Hemoglobin (hb) gene expression in maize seedling roots under low oxygen tensions

By

R. Ileana Silva-Cardenas

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A mis padres

Porque les debo lo que fui...
lo que soy...
y lo que llegare a ser...

ABSTRACT

Anaerobic conditions dramatically modify the pattern of proteins expressed by maize roots (Sachs et al, 1980). Previous experiments showed the upregulation of *hb* message in the roots of flooded maize and barley (Taylor et al, 1994). *Adh* gene is one of the best characterized anaerobic responsive genes. Under oxygen deprivation the gene is strongly induced at both the transcriptional and the translational levels, furthermore the enzymatic activity increases dramatically as well (Dennis et al, 1984).

In order to determine the pattern of expression of *hb* gene in anaerobic maize roots, two experimental systems were tested, a hydroponic and a flooded system. The hydroponic system could not discern between the treatments applied to the plants and the conditions of the system itself. On the other hand, the flooded system revealed that *hb* gene is expressed transiently during the first hours of anaerobiosis. Expression reaches its peak at 2 hrs and then declines. Under the same conditions, the pattern of expression for *adh* gene is also time dependent. The gene is strongly induced after 40 min of flooding and peaks at 12 hrs, then slowly decreases during the following 48 hrs.

Oxygen transported via developing aerenchyma does not seem to have an effect on the pattern of expression of both *hb* and *adh* genes.

Previously, Thompson and Greenway (1991) reported that under anaerobic conditions, maize roots have an anaerobic stele and an aerobic cortex. Bogusz et al (1990) found the expression of reporter genes to be directed by the *Parasponia* promoter in the tip and vascular cylinder of tobacco. Our results show that *hb* and *adh* genes are expressed preferentially in

the stele of flooded maize roots. mRNA production of *hb* and *adh* is 2 and 5 times greater respectively, in the flooded stele than in the cortex.

In terms of the protein, we found Hb to be present in the roots of both flooded and unflooded maize roots. The protein level shows a slight increase after 12 hrs, even though it does not correlate to the levels of the message. The implications of these findings are discussed.

LIST OF ABBREVIATIONS

adh	Alcohol dehydrogenase gene
Adh	Alcohol dehydrogenase protein
ANP	Anaerobic proteins
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
°C	Degrees Celsius
cashb-sym	*Casuarina cunninghamiana* symbotic hemoglobin gene
cashb-nonsym	*Casuarina cunninghamiana* non-symbiotic gene
CAT	Chlorampehicol acetyl aminotransferase
cDNA	Complimentary deoxyribonucleic acid strand
CO	Carbon monoxide
CO_2	Carbon dioxide
cpm	Counts per minute
cv	Cultivar
Da	Daltons
DEPC	Diethyl pyrocarbonate
DNA	Deoxiribonucleic acid
DTT	Dithiotreitol
EDTA	Ethylene-diamine tetraacetic acid
$FADH_2$	Flavin adenine nucleotide (reduced form)
glb	*Sesbania rostrata* leghemoglobin gene
GUS	*E. coli* β-glucuronidase
hb	Hemoglobin gene
Hb	Hemoglobin protein
HCl	Hydrochloric acid
H_2S	Hydrogen sulfide
IgG	Immunoglobulin
kDa	Kilodaltons
$K_m[O_2]$	Michaelis Menten constant for O_2
lb	Leghemoglobin gene
Lb	Leghemoglobin protein
LDH	Lactate dehydrogenase
LiCl	Lithium Chloride
M	Molar
Mb	Myoglobin protein
$MgCl_2$	Magnesium chloride
mL	Milliliters
mM	Millimolar
µM	Micromolar
mRNA	Messenger ribonucleic acid
MW	Molecular weight

NAD+	Nicotinamide adenine dinucleotide (oxydized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
nm	Nanometers
nM	Nanomolar
N_2	Molecular nitrogen
NaCl	Sodium chloride
NO	Nitric oxide
O_2	Molecular oxygen
OD	Optical density
pdc	Pyruvate decarboxylase gene
PDC	Pyruvate decarboxylase protein
[α-^{32}P]dCTP	Deoxyribonucleoside triphosphate
PMSF	Phenylmethylsulphonyfluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TCA	Tricarboxylic acid cycle
TEN	Tris-EDTA Sodium chloride buffer
UV	Ultraviolet light
XET	Xyloglucan endotransglycosylase

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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INTRODUCTION

Plants are exposed to various environmental and biological stresses during their life. The adverse conditions at which they are exposed can interfere with their normal growth and development.

As aerobic organisms, plants require oxygen for their growth and survival. Oxygen deprivation is one of the major stresses plants have to overcome during waterlogging. Every year, millions of acres of crop plants are inundated with water causing very important economic losses.

For the last 30 years, several research groups have been investigating the mechanisms involved in the response of plants to anaerobic stress. The primary model in their studies into flood injury have been the roots, since they are the main organ having to endure oxygen shortage.

Research on plant responses to anoxia has been focused on several different areas. Changes at the molecular, biochemical, physiological, and anatomical levels have all been investigated (Kennedy et al, 1992; Perata et Alpi, 1993, Ricard et al, 1994; Sachs et al, 1996).

Anaerobiosis results in the redirection of the gene expression in plants leading to the accumulation of a new set of proteins. Regulation at both the transcriptional and the translational levels is involved in the anaerobic response (Sachs et al, 1996).

One of the proteins that has been found to redirect its expression under anaerobic conditions is Hb (Taylor et al, 1994), however, its function as well as the mechanisms involved in its regulation have not been elucidated yet.

The research documented in this thesis was intended to determine the pattern of expression of the hemoglobin gene in maize roots under anaerobic conditions. Knowledge gained through this experiments might bring us closer to the understanding of hemoglobin role in the anaerobic response.

LITERATURE REVIEW

Most living organisms depend on the presence of oxygen for their regulatory processes. Oxygen is readily available for organisms living in open air. However, for the organisms that live in water or in soil (eg. plant roots) the supply of oxygen may sometimes be inadequate or insufficient.

The vast majority of agricultural crops are mesophytes requiring an environment that is neither too wet nor too dry to reach their highest growth and productivity. When soil becomes saturated with water (waterlogged), air and particularly oxygen is displaced with water. Depletion of the oxygen remaining in the soil, either dissolved in water or trapped in air cavities, occurs rapidly by respiration of plant roots and soil microorganisms (Kawase, 1981). Oxygen replenishment in the soil is very inefficient because of the slow diffusion of atmospheric oxygen into the waterlogged soil (AIR=0.214 cm^2s^{-1}, H_20=2.67 x 10^{-5} cm^2s^{-1} ~10,000 fold slower in water than in air) and also because of its low solubility in water (Armstrong, 1979; Ricard et al, 1994).

The anaerobic conditions resulting as a consequence of the interruption of the free exchange of gas between the soil and the atmosphere lead to changes in the chemical and biological properties of the soil, that adversely affect the growth of the plant. Examples of these changes are, a reduction in the soil redox potential, changes of the soil pH, decreased levels of oxygen and mineral nitrogen, increased levels of CO_2 and ethylene found in the soil (Atwell et al, 1985). Microbial respiration renders an

accumulation of fatty acids and phenolic compounds (Drew, 1979; Barclay and Crawford, 1982). Changes also occur in the activity and composition of the microflora (Atwell et al, 1985).

Waterlogging places root systems into anaerobic conditions. Oxygen deficiency causes plants to switch their metabolism from aerobic to anaerobic, inevitably resulting in much lower yield of ATP, accumulation of toxic end products of anaerobic respiration, and rapid depletion of organic substrates. Absorption and translocation of water and nutrient in roots are slowed by the limited available energy.

Characteristic symptoms of damage to vegetative growth by waterlogging are: leaf chlorosis, leaf epinasty, leaf abscission, decreased stem growth rate, stem hypertrophy, aerenchyma formation, wilting of leaves and shoots, absence of fruits or decreased yields, adventitious root formation, decreased root growth, death of roots, increased susceptibility to attack by predators and pathogens, and finally death of plants (Kawase, 1981).

Adaptations to waterlogging

The first and major consequence of waterlogging is a reduction in the availability of O_2 to the plant root system. Therefore, any mechanism than can improve the supply of O_2 to the roots and the rhizosphere will be beneficial and increase the chances of survival for plants which must endure waterlogging conditions. Adaptation to waterlogging in hydrophytes is centered mainly in their unique O_2 translocation system

from the above ground portion to the root system (aerenchyma) in the newly formed adventitious roots, an increase in branching of the roots and superficial rooting (Drew et al, 1979; Laan et al, 1989). Anatomical and morphological adaptations are relevant due to their capability in taking up O_2 into the aerial tissues of the plant, translocating and providing it to the root system, and diffusing some of it into the rhizosphere (de Wit, 1979; Justin and Armstrong, 1987).

Plant adaptations to waterlogging are not limited to anatomy and morphology but include also biochemical or metabolic adaptations (securing energy through anaerobic respiration and detoxification of the end products of anaerobic respiration) and physiological adaptations that slow the growth rate of the shoots and redistribute inorganic nutrients until aeration is restored (Drew, 1979; Barclay and Crawford, 1982).

I. Root Morphology.

1. Adventitious roots and aerenchyma development.

When plants are flooded for prolonged periods, adventitious roots (with cortical air spaces denominated aerenchyma) will emerge from the base of the stem and grow into the anaerobic soil or solution. These roots, developed after flooding, are more porous than the primary roots and under persistent stress conditions, constitute the larger part of the root system. The development of aerenchyma has a dual role: first of all, the air spaces may improve the oxygen status of the newly formed roots by reducing the amount of respiring tissue relative to the root volume and, by providing a pathway of low resistance to the internal diffusion of oxygen from the shoot (Williams and Barber, 1961; Armstrong, 1979; Drew, 1979; Mendelssohn and Postek, 1982).

Armstrong (1979) reported that the ability of rice to grow in flooded soils derives from the presence of aerenchyma allowing the efficient transport of air from leaves to the submerged parts of the plant. He also found significant differences in O_2 diffusion in roots of various cultivars of rice. Those cultivars that have lower rates of O_2 flux are susceptible to physiological diseases associated with reduced conditions in the soil. In addition to these findings, Perata and Alpi (1993) showed that the efficiency of oxygen transport is 10 times higher in rice than it is in barley and 4 times that in maize. On the other hand, de Wit (1979) reported the ability of maize roots to maintain equal growth rates in non aerated and aerated media, whereas barley roots are unable to maintain their normal growth rate. In non aerated medium, these results are explained by the difference in the percentage of air spaces. In maize, this percentage accounts for as much as ~30-45% of the root, while in barley corresponds to less than 10 %. Barber et al (1962) showed that waterlogging tolerant species have a better air translocation system from the aerial parts to the root systems than non tolerant species and also noted that oxygen diffusion from the shoots is faster in waterlogging-tolerant rice than in barley.

The main anatomical and morphological changes involved in the development of aerenchyma in adventitious roots include fusion of xylem vessels, deformation of endodermis and parenchyma cells and the formation of intercellular cavities. All these modifications occur in the maturation and elongation zones but not in the meristematic zone and start to be evident after 6-12 hrs of oxygen deprivation (Webb and Jackson, 1986). Extreme treatments where the shoots are also subjected to flooding, cause profound similar modifications of the shoots as well (Grineva et al, 1988; McPherson, 1939; Justin and Armstrong, 1987; Sachs et al 1996).

Factors promoting the development of adventitious roots under anaerobic conditions are poorly understood, but there is evidence indicating that formation of aerenchyma is promoted by the accumulation of endogenous ethylene in flooded roots. In flooded conditions, the presence of stationary water layers around the submerged organs retards ethylene losses, the diffusion coefficient in water is 10^4 times slower than in air. (Drew et al, 1979; Kawase, 1979; Konings, 1982; Justin and Armstrong, 1991; Sachs et al, 1996). Formation of aerenchyma can also be triggered by nitrogen or phosphate starvation in well aerated plants (He et al, 1992). The formation of aerenchyma has also been found associated with increases in the activity of cellulase, which presumably contributes to wall fission and cell lysis (Drew et al, 1979; He et al, 1992). Additional cell wall degradation enzymes however, are likely to be involved in the process. Very recently, a cell wall loosening and degradation enzyme gene (XET= xyloglucan endotransglycosylase) was found to be induced during oxygen deprivation (Saab and Sachs, 1995). XET is a putative cell wall loosening and degradation enzyme that is proposed to play a role in wall metabolism during germination (Fanutti et al, 1993) cell expansion and fruit ripening (Fry et al, 1992; Redgwell and Fry, 1993). The enzyme is supposed to have a role in the cutting and rejoining process of the xyloglucans that cross link adjacent cellulose microfibrils, being one of the first steps on aerenchyma development (Fry, 1989).

2. Meristematic Region.

2.1. Morphology.

There are 4 major zones of development in roots, the cap, the apical meristem, the cell elongation zone and the maturation zone. The root cap protects the apical meristem and has a role in aiding the root during its penetration of the soil by secreting a highly hydrated polysaccharide (mucigel) which lubricates the root during its extension through the soil. In addition to its protective role, the root cap plays an important role in controlling the response of the root to gravity.

The apical meristem consists of small, many sided cells, and corresponds to the area of the root where cell division takes place, leading to the eventual development of the epidermis, cortex, endodermis, pericycle, phloem and xylem. Behind the region of cell division, but not sharply delimited from it, is the elongation zone, that accounts for the lengthening of the root. The region of elongation is followed by the maturation zone, in which most of the cells of the primary tissues mature. Root hairs are also produced in this area (Raven et al, 1992).

As the delimitation of the zones of the root cannot be defined precisely, Ishikawa and Evans, (1995) proposed a new terminology for the transitional region between the division and the elongation zones, where cell division and elongation simultaneously take place. They proposed the denomination of such a region as the distal elongation zone (DEZ) indicating that the cells are near the distal end of the elongation zone and categorizing them, not in terms of mitotic activity, shape or coefficient of expansion but by their physiological properties such as responses to gravity, electrotropic or thigmostimulation, mechanical impedance, water stress or auxin presence. The distal

elongation zone together with the apical meristem and the elongation zone, constitute the root tip and expand for approximately 2 mm in maize roots.

2.2. Acclimation.

Most dry-land plant species do not display a high degree of tolerance to anoxia, maize roots remain viable for only 15 to 24 hr when the environment is strictly anaerobic (Saglio et al, 1988). Maize roots tips have been used as a model to investigate plant response to oxygen shortage in conditions of either anoxia (complete lack of oxygen) or hypoxia (less severe oxygen deficiency) within the tissues (Thompson and Greenway, 1991; Andrews et al, 1994; Fennoy and Bailey-Serres, 1995 and Sachs et al, 1996).

Anoxia tolerance of maize root tips can be greatly increased by exposing the roots tips to a hypoxic pretreatment prior to imposition of anoxia (Xia and Saglio, 1992). One of the most important acclimation consequences is the increase in viability. Non pretreated roots die after 24 hrs of anoxic treatment while roots pretreated under hypoxic conditions remain viable after 24 hrs of anoxia (Saglio et al 1988; Johnson et al, 1989). These roots show, however, a net decline of vigor at 72 hrs. Acclimated roots remain viable and retain turgidity during anoxia, while non acclimated roots lose viability and become flaccid.

Root tips of non-hypoxically pretreated plants, rapidly lose ATP and experience a decrease in their adenine nucleotide pool during the initial hours of anoxia. In contrast, pretreated roots maintain concentrations of ATP and adenylates, close to those of control roots (Johnson et al, 1989).

One of the factors responsible for cell death under anaerobic conditions is the acidification of the cytoplasm. Hypoxically pretreated roots are capable of excreting most of the lactate synthesized under anoxia, in contrast to non-acclimated roots, in which, lactate is retained intracellularly (Xia and Roberts, 1994). In addition to the capacity of acclimated roots to excrete lactic acid into the medium, they also show a lower production of it, rendering higher cytosolic pH values than in non-acclimated roots (Xia and Saglio, 1992). Estimations of cytoplasmic pH indicate that acclimated roots maintain a cytoplasmic pH approximately 0.4-0.5 pH unit less acidic than non acclimated tips (Xia and Roberts, 1994, 1996). The suggestion that the reduction in cytoplasmic acidosis is a potential factor in anoxia tolerance in plants, is borne out on studies of anoxia tolerant plants, compared to anoxia intolerant plants. Anoxia tolerant species, such as *Echinochloa* sp and rice, undergo little or no cytoplasmic acidosis during anoxic conditions (Johnson et al, 1989; Kennedy et al, 1992; Xia and Roberts, 1994, 1996).

Acclimation to anoxia is centered in 2 events: the pH regulation and an increase of the amount of glycolytic key enzymes such as hexokinase and glucokinase to sustain the supply of ATP (Bouny and Saglio, 1996).

Among the metabolic changes associated with the maintenance of energy production during anaerobic conditions, the induction of ADH is probably one of the most important factors. ADH activity is strongly induced in hypoxically pretreated maize roots during the first 24 hrs of anoxia and remains high for up to 96 hrs, but not in non acclimated roots (Johnson et al, 1989).

3. Stele and Cortex.

Morphologically, the structure of primary roots is relatively simple. Along the root, the arrangement of the primary tissues shows very little difference from one level to the next one.

A general arrangement of the tissues in the primary roots of maize shows two major layers, the cortex and the stele. The stele constitutes the inner part of the root and contains the vascular cylinder and the pericycle. The vascular cylinder consists of a variable number of xylem elements, alternating with thin walled phloem cells. Maize roots have a parenchymatous pith surrounded by the xylem and phloem. To the periphery of the vascular cylinder lies the pericycle, a single cell layer of parenchymatous cells, whose function is to give rise to secondary roots (Cutter, 1979).

Just outside the stele is a layer of cells called the endodermis. The endodermis plays an important role in water and ion movement based on the characteristic suberin thickenings of its anticlinal cell walls (Casparian strips). The endodermis is also the physical separation between the stele and the cortex (Raven et al, 1992).

The cortical layer is located outside of the endodermis which, on most roots, is parenchymatous. During development, the size of the differentiating cortical cells increases considerably and in monocotyledonous species, cells develop a secondary wall that becomes lignified. Regardless of the degree of differentiation, cortical tissue contains numerous intercellular spaces essential for the aeration of the root (Cutter, 1979; Raven et al, 1992).

Differences between stele and cortex are not solely based on morphological but also metabolic traits. Oxygen diffusion across the root is determined by the

morphology of the tissue along with its metabolic requirements. Across the cortical region, oxygen moves gradually due to the high porosity and low oxygen demand of the tissue, as well as, its closeness to the oxygen source. On the opposite, in the stele, oxygen gradients are steep due to the low porosity of the tissue itself, the higher respiratory demand per unit of volume and the greater distance of the stele from the oxygen source (Thompson and Greenway, 1991).

Thompson and Greenway (1991) demonstrated that under low oxygen supply, maize roots develop an anoxic stele while the cortex remains aerobic. Metabolic evidence to support the existence of an anoxic stele and aerobic cortex included a dramatic increase of over 40-50% in the activity of ADH and PDC in the stele but not in the cortex of low oxygen treated maize roots. Alanine concentrations in the stele increased over 3 fold and ethanol increased 44% compared with aerated roots. And finally, when shoots of the plants were kept in air, PDC extracted from anaerobically treated roots was inactive in the cortex but active in the stele. However, when the oxygen supply from the shoots to the roots via the aerenchyma was blocked, PDC became active in the cortex as well. The results suggest that aerenchyma provides adequate oxygen supply for respiration to the roots of plants exposed to low oxygen environments.

II. Biochemical Adaptations

Plant tolerance to low oxygen availability differs considerably among tolerant and intolerant species, but despite these differences, the common fact is the production of energy by alternative mechanisms.

1. Respiration overview

Glucose is a source of energy for nearly all organisms. The complete utilization of glucose involves 3 distinct stages: glycolysis, the Krebs cycle and the electron transport chain.

Glycolysis is a ubiquitous pathway that operates under both aerobic and anaerobic conditions. Glycolysis is the initial stage of glucose metabolism, in which each glucose molecule is converted to two molecules of pyruvate, in a series of 10 chemical reactions that occur in the cytosol and do not require the presence of molecular oxygen. If deprived of oxygen, for even short periods of time, cells increase the rate of glycolysis.

During hypoxia, mitochondrial respiration is highly affected, because without oxygen (the final electron acceptor), the NADH and $FADH_2$ reduced during glycolysis and the Krebs cycle cannot be reoxidized. The response then, is a switch to a fermentative metabolism by which the pyruvate is further metabolized to alanine, lactate or ethanol and CO_2 with the concomitant oxidation of NADH and the production of 2 ATP molecules per molecule of glucose metabolized (Perata and Alpi, 1993).

When cells do not have sufficient oxygen supply, there are some shifts in the pathways utilized, which are regulated by pH. NAD^+ is regenerated from NADH by the activity of LDH, reducing pyruvate to lactate. As the pH starts decreasing due to the

accumulation of lactate, LDH activity is inhibited, PDC activity is stimulated and ethanol synthesis predominates via ADH (Crawford, 1978; Hook and Crawford, 1978; Kennedy et al, 1992).

There are two other alternative pathways for NADH reoxidation when the oxygen supply is limited, the formation of succinate from oxaloacetate (Fan et al. 1988; Roberts et al, 1992) and the use of nitrate as an electron acceptor (Lambers, 1976; Garcia-Novo and Crawford,1973; Drew, 1979; Fan et al, 1988). The reduction of oxaloacetate to succinate generates NAD^+ from the NADH produced. In maize roots, the amounts of aspartate and malate degraded during anoxia are enough to account for the succinate accumulation after 90 min of anoxia (Roberts et al, 1992). However, in terms of ATP production, the contribution from this pathway is very small. In maize roots, the amount of succinate accumulated is equivalent to only a 2.5% and 1% of the amount of lactate (Fan et al, 1988) and ethanol (Roberts et al, 1992) respectively accumulated in the tissues after 8 hrs of anoxia.

The other possible pathway for the reoxidation of NADH in the absence of molecular oxygen involves the use of nitrate as an alternative electron acceptor (nitrate respiration) via the mitochondrial respiratory complex I (Garcia-Novo and Crawford, 1973; Fan et al, 1988). This hypothesis is based on the observations that maize and other cereals improve their survival to flooding if pretreated with nitrate (Fan et al, 1988), however, it is still not clear whether nitrate *per se* produces the effect or if the roots become hypoxic during the incubation in the nitrate solution, which may induce an acclimation to anoxia (Garcia-Novo and Crawford, 1973; Fan et al, 1988). In addition to the previous findings, nitrate reductase activity increases in leaves and roots of

waterlogging tolerant species when plants are flooded (Armstrong, 1979). The last evidence comes from the identification of a cytochrome d in the mitochondria of *Echinochloa phyllopogon* seedlings, suggesting that nitrate respiration could take place in this species (Drew, 1989; Ricard et al, 1994).

III. Protein metabolism

Anaerobic treatment has been shown to dramatically affect protein synthesis in maize seedlings. During the onset of anaerobiosis, there is an immediate repression of aerobic protein synthesis as a result of the almost complete dissociation and reassociation of polysomes (Bailey-Serres and Freeling, 1990) and the initiation of the synthesis of a new set of polypeptides with molecular weights around 33 kDa (transition polypeptides, TP's) (Sachs and Freeling, 1978; Sachs et al, 1980)

After 90 minutes of anaerobiosis, the TP's are no longer produced and the synthesis of approximately 20 anaerobically induced proteins (ANP's) commences that account for more than 70% of the total translated proteins after 5 hrs. The synthesis of the ANP's continues at approximately the same rate for up to 72 hrs, when seedlings start to die (Sachs et al, 1980; Okimoto et al, 1980; Sachs and Ho, 1986). Regulation of protein synthesis under anaerobiosis appears to occur at the levels of transcription and translation and several genes involved in this response has been isolated and characterized.

The vast majority of the ANP's identified are enzymes related to the glycolytic and fermentative metabolism (as a mechanism to enable the production of as much ATP as possible) and can be grouped in three categories: 1) enzymes mobilizing sucrose such

as sucrose synthase (Springer et al, 1986), 2) glycolytic enzymes such as glucose-6-phosphate isomerase (Kelley and Freeling, 1984), aldolase (Kelley and Tolan, 1986), enolase (Bailey-Serres et al, 1988), and glyceraldehyde-3-phosphate dehydrogenase (Russell and Sachs, 1989, 1992), and 3) enzymes involved in ethanol or lactate synthesis: alcohol dehydrogenase (Freeling, 1973) and pyruvate decarboxylase in maize, (Laszlo, 1981; Laszlo and St Lawrence, 1983; Kelley, 1989; Peschke and Sachs, 1993) as well as, lactate dehydrogenase in barley (Hoffman et al, 1986). In addition to these findings, the activity of 2 other proteins has been found to be induced by anoxia in barley roots and white spruce cell suspensions, alanine aminotransferase, proposing the role of alanine synthesis as an alternative route for NADH reoxidation (Good and Crosby, 1989, Ricard et al, 1994) and superoxide dismutase as a protective mechanism against oxygen toxicity upon return to aerobic conditions, until aerobic metabolism is switched back on (Monk et al, 1987).

Peschke and Sachs (1994) and Saab and Sachs (1995) reported the anaerobic induction of 3 genes that are not involved in glucose-phosphate metabolism in maize and seem to be related to the development of aerenchymatous tissue: wusl1005 (gfu), wusl1032 and umc217 (gfu) (Peschke and Sachs, 1994; Saab and Sachs, 1995).

Under aerobic conditions, the pattern of polypeptide synthesis in roots, coleoptiles, mesocotyl, endosperm, scutellum and anther wall is tissue specific. Under anaerobiosis, all organs exclusively synthesize ANP's, with the exception of leaves, that practically do not incorporate label under anaerobiosis and do not survive for long periods of stress (Okimoto et al, 1980).

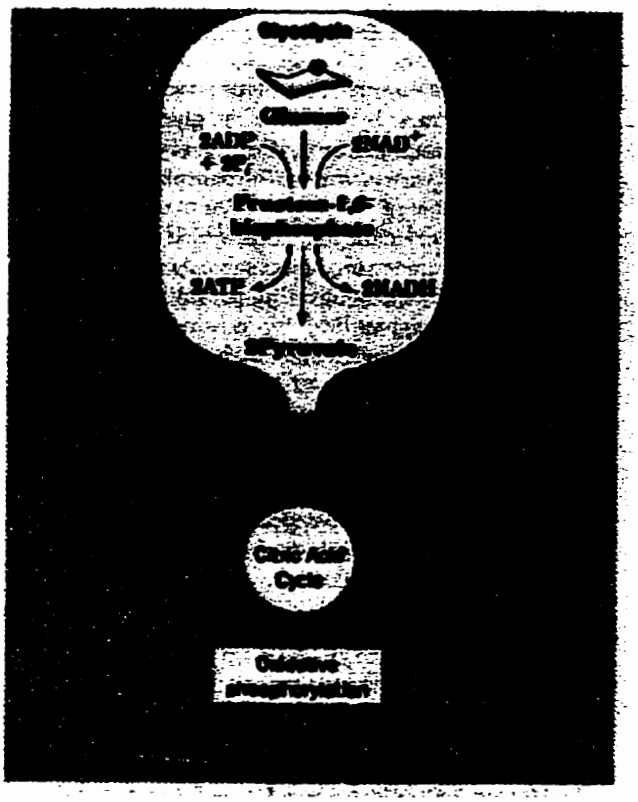


Fig 1. Schematic representation of the pathways of energy production operating under aerobic and anaerobic conditions. Under aerobic conditions carbohydrates are utilized through glycolysis and mitochondiral respiration while under anaerobiosis fermentation prevails with the concomitant production of lactate and ethanol. Enzymes involved in the fermentative metabolism are indicated.
(Taken from Voet and Voet, 1995).

1. Pyruvate decarboxylase

Pyruvate decarboxylase (PDC) catalyzes the conversion of pyruvate to acetaldehyde, functioning as the means of access from glycolysis to fermentation, rather than into the TCA, which is catalyzed by pyruvate dehydrogenase. PDC is thought to be active as an octamer of non-identical subunits with molecular weights of 61 and 62 kDa (Laszlo, 1981). Wignarajah and Greenway (1976) found an increase in PDC activity under anaerobic conditions. Later, Laszlo and St Lawrence (1983), reported the increase of PDC activity associated to *de novo* synthesis of the protein and therefore, identified PDC as another member of the anaerobic peptides of maize.

Under aerobic conditions, PDC is inactive. With the onset of anaerobiosis, glycolysis leads to an accumulation of lactate with a corresponding fall in pH. Lactate dehydrogenase (LDH) has its peak of activity at higher pH than PDC, so, as lactate is being produced, LDH becomes inactive and PDC activity increases considerably (Davis et al, 1974; Roberts et al, 1984, Morell et al, 1990, Peschke and Sachs, 1993).

In maize roots exposed to anaerobic conditions, there is a parallel increase on the activities of PDC and ADH, this increase is dependent on the time of exposure to the stress. The onset of activity for both enzymes is between 8 and 12 hrs of exposure to anoxia. Activity increases linearly for up to 48 hrs. The pattern of activity has been observed among cultivars as well as in other tissues such as the mesocotyl and the plumule, with the same pattern and only slight variations in the magnitude of the response (Laszlo and St Lawrence, 1983).

In maize, PDC has a very long half life, even after returning to aerobic conditions (Laszlo, 1981). Freeling (1978) reported similar findings for ADH, he found that ADH

activity induced under anoxia, turned over slowly, both under anoxia and subsequent exposure to aerobic conditions.

PDC is encoded by a family of three genes, *pdc1*, located in chromosome 8L (Kelley, 1989), *pdc2*, in chromosome 8S and *pdc3* in chromosome 1S (Peschke and Sachs, 1993). The 3 genes are induced at the mRNA level under hypoxic conditions giving products of identical size, however, the kinetics of accumulation for the message for each one of the genes shows a special pattern (Peschke and Sachs, 1993).

2. Alcohol Dehydrogenase

Fermentation of sugars to ethanol is the primary mechanism of energy production in hypoxic roots. Ethanolic fermentation permits continuous production of ATP without cytoplasmic acidosis, in contrast to lactic fermentation. Reduction of acetaldehyde to ethanol is catalyzed by ADH, which in maize, is encoded by 2 unlinked loci, *adh1* and *adh2* (Shwartz, 1966; Freeling and Shwartz, 1971). *Adh1* is located at position 127 in the long arm of the chromosome 1 of maize (Shwartz, 1969; 1971). *Adh2* is located at position 46 on the short arm of chromosome 4 (Dlouhy, 1980). The two genes code for 2 ADH polypeptide products (ADH1 and ADH2 respectively) that dimerize to give a total of 3 isozymes (ADH1.ADH1; ADH1.ADH2; ADH2.ADH2). The ADH proteins are very similar among each other and have a molecular weight of 38 kDa (Sachs et al, 1985).

In maize seed tissue and roots, the ADH1.ADH1 homodimer is predominant and just traces of the heterodimer ADH1.ADH2 and the ADH2.ADH2 homodimer are present. However, under anaerobiosis, the expression and activity for both proteins is

enhanced (Dennis et al, 1984).

The ADH system of barley resembles that of maize with the difference being that ADH in barley is encoded by 3 genes (*adh1*, *adh2* and *adh3*), coding for 3 polypeptide products (ADH1, ADH2 and ADH3) that dimerize to give a total of 6 ADH isozymes. Under aerobic conditions, barley aleurone layers have a high titer for the ADH1 homodimer, however, the rest of the isozymes are only induced by oxygen deficit. Expression for the 3 genes is maximal in the absence of oxygen (Hanson and Jacobsen, 1984).

In maize seedlings, treated under aerobic conditions, *adh1* and *adh2* are expressed at very low levels, however expression of both genes is anaerobically induced. The kinetics of accumulation for both genes during anaerobiosis is very similar. The level of mRNA increases at 90 minutes after imposition of the stress and continues increasing until 5 hrs, at which point the highest level is reached. This level is maintained for up to 48 hrs for *adh1* but starts decreasing after 10 hrs for *adh2* (Dennis et al, 1984; Sachs et al, 1986).

ADH activity shows a time dependent increase in maize roots and other tissues during hypoxia because of an increase of the expression of its genes. ADH activity increases between 4 and 6 hrs after the exposure to anaerobiosis and keeps increasing linearly for up to 48 hrs (Laszlo and St Lawrence, 1983; Roberts et al, 1989). *Adh* gene transcription peaks after 5 hrs under anaerobic conditions. This does not correspond to the highest levels of ADH activity which occurs after 48 hrs, presumably because of the long half-life of the protein (Andrews et al, 1994).

IV. Hemoglobin (Hb)

Hemoglobin presence in both animal and plant kingdoms has been known for many years. In animal systems, this protein belongs to a very sophisticated oxygen delivery system that transports oxygen from the lungs, gills or skin to the capillaries where it is used in respiration (Rawn, 1989).

Mammalian Hb is a tetramer composed of α and β subunits. Each subunit of hemoglobin, has a non-covalently bound heme group. The heme cluster of Hb is a protoporphyrin IX bound to an iron atom. The protoporphyrin group consists of 4 pyrrole rings linked by methene bridges arranged in a heterocyclic ring system as 4 methyl, 2 propionate and 2 vinyl groups. The iron atom, in Hb, remains in the ferrous state of oxidation, whether or not it is oxygenated (Rawn, 1989; Mathews and van Holde, 1990).

The secondary structure of each of the subunits is arranged in 8 α helices (A, B, C, D, E, F, G, and H) connected among them by helical segments. The heme lays between helices E and H and is kept in place by histidine residues 6E and 7H.

The distinctive characteristic of hemoglobin is its reversible oxygen binding ability. The heme itself cannot bind oxygen reversibly, the modulation of the oxygen binding affinity of the heme is regulated by the globin part of the molecule by protecting it in a hydrophobic pocket. Hb binds oxygen in a cooperative fashion, that is, the binding of oxygen increases the affinity of Hb for binding more oxygen. Other molecules are bound by Hb as well, CO, NO and H_2S, although, the affinity of Hb for these molecules is much higher than that for oxygen (Rawn, 1989).

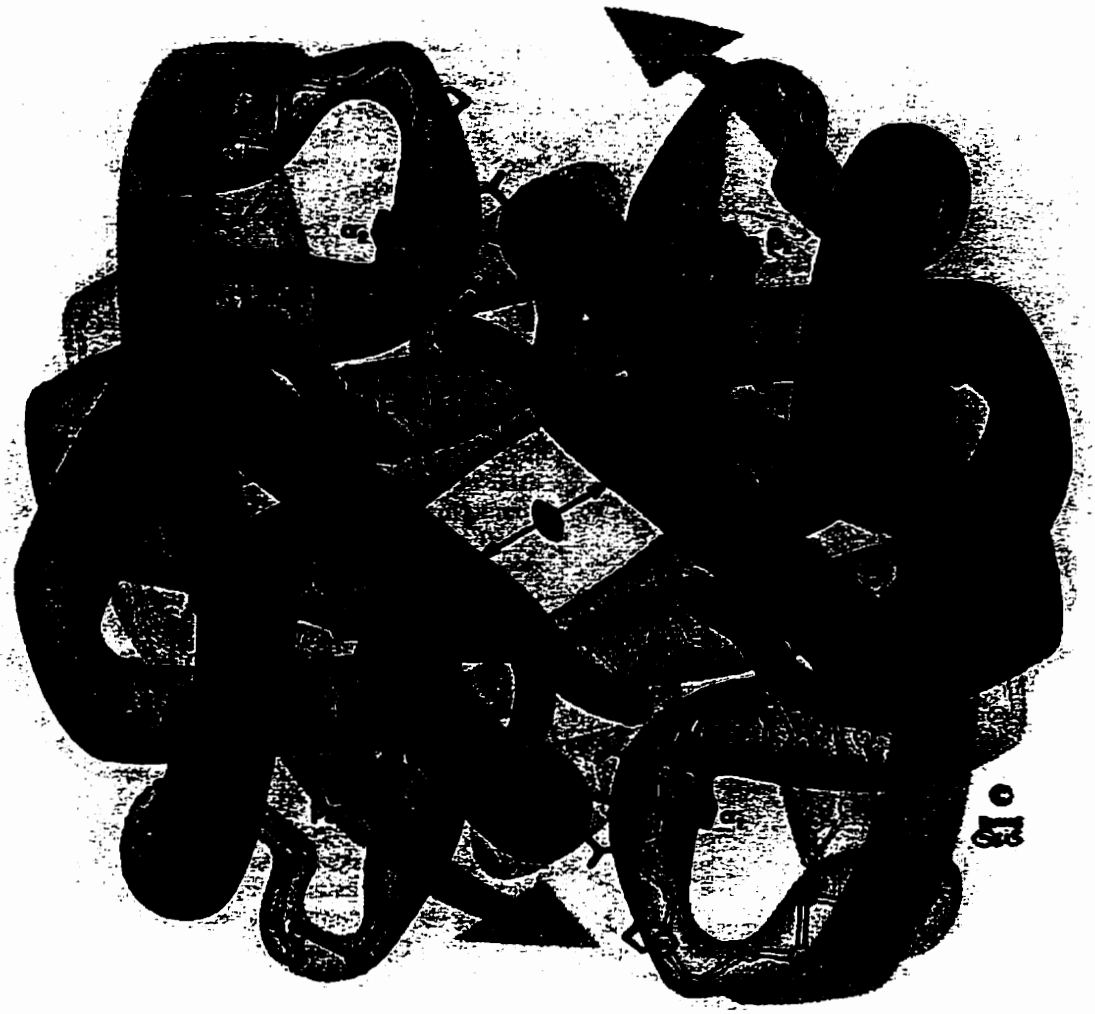


Figure 2. The structure of the hemoglobin tetramer in its oxidized form. The C atoms are numbered from each N-terminus, and the heme groups are shown. [Taken from Voet and Voet (1995)].

1. Hemoglobin presence in the plant kingdom.

In plants, hemoglobin was discovered in the late 1930's in nitrogen fixing legumes (Kubo, 1939) where it facilitates the diffusion of oxygen between the host plant and the bacteroid in an environment where low oxygen tension is needed to avoid the inactivation of the nitrogenase enzyme and at the same time, allow bacterial respiration (Kubo, 1939).

Hb presence in non-legume plants was not detected until 4 decades later, in the nodulating non-legume *Parasponia* where a single gene, coding for a dimeric protein, seems to have two mechanisms of expression, one for roots and another one for nodules (Appleby et al, 1983; Kortt et al, 1985; Landsmann et al, 1986). Later, Hb was also found in *Casuarina*, (an actinorhizal plant, establishing symbiosis with the actinomycete *Frankia*) where a leghemoglobin was found in nodules (Roberts et al, 1985; Kortt, 1988) and a non-leghemoglobin was found in aseptically grown roots (Kortt, 1988). Non-legume hemoglobin was then discovered in *Trema tomentosa*, a non-nodulating relative of *Parasponia* where the gene is transcribed and translated into a monomeric protein found exclusively in the roots (Bogusz et al, 1988). Very recently, non-legume Hb was also discovered in aleurone layers and roots of barley seedlings and plants as well as in roots of other cereals (Taylor et al, 1994) and in the roots of soybean (Andersson et al, 1996).

Analyses of the secondary structure of the Hb protein in plants reveal the presence of 7 α helices (A, B, C, E, F, G, and H), in comparison with the 8 helices present in animals. Helix H is a highly conserved region in the structure of plant hemoglobins, being the most conserved region a hydrophilic one. The most conserved

amino acid residues between plant and animal hemoglobins, are those located in the vicinity of the heme moiety and confer stability to the heme-oxygen complex. In all plant hemoglobins this region is constituted by 50 invariant amino acid residues (Appleby et al, 1988; Arredondo and Escamilla, 1991).

In terms of gene arrangement, all plant hemoglobins have 4 exons and 3 introns, the third intron being unique to plants. The position of two out of the three introns present in plants corresponds to the two introns located in animal hemoglobins. Analyses of animal hemoglobin sequences reveal that the third intron lays in a position predicted as a potential intron position, since it would separate two structural domains if it occurred in animal hemoglobins (Go, 1981; Appleby et al, 1988).

2. Leghemoglobin (Lb).

Leghemoglobin is present in the nitrogen fixing root nodules of legumes, where it is essential to provide an adequate supply of oxygen for the terminal oxidases of the symbiotic bacteria at low free oxygen concentrations (Appleby et al, 1988). The expression of leghemoglobins is induced at a late stage in the development of the nitrogen fixing nodules, just prior to the onset of nitrogen fixation (Appleby, 1984).

The ability to fix nitrogen by the bacteroids has been shown to be related to the presence of leghemoglobin. Experiments performed with *Rhizobium* mutants deficient in heme synthesis show that nitrogen fixation is inefficient in these strains (Appleby, 1984). Furthermore, there are no reports of legume nodules lacking Lb (Appleby, 1984; Roberts et al, 1985).

2.1. Leghemoglobin genes.

Leghemoglobin is present in numerous copies in the genome of legumes. Genes encoding for the diverse Lb components belong to a family of closely related genes, arranged in independent clusters within the different legume genomes (Appleby , 1984). Leghemoglobin genes contain 3 introns, two of which are located in identical positions to those present in animal hemoglobin introns. The third intron interrupts the central exon at the joining point of two structural units in both α and β globin chains (Go, 1981).

Leghemoglobins are encoded by 4 expressed loci (*lba, lbc1, lbc2* and *lbc3*) (Marker et al, 1984). Regulatory elements, presumably involved in nodule specific expression, have been identified in the sequences of leghemoglobins. These elements group *cis*-acting regions located within the first 1200 bp of the 5' upstream region of the genes, (Stougaard et al, 1986; Stougaard et al, 1987), as well as *trans*-acting factors, which interact with two independent sites in the *lbc3* promoter region (Marker et al, 1984; Jensen et al, 1988). Nodule specific expression of the leghemoglobin genes has been studied extensively in transgenic legumes. The results from these studies reveal the involvement of 2 promoter regions from the soybean *lbc3* gene, as well as the *Sesbania rostrata glb3* gene in the nodule specific expression of leghemoglobin (Metz et al, 1988; Stougaard et al, 1986, Szabados et al, 1990).

The elements identified in the leghemoglobin promoters are the OSE (organ specific element) in the *lbc3* promoter and NICE (nodule infected cell expression) in the *glb3* promoter. These elements contain two motifs, 5'-AAAGAT-3' and 5'-CTCTT-3' present in most of the nodulin promoters and located 6 bases apart from each other

(Sandal et al, 1987; Stougaard et al, 1987; Ramlov et al, 1993; Szcyglowski et al, 1994).

2.2. Leghemoglobin protein

Lb consists of 4 major subunits (a, c_1, c_2, c_3) and 4 minor elements (b, d_1, d_2, d_3) corresponding to the n-acetylation products of the major elements (Whittaker et al, 1981).

Leghemoglobin is present differentially in the host cell cytoplasm and the peribacteroid membranes of soybean. Appleby (1974), reported the concentration of Lb in the free space within the soybean peribacteroid membranes as being 200-500 µM in contrast to its concentration in the host cytoplasm (~ 3 mM). These findings reveal that only about 1/6 of the total Lb is present within the peribacteroid space. Furthermore, uninfected plant cells lack the presence of leghemoglobin (Bisseling et al, 1983; Verma and Long, 1983).

During the assembly of the protein, the protoheme moiety is synthesized by the *Rhizobium* symbiont and exported to the plant cytoplasm, where the Lb apoprotein is made and the assembly of the holoprotein takes place. The synthesis of Lb is supposed to occur as a sequence of events, starting with the heme synthesis by the bacteria, export of the heme to the host cytoplasm via the bacterial plasma membrane, periplasmic space, bacterial cell wall, peribacteroid space and peribacteroid membrane. Once the heme is in the cytoplasm, combination of protoheme and apoprotein takes place (Verma et al, 1979).

Lb protein posses various kinetic characteristics that allow it to provide oxygen supply to the bacteria present in the nitrogen fixing nodules of legumes at low free

oxygen levels. These features include a high oxygen affinity (48 nM), an extremely fast O_2 combination rate (116 x 10^6 $Msec^{-1}$), a fast turnover rate with values between 27-135 μMs^{-1} and a moderately slow O_2 dissociation rate constant (5.6 sec^{-1}) (Bisseling et al, 1980 Appleby et al, 1988; Gibson et al, 1989).

3. Non-Legume Hemoglobin.

3.1. In nodules.

The presence of hemoglobin in non-legume plants was not reported until 5 decades after it was found in legumes. It was not until the late nineteen eighties, when hemoglobin was found in non-leguminous plants. Firstly, it was found in the nodules of the non-legume but nitrogen fixing member of the Ulmaceae family *Parasponia andersonii* (Appleby et al, 1988). Subsequently in two of the members of the Actinorhizal family: *Casuarina cunninghamiana* and *Myrica gale L.*, establishing nitrogen-fixing symbiosis with actinomycetes from the genus *Frankia* (Appleby, 1984).

3.1.1. Non legume hemoglobin in *Casuarina* nodules.

Hb presence in non-legume plants was first reported by Davenport (1960), by spectroscopical analyses of whole nodule clusters of *Casuarina cunninghamiana*. He found a total heme concentration in *Casuarina* nodules equal to that in pea nodules. The absorption spectra for *Casuarina* nodules corresponds to the presence of a deoxygenated (562 nm) oxygenated (544 and 580 nm) and CO-bound (572 and 542 nm) hemoglobin.

Twenty years later, Tjepkema (1983) confirmed Davenport's findings and

reported the presence of a soluble Hb in *Casuarina* nodules. But it was not until 5 years later, that Hb protein was finally isolated from the nodules of the non-leguminous *Casuarina-Frankia* symbiosis. Kortt et al (1988) isolated a monomeric Hb protein that contains 151 amino acid residues, corresponding to a MW of approximately 17,856 Da. The secondary structure predicted for this Hb has a 75% content of α helix in the same regions as lupin and soybean hemoglobins.

Casuarina, like legumes, possesses more than one hemoglobin gene (*cashb-sym1 and cashb-sym2*) (Jacobsen-Lyon et al, 1995). The promoter of the *Casuarina* symbiotic hemoglobins contains nodulin motifs similar to those found in leghemoglobin genes. The 5'-AAAGAT-3' and 5'-CTCTT-3' motifs conferring nodule specific expression in legumes, are also present in similar positions in the *Casuarina* promoter, suggesting their involvement in resembled functions as those in legumes. Surrounding the nodulin boxes, there are AT rich regions, comparable to those present in legume promoters as well. In addition to these elements, *Casuarina* promoter has a second copy of the AAAGAT motif, located farther downstream in the promoter. Deletion analysis performed with the promoter of these genes revealed the involvement of other regions (located upstream of the promoter) in the expression of the gene as well (Jacobsen-Lyon et al, 1995).

Casuarina symbiotic genes are expressed at high levels exclusively in nodules and no expression has been detected in roots, leaves or stems (Jacobsen-Lyon et al, 1995).

3.1.2. Non-legume hemoglobin in *Parasponia* nodules.

In 1985, Kortt et al reported the presence of a Hb protein in the nitrogen fixing root nodules of *Parasponia andersonii*, a non-leguminous member of the Ulmaceae family establishing symbiosis with *Rhizobium* strains.

The hemoglobin gene from *Parasponia andersonii* was isolated for the first time by Landsmann et al (1986). *Parasponia* has a single *hb*, strongly expressed in the nodules induced by the CP283 *Rhizobium* strain and also expressed at very low levels in the roots of non-nodulated plants (Landsmann et al, 1986; Bogusz et al, 1988). *Parasponia hb* possesses 3 introns located at the same positions as in *lb*. Introns 2 and 3 are inserted between codons and intron 1 splits a codon after its second nucleotide as in leghemoglobins and vertebrates (Landsman et al, 1986).

Hb from *Parasponia* shows a 40% homology to that of soybean. The most conserved regions between the two genes, being those presumably involved in the regulation of the gene. Non coding regions seem to have diverged the most and as a consequence, there is no cross hybridization between the two genes, *Casuarina* and *Trema* genes though, do cross hybridize with the gene from *Parasponia* (Landsman et al, 1986).

Parasponia Hb protein is a dimer of two identical polypeptide chains comprising 155 amino acid residues, corresponding to a monomer MW of 18,044 Da, including the heme group. The results obtained from alignments of the amino acid sequence of *Parasponia* hemoglobin against other hemoglobins revealed a 54%, 49%, 43% and 17% sequence homology with *Casuarina, Lupinus perennis, Glycine max* and myoglobin sequences respectively. The amino acid sequence also shows the presence of 38 out of

the 50 invariant residues present on other plant hemoglobins, plus 7 conservative substitutions (Appleby et al, 1983; Kortt et al, 1985).

The secondary structure of *Parasponia* Hb indicates the presence of 67% α helix, 10% β sheet and 9% β turns. The helical regions predicted for *Parasponia* are in agreement with those predicted and known for leghemoglobins. Immunological tests show reciprocal cross reaction between *Casuarina* and *Parasponia* hemoglobins, but not with leghemoglobins (Kortt et al, 1985).

Comparisons of the kinetic properties of the hemoglobins from *Casuarina*, *Parasponia* and leghemoglobins are shown in Table 1. Oxygen ON rates (k' "on") for *Parasponia*, *Casuarina* and soybean are extremely fast whereas the OFF rates (k "off") are moderate. The oxygen affinity, as well as the equilibrium dissociation rate ($K' = k:k'$) constants for both O_2 and CO of *Parasponia* hemoglobin are similar to those of other plant hemoglobins (Wittenberg et al, 1986; Fleming et al, 1987; Gibson et al, 1989).

3.2. Non-legume hemoglobins in roots

Until the 1980's the presence of hemoglobin in organs other than the root nodules of the species capable of fixing nitrogen by the establishment of symbiotic associations with bacteria was unknown.

The presence of Hb in the roots of the non-legume nitrogen fixing *Parasponia* was first reported by Appleby (1984). Then, Appleby et al (1988), found a hemoglobin protein in root tissue of *Casuarina*, as well as in the roots of plants which are not involved in any nitrogen fixation symbiosis (*Trema tomentosa*) and in the roots of nodule forming plants, grown under sterile conditions (Bogusz et al, 1988). Finally,

Table 1. Kinetic and equilibrium constants for the reactions of Casuarina Hb with ligands compared with those of Parasponia Hb and Soybean Lb.

	Casuarina Hb	*Parasponia* Hb	Soybean Lb
Oxygen			
k' "on" ($M^{-1}s^{-1}$)	41 x 10^6	165 x 10^6	116 x 10^6
k "off" (s^{-1})	5.5	14.8	5.55
K' (=k:k') (nM)	135	89	48
p_{50} (torr)	0.074	0.049	0.026
Carbon monoxyde			
l' "on" ($M^{-1}s^{-1}$)	2.34 x 10^6	13.7 x 10^6	12.7 x 10^6
l "off" (s^{-1})	0.0027	0.0187	0.0078
L' (=l:l') (nM)	1.2	1.4	0.62
p_{50} (torr)	0.84 x 10^{-3}	1.0 x 10^{-3}	0.45 x 10^{-3}
M=[HbCO]pO_2/[Hb O_2]pCO	87	50	58

Taken from Fleming et al, 1987

Taylor et al (1994) discovered a *hb* gene in the aleurone layers and roots of barley seedlings and maize and rye plants.

3.2.1. Non-legume hemoglobin in *Casuarina* roots.

Casuarina glauca possesses various hemoglobin genes, two of them are expressed exclusively in the nitrogen fixing nodules established in the presence of the ascomycete *Frankia* (*Cashb-symb1* and *Cashb-sym2*) and another one expressed in root tissue (*Cashb-nonsym*) (Christensen et al, 1991).

Casuarina possesses only one functional root hemoglobin gene (*Cashb-nonsym*) which encodes a 159 amino acid protein, 81% and 80% homologous to the *Trema* and *Parasponia* hemoglobins respectively, whereas the homology with the *Cashb-symb1* gene and soybean is only 53% and 44% respectively. The protein contains the invariant residues region present in all plant hemoglobins (Christensen et al, 1991; Jacobsen-Lyon et al, 1995).

The pattern of expression of the non symbiotic gene from *Casuarina* shows that the gene is expressed in roots, leaves and stems and at very low levels in nodules, approximately 100 fold less than that of the symbiotic genes (Jacobsen-Lyon et al, 1995).

The molecular weight of the symbiotic and non-symbiotic hemoglobins present in *Casuarina* differs from one another. Suggesting that the two proteins are the product of two different genes, expressed in a tissue specific manner (Appleby, 1988).

3.2.2. Non-legume hemoglobin in *Parasponia* roots.

The *hb* gene in *Parasponia* has been found to be transcribed and translated in the nodules as well as in roots of non-nodulated plants. Bogusz et al (1988) found the expression of the *hb* at the mRNA level in both nodules and non-nodulated roots of *Parasponia*, even though, the level of mRNA present in roots was 1,000 fold less than that of nodules. At the protein level, nodule and root proteins are both dicers with the same molecular weight. These findings suggest that *Parasponia andersonii* possesses a single *hb* gene with two mechanisms of expression, or an alternative regulation of the gene, one for roots and another for nodules in order to fulfil the functions of each one of the hemoglobins (Landsman, 1986). The gene is expressed at high level in nodules, at low levels in root tissue and is not expressed in leaves.

3.2.3. Non-legume hemoglobin in *Trema tomentosa* roots.

Trema tomentosa is a non-nodulating plant relative to *Parasponia* and *Celtis australis*, members of the Ulmaceae family. *Trema* species do not possesses the ability of establishing nitrogen fixing nodulating symbiosis with neither the members of the *Rhizobium* nor the *Bradyrhizobium* species (Bogusz et al, 1988).

Hb presence in *Trema tomentosa*, was reported in 1988, when Bogusz et al, isolated a single *hb* from *Trema* roots.

Hb from *Trema* presents 4 exons and three introns in identical positions as those present in the hemoglobin genes from nodulating species, and shows a 90% nucleotide sequence homology with the *Parasponia hb* and an 80% similarity when the intron, the

non-translated leader and the 3'-untranslated sequences are compared. The gene is transcribed and translated exclusively in roots, rendering a 161 amino acid monomeric protein with a MW of 18 KDa and containing 30 out the 50 invariable amino acids present in all plant hemoglobins (Bogusz et al, 1988).

4. Transgenic plants and tissue specific expression of hemoglobin.

As an attempt to investigate the mechanisms involved in plant hemoglobin gene expression, Stougaard et al (1987) performed experiments with transgenic plants using the soybean leghemoglobin *lbc3* promoter. In their experiments the *lbc3* promoter was linked to a reporter gene and used to transform *Lotus corniculatus* by the *Agrobacterium*-mediated transformation system. Their results show the specific expression of *lbc3* in the central portion of the nodule of transgenic plants nodulated by *Rhizobium loti*. Despite these results, there was no expression of the reporter gene driven by the *lbc3* promoter found in any other plant tissue.

Further investigation on non-legume hemoglobin gene expression arose from studies performed in transgenic tobacco plants. Appleby et al (1988), transformed *Nicotiana tabacum* with the complete *Parasponia hb* gene and a chimeric gene containing the *Parasponia hb* promoter linked to a bacterial chloramphenicol acetyl transferase reporter gene (CAT). Both the complete and the chimeric gene were found to be expressed in roots of the transgenic tobacco plants (Appleby et al, 1988). Similar experiments performed by Bogusz et al (1990), used the promoters of *Parasponia* and *Trema* non-legume hemoglobins linked to a β-glucoronidase reporter gene to transform a legume species (*Lotus corniculatus*) and a non-legume non-nodulating *Nicotiana*

tabacum, using the *Agrobacterium*-mediated transformation system.

Both promoters directed the expression of the GUS gene in nodules of *Lotus* at very high levels. The expression was localized in the central region of the nodule, corresponding to the *Lotus* endogenous *lb*, as well as to the pattern observed by Stougaard et al in 1987 when transforming *Lotus* with the soybean *lbc3* promoter. Noticeable levels of the GUS gene were also found in the roots of transgenic *Lotus*.

On the other hand, similar experiments performed with tobacco revealed that the expression of the GUS reporter gene driven by both *(Parasponia* and *Trema)* promoters was preferentially found in the roots of transgenic tobacco, similar to the pattern observed for the endogenous *Trema* and *Parasponia* hemoglobins. Expression of both *P-GUS* and *T-GUS* promoters in tobacco was greatest in the meristematic region of the root tips. For *Parasponia*, GUS expression was also found in the vascular cylinder in the region 2-3 mm proximal to the root tip. *Trema* directed GUS expression was exclusively present in the root tips of the transgenic tobacco plants.

A more detailed study on the expression of the non-legume hemoglobins in nodules, revealed that the expression in the mature nodules was confined to the central core tissue of the nodule, corresponding to the area where the bacteroid containing cells are located, in accordance to the results obtained by Stougaard et al (1987) for the *lbc3* promoter. The time course of expression of the non-legume hb promoters was dependent upon size and developmental stage of the nodule. The highest expression being found in the largest nodules that were already pink due to the presence and accumulation of leghemoglobin (Bogusz et al, 1990).

All these findings led to the suggestion that legumes and non-legumes posses a

similar control of gene expression by means of the same regulatory mechanisms, therefore, they possess similar DNA sequence motifs in their promoters.

5. Hemoglobin origin and evolution

Hemoglobin was first discovered in legumes, and not detected in any other plants. The level of similarity between plant and animal hemoglobins is considerable. These factors led to the suggestion that hemoglobin gene came to the plant kingdom through a horizontal transfer from an animal. The later discovery of non-legume hemoglobin led to the suggestion that probably all plants posses a copy of the gene. Recent findings of hemoglobin in cereals, fill the major gap for the presence of hemoglobin in plant kingdom and give strong support to the hypothesis of a vertical evolution from a common ancestral gene by the process of gene duplication (Landsmann et al, 1986; Bogusz et al, 1988; Appleby, 1988; Taylor et al, 1994).

The fact that hemoglobin is widespread in diverse groups of plants, supports the idea that all plants posses at least one copy of the gene and that the protein has a function in normal roots. It is possible that the presence of hemoglobin in the nitrogen fixing nodules of legumes might have originated by the process of gene duplication, from a root expressed *hb* gene, like the one present in *Parasponia* and *Trema* roots. The legume root hemoglobin and nodule hemoglobin, may have diverged considerably during the course of evolution, to the point where they no longer cross react.

The presence of common DNA regulatory sequences in both leghemoglobin and non-legume hemoglobin, as well as the sequence homology strongly supports the idea of a common origin for hemoglobin.

6. Hemoglobin function

For many years, plant hemoglobin was thought to be exclusively found in the nodules of legumes. The findings that hemoglobin was widespread in the plant kingdom, however, raised the question about its function. The function of hemoglobin in non-symbiotic tissue is not clear. There are two major hypotheses, the first one proposes that hemoglobin acts as a sensor of oxygen tension. By changes of conformation, when hemoglobin becomes deoxygenated it may act as a trigger switching between aerobic and anaerobic metabolism. In this case, hemoglobin may cooperate with another molecule that is able to detect it's conformational changes. This hypothesis is based on observations of the concentration of hemoglobin in roots, which is much less than the concentration of free oxygen. The second hypothesis points at the idea of hemoglobin acting as a facilitator of oxygen diffusion in the root tips, based on the upregulation of the message for hemoglobin under oxygen stress (Appleby et al, 1988; 1990; Taylor, 1994).

OBJECTIVES

Several different approaches have been taken in attempts to elucidate the mechanisms plants have to adapt or to overcome anaerobiosis.

Some groups have focused on the switch from aerobic to anaerobic metabolism. Special interest has been put on the isolation and characterization of enzymes involved in the glycolytic and fermentative pathways (Freeling, 1973; Sachs et al, 1980; Laszlo and St Lawrence, 1983; Kelly and Freeling, 1984; Hoffman et al, 1986; Kelly and Tolan, 1986; Springer et al, 1986; Russell and Sachs, 1989, 1990).

Other groups have concentrated on the signal transduction pathways, the role of Ca^{+2} as a second messenger in signalling anaerobic conditions in maize (Subbaiah et al, 1994).

In contrast, other researchers are interested in the molecular mechanisms involved in the switch from aerobic to anaerobic metabolism; investigating the factors involved in the regulation of anaerobic genes (Olive et al, 1990; Ferl, 1990).

Another approach has been the investigation of the morphological changes leading to the development of aerenchyma in the roots and stems of anaerobically treated plants, as well as the formation of adventitious roots (de Wit, 1979; Drew et al, 1979; Justin and Armstrong, 1987; Laan et al 1989).

The role of hemoglobin in non-legume plants has not been elucidated. There are two major theories, one points at *hb* as an oxygen sensor and the other as a facilitator of oxygen diffusion (Appleby et al, 1988; 1990).

Thompson and Greenway (1971) reported the development of an anaerobic stele while

the cortex remained aerobic when maize plants were exposed to anaerobiosis.

Previous findings in our laboratory revealed the upregulation of the *hb* gene in the roots of barley and maize, as well as in barley aleurone layers exposed to anaerobic conditions (Taylor et al, 1994). Hence, the general objective in our laboratory has been to characterize the response of *hb* gene under low oxygen conditions, as well as to elucidate its role in the anaerobic response.

The specific objectives of the present investigation were to:

1. Analyze the expression of *hb* gene at different oxygen levels, thereby establishing the conditions at which the gene switches on.
2. Determine whether *hb* gene is expressed in a time dependent manner in the roots of flooded maize plants.
3. Determine if *hb* gene expression is tissue specific in the roots of flooded plants.
4. Determine if the pattern of expression of the Hb protein in flooded plants corresponds to that for the message.

MATERIALS AND METHODS

Maize seeds (*Zea mays* cv CO316, CO328, and Maisadour) were provided by the Canadian Grain Commission, Ottawa, Ontario.

The Hb probe was isolated from a barley cDNA library in Dr R.D. Hill's laboratory (Taylor et al, 1994).

The *Adh1* probe was a gift from E.S. Dennis (Dennis et al, 1984).

Hb protein and antibodies were obtained as described by Duff et al (1997).

I. Germination and Growth Conditions

Maize seeds were sterilized in 0.1% v/v sodium hypochlorite for 1 hr and rinsed thoroughly with running water for 2 hrs. Germination was achieved in germination boxes containing moisturized filter paper for three days at 20°C in the dark. Once the radicle and the coleoptile emerged, batches of 5 seedlings were either transferred to styrofoam plates or planted in soil. The seedlings were then kept at 30°C day/20°C night with high humidity and a light regime of 16/8 hrs of light/darkness, until the 3 leaf stage was reached (about 3 days after germination).

A preliminary comparison between cultivars CO316 (cold sensitive), CO328 (cold resistant) and Maisadour (hybrid) was performed, in order to select the most suitable cultivar to perform our experiments. Plants were selected on the basis of growth at 30°C

in a hydroponic system, as well as by the intensity of the *adh* gene signal in northern blots, when nitrogen was bubbled into the nutrient solution as described later. Cultivar CO316 was chosen to perform all experiments.

II. Hydroponic system and gas treatments

Once plants reached the 3 leaf stage, they were transferred to styrofoam boards (28 cm long x 28 cm x 3 cm). Each board contained 30 seedlings that were floated onto 5 liters of Hoagland's nutrient solution in a translucent container (50 cm x 39 cm x 12 cm) and the gas treatments commenced. Seedlings were put into holes (1 cm diameter) carved into the styrofoam boards and secured with security pins in a way that allowed only the roots to be in contact with the solution, while the leaves and shoots remained in the gas phase.

In order to avoid the presence of external sources of oxygen into the nutrient solution, two different systems were tested. One was labeled as closed, where a translucent lid was placed on top of the containers with the plants, and the other was labeled open, without the lid.

The gas treatments used to perform this study were: atmospheric air, compressed air, oxygen and nitrogen. The gas mixtures were continuously bubbled into the nutrient solution, at a rate of 54 mL/min during the whole experiment (24 hrs). Temperature was maintained at 30°C during the 16 hrs of light and at 20°C during the night in a CONVIRON growth cabinet.

After the treatments, seedlings were collected, and roots and leaves were excised, frozen in liquid N_2 and kept at -70°C until processed.

III. Flooding system

Pots (10 cm x 9.5 cm) were filled with 360 g of clay loam soil and 5 seedlings were planted per pot. At the beginning of each experiment, an appropriate number of pots, containing the 3 leaf stage seedlings, were transferred to a big container (50 cm x 45 cm x 35 cm) in order to be waterlogged. Tap water was used to slowly (approximately 45 min) fill the container so as to completely submerge the root systems up to the soil level allowing a period of acclimation for the roots. For sampling, individual pots containing 5 plants each, were collected after 0, 20, 40, 60, and 80 min and 2, 4, 6, 12, 24 and 48 hrs intervals, once the plants were submerged. Roots were washed to clear away soil and debris. Primary roots were collected, frozen in liquid nitrogen and used immediately to isolate RNA or protein, as described later.

1. Flooding Whole Plants

To ensure that plants were not translocating oxygen from the leaves and shoots into the roots through the aerenchyma, 40 maize seedlings were flooded up to the level of the leaves. Plants were maintained at 30°C night/20°C light and under a light regime of 16/8 hrs. The waterlogging treatment was imposed at the beginning of the light period. Samples were collected at 0, 2, 4, 6, 12, and 24 hrs flooding intervals as previously described.

2. Plants flooded in the darkness

To eliminate the possibility of photosynthetically-produced oxygen, contributing to the oxygen content of the water used to flood the maize plants, the experiment was also performed in darkness. Plants were flooded completely up to level of the leaves right after the end of the dark period, and were kept in the dark for the following 24 hrs. The temperature was maintained at 30°C during the treatment, due to the nature of the growing cabinet which would not allow us to vary the temperatures when the lights were off. Samples were collected after 0, 2, 4, 6, 12 and 24 hrs intervals as described previously.

3. Whole plants flooded in the dark transferred to light.

To accomplish this experiment, plants exposed to 24 hrs of flooding treatment in the dark, as described previously, were transferred to light, while flooded. Collection of samples was carried out at 2, 4, 6, 12 and 24 hrs after restoration of light conditions.

IV. Stele and cortex isolation

To determine the pattern of expression of *hb* and *adh* in maize flooded roots, 3 leaf stage seedlings were flooded up to the level of the roots over 4 hrs at 30°C. Primary roots were excised and dissected under the microscope to isolate the cortical and vascular tissues. Samples were immediately frozen in liquid nitrogen and kept at -70°C for further processing.

V. RNA isolation and northern blots

Total RNA was isolated from the roots according to Mohapatra et al (1987). Ten roots (approximately 3 g of fresh weight) were frozen and pulverized in a mortar and pestle in the presence of liquid nitrogen. The powder was transferred to 50 ml Corning tubes and 10 ml of pre-warmed phenol (65°C), 10 ml of RNA isolation buffer (1% (w/v) SDS, 200 mM Sodium Acetate, 10 mM EDTA at 65°C) and 78 µL of β mercapto-ethanol were added for each 1 g of tissue (fresh weight). The root samples were homogenised using a blender (Polytron, Brinkman) at top speed for 1 min and centrifuged at 4332 g for 15 min. The aqueous phase was collected, phenol/chloroform extracted once, and chloroform extracted 3 more times. RNA's were precipitated overnight at 4°C in the presence of 2 M LiCl.

To collect the RNA, samples were centrifuged at 4332 g for 45 min at 4°C. Supernatants were discarded and pellets were dissolved in 400 µL of DEPC-treated cold water and transferred to 1.5 mL eppendorf tubes to be phenol/chloroform extracted once and chloroform extracted 4 more times. Samples were precipitated in the presence of 3 M Na-Acetate and 100% (v/v) ethanol at -20°C for 2 hrs. Samples were then centrifuged at 20,384 g for 30 min at 4°C, washed with 70% (w/v) ethanol once, air dried and resuspended in 50 µL of DEPC-treated cold water. Yields obtained from the isolation of RNA were 300 µg RNA/g fresh weight of tissue.

2. Northern blotting

RNA was quantitated by UV absorption spectroscopy and size fractioned on 1.1%

agarose/formaldehyde gels, according to the procedure described by Chomczynski and Sacchi (1989). Fifteen µg of RNA were denatured for 10 min at 65°C in 15 µL of loading buffer, containing 50% (v/v) formamide, 6% (v/v) formaldehyde, 2 mM EDTA, 6% (v/v) glycerol, 0.1% (w/v) SDS, 0.005% (w/v) bromophenol blue and 0.005% (w/v) xyanol blue. Loading equivalence and RNA integrity were confirmed by ethidium bromide staining of gels prior to transfer. RNA was blotted onto Zeta Probe membrane in 20X SSC overnight and fixed by baking at 80°C and UV treatment (one autocross-linker cycle in a Stratalinker). Hybridisations were carried out over 16 hr in a mixture containing 500 mM $NaPO_4$ buffer, 2.5 M NaCl, 7% (w/v) SDS and 50% (v/v) formamide, at 43°C. Washes were carried out at room temperature in 0.1% (w/v) SDS plus varying concentrations of SSC. Washes were initially performed in 2X SSC, then 1X SSC and finally 0.5X SSC. Filters were exposed to Kodak XAR-5 film with screens at -70°C for several days.

For each experiment, the same blot was assayed sequentially against the *hb*, *adh* and ribosomal probes. The ribosomal probe was used to verify equivalent loading.

Relative intensity of the bands was quantified using an image analizer system (image X). Corrected values were obtained by dividing the relative intensity of the bands for the *hb* and *adh* genes by the relative intensity of the corresponding ribosomal bands. Each experiment was repeated three times, corrected values were averaged and standard deviations were caculated individualy for each condition tested. Quantification of the ribosomal probe was not available for the flooded experiments, both those performed by completely submerging the plants in darkness and in the presence of light. Instead, a

picture of the agarose gel was used to compare the relative intensity of the bands obtained for both *hb* and *adh* genes. Averages and standard deviations where then calculated directly from the relative values scanned from the blots.

3. Preparation and use of probes

Purified DNA inserts (hemoglobin, 900 bp; adh, 1200 bp and ribosomal) were radioactively labeled according to the random primer procedure described by Feinberg and Vogelstein (1984). Fifty ng of DNA were labelled each time using [α-^{32}P]dCTP (ICN). Unincorporated label was removed by spinning through a 1 mL BIOGEL P-30 column equilibrated in TEN according to Sambrook et al (1989). Probes were added at concentrations of 1-5 X 10^6 cpm per mL of hybridisation fluid.

VI. Protein isolation and quantification

1. Crude protein extracts

To obtain a crude protein extract from maize seedlings, 5 roots were ground in 20 mL of extraction buffer (20 mM Tris, 50 mM EDTA, 100 µM PMSF, pH 7.4) using the Polytron. Samples were spun at 12,062 g for 10 min. The supernatant was recovered and spun repeatedly until clear. The protein extract was concentrated in an Amicon cell with a 5,000 Da cutoff membrane (YM 5).

2. Protein Quantification

Protein concentration from the root extracts was quantified according to the

method of Bradford (1976), using a BSA standard curve.

VII. Gel electrophoresis and western blots

1. Gel Electrophoresis

Electrophoresis of proteins was carried out in SDS-polyacrylamide gels according to the procedure described by Laemmli (1970). Final monomer concentrations of acrylamide were 5% (w/v) for the stacking gel and 14% (w/v) for the separating gel. Proteins were denatured at 65°C during 10 min in a mixture containing 60 mM Tris-HCl, 7.7% (v/v) glycerol, 1% (w/v) SDS, 1% (w/v) DTT and a trace of bromophenol blue. Gels were run at 100 Volts (constant voltage) for 2 hrs and then electroblotted onto nitrocellulose membranes for 1 hr in 25 mM Tris, 190 mM glycine and 20% (v/v) methanol (pH 8.3) at 0.5 amps (constant current).

2. Western Blots

Nitrocellulose filters were rinsed four times in TBS (10 mM Tris -HCl, 150 mM NaCl, pH 8.0) for 5 min. Blocking of non-specific sites was achieved by incubating nitrocellulose membranes in TBS containing 5% (w/v) milk powder for an hour. This was followed by two, 5 min washes in TBS. The membranes were then incubated for an hour in a 1:500 dilution of the Hb antibody in TBST (TBS containing 0.05% (v/v) Tween 20). Blots were washed four times in TBST and then incubated for one hour in TBST containing a 1:3,000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate. Membranes were washed twice in TBST and then developed in alkaline

phosphatase buffer (100 mM Tris-HCl; pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) containing 0.3 mg/ml of nitroblue tetrazolium and 0.2 mg/ml of 5-bromo-4-chloro-3-indoyl phosphate.

VIII. ADH enzyme assays

ADH activity was assayed in the ethanol to acetaldehyde direction, according to Hanson and Jacobsen (1984). A 3 mL reaction mix was used, containing 500 mM Tris-HCl (pH 9.0), 3 µM of NAD, 100 µL of ethanol, 1.5 mL of water and 100 µL of the enzyme extract containing 100, 200, 300 and 500 µg of protein. The mixture was incubated at 27°C for 36 min and monitored at an OD of 340 nm at the beginning (once the enzyme was added) and at the end of the reaction.

Specific activity was determined using the NADH extinction coefficient.

RESULTS

Expression of hemoglobin mRNA has been found to be induced under low oxygen tensions in *Parasponia* (Appleby, 1983), maize, and barley roots as well as in barley aleurone layers (Taylor et al, 1994).

Maize roots are the most characterized system with respect to physiological and biochemical responses to oxygen shortage (Okimoto et al, 1980; Sachs et al, 1980; Saglio et al, 1988; Thompson and Greenway, 1991; Andrews et al, 1994). Experiments described in this thesis were carried out in order to analyze in detail the induction of the *hb* gene in maize roots. Knowledge gained through these experiments might bring us closer to the understanding of the role of hemoglobin in the anaerobic response. So, the effects of anaerobiosis on the expression of *hb* and *adh* genes were studied in hydroponic and waterlogged soil systems.

I. Expression of *hb* and *adh* genes in maize roots grown in a hydroponic system

The main advantage of the hydroponic system is the ease of handling and control of the experimental conditions.

Northern blot analyses were performed in order to determine the effect of anaerobic conditions on the accumulation of mRNAs for *hb* and *adh* genes, in the roots of maize seedlings grown in hydroponic systems.

To characterize the response of the maize seedlings in the hydroponic system, a

preliminary comparison between the oxygen and nitrogen bubbling treatments was carried out. Open and closed systems were tested on the basis of avoidance of the exposure of aerial parts to atmospheric air, thus creating an external supply of air to the root systems. Closed systems had a transluscent lid on top of the container with the plants and open systems did not have a lid. The treatments were performed over a period of 24 hrs.

After 24 hrs of treatment, plant root tips were generally damaged and root growth was delayed. Cortical cells of these roots had a reddish coloration, presumably from anthocyanin production and accumulation. Development of secondary roots was just incipient, but adventitious roots developed. Leaf growth was normal, even though leaves were wilted and showing chlorosis symptoms in some spots, especially in the closed systems due to the higher temperature prevailing in these systems (33°C in comparison with 29°C in the opened systems).

Northern blot results from this experiment showed that after 24 hrs of either nitrogen or oxygen bubbling, *hb* gene expression was slightly higher in the roots of plants exposed to nitrogen treatment (open and closed environments) as well as in the oxygen closed systems. Similar results were obtained when the accumulation of the *adh* message was tested in the same conditions although the level of expression for the *adh* gene was considerably higher than that for *hb* (Fig 3). Average corrected values for the 3 replicates of the experiment are included in Table 2.

Based on the physical appeareance of the roots and the expression of a diagnostic gene for anaerobiosis (*adh*), our results indicate that the hydroponic system was

FIG 3. Northern blot analysis of the *hb* and *adh* expression in the roots of maize seedlings in a hydroponic system. Closed systems (cl) had a translucent lid on top of the plants, open systems (op) did not have the lid. Gas treatments (oxygen [O_2] and nitrogen [N_2]) were performed over 24 hrs. The same blot was assayed against all probes. A rRNA probe was utilized to verify equivalent loading. Numbers below the blots indicate the relative intensity of the bands obtained from scanning each blot.

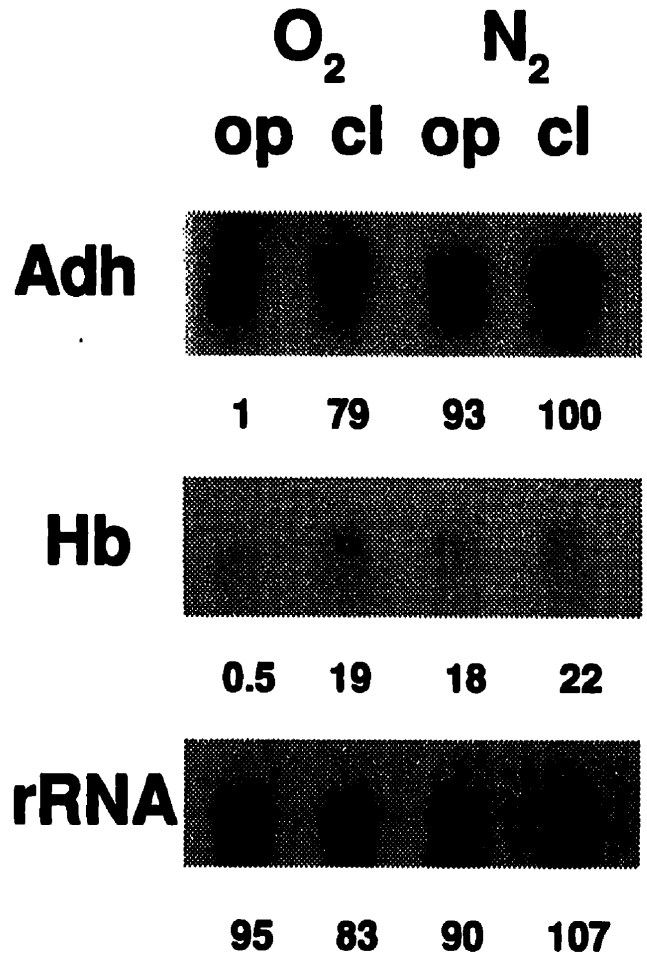

O_2 N_2
op cl op cl
Adh
1 79 93 100
Hb
0.5 19 18 22
rRNA
95 83 90 107

Table 2. Average corrected relative values of *adh* and *hb* transcripts in maize roots in a hydroponic system over 24 hrs of treatment

	***Adh* gene**		***Hb* gene**	
	Average	**Standard deviation**	**Average**	**Standard deviation**
O_2 open	0.030	0.020	0.035	0.050
O_2 closed	1.083	0.189	0.250	0.096
N_2 open	0.863	0.146	0.290	0.182
N_2 closed	1.117	0.265	0.21	0.070

Corrected values are the average of 3 separate experiments

hypoxic and/or exhibited nitrogen deficiency, as indicated by the color of the leaves and the presence of anthocyanin in the base of the stems (Kohl et al, 1979; Salisbury et al, 1992).

II. *Hb* and *adh* gene expression in response to oxygen, nitrogen, atmospheric air and compressed air in hydroponic systems.

To gain insight into the mechanism inducing the response of maize plants to the hydroponic system, two intermediate treatments (atmospheric air and compressed air) were examined, in addition to the oxygen and nitrogen previously tested.

Observations of the plant morphology showed the same results as in the oxygen and nitrogen treatments previously described, indicating that these plants were also hypoxic.

Northern blot results obtained from this comparison showed that regardless of the conditions of the treatment (nitrogen, oxygen, air or compressed air; closed or open systems) there was no evident change of the expression of the *hb* or the *adh* genes, suggesting that the system itself provokes hypoxic conditions and induces a response in the roots of the plants in all treatments. This is evident by the physical appeareance of the roots and leaves as well as by the expression of a diagnostic gene for hypoxia (*adh*) (Fig 4). This made it difficult to differentiate between the response of the plants to the growth conditions *per se*, and the treatments imposed on the plants. Average corrected values obtained from the 3 replicates of the experiment are included in Table 3.

FIG 4. Northern blot analysis of the accumulation of *hb* and *adh* mRNA in maize seedling roots in hydroponic systems over 24 has of treatment. Gas treatments were oxygen, atmospheric air (atm air), compressed air (comp air) and nitrogen. Closed systems (cl) had a translucent lid on top of the plants, and open systems (op) did not have the lid. The same blot was assayed sequentially against all probes. A rRNA probe was used to show equivalence of loading. Numbers shown below the blots indicate the relative intensity of the bands, obtained by scanning the autoradiographies.

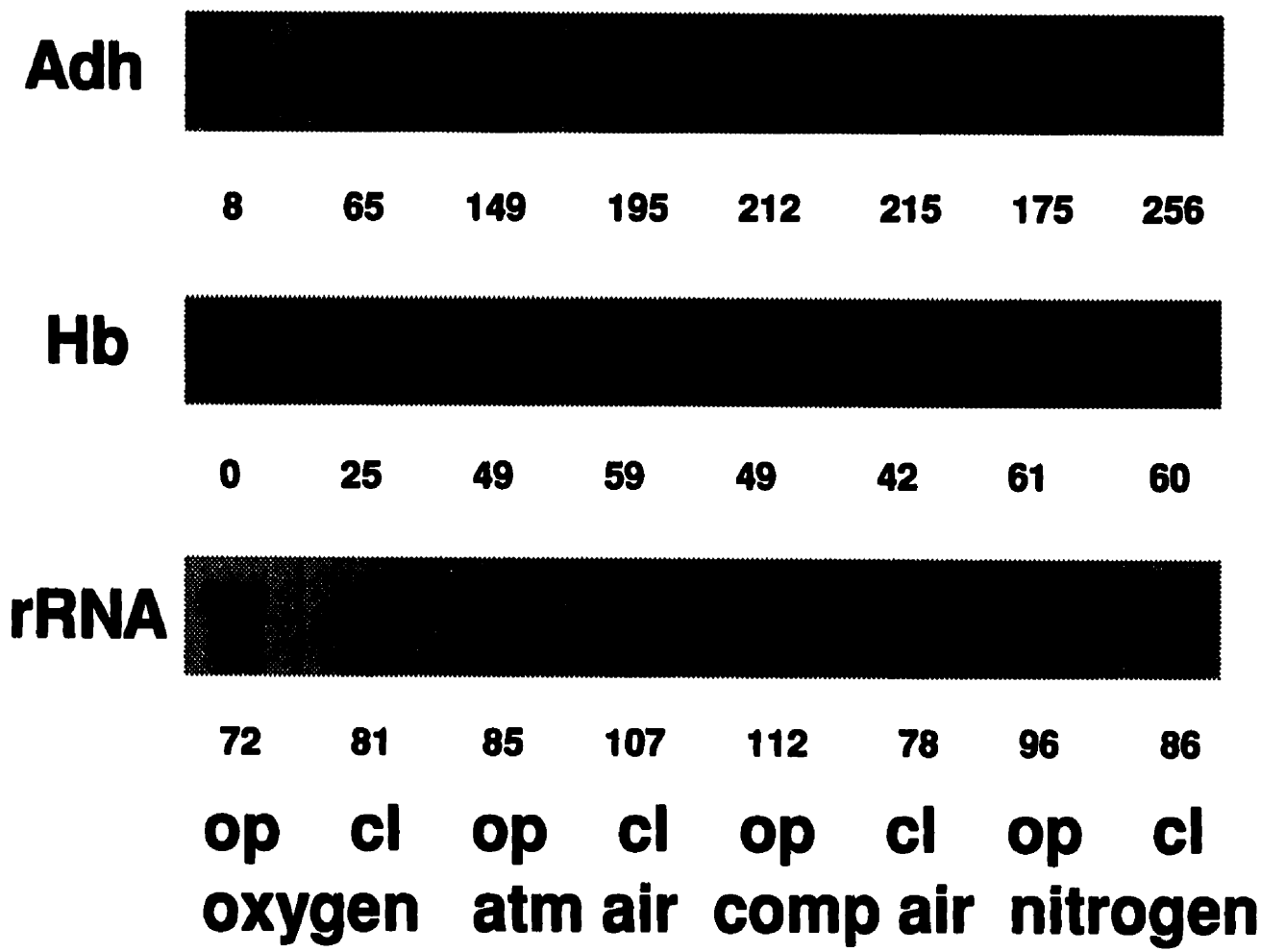

Adh
8 65 149 195 212 215 175 256
Hb
0 25 49 59 49 42 61 60
rRNA
72 81 85 107 112 78 96 86
op cl op cl op cl op cl
oxygen atm air comp air nitrogen

Table 3. Average corrected relative values of *adh* and *hb* transcripts in maize roots in a hydroponic system over 24 hours of treatment

	***Adh* gene**		***Hb* gene**	
	Average	Standard deviation	Average	Standard deviation
O_2 open	0.167	0.208	0	0
O_2 closed	0.967	0.153	0.300	0.100
Atmospheric air open	1.867	0.438	0.560	0.101
Atmospheric air closed	2.307	0.617	0.570	0.076
Compressed air open	1.687	0.188	0.417	0.091
Compressed air closed	2.417	0.382	0.553	0.137
N_2 open	1.843	0.091	0.693	0.137
N_2 closed	3.060	0.131	0.697	0.120

Corrected values are the average of 3 separate experiments

III. Comparative expression of *hb* gene in the roots of plants grown in hydroponic and waterlogged soil systems.

It has been reported that flooding conditions deprive the roots of oxygen, since the water fills the air spaces among the soil particles, creating hypoxic or even anoxic conditions for the roots (Armstrong, 1979; Hook and Crawford, 1978; Drew, 1979; Kennedy et al, 1992; Perata and Alpi, 1993; Ricard et al, 1994; Sachs et al, 1996).

The main purpose of the present research was to elucidate the role of *hb* gene and protein during anaerobic stress conditions in maize seedlings roots. The results obtained with the hydroponic system with N_2 bubbling, did not allowed us to discern between the effects of the treatments imposed and the effects of the system itself, hence, a comparison between growth in hydroponic systems and waterlogged soils was conducted.

Physically, waterlogged plants appeared healthier than those in hydroponic systems; the rate of growth was maintained, leaves and stems were turgid and dark green in color, with the exception of the basal region which had a reddish color. Roots were light yellow and continued to grow for up to 24 hrs of flooding and lateral root development took place. There was a prolific development of adventitious roots, growing near and at the soil/water interface. These roots were coarser and longer than the primary and secondary roots.

Northern blot results from this experiment showed that after 4 hrs of treatment, *hb* mRNA levels are strongly induced in the roots of flooded plants, whereas, in the nitrogen treated plants in a hydroponic system, the mRNA levels were not

substantially different from those of the control plants that were growing in soil (Fig 5). Average relative values for the 3 replicates of the experiment are included in Table 4.

These findings suggest that at least for us, hydroponic system is not suitable to perform studies on the expression of the *hb* gene under anaerobic conditions, eventhough, *adh* gene expression was as expected for an anerobic system. Waterlogged systems in contrast, provided a clear response of the plant under anaerobiosis at the mRNA as well as at the physiological level. In addition to this, the physical conditions of the plants in the waterlogged soils was better, indicating that this system was more suitable to perform long term experiments since the plants remained viable and healthy for the entire duration of the experiment.

In addition to these findings, the rate at which nitrogen gas was bubbled was 6 times slower than the reported experiemental conditions for the measurement of the *adh* gene (Johnson et al, 1989). This fact translated into partially hypoxic conditions for the hydroponic system, while truly anoxic for the waterlogged system.

FIG 5. Comparative expression of *hb* gene in maize seedling roots in hydroponic (lane H) and flooded systems (lane F), control (lane C) is referred to unflooded plants in soil. Treatments were carried out over 4 hrs as described in materials and methods. Numbers shown below the blot indicate the relative intensity of the bands as obtained by scanning the autoradiography. Data from the adh and ribosomal probes was not available.

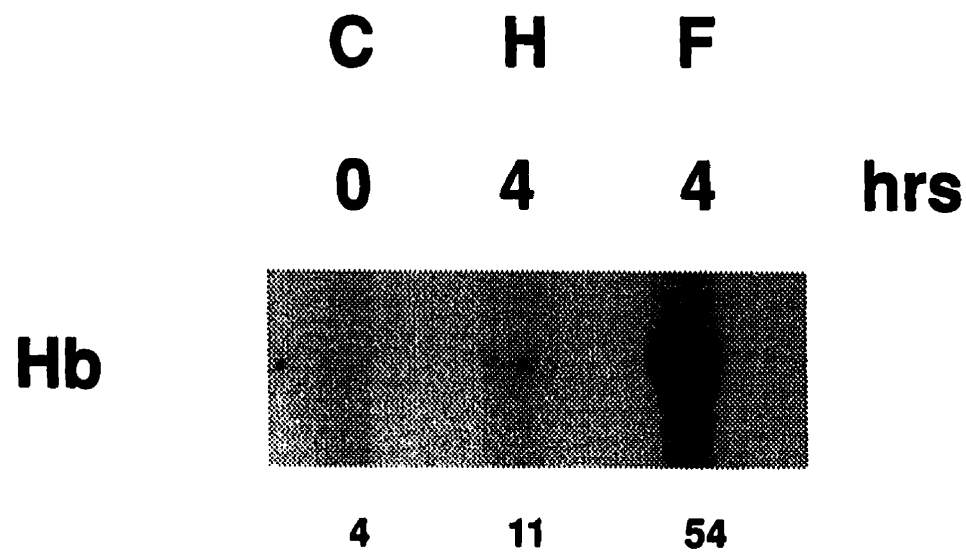

C H F
0 4 4 hrs
Hb
4 11 54

Table 4. Average values for hb gene in maize roots grown in hydroponic and flooded systems

	Hb gene	
	Average	Standard deviation
control	1.333	2.309
hydroponic system	13.000	2.645
flooded system	56.670	3.786

Values are the average of 3 separate experiments

IV. Time course of induction of *hb* and *adh* genes during waterlogging.

In order to determine the effect of flooding on the accumulation of mRNA for *hb* and *adh* genes in maize roots, a time course was performed by submerging the roots, for a period of 48 hrs.

During the experiment, roots remained healthy. Roots were on average 10 ± 0.016 cm long at the beginning of the experiment and reached 20 ± 0.021cm after 48 hrs of flooding. Their color was light yellow and they did not display symptoms of damage. Sub-apical hairs were present on primary roots and secondary roots developed, indicating that the flooding treatment was not lethal or interfering with normal growth and development of the seedlings. However, after 24 hrs of flooding, the development of adventitious roots was evident (Fig 6).

Northern blot results from these experiments showed that in maize roots, the induction of *hb* gene occurred after only 20 min of flooding (Fig 7a). The expression of the gene continued to increase for another hour, to reach a peak at approximately 80-120 min. This was followed by a slow disappearence of the message in the next 46 hrs (Fig 7b).

Under the same conditions, the results showed that the accumulation of the message for the *adh* gene was strongly induced after 40 min of flooding (Fig 7a) and reached a maximum induction at 12 hrs (Fig 7b). By 48 hrs, the expression of the gene had decreased, but remained high compared to unflooded roots. Average corrected relative values for the three repeats performed for each time course are

included in Tables 5 and 6.

Our results showed that the induction of *hb* gene occurs at very early stages of anaerobiosis, that the message is a very short lived one and that its expression precedes the induction of the anaerobic gene *adh*.

Table 5. Average corrected relative values of *adh* and *hb* transcripts in maize roots during a short time course of flooding

	***Adh* gene**		***Hb* gene**	
Minutes	Average	Standard deviation	Average	Standard deviation
0	0	0	0	0
20	0.226	0.115	0.193	0.090
40	0.467	0.130	0.43	0.085
60	0.587	0.174	0.49	0.141
80	2.453	0.474	1.943	0.083
100	2.787	0.720	1.79	0.201
120	2.98	0.650	1.453	0.122
140	3.423	0.609	1.377	0.337

Corrected values are the average of 3 separate experiments

Table 6. Average corrected relative values of *adh* and *hb* transcripts in flooded maize roots over time

	***Adh* gene**		***Hb* gene**	
Hours	Average	Standard deviation	Average	Standard deviation
0	0	0	0	0
2	1.043	0.412	1.68	0.668
4	1.38	0.375	1.143	0.517
6	3.3	0.755	1.22	0.252
12	1.716	0.340	0.667	0.284
24	1.637	0.367	0.213	0.091
48	1.537	0.467	0.133	0.075

Corrected values are the average of 3 separate experiments

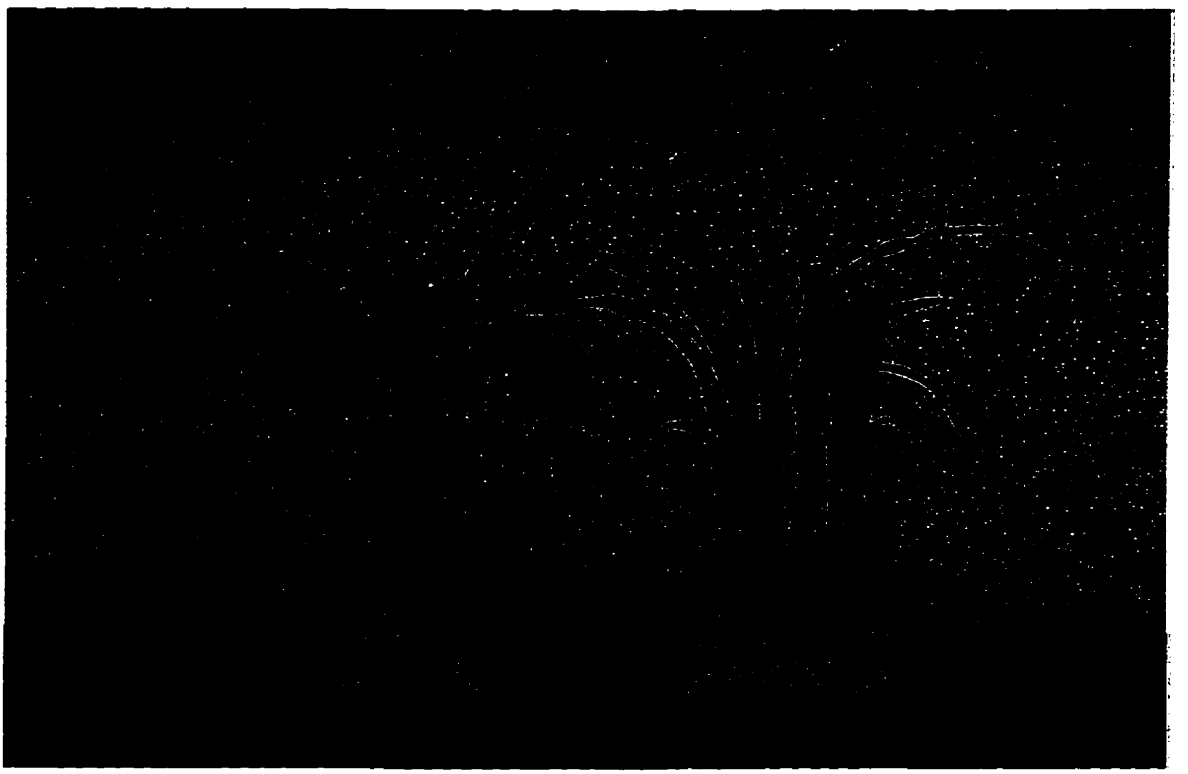
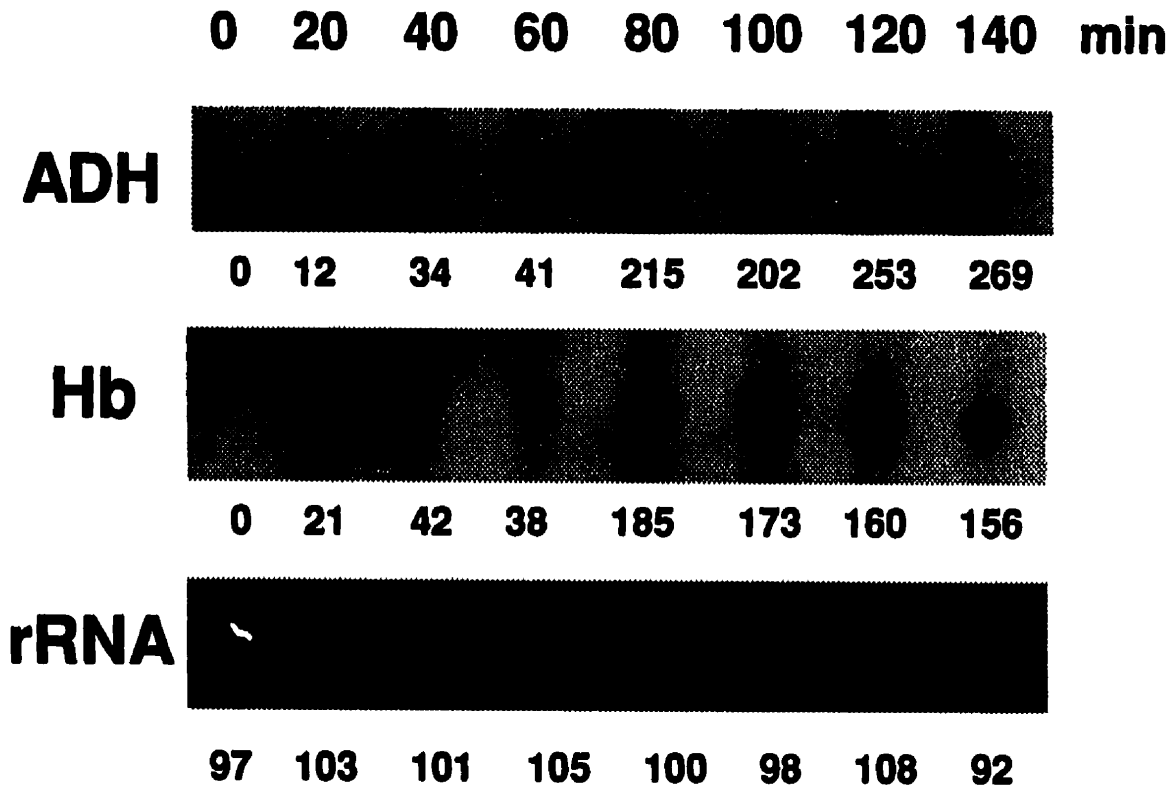


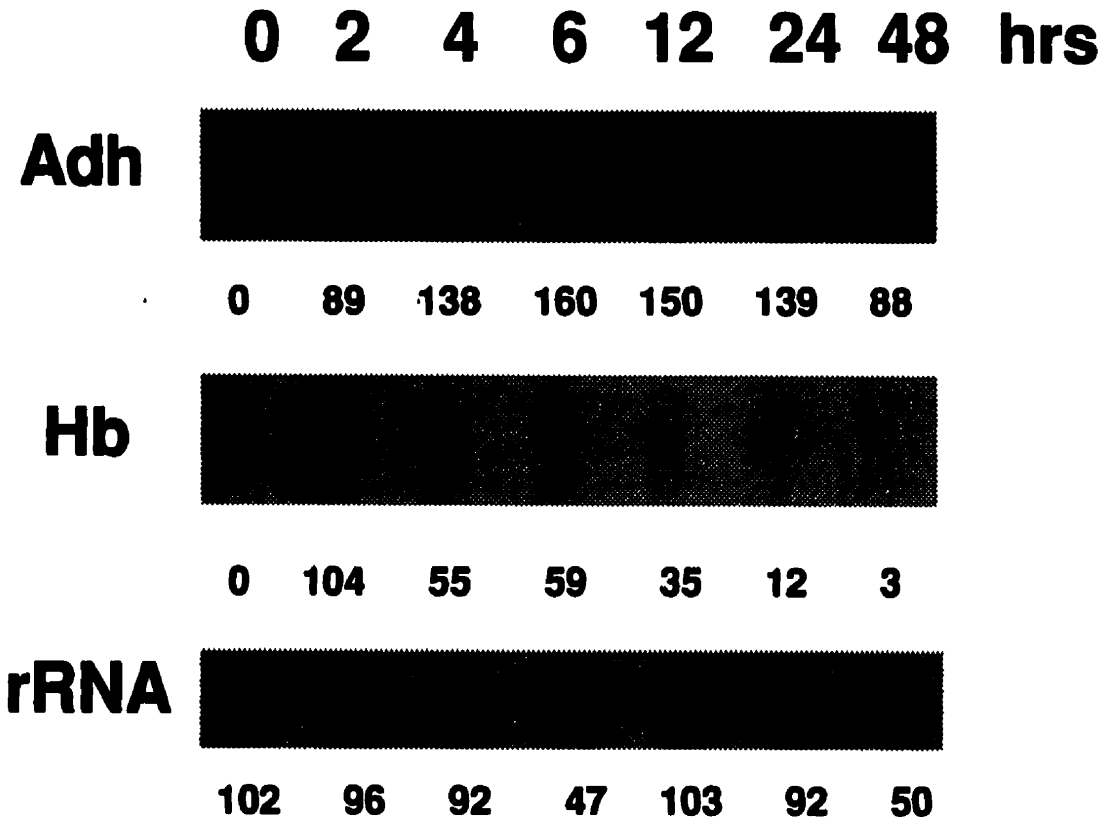
Fig 6. Comparison between flooded and unflooded plants. Shown on the right are the flooded plants after 24 hrs of treatment. Flooded plants show a prolific development of adventitous roots at the interface soil/water, leaves and stems are turgid and green in color. Rate of growth was maintained during the flooding treatments, as evident by the size of the flooded plants in comparison to the unflooded plants.

FIG 7. Northern blot analysis of the time course of mRNA accumulation of *hb* and *adh* genes in flooded maize roots. Fig 7a depicts the time periods between 0 and 140 minutes of the flooding treatment while, fig 7b shows the time periods between 0 and 48 hrs of flooding in 2 hr intervals. A rRNA probe was used to standardise the RNA loading. The same blot was assayed sequentially against all probes. Numbers shown below each blot indicate the relative intensity of the bands as obtained from scanning the autoradiographies.

a)


0 20 40 60 80 100 120 140 min
ADH
0 12 34 41 215 202 253 269
Hb
0 21 42 38 185 173 160 156
rRNA
97 103 101 105 100 98 108 92

b)


0 2 4 6 12 24 48 hrs
Adh
0 89 138 160 150 139 88
Hb
0 104 55 59 35 12 3
rRNA
102 96 92 47 103 92 50

V. The contribution of oxygen from aerial parts to anaerobic expression.

1. Plants grown in light.

Reports in the literature show that maize responds to flooding by developing aerenchyma (as fast as 6 hrs after imposing the stress), which supplies oxygen to the roots, minimizing the anaerobic conditions (Williams and Barber, 1961; Armstrong, 1979; de Wit, 1979; Drew et al, 1979, Mendelssohn and Postek, 1982; Justin and Armstrong, 1987; Laan et al, 1989).

To elucidate the effect of the development of aerenchymatous tissue on the supply of air from the shoots to the roots and as a consequence, on the expression of *hb* and *adh* genes, the present experiment was carried out by submerging the whole plant in water.

After standardization of the RNA amount per lane based on the ribosomal RNA loadings, results from this experiment showed no major differences with the patterns of induction of *adh* and *hb* genes (Fig 8) when compared to experiments performed by solely flooding the roots (Fig 7). *Hb* gene expression reached its peak at 2 hrs and started to decline after 6 hrs. Under the same conditions, the expression of the *adh* gene slowly increased, reaching its maximum at 12 hrs and then declined (Fig 8). Average corrected relative values for the three repeats of the experiment are included in Table 7.

Results from Fig 7 showed that *hb* expression preceded that of *adh* under anaerobic conditions, suggesting a role for *hb* in an environment where O_2 had not

been depleted to a very great extent. The results also suggested 2 possibilites: one that the system was still getting oxygen from another source, possibly, from the photosynthetic activity of leaves, with the concomitant liberation of O_2 into the water surrounding the roots. The second possibility was that *hb* gene is just transiently expressed, in a similar fashion as the transition peptides of maize.

FIG 8. Northern blot analysis of the time course of induction of *hb* and *adh* mRNA in the roots of completely submerged maize seedlings as described in materials and methods. The agarose gel picture is shown to verify loading equivalence. The same blot was assayed sequentially against all probes. Numbers shows below the blots indicate the relative intensity of the bands as obtained by scanning. Intensity values for the gel were not obtained due to methodological problems.

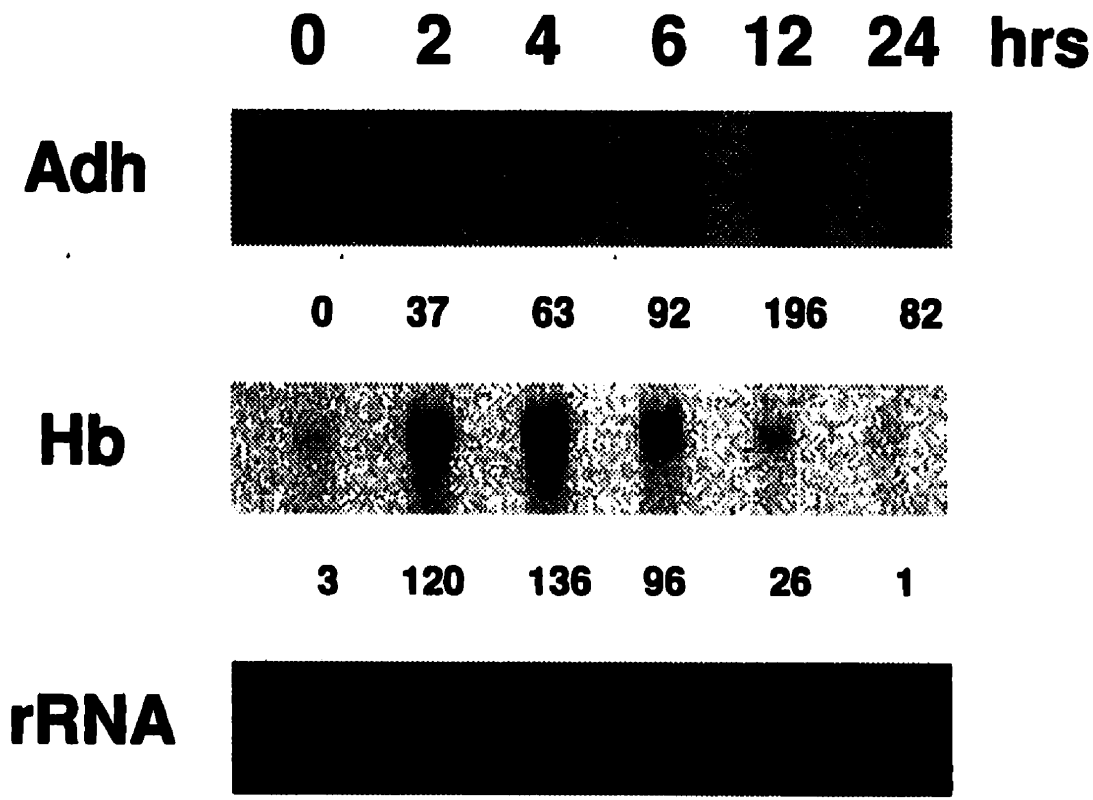

0 2 4 6 12 24 hrs
Adh
0 37 63 92 196 82
Hb
3 120 136 96 26 1
rRNA

Table 7. Average relative values of *adh* and *hb* transcripts in the roots of maize plants entirely flooded

	***Adh* gene**		***Hb* gene**	
Hours	Average	Standard deviation	Average	Standard deviation
0	0	0	3.33	2.517
2	39	5.291	122.33	11.676
4	63.67	7.023	135.33	9.504
6	93.70	8.621	95.67	5.508
12	197.33	9.073	28.67	5.508
24	90.67	9.018	2.9	2.007

Values are the average of 3 separate experiments

2. Plants subjected to darkness

To eliminate the possible contribution of the photosynthetically produced oxygen and the aerenchyma into the experimental system, maize plants were flooded completely and kept in the dark.

Results showed that *hb* gene was induced after 2 hrs of flooding in the dark and its expression was maximal at 6 hrs. After 12 hrs, the message had disappeared from the tissues (Fig 9).

Qualitative results from this experiment were similar to the results obtained from previous experiments where plants were flooded in light. The *hb* message preceded the appearance of the *adh* and remained present for just a short period of time. Average quantitation of the intensity of the bands obtained for the 3 repeats of the experiment are included in Table 8.

Our results showed that light had no effect on the accumulation of *hb* and *adh* messages in maize roots under flooding conditions. They also show that *hb* is trtansiently expressed in the roots of flooded, preceding the expression for the *adh* gene.

FIG 9. Northern blot analysis of the time course of induction of *hb* and *adh* mRNA in the roots of fully submerged and maintained in the dark maize seedlings as described in materials and methods. A photograph of the agarose gel prior to transfer was used to verify equivalence of RNA loading. The same blot was assayed sequentially against all probes. Numbers shown below the blots indicate the relative intensity of the bands as obtained by scanning the autoradiographies. Intensity values for the gel were not obtained.

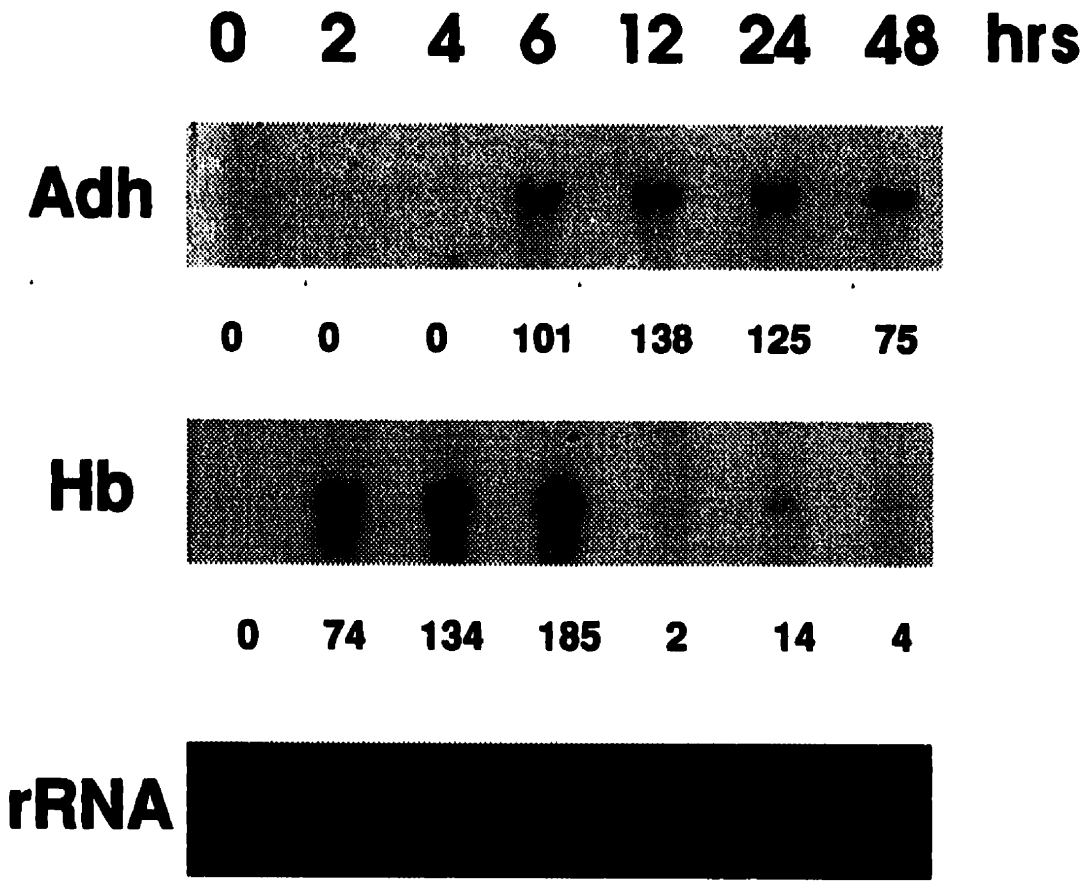

0 2 4 6 12 24 48 hrs
Adh
0 0 0 101 138 125 75
Hb
0 74 134 185 2 14 4
rRNA

Table 8. Average relative values of *adh* and *hb* transcripts in the roots of maize plants entirely flooded and kept in the dark

	***Adh* gene**		***Hb* gene**	
Hours	Average	Standard deviation	Average	Standard deviation
0	0.287	0.163	0	0
2	0.467	0.181	0.077	0.108
4	0.47	0.151	0.143	0.971
6	0.463	0.150	0.283	0.152
9	0.56	0.125	0.367	0.136
12	0.727	0.205	0.273	0.095
24	0.96	0.44	0.2	0.06

Values are the average of 3 separate experiments

3. Whole plant flooded in the dark transferred to light.

With the purpose of determining the response of *hb* and *adh* genes, once the leaves were allowed to restart photosynthesizing, and the oxygen levels restored; maize plants flooded for a 24 hr period in the dark, were transferred to light under continuous complete flooding.

Results from this experiment showed an induction of *hb* at 6 hrs, while *adh* gene transcripts remained constant during the treatment (Fig 10). Average corrected values for the 3 repeats of the experiment are inluded in Table 9.

It is important to note that extreme conditions at which the plants were exposed, provoke severe damage in the root systems.

FIG 10. Northern blot analysis of the time course of induction of *hb* and *adh* mRNA in the roots of maize seedlings flooded in the dark over a period of 24 hrs and then transferred to light while the flooding treatment was maintained, as described in materials and methods. A rRNA probe was used to show equivalence of loading. The same blot was assayed sequentially against all probes. Numbers below the blots indicate the relative intensity of the bands as obtained by scanning the autoradiographs.

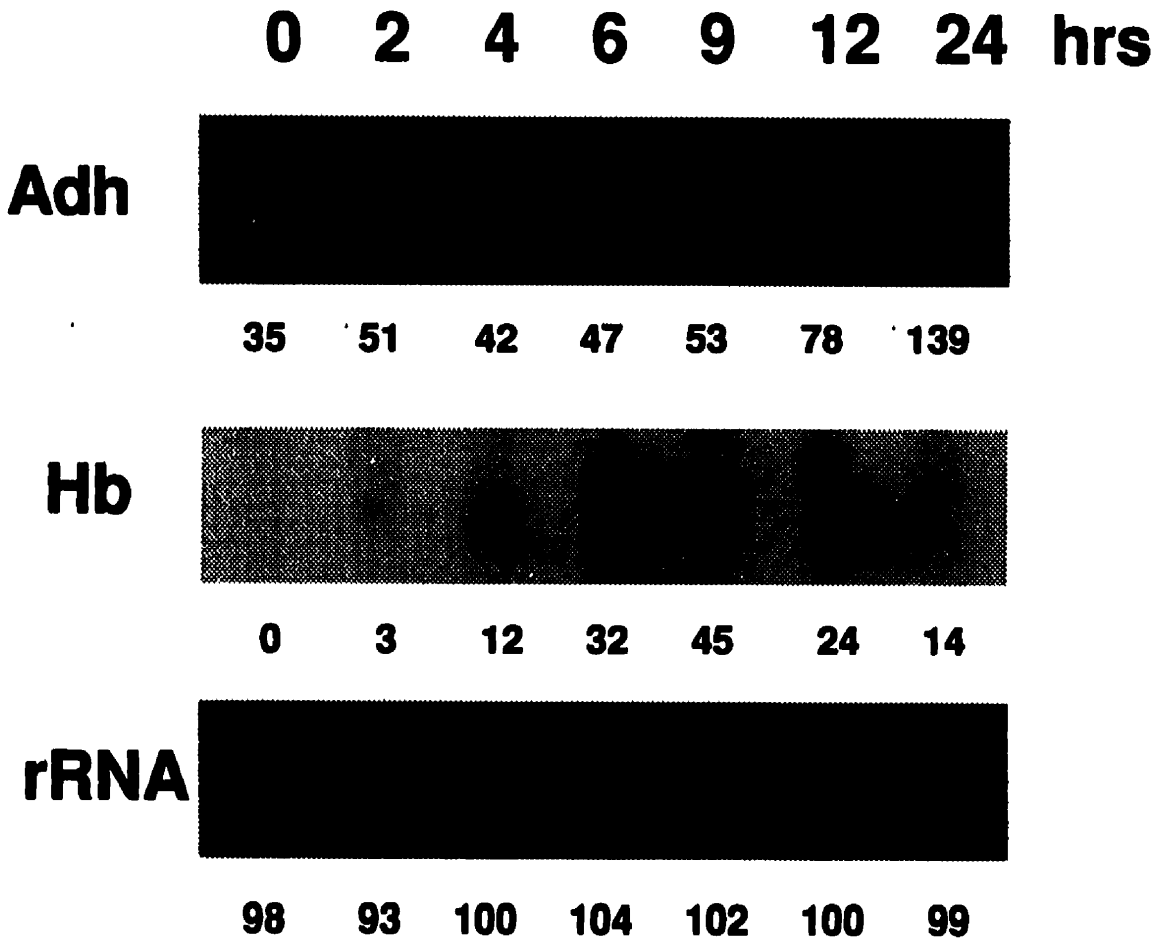

0 2 4 6 9 12 24 hrs
Adh
35 51 42 47 53 78 139
Hb
0 3 12 32 45 24 14
rRNA
98 93 100 104 102 100 99

Table 9. Average corrected relative values of adh and hb transcripts in the roots of maize plants completely flooded in the dark for 24 hrs and then transferred to light while kept flooded

	***Adh* gene**		***Hb* gene**	
Hours	Average	Standard deviation	Average	Standard deviation
0	0.287	0.163	0	0
2	0.467	0.181	0.077	0.108
4	0.47	0.151	0.143	0.097
6	0.463	0.150	0.283	0.152
9	0.56	0.125	0.367	0.136
12	0.727	0.205	0.273	0.095
24	0.96	0.44	0.2	0.06

Corrected values are the average of 3 separate experiments

VI. Tissue specific expression of *hb* and *adh* genes in maize seedling roots under flooding conditions.

Under hypoxic conditions, the metabolism of an organ may be heterogenous since the outer layers receive more oxygen and therefore are less hypoxic than the core of the tissue (Thompson and Greenway, 1991).

In order to investigate whether the *hb* gene in flooded maize roots followed the same pattern of tissue specific expression as ADH and PDC in maize (Thompson and Greenway, 1991), and *hb* in tobacco and *Parasponia* (Bogusz et al, 1990), maize plants were flooded for 4 hrs. The roots were harvested and stelar and cortical tissues were collected. RNA from both tissues was extracted and probed for the presence of *adh* and *hb* messages.

Results showed that in non flooded plants, there was a low constitutive amount of *adh* and *hb* genes in both stele and cortical tissues (Fig 11).

After corrections for variation in loading, *hb* as well as *adh* gene expression is about 1.2 and 1.5 times respectively higher in the stele than in the cortex of flooded plants. The expression of these genes is also prominent in the stele of non flooded plants. However, the level of the transcripts in the roots of flooded plants is much higher than in control plants. Average corrected values for the 3 repeats of the experiment are included in Table 10.

These results are supported by adh activity measurements showing that adh activity increased 17.5 fold in the stele and 13 fold in the cortex of flooded plants. In whole roots, adh activity is induced 8 fold during the flooding treatment (Table 11).

FIG 11. Northern blot analysis showing the expression of the *hb* and *adh* genes in the stele (s) and cortex (c) of unflooded (nf) and flooded (f) maize roots over a period of 4 hrs. A rRNA probe was used to show equivalence of loading. The same blot was assayed sequentially against all probes. Numbers below the blots indicate the relative intensity of the bands as obtained by scannin the x-ray films.

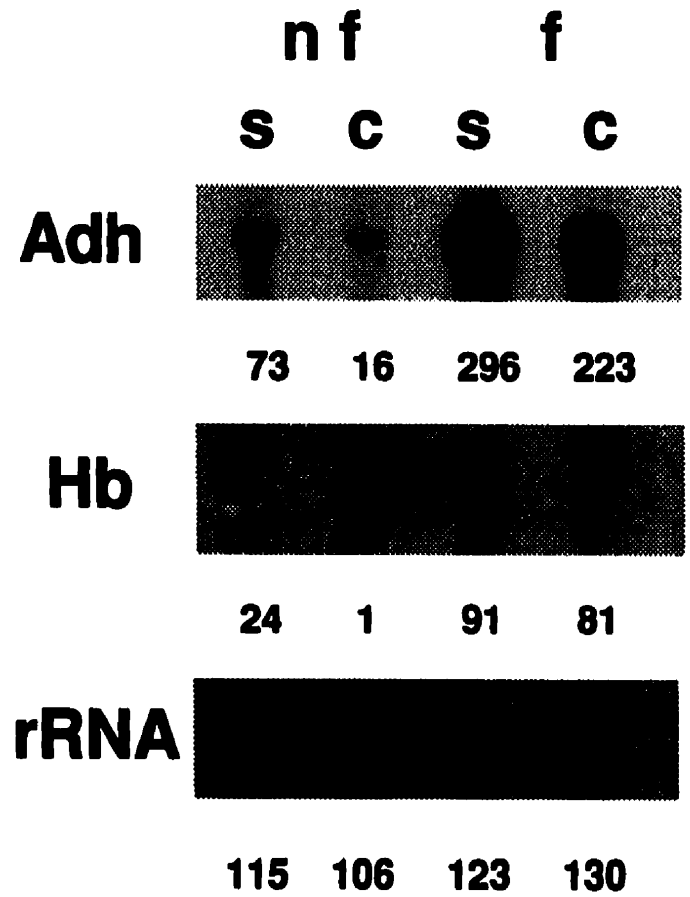

n f
f
s c s c
Adh
73 16 296 223
Hb
24 1 91 81
rRNA
115 106 123 130

Table 10. Average corrected relative values of *adh* and *hb* transcripts in the stele and the cortex of flooded maize plants

	***Adh* gene**		***Hb* gene**	
	Average	Standard deviation	Average	Standard deviation
stele unflooded	0.66	0.079	0.203	0.040
cortex unflood	0.177	0.031	0.008	0.004
stele flooded	2.547	0.263	0.743	0.105
cortex flooded	1.803	0.208	0.62	0.11

Corrected values are the average of 3 separate experiments

Table 11. ADH enzymatic activity (expressed as µmol $min^{-1}g^{-1}$ fresh weight) in maize seedlings roots after 24 hrs of flooding.

tissue	conditions	activity (µmol/min/g)	times of induction
stele	non-flooded	3.46	0
stele	flooded	60.46	17.5
cortex	non-flooded	3.76	0
cortex	flooded	51.07	13
root	non-flooded	24.3	0
root	flooded	206.15	8

All these results are in accordance with the reports of Thompson and Greenway (1991) in terms of enzymatic activity. In terms of gene expression, *hb* was found to be expressed in both the stele and the cortex of flooded maize, with a predominant expression in the stele. The results then indicate that the pattern of expression for the *hb* gene in maize might be different from the one for *Parasponia* and *Lotus* (Bogusz et al, 1990)

VII. Protein experiments

In order to further investigate the regulation of the *hb* gene in relation to anaerobic stress, we examined the accumulation of Hb protein in flooded maize roots.

Results showed the presence of hb protein in maize roots, as a band of ~ 17 kD assayed against Ab raised vs a recombinant hb protein isolated from *E. coli* (Duff et al, 1997) (Fig 12). The amount of protein seemed to be constant during the first 12 hrs after flooding and then it increased slightly (Fig 12). These results indicate that there is a lag period between the increase in mRNA levels and the increase in protein levels. The results can be explained on the basis of post-translational regulation of the gene expression that enable the protein to be recognized by the antibody. Gallie (1993) reported that wide disparity occurs between the levels of mRNA and the accumulation of the protein as a feature of post-translational regulation of the expression of the anaerobic genes.

Our results suggested three possibilities:

A first one indicated that the message was being transcribed but not translated. The

second possibility suggested that the protein was being produced, but required to undergo a series of post-translational modifications in order to be recognized by the antibody. And a third option suggested that modifications in the translational machinery were influencing the rate at which the protein was being produced.

Quantification of the intensity of the bands obtained from the western blot experiments, was not achieved due to the lack of contrast between the background and the protein bands.

FIG 12. Protein-immunoblot with a concentration curve of Hb protein, flooded and unflooded maize roots extracts. Lane MC contains 40 µg of unflooded maize root extract, lane MF contains 40 µg of flooded maize root extract, lane MWM contains 10 µg of protein standards BSA (66kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), tripsin inhibitor (20 kDa) and lysozyme (14.3 kDa). Following lanes contain sequential dilutions of the Hb protein (1:100, 1:50, 1:20, and 1:10). The last lane contains 2.5 µg of pure Hb protein. Relative intensity of the bands was not obtained due to the lack of contrast between the bands and the background.

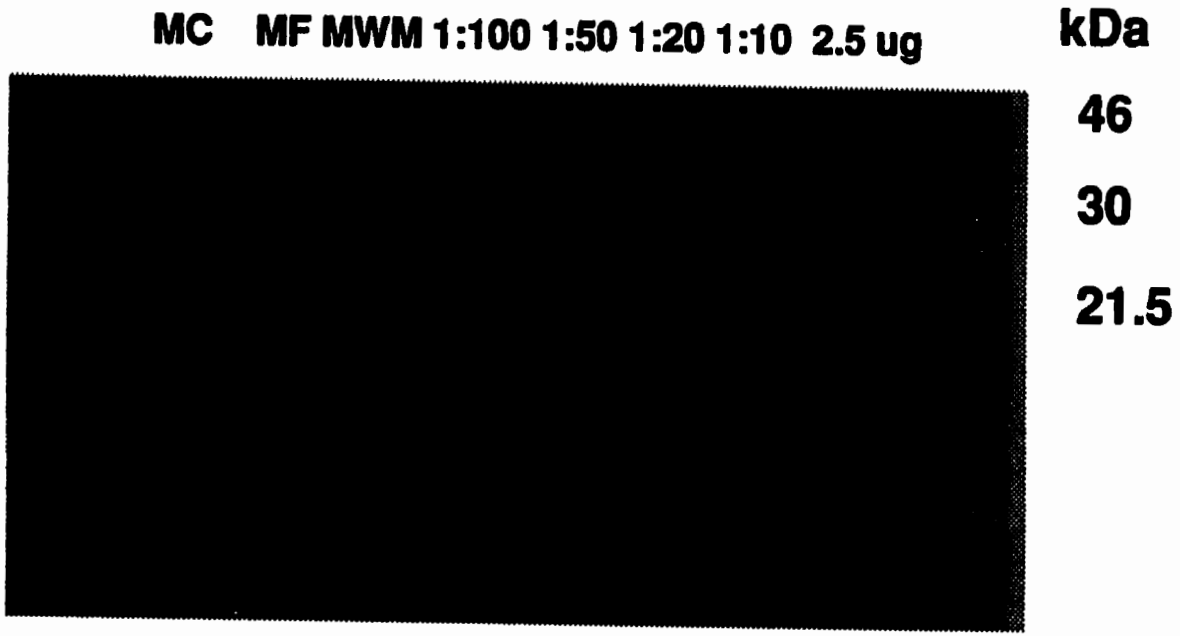
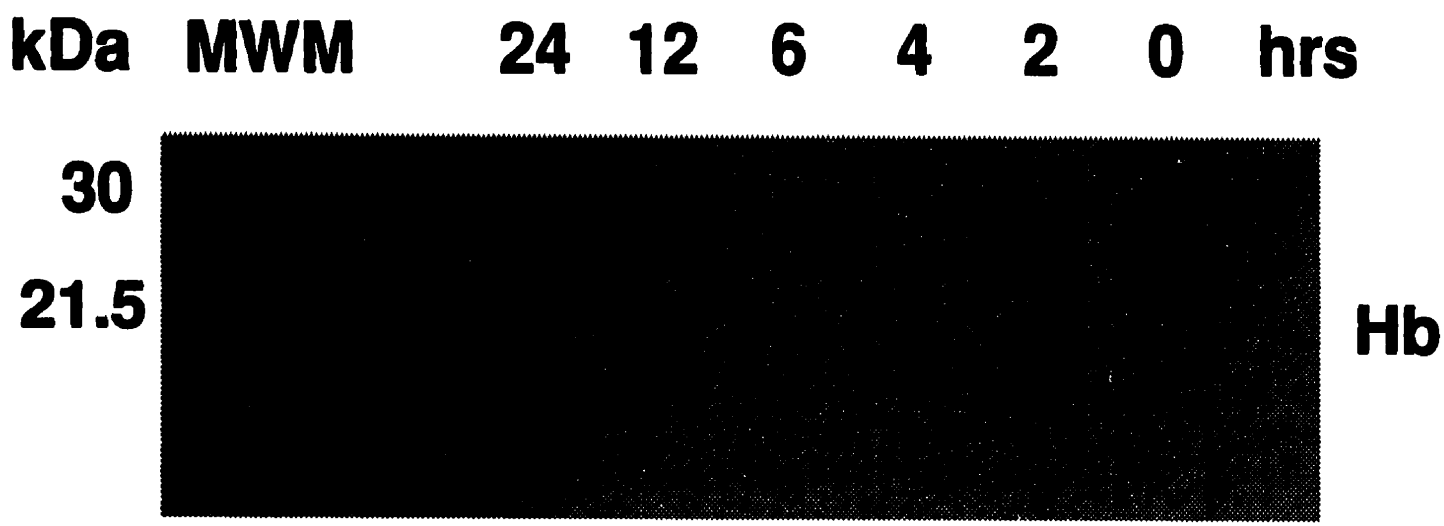

MC MF MWM 1:100 1:50 1:20 1:10 2.5 ug
kDa
46
30
21.5

FIG 13. Protein-immunoblot showing the effect of flooding on the accumulation of the Hb protein in the roots of maize seedlings over time. Maize seedlings were flooded as described in materials and methods. 40 µg of sample were loaded per each well. The left lane contains 10 µg of protein standards BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa). Quantification of the relative intensity of the bands was not obtained due to methodological problems.


kDa
MWM
24
12
6
4
2
0
hrs
30
21.5
Hb

DISCUSSION

Plants as aerobic organisms depend upon the presence of oxygen to survive. However, diverse environmental factors cause changes in the oxygen level available to them. Therefore, in order to survive, plants must first adapt to the hypoxic and then to the anoxic conditions which may prevail in their surrounding environment.

Flooding is one of the principal ways in which anaerobic environments are created. The present work focused on the physiological and molecular mechanisms maize plants develop as a response to low oxygen tensions. Emphasis was placed on the expression of *hb* and *adh* genes.

I. Hydroponic System

The results obtained from the hydroponic system indicated that there was a very slight induction of the *hb* gene in all treatments. *Adh* gene expression also appeared to be induced in all treatments, especially when nitrogen was bubbled in closed systems. Although, the signal for the *adh* gene was much stronger than that for the *hb* gene. These results, along with the necrotic state of the seminal roots, the development of adventitious roots, the general state of the plants and the rate at which the gas mixtures were bubbled, suggested that the hydroponic system was hypoxic. Furthermore, the results also suggested a nitrogen deficiency; the presence of anthocyanin in the roots and in the base of the stems along with the general chlorosis of the leaves are all indicative of a nitrogen deficiency (Kohl et al, 1979; Salisbury et al, 1992). Nitrate supply partly

relieves the growth repression caused by anaerobiosis in the root zone and thus, oxygen starvation is aggravated by nitrogen starvation (Kohl et al, 1979). In our hydroponic systems, the original concentration of nitrate supplied to the plants was 10 mM, sufficient for plant metabolism. However, during the 48 hrs of treatment, the solution was not replenished and therefore, nitrate could have been exhausted by the plants.

Vartapetian et al (1979) reported a topographical distribution of dissolved oxygen in the nutrient solution of hydroponic systems. This distribution was independent of whether or not the nutrient solution was stagnant or vigorously stirred. In these experiments, three electrodes were placed at different points in the nutrient solution: one in the mass of roots, another in the outer region of the roots and a third one in the solution. Results obtained, showed the formation of an oxygen gradient, as a consequence of the consumption rate of oxygen by the roots. Oxygen around the roots was depleted in as little as 30 minutes of exposure to the hydroponic system, while oxygen in the solution remained present for up to three hours. Hence, it appears that even in very well aerated hydroponic systems, roots experience some degree of anaerobiosis. This is due to the slower diffusion of oxygen in water, which results in an inability to compensate for the metabolical requirements of the roots.

Wample and Reid (1975) found that simply surrounding the roots and the stem base with well oxygenated water can stimulate adventitious root initiation and emergence on the stems of sunflower.

Drew (1979) and Konings (1982) supported these findings when measuring the development of adventitious roots and aerenchyma in maize plants in stagnant and

well-aerated solutions. Their results point to ethylene as a main contributor in the anaerobic stress experienced by roots of waterlogged plants. The production of ethylene by roots in liquid medium was found to be three fold higher than in roots grown in filter paper or in soil. Moreover, the application of ethylene to roots grown in air had a retarded and weaker effect on the development of adventitious roots and aerenchyma than in plants grown in aqueous solution. This response is due in part to *de novo* production of endogenous ethylene, which occurs when plants are exposed to low oxygen environments (Drew et al, 1979; Konings, 1982). A second factor involved in the accumulation of ethylene in the vicinity of the roots is the slower diffusion of ethylene in water than in air (Kawase, 1976; Bradford and Dilley, 1978; Jackson and Campbell, 1979; Drew et al, 1979; Kawase, 1979; Konings, 1982, Jackson, 1985; Jackson et al, 1985; Justin and Armstrong, 1991, Sachs et al, 1996).

Further evidence to support the idea of the presence of hypoxic conditions in hydroponic systems came from the enhanced expression of *adh* gene (one of the most characterized and diagnostic genes for hypoxic conditions). Reports in the literature showed the expression of *adh* to be induced at both the transcriptional (Andrews et al, 1993) and translational levels (Laszlo and St Lawrence, 1983; Andrews et al, 1993) when the concentrations of oxygen drops below 10%.

The rate at which the various gas mixtures was bubbled into the nutrient solution was 56 mL/min. This rate was 5 times slower than the values reported for measurements of the *adh* gene (Johnson et al, 1989). When greater quantities of gas were passed through, the resulting pressure built up in the hydroponic tank causing the styrofoam

floats to pop off the top.

Our results suggested that the hydroponic systems experienced oxygen deficiency.

II. Comparative expression of *hb* gene in the roots of plants grown in hydroponic and waterlogged soil systems.

Results from hydroponic systems were ambiguous, as it was unclear whether they were due to the effects of the treatments or whether they were artefacts of the system itself. Hence, it became necessary to find an alternative experimental method. It was previously found in our laboratory that hypoxic conditions can be created by flooding the roots. Under these conditions, the expression of *hb* and *adh* was induced in maize and barley plants (Taylor et al, 1994).

The comparison between waterlogged and hydroponic systems revealed that maize seedlings grown in soil appeared healthier when flooded. At the level of mRNA expression, there was a clear difference between untreated and flooded plants. Non flooded plants had little or no expression of the *hb* gene, while flooded plants had considerable levels of the message.

The ability of maize roots to survive anoxic conditions is greatly improved if the plants are allowed to acclimate to low oxygen conditions (Saglio et al, 1988; Johnson et al, 1989; Andrews et al, 1994). In contrast, when plants are not allowed to undergo the process of acclimation, they experience cell death in about 15 hrs after imposing the anoxic conditions (Roberts et al, 1984; 1992).

In our experiments, the complete coverage of the roots was achieved in approximately 45 min. The oxygen present between the soil particles was slowly replaced by the water, allowing a period of acclimation for the roots. In contrast, hydroponic treatments did not allow any acclimation period because the solution was flushed with the different gas mixes before floating the roots.

The differential intensity between the signals obtained from the hydroponic and the flooded system could be explained on the basis of the presence of some oxygen in the medium. The low rate at which nitrogen was bubbled into the solution created a partially hypoxic system, whereas, the flooded system presented truly anoxic conditions. Furthermore, flooded roots were allowed a period of acclimation, translating into enhanced genetic response and survival to the stress.

III. *Hb* expression in maize seedlings grown in flooded soils

The time course of expression of the *hb* gene in maize flooded roots revealed that the *hb* message is short-lived and precedes that for the *adh* gene. Its accumulation resembled that of the transition peptides reported by Sachs (1980). This group of proteins is strongly expressed during the first hour of anaerobiosis. Hence, our results then suggested two possibilities; either *hb* is involved in the first stages of the anaerobic response, possibly as another transition peptide, or expression is decreased due to the entry of oxygen via developing aerenchyma.

Many reports in the literature described the development of adventitious roots and the formation of air spaces as adaptive responses to low oxygen environments in the

roots of higher plants (Kramer, 1951, 1969; Armstrong, 1979; Wample and Reid, 1975; Coutts and Armstrong, 1976; Drew, 1979). The presence of aerenchyma facilitates the diffusion of oxygen from aerial parts of the plant into the roots, contributing to the survival of flooded plants. Development of root cortical air spaces varies among the plant groups, but is evident after 6-12 hrs of oxygen shortage (Webb and Jackson, 1986). In fully submerged plants, the shoots also develop aerial spaces after 24 hrs (Grineva et al 1988).

IV. The contribution of oxygen from aerial parts to anaerobic expression in entirely flooded maize seedling roots.

The objective of completely submerging the maize seedlings was to further our understanding of the role of the development of aerenchyma in air translocation from the leaves into the roots of flooded plants. Many reports point at the contribution of aerenchyma as a means to compensate for the anaerobic conditions prevailing in the roots. By measuring the levels of mRNA accumulation over time, we found non significant differences between flooded roots and fully submerged plants. Our results then indicated that the development of aerenchymatous tissue does not seem to alleviate the anaerobic conditions. However, all our experiments were performed in primary roots and the development of aerenchyma and its effects occur principally in adventitious roots (Campbell and Drew, 1983; Drew et al, 1979; Thompson et al, 1990).

The results the indicated two possibilities. One suggesting that the expression of the hb gene is transient once plants are exposed to low oxygen conditions. The second

option pointed at the contribution of photosynthetically produced oxygen on the concentration of oxygen dissolved in the water flooding the maize plants.

V. Is there a contribution of the photosynthetically produced oxygen into the anaerobic response of flooded maize seedlings?

McPhersen (1939) found that photosynthetically produced oxygen is not transported to the roots in sufficient quantity to affect the development of aerenchyma and to compensate for the hypoxic conditions in flooded roots. When other conditions remained constant, air space formation was equally pronounced whether the plants grew in complete darkness, in alternating periods of light and dark, or under continuous illumination. Our results are in accordance with his findings. The pattern of expression for both the *hb* and *adh* genes seems to be the same whether the roots or the whole plants were flooded. A similar pattern was also observed when the experiment was carried out in the dark. Therefore, during flooding stress, *hb* mRNA accumulation occurred exclusively at early stages of the anaerobiosis. The onset of *adh* gene expression occurs early during the anaerobic period as well, but it continues for up to 48 hrs.

It is important to note that experiments performed with flooded systems showed a time dependent expression of the *hb* gene. However, the previous experiments (in hydroponic systems) were performed at the 24 hrs point, at which time the level of the message had decreased considerably. Hence, the difference in the level of expression observed between both systems might be due in part to this factor.

VI. Tissue specific expression of *adh* and *hb* genes in maize flooded roots

Under anaerobic conditions, the metabolism of the various tissue of the roots is heterogenous due to their metabolic requirements, morphology and environmental conditions at which they are exposed. The outer layers are in contact with the source of oxygen and therefore, experience low or no oxygen deficiency. The inner portions, on the other hand, will suffer a more severe degree of anaerobiosis. The slower diffusion of O_2 into the compact stellar cells as well as the higher respiratory demand per unit of volume in the stele in comparison with the cortex also have a determinant influence in the differential conditions of stele and cortex. Thompson and Greenway (1991) reported the development of an anaerobic stele while the cortex remained aerobic, in maize roots exposed to low oxygen conditions. Their findings were based on ADH and PDC activity measurements.

Our results are in accordance to Thompson and Greenway findings. They indicated that *adh* transcription is strongly enhanced in a tissue specific fashion under flooded conditions. The accumulation of the a*dh* message in the stele was induced 1.5 fold. At the translational level, the activity of the ADH enzyme was shown to be induced 17 fold in the stele and 13 fold in the cortex, indicating that the increase of activity corresponds to activated gene transcription. Our results for the *adh* gene and gene products support the idea of the development of an anaerobic stele in maize roots under flooding conditions.

In terms of the *hb* gene, the results are not that striking. Our results showed the

induction of *hb* in both the stele and the cortex after 4 hrs of flooding. The induction in the stele is 1.1 fold higher in the stele than in the cortex.

Previous experiments performed by Bogusz et al (1990) with transgenic plants revealed that *Parasponia* and *Trema* promoters directed the expression of a reporter gene preferentially in the root tips and vascular cylinder of tobacco plants. However, experiments to localize the endogenous *hb* for these species have not been performed yet and might show a similar pattern to the one obtained in our experiments.

The induction of the ANP's by low oxygen tensions shows diverse patterns. Each gene is induced at a different rate. The magnitude of the induction and time at which it reaches its maximal level, as well as the stability of the mRNA once it is released into the cytoplasm is different as well. All these events contribute to the overall control of gene expression (Gallie, 1993; Green, 1993; Jackson, 1993).

In our experiments, differences in the level of *adh* and *hb* messages are due in part to the above mentioned factors. However, during the course of this study, differences in the level of *adh* and *hb* messages do not necessarily reflect their real levels in the cell. Homology differences between the probes and the RNA's made it difficult to obtain inter-specific cross-hybridization signals. *Adh* signals were stronger because both the probe and the RNA were obtained from maize, while signals for the hb message were considerably lower due to sequence divergence and therefore, lack of hybridization between the barley probe and maize RNA.

Bogusz et al (1988) were unable to detect hemoglobin in several species because of the absence of cross hybridization as a result of sequence divergence.

VII. Is *hb* gene translated into protein?

A time course of accumulation of the Hb protein in maize flooded roots revealed the presence of the protein in both unflooded and flooded plants. The protein level showed a slight increase after 12 hrs of flooding. The results showed no direct correlation between the relative levels of *hb* mRNA and protein.

To explain our results, there are three possibilities, one that modifications of the translational machinery, such as phosphorylation of ribosomal proteins and/or initiation factors may be required for the selective translation of the hb protein. Therefore, these modifications may be influencing the rate at which the protein is being translated. A second possibility is that hb protein is being produced but needs to undergo some post-translational modifications in order to be recognized by the antibody. The third possibility suggests that hb message is being transcribed but not translated.

It is well known that under stress, mRNA reaches levels in excess of what would be sufficient to account for the activity observed and needed to survive. However, mRNA levels do not always correlate to protein levels or activity (Hoffman et al, 1986; Rivoal et al, 1990; Ricard et al, 1994). Anaerobic mRNA's are not translated in direct proportion to their relative amounts. Russell and Sachs (1992) found no correlation between the accumulation of mRNA for *adh1* and *gpc3* and the levels of protein in maize roots. Sucrose synthase (Taliercio and Chourey, 1989) *pdc3* and enolase (Peschke and Sachs, 1994; Russell and Sachs, 1992) are other examples. They show a very significant increase in mRNA levels without a concomitant production of the protein.

Gallie (1993) reported that the wide disparity between the accumulation of

mRNA and the accumulation of the anaerobic proteins is a feature of post-translational regulation of gene expression. Hence, induction of enhanced levels of transcripts is important at early stages of anaerobiosis. Furthermore, stability of the transcripts, selective translation, a slower turnover and/or post-translational modifications of the products may be important in long term survival during anoxia (Hole et al, 1992; Andrews et al, 1994).

At the enzymatic activity level disparities also occur, ADH transcripts are not correlated with enzyme activities. The mRNA increases rapidly and then declines whereas the enzymatic activity continues rising throughout 48-72 hrs or for as long as the anaerobic conditions prevail (Andrews et al, 1993, 1994a, 1994b).

Very little is known about how plants sense the changes in oxygen availability in their surroundings. As well, very little is known about the sensing mechanism triggering the switch of metabolism from aerobic to anaerobic and the signal transduction pathway involved in this process.

Jackson (1985), Drew (1990), and He (1994) reported the involvement of phytohormones (ethylene and ABA) in the intercellular and long distance signaling of anoxia. However, the intracellular component of the oxygen sensing mechanism as well as its signal transduction pathway are still unclear.

Hemoglobin was suggested to signal low oxygen concentrations in plants (Appleby, 1988). However, Taylor et al (1994) found the *hb* message to be upregulated under low oxygen conditions in barley aleurone layers and maize and barley roots. The kinetics of the upregulation of the *hb* message in barley aleurone layers and roots ruled

out the possibility of hb acting as an oxygen sensor. On the other hand, in maize roots *hb* presents a different response. The message is rapidly induced during the first minutes of anaerobiosis. The kinetics for the induction of *hb* in maize roots resemble that of the TP's (Sachs et al 1980), suggesting the involvement of hb in the early events of the anaerobic response.

Nevertheless, recent findings in our laboratory indicate that hb is involved in the energy production metabolism rather than in the oxygen sensing process (Nie et al, 1997). The kinetics for a recombinant barley hb expressed in bacteria show that hb has an extremely high affinity for oxygen and a very slow dissociation constant (Duff et al, 1997).

However, in spite of these findings, the exact function of hb in maize flooded roots remains to be determined.

CONCLUSIONS

1. Hydroponic systems as tested in our experiments, are not suitable to conduct comparative studies for the *hb* gene expression, due to the hypoxic conditions prevailing in them. However, they are suitable for experiments with the *adh* gene.

2. *Hb* gene expression is induced in the roots of flooded maize plants.

3. The accumulation of the message for *hb* under flooding conditions is short lived and precedes that for the *adh* gene.

4. *Hb* expression is tissue specific in the roots of flooded maize.

5. Hemoglobin protein is present in both flooded and unflooded plants. A slight induction occurs after 12 hrs of flooding.

6. The pattern of accumulation of the protein does not parallel that of the message.

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