In vitro culture of *Camptotheca acuminata* (Decaisne) in Temporary Immersion System (TIS): Growth, development and production of secondary metabolites

DISSERTATION

A thesis submitted for the degree of Dr. rer.nat. (*rerum naturalium*)
to the Biology Department,
the Faculty of Mathematics, Informatics and Natural Sciences,
University of Hamburg

prepared by

Yantree Devi Sankar-Thomas
Republic of Guyana

2009

Genehmigt vom Department Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Herrn Professor Dr. R. LIEBEREI
Weiterer Gutachter der Dissertation:
Herr Professor Dr. G. ADAM
Tag der Disputation: 18. September 2009

Hamburg, den 04. September 2009

Professor Dr. Jörg Ganzhorn
Leiter des Departments Biologie

This thesis is dedicated to my
husband and two children

Table of Contents

Camptothecin (CPT) and its analogue 10-hydroxycamptothecin (HCPT) are two naturally occurring monoterpene indole alkaloids in *Camptotheca acuminata*. Both, CPT and HCPT show potential efficacy as anticancer and antiviral agents. Currently, there is a high demand for CPT and commercial nurseries cannot meet the demand for CPT production. Therefore, the production of these compounds relies primarily on natural grown plants, which is the main reason that *C. acuminata* is facing a steep decimation in its population. Thus, an efficient and reliable micropropagation protocol would aid *Camptotheca's* conservation and at the same time would be a sustainable source for the production of CPT. This thesis has evolved from the profound knowledge of *C. acuminata.*

The key objective of this study was to investigate the growth, development and production of secondary metabolites of *C. acuminata*, using the Temporary Immersion System (TIS). Shoots were cultured in two different TIS vessels, DVS and RITA®. In the course of this study the following results were achieved.

- *Ex situ* germination of shortly stored seeds ranged from 60 to 80%, while fresh seeds did not germinate. Conversely, *in vitro* seeds germination raised up to 100% after pericarp and endosperm were removed. Whereas stem cuttings cultured *ex situ* in a mixture of soil, sand and vermiculite showed a rooting and survival rate of 32 to 38% regardless of the seasons.

- Multiple shoots were successfully initiated *in vitro* from one-year-old greenhouse plants via axillary buds. Each nodal explant produced one shoot but when this shoot was excised and subcultured on MS medium supplemented with 0.5, 1.0 and 1.5 mg l^{-1} BAP, an average of 7.3, 9.8 and 16.8 shoots per explant were obtained, respectively.

- Plant regeneration via organogenesis from matured zygotic embryos and seedlings hypocotyl have been established in both solid and liquid media. Shoot bud formation occurred on radicle, hypocotyl, apical tip and cotyledon explants cultured on MS medium containing high cytokinins (BAP, Kin, 2iP) and low auxins (NAA, 2,4-D and IAA). An average of 17 buds was scored per callus derived from apical tips and 10 buds among calli derived from cotyledons, hypocotyls and radicles. The best shoot bud formation occurred on 2 mg l^{-1} BAP plus 0.1 mg l^{-1} IAA.

- Somatic embryogenesis was induced on hypocotyl segments from 14-day-old *in vitro* seedlings in MS medium containing 35 g l^{-1} sucrose devoid PGR after 16 weeks of incubation in both the DVS and RITA® vessels. Embryo maturation was extremely slow in TIS but when plated on the same solid PGR-free medium, proembryos matured into well-developed cotyledonary embryos within four weeks. Cotyledonary embryos were selected and re-cultured in MS medium supplemented with 0.5 mg l^{-1} BAP in TIS for plantlet conversion. Plant conversion via somatic embryos was successfully achieved in TIS and in sterilised substrates moistened with 0.5 mg l^{-1} BAP.

- Shoot multiplication and *in vitro* rooting of *C. acuminata* was successfully achieved in solid medium and in TIS.

    - After eight weeks in culture a multiplication rate of 7 shoots per explant was obtained in solid MS medium supplemented with 0.5 and 1.0 mg l^{-1} BAP and 9 shoots per explant with 1.5 mg l^{-1} BAP. Shoots cultured with 0.5 mg l^{-1} BAP showed the best quality.

    - Shoot multiplication rate in TIS was influenced by the BAP concentration and the immersion cycles. Shoots cultured in MS medium supplemented with 1.0 and 1.5 mg l^{-1} BAP showed the highest multiplication rate in both DVS (23 ± 3.1, 29 ± 3.0) and RITA® vessels (14 ± 2.8, 20 ± 4.9) respectively, but hyperhydricity and callus formation was obvious, especially on explants in RITA®. The best results on shoot growth and multiplication rate in TIS was achieved with 0.5 mg l^{-1} BAP. However, shoot proliferation in DVS was distinctly affected by the one minute immersion cycles (IC) 4 and 8 times daily (d^{-1}). The highest shoot multiplication rate scored in DVS was 20 and 13 shoots per explant after eight weeks at 4 and 8 IC d^{-1}, respectively. Shoots grown in RITA® were not affected by the IC. An average of 15 shoots per explant was scored in both IC treatments. Shoot height and fresh weight was also not affected by the different IC treatments.
    - *In vitro* rooting of microshoots was more successful with IBA in both solid medium and in TIS than with NAA. The two auxins had no effect on the *in*

vitro rooting on sterilised substrates. Rooting percentage with 0.5 IBA was 90% in DVS, 92% in RITA®, 81% in solid medium, while those on the different sterilised substrates ranged from 60 to 92%. Root induction with NAA was less effective. *Ex vitro* rooting on non-sterile substrates ranged form 27 to 46%.

- The highest survival rate under greenhouse conditions was recorded on plantlets derived from sterilised substrates with 93% followed by those derived from RITA® with 47%, solid medium with 45% and from DVS with 41%.

- *In vitro* cell, tissue and organ cultures of *C. acuminata* were examined for their variation of camptothecin (CPT) and 10-hydroxycamptothecin (HCPT) content. Quantification was carried out on undifferentiated and differentiated cultures grown in TIS and in solid medium. CPT accumulation was also detected in liquid culture media. Among the *in vitro* cultures the highest amount of CPT was found in individual genotypes grown in TIS and in solid medium with an average of 2.5 and 2.2 mg g^{-1} DW, respectively. The maximum CPT content found in *ex vitro* seedlings ranged from 2.3 to 4.8 mg g^{-1} DW and in *in vitro* grown seedlings from 4.1 to 4.5 mg g^{-1} DW. Cotyledonary embryos and regenerated plantlets were marked with 0.87 and 1.23 mg g^{-1} DW, respectively. CPT excretion in liquid media at 8 IC d^{-1} was about 6 µg g^{-1} FW in RITA® (0.048 mg l^{-1}) and 12.6 µg g^{-1} FW in DVS (0.193 mg l^{-1}).

Camptotheca acuminata (Decne) (Xi Shu, der Glücksbaum) gehört zu den Laubbäumen der Familie *Nyssaceae* (*Cornales*, Hartriegelgewächse) und ist der einzige Vertreter seiner Gattung. *C. acuminata* wurde während einer systematischen Prüfung chinesischer Arzneipflanzen vom United States Department of Agriculture (USDA) in den fünfziger Jahren entdeckt. Es hat sich herausgestellt, daß sowohl aus den Blättern als auch aus Früchten, Samen, Rinden und Zweigen verschiedene Indolalkaloide isoliert werden können, darunter Camptothecin (CPT). CPT ist ein Anti-Krebs-Wirkstoff, der zur Behandlung von Lungen- Eierstock-, und kolorektalem Krebs verwendet wird. Aufgrund einer weltweit stetig steigenden Nachfrage für CPT, wird *C. acuminata* durch Wildsammlungen derart ausgebeutet, daß die Art inzwischen vom Aussterben bedroht ist. Auch kommerzielle Baumschulen sind noch nicht in der Lage diesen Bedarf zu decken und den Raubbau zu verhindern. Vor dem Hintergrund, daß *C. acuminata* vom Aussterben bedroht ist und die Nachfrage für den Rohstoff CPT jedoch kontinuierlich steigt, sollte in der vorliegenden Arbeit ein zuverlässiges Protokoll zur *in vitro* Vermehrung von *C. acuminata* erstellt werden, sodaß in einem weiteren Schritt sowohl die Produktion von neuen Pflanzen als auch die Gewinnung des Rohstoffs CPT gewährleistet werden kann.

Eines der Hauptziele dieser Arbeit war die *in vitro* Kultivierung von *C. acuminata* im Temporary Immersion System (TIS), um das Wachstum und die Entwicklung der Pflanzen und damit die Produktion des Sekundärstoffes CPT zu optimieren. Zusätzlich zu der *in vitro* Kultivierung wurde die konventionelle Methode angewendet, *C. acuminata* im Gewächshaus zu vermehren.

In der vorliegenden Arbeit wurden folgende Ergebnisse erzielt.

- Die *ex situ* Keimung kurz gelagerter *C. acuminata* Saaten zeigte im Gewächshaus eine Keimrate von 60 bis 80%, während frisch geerntetes Saatgut nicht keimte. Im Gegensatz dazu wurde *in vitro* eine Keimrate bis zu 100% erzielt, dies jedoch erst nach Entfernung von Perikarp und Endosperm.
- Eine Stecklingvermehrung in einer Substratmischung von Quarzsand, Erde und Vermiculit zeigte im Gewächshaus, unabhängig von der Jahreszeit eine Überlebensrate von nur 32-38%. Parallel hierzu konnte eine *in vitro* Vermehrung von einjährigen Gewächshaus-Pflanzen erfolgreich etabliert werden. Aus jedem Sproßabschnitt entwickelte sich jeweils nur ein Sproß. Nach Abschneiden und Umsetzen dieser Sprosse auf MS Medium unter Zusatz von 0,5; 1,0 and 1,5 mg l^{-1} BAP, wurden pro Explantat im Mittel 7,3; 9,8 bzw. 16,8 neue Sprosse gebildet.

- Aus reifen zygotischen Embryonen und verschiedenen Keimlingsabschnitten wurden über den Weg der Organogenese und somatischen Embryogenese, Pflanzen erfolgreich regeneriert. Organogenese wurde bei verschiedenen Keimlingsexplantaten, sowohl auf festem als auch in flüssigem MS Medium mit hohen Cytokinin- (BAP, Kin, 2iP) und niedrigen Auxinkonzentrationen (NAA, 2,4-D, IAA) erreicht. Es wurden bis zu 17 Knospen am Kallusgewebe von Apikalmeristemen gebildet und im Mittel 10 Knospen am Kallusgewebe von Kotyledonen, Hypocotylen und Wurzeln. Die meisten Knospen wurden bei einer Cytokinin-Auxinkombination von 2 mg l^{-1} BAP und 0,1 mg l^{-1} IAA gebildet. Die Induktion somatischer Embryogenese wurde an Hypocotylexplantaten nur im TIS in phytohormon-freiem MS Medium mit 35 g l^{-1} Saccharose erfolgreich induziert. Die Maturation somatischer Embryonen verlief jedoch sehr langsam. Eine Ausplattierung auf phytohormon-freies Festmedium (Agar) mit 35 g l^{-1} Saccharose führte innerhalb von vier Wochen zu gut ausgebildeten Kotyledon-Embryonen. Diese Embryonen wurden entnommen und im TIS zur Pflanzenregeneration weiter kultiviert. Die Pflanzenregeneration mittels somatischer Embryogenese war erfolgreich im TIS und auf sterilen Substraten, die mit 0,5 mg l^{-1} BAP-haltigem MS-Medium befeuchtet wurden.
- Die *in vitro* Sprossvermehrung und Bewurzelung von *C. acuminata* war sowohl auf Agar-Medium als auch im TIS in zwei verschiedenen Kulturgefäßen (DVS und RITA®) erfolgreich.
    - Bei Zugabe von 0,5 bzw. 1,0 mg l^{-1} BAP wurde auf Agar-Medium nach acht Wochen eine 7 fache Sprossvermehrung erzielt und eine 9 fache Rate bei Zugabe von 1,5 mg l^{-1} BAP. Die mit 0,5 mg l^{-1} BAP kultivierten Sprosse waren jedoch von deutlich besserer Qualität.
    - Die Sprossvermehrung im TIS wurde durch die BAP- Konzentrationen und die Anzahl der Flutungen pro Tag beeinflußt. Die höchste Vermehrungsrate wurde im MS-Medium mit 1,0 bzw. 1,5 mg l^{-1} BAP erreicht. Bei 4 Flutungen pro Tag wurden im DVS 23 ± 3,1 bzw. 29 ± 3,0 und RITA® 14 ± 2,8 bzw. 20 ± 4,9 Sprosse pro Explant gebildet. Diese Sprosse waren jedoch von schlechter Qualität, besonders im RITA® zeigten sich die Pflanzen glasig und neigten zur Kallusbildung. Die besten Ergebnisse beim Sprosswachstum, sowie bei der Vermehrungsrate wurden im TIS mit 0,5 mg l^{-1} BAP erzielt.

- Die höchste Vermehrungsrate in DVS war 20 fach bei 4 Flutungen und 13 fach bei 8 Flutungen pro Tag, während im RITA® die Vermehrungsrate nur 15 fach war. Im Gegensatz zum DVS wurde im RITA® die Vermehrungsrate nicht durch die Flutungshäufigkeiten beeinflußt. Die Sprossgröße und das Frischgewicht waren im TIS flutungsunabhängig.
- Bei der *in vitro* Bewurzelung von Microsprossen wurden mit IBA, sowohl auf Agar-Medium als auch im TIS bessere Ergebnisse erzielt als mit NAA. Im Vergleich zu Fest- und Flüssigkulturen hatte die Verwendung von IBA und NAA für die *in vitro* Bewurzelung auf sterilen Substraten keinen Einfluß. Die Bewurzelung betrug mit 0,5 mg l^{-1} IBA im RITA® 92%, im DVS 90%, auf Agar-Medium 81% und auf den verschiedenen sterilen Substraten 60 bis 92%. Die *ex vitro* Bewurzelung betrug bei nicht sterilen Substraten 27 und 46%.

- Die höchste Überlebensrate unter *ex vitro* Bedingungen zeigten *in vitro* bewurzelte Pflanzen, die aus sterilen Substraten stammten (93%), gefolgt mit 47% aus RITA®, 45% aus Agar-Medium und 41% aus dem DVS.

- Die Sekundärstoffe Camptothecin (CPT) und 10-Hydroxycamptothecin (HCPT) wurden mittels der HPLC-Analyse an verschiedenen *C. acuminata* Pflanzengeweben bestimmt. Die *in vitro* Pflanzen zeigten ein unterschiedliches Verteilungsmuster der CPT- und HCPT-Anreicherung. Im allgemeinen hat sich herausgestellt, daß die Inhaltstoffe genotypisch- und von den Kulturbedingungen abhängig waren. Der höchste CPT-Gehalt auf das Trockengewicht (TG) bezogen lag zwischen 2,5 mg g^{-1} für in TIS kultivierte Sprosse, und bei 2,2 mg g^{-1} für Kulturen auf Agar-Medium. Der maximale CPT-Gehalt betrug bei den *ex situ* Sämlingen 2,3 bis 4,8 mg g^{-1} TG und bei den *in vitro* Pflänzchen 4,1 bis 4,5 mg g^{-1} TG. Eine große CPT- Variation wurden bei den verschiedenen Kalli und somatischen Embyronen festgestellt. Der höchste CPT-Gehalt wurde mit 0,87 mg g^{-1} TG in den Kotyledonen-Embryonen und mit 1,23 mg g^{-1} TG bei den regenerierten Pflänzchen bestimmt. Hohe HCPT-Gehalte kamem nur in den Hypokotylen der *ex situ* Sämlinge vor. Die *in vitro* Sämlinge enthielten sehr wenig bzw. kein HCPT. Die höchste CPT-Absonderung aus den Pflanzenkulturen in das Nährmedium wurde bei 8 Flutungen pro Tag in beiden Systemen gemessen, und zwar im RITA® mit 6 µg g^{-1} FG gefolgt von 12,6 µg g^{-1} FG im DVS.

Camptotheca acuminata (Decne) is a deciduous and endangered tree species endemic to east Tibet and southern China, where it is popularly known as Xi Shu (Happy Tree). The genus *Camptotheca* belongs to the Nyssaceae family (Li and Adair, 1994), which is represented by three other genera, *Nyssa* (tupelos) with about 7-10 species in eastern North America and east to Southeast Asia, *Davidia (D. involucrata,* Dove tree) a monotypic genus in central China and *Camptotheca* with two species in China (*C. acuminata* and *C. lowreyana*). A fourth genus *Diplopanax* with two species (*D. vietnamensis and D. stachyanthus)* in southern China and Vietnam has recently been transferred to the Nyssaceae family (Averyanov and Hiep, 2002). The species *C. lowreyana* seems to be very rare and is mentioned only in few reports. Thus, *C. acuminata* is the only well known species of its genus and it is distributed in south-eastern China mostly along the Yangtze River, growing in forests and on hillsides at altitudes between 250 and 1500 metres (Perdue *et al.,* 1970; Ying *et al*., 1993). In its natural habitat, *C. acuminata* is a fast growing tree. The woods of *C. acuminata* are primarily used as furniture, packaging material, mine timber, pulp and fuel wood (Li, 2000). The genus *C. acuminata* is also cultivated as an ornamental plant but recently its greatest commercial value lies in its secondary metabolites production, which is mainly isolated from seeds and barks. This is one of the reasons why the genus is facing a steep decimation in its wild population. Trees of *C. acuminata* are commonly raised from seeds (Perdue *et al*., 1970) and are the major source for the monoterpene-indole alkaloids camptothecin (CPT) and 10-hydroxycamptothecin (HCPT), which has been identified by Wall *et al.,* (1966). CPT and its analogues have recently emerged as one of the most promising agents for cancer treatment because of their inhibitory activity against tumour cells and the Human Immunodeficiency Virus (HIV) (Priel *et al*., 1991). Unlike Taxol® and other anticancer agents, CPT is the first known inhibitor of topoisomerase (Topo I), a DNA enzyme that plays key roles in DNA replication, transcription, and recombination. As a result of CPT's action, breaks occurred in the DNA single- and double-strand, leading to termination of replication and inhibition of transcription and subsequent death of the cell (Hsiang *et al*., 1985; Ewesuedo and Ratain, 1997; Wu and Liu, 1997; Czuwara-Ladykowska *et al*., 2001). Currently, two semi-synthetic derivatives of CPT, topotecan (trade name Hycamtin®) (TPT) and irinotecan (trade name Camptosar®) (CPT-11) are widely used as a standard treatment against ovarian and colorectal cancer (Cunningham *et al*., 1998; Douillard *et al*., 2000). The worldwide market value for CPT derivatives TPT and CPT-11 was estimated at about US$ 750 million in 2002 which rose to US$ 1 billion by 2003 and has now reached

2.2 billion US$ in 2008 (Lorence and Nessler, 2004; http://www.tremcon.net/trading /biotech/cpt.html, 2009). Thus CPT is becoming increasingly important and it is a valuable starting material for the production of TPT, CPT-11 (Maliepaard *et al*., 2001) and several other camptothecin analogues. There are a few other plant species such as *Nothapodytes foetida* (Icacinaceae) *Ophiorrhiza mungos* (syn. *O*. *pumila,* Rubiaceae) and *Ervatamia heyneana* (Apocyanceae) from which CPT is being isolated. However, *C. acuminata* remains the major source of CPT and in spite of the rapid market growth, CPT is still harvested by extraction from barks and seeds of naturally grown *C. acuminata* trees (Lorence and Nessler, 2004). The few commercial nurseries for *C. acuminata* cannot meet the demand for CPT production. Thus, it is assumed that the extraction of CPT relies primarily on plant materials and fruits that are harvested from naturally grown trees, which are causing a hazard to this plant (Li *et al*., 2002).

In 2000 and again in 2006 *C. acuminata* was proposed for protection in the CITES appendix II (http://www.cites.org/eng/cop/11/prop/58.pdf) but the proposal was withdrawn on both occasions.

The yields of CPT from field trees vary widely and depend on factors that are difficult to control. For instance, plant diseases such as leaf spot and root rot are some of the major fungal diseases that can limit the cultivation of *Camptotheca* plants (Li *et al.,* 2005) and diminish the production of CPT. Cultivation of *Camptotheca* plants is limited to subtropical climates and it takes about ten years for plants to produce a stable fruit yield (Li *et al*., 2005). Currently, the combination of a high demand for CPT and its variable concentration in naturally grown plants has led to a number of strategies to gain plants or cell lines that could produce higher amounts of CPT.

Therefore, the production of *in vitro* grown plants to obtain its natural products (CPT) may be an attractive alternative. Several attempts have been made to produce CPT from cell suspension (Sakato and Misawa, 1974; Wiedenfeld *et al.,* 1997), however, the low yield limits this approach (Lorence *et al*., 2004). Cultures of differentiated tissues and the selection of highly desirable propagated plants may be an alternative source for the production of CPT. To date, only a few reports on the *in vitro* culture of *C. acuminata* have been published using shoot buds (Jain and Nessler, 1996; Li and Liu, 2001, 2005; Wang *et al*., 2006) and plant regeneration through organogenesis (Wang *et al*., 2006). Consequently, the rate of micropropagated plants is still critical to meet the pharmaceutical demand for CPT production. Therefore, the main objectives of this study was to develop an effective method for plant regeneration and multiplication of *C. acuminata* in Temporary Immersion

System (TIS), a technique that allows mass production of plant material within a short period of time which is also considered to be a practical and an efficient method for *in vitro* commercialisation (Aitken-Christie, 1991; Berthouly and Etienne, 2005). Until now there has been only one report on the *in vitro* culture of *C. acuminata* using TIS (Sankar-Thomas *et al*., 2008).

1.1. Aims of the Study

Camptotheca acuminata is an economically important medicinal plant and an endangered species. The extraction of CPT from raw material is variable and consumes a lot of resources (Wang *et al.,* 2005). Therefore, conventional breeding can not meet the demand for its pharmacologically active compounds. Developing a reliable protocol for the *in vitro* cultures of *C. acuminata* could open the door for large-scale production and at the same time supersede the extraction of CPT from plants of wild populations.

Hence, the main objectives of this study were to establish an efficient and reliable protocol for *in vitro* culture of C. *acuminata* in the TIS and the selection of desirable clonally propagated plants according to their *camptothecin* (CPT) production.

In order to study the objectives of this thesis, experiments have been carried out between 2004 and 2007 under controlled environmental conditions (temperature, air humidity and light intensity) in growth chambers and greenhouses. To secure high genotype variability of *C. acuminata* cuttings obtained from various botanical gardens and private growers within the EU and seeds from different geographic locations in China and the USA were used as starting material. This thesis is composed of the following steps.

i. Propagation Methods of *Camptotheca acuminata.*
ii. Regeneration via organogenesis and somatic embryogenesis.
iii. Shoot multiplication in solid media and in TIS.
iv. The *in vitro* and *ex vitro* rooting of microcuttings derived from solid media and TIS
v. The acclimatisation of rooted plantlets to *ex vitro* conditions in greenhouses.
vi. Analysis of CPT content in undifferentiated and differentiated plant tissues and in the exudations of culture media. Finally, plants are selected as desirable clones according to their CPT contents.

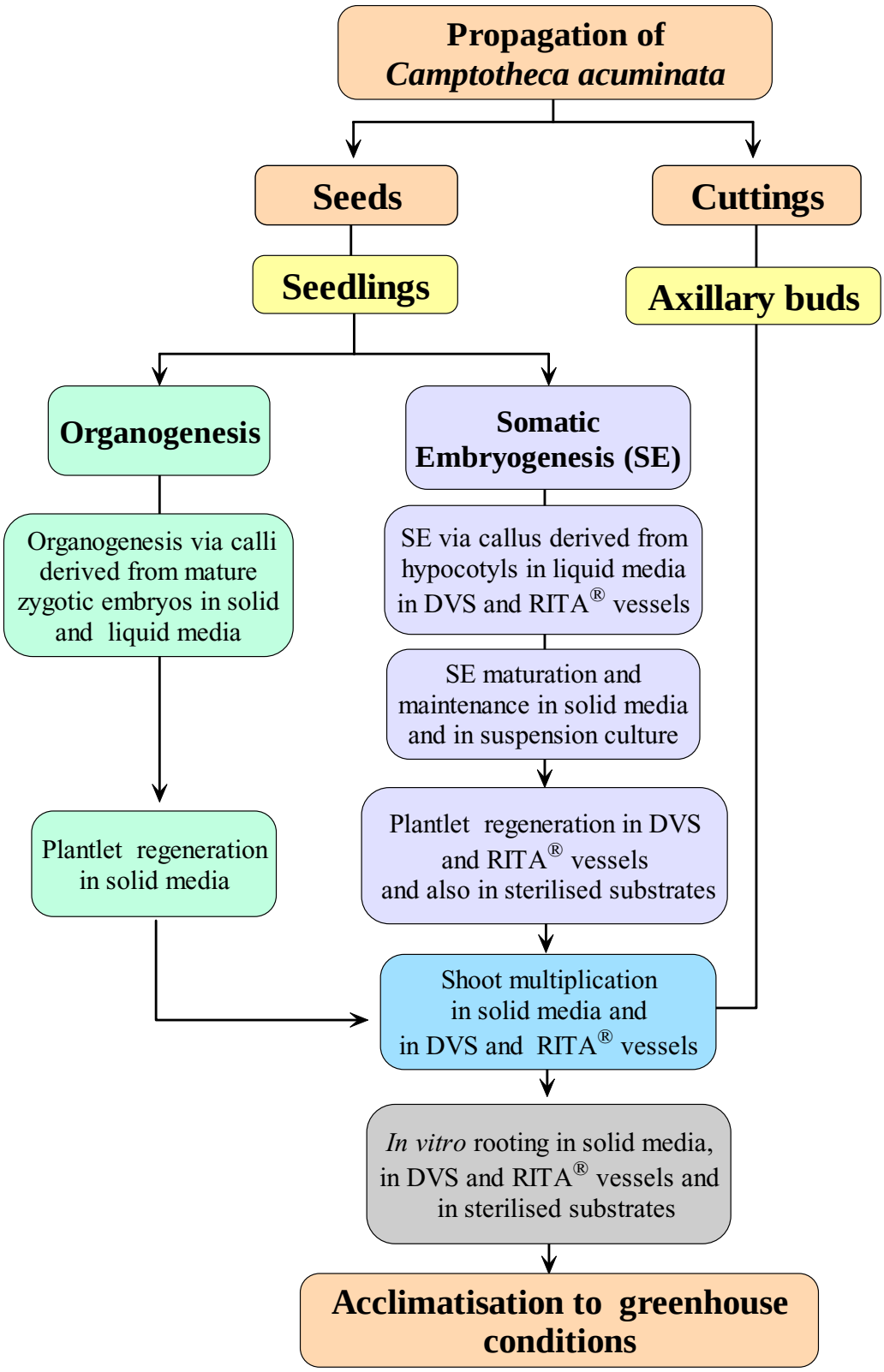

Fig. 1 Flow chart for the micropropagation of *C. acuminata*. The following steps were taken, to establish a reliable protocol for the *in vitro* culture, plant regeneration and shoot multiplication. Brown boxes represent the *ex vitro* conditions and the remaining colours (yellow, green, purple, blue and grey) the *in vitro* conditions. DVS (= Dual Vessel System). RITA® (= Récipient à Imersion Temporaire Automatique)

2.1. *Camptotheca acuminata*

2.1.1. Botany

Camptotheca acuminata Decne (Nyssaceae) is a rapidly growing deciduous tree native to southern China and east Tibet. *Camptotheca* trees exhibit monopodial growth that can reach a height of 20 to 25 m and under favourable conditions their trunks can grow to a diameter of 100 cm. Young twigs are pubescent and purplish while older ones are glabrous. The bark of fully-grown trees is pale-grey with deep furrows. Leaves are petiolate, alternate, pinnately veined, entire or occasionally dentate with a length of 10 to 30 cm and 6 to 15 cm in width.

Fig. 2 (A) One-year old acclimatised *Camptotheca acuminata* plants with an average height of 1 to 1.5 m **(B)** Flowering plant (http://www.botanic.jp/plants-ka/kanren.htm) **(C)** Green fruits (http://had0.big.ous.ac.jp/plantsdic/angiospermae/dicotyledoneae/choripetalae/nyssaceae/kanrenboku/kanrenoboku.htm) and **(D)** Ripe seeds (http://www.hktree.com/tree/Camptotheca%20acuminata1.htm)

Young leaves are dark-red and pubescent, which gradually turn green and then greyish with age. Two to three sessile flowers form a cyme and about 15 to 30 cymes form a globular head. These heads are arranged in terminal or axillary raceme-like inflorescences (Fig. 2B). The white flowers in the upper terminal head are bisexual and bloom first, whereas the lateral heads are male or sometimes also bisexual. Flowers bloom from May to August. The mature fruit is an almost wingless samara about 20 to 30 mm long and 6 to 9 mm wide with one seed, which is ripen between September and December. Usually, trees start to bear fruits 7 to 10 years after germination.

Apart from *C. acuminata* it is reported that there are two other varieties, *C. acuminata var. tenuifolia* and *C. acuminata var. rotundifolia*. *C. tenuifolia,* which was found growing along streams in the province Guangdong has longer fruits, smaller leaves and grow to a height of 5 m only (Fang and Soong, 1975; Fang and Zhang, 1983). *C. rotundifolia* was recently found at a low elevation (45 m above sea level) and differs from *C. acuminata and C. tenuifolia* by the brown bark, sub-round and small leaves and grows to a height of 10 m (Yang and Duan, 1988). Currently, *C. acuminata* is becoming an economically important plant and is now cultured commercially in Asia and North America for its valuable secondary product camptothecin.

2.1.2. Geographical Distribution

C. acuminata is a tertiary relict and a monotypic genus of the family Nyssaceae. *Camptotheca* was first recorded in 1848 and scientifically described and named by Decaisne (1873), Director of the Jardin Des Plantes, Paris. The genus name *Camptotheca* is from the Greek. *Campto* (= bent or curved) and *theca* (= a case) referring to the anthers, which are bent inward. The species name *acuminata* is derived from acuminate, which refers to the tips of leaves. *C. acuminata* was also widely distributed in Japan during the Tertiary Period (Suzuki, 1976; Tanai, 1977). Today it is native only to central, southern and south-eastern China, in the provinces of Anhui, Zhejiang, southern Jiangsu, Jiangxi, Fujian, Hubei, Huna, Guangdong, Guangxi, Guizhou, Sichuan and Yunnan. In China the *Camptotheca* tree is cultivated as an ornamental tree and as a firewood species because of its rapid growth. In this century the species was reintroduced to Japan and today it is also cultivated in South Korea, Taiwan, Europe and the United States (Li and Adair, 1994)

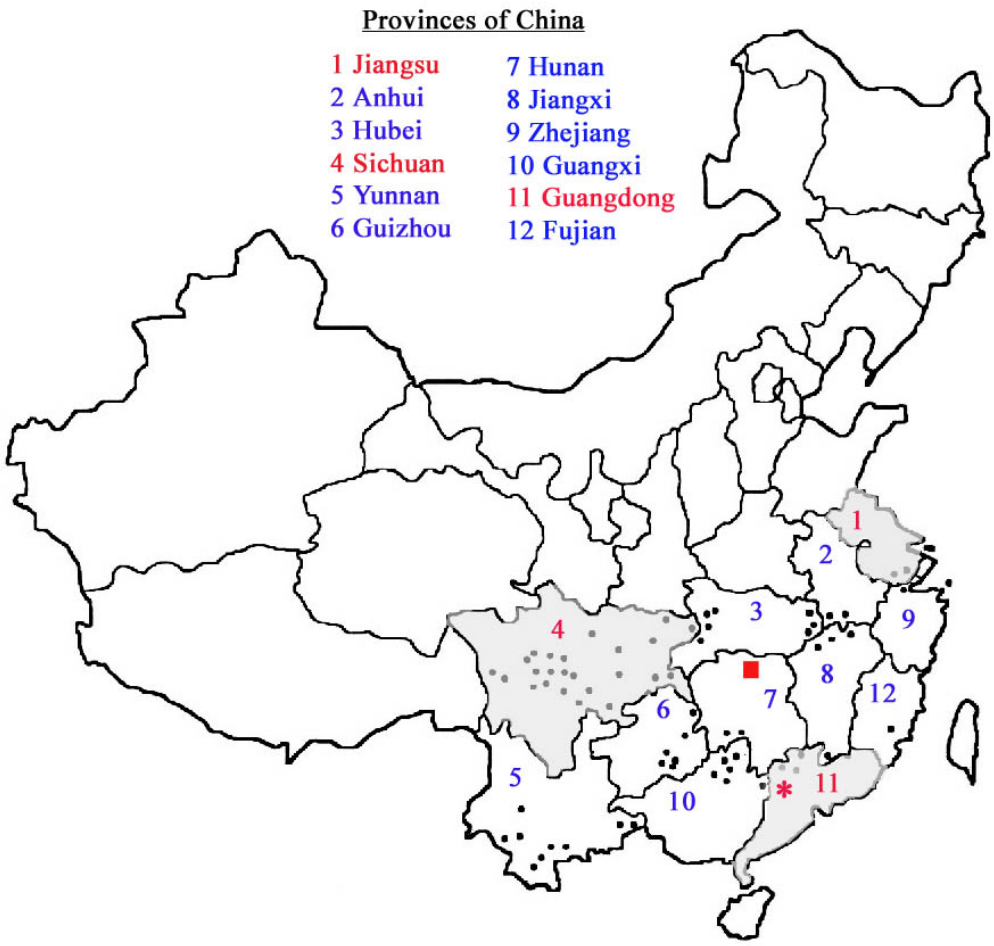

Fig. 3 Natural distribution of *C. acuminata* in China. • C. *acuminata* (Decne, 1973) var. *acuminata*; ✻ *C. acuminata* var. *tenuifolia* (Fang and Soong, 1975); ■ *C. acuminata* var. *rotundifolia* (Yang and Duan, 1988). Seeds used in this study were obtained from three provinces (1, 4, 11; grey underlaid).

2.1.3. Ecology

In its natural environment *C. acuminata* plants are shade-tolerant and are mostly found in thickets. *Camptotheca* plants require an annual mean of 28 to 57% sunshine, a humidity of about 80% and a precipitation of 1000 to 2000 mm per annum of which 75% falls during April to September and a winter drought (Perdue *et al.*, 1970; Chou and Li, 1990). Temperature varies from 15 to 23 °C at low elevation and 2 to 14 °C at higher elevation (above 2000 m). Plants have withstood temperature of -11 °C in California and -13 °C in South Carolina, USA (Perdue *et al.,* 1970) and -12 °C in South Korea without any severe damages (Meyer, 1991). *Camptotheca* plants growing at an elevation above 2000 m in China even survived the coldest month (January), which was recorded with a mean temperature of -20 °C (Chou and Li, 1990). From *C. acuminata* trees growing in Austria and the Netherlands it was reported that only the terminal buds are often damaged at

temperatures below 0°C (Bulk, personal communication 2003; Tod, personal communication, 2004). These cold tolerances may result from different seed sources or possibly different ecotypes. *C. acuminata* trees are also found growing at the edges of forests, in valleys and on slopes along streams in moist, fertile and well drained friable clay soils with a pH ranging from 4.5 to 6.0 (Ying *et al*., 1993; Li and Adair, 1994).

2.2. Camptothecin (CPT)

2.2.1. Plant Secondary Metabolites

Plants have evolved their own unique strategies not only to attract pollinating insects and seed or fruit dispersing animals but also to protect themselves against threats and adversaries. One of these strategies is the production of a wide range of secondary metabolites, which are generally associated with the defense against herbivores and pathogens (Wink, 2003). Many of these compounds (phenols, alkaloids, terpenoids, etc.) represent a large reservoir of chemical structures with biological activity and were apparent in the earliest annals of human history. It is estimated that 80% of the non-industrialised world still relies on plants as the major source of medicines in their primary health care (Farnsworth and Soejarto, 1985; Wijesekera, 1991). Many of these known compounds such as curare, opium, reserpine, paclitaxel or camptothecin are being exploited as poisons, stimulants, narcotics and pharmaceuticals (Wink, 1998).

2.2.2. Camptothecin and its Pharmacological Effects

Camptothecin (CPT) and 10-hydroxycamptothecin (HCPT) are naturally occurring toxic alkaloids (Cao *et al*., 1986).

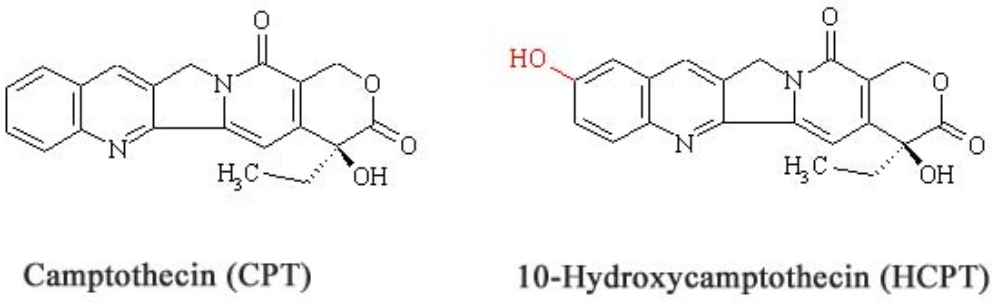

Fig. 4 Molecular structure of camptothecin ($C_{20}H_{16}N_2O_4$) and 10-hydroxycamptothecin ($C_{20}H_{16}N_2O_5$).

CPT is an indole alkaloid first isolated from the leaves of C. *acuminata* (Wall and Wani, 1991) during a phytochemical screening by the United States Department of Agriculture (USDA) in the early 1950s. In 1958 the anticancer properties of this alkaloid were discovered by Wall (USDA) and Hartwell from the National Cancer Institute (NCI) and it was identified as a pyrano-indolizinoquinoline alkaloid, which they termed CPT (Fig. 4) (Wall *et al.* 1966; Perdue *et al.,* 1970). In the early 1970s clinical trials were conducted on CPT as an anticancer agent but were then dropped because of a variety of unacceptable side effects. Renewed interest was revived after Hsiang *et al*., (1985) discovered that CPT directly inhibited topoisomerase I (Topo I), a DNA replication enzyme, and catalysed a cascade of events that lead to cell death (apoptosis) and therefore inhibiting the DNA replication. Apart from its potent anticancer activity, CPT was also reported to show inhibitory and antiviral activity against the fowl plague virus BHK21/13 replication (Kelly *et al*., 1974), Trypanosomes and Leishmania (Bodley and Shapiro, 1995), the Human Immuno Deficiency Virus (HIV) and the equine infectious anemia virus (Priel *et al*., 1991).

2.2.3. Semi-Synthetic Derivatives of Camptothecin

In 1996 topotecan and irinotecan, two semi-synthetic derivatives of CPT (Fig. 5), received approval for human testing and application from the Food and Drug Administration (FDA).

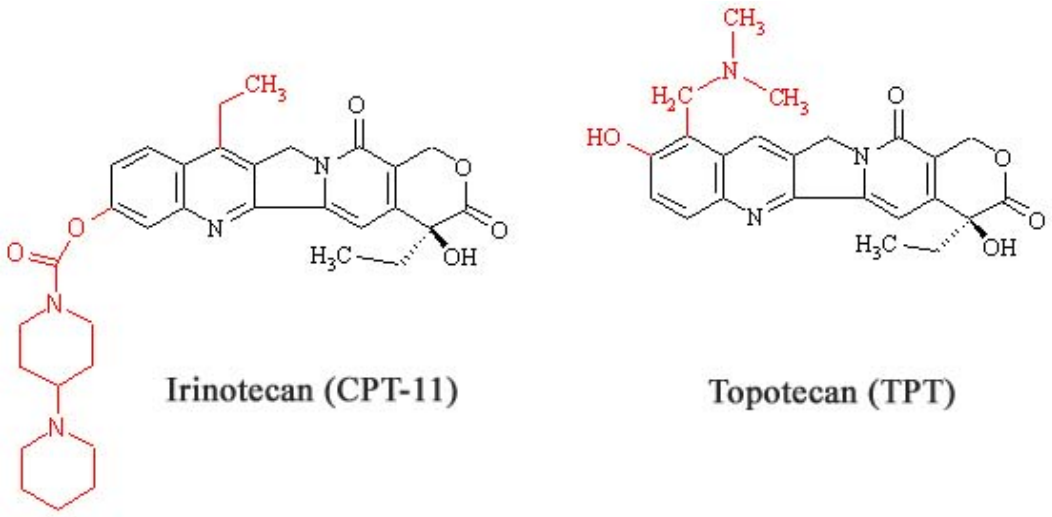

Fig. 5 Molecular structure of the two major semi-synthetic derivatives of CPT (black), irinotecan hydrochloride trihydrate ($C_{33}H_{38}N_4O_6$) and topotecan hydrochloride ($C_{23}H_{23}N_3O_5$), which have been approved for clinical use since 1996.

Topotecan, which is manufactured by SmithKline Beecham Pharmaceuticals (also known as GlaxoSmithKline) is sold under the trade name Hycamtin® and is used to treat advanced

ovarian cancers that have resisted other chemotherapy drugs. The injectable irinotecan HCl was approved as a treatment for metastatic cancer of the colon or rectum; irinotecan is marketed by Pharmacia and Upjohn under the trade name Camptosar® (Heron, 1998; McDonald, 1997). Bodley *et al*., (1998) reported that CPT has cytotoxic effects on *Plasmodium falciparum*. Both the industry and academic research groups worldwide have focused their attention on CPT as an antitumor and antiviral agent (Lorence and Nessler, 2004). Thus CPT has proved to be a powerful drug for several treatments, which have made it a valuable compound for the production of other water-soluble derivatives (Creemers *et al*., 1996) that are less cytotoxic than CPT itself.

2.2.4. Biosynthetic Pathway of Camptothecin

Although much is known about the pharmacological effects of CPT, little is known about its biosynthesis (Silvestrini *et al*., 2002). Several studies on plant cell cultures have indicated that CPT biosynthesis and accumulation, as in the case of many other alkaloids, are under the strict control of cell development and environmental factors (De Luca and St. Pierre, 2000). The biological activity and biosynthesis of CPT has been recently reviewed (Lorence and Nessler, 2004). Although CPT is a quinoline alkaloid lacking the basic indole structure, it is suggested to be of the terpene indole-alkaloid (TIA) family (Wenkert *et al.*, 1967), which belongs to a group of low molecular weight. CPT is a nitrogen-containing molecule and has a pentacyclic structure (Fig. 4). Most alkaloids are derived from the amino acids, phenylalanine (Phe), tryptophan (Tyr), lysine (Lys) and ornithine (Orn) (De Luca and St. Pierre, 2000) and all TIAs including CPT are derived from the precursor strictosidine, a condensation product formed by the reaction between indole tryptamine and terpenoid secologanin, which is then catalysed by the enzyme strictosidine synthase (Kutchan, 1995). Early experiments have confirmed that the tryptamine moiety is completely incorporated into the CPT molecule (Sheriha and Rapoport, 1976). Strictosidine is then converted to strictosamine via intramolecular cyclisation and this compound is a precursor of CPT. The remaining details and precise intermediates between strictosamide and CPT are not completely defined. Therefore, it is postulated that CPT could be formed from strictosamide by three transformations (Hutchinson *et al*., 1979). The steps following strictosamide formation remain somewhat speculative (O'Connor and Maresh, 2005). A series of chemically reasonable transformations have been proposed (Fig. 6) though there is little experimental evidence for these steps (Hutchinson *et al*., 1979; Cordell, 1974).

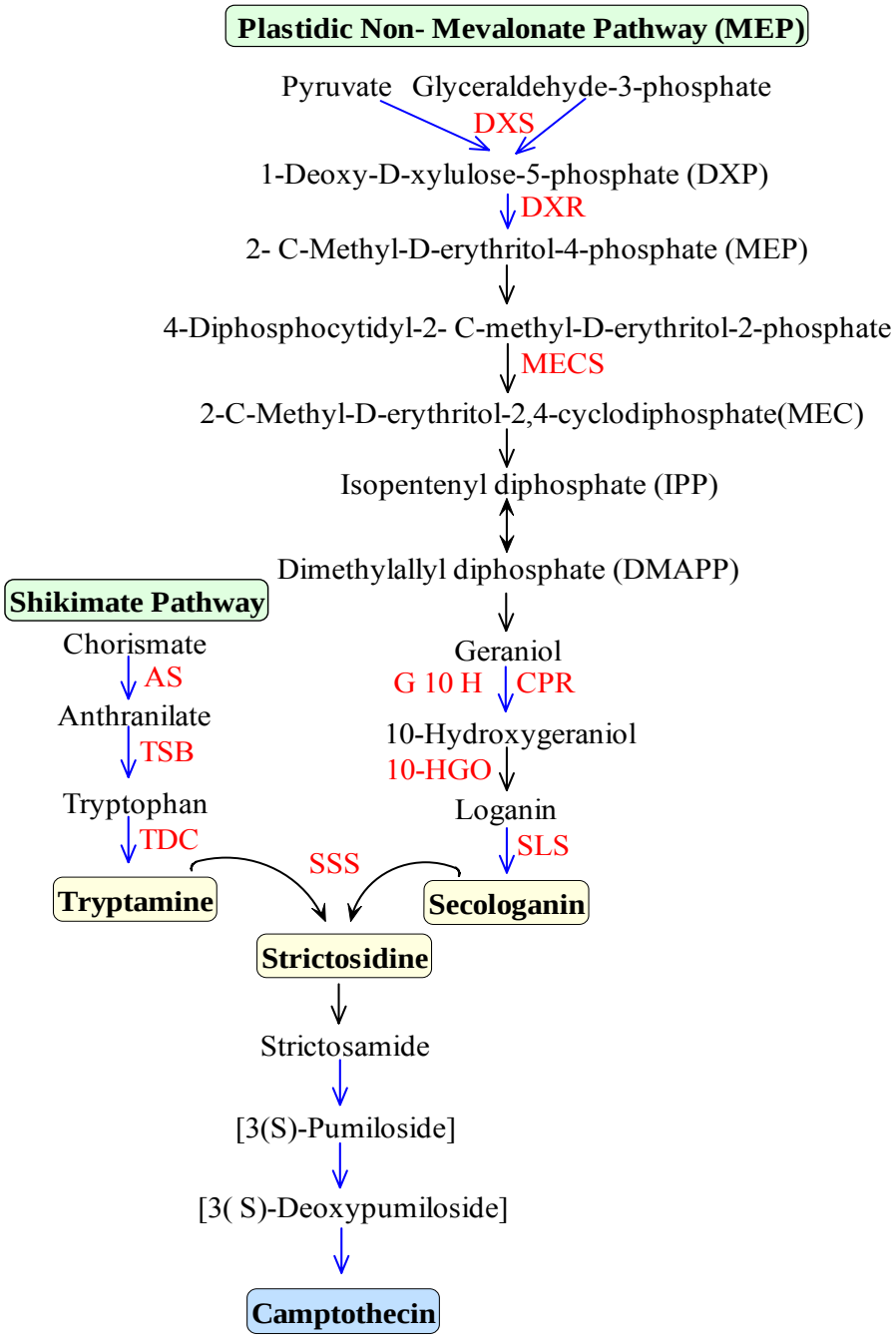

Fig. 6 Biosynthetic pathway for TIAs in Camptothecin producing plants (Lorence and Nessler, 2004). The blue arrows indicate multiple steps between the intermediates. Enzymes in red: **TSB** (β-subunit of tryptophan synthase), **TDC** (tryptophan decarboxylase), **SSS** (strictosidine synthase), and **10-HGO** (10-hydroxygeraniol oxidoreductase) were characterised in either *C. acuminata* or *O. pumila*. **TSB** is abundant in vascular tissues (cambium, primary xylem and primary phloem; Lu and McKnight, 1999). Other enzymes involved in this pathway already cloned in *Catharanthus roseus* the best characterized TIAs-producing model, are also shown: 1-deoxy-D-xylulose-5-phosphate (DXP) synthase (DXS); DXP reductoisomerase (DXR); 2-C-methyl-D-erythritol-2, 4-cyclodiphosphate synthase (MECS); geraniol-10-hydroxylase (G10H), and secologanin synthase (SLS). In *C. roseus* DXS, DXR, MECS, and G10H are expressed in the internal phloem parenchyma, while SLS, TDC and SSS are expressed in the epidermis (Burlat *et al.*, 2004).

Two of these potential biosynthetic intermediates, 3(S)-pumiloside and 3(S)-deoxy pumiloside, have been isolated from another CPT producing plant *Ophiorrhiza pumila* (Carte *et al*., 1990; Kitajima *et al*., 1997; Yamazaki *et al*., 2003). The biosynthetic pathway for TIAs (Fig. 6) after Lorence and Nessler (2004) shows that CPT is derived from both the shikimate and mevalonate pathway since tryptamine is derived from the shikimate and secologanin from the MEP pathway.

2.3. Plant Micropropagation

Plant *in vitro* propagation (micropropagation) techniques are becoming increasingly popular as an alternative method of plant cloning. It is the major and most widely accepted practical application of plant biotechnology (Mehrotra *et al*., 2007). A significant advantage offered by *in vitro* propagation over the conventional methods (*ex situ*) is that, in a relatively short span of time and in relative small rooms, a large number of plants can be produced starting with one single individual. Hence, micropropagation allows plant breeders to introduce new cultivars much earlier than they could through conventional practices and the establishment and maintenance of virus-free plant stocks (Morel and Martin, 1952, 1955).

Other aspects of *in vitro* propagation are the large-scale regeneration for commercially important elite material with desirable and characteristic traits. Presently, micropropagation techniques are being used for plants that are difficult to propagate through conventional practices. Many botanic gardens and institutions are using these techniques for the conservation of genetic resources of elite and rare plant species that are threatened or on the verge of extinction (Normah *et al*., 1997; Wawrosch *et al*., 2001; Michael *et al*., 2001). This applies also to economically important crops that are vegetatively propagated or plants with recalcitrant seeds that cannot be stored under conventional seed bank conditions (George and Sherrington, 1984; Dodds, 1991; George, 1993). In spite of these merits, the major limitation of *in vitro* propagation is the high plant production costs. Generally, “conventional” micropropagation processes are carried out in culture vessels containing medium solidified with gelling agents. Although this method is generally used, it has some disadvantages. Propagation in solid or semi-solid medium requires a large number of culture vessels, agarose media and aseptic division of plant tissues. Cultures need periodic transfer to fresh media at least every 4 to 6 weeks due to exhaustion of the nutrients in the medium, which may alter the continuous tissue growth and proliferation (Debergh and Maene, 1985).

Consequently, the running costs mainly for intensive manual labour and gelling agents increase the production costs. Thus, conventional micropropagation is time consuming, intensive and generates 40-60% of the plant production costs (Etienne and Berthouly, 2002). In order to simplify the *in vitro* process, reducing manual labour and production costs it was necessary to develop simpler and cheaper methods for the mass production of micropropagated plantlets.

Therefore, during the past decades there has been an increased interest to enhance plant production and at the same time to reduce production costs (Andrea-Kodym and Zapata-Arias, 2001). To overcome these limitations a number of strategies have been developed such as the use of shake cultures utilizing liquid medium solely (Weathers and Giles, 1988) or in combination with solid culture medium (Debergh and Maene, 1981; Aitken-Christie and Jones, 1987). Culture in solid medium is inert and hampered automation, whereas utilising liquid medium facilitated automation and reduced intensive labour and consequently the production costs (Debergh, 1988; Aitken-Christie, 1991). Thus, the Temporary Immersion Culture System combines the advantages of solid and liquid media.

2.3.1. Temporary Immersion System (TIS)

The Temporary Immersion system (TIS) is a method that combines the advantages of both the semi-solid and liquid culture media. TIS is a periodical flooding and draining of the entire culture with liquid nutrients for a defined time. The principle of this method was already mentioned by Heller (1965), which was probably the first concept of TIS. He reported that an up-and-down motion of nutrients without renewal showed the same effect as a true renewal in suspension culture. In 1985, Tisserat and Vandercook applied a similar method for the first time in plant tissue culture. They designed a system consisting of a large elevated culture chamber that was drained and then refilled with fresh medium at certain intervals. The first semi-automated culture system was introduced in 1988 by Aitken-Christie *et al*., (Ziv *et al*., 1998; Aitken-Christie, 1991). However, there were some drawbacks. Hyperhydricity was one of the most common disadvantages using liquid media, which results in severe physiological disorder of herbaceous as well as woody shoots (Kevers *et al*., 2004). In order to overcome this physiological disorder in plant shoots and the technical limitations encountered in the liquid medium, a new TIS vessel known as RITA® vessel (Automated Temporary Immersion Apparatus) was developed at the French Laboratory CIRAD (Alvard *et al*., 1993; Teisson and Alvard, 1995). The growth and multiplication rate of plantlets are enhanced by aeration and the close contact of

plantlets with liquid medium, which facilitates the availability of nutrients and phytohormones that promote shoot growth and proliferation (Ziv, 1989; Sandal *et al*., 2001).

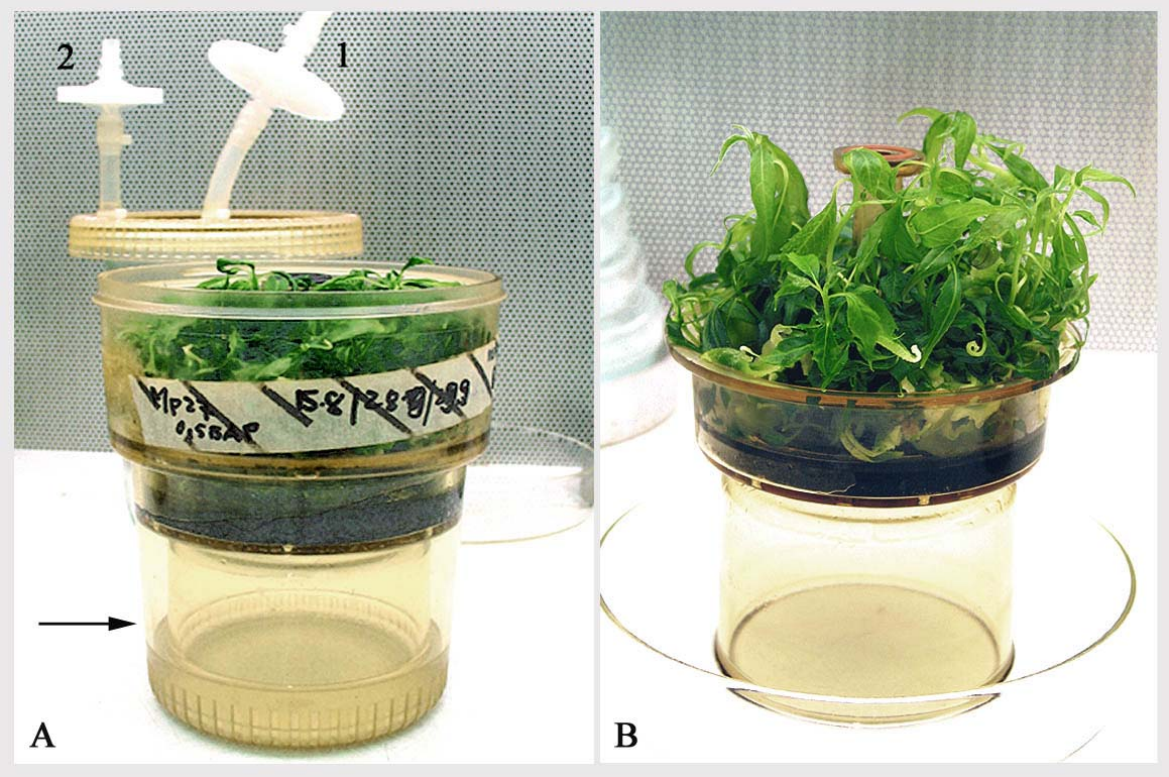

Fig. 7 The RITA®, a one litre vessel comprised of two compartments. **(A)** The complete opened vessel with airflow vents on the cap (air inlet 1 and outlet 2). The culture medium is kept in the lower compartment of the vessel (arrow), while plant material is located in the upper compartment laid out with a polysulfone foam disc. **(B)** The upper compartment with plant material taken out of the vessel for medium renewal.

This new technique has been successfully tested with growth of meristem cultures (Alvard *et al*., 1993), microcuttings and somatic embryogenesis of many tropical crops such as *Ananas comosus* (pineapple), *Musa acuminata* (banana), *Citrus deliciosa* (mandarin), *Coffea arabica* (coffee) and *Hevea brasiliensis* (rubber tree) (Escalant *et al.*, 1994; Cabasson *et al*., 1977; Teisson and Alvard, 1995). and recently with the induction of somatic embryogenesis and plant regeneration of *Camptotheca acuminata* (Sankar-Thomas *et al*., 2008).

The basic RITA® vessel has a capacity of one litre and consists of a two compartment units, which are connected by a central tube. The liquid culture medium is kept in the lower compartment, while explants are located in the upper. As soon as air pressure is

applied through a 0.2µm hydrophobic sterile filter (Midisart® 2000, Sartorius) into the central tube (Fig. 7A 1) the air forces the liquid medium to the upper compartment immersing explants as long as the air pressure is applied. As soon as the air pressure is released, the liquid medium flows back to the lower compartment by gravity. During immersion, the airflow aerates the medium, agitates explants and the atmosphere inside the vessel escapes through the sterile outlet filter in the cap (Fig. 7A 2). Hence, the air inside is simultaneously renewed at every immersion. The RITA® vessel can also be used to produce large number of tissue cultures and could simply be scaled up and automated (Aitken-Christie, 1991). Apart from RITA® a second type of TIS vessel was used in this study, the Dual Vessel System (DVS) (Fig. 8) The DVS is similar to the twin flasks principle of Escolona *et al.* (1999).

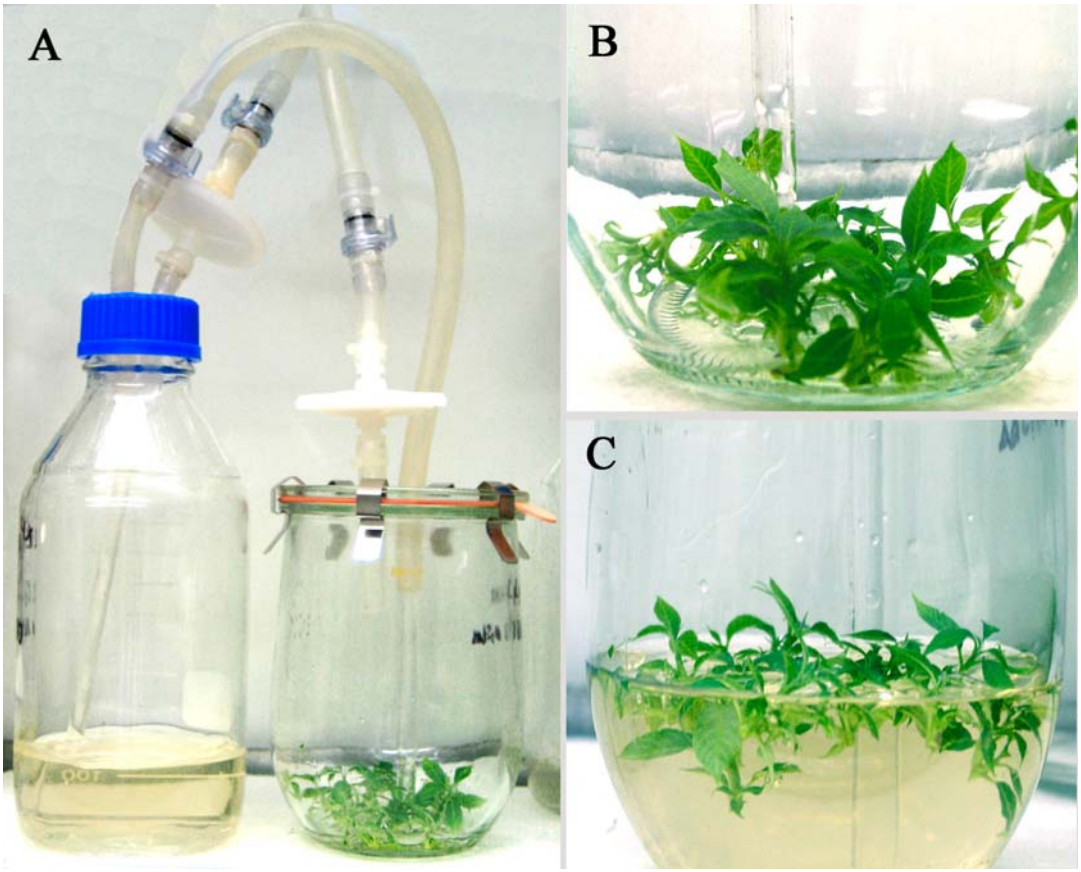

Fig. 8 (**A)** The Dual Vessel System (DVS) consists of two separate vessels connected with silicone and glass tubes. A Schott flask (left) is used as a medium reservoir while explants are located in the second vessel (right). (**B)** Explants during ebb and (**C)** during flood.

As shown in Fig. 8 the DVS consists of two separate chambers, one for growing plants and the other as a reservoir for the liquid medium. The two chambers are connected by silicone

and glass tubes. The air pressure is first applied to the medium reservoir forcing the medium over into the culture chamber to immerse the explants completely. After a defined time of immersion the airflow is reversed to withdraw the medium from the culture chamber.

Thus, with the TIS system manual labour is drastically reduced in comparison to culture and subculture in solid medium. Another advantage is that there is less risk of contamination due to the self-sealing couplings used to disconnect and connect the vessel during culture media renewal without disturbing the plant tissues. Hyperhydricity problems could be solved by adjusting the duration and immersion frequency of cultures. Thus, in general the use of liquid medium for *in vitro* culture simplifies the handling, reduces production costs and is considered to be the most appropriate way to achieve automation (Aitken-Christie, 1991).

3.1. Plant Materials

3.1.1. Cuttings and Seeds

In order to secure a plant stock with a high genotype variability of *Camptotheca acuminata* for this thesis, cuttings were obtained during 2004 from the botanical gardens of Vienna, Austria; Utrecht, The Netherlands; Bonn, Germany and from a private grower in Boskoop, The Netherlands. Seeds of *C. acuminata* were obtained in 2005 from four different geographical locations. Seeds originated from three provinces in China, Sichuan, Jiangsu and Guangdong and from a plantation in Baton Rouge, Louisiana (USA). Seeds from each geographical location were considered as one group (Fig. 9) Thus, seeds originating from Sichuan were grouped as **Ch 1**, from Jiangsu as **Ch 2**, from Guangdong as **Ch 3** and those from Louisiana as **Lou**. Four seedlings from each group **Ch 1** (Bp31, Bp81, Bp101, Bp141), **Ch 2** (Mp27, Mp28, Mp35, Mp36), **Ch 3** (Gz5, Gz7, Gz9, Gz10) and **Lou** (Lp4, Lp13b, Lp18, Lp45) were randomly chosen for further cultivation. Each seedling was considered as an individual genotype and multiple shoots from each seedling as identical clones.

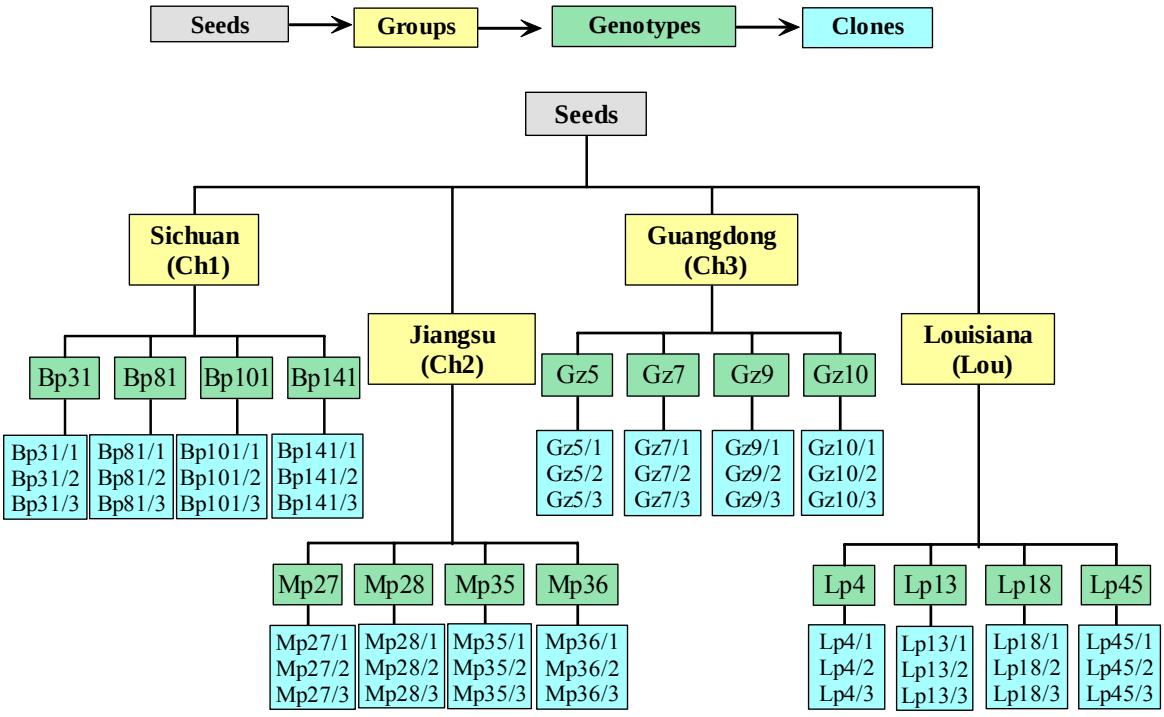

Fig. 9 Flow chart showing the four groups of *C. acuminata* seeds (yellow), the four genotypes (green) and the individual clones (blue) of each genotype.

3.2. Plant Culture Media

Preliminary experiments with B5 basal medium (Gamborg *et al.,* 1968) and MS medium (Murashige and Skoog, 1962), supplemented with various cytokinins (Kin, BAP, GA3, TZD, 2iP) and auxins (IAA, 2,4-D, IBA, NAA) used solely or in combination, showed that full strength MS medium was more appropriate to culture *C. acuminata* cuttings as well as seedlings. For shoot multiplication, a full strength MS medium supplemented with BAP was used. Unless otherwise specified, only a full strength MS medium was used throughout this study for both the solid and liquid cultures.

Tab. 1 Composition and preparation (Macronutrients, Micronutrients, Vitamins and other supplements) of the modified Murashige and Skoog (MS) medium.

Constituent	MS Medium [mg l^{-1}]	Concentrated Stock Solution [mg l^{-1}]	Working Solution [ml l^{-1}]	Storage [°C]
Macronutrients				
Ammonium nitrate	1650	33000	50	4
Calcium chloride dihydrate	440	8800	50	4
Magnesium sulphate heptahydrate	370	7400	50	4
Potassium nitrate	1900	38000	50	4
Potassium phosphate monobasic	170	3400	50	4
Micronutrients (Trace elements)				
Boric acid	6.2	620	10	-18
Cobalt chloride hexahydrate	0.025	25	1	-18
Cupric sulphate pentahydrate	0.025	25	1	-18
Manganese sulphate monohydrate	16.9	338	50	-18
Molybdic acid sodium dihydrate	0.25	250	1	-18
Potassium iodide	0.83	830	1	-18
Zinc sulphate heptahydrate	8.6	172	50	4
Sodium iron (III) ethylene diamine tetraacetic acid	37.5	750	50	4
Organics supplement (Vitamins)				
Glycin	2	40	50	4
Myo-inositol	100	2000	50	4
Nicotinic acid (B3)	0.5	50	10	-18
Pyridoxine hydrochloride (B6)	0.411	41.1	10	-18
Thiamine hydrochloride (B1)	0.1	10	10	-18
Buffer				
Sodium dihydrogen phosphate dihydrate	192.5	3850	10	4
Other Supplements				
Sucrose	30000			
Phyto-Agar	6000			
Gelrite	2500			

Plant culture media preparation was made from self-made concentrated stock solutions. All solutions were prepared using de-ionised water. Working solutions were then prepared by mixing the individual groups of components (macro-, micronutrients, organic compounds and phytohormones) to achieve the required working concentration. After the addition of supplements, the pH was adjusted to the required value using HCl or KOH. For solid media gelling agent (phytoagar) was added before the pH was adjusted. Stock solutions of the major MS inorganic nutrients were prepared at 20-fold and trace elements at 100-fold concentrations. Vitamins and individual plant growth regulator (PGR) for the different components were also prepared in advance (Tab. 1), likewise the stock solutions of other ingredients (e.g. NaFe-EDTA, glycin, *myo*-inositol and sodium dihydrogen phosphate dihydrate). Vitamins, trace elements and PGRs were stored in small volumes at -18 °C, while all other stock solutions were kept in a refrigerator at 4 °C.

3.3. Culture Vessels

Explants in solid media were cultured either in Petri dishes or in home canning jars from Weck® (Wehr, Germany) of various sizes, 250, 500, 750 and 1000 ml, which were sealed with felt rings that allowed aeration due to temperature fluctuation in the growth chambers (Fig. 10A).

3.3.1. Temporary Immersion System (TIS) Vessels

Micropropagation experiments in TIS were carried out in two different types of culture vessel, the Dual Vessel System (DVS) and RITA® vessel (Chapter 2.3.1). DVS explant vessels had a volume of 500 and 1000 ml (Fig. 10C). In order to decimate turbulence of newly cultured explants all treatments for shoot multiplication in DVS were initial with 200 ml liquid medium for the first four weeks, thereafter, 400 ml medium was used. The second vessel type was the one litre RITA® vessel (Fig. 10B) utilising 250 ml liquid medium throughout this study.

For the callus, organogenesis and somatic embryogenesis induction, plant regeneration and rooting in RITA® and DVS explants were immersed for one minute every 6 hours (4 immersion cycles daily = IC d^{-1}), whereas, for shoot multiplication four and eight IC d^{-1} were applied. Electronic timers were used to control the frequencies and duration of the immersion cycles (IC).

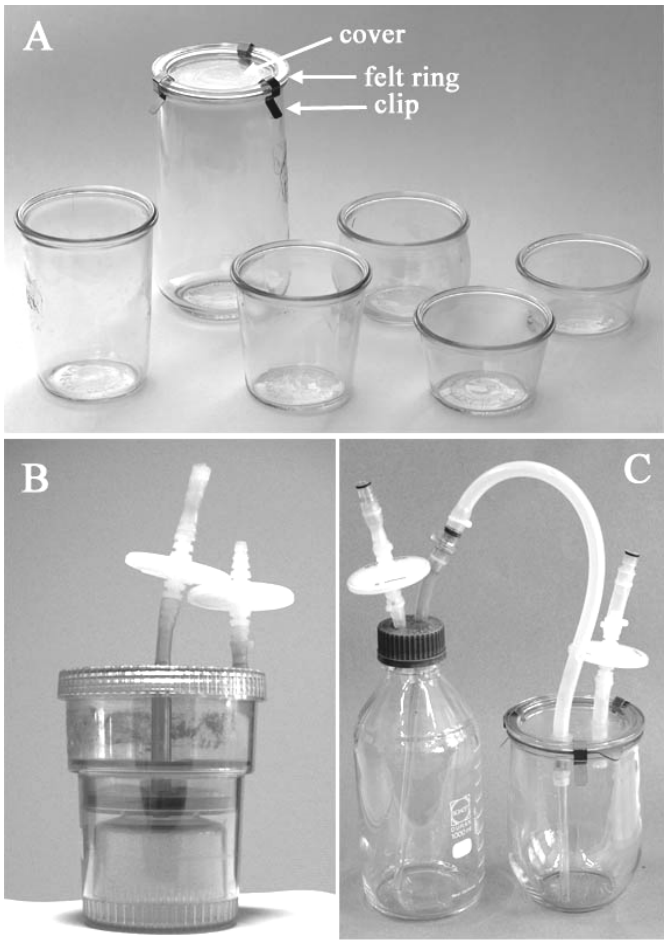

Fig. 10 Type of vessels used in this study. **(A)** Different sizes (250, 500, 750 and 1000 ml) of Weck® glasses used for solid medium culture. **(B)** The RITA® vessel (CIRAD, France) with 2 compartments in one. **(C)** The Dual Vessel System (DVS) with a Schott bottle as medium reservoir and a Weck® glass for the growing culture.

3.4. Sterilisation

3.4.1. Culture Apparatus, Glassware and Equipment

All aseptic processes were conducted under a laminar flow hood, which was decontaminated by turning on the hood and wiping the inwards surfaces with 96% ethanol 15-30 minutes before initiating any operation. Heat-labile components (e.g., polyethylene scintillation vials or conical tubes) were submerged for several minutes in 96% ethanol 15 minutes before use. Glassware such as Petri dishes, Weck® glasses, beakers and Erlenmeyer flasks were sterilised by exposure to 214 °C in a hot air steriliser overnight. All TIS culture vessels, water, paper filters, pipette tips, aluminium foil etc. and culture media were autoclaved at 121°C and 103 kPa for 15 minutes. Working instruments such as scalpels, forceps, scissors and other metal objects were rinsed in 96% ethanol, flamed and allowed to cool down before being used.

3.4.2. Nutrient Media and Substrates

Solid MS media were gelled with either 6 g l^{-1} phytoagar or 2.5 g l^{-1} Gelrite®. (Duchefa Biochemie B.V., Netherlands) and the pH was adjusted to 5.8 for both solid and liquid media before being autoclaved at 121 °C and 103 kPa for 15 minutes. Substrates for the plantlet regeneration via somatic embryogenesis and the *in vitro* rooting were moistened with MS medium supplemented with or without PGR before being autoclaved twice within 48 hours at 121 °C for 45 minutes.

3.4.3. Sterilisation of Plant Material

Tab. 2 Sterilants and tenside used to sanitise plant material.

Sterilants and tenside [a)]	Concentration [%]	Exposure [minute]	Remarks
Ethyl alcohol (ethanol) (Sigma-Aldrich)	70	0.5 to 1	Has a better wetting properties than 96 %; 0.5 minutes for embryos and 1 minute for plant materials
Sodium hypochlorite (NaOCl) *	1	5 to 20	For dissected zygotic embryos 5 minutes is enough; young leaf material 15 minutes and stem cuttings 20 minutes; is efficient cheap and should be prepared fresh
Mercuric chloride (Merck)	0.1 to 0.5	2 to 10	Is toxic and requires special handling and waste disposal; more effective for mature cuttings of *Camptotheca* plant material and seeds before dissection
Triton X-100 [a)] (Sigma-Aldrich)	5	3	A surfactant, which reduces the surface tension and allows a better contact between explants and solution

* Commercial bleach contains about 5% sodium hypochlorite; using a concentration of 20% is equivalent to 1.0% sodium hypochlorite (equal to a 1:5 dilution).

3.5. Growth Chambers

3.5.1. Illumination and Temperature

C. acuminata cultures in solid medium were grown in two different walk-in growth chambers with different environmental conditions. Chamber I (Weiss Technik, Typ LKK +2, 1981) has a dimension of 250 cm width x 200 cm depth x 210 cm height and was equipped with 24 Philips lamps TLD 58W/840 situated vertically behind the culture

vessels (Fig. 11A), with an artificial light radiation of 150 to 300 µmol m^{-2} s^{-1}. All explants cultured in chamber I were grown under 15 h photoperiod, with night and day temperatures of 19 and 24 ± 0.5 ºC, respectively and a humidity of 60%.

Chamber II (Fig. 11B and 11C) with a dimension of 240 cm width x 260 cm depth x 230 cm height and was equipped with 6 Philips TLD 18W/940 and 6 TLD 58W/840 lamps situated horizontally above the culture vessels, with an artificial light radiation of 30-79 µmol m^{-2} s^{-1}. All plants in this chamber were maintained at a 16-hour illumination and a constant temperature of 26 ± 0.5 ºC, with a humidity of 66%. All TIS treatments were conducted in chamber II.

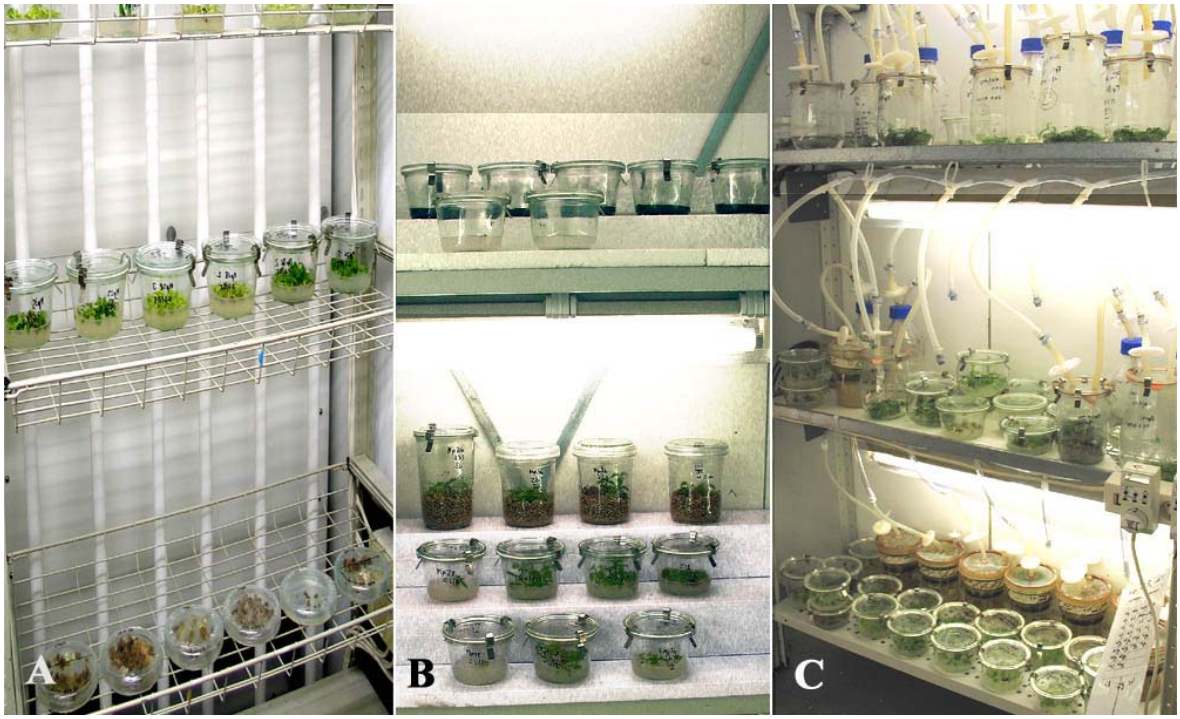

Fig. 11 A view inside the two plant growth chambers. **(A)** Chamber I primarily used for solid and suspension cultures. **(B)** and **(C)** Chamber II for solid, suspension and TIS cultures.

3.6. Plant Propagation Methods of *C. acuminata*

3.6.1. *Ex situ* Seed Germination

A minimum of 50 and a maximum of 100 seeds from each location were cultured in two different substrates, peat overlaid with a mixture of vermiculite and soil (2:1:1) and peat overlaid with quartz sand (2:1). All cuttings and seedlings were maintained at 60 µmol m^{-2} s^{-1} PPFD at a temperature of 24 ± 2 °C under greenhouse conditions. Cultures were visually

checked daily and irrigated with tap water when necessary. Growth and development scoring were carried out at 4, 8 and 12 weeks intervals.

3.6.2. Conventional Propagation via Stem Cuttings

Since plant material within Europe was limited and only seasonally available, stock culture of *C. acuminata* plants were established through conventional propagation (stem cuttings and seeds) to maintain a greenhouse collection of starting material for experimental purposes. For the *ex vitro* culture four different types of cuttings (Fig. 12A), softwood (SW), semi-softwood (SSW), semi-hardwood (SHW) and hardwood (HW) were taken from two to three-year-old plants and cultured (December/January, March/April, June/July) using two different root stimulator powders, Wurzelfix® (0,01% 1-naphthaleneacetic acid (NAA) and Rhizopon® (0,05% indole-3-acetic acid, IAA).

Fig. 12 The four different types of cuttings **(A)**, softwood (SW), semi softwood (SSW), semi-hardwood (SHW) and hardwood (HW). **(B)** Cuttings, propagated *ex situ* in a substrate mixture of sand, soil and vermiculite.

All leaves were removed and stems were cut into pieces of 10-12 cm in length with three to five nodes each. The cut ends of 100 stem cuttings were dipped into Wurzelfix® and another 100 in Rhizopon® before they were cultured in well-drained plastic trays containing a mixture of moist sand, soil and vermiculite 1:2:1 (Fig. 12B). One to two nodes of each stem were buried into the substrate for root induction.

3.6.3. *In vitro* Seed Germination

C. acuminata seeds were obtained from different geographical locations: Baton Rouge, Louisiana (USA) (Fig. 13A) and from the provinces Guangdong, Sichuan and Jiangsu (China) (Fig. 13B).

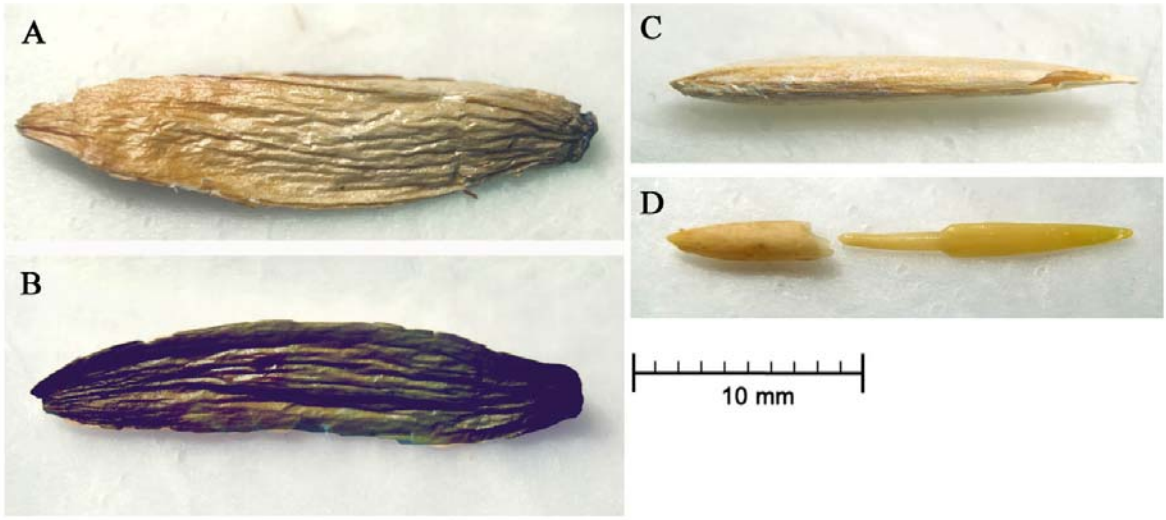

Fig. 13 *C. acuminata* seeds. **(A)** Seed from Louisiana (yellow) and **(B)** from China (dark brown). **(C)** Seed with testa and **(D)** mature zygotic embryo dissected from the endosperm.

Seeds with pericarp were washed in a 5% (v/v) Triton X-100 detergent solution (Biomol GmbH, Hamburg) for 3 minutes then thoroughly rinsed with sterilised water until foam free. The seeds were then dipped into a 70% (v/v) ethanol solution for 1 minute. Thereafter, they were transferred into a 20% (v/v) sodium hypochlorite solution (NaClO) (Clorox® containing 5.0% Chlorine), for 10 minutes, gently agitated once or twice during this time. Subsequently, they were rinsed four times with sterilised deionised water. The zygotic embryos were aseptically removed from the testa (Fig. 13C) and then from the endosperm (Fig.13D). Ten embryos were placed in each vessel (Weckglass®, Ø 100 mm x 69 mm) containing 100 ml PGR-free MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose. All cultures were maintained under a 16-hour photoperiod provided by Philips TLD 58W/840 fluorescent lamps, with a light intensity of 60 µmol m^{-2} s^{-1} PPFD at 25 ± 1°C.

3.6.4 Micropropagation via Apical and Axillary Buds

Young branches from greenhouse and botanical gardens plants of *C. acuminata* were used as starting material for shoot multiplication in solid medium and in TIS. Apical tips and

axillary buds from greenhouse plants were removed by cutting the stem just above and below the node. The cuttings were approximately 1 to 1.5 cm long, each including one node with a bud. These single node explants were washed under running tap water for 15 minutes before surface sterilisation. Explants were surface sterilised with 0,1% mercuric chloride solution for 10 minutes gently agitated once or twice during this time. Hereafter they were washed four times in sterilised deionised water and air dried for 2 to 5 minutes under aseptic condition before culture. Ten explants were cultured in each vessel (Weckglass®, Ø 100 mm x 69 mm) containing 100 ml full strength MS medium supplemented with three different concentrations of BAP (0.5, 1.0 and 1.5 mg l^{-1}) and 30 g l^{-1} sucrose. Cultures were maintained under the same condition as described in chapter 3.6.3.

3.7. Plant Regeneration via Organogenesis and Embryogenesis

3.7.1. Callus Induction in Solid and in Liquid Media

Mature zygotic embryos were divided into four parts (Fig.14), which were then used as explants for embryogenic callus induction. Explants were excised and small pieces of 2 to 5 mm inoculated in solid MS medium supplemented with 30 g l^{-1} sucrose and different combinations and concentrations of cytokinins and auxins (Tab. 3). For each treatment a minimum of three replicates were cultured with five explants per dish. Each genotype was kept separately.

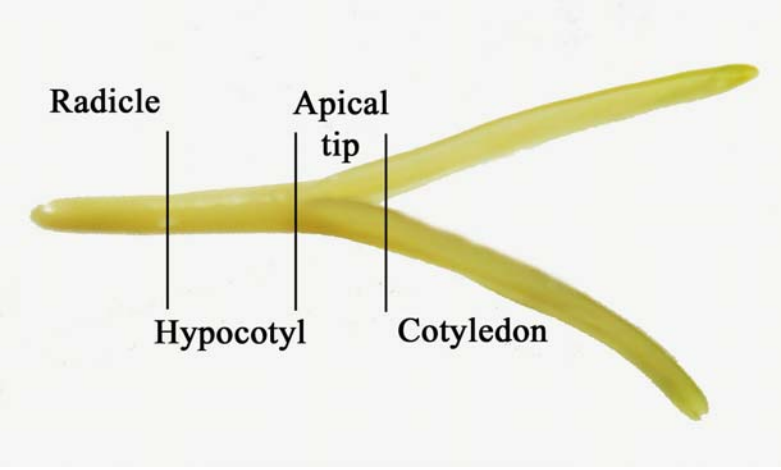

Fig. 14 Division of a mature zygotic embryo into radicle, hypocotyl, apical tip and cotyledon for embryogenic callus induction.

Tab. 3 MS medium supplemented with various plant growth regulators (PGRs) used to induce organogenesis and somatic embryogenesis in solid and in liquid culture.

MS-Medium with various PGR	Concentration of growth regulators [mg l^{-1}]					
	Kin	BAP	2iP	IAA	2,4-D	NAA
BI		2.0		0.1		
B21		2.0			0.1	
B22		2.0			0.5	
K21	2.0				0.1	
K22	2.0				0.5	
NiP			0.1			0.2

For callus induction directly in liquid medium, hypocotyl and leaf segments from twenty-one-days-old *in vitro* grown seedlings were used. Hypocotyls were cut into 2-3 mm discs or into cylindrical pieces of 5 mm length, while each cotyledon was divided into 10-15 mm pieces. Explant segments were then transferred into 250 ml Erlenmeyer flasks containing 50 ml liquid induction medium (Tab. 3). Culture treatments were identical for each seedling explant and a minimum of 3 flasks was set up for each treatment. Flasks were placed on a 2D rotary shaker at 108 rpm for 12 weeks. Cultures were incubated at 25 ± 0.5 °C under 16 hours photoperiod in chamber II at 20 µmol m^{-2} s^{-1} PPFD. Visual and stereo microscope observations were carried out at two-day intervals for explants in solid media and at four-day intervals for explants in liquid media for callus, somatic embryo and organogenesis development. Explants were subcultured at four-week intervals.

3.7.2. Embryogenic Callus Induction in PGR-free Media in TIS

Hypocotyls of fourteen-day-old seedlings (Fig. 15A) with a height of about 20 to 40 mm were dissected and used as primary explants. For the induction of somatic embryogenesis, ten hypocotyl segments of 2-3 mm thickness (Fig. 15B) from each seedling were cultured in 200 ml full-strength hormone free MS medium containing various concentrations of sucrose (20, 25, 30 and 35 g l^{-1}). Each treatment consisted of three replicates. It should be noted that each seedling represents a single genotype. Explants were cultured in two different types of TIS vessels, the Dual Vessel System (DVS, Fig. 15C, D) and the RITA® (Fig. 15E).

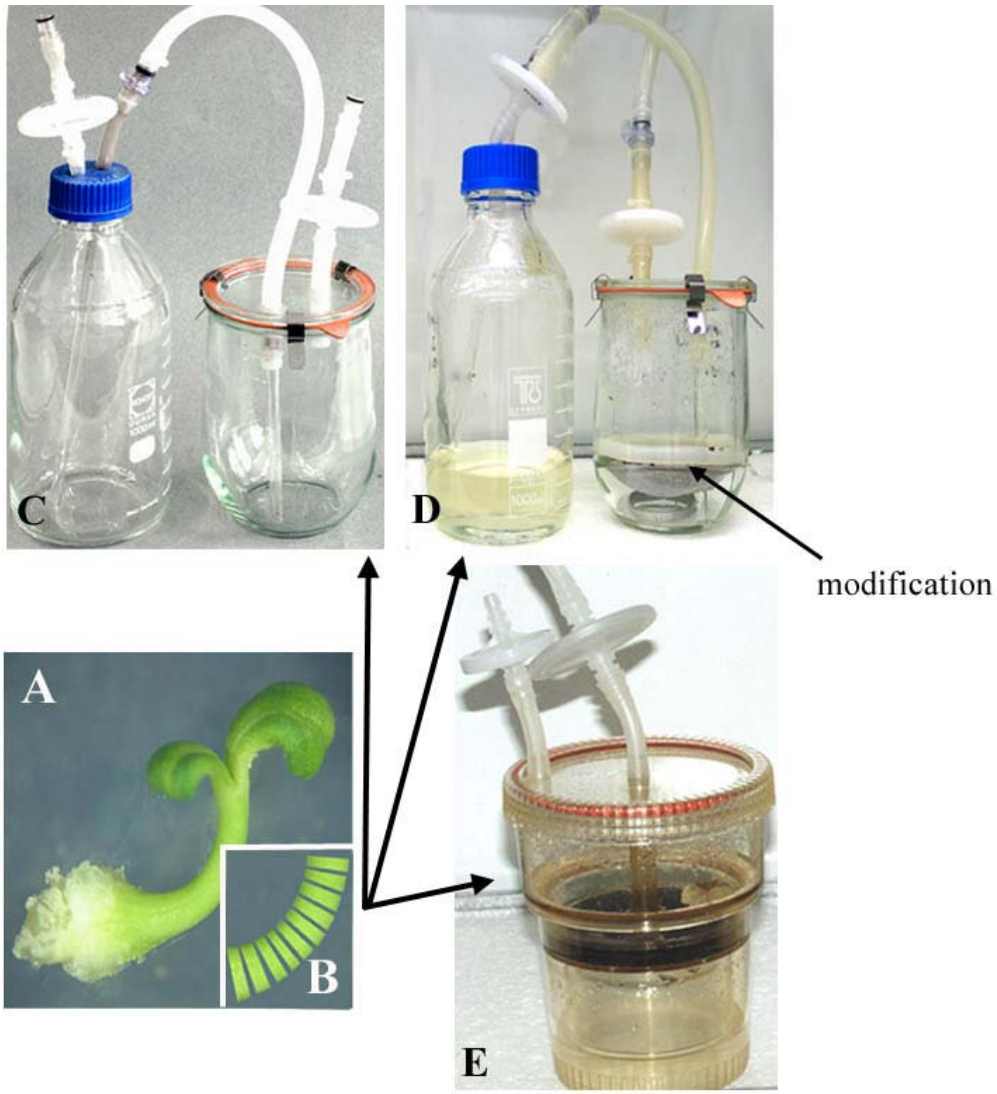

Fig. 15 **(A)** Fourteen-day-old *C. acuminata* seedling. **(B)** Hypocotyl segments 2-3 mm thick for TIS culture. **(C)** Standard DVS, **(D)** modified DVS and **(E)** RITA® vessels.

Explants were cultured in both the standard (Fig. 15C) and modified DVS (Fig. 15D). In the modified DVS a silicone ring and a sieve were placed on a stainless steel ring to prevent direct contact between explants and the liquid layer that remained in the culture chamber after each immersion cycle. Consequently, this modification served at the same time as an orientation device for the explants. Temporary immersion cycle was 1 minute every 6 hours throughout the study unless otherwise indicated.

3.7.3. Plant Regeneration via Organogenesis in Solid and Liquid Media

Friable and nodular calli, which were assumed to be potentially organogenic or embryogenic were selected and cultured on MS medium supplemented with 0.5 mg l^{-1} BAP or in PGR-free medium containing 3% sucrose for maturation and adventitious shoot or somatic

embryo regeneration. All regenerated adventitious shoots of about 10-15 mm induced in solid and in liquid medium were excised and transferred to solidified MS medium fortified with 0.5 mg l^{-1} BAP for plantlet development. Induced somatic embryos were also selected and cultured following the same pattern used for the adventitious shoots.

3.7.4. Plant Regeneration via Somatic Embryogenesis in TIS

To accelerate maturation, embryogenic calli induced in PGR-free medium containing 35 g sucrose in TIS were removed and plated on the same medium gelled with 6 g l^{-1} Phytoagar. After four weeks of maturation in solid medium, embryos at cotyledonary stage were selected and re-cultured in DVS and RITA® in medium fortified with 0.5, 1.0, 1.5 mg l^{-1} BAP and in PGR-free medium. A total of 25 embryos were placed in each vessel and all treatments were conducted nine times. After six weeks in TIS, regenerants were visually evaluated. Only embryos grown in 0.5 mg l^{-1} BAP and PGR-free medium were scored and classified in three morphological categories, adnated, deformed and normal growth. Growth and development were evaluated by recording the conversion rate in percentage, the numbers of leaves and roots of regenerants.

3.7.5. Plant Regeneration via Somatic Embryogenesis in Sterilised Substrates

For the *in vitro* culture in sterilised substrates a total of 10 cotyledonary embryos were dislodged from the solid culture medium and transferred into Weck® glass jars in four different substrates mixtures, with five replicates each: **1)** A mixture of sand and vermiculite (SV, 1:2 v/v); **2)** Sand, soil and vermiculite (SSV, 1:2:2 v/v/v); **3)** Pure sand and **4)** pure soil. All substrates were moistened with MS medium supplemented with 0.5 mg l^{-1} BAP before being autoclaved twice within 48 hours at 121 °C for 45 minutes. Cultures were visually evaluated under aseptic conditions after 4, 8 and 12 weeks. Growth, development and survival rate of regenerants were recorded.

3.8. Shoot Multiplication in Solid Medium and in TIS

3.8.1. Multiple Shoot Induction in Solid Media

In the course of this study preliminary experiments had shown that media supplemented with BAP were more appropriate for the growth and development of *C. acuminata* shoots. To test the effect of different BAP concentrations four seedlings (= genotypes) from each group **Ch1** (Bp31, Bp81, Bp101, Bp141), **Ch2** (Mp27, Mp28, Mp35, Mp36), and **Lou** (Lp4, Lp13b, Lp18, Lp45) were randomly chosen and used as explants for multiple shoot

induction. Shoot tips or axillary buds of *in vitro*-raised seedlings were cultured in solid MS medium supplemented with three different concentrations of BAP (0.5, 1.0 and 1.5 mg l^{-1}) and 30 g l^{-1} sucrose. After a culture period of 4 and 8 weeks explants were harvested and the multiplication rate, number of shoots per explant, shoot height (mm), fresh weight (mg), leaf area (mm^2), chlorophyll (mg g^{-1} FW) and stomata density (mm^{-2} leaf area) were recorded. All treatments consisted of ten replicates and the experiment was repeated at least three times.

3.8.2. Multiple Shoot Induction in TIS (DVS and RITA®)

Shoot tips and axillary buds of *C. acuminata* established in solid medium cultures were excised and subcultured for a period of 8 weeks in TIS. Explants were cultured in both the DVS and RITA® vessel containing full strength MS media fortified with 0.5, 1.0 and 1.5 mg l^{-1} BAP plus 30 g l^{-1} sucrose to compare the effect of BAP concentrations on shoot proliferation among the clones of the different genotypes of the three individual groups Ch1, Ch2 and Lou of *C. acuminata*. During the first four weeks a working volume of 200 ml culture medium was used in DVS and thereafter 400 ml. For the RITA® vessel a volume of 250 ml was used throughout this study. Seven to twelve shoots (20 mm in height) were cultured in each vessel. As a control the same numbers of shoots were grown in PGR free medium. The immersion cycles set to 1 minute every 3 and 6 hours, was controlled by electronic timers for both DVS and RITA®. All treatments consisted of at least five replicates and the experiments were repeated two times. Visual observations of growth and development were done at a weekly interval and evaluation included recording the number of shoots per explant, shoot height (mm), fresh and dry weight (mg), leaf area (mm^2), chlorophyll (mg g^{-1} FW) and stomata density (mm^{-2} leaf area) over a culture period of 4, 6 and 8 weeks.

3.9. Rooting and Acclimatisation

3.9.1. *In vitro* and *Ex vitro* Rooting

In vitro rooting of regenerants and microcuttings were conducted in solid media, in liquid media in TIS (DVS and RITA® vessels) and in three different sterilised substrates: **1)** A mixture of sand and vermiculite (SV, 1:2 v/v); **2)** Sand, soil and vermiculite (SSV, 1:2:2 v/v/v) and **3)** pure commercial sowing soil. Shoots of regenerated plantlets via somatic embryogenesis were rooted in a half strength MS medium, while microcuttings derived from solid medium and TIS were rooted in full strength MS medium. Shoots of 20 to 30 mm in length were excised from proliferating cultures and transferred into the liquid media

systems DVS and RITA® set at 4 IC d^{-1} and in solid media supplemented with either 0.5 mg l^{-1} IBA or 0.5 mg l^{-1} NAA. Shoots cultured in sterilised substrates were also moistened with MS media containing 0.5 mg l^{-1} IBA or 0.5 mg l^{-1} NAA but without sucrose. Approx. 10 shoots were cultured in solid media and in sterilised substrates while 15 to 20 shoots were cultured in the TIS vessels for root induction. All TIS cultures were maintained in growth chamber I, while cultures in solid medium and sterilised substrates in growth chamber II. Visual observations were made every day and the effects of IBA and NAA on rooting were evaluated after 6 weeks in culture.

For the ex *vitro* rooting, shoots were cultured in three different substrates (SSV, SV and commercial sowing soil), which were moistened with 0.5 mg l^{-1} IBA or 0.5 mg l^{-1} NAA without sucrose. Explants were maintained in a greenhouse at 25 ± 2 °C under a 12-hour photoperiod (Philips 400 Watt Son-T Agro HPS lamps) and at 60 - 70% relative humidity. All treatments were performed with a minimum of five replicates and were repeated at least three times.

3.9.2. Acclimatisation to Greenhouse Conditions

Rooted plantlets derived from the *in vitro* systems (solid media, DVS, RITA® and from sterilised substrates) were transplanted into well-drained trays (30 cm x 45 cm) or plastic pots (90 mm x 60 mm) containing commercial sowing soil. Soil mixture comprised of quartz sand, peat, clay and NPK-minerals with pH 5.6. Pots and trays with *in vitro* derived plantlets were kept covered for the first 10 days to avoid dehydration. Hereafter, the traylids were opened periodically for acclimatisation. Plantlets were grown at 60 µmol m^{-2} s^{-1} PPFD, at a temperature of 24 ± 2 °C and 60% humidity. The survival rate was recorded after 6 weeks.

3.10. Camptothecin (CPT) Extraction and Sample Preparation

3.10.1. Callus, Greenhouse Plants, Shoots Grown in TIS Solid Media

The extraction and quantification of CPT and HCPT using the HPLC was done according to the method described by Yan *et al.,* (2003). About 0.5 to 1.0 g freshly harvested or frozen plant tissue was placed into a 50 ml centrifuge glass tube to which 3 to 4 ml of 61% ethanol was added, depending on the amount of tissue used. Tissues were thoroughly homogenised with an Ultra-Turrax T25 Homogeniser before the extract was sonicated for 10 minutes at 50 °C in a Sonorex RK510 Bath (Bandelin, Berlin, Germany). After cooling down to room temperature samples were made up to a volume of 5 ml with 61% ethanol.

The extract was centrifuged at 15,000 *g* for 10 minutes (Model Biofuge, Heraeus Instruments). The supernatant was filtered through a PTFE 0.45 µm filter into a HPLC vial for CPT analysis. CPT content was expressed in mg g^{-1} dry weight.

3.10.2. Cell suspension Cultures

Cell culture was centrifuged for 10 minutes to separate the supernatant from the cells. Thereafter, the supernatant was filtered through a PTFE 0.45 µm directly into HPLC vials for CTP analysis. The remaining cell sediment was similarly prepared for analysis as in 3.10.1.

3.10.3. Embryogenic Callus and Different Stages of Somatic Embryos

Embryogenic callus and somatic embryos of globular, torpedo and cotyledonary stages (0.2 to 1.2 g) were prepared individually and the same procedure as described in chapter 3.10.1 was applied for CPT analysis.

3.10.4. CPT Excretion into Liquid Culture Media

Liquid culture media were filtered through a PTFE 0.45 µm directly into HPLC vials for CPT and HCPT exudation analysis. CPT content was expressed in µg g^{-1} FW for exudation.

3.10.5. CPT Recovery Test

For the recovery test 5 ml CPT at a concentration of 51µg ml^{-1} was spiked to the 200 mg leaf sample, which was extracted and analysed as described above. The same extraction procedure was used on a 200 mg leaf sample without CPT. Both extracts were analysed by HPLC and the recovery was calculated by use of the equation:

Recovery = **[(A-B)/C]** × 100 where **A** is the quantity of CPT in the spiked sample, **B** is the quantity of CPT in the leaf sample without added standard, and **C** is the quantity of CPT added.

3.11. HPLC Analysis of Camptothecin (CPT)

A rapid and sensitive assay for quantitative determination of CPT and 10-Hydroxy-camptothecin (HCPT) in the different tissues of *C. acuminata,* was performed on an isocratic reverse-phase high performance liquid chromatography system (RP-HPLC). The HPLC-system consists of a Knauer Pump 64 (Knauer, Berlin, Germany), an AS-2000 Autosampler (Merck/Hitachi, Darmstadt, Germany), and a Waters Model 474 scanning

fluorescence detector. Chromotographic separation of analytes was carried out on an analytical column LiChroCART 250-4 5 µm Superspher 100 RP-18 (Merck) with a 4 x 4 mm pre-column maintained at 35 °C. The mobile phase consisted of 58% methanol and 42% Water (58 : 42, v/v) with a flow-rate of 1 ml min^{-1} and the injected volume was 20µl. Fluorescence detector was set at an excitation of 370 nm (λe_x) and the emission of 434 nm (λe_m). Retention time for HCPT and CPT were 5.6 and 8.5 minutes, respectively. The CPT and HCPT standards used to prepare the calibration curves (25-102 ng ml^{-1}) were purchased from Sigma-Aldrich (Eisenhower, Germany). All standard solutions were stored at -20°C. The quantifications of CPT and HCPT were calculated based on the area of separated peaks using standard calibration curves.

3.12. Plant Selection According to their CPT Content

To select plants with high CPT content, it was important that all plants had been cultured under the same conditions and harvested at the same stage of development. Clones of four genotypes from each group were selected randomly according to their height, branching pattern, leaves size, and colour. Samples from each clone were analysed individually for their CPT contents. For the selection the following criteria were used: Clones with best growth and multiplication rate and the highest CPT content were considered the best for further culture.

3.13. Fresh and Dry Weight Measurements

It was not appropriate to use a precision balance for small amount (< 1 mg) of tissue material in the laminar flow hood, therefore, fresh weight increment of shoots were measured without sacrificing the samples during the experiment. Culture vessels containing solid medium were weighed outside the laminar flow hood before and immediately after shoots were transferred into it to define the fresh weight. At the end of the experiment each entire culture was taken out and placed in pre-weighed vessels to determine the final fresh weight. The same procedure was repeated for the fresh weight of callus and embryos at different stages. To determine cell suspension fresh and dry weight 5 to 10 ml cell suspension was transferred into a pre-weighed centrifuge tube. Samples were centrifuged for 15 minutes at 2,000 g until no cells were present in the supernatant, which was then carefully pipetted without disturbing the pellet. The centrifuge tube was then weighed again to determine the fresh weight. Hereafter, all samples were oven dried at 105 °C until no change in dry weight was observed.

3.14. Leaf area

Leaf area was measured by a simple tracing technique. Eight to 15 leaves were randomly chosen from each jar and placed between DIN A4 papers and an accurate trace of the leaf outline was captured. Thereafter, the leaf outline was cut out and weighed, likewise a piece of paper of a known dimension (20 x 20 mm). A conversion factor was calculated by dividing the area of the paper by its weight. The units are given in mm^2 mg^{-1}. The conversion factor was used to determine the leaf area according to the following equation:

Leaf area (mm^2) = weight of leaf tracing (mg) $*$ conversion factor (mm^2 mg^{-1})

3.15. Chlorophyll extraction

Chlorophyll content was determined according to Hiscox and Israelstam (1979). Approximately 100 mg leaf tissue was placed in a test tube containing 5 ml of dimethyl-sulphoxide (DMSO). Without damaging the leaf structure chlorophyll pigments were extracted at 65 °C for 30 to 45 minutes depending on the degree of leaf thickness and age. After cooling down to room temperature the extract was transferred to a graduated tube and made up with DMSO to a total volume of 10 ml. The chlorophyll extract was assayed immediately or transferred to vials and stored between 0 - 4 °C until required for analysis.

For the assay 2 ml of chlorophyll extract was transferred into a quartz cuvette and read in a spectrophotometer (LKB Ultraspec Plus, model 4054, Biochrom Ltd, Cambridge, UK) at 470, 646 and 663 nm against DMSO. Three replicates were measured for each sample. Chlorophyll content was calculated following the equation used by Arnon (1949) where **A** represents the absorbance of the sample expressed in mg g^{-1} fresh weight.

Total chlorophyll (µg/ml)	= 20.2 (A_{645}) + 8.02 (A_{663})
Chlorophyll *a* (µg/ml)	= 12.7 (A_{663}) - 2.69 (A_{645})
Chlorophyll *b* (µg/ml)	= 22.9 (A_{645}) - 4.68 (A_{663})

3.16. Determination of Stomata Density

Leaves from plants grown in greenhouses, TIS and in solid medium were harvested to calculate stomata density. To visualise the stomata for counting, nail varnish was applied on the adaxial and abaxial surfaces of fully expanded leaves. After several minutes (air dry) Sellotape® tape was attached to the leaf surface and the epidermal peel was pulled off. The layer was mounted on a microscope slide and stomata density was estimated on at least three randomly selected regions per leaf. All stomata within an area of 0.073 mm^2 were counted using an Olympus BHS microscope at 200 magnification. The obtained values were used to calculate the stomata density per leaf area mm^{-2}.

3.17. Maintenance of Embryogenic Callus

3.17.1. In Suspension Culture and in Solid Media

Somatic embryogenic calli were maintained on same media compositions as the induction media at 4-week subculture intervals. About 1-2 g embryogenic callus from each TIS treatment was transferred to solid medium and into a 250 ml Erlenmeyer flask containing 100 ml PGR-free, BI or NiP (Tab. 3, p 26) liquid media. Flasks were kept on a 2D rotary shaker at 108 rpm. All dishes and flasks with embryogenic calli were incubated at 25 ± 0.5 °C under 16 hours photoperiod (40 µmol m^{-2} s^{-1} PPFD). Upon subculturing cells were separated using a 1 mm mesh stainless-steel sieve and transferred to fresh liquid medium. Cell aggregates were cultured in solid media of the same PGR compositions. Visual observation was conducted using a stereo microscope at 4 week intervals.

3.17.2. Cell Viability test with Fluorescein Diacetate (FDA)

A vital staining method with fluorescein diacetate (FDA) was used to determine cell viability in suspension cultures. To prepare a 0.5% (g/v) FDA stock solution, 5 mg of fluorescein diacetate was dissolved in 1 ml acetone. A working solution of 0.1% was obtained by adding 0.5 ml of the FDA stock solution to 2 ml fresh culture medium. Diluted FDA solution was stored on ice. A volume of 0.5 ml of FDA solution and 0.5 ml cell suspension were mixed and incubated for 10 minutes at room temperature. Thereafter, 1 or 2 drops of the solution containing stained cells were put onto a microscope slide and covered with a cover slip. Stained cells were observed at a magnification of 100 to 200× under an Olympus BHS microscope with a reflected light fluorescence illuminator (Model BH2-RFL) using a mercury burner light source, an exciter U- (UG-1), a Dichroic mirror U (DM400 + L420) and an additional barrier filter L-435.

3.18. Statistical Analysis

Explants were randomly chosen for treatments and data were recorded as means of a minimum of three and maximum of ten to twenty five replicates. Each experiment was repeated at least twice. Analysis of variance (ANOVA) was performed to test the significance of the differences between treatments. When significant differences were found ($p \leq 0.05$), a multiple comparison test of means (Duncan's test) was calculated using Statistica 6.0. Linear regression analysis was used to examine the relationship between the plant growth and the number of shoots per explant.

4.1. Plant Propagation Methods of *C. acuminata*

Two different procedures were employed to establish a culture of *C. acuminata*. Plants were conventionally propagated (*ex situ*) through seeds and stem cuttings. In addition, plants were regenerated from two different explant types, seeds and axillary buds, using *in vitro* culture methods.

4.1.1. *Ex situ* Seed Germination

The first set of seeds was cultured between December and January (2004-2005) followed by the second batch in March / April (2005) and the third batch in June / July (2005). No pre-treatments were applied to enhance germination before seeds were sown in substrates. The germination time was about 13 days. Seeds of *C. acuminata* show an epigeous germination (Fig. 16 A-C). There were no significant differences among the four groups in terms of development, however, there was a substantial differences in germination rate. Seeds from Jiangsu, Guangdong and Sichuan showed germination rates of 80%, 80% and 60% respectively. No *ex situ* seed germination was observed in seeds from Louisiana (Tab. 4).

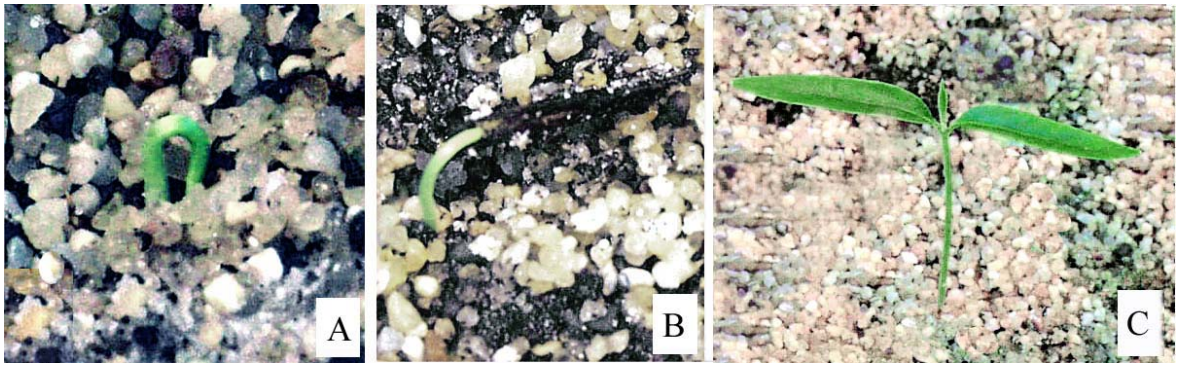

Fig. 16 Epigeous germination of *C. acuminata*

Within six weeks seedlings had reached an aerial stem height of 10 cm showing no significant differences in growth among the three groups, however a number of seedlings died because of stem rot. After six weeks all surviving seedlings were transplanted to commercial soil, during this phase seedling mortality was still high (Tab. 4). After 12 weeks only 40% of the seedling from Ch1, 50% from Ch2 and 20% from Ch3 survived. Hereafter, all *C. acuminata* plants showed a rapid and typical slender growth.

Tab. 4 *Ex situ* germination of *C. acuminata* seeds obtained from four different geographical locations. Sichuan (Ch 1), Jiangsu (Ch 2), Guangdong (Ch 3), China and Louisiana (Lou), USA. Seeds were cultured in substrates under greenhouse conditions. Data shown below are the means of three replicates.

Origin of seeds	Group	Seeds sown [n]	Germination rate [%]	Survival rate of germinated seeds [%]	
			after 2 weeks	6 weeks	12 weeks
Sichuan	Ch 1	150	60	50	40
Jiangsu	Ch 2	150	80	60	50
Guangdong	Ch 3	150	80	40	20
Louisiana	Lou	300	0	0	0

4.1.2 Conventional Propagation via Stem Cutting

Survival rate of *C. acuminata* cuttings was estimated by observation of shoot and root development. Apical and axillary bud development was similar throughout the year which indicated that there was no seasonal variation in terms of growth in the greenhouse. Stems cultured with buds, flushed more rapidly than leafless stems. Nevertheless, after three weeks shoot buds began to shrivel and finally died, mostly because cuttings showed no sign of root development. Treatments with two different auxins, Wurzelfix® and Rhizopon® to enhance rooting did not show any distinctive difference regarding root formation. Figure 17 shows the survival rate of cuttings during a culture period of twelve weeks. At the end of the experiments 38% SW, 32% SSW, 37% SHW and 35% HW survived and developed into strong and healthy normal greenhouse plants. In general, an average survival rate below 50% was considered to be poor.

Roots initialising were observed earlier (after 4 weeks) on semi-softwood (SSW) and semi-hardwood (SHW) without callus formation than on softwood (SW) and hardwood (HW), on which callus was first formed before roots appeared. Statistical analysis showed no significant differences in terms of growth and rooting rate between the four types of stem cuttings. Once roots were induced, cuttings became more vigorous and within three months they reached an average stem height of 70 ± 1.7 cm. Plants were then strong enough to overwinter in a polythene clad tunnel at a temperature above 0° C and an average of 98% survived. In December - January most of the plants lost their leaves but began to flush in early April.

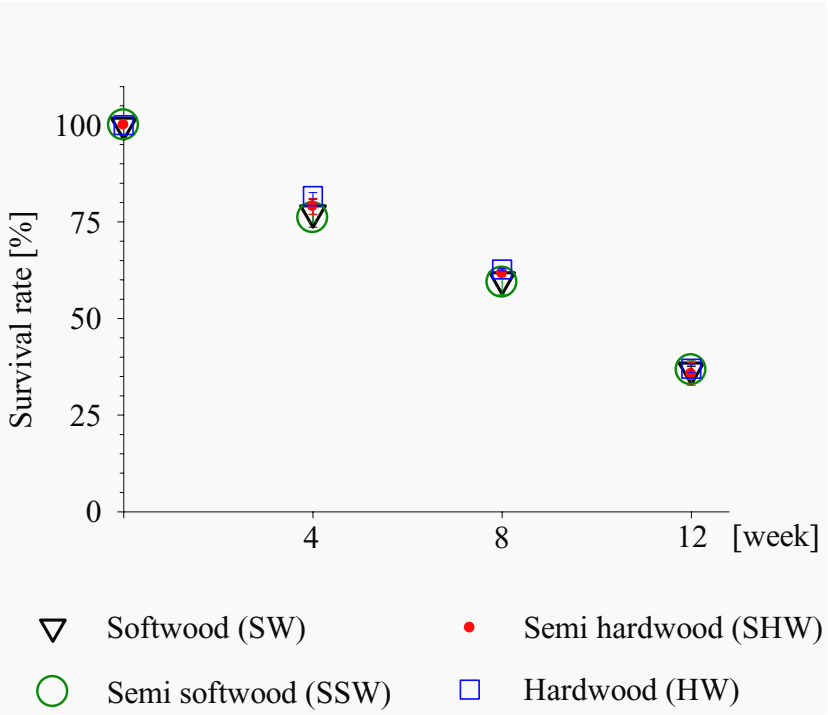

Fig. 17 Survival rate of the four types of stem cuttings, softwood (SW), semi-softwood (SSW), semi-hardwood (SHW) and hardwood (HW) cultured in substrates under greenhouse condition.

4.1.3. *In vitro* Seed Germination

C. acuminata seeds from the four different geographical locations Sichuan (Ch 1), Jiangsu (Ch 2), Guangdong (Ch 3) and Louisiana (Lou) were cultured on PGR-free medium not only to test the germination rate but to secure a broad genetically variable source of explants for further research. Seeds from each location were considered as a group since they were collected from the same parental plant. Seeds varied little in size and weight. The average length and weight was 25 ± 1 mm and 40 ± 0.5 mg respectively. They were however, different in colour, which seems to be regional specific. All seeds from Louisiana were brighter in colour than those from China which were dark brown. Seeds of *C. acuminata* succumbed to contamination due to their furrowed surface and fibrous pericarp. However, when the pericarps were removed seed germination was accelerated up to 81% with endosperm (Fig. 18A) and up to 94% when the mature zygotic embryos were dissected from the endosperm (Tab. 5 and Fig. 18B). Seeds cultured with endosperm germinated after 7 days and no contamination was observed.

Tab. 5 *In vitro* seed germination of *C. acuminata* from the four different geographical locations on PGR-free MS medium. Data shown below are the means of three replicates.

Seed origin		Seeds cultured [n]	Germination [days]	Germination [%]	Abnormal growth [%]	Contamination after 7 days [%]
		with pericarp				
Sichuan	**Ch 1**	20	-	-	-	-
Jiangsu	**Ch 2**	20	-	-	-	-
Guangdong	**Ch 3**	25	21	30	-	100
Louisiana	**Lou**	50	21	1	-	100
		with testa				
Sichuan	**Ch 1**	30	21	50	-	100
Jiangsu	**Ch 2**	20	21	15	-	100
Guangdong	**Ch 3**	25	21	57	-	100
Louisiana	**Lou**	50	21	40	-	100
		with endosperm				
Sichuan	**Ch 1**	170	7	79	21	-
Jiangsu	**Ch 2**	75	7	81	19	-
Guangdong	**Ch 3**	25	7	80	20	-
Louisiana	**Lou**	240	7	77	23	-
		dissected embryos				
Sichuan	**Ch 1**	75	3	90	10	-
Jiangsu	**Ch 2**	75	3	94	6	-
Guangdong	**Ch 3**	25	3	91	9	-
Louisiana	**Lou**	100	3	90	10	-

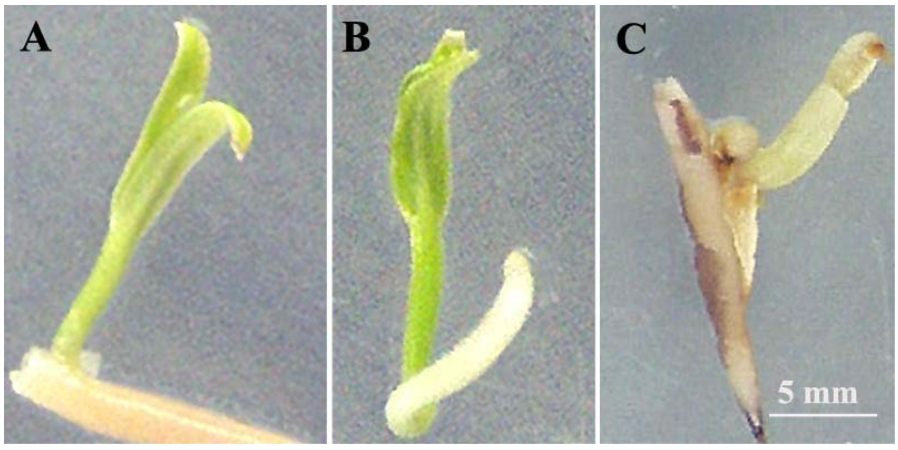

Fig. 18 *In vitro* germination of *C. acuminata* seeds on PGR-free MS medium. **(A)** Seed sown with endosperm **(B)** zygotic embryo excised from the endosperm before cultured. **(A** and **B)** represent a normal germination, whereas **(C)** an abnormal germination.

Dissected mature zygotic embryos with large flat cotyledons became green after 3 days and started to grow immediately thereafter. Once again no contamination was observed. Generally, all zygotic embryos with and without endosperm germinated, however, during germination about 20% failed to push their way entirely through the endosperm, resulting in an abnormal growth (Fig. 18C). Seed abnormality was higher in seeds cultured with endosperm than those without endosperm.

4.1.4. Micropropagation via Apical and Axillary Buds

C. acuminata cuttings obtained from the different botanical gardens and private growers failed to induce shoots, instead profuse calli formation were observed on all cuttings originated from *C. acuminata* plants older than ten years one week after inoculation.. Nodal segments containing apical and axillary buds taken from of one-year old plants were more suitable for establishing shoot buds *in vitro*. All greenhouse *C. acuminata* plants exhibited long internodes (50 mm ± 0.5). Therefore, only single node explants were cultured for shoot multiplication in solid medium. Explants were incubated on MS medium fortified with different concentrations of BAP (0.5, 1.0 and 1.5 mg l^{-1} BAP).

Fig. 19 Three-weeks-old single shoot buds developed on nodal explants cultured on MS medium containing BAP.

Nodal explants showed their first response by enlarging and bursting of the axillary buds within two to three weeks in culture. One single shoot emerged directly from each node of the explants (Fig. 19).

The three BAP concentrations did not affect the flushing of shoot buds induction on cuttings. In all three concentrations only one new shoot was recorded. The success of *C. acuminata* clones establishment from greenhouse plants was rather poor and strongly depended on the genotype.

Out of the three donor group of trees (Ch1, Ch2 and Ch3), only explants from Ch1 and Ch2 could be established *in vitro*. Growth among these two genotypes was very slow and a survival rate of 41% was recorded after four weeks. Genotypes from group Ch3 were axenic for more than six weeks before they became contaminated. Therefore, it was not possible to establish plants originating from group Ch3. Plants from group Ch1 and Ch2 were established successfully already after the first experiment. There were no significant differences among the various BAP treatments used to induce shoots, each node produced just one shoot. However, when shoots were excised and subcultured on fresh medium new shoots development from the axillary bud and proliferation was excellent. After an additional six weeks the number of shoots per explant was found in the range of 7.3 to 16.8 in the different concentrations of BA (Tab. 6).

The highest multiplication rate with 16.8 shoots per explant was obtained on MS medium containing 1.5 mg l^{-1} BAP, followed by 1.0 mg l^{-1} with 9.8 shoots per explant and 0.5 mg l^{-1} with 7.3 shoots per explant. Shoot length was significantly different among the three concentrations (Tab. 6).

Shoots of 20 - 30 mm in length were excised and used for further culture in TIS and for rooting experiments.

Tab. 6 Effect of BAP concentration on shoot multiplication and shoot length in *C. acuminata.* Values represent means ± SE of three independent experiments with ten replicates each.

BAP [mg l^{-1}]	Number of shoots per explant	Shoot length [cm]
0.5	7.3 ± 0.8	4.5 ± 0.5
1.0	9.8 ± 0.8	4.2 ± 0.7
1.5	16.8 ± 1.6	2.3 ± 0.4

4.2. Plant Regeneration via Organogenesis and Embryogenesis

4.2.1. Embryogenic Callus Induction in Solid Media

Callus formations on the apical tips, cotyledons, hypocotyls and radicles of the zygotic embryos were observed 7 days after inoculation on MS medium supplemented with various concentrations and combinations of PGRs shown in Tab. 3 (p. 26) During the first weeks after induction, calli derived from apical tip grew as a white-green compact unit and those from cotyledon explants enlarged without showing a real callus differentiation (Fig. 20). Calli derived from hypocotyl explants exhibited a soft, shiny and pale brown to green texture that gradually turned into a compact grey-reddish mass, while those derived from radicles were soft, hairy, watery and creamy in colour. Calli from apical tip and radicle explants retained their texture over weeks.

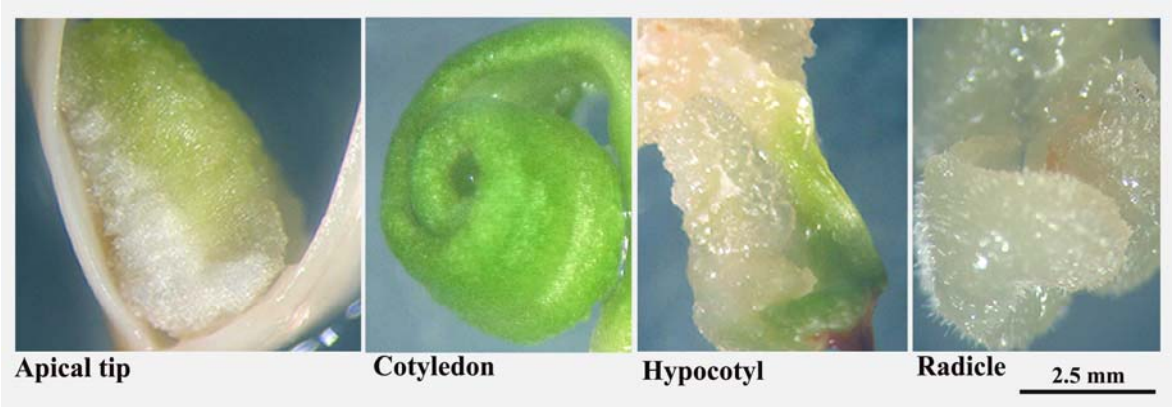

Fig. 20 Callus formation on apical tip, cotyledon, hypocotyl and radicle of zygotic embryos.

The highest frequency of callus induction was obtained from explants cultured on medium supplemented with 2 mg l^{-1} BAP plus 0.1 mg l^{-1} IAA (BI) followed by 2 mg l^{-1} BAP plus 0.1 mg l^{-1} 2,4-D (B21) and 2 mg l^{-1} BAP plus 0.5 mg l^{-1} 2,4-D (B22). In all cases, cotyledon explants were more responsive than apical tip, hypocotyl or radicle explants (Fig. 21). Callus induction on explants cultured on medium containing 2 mg l^{-1} Kin plus 0.1 mg l^{-1} 2,4-D (K21) and 2 mg l^{-1} Kin plus 0.5 mg l^{-1} 2,4-D (K22) was less responsive compared to that on BI, B21 and B22. Similar results were observed on explants cultured on medium with 0.1 mg l^{-1} 2iP plus 0.2 mg l^{-1} NAA (NiP). An average of 25 % was scored for callus formation on NiP and most of these calli turned brown to the end of the second week. No callus formation was observed in PGR-free medium.

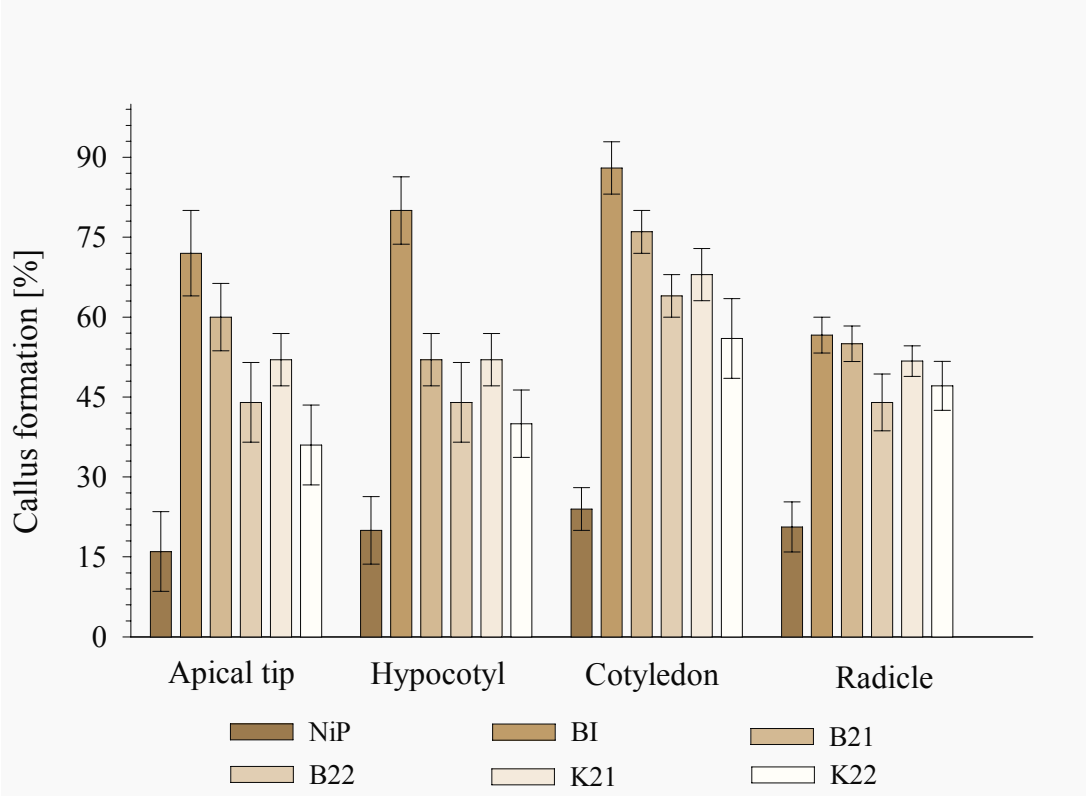

Fig. 21 Effect of PGR compositions on callus formation after 4 weeks in culture. Data shown are the means with ± SE of nine replicates each containing five explants.

4.2.2. Plant Regeneration via Organogenesis in Solid Media

A distinctive response was observed among the different explants tested for their regeneration potential. Adventitious shoot buds were observed six to eight weeks after callus induction on apical tip, hypocotyl and radicle explants (Fig. 22A, C and D). Bud-like structures appeared as small green compact nodules on the callus surface, which finally proliferated into recognisable shoots. In contrast, cotyledon explants exhibited a swelling along the edges and the vicinity of the midribs from which globular structures were formed. Shoot buds apparently formed directly from the subepidermal cell layer without an intervening callus phase. Protuberances and shoot-buds were observed only on cotyledon explants cultured on BI medium. Most of these adventitious shoots were induced along the edge and the midrib of cotyledons (Fig. 22 B). An average of 12 ± 2.1 hairy buds were scored on explant of 25 by 5 mm in size. When shoots were excised and cultured on MS medium supplemented with 0.5 mg l^{-1} BAP and 30 g l^{-1} sucrose they attained a height of about 2 cm within 30 days and finally regenerated into normal and healthy plantlets. These shoots or plantlets were then used for multiplication in TIS.

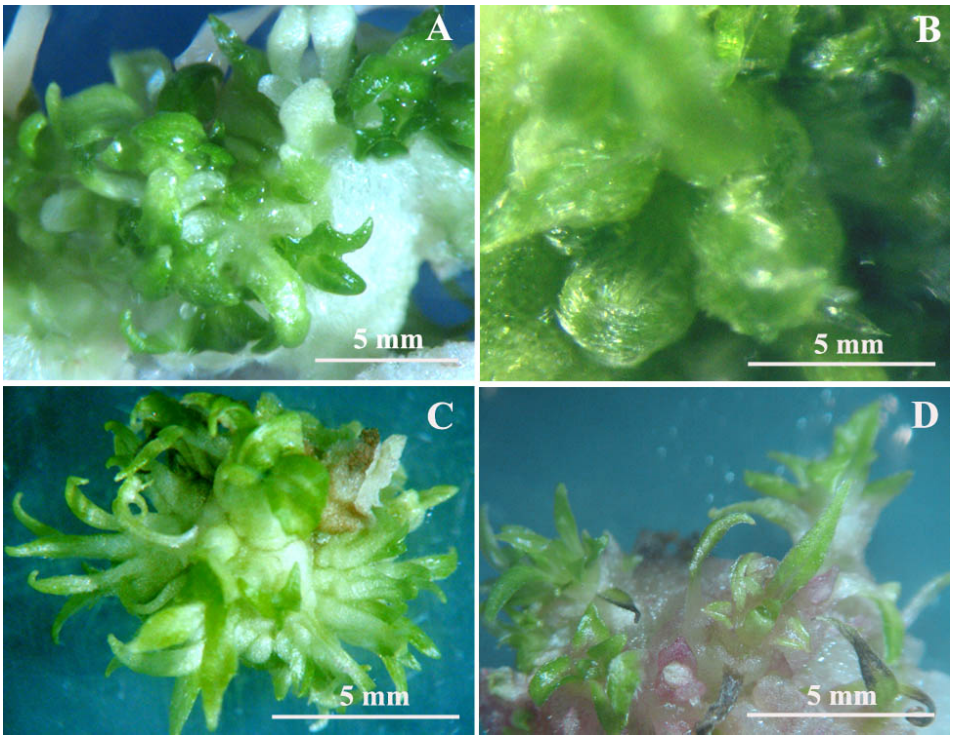

Fig. 22 Organogenesis in *C. acuminata.* **(A)** Shoot bud formation on apical tip. **(B)** Globular structure and adventitious buds on cotyledon explants originated from the subepidermal cell layer without formation of undifferentiated callus mass on BI medium 4-5 weeks after inoculation. **(C, D)** Shoot buds formation on differentiated callus mass derived from radicle and hypocotyl explant, respectively.

Shoot buds formed on leaf explants were pubescent in contrast to those on apical tips, hypocotyls and on radicles, which were smooth and glossy (Fig. 22A, C, D). Significant differences ($\rho \leq 0.05$) were observed among the various cytokinin and auxin combinations and concentrations used. Calli derived from apical tips showed the best response on BI medium in terms of adventitious shoot induction. A mean of 17 buds were scored on callus (15mm x 20 mm) derived from apical tips and about 10 buds among calli derived from cotyledons, hypocotyls and radicles. Comparing the five PGRs combinations and concentrations used for shoot bud induction BI seems to be the most effevtive combination followed by B21 and K21. The combination of high cytokinin (2 mg l^{-1} BAP or Kin) and low auxin concentrations (0.1 mg l^{-1} IAA or 2, 4-D) showed a higher effectivity on bud formation than high cytokinin and auxin concentrations. These observations are shown in Figure 23. Bud formation was significantly lower on B22 and K22 than on BI, B21 and K21 media.

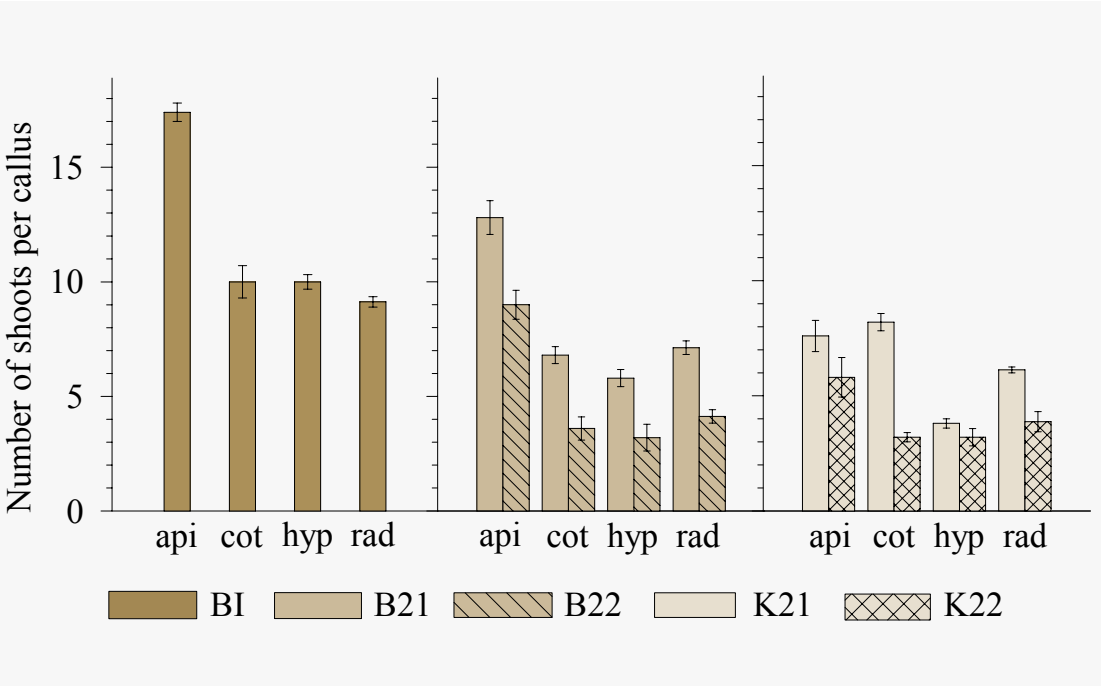

Fig. 23 Effects of cytokinins and auxins combinations used for organogenesis induction in solid medium: **BI** (2 mg l^{-1} BAP + 0.1 mg l^{-1} IAA), **B21** (2 mg l^{-1} BAP + 0.1 mg l^{-1} 2,4-D), **B22** (2 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D), **K21** (2 mg l^{-1} Kn + 0.1 mg l^{-1} 2,4-D), and **K22** (2 mg l^{-1} Kn + 0.5 mg l^{-1} 2,4-D). Bars indicate the means of 10 replicates ± SE.

In general, BI followed by B21 and K21 proved to be the best combination and concentration for adventitious shoot induction. Shoot growth on leaf explants was retarded compared to that on hypocotyl and radicle calli. However, when the shoots were excised and transferred to medium supplemented with 0.5 mg l^{-1} BAP, no growth nor proliferation differences were observed. After 4 to 6 additional weeks regenerated shoots were used for multiplication in solid media and in TIS. Somatic embryogenesis induction was not observed in neither of the PGR compositions used.

4.2.3. Embryogenic Callus Induction in Liquid Media

Explants obtained from young seedlings' leaves and hypocotyl segments (Fig. 24A) were tested for their organogenic and embryogenic callus initiation in liquid media supplemented with various PGR concentrations and combinations used in solid medium (Tab. 3, p. 26). Callus formation on leaf and hypocotyl explants was achieved after four weeks of culture. Before callus initiation, leaf explants began to curl at the edges and all calli derived from leaf explants exhibited a soft, smooth and green texture throughout the whole treatment (Fig. 24B, C). Disc shape hypocotyl explants formed soft and greenish callus on both sides (Fig. 24D, E) and those derived from cylindrical shape hypocotyl

explants did not show distinctive differences in texture and colour among the various PGRs used. They were soft in textures and creamy in colour. Callus was initially formed on one of the cut ends first and after 6 weeks in culture the epidermis dehisced lengthwise and callus began to proliferate from the subepidermal layer (Fig. 24F, G).

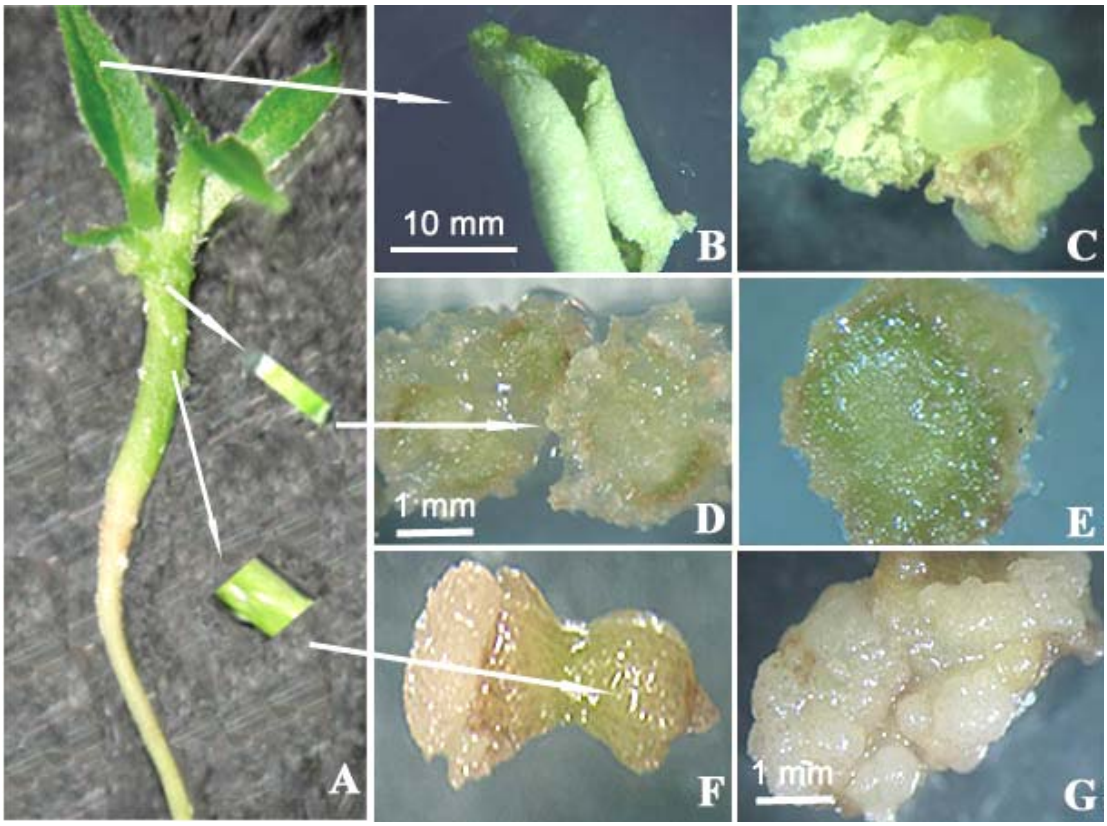

Fig. 24 Callus induction in liquid medium after four weeks. **(A)** Twenty-days-old *in vitro* grown seedlings. **(B, C)** callus derived from leaf explants. **(D, E)** discs shape explants from hypocotyls. **(F, G)** cylindrical shape explant.

The percentage of explants on which callus was formed varied little between the PGRs used. There were no substantial differences except for NiP medium, which showed less than 20% induction rate (Fig. 25). Callus cultures proliferated well and the fresh weight increase was 4 fold after eight weeks.

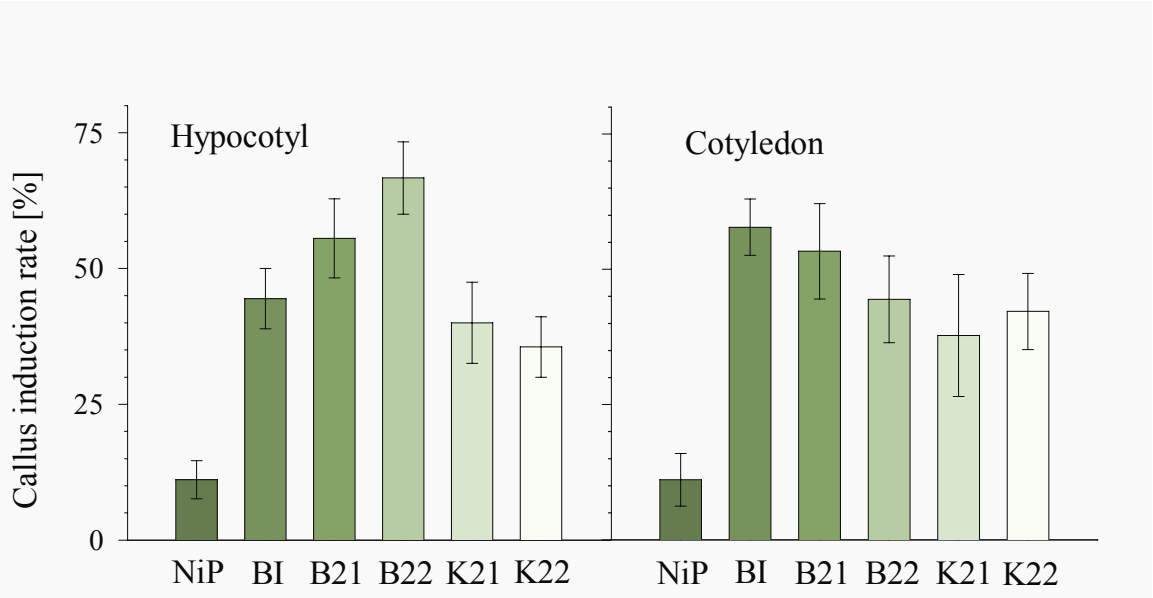

Fig. 25 Effect of cytokinin and auxin combinations and concentrations on callus induction in liquid media after 30 days in culture. Data shown are the means with ± SE of nine replicates containing five explants each.

4.2.4. Plant Regeneration via Organogenesis in Liquid Media

The most remarkable observation made among the various explants cultured in liquid media was the induction of organogenesis on disc shape hypocotyl explants. Adventitious buds were observed on 56% of the disc shape hypocotyl explants cultured in BI medium. Buds showed both the smooth glossy and the pubescent appearance (Fig. 26D, E). An average of 10 ± 0.2 buds per explant was recorded, which originated mostly between the epidermal layer and the vascular bundles (Fig. 26A, arrow). After seven weeks in liquid culture adventitious buds were transferred to solid MS medium supplemented with 0.5 mg l^{-1} BAP, where they developed into normal shoots.

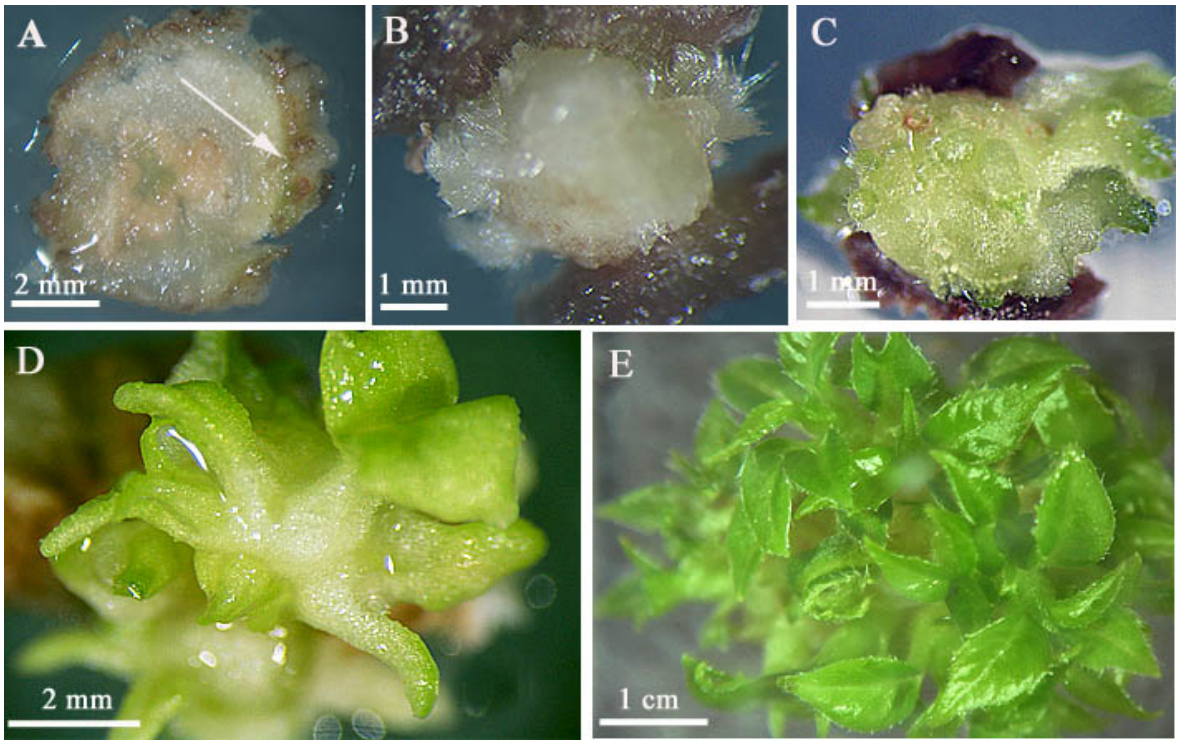

Fig. 26 Organogenesis in *C. acuminata* in liquid culture. **(A)** Disc shape hypocotyl explants cultured in MS medium supplemented with 2 mg l^{-1} BAP and 0.1 mg l^{-1} IAA (BI). **(B)** Shoot buds 4-5 weeks after callus induction at 20 µmol m^{-2} s^{-1} PPFD. **(C)** Five to six days after cultures were exposed to a light intensity of 40 µmol m^{-2} s^{-1} PPFD. **(D)** Seven weeks old shoot buds still in liquid culture. **(E)** Shoot development after 3 additional weeks in liquid medium containing 0.5 mg l^{-1} BAP.

4.2.5. Somatic Embryogenesis Induction in PGR-free Medium in TIS

Hypocotyl segments cultured in PGR-free MS medium containing 20, 25, 30 and 35 g l^{-1} sucrose in the unmodified DVS remained green during the first two weeks. After three weeks in culture explants gradually became brown and died. No callus formation was observed among these explants in neither of the four sucrose concentrations used. Callus induction was also not observed in PGR-free medium containing 20, 25 and 30 g l^{-1} sucrose in the RITA® vessels. Among the same treatment in the modified DVS explants cultured in medium supplemented with 20 and 25 g l^{-1} sucrose showed no callus formation, while those in medium with 30 g l^{-1} sucrose showed a tendency for callus formation with a mean of 40% (4 ± 1.5) but then retarded. Successful callus formation was observed four weeks after inoculation only on segments cultured in medium fortified with 35 g l^{-1} sucrose in the modified DVS. Similar responses were observed in the RITA® vessels in medium supplemented with 35 g l^{-1} sucrose.

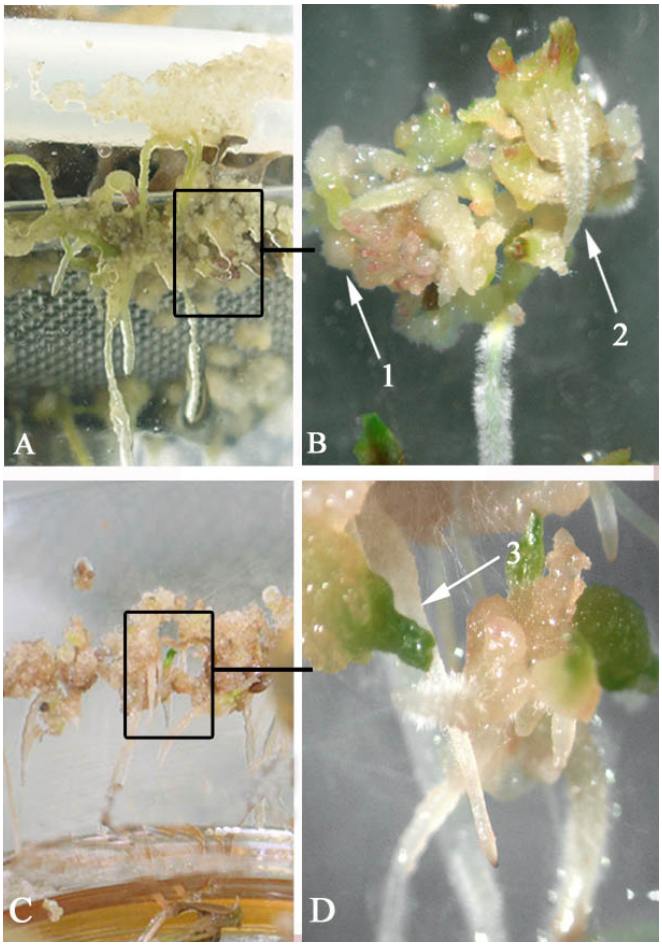

Fig. 27 Induced embryogenic callus in TIS. **(A)** Overview of embryogenic callus in the modified DVS. **(B)** Enlarged view showing the asynchronous development of embryos from globular (arrow 1) to embryo-like structures with the typical bipolar appearance (arrow 2). **(C)** Embryogenic callus in RITA® vessel at flooding height. **(D)** Enlarged view showing cotyledonary stages (arrow 3).

An average of 80% of the explants (eight out of ten segments) in DVS (8 ± 1.5) and 60% (six out of ten) in RITA® (6 ± 1.0) formed callus, upon which embryogenic calli were initiated. Embryogenic calli were observed after 16 weeks in DVS culture (Fig. 27A, B) and after 12 weeks in RITA® (Fig. 27C, D). Calli in DVS were friable and showed a translucent to yellow-green appearance, while those in RITA® were soft and light brownish. Proembryos protruded as green dots on the surface of the callus mass. The development of somatic embryos was asynchronous, and several stages of embryos from globular to embryo-like structures with a typical bipolar appearance and cotyledonary stage were present at the same time in both vessels. Embryo maturation into cotyledonary stage was very slow in TIS but when plated in solid PGR-free medium proembryos matured into well-developed cotyledonary embryos within four weeks. Cotyledonary embryos selected from the solid media were then used for plantlet conversion in TIS.

4.2.6. Plant Development via Cotyledonary Embryo in TIS

The results showed a distinctive difference among the culture media. Cotyledonary embryos cultured in medium fortified with 1.0 and 1.5 mg l^{-1} BAP revealed a high rate of hyperhydricity and callus formation at the shoot base (Fig. 28), which was not associated with root formation. Therefore only embryos grown in 0.5 mg l^{-1} BAP and PGR-free medium were evaluated.

Fig. 28 The effect of BAP concentrations 0.5, 1.0 and 1.5 mg l^{-1} BAP on the conversion and development of cotyledonary embryos in TIS after eight weeks in culture with an immersion of 1 minute every 4 hours.

During the selection of cotyledonary embryos for cultivation in TIS, it was inevitable that immature embryos (arrow Fig. 29A) were also transferred into the vessels. Thus these immature embryos developed and fused to form adnated embryos (Fig. 29B). Adnated regenerants were slow in development but eventually recovered and differentiated into normal

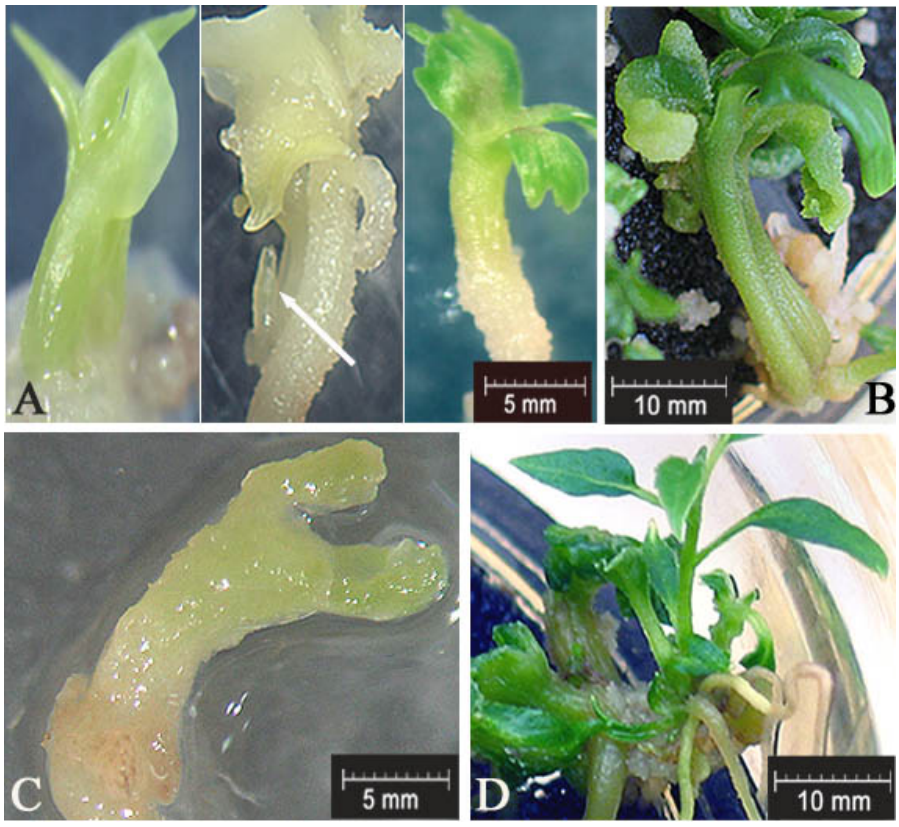

Fig. 29 Cotyledonary embryos **(A)** with attachment (arrow) for plantlet regeneration in DVS and RITA®. After six weeks in culture regenerants were classified in three morphological categories **(B)** adnated (**C)** deformed and **(D)** normal growth.

plantlets, while deformed embryos (Fig. 29C) remained stunted and did not recover. Plantlets with phenotypical normal growth (Fig. 29D) had an average height of 28.6 ± 5.5 mm with 4 leaves in RITA®, while those in DVS had an average of 36.7 ± 4.4 mm in height and 5 leaves per plantlet. Apical tip leaves were not counted. The analysis of variance (ANOVA) showed that there were no significant differences ($\rho > 0.05$) between embryo development in PGR-free medium and medium supplemented with 0.5 mg l^{-1} BAP in DVS. A similar result was observed in the RITA® vessel. However, there was a clear difference between the conversion rate of cotyledonary embryos in DVS and RITA® (Fig. 30). Adnated embryos were recorded with 15% in the RITA®, which was higher in comparison to the 3% in DVS. On the other hand, deformed embryos were recorded with

68% in DVS and only 27% in RITA®. Embryos converted to plantlets in RITA® showed a higher percentage of normal growth (58%) than those grown in DVS (30%).

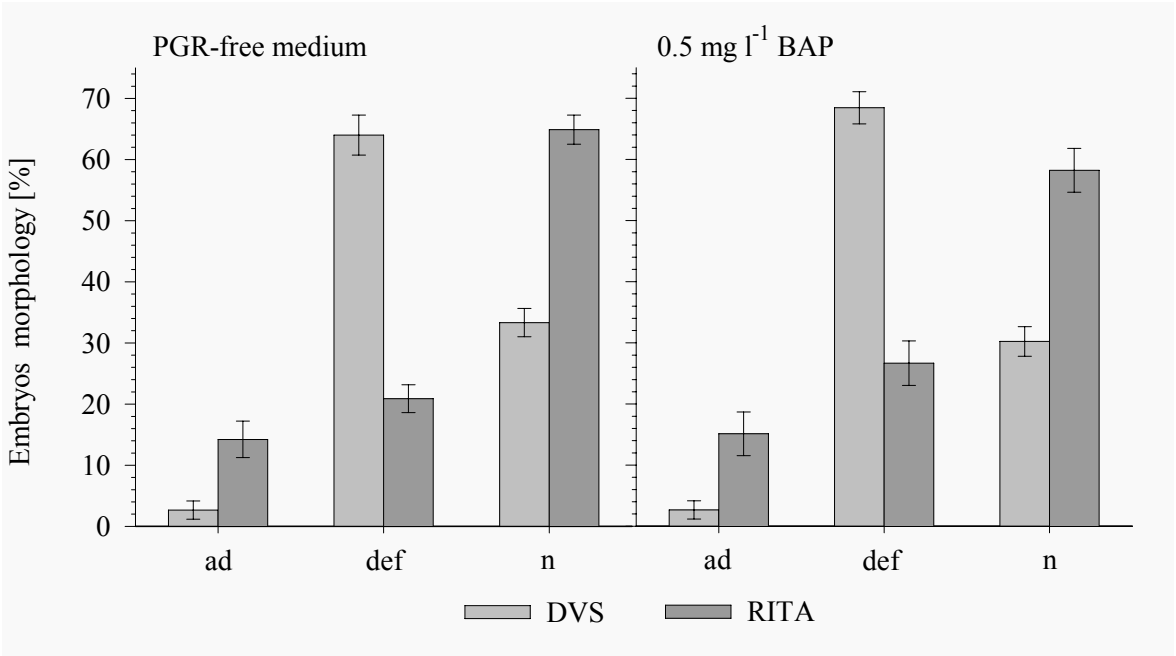

Fig. 30 Comparison of embryos morphology in DVS (light grey bars) and in RITA® (dark grey bars) grown in PGR-free medium and in medium supplemented with 0.5 mg l^{-1}BAP. Regenerants were classified in **ad** = adnated, **def** = deformed and **n** = normal growth. Data shown in this figure are the means of nine replicates containing 25 embryos each.

4.2.7. Plant Conversion via Cotyledonary Embryo in Sterilised Substrates

Figure 31 shows the *in vitro* conversion rate of cotyledonary embryos to plants after 4, 8 and 12 weeks in culture in the four different sterilised substrates. A high frequency of plant conversion was observed in all four substrates mixtures, at least during the first 4 weeks. The highest conversion rate was recorded after 12 weeks in SV followed by SSV with 88% and 80%, respectively. Despite the high plant regeneration rate scored in pure sand and soil during the first 4 weeks, only 62% of the regenerated plantlets in sand and 26% on soil survived towards the end of the 12th week. Desiccation was greater in both sand and soil thus plantlets shrivelled and dehydrated. Regenerated plantlets were typically smaller in size in sand than those in SV, SSV and soil. There was no significant difference between growth and survival of embryos grown in SV and SS. However, those grown in sand and soil, showed a substantial difference. Figure 32 demonstrates the successful *in vitro* conversion into complete rooted plantlets of *C. acuminata* through somatic embryogenesis on the different substrates.

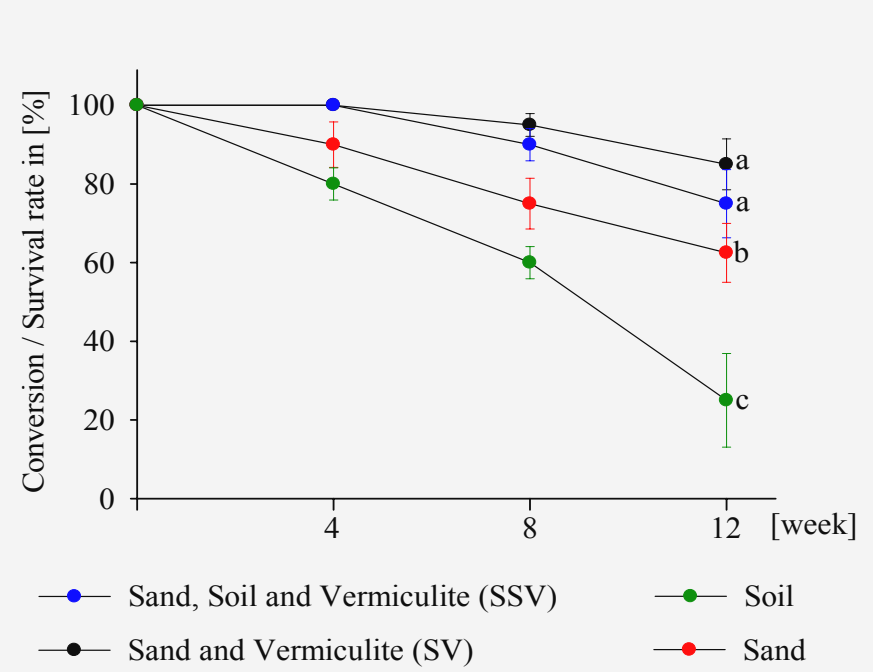

Fig. 31 Conversion / survival rate of cotyledonary embryos after 12 weeks in four different sterilised substrates. Values represent the means ± SE of 5 replicates each containing 10 cotyledonary embryos. The different letters indicate significant differences at $p \leq 0.05$.

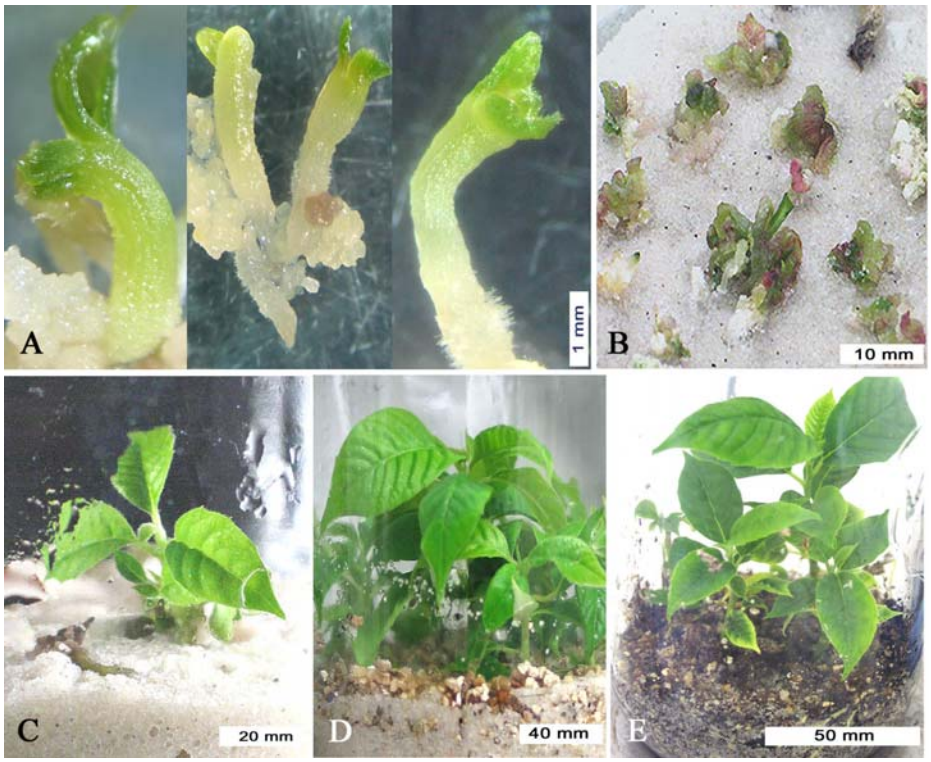

Fig. 32 Sequential plantlet regeneration through somatic embryo in sterilised substrates. **(A)** Selected cotyledonary embryos, after 4 weeks of maturation in solid medium. **(B)** Embryo development in sand after 4 weeks and **(C)** after 8 weeks. **(D, E)** Regenerants after 12 weeks on SV and SSV still under aseptic condition.

4.3. Shoot Multiplication in Solid Medium and in TIS

In vitro cultured plants of *C. acuminata* were established from seeds grown on hormone-free full strength MS medium. However, success has been limited establishing an *in vitro* stock of *Camptotheca* plantlets from the seeds of group Ch3 and cuttings derived from the various botanic gardens. Plants from group Ch3 were extremely sensitive to the varied culture conditions and did not survive the treatments with the different basal media and PGR concentrations used during the tests to find the appropriate growth medium for *Camptotheca* shoots. Seeds of Ch3, as mentioned previously, germinated successfully on MS medium devoid of PGR but seedlings cultured on MS and B5 basal media containing different concentrations of cytokinins (BAP, 2iP, TDZ and Kin) inexplicably became brown after one to two weeks in culture. As for the cuttings, a high rate of contamination (95%) occurred among explants derived from the various botanic gardens after 4 to 6 weeks in culture. In contrast, apical and axillary buds derived form one-year-old *ex situ* plants showed less contamination and more than 80% of the explants survived *in vitro*. Thus, for the establishment of the *in vitro* stock culture shoot buds from greenhouse plants and *in vitro* grown seedlings from group Ch1, Ch2 and Lou were used. Seedlings growth was ideal on full strength MS medium devoid of PGR but without lateral shoot formation (Fig. 33A). However, when the apical tip of a seedling was removed, new axillary buds were formed (Fig. 33B). These new shoot buds were used for the clonal propagation of each genotype.

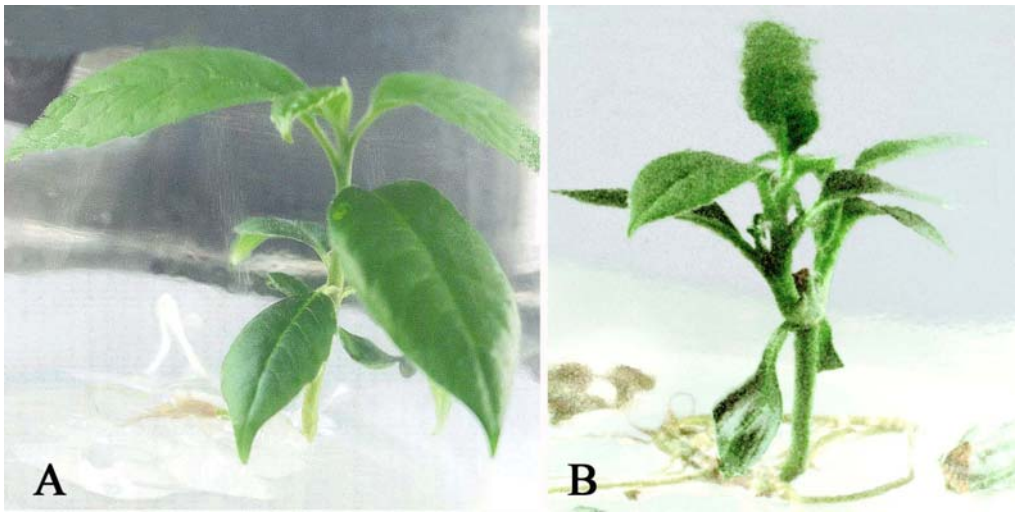

Fig. 33 *S*eedling and axillary buds used to establish an *in vitro* stock of *C. acuminata.* **(A)** Seedling in PGR-free medium without lateral shoot formation. **(B)** Seedling after removal of the apical tip with newly formed axillary buds.

Preliminary experiments revealed that relatively low BAP concentrations (< 2 mg l^{-1}BAP) were more suitable for shoot multiplication in *C. acuminata* than other cytokinins (Kin, TDZ or 2iP) previously tested. Hence, shoots established from seedlings and axillary buds from *ex situ* plants were cultured in solid MS medium containing 0.5, 1.0, 1.5 mg l^{-1}BAP to compare the BAP effect on shoot proliferation.

4.3.1. Shoot Multiplication in Solid Media

Browning caused by oxidation at the cut surface of the explants was observed after five days. Callus formation was observed at the cut ends of explant in all media tested. During the first two weeks no shoot formation occurred on neither of the three BAP concentrations, instead, leaves of explants shrivelled, turned brown and finally dropped off. Towards the third week bright new apical leaves and tiny lateral buds appeared on most of the explants. Multiple shoots were generally initiated from the callus formation at the base of the explants. After four weeks in culture an average of 3 shoots per explant was scored among explants of each group on all three BAP concentrations. Cultures evaluated on the 8th week varied slightly from 7 shoots per explant on 0.5 and 1.0 mg l^{-1} BAP to 9 shoots per explant on 1.5 mg l^{-1} BAP (Fig. 34). As expected, no notable shoot multiplication was observed on explants cultured in PGR-free medium.

Since the number of shoots developed on the individual genotypes from Sichuan (Ch1), Jiangsu (Ch2) and Louisiana (Lou) showed no significant differences at the 5% level, they were considered as replicates but still they were persistently cultured separately. In general, all shoots in solid media exhibited virtually a normal growth with well developed dark green leaves. However, there were recognisable differences in leaf size and shoot length among the three BAP concentrations. Figure 35 A-C demonstrated the morphological appearance among the microshoots on the different BAP concentrations (0.5, 1.0 and 1.5 mg l^{-1} BAP) respectively. Shoots of group Ch1, Ch2 and Lou cultured on medium containing 0.5 mg l^{-1} BAP had an average leaf size of 127 ± 23.5 mm^2; 210 ± 48.7 mm^2; 243 ± 59.1 mm^2, respectively, while leaves of shoots grown in medium containing 1.0 and 1.5 mg l^{-1} BAP were smaller in size (84 ± 14.2 mm^2; 125 ± 46.9 mm^2; 188 ± 57.9 mm^2) and (54 ± 14.8 mm^2; 59 ± 11.9 mm^2; 123 ± 20.1 mm^2, respectively). The standard errors reflect the differences of leaf sizes within the same vessel. This variation was not necessarily associated with the light intensity because similar results were achieved with shoots cultured at different light intensities (96 to 240 µm $s^{-1}m^{-2}$ PPFD).

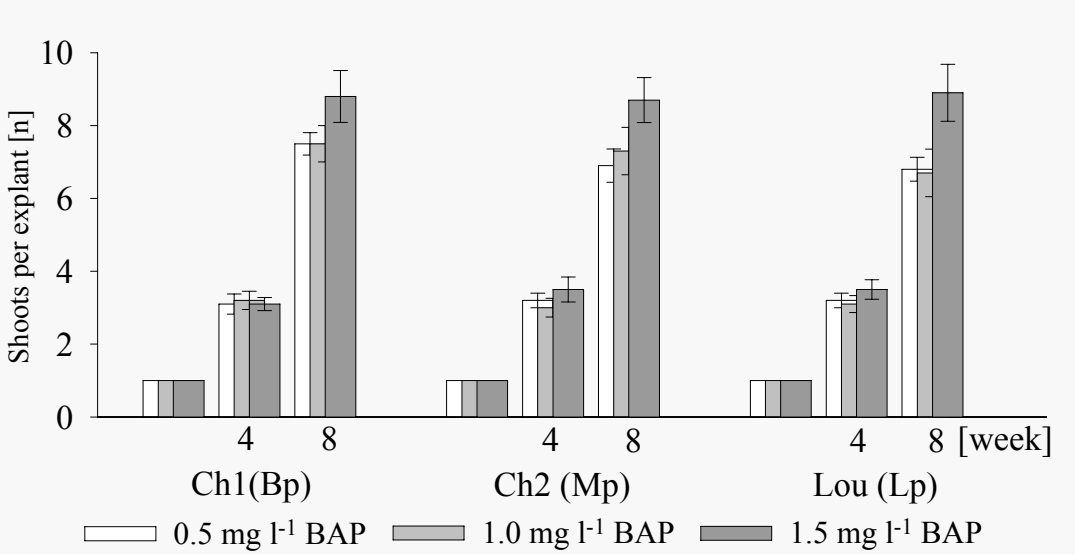

Fig. 34 Shoot multiplication per explant in solid MS medium fortified with various BAP concentrations after four and eight weeks among the groups Ch 1, Ch 2 and Lou. Each bar represents the means ± SE (n = 80).

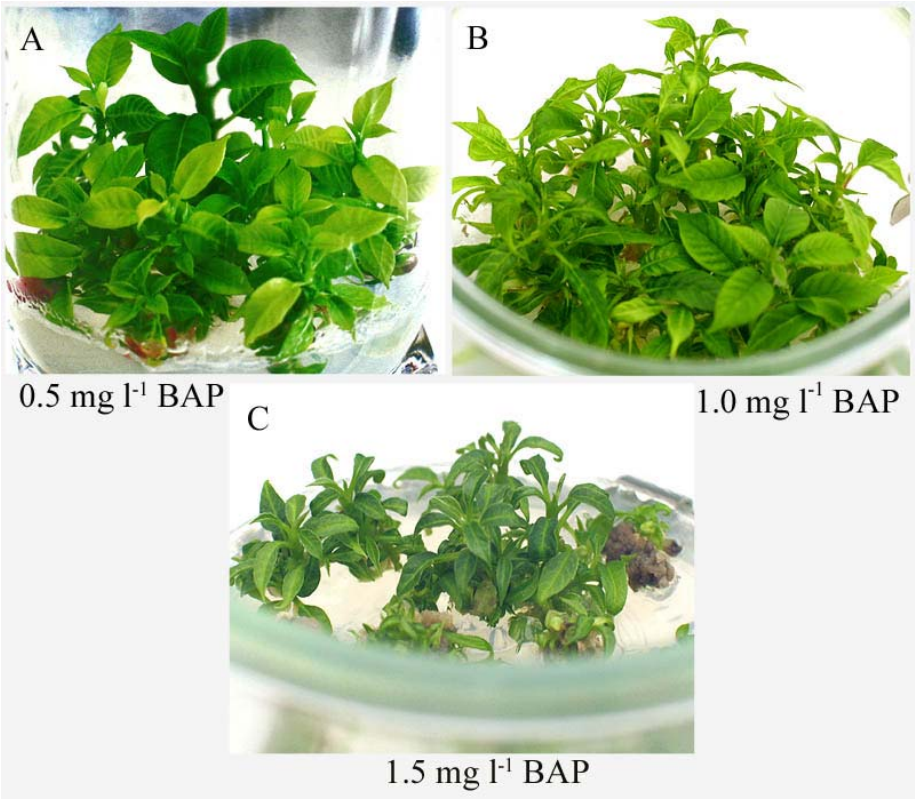

Fig. 35 Morphological appearance of microshoots grown in medium supplemented with different BAP concentrations (0.5, 1.0 and 1.5 mg l^{-1} BAP) after eight weeks.

Chlorophyll content varied according to the BAP concentrations. The highest value of chlorophyll *a* and *b* was found in shoots regenerated in medium fortified with 0.5 mg l^{-1}

BAP with 1.59 ± 0.22 mg g^{-1} fresh weight. Those grown in medium containing 1.0 and 1.5 mg l^{-1} BAP showed a similar amount of chlorophyll *a* and *b* (0.86 ± 0.29 and 0.84 ± 0.32 mg g^{-1} fresh weight, respectively. The leaf area of shoots grown in solid medium was measured with 19 ± 7, 13 ± 6 and 8 ±.3 mm^2 in 0.5, 1.0 and 1.5 mg l^{-1} BAP, respectively.

4.3.1.1. Shoot Height in Solid Medium

Shoot height decreased with an increase in BAP concentrations. Height increment differed significantly among the various BAP concentrations tested. It was obvious that BAP concentration higher than 1.0 mg l^{-1} suppressed shoot growth. Shoots cultured on medium containing 1.5 mg l^{-1} BAP were significantly shorter (approx. 2.9 cm long) than those grown on medium containing 0.5 and 1.0 mg l^{-1} BAP, with an average shoot height of 3.4 and 4.4 cm, respectively after eight weeks (Fig. 36).

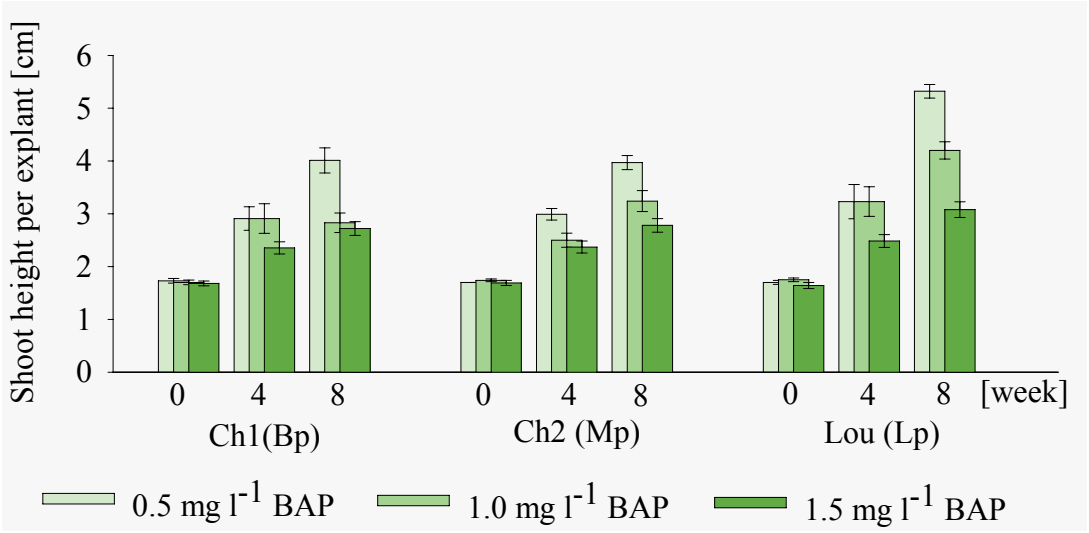

Fig. 36 Shoot height in the three groups: Ch1, Ch2 and Lou after 4 and 8 weeks in culture on the different BAP concentrations. Bars represent the means ± SE of three replicates.

Hence, the maximum shoot height was obtained on medium supplemented with 0.5 mg l^{-1} BAP followed by 1.0 mg l^{-1} BAP (Fig. 37A, B).

During the first 4 weeks no significant differences in shoot height were found among the groups Ch1, Ch2 and Lou cultured in three different BAP concentrations. Towards the end of the culture period (8 weeks) explants in the Lou group showed the maximum growth height in all BAP concentrations tested.

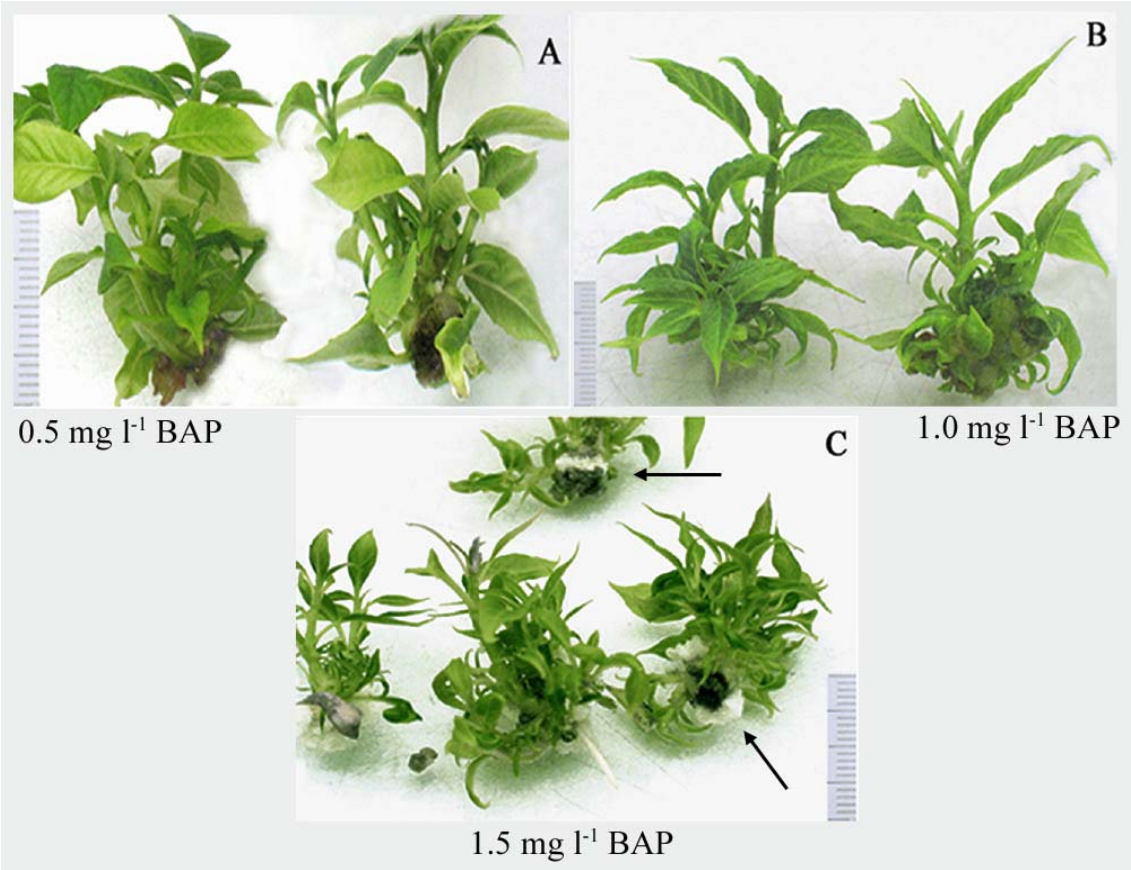

Fig. 37 Effect of BAP concentration on shoot growth. **(A)** Shoots grown on 0.5, **(B)** 1.0 and **(C)** 1.5 mg l^{-1} BAP. Black arrows indicate a dust-like white friable callus that emerged from former brown callus on explants cultured on 1.5 mg l^{-1} BAP.

4.3.1.2. Shoot Fresh Weight in Solid Media

Shoot fresh weight among the explants grown in solid medium fortified with the various BAP concentrations showed comparable results during the first four weeks. Explants cultured on medium containing 0.5 and 1.0 mg l^{-1} BAP demonstrated a similar fresh weight increment during the entire culture period of eight weeks, never exceeding 100 mg per explant.

However, already during the 4th week explants on medium containing 1.5 mg l^{-1} BAP showed a fresh weight increase, which was significantly ($p \leq 0.05$) higher compared to those on medium with 0.5 and 1.0 mg l^{-1} BAP (Fig. 38). This was partially due to increased callus formation at the base of shoot explants. Callus formation on *C. acuminata* shoots cultured in solid medium was characteristic but callus size varied among the different BAP concentrations. Callus growth on explants grown on medium containing 0.5 and 1.0 mg l^{-1} BAP remained relatively small in comparison with those grown in medium with 1.5 mg l^{-1} BAP. Calli proliferated continuously and were either green and compact (0.5 and 1.5 mg l^{-1}

BAP) or brown (1.5 mg l^{-1} BAP). New shoots initially sprouted from the green and compact calli. Brown calli observed on explants on 1.5 mg l^{-1} BAP simple enlarged and showed a white dust-like friable callus (Fig. 37C). No shoot sprouting was observed on these calli.

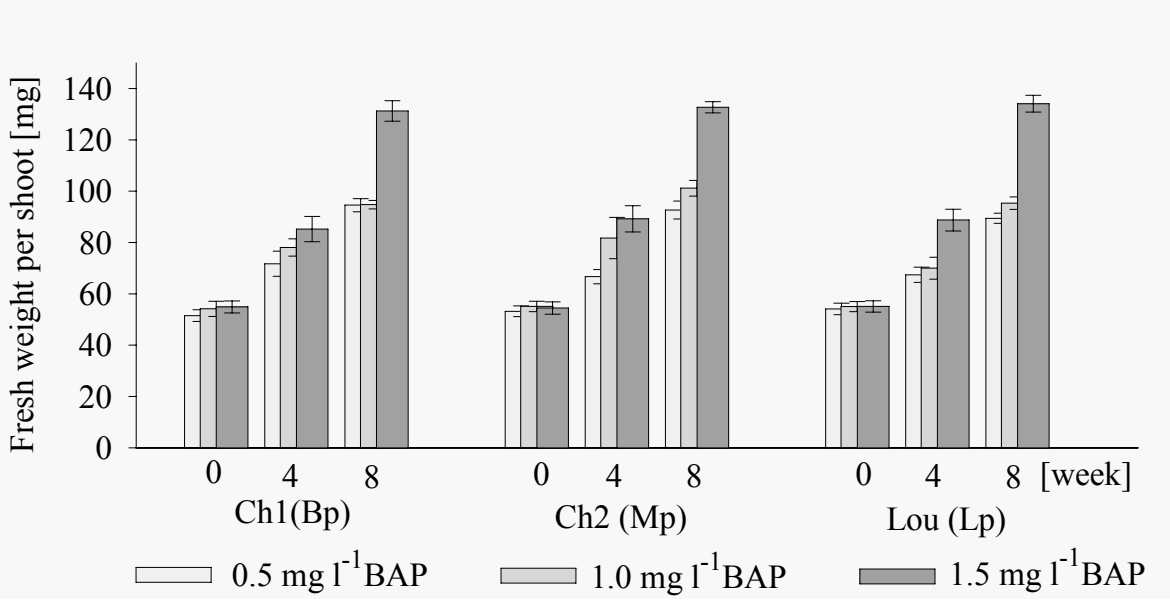

Fig. 38 The effect of BAP concentrations on shoot fresh weight after four and eight weeks among the groups Ch1, Ch2 and Lou. Each treatment consisted of 10 jars with eight shoots at culture begin. Bars represent the means ± SE of three replicates.

4.3.2. Shoot Multiplication in TIS

Shoot tips and axillary buds of *C. acuminata* cultures established in solid medium were excised and subcultured in TIS for shoot multiplication. Since there were no substantial differences found among the three BAP concentrations applied to shoots cultured in solid media, the same experiment was conducted in TIS to compare the effect of BAP concentrations and immersion frequencies on shoot proliferation among the different genotypes of groups Ch1, Ch2 and Lou.

In general, it was found that using 400 ml liquid medium at the beginning of the culture period resulted in leave senescence. More than 90% of the newly cultured explants in DVS lost their leaves after 5 to 7 days in culture. This phenomenon was reduced when a volume of 200 ml culture medium was used for the first four weeks. As multiple shoots began to proliferate and elongate, medium volume was increased to 400 ml, which was adequate for the remaining period of culture.

This phenomenon was not observed on explants cultured in RITA®. Nevertheless, shoot induction was observed in all treatments, excluding the BAP-free control within 2 weeks of culture. Independently of the genotypes, explants cultured in the various BAP concentrations treated with a one minute IC four times d^{-1} initiated shoots within 2 weeks. The results achieved in DVS and RITA® did not follow the same pattern as those cultured in solidified medium. The highest shoot multiplication was scored in media supplemented with 1.0 and 1.5 mg l^{-1} BAP in both DVS (23 ± 3.1, 29 ± 3.0) and RITA® (14 ± 2.8, 20 ± 4.9) respectively. Both lateral and basal buds sprouting were observed on shoots cultured in the different BAP concentrations. However, lateral buds formation were more frequent on shoots cultured in 1.0 and 1.5 mg l^{-1} BAP, whereas basal sprouts were more common on shoots grown in medium containing 0.5 mg l^{-1} BAP.

Shoots grown in medium supplemented with 1.0 and 1.5 mg l^{-1} BAP were translucent, brittle and possessed curled leaves, which are typical signs of hyperhydricity. Callus formation on shoot leaves was also common in these two BAP concentrations in both TIS vessels, especially in RITA®. Figure 39 shows the morphological appearance of shoots after 8 weeks in DVS and RITA®. It has been observed that a rise in BAP concentration in media promoted shoot proliferation, but concentrations above 1.0 mg l^{-1} resulted in hyperhydricity and poor shoot quality.

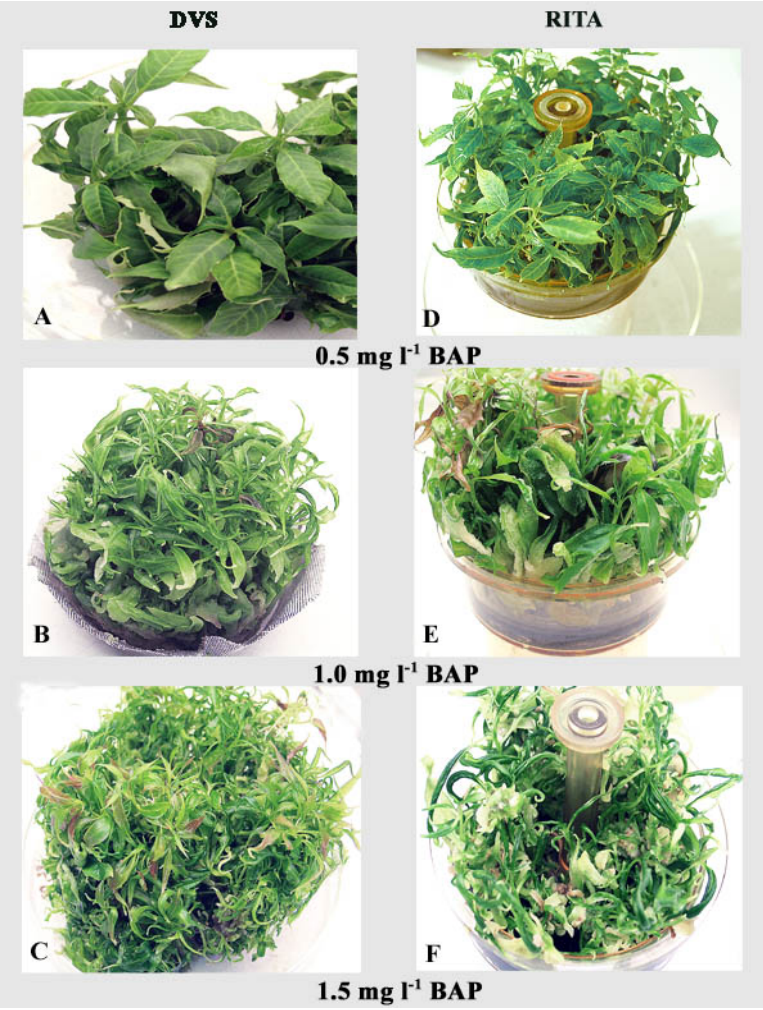

Fig. 39 The morphological appearance of *C. acuminata* shoots grown in the various BAP concentrations (0.5, 1.0 and 1.5 mg l^{-1} BAP) in DVS (A-C) and RITA® (D-F) Explants were treated with one minute IC four times d^{-1} over a period of eight weeks.

Since shoots cultured in 1.0 and 1.5 mg l^{-1} BAP exhibited poor growth quality, hyperhydricity and frequent callus formation, treatments with these two BAP concentrations were no longer used for shoot multiplication. Consequently, all further multiplication treatments in TIS were continued with 0.5 mg l^{-1} BAP. Explants of the different genotypes grown in medium fortified with 0.5 mg l^{-1} BAP showed less shoot proliferation but the quality of shoots was much better (Fig. 39A, D). Hyperhydricity symptoms were scarcely observed during a culture period of 8 weeks. Explants cultured for more than 8 weeks in TIS remained viable like those grown in 1.0 and 1.5 mg l^{-1} BAP but shoot tip necrosis and hyperhydricity became obvious. Moreover, after 10 - 12 weeks in culture explants turned black in the centre of the vessel. Thus, the culture period of *C. acuminata* in TIS was limited to 8 weeks.

4.3.2.1. Multiple Shoot Development in DVS and RITA®

The number of shoots formed in the DVS and RITA® vessels demonstrated a continuous increase over the entire culture period of eight weeks. Figure 40 illustrates the effect of 0.5 mg l^{-1} BAP on the shoot multiplication on explants of the different groups. It should be noted that each group (Ch1, Ch2 and Lou) consists of 4 genotypes and each genotype of at least 5 clones. Generally, explants cultured in TIS developed new shoots earlier compared to those grown in solid medium. The mean numbers of newly formed shoots scored in DVS after two weeks were 7 shoots per explant among the genotypes of group Ch1, 5 shoots per explant in group Ch2 and 6 shoots per explant in the Lou group at an immersion cycle of one minute every 6 hours.

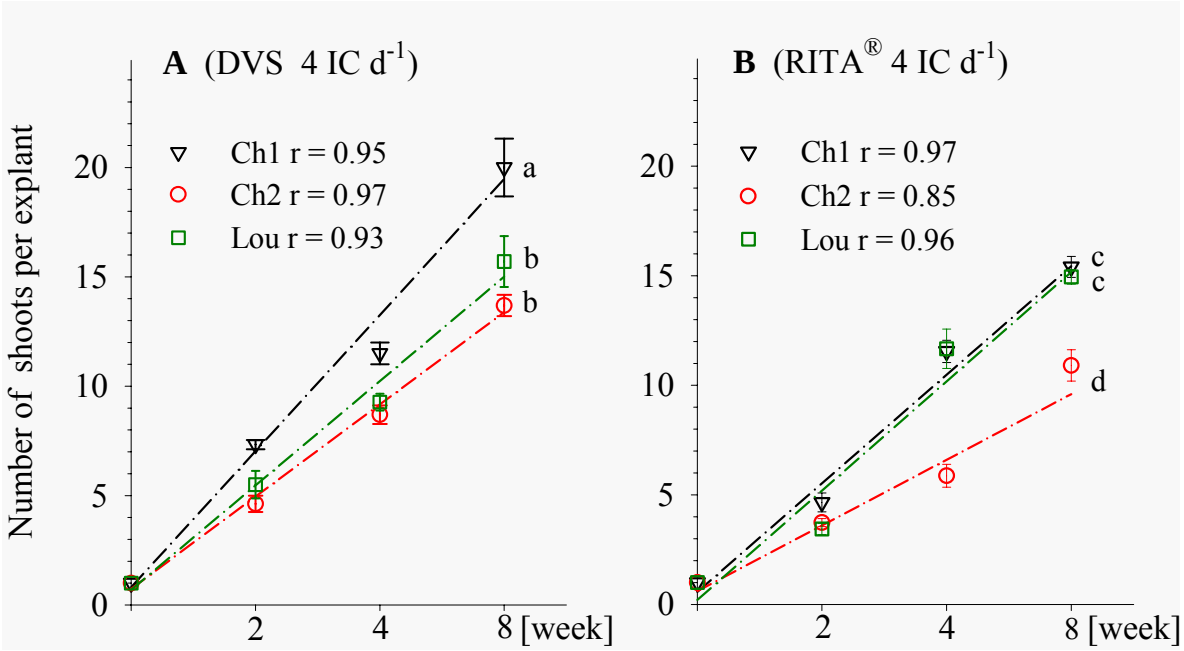

Fig. 40 Mean shoot multiplication of *C. acuminata* in **(A)** DVS and **(B)** RITA® treated with a one minute IC four times d^{-1} over a period of eight weeks. Each data point represents the means ± SE of 6 to 10 vessels with 15 shoots at culture beginning. The straight lines represent linear regression estimate for group Ch1, Ch2 and Lou. The different letters indicate significant differences at $p \leq 0.05$.

Explants of the same genotype cultured in RITA® were scored with 5 shoots per explant in group Ch1 and 4 shoots per explant for Ch2 and Lou after two weeks. There were no significant differences in shoot formation among the clones of the same genotype within each group, in neither of the TIS vessels used. However, there were substantial differences among the three groups after eight weeks. Explants of group Ch2 and Lou in DVS showed

similar results of multiple shoots throughout the whole culture period, whereas Ch1 vs. Ch2 and Ch1 vs. Lou were significantly different at $p \leq 0.05$ and $p \leq 0.01$, respectively. The highest shoot proliferation in DVS was scored with 20 shoots per explant in group Ch1 followed by Lou with 16 and Ch2 with 14 shoots per explant (Fig. 40A) after eight weeks. Regenerated shoots in the RITA® vessels were similar during the first 2 weeks among all groups, but as culture period advanced shoot proliferation was perceivable (Fig. 40B). Ch1 and Lou again showed the highest multiplication rate with 15 shoots per explant compared to Ch2 with 11 shoots per explant after eight weeks. Thus, a comparison between shoots treated with 4 IC d^{-1} over a period of eight weeks reveals that explants cultured in DVS produced a higher amount of shoots than those cultured in RITA®.

A raise to 8 IC d^{-1} resulted in a lower multiplication rate in DVS (Fig. 41A) compared to those attained at 4 IC d^{-1}. Shoot multiplication showed only a marginal variation during the first two weeks of culture among the three groups. However, after eight weeks a maximum of 13 shoots per explant were scored for group Ch1, Ch2 and 11 for Lou. Conversely, explants in RITA® were not influenced by 8 IC d^{-1}. The multiplication rate remained similar to those treated with 4 IC d^{-1} (Fig. 41B).

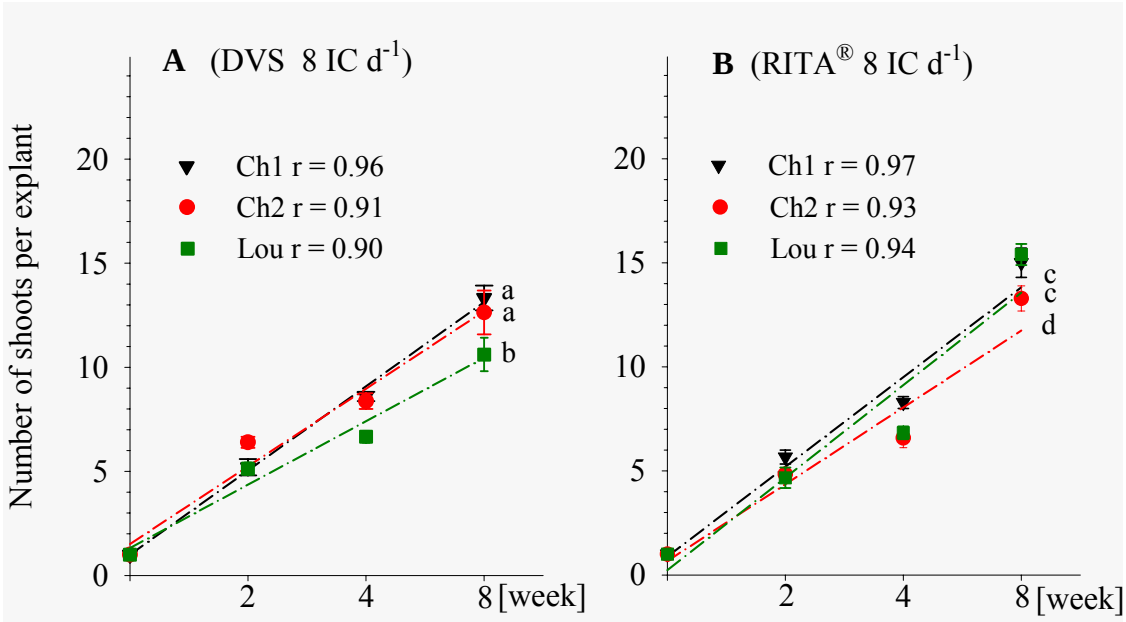

Fig. 41 Mean shoot multiplication of *C. acuminata* in **(A)** DVS and **(B)** RITA® treated with a one minute IC eight times d^{-1}over a period of eight weeks. Each data point represents the means ± SE of 6 to 10 vessels with 15 shoots at culture beginning. The straight lines represent linear regression for groups Ch1, Ch2 and Lou. The different letters indicate significant differences at $p \leq 0.05$.

Fresh weight of individual shoots was less affected by the IC d^{-1} in both the DVS and RITA® vessels. No statistical differences were found between 4 and 8 IC d^{-1} (Fig. 42). After eight weeks in culture shoot fresh weight in DVS showed an increase by a factor of 2.8 and in RITA® of 3.1 for both immersion treatments.

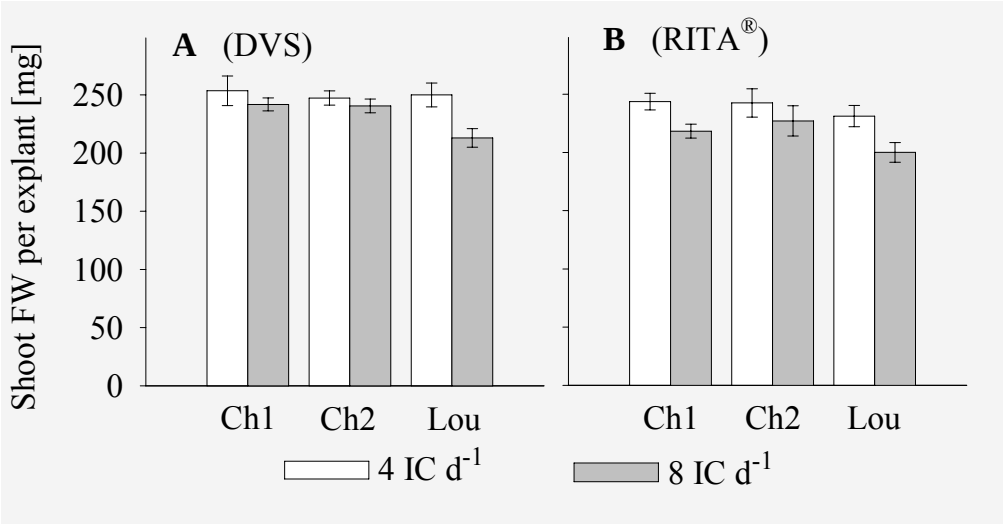

Fig. 42 Shoot fresh weight of *C. acuminata* grown in **(A)** DVS and **(B)** RITA® treated with a one minute IC four (blank bars) and eight (grey bars) times d^{-1} over a period of eight weeks. Bars represent the means ± SE of three replicates.

Similar results were observed for shoot height in DVS and RITA® (Fig. 43). With the exception of Lou in RITA®, which showed an average growth height of 8.8 cm at 4 IC and 8.1 cm at 8 IC d^{-1}, no substantial differences were found among the remaining individuals of other groups. The average shoot height at 4 and 8 IC d^{-1} was 7.6 and 7.3 cm in DVS, respectively, compared to 7.7 and 7.5 cm in RITA®. Apart from a decrease in shoot formation in DVS at 8 IC d^{-1} there was no substantial difference in terms of shoot fresh weight and shoot height.

However, it was obvious that explants submerged eight times a day showed traces of morphological changes when compared with those treated with only 4 IC d^{-1} (Fig. 44). Shoots, that were completely covered by the liquid nutrient during immersion developed stout internodes and brittle stems and leaves were translucent, wrinkled and glossy. These changes were consequently considered as hyperhydricity and were not genotype-dependent. Because these symptoms were scarcely observed on the same genotypes treated with 4 IC d^{-1}.

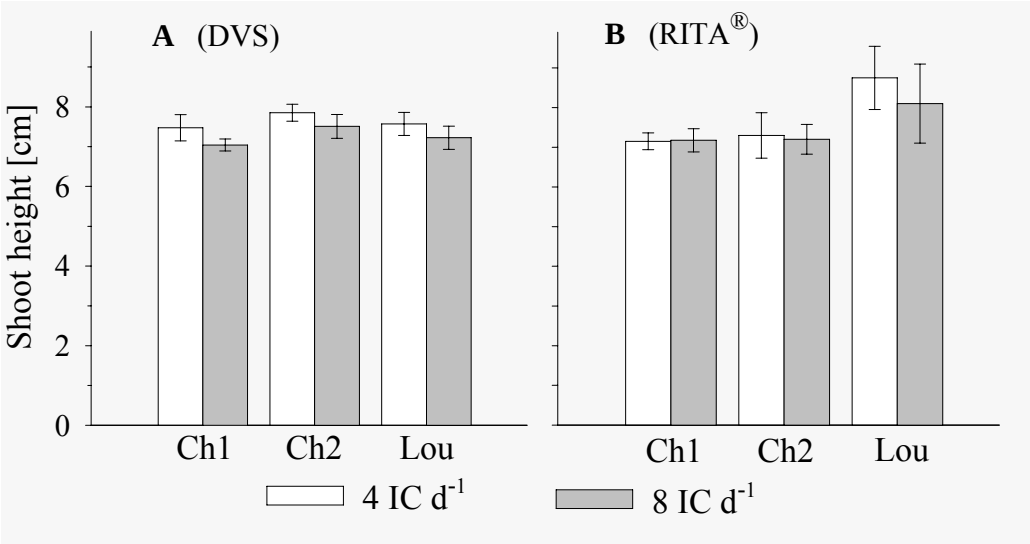

Fig. 43 Shoot height among the three groups: Ch1, Ch2 and Lou in DVS **(A)** and RITA® **(B)** at an immersion of 4 and 8 cycles d^{-1} after 8 weeks. Bars represent the means ± SE of three replicates. Each treatment consisted of at least 5 vessels with 14 to 16 shoots at the beginning of the culture period.

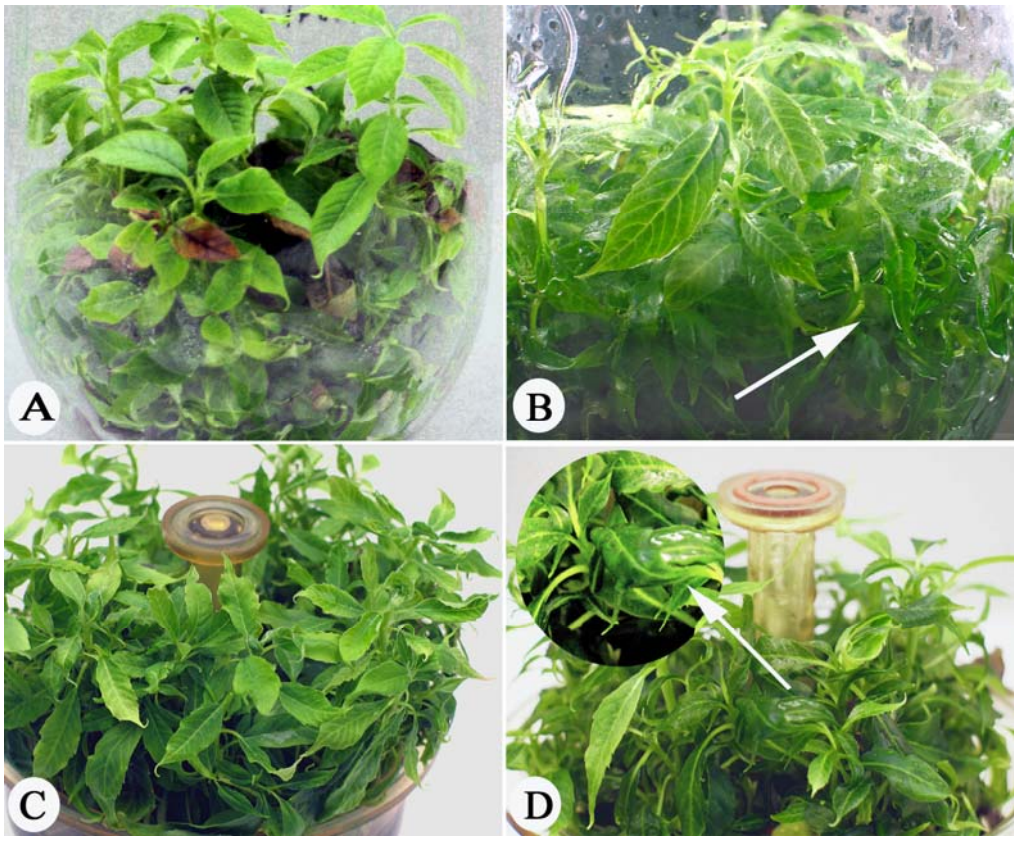

Fig. 44 Hyperhydricity traits in *C. acuminata* shoots cultured in MS medium fortified with 0.5 mg l^{-1} BAP in TIS at a one-min immersions 4 and 8 times d^{-1}. **(A)** Shoots grown in DVS at 4 and **(B)** at 8 immersions d^{-1}. **(C, D)** Shoots grown in RITA® at 4 and 8 immersion d^{-1}, respectively. White arrows indicate the hyperhydricity traits on leaves.

Considering all parameters of growth and development, it was evident that shoot culture in DVS and RITA® treated with 4 IC d^{-1} was optimal to produce quality *Camptotheca* plants. The leaf area of shoots grown in DVS was significantly affected by a higher IC d^{-1}, while those in RITA® did not show any difference between 4 and 8 IC d^{-1} (Fig 45). The average leaf area in DVS was 2.8 to 3.5 cm^2 in the three groups treated with 4 IC d^{-1}, while those treated with 8 IC d^{-1} had an area of 1.9 to 2.8 cm^2. Leaf area of shoots cultured in RITA® were measured as 2.4 to 2.8 cm^2 at 4 IC d^{-1} and 2.4 to 2.7 cm^2 under 8 IC d^{-1}. Leaves in RITA® were smaller and more lanceolate compared to those in DVS, which were larger and more oval in shape.

In general, the highest chlorophyll content based on leaf fresh weight was observed in plantlets grown in RITA® (Fig. 46A). Chlorophyll *a* and *b* contents were 0.97 and 0.4 mg g^{-1} FW, respectively, when plantlets were immersed 4 times a day and 0.95 and 0.41 mg g^{-1} FW at 8 IC d^{-1}. Thus, there were no significant differences between 4 and 8 IC d^{-1}.

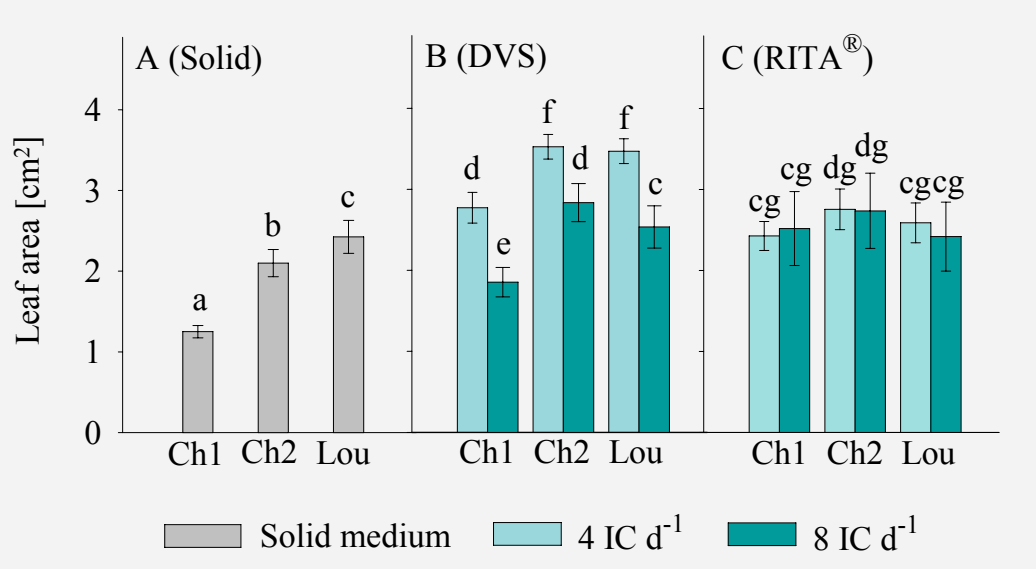

Fig. 45 Comparison of leaf areas among shoots grown in solid medium and in TIS after eight weeks in culture. **(A)** Shoots grown in solid medium. **(B)** Shoot grown in DVS and **(C)** shoots grown in RITA® at 4 (pale blue bars) and 8 (dark blue bars) immersions d^{-1}. Significant differences between treatments at $p \leq 0.05$ are indicated by a, b, c, d, e, f and g.

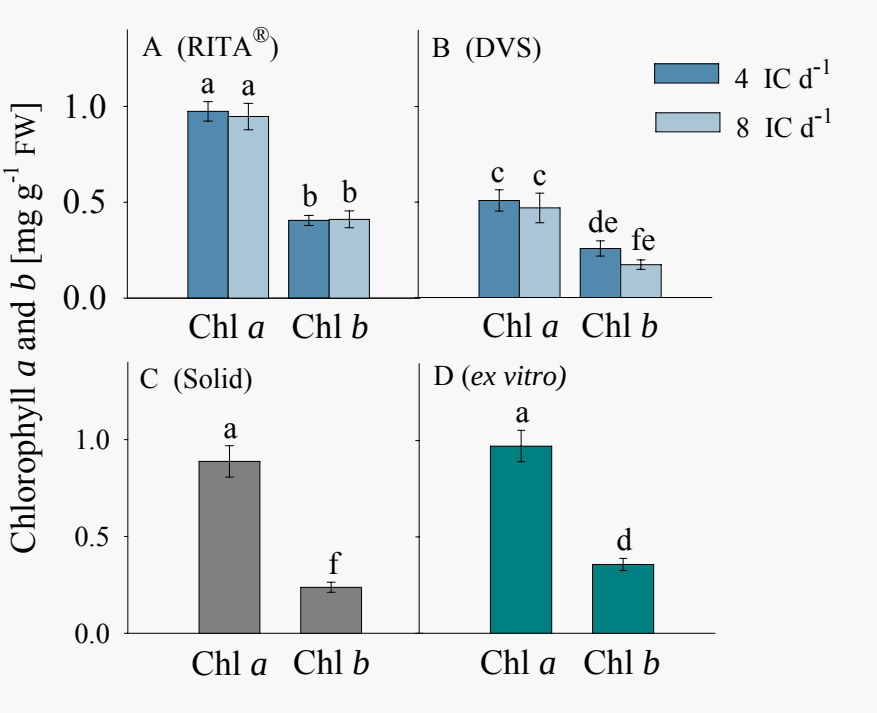

Fig. 46 Chlorophyll *a* and *b* contents in leaves of 8 weeks-old *C. auminata* plantlets grown in the different culture systems. **(A**, **B)** Shoots grown in RITA® and DVS at 1 minute immersion every 6 (dark blue bar) and 3 h (pale blue bar). **(C)** In leaves of shoots grown in solid medium and **(D)** in leaves of *ex vitro* plantlets after 8 weeks. Significant differences between treatments at $p \leq 0.05$ are indicated by a, b, c, d, e and f.

Chlorophyll (Chl) content in DVS was similar in terms of the immersion frequencies (Fig. 46B). However Chl *a* and Chl *b* contents were far higher (1.5 to 2.7 times) in leaves of shoots grown in RITA® than those grown in DVS. Shoots grown in solid medium (Fig. 46C), showed similar Chl *a* and Chl *b* contents to those grown in RITA®. Chlorophyll *a* and Chl *b* contents in leaves of plantlets grown under *ex vitro* conditions (Fig. 46D) were comparable to those grown in RITA® and in solid medium. Chlorophyll content in plantlets grown in the different substrates ranged from 1.59 ± 0.34 mg g^{-1} FW to 1.65 ± 0.42 mg g^{-1} FW.

4.4. Rooting and Acclimatisation

4.4.1. *In vitro* Rooting in Different Culture Systems

In vitro rooting of regenerated plantlets via somatic embryogenesis in sterilised substrates was more effective than in the TIS vessels DVS and RITA®. Regenerants on substrates moistened with sucrose free half strength MS medium containing 0.5 mg l^{-1} IBA developed roots within 12-14 days, whereas those in DVS and RITA® induced roots after 18 to 20 days. As shown in Table 7 rooting of regenerants was not very high in TIS, likewise the survival rate under greenhouse conditions. Whereas regenerants rooted in sterilised substrate, especially those in a mixture of sand and vermiculite (SV) and sand, soil and vermiculite (SSV) showed higher rooting percentage as well as the best survival rate under *ex vitro* conditions. Rooting in pure sand was also relatively higher and showed a better effectiveness than in soil. However, desiccation was greater on both sand and soil than in SV and SSV. Thus, if these two substrates were not moistened frequently (weekly) plantlets just dehydrated and shrivelled.

Tab. 7 The results of regenerated plantlets obtained for the TIS and sterilised substrate culture systems during a twelve week *in vitro* phase including the rooting and acclimatisation. The values in the columns represent the means ± SD of nine replicates with 25 embryos per replicate for DVS and RITA® (n = 225) and 5 replicates each for the four different substrates with 10 embryos per replicate (n = 50).

Culture Systems		*In vitro*		*Ex vitro*
		Regenerants after 8 weeks [%]	**Rooting of regenerants after 4 weeks [%]**	**Survivals after 4 weeks [%]**
TIS	RITA®	58 ± 6.0	52 ± 10.3	27 ± 5.7
	DVS	30 ± 7.2	26 ± 7.2	11 ± 3.5
Sterilised Substrates	SV	88 ± 13.0	86 ± 11.4	82 ± 14.8
	SSV	80 ± 18.7	78 ± 16.4	76 ± 23.0
	Pure sand	62 ± 13.0	62 ± 11.0	60 ± 10.0
	Pure soil	26 ± 20.7	26 ± 20.7	20 ± 18.7

The effects of two auxins (IBA and NAA) on the rooting induction on *C. acuminata* microcuttings in various culture systems, solid media, DVS, RITA® and sterilised substrates were tested. Full strength MS medium supplemented with 0.5 mg l^{-1} IBA and 2% sucrose proved to be more effective for *in vitro* rooting compared to media fortified with 0.5 mg l^{-1} NAA and 2% sucrose, particularly in solid media, DVS and RITA®. Excised shoots cultured in IBA started to initiate roots within 17 to 18 days in agar medium.

Microcuttings in RITA® initiated roots after 14 to 15 days and those in DVS after 18 to 20 days. In all cases, root initiation on microcuttings cultured in media supplemented with NAA was observed about one week later. Microcuttings cultured in sterilised substrates (SV, SSV and pure soil) moisturised with MS medium containing either IBA or NAA without sucrose initiated roots within 12 to 14 days.

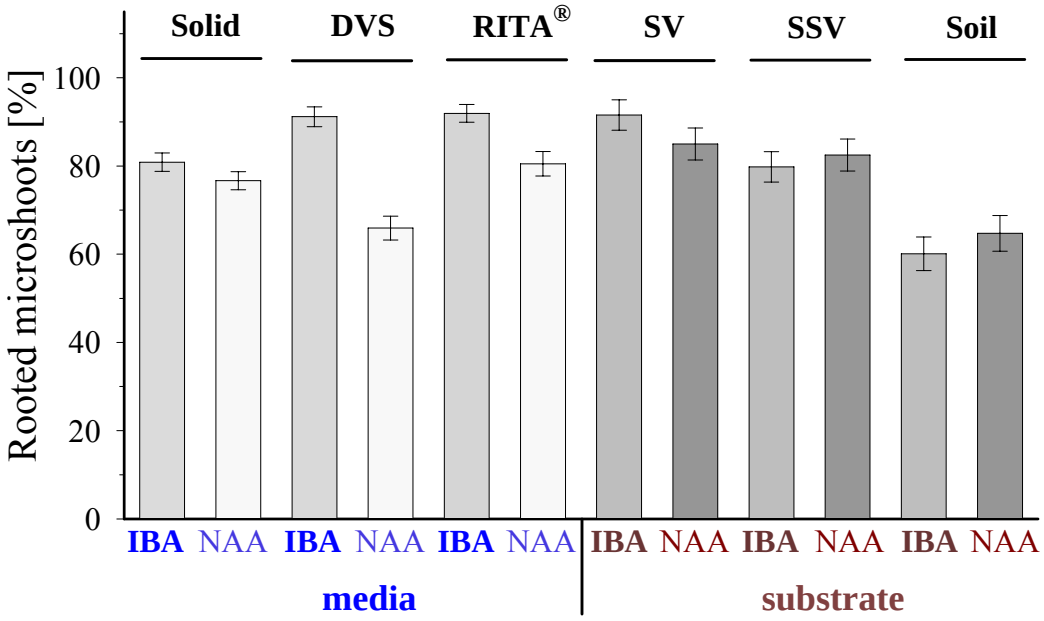

Fig. 47 The effects of 0.5 mg l^{-1} IBA and NAA after 6 weeks on the *in vitro* rooting of *C. acuminata* microcuttings in the different systems, in solid medium, in TIS (DVS and RITA®) and in sterilised substrates (SV, SSV and soil). Bars indicate means ± SE.

Both IBA and NAA showed a high percentage of root-enhancing activity in solid media. Microcuttings that developed roots in IBA were recorded with 81% and those in NAA with 78%. No significant differences were found between IBA and NAA. In DVS and RITA® similar results were observed for media supplemented with IBA. In DVS, 90% of the microcuttings developed roots and 92% in RITA®. Rooting with NAA was less in both vessels. In DVS only 66% of the microcuttings developed roots, while in RITA® an average of 81% was recorded, which was remarkable higher than in DVS. Rooting in sterilised substrate moistened with MS medium containing IBA or NAA did not show any significant differences. However, there were significant differences between the substrates used. The best rooting results were achieved on SV moistened with IBA (91.6%) and NAA (85%), while those cultured on SSV moistened with either IBA or NAA were recorded as 80% and 83%,

respectively. Less inductive were microcuttings cultured on soil. An average of 60% developed roots in soil treated with IBA and 65% with NAA (Fig. 47).

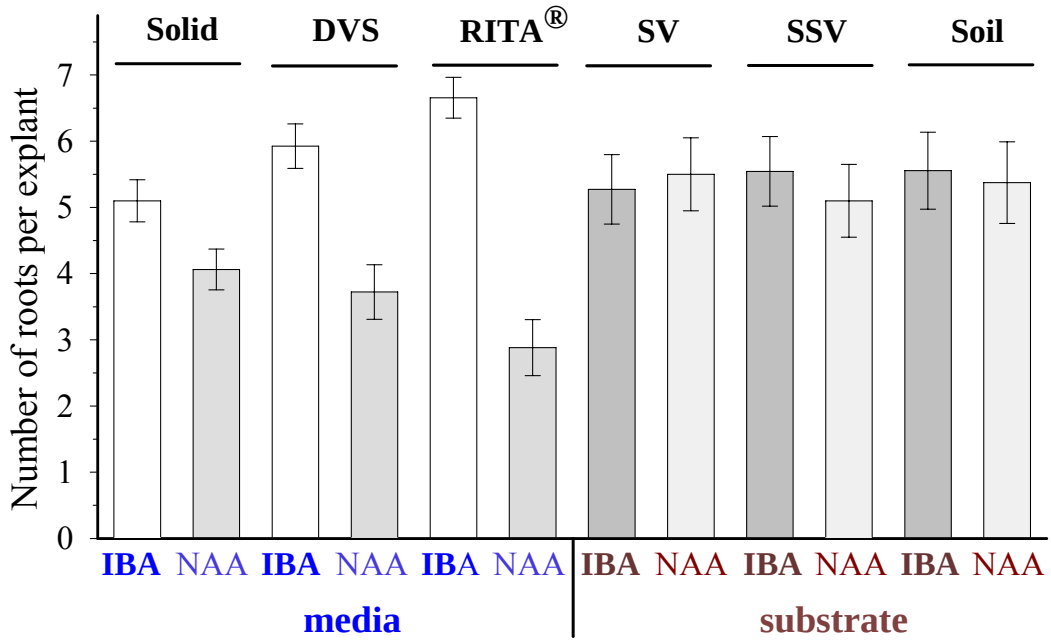

Fig. 48 Number of roots developed after 6 weeks on media and substrates containing 0.5 mg l^{-1} IBA or NAA during the *in vitro* rooting of *C. acuminata* microcuttings in the different systems: Solid medium, TIS (DVS and RITA®) and in sterilised substrates (SV, SSV and pure soil). Bars indicate means ± SE.

Significant differences were marked in terms of the number of roots developed per microcuttings when IBA or NAA was added to the basal medium. As shown in Figure 48, NAA induced significantly fewer roots per explant, compared to IBA. Medium containing 0.5 mg l^{-1} IBA resulted in an average of 5.1 roots per shoot in solid medium, 5.9 in DVS and 6.7 in RITA®, while those on medium fortified with NAA showed an average of 4.1, 3.7 and 2.8 roots per shoot, respectively. The numbers of roots developed on the different sterilised substrates were non-significant. In all treatments a number of 5.1 to 5.5 roots per shoots were scored.

NAA seems not only to have an inhibitory effect on root development but also on the length, particularly on shoots cultured in TIS. Otherwise there were no significant differences found between IBA and NAA in terms of root length among microcuttings rooted in MS solid. A similar response was observed among the different sterilised substrates used for rooting. However, *in vitro* rooting in solid medium has a positive effect on the root length and quality of plantlets (Fig. 49).

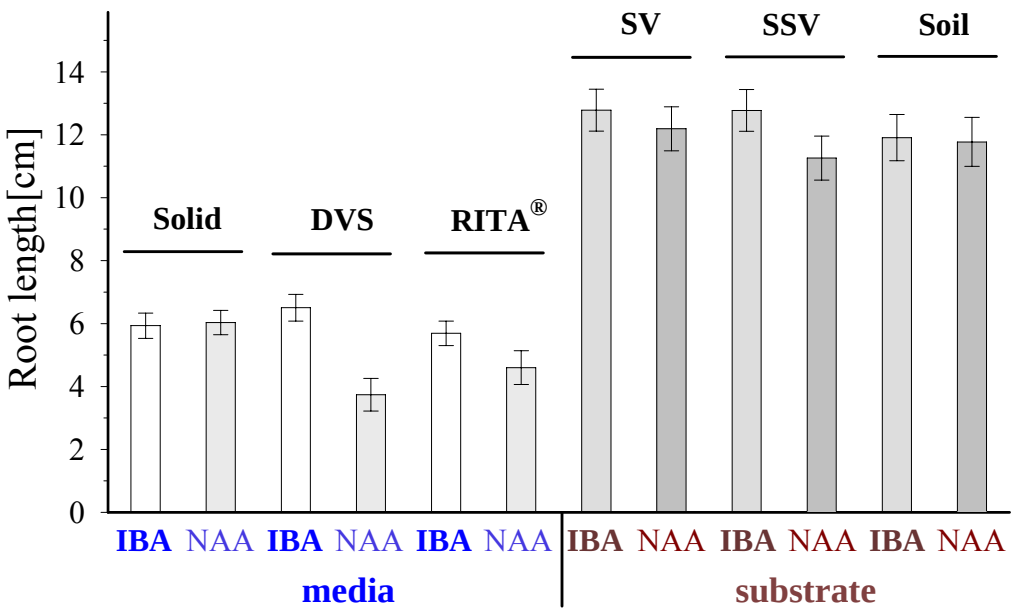

Fig. 49 Root length measured after 6 weeks on media and substrates containing 0.5 mg l^{-1} IBA or NAA during the *in vitro* rooting of *C. acuminata* microcuttings in the different systems: Solid medium, TIS (DVS and RITA®) and in sterilised substrates (SV, SSV and soil). Bars indicate means ± SE.

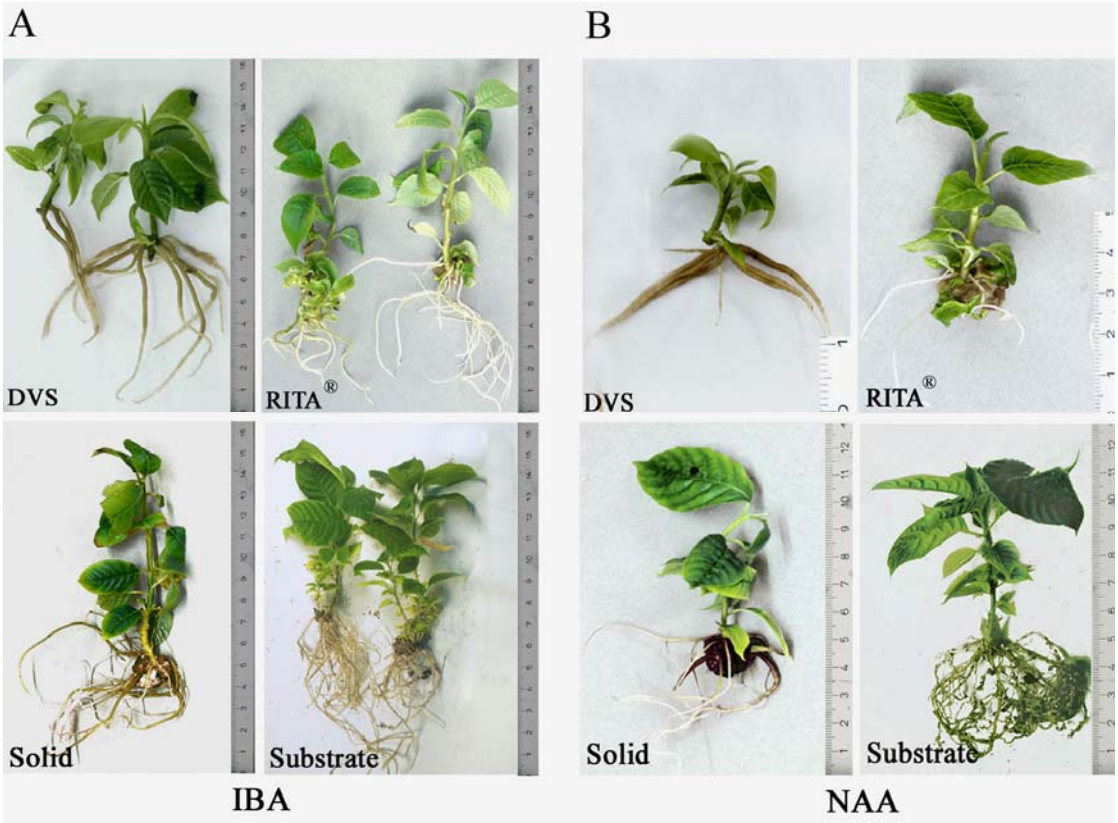

Fig. 50 The influence of IBA and NAA on *in vitro* rooting of microcuttings in different culture systems. Solid medium, TIS (DVS and RITA®) and in sterilised substrates. **(A)** Microcuttings rooted in 0.5 mg l^{-1} IBA, **(B)** microcuttings rooted in 0.5 mg l^{-1} NAA showing the root development in the different culture systems after 6 weeks.

Figure 50 illustrates the differences in morphology among rooted microcuttings derived from the different culture systems. Microcuttings rooted in media supplemented with IBA (Fig. 50A) exhibit elongated and vigorous roots compared to those cultured in media containing NAA (Fig 50B). Shoots in DVS showed roots that were stout but brittle, while roots developed on shoots in RITA® and solid media were longer and robust.

Roots formed on microcuttings treated with NAA were generally similar to those treated with IBA but it was obvious that some shoots produce fewer roots particularly in TIS. Microcuttings cultured in the different sterilised substrates did not show any significant differences in root formation between the two auxin treatments. It was also obvious that plantlets grown in sterilised substrates were more vigorous and healthier.

4.4.2. *Ex vitro* Rooting of Microcuttings in Non-sterile Substrates

Tab. 8 The effect of IBA and NAA on the ex *vitro* rooting of microcuttings derived from two different culture systems, TIS (DVS and RITA® vessels) and solid media. The values in the columns represent the means ± SE of at least three replicates.

Microcuttings derived from	Rooted and survived microcuttings in non-sterile substrates [%]		
	SV	SSV	Pure soil
DVS	39 ± 4.0	42 ± 7.0	33 ± 1.0
RITA®	33 ± 1.0	38 ± 4.0	27 ± 5.7
Solid media	46 ± 1.0	46 ± 1.0	29 ± 8.0

Root formation under *ex vitro* conditions in non-sterile substrate moistened with MS medium containing 0.5 mg l^{-1} IBA or NAA was not enhancing. In all substrates only moderate results were achieved. Table 8 shows the percentage of rooted microcuttings after 6 weeks. No substantial differences were noted between the two auxins used to stimulate rooting.

Compared to the *in vitro* rooting with the same auxins concentrations used, *ex vitro* rooting showed a considerably lower percentage of rooted microcuttings. However, as soon as the newly transferred cuttings (Fig. 51A) had developed roots and new leaves all rooted cuttings recovered into normal healthy *C. acuminata* plantlets as shown in Figure 51B. All rooted *ex vitro* plants survived, showed a vigorous growth and were phenotypically normal.

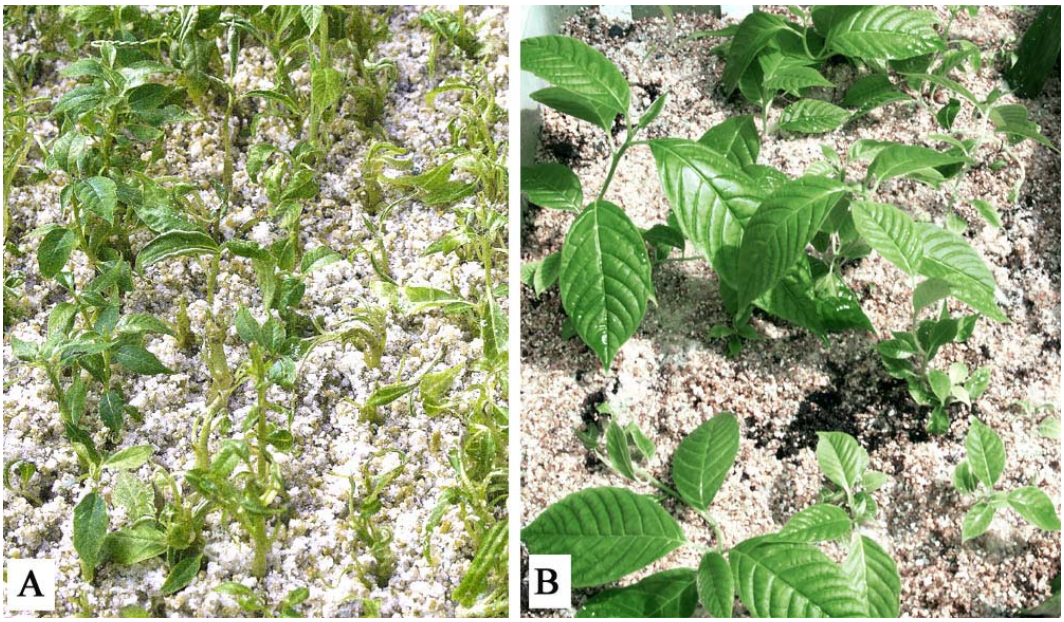

Fig. 51 *Ex vitro* rooting by transferring microcuttings directly to non-sterile substrate. **(A)** Newly transferred microcuttings cultured in SV and **(B)** survived plantlets after 6 weeks.

4.4.3. Acclimatisation to Greenhouse Conditions

Survival rates of rooted plantlets after 6 weeks under greenhouse conditions are shown in Figure 52. The highest survival rate of 93% was recorded for plantlets derived from substrates followed by those derived from RITA® with 47%, solid medium with 45% and from DVS with 41%.

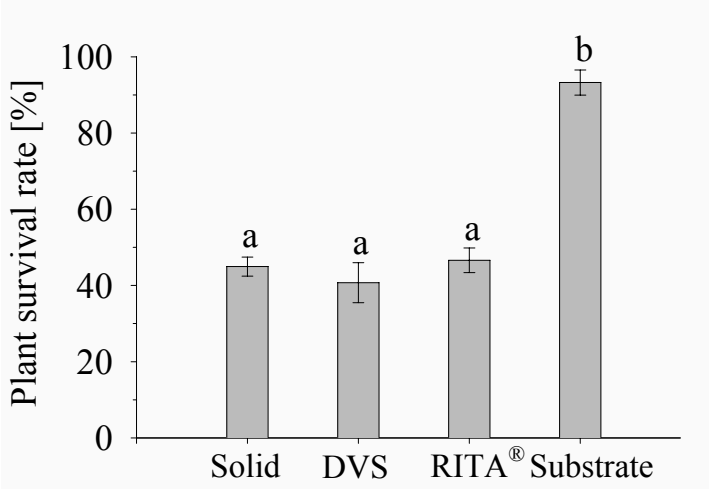

Fig 52 Survival rate of microcuttings rooted *in vitro* after 6 weeks under greenhouse conditions. Bars represent the means ± SE of at least five replicates with 100 plantlets per tray. Different letters indicate significant differences at $p \leq 0.05$.

Rooted plantlets derived from solid medium, DVS and RITA® showed similar results in terms of survival rate after 6 weeks without any significant differences, whereas plantlets derived from substrates showed the best survival rate (93%).

Roots developed in TIS (DVS and RITA®) were fragile and unbranched, while those grown in solid medium and substrates showed branched roots. The roots of most plantlets derived from solid medium and TIS died upon transplantation to commercial soil. Rooted plantlets derived from the different sterilised substrates did not show any changes upon transplantation. This was because plantlets from sterilised substrates had well-developed and robust roots (Fig. 50). Thus as shown in Figure 52 rooted plantlets from sterilised substrates have a better chance to survive *ex vitro* conditions.

Figure 53 shows the stomata density among leaves of plantlets grown in the various culture systems. Among the *in vitro* grown plantlets the highest stomata density was found on leaves of plantlets grown in solid medium and substrate cultures followed by plantlets grown in RITA® and DVS.

Plantlets in DVS showed invariably open stomata, while those in the remaining *in vitro* cultures had both opened and closed stomata. Stomata density in the *in vitro* cultures ranged from 97.6 to 278.9 stomata mm^{-2}. No differences were found in the number of stomata per unit of area between plantlets grown in solid medium, substrates and *ex vitro* plantlets after new leaves had been developed. Plants grown from seed under greenhouse condition showed the greatest number of stomata per unit of area (416.0 stomata mm^{-2}) compared to those from *in vitro* and acclimatised plantlets.

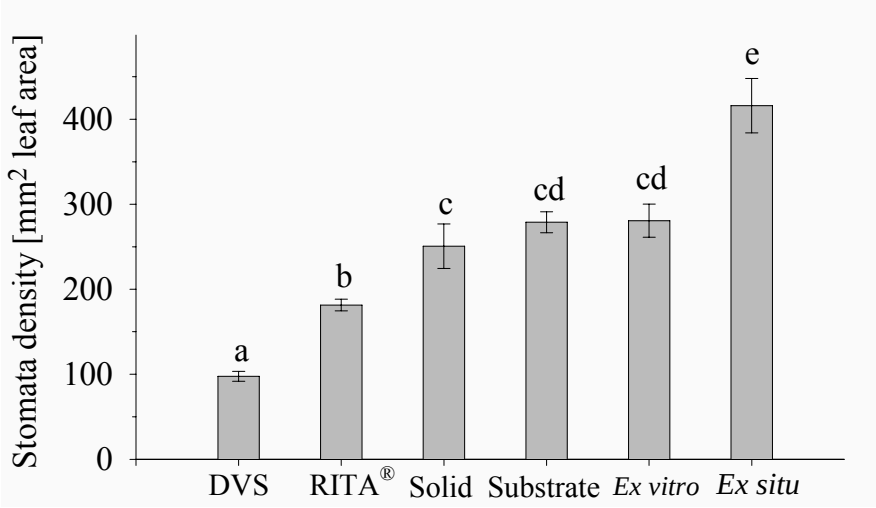

Fig. 53 Stomata density on leaves of *C. acuminata* shoots grown *in vitro* in the different systems, TIS (DVS and RITA®), solid medium, in sterilised substrates (SV, SSV and pure soil), *ex vitro* acclimatised plantlets and *ex situ* plants.

4.5. Maintenance of Embryogenic Callus

Embyogenic calli of *C. acuminata* subcultured at an interval of 4 to 6 weeks turned brown and gradually lost their proliferation capacity. Thus, in order to avoid necrosis, all cultures were frequently subcultured at an appropriate interval of 2 to 3 weeks. Embryogenic calli in solid media were basically grey to white in colour and exhibited a heterogeneous growth. In some dishes globular and heart shaped embryos were obvious, while in others adventitious buds or embryos of torpedo and cotyledonary stages were predominant (Fig. 54A-C). Calli with distinct roots were also frequently observed (Fig. 54D). Secondary callus formations were observed mostly in solid cultures particularly on the surface of already differentiated adventitious buds and cotyledonary embryos.

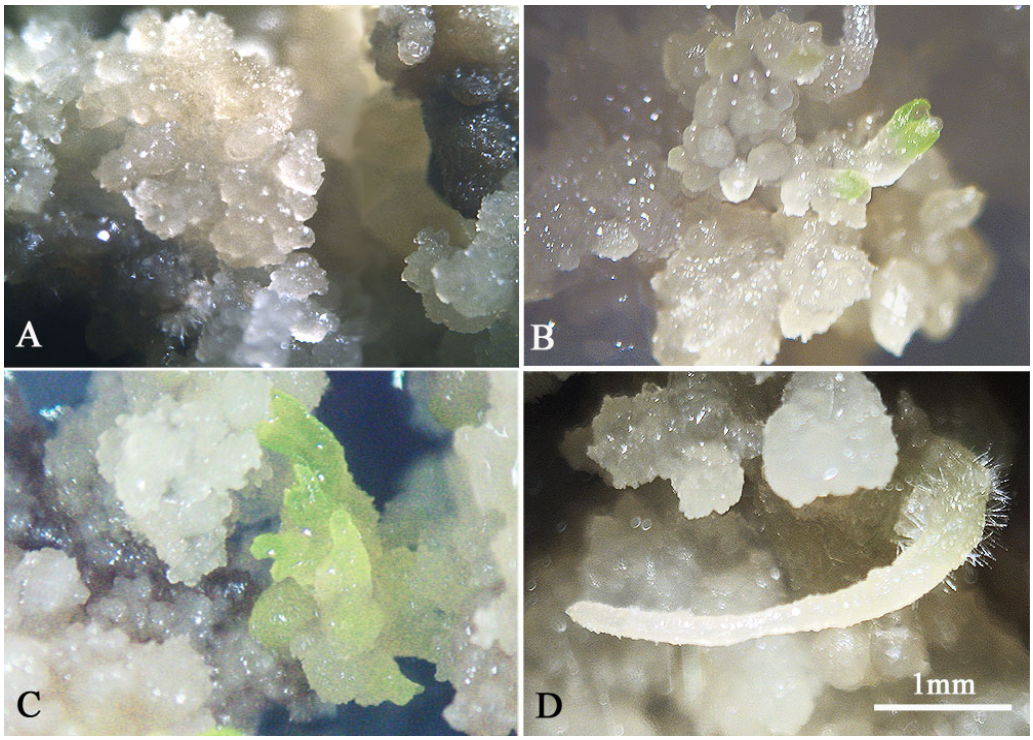

Fig. 54 Callus cultures in solid medium. **(A)** Callus mass with globular and heart shape embryo stages. **(B)** Torpedo and early cotyledonary embryo stages. **(C)** Adventitious buds and **(D)** callus mass with root. Scale applies for **(A - D)**.

Cell suspension cultures were homogeneous and consisted of either cell aggregates (Fig. 55A) or rooted callus (Fig. 55B) with round or elongate cells (Fig. 55C, D). Upon subculturing cell aggregates and rooted callus were separated and re-cultured in solid medium for plant regeneration, while the single cells were sieved and subcultured in fresh liquid medium.

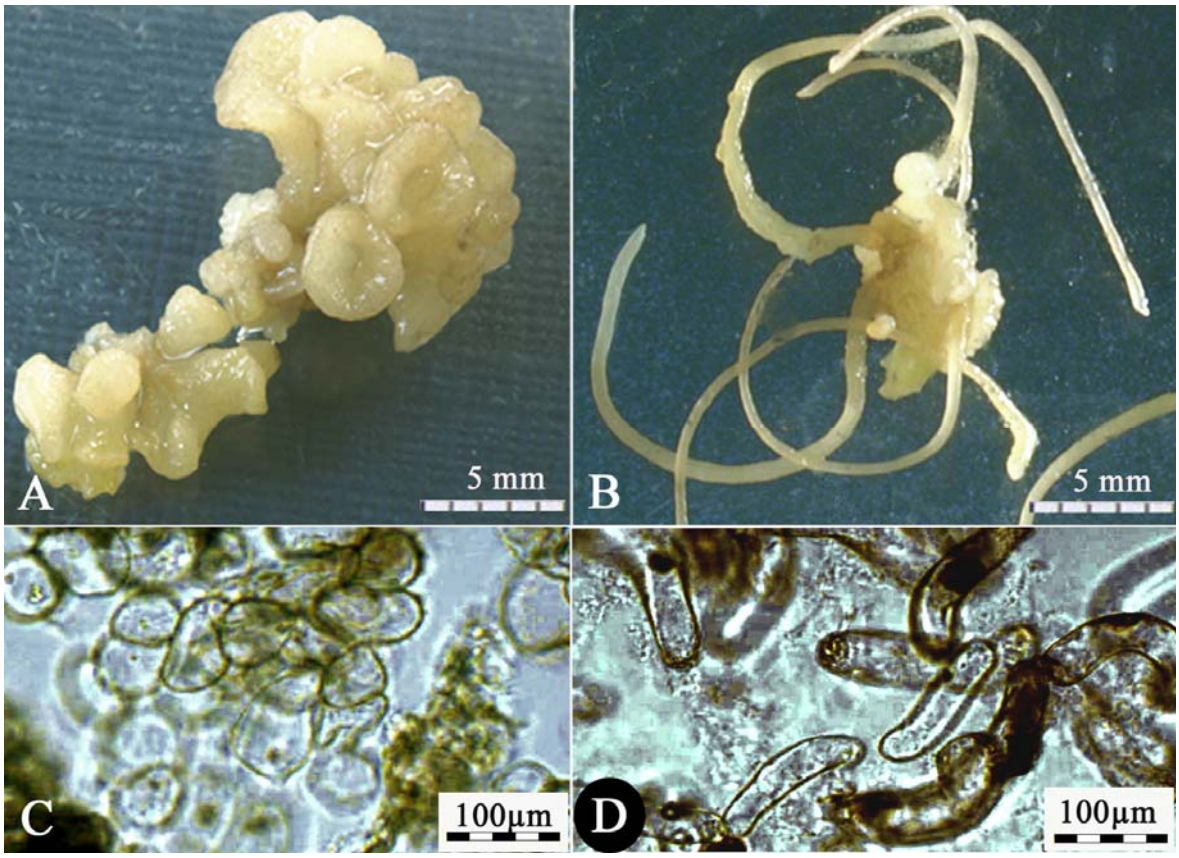

Fig. 55 Typical morphological structures of embryogenic callus in liquid medium. **(A)** Compact clusters of embryos. **(B)** Embryogenic callus with roots. (**C**) Dispersed cell suspension cultures with isodiametric and (**D)** elongate cells.

Embryogenic calli of *C. acuminata* could be maintained in both solid and liquid media for more than 3 years without losing their ability to regenerate into true plantlets. For a long-term maintenance, friable embryogenic callus was selected and periodically subcultured either in solid or liquid **BI** (2 mg l^{-1} BAP + 0.1 mg l^{-1} IAA) or **NiP** (0.2 mg l^{-1} NAA + 0.1 mg l^{-1} 2iP) medium for 2 to 3 weeks and then transferred in PGR-free medium for another 2 to 3 weeks. It was obvious that although embryogenic cells in solid medium and in suspension cultures were cultured on the same media and exposed at the same temperature (25 ± 0.5 °C) and photoperiod (40 µmol m^{-2} s^{-1} PPFD), calli in solid media scarcely showed any changes in colour. In contrast, cell suspension cultures differed in colour depending on the medium regardless of their genotypes or position on the 2D rotary shaker. Calli cultured in PGR-free medium were grey, those in BI medium exhibited a scale from dark red to grey-red, while those in NiP medium were mainly green in colour (Fig. 56A-C respectively).

No histological observation was carried out to differentiate between embryogenic and non-embryogenic character of the cells. It was only assumed that both types of cells were present because when cell clusters were plated in solid media, embryos or adventitious buds were formed only on some clusters, while others showed only cell proliferation. Apart from the two morphological forms shown in Figure 55A-B suspension cultures did not show any real embryo development during the liquid phase. However, a change after 3 to 4 subcultures from liquid NiP to solid NiP media proved to be effective for callus growth and a change from liquid BI to solid BI media was more preferable for somatic embryos or adventitious buds development. During subculturing, all somatic embryos or adventitious buds were selected and transferred for plant regeneration in TIS or in sterilised substrates. Microscopic observation (Fig. 56D-F) and a staining test with fluorescein diacetate (FDA) (Fig. 56G-I) were carried out to control the viability of cells in suspension culture. Viable cells stained green with FDA, while non-viable cells remained uncoloured. Figure 56G-I shows the viable cells.

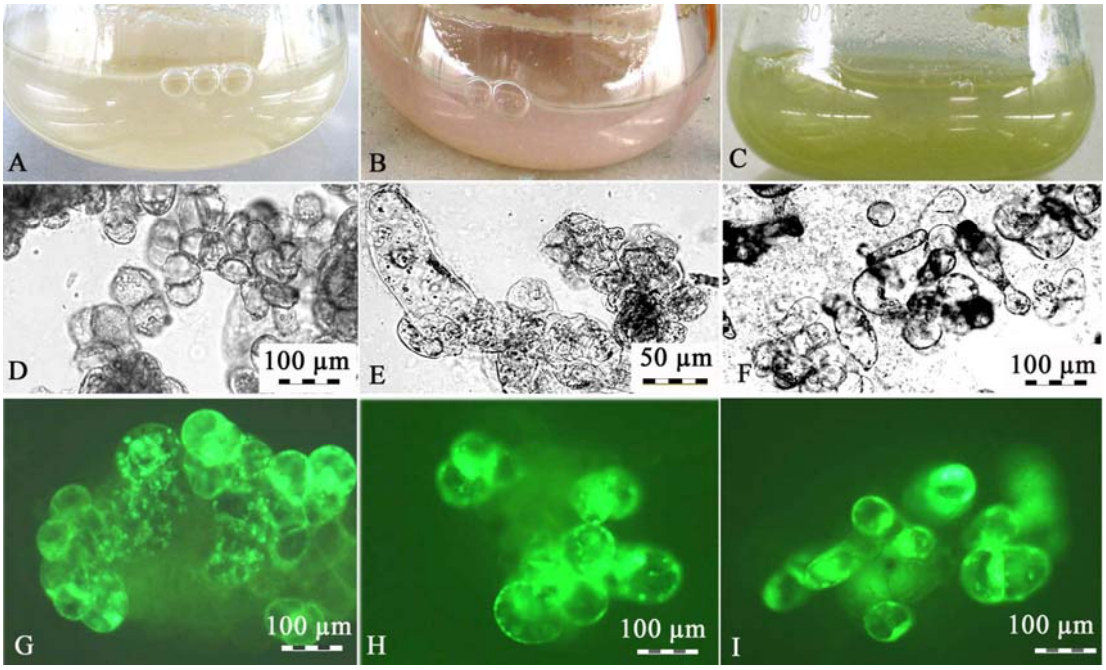

Fig 56. *C. acuminata* cell suspension cultures **(A)** Culture in PGR-free medium, **(B)** in BI and **(C)** in NiP medium. **A** to **C** also show the different colours of suspension culture after 4 weeks of inoculation in MS medium. (**D-F**) Microscopic observation of dispersed cell suspension cultures 4-week after inoculation. (**G-I**) Fluorescence microscopy of FDA stained cells.

4.6. Camptothecin (CPT) Distribution in Plant Organs, Tissues and Liquid Media

Accumulation of CPT was investigated in the intact tissues of *in vitro* grown *C. acuminata* plants at different developmental growth stages and compared with those of *ex vitro* and greenhouse cultures. HPLC analysis has shown that CPT was present in undifferentiated and differentiated tissues of *C. acuminata*. HCPT was detected only in the hypocotyls of *ex vitro* seedlings and in few *in vitro* grown seedlings.

4.6.1. CPT Comparison Between *Ex situ* and *In vitro* Seedlings

The variations in CPT concentrations in different organs of *C. acuminata* seedlings from two sources (Jiangsu and Sichuan, China) were compared. HPLC analysis revealed that CPT was present in all parts of the seedlings in various concentrations. It was obvious that CPT content in *ex situ* and *in vitro* seedlings differed significantly. Between the two provenances, seedlings from Jiangsu (Fig. 57A) showed a higher concentration of CPT in both *ex situ* and *in vitro* compared to those originated from Sichuan (Fig. 57B). Among the various parts analysed, apical tips (at) exhibited the highest CPT content in both *ex situ* and *in vitro* seedlings.

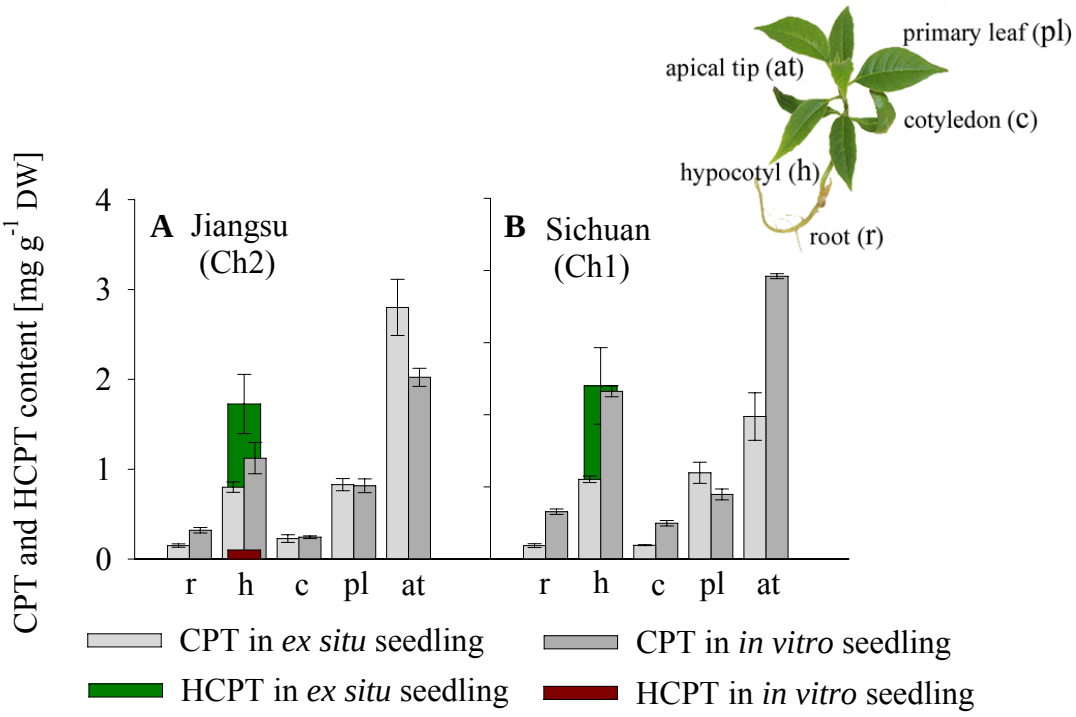

Fig. 57 Comparison of CPT and HCPT concentrations between 6-week-old *C. acuminata* seedlings grown *ex situ* and *in vitro*. Experiments were repeated twice with seedlings from two different sources. **(A)** Seeds originated from Jiangsu and **(B)** from Sichuan, China. Each bar represents the means ± SE (n = 4). Root (r), hypocotyl (h), cotyledon (c), primary leaf (pl) and apical tip (at).

The second-highest CPT concentrations were found in hypocotyls (h) followed by the primary leaves (pl) among *in vitro* grown seedlings, whereas *ex situ* seedlings did not show any significant differences in terms of their CPT concentrations.

Hypocotyls and primary leaves of seedlings from both sources (Jiangsu and Sichuan) showed a similar CPT content (0.80 to 0.83 and 0.55 to 0.60 mg g^{-1} DW, respectively). Cotyledons (c) and roots (r) exhibited the lowest CPT content ranging from 0.09 to 0.33 mg g^{-1} DW.

HCPT was found in the hypocotyls of both *ex situ* grown seedlings originated from Jiangsu and Sichuan in concentrations of 1.7 ± 0.29 and 1.2 ± 0.25 mg g^{-1} DW, respectively. No HCPT was detected in *in vitro* seedlings from Sichuan, while seedling from Jiangsu showed a very low concentration of 0.1 mg g^{-1} DW.

4.6.2. CPT Content in the Different Stages of Somatic Embryos

The evaluation of CPT content in non-embryogenic and embryogenic callus as well as in all stages of somatic embryos (heart-shape, torpedo and cotyledonary) and regenerated plantlets showed a gradual increase with embryo development (Fig. 58). Non-embryogenic callus (n-ec) synthesised trace amounts of CPT (0.002 mg g^{-1} DW), while slightly higher amounts of CPT (0.024 and 0.030 mg g^{-1} DW) were detected in embryogenic callus (ec) and callus containing cream-glossy globular and green heart stage embryos (hse), respectively.

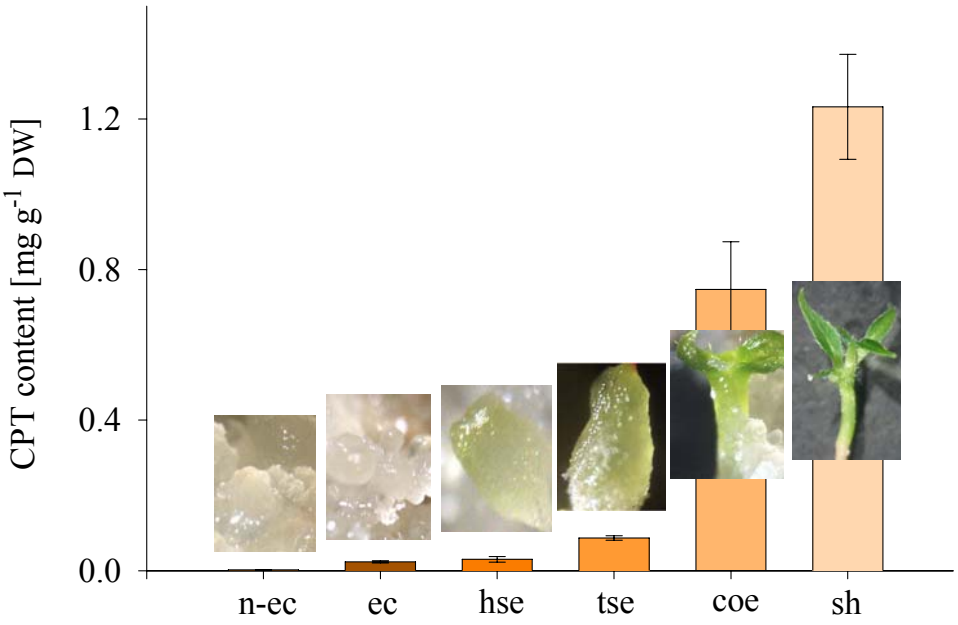

Fig. 58 CPT content in the different developmental stages. Non-embryogenic **(n-ec)** and embryogenic callus **(ec)**, globular and heart shape embryos **(hse)**, torpedo **(tse)**, cotyledonary **(coe)** stage of embryos and shoots **(sh)**. Data represent the means ± SE of 3 replicates (n = 7-10).

Somatic embryos at torpedo stage (tse) showed a high amount of CTP (0.087 mg g^{-1} DW), but the highest was detected in well-developed cotyledonary embryos (coe) (0.75 mg g^{-1} DW) and in regenerated plantlets (sh) (1.23 mg g^{-1} DW). The differences in CPT content among the individual cotyledonary embryos and plantlets were highly significant.

4.6.3. CPT Content in Shoots Grown in Solid Media

Seeds originated from Sichuan (Ch1), Jiangsu (Ch2) and Louisiana (Lou) cultured *in vitro* in solid medium and in TIS were analysed for their CPT content. Four genotypes from each group were employed to study the CPT production. CPT content after four and eight weeks did not show any significant differences. As shown in Figure 59, CPT concentrations amongst these genotypes varied significantly within the three groups. The highest CPT concentrations were found in Mp27, Mp28 and Mp35 of group Ch2, showing mean concentrations of 2.17, 1.77 and 1.93 mg g^{-1} DW, respectively. Remarkably, within group Ch2, Mp36 was the only genotype with an extremely low CPT concentration of 0.026 mg g^{-1} DW. Among the Lou group Lp4 showed the highest content of 0.47 mg g^{-1} DW, whereas the remaining three genotypes (Lp13b, and Lp18 and Lp45) were scored with 0.099, 0.069, and 0.002 mg g^{-1} DW respectively. Genotypes Bp31, Bp 81, Bp101, Bp141 of group Ch1 showed the lowest concentrations with an average from 0.013 to 0.054 mg g^{-1} DW (Fig. 59).

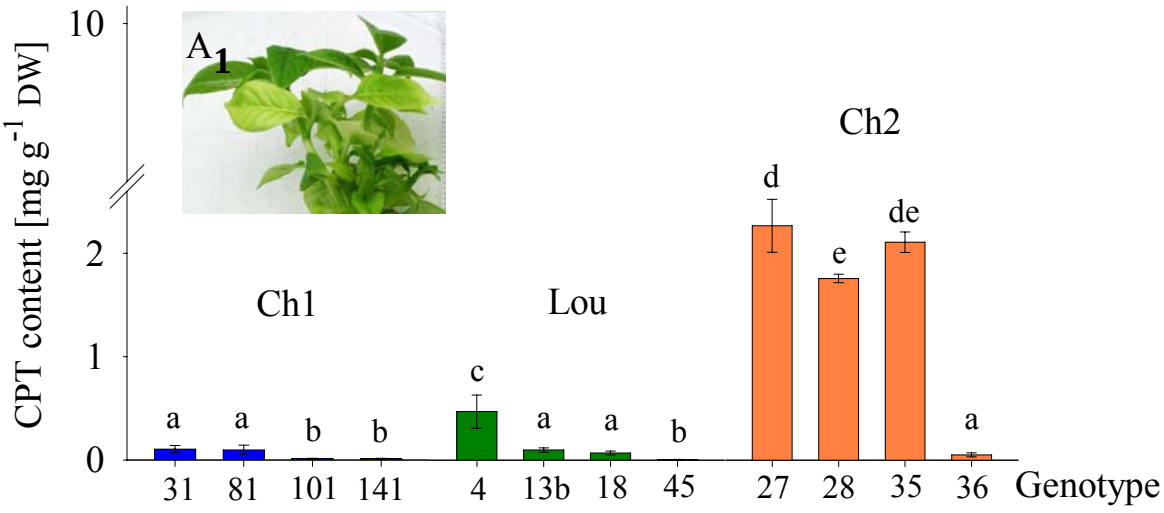

Fig. 59 Variation of CPT content among the different genotypes of *C. acuminata* grown in solid media. **(A_1)** Shoots used for CPT analysis. Each treatment was repeated at least three times and three samples were taken from each culture vessel. Bars represent the means ± SE of three replicates.

4.6.4. CPT content in Shoots grown in TIS Vessels

CPT contents in shoots grown in two types of TIS vessels were compared. After a culture period of 4 and 8 weeks in TIS, CPT levels among the twelve genotypes varied distinctively. As depicted in Figure 60 and 61, CPT concentrations differed significantly between the two immersion treatments. It was obvious that CPT concentrations in some genotypes were unexpectedly higher than in others. In general, shoots immersed eight times a days showed a higher CPT content after eight weeks than those treated with only four immersions. However, there were few a genotypes that showed higher CPT level also after 4 weeks. Genotypes grown in DVS treated with 4 IC d^{-1}, such as Bp81 (0.83 mg g^{-1} DW), Bp141 (0.88 mg g^{-1} DW) and Mp28 (1.65 mg g^{-1} DW) were found to have a significant high level of CPT. The remaining genotypes were scored with CPT levels from 0.5 to a minimum of 0.1 mg g^{-1} DW (Fig. 60A).

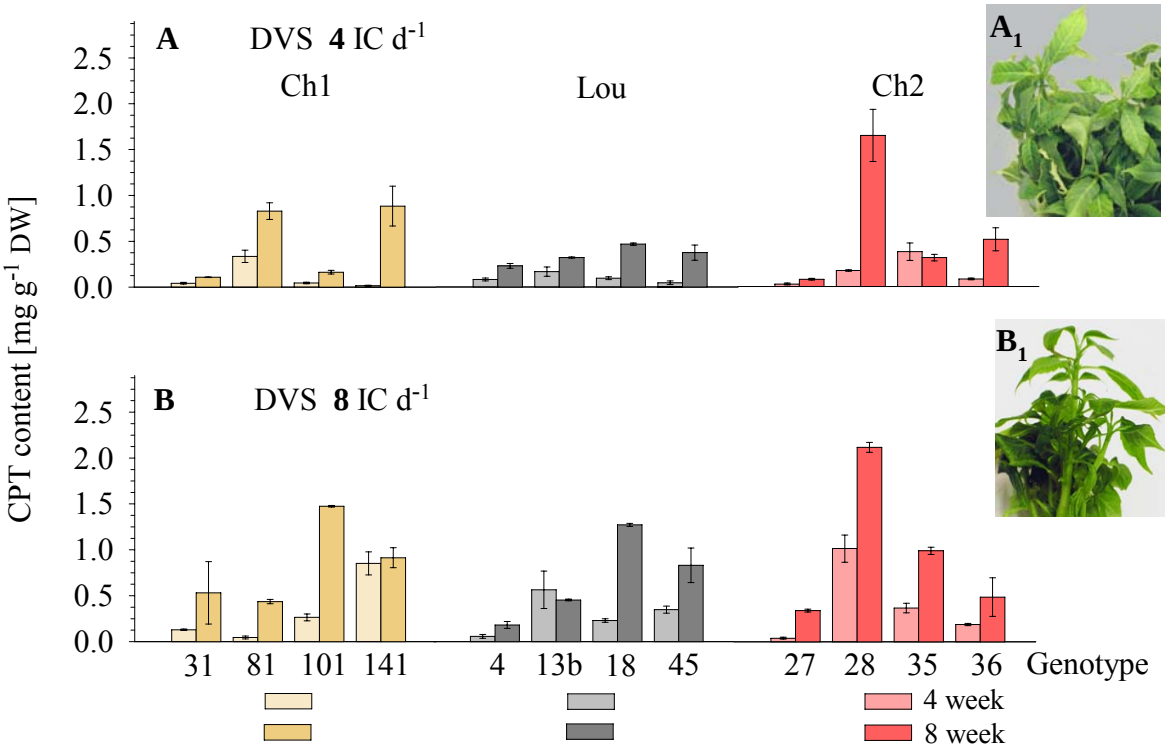

Fig. 60 The effects of temporary immersion cycles on CPT concentrations among the shoots of twelve *C. acuminata* genotypes grown in DVS. **(A)** One minute immersion cycle 4 times d^{-1} and **(B)** 8 immersion cycles d^{-1}. **(A_1, B_1)** Shoots used for CPT analysis. Bars represent means ± SE of three replicates.

The same genotypes treated with 8 IC d^{-1} showed a much better CPT yield except for Bp81, which showed a reduction in CPT content (Fig. 60B). Genotypes Bp101 and Bp141

of group Ch1, Lp13b and Lp45 of group Lou and Mp28 and Mp35 of group Ch2 exhibited substantial increase in CPT after 4 weeks in culture, compared to those treated with only 4 IC d^{-1} after 4 weeks. However, the maximum amount of CPT was found after eight weeks in culture in most genotypes. Among the Ch1 group, Bp101 and 141 showed the highest CPT content with 1.48 and 0.92 mg g^{-1} DW, respectively. In the Lou group Lp18 and Lp45 had substantially higher levels and in the Ch2 group Mp28 once again showed the highest CPT yield of 2.12 mg g^{-1} DW, followed by Mp35 with 0.99 mg g^{-1} DW.

Analysis of CPT contents among the same genotypes cultured in the RITA® vessels (Fig. 61A, B) revealed a different pattern compared to those grown in DVS. Generally, the influence of immersion treatments was similar to that in DVS. Thus shoots treated with 4 IC d^{-1} accumulated less CPT than those treated with 8 IC d^{-1} except Mp 28 and Mp35 (Fig. 61A). It was also noticed that not all of the genotypes treated with 8 IC d^{-1} showed similar high CPT content after 8 weeks in culture as in DVS. There were at least six genotypes in RITA® that showed a substantially higher amount of CPT after 4 weeks at 8 IC d^{-1}. For instance Lp4, Lp18 and Lp45 of the Lou groups showing a CPT level of 1.28, 0.78, 0.66 mg g^{-1} DW, respectively, which was considerably higher than those achieved at 4 immersion cycles (Fig. 61B). Similar results were observed in Mp28, Mp35 and Mp36 of group Ch2 (2.31, 0.91, 0.89 mg g^{-1} DW, respectively). The remaining genotypes exhibited CPT content below 0.5 mg g^{-1} DW. The greatest changes in CPT contents in RITA® were observed in genotypes Bp81, Bp101, Bp141, Lp45 and Mp28 with 1.60, 2.52, 1.18, 0.82 and 2.36 mg g^{-1} DW, respectively after 8 weeks in culture at eight IC d^{-1} (Fig. 61B)

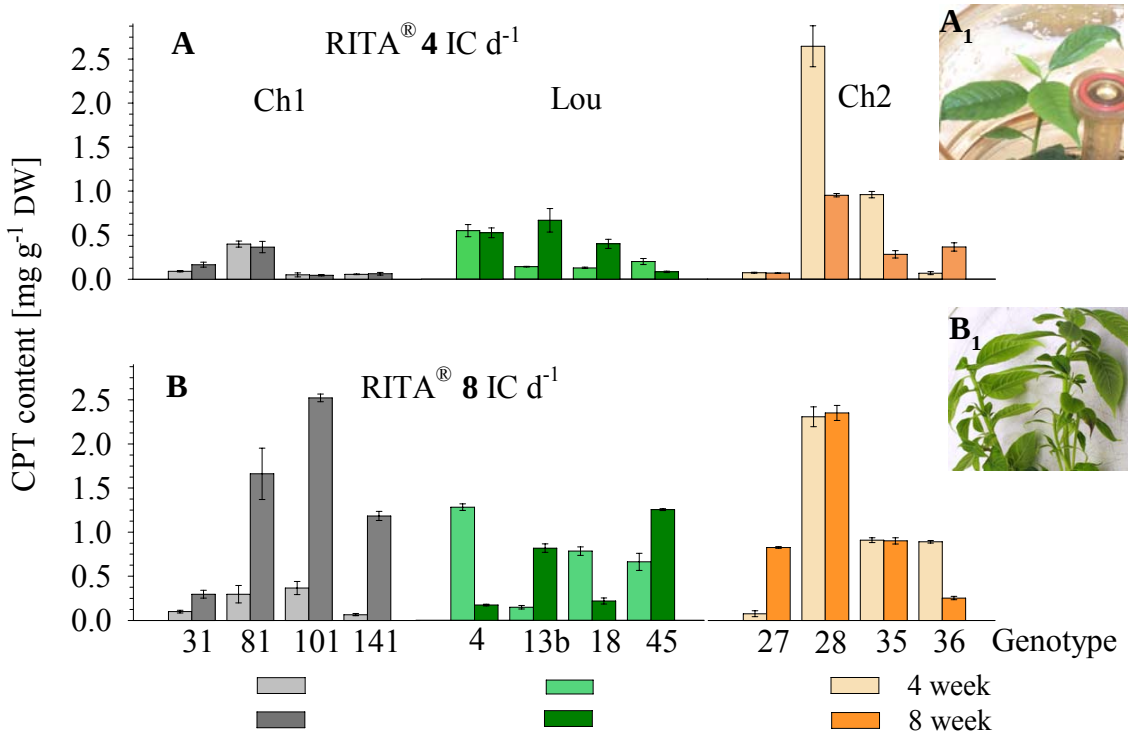

Fig. 61 The effects of temporary immersion cycles on CPT concentrations among the shoots of twelve *C. acuminata* genotypes grown in RITA®. **(A)** One minute immersion cycle 4 times $d^{-1}$and **(B)** 8 immersion cycles d^{-1}. **(A_1, B_1)** Shoots used for CPT analysis. Bars represent means ± SE of three replicates.

4.6.5. CPT Content in Acclimatised Plantlets

Accumulation of CPT was assessed in acclimatised plants of *C. acuminata.* Young leaves of four-month-old plants were harvested and evaluated for their CPT content. The results achieved indicated that CPT contents among the different *ex vitro* genotypes varied significantly, as shown in Figure 62. Genotype Bp31, Lp13b and Mp27 exhibited the highest CPT content (10.1, 9.2, 9.1 mg g^{-1} DW, respectively), followed by Lp4 (7.4 mg g^{-1} DW) and Mp28 (7.5 mg g^{-1} DW) showing similar amounts of CPT. Genotypes Bp81, Lp45 and Mp36 (5.6, 5.5, 3.7 mg g^{-1} DW, respectively) were measured with the lowest CPT concentrations compared to the other genotypes.

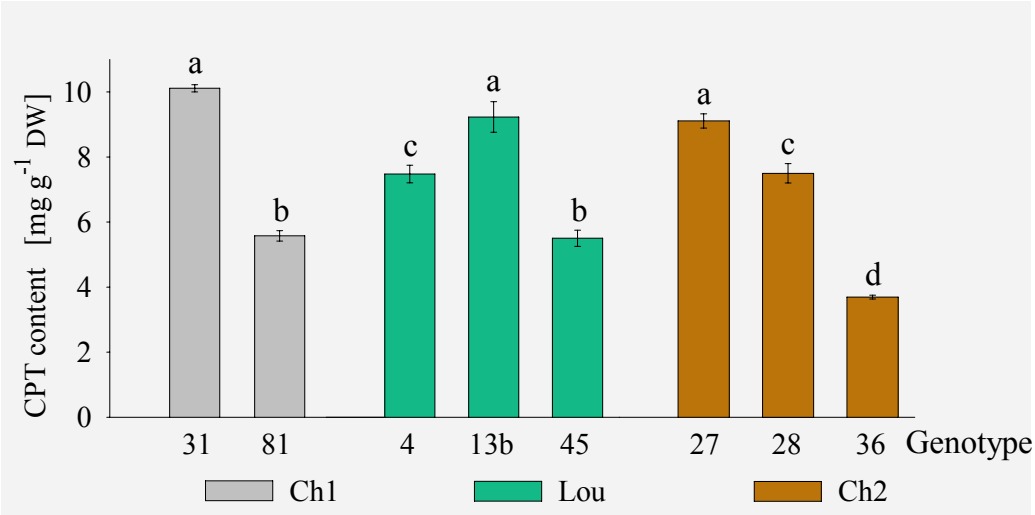

Fig. 62 Variation of CPT contents in young leaves of four-month-old *C. acuminata* plants acclimatised in greenhouse. Mean values ± SE obtained from three independent measurements with (n = 9). Bars with the different letters are significant at $p \leq 0.05$.

4.6.6. CPT Excretion into Liquid Culture Media in DVS and RITA®

HPLC analysis has revealed that intact shoots of *C. acuminata* grown *in vitro* in TIS were capable of excreting CPT into the culture media. At this point it should be noted that no elicitors were applied to enhance CPT excretion and all shoots were rootless. Analysis of CPT secretion into liquid media was carried out on shoots of all twelve individual *C. acuminata* plants. A comparison drawn between the two temporary immersion cycles applied, confirmed that shoots immersed for one minute every three hours (8 IC d^{-1}) secreted higher level of CPT in both the DVS and RITA® vessels compared to those immersed every six hours. The maximum accumulation of CPT secretions was detected after 4 weeks of culture. However, a decline in CPT concentration was observed towards the end of the experiment, even though shoot fresh weight continued to increase. Biomass in RITA® reached an average fresh weight of 34 g at 4 IC d^{-1} and 32 g at 8 IC d^{-1} after eight weeks in culture, while shoots in DVS were scored with 49 g and 38 g at 4 and 4 IC d^{-1}, respectively (Fig 63).

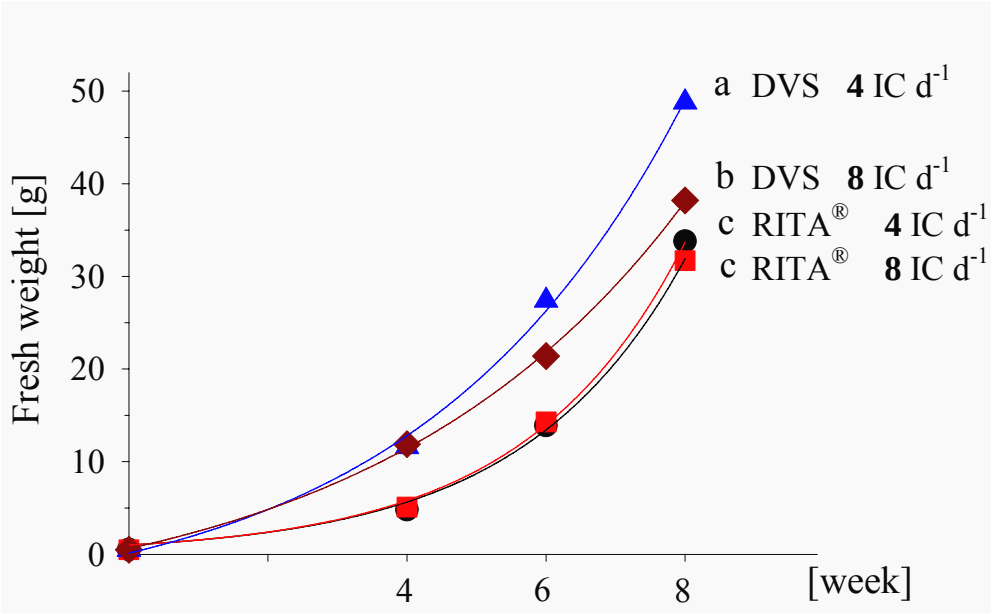

Fig. 63 Fresh weight increment in the TIS vessels, DVS and RITA®. Mean values ± SE obtained for three independent replicates (n = 3-8). Different letters are statistically significant at the 5% level.

The total amount of CPT secreted into the culture medium per gram fresh tissue differed significantly between the two immersion treatments and among the shoots of the different *Camptotheca* plants (Fig. 64). It was obvious that CPT excretions were much higher at 8 IC d^{-1} in both DVS and RITA® than at 4 IC d^{-1}. However, not all genotypes secreted a similar amount of CPT. Treatments with 4 IC d^{-1} in DVS resulted in low CPT secretions ranging from 0.02 to 0.83 µg g^{-1} FW, except in genotype Lp45, which showed a CPT secretion of 3.3 µg g^{-1} FW (Fig. 64A). When shoots were treated with 8 IC d^{-1}, CPT secretions increased significantly among the same genotypes (Fig. 64B). After 4 weeks in culture the highest CPT excretion was recorded with 12.6 µg g^{-1} FW (Fig. 64B) but then a continuous decline followed towards the end of the experiment, showing a minimum CPT content of only 0.2 µg g^{-1} FW. An exception was observed in genotypes Bp31 and Mp28 cultured in DVS, which showed the highest CPT contents after 6 weeks at 8 IC d^{-1}.

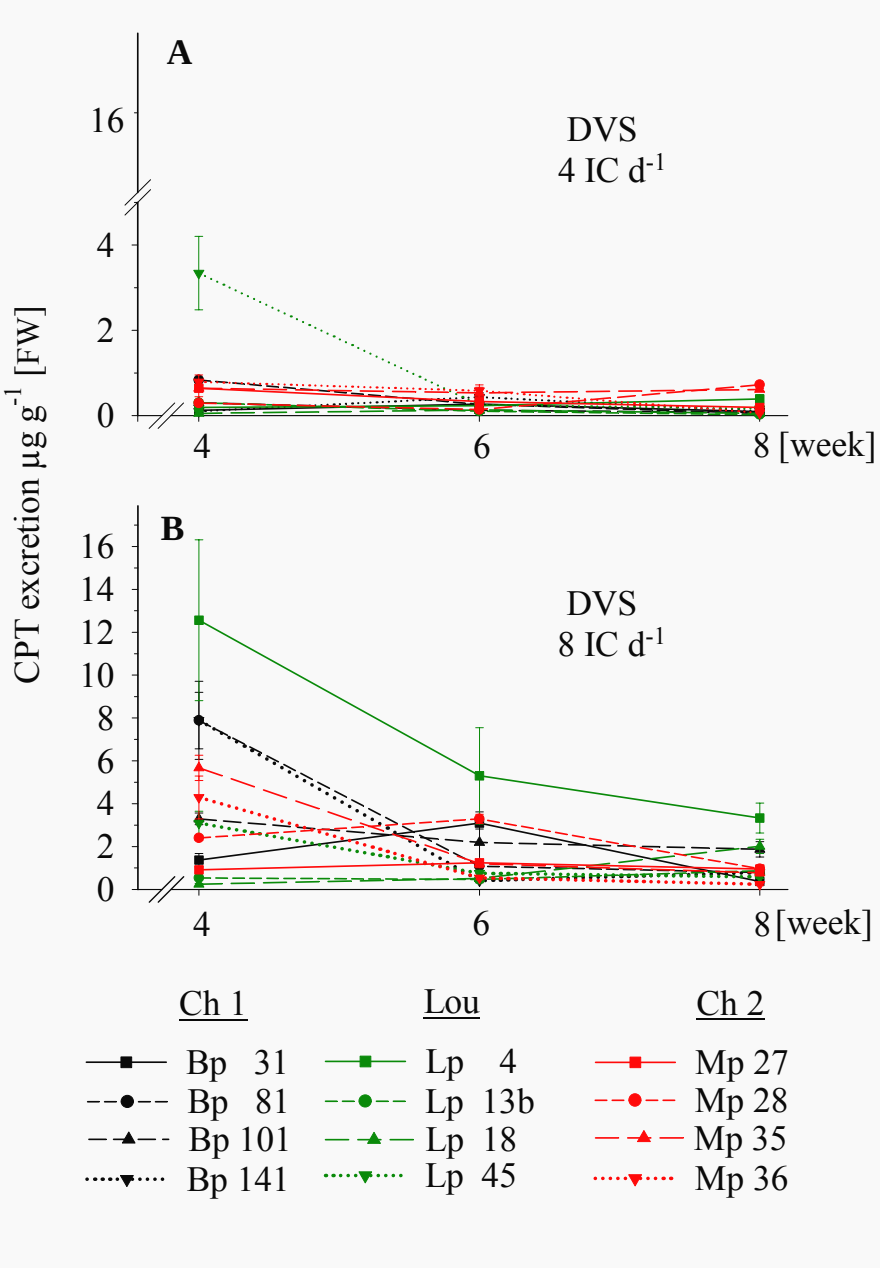

Fig. 64 Effects of immersion cycles on CPT excretion of shoots into the culture media in DVS. **(A)** Shoots of individual genotypes treated with four IC d^{-1} and **(B)** shoots treated with eight IC d^{-1}. Data shown are the average of three replications. Error bars represent ± SE (n = 3-8).

Genotypes cultured in RITA® vessel displayed a similar pattern of CPT secreted into the culture medium. High CPT contents were detected exclusively after 4 weeks in culture at both immersion treatments. However, significant differences were observed on shoots treated with eight IC d^{-1} (Fig. 65A) compared to those treated with only four IC d^{-1} (Fig. 65B). The amount of CPT secreted by shoots treated with four IC d^{-1} reached a maximum after 4 weeks with 3.61 µg g^{-1} FW, which finally decreased to 0.01 µg g^{-1} FW (Fig. 65A).

At the same time shoots treated with eight IC d^{-1} showed a substantially higher CPT level (6.05 µg g^{-1} FW), which also declined towards the end of the experiment to 0.02 µg g^{-1} FW.

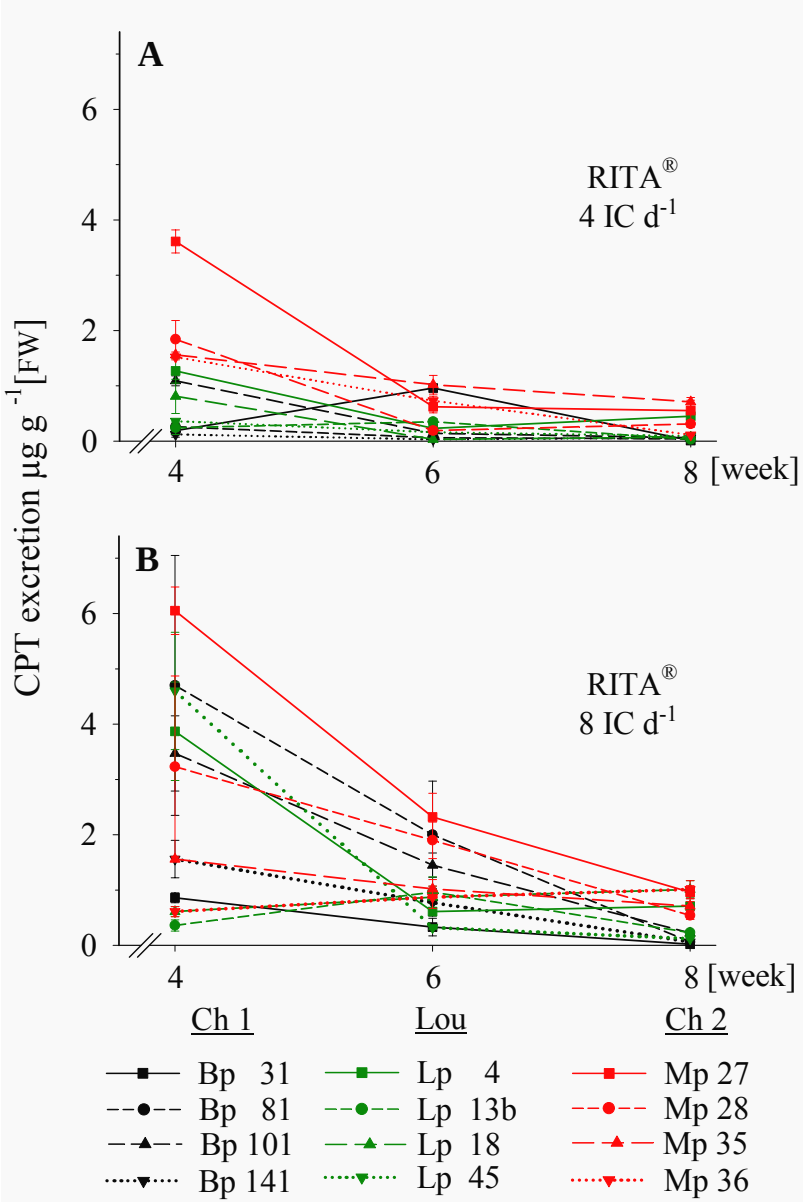

Fig. 65 Effects of the temporary immersion cycles on CPT excretion by the shoots into the culture medium in RITA®. **(A)** Shoots of 12 genotypes treated with four IC d^{-1} and **(B)** those treated with eight IC d^{-1}. Data shown are the average of three replicates. Error bars represent ± SE (n = 3-5).

4.6.7. CPT Content in Callus and Cell Suspension Culture

Calli derived from cuttings of different genotypes (Bn1, Ut1, Bk1, Bl1, and Wn1) obtained from various botanic gardens and private breeders were established in RITA® and as cell suspension cultures in MS medium supplemented with 2 mg l^{-1} BAP + 0.1 mg l^{-1} IAA **(BI)** and 0.2 mg l^{-1} NAA + 0.1 mg l^{-1} 2iP **(NiP)** for eight weeks. Callus growth in the RITA®

vessels were genotype dependent (Fig. 66A) and showed two different growth patterns, regardless of PGR concentrations or combinations. Wn1 and BK1 demonstrated a vigorous growth in all replications, with compact yellowish-green callus of about 2.2 cm in diameter, while the remaining genotypes (Bn1, Ut1 and Bl1) grew very slowly into small, friable, pale green clusters of cells less than 1.1 cm in diameter.

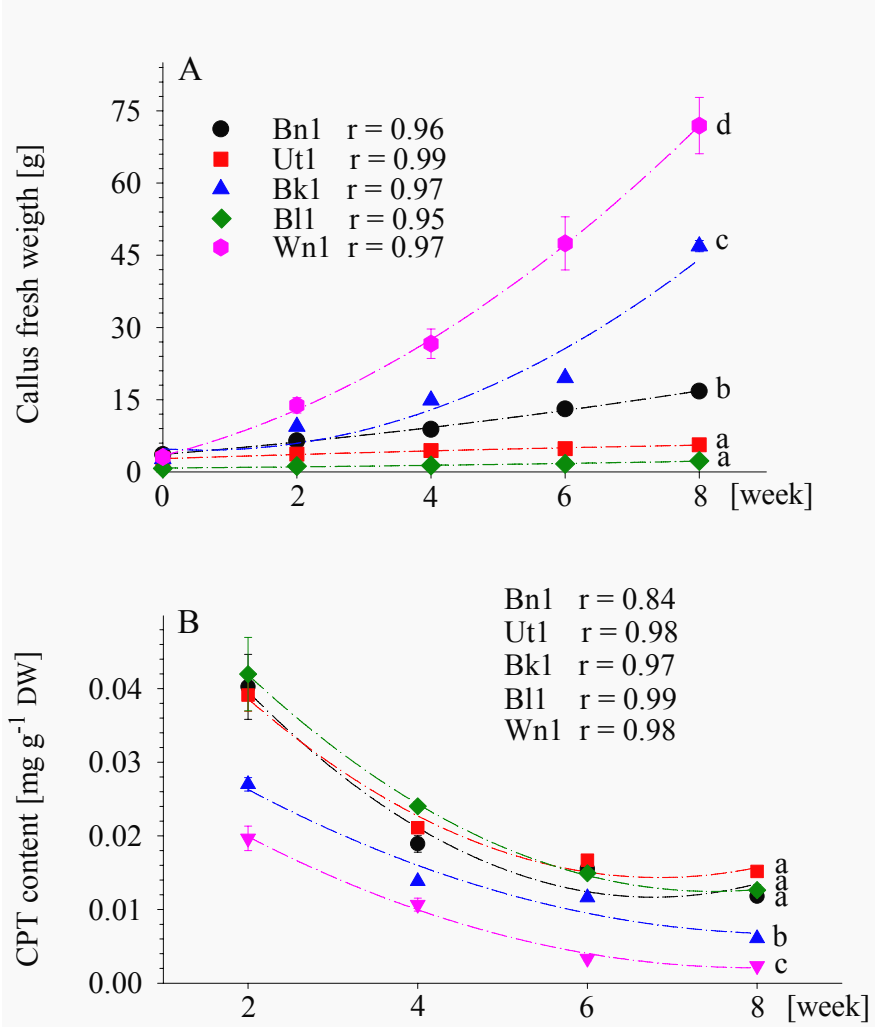

Fig. 66 Callus growth and CPT content in the liquid medium in RITA® of *C. acuminata* genotypes Bn1, Ut1, Bk1, Bl1, and Wn1. **(A)** Increment of callus fresh weight during eight week culture. **(B)** CPT content over a period of eight weeks. Values are expressed as the means ± SE (n = 3-8). Means with different letters are statistically significant at the 5% level.

As shown in Figure 66A, an increase in callus fresh weight resulted in a decrease of CPT content found in the liquid medium (Fig. 66B). The highest CPT content was between 0.020 - 0.042 mg g^{-1} DW after the second week of culture. Towards the end of the experiment CPT contents decreased to 0.002 - 0.015 mg g^{-1} DW in all genotypes. The

lowest CPT content was found in Wn1 and Bk1, which showed a profuse growth (71 and 46 g g^{-1} FW respectively), compared to those with a less vigorous growth but they excreted a higher CPT amount after eight weeks (Fig. 66A). CPT contents were very low in fast growing callus cultures showing reddish, grey and grey-brown colour (Wn1 0.104 ± 0.05, Bk1 0.094 ± 0.07, Bn1 0.116 ± 0.09 mg g^{-1} DW, respectively). In calli of grey-green to green colour the contents were somewhat higher (Ut1 2.45 ± 0.53, Bl1 0.22 ± 0.07 mg g^{-1} DW). Cell suspension cultures were not included in the graph since no CPT content was detected in the liquid media and only traces were found in cells of suspension cultures. HCPT was not detected in any of the callus or cell suspension cultures.

4.7. Plant Selection According to Their CPT Content

Comparative investigations on CPT accumulation in shoots and in liquid culture medium of *C. acuminata* were performed to select plants that could demonstrate a reliable predictive potential for the CPT yield. Genotypes grown in TIS showed considerable CPT variations in both shoots and excretion into liquid media. According to the relatively high CPT amount, five genotypes were selected at the first instance as potential plants (Tab. 9), that could be used for a large-scale CPT production in TIS. However, a second selection showed that genotypes Mp28 and Bp101 are the best CPT producing plants.

Tab. 9 Camptothecin (CPT) accumulation in liquid culture media and shoot cultures grown in DVS and RITA® after a period of 4 weeks with 8 IC d^{-1}. Values are the means of 3-6 replicates ± SE.

TIS vessels	Genotype	CPT content in shoots [mg g^{-1} DW]	CPT secreted into liquid culture medium [mg g^{-1} DW]
DVS	Mp28	2.12 ± 0.05	0.216 ± 0.010
	Bp101	1.48 ± 0.01	0.297 ± 0.025
	Mp35	0.99 ± 0.09	0.754 ± 0.245
	Bp141	0.92 ± 0.11	0.712 ± 0.164
	Lp45	0.84 ± 0.19	0.279 ± 0.051
RITA®	Mp28	2.35 ± 0.09	0.481 ± 0.049
	Bp101	2.25 ± 0.04	0.313 ± 0.062
	Lp45	1.25 ± 0.01	0.415 ± 0.096
	Bp141	1.18 ± 0.05	0.141 ±.0.030
	Mp35	0.90 ± 0.04	0.297 ± 0.052

5.1. Plant Propagation Methods of *C. acuminata*

Conventional regeneration of *C. acuminata* is commonly done by seeds (Perdue and Smith, 1970; Li and Adair, 1994) and large scale propagation for various plantation programmes is mainly achieved by seeds. There is however, a considerable variation in seed germination and very little is known about clonal propagation (Jain and Nessler, 1996). In recent years tissue culture propagation has become a useful alternative for more rapid clonal multiplication compared to conventional methods that are very slow. This study was commenced with the conventional propagation (*ex situ*) through seeds, stem cuttings and continued with *in vitro* plants regenerated from seeds and axillary buds.

Ex situ experiments on seed germination carried out in the greenhouse were not significantly influenced by the seasons. However, it appeared that seeds from China (Sichuan, Jiangsu and Guangdong) germinated readily compared to those from Louisiana. Seeds from Guangdong were obtained in November 2005, from Louisiana in December 2005 and February 2006 and those from Sichuan in May and Jiangsu in June 2006. Since, *C. acuminata* seeds ripen between September and December it is assumed that seeds from Guangdong and Louisiana were possibly too fresh, which may have resulted in a lower or unsuccessful germination rate, respectively. Seeds from Sichuan and Jiangsu obtained later in the year showed a germination rate of 80% compared to those from Guangdong (60%) and Louisiana (0%). Seed dormancy is an extremely widespread phenomenon regulated by many exogenous and endogenous inhibitor substances (Overbeck, 1966), particularly in fresh seeds. Obviously, seed dormancy in *C. acuminata* appears to be also a temperature problem. Shao (1989) reported that germination of fresh *Camptotheca* seeds usually do not occur or is delayed with a low germination rate (5%), when sown at room temperature. In addition, other authors (Smith, 1969; Shao, 1989; Zhou, 1989; Chen *et al.,* 2004) reported that inhibitory substances and the pericarp as a restricted water-uptake-barrier also affect the germination. Fresh *Camptotheca* seeds cultured with pericarp showed only 15% germination after 4 weeks, however, after the removal of the pericarp, germination increased up to 95% (Chen *et al*., 2004). Li and Liu (2005) also reported that higher *in vitro* germination and survival rate were achieved after the removal of the seed coat. Chen *et al.* (2004) reported that seed germination was promoted after a three months cold stratification at 4 °C, increasing the germination rate up to 80% compared to the 15% achieved with fresh seeds. In this study no stratification or pre-treatment was applied to stimulate the germination. Since a high germination rate was observed in seeds from Sichuan and Jiangsu, it is suggested that maturation of the embryo was completed after 5-6

months. However, this assumption does not explain why a germination rate of 60% was observed in seeds from Guangdong, whereas seeds from Louisiana did not germinate, although seeds from both localities were freshly collected.

Seed dormancy is common in a large number of plants, for instance in Rosaceae, in many woody species and in some grasses such as in wild oat (*Avena fatua*), and is caused by several factors, which may delay the whole life cycle of the plants. Regardless of whether seeds are dormant or non-dormant at maturity, the uptake of water plays an important role in the process of germination. Observations in this study on the *in vitro* germination of *C. acuminata* seeds led to a similar assumption that seed coat was impervious to water. Preliminary studies have shown that soaking *Camptotheca* seeds overnight in sterilised water did not improve the process of germination. In addition, surface contamination was very high when seeds were cultured with pericarp. It was possible to minimize surface contamination by using 0.15% mercuric chloride ($HgCl_2$) to disinfect *Camptotheca* seeds but this led to a decrease in germination and as soon as radicles protruded, 100% pericarp contamination occurred. Conversely, excision of the pericarp increased germination significantly and surprisingly, no contamination was observed. Seeds from Louisiana cultured *in vitro* showed high germination with endosperm and even higher when endosperm was removed, whereas under *ex situ* conditions seed germination did not occurred at all. Embryos excised from seeds commenced development after three to seven days without contamination, which shows clearly that the seed coat limits germination. Improvement in germination through removal of the seeds coat has been observed in other woody plants. Jain and Nessler (1996) also report on removing the seed coat before culturing *C. acuminata* in *in vitro*. Barnett (1972, 1976) demonstrated that the seed coat is the major barrier to germination in loblolly pine (*Pinus taeda*).

Rooting of stem cuttings is one of the easiest and economically most valuable methods of vegetative propagation for plants of desired genetic types (Haissig and Davis, 1994; Gaspar *et al*., 1997; Kevers *et al.*, 1997). It is an effective means of multiplying a variety of trees and other woody species (Dirr, 1990; Pierik, 1987) and it is considered to be simple and inexpensive. On the other hand it can be slow, time-consuming and sometimes difficult. However, in most woody plant species an alternative propagation method has not yet been established. In addition, conventional practices of propagation are inadequate for rapid multiplication of selected genotypes (Chalupa, 1990). In most cases *C. acuminata* plantations are established from seeds and sometimes small plantations are raised from cuttings (McDonald, 1997), even though it seems to be a difficult-to-root species. Data

acquired from private growers on conventional propagation of *C. acuminata* recorded a rooting percentage of 5 to 10% and a survival rate of about 2 to 3%. However, once cuttings were rooted, they developed into healthy and normal plants (Bulk, personal communication 2003; Tod, personal communication, 2004). In the present study rooting and survival rate through stem cuttings of *C. acuminata* was below 50%. Maxwell (2003) has reported the opposite, in his thesis he recorded 82% rooting on softwood cuttings of *C. acuminata* after six weeks, when treated with a quick-dip in 29.0 mmol potassium salt of IBA (K-IBA) and mist applied every 6 minutes for 4 seconds. However, Maxwell's (2003) results are not comparable to this study since no K-IBA was used for stem cuttings.

No substantial effect was observed with the application of 0,01% NAA (Wurzelfix®) and 0,05% IAA (Rhizopon®) during the rooting phase in this study, which indicates that the two root stimulator powders used for this experiment were probably too low in concentration or were not appropriate auxins for rooting of *Camptotheca* stem cuttings. Nevertheless, a rooting and survival result of about 38% is far better compared to some private growers, who achieved only 2 to 3%. Hormone application is reported to promote adventitious root development in cuttings (Leakey *et al*., 1982; Hartmann *et al*., 1990; Aminah *et al*., 1995; Tchoundjeu and Leakey, 1996), and IBA is considered to be less toxic and more stable compared to NAA and IAA, which were used in this study for rooting (Blazich, 1988; Hartmann *et al*., 1990). Poor rooting results of cuttings could also be due to the age of the stock plants, as rooting ability of cuttings decreases with age (Hartmann *et al*., 1990). This could be one reason why sprouting was not successful with cuttings obtained from the different botanical gardens and private growers, since all cuttings were taken from *C. acuminata* trees older than ten years. Other possible reasons could be the lack of rooting co-factors, such as endogenous carbohydrates and auxins, or the presence of rooting inhibitors (Hartmann *et al*., 1990). In general, further research on stem cuttings of *C. acuminata* is needed to confirm these assumptions.

Rapid clonal multiplication through axillary buds culture was successfully established in several woody plant species (Ajithkumar and Seeni, 1998; Ahmad and Anis, 2007). However, multiple shoot induction from *C. acuminata* cuttings showed to some extent difficulties similar to the conventional propagation with stem cuttings. Nodal segments taken from cuttings obtained from the different botanical gardens and private growers failed to produce shoots in both *ex situ* and *in vitro* cultures. Callus formation was more obvious on all explants. Callus formation on *C. acuminata* nodal explants was also observed by Maxwell (2003) occurring at high cytokinin concentrations (4.44 to 17.8 µmol l^{-1} BAP).

Since sprouting of cuttings taken from trees older than ten years was not successful in this study, it was speculated that these cuttings were most likely parental or age dependent. Although, Ajithkumar and Seeni (1998) successfully cultured nodal segments from cuttings derived from a 25-year old *Aegle marmelos* tree. Thus, there may be other reasons why shoots were not induced on these *C. acuminata* cuttings. In contrast, nodal segments taken from one-year and two-year old *C. acuminata* greenhouse plants did not show this phenomenon and each node produced one single shoot after 3 weeks. However when excised and cultured on the same medium, shoots proliferated excellently. This result is consistent with those of Jain and Nessler (1996). For higher shoot multiplication the authors pre-soaked freshly excised segments in B5 medium containing 17.74 µmol BAP for 48 hours, which then gave rise to 11-18 shoots per explant. Pre-soaking of explants in cytokinin has been reported to be effective for shoot multiplication in sesame *(Sesamum indicum)* (George *et al*., 1987) and in mulberry (*Morus* sp.*)* (Jain and Datta, 1992). Pre-soaking of explants might help to leach endogenous inhibiting substances that might cause primary dormancy in buds. PGRs were also reported to stimulate shoot proliferation in *Philadelphus* and *Dirca* by placing twigs into BAP solution and a higher percentage of explants produced more shoots after dormant stems of *Vanhoutte's Spirea* were treated with BAP and GA_3 solution before being cultured (Yang and Read, 1993; 1997). Although in this study no pre-treatments were applied on *C. acuminata* nodal segments, an average of 41% produced single shoots. Multiple shoots were successfully achieved on MS medium containing various BAP concentrations. It was obvious that an increase of BAP concentration promoted more shoots, but for quality shoots 0.5 mg l^{-1} BAP was the best. However, further investigations are still needed to understand the detailed mechanism of rooting response and the axillary shoot induction on *Camptotheca* stem cuttings of different age and parental origin.

5.2. Plant Regeneration via Organogenesis

To date there are only few reports on *in vitro* plant regeneration via organogenesis in *C. acuminata* (Li and Liu, 2005; Wang *et al*., 2006) and only one on somatic embryogenesis (Sankar-Thomas *et al*., 2008). Generally, it was found that tissues of juvenile development stage of *C. acuminata* were more appropriate to induce organogenesis or somatic embryogenesis than those from mature tissues. Organogenesis was observed on three different types of callus derived form mature zygotic embryos and from cotyledon and hypocotyl explants of *in vitro* germinated seedlings. Several adventitious shoots were

induced after callus induction on MS medium fortified with various combinations of high cytokinins and low auxins concentrations. The average number of regenerated shoots per callus explant was strongly influenced by the PGR combinations and varied from 10 to 17. Among the PGR combinations used, BI (2 mg l^{-1} BAP and 0.1 mg l^{-1} IAA) appeared to be the best for adventitious shoot induction regardless of the explants or callus types. Comparison between BAP and Kin combined with 0.1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} 2,4-D showed that 0.5 mg l^{-1} 2,4-D decreased the number of regenerative shoots.

Adventitious buds have successfully been established using explants from young *in vitro* grown seedlings and from newly sprouted leaves of greenhouse plants of *C. acuminata* (Li and Liu, 2005; Wang *et al*., 2006). Li and Liu (2005) found that Gamborg's B5 medium supplemented solely with 8.9 µmol BAP (2 mg l^{-1}BAP) gave the best regeneration results, whereas Wang *et al*., (2006) reported that woody plant medium (WPM) in the presence of 19.8 µmol BAP (4.5 mg l^{-1} BAP) and 5.8 µmol NAA (1.1 mg l^{-1} NAA) led to the highest shoot regeneration frequency.

Preliminary experiments in this study have shown that explants cultured in MS medium developed much better than those cultured on Gamborg's B5 and WPM. Regeneration of adventitious shoots was observed neither on B5 nor on WPM medium, hence further studies were carried out using only MS medium. It was also obvious that cytokinin (BAP or Kin) concentrations higher than 2 mg l^{-1} induced profuse callus formation but without shoot induction. The combination of low 2iP and NAA (NiP) concentrations induced a low rate of callus formation without shoot induction.

Apical tips of mature zygotic embryos appeared to be the best sort of explants for shoot regeneration in *C. acuminata*. Adventitious buds were also observed on explants cultured in liquid medium containing the same PGR composition as those in solid medium. However, adventitious buds occurred only on disc shaped hypocotyl explants cultured on BI medium. Apart from callus formation cotyledon explants did not show any further differentiation. These results suggested that the presence of BAP combined with IAA was more effective for adventitious shoot induction than the other PGR combinations used. Hyperhydricity did not occur on adventitious shoots in solid medium as reported by Li and Liu (2005). Regenerated plantlets were normal without any significant visible variability. Thus, it is possible to produce clones of *C. acuminata* for medicinal purposes.

5.3. Plant Regeneration via Somatic Embryogenesis in TIS and in Sterilised Substrates

Induction of somatic embryogenesis is a complex phenomenon, which is regulated by numerous factors. In most cases, treatments with exogenous PGR are required to manipulate cell differentiation (Carman, 1990; Sen *et al.,* 2002). In addition to exogenous PGR, high amounts of carbohydrates (4 to 7% or higher) in media are used as an osmoticum to stimulate induction of somatic embryogenesis (Kamada *et al*., 1993, 1994; Shrikhande *et al*., 1993; Ikeda-Iwai *et al*., 2003). However, in this study somatic embryogenesis was obtained from hypocotyl explants of *C. acuminata* seedlings in TIS in PGR free MS-medium containing only 3.5% sucrose in both the DVS and the RITA® vessels. Since in this case neither exogenous PGR, nor high concentrations of sucrose were used, it is difficult to clarify what mechanisms were involved in the induction of somatic embryogenesis. Based on the results presented in this study two effects may have played an important role during the induction process. Firstly, due to the fact that embryogenic calli were formed only in medium containing 3.5% sucrose in both TIS vessels, it is suggested that perhaps 3.5% sucrose is the critical value for somatic embryogenesis induction in *C. acuminata*. In species such as *Solanum melongena* and *Medicago sativa* 2 to 3% sucrose was enough to achieve somatic embryos (Meijer and Brown, 1987). Conversely, high levels of sucrose (> 6%) were reported to inhibit the induction of somatic embryos (Konan *et al*., 1994). Cunha and Fernandes-Ferreira (1999) reported that a low concentration of 2% sucrose was more effective for the induction of somatic embryos than 4%. Thus the above results indicate that the optimum sucrose concentration is probably species specific and perhaps the given 3.5% of sucrose was just the threshold for somatic embryogenesis induction in *C. acuminata.* It may be concluded that raising the sucrose level may enhance or suppress the harvest.

Secondly, since embryogenic calli occurred only in the modified DVS containing medium supplemented with 3.5% sucrose, it is obvious that the modification as well as the sucrose concentration had some effect on the induction. Explants fixation and orientation play a critical role in the induction of somatic embryogenesis in several species (Chen *et al*., 1987; Lakshmanan *et al*., 2006). Thus perhaps the vessels modification participates in the orientation of the explants, which results in the development of embryogenesis. This assumption is not yet proven, therefore further investigations are necessary to analyse whether there is a relation between the sucrose concentration and/or the mechanical device.

A comparison between *C. acuminata* embryos regenerated in DVS and RITA® demonstrated that culture vessels have a great influence on plant development. In this study a higher percentage of well-developed plantlets were achieved in RITA® than in DVS. Similar results were reported on the development of somatic embryos in RITA® with different plant species such as *Musa* sp. (Alvard *et al*., 1993), *Hevea brasiliensis* (Etienne *et al*., 1997), *Citrus deliciosa* (Cabasson *et al*., 1997) and C*offea arabica* (Etienne-Barry *et al*., 1999). Cabasson *et at.,* (1997) asserted that the temporary immersion in RITA® promotes development and conversion of somatic embryos. This is probably due to the design of the vessel. Thus, the upper chamber is overlaid with open-pores polyurethane foam in which embryos are able to support their geotropism easier than in DVS without such adhering possibility. During immersion and emersion embryos in RITA® were able to maintain their orientation, while those in DVS have no orientation until they reached a certain height and biomass. This could be one reason why 27% of the *Camptotheca* regenerants in RITA® developed roots without any additional exogenous PGR. Hence, the results in this experiment provide clear evidence that the RITA® vessels are more suitable and effective for embryo-to-plantlet development. This vessel has been developed for culturing embryogenic cells and embryos (Afreen *et al*., 2002). Comparing the two vessel types, DVS is easier to handle especially in terms of medium exchange, which is simply done by disconnecting the medium vessel without exposing the culture to the environment outside the vessel like in RITA®. This exposure may increase the risk of contamination.

The consideration of the substrates experiment was not to compare the conversion rate of cotyledonary embryos in the two culture systems (TIS and in sterilised substrates). It was more an approach to compare the survival rate of regenerated plantlets derived from TIS with those derived from sterilised substrates upon exposure to greenhouse conditions. A similar experiment in sterilised substrates was conducted by Jayasankar *et al*., (2001) who achieved a high conversion rate of grapevine via somatic embryos on sand and soil overlaid with sand. However, regenerants mortality was high due to contamination, inadequate moistening and perhaps lack of nutrients because only sterilised tap water was used to moisten the substrates. In contrast to seeds, somatic embryos have to develop without seed coat and maternal tissue that normally supplies nutrition during germination (Gray and Purohit, 1991). Thus, the high survival rate obtained in this study is probably due to extensive autoclaving of substrates and because sterilised MS medium supplemented with 0.5 mg l^{-1} BAP was used to moisten the substrates during the regeneration phase, which facilitated the maturation of cotyledonary embryos. After eight

weeks a shift from full strength to half strength MS medium without sucrose was made, to allow regenerants to become partially autotrophic, which reduces stress and allows plantlets to adapt better to *ex vitro* conditions (Purohit *et al*., 1995).

5.4. Shoot Multiplication in Solid Media and in TIS

The main goal of the current study was to establish an efficient and economic protocol for the *in vitro* multiplication of *C. acuminata* in TIS, which is also applicable for a large scale production of plant material. Until now, limited reports are available on *in vitro* propagation of *C. acuminata* in solid medium (Jain and Nessler, 1996; Wiedenfeld *et al*., 1997; Wang *et al*., 2005, 2007) and to date this is the first report on shoot multiplication of *C. acuminata* using TIS. In this study shoots derived from *in vitro* grown seedlings and axillary buds from greenhouse plants were successfully multiplied on both solid medium and in TIS. However, compared to solidified medium, shoot number and length increased significantly in TIS. Within eight weeks a 2.9-fold difference in shoot proliferation was observed with the same medium (0.5 mg l^{-1} BAP) without affecting the morphological characteristics in TIS. Etienne and Berthouly (2002) reported that TIS generally improves plant quality and production. This is probably due to the fact that explants cultured in TIS are preferential with the availability of nutrients in the liquid. A frequency of four or eight immersions per day for one minute was suitable to stimulate shoot development and growth. Another advantage is that after immersion, enough liquid nutrient remains on the plant surface as a film, which also prevents desiccation during the non-immersed period. In contrast, propagation in solid medium also allows gas exchange, but nutrient uptake is limited to the explants basal surface, which may result in less shoot development and a slower growth rate. There are numerous studies showing satisfactory results for the propagation of various plant species (*Eucalyptus* sp., *Saccharum* sp., *Phalaenopsis*, *Manihot*, *Prunus*, *Malus, Ananas comosus*, *Musa* sp.) using TIS (Escalona *et al.*, 1999; González-Olmedo *et al*., 2005; Damiano *et al*., 2003; Roels *et al*., 2005). The best quality of *C. acuminata* shoots was achieved when MS medium was supplemented with 0.5 mg l^{-1} BAP. However, immersion time is very important, since it determines nutrient and PGR uptake and can influence hyperhydricity of explants (Etienne and Berthouly, 2002). In addition, high cytokinins concentrations can induce hyperhydricity. The use of 1.0 and 1.5 mg l^{-1} BAP has shown a higher shoot multiplication rate, but at the same time an increased callus formation and hyperhydricity in both TIS vessels. This phenomenon was not observed when shoots were cultured in medium supplemented with 0.5 mg l^{-1} BAP. A

similar result was obtained by Zhu *et al*., (2005) in *Malus*. The authors reported that high BAP concentration results in a higher multiplication rate, but were also accompanied by hyperhydricity. In this study shoots treated with 8 IC d^{-1} in DVS showed traits of hyperhydricity and reduced shoot proliferation.

Other types of cytokinins such as zeatin have also caused hyperhydricity (Shibli and Smith, 1996). Obviously, *C. acuminata* shoots were sensitive to BAP only in TIS cultures, because a BAP concentration of 2 mg l^{-1} BAP was necessary for bud regeneration via organogenesis and no hyperhydricity was observed on newly induced shoots. Apart from a few morphological traits such as leaf size and shoot height, BAP concentrations of 1.0 to 1.5 mg l^{-1} did not influence the shoot multiplication rate in solid medium. However the same BAP concentrations showed considerable differences in both morphological appearance as well as shoot multiplication in TIS. Within eight weeks a maximum shoot multiplication rate of 20 in DVS and 15 fold in RITA® was achieved at four IC d^{-1}. Whereas at eight IC d^{-1} the multiplication rate was 13 in DVS and 15 fold in RITA®. Hence, the results presented in this study have demonstrated that *C. acuminata* shoots cultured in 0.5 mg l^{-1} BAP gave the best shoot multiplication rate in TIS and can be recommended for the *in vitro* propagation. The efficiency of shoot proliferation can be improved by optimising nutrient volume, immersion frequencies and duration as well as the vessel capacity.

TIS has been recognised as an efficient tool for plant propagation and reduces production cost per plant compared to culture in solidified media (Lorenzo *et al*., 1998; Escalona *et al.,* 1999). Certainly, it also reduces manual labour and facilitates the changing of culture medium compared to solid media culture, which has been one of the most time and labour intensive procedures in plant tissue culture not only in this study. However, comparing the two vessel types, DVS was more appropriate and simpler to handle than RITA® especially during media exchange.

5.5. Rooting and Acclimatisation in the Greenhouse

5.5.1. *In vitro* Rooting in TIS, in Solid Medium and Sterilised Substrates

Results on stem rooting have shown that *C. acuminata* is difficult to propagate by conventional methods, making the *in vitro* propagation a feasible alternative for multiplication. However, the success of micropropagation systems depends chiefly on a high rooting percentage and the acclimatisation of plantlets to greenhouse conditions, which is one of the most critical phases in the entire micropropagation process (Hutchinson

et al., 1985; Zimmerman, 1988; Kirdmanee *et al*., 1995). This is because *in vitro* grown plantlets are forced to change from being heterotrophic to autotrophic and in most cases mortality of micropropagated plantlets is high under *ex vitro* conditions. The main reason is that *in vitro* grown plants typically show a low photosynthetic efficiency due to malfunctioning of stomata and the lack of epicuticular wax, so that plants are susceptible to dehydration (Preece and Sutter, 1991; Hazarika, 2006). Apart from poor photosynthetic ability rooting is also an important factor and as in most tree species rooting is difficult and capacity is generally reduced in woody species compared to herbaceous plants (Hackett, 1988). Several plant species differ in their requirement of auxins and media strength for root formation (Kooi *et al*., 1999). The first *in vitro* rooting experiment in this study was carried out on regenerated plantlets via somatic embryogenesis from TIS and sterilised substrates using a half-strength MS medium supplemented with 0.5 mg l^{-1} IBA. Rooting percentage of regenerants in sterilised substrates moistened with medium containing 0.5 mg l^{-1} IBA without sucrose was higher than those in TIS. Thus, since the rooting percentage of regenerants in TIS was about 52% it was suggested to root microcuttings in full-strength MS medium fortified with two different auxins. Remarkably was that rooting of microcuttings in a full-strength MS medium with IBA and NAA exhibited in all systems a higher root induction rate than regenerants. In contrast to this result, several reports conclude that most woody plants particularly gymnosperms prefer a low salt concentration for root formation (Anonymous, 1978; George and Sherrington, 1984; Kamil and Umboh, 1992; Bhansali, 1993).

Wang *et al*., (2007) reported that MS medium inhibited root formation in *C. acuminata.* This is not in consistent with the results in this study, in which a high rooting percentage was achieved in both liquid and solid full-strength MS medium. *In vitro* rooting of *C. acuminata* in liquid medium in DVS and RITA® using IBA is in agreement with the results of Pierik (1987), who reported that adventitious rooting of woody plants was generally better in liquid media. Nevertheless, further investigations are needed to prove whether the increased rooting rate here is due to salt strength or is explant dependent. Meanwhile, the findings reported in this study show that rooting percentage exceeded 80% with IBA and 60% with NAA, except for those in soil. The rooting capacity of microcuttings in DVS showed large variations between IBA and NAA. In RITA® and in the other systems there were no substantial differences between IBA and NAA. Differences in the morphology, length and number of developed roots were also observed in both media and substrates. Shoots rooted with NAA produced fewer roots per shoot and varied in length especially in

TIS. Root numbers and length were similar among shoots rooted in sterilised substrates. Comparing the two auxins, IBA performed a better rooting induction than NAA in *C. acuminata* microcuttings. A similar observation was reported by Wang *et al*., (2007). The authors reported that 0.5 mgl^{-1} IBA was more enhancing than NAA. There are several reports concerning the rooting effect of IBA and NAA, but in most cases IBA has been reported as a stronger auxin than NAA in promoting rooting regardless of its concentration (Lê, 1985; Németh, 1986; Gray and Benton, 1991). The results in this study demonstrate that IBA and NAA have a significant effect on *in vitro* rooting in TIS, but show no effect on microcuttings in solid media and in substrates.

It was obvious that microcuttings rooted faster in RITA® than in DVS, which is possibly due to the vessel design. During immersion explants in RITA® do not undergo a vigorous turbulence compared to those in DVS, thus explants can retain their geotropic orientation due to the design of the upper compartment of the RITA® vessel.

5.5.2. *Ex vitro* Rooting of Microcuttings on Non-sterile Substrates

Ex vitro rooting of microcuttings derived from solid media and TIS showed a great aberrancy. Regenerated shoots derived from solid media showed a better root growth and survival rate compared to those derived from DVS or RITA®. A comparison between *in vitro* and *ex vitro* rooting showed that ex *vitro* rooting of *C. acumianta* was not as successful as the *in vitro* rooting. The first assumption is that the low auxin concentration used for rooting was not high enough to stimulate root induction. Secondly, many microcuttings developed callus-like areas at the cut end, which is a disadvantage since it may limit water transport (Grout and Aston, 1977) and may result in a low rooting induction. McClelland *et al*., (1990) assumed that since nutrients, water and oxygen are readily available *in vitro*, it might not be necessary for microcuttings to develop secondary xylem and phloem for transportation, which would be a disadvantage during transplantation and acclimatisation under greenhouse conditions.

In spite of the low auxin concentration, 46% of the microcuttings induced roots, this was still higher than the 38% that was achieved in conventional stem propagation of *C. acuminata.* Plietzsch and Jesch (1998) have shown that shoots derived from micropropagated stock plants rooted far better than cuttings derived from conventional stock plants. For instance rooting of *Syringa vulgaris* showed only 10% from cuttings taken from conventional stock plants, while in those taken form *in vitro* grown stock was up to 85%. The difference between 38 and 46% achieved in this study may not be

particular large but perhaps if a higher IBA concentration is used a higher rooting rate may be achieved. It is likely that *ex vitro* rooting was less satisfactory because of the non-sterile substrate used here. Zhao *et al*., (2006) report a high rooting efficiency using arbuscular mycorrhiza. Thus perhaps mycorrhizal inoculation may help plantlets to resist environmental stress during transplanting from axenic conditions to greenhouse conditions, and this may possibly increase root induction. Results on *ex vitro* rooting and plantlet establishment of *C. acuminata* in the greenhouse are not yet encouraging. However, as soon as roots are developed, all survived *ex vitro* plantlets showed a vigorous growth and were phenotyically normal.

5.5.3 Acclimatisation under Greenhouse Conditions

Acclimatisation of rooted regenerants and microcuttings of *C. acuminata* derived from TIS was crucial. Rooted regenerants showed very low survival rates when transferred to non-sterile soil under greenhouse conditions, whereas rooted microcuttings revealed a higher survival rate of about 40% and no substantial difference between cuttings derived from TIS or solid medium was found. Regenerants were not misted regularly during acclimatisation and this may be one reason why only few plantlets survived. Wang *et al*., (2007) report that rooted microcuttings of *C. acuminata* need high humidity and shade to survive under greenhouse conditions.

Regenerated plantlets in DVS showed the lowest chlorophyll content compared to those cultured in RITA®, in solid medium and substrates. Stomata density on leaves was also the lowest in DVS but continuously increased from RITA®, solid medium to sterilised substrates. The majority of shoots in DVS possess stomata with rounded guard cells, while stomata with elliptical structure dominated on shoots in RITA® and in the other culture systems. Low Chlorophyll content and abnormal shape of stomata indicated poor photosynthetic ability due to heterotrophic mode of nutrition. Thus, these factors hamper plantlets when transferred to greenhouse conditions causing a low survival rate. Regenerated plantlets via somatic embryogenesis and mircrocuttings rooted in sterilised substrates were easily acclimatised, showing a survival rate of 93% compared to those from TIS and solid media with 40%. Drawing a conclusion between the *in vitro* rooting in the different systems, it was obvious that rooted plantlets derived from the various substrates showed higher survival rates under *ex vitro* conditions than those from TIS or solid media. *In vitro* rooting of shoots in solid media is considered to be labour-intensive and expensive (Hazarika, 2003 compared to TIS where rooting is much easier, and cheaper

since agar is not necessary. Rooting in TIS is certainly less time-consuming and plantlets are simply rinsed with sterilised water before transferred to *ex vitro* conditions. Therefore, improvement and optimising measurements are needed to obtain a higher rate of acclimatised *C. acuminata* plantlets from TIS.

5.6. Camptothecin (CPT) distribution in Plant Tissues, Organs and in Liquid Media

5.6.1. *Ex situ* and *In vitro* Seedlings

Several studies have shown that CPT is derived from the shikimate and mevalonate pathways (Sheriha and Rapoport, 1976; Dewick, 1997; Lorence and Nessler, 2004; O'Conner and Maresh, 2005; Zhang *et al*., 2007) and that it is a derivative of strictosidine. Whereas, strictosidine results from the condensation of the amino acid derivative tryptamine and secologanin (Kutchan, 1995; Hutchinson *et al*., 1979; Dewick, 1997). Until now it is not fully understood why CPT and its derivatives, such as HCPT and MCPT are formed or converted, nor why it is needed in the biological system (Zhang *et al*., 2007). Research data have shown that the alkaloid CPT is accumulated in *C. acuminata* and in several other CPT producing plants such as *Ophiorrhiza pumila* and *Nothapodytes foetida.*

CPT accumulation in undifferentiated and differentiated plant tissues of *C. acuminata*, grown in different culture systems, has been examined and compared here. In the last decade a number of reports on CPT production in *in vitro* grown plant tissues have been published (Van-Hengel *et al.,* 1992, 1994; Sakato and Misawa, 1974; Sudo *et al*., 1991; Roja and Heble, 1994; Wiedenfeld *et al.*, 1997; Ciddi and Shuler, 2000). However, to date this is the first study reporting on CPT content in cells and plant tissues grown in TIS.

Alkaloid analyses performed on seedlings grown *ex situ* in greenhouse and on those of *in vitro* origin revealed that CPT concentrations varied within the entire plantlet. HCPT was only found in seedling hypocotyls. It was evident that CPT accumulation was higher in the hypocotyls, primary leaves and apical tips than in roots or cotyledons, in both *ex situ* and *in vitro* seedlings. Root and hypocotyl CPT concentrations in *ex situ* seedlings in this study are consistent with the findings of Valletta *et al*., (2007). The authors reported that CPT concentration in roots decreased as seedlings aged, while CPT content in hypocotyls increased. The highest CPT content in roots was found in the early development of seedlings, which was 4 to 8 days after germination. In contrast they found that the CPT concentration in hypocotyls was very low during the first 12 days, but then increased towards day 20. Observations have shown that CPT accumulation increased from cotyledons, primary leaves to the apical tips. Previous studies from other authors (Li *et al*.,

2002; Lopez-Meyer *et al*., 1994) have also shown that CPT content was higher in young leaves compared to other organs of mature plants. A similar CPT distribution was found in this study for *in vitro* grown seedlings. There are limited studies on CPT accumulation on *in vitro* grown seedlings, therefore, it is difficult to compare the CPT content in different organs. Yuan *et al.,* (2008) found that CPT levels in roots of three-month old seedlings were higher in liquid (7.47 µg g^{-1} FW) than in solid medium (6.96 µg g^{-1} FW). In this study higher concentrations were detected ranging from 23 to 30 µg g^{-1} FW. Hairy root cultures of *Ophiorrhiza pumila*, *O. mungos* and *C. acuminata* showed CPT levels of 0.2, 1.0 mg g^{-1} DW and 80 µg g^{-1} FW, respectively (Saito *et al*., 2001; Yamazaki *et al*., 2003; Lorence *et al*., 2004; Watase *et al*., 2004). Moreover their data are not really comparable with the data of this study, since no *Agrobacterium rhizogenes* was applied.

No HCPT was found in seedlings, roots or leaves and HPCT concentrations in hypocotyls were higher in *ex situ* seedlings compared to those of *in vitro* origin. Lopez-Meyer *et al*., (1994) also reported that HCPT is not always found together with CPT and is the highest in bark of *C. acuminata* plants. Thus results of this study agree with those of Lopez-Meyer *et al*., (1994) and Zhang *et al*., (2007) that HCPT is probably tissue specific for field or greenhouse grown plants.

It is known that many alkaloids, such as scopolamine, hyoscyamine and nicotine are synthesised in roots (Pasqua *et al*., 2004). However, since CPT concentration was very low in roots, it is suspected that the roots of *C. acuminata* are or at least not exclusively the source of CPT production. On the other hand this would support the hypothesis that roots of *C. acuminata* were the source of CPT and a CPT decrease in mature organs could be the result of CPT degradation or a translocation to sink organs such as young leaves, secretory canals and glandular trichomes (Liu, 2004). Alkaloids translocation has been observed in other plant species. In Solanaceae, nicotine and tropane alkaloids are synthesized in the roots and then transported via the xylem to the leaves the main accumulation site (Pasqua *et al*., 2004). In *Catharanthus roseus* catharanthine and tabersonine are produced in the roots and then translocated to leaves where it is converted to vindoline to form vinblastine and vincristine (Zu *et al*., 2003; Pasqua *et al*., 2004). At a cellular level CPT has been localised in the mesophyll, sub-palisade layers and subcellular in the vacuoles of young and older leaves (Nolte, 1999). However, the mechanism of transport and storage of CPT remains unclear.

5.6.2. Different Stages of Somatic Embryos

Despite several studies examining the CPT concentration in bark, leaves and roots of *C. acuminata*, to date there are very few reports available on CPT production in *in vitro* grown plant organs. Comparative studies on CPT of various tissues and organs have indicated that CPT is accumulated in all parts of *C. acuminata*. Analyses of the different developmental stages of somatic embryos and regenerated plantlets revealed the presence of CPT. Non-embryogenic and embryogenic callus showed traces and low level of CPT respectively, but as embryo development progresses, CPT content increases. Cotyledonary embryos and regenerated plantlets showed the highest CPT content compared to embryos of heart and torpedo stage. Traces or low levels of CPT in undifferentiated callus tissues lead to the suggestion that CPT biosynthesis of *C. acuminata* requires, to some extent, tissues or organs of differentiated stages.

Similar results were reported on somatic embryos of another CPT producing plant, *Nothapodytes foetida* (Fulzele and Satdive, 2003). Zygotic embryos of *C. acuminata* showed a CPT content of 1.0 mg g^{-1} DW (Valletta *et al*., 2007). However, they assumed that CPT accumulation occurred before the fruit is detached from the parental plant. In conclusion, this study has demonstrated that somatic embryos of C. *acuminata* can produce a considerable amount of CPT under standard culture conditions without the influence of elicitors.

5.6.3. CPT Content in Shoots Grown in Solid Media, TIS and in Acclimatised Plants

Until now, there are only a few studies concerning the *in vitro* culture of *C. acuminata* for possible CPT production. (Sudo *et al*., 1991; Roja *et al*., 1994; Wiedenfeld *et al*., 1997). In most cases CPT contents found in regenerated shoots or plantlets were very low ranging from 0.86 to 1.99 mg g^{-1} DW. Shoots and plantlets from other CPT producing plants such as *Nothapodytes foetida* showed low level or only traces of CPT (Roja *et al*., 1994; Fulzele and Satdive, 2003). The results presented in this study confirmed that all twelve *C. acuminata* genotypes cultured in TIS and in solid medium contained CPT. However, concentrations varied extremely among the different genotypes. It was evident that most plants originating from Jiangsu (Ch2) showed higher CPT contents in both culture systems. Similar results were observed among the *ex vitro* and *in vitro* seedlings from Jiangsu. It was suggested that differences in CPT content are perhaps genotype dependent or may be an attribute to the geographical origin of plants. Wiedenfeld *et al.,* (1997) and Park *et al*., (2003) concluded that variation in CPT contents might be genotype specific. This would

explain why genotypes of group Ch2 produced immensely more CPT (2.5 mg g^{-1} DW in shoots) than those from the groups Ch1 and Lou. Another possible explanation could be that shoots of the different genotypes were not of the same biological age (stock plants of Ch2 were 12 months, those of Ch1 and Lou 18 months old). Since plants of group Ch2 contained a significantly higher CPT concentration, it would be desirable to use these genotypes for CPT production.

To date this is also the first study to examine the growth and CPT content in *C. acuminata* shoots grown in TIS. Apparently some genotypes have the capability to produce high levels of CPT while others produce a moderate or very low amount. At the end of the growing period, plants grown in TIS treated with 8 IC d^{-1} had significantly higher CPT concentrations in both the DVS and RITA® vessels than plants treated with only 4 IC d^{-1}. Few genotypes also showed an increase in CPT accumulation after four weeks in culture. However, in general CPT accumulation was higher after eight weeks in culture submerged 8 times d^{-1}. Therefore, a more frequent immersions may be associated with a CPT increment.

Concentrations of secondary compounds in plant tissues often increase in response to stress (Mooney *et al*., 1991) or when nutrient availability are replenished or exhausted so that growth is limited (Barrios-Gonzalez *et al*., 2005). It has also been reported that plant secondary metabolites are produced at higher concentrations when plant growth is slow or in non-growing cultures (Addullah *et al*., 1998; Komaraiah *et al*., 2005). Shoot proliferation at 8 IC d^{-1} was less than at 4 IC d^{-1}, thus this could be an explanation why CPT content was higer after eight weeks in shoots treated with 8 IC d^{-1} than those treated with 4 IC d^{-1}. Variability in secondary metabolites production in plant tissue and organ cultures is frequently observed (Gurney *et al*., 1992). Such variation may also occur through genetic changes, tissue age, somaclonal variations or physiological states of explants. External factors such as nutrient levels, light or temperature can easily affect the CPT content. Pan *et al*., (2004a) have found that 70 mmol of nitrate was favourable for cell growth but a decrease to 40 mmol resulted in a higher CPT accumulation in cell suspension culture of *C. acuminata.* In a second report from Pan *et al*., (2004b) the authors have shown that by manipulating the concentration of microelements of MS medium it is possible to influence the CPT yield. Likewise, CPT concentration in callus culture was effected by light and culture conditions (Park *et al*., 2003). However, in this study no CPT was detected in medium of cell suspension cultures and only traces of CPT were found in the biomass of cells. Similar results were obtained by van Hengel *et al*., (1994), which

indicated that CPT is accumulated intracellularly. Li (2002) has also reported that nitrogen deficiency increased the CPT concentration by possibly creating environmental stress.
CPT concentrations vary significantly with genotypes, seasons and plant age in field grown plants (Liu *et al*., 1998, 2002). It was also shown that CPT in leaves varied significantly with age. Young leaves showed a high CPT content which declined dramatically as the leaves aged (Lopez-Meyer *et al*., 1994; Liu *et al*., 1998). In essence, the results of this thesis should help to improve the *in vitro* culture of *C. auminata* as well as the overall yield of CPT.

5.6.4. CPT Excretion into Liquid Culture Media

Data presented here show that both undifferentiated and differentiated tissue cultures of *C. acuminata* are capable of excreting CPT into the liquid culture medium. An interesting difference between the two immersion treatments used was observed. Plants treated with 8 IC d^{-1} released considerably more CPT into the culture medium than those treated with 4 IC d^{-1}. CPT excretion into liquid media at 8 IC d^{-1} was about 6 µg g^{-1} FW in RITA® (0.048 mg l^{-1}) and 12 µg g^{-1} FW in DVS (0.193 mg l^{-1}). It was suggested that the frequent immersion may have excited a stress situation in the shoots leading to the activation of various genes involved in alkaloid biosynthesis. In the natural ecosystems, plant secondary metabolites are generally associated with defense responses against herbivores or pathogens (Bertin *et al*., 2003), to environmental changes, such as drought, increasing nutrients or protection against metal toxicity (Liu, 2000; Dakora and Phillips, 2002; Hawes *et al*., 2003). Hence, *in vitro* grown *C. acuminata* shoots are enriched with an increased amount of nutrient during 8 IC d^{-1} compared to those with 4 IC d^{-1}, this may result in a higher excretion of CPT. However, further study is needed to verify this hypothesis. It was also reported that hairy root culture of *C. acuminata* released CPT up to 1 mg g^{-1} DW into liquid medium (Lorence *et al*., 2004). Production increase of secondary metabolites can be influenced by a variety of strategies including optimisation of the medium salt composition (Pan *et al*., 2004a, 2004b), elicitation or by genetic engineering. PGRs have also a strong influence on secondary metabolites (Arvy *et al*., 1994; Imanishi *et al*., 1998). CPT secretion was analysed after four, six and eight weeks in this study. Considering that the highest amount of CPT was found after four weeks with a decrease thereafter, it would have been perhaps more interesting to follow the CPT secretion at weekly or even daily intervals. It is possible that the CPT content was even higher shortly after inoculation of explants.

CPT yields in callus cultures grown in TIS were approximately 19 - 40 µg g^{-1} FW (0.13 - 1.79 mg g^{-1} DW), regardless of the IC d^{-1}. However, it was observed that fast growing calli excreted less CPT than those with less profuse growth. A few studies supported this observation that the accumulation of secondary products occurs at a maximum when growth rate decreases and cultures exhibit structural differentiation such as roots, shoots or embryogenesis induction (Yeoman *et al*., 1980; Yeoman *et al*., 1982a, 1982b). Thus, there are further studies to be undertaken concerning the CPT content in explants grown in TIS and their CPT excretion into the liquid culture media.

5.7. Plant Selection According to their CPT Content

Plants have always been valuable sources for the production of pharmaceuticals (Fischer *et al*., 2007). However, environmental conditions, diseases and the application of pesticides often lead to a decrease in plant quality and consequently to a fluctuation and heterogeneity of their active substances. Therefore, controlled production appears to be of high priority. CPT is an example of one of these valuable metabolites that showed large seasonal variations (van Hengel *et al*., 1992; Liu *et al*., 1998; Yan *et al.*, 2003). To date the production of CPT via cell suspension and organ cultures have not been very profitable (Wiedenfeld *et al*., 1997; Lorence *et al*., 2004) due to the low CPT yields.

From the twelve *Camptotheca* plants tested in this thesis, five were selected by the mean of their total CPT yields as elite plants. The greatest concentrations were found in shoots derived form genotypes Bp141, Bp101, Mp28, Mp35 and Lp45. A second selection has shown that the genotypes Mp28 and Bp101 were the best CPT producing plants in terms of CPT production in TIS.

To conclude it was evidenced that CPT contents in shoots of the different genotypes grown in DVS and RITA® were the highest at 8 IC d^{-1} after 8 weeks in culture, whereas those treated with 4 IC d^{-1} were considerably lower in their CPT contents. Considering the CPT content in *in vitro* grown seedlings (max. 4.81 mg g^{-1} DW), somatic embryos at cotyledonary stage (0.75 mg g^{-1} DW), regenerated plantlets (1.23 mg g^{-1} DW) and genotype Mp28 and Bp101 grown in TIS (2.35 and 2.25 mg g^{-1} DW, respectively) it might be of great interest to exploit the production of CPT using both culture systems described in this thesis. Indeed, further research is necessary to manifest these results. Another interesting aspect would be to test regenerated plants via somatic embryogenesis and organogenesis for somaclonal variation and their CPT contents.

The results in this thesis have shown that CPT content differed among the genotypes and that cultures can be manipulated for a more efficient production of CPT. Thus, to meet the pharmaceutical demand for CPT, I would propose a large-scale *in vitro* production of differentiated plant material under controlled environmental conditions, which may overcome factors that are difficult to control such as, seasonal variations of CPT, pests and plant deseases and at the same time supersedes the extraction of CPT from plants of wild populations.

Abdullah, M.A., Ali, A.M., Marziah, M., Lajis, N.H., Ariff, A.B., 1998. Establishment of cell suspension cultures of *Morinda elliptica* for the production of anthraquinones. Plant Cell Tiss. Org. Cult. 54, 173-182.

Afreen, F., Zobayed, S.M.A., Kozai, T., 2002. Photoautotrophic culture of *Coffea arabusta* somatic embryos: Development of a bioreactor for large-scale plantlet conversion from cotyledonary embryos. Ann. Bot. 90, 1-29.

Ahmad, N., Anis, M., 2007. Rapid clonal multiplication of a woody tree, *Vitex negundo* L. through axillary shoots proliferation. Agroforest Syst. 71, 195-200.

Aitken-Christie, J., 1991. Automation. pp 363-388 in Debergh, P.C. and Zimmerman, R.H (eds.) "Micropropagation: Technology and application." Kluwer Academic Publishers, Dordrecht/Boston/London.

Aitken-Christie, J., Jones, C., 1987. Towards automation: Radiata pine shoot hedges *in vitro*. Plant Cell Tiss. Org. Cult. 8, 185-196.

Aitken-Christie, J., Singh A.P., Davies, H., 1988. Multiplication of meristematic tissue: a new tissue culture system for radiata pine in Genetic Manipulation of Woody Plants, J.W. Hanover and D.E. Keathley, (eds.) Plenum Press, New York. pp 413-432.

Ajithkumar, D., Seeni, S., 1998. Rapid clonal multiplication through *in vitro* axillary shoot proliferation of *Aegle marmelos* (L.) Corr. a medicinal tree. Plant Cell Rep. 17, 422-426.

Alvard, D., Cote, F., Teisson, C., 1993. Comparison of methods of liquid medium culture for banana micropropagation. Effects of temporary immersion of explants. Plant Cell Tiss. Org. Cult. 32, 55-60.

Aminah, H., Dick, J. Mc.P., Leakey, R.R.B., Grace, J., Smith, R. I., 1995. Effect of Indole butyric acid (IBA) on stem cuttings of *Shorea leprosula*. For. Ecol. Manage. 72, 199-206.

Andrea-Kodym, F., Zapata-Arias, J., 2001. Low cost alternatives for the micropropagation of banana. Plant Cell Tiss. Org. Cult. 66, 67-71.

Anonymous, 1978. In vitro multiplication of woody species. Round Table Conf., Gembloux, Belgium, Centre Rech. Agron. Etat, 1-295.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts, polyphenoxidase in *beta vulgaris*. Plant physiol. 24, 1-15.

Arvy, M.P., Imbault, N., Naudascher, F., Thiersault, M., Doireae, P., 1994. 2,4-D and alkaloid accumulation in *periwinkle* cell suspensions. Biochimie 76, 410-416.

Averyanov, L.V., Hiep, N.T., 2002. *Diplopanax vietnamensis*, a New Species of *Nyssaceae* from Vietnam – One More Living Representative of the Tertiary Flora of Eurasia. Novon 12, 433-436.

Barnett, J.P., 1972. Seed coat influences dormancy of loblolly pine seeds. Can. J. For. Res. 2, 2-10.

Barnett, J.P., 1976. Delayed germination of southern pine seeds (*Pinus* ssp.) related to seed coat constraint. Can. J. For. Res. 6, 504-510.

Barrios-Gonzalez, J., Fernandez, F.J., Tomasini, A., Mejia, A., 2005. Secondary metabolites production by solid-state fermentation. Malaysian J Microbiol. 1, 1-6.

Berthouly, M., Etienne, H., 2005. Temporary immersion system: A new concept for use liquid medium in mass propagation liquid culture systems for *in vitro* plant propagation. Dordrecht, 165-195.

Bertin, C., Yang, X., Weston, L.A., 2003. The role of root exudates and allelochemicals in the rhizosphere. Plant and Soil 256, 67-83.

Bhansali, R.R., 1993. Bud culture for shoot multiplication and plantlet formation of *Tecomella undulata* (Rohida), a woody tree of the arid zone. Trop. Sci. 33,1-8.

Blazich, F.A., 1988. Chemicals and formulations used to promote adventitious rooting, in: "Adventitious root formation by cuttings" In: Davis, T.D., B.E. Haissig, N. Sakhla, (eds.) Advances in Plant Science Series vol 2, p. 312. Dioscorides Press, Portland.

Bodley, A.L., Shapiro, T.A., 1995. Molecular and cytotoxic effects of camptothecin, a topoisomerase I inhibitor, on trypanosomes and *Leishmania*. Procedures of the National Academy of Science USA, 92, 3726 -3730.

Bodley, A.L., Cumming, J.N., Shapiro, T.A., 1998. Effects of camptothecin, a topoisomerase I inhibitor, on *Plasmodium falciparum.* Biochem. Pharmacol. 55, 709-711.

Bulk, M., 2003. Nursery of rare and unknown plants. Rijneveld 115, 2771 XV Boskoop. The Netherlands.

Burlat, V., Oudin, A., Courtois, M., Rideau, M., St-Pierre, B., 2004. Coexpression of three MEP pathway genes and geraniol 10 hydroxylase in internal phloem parenchyma of Catharanthus roseus implicates multicellular translocation of intermediates during biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. Plant J. 38, 131-141.

Cabasson, C., Alvard, D., Dambier, D., Ollitrault , P., Teisson, C., 1997. Improvement of Citrus somatic embryo development by temporary immersion. Plant Cell Tiss. Org. Cult. 50, 33-37.

Cao, G.R., Gao, J.X., Duan, D.X., Li, S.J., Wang, K., 1986. Studies on *Camptotheca acuminata* leaves: main toxic principle, poisoning, and treatment in goats. In L.F. James, R.F. Keeler, E.M. Bailey, Jr., P.R. Cheeke and M.P. Hegarty (eds.), Proc. Third Int. Symp., pp 506-508.

Carman, J.G., 1990. Embryogenic cells in plant tissue cultures: Occurrence and behaviour. In Vitro Cell. Dev. Biol. 26, 746-753.

Carte, B.K., DeBrosse, C., Eggleston, D., Hemling, M., Mentzer, M., Poehland, B., Troupe, N., Westley, J.W., Hecht, S.M., 1990. Isolation and characterization of a presumed biosynthetic precursor of Camptothecin from extracts of *Camptotheca acuminata* Tetrahedron 46, 2747-2760.

Chalupa, V., 1990. Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.) Plant Cell Rep. 9, 398-401.

Chen, S.Y., Kuo-Huang, L.L., Chien, C.T., Hsui, Y.R., 2004. Effect of cold stratification on pericarp structure, and seed germination and storage of *Camptotheca acuminata*. Taiwan Journal of Forest Science 19, 287-95. Contact: Chien, Ching-Te, Div Silviculture, Taiwan Forestry Res. Inst., 53 Nanhai Rd, Taipei, 100, Taiwan.

Chen, T.H.H., Marowitch, J., Thompson, B.G., 1987. Genotypic effects on somatic embryogenesis and plant regeneration from callus cultures of *alfalfa.* Plant Cell Tiss. Org. Cult. 8, 73-81.

Chou, Y.L., Li, S.Y., 1990. Forests of China. Science Press Beijing.

Ciddi, V., Shuler, M.L., 2000. Camptothecine from callus cultures of *Nothapodytes foetida*. Biotechnol. Lett. 22, 129-132.

Cordell, G.A., 1974. Review: Lloydia, 37, 219-298.

Creemers, G. J., Bolis, G., Gore, M., Scarfone, G., Lacave A.J., 1996. Topotecan, an active drug in the second-line treatment of epithelial ovarian cancer: Results of a large European phase II study. J. Clin. Oncol. 14, 3056-3061.

Cunha, A., Fernandes-Ferreira, M., 1999. Influence of medium parameters on somatic embryogenesis from hypocotyl explants of flax (*Linum usitatissimum* L.): Effect of carbon source, total inorganic nitrogen and balance between ionic forms and interaction between calcium and zeatin. Journal of Plant physiol. 155, 591-597.

Cunningham, D., Pyrhonen, S., James, R.D., Punt, C.J., Hickish, T.F., Heikkila, R., Johannesen, T.B., Starkhammar, H., Topham, C.A., Awad, L., Jacques, C., Herait, P., 1998. Randomised trial of irinotecan plus supportive care *versus* supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. Lancet. 352, 1413-1418.

Czuwara-Ladykowska, J., Makiela, B., Smith, E.A., Trojanowska, M., Rudnicka, L., 2001. The inhibitory effects of camptothecin, a topoisomerase I inhibitor, on collagen synthesis in fibroblasts from patients with systemic sclerosis. Arthritis Res. 3, 311-318.

Dakora, F.D., Phillips, D.A., 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant and Soil 245, 35-47.

Damiano, C., Monticelli, S., La Starza, S.R., Gentile, A., Frattarelli, A., 2003. Temperate Fruit Plant Propagation through Temporary Immersion. Acta Hort. (ISHS) 625, 193-200.

De Luca, V., St-Pierre, B., 2000. The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci. 5, 168-173.

Debergh, P., 1988. Improving mass propagation of in vitro plantlets. In: The Organizing Committee Intl. Symp. High Technology in Protected Cultivation (ed). Horticulture in High Technology Era, Tokyo pp 45-57.

Debergh, P.C., Maene, L.J., 1981. A scheme for commercial propagation of ornamental plants by tissue culture. Sci. Hortic. 14, 335-345.

Debergh, P.C., Maene, L.J., 1985. Preparation of tissue cultured plants for rooting and establishment *in vivo*. In: Proc. Int. Symp. Plant. Tissue Cell. Cult. Applic. Crop Improv. (Eds.). F.J. Novak, T. Havel, K. Dolezel, Czechoslovakia Acad. Sci., Prague, 487-495.

Decaisne, J., 1873. Caracteres et Descriptions de trios genres nouveaus de plantes recueilles en Chine par L'abbe A. David Bull. Soc. Bot. France 20, 155 -160.

Dewick, P.M., 1997. Medicinal Natural Products: A Biosynthetic Approach, John Wiley & Sons Ltd., Baffins Lane, England.

Dirr, M.A., 1990. Effects of P-ITB and IBA on the rooting response of 19 landscape taxa. J. Environ. Hort. 8, 83-85.

Dodds, J.H., (ed.) 1991. In vitro methods for conservation of plant genetic resources. Chapman & Hall, London.

Douillard, J.Y., Cunningham, D., Rothm A.D., Navarro, M., James, R.D., Karasek, P., Jandik, P., Iveson, T., Carmichael, J., Alakl, M., Gruia, G., Awad, L., Rougier, P., 2000. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet. 355, 1041-1047.

Escalant, J.V., Teisson, C., Cote, F., 1994. Amplified somatic embryogenesis from male flowers of triploid banana and plantain cultivar (*Musa* sp.). In vitro Cell Dev. Biol. Plant. 30, 181-186.

Escalona, M., Lorenzo, J.C., Gonzales, B.L., Daquinta, M., Borroto, C.G., Gonzales J. I., 1999. Pineapple (*Ananas cosmus* L. Merr) micropropagation in temporary immersion systems. Plant Cell Rep. 18, 743-748.

Etienne, H., Berthouly, M., 2002. Temporary immersion systems in plant micropropagation. Plant Cell Tiss. Org. Cult. 69, 215-231.

Etienne, H.M., Lartaud, M., Michaux-Ferrière, N., Carron, M.P., Berthouly, M., Teisson, C., 1997. Improvement of Somatic Embryogenesis in *Hevea brasiliensis* (Müll. Arg.) Using the Temporary Immersion Technique. In Vitro Cell. Dev. Biol. 33, 81-87.

Etienne-Barry, D., Bertrand, B., Vasquez, N., Etienne, H., 1999. Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and regeneration of plant. Plant Cell Rep. 199, 111-119.

Ewesuedo, R.B., Ratain, M.J., 1997. Topoisomerase I Inhibitors. The Oncologist 2, 359-364.

Fang, W.P., Soong, T.P., 1975. Praecursores flora *Nyssacearum Sinensium* Zhiwu Fenlei Xuebao 13, 83-89.

Fang, W.P., Zhang, Z.R., 1983. Flora Reipublicae Popularis Sinicae Science Press, Beijing, 52.

Farnsworth, N.R., Soejarto, D.D., 1985. Potential consequences of plant extinction in the United States on the current and future availability of prescription drugs. Econ. Bot. 39, 231-240.

Fischer, R., Twyman, R.M., Hellwig, S., Drossard, J., Schillberg, S., 2007. Facing the future with pharmaceuticals from plants. Proceedings of the 11th IAPTC&B Congress, August 13-18, 2006, Beijing, China. Dordrecht: Springer Netherland, pp 13-32.

Fulzele, D.P., Satdive, R.K., 2003, Somatic embryogenesis, plant regeneration and the evaluation of the camptothecin content in *Nothapodytes foetida*. Soc. In Vitro Biol. 39, 212-216.

Gamborg, O., Miller, R., Ojima, K., 1968. Nutrient requirement suspensions cultures of soybean root cells. Exp. Cell Res. 50, 151-158.

Gaspar, T., Kevers, C., Hausman, J.E., 1997. Indissociable chief factors in the inductive phase of adventitious rooting. In: Ahman, A., Waisel, Y., (eds.). Biology of root formation and development. New York and London: Plenum Press, 1997, 55-64.

George, E.F., 1993. Plant propagation by tissue culture. Part 1. The technology. Exegetics Ltd., Basingstoke, UK.

George, E.F., Sherrington, P.D., 1984. Commercial tissue culture laboratories. In: George EF, Sherrington PD (eds.) Plant propagation by tissue culture. Handbook and directory of commercial laboratories. Easter Press, Great Britain, pp 549-551.

George, L., Bapat, V.A., Rao, P.S., 1987. In vitro multiplication of sesame (*Sesamum indicum*) through tissue culture. Ann. Bot. 60, 17-21.

González-Olmedo, J. L., Fundora, Z., Molina, L.A., Abdulnour, J., Desjardins, Y., Escalona, M., 2005. New contributions to propagation of pineapple (*Ananas comosus* L. Merr) in temporary immersion biorreactors. In Vitro Cell. Dev. Biol. Plant 41, 87-90.

Gray, D.J., Benton, C.M., 1991. «*In vitro*» micropropagation and plant establishement of Muscadine grape cultivars, *Vitis rotundifolia*. Plant Cell Tiss. Org. Cult. 27, 7-14.

Gray, D.J., Purohit, A., 1991. Somatic embryogenesis and development of synthetic seed technology. Crit. Rev. Plant Sci. 10, 33-61.

Grout, B.W.W., Aston, M.J., 1977. Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hortic. Res. 17, 1-7.

Gurney, K.A., Evans, L.V., Robinson, D.S., 1992. Purine Alkaloid Production and Accumulation in Cocoa Callus and Suspension Cultures. J. Exp. Bot. 43, 769-775.

Hackett, WP., 1988. Donor plant maturation and adventitious root formation. In: Davies T.M., Haissig, B.F Sankhala N., (eds.). Adventitious root formation in cuttings, vol 2. pp11-28. Dioxcroides, Portland.

Haissig, B.E., Davis, T.D., 1994. An historical evaluation of adventitious rooting research to 1993.In: Davis, T.D., Haissig, B.E. (eds.). Biology of adventitious root formation. New York: Plenum Publishing Corporation. pp 275-331.

Hartmann, H.T., Kester, D.E., Davis, Jr. F.T., 1990. Plant propagation-Principals and Practices. 5th. Edition. Prentice-Hall International Editions. Englewood Cliffs, New Jersey. 647 pp.

Hawes, M.C., Bengough, G., Cassab, G., Ponce, G., 2003. Root caps and rhizosphere. J. Plant Growth Regul. 21, 352-367.

Hazarika, B.N., 2003. Acclimatisation of tissue-culture plants. Current science 85, 1704-1712.

Hazarika, B.N., 2006. Morpho-physiological disorders in *in vitro* culture of plants, Scientia Horticulturae 108, 105-120.

Heller, R., 1965. Some aspects of the inorganic nutrition of plant tissue cultures. In: P. R. White and A. R. Grove, (eds.). Proceedings of an International Conference on Plant Tissue Culture. McCutchan Publishing Corporation, Berkeley, Calif. pp 1-18.

Heron, J. F., 1998. Topotecan: an oncologist's view. The Oncologist 3, 390-402.

Hiscox, J.D., Israelstam, G.F., 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. 57, 1332-1334.

Hsiang, Y.H., Hertzberg, R., Hecht, S., Liu, L.F., 1985. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem. 260, 14873-14878.

http://www.botanic.jp/plants-ka/kanren.htm

http://www.cites.org/eng/cop/11/prop/58.pdf

http://had0.big.ous.ac.jp/plantsdic/angiospermae/dicotyledoneae/choripetalae/nyssaceae/kanrenboku/kanrenoboku.htm)

http://www.hktree.com/tree/Camptotheca%20acuminata1.htm

http://www.tremcon.net/ trading/biotec/cpt.html

Hutchinson, J.F., Beardsell, D.V., McComb, J.A., 1985. Propagation by tissue culture I. Introduction. In: Lamont, B.B., Watkins, P.A., (eds.) Horticulture of Australian Plants. West Aust. Dep. Agric. pp 38-51.

Hutchinson, C.R., Heckendorf, A.H., Straughn, J.L., Daddona, P.E., Cane, D.E., 1979. Biosynthesis of camptothecin. 3. Definition of strictosamide as the penultimate biosynthetic precursor assisted by 13C and 2H NMR spectroscopy. J. Am. Chem. Soc. 101, 3358-3369.

Ikeda-Iwai, M., Umehara, M., Satoh, S., Kamada, H., 2003. Stress-induced somatic embryogenesis in vegetative tissue of *Arabidopsis thaliana*. The Plant Journal 24, 109-114.

Imanishi, S., Hashizume, K., Nakakita, M., Kojima, H., Matsubayashi, Y., Hashimoto, T., Sakagami, Y., Yamada, Y., Nakamura, K., 1998. Differential induction by methyl jasmonate of genes encoding ornithine decarboxylase and other enzymes involved in nicotine biosynthesis in tobacco cell cultures. Plant Mol. Biol. 38, 1101-1111.

Jain, A.K. Datta, R.K., 1992. Shoot organogenesis and plant regeneration in mulberry (*Moms bombycis* Koiz.): factors influencing morphogenetic potential in callus cultures. Plant Cell Tiss. Org. Cult. 29, 43-50.

Jain, K. J., Nessler, C. L., 1996. Clonal propagation of *Camptotheca acuminata* through shoot bud culture. Plant Cell Tiss. Org. Cult. 44, 29-233.

Jayasankar, S., Van Aman, M., Li, Z., Gray, D.J., 2001. Direct seeding of grapevine somatic embryos and regeneration of plants. In Vitro Cell. Dev. Biol. Plant 37, 476-479.

Kamada, H., Ishikawa, K., Saga, H., Harada, H., 1993. Induction of somatic embryogenesis in carrot by osmotic stress. Plant Tissue Cult. Lett. 10, 38-44.

Kamada, H., Tachikawa, Y., Saitou, T., Harada, H., 1994. Heat stresses induction of carrot somatic embryogenesis. Plant Tissue Cult. Lett. 11, 229-232.

Kamil, H., Umboh, M.I.J., 1992. Root induction of *Santalum album* by using IBA and NAA. Seameo Biotrop, Indonesia. BIOTROP Spec. Publ. 49, 67-73.

Kelly, D.C., Avery, R.J., Dimmock, J.N., 1974. Carnptothecin: an Inhibitor of Influenza Virus Replication J. Gen. Virol. 25, 427-432.

Kevers, C., Hausman, J., Faivre-Rampart, O., Evers, D., Gaspar, Th., 1997. Hormonal control of adventitious rooting: Progress and questions. Angewandte Bot. 71, 71-79.

Kevers, C., Franck, T., Strasser, R.J., Dommes, J., Gasper, T., 2004. Hyperhydricity of micropropagated shoots: a typically stress induced change of physiological state. Plant Cell Tiss. Org. Cult. 77, 181-191.

Kirdmanee, C., Kitaya, Y., Kozai, T., 1995. Effects of oxygen enrichment and supporting material in vitro on photoautotrophic growth of *Eucalyptus* plantlets in vitro and ex vitro: anatomical comparisons. Acta Hortic. 393, 111-118.

Kitajima, M., Masumoto, S., Takayama, H., Aimi, N., 1997. Isolation and partial synthesis of 3(*R*)-and 3 (*S*)-deoxypumiloside, structural revision of the key metabolite from the camptothecin producing plant, *Ophiorrhiza pumila*. Tetrahedron Lett. 38, 4255-4258.

Komaraiah, P., Kavi Kishor, P.B., Carlsson, M., Magnusson, K.E., Mandenius, C.F., 2005. Enhancement of anthraquinone accumulation in *Morinda citrifolia* suspension cultures. Plant Sci. 168, 1337-1344.

Konan, N.K., Sangwan, R.S., Sangwan, B.S., 1994. Somatic embryogenesis from cultured mature cotyledons of cassava (*Manihot esculenta* Crantz) Identification of parameters influencing the frequency of embryogenesis Plant Cell, Tissue and Organ Cult. 37, 91-102.

Kooi, L.T., Keng, C.L., Hoe, C.T.K., 1999. In vitro rooting of *Sentang* shoots (*Azadirachta excelsa* L.) and acclimatization of the plantlets. In vitro Cell Dev. Biol. Plant. 35, 396-400.

Kutchan, T.M., 1995. Alkaloid Biosynthesis -The Basis for Metabolic Engineering of Medicinal Plants-. The Plant Cell 7, 1059-1070.

Lakshmanan, P., Geijskes, R., Wang, L., Elliott, A., Grof, C., Berding, N., Smith, G., 2006. Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum* spp. interspecific hybrids) leaf culture. Plant Cell Rep. 25, 1007-1015.

Lê, C.L., 1985. La micropropagation. Revue Suisse Viticultute Arboriculture. Horticulture 17, 347-349.

Leakey, R.R.B., Chapman, V.R., Longman, K.R., 1982. Physiological studies for tropical tree improvement and conservation. Factors affecting root initiation in cuttings of *Triplochiton scleroxylon* K. Schum. For. Ecol. Manage. 4, 53-66.

Li, S.Y., 2000. Utilizing *Camptotheca* products for termite control. U.S. Patent Appl. US20020018762 and PCT Int. Appl. WO0200020.

Li, Z., 2002. Effects of several abiotic and biotic factors and plant hormones on growth morphology, and camptothecin accumulation in Camptotheca acuminate seedlings. Dissertation, Louisiana State University, USA, 117 pp.

Li, S., Adair, K.T., 1994. *Camptotheca acuminata* Decaisne, Xi Shu. A promising antitumor and antiviral tree for the 21st century. Tucker Center, College of Forestry, Stephen F. Austin State University, Nacogdoches, Texas.

Li, Z., Liu, Z., 2001. Micropropagation of *Camptotheca acuminata* decaisne from axillary buds, shoot tips, and seed embryos in a tissue culture system. In Vitro Cell. Dev. Bio. Plant 37, 84-88.

Li, Z., Liu, Z., 2005. Plant regeneration from leaf petiole in *Camptotheca acuminata*. In Vitro Cell Dev Biol 41, 262-265.

Li, S., Yi, Y., Wang, Y., Zhang, Z. and Beasly, R. S., 2002. Camptothecin accumulation and variations in *Camptotheca*. Planta Med. 68, 1010-1016.

Li, S., Zhang, Z., Cain, A., Wang, B., Long, M., Taylor, J., 2005. Antifungal activity of camptothecin, trifolin, and hyperoside isolated from *Camptotheca acuminata*. J. Agric. Food Chem. 53, 32-37.

Liu, W.Z., 2004. Secretory Structures and Their Relationship to Accumulation of Camptothecin in *Camptotheca acuminata* (Nyssaceae). Acta Bot. Sinica, 46, 1242-1248.

Liu, Z. J., 2000. Drought-induced in vivo synthesis of camptothecin in *Camptotheca acuminata* seedlings. Plant Physiol. 11, 483-488.

Liu, Z., Zhou, G., Xu, S., Wu, J., Yin, Y., 2002. Provenance variation in *camptothecin* concentrations of *Camptotheca acuminata* grown in China. New Forests 24, 215-224.

Liu, Z., Carpenter, S.B., Bourgeois, W.J., Yu, Y., Constantin, R.J., Falcon, M.J., Adams, J.C., 1998. Variations in the secondary metabolite camptothecin in relation to tissue age and season in *Camptotheca acuminata*. Tree Physiol. 18, 265-270.

Lopez-Meyer, M., Nessler, C.L., Mcknight, T.D., 1994. Sites of accumulation of the antitumor alkaloid camptothecin in *Camptotheca acuminata*. Planta Med. 60, 558-560.

Lorence, L., Nessler, C.L., 2004. Camptothecin, over four decades of surprising findings. Phytochemistry 65, 2735-2749.

Lorence, A., Medina-Bolivar, F., Nessler, C.L., 2004. Camptothecin and 10-hydroxy-camptothecin from *Camptotheca acuminata* hairy roots Plant Cell Rep. 22, 437-441.

Lorenzo, J.C., González, B., Escalona, M., Teisson, C., Espinosa, P., Borroto, C., 1998. Sugarcane shoot formation in an improved temporary immersion system. Plant Cell Tiss. Org. Cult. 54, 197-200.

Lu, H., McKnight, T.D., 1999. Tissue specific expression of the beta subunit of tryptophan synthase in *Camptotheca acuminata*, an indole alkaloid producing plant. Plant Physiol. 120, 43-52.

Maliepaard, M., van Gastelen, M.A., Tohgo, A., Hausheer, F.H., van Waardenburg, R.C.A.M., de Jong, L.A., Pluim, D., Beijnen, J.H., Schellens, J.H.M., 2001. Circumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins *in Vitro* using non-substrate drugs or the BCRP inhibitor GF120918. Clinical Cancer Res. 7, 935-941.

Maxwell, D.W., 2003. Propagation of *Camptotheca acuminata*. Dissertation, Texas A&M University, USA, 49 pp.

McClelland, M.T., Smith, M.A.L., Carothers, Z.B., 1990. The effects of in vitro and ex vitro root initiation on subsequent microcutting root quality in three woody plants Plant Cell Tiss. Org. Cult. 23, 115-123,.

McDonald, C., 1997. China's tree of joy offers hope in the US. Pacific West Cancer Fund. pp 37-49.

Mehrotra, S., Goel, M, K., Kukreja, A.K., Mishra, B. N., 2007. Efficiency of liquid culture systems over conventional micropropagation: A progress towards commercialization. Afr. J. Biotechnol. 6, 1484-1492.

Meijer, E.G.M., Brown, D.C.W., 1987. Role of exogenous reduced nitrogen and sucrose in rapid high frequency somatic embryogenesis in *Medicago sativa*. Plant Cell Tiss. Org. Cult. 10, 11-19.

Meyer, PW., 1991. *Camptotheca acuminata* Publ.Gard. April, 39.

Michael, E., Compton, B.L., Pierson, J.E., 2001. Staub. Micropropagation for recovery of *Cucumis hystrix*. Plant Cell Tiss. Org. Cult. 64, 63-67.

Mooney, H.A., Winner, W.E., Pell, E.J., 1991. Response of plants to multiple stresses. Academic Press, San Diego, California, USA.

Morel, G., Martin, C., 1952. Guerison de Dahlias atteints d' une maladie a virus. C.R. Acad. Sci. Ser. D. 235, 1324-1325.

Morel, G., Martin, C., 1955. Guerison de pommes de terre atteintes de maladies a virus. CR Seancer Acad Agric Fr. 41, 472-475.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with Tobacco tissue cultures. Physiol. Plantarum. 15, 473-497.

Németh, G., 1986. Induction of rooting. In Biotechnology in Agriculture and Forestry, vol, 1. Trees (Bajaj YPS, ed). Berlin Heidelberg: Springer-Verlag, pp 49-64.

Nolte, B.A., 1999. Micro-analysis and localization of camptothecin in *Camptotheca acuminata*. M.S. Thesis, Texas A&M University, College Station, TX.

Normah, M.N., Hamidah, S., Ghani, F.D., 1997. Micropropagation of *Citrus halmii* - an endangered species of S.E. Asia. Plant Cell Tiss. Org. Cult. 50, 225-227.

O'Connor S.E., Maresh J.J., 2005. Chemistry and biology of monoterpene indole alkaloid biosynthesis. Nat. Prod. Rep. 23, 532-547.

Overbeek, J.V., 1966. Plant hormones and regulators. Science 152, 721-731.

Park, Y.G., Kim, M.H., Yang, J.K., Chung, G.Y., Choi, M.S., 2003. Light-susceptibility of Camptothecin Production from In Vitro Cultures of *Camptotheca acuminata* Decne. Biotechnol. Bioprocess Eng. 8, 32-36.

Pan, XW., Xu, H.H., Gao, X., Liu, X., Lu, Y.T., 2004a. Improvement of growth and camptothecin yield by altering nitrogen source supply in cell suspension cultures of *Camptotheca acuminata*. Biotechnol. Lett. 26, 1745-1748.

Pan, X.W., Shi, Y.Y., Liu, X., Gao, X., Lu, Y.T., 2004b. Influence of inorganic microelements on the production of camptothecin with suspension cultures of *Camptotheca acuminata*. Plant Growth Regul. 44, 59–63.

Pasqua, G., Monacelli, B., Valletta, A., 2004. Cellular localisation of the anti-cancer drug camptothecin in *Camptotheca acuminata* Decne (*Nyssaceae*). European journal histochemistry ISSN 1121-760X 48, 321-328.

Perdue, R.E., Smith, R.L., 1970. *Camptotheca acuminata* Decaisne (*Nyssaceae*), source of camptothecin, an anti-leukemic alkaloid. USDA Tech. Bull. 1415, 1-26.

Perdue, R.E., Smith, Jr. R.L., Wall, M.E., Hartwell, J. L., Abbott, B.J., USDA, 1970. Agr. Research Service Technical Bulletin No. 1415.

Pierik, R.L.S.M., 1987. *In vitro* culture of higher plants. Martinus Nijhoff Publishers. Dordrecht. Netherlands. 344 pp.

Plietzsch, A., Jesch, H.H., 1998. Using in vitro propagation to rejuvenate difficult-to-root woody plants. Comb. Proc. Intl. Plant Prop. Soc. 48, 171-176.

Preece, J.E., Sutter, E.J., 1991. Acclimatization of micropropagated plants to the greenhouse and field. In: Debergh, P.C., Zimmerman, R.H., (eds.) Micropropagation, technology and application. London: Kluwer Academic, 1991. pp 71-93.

Priel, E., Showalter, S.D., Blair, D.G., 1991. Inhibition of human immunodeficiency virus (HIV-1) replication by non-cytotoxic doses of camptothecin, a topoisomerase I inhibitor. AIDS Res. Hum. Retroviruses 7, 65-72.

Purohit, S.D., Tak, K., Kukda, G., 1995. In vitro propagation of *Boswellia serrata* Roxb Biol. Plant. 37, 335-340.

Roels, S., Escalona, M., Cejas, I., Noceda, C., Rodriguez, R., Canal, M.J., Sandoval, J., Debergh, P., 2005. Optimization of plantain (*Musa* AAB) micro-propagation by temporary immersion system. Plant Cell Tiss. Org. Cult. 82, 57-66.

Roja, G., Heble, M.R., 1994. The quinoline alkaloids camptothecin and 9-methoxy camptothecin from tissue cultures and mature trees of *Nothapodytes foetida.* Phytochemistry 36, 65-66.

Saito, K., Sudo, H., Yamazaki, M., Koseki-Nakamura, M., Kitajima, M., Takayama, H., Aimi, N., 2001. Feasible production of camptothecin by hairy root culture of *Ophiorrhiza pumila* Plant Cell Rep. 20, 267-271.

Sakato, K., Misawa, M., 1974. Effects of chemical and physical condition of growth of *Camptotheca acuminata* cell culture. Arg. Biol.Chem. 38, 491- 497.

Sandal, I., Bhattacharya, A., Ahuja, P.S., 2001. An efficient liquid culture system for tea shoot proliferation. Plant Cell Tiss. Org. Cult. 65, 75-80.

Sankar-Thomas, Y.D., Saare-Surminski, K., Lieberei, R., 2008. Plant regeneration via somatic embryogenesis of *Camptotheca acuminata* in temporary immersion system (TIS) Plant Cell Tiss. Org. Cult. 95, 163-173.

Sen, J., Kalia, S., Guha-Mukherjee, S., 2002. Level of endogenous free amino acids during various stages of culture of *Vigna mungo* (L.) Hepper - Somatic embryogenesis, organogenesis and plant regeneration. Current Science 82, 429-433.

Shao, B.B., 1989. Effects of stratification and temperature variation on the germination of seeds of ten different trees. Seed Sci. Technol. 4-7.

Sheriha, G.M., Rapoport, H., 1976. Biosynthesis of *Camptotheca acuminata* alkaloids. Phytochemistry 15, 505-508.

Shibli, R.A., Smith, M.A.L., 1996. Direct shoot regeneration from *Vaccinium pahalae* (ohelo) and *V. myrtillus* (bilberry) leaf explants. HortScience 31, 1225-1228.

Shrikhande, M., Thengane, S.R., Mascarenhas, A.F., 1993. Somatic embryogenesis and plant regeneration in *Azadirachta indica* A. Juss. In Vitro Cell. Dev. Biol. 29, 38-42.

Silvestrini, A., Pasqua, G., Botta, B., Monacelli, B., van der Heijden, R., Verpoorte, R., 2002. Effect of alkaloid precursor feeding on a *Camptotheca acuminata* cell line. Plant Physiol. Biochem. 40, 749-753.

Smith, R.L., 1969. *Camptotheca acuminata*, biography of camptothecin, a promising cancer drug. Lasca leaves September-December, 55-59.

Sudo, H., Hasegawa, Y., Matsunaga, J., 1991. Jpn. Pat. 03,272,628. Tafur S, Nelson J.D, DeLong, D.C., Svoboda, G.H., 1976. Antiviral components of *Ophiorrhiza mungos*. Isolation of camptothecin and 10-methoxycamptothecin. Lloydia 39, 261-262.

Suzuki , M., 1976. Some fossil woods from the Palaeogene of Northern Kyushu. Bot. Mag. Tokyo 89, 59-71.

Tanai, T., 1977. Fossil Leaves of the *Nyssaceae* from the Miocene of Japan. J. Fac. Sci. Hokkaido Univ., Ser. 4, 17, 505-516.

Tchoundjeu, Z., Leakey, R.R.B., 1996. Vegetative propagation of African mahogany: Effect of auxin, node position, leaf area and cutting length. New For. 11, 125-36.

Teisson, C., Alvard, D., 1995. A new concept of plant *in vitro* cultivation liquid medium: temporary immersion. In: M. Terzi et al. (eds.), Current issues in plant molecular and cellular biology, pp 105-110 Kluwer Acad. Publ. Netherlands.

Tisserat, B., Vandercook, C.E., 1985. Development of an automated plant culture system. Plant Cell Tiss. Org. Cult. 5, 107-117.

Tod, F., 2004. Botanical Garden University of Vienna, Austria.

Valletta, A., Santamaria, A.R., Pasqua, G., 2007. CPT accumulation in the fruit and during early phases of plant development in *Camptotheca acuminata* Decaisne (*Nyssaceae*). Nat. Prod. Res. 21, 1248-55.

Van Hengel, A.J., Buitelaar, R.M., Wichers, H.J., 1994. *Camptotheca acuminata* Decne: *In vitro* culture and the production of camptothecin. In: Bajaj YPS (Ed). Biotechnology in agriculture and forestry, vol 28. Medicinal and aromatic plants VII pp 98-112. Springer-Verlag Berlin Heidelberg New York.

Van-Hengel, A.J., Harkes, M.P., Wichers, H.J,, Hesselink, P.G.M., Buitelaar, R.M., 1992. Characterization of callus formation and camptothecin production by cell lines of *Camptotheca acuminata*. Plant Cell Tiss. Org. Cult. 28, 11-18.

Wall, M.E., Wani, M.C., 1991. Chemistry and Antitumor Activity of Camptothecins, DNA Topoisomerases in Cancer, M. Potmesil and K. W. Kohn, (eds.). Oxford University Press, New York, NY, 93 pp.

Wall, M.E., Wani, M.C., Cook, C.E., Palmer, K.H., McPhail, A.T., Sim, G.A., 1966. Plant antitumor agents. I. Isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. J. Am. Chem. Soc. 88, 3888-3890.

Wang, H.M., Zu, Y.G. Liu, H., 2007. Efficient rooting and root development after transfer of regenerated plantlets of *Camptotheca acuminata*. Eurasian J. For. Res.10, 179-184.

Wang, H.M., Zu, Y.G., Dong, F., Zhao, X.J., 2005. Assessment of factors affecting adventitious shoot regeneration from axilliary bud of *Camptotheca acuminata.* J. For. Res. 16, 45-49.

Wang, H., Zu, Y., Wang, W., Wu, S., Dong, F., 2006. Establishment of *Camptotheca acuminata* regeneration from leaf explants. Biologia Plantarum 50, 725-728.

Watase, I., Sudo, H., Yamazaki, M., Saito, K., 2004. Regeneration of transformed *Ophiorrhiza pumila* plants producing camptothecin. Plant Biotechnol. 21, 337-342.

Wawrosch, C., Malla, P.R., Kopp, B., 2001. Clonal propagation of *Lilium nepalense* D. Don., a threatened medicinal plant of Nepal. Plant Cell Rep., 4, 285-288.

Weathers, P.J., Giles, K.L., 1988. Regeneration of lentils utilizing nutrient mist culture. In vitro Cell Dev. Biol. Plant. 24, 727-732.

Wenkert, E., Dave, L.G., Lewis, R.G., Sprague, P.W., 1967. J. Am. Chem. Soc. 89, 6741-6745.

Wiedenfeld, H., Furmanowa, M., Roeder, E., Guzewska, J., Gustowski, W., 1997. camptothecin and 10-hydroxycamptothecin in callus and plantlets of *Camptotheca acuminata*. Plant Cell Tiss. Org. Cult. 49, 213-218.

Wijesekera, R.O.B., 1991. The Medicinal Plant Industry 280 pages, Taylor & Francis Ltd., United States ISBN 9780849366697.

Wink, M., 1998. Modes of action of alkaloids. In: Roberts, M.F., Wink, M. (eds.), Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum Press, New York, pp 301-326.

Wink, M., 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64, 3-19.

Wu, J., Liu, L.F., 1997. Processing of topoisomerase I cleavable complexes into DNA damage by transcription. Nucleic Acids Res. 25, 4181-4186.

Yamazaki, Y., Urano, A., Sudo, H., Kitajima, M., Takayama, H., Yamazaki, M., Aimi, N., Saito, K., 2003. Metabolite profiling of alkaloids and strictosidine synthase activity in camptothecin producing plants. Phytochemistry 62, 461-470.

Yan, X.F., Wang, Y., Yu, T., Zhang, Y.H., Dai, S.J., 2003. Variation in camptothecin content in *Camptotheca acuminata* leaves Bot. Bull. Acad. Sin. 44, 99-105.

Yang, G., Read, P.E., 1993. In vitro culture of Vanhoutte's *spirea* explants from secondary cultures and dormant stems forced in solutions containing plant growth regulators. Plant Cell Tiss. Org. Cult. 33, 25-30.

Yang, G., Read, P.E., 1997. Plant growth regulators in the forcing solution influenced bud break and shoot elongation of dormant woody plant species. PGRSA Quarterly 25, 145-152.

Yang, B.M., Duan, L.D., 1988. One new plant of Nyssaceae from Hunan. Nat. Sci. J hunan Norm, Univ. 11, 63-64.

Yeoman, M.M., Lindsey, K., Hall, R.D., 1982a. Differentiation as a prerequisite for the production of secondary nretabolites. In: Proc. Plant Cell Cult. Conf., Sudbury House, London, pp 1-7. Oyez Scientific and Technical Services Ltd., London.

Yeoman, M.M., Miedzybrodzka, M.B., McLauchlan, W.R., 1982b. Accumulation of secondary products as a facet of differentiation in plant cell and tissue cultures. In: Differentiation *in vitro*. British Society for cell biology symposium vol. 4. pp 65-82. (Eds.) M. M. Yeoman and D. E. S. Truman.Cambridge University Press.

Yeoman, M.M., Miedzybrodzka, M.B., Lindsey, K., McLauchlan, W.R., 1980. The synthetic potential of cultured plant cells. In: Plant Cell Cult: Results and perspectives, pp 327-343. (Eds.) F. Sala, B. Parisi, R. Cella and O. Cifferi, Elsevier-North Holland. Amsterdam.

Ying, T.S., Zhang, Y.L., Boufford, D.E., 1993. The endemic genera of seed plants of China. Beijing: Science Press, 278-280, 467-469.

Yuan, Y., Liu, Z., Yang, Y., Wu, H., 2008. Studies on the production of comptothecin in *in vitro* cultured roots of *Camptotheca acuminata.* The 2nd International Conference on Bioinformatics and Biomedical Engineering (iCBBE 2008).

Zhang, J., Yu, Y., Liu, D., Liu, Z., 2007. Extraction and composition of three naturally occurring anti-cancer alkaloids in Camptotheca acuminata seed and leaf extracts Phytomedicine, 14, 50-56.

Zhao, X., Y.U, T., Wang, Y., Yan, X.F., 2006. Effect of arbuscular mycorrhiza on *Camptotheca acuminata* seedlings. J. For. Res. 17, 121-23.

Zhou, Y.X., 1989. Study on the characteristics of seed dormancy and germination of *Camptotheca acuminata.* Linye Keji 8, 22-25.

Zhu L. H., Li X. Y., Welander M., 2005. Optimisation of growing conditions for the apple rootstock M26 grown in RITA containers using temporary immersion principle. Plant Cell Tiss. Org. Cult, 81, 313-318.

Zimmerman, R.H., 1988. Micropropagation of woody plants: post J.R., Davies, W.J., (eds.) Physiology, Growth and Development of tissue culture aspects. Acta Hortic. 227, 489-499.

Ziv, M., 1989. Enhanced shoot and cormlet proliferation in liquid cultured gladiolus buds by growth retardants. Plant Cell Tiss. Org. Cult. 17, 101-110.

Ziv, M., Ronen, G., Raviv, M., 1998. Proliferation of meristematic clusters in disposable presterlized plastic bioreactors for large scale micropropagation of plants. In vitro Cell Dev. Biol. Plant 34, 152-158.

Zu, Y., Tang, Z., Yu, J., Liu, S., Wang, W., Guo, X., 2003. Different responses of camptothecin and 10-hydroxycamptothecin to heat shock in *Camptotheca acuminata* seedlings. J Integrative Plant Biol. 7, 809-814.

7.1. Abbreviations

λ_{em}	wavelength emission
λ_{ex}	wavelength excitation
µg	microgram
µm	micrometre
µmol	micro mole
10-HGO	10-hydroxygeraniol oxidoreductase
2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-(γ,γ-dimethylallylamino)-purine
ANOVA	analysis of variance
Approx.	approximately
aqua dest.	distilled water
B21	2 mg l^{-1} BAP plus 0.1 mg l^{-1} 2,4-D
B22	2 mg l^{-1} BAP plus 0.5 mg l^{-1} 2,4-D
B5	Gamborg B5 Medium
BAP	6-benzylaminopurine
BI	2 mg l^{-1} BAP plus 0.1 mg l^{-1} IAA
Chl	chlorophyll
CIRAD	Centre de coopération internationale en recherche agronomique pour le développement
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
cm	centimetre
CPT	camptothecin
CPT-11	irinothecan (Camptosar®)
Decne	Decaisne
DNA	desoxyribonucleic acid
DVS	Dual Vessel System
DW	dry weight
DXP	1-deoxy-D-xylulose-5-phosphate
EDTA	ethylenediaminetetraacetic acid
FDA	fluorescein diacetate
Fig.	figure
FW	fresh weight
G10H	geraniol-10-hydroxylase
GA_3	gibberellic acid
HCPT	10-hydroxcamptothecin
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HW	hardwood
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IC d^{-1}	immersion cycles per day
K21	2 mg l^{-1} Kin plus 0.1 mg l^{-1} 2,4-D
K22	2 mg l^{-1} Kin plus 0.5 mg l^{-1} 2,4-D
Kin	*N*-furfuryl-7-*H*-purin-6-amine (Kinetin)
Lys	lysine
m	metre
m^{-2} s^{-1}	square metre per second

MCPT	9-methoxycamptothecin
MECS	2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase
mm	millimetre
MS	Murashige and Skoog's Medium (1962).
NAA	1-naphthaleneacetic acid
NCI	National Cancer Institute
NiP	0.1 mg l^{-1} 2iP plus 0.2 mg l^{-1} NAA
Orn	ornithine
PGR	plant growth regulator
Phe	phenylalanine
PPFD	photosynthetic photon flux density
RITA®	Récipient à Imersion Temporaire Automatique
rpm	revolutions per minute
SD	standard deviation
SE	standard error
SE	somatic embryogenesis
SHW	semi-hardwood
SLS	secologanin synthase
SSS	strictosidine synthase
SSV	sand, soil and vermiculite
SV	sand vermiculite
SW	softwood
Tab.	table
TDC	tryptophan Decarboxylase
TDZ	1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea
TIA	terpene indole-alkaloid
TIS	Temporary Immersion System
Topo I	topoisomerase I
TPT	topothecan (Hycamtin®)
TSB	β-subunit of tryptophan synthase
Trp	tryptophan
USDA	United States Department of Agriculture
UV	ultraviolet radiation
v/g	volume to gram
v/v	volume to volume
v/v/v	volume to volume to volume
w/v	weight by volume (concentration)
WPM	Woody Plant Medium (Lloyd & McCown)

7.2. List of Figures

7.3. List of Tables

Sankar-Thomas, Y.D., Saare-Surminski, K., Lieberei, R., 2008. Plant regeneration via somatic embryogenesis of *Camptotheca acuminata* in temporary immersion system (TIS) Plant Cell Tiss. Org. Cult. 95, 163-173.

I am most grateful to my supervisor Prof. Dr. Reinhard Lieberei for accepting me as Ph.D. student and for giving opportunity to work in the fascinating world of plant tissue cultures. I also thank him for his continuous guidance and advice throughout my scientific work and for the review of this manuscript. I sincerely thank Dr. Katja Saare-Surminski for her supports during the tenure of my thesis, for her critically review of this manuscript and the valuable comments on my work.

I also express my thanks to Dr. Helmut Kassner and the remaining colleagues of the department for their valuable tips, practical help and support during my PhD thesis.

I am also grateful to Nicolas Niemenak and Douglas Steinmacher from whom I have gained a great knowledge and enthusiasm for somatic embryogenesis.

My special thanks to Detlef Boehm and Thomas Tumforde, the most friendly and helpful laboratory technicians I have worked with. It was great to have someone to turn to, at any time with any kind of chemical or technical questions, which were then readily solved. I also thank Thomas Tumforde for his collaboration with the camptothecin analyses.

I sincerely thank Mike Cummins and Prof. Dr. Jens G. Rohwer for the proofreading of my manuscript.

I specially thank Mark Bulk, (Tree Nursery, Boskoop, NL) who twice gave me his last *Camptotheca* plants and to Franz Tod, Botanic Garden of Vienna for sending me without hesitation *C. acuminata* cuttings when ever it was necessary. I also thank Eric Gouda (Botanic Garden of Utrecht, NL) and Dr. Wolfram Lobin (Botanic Garden of Bonn) for providing me cutting material. I am also thankful to Prof. Dr. Liu Zhijun of the School of Renewable Natural Resources Louisiana State University, USA who has provided me many times with fresh harvest *C. acuminata* seeds from the plantation in Louisiana.

Finally, I am deeply grateful to my husband, without whose encouragement, patience and perseverance, this thesis would not have been completed. He successfully helped me to overcome all dolefulness and difficulties during the tenure of my work. I also hope that my daughter Chandra and son Maart would not want to remember the times I have stolen from them during the course of my studies and writing this thesis. As a token of my appreciation I herewith dedicate this thesis to my husband and two children. I sincerely thank my in-laws for their continuous private and financial support.