International effort to induce somatic embryogenesis in adult pine trees

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Abstract

The genus *Pinus* includes several species that are economically important and planted outside their natural ranges as plantation species. Somatic embryogenesis (SE), a biotechnological tool for mass propagation of pines, has been reported for many species but only from seed embryos. Cloning the individual adult trees, through SE from vegetative explants, could potentially benefit the forest industry in that only trees with elite characteristics would be planted commercially. The attributes of conifer trees may only be evaluated after many years of growth and often not until the reproductive growth phase. This chapter describes a concerted effort by several research teams in five countries to initiate SE in primordial shoot explants of six pine species, each commercially important in its respective country. In spite of the multi-year experiments, SE was induced in only one species (*Pinus sylvestris*), but embryogenic lines showed some instability at microsatellite loci and the somatic embryos did not germinate. Some cell lines

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initiated in different species showed embryogenic-like characteristics at the microscopic level. Expression of embryogenesis specific genes (*LEC1*/*CHAP3A*, *WOX2*, *VP1*) was detected in such calli/cell aggregates of all three tested pine species (including those with embryogenic-like characteristics) even when the presence of early somatic embryos could not be confirmed. Overall, the results presented in this chapter are indicative of the existing challenges in propagation of adult pines as in other conifers.

Keywords: callus, embryonal mass-like, explant pre-treatment, gene expression, needle fascicles, *Pinus* spp., primordial shoot explants, vegetative shoot buds

1. Introduction

The ability to vegetatively propagate adult conifer trees as opposed to zygotic embryos or seedlings, or juvenile trees, all of which are of unproven genetic potential, would be more advantageous in that only field-proven trees would be propagated for commercial plantings. Although rooted plants have been produced from axillary buds of adult trees of some pine species, such as *P. pinea, P. radiata, P. pinaster* and *P. sylvestris* on a laboratory scale, the method has many limitations including the lack of potential for scale-up as only a limited number of plants could be established (Cortizo et al. 2009; De Diego et al. 2008, 2010; Montalbán et al. 2013, respectively). Somatic embryogenesis (SE) of adult trees would provide not only a potential for scale-up production of clonal trees but coupled with the ease of cryopreservation of embryonal masses (EMs) would ensure a continuous supply of elite genotypes for plantings (*see also the chapter in this book "**Is there potential for propagation of adult spruce trees through somatic embryogenesis?**" by Klimaszewska and Rutledge*). Moreover conventional breeding is likely to benefit in the near future from the synergistic application of both genome-wide (genomic) selection and vegetative propagation (cloning) through SE of elite varieties to implement multivarietal forestry (Klimaszewska et al. 2007, El-Kassaby and Klápště 2015).

Starting in 2003 and onwards, a number of publications by Malabadi and collaborators claimed that SE could be initiated from adult trees (10-20 years old) of different tropical and subtropical pine species of high economic interest in South Africa (*Pinus patula,* Malabadi and van Staden 2003, 2005a) and northeastern India (*P. kesiya*, *P. roxburghii*, *P. wallichiana*, Malabadi et al. 2004, Malabadi and Nataraja 2006, 2007a, b). The authors described relatively similar tissue culture procedures for all the tested species to achieve the initiation of SE from shoot apical domes (primordial shoot, PS) and, in one case, from secondary needles (Malabadi and Nataraja 2007a). Positive results were presented for all three

genotypes tested in each species suggesting that the method is sufficiently refined to be considered generic, i.e., most genotypes would be responsive within a species. The SE initiation protocol was based on a modified DCR (Gupta and Durzan 1985) medium formulation (mDCR) and involved three main steps taking place in the dark (Table 1): (1) cold pre-treatment of explants for 3 days on a pre-treatment medium (PM) including activated charcoal but no plant growth regulators (PGR), (2) culture of explants on an initiation medium (IM) with high PGR content until white mucilaginous embryogenic tissue (ET) was detected (28-42 days) and (3) ET proliferation on maintenance medium (MM) with reduced PGR content.

Table 1. *Description of cold pre-treatment (PM), initiation (IM) and maintenance (MM) steps and media for inducing SE in explants of adult trees in different Pinus spp. (according to Malabadi et al.). Note: "?" indicates lack of information in the publication.*

Step - Medium	1- PM					2 - IM					3 - MM				
Pinus species[a]	Pp	Pk	Pr	Pr	Pw	Pp	Pk	Pr	Pr	Pw	Pp	Pk	Pr	Pr	Pw
Reference[b]	[1-3]	[4]	[5]	[6]	[7]	[1-3]	[4]	[5]	[6]	[7]	[1-3]	[4]	[5]	[6]	[7]
Explants source[c]	AD	AD	AD	ND	AD	AD	AD	AD	ND	AD	TS	TS	TS	LS	TS
Explants type[d]	TS	TS	TS	LS	TS	TS	TS	TS	LS	TS	EM	EM	EM	EM	EM
Ø 25 glass tube size (mm)	75	145	145	145	145	?	?	?	?	?	?	?	?	?	?
Medium volume/tube (ml)	10	15	15	15	15	?	?	?	?	?	?	?	?	?	?
Temperature (°C)	2	4	4	4	2	25	25	25	25	25	25	?	?	?	?
Incubation time (days)	3	3	3	3	3	28	28	42	?	?	30	?	28	30	30
Number of subculture	0	0	0	0	0	3	?	?	?	?	2	?	?	2	2
DCR macronutrients[e]	1X	0.5X	1X	1X	1X	1X	0.5X	1X	1X	1X	1X	0.5X	1X	1X	1X
DCR micronutrients[e]	1X	0.5X	1X	1X	1X	1X	0.5X	1X	1X	1X	1X	0.5X	1X	1X	1X
DCR vitamins[ef]	1X	?	?	?	?	1X	?	1X	1X	1X	1X	?	1X	1X	1X
Myo-inositol (g l^{-1})	?	?	?	?	?	1	1	1	1	1	?	?	?	?	?
PVP-40 (g l^{-1})	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	/	/	/	/	/
Activated charcoal (g l^{-1})	3	3	3	3	3	/	/	/	/	/	/	/	/	/	/
Maltose (mM)	90	83	83	83	83	90	/	/	/	/	120	111	167	83	167
2,4-D (µM)	/	/	/	/	/	20	22.6	22.6	22.6	22.6	2	2.26	2.26	2.26	2.26
NAA (µM)	/	/	/	/	/	25	26.8	26.8	26.8	26.8	2.5	2.68	2.68	2.68	2.68
BA (µM)	/	/	/	/	/	9	8.9	8.9	/	8.9	1	0.88	0.88	/	0.88
Triacontanol (µM)	/	/	/	/	/	/	/	/	5	/	/	/	/	2	/
pH[g]	?	?	?	?	?	5.8	5.8	5.8	5.8	5.8	?	?	?	?	?
Phytagel (g l^{-1})	1.5	/	/	2	2	1.5	2	2	2	2	2	4	4	1.8	4
Bacto-Agar (g l^{-1})	/	7	7	/	/	/	/	/	/	/	/	/	/	/	/
Casein hydrolysate (g l^{-1})[h]	/	?	?	?	?	1	1	1	1	1	/	/	/	/	/
L-Glutamine (g l^{-1})[h]	/	?	?	?	?	1	1	1	1	1	/	/	/	/	/

[a]Pp: *P. patula*; Pk: *P. kesiya*; Pr: *P. roxburghii*; Pw: *P. wallichiana*

[b][1] Malabadi and van Staden (2003); [2] Malabadi and van Staden (2005a); [3] Malabadi and van Staden (2005b); [4] Malabadi *et al.* (2004); [5] Malabadi and Nataraja (2006); [6] Malabadi and Nataraja (2007a); [7] Malabadi and Nataraja (2007b).

[c]AD: apical domes from shoot apices; ND: secondary needles; [d]TS: transverse sections (0.5-1 mm thick); LS: longitudinal sections of basal ND part (1-1.5 cm long); [e]Gupta and Durzan (1985); [f]Original vitamins excluding myo-inositol; [g]Medium pH was adjusted to 5.8 before gelling agent was added and autoclaving (121°C, 15 min, 103 kPa). [h]Filter-sterilized and added after autoclaving to the warm cooled medium (50°C).

Mean initiation rates of SE obtained by using this basic procedure (Table 2) were in the range of 2-7% (standard treatment) or 4-8% (treatment with dithiothreitol, DTT), in *P. patula*, 3-6% (standard) or 13-27% (treatment with smoke-saturated water, SSW) in *P. wallichiana*, 34-63% (apical domes of PS) or 46-65% (secondary needles) in *P. roxburghii,* and as high as 86% in *P. kesiya*. It was therefore conceivable that such a simple procedure could be successfully

applied, with potentially minor modifications, to other pine species of commercial interest. Based on the published studies, there were two key complementary factors to obtain initiation of SE within explants of PS of mature pines: (1) cold pre-treatment for 3 days at 2°C (*P. patula*, *P. wallichiana*) or 4°C (*P. kesiya*, *P. roxburghii*) on mDCR supplemented with 0.3% (w/v) activated charcoal and (2)

Table 2. *The best initiation rates of SE obtained from mature pine genotypes (Malabadi et al., procedure and media described in Table 1). Note: "?" indicates lack of information in the publication.*

Pinus spp.	Genotype	Age (years)	Explant source (type)[a]	Collection date (developmental stage)	Mean initiation rate ± standard error (%)	Ref.[a]
patula	PP3	15	AD (TS)	May (?)	4.5 ± 1.2 6.2 ± 0.5 (DTT)[b]	[1-2] [3]
	PP13	15	AD (TS)	March (?)	7.0 ± 1.2 8.2 ± 0.5 (DTT)[b]	[1-2] [3]
	PP18	15	AD (TS)	June (?)	2.0 ± 0.6 4.2 ± 0.2 (DTT)[b]	[1-2] [3]
kesiya	PK08	15-20	AD (TS)	April-July (?)	86.3 ± 7.4	[4]
	PK351	15-20	AD (TS)	April-July (?)	86.3 ± 7.4	[4]
	PK934	15-20	AD (TS)	April-July (?)	86.3 ± 7.4	[4]
roxburghii	PR11	14	AD (TS)	April (bud break)	57.0 ± 1.3	[5]
	PR105	14	AD (TS)	April (bud break)	63.0 ± 5.6	[5]
	PR521	14	AD (TS)	April (bud break)	34.5 ± 1.2	[5]
	PR17	10	ND (LS)	May (needle sprouting)	51.5 ± 4.1[c]	[6]
	PR100	10	ND (LS)	May (needle sprouting)	46.2 ± 3.2[c]	[6]
	PR321	10	ND (LS)	May (needle sprouting)	65.0 ± 1.9[c]	[6]
wallichiana	PW10	13	AD (TS)	May (bud break)	3.0 ± 0.2 13.0 ± 1.2 (SSW)[d]	[7]
	PW39	13	AD (TS)	May (bud break)	4.0 ± 0.6 21.0 ± 1.4 (SSW)[d]	[7]
	PW120	13	AD (TS)	May (bud break)	6.0 ± 0.3 27.0 ± 2.1 (SSW)[d]	[7]

[a]See Table 1. [b]Explants were submerged in 0.1% (w/v) dithiothreitol (DTT) for 10 min before cold pre-treatment (PM) and initiation (IM). [c]BA replaced by 5 µM triacontanol (TRIA) in IM medium. [d]10% (v/v) smoke-saturated water (SSW) in cold pre-treatment (PM) and initiation (IM) media.

the developmental stage of PS or secondary needles. Very low initiation rates or only hard non-embryogenic calli (NEC) were obtained if shoot explants were not exposed to cold pre-treatment (Malabadi and van Staden 2003, Malabadi et al. 2004, Malabadi and Nataraja 2006, Malabadi and van Staden 2005a), or if incubated at temperatures above 2-4°C (Malabadi et al. 2004, Malabadi and van Staden 2005a), or if cultured on medium with activated charcoal at a lower or higher concentration than 0.3% (Malabadi and van Staden 2005a, Malabadi and

Nataraja 2006, Malabadi et al. 2004, Malabadi and Nataraja 2007b) or if the explants were cultured on charcoal medium for longer than 3 days (Malabadi and van Staden 2005a, Malabadi and Nataraja 2006). Cold pre-treatment for only 1 or 2 days resulted in very low initiation rate of SE (Malabadi and van Staden 2005a). The optimized cold pre-treatment conditions, i.e., cold incubation for 3 days on half-strength (*P. kesiya*) or full-strength (other pine species) DCR supplemented with 0.3% activated charcoal, 0.2 g l^{-1} PVP-40, 83-90 mM maltose and 1.5-2.0 g l^{-1} gellan gum (Phytagel™) or 7 g l^{-1} agar (Difco-bacto), apparently fulfilled the requirement for SE initiation in all four species tested. Only the optimal cold incubation temperature appeared to be slightly variable among the species (2 to 4°C). It was found that PS explants produced ET only at specific collection dates from March to July depending on the pine species, the genotype, and the explant source (Table 2). Only white, hard NEC was obtained on other collection dates. It was noteworthy that all tested genotypes were able to produce ET during a relatively short period of competence of 1 (*P. patula*, *P. roxburghii* and *P. wallichiana*) to 3 months (*P. kesiya*). The "right" developmental stage of the shoot buds was apparently determined as being the early stages of elongation, i.e., immediately after bud break (Malabadi and Nataraja 2007b) or at the stage of needle fascicle sprouting (Malabadi and Nataraja 2007a).

Notwithstanding, none of the publications presented sufficient and unambiguous photographic evidence to illustrate progression of the SE initiation within the pine explants nor did the authors present regenerated plants from the somatic embryos established *ex vitro* except apparently in *P. kesiya* (Malabadi et al. 2004). It is also unclear whether the experiments were repeated in the consecutive years and if the same genotypes responded or whether the described responses were single events. However, owing to the importance of the subject matter for commercially important conifers, the above published results triggered the commencement of an international project, initiated in 2006 and coordinated by K. Klimaszewska, on SE induction in adult pine trees in two laboratories in Canada, two laboratories in France, and one each in Finland, Spain and New Zealand. Each laboratory worked on locally important pine species with specific protocols that were adapted following common discussion of the protocols published by Malabadi and collaborators from 2003 to 2007.

In this chapter, we present the results obtained in the above-listed laboratories for *P. pinaster, P. sylvestris, P. radiata, P. patula, P. strobus* and *P. contorta,* followed by conclusions. Most of these results have either not been published at all or have been included in conference abstracts and proceedings. Only the results on *P. contorta* and *P. radiata* were published by Park et al. (2010) and Garcia-Mendiguren et al. (2015), respectively and are also included in this chapter.

2. *Pinus pinaster* Ait. (Maritime pine) - Trontin J-F, Quoniou S, Lelu-Walter M-A

At FCBA and INRA, the research mainly focused on the determination of the responsive developmental stage of the shoot buds, applying the cold pre-treatment to the explants for 3 days at 2 or 4°C on mDCR supplemented with 0.3% (w/v) activated charcoal. Two genotypes of 5-year-old trees were used at INRA and four genotypes of 11- to 34-year-old trees at FCBA. We report here only the more advanced results obtained at FCBA from cultured explants. The shoot buds were collected in 2007 (10 experiments), 2008 (7 experiments) and 2009 (3 experiments).

2.1 Donor trees and PS explant sources

Three F1 genotypes of adult trees selected from elite G0 families (FCBA breeding populations) were compared in the SE initiation experiments: 1443 (0041 x 0022), 2599 (4301 x 3110) and 2849 (1337 x 0243). One genotype of a somatic tree (0136) was also introduced in several experiments as a tissue culture derived material. In the microscopic analyses, an embryogenic line initiated in 1999 (PN519) from an immature zygotic embryo (4304 x 4301) was included as the reference for embryogenic culture characteristics.

In March 2007, genotypes 1443, 2599 and 2849 were available as dozens of 4-year-old grafts; potted plants in a greenhouse or plants established outdoors in soil containers. The ortet age was 34 ± 1 year. This grafted mature material (producing female cones) has also been involved in rejuvenation experiments using micrografting techniques (Trontin et al. 2005). Significant knowledge has thus been gained about meristem activity and shoot growth during several growing seasons. The somatic genotype 0136 was acclimatized in 1996, grown outside since 1997 and planted in the field in the spring of 1999. This tree was 11 years old at the time of the first experiment and had just started its reproductive phase (first female cones were observed in spring 2007).

2.2 Preparation of PS explants

The grafts and somatic plants were treated monthly with fungicides by alternating aluminum ethylphosphite and thirame treatment, and weekly application with iprodione or vinchlozoline. Branch tips with shoot buds (about 20 cm long) were collected from mother plants 1-2 days after the foliar fungicide treatment. Unless specified otherwise, the shoots were randomly sampled within clone and individual plants. Secondary needles were removed from the subapical zone and the shoot bud (about 5-10 cm long) was excised, washed with a home

detergent solution and thoroughly rinsed with running tap water. Shoot buds were

Table 3. *Sampling, tested factors and success in decontaminating PS slices (SAS) during initiation experiments from four genotypes of mature P. pinaster (1443, 2599, 2849, 0136) launched at FCBA in 2007-2009.*

Exp.	Collection date	Plant setting[a]	Nb of genotypes	Buds Nb / genotype	Tested factors[b]	SAS (Nb)[c]	Decontaminated SAS (%)
1	09/03/07	G	3	2	Bud type	121	34.7
2	27/03/07	G	1	15	Decontamination + subculture	150	36.0
3	17/04/07	G	3	6	Bud type + decontamination	180	41.7
4	15/05/07	O	1	15	Decontamination	150	59.3
5	12/06/07	O	1	15	Decontamination	150	32.7
6	29/06/07	O	1	10	Decontamination	100	56.0
7	17/07/07	O	3	5-10	Decontamination	200	65.0
8	27/08/07	O	3	5	SAS position	295	39.3
9	02/10/07	O	3	5	SAS thickness	270	38.5
10	06/11/07	O	3	5	/	157	31.8
11	22/01/08	G	3	2	Bud stage + basal	177	95.5
		O	4	2-4	medium	266	80.8
12	04/02/08	G	3	4-8	Bud stage + basal	235	67.2
		O	4	4	medium + SAS thickness	264	59.1
13	19/02/08	G	3	4-6	Bud stage + basal	255	80.4
		O	4	4-6	medium	327	53.8
14	31/03/08	G	3	4	Bud stage + basal	230	90.9
		O	4	4	medium	312	56.7
15	12/05/08	G	3	7	Bud stage + initiation	144	100
		O	4	7	method	192	77.1
16	06/06/08	G/O	4	4	Medium composition (IM)	164	96.9
17	04/07/08	G/O	4	4	Medium composition (IM)	160	98.7
18	17/03/09	G	2	8	SAS pretreatment	166	100
		O	2	13-16		283	73.1
19	20/04/09	O	4	8	SAS pretreatment	320	98.1
20	11/05/09	O	4	4	Bud and SAS pretreatment	163	97.5
		O 4°C	4	9		370	98.1
		O Cryo	4	3		120	94.2
1-20	Total	/	4	495	/	5921	70.3

[a]*As genotypes 1443, 2599 and 2849 were available as dozens of potted plants (clones), some were established outdoors (O) in a nursery in soil containers during May 2007, whereas a second plant lot was put back in the greenhouse (G) in November 2007. Genotype 0136 was available as one tree planted outdoors (O), i.e., without any soil containers. O 4°C: buds collected at the time of experiment 18 (17/03/09) and stored at 4°C in dark (50 cm-long twigs in water + aluminum ethylphosphite; weekly sprayed with iprodione/vinchlozoline). O Cryo: buds collected in April (08/04/09) and immediately cryopreserved.*
[b]*In addition to genotype;* [c]*Sample size = total number of SAS investigated (nb of SAS x buds x genotypes).*

then surface-sterilized. Four methods were tested: (1) calcium hypochlorite: 90 g l^{-1} $Ca(OCl)_2$ with 70% active chlorine + 0.01% (v/v) wetting agent (home detergent) for 20 min (with stirring). Shoot buds were rinsed three times in large volumes of sterile water (exp. 1-7, 16, Table 3). (2) Hydrogen peroxide: H_2O_2 30% for 20 min

(with stirring). Shoot buds were rinsed three times in large volumes of sterile water (experiments 5-15, 17-18). In experiments 19-20, the H_2O_2 treatment lasted 60 min and was immediately followed by 70% (v/v) ethanol for 15 min before the three final rinses in sterile water. (3) Bleach/ethanol/$HgCl_2$: NaOCl containing 2.6% active chlorine + 0.01% (v/v) wetting agent for 5 min. Shoot apices were rinsed three times in a large volumes of sterile water, then immersed in 70% (v/v) ethanol for 5 min followed by 0.2% (w/v) $HgCl_2$ for 2 min. Shoot buds were finally rinsed four times in large volume of sterile water (experiment 2). (4) Sodium dichloro (iso) cyanurate: 5 g l^{-1} NaDCC + 0.01% (v/v) wetting agent for 2 min (with stirring). Shoot buds were rinsed two times in a large volume of sterile water. Bud scales were removed and shoot buds were further soaked for 1 min in 0.167 g l^{-1} NaDCC without rinsing (experiments 4-5).

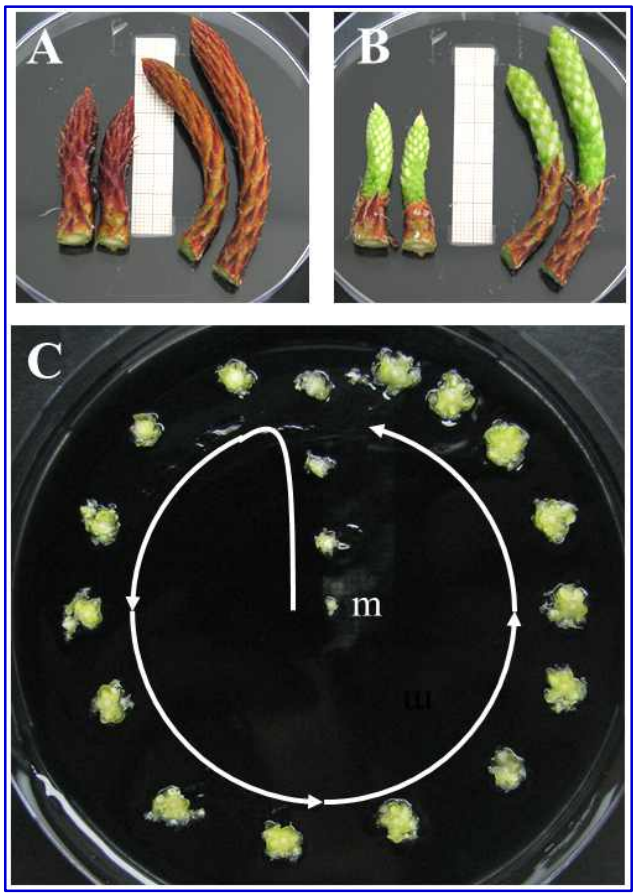

Figure 1. *Preparation of PS explants for initiation experiments in P. pinaster. Shoot buds were surface sterilized (**A**) and the bud scales were aseptically removed (**B**). Transverse PS apex slices (10-20 per shoot, 0.5-1 mm thick) were arranged in Petri dishes (**C**) from the top to the bottom of the PS apex (arrows). The first slice including the apical meristem is indicated (m).*

Transverse PS apex slices (SAS) were the final explants used in the initiation experiments with different medium formulations (Table 4). After removing the shoot bud sheath, the PS apex was transversely cut into slices (SAS) using sterile surgical blades #11 (slice thickness: 0.5-1 mm, exp. 1-8, 13-20) or razor blades (slice thickness ≤ 0.5 mm, experiments 9-12). An effort was made to cut thin slices (≤ 1 mm) because thick slices (1-2 mm) were reported to have a reduced SE initiation rate (Malabadi et al. 2004). A maximum of 10 (up to 20 in some experiments) transverse slices per PS, beginning from the top of the shoot bud (one slice including the apical meristem) to the subapical region (1-2 cm below the former) were cut. Slices were arranged on a culture medium according to their original position in the shoot bud (Figure 1). Overall (in 20 experiments, Table 3) and on average, we sampled about four shoot buds (up to 20) and 36 SAS per treatment (up to 164) from different shoot developmental stages (Figure 2), types and positions (Table 5).

Figure 2. *Developmental stages of one PS apex in P. pinaster monitored during 6 months from autumn 2006 to spring 2007.* ***D****: Dormant buds (arrested shoot growth); apical bud length ~ 10 cm.* ***S****: Bud swelling; terminal shoot length ~ 11 cm.* ***E****: elongating bud; terminal shoot length ~ 16 cm.* ***EF****: elongating shoots with female cones emergence (****EF1****, red arrows) and growth (****EF2****); terminal shoot length ~ 18 cm (EF1) or 27 cm (EF2).* ***NS****: secondary needles sprouting from elongated shoots; terminal shoot length ~ 34 cm.*

Of the different methods for disinfection of shoot buds, the H_2O_2 method consistently yielded the best results (68% of non-contaminated SAS) followed by NaOCl/ethanol/$HgCl_2$ (44%), $Ca(OCl)_2$ (41%) and NaDCC (34%). Removing the scales prior to disinfection did not improve the sterility of SAS, while explant viability was significantly reduced. In individual shoot buds from the same clone, the sterility varied from 0 to 100% suggesting that most (if not all) contaminants were endophytes. Subsequently, the H_2O_2 method was used in experiments 8 to 18 (excluding experiment 16) and the overall sterility of SAS reached 68% (N = 3733). Significant differences were found among genotypes 2599 (51%, N = 910), 2849 (60%, N = 1250), 1443 (80%, N = 1148) and 0136 (97%, N = 425). The sterility of the cultures was also very good in experiments 19-20 using a H_2O_2 modified protocol (97%, N = 973). Large fluctuations were observed among collection dates (Table 3), from 31% in experiment 10 (06/11/07), 86% in experiments 11 (22/01/08) and 15 (12/05/08), and 98% in experiments 17 (04/07/08) or 19 (20/04/09), suggesting that the endophytes within a plant are highly variable and poorly controllable by pesticides. It was clear however from experiments 11 to 15 and 18 (clones 1443, 2599 and 2849) that potted plants in the greenhouse yielded more sterile explants (87%, N = 1207) than the plants established outdoors in soil containers (only 56%, N = 1259).

2.3 Initiation of cultures from PS explants

The PS explants (SAS) were subjected to two initiation procedures: IP1 (experiments 1-18) and IP2 (experiments 19-20). In both IP1 and IP2 the standard cold pre-treatment of SAS was for 3 days in the dark at 2°C or at about 4°C (experiments 18-20) on PM medium. In one IP1 experiment we also tested 4°C for 17 days, 23°C for 3 days or no pre-treatment (experiment 18). In two IP2 experiments we investigated whether high *versus* low temperature pre-treatment could be beneficial for maritime pine using the following temperature sequences: 4°C for 1 day, 40-53°C (heat shock) for 4 hours, 4°C for 2 days (experiments 19-20), 23°C for 1 day, 40°C for 4 hours, 23°C for 2 days (experiment 19) or 40°C for 3 days (experiment 20). After pre-treatment, the PS sections were incubated on the initiation medium (IM) at 25 ± 2°C in the dark for 4 weeks or until soft, translucent to whitish mucilaginous tissue was detected (no subcultures were carried out). This potentially EM was then separated from the surrounding tissue and subcultured biweekly onto maintenance medium (MM) at 25 ± 2°C in the dark. PS explants were cultured in Petri dishes (9 x 1.5 cm) containing 23.5 ml medium and sealed with two layers (experiments 1-12) or only one layer (experiments 13-20) of cling film.

The recipes for pre-treatment (PM), initiation (IM) and maintenance media (MM) used in IP1 and IP2 are listed in Table 4. In IP1, the formulation is mDCR

including full-strength macronutrients, micronutrients and "vitamins" (meso-inositol, nicotinic acid, pyridoxine and thiamine hydrochloride) from Gupta and Durzan (1985) with the modifications proposed by Malabadi and van Staden (2005a). We also tested a modified Litvay (mLV) medium (Litvay *et al.* 1985) with modifications as above. Compared with DCR, Litvay-based media were found best-suited for initiation of SE from immature zygotic embryos in maritime pine (Park et al. 2006, Trontin et al. 2009, see also Trontin et al., Chapter on Maritime pine in this book). Unless specified otherwise, in the text and in Table 5 (mDCR2,3,4), all media were supplemented with vitamins Gupta and Durzan (1985) or Litvay et al. (1985) to obtain mDCR1 or mLV1 recipes, respectively (Table 4). In IP2, substantial modifications of the original mDCR1 recipe were made to obtain mDCR5 (Table 4). The main change was the high calcium content in mDCR5 (2.5 g l^{-1} $CaCl_2$ x 6 H_20) compared with mDCR1 (0.085 g l^{-1}). High calcium was found to mediate cold-enhanced SE in *P. patula* (Malabadi and van Staden 2006).

Table 4. *Composition of the pre-treatment (PM), initiation (IM) and maintenance media (MM) for SE initiation in P. pinaster (standard IP1 and IP2 procedures, this study).*

Basal medium	mDCR[a]						mLV[b]		
Basic procedure (medium)	IP1 (mDCR1)			IP2 (mDCR5)			IP1 (mLV1)		
Medium	PM	IM	MM	PM	IM	MM	PM	IM	MM
Macronutrients	DCR 1X			DCR 1X with 2.5 g l^{-1} $CaCl_2$, 2 H_20			LV 0.5X		
Micronutrients	DCR 1X			DCR 1X without $NiCl_2$, 6 H_20			LV 1X		
Vitamins[c]	DCR 1X			DCR 1X			LV 10X		
Meso-inositol (g l^{-1})	0.2	1	1	/	1	1	0.2	1	1
PVP-40 (g l^{-1})	0.2	0.2	/	0.2	0.2	0.2	0.2	0.2	/
Activated charcoal (g l^{-1})[d]	3.0	/	/	3.0	/	/	3.0	/	/
Casein hydrolysate (g l^{-1})	/	1	1	1	1	1	/	1	1
Maltose (mM)	90	90	120	83.3	83.3	83.3	90	90	120
2,4-D (µM)	/	20	2	/	22.6	2.3	/	20	2
NAA (µM)	/	25	2.5	/	26.8	2.7	/	25	2.5
BA (µM)	/	9	1	/	8.9	0.9	/	9	1
pH[e]	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Phytagel (g l^{-1})	1.5	1.5	2	2	2	2	1.5	1.5	2
L-Glutamine (g l^{-1})[f]	/	1	1	1	1	1	/	1	1
Gel strength (N)[g]	0.20	0.29	0.34	0.12	0.09	0.08	0.17	0.22	0.26

[a]Gupta and Durzan (1985); [b]Litvay *et al.* (1985); [c]Original vitamins excluding myo-inositol; [d]Purchased from Sigma Ca. N°C6289; [e]Medium pH was adjusted to 5.8 before gellan gum was added and autoclaving (121°C, 15 min, 1.05 kg cm^{-2}). [f]pH adjusted to 5.8, filter-sterilized and added after autoclaving to the cooled medium (50°C for IP1, 60-65°C for IP2). [g]Mean gel strength (in Newton) measured 24 h after autoclaving and pouring into Petri dishes.

Other major changes included nickel deprivation in all media, no myo-inositol in PM, PVP-40 added to MM, PM supplemented with casein hydrolysate and glutamine, higher maltose content in MM, higher gellan gum in PM and IM.

The gel strength of media was much lower in this IP2 protocol, around 0.10 Newton (N) versus 0.20-0.34 N, owing to the 29-fold higher calcium concentration.

2.4 Generalized morphogenetic response of SAS to the initiation protocols (IP1 and IP2).

Table 5. *Mean number of white tissues (WT) produced per SAS explant of P. pinaster after 4-week culture on IM medium.*

Exp.	Shoot stage[a]	Shoot type[b]	SAS position[c]	Mineral base[d]	SAS pretreatment on PM (temperature, duration)	N[e]	Necrotic SAS (%)[f]	Reactive SAS (%)[f]	WT
1	E	S	A+SA	mDCR1	2°C 3 d	22	0.0	95.5	0.45
		L				20	0.0	95.0	0.60
2	EF2	L	A	mDCR1	2°C 3 d	54	27.8	96.3	0.52
3	EF2	LT	A	mDCR1	2°C 3 d	43	0.0	100	0.84
		LA				32	0.0	93.7	0.87
4	NS	L	A	mDCR1	2°C 3 d	89	0.0	100	0.73
5	D	L	A	mDCR1	2°C 3 d	49	6.1	93.9	0.94
6	S	L	A	mDCR1	2°C 3 d	56	0.0	100	1.00
7	E	L	A	mDCR1	2°C 3 d	130	0.0	100	0.91
8	NS	L	A	mDCR1	2°C 3 d	67	3.0	97.0	0.82
			SA			49	32.7	81.6	0.29
9	D	L	A	mDCR1	2°C 3 d	104	1.0	91.3	0.81
10	D	L	A	mDCR1	2°C 3 d	50	10.0	76.0	0.58
11	S	L	A+SA	mDCR1	2°C 3 d	86	3.5	96.5	0.72
				mLV1		83	22.9	69.9	0.58
	D			mDCR1		98	8.2	89.8	0.57
				mLV1		117	73.5	18.8	0.13
12	S	L	A	mDCR1	2°C 3 d	94	8.5	83.0	0.64
				mLV1		64	29.7	57.8	0.48
	D			mDCR1		75	0.0	92.0	0.55
				mLV1		81	70.4	23.5	0.10
13	E	L	A+SA	mDCR1	2°C 3 d	111	10.8	85.6	0.59
				mLV1		94	24.5	78.7	0.63
	D			mDCR1		104	5.8	94.2	0.80
				mLV1		82	29.3	73.2	0.61
14	EF2	L	A+SA	mDCR1	2°C 3 d	116	0.0	100	0.33
				mLV1		93	25.8	69.9	0.26
	S			mDCR1		90	3.3	96.7	0.92
				mLV1		86	60.5	34.9	0.23
15	NS	L	A	mDCR1	2°C 3 d	72	1.4	98.6	0.28
				mDCR2		72	87.5	1.4	0.00
	EF2			mDCR1		83	1.2	98.8	0.30
				mDCR2		65	92.3	0.0	0.00
16	NS	S	A	mDCR1	2°C 3 d	81	1.2	96.3	1.14
				mDCR3		78	85.9	10.3	0.00
17	D	S	A	mDCR1		80	0.0	93.7	0.89
				mDCR4	2°C 3 d	79	0.0	91.1	1.03
18	E	S	A	mLV1	2-4°C 3 d	84	34.5	61.9	0.54
					2-4°C 17 d	82	84.7	13.4	0.01
	S	S	A	mLV1	2-4°C 3 d	100	53.0	51.0	0.26
					23°C 3 d	86	61.6	54.7	0.14
					No pre-treatment	21	71.4	38.1	0.29

19	E/EF1	S	A	mDCR5	2-4°C 3 d	79	84.8	11.4	0.00
					4°C 1 d, 40°C 4 h, 4°C 2 d	158	75.9	11.4	0.00
					23°C 1 d, 40°C 4 h, 23°C 2 d	77	83.1	16.9	0.00
20	EF2	S	A	mDCR5	4°C 1 d, 53°C 4 h, 4°C 2 d	84	100	0.00	0.00
					4°C 3 d	75	4.0	6.7	0.00
	E cryo	S	A	mDCR5	4°C 1 d, 53°C 4 h, 4°C 2 d	80	100	0.00	0.00
					4°C 3 d	33	66.7	0.00	0.00
	S 4°C	S	A	mDCR5	4°C 1 d, 53°C 4 h, 4°C 2 d	122	100	3.3	0.00
					4°C 3 d	118	5.1	5.1	0.00
					40°C 3 d	123	100	0.00	0.00
1-20	/	/	/	/	/	4171	35.7	57.9	0.41

[a]Shoot developmental stages defined in **Fig. 2**.
[b]S = Short shoot (≤ 5 cm in length); L = Long shoot (≥ 10 cm in length); T = Terminal shoot; A = Axillary shoot.
[c]A: apical zone (< 10 mm from meristem); SA: subapical zone (10-20 mm from meristem).
[d]mDCR1 standard procedure and media (IP1, **Table 4)** ; mDCR2: explants were individually cultivated in glass tubes (25 x 200 mm; medium volume = 20 ml) instead of Petri dishes with some modification of PM (no vitamins and meso-inositol; gel strength = 0.02 N) and IM media (no maltose; casein hydrolysate was filter-sterilized and added after autoclaving; gel strength = 0.17 N); mDCR3: mDCR standard procedure but without maltose added to IM ; mDCR4: mDCR standard procedure with 10 g l^{-1} maltose added to IM; mDCR5: mDCR1 modified procedure (IP2, **Table 4**).
[e]N: sample size (decontaminated SAS, see **Table 3**).
[f]Evaluated after 2 weeks on IM medium. Necrotic SAS: black localized areas or affecting the whole explant are observed. SAS are reactive when swelling, healthy tissues are observed at least in some parts of the explant (**Fig. 3B, 3D**). Note that reactive SAS could also be classified as necrotic in the case of localized necrosis.
Notes: SAS thickness is 0.5-1 mm except during experiments 9-12 (< 0.5 mm); Petri dishes were sealed with 2 (experiment 1-12) or only 1 ring (experiment 13-20) of cling film.

In the case of IP1 (mDCR1 or mLV1 media), the SAS integrity appeared to be largely preserved after cold pre-treatment on PM medium (Table 4) for 3 days at 2°C and incubation for 1-5 days on IM medium (Figure 3A). The whole structural organization of the sections remained recognizable with an epidermis, a broad cortex parenchyma containing resin ducts and a ring of yellowish vascular bundles surrounding the whitish pith. Some enlargement and elongation of young wounded needle fascicles or primordia surrounding the SAS usually started after 6-9 days on IM medium. We also observed some translucent tissues mainly located in the cortex but not in the central pith (Figure 3D). At the same time, some SAS became yellowish to brownish in colour, which started in the central parenchyma pith zone (Figure 3B) eventually affecting other tissues within only 1 week (Figure 3C). In other cases, a white, often translucent and soft tissue (WT) morphologically resembling EM was growing out from both SAS edges (needle fascicles) and cortical zone. The WT could rapidly, within 1-2 weeks, cover the entire SAS surface (Figure 3E) and/or more frequently underwent localized profusion, especially at the contact site between the explant and the gelled IM medium (Figure 3F, arrows). Some signs of likely oxidative stress (browning) were already detectable at this step and WT usually turned hard and entirely brown within 4 weeks after the transfer to MM medium (Figure 3G). Some WT reappearance (usually hard tissue) was consistently observed after biweekly subculture of

browning calli for 6 weeks (Figure 3F) but, again, sustained proliferation of these cells could not be achieved. In IP2, most explants turned brown (Figure 3B, 3C) and finally died within 2-3 weeks following either low or high temperature pre-treatment. None or very discrete WT production could be observed. The high calcium content of mDCR5 (2.5 g l^{-1} $CaCl_2$, $2xH_2O$) was most likely toxic to maritime pine.

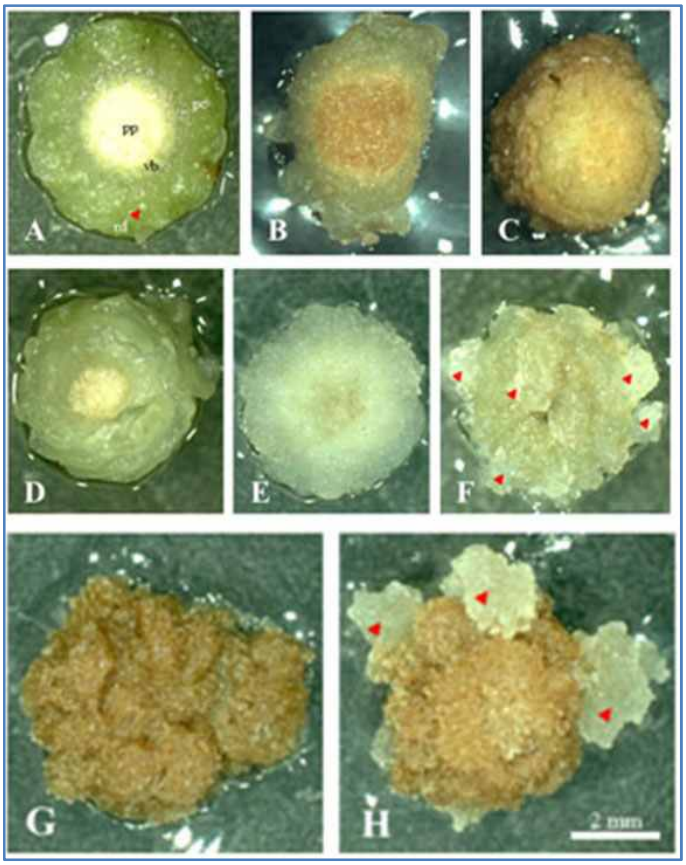

Figure 3. *General morphogenetic response of SAS from P. pinaster subjected to the initiation procedure (see Table 1 and Table 4) after 1-5 (**A**), 6-9 (**B, D**) or 10-14 days (**C, E**) on initiation medium (IM), and 1-2 (**F,** note the localized profusion of whitish soft tissue, arrows), 3-4 (**G**) or 5-6 more weeks (**H,** note the resurgence of white hard tissue, arrows) on maintenance medium (MM). **e**: epidermis; **pc**: broad parenchymatous cortex; **pp**: parenchymatous pith; **rd**: resin ducts (arranged in a ring in the cortex, arrow); **vb**: vascular bundles arranged in a ring around the pith.*

2.5 Factors affecting production of white tissue (WT) in IP1

Culture medium - The occurrence of WT was scored 4 weeks after SAS transfer onto IM (Table 5). The medium (mDCR, mLV) strongly affected SAS

response and necrosis, and ultimately WT production. Medium mDCR1 (N = 774) consistently supported a high SAS response (92.2%), low necrosis (5.2%) and a high WT production rate per SAS (0.63). By comparison, only 52.1% SAS were reactive using mLV1 (N = 700), with 43.4% explants showing large necrotic areas and a low mean of 0.36 WT per SAS. This medium interacted with the genotype. On mDCR1, a similar SAS response (91.4-94.6%) and necrosis (3.8-5.9%) were obtained and quite low differences in WT production rate per SAS (0.57-0.76) were detected among genotypes (Table 6). Conversely, higher variability was observed using mLV1. Genotypes 2599 and 0136 were poorly responsive (15.9-36.7%), highly necrotic (51.1-79.9%) and sporadic WT producers (0.07-0.09). Genotype 1443 was moderately responsive (51.2% response; 46.8 % necrosis) with low WT (0.36) produced per SAS (as compared with 0.74 on mDCR1). Only genotype 2849 showed a similar and quite high WT production rate on both mLV1 (0.64) and mDCR1 (0.67) but with slightly decreased responsiveness (81.7 vs. 94.5%) and increased necrosis (16.5 vs. 5.9%).

In experiment 15, we compared mDCR1 medium and procedure (Table 4) with a new DCR method (mDCR2, see Table 5) considered to be experimentally closer to the procedure described by Malabadi and collaborators (Table 1). Surprisingly, most decontaminated explants (89.8%) died within 2 weeks on IM

Table 6. *Mean number of white tissues (WT) produced per SAS explant in P. pinaster as influenced by the medium (mLV1, mDCR1) and genotype.*

Genotype	1443		2599		2849		0136	
Basal medium	mDCR1	mLV1	mDCR1	mLV1	mDCR1	mLV1	mDCR1	mLV1
N*	755	346	387	90	492	284	221	164
Necrotic SAS (%)	3.8	46.8	4.6	51.1	5.9	16.5	4.1	79.9
Reactive SAS (%)	94.2	51.2	94.6	36.7	94.5	81.7	91.4	15.9
WT	0.74	0.36	0.57	0.07	0.67	0.64	0.76	0.09

*N: sample size (decontaminated SAS, see **Table 3**), experiments 1-18, cold pre-treatment 2-4°C 3 days.

medium (experiment 15, Table 5). As IM was not supplemented with maltose in mDCR2 (in contrast with PM medium), we hypothesized that the use of such a carbon source during both pre-treatment and initiation was critical for SAS responsiveness. General SAS necrosis could also result from the very low gel strength of mDCR2 PM medium in glass tubes (0.02 N) compared with mDCR1 PM medium in Petri dishes (0.20 N). In experiment 16, we thus tested the standard mDCR1 in Petri dishes (Table 1) with (mDCR1) or without maltose (mDCR3) added to IM (Table 5). The very low survival rate of SAS on IM medium, when deprived of maltose, was confirmed (85.9% vs. 1.2% necrosis). SAS from the top position were much more affected by necrosis than SAS from a more basal position. SAS response was also significantly reduced (10.3% vs. 96.3%) and no WT

production was observed on mDCR3, as compared with 1.14 for mDCR1 (data not shown). We thus concluded that IM must be supplemented with maltose (32.4 g l^{-1}) to prevent necrosis and to achieve a high SAS response and WT production. By reducing the maltose concentration from 32.4 g l^{-1} (mDCR1) to 10 g l^{-1} (mDCR4) in IM medium (experiment 17, Table 5) we obtained a very similar SAS response (93.7 vs. 91.1% with no necrosis) and WT production rate (0.89 vs. 1.03) after 4 weeks induction. However WT induction and growth was much more discrete on mDCR4 than on mDCR1 and sometimes originated from the central part of the SAS (vascular bundle and pith zones) instead of on the cortex parenchyma (data not shown). This is an interesting point because WT production in *P. patula*, *P. roxburghii* and *P. wallichiana* seems to exclusively arise from the vascular bundle/cambial cells of SAS (Malabadi and van Staden 2005a, Malabadi and Nataraja 2006, 2007b).

There are thus some troubling similarities between the SAS reaction figures obtained in *P. pinaster* with reduced maltose concentration and the SE initiation figures reported by Malabadi and collaborators. As already mentioned, there are some inconsistencies in IM medium formulation proposed by these authors, especially in maltose concentration (0-90 mM, Table 1). We concluded that a more detailed spatiotemporal study of WT induction from SAS as a function of maltose concentration in IM medium is required in maritime pine.

SAS position - In addition to the medium type and genotype, SAS position relative to the meristem was identified as another factor affecting WT production in *P. pinaster*. We consistently observed that the 4-5 first slices (including the meristem) were more prone to WT production. This was specifically tested during experiment 8 (Table 5) by comparing SAS located in the apical (< 10 mm) and subapical zones (10-20 mm below the meristem). Apical SAS produced more WT (0.82 per SAS) than the subapical slices (0.29), with the latter ones being also more necrotic in this experiment (32.7 vs. 3.0%) and less reactive (81.1 vs. 97.0%). Pooling the data from all the experiments (mDCR1 basal medium only) we calculated that SAS restricted to the apical region of the shoot (N = 1159) produced more WT (0.74) than SAS originating from both apical and subapical zones (0.61 N = 696).

SAS thickness - Although not specifically tested in this work, our results suggested that slice thickness affected WT production. Considering only the data collected under the most favourable conditions (mDCR1, SAS belonging to the apical zone), we found that very thin slices (< 0.5 mm thick, N = 323, experiments 9-12, Table 5) were less reactive (86.7 vs. 97.7%), slightly more necrotic (4.3 vs. 2.8%) and produced less WT (0.66 vs. 0.77) than slices 0.5-1 mm thick (N = 836).

Shoot developmental stage - The effect of shoots developmental stages (D, S, E, EF, NS Figure 2) on SAS responsiveness and necrosis and incidence of WT production were studied over the 18 IP1 collection dates from 9/03/2007 to

17/03/2009 (mDCR1 or mLV1 media, cold pre-treatment for 3 days at 2-4°C). Using the mDCR1 standard procedure (all genotypes) we obtained low necrosis (4.1-5.6%) and a high response (90.9-98.5%) of SAS (Figure 4A). We observed high WT occurrence (mean of 0.69/SAS) but with significant variation as a function of the shoot developmental stage (0.47-0.80, Figure 4A). Similar results (0.69-0.80 WT/SAS) were observed at the D (dormant buds), S (swelled buds), E (elongating buds) and NS (needle sprouting) stages with a maximum at the S stage (0.80). In contrast a strong decrease was observed at the time of elongating shoots with female cone growth (EF2, 0.47 WT/SAS), but without any clear correlation with SAS necrosis and response. Similar conclusions were drawn by analyzing the data from independent genotypes, especially 1443 (Figure 4A) and 2599 (data not shown). A slow decrease in WT production from stage D (0.76) to NS (0.60, Figure 4A) was however observed in the case of genotype 2849.

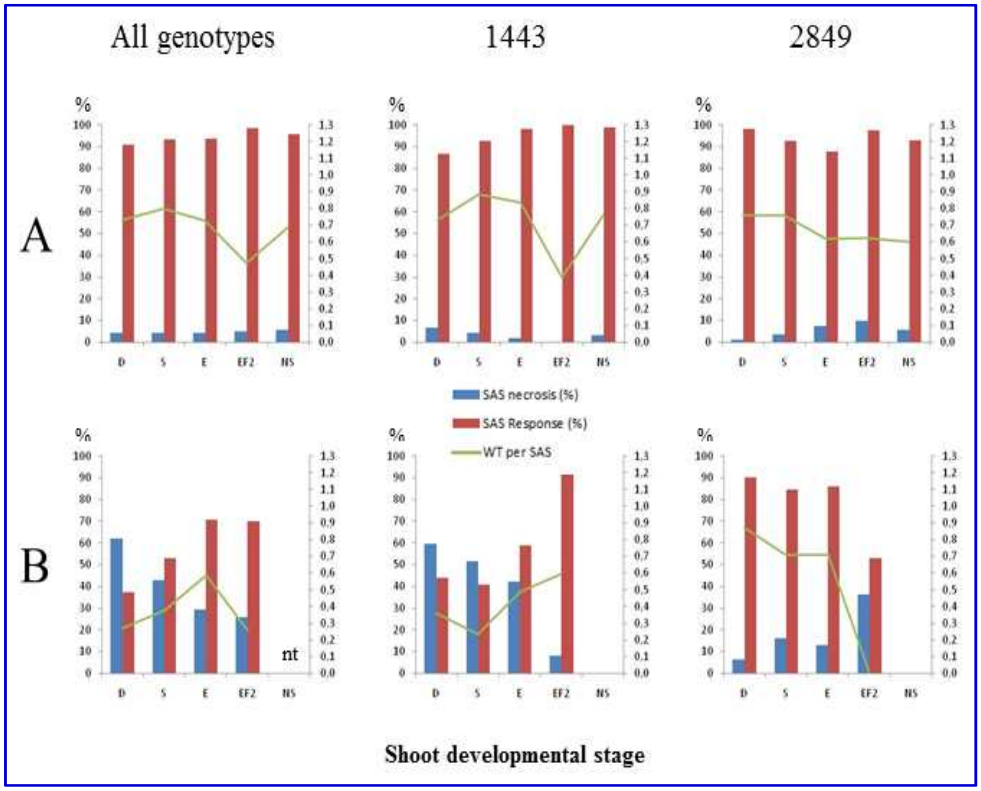

Figure 4*. SAS necrosis and responses (%, left vertical axis) and WT occurrence per explant (WT index, right vertical axis) in P. pinaster as a function of shoot developmental stage and culture medium.*
A. *mDCR1 medium;* ***B.*** *mLV1 medium. See* ***Table 4*** *for medium composition. Shoot developmental stages (D, S, E, EF2 and NS) are defined and illustrated in* ***Figure 2****. Data were computed from all genotypes or from the highly responsive genotypes 1443 and 2849. Nt: not tested.*

Different results were obtained using mLV1 (data from all genotypes, Figure 4B). The high necrosis rate of SAS observed on this medium decreased with advanced developmental stages, from 61.9% at D stage to 25.8% at the EF2 stage. The SAS response concomitantly increased from 37.4 to a mean of 70.5% at the E and EF2 stages. Interestingly, the WT occurrence increased with SAS response from the D (0.27) to E stages (0.58) before decreasing at the EF2 stage (0.26). This general trend was however not representative of individual genotypes (Figure 4B), especially responsive genotypes on mLV1 (1443, 2849). WT production was found to increase from D (0.36) and S stages (0.23) to EF2 (0.59) in genotype 1443 but a strong decrease was observed for genotype 2849 (from 0.87 to 0). In both cases a negative correlation with SAS necrosis and positive correlation with SAS response was observed. We finally concluded that WT occurrence was affected by the shoot developmental stage on both mDCR1 and mLV1 media. Early stages (D, S, E) produced better results in most genotypes in the case of mDCR1. In contrast, the SAS response to developmental stages on mLV1 appeared much more genotype-dependent with the optimal stages being D, S, E in 2849 but EF2 in 1443.

2.6 Microscopic observations

Potential EMs collected from explants on IM and/or MM media were stained with acetocarmine (1.5% w/v) directly on the glass slides for 3-4 min. Stained samples were gently rinsed with water and mounted (with cover slide) in a fructose syrup (150 g fructose + 100 ml water). Squashes were then incubated overnight at 37°C and sealed with two layers of nail varnish. A light microscope (Optiphot, Nikon, Kogaku, Japan) equipped with a digital camera (DX20N, Kappa opto-electronics, Gleichen, Germany) was used for observations and picture taking.

For cellular organization (CO) analysis of squashes, we used the nomenclature (five classes CO1 to CO5) defined by Breton et al. (2005) for maritime pine. Classes CO1 and CO2 refer to numerous, loosely aggregated, small and cytoplasmically dense cells with rare (CO1) or some occurrence (CO2) of clustering (less than 50 µm cluster size) and/or organized cell divisions. Class CO3 is characterized by large clusters of cytoplasmically dense cells (up to 100-200 µm in size) forming early EM (usually shapeless) with differential growth (somatic polyembryony). In this CO3 class, EMs were sometimes attached to unequally elongating, loosely aggregated vacuolated cells forming early secondary suspensors (embryonal tube). Such early embryogenic structure with a clear bipolarity, i.e., apically situated EM sustained by a well-organized (compact) secondary suspensor, are found in class CO4 (small embryoids with EM of about 100 µm in size) and in CO5 (well-developed embryoids with EM up to about 500 µm in size). In both CO4 and CO5 (late stages of early embryogeny) differential growth of regions of the embryoid EM is possibly observed (delayed cleavage

polyembryony). CO1 and CO2 cells and structures are frequently observed in CO3, CO4 and CO5. The occurrence of each CO class, early embryogenic structures and somatic embryos is largely affected by culture aging (Breton et al. 2005, 2006).

We were unable to promote sustained growth of soft WT resembling EM on either mDCR- or mLV-based IM or MM media (IP1 protocol) using various subculture methods and frequencies. Similar results were obtained with adult *P. pinaster* genotypes growing in Spain (Humánez et al. 2012). WT usually became yellowish to brownish shortly after initiation, i.e., within a few days regardless whether the tissue was kept attached (see Figure 3F-G) or removed from the explant. In the latter case, browning could sometimes be observed within a few minutes after excision suggesting a severe wounding stress response. As a result, soft WT rapidly ceased to grow and reversed towards hard, yellowish callus later producing hard WT with similar morphological progression (data not shown). No more than a few mm^3 of apparent volume growth could be obtained for most sampled soft WT. The non-destructive morphological observation of such a tiny amount of WT was ambiguous (Figure 5A) and micromorphological analysis was required to differentiate potential embryogenic from non-embryogenic tissue (Figure 5B). WT were thus usually sacrificed shortly after initiation (usually at the time of collection) to prepare squashes stained with acetocarmine. A total of 187 WT were analyzed from experiments 7 to 18 (IP1, cold pre-treatment for 3 days, mLV1 or mDCR1 media). As expected from the response and WT production data (Table 6, Figure 4) most squashed WT were from genotypes 1443 (40.1%, $8.7*10^{-2}$

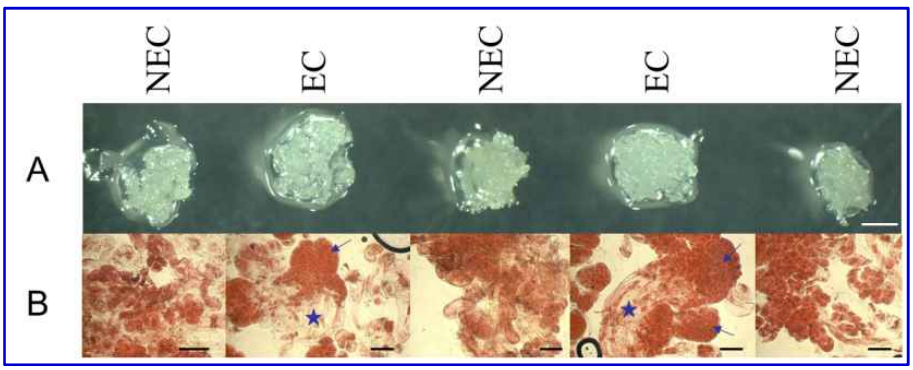

Figure 5. *Macro- (**A,** bar = 1 mm) and micromorphological (**B,** bar = 100 µm) observations of small amount of soft WT (a few mm^3, less than 10 mg) collected from embryogenic-like (EC) or non-embryogenic (NEC) cultures in P. pinaster.*
The macromorphological observation of small, soft WT initiated from SAS on IM medium was unsuitable to distinguish EC vs. NEC. Microscopic observations were required to detect EM (blue arrows) and secondary suspensor cells (embryonal tube, blue stars) forming early embryogenic structures in EC.

squashes/SAS) and 2849 (38.5%, $10.8*10^{-2}$ squashes/SAS). Genotypes 2599 and 0136 accounted for only 17.6% ($7.1*10^{-2}$ squashes/SAS) and 3.7% ($1.8*10^{-2}$ squashes/SAS) of slides, respectively. Most squashes (88.2%, 165 WT) only revealed quite hard, highly difficult to disaggregate clusters of cells. These compact tissues were usually accompanied by numerous loosely aggregated, cytoplasmically dense spherical (Figure 6, WT3) or elongated cells (Figure 6, WT4). We therefore concluded that the sampled tissue did not exhibit any advanced cellular organization of EM (CO3 to CO5 classes, Breton et al. 2005) and was best classified in this work as NEC. Interestingly, the cellular organization consisting of loosely aggregated (unorganized divisions), oval cells illustrated in Figure 6 (WT3) was similar to the CO1 class usually found largely interspersed with the CO3, CO4 and CO5 in EC. The frequency of CO1 increased with culture aging or in response to an unsuitable treatment (Breton et al. 2005).

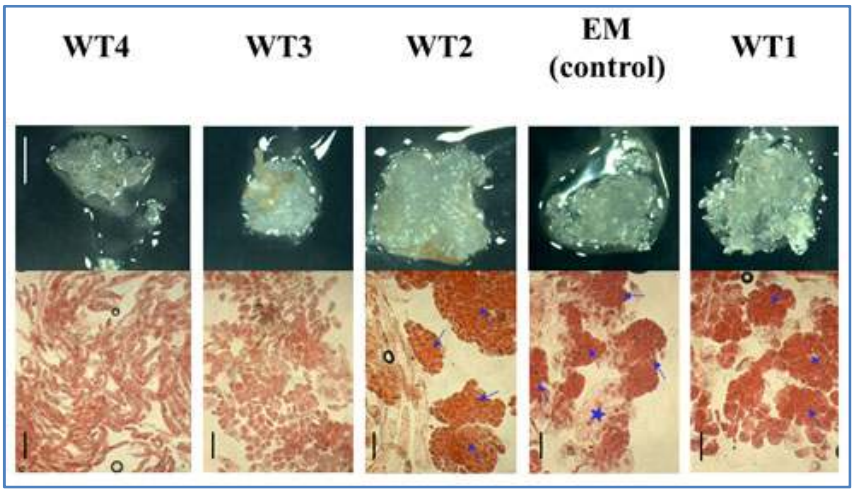

Figure 6. *Micromorphological observation (lower panel, bar = 100 µm) of several WT (upper panel, bar = 1 mm) initiated on SAS explants collected from mature P. pinaster trees compared with control EM. Compact aggregates of cytoplasmically dense cells (blue arrows) detected in some WT are very similar to early EM forming suspensor cells (blue stars) in embryogenic culture.*

In a significant number of squashes (11.8%, 22 WT), we detected (after about 4 weeks on IM medium) more advanced and/or intriguing structures with cellular organizations resembling the CO3, CO4 or CO5 classes found in embryogenic culture. Large clusters of cytoplasmically dense cells actively dividing in a cohesive way and forming compact cell aggregates (Figure 6, WT1, WT2) were observed. Usually shapeless but with apparent differential growth (e.g. WT2), those cell aggregates 100-500 µm in size were found very similar to early

EM undergoing somatic polyembryony in SE cultures from seed embryo (Figure 6) with CO3 cellular organization. Very similar figures were obtained from mature trees in *P. contorta* (Park et al. 2010, see *Figure 3c, d* in that paper) and *P. pinaster* (Humánez et al. 2012, see *Figure 8a, b* in that paper). Interestingly, elongating cells were usually (86.4%, 19 WT) found in the vicinity of such EM-like tissues, especially big clusters, and often (68.2%, 15 WT) in direct connection suggesting early embryonal tube formation. This hypothesis was further supported in a few squashes (22.7%, 5 WT) where more advanced and quite well-organized embryonal tubes were found connected to potential EM (Figure 7A, B, D). The

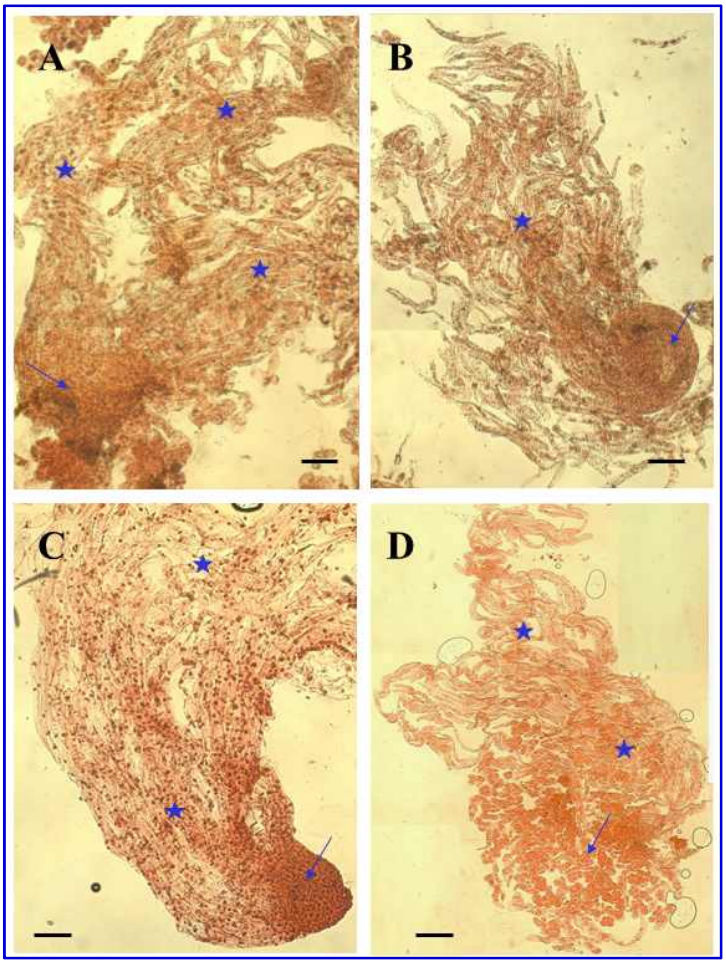

Figure 7. *Examples of EM-like structures (**A, B, D**) obtained from SAS of mature tree of P. pinaster (clone 1443, experiment 11, see Table 5). Typical well-developed early immature embryo in proliferating control embryogenic culture (clone PN519) is shown for comparison of both size and structure (**C**). Putative embryonal tubes cells (blue stars) were found connected to potential EM (blue arrows). Scale bars = 200 µm.*

resulting bipolar structures are similar to the early embryogenic structures observed in the CO4 and/or CO5 cellular organization of embryogenic culture both in size (200-500 μM) and micromorphology (Figure 7C). No differential growth of the putative EM region of such embryoids (delayed cleavage polyembryony) was observed in contrast to what is usually the case during the late development stages of early embryogenic structures. This is in close agreement with the very low growth ability of WT. We thus concluded that under our experimental conditions EMs were capable of some development towards the late stages of early embryogeny but were apparently unable to propagate through cleavage polyembryony. In contrast, some proliferation of early somatic embryos was obtained from mature trees of *P. sylvestris* (Aronen et al. 2009), as described below in this chapter.

The majority of EM-like structures were obtained from genotypes 1443 (68.2%, 15 WT) and 2849 (27.3%, 6 WT). In contrast, genotype 0136 did not produce any promising WT whereas only one was observed in 2599 (4.5%). EM-like tissues were initiated on both mLV1 (59.1%, 15 WT) and mDCR1 (40.9%, 9 WT) media but with apparently some interaction with genotype, with 2849 being more "productive" on mLV1 (5 out of 6 squashes) compared with 1443 (8 out of 15 squashes). Considering the developmental stage, no conclusions could be drawn because of the small sample size (N = 22). Most potential ET (77.3%, 17 WT) were initiated from SAS collected at the early stages (D/S/E, N = 862 SAS) rather than later stages (EF2/NS, N = 303 SAS). However, estimation of EM-like tissue occurrence per SAS (thus correcting for sample size) was similar at early ($1.9*10^{-2}$) and late developmental stages ($1.6*10^{-2}$).

3. *Pinus sylvestris* L. (Scots pine) - Aronen T, Ryynänen L

All the experiments described below were performed in 2008, 2009 and 2010 at the Finnish Forest Research Institute (METLA), Punkaharju Unit. On 01 January 2015, METLA became a part of the Natural Resources Institute Finland (LUKE).

3.1 Plant material

Two progeny trials of Scots pine (*Pinus sylvestris* L.) located at Punkaharju, Finland (61° 49' N; 29° 19' E) were used as a source of donor trees. Trial 1323/3 was planted in 1991 using 1-year-old seedlings, and trial 1801/7 in 1995 using 3-year-old seedlings, both with F1 progenies of selected plus trees. From the trial 1323/3 families 22/G01-86-0178 (trees A-F) and 36/G01-84-0071(trees A-F), and from the trial 1801/7 families 123/G04-86-0474 (trees 25-30), 136/G04-85-0392 (trees 31-36), and 141/G01-87-0418 (trees 37-42) were used for

SE initiations in 2008 when the donor trees were 18 or 16 years of age. In 2009, the same trees from trial 1801/7, now 17-year-old, were used. In 2010, only nine trees from trial 1801/7 (26, 28, 29, 34, 35, 36, 37, 38, and 39), now 18-year-old, were used.

In addition to the field-grown trees, somatic trees regenerated from the embryogenic lines 13, 51 and 76, all originating from open-pollinated seed embryos of donor K374, were used as explant source in 2009 and 2010. The somatic trees were 2 year-old in 2009, 3 year-old in 2010, and grew in pots in a nursery.

3.2 Shoot bud collection and surface sterilization

Tips of the branches with pre-flush shoot buds (Figure 8a) or flushing shoot buds (Figure 8b) were collected from the donor trees in the spring, when the temperature sum was between 35-180 d.d. (day degrees, i.e., the sum of daily average temperatures with a threshold of 5°C). Collections were performed once or twice a week, 5-10 collections per year (Table 7). Branch tips were brought immediately to the laboratory, where long needles were carefully removed. Then the shoot buds attached to a few centimeters long stem were immersed into 10% H_2O_2 overnight (approximately 16 h). Next morning the shoot tips were immersed in sterile water until being used for explant excision.

Surface-sterilization of the explants using H_2O_2 proved successful and explant contamination rates varied from 3.6 to 9.0% in different experiments.

3.3 Culture initiation

Bud scales of the surface-sterilized buds were first peeled aseptically. Then thin (approximately 1 mm) cross-sectioned slices (Figure 8c), beginning at approximately 1-2 mm below the bud tip, were cut and placed on the medium with the upper surface upwards. Each slice was considered as a separate explant, and numbered. A subgroup of the explants was subjected to heat shock at +37°C on different pre-treatment media for 3 days prior to placement onto initiation medium (Table 7). In 2008-2009, the explants were subcultured once from the initiation medium onto the same, fresh medium within 2 weeks of culture. In 2010, the explants were kept on the original initiation medium without subculturing. No cold pre-treatment was tested in Scots pine, since the temperature in Finland drops close to 0°C during the bud break period. Instead a heat shock of +37°C for 3 days was tested.

3.4 Subculture and proliferation of the induced tissues

Only the tissues considered potentially embryogenic, i.e., whitish or light in colour, translucent and soft, were separated from the explants and subcultured. In 2008-2009, the separated tissues were first placed on initiation medium and cultured on that for 4-8 weeks, with 2-week subculturing intervals.

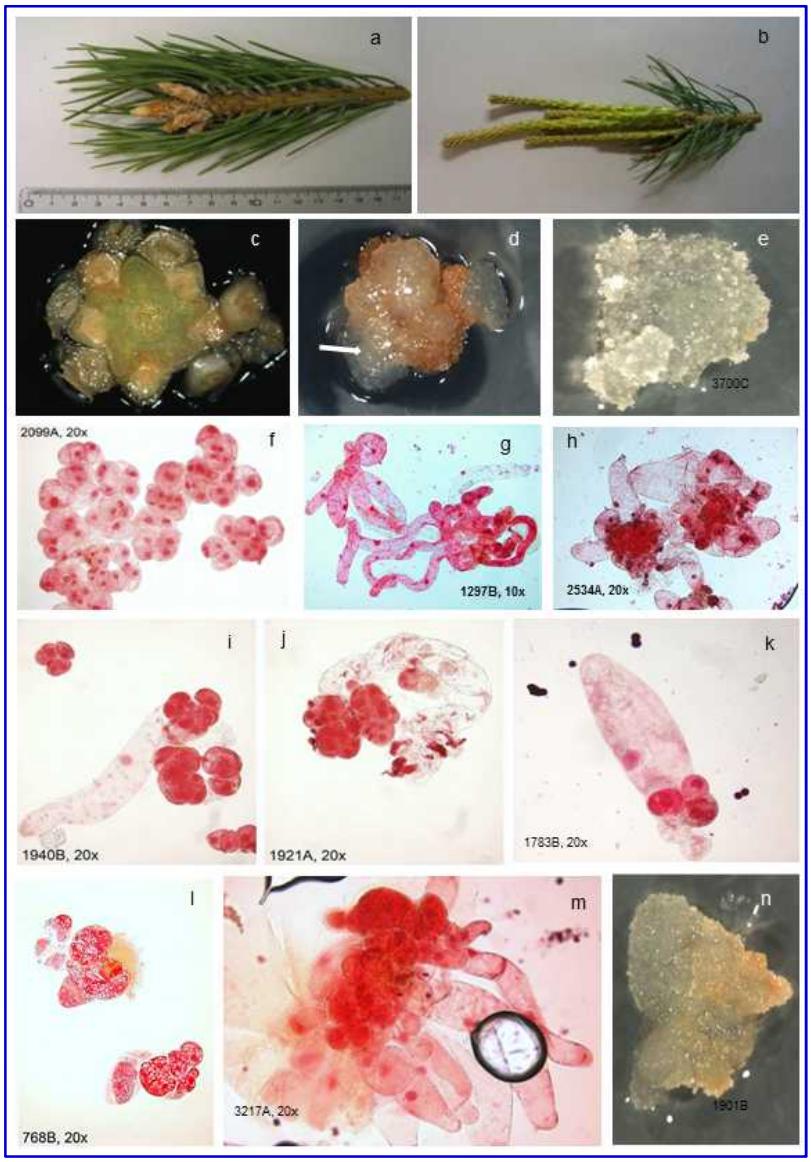

Figure 8. *Initiation of the cultures from PS explants in P. sylvestris. Developing buds (**a**) or flushing buds (**b**) collected between 35-180 d.d. were used as source of explants. Following surface-sterilization, thin cross-sectioned PS slices (**c**) were placed on an initiation medium, on which proliferation of both callus and potentially embryogenic tissue (arrow) was induced (**d**). Acetocarmine staining and microscopic examination of induced tissues that have been isolated as potentially embryogenic i.e., being soft,*

*white, and/or translucent (**e**) revealed ovoid cells (**f**), elongated cells (**g**), or cell clusters consisting of both small cells with dense cytoplasm and bigger cells having distinguishable nucleus and lightly stained cytoplasm (**h**). Often EM-like structures (**i, j, k**) are found in the same samples with callus cells. Presence of endophytes in the induced tissues is common and can be observed as brownish or orange growth (**l, m**), often resulting later in visible browning (**n**) and loss of the cultures.*

***Table 7.** Initiation treatments and responses of PS explants of P. sylvestris.*

Explants					**Initiation treatment**			**Response of explants**		
Year	Nb of families	Nb of genotypes	Age & type of donors	Nb and timing of collections	Nb of explants	Pretreatment [1]	Medium [1]	Number of isolated tissues (translucent, whitish, soft)	Potentially embryogenic, microscopy	Remaining cultures after 6 months (genotypes)
2008	5	30	15/17-year-old F1 progenies	10x, 50-178 dd	4500	none	MB2+Ca	253	56 / 9[2]	0
	5	30	15/17-year-old F1 progenies	10x, 50-178 dd	4500	3d +37°C, MB1	MB2+Ca	3185	1000 / 11[2]	5 (2)
					total 9000			**total 3438**	**20 (0.6 %[3])**	**0.1 %[3] 5 (2)**
2009	3	18	16-year-old F1 progenies	10x, 35-180 dd	900	none	MB2+Ca	900	16	2 (1)
	3	18	16-year-old F1 progenies	10x, 35-180 dd	900	3d +37°C, MB1	MB2+Ca	777	11	0
	3	18	16-year-old F1 progenies	10x, 35-180 dd	900	3d +37°C, MB1+Ca	MB2+Ca	744	13	4 (3)
	3	18	16-year-old F1 progenies	10x, 35-180 dd	900	none	mLV+Ca	695	2	1 (1)
	1	3	2-year-old emblings	10x, 35-180 dd	150	none	MB2+Ca	98	2	0
	1	3	2-year-old emblings	10x, 35-180 dd	150	3d +37°C, MB1	MB2+Ca	82	0	0
	1	3	2-year-old emblings	10x, 35-180 dd	150	3d +37°C, MB1+Ca	MB2+Ca	80	1	0
	1	3	2-year-old emblings	10x, 35-180 dd	150	none	mLV+Ca	115	0	0
					total 4200			**total 3491**	**45 (1.3 %[3])**	**0.2 %[3] 7 (4)**
2010	3	9	17-year-old F1 progenies	5x, 50-170 dd	450	none	MB2+Ca	267	15	1 (1)
	3	9	17-year-old F1 progenies	5x, 50-170 dd	450	3d +37°C, MB1+Ca	MB2+Ca	268	11	3 (2)
	3	9	17-year-old F1 progenies	5x, 50-170 dd	450	none	DCR	64	6	0
	3	9	17-year-old F1 progenies	5x, 50-170 dd	225	none	DCR+Ca	46	1	0
	1	3	3-year-old emblings	5x, 50-170 dd	150	none	MB2+Ca	99	1	1 (1)
	1	3	3-year-old emblings	5x, 50-170 dd	150	3d +37°C, MB1+Ca	MB2+Ca	77	14	1 (1)
	1	3	3-year-old emblings	5x, 50-170 dd	150	none	DCR	12	0	0
	1	3	3-year-old emblings	5x, 50-170 dd	75	none	DCR+Ca	19	0	0
					total 2100			**total 852**	**48 (5.6 %[3])**	**0.7 %[3] 6 (3)**

[1] Media used during pretreatment and initiation:

MB1 = Original DCR macro- and micronutrients and vitamins (Gupta and Durzan 1985), except myo-inositol 1 g l^{-1}; with added L-glutamine 1g l^{-1}, casein hydrolysate 1g l^{-1}, PVP 200 mg l^{-1}, activated charcoal 3 g l^{-1}, 90 mM maltose, no PGR; 2g l^{-1} gellan gum (Phytagel™) (Malabadi and van Staden 2003, 2005a,b)

MB1+Ca = MB1 with addition of extra 2.5g l^{-1} of $CaCl_2 \times H_2O$

MB2+Ca = Original DCR macro- and micronutrients and vitamins (Gupta and Durzan 1985), except myo-inositol 1 g l^{-1} with added L-glutamine 1g l^{-1}, casein hydrolysate 1g l^{-1}, PVP 200 mg l^{-1}, 90 mM maltose, 9µM BA, 20 µM 2,4-D, 25 µM NAA; 2g l^{-1} gellan gum (Malabadi and van Staden 2003, 2005a,b); with addition of extra 2.5g l^{-1} of CaCl2 x H2O

mLV+Ca = Litvay's medium (Litvay et al. 1985) modified according to Lelu-Walter et al. (2008) i.e. containing half-strength macroelements, 90 mM sucrose, 2.2 µM 2,4-D and 2.3 µM BA; 4g l^{-1} gellan gum with addition of extra 2.5g l^{-1} of CaCl2 x H2O

DCR = Original DCR macro- and micronutrients and vitamins (Gupta and Durzan 1985); with added L-glutamine 250 mg l^{-1}, casein hydrolysate 500 mg l^{-1}, 90 mM sucrose, 13.5 µM 2,4-D and 2.2 µM BA; 2.5 g l^{-1} gellan gum

DCR+Ca = DCR as above; with addition of extra 2.5g l^{-1} of CaCl2 x H2O

[2] Microscopic examination repeated; first sampling within a month from separation, second one 1-2 months later. Results from 1st / 2nd observations shown.

[3] Percent of initial number of explants

Afterwards and if still growing and considered potentially embryogenic, based on the acetocarmine staining and microscopic examination (Latutrie and Aronen (2013)), the cultures were transferred onto proliferation medium. In 2010, the separated tissues were placed directly onto proliferation medium. From the same explant, tissue could be isolated several times, and the cultures originating from the same explant were distinguished by adding the same letter to the line number. The first separations were done approximately 2-3 weeks from the onset of initiation, and the last ones over 3 months later.

Different proliferation media were used. In 2008, we used MB3 containing original DCR macro- and micronutrients and vitamins (Gupta and Durzan 1985), with added L-glutamine 250 mg l^{-1}, casein hydrolysate 500 mg l^{-1}, PVP 200 mg l^{-1}, 70 mM maltose, 1 µM BA, 2 µM 2,4-D, 2.5 µM NAA, and gelled with 2g l^{-1} of gellan gum (Phytagel™). MB3 was based on Malabadi and van Staden (2003, 2005 a,b) formulations but with various modifications (see Table 1). In 2009, the initiated tissues were subcultured onto MB2 medium (Table 7) and were proliferated on MB3 with the addition of extra 2.5g l^{-1} of $CaCl_2$ x H_2O for the first 4 weeks. The tissues initiated on mLV+Ca medium (see Table 7) were isolated and proliferated on mLV (Lelu-Walter et al. 2008) containing half-strength macrosalts, 90 mM sucrose, 2.2 µM 2,4-D and 2.3 µM BA, and gelled with 4g l^{-1} of gellan gum. Likewise, in 2010, MB3 was used as proliferation medium for tissues initiated on MB2+Ca. In the case of tissue initiated on DCR (see Table 7), proliferation medium was a DCR formulation (Gupta and Durzan 1985) supplemented with L-glutamine 250 mg l^{-1}, casein hydrolysate 500 mg l^{-1}, 90 mM sucrose, 9.1 µM 2,4-D and 2.2 µM BA and gelled with 2.5g l^{-1} of gellan gum.

The cultures remained on proliferation medium for over 6 months (Table 7); afterwards they were cryopreserved using slow-cooling in the PGDI (aqueous solution of 10% polyethylene glycol 6000, 10% glucose and 10% dimethylsulfoxide) cryoprotectant mixture (Latutrie and Aronen 2013), using MB3 medium supplemented with sucrose instead of maltose.

The results of the SE initiation experiments performed at the Finnish Forest Research Institute in 2008-2010 with bud explants is summarized in Table 7. Based on the visual examination, a huge number of EM-like tissues i.e., whitish, translucent and soft (Figure 8d), were separated and subcultured (Figure 8e). Visual discrimination of EM-like tissue from non-embryogenic tissue parts proved impossible, and most of the isolated tissues were found non-embryogenic (Figure 8f) when acetocarmine stained samples were examined under a microscope. There were, however, also tissues (a tiny percent of the examined ones, see Table 7) containing early EM-like structures with densely stained small, dividing cells and longer suspensor-like cells (Figure 8g-k).

The majority of the tissues considered potentially embryogenic degenerated during the proliferation stage. Most of them were from the start a

mixture of both potentially embryogenic cells and callus cells, and the latter contributed to the change in the morphology of the tissues to yellowish and hard. This took place also in old cultures in which predominantly callus cells were found in microscopic samples (Figure 9a, e.g. lines 1901B, 2382C). Another big problem was caused by endophytic contaminants, observed as orange-stained growth or film in the acetocarmine-stained samples (Figure 8 l, m). The cultures suffering severely

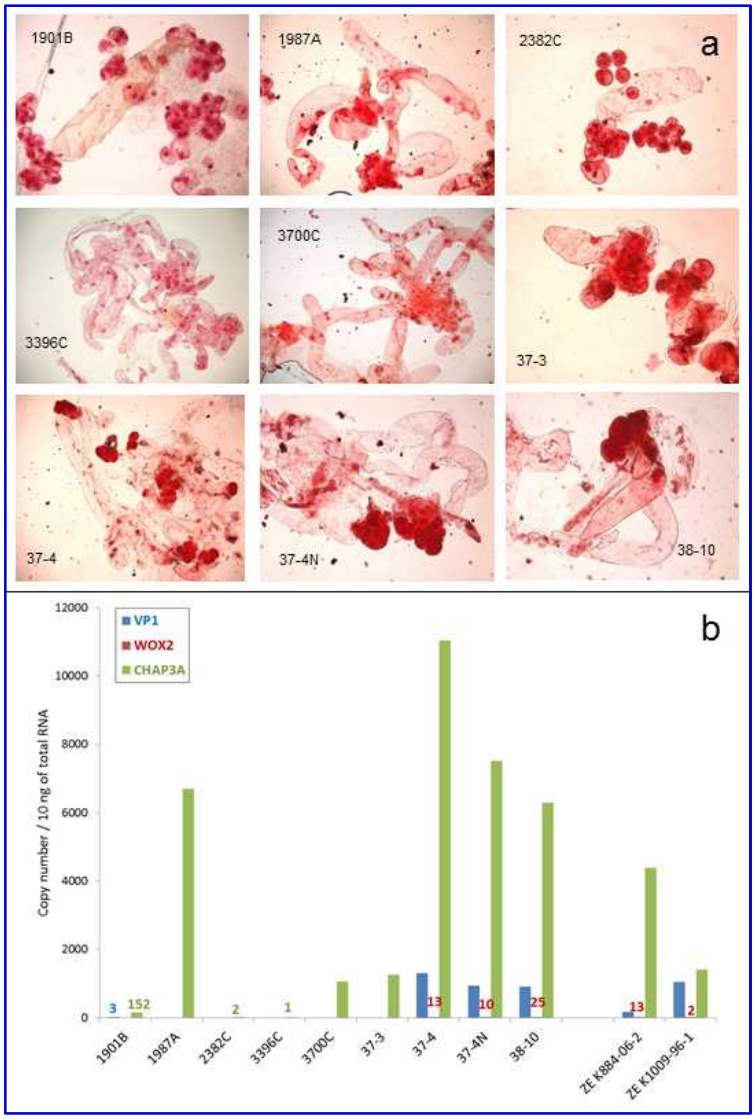

Figure 9. *Microscopic observations of acetocarmine stained samples (**a**) versus expression of the VP1, WOX2, and CHAP3A genes (**b**) in P. sylvestris lines of PS explant origin: 1901B, 1987A, 2382C, 3396C, 3700C, 37-3, 37-4, 37-4N, and 38-10 remained under long-term culture. For gene expression, results from the control lines of ZE origin, K884-06-2 and K1009-96-1, are shown.*

from endophytes remained soft in structure but gradually turned brown (Figure 8n) and ceased growing. However, in total 18 lines (Table 7) appeared embryogenic and these were subjected to the maturation treatment (see below).

The pre-treatment at +37°C, or the initiation media tested did not affect number of isolated tissue pieces (Table 7). However, when the remaining cultures after 6 months were examined, most of them were initiated from the pretreatment and MB2 medium supplemented with extra calcium. The majority of the remaining cultures originated from the PS belonging to one family, 141/G01-87-0418. The collection time (developmental stage of the shoot bud) did not affect the number of isolated tissue pieces, and the remaining cultures originated from the explants collected at various times, from 52 to 176 d.d.

3.5 Maturation and germination results

Maturation of the Scots pine lines initiated within the PS explants was started with methods developed in collaboration with INRA, and the procedure was based on Lelu-Walter et al. (2008). Prior to maturation, the cultures were proliferated on mLV as described above, with 200 mg l^{-1} PVP to prevent browning of the tissues. Tissue was then suspended in liquid maturation medium containing 10 g l^{-1} of activated charcoal but no PGR, and spread on a Whatman #2 filter paper placed on mLV maturation medium containing L-glutamine 500 mg l^{-1}, casein hydrolysate 1 g l^{-1}, myo-inositol 100 mg l^{-1}, 0.2 M sucrose, 80 µM ABA with 10 g l^{-1} of gellan gum or 120 µM ABA with 12 g l^{-1} of gellan gum. No subculturing took place during the 12-week maturation. Filter maturation on MB4 medium [original DCR macro-, micronutrients and vitamins, from Gupta and Durzan (1985), with added L-glutamine 250 mg l^{-1}, casein hydrolysate 500 mg l^{-1}, 0.18 M maltose, 80 µM ABA, gellan gum 9 g l^{-1}] was also tested with subcultures onto fresh medium every 2 weeks. Germination of the mature somatic embryos was performed on MB5 medium according to Aronen et al. (2009).

In the maturation tests, only two lines, 37-4N and 38-10, showed some kind of somatic embryo development **(**Figure 10**)** on mLV medium. The number of normal-looking somatic embryos was small; most of them turned brown before reaching the cotyledonary stage. On the germination medium, none of these somatic embryos developed a root, and only a few developed cotyledons. None of the produced somatic embryos survived.

3.6 Analysis of microsatellite loci in initiated lines

To study genetic fidelity of the induced lines, genomic DNA was extracted from the tissue cultures and from the buds of the corresponding donor trees using the modified method of Lodhi et al. (1994), as described in Valjakka et al. (2000), and the microsatellite loci were analyzed as described by Varis et al. (2008).

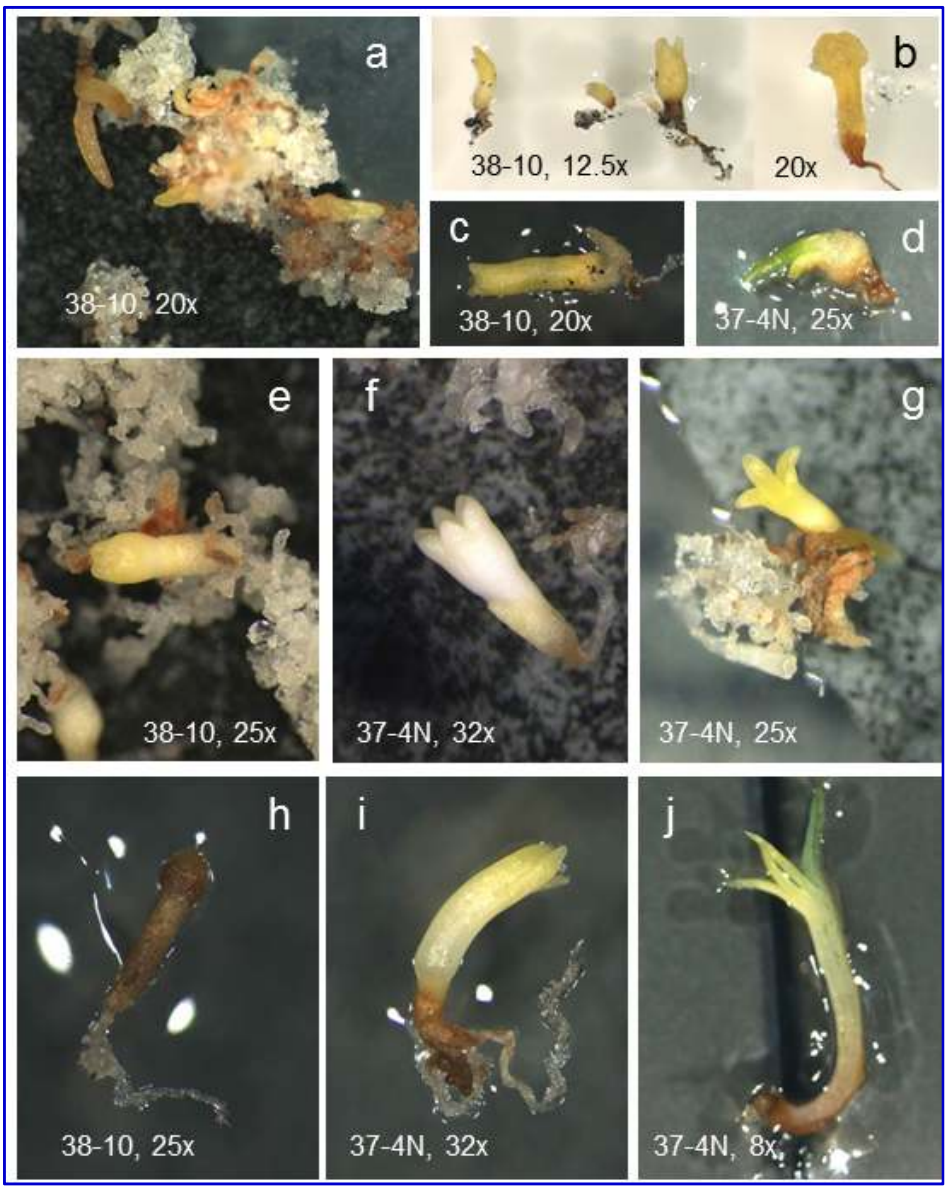

Figure 10. *Somatic embryo maturation in P. sylvestris lines originating from PS explants.* ***a)*** *Tissue spread on maturation medium often continued proliferation while development of somatic embryos was blocked and necrosis ensued. Photo taken after 11 weeks on maturation medium. Most of the developed somatic embryos were abnormal (**b, c, d**). Occasionally development of normal-looking somatic embryos was observed: photos taken after 8 weeks (**e, f**) or 11 weeks (**g**) on maturation medium. All the embryos, however, died following transfer onto germination medium: either without showing any further development (**h**), or after developing cotyledons but no root (**i, j**). Photos **h** and **i** taken after 3 weeks, and **j** after 1 month on germination medium.*

Table 8. *Alleles detected at three microsatellites loci in PS derived cell lines of P. sylvestris in comparison with the corresponding donor trees.*

	Microsatellite locus		
Genotype	S125	S714	P2146
Donor 29	174/197	204/204	181/220
Line 3217A	174/197	204/204	181/220
Line 3700C	174/197	204/204	181/220
Donor 36	163/163	184/214	166/220
Line 1901B	163/163	184/214	166/220
Line 2382C	163/163	184/214	166/220
Donor 37	147/166	202/228	209/235
Line 37-3	147/166	202/228	209/235
Line 37-4	147/166	202/228	209/235
Line 37-4N	**166**/166	202/**202**	**189**/235
Donor 38	147/147	190/228	195/220
Line 38-10	**166/166**	**205/205**	**189**/220
Line 3396C	147/147	190/228	**193**/220
Donor 40	147/147	204/229	203/220
Line 1987A	147/147	204/229	203/**203**
Line 2958A	147/147	204/229	**220**/220

Note: differences in allele size between initiated lines and donor tree are highlighted in bold.

Examination of three microsatellite loci in lines originating from PS explants showed some deviance from the donor trees (Table 8), especially in the lines that had shown some somatic embryo production ability (37-4N, 38-10), but also in other lines (3396C, 1987A, 2958A). In an earlier study, Burg et al. (2007) had shown variations of microsatellite markers (four loci) taking place both during zygotic and somatic embryogenesis of Scots pine, and found some families having higher mutation rates in tissue culture than in seed embryos. Interestingly, families with low genetic stability during establishment of embryogenic culture had higher maturation ability than those that were genetically more stable. In the present study, the pretreatment of the explants at +37°C, and a high concentration of calcium in the medium during initiation of the cultures could have imposed stress, hence potentially increasing cellular and genetic instability. This result may indicate some genetic plasticity of tested genotypes to cope with stressful culture condition.

3.7 Expression of embryogenesis-related genes in initiated lines

Expression of *VP1*, *WOX2*, and *CHAP3A* genes in nine proliferating cultures of PS origin and in two embryogenic lines of zygotic embryo origin was analyzed as described in Klimaszewska et al. (2011). These genes were chosen for their potential to be markers of embryogenicity (Klimaszewska et al. 2011, Park et al. 2010, Uddenberg et al. 2011). A variable level of *CHAP3A* expression was revealed in all the studied lines. The expression of both the *VP1* and *WOX2* genes, on the other hand, was observed not only in the lines of zygotic embryo origin, but also in three lines of PS origin (37-4, 37-4N, 38-10, Figure 9b). When samples of these three lines were observed under a microscope, the presence of early stage somatic embryos, although not well-structured, were detected (Figure 9a). Two of these three lines (37-4N, 38-10) also showed some kind of embryo maturation (Figure 10).

4. *Pinus sylvestris* L. (Scots pine) - Supplementary short note *(*Lelu-Walter MA)

4.1 Plant material

In 2007, PS buds were collected from one adult 17-year-old tree (818, INRA, Orleans, France) known to be responsive to SE induction from seed embryos in previous years (25% initiation rate from control pollinated seeds, the highest initiation frequency ever obtained for Scots pine). Pre-flush shoot buds were 3-5 cm in length and both apical and lateral shoot buds (16 in total) were pooled for the experiment.

4.2 Shoot bud disinfection and PS explant excision and culture

PS buds covered with scales were disinfected for 20 min in 0.5% $HgCl_2$ solution followed by stirring in $CaCl_2$ solution twice to neutralize the residual $HgCl_2$. Each $CaCl_2$ treatment lasted 10 min and then PS buds were rinsed three times, each for 10 min in sterile water. The scales were kept in order to minimize the effect of the sterilization agent on the shoot tissue (we assumed that these pre-flush elongated PS were aseptic). The scales and basal parts of the shoot buds were removed and transverse slices were cut from the entire PS under the binocular. The slices were positioned with the basal part on the medium, and the slices were marked from the base to the apex of a PS. The disinfection method proved very effective and no explant contamination was observed.

Two media were used for both pre-treatment and initiation: mDCR (Malabadi and van Staden 2005a, Table 1) and mLV (Lelu-Walter et al. 2008). The explants were pre-treated at 2 or 4°C for 3 days on the two pre-treatment media

(Table 1) and then cultured for 12 weeks on the initiation medium (Table 1) in darkness at 24°C.

4.3 PS explant responses

After the first 2 weeks, explants produced translucent calli appearing to arise from the cambium. There was also some cell proliferation at the base of the needle fascicles. After 1 month, the calli were still white but after two months they turned brown on mDCR, whereas on mLV the calli remained white. However, in spite of the explant necrosis on mDCR medium, the growth of white callus was observed, which upon microscopic observation did not display any EM characteristics. Callus growth was greater on mLV than on DCR. After 12 weeks of culture a total of 590 PS explants produced calli and remained alive without any noticeable trend regarding the length of the shoot buds. In order to promote proliferation of the white-translucent EM-like tissue, tiny pieces were isolated from the surrounding callus as soon as they were identified, and transferred onto nylon mesh placed over the maintenance medium (Table 1), a procedure developed to rescue EM of Scots pine (Lelu-Walter et al. 2008). However, the white calli that grew on the nylon mesh did not produce EM.

5. *Pinus radiata* D. Don (Monterey pine, radiata pine) and *Pinus patula* Schiede ex Schltdl. & Cham. (patula pine) - Hargreaves C, Reeves C

5.1 Plant material

Five shoot bud collections were made in 2007 at weekly intervals between early May and mid-June, from one *Pinus patula* tree and 20 *Pinus radiata* trees from the Long Mile Archive, at Scion in Rotorua, New Zealand. The *P. radiata* buds were from 10-20-year-old grafted clones and these buds had whorls of small, developing female cones. The buds were on average 5 cm long. The *P. patula* buds were from the lower crown of the approximately 15-year-old tree. The *P. patula* buds were approximately 3 cm long and with a smaller diameter than those of *P. radiata*. At collection time, the buds were near their strongest period of winter dormancy and the buds were enclosed in tightly packed brown scales. A further three collections were made of a subset of the *P. radiata* genotypes in August-September and this is detailed in the results and discussion section (see below).

5.2 Shoot bud disinfection, treatments and media

The buds were disinfected in commercial bleach 50:50 v/v (Chlorodux 5% sodium hypochlorite) plus surfactant (0.1 ml Silwet L-77.L–1), for 10-15 minutes.

The buds were then rinsed three times in sterile water, and the brown scales were peeled off aseptically, which was more difficult with the smaller *P. patula* shoot buds. Contamination was very low for these five collections (< 5%) and was likely to have been positively influenced by the tight sheath of scales on the bud material.

Transverse slices of the PS buds were then made and were about 1 to 2 mm thick. These PS explants were divided between a 2°C pre-treatment, a 4°C pre-treatment and a control pre-treatment (22°C), with larger numbers exposed to cold pre-treatments. The medium used for these pre-treatments was a modified Quoirin and Lepoivre medium (Quoirin and Lepoivre 1977; modification of Aitken-Christie et al. 1988) and included 5 g l^{-1} activated charcoal (Merck). Care was taken to ensure the PS slices were orientated in an upright position (small needle fascicle primordia can be seen on the bud slices and this also helps with regard to correct positioning on the medium). Following incubation, all explants were transferred to three initiation media: DCR (Gupta and Durzan 1985), EDM (Smith 1996) and Glitz (Litvay et al. 1985). The modifications to both EDM and Glitz are detailed in Hargreaves et al (2009). Cultures were incubated in the dark at 22°C and then assessed for callus formation.

In total there were 350, 680, and 301 *P. radiata* PS explants, whereas in *P. patula* there were 320, 160 and 170 explants cultured on DCR, EDM and Glitz media, respectively.

5.3 PS explant responses

Calli were obtained on all explants irrespective of species, genotype, pre-treatment and medium. It should be noted that both EDM and Glitz are extremely good media for SE in *P. radiata* (Hargreaves et al. 2009, 2011). The callus was generally glassy and friable and sometimes with a yellowish colouration. In the case of the smaller *P. patula* explants the calli formed all over the explant but with the *P. radiata* there were often two distinct areas of growth, one from around the outer edges of the explant, where the needle primordia were, and from the centre of the slice, in what is the early cambial tissue. We also observed translucent callus being produced from the edges of explants (needle fascicle primordial regions) that contain long "stringy" cells, visible after staining with acetocarmine. These cells looked like elongating epidermal cells, which of course may be exactly what they were. The bulk of the callus tissue tended to be a mixture of highly vacuolated cells, some quite elongated and a little like suspensor cells with some darker staining nucleic material and starch granules scattered through the cells. Isolated callus tissue grew with variable success on the three media irrespective of medium formulation but subsequent staining of this showed it all to be non-embryogenic. Further collections (3) were made at 3-week intervals with 10 genotypes rather than 20 of *P. radiata* starting six weeks after the shortest day in 2007 (August-

September). The elongation of the PS buds was rapid and the contamination of the explants increased from 13.4% to 52.6% for the last collection and probably can be attributed to the open nature of the buds at this stage (very elongated and only a few sparse brown scales at the very tip of the bud). Only two pre-treatments (2 ^{o}C and 4 ^{o}C) were tested with this material due to the results observed in the samples taken earlier in the year. The same initiation media were used (DCR, EDM and Glitz). From each genotype, 15 to 50 transverse PS slices were placed on each of the test media for each of the pre-treatments. Care was taken to observe the tissue every few days in case EM was appearing and being overgrown by the proliferating callus cells. As with the earlier work, callus growth was in general prolific and WT was easily isolated and in most cases continued to proliferate. However, in all cases microscopic examination of the WT revealed regions of large vacuolated cells resembling suspensor cells with some densely staining cells, perhaps meristematic in origin, but nothing that was convincingly embryogenic, or that on isolation and subsequent proliferation retained an 'interesting' morphology. We have to conclude from this work, somewhat surprisingly, that both our *P. patula* and *P. radiata* genotypes were not as responsive to similar treatments tested for *P. patula* by Malabadi and co-workers as previously discussed in this chapter.

6. *Pinus radiata* D. Don (Monterey pine, radiata pine) - Moncaleán P, Montalbán IA, Garcia-Mendiguren O

6. 1 Experiment 1

In 2008, seven 19-year-old trees were selected in the seed orchard established by Neiker-Tecnalia in Deba, Spain. Shoot buds (3–5 cm long) were taken from the mid-basal part of the trees. The buds were collected fortnightly from February 18 (Figure 11a) to April 29 (Figure 11b), wrapped in moist paper to prevent dehydration and stored in polyethylene bags at 4ºC for a maximum of a week.

Buds were sprayed with 70% (v/v) ethanol, and then rinsed with sterile distilled H_2O. Afterwards, the buds were submerged in 50% (v/v) commercial bleach (active chloride >5%) plus two drops of Tween 20® and agitated for 10 min. Finally, they were rinsed three times in sterile distilled H_2O in aseptic conditions. When possible bud scales were removed, explants were cut transversely into 1–1.5 mm thick slices with a surgical scalpel blade and were laid on the culture medium (Figure 11c).

On the first and the second collection dates (February 18 and March 3) bud slices were cultured on two initiation media. The first medium was embryo development medium (EDM) (Walter et al. 1994) with 30 g l^{-1} sucrose, 1 g l^{-1} inositol and a combination of 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and

2.7 µM BA. Before autoclaving, the pH of the medium was adjusted to 5.7 and then 3 g l^{-1} gellan gum (Gelrite®) was added. Medium was autoclaved at 121ºC for 20 min. After autoclaving, filter-sterilized solutions (pH = 5.7) of 550 mg l^{-1} L-glutamine, 525 mg l^{-1} asparagine, 175 mg l^{-1} arginine, 19.75 mg l^{-1} L-citrulline, 19 mg l^{-1} L-ornithine, 13.75 mg l^{-1} L-lysine, 10 mg l^{-1} L-alanine and 8.75 mg l^{-1} L-proline were added to the cooled medium.

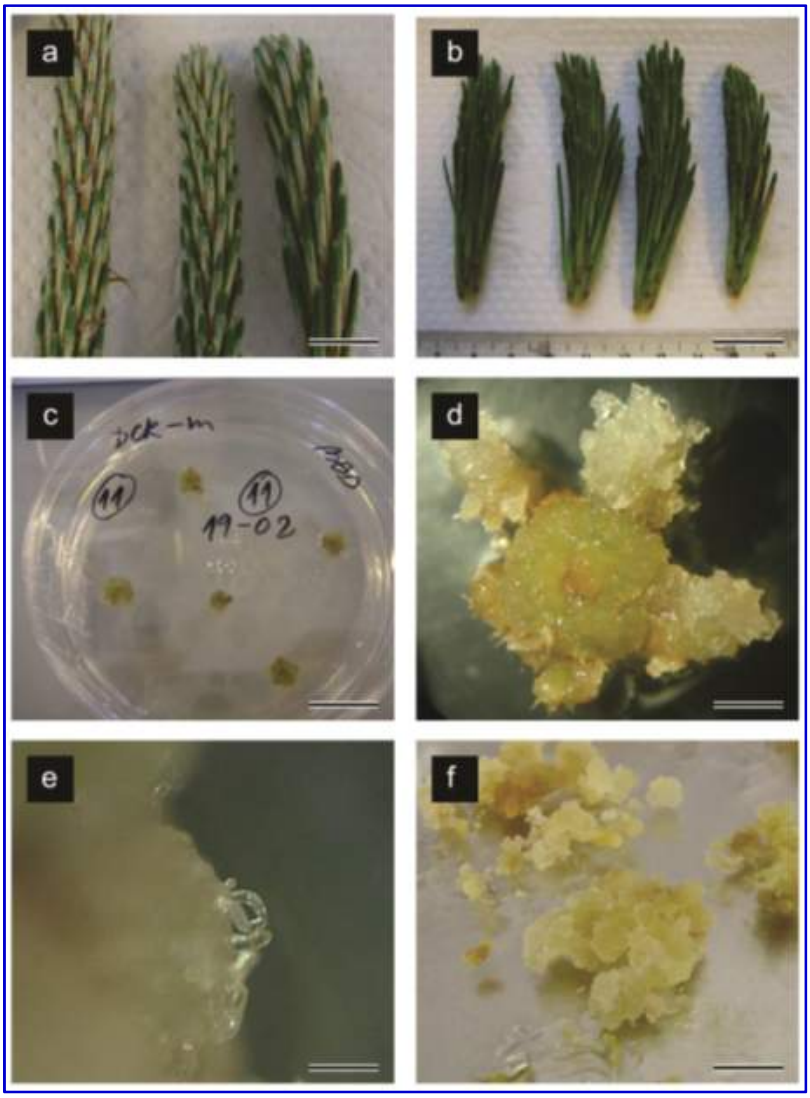

Figure 11. *Pinus radiata cultures initiated from PS explants (Experiment 1).* ***a*** *Shoot buds collected at the end of February (bar=12 mm).* ***b*** *Shoot buds collected at the beginning of April (bar=19 mm).* ***c*** *PS explants cultured on DCRI (February collection) (bar=14 mm).* ***d*** *PS explant cultured on DCRI for 3 weeks (bar=4 mm).* ***e*** *Elongated cells in the proliferating tissue (bar=1 mm).* ***f*** *Tissue proliferating on DCRM (bar=9 mm).*

The second medium was full-strength DCR medium (DCRI, Gupta and Durzan (1985) modified by Malabadi and Van Staden (2005a), see Table 1) containing 0.2 g l^{-1} polyvinylpyrrolidone-40 (PVP-40), 3.24 % (w/v) maltose, 1 g l^{-1} inositol and supplemented with 20 µM 2,4-D, 25 µM 1-naphthaleneacetic acid (NAA) and 9 µM BA. Before autoclaving, the pH of the medium was adjusted to 5.7 and then 1.5 g l^{-1} gellan gum was added. After autoclaving, filter-sterilized solutions (pH= 5.7) of 1 g l^{-1} casein hydrolysate and 1 g l^{-1} L-glutamine were added to the cooled medium prior to dispensing into gamma-irradiated Petri dishes (90 x 20 mm).

From the third to the sixth collection date (from March 17 to April 29) half of the PS explants were cultured on DCRI, and the other half were subjected to cold pre-treatment. This pre-treatment (see Table 1) consisted of culturing the explants at 4ºC on full-strength DCR medium containing 0.2 g l^{-1} PVP-40, 3.24 % (w/v) maltose, 0.3% (w/v) activated charcoal (AC), and 1.5 g l^{-1} gellan gum (Phytagel); after 3 days, these explants were subcultured on DCRI.

At each collection date, five PS slices per Petri dish were cultured; seven Petri dishes per treatment were laid out randomly on the shelves of the growth chamber. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks. Then the explants and/or the proliferating tissues were transferred to maintenance medium. Maintenance medium for explants cultured on EDM was the same as for initiation. Maintenance medium for explants cultured on DCR (DCRM) had the same basal composition but contained 4.32 % (w/v) maltose, 1 g l^{-1} inositol and was supplemented with 2 µM 2,4-D, 2.5 µM NAA and 1 µM BA. This formulation is similar to that of Malabadi et al. (2005a) (see Table 1). The amino acid mixture was the same as for initiation. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks on maintenance medium.

On the first and second collection dates and in all the genotypes tested, the PS slices cultured on EDM produced tissue from the margins (Figure 11d). These cell proliferations were formed by white-translucent tissue formed by elongated cells (embryogenic-like cells, Figure 11e) and a small population of round cells (callus cells). The explants cultured on DCRI showed the same growth of tissue but it grew less and more slowly than on slices cultured on EDM. When transferred to maintenance medium, the tissues continued proliferating rapidly but the population of round cells increased and the tissue became yellow-brown (Figure 11f).

In all the genotypes from the third to the sixth collection dates, the growing tissues on DCRI showed the same trend as described above for the initiation and maintenance. The cold pre-treatment did not have any effect on the responses of the PS slices and half of these slices had no cell proliferation and necrotized. The other explants, when transferred to DCRI, had cell proliferation in the needle primordia areas. Although this tissue was white-translucent and EM-like cells were

identified (Figure 11e), when proliferated on DCRM, the tissue became yellowish and after 1 month was predominantly composed of round cells (Figure 11f).

6.2 Experiment 2

Ten trees over 20 years old were selected from a seed orchard established by Neiker-Tecnalia in Amurrio, Spain. Apical shoot buds (3–5 cm long) were taken from the mid-basal part of the trees (Figure 12a). The shoot buds were collected fortnightly from December 2009 to January 2010. The buds were stored

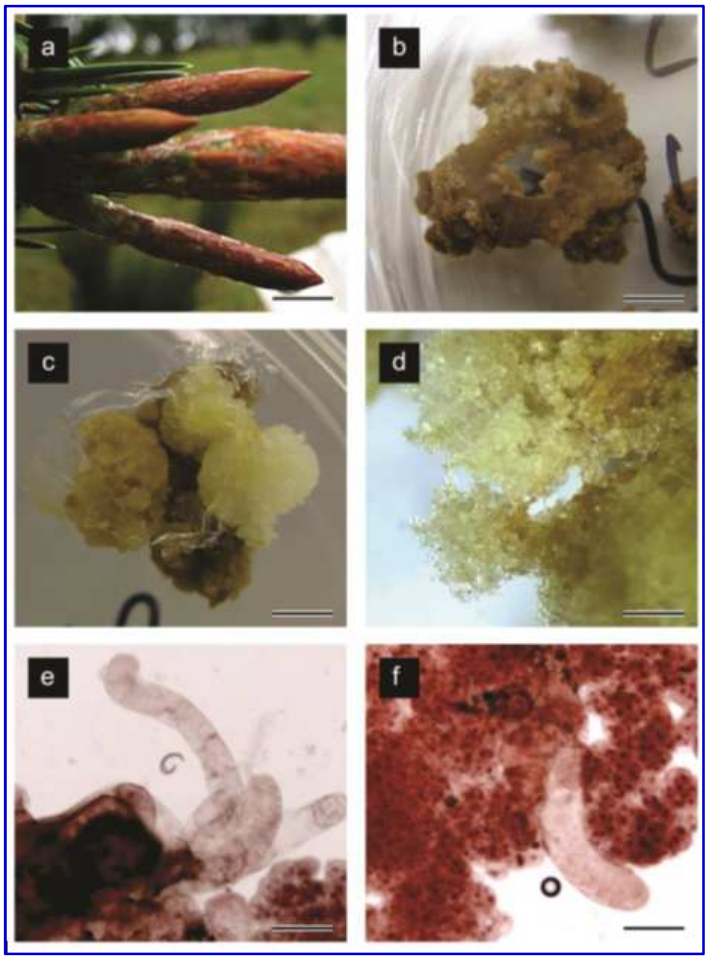

Figure 12. *Pinus radiata cultures initiated from PS explants (Experiment 2).* ***a*** *Shoot buds collected at the end of December (bar=17 mm).* ***b*** *PS slice (1.5 cm thick) cultured on EDM (bar=4 mm).* ***c*** *Tissue growing on 7 mm PS slices cultured on LPI medium (bar=4 mm).* ***d*** *Elongated and round cells in the proliferating tissue (bar=3 mm).* ***e*** *Micro-morphology of elongated cells in the proliferating tissue (bar=0.15 mm).* ***f*** *Micro-morphology of elongated and round cells in the proliferating tissue (bar=0.2 mm).*

and disinfected as described in *Experiment 1*. PS were cut transversely into 1-1.5 mm thick slices and 6-7 mm thick slices. These slices were cultured on EDM and on modified LP basal medium (LPI, Quoirin and Lepoivre (1977), modified by Aitken-Christie et al. (1988)). This LPI medium was supplemented with 30 g l^{-1} sucrose, 1 g l^{-1} inositol, 20 µM 2,4-D, 25 µM NAA and 9 µM BA; before autoclaving the pH of the medium was adjusted to 5.8 and 3 g l^{-1} gellan gum (Gelrite®) were added. After autoclaving the same amino acid mixture as used in EDM medium was added. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks.

The PS slices cultured on EDM were transferred to the same medium. The slices cultured on LPI were subcultured either on LPI or on EDM. The slices cultured on LPO (the same as LPI without PGRs) were subcultured to LPI.

The 1-1.5 mm thick slices necrotized rapidly and did not produce any tissue (Figure 12b). When the initial explants were 6-7 mm thick slices and cultured on EDM, a developing white-translucent tissue was observed with embryogenic-like cells (Figure 12e); this tissue was subcultured onto the same medium and became yellowish with a higher proportion of round cells.

On explants cultured and subcultured on LPI, the tissue grew slower than on those subcultured on EDM or cultured from the beginning of the experiment on EDM. But after 4 to 8 weeks, the tissue growing on LPI showed the same macro- and micro-morphological features as the ones growing on EDM (Figure 12f). When cultured on LPO most explants displayed cell proliferation in the brachyblast meristems surrounding the PS slices; when transferred to LPI, 50% of them necrotized; the others developed globular structures (Figure 12c) that proliferated into the tissue type previously observed on the other culture media (Figure 12d).

6.3 Experiment 3

In 2010, the explants were from *in vitro* adventitious shoot buds. These were obtained from shoot buds of 10 trees that were over 20 years old and from the same seed orchard mentioned in *Experiment 2* (Figure 13a). These explants were obtained by culturing the shoot buds collected from the field on LPO for 4 weeks to induce axillary shoots and adventitious shoots and then transferring the explants to LP medium lacking PGRs and supplemented with 0.2% (w/v) AC (LPAC). The explants were subcultured every month. After 1 year, *in vitro* buds were cut into halves, quarters or slices (Figure 13b) and cultured on EDM or on EDM supplemented with 20 µM 2,4-D, 25 µM NAA and 9 µM BA (EDM2) (Figure 13). After 4 to 8 weeks, when proliferation of tissue was observed, the explants were transferred to maintenance medium. The maintenance media were those used for initiation. Cultures were maintained in the dark at 21±1ºC.

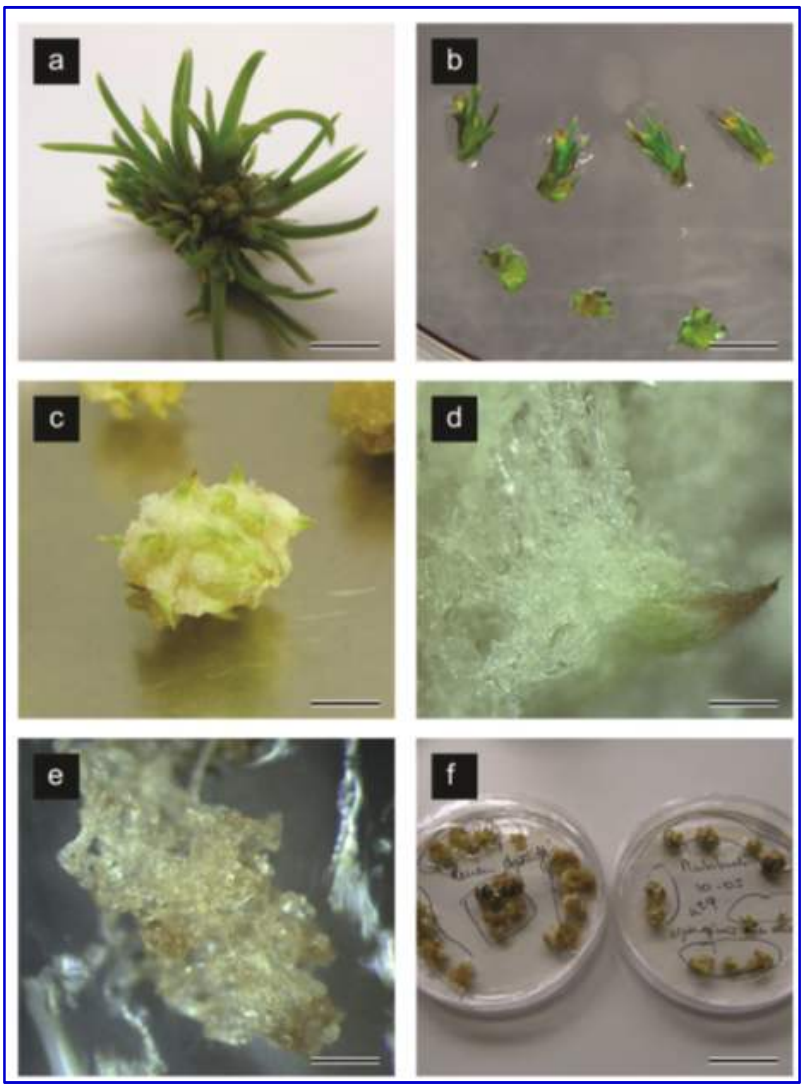

Figure 13. *Pinus radiata cultures initiated from sections of adventitious shoots (Experiment 3).* ***a*** *Adventitious shoots and shoot buds regenerated in vitro (bar=7 mm).* ***b*** *Shoots cut into quarters, halves and small sections (bar=5 mm).* ***c*** *Shoot explants after culture on EDM for 4 weeks (bar=6 mm).* ***d*** *Long cells within proliferating tissue cultured on EDM for 4 weeks (bar=0.8 mm).* ***e*** *A tissue piece with EM-like morphology cultured on EDM for 6 weeks (bar=0.7 mm).* ***f*** *Explants cultured on EDM on the left, and on EDM2 on the right (bar=30 mm).*

Small pieces of proliferating tissue were stained with acetocarmine (2% w/v) directly on glass slides for 4 min. Then, the samples were rinsed with water and mounted with a cover slide. Samples were observed with an inverted microscope (Leica DM4500) using a 40-fold magnification.

Tissue proliferation was observed in the three types of explants tested (halves, quarters and slices) and this tissue growth was most abundant in shoot buds cut into quarters (Figure 13c). In the explants cultured on EDM, the tissue grew at a higher rate than in the explants cultured on EDM2 (Fig 13d). As observed in previous experiments, when transferred to maintenance medium the tissue became yellowish (Figure 13f) and a large population of cells was round despite that initially, the proliferating tissue was white-translucent and that EM-like cells were observed (Figure 13 d, e).

6.4 Gene expression profiling of callus lines

Through the collaboration with NRCan-CFS, Canada, a project was undertaken to analyze gene expression profiles in six callus lines: two derived from shoot buds (PS) of somatic trees and three derived from axillary shoots generated *in vitro* from shoots collected from four seed-derived trees (Garcia-Mendiguren et al. 2015). In addition, expression profiles of three embryogenic lines derived from seed embryos were also generated for comparison. The explants were cultured on media with four different PGR compositions. The analysis revealed that culture medium had no significant impact on gene expression. High level expression of two *Knotted1*-like genes further reflected the vegetative character of these callus lines along with the expression of *WOX4*, a marker of vascular procambium tissue. Most notable was expression of embryogenesis-related gene *LEC1* in all five callus lines, although expression of two other embryogenic markers (*ABI3* and *WOX2*) was undetectable. Whether *LEC1* expression could be reflective of some level of embryogenic character, which might have progressed in the presence of additional embryo inducing factors, remains to be studied.

7. *Pinus strobus* L. (eastern white pine) - Klimaszewska K, Overton C

At the Laurentian Forestry Centre of the Canadian Forest Service, the SE induction experiments were conducted with clones of four genotypes (1053-8, 1053-12, 1073-12 and 1145-23) of somatic trees planted in Valcartier, Quebec in 2003. The trees were regenerated through SE induced in seed embryos (Klimaszewska et al. 2001). The first experiments started in November 2007 with dormant shoot buds, and in the spring of 2008, 2009, 2011 and 2014 with pre-flush buds when the trees were 7, 8, 9, 11 and 14 years old, respectively. Apical and subapical buds were pooled for the experiments.

7.1 PS bud disinfection and explant excision

The shoot bud disinfection protocol was the same as described for white spruce (Klimaszewska and Rutledge 2015 in this volume). Prior to disinfection, the

majority (not all) of the scales were removed together with the young needle fascicles, which invariably left small wounds on the stem. This protocol resulted in 70 to 80% explant sterility. In November 2007, PS was transversely sliced or cut longitudinally into four pieces and cultured (Figure 14 a, b, c). At least 100 transverse 1 to 2 mm thick PS slices, including PS apices, were cultured per genotype and pre-treatment. In 2008, shoot buds were collected in May (Figure15 a, b) and in June (Figure 15 g). In May, the PS were transversely sliced as described above (Figure 15 d); small PS were also cut longitudinally in four parts (Figure 15 c). In June the buds were longer and the primordia of needle fascicles were also elongated (Figure 15 g). These needle fascicles were separated from the buds, the basal scales were removed (Figure 15 h) and 30 were cultured per Petri dish. In 2009, 2011 and 2014, shoot buds were collected in May.

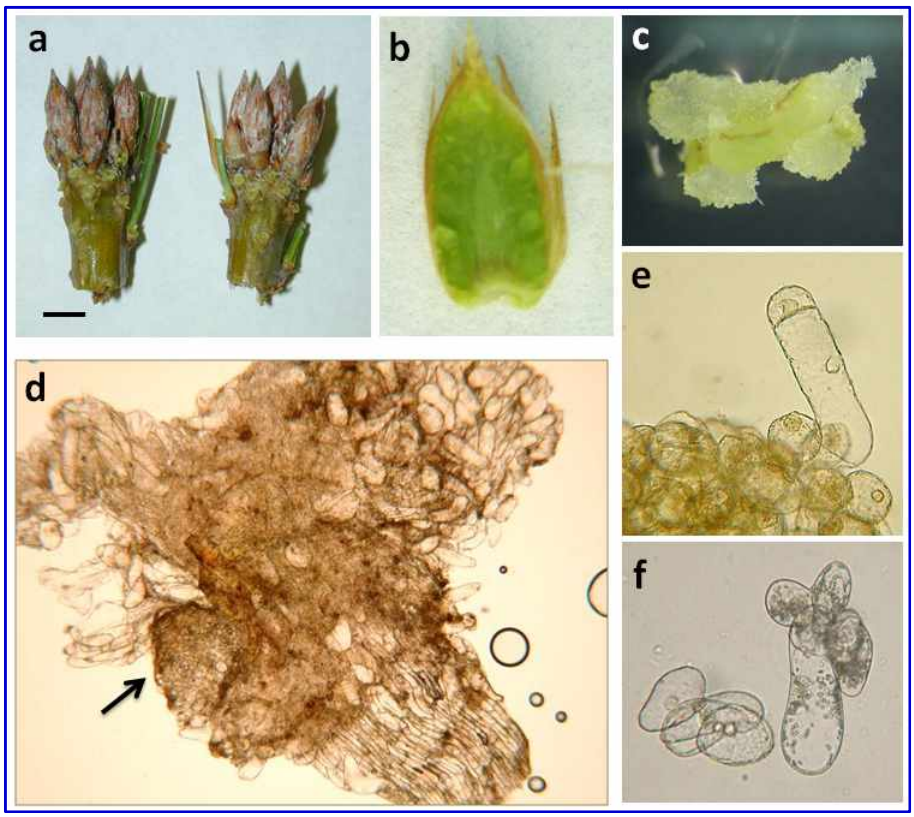

Figure 14. *Pinus strobus cultures initiated from PS longitudinal and transverse sections collected in November 2007 from 7-year-old somatic trees.* ***a*** *Dormant shoot buds prior to excision and disinfection (bar=1cm).* ***b*** *Longitudinal section through the disinfected shoot bud (bar=0.36cm).* ***c*** *White, partially translucent callus growing from the longitudinal section of entire PS after 2 weeks of culture (bar=037cm).* ***d*** *Slight squash of the PS explant showing callus cells and a small protuberance (arrow) (bar=60µm).* ***e, f*** *Asymmetrically dividing cells identified within callus cells (bar=30 µm).*

7.2 Culture medium and pre-treatments

In 2007, the PS explants were cultured according to the experimental design listed in Table 9, whereas in the following years only MLV-S medium was used. Explants were cultured in 90 x 10 mm Petri dishes, in darkness at 24°C.

Table 9. *Pre-treatment temperatures and culture media for PS explants of P. strobus in 2007.*

Pre-treatment conditions (medium composition)	Initiation medium (composition)
None	MLV-S (MLV + 3% sucrose, 0.5 gl^{-1} glutamine, 1 gl^{-1} CH, 100 mgl^{-1} inositol, 9.5 µm 2,4-D + 4.5 µM BA)
None	MLV-HPGR (MLV + 3% sucrose, 0.5 gl^{-1} glutamine, 1 gl^{-1} CH, 100 mg l^{-1} inositol, 20 µM 2,4-D, 25 µM NAA, 9 µM BA)
2/4°C, 3 days, dark (MLV (no N, no sucrose) + 100 mg l^{-1} inositol, 0.3% charcoal, 0.2 % gellan gum, no PGR)	MLV-S
2/4°C, 3 days, dark (same medium as above)	MLV-HPGR
32°C, 2 days, dark (same medium as above)	MLV-S
32°C, 2 days, dark (same medium as above)	MLV-HPGR

Note: Explants were split in equal numbers between 2 and 4°C pre-treatment. In the following year's experiments, only MLV-S was tested as a culture medium.

Explant responses- Regardless of the experimental design, within the first 2 weeks of culture, the majority of PS slices produced some calli from the cambial region (Figure 15 e) and also from the edges (Figure 15 f). The calli arising from the edges of explants had initially a white/translucent phenotype and might have originated from the wound areas / needle primordia. However, invariably after 4-6 weeks of culture and regardless of the subcultures on the "SE maintenance medium", the calli changed the phenotype and while still white they became a typical callus composed of spherical loose cells without any degree of organization. No discernible embryo structures were detected upon microscopic examination except sporadic cells undergoing asymmetric division (Figure 14 e) and a few cell aggregates composed of small cells and a large, elongate cell (Figure14 f). After

each subculture the callus pieces grew more slowly, became hard in texture and finally necrotized after several weeks of culture. Occasionally, the PS slices became necrotic with one or two distinct areas of white tissue growth, which, when isolated and subcultured, did not survive.

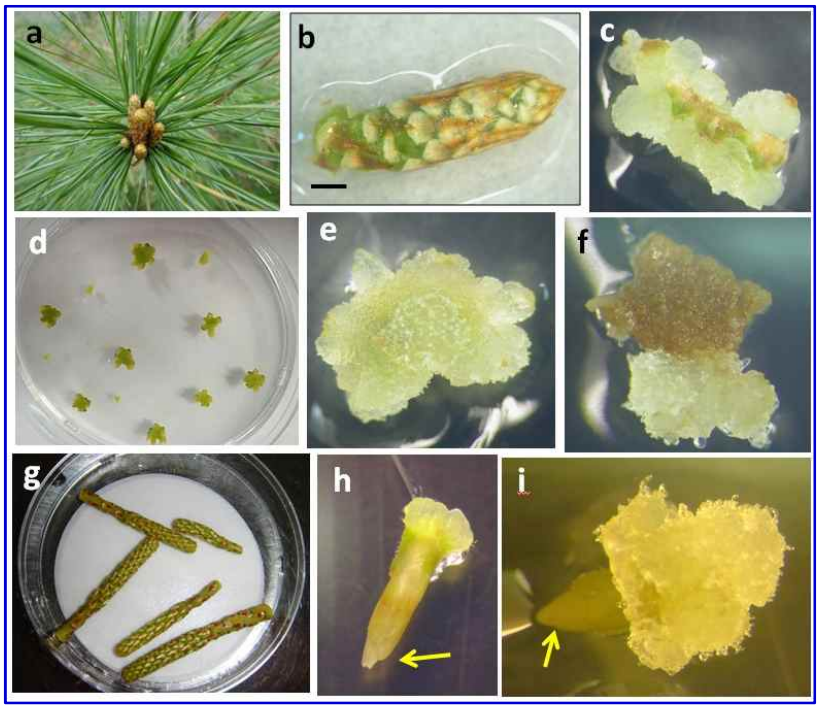

Figure 15. *Pinus strobus cultures initiated from spring PS explants collected in May (a – f) and June (g – i) 2008, 7.5- year-old trees.* ***a*** *Pre-flush shoot buds on a tree prior to collection (bar=2.5cm).* ***b*** *Disinfected pre-flush shoot bud (bar=3.25cm).* ***c*** *Callus growing from a longitudinal section of PS (bar=1cm).* ***d*** *Transverse slices of PS at the onset of cultures (bar=1.2cm).* ***e, f*** *Callus growing on the transverse slice of PS after 2 weeks (**e**) and on another explant after 6 weeks (**f**) (bar=2.4cm).* ***g*** *Disinfected shoot buds with developing needle fascicles (bar=1.38cm).* ***h, i*** *Isolated needle fascicle after 1 week (**h**) (bar=2mm) and 3 weeks of culture (**i**) (bar=1mm). Arrows indicate the tips of the needle fascicles. Note the calli growing from the bases of the fascicles.*

8. *Pinus contorta* Doug. ex Loud. (lodgepole pine)*:* Brief description of the published results (Park et al. 2010)

8.1 Plant material

Branches of fifteen 20-year-old lodgepole pine (*P. contorta* Dougl. ex Loud. Engelm.) genotypes, which had previously been selected based on the level of resistance to mountain pine beetle (MPB) attack, from the British Columbia

Ministry of Forests and Range seed orchard 307 (British Columbia, Canada), were collected bi-weekly from February to July in 2008 and 2009 (Park et al. 2010).

8.2 Explant preparation

The shoot bud disinfection and pre-treatment of explants were as described in Malabadi and van Staden (2005a) with some modifications (Park et al. 2010). Five transverse slices (0.5–1 mm thick) were taken sequentially from the tip of each PS. Approximately 25 slices were placed in Petri dishes (90 × 15 mm) containing a Gupta and Durzan (DCR) pre-treatment medium (Gupta and Durzan 1985) supplemented with 3 g l^{-1} activated charcoal (AC) and 0.2 g l^{-1} polyvinylpyrrolidone (PVP) for pre-culture at 38°C for 4 h followed by 4°C for 3 days in the dark (Park et al. 2010). The control PS explants had no pre-treatment and were cultured on DCR induction medium with high PGR concentrations (Table 1).

After 8 weeks of initial culture, calli originating from the cambial region of a PS slice were recorded. The calli were then proliferated and cultured on maintenance medium with reduced PGR concentrations (Table 1) and subcultured every 2 or 4 weeks for further development. The putative embryogenic cultures were preliminarily identified by microscopic observation.

8.3 Explant responses

In both 2008 and 2009, the highest number of explants producing EM-like white tissue was obtained between 27 March and 2 April. The survival rate of explants from later collections decreased and EM-like tissue could not be recovered. Genotypic specificity was also observed, as genotypes 1506 and 1537 produced EM-like tissue from more than 10% of all explants with pre-culture treatment. The same two genotypes consistently showed a similar response throughout the experiment. In contrast, two additional genotypes (1520 and 1530) were strongly recalcitrant and did not produce EM-like tissue at any sampling time. Shoot bud growth ceased in the middle of May, and needles flushed. More than 50% of the explants taken from apical PS collected from these latter time points became necrotic, whereas the remainder of the explants produced only brownish callus. Throughout the entire collection series, less than 2% of the PS explants produced callus from the cambial region, regardless of genotype. The calli that did develop grew slowly compared with those originating from tissues other than the cambium and displayed a characteristic transparent and white morphology. Staining the isolated calli with 2% aceto-carmine clearly showed the presence of EM-like cells that were small and dense with cytoplasm. These calli initially grew quickly, but after 6 months began to grow slowly and eventually turned brown and subsequently necrotized.

The calli originating from the cambial region were carefully separated from the explants after 2 months of culture and were transferred to fresh initiation medium. After 2 months, the proliferating calli were transferred to maintenance medium for further development and proliferation, and were sub-cultured every 2–4 weeks depending on their growth. When the calli were substantially proliferated, sub-samples were observed under the microscope to investigate the developmental stage of the EM-like aggregates. Some calli clearly revealed the presence of EM-like aggregates, which consisted of dividing cells and elongated suspensor-like cells. Despite the abundance of EM-like cells, the cultures were developmentally arrested even after culturing on spent medium or on the embryo development medium, which contained half the amount of PGRs compared with maintenance medium. These cultures also failed to produce somatic embryos on maturation medium with abscisic acid (ABA). Expression of embryogenesis-related genes *WOX2* and *LEC1/HAP3A* was studied in a line derived from PS explants as well as in a control EM and NEC. *WOX2* was expressed in all lines although at low levels compared with EM. Very low *WOX2* expression was detected in NEC. Similarly *LEC1/HAP3A* was expressed in all lines but at variable levels similar to either NEC or EM. Therefore, the cell lines derived from PS buds could be EM (Park et al. 2010), similarly to those in *P. sylvestris* described above.

9. Conclusions

The experiments carried out in different labs with six pine species (both somatic and zygotic embryo-derived trees) failed to produce SE from PS slices except for *P. sylvestris* in which two cell lines were embryogenic, but maturation yield was very low and the resulting somatic embryos were mostly abnormal and failed to grow into plants. Such behaviour of embryogenic line is often seen in pine EM of seed origin after continuous, prolonged subcultures. Because genetic variation was observed at several microsatellite loci for these two lines and others, follow up experiments with the same trees will be required to confirm these positive results and to establish a final protocol.

Otherwise, all authors observed very similar explant responses in culture, which initially produced translucent/white and embryogenic-like in appearance proliferating cell aggregates during the first 2-3 weeks and later, after a few subcultures, displayed reduced growth rate, changed appearance with respect to colour and consistency and eventually necrosis ensued. These changes were reflected in the cell shapes, the majority of which became round and formed aggregates of various sizes. On some necrotic explants, outgrowth of white tissue was observed later in culture. It was technically very difficult to separate and culture tiny pieces of embryogenic-like tissue that could only be identified through

staining and microscopic viewing, thus inadvertently rendering them non-viable for culture. Based on the micromorphology, seemingly EM-like cell aggregates were observed in *P. sylvestris*, *P. contorta*, *P. pinaster* and *P. radiata*. Interestingly, *P. radiata*, *P. contorta* and *P. sylvestris* cell lines obtained from PS explants expressed the embryogenesis-related gene *LEC1* at various levels. Similarly, *WOX2* expression was detected in *P. contorta* and *P. sylvestris* whereas *ABI3/VP1* was detected in *P. sylvestris*. These results suggest that activation of some prerequisite stages for SE might have occurred but did not progress further.

We conclude that the positive results on SE induction in adult pines attributed to the methods of Malabadi et al. (see the references) have largely proven unrepeatable with other pine species, particularly regarding the ease and high frequency inductions of SE. Furthermore, the insufficient details documenting the initiation and progression of SE from PS domes and slices, missing or confusing information detailing medium composition (see Table 1**,** e.g. maltose concentration in IM) and no proof, in most species, of normal germination and of established somatic embryo-derived plants bring to doubt the authors' claims that SE was achieved in explants of adult pines. Various media modifications were cited by the same authors as beneficial for particular pine species. Unfortunately, no comparative work was done simultaneously between species. These modifications included calcium, antioxidants, triacontanol and the addition of extracts from smoke-saturated water. In all cases, the discussion cited very solid research with other plant species by other researchers. However, the links of the effect observed in the *Pinus* spp. SE with the wider reported literature were often tenuous.

Certainly, the work by this chapter's international group of scientists negates the general applicability of the published methods together with the claim that SE in adult pine explants is feasible at high efficiency. Indeed, the challenge still exists in pines and other conifers as exemplified by the multiyear study on induction of SE in adult *Picea glauca* somatic trees (Klimaszewska et al. 2011, see also Klimaszewska and Rutledge 2015 in this volume).

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