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# DIPLOMARBEIT

Somatische Embryogenese bei Edelkastanie (*Castanea sativa* Mill.)

Magister/Magistra der Naturwissenschaften (Mag. rer.nat.)

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## KAPITEL 1

### EINLEITUNG

#### 1.1. Allgemeines zur Edelkastanie

##### 1.1.1. Systematische Einordnung, Herkunft und Verbreitung der Gattung *Castanea*

Die Gattung *Castanea* gehört gemeinsam mit *Fagus* (Buche), *Nothofagus* (Südbuche) und *Quercus* (Eiche) zur Familie der **Fagaceae** (Buchengewächse). *Fagaceae* und *Betulaceae* bilden die Ordnung der *Fagales*, ein Taxon der *Dicotyledonen*-Unterklasse der *Hamamelididae* (=Amentiferae). Diese nach Artenzahl kleine Ordnung enthält die wichtigsten Laubbäume der nördlich temperierten Regionen [Frohne 1992].

Es gibt sieben *Castanea*-Arten (*C. mollissima* Bl.; *C. seguinii* Dode.; *C. crenata* Sieb. & Zucc.); *C. dentata* (Marsh.) Borkh.; *C. sativa* Mill.; *C. pumila* Mill.; *C. henryi* (Skan) Rehder & Wilson), die **nordhemisphärisch**, in den temperaten Zonen Asiens und Europas und im Osten der Vereinigten Staaten beheimatet sind [Dane 2003]. Abbildung 1 zeigt die **interkontinental disjunkte Verbreitung** der Gattung *Castanea*. DNA Sequenzierungsdaten des Chloroplastengenoms bestätigen den monophyletischen Charakter der Gattung und legen den Ursprung des Taxons in Ostasien nahe [Lang 2007]. Die somatische Chromosomenzahl beträgt in allen Arten 24 [Jaynes 1975].

*Castanea sativa* ist eine **pontisch-kaukasische** Art. Seit der Zurückdrängung aus Nordeuropa in der letzten Eiszeit liegt die Hauptverbreitung der europäischen Edelkastanie (*Castanea sativa* Mill.) in Südeuropa und Westasien. In Österreich ist die Edelkastanie wohl nirgends ursprünglich heimisch, sie ist ein aus Kleinasien stammender Archäophyt. Als Obstbaum kultiviert kann sie in naturnahen Waldgesellschaften in milden Klimatalagen verwildern [Adler 1994].

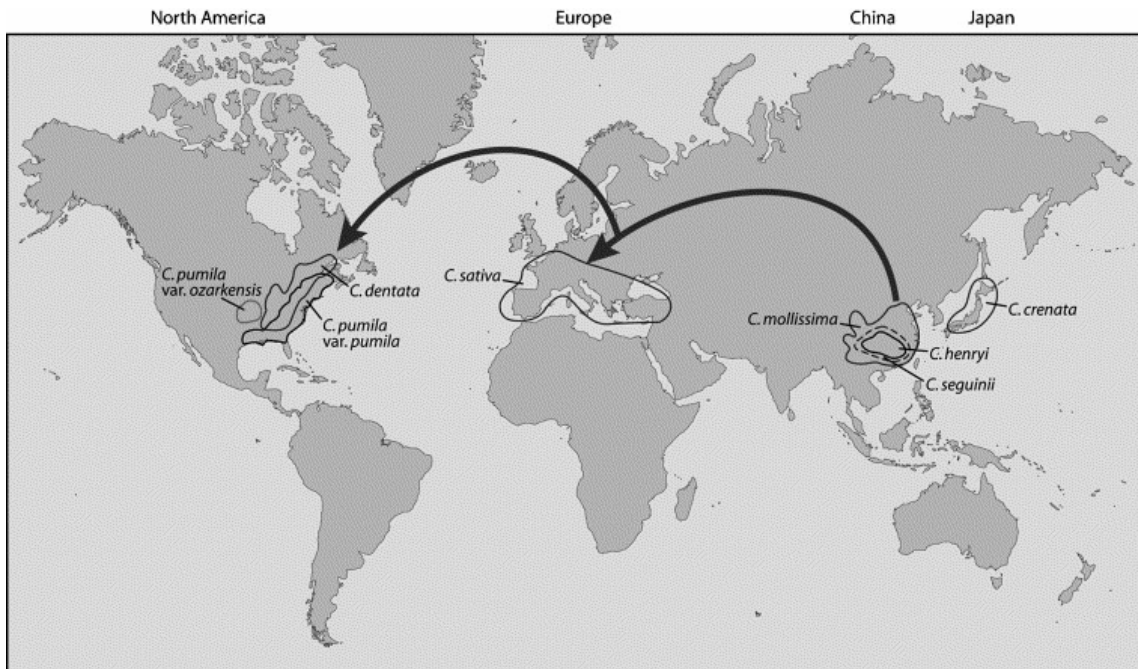


Abbildung 1. Distribution of the genus *Castanea* and a hypothesized westward migration route indicated by arrows based on cpDNA sequence information [Lang 2007].

Neben der hier behandelten europäischen Edelkastanie sind verbreitete Arten der Gattung die amerikanischen Edelkastanien *C. dentata* und *C. pumila* (American Chinkapin), die asiatischen Arten *C. crenata* aus Japan und Korea und *C. mollissima* aus China (siehe Abb. 1.).

#### 1.1.2. Biologie und Ökologie der Edelkastanie

Die 15 – 35 m hohen Bäume haben breite Kronen und sind sehr raschwüchsig. Die Borke ist längsrissig und hat typische Lentizellen (Abbildung 2). Die Laubblätter sind 10-20 cm lang, lanzettlich und grob gezähnt, die Stipel sind hinfällig.

Die **monöcischen Blüten** erscheinen nach den Laubblättern. Am Grunde der männlichen Kätzchen befinden sich die weiblichen Blütenstände. Sie bestehen meist aus drei Blüten mit ebenfalls unscheinbarer Blütenhülle (Abbildung 3) und sind von einer Cupula umgeben. Es finden sich Ansätze von Zwitterblütigkeit und Zoogamie. Der Pollen ist zunächst von



klebrigem Pollenkitt überzogen und wird von Insekten (Käfer, Bienen) aufgesucht, trocknet dann aber aus und wird vom Wind vertragen [Strasburger 1983]. Die Blütezeit liegt in den Monaten Juni bis Juli. Die Samenanlagen haben zwei Integumente, wovon eines zur Testa wird. *Castanea* ist autosteril. Der Fruchtbecher (**Cupula**) ist eine Zusatzbildung aus Achsengewebe, er verholzt und ist mit Stacheln bewehrt. Er ist den Fruchtbechern der Eichen und Buchen **homolog**. Bei der Fruchtreife springt die Cupula vierklappig auf und gibt ein bis drei Samen (Maronen) frei (Abbildung 4). Der Embryo ist durch eine dünne behaarte Pellicula und eine braune Testa geschützt.

Bevorzugte Standorte sind kalkarme, meist bodensaure Laub- und Eichen-Föhrenwälder in **colliner bis submontaner** Höhenstufe [Adler 1994]. Relativ tiefe Wintertemperaturen werden zwar ertragen, Jungtriebe sind aber gegenüber Frühjahrsfrösten empfindlich. Die Edelkastanie verlangt viel Licht und Wärme. In nördlicheren Lagen kommen vor allem Weinbaugebiete den Ansprüchen der Kastanie entgegen [Rusterholz 1999].

**Streuobstbestände** gehören wegen ihrer Strukturvielfalt zu den wertvollsten Lebensräumen. Sie werden von einer enormen Anzahl von Insekten und anderen Tieren genutzt. Das an älteren Bäumen starke Auftreten von Totholz, und damit verbunden von Hohlräumen und Baumhöhlen, führt zu einem verstärkten Angebot von Nistgelegenheiten und Lebensräumen für Höhlenbrüter und –bewohner [www.gehoelze.ch]. Für Wildtiere sind die Nüsse der Edelkastanie eine bedeutende Futterquelle, Vögel und Nager sorgen auch für die Verbreitung der Früchte. Die Bäume sind eine wichtige Bienenweide.



Abbildung 2. Borke mit Lentizellen © Sauer



Abbildung 3. weibliche Blütenstände am Grunde eines Kätzchens © Sauer



Abbildung 4. Reife Früchte und vierklappig geöffnete Cupula. © Sauer

### 1.1.3. Nutzung und wirtschaftliche Bedeutung der Edelkastanie

Die vielseitigen Verwendungsmöglichkeiten machen die Edelkastanie zum typischen "**multiple option crop**". Die Geschichte der Edelkastanienkultur (*C. mollissima*) reicht in China 6000 Jahre zurück. Der Ursprung der europäischen Edelkastanienkultur wird im Mittelmeerraum oder in Kleinasien vermutet. In Südeuropa wird die europäische Edelkastanie seit der Antike in den Speiseplan eingebaut. Aus Kastanienmehl gebackenes Brot half in den traditionellen Anbauländern über so manche Hungersnot hinweg. Ein Indiz für die frühe Bedeutung als Nutzpflanze ist die Anordnung Karls des Großen in seiner Landgüterverordnung (Capitulare de villis) **Castanearios** anzulegen. Wie Eicheln werden Kastanien in der Schweinemast verwendet. Mit der Einführung der Kartoffel schwand die Bedeutung der Kastanie vor allem nördlich der Alpen wieder.

Aufgrund der Toleranz gegenüber pH-Werten zwischen 5 und 6,9 können Kastanienplantagen auf Randertragsböden gepflanzt werden. Weiters zeichnet die Bäume Schattentoleranz und Trockenresistenz aus. Auch in sehr trockenen Jahren werden Früchte produziert.

Ein adulter Baum kann pro Jahr über **100 kg Nüsse** liefern [Vieitez 1995]. Diese sind mit 10 - 15% Proteinanteil und 40% Kohlehydratanteil ernährungsphysiologisch wertvoll. Der Fettanteil liegt bei nur 2-3%. Mandeln, Haselnüsse und Walnüsse haben im Vergleich dazu über 50% Fett. Die Früchte können roh, geröstet oder gekocht gegessen werden. Es werden Mehl, Püree und Konfiserieartikel hergestellt.

Der verglichen mit anderen Nüssen hohe Zuckergehalt macht sie allerdings für die Besiedelung mit **Schimmelpilzen** höchst anfällig. Eine Untersuchung in Kanada zeigte, dass besonders Nüsse, deren Samenschale von Insekten aufgebrochen wurde, einen hohen Befall mit Pilzen, die gefährliche Mykotoxine produzieren, aufweisen [Overy 2003].

Die Vermarktung von essbaren **Mycorrhiza** – Pilzen wie *Boletus edulis* kann den Gewinn einer Plantage erheblich steigern. Die Kultivierung von Steinpilzen durch eine stabile Kolonisierung der Pflanzen ist bisher allerdings nur *in vitro* und im Glashaus gelungen.

Nach Auspflanzung werden die Mycelien von anderen Ectomycorrhizapilzen verdrängt [Peintner 2007].

Heute sind vier Edelkastanien - Arten wirtschaftlich bedeutsam, nämlich. *C. dentata* Borkl. in den USA, *C. crenata* Sieb. & Zucc. in Japan, *C. mollissima* Bl. in China und *C. sativa* Mill. in Europa. Die jährliche Weltproduktion beträgt circa eine halbe Million Tonnen Früchte, wobei China mit geschätzten 80 000 - 120 000 Tonnen pro Jahr Marktführer ist, vor Korea mit etwa 80 000 Tonnen. Japan steht an erster Stelle beim Kastanienimport, vor allem aus China und Korea. In Europa sind die Hauptproduzenten Kroatien, Italien, Spanien, Frankreich, Portugal und die Türkei (Tabelle 1). Sozioökonomische Veränderungen, mangelnde Mechanisierungsmöglichkeiten, vor allem aber zwei Pilzkrankungen führten in Europa seit Beginn des 20. Jahrhunderts zum Niedergang des Edelkastanienanbaus. So ging die Produktion in Italien und Frankreich von 1900 bis 1965 um 85% zurück [Chua 1996]. Die Züchtung resistenter Kultivare, Schädlingskontrolle und bessere Kultivierungstechniken haben in den letzten Jahren wieder zum stetigen Anstieg der Anbauflächen geführt. Neben den traditionellen Anbauländern zeigen auch Brasilien, Chile, Argentinien, Australien und Neuseeland vermehrt Interesse, da Kastanienprodukte vor allem in den USA und Japan hohe Preise erzielen. 3-4 Jahre nach dem Anlegen einer Plantage können die ersten Nüsse geerntet werden, positiver cash flow ist nach 5-8 Jahren zu erwarten.

Tabelle 1. Jährliche Produktionszahlen einiger europäischer Anbauländer nach [Chua 1996] und Zahlen der FAO 1999 [<http://www.fao.org/DOCREP/006/AD235E/ad235e04.htm#TopOfPage>].

	Chua 1996	FAO 1999
<b>Kroatien</b>	70 000 t	
<b>Türkei</b>	52 000 t	70 000 t
<b>Italien</b>	50 000 t	78 000 t
<b>Frankreich</b>	20 000 t	13 000 t
<b>Spanien</b>	20 000 t	20 000 t
<b>Griechenland</b>	18 000 t	11 000 t
<b>Portugal</b>		20 000 t

Neben den Früchten wird auch das dauerhafte, gerbstoffreiche, mit Eiche vergleichbare Holz genutzt (Tabelle 2). Dieses ist ringschällig und wird unter anderem beim Möbel- und Brückenbau und im Lawinenverbau geschätzt. Das Holz wird nach DIN 68364 (EN 350) in Resistenzklassen eingeteilt, die anzeigen, wie lange Holz ohne Konservierungsmaßnahmen Widerstandskraft gegen holzerstörende Organismen zeigt. Dabei werden 5 Stufen unterschieden: 1 = sehr resistent; 2 = resistent; 3 = mäßig resistent; 4 = wenig resistent; 5 = nicht resistent.

Tabelle 2. Eigenresistenz von Holz nach DIN 68364

Resistenzklasse	Haltbarkeit	Baum-/Holzart
R 1-2	15-25 Jahre	Robinie
R 2	15-25 Jahre	Edelkastanie ( <i>Castanea sativa</i> ), Eiche
R 3-4	10-15 Jahre	Douglasie, Lärche, Kiefer
R 4	unter 10 Jahre	Fichte, Tanne, Ulme
R 5	gar nicht	Erle, Buche, Birke, Pappel

Blätter und Rinde der Edelkastanien enthalten **Tannine**, die in der Lederindustrie Anwendung finden. Heißwasserextrakte der Edelkastanie sind zusammen mit Mimosen- und Quebracho-Extrakten die wichtigsten pflanzlichen Tanninquellen für die Lederindustrie [Krisper 1992]. Die Hauptkomponente ist Castalagin, zusammen mit kleineren Mengen Vescalagin, Castalin, and Vescalin. Das Hamamelitannin der Rinde kommt auch in Hamamelis vor und war einer der ersten rein dargestellten und strukturell aufgeklärten Gerbstoffe [Frohne 1992]. Blattextrakte von *Castanea sativa* enthalten **phenolische Komponenten**, die eine antioxidative Wirkung zeigen, vergleichbar mit *Ginkgo biloba*, *Pinus maritima* und *Vitis vinifera*, und die in Medikamenten und Kosmetika eingesetzt werden [Calliste 2005].

In der Kräuterheilkunde werden getrocknete Blätter als **hustenstillendes** und leicht **antiseptisches** Mittel für die Atemwege verwendet (*Folia Castaneae*). Die Rinde hat adstringierende Wirkung auf die Haut und den Magen- und Darmtrakt.

#### 1.1.4. Krankheiten und Schädlinge

##### 1.1.4.1. Kastanienrindenkrebs

*Castanea dentata* war die bestimmende Baumart von den Appalachen bis ins Mississippi-Becken. Anfang des 20. Jahrhunderts wurde der Pilz ***Cryphonectria parasitica*** Murr (Barr) (Ascomycetes), der Erreger von Kastanienrindenkrebs, in die USA eingeschleppt, wo er in nur 50 Jahren die ausgedehnten Edelkastanienbestände fast ausrottete. Bis in die 40er Jahre vernichtete das gefürchtete Forstpathogen etwa 3 Milliarden Bäume. In den 30er Jahren schließlich griff die Epidemie auf Europa über. Von Norditalien her breitete sich das Pathogen über ganz Europa aus. 1938 wurde der Pilz erstmals in der Nähe von Genua beschrieben [Heiniger 1994], in den 60er Jahren gelangte er auch nach Österreich und breitete sich stark aus. Eine Katastrophe wie in den USA blieb aber aus, da sich die europäische Edelkastanie als etwas weniger anfällig erwies [www.waldwissen.net]. Ursprünglich ist der Pilz ein Pathogen an *C. mollissima* und *C. crenata*. Die beiden asiatischen Arten haben aber hohe natürliche Resistenz gegen die Pilzerkrankung. Die aus *Castanea mollissima* isolierten Substanzen Mollisin und Castamollin wurden als natürliche Fungizide identifiziert. [Chu 2003, Wang 2003].

Der Pilz dringt über Wunden in den Baum ein und zerstört zunächst das umliegende Gewebe. Nekrosen, Kollaps des Rinden- und Kambiumgewebes, Welken der Blätter Absterben von Zweigen und schließlich Absterben der Bäume sind die weiteren Folgen [Seemann 1995] (Abbildungen 5 und 6).

Bei der Bekämpfung kommen Fungizide, die Züchtung resistenter Hybriden und die **biologische Schädlingskontrolle** mittels **hypovirulenter Pilzstämme** zum Einsatz. Stellenweise wurden untypische, leichtere Krankheitsbilder – also verminderte Virulenz - beobachtet, die Bäume überlebten [Grente 1965]. Die aus ihnen isolierten Pilze waren von einem Microvirus befallen der sich in Form von dsRNA nachweisen lässt und den Pilz schwächt. Die dsRNA kann auf virulente Pilzstämme übertragen werden, die dann als hypovirulente Stämme bezeichnet werden [Bazzigher 1981]. Die Hypovirulenz breitet sich auch natürlich in Europa aus, wahrscheinlich über Konidien, die dsRNA enthalten und mit dem Wind oder von Insekten verbreitet werden.

Die Übertragung der Hypovirulenz funktioniert nur innerhalb gleicher so genannter vegetativer Kompatibilitätsgruppen (vc-Gruppen), was den Einsatz dieser Bekämpfungsmaßnahme in Nordamerika auf Grund vieler verschiedener vc-Gruppen schwierig macht. In Österreich wurde auch schon natürliche Hypovirulenz gefunden, es gibt mindestens 7 vc-Gruppen [Wronski 1997]. Die biologische Bekämpfung mittels Hypovirulenz wird in Österreich auch zur Erhaltung von wertvollen Einzelbäumen eingesetzt [Wilhelm 2001].



Abbildung 5: Läsion, Agfalva (H) © Sauer



Abbildung 6. Teilweise abgestorbener Baum in Cac (H) © Sauer

Neben der kurativen Behandlung mit Hypovirulenz ist auch ein **präventives** Verfahren im Teststadium:

***Bacillus subtilis*** ist ein endophytisches Bakterium aus dem Gefäßsystem von Edelkastanie, das *in vitro* eine starke Hemmwirkung auf das Pilzwachstum ausübt. In Inokulationsversuchen im Labor an mikrovegetativ vermehrten Kastanienpflänzchen zeigte sich, dass es zu einer Verzögerung des Krankheitsverlaufes kommt. Die Freilandversuche brachten allerdings bis jetzt keine positiven Ergebnisse [Arthofer 1996, Wilhelm 1997].

#### 1.1.4.2. Tintenkrankheit

Das Forstpathogen *Phytophthora* (*Phytophthora cinnamomi* und *P. cambivora*) wurde 1838 zum ersten Mal an portugiesischen Edelkastanien beschrieben, es ist vor allem in den Mittelmeerländern stark verbreitet. Ein befallener Baum zeigt dunkle Verfärbungen am Wurzelkambium und an der Stammbasis. Schließlich wird die Krone schütter und der Baum stirbt ab. Kreuzungen mit den beiden asiatischen Arten *C. crenata* und *C. mollissima* führten zu resistenten Hybriden [Anagnostakis 1995].

#### 1.1.4.3. Insektenbefall

Befall mit dem Esskastanienbohrer *Curculio elephas* (Coleoptera: Curculionidae) und verschiedenen *Cydia* – Arten (Lepidoptera: Tortricidae), den Kastanienwicklern, kann die Zahl der vermarktbareren Nüsse erheblich vermindern [www.waldwissen.net]. Darüber hinaus spielen die Schädlinge auch bei der Besiedlung der Nüsse mit Pilzen eine Rolle.

Larven von *Spulerina simploniella* (Lepidoptera: Gracilariidae) minieren die Borke unter der Epidermis und bilden so neue Infektionsstellen für *Cryphonectria parasitica*.

Bei der Bekämpfung kommen neben Insektiziden auch *Bazillus thuringensis* und *Baculovirus* zum Einsatz.

## 1.2. Vegetative Vermehrung der Edelkastanie

### 1.2.1. Traditionelle forstliche Vermehrungsmethoden

Die wurzelechte vegetative Vermehrung über Stecklinge oder Ableger, also die Regeneration ganzer Pflanzen aus Teilstücken, ist bei der Edelkastanie nicht leicht zu bewerkstelligen. Wie andere großsamige Hölzer sind auch Edelkastanien sehr schwer vegetativ zu vermehren. Erfolg versprechender als die Stecklingsvermehrung ist die Methode des „Anhäufelns“ und die „Ablegermethode“ [Bazzigher 1982], beide sind allerdings sehr zeit- und arbeitsaufwändig. Stockausschläge kommen hingingen häufig vor, deshalb wurden Kastanienwälder oft als Niederwälder bewirtschaftet. Diese lieferten Rebstecken und leichtes Bauholz, sind aber heute oft unrentabel und aufgelassen.



Als heterovegetative Vermehrung bezeichnet man das Pfropfen von Reiseren oder Okulieren von Knospen ausgewählter Mutterpflanzen auf bewurzelte „fremde“ (also einer anderen Sorte) Unterlagen. In der Forstpflanzenzüchtung dienen Pfropfplantagen der Gewinnung von genetisch hochwertigem Saatgut [Aas 1992].

Die generative Vermehrung hat vor allem Bedeutung für die Züchtung neuer hochwertiger Sorten und für den Erhalt der genetischen Variabilität der Populationen.

### 1.2.2. *In vitro* Vermehrung

#### 1.2.2.2. Stecklingsvermehrung

Die vegetative Vermehrung über Mikrostecklinge dient der Massenvermehrung von herausragenden Genotypen, vor allem solchen die Resistenz gegen *Cryphonectria parasitica* und *Phytophthora cinnamomi* zeigen. Die Methode stellt eine viel versprechende Alternative zur konventionellen forstlichen Vermehrung dar, wenn optimierte Protokolle eine hohe Reproduzierbarkeit garantieren können, und Probleme mit Spitzennekrosen und dem Fehlschlagen der Bewurzelung bewältigt werden. Dazu müssen Faktoren wie Kohlenstoffquellen, geeignete Basalmedien, Kulturbedingungen und die Akklimatisierungsphase optimiert werden. Mikrostecklingsvermehrung ist über Adventivsprosse oder axilläre Sprosse möglich.

#### 1.2.2.2. Somatische Embryogenese

Neben Mikrostecklingsvermehrung, Vermehrung über axilläre Knospen und Adventivknospen, gewinnt die somatische Embryogenese seit Mitte der 80er Jahre verstärkt an Bedeutung. Mit somatischer Embryogenese bezeichnet man die Bildung von Embryonen aus **vegetativen Zellen** im Gegensatz zur Entstehung von Embryonen aus einer Gametenkopulation.

Somatische Embryonen sind an ihrer **bipolaren Organisation** und dem Fehlen von Gefäßverbindungen zum Muttergewebe zu erkennen und von Adventivsprossen zu unterscheiden. Die Embryo-Initialen sind durch dichtes Zytoplasma und große Kerne charakterisiert, also typisch meristematisch. Es werden globuläres-, Torpedo- und Herzstadium bis hin zum reifen Kotyledonenstadium mit deutlich erkennbarem Wurzel- und

Sprosspol unterschieden. Somatische Embryonen durchlaufen also ähnliche morphologische Stadien wie zygotische Embryonen.

Die Bildung somatischer Embryonen kommt in einigen Pflanzengattungen natürlich vor. Die Teilung von jungen Embryonen bezeichnet man als *cleavage polyembryony*. *Apomixis* nennt man die asexuelle Bildung von Embryonen aus Zellen des Embryosacks (Synergiden und Antipoden), des Nucellus oder der Integumente [George 1993]. Bei *Kalanchoe* entstehen somatische Embryonen spontan an Blatträndern.

Somatische Embryogenese *in vitro* gelang erstmals bei Karotte 1958 [Steward 1958]. Für die somatische Embryogenese bei Holzpflanzen wurden in den 60er Jahren erste Protokolle entwickelt, für Koniferen erstmals 1985.

Im Unterschied zur zygotischen Embryogenese ist eine **Induktion** der embryogenen Kompetenz der Zellen nötig, das Programm der **Genexpression** des Explantats muss auf embryogene Genexpression umgeschaltet werden. Das geschieht mittels Zugabe geeigneter Phytohormone und Kulturbedingungen. Außerdem hängt der Erfolg von der Art, dem physiologischen Alter und nicht zuletzt vom Genotyp des Explantats ab. Die Entwicklung von zygotischen Embryonen durchläuft zahlreiche verschiedene Phasen mit speziellen Mustern der Genexpression. Um die normale Embryoentwicklung bis zum lebensfähigen Keimpflänzchen *in vitro* zu erreichen, gilt es Medien- und Kulturbedingungen den verschiedenen Embryostadien anzupassen, um die Bedingungen *in planta* zu simulieren.

Generell begünstigen **Auxin** die Induktion, hemmen aber die Weiterentwicklung der Embryonen aber, während **Cytokinine** sich günstig auf die Ausdifferenzierung auswirken.

Man unterscheidet zwischen direkter und indirekter somatischer Embryogenese. Bei der **direkten somatischen** Embryogenese entstehen Embryonen am Explantat ohne zwischengeschaltete Kallusphase. Dies wurde vor allem an gametophytischen Gewebe beobachtet, beziehungsweise an Geweben, die in enger Verbindung mit dem Gametophyten stehen (Fruchtknoten, Samenanlagen, zygotische Embryonen, Keimpflänzchen). Laut einer Hypothese von Evans und Sharp können sich direkte somatische Embryonen nur aus PECDs (pre-embryogenically determined cells) bilden. [George 1993]. Die **indirekte somatische Embryogenese** ist durch eine Kallusphase

charakterisiert, wobei einzelne Zellen embryogen werden. Sharp nennt sie IEDCs (induced embryogenic determined cells) [George 1993]. PEMs (proembryogenic masses) werden die Zellaggregate genannt, aus denen Embryonen entstehen. Die Grenzen zwischen direkter und indirekter somatischer Embryogenese sind in der Praxis nicht leicht zu ziehen.

Die somatischen Embryonen können in einen Zyklus **repetitiver** oder **sekundärer** Embryogenese eingebracht werden. Das ist die Produktion von Embryonen aus primären somatischen Embryonen oder indirekt aus proliferierendem embryogenem Kallus. Dieser Prozess geht auf Kosten der Weiterentwicklung der Embryonen zu Keimpflänzchen.

Für normale **Reifung** und **Keimung** sind meist die Ernährungs-, Hormon- und Milieubedingungen am zielführendsten, die jene bei der Entwicklung eines zygotischen Samens nachahmen. Die natürliche Samenbildung ist geprägt von Wachstum, Reservestoffakkumulation, Austrocknung und oft Samenruhe (Dormanz). Bei den Hamamelididae wird nur wenig Endosperm gebildet, Reservestoffe werden vorwiegend im Embryo selbst gespeichert (Speicher cotyledonen). Eine Reifungsphase, in der die Akkumulation der Reservestoffe erfolgt, begünstigt die Keimung somatischer Embryonen. Manchmal sind zusätzliche Maßnahmen zur Brechung der Dormanz erforderlich, wie Kältebehandlung, Trocknung und Hormonzugaben.

Nach der Umwandlung in Jungpflänzchen findet die Umstellung auf Erdkultur statt. Da der Photosyntheseapparat in vitro im Allgemeinen nicht vollständig funktionsfähig ist, ist eine allmähliche Abhärtung des Keimpflänzchens nötig.

Abbildung 7 fasst schematisch den Ablauf eines Protokolls für die somatische Embryogenese bei *Castanea sativa* zusammen.

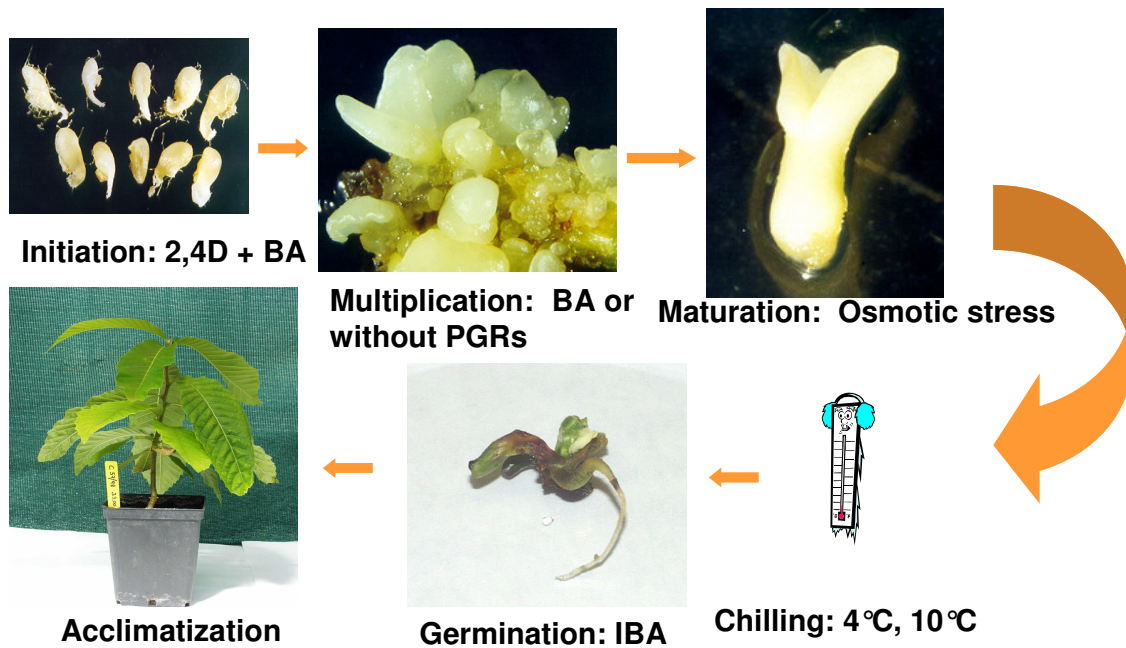


Abbildung 7. Ablauf der somatischen Embryogenese bei *Castanea sativa*. © Sauer

### 1.2.2.3. Somatische Embryogenese bei *Castanea* Arten

Seit den ersten Versuchen 1985 in Spanien (Tabelle 3) konnten einige wesentliche Fortschritte bei der somatischen Embryogenese von Edelkastanien verzeichnet werden.

Die Keimung erster Pflanzen gelang Vieitez et al. 1992. Allgemein waren die Konversionsraten von Embryonen in lebensfähige Pflänzchen sehr niedrig: Xing erreichte 9 % bei *C. dentata*, Vieitez (1995) nach Kältebehandlung 30 % bei *Castanea sativa* x *C. crenata* Hybriden, und Corredoira (2003) berichtete von 39 % gekeimten Pflänzchen. Allerdings wurden in diesen Studien die unvollständig gekeimten Embryonen mit eingerechnet, bei einem Großteil der Embryonen keimten nur die Sprosse, die anschließend bewurzelt werden mussten.

Ein sehr wichtiger Schritt gelang 2002 Corredoira mit der Induktion embryogener Kulturen aus adultem Material, nämlich an Blättchen von *in vitro* Stecklingen. Schließlich wurde mit der Etablierung von Suspensionskulturen auch der Grundstein für eine Massenvermehrung der somatischen Embryonen gelegt [Andrade 2005]. Erste Versuche mit der Kryokonservierung von Kastanienembryonen wurden bereits unternommen. 68% der in

flüssigem Stickstoff kryokonservierten somatischen Embryonen von *Castanea sativa* [Corredoira 2004] überlebten und proliferierten nach dem Auftauen.

Tabelle 3. Zusammenfassung der Literatur

Zitat	Art	Explantat	Ergebnis
Gonzales et al. [1985]	<i>C.sativa</i> x <i>C.crenata</i>	Kotyledonenscheiben	embryogener Kallus
Vieitez et al. [1990]	<i>C.sativa</i> x <i>C.crenata</i>	zygotische Embryonen	somatische Embryonen
Merkle et al. [1991]	<i>C.dentata</i>	zygotische Embryonen Samenanlagen	somatische Embryonen
Vieitez et al. [1992]	<i>C.sativa</i> x <i>C.crenata</i>		Pflänzchen
Carraway et al. [1994]	<i>C.dentata</i>		transgener Kallus
Xing et al. [1999]	<i>C.dentata</i>	zygotische Embryonen	Pflänzchen
Corredoira [2002]	<i>C. sativa</i>	Blätter von <i>in vitro</i> Stecklingen	somatische Embryonen
Corredoira [2003]	<i>C. sativa</i>	Blätter von <i>in vitro</i> Stecklingen	Pflänzchen
Corredoira [2004]	<i>C. sativa</i>		Transgene Pflanzen
Robichaud [2004]	<i>C.dentata</i>	zygotische Embryonen	Pflänzchen
Andrade [2005]	<i>C. dentata</i>		Pflänzchen Suspensionskulturen
Maynard [2006]	<i>C. dentata</i>	Embryogene Kulturen	Transgene Pflänzchen

Carraway (1994) transformierte erstmals embryogene Kulturen von *Castanea dentata* mittels „Particle Bombardment“. Allerdings konnte der Gentransfer nur an Kallus nachgewiesen werden, der in Folge keine Embryonen mehr bildete. Corredoira (2004) und

Maynard (2006) berichten über erfolgreiche Transformationsversuche mittels *Agrobacterium tumefaciens*. Transformierte Embryonen konnten in beiden Arbeiten zur Keimung gebracht werden, wenn auch nur in geringem Ausmaß und meist ohne Keimung des Wurzelpols.

#### 1.2.2.4. Anwendungsmöglichkeiten der Somatischen Embryogenese

Mit dem Schädlingsdruck auf *Castanea* stiegen auch die Anstrengungen den Bedarf an resistenten Bäumen zu decken. Neben den traditionellen forstlichen Vermehrungs- und Züchtungsmethoden, die aufgrund des langen Lebenszyklus von Bäumen viel Zeit kosten, kommen seit den Mitte der 80er Jahre auch *in vitro*-Techniken zum Einsatz. Die Möglichkeit zur Automatisierung (*scale-up*) und zum Gentransfer sind Vorteile der Gewebekulturtechniken.

Neben Mikrostecklingsvermehrung, Vermehrung über axilläre Knospen und Adventivknospen, gewinnt die somatische Embryogenese als *in-vitro* Technik verstärkt an Bedeutung. Sie stellt eine effiziente und vor allem schnelle Methode dar, Holzpflanzen im großen Maßstab zu klonen. Durch den Einsatz von Bioreaktoren könnte die Produktivität noch einmal gesteigert werden. So werden zum Beispiel in Kanada schädlingsresistente *Picea sitchensis* Pflanzen in großem Maßstab aus somatischen Embryonen erzeugt [Thompson 2005]. Die kanadische Biotech Firma Cellfor® Inc. produzierte weltweit von 1996 bis 2003 etwa 8 Millionen Koniferenkeimlinge, vor allem *Picea sitchensis*, *Pinus radiata*, *Pinus taeda* und *Pseudotsuga menziesii*.

Kryokonservierung und künstliches Saatgut dienen zur Erhaltung von Genressourcen und können einen Beitrag zu Wiederaufforstungsprogrammen leisten. Somatische Embryonen können im Labor das ganze Jahr über in großem Maßstab produziert werden, das Ausbringen der Setzlinge ins Freiland ist aber nur einige Wochen im Jahr möglich.

Kryokonservierte somatische Embryonen sichern die langfristige Erhaltung von wertvollen Zelllinien. Dabei werden die vorher mit „Cryoprotectants“ (Substanzen die die intrazelluläre Eisbildung hintanhaltend) behandelten Kulturen langsam abgekühlt und schließlich in flüssigem Stickstoff bei -196 °C gelagert. Nach dem Auftauen regenerieren die Kulturen und bilden neue somatische Embryonen.

Unter künstlichem Saatgut versteht man somatische Embryonen die in Gele eingeschlossen (zum Beispiel Natrium-Alginat) werden. Das Gel kann auch mit Nährstoffen,

Zucker und Wuchsstoffen versetzt werden, also sozusagen als künstliches Endosperm dienen [Prewein 2003].

Ein wichtiges Anwendungsgebiet der somatischen Embryogenese ist die Gentechnik, deren Erfolg von einem verlässlichen Regenerationssystem abhängt. Sie eröffnet die Möglichkeit der Aufklärung von Pflanze-Pathogen-Interaktionen durch Gentransfer oder des Einbringens von Pilzresistenzgenen in die Pflanze.

### 1.3. Zielsetzung

Das Ziel der Untersuchung ist ein Protokoll zur somatischen Embryogenese von Edelkastanien zu entwickeln. Dies beinhaltet die **Induktion somatischer Embryonen** bei *Castanea sativa*. Explantate von drei verschiedenen Mutterbäumen werden verglichen. Von besonderem Interesse ist der Einfluss des **Entwicklungsstadiums**, also des Erntezeitpunktes der Explantate. Der für die Bildung somatischer Embryonen günstige Zeitraum, das Zeitfenster der Induktion, soll bestimmt werden. Erntedatum, Größe der Früchte und Größe und Wassergehalt der zygotischen Embryonen werden als Parameter die diese Entwicklungsstadien herangezogen.

Weiters wird die **embryogene Kompetenz** von verschiedenen Gewebetypen beziehungsweise Organen (Fruchtknoten, Samenanlagen, ganze Embryonen, Keimblätter, Wurzelanlagen) vergleichend untersucht.

Die Bedingungen für Erhaltung und **Vermehrung embryogener Kulturen** sollen optimiert werden, mit dem Ziel hochwertige Embryonen ohne Anomalien zu produzieren. Weiters werden Protokolle für die **Reifung und Keimung** der Embryonen getestet. Schließlich wird gezeigt, dass eine Umwandlung der somatischen Embryonen in lebensfähige Pflänzchen und eine Akklimatisierung im Glashaus möglich ist.





## KAPITEL 2

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### **IMPROVING CHESTNUT MICROPROPAGATION THROUGH AXILLARY SHOOT DEVELOPMENT AND SOMATIC EMBRYOGENESIS.**

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# Improving chestnut micropropagation through axillary shoot development and somatic embryogenesis

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## Abstract [Review article]

The effects of the mineral media and the carbon source on the proliferation capacity of different *Castanea sativa* x *C. crenata* cultivars were the focus of the research reported here. Using the commercial cultivar Marigoule, the addition of riboflavin to the rooting medium did not improve the rooting rates recorded. It did, however, positively affect the survival of regenerated plantlets after weaning. By measuring certain physiological parameters, the beneficial effect of high concentrations of CO<sub>2</sub> on the acclimatization of chestnut regenerated plantlets was recorded. However, general protocols for large-scale micropropagation of specific cultivars could not be defined.

Our research has determined for the first time the developmental window in which somatic embryogenesis induction is possible from ovaries, ovules and/or zygotic embryos in *C. sativa*. Induction is possible between the 2<sup>nd</sup> and the 10<sup>th</sup> weeks post-anthesis, giving an overall frequency of 4.5%. Somatic embryogenesis induction in chestnut was not possible from mature tissues; however, embryogenesis was achieved using leaf tissue from shoot multiplication cultures. This indicates, for the first time, that material from explants other than zygotic chestnut embryos is competent for somatic embryogenesis.

The effect of thidiazuron on the ability of different seedling explants of chestnut to induce multiple shoots was also evaluated: cotyledonary node explants, which contain preformed meristematic tissue, were the only responsive explants.

Keywords: Axillary shoots, *Castanea sativa*, *C. sativa* x *C. crenata*, chestnut, micropropagation, somatic embryogenesis

## 1 Introduction

The two chestnut species, *Castanea sativa* (European chestnut) and *C. dentata* (American chestnut), have, for a long time, suffered from ink disease and chestnut blight. Ink disease is the result of attacks by the fungus *Phytophthora cinnamomi*, and chestnut blight by *Cryphonectria parasitica*. A substantial body of research on the chestnut still focuses on the development of vegetative propagation systems capable of satisfying the demand for elite genotypes, and more specifically, genotypes resistant to both diseases. As an alternative to conventional vegetative propagation methods, efforts are being made to establish reliable *in vitro* regeneration systems that allow the clonal propagation of these materials.

*In vitro* tissue techniques have been applied to chestnut regeneration since the 1980's (VIEITEZ and VIEITEZ 1980a, 1980b; VIEITEZ *et al.* 1983). However, certain points need to be addressed in order to apply the technique more widely. Bearing in mind that *in vitro* establishment in chestnut is possible from both juvenile and mature material (VIEITEZ and VIEITEZ 1980a, SÁNCHEZ *et al.* 1997), points such as the selection of culture medium, carbon source, rooting stage and acclimatisation period need investigation.

In addition, several papers have shown the potential of somatic embryogenesis not only as an alternative clonal system for chestnut micropropagation (VIEITEZ *et al.* 1990, VIEITEZ 1995, CARRAWAY and MERKLE 1997, XING *et al.* 1999) but also as a tool in genetic engineering programmes (CARRAWAY *et al.* 1994, SEABRA and PAIS 1998). For these purposes, the development of adventitious shoots would be an alternative. However, systems based on adventitious shoot regeneration (SAN-JOSÉ *et al.* 1984) have not yet proved to be reliable. The use of meristematic tissues of apical or axillary buds has been reported as a target tissue that is useful for genetic transformation in species recalcitrant for somatic embryogenesis and/or adventitious shoot regeneration (MORRE *et al.* 1998, SAN-JOSÉ *et al.* 2001).

In this paper we summarise the work carried out by the Working Group 1 "Tree Physiology" within the scope of the COST G4 Action "Multidisciplinary Chestnut Research". We focus on three aspects of *in vitro* chestnut tissue culture:

- 1) optimising micropropagation protocols based on axillary shoot development;
- 2) defining protocols for induction, maturation and germination of somatic embryos; and
- 3) inducing multiple shoot formation from cotyledonary nodes.

## 2 Material and methods

### 2.1 Micropropagation through axillary shoot development

Effect of basal medium: In order to select the best basal medium, axillary shoots of five different clones were induced to proliferate on the following media: HELLER (1953) (H), MURASHIGE and SKOOG (1962) with half-strength nitrates (MS 1/2N), GRESSHOFF and DOY (1972) (GD), LEPOIVRE (QUOIRIN and LEPOIVRE 1977) (Lp), BLAYDES (1966) (Bl), SCHENK and HILDEBRANDT 1972) (SH), DRIVER and KUNIYUKI (1984) (DKW) and P24 (TEASDALE 1992). All media were supplemented with 0.2 mg/l BA. After 4 weeks of subculture under photoperiodic conditions (16 h light under 30  $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ , 25 °C temperature), the following parameters were recorded: number of shoots per explant (NSH), number of subculturable segments per explant (NS), percentage of responsive explants (R) and multiplication coefficient (mc). Data were analysed by analysis of variance.

Effect of carbon source: To study the effect of different carbon sources on the proliferation of chestnut explants from the *C. sativa* x *C. crenata* cultivars Verdesa, Torcione, SF43I and SP125 were cultivated on GD basal mineral medium supplemented with 0.2 mg/l BA and 0.0876M of sucrose, fructose, glucose or sorbitol. After a 4-week subculture period, the number of shoots produced per explant was recorded.

Rooting: The valuable commercial cultivar, Marigoule (*C. sativa* x *C. crenata*), was used for rooting experiments. Two methods were tested: a) The use of BdR (BOURRAIN *et al.* 1998) medium supplemented with 2 mg/l IBA for seven days and subsequent transfer of the shoots to an auxin-free MS medium (macronutrients diluted 1/4) plus vermiculite; and b) Riboflavin technique: BdR medium supplemented with 2 mg/l IBA, 30 g/l sucrose, and 2 mg/l riboflavine diluted in a few drops of ethanol. The shoots

were kept in the same medium for the whole rooting period. Four weeks after the beginning of experiments, the percentage of rooting and the mean number of roots per rooted shoot were recorded.

Acclimatisation: Rooted shoots of the clone MI (*C. sativa* x *C. crenata*) were transplanted to plastic pots filled with 200 cm<sup>3</sup> peat:perlite (1:2 v/v). The plantlets were acclimatised over the following four weeks in controlled chambers and subjected to CO<sub>2</sub> concentrations of either 350 or 700 µl<sup>-1</sup>. The dry weight of leaves, stem and roots were recorded. Leaf and root areas were obtained by computer image analysis.

## 2.2 Somatic embryogenesis

Induction from embryonic tissues: Chestnut burs were harvested weekly from four *C. sativa* trees at two different sites in Austria during July and August for two consecutive years. After surface sterilisation, the cupula was removed and the seeds were sectioned and separated into several tissues. Explants were cultured on solid induction P24 medium with 0.8% agar, 3% sucrose, 1 mg/l 2,4-D and 0.1 mg/l BA. After three weeks, the cultures were transferred to P24 medium supplemented with 0.2 mg/l BA but no 2,4-D.

Induction from leaf sections: Leaf and internode explants from stock shoot cultures of juvenile origin (clone 12, *C. sativa* x *C. crenata*) were used as explants. The two to three uppermost internodes (2-3 mm in length) and proximal leaf of unfurled expanding leaves were cultured on MS basal medium, supplemented with different concentrations of BA in combination with IAA or NAA. The cultures were transferred after six weeks to a medium supplemented with 0.1 mg/l BA plus 0.1 mg/l NAA for a further four weeks.

## 2.3 Induction of multiple shoot formation from cotyledonary nodes

Chestnut embryonic axes were aseptically germinated for 14 days in MS basal medium supplemented with 0.1 mg/l thidiazuron (TDZ) or 1 mg/l BA. Then the hypocotyl, epicotyl and cotyledonary node explants were cultured on MS 1/2N medium containing 0.01 mg/l NAA in combination with different TDZ concentrations. After four weeks of culture, the explants were transferred for a further eight weeks to media with low concentrations of BA. The number of explants forming callus, shoot buds and shoots longer than 5 mm were recorded every four weeks (SAN-JOSÉ *et al.* 2001).

# 3 Results and discussion

## 3.1 Micropropagation through axillary shoot development

The responses of five chestnut clones to different mineral media are shown in Table 1. According to the ANOVA results (data not shown), the model

$$X_{ijk} = \mu + C_i + M_j + C^*M_j + \varepsilon_{k(ij)}$$

(X = the observed value of each explant affected by the factor clone C; culture medium M; and their interaction M\*C) is highly significant for all parameters evaluated, both for main factors (clone and medium) and for the interaction media-clones. But the value for this interaction is comparatively much less important than the media and clone values. The media comparison results indicate that the GD medium was the best for the variable NSH, but MS (1/2 nitrates) was the most valuable mineral medium for the variable NS. Most of the shoots used for the experiment responded to the culture conditions, regardless of the medium composition used, and the multiplication coefficient (the most useful parameter for evaluating multiplication capacity) mainly ranged from two to five. In addition to the measurable parameters, the quality of the shoots should be also taken into consideration, as the success of the rooting step clearly depends on the physiological state of the shoots produced in the multiplication stage.

The results indicate that it is very difficult to recommend a mineral medium for general application; nevertheless, GD and MS (1/2 N) media appear to be the most suitable for multiplication.

Table 1. Effect of six mineral media on the proliferation of five chestnut clones. Data were recorded at the end of the multiplication stage. NSH: number of shoots per explant; NS: number of segment of explant; RE: % of responsive explants; MC: multiplication coefficient. Within each clone and column, values followed by the same letter are not significantly different at the 5% level. Student-Newman-Keuls test.

Clone	Medium	NSH	NS	%RE	MC
CIIR39 ( <i>Castanea crenata</i> <i>x C. sativa</i> )	MS	3.33 b	4.60 a	100.00 a	4.60 a
	CD	3.65 b	3.21 b	100.00 a	3.21 b
	Hm	3.21 b	3.46 b	98.33 a	3.40 b
	Lp	4.31 a	4.10 a	93.33 ab	3.82 ab
	Bl	2.28 c	2.41 c	88.33 b	2.12 c
	SH	3.60 b	3.30 b	93.33 ab	3.07 b
CHR121 ( <i>Castanea crenata</i> <i>x C. sativa</i> )	MS	2.05 c	3.55 c	100.00 a	3.55 ab
	GD	3.55 a	5.21 ab	98.33 a	5.13 ab
	Hm	2.83 b	4.31 bc	100.00 a	4.31 ab
	Lp	2.91 b	5.46 a	98.33 a	5.36 a
	Bl	2.20 c	3.38 c	93.33 b	3.15 b
	SH	2.81 b	4.40 bc	95.00 ab	4.15 ab
CIIR155 <i>Castanea sativa</i>	MS	2.31 b	4.58 a	98.33 a	4.47 a
	GD	2.71 ab	4.41 ab	95.00 a	4.20 a
	Hm	2.68 ab	4.45 ab	100.00 a	4.45 a
	Lp	3.13 a	5.01 a	100.00 a	5.01 a
	Bl	2.23 b	3.71 b	98.33 a	3.65 a
	SH	2.56 b	4.66 a	98.33 a	4.50 a
CHR162 <i>Castanea mollissima</i> <i>x C. sativa</i> )	MS	3.11 b	7.25a	100.00a	7.25 a
	GD	3.00 b	4.61 c	93.33 a	4.30 a
	Hm	3.36 b	4.83 c	98.33 a	4.74 a
	Lp	4.15 a	6.10b	98.33 a	5.99 a
	Bl	2.95 h	4.66 c	98.33 a	4.58 a
	SH	3.60 b	5.33 bc	93.33 a	4.97 a
Marigoule ( <i>C. sativa x</i> <i>C. crenata</i> )	GD	4.0	4.2	95.00	4.0
	DKW	4.3	4.4	97.00	4.3
	P24	3.2	3.3	75.00	2.5

The results obtained when four carbon sources were tested on shoot multiplication cultures of four chestnut cultivars are shown in Figure 1. The proliferation capacity of axillary shoots varied according to cultivar. Compared to fructose and glucose, a sucrose-containing medium seemed to stimulate the proliferation of Torcione, while there was little difference between the sugars in the cultivars Verdesa SP431 and SP125. When sorbitol was used as the main carbon source, the proliferation rate was significantly reduced in all cultivars. Here again, not only is the number of shoots produced important, but also the quality of the usable shoots. In this regard, sucrose was highly beneficial for the cultivars SP431 and SP125, whereas glucose had a promotive effect in Torcione and fructose in Verdesa.

There were no conclusive results on the rooting of the commercial cultivar Marigoule, as the two methods defined in Material and methods produced similar data, namely, 86% rooting rate using the BdR system and 90% with the riboflavin method. The percentage of plantlets of good quality (vigour, expanded leaves, healthy appearance) that developed after the

acclimatisation phase was higher in the riboflavin medium than with the BdR technique (72% vs 64%), but the data were not significantly different. Furthermore, the BdR technique required a secondary medium, increasing the costs of the process.

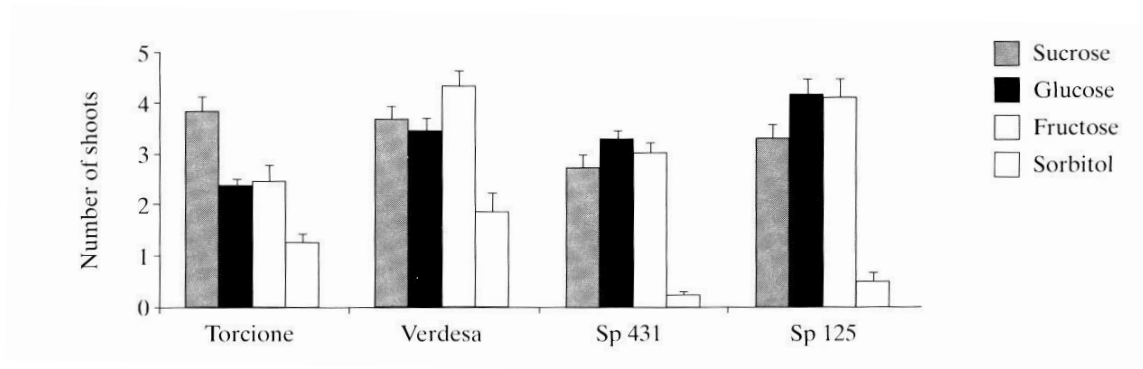


Fig. 1. Influence of carbon source on the proliferation of axillary shoots of three *C. sativa x C. crenata* cultivars grown on GD medium supplemented with 0.2 mg/l BA and 0.0876M of sucrose, fructose, glucose or sorbitol. 20 shoots were used per treatment and the experiment was repeated at least twice. Bars give the standard deviation.

In the acclimatization studies, the elevated CO<sub>2</sub> concentration did not affect the survival rates of the plantlets but gave rise to a significant increase in their relative growth, the shoot/root ratio and the leaf area ratio (see data in GONCALVES *et al.* 1999). WOLFE (1995) reports that temperate tree saplings show either little response or an increase in shoot/root ratio at high CO<sub>2</sub> levels. We recorded similar findings with the chestnut. The shoot/root ratio showed a less developed root system for the plants under the elevated CO<sub>2</sub>, in spite of the greatest diameter of their roots. This was confirmed by the root system analysis. Although the results achieved are promising, more significant gains could be expected using a higher light intensity, since the saturation light level of chestnut plantlets is near 400 μmol.m<sup>-2</sup>.s<sup>-1</sup> (GONCALVES *et al.* 1999).

As mentioned in the Introduction, *in vitro* chestnut regeneration was achieved many years ago (VIEITEZ and VIEITEZ 1980a). Large-scale propagation of chestnut species using *in vitro* techniques is still difficult. Nevertheless, around 50 000 plants per year are produced *in vitro* by a European company, which is the largest number of *in vitro* chestnut plants produced world-wide (GARCIA-NIMO 1998). We were not, however, able to define a single, general protocol for chestnut micropropagation, which means that to micropropagate a specific cultivar, the protocol will probably have to be adapted to the characteristics of each cultivar.

### 3.2 Somatic embryogenesis

For the first time it has been possible to define the developmental window for *Castanea sativa* during which somatic embryogenesis (SE) may be induced. In total, 142 SE cell lines out of more than 3000 juvenile explants were initiated during 1998 and 1999, corresponding to an overall frequency of 4.5% (5.1 % for ovaries, 3.0% for ovules and 27% for zygotic embryos). In Table 2, the frequencies of SE induction are shown from explants obtained 1 to 10 weeks post-anthesis (WPA). The embryogenic induction rate is related to the size (and water content) of the original zygotic embryo. It was demonstrated that SE induction was possible from the second to the tenth WPA, regardless of the climatic conditions of the year. The ability for proliferation via repetitive SE differed among cell lines, and direct formation of secondary embryos was frequently observed at the hypocotyl region of primary somatic embryos. The inclusion of BA in the culture medium promoted indirect secondary embryogenesis from cortical parenchyma cells, indicating a multicellular origin. This may explain the occurrence of polycotyledonary embryos, fused embryoids and other anomalous formations of somatic embryos, which were frequently observed. The fresh weight was recorded at 4-, 8-, and 12-week intervals (data not shown), and the highest fresh weight was reached after eight weeks. Cell line cultures can be cold stored (at 10°C) without subculture for at least three months, regardless of the cell line and the culture medium. In maturation assays, the positive effect of increasing the agar concentration (up to 1.1% instead of the standard concentration of 0.8%) was observed, giving a mean number of somatic embryos of 11.3 per original cluster. Five somatic-embryo-derived plants are currently growing in the greenhouse.

Table 2. Frequencies of embryogenic induction in relation to both the water content and size of the explant during two consecutive years, a1: percentage of embryo induction from ovaries; a2: percentage of embryo induction from ovules; a3: percentage of embryo induction from zygotic embryos; b: number of explants; c: percentage (%) of moisture was calculated on a fresh weight basis (FW-DW)/FWx100; DW dry weight, determined after drying at 104 °C for 24 hours.; d: length x width; e: mean value.

Date	Weeks Post-anthesis	Ovary		Ovule		Embryo		Moisture content <sup>c</sup> %	Size index <sup>d</sup> of cupula x'	Size index <sup>d</sup> of embryo x'
		n <sup>b</sup>	a1	n <sup>b</sup>	a2	n <sup>b</sup>	a3			
28.06.98	2	20	0	-	-	-	-		93.63	
13.07.98	4	43	16.3	-	-	-	-		157.0	
20.07.98	5	45	8.9	-	-	-	-		237.1	
27.07.98	6	21	19.1	33	0	1	0		269.5	13.5
03.08.98	7	20	5.0	-	-	1	0		346.3	53.0
10.08.98	8	9	11.1	7	0	18	27.8	82.2	331.7	61.3
17.08.98	9	-	-	28	7.2	17	41.2	81.6	423.9	160.0
24.08.98	10	-	-	2	0	17	47.1	81.7	571.2	254.0
21.06.99	1	52	0	-	-	-	-		39.0	
28.06.99	2	39	0	85	0	-	-		85.1	
05.07.99	3	60	0	364	0.8	-	-		164.6	
12.07.99	4	49	2.0	400	4.0	-	-		294.4	
19.07.99	5	57	5.3	501	7.8	13	30.8	81.6	339.0	6.3
26.07.99	6	48	8.3	589	2.7	17	58.8	81.6	374.3	11.0
02.08.99	7	28	0	455	0	19	10.5	80.3	432.1	40.5
09.08.99	8	-	-	56	0	22	13.6	81.5	460.7	150.5
16.08.99	9	-	-	-	-	24	25.0	81.4	579.4	262.3
23.08.99	10	-	-	-	-	23	21.7	74.2	802.6	432.5

This is the first report demonstrating that explants other than zygotic embryos are able to produce embryogenic chestnut cultures. Somatic embryogenesis induction in hybrids *C. sativa* x *C. crenata* (VIEITEZ *et al* 1990, VIEITEZ 1999) as well as in *C. dentata* (CARRAWAY and MERKLE 1997) has previously been reported. In both cases, the original explant was derived from immature zygotic embryos. In many forest tree species, the only response to embryogenic induction occurs with explants derived from juvenile tissue. In this report, the SE induction was achieved from juvenile tissues of *C. sativa*, but attempts to induce SE from adult material failed. In addition, the developmental window during which SE induction is possible has been defined for this chestnut species. Furthermore, the results reported in our work demonstrate, for the first time in the chestnut, that the induction of SE is also possible in tissue other than zygotic embryos. We found that leaf sections of *in vitro* clonal material responded to the embryogenic stimuli. Induction of SE from leaf sections has been reported for other members of the Fagaceae (CUENCA *et al.* 1999).

### 3.3 Induction of multiple shoot cultures from cotyledonary nodes

The objective of this work was to act on cotyledonary node meristematic tissue before its organisation into axillary buds. The cotyledonary nodes of preconditioned germinated plantlets showed a high ability to develop shoot buds when cultured on media supplemented with TDZ. These buds were developed in the cotyledon axil zone, generally in compact clusters (see SAN-JOSÉ *et al.* 2001 for details). The methodology developed would be useful for efficient genetic transformation programmes since it means that there is a reasonably high probability of a large number of transformed cells producing transformed buds.



Some attempts have been carried out to transform chestnut via genetic engineering (SEABRA and PAIS 1998, XING *et al.* 1999), but the results were disappointing, mainly due to the poor regeneration obtained. However, the results reported in this paper and those reported by SAN-JOSÉ *et al.* (2001) open up new possibilities for using preconditioned, cotyledonary nodes as a material with a high capacity for regeneration.

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## KAPITEL 3

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### **SOMATIC EMBRYOGENESIS FROM OVARIES, DEVELOPING OVULES AND IMMATURE ZYGOTIC EMBRYOS, AND IMPROVED EMBRYO DEVELOPMENT OF CASTANEA SATIVA.**

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#### **Abstract**

Somatic embryogenesis of European chestnut (*Castanea sativa* Mill.) was obtained using juvenile tissue cultured on P24 medium with 5 µM 2,4-dichlorophenoxyacetic acid plus 0.5 µM 6-benzylaminopurine (BA) for three weeks and then cultured on 0.89 µM BA. Induction frequency with ovaries ranged from 2.0 to 19.1 % and was observed in tissue collected 2 to 8 weeks postanthesis, ovules used as a starting tissue gained 0.8 to 7.8 %, 3 to 9 weeks postanthesis. Zygotic embryos collected 5 to 10 weeks postanthesis formed 10.5 to 57.1 % somatic embryos, respectively. The culture lines were maintained *via* secondary embryogenesis on P24 medium with 0.89 µM BA. Development and maturation were stimulated on P24 medium with increased agar concentration (1.1 %). Five plantlets were transferred to substrate and acclimatized successfully in greenhouse.

*Additional key words:* 6-benzylaminopurine, 2,4-dichlorophenoxyacetic acid, European chestnut, *Fagaceae*.

*Abbreviations:* ABA - abscisic acid; BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA3 - gibberellic acid;

GD - Gresshof and Doy; IBA - indole-3-butyric acid; LSD - least significant difference; PGR - plant growth regulator; SE – somatic embryos, somatic embryogenesis; WPM - woody plant medium.

## Introduction

From the Black Sea to the Iberian Peninsula, European chestnut (*Castanea sativa* Mill.) is an important tree species for both timber and nut production. However, this tree species is threatened by pollution, social and economic changes, and two major fungal diseases; ink disease (*Phytophthora* ssp.) and chestnut blight [*Cryphonectria parasitica* (Murr.) Barr.].

In the area of tree biotechnology, propagation *via* somatic embryogenesis (SE) is regarded as a system of choice for mass propagation of superior tree genotypes (Cervelli *et al.* 1995). The advantages of somatic embryos (SE) include high multiplication rates and the potential for scale-up in bioreactors (Eeva *et al.* 2003) and for direct delivery to the greenhouse or field as artificial seeds. Production of artificial seeds has been reported in many species, in conifers as well as in angiosperms (Gupta *et al.* 1993). For the genus *Castanea*, SE has been reported for *C. mollissima* Blume × *C. dentata* (Marsh.) Borkh. hybrids (Skirvin 1981), *C. dentata* (Merkle 1991, Xing *et al.* 1999) and *C. sativa* × *C. crenata* Sieb. & Zucc. hybrids (Vieitez *et al.* 1990). Regeneration of plants *via* somatic embryogenesis in *C. sativa* × *C. crenata* hybrids was achieved by Vieitez *et al.* (1994). Xing *et al.* (1999) recovered plants from developing ovules through somatic embryogenesis in American chestnut (*C. dentata*). Carraway *et al.* (1994) transferred neomycin phosphotransferase and β-glucuronidase genes into American chestnut embryogenic cultures via particle bombardment. However, the application of somatic embryogenesis for the improvement of chestnut is limited as a result of problems with low initiation frequencies, maintenance of embryogenic cell lines and low conversion rates. In the present study, we investigated the frequencies for initiation of SE from different juvenile explants of European chestnut: ovaries, ovules and immature zygotic

embryos. To stimulate secondary somatic embryogenesis, two basal media, GD (Gresshoff and Doy 1972) and P24 (Teasdale 1992) were tested. The effect of glutamine was also tested. In order to improve development and maturation of SE, an experiment with increased agar concentration was performed.

## Materials and methods

**Plants:** Burs were collected weekly starting from the end of July until the end of August (2 to 10 weeks postanthesis) during 1998 and 1999, from three open pollinated European chestnut (*Castanea sativa* Mill.) trees growing in the park of Castle Schönbrunn in Vienna, Austria. Spikes were removed and burs were surface sterilized (2 × 10 min) with a commercial bleach containing 2.5 % NaOCl in an ultrasonic bath (Wilhelm 1997), with an intermittent washing step with 70 % ethanol for 15 s. Burs were rinsed twice in a sterile solution of 300 mM Na<sub>2</sub>HPO<sub>4</sub> + 42 mM citric acid + 100 mM KI + soluble starch (5 g dm<sup>-3</sup>), then transferred to a sterile solution of starch (5 g dm<sup>-3</sup>) and rinsed twice in sterile distilled water (Dirks *et al.* 1991). The cupule was removed aseptically and nuts were excised. Longitudinally halved nuts (ovaries), clusters of ovules, single ovules and developing embryos were tested as explants. Sizes of burs, ovules and zygotic embryos were measured by determining length (l) and width (w) in mm and a size index was calculated as  $l \times w$  for morphological description. Seventy seven zygotic embryos with a size index exceeding 40 were separated into axes and cotyledons. Developing ovules with a size index exceeding six were defined as immature zygotic embryos, as the remaining ovules stopped growing beyond this size.

**Induction of somatic embryogenesis:** To induce SE, explants were placed in Petri dishes containing 25 cm<sup>3</sup> P24 medium (Teasdale 1992) with 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) plus 0.5 μM 6-benzylaminopurine (BA), and 2 cm<sup>3</sup> dm<sup>-3</sup> *Preservative for Plant Tissue Culture Media* (PPMTM, *Plant Cell Technology, Inc.*, Washington, USA), supplemented with 3 % sucrose and gelled with 0.8 % agar (*Daishin, Brunschwig Chemie*, Amsterdam, The Netherlands). The pH of the medium was adjusted to 5.6 - 5.7 prior to autoclaving at 121 °C for 20 min. To avoid the inhibitory influence of polyphenols, the explants were transferred to fresh induction medium within the first week. After 3 weeks explants were transferred to P24 medium with 0.89 μM BA. The latter medium was used for maintenance of embryogenic cell lines. Subculture interval was 4 weeks. Cultures were incubated in the growth chamber at temperature of 24 ± 2 °C, irradiance of 50 μmol m<sup>-2</sup> s<sup>-1</sup> (white fluorescent light) and a 16-h photoperiod. After 6 weeks, cultures were scored for induction of embryo like structures (white and yellow globular objects).

**Development of embryogenic lines:** To stimulate secondary SE formation, two basal media, GD (Gresshoff and Doy 1972) and P24 (Teasdale 1992) were tested. Experiments with 300 and 1 000 mg dm<sup>-3</sup> L-glutamine were also performed. L-glutamine was autoclaved with the medium. Three embryogenic lines, B8/117, B7/65 and B7/89 were cultured on P24 or GD with 0.89 µM BA. For the experiment with glutamine, P24 medium with 0.89 µM BA was used with the cell lines C6/57, B7/65 and B7/89. Six plates, 0.5 - 0.7 g of tissue per plate, were used for each line and treatment and the entire experiment was repeated twice. Percentages of cultures with proliferating embryogenic tissue were evaluated after 4 weeks.

**Maturation and germination:** For maturation, the effect of two agar concentrations (0.8 and 1.1 %) was tested. Clusters of SE of cell line C6/57, 0.5 - 0.7 g per Petri dish, were placed on P24 medium with 0.89 µM BA, solidified with 0.8 or 1.1 % agar. After 5 weeks the number of well formed embryos (size < 3 mm, Fig. 2c), was scored and calculated as numbers of SE produced per g tissue. Data were compared with a one-sided *t*-test. For germination, harvested SE of two cell lines (C6/57 and B7/89) were cultured on P24 medium with 0.1 µM indole-3-butyric acid (IBA) and 0.89 µM BA or on P24 medium without plant growth regulators (PGRs). After 5 weeks the germination response was classified as either conversion (both root and shoot development) or as shoot or root formation only. Seedlings were transferred to *Magenta*<sup>TM</sup> GA-7 vessels containing PGR-free P24 medium supplemented with 1 % (m/v) activated charcoal.

**Data analysis:** Statistical analysis was performed with *SPSS for Windows 6.0.1* ( $\chi^2$  contingency table) and *Microsoft Excel 97* (*t*-test). Data from proliferation and germination experiments were compared with  $\chi^2$  contingency table.

## Results and discussion

**SE induction:** Data were pooled for all three source trees per sampling date, as source tree did not effect embryogenic response ( $\chi^2 = 2.4$ ,  $P = 0.3$ ). In total, 142 embryogenic lines out of 3163 explants were initiated during 1998 and 1999, which corresponded to an overall induction frequency of 4.5 %. Twenty-five embryogenic cell lines were formed from 491 ovaries (5.1 %), 76 lines were derived from 2520 ovules (3.0 %) and 41 lines were induced from 152 zygotic embryos (27.0 %). According to  $\chi^2$  contingency table embryogenic response of ovules and ovaries did not differ significantly, whereas induction frequencies of immature zygotic embryos were significantly higher than of ovules ( $P < 0.01$ ) and ovaries ( $P < 0.05$ ).

Embryogenic tissue from halved nuts (ovaries) was creamy-white, nodular and friable and developed after 3 to 4 weeks. In contrast, yellow and green compact callus showed no further organized development. SE were initiated from bisected ovaries 4 to 8 weeks postanthesis in 1998, and 4 to 6 weeks postanthesis in 1999 (Fig. 1).

In 1998, clusters of ovules turned brown and necrotic; therefore single ovules were explanted in 1999. After 2 to 3 weeks in culture, direct SE was observed from several isolated ovules, where SE formed without any intervening tissue. Size index of isolated ovules ranged between 1.5 and 5. One ovary contained 10 to 18 ovules. Up to six ovules out of them showed an embryogenic response (Fig. 2a,b), indicating that either at least six ovules per ovary were fertilized, or that non zygotic tissue was responsive as well.

In 1998, SE frequencies from zygotic embryos ranged between 41 and 47 % from immature zygotic embryos collected 9 and 10 weeks after anthesis (Fig. 1), whereas the highest induction rate (57.1 %) was obtained from material collected only 6 weeks postanthesis in 1999 (Fig. 1). Twenty one % of isolated axes from immature zygotic embryos gave rise to direct SE, while only 1 % of cotyledon tissue responded.

In the present study we were able to induce high frequencies of SE in *C. sativa* using immature zygotic embryos. Xing *et al.* (1999), using developing ovules, achieved initiation rates of 1.6 %. In *C. sativa* × *C. crenata* hybrids, formation of SE was restricted to 15 - 20 mm immature nuts, collected 10 to 12 weeks post fertilisation, with a frequency of 2 % (Vieitez 1995). This variation may be explained by different protocols, such as culture regimes, media and PGRs as well as by species. Merkle *et al.* (1991) and Xing *et al.* (1999)

induced SE in the dark, on woody plant medium (WPM) with high auxin content (18.1  $\mu\text{M}$  2,4-D). Carraway and Merkle (1997) tested the embryogenic response of different genotypes and explant types in *C. dentata* and reported an induction frequency of 2.8 %. Initiation of SE was only possible from *C. dentata* zygotic embryos less than 5 mm.

The frequency of SE initiation was related to the developmental stage of the zygotic embryo in European chestnut. This phenomenon has also been observed in several broad-leaved tree species such as *Quercus* ssp. (Endemann and Wilhelm 1999, Wilhelm 2000) and *Liriodendron tulipifera* (Sotak *et al.* 1991). The optimal stage for formation of SE in *C. sativa* was between 5 and 10 weeks post anthesis and a size index ranging from 6 to 350 (Fig. 3). Sizes of seeds differed from year to year and even within one harvesting date. The growth of the cupule and the zygotic embryo started at the same time during both years, but was retarded in 1998. Therefore seasonal influences have to be considered when choosing the optimal induction time.

**Development of embryogenic lines:** The ability for proliferation *via* repetitive SE differed among the cell lines. Stable embryogenic cell lines were initiated from all types of explants 6 and 7 weeks postanthesis in 1998, and 4, 5 and 6 weeks postanthesis in 1999. Twenty-one SE lines were selected for their proliferation capacity and subcultured on P24 with 0.89  $\mu\text{M}$  BA (Fig. 2d). High ratios of vitamins and amino acids, especially glutamine and serine, stimulated androgenesis in *Quercus petraea* and *Fagus sylvatica* (Jørgensen 1988). In chestnut hybrids, glutamine was necessary for repetitive embryogenesis (Vieitez 1995). However, in our experiments, glutamine had no significant beneficial effect on secondary embryogenesis for any of the tested cell lines (Table 1). Embryogenic cell lines were maintained without glutamine for 2 years and did not lose embryogenic competence. Carraway and Merkle (1997) considered addition of an auxin necessary to maintain SE in *C. dentata*, whereas formation of *C. sativa* SE was halted by 1  $\mu\text{M}$  2,4D.

The tested basal media, GD and P24, produced no differences in proliferation capacity of the tested cell lines, and differences among cell lines were also not significant (Table 2).

In many broadleaved tree species such as oak (Wilhelm 2000), pecan, black locust and bigleaf magnolia (Merkle 1995, Merkle *et al.* 1995) the embryogenic lines are normally maintained *via* repetitive secondary embryogenesis. However the proliferation of SE was found to be difficult in European chestnut as a result of development of many different



tissue types. As described by Carraway *et al.* (1994) explants produce a mixture of callus types and continuous selection of embryogenic cells is necessary. Direct formation of secondary embryos was frequently observed at the hypocotyl region of a germinating SE (Fig. 2e). Unicellular or multicellular pathways are possible origins of secondary embryogenesis. Zegzouti and Favre (1999) revealed that both pathways were possible in *Quercus robur*, depending on the applied PGRs. The application of BA promoted indirect secondary embryogenesis from cortical parenchyma cells, indicating multiple cell origin in oak, which may explain the polycotyledonary embryos and fused embryoids. In *C. sativa* we also frequently observed anomalous formations of SE, which may have been evoked by the use of BA.

**Maturation and germination:** Maturation and germination are considered to be major bottlenecks in SE of many broadleaved species. Spontaneous germination of *C. sativa* SE was rarely observed on proliferation medium after one year in culture. With a higher agar concentration (1.1 %), the average number of single cotyledonary SE produced per gram increased significantly (Table 3).

Within the *Fagaceae*, a wide range of treatments and culture conditions lead to regeneration of plants *via* SE. Most often, radicles elongate first and epicotyls form with delay or fail to elongate. Increasing sucrose concentration from 20 to 60 g dm<sup>-3</sup> enhanced embryo maturation and conversion in American chestnut (Xing *et al.* 1999). Vieitez (1999) employed a cold treatment (4 °C) to improve germination of *Castanea sativa* × *C. crenata* hybrids. The combination of increased agar concentration (1.1 %) and partial desiccation treatment improved conversion rates in *Quercus robur* (Wilhelm *et al.* 1999) Additionally Sunderlikova and Wilhelm (2002) showed that high expression of storage protein genes during maturation of oak somatic embryos was associated with increased conversion frequency. Although we did not determine the transcript accumulation of storage protein genes in chestnut somatic embryos, we also found significantly more embryos converted in both cell lines when harvested from maturation media with the higher agar concentration, followed by culture on P24 with 0.1 M IBA and 0.89 µM BA or P24 without PGRs. This result suggests that the regulation of storage proteins is under developmental control and can be influenced by manipulation of the culture conditions also in chestnut somatic embryos. No differences in shoot formation were observed between the different treatments (Table 4).

SE derived plantlets were transferred to PGR-free medium supplemented with 1 % m/v activated charcoal. Five plants with well developed shoots and roots were transplanted into pots containing a 1:1 peat moss:perlite mixture and acclimatized in a growth chamber under artificial light with a 14-h photoperiod at 20 - 22 °C and a relative humidity of 97 %. After 4 weeks the plants were kept under daylight conditions at 20 to 25 °C in the greenhouse (Fig. 2f).

**Conclusion:** We were able to induce somatic embryogenesis from ovules and immature zygotic embryos at high frequencies. The conversion rate of chestnut somatic embryos was improved by using a maturation medium containing 1.1 % agar. However, the number of resultant plantlets was low. Further work is needed to elucidate the biochemical and molecular regulation of chestnut somatic embryo development with the aim of adjusting maturation and germination treatments for the production of high quality plants.

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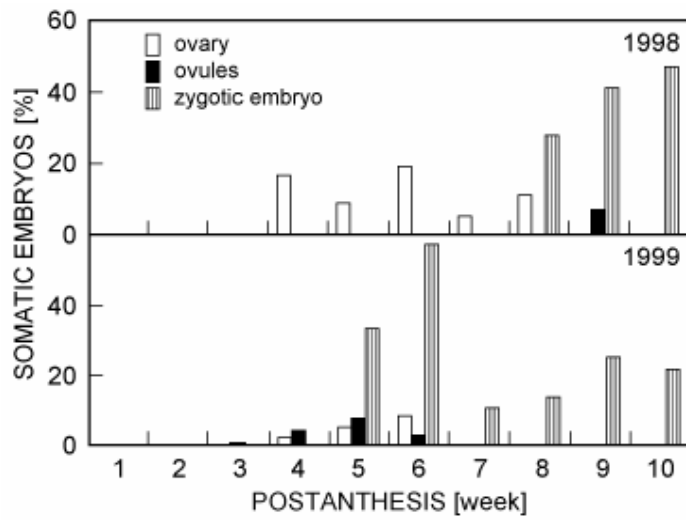


Fig. 1. Effect of collection date on formation of somatic embryos [%] in ovaries, ovules and immature zygotic embryos from *Castanea sativa* in 1998 and 1999.

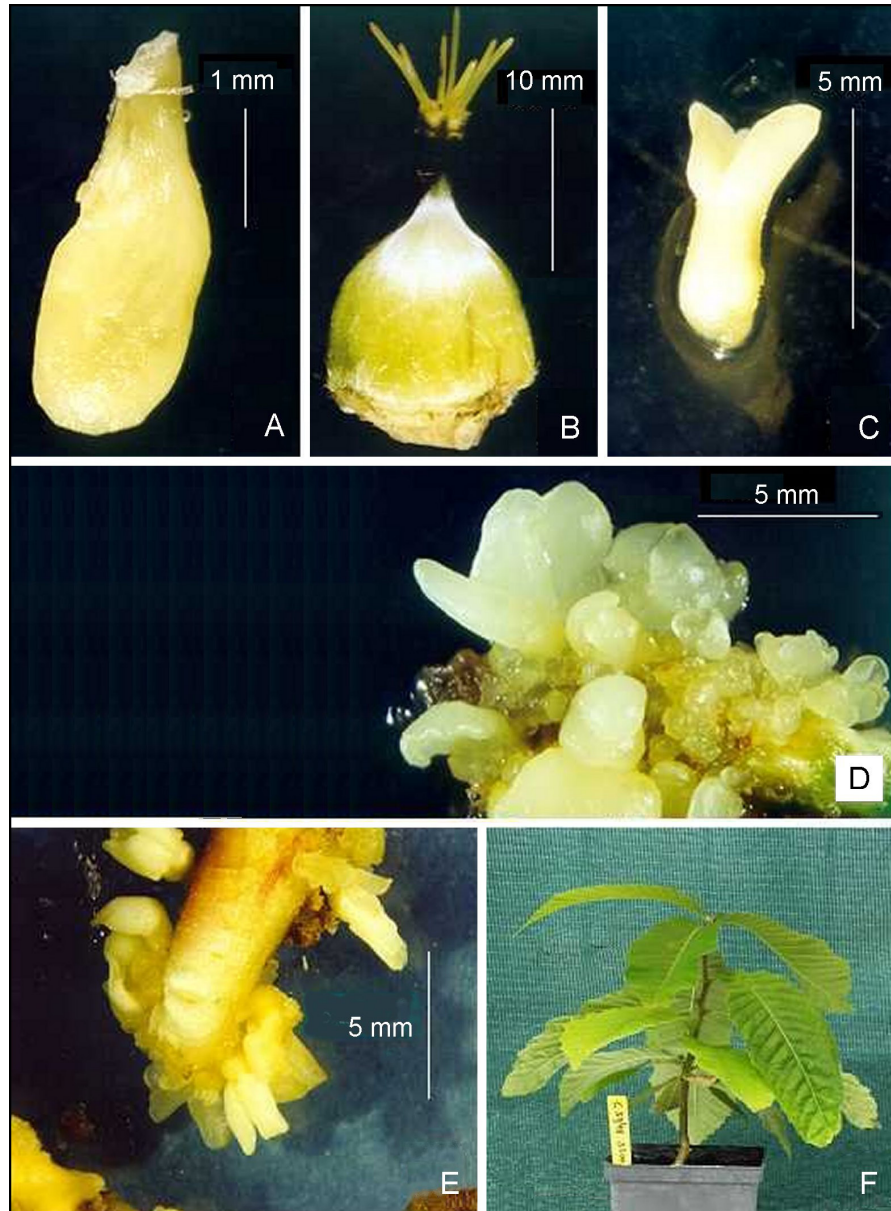


Fig. 2. Somatic embryogenesis in *Castanea sativa*. *A* - single ovule as explant, *B* - ovary as explant, *C* - isolated cotyledonary somatic embryo on maturation medium with 1.1 % agar, *D* - secondary embryos at various stages of development on proliferation medium, *E* - secondary embryos developed from hypocotyl of a germinated somatic embryo, *F* - plant regenerated from somatic embryo.

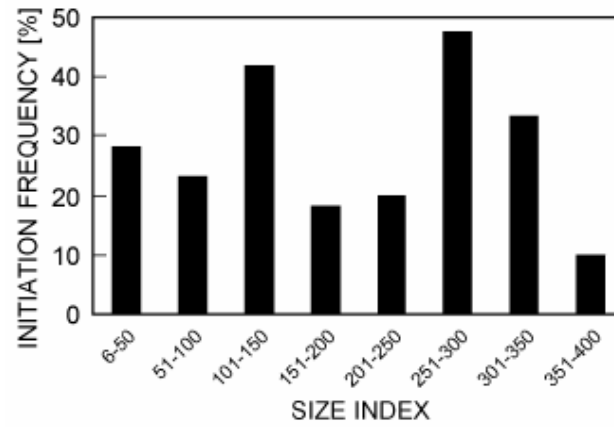


Fig. 3. Initiation frequencies [%] of zygotic embryos of *C. sativa*, explants grouped into nine classes by size index (l × w).



Table 1. Effect of addition of glutamine to P24 medium with 0.89  $\mu\text{M}$  BA on proliferation of embryogenic cell lines of *Castanea sativa*. Values are percentages of samples with embryogenic proliferation. According to  $\chi^2$  contingency table, cell lines ( $\chi^2 = 1.2$ ,  $P = 0.5$ ), and treatments ( $\chi^2 = 2.1$ ,  $P = 0.3$ ) do not differ significantly.

Cell line	Glutamine [ $\text{mg dm}^{-3}$ ]		
	0	300	1000
C6/57	67	75	58
B7/65	58	58	50
B7/89	75	17	75

Table 2. Effect of basal media P24 and GD on proliferation of SE [%] of *C. sativa*. Values are percentages of samples with embryogenic proliferation. GD versus P24  $\chi^2 = 2.0$ ,  $P = 0.2$ ; B8/117 versus B7/89  $\chi^2 = 3.0$ ,  $P = 0.1$ ; B8/117 versus B7/65  $\chi^2 = 2.3$ ,  $P = 0.1$ ; B7/89 versus B7/65  $\chi^2 = 0.3$ ,  $P = 0.6$ .

Cell line	Medium	
	GD	P24
B8/117	33	33
B7/65	42	58
B7/89	42	75

Table 3. Effect of 0.8 and 1.1 % agar on maturation of SE of *C. sativa*. Means  $\pm$  SE; values are significantly different as determined by  $t$ -test (one-tail):  $P = 0.01$ ,  $n = 24$ .

Cell line	Agar [%]	Number of SE [ $\text{g}^{-1}$ ]
C6/57	0.8	$4.5 \pm 0.5$
C6/57	1.1	$6.8 \pm 0.8$

Table 4. Effect of different concentrations of agar in maturation media (1: P24 + 0.89  $\mu$ M BA + 0.8 % agar; 2: P24 + 0.89  $\mu$ M BA + 1.1 % agar) followed by culture on two different germination media (I: P24 without PGRs; II: P24 + 0.89  $\mu$ M BA + 0.1  $\mu$ M IBA), tested with cell lines C6/57 and B7/89. C6/57 *versus* B7/89  $\chi^2 = 3.0$ ,  $P < 0.1$ ; cell line C6/57 1 *versus* 2  $\chi^2 = 10.3$ ,  $P < 0.01$ ; I *versus* II  $\chi^2 = 3.5$ , ns; cell line B7/89 1 *versus* 2  $\chi^2 = 7.3$ ,  $P < 0.01$ ; I *versus* II  $\chi^2 = 0.005$ , ns.

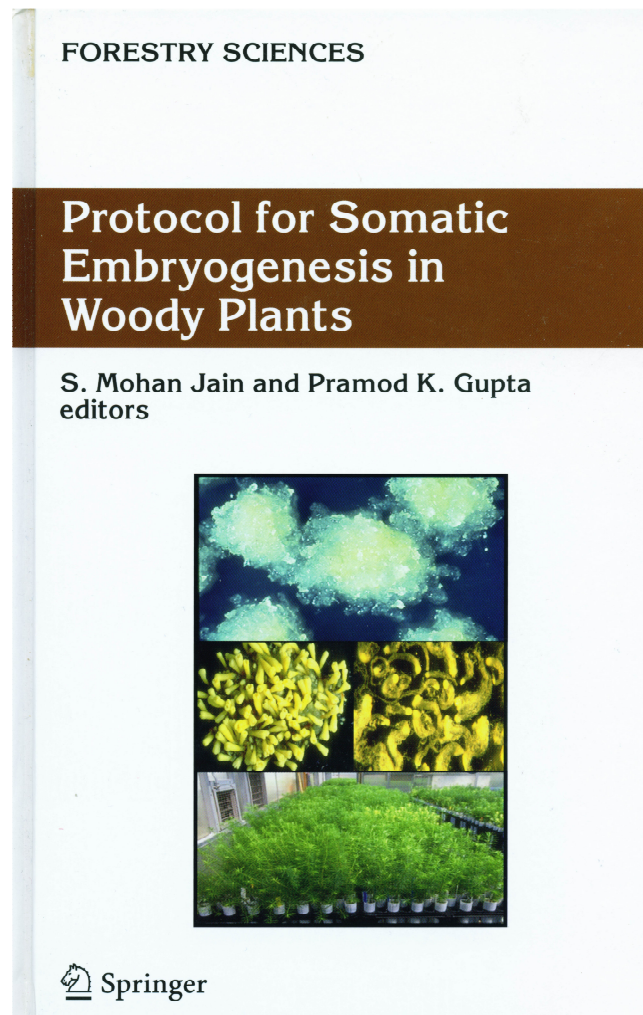
Cell line	Agar [%]	Germination medium	Number of embryos tested	Number of plantlets	roots only	shoots only
C6/57	0.8	-PGR	86	2	12	2
C6/57	0.8	+PGR	57	3	6	3
C6/57	1.1	-PGR	76	8	17	5
C6/57	1.1	+PGR	89	15	4	3
B7/89	0.8	-PGR	36	2	7	3
B7/89	0.8	+PGR	32	2	8	3
B7/89	1.1	-PGR	32	8	7	3
B7/89	1.1	+PGR	52	10	10	4

## KAPITEL 4

In: Protocol for Somatic Embryogenesis in Woody Plants, Forestry Sciences Vol 77, 2005. S Mohan Jain and Pramod K. Gupta (eds), Springer, Dordrecht.

### **PROTOCOL OF SOMATIC EMBRYOGENESIS: EUROPEAN CHESTNUT (*CASTANEA SATIVA* MILL.)**

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## 1. INTRODUCTION

European chestnut (*Castanea sativa* Mill.) is an important tree species for both landscaping and multifunctional plantations. Besides their main role in nut production in grafted orchards, the trees are found in coppices for production of small pieces of wood or in high forests supplying a variety of valuable timber highly resistant to decay (Fernández-López (1999). Interesting by-products are mushrooms and tannins for leather processing. Up to 500,000 tonnes of chestnuts are produced each year around the world from four commercially important species of the genus, a large part coming from European chestnut. Nowadays chestnuts are grown not only in the traditional Mediterranean areas of cultivation such as Spain, Turkey and Italy, but also in South America, Australia, and New Zealand.

However, this tree species is threatened by pollution, social and economic changes, and by two major fungal diseases: ink disease (*Phytophthora* ssp.) and chestnut blight (*Cryphonectria parasitica* (Murr.) Barr.). With its high nut and timber quality the European chestnut is an important breeding partner in hybridisation programmes with *Castanea crenata* Sieb. & Zucc. undertaken to produce blight-resistant trees.

As in other recalcitrant, large-seeded temperate tree species, storage of seeds is limited and rooting is a non-trivial task, making somatic embryogenesis an interesting alternative for clonal propagation of superior genotypes. Cryostored somatic embryos may serve the end of preserving the germplasm of old landraces as well as of new superior varieties. In addition, somatic embryogenesis is a potential tool for regenerating transgenic trees. Carraway *et al.* (1994) transferred neomycin phosphotransferase and  $\beta$ -glucuronidase genes into American chestnut embryogenic cultures via particle bombardment. However, the application of somatic embryogenesis for the improvement of chestnut is still limited as a result of problems with low initiation frequencies, maintenance of embryogenic cell lines and low conversion rates.

## 2. INDUCTION OF EMBRYOGENIC TISSUE

Induction of sweet chestnut somatic embryos has been described for juvenile reproductive tissues and for leaves of *in vitro* stock shoot cultures. Besides the physiological status of the explant tissue the genotype also greatly influences the induction frequency.

2.1. Induction of somatic embryos from ovaries, ovules and zygotic embryos (Sauer & Wilhelm, 2005):

Spikes of burs are removed and burs are surface-sterilised (2 x 10 minutes) with a commercial bleach (containing 2.5 % NaOCl) in an ultrasonic bath with an intermittent washing step using 70 % ethanol for 15 s (Wilhelm 1997). Burs are rinsed twice in a sterile solution of 300 mM Na<sub>2</sub>HPO<sub>4</sub> + 42 mM citric acid + 100 mM KI + soluble starch (5 g/l), then transferred to a sterile solution of starch (5 g/l) and rinsed twice in sterile distilled water (Dirks *et al.* 1991). By employing these disinfection and washing procedures, losses of not more than 5 % are observed. The cupule is removed aseptically and nuts are excised. Induction is possible from ovaries (longitudinal sections), ovules or zygotic embryos in a developmental window between the second and tenth week postanthesis.

To induce somatic embryos, explants are placed in Petri dishes containing 25 ml basal medium with 5 µM 2,4-D plus 0.5 µM BA, and 2 ml/l Preservative for Plant Tissue Culture Media (PPM™, Plant Cell Technology, Inc., Washington, DC), supplemented with 3 % sucrose and gelled with 0.8 % agar. The pH of the medium is adjusted to 5.5 – 5.6 prior to autoclaving at 121 °C for 20 min. To avoid the inhibitory influence of polyphenols, the explants are transferred to fresh induction medium within the first week. After 3 weeks explants are transferred to basal medium with 0.89 µM BA. As basal media P24 (Teasdale 1992) or GD (Gresshoff and Doy 1972) are suitable. Embryogenic tissues usually can be observed after 3 weeks. Cultures are incubated in the growth chamber at 24 °C (+/- 2 °C) under white fluorescent light at 50 µmol m<sup>-2</sup> s<sup>-1</sup> at a 16h photoperiod. The induction frequency when using ovaries ranges from 2.0 to 19.1 % and somatic embryos are observed in tissue collected 2 to 8 weeks postanthesis, while the yield is 0.8 to 7.8 % when ovules are used as starting tissue 3 to 9 weeks postanthesis. Zygotic embryos collected 5 to 10 weeks postanthesis form somatic embryos at a rate of 10.5 to 57.1 %.

## 2.2. Induction of somatic embryos from leaf explants (Corredoira *et al.* 2001):

Starting material is the 2-3 uppermost unfurled expanding leaves originated from in vitro shoots multiplied in tissue culture.

Explants are cultured on solid MS medium supplemented with 1 mg/l BA and 1 mg/l NAA, and incubated under a 16h photoperiod and 25 °C light/20 °C dark temperatures. After eight weeks somatic embryos form on the leaf explants at an induction rate of 0.5 % and are transferred to maintenance medium.

## 3. MAINTENANCE OF EMBRYOGENIC TISSUE

In general, low salt concentrations, as found in WPM, MS with half strength of macronutrients, GD and P24, as well as low hormone levels are thought to be beneficial for chestnut somatic embryo proliferation (Vieitez 1995). Repetitive somatic embryogenesis occurs as direct secondary embryogenesis in the hypocotyl region or as indirect proliferation via a nodular callus. As described by Carraway *et al.* (1997) explants produce a mixture of callus types and continuous selection of embryogenic cells is necessary.

3.1. Somatic embryo cultures derived from ovaries, ovules and zygotic embryos are maintained on solid medium with 0.89 µM BA (0.5 - 0.7 g of tissue per plate). Hormone-free basal medium is suitable for maintenance as well (Sauer, 2001). Omitting BA from multiplication medium has no significant effect on fresh weight increase and no differences in multiplication of somatic embryos are observed. While during long-term culture of SE on hormone-free medium the beneficial effect of BA on cell division may be missing, embryo quality should nonetheless improve in the absence of BA, as described by Zegzouty and Favre (1999), and less somaclonal variation should occur. We recommend adding BA to the multiplication medium once within four to five subculture cycles. Subculture intervals are 5 weeks. Cultures are incubated in the growth chamber under the same conditions as for induction.

3.2. For maintenance of somatic embryos from leaf explants by repetitive embryogenesis, solid MS medium (half-strength macronutrients; 3 g/l sucrose; 8 g/l agar) plus 0.1 mg/l BA

and 0.1 mg/l NAA is employed. Subculture intervals are 6 weeks. (Ballester *et al.* 2001)

3.3. Cold storage of embryogenic tissue: as induction from zygotic embryos is restricted to a certain season and handling of the cultures is enormously time-consuming, we looked for a method of storing embryogenic tissue for some months without subculture. Seven days after the last subculture material is transferred to an environment of 10 °C under dim light in which it may be stored for at least three months. To avoid losses due to excessive condensation, Petri dishes are stored vertically, so that water gathers in the lower part of the dish.

#### **4. MATURATION**

During the maturation phase, the embryos must accumulate nutrient reserves. Translucent somatic embryos regularly fail to germinate, in contrast to embryos with thickened opaque cotyledons. Various types of stress, such as use of osmotic compounds, desiccation and the application of ABA have been investigated as switches for accumulation of storage products (Wilhelm 2000). Increasing the agar concentration up to 1.1 % or application of an osmoticum such as 6 % sorbitol (Sauer 2001) or 3 % maltose (Corredoira *et al.* 2003) enhance embryo maturation and conversion.

Maturation treatment 1 (Sauer & Wilhelm in press): clusters of somatic embryos, 0.5 - 0.7 g per Petri dish, are placed on maintenance medium solidified with 1.1 % agar instead of the standard concentration of 0.8 %. After 5 weeks cotyledonary-stage embryos (size > 3 mm) are collected and subjected to the chilling treatment or transferred to germination medium directly.

Maturation treatment 2 (Sauer 2001): instead of the water stress in maturation treatment 1, an osmoticum is applied: standard maintenance medium is supplemented with 6% sorbitol.

Maturation treatment 3 (Corredoira *et al.* 2003): cotyledonary embryos are cultured individually on maturation medium consisting of PGR-free basal medium supplemented with 3 % maltose.

## 5. GERMINATION AND TRANSFER TO SOIL

As pregermination treatments, desiccation and/or chilling are applied in order to brake dormancy. Lipid and protein reserves are mobilised to enable root and shoot growth. Most often radicles germinate first, while epicotyls form only after delay or fail to germinate. Conversion rates range from 6 % (maturation treatments 1 and 3) to 13 % (maturation treatment 2) when combined with a chilling treatment.

Chilling treatment: after 4-5 weeks on maturation medium, somatic embryos are cultured on maintenance medium and stored at 4 °C in the dark for 2 months.

For germination, somatic embryos are cultured in Petri dishes on basal medium with 0.1 µM indole-3-butyric acid (IBA) and 0.89 µM BA. After 5-6 weeks the seedlings are transferred to test tubes or Magenta™ GA-7 vessels containing PGR-free medium supplemented with 1 % m/v activated charcoal.

Plants with well developed shoots and roots are transplanted into pots containing a 1:1 perlite : soil mixture and acclimatised in a growth chamber under artificial light at a 14h photoperiod, a temperature of 20 - 22 °C and a relative humidity of 97 %. After 4 weeks the plants are kept under daylight conditions at 20 to 25 °C in the greenhouse.

## 5. CONCLUDING REMARKS

The first records on somatic embryogenesis in *Castanea sativa* date back to Piagnani *et al.* in 1990, and Leva *et al.* in 1993, but conversion into plantlets was not achieved before 2000 (Sauer & Wilhlem). For the genus *Castanea*, somatic embryogenesis has been reported for *C. mollissima* Blume x *C. dentata* (Marsh.) Borkh. hybrids (Skirvin 1981), *C. dentata* (Merkle 1991, Xing *et al.* 1999) and *C. sativa* x *C. crenata* Sieb. & Zucc. hybrids (Vieitez *et al.* 1990). Regeneration of plants via somatic embryogenesis in *C. sativa* x *C. crenata* hybrids has been achieved by Vieitez *et al.* (1992).

A main focus for the future is on the development of propagation systems for initiating somatic embryos from mature trees, in order to establish cell lines with known phenotypes.



The induction from juvenile leaves of sweet chestnut and the protocols for somatic embryogenesis from mature tissues of cork oak (Hernandez *et al.* 2003), pedunculate oak (Cuenca 1999) and elm (Conde *et al.* 2003) are promising starting points.

In the face of problems with somaclonal variation, a system for cryopreservation of somatic embryos has to be addressed next. Holliday *et al.* (2000) have defined a protocol for cryostorage of embryogenic cultures of American chestnut, and Pence (1992) showed that zygotic embryo axes of European chestnut survived and elongated after exposure to liquid nitrogen.

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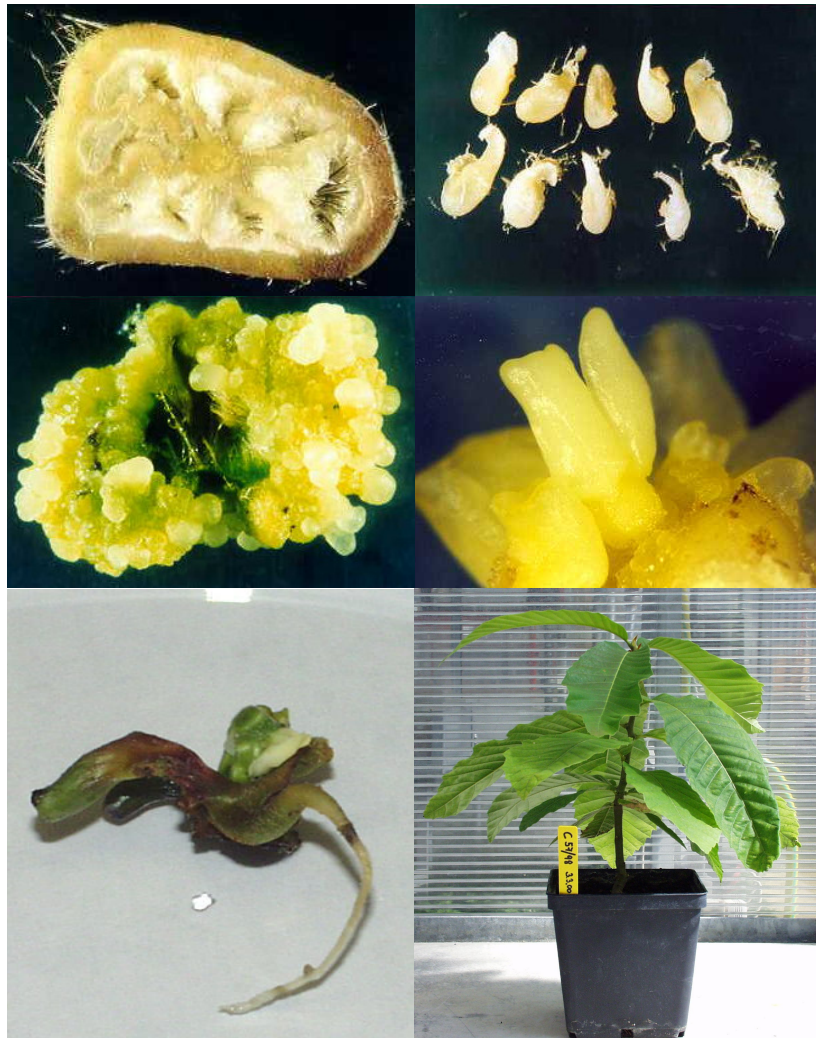


Figure 1. Somatic embryogenesis in European chestnut: a) explant type: ovary b) explant type: ovules c) mixture of callus types produced from ovary on induction medium d) secondary embryos produced on maturation medium e) germinated somatic embryo f) plantlet regenerated from somatic embryo under greenhouse conditions.



## KAPITEL 5

Xth International Conference on Plant Embryology, September 5-8, 2001, Nitra, Slovakia. Posterpräsentation

### **IMPROVING MATURATION AND GERMINATION CONDITIONS FOR SOMATIC EMBRYOS FROM EUROPEAN CHESTNUT (CASTANEA SATIVA MILL.)**

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#### **ABSTRACT**

Somatic embryogenesis (SE) is a process by which differentiation is induced in somatic cell material, closely paralleling the natural development of an embryo from a zygote without fertilization. The advantages of this vegetative plant propagation technique include cryopreservation, artificial seed production, large scale culture in bioreactors and it can provide the basis for further genetic improvement. Technically the process goes through the stages of initiation, multiplication and maturation, germination and plantlet conversion. The low germination frequency is considered to be one of the major bottlenecks. During 1998 and 1999 142 (SE) cell lines out of 3163 juvenile explants were initiated from *Castanea sativa*, which resulted in an induction frequency of 4.5%. Embryogenic cell lines were maintained on P24 medium with 0.9  $\mu\text{M}$  BA. In order to evaluate development- and maturation-conditions, experiments with increased agar (1.1%) concentration and sorbitol (6%) were performed on both hormone free and BA-media. The moisture content of the developing embryos, water potential from the media and the plant tissue was determined. Numbers of well formed SE were scored and cultivated on PGR free medium and stored at 4°C, 10°C or at room temperature. Maturation media influenced water content and osmotic potential of SE. Highest embryo yield was achieved on hormone free media plus 6% sorbitol. Highest germination frequency (up to 40%) was obtained on 6% sorbitol medium without PGRs, after a cold treatment (8 weeks, 4°C). Plantlets were transferred to substrate and acclimatized in the greenhouse.

## POSTER

### Introduction

Application of SE for the improvement of chestnut is limited due to problems with low initiation frequencies, maintenance of embryogenic cell lines and low conversion rates. In the present study experiments with increased agar (1.1%) concentration and sorbitol (6%) were performed on both hormone free and BA-media. Conversion frequencies of SE derived from maturation media and the effect of a chilling treatment were investigated. In order to characterise the effect of maturation treatments, moisture content and osmotic potential of developing embryos were determined.

### Material and Methods

#### *Maintenance and multiplication*

SE cell lines of European chestnut, initiated in 1998, were maintained on P24 medium [1] plus 0.89  $\mu\text{M}$  BA (P1) or on hormone free medium (P2) in a four week subculture cycle. Increase in fresh weight was calculated after 4, 8 and 12 weeks.

#### *Maturation and germination*

Clusters of SE were placed on different maturation media (Table 1). After five weeks numbers of well formed embryos (size > 3 mm), which were only loosely attached, were scored and calculated as numbers of SE produced per g tissue. Harvested SE were transferred to germination treatment (P24 + 0.8% agar, 3% sucrose, 0.1  $\mu\text{M}$  IBA; room temperature) immediately, or underwent a cold treatment first (P24 + 0.8% agar, 3% sucrose; 4°C or 10°C for 8 weeks). Moisture content of SE tissue after maturation treatments was determined gravimetrically. SE and media were frozen for osmotic potential measurement with a digital micro-osmometer. After 6 weeks on germination media the conversion response was evaluated. Converted plantlets were transferred to Magenta™ GA-7 vessels containing hormone free P24-medium supplemented with 1% w/v activated charcoal.



## **Results and discussion**

### ***Multiplication of SE:***

Multiplication media strongly influence the quality of SE and the final plant yield. Application of BA or 2,4 D, which are employed for proliferation regularly, may induce anomalous formation of SE [2, 4]. Therefore the ability for proliferation of SE without PGR's is an important step toward high quality embryo production. Omitting BA from multiplication media had no significant effect on fresh weight increase and multiplication of somatic embryos (Table 2).

### ***Maturation and Conversion***

Maturation and germination are considered to be the major bottleneck in SE of many broadleaved species [3]. Application of 1.1% agar and addition of sorbitol increased high-quality-embryo yield (Figure 1 and 5). This coincides with more negative osmotic potential (Figure 2) and lower moisture content (Figure 3) of tissue grown on these media. Conversion frequencies were affected by the use of BA in multiplication and maturation phases (Figure 4). Water stress, induced by application of osmotic compounds, in combination with a 4°C chilling treatment, exhibited a positive effect on conversion frequencies in combination with hormone free media only. Highest germination frequency was obtained on 6% sorbitol medium without PGRs, after a cold treatment (8 weeks, 4°C). Ten plants with well developed shoots and roots were transplanted into containers with a 1:1 peat moss : perlite soil mixture and acclimatized under 90% humidity in the greenhouse for three weeks (Figure 6).

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Table 1: Experimental design

Pretreatment 12 wks	Maturation - treatment 5 wks	Temperature regime 8 wks
P24 hormone free	0.8% Agar	4°C RT
	1.1% Agar	4°C RT
	6% Sorbitol	4°C RT
P24 + BA	0.8% Agar	4°C RT
	1.1% Agar	4°C RT
	6% Sorbitol	4°C RT

Table 2: Proliferation of chestnut SE with and without 0.89 µM BA

Medium	% FW increase <sup>c</sup>		
	4 weeks	8 weeks	12 weeks
P1 <sup>a</sup>	172.09 ± 15.87	287.79 ± 24.46	221.64 ± 21.89
P2 <sup>b</sup>	140.03 ± 10.98	268.08 ± 13.29	207.02 ± 20.11

a P 1 P24 + 3 % sucrose, 0.8 % agar, 0.89 µM BA

b P 2 P24 + 3 % sucrose, 0.8 % agar

c mean value of %fresh weight increase±standard error (18 samples)

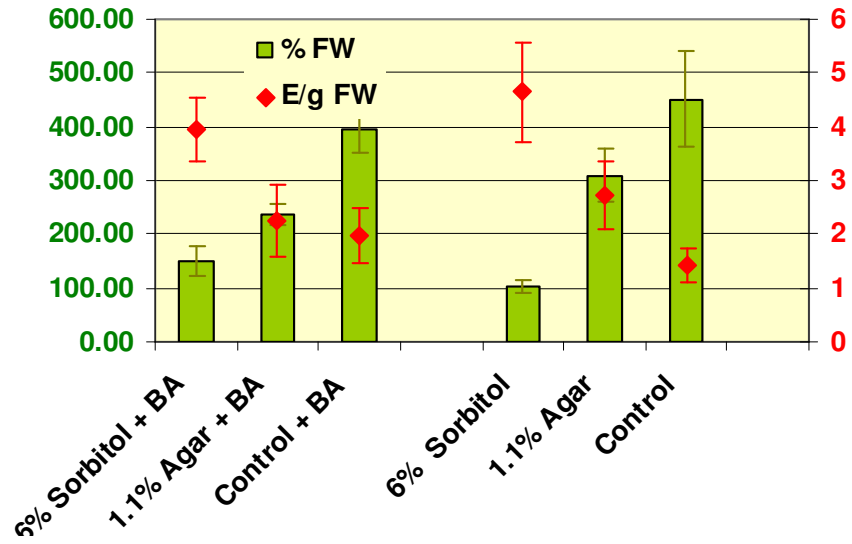


Figure 1: Maturation treatments in relation to embryo yield and fresh weight increase.

Control: cultured on P24 + 3% sucrose + 0.8% agar

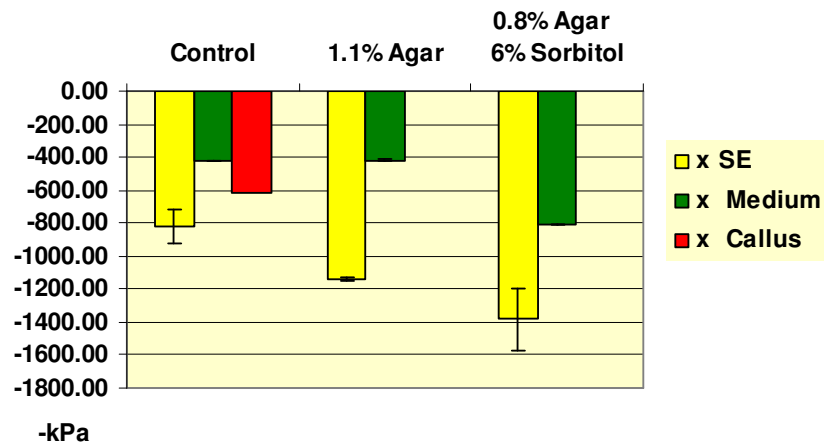


Figure 2: Osmotic potential of maturation media and somatic embryos cultivated on these.

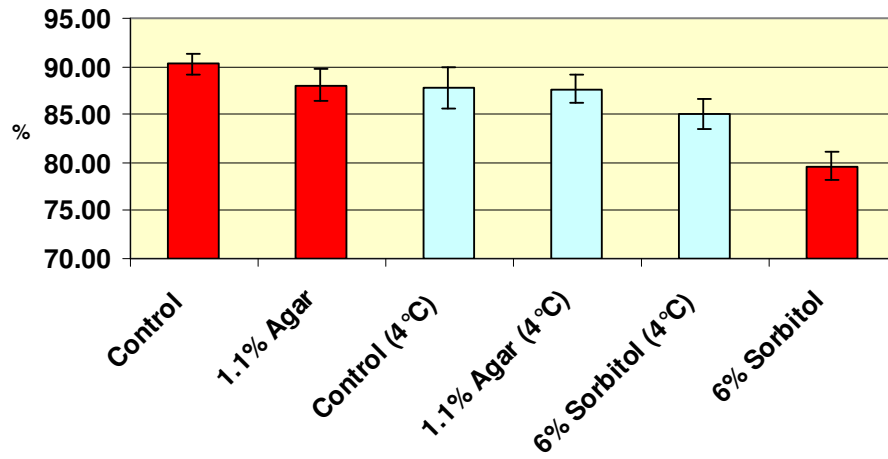


Figure 3: Moisture content of SE derived from different maturation treatments.

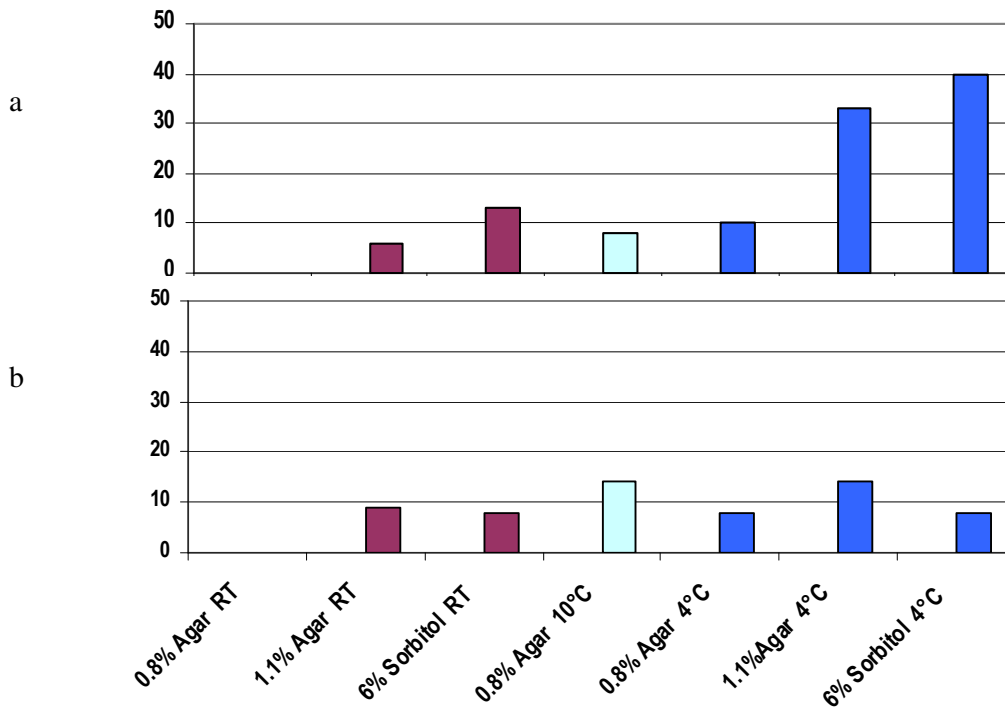


Figure 4: Effect of maturation treatments on conversion rates (%)

- a) multiplication- and maturation media are hormone free
- b) multiplication- and maturation media are supplemented with BA



Figure 5: Cotyledonary somatic embryo



Figure 6: Chestnut plantlet, regenerated from a somatic embryo.

## KAPITEL 6

### ZUSAMMENFASSUNG

Die Edelkastanie verzeichnete in den 70er und 80er Jahren des vorigen Jahrhunderts einen starken Rückgang sowohl bezüglich Holz- als auch Fruchtproduktion. Seit den 90er Jahren gibt es europaweit verstärkt Interesse an der Erhaltung der genetischen Ressourcen dieser Baumart und auch wieder Aufforstungsprogramme. Daher werden auch neue Methoden zur vegetativen Vermehrung von speziellen Genotypen (zum Beispiel alte Landsorten oder pilzresistente Sorten) untersucht. Die somatische Embryogenese als vegetative Vermehrungsmethode eignet sich zur Produktion von Edelkastanien im großen Maßstab und bietet somit viele Vorteile als Klonierungsmethode; für die Kryokonservierung als *ex-situ* Erhaltungsmöglichkeit; für die Produktion von künstlichem Saatgut oder als potentiell Verfahren zum Gentransfer.

In der vorliegenden Arbeit wurde erfolgreich ein System zur Induktion somatischer Embryogenese bei Edelkastanie entwickelt, ein Zeitfenster der Induktion bestimmt und die Multiplikation von somatischen Embryonen erreicht.

Die Embryonenausbeute der Induktion und das Entwicklungsstadium des Explantats standen in engem Zusammenhang. Als optimales Stadium der Explantate zur Initiierung des Prozesses der Embryogenese wurde der Zeitpunkt mit 5-10 Wochen nach der Anthese bestimmt. Zelllinien, die 4-7 Wochen nach der Anthese induziert wurden, konnten auch in einen Zyklus der Weitervermehrung eingebracht werden. Durchschnittlich konnten in zwei Jahren 4.5% von 3.000 getesteten Explantaten embryogene Zelllinien induziert werden. Dabei war die Ausbeute bei den Ovarien (5.1 %) und bei den Samenanlagen (3 %) wesentlich niedriger als bei den jungen zygotischen Embryonen (27 %). Verglichen mit den Kotyledonen waren die Achsen die embryogen aktiveren Gewebe, was auf die Beteiligung meristematischer Zellen hinweist.

Das Vermehrungsstadium der embryogenen Kulturen erwies sich als wichtig für die Qualität der produzierten Embryonen und somit für den nachfolgenden Keimungserfolg.

21 ausgewählte Zelllinien wurden über 2 Jahre auf P24 Medium mit 0.89  $\mu\text{M}$  6-Benzylaminopurin weitervermehrt. Dabei war eine ständige Trennung von embryogenen und nicht-embryogenen Geweben notwendig, da neben den sekundären Embryonen auch Kallus produziert wurde. Weiters wurde gezeigt, dass die Vermehrung der Kulturen auch auf hormonfreiem Medium möglich ist, was in Folge zu hochwertigeren weil keimungsfähigeren Embryonen und weniger Missbildungen führte.

Erste Erfolge konnten in dieser Arbeit auch bei Reifung, Keimung und Akklimatisierung erzielt werden. Pflanzen konnten ins Glashaus transferiert und erfolgreich akklimatisiert werden. Der Erhalt der embryogenen Kompetenz der induzierten Zelllinien ist wenig problematisch, das Umschalten von repetitiver Embryogenese auf Reifung und Keimung stellt hingegen eine Herausforderung dar. Stressbehandlungen (Kälte, osmotischer Stress, Trocknung) sollen bei somatischen Embryonen die Reservestoffakkumulation einleiten und die Dormanz brechen. Auf salzarmen Medien (P24, GD) mit erhöhten Agarkonzentrationen (1.1 %) konnten höhere Keimungsraten erzielt werden als auf den Referenzmedien mit 0.8 % Agar. Hormonfreies Reifungsmedium mit 6% Sorbitol und eine nachfolgende Kältebehandlung von 4°C für 8 Wochen führte zu den höchsten Keimungsraten. Es wurde gezeigt, dass der durch Sorbitolzugabe erzielte Trockenstress zur Ernte von 2- bis 3-mal mehr qualitativ hochwertiger Embryonen mit niedrigerem Frischgewicht führte als die Kontrollbehandlung.

Keimlinge wurden in Magenta GA-7 Dosen auf hormonfreiem Aktivkohlemedium bis zu einer Größe von etwa 5 cm gezogen und anschließend im Glashaus akklimatisiert.

Wie andere recalcitrante Samenpflanzen sind auch Edelkastanien sehr schwer vegetativ zu vermehren, deshalb sind *in vitro* Methoden zum Klonen von ausgewachsenen Bäumen mit bekannten Eigenschaften eine interessante Ergänzung zur forstlichen Vermehrung.

Die Induktion somatischer Embryogenese bei Holzpflanzen gelingt aber meist nur ausgehend von juvenilem Gewebe, insbesondere von unreifen Früchten und mit dem Gametophyt in Zusammenhang stehenden Geweben. Immerhin konnten somatische Embryonen der Edelkastanie bereits an jungen Blattgeweben von *in vitro* Stecklingen induziert werden [Corredoira 2002].

Um als Routineverfahren für die Massenproduktion von Klonen einsetzbar zu sein, sind vor allem bei Reifung, Keimung und Akklimatisierung weitere Optimierungsarbeiten nötig. Als



Basis dazu sind Untersuchungen der Effekte verschiedener Reifungs- und Keimungs - Behandlungen mittels anatomischer, histologischer, physiologischer und molekularbiologischer Methoden nötig. Reifungsprotokolle könnten dann gezielter auf die Bedürfnisse der Embryonen abgestimmt werden.

Eine weitere Herausforderung für zukünftige Arbeiten ist es, das Potenzial zur Automatisierung auszunutzen. Dies ist oft schwer zu bewerkstelligen ist, da Flüssigkulturen für Bioreaktoren nicht erfolgreich etabliert werden konnten [Vágner 2005]. Andrade [2005] konnte für die amerikanische Edelkastanie bereits ein System für Suspensionskulturen entwickeln, das eine wesentliche Effizienzsteigerung bei der Keimlingsproduktion ermöglichte.

Trotz der hohen Kosten, die mit der Entwicklung der *in vitro* Methoden einhergehen, könnte ein funktionierendes System für die somatische Embryogenese den steigenden Bedarf decken, Züchtungsprogramme beschleunigen und somit einen Beitrag zur Erhaltung der Edelkastanie in unserer Kulturlandschaft leisten.



## KAPITEL 7

### SUMMARY

Sweet Chestnut is not only a commercially valuable tree species for nut and timber production, but also a valued element of our landscape. The traditional chestnut culture in coppices is no longer important, the demand for poles, sticks and fuel wood decline and nuts are no longer „bread of the poor“. Not only the social and economic changes threatened the species in Europe in the last century; pollution, climate change and introduced pathogens were responsible for the decline of stands. A sign for the raising interest in this species was the COST Action (European Cooperation in the field of Scientific and Technical Research) *G4 Multidisciplinary Chestnut research* 1996 to 2001, initiated by Austria, a framework for European experts in tree physiology, pathogens, genetics and silviculture. A wide range of research topics was studied, such as basic research regarding blight, resistance to the disease and blight control, but also development of new management practice for high quality timber production, assessing genetic resources of European chestnut, breeding and mass production of trees.

The scope of this work was to develop a protocol for somatic embryogenesis (SE) in European chestnut (*Castanea sativa* Mill.).

During 1998 and 1999 142 SE cell lines out of 3163 juvenile explants were initiated from *C.sativa*, which corresponded to an overall induction frequency of 4.5%. For the first time the developmental window in which SE induction is possible was determined. The optimal stage was between 5 and 10 weeks postanthesis, cell lines initiated 4-7 weeks postanthesis could be maintained via secondary embryogenesis on P24 medium with and without 0.89  $\mu$ M 6-benzylaminopurine (BA). Since proliferation media influence quality of SE and subsequently conversion frequencies and anomalous formations of SE were frequently observed on BA supplemented media, hormone free medium was preferred. In order to improve development- and maturation-conditions, experiments with increased agar (1.1%) concentration and sorbitol (6%) were performed on both hormone free and BA-media.

Subsequently SE were cultivated on media without PGR and stored at 4°C, 10°C, or at room temperature. Highest embryo yield as well as highest germination frequency (40%) was achieved on hormone free media plus 6% sorbitol, after a cold treatment (8 weeks, 4°C). Germinated plantlets were transferred to Magenta™ GA-7 vessels containing hormone free P24-medium supplemented with 1% w/v activated charcoal. Plantlets were transferred to substrate and acclimatized in the greenhouse.

The data generated from this study and also from literature are suggesting that it is not possible to define a single, general protocol for chestnut micropropagation. Consequently, the protocol will have to be adapted to the characteristics of each cultivar. *In vitro* chestnut regeneration was achieved many years ago; nevertheless, large-scale propagation of chestnut species using *in vitro* techniques is still difficult. However, around 50 000 plants per year are produced *in vitro* by a company in Spain. This is the largest number of *in vitro* chestnut plants produced world-wide (Garcia-Nimo 1998), but it includes only several selected genotypes.

Further work is needed to elucidate the biochemical and molecular regulation of chestnut somatic embryo development with the aim of adjusting maturation and germination treatments for the production of high quality plants.

Although high costs can be involved in the development of tissue culture methods, there are several advantages of *in vitro* culture, such as potential for scaling up in bioreactors, cryopreservation, method for gene transfer etc. A system for somatic embryogenesis can be a useful tool integrated in traditional breeding programmes and as such be a valuable contribution for the maintenance of European chestnut in the traditional cultural landscape.

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## CURRICULUM VITAE

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### Further training

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4/04	EURISKED / CREDO Cluster Workshop "Multi-organic Risk Assessment of Endocrine Disrupters", Mallorca
2/03	Workshop on statistical analysis of microarray data University of Aarhus, Denmark
6/02	Bioinformatics Summer School National University of Ireland, Maynooth
6/99	Project: „Induction of Androgenesis in Chestnut Hybrids“ Instituto de Investigaciones Agrobiologicas de Galicia, Santiago de Compostella, Spain

### Collaboration in Projects

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since 8/01	„DNA Chip/ Diagnostik“ – Development and evaluation of new reactive coatings for biochips; assay development for protein biochips
	<b>Austrian Research Centers</b>
7/00-10/00	„Publish reproducible operating procedures on tissue culture and mutation techniques“
	<b>International Atomic Energy Agency (IAEA)</b>
6/00	„Cryopreservation of Oak“
	ARC Seibersdorf research GmbH, Biotechnology
6/99-12/99	„Biocontrol of Chestnut Blight“
	ARC Seibersdorf research GmbH, Biotechnology

## Peer reviewed Papers

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Invited Lecture: Assessment of the Quality of Microarray Data; MolPAGE workshop: Statistical Analysis of Genetic and Gene Expression Data, Pavia, Italy, March 20.-24. 2006

U. Sauer, C. Preininger: Protein Chips zur Bestimmung Endocriner Disruptoren, Deutsches Biosensor Symposium, Regensburg. March 13. -16. 2005

U. Sauer, Preininger C, Trombitas M, Obersriebnig S, Krumpel G, Kern W: Signal Enhancement of Protein Chips. 7th European Conference on Optical Chemical Sensors and Biosensors, 4.-7. April 2004, Madrid.

## Poster presentations

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Advances in Microarray Technologies, 7-8 May 2008, Barcelona, U. Sauer, J. Pultar, P. Domnanich, C. Preininger, A biochip for the detection of C-reactive protein, procalcitonin and interleukin-6, the major biomarkers for inflammation.

The 11<sup>th</sup> Annual European Conference on Micro and Nanoscale Technologies for Biosciences 2007, Montreux, U. Sauer, L. Schorn & C. Preininger: Polymer nanostructures as immobilization matrix in protein chips for detection of inflammation and sepsis.

The Ninth World Congress on Biosensors 2006, Toronto, U. Sauer, C. Preininger, K. Derwinska, J. Pultar, C. Martin: Protein Biomarker Chip for TNF alpha, IL-6, IL-8, IL-10, IL-13, Fibrinogen, and Albumin.

Stusemiar Chiptechnolgien 2005, Frankfurt am Main, Germany, U. Sauer, C. Preininger. Strategies for signal enhancement of antibody arrays.

ÖGBM Joint Meeting 2002, Salzburg, Austria, U. Sauer, C. Preininger, Experimental Design and Quality Control of Oligonucleotide Microarrays.

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