2nd International Conference of the IUFRO WORKING PARTY 2.09.02
June 25–28, 2012 • Brno, Czech Republic

Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management

book of abstracts

SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES
IUFRO WORKING PARTY 2.09.02

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Welcome Message

Welcome to our second conference of IUFRO working party 2.09.02: Somatic embryogenesis and other vegetative propagation (VP) technologies.

Above all, I am very happy to see a large number of colleagues attending this conference despite the hard economic times.

I am particularly pleased to hold this conference here in Brno, where Gregor Mendel founded modern genetics, although in our efforts to achieve clonal propagation, we try to defy Mendel's law of segregation.

Two years ago, we met in Korea with a primary interest in somatic embryogenesis and its application in tree improvement programs. However, this group has been extended to include all vegetative propagation technologies of woody plants as well as related tree physiology and genetics.

Early work on the vegetative propagation of trees goes back to the 1940s but the active research started in the 1960s, which led to the discovery of SE in conifers in 1985. The development of vegetative propagation technology in some trees is at the point where it can be implemented industrially. Therefore, an objective of this conference is to discuss this possibility, and I think this conference is an appropriate forum to help accomplish this task.

Attending this conference are several pioneering researchers from the 1960s as well as new breeds of tissue culturist, physiologists, geneticist, biotechnologists, foresters, etc. During the conference, we will hear about the current state and future prospects of VP technology, offering an excellent opportunity to connect and exchange ideas. I think that we also have a nice social program as well. I would encourage you to meet and get acquainted new and reacquainted with old colleagues during the conference.

On behalf of organizing committee, I would like to thank all the contributors. My special thanks go to Professor Horáček, the Dean of Forestry, for graciously agreeing to host this conference. Without his support, having this conference in beautiful Brno would not have been possible! Thank you. We are also grateful to the Rector of Mendel University, Prof. Hlušek for his support. Thanks to Professor Brzobohatý for bringing the Mendel Lectures to the conference. Many thanks to the South Moravian Region for providing financial assistance, which has helped PhD students to attend this conference.

I also would like to take this opportunity to introduce the members of the organizing committee: Jana, Mariano, Jean-Francois, So-Young, and Professors Jankovsky and Reinohl, and Martin Vagner (IEB-Prague), Martina Šatinská (South Moravian Region for Mobility) and Mrs. Levová (Barcelo Brno Palace Hotel) for local arrangements. Thank you.

Once again, have a great conference!

Yill-Sung Park
Coordinator, IUFRO 2.09.02

DETAILED SCIENTIFIC PROGRAMME

Sunday, June 24, 2012		
12:00		Registration desk opens
15:30 – 17:30		Brno Sightseeing tour
19:00 – 21:00		Welcome drink

Monday, June 25, 2012		
7:45		Registration desk opens
8:45 – 9:30		Opening messages / Plenary speech
Session MLP: Mendel Lectures and Plenary		
		Moderator: B. Brzobohatý
9:30 – 10:20 Mendel lectures	D. J. Durzan	Parthenogenetic apomixis, androsporogenesis, progenesis and asexual heterospory in a gymnosperm artificial sporangium
10:20 –10:40		Coffee Break
10:40 – 11:30 Mendel lectures	K. Klimaszewska	Cloning of adult conifer trees via somatic embryogenesis: are we close to understand and overcome recalcitrance?
11:30 – 12:20 Mendel lectures	H. Häggman	Biosafety of genetically modified forest trees (GMTs)– COST action P0905 – a common action of European scientists
12:20 – 13:20		Lunch
		Moderator: S. Merkle
13:20 – 13:50	J. M. Bonga	Recalcitrance is still a major issue
13:50 – 14:20	S. von Arnold	Patterning during somatic embryogenesis in conifers
14:20 – 14:50	A. Ballester_	Alleviating recalcitrance in clonal propagation of mature trees through somatic embryogenesis in *Fagaceae* species. The example of pedunculate oak
		Moderator: Organizing committee
14:50 – 16:00	Poster Introduction: Various authors	Poster presentations (slide show)
16:00 – 18:30		Poster Session – Coffee served at 16:00
19:00 – 22:30		Conference gala dinner

Tuesday, June 26, 2012		
Session 1: Somatic Embryogenesis		
		Moderator: K. Klimaszewska
8:20 – 8:50	J. M. Canhoto	Somatic embryogenesis: great achievements from small temperate and subtropical trees
8:50 – 9:10	K. Zoglauer	Somatic embryogenesis in *Abies nordmanniana*: present status and future application
9:10 – 9:30	Y. W. Kim	Initiation of embryogenic suspensor mass (ESM) and somatic embryogenesis in Japanese red pine (*Pinus densiflora*)
9:30 – 9:50	J. H. Wang	Initiation of embryogenic suspensor mass in *Picea balfouriana*
9:50 – 10:10	A. Ivanitskaya	Embryological Aspects of Somatic Embryogenesis in *Larix sibirica* and *Larix gmelinii*
10:10 – 10:30		Coffee Break

		Moderator: J. M. Canhoto
10:30 – 11:00	C. Miguel	An integrated approach to the study of embryogenesis in maritime pine
11:00 – 11:20	P. Moncaleán	A combined method to increase somatic embryogenesis efficiency in valuable cell lines of *Pinus* spp.
11:20 – 11:40	I. Tretiakova	The Embryogenic Lines and Somatic Embryogenesis of Coniferous Species in Siberia
11:40 – 12:00	H.-K. Moon	Somatic embryogenesis and plantlet regeneration from *Oplopanax elatus* – effect of genotype and physical culture conditions
12:00 – 12:20	C. Reeves	It isn't all about Rugby: New Zealand and France unite to give Douglas-fir somatic embryogenesis protocols a workout.
12:20 – 13:20		Lunch
13:20 – 13:40		Bus loading
14:00 – 18:00		Visit Botanical Gardenin Brno / Arboretum Krtiny
18:00 – 19:30		Visit cave in Moravian Karst
19:30 – 22:30		Social Dinner Cerna Hora offered by Mendel University / beer taster session

Wednesday, June 27, 2012		
Session 2: Other vegetative propagation techniques including rooted cuttings, micropropagation by organogenesis, etc.		
		Moderator: O. Monteuuis
8:20 – 8:50	C. Díaz-Sala	Maturation, developmental transitions and reprogramming during adventitious regeneration in forest species
8:50 – 9:20	C. Hargreaves	Laboratory and field performance of plants derived from adventitious and axillary meristems of the same genotypes of radiata pine. Big surprises, little surprises or no surprises?
9:20 – 9:40	S. Parlak	Clonal propagation of bay tree (*Laurus nobilis* L.) using cuttings
9:40 – 10:00	A. Sinha	Integrated vegetative propagation of *Schleichera oleosa*
10:00 – 10:20		Coffee Break
		Moderator: C. Díaz-Sala
10:20 – 10:40	M. L. Robert	Tissue culture systems for the efficient propagation of red cedar (*Cedrella odorata* L.)
10:40 – 11:00	C. Ragonezi	Micropropagation of recalcitrant pine (*Pinus pinea* L.). An overview of the effects of ectomycorrhizal inoculation
11:00 – 11:20	P. von Aderkas	Culturing nucellus as a way to produce proteins involved in conifer reproduction
11:20 –12:20		Business meeting
12:20 – 13:20		Lunch
Session 3: Implementation / mass propagation into commercialization		
		Moderator: P. Gupta
13:20 – 13:50	D. K. S. Goh	Mass clonal propagation of teak plus trees for high yield and superior quality plantations
13:50 – 14:10	S. Kuusienė	Practical application of tissue culture for the mass production of the clones of selected hybrid aspen trees in Lithuania
14:10 – 14:30	C. K. Aidun	Fluidics-Based Automation of Clonal Propagation via Somatic Embryogenesis: SE-Fluidics System

14:30 – 14:50	R. J. Licea-Moreno	Towards scaling-up the micropropagation of *Juglans major* var. 209 × *J. regia*, a hybrid walnut of commercial interest.
14:50 – 15:10	M. Lstibůrek	Mathematical programming framework to clonal deployment in multi-varietal forestry
15:10 – 15:30		Coffee Break
		Moderator: T. Fenning
15:30 – 15:50	K.-A. Högberg	SE propagation and genetic diversity – example from a practical case
15:50 – 16:10	A. McCartney	Building toward commercial scale implementation of multi-varietal forestry
		Moderator: T. Fenning, A. McCartney, Expert Panel
16:10 – 18:30		Workshop and General Discussion on commercial Implementation of Vegetative Propagation
18:30		Free evening to enjoy Brno

Thursday, June 28, 2012		
Session 4: Physiology, genetics, epigenetics, biotechnology, cryopreservation, etc.		
		Moderator: M. Vágner
8:20 – 8:50	B. Brzobohatý	Cytokinin: Novel roles for an old hormone
8:50 – 9:10	V. Neděla	Native state of extracellular matrix of early conifer embryogenic tissue imaged by environmental scanning electron microscope
9:10 – 9:30	C. Teyssier	Reduce water availability during hybrid larch somatic embryo maturation is not stressful
9:30 – 10:00	M. Vágner	The key role of phytohormones in somatic embryogenesis of Norway spruce
10:00 – 10:20		Coffee Break
		Moderator: P. von Aderkas
10:20 – 10:50	S. A. Merkle	Building the "pipeline": Applying somatic embryogenesis, bioreactors and transgenic technology to restore the American chestnut
10:50 – 11:10	C. Sánchez	Gene expression patterns during the acquisition of embryogenic competence and embryo development in Fagaceae species
11:10 – 11:30	J. Krajňáková	Involvement of mitochondria in the programmed cell death during somatic embryogenesis of *Abies alba*
11:30 – 11:50	Y. K. Lee	Climatic adaptation in Norway spruce – Molecular dissection of a novel epigenetic memory mechanism
11:50 – 12:20	T. Fenning	Forest biotechnology and climate change
12:20 – 13:20		Lunch
13:20 – 13:40		Bus loading
13:40 – 19:00		Visit Research Station at Kunovice
19:00 – 22:30		Social Dinner in Uherske Hradiste / Local wine taster session

Friday, June 29, 2012	
7:00	Departure for Prague trip

Abstracts for oral presentations

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Session

Mendel Lectures and Plenary

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Mendel lectures are a part of the project "Advanced professional education as a mean to improve quality of personal available for biotechnological R & D" co-financed by the European social fund and national budget of the Czech Republic

IUFRO WORKING PARTY 2.09.02

SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES

MLP–1

Interpolated progenesis and heterospory in gymnosperm artificial sporangium

D. J. Durzan

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Female parthenogenetic apomixis (fPA) and androsporogenetic parthenogenesis (mAP) in an artificial sporangium (AS) was demonstrated in three dioecious genotypes viz. Norway spruce (Finland), Douglas-fir & *Araucaria angustifolia* (Brazil). An AS is any process-controlled bioreactor which removes the supporting structures for ovule and microspore (androspore) formation in mature trees and replaces them with nutrients and hormones in an aqueous environment under controlled temperatures.

In fPA, uniform cell suspensions of embryonal initials were transdifferentiated into archegonial tubes with nuclei originating by apomixis (endoreduplication & amitosis). Archegonial tubes comprised a diploid egg-equivalent and ventral nuclei (vcn). The vcn becomes apoptotic. Female parthenospores were discharged from archegonial tubes and dispersed into the aqueous medium In Norway spruce, Douglas-fir and *Araucaria angustifolia* the diploid egg-equivalent nuclei recapitulated free-nuclear proembryogenesis. Norway spruce and Douglas-fir proembryos, when were removed from the AS, were regenerated into clones. Proembryogenesis in *Araucaria* recapitulated the primitive features of *Araucariaceae*.

In mAP, nuclei in embryonal initials underwent automixis (meiotic parasexual reproduction) forming androsporangial tubes which discharged and dispersed male parthenospores (monads, dyads, triads, tetrads, polyads) into the aqueous medium.

Scaled-up suspension cultures of eggs from *Taxus brevifolia* and female *Ephedra californica* (female monoecious genotypes) exhibited free-nuclear mitotic replications before discharging and dispersing mitospores into the aqueous medium. Gametic genome doubling (diploidy) was not ruled out.

Reproduction in monoecious and dioecious genotypes was brought to an earlier ontogenetic stage (progenesis). Expressions of asexual heterospory emulated reports for spore formation in some modern and extinct algae. Observations have significance for exploring the adaptive evolution of the alternation of generations in gymnosperms under controlled laboratory conditions.

Durzan DJ (2012) Female parthenogenetic apomixis and androsporogenetic parthenogenesis in embryonal cells of *Araucaria angustifolia*: Progenesis and asexual heterospory in an artificial sporangium. Sexual Plant Reproduction (in press)

Durzan DJ (2011) Parthenogenetic apomixis and androsporogenesis in a Douglas-fir artificial sporangium. Sexual Plant Reproduction 24: 283–296, DOI 10.1007/s00497-011-0171-2

Durzan DJ, Jokinen K, Guerra M, Santerre A, Chalupa V, Havel L (1994) Latent diploid parthenogenesis and parthenote cleavage in egg-equivalents in Norway spruce. Intl J Plant Science 155: 677–688

Keywords: partenogenetic apomoxis, androsporogenesis, artificial sporangium, asexual heterospory, gymnosperms

MLP–2

Cloning of adult conifer trees via somatic embryogenesis: are we close to understand and overcome recalcitrance?

K. Klimaszewska[1], B. Boyle[2], S. Caron[2], C. Overton[1], D. Stewart[1], J. Mackay[2] & R. G. Rutledge[1]

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Conifer somatic embryogenesis (SE) from mature or immature zygotic embryos has become an established biotechnology within the forest industry, providing the capability for commercial scale production of genetically defined seedlings. However, cloning of adult conifers via SE is still challenging. If possible, this would not only allow capture and propagation of individual trees with elite characteristics, but would also help circumvent the need for long-term performance testing before initiating large-scale propagation, as is required for zygotic embryo-derived genotypes.

Identification of a white spruce genotype (G6) whose shoot bud primordia have remained responsive to SE induction for over a decade (Klimaszewska *et al*. 2011) provided a unique opportunity to determine if molecular differences between this and a nonresponsive genotype (G12) could be identified. Based on the presumption that differences in gene expression during early stages of SE induction would reflect differences in response, a 32K oligonucleotide microarray developed by the Arborea genomics project (*www.arborea.ca*) was used to select four candidate genes for each genotype, based on the greatest difference in expression relative to the other genotype at day 7 of induction. Referred to G6UP and G12UP, fold differences ranged from 3.6–5.5X and 6.9–9.6X (linear scale) for each group, respectively. In contrast, no differences in expression were detected among any of the eight candidate genes at the point when the buds were collected, indicating that all eight of the candidate genes were activated by the induction treatment. This was found to range from 3.6–17.0X for the G6UP candidates and 9.2–46.1X for the G12UP candidates, and indicated that differences between the two genotypes at day 7 were a result of a higher level of activation, rather than reduction of expression within the other genotype.

To increase the scope and resolution of the study, three additional time points were included that extended the analysis to day 21 of induction, along with using LRE qPCR to determine the absolute quantities of these transcripts at each of the five induction time points. The resulting expression profiles not only confirmed the trends predicted by the microarray analysis, but also revealed extensive genotype-specific differences in expression of all the candidate genes throughout the induction treatment. Most notable was a progressive increase in expression in the nonresponsive G12 buds (>5000X), of two G12UP genes encoding for small molecular weight proteinase inhibitors, producing some of the highest expression levels for any gene we have encountered to date. Although the expression of both genes increased in the responsive G6 buds, this activation was moderate, reaching only about 15% of that in the G12 buds, and only during last six days of induction. A very similar expression profile was produced by another G12UP candidate gene that has homology to peroxidase, but with lower levels of expression.

While resolving how they are correlated to SE responsiveness will require additional work, this study does reveal substantive genotype-specific differences in gene activity, which among other things, provide targets for future research.

Keywords: white spruce, genotypes, microarrays, gene expression, plant regeneration

MLP–3

Biosafety of genetically modified forest trees (GMTs)– COST action P0905 – a common action of European scientists

H. Häggman[1], J. M. Find[2], G. Pilate[3], F. Gallardo[4], M. Ruohonen-Lehto[5], V. Kazana[6], F. Migliacci[7], L. Ionita[8], M. Sijacic-Nikolic[9], F. Donnarumma[10], A. Harfouche[11], S. Biricolti[12], B. Glandorf[13], L. Tsourgiannis[14], K. Minol[15], D. Paffetti[16], M. Fladung[17] & C. Vettori[10]

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After more than 20 years experience of confined transgenic forest tree field trials and millions of hectares of commercial transgenic crop fields, the only commercialized GM tree plantations are in China. Concidering the increasing population size and more area needed for food production in the future, it has been proposed that only the marginal areas will be available for wood production. Thus it has to include effective tree breeding combined with propagation of specific elite or tailor-made genotypes for plantation forestry. In addition the plantation forestry needs efficient but sustainable management.

The ongoing COST Action FP0905 "Biosafety of forest transgenic trees: improving the scientific basis for safe tree development and implementation of EU policy directives" focuses on four key aspects related to the biosafety of GMTs: (a) analyses of the efficiency of existing gene containment strategies to avoid or if not possible to minimize gene flow; (b) facilitate efforts to develop site-specific integration of transgenes in tree genomes to minimize variability of transgene expression and pleiotropic effects, (c) evaluate possible methods to monitor GMTs in the whole production chain, and (d) conduct socio-economic and cost/benefit analyses in relation to the use of GMTs in plantations.

We are looking forward that the information gathered on GMTs will contribute to strengthen the scientific basis for the execution of the EU policy directives related to transgenic trees intended for cultivation in Europe. Moreover our aim is to evaluate the scientific knowledge relevant for GMT biosafety protocols by combining the already existing information generated in various EU and Non-EU countries as a basis for future EU policy and regulation for the environmental impact assessment and the safe development and practical use of GMTs.

Keywords: genetically modified trees, biosafety, field trials, COST action P0905

MLP–4

Recalcitrance is still a major issue

J. M. Bonga

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After almost 80 years of in vitro culture recalcitrance is still a common problem. In my presentation I will deal briefly with some historical observations of recalcitrance and attempt to define it. Subsequently I will describe the often early onset of recalcitrance and will explain potential measures that could, in some instances, possibly resolve the problem. Because the issue is complex and involves a large number of factors only a few relevant aspects will be dealt with. Among the measures discussed are miniaturization, selection of explants and paying attention to asymmetric divisions. If time permits, a few aspects of the involvement of stress in overcoming recalcitrance will be touched upon.

Keywords: recalcitrance, *in vitro* culture, stress, asymetric division, selection of explant

MLP–5

Patterning during somatic embryogenesis in conifers

S. von Arnold, D. Uddenberg & E. Larsson

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Improvement of forest trees by breeding is a slow process in which economically significant characteristics are continuously improved. The improvement is obtained by testing and selection. Each breeding cycle for Norway spruce and Scots pine takes in Sweden 20 to 25 years. The selected trees are used as parents in the next breeding cycle. The selected genotypes are also used for mass-propagation. This is done by establishing seed orchards which starts to produce seeds after 15 to 20 years. Consequently, seed orchards are 20 years behind current breeding front. Can somatic embryos replace seed orchards in the future?

The early events in embryo development are the most critical for plant body formation. We are using somatic embryos of Norway spruce as a model for studying embryology in conifers. Polar auxin transport (PAT) is of major importance for the correct patterning of the embryonal shoot and root meristems. PAT is inhibeted when embryogenic cultures and developing embryos are treated with the PAT inhibitor NPA. Blocked PAT caused fused cotyledons, aborted embryonal shoot apical meristem (SAM) and an irregular root meristem. The establishment of the embryonal SAM in Arabidopsis is dependent on the expression of the homeodomain containing transcription factor *SHOOT MERISTEMLESS* (*STM*). STM is one of four class 1 *KNOTTED-like homeobox* (*KNOX1*) genes in Arabidopsis. Four *KNOX1* genes have been identified in Norway spruce, *HBK1*, *HBK2*, *HBK3* and *HBK4*. During embryo development the *HBK2* and *HBK4* genes were significantly up-regulated concomitantly with the formation of an embryonic SAM, the up-regulation was delayed by NPA treatment. In contrast, *HBK1* and *HBK3* were up-regulated prior to SAM formation, and their temporal expression was not affected by NPA treatment. Together our results suggest that *HBK2* and *HBK4* are essential for somatic embryogenesis and the formation of SAM.

Embryogenic cultures can be established from mature zygotic embryos of Norway spruce. The embryogenic potential decreases after germination. However, after exposure to the histone deacetylase inhibitor, TSA, germinating somatic embryos maintained the competence to differentiate embryogenic tissue simultaneously as the germination progression was partially inhibited. In Arabidopsis, *LEAFY COTYLEDON* (*LEC*) genes are expressed during the embryonic stage, and must be repressed to allow germination. Furthermore, treatment with TSA causes de-repression of *LEC* genes. We have isolated two LEC1-type *HAP3* genes (*PaHAP3A* and *PaHAP3B*) from Norway spruce. The expression of *PaHAP3A* was high during early embryo development, but decreased during late embryogeny. When embryogenic cultures were exposed to TSA during embryo maturation, the maturation process was arrested and the expression level of *PaHAP3A* was maintained, suggesting a possible link between chromatine structure and expression of embryogenesis-related genes in conifers.

Keywords: Embryo development, Embryogenic potential, Norway spruce, Somatic embryogenesis.

MLP–6

Alleviating recalcitrance in clonal propagation of mature trees through somatic embryogenesis in *Fagaceae* species. The example of pedunculate oak.

A. Ballester & A. M. Vieitez

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Mature trees, exhibiting desirable traits, are generally recalcitrant to clonal propagation. In hardwoods, the use of plant material retaining juvenile physiological characteristics, such as epicormic shoots, root suckers or stump sprouts, may facilitate the in vitro propagation of elite genotypes. Even using this approach, micropropagation of mature trees may be hindered by relatively low rates of multiplication and rooting abilities, decline of proliferation capacity over time, propagation limited to specific genotypes within species, etc. Theoretically, somatic embryogenesis (SE) is the most efficient procedure for mass propagation of forest trees. However, major difficulties must still be overcome, as the main problem reported for many hardwood species, including *Fagaceae*, is the relatively low conversion rate of the somatic embryos into plantlets. According to Bonga *et al*. (2010) the induction of SE may be the only method of rejuvenating truly juvenile propagules. If this is true, plants derived from somatic embryos should exhibit juvenile characteristics, including a high capacity for micropropagation through organogenesis.

The main objective of the present study was to increase the efficiency of in vitro clonal propagation of mature *Quercus robur* (100–300 years old), by induction of somatic embryogenesis as rejuvenation step prior to establishment of shoot culture through micropropagation of somatic embryo-derived plantlets. Shoot culture lines of "mature" origin were established from epicormic shoots of two centenarian oak genotypes (Sainza and CR-0) and maintained by axillary shoot proliferation. Embryogenic lines were also initiated from epicormic leaf explants of the same genotypes and maintained by secondary somatic embryogenesis. Although the frequency of somatic embryo conversion into plantlets was low in pedunculate oak, shoot culture lines could be established and maintained by axillary branching from several germinated somatic embryos. For each genotype and shoot culture line of the two origins (mature tree and somatic plantlets), shoot proliferation rate, elongation and rooting ability parameters were determined. Compared with "mature-origin" shoot cultures and after more than one year propagation in vitro, shoot lines established from somatic plantlets produced a significantly higher proportion of elongated, rootable shoots with increased rooting capacity. The results (Martínez *et al*., 2011) provided evidence that some rejuvenation occurred during the process of somatic embryogenesis and resulted in improved shoot growth and rooting of somatic embryo-derived culture compared with "mature" shoot culture. The results reported in this study might be useful in embryogenic systems with low plant conversion rates. The proposed experimental model might also be useful in finding molecular markers of plant ontogeny.

Bonga JM, Klimaszewska KK, von Aderkas P (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tissue Organ Cult 100: 241–254

Martínez T, Vidal N, Ballester A, Vieitez AM (2011) Improved organogenic capacity of shoot cultures from mature pedunculate oak trees through somatic embryogenesis as rejuvenation technique. Trees (doi 10.1007/s00468-011-0594-2)

Keywords: recalcitrans, clonal propagation, mature trees, somatic embryogenesis, *Quercus robur*

Session

Somatic Embryogenesis

IUFRO WORKING PARTY 2.09.02
SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES

Somatic embryogenesis: great achievements from small temperate and subtropical trees

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Somatic embryogenesis is a powerful tool for plant breeding and analysis of plant development. In recent years, we have developed and optimized protocols for plant regeneration of several small temperate and subtropical trees of economic and/or environmental relevance, including *Arbutus unedo* (strawberry tree), *Ceratonia siliqua* (carob), *Cyphomandra betacea* (tamarillo), *Feijoa sellowina* (pineapple guava), *Laurus nobilis* and *L. azorica* (laurels) and *Myrtus communis* (true myrtle). Regeneration from adult tamarillo and strawberry tree was achieved, opening the way for large scale cloning of selected trees. In the others, somatic embryogenesis was only obtained from juvenile tissues, such as zygotic embryos. In spite of the different patterns of somatic embryo formation – direct or indirect, one step or two steps, repetitive or through the maintenance of embryogenic calli – cytological, anatomical and ultrastructural studies have shown that somatic embryos are always originated from meristematic-like cells presenting a dense cytoplasm and large nucleus. Moreover, a suspensor-like structure was found in all species and its formation characterized in pineapple guava. The data have also shown that in embryogenic tissues kept in culture for periods over two years, modifications in DNA content and chromosome number usually appear in the regenerated plantlets, especially in tamarillo. Attempts to use embryogenic calli to obtain tetraploid plants following treatments with c-mitotic agents are being made. Morphologically abnormal embryos are a common feature in all species but manipulation of the culture conditions (light) and media (sucrose and growth regulators) greatly reduced its formation. In all species, somatic embryos have been induced by auxin treatments. However, they could be also induced by stress conditions such as high osmolarity treatments, extreme pH pulses, cold and wounding in an auxin-free medium. Among the studied species tamarillo seems to be the more appropriate to perform molecular studies in order to understand totipotency acquisition and somatic embryo formation. In this case embryogenic and non-embryogenic calli can be obtained from the same explant (young leaves) and in large amounts making them a useful system to compare embryogenic and non-embryogenic mRNA and protein profiles. Based on this system a putative member of the SpoU Methylase protein family has been identified in non-embryogenic calli. Genetic transformed lines of tamarillo, in which this gene was silenced, have been obtained and propagated. Investigation is going on to assess their ability to produce somatic embryos in parallel with knocked-out lines of *Arabidopsis* for this gene. New lines of inquiry were open and deserve our attention.

Keywords: genetic transformation, proteomic, tamarillo, totipotency, woody plants.

S1–2

Somatic embryogenesis in *Abies nordmanniana*: present status and future application

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The biotechnical procedure for clonal mass propagation of Nordmann fir (*Abies nordmanniana*) has been studied for more than 10 years and improved with regard to the critical steps as long-term propagation of embryogenic cultures, loss of maturation ability, storage of mature somatic embryos, conversion, acclimatisation and field establishment.

80 to 90% of the embryos with normal morphology could be converted, independently on their genotype. After approx.16 days of conversion, seedlings are ready for acclimatisation. Various substrates, potting systems, humidification systems, plant protection etc. have been compared resulting in a survival of 90% of the plants under appropriate conditions. In contrast to other genera, *Abies* seedlings go into dormancy in the cotyledonary stage. However, 2 to 3 months after acclimatisation dormancy could be broken using artificial cold treatment and followed by a second flush in the same season. Although this may speed up their growth somatic seedlings developed slower than and lost up to 1 year compared to plantlets from seeds. Reasons, consequences and possible solutions for breeding of clonal varieties will be discussed.

Keywords: somatic embryogenesis, *Abies nordmanniana*, clonal mass propagation, clonal varieties

S1–3

Initiation of embryogenic suspensor mass (ESM) and somatic embryogenesis in Japanese red pine (*Pinus ensiflora*)

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The best ESM initiation frequency was obtained from 0.88% (June 28, 2004, Suwon), 1.4% (July 1st, 2005, Suwon), 2.31% (July 1st, 2005, Anmyeon) and 0.91% (July 1st, 2006, Suwon), respectively and the all embryos in the seeds were at the proembryo stage regardless seed collection year (2004, 2005 or 2006) or location (Suwon or Anmyeon). Albeit it has well known that seed development may vary in climate, from year to year by latitude and elevation, the initiation frequency of ESM in relation with histological result suggests that the optimum yearly collection time for seeds can be based on the collection dates (June 28, July 1st and July 5), at least for *Pinus densiflora*, in Korea. The highest proliferation rate (9.8-fold) of ESM was obtained from ½LM medium supplemented with 3.42 mM L-glutamine. For somatic embryo maturation with 0.05% activated charcoal (AC), the highest number (798/g^{-1} FW) of cotyledonary somatic embryos (line 06–29) was obtained. In germination of somatic embryos from ESM line 05–3 with light-emitting diodes (LED), the frequency was strongly inhibited by both fluorescent lamp and red + blue light (0%, respectively) for that. On the other hand, other lines (05–12, 05–29 and 05–37) showed similar germination patterns to five LED sources.

Keywords: *Pinus ensiflora*, somatic embryogenesis, initiation frequency

S1–4

Initiation of embryogenic suspensor mass in *Picea balfouriana*

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Abstract Somatic embryogenesis of *Picea balfouriana* were initiated from zygotic embryos in different developmental stages sampled from three different genotypes. Overall average initiation frequency of SE was 25.37% and 267 embryogenic lines were established, representing only 6.5% of the initial explants. The highest induction rate of 71.2% was obtained with the best explants and 1/2 LM basal medium supplemented with 2.2 mg·L^{-1} 2,4-D, 1.1 mg·L^{-1} BAP and 0.2 mg·L^{-1} KT. Genotype 2 was significantly superior than other genotypes, suggesting that initiation was under strong genetic control. Zygotic embryos at stage 9.1 were the most optimized for initiation SE.

Keywords: *Picea balfouriana*, Somatic embryogenesis, Initiation, Embryogenic suspensor mass

Embryological Aspects of Somatic Embryogenesis in *Larix sibirica* and *Larix gmelinii*

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Larch trees growing in Siberia are damaged by larch bud midge which influences larch stands state negatively. Therefore propagation of improved, resistant to larch bud midge larch trees is actual. Mass propagation of progeny with defined heredity could be achieved with the help of somatic embryogenesis.

Successful micropropagation *in vitro* should be closely connected with cytological control of the embryological structures, formed *de novo*, so we studied cyto-embryological peculiarities of somatic embryogenesis. At present time we obtained somatic embryogenesis in two larch species, growing in Siberia – *Larix sibirica* and *Larix gmelinii*. The material for the experiments on somatic embryogenesis was immature zygotic embryos obtained as a result of open and control pollination of cones.

Somatic embryogenesis passed most perspective from immature zygotic embryos at the stage of cotyledon formation on special developed АИ medium (patent). After a week of culturing at the area of pericolumn of zygotic embryo intense elongation of cells were observed. Asymmetric division of the elongated cells leads to the formation of a small spheric cell at one of the poles. Division of spheric sells gave rise a globule of somatic embryo whereas division of elongated cells lead to the formation of suspensor. Then somatic embryos differentiated identical to zygotic embryos of investigated species. Investigations have indicated common regularities of somatic embryogenesis process in *Larix sibirica* and *Larix gmelinii*.

After two month of culturing somatic embryos were capable to mature at transfer on medium with ABA. From such somatic embryos well-developed plantlets were formed. However, it should be noted, that not all of obtained embryogenic lines can produced somatic embryos capable to pass maturation. Thus, somatic embryos of one embryogenic line (Kl-3) stopped their developing at the torpedo stage in spite of additional treatments.

Emryogenic lines of *Larix sibirica* obtained from immature zygotic embryos can proliferate for a long time, and could be used as a material for further embryological and genetic research.

This work was supported by RFBR grants № 11-04-00281

Keywords: somatic embryogenesis, embryology, *Larix sibirica*, *Larix gmelinii*

An integrated approach to the study of embryogenesis in maritime pine

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Members of the *Pinaceae* family, including spruces and pines, dominate many of the northern hemisphere temperate and boreal forests. These organisms adapted to a range of diverse climates and habitats and are of critical importance for global ecosystem stability and biodiversity. They are also a major source of raw material for many essential human needs providing the majority of world's wood and fibre supply. Maritime pine (*Pinus pinaster* Ait.) is the most important tree species in South-western Europe (Portugal, Spain and France) covering approximately 4 million hectares. Understanding the biology of pines is crucial to maintaining the European competitiveness in the global forest products market.

Embryogenesis is a crucial phase of plant life cycle as it establishes the basic body plan with shoot and root poles, hypocotyls and cotyledons, and prepares the embryo for subsequent germination and early plant growth. Although important insights into plant embryogenesis have been given through the investigation of this stage of plant development in the model species *Arabidopsis*, still much less is known about the molecular mechanisms controlling conifer embryo development. In our lab we first started working in maritime pine embryogenesis as a potential tool for clonal propagation within breeding strategies through somatic embryogenesis. At present, somatic embryogenesis is also an essential experimental system to successfully achieve genetic transformation in pine. A system for somatic embryo induction and development has been established; however the lack of knowledge concerning the regulation of embryo development has hampered the full exploitation of somatic embryogenesis potential. Therefore, in our lab we engaged in a search for molecular regulators of embryo development. Several transcriptomic approaches for identifying genes that are differentially expressed in consecutive stages of development have been followed including microarray analysis. Differentially expressed genes could be clustered into distinct expression profiles along embryo development. The grouping of these profiles in early, mid-embryogenesis and embryo maturation, according to the developmental period where most of the sequences were up-regulated, indicates that characteristic transcriptional changes are associated to each developmental period. We confirmed that different molecular mechanisms regulating zygotic embryo development are common to both angiosperms and gymnosperms, in particular signaling pathways regulated by phytohormones. We also identified the putative involvement of genes for which no previous relation to conifer embryogenesis had been reported contributing to our knowledge on a fundamental process of development in species that are essential components of terrestrial ecosystems and a unique resource for sustainable growth.

Keywords: conifer, maritime pine, embryogenesis, transcriptomics

A combined method to increase somatic embryogenesis efficiency in valuable cell lines of *Pinus* spp.

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Radiata pine (*Pinus radiata* D. Don), is one of the most widely grown exotic timber species in the world. In our lab, the two main in vitro techniques used routinely for *Pinus* spp. micropropagation are somatic embryogenesis (SE) (Montalbán *et al*. 2010, 2012) and organogenesis (De Diego *et al*. 2008, 2010, 2011; Cortizo, 2009; Montalbán *et al*. 2011a). Studies on organogenesis and SE have contributed to the extension of tissue culture for commercial applications (Stasolla and Thorpe 2010). Although propagation via SE is an effective method in propagating elite plants when it is combined with other technologies, such as cryopreserving the embryogenic tissue and selecting elite clones in field tests (Park 2002), maturation of the embryogenic tissue into cotyledonary, normal somatic embryos is not always successful, in several pine species. Our research team has developed a maturation system that provides the best maturation yields reported to date in *Pinus* species (Montalbán *et al*. 2010). But there are some cell lines that still produce a low number of somatic embryos, and make large-scale production of these genotypes too expensive and therefore eliminate them from production; consequently, the number of genotypes that can be candidates for clonal forestry decreases (Davis and Becwar 2007). This is particularly important issue in the case of genetically transformed embryogenic tissues. This work describes a combined plant regeneration system that includes somatic embryogenesis and organogenesis from immature seeds of radiata pine (Montalbán *et al*. 2011b) as well as a cold storage step. In this sense, recently, we have developed a preservation method for somatic embryos at non-freezing temperatures (5ºC) which maintained the viability of the explants for more than a year, and a multiplication system of these somatic embryos through organogenesis. By means of cold preservation and *in vitro* organogenesis somatic plantlet production is not restricted to a certain moment of the year and the material obtained can be multiply and rooted when demanded, with no detrimental effect on the explants produced.

Acknowledgments: This research was funded by Ministerio de Ciencia y Tecnología-Spain (AGL2005-08214-CO2-02) and Departamento de Agricultura y Pesca-Gobierno Vasco (VEC2004021and VED2007014), who granted Itziar A. Montalbán a PhD scholarship. The authors are grateful to C. Hargreaves and her team from Scion (New Zealand) and K. Klimaszewska from Canadian Forest Service-Laurentian Forestry Center (Canada) for giving I.A. Montalbán the opportunity of learning in their respective laboratories and for enriching discussions during the course of this work.

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Keywords: *Pinus radiata*, micropropagation, somatic embryogenesis, organogenesis

The Embryogenic Lines and Somatic Embryogenesis of Coniferous Species in Siberia

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In the beginning XXI century somatic embryogenesis in culture in vitro of *Larix*, *Pinus*, *Picea* growing in Siberia have began studied. Experiments of culturing the immature isolated embryos and megagamethophytes of Siberian coniferous species were carried out on different modified media: *Pinus sibirica*, *Pinus pumila* on ½ LV medium, *Larix sibirica*, *Larix gmelinii* on MSG and АИ (patent) media, *Picea ajanensis* on DCR medium. For induction of embryogenic callus every species needs the optimized medium supplemented with L-glutamine, casein hydrolysate, ascorbic acid and hormones with different concentrations and their different proportions. The active proliferation of embryonal masses is observed on the same medium with reduced concentration of cytokinins. The proliferation of embryonal masses was significantly improved when they were subcultured after dispersing in liquid medium. The somatic embryos from embryonal masses are matured on basal medium with ABA (60–120 mM) and PEG.

In spite of species specificity the embryogenesis of morphogenic structures had the same scheme: elongation of somatic cells, formation of initial cells and embryonal tubes, development of globular, torpedo and bipolar somatic embryos, embryos maturation and germination. However, not all donor-plants of coniferous species can form the embryogenic cell lines and somatic embryos. The active development of embryonal masses and formation of somatic embryos are observed from zygotic embryo of hybrid seeds of *Pinus sibirica* and *Larix sibirica*. The obtained embryogenic lines were characterized by different proliferative activity. During 10 months cultivation the value of embryonal masses in different lines was 140–570 g. The number of somatic embryos varies from 210 to 410 per 100 mg of callus fresh weight. Decreasing proliferation activity did not observed during 24–45 months cultivation. However, development of somatic embryos in long cultivated lines decreased. Maturation of somatic embryos and development of plantlets were established in *L. sibirica* and *P. pumila* 50–60 somatic embryos were matured per 1 g of callus fresh weight.

Somatic embryogenesis pass over the strong genetic control. Only donor tree genotypes with high reproductive potential form embryogenic cell lines and somatic embryos. The studying molecular mechanisms involved in the control regulation of embryo development (embryo maturation, desiccation and germination) allow to understund of many aspects of molecular biology of gymnosperms.

This work was supported by RFBR grants № 11-04-00281 and Integration project № 140.

Keywords: embryogenic lines, somatic embryogenesis, *Pinus sibirica*, *Pinus pumila*, *Larix sibirica*, *Larix gmelinii*, *Picea ajanensis*

Somatic embryogenesis and plantlet regeneration from *Oplopanax elatus* – effect of genotype and physical culture conditions

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Oplopanax elatus, a rare and endangered species, is one of important medicinal trees to be recommended for research as a ginseng-type preparation source. Because of its highly medicinal properties against such maladies as asthenia, depression, and hypertension, demand for the species has increased, but its distribution in Korea is quite limited, occurring only on several high mountains and within restricted areas. In an attempt to establish mass production system of *O. elatus* by increasing productivity of somatic embryogenesis, various factors affecting somatic embryogenesis was studied. Embryogenic cell lines were induced total 13 mature embryos collected from 4 different mountains, and these calli were used for simple sequence repeat (SSR) to investigate the genetic diversity and genotype effect. Embryogenic capacity was affected by genotype, but little correlation between embryogenic capacity and the location which is seeds were collected was founded. Root segments of in vitro plantlets was the best explants to induce embryogenic callus by showing good embryogenic capacity compare to petiole and leaf segments. Sucrose among carbon sources, especially 5% in concentration, effectively produced somatic embryos from embryogenic callus (around 180 SEs per petri-dish), while glucose and maltose produced SEs less than 20 per petri-dish, and sorbitol and mannitol completely inhibited SE formation. Culture density was not significantly effective in promoting somatic embryo formation when cells were cultured 0.007~0.02g per petri-dish, with over 0.02g of culture density actually inhibiting it. Over 50% of SEs were converted into plantlets on MS medium solidified with 0.8% agar or agar and gelrite mixture (0.6% gelrite + 0.4% agar), and the medium containing GA_3 0.2 mg/L enhanced conversion rate up to 80%. Around five hundred somatic embryo-derived plantlets were transferred to artificial soil mixture and cultivated in greenhouse. After two months, approximately 80% of transplanted plantlets were successfully acclimatized.

Keywords: somatic embryogenesis, *Oplopanax elatus*, genotype, sucrose, conversion

S1–10

It isn't all about Rugby: New Zealand and France unite to give Douglas-fir somatic embryogenesis protocols a workout

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Douglas-fir is the second most important exotic forest species in New Zealand next to radiata pine which is currently 89% of the planted estate. There is significant interest in genetically improving this species to provide diversification of the national planted forest estate away from radiata pine. Benefits include; the better matching of species to sites at higher altitude and cooler climates; minimising exposure to disease; timber would produce a wider range of forest products. Additionally it would give us a contingency species if radiata pine was to fail in some significant way. Breeding programs to improve and select the best Douglas-fir genes for New Zealand conditions are advanced and two seed orchards have been established in the South Island.

Propagation of this species is challenging, seed production is affected by climatic factors and production of control-pollinated seed is not straight forward. The combination of irregular seed crops, plagiotrophism and dormancy in cuttings as well as the limited life of stool beds (approximately 2 years as compared to 4 plus years, for radiata pine) make producing improved genetic planting stock problematic. Somatic embryogenesis could potentially address some of these issues via amplification of control-pollinated seed, production of non-plagiotrophic plants and arrest of maturation in clonal material while field testing takes place. There is potential to integrate somatic embryogenesis with nursery stoolbed production methods.

Somatic embryogenesis methods are established for this species overseas and part of the Weyerhaeuser forest strategy. We wanted to develop independent capability with this species using *P. radiata* protocols. Preliminary investigations were started in New Zealand to develop SE capability with Douglas-fir essentially adapting the *P. radiata* protocols we were most familiar with. We got to know our French collaborators and the importance of Douglas-fir in France (2.6 million m^3 harvested/year) via the two previous IUFRO Somatic Embryogenesis of Forest Trees conferences held in Whistler (Canada) and Suwon (Korea). INRA and FCBA have been developing breeding programs for growth rate, branching and other traits of interest for more than 40 years. Joint funding was obtained for two years from both the French and New Zealand governments via the Dumont d'Urville scheme to develop the collaboration. Progress with this species is a direct result of happy and vigorous discussions (and a bit of mutual rugby appreciation through 2011).

Using only slightly modified radiata pine protocols we were able to initiate cell lines, cryopreserve tissue, mature embryos and establish plants in the greenhouse. But, there were a few challenges. Culture initiation rates using pine methods were satisfactory but the resultant tissue grew slowly and many of the cell lines had a mixture of both embryogenic and non embryogenic tissue. This was the most limiting step with regard to developing capability with Douglas-fir at Scion. We are pleased to report that progress has been made with proliferation and this is the data we would like to concentrate on in this presentation. However, all the methods we are using will be discussed and data presented as appropriate on initiation, proliferation, cryopreservation, maturation and organogenesis.

Keywords: somatic embryogenesis, Douglas-fir, initiation, proliferation

Session

Other vegetative propagation techniques including rooted cuttings, micropropagation by organogenesis, etc.

S2–1

Maturation, developmental transitions and reprogramming during adventitious regeneration in forest species

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The possibility of regenerating whole plants from somatic differentiated cells emphasizes the plasticity of plant development. Cell-type respecification during regeneration can be induced in adult tissues as a consequence of injuries, changes in external or internal stimuli or changes in positional information. However, in many plant species, switching the developmental program of adult cells prior to organ regeneration is difficult, especially in forest species. In these species, a decline in the capacity of regenerating shoots, roots or embryos from somatic differentiated cells is associated with tree age and maturation. The loss of regeneration capacity associated with the juvenile-adult transition makes forest species representative and reliable systems to study how cell fate becomes fixed during development and how plant cells can manage to retain developmental plasticity. Besides its impact on forest productivity, basic information on the flexibility of cell differentiation is necessary for a comprehensive understanding of the epigenetic control of cell differentiation and developmental plasticity. Studies of reprogramming adult cells in terms of regulative expression changes of selected genes will be of great interest to unveil basic mechanisms regulating cellular plasticity. Cell fate switches both in plants and animals are characterized by remarkable changes in the pattern of gene expression, as cells switch from an expression pattern typical of a somatic cell to a new one directing a new developmental pathway. Thus, determining the way by which cells reset their gene expression pattern, especially for the timetable and repertoire of gene expression characteristic of the earliest stages of development, is crucial to understand cellular plasticity. In addition, the presence of specific cellular signalling pathways or specific factors, perhaps distributed in a localized- or developmental-specific manner, in the tissues involved in regeneration could be crucial for cell fate switch. Factors such as asymmetric distribution of plant growth regulators, asymmetric gene expression or other tissue specific-factors underlying establishment, maintenance and redirection of different gene expression patterns could be involved in the control of age-dependent cellular plasticity.

Keywords: adventitious rooting, age, asymmetric gene expression, auxin gradients, cell fate, developmental plasticity, epigenetics, maturation, pluripotency, somatic embryogenesis

S2–2

Laboratory and field performance of plants derived from adventitious and axillary meristems of the same genotypes of radiata pine. Big surprises, little surprises or no surprises?

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Adventitious shoot formation from immature cotyledons of *P. radiata* was the most promising technology we had in New Zealand through the early 1980's. This method was used to amplify scarce and expensive control pollinated seed produced from elite crosses. We could in some genotypes, get sustained meristematic tissue production in both liquid and solid media. The resultant shoots were amplified *in-vitro*, set and the subsequent plants could be used as stoolbeds for further cutting production. *In-vitro* shoots were also cool stored to delay maturation while the initial field plantings were assessed for performance. Fletcher Challenge Forestry (now Arborgen owned) established a pilot commercial laboratory to test and improve these protocols which had initially been developed at the Forest Research Institute (now Scion). Our propagation science evolved and by the late 80's it was clear that the new methods established for somatic embryogenesis offered some distinct advantages over adventitious shoot formation. These advantages included cryopreservation of embryogenic tissue (arresting all maturation of stored tissue), potential for exponential embryo production and a plantlet that had a preformed root initial.

Somatic embryogenesis was not without its challenges; the most significant of these was the limited number of elite crosses and genotypes that made it through the entire process to the field. Initiation success for adventitious shoot cultures was high, all crosses responded and it was not unusual to have in excess of 75% of genotypes responding for any one cross. Subsequent research had shown that cotyledons prior to adventitious shoot production could be cryopreserved successfully thus delivering effective long-term storage of juvenile germplasm. Like somatic embryogenesis, the adventitious method had some mysteries and the most significant of these was the variable physiological age observed in propagules when established in the field. It appeared that some genotypes accumulated extra age, some were unaffected and others appeared more juvenile than comparisons with either seedlings from the same control crosses or epicotyl derived shoots from the same genotypes. We needed to establish what percentage of crosses/genotypes would produce useful planting stock and if we could incorporate these stocks into our family forestry approach.

An experiment was designed using 10 genetically diverse elite crosses with multiple genotypes from each cross. Two methods of shoot production were compared, adventitious shoots from cryopreserved cotyledons and epicotyl derived shoots from the same genotype. Data was collected for culture initiation success, shoot amplification and root initiation. Physiological age was assessed in the nursery bed. Growth and male and female cone production were measured in the field. Results of this work will be presented and the implications of these results in the context of what we do today at Scion and conifer biotechnology in general.

Keywords: *Pinus radiata*, adventitious, physiological age, flowering embryogenesis, Douglas-fir, initiation, proliferation

S2–3

Clonal Propagation of Bay Tree (*Laurus nobilis* L.) Using Cuttings

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This study was carried out in Aegean Forestry Research Institute's greenhouse between 2003-2008 years to determine the most suitable vegetative propagation method of Bay laurel *(Laurus nobilis* L.).
Effecting factors on rooting, such as cutting time, rooting media and rooting hormone doses were admitted as treatments. The study was carried out on rooting of cuttings taken in 11 periods from summer to winter, using four different rooting media and five different doses of IBA.

According to the results, considering root length, cuttings taken 30 July or 30 September should be prefered. The rooting ratio was high in sand media from cuttings taken on 30 July, however rooting ratio was high in all media for cuttings from 30 September. The highest rooting ratio for the cuttings from 30 July in 5,000 ppm IBA media was 63.3% and from 30 September cuttings in 10,000 or 20,000 ppm IBA medium was 56.7%. The lowest rooting ratio was from the samples provided on 30 July, and therefore less suitable for cutting collection.

Although the effect of media on rooting ratio is important, it was found that its effect on root number is not significant. Excess fringe root formation on cuttings are relative to successful planting after the rooting period because these cuttings have more adaptative capability in alternate environmental situations than others. Fringe root formation in perlite-peat media appear thinner structured than in other media. Auxiliary roots in sand, perlite and pumice media are pulpy and thick in formation.

Except in control samples, there are no statistically differences in rooting ratio among 3,000, 5,000, 10,000 and 20,000 dosages of IBA. When the number of roots are considered, the samples treated with 3,000 and 5,000 ppm showed to form one gruop, those treated with 10,000 and 20,000 ppm dosages formed another group with more roots. Control cutting samples formed a different group.

With respect to cutting collection period, if it is done on 30 July, a dosage of 5,000 ppm IBA, and for those from 30 September, 10,000 ppm IBA are prefered.

Keywords: Laurel, *Laurus nobilis* L. cutting propagation, IBA hormone dosages, cutting periods, rooting media.

S2–4

Integrated Vegetative Propagation of *Schleichera oleosa*

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Schleichera oleosa is a multipurpose and commercially important tree particularly in South and Southeast Asia. Immature fruits are edible. Seed kernel contains 40% oil which is presently used as hair oil, body massage oil and lubricants. It also has potential for using as biodiesel. Plants extracts have several medicinal properties. In eastern part of India, this tree is used mostly for lac cultivation. This tree produces the best quality lac and gives higher yield per tree basis than other host trees. Lac is a natural resin recreated by an insect *Kerria lacca* (Kerr.). The raw lac is the source of resin, dye and wax. India is the largest producer of lac in the World. Major importing countries of Indian lac are Arab, Bangladesh, Egypt, Germany, Indonesia and USA. However, the area of lac cultivation as well as the production of quality lac has been eroded due to several reasons and loss of biodiversity of lac insects as well as their host plants is a major point. So far, very limited research activities on improvement of lac host plants particularly *S. oleosa* has been carried out.

In the present paper, the efforts since more than a decade to explore vegetative means of propagation for ensuring the regular supply of quality planting stocks have been depicted. The extensive exploration was carried out for the collection of superior trees from the whole lac producing region in eastern part of India. Techniques were standardized for plant establishment from stem cuttings, grafting and air-layering. Stem cutting studies were carried out on source plants of different ages, length of stem cuttings, collection seasons and hormonal effects. Higher rooting (more than 60%) was observed in cuttings collected from seedlings. For propagation of mature trees, maximum success (56.7%) in grafting was achieved by cleft grafting. However, variability in success of graft union was observed among selected trees.

Combined treatment of auxins induced rooting in air-layering of mature trees. Twig diameter of smaller than 2 cm was found to be unsuitable for successful plant establishment. For the first time in vitro shoot proliferation of the species was reported by first author. Like other woody plant tissue culture, culture of *S. oleosa* also faced the problem of browning. The exudation of phenolic substances was checked by suspending explants in a chilled solution of ascorbic acid and by the addition of antioxidants in medium. Dropping of leaflets, shoot tip necrosis and death of cultures were observed even after 6–12 months of establishment of cultures. In vitro seed germination, indirect organogenesis and integration of other vegetative propagation for mass multiplication of selected clones have been approached to solve the problem.

Keywords: stem cutting, grafting, air-layer, lac host, browning

S2–5

Tissue culture systems for the efficient propagation of red cedar (*Cedrella odorata* L.)

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Cedrela odorata L. is a valuable tropical tree widely appreciated for its wood. This species confronts serious problems due to both overexploitation of its natural populations and its susceptibility to the *Meliaceae* borer *Hypsipyla grandella*, which destroys the apical meristems and produces structural deformations. The establishment of commercial plantations, on the other hand, is hampered by the lack of high quality homogeneous plants

The rapid introduction of cloned selected materials is the most effective way to improve the production of perennial plantation species. In this work, we report a protocol for the rejuvenation of elite mature trees of *C. odorata* and the optimization of an in vitro culture system to scale up micropropagation.

Several media formulations and the use of temporary immersion culture in bioreactors were evaluated. The addition of 20% coconut water to TY17 medium increased the number of adventitious shoots from hypocotyl segments to an average number of 4.68 shoots per explant. To replace coconut water and to define the culture medium, several cytokinins were tested at various concentrations; however, none of them produced the effect of coconut water. Rejuvenation of elite mature individuals was investigated by *ex vitro* grafting of mature tree twigs onto 3-mo-old juvenile trees.

Although the grafting had a positive effect on the micropropagation of mature material, the multiplication rate of 1.5 new shoots per explant did not compare to the organogenic capacity of younger materials. Shoot and root elongation as well as acclimatization to *ex vitro* conditions of juvenile material were carried out in a temporary immersion culture bioreactor (BioMINT®). A 3.5-fold increase in shoot elongation and a 4-fold increase in root elongation were achieved compared to material cultured on semisolid media. Furthermore, this culture system allowed for 98% survival when the *in vitro* grown plants were transferred to soil. The overall multiplication capacity of this system, over a period of 6 months is around 16,000 plants per mother plant when young plants (derived from seed) are used, but only 125 when selected mature mother plants were used as the source for explants.

Keywords: Red cedar, Clonal forestry, BioMINT® Bioreactor, Rejuvenation.

S2–6

Micropropagation of recalcitrant pine (*Pinus pinea* L.) – An overview of the effects of ectomycorrhizal inoculation

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Stone pine *(Pinus pinea* L.) is an economically important forest species in some regions of Iberian Peninsula. Portugal and Spain have nearly 500,000 ha of stone pine stands, representing 85% of worldwide distribution. The main use of this species is for the production of seeds (pinion) for food industry. In addition to its enormous profitability as a producer of seeds, it has beneficial impact on soil protection, dunes fixation and is a pioneer species particularly for cork and holm oaks degraded ecosystems. Stone pine plantations are today a major source of income for forestry holdings. Investments have targeted breeding, reforestation, forest management and harvesting. The maternal inheritance of desirable characteristics such as cone weight, number of seeds per cone and seed length is considerably high in this species thus encouraging the selection of seeds from "plus" trees. The selected trees have been propagated by grafting and micropropagation. However, grafting generates high variability due to scion-rootstock interaction that varies production levels. The production of clonal plants from selected seeds by micropropagation techniques has advanced very slowly due to the recalcitrance of this species in tissue culture and particularly to adventitious rooting of microshoots. Due to the tremendous importance of developing a reproducible tissue culture method for clonal propagation, a study has been carried out for over a decade to enhance rooting and acclimation. During this period of time, continuous increments in the multiplication rate and rooting frequency were achieved by introducing variations in culture media composition and conditions. Auxins, carbohydrates, light quality and duration, temperature at different concentrations and levels as well as compounds such as coumarin; salicylic acid, polyamines, etc. were tested for induction and expression phases of adventitious rooting. Despite these efforts, microshoots regenerated through organogenesis from mature embryo cotyledons failed to root or to have sustained root growth. At this point, an *in vitro* co-culture technique of stone pine microshoots with ectomycorrhizal-fungi was introduced to overcome the adventitious root growth cessation *in vitro* and improve root development during acclimation phase. An overview of the results showing the positive effect of fungal inoculation in promoting root growth *in vitro* and on plantlet survival during acclimation will be presented. Preliminary results of biochemical signals between *Pinus pinea*/*Pisolithus arhizus* during early steps of *in vitro* culture detected by liquid chromatography-mass spectrometry that might be responsible for the positive effect on root growth will be also presented.

Keywords: acclimation, co-culture, ectomycorrhiza, *in vitro* adventitious rooting, micropropagation, stone pine.

S2–7

Culturing nucellus as a way to produce proteins involved in conifer reproduction

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The nucellus has many roles during conifer ovule development. Initially, it is a sporogenous tissue that undergoes meiosis to produce a megagametophyte and its eggs. Next, during pollination it produces a pollination drop that traps pollen. Afterwards, it interacts with pollen tubes. In a mature seed, the nucellus dies, leaving only a wax-rich hydrophobic layer. We sampled Douglas-fir (*Pseudotsuga menziesii*) nucellus at the time of pollination drop secretion. Nucellar cultures were easily induced on different media, then multiplied in suspension. Proteins that were secreted into the growth medium were harvested and analyzed by mass spectrometry. We were able to confirm that many of these proteins were the same as those produced by ovular secretions during reproduction. Nucellar culture represents a significant advance in the study of the genesis of pollination drop constituents. Nucellar tissue culture not only overcomes the restrictions imposed by *in situ* studies such as short collecting seasons and low volumes, but is potentially a tractable experimental system for studying pollen-ovule interactions in controlled conditions.

Keywords: *Pinus radiata*, micropropagation, somatic embryogenesis, organogenesis

Session

Implementation / mass propagation into commercialization

IUFRO WORKING PARTY 2.09.02
SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES

S3–1

Mass Clonal Micropropagation of Teak Plus Trees for High Yield and Superior Quality Plantations

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The dramatic reduction of high value timber supplies from natural stands facing an increasing worldwide demand has accounted for a greater interest in teak (*Tectona grandis*) plantation establishment. Production of high yield of top quality teak wood in short rotations is now becoming a priority for a lot of land owners and investors in many humid tropical countries. This trend warrants the current attractiveness for superior teak clones that can be planted either as monocultures or in combination with other crops. During the early 1990's in Sabah (East Malaysia), the company Yayasan Sabah Group Biotech ("YSG Biotech"), jointly with CIRAD-Forêt (France), had developed a very efficient method for mass cloning superior teak trees of any age by micropropagation. The outstanding field behaviour of the first clonal offspring produced *in vitro* from the mature teak "Plus" trees locally selected has led to their rapid mass production to meet local and international demands. To date, several millions of these clonal offspring have been produced worldwide and the demands keep increasing due to the superiority of these materials. Meanwhile, efforts aiming at enriching YSG Biotech teak genetic base have been pursued, and today the company owns the world widest teak gene pool. This richness is essential for genetic improvement through wisely established breeding populations, from which advanced generations of new clones can be produced, resorting to non destructive wood analyses methods and DNA markers for upgrading the clonal selections. The advantages of the micropropagation technique developed for utilizing at best these valuable genetic resources, including the true-to-type mass cloning of any mature selected teak individual, and for exporting these clones all around the world bypassing even very stringent phytosanitary requirements, are presented.

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cont. **S3–1**

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Keywords: mass clonal propagation, *Tectona grandis*, genetic improvement

S3–2

Practical application of tissue culture for the mass production of the clones of selected hybrid aspen trees in Lithuania

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Plant biotechnology methods used for mass production of hybrid aspen clones in Lithuania will be reviewed. Micropropagation of hybrid aspen (*Populus tremuloide*s Michx. × *P. tremula*) involves the establishment of explants from shoot segments carrying vegetative buds, the initial growth and multiplication of microshoots in tissue culture and their subsequent transfer into ex vitro conditions.

Growing demand for biomass revealed the advantages of fast-growing hybrid aspen under Lithuanian conditions. The first hybrid aspen (*Populus tremuloides* Michx. × *P. tremula*) stands in Lithuania were established by Dr. Murkaitė in 1968. In the present Laboratory of Forest Plant Biotechnologies of Lithuanian Research Centre for Agriculture and Forestry (LRCAF), the technology of hybrid aspen micropropagation through tissue culture was developed in 2003, including *ex vitro* acclimatization of microshoots and production of saplings with closed root system as well as the establishment of the first experimental clonal stands. However, at that moment, a more extensive practical application was not achieved and the shortage of hybrid aspen saplings remained the main limiting factor for the development of hybrid aspen stands in Lithuania. Since 2009, the cooperation between the scientists of Institute of Forestry of LRCAF and a private enterprise (joint-stock company "Euromediena") brought more positive results in the development of large-scale micropropagation of hybrid aspen. After additional studies, Woody Plant Medium developed by Lloyd and McCown (1980) and 400-ml vessels of transparent plastic were applied for the culture of hybrid aspen explants. Currently, microshoot segments with axillary buds are routinely cultured for the period of three weeks under standard light and temperature conditions. LED lamps and some plant hormones have been tested for the optimization of *ex vitro* acclimatization conditions, leading to the rate of rooted and vigorously growing clones as high as 90–98%. As for the year 2012, 100 thousand saplings per month are produced. After adaptation, the saplings are transferred to containers and grown in a nursery until their are high enough (0.6–1.0 m) for the establishment of a forest stand. The technology for the production of hybrid aspen artificial seeds is also under development. It was shown so far that the time period required for the *ex vitro* acclimatization of encapsulated microshoots can be shortened to 14 days and that the saplings obtained in such a way are firmer. This technology can be advantageous also for the clonal propagation of other forest tree species.

Acknowledgments: The presented material includes research findings, which have been obtained through long-term research programme "Sustainable forestry and global changes" implemented by Lithuanian Research Centre for Agriculture and Forestry.

Keywords: clone, tssue culture, hybrid aspen, microshoot, propagation, technology

S3–3

Fluidics-Based Automation of Clonal Propagation via Somatic Embryogenesis: SE-Fluidics System

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High cost of production due to tedious manual handling is the obstacle preventing large-scale commercial plant production through somatic embryogenesis (SE). It is widely accepted that SE is the only viable method for mass clonal propagation of many plant species. Several attempts to replicate human hand-eye movements in SE processing by smart robots have been underway for some years. In our view, a completely different approach with new way of thinking is required. We present a 'fluidics-base' approach with several unique and innovative features, referred to as the "SE-Fluidics System". In this process, we no longer follow the conventional laboratory procedure. The entire SE process takes place in liquid so to allow easy transfer from one stage to another without human handling of the embryos. It turns out that not only this approach allows automation, some developmental steps such as maturation and germination are improved when processing in liquid instead of solid medium. The entire process can be divided into the following steps. Proliferation and Maturation take place in partially immersed bioreactors designed with special features for automated transfer of embryos and proembryogenic masses (PEM) to the SE-Fluidics System. The entire content of the BRs are transferred and processed in the SE-Fluidics System through the following steps: (1) Extraction system where embryos and PEMs are transferred from the BRs into sterile water ready for processing in the (3) Dispersion system; at this stage the embryos and PEMs are dispersed in liquid. The next processes are (3) Separation, where the embryos are isolated from the PEMs, (4) Regulation, where the embryos are lined up in a liquid tube with regular spacing, (5) Inspection, where the embryos are imaged by a high-speed camera followed by image analysis, (6) Selection, where the embryos are examined against a pre-programmed 'selection criteria', and (7) Orientation and Deposition, where the embryos that pass the selection criteria are oriented in the right direction and deposited into the substrate.

We demonstrate the SE-Fluidics System outlined above by presenting the 2-Line Pilot System (model number U-P2) at SweTree Technology's facilities in Uppsala, Sweden. We show that with this system the matured embryos are deposited into a substrate without ever being handled by human hands. The proliferation and maturation are in partially immersed bioreactors designed for rapid attachment and extraction into U-P2. The material inside the bioreactors is extracted where the mature embryos are automatically isolated and deposited into the substrate. U-P2 is designed and built with instrumentation speed allowing for isolation and deposition of 4 embryos per second when both lines operate in parallel. However, the actual rate of deposition depends on synchronization of the embryo development in the bioreactors. For now, our preliminary results show that on average one mature embryo can be deposited per second. The focus of this presentation will be to introduce the SE-Fluidics System and to outline the key steps.

Keywords: automation, somatic embryogenesis, liquid handling, dispersion, separation, orientation

S3–4

Towards scaling-up the micropropagation of *Juglans major* var. 209 × *J. regia*, a hybrid walnut of commercial interest

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As a result of the program for quality-wood production conducted by Bosques Naturales SA, several walnut *èlite* genotypes have been selected on the basis of chemical and mechanical wood properties, as well as overall morphology and growth under plantation conditions. Whereas it is important to establish an efficient clonal propagation system for such hybrids, doing it has been hampered by the well-known recalcitrance of walnut to *in vitro* manipulation. In addition, many walnut hybrids have difficulties in shedding viable seeds. Whereas a few general protocols are available, they must be adapted to the specific genotypes and conditions of each laboratory. Here, we present some recent findings that have allowed us to improve current micropropagation strategies for *Juglans major* var. 209 × *J. regia*, a hybrid of commercial interest. These improvements focus mainly on microshoot quality, rooting capacity, and tolerance to *ex vitro* conditions. DKW formulation was used as basal culture *medium* and field-grown trees were used as explant donors. We specifically investigated (i) the influence of phloroglucinol (Phl), during multiplication, on rooting; (ii) the possible benefits of using FeEDDHA instead of FeEDTA; (iii) the influence of subculture length; (iv) the optimal doses of sucrose and other carbon sources for pre-induction and root expression, respectively; and (v) the appropriate conditions for expression phases. The use of a Temporal Immersion System (TIS) to promote elongation was also evaluated. Eight genotypes were chosen for these experiments, based on their differential behaviour. Inducing the sprouting under controlled laboratory conditions allowed us to rise up the success of *in vitro* introduction once the explants do not release phenols to culture *medium*, probably as a consequence of the first *ex vitro* rejuvenation of explants. The use of Phl was a key factor to promote basal-calli formation, to increase microshoot length and to enhance the formation of auxiliary shoot. Only apical microshoots at least 20 mm in size were used for root induction. These were inoculated in culture medium supplemented only with IBA as hormone. After incubation in the dark, the expression phase started under photoperiod conditions. There was no statistically significant interaction between genotype and the iron source used, a relevant finding taking into account that substitution of FeEDTA by FeEDDHA was determinant for the rooting of all genotypes. The subculture length prior to root pre-induction was also found to be important for rooting. Despite genotype-specific effects on in vitro behaviour, there was a high influence of sucrose concentration for root pre-induction for all clones. Both sucrose and D-glucose were found to be more suitable than D-fructose for root expression and ex vitro acclimatization. Microshoots growing on TIS were taller than those multiplied on gelled culture media. Forty seconds of immersion per day was enough to increase shoot height. More than 90% of the vitroplants that survived the first 4 weeks under *ex vitro* conditions could reach nursery and field plantation. These results allowed us to clone 6 *èlite* genotypes during 2011.

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Keywords: acclimatization, explant quality, rooting, phenolization, temporal immersion system.

S3–5

Mathematical programming framework to clonal deployment in multi-varietal forestry

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Clonal deployment is methodically presented as a standardized mathematical programming problem. Using a simple dataset, we present optimum deployment solutions under more complex scenarios, such as relatedness or differential success rate (propensity) in vegetative propagation. We demonstrate that non-linear solutions are optimum under the majority of practical scenarios. Next, we provide recommendations to operational breeding and deployment programs worldwide.

Keywords: deployment populations, clonal mixtures, genetic gain and diversity

S3–6

SE propagation and genetic diversity – example from a practical case

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Somatic embryogenesis is notorious for the uneven distribution of cell lines among families and uneven distribution of plants per cell line. The uneven structure is generated already at initiation and is then present throughout the propagation.

In a practical propagation, starting with 37 families entering initiation, there were 28 families left at the stage where plantlets were acclimatised to nursery conditions. The uneven representation among families gave a status number of 15.2 with the restriction that the number of plants per cell line was equal. After taking into account also the uneven distribution of plants among cell lines, the status number increased slightly to 16.

If only the 16 most productive cell lines were included, the status number dropped to 8.1 due to the unevenness.

In a practical production of untested clones, uneven family and clone representation can lead to considerably lower genetic diversity than the number of families entering the propagation indicates.

Keywords: somatic embryogenesis, genetic diversity, propagation, practical production, *Picea abies*

Building toward commercial scale implementation of multi-varietal forestry

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J. D. Irving, Limited (JDI) is a family owned group of businesses based in eastern Canada. The company includes a diversified and vertically integrated mix of forest products companies, including sawmills, pulp and paper mills, tissue mills, and a corrugated paper mill. JDI manages 1.2 million hectares of freehold land and 1 million hectares of provincially owned leased land in the Canadian provinces of New Brunswick, Nova Scotia, and the State of Maine. The company has a long history of establishing planted forests and since 1957 has planted over 850 million trees. Maintaining a sustainable supply of fibre from these woodlands is vital for the growth of company operations over the long term. We recognized early on that multi-varietal forestry (MVF) through somatic embryogenesis (SE) is an effective tool to capture genetic gain, providing a means to contribute to the company's vision for sustainability while securing wood fibre supply to meet future demand. We, therefore, began developing SE capabilities in the mid 1990's by focusing primarily on white spruce and Norway spruce. To date, we have initiated and evaluated over 1600 unique varieties. By selecting the top 20% of varieties in terms of growth, insect resistance, and adaptability, we have been able to capture significant genetic gain from a diverse number of full-sib crosses. More recently, however, our focus has shifted to strategies aimed at maximizing the value of MVF technologies. Our strategy has two primary goals: (1) to implement technologies that reduce the cost of SE production through efficiencies of scale and (2) to integrate MVF within the company's overall silviculture management plan in order to maximize both the genetic and economic value of the trees produced. Through strategic partnerships we are interested in expanding SE production of well characterized varieties that are targeted to appropriate sites.

Keywords: multi-varietal forestry, somatic embryogenesis, genetic gain

Session

Physiology, genetics, epigenetics, biotechnology, cryopreservation, etc.

IUFRO WORKING PARTY 2.09.02
SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES

S4–1

Cytokinin: Novel roles for an old hormone

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Cytokinins are plant hormones involved in the regulation of diverse developmental and physiological processes in plants. Over the last decade, much progress has been made in our understanding of cytokinin biosynthesis, metabolism, perception and signal transduction. These achievements have paved the route for recent progress in our understanding of the interactions between the function of cytokinin and environmental inputs. Thus, a network of molecular interactions between various environmental signals and cytokinin biosynthesis, metabolism, and signaling is emerging.

Plants integrate a range of environmental cues to regulate morphology according to their habitat and the time of year. Of these cues, light has the most profound effects on plant development. Morphological responses of plants to shading have long been studied as a function of light quality, in particular the ratio of red to far red light that affects phytochrome activity. However, responding to mere changes in light intensity is of considerable importance in Arabidopsis and other plant species which produce a large number of small seeds as part of their survival strategy. As a consequence, seedlings are small and often experience shading caused by soil surface irregularities. Previously, ethylene and auxin have been shown to control the Arabidopsis response to decreased white light intensity. Here, we report stimulation of hypocotyl elongation by cytokinins when the seedlings are cultivated at a decreased white light intensity. The stimulation is dose-dependent already in the nanomolar range of cytokinin concentration. Mutant and transgenic plant analysis indicated that the canonical two-component response pathway is necessary for this process with prevailing contribution of the cytokinin receptors AHK2 and AHK3. This cytokinin action was independent of ethylene signaling and partially inhibited by auxin.

Plants must adapt to changing environmental conditions that often include dramatic alterations in temperature during diurnal cycles. The degree of tolerance to heat- and cold-shock has a direct impact on plant fitness, and, consequently, plant productivity. Still, temperature perception remains poorly understood. Cytokinin levels were reportedly found decreased in heat-shock treated plants, responses to cytokinin treatment were enhanced in heat-shocked Arabidopsis, and a role for a subset of cytokinin two-component signaling system in cold temperature stress response was recently shown. Our proteome and phosphoproteome profiling of cytokinin action in Arabidopsis resulted in identification of early cytokinin response proteins and phosphoproteins, and a fraction of those evidenced hitherto unrecognized links between temperature and cytokinin signaling. Further, we investigated mutual modulation of cytokinin and temperature responses at the proteome and phosphoproteome levels. We have identified over 100 temperature-shock responsive (phospho)proteins of which more than 70% are cytokinin dependent and/or modulated. We propose that these (phospho)proteins might be functionally relevant for cytokinin-temperature cross talk.

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Keywords: cytokinins, signal transduction, proteome and phosphoproteome prolifiling, light, stress

S4–2

Native state of extracellular matrix of early conifer embryogenic tissue imaged by environmental scanning electron microscope

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Environmental scanning electron microscopy (ESEM) opens up a wide range of new applications in the field of electron microscopy which rely on being able to image sample in, or close to, their native state [1, 2]. In the ESEM the specimens can be observed in a wide range of pressure from 0,001 Pa (comparable with the SEM) to over a thousand Pa in the specimen chamber [3]. In high pressure conditions very wet non-conductive samples can be observed free of charging artefacts without a conductive coating covering their surface. If the gas pressure is sufficiently increased or the sample's temperature reduced, its natural and fully hydrated surface structure is preserved [4, 5, 6].

This study is focused on introduction on methodology enabling the creation of suitable condition for the study of ECM in situ. Early somatic embryogenic tissues of selected conifers (*Abies alba*, *Abies numidica* and *Pinus sylvestris*) were observed free of sputter coating with an electrically conductive layer, without use of any chemical fixation or preparation technique which means in really native state. Method was following: our samples were placed on a cooled Peltier stage with temperature from –18 °C to –22 °C in high pressure conditions 550 Pa of the specimen chamber of an ESEM. For comparison of natural surface of early conifer somatic embryogenic tissues a specially designed ionization detector of secondary electrons and yttrium aluminium garnet activated with trivalent cerium (YAG: Ce^{3+}) detector of backscattered electrons were used. Our results show native early embryogenic tissue of *Pinus sylvestris*, *Abies alba* and *Abies numidica* covered by a network of fibrillar material forming ECM layer.

We found that environmental scanning electron microscope operated in above described conditions is useful and very convenient for observing of native state plant tissue, even though we suppose that this method is unsuitable for observing of animal tissue without frees damages. Additionally, plant tissue free of chemical fixation procedures allows observing extracellular matrix in its native state. This method is fast and simple, more over relatively inexpensive. We suppose it will be generally applicable tool in the field of plant research [7].

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Keywords: environmental scanning electron microscopy, early somatic embryos, extracelullar matrix, conifers

S4–3

Reduce water availability during hybrid larch somatic embryo maturation is not stressful

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Development of clonal propagation method, such as somatic embryogenesis, has many applications including mass production of genetically improved plants. Since the 90's, INRA engaged researches on somatic embryogenesis of important species such as *Larix*, *Pinus sylvestris* and *P. pinaster* (Lelu-Walter *et al*. 2006; 2008; Lelu-Walter and Pâques, 2009). For these pine species, it is well established that somatic embryo maturation requires an increase of the gellan gum concentration of the culture media (Klimaszewska e*t al.* 2007). This high gellan gum is associated with reduced water availability from the culture medium to the embryonal masses (Klimaszewska *et al*., 2000).

In hybrid larch, increase of gellan gum concentration (from 4 to 8gL^{-1}) improved both the quantity and the quality of cotyledonary somatic embryos that subsequently germinate and develop into plantlets (Lelu-Walter and Paques 2009). Indeed, on high gellan gum somatic embryos 8 weeks old had lower water content and higher dry weight compared with those matured on standard medium. This increase in dry weight is consistent with an increase in protein content (total and soluble parts).

To better understand this benefic effect of high gellan gum on hybrid larch somatic embryos maturation, a proteomic study was performed via 2D gels. The proteome of soluble proteins were compared after one week of maturation on both culture media. The 62 proteins with significant differences in abundance were identified by LC-MS/MS analysis. Their functional classes correspond to the "carbohydrate metabolism", "genetic information processing" or "information processing environment." Interestinlgy, physiological parameters and identified proteins suggested that somatic embryos were stressed when they were cultured on 4 gL^{-1} ie on standard medium.

In conclusion, this is the first report of a 2-DE proteomic analysis of conifer somatic embryo maturation in presence of gelling agent at high concentration. These results provides information related to conifer somatic embryo maturation at the protein level giving rise to a better understanding and control of the maturation process.

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Keywords: Somatic embryogenesis; maturation; conifer; larch; water availability; dry weight; 2-DE proteomic analysis.

S4–4

The key role of phytohormones in somatic embryogenesis of Norway spruce

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Somatic embryogenesis is a dynamic process roughly driven by the changes of exogenous plant growth regulators. Subtle changes in quantity and quality of endogenous phytohormones are shown to govern metabolic and anatomic events and are linked to particular developmental stages from the proliferation of embryogenic suspensor mass (ESM) on primary explant till the germination of emblings.

The proliferation of ESM was promoted by exogenously supplied cytokinins and 2,4-D. Proliferating cultures were characterized by steady and relatively low levels of IAA, ABA, cytokinins and a low production of ethylene.

The development of somatic embryos (SE) was triggered by the change of exogenous regulators: auxin and cytokinins were replaced by ABA (for 5–6 w). The response of ESM was almost immediate: meristematic cells of embryos started their division and thus marked the launch of SE development. Accumulation of ABA in ESM and embryos was almost immediate. Within a few days, we recorded a transient increase of polyamines and a permanent increase of IAA. The IAA peak coincided with cotyledon formation after 2-3 w on ABA medium, followed by a marked decrease of IAA during further embryo development. Despite a continuous supply of ABA, the endogenous ABA level plateaued after 4–5 w and decreased thereafter. Elevated ethylene production was found at the ESM kept on an ABA-enriched medium.

No exogenous regulators were applied during a high relative humidity treatment of SE (3 w). Surprisingly, in this period before germination, the changes of endogenous phytohormones in SE were the most prominent. Embryos became steadily prepared to germinate, which was joined by a marked increase of several types of cytokinins (mainly *cis*-zeatine-9-riboside and *cis*-zeatine-9-riboside-*o*-glucoside) and IAA, whereas ABA was metabolized to low level.

Changes in the levels of phytohormones preceded anatomical development during the early stages of SE germination. During the first day of germination, IAA and ABA decreased manifold. Different trends were seen in cytokinins: a decrease in cis-zeatine-9-riboside was compensated by an increase in N^6-isopententyladenosine-9-riboside. *Cis*-zeatine-9-riboside-o-glucoside – the most abundant cytokinin in later stages of SE – remained high during germination.

Keywords: somatic embryogenesis, ABA, IAA, cytokinins, polyamines, ethylene, embryo development, desiccation, germination

S4–5

Building the "pipeline": Applying somatic embryogenesis, bioreactors and transgenic technology to restore the American chestnut

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For the past three years, our lab has been part of the Forest Health Initiative (FHI), a multi-institution research project that has as its mission to demonstrate the application of biotechnological tools to address forest health threats in the U.S. The first FHI target species is American chestnut (*Castanea dentata*) and we have been employing embryogenic chestnut cultures to address several FHI objectives focused on restoration of the species. Somatic embryogenesis (SE) is being used to propagate chestnut blight-resistant hybrid backcross-derived material from The American Chestnut Foundation's (TACF) breeding program for clonal testing. SE is also providing target material to test candidate genes (CGs) from Chinese chestnut and heterologous sources that may provide resistance to the blight fungus (*Cryphonectria parasitica*) and/or *Phytophthora*. New embryogenic cultures have been initiated from several American chestnut full-sib and half-sib families and, for the first time, from hybrid backcross material, including TACF B3F3 families. Airlift bioreactors, tested as an alternative to growing embryogenic suspension cultures in shaken flasks, have greatly accelerated the production of embryogenic material for both somatic embryo production and Agrobacterium-mediated genetic transformation, producing sufficient target material for transformation experiments every two weeks. Using new modular vectors constructed specifically for the FHI project, over 25 CGs are already in the transformation "pipeline". Transformation frequencies for some target lines have been very high, producing almost 700 putative transformation events per 50 mg of inoculated tissue. The first somatic seedlings carrying the FHI CGs from Chinese chestnut and from heterologous sources are currently in production.

Keywords: *Castanea dentata*, American chestnut, somatic embryogenesis, bioreactors, gene transfer, somatic seedlings

S4–6

Gene expression patterns during the acquisition of embryogenic competence and embryo development in *Fagaceae* species

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Somatic embryogenesis is a powerful method of tree regeneration that potentially offers an efficient system for mass clonal propagation, germplasm conservation and genetic improvement. Somatic embryos develop through a sequence of stages associated with morphological and biochemical changes related to genomic activity. Reprogramming of the current gene expression pattern of differentiated somatic cells is required for cellular dedifferentiation and the acquisition of embryogenic competence of certain responsive cells to allow them to establish new development programmes. This developmental switching involves activation and repression of specific genes that confer on somatic cells the ability to initiate the embryogenic pathway (Chug and Khurana, 2002). Moreover, the different developmental stages of somatic embryos are also controlled by the temporal expression of specific genes. However, the molecular mechanisms underlying the switch of the somatic cell fate towards embryogenic competence and the regulation of embryo development remain unclear.

In our laboratory, somatic embryos have been induced from different types of oak and chestnut material (Corredoira *et al*., 2003; Toribio *et al*. 2004; Valladares *et al*., 2006). The induction system and the different embryogenic lines are highly valuable for studying the molecular events that control the onset and development of somatic embryos.

The objective of this study was to analyse the spatio-temporal expression of four genes (*SCL1*, *ERF1*, *SERK-like* and *CPE*), associated with morphogenetic processes in oak and chestnut, which have been isolated by our research group. The *SCL1* gene encodes a GRAS family transcription factor (Sanchez *et al*., 2007), the *ERF1* gene belongs to the AP2/ERF family, the *SERK-like* gene encodes a protein containing characteristics domains of the SERK proteins, and the *CPE* gene encodes for a glycine rich protein (Gil *et al*., 2003).

Although we found differences in the gene expression pattern in different embryogenic lines, the *SCL1* and *SERK-like* transcripts accumulated at the highest levels in globular stage somatic embryos. Transcript levels of ERFl and CPE increased during embryo development, and the highest levels were detected in cotyledonary somatic embryos. In situ hybridization experiments also revealed the presence of high mRNA levels of *QrSCL1*, *QrSERK-like* and *QrERF1* in oak proembryogenic structures originated during the induction process.

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Keywords: somatic embryogenesis, gene expression, morphogenetic processes, oak, chestnut

S4–7

Involvement of mitochondria in the programmed cell death during somatic embryogenesis of *Abies alba* (Mill.)

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For a long time, mitochondria were known mainly as "cellular power" of eukaryotic organisms, although it became clear that their role in cellular physiology is not only restricted to ATP production for metabolic demands. Mitochondria were now shown to be crucial for the regulation of intracellular Ca^{2+} homeostasis, especially under pathological conditions; they produce reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), which are involved in the regulation of various physiological processes, but ROS become harmful to the cell if produced excessively [1]. In addition, mitochondria are considered central to regulate apoptosis in animal cells, and a similar regulatory role has been suggested by several plant studies related to programmed cell death (PCD) [2]. In particular, they appear to control, also in plants, the intrinsic pathway leading to PCD [3].

During the development of *Picea abies* (L.) Karst. somatic embryos, two ways of PCD were described [4] and several molecular and physiological aspects were studied in details [5]. For studies aiming at elucidating cell bioenergetics and involvement of mitochondria in PCD, it is a crucial prerequisite to have a valuable method for isolation and purification of mitochondria from embryogenic cultures. We have described a method to isolate and purify mitochondria from proliferating embryogenic cells of two coniferous species (*P. abies* and *Abies cephalonica* Loud.) [6]. Afterwards, the changes in some bioenergetic parameters and mitochondrial activities during the manifestation of two events of PCD, linked to *Abies alba* somatic embryogenesis (proliferation and maturation phases), were studied [7]. PCD, evidenced by *in situ* nuclear DNA fragmentation (TUNEL assay), DNA laddering and cytochrome *c* release, decreased in maturing embryogenic tissue with respect to the proliferation stage. In addition, the major cellular energetic metabolites (ATP, NAD(P)H and glucose-6-phosphate) were higher during maturation. The main mitochondrial activities changed also during these two developmental stages. Mitochondria, isolated from maturing, with respect to proliferating cell masses, showed an increased activity of the alternative oxidase, external NADH dehydrogenase and fatty-acid mediated uncoupling. Conversely, a significant decrease of the mitochondrial K^+_{ATP} channel activity was observed. The results suggest a different regulation of mitochondrial alternative energetic activities and inner membrane permeabilization during the manifestation of PCD that occurs in the development of somatic embryos.

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Keywords: *Abies alba*, somatic embryogenesis, mitochondria, PCD, mitochondrial K^+_{ATP} channel

S4–8

Climatic adaptation in Norway spruce – Molecular dissection of a novel epigenetic memory mechanism

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Forests are ecologically and economically important in our ecosystem. Upon world climate changes, it is worthy to understand climatic adaptation of trees. The temperature level experienced during zygotic embryogenesis and seed maturation in Norway spruce (*Picea abies*) affects an epigenetic memory and vital phenological traits in the progeny. Timing of bud burst as well as growth cessation and bud set occurs early if the embryo temperature is low whilst late if temperature is high. We intend to identify and characterise genes involved in this epigenetic memory upon temperature, as well as in traits where the epigenetic memory is expressed in plants and embryos. To recognize molecular mechanisms in epigenetic phenomenon, we isolate micro RNA from seedlings and embryos which shows distinct differences in epigenetic phenotypes. In seedlings, four selected genes *PaLPT4*, *PaGaMYB*, *PaMYB10* and *PaSPB13* regulated by miRNAs may be involved in epigenetic memory regulation. Although the functions of these genes are not elucidated yet, these findings imply these miRNAs may involve or at least affect the molecular mechanisms underlying the temperature sensitive epigenetic memory in Norway spruce. Using the Illumina based MACE (massive analysis of cDNA ends) approach transcriptome changes were monitored in *in vitro* propagated somatic embryos from two full-sib genotypes during early maturation stage in two different temperatures (18° vs. 30°C). MACE results were validated for a variety of candidate genes using qRT-PCR. We revealed striking differences in transcriptomes between genetically identical embryogenic tissues grown under different temperatures as well as between genotypes originating from crossings of the same parental trees under cold (outdoors) and warm (greenhouse) conditions. Also, a common pattern was observed for both genotypes in common temperature conditions. A large amount of candidate genes thought to be involved in epigenetic regulation was found. Formation of different epitypes under warm and cold conditions during embryogenesis is linked to expression of different sets of genes, which provide candidate genes fit for further studies of the epigenetic memory initiation.

Keywords: Climate adaptation, Epigenetic memory, Somatic embryogenesis

S4–9

Forest biotechnology and climate change

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Climate change represents an unprecedented threat to managed and natural forests the world over, as well as to how forestry operations are conducted, now and in the future. However, although much of the debate about climate change and other human induced environmental problems has focused on the threat that these challenges pose to or by forests and forestry operations worldwide, much less attention has been given to the potential for using natural and managed forests to mitigate these effects.

This presentation will summarise the available data about forests and climate change and will make the case that far from being merely part of 'the problem', how we manage and utilise the world's forests needs to be central to any plans to mitigate these problems, if they are to be successful.

In particular, natural and managed forests in combination have the potential to sequestrate much of the alarming rise in anthropomorphic carbon emissions, as well as to displace the use of fossil fuels, due to their ability to provide large quantities of timber, fibre and biofuels that the world needs on a long term sustainable basis, and in a manner that does not conflict with agricultural or conservation demands.

The adoption of modern forest biotechnological methods will be crucial to success in this area, because it is only by these means that the necessary gains in productivity can be secured in the time available, as well as greatly increasing the speed and flexibility of response to the effects of these pose environmental problems pose to forests themselves, and lastly through the provision of essential R&D support to the sector.

Keywords: forest biotechnology, climate change, natural and managed forests

Abstracts for poster presentations

2nd International Conference of the IUFRO WORKING PARTY 2.09.02

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Session

Somatic Embryogenesis

S1–1P

Origin and expression of embryogenic competence in *Prunus* sp.

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Within the *Prunus* species, *P. incisa* × *serrula* is one of the few embryogenic genotypes. The tests on the parents of that cherry rootstock hybrid and on its progenies issued from open pollination on one hand or obtained as a result of manual crosses with *P. dawyckensis*, a not embryogenic genotype, showed that the competence for somatic embryogenesis clearly came from the single genome of *P. incisa*.

This competence is expressed directly on isolated roots whatever they are collected from shoots and leaves or they are regenerated after protoplasts culture. Somatic embryos appear more frequently at the proximal area of the initial roots. However, additional ones sometimes occur at the middle as well as the apical areas of newly formed lateral roots. On the other hand, leaves require auxin treatment (IBA, Picloram) before somatic embryogenesis expression. The embryogenic structures are formed at injuries made in the proximal area of the lamina.

Keywords: *Prunus*, somatic embryogenesis, embryogenic competence

S1–2P

Somatic embryogenesis from clonal sources of *E. camaldulensis*

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Somatic embryogenesis in eucalyptus has been studied since the early 1980s. Embryogenesis has been reported in various eucalyptus species including *E. citriodora*, *E. nitens*, *E. dunnii*, *E. grandis*, *E. tereticornis*, *E. camaldulensis* and *E. globulus*. All these studies have been done using mature seeds as the source of explants, which reduces the homogeneity of the material and manifests vast variation. For commercial use, clonal genetic material would be the preferred source for mass propagation of elite lines. Reproduction of clones by somatic embryogenesis could also be significant for overcoming rooting problems of recalcitrant species, and may facilitate the cryopreservation of desired lines. Additionally, embryogenic callus can be used to introduce new traits into elite clones via transformation, for the development of yet further improved transgenic clones.

We report here embryogenesis from a clonal source of *E. camaldulensis*. By using leaf explants from an *E. camaldulensis* clone, we have established a protocol to obtain embryogenic callus. Different stages of embryos could be observed on the callus and we have succeeded in germinating the embryos. We are currently planning further work to optimize the procedure.

Keywords: Somatic Embryogenesis, *Eucalyptus camaldulensis*, clone propagation

S1–3P

Somatic embryogenesis as a method of conservation of endangered subendemit bog pine

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Bog pine (*Pinus uncinata* DC. subsp. *uliginosa* (Neumann) Businsky) is a subendemic species and appears to be one of the most endangered tree species of the Czech Republic. Recently its rare populations are greatly endangered namely by spontaneous interspecific hybridization with the ecologically more flexible Scots pine (*Pinus sylvestris* L.). Even if the rest of population is preserved in national nature reserves, the passive protection of this species failed. One of the negative factors is decrease of the groundwater level in some localities after removing of peat from surroundings. This change of environmental conditions support Norway spruce and white birch and suppress bog pine. The development of bog pine seedlings stopped. Regarding the fact that its protection is insufficient and the classical propagation by cuttings is problematic, modern methods were adopted for a long-term preservation of the taxon. Growth regulation conditions were investigated for the induction of organogenesis and somatic embryogenesis. Somatic embryogenesis was induced on immature megagametophytes from one selected tree of bog pine, growing in Dendrological garden of Silva Tarouca Institute for Landscape and Ornamental Gardening, Pruhonice, Czech Republic. The green cones were collected in two data (20th June and 30th June) from North and South side of tree. Together 1000 megagametophytes were excised in the first and 160 in the second date. The basal induction medium was DCR (Gupta, Durzan, 1985). Three variant of medium were tested: 5 µM 2.4-dichlorophenoxyacetic acid (2,4-D) with 2.5 µM benzyladenine (BA) with and without active charcoal (AC 100 mg.l^{-1}) and with half concentration of growth regulators. After 3 weeks the first somatic embryos were observed. Together 11 lines were established – 9 in full concentration of BA (4 with AC) and 2 in half concentration (total frequency of initiation 0.95%). During proliferation effect of concentration of gelling agent was studied. Even if all the lines come from one mother tree, they differ in their growth activity and also in their maturation capacity and could be used mainly for optimization of this method. Somatic embryogenesis could be the helpful tool for empowerment of week populations of rare bog pine however controlled pollination of selected mother trees is demanded for development of verified cultures.

Gupta, P. K. and Durzan, D. J., 1985: Plant Cell Reports, 4: 177–179.

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Keywords: somatic embryogenesis, *Pinus uncinata*, endangered species, preservation

S1–4P

Somatic Embryogenesis of Black saxaul (*Haloxylon aphyllum*), a Plant for Saline Soil Reclamation of the Dry Aral Seabed

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The Aral Sea problem is already in the center of researches' attention for 30 years. As a result of the irrational economic activity in the Aral Sea basin national level priority environmental problems have arisen.

One solution to the problems of the Aral Sea is phytoremediative works, which involve the use of salt-tolerant plants - halophytes to improve environmental remediation, retention of soil, and overall reduction of wind erosion.

Huge experimental plantings with plots up to 200 ha have shown, that only very few species are suitable for this purpose. One of them is black saxaul (*Haloxylon aphyllum*).

At present the majority of plantings of black saxaul are affected by various injurious organisms (pests) and diseases. High contamination by injurious organisms (pests) and diseases (various cecidomyids (gall-gnats)) interfere with establishment of forests and production of viable seeds. Selection and in vitro propagation of *Haloxylon aphyllum* will allow production of the improved genetically homogeneous planting material.

There is need for study on biology and propagation of this plant for reclamation of saline soils. Somatic embryogenesis has been a great resource for cloning trees.

Seeds of *Haloxylon aphyllum* were collected from the plantings of the Dry Aral Seabed. Sterilized top parts of seedlings inoculated into medium containing Murashige and Skoog (MS) medium, supplemented with 20 g/l sucrose, 6 g/l Difco Bacto-agar, 100 mg/l myoinositol, 25 mg/l glutamine. For callus induction into medium supplemented phytohormones with concentrations 0.5, 1.0, 1.5 mg/l of BAP (6-benzylaminopurine), K (kinetin), GA3 (gibberellic acid), 2,4-D (2.4-dichlorophenoxyacetic acid) alone. pH of the medium adjusted to 5.6-5.8 before autoclaving. Cultures were incubated at 26±2°C with a 16 hour illumination.

MS medium, supplemented with 1.0 mg/l BAP, was found to be the most suitable for induction of embryogenic callus from top parts of seedlings of *H. aphyllum* in comparison with K, GA3, 2,4-D. Induction of callus began in 3–4 weeks after inoculation of explants. Growth of callus was observed during 2–3 weeks.

Keywords: *Haloxylon aphyllum*, somatic embryogenesis, embryogenic callus, phytohormones

Somatic embryogenesis from Spanish provenances of maritime pine

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Maritime pine (*Pinus pinaster* Ait.) covers more than 4 million hectares in southwestern Europe (Spain, Portugal, Italy and France) where contrasting climatic environments prevail. Three maritime pine groups with differences in their genetic characteristics and in their morphological and adaptive traits have been described. Two of these groups can be found in the Iberian Peninsula: the Atlantic group, which coincides with the so-called ssp. *atlantica* H. del Villar; and the European Mediterranean group, that is part of the Mediterranean subspecies, ssp. *pinaster* (*P. mesogeensis* F. et Gaussen) (see Humanez *et al.* 2011, *In vitro*-P and references therein). In Spain these two groups are distributed in twenty provenance regions. This work examines the capacity of megagametophytes from provenances of both Spanish groups for the induction and establishment of somatic embryogenesis (SE). The effect of 24-epibrassinolide on SE response is also assayed.

Cones were collected in mid July from five open pollinated plus mother trees, from 4 provenances of the species: The Noroeste interior provenance collected in Anllo (Lugo, Spain); the Soria-Burgos provenance collected in Valsain (Segovia, Spain); The Serrania de Cuenca provenance collected in Sinarcas (Valencia, Spain) and the Levante provenance collected in Sierra Calderona (Valencia, Spain). Anllo and Valsain cones belong to the Atlantic group, whereas Sinarcas and Calderona cones belong to the Mediterranean group.

Cones were surface sterilized, seeds isolated, and megagametophytes containing the immature zygotic embryos were cultured on a modified Litvay's medium as described by Lelu *et al.* (2006, Plant Cell Rep). Alternatively, medium was supplemented with 0.1 µM 24-epibrassinolide as the only plant growth regulator. The percentage of somatic embryogenesis initiation was recorded after 45 days and the percentage of established embryogenic lines was recorded after 4 months.

Embryogenic response of megagametophytes was affected by their group rather than their provenance, by the culture medium and by the mother tree. Thus, megagametophytes from provenances of the Atlantic group (Anllo and Valsain) showed higher capacity for SE induction and establishment than those of the Mediterranean group (Sinarcas and Calderona; $p < 0.05$) and there were not significant differences between provenances within the same maritime pine group. Irrespective of the provenance, epibrassinolide was superior to 2,4-D/BA combination for SE induction and establishment. Irrespective of provenance, an important effect of the mother tree on SE response was also observed. Maturation experiments with several ABA treatments are in course.

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Keywords: epibrassinolide, maritime pine provenances, somatic embryogenesis

S1–6P

Induction of Friable Embryogenic Callus in Cassava Clones and Plant Regeneration Using Temporary Immersion

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Cassava (*Manihot esculenta* Crantz) is the third most important source of calories in the tropics, after rice and maize. Millions of people depend on cassava in Africa, Asia and Latin America. It is grown by poor farmers, many of them women, often on marginal land (FAO 2012). Cassava is vegetatively propagated where key demand is to propagate disease free planting material. Planting cassava cuttings from the infected cassava plants are the major reason of spreading virus disease like CBSD (cassava brown streak disease) a devastating disease that causes loss of cassava root (tuber) production and quality in several African courtiers. Cassava diseases in Africa are the major thread of the food security of millions peoples in this continent (FAO 2010).

We studied the induction of Friable Embryogenic Callus–FEC in three commercial cassava clones. FEC induction and proliferation was genotype-dependant. Somatic embryos, derived from FEC, matured on semisolid media with Nafthaleneacetic acid or 6-Benzilaminopurine, depending upon the clone. When compared with semisolid media, plant recovery was significantly enhanced using the Temporary Immersion System known as RITA®. Statistical analysis allowed the conclusion that the greater proportion of embryo germination to produce plants occurred in RITA® with liquid medium, regardless of clone and plant growth regulator. However, individual clones responded differently to the type of plant growth regulators, i.e., Mcol2215 and CM3306-4 produced more plants with 6-Benzylaminopurine, while TMS60444 produced more with Naphthalene acetic acid. We recommend using RITA® to improve embryo-to-plant conversion in cassava.

Keywords: cassava, CBSD, embryogenic callus, somatic embryos, RITA®

S1–7P

Somatic embryogenesis induction on male catkins of Holm oak (*Quercus ilex* L.)

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Mediterranean forests are currently dominated by evergreen oak species, the holm oak (*Quercus ilex* L.) being the commonest in the Iberian Peninsula. Traditionally the species has been used as a source of corns for animal feeding and lately as one of the top trees for the establishment of truffle orchards (mainly black truffle, Tuber melanosporum). Then, holm oak trees have become of great economical importance and would contribute to rural development in depressed areas. Traditional breeding of the species is hampered due to the low survival of the seeds, poor growth in plantations, especially under stressful conditions, and also because their recalcitrance to vegetative propagation. During the last years our group is being working on the development of a suitable somatic embryogenesis (SE) protocol to propagate adult *Q. ilex*. Here we present our first results on the effect of male catkins developmental stage on the somatic embryogenesis induction.

Male catkins of *Q. ilex* were sampled at different developmental stages in five locations of the Valencia area (Spain) named Ayora, Hunde, Portera, Remedio and Villar del Arzobispo. Three different developmental stages were defined. Stage A) Floral buds up to 2–4 mm with varying number of meristematic structures developing at the same points. Stage B) Catkin starting to develop with a short bare pedicel with distinguible flowers (catkin size no larger than 1 cm); and stage C) Elongated catkin (up to 2 cm in length) where erect catkins and closed flowers were distinguishable along the axis.

Catkins and isolated flowers (from stages B and C) were cultured in the dark on a modified MS medium supplemented with 10µM BA and 50µM NAA for 20 days, and then transferred to the same medium with growth regulators at half concentrations and cultured under dim light. After 60 days, explants were subcultured on MS medium with ammonium nitrate reduced to a half of its standard concentration, 0.5 µM BA and 0.5 µM NAA.

Callus development was observed in all genotypes and catkin developmental stages. However somatic embryos (SE) were obtained only from Hunde genotype sampled at stage C (6.7% and 1.5% of explants producing SE for catkins and isolated flowers, respectively). SEs are being cultured to induce secondary embryogenesis that allows establishing embryogenic lines. Ploidy level of somatic embryos will be determined.

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Keywords: adult trees, catkin, oak, *Quercus*, somatic embryogenesis

S1–8P

Induction of Somatic Embryogenesis in Culture *in vitro* and Content of Phytohormones in Callus and Seeds of *Pinus sibirica*

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Pinus sibirica is one of the main forest-forming species in Siberia. It is characterized by long-generative cycle (2,5 years), long-stratification seeds and the occurrence of trees-"akselerate", in which the period from pollination to seed maturation is 2 months (instead 1 year). The main objective of this study was to improve protocol of *Pinus sibirica* somatic embryos obtaining. Experiments were performed on clones of *Pinus sibirica*, growing in clonal graft plantations in the West Sayan. Trees of clones were subjected to control pollination of trees-"akselerate" pollen. As explants for induction of somatic embryogenesis were used isolated zygotic embryos obtained from control pollination. We used embryos at the globular stage of development, the cotyledons formation and mature seeds. The embryos were placed on basal medium ½ LV with 2,4D (9 μM) and 6-BA (4,5 μM).

It was show that morphological response of explants was already evident by 4–8 days and expressed in formation callus in the area of embryonic root. After 30 days of cultivation, callus formed on hypocotyl. Only embryos of some clones which were pollinated with pollen from trees-"akselerates" pass over to the stage of proliferation. After 50–60 days of cultivation in embryonal mass, torpedo embryos were observed.

Hormone levels (auxins, cytokinins, ABA) were measured in zygotic embryos and megagametophytes of *Pinus sibiric*a. It was shown, that IAA and ABA in megagametophytes of *Pinus sibirica* were less than in zygotic embryos (44,840 and 83,643, and 269,290 and 346,502 ng/g dry weight). The content of cytokinins in megagametophyte and zygotic embryos was 92,724 and 103,551 ng/g dry weights, respectively.

During 2–3 months, cultivation of callus, content of cytokinins increased in 2–3 times compared to zygotic embryos, while content of auxin in the callus was the same in zygotic embryo (88,348 ng/g dry weight). The content of ABA in the callus increased in two time in comparison with zygotic embryos.

So, somatic embryogenesis of *Pinus sibirica* was induced in culture in vitro for the first time. Embryogenic callus was characterized by high levels of cytokinins and ABA.

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Keywords: somatic embryogenesis, hormones, *Pinus sibirica*

S1–9P

Induction of somatic embryogenesis in developing ovules of *Quercus ilex* L.

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The implementation of multivarietal forestry as part of breeding strategies is expected to provide more productive forest plantations. In order to achieve this, a reliable and effective method of mass production of clonal plants is needed. Somatic embryogenesis is considered as a suitable way of vegetative propagation for this purpose. Moreover techniques able to clone adult trees are desired because they will improve these strategies by maximizing the performance of clonal tests and excluding the need of cryopreservation while tests are in progress. The holm oak (*Quercus ilex* L.) is a Mediterranean evergreen tree with economic interests because of the acorn production and edible fungi mycorrhization. The aims of this work were to obtain embryogenic lines in teguments of ovules from mature trees of this species, assessing the influence of the explant developmental degree along with genotype and media composition, and evaluate the effect of arabinogalactan proteins (AGP), on the induction rate of somatic embryogenesis. Developing flowers of four trees (genotypes E00, E0, E2, E4) were collected in El Encín (Madrid) at three developmental points: containing six ovules of the same size (putatively unfertilized); one of the ovules predominates in size (putatively fertilized); the predominant ovule reaching around 3 mm width and 5 mm length. After surface sterilization, ovules were removed from ovaries (zygote embryos were excised from the larger ovules and discarded) and were cultured on G-macronutrients medium lacking plant growth regulators (PGRs). Teguments from ovules of genotypes E00, E0, and E4 at the third stage of development were also cultured on SH-macronutrients medium without PGRs. They were maintained for 5 months at 25 ± 2 ºC in darkness with monthly subcultures. To test the influence of arabinogalactans and their interaction with plant growth regulators a mix of immature acorns from seven trees were collected in Sotolargo (Guadalajara). Ovules ranging 3-5 mm width and 5-10 mm length (without zygotic embryos) were cultured on SH-macronutrients media containing either 0, 2, 6 mgl^{-1} of *Larix*-AGP or 0, 10, 30 mgl^{-1} of *Acacia*-AGP, both groups of treatments in factorial combination with presence (10 µM of NAA plus 10 µM of BAP) or absence of PGRs. After 30 days at 25 ± 2 ºC in darkness, they were subcultured to the same media, but reducing the PGR concentrations to 0.5 µM of both NAA and BAP. They were placed in a 16-hour photoperiod growth chamber and after 30 days transferred to media lacking PGRs. No embryogenic response was observed in explants collected at the first and second developmental points. However somatic embryos developed from explants of the third developmental point. Two out of the four genotypes were captured when G medium was used, while all three tested genotypes formed somatic embryos when SH medium was used. Induction frequencies ranged between 1.2 and 3.2 %. Somatic embryos were obtained in the *Larix*-AGP treatments only when no PGRs were supplied, although induction frequencies were not improved. No response was observed when the effect of AGP from the *Acacia* genus was tested.

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Keywords: Adult trees, Arabinogalactan protein, Holm oak, *Quercus ilex*, somatic embryogenesis

S1–10P

Effect of size of inoculum, density of inoculation and shaking on growth and differentiation of *Quercus suber* embryogenic suspensions

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The cork oak (*Quercus suber* L.) is one of the most important forest species of the Mediterranean ecosystem. This evergreen tree forms along with other *Quercus* species the Iberian "dehesas" and "montados", and its main products are cork, which is used for several industrial applications, and acorns, to feed the Iberian race of pigs. Somatic embryogenesis is considered the most suitable way of vegetative propagation for implementing multivarietal forestry. To perform it mass production of cloned plants at profitable cost is compulsory, and therefore protocols for culturing embryogenic tissues in liquid medium are required. The induction of SE in mature cork oak trees has been achieved, and too the establishment of embryogenic suspension cultures. In this study the effect of density of inoculation and shaking of two size fractionated inoculums on proliferation and somatic embryo differentiation was evaluated. These fractions represented two gross developmental stages: the smallest one was comprised of isolated cells and unstructured aggregates (PEMs) and the largest one contained mainly structured embryogenic clumps. Suspension cultures were initiated from embryogenic cultures of ALM80 and TRG3 lines, which were maintained on semi-solid SH medium without plant growth regulators (PGRs) for more than 4 years retaining embryogenic ability, in the same liquid medium. Two fractions were collected, 41–180 µm (S) and 180–800 µm (L) from this suspension. The S-fraction was inoculated into 250 ml baffled Erlenmeyer flasks with 50 ml of fresh medium at 0.5 and 1 gl^{-1}, while the L-fraction was at 0.5, 1, 2, 4 and 6 gl^{-1}. All these suspension cultures were maintained static or agitated on an orbital shaker at 110 rpm, in a growth chamber at 25 ± 2 ºC under a 16 h light photoperiod (180 µE m^{-2} s^{-1}) for 30 days. Growth was determined by fresh weight quantification of fractions of 41–180, 180–800 and larger than 800 µm, and the total biomass. Cultures were qualitatively assessed by photographic patterns and a scale to score different features: necrosis, proportion of structured vs unstructured aggregates, morphology of structures, and presence of cotyledonary embryos. Regardless of genotype, the size of inoculum had a dramatic effect on growth. When the S-fraction was inoculated at 0.5 gl^{-1} multiplied the initial weight by 20 when it was shaken and half when remained static. However the L-fraction inoculated at the same density showed a 70-fold increase when cultured agitated and a 24-fold increase when maintained static. For the L-fraction a clear inverse relationship was observed between density of inoculation and multiplication rate, both in shaking culture as static. The structures contained in the S-fraction showed a limited development. Few structures larger than 800 µm were observed, slightly more when the lower density of inoculation was used. The L-fraction mainly produced structured and compact embryogenic clumps, and some polar forms were detected. Genotype, density of inoculation and shaking showed some minor differences in qualitative characteristics. Genotype ALM80 exhibited higher frequency of structures with confined necrosis, and lower ability to disaggregate than genotype TRG3. Cotyledonary embryos were not observed in any treatment.

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Keywords: Cork oak, embryogenic cultures, inoculum density, *Quercus suber*, suspension culture

S1–11P

Effect of shaking on growth, morphology and maturation ability of embryogenic suspension cultures of *Pinus pinea* L.

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Plant regeneration by somatic embryogenesis in Stone pine (*Pinus pinea* L.) has the potential to enable mass propagation of the best performing genotypes for pine nut production or suitable rootstocks in agroforest plantations. The large scale production of somatic embryos requires the establishment of an efficient system for growth and plant formation in liquid culture medium. Liquid culture conditions may support growth of cell clusters and early stage embryos at several stages of development. Patterns of the number and sizes of clusters in the suspension cultures could be associated with the prospective development of somatic embryos. Several physical factors of the culture that affect oxygen transfer and hydrodynamic stress may influence these developmental patterns. This study reports on the orbiting speed effects on somatic embryo development, which were evaluated by growth parameters in liquid culture system as well as qualitative differences among cultures. Relation between the morphology of suspensions and their maturation ability was also examined. Suspension cultures were initiated from proliferating embryogenic tissue of Stone pine lines 2F47, 1F11 and 7F11, by transferring 500 mg of embryonal-suspensor masses (ESM) to 200 ml Erlenmeyer flasks containing 25 ml of M-mLV liquid medium. They were cultured on an orbital shaker at 50, 100 and 150 rpm in the dark at 23 ± 1ºC, for three weeks. The growth of embryogenic lines was determined by measuring the settled cell volume (SCV), and the fresh (FW) and dry weight (DW) after filtration. Qualitative characteristics of embryogenic tissue were determined by microscope examination of samples of 1 ml of suspension placed on Petri dishes. Growth parameters were significantly affected by the orbiting speed. The highest SCV rate was obtained at the lower speed. However higher orbiting speeds decreased biomass production (FW and DW). Significant effects of line and their interaction with orbiting speed for growth parameters were found. Line 2F47 showed the maximum increase of SCV; however line 1F11 produced higher FW. No significant differences among embryogenic lines were recorded for DW. Cultures at 50 rpm most commonly grew as accumulation of clusters of embryonal cell masses, whereas at 150 rpm showed a higher frequency of freely suspended cell structures. Moreover, the larger clumps were disaggregated into smaller ones at 100 rpm. Somatic embryo proliferation of line 1F11 was characterized by more organized embryonal-suspensor structures. This organization was reduced at the highest orbiting speed, showing less organized bipolar structures from suspension culture with higher visible elongated single cells and proembryonal heads. Cotyledonary mature somatic embryos were recovered from the three embryogenic lines on ABA-containing semi-solid medium. Production of somatic embryos depended on the orbiting speed. When 100 rpm was applied, 54 mature embryos were obtained per gram of FW from all embryogenic lines, while 34 mature embryos were obtained under 50 rpm. Lines 2F47, 7F11 and 1F11 produced 78, 41 and 6 mature embryos per gram of FW, respectively.

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Keywords: Agroforestry, embryogenic cultures, *Pinus pinea*, Stone pine, suspension cultures

S1–12P

Plant regeneration in *Pinus pumila* (Pall.) Regel by somatic embryogenesis

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Reproductive biology of *Pinus pumila* which grows at the north-eastern region of Siberia is not enough studied. Researches of micropropagation in vitro for this species are absent.

To induce somatic embryogenesis of *Pinus pumila* it was used the basal medium ½ LV1 with added plant growth regulators (PGR) 2,4-D and 6-BAP. The medium consisted of half-strength macroelements and full-strength microelements also it included Fe-EDTA and vitamins. Somatic embryogenesis was initiated from immature zygotic embryos of Pinus pumila on precotyledon stage of development.

The embryonic mass (EM) from zygotic embryos were obtained after one month of cultivation. This EM was transferred on the medium for proliferation ½ LV2 with the reduced concentrations of PGR. The somatic embryos were found in EM after two months of cultivation. As a result, seven actively proliferating embryogenic lines were obtained. For each embryogenic line approximately 300 mg of embryogenic tissue were suspended in liquid medium of ½LV3 without PGR. The embryogenic tissue was cultivated during seven days in a shaker (60 revolutions per minute). This procedure was necessary for transition of the somatic embryos to maturation. For maturation somatic embryos, the embryonic tissues were transferred on medium ½ LV4 with added various concentrations of ABA, PEG and sucrose. One part of EM (experiment) at the stage of maturation somatic embryos was subjected to low positive temperature (+40°C) influence. Another part of EM (control) has been cultured in conditions of thermostat in the darkness at 24 ± 10°C. Both groups (control and experiment) were transferred at the light after two weeks of treatment. The somatic embryos began to visible on surface of EM after one month of cultivation on light. The somatic embryos were transferred on the media for germination AFC and ¼ LV (one quarter of full-strength macroelements), when they reached length 5–8 mm. The somatic embryos were grown in the conditions of climate camera (AWTech, GC-300). After two months, germinated somatic embryos were transplanted to a soil mix (3 : 1 vermiculate : peat).

Thus a possibility of *Pinus pumila* micropropagation by somatic embryogenesis has been shown. It was determined that concentrations PGR and culturing conditions necessary for realization this method of *Pinus pumila* micropropagation. It was noted that more quantity of mature somatic embryos were formed after treatment of a low positive temperature. So, the plantlets of *Pinus pumila* were obtained for the first time.

Keywords: somatic embryogenesis, *Pinus pumila*, plantlets, low positive temperature, embryonic line, maturation

S1–13P

Advances in somatic embryogenesis of *Acacia caven* Mol. (roman cassie)

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In vitro somatic embryogenesis and subsequent plant conversion was achieved in cultures derived from cotyledons of *Acacia caven* (roman cassie, espinillo). This leguminous tree is part of the xeromorphic forest in Chile and Argentina. Due to its high plasticity can be used for reforestation of degraded ecosystems, and present a high resistance to environmental pollution, saline soils and climatic tolerance and a remarkable ecological adaptability. Previously we developed a protocol for somatic embryogenesis of acacia, which must be optimized to achieve a higher rate of plant regeneration and allow the production of synthetic seeds. Therefore, the aim of this study was to optimize a strategy for somatic embryogenesis. Cotyledon explants obtained from mature seeds were used as explants. These seeds were treated with 98% sulfuric acid for two hours and washed with water (10 min). Then disinfected with 70% ethanol (5 minutes) and 20% sodium hypochlorite (30 minutes), they were 3 rinses with distilled water under laminar flow hood and then were left for 7 days in sterile water to allow softening the seed coat and obtain the cotyledons. The explants were grown in Murashige-Skoog (MS) half strength concentration, supplemented with sucrose (3%), agar, and 2, 4-D (1 mg/l) and BAP (0.1 mg/l).The cotyledons were placed with their abaxial surface in contact with the culture medium and incubated at 25 ± 2 ºC in darkness. Somatic embryogenesis occurs directly on the cotyledons, in your adaxial face or indirectly from callus formation after 6 months of culture. Light-microscopy showed that the somatic embryos originated from single cells of the cotyledon epidermal layers or from embryogenic callus. The globular stage somatic embryos were sub cultured PGR-free MS medium. Germination and conversion into whole plants was low (30%). The embryo-derived plantlets were acclimatized in the greenhouse and subsequently showed normal growth. For synthetic seed the protocol must be optimized even more to support propagation and conservation programs.

Keywords: *Acacia caven*, somatic embryogenesis, regeneration, synthetic seeds

S1–14P

Effects of cellulose acetate semi-permeable membranes on prematuration of avocado somatic embryos

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Application of transformation and other biotechnological tools in avocado (*Persea americana* Mill.) is hampered by difficulties in obtaining mature somatic embryos with an acceptable germination capacity. In Citrus (Niedz *et al.*, 2002) and olive (Cerezo *et al.*, 2011), a normalized development was observed when somatic embryos were matured on cellulose acetate semi-permeable membranes; moreover, these embryos showed an increased germination rate.

In avocado, one hundred mg of filtered embryogenic calli were cultured for 5 weeks on top of 4 × 4 cm dialysis tubing cellulose acetate membranes (MW cut-off 12,000, Sigma D9777) in jars containing B5m10A embryo maturation medium (Márquez-Martín *et al.*, 2011). Afterwards, the obtained white-opaque somatic embryos (WOSEs) were cultured in the same medium without membrane for 5 weeks, followed by another culture period in a medium with 20% (v/v) coconut water and 45 g/l sucrose. Once maturation was completed, somatic embryos larger than 4 mm, were germinated using a modified procedure from that of Witjaksono and Litz, (1999); e.g. three successive recultures, each consisting of a 3 day-preculture in liquid MS medium with 4,44 µM BA and 2,89 µM GA3 followed by 4 weeks culture on solid medium of the same composition. Using this protocol, a higher number of better quality WOSEs were obtained. These embryos also showed an increased germination rate (39.13%) in comparison to the embryos obtained without membranes (7.65%).

To determine the effects of cellulose membranes on somatic embryo physiology, water potential using a Wescor Dew Point Microvoltmeter HR-33T in dew-point mode and ABA content with ELISA Phytodetek® ABA Test Kit, were estimated. In addition, water availability from the medium was estimated as the amount of water absorbed by filter paper laid on the medium surface for 24 h.

Significantly lower water potential as well as a lower ABA content were observed in embryos matured on top of cellulose membranes. In addition, water availability from the medium was lowered to half through the use of membranes. This controlled embryo desiccation and the decreased ABA content could be the reasons for the improved WOSE quality and the higher germination percentages.

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Keywords: somatic embryo water potential, *Persea americana*, cellulose acetate membranes, water availability from culture medium, somatic embryo germination

S1–15P

Somatic embryogenesis from *in vitro* leaf callus cultures of Caspian honey locust (*Gleditsia caspica*)

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Caspian honey locust (*Gleditsia caspica*) is one of the valuable species that ecologically and socially plays a significant role in northern forests of Iran. This investigation is aimed to study of somatic embryogenesis process using different plant growth regulators (PGRs). The leaves of in vitro seedlings used as explant for callus induction on half-strength Murashige and Skoog (MS) medium containing different PGRs. The results showed that the used plant growth regulators in different concentrations had significant effects on callus induction from leaf explants, somatic embryogenesis, the somatic embryos development and maturity, and also shoot regeneration. Half-strength MS medium containing 4.5 µM 2,4-dichlorophenoxyacetc acid (2,4-D) and 2.2 µM benzyl adenine (BA) was the most effective PGR treatment for callus induction. The morphologically embryogenic calli and formation of globular embryos were achieved on medium containing 0.54 µM naphthalene acetic acid (NAA) along with 2.3 µM Kinetin (KIN), and the medium containing 2.7 µM NAA in combination with 2.3 µM KIN. The results showed that half-strength MS medium containing 60 mg l^{-1} sorbitol, 2.3 µM 2,4-D and 1.3 µM BA was the best medium for development and maturation of somatic embryos. The highest shoot regeneration of somatic embryos was obtained on half-strength MS medium supplemented with 9.2 µM KIN.

Keywords: *Gleditsia*, *In vitro* culture, Callus induction. Murashige and Skoog (MS) medium.

S1–16P

Horizontal Disposable Bioreactor for the Pre-Germination of Coffee Somatic Embryos

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The pre-germination step corresponds to the conversion from the torpedo to the cotyledonary stage. It is achieved in large temporary immersion bioreactors (TIB). Up to 25,000 cotyledonary coffee embryos (5 to 10 mm) can be collected per 10 L glass bottles, then directly transferred to the greenhouse. However, light distribution becomes rapidly a major constraint during embryo pre-germination as it can only penetrate the first few centimetres in the biomass. We hypothesized that a horizontal design will be more convenient than a vertical one, which is the usual shape of conventional vessels. As a result of a higher area/volume ratio, the embryos are more dispersed; consequently, the light transmittance to the embryos is increased. A very simple and cheap way to develop horizontal TIB is to include a rigid box inside a plastic bag. A higher growth performance is obtained which could be attributed to a better light distribution. This new TIB ("Box-in-Bags") is disposable allowing to reduce the production cost. Its advantages are listed.

Keywords: box-in-bags, cloning, ebb and flood, *in vitro* multiplication, liquid medium, TIB, TIS, scale up

S1–17P

Somatic embryogenesis as an effective regeneration support for reverse genetics in maritime pine: the Sustainpine collaborative project as an illustration

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Reverse genetics, defined as ectopic candidate gene expression or silencing, has become an indispensable tool for functional dissection of traits of interest in forest trees. In maritime pine as in other conifers, long generation cycle, large genetic loads as well as high genetic redundancy are major obstacles to performing standard genetic approaches including association genetics. Validating marker associations with specific properties before transfer into breeding selection models is still challenging. Agrobacterium-mediated genetic transformation of maritime pine was first reported by Trontin *et al*. (2002, Ann. For. Sci. 59: 687) and developed in both France (FCBA, INRA) and Portugal (IBET) with sufficient refinement (reviewed in Trontin *et al*. 2007, Trans. Plant J. 1: 314) to envisage practical application in reverse genetics as an attractive alternative to association studies. The technology has been implemented in French (GenoQB, 2006-2009) and multilateral European initiatives (Sustainpine, 2010-2013; Procogene 2012-2016). Somatic embryogenesis was revealed as a key tissue culture system for achieving genetic transformation, easy cryopreservation of transgenic tissue and efficient transgenic plant regeneration in maritime pine. Much consideration is given in France, Portugal and Spain to applying somatic embryogenesis as a clonal propagation system for maritime pine improvement and deployment strategies in multivarietal forestry (reviewed in Klimaszewska *et al*. 2007, Tree & For. Sci. Biotech.1: 11). Both somatic embryogenesis and Agrobacterium-mediated transformation methods developed at FCBA, INRA or IBET were successfully transferred to partners in the frame of the running Sustainpine project (*http://www.scbi.uma.es/sustainpine/*). Using reference, control embryogenic line (PN519) and binary vector for constitutive overexpression (pCbar, phosphinothricin or hygromycin selection), both provided by FCBA, we consistently obtained at different labs 80–120 events g^{-1} with most lines confirmed to be transgenic by a combination of GUS and PCR tests (typically > 80%). The whole steps of somatic embryogenesis could be achieved with minor technical problems for the non-transformed, control line, i.e. 100% recovery from crypreserved stocks, 70–120 somatic embryos (SE) g^{-1} maturing tissue and 30–60% SE conversion into plantlets. Maturation rate of transgenic lines is decreased compared to non-transgenic control (usually < 50 SE g^{-1}), ageing during the long selection process being the main factor suspected. Efficient handling of embryogenic line post-reactivation using a combination of adapted culture practices is a key point for successful regeneration of transgenic plants. Transformation yield with new overexpression and RNAi-induced silencing vectors can be severely decreased

(< 20 events g^{-1}) compared to pCbar as a result of variation in selection efficiency. The number of transgenic lines produced in standard transformation experiment (1 g target tissue) is however sufficient (5–20 events) to fulfil the requirement for biological repeats, i.e. 3–10 independent transgenic lines per construct. A number of improvements of the transformation toolbox will be developed during Sustainpine. Reverse genetic studies will be initiated for more than 60 constructs from 38 genes involved in wood formation, C/N metabolism, stress resistance and plant or embryo development. This is one of the greatest efforts for gene functional analysis of conifers worldwide.

Keywords: maritime pine, somatic embryogenesis, transgenesis, reverse genetics, functional gene validation

S1–18P

Somatic embryo maturation in maritime pine (*Pinus pinaster*): contribution of a 2-DE proteomic analysis for a better understanding

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Large-scale propagation of selected conifers, those are ecologically and economically important, could be achieved via vegetative propagation method especially somatic embryogenesis due to its high efficiency in plant regeneration. Somatic embryogenesis has reached an application stage for *Picea* and *Larix* species; however it remained not yet optimized in pine species. For maritime pine maturation needs improvements.

Maturation protocols lead to a development of mature somatic embryos defined by their ability to germinate and to convert to plant. These embryo that morphologically resemble to zygotic embryos are harvested after arbitrarily chosen periods of time (usually 12 weeks), and are germinated. Determination of the harvest time is decided on the basis of somatic embryo morphology. However, such an empirical approach does not give any information of the quality of somatic embryos with respect to storage reserves accumulation.

Therefore, to optimize maturation, it is necessary to develop markers that can be used to verify or monitor the quality of somatic embryos. One approach is to follow the accumulation of storage proteins in the somatic embryos during maturation and to compare them to those of mature zygotic embryos. This was done by SDS-PAGE from total proteins extract of somatic embryos, and HPLC respectively. Another developed approach was the comparison of two maturation stages by proteomic study. The significantly expressed proteins were about 140 (Student's test, $p<0.01$). They were mainly involved in carbohydrate or lipid metabolism and genetic information processing. In addition many storage proteins are identified and represented interesting markers of developmental stage of embryos (vicillin-like, legumin-like, LEA proteins). Ultimate goal is to have a better understanding of the maturation of *Pinus pinaster*. The description of the somatic embryogenesis would help to optimize the process of maturation and *in vitro* production of plants.

Keywords: somatic embryogenesis, maritime pine, maturation, 2DE-gels, storage protein, molecular markers, dry mass, water content

S1–19P

A Review of somatic embryogenesis work done on prioritized medicinal plants species identified by National Medicinal Plant Board, New Delhi, India

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There are about 32 medicinal plant species which have been identified as priority species for cultivation and conservation by National Medicinal Plant Board (NMPB) set up under the Union Ministry for Health & Family Welfare, Government of India. In vitro techniques have great potential for their propagation and conservation of prioritized species of medicinal importance. The work carried out on somatic embryogenesis of some of the prioritized plant species as identified by NMPB viz. *Aegle marmelose*, *Chlorophytum brovillianum*, *Commiphora weightii*, *Santalum album*, *Swertia chirata* has been reviewed and presented in this paper. The work on somatic embryogenesis on *Aegle marmelose* has been reported using embryo as explant, *Chlorophytum brovillianum* via callus, *Commiphora weightii* using immature zygotic embryos as explant, *Santalum album* using shoots and endosperm via callus, *Swertia chirata* using immature seeds as explants.

This review article will help researchers and agencies to get consolidated information at one place of the species which have been subjected to tissue culture studies and those which need attention to be focused on them for such studies.

Keywords: Somatic embryogenesis, explant, Callus, *In vitro* techniques, endosperm

S1–19P

Long term analysis of the proliferation abilities of *Abies alba* Mill. embryogenic cell lines

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European silver fir (*Abies alba* Mill.) is the most productive native tree species of European forests and it has traditionally been involved in conventional tree improvements programmes. However, in recent years is suffering dieback in a large part of its distribution. A lot of effort has been put into the development of vegetative propagation methods for firs, in order to rapidly gain the benefits of traditional breeding to be utilized in reforestation. Somatic embryogenesis has been proved to be a suitable method for vegetative propagation of silver fir.

During the summer 2010 and 2011, altogether 1,573 and 419 immature zygotic embryos were isolated and 191 and 42 *Abies alba* embryogenic cell lines were yielded, respectively. Mucilaginous embryogenic cell masses (ECMs) induced on induction medium were visible at 3 – 8 weeks. ECMs with a clear, glassy appearance were subsequently transferred on proliferation medium and sub-cultured regularly. The intensive proliferation of more than 107 cell lines was stabilized over a period of 16 months in case of *A. alba* embryogenic cell lines induced during summer 2010 and 20 cell lines induced during summer 2011 are intensively proliferating.

As proliferation stage is very important for further consecutive steps in development of somatic embryos, the present study has focused on long term analysis of proliferation and morphological characteristics of different embryogenic cell lines. Loss of embryogenic potential and changes in their morphological features, genetic stability is often the result of prolonged sub-culturing. During proliferation stage variability in morphological appearance is not noticeable, but in case of a large number of different embryogenic cell lines differences are evident. Analyzing the proliferation dynamics in embryogenic cell lines it was possible to distinguish five different types of proliferation categories which were characterized according to their proliferation rate and microscopic observations based on staining with FDA and PI. The experiments which monitored the genetic fidelity during the proliferation period are in progress.

The research is part of SIGA 591 project and was financed by the European Community within the Seventh Framework Programme (FP/2007–2013) under Grant Agreement No. 229603. The research is also co-financed by the South Moravian Region.

Keywords: somatic embryogenesis, *Abies alba* Mill., proliferation

Session

Other vegetative propagation techniques including rooted cuttings, micropropagation by organogenesis, etc.

IUFRO WORKING PARTY 2.09.02
SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES

S2–1P

Results of hybrid aspen (*Populus tremula* × *P. tremuloides*) root cutting propagation experiments in Latvia

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Hybrid aspen breeding is carried out for last two decades in Latvia with the aim to select clones for afforestation of abandoned agricultural lands. Commercial plant production of selected best clones are carried out in facilities of JST "Latvia's State Forests" using microclonal propagation. This propagation method is reliable, but leads to high plant costs and therefore low demand of plants, as there are no subsidies for plantation establishment in Latvia.

Number of experiments has been carried out to assess possibilities to use root cuttings as alternative propagation method in order to reduce plant costs. Results from altogether 5 tests, containing on average 16 clones, represented by 200 – 3161 cuttings are analyzed.

Strong and significant correlation was found among size of on year old mother tree (weight, diameter at root collar) and number of cuttings ($r = 0.64$), that in turn affected the rooting percentage ($r = 0.67$). There was no direct correlation between weight of mother plant and rooting success. Successful rooting was observed for 23% of cuttings on average, significantly differing among experiments (from $49 \pm 8.7\%$ to as little as $2 \pm 2.6\%$). Lowest result was achieved placing cutting vertically, in setting without ground heating. Correlation of rooting percentage in clone mean level among years (repeated experiments) was generally low, indicating rather un-predictable results of the method; however in some cases correlation as high as 0.91 was achieved.

Using one or two years old mother plants yielded the same number of cuttings per root (67 on average), but the total number of plants and rooting percentage was significantly higher for older mother trees (74 vs. 386 and 3 ± 2.9 % vs. 12 ± 4.3 % respectively).

Shoot formation started on average after 14 days and no significant differences among experiments or clones were found in this trait, indicating rather similar production cycle in nursery, if number of clones is propagated. On average only 48% of cuttings forming shoots achieved also successful rooting. Successful rooting did not always lead to production of desired size of plant (40 cm or higher) – on average only 17% of cuttings reached the target. Results differ notably between experiments: from 10 ± 5.2 to $24 \pm 6.7\%$.

Relative low rooting percentage and share of plants of desired size as well as high variation among experiments and years for the same clones indicates that notable improvements of the method are needed before it can be used in mass propagation of hybrid aspen.

Keywords: vegetative propagation; root cuttings; rooting conditions

S2–2P

Vegetative Propagation of Minicuttings of *Citharexylum montevidense* (Spreng) Moldenke, A Native Species of Argentine

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Citharexylum montevidense (Spreng) Moldenke is a native tree species of Argentine which belongs to the *Verbena* Family. This thorny tree is about 8–12 m tall and grows in the south of Brazil, Paraguay, Uruguay and Argentina. *Citharexylum montevidense* is part of the biological diversity of the province of Buenos Aires. This territory has a high level of anthropogenic pollutants. This jeopardizes the preservation of its biological diversity, therefore, all the necessary measures will have to be implemented in order to assure the vegetable genetic patrimony in the future. Traditionally, the propagation of trees has been mainly made with plants of seminal origin. An interesting alternative is to multiply the selected phenotypes in a vegetative way. The aim of this study was to determine the incidence of the diameter of the *Citharexylum montevidens*e juvenile cuttings in the rooting ability. In order to do this, an experiment was made near the end of winter in which thin cuttings (3 to 5 millimeters wide), medium cuttings (5 to 7 millimeters wide), and thick cuttings (7 to 10 millimeters wide) were used. Their sprouting and rooting were analyzed, as well as the amount of shoots and their length, and the amount of roots and their length. As a result, a high percentage of survival of medium and thin cuttings was obtained (51.3% and 48.7% respectively) and they could be easily reproduced in an agamic way. Very good results were also obtained with the rooting of live thin and medium cuttings: 100% in the first case and 85.7% in the second case. This study showed that the vegetative propagation of *C. montevidense* was possible by using cuttings which are not bigger than 7 mm in diameter and which come from vegetative material that is not more than one year old.

Keywords: Espina de Bañado, agamic reproduction, gallery forest, clone

S2–3P

New ornamental conifers for harsh Northern conditions trough vegetative propagation of special forms of Norway spruce

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Landscaping is a growing business, both in private and public sectors in modern societies. In the Northern Europe, the market now demands consistent and sustainable production of hardy, ornamental conifers to replace the less hardy imports. There are ornamental forms of native conifers that are well adapted to harsh Northern conditions. These naturally born forms found in forests have been collected and registered by Finnish Forest Research Institute. Recently, also a small number of crossings between certain forms have been produced in order to find new hybrids for ornamental use.

The EU-funded project "Vegetative propagation – knowhow and technology for enhancing bioeconomy" was launched in Finland 2011 and is carried out collaboratively with Finnish Forest Research Institute as a main performer, and University of Eastern Finland and a commercial company Taimityllilä Ltd as partners. One of the project aims is to facilitate propagation of the selected special forms of Norway spruce (*Picea abies*) to meet the increasing demand of hardy conifers for ornamental purposes and landscaping. In all, two methods including cutting and tissue culture technologies are tested. The suitability of the methods for commercial plant production is piloted in cooperation with a company partner. User right and royalty issues for both the natural mutants found and the special forms created by breeding are also covered.

For easily accessible and low-cost cutting propagation, 17 taxa of Norway spruce have been examined so far. The effects of donor age, timing of propagation, type of cutting, and rooting substrates on the rooting success of shoot cuttings are studied. The rooting success varies highly among the taxa. Generally, the forms having normal height growth but coloured needles root more easily than the forms without apical dominance or the ones showing reduced or pendulous growth. The overall rooting success of the winter cuttings (16.7%) has highly exceeded that of the summer cuttings (0.7%). Amongst the winter cuttings, the use of peat-vermiculite as substrate clearly increased the average rooting (22.6%) compared with that of Spruce-Rhododendron soil™-bark-vermiculite (10.6%). The relatively high rooting success of the cuttings originating in the 45–50-year-old donor trees, 51–53%, was achieved with the best combination of the treatments and the clones (*P. a.* f. *aurea* clone K219, *P. a.* f. *cruenta* clone U2080, and the regular and dense-crowned clone K359). The satisfactory rooting success of 52 and 42% was also achieved in the 20-year-old hybrids of P. *a.* f. *globosa* × *P. a.* f. *cruenta* and *P. a.* f. *cruenta* × P. *a.* f. *pendula*, respectively. The use of rejuvenilised donor plants and modified rooting substrates is currently studied.

For tissue culture approach, altogether 119 seed embryo explants originating in the controlled crossings between special forms of Norway spruce has so far been used for initiation of somatic embryogenesis, and 96 embryogenic lines have been obtained. Production and evaluation of emblings from these lines are currently under way, and the lines showing desired ornamental characteristics will be selected for mass- propagation.

Keywords: ornamental conifer, propagation time, rooted cuttings, rooting substrate, somatic embryogenesis

S2–4P

Wavy grain maple – gene conservation by use: attempts to start tissue culture from adult material

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Top-quality logs of wavy grain sycamore maple, a variety of *Acer pseudoplatanus* L., are sold frequently during annual timber auctions in Germany. The conservation of this valuable material as a genetic resource and the commercial exploitation, e.g., as a clone mixture, depends on the resolution of different scientific questions. It has to be shown that this special wood pattern (wavy grain) will be transmitted via auto- or hetero-vegetative propagation. The tree logs are often cut in early winter but are usually auctioned off in February or March. As soon as the high value becomes obvious, shoot material from the crown is collected for gene-conservation. The long-term storage of the material in the forest under changing environmental conditions is the main disadvantage and difficulty. In this study, the conservation and propagation of clones by grafting were carried out as the first step. Testing the tissue culture ability and optimizing the micropropagation will be the second step. Whereas rooting of cuttings from grafts often failed, micropropagated shoots have shown an almost complete root formation up to now. To our knowledge, this will be the first time that tissue cultures are established from already felled and sold wavy grain sycamore maples. The genetic identification of clones by microsatellite markers is necessary to ensure the quality of the material (genetic fingerprint) and will be included in the investigation.

Keywords: *Acer pseudoplatanus*, grafting, micropropagation

S2–5P

In vitro propagation of *Uncaria rhynchophylla* – a medicinal woody plant

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Uncaria rhynchophylla (Kagikazura or the cat's claw herb is a plant species used in traditional Chinese medicine) is an medicinal woody plant observed widely in Japan and China. It contains alkaloids (rhynchophylline[1], iso-rhynchophylline[1], hirstine and so on) which are good for remedy of high blood pressure and dementia. (+)-Catechin and (–)-epicatechin are found in the plant[2]. It is also used in Kampo medicine which is the Japanese study and adaptation of traditional Chinese medicine. It is in 4 of the 148 Kampo medicine formulae. Kampo does not incorporate any human body parts nor animal parts, thus avoiding issues with animal cruelty prevalent in traditional Chinese medicine. Kampo herbal medicines are regulated as pharmaceutical preparations and their ingredients are exactly measured and standardized. Access to Kampo herbal medicines is guaranteed as part of Japan's national health plan for each of its citizens. For the purpose of in vitro propagation and development of basis of useful substance production by cell culture, tissue culture procedure was developed for this species.

Shoots were induced from stem spine (thorn) of kagikazura in the 1/2MS medium containing BAP or Zeatin. Callus induced around the stem segments were continuously subcultured in the fresh 1/2LP medium containing 0.5 uM BAP and 1 uM 2,4-D. These cell lines can be used for the possible secondary metabolite production and for chemical breeding by somaclonal variation or molecular genetics technology. Regenerated plants were obtained by rooting of these shoots on 1/2MS medium containing 1 uM IBA. Rooted plantlets were cultured in giffy 7® with 60 ml of 0.1 % Hyponex® medium in plant boxes. Each plant box contained 1 regenerated plantlet. Culture condition was at 25 °C constant temperature under 16 h photoperiod of 70 $\mu Mm^{-2}s^{-1}$ by fluorescent lamp. Then after 2 months, they were habituated under nursery terrace® system (MKB Dream Co., Japan) which contain 100% humidity and automatic watering for 1 month, then grown in greenhouse for 6 months. Field plantation was successful. Selection of clones with higher chemical content is planned.

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Keywords: tissue culture, *Uncaria*, rhynchophylline

S2–6P

In vitro plant regeneration via callus derived from cotyledonary explants of Caspian honeylocust (*Gleditsia caspica* Desf.)

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An *in vitro* plant regeneration system through callus induced from cotyledonary explants of *Gleditsia caspica* was established. Calli were induced on Murashige and Skoog (MS) medium containing 3% sucrose, 0.8% agar, and different concentrations of indole-3-butryc acid (IBA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetc acid (2,4-D) alone, or in combination with 4.4 µM benzyl adenine (BA). The highest frequency of compact, nodular, and regenerative callus formation was obtained on MS medium supplemented with either 13.3 µM 2,4-D alone, or in combination with 4.4 µM BA. Shoot regeneration successfully occurred when calli were transferred onto MS medium supplemented with BA alone (2.2, 4.4, 8.8, or 17.7 µM), or in combination with 2,4-D (2.3 µM). The highest shoot regeneration was achieved on MS medium containing 8.8 µM BA alone, with an average of 13.7 microshoots per callus. To further elongation of the formed shoots, these were transferred to MS medium supplemented with gibberellic acid (GA_3) (1.4, 2.9, 5.4, or 8.2 µM) alone or in combination with 4.4 µM BA. The highest elongation growth was recorded on MS medium containing 8.2 µM GA3 plus 4.4 µM BA. To induce root formation, the regenerated shoots were transferred onto half-strength MS medium containing different concentrations of IBA alone, or in combination with kinetin (KIN). Maximum rooting (93.8%) was obtained on medium containing 9.8 µM IBA along with 0.92 µM KIN. After acclimatization, the regenerated plants were successfully transferred to soil under greenhouse conditions.

Keywords: Organogenesis, Adventitious shoots, Root formation, Leguminosae, Murashige and Skoog medium, Tissue culture

S2–7P

Plant regeneration from *in vitro* leaf and stem-derived callus of *Cercis siliquastrum* L.

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An efficient method was developed for organogenic plant regeneration of *Cercis siliquastrum* through callus cultures. The leaves as well as stems of *in vitro* grown seedlings were cultured on Murashige and Skoog (MS) medium containing 3% sucrose, 0.8% agar, and different concentrations of 2,4-Dichlorophenoxyacetc acid (2,4-D) and naphthalene acetic acid (NAA) in combination with 4.43 µM benzyl adenine (BA). The highest frequency of white, compact and nodular calli (48%) was induced from leaf explants cultured on MS medium containing 9.04 µM 2,4-D and 4.43 µM benzyl adenine (BA). According to calli weighing, however, the most callus growth and proliferation (2.4 gram) was achieved on MS medium supplemented with 18.09 µM 2,4-D and 4.43 µM benzyl adenine (BA). Shoot regeneration was occurred when the nodular calli were transferred onto MS medium supplemented with 8.87 µM BA and 4.54 µM thidiazorun (TDZ). Addition of 5.77 µM GA_3 along with 4.43 µM BA to the medium promoted the shoot elongation. For rooting experiments, the regenerated shoots were transferred onto MS medium containing different concentrations of IBA alone, or in combination with kinetin (KIN). The plants were acclimatized and successfully transferred to soil under greenhouse conditions.

Keywords: Organogenesis, Adventitious shoots, Woody plant, Plant tissue culture, *Cercis*

S2–8P

Callus induction and *in vitro* regeneration of Catalpa through organogenesis

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To determine the optimum *in vitro* conditions for callus induction and plant regeneration from *in vitro* leaf and stem of Catalpa (*Catalpa bignonioides*) seedlings, the explants were cultured on Murashige and Skoog (MS) or woody plant medium (WPM) medium supplemented with different concentrations (4.5, 6.7, 9.0, 18.0, and 27.1 µM) of 2,4-dichlorophenoxyacetc acid (2,4-D) or naphthalene acetic acid (NAA) (5.4, 8.1, 10.7, 21.4, and 32.2 µM) along with 2.2 µM benzyl adenine (BA). The highest rate of whitish, compact, and nodular calli were induced when the leaf explants cultured on MS medium supplemented with 32.22 µM NAA plus 2.2 µM BA (74.5%). Weigh calluses out showed that the callus produced from this medium had the most weigh (2.9 gram). The nodular, compact and organogenic callus tissues regenerated into shoots following 5 weeks on MS medium supplemented with 17.7–26.6 µM BA and then these were transferred for an additional 3 weeks on MS medium with Gamborg B5 vitamins supplemented with same plant growth regulators and 20% coconut water for shoot elongation by culture under 16-h photoperiod. The adventitious formed shoots then were rooted on MS medium containing four different concentrations of IBA or NAA. The adventitious root formation as high as 82.5% was occurred on MS medium supplemented with 4.9 µM BA. The regenerated plants were successfully acclimatized under greenhouse conditions.

Keywords: *Catalpa bignonioides,* Adventitious shoot organogenesis, Tissue culture, *In vitro* regeneration.

S2–9P

Rooting microcuttings of *Fraxinus pennsylvanica* Marsh.

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Forest industry in Argentina depends of plantations with exotic trees (mainly *Eucalyptus* sp., *Pinus* sp., Poplar and Willows) but to a lesser extent there are plantations with exotic species as *Melia* sp., *Robinia* sp., *Fraxinus* sp. and *Quercus* sp. Among the exotic cultivated species *Fraxinus pennsylvanica* Marsh. has great interest because their good timber features. On the other hand, *Fraxinus* is a genus widespread in urban forestry used as alignment. The male trees have great potential as urban tree because their shape and absence of fruit. In order to have selected phenotypes of male specimens of Fresno, it is necessary to adjust vegetative propagation techniques in order to reproduce clones quickly and efficiently. The aim of this study was to adjust a system for vegetative propagation by microcuttings from male individuals of *Fraxinus pennsylvanica* Marsh. The influencing of rooting promotion substances in cuttings were tested under greenhouse conditions. The experiments consisted of six concentrations of NAA: control, 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm. with slow solution The results showed that the average rooting rate was 60% in NAA 80 ppm. In this concentration we obtained the largest number of roots per cuttings, as well as the increased number of shoots, the same with respect to its length.

Keywords: *Fraxinus pennsylvanica*, rooting, microcuttings, vegetative propagation

S2–10P

Propagation of American chestnut using non-germinable somatic embryos

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In our efforts to produce blight-resistant American chestnuts (*Castanea dentata*) using genomics, *in vitro* propagation and gene transfer, embryogenic cultures have been proven to be good targets for genetic transformation. However, constitutive expression of some transgenes may disturb somatic embryo development, resulting in a high percentage of abnormal embryos, such as segmented embryos or embryos with single or fused cotyledons. Plant regeneration through germination of such abnormal embryos is either impossible or occurs only at very low frequencies. In this study, a micropropagation method was developed to regenerate plants from non-germinable transgenic embryos. Multiple adventitious buds were induced from the apical meristem areas of the abnormal embryos. Buds could be induced from these embryos at early developmental stages, although a better induction rate was obtained with more mature embryos. Bud induction success varied with genotype and concentration of 6-benzylaminopurine (BAP). After bud induction and multiplication, individual buds were removed and cultured on shoot elongation medium with reduced BAP for 2 weeks, then transferred to root induction medium containing 5 mg l^{-1} indole butyric acid for one day before culturing on root development medium with no plant growth regulators. All cultures were maintained under light (16/8 h). Rooted plants were obtained from non-germinable transgenic embryos of American chestnut genotype WB484-3 engineered with constructs carrying a GUSi-YFP gene fusion or a Chinese chestnut deoxy-arabino-heptulosonate phosphate synthase gene. This study demonstrates a novel system, through which plant regeneration was enhanced, for producing phenotypically normal plants from genetically engineered tissue.

Keywords: American chestnut, adventitious buds, genetic transformation, non-germinable somatic embryos, plant regeneration

S2–11P

Micropropagation of Yellow-Poplar (*Liriodendron tulipifera*) via indirect secondary somatic embryogenesis

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Yellow-poplar is one of the most valuable hardwoods for plantations of fast-growing forest tree species to be used for such purpose as biomass energy. Somatic embryogenesis (SE) is known as a useful technique in large-scale propagation of superior yellow-poplar trees. However, progressive decreasing and final missing of embryogenesis capacity have been observed in yellow-poplar embryogenic cell lines. In the present study, effective methods of increasing the productivity of somatic embryogenesis in yellow-poplar were investigated for biotechnological applications in clonal forestry. In yellow-poplar somatic embryogenesis, embryogenic cell lines maintained on MS medium with 1.0 mg/L 2,4-D lost embryogenic capacity during the subculture. These low-embryogenic cells resulted in decreased SE efficiency, and produced only 3,000 somatic embryos per 0.5g of ECs. Enhancement of somatic embryogenesis was achieved through indirect secondary somatic embryogenesis. To initiate secondary embryogenic cells, cotyledonary somatic embryos induced from the first embryogenic cultures were cultured on MS medium with 1.0 mg/L 2,4-D. The newly initiated secondary embryogenic cells produced more than 13,000 somatic embryos per 0.5g of ECs. This result indicates that newly initiated secondary ECs are high-embryogenic.

Keywords: *Liriodendron tulipifera*, hardwoods, indirect secondary somatic embryogenesis

S2–12P

Somaclonal variation among calli and derived plants of *Melia azedarach* L.

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Frequency of somaclonal variation in organogenic calli and plants of *Melia azedarach* L (chinaberry tree; persian lilac) derived from indirect organogenesis were examined by amplified fragment length polymorphism (AFLP) analysis. Cotyledon of mature zygotic embryo were cultured in MS medium supplemented with BAP (1 mgL^{-1}) and NAA (0.5 mgL^{-1}). After culturing the organogenic calli on MS medium in the presence of BAP (1 mgL^{-1}), putrescine (80 mgL^{-1}) and adenine (40 mgL^{-1}), regenerated plants were obtained. DNA samples from the organogenic calli maintained under cultivation for 48 months, with morphogenic capacity and plants regenerated from organogenic calli (10 each) were subjected to AFLPs analysis. Four combinations were used as "primers" for amplification products. A dendrogram, based on the unweighted pair group mean average (UPGMA) method of cluster analysis, were constructed using a similarity matrix derived from the AFLP amplification generated by all primers. The estimation of genetic similarity coefficient based on AFLP data indicated that similarity were more than 80% demonstrating the existence of a low level of variability between them. Several types of polymorphisms were observed. There weren't coefficients equal to unity, which means that AFLP profiles of regenerated plants no were equal to the mother line. However, the detected variability did not affect the phenotype of the regenerated plants. This study may provide information that will be used to assist the development of techniques for CTV with less somaclonal variation.

Keywords: micropropagation, indirect organogenesis, variability, AFLPs, Persian lilac, chinaberry

S2–13P

In vitro techniques for large scale propagation of *Oroxylum indicum* Vent. using different explants

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In vitro propagation of *Oroxylum indicum* is possible through indirect organogenesis via callus. Seeds were germinated in vitro on hormone free MS medium. Roots of *in vitro* grown seedlings was used as explants. Callus was produced in full strength MS medium containing different concentrations of NAA & 2,4-D.

Shoot initiation was achieved in full strength MS medium supplemented with IAA 0.1 mg/l + BA 2.5 mg/l. Shoot elongation was achieved in half strength MS medium supplemented with BA 1.0 mg/l. Maximum shoot multiplication was observed in full strength MS medium containing IAA 0.1 mg/l + BA 0.5 mg/l +Adenine sulphate 50mg/l. However, when medium fortified with IAA 0.1 mg/l + BA 5.0 mg/l +Adenine sulphate 50mg/l rosette clump of shoots was formed with more calluses. Addition of adenine sulphate promoted high rate of shoot multiplication.

Maximum rooting (50%) of *in vitro* grown shoots was observed in ¾ MS medium supplemented with IBA 1mg/l. Plantlets were successfully hardened in *in vitro*.

Keywords: *Oroxylum indicum*, *In vitro* propagation, Indirect organogenesis, Callus, Root

S2–14P

Effects of Plant Growth Regulators and Cutting Dates on Rooting of Hardwood Cutting in *Vaccinium uliginosum* L.

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Vaccinium uliginosum (Northern Bilberry) is a small deciduous shrub which is a rare species and grows in high latitude regions of Eurasia including high mountains in Korea and North America. This study was performed to investigate the effects of plant growth regulators and cutting times on hardwood cuttings (2nd year-branches). Rooting percentage was highest (76.7%) at treatment with 500 mg/l IBA than other treatments after 3 months on hardwood cutting. Number of roots and length of major roots with 1,000 mg/l IBA were superior to other treatments (3.4ea and 7.8cm, respectively). New shoots were developed with NAA 100 mg/l (1.5ea) more than other treatments, and the length of new shoots was the longer at control treatment (1.7 cm). But growth velocity of new shoots was very slow in control treatment. Cutting date was very important factor in rooting and survival in *V. uliginosum*. For the rooting percentage by cutting date, all treated groups demonstrated a higher rooting percentage and early growth for cuttings performed in early May than in late July. The rooting rates of May-rooting with 500 mg/l and 1,000 mg/l IBA groups were 76.7% and 75.6%, and rates were 62.3% and 54.5% at July-rooting. Survival rates of May-rooting were 82.2%, but those were 22.2~23.3% at July-rooting. The number of roots with 500 mg/l IBA was different by rooting time, but was not significantly different with 1,000 mg/l. Root length of May-cutting was longer than that of July-cutting in both regulators. These results revealed that optimum conditions of asexual propagation for *V. uliginosum* by hormone regulators and cutting time.

Keywords: *V. uliginosum*, growth regulators, cutting dates, hardwood cutting

S2–15P

The effect of different substrate compositions on the acclimation of micropropagated plants to *ex vitro* conditions

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One means of production of high quality forest seedlings is micropropagation. The terminal events of micropropagation are rooting and acclimatization of micropropagated plants. Optimization of these events enables an increase in the survival percentage and a reduction in the period of the raising of transplants. Hence the objective of this study was to investigate the influence of different culture methods on the growth of microplants in soil conditions.

In our study we used a shoot culture of *Populus tremula*. In the initial stage of the investigation two substrata were used (1) modified woody plant medium (WPM, Lloyd & McCown, 1980) and (2) pearlite saturated with mineral salts of the same medium. To encourage rooting we used strain *Pseudomonas mendocina* 9–40 provided by the biological department of Byelorussian State University. We tested the following treatments for the microplants raising: I) sterile conditions, glass culture jars, medium 1; II) treatment I + bacteria of the strain 9–40 inside the medium; III) treatment I + bacteria of the strain 9–40 on the surface of the medium; IV) sterile conditions, plastic containers, medium 1; V) sterile conditions, plastic containers, pearlite; VI) unsterile conditions, plastic containers, pearlite; VII) the conditions of treatment I for a fortnight followed by transplantation into the conditions of treatment VI; VIII) treatment VI + addition of strain 9–40; and IX) treatment VII + addition of strain 9–40 to pearlite. After 30 days all the microplants were transplanted into the mixture of peat and sand and were grown during six weeks. In a greenhouse the plants were grown in substrata composed of either peat and sand or peat, pearlite and fertilizers. The total of 540 microplants was used in the current study.

Within 75 days after the start of the experiment the survival percentage was high (85–98%), the aboveground parts of the plants averaged 5.1–7.8 cm long. Plants of treatment IX therewith exhibited the highest values, namely, 98% survival and their aboveground parts averaged 7.8 ± 1.4 cm long. Co-cultivation with *Ps. mendocina* 9–40 increased average dimensions of the aboveground parts of the plants by 8.4–19.7%.

After three months' raising in the greenhouse the survival percentage varied from 48% to 100% and the leading shoots ranged in average height from 14.7 to 27.7 cm depending on the test group. Both the substratum used and means for transferring the microplants into *ex vitro* conditions had a pronounced effect. With a mixture of peat and sand, the survival percentage was 5–40% higher than those of the plants grown in peat mixed with pearlite.

The generalization of the results obtained demonstrated that the highest 75–80% survival was typical for the plants of test groups IV, VI, VII and VIII. The use of these methods shows considerable promise.

Keywords: micropropagation, acclimatization to *ex vitro* conditions, co-cultivation

Session

Implementation / mass propagation into commercialization

IUFRO WORKING PARTY 2.09.02
SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES

S3–1P

The prospects for using somatic embryogenesis to propagate Sitka spruce in the UK

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Although native to the west coast of North America, Sitka spruce (*Picea sitchensis* (Bong.) Carr.) has become the predominant commercial forest tree species within the UK, accounting for 29% of the GB forested area or 49% of all conifers.

Forest Research (part of the UK Forestry Commission) maintains an active breeding programme for this species and provides seed lots from controlled crosses (family forestry) to the UK nursery industry, which are in turn used to produce large numbers of cuttings, but to date this has not included the use of tissue culture or SE approaches at any stage of the process. In order to maximise the breeding gains that are potentially available for the UK forest sector, specific efforts have recently been made to replicate the approach and methods that have been successfully applied to White spruce (*P. glauca* (Moench) Voss.) by the Canadian Forest Service.

This presentation will describe the current levels of success that have been achieved for initiating and proliferating embryogenic cell cultures of Sitka spruce from immature and mature seeds; the production of somatic embryos and plants from these cultures; as well as the cryo-preservation of selected cell lines, such that multi-varietal forestry (or MVF) is now a viable prospect for Sitka spruce in the UK.

This presentation will conclude with a brief discussion of the likely contribution of this technology to the UK forest sector, including for meeting the pressures and threats posed to the sector by climate change, as well as assisting with the UK Government's carbon reduction obligations.

Keywords: *Picea sitchensis*, somatic embryogenesis, breeding programme

S3–2P

Incorporating wood quality traits in multi-varietal forestry of white spruce

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Genetic effects of wood quality traits (latewood proportion, wood density, and microfibril angle) and their relationship with growth were studied using a clonally replicated genetic test of white spruce at age 19 years. Both growth and wood quality traits appear to be under moderate genetic control. The main contributor of variation in growth traits was variation due to clones within family. For wood quality traits, variation due to families was greater than the clonal variation. Generally, faster growth resulted in a significantly lower overall wood density. Importantly, this study demonstrated that, despite the negative correlation, clones that break such negative correlation may be found through multi-varietal forestry aimed at improving growth without compromising wood quality.

Keywords: multi-varietal forestry, white spruce, wood quality traits

S3–3P

Towards mass-propagation of Norway spruce in Finland

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A new project "Vegetative propagation – knowhow and technology for enhancing bioeconomy" has been launched in Finland. The aim of the project is to deepen knowhow and develop technology for vegetative propagation of forest trees in Finland, and to enhance collaboration among research institutions and practical plant producers. The project is realized by Finnish Forest Research Institute (Metla) with the University of Eastern Finland and a commercial company Taimityllilä Ltd as partners. The project is funded with 600 000 € for the years 2011 to 2014 by the European Regional Development Fund of EU.

In Finland, there is a lack of high-quality Norway spruce seed for forest regeneration. Seed orchards, established for production of genetically superior seed, suffer from irregular flowering of the species, as well as problems caused by plant pathogens and pests. Thus, the primary task of the present project is to develop a vegetative mass propagation method, suitable for commercial production of Norway spruce. Tissue culture approach using somatic embryogenesis, potentially combined with cutting technology, is developed and applied. Also issues related to commercial production of selected clones or bred families originating in the national tree breeding programme, will be solved.

In order to get high-quality forest regeneration material, controlled crosses among the top trees of the Norway spruce breeding programme are used as a source of explants for somatic embryogenesis (SE). Both immature and mature seed embryos are tested as explants. Embryogenic lines obtained are tested for their capacity to produce embryos, and selected to be stored using cryopreservation until they can be delivered to plant producers. Field tests are established with the emblings regenerated. During the first year of the project, 12 Finnish full-sib families have been tested for SE using 3198 immature embryo explants. The SE initiation rate among these families vary from 22 to 93% on LM-based medium, and from 1 to 36% on LP-medium. Around 900 embryogenic lines have been tested for their embryo production, with the best lines showing the capacity to produce 600–700 embryos per gFW. The project continues by evaluation of the regenerated emblings, new SE initiations, and further development of the technology, such as cold storage of mature embryos, and finally, by a commercial pilot with the company partner.

Concurrently, with the propagation materials (SE lines) being produced, a service model is created for future dissemination of these materials for commercial use. The idea is to describe and rationalize the government funded parts of the process that are performed by Metla, as well as user rights and possible royalties paid by commercial producers. It is suggested that creation, testing, and storage of the propagation materials, i.e. controlled crossings, SE initiations, clone selection, and cryopreservation would be carried out by Metla, in order to ensure materials being equally available for all the plant producers in Finland. To support these actions, the commercial producers are suggested to pay small royalties based on the amount of emblings sold on the market.

Keywords: mass-propagation, Norway spruce, somatic embryogenesis, tree breeding

S3–4P

Tree breeding and mycorrhizal symbiosis as important tools in forestation processes

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Higher growth rate and morphological traits have been the major criteria for selecting trees classified as improved in breeding programs. The symbiotic associations between *P. pinaster* and ectomycorrhizal fungi can be an effective approach to enhance plant development. The aim of this work was to assess whether the establishment of mycorrhizal symbiosis at nursery stage was affected by tree breeding.

P. pinaster improved and non-improved seedlings were inoculated with compatible ectomycorrhizal fungi: *Suillus bovinus*, *Pisolithus tinctorius* or *Rhizopogon roseolus*, and grown in individual cells containing forest soil, in a commercial forest nursery. Growth and nutritional traits, colonisation parameters and the fungal community established were assessed. *R. roseolus* and *P. tinctorius* were the most efficient isolates in promoting plant development. Inoculated improved saplings had an overall superior development than their non-improved counterparts, with up to a 4.9-fold in root dry weight and a 13.6-fold increase in the total number of ectomycorrhizal root tips. Differences in fungal community were revealed through the denaturing gradient gel electrophoresis profile of each treatment. The results from our study suggest that improved seedlings benefit more from the mycorrhizal association and therefore this could be a valuable biotechnological tool for the nursery production of improved *P. pinaster*.

Keywords: Tree breeding, improved trees, ectomycorrhiza, forest nursery inoculation, maritime pine

S3–5P

Two genotypes of mycorrhizal *Pinus pinaster* respond differently to cadmium contamination

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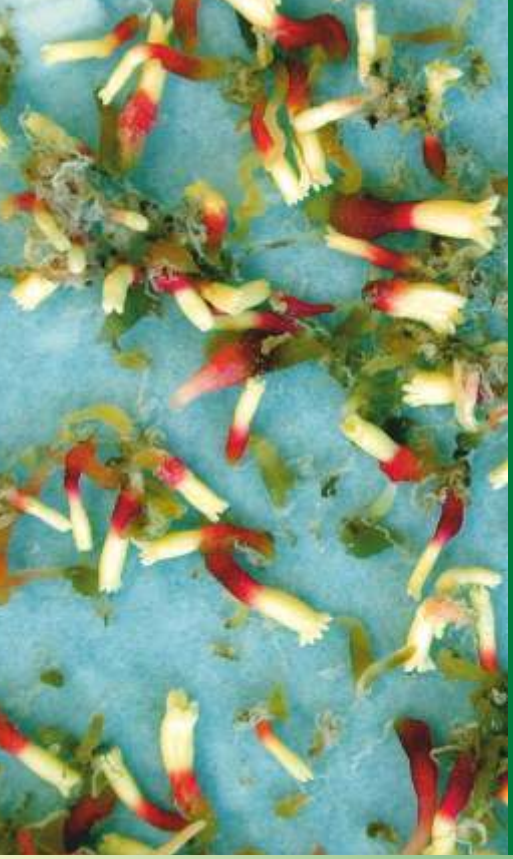
Fertilization is one of the main anthropogenic sources of Cd accumulation in agricultural soils and when toxic levels have been reached, food crop production is no longer viable. Adequate strategies for the forestation of agricultural metal contaminated sites are of vital importance. The aim of this work was to evaluate the response of two different genotypes of *P. pinaster* (A and B) to Cd contamination and to assess how inoculation with ectomycorrhizal fungi influenced each genotype. Seedlings were exposed to soil contaminated at different levels of Cd. At 30 mg Cd kg^{-1} non-inoculated genotype A accumulated more Cd in the shoots. At the lowest Cd concentration *S. bovinus* decreased Cd shoot concentration and increased aboveground development in both genotypes. At the highest Cd dosage inoculation with *R. roseolus* decreased Cd concentration in the roots of genotype B whereas the opposite occurred in genotype A. The results from this study suggest that the selection of an adequate combination between genotype and associated mycobionts may be an important biotechnological tool to enhance the efficiency of forestation and phytoremediation processes of degraded land using *P. pinaster*.

Keywords: *Pinus pinaster*, cadmium, ectomycorrhizal fungi, phytoremendation



Session

Physiology, genetics, epigenetics, biotechnology, cryopreservation, etc.

IUFRO WORKING PARTY 2.09.02
SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES

S4–1P

Secondary phenolic compounds in somatic embryogenesis of *Pinus sylvestris* L. – a preliminary study

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The increasing concern about the ecological impacts of wood preservation chemicals has raised the interest on the natural durability of Scots pine (*Pinus sylvestris* L.) heartwood. Phenolic compounds such as stilbenes as well as monoterpenes have been found to inhibit fungal growth and thus make the wood more resistant to decay (Venäläinen *et al*. 2003). There is a strong genetic component in the decay resistance of the heartwood in *P. sylvestris* (Harju & Venäläinen 2002), and positive genetic correlation has been found between the concentration of constitutive stilbenes in the heartwood of mother trees and in their seedling progenies (Harju *et al.*, 2009).

Vegetative propagation of Scots pine individuals with high content of phenolics could provide a way to produce more durable timber than those with low phenolics. A new EU (ERDF) – funded project "Vegetative propagation – knowhow and technology for enhancing bioeconomy" was launched in Finland 2011 and is carried out at Finnish Forest Research Institute and cooperated with the University of Eastern Finland and a commercial company Taimityllilä Ltd. One of the project aims is to study potentials of vegetative propagation in improving the heartwood quality in Scots pine. This is done by comparing success of somatic embryogenesis (SE) in Scots pine families producing high concentration of phenolics with those producing small amounts of these compounds. Furthermore, analyses of phenolics induced in embryogenic cultures will be carried out and their suitability for in vitro selection will be studied.

Three mother trees with either high or low content of phenolic compounds were chosen and crossed with each other in 2011, to provide seeds for SE initiation in 2012. In 2011, open-pollinated immature cones were collected from the same trees at five different degree days for testing the success of SE initiation. After ten weeks of initiation, 4.7% of the explants produced the embryogenic culture (ECs) in two different media (LM and DCR). The LM-based initiation medium proved to be better than the DCR-based one, and most the lines were initiated from the explants collected between 526 and 576 dd. The effect of genotype on the initiation rate was significant on both media. However, the phenolic compounds of mother trees had no clear effect on the initiation frequency, suggesting that propagation through SE would be possible originating both from the trees having high and low content of phenolic compounds. We also analyzed the secondary phenolic compounds by HPLC/DAD in responding (able to initiate ECs) and non-responding (not able to initiate ECs) explants after the initiation. In these analyses, 17 different phenolic compounds were determined according to their retention time and UV-spectra. Most of the compounds were found to be more abundant in the non-responding explants. Embryo production capacity, and the phenolic compounds of the SE lines obtained are currently being studied. Experiments will be repeated with the explants originating in the controlled crossings in 2012.

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Keywords: *Pinus sylvestris*, somatic embryogenesis, secondary phenolic compounds

S4–2P

From angiosperm models to forest trees: A study on expression behavior of Arabidopsis homologous genes during embryogenesis of *Larix decidua*

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The organization of a plant embryo is defined by the first division of the zygote. In Arabidopsis, members of the *WUSCHEL-related HOMEOBOX* family (*WOX*) determine the apical and basal part of the developing embryo. Auxin transport and with it organogenesis is mediated by the family of *PIN-FORMED* (*PIN*) auxin efflux carriers. The shoot and root meristems are established, whereupon the transcription factor *SHOOTMERISTEMLESS* (*STM*) is required. *LEAFY COTYLEDON1* (*LEC1*) is necessary to prevent an early maturation. *BABYBOOM* (*BBM*) and *SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE* (*SERK*) are known to promote embryogenicity as their overexpression leads to the formation of somatic embryos.

Our intention was to study genes in *Larix decidua* that are homologous to the factors mentioned above during Arabidopsis embryogenesis. Furthermore, we were interested in the correlation between those genes and the hormonal control of the master regulator auxin. Accordingly, we analyzed expression patterns of selected genes depending on auxin distribution within the embryo. To alter auxin availability during maturation we supplemented maturation media with the polar auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA; 20 µM).

Full length or at least partial length sequences of homologous genes were identified before by screening a cDNA library and using PCR-based methods. For expression studies we used somatic embryos of an established line (in regard to maintenance, maturation, conversion and transformation) taking advantage of easy available and accessible starting material as well as the controllability of the *in vitro* system itself. Relying on those benefits we analyzed the localization of several genes of interest in mature somatic embryos by the means of in-situ-hybridization.

Further we aimed to assess embryo-specificity of the genes of interest in order to use an appropriate tool for the indication of early differentiation between embryogenic and non-embryogenic tissues in the process of induction of somatic embryogenesis. So far confirmation of embryogenicity to select positive induction events is done by microscopic analysis of cultures of a certain age (several weeks). Callus is characterized by a loose structure and globular cells, whereas somatic embryos are subdivided in suspensor and embryo head. The use of expression markers is necessary to prove the embryogenic competence as soon as cells are reprogrammed. According to this we analyzed transcription patterns of several genes of different tissue types.

Identification and vital expression studies point to the relevance of the genes of interest (*LdBBM, LdLEC1, LdPIN, LdWOX2, LdSTM, LdWOX2*) during somatic embryogenesis of *Larix decidua* and the results further suggest a conserved role of principal regulators during land plant embryogenesis. The interaction of those genes is sensitive to external influences and hormonal control as shown by the NPA assay. Functional studies are necessary to confirm the assumed roles for the individual genes. Embryo-specificity could be demonstrated for several genes and possibly introducing a potent tool to improve the induction of somatic embryogenesis.

Keywords: *Larix decidua,* embryogenicity, embryo-specificity, *WUSCHEL-related HOMEOBOX2 (WOX2), PIN-FORMED (PIN), SHOOTMERISTEMLESS (STM), LEAFY COTYLEDON1 (LEC1), BABYBOOM (BBM), SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE (SERK)*

S4–3P

Differences in morphogenesis of horse chestnut resistant and non-resistant to *Cameraria ohridella*

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The horse chestnut (*Aesculus hippocastanum* L.) is a large deciduous tree from family *Aesculaceae*, originated in Balkan Peninsula that has been cultivated in all Europe. It is used for horticultural qualities and also for pharmaceutical industry. Recently, the successfulness of its planting in Europe is decreased due to an invasive pest (the horse chestnut leaf miner *Cameraria ohridella*). From this point of view is very important to find resistant genotypes and develop methods for their fast propagation. Classical methods have strong seasonal and production limits. The aim of presented work was to develop methods of fast horse chestnut micropropagation. The morphological responses of two genotypes have been compared. One of them showed resistance to horse chestnut leaf miner (R), the other one was non-resistant (N). The behaviour of these two clones showed the big differences, the resistant clone seemed to be nearly recalcitrant. Organogenesis was induced in summer buds of 5-year-old trees on WPM medium with vitamins supplemented with 0.5 mg l^{-1} BA, N clone showed shoot formation also on petiole and leaf explants, with optimal concentrations 10 µM of BA/0,1 µM NAA. On the contrary R clone showed slow growth and produced two times less shoots then N and hardly any callus and buds on petioles and leaves. Somatic embryogenesis was achieved in N clone on medium with 10 µM of each: kinetine, 2,4-D and NAA, where R clone showed only rare callus formation, hardly any shoot formation and none somatic embryogenesis. The probable reason of a very low response of R in compare to N clone could be different cytokinin metabolism. Fortunatelly it could be overcome by induction of embryogenic culture from filaments of older trees. Successful micropropagation protocol of of *A. hippocastanum* contributes to mass production of selected resistant clones.

Acknowledgement: This work was supported by the Ministry of Agriculture of the Czech Republic (project NAZV QH81101).

Keywords: organogenesis, somatic embryogenesis, *Aesculus hippocastanum*, resistance, *Cameraria ohridella*

S4–4P

PeakForce quantitative nanomechanical mapping of vascular cell wall stiffness in the early years of micropropagated hybrid poplar stem development

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Mechanical properties of the cellular microenvironment, notably its rigidity and stiffness, play a critical regulatory role for a variety of fundamental cell behaviors and responses. In the case of secondary xylem vessels, knowledge of the in situ cell wall stiffness quantified by a modulus of elasticity should be of great importance as it can be used not only to assess stress values resulting in cell wall deformations but also to evaluate the risk of vessel implosion when an embolism spreads and cavitation occurs under stressful environmnental conditions. Mechanical properties of secondary xylem are also influenced by the ontogenetic stage of a tree due to cambial ageing. In this study, the newly developed atomic force microscopy technique, PeakForce quantitative nanomechanical mapping, was used to extract quantitative nanomechanical data such as the reduced Young's modulus of elasticity, adhesion, deformation and dissipation. We assessed the nanomechanical properties of secondary xylem vessel walls for the micropropagated hybrid poplar in the early years of stem development, including ex vitro acclimatized, 1-year-old and 2-year-old plants.

Keywords: *Populus tremula* × (*Populus* × *canescens*); modulus of elasticity; vascular anatomy.

S4–5P

Analysis of different promoters and reporter genes in somatic embryos of *Pinus pinaster* Ait. and *Larix decidua* Mill.

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Reporter gene systems play a key role in many gene expression studies and in detection or localization of genes of interest. We analyzed reporter gene expression in somatic embryos of *Pinus pinaster* and *Larix decidua* to develop a screening strategy for accurate T-DNA integration into the plant genome and to identify false positive lines which frequently occur during the transformation process of somatic embryos from conifers. Also, the right choice of the promoter that drives the expression of a foreign gene, a reporter gene and the selection cassette is of importance considering the level of expression, not least in regard to overexpression studies. Hence, β-*GLUCURONIDASE (GUS)* activity regulated by either the cauliflower mosaic virus *(CaMV) 35S* or the maize-derived *UBIQUITIN 1 (UBI-1)* promoter during somatic embryogenesis of L. decidua was analyzed quantitatively and qualitatively.

Transformation was conducted with the plasmids pCAMBIA1305.2 (*35S::GUSPlus*), pGH217 (*35S::GUSA*), pSB241 (*UBI-1::GUSA*), modified pCAMBIA1301 (*35S::GUSA*), pLH6000 (*UBI-1::sGFP*) and mt-rb CD3-992 (*35S::mCherry*) comprising the respective reporter gene and the selectable markers *HYGROMYCIN PHOSPHOTRANSFERASE (HPT)* and/or *PHOSPHINOTRICIN ACETYL TRANSFERASE (PPT)*. For Agrobacterium- mediated transformation of embryogenic tissue of *P. pinaster* and *L. decidua* the 'droplet method' was used. Stable integration of the T-DNA into plant genomes was confirmed by polymerase chain reaction (PCR). The transgenic lines harboring green or red fluorescent proteins (GFP or mCherry) were tested for reporter gene activity by fluorescence microscopy. The activity of the reporter gene *GUS* was observed by quantitative (fluorometric assay) and qualitative (X-gluc staining) detection during development of somatic embryos.

The promoter analysis demonstrated that expression of *GUS* regulated by the *UBI-1* promoter is tenfold higher than driven by the *CaMV 35S* promoter during embryogenesis of *L. decidua*. The *GUS* assay showed high activity in the proliferation stage of somatic embryos in *P. pinaster* and *L. decidua*. Then the *GUS* activity decreased significantly in early stage of embryo maturation and increased again after 20 days of embryogenesis in both species. However, in general, the *GUS* expression regulated by *CaMV 35S* was much higher in *P. pinaster* compared to *L. decidua*. These results indicate that the *CaMV 35S* promoter activity depends on species and the respective developmental stage.

The GFP fluorescent signals were detectable in somatic embryos of *P. pinaster* that were tested positively by PCR. A clear assignment of these signals was however impaired by their low intensity in comparison to that exhibited by model plants and the considerably high background fluorescence of wild type tissue. So far fluorescence screening by itself is unreliable for gene expression studies in conifers because of difficulties in the identification of proper signals. It has been shown that the *GUS* reporter gene system was more efficient to monitor gene activity in somatic embryos compared to the GFP system. However, further studies on expression of fluorescent reporter genes as vital markers in conifers are necessary.

Keywords: reporter genes, promoter analysis, *Pinus pinaster*, *Larix decidua*

S4–6P

Cryopreservation of embryogenic tissues of selected spruce species after sucrose preculture and air desiccation, and genetic stability of the tissues

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One- and two-year-old embryogenic tissues (ETs) of *Picea abies* and *P. omorika*, respectively, were successfully cryopreserved after sucrose preculture and air desiccation. ET of *P. abies* was precultured for 7 days on a medium with increasing concentrations of sucrose (0.25 M sucrose for 24 h, 0.5 M for 24 h, 0.75 M for 2 days, and 1.00 M for 3 days; preculture S) or with sucrose, as above, and 10 µM abscisic acid (preculture SA). ET of *P. omorika* was precultured on a medium with increasing concentrations of sucrose, as above (preculture S) and in reverse time of treatment (0.25 M sucrose for 3 days, 0.5 M for 2 days, 0.75 M for 24 h, and 1.00 M for 24 h; preculture ST). Next the clumps were air-dried over silica gel, down to a water content of 20%, placed in cryovials, and rapidly immersed in liquid nitrogen for 24 h. Then the clumps were thawed at 42°C and placed on a proliferation medium, where they started to grow after a week (*P. omorika*) or 3 weeks (*P. abies)*. Using this method, we have obtained maximal ET survival of 99% for *P. omorika* (after preculture S) and 54.4% for *P. abies* (after preculture SA). Surviving clumps were friable and white, like before cryostorage. They were able to proliferate and to form normally developed somatic embryos. Genetic analysis of 5 microsatellite regions (SpAGC1, SpAGC2, SpAGG3, SpAC1H8, SpAC1F7) in the DNA of cryopreserved *P. abies* ET and somatic embryos obtained from restored ET after freezing in liquid nitrogen indicate genetic stability of the plant material stored using this method. Our results have shown that the presented method is effective for cryopreservation of embryogenic cultures of both tested *Picea* species, although in *P. abies* the survival rate and growth was lower than in *P. omorika*. Addition of abscisic acid in future experiments may help to increase the efficiency of cryopreservation of *P. omorika*.

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Keywords: cryostorage, ABA , microsatelitte markers, *Picea*

S4–7P

Suitability of cryopreservation for the long-term storage of conifer embryogenic tissues

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Cryopreservation – storage of biological material at ultralow temperatures (usually at –196°C in liquid nitrogen) is a suitable method for the conservation of plant tissues and organs since it has already been applied for wide range of plant species with success. In recent years protocols have been developed for the long-term storage of conifer embryogenic tissues by cryopreservation. Somatic embryogenesis in plants represents valuable tool for the study of plant early development and is considered as an efficient plant propagation system.

Embryogenic tissues of *Pinus nigra* Arn. and *Abies* hybrids (*Abies alba* × *A. cephalonica*, *Abies alba* × *A. numidica*) were cryopreserved by slow-freezing method and their growth, maturation capacity and genetic background were followed during the post-thaw period.

Tissues regeneration (recovery) started relatively soon, around the 4 to 9th day after thawing. In *Abies* all the four tested cell lines regenerated with individual frequencies 37 to 100%. The recovery in *Pinus nigra* cell lines was more cell line dependent. Altogether 46 cell lines were cryopreserved and 35 of them regenerated after exposure to liquid nitrogen storage. The individual regrowth percentages reached 10 to 100%. In *Pinus* the duration of storage in liquid nitrogen (1 hour versus 1 year) had no significant impact on tissues recovery (75% for tissues stored for 1 hour and 70% for tissues stored for 1 year). Similarly growth parameters that were measured (fresh and dry mass accumulation) were not negatively influenced by cryopreservation and no statistically significant differences were observed when compared to controls (non-cryopreserved tissues). The maturation capacity of cryopreserved cell lines was also tested. The cryopreserved tissues produced somatic embryos capable of maturaton and plantlet regeneration. RAPD analysis of 88 genomic regions per cell line (*Abies* hybrids) did not reveal any changes in genetic fidelity of cryopreserved tissues compared to non-cryopreserved control. Similarly for *Pinus*, no genetic variation was observed in cryopreserved tissues using the RAPD approach. The most important characteristic of embryogenic tissues is the presence of bipolar somatic embryos. Therefore special attention was paid to the study of their structure during the complete cryopreservation procedure (pretreatment and post-thaw regeneration). The preatreatment and liquid nitrogen storage caused desintegration of bipolar stuctures. After thawing, especially the meristematic embryonal cells were alive and the suspensor cells were almost completely disrupted. In the post-thaw period by repetitive cell divisions in survived cells, meristematic cell clusters differentiated and by vacuolisation the suspensor was formed. Finally the bipolar organisation of somatic embryos was restored. These results suggest the suitability of the slow-freezing cryopreservation method for long-term storage of mentioned conifer embryogenic tissues.

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Keywords: somatic embryogenesis, cryopreservation, *Pinus nigra*, *Abies* hybrids

S4–8P

The biochemical characteristics of the physiological activity of beech and spruce embryos

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We try to estimate the properties of seeds harvested from different sources and/or the properties of the somatic embryos developed in the different embryogenic cultures. Usually the quality of embryos or seeds is established according to the yield and viability of seedlings, i.e. after the long process of preparation (stratification, desiccation etc.) and germination of seeds or embryos. Our experiments can bring the information about the depth of seed dormancy and/or the ability of embryos to continue their development just before germination using biochemical methods.

Plant material: Zygotic embryos of beech; spruce embryogenic culture during the process of somatic embryogenesis.

Methods: We selected 3 substances to analyse - ABA (abscisic acid), which controls the dormancy and regulates the maturation of somatic embryos; IAA (indolyl-3-acetic acid), which regulates the growth and the development of embryos and whole seedlings; fumarase, which indicates the ability of seeds to mobilise reserves and to produce energy for germination and seedlings growth.

IAA and ABA: The plant material (around 0.1 g FW) was milled on a DNA mill and extracted in a modified Bieleski solution. The extract was centrifuged and dried on a rotary vacuum concentrator at room temperature. Samples were dissolved in solution of acetonitrile in water (15 vol %), injected into HPLC and precleaned on C-18 with gradient elution and fractionation on fraction collector. Fraction at time 23.05 min was collected for 1 min and dried. Collected fraction was derivatized by diazomethane solution in ether, dried, and dissolved in 100 µl of acetone. 8 µl of redissolved sample was injected into GC-MS/MS and analyzed by Ion trap in MS/MS scan mode.

Fumarase: The plant material (around 0.1 g FW) was milled on a DNA mill and extracted by extraction buffer (HEPES, Dithiotreithol, Triton X-100). Solution was centrifuged and pure extract was filtered by a 0.2 µm microfilter. 150 µl of filtered extract was added to a reaction mixture (HEPES, $MgCl_2$, KH_2PO_4, NADP, malat dehydrogenase, fumarate). After 45 minutes of reaction absorbance at 340 nm (growth of NADPH) is measured in 50 mm cuvette which is proportional to the amount of fumarase in sample.

Results: ABA only is the good marker of the depth of seeds dormancy and the ability of somatic embryos to germinate. The content of ABA and/or IAA in embryo axis of beech is 10× higher than in cotyledons. ABA decreases during stratification to ½ of the level in dormant embryos; analogous to decrease of ABA content necessary for germination of somatic embryos. Low IAA level fluctuates during stratification of beech embryos as well as in somatic embryos before germination. Fumarase activity correlates especially with the viability of seeds and embryos.

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Keywords: somatic embryogenesis, seeds quality, ABA, IAA, fumarase

S4–9P

Gene expression and proteomic analysis in embryogenic cultures of Brazilian pine (*Araucaria angustifolia*) with different embryogenic potential

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Brazilian pine is a subtropical woody tree species for which traditional seed storage is a problem due to its recalcitrancy. In this sense, survival of naturally occurring population is at risk. Biotechnological tools comprise efficient methods for genetic improvement and germoplasm conservation of woody trees. Among them, somatic embryogenesis is one of the most promising techniques in forest biotechnology, and it has brought a significant progress to the mass production of genetically improved plants in the past two decades. As observed for other conifers, the presence of well-developed early somatic embryos (SE) of Brazilian pine can be considered the pre-requisite for embryo maturation in a medium supplemented with abscisic acid (ABA) and osmotic agents. However, in some genotypes even the presence of bipolar SE does not guarantee embryo maturation. Since SE morphology cannot be used as the only factor for embryogenic culture (EC) selection, the development of molecular markers for early detection of EC responsive to maturation promoters (ABA and osmotic agents) is highly desirable. In order to develop molecular markers for early detection of EC with high embryogenic potential in Brazilian pine, we evaluated the expression of *SERK1* and *PP2C* transcripts by qPCR and carried out a differential proteome analysis of ECs with different maturation capabilities. Concerning gene analysis during the proliferation phase, *SERK1* expression was significantly higher in EC responsive to maturation medium when compared to non-responsive EC. On the contrary, *PP2C* expression did not show vary significantly between EC responsive and non-responsive to maturation treatments. Differential proteome analysis revealed the exclusive presence of proteins related to polyamine metabolism (SAM synthase), protein biosynthesis (elongation factor 2), and energy metabolism associated proteins (mitochondrial ATPase beta subunit and enolase) in EC responsive to maturation medium. In non-responsive EC to maturation promoters, MS/MS analyses displayed the presence of a plant porin (outer membrane protein). Our findings, besides providing a basis for the early selection of ECs with high embryogenic potential, may also become a useful tool in the improvement of a somatic embryogenesis protocol for Brazilian pine.

Financial support: PETROBRAS, CAPES, and FAPESP

Keywords: *Araucaria angustifolia*, gene expression, proteomic analysis, embryogenic potential

S4–10P

Effect of auxins on the induction of somatic embryos from immature zygotic embryonic axes of *Ocotea porosa* (Nees ex Mart.) Barroso

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Ocotea porosa, known as "imbuia", belongs to the *Lauraceae* family and is native to the Mixed Ombrophilous Forest (Araucaria Forest) of Brazil where it was heavily exploited due to the high quality and worldwide value of his hardwood. The sexual propagation of *O. porosa* at its natural occurrence area is difficult, due to the strong tegumentary dormancy and its irregular germination. Moreover, the seed viability is short, for presenting recalcitrant behavior; the seeds have high water content at physiological maturity, rendering difficult their storage. Its vegetative propagation is limited by the low response of cuttings to the induction of adventitious roots. The aim of this study was to test the effect of other auxins (NAA or picloram) beyond 2,4-D, considering that prolonged use of 2,4-D in the induction phase may inhibit the progression of somatic embryos to later stages and cause abnormalities in embryos. Zygotic embryonic axes obtained from immature seeds were used as explants. The seed disinfestation was performed through immersion in ethanol 70% (v/v) for 5 min, followed by 20 min in NaOCl 4% (v/v) supplemented with 0.1% Tween 20®. The seeds were then rinsed five times with sterile water. Immature zygotic embryonic axes were inoculated on WPM culture medium, supplemented with sucrose (20 g L^{-1}), activated charcoal (1.5 g L^{-1}), Vetec® agar (3.5 g L^{-1}) and 2,4-D, NAA or picloram (200 or 400 µM) during 120 days. The cultures were maintained in the dark and the experiments repeated twice. The induction of somatic embryos from immature zygotic embryonic axes occurred in the presence of all auxin types and was observed only after 90 days of culture. The induction and expression of somatic embryogenesis occurred indirectly from embryogenic masses with yellowish white color. The highest percentage of induction and initiation of somatic embryos (30%) was obtained in WPM medium containing 200 µM 2,4-D. In this treatment also the highest mean number per explant of somatic embryos at globular stage (19 embryos) was recorded. In the other treatments, the percentage of induction of somatic embryos varied between 10 and 14% with a mean number of 15 globular embryos per explant. Embryos in cordiform stage were observed only in the medium supplemented with 200 µM picloram. Formation of yellowish, white and black callus occurred in all treatments and the highest percentage of white calli was obtained in the culture medium containing 400 µM picloram. Thus, we conclude that addition of NAA or picloram as well as 2,4-D to culture medium induced somatic embryos formation. It is suggested to carry out further investigations with these auxins, in order to observe its effects on the subsequent development of somatic embryos.

Keywords: Tropical forest species, NAA, picloram, 2,4-D.

S4–11P

Effect of picloram and silver nitrate on callogenesis and embryogenesis induction in *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. thin cell layer culture.

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Acrocomia aculeata, known as "bocaiuva", is a native palm of South America. Its fruits have a great potential for oil production. The quality of this oil is similar to that of olive oil. The oil extraction, moreover, generates by-products, such as the mesocarp cake, rich in soluble fibers of high value, and the kernel bran, that has high proteinic value. Its propagation by seeds is difficult, as germination rate is low. Somatic embryogenesis could be a valuable tool for propagation of *A. aculeata*. Therefore, the purpose of this study was the effect of nitrate silver and picloram on callogenesis and somatic embryogenesis induction from leaf explants. The leaf of 90 day old plants from in vitro culture was sectioned transversally in five TCLs (thin cell layers) from the base and cultured in Petri dishes containing 40 ml of culture medium. The induction culture medium contained Y3 (Eeuwens, 1978) salts, vitamins of Morel and Wetmore, 1.5 g.L^{-1} active charcoal and 500 mg.L^{-1} glutamine, 150 or 300 µM picloram, with or without 1 µM silver nitrate, 30 g.L^{-1} sucrose and 2 g. L^{-1} Gelzan (Sigma). The calluses appeared after 3 weeks of culture in the dark. On medium containing 150 µM picloram without silver nitrate, 68% of the explants developed callus, while on medium containing silver nitrate, this percentage was only 26%. On medium supplemented with 300 µM picloram and 1 µM silver nitrate, callus appeared in 34% of them. Explant oxidation was high when silver nitrate was added to the media. After 12 weeks, calluses were transferred into somatic embryo formation medium. In this step, the active charcoal was reduced to 0.3 g.L^{-1}, picloram to 75 µM, 500 mg.L^{-1} hydrolyzed casein and 25 µM 2-iP were added and silver nitrate was omitted. After 4 weeks, embryogenic calluses appeared and, after 8 weeks, the first clusters of globular somatic embryos in 6% of calluses. They will be further transferred to maturation and conversion medium till entire development. In conclusion, picloram is necessary for callus induction and its concentration must be reduced in the second medium and silver nitrate must be omitted.

Keywords: macaw palm, macauba, bocaiuva, somatic embryogenesis

S4–12P

Molecular Characterization of *Robinia pseudoacacia* L. Varieties in Hungary

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The Black locust (*Robinia pseudoacacia* L.) was introduced from North America and successfully acclimatized in Europe in the 17th century. For today it has been well-known and widely planted throughout the world at the subtropical and temperate climatic areas. Nowadays 23% of the afforested areas of Hungary is wooded by this species.

The *R. pseudoacacia* L. is primarily utilized in the forest industry, but it is very popular also in other areas, like: fuel, food, lumber and feral forage. Beside these it is known also as medical plant.

Our attention has been attracted by the multilateral possibility of usage to this species and we have started to analyze *R. pseudoacacia* L. varieties with molecular markers.

Our purpose is:

- Identifying varieties /clones by using SSR (microsatellite) markers.
- Determining DNA fingerprint of the Hungarian varieties and to create a database from the results.
- Estimating the *R. pseudoacacia* L. variety composition of Hungary's forested territories with SSR markers.

We analyzed 12 varieties of *Robinia pseudoacacia* L. conserved in the Arboretum of Gödöllő, with 13 SSR (Single Sequence Repeat) primers designed for this species.

Based on our results, these SSR primers were enough for genotyping and discriminating the studied varieties.

The next aim of our team is to create a Hungarian *R. pseudoacacia* L. microsatellite database.

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Keywords: acacia, *Robinia pseudoacacia*, SSR markers, molecular analysis

S4–13P

The role of polyamines in embryogenic and organogenic capacity of yellow polar (*Liriodendron tulipifera*)

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In plants, polyamines (PAs) have been implicated in the regulation of developmental processes; cell division, proliferation, morphogenesis, growth and environmental stress responses in and *ex vitro* plants. In addition to a multitude of potential functions of polyamines in plants, it has been suggested that polyamines play a critical role in morphogenesis in plant cell and tissue cultures, especially somatic embryogenesis and organogenesis. In woody plants, recalcitrant characteristic of *in vitro* culture has been often considered as species-specific characteristic because of its difficulty to identify the reasons as it's a consequence of the complex interplay of several physiological factors and the regulation at the transcription level. To approach in this notorious characteristic, we study metabolites, mainly PAs, to investigate its role for embryogenic and organogenic capacity in yellow poplar (*Liriodendron tulipifera*). Embryogenic (EC) and non-embryogenic cells (NEC) of yellow poplar were investigated for its metabolic compositions including PAs. The PAs content were remarkably different from the cell types, the highest levels occurring in the NEC on proliferation medium, when putrescine and spermidine were most abundant. However, the putrescine/spermidine (Put/Spd) ratio was higher in EC of yellow poplar. A comparison of metabolic compositions of NEC and EC using GC/MS identified around 50 compounds, partly displaying significant changes in metabolite levels, e.g., highly elevated levels of xanthosine and methyloxazole in EC compared to NEC. From this analysis, we have identified numerous compounds including PAs involved with embryogenic state. In organogenesis, yellow poplar (YP) with recalcitrant species (YP1 & YP2 – recalcitrant & less recalcitrant) and Italy poplar (IP) with non-recalcitrant one (IP1 & IP2 – less & high regeneration ability) were used. Stem discs cultured on regeneration medium were sampled and the contents of three PAs were analyzed during entire culture period. Total PA contents were always higher in recalcitrant species (YP) and more recalcitrant genotype within the species, and the tendency was similar in the spermidine/spermine (Spd/Spm) ratio. Interestingly the Put/Spd ratio was higher in non-recalcitrant species (IP) and genotype (IP2) than that of YP. These results showed a close relationship between cellular PA levels and their Put/Spd ratio with in vitro regeneration capacity in yellow poplar and suggest that the cellular PAs and Put/Spd ratios are important indicators of regeneration ability in yellow poplar, and morphogenetically poor and recalcitrant species/genotype could be found by investigation of the PAs analysis.

Keywords: yellow poplar, *Liriodendron tulipifera*, embryogenic capacity, regeneration, polyamine

S4–14P

Norway spruce somatic embryogenesis is accompanied by characteristic carbohydrate dynamics irrespective of various exogenous sugar supply

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Somatic embryogenesis, besides its potential to serve in improving plant material and mass propagation of elite genotypes of conifers, represents an excellent tool for studying structural and biochemical changes accompanying embryonal development. Exogenous saccharides together with phytormones are two main medium components that play crucial roles in the process. In plants, saccharides are known to serve as carbon and energy sources, osmotic agents, stress-protectants, and importantly as signal molecules controlling wide range of different processes.

The present study, aimed to gain detail knowledge about the effect of exogenous carbohydrate supply on embryogenic cultures of *Picea abies* (line AFO 541, AFOCEL, France), took advantage of cultivation on rafts floating on liquid medium surface, which enable precise manipulation with carbohydrate availability during embryo maturation. The most significant results are connected with the finding that irrespective of the type of sugar provided from the cultivation medium (sucrose, hexoses or their mixture) the cultures exhibit similar endogenous saccharide dynamics profile with sucrose representing dominant part of carbohydrate spectrum at the end of maturation. This finding indicates strict regulation of carbohydrate metabolism accompanying regular structural embryo development. However, the exchange of solid for liquid media itself induced the considerable differences in other embryo characteristics. The use of liquid media resulted in bigger embryos that often exhibited hyperhydric appearance and overall lower embryo yields.

The presented data clearly show that somatic embryogenesis of Norway spruce proved to be a very delicate process substantially reacting to changes in cultivation conditions including type of medium (solid or liquid) and medium composition. Arrangement leading to enhanced sucrose availability (a frequent exchange of cultivation medium preventing sucrose cleavage) has positive effect on somatic embryo yield. Importantly, the endogenous carbohydrate dynamics pattern stability under broad spectrum of cultivation conditions indicates carbohydrate status to be a robust characteristic of regularly developing somatic embryos of Norway spruce.

This work supported by the Ministry of Education, Youth and Sports of the Czech Republic (Grants MSM 0021620858 and GAUK 656512).

Keywords: hexoses, liquid media, membrane rafts, *Picea abies*, sucrose

S4–15P

Intersectional hybridization in poplar – new ideas for an old problem

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Intersectional hybridization in poplar is an old idea, but it failed in cases involving members of section *Populus* (former *Leuce*). These findings were fixed and reported in a scheme by Zufa (1975). With the need to create new material appropriate for biomass production under changing climatic conditions, hybrid poplars again came into the focus of breeders. Based on the fact that parts of Germany will have drier conditions in the near future and that the availability of very fertile soils is limited, poplars of the section *Populus* are being discussed. The disadvantage of this section is the limited ability of vegetative propagation (poor rooting of cuttings) as well as the limited growth compared with poplars of the sections *Tacamahaca* and *Aigeiros*. Again, the idea appeared to combine the characteristics of different sections. Although it seemed impossible, there were some indications for real chances in the literature (Ronald 1982). Some controlled crosses were carried out based on these conclusions and on the existence of biotechnological methods,. In most cases, the early stage zygotic embryos were extracted and germinated in vitro. Micropropagation was carried out for the first multiplication of the material. With the help of such methods, it was possible to combine hybrid aspen (*P. tremula* × *P. tremuloides*) with *P. deltoides* and with *P. pseudosimonii*. Molecular markers (nuclear microsatellites) were used to describe and confirm the crossing success.

Some such hybrids showed normal growth behaviour. The first experiments concerning the improvement of rooting cuttings were successful. Further tests will inform about the growth, resistance behaviour and rooting ability of the hybrids as well.

Keywords: *Populus* spec., controlled crossing, embryo rescue

Excursion guide for experimental plot

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Vegetative propagation of forest tree species – poplar, aspen, oak

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Experimental plots were established at the locality Kostelany in south-east part of the Czech Republic (GPS 49°02'71.658"N, 17°25'32.206"E , elevation 176 m a. s. l.) along the Morava river in 2003. Soil type is fluvisol with good water supply.

The site was prepared with a single pretreatment cultivation with plow, disc and rotary tiller. One-year-old rooted plants were planted, each clone in three repetitions. Weed control was applied without herbicides with a disc and rotary tiller twice during each growing season. Individual plants were first hoed and then grass was only mowed.

Micropropagation of aspen and hybrid aspen

Populus tremula × *P. tremula*, *P. tremula* × *P. tremuloides*
Research was aimed to verify the possibility to regenerate aspen mature trees by means of micropropagation. The primary cultures were founded by 24 years old trees from the progeny test or from the clone archive. Dormant buds were sampled from the selected trees in spring and autumn. The extirpated full-grown tops were sterilized and put on the nutrient medium. MS medium with higher concentration of BAP (1.0 mg/l) and IBA (0.1 mg/l) shows to be suitable for induction of organogenesis. MS medium with lower concentration of BAP (0.2 mg/l) and higher concentration of glutamine (100 mg/l) in agar medium was used for multiplication. The high number of adventitious shoots (20–30) occurred in one multi-apex culture. The losses were minimal, around 2%, during rooting and acclimatization occurred. The plantlets were growing on the outside bed of the experimental nursery.

Research plot was established in autumn 2004 with 20 clones of *P. tremula* × *P. tremuloides* and 10 clones of *Populus tremula* × *P. tremula*. Clones were selected in progeny test in Ore Mountains which was established with progenies from hybridization programme of hybrid aspen.

Vegetative propagation by woody cuttings

P. alba, P. alba × *P. grandidentata, P. tremula* × *P. alba, P.* × *canescens*
Research plot was established in spring 2003 with 24 clones of *P. tremula* × *P. grandidentata* and 3 clones of *Populus alba* × *P. tremula* improved in breeding programme. *Populus alba* and *Populus* × *canescens* clones were found in forest stands and taken to clonal archive in research station. All tested clones were propagated by hard woody cuttings in greenhouse and α-naphtalenacetic acid as growing stimulator was used.

Research is aimed to test growth and select clones with the highest yield for forestry.

P. × *euroamericana, P. deltoides*
Research plot was established in spring 2003 with 28 clones of *P.* × *euroamericana* and 4 clones of *Populus deltoides*. Spacing of trees was 4 × 4 m. A set of hybrid clones was derived from 17 clones newly bred in FGMRI in the Czech Republic, 11 well-known cultivars of *Populus* × *euroamericana*

planted in Europe and imported to the Czech Republic, two standard cultivars ´I – 214´ and ´Robusta´ (*Populus* × *euroamericana*) were used.

Growth and timber production of poplar clones were evaluated at the age of 6 years. Stem diameters, heights of trees, and stem volume production were evaluated. In the group of candidate clones with estimated mean stem volume higher than that of standard registered cultivar ´I-214´ (*P.* × *euroamericana*) were ranked clones P-798 (*P. deltoides*), P-781 (*P.* × *euroamericana* ´Koltay´), P-447 (*P.* × *euroamericana* ´CZ-144´) originated from open pollination and P-789 (*P. deltoides*) maintained in Czech poplar germplasm collection. The cultivar ´I-214´ was included into this group. Mean DBH of the top five clones varied within a range 16.50 – 19.86 cm, mean tree height within a range 13.87–15.29 m, mean annual increment 2.30–2.65 m, mean stem volume 0.121–0.196 m^3, estimated standing volume 76–121 m^3. ha^{-1}.

In the group of perspective clones were ranked clones comparable with cultivar ´I-214´ which can promise high yield during next growing period, their growth reached more than 90% of growth of the standard cultivar ´I-214´. Results attained for clone ´Pannonia´ were near under that of cultivar ´I-214´. Clonal ranking was based on comparison of diameters and stem volumes. In this group were presented five new clones from breeding programme (four clones from progenies *P. angulata* ´Törökfay´ × *nigra*, one clone from open pollination *P. angulata* ´Törökfay´ × wind) and three registered cultivars of *P.* × *euroamericana* (´Blanc du Poitou´, ´I-154´, ´Boccalari´). It is supposed that growth of 8 perspective clones with stem volume 0.098–0.119 m^3 will significantly increase during next 5 years but clones with stem volume less than 0.667 m^3 will be eliminated from the next evaluation.

According to results, it was concluded that yield of four clones was higher than that of standard cultivar ´I – 214´. Standing volume of two P. deltoides clones and one new Czech *P.* × *euroamericana* clone varied within a range 77–121 m^3. ha^{-1}.

Research is aimed to test growth and pest resistance and select clones with the highest yield for forestry.

Salix alba, Salix × *rubens*

Research plot was established in spring 2005 with 39 clones of *Salix alba* including 4 clones of *Salix* × *rubens*. A set of clones was derived from clones founded in forest stands from mature trees and 12 clones were selected in progenies planted from seeds of open-pollination origin.

Research is aimed to test growth and select well-shaped clones with the highest yield for forestry.

Vegetative propagation by soft cuttings

Quercus robur

Research was aimed to verify the possibility to regenerate pedunculate oak by means of simple vegetative propagation. All tested clones were propagated by soft cuttings sampled from fresh shoots in June. Cuttings were rooted in greenhouse and indole-3-butyric acid as growing stimulator was used. The losses were different between clones, approximately 33–65%. Well rooted clones are suitable for forest nurseries in case of lack of seeds. Tested methodology can be used in each type of greenhouse.

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Appendix 1

PRACTICAL INFORMATION

Prepared by Martina Šatinská and Jana Levová

Time zone and time

The 24-hour clock is generally used in the Czech Republic in printed materials and on digital clocks. The 12-hour clock is also used when speaking colloquially or in relation to analogue clocks.

The week starts on Monday and ends on Sunday. Saturday and Sunday are not working days.

The time zone GMT (UTC) +1 applies throughout the territory of the whole Czech Republic, i.e. CET (+0). Winter and summer time are used in the Czech Republic. The clocks go forward on the last Sunday in March at 2:00 CET to 3:00 CEST. The clocks then go back on the last Sunday in October from 3:00 CEST to 2:00 CET. Summer time (an hour more) thus applies here from roughly April to October.

Climate and weather

This landlocked country in the centre of Europe does not abound in extremes. The climate is moderate with four seasons. People ski in the mountains in winter and the hot summer is excellent for bathing.

Weather forecast on:
http://www.czechtourism.com/weather.aspx?lang=en-GB&selectedculture=en-GB

Currency

Although the Czech Republic is a member of the EU, it is not so far a member of the Eurozone and for this reason, the Euro is not the official currency here (yet despite this it is possible to exchange Euro for Czech crowns without any problems).

The official currency is called the crown, which is made up of 100 hellers. Small coins start at 1 crown coin, followed by the 2 crown coin, 5 crown coin, 10 crown coin, 20 crown coin and 50 crown coin. Banknotes begin with the 100 crown note, followed by the 200 crown note, 500 crown note, 1,000 crown note, 2,000 crown note and 5,000 crown note.

You can find out the crown exchange rate from the European Central Bank

Exchanging money

There are three basic methods of changing money:

- banks – they have a good exchange rate, but are not usually open in the evening or at weekends,
- hotels – they have worse exchange rates, but smaller amounts in euro are not usually a problem almost any time of the day or night
- bureaux de change – there are relatively large differences between them. For example, some bureaux de change do not charge a fee for the exchange, but have a worse exchange rate. The best idea is to first ask how much money you will get and calculate the actual exchange rate yourself.

If you have an international payment card, you can of course pay directly using this or withdraw cash from a bank machine.

Payments

Payment cards are regularly accepted in shops and also in some restaurants in large cities. Traveller's cheques issued by internationally acknowledged companies are mostly accepted by Czech banks without any problems.

Tips

It is usual to leave a tip in restaurants – especially as an expression of your satisfaction with the services of the establishment. A member of staff usually brings the bill and leaves. When he or she returns, it is up to you to say how much you actually want to pay. Another option is to pay the precise amount and to leave the tip on the table. Tips are usually left at the level of roughly 10 percent of the bill.

Opening hours

Shops

In smaller towns or smaller shops, opening hours are usually Monday to Friday from 8 or 9 am until 6 pm. Shops are usually only open in the morning on Saturday. In larger cities, shops are usually open later, for example until 8 or 9 pm. The smallest shops very rarely have a break for lunch and if so, usually around 12 noon to 1 pm.

Hypermarkets, shopping centres and similar establishments usually have long opening hours, for example 7 days a week until 9 pm. Some larger shops operate almost nonstop (other than the early hours of the morning).

There are also small shops which are called "večerka", which are open until late at night and often at weekends. However their disadvantage is their higher prices than in other shops and a limited choice of goods.

Banks

Banks are regularly open on weekdays during working hours. Some banks are open later, for example until 8 pm. Busy branches in the city centres are usually open longer. Access to cash machines is ensured 24 hours a day.

Authorities

Monday and Wednesday are standard office days for authorities, from 9 am until 5 pm. You will always find somebody at the authority on these days. Some authorities provide services for the public on other days too. Offices are usually closed over lunch (often 12:00 noon – 1:00 pm).

Post Offices

Post Offices are regularly open from 8 am to 7 pm. There is a Post Office on Jindřiška in Prague, which is open almost nonstop.

Restaurants

Restaurants, pubs or cafes are often open from late afternoon until late at night, often until 11:00 pm. During the summer, restaurants open gardens for you to sit outside, which are usually open until 10:00 pm (due to regulations relating to a ban on loud noise at night, which usually lasts until 6 am). Bars or clubs are often open even long after midnight, especially at weekends.

Telephoning

The GSM network works on frequencies of 900 MHz and 1800 MHz. If you are taking your mobile phone with you, make sure that it is able to work on these frequencies.

The international dialling code for the Czech Republic is +420 (00420).

Operators offer prepaid telephone cards which can even be purchased in large hypermarkets (often at the checkout). Be careful to check whether your telephone can only be used for one operator (a so-called blocked device).

Calling to the telephone info lines of various companies and government institutions in the Czech Republic, which start with 800, is free.

If you send an SMS, by means of which you wish to pay for a service, the price of the service is generally the last two numbers. You can for example even buy a ticket for public transport in Prague by SMS. If you do not have a mobile phone with you, you can call from one of the telephone booths which can generally be found on squares, at stations, in Post Offices and similar. You will need small change or a prepaid card to use these.

Electricity network

The electricity network in the Czech Republic has a voltage of 230 V and frequency of 50 Hz. Plug sockets have two round holes and one round pin. If for example you have a universal recharger, all you will need is a simple connector with your system and with the Czech system on the other end. If your appliance works on another voltage or frequency, you will need a more complicated adapter. This can easily be purchased in the Czech Republic or borrowed in a hotel.

Internet

Technology for broadband connection to the Internet is widespread in the Czech Republic. You can use all regular technical connection standards.

Wi-Fi

Wireless connection via Wi-Fi is commonly used. You can easily connect up with a netbook, notebook or smartphone via Wi-Fi in restaurants, cafes, hotels and in many other locations.

Broadband, ADSL, fixed connection

Companies, households and practically all hotels in the Czech Republic commonly have a fast fixed connection available.

Mobile technology

You can also connect to the Internet in the Czech Republic with the aid of mobile technologies. Most large cities are covered with a signal and the level of coverage is gradually increasing. The following technologies are available: UMTS (3G, 4G), EDGE, GPRS and CDMA.

Internet if you do not have a computer or smartphone

Several cafes offer connection to the Internet for a fee, mostly by the hour. Shopping centres sometimes have gaming centres where you can connect up to the Internet. Connections can also be founds in public libraries. Almost all hotels have a computer with Internet connection.

Services on the Internet

You can easily do the following over the Internet in the Czech Republic:

- purchase goods
- reserve tickets to cultural events
- search for travel connections
- communicate with the authorities

A few tips for Czech websites

- local search engines: Google.cz, Seznam.cz, Centrum.cz
- ticket reservation: TicketPro, TicketPortal, TicketStream
- transport: IDOS.cz
- authorities: Ministry of Foreign Affairs of the Czech Republic

Moving around the city

Large cities have carefully designed tram, bus, trolleybus or metro routes in terms of the local public transport company. You can regularly purchase individual tickets for individual journeys, but if you are staying for longer, be sure to buy the more advantageous day tickets, two-day tickets or week tickets etc.

You will avoid needless problems in the metro, tram or bus if you have purchased a ticket and stamped it. In the case of the metro, you stamp the ticket when entering by inserting it into the stamping machine. In other means of public transport, these machines are located throughout the whole vehicle.

You can use taxi services all over the country, but it is better to use the services of larger renowned companies and before setting off, ask for a rough final price.

BRNO TAXI IMPULS: call 14014 (*http://www.taxi-impuls.cz/en/*)

Important telephone numbers

You should have certain telephone numbers with you at all times or know them by heart. The numbers of the most important institutions, which you might need, are mostly three digits. You can get through to these wherever you are at any time free of charge.

112 emergency calls (this number works throughout the whole of Europe and includes universal medical aid, the police and the fire brigade – but it need not necessarily work on older mobile telephones without SIM cards)

- **Fire Brigade 150**
- **Medical First Aid 155**
- **Police 158**
- **Municipal Police 156** (they have limited authority and resolve smaller, local problems)

Embassies

In the event of any problems or in complicated situations, you can get help from your country's embassy. If your country does not have any representation or an embassy here, contact the consulate. A list of embassies and consulates can be found on the following website: Ministry of Foreign Affairs of the Czech Republic

The health service

State-run and private medical facilities exist in the Czech Republic. Most of them have concluded a contract with an insurance company on provision and settlement of costs for healthcare and only provide insured patients with the essential care subject to settlement of the excess as stipulated by law – this relates to EU citizens who have a European health insurance card.

First aid, emergency and rescue service

Sudden illness, accidents and similar can be resolved using the emergency services. These are open as special departments in hospitals. In very serious cases, it is possible to call the rescue service on the emergency number 112.

Pharmacies

Medicines to relieve flu or a cold and similar preparations can be purchased without a prescription. A prescription from a doctor is required to purchase other medicines.

How to get to Hotel Barceló Brno Palace

The hotel is situated in the city center. Follow the indicators to "Barceló Brno Palace hotel" from all main directions.
• from Prague (Capital of Czech Rep.) – D1 exit 190
• from Vienna (Austria) – E4
• from Bratislava (Slovak Rep.) – D2 exit 194 AB

By public transport from Airport Tuřany (Brno) to Main Railway station:
Take bus line N° 76 (direction – Hlavní nádraží) from the stop Letiště Tuřany (airport Tuřany) to the stop Hlavní nádraží (10th stop).

By public transport from Bus Station at Hotel Grand (lines of STUDENT AGENCY) and from Main Railway Station:
Take tram line N° 12 or 13 (direction – Technologický park) from the stop Main railway station (Hlavní nádraží) to the stop Šilingrovo náměstí/square (2nd stop). The hotel is right next to the tram stop.

By public transport from Main Bus Station (Zvonařka):
Take tram line N° 12 (direction – Technologický park) from the stop Main bus station (Zvonařka) to the stop Šilingrovo náměstí/square (4th stop). The hotel is right next to the tram stop.

Tickets for Brno City Transport:
All tickets for Brno City Transport can be used for both tram, bus and trolleybus. Tickets are available in kiosks and in automatic ticket machine. A transfer ticket allows you to change all tram, bus and trolleybus lines in Brno as you like.
The validation of these tickets is either 15 (20 CZK) or 60 minutes (25 CZK).

Recommended restaurants

Restaurant La Bouchée
Údolní 33, 602 00 Brno
Monday – Saturday: 11a.m. –11 p.m., Sunday: 11 a.m. – 10 p.m.
+ 420 542 212 56

KOISHI – fish and sushi restaurant Brno
Údolní 11, 602 00 Brno
Monday – Friday 11 am – 11 pm, Saturday and Sunday: 9 am – 11 pm
+420 777564744

Restaurant Hansen, Municipal House
Komenského náměstí 8, 602 00 Brno
Mo–Thu: 11–22 h, Fri–Sat: 11–23 h, Sun: 11–22 h
+ 420 737 364 000

Borgo Agnese Restaurant
Kopečná 43 Street, 602 00 Brno
Monday to Saturday 12–24 h, Sundays are closed
+420 515 537 500

Il Mercato
Zelný trh 2, 602 00 Brno
new restaurant close to the hotel
+420 542 212 156

Baroko
Orlí 469/17, 602 00 Brno
11:00 – 23:00 (Mon –Tue), 11:00 – 00:00 (Wed – Thu), 11:00 – 01:00 (Fri), 11:00 – 00:00 (Sat), Closed (Sun)
+420 544 213 845

Mazaný Anděl
Šilingrovo nám. 4/5, 602 00 Brno
+420 724 984 167

Restaurant Pod Radničním kolem
Mečová 5, Brno
Monday - Saturday 11.00 - 24.00 , Sunday 11.00 - 22.00
+420-542211135

Restaurant císaře Leopolda
Galerie Orlí 3 602 00 Brno
Mon - Thu 11:00 AM - 11:00 PM, Fri - Sat 11:00 AM - 12:00 PM, Sunday 12:00 AM - 10:00 PM
+420 542 516 606

Café Onyx
Zámečnická 87/1, 602 00 Brno
Mon -Thu: 8:00–22:00, Fri: 8:00–0:00, Sat: 9:00–22:00, Sun: 11:00– 22:00
+420 542 211 406

Pizza Coloseum
Dominikánská 3, (Velký Špalíček), 602 00 Brno-město
Sun-Thu: 11:00 - 23:00, Fri-Sat: 11:00 - 23:30
+420 543 237 318

Ristorante Piazza
OC OMEGA nám. Svobody, Brno
Mon-Sat: 11 – 24, Sun: 12 - 23
+420 542 211 731

Středověká krčma
Česká 6, 602 00 Brno
daily from 11:00 to (until the last guest leaves)
+420 542 21 00 21

Stopkova plzeňská pivnice
Česká 5, Brno, 602 00
Mon - Sun : 11:00 - 24:00
+420 517 070 080

Enjoy your stay in Brno!!!

TAXI IMPULS BRNO: call 14014 or 542 216 666, LIDO TAXI BRNO: call 542 214 221

Appendix 2

SOCIAL PROGRAMME

Prepared by Martina Šatinská

Brno Sightseeing Tour

Sunday 24th June / 15:30–18:00

There will be the possibility to join the guided tour of the city on the arrival day. The tour will start by the Hotel Barceló Brno Palace and will bring you to St. Peter and Paul Cathedral, Cabbage Market, Špilberk Castle, Old Brno Abbey and Mendel Museum of Genetics (*http://www.mendelmuseum.muni.cz/en/*). The best way to learn about the city.
DRESS CODE: comfortable

Welcome Drink

Sunday 24th June / 19:00

The Welcome Drink will be offered .....
DRESS CODE:

Conference GALA DINNER

Monday 25th June / 18:30–22:30, Hotel Barceló Brno Palace

The Hotel Barceló Brno Palace, Šilingrovo nám. 2, Brno, Telephone number: +420 532 156 777
The Conference Gala Dinner will take place in the Hotel Barceló Brno Palace (*http://www.barcelo.com/BarceloHotels/en_GB/hotels/Czech-Republic/Brno/hotel-barcelo-brno-palace/practical-information.aspx*) built in the middle of the 19th century in the historic and commercial centre of the city. It is next to St. Peter and Paul Cathedral with the views of the Špilberk Castle. The Brno Palace is an old Jewish palace, which has undergone full restoration and been transformed into a hotel. During the evening the honored researchers will receive the Medals of the Mendel University.
DRESS CODE: evening wear

Social Dinner Černá Hora

Tuesday 26th June / 18:30–22:30, The Černá Hora Brewery

Before coming to the famous local Černá Hora Brewery the delegates will have the opportunity to visit Botanical Garden of Mendel University in Brno and Arboretum Křtiny (passing by the beautiful baroque church in Křtiny, the old pilgrimage place built by Santini) *http://en.wikipedia.org/wiki/K%C5%99tiny_(Blansko_District)* and also to enjoy the fantastic accoustics of the Sloupsko-sosuvske Caves (*http://www.cavemk.cz/sloupsko-sosuvske-caves*) in the Moravian Karst. The Moravian Karst is the most famous and best developed Karst area in the Czech Republic. It is located to the north of Brno and it is created from Devon limestone on the area of 100 km^2. There are more than 1100 caves, only five of them are open to public. The short visit to the Sloupsko-sosuvske Caves (about 30 minutes) will introduce the entrance part of the cave with the beautiful decoration and accoustics. Coming to Černá Hora Brewery the delegates will start with the dinner served together with 3 different kinds of beer (beer taster session) and shortly visit the brewery (*http://www.pivovarcernahora.eu/*). The Social Dinner will be offered by Mendel University in Hotel Sladovna Černá Hora.
DRESS CODE: comfortable (pullover and waterproof jacket to the cave, good shoes)

Social Dinner Uherské Hradiště

Thursday 28th June / 18:00–23:00

This trip will be directed in very interesting part of South Moravia passing by Austerlitz Battlefield – "The Battle of Three Emperors Slavkov/Austerlitz 1805" (*http://en.wikipedia.org/wiki/Battle_of_Austerlitz*). Vitit of the open-air Strážnice Museum of the Villages of South-east Moravia. The museum is very unique, besides the presentation of folk architecture and the way people used to live in the particular regions, it also focuses on the viticulture and wine growing that has been typical for the region for ages. The exposition of a vineyard demonstrates the history and the development of wine growing from the past until the present time. It is the only exposition of its kind in the Czech Republic (*http://skanzen.nulk.cz/*). Excursion to experimental plot in Kunovice. Dinner will be offered in "Pension U konicka" in Uherské Hradiště. There will be wine tester session and during the evening the traditional cimbalom music will entertain us.
DRESS CODE: comfortable, raincoats or umbrellas

Tour to Prague

Friday 29th June / 7:00 bus departure

The full day optional excursion to Prague, the capital of the Czech Republic. Visit of the Institute of Experimental Botany and guided sightseeing tour by walk. Prague Castle, St. Vitus Cathedral, Strahov Monastry, Charles Bridge, Old Town Square etc.
DRESS CODE: comfortable