Department of Forest Sciences, Department of Agricultural Sciences
Faculty of Agriculture and Forestry
and
Viikki Graduate School in Molecular Biosciences
University of Helsinki

The effects of abiotic and biotic factors on somatic embryogenesis and seedlings of *Pinus sylvestris* (L.)

Jinrong Lu

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in lecture room B2, B-building (Latokartanonkaari 7), on April1 5th, 2011, at 12 noon.

Helsinki 2011

Supervisors: Professor Fred O. Asiegbu
Department of Forest Sciences
University of Helsinki, Helsinki

Professor Ari Pappinen
Faculty of Science and Forestry
University of Eastern Finland, Joensuu

Pre-examiners: Docent Karoliina Niemi
The Central Union of Agricultural Producers and Forest Owners (MTK)
Helsinki, Finland

Docent Malin Elfstrand
Department of Forest Mycology & Pathology
Swedish University of Agricultural Sciences

Opponent: Adjunct professor Yill Sung Park
Candian Wood Fibre Centre
PO Box 4000, Fredericton, New Brunswick
E3B 5P7 CANADA

Cover: Somatic embryos of Socts pine at the prematuring, maturing and germinating stage.

ISSN 1795-7079
ISBN 978-952-10-6824-9 (paperback)
ISBN 978-952-10-6825-6 (PDF)
http://ethesis.helsinki.fi

Helsinki University Printing House
Helsinki 2011

TABLE OF CONTENTS

ABBREVIATIONS USED

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
AdoMet	S-adenosylmethionine
AFAP	actin filament-associated protein
AOA	amino-oxyacetic acid
AspRS	aspartyl-tRNA synthetase
BAP	6-benzylaminopurine
BLAST	basic logical alignment search tool
cDNA	complementary deoxyribonucleic acid
DCR	Douglas-fir cotyledon revised medium
ECM	ectomycorrhiza / ectomycorrhizal
ECs	embryogenic cultures
EF-1A	elongation factor 1 alpha
EF-Tu	elongation factor thermo unstable
HBK3	homeobox of knox3
JA	jasmonic acid
LEA	late embryogenesis abundant protein
LP	von Arnold and Eriksson medium
MAMP	microbe-associated molecular patterns
MS	Murashige and Skoog medium
NAA	α-naphthaleneacetic acid
NCBI	national center for biotechnology
P6	*Pinus taeda* basal medium
PAMP	pathogen-associated molecular patterns
PAs	polyamines
PDT	partial drying treatment
PEG	polyethylene glycol (4000)
PGR	plant growth regulator
PRRs	pattern-recognition receptors

PUT	diamine putrescine
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SBH1	*Suillus bovinus* isolate1 in Helsinki
SE	somatic embryogenesis
Spd	spermidine
Spm	spermine
tRNA	transfer RNA

LIST OF PUBLICATIONS

The thesis is based on the following publications, which will be referred to in the text with their Roman numerals (I-IV).

I. Niskanen AM, **Lu J,** Seitz S, Keinonen K, Pappinen A, von Weissenberg K (2004). Effect of parent genotype on somatic embryogenesis in Scots pine (*Pinus sylvestris* L.). Tree Physiology 24:1259–1265.

II. **Lu J,** Vahala J, Pappinen A (2011). Involvement of ethylene in somatic embryogenesis in Scots pine (*Pinus sylvestris* L.) (Plant Cell, Tissue and Organ Culture; Manuscript, Accepted).

III. **Lu J**, Aronen T, Pappinen A, Asiegbu FO (2010). Response of somatic embryos of Scots pine to fungal cell wall elicitors. Forest Pathology. doi: 10.1111/j.1439-0329.2010.00641.x

IV. **Lu J**, Pappinen A, Timonen S (2011). Scots pine seeding-microbe interactions in the absence of physical contact (Manuscript, Submitted).

ABSTRACT

Somatic embryogenesis (SE) is an asexual form of plant propagation that occurs in nature and mimics many of the events of sexual reproduction. *Pinus sylvestris* (L.) is an important source of timber in Northern Eurasia but it is recalcitrant to somatic embryogenesis. Several factors important for the success of the *P. sylvestris* embryogenic cultures have not been thoroughly investigated. In this study, we examined the effects of parental genotypes on the SE in *P. sylvestris*, the involvement of the gaseous plant growth regulator, ethylene in SE, and also biotic effects on somatic embryos as well as on seedlings. We tested parental effects on immature embryo initiation for different media, storage periods, and on the maturation process. Maternal effects were found to be crucial for SE in the absence of paternal effects. No maternal-paternal interaction was observed at any stage of somatic embryo production. Additionally the role of ethylene at different developmental stages of SE was investigated. Two ACC synthase genes, *PsACS1* and *PsACS2*, were isolated and characterized. *PsACS1* was expressed during the proliferation stage in all tested genotypes, whereas *PsACS2* was only expressed in somatic embryos of each genotype. Ethylene production in embryos at stage 3 was significantly higher than the other stages. In a parallel study, the response of somatic embryos to fungal elicitors was investigated. Three fungi, a mutualistic ectomycorrhizal (ECM) fungus (*Suillus bovinus)*, a weak Scots pine pathogen (*Heterobasidion parviporum*) and a strong pathogen (*H. annosum*) were used. The gene expression patterns for embryos exposed to the *H. parviporum* elicitor were found to be similar to that documented for *S. bovinus* among the tested genes. By contrast somatic embryos exposed to the *H. annosum* elicitor had a different pattern of regulation which was marked by a delayed response, and in some cases death of the embryos. Furthermore, interaction without direct contact between *P. sylvestris* seedlings and microbes (mutualistic and pathogenic fungus, cyanobacterium) were investigated. Several novel genes expressed in seedlings treated with ECM fungus were isolated which suggested that physical contact is not necessary for elicitation of host responses.

The results suggest that somatic embryos and seedlings of *P. sylvestris* are genetically well equipped to respond to fungal elicitor/exudates and could serve as a suitable model for reproducible molecular studies in conifer tree patho- and symbiotic systems.

1. INTRODUCTION

1.1. Somatic embryogenesis-a potential *in vitro* propagation method for conifers

1.1.1. Development of somatic embryogenesis in conifers

Clonal propagation is a technique used for producing large amount of genetically identical individuals by means of organ, tissue or cell culture (Nehra *et al.,* 2005, Bonga *et al.,* 2010). Clonal propagation methods include somatic embryogenesis (SE), micropropagation and macropropagation. The main difference is in the tissue explants used. Macropropagation involves mainly the rooting of cuttings, whereas micropropagation is based on the induction of either pre-existing meristems (auxiliary buds) or adventitious buds (Nehra *et al.,* 2005). However, the age of donor plant has been the obstacle preventing its wide application in conifers (Park 2002). This is a serious limitation for forest trees because, by the time the genetic superiority of a clone has been genetically tested and determined, the donor plant would have been too old to be used for mass propagation (Park 2002). Somatic embryogenesis is an asexual form of plant propagation, which mimics many of the events of sexual reproduction in nature (Santacruz-Ruvalcaba *et al.,* 1998). The application of SE technology makes it possible to circumvent the age problem, at least for some conifer species (Klimaszewska & Cyr 2002, Nehra *et al.,* 2005, Bonga *et al.*, 2010).

Since the early descriptions of the process of SE in carrot (Steward *et al.,* 1958) and the „totipotency' theory by Schwann and Schleiden (Gautheret 1983), somatic embryo formation has been achieved for a variety of plant species, including angiosperms and gymnosperms (Breton *et al.*, 2004, Park *et al*, 2006, Szczygiel *et al*. 2007, Aronen *et al*. 2009). The theoretical basis is that cells are capable of regeneration to give a whole plant. Plant tissue culture techniques developed rapidly after the discovery of plant growth regulators (PGRs). Unlike flowering plants, regeneration through SE in conifers is a more difficult process as many species are recalcitrant to *in vitro* conditions (Bonga *et al.,* 2010). The first hints of SE in gymnosperms occurred in the late 1970s and early 1980s with the descriptions of embryolike structure in *Pinus banksiana* (Durzan & Chalupa 1976). These structures were unable to undergo further development. However, it was only a few years later that the mature somatic embryos were converted into plantlets from immature embryos of *Picea abies* (Chalupa 1985, Hakman *et al.,* 1985). In the last two decades, the technology of conifer somatic embryogenesis has developed quickly due to its potential impact on the forest

industry. Somatic embryogenesis has been successfully induced in many coniferous species (Attree & Fowke, 1993, Stasolla *et al.,* 2002, Stasolla & Yeung 2003, Park *et al.*, 2006, Szczygiel *et al*., 2007, Aronen *et al*., 2009, Pullman *et al.,* 2009, Bonga *et al.,* 2010). Since SE has a vast potential for propagating large-scale plant production, a number of conifer species such as lobloly pine (*Pinus taeda*) Monterrey pine (*Pinus radiata*) and Norway spruce (*P. abies*) have been commercialized to provide the plant materials with desired characteristics and breeding goals (Bozhkov & von Arnold 1998, Pullman *et al*., 2003b, Aquea *et al.,* 2008). Besides, SE also could be used for conserving endangered plant species (Maruyama *et al.,* 2007).

The most important advantage of conifer propagation by SE is that the embryogenic tissue can be cryopreserved, in liquid nitrogen indefinitely without changing its genetic characters (Ford *et al.,* 2000a, Ford *et al.,* 2000b, Häggman *et al.,* 2000), while the corresponding trees are tested in the field. This advantage offers great flexibility in genetic studies to propagate desired genotypes consistently at any time (Häggman *et al.,* 2000).

1.1.2. Four main phases of the somatic embryogenesis and conditions

The establishment and regeneration of somatic embryos is successfully achieved through a continuous series of developmental steps including: the initiation and proliferation of embryogenic tissue, embryo maturation and germination.

Initiation and proliferation of embryogenic cultures

In coniferous species, asymmetric cell divisions within the embryogenic tissue mark the initiation of somatic embryo formation. The two cells originating from such divisions are morphologically distinct; one is small and densely cytoplasmic, whereas the other is larger and vacuolated. The densely cytoplasmic cell will develop into the embryo proper, whereas the vacuolated cell will form the suspensor (Nagmani *et al.,* 1995). Initiation of embryogenic tissues in coniferous species is generally achieved from juvenile tissues, such as immature or mature zygotic embryos, cotyledons or the entire female gametophyte.The most successful explants used for pine are precotyledonary immature and cotyledonary mature embryos (Gupta & Durzan, 1986b, Keinonen-Mettälä *et al*., 1996, Pullman *et al.,* 2009). In addition, megagametophytes, young seedlings, and re-induced cotyledon somatic embryos have been

used to initiate somatic embryogenesis in different coniferous species (Becwar *et al.,* 1990, Lelu & Bornman 1990, Malabadi &VanStaden, 2005).

In SE of pine, several basal media are often used (modified Murashige and Skoog medium (MS), LP [AE], Douglas-fir cotyledon revised medium (DCR), and *P. taeda* basal medium (P6)) (Murashige & Skoog 1962, von Arnold & Eriksson 1981, Chalupa 1985, Gupta & Grob 1995) with low concentrations of carbohydrates, auxin (2,4-dichlorophenoxyacetic acid (2,4-D) or a-naphthaleneacetic acid (NAA)) and cytokinin (BAP). Other factors including pH, nitrogen level and light regime can also affect the induction process (Pullman *et al.*, 2002). Moreover, different genotypes of the same species may require different culture conditions (Becwar & Pullman 1995, Lipavska & Konradova 2004).

Somatic embryogenesis begins with a plant embryo excised from a seed and incubated on the initiation medium in darkness. Embryogenic cultures began to grow from the micropylar end of the megagametophyte with a unique white, fluffy and translucent appearance (Becwar & Pullman 1995). This is the initiation phase of SE and as long as the embryogenic tissue is maintained on the initiation medium, it will continue to proliferate indefinitely. During the early filamentous stage, embryo proper cells and suspensor cells develop continuously on the proliferation medium (Keinonen-Mettälä *et al.,* 1996). Maintenance of embryogenic tissues can occur in a liquid or on solid medium with the same composition as the induction medium (Gupta & Durzan 1987, Tautorus *et al.,* 1991). At this stage, ECs could be cryopreserved in liquid nitrogen (Ford *et al.,* 2000a, Häggman *et al.,* 2000) and also could be used to continue the somatic embryogenesis process - maturation.

Somatic embryo maturation

Upon transfer onto the maturation medium, the embryo increases in size with a more globular head, and attains an elongated filamentous shape. Maturation stage could be divided into five according to the differentiation of cotyledon. Stage 1 refers to small embryos consisting of an embryonic region of small, densely cytoplasmic cells subtended by a suspensor. Stage 2, embryos have a prominent embryonic region that becomes more opaque. Stage 3 represents somatic embryos in which the cotyledons are visible and characterized by distinct shoot and root apical meristems. Stage 4 is characteristic of germinating embryos, and at stage 5 the embryos are fully mature (Hakman & Fowke 1987).

It is well documented that a PGR, abscisic acid (ABA), has a major role in stimulation of somatic embryo maturation stage in many conifer species (Stasolla *et al.,* 2002, Pullman *et al.,* 2003a, Lipavska & konradova 2004, Aronen *et al*. 2009). ABA consumption differs from species to prevent precocious germination. For example, in *Picea likiangensis*, higher amount of ABA was used than that in *P. sylvestris* to give the best result (Aronen *et al., 2009*, Chen *et al*., 2010). The function of ABA on embryo maturation at molecular level will be discussed later.

Besides ABA, polyethylene glycol (PEG4000) is often used during the maturation stage to maintain a high osmolarity in the medium. Effect of PEG mimics the naturally occurring water stress on seeds during late stages of maturation and improves the conversion of proembryonic masses into torpedo-shaped embryos (Attree *et al.,* 1991, Ramarosandratana *et al.,* 2001, Stasolla *et al.,* 2003). The combination of ABA and PEG is the most commonly used factor for promoting maturation of somatic embryos of several genera of conifers (Li *et al.,* 1997, Klimaszewska & Smith, 1997, Pullman *et al.*, 2003a, Aronen *et al*., 2009). However, it was also found that PEG promoted maturation but inhibited further development of somatic embryos in *P. abies* (Bozhkov & Von Arnold, 1998) and in *Picea glauca* (Stasolla *et al.,* 2003).

The use of carbohydrate and desiccation treatment has been examined for conifer somatic embryos maturation (Ramarosandratana *et al.,* 2001, Bomal *et al.,* 2002, Malabadi *et al.,* 2004, Pullman & Buchana, 2008). In addition to being an important energy source and osmotic agent, sucrose may serve a developmental signaling or regulatory role in conifer SE development (Lipavska & Konradova 2004, Iraqi *et al.,* 2005, Pullman & Buchana, 2008). It has also been shown that reduced amounts of amino acids and peptides such as tripeptide glutathione (Stasolla *et al.,* 2004) or higher percentages (to 1%) of the gelling agent Gelrite may decrease the water content of the somatic embryos. Consequently, the embryos do not need further drying treatment (Klimaszewska & Smith 1997, Klimaszewska *et al.,* 2000). Such effect is similar to that caused by non-plasmolyzing osmotica, such as PEG4000.

Germination of somatic embryos

Individual mature somatic embryos are picked from the callus and placed onto germination medium. In general, the structure of the shoot apical meristems of cotyledonary somatic

embryos appears less organized than those observed in the zygotic counterparts (Yeung *et al.,* 1998). Numbers of cotyledons varied from 5 to 8 even within the same genotype.

Efficient germination of somatic embryos is dependent on the quality of embryos. Only those mature embryos that have accumulated enough storage materials and acquired desiccation tolerance at the end of maturation develop into plantlets (Konradova *et al.,* 2003, Lipavska & Konradova 2004). Partial drying treatment (PDT) together with high percentage of gellan gum has been shown to produce more high quality embryos (Klimaszewska & Smith 1997, Malabadi *et al.,* 2004) as well as improve the germination of somatic embryos in conifers (Bomal & Tremblay 1999, Bomal *et al.,* 2002). However, PDT was not found to be essnetial for germination in some species if the embryos were picked up at the cotyledon stage (Choudhury *et al*., 2008, Aronen *et al*., 2009).

Another factor reported to affect germination and conversion of conifer somatic embryos is light quality. A discrete, light-sensitive pre-germination stage and a later germination (radicle emergence) stage were identified by the differential response of somatic embryos to light of different wavelengths (Merkle *et al.,* 2006). Kvaalen and Appelgren (1999) reported that red wavelengths improved the germination frequencies of *P. abies* somatic embryos and enhanced hypocotyl and taproot lengths. While blue or ultraviolet has no direct effect on hypocotyls growth (Fernbach & Mohr 1990) but affects glutamine synthetase during seed germination of *P. sylvestris* (Canovas *et al.,* 1998).

1.1.3. Biochemical and molecular aspects of conifer somatic embryogenesis

Physiological and biochemical study during somatic embryogenesis

Most studies have focused on the morphological and morphogenic aspects of conifer SE, as described above. However, few have dealt with the physiology of the process. The reason is partly due to the fact that physiological responses often differ among genotypes within the same species (Stasolla *et al.,* 2002). The success of SE depends on the quality of somatic embryos. Knowledge of the physiological and biochemical environments associated with zygotic embryo development will improve somatic embryogenesis (Silvera *et al.*, 2004, Tereso *et al*., 2007, Klimaszewska *et al.,* 2004, 2009).

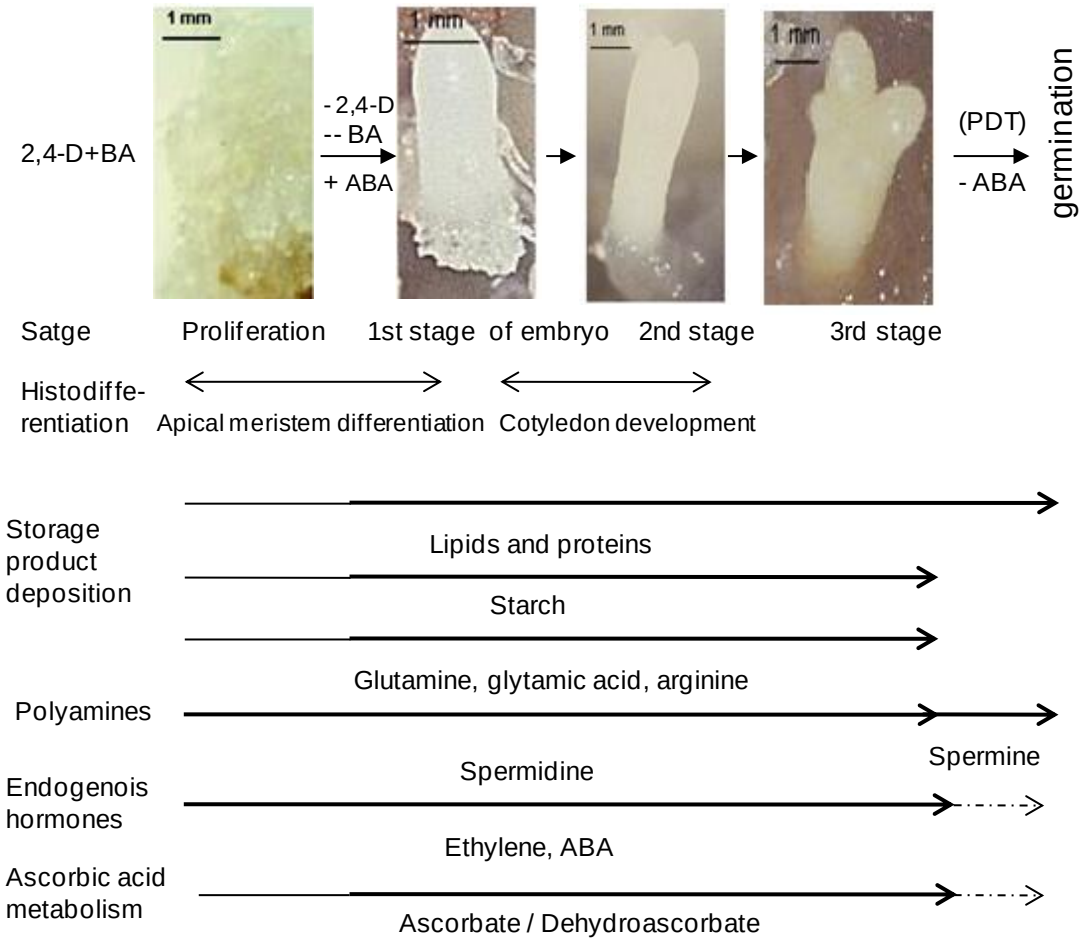

Fig. 1. Summary of the major physiological events during somatic embryo maturation of pine from the early-filamentous stage to germination stage.

steady values; increasing values; decreasing values.

During maturation the embryos undergo structural development and also accumulate compounds that are necessary for further embryo development (Brownfield *et al*., 2007, Tereso *et al*., 2007). Such compounds include storage compounds that provide carbon and energy sources for the maturation phase and for germination (Lipavska &Konradova 2004, Brownfield *et al*., 2007). From proliferation to maturation of somatic embryos, morphological changes, storage products, PGRs, as well as antioxidants changes have been demonstrated (Fig. 1).

Storage product accumulation during somatic embryogenesis has been studied in pine (Brownfield *et al*., 2007, Tereso *et al*., 2007). Somatic embryo accumulates starch first, followed by lipids and proteins (Silveira *et al.*, 2004, Brownfield *et al*., 2007) (Fig.1.). The

differences between somatic embryo and zygotic embryo in storage starch and protein showed that storage protein might be a marker for embryo quality (Tereso *et al.,* 2007). Changes in PGR levels, especially those of ABA, have been observed during embryogenesis of both angiosperms (Sghaier *et al.,* 2009, 2010) and gymnosperms (Silveira *et al.,* 2004, Vales *et al.,* 2007, Wang *et al.,* 2009, Chen *et al*., 2010).

Unlike somatic embryos of many angiosperm crops, it has been shown that early-stage somatic embryos of most conifers require high concentration of exogenous ABA in order to develop into cotyledon-stage embryos (Stasolla *et al.,* 2002, Lelu-Walter *et al.,* 2008). In a developing zygotic embryo, endogenous ABA is relatively low during the initial stages of development. It reaches maximum level at stage 3 during maturation and declines again during the late stages, as the seed dries (Silveira *et al.*, 2004). The dynamic expression of ABA-responsive gene during embryo maturation in *P. taeda* was a further proof that high concentration of ABA was crucial for embryo maturation (Vales *et al*. 2007). The ABA-responsive LEA-like (late embryogenesis abundant protein) gene expression pattern in zygotic embryos provides reasonable evidence for producing high quality somatic embryos (Vales *et al.,* 2007).

High levels of ABA in maturing embryos have been associated with increased storage deposition and inhibition of precocious germination (Hay & Charest 1999, Lipavska & Konradova 2004). Storage proteins of coniferous species have also been found to be high in arginine and glutamine (Rodriguez *et al.,* 2006). Some studies on glutamine, glutamic acid, and arginine revealed that the changes in amino acid metabolism were associated with the storage protein deposition during somatic embryo maturation (Garin *et al.,* 2000, Klimaszewska *et al.,* 2004, Malabadi & Van Staden 2005). Glutathione redox state improved somatic embryo quality (Belmonte *et al.,* 2005, 2007). Application of D buthionine-sulfoximine, which inhibits the biosynthesis of reduced glutathione, could improve somatic embryo yield and quality (Belmonte *et al*., 2007).

The polyamines (PAs) spermidine (Spd) and spermine (Spm) and their precursor diamine putrescine (Put) involved in modulating many plant metabolisms are considered as plant hormones (Silveira *et al.,* 2004, El Meskaoui & Trembaly, 2009, Noceda, *et al.* 2009). It was found that PA activities steadily increased during the development of somatic embryos, from embryogenic suspensor mass until early cotyledonary stages (Monocha *et al.*, 1999, Niemi *et*

al., 2002a, Noceda *et al*., 2009) (Fig. 1). Polyamines played an important quantitative and qualitative role during the maturation of *P. taeda* somatic embryos (Minocha *et al.,* 1999, Silveira *et al.,* 2004) and *P. sylvestris* (Niemi *et al.,* 2002b, 2007). The ratio of Spd/Put from mature somatic embryos is similar to that from zygotic embryos, while Spm activity increased from maturation to germination stage (Niemi *et al*., 2002a, Gemperlova *et al.,* 2009). It was well known that polyamines and ethylene derive from the same precursor, S-adenosylmethionine and polyamines regulate plant metabolism by influencing the biosynthesis of ethylene (Adams & Yang, 1979). However, few studies were done on the function of ethylene in somatic embryogenesis of *Pinus*.

Changes in ascorbic acid (vitamin C) metabolism were also investigated during the maturation process (Malabadi & Van Staden 2005). Application of ascorbic acids together with other vitamins and organic acids produced statistically significant increases in early-stage embryo growth (Pullman *et al.*, 2006*)*. The study showed that the ascorbate (reduced form), dehydroascorbate (oxidized form) ratio increased sharply at the beginning of the maturation period in embryogenic tissues, whereas it remained unaltered in non embryogenic tissue (Pullman *et al.*, 2006).

Partial drying treatment of somatic embryos is needed in some conifers for the physiological and biochemical changes before germination (Stasolla *et al.,* 2001, 2002, Jones & Staden 2001). The initial water loss during desiccation is thought to be a signal setting up the events by which desiccation tolerance is established (Bomal *et al.,* 2002, Konrádová *et al*., 2003). It has been suggested that the desiccation reduces endogenous ABA content (Vales *et al.,* 2007) and ethylene level (Kong & Yeung 1994, Stasolla *et al.,* 2002).

Molecular markers during induction and maturation of somatic embryos

Somatic embryogenesis has been studied at the molecular level in conifers. Many processes of plant development and differentiation, including embryogenesis, are directly or indirectly regulated by alterations of gene expression patterns. Transcript profile in somatic embryo development of *P. taeda* has provided a basis for manipulation of embryo development in tissue culture (Cairney *et al.,* 2000). When compared with *Arabidopsis thaliana*, many developmental pathways and signaling pathway were found to be conserved between angiosperms and gymnosperms (Cairney & Pullman 2007).

During *in vitro* embryogenic process, two major processes that require the reprogramming of gene expression pattern are the induction and the maturation stages. Identification of embryo-specific and embryo-stage-specific molecular markers during embryogenesis would provide a new approach for improvement of somatic embryo production (Fischerova *et al.,* 2008, Wang *et al.,* 2009). Many genes involved in the early stages of somatic embryo development have been studied by comparing embryogenic and non-embryogenic cultures in *P. radiata* (Bishop-Hurley *et al.*, 2003, Aquea & Arce-Johnson 2008). OTUBAINS family participating in the ubiquitin pathway was also detected during early somatic embryo development in *P. radiata* (Aquea & Arce-Johnson 2008). The meristem regulation gene family, *WOX* (Wuschel related homeobox), its transcription factors were expressed in earliest somatic embryo stage and can be used as a possible marker for embryo quality in *P. abies* (Palovaara & Hakman 2008). *PgAGO*, isolated from *P. glauca*, is required for shoot and root apical meristem differentiation. Suppression of *PgAGO* will result in the abnormal embryo development (Tahir *et al.,* 2006). ABA sensing gene*VIVIPAROUS* 1 in maize (Wang *et al.,* 2009), ABI 3 in *A. thaliana* (Fischerova *et al.,* 2008) and *PaVP1* in *P. abies* are transcriptional factors expressed in early embryo maturation stage (Fischerova *et al.*, 2008, Wang *et al.,* 2009).

Several efforts have been made to obtain high quality somatic embryos at the maturation stage (Rodriguez *et al.,* 2006, Pullman *et al.,* 2005, Belmonte & Stasolla 2009). ABA-mediated late embryogenesis genes, for example, LEA-like gene were isolated and studied (Vales *et al.,* 2007, Wang *et al.,* 2009). HBK3 (homeobox of Knox 3) gene, which play an important role in plant development, was also found to be involved in somatic embryo maturation through alteration in glutathione and ascorbate metabolism (Belmonte *et al.,* 2007, Belmonte & Stasolla 2009). Embryo-specific patterns of glutamine synthetase genes could be useful as a molecular marker for early stages of vascular differentiation and the quality of somatic embryos in pine (Rodriguez *et al.,* 2006). Late embryo development marker gene homeodomain-leucine zipper (*PgHZ1*) was isolated from *P. glauca*, which was related to storage product accumulation in embryos.

Other genes have also been found to be developmentally regulated during somatic embryogenesis. These include genes encoding pathogenesis related proteins encoding a basic class IV chitinase and a beta-1,3-glucanase (Dong & Dunstan 1997), heat-shock proteins (Dong & Dunstan 1996) and stress related genes (Frederico *et al.,* 2009, Mathieu, *et al.,* 2009). Transcript profiling studies are expected to provide insight into somatic embryo

development in conifers as well as make mass propagation applicable for the forest industry (Cairney *et al.,* 2000, 2006).

1.1.4. Somatic embryogenesis in pine

Somatic embryogenesis of conifers for clonal propagation has become an integral component of tree improvement strategies. With its capacity for long-term preservation and technology method employed, it is seen to be the preferred technology to accelerate selection and operational deployment of value-added genotypes. However, pine species are often more recalcitrant toward somatic embryogenesis tissue culture methods due to their specific physiological and biochemical properties (Bonga *et al*., 2010). At present, SE has been applied to 23 species of *Pinus*. The whole developmental process has been successful in production of plantlets in several species. These species include *Pinus banksiana* (von Aderkas *et al.*, 2005, Park *et al*., 2006) *P. elliottii* (Merkle *et al.,* 2006), *P. kesiya* (Malabady *et al.,* 2004), *P. monticola* (Percy *et al.,* 2000), *P. patula* (Malabadi & Van Staden 2005), *P. pinaster* (Lelu *et al.,* 1999, Miguel *et al.,* 2004), *P. radiata* (Aquea *et al.,* 2008), *P. strobus* (Park *et al.,* 2006), *P. sylvestris* (Keinonen-Mettälä *et al.*, 1996, Häggman *et al.,* 1999, Lelu *et al.,* 1999, Aronen *et al.,* 2009) and *P. taeda* (Pullman *et al.,* 2006, Pullman & Buchanan 2008).

The culture medium composition is important for the success of SE. It should provide all the nutrients and PGRs for each stage. Information from analyses of zygotic embryogenesis may help improve SE protocols for initiation, maintenance, and maturation (Brownfield *et al*., 2007, Pullman & Buchanan 2008, Pullman *et al*., 2009). Recently, some stage-specific condition has been analyzed by comparison with zygotic embryos, such as carbohydrates (Pullman *et al*., 2008). During initiation stage, adding D-xylose and D-chiro-inositol could increase initiation rate (Pullman *et al*., 2009). And sucrose, raffinose, melibiose, stachyose and fructose were the major contributors to osmotic potential at late seed development (Pullman & Buchanan 2008). These data suggest stage-specific media composition for each step in the somatic embryogenesis protocol. Additionally, adjusting ABA concentration according to embryo development is equally necessary (Vales *et al.,* 2007).

Current state of knowledge of somatic embryogenesis in P. sylvestris

Pinus sylvestris (L.) is an important source of timber in Northern Europe and is often used in the pulp and paper industry. Clonal forestry in *P. sylvestris* has generated keen interest in

Finland and Sweden (Keinonen-Mettälä *et al.,* 1996, Häggman *et al.,* 1999). However, study of SE in *P. sylvestris* lags far behind parallel study in *P. taeda*, which has been used for industry application. Embryogenic cultures have been established both from precotyledonary embryos isolated from immature seeds (Keinonen-Mettälä *et al.,* 1996, Sarjala *et al.,* 1997, Häggman *et al.,* 1999) and from mature embryos (Hohtola 1995). Several factors affect the success of SE in *P. sylvestris*, which include seed collection time (KeinonenMettala *et al.,* 1996), explant storage time and temperature (Häggman *et al.,* 1999), culture medium condition include auxin and cytokinin concentration, ABA level and polyamines (Niemi *et al.,* 2007, Lelu-Walter *et al.,* 2008), and the genotype of the parent trees (Lelu-Walter *et al.,* 2008). These factors have to be modulated specifically for initiation and embryo maturation.

A successful protocol for SE of Scots pine has recently been developed (Lelu *et al.,* 2008, Aronen *et al.,* 2009). The age of immature zygotic embryo as explants was found to be two weeks after fertilization, which gave the best ratio for initiation of somatic embryogenic cultures (Keinonen-Mettälä *et al.,* 1996). The media used for the induction and maturation of somatic embryogenesis include: DCR basic medium (Gupta & Durzan 1986a), MSG (Becwar *et al.,* 1990) and W-pat (Pullman & Gupta 1991). For induction and proliferation, DCR medium can be supplemented with casein hydrolysate, glutamine, 2,4-D, BA and maltose (Keinonen-Mettälä *et al.,* 1996, Häggman *et al.,* 1999, Lelu *et al.,* 1999, Aronen *et al.,* 2009). Embryogenic cultures could be cryopreserved at the proliferation stage for long-term storage (Häggman *et al.,* 1998, 2000). For the somatic embryo maturation, high molecular weight PEG 4000 along with a high ABA and low water content in the maturation medium have been reported to yield high quality mature somatic embryos (Lelu-Walter *et al.,* 2008, Aronen *et al.,* 2009). However, recalcitrant genotypes still exist throughout the processes (Aronen *et al.,* 2009).

Apart from those factors caused by the medium, donor genotype has significant effects on SE initiation in Scots pine (Häggman *et al.,* 1999, Lelu-Walter *et al.,* 2008, Aronen *et al.,* 2009). The genotype phenomena have been investigated in cotton (Michel *et al.,* 2008). The differences between clonal genotypes were caused by the genetic integrity of megagametophytes. In pines, megagametophytes commonly contain multiple archegonia, which are capable of producing multiple genotypes within a megagametophyte. Differences were also observed for the ECs of the dissected immature seeds but without the megagametophyte effect (Yildirim 2005, Özkurt *et al.,* 2008). Microsatellite results have

shown that genotypic instability causes mutation in SE of *P. sylvestris* (Burg *et al.,* 2007). The reasons for genotypic instability are partially due to the immature zygotic embryo, which carry not fully developed genomic information. Stress from PGRs or unsuitable medium conditions might be another reason for genotypic instability (Lopes *et al.,* 2006, Burg *et al.,* 2007). In this case, genotype screening still should be done to achieve successful somatic embryos production (Burg *et al.,* 2007, Marum *et al.,* 2009).

Plant hormonal factors have been studied in *P. sylvestris*. Apart from 2,4-D and BA in the proliferation medium (Lelu *et al.,* 1999), PAs and ethylene have been tested for the growth of ECs (Sarjala *et al.,* 1997, Niemi *et al.,* 2002a, Niemi *et al.,* 2007). A general decrease in the endogenous PA pool occurred towards the later stages of embryo development. Moreover, the Spd/Put ratio transiently increased after the transfer from the proliferation medium to the maturation medium (Niemi *et al.,* 2002a).The changes in PA transcription level at early stage of zygotic embryos (Vuosku *et al.,* 2006) were a prerequisite for the success of somatic embryogenesis. Ethylene also has been reported to affect the processes of SE in pine (Pullman *et al.,* 2003b, Kumar *et al.,* 2009). More information of ethylene on SE will be discussed separately. Integrated studies in *P. sylvestris* with an emphasis on biochemical and physiological aspects of each stage of somatic embryo development are needed. Understanding the variability between zygotic and somatic embryos should provide a feasible basis to develop SE culture protocols that normalize response among genotypes at each step of the process (Brownfield *et al*., 2007, Pullman & Buchanan 2008).

Applications of somatic embryogenesis in P. sylvestris

Genetically identical study material of an individual tree has great value for many scientific studies (Sutton 2002). Genetic improvement of forest trees is at an early stage especially in SE (Häggman *et al.,* 2006). So far most of the research on pine species has focused on the development of genetically improved genotypes through DNA-transformation protocols. There are reports on studies of genes involved in the improvement of embryo development in conifers, such as germin-like gene and *PgAGO* gene (Bishop-Hurley *et al*., 2001, Mathieu *et al.,* 2006, Tahir *et al.,* 2006, Klimaszewska *et al.,* 2010). A range of genes that are of commercial interest have been studied by genetic transformation, for example, alteration of cellulose and hemicelluloses composition by manipulation of lignin biosynthesis (Diouf 2003, Henderson *et al*., 2006), as well as genes to enhance resistance to herbicide and insects (Brukhin *et al*., 2000, Bishop-Hurley *et al*., 2001, Wang *et al*., 2010). In *P. sylvestris*,

cotyledons and embryogenic cultures have successfully been used as targets for genetic transformation (Häggman & Aronen 1998). Later on a transgenic line was achieved by particle bombardment of pollen before fertilization (Aronen *et al*., 2003). The frequency of transgenic progenies was very low by this method. In this case, combining recently improved SE techniques with transgenic method would be an option to obtain more transgenic progenies (Aronen *et al.,* 2003). However, the cumbersome nature of DNA transformation in SE of conifer, together with costs and public opinion toward transgenic plant make the technique less attractive for breeding purposes (Häggman *et al.,* 2006).

Embryogenic suspension cells usually give uniform growth condition. The suspension cells could respond more rapidly to pathogens or their elicitors (Schwacke & Hager 1992). Utilization of this system to study the response reaction has been carried out on agricultural and horticultural plants, but very few on conifers (Kvaalen & Solheim 2000, Elfstrand *et al.,* 2001). A defensin-like gene *spi 1* with enhanced CaMV 35S promoter has been transformed to embryogenic cultures in *P. abies* (Elfstrand *et al*., 2001). The transformed plantlet had reduced fungal growth in the sapwood after inoculation of *H. annosum* (Elfstrand *et al.,* 2001). Interactions between embryogenic cultures and plant pathogen in *P. sylvestris* (Terho *et al.,* 2000) and in *P. abies* (Kvaalen & Solheim 2000) have also been tested in order to select the resistance genotypes.

Some limitations should be considered in studies of host-parasite interactions. The effects of the medium, the lack of tissue structures, unnatural environments, somaclonal variations and genetic instabilities are important factors to be considered when the results of experiments with tissue cultures are evaluated (Cry & Klimaszewska 2002, Marum *et al.,* 2009). However, together with gene cloning and genomics technology applied in SE of conifers, the discovery and introduction of value-added traits, such as high wood quality and resistance to biotic and abiotic stresses, will facilitate wider application of SE propagation in forests plantation (Nehra *et al*., 2005).

1.2. Ethylene in somatic embryogenesis

1.2.1. Ethylene biosynthesis

Gaseous ethylene has been recognized as a plant hormone for almost a century (Neljubov 1901; Crocker & Knight 1908). It influences a diverse array of plant growth and developmental processes, such as germination, flower senescence, cell elongation, fruit ripening and the response to a wide variety of biotic and abiotic stresses (Yang & Hoffman 1984, Kende1993, Bleecker & Kende 2000, Klee & Clark 2004).

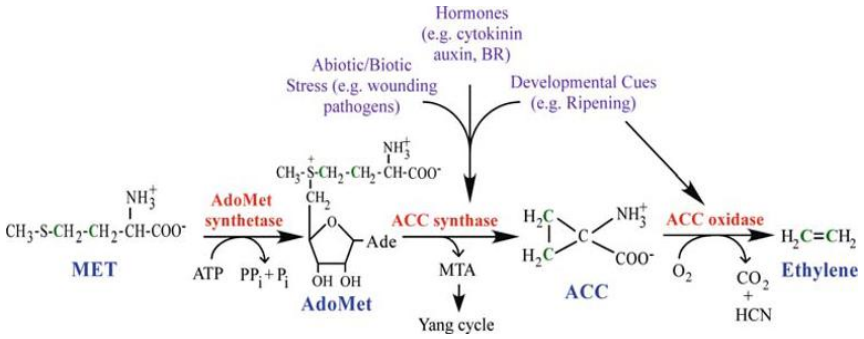

Figure 2. The ethylene biosynthetic pathway

The enzymes that catalyze each step are shown above the arrows. AdoMet: S-adenosyl-methionine; Met: methionine; ACC: 1-aminocyclopropane-1-carboxylic acid; MTA: methylthioadenine. Inputs that regulate the enzymes are also shown above the pathway, either via a transcriptional or post-transcriptional mechanism. See text for further details (Argueso *et al.*, 2007).

The biosynthesis of ethylene occurs through a relatively simple metabolic pathway (Fig. 2) that has been extensively studied and well documented in plants as reviewed by Yang & Hoffman (1984), Kende (1993) and Zarembinski & Theologis (1994). Ethylene is derived from the amino acid methionine, which is converted to AdoMet by AdoMet synthetase. AdoMet is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) and 5´-deoxy-5´methylthioadenosine (MTA) by the enzyme ACS (Adams & Yang 1979, Argueso *et al.*, 2007), which is the rate-limiting step in ethylene biosynthesis. Methylthioadenine is recycled to methionine through the Yang cycle, which allows high rates of ethylene production without depletion of the endogenous methionine pool (Miyazaki & Yang 1987). ACC is converted to ethylene by ACC oxidase (ACO). ACO has a small gene family and is found as a rate limiting enzyme in ethylene biosynthesis (Peck & Kende 1995, Argueso *et al.*, 2007).

1.2.2. ACC Synthase—the key enzyme in ethylene biosynthesis pathway

In most plant species, ACS is encoded by multigene families, which are differentially regulated by various environmental and developmental factors. The genes that encode ACS were first isolated from zucchini (*Cucurbita pepo*) (Sato & Theologis 1989) and numerous ACS genes have since been identified and characterized in various plant species since then (Barry *et al.,* 1996, Arteca & Arteca 1999, Yamagami *et al.,* 2003, Chae & Kieber 2005). In *A. thaliana,* 12 putative genes that encode eight functional proteins have been analyzed. They can be divided into three main groups based on their C-terminal sequences and act as homo- or hetero-dimeric proteins (Yamagami *et al.,* 2003). The form of heterodimers might act to increase the versatility of ethylene responses, enhance the capacity to regulate ethylene production during *A. thaliana* growth, development and response to different stresses (Tsuchisaka & Theologis 2004a, Argueso *et al.,* 2007). Each member of this protein family has the unique and overlapping expression in response to different conditions (Tsuchisaka & Theologis 2004b, Tsuchisaka *et al.,* 2009).

Another well-studied case of transcriptional regulation of *ACS* is that of fruit development in the *Lycopersicon esculentum*. The *L. esculentum ACS* family consists of at least eight genes, and these are differentially regulated by various biotic and abiotic factors (Barry *et al.,* 2000, Alexander & Grierson 2002, Bartley & Ishida 2007). Apart from herbal plants, eight *ACS*s have also been obtained from poplar (*Populus trichocarpa*) and deposited in the GenBank (*POPTR05028*, *POPTR12462*, *POPTR16947*, *POPTR20081*, *POPTR31859 POPTR32359*, *POPTR39736* and *POPTR40659*). However, a few *ACSs* have been reported for gymnosperms. Two *ACS* genes related to somatic embryogenesis of Scots pine were first isolated and registered with the GenBank in 2005 (accession number: AY366205 and AY366206). Several *ACS*s associated to wound, insect infestation and wood formation were reported for other conifer species (Ralph *et al.,* 2007, Barnes *et al.,* 2008).

1.2.3. Regulation of ethylene biosynthesis

Abiotic Stress

Almost all plant tissues have the capacity to synthesize ethylene, although in most cases the amount of ethylene produced is very low. Expression of the *ACS* family in higher plants is induced by a diverse group of abiotic stresses, such as: wounding, anaerobiosis, chilling, drought, auxin, ripening and senescence processes (Chae & Kieber 2005), and also by multi stimuli including: LiCl, $CuCl_2$, auxin, cycloheximide, aminooxyacetic acid (AOA) and

ethylene (Arteca & Arteca 1999, Chae & Kieber 2005, Wi *et al.,* 2010).

One of the most studied abiotic stimulus involving stress-ethylene responses is wounding. In *A. thaliana*, the expression of multiple *ACS* genes increases after wounding (Tsuchisaka & Theologis 2004b, Ralph *et al.,* 2007). Both jasmonic acid (JA) and ethylene are increased synergistically when plants are wounded by pests or pathogen (Turner *et al.,* 2002, Onkokesung *et al.,* 2010). However, ethylene has been shown to be antagonist to JA induced defence reaction (Shoji *et al.,* 2000, Ankala *et al.,* 2009). In *L. esculentum*, JA is a major regulator of wound responses, and ethylene seems to act together with JA to regulate wound-responsive genes encoding proteinase inhibitor (*pin*) proteins (Wasternack *et al.,* 2006, Onkokesung *et al.,* 2010). Ankala *et al.,* (2009) pointed out that JA functions upstream of ethylene in maize insect resistance 1-cysteine protease (Mir1-CP) pathway after herbivory wounding. The balance of these two pathways is critical depending on the plant species and type of stress (Ankala *et al.,* 2009, Wi *et al.,* 2010).

Biotic factors

The involvement of ethylene in response to pathogen attack has long been recognized (Boller 1991). Plant-derived ethylene is associated with a resistance response when plants are attacked by pathogens (van Loon *et al.,* 2006). Ethylene was also observed in the roots of *Medicago sativa* inoculated with *Rhizobium*, and this is associated with defence responses (Ligero *et al.,* 1987).

Some micro-organisms, such as plant-growth promoting rhizobacteria, are also able to modulate ethylene responses by altering the levels of *ACC* produced by plants (Naveed *et al.,* 2008, Govindasamy *et al.,* 2009). Through this reaction, which is catalyzed by the microbial-encoded enzyme *ACC* deaminase, *ACC* is hydrolyzed to α-ketobutyrate and ammonia. This in turn decreases the levels of *ACC* that are available for ethylene production and improves growth and yield of the plant (Naveed *et al.,* 2008).

Transcriptional and post transcriptional regulation of ACC synthase

Transcripts of *ACS* genes could be regulated during the course of development and in response to various external cues. As discussed above, various biotic and abiotic stresses can influence the transcription of different *ACS* genes. In *A. thaliana*, diverse patterns of *ACS* transcripts have been detected in roots, leaves, flowers, siliques, stems, and etiolated seedlings

(Yamagami *et al.,* 2003, Tsuchisaka & Theologis 2004b, Wang *et al.,* 2005). The genes *ACS2*, *4*, *5*, *6*, *7*, *8* which are expressed in the flower are also observed in other tissues. A well-studied case is the transcriptional regulation of *ACS* in *L. esculentum* fruit development. Two systems of ethylene production have been proposed (Barry *et al.,* 2000, Klee & Clark 2004). System 1 is functional during normal vegetative growth; it is ethylene auto-inhibitory and responsible for producing the basal levels of ethylene detectable in all of the tissues including non-ripening fruit. System 2 operates during the ripening of climacteric fruit and during petal senescence when ethylene is autostimulatory and requires the induction of both of the ACS and ACO. By this theory, when the fruit ripening process commenced, the *ACS* family becomes integrated in it and responds to different conditions (Barry *et al.,* 2000).

ACS protein turnover is also important for regulating the production of ethylene. Various studies are consistent with a model in which the C-terminal region of *ACS* proteins plays a crucial role in regulating their turnover (Chae & Kieber 2005) (Fig. 3), for example, the phosphorylation of *AtACS2* and *AtACS6* (Type 1) by *MPK6* leads to the accumulation of *ACS* protein (Kim *et al*., 2003). This, in turn, causes elevated levels of cellular *ACS* activity, which leads to increased ethylene production (Kim *et al*., 2003, Liu & Zhang 2004). Induction of *ACS* activity through a phosphorylation-dependent mechanism was observed in *L. esculentum*, which involves increased turnover of the *ACS* protein (Spanu *et al.,* 1990).

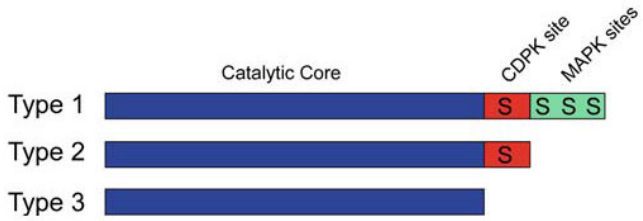

Figure 3. Cartoon representing type 1, 2, and 3 *ACS* proteins

ACS proteins in *A. thaliana* can be divided into three main groups, based on their C-terminal sequences. The conserved catalytic core, present in all *ACS* proteins, is shaded, whereas putative *CDPK* (calcium-dependent protein kinase) (Sebastia *et al.,* 2004, Tsuchisaka & Theologis 2004a) and *MAPK* (mitogen-activated protein kinase) phosphorylation sites (Liu & Zhang 2004) are represented by "S" in Type 1 and 2 *ACS* proteins.

1.2.4. Ethylene in somatic embryogenesis

Effect of ethylene on the growth of ECs

The gaseous PGR, ethylene, regulates many aspects of plant morphogenesis. Modification of ethylene composition and its interaction in the nutrient medium has been the primary strategy to manipulate morphogenesis *in vitro* (Kumar *et al.,* 1998, Yoo *et al.,* 2009). In the model plant of Alfalfa, the requirement for ethylene is optional during the induction of somatic embryogenesis; too much ethylene production might be inhibitory for the initiation stage (Huang *et al.,* 2001). The similar function was also observed in conifers (Pullman *et al.,* 2003b). At the proliferation stage, auxin is often required for cell differentiation (Find *et al.,* 2002). However, increased ethylene production at this stage was also detected (Kvaalen 1994). High ethylene production in the ECs will decrease the initiation rate and embryo maturation (El Meskaoui & Tremblay 2001, Pullman *et al.,* 2003b). A decrease in ethylene production during proliferation, for example, by silver nitrate ($AgNO_3$), was found to increase the induction rate and growth in lobloly pine (Pullman *et al.,* 2003b, Kumar *et al.,* 2009). Differences in ethylene production were observed among ECs with varying maturation capacities (El Meskaoui & Tremblay 2001). Regulation of ethylene biosynthesis is considered necessary for embryo production.

Effect of ethylene biosynthesis on somatic embryo maturation

At the maturation stage, several studies have revealed that high ethylene production inhibit somatic embryo development (Stasolla *et al.,* 2002, Minocha *et al.,* 2004, Kumar *et al.,* 2009). Non-embryogenic lines, which will not produce somatic embryos, accumulate more ethylene than its corresponding embryogenic lines (Kumar *et al.,* 1989). Higher accumulation of ethylene has been found to reduce the number of cotyledonary embryos and increase morphological abnormal embryo production (Kong & Yeung 1994, Kong *et al.,* 1999).

Since the function of endogenous ethylene is crucial in somatic embryo development, optimal ethylene level should be determined for *in vitro* culture (Tsuchisaka *et al.,* 2009). ECs grown on artificial medium, might contain components that can increase ethylene production, control of ethylene production level during the maturation stage will help to improve somatic embryo formation. Regulation of ethylene biosynthesis is well studied in *A. thaliana* and in *L. esculentum* (Yamagami *et al.,* 2003, Bartley & Ishida 2007). Inhibiting ethylene production by 1-Methylcyclopropene, aminoethyoxyvinyl glycine, and NAA could prolong the fruit storage time (Martinez-Romero *et al.,* 2007, Yuan & Li 2008). In somatic embryogenesis of conifers, the use of $AgNO_3$, an ethylene antagonist was found to increase embryos production in black spruce (El Meskaoui & Tremblay 2001, Huang *et al.,* 2001, Pullman *et al.,* 2003a,

Jimenez 2005, Kumar *et al.,* 2009). Modifying the concentration of ACC, the immediate precursor of ethylene, was reported to increase endogenous ACC content and in the process improve the embryo production in the genotype lines with low ethylene production (El Meskaoui & Tremblay 2001). AOA, an inhibitor of ethylene synthesis have been used to decrease ethylene production during maturation stage to obtain high embryo production (El Meskaoui & Tremblay 2001, Huang *et al.,* 2001). However, the effect of ethylene on somatic embryo maturation in *P. sylvestris* has not been thoroughly studied.

1.3. Interactions between rhizosphere microbes and development of *P. sylvestris* roots

In nature, plants including forest trees are exposed to a diverse array of microbes, fungi, bacteria and viruses at every stage of their life time (Pearce 1996). The relationship between *P. sylvestris* and their microbial partners could range from latent entophytic to mutualistic associations and fatal infections.

1.3.1. Ectomycorrhizal fungi and its interaction with host trees

Ectomycorrhizal fungi are group fungi that form a mutualistic association with plant roots (Smith & Read, 2008). ECM fungi form symbiotic association with trees, which include species in the *Pinaceae*, *Betulaceae*, and *Fagaceae* genera (Smith & Read, 2008, van der Heijden *et a*l., 2008). During ECM symbiosis, the fungal partner and host plant could establish an efficient mechanism for water and nutrient uptake (Nehls 2008). An ECM root is characterized by the presence of three structural components: a sheath or mantle of fungal tissues around the root, a Hartig net is formed by ECM hyphae between the epidermal and cortical cells and external hyphal elements which form essential connections with soil and sporocarps of the fungi forming the ectomycorrhizas (Smith & Read, 2008). The ECM increases the contact area of plant roots in soil. The symbiosis facilitates the uptake of nutrients such as N and P to the plant root efficiently while the ECM fungi obtain carbohydrates from the tree.

During the symbiotic interaction, the ECM fungi partner releases PGRs including auxins, cytokinins, ethylene and PAs (Niemi *et al.,* 2003, Felten *et al*., 2009, Splivallo *et al.*, 2009, Sarjala *et al.*, 2010). ECM fungus has been used to induce root formation of cuttings and somatic embryo germination (Niemi & Häggman 2002, Niemi *et al.,* 2004). Use of ECM

fungus *P. tinctorius* in somatic embryogenesis of *P. sylvestris* has been reported to increase somatic embryos germination (Niemi & Häggman 2002).

Cyanobacteria are aquatic, photosynthetic and often called "blue green algae" (Whitton & Potts 2000). They form a wide variety of symbiotic associations with eukaryotic hosts such as plants, sponges and fungi (Adam & Duggan 2008). It is found that cycads are the only group among gymnosperms that have been shown to form symbiosis with cyanobacteria (Lindblad *et al.,* 1987; Osborne 2007). Plant cyanobionts normally belong to the genus *Nostoc*, which is commonly found free- living in nature (Rai *et al.,* 2002). Nostoc cyanobacteria fix nitrogen in special cells called heterocyst (Zhang *et al.,* 2006). During symbiosis, cyanobacteria provide organic nitrogen to the plant and in return get water, minerals and protection from ultraviolet (light) damage (Honegger 1991, Kneip *et al.,* 2007). Unlike other nitrogen-fixing *Rhizobia* or *Frankia sp*. that has specific host preference, cyanobacteria show a broad host range. Another remarkable difference is plant cyanobionts are always endosymbionts, form symbiotic cavities in the plant (Rai *et al.,* 2002, Kneip *et al.,* 2007).

The biological and molecular nitrogen-fixation mechanism in *Nostoc* cyanobacteria has been studied (Fay 1992, Singh *et al.,* 1994, Berman-Frank *et al.,* 2003). Since nitrogenase enzyme complex is very sensitive to oxygen, nitrogen fixation is restricted to the dark period in unicellular cyanobacteria, when oxygen levels are low (Berman-Frank *et al.,* 2003). Some organism such as the oceanic picoplanktion has been found to lack photosysten II of photosynthesis, which enables it to perform nitrogen fixation during the day (Bothe *et al.,* 2010). In addition to this protection, concentration of oxygen can be decreased via biochemical pathway such as cyanoglobin, an oxygen scavenging molecules (Hill *et al.,* 1996).

In forest soil, the biological nitrogen is always a limiting factor in forest tree growth. The exogenous source of nitrogen into boreal forest is important. The largest natural input of nitrogen to the forest is through cyanobacteria that form symbionts with moss (Adams & Duggan 2008). However, litter chemical components and moisture are often the limitations to nitrogen fixation through cyanobacteria association (Gundale *et al.,* 2009).

1.3.2. Pathogenic fungus - host interaction

In the forest, many fungi cause diseases to trees. In tree-pathogen interaction, the host tree could be extremely susceptible or completely resistant to pathogenic fungi. One of the major pathogens of forest trees is the root and butt rot fungus, *Heterobasidion spp.* which causes significant decay to conifer trees in Europe and North America (Korhonen & Stenlid 1998, Asiegbu *et al.,* 2005). According to their host preferences and physiological and biochemical characters, *Heterobasidion* spp. has been devided to three different species in Europe (Karlsson and Stenlid 1991, Korhonen & Stenlid 1998). *H. annosum* s.s. (P-type) which mainly attacks *Pinus* species, although it has a broad host range including species of *Picea, Larix, Juniperus* and sometimes even deciduous trees like *Betula* and *Alnus*. *H. parviporum* (S-type) attacks mainly Norway spruce and has only occasionally been found on other native trees in most parts of Europe. And *H. abietinum* (F group) infects firs (Korhonen & Stenlid 1998, Maijala *et al.,* 2003). The pathogen is spread through root contacts. The tree cell wall is the first barrier against infection by a pathogen. *Heterbasidion annosum* can secret a number of cell wall degrading enzymes to degrade the root cell walls (Korhonen & Stenlid 1998, Maijala *et al.,* 2003, Asiegbu *et al.,* 2004); whereas *H. parviporum* can kill young growing close to infected *Picea* stumps, but obviously its pathogenicity towards pine is relatively weak.

Plant resistance mechanisms induced by microbes include basal resistance (Jones & Dangl 2006, Kiraly *et al.,* 2007) and acquired or induced resistance (Shoresh *et al.,* 2005, Jones & Dangl 2006). Several signalling molecules are known to regulate the plant defence responses. Normally plant reacts to biotrophic pathogen infection by inducing cell death, which is regulated by a SA dependent pathway (Glazebrook 2005). In contrast, necrotrophic pathogens benefit from host cell death which implies that SA regulated cell death does not limit their growth. Plants respond to necrotrophic pathogens by different defence pathways activated by JA/ethylene molecules (Glazebrook 2005, Jones & Dangl 2006).

1.3.3. Fungal elicitors, effectors and host-microbes recognition

The term “elicitor” was originally used to refer to molecules and other stimuli that induce the synthesis and accumulation of antimicrobial compounds (phytoalexins) in plant cells (Keen 1975). It is now commonly used to describe molecules that stimulate any plant defence mechanism (Dixon 1986, Dixon & Lamb 1990). Many of these molecules have the ability to induce plant defence responses and are referred to as elicitors (Leitner *et al.,* 2008). Microbe

associated molecular patterns (MAMPs) are released in plants as complex mixtures of elicitors (Nürnberger & Brunner 2002, Jones & Dangl 2006). The skeleton of fungal cell walls contains 80-90% polysaccharides including chitin, cellulose, non-cellulosic β-glucans and α-glucans (Bartnick 1968, Mazan *et al.,* 2006). Many fungal elicitors, such as chitin, α-1, 3-Glucan, a polysaccharide oligochitin and glycoprotein from the cell wall, elicit the innate immunity for the defence of the plant (Hahn 1996, Wolski *et al.,* 2007). The use of cell-wall components from the ECM fungi *Amanita muscaria*, *Hebeloma crustuliniforme* and from the conifer pathogen *H. annosum* have been described by Schwacke & Hager (1992). Usually, when a fungus comes into contact with a plant, certain molecular and chemical components of the fungal cell wall play key roles in the recognition process.

Effectors are microbe-derived molecules whose intrinsic activities are to interact with and thus affect the host (de Wit *et al.,* 2009). Plant pathogens secrete a suite of effectors that collectively promote their virulence (Lee & Schneewind 2001). In resistant plants, effectors are directly or indirectly recognized by cognate resistance proteins that reside either inside the plant cell or on plasma membranes (Jones & Dangl 2006, Ellis *et al.,* 2009). To date, 10 fungal effectors, four *avr* genes and six extracellular proteins (ECPs) have been isolated from *Cladosporium fulvum*. All avrs and ECPs proved to be virulent factors in *C. fulvum* - *L. esculentum* pathosystem (Bolton *et al.,* 2008, de Wit *et al.,* 2009). However, necrotrophic fungi for which no gene-for-gene relationship has been established produce several necrogenic host-specific peptide effectors. ToxA was the first peptide toxin produced by *P. tritici-repentis* and caused necrosis in particular genotypes of wheat (Ciuffetti *et al.,* 1997). Similar to the effectors of biotrophic fungal pathogens, toxic peptides from necrotrophic pathogens represent effectors that interact with host targets. Little is known about effectors from ECM fungi, it is hoped that the availability of genome sequences of fungal symbionts will facilitate identification of potential effectors (Martin & Selosse 2008).

During the process of plant interaction with microbes, recognition plays a central role in the contacts between plants and their pathogens. Pathogens must be able to recognize the presence of their host plants in their environment, detect their host features in order to colonize/invade the plant and cause infection (Jones & Dangl 2006, Boller & Felix 2009). Successful pathogens should also be able to overcome plant defence responses (Jones & Dangl 2006).

The plant partners release beneficial and also harmful compounds to the rhizosphere, such as amino acids, organic acids, sugar, phenolics, polysaccharides and proteins (Bais *et al.,* 2006). These compounds will either stimulate or inhibit the surrounding microbes (Bais *et al.,* 2006, Hartmann *et al.,* 2009). The host plant also release some molecules, such as flavonoids or related compounds derived from plant phenylpropanoid metabolism, which are fundamental molecules involved in plant defence (Eckardt 2006). On the other hand, pathogenic fungi secrete some chemical compounds or a large array of enzymes to suppress the host defence responses and cause desease (Jones & Dangl 2006, de Wit *et al.,* 2009).

Pattern recognition emerged as a fundamental process in the immune response of plants during the past 10 years (Boller & Felix 2009). Podila *et al*. (2002) showed that early symbiotic interactions occurred during pre-infection stage. Others reported that plants possessed pattern recognition receptors (PRRs) that enable it to perceive molecular signatures for easy of identification of different classes of microbes (Jones & Dangl 2006, Boller & Felix 2009). For example, one identified β-glucan binding protein in legumes was proved as the specific binding site for 1,6-β-linked and 1,3-β-branched heptaglucoside (HG) in the cell wall of the oomycete *Phytophthora sojae* (Fliegmann *et al.,* 2004). Very few studies have described the response of gymnosperm species to fungal elicitors (Schwacke & Hager 1992, Salzer *et al.,* 1997). In cyanobacteria plant-microbe interaction, the MAMPs recognized by PRRs are typically associated with a specific class of microbes regardless of pathogenicity (He *et al.,* 2007, Boller & Felix 2009).

2. AIMS OF THE STUDY

Somatic embryogenesis is a potential vegetative propagation method for conifers, including *P. sylvestris*. The aim of this study was to improve SE propagation method of *P. sylvestris* through biotic and abiotic methods and to study the plant-microbe interaction using somatic embryos and seedlings.

The objectives of the present study were as follows:

1) Investigate the effect of genotypes and initiation media on somatic embryogenesis of Scots pine (Paper I).

2) Isolate and characterize *ACS* genes from somatic embryogenic tissues of Scots pine and study the role of ethylene in embryo maturation (Paper II).

3) Investigate effects of fungal cell wall elicitors secreted from pathogenic (*Heterobasidion spp.*) and ECM (*S. bovinus)* fungus on somatic embryogenesis on *P. sylvestris* (Paper III).

4) Study the interactions between *P. sylvestris* seedling roots and microbes without direct physical contact (Paper IV).

3. MATERIALS AND METHODS

3.1. Plant material and microbes used

Immature female cones from controlled crosses of seven Scots pine elite trees were collected and stored at 4 °C for different periods of cold treatment (I). All the initiated lines were used for the study of genotype effects on the success of somatic embryo production (I). The selected lines from these sets of ECs were subsequently used for ACS isolation and ethylene regulation treatments (II). Newly sprouted needles, roots or shoots from four-year-old Scots pine emblings were collected for ribonucleic acid (RNA) extraction for the study of ACS expression (II). Six embryogenic lines with abundant embryo production provided by Finnish Forest Research Institute, Punkaharju Research Forests were used for the study of elicitor effects (paper III). Scots pine seeds (E2866 × E2350, Asikkala × Anjalankoski, Punkaharju Research Unit, Finnish Forest Research Institute) were surface sterilized, germinated and used for the study of indirect interaction with microbes (IV).

Heterobasidion annosum (Fr.) Bref. sensu stricto (isolate 03012) (III, IV) and *H. parviporum* Niemelä & Korhonen (isolate 03014) (III) were cultured on 2% malt extract medium. *Suillus bovinus* (L.: Fr.) O. Kuntze (isolate SBH1) (III, IV) grew on a Melin-Norkrans medium (Molina and Palmer 1982). Cyanobacteria *Nostoc spp*. (JR 990770) (IV) was cultured on Z8X medium (Kotai 1972).

3.2. Initiation of embryogenic tissue cultures (I)

The immature female cones from 7x7 cross lines were surface sterilized. Seed coats were removed and the whole megagametophytes with immature embryos were placed horizontally on the initiation medium in 90-mm diameter Petri dishes. The following media were used: Modified MSG, Modified W-pat and Modified DCR20 (I). One to three cones per family were used for each cold-storage treatment (I). The plates were left to grow in the dark at 23°C. The total number of immature zygotic mega-gametophytes used in the experiment was 5264.

3.3. Maintenance and production of mature somatic embryos

After initiation, cultures were transferred to DCR proliferation medium supplemented with 500 mg l^{-1} casein hydrolysate, 13.6 μM 2,4-D, 2.2 μM BA and 2% maltose (I, II, III). The somatic embryogenic tissues were sub-cultured at two week intervals, with nine of the

embryogenic tissues sub-cultured on a 9-cm-diameter Petri dish with 20 ml of culture medium. The cultures were proliferated for two months. To mature the embryo cultures, the tissues were first cultured on pre-maturation medium for two weeks. The cultures were moved to 0.1% active charcoal medium without growth regulators for a week. Thereafter they were transferred to maturation medium, DCR with 8% PEG 3500 and 15.1 μM ABA (I, II) or with 80 μM of ABA (III).

3.4. Induction treatment of somatic tissues (II)

The ECs of the genotype BG8 (I) grown on DCR proliferation medium was used for induction treatment (II). Six pieces (0.2 g each) of ECs were randomly sampled, dipped into 100 ml of one of the following: 500 μM $CuCl_2$, 100 μM 2,4-D, 100 μM BAP) or 100 μM ABA in DCR medium for 6 h. ECs in DCR medium without treatment were used as the control. After each treatment, three pieces of the ECs were collected and frozen in liquid nitrogen and stored at -70 °C for RNA extraction. The remaining three pieces were used for the measurement of ethylene production.

3.5. Treatment of ECs and determination of ethylene production (II)

ECs were grown on maturation medium and treated with either 10 μM ACC, 5 μM AOA, or 1 mM $AgNO_3$ for ethylene production measurement and the somatic embryo maturation test. Ethylene was measured by gas chromatograph (Varian 3700) equipped with porapak Q column (80-100 mesh, 1 m × 3.2 mm) according to Vahala et al. (1998). Oven, injector and detector temperatures were set at 40 °C, 150 °C and 200 °C, respectively.

3.6. Elicitor preparations and sample treatment (III)

Fungal mycelia collected from fresh cultures of *S. bovinus, H. parviporum* and *H. annosum* were used for crude cell wall elicitor preparation (Ayers *et al.*, 1976, Schwacke and Hager 1992). The final products were autoclaved, lyophilised and stored at -20°C. Six mature somatic embryos were treated with fungal cell wall elicitors for one month to investigate their effects on the root germination rate. The embryos of cell line L76 were subsequently chosen for detailed molecular biological investigation at 1, 7 and 15 days after elicitor treatment. The germinated roots were collected for RNA extraction and quantitative real time PCR.

3.7. RNA isolation, reverse transcription PCR and quantitative PCR

Total RNA was extracted according to Chang et al. (1993) (II, III, IV). Reverse transcription polymerase chain reaction (RT-PCR) was used for gene expression study. Complementary deoxyribonucleic acid (cDNA) was reverse transcribed with avian myeloblastosis virus (Promega, Madison, WI, USA) (II, IV) or with Superscript III (Invitrogen) reverse transcriptase and with OLE4 reverse primer (II) or anchor T (5′-$(T)_{20}$VN-3′) as primers (III, IV). Normal PCR was performed according to each primers melting temperature (II, IV). Quantitative real time PCR was made with SYBR Green I Master Mix (Roche, Germany) according to the manufacturer's recommendations (III).

3.8. Sequences and phylogenetic analyses of *PsACS1* and *PsACS2*

The cloned sequences were analyzed by NCBI BLAST (national center for Biotechnology, basic logical alignment search tool) (II, IV). Amino acid sequence of *PsACS1* and *PsACS2* alignments were made using ClustalW (II). The evolutionary history of 29 taxa from *A. thaliana*, *L. esculentum*, *P. glauca*, *Picea engelmannii* × *P. glauca*, *Pseudotsuga menziesii* and *P. taeda* was inferred using the Neighbour-Joining method. Phylogenetic analyses were conducted with MEGA4 (II).

3.9. Induction of *P. sylvestris* seedlings by different microbes (IV)

The pine seeds were surface sterilized and then germinated on water agar for two weeks at room temperature in the dark until the radicles were 0.5-2 cm long. The germinating seedlings were transferred to their respective 100 ml test tubes in which the medium had been set diagonally sloping against the side of the tube. A sterile membrane tube which had one end sealed was placed in the test tube and filled with the sterile light gravel. The tubes were sealed with cotton wool. The seedlings were incubated in the growth chamber for four weeks. Then two agar cubes were inoculated inside the membrane tube which was placed adjacent to the roots of the seedling. Seedlings without inoculation were used as control. After six weeks post inoculation, the weight of the root and number of root tips were determined. Total RNA was isolated from the root tips and used for RT-PCR.

3.10. Statistical analyses

Data obtained for the survival of the cell lines, ethylene production and embryo production was statistically analyzed using analysis of variance (ANOVA) (I, II). The difference in root tip numbers, germination between fungal cell wall elicitor treatments, cell lines and treatment

times were analyzed using independent sample one-way ANOVA and Tukey's test (III, IV)). All the tests were performed using SPSS for Windows release 15.0 (SPSS, Chicago, IL, USA).

4. RESULTS AND DISCUSSION

4.1. Parental effects on the success of the somatic embryogenesis of *P. sylvestris* (Paper I)

The effect of parental genotypes on the cultures was evaluated at the initiation of the tissue culture period, after six months incubation on maintenance culture and at embryo maturation. The maternal effect was more significant than the paternal effect at the initiation stage of *P. sylvestris* (I). Lelu *et al*. (1999) observed a significant maternal tree effect in both *P. sylvestris* and *P. pinaster* at the initiation stage. Different initiation rates were found among different species (Park *et al.,* 2006). The overall percentage of culture initiation in this study was 13% and the variation between families was 1 to 42%. The initiation success was about three times higher for cross-pollinated (14%) families than for self-pollinated (4%) families. Four of the trees in our experiment (P451, K801, K828 and K1005) were included in earlier somatic embryo production experiments (Keinonen-Mettälä *et al.,* 1996, Häggman *et al.,* 1999) and were the most responsive of 138 tested trees. This pre-selection of material may explain the high initiation percentage in the study of Aronen *et al.,* (2009).

After six months culturing in maintenance culture, the genotype of both maternal and paternal lines had a significant effect on the survival of the offspring (paper I, Table 4). The significance of the maternal genotype diminished and that of the paternal genotype correspondingly became detectable among the surviving lines. Klimaszewska *et al*. (2001) reported that two thirds of the initiated *P. strobus* lines survived the proliferation stage. Similar result was also observed in *P. sylvestris*, 67 % of initiated lines survived for at least one year in maintenance culture (Häggman *et al.,* 1999). A small percentage of the lines were eliminated during the proliferation stage (Häggman *et al.,* 1999).

Survival of embryogenic cultures may partly depend on factors related to tissue culture techniques. For example, the two-week subculture period and culture medium may not have been optimal for all the genotypes selected. Chandler & Young (1995) studied *P. radiata* whereas Lelu *et al*. (1999) investigated *P. sylvestris*, both groups reported successful proliferation on hormone free culture medium. The uses of different carbohydrates at proliferation and maturation stages as well as adjustment of ABA concentration have also been tested (Aronen *et al*., 2009).

Only the maternal genotype significantly influenced the production of mature somatic embryos (paper I, Table 5). The mature somatic embryos production rate was 69% (paper I, Fig. 3), which indicated that fewer lines were eliminated at the maturation stage than at either the initiation stage or the proliferation stage. The differences in somatic embryo production varied among cell lines within families. The most and least productive lines were scattered within different families. Large variation in mature embryo production among lines was observed recently in self- and cross- pollinated seed families (Lelu *et al.,* 2008). The percentage in producing mature somatic embryo is rather low in *P. sylvestris* (Häggman *et al.,* 1999). However, recalcitrant lines did not produce or produced very limited amount of embryos, which have low germination rate (Aronen *et al.,* 2009). In a word, selection of elite maternal genotypes is crucial for initiation, proliferation and embryo maturation in SE of *P. sylvestris*.

4.2. Ethylene synthesis genes in somatic embryogenesis of *Pinus sylvestris* (Paper II)

Genes those encoding two ethylene synthases, *PsACS1* and *PsACS2*, were isolated from the ECs. These two *ACS* genes contain seven conserved boxes, comparable with the known *A. thaliana AtACS7* and one coniferous *PgACS1* (II, Fig. 1 and S1). The *ACS* genes are known to belong to a multi-gene family. In *A. thaliana*, 12 putative *ACS* genes encoding eight functional proteins have been analysed (Yamagami *et al.,* 2003). However, only few studies have been carried out on Gymnosperms. *PgACS1* and *PgACS4* were cloned from *P. glauca* in response to wounding and insect infestation damages which induced expression (Ralph *et al.,* 2007) and one *PtaACS*1 gene was isolated from *P. taeda* in response to bending stress (Barnes *et al.,* 2008).

In this study, *PsACS1* was found to be expressed in the ECs at the proliferation and the maturation stages. At the proliferation stage, both 2,4-D and BAP could induce *PsACS1* expression but only 2,4-D treated samples induced high ethylene production (paper II, Fig. 2A and 2B); whereas 2,4-D decreased the *PsACS2* transcript level thereby supporting results from earlier observations (Lelu *et al.,* 1999). Wochok and Wetherell (1971) showed that 2,4-D inhibited carrot embryos maturation by inducing the production of ethylene. At the maturation stage, differences in expression of *PsACS1* were not detectable within the tissues

and also within the cell lines (paper II, Fig. 3 A). Thus it probably functions like a house keeping gene to keep ECs differentiation under stress conditions.

The *PsACS2* transcript was expressed in embryos at the maturation stage. The high *PsACS2* transcript abundance was correlated with the higher ratio of cotyledonary stage embryos, and the production of ethylene (II, Fig. 3A and B, Fig. 4). This probably suggests that *PsACS2* started to express at the time when ethylene production increased. Since *ACS* is the rate limiting enzyme to ethylene production, it was presumed that the expression of *PsACS2* is coincident with ethylene production during somatic embryo maturation (Fig. 4). Cytokinin and Cu^{+} could induce the expression of *PsACS2* in plant regulation free medium. *PgACS1*, shared 93% similarity at the amino acid level with *PsACS2*, was reported to be expressed at the embryo stage and was not induced by wound or insect infestation (Ralph *et al.,* 2007). It was concluded that both *PsACS2* and *PgACS1* are expressed during embryo development. The sequenced *PgeACS2* and *PgeACS3* in *Picea engelmannii* × *P. glauca* and *PmACS3* in *P. menziesii* was induced by insects (Ralph *et al.,* 2007). It would be useful to identify additional *ACS*s in *P. sylvestris* for a better understanding of the involvement of ethylene in development and in responses to stresses.

Further study on ethylene biosynthesis showed that ACC increased ethylene production significantly in both BG8 and DA5 cell line and decreased slightly the number of mature embryos. However, $AgNO_3$ and AOA had no significant effects on either ethylene output or embryo production (paper II, Fig. 5A, B & C). Similar results were observed for *Picea mariana* (El Meskaoui & Tremblay 1999, El Meskaoui & Tremblay 2001). However, a contrasting result was reported in *P. taeda* for which AgNO3 was found to increase ethylene production, improved culture initiation and embryo maturation (Pullman *et al.,* 2003a). A genotypic effect might be one explanation, since the cell capacities in producing embryos were different in response to ethylene (El Meskaoui & Tremblay 2001).

4.3. Response of somatic embryos to fungal cell wall elicitors (paper III)

Germination was tested among six cell lines with elicitor treatment. Two cell lines had the highest rooting ability. Under the elicitor treatment, the ability of germination in two cell lines appeared to be favoured by the *H. parviporum* or the *S. bovinus* elicitor. The elicitor from *H.*

annosum decreased the germination and subsequent root development in all the genotypes (III, Fig. 2). It was interesting to observe that *H. parviporum* elicitor increased the germination in some genotypes. Generally, when an ECM fungus interacts with the host plant, it secretes different PGRs including auxins, cytokinins, ethylene and PAs to favour colonization and also nutrient intake from the host plant (Felten *et al.,* 2009, Splivallo *et al.,* 2009). The differences between the two pathogens *H. annosum* and *H. parviporum* in the induction of germination / root growth could lie on the apparent differences in their host preferences (Korhonen & Stenlid 1998) or their cell wall components (Schwacke & Hager 1992). However, only one isolate from each fungus was tested with somatic embryos. This study proved that fungal cell wall elicitors could elicite similar defense responses in the emblings as well as living fungal hyphae.

The effects of the fungal cell wall elicitors on a subset of genes that encode proteins involved in cell wall synthesis and modification, and the role in mediating stress responses were further examined. Gene transcripts that encoded actin (*ACT-1*) and peroxidase (*Pero-1*) were up-regulated at 7-day after exposure of the emblings to *S. bovinus* or to *H. parviporum* cell wall elicitors. (Paper III, Fig. 3). Actin cytoskeleton forms a dynamic network that rapidly reorganizes in response to a variety of stimuli and regulated by JA mediated plant defense responses (Staiger 2000, Turner *et al.,* 2002, Shimada *et al.,* 2006). In this study, the results also indicated that the weak fungal pathogens had similar defence responses to ECM fungus, but a little higher expression for each tested genes. The weak pathogenic fungus may act as non pathogenic fungus to *P. sylvestris* since the ethylene synthase gene was not accumulated as high as that induced by *H. annosum*. Hence we concluded that in *P. sylvestris–H. parviporum* pathosystem, JA signaling pathway but not ethylene regulates the defense responses. This reaction is similar to the wound induced defence responses in maize as described by Ankala *et al.* (2009).

Similar responses were also detected in genes involved in cell replication, hormone, signal transduction and metabolism, such as *EF-1A* (elongation factor-1 alpha) and *Ps-ACS1* (ethylene synthase) (II) (III, Fig. 3). *EF-1A* will be discussed in paper IV since we also isolated a similar gene. The significantly increased transcripts of *PsACS1* indicated that ethylene was involved in the defence against necrotrophic fungi. This result was in line with

the observation that necrotrophic fungus elicits defense response regulated by ethylene and JA signaling pathway (Glazebrook 2005).

The fungi used in this study also appear to have possessed some characteristic features in their cell walls that activated plant innate immunity. Recently, the term „MAMPs' and „PAMPs' (microbe- or pathogen-associated molecular patterns) have been used to describe fungal cell wall elicitors such as oligosaccharide (Leitner *et al.,* 2008) and α-glucan (Wolski *et al.,* 2007). The defense responses in somatic embryos to fungal elicitors indicated that the young emblings are already well equipped with innate immunity to respond to stress.

4.4. Induced defence responses in *P. sylvestris* seedlings (paper IV)

Ectomycorrhizal fungus *S. bovinus* (SBHI) (III, IV), cyanobacteris *Nostoc spp*. and pathogenic fungus *H. annosum* (III, IV) were used to study the plant-microbe interaction (IV, Fig.1). After one month incubation, *S. bovinus* treated seedling grew as well as the uninoculated control. The number of root tip was similar as that of the control, but with higher percentage of dichotomous roots (IV, Fig. 2 & 3). Similar results have previously been observed by Laajanen *et al*. (2007) that ECM fungi did not induce lateral root formation. The numbers of dichotomous roots obtained in this study were similar to that observed by direct fungus inoculation in pine (Niini & Raudaskoski 1998, Sarjala *et al.,* 2010). The genetic change may be the reason for the morphological change. We have observed that *act1* increased (paper III) during treatment with *S. bovinus* cell wall extracts. The host plant manipulated cell wall regulation gene, such as *act1,* against ECM infection. Chitinase genes, required for root cell wall reorganization, are known to be expressed when host plants encounter ECM infection (Martins *et al.,* 1999, Tagu *et al.,* 2002 Frettinger *et al.,* 2007). Herrman *et al.* (2007) reported that chitinase was only expressed in the lateral roots, where dichotomous roots form. The host plant often responds to ECM fungi by changing root morphological and genetical state to sustain the ECM symbiosis.

Increased number of the root tips and weight of the seedlings were observed in cyanobacteria treated seedlings. Cyanobacteria provide biological nitrogen to seedling during the incubation period. However, the nodule-like structure needs to be investigating further since *Nostoc* cyanobacteria usually form the cyanbiont cavities inside the plant (Adams 2008). The *H.*

annosum treated seedlings were smaller than the control and the needle tips turned brown and necrotic (Fig. 3).

Six gene fragments were cloned and sequenced from the SBH1 treated samples in addition to the control. The isolated gene products encoded five different proteins (paper IV, Table1.). *Ps-Con1* and *Ps-Con2* were isolated from the control and were found to encode aspartyl-tRNA synthetase (*AspRS*). *AspRS*, a class IIb aminoacyl tRNA synthetase (*AARS*), may be involved in large complexes of aaRS, the *EF-1A* elongation factor, and ribosomes (Stapulionis & Deutscher 1995). The gene encoding elongation factor-1A (*EF-1A*) is functionally homologous to the prokaryotic elongation factor EF-Tu (Kidou & Ejiri 1998), one of the well known PAMPs that trigger the defence response in plant (Hahn 1996, Zipfel *et al.,* 2006, Schwessinger & Zipfel 2008). The ECM fungus and weak pathogenic fungus could induce expression of *EF-1A* in emblings (Paper III) while downregulated by *H. annosum*.

Another gene, *Ps-Sbe1*, encodes the GTP-binding protein, which shares homology to domain II of the elongation factors (EFs) EF-G and EF-Tu. EF-Tu is the most abundant bacterial proteins and is actively used as a PAMP for recognition by *A. thaliana* plants (Kunze *et al.,* 2004).

The *Ps-Sbe2* was also found to have a 99% similarity to a human gene that encodes *rag C* protein, NACA (nascent-poly-peptide-associated complex α-polypeptide) pseudogene. Several lines of evidence suggested that the α-NAC could function as a transcriptional coactivator (Akhouayri *et al.* 2005) and may play a role in proliferation and differentiation of the cells (Al-Shanti & Aldahoodi 2006).

The *Ps-Sbe3* fragment contains putative conserved domain, SGA1 (Glucoamylase and related glycosyl hydrolases), which function in carbohydrate transport and the related metabolism. *Ps-Sbe3* also contains a small part encoding actin filament-associated protein (AFAP) with 86% identity. AFAP acts as an adaptor protein that links signalling molecules to actin (III) filaments (Baisden *et al.*, 2001).

Fragment *Ps-Sbe4* (Table1.) was similar to bilirubin oxidase / multicopper oxidase (MCOs). MCOs such as CueO, bilirubin oxidase, and laccase contain four Cu sites, type (I) Cu, type (II) Cu, and a pair of type (III) Cu's in the protein molecule. Cu (III) oxidase converts the final electron acceptor O_2 to H_2O without releasing activated oxygen species (ROS) (Sakurai &

Kataoka 2007). It is found that increased ROS also increased ethylene production during plant–fungus interaction (Wi et al., 2010). The expression of *Ps-Sbe4* probably indicates that fewer oxygen species and less amount of ethylene will be released during ECM fungus-plant interaction. Possibly this could explain how the system regulate ethylene, an antagonist of JA signalling pathway, during plant-ECM fungus interaction.

5. CONCLUSIONS

Many factors including medium conditions, cold storage time, paternal and maternal effects on the success of SE in *P. sylvestris* were investigated. We demonstrated that maternal effects occurred in the absence of paternal effects. Moreover, the absence of no maternal × paternal interaction suggests that selecting elite maternal genotypes were crucial for the success of SE. Effects of plant growth regulator ethylene were investigated for embryo maturation from various genotypes. Two ACC synthase genes, the precursor of ethylene synthesis, were isolated from ECs in *P. sylvestris*. They were related to different developmental stage of somatic embryo development. *PsACS2* was expressed in embryo stage in all the genotypes, which indicated that *PsACS2* could be a genetic marker for early maturation stage. Since ethylene requirements differs from genotypes and developmental stages, improving somatic embryo production by modulating ethylene production should be genotype-specific and stage-specific in the application.

In parallel to the above studies, the effects of biotic factors (fungal cell wall elicitors) on somatic embryos were also studied. Fungal cell wall elicitors from *S. bovinus* or *H. parviprum* induced different plant responses by compared to that from pathogenic *H. annosum*. The study demonstrates that mature somatic embryos of *P. sylvestris* are already well equipped with innate immune system to respond to defend the environmental/biological stress and could serve as suitable model plants to study tree-fungal interaction at molecular level.

Finally, the results of the interaction experiments with seedlings indicate that elicitation of conifer host defences could be provoked without direct physical contact with the associating microbe.

6. ACKNOWLEDGEMENTS

The present study was carried out at the University of Helsinki, Faculty of Agriculture, Department of Agricultural Sciences (former Department of Applied Biology) and Department of Forest Sciences. The project was supported by Helsinki University Research Foundation, Project No. 2108012. European Community project No. QLK5-CK-1999-00679, forest breeding foundation in 2007 and in 2008 and Suomen Metsätieteellinen Seura 2010.

I wish to express my sincere gratitude to emeritus Prof. Kim von Weissenberg, former supervisor, who accepted me as a PhD student. I am also grateful to my advisor Dr. Ari Pappinen for leading me to my PhD thesis topic „somatic embryogenesis of Scots pine' and patiently arranging funding for me. I would like to give my thanks to Prof. Fred O. Asiegbu, head of the forest pathology division and the most recent supervisor of this work, for his encouragement, helpful supervision and valuable comments. I wish to thank members of my thesis follow-up group (Prof. Teemu Teeri and Dr. Tuija Aronen) for their support and encouragement over all these years.

My sincere thanks go to all the co-authors, especially Dr. Jorma Vahala, who led me to the "Ethylene" work in addition to his contribution in the lab, his editing and critical remarks on the manuscript. Thanks also go to Anna-Maija Niskanen, my coworker and dear friend, for her support, in editing and commenting on my manuscripts. Special thanks also for your kindness, willingness to help during this journey.

I am indebted to Dr. Hanna Pasonen for her thoughtful comments on my manuscripts and friendly support. I learned a lot from your attitude towards life. You are not only a colleague to me, but more than that, a dear friend, and mentor. I am also grateful to my former colleague, Dr. Sanna Seppänen for your friendship, sharing laughter and tears of life. I wish to gratefully acknowledge the useful comments I received from Dr. Kari Korhonen, you are my hero as a scientist.

I wish to express my appreciation to all my colleagues and friends in KPAT and MPAT, especially to Marjo Kilpinen, Dr. Anna Kärkönen, Dr Laura Mattinen, Dr. Timo Hytönen, Lahja Pesonen, Terttu Parkkari and Jouko Närhi for your assistance in addition to creating a

friendly environment at work. Docent Risto Kasanen, Dr. Yeshtila Degefu, Dr. Nora Garcia, Hui Sun and all the other former and present members of Forest pathology group are heartily thanked for being such good friends and colleagues, and sharing the ups and downs of both professional and private life.

I also wish to thank my dear friends, inside and outside of the University, especially to Huang Hua (Xu), Lu Wei, Yu Liying, Chang Wei, Wang Limei, Liu Changli, Katariina Mustakallio and Oili Lähelmä. Without you, my life in Finland would have been lonely.

It has been a long and hard trip to this point. I wish to dedicate this thesis to my husband Xing, without you, I wouldn't have found my lost files in the new computer system. Thanks for your patience, encouragement and sometimes criticism to keep me going in life. My dear honeys, Huan and Ying, you both are my heart and my all. I feel grateful that Ying understands that I have to work late. My sincere gratitude also goes to my father, mother-in-law, brother and sisters back in China.

Espoo, May, 2010

7. REFERENCES

Adams DG, Duggan PS, 2008. Cyanobacteria-bryophyte symbioses. *Journal of Experimental Botany* 59, 1047-58.

Adams DO, Yang SF, 1979. Ethylene biosynthesis identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proceedings of the National Academy of Sciences of the United States of America* 76, 170-4.

Akhouayri O, Quélo I, St-Arnaud R, 2005. Sequence-specific DNA binding by the α_NAC coactivator is required for potentiation of c-jun-dependent transcription of the osteocalcin gene. *Molecular Cell Biology* 25, 3452-60.

Alexander L, Grierson D, 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *Journal of Experimental Botany* 53, 2039-55.

Al-Shanti N, Aldahoodi Z, 2006. Inhibition of alpha nascent polypeptide associated complex protein may induce proliferation, differentiation and enhance the cytotoxic activity of human $CD8^{(+)}$ T cells. *Journal of Clinical Immunology* 26, 457-64.

Ankala A, Luthe DS, Williams WP, Wilkinson JR, 2009. Integration of ethylene and jasmonic acid signaling pathways in the expression of maize defense protein *Mir1-CP*. *Molecular Plant-Microbe Interactions* 22, 1555-64.

Aquea F, Arce-Johnson P, 2008. Identification of genes expressed during early somatic embryogenesis in *Pinus radiata*. *Plant Physiology and Biochemistry* 46, 559-68.

Aquea F, Poupin MJ, Matus JT, Gebauer M, Medina C, Arce-Johnson P, 2008. Synthetic seed production from somatic embryos of *Pinus radiata*. *Biotechnology Letters* 30, 1847-52.

Argueso CT, Hansen M, Kieber JJ, 2007. Regulation of ethylene biosynthesis. *Journal of Plant Growth Regulation* 26, 92-105.

Aronen TS, Nikkanen TO, Häggman HM, 2003. The production of transgenic Scots pine (*Pinus sylvestris* L.) via the application of transformed pollen in controlled crossings. *Transgenic Research* 12, 375-8.

Aronen T, Pehkonen T, Ryynänen L, 2009. Enhancement of somatic embryogenesis from immature zygotic embryos of *Pinus sylvestris*. *Scandinavian Journal of Forest Research* 24, 372-83.

Arteca JM, Arteca RN, 1999. A multi-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase (*ACS6*) in mature *Arabidopsis* leaves. *Plant Molecular Biology* 39, 209-19.

Asiegbu FO, Abu S, Stenlid J, Johansson M, 2004. Sequence polymorphism and molecular characterization of *laccase* genes of the conifer pathogen *Heterobasidion annosum*. *Mycological research* 108, 136-48.

Asiegbu FO, Adomas A, Stenlid J, 2005. Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. s.l. *Molecular Plant Ppathology* 6, 395-409.

Attree SM, Moore D, Sawhney VK, Fowke LC, 1991. Enhanced maturation and desiccation tolerance of White Spruce [*Picea glauca* (Moench) Voss] Somatic embryos - effects of a non-plasmolysing water stress and abscisic acid. *Annals of Botany* 68, 519-25.

Attree SM, Fowke LC, 1993. Embryogeny of Gymnosperms - Advances in Synthetic Seed Technology of Conifers. *Plant Cell Tissue and Organ Culture* 35, 1-35.

Ayers AR, Ebel JF, Berger N, Albersheim P, 1976. Host-Pathogen Interactions: IX. Quantitative Assays of elicitor activity and characterization of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. *Plant Physiology* 57, 751-9.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM, 2006. The role of root exudates in rhizosphere interations with plants and other organisms. *Annual Review of Plant Biology* 57, 233-66.

Baisden JM, Qian Y, Zot HM, Flynn DC, 2001. The actin filament-associated protein AFAP-110 is an adaptor protein that modulates changes in actin filament integrity. *Oncogene* 20, 6435-47.

Barnes JR, Lorenz WW, Dean JFD, 2008. Characterization of a 1-aminocyclopropane-1-carboxylate synthase gene from loblolly pine (*Pinus taeda* L.). *Gene* 413, 18-31.

Barry CS, Blume B, Bouzayen M, Cooper W, Hamilton AJ, Grierson D, 1996. Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. *Plant Journal* 9, 525-35.

Barry CS, Llop-Tous MI, Grierson D, 2000. The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. *Plant Physiology* 123, 979-86.

Bartley GE, Ishida BK, 2007. Ethylene-sensitive and insensitive regulation of transcription factor expression during in vitro tomato sepal ripening. *Journal of Experimental Botany* 58, 2043-51.

Bartnick S, 1968. Cell wall chemistry morphogenesis and taxonomy of fungi. *Annual Review of Microbiology* 22, 87-108.

Becwar MR, Nagmani R, Wann SR, 1990. Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). *Canadian Journal of Forest Research-Revue canadienne de Recherche Forestiere* 20, 810-7.

Becwar MR, Pullman GS, 1995. Somatic embryogenesis in loblolly pine (*Pinus taeda* L.). In: Jain S, Gupta PK and Newton RJ, eds. *Somatic embryogenesis in woody plants.* The Netherlands: Kluwer academic publishers, 287-301.

Belmonte MF, Donald G, Reid DM, Yeung EC, Stasolla C, 2005. Alterations of the glutathione redox state improve apical meristem structure and somatic embryo quality in white spruce (*Picea glauca*). *Journal of Experimental Botany* 56, 2355-64.

Belmonte MF, Tahir M, Schroeder D, Stasolla C, 2007. Overexpression of *HBK3*, a class *IKNOX* homeobox gene, improves the development of Norway spruce (*Picea abies*) somatic embryos. *Journal of Experimental Botany* 58, 2851-61.

Belmonte MF, Stasolla C, 2009. Altered *HBK3* expression affects glutathione and ascorbate metabolism during the early phases of Norway spruce (*Picea abies*) somatic embryogenesis. *Plant Physiology and Biochemistry* 47, 904-11.

Berman-Frank I, Lundgren P, Falkowski P, 2003. Nitrogen fixation and photosynthetic oxygen evolution in *cyanobacteria*. *Research in Microbiology* 154, 157-64.

Bi HH, Song YY, Zeng RS, 2007. Biochemical and molecular responses of host plants to mycorrhizal infection and their roles in plant defence. *Allelopathy Journal* 20, 15-27.

Bishop-Hurley SL, Zabkiewicz RJ, Grace L, Gardner RC, Wagner A, Walter C, 2001. Conifer genetic engineering: transgenic *Pinus radiata* (D. Don) and *Picea abies* (Karst) plants are resistant to the herbicide *Buster*. *Plant Cell Reports* 20, 235-43.

Bishop-Hurley SL, Gardner RC, Walter C, 2003. Isolation and molecular characterization of genes expressed during somatic embryo development in *Pinus radiata*. *Plant Cell Tissue and Organ Culture* 74, 267-81.

Bleecker AB, Kende H, 2000. Ethylene: A gaseous signal molecule in plants. *Annual Review of Cell and Developmental Biology* 16, 1-18.

Boller T, 1991. Ethylene in pathogenesis and disease resistance. In: Mattoo AK SJ, ed. *The Plant Hormone Ethylene.* Boca Raton, FL, USA: CRC Press, 293-314.

Boller T, Felix G, 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology* 60, 379-406.

Bolton MD, Van Esse HP, Vossen JH, De Jonge R, Stergiopoulos I, Stulemeijer IJE, Van Den Berg GCM, Borrás-Hidalgo O, Dekker HL, de Koster CG, de Wit PJGM, Joosten MHAJ, Thomma BPHJ, 2008. The novel *Cladosporium fulvum* lysin motif effector *Ecp6* is a virulence factor with orthologues in other fungal species. *Molecular Microbiology* 69, 119-36.

Bomal C, Tremblay FM, 1999. Effect of desiccation to low moisture content on germination, synchronization of root emergence, and plantlet regeneration of black spruce somatic embryos. *Plant cell tissue and organ culture* 56, 193-200.

Bomal C, Le VQ, Tremblay FM, 2002. Induction of tolerance to fast desiccation in black spruce (*Picea mariana*) somatic embryos: relationship between partial water loss, sugars, and dehydrins. *Physiologia Plantarum* 115, 523-30.

Bonga JM, Klimaszewska KK, von Aderkas P, 2010. Recalcitrance in clonal propagation, in particular of conifers. *Plant cell Tissue and Organ Culture* 100, 241-54.

Bothe H, Tripp HJ, Zehr JP, 2010. Unicellular *cyanobacteria* with a new mode of life: the lack of photosynthetic oxygen evolution allows nitrogen fixation to proceed. *Archives of Microbiology* 192, 783-90.

Bozhkov PV, von Arnold S, 1998. Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. *Physiologia Plantarum* 104, 211-24.

Breton C, Cornu D, Chriqui D, Sauvanet A, Capelli P, Germain E, Jay-Allemand C, 2004. Somatic embryogenesis, micropropagation and plant regeneration of "Early Mature" walnut trees (*Juglans regia*) that flower in vitro. *Tree physiology* 24, 425-35.

Brownfield DL, Todd CD, Stone SL, Deyholos MK, Gifford DJ, 2007. Patterns of storage protein and triacylglycerol accumulation during loblolly pine somatic embryo maturation. *Plant Cell Tissue and Organ Culture* 88, 217-23.

Brukhin VD, Elfstand CM, vonArnold S, 2000. Basta tolerance as a selectable and screening marker for trandgenic plants of Norway Spruce. *Plant Cell Reports* 19, 899-903.

Burg K, Helmersson A, Bozhkov P, von Arnold S, 2007. Developmental and genetic variation in nuclear microsatellite stability during somatic embryogenesis in pine. *Journal of Experimental Botany* 58, 687-98.

Cairney J, Pullman GS, 2007. The cellular and molecular biology of conifer embryogenesis. *New Phytologist* 176, 511-36.

Cairney J, Xu NF, Mackay J, Pullman J, 2000. Transcript profiling: A tool to assess the development of conifer embryos. *In vitro Cellular & Developmental Biology-Plant* 36, 155-62.

Cairney J, Zheng L, Cowels A, Hsiao J, Zismann V, Liu J, Ouyang S, Thibaud-Nissen F, Hamilton J, Childs K, Pullman GS, Zhang Y, Oh T, Buell CR, 2006. Expressed Sequence Tags from loblolly pine embryos reveal similarities with angiosperm embryogenesis. *Plant Molecular Biology* 62, 485-501.

Canovas FM, Canton FR, Garcia-Gutierrez A, Gallardo F, Crespillo R, 1998. Molecular physiology of glutamine and glutamate biosynthesis in developing seedlings of conifers. *Physiologia Plantarum* 103, 287-94.

Carneros2009

Chae HS, Kieber JJ, 2005. Eto Brute? Role of ACS turnover in regulating ethylene biosynthesis. *Trends in Plant Science* 10, 291-6.

Chalupa V, 1985. Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. *Comm. inst. Forest Chech.* 14, 57-63.

Chandler SF, Young R, 1995. Somatic embryogenesis in *Pinus radiata* Don. In: Jain SM, Gupta PK and Newton RJ, eds. *Somatic embryogenesis in woody plants.* Dordrecht: Kluwer academic publishers, 243-56.

Chang S, Puryear J, Cairney J, 1993. A simple and efficient method for isolation RNA from pine trees. *Plant Molecular Biology Reports* 11, 113-6.

Chen S, Chen S, Chen F, Wu T, Wang Y, Yi S, 2010. Somatic embryogenesis in mature zygotic embryos of *Picea likiangensis*. *Biologia* 65, 853-8.

Choudhury H, Kumaria S, Tandon P, 2008. Induction and maturation of somatic embryos from intact megagametophyte explants in Khasi pine (*Pinus kesiya* Royle ex. Gord.). *Current Science* 95, 1433-8.

Ciuffetti LM, Tuori RP, Gaventa JM, 1997. A single gene encodes a selective toxin causal to the development of tan spot of wheat. *Plant Cell* 9, 135-44.

Courdavault V, Burlat V, St-Pierre B, Giglioli-Guivarc'h N, 2009. Proteins prenylated by type I protein geranylgeranyltransferase act positively on the jasmonate signalling pathway triggering the biosynthesis of monoterpene indole alkaloids in *Catharanthus roseus*. *Plant Cell Reports* 28, 83-93.

Crocker W, Knight LI, 1908. Effect of illuminating gas and ethylene upon flowering carnation. *Botanical Gazette* 46, 259-76.

Cyr DR, Klimaszewska K, 2002. Conifer somatic embryogenesis: II. Applications. *Dendrobiol* 48, 41-9.

Day B, Graham T, 2007. The plant host-pathogen interface - cell wall and membrane dynamics of pathogen-induced responses. *Stress Responses in Biology and Medicine* 1113, 123-34.

de Wit PJGM, Mehrabi R, van den Burg HA, Stergiopoulos I, 2009. Fungal effector proteins: past, present and future. *Molecular Plant Pathology* 10, 735-47.

Debaud JC, Marmeisse R, Gay G, 1995. Intraspecific genetic variation in ectomycorrhizal fungi. In: Varma A and Hock B, eds. *Mycorrhiza: structure, function, molecular biology and biotechnology.* Heidelberg: Springer-Verlag, 79-113.

Diouf D, 2003, genetic transformation of forest trees. *Africa Journal of Biotechnology* 2: 328-333.

Dixon RA, 1986. The phytoalexin response - elicitation, signaling and control of host gene-expression. *Biological Reviews of the Cambridge Philosophical Society* 61, 239-91.

Dixon RA, Lamb CJ, 1990. Molecular communication in interactions between plants and microbial pathogens. *Annual review of plant physiology and plant molecular biology* 41, 339-67.

Dong JZ, Dunstan DI, 1996. Characterization of three heat-shock-protein genes and their developmental regulation during somatic embryogenesis in white spruce [*Picea glauca* (Moench) Voss]. *Planta* 200, 85-91.

Dong JZ, Dunstan DI, 1997. Endochitinase and β-1,3-glucanase genes are developmentally regulated during somatic embryogenesis in *Picea glauca*. *Planta* 201, 189-94.

Durzan DJ, Chalupa V, 1976. Growth and metabolism of cells and tissue of Jack pine (*Pinus banksiana*). 3. Growth of cells in liquid suspension cultures in light and darkness. *Canadian Journal of Botany-Revue Canadienne de Botanique* 54, 456-67.

Eckardt NA, 2006. The role of flavonoids in root nodule development and auxin transport in *Medicago truncatula* . *Plant Cell* 18, 1539-40.

El Meskaoui A, Tremblay F, 1999. Effects of sealed and vented gaseous microenvironments on the maturation of somatic embryos of black spruce with a special emphasis on ethylene. *Plant Cell, Tissue and Organ Culture* 56, 201-9.

El Meskaoui A, Tremblay FM, 2001. Involvement of ethylene in the maturation of black spruce embryogenic cell lines with different maturation capacities. *Journal of Experimental Botany* 52, 761-9.

Elfstrand M, Fossdal CG, Swedjemark G, Clapham D, Olsson O, Sitbon F, Sharma P, Lonneborg A, von Arnold S, 2001. Identification of candidate genes for use in molecular breeding - A case study with the Norway spruce defensin-like gene, Spi 1. *Silvae Genetica* 50, 75-81.

Ellis JG, Rafiqi M, Gan P, Chakrabarti A, Dodds PN, 2009. Recent progress in discovery and functional analysis of effector proteins of fungal and *oomycete* plant pathogens. *Current Opinion in Plant Biology* 12, 399-405.

Fay P, 1992. Oxygen relations of nitrogen-fixation in *Cyanobacteria*. *Microbiological reviews* 56, 340-73.

Felten J, Kohler A, Morin E, Bhalerao RP, Palme K, Martin F, Ditengou FA, Legue V, 2009. The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in *Poplar* and *Arabidopsis* through auxin transport and signaling. *Plant Physiology* 151, 1991-2005.

Fernbach E, Mohr H, 1990. Coaction of blue ultraviolet-a light and light absorbed by phytochrome in controlling growth of pine (*Pinus sylvestris* L.) seedlings. *Planta* 180, 212-6.

Find J, Grace L, Krogstrup P, 2002. Effect of anti-auxins on maturation of embryogenic tissue cultures of Nordmanns fir (*Abies nordmanniana*). *Physiologia Plantarum* 116, 231-7.

Fischerova L, Fischer L, Vondrakova Z, Vagner M, 2008. Expression of the gene encoding transcription factor *PaVP1* differs in *Picea abies* embryogenic lines depending on their ability to develop somatic embryos. *Plant Cell Reports* 27, 435-41.

Fliegmann J, Mithofer A, Wanner G, Ebel J, 2004. An ancient enzyme domain hidden in the putative beta-glucan elicitor receptor of soybean may play an active part in the perception of pathogen-associated molecular patterns during broad host resistance. *Journal of Biological Chemistry* 279, 1132-40.

Ford CS, Jones NB, van Staden J, 2000a. Cryopreservation and plant regeneration from somatic embryos of *Pinus patula*. *Plant cell reports* 19, 610-5.

Ford CS, Jones NB, Van Staden J, 2000b. Optimization of a working cryopreservation protocol for *Pinus patula* embryogenic tissue. *In vitro Cellular & Developmental Biology-Plant* 36, 366-9.

Frederico AM, Zavattieri MA, Campos MD, Cardoso HG, McDonald AE, Arnholdt-Schmitt B, 2009. The gymnosperm *Pinus pinea* contains both *AOX* gene subfamilies, *AOX1* and *AOX2*. *Physiologia Plantarum* 137, 566-77.

Frettinger P, Derory J, Herrmann S, Plomion C, Lapeyrie F, Oelmueller R, Martin F, Buscot F, 2007. Transcriptional changes in two types of pre-mycorrhizal roots and in ectomycorrhizas of oak microcuttings inoculated with *Piloderma croceum*. *Planta* 225, 331-40.

Friml J, 2003. Auxin transport - shaping the plant. *Current Opinion in Plant Biology* 6, 7-12.

Garin E, Bernier-Cardou M, Isabel N, Klimaszewska K, Plourde A, 2000. Effect of sugars, amino acids, and culture technique on maturation of somatic embryos of *Pinus strobus* on medium with two gellan gum concentrations. *Plant Cell Tissue and Organ Culture* 62, 27-37.

Gautheret RJ, 1983. Plant tissue culture - a history. *Botanical Magazine-Tokyo* 96, 393-410.

Gemperlova L, Fischerova L, Cvikrova M, Mala J, Vondrakova Z, Martincova O, Vagner M, 2009. Polyamine profiles and biosynthesis in somatic embryo development and comparison of germinating somatic and zygotic embryos of Norway spruce. *Tree physiology* 29, 1287-98.

Glazebrook J, 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* 43, 205-27.

Govindasamy V, Senthilkumar M, Mageshwaran V, Annapurna K, 2009. Detection and characterization of ACC deaminase in plant growth promoting rhizobacteria. *Journal of Plant Biochemistry and Biotechnology* 18, 71-6.

Gundale MJ, Gustafsson H, Nilsson MC, 2009. The sensitivity of N-fixation by a cyanobacteria-feathermoss association to litter and moisture variability in young and old boreal forests. *Canadian Journal of Forest Research* 39, 2542-9.

Gupta PK, Durzan DJ, 1986a. Plantlet regeneration via somatic embryogenesis from subcultured callus of mature embryos of *Picea abies* (Norway Spruce). *In vitro Cellular & Developmental Biology-Plant* 22, 685-8.

Gupta PK, Durzan DJ, 1986b. Somatic polyembryogenesis from callus of mature sugar pine embryos. *Bio/Technology* 4, 643-5.

Gupta PK, Durzan DJ, 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. *Bio-Technology* 5, 147-51.

Gupta PK, Grob JA, 1995. Somatic embryogenesis in conifers. In: Jain S, Gupta PK and Newton RJ, eds. *Somatic Embryogenesis in Woody Plants.* p81-98.

Häggman H, Aronen T, 1998. Transgene expression in regenerating cotyledons and embryogenic cultures of Scots pine. *Journal of Experimental Botany* 49, 1147-56.

Häggman HM, Ryynanen LA, Aronen TS, Krajnakova J, 1998. Cryopreservation of embryogenic cultures of Scots pine. *Plant Cell Tissue and Organ Culture* 54, 45-53.

Häggman H, Jokela A, Krajnakova J, Kauppi A, Niemi K, Aronen T, 1999. Somatic embryogenesis of Scots pine: cold treatment and characteristics of explants affecting induction. *Journal of Experimental Botany* 50, 1769-78.

Häggman HM, Aronen TS, Ryynanen LA, 2000. Cryopreservation of embryogenic cultures of conifers. *Somatic Embryogenesis in Woody Plants, Vol* 667, 707-28.

Häggman H, Vuosku J, Sarjala T, Jokela A, Niemi K, 2006. Somatic embryogenensis of pine species: from functional genomics to plantation forestry. In: Mujib A and Samaj J, eds. *Somatic Embryogenesis.* Berlin: Springer, 119-40.

Hahn MG, 1996. Microbial elicitors and their receptors in plants. *Annual Review of Phytopathology* 34, 387-412.

Hakman I, Fowke LC, Von Arnold S, Eriksson T, 1985. The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). *Plant Science* 38, 53-9.

Hakman I, Fowke LC, 1987. Somatic Embryogenesis in *Picea glauca* (White Spruce) and *Picea mariana* (black spruce). *Canadian Journal of Botany-Revue Canadienne de Botanique* 65, 656-9.

Hartmann A, Schmid M, van Tuinen D, Berg G, 2009. Plant-driven selection of microbes. *Plant and Soil* 321, 235-57.

Hay EI, Charest PJ, 1999. Somatic embryo germination and desiccation tolerance in conifers. In: Mohan Jain S, Gupta PK and Newton RJ, eds. *Somatic Embryogenesis in Woody Plants.* Dordrecht, The Netherlands: Kluwer academic publishers, 61-9.

He P, Shan L, Sheen J, 2007. Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. *Cellular Microbiology* 9, 1385-96.

Herrmann S, Buscot F, 2007. Cross talks at the morphogenetic, physiological and gene regulation levels between the mycobiont *Piloderma croceum* and oak microcuttings (*Quercus robur*) during formation of ectomycorrhizas. *Phytochemistry* 68, 52-67.

Henderson AR, Walter C, 2006. Genetic engineering in conifer plantation forestry. *Silvae Genetica* 55, 253-62.

Hill DR, Belbin TJ, Thorsteinsson MV, Bassam D, Brass S, Ernst A, Boger P, Paerl H, Mulligan ME, Potts M, 1996. GlbN (cyanoglobin) is a peripheral membrane protein that is restricted to certain *Nostoc spp*. *Journal of Bacteriology* 178, 6587-98.

Hohtola A, 1995. Somatic embryogenesis in Scots pine (*Pinus sylvestris* L.). *Somatic Embryogenesis in Woody Plants* 3, 269–285.

Honegger R, 1991. Functional-Aspects of the Lichen Symbiosis. *Annual Review of Plant Physiology and Plant Molecular Biology* 42, 553-78.

Huang XL, Li XJ, Li Y, Huang LZ, 2001. The effect of AOA on ethylene and polyamine metabolism during early phases of somatic embryogenesis in *Medicago sativa*. *Physiologia Plantarum* 113, 424-9.

Iraqi D, Quy Le V, Lamhamedi MS, Tremblay FM, 2005. Sucrose utilization during somatic embryo development in black spruce: involvement of apoplastic invertase in the tissue and of extracellular invertase in the medium. *Journal of Plant Physiology* 162, 115-24.

Jimenez VM, 2005. Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. *Plant Growth Regulation* 47, 91-110.

Johansson SM, Lundgren LN, Asiegbu FO, 2004. Initial reactions in sapwood of Norway spruce and Scots pine after wounding and infection by *Heterobasidion parviporum* and *H. annosum*. *Forest Pathology* 34, 197-210.

Jones JDG, Dangl JL, 2006. The plant immune system. *Nature* 444, 323-9.

Jones NB, van Staden J, 2001. Improved somatic embryo production from embryogenic tissue of *Pinus patula*. *In vitro Cellular & Developmental Biology-Plant* 37, 543-9.

Karlsson JO, Stenlid J, 1991. Pectic Isozyme Profiles of Intersterility Groups in Heterobasidion-Annosum. *Mycological Research* 95, 531-6.

Kasprzewska A, 2003. Plant chitinases - Regulation and function. *Cellular & Molecular Biology Letters* 8, 809-24.

Keen NT, 1975. Specific elicitors of plant phytoalexin production - determinants of race specificity in pathogens. *Science* 187, 74-5.

Keinonen-Mettälä K, Jalonen P, Eurola P, VonArnold S, VonWeissenberg K, 1996. Somatic embryogenesis of *Pinus sylvestris*. *Scandinavian Journal of Forest Research* 11, 242-50.

Kende H, 1993. Ethylene biosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 44, 283-307.

Kidou S, Ejiri S, 1998. Isolation, characterization and mRNA expression of four cDNAs encoding translation elongation factor 1A from rice (*Oryza sativa* L.). *Plant Molecular Biology* 36, 137-48.

Kim CY, Liu YD, Thorne ET, Yang HP, Fukushige H, Gassmann W, Hildebrand D, Sharp RE, Zhang SQ, 2003. Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants. *Plant Cell* 15, 2707-18.

Kiraly L, Barnaz B, Kiralyz Z, 2007. Plant resistance to pathogen infection: Forms and mechanisms of innate and acquired resistance. *Journal of Phytopathology* 155, 385-96.

Klee HJ, Clark DG, 2004. Ethylene signal transduction in fruits and flowers. In: Davies PJ, ed. *Plant hormones*: Springer Netherlands, 369-90.

Klimaszewska K, Smith DR, 1997. Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. *Physiologia Plantarum* 100, 949-57.

Klimaszewska K, Bernier-Cardou M, Cyr DR, Sutton BCS, 2000. Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. *In vitro Cellular & Developmental Biology-Plant* 36, 279-86.

Klimaszewska K, Park YS, Overton C, Maceacheron I, Bonga JM, 2001. Optimized somatic embryogenesis in *Pinus strobus* L. *In vitro Cellular & Developmental Biology-Plant* 37, 392-9.

Klimaszewska K, Cyr DR, 2002. Conifer somatic embryogenesis: I. Development. *Dendrobiol* 48, 31-9.

Klimaszewska K, Morency F, Jones-Overton C, Cooke J, 2004. Accumulation pattern and identification of seed storage proteins in zygotic embryos of *Pinus strobus* and in somatic embryos from different maturation treatments. *Physiologia Plantarum* 121, 682-90.

Klimaszewska K, Noceda C, Pelletier G, Label P, Rodriguez R, Lelu-Walter MA, 2009. Biological characterization of young and aged embryogenic cultures of *Pinus pinaster* (Ait.). *In vitro Cellular & Developmental Biology-Plant* 45, 20-33.

Klimaszewska K, Pelletier G, Overton C, Stewart D, Rutledge RG, 2010. Hormonally regulated overexpression of *Arabidopsis* WUS and conifer LEC1 (*CHAP3A*) in transgenic white spruce: implications for somatic embryo development and somatic seedling growth. *Plant Cell Reports* 29, 723-34.

Kneip C, Lockhart P, Voss C, Maier U, 2007. Nitrogen fixation in eukaryotes - New models for symbiosis. *Bmc Evolutionary Biology* 7, 55.

Kong LS, Yeung EC, 1994. Effects of ethylene and ethylene inhibitors on white spruce somatic embryo maturation. *Plant Science* 104, 71-80.

Kong L, Attree SM, Evans DE, Binarova P, Yeung EC, Fowke LC, 1999. Somatic embryogenesis in white spruce: studies of embryo development and cell biology. In: Jain SM, Gupta PK and Newton RJ, eds. *Somatic embryogenesis in woody plants.* Dordrecht, The Netherlands: Kluwer academic publishers, 1-28.

Konradova H, Grigova M, Lipavska H, 2003. Cold-induced accumulation of raffinose family oligosaccharides in somatic embryos of Norway spruce (*Picea abies*). *In vitro Cellular & Developmental Biology-Plant* 39, 425-7.

Korhonen K, Stenlid J, 1998. Biology of *Heterobasidion annosum*. In: Woodward S, Stenlid J, Karjalainen R and Hütterman A, eds. *Heterobasidion annosum: biology, ecolgy, impact and control.* London: CAB international, 43-71.

Kotai J, 1972. Instructions for preparation of modified nutrient solution Z8 for algae. Norwegian institute for water research publication B: 11-69.

Kumar PP, Joy IVRW, Thorpe TA, 1989. Ethylene and carbon dioxide accumulation and growth of cell suspension cultures of white spruce (*Picea glauca*). *Journal of Plant Physiology* 135, 592-6.

Kumar PP, Lakshmanan P, Thorpe TA, 1998. Regulation of morphogenesis in plant tissue culture by ethylene. *In vitro Cellular & Developmental Biology-Plant* 34, 94-103.

Kumar V, Parvatam G, Ravishankar GA, 2009. AgNO3 - a potential regulator of ethylene activity and plant growth modulator. *Electronic Journal of Biotechnology* 12.

Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G, 2004. The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. *Plant Cell* 16, 3496-507.

Kvaalen H, 1994. Ethylene synthesis and growth in embryogenic tissue of Norway spruce - effects of oxygen, 1-aminocyclopropane-1-carboxylic acid, benzyladenine and 2,4-dichlorophenoxyacetic acid. *Physiologia Plantarum* 92, 109-17.

Kvaalen H, Appelgren M, 1999. Light quality influences germination, root growth and hypocotyl elongation in somatic embryos but not in seedlings of Norway spruce. *In vitro Cellular & Developmental Biology-Plant* 35, 437-41.

Kvaalen H, Solheim H, 2000. Co-inoculation of Ceratocystis polonica and Heterobasidion annosum with callus of two Norway spruce clones with different in vivo susceptibility. *Plant Cell Tissue and Organ Culture* 60, 221-8.

Laajanen K, Vuorinen I, Salo V, Juuti J, Raudaskoski M, 2007. Cloning of *Pinus sylvestris* SCARECROW gene and its expression pattern in the pine root system, mycorrhiza and NPA-treated short roots. *New Phytologist* 175, 230-43.

Lee VT, Schneewind O, 2001. Protein secretion and the pathogenesis of bacterial infections. *Genes & Development* 15, 1725-52.

Leitner M, Kaiser R, Rasmussen MO, Driguez H, Boland W, Mithoefer A, 2008. Microbial oligosaccharides differentially induce volatiles and signalling components in *Medicago truncatula*. *Phytochemistry* 69, 2029-40.

Lelu MAP, Bornman CH, 1990. Induction of somatic embryogenesis in excised cotyledons of *Picea glauca* and *Picea mariana*. *Plant Physiology and Biochemistry* 28, 785-91.

Lelu MA, Bastien C, Drugeault A, Gouez ML, Klimaszewska K, 1999. Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators. *Physiologia Plantarum* 105, 719-28.

Lelu-Walter MA, Bernier-Cardou M, Klimaszewska K, 2008. Clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis. *Plant Cell Tissue and Organ Culture* 92, 31-45.

Leon-Reyes A, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S, Millenaar FF, Welschen RAM, Ritsema T, Pieterse CMJ, 2009. Ethylene modulates the role of Noneexpressor of Pathogenesis-related Genes 1 in cross talk between salicylate and jasmonate signaling. *Plant Physiology* 149, 1797-809.

Li GS, Asiegbu FO, 2004. Use of Scots pine seedling roots as an experimental model to investigate gene expression during interaction with the conifer pathogen *Heterobasidion annosum* (P-type). *Journal of Plant Research* 117, 155-62.

Li XY, Huang FH, Gbur EE, 1997. Polyethylene glycol-promoted development of somatic embryos in loblolly pine (*Pinus taeda* L.). *In Vitro Cellular & Developmental Biology-Plant* 33, 184-9.

Ligero F, Lluch C, Olivares J, 1987. Evolution of ethylene from roots and nodulation rate of Alfalfa (*Medicago sativa* L.) plants inoculated with *Rhizobium meliloti* as affected by the presence of nitrate. *Journal of Plant Physiology* 129, 461-7.

Lindblad P, Rai AN, Berbman B, 1987. The *Cycas rettoluta-nostoc* symbiosis: enzyme activities of nitrogen and carbon metabolism in the cyanobiont . *Journal of General Microbiology* 133, 1695-9.

Lipavska H, Konradova H, 2004. Somatic embryogenesis in conifers: The role of carbohydrate metabolism. *In Vitro Cellular & Developmental Biology-Plant* 40, 23-30.

Liu YD, Zhang SQ, 2004. Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in *Arabidopsis*. *Plant Cell* 16, 3386-99.

Lopes T, Pinto G, Loureiro J, Costa A, Santos C, 2006. Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. *Tree Physiology* 26, 1145-52.

Maijala P, Harrington TC, Raudaskoski M, 2003. A peroxidase gene family and gene trees in *Heterobasidion* and related genera. *Mycologia* 95, 209-21.

Malabadi RB, Choudhury H, Tandon P, 2004. Initiation, maintenance and maturation of somatic embryos from thin apical dome sections in *Pinus kesiya* (Royle ex. Gord) promoted by partial desiccation and Gellan gum. *Scientia Horticulturae* 102, 449-59.

Malabadi RB, Van Staden J, 2005. Somatic embryogenesis from vegetative shoot apices of mature trees of *Pinus patula* . *Tree Physiology* 25, 11-6.

Martin F, Laurent P, de Carvalho D, Voiblet C, Balestrini R, Bonfante P, Tagu D, 1999. Cell wall proteins of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*: Identification, function, and expression in symbiosis. *Fungal Genetics and Biology* 27, 161-74.

Martin F, Selosse M, 2008. The *Laccaria* genome: a symbiont blueprint decoded. *New Phytologist* 180, 296-310.

Martinez-Romero D, Bailen G, Serrano M, Guillen F, Valverde JM, Zapata P, Castillo S, Valero D, 2007. Tools to maintain postharvest fruit and vegetable quality through the inhibition of ethylene action: A review. *Critical Reviews in Food Science and Nutrition* 47, 543-60.

Marum L, Rocheta M, Maroco J, Oliveira MM, Miguel C, 2009. Analysis of genetic stability at SSR loci during somatic embryogenesis in maritime pine (*Pinus pinaster*). *Plant Cell Reports* 28, 673-82.

Maruyama E, Hosoi Y, Ishii K, 2005. Propagation of Japanese red pine (*Pinus densiflora* Zieb. et Zucc.) via somatic embryogenesis. *Propagation of Ornamental Plants* 5, 199-204.

Mathieu M, Lelu-Walter MA, Blervacq AS, David H, Hawkins S, Neutelings G, 2006. Germin-like genes are expressed during somatic embryogenesis and early development of conifers. *Plant Molecular Biology* 61, 615-27.

Mazan M, Mazanova K, Farkas V, 2006. Fungal cell wall - Challenge for the search of new antimycotics. *Chemicke Listy* 100, 433-9.

Merkle SA, Nairn CJ, 2005. Hardwood tree biotechnology. *In vitro cellular & developmental biology-plant* 41, 602-19.

Merkle SA, Montello PM, Xia XQ, Upchurch BL, Smith DR, 2006. Light quality treatments enhance somatic seedling production in three southern pine species. *Tree Physiology* 26, 187-94.

Michel Z, Hilaire KT, Mongomake K, Georges AN, Justin KY, 2008. Effect of genotype, explants, growth regulators and sugars on callus induction in cotton (*Gossypium hirsutum* L.). *Australian Journal of Crop Science* 2, 1-9.

Minocha R, Minocha SC, Long S, 2004. Polyamines and their biosynthetic enzymes during somatic embryo development in Red spruce (*Picea rubens* Sarg.). *In vitro Cellular & Developmental Biology-Plant* 40, 572-80.

Minocha R, Smith DR, Reeves C, Steele KD, Minocha SC, 1999. Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. *Physiologia Plantarum* 105, 155-64.

Miyazaki JH, Yang SF, 1987. The methionine salvage pathway in relation to ethylene and polyamine biosynthesis. *Physiologia Plantarum* 69, 366-70.

Murashige T, Skoog F, 1962. A revised medium for rapid growth and bioassays with tobacco culture. *Physiologia Plantarum* 15, 473-97.

Nagmani R, Diner AM, Garton S, Zipf AE, 1995. Anatomical comparison of somatic and zygotic embryogeny in conifers. In: Jain S, Gupta PK and Newton RJN, C. H, eds. *Somatic embryogenesis in woody plants.* Dordrecht, The Netherlands: Kluwer Academic Publishers, 23-48.

Naveed M, Zahir ZA, Khalid M, Asghar HN, Akhtar MJ, Arshad M, 2008. Rhizobacteria containing ACC-Deaminase for improving growth and yield of wheat under fertilized conditions. *Pakistan Journal of Botany* 40, 1231-41.

Nehls U, Ecke M, Hampp R, 1999. Sugar- and nitrogen-dependent regulation of an *Amanita muscaria* phenylalanine ammonium lyase gene. *Journal of Bacteriology* 181, 1931-3.

Nehls U, 2008. Mastering ectomycorrhizal symbiosis: the impact of carbohydrates. *Journal of Experimental Botany* 59, 1097-108.

Nehra NS, Becwar MR, Rottmann WH, Pearson L, Chowdhury K, Chang S, H. Dayton Wilde, Kodrzycki RJ, Zhang C, Gause KC, Parks DW, Hinchee MA, 2005. Forest biotechnology: innovative methods, emerging opportunities. *In vitro Cellular & Developmental Biology-Plant* 41, 701-717.

Niemi K, Häggman H, 2002. *Pisolithus tinctorius* promotes germination and forms mycorrhizal structures in Scots pine somatic embryos *in vitro*. *Mycorrhiza* 12, 263-7.

Niemi K, Sarjala T, Chen XW, Haggman H, 2002a. Spermidine and methylglyoxal bis (guanylhydrazone) affect maturation and endogenous polyamine content of Scots pine embryogenic cultures. *Journal of Plant Physiology* 159, 1155-8.

Niemi K, Häggmän H, Sarjala T, 2002b. Effects of exogenous diamines on the interaction between ectomycorrhizal fungi and adventitious root formation in Scots pine *in vitro*. *Tree Physiology* 22, 373-81.

Niemi K, Häggman H, Sarjala T, 2003. Ectomycorrhizal fungal species and strains differ in their ability to produce free and conjugated polyamines. *Mycorrhiza* 13, 283-7.

Niemi K, Scagel C, Häggman H, 2004. Application of ectomycorrhizal fungi in vegetative propagation of conifers. *Plant Cell Tissue and Organ Culture* 78, 83-91.

Niemi K, Sarjala T, Chen X, Häggman H, 2007. Spermidine and the ectomycorrhizal fungus Pisolithus tinctorius synergistically induce maturation of Scots pine embryogenic cultures. *Journal of Plant Physiology* 164, 629-35.

Niini S, Raudaskoski M, 1998. Growth patterns in non-mycorrhizal and mycorrhizal short roots of *Pinus sylvestris*. *Symbiosis* 25, 101-14.

Noceda C, Salaj T, Perez M, Viejo M, Jesus Canal M, Salaj J, Rodriguez R, 2009. DNA demethylation and decrease on free polyamines is associated with the embryogenic capacity of *Pinus nigra* Arn. cell culture. *Trees Structure and Function* 23, 1285-93.

Nürnberger T, Brunner F, 2002. Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. *Current Opinion in Plant Biology* 5, 318-24.

Onkokesung N, Galis I, von Dahl CC, Matsuoka K, Saluz HP, Baldwin IT, 2010. Jasmonic acid and ethylene modulate local responses to wounding and simulated herbivory in *Nicotiana attenuata* Leaves. *Plant Physiology* 153, 785-98.

Osborne B, 2007. The Gunnera-Nostoc symbiosis in context. *Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology* 146, 5.

Özkurt Z, Yildirim T, Önde S, Kaya Z, 2008. Induction of embryogenic tissue from immature zygotic embryos in *Pinus nigra* J.F.Arnold subsp. *nigra* var. *caramanica* (Loudon) Businsky. *Turkish Journal of Botany* 32, 179-83.

Palovaara J, Hakman I, 2008. Conifer WOX-related homeodomain transcription factors, developmental consideration and expression dynamic of WOX2 during Picea abies somatic embryogenesis. *Plant Molecular Biology* 66, 533-49.

Park YS, 2002. Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. *Annals of Forest Science* 59, 651-6.

Park YS, Lelu-Walter MA, Harvengt L, Trontin JF, MacEacheron I, Klimaszewska K, Bonga JM, 2006. Initiation of somatic embryogenesis in *Pinus banksiana*, *P. strobus*, *P. pinaster*, and *P. sylvestri*s at three laboratories in Canada and France. *Plant Cell Tissue and Organ Culture* 86, 87-101.

Pearce RB, 1996. Effects of exposure to high ozone concentrations on stilbenes in Sitka spruce (*Picea sitchensis* (Bong) Carr) bark and on its lignification response to infection with *Heterobasidion annosum* (Fr) Bref. *Physiological and Molecular Plant Pathology* 48, 117-29.

Peck SC, Kende H, 1995. Sequential induction of the ethylene bosynthetic-enzymes by indole-3-acetic-acid in etiolated peas. *Plant Molecular Biology* 28, 293-301.

Percy RE, Klimaszewska K, Cyr DR, 2000. Evaluation of somatic embryogenesis for clonal propagation of western white pine. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* 30, 1867-76.

Pieterse CMJ, van Wees SCM, van Pelt JA, Knoester M, Laan R, Gerrits N, Weisbeek PJ, van Loon LC, 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* 10, 1571-80.

Podila GK, Zheng J, Balasubramanian S, Sundaram S, Hiremath S, Brand JH, Hymes MJ, 2002. Fungal gene expression in early symbiotic interactions between *Laccaria bicolor* and red pine. *Plant and Soil* 244, 117-28.

Pullman GS, Gupta PK, 1991. Method for reproducing coniferous plants by somatic embryogenesis using absorbent materials in the development stage media. USA patent, No. 5 034 326.

Pullman GS, Johnson S, Peter G, Cairney J, Xu N, 2003a. Improving loblolly pine somatic embryo maturation: comparison of somatic and zygotic embryo morphology, germination, and gene expression. *Plant Cell Reports* 21, 747-58.

Pullman GS, Namjoshi K, Zhang Y, 2003b. Somatic embryogenesis in loblolly pine (*Pinus taeda* L.): improving culture initiation with abscisic acid and silver nitrate. *Plant Cell Reports* 22, 85-95.

Pullman GS, Gupta PK, Timmis R, Carpenter C, Kreitinger M, Welty E, 2005. Improved Norway spruce somatic embryo development through the use of abscisic acid combined with activated carbon. *Plant Cell Reports* 24, 271-9.

Pullman GS, Chopra R, Chase KM, 2006. Loblolly pine (*Pinus taeda* L.) somatic embryogenesis: Improvements in embryogenic tissue initiation by supplementation of medium with organic acids, Vitamins B-12 and E. *Plant Science* 170, 648-58.

Pullman GS, Buchanan M, 2008. Identification and quantitative analysis of stage-specific carbohydrates in loblolly pine (*Pinus taeda*) zygotic embryo and female gametophyte tissues. *Tree Physiology* 28, 985-96.

Pullman GS, Chase KM, Skryabina A, Bucalo K, 2009. Conifer embryogenic tissue initiation: improvements by supplementation of medium with D-xylose and D-chiro-inositol. *Tree Physiology* 29, 147-56.

Rai AN, Bergman B, Rasmussen U, 2002. *Cyanobacteria in symbiosis.* Dordrecht: Kluwer Academic Publishers.

Ralph SG, Hudgins JW, Jancsik S, Franceschi VR, Bohlmann J, 2007. Aminocyclopropane carboxylic acid synthase is a regulated step in ethylene-dependent induced conifer defense. Full-length cDNA cloning of a multigene family, differential constitutive, and wound- and insect-induced expression, and cellular and subcellular localization in spruce and Douglas fir. *Plant Physiology* 143, 410-24.

Ramarosandratana A, Harvengt L, Bouvet A, Calvayrac R, Paques M, 2001. Effects of carbohydrate source, polyethylene glycol and gellan gum concentration on embryonal-suspensor mass (ESM) proliferation and maturation of maritime pine somatic embryos. *In vitro Cellular & Developmental Biology-Plant* 37, 29-34.

Rodriguez MJP, Suarez MF, Heredia R, Avila C, Breton D, Trontin JF, Filonova L, Bozhkov P, von Arnold S, Harvengt L, Canovas FM, 2006. Expression patterns of two glutamine synthetase genes in zygotic and somatic pine embryos support specific roles in nitrogen metabolism during embryogenesis. *New Phytologist* 169, 35-44.

Sakurai T, Kataoka K, 2007. Basic and applied features of multicopper oxidases, CueO, bilirubin oxidase, and laccase. *The Chemical Record* 7, 220-9.

Salzer P, Hubner B, Sirrenberg A, Hager A, 1997. Differential effect of purified spruce chitinases and beta-1,3-glucanases on the activity of elicitors from ectomycorrhizal fungi. *Plant Physiology* 114, 957-68.

Santacruz-Ruvalcaba F, Gutiérrez-Mora A and Rodríguez-Garay B, 1998. Somatic embryogenesis in some cactus and agave species**,** *Professional Association for Cactus Development Journal* 3, 15–26.

Sarjala T, Häggman H, Aronen T, 1997. Effect of exogenous polyamines and inhibitors of polyamine biosynthesis on growth and free polyamine contents of embryogenic Scots pine callus. *Journal of Plant Physiology* 150, 597-602.

Sarjala T, Niemi K, Häggman H, 2010. Mycorrhiza formation is not needed for early growth induction and growth-related changes in polyamines in Scots pine seedlings in vitro. *Plant Physiology and Biochemistry* 48, 596-601.

Sato T, Theologis A, 1989. Cloning the mRNA encoding 1-aminocyclopropane-1-carboxylate synthase, the key enzyme for ethylene biosynthesis in plants. *Proceedings of the National Academy of Sciences of the United States of America* 86, 6621-5.

Schwacke R, Hager A, 1992. Fungal elicitors induce a transient release of active oxygen species from cultured spruce cells that is dependent on Ca^{2+} and protein kinase activity. *Planta* 187, 136-41.

Schwessinger B, Zipfel C, 2008. News from the frontline: recent insights into PAMP-triggered immunity in plants. *Current Opinion in Plant Biology* 11, 389-95.

Sebastia CH, Hardin SC, Clouse SD, Kieber JJ, Huber SC, 2004. Identification of a new motif for CDPK phosphorylation *in vitro* that suggests ACC synthase may be a CDPK substrate. *Archives of Biochemistry and Biophysics* 428, 81-91.

Sghaier B, Kriaa W, Bahloul M, Novo JVJ, Drira N, 2009. Effect of ABA, arginine and sucrose on protein content of date palm somatic embryos. *Scientia Horticulturae* 120, 379-85.

Sghaier B, Jorrin-Novo JV, Gargouri-Bouzid R, Drira N, 2010. Abscisic acid and sucrose increase the protein content in date palm somatic embryos, causing changes in 2-DE profile. *Phytochemistry* 71, 1223-36.

Shimada C, Lipka V, O'Connel R, Okuno T, Schulze-Lefert P, Takano Y, 2006. Nonhost resistance in *Arabidopsis*-colletotrichum interactions acts at the cell periphery and requires actin filament function. *Molecular Plant-Microbe Interactions* 19, 270-9.

Shoji, T., Nakajima, K., and Hashimoto, T. 2000. Ethylene suppresses jasmonate-induced gene expression in nicotine bio-synthesis. *Plant Cell Physiol*. 41:1072-1076.

Shoresh M, Yedidia I, Chet I, 2005. Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. *Phytopathology* 95, 76-84.

Silveira V, Floh EIS, Handro W, Guerra MP, 2004. Effect of plant growth regulators on the cellular growth and levels of intracellular protein, starch and polyamines in embryogenic suspension cultures of *Pinus taeda*. *Plant Cell Tissue and Organ Culture* 76, 53-60.

Singh S, Hasija SK, Negi S, Singh HN, 1994. Mutational analysis of the NH^{4+}-nitrogen controls that regulate ammonium transport activity, heterocyst differentiation, nitrogenase activity and the heterocyst-spacing pattern in the cyanobacterium *Nostoc muscorum*. *Biochemistry and Molecular Biology International* 32, 359-70.

Smith SE, Read DJ, 2008. *Mycorrhizal Symbiosis.* London: Academic Press.

Spanu P, Felix G, Boller T, 1990. Inactivation of stress-induced 1-aminocyclopropane carboxylate synthase *in vivo* differs from substrate-dependent inactivation *in vitro*. *Plant Physiology* 93, 1482-5.

Splivallo R, Fischer U, Goebel C, Feussner I, Karlovsky P, 2009. Truffles regulate plant root morphogenesis via the production of auxin and ethylene. *Plant Physiology* 150, 2018-29.

Staiger CJ, 2000. Signaling to the actin cytoskeleton in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 51, 257-88.

Stapulionis R, Deutscher MP, 1995. A channeled transfer-RNA cycle during mammalian protein-synthesis. *Proceedings of the National Academy of Sciences of the United States of America* 92, 7158-61.

Stasolla C, Loukanina N, Ashihara H, Yeung EC, Thorpe TA, 2001. Purine and pyrimidine metabolism during the partial drying treatment of white spruce (*Picea glauca*) somatic embryos. *Physiologia Plantarum* 111, 93-101.

Stasolla C, Yeung EC, 2001. Ascorbic acid metabolism during white spruce somatic embryo maturation and germination. *Physiologia Plantarum* 111, 196-205.

Stasolla C, Kong LS, Yeung EC, Thorpe TA, 2002. Maturation of somatic embryos in conifers: morphogenesis, physiology, biochemistry, and molecular biology. *In vitro Cellular & Developmental Biology-Plant* 38, 93-105.

Stasolla C, van Zyl L, Egertsdotter U, Craig D, Liu WB, Sederoff RR, 2003. The effects of polyethylene glycol on gene expression of developing White spruce somatic embryos. *Plant Physiology* 131, 49-60.

Stasolla C, Yeung EC, 2003. Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. *Plant Cell Tissue and Organ Culture* 74, 15-35.

Stasolla C, Belmonte MF, van Zyl L, Craig DL, Liu WB, Yeung EC, Sederoff RR, 2004. The effect of reduced glutathione on morphology and gene expression of white spruce (*Picea glauca*) somatic embryos. *Journal of Experimental Botany* 55, 695-709.

Stein E, Molitor A, Kogel KH, Waller F, 2008. Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of *NPR1*. *Plant and Cell Physiology* 49, 1747-51.

Steward FC, Mapes MO, Mears K, 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *American Journal of Botany* 45, 705-8.

Sutton B, 2002. Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis. *Annals of Forest Science* 59, 657-61.

Szczygiel K, Szczygiel K, Hazubska-Przybyl T, Bojarczuk K, 2007. Somatic embryogenesis of selected coniferous tree species of the genera *Picea*, *Abies* and *Larix*. *Acta Societatis Botanicorum Poloniae* 76, 1, 7-15

Tahir M, Law DA, Stasolla C, 2006. Molecular characterization of *PgAGO*, a novel conifer gene of the *ARGONAUTE* family expressed in apical cells and required for somatic embryo development in spruce. *Tree physiology* 26, 1257-70.

Tagu D, Lapeyrie F, Martin F, 2002. The ectomycorrhizal symbiosis: genetics and development. *Plant and Soil* 244, 97-105.

Tautorus TE, Fowke LC, Dunstan DI, 1991. Somatic embryogenesis in conifers. *Canadian Journal of Botany-Revue Canadienne de Botanique* 69, 1873-99.

Tereso S, Zoglauer K, Milhinhos A, Miguel C, Oliveira MM, 2007. Zygotic and somatic embryo morphogenesis in *Pinus pinaster*: comparative histological and histochemical study. *Tree Physiology* 27, 661-9.

Terho M, Pappinen A, von Weissenberg K, 2000. Growth reactions of a *Gremmeniella abietina* isolate and Scots pine embryogenic tissue cultures differ in a host-parasite *in vitro* system. *Forest Pathology* 30, 285-95.

Timonen S, Finlay RD, Soderstrom B, Raudaskoski M, 1993. Identification of cytoskeletal components in pine ectomycorrhizas. *New Phytologist* 124, 83-92.

Tsuchisaka A, Theologis A, 2004a. Heterodimeric interactions among the 1-amino-cyclopropane-1-carboxylate synthase polypeptides encoded by the *Arabidopsis* gene family. *Proceedings of the National Academy of Sciences of the United States of America* 101, 2275-80.

Tsuchisaka A, Theologis A, 2004b. Unique and overlapping expression patterns among the *Arabidopsis* 1-amino-cyclopropane-1-carboxylate synthase gene family members. *Plant Physiology* 136, 2982-3000.

Tsuchisaka A, Yu GX, Jin HL, Alonso JM, Ecker JR, Zhang XM, Gao S, Theologis A, 2009. A combinatorial interplay among the 1-aminocyclopropane-1-carboxylate isoforms regulates ethylene biosynthesis in *Arabidopsis thaliana*. *Genetics* 183, 979-1003.

Turner JG, Ellis C, Devoto A, 2002. The jasmonate signal pathway. *Plant Cell* 14, S153-64.

Vahala J, Schlagnhaufer CD, Pell EJ, 1998. Induction of an ACC synthase cDNA by ozone in light-grown *Arabidopsis thaliana* leaves. *Physiologia Plantarum* 103, 45-50.

Vales T, Feng XR, Ge L, Xu NF, Cairney J, Pullman GS, Peter GF, 2007. Improved somatic embryo maturation in loblolly pine by monitoring ABA-responsive gene expression. *Plant Cell Reports* 26, 133-43.

van Loon LC, Geraats BPJ, Linthorst HJM, 2006. Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science* 11, 184-91.

van Wees SCM, Van der Ent S, Pieterse CMJ, 2008. Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology* 11, 443-8.

van der Heijen MGA, 2008. The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11, 651-651.

von Arnold S, Eriksson T, 1981. *In vitro* studies on adventitious shoot formation in *Pinus contorta*. *Canadian Journal of Botany-Revue Canadienne de Botanique* 59, 870-4.

Vuosku J, Jokela A, Laara E, Saaskilahti M, Muilu R, Sutela S, Altabella T, Sarjala T, Häggman H, 2006. Consistency of polyamine profiles and expression of arginine decarboxylase in mitosis during zygotic embryogenesis of Scots pine. *Plant Physiology* 142, 1027-38.

Wang NN, Shih MC, Li N, 2005. The GUS reporter-aided analysis of the promoter activities of *Arabidopsis* ACC synthase genes *AtACS4*, *AtACS5*, and *AtACS7* induced by hormones and

Wang W, Park YS, Riding R, Beardmore T, 2009. Norway spruce somatic embryogenesis: improvement of somatic embryo maturation. *Propagation of Ornamental Plants* 9, 185-97.

Wang Y, DiGuistini S, Wang TT, Bohlmann J, Breuil C, 2010. Agrobacterium-meditated gene disruption using split-marker in *Grosmannia clavigera*, a mountain pine beetle associated pathogen. *Current Genetics* 56, 297-307.

Wasternack C, Stenzel I, Hause B, Hause G, Kutter C, Maucher H, Neumerkel J, Feussner I, Miersch O, 2006. The wound response in tomato - role of jasmonic acid. *Journal of Plant Physiology* 163, 297-306.

Whitton B, Potts M, 2000. Introduction to cyanobacteria. In: Whitton B and Potts M, eds. *The Ecology of cyanobacteria: the diversity in time and space.* Dordrecht: Kluwer Academic Publishers, 1-11.

Wi SJ, Jang SJ, Park KY, 2010. Inhibition of biphasic ethylene production enhances tolerance to abiotic stress by reducing the accumulation of reactive oxygen species in *Nicotiana tabacum*. *Molecules and Cells* 30, 37-49.

Wochok ZS, Wetherell DF, 1971. Suspension of organized growth in cultured wild carrot tissue by 2-chloroethylphosphonic acid. *Plant cell Physiology* 12, 771-4.

Wolski E, Maldonado S, Daleo G, Andreu A, 2007. Cell wall alpha-1,3-glucans from a biocontrol isolate of *Rhizoctonia*: immunocytolocalization and relationship with alpha-glucanase activity from potato sprouts. *Mycological Research* 111, 976-84.

Yamagami T, Tsuchisaka A, Yamada K, Haddon WF, Harden LA, Theologis A, 2003. Biochemical diversity among the 1-amino-cyclopropane-1-carboxylate synthase isozymes encoded by the *Arabidopsis* gene family. *Journal of Biological Chemistry* 278, 49102-12.

Yang SF, Hoffman NE, 1984. Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 35, 155-89.

Yeung EC, Stasolla C, Kong LS, 1998. Apical meristem formation during zygotic embryo development of white spruce. *Canadian Journal of Botany-Revue Canadienne De Botanique* 76, 751-61.

Yildirim T, 2005. Induction of embryogenic tissue and development of somatic embryos in *Pinus brutia* Ten. Middle East Technical University (thesis)

Yoo SD, Cho YH, Sheen J, 2009. Emerging connections in the ethylene signaling network. *Trends in Plant Science* 14, 270-9.

Yuan R, Li J, 2008. Effect of sprayable 1-MCP, AVG, and NAA on ethylene biosynthesis, preharvest fruit drop, fruit maturity, and quality of 'Delicious' apples. *HortScience* 43, 1454-60.

Zarembinski TI, Theologis A, 1994. Ethylene biosynthesis and action - a Case of conservation. *Plant Molecular Biology* 26, 1579-97.

Zhang CC, Laurent S, Sakr S, Peng L, Bedu S, 2006. Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. *Molecular Microbiology* 59, 367-75.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G, 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125, 749-60.