Abstract The prerequisite for shoot, root or somatic embryo formation in plant in vitro culture is the development of meristem from dedifferentiated cells of the explant tissue. Auxin and cytokinin levels and their relative ratios play a decisive role in inducing the morphogenetic pathways leading to shoot, root or somatic embryo formation in plant in vitro cultures. Exogenous auxin is required to maintain the high rate of an unorganised growth in plant cell suspension cultures. On the other hand, the proliferation of hairy root cultures is usually dependent on endogenous hormonal factors. Auxin and cytokinin execute their regulatory role by being involved in a cross-talk with numerous endogenous factors affecting cell division and differentiation. Among them, ascorbate/dehydroascorbate (ASC/DHA), glutathione/glutathione disulphide (GSH/GSSG) redox pair, \( \text{H}_2\text{O}_2 \) and other components of cellular redox systems play an important role in triggering developmental responses in plant in vitro culture. Ascorbate, glutathione and related enzymes participate in the responses to auxin/ cytokinin treatments. In addition, they can even directly affect hormone metabolism in tissue. Ascorbate and glutathione have important regulatory roles in the process of cell-cycle progression within the meristems, where they participate in redox-dependent determination of proliferation and quiescence patterns. The mechanism underlying the regulatory effects of ascorbate and glutathione in cell divisions is not fully elucidated; however, it seems to be related to the regulation of nucleotide synthesis. Ascorbate levels in apoplast modulate the rate of organ elongation by increasing cell wall extensibility. Besides the effects on cell proliferation and growth, ascorbate and glutathione concentrations as well as the enzymes of their metabolism protect the in vitro cultured tissues against oxidative stress. This function is of particular importance during root regeneration and the elicitation of metabolite production by hairy root cultures, where increased levels of oxidising agents are often required to stimulate both processes. In this review, we report recent studies on the involvement of ascorbate and glutathione in the processes of regeneration and proliferation in plant tissue culture.
Keywords  Ascorbate • Glutathione • In vitro • Rooting • Shoot regeneration • Somatic embryogenesis

1 Regenerating Plants from In vitro Cultured Cells, Tissues and Organs

The capacity of plant cells to undergo inducible morphogenetic pathways is essential for various biotechnological applications of plant cell culture, such as clonal propagation and genetic transformation. Morphogenesis in plant tissue culture may occur in two ways. It may result in the formation of bipolar structures called somatic embryos, equipped with shoot and root meristem. Alternatively, organogenesis may occur leading to the formation of unipolar structures, i.e. shoots or roots. Whereas, a somatic embryo directly develops into a complete plantlet, shoot organogenesis must be followed by root organogenesis at the base of a shoot before the newly regenerated plant is transferred to ex vitro conditions (Warren 1993) (Fig. 1).

The process of somatic embryogenesis may be divided into two phases: induction and expression. During the induction phase, differentiated somatic cells of an explant, undergo de-differentiation, acquire embryogenic competence and proliferate as embryogenic cells. In the expression phase, the embryogenic cells differentiate to form somatic embryos (Namasivayam 2007). In few experimental models, somatic embryos follow a sequence of development comprising the stages of embryo formation similar to those, which are observed during zygotic embryogenesis, i.e. globular, heart shape and torpedo. Such developmental pattern occurs during embryogenesis of carrot callus cells cultured as a suspension in liquid medium. This system is one of the most controllable somatic embryogenesis processes, and is often used as a model system in studies on cell differentiation. However, usually, developmental events that give rise to a somatic embryo in culture show far more variation than the equivalent process in the ovule. Somatic embryos exhibit a much greater range of sizes and shapes than the zygotic embryos (Warren 1993).

Fig. 1  Most important developmental pathways in plant tissue culture. See text for details
Somatic embryogenesis can proceed as a direct or indirect process. In direct embryogenesis, which is a relatively rare event, embryos develop directly on the surface of organised tissue such as leaf, stem, zygotic embryo, etc. Alternatively, the much more common indirect somatic embryogenesis requires an intermediary step of meristematic cluster formation preceding the embryo development. This kind of embryogenesis often occurs in cell suspensions and callus culture (Namasivayam 2007). Typically in meristematic clusters, only a few surface cells give rise to embryos; however the presence of cells that do not undergo embryogenesis is necessary within the cluster to complement cells, which are directly involved in embryo formation (Kreuger and van Holst 1993).

Induction of plant explants for somatic embryo formation generally requires a pretreatment on auxin-supplemented medium. Among auxins, the most frequently used was 2,4-diphenoxycetic acid (2,4-D). However, other auxins like β-naphthoxyacetic (NAA) and indole butyric acid (IBA) were also used for this purpose (Razdan 2003). The formation of meristematic clusters containing embryogenically competent cells occurs during the period of auxin treatment. On the other hand, the following phase, when competent cells develop into embryos, requires the reduction or removal of auxin from the medium. This is achieved by transferring cells to a new medium with a very low level of auxin or no auxin at all (Razdan 2003). Nevertheless, auxin treatment is most frequently used to induce somatic embryogenesis. However, the effect of other plant growth regulators should not be overlooked (Jimenez 2001). For example, in the case of nucellus cultures of *Vitis*, simultaneous presence of NAA and benzylaminopurine (BAP) were inductive for embryogenesis. In some systems, somatic embryogenesis was induced by abscisic acid (ABA) (Nishiwaki et al. 2000) or even occurred on hormone-free medium (Choi et al. 1998).

Plant organogenesis in vitro is a more controllable process than embryogenesis (Warren 1993). Different morphogenetic pathways, i.e. shoot or root formation, may be induced in culture by application of the appropriate hormones in the medium. Although the exact nature of these hormonal signals may vary between species, the balance of auxin to cytokinin has been found to have a consistent effect on the type of regenerated organs. A relatively high ratio between auxin and cytokinin promotes the regeneration of roots. Whereas, shoot organogenesis is a preferred type of differentiation on culture media supplemented with high cytokinin and low auxin concentration (Skoog and Miller 1957; Christianson and Warnick 1983). The explants cells proliferate to form callus when the same concentrations of auxin and cytokinin are added to the medium. Callus cells are usually grown on solid media. Alternatively, long term cultures of friable callus may be grown on liquid media as cell suspension cultures (Zhao et al. 2008) (Fig. 1).

Root cultures may be derived without the use of exogenous growth regulators by infecting plant explants with *Agrobacterium rhizogenes*. This gram-negative soil bacterium transfers a DNA segment (T-DNA) from its large root-inducing (Ri) plasmid into the genome of the infected plant. This T-DNA carries a set of genes that encode enzymes which control auxin and cytokinin biosynthesis. The new hormonal balance induces the formation of proliferating roots, called hairy roots, that emerge at the wounding site. The hairy root phenotype is characterized by fast
hormone-independent growth, increased lateral root branching and genetic stability (Guillon et al. 2006) (Fig. 1).

Temporal requirements for a specific balance of phytohormones for the organogenesis process indicate that organ regeneration is accomplished in three phases (Christianson and Warnick 1983). In the first phase, cells of the explants acquire competence, which is defined as the ability to respond to hormonal signals. The competence acquisition involves dedifferentiation of explants cells, which re-enter cell cycle. Competent cells are then canalized and determined for specific organ formation under the influence of phytohormone balance through the second phase, referred to as induction phase. Organ primordia differentiate from induced explants cells and their further development and outgrowth occurs during the third phase. These processes usually proceed independently of the exogenously supplied phytohormones (Christianson and Warnick 1983; Sugiyama 1999). Studies using Arabidopsis temperature sensitive mutants (srd1, srd2, srd3) defective for shoot and/or root redifferentiation revealed further complexity of the process of competence acquisition (Ozawa et al. 1998). It has been found that hypocotyl explants grown on callus-inducing medium first become competent in root organogenesis and then gain competence in shoot organogenesis. Therefore, dedifferentiation stage is divided into two sub-phases occurring sequentially one after another. Finally, explants cells are competent in regeneration of both roots and shoots. The transition from an incompetent state to competence in root organogenesis and from competence in root organogenesis to competence in root and shoot organogenesis requires the functions of SRD2 and SRD3 gene, respectively (Ozawa et al. 1998).

In spite of more than 50 years of studies, molecular and biochemical processes underlying morphogenesis in tissue culture are not fully understood. However, the growing list of genes which are known to be specifically involved in organogenesis and/or somatic embryogenesis mark significant advances in the field of elucidating the mechanism of plant regeneration in vitro (Philips 2004). It was found that the SRD2 gene, involved in control of proliferation competence and dedifferentiation, encodes for a nuclear protein responsible for activation of snRNA transcription. At present, it remains unclear which molecular event, subsequent to the activation of snRNA transcription, is responsible for the elevation of cell proliferation competence (Ohtani and Sugiyama 2005). Re-entering the cell cycle by quiescent cells during dedifferentiation is correlated with expression of cell cycle-related genes such as cyclins and cyclin-dependent kinases (CDK). Among them, gene cdc2At coding PSTAIRE domain-containing CDK and CYCD3 coding a D-type cyclin are possibly involved in committing the dedifferentiating cells to the cell cycle (Sugiyama 1999).

A developmental pathway leading to shoot organogenesis was found to be related to genes involved in cytokinin perception and signalling (Sugiyama 1999, 2000). The establishment and maintenance of shoot meristem is dependent on meristem identity genes such as SHOOT MERISTEMLESS (STM), WUSHEL (WUS) and CLAVATA (CLV). These genes function for the establishment of shoot apical meristems only after dedifferentiated cells are determined for shoot organogenesis (Philips 2004). In accordance with the essential role of auxin in root regeneration, an important role of genes engaged in auxin perception and signalling was also
identified. The *ROOTING AUXIN CASCADE (RAC)* gene coding for Rac/Rop GTPase (Tao et al. 2002) mediating an auxin-signalling pathway is involved in an early stage of auxin perception specific to the formation of adventitious roots (Sugiyama 1999). Similar to organogenesis, the process of somatic embryogenesis involves reprogramming of gene expression patterns. Vegetative-embryonic transition is marked by changes in expression of gene coding for somatic embryogenesis receptor-like kinase (SERK), which was identified as a specific marker distinguishing individual embryo-forming cells from non-embryogenic cells in carrot suspension culture. The transcription factors *LEAFY COTYLEDON 1 (LEC1)* and *WUSHEL (WUS)* are possibly involved in inducing and maintaining embryogenesis in culture (Namasivayam 2007).

## 2 Interaction of Ascorbate and Glutathione with Auxin and Cytokinin

A decisive role in regulating the morphogenetic pathways in plant tissue culture is attributed to auxin and cytokinin. Below, we describe possible links between these growth regulators and several aspects of ascorbate and glutathione metabolism. These interactions may possibly be important for understanding the roles of these antioxidants in differentiation and growth in plant in vitro culture.

### 2.1 Auxin

An apoplastic enzyme ascorbate oxidase (AOX) provides a clear link between regulatory action of auxin and ASC. Highest levels of AOX activity were detected in young and growing parts of the plant. For example, AOX activity in rapidly growing immature pumpkin fruits was 15–20 times higher than that in mature fruits, which no longer increase in size (Esaka et al. 1992). In tobacco plants, high levels of AOX transcript were detected in young and growing parts like upper leaf, upper stem and root but little or none in old tissues such as lower leaf and lower stem (Kato and Esaka 1996). Similarly, in tomato seedling roots, AOX activity was stimulated by auxin treatment in the apical part of the organ, whereas no stimulation was observed in the proximal part of root (Tyburski et al. 2008).

The relation between auxin and AOX expression and activity in pumpkin fruits was demonstrated by Esaka et al. (1992). AOX activity in pumpkin fruit tissue, cultured in vitro, strongly increased when culture medium was supplemented with synthetic auxin, 2,4-D. It was shown that the enzyme’s activity is regulated by auxin at the level of gene expression. Gene expression analysis showed that AOX mRNA level also increased after transfer onto the culture medium in the presence of 2,4-D. The highest steady-state level of AOX mRNA appeared 2 days after transfer, and decreased thereafter. In the absence of 2,4-D, no increase in AOX transcript
level was detected in fruit tissue (Esaka et al. 1992). AOX expression was also induced by auxin in tobacco leaves and this induction was associated with stimulation of plant growth (Pignocchi et al. 2003).

AOX is able to react with indole-3-acetic acid (IAA) as a substrate at least in vitro. It was demonstrated that AOX may effectively decrease IAA concentration in radish roots via oxidative decarboxylation forming, as a main product, oxindole-3-methanol (Kerk et al. 2000). While the principal mechanism of auxin turnover in shoots engages non-decarboxylative pathway (Woodward and Bartel 2005), the analysis of IAA metabolite in various parts of root tissue shows that decarboxylation is a main IAA-degradation pathway in roots. Oxidative decarboxylation occurred almost exclusively in the root tip with possible participation of AOX. This finding implicates an existence of a regulatory loop involving AOX, IAA and ASC in the root tip cells (Kerk et al. 2000).

Further insight into the relation between AOX and auxin was provided by studies on AOX-over-expressing tobacco plants. It was demonstrated that over-expression of AOX results in oxidation of the redox state of apoplast, which is followed by the reduction in sensitivity to exogenous auxin. While wild-type seedlings reacted to spraying with 0.5 μM NAA with enhancement of shoot growth (i.e. advanced development of cotyledon and leaves) and increase in fresh weight, AOX–over-expressing seedlings, submitted to the same treatment, accumulated 25–30% less biomass. Although lateral root proliferation following auxin treatment was observed in both, control line and AOX–over-expressing line, this effect was much more evident in wild-type seedlings. Desensitisation to auxin in AOX-over-producing plants was related to constitutive induction of auxin signalling pathway in these plants (Pignocchi et al. 2006). Auxin stimulates ROS production in apoplast and employs them as mediators in the regulation of plant growth reactions (Joo et al. 2001; Schopfer et al. 2002; Liszkay et al. 2004). Because apoplast is already oxidised in AOX-over-expressing plants, a further stimulation of ROS production by auxin has minimal effects on oxidative signalling processes leading to auxin insensitivity. Moreover, the activity of auxin-dependent MAP kinase pathway was doubled in leaves of AOX-over-expressing seedlings when compared to control wild-type seedlings. These data emphasise the role of apoplast redox-mediated changes in developmental reactions of plants to auxin treatments (Pignocchi et al. 2006).

Besides AOX, the effects of auxin treatment on the activities of other ASC metabolising enzymes were reported. Changes in the activities of AOX and Halliwell–Asada cycle enzyme activity were analysed in the proximal and distal parts of tomato seedling roots grown in vitro (Tyburski et al. 2008). Roots respond to auxin treatment with an increase in lateral root formation which occurs primarily in the proximal part of root and concurrent inhibition of root elongation, which affects root elongation zone localised in proximity of the root apex (Cleland 2004; Tanimoto 2005). It was demonstrated that a 3-day-long treatment with 1 μM IAA decreased the activities of ASC-regenerating enzymes, i.e. monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) within both, distal (elongating) and proximal (lateral root producing) part of the root. ASC-oxidising
enzymes were stimulated by IAA. However, auxin’s stimulatory effect on ascorbate peroxidise (APX) activity was restricted to proximal part of the root, whereas, AOX activity increased exclusively within apical part of the root. These changes were accompanied with an increase in the participation of DHA in total pool of ascorbate. These findings show that the effects of exogenous auxin on ascorbate metabolism depend on the zone of root which is exposed to an auxin stimulus (Tyburski et al. 2008). Generally, auxin application is usually followed by increased oxidation of the ascorbate pool due to stimulatory effects on AOX or ascorbate peroxidase (APX) activity and/or expression. This finding is consistent with an overall mode of auxin action, which triggers auxin-specific signalling pathways, oxidising the cellular environment (Joo et al. 2001; Schopfer et al. 2002).

2.2 **Cytokinin**

A role of endogenous cytokinin in regulation of ASC and GSH enzyme activity was studied using transgenic tobacco plants expressing supplementary ipt – gene under a control of the constitutive promoter for small subunit of RUBISCO (Pssu-ipt). Over-expression of bacterial ipt gene encoding isopentenyl transferase, a key enzyme in cytokinin synthesis, in plant cells led to an increase in endogenous cytokinin content in transgenic plants. When compared to non-transformed control, active forms of cytokinins increased seven times in transgenic shoots grafted onto nontransgenic rootstock and 12 times in transgenic shoots propagated in vitro. Tobacco plants over-expressing ipt, exhibited typical traits of cytokinin over-production syndrome, including reduced root growth, reduced apical dominance, and retarded plant and leaf senescence. Transgenic plants were also characterised by alterations in water regime, disturbance in photosynthesis and over-expression of PATOGENESIS RELATED PROTEIN 1 (PR1). These findings were interpreted as symptoms of permanent stress, which is caused directly and indirectly by cytokinin over-production (Synková et al. 2004. Transgenic plants produced more H₂O₂ than control plants; however, higher activities of ascorbate/glutathione enzymes were detected in cytokinin over-producing plants as well. APX activity was two times higher in transgenic plants grown either in vitro or in vivo conditions when compared to the control. Glutathione reductase (GR) activity was also doubled in ipt transformants, however, this increase was observed only in in vitro cultured plants, whereas there was no difference in GR activity between control and transgenic plants grown in ex vitro conditions. In addition, catalase (CAT) activity was stimulated in transgenic plants; on the other hand, superoxide dismutase activity was halved. These findings suggest that cytokinins may regulate H₂O₂ levels by changing the activities of principal enzymes of H₂O₂ turnover (Synková et al. 2004, 2006).

Cytokinin over-expression had important consequences for redox metabolism during senescence. This process is, to a large extent, dependent on ROS accumulation in chloroplasts, which causes chlorophyll bleaching, lipid peroxidation and eventual loss of chloroplast integrity (Navabpour et al. 2003; Bhattacharjee 2005).
Control plants exhibited high activities of antioxidant enzymes in early stages of plant development, i.e. until the onset of flowering, and a decline in later stages. Contrary to the control, transgenic plants showed increase in CAT, GR and APX in later stages of development, i.e. during flowering and forming seeds. These data suggest that cytokinin control of senescence involves the regulation of the activities of aforementioned H$_2$O$_2$-processing enzymes (Synková et al. 2006).

Similar effects of exogenously applied cytokinin were demonstrated. Wheat leaf segments treated with $10^{-4}$ M BAP exhibit delayed senescence, which manifests itself in the retention of chlorophylls and chloroplast proteins (Zavaleta-Mancera et al. 2007). Consequently, during incubation period following exposure to BAP, chloroplasts in the cells of treated leaf segments remained intact significantly longer, whereas those of control segments exhibited various symptoms of degradation. BAP-treated leaves accumulated significantly lower levels of H$_2$O$_2$. This finding together with increasing the content of xanthophylls, at least partly, explains delayed senescence of leaves pre-treated with BAP. Decrease in H$_2$O$_2$ was due to enhanced activity in CAT and APX enzymes. During 6-day-long incubation period, in control leaves CAT activity decreased from the fourth day on and APX activity decreased on the sixth day. BAP treatment prior to incubation period efficiently prevented the decrease in the activities of both enzymes. It was concluded that the mechanism of cytokinin-dependent delay in leaf senescence involves the reduction in H$_2$O$_2$ levels due to the hormone’s stimulatory effect on CAT and APX activities (Zavaleta-Mancera et al. 2007).

3 Ascorbate and Glutathione as Regulators of Cell Division in the Root Apical Meristem

There is a growing body of evidence indicating that the role of ascorbate and glutathione in plants extends beyond their intensively explored antioxidant function (De Pinto and De Gara 2004). It has been demonstrated that high ascorbate and glutathione levels are required for normal progression of the cell cycle in meristematic tissues (Liso et al. 1984, 1988; Potters et al. 2000; Vernoux et al. 2000; Jiang et al. 2003). Ascorbate is directly involved in the regulation of two processes that mediate morphogenetic responses in plants: cell division and elongation. The reduced ascorbate (ASC) as well as oxidised form of this compound (dehydroascorbate, DHA) play an important role in the regulation of mitotic activity in the meristems (De Tullio et al. 1999; De Pinto et al. 2000; Potters et al. 2002). It was observed that ASC promotes cell-cycle progression in the root apical meristem by stimulating G1-S transition. If ASC is added to the cells of the root quiescent centre (QC), it induces these normally non-dividing cells to pass from G1 into the S phase (Liso et al. 1988).

A direct link between ASC/DHA and GSH/GSSG redox states and auxin-independent regulation of mitotic activity in discrete parts of the apical root meristem
has been demonstrated. The apical root meristem comprises of proximal meristem (PM), which produces the tissues of the root proper and the distal meristem (DM) that produces root cap. Between the actively dividing cells of PM and DM, the population of slowly dividing cells, called quiescent centre (QC) is localised. QC cells spend extended periods of time in G1 phase, dividing, on average, every 200 h (Jiang et al. 2006). It was demonstrated that laser ablation of QC cells leads to differentiation of the initial cells previously attached to it. Therefore, it was concluded that the function of QC is maintaining the dedifferentiated state of initial cells in adjacent meristems in a cell-contact-dependent manner (Bonke et al. 2005).

Differences in the cell division rate between the meristems and QC depend on the polar transport of auxin from shoot apex, where the hormone synthesis takes place, to the root tip. Directions of hormone transport in the root are determined by the patterns of PIN family auxin – efflux carrier protein expression (Friml 2003). Auxin is transported acropetally towards the root tip through the root stele tissue employing cells that express PIN1 auxin efflux carrier (Friml 2003). The hormone is transported to QC and columella initials where high levels of auxin accumulate (Kerk and Feldman 1995). Part of auxin is further transported from QC to columella by PIN4 protein. An auxin efflux protein PIN3, specifically expressed by columella cells is localised in lateral sides of plasma membrane that enables basipetally directed lateral auxin transport through lateral parts of the root cap and cortex/endoderm cells expressing PIN2 auxin carrier (Benkova et al. 2003; Friml 2003; Blilou et al. 2005).

It was demonstrated that auxin accumulation in QC switches the redox balance in the cells towards a more oxidised state (Jiang et al. 2003). When compared to rapidly dividing PM initials, cells of the QC are characterised by the elevated levels of DHA (×1,000), GSSG (×10), O$_2^-$ (×15.6) and H$_2$O$_2$ (×34). On the other hand, rapidly dividing cells of PM contain reduced forms of ASC and GSH and do not accumulate ROS. Auxin-induced oxidised intracellular environment plays a decisive role in maintaining the low cell division rate in QC (Kerk and Feldman 1995; Jiang et al. 2003). Treating roots with an inhibitor of auxin polar transport, such as NPA, results in a relocation of auxin maximum from QC to cortical and procambial region of proximal meristem. This alteration in auxin distribution was followed by mitotic activation of QC. This correlated with the development of less oxidised status in the QC and more oxidised status in the adjacent PM, to which the auxin maximum was shifted (Jiang et al. 2003).

An increase in ASC oxidation in QC is attributed to high AOX activity. Both, AOX transcript and protein accumulate very distinctly in QC, whereas proximal and distal meristem cells were characterised by much lower AOX activity. Because AOX expression is stimulated by auxin (Kato and Esaka 1999; Kerk and Feldman 1995; Liso et al. 2004), high levels of enzyme activity are maintained in QC by IAA accumulating within this structure. Consequently, high rate of ASC oxidation keeps the oxidised ascorbate redox balance (Kerk and Feldman 1995). QC cells exhibit lower abilities for regenerating ASC from DHA because of the absence of DHAR activity. However, the activity of MDHAR was similar in QC
and adjacent meristem cells, which possibly prevents the ascorbate pool in QC from total oxidation. QC cells were equipped with several times lower levels of GR activities, which explains decreased abilities for recycling GSH from GSSG (Jiang et al. 2003).

Highly oxidised intracellular environment of QC seems to slow down cell division rate by reducing mitochondrial activity. QC cells are characterised by lowered mitochondrial membrane potential, which indicates the decrease in the production of ATP and NADH. Critical levels of these compounds are necessary to satisfy the G1-S checkpoint energy requirement. Therefore, reduced ATP/NADH levels in QC cells result in a decrease in the energy supply, which may cause the decrease in the cell division rate in QC (Jiang et al. 2006). Alteration in mitochondrial activity in QC may be related to inhibition of expression of some nuclear-encoded mitochondrial proteins. Among them, the reductions in tricarboxylic acid cycle enzymes have been demonstrated (Jiang et al. 2006). On the other hand, transcripts encoded by the mitochondrial genome were not decreased in QC cells when compared to adjacent meristem cells. It was also demonstrated that, in spite of constitutive oxidative stress conditions, which often result in a loss of mitochondrial membrane integrity, followed by the induction of apoptosis pathway, the overall cellular ultra-structure of the QC, including that of the mitochondria is typical of that found in unstressed plant cells. The intactness of the mitochondria, and other cell organelles of QC cells is possibly due to protective functions of residual levels of reduced ascorbate and glutathione still present within these cells (Jiang et al. 2006).

The mechanism of ASC-dependent stimulation of cell divisions is still not sufficiently explained. Some data suggest that the stimulatory effect of ASC on cell divisions in root apical meristem results from the involvement of ASC in hydroxyproline synthesis. Hydroxyproline-containing proteins play a decisive role in cell cycle regulation and ASC is a co-factor of peptidyl-prolyl hydroxylase – a hydroxyproline-synthesising enzyme (De Tullio et al. 1999). Another hypothesis indicates the role of ASC in inducing the activity of ribonucleotide reductase (RNR). The enzyme reduces ribonucleotides to deoxyribonucleotids and therefore is an essential enzyme for DNA synthesis during the S phase. ASC is supposed to be required for effective reconstitution of an iron centre of RNR by increasing the release of Fe$^{2+}$ from intracellular stock such as ferritin (Potters et al. 2002). A complementary explanation stresses the role of AOX in oxygen management and the regulation of metabolic rate in plant cells. According to this idea, ASC oxidation catalysed by AOX as an oxygen-consuming reaction decreases the oxygen availability in the cell. This results in slowing down of the metabolism and is followed, as observed in QC cells, by a decrease in the rate of cell divisions (De Tullio et al. 2007).

Finally, ascorbate affects plant development being engaged in the synthesis of several growth regulators, such as ethylene, abscisic acids and gibberellins. It functions in these processes as a co-factor of dioxygenases – enzymes playing a decisive role in the synthesis of aforementioned hormones (Dong et al. 1992; Liu et al. 1999; Arrigoni and De Tullio, 2000; Lopéz-Carbonell et al. 2006).
4 Glutathione as a Regulatory Factor in Plant Development

It has been previously demonstrated that GSH has many functions in plants including an important role in the antioxidant system, sulphur metabolism and detoxification of xenobiotics (Noctor and Foyer 1998). In addition, similar to ASC, a regulatory role of GSH in some aspects of plant development has been reported. It was demonstrated that the timing of the development of inflorescence and flowering in the rosette plants: Arabidopsis thaliana (Ogawa et al. 2001) and Eustoma grandiflorum (Yanagida et al. 2004) is regulated by changes in the rate of GSH biosynthesis. Ogawa et al. (2001) have shown that the effects of endogenous GSH levels on flowering in Arabidopsis are dependent on the stage of plant development. Depleting GSH by treating plants with a specific inhibitor of GSH biosynthesis, buthionine-sulfoximine (BSO), promoted flowering when it was applied at the onset of transition to flowering. On the other hand, when plants were exposed to BSO from the beginning of culture, flowering was delayed. The GSH-deficient Arabidopsis mutant cad2 also exhibited delayed flowering when compared to wild type plants (Ogawa et al. 2001). Later it was demonstrated that levels of endogenous GSH, decisive for the proper timing of flowering, are regulated by the availability of ATP synthesised during photosynthesis and therefore are dependent on the intensity of photosynthesis (Ogawa et al. 2004). GSH is also required for vernalization-induced flowering of rosette plant Eustoma grandiflorum. It was shown that vernalization was efficiently replaced by feeding with GSH, or its precursor cysteine (however not by other thiols) in promoting flower induction. On the other hand, the inductive effect of vernalization on bolting was suppressed by BSO, without decreasing the plant growth rate (Yanagida et al. 2004).

GSH is also involved in the regulation of the phytohormone-induced differentiation of tracheary elements (TE) in Zinnia and Arabidopsis (Henmi et al. 2001). This process was promoted by GSSG, if the substance was applied during the early period of culture, whereas the effect was completely reversed when GSSG was applied at a later period of culture. The expression of glutathione reductase (GR) was down-regulated during TE development and exogenous GSH suppressed TE formation. Over-expressing GR in Arabidopsis had the same effect. Because GSSG stimulated TE differentiation only in the presence of appropriate growth regulators, the authors conclude that GSSG-dependent regulation cooperates with phytohormones to induce TE differentiation (Henmi et al. 2001).

Presence of the reduced form of glutathione (GSH) in tissue was found to be necessary to maintain cell divisions in Arabidopsis root meristem (Sánchez-Fernández et al. 1997; Vernoux et al. 2000). GSH was localised in actively dividing initial cells while it was absent in slowly dividing cells of the quiescent centre, that suggests that growing tissues have a requirement for glutathione. Cell divisions of meristem initials were stimulated by exogenous GSH and inhibited by treatment with the inhibitor of GSH biosynthesis BSO (Sánchez-Fernández et al. 1997). Moreover, Arabidopsis plants homozygous for a mutation in the ROOT MERISTEMLESS I (RML1) gene, coding for γ-glutamylcysteine synthetase
(enzyme of GSH biosynthesis), accumulating only 3% of the wild type GSH level, were unable to maintain cell divisions in the root apical meristem. On the other hand, root cells of the mutant plants divided normally when fed with exogenous GSH. Cells in the root meristem of the mutant plant were arrested at the G1-S transition of the cell cycle, which resulted in inhibition of root growth. However, while post-embryonic root development was blocked due to cell division arrest, the overall organisation of cell types in the root apex was not changed when compared to wild type plants. The *rml1* phenotype can be induced by treating wild-type seedlings by lowering GSH levels with BSO. Similar to *rml1* plants, cells in the root apical meristem of BSO-treated seedlings are arrested in G1 phase. It should be noted that the GSH requirement of cell division process is a root-specific phenomenon. The shoot meristem of *rml1* seedlings is able to produce all the above-ground organs with timing similar to that of wild type plants (Vernoux et al. 2000).

5 Plant Cell Suspension Cultures as Model Systems in Studies on the Mechanism of Ascorbate and Glutathione Role in Cell Proliferation

The long-term cultures of plant cells and small cell aggregates named cell suspension cultures, are characterised by the highest structural and metabolic homogeneity in plant in vitro culture. Cell suspension cultures are usually derived from callus cultures and grown in liquid media under constant shaking and aeration. As quickly dividing and easily synchronizable cell populations, which follow specific growth kinetics, cell suspensions are frequently used in studies on the mechanism of cell cycle regulation (Menges and Murray 2002). Cell suspension cultures of *Arabidopsis* and tobacco cell line BY2 were used as model systems in studies on ascorbate and glutathione involvement in the regulation of cell divisions.

Timing of phases during cell suspension growth kinetics was found to be synchronized with changes in cellular ASC and GSH levels. A three- to fourfold increase in the endogenous ASC content was observed during the exponential growth phase, and a peak in ASC coincided with a peak in the mitotic index in BY2 culture (De Pinto et al. 1999). Ascorbate was also abundant in the exponential phase of *Arabidopsis* cell culture growth cycle. When the growth rate in culture declined, ascorbate levels decreased to about half its original value (Pellny et al. 2009). In addition, an increase in GSH levels was observed during a proliferation phase of the growth cycle (De Pinto et al. 1999).

Cell proliferation in tobacco cell culture was strongly stimulated by exogenous GSH, however, was not affected by GSSG (De Pinto et al. 1999, 2000). Stimulation of ASC biosynthesis in BY-2 by adding ascorbate precursor galactono-γ-lactone (GalL) to the culture medium accelerated culture growth by promoting cell division. In contrast to the reduced form of ascorbate, exogenous DHA strongly blocked cell divisions in BY2 culture (De Pinto et al. 1999). Studies using synchronized BY-2 cells revealed that addition of 1 mM DHA to cells in G1 phase induced a delay in
cell cycle progression. DHA-treated cells reached the same value of mitotic index as untreated cells but several hours later. DHA added to the medium was quickly reduced after the uptake by the cells, leading to a strong increase in ASC intracellular level. Because oxidative stress induced by various environmental factors favours oxidation of apoplastic ASC to DHA, it was concluded that slowing down the cell cycle progression in the presence of high DHA levels may function as an adaptation strategy to surveillance under stress condition. (Potters et al. 2000).

Studies on BY2 cell culture revealed that the DHA effect on cell proliferation could not be reproduced by GSSG treatment (De Pinto et al. 1999). However, total depletion of cellular GSH with BSO resulted in a cell cycle delay similar to that caused by DHA treatment. Simultaneous application of both compounds completely blocked mitotic activity. On the other hand, combined addition of GSH and DHA resulted in delay similar to DHA alone. This finding suggests that the ASC/DHA pair has a specific regulatory role on cell division rather than merely acting as a general redox pair and that ascorbate and glutathione control cell cycle using independent pathways (Potters et al. 2004).

It is noteworthy that DHA is only capable of slowing down the cell cycle when added during G1 phase. Addition of DHA during G2 phase did not affect the cell cycle progression. This suggests that DHA influences cell cycle through processes that are specific for G1 or S phase. However, the precise mechanism of DHA-dependent cell cycle regulation still remains to be deciphered (Potters et al. 2004).

Changes in the activities in ASC and GSH metabolising enzymes were synchronized with ASC, GSH levels and rate of cell division in BY2 cell culture. Similar to ASC and GSH content the activities of the Halliwell–Asada cycle enzymes rise during exponential phase of the cell culture growth cycle. These finding suggests that cell division process is marked by the intensification in ascorbate and glutathione turnover. Apart from the ASC-dependent H$_2$O$_2$ scavenging that is particularly high in dividing cells, other ascorbate-consuming processes like hydroxyproline-rich protein or nucleotide synthesis are responsible for an increased ascorbate recycling (De Pinto et al. 2000).

A regulatory role for glutathione with respect to cell proliferation in Arabidopsis cell culture was demonstrated to be linked to pyridine nucleotide metabolism and activity of poly (ADP-ribose) polymerase (PARP) activity (Pellny et al. 2009). PARP is a nuclear enzyme which transfers ADP-ribose units from NAD$^+$ residues on target proteins, resulting in branched chains of ADP-ribose polymers. ADP-ribosylation is important in regulating processes such as DNA repair, modification of histone structure and chromatin remodelling (Kraus and Lis 2003). PARP activity level correlated with the rate of cell division in Arabidopsis cell culture; lower activity was observed on the first day after inoculation but values increased rapidly during the exponential growth phase. Subsequently, when the growth rate in culture declined, PARP activity decreased (Pellny et al. 2009). Expression patterns of the major Arabidopsis PARP-coding genes (PARP1 and PARP2) increased progressively during the exponential phase, giving a peak when growth was the highest and declined thereafter. High PARP1 and PARP2 expression levels were associated with increases in the NAD$^+$ NADH pool and oxidised state
of NAD/NADH redox pair. Because PARP is a NAD-cleaving enzyme, this reflects the requirement for the pyridine nucleotide substrate. Intracellular glutathione (but not ascorbate) levels increased in parallel with PARP activity and expression. The putative reason for glutathione increase during exponential phase is the necessity to buffer cellular oxidation caused by enhanced oxidation of NAD pool. At this phase of the growth cycle, glutathione is present in the cytoplasm. However, almost total cellular glutathione is recruited to the nucleus at the end of the phase of exponential growth. GSH movement between cytoplasm and nucleus may possibly have important consequences for both, redox buffering in the cytoplasm and direct regulation of gene expression. A close correlation between glutathione levels and PARP activity suggests that there is a relationship between these parameters. PARP is a redox-sensitive enzyme whose activity could be regulated by the GSH in the nucleus, via thiol-disulphide exchange mechanism or glutathionylation (Pellny et al. 2009). However, these ideas should be supported by further experiments.

6 Regulation of Cell Growth by Ascorbate

Besides the effects on cell division rate, ascorbate affects organ growth by participating in the process of cell wall stiffening, which regulates the cell expansion within the organ elongation zones (Green and Fry 2005a). An increase in activity of apoplastic AOX, detected in rapidly elongating tissues, results in the production of monodehydroascorbate (MDHA) in the plant cell wall. This compound activates plasma membrane H⁺-ATPase (Gonzales-Reyes et al. 1992; Asard et al. 1995). An increase in the activity of this enzyme is followed by an acidification of apoplast that according to the acid growth theory leads to the loosening of cell wall structure and facilitates cell growth (Cosgrove 2001). Moreover, MDHA in apoplast functions as an acceptor in transmembrane electron transport. The plasma membrane redox system, involving cytochrome B, transports electrons from NADH across the plasma membrane onto MDHA, which is reduced to ASC. The intensification of this process results in plasma membrane hyperpolarisation which is followed by activation of plasma membrane H⁺-ATPase that promotes cell growth as mentioned above. Simultaneously, local cytoplasm acidification resulting from NADH oxidation activates vacuolar H⁺-ATPase. Consequently, an increase in cell vacuolization occurs which further enhances cell expansion (Gonzales-Reyes et al. 1992; Horemans et al. 2000). MDHA generated by AOX activity in the apoplastic spaces may undergo further spontaneous oxidation to DHA (Noctor and Foyer 1998). This compound as well as a product of its degradation – oxalate – may directly cause cell wall loosening and/or enhance cell vacuolization (Lin and Varner 1991).

Another mechanism linking apoplastic ASC with loosening of cell walls in growing segments of plant organs involves the inhibitory effect of this antioxidant on peroxidase-dependent processes of cross-linking cell wall polymers. ASC prevents oxidative formation of diferulate bridges between cell wall polysaccharides in elongating onion roots (Córdoba-Pedregosa et al. 1996; Takahama and Oniki 1992).
and pine hypocotyls (Sánchez et al. 1997). ASC inhibited the polymerisation of monomers of cell wall structural protein extension by blocking isodityrosine cross-linking (Córdoba and Gonzales-Reyes 1994) and delayed cell wall lignification by reducing phenoxy radicals, which serve as precursors in peroxidase-dependent lignin synthesis (Takahama and Oniki 1991; Padu 1999).

Ascorbate may also stimulate a pro-oxidative process within the plant cell wall, which results in local production of hydroxyl radical (\(^{\cdot}\)OH) (Fry 1992; Green and Fry 2005a, b). This highly reactive compound is able to cause an oxidative scission of cell wall polysaccharide chains, which is followed by loosening the cell wall structure that again promotes cell growth (Schopfer et al. 2001; Schopfer 2002). The mechanism of ASC-dependent \(^{\cdot}\)OH was proposed to have a non-enzymatic nature and to require \(O_2\) and a transition metal such as \(Cu^{2+}\). It was demonstrated that in the presence of traces of \(Cu^{2+}\), ASC reduces \(O_2\) to \(H_2O_2\). ASC also reduces \(Cu^{2+}\) to \(Cu^{+}\). These two products: \(H_2O_2\) and \(Cu^{+}\), can participate in a Fenton reaction. In the course of this reaction, \(Cu^{2+}\) is regenerated with concurrent \(^{\cdot}\)OH formation (Fry 1992).

Besides ascorbate itself, the components of its breakdown pathway may also act as pro-oxidative agents that stimulate the generation hydroxyl radical (\(^{\cdot}\)OH) in the cell wall. DHA generated by ASC oxidation may undergo an apoplast-specific degradation pathway via oxidase-dependent or non-enzymatic, conversion of DHA to 4-O-oxalyl-L-threonate. The latter compound is further converted to L-threonate and oxalate as final degradation products. This reaction is catalysed by apoplastic oxalyl esterases but may also occur non-enzymatically. During these steps additional \(H_2O_2\) molecules are formed, which can take part in a Fenton reaction. Moreover, in many plants L-threonate is oxidated to L-threarate in a reaction, which uses two \(O_2\) and generates two \(H_2O_2\) molecules. In tissues possessing apoplastic oxalate oxidase, the oxalate can also yield \(H_2O_2\) when oxidised (Green and Fry 2005a, b).

### 7 A Role of Ascorbate in Somatic Embryogenesis

Studies on the roles of ASC in the formation of somatic embryos were conducted using white spruce (\textit{Picea glauca}) embryogenesis as a model system. An embryogenic tissue for the induction of somatic embryogenesis was generated from zygotic embryos (Stasolla and Yeung 1999). A well-characterised process of somatic embryogenesis in this plant generally consists of proliferation and maturation stages. These stages are characterised by different hormonal requirements. The first stage is characterised by proliferating tissue on the auxin- and cytokinin-containing medium. At this stage, the formation of filamentous embryos occurs. These structures are composed of a suspensor region subtending clusters of small, non-vacuolated cells of the embryo proper. Then, the tissue is transferred onto the ABA-supplemented maturation medium. During the culture in the presence of ABA, cell divisions occur which leads to an increase in the size of the embryo proper. Subsequently,
a well-developed shoot and root pole become visible. Finally, the embryos develop a ring of cotyledons at the shoot apical region. Mature embryos are transferred onto the germination medium where, root and shoot emergence occurs (Stasolla et al. 2002).

White spruce somatic embryo maturation and germination were marked by important changes in ASC metabolism. The differences in ASC content, ASC/DHA redox state, and the activities of ascorbate-metabolising enzymes were analysed using both embryogenic and non-embryogenic cell lines. While no differences in ASC metabolism were observed between embryogenic and non-embryogenic cell lines in the proliferation medium, after the transfer onto the maturation medium, the rate of ASC synthesis sharply increased in embryogenic lines, whereas it remained constant in the non-embryogenic line. Gradual decrease in the APX activity followed by a shift within the total ascorbate pool towards the reduced form was observed in embryogenic lines. On the other hand, the non-embryogenic line was characterised with significantly higher APX activity and oxidised state of ascorbate pool. Switching of the ASC/DHA ratio towards the reduced state and increase in ASC levels in embryogenic lines are possibly required for the progression of cell divisions within developing embryos (Stasolla and Yeung 2001).

In contrast to zygotic embryos, which undergo desiccation before subsequent germination preceded by imbibition, mature somatic embryos usually directly develop to plantlets. However, the germination frequency of somatic embryos of coniferous species, including white spruce, was found to be greatly increased by partial drying treatment (PDT) preceding transfer onto germination medium. Stimulatory effect of PDT results from an alteration in storage product deposition, decreased synthesis of ethylene and ABA, and changes in the pattern of nucleotide synthesis and utilization (Stasolla et al. 2002). PDT applied to the mature embryos was characterised by several changes in ASC metabolism. Firstly, ASC levels in the embryos as well as the activities of its redox enzymes significantly declined. Secondly, ASC/DHA ratio shifted to a more oxidised state, with equal participation of ASC and DHA in total ascorbate pool observed at the end of PDT. After PDT, white spruce embryos are transferred onto a hormone-free germination medium, where the induction of root and shoot meristem activity occurs. Upon the transfer onto the germination medium a restoration of ASC synthesis and ASC metabolism were observed. Besides the onset of ASC synthesis, a reduction of DHA accumulated during PDT occurs which contributes to an increase in the ASC level. Elevated ASC levels are required for activation of meristematic activity in germinating embryo. Moreover, an increase in ASC levels are followed by rise in APX activity necessary for detoxification of \( \text{H}_2\text{O}_2 \) generated after the recovery of oxidative metabolism (Stasolla and Yeung 2001).

The process of shoot emergence was found to be significantly affected by ASC added to the germination medium (Stasolla and Yeung 1999). An optimised concentration of ASC significantly enhanced shoot development. Moreover, ASC-treated embryos were larger and produced dark green leaves at the shoot pole. Control embryos, germinated on ascorbate-free medium were smaller than and not as green
Ascorbate and Glutathione in Organogenesis

As those cultured in the presence of ASC. In contrast to shoot meristem, the root pole of the embryo did not react with an increase of root growth to ASC treatments (Stasolla and Yeung 1999). An importance of ASC for embryo meristem reactivation at germination was confirmed by a complementary approach using lycorine – an inhibitor of ASC biosynthesis. Lycorine inhibits the last step of ASC synthesis, i.e. conversion of GalL to ascorbate (Arrigoni 1994). It was demonstrated that ASC depletion following the lycorine administration to the germination medium prevents cellular divisions in the shoot apical meristem (Stasolla and Yeung 2007).

The mechanism of ASC stimulatory effect on the shoot apex development involves prevention of the peroxidase-dependent cell wall stiffening within shoot apical meristem. Experimental manipulations resulting in an increase in ASC content, i.e. supplementing germination medium with ASC or GalL, decrease the activities of ferulic acid peroxidase (FPOX) and guaiacol peroxidase (GPOX). On the other hand, activities of the aforementioned enzymes increase in lycorine-treated embryos. FPOX and GPOX are responsible for cross-linkage of cell wall compounds, which leads to cell wall stiffening and prevents cell division and cell growth. Therefore, the critical levels of endogenous ASC in the apical meristem are necessary to maintain cell wall plasticity prerequisite for both cell growth and proliferation (Stasolla and Yeung 2007).

A Role of Glutathione in Somatic Embryogenesis

A series of studies revealed an importance of glutathione concentration and redox state for the process of somatic embryo formation in the white spruce tissue culture. In contrast to ASC treatments, which stimulated mainly postembryonic developmental events within the shoot meristem, altering glutathione level affected all stages of somatic embryo formation and further development: cell proliferation on maintenance medium, embryo maturation and germination. GSH supplementation to the maintenance medium had a stimulatory effect on cell divisions in the embryogenic tissue resulting in higher fresh weigh increase when compared to untreated control. On the other hand, the rate of growth of GSSG-treated tissue was comparable to that of control (Belmonte et al. 2003).

The addition of exogenously supplied GSH or GSSG to the ABA-containing maturation medium affected the number of embryos formed in culture as well as their quality. An optimised GSH concentration in the medium resulted in an increase in the number of embryos formed, this increased number was, however, mostly due to the production of low quality embryos, unable to regenerate viable plants. In contrast to GSH-treatment, supplementing medium with GSSG had a marginal effect on total embryo population. However, supplementing with GSSG strongly increased embryo quality, which manifested in higher number of embryos equipped with four or more cotyledons and possessing high germination potential. It should be noted that the aforementioned effect of GSH or GSSG applications were limited to the narrow ranges of concentration. Higher level of
both glutathione redox forms inhibited overall embryo-forming capacity in cultured tissue and impeded embryo development (Belmonte and Yeung 2004; Belmonte et al. 2005a).

GSSG treatment caused an increased accumulation of starch granules, lipids and protein bodies in cortical and procambial cells of GSSG-treated embryos. Storage product deposition in cells of treated embryos may be required for the acquisition of desiccation tolerance, thus enhancing the viability of embryos. Another striking feature of embryos exposed to GSSG was the proper structure of meristems, whereas meristems of untreated embryos were frequently disrupted by intercellular spaces, which led to a failure in meristem reactivation during postembryonic development (Belmonte et al. 2005a). The improved architecture of shoot apical meristems in GSSG treated embryos may be due to the reduction of ethylene production. Disorganisation of cell division patterns in meristem resulting in intercellular spaces formation was related to the accumulation of this hormone in the culture vessel. In embryos grown on GSSG-supplemented medium endogenous ethylene level did not vary throughout the culture period. On the other hand, in control cuttings ethylene production increased markedly after 10 days of culture and remained high until the end of culture period (Belmonte et al. 2005a).

Ethylene synthesis in white spruce seems to be under control of GHS/GSSG redox status. It was demonstrated that the gene encoding aminocyclopropane-1-carboxylate (ACC) oxidase, an enzyme responsible for the final step of ethylene biosynthesis is induced by GSH treatment at all the stages of somatic embryo development. A switch of the glutathione pool towards oxidised form by GSSG administration is believed to have inhibitory effect on ACC oxidase expression that consequently decreases ethylene levels (Stasolla et al. 2004). However, the down regulation of ACC oxidase transcript level imposed by GSSG application still needs to be demonstrated to prove this hypothesis.

Given that supplementing GSH to the medium favours cell proliferation during early stages of somatic embryogenesis and increases total embryo yield, whereas effect of GSSG treatment manifests itself at embryo maturation period, a protocol of sequential treatments with GSH and GSSG was developed to profit the beneficial effects of both glutathione redox forms. An optimised procedure that resulted in the highest embryo yield, containing the highest percentage of high quality embryos consisted of 7 days of GSH treatment followed by culture in the presence of GSSG during the remaining part of 40-days-long culture period (Belmonte et al. 2005a). Similar stimulation of somatic embryogenesis was achieved by replacing sequential GSH/GSSG treatment with supplementing maturation medium with optimised concentration of BSO. It was observed that the glutathione redox state turned towards a more oxidised one with 0.01 mM or higher concentration of BSO. Compared to control embryos, the GSH/GSH + GSSG ratio declined quickly in BSO-treated embryos over the course of culture period, reaching its minimal value in fully mature embryos. In contrast to BSO-treated embryos, control ones were characterised by reduced glutathione redox status throughout the culture period (Belmonte and Stasolla 2007; Stasolla et al. 2008).
Oxidation of glutathione pool in tissues grown in the presence of BSO was beneficial for the efficiency of embryogenesis and the quality of embryos formed. In contrast to control embryos, those formed in the presence of 0.01 mM BSO were characterised by proper organisation of shoot apical meristem with its sub-apical domain composed by tightly packed cells. Moreover, BSO-treated embryos contained more initial cells in root apical meristem when compared to the untreated control (Belmonte and Stasolla 2007; Stasolla et al. 2008). This finding is consistent with the present knowledge which emphasises the importance of an oxidised environment for the establishment of quiescence and cell division patterns in the apical root meristem (Jiang et al. 2003).

Since glutathione levels and redox state are coupled to ascorbate through ASC–GSH cycle, BSO treatment affected both ascorbate levels and the activities of several ascorbate enzymes. Generally, BSO treatment decreased total ascorbate levels in embryos and transiently stimulated the expression of genes coding for APX and MDHAR, which resulted in an increase in the activities of these enzymes. A stimulatory effect of BSO on the APX activity during the early phase of embryo development may have particular importance for somatic embryo development due to decreasing toxic levels of $H_2O_2$ generated during active embryonic growth (Stasolla et al. 2008).

9 Glutathione-Dependent Changes in Nucleotide Metabolism During Somatic Embryogenesis

Studies on nucleotide metabolism in cultures grown on GSH-supplemented media revealed that stimulatory effect of GSH treatment on cell proliferation in white spruce culture grown on maintenance medium is possibly related to alterations in purine and pyrimidine metabolism (Belmonte et al. 2003, 2005b). Both purine and pyrimidine nucleotides serve as building blocks for nucleic acid synthesis. Moreover, purine nucleotides participate in bio-energetic processes. In white spruce cells, both purine and pyrimidine nucleotides can be synthesised de novo from precursor molecules, or through the salvage mechanism, that utilises bases and nucleosides as substrates (Stasolla et al. 2003).

The effects of GSH or GSSG supplementation to the maintenance medium on pyrimidine metabolism was studied by following the metabolic fate of radiolabelled orotic acid, precursor of the de novo synthesis pathway and uridine and uracil, respective intermediates of the salvage and degradation pathway. Inclusion of either GSH or GSSG to the medium increased the levels of pyrimidine nucleotide triphosphates and nucleic acids in cultured cells. It was demonstrated that elevated GSH levels result in activation of the de novo synthesis and reduction of the degradation pathway, whereas GSSG increases the activity of salvage pathway. Compared to the control tissue, grown on GSH-free medium, tissues cultured in the presence of 0.2 mM GSH were characterised by increased production of UMP from orotic acid leading to high cellular levels of UTP and CTP. The enlargement of
pyrimidine nucleotide pool observed in GSH-treated tissue was due to an increase in the activity of orotate phosphoribosyl transferase, an enzyme that converts orotic acid to orotidine-5'-monophosphate during the first step of pyrimidyne nucleotide synthesis pathway (Bellmonte et al. 2005b).

Induction of the de novo synthesis of pyrimidine nucleotides by GSH was accompanied by a reduction of the degradation pathway. Pyrimidine nucleotides undergoing degradation are converted to uracil, which in further steps is degraded to β-ureidopropionate and CO₂ (Stasolla et al. 2003). If the tissue was grown in the presence of GSH, substantially less radiolabelled uracil was converted to these degradation products. Instead, uracil was recovered into nucleotides and nucleic acids (Belmonte et al. 2005b).

In contrast to GSH, in the GSSG-treated tissue, the activities of synthesis and degradation pathways of pyrimidine nucleotides were at control level. On the other hand, GSSG increased the efficiency of the salvage pathway by stimulating the phosphorylation of uridine to uridine monophosphate (UMP). Following further phosphorylation UMP converts to UTP, which may directly be incorporated to RNA synthesis or serve as a substrate in CTP- or UDP-glucose synthesis. The reaction of UMP synthesis from uridine may be catalysed by uridine kinase or nucleoside phosphotransferase. Given that both enzymes respond to GSSG treatment with a simultaneous rise in their activities, indicates the possible mechanism of GSSG-dependent increase in the rate of uridine salvage (Belmonte et al. 2005b).

Similar approach was applied to test the effects of GSH or GSSG supplementation on purine nucleotide metabolism in white spruce embryogenic tissue grown on maintenance medium. The activity of synthesis pathway was studied determining the rate of incorporation of purine synthesis precursor AICAR to AMP, ATP + ADP and nucleic acids. It was observed that GSH or GSSG treatments did not significantly increase the efficiency of AICAR conversion into aforementioned compounds. This finding suggests that GSH and GSSG do not affect purine synthesis pathway (Belmonte et al. 2003). On the other hand, the differential salvage activity was observed between control and GSH-treated tissue manifested in an increased incorporation of the salvage pathway intermediates, adenine and adenosine, during ATP synthesis in GSH-treated tissue. High levels of ATP during exponential growth phase of the embryogenic tissue may be required not only as a building block for nucleic acid synthesis but also as an intermediate involved in bio-energetic processes related to cell proliferation. Finding that only low levels of labelled adenine and adenosine was recovered in nucleic acids, suggests that increased ATP synthesis is the principal outcome of GSH-dependent stimulation of purine salvage pathway (Belmonte et al. 2003). The rate of the degradation of purine nucleotides was not affected by GSH or GSSG treatments. However, in GSSG-treated tissue, a substantial amount of degradation pathway intermediate – inosine – was recruited for the ATP production compared to untreated control and GSH-treated tissue (Belmonte et al. 2003).

The data reported above show that the possible mechanism underlying the stimulation of the cell proliferation in white spruce embryogenic tissue by glutathione is related to an enhancement of the production of nucleotides serving as “building
blocks” for nucleic acid synthesis of substrates in bio-energetic reactions (Belmonte et al. 2003, 2005b). It was demonstrated that GSH availability regulates the activities of enzymes of nucleotide metabolism at the level of gene expression. The level of transcript coding for adenosine kinase was markedly higher in GSH-treated embryos during embryo maturation phase and germination. GSH supplementation to the medium reduced the expression of genes encoding for uracil phosphotransferase and nucleoside diphosphate kinase during the maturation phase. On the other hand, GSH stimulated the expression of uridinylate kinase in mature embryos of white spruce (Stasolla et al. 2004).

10 Glutathione-Induced Changes in Patterns of Gene Expression During Somatic Embryo Formation

The beneficial effect of GSH on embryo conversion is due to profound changes in gene expression patterns observed upon GSH administration. Microarray studies revealed that genes involved in large number of metabolic and regulatory pathways are differentially expressed between control and GSH-treated tissue. Compared with early stages of somatic embryo development, the total number of differentially expressed genes increased during embryo maturation (Stasolla et al. 2004). Many genes encoding for proteins involved in protein synthesis like ribosomal proteins, initiation and translation factors as well as late embryogenic abundant proteins were down-regulated in the presence of GSH during embryo maturation phase. The lower transcript level of genes related to protein synthesis, together with the reduced accumulation of protein bodies in embryos grown on GSH-supplemented medium is interpreted to be directly related to the switch to germination mode without PDT (Stasolla et al. 2004).

Some genes involved in carbohydrate metabolism, such as several glucanases, acetyl-CoA synthase, phosphoenolpyruvate carboxylase were repressed by the presence of GSH. On the other hand, expression levels of other group of genes involved in carbohydrate processing, represented by transketolase, fructokinase, aldolase and aconitase hydratase were elevated in GSH-treated embryos (Stasolla et al. 2004). Changes in the expression of aforementioned genes may account for an increased starch deposition in the cell of GSH-treated embryos (Stasolla et al. 2004).

An establishment of functional shoot and root apical meristem during somatic embryo maturation is dependent on expression of genes involved in meristem identity and organisation (Golz 2005). It was observed that several genes falling into that category, including the homologues of CLAVATA 1 (CLV 1), NO APICAL MERISTEM (NAM) and ARGONAUTE (AGO) were substantially stimulated by GSH treatment during the late phases of embryo development (Stasolla et al. 2004). The aforementioned genes regulate division patterns within shoot apical meristem (Golz 2005). Among genes that regulate differentiation within root apical meristem, a SCARECROW (SCR) gene was found be up-regulated in mature embryos grown on GSH-supplemented medium (Stasolla et al. 2004).
Relation between expression patterns of genes involved in meristem patterning and glutathione levels was studied with regard to spruce KNOTTED-like homeobox (KNOX) gene HBK 1. The HBK 1 gene is preferentially expressed in the shoot apical meristem of spruce where, similar to its Arabidopsis homologue SHOOT MERISTEM ELESS (STM), plays decisive role in meristematic cell specification (Sundås-Larsson et al. 1998; Hjortswang et al. 2002). In situ hybridisation experiments using probes specific to HBK1 mRNA revealed that long-term culture in the presence of GSSG extended the HBK 1 expressing zone within an apical shoot pole. Localisation pattern of HBK 1 was similar for both control and GSSG-treated embryos during the first 10 days of culture. HBK 1 transcript was restricted to the apical pole of embryos. Differences in the expression pattern were visible at day 20 when HBK 1 expressing zone was still restricted to the apical cells in control embryos, whereas was extended to the sub-apical cells in treated embryos. Moreover, less cells expressed HBK 1 gene if meristem was disrupted by intercellular spaces. These findings raise a possibility that the improvement of the meristem organisation by GSSG is possibly related to conferring stem cell identity to larger population of cells through stimulation the HBK 1 expression (Belmonte et al. 2005b).

A transformation approach using white spruce cells transformed with HBK 3 gene in sense or antisense orientation revealed that representatives of HBK gene family control both early and late stages of somatic embryogenesis in spruce. Overexpression of HBK 3 gene in cells of HBK 3 – sense line promoted the initiation of embryo formation in embryogenic tissue. On the other hand, embryo formation was strongly reduced in transgenic lines where down-regulation of HBK 3 expression was imposed due to antisense HBK 3 transformation. Compared to the control embryos, those over-expressing HBK 3 formed larger shoot apical meristems and exhibited higher competence for generating viable plants with no phenotypic aberrations (Belmonte et al. 2007). Further studies on HBK 3 over- or under-expressing lines of embryogenic cells revealed an intimate relationship between HBK 3 expression level and ASC and GSH metabolism. Both, GSH and ASC levels were significantly higher in cells over-expressing HBK 3 especially after the onset of somatic embryo formation from embryogenic tissue induced by transfer from plant growth regulator-supplemented medium onto plant growth regulator-free medium. This increase was due to the activities of ASC and GSH regenerating enzymes. The activities of DHAR, MDHAR and GR were elevated in cells of HBK 3 over-expressing line. These findings suggest that HBK 3 expression might regulate the transition from cell proliferation to embryo formation in embryogenic tissue through alterations in ascorbate and glutathione metabolism (Belmonte and Stasolla 2009).

Similar to GSH/GSSG treatments, an application of BSO, resulting in a shift in the glutathione pool towards its oxidised form, induced the expression of genes involved in shoot meristem formation. Treatment of Brassica napus somatic embryos with 0.1 mM BSO resulted in an increase in the expression levels of ARGONAUTE 1 (AGO 1) transcript during the globular and early torpedo stages of development (Stasolla et al. 2008). AGO 1 is a protein required for both shoot meristem formation and identity, characterised by conserved PAZ and PIWI domains.
that are engaged in protein–protein interactions. This protein is closely related to CLAVATA 3 – a well recognised factor engaged in meristem patterning and maintenance (Lynn et al. 1999). During the same stage of embryo development, BSO treatment increased the expression of gene coding for CLE 27 protein (Stasolla et al. 2008), engaged in meristem size regulation (Fiers et al. 2007). During next stages of embryo formation, i.e. when embryos complete their histodifferentiation programme and switch to post-embryonic development, besides AGO 1 and CLE 27, the level of ZWILLE transcript increased in BSO-treated embryos (Stasolla et al. 2008). ZWILLE gene codes for protein specifying the expression pattern of STM, which is also involved in the maintenance of apical shoot meristem (Moussian et al. 1998). Finally, in embryos cultured on BSO-supplemented medium, the expression of B. napus STM homologue increased in a late stage of embryo development (Stasolla et al. 2008). The data mentioned above, demonstrate that the imposition of an oxidised environment, effected by BSO application results in a sequential induction of a set of genes responsible for proper organisation of shoot apical meristem. This finding accounts for a beneficial effect of BSO treatment on the structure of the apical meristem.

11 Ascorbate and Glutathione Involvement in Adventitious Root Formation In vitro

Adventitious rooting consists of two stages: formation of root primordia and its subsequent outgrowth. The phase of primordia formation can be subdivided on the induction phase, when molecular and biochemical events related to dedifferentiation and competence acquirement occur, and initiation phase, characterised by organised cell divisions in the developing root primordium (Gaspard et al. 1997; Li et al. 2009a). Rooting is affected by numerous endogenous and exogenous factors, with the principal role of auxin as a chief regulator of adventitious root formation. Blocking the transport of endogenous auxin to seedling rooting zone inhibits rooting (De Klerk et al. 1999). In the regulation of adventitious rooting process, ascorbate and glutathione seem to be involved in a complex interplay between auxin and other components of cellular redox systems.

It was demonstrated that auxin stimulatory effect on rooting is mediated by NO (Pagnussat et al. 2002) and the NO-dependent signalling pathway, leading to root formation, is mediated by H$_2$O$_2$ (Liao et al. 2009). The signalling role of H$_2$O$_2$ in root formation was explored in studies on rooting of cucumber (Li et al. 2007) and Mung bean seedling cuttings (Li et al. 2009a). It was found that the culture in the presence of 20–40 mM H$_2$O$_2$ significantly increased the number of adventitious roots per explant formed by cucumber cuttings, when compared to the untreated control (Li et al. 2007). Mung bean cuttings reacted with increased rooting to H$_2$O$_2$ concentrations ranging from 1–100 mM. Cuttings underwent 8–24-h-long pulse H$_2$O$_2$ treatment followed by culture on the H$_2$O$_2$-free medium. Stimulatory effect of H$_2$O$_2$ treatment was observed both in the absence and in the presence of exogenous...
IBA as an auxin source. Promoting effect of H$_2$O$_2$ on rooting decreased if a relatively higher H$_2$O$_2$ concentration and longer treatment times were applied (Li et al. 2009a). Decreasing H$_2$O$_2$ content, with diphenyloiodonium (DPI, NADPH oxidase inhibitor), catalase or ASC prevented the stimulation of rooting. Application of 4 mM ASC significantly decreased the number of regenerated roots and eliminated the stimulatory effect of H$_2$O$_2$ if these two substances were applied simultaneously (Li et al. 2009a). In contrast to H$_2$O$_2$, IBA still stimulated rooting in the presence of 4 mM ASC in Mung bean, and IAA stimulated rooting in the presence of 2 mM ASC in cucumber (Li et al. 2007, 2009a). On the other hand, ASC in concentration of 6 mM completely prevented rooting in the absence or presence of either H$_2$O$_2$ or IBA. These results suggested that H$_2$O$_2$ or IBA promotion of adventitious root formation was blocked by certain concentrations of ASC (Li et al. 2009a).

Measurements of H$_2$O$_2$ concentration in cucumber seedling explants during subsequent phases of rooting on auxin-free medium revealed that H$_2$O$_2$ peaks during the early induction phase. It may suggest that elevated H$_2$O$_2$ levels may possibly be required in early events of rooting, i.e. dedifferentiation and acquiring competence (Li et al. 2007). In Mung bean cuttings cultured on basal medium an induction phase of rooting was marked by a progressive increase in POX and APX activities. At the switch from induction to initiation phase, activities of both enzymes strongly decrease. It was demonstrated that IBA treatment transiently decreased POX and APX activities during induction phase of rooting, which may be correlated with an increase in endogenous H$_2$O$_2$ level, required for the induction of adventitious roots. Therefore, APX activity may be involved in an auxin-dependent mechanism of regulation of H$_2$O$_2$ level during adventitious rooting and the early decrease in its activity may be one mechanism by which IBA and H$_2$O$_2$ promote adventitious rooting (Li et al. 2009b). It should be noted that although the moderate oxidation stimulates rooting, H$_2$O$_2$-overproduction, resulting in severe oxidative stress inhibited root formation (Pal Singh et al. 2009).

Imin and co-workers (2007) show that both reduced (GSH) and oxidised (GSSG) form of glutathione markedly enhance the number of roots formed by callus derived from leaf explants of *Medicago truncatula* cultured on auxin-supplemented medium. In their experiments leaf segments were grown for 3 weeks on proliferation medium supplemented with NAA or NAA in combination with either GSSG or GSH. Significantly, more roots were produced by explants grown on NAA-containing media supplemented with GSH or GSSG than on media supplemented with NAA alone, which suggests that glutathione may act synergistically with exogenous auxin in the stimulation of root regeneration (Imin et al. 2007). Because in the absence of NAA, root formation was negligible (Imin et al. 2007), it would be interesting to find out if GSH or GSSG may, to some extent, replace the hormone in the stimulation of root formation. However, the authors do not include any data on effects of GSH or GSSG on root formation on NAA-free media (Imin et al. 2007).

During early stages of rooting in tomato seedling cuttings, when the root primordia are formed, total glutathione pool is characterised by higher participation of GSSG in the total glutathione pool in comparison to the later stages when the outgrowth
Ascorbate and Glutathione in Organogenesis

and elongation of newly formed roots occurs (Tyburski and Tretyn 2010). Higher oxidation of glutathione pool during the formation of root primordia by tomato seedling cuttings may result from an increase in the rate of ascorbate turnover in Halliwell–Asada cycle and a strong rise in the activity of dehydroascorbate reductase at this stage of root formation. Subsequent days of rooting are characterised by a decline in the activity of dehydroascorbate reductase and other enzymes of ascorbate metabolism (Tyburski et al. 2006). Consequently, higher amounts of reduced glutathione can be accumulated in the rooting zone (Tyburski et al. 2006).

Supplementing the rooting medium with GSH increased the number of roots formed by tomato seedling cuttings grown on an auxin-free medium. However, the stimulatory effect of GSH was restricted to a narrow range of concentrations spanning from 1 or 2.5 mM GSH. Strongest stimulation of root formation occurred when plants were simultaneously treated with auxin and GSH. Treatments with GSSG did not affect root formation if cuttings were grown on the basal medium, however, an optimised GSSG concentration strongly enhanced the rooting-stimulatory effect of auxin treatment. BSO did not affect the number of roots formed by cuttings grown on BM and only slightly decreased the efficiency of rooting in the presence of IAA, which shows that depletion of GSH from rooting zones does not inhibit rooting. This finding suggests that root formation may occur in the absence of GSH but the process is stimulated by its presence (Tyburski and Tretyn 2010).

Root regeneration by tomato seedling cuttings proceeds on a highly synchronized manner with a 3-day-long period of primordia formation encompassing initiation and induction phase and subsequent phase of primordia elongation, which manifests in root emergence on the fourth day of culture. Induction and initiation phases of rooting in tomato seedling cuttings are characterised by rapid increase in the content of ASC in the explant rooting zone. The accumulation of ASC in the tissue correlates with the biosynthetic capability from GalL and with the activities of enzymes regenerating ASC from its oxidised forms (MDHAR and DHAR). Simultaneously, the sharp increase in \( \text{H}_2\text{O}_2 \) content and in the activities of APX and AOX were observed. ASC peaks on the third day of rooting, i.e. at the switch from the phase of primordia organisation to the phase of primordia elongation. With the beginning of elongation phase a dramatic decrease in ASC content was observed. In contrast to ASC, DHA levels remained constant during root formation. Similar to ASC, the \( \text{H}_2\text{O}_2 \) level, as well as, the activities of ASC-metabolising enzymes dropped at the onset of the root elongation phase. These finding suggest that the increase in the endogenous level of ASC observed at the beginning of rooting may be explained by the necessity of the regulation of \( \text{H}_2\text{O}_2 \) level in the rooting zone during the formation of root primordia. This point of view is supported by the finding that the activities of \( \text{H}_2\text{O}_2 \) scavenging enzymes: APX, POX and CAT rise simultaneously with the ASC (Tyburski et al. 2006).

Functions of ascorbate in adventitious rooting seem to extend beyond the regulation of \( \text{H}_2\text{O}_2 \) levels (Tyburski et al. 2006). It has been observed that the addition of exogenous GalL, ASC or DHA to the rooting medium affect the root formation by tomato seedling cuttings. The ascorbate precursor (GalL) as well as ASC and DHA modified the rooting response in a similar way, i.e. stimulated the formation
of roots but inhibited their elongation. Therefore, their effect was similar to that of exogenous auxin, which also increased the number of regenerated roots, simultaneously reducing their length (Tyburski et al. 2006) (Fig. 2). It is noteworthy that DHA was more effective in inducing abundant root formation than ASC and GalL, having higher stimulatory effect than auxin. Moreover, because ASC added to the medium, being an unstable molecule, is partly oxidised to DHA and GalL after being converted to ASC is subsequently oxidised to DHA. It has been found that treatments with DHA and GalL that induced more roots than ASC, resulted in a significant increase in DHA content in the rooting zones in comparison with ASC-treated cuttings. Therefore, the oxidised form of ascorbate is supposed to have a decisive role in the stimulation of rooting (Tyburski et al. 2006).

Rooting of cuttings does not occur, or is severely reduced, if auxin transport to the rooting zone is blocked by application of auxin transport inhibitors like NPA or TIBA. These treatments result in a suboptimal auxin level in explant rooting zone that block root formation (Visser et al. 1995; Ludwig-Müller et al. 2005). Several redox agents were able to restore root development in NPA or TIBA treated explants. NO and H$_2$O$_2$, which are believed to mediate in the auxin signalling pathway, were able to trigger adventitious root formation in Tagetes erecta seedling cuttings cultured on medium supplemented with 10 mM NPA (Liao et al. 2009). On the other hand, an inhibitory effect of 1 μM TIBA on rooting of tomato seedling cuttings was reversed by 4 mM ASC. It has been found that only ASC (but neither GalL nor DHA) was able to reverse the stimulatory effect of TIBA on root formation, although the stimulatory effect of GalL and DHA on rooting of cuttings grown on medium without TIBA was comparable to the one of ASC or even stronger. The question of why ASC added to rooting the medium is able to reverse the inhibition of rooting by TIBA while the GalL administration, which actually efficiently increases the ASC content in the root-forming tissue remains unresolved (Tyburski et al. 2006).

Fig. 2 The effect of ASC, DHA and IAA on rooting of tomato seedling cuttings. The cuttings were cultured 7 days on basal medium (a), or on the same medium supplemented with 2 mM ASC (b), 2 mM DHA (c) or 1 μM IAA 9 (d). Similar to exogenous auxin, ASC and DHA stimulate the formation of roots but inhibit their elongation.
Isolated root systems grown in bioreactors as suspensions on liquid media serve as important source of metabolites for commercial purposes (Flores et al. 1999; Guillon et al. 2006). It was demonstrated that changes in activities of the enzymes of ASC and GSH metabolism may influence both root development in culture as well as the rate of metabolite production, which often needs elicitation by oxidative stress-inducing factors. The production of ginsenoside by adventitious cultures of *Panax ginseng* was enhanced by increasing O$_2$ concentration from 20% to 40%. The same effect was achieved when roots were treated with optimised concentrations of H$_2$O$_2$. An increase in metabolite production was observed between 15 and 45 days after the exposition to O$_2$ started. On the other hand, treatments with 25 and 50 μM H$_2$O$_2$ resulted in increased ginsenoside levels after only 7 days after treatment. As inferred from malonyl dialdehyde (MDA) content and H$_2$O$_2$ levels in O$_2$-treated roots, this treatment imposed a mild oxidative stress, which seems to have a stimulatory effect on the induction of ginsenoside production. To counter the oxidative damage, antioxidant enzymes of ascorbate–glutathione cycle were induced by O$_2$ application. An increase in APX activity was detected after 15 days of treatment and from that day on high enzyme activity was observed during the rest of culture period with maximum after 45 days. Other enzymes, i.e. MDHAR, DHAR and GR behaved in a similar manner. Increase in their activities was coordinated with increases in the activities of other constituents of antioxidant system such as superoxide dismutase (SOD), CAT, glutathione peroxidase (GPX) and guaiacol peroxidase (POX). These finding suggest that during O$_2$/H$_2$O$_2$-elicited ginsenoside production, the intensity of oxidative stress is regulated in a coordinated manner by synchronized changes in the activities of antioxidant enzymes (Ali et al. 2005a).

Redox agents played a decisive role also in the regulation of root proliferation in culture. The proliferation of *Panax* isolated adventitious roots was stimulated by NO. It was demonstrated that supplementing media with NO producing producers like sodium nitroprusside (SNP) increased the number of rootlets per explant. On the other hand, decreasing NO levels with NO-scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl3-oxide (PTIO), prevented root proliferation (Tewari et al. 2007). NO stimulatory effect was blocked if roots were treated with DPI – an inhibitor of O$_2^-$-producing enzyme: NADPH-oxidase. This observation together with the finding that NO stimulates NADPH oxidase in ginseng roots suggest that NO stimulatory effect on root proliferation is mediated by O$_2^-$.- However, along with increasing the pro-oxidant activity of NADPH oxidase, NO stimulated the activity of the set of antioxidant enzymes including SOD, CAT, POX, APX, DHAR and GR. Treatment with SNP also increased the levels of total ascorbate and the participation of ASC in the pool of total ascorbate. These effects were prevented by the application of NO scavenger. Therefore, a coordinated stimulation of pro-oxidant and antioxidant systems by NO impose a moderate oxidative stress conditions which promote root proliferation in culture (Tewari et al. 2008).
The onset of secondary metabolite production in hairy root culture often requires the treatments with elicitors; the signalling molecules of plant defence responses (Guillon et al. 2006). Elicitor treatments frequently induce oxidative stress, which sometimes is a direct factor triggering metabolite synthesis (Huerta-Heredia et al. 2009). When biotransformation process in a hairy root culture is dependent of ROS production, antioxidant activity of ASC may have an inhibitory effect, as it was shown for the tetracycline phytoremediation by hairy roots of sunflower (Gujarathi et al. 2005). However, in other experimental systems, ASC, GSH and associated enzymes play an important role in protecting cultured roots from oxidative damage during elicitation (Ali et al. 2005b). The stimulation of saponin production by hairy root cultures of *Panax ginseng* and *Panax quinquefolium* by methyl jasmonate (MeJA) was accompanied by a gradual increase in \( \text{H}_2\text{O}_2 \) content. In addition, *P. quinqufolium* root culture showed a marked increase in lipooxygenase activity upon MeJA treatment, which usually results in increased lipid peroxidation. However, the markers of lipid peroxidation declined upon elicitation in roots of both species of ginseng, which suggests that in spite of increased oxidation no severe oxidative damage occurred in treated roots. It was shown that MeJA-induced oxidative stress was mitigated by an increase in the activities of various components of the antioxidant system. Upon elicitation, ASC content and ASC/DHA ratio as well as SOD and POX activities increased in both species. On the other hand, a decrease in CAT activity was observed. APX activity increased after MeJA treatment in *P. ginseng* but not in *P. quinqufolium*. In contrast, *P. quinqufolium* was characterised by increased DHAR activity but the activity of this enzyme decreased in *P. ginseng*. These findings indicate that the enzymes of ASC–GSH cycle play a protective role against oxidative stress during the elicitation of metabolite production in hairy root culture (Ali et al. 2005b).

13 **Ascorbate and Glutathione Roles in Shoot Organogenesis**

**In vitro**

Relatively less attention, when compared with somatic embryogenesis and root formation, was paid on the role of ascorbate and glutathione in shoot differentiation in plant tissue culture. Early reports suggest that ASC may enhance shoot organogenesis in tobacco callus culture (Joy et al. 1988). Addition of 0.8 mM ASC to the shoot-forming medium increased shoot formation by 45% over the control in young callus, having a high organogetic potential. Different concentrations of exogenous ASC (0.4–0.8 mM) were able to restore regenerative capacities of old callus tissue, characterised by reduced ability to regenerate shoots. Treatment with ASC also speeded up the shoot-forming process with the appearance of primordia as early as day 10 in culture, compared to more than 12 days in control tissue. It has also been shown that ASC treatment reversed the inhibitory effect of exogenous gibberelic acid on root formation. The stimulatory effect of ASC on shoot differentiation in tobacco callus was linked to the content of soluble sugars, which increased in tissue
Ascorbate and Glutathione in Organogenesis

during ASC treatment. However, the mechanism of ascorbate involvement in shoot organogenesis in tobacco has not been explained (Joy et al. 1988).

Treatments with reduced glutathione improved the development of isolated shoot tips of apple (Nomura et al. 1998). Isolated shoot meristems were grown on basal medium supplemented with BAP for shoot development and then transferred onto the medium containing IBA for rooting the differentiated shoots. Efficiency of shoot tip culture was strongly reduced by explant browning observed within 1 day after the start of culture. Explant browning was prevented by dipping the shoot tips in 0.1 mM GSH, prior to inoculation on a solid medium. Besides inhibiting browning, dip treatment increased the number of shoot tips that developed into normal shoots (Nomura et al. 1998). Treated shoots developed into normal shoots with an efficiency of 100%. In the untreated control, rates of shoot development from untreated shoots did not exceed 60%. Promotion of normal shoot development by the dip treatment was also expressed in terms of the length of first leaves from cultured shoot tips. The dip-treated shoot tips produced longer first leaves when compared to untreated controls. Interestingly, the promotion of shoot development was observed only if pulse GSH treatment was applied before the start of culture and after that, shoot tips were grown on basal medium. If instead of GSH pulse treatment, shoot tips were cultured on medium supplemented with GSH, shoot development was not stimulated. On the other hand, cultures on medium containing GSH promoted callus proliferation at the base of shoot tips (Nomura et al. 1998).

Reducing conditions imposed by adding antioxidants (ASC, GSH and \(\alpha\)-tocopherol) stimulated shoot organogenesis from leaf segment of gladiolus (Dutta Gupta and Datta 2003). Both, frequency of shoot organogenesis as well as the number of number of shoots per explant were increased with the addition of antioxidants at 0.5 mM concentration. However, higher concentrations were found to be inhibitory. Compared to \(\alpha\)-tocopherol and ASC, GSH was noted to be most effective. On the other hand, \(\text{H}_2\text{O}_2\) added to the culture medium in concentrations ranging from 0.005 to 0.15 mM strongly inhibited shoot organogenesis. Moreover, the process of shoot formation was marked by a gradual increase in the CAT and POX activities with a concurrent decrease in the SOD activity. Given that, CAT and POX are \(\text{H}_2\text{O}_2\)-consuming enzymes, whereas high SOD activity results in elevated \(\text{H}_2\text{O}_2\) levels, it was concluded that shoot organogenesis requires a reducing environment (Dutta Gupta and Datta 2003).

In contrast to shoot organogenesis, somatic embryo formation on the gladiolus leaf explants was promoted by oxidising factors. The frequency of somatic embryogenesis and the number of embryos per responding culture were decreased by ASC, GSH and \(\alpha\)-tocopherol in a dose-dependent manner. On the other hand, \(\text{H}_2\text{O}_2\) at 100 \(\mu\text{M}\) concentration increased the frequency of somatic embryogenesis by about 18%. The process of somatic embryo formation was accompanied, in its initial phase, by an increase in SOD activity and progressive reduction in CAT and POX activities, which creates conditions favourable for \(\text{H}_2\text{O}_2\) accumulation (Dutta Gupta and Datta 2003). Similar to gladiolus, initial phase of somatic embryo formation in the callus of \textit{Lycium barbarum} was characterised by increase in SOD activity accompanied by decrease in POX and CAT activities, which resulted in a marked increase in intracellular \(\text{H}_2\text{O}_2\) levels. Moreover, the treatments that increase \(\text{H}_2\text{O}_2\) levels, such as inhibiting
J. Tyburski and A. Tretyn

CAT activity with aminotriazole or adding \( \text{H}_2\text{O}_2 \) to the culture medium promoted somatic embryogenesis. On the other hand, inhibiting SOD activity with \( \text{N},\text{N}'-\text{diethyldithiocarbamate} \) caused a decrease in the frequency of somatic embryogenesis (Kairong et al. 1999). The aforementioned data suggest that shoot organogenesis and somatic embryogenesis differ in their redox requirements, being promoted by reducing or oxidising environment, respectively. However, it should be kept in mind that in some shoot regeneration systems, similar to somatic embryogenesis, oxidising processes may also have a stimulatory effect on organogenesis. This was the case for shoot organogenesis in strawberry callus, where \( \text{H}_2\text{O}_2 \) levels and SOD activity increased during early phases of shoot regeneration when meristemoid formation and vascular tissue occurs in calli grown on regeneration medium. Simultaneously, a decline in \( \text{H}_2\text{O}_2 \) scavenging enzymes; CAT and POX was observed (Tian et al. 2003). Similar to somatic embryogenesis systems (Kairong et al. 1999; Dutta Gupta and Datta 2003), shoot organogenesis percentage in strawberry was stimulated by exogenous \( \text{H}_2\text{O}_2 \) and decreased by SOD inhibitor – \( \text{N},\text{N}'-\text{diethyldithiocarbonate} \). Moreover, it was shown that types of strawberry calli, which showed high regeneration capacity, had several times higher \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) levels compared to calli with low-regeneration capacity (Tian et al. 2004).

14 Conclusion

The studies reported in this review demonstrate that ascorbate- and glutathione-dependent biochemical systems play important roles in many aspects of plant tissue culture, affecting growth, differentiation and metabolism. Ascorbate and glutathione participate in plant regeneration, being involved in the mechanisms regulating cell divisions in newly formed meristems and participating in hormone metabolism and signalling. Moreover, an antioxidant activity of these molecules manifested in Halliwell–Asada cycle protects in vitro cultured tissues against oxidative stress. Diverse functions of these antioxidants open vast possibilities of using them for the improvement of tissue culture and plant regeneration methods. However, further studies are required to fully exploit the properties of ascorbate and glutathione for manipulating developmental processes in plant tissue culture.

References


Ascorbate and Glutathione in Organogenesis


Ascorbate and Glutathione in Organogenesis


Ascorbate-Glutathione Pathway and Stress Tolerance in Plants
Anjum, N.A.; Umar, S.; Chan, M.-T. (Eds.)
2010, XVII, 443 p., Hardcover