Analysis of Seed Redox Chemicals in Loblolly Pine to Improve Somatic Embryo Growth and Germination

Gerald S. Pullman[1,2], Brandi Copeland[1], and Xiaoyan Zeng[2]

Abstract: Glutathione/ glutathione disulfide and ascorbic acid /dehydroascorbate are two major redox pairs that control the redox-state in a developing seed. Recently these redox compounds have been shown to exert strong positive effects on embryo development in several plants including white spruce. A picture is emerging that early-stage embryo development occurs best in the presence of a reducing environment while late-stage development requires a shift to an oxidizing environment. Knowledge of physiological concentrations of these compounds during pine embryo development would allow us to better mimic the seed environment and improve somatic embryo development.

Triplicate analysis for the seed redox chemicals ascorbic acid (AsA), dehydroascorbate (DHA), glutathione (GSH) and glutathione disulfide (GSSG) were completed for developmentally staged zygotic embryos and female gametophytes over the sequence of loblolly pine (*Pinus taeda* L) seed development. The two trees tested generally showed similar concentrations and patterns of change through time. The redox potential changed significantly through the progression of seed development. These profiles suggest that early-stage embryo development occurs best in the presence of a reducing environment while late-stage development requires a shift to an oxidizing environment. The redox chemical profiles obtained provide guidelines and targets to mimic in the tissue culture environment.

Recent and ongoing tests have shown that alteration of the *in vitro* redox environment through medium supplementation with glutathione or glutathione disulfide during specific developmental stages can indeed enhance loblolly pine somatic embryo development and germination.

INTRODUCTION

Loblolly pine (LP, *Pinus taeda* L.) and its close relatives are among the most commercially important species grown in the U.S., composing about 23% of the total U.S. harvest of all species, 37% of the U.S. softwood harvest, and 58% of the total Southern harvest of softwoods (Forest Service FIA Data Retrieval System 2004). LP is the major species planted across the southern U.S. ranging from New Jersey to Texas with 1 to 1.5 billion trees planted annually (Schultz 1999). Since pine plantations in the South are expected to increase 67% from 1999 to 2040, methods to provide the best planting stock will become increasingly important (Prestemon and Abt 2002).

[1]School of Biology, Georgia Institute of Technology, Atlanta, GA 30332-0620
[2]Institute of Paper Science and Technology, Georgia Institute of Technology, Atlanta, GA 30332-0620

Forest productivity can be increased by planting tree farms with large numbers of elite, high-value trees. To better take advantage of long-term breeding programs, genetic engineering advances, product improvements based on selected fiber characteristics, and to produce chemicals from renewable tree biomass, methods to propagate large numbers of superior conifer trees are needed. Clonal propagation by somatic embryogenesis (SE) can capture the benefits of breeding or genetic engineering programs to multiply high-value trees and improve wood quantity, quality, and uniformity. Factors limiting commercialization of SE for loblolly pine include low culture initiation from recalcitrant crosses, low culture survival, culture decline causing low or no embryo production, and inability of many somatic embryos to fully mature resulting in low germination and slow initial growth of somatic seedlings. Each of these barriers contribute to high somatic seedling costs and must be removed for SE technology to reach full potential value.

In pine seed, the zygotic embryo grows and develops within the megagametophyte or female gametophyte (FG). Somatic embryos, however, are cultivated in the absence of megagametophyte, and the culture medium provides nutrients and developmental signals. Optimization of the nutritional, physical, hormonal, and gaseous environments in vitro is critical for the growth and development of high-quality, robust somatic embryos. We have used analyses of zygotic tissues and the seed environment to suggest targets for the growth medium. We extend this approach to include understanding the oxidation reduction environment in developing LP seeds and the major redox chemicals that create this environment with a view to use this knowledge to modify the in vitro environment.

MATERIALS AND METHODS

Plant material. LP pinecones were collected and their embryos' and female gametophytes' were separated by stage and were catalogued and stored at -80°C. 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. Seeds were collected using the methods described by Pullman and Buchanan (2006).

Redox chemical analysis using Ultra-Violet Spectroscopy. The sorghum seeding procedure of Zhang and Kirkham (1995) 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 made daily by adding to 25 mL DI H_2O; 0.0129 g of dithiothreitol (DTT) 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. Between 0.1 and 0.15 gram samples were weighed. Not all samples had a mass large enough for this weight due to small embryo size for early stages. 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 minutes.

For total ascorbate (TAsA), 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 minutes at room temperature. 0.15 mL of 0.5% N-ethylmaleimide was added to remove the excess DTT. 2.1 mL of the color developer mixture was added. This was vortexed for 20 seconds and then incubated in a water bath for 40 minutes at 40°C. These were then put in cool water to stop the reaction and centrifuged for five minutes. The extract was tested in a UV 2450 UV-visible spectrometer at a wavelength of 525 nm.

For ascorbic acid (AsA), 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 was then incubated for 10 minutes at room temperature. 2.1 mL of the color developer mixture was added. This was vortexed for 20 seconds and then incubated in a water bath for 40 minutes at 40°C. These were then put in cool water to stop the reaction and centrifuged for five minutes. 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 ascorbic acid.

The glutathione (GSH) and glutathione disulfide (GSSG) testing procedure was loosely adapted from the procedures of Smith (1985) and Griffith (1980). Total glutathione (GSH + GSSG) were 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_2PO_4 (pH 7.5), and 50 µl dH_2O and shaken until an emulsion formed. This was then incubated for 60 minutes at room temperature (25°C). The following reaction occurred rapidly, so the following chemicals were added directly to a glass cubit: 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. This 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 was recorded for 70 seconds. To standardize absorbencies, the reading at 1 second was subtracted from the reading at 61 seconds.

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_2PO_4 (pH 7.5), and 50 µl 2-vinyl pyridine and shaken until an emulsion formed and incubated for 60 minutes at room temperature (25°C). The following reaction occurs rapidly, so the following chemicals were added directly to the glass cubit: 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. This 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 was recorded for 70 seconds. To standardize absorbencies, the reading at 1 second was subtracted from the reading at 61 seconds. Glutathione (GSH) was estimated from difference between total glutathione and glutathione disulfide.

Growth of early-stage somatic embryos on medium varying in redox chemical content. Early-stage somatic embryos of LP, grown in suspension culture in liquid medium 1133 (Pullman et al. 2006), were used as explants for growth bioassays to evaluate a medium's potential to support growth of embryogenic tissue. SE cultures were established and grown in liquid medium 1133 (Pullman and Webb 1994, Pullman et al. 2003). Single stage-2 embryos were isolated with forceps from suspension culture and placed on 2 ml of medium 1250 (Pullman et al. 2006) or 1250 + redox chemical contained in Costar #3526 Well Culture Cluster Plates. Explants were grown for 4-7 wks in the dark at 23-25°C and then measured for embryogenic tissue diameters. Three genotypes each with forty single early-stage somatic embryos per genotype were grown on test medium arranged in four replicates of 10 embryos.

Somatic embryo germination on medium varying in redox chemical content. The germination protocol of Vales et al. (2007) was used. Medium 397 was supplemented with 220 mg/l $CaCl_2 \bullet 2H_2O$ (an error in the original publication) and used as a control or supplemented with glutathione or glutathione disulfide.

RESULTS AND DISCUSSION

Redox chemical analysis using Ultra-Violet Spectroscopy. Profiles for the four redox chemicals analyzed are shown in Figures 1-8. Ascorbic acid and dehydroascorbate peaked in mid-development and then decreased in the embryo (Figures 1, 3). Pullman and Buchanan (2006) saw similar ascorbic acid peaking around stage 7-9.1 in organic acid analyses performed with mass spectroscopy. Ascorbic acid and dehydroascorbate in female gametophyte did not show a consistent pattern during development (Figures 2, 4). Both glutathione and glutathione disulfide in both embryo and female gametophyte increased until mid-development and then decreased. This suggests the pool of glutathione + glutathione disulfide is maximal at mid development but the ratio of glutathione to glutathione disulfide is about 5:1 during mid development and continues to increase until the end of development.

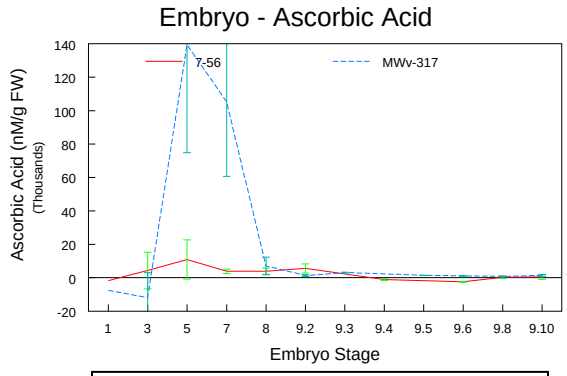


Figure 1: Embryo ascorbic acid in nmoles/g Fresh Weight for stages 1 through 9.10 for tree 7-56 and tree MWv-317.

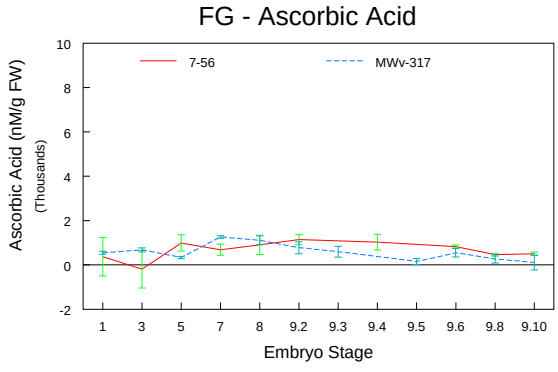


Figure 2: Female gametophyte ascorbic acid in nmoles/g Fresh Weight for stages 1 through 9.10 for tree 7-56 and tree MWv-317.

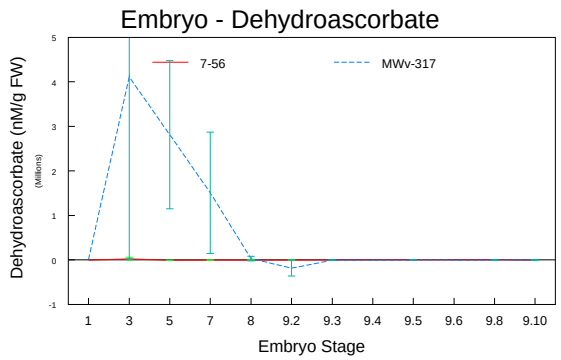


Figure 3: Embryo dehydroascorbate in nmoles/g Fresh Weight for stages 1 through 9.10 for tree 7-56 and tree MWv-317.

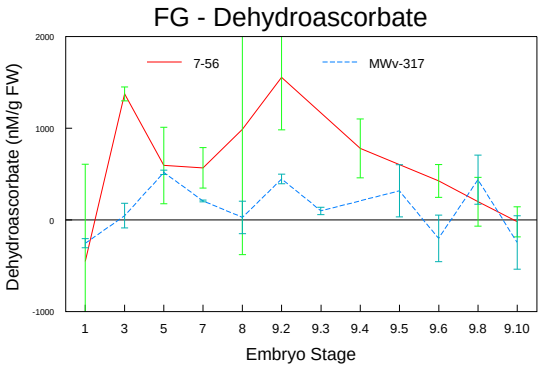


Figure 4: Female gametophyte dehydroascorbate in nmoles/g Fresh Weight for stages 1 through 9.10 for tree 7-56 and tree

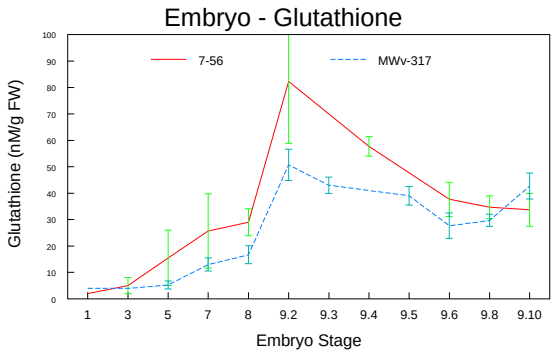


Figure 5: Embryo glutathione in nmoles/g Fresh Weight for stages 1 through 9.10 for tree 7-56 and tree MWv-317.

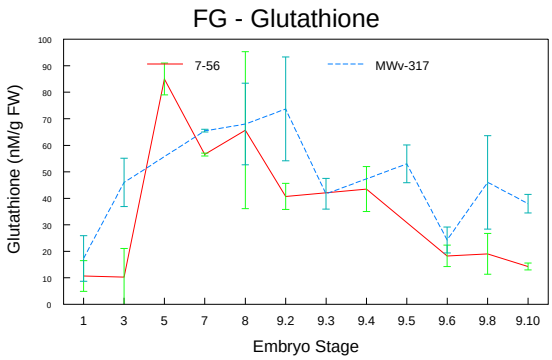


Figure 6: Female gametophyte glutathione in nmoles/g Fresh Weight for stages 1 through 9.10 for tree 7-56 and tree MWv-

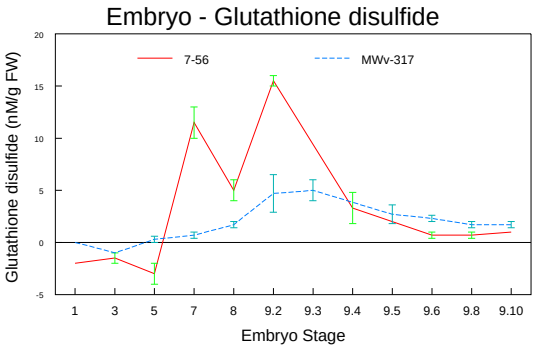


Figure 7: Embryo glutathione disulfide in nmoles/g Fresh Weight for stages 1 through 9.10 for tree 7-56 and tree MWv-317.

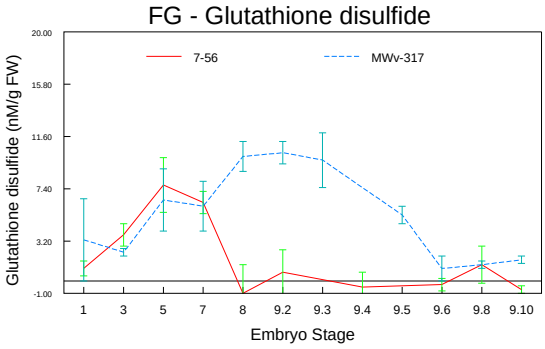


Figure 8: Female gametophyte glutathione disulfide in nmoles/g Fresh Weight for stages 1 through 9.10 for tree 7-56 and

Somatic embryo growth and germination. Early-stage somatic embryo growth tests with ascorbic acid, glutathione and glutathione disulfide indicate that medium supplementation with reduced ascorbic acid or reduced glutathione increases growth (Tables 1 and 2). Ascorbic acid increased LP early-stage somatic embryo when added to medium at 25 mg/l (0.14 mM) (Table 1). Glutathione also increased somatic embryo growth when added to medium at 0.5 mM (Table 2). Medium containing glutathione disulfide at 0.1 or 0.5 mM did not alter somatic embryo growth and medium containing 1.0 mM glutathione disulfide decreased growth.

Table 1. Average colony diameter (mm) of loblolly pine embryogenic tissue grown on medium with four concentrations of ascorbic acid.

Media	Ascorbic acid (mg/l)	Genotype and embryogenic tissue colony diameter + SE (mm)			
		249	351	500	Average (%)*
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

*Four reps of ten 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 2. Average colony diameter (mm) of loblolly pine embryogenic tissue grown on control medium and medium with three concentrations of glutathione (GSH) or glutathione disulfide (GSSG).

Media	Redox chemical (mg/l)	Genotype and embryogenic tissue colony diameter ± SE (mm)			
		132	203	500	Average (%)*
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

*Four reps of ten 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.

Germination tests with somatic embryos showed a surprising result. Germination medium supplementation with glutathione disulfide at 1.0 mM increased shoot development in germination tests with genotypes that did not form roots (Table 3). The same treatment also increased germination (shoot and root development) in genotypes that were able to germinate (Table 4). In both cases increases were statistically significant at P = 0.10 or less.

Table 3. Average shoot production during germination of loblolly pine somatic embryos from recalcitrant genotypes that do not normally germinate. Embryos were tested on control medium and medium with three concentrations of glutathione (GSH) or glutathione disulfide (GSSG).

Media	Redox chemical (mg/l)	Genotype and somatic embryo shoot production (%)			
		132	178	186	Average (%)*
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

*Five reps of ten 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 4. Average germination of loblolly pine somatic embryos tested on control medium and medium with three concentrations of glutathione (GSH) or glutathione disulfide (GSSG).

Media	GSSG (mg/l)	Genotype and somatic embryo germination (%)				
		200	222	433	464	Average (%)*
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

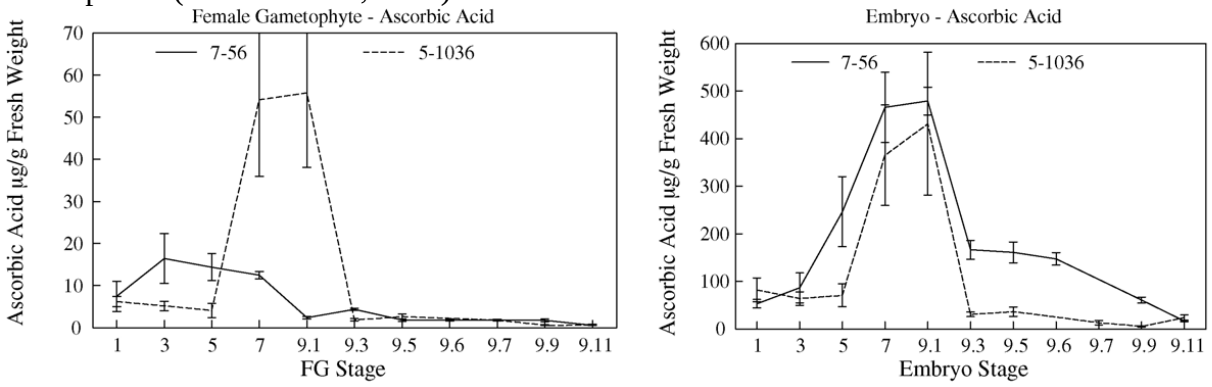
*Five reps of ten embryos were tested per medium per genotype. Percentages were transformed by arcsine √%. Values followed by same letters are not statistically different by Multiple Range Test at P=0.08. SE = standard error.

Glutathione/ glutathione disulfide and ascorbic acid /dehydroascorbate are two major redox pairs that work together in the Ascorbate-Glutathione System to control the redox-state in a developing seed (Belmonte et al. 2005b). Redox regulation is well established to control many aspects of cell growth (Buchanan and Balmer 2005).

Ascorbic acid (vitamin C) 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 ascorbic acid has been shown to play an important role in seed development (Arrigoni et al. 1992). In *Vicia faba* seed, changes in ascorbic acid were correlated with different stages of natural embryogenesis. Early embryo development showed high ascorbic acid/ dehydroascorbate ratios that declined as embryo development continued. When analyzed in white spruce, ascorbic acid (reduced form) peaked during mid embryo development and then rapidly declined as the tissue shifted to the more oxidized member of the pair (dehydroascorbate) (Belmonte et al., 2005b). In our prior work on organic acids, ascorbate peaked during mid-development for both FG and embryo tissue, correlating with the initial accumulation of oxalate (Figure 9, Pullman et al. 2006). In our tests, 0.14–0.57 mM ascorbic acid increased early-stage somatic embryo growth. Ascorbic acid (0.1 mM) also has been shown to increase somatic embryo germination of white spruce by enlarging apical regions so more leaf

primordia and larger shoots form (Stasolla and Yeung, 1999). Our redox measurements in LP FG and embryos begin to document redox changes during embryo development in LP and supports the hypothesis that manipulation of the in vitro redox environment will improve somatic embryo growth.

Figure 9. Loblolly pine female gametophyte and embryo ascorbic acid profiles across seed development (Pullman et al., 2006).



Glutathione / glutathione disulfide, the second redox pair of the Ascorbate–Glutathione System, are also important in creating the redox environment. Manipulation of the tissue culture environment with glutathione (reduced) or glutathione disulfide (oxidized) has also been shown to improve embryo development in angiosperms and white spruce (De Gara et al., 2003, Yeung et al. 2005, Belmonte et al. 2005a, Belmonte et al. 2006).

The ratio of GSH: GSSG seems to be more important than the actual amounts of GSH and GSSG (Yeung et al. 2005). The increasing ratio of GSH: GSSG improves total number of embryos produced and a decreasing ratio of GSH: GSSG produces better roots and shoot tips. Ascorbic acid has been found in high concentrations in early stage embryos and dehydroascorbate in late stage embryos (Stasolla et al. 2001).

Our recent LP somatic embryo experiments using the above information indeed show that we are able to increase early-stage embryo growth by supplementing the medium with ascorbic acid (reduced) or glutathione (reduced). In addition, we have been able to improve embryo germination by supplementation with glutathione disulfide (oxidized). Taken together these results show promise in optimizing the embryo development and germination environments for loblolly pine somatic embryos through medium supplementation with stage-specific redox chemicals.

Acknowledgements. Authors thank the member companies of the Institute of Paper science and Technology at Georgia Tech and the Consortium of Plant Biotechnology Research, by DOE Prime Agreement No. 14 DEFG36-02GO12026 for financial support. We also thank MeadWestvaco Corporation and Weyerhaeuser Company for cone collections. In addition, the authors are grateful for the valuable technical assistance of Madeline Brumby, Kylie Bucalo, Mike Buchannan, Erin Clark, Molly Clark, Xiying He, Daniel Ma and Shadeah Suleiman.

REFERENCES

Arrigoni O, L. De Gara, F. Tommasi and R. Liso. 1992. Changes in the ascorbate system during seed development in *Vicia faba* L. Plant Physiology 99: 235-238.

Belmonte, M.F., G. Donald, D.M. Reid, E.C. Yeung, and C. Stasolla. 2005a. Alterations of the glutathione redox state improve apical meristem structure and somatic embryo quality in white spruce (*Picea glauca*). J Exp Bot 56: 2355-2364.

Belmonte, M.F., J. Macey, E.C. Yeung, and C. Stasolla. 2005b. The effect of osmoticum on ascorbate and glutathione metabolism during white spruce (*Picea glauca*) somatic embryo development. Plant Physiology and Biochemistry 43: 337-346.

Belmonte, M.F., S. J. Ambrose, AR.S. Ross, S.R. Abrams and C. Stasolla. 2006. Improved development of microspore-derived embryo cultures of *Brassica napus* cv Topaz following changes in glutathione metabolism. Physiologia Plantarum 127: 690-700.

Buchanan, B.B. and Y. Balmer. 2005. Redox regulation: A broadening horizon. Ann Rev Plant Biol 56: 187-220.

De Gara, L., M.C. De Pinto, V.M.C. Moliterni and M.G. D'Egidio. 2003. Redox regulation and storage processes during maturation in kernels of *Triticum durum*. J Exp Bot 54: 249-258.

Forest Service FIA Data Retrieval System. 2004. http://ncrs2.fs.fed.us/4801/fiadb/index.htm (courtesy of A. Goetzl, AF&PA).

Griffith, O.W. 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-Vinylpyridine. Analytical Biochemistry 106: 207-212.

Pullman, G.S., S. Johnson, G. Peter, J. Cairney, N. Xu. 2003. Improving loblolly pine somatic embryo maturation: Comparison of somatic and zygotic embryo morphology, germination, and gene expression. Plant Cell Rep. 21:747-758.

Pullman, G.S. and M. Buchanan. 2006. Identification and quantitative analysis of stage-specific organic acids in loblolly pine (*Pinus taeda* L.) zygotic embryo and female gametophyte. Plant Sci 170:634-647.

Pullman G.S., R. Chopra and K-M. Chase. 2006. Loblolly pine (*Pinus taeda* L.) somatic embryogenesis: Improvements in embryogenic tissue initiation by supplementation of medium with organic acids, Vitamins B_{12} and E. Plant Sci 170:648-658.

Pullman, G.S. and D.T. Webb. 1994. An embryo staging system for comparison of zygotic and somatic embryo development, Minneapolis, MN, Oct 3-6, 1994, Proc. TAPPI Biol. Sci. Sym., TAPPI Press, pp 31-34, ISBN 0-89852-930-1.

Prestemon, J.P. and R.C Abt. 2002. Timber products supply and demand. In: Southern Forest Resource Assessment. Eds. DN Wear and JG Greis. Gen. Tech. Rep. SRS-53. Asheville, NC: U.S. Department of Agriculture, Forest Service, Southern Research Station, pp 299-325.

Schultz, R.P. 1999. Loblolly – the pine for the twenty-first century. New For 17:71-88.

Stasolla, C. and E.C. Yeung. 1999. Ascorbic acid improves conversion of white spruce somatic embryos. In Vitro Cell Dev Biol Plant 35: 316–319.

Zhang, J and M.B. Kirkham. 1996. Antioxidant responses to drought in sunflower and sorghum seedlings. New Phytologist 132: 361-373.

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

Smirnoff, N. and G.L. Wheeler. 2000. Ascorbic acid in plants: biosynthesis and function. Crit Rev Biochem Mol Biol 35: 291–314.

Smith, I.K. 1985. Stimulation of glutathione synthesis in photorespiring plants by catalase inhibitors. Plant Physiol 79: 1044-1047.

Yeung, E.C., M.F. Belmonte, L.T.T. Tu and C. Stasolla. 2005. Glutathione modulation of in vitro development. In Vitro Cellular Dev Biol – Plant 41: 584-590.

Vales, T., X. Fang, L. Ge, N. Xu, J. Cairney, G.S. Pullman and G.F. Peter. 2007. Improved somatic embryo maturation in loblolly pine by monitoring ABA-responsive gene expression. Plant Cell Rep 26:133-143.