion of *rol* genes during regeneration (SÉVON *et al.*, 1997). Failure of *rol* amplification in some cases is due to rearrangements and point mutations occurring at the anneal-sites of primers (SÉVON *et al.*, 1997).

TANAKA *et al.* (2001) recently reported on the transformation of Egyptian clover with the Ri genes. This species has previously been shown by this group to be amenable to transformation but at low incidence, however, prolific root growth was never realized in culture. Addition of NAA (1 mg l^{-1}) resulted in dedifferentiation but also production of new adventitious roots. Investigation into the establishment of liquid cultures upon transformation in that study showed that transformed Egyptian clover lines are variable in their growth capacity and the different levels of *rol* B and *rol* C transcripts were also noted. When *rol* B was not sufficiently expressed active hairy root growth was lost at times. These authors could not always correlate the expression of the *rol* genes after analysis of mRNA transcripts to the growth capacity of transgenic roots. In another study, over-expression of *rol* B resulted in unexpected reduced growth rate of hairy roots. Although *rol* B has a key role in the induction of hairy roots, other genes such as *rol* A, ORF 13 and ORF 14 of the TL-DNA also function significantly in growth characteristic of Ri-transgenic roots.

Relatively little is known regarding the mechanisms that precisely control integration of T-DNAs into the host genome. In a majority of cases, assays for transient expression fail to correlate with expression of stably integrated transgenes. The levels of transient expression often do not match expression of stably integrated genes thus indicating that most T-DNA successfully transferred to the host genome do not stably integrate and subsequently express (GELVIN, 2000).

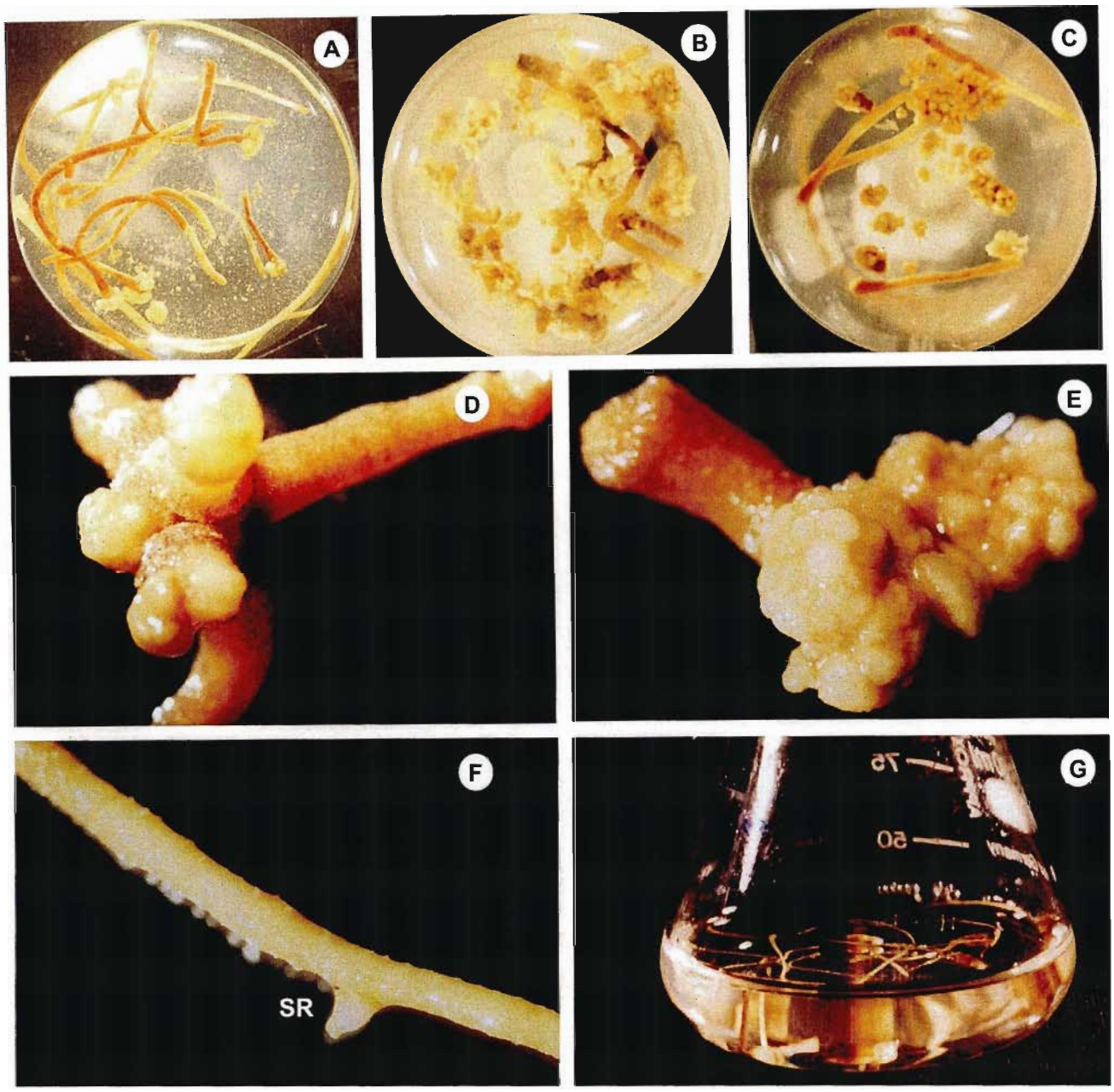


Figure 7.3: Response of transformed roots upon transfer to liquid-shake culture. **(A)** Elongation of putative *rol* transgenic roots grown in ½ MS liquid medium. **(B)** Some of the roots formed secondary roots and produced somatic embryos in modified Miller's medium. **(C)** Proliferation of somatic embryos produced from putatively transformed root tips. **(D)** Enlargement of roots was concomitant with secondary root formation and production of globular embryogenic tissues, at times. **(E)** Globular callus produced from putative transformed roots when grown in the presence of an auxin (1 mg l^{-1}) in modified Miller's medium. **(F)** Incidence of secondary root (SR) production was higher in Miller's medium compared to MS liquid medium. **(G)** Suspension cultures of transgenic roots in MS medium resulted in root elongation without mass propagation.

In this study, establishment of a true hairy root liquid culture for *T. garganica* became complicated as putative transgenic roots failed to response positively and mass propagate (Figure 7.3G). Initially MS, ½ MS and ¼ MS (quarter strength MS) were tested for their effect on growth of normal roots in liquid culture. These media did not stimulate the growth of *T. garganica* roots. Miller's medium (MILLER, 1965) had the most significant effect on root cultures as some of the roots started to initiate secondary roots (Figure 7.3F) and optimization of nutrient composition in the future is needed.

Figure 7.3 illustrates the morphological characteristics of transgenic lines after attempts to initiate a liquid suspension system. Other researchers who have also found the establishment of hairy roots in tissue culture problematic include HANDA (1991) who noted the formation of roots on *Dianthus chinensis*, *Brassica campestris*, *Prunus incisa*, *Lupinus polyphyllus*, *Curcubita pepo*, *Lagenaria siceraria* and *Solanum melongena* after transformation with *A. rhizogenes* but unexpectedly these roots failed to respond favourably as root cultures *in vitro*. HAMILL and LIDGETT (1997) advised the use of other recombinant strains together with optimized tissue culture media for the cultivation of such challenging species.

In this study, the use of modified Miller's medium (MILLER, 1965) without auxin was the most encouraging medium for secondary root formation. Even though, the growth rate was not particularly high compared to control roots, elongation was not the only response noted in culture. The roots increased in size and some secondary branching was observed (Figure 7.3F, SR) although limited to a few cases. The inclusion of IBA (1 mg l^{-1}) in Miller's medium promoted enlargement of roots in the first two weeks of liquid culture and it was hoped that secondary root formation would follow. However, cellular dedifferentiation of *T. garganica* roots occurred with single cells being released into the suspension medium after four weeks and some of these lines (25 %) lost their rhizogenic ability; instead the cells regenerated into somatic embryos (Figure 7.3A-E). Occasionally the root tips regenerated into globular clumps and the ability for generating somatic embryos in liquid suspension culture became apparent (Figure 7.3D-E). Regeneration of

transgenic hairy roots in *T. garganica* remains a challenge as constraints imposed by tissue culture were most probably one of the limiting factors to the occurrence of true hairy roots. It is also possible that insufficient production of the *rol* gene products may be limiting the capacity for establishment of a proliferating hairy root culture for *T. garganica*. A more suitable medium for the regeneration of hairy roots requires optimization and such empirical studies are time-consuming. However, for the intention of establishing a transgenic culture line for thapsigargin production, a prolific regenerating root system would prove more beneficial for adaptation in a bioreactor system.

The histochemical analysis for the GUS reporter as well as genomic inspection through application of the PCR technique conclusively indicated that mechanisms of transgene delivery via *Agrobacterium* were functioning effectively and that *T. garganica* lends itself to genetic transformation upon more penetrative wounding as assisted by the particle transfer process. However, several mechanisms are probably key factors in hindering the true-to-phenotype expression of hairy roots. These include 1) establishment of a liquid suspension system enabling for secondary root formation. The *rol* genes were assumed not to express their rooting function in *T. garganica* fully as surviving roots after selection on kanamycin medium unexpectedly did not present the apparent features of the hairy root syndrome as conveyed in the literature, albeit elongating but failing to propagate *en masse* upon submersion in liquid medium; 2) transgene silencing mechanisms imposed by the use of the BAAT 2 which may have resulted in more than one copy being integrated into the genome (not determined). Particle bombardment based methods are often plagued by transgene rearrangement and as a result tissues transformed using such methods are prone to transgene silencing; and 3) the possibility of the incorporation of transgenes into quiescent areas of the genome. Genetic transformation does not necessarily target transgenes into transcriptionally active specific regions where gene expression is guaranteed to take place.

Transgene silencing mechanisms resulting from transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS) can affect single copy transgenes, endogenous genes and viruses through homology-dependent

processes (CHANDLER and VAUCHERET, 2001). Silencing via TGS is associated with multiple copy integration and complex integration patterns. However, single-copy and simply integrated transgenes are also prone to silencing (CHANDLER and VAUCHERET, 2001). A massive amount of peer-reviewed literature is now accessible on this subject and for example of review publications, the reader is directed to the recent papers by MATZKE and MATZKE (1998); FAGARD and VAUCHERET (2001); CHANDLER and VAUCHERET (2001).

7.3.3 Means of improving gene transfer

CHRISTOU (1995) advised for better understanding of explant-bombardment parameters for optimization of particle gene transfer processes in plant tissues. A better understanding of the biology of pre- and post-bombardment explants would increase the competence of cells for foreign DNA uptake and regeneration. For the last decade, the use of various direct transgene technologies has enabled for transfer of genes into a wide variety of plants but still little is known about the fate of DNA once introduced into plant cells. Methylation due to higher copy integration as a result of particle bombardment leads to transgene silencing (SRINIVASA-REDDY *et al.*, 2003) and even single copy T-DNAs are prone to methylation. Our knowledge of the complicated integration mechanisms often leading to transgene silencing is still limited. There is no sequence specificity within the plant host for integration of T-DNA even though it is thought that with *Agrobacterium*-mediated transformation mechanisms of illegitimate recombination governing integration of T-DNA into the genome supposedly select for potentially transcriptionally active regions. Accuracy of T-DNA transfer into the host remains a problem following the transformation, even including aberrant gene expression resultant from extraneous vector DNAs. It has also been shown that vector DNA is transferable due to the left border being prone to incomplete nicking (HELLENS *et al.*, 2000). Both *Agrobacterium* transformation and direct transfer methods result in vector backbone incorporation as whole circular plasmids are generally applied for genetic modification in plants. Expression of the transgene is adversely affected in most cases as a consequence of vector backbone incorporation into the host genome and is often accompanied by transgene rearrangements. The use of

linearised chimaeric constructs, lacking a vector backbone, is fast gaining acceptance (SRINIVASA-REDDY *et al.*, 2003) and in times to come may become standard practice in order to avoid complex integration patterns.

7.4 CONCLUSION

In summary, methods applied in this Chapter were able to validate that *T. garganica* is amenable to genetic transformation. Positive GUS histochemical assay and molecular analysis using PCR provided proof of the transgenic status of *T. garganica* following transformation. It is evident that upon effective wounding, physiological and genetic processes within *Agrobacterium* were functioning with efficiency to enable for *T. garganica* transformation. These processes include bacterial attachment, induction of *vir* genes, ss T-DNA generation, its subsequent transfer, delivery and integration into the genome of *T. garganica* occurs with sufficient efficacy to enable stable *rol* transformation. Therefore, we conclude that these steps are not limiting for successful *Agrobacterium* transformation with the application of BAAT 2 in *T. garganica*. Although the transformation of this species is possible, it is still problematic as regeneration of true-to-type hairy roots are a challenge. Optimisation of the media components is necessary in order to overcome tissue culture-related limitations for the proliferation of hairy roots of this particular species and for Ri- transformation to become routine in this species. The possibility of transgene silencing mechanisms occurring at the transcriptional level which may be hindering the expression of the *rol* operon upon stable integration cannot be ruled out or disregarded. It is thus speculated that some mechanisms acting at the transcriptional-translational level may be causing a block in the realization of roots phenotypically characteristic of Ri-expression. Such speculation raises interesting questions for which the current study is unable to provide the answers. Nonetheless a more comprehensive molecular investigation into the mechanisms of transgene silencing becomes attractive for future consideration in our laboratories.

CHAPTER 8
GENERAL CONCLUSIONS

Prior to the initiation of this thesis, the only report on an *in vitro* system for *T. garganica,* was reported by JÄGER and co-workers (1993). These authors identified one disadvantage of that particular system and this was the lack of uniformity in the phenotype of somatic embryos produced - an indication of somaclonal variation. Also, as part of that study, generation of plantlets from somatic embryos was not investigated. Generally in apiaceous plants, *in vitro* regeneration without somatic embryo production and acclimatisation has been successful with a few species (EKIERT, 2000).

A reliable and efficient system for micropropagation of *T. garganica* that excluded a somatic embryo stage, has been established successfully, in this study. One of the advantages of this protocol was ready-available leaf material proving most valuable for initiation of cultures as leaf based tissue culture methods are generally favoured for genetic improvement of plants. Decontamination procedures were performed with relative ease and a medium with a high rate of multiplication was established for *T. garganica* micropropagation. Methods applied in Chapter 3 demonstrated that development of *in vitro* propagules in this species may be hindered by several factors including – (1) hyperhydrification of plantlets on medium that is generally favoured for its regenerative potential; (2) rooting is not always reliable on PGR-free medium and (3) acclimatisation proved the most difficult stage of the tissue culture regime. This study attempted to deal with these problems by evaluating other PGR combinations for plantlet regeneration as well as rooting procedures in a solid or liquid system and a variety of transplantation techniques as part of the acclimatisation protocol.

Investigating methods for optimal shoot regeneration coupled with the intention to produce healthier plants proved advantageous for *T. garganica in vitro* culture as an alternative medium for regeneration was attained (demonstrated in Chapter 4).

Furthermore, inclusion of PEG or growth of plantlets in culture jars allowing for better gaseous exchange and reduction of humidity resulted in plantlets that were fitter for *ex vitro* transfer (see Chapter 5). As rooting was difficult, inclusion of an extra step to the micropropagation regime was clearly justified even though extension of culture time can exacerbate the frequency of somaclonal variation. In this particular case, the benefits far outweighed the risks as transplantation rates were significantly improved (refer to Chapter 5). Although, it is often more beneficial to root microplant *ex vitro*, application of rooting powder in this particular investigation was not determined as *T. garganica* shoot bases were an easy target for fungal phytopathogens. This further complicated the micropropagation of this herbaceous plant as weaning of microplants was only possible with the extensive use of fungicides to deter fungal diseases. This research has thus established a regime that allows for micropropagation of a threatened plant species– *T. garganica* as wild populations are limited for attainment of medicinally important thapsigargins. For the application of the regime presented in this thesis to become practically viable for conservation purposes, further improvement of the acclimatisation frequency is necessary. It is thus proposed that future studies should focus on determining at a species-level, the most dominant pathogens damaging tissue culture-derived plantlets of *T. garganica*. This would ensure for better disease management by the utilisation of species-specific fungicides.

Steps for successful *in vitro* propagation of *T. garganica* without a somatic embryogenesis phase are outlined as a schematic illustration in Figure 8.1. This figure also illustrates where the most appropriate stage for instigation of genetic transfer is recommended. The generation of *in vitro* plants also provided sufficient target material for utilisation in genetic modification of *T. garganica* shoots where the base of the shoots were the most amenable to Ri-transformation using BAAT 2 methodology, as demonstrated in Chapters 6 and 7. This explant is easily obtainable and renewable as continuous culture practices on 0.5:1.5 NAA: BA medium provided sufficient material for experimentation. Although the frequency of transformation is low, genetic modification of a large number of shoots bases would increase the number of hairy root clones obtained via this method.

***IN VITRO* METHODS**

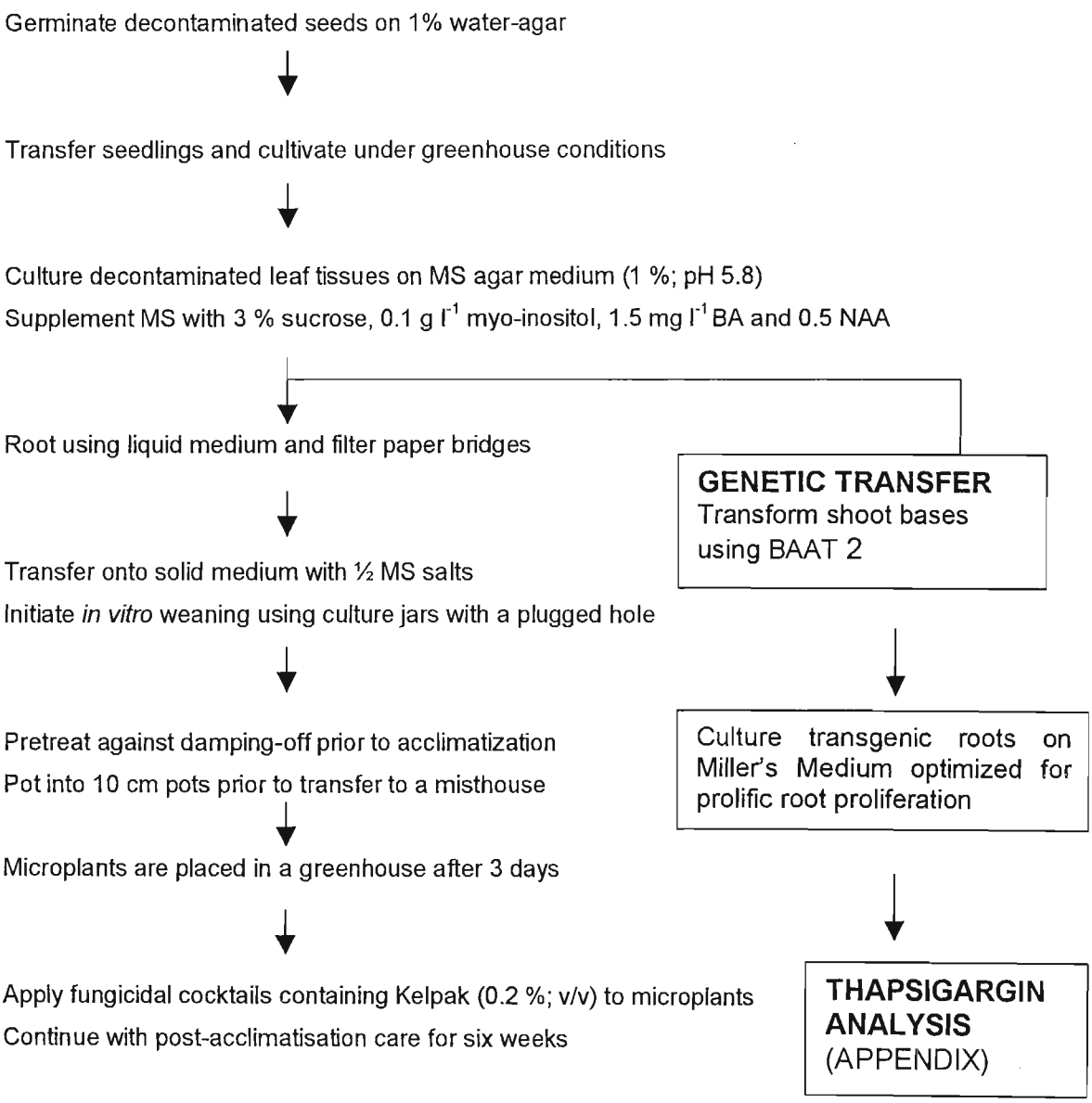


Figure 8.1: A schematic illustration for *in vitro* propagation and genetic transformation of *T. garganica*

Molecular analysis through PCR amplification (Chapter 7) as well as selection procedures on kanamycin supplemented medium plus GUS assaying, convincingly showed that *T. garganica* may be modified genetically. These results draw assumption that the mechanisms of excision and transfer of introduced genes of the T-DNA as regulated by *Agrobacterium* function with credibility albeit *T. garganica* being one of the more recalcitrant dicotyledonous species to transformation. The major stumbling block of the transformation process appears to be at the transcriptional-translational level concomitantly with tissue culture methods not meeting the demands of providing a suitable microenvironment for hairy root establishment. This is not a unique result as other researchers have found inherent difficulties in producing phenotypically true hairy roots in culture in other plant species (as discussed in Chapter 7). It is also speculated that expression of the Ri-genes in this species is probably hindered by mechanisms governing the phenomenon of transgene silencing. It remains a possibility that BAAT, due to the nature of the technique, inserted several copies of the transgene into the genome. This in turn, exacerbated the probability for silencing of transgenes, a phenomenon that requires future inspection in *T. garganica.* Comparative inspection of thapsigargins in transgenic roots versus untransformed roots could not be examined. Future studies of that nature would provide further insight into regulation of thapsigargin synthesis in transgenic cultures.

As a final point, this thesis aimed at developing a micropropagation protocol for *T. garganica* L. and this has been achieved. The second major objective of this study has been met as genetic modification of this plant *T. garganica* was attained and thus concluding that this umbelliferous plant of medicinal importance lends itself to this type of transformation – a first time successful attempt in this species.

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APPENDIX

A1. THAPSIGARGIN ANALYSIS FOR MOTHER PLANT SELECTION

Plant material (100 mg) was ground to a fine powder and extracted in 3 ml ethylacetate in a sonication bath for 30 min. This step was repeated. The extract was centrifuged at 12 000 rpm and the supernatant was air-dried. The residue was then dissolved in 1 ml dichloromethane and loaded onto a Bond-Elut column (Analytichem International, USA) and eluted with 1 ml of a solvent combination of dichloromethane: ethylacetate (19:1; v/v). The fraction was collected and this was denoted Fraction I. A second fraction was collected by eluting the column with 2 ml dichloromethane: ethylacetate (1:1; v/v). This fraction was dried completely and the residue was dissolved in 1 ml HPLC-grade methanol (Merck Products, Germany) and the dissolved residue was denoted Fraction II.

The extracted thapsigargins (10 µl) were assessed using thin-layer chromatography (TLC) and high-performance liquid chromatography according to JÄGER *et al.* (1993). The extracted compounds were compared to a thapsigargin standard (Sigma, USA) and Fraction II with various trilobolides and thapsigargin compounds, previously isolated by Dr A.K. JÄGER from fruits of *T. garganica* L. collected from Ibiza in 1988.

A2. PLANT TISSUE CULTURE MEDIA

MILLER'S MEDIUM
(adapted from Miller,1965)

Chemical components	**mg l^{-1}**
Macronutrients	
KH_2PO_4	300
KNO_3	1000
NH_4NO_3	1000
$Ca(NO_3)_2.4H_2O$	500
Micronutrients	
$MgSO_4.7H_2O$	71.5
KCl	65
$MnSO_4.4H_20$	14
KI	0.8
$Cu(NO_3)_2.3H_2O$	0.35
$(NH_4)Mo_7O_{24}.4H_2O$	0.1
Organic supplements	
myo-inositol	100
nicotinic acid	2
pyridoxine HCl	0.8
thiamine HCl	0.8

MURASHIGE AND SKOOG MEDIUM (1962)

COMPONENTS	Amount (mg l^{-1})
Macronutrients	
$MgSO_4.7H_2O$	370
KH_2PO_4	170
KNO_3	1900
NH_4NO_3	1650
$CACl_2.2H_20$	440
Micronutrients	
H_3BO_3	6.2
$MnSO_4.4H_20$	22.3
$ZnSO_4.7H_20$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.83
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organic supplements	
Thiamine-HCl	0.5
Pyridoxine-HCl	0.5
Nicotinic acid	0.5
Myo-inositol	100
Glycine	2
Sucrose	30

A3. BACTERIAL MEDIA

The pH was adjusted prior to addition of agar when solid medium was required. In cases where bacterial cells were grown in a nutrient broth, the agar was omitted.

(i) LB MEDIUM (pH 7.0)

5 g l^{-1} yeast extract
10 g l^{-1} tryptone
10 g l^{-1} sodium chloride
10 – 15 g l^{-1} Agar

(ii) YMA MEDIUM (pH 6.6)

5 g l^{-1} yeast extract
0.5 g l^{-1} casein hydrolysate
8 g l^{-1} mannitol
2 g l^{-1} ammonium sulphate
5 g l^{-1} NaCl
15 g l^{-1} Agar

(iii) YMB MEDIUM (pH 7.0)

5 g l^{-1} yeast extract
10 g l^{-1} mannitol
0.1 g l^{-1} $MgSO_4$
0.5 g l^{-1} KH_2PO_4
15 g l^{-1} Agar

(iv) YEB MEDIUM (pH 7.4)

1 g l^{-1} yeast extract
5 g l^{-1} beef extract
1 g l^{-1} peptone
5 g l^{-1} sucrose
0.049 g l^{-1} $MgSO_4.7H_2O$

A4. SOUTHERN ANALYSIS

Restriction digestion of DNA

Genomic DNA was isolated from putative transgenic clones and 10 µg DNA was digested with 2.5-5 U of *Hind*III or *Eco*R1. Digestions (6 h) were performed according to manufacturer's recommendation except that 0.5 µl of 0.1 M spermidine was added in each 50 µl reaction to increase reaction efficiency. Subsequent to restriction digestion, the DNA was electrophoresed.

Downward Southern transfer

After electrophoresis, the gel was trimmed and then soaked in 250 ml 0.25 M HCl for 10 min and thereafter rinsed with distilled water. It was then submerged in transfer solution (0.3 M NaOH/ 0.3 M NaCl) until it was ready for Southern blotting. A Zetaprobe membrane was cut to the size of the gel (7 cm x 10 cm) and pre-wetted in de-ionised distilled prior to soaking in transfer solution for 10 min. Filter paper (3 mm) were also wetted in transfer solution and a downward Southern transfer unit was assembled. The DNA from the gel was transferred onto the membrane for 2 to 3 h. The membrane was then rinsed in a solution of 0.5 M Tris-HCl and 1 M NaCl for 10 min afterwards, blotted dry with paper towels and baked for 30 min at 80 °C. The DNA was then fixed onto the membrane by UV-crosslinking for (Biorad GS Genelinker ™).

Probe preparation

The probe was labeled by nick translation (Promega) according to the manufacturer's instruction. The protocol was followed without modifications and α^{35}S ATP (Amersham, 1000 Ci/mmol) was used as radioactive label. The reaction was incubated at 15 °C for 60 min and stopped by the addition of 5 µl stop solution (Promega). The probe was used immediately and unincorporated nucleotides were removed by chromatography centrifugation via a Sephadex® G-50 mini-spin column (Roche), as advised by the manufacturer. All centrifugation steps were carried out at 3.5 000 rpm (Sigma 113 desk top centrifuge). Once prepared, the probe was used immediately for Southern hybridization.

Southern hybridization and signal detection

While the probe was being radioactively labeled, hybridisation was initiated by prewashing of the nylon/nitrocellulose membrane with 0.1 % SSPE/1 % SDS (v/v) for 1.5 h, then submerged in 0.25 M Na_2PO_4/7% SDS (pH 7.2) for 5 – 30 min at 65 °C in a rotating hybridization tube under controlled temperature conditions (Hybaid hybridization oven). The probe was incubated in boiling water for 5 min to denature it and thereafter placed on ice to prevent reannealing. For hybridization, fresh hybridization buffer (0.25 M Na_2PO_4/7% SDS [pH 7.2]) was added to the membrane and hybridized with continuous agitation overnight at 65 °C. During hybridization, 170 µl solution cm^{-2} Zeta-Probe GT membrane (Biorad) was used. The following day, the probe was washed twice with 100 ml primary wash buffer (2 X SSC/ 1 % SDS) for 30 min and then washed with secondary wash buffer (1X SSC/ 0.5 % SDS) for 30 min twice and blotted, and subsequently dried at 37 °C for 10 min in preparation for signal detection. The membrane was placed with the DNA side facing the emulsion side of Hyperfilm™βmax (Amersham International, United Kingdom) under red safe light conditions (in the darkroom) and exposed for as long as was necessary. The film was processed using standard procedures.

HYBRIDISATION SOLUTIONS

(i) 20X SSC

0.3 M sodium citrate

3 M NaCl

Adjust pH 7.0

(ii) 20X SSPE

3.6 M NaCl

0.2 M Na_2HPO_4

0.02 M EDTA