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# Stress-induced nitric oxide and adaptive plasticity in conifers

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**ABSTRACT**: The excitable properties of conifer protoplasm consist of nitric oxide (NO) bursts that prime and prepare chemical messengers for the transmission of stressful environmental signals. NO in somatic and reproductive cells is produced in response to mechanical forces, gravity, wounding, changes in nutrition, hypoxia, drought, salinity, temperature shock, pollutants, and pathogen attack. NO arises primarily from nitrite via nitrite:nitric oxide reductase and nitrate reductase. It also arises from arginine N and oxygen via putative nitric oxide synthase activity. NO rapidly reacts with, oxygen species, hemes, thiols, and proteins to produce biochemical signals that directly and indirectly regulate enzymatic activity. The effects of NO depend on its location and concentration. Beneficial reactions counteract oxidative and nitrosative stresses, while damaging reactions, due to high levels of NO, cause oxidative and nitrosative damage, and cell death. NO contributes to structural and functional adaptive plasticity, and to the habituation of trees to their sites. The use of NO donors and traps, and enzyme inhibitors offers a new experimental approach and countermeasures to control stress signals throughout conifer life histories.

**Keywords**: nitric oxide; adaptive plasticity; conifers; programmed cell death; embryogenesis; tree decline; nitrogen assimilation; oxidative and nitrosative stress

NO is a lipophilic, free radical, and a paramagnetic gas that diffuses and reacts rapidly with molecular oxygen in the gas and aqueous phase. NO also reacts with hemes and thiols to produce other transient compounds whose functions remain largely uninvestigated. In conifer cells, NO bursts comprise the excitability and irritability of protoplasm by 'priming' and producing transient chemical messengers that are rapidly transmitted throughout the cell and, and in some cases, to other cells. Three significant outcomes are **1**: protection against oxidative and nitrosative stresses, **2**: the signaling of adaptive structural and functional changes for survival and habituation, and **3**: damaging reactions requiring repair or resulting in cell death and necrosis (DURZAN, PEDROSO 2002). The association of NO with the excitability of stressed cells was reaffirmed by the development of methods that could visualize and control levels of NO (MAGALHAES et al. 1999; PEDROSO et al. 2000a,b). NO is formed in subcellular organelles, e.g., chloroplasts, peroxisomes, amyloplasts, and in nearly all tissues, e.g., pollen, embryos, guard cells, wounded tissues, etc. The role of NO in postharvest physiology has been reviewed by LESHEM (2000), in plant pathology by WENDEHENNE et al. (2001), and in animal cell physiology by MONCADA, ERUSALIMSKY (2002).

## SOURCES OF NO

NO arises from at least four enzymatic sources (DURZAN, PEDROSO 2002). **1**. nitrate reductase (NR) activity where *nitrite* becomes a substrate for NO in the presence of NADH (YYAMASAKI et al. 2001), **2**. nitrite accumulation arising from nitrite reductase (NiR) activity, **3**. the conversion of nitrite to NO by nitrite:nitric oxide reductase (NI-NOR), and **4**. from putative nitric oxide synthase (NOS) activity. NOS substrates (L-arginine, NADPH, and oxygen), products (NO, NAD, and L-citrulline) are ubiquitous in plants, and pivotal components of the intermediary N metabolism of conifers. NO can also form *nonenzymatically* from nitrite depending on pH and oxygen availability and the presence of reducing agents.

Many angiosperms and gymnosperms show putative NOS activity (DURZAN, PEDROSO 2002; WENDEHENNE et al. 2001). It is still unclear how many endogenous sources exist, and if, when and where, they coexist. NOS activity was not detected in tobacco plasma membranes. Only NI-NOR activity was present (STÖHR et al. 2001). Double NR mutants of *Arabidopsis* survived only with ammonium as a N source, and did not emit NO into the atmosphere (MAGALHAES et al. 1999). However, the same mutants produced NO inside stressed cells. This

indicated that NOS activity was responsible for the endogenous NO (GARCĘS et al. 2001). Exposure of plants to a guanidino compound that is a universal NOS inhibitor, *viz*. *N*-monomethyl-L-arginine (NMMA), completely blocked endogenous NO production. This response also ruled out the possibility that any residual nitrite and NR activity were sources of the NO. In the non-mutant plants grown with nitrate and ammonium as N sources, NO bursts always preceded the release of ethylene.

Conifers convert arginine to a wide range of guanidino compounds (BIDWELL, DURZAN 1975; DURZAN 1968, 1969). Arginine analogues and guanidino compound (also called guanidines) are potent NOS inhibitors (HOBBS et al. 1999). In *Taxus*, *Araucaria*, and *Picea* sp. NMMA completely blocked NO production (PEDROSO et al. 2000a,b; unpublished data). When nitrate was supplied in sand cultures to white spruce and jack pine as a sole N source, the guanidino compounds were almost undetectable. By contrast, ammonia supplied as a sole N source under the same conditions, contributed to a significant increase in γ-guanidinobutyric acid and several yet unidentified guanidines (DURZAN, STEWARD 1967). Many guanidines were detected in a worldwide conifer seed collection at the Petawawa Forest Experiment Station, Ontario, Canada. White spruce (shade-tolerant) and jack pine (shade-intolerant) saplings grown under lath houses at low light to 13% natural light reduced their arginine N levels, and significantly increased the proportion of guanidino compounds as biomass was adaptively redistributed in shoots and roots (DURZAN 1971, unpublished).

Many plants and soil microorganisms release NO into the air especially at night (WILDT et al. 1997). The emitted NO reacts rapidly with oxygen and carbon dioxide to produce $NO_x$. Elevated levels of carbon dioxide impede nitrite translocation into chloroplasts of some agronomic species (BLOOM et al. 2002). This has major implications for the ability of some plants to use nitrate as an N source under elevated carbon dioxide. While not yet recognized, it is possible that the availability of nitrate and nitrite could contribute to the overproduction of NO in conifers exposed to elevated levels of carbon dioxide. The responsible plant enzymes and the genes for the endogenous production and emission of NO have not been isolated nor fully characterized.

## NO BURSTS

NO priming reactions are far-from-equilibrium, chaotic, dissipative, and create new levels of protoplasmic organization. Repeated NO bursts may not always produce the same response because of hysteresis in the stressed cellular response system. A postresponse reduction in strength or magnitude of the NO bursts may be due to the length of the interstimulus interval, and system fatigue. Rapid subcellular changes were seen in the time-lapse photography of jack pine cells exposed to D- and L-glutamine (DURZAN, BOURGON 1976). Added L-glutamine stimulated peristaltic streaming in transvacuolar strands and increased the interactions among organelles at rates that were similar to the diffusion of NO. L-Glutamine provides N for the biosynthesis of arginine, and is a product of nitrate and ammonia assimilation via NR and NiR. D-Glutamine acted as a poison, stopped protoplasmic streaming, enlarged the nucleolar vacuoles, and led to cell death.

The shoot growing tips of plants rotate, growth upward, and show phototropism. These processes comprise a simple search process to find light. If a leader shoot tip gets mechanically blocked or damaged by an insect, a NO signal may allow the tip to 'escape' and continue its growth. In the case of many conifers a new leader shoot may be initiated. This illustrates how adaptive growth involves the interaction between the variations in tropic behavior and positional orientations through trial and error.

## REACTIONS OF NO

The main direct biological targets of NO are the hemes that shuttle three gases key to plant life, *viz*. NO, carbon dioxide, and oxygen. Hemes use NO to control oxygen levels; protect against sulfides; sense and scavenge oxygen radicals; and are useful in the detoxification of halogens. Hemes have the highest association rate constant for NO in forming a metal nitrosyl complex. The rapid *oxidation* and *addition* reactions of NO with hemes disarm and preserve NO bioreactivity, respectively. This bioactivity extends the inhospitable environments available to organisms.

NO reacts with thiols (*S*-nitrosation) to produce *S*-nitrosothiols (RSNOs) (Fig. 1). NO may be stored and shuttled by transnitrosation (also known as transnitrosylation) of proteins with thiol groups. The sites and spatial distribution of NO formation, fluxes of NO, and the duration of NO production are important physiological factors. At NO concentrations below 1 μM, and distant from the site of production, the *direct effects* of NO predominate. Direct NO targets are metal complexes (e.g., hemes, guanylate cyclase, cytochrome P450), nonheme iron proteins (iron-sulfur proteins), zinc and copper proteins, and powerful free radicals (hydroxyl, tyrosyl) that are lipid and carbon-centered (WINK et al. 1999).

At sites where high and sustained amounts of NO are produced, the *indirect effects* prevail. They arise rapidly and non-enzymatically from interactions of NO with oxygen and superoxide giving reactive nitrogen oxide species (RNS) and ROS. These products cause nitrosative and oxidative stress (GUTTERIDGE, HALLIWELL 1999). The stressful chemical reactions comprise *nitrosation* when the nitrosonium cation ($NO^+$) is added to an amine, thiol, or hydroxy aromatic group; *oxidation* when electrons are removed from a substrate; and *nitration,* when $NO_2^+$ (nitryl, nitronium) is added to a molecule. NO directly nitrates tyrosyl radicals in ribonucleotide reductase and photosystem II (DAVIS et al. 2001).

NO reacts with superoxide to produce peroxynitrite ($ONOO^-$). Peroxynitrite decay depends on pH giving peroxynitrous acid (OHNOOH) and nitrate. Both perox-

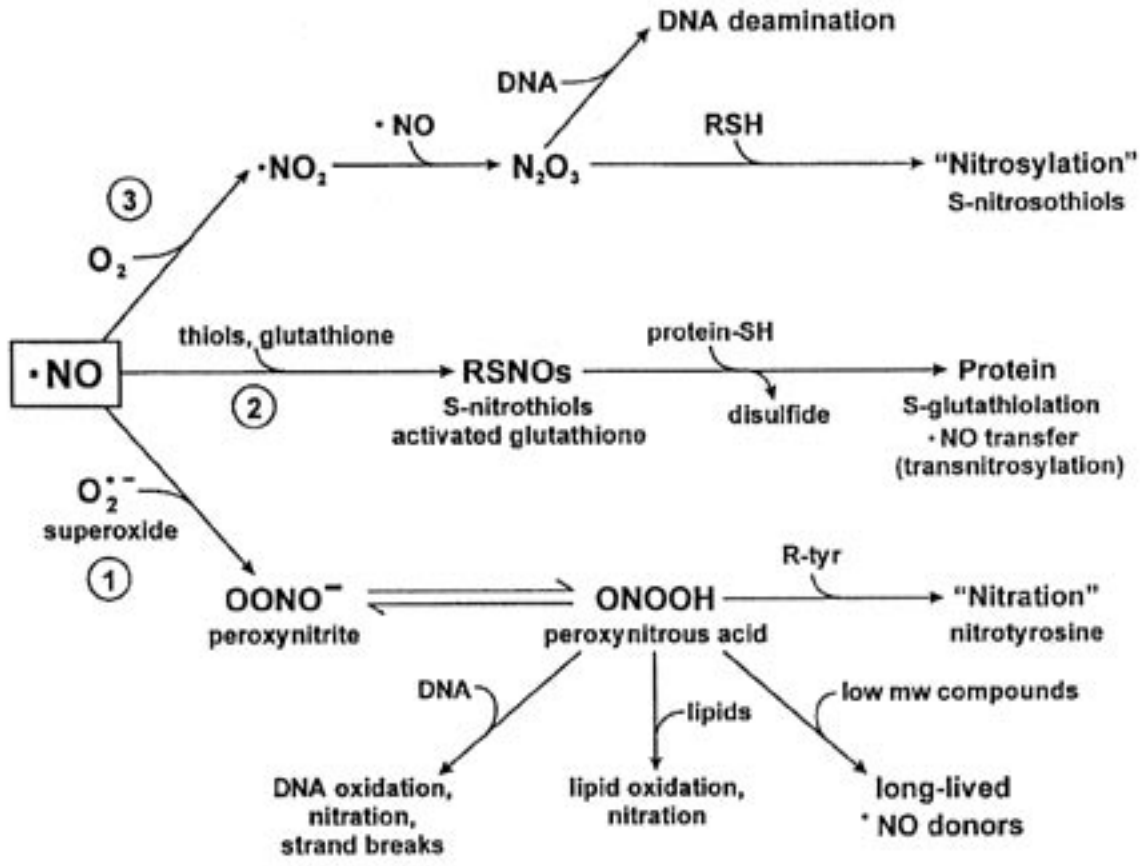


Fig. 1. Reactions of NO and RNS. **1.** NO shown as a free radical (NO) reacts with superoxide to form peroxynitrite and peroxynitrous acid leading indirectly to cellular damage. The protonation of peroxynitrite is enhanced by acidification resulting in peroxynitrous acid and nitrate. Peroxynitrite may also react with carbon dioxide and catalytically decompose via nitrosoperoxycarbonate ($ONOOCO_2^-$) releasing carbon dioxide and nitrate (not shown). Nitrosoperoxycarbonate can nitrate phenols and the tyrosine residues in proteins faster than either peroxynitrite or ONOOH. These reactions represent a sink for NO. **2.** The bioactive formation of *S*-nitrosothiols (RSNOs) for the storage, transfer, and production of NO. Proteins are regulated by thiols and *S*-glutathiolation to transfer and shuttle NO from protein to protein (transnitrosation, transnitrosylation), deliver NO to oxygen-poor tissues, and to elicit specific biological responses. NO from RSNOs is transferred by sulfur-to-nitrogen and sulfur-to-sulfur transnitrosation. This may involve the formation of carcinogenic N-nitroso derivatives. RNSOs also activate guanylyl (guanylate) cyclase (Fig. 2). *S*-Nitrosoglutathione reductase may control the intracellular levels of GSNO and *S*-nitrosylated proteins independently of oxidative stress. **3.** Reactions with oxygen producing $N_2O_3$ lead indirectly to DNA deamination. $N_2O_3$ also reacts with thiols producing S-nitrosothiols (RSNOs) that contribute to nitrosylation. These reactions balance the damaging effects of RNS, ROS, and the regenerative repair capacity of tissues. The damaging reactions contribute to aging and cell death. The beneficial reactions contribute to survival, adaptation and habituation throughout plant life histories

ynitrite and peroxynitrous acid damage cells indirectly. The peroxynitrite may react with carbon dioxide giving nitrosoperoxycarbonate ($ONOOCO_2^-$). The later can nitrosate phenolics such as the tyrosine residues in proteins or decompose giving carbon dioxide and nitrate. In conifers, the nitrosation of tyrosine to 3-nitrotyrosine in acid protein hydrolysates serves as a useful marker for reactions predisposing cell damage, differentiation, e.g., xylogenesis, and apoptosis (PEDROSO et al. 2000b).

*S*-Nitrosothiols (RSNOs) contribute to *S*-nitrosation (also known as *S*-nitrosylation). The *S*-nitrosylation of proteins in tissues or protein mixtures can be detected by gel electrophoresis and chemiluminescent imaging. The reactions of NO with glutathione produce *S*-nitrosoglutathione (GSNO) for the storage, transfer, and the subsequent production of NO in oxygen-poor tissues. NO transfer may be important for tissue development inside seeds, and in the development of moribund cells deep in a callus. NO from RSNOs can be transferred by sulfur-to-nitrogen and sulfur-to-sulfur transnitrosation. *S*-Nitrosoglutathione reductase, known from mammalian studies, is thought to control the intracellular levels of S-nitrosoglutathione (GSNO) and levels of *S*-nitrosylated proteins independently of oxidative stress. These reactions have not yet been examined in conifers.

The chemical reactions of NO with oxygen to produce dinitrogen trioxide ($N_2O_3$) will indirectly deaminate DNA. At physiological NO concentrations, $N_2O_3$ forms inside

hydrophobic cores and causes protein nitration. Dinitrosyl adducts of aconitase formed by NO disrupt the citric acid cycle (WINK et al. 1999). Aconitases are a family of dehydratases that catalyze the reversible isomerization of citrate and isocitrate via *cis*-aconitate and are important targets for NO in plants (WENDEHENNE et al. 2001).

The interactions of NO with components of the electron transport chain in chloroplasts and mitochondria have the potential to regulate stress-related photosynthesis and respiration (DURZAN, PEDROSO 2002). The production of NO in guard cells also adds a new but unexplored dimension to models involving photosynthesis, respiration and stress. High levels of NO contribute to aging but by themselves do not necessarily cause death. Aging is not a programmed process in the sense that genes have evolved specifically to cause damage and aging.

## NO AS A MOLECULAR SIGNAL

The first and crucial step in molecular sensing is the transduction of diverse stimuli into a cellular response (Fig. 2). NO signal transduction pathways comprise guanylyl cyclase (GC) and enzymatic reactions that are *dependent* or *independent* of cyclic guanosine 3‘,5‘-monophosphate (cGMP) (WENDEHENNE et al. 2001; DAVIS et al. 2001). NO increases the activity of soluble guanyl cyclases (sGCs) by interacting with its heme moiety. sGC converts GTP to cGMP. In spruce needles NO stimulates cGMP production (PFEIFFER et al. 1994). The binding of NO to sGC activates a rise in cGMP. A NO signal is transmitted by sGC to downstream elements of the signaling cascade *viz.* the cGMP-dependent protein kinases, cGMP-gated cation channels, and cGMP-regulated phosphodiesterases.

NO reacts with proteins having transition metals, hemes, thiols, and tyrosine residues through *cGMP-independent* reactions (WENDEHENNE et al. 2001). NO binds reversibly with cytochrome oxidase, a terminal enzyme in the mitochondrial electron chain. This binding has the potential to control cell respiration and cell death (MONCADA, ERUSALIMSKY 2002). NO can modulate the activities of calcium-dependent potassium channels, transcription factors, phosphatases, kinases, signaling proteins, iron homeostasis, and cytosolic aconitase. Enzymes inhibited are lipoxygenase, iron-metabolizing proteins, aconitase, proton ATPase, cytochromes, and ribonucleotide reduc-

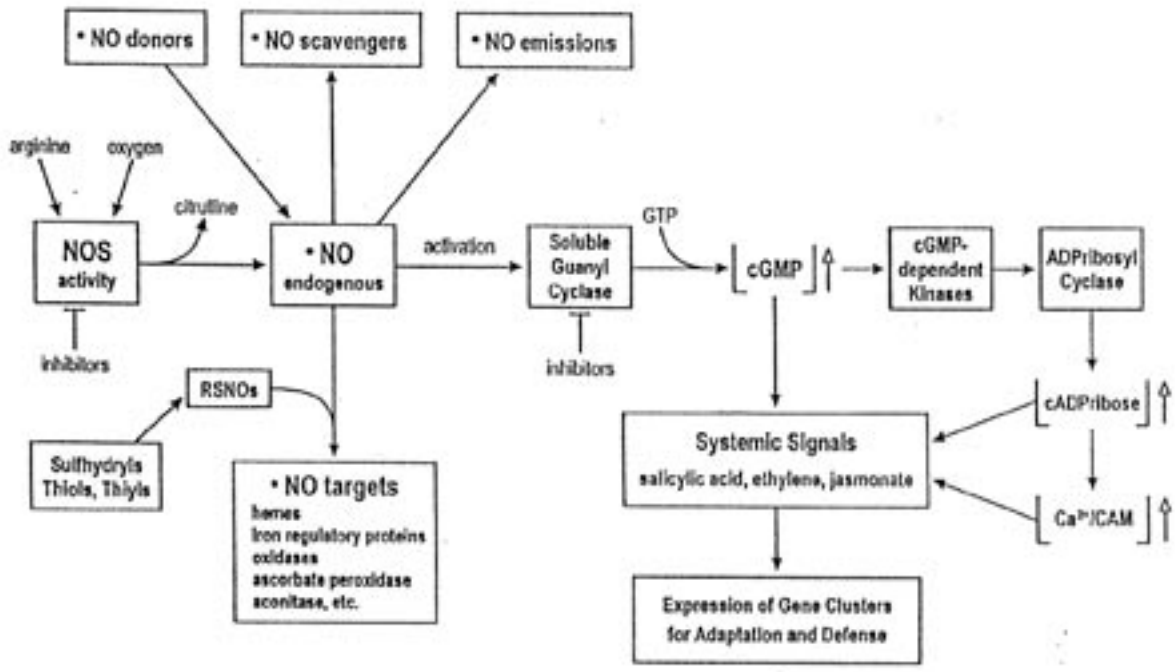


Fig. 2. NO bursts prime and prepare cell membrane-bound receptors to initiate a multitude of specific stimuli in response to a wide range of stresses. The sensitivity, speed, and reliability of the response is facilitated by shuttling NO via RSNOs and by the transnitrosylation of proteins. The reactions elicited by cellular NO are dependent and independent of the activation of soluble guanylyl cyclase (sGC) and/or cyclic guanosine 3‘5‘-monophosphate (cGMP). The activation of sGC drives inter and intra-cellular signal transduction cascades in systemic responses to mechanosensory transduction, pathogens, and environmental stresses. Activation mechanisms comprises kinases, ADP ribosyl cyclase, changes in cellular calcium levels, and other protective responses. The control of upregulated reactions (upward arrows) are important in post-harvest biology. Hypersensitive responses to invading organisms are modulated by ethylene, jasmonate, salicylic acid, and by changes in intracellular free calcium. These reactions activate defense genes and may contribute to apoptosis. When local calcium stores are depleted, calcium cannot deliver sustained calcium signals. This model implies that calcium spark frequency may be altered by inhibition of NR, NiR, NI-NOR, and NOS activity. NO reactions, independent of cGMP, target the hemes, ion-channels, proteins and enzymes and also alert conifers to real or impending injury

tase. The recovery of covalently bound tritium from the deoxyribose sugar in deoxyribonucleotides indicated that ribonucleotide reductase activity could have accounted for the inhibition of jack pine seed germination at high levels of tritiated water (DURZAN 1983). In retrospect, this inhibition may also have been mediated by stress-induced NO.

When added to plants, sildenafil (Viagra®) potentiates the activity of NO by selectively inhibiting a cGMP-specific phosphodiesterase that is responsible for the degradation of cGMP. The duration of action of cGMP is controlled by the action and tissue distribution of this enzyme. Sildenafil inhibited ethylene production, doubled the half-life of cut flowers, and kept them 'straight' for a week beyond their natural life span LESHEM (2000). Leshem considers NO as a newly defined gaseous endogenous hormone, and should be consulted for the role of NO in postharvest physiology. The control of NO production and its effects on the survival of Christmas trees is unknown.

NO was released within three mins from plant cell cultures exposed to cytokinins (TUN et al. 2001). Other plant hormones and an inactive cytokinin analog did not release NO. How NO relates to phytohormone activity for the initiation of new meristems, apart from protection against oxidative and nitrosative reactions, remains unclear. Four structurally diverse polypeptides are now classified as plant hormones in terms of their action as intercellular chemical messengers (PARCE et al. 2001). These polypeptide hormones are defensive proteinase-inhibitors that may help shuttle NO through cell populations.

Tree pruning, and the excision of tissues for propagation elicit NO bursts. If multiplication rates and whole plant correlations are not reestablished, development commonly fails with the 'browning' and death of tissues. Thiols and guanidines as NOS inhibitors may offer countermeasures that target NO-dependent browning reactions, and protect against genomic decay (DURZAN, PEDROSO 2002). NO-mediated stresses induce DNA fragmentation in subcellular organelles, e.g., chloroplasts, amyloplasts, and nuclei (GARCĘS et al. 2001). The NO-dependent activation of cell divisional checkpoints would normally correct for errors in genes expression, DNA and chromosome replication, and in cell division (HAVEL, DURZAN 1999). Where repeated stress-related NO bursts occur, the checkpoints may become error-prone and stop cell cycling. A moderate decline in ATP would favor apoptosis, while rapid depletion of ATP could result in necrosis.

## NO BURSTS IN APOMIXIS AND EMBRYO DEVELOPMENT

Apomictic parthenogenesis was considered absent or rare in gymnosperms (MOGIE 1992; MININA, LARIONOVA 1979). Mogie's theoretical contributions postulated the limiting factors that kept parthenogenesis from being expressed. When these were experimentally removed, a latent expression of diploid parthenogenesis (LDP) was observed in Norway spruce embryonal suspensor masses (ESMs) (DURZAN et al. 1994). LDP has remained an elusive concept, especially for those not familiar with the complexities of asexual evolution, and the details of conifer seed development. ESMs rescued from endangered *Fitzroya* sp. when grown in cell suspension are able to express LDP (ARCE et al. in preparation). More unexpectedly, the diploid pollen of *Cupressus sempervirens* naturally produces an apomictic embryo without fertilization, i.e., paternal apomixis (PICHOT et al. 2001, 2000). Embryos are produced that are genetically unrelated to other seed components (*viz*. maternal sporophyte and gametophyte).

ESMs have the capacity for embryo multiplication by monozygotic cleavage polyembryony without the need to *induce* and redifferentiate a callus (DURZAN 1988; GUPTA, DURZAN 1987; DURZAN et al. 1994; CHALUPA 1985). DOGRA (1978) classified Norway spruce seeds as representative of a non-cleavage species. However, cleavage was clearly expressed when rescued ESMs were grown *in vitro* and reaffirmed at Davis by a visit from Dogra. The recognition and acceptance of monozygotic cleavage polyembryony as a process distinct from somatic embryogenesis was and in some cases not widely recognized. NO bursts occur in cells destined to initiate the cleavage process. These cells can become apoptotic (DURZAN, PEDROSO, HAVEL unpublished data). Apoptotic cells at the cleavage site are also seen in histological cross sections of developing zygotic embryos in conifer seeds.

Mitotically active cells of a *Cupressus* callus were characterized by expression of the proliferating cell nuclear antigen (PCNA) (HAVEL et al. 1997). PCNA is important for DNA replication and repair. NO bursts that disrupt PCNA activity may account for cell death in mega- and microsporogenesis. The recognition of apomictic variations, NO bursts, apoptosis, and adaptive plasticity are not evident in recent models for conifer somatic embryogenesis (e.g., FILONOVA et al. 2000; CIAVATTA et al. 2001). The tendency has been to adopt models from angiosperm embryonic development rather than concepts based on model-referenced zygotic 'process controls' in the species under investigation (DURZAN, DURZAN 1991).

Apoptosis is involved in the differentiation of suspensors along the axial tier of Norway spruce early embryos (HAVEL, DURZAN 1996a). During differentiation, the cell regulatory proteins in the proembryonal group became ubiquitinylated (also known earlier as ubiquitation, ubiquintination). The breakdown products of the ubiquitinylated proteins were released into the culture medium as mucilage, which coated the surface of proembryonal cells, possibly with feedback control implications (DURZAN 1996). The ubiquitylation process has two important aspects: one determines the turnover of cell regulatory proteins during development; another connects auto-ubiquitylation with cell death (apoptosis and necrosis). This coordinates the timing of divisional cycles with the regulation of cell death.

The use of NO donors, traps, and NOS inhibitors can now be used to evaluate NO transfers for regulatory

protein turnover, and apoptosis, e.g., in seeds developing abnormally due to climatic constraints at tree lines (SIMAK 1973). It is not clear if and how tightly NO production actually controls axial tier differentiation. Most surgically cut segments along the axial tier of the early embryos of Norway spruce can regenerate the missing parts, except for the differentiated suspensors (HAVEL, DURZAN unpublished). The wounded cells are able to produce NO bursts in the dark and in the absence of nitrate and nitrite. In light and in the presence of nitrate and nitrite, it is not known if NO can arise via NR and NI-NOR activities, as well as from arginine and oxygen via NOS activity.

## GERMINATION

Nitrites and nitrates stimulate the germination of many seeds, while ammonia salts are usually ineffective. Nitrites release NO at pH 3 or lower by the dismutation of nitrous acid. In several plants, germination is dependent on nitrite, or on hydroxylamine (HENDRICKS, TAYLORSON 1974). Hydroxylamines, nitrites, and other nitrogenous compounds yielded NO under strong oxidation and promoted germination. NO was produced from nitrite, rather than from nitrate reduction *per se*. Peroxidase was thought not to contribute to the oxidation of these compounds to NO. The chelation of hydroxylamine(s) with iron atoms of hemes was suggested as a source of NO.

The inhibition of catalase was required for the breaking of seed dormancy (HENDRICKS, TAYLORSON 1975). Catalase is a ferric heme enzyme that is critical for the degradation of hydrogen peroxide. NO at 1 mM, when fully absorbed, completely inhibited catalase activity as did substituted nitrogen dioxides. This indicated that the NO produced in germination contributed to the NO-dependent inhibition of catalase. Changes in low levels of hydrogen peroxide consist of reversible biochemical signals in cellular defense. Higher concentrations of hydrogen peroxide would lead to the formation of peroxynitrite from NO and superoxide with deleterious consequences, *viz.* apoptosis or necrosis.

The arginine-rich seeds of jack pine are commonly released during forest fires. Seed composition reflects climate at the seed source and preconditions germination (DURZAN, CHALUPA 1968; CHALUPA, DURZAN 1973b). Forest fires release NO that stimulates germination (HAYHURST, MCLEAN 1974). In several plants, endogenous NO counteracts ROS damage, stimulates de-etiolation, and inhibits hypocotyl elongation (BELIGNI, LAMATTINA 2000). The exposure of *Arabidopsis* to ultraviolet-B radiation increased NOS activity (MACKERNESS et al. 2001). All of these observations point to the need for new models for seed dormancy and germination.

## MECHANICAL FORCES, GRAVITY, AND WOUNDING

Centrifugation, clinorotation, and wounding of angiosperm and gymnosperm cells elicit early and rapid endogenous NO bursts (GARCĘS et al. 2001; PEDROSO, DURZAN 2000; PEDROSO et al. 2000a,b). NMMA prevented these NO bursts and stopped the subsequent DNA fragmentation in chloroplasts and nuclei. The addition of a NO donor (sodium nitroprusside) promoted DNA fragmentation. NMMA also significantly reduced NO-dependent apoptosis.

Mechanical stimuli increase the production of 'reaction wood' or 'compression wood' through changes in peroxidase activity, and ethylene and auxin levels (SAVIDGE 1988). The major oxidase in lignifying compression wood is a laccase-type polyphenol oxidase. Three copper sites of laccases are targets for NO (MARTIN et al. 1981). Another pivotal intermediate in mechanotransduction is 12-oxo-phytodienoic acid (OPDA) (STELMACH et al. 1998). OPDA derives from linoleic acid and yields 3-hydroperoxylinoleic acid, a precursor for traumatic acid. The latter was observed in the traumatotropic curvature reactions during plant wounding. Lipoxygenase acts on 3-hydroperoxylinoleic acid to program organelle degradation. It has a nonheme iron that reacts with NO (LESHEM et al. 1997).

We do not yet know exactly how NO affects touch (*TCH*) gene expression when conifers are subjected to mechanical forces. In other plants, the *TCH* family comprises *TCH1* encoding calmodulin (CaM); *TCH2* and *TCH3* encoding CaM-related proteins; and *TCH4* that encodes a xyloglucan endotransglycosylase (JOHNSON et al. 1998; XU et al. 1995). CaM is also a commonly implicated cofactor for NOS. NO bursts from NR, NI-NOR, and NOS activities must now be considered as early and rapid signals in response to gravitational and mechanical forces. A role for ethylene in the *TCH* response was proposed in *Arabidopsis*. However, ethylene-insensitive mutants (*etr1-3, ein2-1*), and the ETR1 and EIN 2 protein functions were not evident in responses to mechanical stimulation (JOHNSON et al. 1998). NO bursts occur prior to ethylene release (LESHEM 2000; MAGALHAES et al. 2000). Together with the TCH proteins, NO and other gaseous signals have the potential to shape new functional phenotypes through redox changes and apoptosis.

Paclitaxel (Taxol®), is an anti-microtubule agent produced by *Taxus* cells for the effective treatment of human cancers. Its complex taxane ring is synthesized in amyloplasts and chloroplasts of gravisensing *Taxus* cells. Side chain modifications are completed in part by cytochrome P-450 in cytoplasm. Inhibitors of NR and NOS blocked both NO bursts and paclitaxel formation (unpublished). Mechanical actions, NO bursts, isoprenoid metabolism, and paclitaxel formation are important parameters in the design of 'process control models' for the metabolic engineering of plant cells in simulated microgravity (DURZAN 2000).

## ARGININE AND NO IN CONIFER 'RED' DECLINE

Coniferous forests are usually established on acidic podsolic forest soils that lack N and are low in nitrate and

nitrite. The soluble N of tissues is often rich in arginine especially when trees enter winter dormancy. The 'red' decline of conifers has now been related to climatic stress, and ammonia overload from arginine (ENGVILD 1998). Increased contents of arginine, ornithine, and urea are characteristic of the syndrome. The fact that arginine is an endogenous source of stress-related NO, or a source of urea (ammonia via urease), or a substrate for naturally occurring guanidino compounds (NOS inhibitors), complicates the role of arginine in the red decline.

The 'red' decline of forests also involves chlorophyll breakdown. During leaf senescence, a red catabolite of chlorophyll (RCC) is accumulated (WÜTHRICH et al. 2000). RCC is formed by an oxygenase and a RCC reductase (RCCR). This reaction requires reduced ferredoxin, and is sensitive to oxygen and NO. The accelerated death gene *(ADC*) encodes the RCCR and suppresses the spread of disease symptoms (MACH et al. 2001). Other hemes are sites for NO binding with potentially damaging effects. Arginine, NO formation, putative NOS activity, RCC formation, and apoptosis are probably important components the 'red' decline syndrome. The red decline differs from "Waldsterben" caused primarily by air pollution, acid rain, nutrition, growth-altering substances, and many other interacting factors (FINK 1999). Plant pathologists have contributed significantly to our understanding of NO burst especially during the initiation of the hypersensitive reaction and to the downstream signaling of NO under these conditions (Fig. 2; WENDEHENNE et al. 2001).

## PHARMACOLOGY OF ARGININE, GUANIDINES, AND NITRIC OXIDE

Conifer seeds were and still are important food sources in middle Eastern populations and are gaining in popularity in North America. Nutritional and therapeutic sources of arginine and guanidino compounds were available to American native Indians who widely collected conifer seeds (MOERMAN 1998). In humans, L-arginine is a semi-essential amino acid in the diet and a precursor of ·NO for a variety of physiological effects in the vascular system (BÖGER, BODE-BÖGER 2001). Side effects from arginine doses are rare, mostly mild, and dose-dependent. Arginine stimulates wound healing and immune functions in elderly people (KIRK et al. 1993). Pine bark extracts that contain complex guanidines and procyanidins are used to treat several human pathologies. Procyandin's antioxidant properties modulate inducible NOS, enhance immune and haemopoietic functions, counteract the constriction of blood vessels through angiotensin-converting enzyme (ACE) inhibition, promote anti-inflammatory activity, and protect against ultraviolet-B radiation (e.g., FITZPATRICK et al. 1998; VIRGILI et al. 1999).

Extracts of gymnosperms, e.g., *Ginkgo biloba* have NO scavenging properties (MARCOCCI et al. 1994). They also produce neurological properties, memory enhancement, and have been shown to sequester amyloid-β-proteins relevant to Alzheimer's disease. Flavanoids and other phenolics scavenge the damaging free radicals generated under acid conditions in the stomach (HALLIWELL 1999). Ascorbate, glutathione, vitamin E, and β-carotene react with peroxynitrite to inhibit oxidation and nitration reactions. Guanidinosuccinate relaxed rat aortas and mimicked the action of L-arginine as a source of NO (NORRIS, REMUZZI 1999). Some guanidino compounds are free radical generators (MORI et al. 1996). In humans, diguanidines have biological and clinical significance in the treatment of diabetes (DE DEYN et al. 1992). NOS has become a therapeutic target for inhibition in human pathology (HOBBS et al. 1999).

Numerous recent reports have shown that a wide range of plant secondary products affect human physiology through the control of NOS activity and apoptosis. The induction of ·NO-dependent apoptosis by dietary factors may provide a mechanism by which diet can protect against certain diseases associated with aging (WATSON et al. 2000). For example, an agent that activates apoptosis in precancerous cells may be good for an individual at risk for tumor development, but may not be so good for overall health if it also activates apoptosis in postmitotic cells and contributes to degenerative diseases. Conversely, a compound that inhibits apoptosis in postmitotic cells may not be useful *in vivo* if it also inhibits the normal physiological mechanisms to eliminate precancerous cells. Care should be taken with the interpretations of studies involving the beneficial and harmful effects of guanidines, NO and RNS especially when apoptosis is involved. The same care is needed when developing hypotheses for the role of arginine, guanidines, and NO for the maintenance of viability and vigor of trees.

## GROWTH AND DORMANCY

NO formation has the potential to initiate a 'metabolic hypoxia' for winter rest and dormancy. When arginine N accumulates, it removes much of the soluble N in tissues targeted for protein and nucleic acid synthesis. NO formation from arginine and oxygen by NOS appears to predispose rest and dormancy characterized by an increase in proline N (DURZAN 1973a). The metabolic diversion of arginine to guanidino compounds that regulate NOS activity protects against the damaging reactions of NO during environmental extremes. When the limits to growth are removed, as in spring, the conversion of proline to glutamic acid would yield transferable hydrogen for NAD(P)H or NADH-dependent reactions. Arginine and the guanidines are now metabolized to urea and polyamines. Urea carbon and N contribute to the formation of amides, amino acids, and factors that support protein and nucleic acid synthesis (DURZAN 1973b,c).

Seasonal changes in spruce shoots (DURZAN 1968; CHALUPA, DURZAN 1973a), and the autocatalytic growth of jack pine cell suspensions (DURZAN, CHALUPA 1976a,b,c) show a common sequence for amide, amino acid, arginine, and guanidino compound accumulation. The formation of NO from arginine and the nitrate

(nitrite) would contribute to production of superoxide, peroxynitrite, and guanidines as the relative growth rate falls. *Cryptomeria* sp. accumulate citrulline, which is a product of NOS activity. The metabolism of citrulline has not been fully studied in conifers.

## CONCLUSIONS

NO bursts are now recognized as priming, integrative, protective, and in some cases damaging signals in conifers. Our understanding of NO comes at a time when proteomics, gene knock-outs, mutations, and transgenics may be employed to explain how NO metabolism, signaling, and transduction contribute to resting metabolic rates, irritability, and to oxidative and nitrosative stresses during prolonged genomic × environmental interactions, and throughout development. The reactions of NO with hemes and thiols may suggest new selectable markers and the countermeasures open new directions that may impact how tree breeding and improvement practices are managed.

Future progress is dependent on finding the responsible genes, the endogenous sources of NO, and their targeted biological functions. Experiments will have to determine how many of these truly new concepts are proven and evolve into new models.

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## Stresem indukovaný oxid dusnatý a adaptivní plasticita u konifer

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**ABSTRAKT**: V práci je pojednáno o vlivu oxidu dusnatého (NO) na metabolismus rostlin se zaměřením na jehličnaté dřeviny. Nejprve jsou krátce popsány významné chemické vlastnosti NO a vznik NO v rostlinách. NO je produkován v somatických a generativních buňkách jako odpověď na mechanické síly, gravitaci, poranění, změny ve výživě, nedostatek kyslíku, sucho, zasolení, rychlé teplotní změny, znečištění ovzduší a napadení rostlin patogeny. NO vzniká primárně z dusitanů a také z dusíku argininu. NO rychle reaguje s různými druhy kyslíku, hemy, thioly a bílkovinami, čímž jsou vytvářeny biochemické signály, které přímo nebo nepřímo regulují aktivitu enzymů. Jsou popsány reakce NO, kterými tato látka ovlivňuje metabolismus rostlin, a je uveden popis NO jako molekulového signálu či nového typu fytohormonu. Dále je uveden vliv vzplanutí NO na apomixis a vývoj embrya a jeho vztahu k programované buněčné smrti. Vliv NO závisí na místě, kde působí, a na koncentraci, v jaké působí. Kladné reakce působí proti kyslíkatému a dusíkatému stresu, naopak vysoké koncentrace NO vyvolávají poškození tím,

že způsobují kyslíkatý a dusíkatý stres a buněčnou smrt. NO přispívá ke strukturální a funkční plasticitě a schopnosti stromů přizpůsobit se dané lokalitě. Využití donorů a vychytávačů NO a inhibitorů enzymů může být novým experimentálním přístupem při regulaci stresových signálů v životních procesech jehličnanů.

**Klíčová slova**: oxid dusnatý; adaptivní plasticita; konifery; programovaná buněčná smrt; embryogeneze; hynutí stromů; asimilace dusíku; kyslíkatý a dusíkatý stres

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