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Research paper

Conifer somatic embryogenesis: improvements by supplementation of medium with oxidation–reduction agents

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A major barrier to the commercialization of somatic embryogenesis technology in loblolly pine (*Pinus taeda* L.) is recalcitrance of some high-value crosses to initiate embryogenic tissue (ET) and continue early-stage somatic embryo growth. Developing initiation and multiplication media that resemble the seed environment has been shown to decrease this recalcitrance. Glutathione (GSH), glutathione disulfide (GSSG), ascorbic acid and dehydroascorbate analyses were performed weekly throughout the sequence of seed development for female gametophyte and zygotic embryo tissues to determine physiological concentrations. Major differences in stage-specific oxidation–reduction (redox) agents were observed. A simple bioassay was used to evaluate potential growth-promotion of natural and inorganic redox agents added to early-stage somatic embryo growth medium. Compounds showing statistically significant increases in early-stage embryo growth were then tested for the ability to increase initiation of loblolly pine. Low-cost reducing agents sodium dithionite and sodium thiosulfate increased ET initiation for loblolly pine and Douglas fir (Mirb) Franco. Germination medium supplementation with GSSG increased somatic embryo germination. Early-stage somatic embryos grown on medium with or without sodium thiosulfate did not differ in GSH or GSSG content, suggesting that sodium thiosulfate-mediated growth stimulation does not involve GSH or GSSG. We have developed information demonstrating that alteration of the redox environment in vitro can improve ET initiation, early-stage embryo development and somatic embryo germination in loblolly pine.

Keywords: ascorbic acid, Douglas fir, embryo development, female gametophyte, glutathione, glutathione disulfide, loblolly pine, *Pinus taeda*, *Pseudotsuga menziesii*, redox agents, sodium dithionite, sodium thiosulfate.

Introduction

Forest productivity can be increased by planting tree farms with elite, high-value trees. Methods to propagate large numbers of genetically superior coniferous trees are needed. Clonal propagation by somatic embryogenesis (SE) can meet these needs and capture the benefits of breeding or genetic engineering to improve wood quantity, quality and uniformity. SE technology has yet to make a significant contribution to the nearly 1.5 billion loblolly pine (*Pinus taeda* L.) seedlings planted annually in the USA (Gupta and Durzan 1987, Schultz 1999). Factors currently limiting commercialization of SE for loblolly pine include: (i) low initiation of recalcitrant high-value seed sources; (ii) inability to maintain culture growth for many genotypes once initiation has occurred; (iii) decline of cultures resulting in loss of plant regeneration potential; and (iv) poor embryo quality resulting in slow initial growth and low germination percentages. While the barriers listed above may be overcome for a few of the most advanced genotypes, they raise the overall cost of production

and must be removed before SE technology can reach widespread use.

The development and improvement of tissue culture protocols is a lengthy and costly process. A literature-based trial-and-error approach has traditionally been used for this process; however, yield and quality improvements become increasingly difficult to obtain. Additional approaches such as studying natural embryo development to mimic the hormonal (Kapik et al. 1995, Pullman et al. 2003*b*), nutritional (Carpenter et al. 2000*a*, 2000*b*, Pullman and Buchanan 2003, Pullman et al. 2003*c*, 2006, 2008, Silveira et al. 2004), gene expression patterns (Xu et al. 1997, Cairney et al. 1999, 2000, Cairney and Pullman 2007, Vales et al. 2007, Oh et al. 2008) and physical (Pullman 1997, Pullman and Johns on 2009*a*, 2009*b*) conditions found in vivo, or understanding how medium changes over time, such as activated carbon adsorption and pH effects (Pullman and Johnson 2002, Van Winkle et al. 2003, Van Winkle and Pullman 2003), can facilitate protocol development. Since the female gametophyte (FG) tissue that normally surrounds and feeds the embryo in vivo is not present during SE in vitro, the addition of compounds that normally are provided by the FG may be necessary for normal zygotic-like development and maturation.

Glutathione (GSH)/glutathione disulfide (GSSG) and ascorbic acid (ASC, vitamin C)/dehydroascorbate (DHA) are two major redox pairs that control the redox state in a developing seed. Recently these redox compounds have been shown to exert strong effects on embryo development in several plants including white spruce. A picture is emerging that shows that optimal early-stage embryo development occurs in the presence of a reducing environment while a shift to an oxidizing environment is required for late-stage development (De Gara et al. 2003, Stasolla 2010). The ratio of GSH:GSSG seems to be more important than the actual amounts of GSH and GSSG (Yeung et al. 2005). A more oxidized GSH environment induces changes in ascorbate metabolism, abscisic acid and ethylene synthesis, as well as alterations in storage product deposition patterns (Stasolla 2010).

We hypothesize that the FG induces an oxidation–reduction environment that assists in embryo growth and therefore manipulation of the in vitro environment to more closely simulate the redox environment in vivo will improve somatic embryo growth and development. We have previously used analyses of zygotic tissues and the seed environment to identify targets for optimization of the growth medium (Pullman and Bucalo 2014). We now extend this approach to include understanding the oxidation–reduction environment in developing loblolly pine seeds and the major redox agents that create this environment in order to use this knowledge to modify the in vitro environment. Preliminary reports on parts of this work are presented in Pullman et al. (2009*b*) and Pullman and Bucalo (2014). Here we demonstrate that medium redox potential adjustment can increase embryogenic tissue (ET) initiation and somatic embryo germination.

Materials and methods

Plant material

For analytical work, loblolly pine seeds from Tree 7-56 were collected from a Weyerhaeuser seed orchard near Lyons, GA in 2002 and from Tree M-317 from a MeadWestvaco seed orchard near Summerville, SC in 2003. Cones were collected weekly and their embryos and FGs were separated by stage, cataloged and stored at −80 °C. Seeds were collected using the methods described by Pullman and Buchanan (2006). Seeds were cracked and the integument and nucellus tissue removed, exposing the FG. The FG was slit, pried open and the dominant embryo or mass of embryos removed. Individual embryos were quickly observed through a dissecting microscope, evaluated for stage of development (Pullman and Webb 1994, Figure 1), sorted by stage and tissue type and placed in vials partially immersed in liquid nitrogen. Stage 9 embryos were categorized by the week they were collected: 9.1 (Stage 9, Week 1), 9.2 (Stage 9, Week 2), etc. The value of staging embryos to provide an estimate of physiological age is discussed by Pullman and Buchanan (2003).

Redox chemical analysis of seed tissues using ultra-violet spectroscopy

A step-by-step design of how redox chemical analyses of zygotic embryo and FG tissue were carried out and used to improve our SE protocol is as follows.

(i) Phase 1. Analyze FG and zygotic embryos from two different trees through a sequence of developmental stages to provide stage- and tissue-specific developmental profiles for major redox agents.
(ii) Phase 2. Test individual redox agents at approximate physiological concentrations in an early-stage loblolly pine somatic embryo growth bioassay.
(iii) Phase 3. If any redox agents are identified that cause statistically significant increases in early-stage somatic embryo growth, test them for the ability to improve ET initiation for immature loblolly pine seed.

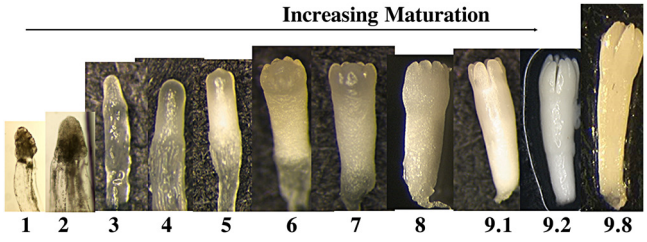

Figure 1. Developmental stages for zygotic embryogenesis in loblolly pine. Stages 2–4 are optimal for loblolly pine ET initiation. Adapted from Cairney and Pullman (2007).

(iv) Phase 4. If loblolly pine ET initiation is improved with specific redox agent addition, then test same redox agent for ability to improve initiation for other coniferous species.

The sorghum seedling procedure of Zhang and Kirkham (1996) was adapted to suit the purposes of this experiment. Solutions were made according to the following procedures: 3% iron(III) chloride was made daily by weighing 0.75 g $FeCl_3$ and adding to 25 ml of deionized water; 0.125 g *N*-ethylmaleimide (NEM) was added to 25 ml of deionized water and solutions were prepared daily; 0.0129 g of dithiothreitol (DTT) was added to 50 ml to make 0.15 mM; 0.125 M sodium phosphate was prepared and pH adjusted to 7.5; 6.3 mM ethylenedinitrilotetraacetic acid (EDTA) was prepared; 0.3 mM of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) was prepared; 125 mM of sodium phosphate was prepared from the 6.3 mM EDTA and was pH adjusted to 7.5; 6 mM of 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's Reagent or DTNB) was prepared using the 125 mM sodium phosphate/6.3 mM EDTA mixture; and 150 mM sodium phosphate was prepared with 5 mM EDTA and pH adjusted to 7.4. The color developer solution was prepared from a 2:2:2:1 ratio of the following: 10% trichloroacetic acid (TCA), 44% ortho-phosphoric acid, 4% α,α′-dipyridyl in 70% ethanol (EtOH) and 3% $FeCl_3$. The following chemicals are only stable for 2 weeks stored at 0–4 °C: 0.3 mM NADPH, the glutathione reductase (GR) solution and 6 mM DTNB.

All tissue samples were stored in separate vials at −80 °C. Liquid nitrogen was used during the weighing process to keep samples cold. Target tissue weights were between 0.1 and 0.15 g for all but the earliest embryo stages which contained less mass. Tissue was transferred into a small vial and 1.5 ml of cold 5% meta-phosphoric acid was added. The tissue was then ground to enhance chemical concentration and centrifuged at 22,000*g* at 4 °C for 15 min.

For total ascorbate, 0.75 ml of 150 mM sodium phosphate and 0.15 ml of 10 mM DTT were added to 0.3 ml of the centrifuged tissue supernatant and incubated for 10 min at room temperature. To remove the excess DTT, 0.15 ml of 0.5% *N*-ethylmaleimide was added. An aliquot of 2.1 ml of the color developer mixture was added. This mixture was vortexed for 20 s and then incubated in a water bath for 40 min at 40 °C. Vials were then put in cool water to stop the reaction and centrifuged for 5 min. The extract was tested in a Shimadzu (Kyoto, Japan) UV 2450 UV-visible spectrometer at a wavelength of 525 nm.

For ASC, 0.75 ml of 150 mM sodium phosphate and 0.3 ml of deionized water were added to 0.3 ml of the supernatant. This mixture was then incubated for 10 min at room temperature and then 2.1 ml of the color developer mixture was added. The solution was vortexed for 20 s and then incubated in a water bath for 40 min at 40 °C. Vials were then put in cool water to stop the reaction and centrifuged for 5 min. The extract was tested in the UV 2450 UV-visible spectrometer at a wavelength of 525 nm. Concentrations were determined using a standard curve. The concentration of dehydroascorbate was estimated from the difference between total ascorbate and ASC.

The GSH and GSSG testing procedures were loosely adapted from the procedures of Griffith (1980) and Smith (1985). Total glutathione (GSH + GSSG) was tested by removing 0.25 ml of the original extract and adding 0.75 ml cold H_3PO_4, 1.5 ml of 0.5 M NaH_2PO4 (pH 7.5) and 50 µl dH_2O and shaking until an emulsion formed. The mixture was then incubated for 60 min at room temperature (25 °C). The reaction occurred rapidly, so the following chemicals were added directly to a glass cuvette: 0.7 ml NADPH followed by 0.1 ml 0.6 mM DTNB, then 0.2 ml of previously incubated sample and finally 10 µl GR. The mixture was shaken a uniform 10 times in order to allow complete mixing and immediately put into the UV 2450 UV-visible Spectrometer with a wavelength setting of 412 nm. The kinematics data were recorded for 70 s. To standardize absorbencies, the reading at 1 s was subtracted from the reading at 61 s.

Glutathione disulfide was tested by removing 0.25 ml of the original extract and adding 0.75 ml cold H_3PO4, 1.5 ml of 0.5 M NaH_2PO4 (pH 7.5) and 50 µl 2-vinyl pyridine and shaken until an emulsion formed and incubated for 60 min at room temperature (25 °C). The reaction occurs rapidly, so the following chemicals were added directly to the glass cuvette: 0.7 ml NADPH followed by 0.1 ml 0.6 mM DTNB, then 0.2 ml of previously incubated sample and finally 10 µl GR. The mixture was shaken a uniform 10 times in order to allow complete mixing and immediately put into the UV 2450 UV-visible Spectrometer with a wavelength of 412 nm. The kinematics data were recorded for 70 s. To standardize absorbencies, the reading at 1 s was subtracted from the reading at 61 s. Glutathione was estimated from difference between total GSH and GSSG.

Growth of loblolly pine ET on medium varying in organic or inorganic redox agents

Early-stage somatic embryos of loblolly pine, grown in suspension culture in liquid medium 1133 (Table 1), were used as explants for growth bioassays to evaluate a medium's potential to support the last phase of initiation, multiplication of ET (Pullman and Webb 1994, Pullman et al. 2003*a*). Single-Stage 2 embryos were isolated with forceps from suspension culture and placed on 2 ml of test growth medium contained in Sigma-Aldrich (St Louis, MO, USA) Corning Costar #3526 Well Culture Cluster Plates. Explants were grown for 4–7 weeks in the dark at 23–25 °C and then measured for ET diameters. Somatic embryo growth bioassays typically employed three genotypes and 40 single early-stage somatic embryos per genotype grown on a test medium arranged in four replicates of 10 embryos each.

Experiment Series 1 Embryogenic tissue on initiation or multiplication medium contained somatic embryos similar in

Table 1. Media components for initiation experiments. LP, loblolly pine; DF, Douglas fir; NS, Norway spruce; TC, tissue culture.

Components	Media and components (mg l^{-1})						
	1133	1250	2212	2305	2301	2433	397
	LP	LP	LP	LP	DF	DF	LP
NH_4NO_3	603.8	603.8	200.0	200.0	–	–	206.3
KNO_3	909.9	909.9	909.9	909.9	1250	1250	1170
KH_2PO_4	136.1	136.1	136.1	136.1	340	340	85
$CaCl_2{\cdot}2H_2O$	–	–	–	–	134.2	134.2	220
$Ca(NO_3)_2{\cdot}4H_2O$	236.2	236.2	236.2	236.2	–	–	0
$MgSO_4{\cdot}7H_2O$	246.5	246.5	246.5	246.5	400	400	185.5
$Mg(NO_3)_2{\cdot}6H_2O$	256.5	256.5	256.5	256.5	–	–	0
$MgCl_2{\cdot}6H_2O$	101.7	101.7	101.7	101.7	–	–	0
KI	4.15	4.15	4.15	4.15	1.0	1.0	0.415
H_3BO_3	15.5	15.5	15.5	15.5	5.0	5.0	3.1
$MnSO_4{\cdot}H_2O$	10.5	10.5	10.5	10.5	15.8	15.8	8.45
$ZnSO_4{\cdot}7H_2O$	14.4	14.4	14.668	14.668	8.0	8.0	4.3
$Na_2MoO_4{\cdot}2H_2O$	0.125	0.125	0.125	0.125	0.2	0.2	0.125
$CuSO_4{\cdot}5H_2O$	0.125	0.125	0.1725	0.1725	0.025	0.025	0.25
$CoCl_2{\cdot}6H_2O$	0.125	0.125	0.125	0.125	0.025	0.025	0.0125
$AgNO_3$	–	–	3.398	3.398	–	–	0
$FeSO_4{\cdot}7H_2O$	6.95	6.95	13.9	13.9	27.8	27.8	13.93
Na_2EDTA	9.33	9.33	18.65	18.65	37.3	37.3	18.65
Maltose	–	–	15,000	15,000	15,000	15,000	0
Sucrose	30,000	30,000	–	–	–	–	20,000
Myo-inositol	1000	1000	20,000	20,000	1000	1000	100
Casamino acids	500	500	500	500	500	500	0
l-Glutamine[1]	450	450	450	450	450	450	0
Thiamine·HCl	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Pyridoxine·HCl	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glycine	2.0	2.0	2.0	2.0	2.0	2.0	2.0
d-xylose	–	–	100	100	100	100	0
MES	–	250	250	250	250	250	0
Biotin	–	0.05	0.05	0.05	5.0	5.0	0
Folic acid	–	0.5	0.5	0.5	50	50	0
Vitamin B_{12}[1]	–	–	0.1	0.1	–	–	0
Vitamin E[1]	–	–	0.1	0.1	–	–	0
α-Ketoglutaric acid[1]	–	–	100	100	–	–	0
Pyruvic acid	–	–	–	–	60.7	60.7	0
NAA	–	–	2.0	0.3	–	–	0
2,4-D	1.1	1.1	–	–	110	110	0
BAP	0.45	0.45	0.63	0.63	45	45	0
Kinetin	0.43	0.43	0.61	0.61	43	43	0
Activated charcoal	–	–	50	50	2500	2500	2500
Abscisic acid[1]	1.3	1.3	–	9.0	1.0	1.0	0
Brassinolide[1]	–	–	0.1 μM	0.1 μM	0.1 μM	0.1 μM	0
Gelrite	–	2500	2000	–	1800	–	0
TC agar							8000
pH	5.7	5.7	5.7	5.7	5.7	5.7	5.7

[1]Filter-sterilized stock solution was added after autoclaving and cooling to 55–60 °C.

appearance to zygotic embryos at early Stages 1–3. To determine if individual supplemental redox agents had a beneficial effect on early-stage somatic embryo growth, GSH, GSSG and ASC were added individually to medium 1250 (Pullman et al. 2006) at approximate concentrations of 0.1–1 mM.

Experiment Series 2 With early-stage somatic embryo growth stimulation seen in tests with two natural reducing agents, five low-cost inorganic reducing agents with known biological tolerance were individually tested for growth effect using the early-stage somatic embryo bioassay: sodium

bisulfite, sodium dithionite, sodium metabisulfite, sodium sulfite and sodium thiosulfate. Sodium dithionite was added to medium 1250 (Table 1) at concentrations of 0, 1, 10 and 100 mg l^{-1}. Other reducing agents were individually added to medium 1250 at concentrations of 0, 0.1, 0.5 and 1.0 mM. A positive control of 0.5 mM GSH was often added to the experiment.

Loblolly pine initiation on media supplemented with redox agents

Open- and cross-pollinated cones were collected in 2009, 2011 and 2012 in early to mid-July from clonal seed orchards, shipped on ice and received within 24–48 h. Cones were stored at 4–5 °C for 1–5 weeks. Cones containing seeds with embryos mostly at Stages 2–4 (Pullman and Webb 1994) were prepared for initiation experiments as described by Pullman et al. (2005).

Medium 2212 (Table 1) overlaid after 14 days with 0.25 ml of liquid medium 2305 (Table 1) was a starting point for this research (Pullman et al. 2009*a*). Medium pH was adjusted to pH 5.7 with KOH or HCl after the addition of all ingredients except gelling agent and filter-sterilized materials. Media were autoclaved at 121 °C for 20 min. Aqueous stock solutions of L-glutamine, abscisic acid, brassinolide and α-ketoglutaric acid (pH adjusted to 5.7) were filter-sterilized and added to medium cooled to ~55 °C. Explants for initiation experiments with zygotic embryos were cultured on 2 ml of medium contained in individual wells of Costar #3526 Well Culture Cluster Plates wrapped in two layers of Parafilm and incubated at 23–25 °C in the dark. Experiments were usually composed of 10 replications of 10 explants per test medium and seed source. Treatments were applied in a completely randomized design.

The initiation process for loblolly pine is pictured in Pullman et al. (2003*d*) and described in more detail by Becwar and Pullman (1995). Loblolly pine initiation occurs in three steps. Extrusion occurs at 1–4 weeks when one or more usually subordinate zygotic embryos expand out of the FG micropylar end. At 5–7 weeks, proliferating cells and somatic embryos appear in the extruded tissue. During the final step, these multiply to form a mass of ET. These phases can be evaluated as percent extrusion, percent of explants that form somatic embryos visible through a dissecting microscope and percent of cultures achieving a target mass or size. Percent extrusion and explants with three or more somatic embryos visible under a dissecting scope (i.e., initiation) were routinely evaluated 9–10 weeks after placement of FGs on media. Data were evaluated by analysis of variance and significant differences between treatment means were determined by the Multiple Range Test at the 5% level of significance using Statgraphics Plus V4.0 (Manguistics Inc., Rockville, MD). Analyses of extrusion and initiation percentage data were done after arcsine $\sqrt{(\%)}$ transformation.

Loblolly pine initiation during 2009 Based on statistically significant increases in ET growth resulting from medium supplementation with GSH or sodium dithionite (see Results), the next step was to test ET initiation from loblolly pine FGs containing early-stage embryos. An ET initiation experiment was conducted with 80–100 immature seeds per cross and treatment from four cross-pollinated seed sources collected in July 2009 and placed onto gelled medium overlaid with 0.25 ml liquid medium at 14 days (Pullman and Skryabina 2007). Treatment 1 was control media 2212/2305 (Table 1), Treatment 2 was control medium plus 0.5 mM GSH, Treatment 3 was control medium plus 10 mg l^{-1} sodium dithionite. In each case the supplement was added to both the solid and liquid overlay medium.

Loblolly pine initiation during 2011 Immature seed from five crosses were collected in July. Each cross was placed onto two treatments (100 per treatment). Treatment 1 consisted of gelled medium 2422 (2212 with brassinolide replaced with 2.0 μM epibrassinolide) (E1641, Sigma-Aldrich) overlaid with 0.25 ml liquid medium 2305 with brassinolide replaced with 2.0 μM epibrassinolide (=2879). An error occurred in medium 2879 and NAA was present at 2.0 mg l^{-1} instead of the 0.3 mg l^{-1} and ABA was absent instead of 9.0 mg l^{-1} (labeled as 2879M). Treatment 2 added 1.0 mM sodium thiosulfate to the gelled and liquid medium.

Loblolly pine initiation during 2012 Immature seed from two crosses with 60 and 100 seeds per treatment and an open-pollinated seed source with 100 seeds per treatment were collected in July and placed onto three treatments. Treatment 1 consisted of gelled medium 2422 overlaid with liquid medium 2879. Treatment 2 added 10 mg l^{-1} sodium dithionite to the gelled and liquid media. Treatment 3 added 1.0 mM sodium thiosulfate to the gelled and liquid media.

Loblolly pine somatic embryo germination on medium supplemented with redox agents

The germination protocol of Pullman and Bucalo (2011) was used. Medium 397 was used as a control or supplemented with GSH or GSSG. After two and a half months on maturation medium 1562 (Pullman and Bucalo 2011), somatic embryos were selected that exhibited normal embryo shape. Ten embryos were placed horizontally on 20 ml of either germination medium 397 or medium 397 supplemented with a redox agent. Experiments contained five replications of 10 shoots per medium and three to four genotypes. Plates were incubated for 7 days in the dark at 25 °C and then placed under a 16/8-h (day/night) photoperiod with light supplied by cool white fluorescent lamps at an intensity of ~7 μmol photons m^2 s^{-1} at 25 °C. After 12 weeks in the light, the embryos were scored for the presence of roots and shoots. An embryo was considered to have germinated when it possessed both root and shoot.

Experiment 1. Medium 397 alone or with 0.1, 0.5, 1.0 mM GSH or 0.1, 0.5, 1.0 mM GSSG was tested for germination using three genotypes.

Experiment 2. Medium 397 alone or with 0.5, 0.75, 1.0, 1.25 mM GSH or 0.5, 0.75, 1.0, 1.25 mM GSSG was tested for germination using four genotypes.

Analyses of somatic ET for GSH and GSSG using Mass spectrometry and HPLC

Somatic embryo growth and tissue extraction As described earlier, 50 Stage 2 loblolly pine somatic embryos grown in liquid suspension culture were individually selected and placed each onto 2 ml of test growth medium. Medium 1250 with or without 1 mM sodium thiosulfate was compared. After 5 weeks, individual tissue colony diameters were measured. ET extraction was performed as described in the literature, with some modifications (Rellan-Alvarez et al. 2006). ET (30 mg) was frozen in liquid N_2, stored at –80 °C until analysis, and ground with mortar and pestle in liquid N_2. The dry powder was homogenized with 200 µl of 4 °C phosphoric acid (5% (w/v)) to give a final ET concentration of 150 mg ml^{-1}. Homogenates were centrifuged at 15,000*g* for 20 min at 4 °C. The supernatants were filtered through 0.22 µm polyvinylidene fluoride filter and immediately analyzed or frozen in liquid N_2 and stored at –80 °C until analysis. All steps were done in a cold chamber at 4 °C. All instruments (e.g., mortar, pestle) were also pre-cooled.

In initial experiments, we found that the matrix of the ET extract suppressed the ionization of GSH and GSSG standards by ~30%. To compensate, we measured the GSH content of ET extracts by using a standard curve of isotopically labeled GSH* (*m/z* 310.26, Sigma-Aldrich), in a matrix of ET extract. We found that standard curves of pure GSH and GSH* yielded identical results, thereby confirming that it is appropriate to measure GSH content using a GSH* standard curve. Stock solutions of 450 µM GSSG, 450 µM GSH and 450 µM GSH* were prepared in 5% (w/v) phosphoric acid. Aliquots of the stock solutions were conserved at –80 °C. Aliquots were thawed only once to prepare the standards and then were discarded.

HPLC–ESI/MS(MRM) Analyses were carried out with a Waters Corporation (Milford, MA, USA) Micromass Quatrro LC multiple reaction monitoring mass spectrometer (ESI/MS(MRM)) coupled to an Agilent 1100 HPLC system (HPLC–ESI/MS(MRM)). The ESI/MS(MRM) was operated with source capillary voltage 3.50 kV, respectively, in negative ion mode. Drying and nebulizer (N_2) flow rates were kept at 592 and 93 l min^{-1}, respectively.

For the MRM analyses, the $[M+H]^+$ ion of each compound was selected in the first quadrupole, and that ion was passed through a collision cell containing argon. The collisions caused fragmentation, and a characteristic product ion was selected in the second quadrupole. A signal is observed only when the $[M+H]^+$ ion passes through the first quadrupole, and when the characteristic product ion passes through the second quadrupole. Chemical noise is removed, and the trace is specific for the compound chosen, even in a complex mixture.

Before the MRM analyses were run, the conditions were optimized for GSH, GSH* and GSSG by infusing a solution of each into the instrument. The source parameters were adjusted to give the highest intensity for the $[M+H]^+$ precursor ion of each compound. The instrument was then set to pass only the precursor ion through the first quadrupole into a collision cell filled with argon at ~3.5×10^{-4} mBar. Product ion spectra were acquired in the second quadrupole as the collision energy was varied. The most intense product ion observed as the collision energy was varied, was selected and the collision energy for that ion was used for the MRM analyses. For the MRM analyses, the instrument parameters were set for the detection of each compound in series as the separations progressed. For GSH the 308–179 *m/z* transition was chosen; for GSH* the 310.9–182 *m/z* transition was chosen; and the 613–355 *m/z* transition was chosen for GSSG.

Aliquots of 20 µl standard solutions and sample extracts were injected in a Phenomenex Gemini C18 column. Samples were eluted at a flow rate of 0.2 ml min^{-1}. The mobile phase was built using two solvents: A (0.1% formic acid in 5% acetonitrile) and B (0.1% formic acid in 95% acetonitrile). For separation of the analytes, 100% A was used for 5 min, then a linear gradient from 0 to 100% B (5.1–13 min) was used. Then, to wash the column, the concentration of A was ramped from 0 to 100% in 0.1 min, and this solvent composition was maintained until 20 min.

To compensate for any potential matrix effects from the ET extract on GSH ionization, we used GSH*, prepared in an equal amount of ET extract as the samples, as a standard for GSH. Two replicates of each extract were analyzed. All the data were evaluated by Student's *t*-test and reported as the mean ± SEM.

Douglas fir plant materials, experimental design and initiation on media supplemented with reducing agents

Douglas fir initiation 2009 With several experiments showing benefit of addition of reducing agents during loblolly pine initiation (see Results), we proceeded to investigate the effect of reducing agents on other coniferous species. The Douglas fir (*Pseudotsuga menziesii* (Mirb) Franco) initiation medium of Pullman et al. (2009*c*) in which 100 mg l^{-1} d-xylose was added to medium 2207 was used as Treatment 1 (medium 2301, Table 1) along with a 2.0 ml liquid overlay of the same medium without gelling agent, added 3 weeks after placement of the explant (2433, Table 1). Douglas fir cones containing embryos at Stages 3–5 (Pullman and Webb 1994) were collected in early July 2009 from five crosses. Cones were opened and seeds still attached to the ovuliferous scale were cut from the full ovuliferous scales. Seeds and attached scale were sterilized as indicated in Pullman et al. (2009*c*). Under aseptic dissection, the seed coat, integument and nucellus were removed, FGs were carefully cut

open and the dominant embryo lifted out while still attached to the FG. The exposed FG and embryo were placed on 7 ml of test medium contained in VWR (Radnor, PA) #29442-036 6-Well Cluster Plates. Plates were wrapped in parafilm and incubated at 23–24 °C in the dark for 7–8 weeks. Five crosses were tested on each medium using 10 replicates of six explants per cross.

Douglas fir initiation 2011 Nine crosses containing mainly Stage 3–5 embryos were collected in early July and tested for initiation on medium 2301 in Treatment 1. Treatments 2 and 3 consisted of adding 10 mg l^{-1} sodium dithionite (Treatment 2) or 1 mM sodium dithionite (Treatment 3), respectively, to medium 2301. Crosses ranged from 5 to 17 replications of six explants per cross and treatment. Liquid overlays were not added.

Douglas fir initiation 2012 Six crosses with embryos mainly at Stages 3–5 were tested for initiation on control medium 2301, 2301 + 10 mg l^{-1} sodium dithionite (medium 2578) and 2301 + 1.0 mM sodium thiosulfate. Crosses ranged from 9 to 15 replications of six explants per cross and treatment.

Douglas fir initiation 2013 Five crosses with embryos at Stages 3–5 were tested for initiation on control medium 2301 and 2301 + 10 mg l^{-1} sodium dithionite (medium 2578). Crosses ranged from 3 to 17 replications of six explants per cross and treatment.

Results

Redox chemical analysis of seed tissues using ultra-violet spectroscopy

Profiles for the four redox agents analyzed are shown in Figure 2. Ascorbic acid and DHA peaked in mid-development and then decreased in the embryo. Pullman and Buchanan (2006) saw a similar pattern for ASC with levels peaking around Stages 7–9.1 in organic acid analyses performed with mass spectroscopy (Figure 3). Ascorbic acid and DHA in FG tissue did not show a consistent pattern during development, although concentrations were generally highest during mid-development and DHA was not detected after mid-development (Figures 2 and 3). Both GSH and GSSG showed similar patterns for the two trees tested. Concentrations of GSH and GSSG in both embryo and FG tissues increased until mid-development and then decreased. During early embryogenesis the ratios of GSH:GSSG were generally positive in both FG and embryo tissues. This suggests the pool of GSH + GSSG was maximal at mid-development. GSSG was low in both embryo and FG tissues toward the end of seed development.

Growth of loblolly pine ET on medium varying in organic redox agents

Early-stage somatic embryo growth tests with ASC, GSH and GSSG indicate that medium supplementation with reduced ASC or reduced GSH increased growth (Tables 2 and 3). Ascorbic acid increased loblolly pine early-stage somatic embryo growth when added to medium at 25 mg l^{-1} (0.14 mM) and 50 mg l^{-1} (0.28 mM) (Table 2). Glutathione also increased somatic embryo growth when added to medium at 0.5 mM (Table 3). Medium containing GSSG at 0.1 or 0.5 mM did not alter somatic embryo growth and medium containing 1.0 mM GSSG decreased growth.

Growth of loblolly pine ET on medium varying in inorganic redox agents

Tests with all reducing agents showed statistically significant increases in ET growth when supplemented with optimal concentrations of inorganic reducing agents (Table 4). ET masses appeared similar, but larger with the addition of reducing agents. As concentrations of the reducing agent were increased, growth reductions often occurred.

Loblolly pine initiation on media supplemented with redox agents

Loblolly pine initiation during 2009 Percent extrusion did not differ significantly among treatments (data not shown). Initiation percentages averaged 33.4 for the control treatment and 36.0 for the addition of sodium dithionite in Treatment 3, but these differences were not statistically significant (Table 5).

Loblolly pine initiation during 2011 Percent extrusion did not differ significantly between treatments (data not shown). Addition of sodium thiosulfate to initiation medium increased average initiation across crosses from 21.7% to 30.7% (Table 5); differences were statistically significant at $P = 0.01$. Four of the five crosses tested showed increased initiation percentages on medium containing sodium thiosulfate.

Loblolly pine initiation during 2012 Percent extrusion averaged 22.3, 31.2 and 32.8% for the control, sodium dithionite and sodium thiosulfate treatments, respectively; differences were statistically significant ($P = 0.05$) for the two media containing redox agents. Percent initiation was also increased by redox agents and differences were statistically significant ($P = 0.05$, Table 5). Two of the three seed sources tested doubled or nearly doubled initiation when redox agents were included in the medium.

Loblolly pine somatic embryo germination on medium supplemented with redox agents

Experiment 1 Germination tests with somatic embryos showed a surprising result. Germination medium supplementation with GSSG at 1.0 mM increased shoot development in germination tests with genotypes that did not form roots (Table 6). Differences had borderline statistical significance at $P = 0.10$.

Experiment 2 Glutathione disulfide at 1.0 mM showed higher germination percentages (shoot and root develop-

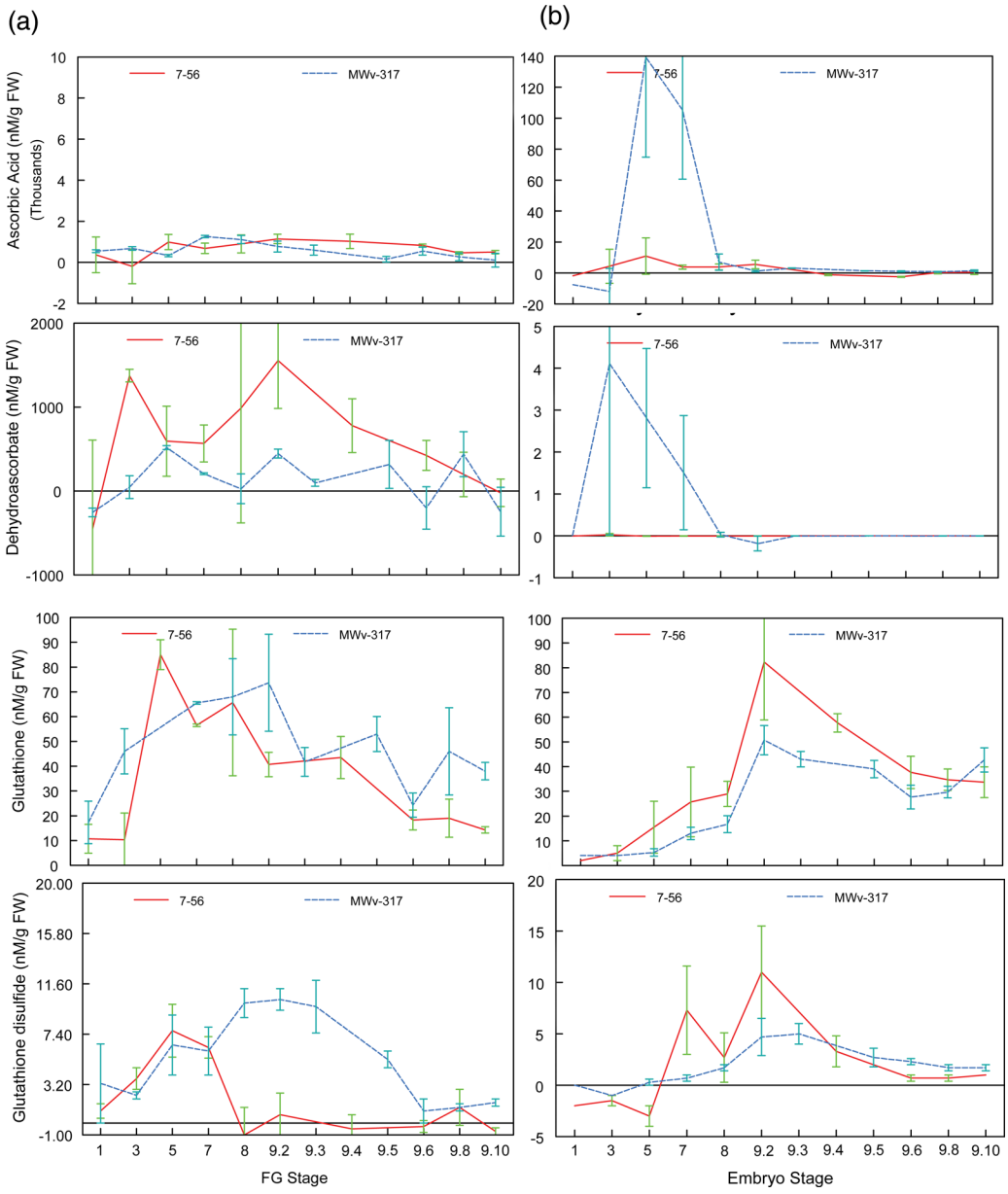

Figure 2. Loblolly pine FG and embryo redox chemical profiles in nmol g^{-1} fresh weight across seed development for two mother trees. (a) Female gametophyte. (b) Embryo. Bars represent standard error. FW, fresh weight.

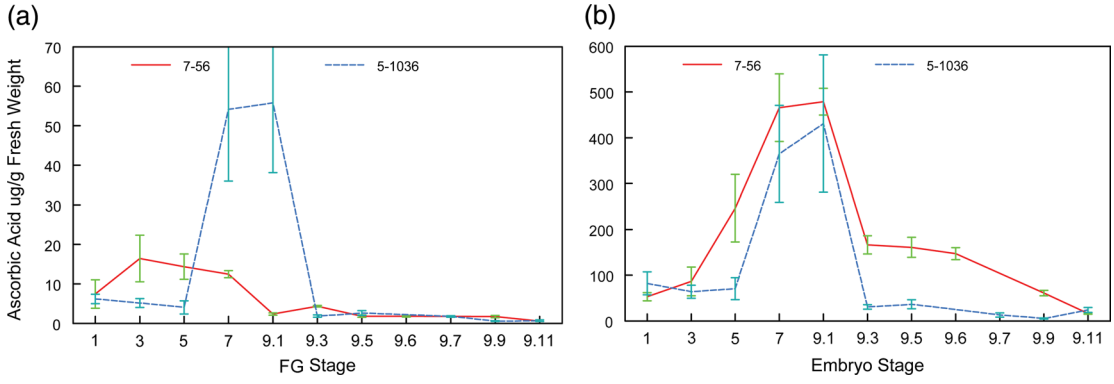

Figure 3. Ascorbic acid in µg g^{-1} fresh weight for trees 7-56 and UC5-1036 (Pullman and Buchanan 2006). (a) Female gametophyte. (b) Embryo. Bars represent standard error.

Table 2. Average colony diameter (mm) of loblolly pine ET grown on medium with four concentrations of ASC.

Media	ASC (mg l^{-1})	Genotype and ET colony diameter + SE (mm)			
		249	351	500	Average (%)[1]
1250	0	3.8 ± 0.5	8.5 ± 0.5	8.9 ± 0.5	7.1 a
2013	25	6.7 ± 0.5	9.8 ± 0.5	9.9 ± 0.5	8.8 b
2014	50	7.1 ± 0.5	9.7 ± 0.5	8.0 ± 0.5	8.3 b
2015	100	6.6 ± 0.5	8.5 ± 0.5	6.9 ± 0.5	7.3 a

[1]Four replicates of 10 explants were tested per medium per genotype. Values followed by the same letters are not statistically different by Multiple Range Test at $P = 0.05$. SE, standard error.

Table 3. Average colony diameter (mm) of loblolly pine ET grown on control medium and medium with three concentrations of GSH or GSSG.

Media	Redox chemical (mM)	Genotype and ET colony diameter ± SE (mm)			
		132	203	500	Average (%)[1]
1250	0	7.5 ± 0.3	1.8 ± 0.3	2.6 ± 0.3	4.0 b
2231	0.1 mM GSH	7.5 ± 0.3	1.8 ± 0.3	3.3 ± 0.3	4.2 b
2232	0.5 mM GSH	7.2 ± 0.3	3.9 ± 0.3	3.8 ± 0.3	4.9 c
2228	1.0 mM GSH	3.8 ± 0.3	1.8 ± 0.3	2.1 ± 0.3	2.5 a
2233	0.1 mM GSSG	7.5 ± 0.3	1.9 ± 0.3	3.6 ± 0.3	4.3 b
2234	0.5 mM GSSG	5.2 ± 0.3	4.7 ± 0.3	3.2 ± 0.3	4.4 b
2235	1.0 mM GSSG	3.6 ± 0.3	3.4 ± 0.3	2.1 ± 0.3	3.0 a

[1]Four replicates of 10 explants were tested per medium per genotype. Values followed by the same letters are not statistically different by Multiple Range Test at $P = 0.05$. SE, standard error.

ment) in genotypes that were able to germinate (Table 7). Differences again showed borderline statistical significance at $P = 0.08$.

Analyses of somatic ET for GSH and GSSG using mass spectrometry and HPLC

Sodium thiosulfate enhances early-stage somatic embryo growth In this test, tissue collected for analyses also confirmed results described earlier. Incubation on medium containing 1 mM sodium thiosulfate increased growth of ET compared with control ET ($n = 100$, $P < 0.01$). Sodium thiosulfate-treated ET grew to 5.1 ± 0.2 mm while control ET grew to 4.2 ± 0.2 mm.

ESI/MS(MRM) analysis We confirmed that neither oxidation of GSH nor reduction of GSSG standards occurred during the ionization process of ESI/MS(MRM) analysis. In addition, we also determined that when either GSH or GSSG standards were used in a mock ET extraction procedure, neither oxidation of GSH nor reduction of GSSG occurred during HPLC–ESI/MS(MRM) analysis.

HPLC–ESI/MS(MRM) analysis Analytes were separated with a linear solvent gradient with a C18 column, and mass spectra were acquired by ESI/MS(MRM) to obtain 3D (time, m/z and intensity) chromatograms. MRM transitions were determined for the m/z values corresponding to the unique product ions of the $[M+H]^+$ ions of GSH, GSH* and GSSG. Using our HPLC methodology, GSH, GSH* and GSSG present in a mixed standard solution, elute at 2.9–3.1 min. However, MRM analysis allows each compound to be separately detected despite this overlap of retention time. A GSH* standard curve, in a matrix of sodium thiosulfate-treated ET extract (150 mg ml^{-1}), was generated with an R^2 value of 0.9984. During initial optimization experiments, we did not detect GSSG in either the control or sodium thiosulfate-treated ET, even with ET concentrations as high as

Table 4. Embryogenic tissue growth in response to reducing agents added to growth medium 1250. Four replicates of 10 explants were tested per medium per genotype. Tissue diameters for all genotypes are averaged. Values followed by the same letters are not statistically different by Multiple Range Test at $P = 0.05$. Values for sodium dithionite trial 2 were only statistically significant at $P = 0.10$.

Reducing agent and concentration	Genotypes tested (#)	Average of tissue diameter (mm)	Growth change (%)
Sodium dithionite trial 1 ($P = 0.05$)			
0	3	7.1 b	
1 mg l^{-1}	3	7.4 bc	
10 mg l^{-1}	3	8.2 c	+15
100 mg l^{-1}	3	1.9 a	
0.5 mM GSH	3	10.8 d	+52
Sodium dithionite trial 2 ($P = 0.10$)			
0	3	5.1 ab	
1 mg l^{-1}	3	5.7 bc	
5 mg l^{-1}	3	5.3 abc	
10 mg l^{-1}	3	5.7 c	+12
25 mg l^{-1}	3	4.9 a	
Sodium metabisulfite ($P = 0.05$)			
0	3	11.6 a	
0.1 mM	3	11.9 ab	
0.5 mM	3	12.1 b	+4
1.0 mM	3	12.2 b	+5
0.5 mM GSH	3	11.6 a	
Sodium bisulfite ($P = 0.05$)			
0	3	9.8 c	
0.1 mM	3	10.5 d	+7
0.5 mM	3	7.8 b	
1.0 mM	3	3.1 a	
0.5 mM GSH	3	9.7 c	
Sodium sulfite ($P = 0.05$)			
0	3	9.9 a	
0.1 mM	3	11.2 c	+14
0.5 mM	3	10.8 bc	+7
1.0 mM	3	11.0 c	+11
0.5 mM GSH	3	10.6 b	+7
Sodium thiosulfate ($P = 0.05$)			
0	3	10.4 a	
0.1 mM	3	12.2 c	+17
0.5 mM	3	12.2 c	+17
1.0 mM	3	12.3 c	+18
0.5 mM GSH	3	11.8 b	+13

Table 5. Loblolly pine initiation on gelled and liquid overlay media varying in addition of redox agents. LOS, liquid overlay added to gelled medium after 14 days; Cr, cross; Op, open pollinated. See text for compositions of media 2422, 2879 and 2879M.

Medium	Seed source cross and initiation percentages					
2009 Tests using medium 2212 LOS and modifications						
	Cross 152	Cross 153	Cross 154	Cross 155		Average[1]
Control (2212/2305)	42.0 ± 4.7	30.0 ± 4.1	22.5 ± 6.7	39.0 ± 4.6		33.4 a
+0.5 mM GSH	35.4 ± 5.2	23.6 ± 6.0	36.8 ± 9.0	44.0 ± 5.2		33.5 a
+10 mg l^{-1} Na dithionite	43.4 ± 5.3	27.8 ± 2.8	35.4 ± 6.5	37.4 ± 8.7		36.0 a
2011 Tests using medium 2422 LOS and modifications						
	Cross 159	Cross 160	Cross 161	Cross 162	Cross 164	Average[1]
Control (2422/2879M)	6.5 ± 1.8	40.0 ± 7.3	37.4 ± 6.3	22.7 ± 3.9	2.0 ± 1.3	21.7 a
+1.0 mM Na thiosulfate	36.7 ± 8.2	34.0 ± 2.2	37.4 ± 3.9	30.3 ± 3.8	15.0 ± 3.4	30.7 b
2012 Test using medium 2422 LOS and modifications						
	Cr 081113	Cr 71105	Op 7–56			Average[1]
Control (2422/2879)	8.5 ± 4.0	7.1 ± 2.6	11.0 ± 2.8			8.7 a
+10 mg l^{-1} Na dithionite	15.8 ± 3.8	7.1 ± 2.2	32.5 ± 4.3			16.7 b
+1.0 mM Na thiosulfate	20.0 ± 5.8	6.1 ± 3.0	24.0 ± 5.4			17.3 b

[1]Values followed by the same letter are not statistically different by the Multiple Range Test at $P = 0.01$ (2011 tests) and $P = 0.05$ (2012 tests), analyses are based on arcsine √% transformation of 8–10 replications of 10 explants.

Table 6. Average shoot production during germination of loblolly pine somatic embryos from recalcitrant genotypes that do not germinate normally. Embryos were tested on control medium and medium with three concentrations of GSH or GSSG.

Media	Redox chemical (mM)	Genotype and somatic embryo shoot production (%)			
		132	178	186	Average (%)[1]
397	0	34.0 ± 6.1	12.0 ± 6.1	15.0 ± 6.9	20.3 ab
2281	0.1 mM GSH	34.0 ± 6.1	0 ± 6.1	12.0 ± 6.1	15.3 a
2282	0.5 mM GSH	40.0 ± 6.1	0 ± 6.1	16.0 ± 6.1	18.7 a
2283	1.0 mM GSH	40.0 ± 6.1	14.0 ± 6.1	26.0 ± 6.1	26.7 bc
2284	0.1 mM GSSG	44.0 ± 6.1	0 ± 6.1	10.0 ± 6.9	18.0 a
2285	0.5 mM GSSG	60.0 ± 6.1	16.0 ± 6.1	16.0 ± 6.1	30.7 bc
2286	1.0 mM GSSG	50.0 ± 6.1	22.0 ± 6.9	26.0 ± 6.1	32.8 c

[1]Five replicates of 10 embryos were tested per medium per genotype. Percentages were transformed by arcsine √%. Values followed by the same letters are not statistically different by Multiple Range Test at $P = 0.10$. SE = standard error.

Table 7. Average germination of loblolly pine somatic embryos tested on control medium and medium with GSSG.

Media	GSSG (mg l^{-1})	Genotype and somatic embryo germination (%)				
		200	222	433	464	Average (%)[1]
397	0	20.0 ± 5.9	0 ± 7.6	24.0 ± 5.9	2.0 ± 5.9	11.5 a
2286	1.0 mM	38.0 ± 5.9	2.5 ± 6.6	17.5 ± 5.9	16.0 ± 5.9	18.5 b

[1]Five replicates of 10 embryos were tested per medium per genotype. Percentages were transformed by arcsine √%. Values followed by same letters are not statistically different by Multiple Range Test at $P = 0.08$. SE = standard error.

640 mg ml^{-1}, as compared with our standard ET concentration of 150 mg ml^{-1}. Using a GSSG standard we determined the GSSG limit-of-detection for our method to be 60 pmol; therefore the smallest amount of GSSG that we were capable of measuring would have been 5 nmol per gram of ET.

Analysis of plant tissue extracts For sodium thiosulfate-treated ET, the extracted MRM transitions for GSH and GSSG (not detected) are shown in Figure 4a and b. Essentially identical results were obtained from control ET (Figure 4c and d). Control ET extracts contained 37 ± 5 nmol GSH per gram of ET, while sodium thiosulfate-treated ET extracts were found to contain 38 ± 5 nmol GSH per gram of ET. Six samples per treatment group, measured in duplicate, were analyzed over two separate days. Therefore, we conclude that sodium thiosulfate-treatment has no statistical effect on GSH or GSSG content in early-stage loblolly pine ET, as determined by the *t*-test ($n = 6$, $P = 1$).

Douglas fir initiation on media supplemented with reducing agents

Douglas fir initiation 2009 Douglas fir initiation averaged 38.0% across the five crosses for control in Treatment 1 compared with 42.9% and 43.05%, when GSH in Treatment 2 or

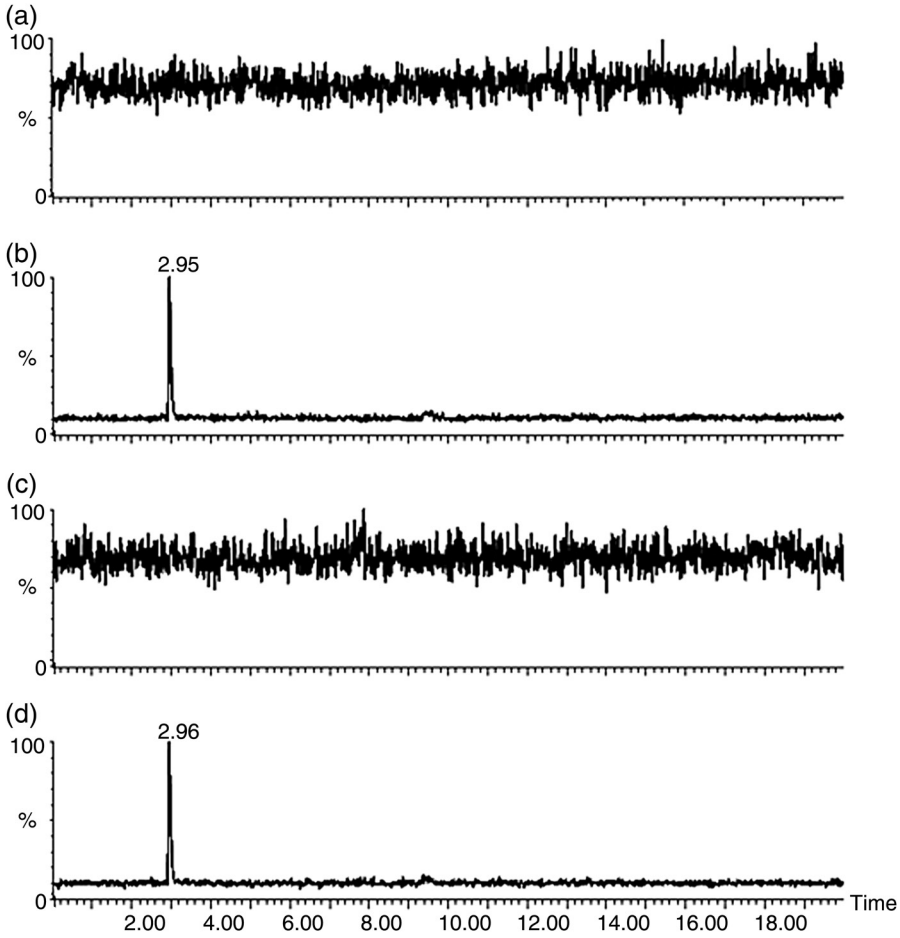

Figure 4. MRM chromatographs of sodium thiosulfate-treated somatic embryo extracts, 150 mg ml^{-1} (a and b) and control ET extracts, 150 mg ml^{-1} (c and d). Samples were analyzed by an HPLC–ESI/MS(MRM) method, see Materials and methods. (a) GSSG (MRM transition 613–355 *m/z*) was not detected. (b) GSH (MRM transition 307.8–179 *m/z*). (c) GSSG (MRM transition 613–355 *m/z*) was not detected. (d) GSH (MRM transition 307.8 to 179 *m/z*). Retention times are displayed above integrated areas. Samples, $n = 6$, were analyzed in duplicate. The data presented are representative of a typical result.

sodium dithionite in Treatment 3 was added (Table 8), but differences were not statistically significant.

Douglas fir initiation 2011 Sodium dithionite initiation percentages were higher for five of nine crosses. Sodium thiosulfate initiation percentages were higher for six of nine crosses tested. However, differences were not statistically significant for either redox supplement (Table 8).

Douglas fir initiation 2012 Initiation percentages averaged 37.4 for the control treatment compared with 41.1 with the addition of sodium dithionite. Differences were not statistically significant at $P = 0.05$ (Table 8).

Douglas fir initiation 2013 Addition of sodium dithionite increased average initiation percentages from 34.2 to 44.5%. All five crosses showed increased initiation in the presence of sodium dithionite. Differences between treatments were statistically significant at $P = 0.05$ (Table 8).

Discussion

Redox changes in the seed and embryo resulting from genetic or environmental factors control plant growth and development (Kocsy et al. 2013). Ratios of ASC:DHA and GSH:GSSG work together in the Ascorbate–Glutathione System to control the seed redox environment (Stasolla 2010, Tyburski and Tretyn 2010). Recently, these compounds have been shown to exert strong effects on embryo development in several plants including *Picea glauca* (Moench) Voss (Stasolla 2010).

The pine seed FG provides nutritional and regulatory materials to the developing embryo. In the in vitro environment, however, many of these natural components may be absent. To better understand the role of redox potential in developing *P. taeda* seed, we began a program of analytical studies for GSH, GSSG, ASC and DHA in seed tissues during development with the goal to supplement the in vitro environment with these chemicals during appropriate developmental stages. We found that medium supplementation with ASC, GSH or inorganic reducing agents increased early-stage somatic embryo growth and ET culture

Table 8. Douglas fir initiation on medium varying in addition of redox agents.

Summer 2009 initiation tests using medium 2301 LOS and modifications										
Medium[1]	Cross 84	Cross 88	Cross 100	Cross 93	Cross 102					Initiation % Average[2]
Control (2301/2433)	61.0 ± 5.0	50.0 ± 6.6	5.2 ± 2.6	60.0 ± 8.3	27.3 ± 5.8					38.0 a
+0.5 mM GSH	54.2 ± 8.9	51.7 ± 5.8	17.0 ± 5.3	52.4 ± 6.9	45.6 ± 4.4					42.9 a
+10 mg l^{-1} Na dithionite	67.3 ± 7.1	55.0 ± 6.1	1.7 ± 0.7	54.0 ± 9.3	48.5 ± 2.5					43.0 a
2011 Initiation tests using medium 2301 and modifications										
	Cross 115	Cross 117	Cross 119	Cross 121	Cross 122	Cross 123	Cross 124	Cross 125	Cross 127	Initiation % Average[2]
Control (2301)	63.3 ± 5.0	9.3 ± 7.2	37.9 ± 7.8	33.1 ± 8.9	42.1 ± 7.9	62.2 ± 12.5	57.4 ± 9.4	36.5 ± 7.5	66.0 ± 7.6	44.4 a
+10 mg l^{-1} Na dithionite	60.0 ± 5.1	15.2 ± 6.1	58.6 ± 7.6	33.2 ± 7.5	21.1 ± 8.5	63.2 ± 11.8	56.5 ± 10.4	25.8 ± 4.0	65.3 ± 9.1	44.2 a
+1.0 µM Na thiosulfate	70.0 ± 7.4	15.6 ± 2.9	41.3 ± 15.5	48.9 ± 10.8	23.1 ± 10.8	63.2 ± 10.3	53.3 ± 6.2	27.0 ± 5.7	72.0 ± 6.3	46.6 a
2012 Initiation tests using medium 2301 and modifications										
	Cross 126	Cross 130	Cross 132	Cross 133	Cross 134	Cross 135				Initiation % Average[2]
Control (2301)	46.0 ± 5.6	48.9 ± 5.8	18.6 ± 7.6	32.9 ± 3.5	38.9 ± 9.2	42.4 ± 9.0				37.4 a
+10 mg l^{-1} Na dithionite	46.0 ± 6.2	46.0 ± 7.7	41.4 ± 7.2	20.3 ± 5.0	36.5 ± 8.8	59.7 ± 4.7				41.1 a
+1.0 µM Na thiosulfate	44.4 ± 5.6	43.8 ± 4.9	33.3 ± 7.2	15.7 ± 5.0	33.3 ± 6.2	52.9 ± 7.7				37.1 a
2013 Initiation tests using medium 2301 and modifications										
	Cross 136	Cross 137	Cross 139	Cross 140	Cross 141					Initiation % Average[2]
Control (2301)	46.0 ± 7.3	65.7 ± 5.2	11.8 ± 7.6	24.5 ± 3.5	19.2 ± 9.2					34.2 a
+10 mg l^{-1} Na dithionite	58.4 ± 4.4	72.9 ± 5.5	35.3 ± 7.2	28.6 ± 5.0	22.7 ± 8.8					44.5 b

[1]2009, Treatment 2—0.5 mM GSH is added to media 2301 and 2433 (Table 1). 2009, Treatment 3—10 mg l^{-1} sodium dithionite is added to media 2301 and 2433. 2011, Treatment 2—10 mg l^{-1} sodium dithionite is added to media 2301. 2011, Treatment 3—1 mM sodium thiosulfate is added to media 2301.[2]Values followed by the same letter are not statistically different by the Multiple Range Test at $P = 0.05$. Analyses are based on arcsine $\sqrt{\%}$ transformation.

initiation for cultures of *P. taeda* as well as increasing initiation for *P. menziesii*. Medium supplementations with GSSG also increased *P. taeda* somatic embryo shoot only or shoot and root growth, with germination increasing from 11.5 to 18.5% when medium was supplemented with 1.0 mM GSSG.

Media improvements have traditionally been based on empirical modifications of existing basic formulations. This approach can be inefficient, costly and does not always produce the desired improvement. Several media for coniferous plants have been developed with the aid of tissue analyses (Pullman and Bucalo 2014). Here, we formulate several new ET initiation media for two coniferous species based partly on analyses of loblolly pine seed tissues for redox chemical content. The data presented here support the concept that fundamental analyses of seed and embryo tissues during the course of development can lead to tissue culture protocol improvements that are more efficient, less costly and potentially more effective.

Ascorbic acid is an important metabolite that is involved in many critical processes in animals and plants. Ascorbic acid functions in plants as an antioxidant, a cofactor for important biochemical reactions and as a precursor for oxalic and tartaric acids (Smirnoff and Wheeler 2000). Recently ASC has been shown to play an important role in seed development (Arrigoni et al. 1992). In *Vicia faba* seed, changes in ASC levels were correlated with different stages of natural embryogenesis. Early embryo development showed high ASC/DHA ratios that declined as embryo development continued. When analyzed in white spruce seeds, ASC peaked during mid-embryo development and then rapidly declined as the tissue shifted to DHA, the more oxidized member of the pair (Stasolla and Yeung 2003, Belmonte et al. 2005*a*). In our analyses of organic acids using mass spectroscopy, ascorbate peaked during mid-development for both FG and embryo tissue of loblolly pine, correlating with the initial accumulation of oxalate (Pullman et al. 2006). In our current research using spectroscopic methods, ASC was also shown to peak during mid-development.

Plants cannot function without GSH and mutations that eliminate the ability to synthesize GSH are lethal (Cairns et al. 2006). Glutathione is an important antioxidant that protects cells against oxidative stress and functions in biosynthetic pathways, antioxidant biochemistry and redox homeostasis (Noctor et al. 2012). Glutathione also appears to be essential for SE, as silencing GSH biosynthetic pathways in wheat inhibited SE (Bossio et al. 2013). Our profiles of GSH and GSSG in zygotic FG and embryo tissues for seed from two trees were similar, with redox agents peaking during mid-development in both. Levels of GSH in the embryo declined sharply after Stage 9.2, suggesting a major shift in the physiology of embryo development.

These data begin to illuminate redox changes during zygotic embryo development and suggest that ASC, GSH and other reducing agents may stimulate growth when applied in concentrations that match physiological concentrations of natural redox

buffers during specific developmental stages. For example, during Stage 1, ASC and GSH are present in FG or zygotic embryo at low concentrations, but DHA and GSSG are not present at all or are barely detectable. Thus, in vitro early-stage somatic embryo growth, such as would occur during ET initiation or maintenance, may benefit from addition of ASC, GSH or other non-toxic reducing agents. Indeed, in our studies, reducing agents increased early-stage somatic embryo growth and ET initiation for *P. taeda* and ET initiation for *P. menziesii*. *Picea glauca*, *P. taeda* and *Araucaria angustifolia* also showed increases in ET initiation or proliferation when medium was supplemented with ascorbic acid, a combination of vitamins including the antioxidant tocopherol (vitamin E), or GSH (Stasolla and Yeung 1999, Pullman et al. 2006, Vieira et al. 2012).

In our studies over 4 years, reducing agents increased loblolly pine initiation averages by 8–99% and *P. menziesii* initiation by 5–30%. Across loblolly pine experiments, 7 of 12 seed sources showed higher initiation percentages when grown on media containing reducing agents. Across Douglas fir experiments, addition of sodium dithionite showed higher initiation percentages for 15 of 25 crosses. Several seed sources more than doubled initiation percentages when reducing agents were included in the medium. Initiation responses to reducing agents varied in different trials and with different seed sources. Some variability in response may be due to oxidation of medium in air over time.

In studies by Stasolla and Yeung (1999), white spruce ET proliferation and early-stage somatic embryo numbers were more than doubled by a reduced environment. ASC (0.1 mM) enlarged apical regions so they produced more leaf primordia and larger shoots, increasing somatic embryo germination of white spruce from 34 to 58%. Embryogenic cell lines of white spruce also had higher ASC:DHA ratios than a non-embryogenic cell line and ASC metabolism was restored as somatic embryos began to germinate (Stasolla and Yeung 2001). Manipulation of the GSH:GSSG ratio in *A. angustifolia* early somatic embryos improved embryo quality through altering nitric oxide emission (Vieira et al. 2012).

Antioxidants such as ASC, cysteine, polyvinylpyrollidone (PVP, PVPP), silver nitrate or tocopherol, are often added to plant tissue culture media to reduce detrimental effects of phenolics released from plant tissue or to improve plant transformation (Trigiano and Gray 2000, Dan 2008). Ascorbate and GSH may also be used in vitro to regulate organogenesis, regeneration and differentiation in plant cultures (Belmonte et al. 2006, Tyburski and Tretyn 2010). However, the relatively high costs of GSH may prohibit its use in many tissue culture protocols. We report here on the feasibility and effectiveness of low-cost antioxidants including sodium bisulfite, sodium dithionite, sodium metabisulfite, sodium sulfite and sodium thiosulfate to increase ET growth or induction of coniferous ET. It is interesting to note that addition of sodium dithionite or sodium thiosulfate further increased loblolly pine ET initiation in a medium already optimized for vitamin E content, suggesting that these antioxidants may have multiple mechanisms of initiation stimulation.

A few reports appear in the literature on the use of alternative antioxidants to stimulate plant growth in vitro. Wang and Janick (1986) included 0.2 mM sodium bisulfite as an antioxidant in their maintenance medium for embryogenic callus of Jojoba. Silver thiosulfate is often used to inhibit ethylene action (Biddington 1992), but sodium thiosulfate was reported to stimulate *Corymbia maculata* (spotted gum) IBA-mediated rooting and plant development as well as tomato root growth in vitro (Steinitz et al. 2010, Steinitz and Bilavendran 2011). Shin and Park (2006) enhanced *Agrobacterium*-mediated transformation efficiency in 3-day-old seedlings of *Brassica juncea* using sodium hydrosulfite, also known as sodium dithionite, as a chemical abrasive. Antioxidants are thought to enhance *Agrobacterium*-mediated transformation through control of reactive oxygen species induced by plant defense responses to pathogen attack along with induction of antioxidant enzymes such as superoxide dismutase and catalase (Zheng et al. 2005, Dan 2008).

Germination in white spruce increased from 22 to almost 70% when conditions favored a more oxidized state (Belmonte et al. 2005*b*, Stasolla 2010). Increasing the ratio of GSH:GSSG improved the total number of embryos produced and a decreasing ratio of GSH : GSSG produced better roots and shoot tips (Stasolla 2010). An explanation for improved germination with applied GSSG may occur through the more oxidized environment increasing activity of monodehydroascorbate reductase that reduces monodehydroascorbate to ascorbate (Stasolla 2010). Somatic embryo maturation in loblolly pine did not improve when maturation media were supplemented with GSH or GSSG (data not shown). However, when germination experiments were carried out, statistically significant increases in shoot emergence or shoot and root growth occurred with addition of 1.0 mM GSSG. This was a surprise as germination is reported to be stimulated by GSH and not GSSG (Tommasi et al. 2001). A possible explanation may be in the immaturity of loblolly pine somatic embryos that resemble Stages 8–9.1 (Pullman et al. 2003*a*). Immature pine somatic embryos may respond to the oxidized environment of GSSG by developing slightly further, resulting in increased germination.

Our analysis of early-stage loblolly pine ET extracts found no detectable GSSG and nanomolar concentrations of GSH per gram of ET, in both control and sodium thiosulfate-treated populations. Experiments with standards confirmed that no conversion of either GSH or GSSG occurred during the extraction or ionization processes. Thus, we are confident that our data represent the true values of GSH and GSSG present in the ET. In agreement with these findings, our analyses of early-stage zygotic embryos, which had been dissected from their FGs, contain no detectable GSSG, as measured by a colorimetric assay adapted from the literature (Zhang and Kirkham 1996,

Figure 2). In further agreement, our analyses of zygotic tissues show that early-stage zygotic loblolly pine embryos contained GSH concentrations of 5–15 nmol per gram of ET. We note that somatic and zygotic embryos may differ in metabolite concentrations and estimation of growth stage is not precise.

We had hypothesized that treatment with sodium-thiosulfate would increase the amount of GSH and decrease the amount of GSSG in early-stage ET. However, we observed no effect of treatment with sodium thiosulfate on GSH or GSSG content. Given that we now report that GSSG was not detected in early-stage zygotic or somatic loblolly pine embryos it would be unexpected that treatment with a reducing agent would alter the GSH : GSSG ratio. We note that the values obtained for GSH content in control and sodium thiosulfate-treated ET are virtually identical. We therefore conclude that even if an amount of GSSG below our limit of detection was being reduced by treatment with sodium thiosulfate, this amount would have to be so small that it is indistinguishable from the statistical noise present in our GSH content measurements.

It is well-known many types of cells possess both GSH-based and thioredoxin-based antioxidant systems. These antioxidant systems protect cells against harmful oxidizing agents and also maintain a redox balance among cellular components. Thioredoxins have now been identified in plants. Thioredoxins are encoded by a multigenic family consisting of eight genes in *Arabidopsis thaliana*, and a family of at least five genes in *Populus* sp. (Gelhaye et al. 2004). At least 20 thioredoxin isoforms have been found in *A. thaliana* (Gelhaye et al. 2005). There is evidence that, at least in some cell types, crosstalk occurs between the GSH- and thioredoxin-based antioxidant systems (Casagrande et al. 2002), and this underscores the complexity of the metabolic relationship between the various cellular redox agents which would be expected to affect many cellular processes, including embryonic development. For example, Belmonte and Stasolla (2009) reported that expression of one of the major embryogenesis control genes, HBK3 (a class I homeobox knox 3 gene in *P. abies*) is associated with ascorbate and GSH metabolism. This gene is required for the proper differentiation of proembryogenic masses into somatic embryos. Clearly, much more information will be needed to unravel the mechanisms that underlie the effects of redox agents such as sodium-thiosulfate on SE.

The analyses of redox agents in developing loblolly pine tissues not only provided valuable clues that assisted in improving initiation and germination media, but also identified a major change as zygotic embryos reach Stage 9.1, when cotyledons have curved at their tip and expanded to enclose the shoot apical meristem. Loblolly pine zygotic embryos continue development after Stage 9.1, but somatic embryos stop at this point (Pullman et al. 2003*a*). Physiologically and biochemically, Stage 9.1 appears to represent a turning point in embryo development. A transition occurs between Stages 8 and 9.1 in the ability of zygotic embryos to germinate. At Stage 8 and earlier, few embryos germinate to form a root and shoot, whereas at Stage 9.1 and later, embryos germinate increasingly to form both root and shoot (Pullman et al. 2003*a*). Redox agents appear to rapidly decline at this point along with other major physiological changes. The osmotic potential of FG tissue begins to rise rapidly at this point indicating a shift to accumulation of storage products (Pullman and Johnson 2009*b*). At the same time, embryo maltose content plunges, indicating a shift from maltose to other carbohydrates including d-*chiro*-inositol, fagopyritol B1, melibiose, melezitose and raffinose (Pullman and Buchanan 2008). Sugars are also known to act as signaling agents and antioxidants involved in plant ROS balance (Couee et al. 2006, Bolouri-Moghaddam et al. 2010). Somatic embryo quality improvement research in conifers should now focus on this developmental point to further improve protocols for embryo development and improved embryo quality.

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Conflict of interest

None declared.

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