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review

Cyanide action in plants - from toxic to regulatory

Irena Siegień[1*], *Renata Bogatek*[2]

[1] Institute of Biology, The University of Białystok, Świerkowa 20B, 15-950 Białystok, Poland, e-mail: siegien@uwb.edu.pl
[2] Department of Plant Physiology, Warsaw Agricultural University, Nowoursynowska 159, 02-776 Warszawa, Poland, e-mail: bogatek@delta.sggw.waw.pl
[*] Corresponding author

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Abstract

Recent biochemical and genetic studies on hydrogen cyanide (HCN) metabolism and function in plants were reviewed. The potential sources of endogenous cyanide and the pathways of its detoxification are outlined and the possible signaling routes by which cyanide exerts its physiological effects are discussed. Cyanide is produced in plant tissues as the result of hydrolysis of cyanogenic compounds and is also released as a co-product of ethylene biosynthesis. Most cyanide produced in plants is detoxified primarily by the key enzyme -cyanoalanine synthase. The remaining HCN at non-toxic concentration may play a role of signaling molecule involved in the control of some metabolic processes in plants. So, HCN may play a dual role in plants, depending on its concentration. It may be used in defense against herbivores at high toxic concentration and may have a regulatory function at lower concentration. Special attention is given to the action of HCN during biotic and abiotic stresses, nitrate assimilation and seed germination. Intracellular signaling responses to HCN involve enhancement of reactive oxygen species (ROS) generation and the expression of cyanide-insensitive alternative oxidase (*AOX*) and ACC synthase (*ACS)* genes. The biochemical and cellular mechanisms of these responses are, however, not completely understood.

List of abbreviations: ABA - abscisic acid, ACC - 1-aminocyclopropane-1-carboxylic acid, ACO - ACC oxidase, ACS - ACC synthase, *ACS6* - ACC synthase gene, AFGC - Arabidopsis Functional Genomic Facility, AOX - alternative oxidase, APX - ascorbate peroxidase, AtRDH - *Arabidopsis thaliana* rhodanese homologue protein, -CAS - -cyanoalanine synthase, CTR1 - Raf-like ser/thr kinase, CS - cysteine synthase (*O*-acetylserine sulfhydratase), 2,4-D - 2,4-dichlorophenoxyacetic acid, GA - gibberellins, *ga-1*- GA deficient mutant, GR - glutathione reductase, HR - hypersensitive response, IAA - indole acetic acid, MAPK - mitogen activated protein kinase, N*F-*κB - redox-sensitive nuclear factor, NR - nitrate reductase, PCD - programmed cell death, PPP - pentose phosphate pathway, ROS - reactive oxygen species, RuBP - ribulose 1,5-bisphosphate, TMV - tobacco mosaic virus, TVCV - turnip vein clearing virus, SAM - S-adenosylmethionine

Introduction

Hydrogen cyanide (HCN) is a small gaseous molecule that has received special attention from scientists since the beginning of the 19th century, due to its toxic effect on living organisms. Biological activity of HCN was mostly correlated with the inhibition of the terminal cytochrome oxidase in the mitochondrial respiratory pathway, whereas its regulatory role in plant metabolism was rarely discussed. Some experimental data reported a protective effect of cyanide in plants against predators such as herbivores (*e.g.* Nahrstedt 1985). This role of cyanide is now better documented *(e.g.* Gleadow and Woodrow 2002, Zagrobelny *et al.* 2004), and additionally supported by the most recent experiments with some transgenic plants (Wittstock and Gershenzon 2002, Siritunga and Sayre 2004). Some data also indicate that HCN, apart from being toxic, plays a regulatory (maybe signaling) function in many physiological processes, *e.g.* seed germination (Bogatek *et al.* 1999), nitrate assimilation (Solomonson and Barber 1990) or in plant responses to some environmental stimuli (Grossmann 1996).

In this paper, we attempt to describe the current understanding of the control of HCN level and metabolism in plants, focusing particularly on its possible cellular effects and highlighting the biological processes likely to be involved in the dual - toxic to regulatory - roles of this molecule.

The sources of hydrogen cyanide in plants

Cyanogenesis, the ability of plants and other living organisms to produce HCN, has been known for several centuries in apricots, peaches, almonds and other important food plants (Nahrstedt 1993). Plants which exhibit this phenomenon usually contain one or more compounds as precursors, which liberate HCN upon hydrolysis. In certain sapindaceous and hippocastanaceous seeds, HCN is derived from the cyanogenic lipids (Seigler 1991). Most frequently, however, HCN production in higher plants results from the catabolism of cyanogenic glycosides. Cyanogens are found in more than 3000 species of higher plants including ferns, gymnosperms and angiosperms. These cyanogens are glycosides of -hydroxynitriles (cyanohydrins); all known compounds are *O*- -linked, mostly with D-glucose (Seigler 1991). Depending on their precursor amino acid, they may be aliphatic, aromatic, or cyclopentenoid in nature (Seigler 1991, Moller and Seigler 1999). All biosynthetic steps, except the final glycosylation of the -hydroxynitrile, are catalysed by membrane-bound enzymes (Fig. 1) and were previously described in detail (Siegień 1998, Moller and Seigler 1999).

Degradation of cyanogenic glycosides in plants is initiated by cleavage of the glycosidic linkage(s) by one or more soluble -glycosidases. The resulting cyanohydrins are relatively unstable and decompose either spontaneously or enzymatically, in reaction catalysed by a -hydroxynitrile lyase to yield HCN and an aldehyde or a ketone (Jones *et al.* 2000) (Fig. 1). In intact plants, the substrates and hydrolytic enzymes appear to be separated by compartmentation at either tissue or subcellular levels (Seigler 1991, Selmar 1993), so cyanide is not evolved continuously from cyanogenic plant tissues. Generally, cyanoglycosides are stored in vacuoles, whereas -glycosidases are apoplastic (Selmar 1993, Gruhnert *et al.* 1994). When tissues are disrupted (*e.g.* by herbivore attack), the glycosides are brought into contact with -glycosidases and hydroxynitrile lyases, which release the toxic HCN (Fig. 1). In contrast to the processes involving injury, many plant cyanoglycosides are also metabolized in intact cells and transported in the plant (Selmar 1993). This situation is observed during seed germination of some rubber tree (*Hevea*) species (Selmar *et al.* 1988), where the majority of cyanogens stored in endosperm is transported into the cotyledons and primary leaves and metabolized to noncyanogenic nitrogen-containing compounds. HCN is also liberated from cyanogenic glycosides during the break of dormancy (early stages of cold stratification) of apple seeds (*Malus domestica*) (Dziewanowska *et al.* 1979b). In this case, the relatively large amounts of free HCN could, however, be produced as a result of damage to the tonoplast during rehydration, that allows the direct contact of cytoplasmic glycosidase with the vacuolar cyanogen - amygdaline. Moreover, it cannot be excluded that cyanogenic glycosides are metabolized at some other stages of growth of certain cyanogenic

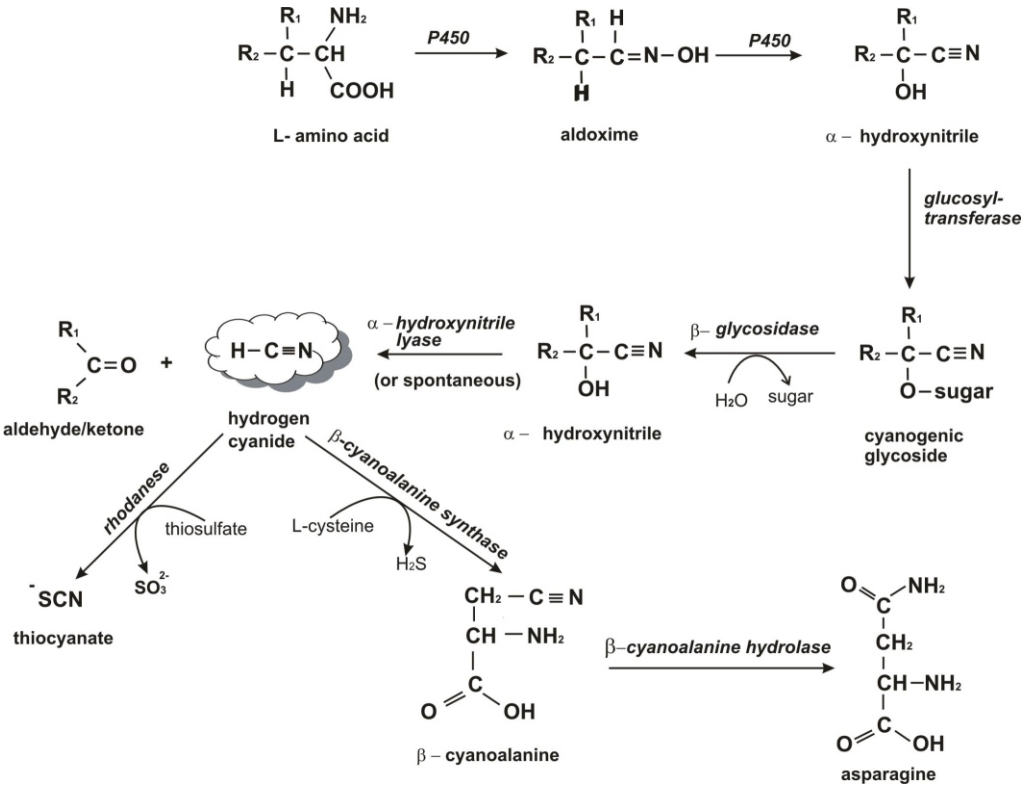

Fig. 1. Biosynthesis and catabolism of cyanogenic glycosides. Enzymes involved are shown in italic; *P450* - P_{450} linked enzyme. Modified according to Zagrobelny *et al.* (2004).

plants thus evolving free cyanide. The fact that the concentration of cyanogenic glycosides (simultaneously the cyanogenic potential) seems to fluctuate diurnally, and varies greatly during the growing season (Niedźwiedź-Siegień 1998, Niedźwiedź-Siegień and Gierasimiuk 2001, Stochmal and Oleszek 1997), may support this assumption.

Cyanogenesis is not exclusive to cyanolipid- and cyanogenic glycoside- containing plant species. Peiser *et al.* (1984) were among the first to show that cyanide was a co-product of the ethylene biosynthesis pathway, where it is produced in stoichiometrically equal amounts to ethylene (Grossmann 2003) (Fig. 2). Cyanide can also be formed in plant tissues from glyoxylate, the product of photorespiration, and hydroxylamine, the possible intermediate of nitrate assimilation (Hucklesby *et al.*1982).

The existence of several potential sources of cyanide in plants supports the hypothesis that this gaseous molecule is present in almost all plant tissues and may contribute in the control of many important physiological events.

Toxicity of hydrogen cyanide and routes of its detoxification in plant tissues

Cyanide is very toxic to all living cells including those of plants. Application of cyanide to *Arabidopsis thaliana* caused a marked growth inhibition, a reduction in overall plant size, and a decrease in chlorophyll content (Smith and Arteca 2000). It caused necrotic spots on tobacco (*Nicotiana tabacum)* leaves (Siefert *et al.* 1995), and induced nuclei degradation in pea (*Pisum sativum*) leaves (Samuilow *et al.* 2000). Various intracellular enzymes involved in many important metabolic pathways are known to be inhibited by cyanide. The most sensitive enzymes include Cu/Zn superoxide dismutase, catalase, cytochrome-c oxidase, nitrate/nitrite reductase, nitrogenase, and peroxidase (Grossmann 2003). In green plant tissues, ribulose-bisphosphate carboxylase is quite sensitive to cyanide as well. Cyanide also interacts with the Cu-protein plastocyanin that is involved in photosynthetic electron transport (Liang 2003). The concentrations of endogenous cyanide required to cause 50 % inhibition of sensitive enzymes are

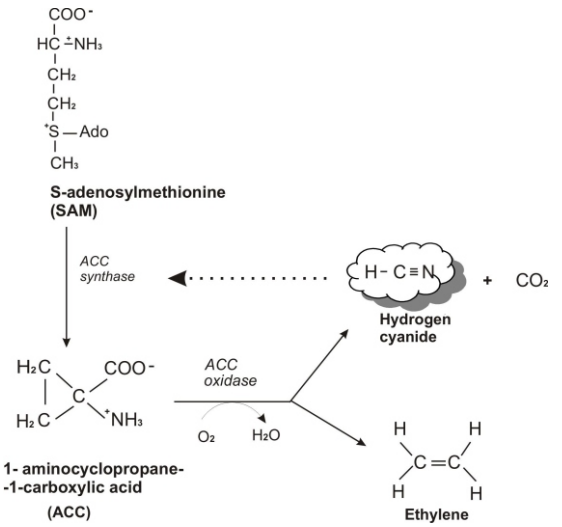

Fig. 2. Generation of HCN and ethylene from S-adenosylmethionine (SAM). Dotted arrow indicates on step stimulated by HCN. Modified according to Grossmann (2003).

mostly in the range of 5 - 10 M (Grossmann 1996). Thus, in order to protect these systems, the concentration of HCN in plant tissues is tightly regulated by different pathways (enzymes) that may quickly detoxify and re-metabolize cyanide.

The pivotal role in detoxification of cyanide is played by -cyanoalanine synthase (-CAS), a pyridoxal phosphate-dependent enzyme. It utilizes cysteine and cyanide to form hydrogen sulfide and -cyanoalanine. The latter is subsequently converted to asparagine in a reaction catalysed by b-cyanoalanine hydrolase (Miller and Conn 1980) (Fig. 1). The activity of -cyanoalanine synthase in plants is primarily located in mitochondria - the organelles most vulnerable to HCN (Meyers and Ahmad 1991). This enzyme appears to be ubiquitous in higher plants, as it plays a general role in assimilation of HCN liberated from cyanogenic compounds, and also cyanide produced concomitantly with ethylene (Grossmann 1996). In apple slices and mungbean (*Vigna radiata*) hypocotyls, for example, the application of a -CAS inhibitor (aminooxyacetic acid) caused an accumulation of endogenous HCN (Yip and Yang 1988). High activity of -CAS has been detected in plant tissues that produce ethylene at high rates (*e.g.* 1650 nmol $g^{-1} \cdot h^{-1}$ in ripe apples), therefore the concentration of HCN appears to be maintained below toxic levels (1 M or less) that are safe for sensitive enzymes (Yip and Yiang 1988). Such situation was observed in barley (*Hordeum vulgare*) seedlings treated with herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) (Grossmann and Kwiatkowski 1995), and in tobacco under drought stress conditions (Liang 2003). Both plants, because of high activity of -CAS, were shown to be resistant to the stress caused by cyanide. It is interesting that activity of -CAS may be enhanced itself by cyanide (Liang 2003), and also by ethylene (Maruyama *et al.* 2001).

Cysteine synthase (CS or *O*-acetylserine sulfhydratase) catalyzes cysteine formation from *0*-acetyl-L-serine and sulfide, but also possesses -CAS activity in plants (Maruyama *et al.* 2001). It is located in cytosol, chloroplasts and mitochondria, and in recent years, corresponding cDNA clones have been isolated from a variety of plants (Saito 2000). However, CS may lose its activity during tissue disruption, due to the enzyme high sensitivity to oxidation, which often happens under different stress conditions (Liang and Li 2001).

In most animals, gaseous HCN may combine with thiosulfate to form less toxic thiocyanate in a process catalysed by rhodanese (Beesley *et al.* 1985), (Fig. 1). However, in contrast to -CAS, rhodanese is not present ubiquitously in plants, and because of very low activity (Kakes and Hakvoort 1992), the existence of this enzyme is highly controversial. Recently, Hatzfeld and Saito (2000) have, for the first time, isolated and characterized two cDNAs encoding rhodanese isoforms in *A. thaliana: AtRDH1 and AtRDH2* (*A. thaliana* rhodanese homologue 1 and 2, respectively). *O*ver expression of both of these genes increased the rhodanese activity of transgenic yeast. AtRDH1 protein is located in the mitochondria, whereas AtRDH2 is found in the cytosol. Rhodanese may also have other functions, the most important of which is to donate sulfur to proteins (Bordo and Bork 2002).

It seems that plant tissue has potentially ample capacity to detoxify cyanide, mainly by b-CAS activity. It has been recently suggested that because of this property, some plants might be useful in phytoremediation processes of soil and groundwater polluted by cyanide (Larsen *et al.* 2004). It is not a rule, however, that the concentration of HCN in plant tissues appeared to be maintained below toxic levels for other organisms.

Dual effect of hydrogen cyanide in plant responses to stress

Plants are known to release ethylene in response to biotic stresses (such as those induced by pathogens) and to abiotic stresses (such as those induced by mechanical stimuli, flooding or drought) (Chen *et al.* 2005). The question arises regarding the physiological significance of cyanide (Grossmann 1996), which is formed as a co-product of ethylene synthesis (Fig. 2). Some evidence support a dual - toxic and signaling - function of cyanide produced in stressed plants. The dual effect might depend on concentration of HCN, as well as on the status of a plant and its growth conditions.

It is generally accepted that most cyanide produced during ethylene synthesis is effectively detoxified, so the possibility of a toxic role for cyanide in the plant tissue is rarely considered. In recent years, attention has once again focused on the mode of action of auxin herbicides in sensitive plants, in which the involvement of cyanide in the induction of herbicide phytotoxicity is postulated (Grossmann 1996). These situations are observed in soybean (*Glycine max*) seedlings exposed to 2,4-dichlorophenoxyacetic acid (2, 4-D), and barnyardgrass (*Echinochloa crus-galli*), exposed to quinclorac (Grossmann and Kwiatkowski 1995, Grossmann 2003). The concentration of endogenous cyanide in these plants, which is derived ultimately from the herbicide-stimulated ACS activity in ethylene biosynthesis, may increase many times, reaching a maximum of nearly 30-50 M (Grossmann 2003). -CAS activity in these plants was too low to detoxify cyanide to physiologically safe levels, causing death of soybean seedlings and growth inhibition, chlorosis and necrosis of barnyard grass (Grossmann 2003). The process appears to be self-amplifying because ACC and its product cyanide promote ACS activity in the shoot tissue (Fig. 2). In this way, the concentration of toxic cyanide is enhanced (Grossmann 2003). The results of some experiments with application of KCN or inhibitors of ethylene biosynthesis, and in transgenic plants with antisense construct to the *ACS* gene, provide additional evidence that cyanide is a toxic agent in herbicide action (Grossmann 1996, 2003).

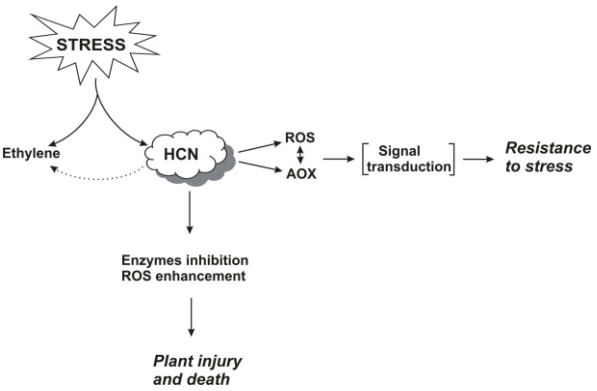

Fig. 3. Dual action of cyanide under stress conditions. Dotted arrow indicates on stimulation of ethylene synthesis by HCN.

The increased level of cyanide in stressed tissue may be caused not only by low activity of -CAS, but also by different intracellular compartmentation of cyanide release (ACC oxidase is in the cytoplasm) and cyanide detoxification (-CAS is predominantly localized in the mitochondria) (Grossmann 1996). Thus, cyanide removal in cell compartments other than mitochondria may be less efficient, and consequently could result in its transiently elevated levels in cell compartments such as cytoplasm, chloroplasts and peroxisomes (Grossmann 1996). On the other hand, most cyanide released during the ACC oxidase reaction exists in the undissociated form, and could therefore diffuse easily through membranes, to reach different compartments of the cell (Meyer *et al*. 2003).

Under certain stress conditions, cyanide concentration that accompanies rapid ethylene production seems to be under the control of detoxifying enzymes. Thus, transient increase in HCN concentration in a small region of plant tissue may take place, and in this case cyanide may act as a signaling cellular molecule, which triggers the events consequently leading to acquisition of stress resistance (Fig. 3). It has been demonstrated that non-lethal concentration of cyanide enhances the resistance of tobacco leaves to tobacco mosaic virus (TMV) (Chivasa and Carr 1998). Subsequent experiments with *A. thaliana* have also shown that cyanide can induce resistance to turnip vein clearing virus (TVCV) (Wong *et al.* 2002). Some events leading to resistance are not yet known, but most likely involve the induction of *AOX* gene and increase AOX activity (Chivasa and Carr 1998, Wong *et al.* 2002).

It was also found that the enzyme may be activated only in the cells infected with the virus or in the cells close to the hypersensitive lesions. This observation is consistent with findings that *AOX* gene expression correlates positively with lesion numbers, which suggests that cells close to lesions express higher levels of AOX (Chivasa and Carr 1998). Thus, the question arises which of AOX-dependent events are involved in virus resistance. We postulate that the rapid and transient oxidative production of ROS is induced by cyanide, as it is known to take place at the early stage of viral infection (Wojtaszek 1997), as well as under other stresses (Vranova *et al.* 2002, Gniazdowska and Bogatek 2005), So, AOX may be involved in removal of ROS in infected plant tissues, possibly by activation of antioxidant systems. It has been proposed that AOX plays a role in avoidance of damages to the cell by ROS, produced in many stresses (Wagner and Moore 1997, Juszczuk and Rychter 2003). This is consistent with some data obtained by Ordog *et al.* (2002) who found that overexpression of AOX in tobacco reduced the size of hypersensitive lessions (HR). Moreover, some metabolic processes in susceptible plants treated with auxin herbicides could be affected by cyanide through ROS production. Uncontrolled synthesis of cyanide in these plants (Grossmann 2003) may lead to uncontrolled accumulation of ROS, which are highly toxic for the cell. ROS can react with the majority of biomolecules, thus resulting in oxidative stress that causes irreversible cellular damages (Vranova *et al.* 2002).

Furthermore, ROS itself may also act as signal molecules in plant transduction cascade, leading to stress resistance (Vranowa *et al.* 2002). It has been shown in animal cells that cyanide mediates the development of stress symptoms, *via* generation of ROS (Gunasekar *et al.* 1998). Moreover, ROS generation was observed in dormant apple embryos treated with cyanide (Bogatek *et al.* 2003).

Cyanide may be involved in alleviation of stress through its effect on synthesis of ethylene (Fig. 2). Recently, Smith and Arteca (2000) showed that in *A. thaliana,* ACS gene (*ACS6*) is rapidly activated after treatment with cyanide. Moreover, the amount of *ACS6* transcript seen on Northern blots is dependent not only upon the concentration of cyanide, but also upon duration of the stress (Smith and Arteca 2000). Some data indicate that the ACS6 gene, whose expression is induced by cyanide treatment, is also transcriptionally activated after mechanical stimulation, IAA, salt, and ozone treatments (Arteca and Arteca 1999, Smith and Arteca 2000). It is intriguing that physiologically relevant and metabolically "safe" concentration as low as 1 M HCN, acting for 20 min is capable of initiating *ACS6* transcription. Ethylene (produced after cyanide treatment) may possibly influence the rate of plant growth under stress. Achard *et al*. (2003) recently reported that at least part of the growth regulatory action of ethylene is mediated *via* its effects on the DELLA proteins, which act as a repressors of growth in response to ethylene. The slowing down of the growth is one of the strategies which plants have adopted under stress conditions. The ability to reduce cell growth under unfavorable conditions may not only allow conservation of energy for defense purposes, but also limit the risk of heritable damage (May *et al.* 1998). Moreover, stunted growth was observed in cyanide-treated *A. thaliana* plants (Smith and Arteca 2000).

It is also possible that sub lethal levels of cyanide produced from ACC together with ethylene can play a role in acclimation of plants to biotic and abiotic stresses. Data obtained until now with mammalian cells indicate that their preconditioning with sub lethal concentration of sodium cyanide (NaCN) protected the neurons against subsequent NaCN-induced damages (Jensen *et al.* 2002).

Thus, cyanide may trigger many events which lead to the acclimation of plants growing under adverse conditions. More experimental data is needed to confirm that these events are common for many biotic and abiotic stresses accompanied with the co evolution of ethylene and cyanide.

The regulatory role of hydrogen cyanide in nitrate assimilation

Nitrate reductase (NR), which catalyses the conversion of nitrate to nitrite, is considered to be the key enzyme in the process of nitrate assimilation. Its activity may be a limiting factor in the growth and yield of grain protein in several cereals. NR

from alga *Chlorella* and several other organisms can exist *in vitro* in two interconvertible forms: active and inactive. The active form, which corresponds to an oxidized state of the enzyme, is converted into a reduced inactive form when incubated with NADH and cyanide (Echevarria *et al.* 1984). Cyanide reacts stoichiometrically with the NADH--reduced enzyme to produce a stable enzyme-cyanide complex having a dissociation constant of about 10^{-10} M, which is in the same range as that of hormone-receptor complexes (Solomonson and Barber 1990). These results indicate that concentration of HCN required for enzyme inactivation is of several orders of magnitude smaller than the one which would inhibit other vital processes such as respiration. Conversely, the reduced inactive form of NR is rapidly reactivated when oxidized and this causes the release of bound cyanide.

Several reports suggest the connection between cyanide generated *in vivo* and photorespiration, as light and oxygen are required for the efficient conversion of NR to its inactive form (Solomonson and Barber 1990). It has been shown that crude extract of *Chlorella* catalyzes the formation of cyanide from glyoxylate (a product of photorespiration) and hydroxylamine (a possible intermediate of nitrate assimilation). The enzyme that catalyzes this reaction was subsequently identified in *Chlorella,* spinach (*Spinacia oleracea*) and also in corn (*Zea mays*) and barley leaves (Hucklesby *et al.* 1982). It has a molecular mass of about 40 kDa, requires Mn^{2+}, and has an absolute requirement for ADP or a combination of ADP and ATP.

Solomonson and Spehar (1977) proposed a model showing regulation of photosynthetic CO_2 fixation and nitrate reduction by HCN, which is generated during these processes (Fig. 4). Under the conditions of high intracellular $[O_2]/[CO_2]$ratio, phosphoglycolate is generated in the reaction of RuBP with oxygenase. Phosphoglycolate can be converted to glycolate and then to glyoxylate, which is the source of HCN. HCN generated in this way can inactivate NR when the rate of CO_2 fixation is low, and carbon skeletons for the fixation of ammonia produced by assimilatory nitrate reduction are insufficient. Also the accumulation of potentially toxic intermediates of nitrate assimilation

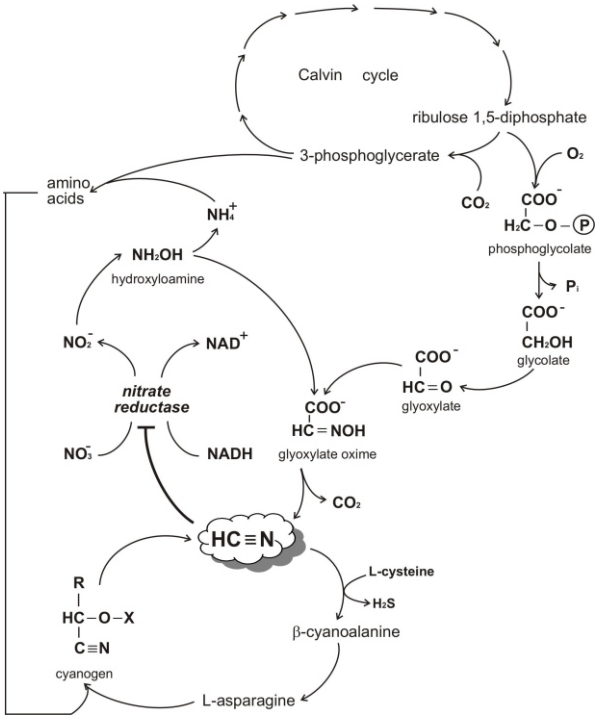

Fig. 4. Cyanide-mediated assimilation of nitrate . Modified according to Dziewanowska (1983).

such as nitrite, hydroxylamine, and ammonia is prevented.

Cyanide as a regulator of seed germination

Dormancy removal and seed germination appear to be one of the best known processes regulated by cyanide. However, the mechanisms of these regulations are not as yet understood.

Cyanide at millimolar concentrations stimulates the germination of seeds of different species including rice (*Oryza sativa*), barley (Roberts 1969), lettuce (*Lactuca sativa*) (Zagórski and Lewak 1985), phalaris grass (*Phalaris*) (Junttila and Nilsen 1980), apple (Dziewanowska *et al.* 1979a, Bogatek and Lewak 1991) and cocklebur (*Xanthium pennsylvanicum*) (Maruyama *et al.* 1996). It is interesting that the stimulatory effect of cyanide was observed only when it has been subsequently eliminated from germination medium. The germination of most of these seeds was, however, completely blocked in the continued presence of cyanide when its volatility and chemical instability were controlled. These observations indicate a dual action of cyanide, similar to that suggested earlier

for stresses. HCN, depending on concentration, inhibits or stimulates seed germination.

The emission of HCN was observed during the pre-germination period of many seeds, including some that did not contain cyanogenic glycosides (Esashi *et al.* 1991). It has been suggested that the partial blocking of the cytochrome system might be necessary for the successful germination of seeds. Possibly, liberated endogenously cyanide may act on germination through the regulation of oxidative electron transport, *i.e.* partial inhibition of the cytochrome pathway and/or induction of alternative, cyanide-resistant one (Hasegawa *et al.* 1994). The alternative cyanide-resistant respiration operates in many plant seeds during imbibition, in addition to cyanide-sensitive one (Morohashi and Matsushima 1983). However, the involvement of cyanide insensitive, AOX pathway in the control of germination of pear (*Pirus communis*) (Alsher-Herman *et al.* 1981) and after-ripened apple seeds (Bogatek and Rychter 1984) was not confirmed. It has been detected that in isolated embryos (apple and pear) and mitochondria (apple) an AOX path does not operate during cold stratification, and the oxygen requirement under these conditions is low enough to be met even by the partly KCN inhibited cytochrome pathway.

The reactivation of metabolism following seed imbibition may be an important source of ROS, causing stress that might affect the successful germination (see for review, Bailly 2004). Thus, the induction of AOX pathway has to be associated with stimulation of some detoxifying enzymes, involved in ROS elimination. It was confirmed by Nkang (2001), who demonstrated that cyanide stimulated the germination of *Guilfoylia monostylis* seeds by enhancing the activity of enzymes capable of degrading hydrogen peroxide. Recent report (Nun *et al.* 2003) emphasizes the significance of the AOX pathway in removing of ROS in seeds of Egyptian broomrape (*Orobanche aegyptica*), which could arise during conditioning and causes damages to the seeds.

Another possibility to explain the effects of cyanide on seeds germination may be the prevention of degradation of the active form of phytochrome, which is required for initiation of some essential germination processes (Junttila and Nilsen 1980). There are also some data on involvement of cyanide in regulation of protein metabolism in seeds, through protease activation (Dziewanowska 1983).

Some interesting results concerning HCN-mediated seed germination have been obtained in experiments with dormancy removal and germination of apple seeds. Germination of apple embryos isolated from dormant seeds is slow, but can be accelerated by light or gibberellins treatment (Smoleńska and Lewak 1971). However, seedlings grown from dormant embryos show a number of morphological and metabolic anomalies. The most important are inhibition of hypocotyl and internode elongation growth, as well as asymmetric growth and greening of cotyledons (Bogatek and Lewak 1991). All these anomalies are not observed in the seedlings grown from non-dormant seeds, submitted to cold stratification, when endogenous cyanide in relatively high amounts is produced and secreted (Dziewanowska *et al.* 1979b). Also, short pre-treatment of dormant apple embryos with gaseous HCN markedly stimulated germination and eliminated all above symptoms of dormancy (Bogatek and Lewak 1991, Lewak *et al.* 2000). Many experimental results indicate several putative modes of cyanide action:

1. Removal of embryonic dormancy in apple seeds requires consecutive activation of glycolysis and pentose phosphate oxidative pathway (PPP) in sugar catabolism. HCN treatment accelerated the appearance of glycolysis (Bogatek and Lewak 1988, Lewak *et al.* 2000). Since glycolysis provides the cells with ATP, and PPP results in production of NADPH as well as several specific intermediates (*e.g.* pentoses), it is tempting to assume a sequence of metabolic events requiring the supply of these products. The crucial role of PPP in dormancy breaking and seed germination has also been postulated for other seeds (Fontaine *et al.*1994).

2. HCN action on dormant apple embryos is related to stimulation of sugar catabolism (Bogatek *et al.* 1999, Lewak *et al.* 2000). Cyanide pretreatment resulted in a decrease of sucrose content, by the stimulation of alkaline invertase activity in upper cotyledon of isolated embryo. Growth, greening, and

sucrose hydrolysis in the upper cotyledon were stimulated by HCN pre-treatment to the levels observed in a lower cotyledon, growing and greening faster (Bogatek *et al.* 1999).

3. HCN markedly enhanced ethylene emission by the stimulation of *ACS6* expression (Bogatek *et al.* 2004). Although ethylene has been implicated in breaking dormancy and germination of many seeds (Kępczyński and Kępczyńska 1997), its role in regulation of these processes is still poorly understood (Calvo *et al.* 2004). There is some evidence that certain components of ethylene signaling pathway are involved in the regulation of germination by gibberellins (GA). Ethylene can fully rescue the germination defect of the *A. thaliana* GA-deficient mutant *ga-1* (Koornneef and Karssen 1994). Calvo and others (2004) showed that ethylene and gibberellins (GA) are involved in breaking dormancy of beech tree (*Fagus silvatica*) seeds, by influence on expression of ACC oxidase gene (*ACO*). There is no data, however, that indicate the existence of similar cross-talk regulation between both hormones in dormant apple embryos after cyanide-induced synthesis of ethylene.

4. Cyanide-pretreatment leads to induction of oxidative stress (accumulation of H_2O_2) in dormant apple embryos and to the increase in the activity of antioxidative enzymes, especially glutathione reductase (GR) (Bogatek *et al.* 2003). It may lead to induction of the pentose phosphate pathway through oxidation – reduction of glutathione and of NADP (Bogatek *et al.* 2003). Glutathione reductase may also be involved in ROS alleviation in apple embryos, similarly as in other seeds (Tommasi *et al.* 2001). Moreover, H_2O_2 itself may act as a signal, triggering some events that lead to breaking the deep dormancy of these embryos. Microarray analysis using the *A. thaliana* Functional Genomic Facility (AFGC) identified a large number of genes up-regulated by H_2O_2, some of them encoding for antioxidant enzymes, defense and stress-related proteins (Desikan *et al.* 2001).

Thus, the results presented above indicate that cyanide acts as a signal molecule triggering many pathways, which in consequence lead to alleviation of seed dormancy and stimulation of germination. These pathways do not operate independently, but are rather linked together in a complex web of interactions. A better knowledge of these events (some elements of cross-talk signal transductions) is needed for a full understanding of cyanide action in these crucial processes.

Possible modes of cyanide action as signaling molecule

It is demonstrated in the previous sections that HCN is involved in the control of different metabolic, physiological and developmental processes in plants. A number of them consist in direct or indirect effects upon enzyme activities, as it has been shown earlier. Other modes of HCN action imply changes in specific gene expression. First, it was reported that cyanide treatment of carrot roots induced several new not yet identified translation products (Tucker and Laties 1984). More recently the attention was paid to the *AOX* gene, and to the ACS gene (*ACS6*), whose expression was induced by cyanide (Chivasa and Carr 1998, Smith and Arteca 2000).

There is still no direct experimental data concerning the receptor(s) of cyanide in the systems leading to selective protein synthesis, as well as some elements of signal transduction that lead to this induction. On the other hand the observations that ethylene and cyanide elicit some similar physiological responses, show some similar physical and chemical properties, including the ability to permeate membranes as a gas and to bind to certain metalloproteins (Smith and Arteca 2000), and both compounds are produced on the same pathway, allows to speculate that both molecules share certain common steps in the control of protein synthesis. The observation that some mutants of *A. thaliana*, which exhibit reduced response to ethylene, also show a lowered response to cyanide treatments (Smith and Arteca 2000), may supports this assumption. However, there is no experimental data indicating that cyanide shares the same receptors as ethylene. The known membrane-bound receptors of ethylene (Chen *et al.* 2005) and the next downstream component identified in the ethylene signaling pathway CTR1 - Ser/Thr kinase (Huang *et al.* 2003), that might suggest the involvement of a

MAP-kinase-like signaling cascade, seems not to operate during HCN induced protein synthesis.

There is, however some evidence that HCN-induced gene expression might be, at least partly, shared with other signal transduction pathways. Calcium mobilization and reversible protein phosphorylation cascade are ubiquitous components of eukaryotic signaling pathways and are both required for the controlled generation of ROS, especially H_2O_2 (Neill *et al.* 2002). We have mentioned earlier that H_2O_2 generation has been observed in cyanide treated dormant apple embryos (Bogatek *et al.* 2003).

According to the suggestion of Ordog *et al.* (2002), cyanide-induced AOX in some plants infected with viruses, may play an active role in HR cell death, and ROS seems to act primarily as signal molecule in this form of programmed cell death (PCD) (Overmeyer *et al.* 2003). Keeping all reservations, analogies with previously characterized animal signaling pathways can help to direct initial investigation into plant processes (see Hippeli *et al.* 1999, Neill *et al.* 2003). Thus, elevation of cytosolic Ca^{2+}, which is due to both influx of extracellular Ca^{2+}, and mobilization of intracellular calcium stores, plays an important role in cyanide-induced apoptosis in cortical neurons (Shou *et al.* 2000, Mathangi and Namasivayam 2004). These results (Shou *et al.* 2000) and others (Li *et al.* 2002) also indicated that ROS was an early signal in cyanide-induced apoptosis, and that a nuclear redox sensitive transcription factor κB (NF-κB), was implicated in this process. Interestingly, there are also some indications for the existence of a similar transcription factor NF-κB in plant cells (Vranova *et al.* 2002). Moreover, a sequence similar to the NF-κB recognition site was identified in the promoters of several plant defense genes (Desikan e*t al.* 2001, Vranova *et al.* 2002). Delocalisation of a redox-sensitive factor(s) in plants, from cytoplasm to nucleus upon cyanide treatment would be the culminating event, that transfer signal to the nucleus, resulting in gene expression. Is it possible that some of them could be expressed in response to cyanide treatment?

It would be difficult to imagine that cyanide acts as the only signal alone responsible for the orchestration of the diverse responses described above. Multiple interactions with other signaling molecules, such as nitric oxide (NO), H_2O_2 and ethylene, in induction and control of different cross-liked pathways is a more realistic scenario. Many of these molecules appear to be produced in some processes regulated by cyanide (Smith and Arteca 2000, Bogatek *et al*. 2004, Dobrzyńska *et al.* 2005, Bogatek and Gniazdowska 2006).

Calcium and MAPK cascades involved in signaling and up-regulation of redox-sensitive transcription factors seem to be common for cyanide, NO, H_2O_2, and also for hormone ethylene. Moreover, cyanide (Chivasa and Carr 1998), H_2O_2 (Moore *et al.* 2002), NO (Zottini *et al.* 2002) and ethylene (Simons *et al.*1999) are known to induce the expression of *AOX* gene. Earlier, the interaction of cyanide with other hormones such as gibberellins (Dziewanowska and Lewak 1982, Zagórski and Lewak 1985), and more recently with ABA (Bogatek *et al.* 2003), in regulation of seed germination was noted.

Examples of cyanide actions in plants discussed in the preceding sections allow to state that the concentration of HCN plays a role of determining factor for these responses. Thus, the question arises, how cyanide can be both recognized as a signal and transduced in the target cell, if it induces different responses (signaling or toxic). Since HCN, similar to NO, is a simple, small and diffusible molecule, it is highly improbable that its transduction involves specific receptors (Neill *et al.* 2003). Taking into account our earlier considerations that cyanide induced signal transduction events, one may suppose that cyanide, depending on its concentration, causes a differential disturbance of the cell redox homeostasis, and thus up-regulates different redox sensitive transcription factors, which in turn activate different genes.

Recent data concerning some plants subjected to osmotic stress indicate that different receptors and stress-induced signal transduction pathways may be activated in cells responding to mild or severe stressors (Munnik and Meijer 2001). These problems has been recently discussed in details and reviewed by Kacperska (2004). There appears to be two groups of membrane-dependent stress-sensing

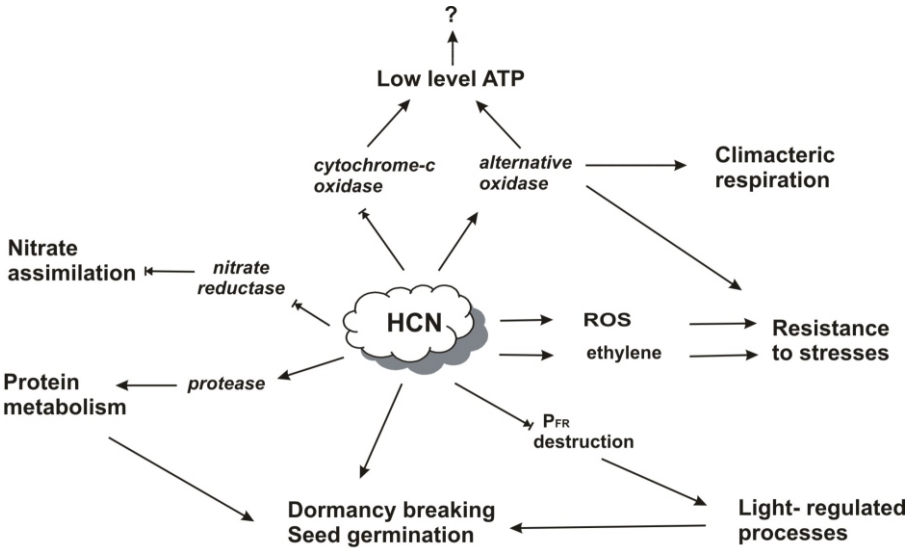

Fig. 5. Possible routes of signaling action of cyanide in plants.

systems in plant cells: redox/H_2O_2-dependent systems, and those dependent on perturbation in cell wall-plasma membrane interaction (Kacperska 2004). Is it possible to explain the mode of cyanide action based on this model? Genetic analysis and further physiological studies will improve our understanding how cyanide is perceived and transduced into specific downstream responses.

Conclusions and future developments

Data presented in this paper indicate that the studies of HCN mode of action have become a new exciting field in plant biology. The question about its toxic or protective role has been stated previously. The regulatory (signaling) function in plant metabolism is now underlined. It seems that cyanide possesses some properties characteristic for signaling molecules: **a)** is produced quickly and efficiently on demand; **b)** induces specific responses within the cell at low concentrations, sometimes as low as 1 M; **c)** and can be metabolized (removed) rapidly subsequently to signaling events. It is worth to note that cyanide as a gaseous molecule may easily diffuse not only through cyanogenic plant tissues, but also may be released into the surrounding atmosphere, similarly to ethylene, methyl jasmonate and fungal or bacterial substances. Therefore, it acts not only as deterrent for herbivores, but it may also affect the growth and metabolism of neighbouring plants. HCN and cyanogenic glycosides have been recently identified as allelopathy compounds (Tretyn 2002, Inderjit and Duke 2003).

Although there are an ever-increasing number of HCN responses recognized in plants, many questions remain to be answered. There are no data concerning specific receptor(s) of HCN in cells. We still know relatively little about the signal transduction processes, involved in HCN regulation of many processes within the plant cell. It is known for animal cells that cyanide mobilizes Ca^{2+} from intracellular stores, which alters plasma membrane function *via* the activation of Ca^{2+}-sensitive K^+ channels (Latha *et al.* 1994). Do similar mechanisms exist in plants? Genetic analysis in addition to physiological studies will be required to support the existence of other elements, detected in mammalian cells.

Little is also known about cyanide interaction with other signaling molecules, as ethylene, gibberellins or H_2O_2. NO has in recent years emerged as a key signaling molecule in plants (*e.g.* Wojtaszek 2000, Neill *et al.* 2003), and similarly to H_2O_2, it is known to be a part of an integrated network of cell signaling. Cyanide as well as nitric oxide and ethylene, are small gaseous molecules, with similar chemical properties and simple structure. More-

over, cyanide seems to be involved in regulation of many processes, which are controlled by NO and ethylene.

The signaling role of the HCN in plant metabolism is proposed in the schematic diagram (Fig. 5). We may expect, however, that further research will prove the significant regulatory role of cyanide in such processes as growth, flowering or senescence of plants.

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