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REVIEW

Physiology and biochemistry of waterlogging tolerance in plants

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Abstract

Waterlogging is a serious problem, which affects crop growth and yield in low lying rainfed areas. The main cause of damage under waterlogging is oxygen deprivation, which affect nutrient and water uptake, so the plants show wilting even when surrounded by excess of water. Lack of oxygen shift the energy metabolism from aerobic mode to anaerobic mode. Plants adapted to waterlogged conditions, have mechanisms to cope with this stress such as aerenchyma formation, increased availability of soluble sugars, greater activity of glycolytic pathway and fermentation enzymes and involvement of antioxidant defence mechanism to cope with the post hypoxia/anoxia oxidative stress. Gaseous plant hormone ethylene plays an important role in modifying plant response to oxygen deficiency. It has been reported to induce genes of enzymes associated with aerenchyma formation, glycolysis and fermentation pathway. Besides, non-symbiotic-haemoglobins and nitric oxide have also been suggested as an alternative to fermentation for maintaining lower redox potential (low NADH/NAD ratio), and thereby playing an important role in anaerobic stress tolerance and signaling.

Additional key words: anoxia, antioxidative enzymes, ethylene, fermentation, flooding, glycolysis, hypoxia, nitric oxide, non-symbiotic haemoglobin, oxidative stress, sugars.

Introduction

Although all higher plants require access to free water, excess water in the root environment of land plants can be injurious or even lethal because it blocks the transfer of oxygen and other gases between the soil and the atmosphere. Crop plants require a free exchange of atmospheric gases for photosynthesis and respiration. Like animals, plants can be easily suffocated if this gas exchange is impeded. Oxygen status of cells and tissues varies significantly during ontogenesis, and depends on environmental oxygen supply. The most common impediment to gas diffusion is water that saturates the root environment in poorly drained soils or that accumulates above soil capacity as a result of the overflow of rivers, excessive rainfall or excessive irrigation. Flooding and submergence are major abiotic stresses and rank alongside water shortage, salinity and extreme temperatures as major determinants of species distribution worldwide. During waterlogging or submergence plants are exposed to a reduction in oxygen supply because of the slow diffusion rate of oxygen in water and its limited solubility (Armstrong 1978). Turbid flood-water can become anaerobic, especially during the night (Setter *et al.* 1987). Growth is greatly inhibited in the deficiency (hypoxia) or complete absence (anoxia) of oxygen. In water-saturated soils roots grow only in a small region near the surface and do not exploit a large soil volume as they would under aerated conditions. Plants invariably wilt within few hours to 2 - 4 d of imposing a flooding stress (Jackson and Drew 1984). This is a consequence of higher resistance to mass flow of water through the root. Wilting is caused by the inhibition of respiration and loss of ATP synthesis in the roots. This blocks the ion transport systems that normally create the gradient in water potential across the root endodermis. Plants react to an absence of oxygen by switching from an oxidative to a solely substrate-level

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Abbreviations: AA - ascorbate; ACC - 1-aminocyclopropane-1-carboxylic acid; ADH - alcohol dehydrogenase; AEC - adenylate energy charge; ANP - anaerobic protein; APX - ascorbate peroxidase; CAT - catalase; DHAR - dihydroascorbate peroxidase; GR - glutathione reductase; GSH - glutathione; Hb - haemoglobin; cNR - cytosolic nitrate reductase; LDH - lactate dehydrogenase; MDHAR - monodihzdroascorbate peroxidase; PM-NR - plasma membrane nitrate reductase; PM-Ni-NOR - plasma membrane nitrite nitric oxide reductase; NAD(P)H - nicotine amide adenine dinucleotide phosphate-reduced; NiR - nitrite reductase; NO - nitric oxide; PDC - pyruvate decarboxylase; SOD - superoxide dismutase; SuSy - sucrose synthase; XET - xyloglucan endo-trans-glucosylase

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phosphorylation of ADP to ATP, the latter reactions predominantly involve glycolysis and fermentation. An important adaptive response is formation of aerenchyma, specialized tissues in roots, which allow diffusion of gases like O_2 from aerobic shoot to hypoxic/anoxic roots. It has been suggested that physiological and molecular studies of the mechanisms of anoxia tolerance in wild plants that still possess long-term anoxia-tolerance are more likely to provide evidence of physiological mechanisms than studies of crop plants (Crawford and Braendle 1996). Therefore, reference is made to some of these studies, but the emphasis of the following is on crop plants.

Hypoxia and anoxia

Normally plant roots are in contact with oxygen at a partial pressure equivalent to the gaseous atmosphere. The reduction of oxygen below optimal levels, termed *hypoxia*, is the most common form of stress in wet soils and occurs during short-term flooding when the roots are submerged under water but the shoot remains in the atmosphere. Hypoxia will also occur in roots near the surface of longer-term flood water. The complete lack of oxygen, termed *anoxia*, occurs in soils that experience long-term flooding, in plants completely submerged by water, in deep roots below flood waters. Long-term flooding shifts the microbial flora in the soil in favour of anaerobic micro-organisms that use alternative electron acceptors to oxygen. As a consequence the soil tends to accumulate more reduced and phytotoxic forms of mineral ions such as nitrite (from nitrate) and ferrous (from ferric) ions and few plants are adapted to growth in these soils (Ponnamperuma 1972).

Flooding and ethylene production

Many of the adaptive growth response in hypoxic roots and shoots occur in response to ethylene. Ethylene accumulates in flooded soils and in submerged plant parts to concentrations of 10 cm^3 dm^{-3} (Musgrave *et al.* 1972). This is accomplished by two mechanisms. First the diffusion of ethylene from the root into the water is 10 times slower than its diffusion into air (Stünzi and Kende 1989). This ethylene may be released into the internal aerenchyma channels and diffuse from the root to the shoot. Secondly, the synthesis of ethylene in the hypoxic root and in the aerobic shoot is increased (Jackson 1985). The immediate precursor of ethylene is 1-amino cyclopropane 1-carboxylic acid (ACC), which is synthesized to a large extent in roots (Bradford and Yang 1980). Because the conversion of ACC to ethylene has an obligate requirement for oxygen, this reaction is blocked in an anaerobic root cell. The ACC is therefore, translocated from the anaerobic root cells towards the more aerobic portions of the root or to the shoot. The previous biochemical steps in the synthesis of ACC do not require oxygen, and in fact, ACC synthase activity is stimulated in roots under flooding conditions (Cohen and Kende 1987). As a consequence the quantity of ACC transported to the shoot increases. The lower portions of the stems are usually the site of highest ACC accumulation and in the presence of oxygen ethylene is released.

Van Der Straeten *et al.* (2001) isolated a genomic cDNA clone (*OS-ACS5*) encoding ACC synthase from rice, which catalyzes a regulatory step in ethylene biosynthesis upon short- and long-term submergence. Rieu *et al.* (2005) reported cloning of a *Rumex palustris* cDNA corresponding to an ACC synthase gene (*RP-ACS1*), whose expression is induced by long term submergence. Vriezen *et al.* (1999) suggested a more prominent role of ACC oxidase in ethylene synthesis and anoxia tolerance during flooding. They isolated a cDNAs from *R. palustris* corresponding to a ACC oxidase gene (*RP-ACO1*) in submerged *Rumex palustris*. An increase in *RP-ACO1* mRNA was observed 2 h after the start of submergence, and ACC oxidase enzyme activity was highest after 24 h. It was earlier reported by these workers that the ethylene production rate of submerged shoots does not increase significantly during the first 24 h of submergence (Voesenek *et al.* 1993), suggesting that under these conditions ACC oxidase activity is inhibited *in vivo*. They suggested that this inhibition is caused by a reduction of oxygen levels, and hypothesized that an increased ACC oxidase enzyme concentration counterbalances the reduced enzyme activity caused by low oxygen concentration during submergence, thus sustaining ethylene production under these conditions. Therefore, ethylene biosynthesis seems to be limited at the level of ACC oxidase activity rather than by ACC synthase in *R. palustris* during submergence (Voesenek *et al*. 1993).

Ethylene and aerenchyma formation

Ethylene initiates and regulates many adaptive molecular, chemical and morphological responses that allow the plant to avoid anaerobiosis by increasing oxygen availability to the roots in a flooded or waterlogged soil, such as development of aerenchyma. Aerenchyma are soft tissues with large intercellular spaces to provide low

resistance internal pathway for the exchange of gases between aerobic shoot to the anaerobic root (Jackson and Armstrong 1999).

Aerenchyma formation under waterlogged condition have been reported in a wide range of crop species such as, *Trifolium subterraneum* (Aschi-Smiti *et al.* 2004), soybean (Bacanamwo and Purcell 1999), wheat (Watkin *et al.* 1998), barley (Arikado and Adichi 1955), rice (Justin and Armstrong 1991), maize (Gunawardena *et al.* 2001), *Carex spp*. (Visser *et al.* 2000) and *Vigna luteola* (Sairam *et al.* - unpublished).

Some of the oxygen transported through the aerenchyma to plant root tips leaks out of pores in the root and into the surrounding soil. This can result in a small zone of oxygenated soil around individual roots providing an aerobic environment for microorganisms that can prevent the influx of potentially toxic soil components (Armstrong and Armstrong 1988) such as nitrites and sulphides of Fe, Cu and Mn.

Ethylene synthesis was strongly enhanced in roots under hypoxia, coincident with the development of aerenchyma (Visser *et al.* 1997). Aerenchyma formation in maize roots was also stimulated by exogenous application of ethylene at rates as low as 0.1 cm^3 dm^{-3} and was inhibited in the presence of Ag^+ ions, an inhibitor of ethylene action (Drew *et al.* 1981). In the presence of ethylene synthesis inhibitors, flooding failed to induce aerenchyma formation (Konings 1982). The presence of a growing root tip is also essential for the formation of aerenchyma. The root tip may serve as the site of ACC synthesis, with subsequent ACC transport to more mature, better aerated tissue leading to ethylene synthesis and aerenchyma formation (Drew *et al.* 1981).

However, there is little information to date on the molecular regulation of aerenchyma formation or the identity of other enzymes involved. In maize, a flooding-induced gene (*xet1*) encodes a xyloglucan *endo*transglycosylase (XET1), a putative cell wall loosening enzyme (Peschke and Sachs 1994, Saab and Sachs 1996). O_2 deprivation induces expression of XET1 in the primary root, mesocotyl, and coleoptile of maize seedlings. The induction of *xet1* appears to be specific to O_2 deprivation, since other stresses do not induce the gene (Peschke and Sachs 1994). The *xet1* gene appears to be a member of a large multigene family in which only *xet1 is* inducible by oxygen deprivation. The induction of *xet1* by hypoxia was associated with aerenchyma development. Hypoxic induction of XET1 mRNA is repressed by ethylene antagonists [(aminooxy)acetic acid, 2-amino-ethoxyvinyl-glycine, $AgNO_3$]. XET1 is also induced under aerobic conditions by exogenous ethylene, as is aerenchyma.

Ethylene accumulation and adventitious roots formation

Adventitious roots emerge from the submerged part of the stem in flooded plants and grow horizontally (diageotropism). Presumably, this is also an adaptive mechanism allowing these new roots to replace the function of the original root system (Jackson and Drew 1984). Since these roots emerge and grow close to the water surface, and since they are connected to the stem close to the site of aerenchyma formation, oxygen is more available to these roots than the original root system. The flood tolerant *Rumex* species *R. crispus* and *R. palustris* developed new flood-resistant roots in the upper 10 cm of the waterlogged soil, whereas the flood susceptible *R. acetosa* did not change its vertical root distribution (Voesenek *et al.* 1989).

Visser *et al.* (1996) reported that accumulation of ethylene has a role in the formation of flooding-induced adventitious roots formation. The large air-spaces in these roots enable diffusion of gases between shoots and roots. Application of ethylene to non-flooded *Rumex* plants resulted in the formation of adventitious roots. Inhibition of ethylene production in *R. palustris* by L-α-(2-amino-ethoxyvinyl)-glycine (AVG) or α-iminobutyric acid (AIB) decreased the number of adventitious roots induced by flooding, indicating that high ethylene concentrations may be a prerequisite for the flooding-induced formation of adventitious roots in *Rumex* species. Bragina *et al.* (2003) reported in maize seedlings that hypoxia resulted in the accelerated ethylene production and the activation of enzymes destroying cell walls in the adventitious roots and changed their growth pattern. The conclusion is that the interrelated responses are adaptive ones and the adventitious roots play a key role in plant adaptation

Mergemann and Sauter (2000) reported that in deep-water rice, adventitious root primordia initiate at the nodes as part of normal development but emergence of the root is dependent on flooding induced ethylene mediated death of nodal epidermal cell covering the tip of the primordia. This facilitates adventitious root emergence and prevents injury to the growing root. Cell death was inducible not only by submergence but also by application of ACC precursor of ethylene, and it was suppressed in the presence of 2,5-norbornadiene, an inhibitor of ethylene action. Adventitious root growth and epidermal cell death are therefore, linked to the ethylene signaling pathway, which is activated in response to low oxygen stress.

Shift in energy metabolism and anaerobiosis induced proteins (ANP)

The anaerobic response of plant cells was first studied in maize roots. Using two-dimensional electrophoresis, it was shown that a set of about 20 anaerobic proteins were synthesized during low oxygen treatment, while synthesis

of the normal aerobic proteins was drastically repressed (Sachs *et al.* 1980). Many of these induced proteins were subsequently identified as enzymes of the glycolytic and fermentation pathways (Dolferus *et al.* 2003). The identified ANP include sucrose synthase, phosphohexose isomerase, fructose-1,6-diphosphate aldolase, pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH), and alcohol dehydrogenase (ADH) (Chung and Ferl 1999, Zeng *et al.* 1999).

The second major effect of oxygen depletion in flooded roots occurs because oxygen serves as the terminal electron acceptor of mitochondrial electron transport. Lack of oxygen thus effectively blocks ATP synthesis in the mitochondria (Pradet and Bomsel 1978). In the absence of an electron acceptor, NADH oxidation is blocked. Once the mitochondrial respiration stops, the adenylate energy charge of the cell (ratio of ATP to ADP and AMP) declines. In the absence of an adaptive response, the flooded root cell rapidly depletes its available supply of ATP. One supplemental source of ATP for the cell is accessed through a stimulation of glycolysis and fermentation, known as the Pasteur effect.

However, glycolysis is relatively inefficient at energy production compared to mitochondrial respiration. It also generates large quantities of pyruvate as an end-product that must be converted to alternative products to recycle NADH to NAD. These end-products of glycolysis and fermentation pathway, such as ethanol, lactic acid and carbon dioxide pose an additional hazard to the cell. Since many of the enzymes of the Krebs cycle are regulated allosterically by the NADH/NAD ratio, the entire Krebs cycle is blocked and glycolysis is stimulated by an increase in redox potential [NAD(P)H/NAD(P)]. The pyruvate that accumulates from glycolysis is converted initially by LDH to lactic acid. Cytoplasmic pH consequently declines as a result of lactic acid accumulation, a process known as cytoplasmic acidosis. Low pH inactivates LDH, which has a pH optimum above 7.0 and activates PDC and ADH that are normally inactive above pH 7. Therefore, pyruvate is shunted to the production of either ethanol or lactic acid in a pH dependent manner that allows tight regulation of cytoplasmic pH around 6.8.

ADH activity is critical for the recycling of NADH and thus for the continuation of glycolytic pathway (Johnson *et al.* 1994). Peng *et al.* (2001) demonstrated that *ADH* gene induction is linked to ethylene production. They demonstrated that hypoxic induction of *ADH* could be partially inhibited by aminooxyacetic acid, an inhibitor of ethylene biosynthesis. This partial inhibition can be reversed by the addition of ACC. In addition, the hypoxic induction of the *ADH* is also reduced in *etr*1-1 and *ein*2-1, two ethylene insensitive mutants (Peng *et al.* 2001).

The importance of ADH in flooding tolerance has been emphasized in the study of a maize mutant deficient in one of its *ADH* genes, and therefore, unable to produce a functional ADH enzyme. When this mutant was flooded, LDH synthesized lactic acid, pH declined, but ADH was not able to synthesize ethanol. As there was no counterbalance to LDH and the pH continued to decline to very low levels, consequently this mutant was more sensitive to flooding injury than the wild type plant and died after 3 d of submergence (Roberts *et al.* 1984a,b).

Other enzymes of glycolytic pathway are also over-expressed under waterlogged condition and reported to confer tolerance to hypoxic/anoxic condition. Hänsch *et al.* (2003) reported maize glyceraldehyde-3-phosphate dehydrogenase 4 (*GapC4*) gene expression in tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*). They showed that the gene is also anaerobically induced in *Arabidopsis thaliana* roots, leaves, stems and flower organs.

Due to shift in energy metabolism from aerobic to anaerobic mode the energy requirements of tissue is greatly restricted because of very few ATPs generated per molecule of glucose. This necessitates availability of comparatively higher amount of readily metabolizable sugar pool. Recard *et al.* (1998) provided evidence for the critical role of sucrose synthase, an enzyme involved in sucrose hydrolysis, in anoxic tolerance of maize roots using sucrose synthase double mutants, *sh1sus1*. Zeng *et al.* (1999) reported in case of hypoxic maize seedlings that of the two enzymes involved in sucrose hydrolysis, the activity of invertase is down-regulated, while that of sucrose synthase is up-regulated. Aschi-Smiti *et al.* (2004) reported that in case of 30-d-old plants of *T. subterraneum* 15 d of hypoxia showed induction of sucrose synthase, fructose kinase, lactate dehydrogenase and alcohol dehydrogenase, enhanced ethanol production, and improved energy charge in association with haemoglobin induction. Our studies in pigeon pea have revealed that roots of comparatively tolerant (ICPL 84023 and ICP 301) genotypes have greater total, reducing and non-reducing sugars content than susceptible genotypes (ICP 7035 and Pusa 207). Waterlogging resulted in decline in total and non-reducing sugars in all the genotypes, while the content of reducing sugar increased only in ICPL 84023 and ICP 301. The variation in reducing sugar content was parallel to sucrose synthase activity. The tolerant genotypes also showed lower decline in total and non-reducing sugars and greater increase in reducing sugar and sucrose synthase (SuSy) activity than susceptible genotypes. Susceptible genotype (ICP 7035) showed very little *SuSy* mRNA expression in control and waterlogged condition, while the gene expression increased significantly in tolerant genotype under waterlogged condition (Sairam *et al.* - unpublished data). The results further confirm the significance of root sugar reserve and sucrose hydrolyzing enzyme in waterlogging tolerance of crop plants.

Chang *et al.* (2000) reported expression of 48 anoxia induced proteins in maize root tips by using two-dimensional gel electrophoresis. Proteomic studies have identified 46 oxygen stress induced proteins, which are associated with processes other than sugar breakdown, glycolysis and fermentation, suggesting that many other biochemical and metabolic processes are modulated during low oxygen stress. For instance, xyloglucan

endotransglycosylase, a cell wall loosening enzyme (Sachs *et al.* 1996) is induced, as is the ethylene biosynthetic enzyme ACC synthase (Olson *et al.* 1995), antioxidant enzyme superoxide dismutase, SOD (Monk *et al.* 1987), and non-symbiotic haemoglobin (Hill 1998).

Hypoxia and non-symbiotic haemoglobins

It is now known that there are several classes of haemoglobins in plants. Non-symbiotic haemoglobins, as the name implies, are not involved in symbiotic nitrogen fixation (Hill 1998). There are two classes of nonsymbiotic haemoglobins, one has oxygen-binding properties similar to symbiotic haemoglobins (class 2), the second with dramatically different oxygen-binding properties (class 1). Class 1 haemoglobins are induced by hypoxic stress and oversupply of some nutrients, and are generally referred as stress-induced haemoglobin.

Expression of stress-induced haemoglobins has also been reported in callus, cell suspension, seed, root and stem tissue of both dicot and monocot plants (Hill 1998, Dordas *et al.* 2003a, 2004). They are generally found at low concentrations [1 - 20 nmol g^{-1}(f.m.)] in plant organs. In addition to their induction by hypoxic stress (Taylor *et al.* 1994), these are also induced by elevated sucrose content in *Arabidopsis* (Trevaskis *et al.* 1997), nitrate ions in barley aleurone layer and *Arabidopsis* roots (Wang *et al.* 2000) and have also been reported in rapidly growing root tips of germinating seeds (Hill 1998). There is reason to believe that their appearance in rapidly growing tissues may be due to hypoxic stress as well (Guy *et al.* 2002). Respiratory chain inhibitors (*e.g.* cyanide, dinitrophenol and oligomycin) that inhibit ATP production also induce haemoglobin expression, suggesting that expression is not directly influenced by the O_2 content, but is influenced by content of ATP or some consequence of ATP action (Nie and Hill 1997).

Constitutive expression of barley haemoglobin in wild-type and transformed maize cells lines maintained cell adenine nucleotide levels and energy charge under hypoxic conditions, whereas wild-type cells and cells in which haemoglobin expression is suppressed had lowered adenine nucleotide levels and energy charge (Sowa *et al.* 1998). In similarly transformed alfalfa root cultures, lines constitutively expressing barley haemoglobin maintained root growth during hypoxic treatment, whereas wild-type and lines with suppressed stress-induced haemoglobin expression had slower root growth (Dordas *et al.* 2003a, 2004). The low dissociation constant of the oxyhaemoglobin complex (Duff *et al.* 1997) may preclude functions for stress-induced haemoglobins as oxygen stores, carriers or signal molecules, however, the molecule is a highly efficient scavenger of oxygen at low oxygen tensions. There is, thus, the possibility that it may act in a metabolic reaction involving oxygen, where it could potentially interact with other enzyme proteins or molecules in an oxygen-consuming reaction in low oxygen environments.

Haemoglobin and nitric oxide interaction

While hypoxic stress-induced haemoglobins (Hb) are widespread in the plant kingdom, their function has not been elucidated. Dordas *et al.* (2003b) proposed that nitric oxide is an important metabolite in hypoxic plant cells and that at least one of the functions of hypoxic stress-induced Hb is to modulate nitric oxide levels in the cell. Dordas *et al.* (2003b) demonstrated the presence of NO/haem complexes in both hypoxic maize cell cultures and alfalfa root cultures using electron paramagnetic resonance (EPR) spectroscopy. The characteristic signal for the complex is not evident in aerobic systems, even though Hb is present. Furthermore, using NO traps, Dordas *et al.* (2003b) showed that significant amounts of NO are formed in hypoxic maize cells during the first 24 h of hypoxic treatment. Transformed lines with reduced stress-induced Hb expression produced greater amounts of NO than wild-type or over expressing-Hb lines, suggesting that Hb may be involved in turnover of the NO. There is also the possibility that NO may be activating guanylate cyclase, as it is reputed to do in defence gene induction (Durner *et al.* 1998). The induction of stress-induced Hb in *Arabidopsis* in the presence of elevated nitrate may also relate to a requirement to modulate NO levels (Wang *et al.* 2000). Stress-induced Hb have also been implicated in regeneration of NAD^+ during hypoxia (Hill 1998) based on the observations that alcohol dehydrogenase activity and CO_2 production is reduced under hypoxia in maize cells constitutively expressing barley Hb (Sowa *et al.* 1998).

Nitric oxide synthesis in roots may involve nitrate reductase (NR) and nitrite oxide synthase or nitrite-nitric oxide reductase (NiR-NOR). In roots, two distinct types of nitrate reductase are present, one located in the cytosol (cNR) and the other attached to the plasma membrane and facing the apoplast (PM-NR) (Stöhr and Ullrich 1997, Stöhr and Mäck 2001). There is a 2.5-fold activation of cNR during exposure of plant roots to hypoxia (Botrel and Kaiser 1997), with nitrite reduction being suppressed at the nitrite reductase step (Botrel *et al.* 1996). The limitation of nitrite reduction is connected both with cellular acidification and with increased flux through nitrate reductase (Botrel and Kaiser 1997, Botrel *et al.* 1996). The potential maximum activity of activated nitrate reductase, although lower than alcohol dehydrogenase, exceeds the rate of hypoxic ethanol

formation by more than 3-fold (Botrel and Kaiser 1997). In *Arabidopsis* root cultures, two nitrate reductase genes were induced under low-oxygen (5 %). The *NR1* gene showed moderate induction after 0.5 - 4 h of hypoxia and strong induction after 20 h. The *NR2* gene was strongly activated in 2 - 4 h and even more after 20 h (Klok *et al.* 2002).

Yamasaki *et al.* (1999) using purified cNR from maize showed that a side-reaction of cNR is the reduction of nitrite to NO with NADH as an electron donor, probably catalyzed by the same molybdenum cofactor-containing domain as in nitrate reduction. NO formation by cNR requires high nitrite concentration, as the K_m of cNR for nitrite is about 300 mM (Yamasaki and Sakihama 2000). Yet the accumulation of nitrite to high concentration within the cell seems unlikely under natural conditions, since nitrite as well as its acid form, nitrous acid, are highly toxic (Sinclair 1987), and nitrite is rapidly reduced by nitrite reductase (NiR). However, under anaerobic conditions nitrite does accumulate *in vivo* (Botrel *et al.* 1996). This may suggest that NO can be formed by cNR and accumulates only when the cells are in transition to the unfavourable anaerobic conditions.

PM-NR activity was initially demonstrated by Ward *et al.* (1989) and Meyerhoff *et al.* (1994). It is present only in root tissue where it exceeds the activity of cNR, particularly during the night (Stöhr and Mäck 2001). It can use both succinate and NADH, but succinate is the preferred electron donor. Taking into account succinate accumulation during hypoxia (Fan *et al*. 2003) and the possibility of fumarate reduction back to succinate by succinate dehydrogenase in co-operation with complex I under the accumulation of reduced ubiquinone (Cecchini 2003), there is the possibility that the plasma membrane may have an important role in nitrate reduction during hypoxic conditions. Plasma membrane-bound nitrite-NO reductase (Ni-NOR) is the likely enzyme that converts nitrite to NO rather than PM-NR. Ni-NOR faces the apoplast and has an activity sufficient to convert all of the nitrite formed by PM-NR to NO (Stöhr and Ulrich 2002). Ni-NOR uses reduced cytochrome *c* for nitrite conversion to NO (Stöhr *et al.* 2001). Since participation of cytochrome *c* at the plasma membrane is unlikely, it is possible that the physiological electron donor for this reaction could be either another cytochrome or Hb, induced under hypoxic condition. A haem protein oxidized during this reaction can be reduced by a protein possessing cytochrome reductase activity. The pH optimum of Ni-NOR is favourable for hypoxic conditions (pH 6.1), and it can utilize even low amounts of nitrite (V_{max} is reached at a nitrite concentration of 100 μM) (Stöhr *et al.* 2001).

Regeneration of nitrate is essential under nitrate limiting conditions of anaerobic roots for the continuation of Hb-NO cycle. It has been suggested that oxyhaemoglobin would donate negatively charged dioxygen to NO, forming nitrate and methaemoglobin, a known reaction of oxyhaemoglobin (Di Iorio 1981). The reduction of methaemoglobin to Hb can occur in a number of ways. A methaemoglobin reductase has been demonstrated in nodules of leguminous plants (Topunov *et al.* 1980). A number of diaphorase-type enzymes, such as cytochrome b_5 reductase of the endoplasmic reticulum (Hagler *et al.* 1979) or dihydrolipoamide dehydrogenase (Moran *et al.* 2002, Igamberdiev and Hill 2004) have methaemoglobin reductase activity. Another possibility is the presence of this reaction in the Hb molecule itself.

Hb may be pivotal in the short-term survival of plant root cells by regulating the levels of NO. Plant roots that express sufficient Hb soon after exposure to hypoxic stress may modulate levels of NO, produced as a result of the stress, either through reaction of the NO with oxyhaemoglobin or through formation of nitrosyl-haemoglobin. This would prevent the onset of cell death, maintaining ATP levels and energy charge, as has been observed in hypoxic maize cells overexpressing Hb (Sowa *et al.* 1998). In primary roots, this may provide sufficient time for the plant to develop adventitious roots, needed for prolonged survival under hypoxia.

NO is also an attractive candidate for involvement in aerenchyma formation. It has been suggested that NO may interact with reactive oxygen species to produce peroxynitrite ($ONOO^-$), which may directly kill plant pathogens (Durner and Klessig 1999). There is an abundance of literature on NO and programmed cell death in many mammalian tissues. Depending on NO concentration and other factors, NO may either accelerate or inhibit apoptosis (Kim *et al.* 2001). The effect may be either direct, through cell necrosis, or through regulatory pathways and it may also be selective in relation to the cells that do respond. A similar type of reaction could be responsible for selected cell death during aerenchyma formation in roots exposed to waterlogging (Drew 1997). Similarly, NO has been implicated in programmed cell death of *Arabidopsis* cell suspension cultures through its action on signal transduction pathways involving guanylate cyclase (Clarke *et al*. 2000).

Waterlogging, ROS production and antioxidant activity

Excessive generation of reactive oxygen species (ROS) or oxidative stress is an integral part of many stress situations, including hypoxia. Hypoxic tissues exhibit enhanced mitochondria-dependent ROS generation, acetaldehyde dependent superoxide formation *via* xanthine oxidase, lipoxygenase action on membrane lipids and finally lipolytic acyl hydrolase-catalyzed liberation of FFA, which underpins a burst in lipid peroxidation on return to normoxia. The main cellular components susceptible to damage by free radicals are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), sugars and nucleic

acids. Consequences of hypoxia-induced oxidative stress depend on tissue and/or species (*i.e.* their tolerance to anoxia), on membrane properties, on endogenous antioxidant content and on the ability to induce the response in the antioxidant system.

Anaerobic tissue has a very high redox potential and the soil environment surrounding the roots contains highly reduced forms of metal ions such as Fe^{2+}, which can readily reduce atmospheric oxygen to superoxide. Therefore, in the interim between return to high oxygen partial pressures and reactivation of the mitochondrial electron transport system, conditions are ideal for activation of oxygen.

It was observed in a study of soybean roots that a short anoxic stress (1 - 2 h) increased the potential for superoxide production (Van Toai and Bolles 1991). With longer durations of anoxia (3 - 5 h) the roots developed an increased ability to cope with oxygen free radicals, and therefore exhibited less post-anoxic injury. Incubation in the presence of exogenous ascorbic acid alleviated post-anoxic injury in these roots. Hydrogen peroxide accumulation under hypoxic conditions has been shown in the roots and leaves of *Hordeum vulgare* (Kalashnikov *et al.* 1994) and in wheat roots (Biemelt *et al.* 2000). The presence of H_2O_2 in the apoplast and in association with the plasma membrane has been visualized by transmission electron microscopy under hypoxic conditions in four plant species (Blokhina *et al.* 2001). Indirect evidence of ROS formation (*i.e.* lipid peroxidation products) under low oxygen has also been reported (Blokhina *et al.* 1999). Using diphenylene iodonium chloride, a specific inhibitor of membrane bound NADPH oxidase, and gene expression studies we have shown increase in NADPH oxidase activity and increased *NADPH oxidase*-mRNA expression under hypoxic condition in pigeon pea genotypes (Sairam *et al*.- unpublished data). These studies suggest that there is ROS production even during the hypoxia, which probably is due to the induction of membrane bound NADPH oxidase.

There are only a few reports of investigations of changes in activities of components of antioxidative system in response to anoxic or hypoxic conditions. Monk *et al.* (1987) reported increase in superoxide dismutase (SOD) activity when the rhizomes of *Iris pseudacorus* were flooded. Van Toai and Bolles (1991) suggested that it probably has a critical role in the survival of the plant when oxygen levels increase as the flooding stress abates. Induction of enzymes involved in the ascorbate-dependent antioxidative system (ascorbate peroxidase – APX, monodehydroascorbate reductase – MDHAR, and dehydroascorbate reductase – DHAR) has been shown for anaerobically germinated rice seedlings after transfer to air (Ushimaru *et al.* 1997). Roots of wheat (*Triticum aestivum*) seedlings could cope with the deleterious effects of oxygen radical generation due to post-hypoxia by increasing glutathione reductase (GR) activity and the content of glutathione (Albrecht and Wiedenroth 1994).

Investigations involving 11 species with contrasting tolerance to anoxia have revealed an increase in MDHAR and/or DHAR in the anoxia-tolerant plants after several days of anoxic treatment. In the intolerant plants activities were very low or without any changes. Glutathione (GSH) content decreased significantly during the post-anoxic period, while ascorbate (AA) showed increased values in the tolerant species (Wollenweber-Ratzer and Crawford 1994). Biemelt *et al.* (1998) reported a slight decrease in the activities of MDHAR, DHAR and GR or no change in the roots of wheat seedlings under hypoxia, while anoxia caused a significant inhibition of enzyme activities, and a significant increase in the reduced forms of AA and GSH. Nevertheless, a rapid decrease in the redox state of both antioxidants was observed during reaeration. Inhibition of GR, APX, catalase (CAT) and superoxide dismutase (SOD) activities has been shown by Yan *et al.* (1996) in maize leaves under prolonged flooding, while a short-term treatment led to an increase in the activities.

Hypoxic signaling and gene regulation

Hypoxia/anoxia is one of the most important abiotic stresses encountered by most of the higher organisms. Studies are required to isolate and characterize the genes involved in tolerance to anaerobic stress and to determine the molecular mechanisms and analysis of genes that confer increased flooding and anaerobic tolerance in crop plants. Genetic analysis indicates that tolerance is a fairly simple dominant trait. A recessive factor that increases anaerobic tolerance in plants that are null for ADH activity has been reported in maize (Lemke-Keyes and Sachs 1989a,b). Subbaiah and Sachs (2003) have also demonstrated how a simple post-translational modification of sucrose synthase by the addition/removal of phosphate can lead to potent changes in the tolerance of seedlings to anoxia. Discovery of genes and proteins likely to be involved in structural modifications (aerenchyma formation and root tip death) indicate further that these mechanisms are multi-pronged and multi-component, perhaps tailored to adapt to different levels of stress.

The co-ordinated expression of the anaerobic protein (ANP) is accomplished by a common trans-acting factor that interacts with an anaerobic responsive element (ARE) in the promoter region of each gene (Olive *et al.* 1991). Oxygen deficiency changes its conformation, or the natures of its binding to ARE, and thereby promotes transcription. Aerobic mRNA is not translated under anaerobic condition in maize roots, whereas those for ANP are translated, presumably reflecting the recognition of a specific anaerobic signal on the mRNA. Finally, the

anaerobic mRNA is much more stable and has a longer half-life under oxygen deficiency (Drew 1990).

The anaerobic stress-response of maize offers an opportunity to characterize the regulatory components of a family of 20 genes that are coordinately expressed. The anaerobically induced proteins appear to be encoded by a set of genes, whose expression is stimulated by a deprivation of oxygen, a condition that would occur in nature during flooding. Regulation of protein synthesis under anaerobiosis appears to occur at multiple levels. Subbaiah and Sachs (2003) have characterized several genes involved in the anaerobic response and provided some insight into a few components of the signal transduction pathway to understand how maize perceives the changes in external O_2 concentration and adapts its growth and metabolism over the short- and long-term. They demonstrated that Ca^{2+} acts as a key transducer of changes in O_2 availability. An additional aim should be to characterize the promotor elements of the anaerobically induced genes as well as the signalling components down-stream to calcium that trigger gene induction.

Though the molecular basis of the adaptation to transient low oxygen conditions has not been completely characterized, but progress has been made towards identifying genes and gene products induced during low oxygen conditions. Promoter elements and transcription factors involved in the regulation of anaerobically induced genes have been characterized. Transgenic plants may clarify the physiological role of the fermentation pathways, and their contribution to flooding tolerance (Dennis *et al.* 2000). Dennis *et al.* (2000) reported first results with the inducible *AtMYB2* transcription factor. Sequencing of the *Arabidopsis* anaerobically-induced root cDNA library may identify novel genes concerned with the low oxygen response.

Lee *et al.* (2007) investigated the transcriptional expression *in vitro* for low-oxygen treatment. Dramatic increases in the transcripts of a *TaMyb1* (*Triticum aestivum Myb transcription factor 1*) gene occurred under hypoxia. The transcriptional expression of *TaMyb1* was enhanced by light under hypoxia. The *TaMyb1* expression was high in the epidermis, endodermis and the cortex adjacent to the endodermis under hypoxia but undetectable in the vascular tissues or cortex, which contained aerenchyma. *TaMyb1* transcription levels in roots also gradually increased as the result of treatment with abscisic acid (ABA), polyethylene glycol (PEG) and NaCl. They suggested that the expression of *TaMyb1* in roots could be strongly related to the oxygen concentration in root environment.

In plants, the involvement of Rop signaling in response to hypoxia was revealed by a screen of *Arabidopsis* seedlings that possess a Ds-GUS transposon gene-trap element for genes induced by low oxygen levels (Baxter-Burrell *et al.* 2003). The screen yielded *ropgap4-1*, a loss-of-function mutant generated by a Ds-GUS insertion in the first exon of ROPGAP4. The *ropgap4-1* seedlings induced significantly higher levels of ADH mRNA and specific activity than wild-type seedlings, hinting that ROPGAP4 negatively regulates ADH induction. Rop activation is a prerequisite for hypoxic induction of ADH because a line overexpressing a dominant negative mutant of *ROP2* (*35S:DN-rop2*) that stably binds GDP showed no detectable increase in ADH mRNA and enzymatic activity in response to the stress. By contrast, a line producing a mutant *ROP2* that constitutively binds GTP (35S:CA-rop2) had elevated ADH activity under control growth conditions (Baxter-Burrell *et al.* 2002). Consistent with this hypothesis, Rop-GTP levels increased dramatically within 1.5 h of hypoxia and then decreased between 12 and 24 h of oxygen deprivation in wild-type seedlings. The ropgap4-1 seedlings had a brownish, water-soaked appearance after oxygen deprivation, similar to that caused by oxidative stress. This led to the unexpected finding that hypoxia promoted an increase in H_2O_2. Significantly higher levels of H_2O_2 were measured in extracts from *ropgap4-1* mutants, whereas no rise in H_2O_2 was detected in *35S:DN-rop2* seedlings. In wild-type, *ropgap4-1* and *35S:CA-rop2* seedlings, increases in H_2O_2 and ADH activity were inhibited by diphenylene iodonium chloride (DPI), an inhibitor of flavin-binding proteins. By contrast, ADH activity was induced under aerobic conditions when H_2O_2 was enzymatically generated on the surface of seedlings (Baxter-Burrell *et al.* 2003). These observations support the conclusion that ROS production is a component of the pathway that induces ADH expression under low oxygen (Fukao and Bailey-Serres 2004). Role of ROS signaling in the induction of transcription factors associated with induction of genes of antioxidant enzymes has also been reported by various workers (Pastori and Foyer 2002, Agarwal *et al.* 2005). It is thus possible that hypoxia generated ROS also induce the genes of antioxidant enzymes, resulting in the observed increase in the activity of various antioxidative enzymes.

Nitric oxide has been reported as a signaling molecule in a wide range of responses in animals and plants. The accumulated evidence suggests that a metabolic pathway involving NO and Hb provides an alternative type of respiration to mitochondrial electron transport under limited oxygen. Hb in hypoxic plants acts as part of a soluble terminal NO dioxygenase system, yielding nitrate ion from the reaction of oxyHb with NO. NO is mainly formed due to anaerobic accumulation of nitrite. The overall reaction sequence, referred to as the Hb/NO cycle, consumes NADH and maintains ATP levels *via* an as yet unknown mechanism (Igamberdiev *et al.* 2005). Hb gene expression appears to influence signal transduction pathways, possibly through its effect on NO, as evidenced by phenotypic changes in normoxic Hb-varying transgenic plants. Ethylene levels are elevated when Hb gene expression is suppressed, which could be a factor leading to root aerenchyma formation during hypoxic stress. Interestingly, both NO and H_2O_2 have recently been found to function as localized and long-range root-derived signals capable of rapidly communicating the redox status and indirectly activating MAP kinase-like activity in the shoots of *A. thaliana* (Capone *et al*. 2004).

Conclusions and perspectives

Examination of regulatory mechanisms and signaling events responsible for triggering responses to oxygen deficient conditions in plants is an interesting area of research. Advances in genome biology, genetic resources and high throughput technologies provide excellent resources for the exploration of oxygen sensing mechanisms in plant cells. It is imperative to identify sensors and dissect the signaling pathways that occur at the cellular, tissue, organ and whole plant level. It will be interesting to investigate the participation of an ROS sensing mechanism involving PM-NADPH oxidase in different plants species. Again the paradoxical ROS may prove to be second messenger in the response mechanism. Further it will be interesting to determine whether observed increases in NO evolution under flooding condition from roots or soils can contribute as a positive message in root-to-shoot communication. Analyses of near isogenic genotypes that differ in the adaptive response to oxygen deprivation are likely to yield critical information on regulatory mechanisms. Alterations in cytosolic pH and calcium may also have a role in the signaling processes. The importance of changes in adenylate charge, redox status and carbohydrate levels must also be considered. Many questions remain to be answered about the response of individual cells. What could be the basis of differential response between stress-tolerant and intolerant organs and species? Do these differ in cellular signaling and response mechanisms? Again we need to understand what signaling transduction pathways are activated or inhibited, how do multiple and interacting pathways control adaptive responses? The involvement of growth regulators such as ethylene, auxin, gibberellins and ABA in hypoxic regulation is also an interesting possibility. The manner in which the energetic needs of meristematic cells are safeguarded and how is programmed cell death promoted or avoided, also needs examination? How do cells in roots and aerial organs communicate over a long distance when there is an oxygen crisis in the roots? Understanding the cell to cell and long-distance signaling mechanisms that determine the organ and whole plant response to oxygen deprivation, *viz.*, regulation of leaf and internode elongation, petiole curvature, aerenchyma formation and adventitious root growth is another inviting area for research. So far we only know a part of the unfolding story, with many more questions still unanswered. Answering these questions will be of relevance to agriculture and will provide knowledge of the fundamental nature of anaerobic life.

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