Xylem Formation in *Pinus radiata* D. Don Callus under *In Vitro* Conditions

A thesis submitted in partial fulfilment of the requirements for the

Degree of Doctor of Philosophy in Plant Biotechnology

By

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Dedication

This Doctoral thesis is dedicated to my mother, Zhila Kalantari, my father, Khosrow Pezhman and my brother, Dr. Khashayar Pezhman. Without their endless love and encouragement I would never have been able to complete my graduate studies. I love you all and I appreciate everything that you have done for me.

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List of acronyms and abbreviations

Word/Acronym/Abbreviation	Full Description
AC	Activated carbon
2,4-D	2,4-dichlorophenoxyacetic acid
4CL	Coenzyme A ligase
BAP	6-benzylaminopurine
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CCoAOMT	Caffeoyl coenzyme A O-methyltransferase
cPTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
EDM	Embryo development medium
EDTA	Ethylenediamine tetra acetic acid
ESTs	Expressed sequence tags
JA	Jasmonic acid
H_2O_2	Hydrogen peroxide
IBA	Indole-3-butyric acid
MetE	Cobalamin-independent methionine synthase
MS	Murashige and Skoog medium
NAA	Naphthaleneacetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NO	Nitric oxide
PAL	Phenylalanine ammonia lyase
PCD	Programmed cell death
PGRs	Plant growth regulators

POD	Peroxidase
PVP	Polyvinylpyrrolidone
qPCR	Real-time polymerase chain reaction
ROS	Reactive oxygen species
SAMS	S-Adenosylmethionine synthetase
SNP	Sodium nitroprusside
T1	P6-SHv medium + 1.0 mg/L BAP + 1.0 mg/L 2,4-D
T2	MS medium + 3.0 mg/L BAP + 3.0 mg/L 2,4-D
T3	MS medium + 0.58 mg/L BAP + 1.0 mg/L 2,4-D
Tes	Tracheary elements
TGAL	Thioglycolic acid lignin
TIM	Tracheary element induction medium

Abstract

A central question in plant cell differentiation is how xylogenesis (the formation of xylem or tracheary elements) is regulated in plants. Plant tissue culture, particularly callus culture, has been applied to assist the investigations of this question. The assessments of the controlling factors such as different sources of explants and hormonal treatments have contributed to a better understanding of the *in vitro* tracheary element (TE) formation in coniferous gymnosperms and angiosperms. Differentiation of tracheary elements (TEs) in the callus cells of *Pinus radiata* under *in vitro* conditions was studied in this project. The main aim of this investigation was to increase the rate of differentiation of TEs in the *P. radiata* calli using a combination of plant growth regulators (PGRs) such as 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP), as well as different sources of explants (genotype effects). The calli derived from the different explants cultured on the media supplemented with or without a particular PGR combination were analysed. The planned analyses included histology, lignin analysis and Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) in order to evaluate the presence of morphological, biochemical and molecular (gene expression) markers associated with the TE formation in the xylogenic *P. radiata* callus culture, although the unprecedented times of the Covid-19 pandemic had impacted on this planned research including the exclusion of the RT-qPCR analysis. Initially, to induce xylogenic *P. radiata* calli we tried to use a medium used in several prior studies on xylem formation in *P. radiata* calli. A number of small and weak calli were induced after 6 weeks of culturing on this medium, but the calli died quickly before any further development. The experiment was then repeated two more times but we were unsuccessful to establish subculturable calli for further TE induction experiments. Each time around 45 days were spent to test the method (a total of 90 days). We concluded that we had to modify this project to include an evaluation of the effects of PGRs on the callus induction, TE induction and

biochemical changes associated with xylem formation in *P. radiata* callus cultures. The hypothesis that different concentrations and combinations of PGRs and a nitric oxide donor, sodium nitroprusside (SNP), could influence TE formation in *P. radiata* callus culture was investigated. The highest percentages of TEs, 13%, 23% and 28%, were observed when the hypocotyl-derived calli were cultured on the medium containing 1.0 mg/L jasmonic acid (JA) and 0.025 mg/L SNP on 5th, 10th and 15th day of culture, respectively. Furthermore, TE induction was significantly increased from 12% to 33% on the media supplemented with 0.025 or 0.05 mg/L SNP in addition to 1.0 mg/L NAA and 1.0 mg/L BAP. The xylogenic *P. radiata* callus cultures were characterised as a model for studying the formation of xylem cells of coniferous gymnosperms at the morphological and histological level. The confocal images obtained showed that the TEs appeared to be joined together as clusters in the xylogenic *P. radiata* calli. This study also showed the increased activities of peroxidase and cinnamyl alcohol dehydrogenase in the xylogenic *P. radiata* calli. The current study extends our knowledge in the involvement of nitric oxide in the process of TEs formation in *P. radiata* callus furthering our understanding of the control of xylogenesis in this plant.

Chapter 1. General Introduction

1.1 Wood industry

Trees are considered as the main component of the biosphere. Their wood is a sustainable source of energy as well as a raw material for world trade. In the new millennium, there is a high demand for wood, and application of biotechnology to forestry practices has a remarkable potential to develop wood for specific purposes. Undoubtedly, applying forest biotechnological methods such as marker-assisted breeding, genetic engineering, and *in vitro* propagation, is inevitable for the management and conservation of the world's forests (Gullison et al., 2007).

Pine trees are important as model organisms, because they are one of the two major groups of seed plants, the best characterised gymnosperms. Their development, reproduction, ecology and genetics are well documented. The haploid mega gametophyte and the ability to perform intensive genetic analyses on individual trees in natural populations or in breeding programmes provide key features for genetic analysis. Pines also provide a biochemical model for the biosynthesis of plant cell walls because of the large amount of xylem differentiation that can be obtained and the highly complex pathway of wood formation (xylogenesis).

Gymnosperms are a taxon with approximately 600 perennial species, representing an ancient and extant relation between seedless ferns and flowering angiosperms. The *Pinus* genus includes at least 111 species of trees and belongs to the Pinaceae, a family of about 200 species of trees in 10 to 11 genera (Figure 3). In vast regions of Europe, Asia and North and Central America, pines are dominant or popular vegetation members (Richardson & Rundel, 1998). Most species of conifers are evergreen, regardless of whether they grow in subpolar zones, with winter temperatures approximating to -50°C, or in subtropical regions with summer temperatures above 40°C. Their leaves are long needles, held in one to five fascicles. Pines

grow naturally or are planted in significant parts of the world and are of major economic value, possibly second only to cereals.

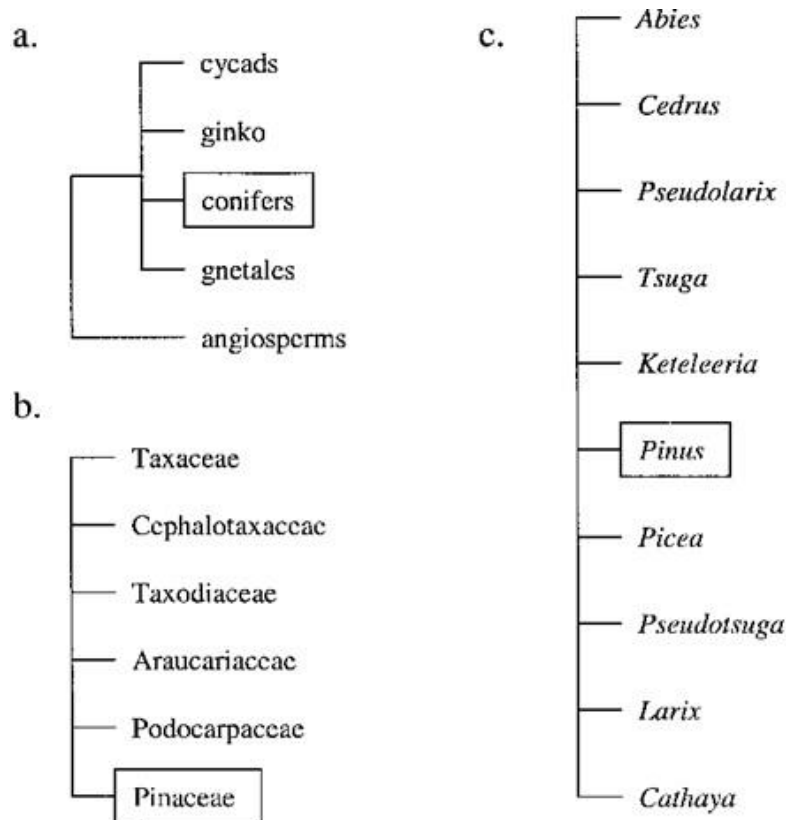

Figure 1. Pines relationships. (a) Relationships of gymnosperms and angiosperms; (b) families of conifers; (c) genera of the Pinaceae. Summarised from Shaw (1914), Mirov (1967), Gifford and Foster (1989) and Price et al. (1998).

Forestry is a significant industry in New Zealand and wood exports are the third top performer of export earnings (Ministry for Primary Industries, 2017). Forests are a valuable resource as they constitute an important component of the terrestrial environmental system and a large natural resource base. They provide fuel, timber, pulpwood, fibre grass fodder and non-wood forest products, and support industrial and commercial activities (Patil, 2019). Wood is the most significant natural resource for the timber and bioenergy industry. Growing trees for wood

may be a way to help to counteract the emission of carbon dioxide into the atmosphere, which is the main contributor to global warming.

1.2. Industrial uses of radiata pine

Radiata pine (*Pinus radiata* D. Don) is the main commonly used conifer in sawmill industry in Australia, New Zealand and some other countries. The wood quality of radiata pine is very variable compared to many other plantation softwood species (McArthur et al., 2019; Wu et al., 2007). About 90% of New Zealand's plantation forests are comprised of *Pinus radiata* (D. Don) and the NZ wood processing sector is advanced and highly productive (Downham & Gavran, 2017; Mauri & Manzanera, 2011).

The construction industry demands continues improvements of building materials which are quick and safe to erect, cost effective and sustainable (Gunawardena et al., 2016; He et al., 2018; Navaratnam et al., 2019; Ngo et al., 2016). Cross Laminated Timber (CLT) shows potential to address many of these requirements in the industry, with a high degree of prefabrication, high in-plane and out-plane strength and stiffness, and good acoustic and thermal performance (He et al., 2018; Nguyen et al., 2016). The engineered wood panel is being used more widely in infrastructure building projects (Block, 2017; He et al., 2018). These panels are made from different timber species that depend on local resources such as Kiri, Katsura, Sugi, Hinoki, Buna spruce pine (Europe and Canada) and radiata pine (Australia and New Zealand).

1.3. Wood formation in trees

The study of xylem formation in woody plants is often justified because of the economic importance of wood. Though, other aspects, for instance the highly organised nature of xylem differentiation during secondary growth in trees, provide a useful model for studying the structure and organisation of the plant body and its architecture. The development from undifferentiated cambial initials through to mature xylem is often clearly displayed. Wood formation involves programmed cell death (PCD) during tracheary element differentiation. The xylem vessels consist of water-conducting cells known as tracheary elements which are hollow cells with lignified secondary cell walls. They are derived from the differentiation of the cambium cells and form highly organised structures as depicted in the model plant *Zinnia elegans* (Figures 2 and 3).

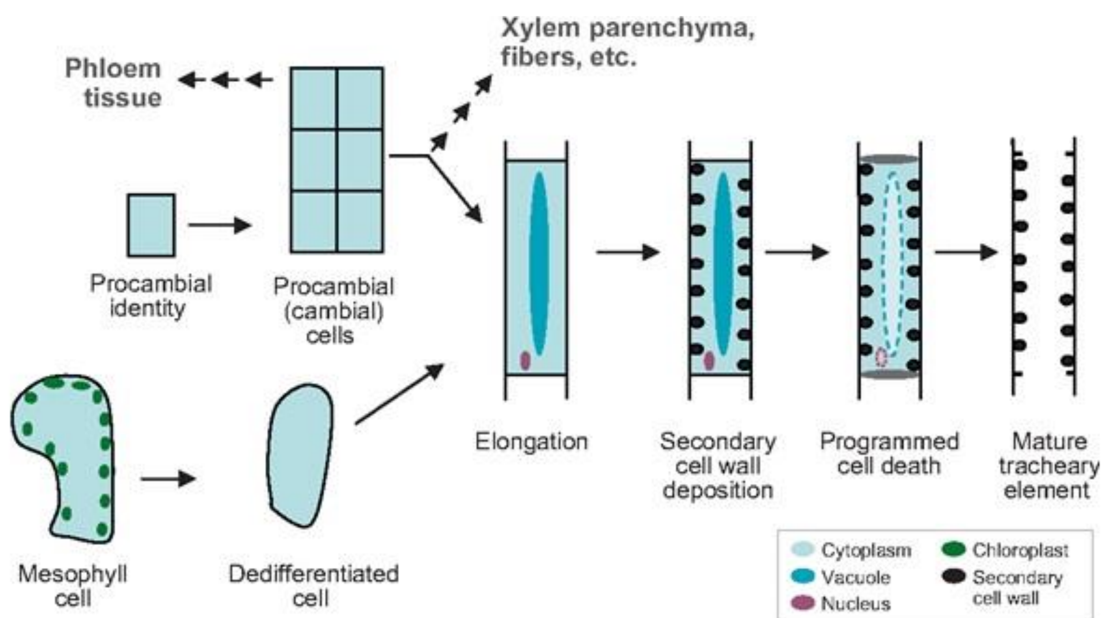

Figure 2. Tracheary element (TE) formation. Procambial cell identification is the first sign of primary vascular growth. These cells then divide and differentiate to become both the xylem and phloem. In secondary growth the equivalent cambial cells continue to divide and differentiate over many years. During xylem differentiation procambial/cambial cells form different cell types in addition to TEs. In *Zinnia* mesophyll cell culture or following wounding, differentiated cells can transdifferentiate to develop TEs (Turner et al., 2007).

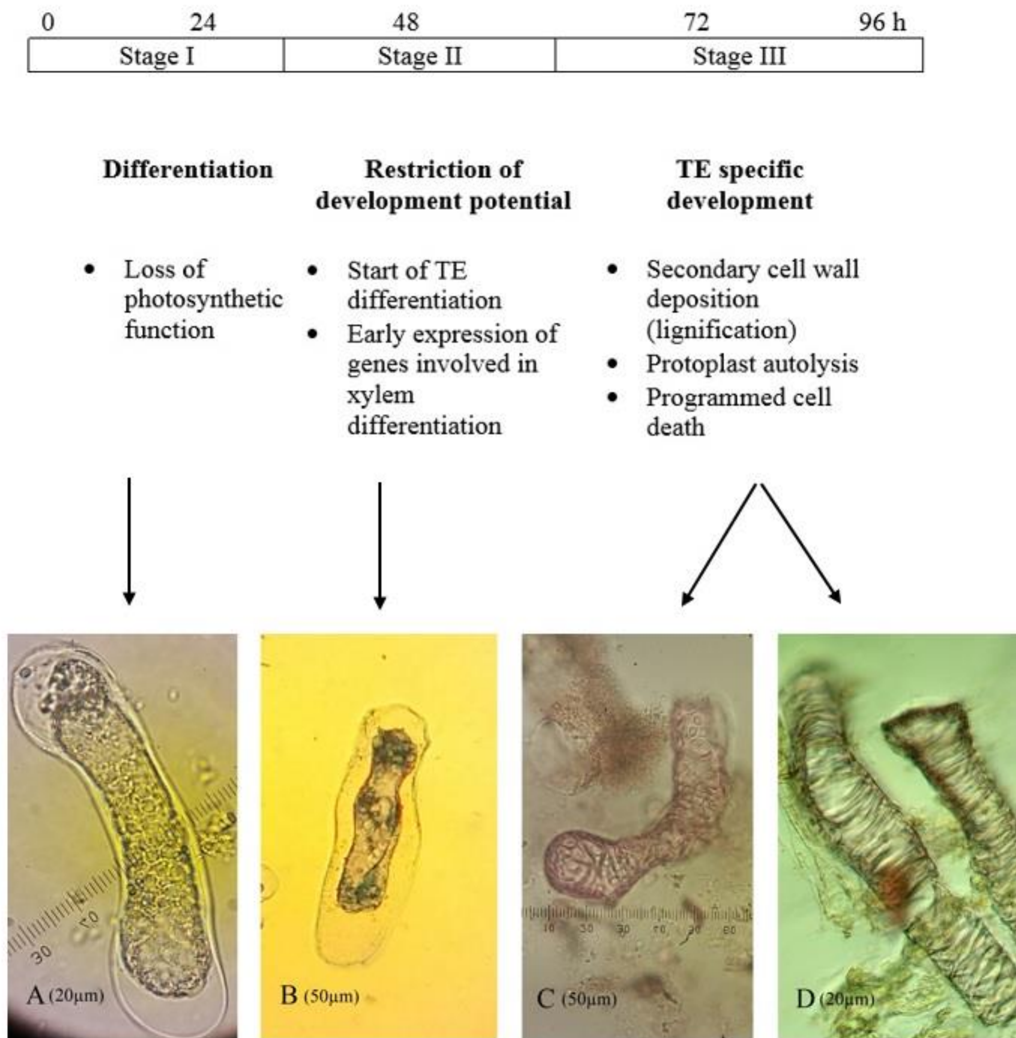

Figure 3. Stages of tracheary element (TE) differentiation in *Pinus radiata* callus cells. (A) Callus cells in stage I. (B) Cells undergoing stage II differentiation. (C and D) Cells in stage III where programmed cell death has been triggered and cell wall lignification has started.

Figure 2 displays a summary of TE induction showing the different stages of vessel development. Tracheary elements are particularly one of the several xylem cell types. They are classified by their characteristic patterned secondary cell walls (SCW), which are confined to the lateral sides of the cell and excluded from the ends. SCW not only is necessary to resist the

negative pressures generated by transpiration in the xylem but also provides mechanical strength vital for structural maintenance and protection to the plant body. The final stages of differentiation are characterised by programmed cell death (PCD), a process that eliminates the cell contents and leaves the cell empty, resulting in what has been called a "functional corpse." Mature TEs in the angiosperms have perforations in the end wall and pits in the lateral wall forming a tube specialised for unimpeded water flow, a process for which coordinated cell-to-cell communication between adjacent cells is clearly vital (Figures 2 and 3). On the other hand, the structural constitution of tracheary element in gymnosperms are basically the same, but every species has its own detailed morphological characteristics.

The lignin in TEs are deposited in different secondary wall thickening patterns of annular, pitted, reticulate, scalariform or spiral appearances (Begum et al., 2013). An analysis of TE formation is notably complicated to study due to the intimate association of vessels with other cells, such as fibers and xylem parenchyma (Figure 5 a). Though, there is an increasing body of evidence showing that surrounding cells play a role in TE induction.

Pinus radiata D. Don, family Pinaceae, is of noticeable economic importance for plantation forestry in the world, known for growing fast and the quality of its lumber and pulp. It was imported into New Zealand in 1859 and today a huge amount of the country's plantation forests has been devoted to this species for commercial purposes (Retrieved from http://www.maf.govt.nz/). Exporting radiata pine log has resulted in about NZD $2.7 billion annually (Ministry for Primary Industries, 2017). Most pine wood is composed of tracheids and a minor component of ray cells and resin ducts mainly determines softwood properties (O'Connell et al., 1998). Hence, understanding cell wall biosynthesis process, particularly that of the secondary cell wall, is necessary for the aim of ameliorating the quality and quantity of wood produced and providing benefits for the pulp and paper industry (Oda & Fukuda, 2012).

The study of cell development in plants is complicated as the process of differentiation occurs inside complex tissues in the plant body. Studying formation of tracheary elements is more accessible and advantageous under *in vitro* conditions. For instance, a huge amount of differentiated TE can be collected from cell cultures at different stages of development. Moreover, the changes in cell wall structure, gene expression, and the proteome or metabolome can be determined (Möller, Ball, et al., 2006). *In vitro* TE systems have been studied in both angiosperm and gymnosperm species such as *Arabidopsis thaliana*, *Pinus* spp., *Populus* spp., *Syringae vulgaris*, *Cupressus sempervirens*, *Centaureae cyanus*, *Daucus carota*, *Cucumis sativus*, Parthenocissus sp., and *Helianthus tuberosus*. However, most research studies have focused on angiosperms (Devillard & Walter, 2014; Iakimova & Woltering, 2017). Yamagishi et al. (2015) reported TE induction in cell suspension cultures developed from the mesophyll cells of young *Cryptomeria japonica* needles. In a previous study, callus was initiated from xylem strips of *Pinus radiata* on a medium called P6-HSv (Hotter, 1997) which was supplemented with 1 mg/L BAP and 1 mg/L 2,4-D. When the xylem strips were transferred to P6-HSv medium enriched with 2 g/L of activated charcoal, TEs were induced (Möller et al., 2003). A high rate of TE induction was also found in callus cells grown on agar-gelled embryo development medium (EDM) containing 5 g/L of activated charcoal without PGRs (Möller, McDonald, et al., 2006a). The rate of TE induction depends on the physical culture conditions (temperature and light) and chemical factors including PGRs, sucrose and nutrients (Möller, Ball, et al., 2006). Pillai et al. (2011) reported TE induction in *Pseudostuga menziesii* calli initiated from cambial strips when subcultured several times on solidified Murashige and Skoog medium (MS) (Murashige & Skoog, 1962) supplemented with 3 mg/L 2,4-D and BAP. Significantly, a high percentage of calli differentiated into TE-like cells when they were cultured on a liquid medium containing BAP for 6-7 weeks without subculture. Only one PGR, 2,4-D, was used for *in vitro* TE development in *Pseudostuga menziesii* cell suspension.

1.4. The advantages of studying tracheary elements *in vitro*

In order to develop a mature TE, a sequence of specific cellular activities involving cell differentiation is needed and the analysis of this procedure in plants is complicated because differentiation occurs within different tissues in the plant body. During secondary xylem formation, TEs development in plants are not easily accessible. Callus grown *in vitro* are more accessible to study the differentiation processes associated with xylogenesis. For instance, cell cultures that initiate a large number of TE cells can be collected at different time points after induction of TE induction, and the changes in cell-wall biochemistry, gene expression, and the proteome or metabolome can be investigated (Demura et al., 2002; Kubo et al., 2005; Milioni et al., 2002; Möller, Koch, et al., 2006; Möller et al., 2005). Theoretically, this would involve *in vitro* cell cultures that synchronously develop TEs to provide access to high percentages of TEs that are in specific developmental stages. *Zinnia elegans* L. or *Arabidopsis thaliana* L. provides examples of successfully established *in vitro* TE systems. These model systems were instrumental in order to gain an improved understanding of cell wall architecture, composition and molecular organisation of cell walls in TEs (Höfte, 2010; Lacayo et al., 2010; Oda & Fukuda, 2012).

1.5. Special problems and prospects in the propagation of woody species

Woody plants represent a vast array of types relative to their taxonomy or use and include both angiosperms and gymnosperms. In general. These are more difficult to propagate asexually than herbaceous species. Which in part is related to the phase change from juvenility to maturation that most of them undergo. Thus, with few exceptions, methods for the large-scale regeneration of true-to-type clones are limited (Thorpe & Harry, 1990). In woody plant tissue cultures, browning or blackening is frequently reported. The oxidation of phenols after cellular

disorganisation causes browning. The degradation of membranes by the toxic sources of oxygen is the result of cellular components disorganisation (Whitaker & Lee, 1995). Phenol oxidation may occur nonenzymatically, or may be catalysed by phenol oxidases or peroxidases (Ke & Saltveit, 1988; Rhodes & Wooltorton, 1978; Vaughn & Duke, 1984). Laukkanen et al. (1999) showed the ability of callus culture derived from shoot tips of mature Scots pine (*Pinus sylvestris* L.) to regenerate is low because of the visible browning. They concluded that browning is associated with cell disorganisation and eventual cell death, making tissue culture of mature pine especially difficult.

The earliest reports of successful regeneration of forest tree plantlets via organogenesis in hardwoods were on *Populus tremuloides* (Winton, 1970) and in softwoods on *Pinus palustris* (Sommer et al., 1975). Regeneration via somatic embryogenesis was achieved with a hardwood, (Durzan & Lopushanski, 1975), and with a softwood, *Picea abies* (Hakman et al., 1985).

Sealing the cut ends of *Dioscorea alata* L. explants with paraffin wax were found to control browning by preventing exudation (Bhat & Chandel, 1991). In another study, the branches of *Platanus occidentalis* L. were soaked with various anti-oxidants and absorbents such as activated carbon (AC) and polyvinylpyrrolidone (PVP) as a pretreatment to prevent contamination and browning of *in vitro* cultures (Tao et al., 2007).

1.6. Callus formation in tissue culture of woody plants

Since the 1930s, woody plants have been cultured *in vitro*. Since then, much study has been done in the culture of woody plant tissues, organs, cells and protoplasts. That is not to consider that all woody plants, particularly forest trees, can be induced to grow and differentiate *in vitro.* Some do, although others are still recalcitrant. In fact, juvenile tissues from woody plants are

more responsive than mature tissues to *in vitro* manipulation. Woody plants have long generation phases. They have an extended vegetative phase, which ranges between 10 and 50 years. During the juvenile phase, beginning from the embryo and perhaps lasting up to ten years, tissues from woody plants are responsive to *in vitro* conditions. Tissues from mature trees become less responsive in tissue culture. Other than the age of a tree, the reaction of an explant / tissue can be determined by the genotype, physiological state of the tissue, time of year when the explant is cultured, and medium composition. The term 'response' means that the tissues will be able to grow, differentiate and germinated seedlings can be developed *in vitro*. By using juvenile tissues, clonal propagation has been accomplished in a significant number of woody plant species. In contrast, mature trees have only been clonally propagated by tissue culture methods in a handful of tree species (Ahuja, 2013).

Callus is comprised of unorganised cell masses formed over a wounded or cut plant surface, resulting from a defensive reaction to seal off damaged tissues. The induction of wound calli has been observed in almost all types of living plants. In culture, callus is developed by placing a piece of plant tissue (an explant) on solid culture media under sterile condition. Callus is formed and initiated from proliferating cells at the cut surface of the explant tissue. Based on the species, the callus can be formed from a variety of tissues on a suitable growth medium. In some samples, however, rapid cell division can be more easily induced than in others. The *in vitro* formation and proliferation of callus is improved by the presence in the medium of PGRs that stimulate cell division and elongation.

1.6.1. Origin of callus

Through the *in vitro* callus formation, the cell differentiation and specialisation that developed in the parent plant is reversed and cells of the explant become dedifferentiated. The procedure of initiation is characterised by changes in metabolic activity, the disappearance of storage

products and rapid division of cells that gives rise to undifferentiated and unorganised parenchyma cells. The lack of structural organisation remains as the callus develops, although a homogeneous callus consisting entirely of parenchyma cells is hard to observe (Evans et al., 2003). Areas of meristematic activity are developed as formation of the callus proceeds. In the form of sieve elements, suberised cells or tracheary elements, random and rudimentary cambial zones can give rise to areas showing vascular differentiation. Some of the meristematic activity centres form nodules that may be precursors of shoot apices, root primordia, or incipient embryos which, if placed on appropriate culture media, are able of further growth (Evans et al., 2003).

1.7. Plant cell suspension cultures

In plant biology, plant cell suspension cultures are usually used as a convenient method for studying a wide variety of phenomena, bypassing the structural complexity of the entire plant organism (Figure 4). Suspension-cultured cells are useful for the study of complex physiological processes at the cellular and molecular levels because of the homogeneity of an *in vitro* cell population, the high material availability, and the high rate of cell growth and the strong reproducibility of conditions. In addition, plant cell cultures provide a valuable source for the production of high value secondary metabolites and other products of commercial interest. Ahuja (2013) described how to initiate and maintain plant cell cultures starting from explants extracted from *in vitro* germinated seedlings of woody plants. Moreover, the isolation of protoplasts from plant cell suspension cultures and regeneration of plants via organogenesis and somatic embryogenesis were also described.

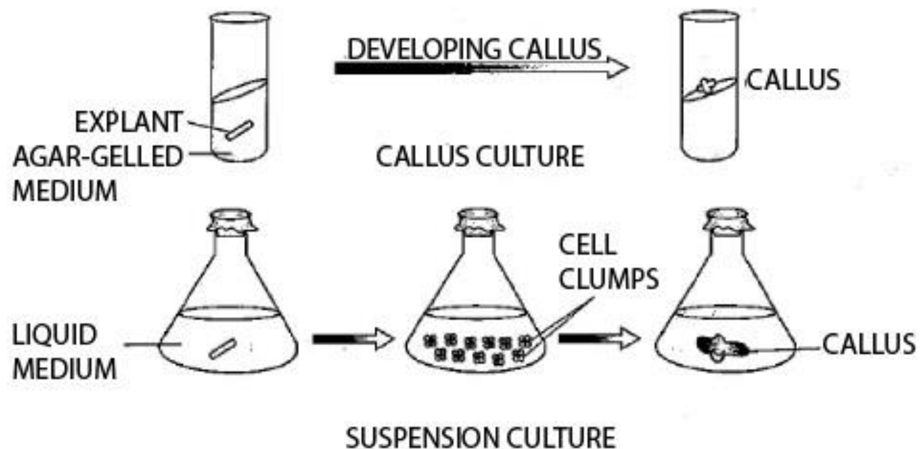

Figure 4. Schematic representation of procedures for establishment of callus and cell suspension cultures.

An optimised medium has been established for the initiation of tracheary elements in the suspension cultures of *Zinnia elegans* L. mesophyll cells (Kohlenbach & Schmidt, 1975; Roberts et al., 1992). In another study, callus cells were derived from cambial strips of Douglas-fir (*Pseudostuga menziesii*) trees and transferred to liquid MS medium supplemented with 2,4-D to differentiate tracheary element (TE) like cells (Pillai et al., 2011).

1.8. Factors affecting early xylem differentiation

1.8.1. Plant growth regulators (PGRs)

1.8.1.2. Auxins

Plants growing in the outdoor environments may face a combination of different separate or concurrent biotic and abiotic stresses. The primary factors or stressors that can impose stress on plant growth include light, temperature, salt, carbon dioxide, water, ozone, and soil nutrient content and availability (Isah, 2019), where the fluctuation of any of these has negative impact on the normal physiological processes. Being static organisms, plants are unable to avoid abiotic stresses simply by moving to an appropriate environment. Therefore, they have developed a suite of adaptive mechanisms to respond to the unwanted stressful conditions by changing their own developmental and physiological processes.

Plant growth regulators (PGRs), are chemicals produced by plants that regulate cellular activities. reproductive processes and coordinate responses to various environmental conditions, such as osmotic stress, drought, chilling injury, heavy metal toxicity, etc. (Lymperopoulos et al., 2018). There are nine major classes of PGRs: (auxins, cytokinins, gibberellins, abscisic acid (ABA), ethylene (ET), brassinosteroids, jasmonic acid (JA), salicylic acid (SA), and strigolactones. All capable of regulating many different plant physiological responses.

Auxis and cytokinins were essential in development of the different phases of cell (Elo et al., 2009; Nieminen et al., 2012). Auxin was a main hormone controlling cell division and differentiation of cambial initials during xylem formation. Indeed, IAA has been shown to be involved in the regulation of anticlinal and periclinal divisions of cambial initials in transgenic

hybrid aspen and could only affect xylem production. When auxin signalling was perturbed, the fibers and vessels in hybrid aspen were shorter and slimmer (Nilsson et al., 2008).

Several studies have suggested that auxin accumulates in a radial gradient pattern through wood-developing tissues with high concentration in cambium and bilateral decay in the direction of differentiating secondary xylem and phloem (Immanen et al., 2016; Tuominen et al., 1997; Uggla et al., 1998). Consequently, auxin is known as a major regulator of secondary xylem induction possibly acting as a signalling molecule in control of morphogenesis (Sundberg et al., 2000; Uggla et al., 1996). An absence of auxin supply from pine shoot apex causes a loss of fusiform shape of cambial derivatives (Savidge, 1983b). Therefore, auxin not only coordinates cambial identity but also controls proliferation activity. Previously, identification of auxin signals through a high-affinity sensor shows its medium level in cambial stem cells, whereas enhanced level in differentiating cambial descendants (Brackmann et al., 2018).

The main role of auxin signalling in cambial differentiation is suggested by these findings. Exogenous auxin is clearly able to initiaTE induction of intact and functionally normal secondary xylem cells (Björklund et al., 2007). Local increase of auxin signalling is important for wood formation and also supports the role of auxin in cell differentiation (Bargmann et al., 2013; Müller et al., 2016). Nevertheless, the precise regulation of auxin signalling for wood differentiation on these coordinated cellular events remains unclear in trees.

1.8.1.3. Cytokinins

Cytokinins have functions in various developmental processes in plant life cycle and are considered as significant regulators of cambium development (Hwang et al., 2012; Jang et al., 2015; Matsumoto-Kitano et al., 2008; Matsuo et al., 2012). As previously reported cytokinins have one of several forms of isoprene-derived side chain, such as N6-(Δ2-isopentenyl) adenine (iP), transzeatin (tZ), cis-zeatin (cZ), and dihydrozeatin (DZ) (Kiba et al., 2013; Sakakibara, 2006). Adenosine phosphate-isopentenyltransferase (IPT) catalyses the first step in their biosynthesis. In *Arabidopsis thaliana*, knocking out expression of genes contributing cytokinin biosynthetic IPT by T-DNA insertions negatively affected cambium development and declined xylem induction in the roots and shoots. The application of exogenous cytokinin restored the mutant xylem differentiation (Matsumoto-Kitano et al., 2008). Moreover in Arabidopsis, cytokinin receptor loss-of-function mutants, Arabidopsis Histidine Kinase 2 and 3 (ahk2 and ahk3) mutants and plants with low concentration of endogenous cytokinins showed defects in metaxylem differentiation (Hejátko et al., 2009). In poplar (*Populus trichocarpa*), overexpressing the cytokinin catabolic gene Cytokinin Oxidase 2 lowered the cytokinin level and reduced secondary growth (Nieminen et al., 2008). The findings of the study involving the manipulation of exogenous cytokinin in transgenic hybrid aspen with attenuated cytokinin signalling in the cambial zone are consistent with the notion that cytokinin played a main role in cell division (Nilsson et al., 2008), even though cytokinins are mainly considered to be essential regulators of cell division (Immanen et al., 2016).

Differences in xylem induction between Japanese and Dutch tomato (*Solanum lycopersicum*) provided evidences that stems of the Dutch cultivars were more developed than those of the Japanese cultivars at the early and late stages of tomato growth due to the level of iP-type cytokinins which are locally synthesised in the hypocotyl (Qi et al., 2020). Previously it was

observed that lignocellulosic biomass production in woody plants can be increased through enhanced cytokinin signalling. These results strongly demonstrate that cytokinins are significant regulators of vascular development. Understanding regulatory mechanisms controlling tree development has immense value for forest industry through biotechnological applications (Immanen et al., 2016).

1.8.1.4. Jasmonic acid

The JAs concentrations are high in the reproductive tissues and flowers, while very low in the mature leaves and roots (Dar et al., 2015; Wasternack & Hause, 2013). JAs control many essential processes in plant development, such as vegetative growth, cell cycle regulation, anthocyanin biosynthesis, stamen and trichome development, fruit ripening, senescence, rubisco biosynthesis inhibition, stomatal opening, nitrogen and phosphorus uptake, and glucose transport (Browse, 2005; Campos et al., 2014; Creelman & Mullet, 1995; Koda et al., 1992; Parthier, 1991; Reinbothe et al., 2009; Sembdner & Parthier, 1993; Wasternack, 2007; Wasternack & Hause, 2002; Wasternack & Hause, 2013; Y. Yoshida et al., 2009; Zhang & Turner, 2008).

1.8.1.4.1. Functions of jasmonic acid in plant species other than angiosperms

Biosynthesis and action of JA compounds in angiosperms has been shown, including the precursor 12-oxo-phytodienoic acid derive from oxygenated polyunsaturated fattyacids that are collectively named oxylipins (Stumpe et al., 2010; Yamamoto et al., 2015). Jasmonates besides act as cellular signaling compounds in gymnosperms (Franceschi et al., 2002; Thaler et al., 2001). As reported in several studies, the application of MeJA enhanced the resistance of the

Norway spruce (*Picea abies*) to the root pathogen *Pythium ultimum* Trow (Kozlowski et al., 1999), stimulated the expression of the 14-3-3 gene in the spruce plant [*Picea glauca* (Muench) Voss] (Lapointe et al., 2001), as well as accumulated a high concentration of paclitaxel in several *Taxus* species (Ketchum et al., 1999). The results of a study showed that both mosses and gymnosperms, not only, provoke similar defense pathways through infection with necrotrophic pathogens, but also that non-flowering land plants induced immune responses similar to those studied in angiosperms (de Vries et al., 2018). Wound-Induced Accumulation of JA in vascular plant species is a common physiological feedback (Isah, 2019). Hence, JA has evolved as a phytohormone in the face of potentially stressful challenges, beginning with the emergence of vascular plants.

1.8.2. Light and temperature

The effect of temperature and light on tracheary element differentiation in cell suspension cultures of 11-day-old *Picea glauca* derived from hypocotyl explants was investigated (Durzan et al. 1973). When the cultures were developed under continuous light at 22.5°C, clumps of cells differentiated that contained tracheary elements in their centres (White and Gilbey (1966). When the cultures were kept under continuous light and dark conditions, differentiation was not observed. Tracheary element differentiation was initiated in *Picea glauca* callus cultures grown under a 16-h photoperiod. However, the rate of tracheary element differentiation was not reported in this study. Möller, Ball, et al. (2006) investigated differential impact of light on increasing tracheary element differentiation in *Pinus radiata* callus cultures. In this investigation, tracheary elements differentiated in *P. radiata* callus culture grown under light after only 2 days as compared to 5 days in callus cultures grown in the dark. The rates of tracheary element differentiation under light and in the dark were 45% and 20%, respectively.

1.8.3. Nitric Oxide

Nitric oxide (NO) is a secondary gaseous messenger molecule playing a key role in different plant growth and development processes (Astier et al., 2018). The function of NO is not restricted to stress responses as this molecule is also engaged in many physiological events occurring during plant cell differentiation and programmed cell death (PCD) (Sanz et al., 2015). Kapoor et al. (2018) have demonstrated that suppression of *Zea mays* phytoglobins induces PCD by elevating NO during somatic embryogenesis. There was a signal for a spatial NO gradient inversely related to the stages of xylem differentiation and a protoplastic NO burst was associated with the single cell layer of pro-differentiating thin-walled xylem cells (Gabaldón et al., 2005). Another important finding was that NO seemed to be involved in the processes of secondary cell wall (SCW) lignification and trans differentiation of *Zinnia* vessel elements (Gabaldón et al., 2005; Gómez Ros et al., 2006). This free radical inorganic gaseous compound was involved in diverse physiological processes, abiotic and biotic stress responses and PCD. The microscopic observations with NO-sensitive fluorescent probes and pharmacological studies with NO releasing and scavenging agents have supported the idea that NO contributed to lignification and cell death of *Zinnia* TEs (Novo-Uzal et al., 2013). In differentiating xylem of *Populus* roots, Bozhkov et al. (2005) have identified contribution of NO signalling in the cell differentiation and TE maturation process. Possible targets of NO include transcription factors and activity of some of the enzymes in lignin biosynthesis (Gabaldón et al., 2005). However, more molecular analysis as well as gene transcriptional profiling are essential to confirm the NO effects.

Nitric Oxide was considered a significant signalling molecule which regulates root growth and plant architecture (Corpas & Barroso, 2015) produced by plants through arginine-dependent (catalysed by nitric oxide synthase) and nitrate / nitrite-dependent pathways (catalysed by

nitrate reductase); but, recent studies have indicated that reduction in nitrite is the main factor of its production (Jeandroz et al., 2016).

Nitric Oxide is involved in regulating the different responses to biotic and abiotic stress in plants (Corpas et al., 2011; Groß et al., 2013). It leads to redox homeostasis in cells through reducing oxidative stress from excessive reactive oxygen species (ROS) (Correa-Aragunde et al., 2015; Groß et al., 2013; Singh et al., 2009). In its microenvironment NO interacts with free radicals and transition metals that contributes to its regulatory mode of action (Thomas, 2015) and causes significant changes in the phenylpropanoid pathway to confer mechanisms of resistance to stress conditions (Li et al., 2017).

The lignification process is an important step during root growth, as it involves sealing cells by deposition of lignin. Lignin, a phenolic heteropolymer composed of polysaccharides and proteins, is a major component of the secondary cell wall, providing structural strength. (Vanholme et al., 2010). It is produced through phenylpropanoid pathway involving well-coordinated actions of phenylalanine ammonia lyases (PAL), peroxidases (PODs), polyphenol oxidases (PPO) and many other related enzymes. Phenylalanine ammonia lyase activity has been associated in monolignols biosynthesis and is the first-rate limiting enzyme of lignin synthesis (Vanholme et al., 2010). Phenylalanine ammonia lyases catalyse the formation of trans-cinnamic acid from phenylalanine, an essential amino acid (Bonawitz & Chapple, 2010). Nitric oxide at higher concentrations decreased root formation with reduced lignin content and PAL activity (Böhm et al., 2010). In comparison, NO induced root growth at lower concentrations and increased the activities of cell wall bound PPO and PAL (Böhm et al., 2010). Peroxidases are highly distributed in the plant kingdom and involved in different processes including lignification, healing of infectious wounds and auxin catabolism (Hiraga

et al., 2001). Polymerisation of monolignols (coniferyl alcohol, sinapyl alcohol, paracoumaryl alcohol) has been proposed to be catalysed by POD, while PPO use phenolic compounds as substrate in lignin formation pathway (Boudet et al., 2003). An increase in POD activity is regarded as a signal for root initiation, while PPO's role in root induction has been related to phenolic metabolism (Liao et al., 2010). Nitric oxide acts as an essential upstream signalling molecule in the monolignol assembly, part of the pathway for lignin biosynthesis (Ferrer & Ros Barceló, 1999) and has regulatory role through lignification of xylem vessels (Ros Barceló et al., 2004). Nitric Oxide prevented POD activity and impacted the activity of lignin synthesising enzymes and increased gene transcription (Gabaldón et al., 2005). Lignin composition has been controlled by endogenous NO in roots of *Helianthus annuus* (Monzón et al., 2014). Nitric Oxide controls the expression of genes involved in the lignin biosynthesis process (4-coumarate-CoA ligase, cinnamyl alcohol, Caffeoyl-CoA O-methyltransferase, etc.) and the treatment with NO scavenger cPTIO leads to variable lignin composition (Monzón et al., 2014). Smart et al. (2002) demonstrated that IAA degradation-involved oxidoreductases such as POD, PPO and IAAO (indole acetic acid oxidase) play a number of roles in root organogensis. During the formation of adventitious roots (AR), POD and IAAO modify the auxin content of the cells (Rama Devi & Prasad, 1996). Treatment of NO and H_2O_2 increased the formation of AR in Chrysanthemum by inducing PPO and IAAO activities (Liao et al., 2010).

Adventitious rooting (AR) is an important physiological process playing a significant role in regular and stressed environments (Steffens & Rasmussen, 2016). Interestingly, the role of NO in mediating AR formation by modulating lignin and enzymes involved in lignification per se has not been known. Therefore, NO involvement in mediating AR growth, lignification, as well as related enzymatic changes in *Vigna radiata* hypocotyls has been explored. Changes in AR development, lignin content, and enzyme activities (POD, PPO, and PAL) were monitored with

2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), NO scavenger, to assess the progress. The experimental conditions for the study were chosen because the AR formation process shows high metabolic rate and initiation of lignin formation during the primary root development phases (Steffens & Rasmussen, 2016).

1.9. Secondary cell wall biosynthesis

1.9.1. Lignin

Lignin is an aromatic polymer that is present mainly in secondarily thickened plant cell wall and is formed from the monolignol oxidative cross-linking. Biosynthesis of lignin has been reviewed several times in recent years (Boerjan et al., 2003). Candidate genes have been identified from microarray data in cases where the genes encoding individual steps in lignin biosynthesis are not known. Lignification defined an interesting link between the vessels and the xylem parenchyma surrounding them. Research from *Zinnia* TEs indicates lignin formation proceeds after cell death (Ehlting et al., 2005). Findings demonstrated that TEs are able to use monolignols or dilignols supplied by other cells or added to the media, and these can be processed into the cell walls of TEs that have already undergone cell death (Hosokawa et al., 2001; Tokunaga et al., 2005). Accordingly, in developing *Zinnia* TEs, Ros Barceló (2005) found the site of production of H_2O_2, which is supposed to be necessary for lignin polymerisation. Hydrogen peroxide was produced in both vessels and thin walled cells not inducing lignification at the plasma membrane. Barcelo concluded that these latter cells provide the H_2O_2 required for lignification in the development of TEs (Ros Barceló, 2005). This study also showed that the limitation of lignin to secondary wall thickening (SCW) sites in vessels is affected by the enzymes location that polymerise the cross-linking of lignin. It is consistent with a recent study specifically localised to the SCW on a peroxidase from *Zinnia* (Sato et al., 2006).

1.9.2. Cell death

Through the cell death the end walls linking adjacent cells are eliminated to generate the perforation plate whereas extensively modified areas of the lateral primary cell wall not covered by the SCW (Figures 2 and 3). This is especially obvious in protoxylem, in which the SCW deposition regions are well spaced. In protoxylem, vessels maintain the vessel integrity for water transport, while being stretched passively as the growing plant elongates. Most of the elements of the primary cell wall are digested through cell death, with only the cellulose microfibrils and an electron-dense material remaining (Figure 5b). Glycine-rich proteins (GRPs) are rich components of this electron-dense material and link SCW thickening regions together with the adjacent protoxylem SCW (Ryser et al., 1997). It has been reported that these GRPs are extremely specific to act as loadbearing components that stabilise the hydrolysed primary cell wall. Therefore, they preserve the integrity of the protoxylem vessels for transport of water even as the vessels expand during plant growth (Ringli et al., 2001).

1.9.3. Programmed cell death (PCD)

Programmed cell death represents an important field of plant research. In the past few years, several molecular components have been characterised but there is no coherent picture is emerging as yet. Plants seem to have evolved PCD mechanisms, but it is not known what these processes have in common with animal PCD hence it is unacceptable to relate to plant PCD as plant apoptosis. Tracheary element cell death has been identified as a typical example of plant developmental PCD. It takes place in a predictable pattern among healthy cells which indicates regulation by a developmental process. It is a dynamic mechanism by which a TE cell up-regulates genes that cause their destruction. Specific protein synthesis is needed, since inhibiting translation by cyclohexamide blocks cell death of TE in the *Zinnia* system (Kuriyama, 1999). The destruction of vacuoles is the most remarkable feature of xylem cell death that coincides with the degradation of various organelles, such as the nucleus. (Fukuda, 1996; Groover et al., 1997). Vacuolar membrane collapse activates or releases hydrolytic enzymes, such as proteases (Funk et al., 2002), RNases (Lehmann et al., 2001), and DNases (Ito & Fukuda, 2002) into the cytosol, some of which may cause its destruction. Vacuolar collapse is not possible for PCD without the accumulation of such enzymes, as it does not cause nucleus digestion in non-TE cells in *Zinnia* cultures (Obara et al., 2001). Moreover, the clearest proof of the basic function of the hydrolytic enzymes during PCD is demonstrated by the introduction of an antisense for the DNase ZEN1 gene which suppresses digestion of the nucleus (Ito & Fukuda, 2002). After the accumulation stage, at the time of the vacuolar collapse the enzymatic activity of these hydrolytic enzymes increases significantly, likely due to their release from the vacuole. A vacuolar localisation for the XCP1 protease has been demonstrated in support of this (Funk et al., 2002). In comparison, at least one RNase accumulates in the TEs endoplasmic reticulum (ER) and not in the vacuole (Lehmann et al., 2001). Indeed, some of the hydrolytic enzymes detected may be cytosolic, and may be activated by cytosol

acidification resulting from vacuolar collapse. The proteome of maize xylem sap has also identified hydrolytic enzymes associated with TE PCD (Alvarez et al., 2006) and this may substantiate the theory that some of them are involved in protection rather than PCD, as proposed for the xylem protease XCP1 (Funk et al., 2002) of the several xylem-specific proteases detected, none have yet been demonstrated to be required in the process of cell death.

Recent findings have shown the significance of caspase-like proteases in this mechanism and the function of proteases in plant PCD (Rotari et al., 2005). Two major classes of plant caspase-like protease exist: metacaspases and caspase-like proteases. Metacaspases are the plant proteases most closely related to caspases, which have no caspase activity. One antisense research showed that a metacaspase is necessary in Norway spruce embryogenic cell cultures for the suspensor cell death (Bozhkov et al., 2005). It is important to note that during the late stages of TE induction only one of the nine metacaspases of Arabidopsis is upregulated, and thus this member of the gene family may be one mediator of the PCD procedure. So far few plant proteases have been reported with caspase-like activity (Rotari et al., 2005). One of these is the protease vacuolar processing enzyme (VPE), which has caspase 1 activity. Silencing of VPEs suppresses the collapse of the vacuole in tobacco mosaic virus (TMV) infected leaves. Therefore, VPE can be required to activate different vacuolar proteins involved in disintegrating the vacuoles that occur through plant PCD (Hatsugai et al., 2004). During TE development, one member of the Arabidopsis VPE family is upregulated and so might be involved in vacuole collapse. Although the absence of any effects on the *Zinnia* system following the addition of caspase inhibitors (Fukuda, 1996; McCann et al., 2000) was reported, the details were not released. This indicates that the PCD of TE can vary from other plant PCD mechanisms which are specifically sensitive to inhibitors of caspase (Rotari et al., 2005). In this context, a study using the full range of available caspase inhibitors would prove to be very

insightful, as would investigations of metacaspase and caspase-like protease knockout and overexpression .

The death of a TE cell was suggested as being a form of autophagy (Weir et al., 2005). There is no evidence supporting that. Autophagy can, on the contrary, restrict PCD, as can be seen in cell death induced by TMV (Liu et al., 2005). Additionally, the upregulation of a suite of genes is a marker for autophagy, but none of the 13 autophagy genes from Arabidopsis are upregulated in xylem induction at any point in a microarray study (Figure 5). This indicated that autophagy through TE induction is not a component of PCD.

The knowledge of mitochondria's role has been another major development in plant PCD. The release of cytochrome C from mitochondria in particular is an early sign of cell death regardless of the fact that this does not seem to activate PCD, as reported in animal models (Balk et al., 2003). One research suggested that themitochondria in TEs are involved in the PCD process and cytochrome C is released before the rupture of the vacuole (Yu et al., 2002). This indicated that vacuole collapse associated with PCD of a TE cell is not a main factor but is more likely a final phase of execution. Vacuole collapse is such a significant and visible event that it has caused a lot of interest, perhaps to the detriment of what remains to be described earlier PCD events. Despite a wide knowledge of the TE death, it is surprising that practically nothing at the biochemical or molecular level of the initiation pathway for PCD of TEs is known. Several researchers have suggested that there might be an involvement of calcium (Groover & Jones, 1999) and nitric oxide (Gabaldón et al., 2005), two main plant PCD regulators. Besides this, less is known about which genes control the process with such contained location of tissue. The mutant gpx showed that PCD can proceed without SCW synthesis and it is possible that SCW deposition in other mutants may occur as a result of PCD absence (Mitsuda et al., 2005; Turner & Hall, 2000). Obviously, no TE induction mutants have been found in which only the

PCD step is absent or has been delayed. These mutants will represent a significant breakthrough and would possibly require specific genetic screens to identify them.

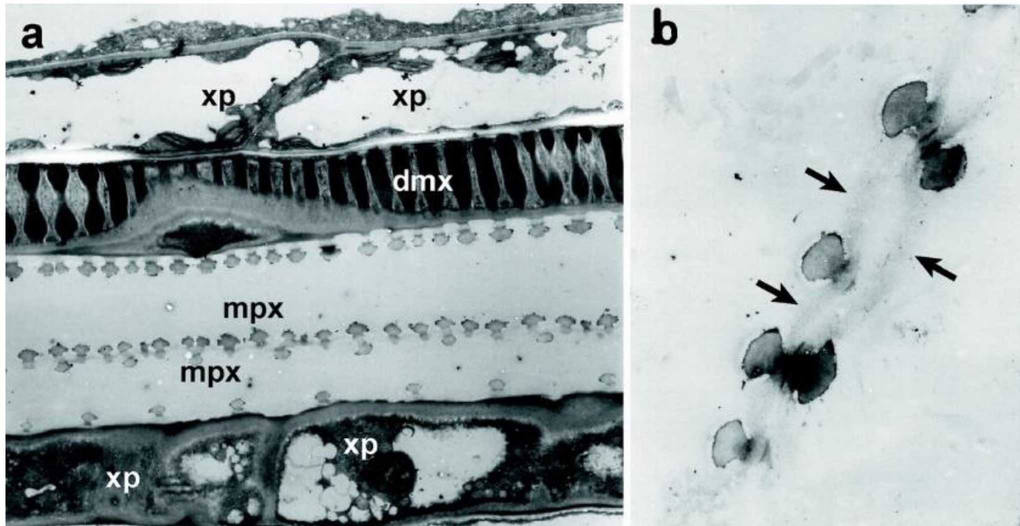

Figure 5. Tracheary element (TE) development in Arabidopsis. (a) Stem vascular bundle displaying primary mature protoxylem (mpx) that is completely differentiated and has lost the cell contents, and developing metaxylem (dmx) that still maintains cytoplasm. In xylem tissue, TEs are intimately associated with xylem parenchyma (xp) that retain their contents and develop nonpatterned secondary cell walls (SCWs) (b) Closer view of the cell wall between two mature protoxylem cells adjacent to it. The SCW is easily identifiable, and the arrows mark the remains of the primary cell wall remaining after the programmed cell death (Turner et al., 2007).

1.10. The objectives of this study

The main aim of this project is to unpack the process of xylogenesis in *Pinus radiata* callus under *in vitro* conditions. The outcomes of this research may help develop a better understanding of the control mechanisms of xylogenesis and provide information on xylem cell differentiation in *Pinus radiata*. This aim is achieved through the following main objectives:

1- To ascertain the involvement of plant growth regulators and a nitric oxide donor, sodium nitroprusside, and its interaction with plant growth regulators in the process of xylogenesis (cell differentiation and tracheary element induction) in the model plant *Pinus radiata* (Chapters 2 and 3).

2- To investigate the process of xylogenesis in *Pinus radiata* at the biochemical level via measuring key enzymes such as POD, CAD, etc. (Chapter 4)

3- To examine the process of xylogenesis in *Pinus radiata* at morphological and histological levels. (Chapter 4)

Chapter 2. Callus Induction in *Pinus radiata*

2.1. Introduction

2.1.1. Biotechnology applications of plant callus cultures

Using new technologies including plant genetic engineering to develop woody plants with desirable features adds a new and increasing dimension to plant use for humanity. Plant biotechnology has come of age, and over the past few decades a plethora of bioengineering applications have been delineated in this context. Callus cultures and suspension cell cultures provide a wide variety of uses in pharmacology and pharmacy (including Chinese medicine), as well as in agricultural and horticultural applications. Genetically engineered callus cultures, for example, can be used to synthesise bioactive secondary metabolites and to produce plants with increased resistance to salinity stress, drought, pests and diseases. While the potential value of the callus culture technology has not been fully exploited yet, the time has come to develop and market more products based on callus culture (Efferth, 2019).

The culture of plant tissues provides an important method for basic science and commercial use. Wounded tissue is recovered by non-differentiated callus cells in every major family of terrestrial plants (Efferth, 2019). For biotechnological applications, these callus cells can be cultured *in vitro*. Callus cultures can be induced from almost any part of the plant. Explants derived from plant tissues grow *in vitro* into a cell mass that ranges from an amorphous and colourless to a pale-brown mass. The explants need to be isolated under sterile conditions to avoid any microbial contamination and grown on solid gel containing plant growth regulators (mainly auxin and cytokinin). Callus cultures can be maintained *in vitro* for an indefinite period by transferring the cells to a new medium regularly. Differentiated plant cells and cultured callus cells are significantly different. Calli are generally similar to non-differentiated cells

accompanying by some meristematic cells with small vacuoles and chloroplasts for photosynthetic process. Whole plants can be regenerated from callus cultures if they are induced under optimal growth conditions and media designed for plant regeneration. Although some callus cultures grow well in the dark, others require different day-night conditions (e.g., 16 h light, 8 h dark). In many plants, such as barley (Haque & Islam, 2014), Vicia faba (Almaghrabi, 2014), maize (Morshed et al., 2014; Pathi et al., 2013), wheat (Islam, 2010) and taro (Paul et al., 2014) callus is induced in dark; while in tomato (Sherkar & Chavan, 2014), tobacco (Yanjie, n.d.), chilli (Aniel Kumar et al., 2010) callus induction occurred under light conditions. Two types of calli with differing textures may be formed from explants and during subculture: compact and friable calli. Friable callus cultures may be used to grow single-cell cultures which are placed in a slowly shaken suspension medium. The *in vitro* culture of forest tree calluses and organs was introduced mainly as a forest tree breeding technique. Pine and *Pciulownia* calli were used for biosynthesis study and biochemical analysis of the lignin formation pathway (Isikawa, 1984).

Plant tissue culture was promoted by Gottlieb Haberlandt (1854–1945), who in the early 20th century established the first root-callus or embryo cultures (Bonner, 1936). Technological advances in the 1940s and 1960s led to the further development of plant tissue culture methods to assess cell behaviour (including cytology, nutrition, metabolism, morphogenesis, embryogenesis, and pathology), pathogen-free plant generation, and conditions of germplasm storage and clonal propagation. The biosynthesis of the secondary metabolites has been a topic of interest since the 1960s. Following the advent of the technological gene-based methods, new and more effective applications of callus cultures and other plant tissue techniques have been established (Thorpe, 2007). Plant cell cultures provide an efficient means for the development

of therapeutically important secondary metabolites (e.g., anticancer drugs) on an industrial scale utilising bioreactors (Georgiev et al., 2009).

Compared with traditional whole-plant cultivation, the main advantages of cell culture systems include: (1) plant compounds of choice can be produced independently of external factors (e.g. composition of soil or climate); (2) the attacks of microorganisms or insects do not affect cultured cells; (3) cells of any plant (even rare or threatened) can be easily cultured for production of their secondary metabolites; and (4) robotic-driven regulation of secondary metabolite production reduces costs and enhance productivity (Efferth, 2019). Callus cultures can be used in pharmaceuticals, cosmetic foods, and related industries for the sustainable and large-scale processing of secondary metabolites. As phytochemicals can be directly obtained from calli without destroying the entire plant, the callus technology can help to protect rare and endangered plant species, and huge amounts of secondary metabolites can be generated *in vitro*. In addition, callus cultures can be converted to single-cell suspension cultures growing in flasks on shakers or in biofermentors in order to produce the desired secondary metabolites (Fischer et al., 1999). This enables growth under controlled conditions without the effect of changing environmental factors, seasonal variation of microbial diseases, pests, and geographical constraints. Therefore, secondary metabolites with high quality can be generated. However, there are only few research papers published on tracheid differentiation in callus and suspension cultures of coniferous gymnosperms. Different growth factors and other media components are known to influence differentiation of *in vitro* tracheids (Ramsden & Northcote, 1987b; Savidge, 1983b; Washer et al., 1977). In *Pinus radiata* D. Don callus cultures, tracheid and sclereid development were induced during culturing on a basal medium without any phytohormones but containing activated charcoal.

2.2. Callus formation

It has been reported that stem cells in animals can initiate into the development of other cell types/tissues that are inevitably terminated. It is expected, however, that differentiated cells in plant tissues can dedifferentiate and regenerate new tissue or even the whole plant (Burris et al., 2009; Chavez et al., 1998; Chen et al., 2000; Finer et al., 1989). A more recent hypothesis suggests that plant cells do not dedifferentiate but instead the callus is formed mostly from pre-existing stem cells (Sugimoto et al., 2011; Wang et al., 2011).

The underlying molecular mechanisms of action leading to the differentiation of stem cells and/or the differentiation – dedifferentiation of somatic plant cells are not clearly known. The stem cell-related genes are important for processes of dedifferentiation. Their expression is regulated not only by transcription factors but also by epigenetic processes such as histone modification and methylation of DNA (Jiang et al., 2015).

Arabidopsis thaliana (L.) Heynh has been used as a model organism for a large variety of different plant biology studies. In Arabidopsis, auxin gradients in embryonic callus seemed to be related to stem cell formation and could be induced by regulation of the Pin-formed 1 (PIN1) protein (Su & Zhang, 2009).

Microscopic live-cell imaging has been widely used to evaluate the kinetics of the spatial and temporal distribution of hormonal and developmental meristem regulators. The following growth determinants are of interest, including microtubules, transcription factor networks, the cytokinin pathways that regulate expression of genes encoding the transcription factor Wuschel (WUS), the auxin-mediated positioning of new primordia, etc. (Sijacic & Liu, 2010).

Many transcription factors regulate the development and dedifferentiation of a meristem. The WUS and Wound Induced Dedifferentiation (WIND) transcriptional repressors are driving factors to maintain stem cell totipotency, while the Teosinte Branched1 (Cycloidea) Proliferating Cell Factor (TCP) is a transcriptional activator that prevents stem cell totipotency in the shoot meristem (Ikeda & Ohme-Takagi, 2014).

2.3. Aim and experimental approach

The main aim of this chapter is to establish a reproducible callus culture production system in *Pinus radiata*. The objectives of this chapter are to:

1. Investigate the impact of starting plant material on callus production.
2. Examine the influence of different genotypes (called lines 32-2, 26-2, 108-3, 103-4, 59-4 and 99-5) on callus production.

2.4. Materials and Methods

2.4.1. Callus induction

All trees and seeds were purchased from Proseed Ltd. (Amberley, New Zealand). Different genotypes of *Pinus radiata* trees (32-2, 26-2, 108-3, 103-4, 59-4 and 99-5) were grown in the glasshouse of University of Canterbury. Cotyledons and hypocotyls from *in vitro* germinated seedlings were used to investigate callus and TE induction as well as for light microscopy studies.

Calli were derived from stem strips obtained from one-year-old shoots of the 3-year-old *P. radiata* trees grown at the glasshouse, University of Canterbury. Segments of young shoots (8 cm long) were surface sterilised using 30% bleach (with 5% sodium hypochlorite as the active ingredient). The bark was then peeled off and the stem strips (1 cm long x 0.8 cm wide) were isolated. The strips were transferred to the P6-SHv medium (Hotter, 1997) containing 1.0 mg/L BAP and 1.0 mg/L 2,4-D (T1) at pH 5.6. Five strips with three replicates on the medium were kept at 24ºC under light and dark. After 5-6 weeks of culture, the explants and any calli formed were visually inspected for any change in morphology and growth. As a different result was obtained from that reported by Hotter (1997), this experiment was repeated two more times. In a second experiment, stem strip explants were cultured on the T2 medium which was MS medium solidified with 7 g/L agar, and supplemented with 3 mg/L (BAP), 3 mg/L 2,4-D and 20 mg/L PVP (Figure 10).

Seeds used in this experiment were collected from the same population of open-pollinated *Pinus radiata* D. Don trees grown in Canterbury, New Zealand. Seeds were surface-sterilised in 70% (v/v) ethanol for 30 s, rinsed in sterile water, and then soaked in 50% (v/v) of a commercial bleach (with 5% sodium hypochlorite as the active ingredient) for 30 min before

being rinsed thoroughly with sterile water. The sterilised seeds were then transferred to MS medium and placed in a plant growth room at 24±2°C with continuous lighting at 80 µmol m^{-2} s^{-1}. Root, cotyledon and hypocotyl explants were excised from the seedlings germinated under aseptic conditions after 7 weeks (Li & Leung, 2000). Five explants were placed on MS medium supplemented with 0.58 mg/L BAP + 1.0 mg/L 2,4-D + 20 mg/L PVP in sterile 9 cm Petri plates (T3). All the media were set at pH 5.7, solidified with 0.8% (w/v) agar. Media were generally sterilised by autoclaving at 121 °C and 1.05 kg/cm^2 (15-20 psi) for 20 min. The cultures were incubated in a dark growth room at 24±2°C. After 8 weeks, calli were subcultured on fresh MS medium of the same composition as used in callus induction (Figure 12).

2.5. Statistical analysis

All the experiments were repeated at least two times and each treatment had at least three replicate plates. The Petri plates were arranged in the growth room using a randomised complete block design. The data obtained were subject to analysis of variance (ANOVA, $p \leq 0.05$) followed by comparison of mean values of the treatments using Fisher Unprotected LSD using Genstat for Windows 19th Edition software.

2.6. Results and Discussion

2.6.1. Callus induction in *Pinus radiata* explants using the T1 medium (P6-SHv medium supplemented with 1.0 mg/L BAP + 1.0 mg/L 2,4-D) as described in Hotter (1997)

The T1 medium was prepared according to the procedure described by Hotter (1997). The percentages of callus formation in the stem strip explants of the different genotypes varied from 22% to 60% (Figures 6 & 7). The genotypes 26-2 and 32-2 showed the best callus induction response, while the genotypes 59-4 and 99-5 showed the lowest callus induction response (Figures 6 & 7). In the juvenile explants cultured on the T1 and kept in a dark growth room medium, the best callus induction response (28%) was observed in the hypocotyl and cotyledon explants, while the lowest percentage of callus formation (12%) was recorded in the root explants (Figure 8). The three types of explants cultured on the T1 medium under continuous lighting exhibited even lower percentages of callus induction, while the root explants did not initiate any callus at all (Figure 8). These experiments were repeated three times for both stem strip and explants of *in-vitro* grown seedlings exactly as described by Hotter (1997) and Möller et al. (2003). The results obtained in the present study, however, were not the same as in the two prior studies, suggesting that their results were not repeatable. Moreover, the calli obtained in the present experiment died later before any further development. This experiment was repeated three times with the same outcome. Callus culture could not be established for the planned experiments using the published *P. radiata* callus formation protocol. The authors of the two previous studies reported that they were able to establish *P. radiata* callus culture which they were able to carry out further study. Their protocol was, therefore, chosen initially as a shortcut approach so that there was no need to establish a *P. radiata* callus induction protocol before the research question using *P. radiata* callus in the present study could be addressed. All efforts to approach the authors to discuss any possible reasons for this discrepancy was not

successful as the authors had left New Zealand some years ago and were no longer involved in this line of research.

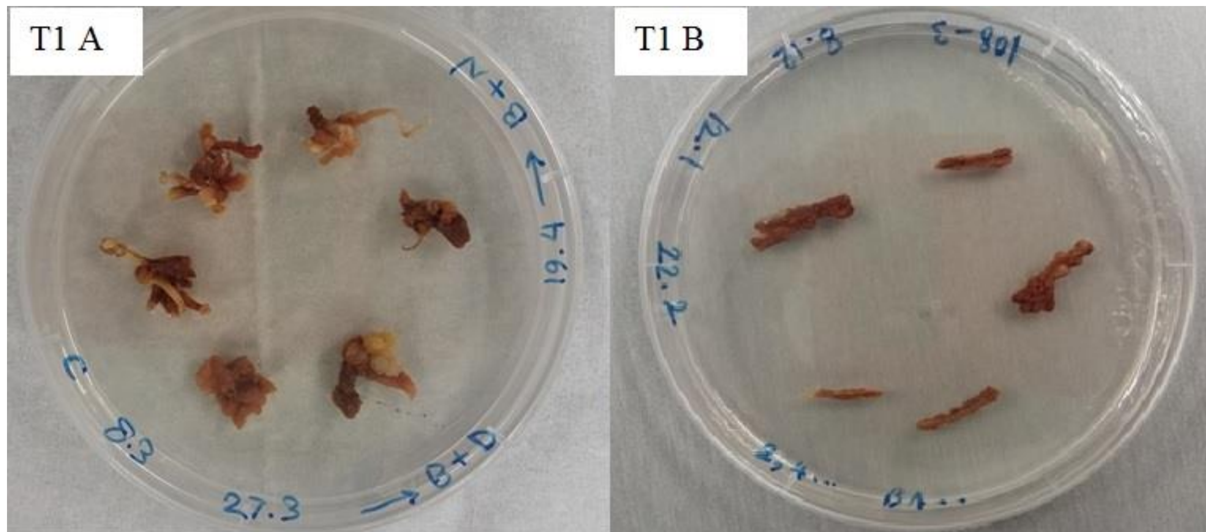

Figure 6. P6-SHv medium + 1.0 mg/L BAP + 1.0 mg/L 2,4-D used to induce callus formation in hypocotyl (T1 A) and stem strips cultured in darkness and light for 8 weeks (T1 B). The explants were isolated from *Pinus radiata* cultured *in vitro* (Hotter, 1997; Möller et al., 2003).

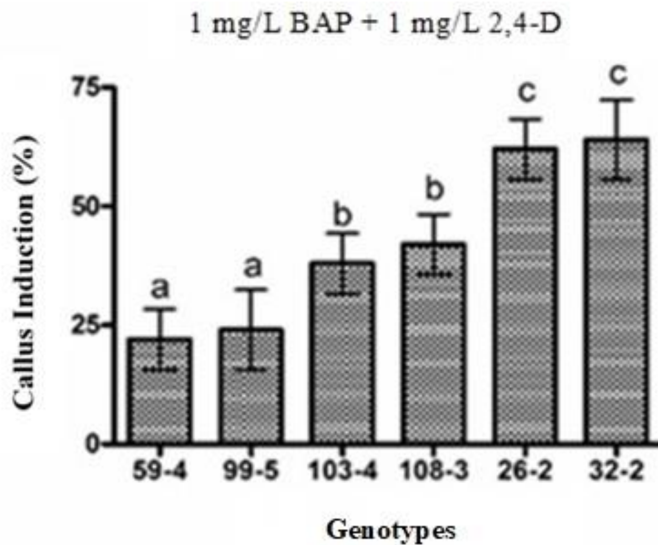

Figure 7. Effect of P6-SHv medium supplemented with 1.0 mg/L BAP + 1.0 mg/L 2,4-D on callus induction in stem strip explants of different genotypes cultured in the dark for 8 weeks. The explants were isolated from one-year-old shoots of 3-year-old *Pinus radiata* trees grown in the glasshouse at the University of Canterbury. Means ± SD with different letters were significantly different ($p \leq 0.05$).

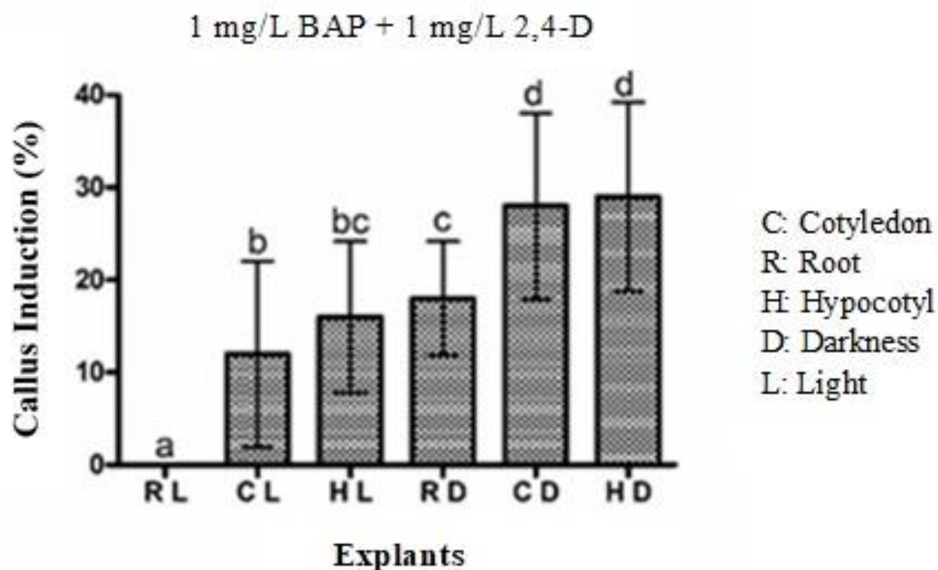

Figure 8. Effect of P6-SHv medium + 1.0 mg/L BAP + 1.0 mg/L 2,4-D on callus induction in cotyledons hypocotyl and root explants cultured in darkness or under continuous light for 8 weeks. The explants were isolated from *Pinus radiata* eight-week-old seedlings grown *in vitro*. Means ± SD with different letters were significantly different ($p \leq 0.05$).

2.6.2. Search for useful culture media for callus induction in *Pinus radiata* explants

Woody plant basal media (WPM) generally contain lower levels of macronutrient salts compared to ½ basal MS andMMS (modified MS) media, but there are many studies showing that basal MS medium can be used for tissue culture of explants from woody plants. It has also been found that MS medium is commonly used in 82% of the 5-y set of citations in the plant tissue culture research literature and may be recommended for most plant tissue culture applications (Herman, 2005; Phillips & Garda, 2019). Yamagishi et al. (2017) used MS medium with 3 % sucrose, 100 mg/L myo-inositol and 1.0 mg/L 2,4-2,4-D, with the pH adjusted to 5.8 and solidification with 0.3
% gellan gum, to initiate hybrid poplar *(Populus sieboldii* x *P. grandidentata*) calli. Callus of *Citrus limon* (L) Burmann var were grown on a liquid MS basal medium supplemented with MS vitamins, indoleacetic acid (IAA) (10 mg/L), kinetin (0.2 mg/L), and sucrose (3% w/v) with an initial pH 5.0–6.0 (Khan et al., 1986). Callus was initiated successfully from the cotyledons of *Cryptomeria japonica* plantlets on MS medium supplemented with 4 mg/L IAA (Mehra & Anand, 1979). Phenylalanine ammonia lyase (PAL) activity in a MS growth medium contained 10 mg/L naphthaleneacetic acid, 2 mg/L kinetin and 6% sucrose (Ramsden & Northcote, 1987b).

The callus formation rates in all the explants were increased to at least 60% when the explants were grown on MS basal medium supplemented with 0.58 mg/L BAP and 1 mg/L 2,4-D (T3). After 8 weeks incubation in dark, 100% of the cotyledon and hypocotyl explants formed callus (Figure 9). This suggests that T3 medium could be used to establish callus cultures in explants from *Pinus radiata* seedlings.

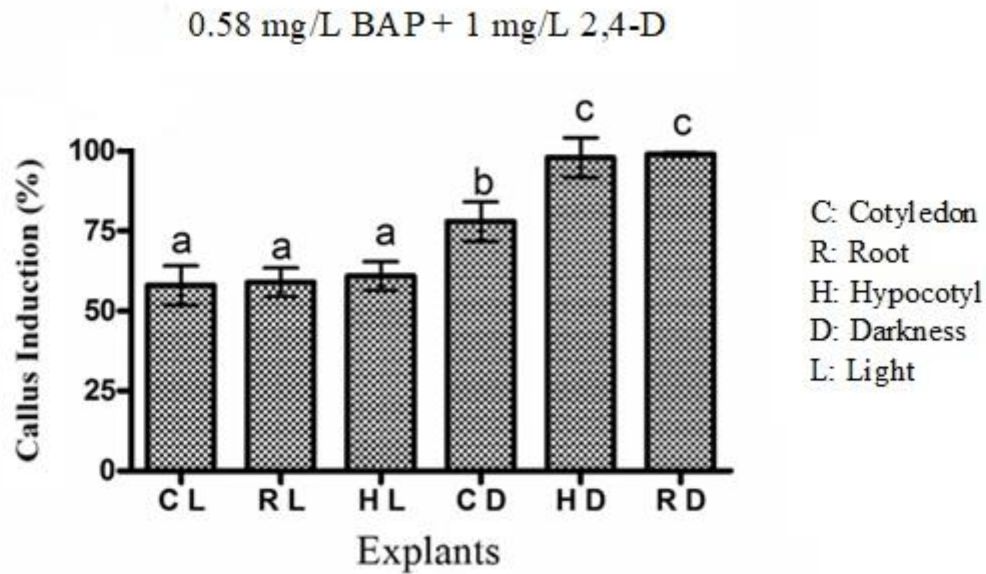

Figure 9. Effect of MS medium + 0.58 mg/L BAP + 1.0 mg/L 2,4-D on callus induction in cotyledon, hypocotyl and root explants from *in-vitro* grown seedlings of *Pinus radiata* cultured in darkness and under continuous lighting for eight weeks. Means ± SD with different letters were significantly different ($p \leq 0.05$)

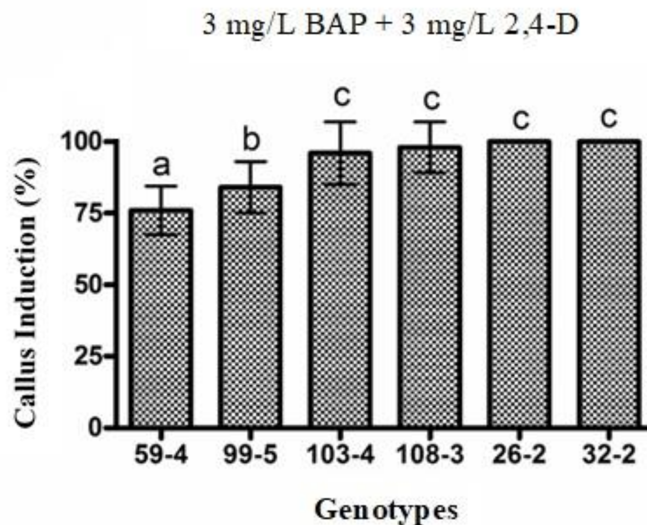

Figure 10. Effect of MS medium + 3.0 mg/L BAP + 3.0 mg/L 2,4-D on callus induction from *in vitro* stem strips of different genotypes of *Pinus radiata* cultured in the dark for 10 weeks. Means ± SD with different letters were significantly different ($p \leq 0.05$).

Callus was formed in 100% stem strips explants of genotypes 26-2 and 32-2 after 8 weeks of culture. The stem strips in the other four genotypes exhibited callus formation of more than 75% (Figure 10). This suggests that a high frequency of callus induction in stem strip explants of 1-year-old shoots from 3-year-old *P. radiata* trees resulted when cultured in the dark on MS medium supplemented with a higher 2,4-D concentration in combination with BAP compared to cotyledon, hypocotyl and root explants (Figure 10).

Based on the results obtained so far, the T3 medium was selected as a promising initial callus induction medium for hypocotyl and cotyledon explants from the 3-week-old *P. radiata* plantlets. Furthermore, calli in stem strips excised from 1-year-old shoots of 3-year-old *P. radiata* trees cultured on T2 were shown to be the best sources of calli to transfer to TE induction experiments (Figures 11, 12 and 13).

Similarly to several other pine species, *Pinus radiata* is known for its recalcitrance in tissue culture. A major challenge to maintain *in vitro* culture initiated usingmature tissues and also many explants from seedlings is browning of culture medium. This is the result of oxidation of mono and diphenols released from the cultured plant tissues into the surrounding medium (Laukkanen et al., 1999; Saxena & Gill, 1986). In the present study, callus induction was stimulated when the explants were cultured in the dark compared to the culture kept under light. The positive effect of the dark culture condition might be explained by suppression of photooxidative damage. The callus induction rate was also influenced by the concentration of PVP added to the induction medium as a browning control for absorption of toxic phenolic substances, and the optimum PVP concentration was 20 mg/L. Exclusion of PVP from the induction medium decreased callus induction rate. The intention of our studies was to allow

stimulating effects of dark but prevent the accumulation of phenolic compounds to toxic concentrations by application of PVP at different stages of callus development. In accordance to Laukkanen et al. (1999), the high POD level in Scots pine callus tissues started from mature trees caused rapid and early browning and possibly subsequent cell death. Efficient callus formation in darkness was also observed when the calli were cultured on the T2 or T3 medium. In contrast, callus formation was decreased when the callus was cultured under continuous light. These results support those of previous studies (Reustle & Natter, 1994; Saxena & Gill, 1986).

Inthe present study, the P6-SHv medium containing 1.0 mg/L 2,4-D + 1.0 mg/L BAP was found to be not suitable for induction of subculturable callus from both seedling and stem explants of *Pinus radiata*.In contrast, MS medium supplemented with 1.0 mg/L 2,4-D + 0.58 mg/L BAP was useful for callus induction and callus proliferation. Similar findings on callus growth of *Pinus radiata* using similar combinations of NAA and BAP added to medium have also been reported (Schestibratov et al. (2003). Combinations of auxin and cytokinin have also been reported to support efficient callus growth in other woody species such as *Jatropha curcas* (Sujatha & Mukta, 1996) and *Lithospermum erythrorhizon* (Yu et al., 1997). In contrast, Cardoso and de Oliveira (1996) reported callus initiation in *Hypericum brasiliensis* when explants were incubated only with either NAA or BA. In general, only recently have studies begun to understand the molecular mechanisms through which these two hormones interact to produce a specific developmental output.

In conclusion, in the present study a useful protocol for *Pinus radiata* callus induction and proliferation has been established. There are many factors including the choice of explants and growth regulators added to medium could influence callus induction and subculture.

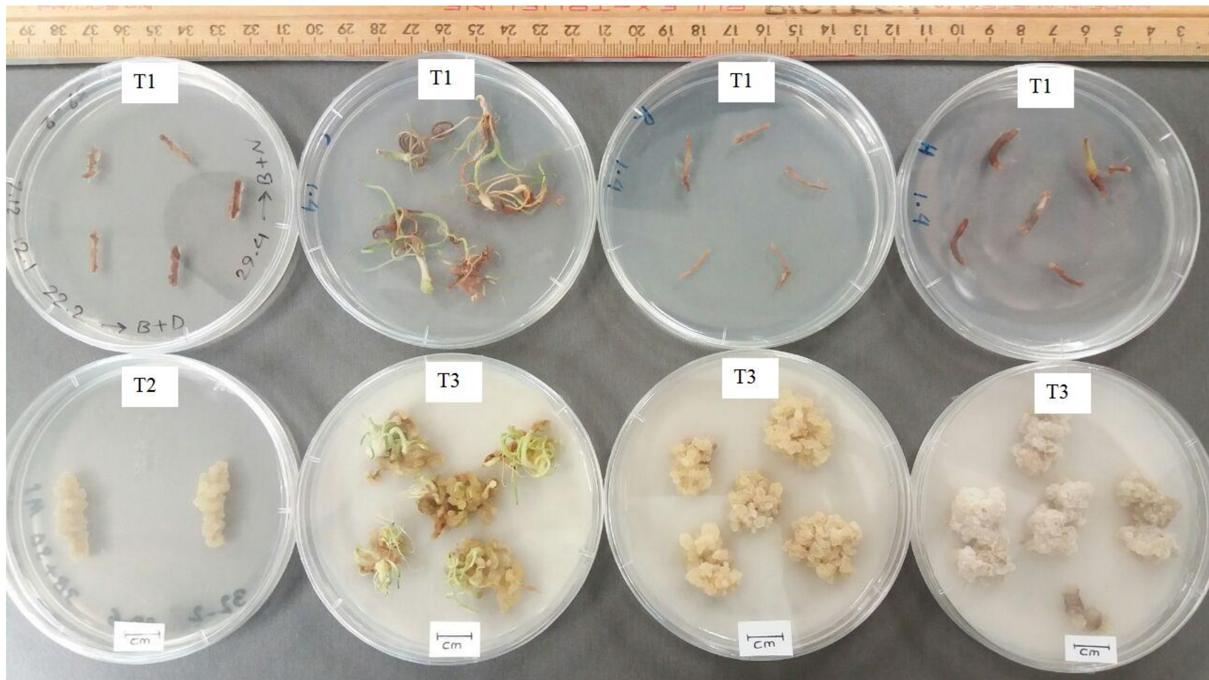

Figure 11. Callus induction in different explants from *Pinus radiata* cultured in a dark growth room at 24±2°C for eight weeks. P6-SHv medium + 1.0 mg/L BAP + 1.0 mg/L 2,4-D (T1). MS medium containing 3.0 mg/L 2,4-D, 3.0 mg/L BAP and 20 mg/L PVP (T2). MS medium containing 1.0 mg/L 2,4-D, 0.58 mg/L BAP and 20 mg/L PVP (T3).

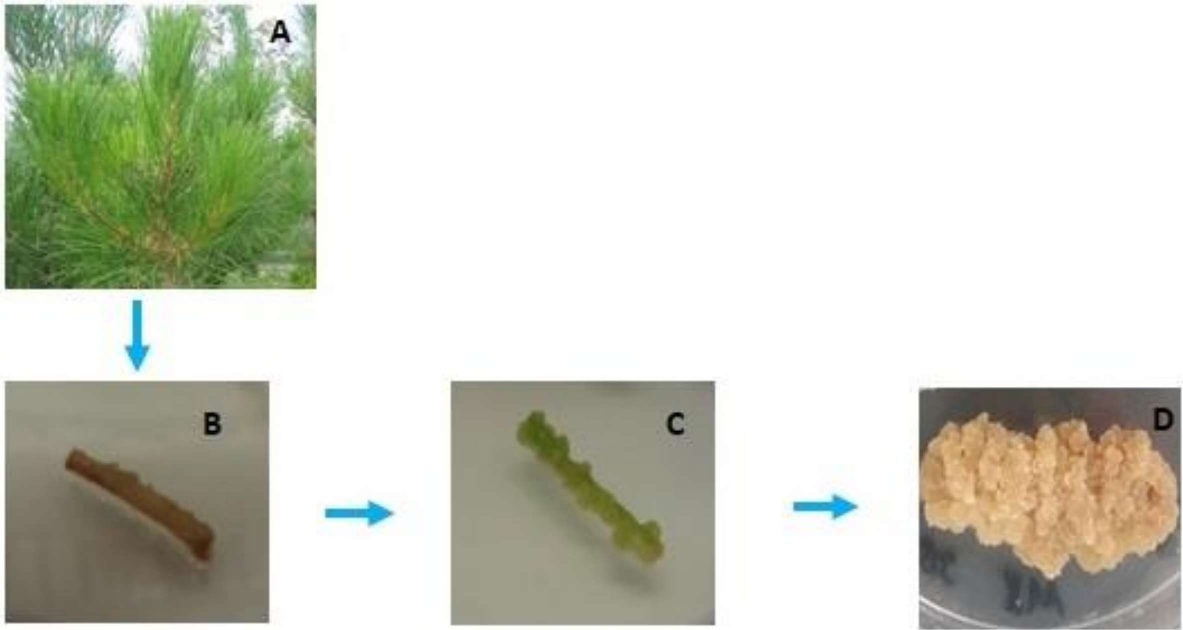

Figure 12. Shoot cutting from a 3-year-old greenhouse plant (A, B), callus initiation after 4 weeks (C), and callus initiation after 8 weeks (D).

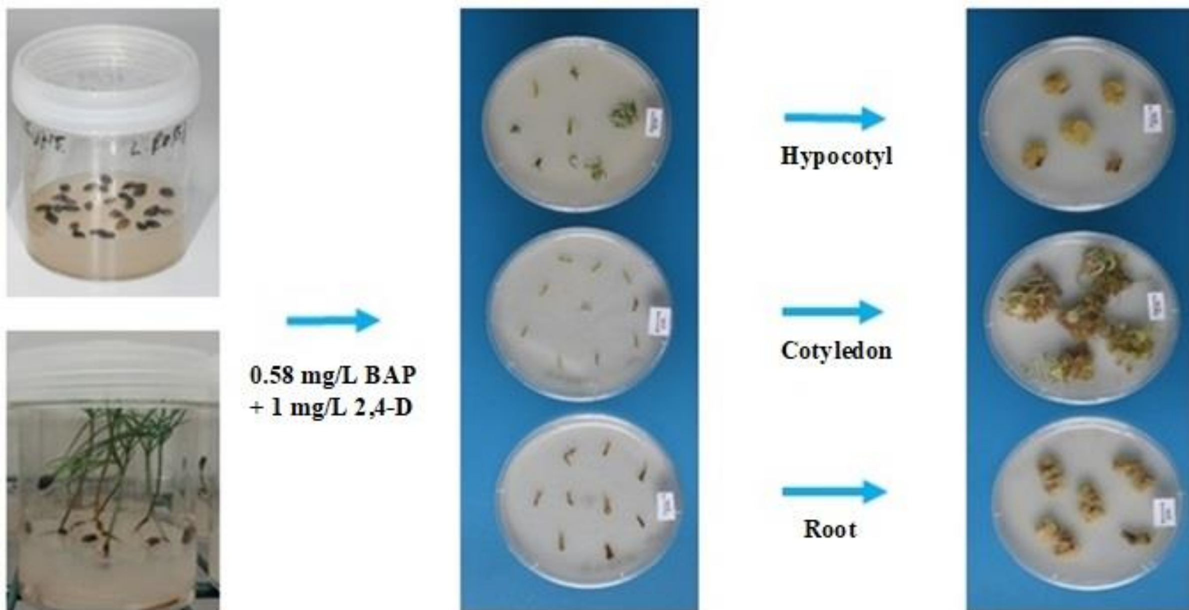

Figure 13. Hypocotyl, cotyledon and root explants from 3-week-old *in vitro* germinated *Pinus radiata* seedlings were placed on MS medium containing 1.0 mg/L 2,4-D and 0.58 mg/L BAP. The cultures were kept in a dark growth room at 24±2°C.

Chapter 3. *In Vitro* Differentiation of Tracheary Elements in *Pinus radiata* Callus Cultures

3.1. Introduction

Tracheary elements (TEs) are specialised plant cells called xylem vessels and tracheids found in the xylem of Angiosperms and Gymnosperms, respectively, which have the main purpose of transporting water and minerals (Schuetz et al., 2013). The negative pressure produced in the xylem as a result of differences in water potential between the soil and the atmosphere drives this transport (Sperry, 2004). Tracheary elements have special and characteristic evolutionary modifications to maintain this pressure for promoting transportation of xylem sap. Initiation of tracheary element formation is associated with the execution of programmed cell death (PCD), so that mature TEs are "hollow dead cells", in which the PCD process leads to the autolysis of the symplast components creating an empty lumen to lessen resistance to sap conduction (reviewed by Ménard and Pesquet [2015]). Tracheary elements have lateral walls to help to withstand the negative water pressure inside, as they have thick secondary wall materials of heterogeneous compositions including complex phenolic compounds, cellulose and hemicellulose polymers, and lignin. During TE induction, secondary cell walls are deposited in different patterns exhibiting spiral, reticulated, and pitted appearances (Schuetz et al., 2013).

Tracheary element development can be initiated *in vitro*. For example, it was first reported by Fukuda and Komamine (1980) that in *Zinnia elegans* leaf cell cultures there was trans-differentiation of single mesophyll cells into TEs in a semi-synchronous manner. Consequently, this method was developed and used to analyse the cellular, biochemical, and molecular processes that occur during TE formation (Ménard & Pesquet, 2015). Previous

studies have suggested that plant growth regulator-induced differentiation of TEs from parenchyma cells in Arabidopsis (Oda et al., 2005; Pesquet et al., 2010). *In vitro* system development has led to a better understanding of the molecular network regulating TE formation, in conjunction with research findings on xylem development *in vivo*. The early molecular markers include the homeodomain-leucine zipper (HD-Zip) III gene *ATHB8*, which specifies procambial cell fate (Donner et al., 2009), and Vascular-Related Nac Domain6 (VND6) and 7 (VND7), that stimulate concomitant activation of PCD and cell wall biosynthesis via the regulation of direct downstream targets (Ohashi-Ito et al., 2010; Zhong et al., 2008). Plant growth regulators and signal molecules also mediate the execution of these two procedures *in vitro*, while nitric oxide (NO) (Gabaldón et al., 2005) and ethylene (Pesquet & Tuominen, 2011) also play a key role.

Phytoglobins (Pgbs) or plant hemoglobins (Hill et al., 2016) similar to mammalian hemoglobins (Hill, 2012) are main PCD regulators through NO and ethylene mediation. Phytoglobins contribute to protecting cells, tissues and organs from stress conditions (Perazzolli et al., 2004) through their ability to bind oxygen (Dordas, 2009; Hoy & Hargrove, 2008) and scavenge NO at low levels of oxygen (Dordas et al., 2003; Dordas et al., 2004). Using maize somatic embryogenesis as a model system, Huang et al. (2014) showed that suppression of *ZmPgb1.1* and *ZmPgb1.2* was enough to initiate PCD in specific cells. The cells destined to die accumulated NO resulting in cell dismantling through the metacaspase activity (Huang et al., 2014). A model resulting from these studies (Huang et al., 2014; M. M. Mira et al., 2016) suggested that phytoglobins (Pgbs) may act as major regulators ensuring plant cell survival under adverse circumstances, and that their suppression triggers the death programme by increasing NO and ethylene concentrations. One possible intermediate in Pgb responses is the transcription factor MYC2, which is suppressed under conditions of low Pgb (or high NO) levels (Elhiti et al., 2013). MYC2 could also interfere with the signalling of multiple plant

growth regulators such as ethylene (Kazan & Manners, 2013). The findings that Pgbs is highly expressed in uninitiated meristematic cells (reviewed by Stasolla and Hill (2017), and that the death of the same cells in response to suppression of *Pgbs* is followed by vacuolation, a sign of cell differentiation (M. Mira et al., 2016) showed that the Pgb-mediated cell death programme is a consequence of "terminal differentiation." That is, the suppression of *Pgbs* triggers the process of cell differentiation as the terminal event resulting in cell death.

In order to set up a cause-effect relationship between Pgbs and cell differentiation and to determine the involvement of NO and ethylene, *in vitro* TE trans-differentiation of Arabidopsis cells over-expressing or down-regulating two *Pgbs* (*Pgb1* and *Pgb2*, (Hebelstrup et al., 2006) was assessed for testing whether suppression of *Pgbs* stimulates cell differentiation. They showed that TE induction was delayed in cells that over-expressed *Pgb1* and *Pgb2*, and encouraged in those cells where the expression of the two genes was decreased. This effect meets a system in which Pgbs reduce the level of NO, a MYC2 repressor that inhibits accumulation of ethylene. Ethylene acts as an effective regulator of TE development by inducing gene expression needed for specifying procambial cell fate (Mira et al., 2019).

3.2. Aim and objectives

The aim of this chapter is to achieve cell differentiation and tracheary elements induction in *Pinus radiata* callus derived from hypocotyl segments, cotyledon segments and stem strips (described in Chapter 2). The objectives are to:

- Evaluate the effects of plant growth regulators on cell differentiation and tracheary element induction.
- Apprise the effect of a nitric oxide (NO) donor, sodium nitroprusside (SNP), and its possible interactions with plant growth regulators on cell differentiation and tracheary element induction.
- Assess the effect of 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a specific scavenger of nitric oxide (NO), on induction of xylem-like cells.
- Establish optimised protocols for tracheary element induction.

3.3. Materials and Methods

3.3.1. Media preparation and tracheary element formation assays

To investigate the induction of TEs and lignified cells in *P. radiata* callus, pieces of 8-week-old calli from a previous subculture for three weeks on the callus induction medium were transferred to TE induction medium (TIM). Tracheary element induction medium was basal MS medium supplemented with a combination of NAA (1.0 mg /L), BAP (0, 0.5 and 1.0 mg/L) and sodium nitroprusside (SNP) (0, 0.005, 0.025, 0.05 mg/L) as a nitric oxide donor, 3% sucrose and 8 g /L of agar. Media were generally sterilised by autoclaving at 121 °C and 1.05 kg/cm^2 (15-20 psi) for 20 min. All cultures were kept at 24±2°C under continuous lighting and were arranged as a randomised complete block design in the culture room. There were five pieces of calli in each Petri dish and three replicate dishes in each treatment. A piece of callus from each treatment was randomly taken out and macerated with a mixture of H_2O_2 and acetic acid (0.5 µL) (one part of 30% H_2O_2, four parts of deionised water, and one part of acetic acid, v/v/v) for 5 hours at 60°C. The cells were then washed three times with deionised distilled water and the percentage (%) of cell viability was determined after staining with 0.025% (w/v) Evans Blue (Baker & Mock, 1994). Lignified cell (sclereid and TE) formation (%) was determined by the phloroglucinol-HCL test (0.5 µL). Red colour indicated the presence of cinnamyl aldehyde residues in lignin (Davidson et al., 1995). Tracheary elements cells were also observed based on their secondary cell wall thickening patterns after staining with toluidine blue-O staining (O'Brien et al., 1964). Tracheary elements can be distinguished from other cell types under polarised light because the cellulose deposited in the reticulate cell-wall thickenings produced strong birefringence. The percentages of cell viability, lignified and TE cells were calculated in relation to the total number of cells counted under a light microscope (40X) using a hemocytometer. After one week, five calli from each treatment were assessed for the presence of tracheids and sclereids. Pieces of callus were chosen randomly, suspended

in water, squash preparations prepared, and observed under a light microscope. The number of tracheids, which had thickened secondary walls , was determined as a percentage of the total number of cells. The means were compared in the Least Significant Difference test at 95% level using Genstat for Windows 19th Edition software.

Hypocotyl segments of *Pinus radiata* seedlings were cultured on MS medium or medium treated with 1 mg/L JA and grown for 5, 10 and 15 days in the presence of either the NO donor sodium nitroprusside (SNP) or the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). The percentages of TE cells were calculated in relation to the total number of cells counted under a light microscope (40X) using a hemocytometer. The means were compared in the Least Significant Difference test at 95% level using Genstat for Windows 19th Edition software.

3.4. Results and Discussion

3.4.1. Effects of SNP on lignin content and TE induction of *Pinus radiata* callus culture

3.4.1.1. Nitric oxide induced lignification in *Pinus radiata* callus culture

The callus cells with lignin appeared red after staining with phloroglucinol HCL (Figure 14). The most commonly used test for the presence of lignin in plant tissue is based on staining with phloroglucinol HCL (Molisch, 2013; Wiesner, 1878). This technique has become particularly significant in plant pathology where it shows changes in the lignification of tissue that has been attacked by fungi (Speer, 1981). The major advantage of this method is that it is technically easy and gives relatively fast and reliable results (Speer, 1987). Lignification of the secondary wall is an essential morphogenic stage in the differentiation of certain cell types such as the TEs, sclerenchyma fibres and sclereids. Tracheids and sclereids were produced in the callus derived from stem strip explants during differentiation, but only tracheids were produced in the cotyledon and hypocotyl-derived calli. Similar to the tracheids from the cotyledon and hypocotyl-derived calli, the walls of the tracheids and sclereids showed a positive colour reaction for lignin with phloroglucinol–HCl. In the stem strips-derived calli, the sclereids were also differentiated with irregular outgrowths in xylogenic callus. Lignification of the cells took place at 7-10 days after the *P. radiata* hypocotyl-derived calli were cultured on MS medium supplemented with NAA (1.0 mg /L), BAP (1.0 and 1.5 mg/ L) and SNP (0, 0.005, 0.025, and 0.05 mg/L). The calli cultured on TIM supplemented with the higher SNP concentrations tested here (0.025 and 0.05 mg/L) exhibited higher percentages of lignified cells than those cultured on TIM supplemented with the lower SNP concentrations (Figure 15). The increase in lignified cells ranged from 16% to 48% in the hypocotyl-derived calli grown on the media supplemented with NAA, BAP and SNP. The highest percentage (48%) of lignified cells was found in the calli cultured on the medium supplemented with 1.0 mg/L NAA, 1.0 mg/L BAP and 0.05 mg/L

SNP (Figure 15). The lowest percentage (around 10%) of lignified cells was found in the calli cultured on the medium without SNP or with a lower SNP concentration (0.05 mg/L) (Figure 14). The percentages of lignified cells were not different in the 0.05 mg/L SNP treatments in combination with the different concentrations of BAP and NAA (Figure 15).

Lignified cells were initiated 7-10 days after culturing cotyledon-derived calli on the TE induction media supplemented with 1 mg/L NAA, 1.0, 1.5 mg/L BAP and 0, 0.005, 0.025, 0.05 mg/L SNP. The highest percentage (21%) of lignified cells was found in the calli cultured on the medium supplemented with 1.0 mg/L NAA,1.0 mg/L BAP and 0.05 mg/L SNP (Figure 16).

In order to obtain a high percentage of lignified cells, the appropriate tissue sources should be utilised. From our studies it may be concluded that lignification was significantly affected by the different SNP concentrations as well as the type of the explants for establishing the calli. Thus, the hypocotyl explants from 3-week-old *in-vitro* germinated *Pinus radiata* seedlings were the best for the formation of lignified cells.

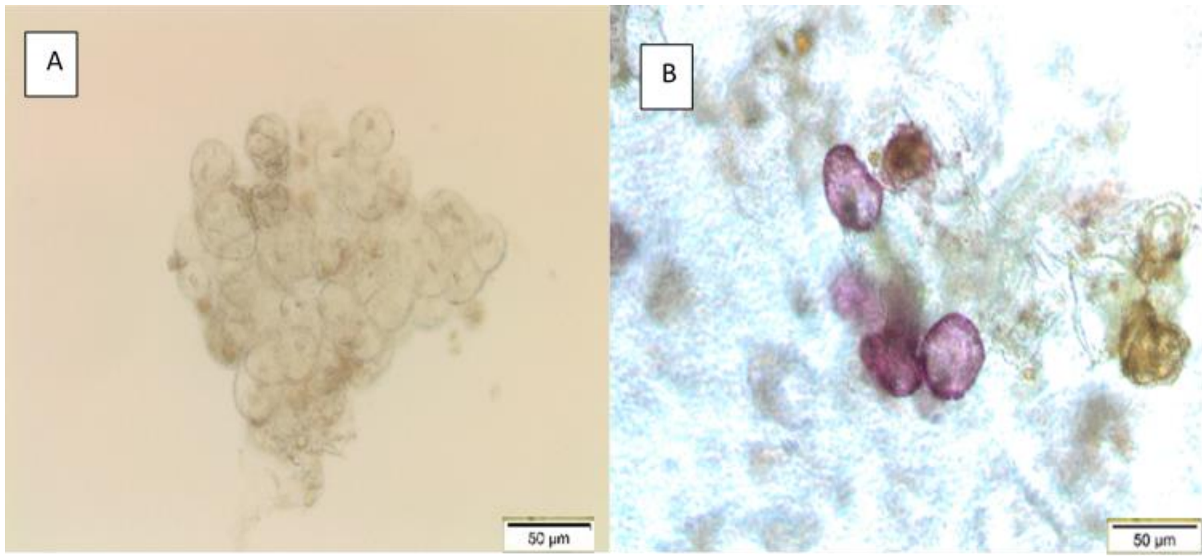

Figure 14. (A) Non-lignified cells, and (B) lignified cells stained with phloroglucinol HCL in *Pinus radiata* callus observed under a light microscope (40X). The callus was cultured on a TE induction medium supplemented with NAA (1.0 mg/L) + BAP (1 mg/L) + SNP (0.05 mg/L)) for 2 weeks before it was taken out of culture and macerated in a mixture of hydrogen peroxide and acetic acid for 6 hours at 60°C.

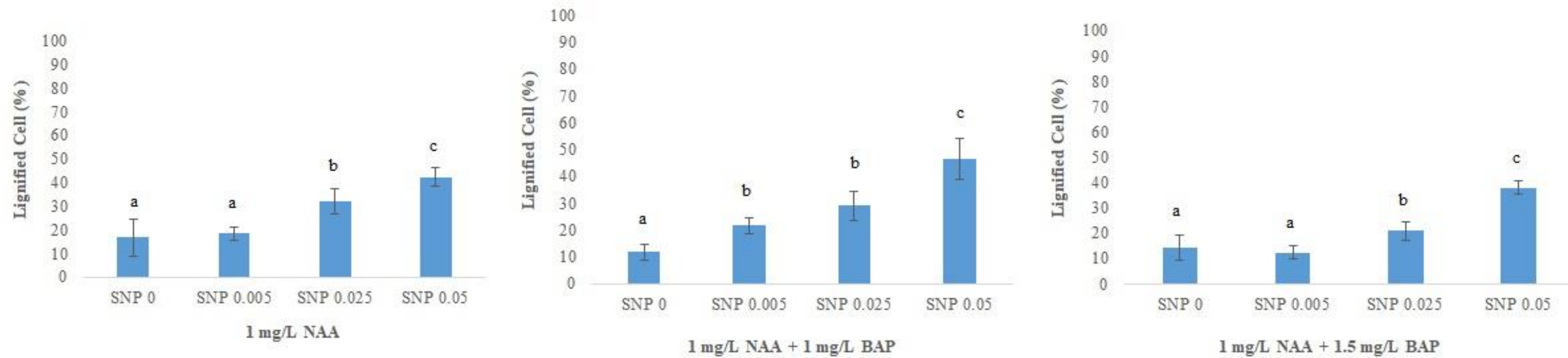

Figure 15. Percentages of lignified cells in hypocotyl-derived calli of *Pinus radiata* at 10 days after culturing on MS medium supplemented with NAA (1.0 mg /L), BAP (1 or 1.5 mg/ L) and SNP (0, 0.005, 0.025, or 0.05 mg/L). Means ± SD with different letters were significantly different ($p \leq 0.05$).

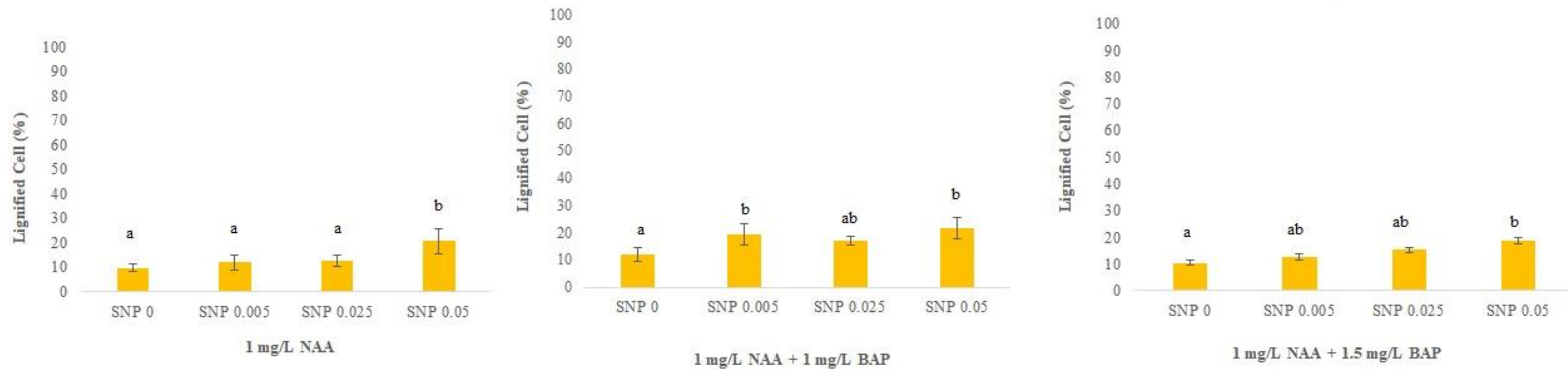

Figure 16. Percentages of lignified cells in cotyledon-derived calli of *Pinus radiata* at 10 days after culturing on MS medium supplemented with NAA (1.0 mg /L), BAP (1.0 or 1.5 mg/ L) and SNP (0, 0.005, 0.025, or 0.05 mg/L). Means ± SD with different letters were significantly different ($p \leq 0.05$).

In Figures 17-19, the percentages of lignified cells in the stem strip-derived calli of different *Pinus radiata* genotypes are shown. The calli were cultured on the TE media with different concentrations of NAA, BAP as well as SNP. Lignified cells appeared after 20-25 days. The percentage of lignified cells generally increased as the SNP concentrations increased up to 0.025 mg/L. The genotypes 32-2 and 103-4 exhibited the highest percentages (48% and 47%, respectively) of lignified cells in their stem strip-derived calli cultured on the medium containing 1.0 mg/L NAA and 0.025 mg/L SNP (Figure 16). The percentage of lignified cells in the genotype 32-2 calli cultured on the medium supplemented with 1.0 mg/L NAA, 1.0 mg/L BAP and 0.025 mg/L SNP was increased to 58% (Figure 18).

Many previous studies have established pine callus cultures (Hohtola, 1988). To investigate tissue browning in callus cultures of Scots pine (*Pinus sylvestris* L.), shoot tips were mounted on modified MS medium supplemented with growth regulators such as 1.0 mg/L 2,4-D, 0.2 mg/L BAP, and 0.4 mg/L kinetin (Laukkanen et al., 1999). Hypocotyl and cotyledon explants excised from *Pinus pinaster* seedlings were maintained on multiplication medium (SHm), that is, on solid Schenk and Hildebrandt culture medium (SH) (Schenk & Hildebrandt, 1972) supplemented with 30 g/L sucrose, 0.1 mg/L indole-3-butyric acid (IBA) and 0.5 mg/L BAP (Faria et al., 2015).

In the present research, it was found that stem strip-derived calli of the genotypes 32-2, 26-2, 108-3 and 103-4 were superior to those of the other genotypes as far as formation of lignified cells is concerned. Although sclereids were only a minor proportion of the differentiated cells in the calli, lignified cells were observed in calli cultured on media without SNP and BAP. The stimulatory effects of NAA and BAP in combination with SNP added to the medium increased the percentages of lignified cells..

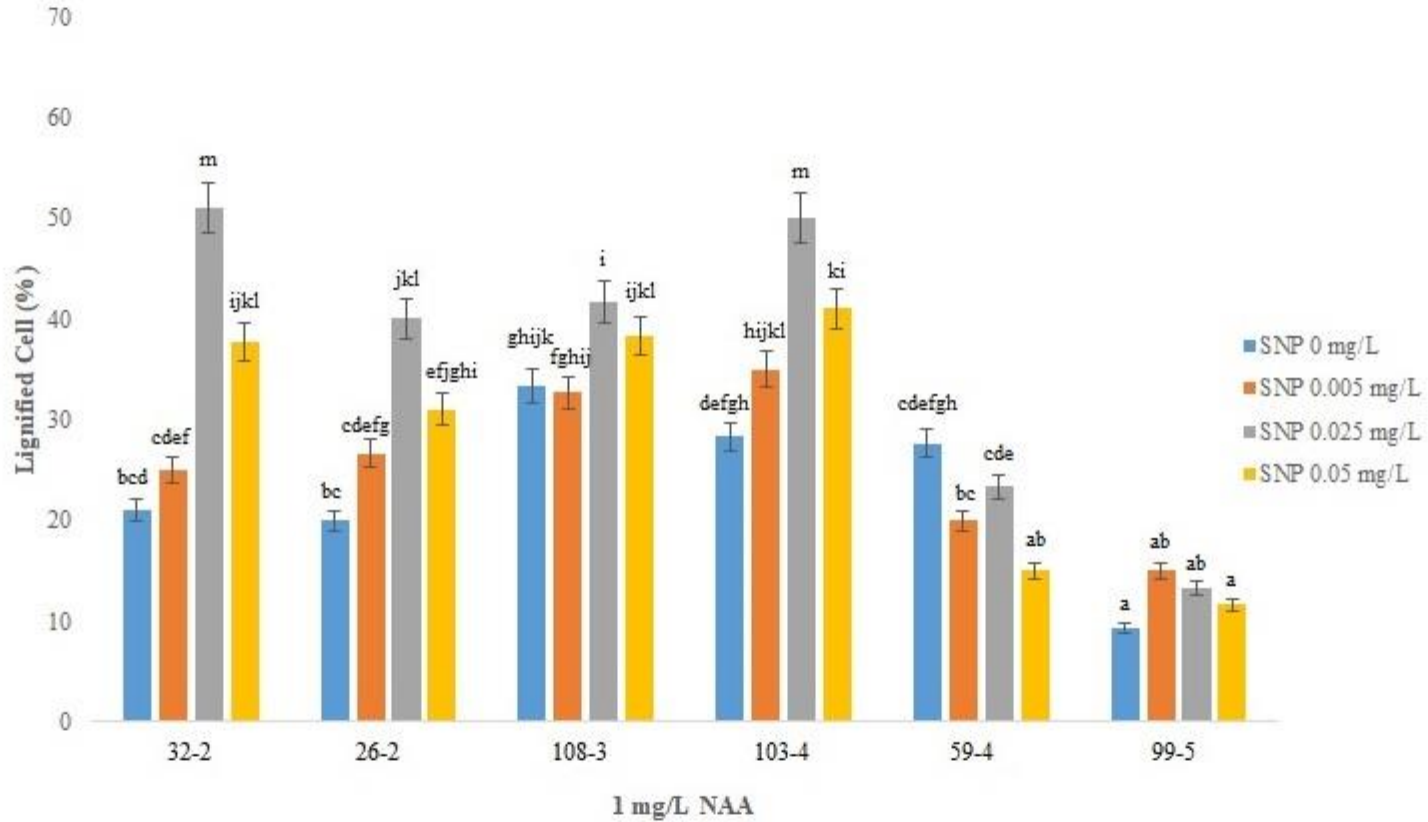

Figure 17. Percentages of lignified cells in the stem strip-derived calli of different *Pinus radiata* genotypes after 20-25 days of culturing on MS medium supplemented with 1.0 mg/L NAA and SNP (0, 0.005, 0.025 or 0.05 mg/L). Means ± SD with different letters were significantly different ($p \leq 0.05$).

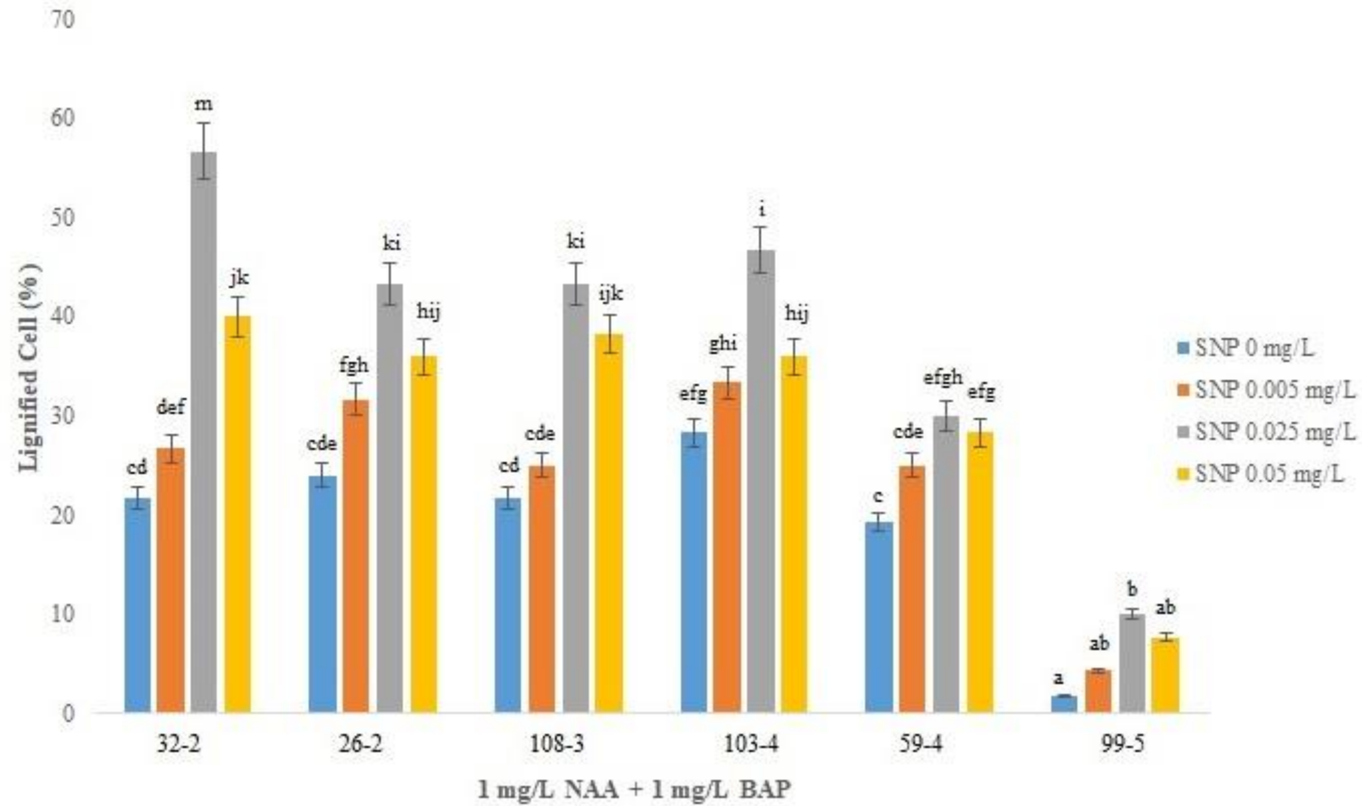

Figure 18. Lignin formation in stem strip-derived calli of different genotypes of *Pinus radiata* on MS medium supplemented with 1.0 mg/L NAA, 1.0 mg/L BAP and SNP (0, 0.005, 0.025 or 0.05 mg/L) after 20-25 days of initial culture. Means ± SD with different letters were significantly different ($p \leq 0.05$).

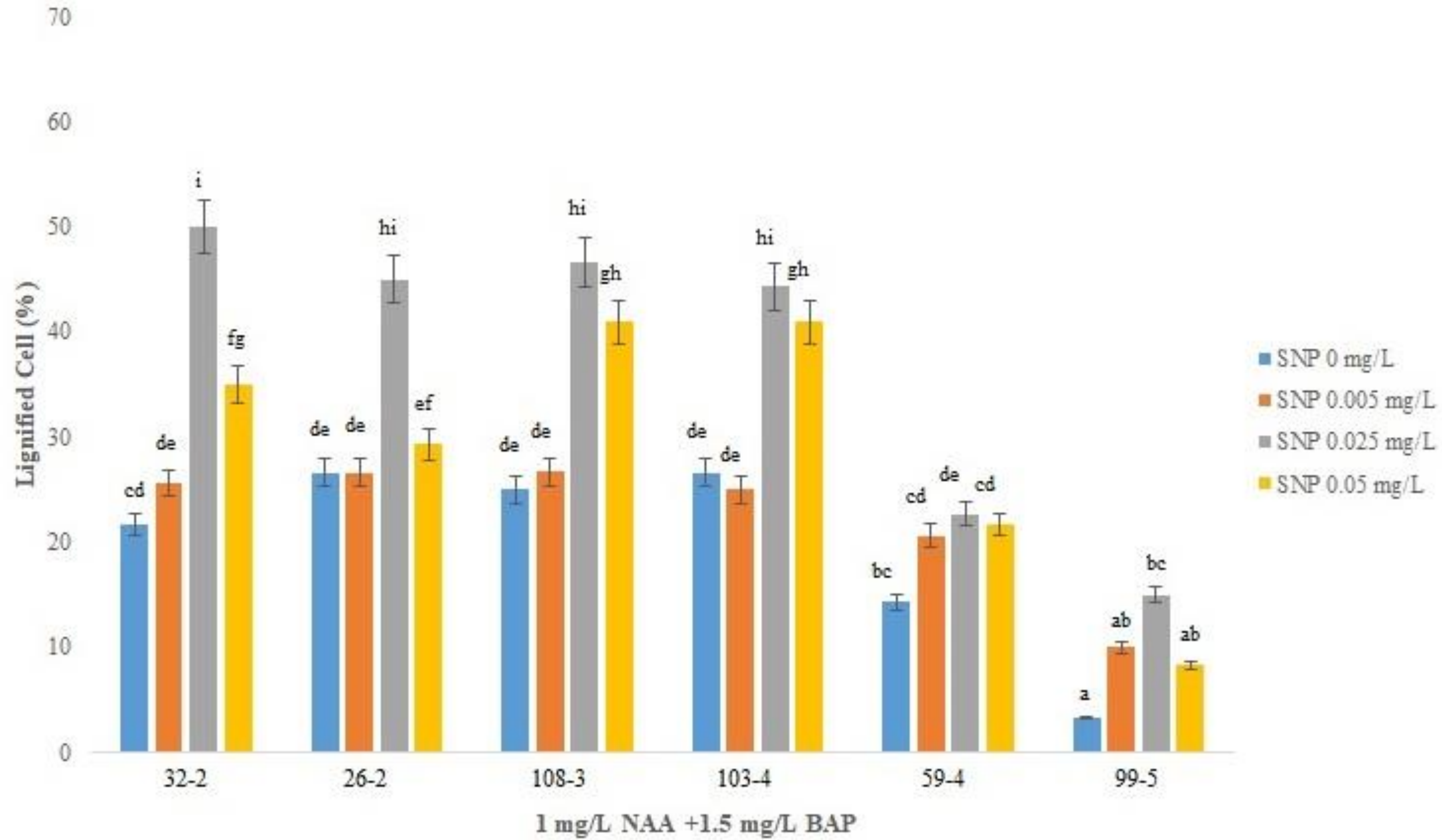

Figure 19. Lignin formation in stem strip-derived calli of different genotypes of *Pinus radiata* on MS medium supplemented with 1.0 mg/L NAA, 1.5 mg/L BAP and SNP (0, 0.005, 0.025 or 0.05 mg/L) after 20-25 days of initial culture. Means ± SD with different letters were significantly different($p \leq 0.05$).

3.4.1.2. Nitric oxide induced TEs in *Pinus radiata* callus culture

After tissue maceration, TE cells can be distinguished from sclereids and non TE callus cells according to their distinct thickening patterns of secondary cell wall similar to those found in the xylem within the plant body. The shapes of the *Pinus radiata* TE cell in callus cultures were highly variable. These include spiral cell wall patterns in protoxylems, reticulated and pitted patterns in hypocotyl-derived callus. The TEs in stem strip-derived callus were different and had a reticulate or pitted pattern (Figure 20). Even though some TEs were found scattering, most appeared to be joined into clusters. The TEs were mostly organised in parallel, but some were also observed to connect end to end.

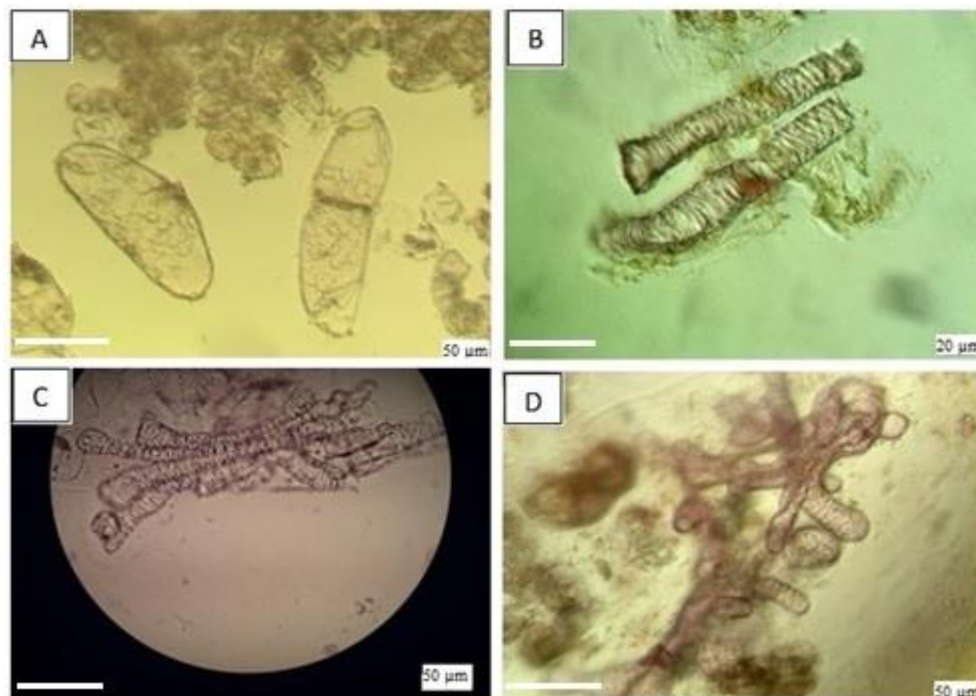

Figure 20. Secondary cell wall thickening patterns of the TEs in *Pinus radiata* callus cells observed following maceration and staining with phloroglucinol HCL. (A) non-differentiated callus cell, and TE with (B) spiral, (C) reticulate and (D) pitted cell wall patterns observed under a light microscope. Bars indicate 50 µm (A, C, D), or 20 µm (B).

In this experiment, TE induction was first observed at 3 days after induction in juvenile-tissue-derived calli (hypocotyl and cotyledon explants). For mature -tissue-derived calli (stem strip explants), it seems to require more time (at least 20 days) for TE induction. Another difference between TE induction in juvenile explants and stem strip explants was the percentage of TE induction (Figures 21 and 23). A previous study demonstrated that *Pinus radiata* calli were induced from xylem strips obtained from 1-year-old shoots of 3-year-old *P. radiata* trees (genotypes 532/5, 532/6 and 532/7) needed at least 20 days for TE induction (Möller et al., 2003). It was reported that *Zinnia* mesophyll cell culture took only 3 days to form TE (Iakimova & Woltering, 2017). The results obtained in the present study are consistent with the previous studies.

Even though TE development was observed in the calli from the juvenile explants (hypocotyl explants) on medium without SNP, TE induction was significantly increased from 12% to 33% on media supplemented with 0.025 or 0.05 mg/L SNP in addition to NAA and BAP (Figure 21). In comparison, the calli generated from the cotyledon explants (another juvenile explant) were less responsive in forming TE on the same media (Figure 22). This finding suggested that while using SNP was not necessary to induce TE formation, it could enhance TE induction in a greater proportion of the callus cells. No TE formation was observed in the calli from the stem strip explants (more mature tissue) cultured on the medium without SNP (Figure 23).

In the calli from the stem strip explants of three genotypes (32-2, 103-4 and 108-3), only small amounts (about 10%) of TE induction were formed after 20 days of culture on medium supplemented with 1 mg/L NAA in combination with 0.025 or 0.05 mg/L SNP (Figure 23). The genotype 26-2 only responded to form a small amount TE on medium supplemented with NAA and 0.05 mg/L SNP.

In the present study, the use of NAA in combination with BAP in addition to SNP was found to induce TE induction in the calli induced from the different explants used. In the study of Eberhardt et al. (1993), cell-wall thickening and lignification were observed in a suspension culture of *Pinus taeda* when the auxin in the medium was changed from 2,4-D to NAA. A similar result was found with a hypocotyl-derived suspension culture of *Pinus sylvestris* when the maintenance medium containing 2,4-D was changed to an induction medium containing NAA and kinetin (Ramsden & Northcote, 1987a).

In conclusion, comparing calli induced from the different explants used, those from hypocotyl explants of 3-week-old aseptically-grown *Pinus radiata* seedlings were the best source of calli for TE induction. The best TE induction medium seems to be MS basal medium supplemented with NAA, BAP and SNP. The work done so far moved forward beyond the prior publications on callus culture and TE induction in *P. radiata* callus culture which were not repeatable in our lab. Many months of this PhD study were lost in attempting to use these prior published methods to obtain subculturable and TE-forming *P. radiata* calli that were required for the main focus of this project, namely, to obtain a better understanding of the regulation of xylogenesis *in vitro*. In the next phase of this project, this primary focus was back on track.

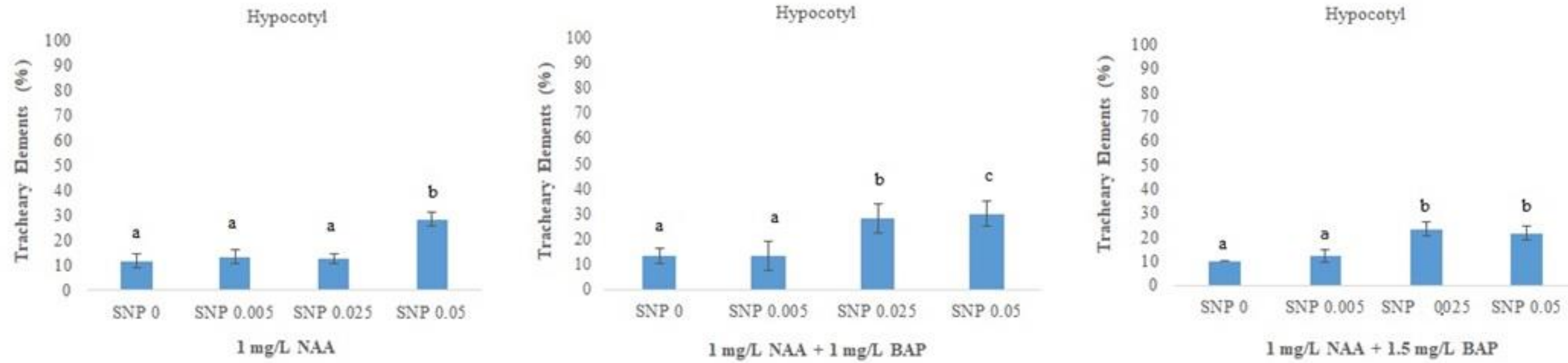

Figure 21. Percentages of tracheary elements in the calli derived from hypocotyl explants of 3-week-old *Pinus radiata* seedlings. The calli were subcultured on MS medium supplemented with 1.0 mg/L NAA, BAP (0, 1.0, and 1.5 mg/L) and SNP (0, 0.005, 0.025, and 0.05 mg/L) for 3-5 days. Means ± SD with different letters were significantly different ($p \leq 0.05$).

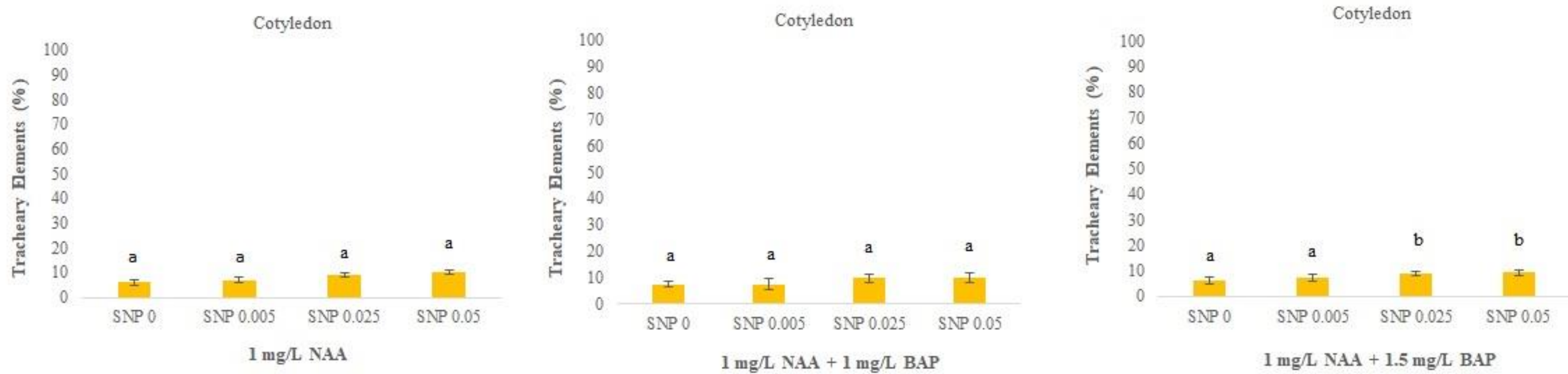

Figure 22. Percentages of TE cells in cotyledon explants calli at 3-5 days after culturing on MS medium supplemented with NAA (1.0 mg/L), BAP (1.0, and1.5 mg/L), and SNP (0, 0.005, 0.025, and 0.05 mg/L). Means ± SD with different letters were significantly different ($p \leq 0.05$).

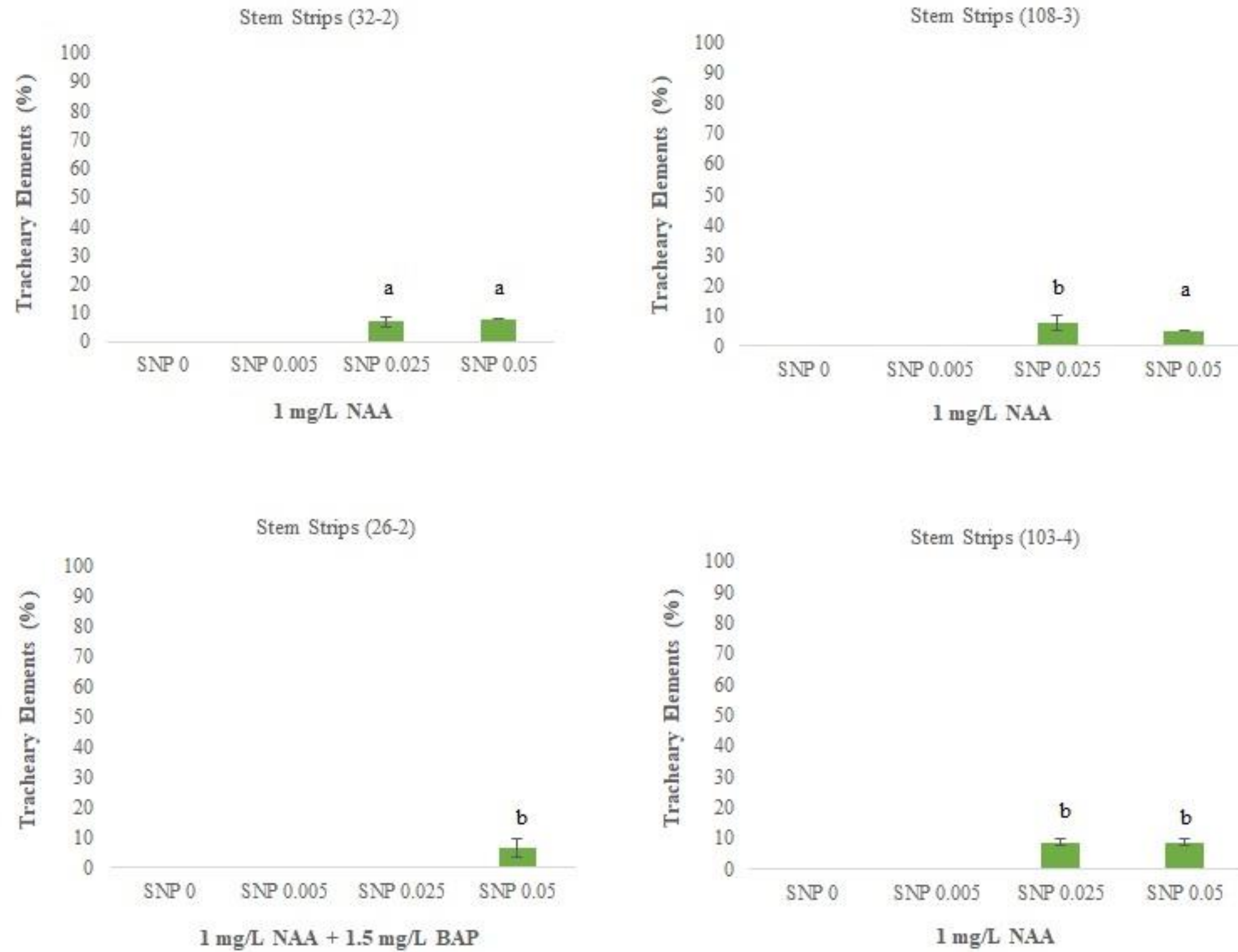

Figure 23. Percentages of tracheary elements in the stem strip-derived calli of different genotypes at 20 days after culturing on MS medium supplemented with 1.0 mg/L NAA, 1.5 mg/L BAP , and SNP (0, 0.005, 0.025, and 0.05 mg/L). Means ± SD with different letters were significantly different ($p \leq 0.05$).

3.4.2. Effects of SNP and cPTIO on the percentages of lignified cells and TEs in *Pinus radiata* callus culture

3.4.2.1 Effect on the percentages of lignified cells

The percentages of lignified cells in the calli derived from the hypocotyl, cotyledon and stem strip (genotype 32-2) explants were 23%, 20% and 52%, respectively, higher when cultured in the medium supplemented with 0.025 mg/L SNP ($p \leq 0.05$) than the control cultured on the medium without added SNP (Figure 24). Addition of 0.025 mg/L SNP together with a NO scavenger, 27.73 mg/L cPTIO, to the medium caused a reduction in the percentages of lignified cells by 17%, 16% and 22% in the calli derived from the hypocotyl, cotyledon and stem strip explants, respectively, compared to the control. The results suggested that cPTIO application reversed the promotive effects of SNP on the percentages of lignified cells and therefore the lignin content in *P. radiata* callus culture (Figure 24).

3.4.2.2. Effect on TE induction

In response to 0.025 mg/L SNP added to the medium, TE induction was promoted as the percentages of TEs increased by 35%, 28%, and 8% in the calli derived from the hypocotyl, cotyledon and stem strip (32-2) explants, respectively, over the control without SNP addition ($p \leq 0.05$) (Figure 24). In contrast, TE induction declined when cPTIO was added together with SNP to the culture medium ($p \leq 0.05$). The percentages of TEs formed were decreased by 13%, 12% and 4% in the calli derived from the hypocotyl, cotyledon and stem strip (32-2) explants, respectively, compared to the control. It was lowered further when cPTIO was added to the medium without SNP (Figure 25).

The important inference that can be drawn from the present study is that NO supplementation in the form of SNP has a significant and dual (promotive or inhibitory) impact on lignification

and TE induction. At a low concentration, SNP could promote lignification, whereas high SNP (NO) concentration and its scavenger (cPTIO) decreased lignification. In contrast to these results, Sharma et al. (2019) revealed that NO supplementation induces prominent alterations in lignin level during adventitious root (AR) formation and this might be due to an alteration in the activity of lignin biosynthetic enzymes, which further affected the polymerisation of monolignols and AR growth. A general up-regulation of genes involved in the lignin biosynthetic pathway was observed which was induced by cPTIO. While there were no significant changes in the overall lignin content, analyses of the cell walls showed that the G/S (guaiacyl ratio/syringyl) lignin ratio increased in roots treated with cPTIO. That means endogenous NO may regulate the composition of lignin in plants as described for sunflower (Monzón et al., 2014).

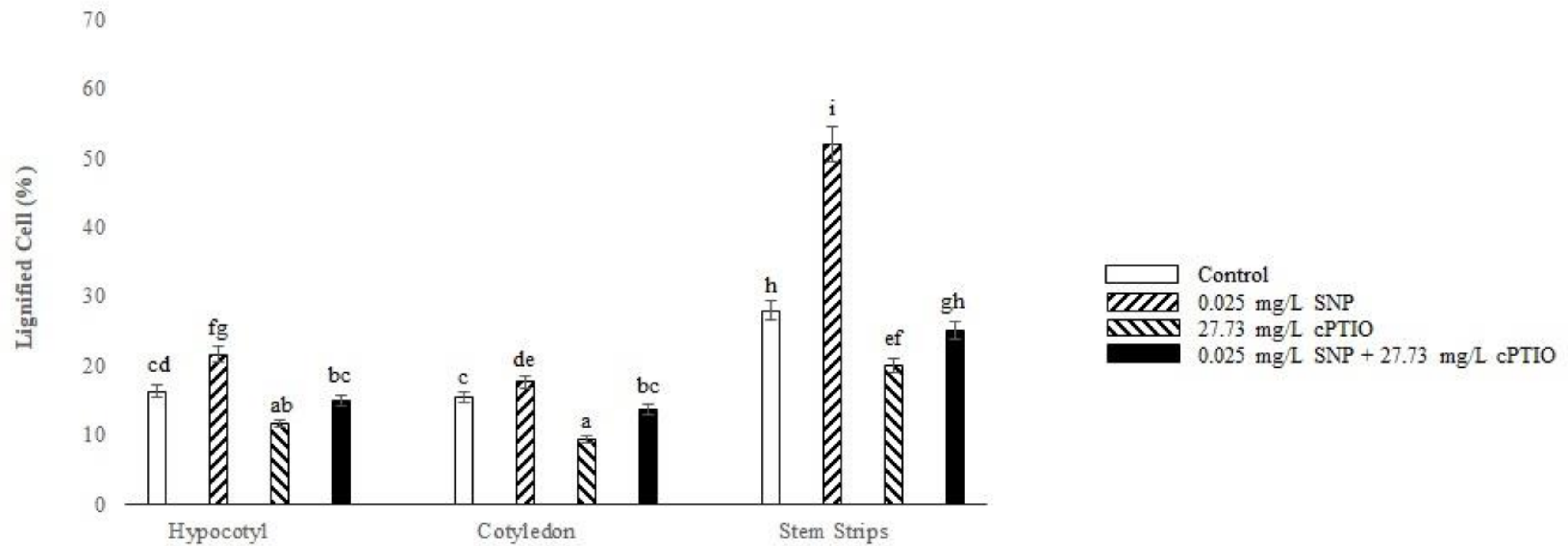

Figure 24. Percentages of lignified cells in the calli derived from various *Pinus radiata* explants including strip explants of genotype 32-2 at 15 days after culturing on MS medium supplemented with 1.0 mg/L NAA and 1.5 mg/L BAP, with or without, 0.025 mg/L SNP and 27.73 mg/L cPTIO. Means ± SD with different letters were significantly different ($p \leq 0.05$).

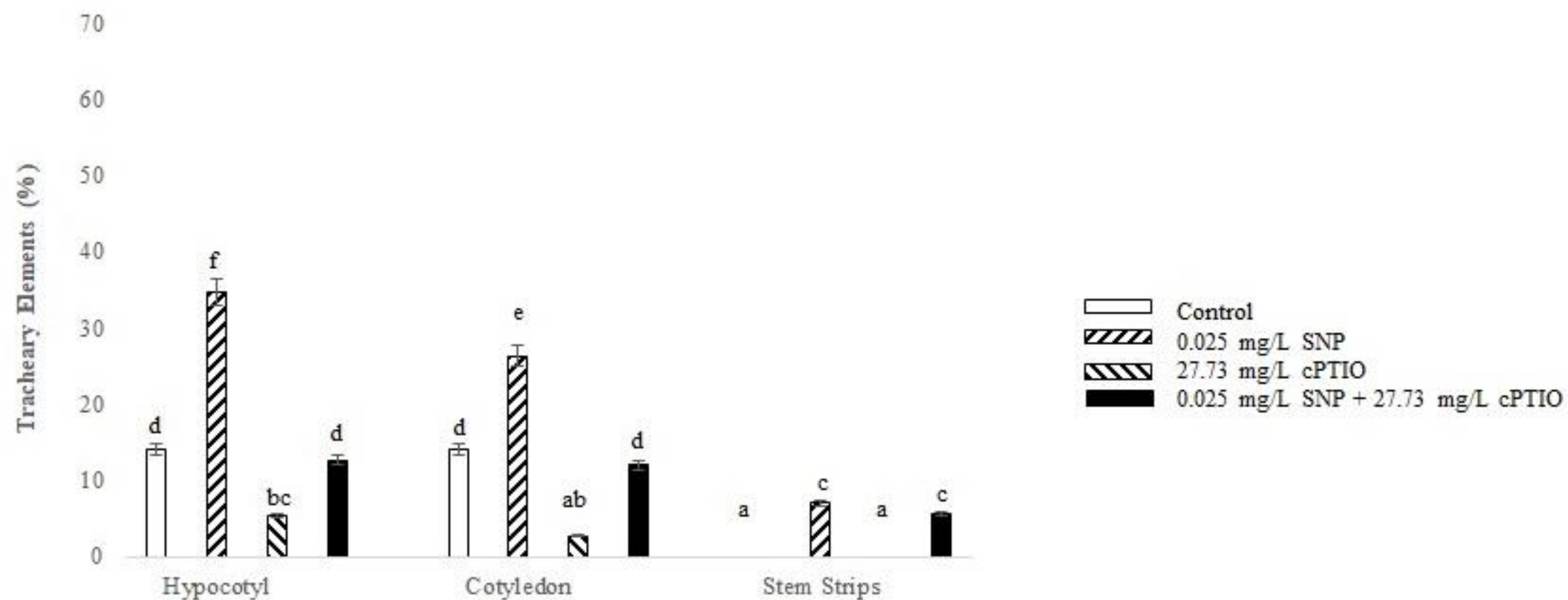

Figure 25. Percentages of tracheary elements in the calli derived from various *Pinus radiata* explants including strip strips from genotype 32-2 at 15 days after culturing on MS medium supplemented with 1.0 mg/L NAA and 1.5 mg/L BAP, with or without, 0.025 mg/L SNP and 27.73 mg/L cPTIO. Means ± SD with different letters were significantly different ($p \leq 0.05$).

3.4.3. Jasmonic acid induces xylogenesis with nitric oxide (NO) involvement

The hypocotyl explants cultured on the medium without jasmonic acid nor SNP (control) showed low TE initiation (Figure 26). This might be attributed to a very low NO production in the control callus culture derived from hypocotyl explants. Tracheary elements were increased dramatically by adding 1 mg/L JA in comparison with control. With the addition of SNP to the medium, TE formation was significantly different from the control (Figure 26). The TE cells were also almost absent in the presence of cPTIO alone (Figure 26). In contrast, TE cell production was enhanced in explants cultured in the presence of JA and SNP. The highest percentages of TEs, 13%, 23% and 28%, were observed when the hypocotyl-derived calli were cultured on the medium containing 1 mg/L JA and 0.025 mg/L SNP on 5th, 10th and 15th day of treatment, respectively (Figure 26). This stimulatory effect on TE induction was significantly diminished by the cPTIO application in combination with SNP. In general, TE induction significantly increased as time in culture increased to 15 days (Figure 26). Tracheary elements induction caused by 1 mg/L JA and 31.5 mg/L cPTIO application did not change when 0.025 mg/L SNP was also present. Xylogenesis increased in callus cultures in the presence of applied SNP, and increased after treatment with JA (1 mg/L) singly or in combination with 0.025 mg/L SNP. Taken together, the present results showed that xylogenesis was induced by JA and NO positively regulates this process.

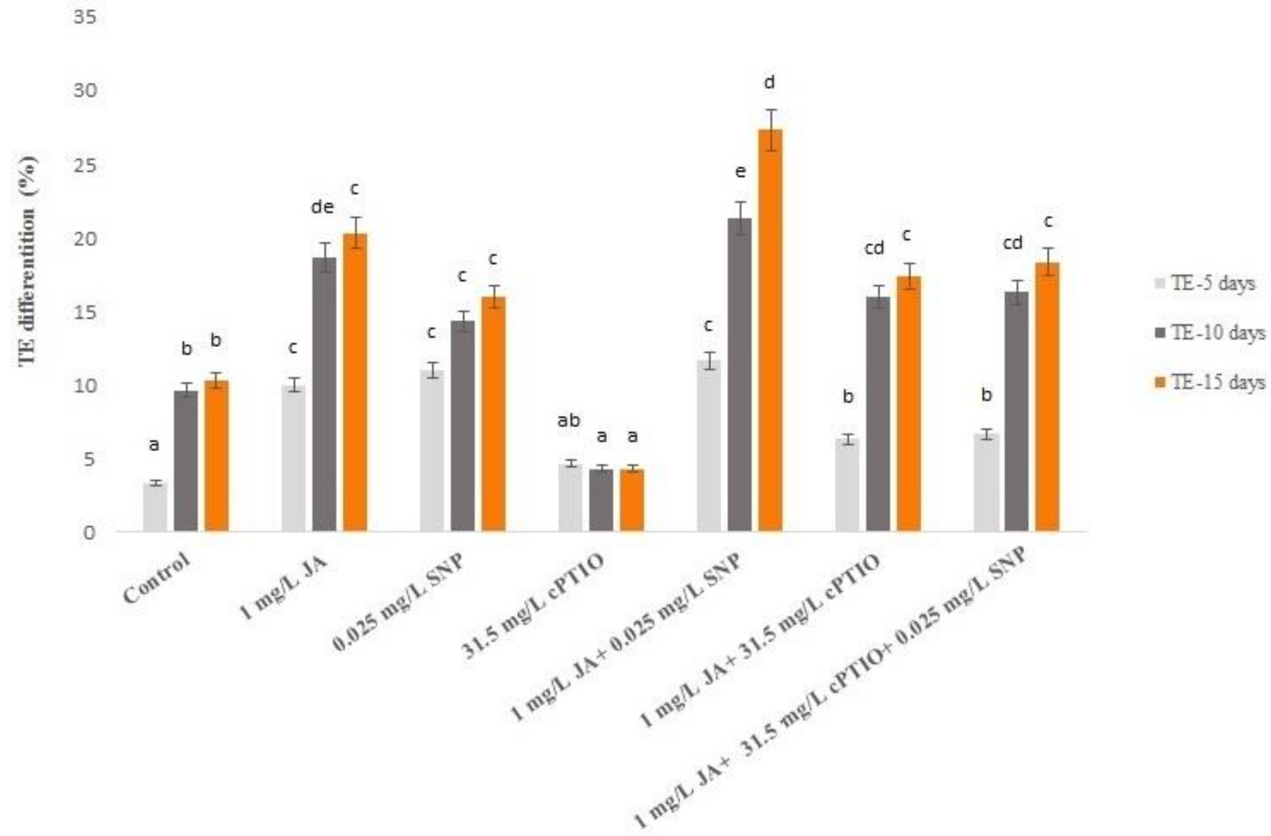

Figure 26. Tracheary element induction (% of TE cells) in calli derived from the hypocotyl explants of *Pinus radiata* seedlings on the 5th day, 10th day and 15th day after culturing on the media supplemented with sodium nitroprusside (SNP, a nitric oxide or NO donor), cPTIO (a NO scavenger) and jasmonic acid (JA). Means ± SD with different letters were significantly different ($p \leq 0.05$).

3.5. Conclusions

The results in the present study showed that a specific application of JA to MS medium initiated xylogenesis in the callus cultures derived from the explants of *Pinus radiata* seedlings. Moreover, JA seemed to interact with NO positively which was counteracted by cPTIO. The formation of tracheary elements in the hypocotyl-derived calli was boosted by 1 mg/L JA added to the medium. Interestingly, JA has recently been shown to induce extra xylem production in Arabidopsis roots on medium supplemented with 1 mg/L jasmonic acid methyl ester (MeJA; Jang et al., 2017). Consistent with our results, they showed that MeJA enhanced xylem production in Arabidopsis roots grown on a medium. There has been a discrepancy regarding the effect of cytokinin on xylem formation. While some studies have considered cytokinin as a necessary material for xylogenesis to occur (Fukuda, 1997), others reported it as a negative regulator of xylem formation (Jang et al., 2017).

Jasmonic acid methyl ester treatments have been shown to reduce the effects of cytokinin on the Arabidopsis root vasculature, and cytokinin treatments nullified MeJA promotion of ectopic xylem formation (Jang et al., 2017). Fattorini et al. (2017) showed that xylogenesis occurs in Arabidopsis hypocotyls of dark-grown seedlings in the absence of exogenous cytokinin, and that this condition may favour the JA inductive action.

Working with Arabidopsis (Saito et al., 2009) demonstrated that addition of 1 mg/L MeJA to the medium enhanced NO production which in turn significantly increased stomatal closure. They concluded that NO is involved in signal transduction resulting in stomatal closure in Arabidopsis. Previous studies also indicated that exogenous addition of MeJA stimulated NO synthase to initiate NO production in *Taxus* cells (Wang & Wu, 2005), and that JA and NO modulated each other's production (Zhou et al., 2015). Della Rovere et al. (2019) also found that NO signalling contributes to the onset of xylem differentiation and also to further

maturation of *Populus* roots, but not in the mature vessels (Bagniewska-Zadworna et al., 2014).

Auxins (natural or synthetic) are needed to initiate xylogenesis (S. Yoshida et al., 2009) and IAA and its precursor IBA are present in the hypocotyls of dark-grown Arabidopsis seedlings (Veloccia et al., 2016). In addition, it is known that endogenous auxin accumulates in numerous plant / culture systems before the formation of xylem cells, and this occurs either after a dedifferentiation in the target cells or after their direct trans-differentiation (Fattorini et al., 2017). In particular, endogenous auxin accumulates in the basal pericycle in Arabidopsis hypocotyls before first divisions occur (Della Rovere et al., 2013). While a synergism of auxin and NO has been reported during cytokinesis activation (Ötvös et al., 2005), it remains to be explained how the endogenous auxin with exogenous MeJA directs the basal pericycle cells towards xylogenic programme realisation and not root development, using the same NO signalling molecule.

Auxins are known to be important for determining xylem cell identity (Bishopp et al., 2011). MeJA changed auxin-induced ectopic xylem cell identity. This was the first report of JAs role in changing xylem cell identity (Della Rovere et al., 2019)

In conclusion, the basic findings of this study have added new insights about the factors involved in the intricate switching on xylogenesis in *Pinus radiata* callus culture. jasmonic acid appears to be invovled in both developmental programmes and xylem cell identity modulation and there was a significant influence from NO mediation.

Chapter 4. Biochemical and Microscopic Studies on the *In Vitro* Callus Culture of *Pinus radiata*

4.1. Introduction

Lignin is a complex heteropolymer consisting of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units produced by the oxidative coupling, respectively, of p-coumaryl, coniferyl, and sinapyl alcohols, which are phenylpropanoid pathway products (Vanholme et al., 2010). Lignin provides a covering for cellulose-hemicellulose microfibrils after deposition in the secondary cell walls, thereby ensuring the lignified tissues of rigidity, strength, and impermeability. These mechanisms protect the plants from pathogen and herbivore attacks (Zobiole et al., 2010). In coniferous gymnosperms, lignin is found as an integral component of the primary and secondary walls of the tracheary elements, sclereids, and phloem. Stress-induced biosynthesis of lignin also occurs in the parenchyma cells near the sites of wounding or pathogen attack and lignin has been found in the primary cell walls and the lumen of these cells (Hawkins & Boudet, 2003). Because of the importance of lignin for water transport in plants, mechanical grain resistance, the textile and paper industries, and as a major obstacle to bioethanol generation (Li et al., 2008; Zobiole et al., 2010), there is a growing interest in improving the assays used to quantify it. Although different techniques have been described for measuring the lignin content in biomass samples (Fukushima & Kerley, 2011; Jung et al., 1999), there is no standard method for defining lignin. This is a significant downside as for the same sample, each technique can show different results. The more common techniques used to measure lignin are the acetyl bromide and thioglycolic acid (Hatfield & Fukushima, 2005). The first two methods are based on lignin content determination at 280 nm absorbance after solubilisation.

The acetyl bromide method was specifically developed to provide a sensitive method for small samples of forage plants. It is based on the formation of acetyl derivatives in non-substituted OH groups and bromide replacement of the α-carbon OH groups to develop lignin solubilisation under acidic conditions. Because it is not a direct process, the lignin content may be overestimated due to the oxidative degradation of structural polysaccharides (e.g. xylans) during cell wall incubation with the acid solution (Hatfield et al., 1999). By its name, the thioglycolic acid process is based on the development of thioethers of benzyl alcohol groups found in lignin, resulting in this polymer being solubilised under alkaline conditions. However, the thioglycolic acid technique can underestimate the lignin content, due to the specificity of the reaction with ether bond types of lignin (Brinkmann et al., 2002).

Lignothioglycolic acid (LTGA) preparations is generally considered appropriate for the isolation and quantitative assay of lignin in herbaceous plants (Bruce & West, 1989; Sarkanen & Ludwig, 1971). In this process, the derivatisation of thioglycolic acid displaces lignin from its normal covalent connections to the cell wall and enables alkali to be removed from cell walls. Acidification of the alkaline extract causes LTGA to precipitate. Precipitated LTGA can be used for lignin structure characterisation or, after being resolubilised, LTGA can be determined quantitatively by measurement of absorbance at 280 nm (Bruce & West, 1989). The formation of LTGA has beenconsidered as a criterion for lignin presence in a sample (Freudenberg & Neish, 1968). The LTGA method was developed in wood for the determination of lignin, which normally contains little detectable proteins. Though LTGA preparations from herbaceous samples have long been suspected of contamination with proteins (Vance et al., 1980; Venverloo, 1969; Whitmore, 1978), most recent investigators believed that their LTGA preparations were not noticeably contaminated with proteins (Aerts & Baumann, 1994; Bruce & West, 1989; Graham & Graham, 1991). Characterisation and quantitative determination of LTGA preparations are usually limited to spectra of UV

absorbance. The spectra of LTGA preparations from bamboo cane, pine wood and maize coleoptiles are very similar, showing a slight peak at about 280 nm, a shoulder peak at about 240 nm, and a major peak at about 225 nm (Müse et al., 1997). In our preliminary experiments, LTGA was prepared from either cotyledons or hypocotyls plus stem strips of germinated seedlings and one-year-old *Pinus radiata* trees. The UV spectra of LTGA of germinated pine seedlings in 0.5 M sodium hydroxide was similar to the LTGA prepared from pine wood (Müse et al., 1997).

Biochemical studies on lignin biosynthesis have been carried out using plant cell cultures. For this aim, cell cultures have several benefits over whole plants because: firstly, they are available in large amounts, regardless of the season; secondly, large amounts of cells, cell walls or conditioned medium can be isolated for chemical analysis from cell cultures; and thirdly, enzymes can be extracted from cell cultures in sufficient quantities for biochemical characterisation. However, lignification of coniferous gymnosperms in most cell cultures has been initiated by stress factors, like treatment with UV-light and fungal elicitors, or media supplemented with growth regulators or high concentrations of sucrose (Anterola & Lewis, 2002; Brunow et al., 1990; Campbell & Ellis, 1992; Eberhardt et al., 1993). Besides this, induced lignification in these cell culture systems was unaccompanied by the development of secondary cell walls. In addition, the secreted "extracellular lignin" was found to have a more condensed structure in comparison with lignin from the same species in the secondary xylem (Brunow et al., 1990).

Lignin is generally synthesised with three major forms of monolignols (sinapyl alcohol, S unit; coniferyl alcohol, G unit and p-coumaryl alcohol, H unit) by peroxidase (POD) and laccase (LAC) in the secondary cell wall as depicted in Figure 27 (Alejandro et al., 2012; Bonawitz & Chapple, 2010; Liu et al., 2011; Miao & Liu, 2010). Moreover, several other

compounds including hydroxycinnamaldehydes, tricin flavones, hydroxystilbenes and xenobiotics etc. were also recognised as lignin subunits (del Río et al., 2017; del Río et al., 2012; Eloy et al., 2017; Mottiar et al., 2016; Ralph, 2010; Singh et al., 2016). During protoxylem differentiation in Arabidopsis, lignin monomers can be free to diffuse in the extracellular space, and are only polymerised in the secondary cell walls (Schuetz et al., 2014).

Peroxidases (POD) (EC 1.11.1.) belong to a group of hemeproteins observed in plant, animal and micro-organism cells. They are considered as metal stress-related biomarkers, because their levels are usually changed under heavy metal (HM) stress (Jouili et al., 2011). Although they, like catalases (CAT), work as hydrogen peroxide scavengers, they are polymeric and also have a larger range of substrates like mono and dihydroxy alcohols and phenols, dihydroquinones, amines, as well as many other donors of hydrogen (Ahmad, 1995). Therefore, their names are mainly based on substrates that they use. Ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (GPX; EC 1.11.1.7) are the two major groups of peroxidases found in higher plants using ascorbate and guaiacol as reducing agents, respectively (Jouili et al., 2011; Martins et al., 2013)

Möller et al. (2003) found differentiated cells with lignified secondary cell walls in a *Pinus radiata* cell culture system. The activities of the first enzyme in the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL) (EC 4.3.1.5), and of the last enzyme involved in the biosynthesis of monolignols, cinnamyl alcohol dehydrogenase (CAD) (EC 1.1.1.195), were studied, in order to investigate the suitability of this cell culture system for studying the development of secondary cell walls and lignification. Activities of PAL and CAD were enhanced concomitantly with cell differentiation in the cell cultures of isolated mesophyll cells of *Zinnia elegans* L. which differentiated tracheary elements (Fukuda & Komamine, 1982; Lin & Northcote, 1990; Sato et al., 1997). This seems to suggest that these enzymes

may be useful markers for tracheary element differentiation in angiosperm cell cultures (Northcote, 1995).

However, more research in cell cultures of the evolutionarily distant coniferous gymnosperms needs to be undertaken. This study presented evidence that *P. radiata* cell culture system is useful for studying lignin formation in the cells with secondary walls in coniferous gymnosperms.

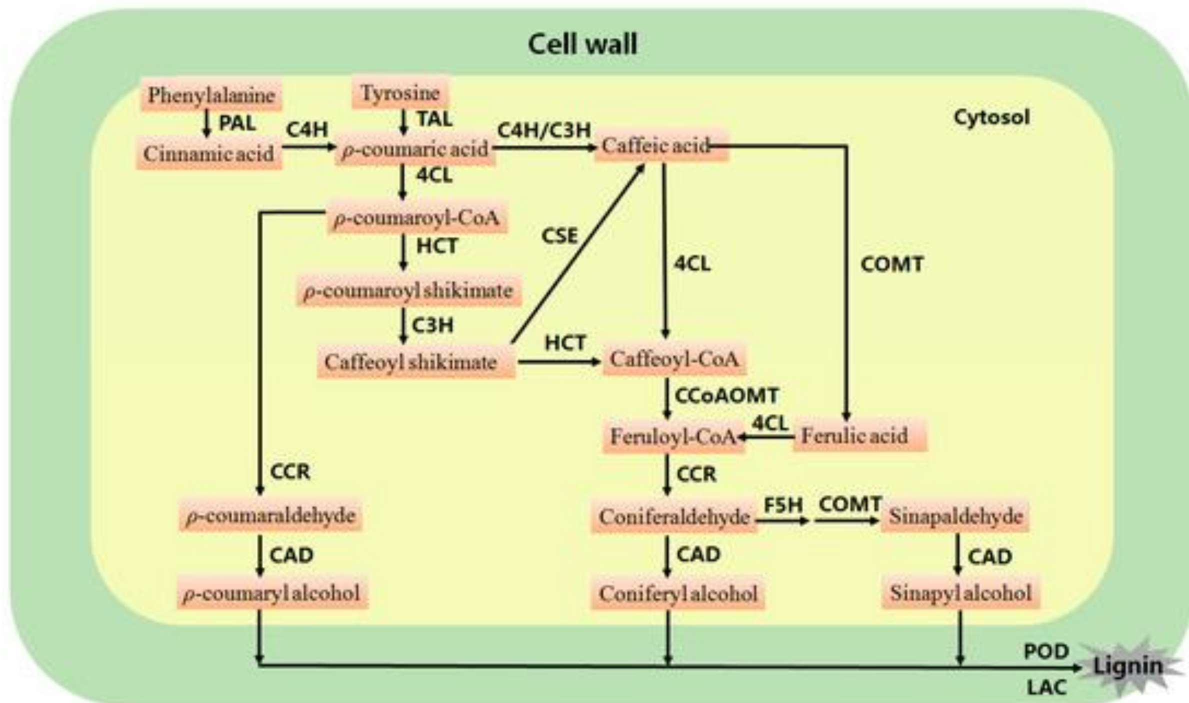

Figure 27. Lignin biosynthesis pathway in higher plants. PAL, phenylalanine ammonia-lyase; TAL, tyrosine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CCR, cinnamoyl-CoA reductase; HCT, hydroxycinnamoyl-CoA shikimate/Quinatehydroxycinnamoyl transferase; C3H, p-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyl transferase; F5H, ferulate 5-hydroxylase; CSE, caffeoyl shikimate esterase; COMT, caffeic acid O-methyl transferase; CAD, cinnamyl alcohol dehydrogenase; LAC, laccase; POD, peroxidase (Liu et al., 2018).

4.2. Aims

The aim of this chapter is to investigate the biochemical markers involved in *Pinus radiata* xylogenesis. This aim is achieved through:

- Determining the amount of lignin using the thioglycolic acid (TGA) method
- Determining enzymes involved in xylogenesis in *Pinus radiata* callus
- Undertaking biochemical and confocal microscopy to assess tracheary elements induction in the calli (callus induction as described in Chapter 3)

4.3. Materials and Methods

4.3.1. Thioglycolic acid lignin (TGAL) assay

4.3.1.1. Plant material

Calli derived from culturing explants of stem strips of from greenhouse-grown *Pinus radiata* plants as well as of hypocotyl and cotyledon of 3-week-old seedlings following seed germination *in vitro* were used. The calli derived from the hypocotyl explants, cotyledon explants and stem strip explants were cut into small pieces, dried at 60 or 70 ± °C, and were then grinded to a fine powder (< 0.08 mm) using a ball mill (Mikrodismembrator U, Braun, Melsungen, Germany).

4.3.1.2. Isolation of structural biomass for lignin analysis

Dry ground callus powder (200 mg) was suspended in 20 mL of washing buffer (100 mM K_2HPO/KH_2PO_4, pH 7.8, and 0.5% Triton X-100), gently stirred for 30 min at room temperature, and centrifuged (20 min, 5500g, swing rotor). The pellet was resuspended in washing buffer and washed as above. The pellet was then washed 4 times (30 min) in 100% methanol (MeOH). The resulting pellet consisted mainly of cell walls, i.e., structural biomass (SBM). The SBM pellet was dried (12 hr at 80 ± °C), weighed, and used to analyse lignin using the thioglycolate method (Brinkmann et al., 2002).

4.3.1.3. TGAL method

In a 2-mL Eppendorf tube, 1–2 mg of a SBM pellet were mixed with 1.5 mL of 2 M HCl and 0.3 mL thioglycolic acid (adapted after Bruce and West, 1989). The mixtures were incubated at 95 ± °C for 4 h and vortexed frequently. At the end of incubation, the mixtures were immediately cooled on ice and then centrifuged for 10 min at 15,000g (desktop centrifuge).

The supernatant was discarded. Pellets were washed 3 times with 1 mL of distilled water each time. Thereafter, the pellets were incubated with 1 mL of 0.5 M NaOH for 18 h on a shaker at room temperature. The suspension was centrifuged for 10 min at 15,000g. The supernatant was carefully transferred into a 2-mL Eppendorf tube. The pellet was resuspended in 0.5 mL 0.5 M NaOH, vortexed, and centrifuged. The resulting supernatant was combined with the first alkaline supernatant and mixed with 0.3 mL conc. HCl. The mixtures were incubated for 4 h at 4 ± °C to precipitate the lignothioglycolate derivate. After centrifugation, the supernatant was discarded, and the pellet solubilised in 1 mL of 0.5 M NaOH. The absorbance of the resulting solution was measured at 280 nm.

4.3.2. Preparation of enzyme extracts

The calli were ground in liquid nitrogen. The extraction buffer used was the same as the one described by Goffner et al. (1992) except that polyvinylpolypyrrolidone (PVPP) was replaced with 0.4 Polyclar AT (Serva, Heidelberg, Germany). The extraction buffer was added to the powder after evaporation of liquid nitrogen. The mixture was centrifuged at 4 °C for 15 min. The supernatant was used for the CAD enzyme assays. The protein quantity was determined using the protein-dye method (Bradford, 1976) and the dye-binding reagent was supplied by Bio-Rad.

4.3.2.1. Peroxidase assay

Activity of peroxidase was quantified as described by Kim and Yoo (1996). For this assay, 0.1 g of fresh callus were homogenised in 1 mL of phosphate buffer (pH 6.0, 0.1 M) for 5 min using a sonicator at 200 W cm^{-2}. The homogenates were then centrifuged at 13,000 rpm for 5 min at 4°C and the supernatants were assayed for peroxidase activity. Peroxidase activity was

determined at 30$^{\circ C}$ with a spectrophotometer following the formation of tetraguaiacol (A_{max} = 470 nm) in a 3 mL reaction mixture containing 1 mL of 0.1 M phosphate buffer, pH 6.0; 1 mL of 1.5 mM 2-methoxyphenol (guaiacol); 1 mL of 3 mM H_20_2; and 50 µL of enzyme extract. One unit of peroxidase activity (U) represents the amount of enzyme catalysing the oxidation of 1 µM of guaiacol in 1 min (Kim & Yoo, 1996).

4.3.2.2. Assay for CAD activity

Preparation of extracts for assay of CAD activity was carried out at 4°C. Pieces of callus (1 mg) were homogenised with a cell disrupter (Fast Prep) in Tris-HCl buffer (100 mM, pH 8.8) containing 10 mM dithiothreitol (DTT), 25 mg mL^{-1} PVPP and 0.5% (w/v) polyethylene glycol (PEG) 8000. The extract was centrifuged (14,926 g, 2 min) and the total protein in extracts was quantified using the Protein Assay Dye Reagent Concentrate (Bio-Rad, CA) according to Bradford (1976). Standard curves were constructed using bovine serum albumin (BSA) (Sigma Chemical). Extracts containing 20 µg of total protein were assayed spectrophotometrically for CAD activity at 30°C using the method of Wyrambik and Grisebach (1975). The assay mixture (1 mL) contained 2 mM coniferyl alcohol and 2 mM NADP in Tris HCl buffer (100 mM, pH 8.8). The production of cinnamyl aldehyde was measured as a change in the absorbance at 400 nm (Möller et al., 2003).

4.3.2.3. Confocal laser scanning microscopy

Using confocal microscopy, three dimensional images of biological and non-biological specimens may be obtained. The importance of this technique lies in the elimination by spatial filtering of out-of-focus glare, utilising a point source of light for excitation, and a pinhole confocal with the excitation pinhole in front of the detector. A combination of transverse resolution and non-invasive optical sectioning result in very high-quality images of biological specimens. Several laser combinations can be associated with the fibre optics of the scanning unit in order to increase the number of excitation wavelengths. Powerful softwares can be used to show and analyse 3-D data. Laser scanning confocal microscopy has shown to be particularly suitable for analysing structural features of thick specimens and promises to be of great potential in providing 3-D volume renderings of living cells and tissues over time (Singh & Gopinathan, 1998).

4.3.2.3.1. Fixation

A formaldehyde-based solution was found to provide the best fixation. This fixation solution comprised of PME solution (50 mM Pipes, pH 7.2, 2 mM EGTA, and 2 mM $MgSO_4$) supplemented with 0.1% (v/v) Triton X-100), 3.7% (v/v) formaldehyde, 0.5% (v/v) glutaraldehyde, and 0.1% (v/v) dimethyl sulfoxide (Singh & Gopinathan, 1998). Fresh, whole calli were fixed in this solution for 1-2 h in vacuum or overnight if not in vacuum. Calli were washed several times for 5 min in the PME solution and then incubated for 1 h in the PME solution with addition of 1% (v/v) Triton X-100. Permeabilisation and extraction of the tissue was done with methanol at -20°C for 15 min, with tissue then rehydrated in phosphate-buffered saline (PBS; 131 mM NaCl, 5.1 mM Na_2HPO_4, and 1.56 mM KH_2PO_4, pH 7.2).

4.3.2.3.2. Vibratome sectioning

The fixed tissue was sectioned, either longitudinally or in cross-section, with a vibratome 3000 Plus sectioning system (Vibratome, St. Louis, MO, USA). To hold the tissue into place for vibratome sectioning, the pine calli were embedded in acrylamide using a method modified from Germroth et al. (1995). Samples were embedded in a polyacrylamide solution (21% (v/v) acrylamide (Biorad, Hercules, CA, USA)), 0.01% (v/v) TEMED (tetramethylethylenediamine) and polymerised with 0.1% (w/v) ammonium persulfate in phosphate buffered saline buffer (PBS) (Figure 28). The thickness of each section was 4 µm. Each sample was represented by 2–4 sections, depending on the ability to obtain the material and the technical appropriateness of the sections before the assessment. This embedding method was preferred to the standard embedding matrix of 3% agar (Collings and Harper 2008) which was insufficient to keep the sample in place for sectioning in the vibratome. This improved grip might be because of the rather large and hard nature of the sample or due to the fact that the acrylamide might enter the velamen layer before it polymerised, thus resulting in a better support matrix.

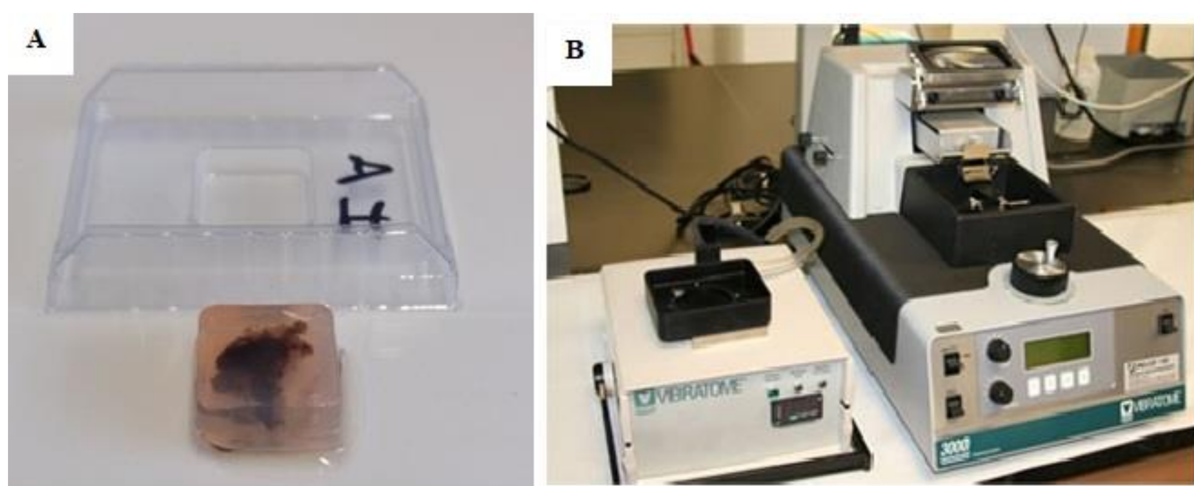

Figure 28. Fixed callus tissue of *Pinus radiata* (A). A fully automated Vibratome 3000 Plus with integrated controlled refrigerated bath (B).

4.4. Results and Discussion

4.4.1. Thioglycolic acid lignin (TGAL)

The lignin contents in various explants from *Pinus radiata* trees and seedlings were measured on the 5th, 10th and 15th days after culturing on TE induction medium (Figure 29). The lignin content in the cell walls of the stem strip-derived calli was higher than in those of the calli derived from the hypocotyl and cotyledon explants (Figure 29). However, the lignin contents of the radiata pine TE-forming calli was higher than that reported in Möller, Koch, et al. (2006).

The TGAL content in the stem strip-derived calli at day 15 of culture on the TE-induction medium was higher from those in the calli at day 5 and 10. The TGAL content in the cell walls of the control cultures of hypocotyl on the callus proliferation medium remained low and showed little change over the 15 days of culture. However, in the callus cultures on the TE-induction medium, the TGAL content in the cell walls of the TE-forming calli increased concomitantly with the increases in POD and CAD activities (Figures 29, 30 and 31). The cotyledon-derived calli had lower lignin contents in comparison with the calli derived from the hypocotyl and stem strip explants (Figure 29).

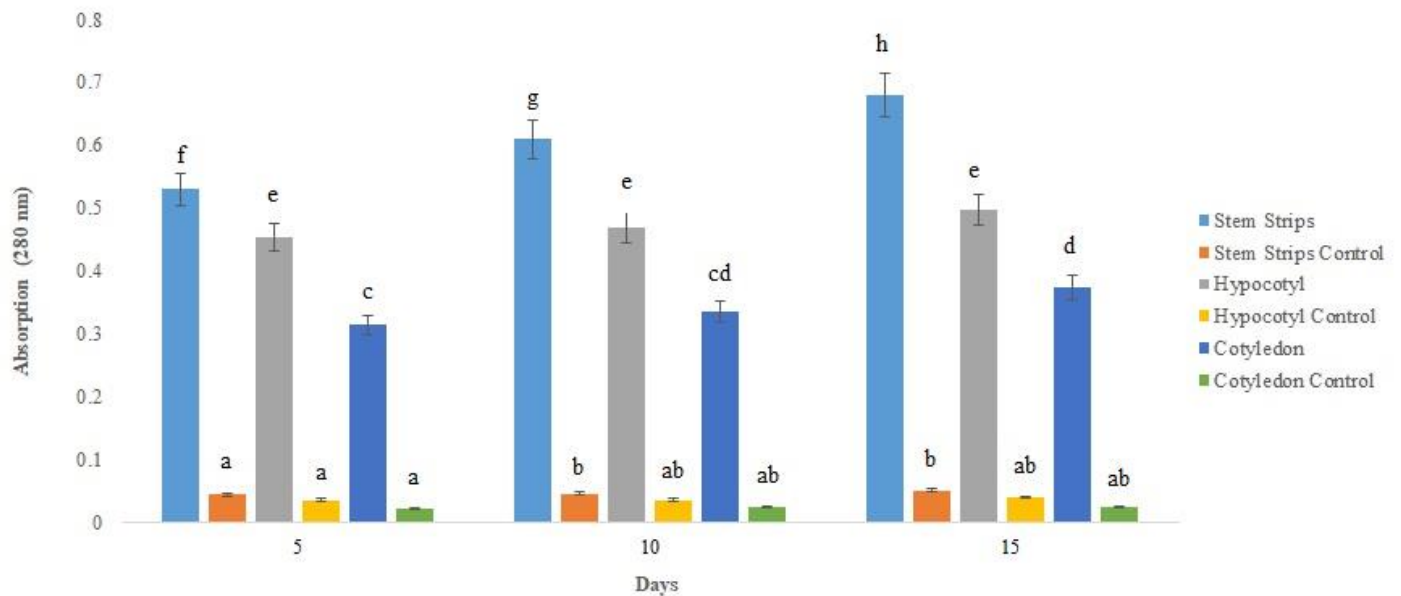

Figure 29. The thioglycolic acid lignin (TGAL) contents in *Pinus radaita* calli on day 5, 10 and 15 after culturing on tracheary element induction medium or the control culture on callus proliferation medium. The absorbance of the TGAL-containing callus extracts was measured at 280 nm using a UV-1600PC spectrophotometer. Means ± SD with different letters were significantly different ($p \leq 0.05$).

4.4.2. Peroxidase

In order to determine the association of the antioxidant responses in TE-induced-calli during differentiation, we measured the POD activity in the calli derived from hypocotyl explants. In this study, horseradish peroxidase was used for validation as a positive control. Generally, POD activity was lower in the hypocotyl-derived callus cultured on callus proliferation medium (control) than that in the calli cultured on TIM (Figure 30). In the first three days of culture, there was no change and no significant difference in the levels of POD activity between the control and the calli on TIM. The level of POD activity was significantly increased in the calli after 2 weeks of culturing on TIM compared to the control (Figure 30). As shown in Figure 30, the increase at day 15 was about double of that at 3 day.

To investigate the correlation between the activity of antioxidant enzymes and xylogenesis we subsequently measured it over the 15 days of culture. Interestingly, xylem formation was associated with increased activities of antioxidant enzymes. A possible explanation for this might be that lignification and environmental stresses could cause oxidative stress via the production and accumulation of ROS. Hydrogen peroxide is a ROS that can be adequately controlled by antioxidants and antioxidant enzymes, under normal conditions. However, a risk for serious cellular damage may arise when ROS are overproduced under stress conditions (Zhou et al., 2008). To cope with oxidative damage under extremely adverse conditions, plants have developed an antioxidant defense system that includes the antioxidant enzymes such as POD (Foyer & Noctor, 2005). Therefore, the levels of POD were higher in xylogenic calli on day 15 than earlier in culture, probably due to formation of a higher percentage of xylem cells by the later date of culture.

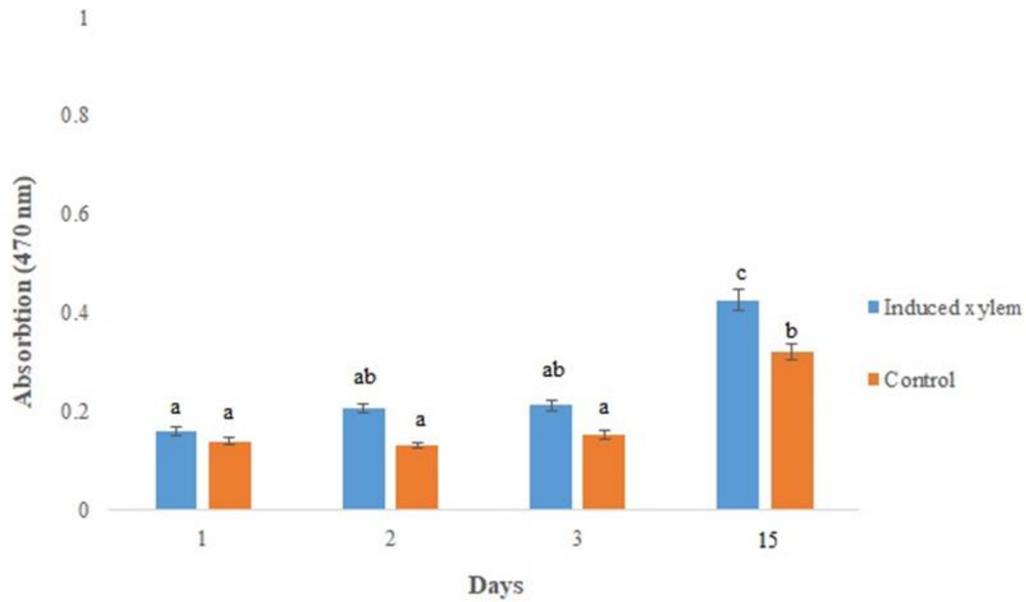

Figure 30. Changes in the levels of peroxidase (POD) activity in the extracts of the xylogenic callus cultured on MS medium supplemented with 1.0 mg/L NAA and 1.5 mg/L BAP, with 0.025 mg/L SNP, and control callus derived from *Pinus radiata* hypocotyl explants for 15 days. Means ± SD with different letters were significantly different ($p \leq 0.05$).

4.4.3. Cinnamyl alcohol dehydrogenase (CAD)

4.4.3.1. CAD activities in the control cultures and TE-forming cultures

The CAD activities in the *P. radiata* calli were monitored at 24 h intervals during the first 5 days of culture and at 5 days thereafter. There were transient increases in the activities of CAD in the calli derived from hypocotyl and cotyledon explants of *P. radiata* during the first day after transfer of the calli to fresh callusmaintenance or induction medium (Figure 31).

The levels of the CAD activities in the *P. radiata* callus cultures were relatively similar and remained steady in the second and third days of culturing on TE induction or callus proliferation medium (Figure 31). In the xylogenic hypocotyl-derived callus cultures, the enzymatic activity increased further (2.1 pkat mg^{-1} protein) after 10 days of culture. This increase was more than twice that of the control cultures (Figure 31).

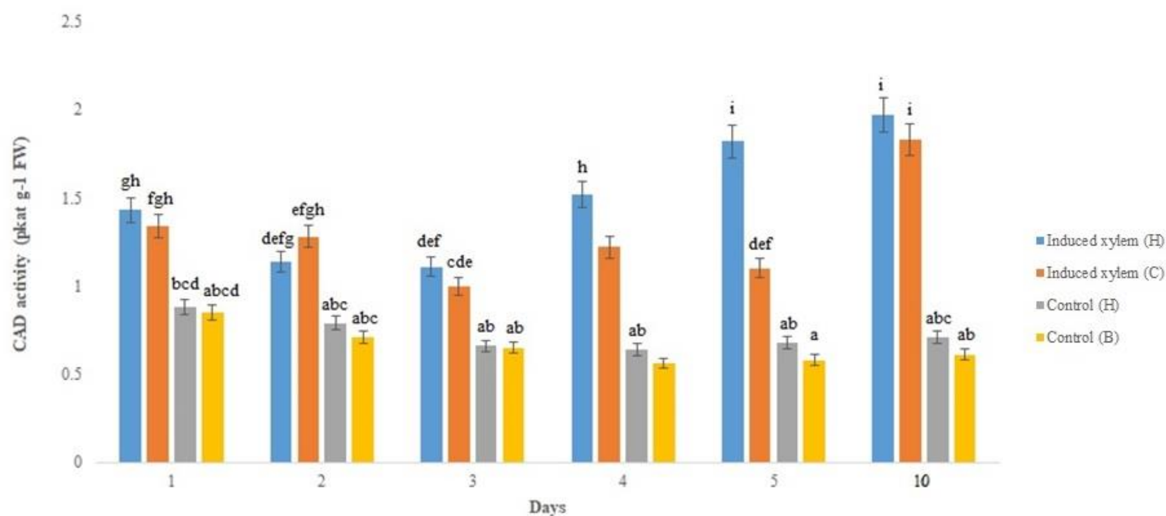

Figure 31. Changes in the levels of cinnamyl alcohol dehydrogenase (CAD) activity in the extracts of the xylogenic callus cultured on MS medium supplemented with 1.0 mg/L NAA and 1.5 mg/L BAP with 0.025 mg/L SNP and control callus derived from *Pinus radiata* hypocotyl and cotyledon explants for 10 days. Means ± SD with different letters were significantly different ($p \leq 0.05$).

4.4.4. Confocal laser scanning microscopy

4.4.4.1. Tracheary elements in *Pinus radiata* calli

Soft green calli were initiated from hypocotyl and cotyledon explants of *Pinus radiata* seedlings after 2 weeks of culture. After 4 weeks, the calli were no longer soft and green but were brown and hard. Tracheary elements (TEs) were frequently found as cell clusters in these calli (Figure 32). Almost all of them had a thick secondary cell wall around the entire cell. Some tracheary elements formed spiral thickening which are a characteristic feature of secondary tracheids (Figures 33 & 36). Other tracheary elements formed helical thickenings on the most inner surface of the secondary walls (Figures 35 & 37). In some tracheary elements, the thickening of the secondary walls was complex, being a mixture of the helical and reticulate types (Figure 34).

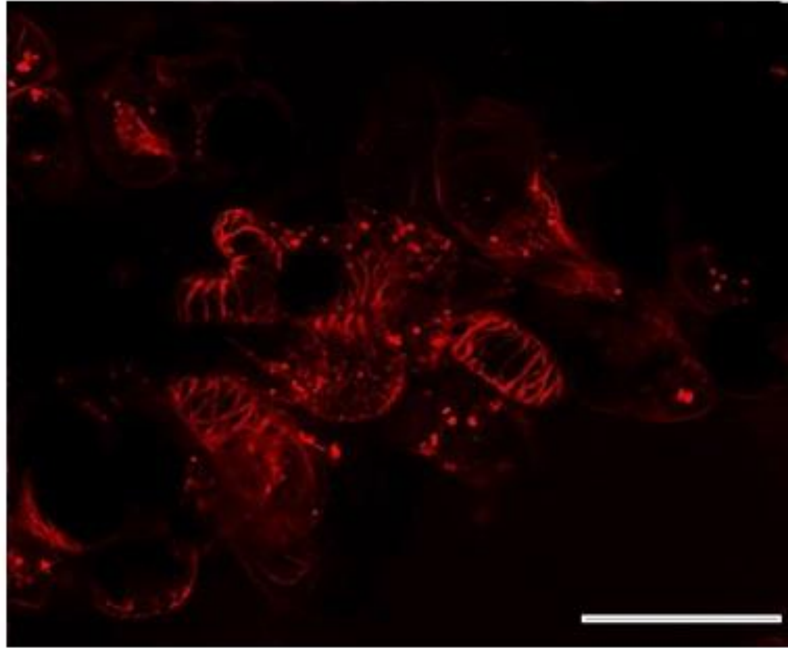

Figure 32. Confocal laser scanning micrographs showing tracheary elements in cell clusters in callus culture of *Pinus radiata*. Bar = 100 µm

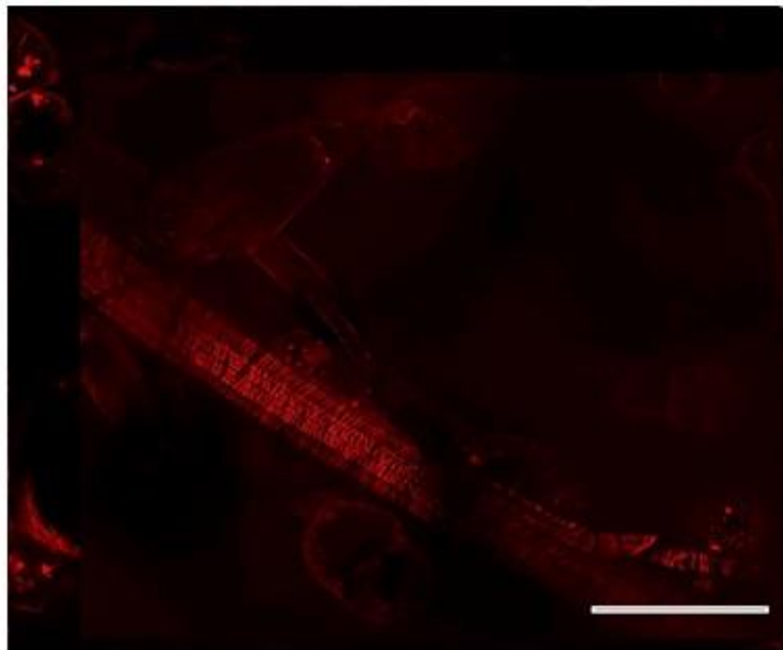

Figure 33. Confocal laser scanning micrographs of tracheary elements in cell clusters produced in callus culture of *Pinus radiata*. Elongated tracheary element with spiral cell-wall patterns. Bar = 200 µm

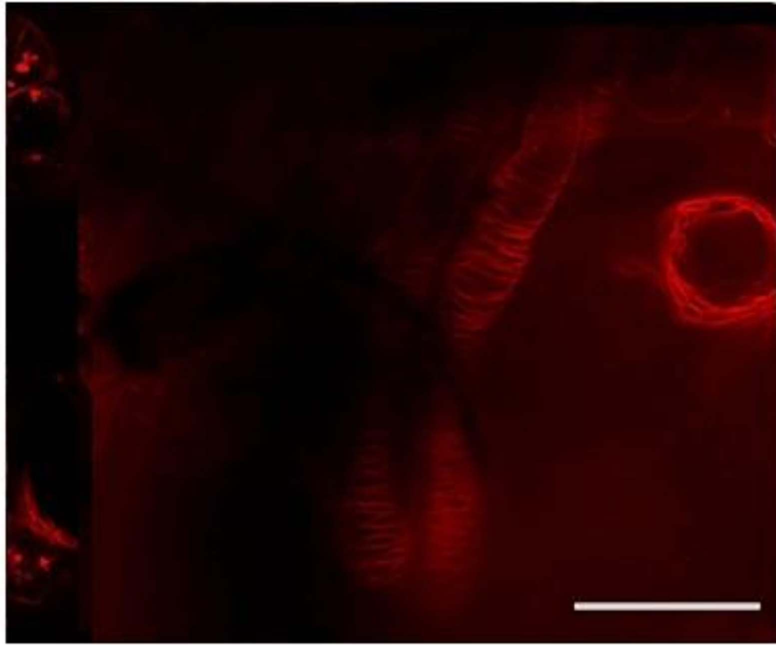

Figure 34. Confocal microscopy showing helical and reticulate cell-wall patterns of *Pinus radiata* callus culture. Bar = 100 µm

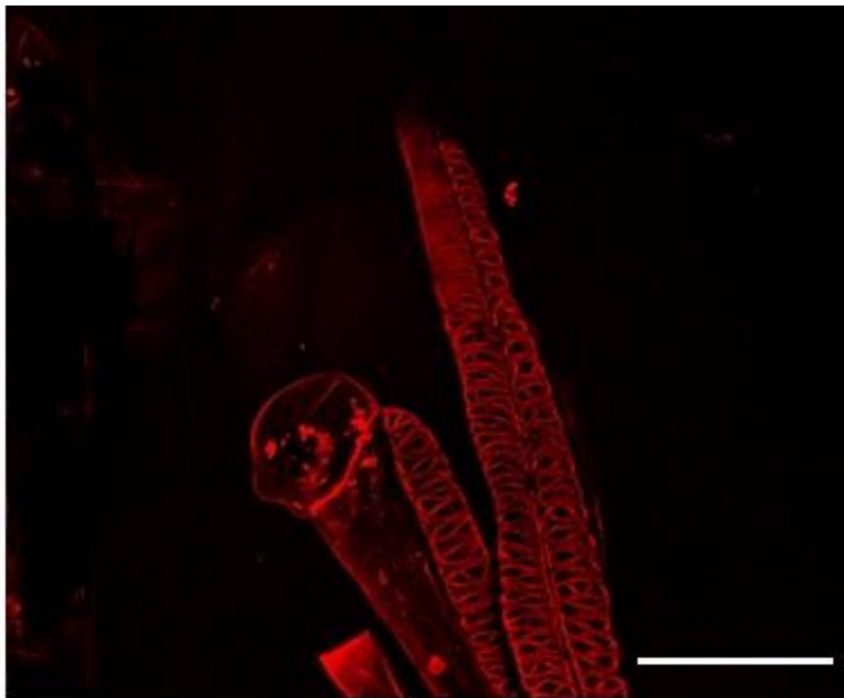

Figure 35. Confocal micrograph of tracheary elements differentiated in *Pinus radiata* callus cultures. TEs showing reticulate secondary cell-wall patterns which had different sizes and shapes. Bar = 150 µm

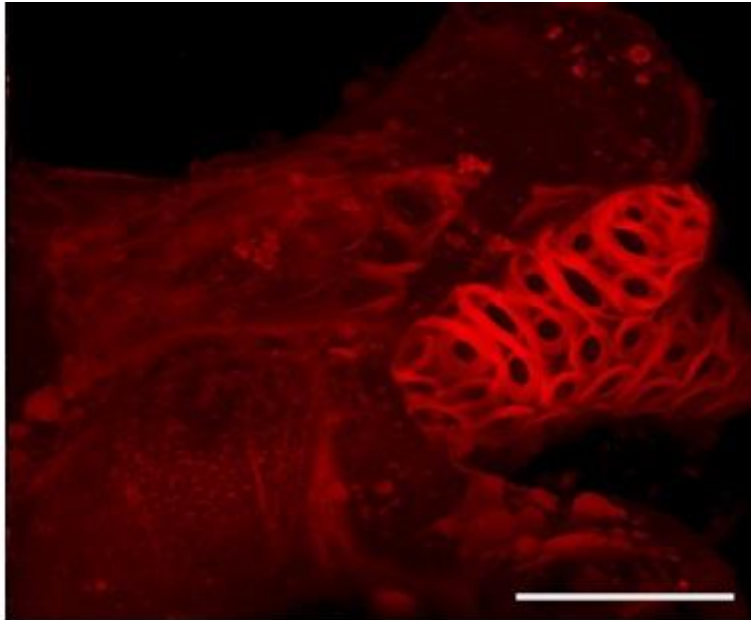

Figure 36. Confocal micrograph of tracheary elements differentiated in *Pinus radiata* callus cultures. Tracheary elements showing reticulate secondary cell-wall patterns. The cells without cell-wall pattern are parenchymatous callus cells. Bar = 25 µm

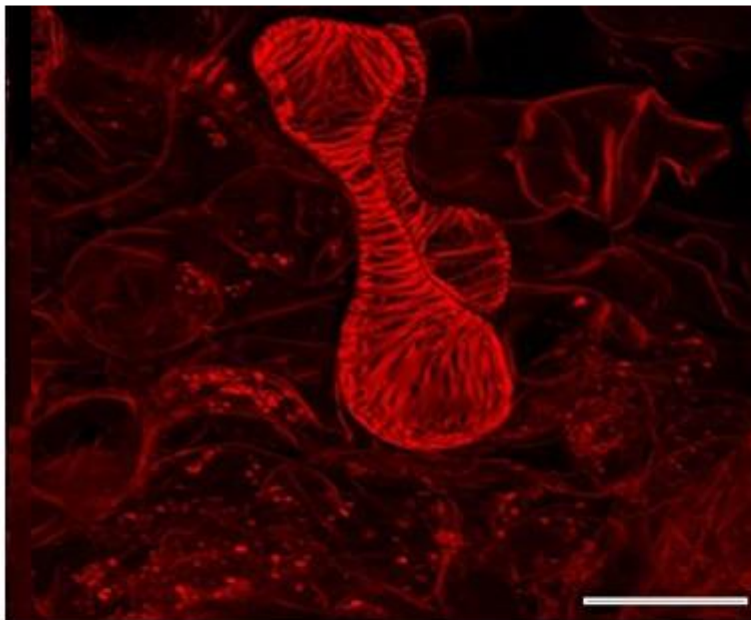

Figure 37. Confocal micrograph of a tracheid in xylem-derived callus showing a helical pattern of secondary cell-wall thickenings. Bar = 25 µm

4.5. Conclusions

Enzymatic aspects of lignification were assessed in a *Pinus radiata* cell culture system that was induced to differentiate TEs and sclereids with lignified secondary cell walls. The activities of the lignin-related enzymes, peroxidaes (POD) and cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195), increased simultaneously with cell differentiation, suggesting that the rise in these enzyme activities was related to lignification of the cell walls (Figure 30).

In this study, there was a lower and constant level of CAD activity in the control callus which did not react with the phloroglucinol-HCl stain for the presence of lignin. Similarly, CAD activities have been found in *P. radiata* and *P. banksiana* Lamb. (Jack pine) suspension cultures (Campbell & Ellis, 1992; Hotter, 1997) and in *Pinus taeda* L. (loblolly pine) megagametophytes, that also have unlignified cell walls (MacKay et al., 1995). Cinnamyl alcohol dehydrogenase is responsible for reducing hydroxycinnamyl aldehydes to lignin-precursor hydroxycinnamyl alcohols (monolignols) (Boerjan et al., 2003). Hydroxycinnamyl alcohol is also essential for the lignans biosynthesis such as dehydrodiconiferyl alcohol (Orr & Lynn, 1992). It can be concluded that this may be a result of CAD activity in the control cultures. The CAD activity increased in the xylogenic callus cultures after 3 days of subculture associated with the lignification of the cell walls in the developing tracheary elements and sclereids. After 10 days of culture, there was a large increase in CAD activity associated with TE formation in the xylogenic callus culture. A similar rise in CAD activity has been reported in *Z. elegans* mesophyll suspension cultures that initiated tracheary elements with lignified secondary cell walls (Sato et al., 1997). We can conclude that the CAD activity is a useful marker for *in vitro* studies of celldevelopment with lignified secondary cell walls in

gymnosperms. However, this enzyme is also involved in the phenolic compound biosynthesis other than lignin and can be stimulated by treatment with elicitors (Brinkmann et al., 2002).

In previous studies, cell cultures of coniferous gymnosperms have been useful model systems for studying various aspects of lignin formation (Anterola & Lewis, 2002; Brunow et al., 1990; Eberhardt et al., 1993; Nose et al., 1995; Stasolla et al., 2003). The *Pinus radiata* callus culture used in the present study is, however, particularly useful due to the TE induction with lignified secondary cell walls. Since the callus culture system of *Pinus radiata* can be genetically transformed before secondary cell-wall induction and lignin biosynthesis (Möller et al., 2003), it can therefore be concluded that studying the effects of altered gene expression on phenylpropanoid biosynthesis and lignification processes in this xylogenic cell culture system would be useful in the future studies.

Chapter 5. Summary, Conclusions and Future Directions

5.1. Key objectives, strategies, major findings and limitations

In vitro TE systems have been developed in both plant species from angiosperms and gymnosperms, but most focus has been placed on angiosperms rather than gymnosperms. A few studies of *in vitro* culture technology for TE differentiation in *Pinus radiata* has been reported (Möller, McDonald, et al., 2006b). In this study, effect of different factors on tracheary element differentiation and TE differentiation rate in callus cultures of *Pinus radiata* were assessed. Furthermore, antioxidant enzyme activities and phenolic contents of pine trees that can be influenced in response to TE differentiation were quantified. This study has confirmed the potential of using callus culture to induce xylem, and also established a useful protocol in *Pinus radiata* xylem formation. The efficient protocol may well impact on future research designs.

In this study, microscopic studies were carried out to understand the microstructure of TEs and the cytological characteristics of TE induction *in vitro*. Tracheary elements formed in callus was visually detected as cells with distinctive secondary cell-wall thickening usually associated with xylem observed under a light microscope (Sato et al., 2011). The TEs formed *in vitro* were then assessed qualitatively using morphological features and quantitatively by evaluating the rate of differentiation based on cell counting.

Tracheary element formation was induced through controlled culture conditions from callus grown on solid medium or as cell suspension cultures (Table 1). In the study, callus culture on solid medium was used and two different media P6-SHv (Hotter, 1997; Möller, McDonald, et al., 2006b) and MS-based media were compared. Stem strips from one-year-old shoots of 3-year-old *P. radiata* trees, hypocotyls and cotyledons from germinated seedlings were

excised and were placed on the P6-SHv medium containing 1.0 mg/L BAP + 1.0 mg/L 2,4-D (T1) under light and in the dark at 24ºC. After 5-6 weeks of incubation, the calli were visually examined. This experiment was repeated two more times because we were not able to establish subculturable calli as reported in the previous studies (Hotter, 1997; Moller, 2006; Möller et al., 2003).

Tracheary elements formation in callus culture has been studied in calli of different plant species previously (Fukuda, 1996; Fukuda & Komamine, 1982; Leitch & Savidge, 2000; McCann et al., 2000; Savidge, 1983a) and is regulated by multiple factors (Möller, Ball, et al., 2006). The time needed for induction and differentiation of TEs differed between systems (Table 1). The differentiation rates of TEs differed between the genotypes of the same plant species. The studies on different species used different *in vitro* TE induction protocols (Table 1).

A xylogenic cell culture from *Pinus radiata* hypocotyl and cotyledon explants was established *in vitro*. Following subculture on fresh medium, the calli derived from the explants of the hypocotyl, cotyledon and stem strips were able to proliferate on a MS medium supplemented with 1 mg/L 2,4-D, 0.58 mg/L BAP and 1 mg/L NAA. The calli were transferred to a fresh medium every 2 weeks. TE induction was activated by the addition of jasmonic acid, auxin, cytokinin, and SNP of different concentrations. Tracheary element induction was up to 28 percent of the cells in the calli derived from hypocotyl explants after 3-5 days of culturing on the latter medium. High TE differentiation rates were observed when calli derived from hypocotyl explants were grown under a 16 h or 24 h photoperiod on solid TIM medium containing 1 mg/L NAA, 1 mg/L BAP and 0.025 mg/L SNP.

In this study, lignified and TE cells were counted using a haemocytometer under either normal light or polarised light. Tracheary elements formed in xylogenic callus appeared as an

assembly of cells in the form of a cell clump, both after tissue maceration or observed after sectioning and observation under confocal microscopy. TE cell clumps of *Pseudostuga menziesii* (Mirb.) Franco were separated using a glass grinder followed by filtration on a nylon mesh before microscopic observations (Pillai et al., 2011). Tracheary elements can be easily recognised under polarised light from other cell types because the deposited lignin in cell wall thickenings causes strong birefringence. Although many studies (Table 1) mainly relied on cells showing birefringence as TEs, sclereids could also exhibit birefringence as they also have secondary cell walls. Therefore, the reported percentage of TEs in many studies might be over-estimated values. In addition, only images of isolated TEs were presented in these studies, whereas in the present study, the notion that TEs induced in *P. radiata* callus appeared as cell clusters was supported by the confocal images obtained.

Table 1. *In vitro* culture techniques, induction treatment duration and tracheary elements percentage in various gymnosperms species.

Gymnosperm species	***In vitro* culture technique**	**Percentage or mean TEs cell count***	**Duration on induction medium**	**References**
Cryptomeria japonica	Callus	2 to 34%	Not reported	Mehra & Anand (1979)
Cupressus sempervirens	Callus	30%	Not reported	Havel et al. (1997)
Pinus contorta	Cell suspension	5 to 40%	Not reported	Webb (1981)
Pinus radiata	Callus	2 to 45%	10 days	Möller et al. (2006)
Pinus sylvestris	Cell suspension	16%	25 days	Ramsden & Northcote (1987)
Pseudostuga menziesii	Cell suspension	65%	42 to 49 days	Pillai et al. (2011)
Pinus radiata	Callus	2 to 35%	3 to 5 days	Pezhman et al. (this study has not been published yet)

*: mean number per callus observed.

Although light has been reported to increase the rates of cell division (Fraser et al., 1967) and the evolution of ethylene (Huxter et al., 1981), that may in turn affect caulogenesis (Pérez-Bermúdez et al., 1985) in this study, calli grew rapidly in the dark conditions (Figures 7 & 8). The calli grown in darkness for 8-10 weeks were 2 times larger than the calli grown in darkness for 4 weeks followed by 4 weeks of light (Figures 7 & 8). In previous studies, it was shown that an alternating period of dark–light exposure using white light is optimal to a continuous photoperiod or monochromatic red light. For instance, dark-treated *Datura* anthers induced more embryoids (Sopory & Maheshwari, 1976) after transferring to white light. The rice anthers (*Oryza sativa* L.) must be kept in dark for at least three weeks prior to light exposure. Though, in some species, dark exposure can be associated with negative effects. *Nicotiana tabacum* anthers developed less callus and fewer embryoids when incubated in the dark than in light.

In spite of lower percentage of TEs in xylogenic callus culture derived from the stem strips of *Pinus radiata* compared to calli derived from hypocotyl- and cotyledon explants, the stem strip-derived callus cultures had a relatively higher lignin content after 20-25 days. One reason for this is that the lignin concentration in the cell walls of the sclereids the stem strips-derived callus cultures was much higher than that of the tracheids of hypocotyl-derived callus cultures of *P. radiata* as determined by UV-microscopy (Fergus et al., 1969). It is also possible that lignin was present in the primary cell walls of parenchymatous cells in the induced callus culture. The results of Fergus et al. (1969) showed that the mean lignin level in the compound middle lamella of tracheids of *Picea mariana* Mill. (Black spruce) was about twice that in the secondary wall. On the other hand, the concentration of lignin in the cell walls of sclereids was variable and generally higher than in the cell walls of tracheary elements. Areas of higher and lower level of lignin were monitored in the secondary cell walls of the sclereids in the previous study, although we did not study lignin distribution matching the pattern of the

layered secondary cell walls of these cells in *P. radiata* callus culture. Future work can focus on this pattern.

Our biochemical studies showed that lignin was present in the secondary cell walls of the tracheids and sclereids in the differentiated callus, but absent from the primary cell walls of the parenchyma cells in the undifferentiated callus. The concentration of lignin in the cell walls of the initiated calli can also be inferred from the results of the TGAL assay, although cell walls from undifferentiated calli also had a significant TGAL content, possibly resulting largely from cell-wall proteins (Eberhardt et al., 1993). Results from the present study indicated that peroxidase appeared remarkably in TE-induced cultures and is in agreement with previous reports (Church & Galston, 1988; López-Serrano et al., 2004; Masuda et al., 1983; Novo-Uzal et al., 2013; Sato et al., 1993). Sato et al. (2006) found that undifferentiated calli contained significant CAD activity. Similar findings have also been reported in unlignified cells of suspension cultures of *P. radiata* (Hotter, 1997) and in unlignified cells of the megagametophyte of *P. taeda* (MacKay et al., 1995). Cinnamyl alcohol dehydrogenase is not only involved in the lignin biosynthesis, it catalyses the biosynthesis of coniferyl alcohol, which, in addition to lignin, is the precursor of lignans and some other biochemical compounds, such as dehydrodiconiferyl alcohol.

5.2 Conclusions and directions for future work

Our findings showed that MS medium with combinations of 2,4-D and BAP produced efficient callus growth in *P. radiata* hypocotyl explants derived from germinated seedlings. Moreover, a huge amount of calli were induced from stem strip explants of different genotypes of *Pinus radiata* cultured on medium supplemented with high concentrations of BAP and 2,4-D in the dark. We could establish optimised protocols for callus formation and TE induction in *P. radiata* callus culture. We found that BAP only promoted TE formation in *Pinus radiata* callus cultures in the presence of NAA. The present results alsosuggested that a high proportion of *P. radiata* callus cells were induced to differentiate into TEs in calli derived from hypocotyl explants cultured in the presence of a combination of a nitric oxide (NO) donor, sodium nitroprusside (SNP) with NAA and BAP. It has been evaluated that application of cPTIO, a specific scavenger of nitric oxide (NO), reversed the promotive effects of SNP on the percentages of lignified cells and TE induction in *P. radiata callus* culture. This stimulatory effect on TE induction was significantly diminished by the cPTIO application in combination with jasmonic acid (JA). A specific application of JA to MS medium induced TEs in the callus cultures derived from the explants of *Pinus radiata* seedlings. While JA positively interactedwith NO, this effect was counteracted by cPTIO.

In general, *Pinus radiata* callus system can be used as a model for the functional testing of genes and promoters, specifically those associated with the primary and secondary cell wall formation in coniferous gymnosperms. This thesis evaluated the undifferentiated callus induced to differentiate to TEs and the effects on the cell walls monitored chemically. Stable transformation of callus on non-TE induction medium, followed by transfer to medium for induction of tracheids and sclereids, will provide us with a better understanding of the specific genes effects on the secondary cell walls of these cell types in future. The amount of TEs can

be further increased by fractionation of TEs from callus cells, which will be necessary for subsequent chemical analysis of cell walls. A purification process needs to be developed to obtain pure TE fractions separated from contaminating primary walls of parenchymatic cells, before detailed chemical analysis of the secondary cell walls can be undertaken. Future molecular work including transcriptome analysis is also worthwhile to pursue to elucidate the overall participation of the relevant genes, metabolic and hormonal pathways involved in the tracheid formation in *Pinus radiata* callus culture.

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Appendix

Murashige & Skoog stock solutions

Major salts (10 x)	1 litre
NH4NO3	16.5 g
KNO3	19.0 g
CaCl2.2H2O	4.4 g
MgSO4.7H2O	3.7 g
KH2PO4	1.7 g
KI	0.083 g
Minor salts (100 x)	1 litre
H3 BO3	0.620 g
ZnSO4 .7H2 O	0.860 g
MnSO4. 4H2O	2.230 g
CuSO4 .5H2 O	0.0025 g
CoCl2 .6H2 O	0.0025 g
Na2 MoO4 .2H2 O	0.025 g
Organic supplement (100 x)	1 litre
Myo-inositol	10 g
Nicotinic acid	0.05 g
Pyridoxine–HCl	0.05 g
Thiamine–HCl	0.01 g
Glycine	0.2 g
Iron stock	0.5 litre
FeSO4 .7H2 O	1.39 g in 200 mL dH2O
Na2 EDTA.2H2 O	1.865 g in 200 mL dH2O

P6-SHv Stock solution

Major salts (10 x)	1 litre
NH_4NO_3	16.5 g
KNO3	19.0 g
$CaCl_2.2H_2O$	2.94 g
$MgSO_4.7H_2O$	4.93 g
KH_2PO4	2.72 g
MgCl2 .6H2O	6.09 g
MnSO4	0.15 g
KI	0.08 g
Minor salts (100 x)	1 litre
H_3BO_3	0.31 g
$ZnSO_4$ $.7H_2O$	0.28
$CuSO_4$ $.5H_2O$	0.0025
$CoCl_2$ $.6H_2O$	0.0025
Na_2 MoO_4 $.2H_2O$	0.0025
Organic supplement (100 x)	1 litre
Myo-inositol	10 g
Nicotinic acid	0.05 g
Pyridoxine–HCl	0.05 g
Thiamine–HCl	0.05 g
Iron stock	0.5 litre
FeSO4 .7H2 O	1.39 g in 200 mL dH2O
Na2 EDTA.2H2 O	1.865 g in 200 mL dH2O