Production of Nitrate Reductase from Corn (*Zea mays* L.) Cell Suspension Culture

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Thesis Title	Production of Nitrate Reductase from Corn (*Zea mays* L.) Cell Suspension Culture.
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ชื่อวิทยานิพนธ์ การผลิตเอ็นไซม์ไนเตรตรีดักเทสจากการเพาะเลี้ยงเซลล์แขวนลอยข้าวโพด

ผู้เขียน นางสาวจิราวดี พูลสุข

สาขาวิชา ชีวเคมี

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บทคัดย่อ

การชักนำแคลลัสข้าวโพดหวานเพื่อใช้เตรียมเซลล์แขวนลอยสำหรับศึกษาการควบคุมการทำงานของเอนไซม์ไนเตรตรีดักเทส (NR; EC 1.6.6.1) พบว่าคัพภะอ่อนขนาด 1-2 มิลลิเมตร เหมาะต่อการชักนำ โดยนำมาเพาะเลี้ยงบนอาหารสูตร N6 ที่มี 2,4-D ความเข้มข้น 2 มิลลิกรัมต่อลิตร และเก็บรักษาไว้ในที่มืด นอกจากนี้พบว่าแนวการวางคัพภะในแนวราบหรือแนวตะแคงข้างบนอาหารแข็งไม่มีผลต่อการชักนำแคลลัสจากคัพภะอ่อนแต่มีผลต่อการชักนำแคลลัสจากคัพภะที่เจริญเต็มที่แล้ว เมื่อเพาะเลี้ยงเซลล์แขวนลอยโดยเพิ่มปริมาณน้ำตาลซูโครสในอาหารเหลวเป็น 3% ทำให้แคลลัสเจริญเติบโตดีที่สุด

เมื่อศึกษาวิธีที่เหมาะสมในการหาค่าความว่องไวของเอนไซม์ไนเตรตรีดักเทส พบว่าทั้งแบบ *in vitro* และ *in vivo* ให้ผลใกล้เคียงกัน และจากการศึกษาความสเถียรของเอนไซม์พบว่าการสกัดเอนไซม์ในบัฟเฟอร์ที่มีทั้ง chymostatin และ PMSF ซึ่งเป็นสารยับยั้งการทำงานของเอนไซม์โปรติเอส ให้ค่าความว่องไวของเอนไซม์และมีความเสถียรสูงที่สุด นอกจากนี้ค่าความว่องไวของเอนไซม์ไนเตรตรีดักเทสถูกยับยั้งได้ด้วย Mg^{2+}

ในระหว่างการเพาะเลี้ยงเซลล์แขวนลอยในอาหารสูตร N6 ที่มีไนเตรต พบว่าความว่องไวเอนไซม์ไนเตรตรีดักเทสของสูงสุดในวันที่ 4 ซึ่งเป็นช่วงที่แคลลัสเริ่มมีการเพิ่มของน้ำหนักอย่างรวดเร็วหลังจากการเปลี่ยนอาหาร หลังจากนั้นความว่องไวของเอนไซม์จะลดลงอย่างรวดเร็ว ขณะที่ปริมาณโปรตีนเพิ่มขึ้นเพียงเล็กน้อยในวันแรกต่อจากนั้นจะคงที่ ปริมาณไนไตรต์ที่สะสมในเซลล์เพิ่มขึ้นอย่างมากในวันที่ 4 หลังจากเปลี่ยนอาหาร ซึ่งมีผลยับยั้งการทำงานของเอนไซม์ไนเตรตรีดักเทสและเป็นพิษต่อเซลล์ การศึกษาผลของช่วงแสงต่อการทำงานของเอนไซม์ไนเตรตรีดักเทสพบว่าเมื่อได้รับแสง 16 ชั่วโมง/8 ชั่วโมงในที่มืด ค่าความว่องไวของเอนไซม์เพิ่มขึ้นสูงสุดหลังจากได้รับแสง 4 ชั่วโมง จากนั้นจะลดลงและคงที่เมื่อเข้าสู่ช่วงมืดในรอบวัน และพบว่าน้ำตาลลดการยับยั้งการทำงานของเอนไซม์ในช่วงมืดของวัน ไม่พบการทำงานของเอนไซม์ไนเตรตรีดักเทสเมื่อไม่มีไนเตรต การศึกษาปริมาณไนเตรต แอมโมเนียม น้ำตาลชนิดต่างๆ ในอาหารเหลวสูตร N6 และความเข้มแสงในการชักนำเอนไซม์ไนเตรตรีดักเทสระหว่างการเลี้ยงเซลล์แขวนลอย พบว่าเมื่อเลี้ยง

แคลลัสในอาหารเหลวที่มี KNO_3 ความเข้มข้น 30 มิลลิโมลาร์ และไม่มีแอมโมเนียม ให้ค่าความว่องไวของเอนไซม์สูงสุด แสดงให้เห็นว่าการทำงานของเอนไซม์ถูกยับยั้งโดยแอมโมเนียม เมื่อได้รับน้ำตาลต่างชนิดกันได้ความว่องไวของเอนไซม์สูงที่สุดในแคลลัสที่เลี้ยงในอาหารเหลวสูตร N6 ที่มีน้ำตาลฟรุกโตสเป็นแหล่งคาร์บอนที่ความเข้มข้น 3%ในอาหารเหลวสูตร N6 และเมื่อได้รับความเข้มแสงที่ระดับ 345 $\mu mole\ m^{-2}\ s^{-1}$ ให้ค่าความว่องไวของเอนไซม์สูงที่สุด

การเปรียบเทียบความว่องไวของเอนไซม์ไนเตรตรีดักเทสในต้นกล้าข้าวและข้าวโพดอายุ 1 สัปดาห์ พบว่าต้นกล้าข้าวมีค่าความว่องไวของเอนไซม์ไนเตรตรีดักเทสสูงกว่าข้าวโพดเมื่อได้รับไนเตรตที่ความเข้มข้นเดียวกัน และแอมโมเนียมมีผลยับยั้งการทำงานของเอนไซม์ไนเตรตรีดักเทส เมื่อเปรียบเทียบการทำงานของเอนไซม์ไนเตรตรีดักเทสในต้นกล้าข้าวโพดและในเซลล์แขวนลอย พบว่า เซลล์แขวนลอยให้ค่าความว่องไวของเอนไซม์สูงกว่า เอนไซม์ไนเตรตรีดักเทสในแคลลัสข้าวโพดมีคุณสมบัติ NAD(P)H-bispecific เช่นเดียวกับในราก สามารถใช้ NADH และ NADPH เป็นตัวให้อิเล็กตรอน โดยมีค่า K_m ของ NADH เท่ากับ 0.27 มิลลิโมลาร์ และค่า K_m ของ NADPH เท่ากับ 0.302 มิลลิโมลาร์ เมื่อใช้ความเข้มข้นของ KNO_3 เป็น 10 มิลลิโมลาร์

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Abstract

Friable calluses (Type II) were induced from 1-2 mm immature embryos of sweet corn with 2,4-dichlorophenoxyacetic acid (2,4-D) at 2 mg. l^{-1} under dark condition, in order to establish a cell suspension culture in N6 medium. The orientation of immature corn embryos, both horizontal and vertical placement on N6 medium culture had no effect on callus induction. The callus growth was highest in N6 liquid medium supplemented with 3% sucrose.

NR activity measured by the *in vitro* and *in vivo* assays showed similar trends. The NR activity of enzyme extracted in the presence of both chymostatin and PMSF, without Mg^{2+}, was very stable and highest among other buffers. In contrast, corn NR activity was sensitive to Mg^{2+} inhibition, even in the presence of both chymostatin and PMSF; the NR activities of that remained lower than other extraction buffers and decreased slightly throughout the incubation on ice.

During the preliminary study, rapid increase of NR activity was observed on the 4^{th} day after the transfer of the calluses to the liquid medium, which coincided with the callus growth. There was also a direct relationship between callus growth and NR activation. The protein content was increased very little on the 1^{st} day, then constant throughout culture period. However, the increase of nitrite accumulation in the calluses inhibited the callus growth and NR activation. Analysis of NR activity in cultured calluses over the 16 h light: 8 h dark photoperiod showed light/dark-dependent variations. However, no NR activity was detected in the absence of nitrate. In dark condition, sucrose supply in the presence of nitrate partially prevented the inhibition of NR

activation. Factors regulating the expression of NR activity in corn cell suspension culture such as concentrations of sugar (sucrose, glucose and fructose), nitrate and ammonium ions in the medium were investigated. In N-free media no NR activity was detected in the cells. The optimal nitrate concentration for induction of high NR activity was about 30 mM of KNO_3 in N6 medium in the absence of 3.5 mM $(NH_4)_2SO_4$. The presence of ammonium ions in the culture medium resulted in low levels of NR activities. The effect of nitrate and ammonium ions on NR activity in callus suspension culture suggested that ammonium inhibited NR activity. The role of sugars on the induction of NR was inferred from the observation that exogenously supplied sugars led to increasing NR activation. It was shown that fructose, in the presence of nitrate, markedly increased the levels of NR activity more than sucrose and glucose.

The values of NR activities were determined in corn calluses illuminated with 354 μmol m^{-2} s^{-1}. The activities of NR and protein levels were largely correlated under the same light intensity. When light intensity was increased, the NR activity and protein level increased. It is concluded that, in corn callus suspension, light, nitrate and sugar (especially fructose) could induce NR.

Rice seedlings showed markedly higher nitrate reduction than corn seedlings at the same concentration of nitrate. NR activity increased with increasing nitrate concentrations in corn seedlings. However, the NR activity of both species was greatly reduced in the presence of ammonium ion. The NR activity in corn callus was higher than that in corn seedlings. However, the NAD(P)H-NR activity of corn calluses was estimated as NADH-NR activity of shoot plus NAD(P)H-bispecific NR activity of root of corn seedlings. The response of corn calluses to NR induction was similar to that of corn seedling tissues, especially roots. In corn calluses crude extract, the apparent K_m-values of NR are 0.27 mM for NADH and 0.302 mM for NADPH. This showed that in corn callus, NR was NAD(P)H-NR bispecific.

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I wish to thank the teaching staff for providing the necessary advice. I also would like to acknowledge the Graduate School and the University Academic Excellence Strengthening Program, which made this study possible. My special appreciation goes to my friends, who helped in everything, all colleagues who gave the invaluable advice and finally, staff of the Department of Biochemistry, Faculty of science, Prince of Songkla University as well as colleagues at the laboratory room ST. 418 for their continuous supports.

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List of Abbreviations

A	= Absorbance
BSA	= Bovine serum albumin
°C	= Degree Celsius
2,4-D	= 2,4-dichlorophenoxyacetic acid
DTT	= Dithiothreitol
EDTA	= Ethylenediaminetetra acetic acid
FAD	= Flavin adenine dinucleotide
g	= Gram
h	= Hour
kDa	= Kilodalton
K_m	= Michaelis constant
L	= Litre
M	= Molar
m	= Meter
mg	= Miligram
min	= Minute
ml	= Milliliter
mm	= Millimeter
MOPS	= 3-[N-Morpholino]propane-sulfonic acid
µg	= Microgram
µl	= Microliter
µmole	= Micromole
µM	= Micromolar
µmole/min/ml	= Micromole per minute per milliliter
NADH	= reduced nicotinamide adenine dinucleotide
NAD(P)H	= reduced nicotinamide adenine dinucleotide phosphate
nm	= Nanometer
NiR	= nitrite reductase

NNEDA	= N-(1-napthyl)ethylenediamine dihydrochloride
NR	= nitrate reductase
OD.	= Optical Density
pH	= -log hydrogen ion concentration
PMSF	= Phenylmethylsulfonylfluoride
pNR	= Phosphorylated nitrate reductase
rpm	= Revolutions per minute
RT	= Room temperature
v/v	= Volume/volume
wt.	= Weight
w/v	= Weight/volume
%	= Percent

Chapter 1

INTRODUCTION

Introduction

Nitrate is a common contaminant of ground and surface water worldwide. The potential for high nitrate concentrations occurs when crops such as corn, sorghum, cereal grains and some grasses are exposed to drought, hail, frost, cloudy weather, or soil fertility imbalance (Campbell *et al.*, 2002). Nitrates accumulate in the lower portion of the plant when stresses reduce the crop yield to less than that expected based on the supplied nitrogen fertility level. Consumption of water containing high nitrate levels is harmful for livestock. Nitrate interferes with the ability of the blood to carry oxygen and can be toxic to humans, causing methemoglobinemia in infants (blue baby syndrome) and oxygen transport problems for the elderly and others with compromised hemoglobin levels (Campbell *et al.*, 2001). The standard method for nitrate analysis by using flow injection analysis (Alves *et al.*, 2002) has at least one negative attribute (cost, difficulty, potential interferences, or handling of hazardous materials), and cannot provide data of reliable accuracy for samples with colored contaminants (Glazier *et al.*, 1998).

A major trend in environment analysis today is the drive toward on site measurement techniques. The result of this trend is increased utilization of field test kits in place of traditional laboratory methods. Bioanalytical methods, including immunoassays and enzyme-based assays have the additional benefit of being safer for the user. Enzyme-base nitrate analysis method is the developed method that is easy to perform on site while retaining the ability to provide high-quality data. The methods for quantification of nitrate based on nitrate reductase (NR) driven reduction to nitrite and colorimetric nitrite analysis were described, in plants, for example, corn (*Zea mays* L.) leaf NADH-nitrate reductase (NR; EC 1.6.6.1) is utilized for nitrate testing in environment (Campbell *et al.*, 2001). The NR from corn leaves, purified by immonoaffinity

chromatography, can replace the cadmium or zinc reagents used in colorimetric nitrate test kits. The nitrate biosensors based on NR are accurate and specific for nitrate, moreover, produce quantitative results in the low-range format, in most aqueous samples. In addition, nitrate testing based on NR is less polluting alternative to tests based on toxic heavy metals and has become a standard in biochemical research where nitrate and nitrite are often monitored as indicators of nitric oxide production (Campbell, 1999). NECi purified NADH-NR from corn leaves by monoclonal antibody-based immunoaffinity chromatography (Campbell, *et al.*, 2002). Corn NR has a high specific activity (40 to 60 units/mg NR; unit = µmol/min). This is the highest specific activity NR (i.e. most highly purified) commercially available anywhere in the world. Corn NR is highly stable and can be shipped at room temperature. Since corn leaf NR uses NADH as electron donor, it is less expensive to use than NADPH dependent NR forms, such as those purified from fungi (Campbell,1999).

NR is one of the key enzymes involved in nitrogen assimilation in plants. It is considered to be a limiting factor for higher plant growth, development and protein production (Ogawa *et al.*, 2000). Nitrate uptake and assimilation are regulated both on a transcriptional and post-translational level. Moreover, NR is an inducible enzyme, depending on the availability of nitrate, light and interaction with carbon metabolism. In all plants, NR activity increases dramatically in response to an addition of nitrate. As nitrate is depleted or removed, NR activity decreases. Increased NR activity results from increased synthesis of NR mRNA and protein. Several studies have shown that NR mRNA is very low prior to the addition of nitrate (Su *et al.*, 1996). Although nitrate is the primary inducer of NR, light is also required for maximum effect. NR activity is level low in dark treated plants exposed to nitrate. Upon exposure to light, nitrate treated plants accumulate maximum amounts of NR activity (Li and Oaks, 1995). Nitrate assimilation is dependent on light not only for the generation of reducing power and ATP but also for the supply of carbon skeletons (sugar) which are synthesized from carbon metabolism (Larios *et al.*, 2001). In this study, NR activities were investigated in corn cultures in

various conditions such as nitrate content, light intensity and sugar (sucrose, fructose and glucose) contents.

Plant tissue culture involves the culture of plant cells, tissues and organs on an artificial nutritional medium. Tissue cultures make use of the fact that the forms and functions of plant cells can be influenced by changes in their chemical and physical environments. Since one can control both the chemical and physical environments of tissue cultures, it is possible to achieve significant control over plant cell growth and development. Tissue culture can be used as a tool for both basic scientific experimentation and in practical applications such as propagation and production of enzymes from cell cultures.

In vitro plant culture systems have been developed that facilitate morphological, physiological, cytological, biochemical and molecular work on differentiation. Such cultures approximate the responses of their whole plant counterparts genetically and morphologically, and are highly suitable for growth and differentiation studies in terms of generation of material at the required stage of morphogenesis and manipulation of nutritional and environmental parameters (Roberts *et al.*, 1996). Cultures of friable (Type II) calluses initiated from corn explants remain the most important source of tissue for production of the enzyme NR. In this study, corn cell suspension culture system was used to examine the regulation of NR expression, particularly the effects of metabolites on NR activity. Culture conditions for induction of corn callus, appropriate methodologies for extraction and assay of NR during callus growth and development in suspension culture were established.

Literature Review

1. Nitrate reductase

1.1 Nitrogen assimilation in higher plants

The mineral nutrient required in greatest abundance by plants is nitrogen. Nitrogen is needed mostly for the synthesis of amino acids and nucleotides. Elemental analysis reveals that 2 to 6 % of plant dry matter is composed of N in the form of amino acids, proteins and nucleic acids. In these compounds nitrogen exists in its most reduced state (-3). Inorganic forms of nitrogen can serve as sources of nitrogen. The principal inorganic form of nitrogen, which is absorbed by agricultural plants, is in the form of nitrate (NO_3^-) which is in its most oxidized form (+5) (Kaiser and Huber, 1994). Nitrate plays a key role in the biochemical nitrogen cycle, which is contributed to by both prokaryotic and eukaryotic organisms (Campbell, 2001). Many bacteria, plants and fungi are capable of this assimilatory nitrate reduction. Nitrate is reduced to nitrite by the enzyme nitrate reductase (NR) and nitrite is then reduced to ammonia in a series of two electron transfers by nitrite reductase (NiR), these pathways which are diagrammed in Figure 1.

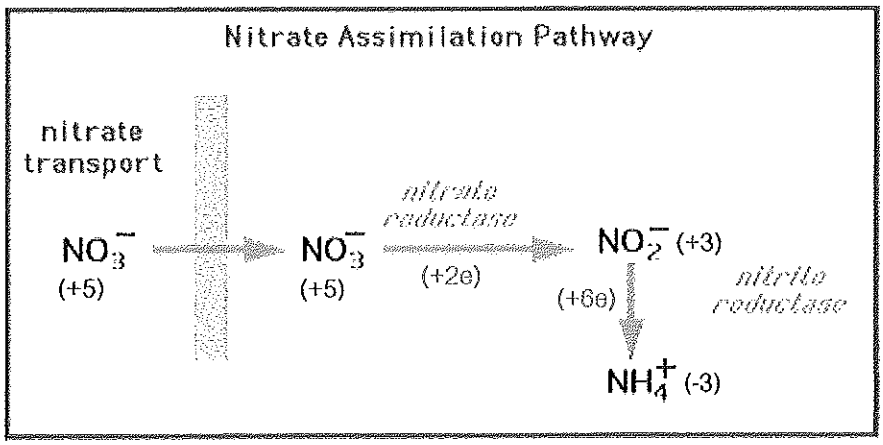

Figure 1 Nitrate assimilation pathway.

The final step of the pathway is the incorporation of ammonia into glutamine by glutamine synthetase and the synthesis of amino acids, proteins and nucleic acids (Der Leij *et al.*,1998). Reducing energy is provided in the form of either NADH or NADPH for

NR and reduced ferredoxin for NiR. Glutamate provides the immediate carbon skeleton. Coordinating these steps is a regulatory network that is responsive to both internal and external signals. In addition, nitrate is not only the major N-source for plants but it is an important signaling molecule that influences plant growth, differentiation, root morphogenesis, shoot: root balance and carbon metabolism adaptation (Campbell, 2001).

1.2 Structure and function of the NAD(P)H: nitrate reductase

In plants, NR is a soluble enzyme composed of 2 polypeptides of about 100 kDa polypeptide each binds 1 equivalent of molybdenum-molybdopterin (Mo-MPT), heme-Fe and FAD (Figure 2a). The structure of the NR monomeric unit, which is generally assumed to be catalytically independent of the other subunit in the enzyme complex, is built from modular sections of the polypeptide folded into quasi-independent conformations to house the redox cofactors and form the two active sites as well as the dimer interface region. Overall, the monomer of NR can now be viewed as having eight distinct sequence regions as shown in figure 2a: (Campbell, 2001)

(i) An N-terminal sequence which is variable in length for different NR forms and may be involved in activity regulation in plant NR forms.

(ii) A Mo-MPT domain with nitrate binding and reducing active site.

(iii) Dimer interface domain, which also contributes to the nitrate reducing active site.

(iv) Hinge 1 with a variable sequence, a highly sensitive proteolytic site and the site of the regulatory Ser residue in plant NR which becomes phosphorylated.

(v) A Cyt *b* domain where heme-Fe is bound.

(vi) Hinge 2 which is variable in length and contains a proteolytic site in many NR forms.

(vii) An FAD binding domain, which contributes along with the pyridine nucleotide binding domain to form the NAD(P)H reaction active site where the enzyme is reduced.

(viii) An NAD(P)H binding domain at the C-terminus which is joined to the FAD domain by a short linker containing a three-strand β sheet.

Dimerization is required for activity, and the native NR is a homodimer with a tendency to further dimerize to a homotetramer (dimer of dimers), with this difference expected to make only a slight impact on functionality. NR transfers two electrons from NAD(P)H to nitrate via three redox centers composed of two prosthetic groups (FAD and heme) and a MoCo cofactor (Figure 2b), which is a complex of molybdate and pterin. Each redox center is associated with a functional domain of the enzyme that has activity independent of the other domains (Solomonson and Barber, 1990). It has two active sites, which are joined by the internal electron transport pathway from FAD via heme-Fe to the Mo-MPT (Figure 2a). At the first active site, electrons are donated to the enzyme at the FAD by NADH, or NADPH in specific isoforms or both in various bispecific isoforms of NR, with this difference in pyridine nucleotide specificity being the major functional feature distinguishing the family members. At the second active site, two electrons are transferred from the reduced Mo IV to nitrate, reducing it to nitrite and hydroxide (Figure 2a). Both products are weak inhibitors with millimolar K_i values, whereas K_m values for NADH and nitrate are in the low micromolar range (Campbell, 2001). Eukaryotic NR has only limited similarity to prokaryotic NR, which also contains Mo and a pterin cofactor (Kramer *et al.*, 1987) and differs from eukaryotic MPT in having an additional nucleotide; in some cases, two pterins are coordinated to the Mo. The structural models of FAD-containing fragment and cytochrome *b* reductase fragment of maize are shown in Figure 2c and 2d, respectively.

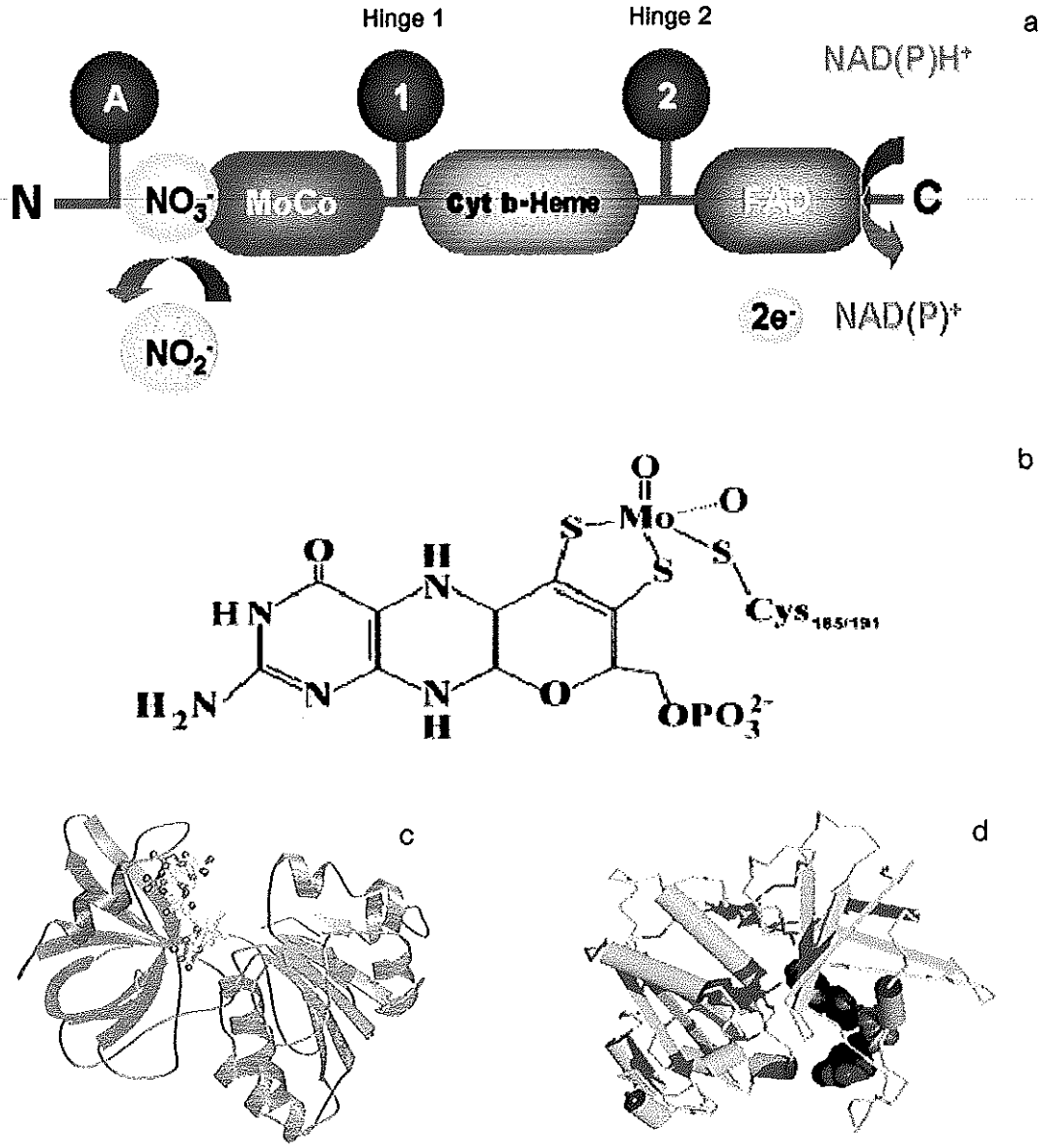

Figure 2 Models of eukaryotic nitrate reductase and the Mo-molybdopterin (Mo-MPT) cofactor. (The above image was modified form Campbell, 2001; Lu *et al.*, 1994 and Lu *et al.*, 1995)

a. Functional schematic for NR showing the three domains containing FAD, heme-Fe and Mo-MPT cofactor.

b. Structure of the Mo-MPT cofactor.

c. 3D structure of FAD-containing fragment of corn nitrate reductase.

d. 3D structure of cytochrome *b* reductase fragment.

1.3 Location of the enzyme

In plants, nitrate is an important source for nitrogen (Forde, 2002). Nitrate assimilation begins with the uptake of nitrate from the soil solution by the root. Root epidermal and cortical cells are the first to actively transport nitrate into their cytosols (Crawford, 1995). Nitrate then crosses the endodermis of the root and is released into the xylem. After long distance transport up the xylem, nitrate is again actively transported into cells of the leaf (Figure 3a). The H^+-ATPase in the plasma membrane pumps protons out of the cell, producing pH and electrical gradients. The nitrate transporter cotransport two or more protons per nitrate into the cell. Nitrate can be transported across the tonoplast and stored in the vacuole. Nitrate in the cytosol is reduced to nitrite, which enters the plastid and is reduced to ammonia (Crawford and Glass, 1998). Ammonia is fixed into glutamate (Glu) to produce glutamine (Gln) by the action of glutamine synthetase (GS) (Figure 3b). Nitrate also acts as a signal to increase the expression of nitrate reductase (NR), nitrite reductase (NiR), and nitrate transporter (NRT) genes. There is general agreement that the NADH and NAD(P)H nitrate reductase enzymes are localized in the cytoplasm (Datta and Sharma, 1999). Several plants reduce nitrate both in the root and in the shoot (Crawford, 1995), with the bulk of the reduction occurring in the shoot. Other species limit their reduction to the shoot, while in other species most of the reduction occurs within the root. Interestingly, in barley when the NADH requiring NR is mutated the NAD(P)H requiring enzyme is expressed in shoots as well as in roots. Thus, it serves like a back-up enzyme when the main enzyme do not function in the shoot. In maize, the shoot NR is NADH dependent, whereas the root posses an NADH-NR as well as an NAD(P)H-bispecific NR (Feil *et al.*, 1993).

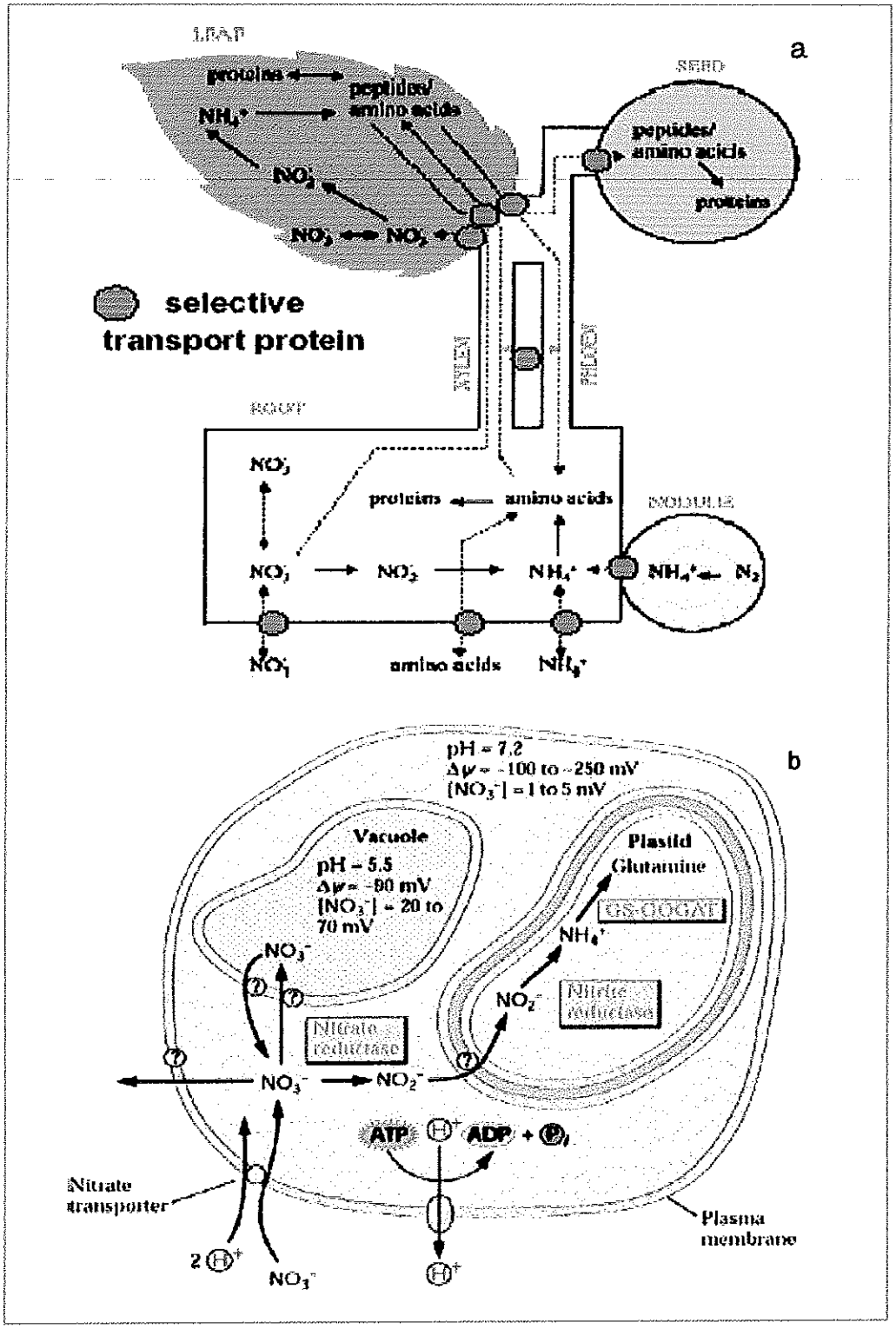

Figure 3 Transports of nitrate (NO_3^-), ammonium (NH_4^+) and amino acid in plants (a) and schematic diagram of the Nitrate Assimilation Pathway in cells (b). (Crawford and Glass, 1998)

1.4 Nitrate transport in plant

Nitrate concentration in the cytosol can reach values of between 1 to 30 mM via the activity of the nitrate transporters. Several fates may await the absorbed NO_3^-:

a. Transport across the tonoplast and storage in the vacuole.

b. Transport across the root and delivery to the xylem to be translocated to the shoot.

c. Biochemical reduction to NO_2^- in the root or the shoot through the activity of the enzyme nitrate reductase.

The nitrate uptake system in plants must be versatile and robust because plants have to transport sufficient nitrate to satisfy total demand for nitrogen even at low external nitrate concentrations. To function in efficiently such environmental variation, plants have evolved a transport system that drives nitrate uptake from the proton gradient maintained across the plasma membrane by the H^+-ATPase. The nitrate uptake system of higher plants consists of a constitutive, low affinity nitrate transport system (LANTS) (possibly a carrier system or an anion channel), and an inducible, high affinity nitrate transport system (HANTS) regulated by cellular energy supply, and by intracellular nitrate consumption, whose activity depends on the proton electrochemical gradient (Forde, 2000). The latter system is regarded as H^+/anion co-transport carrier mechanism that produces transient plasma membrane depolarization upon addition of nitrate. The depolarization is counteracted by the plasma membrane H^+-ATPase (Crawford, 1998). The plasma membrane proton ATPase is induced by nitrate (Santi *et al*, 1995).

1.4.1 Classification of Nitrate Transporter systems

Nitrate transporter systems can be classified on the basis of physiological data (substrate affinity or induction) and gene sequence analysis (Aslam *et al.*, 2001b): inducible and constitutive components

i. LANTS (Low-Affinity Nitrate Transport System) operates when external nitrate concentrations 10-100mM and regulated by NRT2.

ii. HANTS (High-Affinity Nitrate Transport System) operates when external nitrate concentrations > 0.5 mM and regulated by NRT1.

1.4.2 Regulation of the nitrate transport

Nitrate uptake by root cells is a key step of nitrogen metabolism. The regulation of the individual components of the nitrate transporter systems is complex, and both upregulation by nitrate and feedback-repression by N-metabolites resulting from nitrate reduction have been shown for several transporters (Glass *et al.*, 2001). The complexity of the molecular basis of nitrate uptake has been enhanced by the finding that in many plants both NRT1 and NRT2 classes are represented by multigene families. Molecular, genetic and biochemical approaches to the study of eukaryotic nitrate/nitrite transporters allow an initial understanding of this step, which is much more complex and structured than previously suspected. At the plasma membrane level, two gene families, *Nrt2* and *Nrt1*, account for high- and low-affinity nitrate transporters. Functionality of NRT1 from *Arabidopsis* and NRT2 proteins from *Aspergillus* and *Chlamydomonas* has been demonstrated in figure 4a and 4b, respectively. However, redundancy of these systems makes it difficult to assign particular physiological roles to each. Data on genes involved in the regulation of nitrate transport and reduction are still scarce. Information on nitrite transporters to the chloroplast is biased by the belief that *in vivo* nitrous acid diffuses freely to this organelle (Galvan and Fernandez, 2001). The nitrate transport control mechanisms that lead to an active protein at the plasma membrane act on gene transcription, modulating the steady-state levels of mRNA, and on the activation of the protein, possibly by a phosphorylation/ dephosphorylation process (Orsel *et al.*, 2002). The effect of external factors such as external nitrate concentration on N absorption led to the identification of two physiological processes, inducible high affinity transporter system (iHANTS) and consitutive high affinity transporter system (cHANTS) (Forde, 2000). The use of inhibitors of protein synthesis demonstrates that independent components set up these two systems and that iHANTS components were not derived from some cHANTS element expressed at the basal level prior to induction by NO_3^- (Crawford, 1998). There is overwhelming evidence that NO_3^- uptake by plant roots depends not only on external factors but also on the N status of the whole plant (Glass *et*

al., 2001). The rate of N acquisition is controlled by these internal factors so that it matches tightly to N demand during plant growth (Orsel *et al.*, 2002).

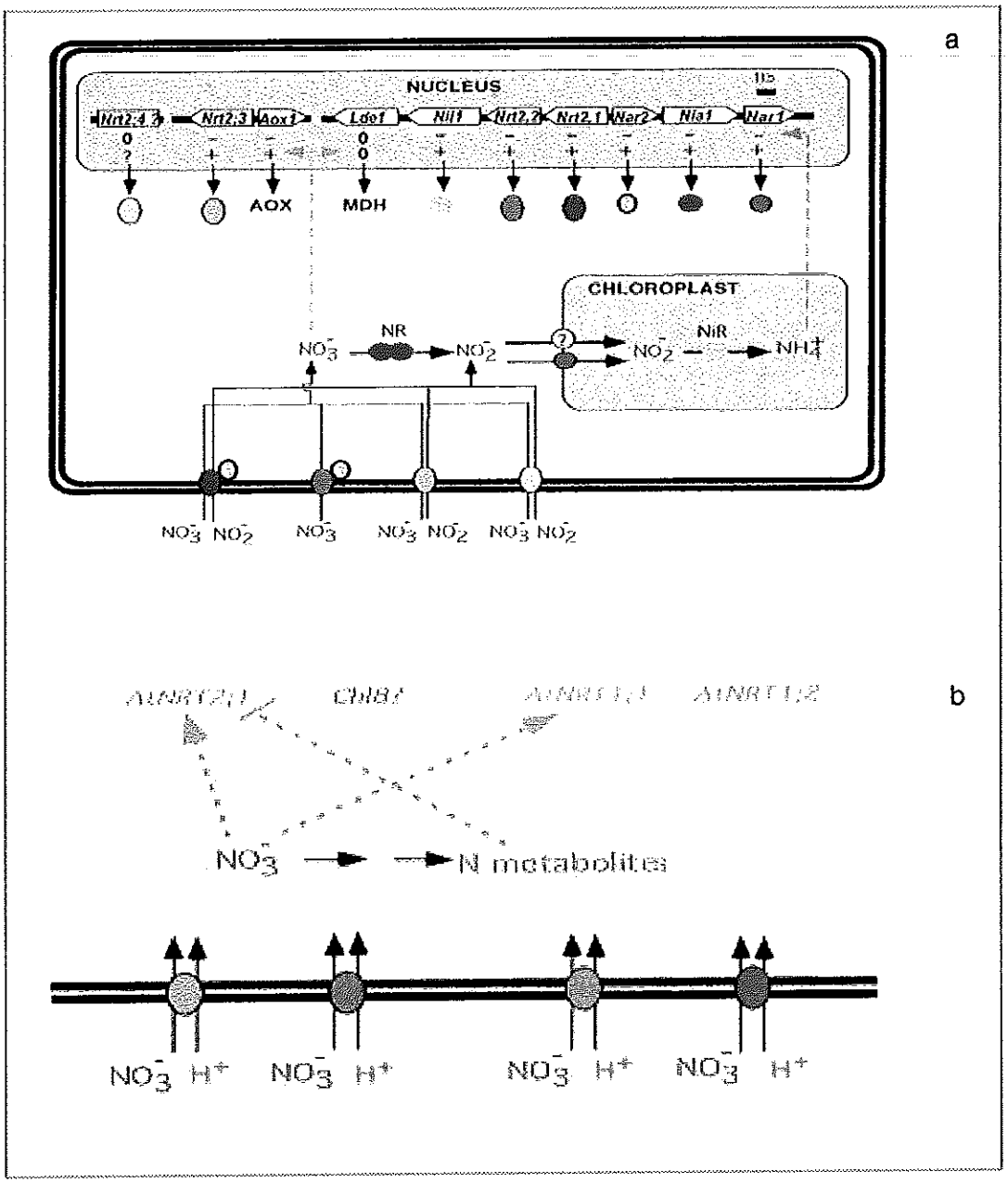

Figure 4 Nitrate: Nitrite transport systems in *Chlamydomonas* (a) and in *Arabidopsis* (b) (Galvan and Fernandez, 2001).

1.5 Regulation of nitrate reductase enzyme expression

The assimilation of nitrate is a highly regulated process (Crawford, 1995). NR regulation in several plant species revealed multiple control mechanisms that control by several factors such as light, plastids, sugars, and reversible phosphorylation.

1.5.1 Transcriptional regulation of nitrate reductase

The NR enzyme is inducible by NO_3^- (Ahmad, and Abdin, 1999). It is also inducible by NO_2^- in barley (Aslam *et al.*, 2001a). Induction occurs at the level of transcription ; within 40 min of exposure to NO_3^-, increased levels of mRNA for NR could be detected in barley roots (Sommers *et al.*, 1983). Enzyme levels depend on the balance between synthesis and degradation. According to Oaks *et al.*, (1994), the half-life of the enzyme is but a few hours. For maximum induction, light, nitrate and carbon skeletons are needed for assimilating the NH_4^+ resulting from nitrate reduction and that 20% of the electrons resulting from the light reaction are consumed in reducing NO_3^- to NH_4^+, it is not surprising that induction of the enzyme should be responsive to light. Nitrate, the inducer, causes the synthesis of nitrate reductase enzyme (Sommers *et al.* 1983 and Buljovcic; Engels, 2001), while light, via phytochrome, modifies the overall expression of the enzyme either at transcriptional or post-transcriptional levels. Regulation of NR at the transcriptional level was demonstrated by inhibitor studies (Campbell *et al.*, 1987) and by in vitro translation of mRNA (Cheng *et al.* 1992). Transcriptional regulation of NR by phytochrome (Pfr, an active form) in maize was shown by Appenroth *et al.*, 2000). In Figure 5, the model showed that phosphorylation of a transcription factor by a PKC (Protein kinase C)-type enzyme (either directly or via a cascade of phosphorylation steps) leads to an increase in NR transcript levels. NR in the dephosphorylated form is active and Pfr mediates either inactivation of protein kinases or activation of phosphatases to stimulate NR activity. Regulation of Pfr at the transcriptional and post-translational levels may occur independently (Chandok and Sopory, 1996).

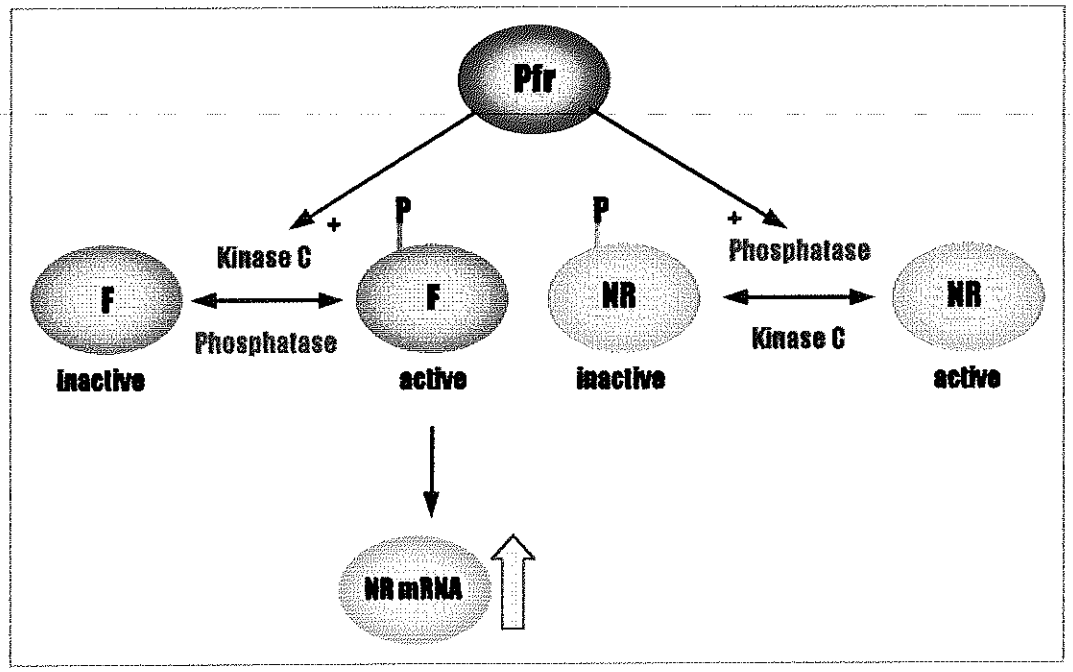

Figure 5 A model for the role of phosphorylation and dephosphorylation in Pfr-mediated enhancement of nitrate reductase transcript levels and NRA. F; transcription factor, NR; nitrate reductase (Chandok and Sopory, 1996).

1.5.2. Post-translational regulation of nitrate reductase

Nitrate reductase enzyme is inducible, depending on the availability of nitrate and light. The activity of enzyme is rapidly reversibly modulated. Fast and reversible regulation, which e.g. causes 3-10 x reduction of NR activity when plants are transferred from light to darkness (Lillo *et al.*, 1997). Nitrate uptake and assimilation are regulated both at transcriptional and post-translational levels. Phosphorylation/dephosphorylation of serine in MoCo-domain of NR is important mechanism in the post-translational regulation of NR. Investigation with ^{32}P-labelling techniques showed that NR was inactivated by phosphorylation on a serine residue (ser 543), which is located in the hinge-1 region of the molecule (Huber *et al.*, 2002). Inactivation of NR requires not only a kinase, but also an inhibitory 14-3-3 protein. Without 14-3-3, even phosphorylated NR (pNR) is active. The protein factor was

initiatially termed "inhibitor protein", or "IP" or "NIP" (nitrate reductase inhibitor protein). It was later identified to belong to the large group of 14-3-3 protein, which are binding proteins with multiple functions in all eukaryotes (Athwal *et al.*, 2002). Accordingly, NR exists basically in three states: free NR (active), phospholylated NR (pNR; active), and pNR: 14-3-3 complex (inactive). The ratio of these three NR-forms is variable, depending on external condition. The percentage of remaining active NR (NR + pNR) measured in the presence of excess Mg^{2+} to the total NR activity measured in the presence of excess EDTA was termed the activation state. For example, if one half of the existing NR would be phosphorylated and bound to 14-3-3 proteins in the presence of millimolar concentration of Mg^{2+}, the activation state (base on activity measurements) would be about 50%. It is important that phosphorylation plus 14-3-3 protein-binding does not change the substrate affinities of NR in a crude extract (Kaiser and Spill, 1991). The pNR: 14-3-3 protein complex is simply switched off, whereas the remaining free NR in the extract works normally as indicated schematically in Figure 6. The formation of NR: 14-3-3 complex may cause a change in NR conformation that interrupts electron transport between the heme and the MoCo-domain (Bachmann *et al.*, 1996). Divalent cations effect several purposes or whether they are needed for the complex to switch from an active into an inactive form or both (Weiner and Kaiser, 2000):

(1) Protein kinase itself is a Ca-dependent enzyme

(2) Substrate for the kinase is Mg-ATP

(3) Divalent cations are required for 14-3-3 binding to pNR

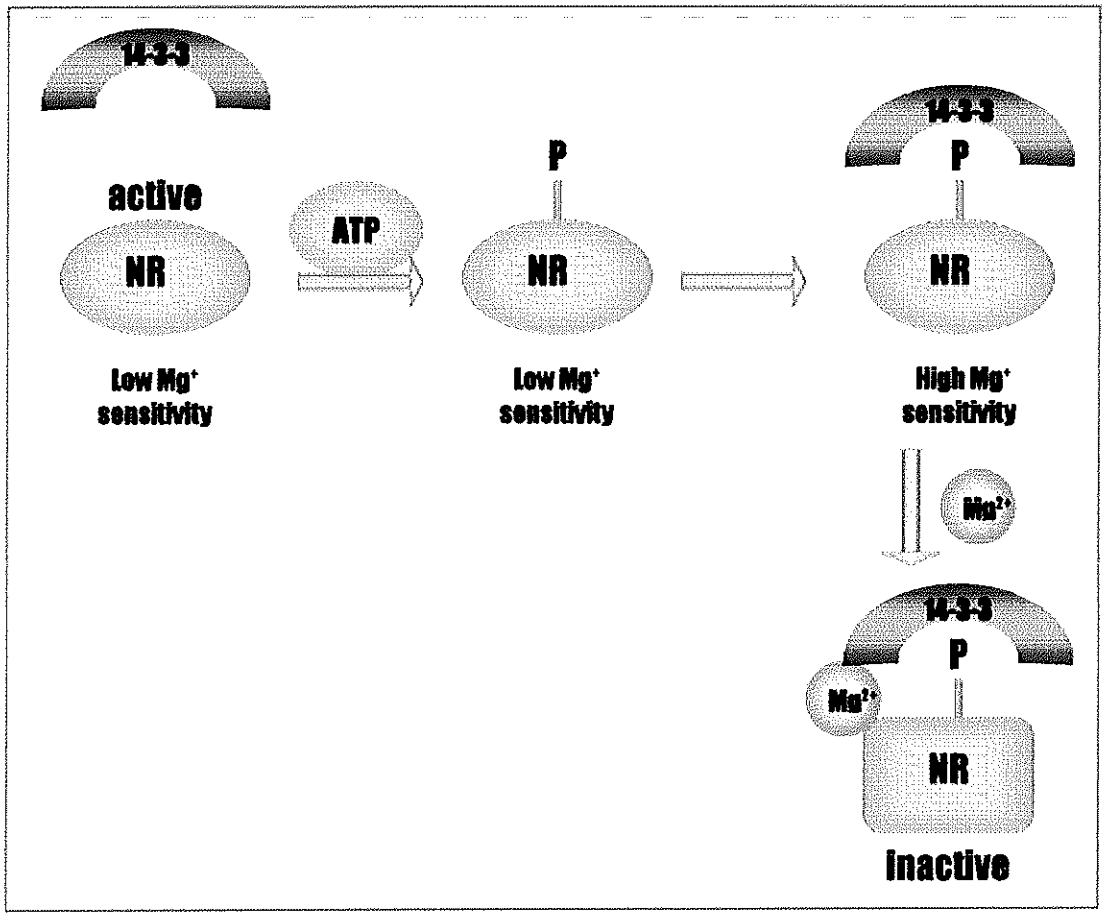

Figure 6 Schematic diagram summarizing the Mg^{+}-dependent modulation of NR by phosphorylation and 14-3-3 binding (Kaiser, *et al.*, 2002).

1.6 Interactions of nitrogen and carbon metabolism

An important biological mechanism that control primary carbon and nitrogen metabolism in plants is protein phosphorylation, which can affect enzymatic activity, protein localization and protein:protein interactions. The assimilation of nitrate is a highly regulated process (Crawford, 1995). Plants provided with a high nitrate supply transport the majority of nitrate to leaves where photosynthesis provides energy and C-skeletons for nitrate assimilation (Cotelle *et al.*, 2000). Carbon metabolites signal changes in nitrate assimilation and can replace the light signal. Carbon metabolites are critical for nitrate reduction because carbon skeletons are required for ammonia fixation and the energy from reduced carbon is needed for nitrate reduction in nongreen tissue (Larios *et al.*, 2001).

1.6.1 Effect of sugar on NR activity

When excised leaves or cells in culture are treated with sucrose in the dark, NR activity and mRNA increase (Morcuende *et al.*, 1998). Moreover, NR promoter elements confer on reporter genes sucrose induction in the dark (Cheng *et al.*, 1992; Vincentz *et al.*, 1993). In sugar-starved *Arabidopsis* suspension cells, NR and many other proteins were rapidly degraded, pointing to the important role of sugars and of sugar sensing for cellular signaling (Cotelle *et al.*, 2000). Cotelle *et al.* (2000) suggested that sugar starvation and 14-3-3 release may be a signal initiating proteolysis, whereas it is suggested that 14-3-3 binding initiates degradation. In darkened aerobic leaf tissues, where NR was degraded, there was induced a rapid consumption mainly at sucrose, but also of hexoses, consistent with the above role of sugar signaling (Huber *et al.*, 1992b). Under anoxia where NR was stabilized and active, sucrose levels decreased, yet at the same time hexose levels increased dramatically (Kaiser and Huber, 2001). Thus, whenever NR was stabilized, cells were sugar sufficient, eventually changing from high sucrose cells to high hexose cells, the multiple roles of sugars in the regulation of NR expression, NR modulation and NR degradation are summarized in Figure 7. High sugar concentrations promote NR expression (together with nitrate; block (eventually as sugar phosphates) the protein kinases), thereby activating NR and prohibiting NR degradation.

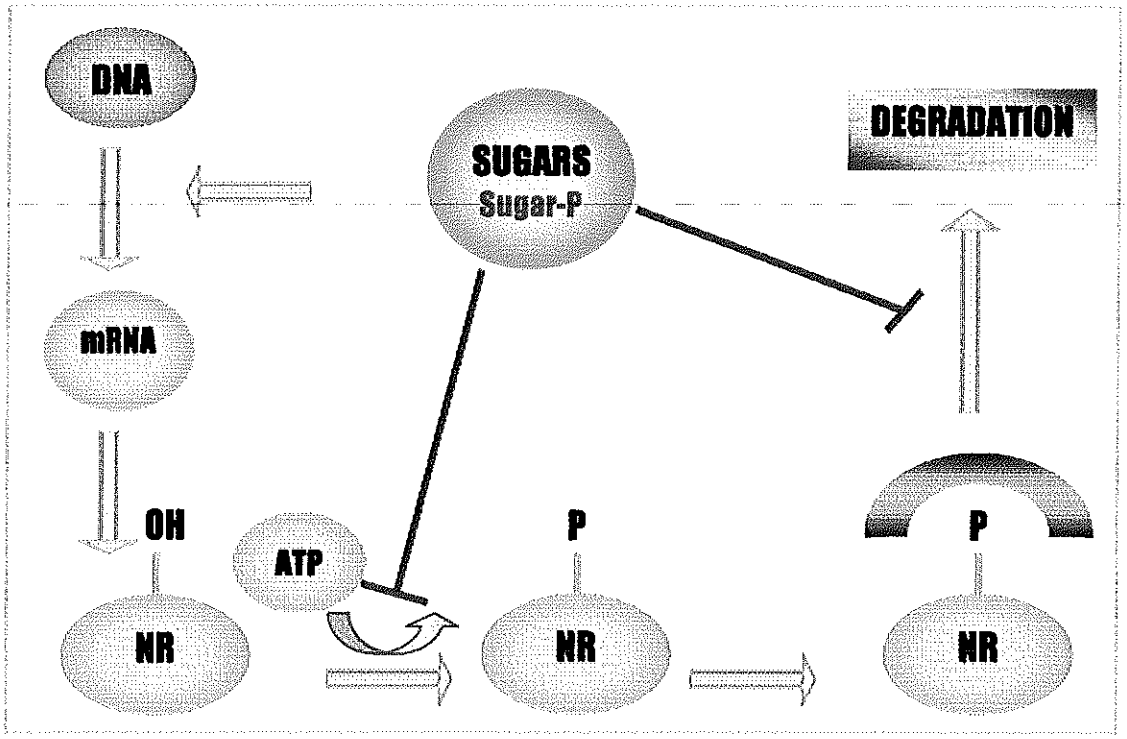

Figure 7 Schematic diagram summarizing the multiple effects of sugar and sugar phosphates on nitrate reductase expression, NR activity and NR degradation (Kaiser and Huber, 2001).

1.6.2 Effect of sugar on nitrate transporters regulation

Diurnal regulation of NO_3^- uptake has been reported in a number of plant species, including *Arabidopsis*, with activities generally being highest during the light period and lowest in the dark (Lebot and Kirkby,1992; Delhon *et al.*,1995) and these diurnal fluctuations in uptake rate are generally correlated with changes in NRT1 and NRT2 transcript levels (Lejay *et al.*, 1999 and Matt *et al.*, 2001). In *Arabidopsis*, both the *AtNR72.1* and *AtNRT1.1* genes are diurnally regulated in a similar manner to the HANTS activity. In each case, the decline in transcript levels during the dark period could be delayed by supplying sucrose to the medium (Lejay *et al.*, 1999). The finding that AtNRT2.1 and AtNRT1.1 are both positively regulated by the sucrose supply but are differentially responsive to feedback regulation by reduced forms of N indicates that the effect of sucrose is not mediated through its influence on the overall N/C balance of the plant (Lam *et al.*, 1996). A role for sucrose in regulating expression of NO_3^- transporter

genes was also surmised from studies with tobacco, which showed that diurnal changes in *Nrt2* expression were correlated with changes in the sugar content of the root, whereas there were no major fluctuations in the overall amino acid content of the root (Matt *et al.*, 2001). Sucrose acts as a signal regulating the expression of a variety of plant genes, and there is evidence for both sucrose-specific and glucose-specific signal transduction pathways operating in plants (Coruzzi and Bush, 2001). Thus it seems likely that diurnal fluctuations in the supply of sugars from the shoot are acting as long range signals to regulate expression of the NO_3^- transporter genes, so helping to coordinate the processes of NO_3^- uptake and photosynthesis (Forde, 2002). A model for the regulation of *AtNRT2.1* and *AtNRT1. 1* expression by local and long-range signaling pathways is presented in Figure 8.

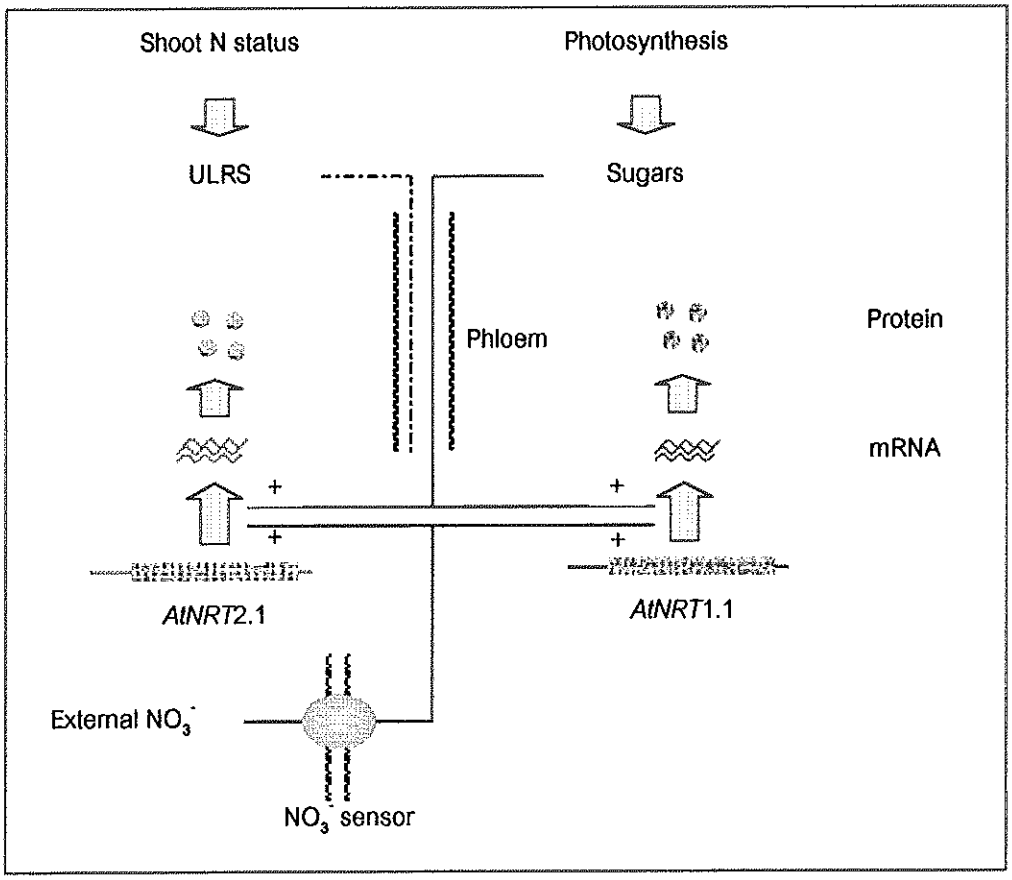

Figure 8 Local and long range signaling pathways regulating expression of NO_3^- transporter genes in *Arabidopsis* (Forde, 2002).

ULRS: unidentified long-range signal

2. Nitrate reductase application

2.1 Nitrate problem

Nitrate is a common contaminant of ground and surface waters worldwide. Consumption of excess nitrate is considered to be dangerous for infants and a potential health hazard to human (Campbell *et al.*, 2002). Effective technology for monitoring and determination of nitrate is available. The flow injection analysis and other standard methods for nitrate analysis manually use cadmium. Cadmium-based methods expose the user to the toxic heavy metal and generate large volumes of hazardous waste. NECi has begun adapting the enzyme-based test kit technology for on-line nitrate analysis in autoanalyzers as shown in Figure 9; this would eliminate the cadmium problem (Campbell *et al.*, 2001).

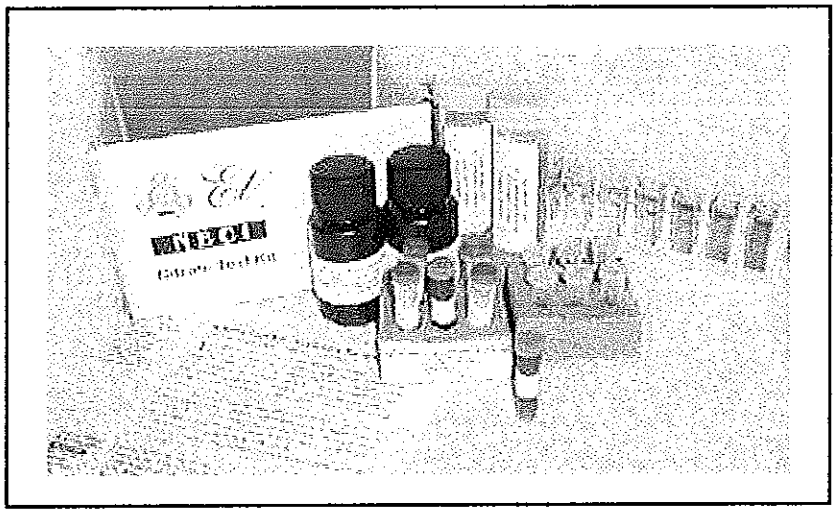

Figure 9 NECi enzyme-base nitrate test kit (www.gentaur.com/nitrate.htm)

2.2 Enzyme-based nitrate test kit

The enzyme used in nitrate test kits is the enzyme nitrate reductase (NR), which has been purified from corn seedlings or yeast. Nitrate analysis based on the NR combines the most desirable features of good analytical tool. Moreover, it provides the ease of use and low capital and per-sample cost of conventional nitrate test kits, plus the sensitivity, selectivity, and accuracy of ion chromatography or flow injection analysis type instrumentation. In addition, no hazardous materials are required (Campbell *et al.*, 2002).

3. Sweet corn

3.1 History and its importance

Corn (*Zea mays* L.), *Zea* (from a Greek name for a related plant) *mays* (from the Mexican name for corn, maize), is one of 4 or 5 species of mostly annual grasses that inhabit disturbed areas and field margins of Central America (Cobley and Stell, 1976). The plant is used to produce grain and fodder that are the basis of a number of food, feed, pharmaceutical and industrial manufactures (Jugenheimer, 1976). Corn is currently produced in most countries of the world and is the third most planted field crop (after wheat and rice). Sweet corn probably originated from a mutation of a Peruvian corn called *Chuspillo* or *Chullpi* . Sweet corn (*Zea mays* L. *saccharata*) is a sugary-seeded kind of corn, as the "saccharin" part of its scientific name indicates. The sugary character in corn positively occurred innumerable times as a mutation, but many Indian tribes either disliked it and threw it away or had trouble in perpetuating it. The wrinkled, glassy appearance of sweet corn kernels is a result of a sugary gene that retards the normal conversion of sugar to starch during endosperm development (Tracy, 1994).

3.2 Taxonomy classification

Class: Angiospermae

Subclass: Monocotyledoneae

Family: Poaceae (Graminae)

Sub-family: Panicoideae

Tribe: Maydeae

Genus: *Zea*

Species: *Zea mays*

Subspecies: *saccharata*

Scientific name: *Zea mays* L. *saccharata*

Common name: sweet corn

3.3 Morphology

Corn (corn) has the basic structure of the grass family, with conspicuous nodes and internodes on the stem. The leaves grow on opposite sides; one leaf per node. Corn is botanically unique among cereal crops. The corn plant is monoecious, bearing male

flowers in the tassel (male inflorescences or staminate flowers) and female flowers (female inflorescence or carpellate flower) on the lateral ear shoots of the same plant (Figure 10). The tassel is a branched inflorescence located in the tip of the main stem. It consists of a central spike (rachis) and about 10-50 lateral branches. The filaments of corn silk are styles. The corn pollen tube has to travel the length of the style to reach the egg. Corn is a cross-pollinating (allogamous) species; therefore, a natural population is usually heterogeneous and produces grains on lateral rather than terminal branches (Cheng and Pareddy, 1994).

Figure 10 Corn plants separate male and female inflorescence (A). Mature tassel at anthesis (B). Ear inflorescence showed silk (long styles with stigmas emergence (C and D). www.Grass Flower.com.

3.4 Classification

Corn is classified as sweet, pop, flour, silage, and feed corn, depending on the type of carbohydrate stored in the ear. Sweet corn gets its name from a special gene that prevents or retards the normal conversion of sugar to starch during kernel development. Four sweet corn genotypes determine the sugar content of commercially available cultivars (Tracy, 1994):

3.4.1 **Normal sugar** [*sugary* (*su1*)] Kernels contain moderate but varying degrees of sugar, depending on the variety. Sugars convert to starch rapidly after harvest. Most available varieties are of this type.

3.4.2 **Sugary-enhanced** [*sugary enhancer* (*se*)] This gene is modified from the normal sugary (*su*) gene. The result is increased tenderness and to a varying degree, sweetness. The conversion of sugar to starch after harvest is slow.

3.4.3 **Sugary2** [(*su2*); located on chromosome 6L] This gene is thought to encode a starch branching enzyme. The recessive *su2* allele causes a sugary, translucent kernel with a glassy, opaque endosperm. The kernel is sometimes wrinkled like *su1*.

3.4.4 **Supersweet, shrunken gene** [*shrunken2* (sh_2)] This gene's name is descriptive of its effect on the dry kernel. Its presence creates greatly heightened sweetness and slow conversion to starch after harvest.. Common names for this type are "Supersweet" and "Shrunken". Cross pollination between a "Supersweet" and "Normal" or "Sugar Enhanced" variety will result in tough, starchy kernels in both types.

3.5 **Corn tissue culture**

Green and Phillips first reported plant regeneration from *in vitro* cultures of corn in 1975. Steady improvements have been made since then in the culture systems and in applying them in genetic studies and other applications. Successful applications of corn tissue culture technology have been largely limited to large research groups. The corn callus cultures are generally classified as Type I or type II. The culture types are distinguished by (1) friability (degree of physical association between cells), (2) mode of regeneration (embryogenesis or organogenesis), and (3) degree of differentiation. Type I culture are nonfriable, regenerate through both somatic embryogenesis and organogenesis, and are highly differentiated (often contain leaflike structures and advanced-stage somatic embryos on maintenance medium). Type II calluses are very friable, regenerate almost exclusively via somatic embryogenesis, and are relatively undifferentiated. Numerous globular-stage somatic embryos are present that does not develop further on maintenance medium. Type II callus cultures are preferred for many applications, primarily because of reduced differentiation and enhanced friability. These

characteristics make *in vitro* selection and establishment of liquid suspension cultures much easier (Duncan, 1985).

Objectives

1. To determine the optimal conditions for induction of Type II corn callus from embryo culture for the establishment of cell suspension culture.
2. To identify and optimize the most appropriate assay for quantification of NR activity in corn callus materials
3. To examine the diurnal changes and the effect of photoperiod on NR activity in the cultured corn calluses.
4. To study the influence of external environmental factors such as nitrate and ammonium supplements, concentrations of sugar (sucrose, glucose and fructose) and light intensity on NR activity of corn callus culture.
5. To compare NR specific activities of rice and corn seedlings and cultured corn calluses.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

Instrument	Model	Company
Autoclave	HA-300	Hiryama
Digital camera	P8	Sony
Homogenizer	-	Wheaton
Lamina air flow cabinet	CF43S	Gelman Sciences Australia
Light meter	-	Extech
Magnetic stirrer	MS101	GEM
Microcentrifuge	5415C	Eppendorf
Micropipette	-	Nichipet *EX*, Biopette
Orbital shaker	-	LB science
pH meter	240	Corning
Refrigerated centrifuge	5804R	Eppendorf
Spectrophotometer	-	Hewlet packard
Stirring hot plate	-	Thermolyne
Timer	TB179	National
Vortex mixer	K-550-GE	Scientific industry
Weight instrument 3 position	GT410	Ohaus
Weight instrument 4 position	AB204-5	Mettler

2.1.2 Chemicals (Analytical grade)

Chemical	Company
Ammonium sulphate	Carlo Erba
Boric acid	AnalaR
BSA	Sigma-Aldrich
Calcium choride	Merck
Casein enzyme hydrolysate	Sigma-Aldrich
Chymostatin	Sigma-Aldrich
Cobalt choride	Univar
Deoxycholic acid Sodium salt	Fluka
2,4-D(2,4-dichlorophenoxyacetic acid)	Fluka
DTT	Sigma-Aldrich
Ethanol	Merck
Ferrous sulfate	Sigma-Aldrich
Fructose	Fluka
Folin reagent	Merck
Glucose	Carlo Erba
Glycine	Sigma-Aldrich
Hydrochloric acid	Merck
Magnesium sulfate	Fluka
Manganese sulfate	BDH
MOPS	Fluka
Myo-inositol	Sigma-Aldrich
NADH	Sigma-Aldrich
NADPH	Sigma-Aldrich
N-(1-naphthyl)-ethylene -diaminehydrochloride	Sigma-Aldrich
Nicotinic acid	Sigma-Aldrich
Plytagel	Sigma-Aldrich
PMSF	Sigma-Aldrich

2.1.2 Chemicals (continued)

Chemical	Company
Potassium dihydrogen phosphate	Merck
Potassium iodide	AnalaR
Potassium nitrate	Carlo Erba
Potassium nitrite	Carlo Erba
Potassium sodium tartrate	Fluka
Proline	Sigma-Aldrich
Propanol-(2)	AnalaR
Salicylic acid	BDH
Sodium carbonate	Merck
Sodium ethylene diamine tetraacetic acid (Na -EDTA)	Fluka
Sodium hydroxide	BDH
Sodium molybdate	Fluka
Sucrose	Carlo Erba
Sulfanilamide	Sigma-Aldrich
Sulfuric acid	Merck
Thiamine HCl	Sigma-Aldrich
Tryptophan	Sigma-Aldrich
Zinc acetate	AnalaR
Zinc sulfate	HW

2.2 Methods

2.2.1 Induction of callus and suspension culture from sweet corn

2.2.1.1 Establishment and culture of Type II calluses cultures

A. Plant material

The sweet corn seeds (inbred lines Olympia99) were germinated in pots (30x30 cm.) containing a mixture of sand and soil in the greenhouse. The corn plants were watered every day with tap water for 8 to 12 weeks. The ears were collected 12-15 days after pollination, when the embryos were at the proper developmental stage. The harvested ear could be used either immediately for embryo dissection and culture initiation or could be stored in plastic storage bags and kept at 4 ^{0}C for later use. Overnight storage at 4 ^{0}C had no known detrimental effect on culture response. Reasonably good culture response was obtained even after 1-2 weeks of refrigeration.

B. Selection of immature embryo

Embryo size was determined by peeling back the husks of an ear still attached to the plant, the tips of several kernels in the center portion of the ear could be sliced off using a knife, and the developing endosperms were removed. The embryos, which were located on the side of the kernel facing the tip of the ear, were removed and placed on a ruler with millimeter grid markings. The embryo size range can be easily determined visually without actual measurement.

C. Sterilization

Corn ears were surface-sterilized prior to embryo isolation by rinsing with tap water plus a few drops of Tween 20. The tip of ear was removed, cut in halves and placed in a storage dish. The kernels were isolated from the ear by sterile blade and placed in an autoclaved 100 ml Duran bottle containing 50 ml 70% ethanol for 90 seconds with continuous stirring. Followed by carefully decanting the 70% ethanol solution and rinsing the kernels with 100 ml of sterile water 3 times for 15 seconds with shaking. The kernels were sterilized for 20 minutes in 50 ml of liquid bleach (1:5 dilution of household bleach (Haiter): water plus 1-2 drops of Tween 20) in the Duran bottle with continuous stirring. After the bleach had been decanted the kernels were rinsed 3 times by shaking for 15 seconds with 100 ml sterile water. The kernels were transferred to

autoclaved storage Petri dishes containing sterile tissue papers. The whole process of the sterilization was performed in a laminar flow hood.

D. Embryo isolation

The tip of the kernel was cut off with sterile blade; the cutting was close to but not touching the embryo as this would damage the embryo. Using the sterile forceps, the embryo was carefully removed from the kernel and placed scutellum-side up on the surface of the culture medium. The scutellum is the rounded side of the embryo that was embedded in the endosperm (Figure 11b). It was essential that the embryo must not be cut or damaged during this process.

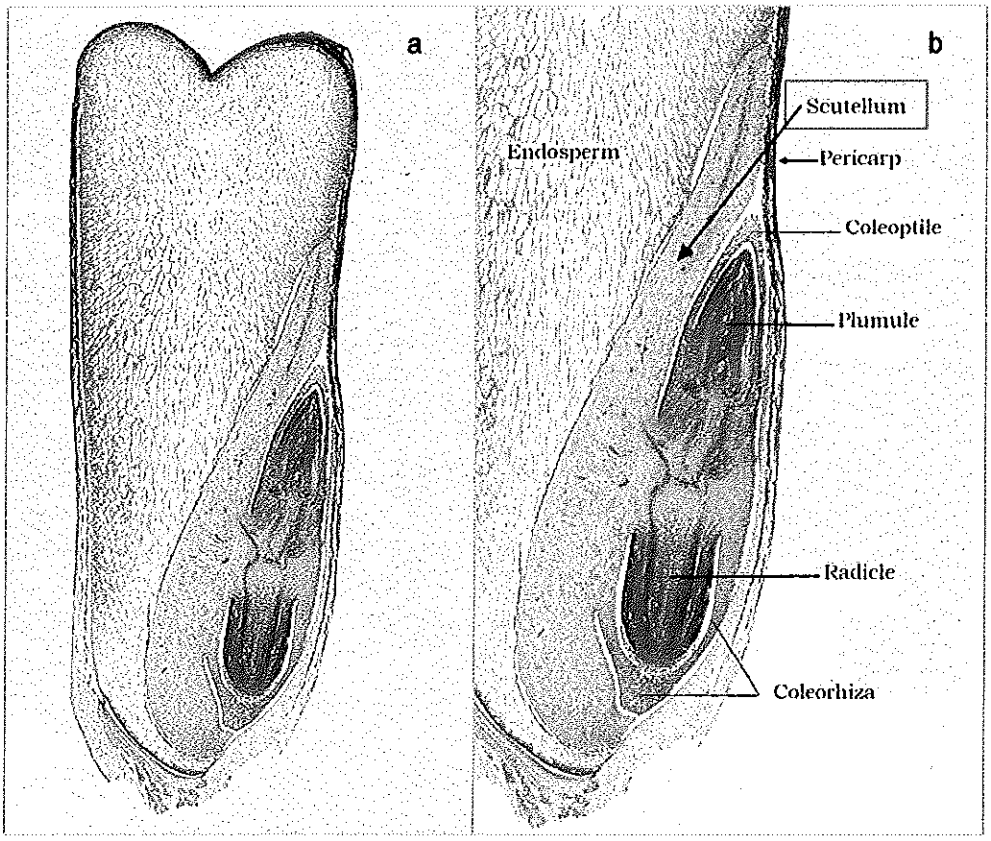

Figure 11 Longitudinal section of a corn grain (a). Enlarged section of the embryo showing the scutellum (b). (www.botit.botany.wisc.edu)

E. Solid culture medium

A type II callus initiation medium was modified and developed by Chu *et al.* in 1975. The induction medium consisted of N6 medium supplemented

with 2.3 g l^{-1} L-proline, 200 mg l^{-1} casein hydrolysate, 2% sucrose, and 2 mg l^{-1} 2,4-D, pH 5.8. The medium was solidified with 0.25% w/v Phytagel. All components were added prior to autoclaving. Aseptically, after autoclaving and cooling to 50 °C, the medium was poured into petri dishes (100 x 25 mm) or bottles (about 10 ml per plate or bottle), then left to solidify at room temperature.

F. Culture incubation conditions

Corn immature embryos were cultured in modified N6 media in petri dishes or bottles in the dark at 26-28 °C. Every 2 weeks, Type II calluses were subcultured onto fresh modified solid N6 medium.

2.2.1.2 Suspension cell culture initiation

Suspension cultures were initiated from the friable Type II callus cultures aged 7 days as this short subculture cycle reduced embryoid development. Modified N6 liquid suspension medium was prepared by adding 2.3 g l^{-1} L-proline, 200 mg l^{-1} casein hydrolysate, 2% sucrose, and 2 mg l^{-1} 2,4-D, pH 5.8 and autoclaved. One gram of friable Type II calluses (weighed on a sterile plate) was transferred to 10 ml modified N6 liquid medium in a 250 ml Erlenmeyer flask. Culture flasks were covered with two layers of aluminum foil to ensure sterility and yet allow air exchange. The flasks were shaken at 120 rpm in the dark at 26-28 °C. After one week, the cell suspension were subcultured into fresh modified N6 medium. For NR induction, the cultures were exposed to 345 μmole m^{-2} s^{-1} of light intensity at 16 h light/ 8 h dark period.

2.2.1.3 Effect of 2,4-D on corn callus induction

Different sizes (stage of embryogenesis) of immature embryos and several concentrations of 2,4-D were used for induction of corn callus. Petri dishes containing 10 ml of callus initiation medium (N6 medium with 2.5 g l^{-1} phytagel) were prepared. Immature ears (Figure 12b) and mature ears (Figure 12c) were used for callus induction. The immature seeds were collected and sterilized as previously described in 2.2.1.1. The mature (5.0 mm) and immature (2.0 mm) embryos were separated and cultured. The initiation medium contained N6 salts and vitamins (Chu *et al.*, 1975), 3% sucrose, and several 2,4-D concentrations (1, 2, 5, 10 mg l^{-1}). The edges of culture plates were sealed with parafilm and incubated in the dark at 26-28 °C for 2 weeks. The

initiation and differentiation of calluses from immature embryos were investigated every second day.

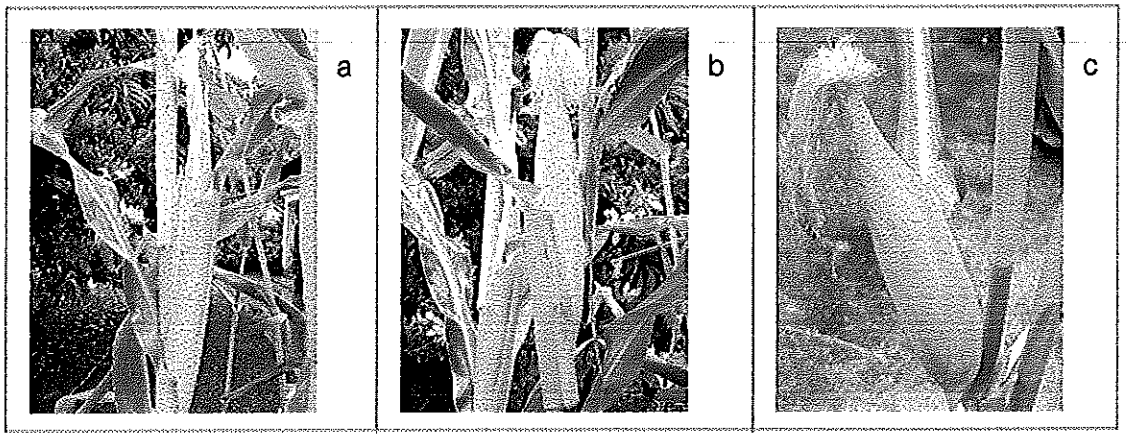

Figure 12 Reproductive Stages and Kernel Development.

a. Silking stage

b. Blister stage (10-14 days after silking)

c. Milk stage (18-22 days after silking)

2.2.1.4 Determination of callus formation

The corn immature ears were collected and sterilized according to the same method as described in 2.2.1.1. The immature embryos were separated and cultured in N6 medium containing 2 mg l^{-1} 2,4-D. The edges of culture plates were sealed with parafilm and incubated in the dark at 26-28 °C for 2 weeks. The initiation, differentiation and growth of calluses from immature embryos were investigated every second day.

2.2.1.5 Callus induction in light and dark conditions

The immature ears and seeds were selected and then sterilized as previously described in 2.2.1.1. The immature embryos were cultured in N6 media containing 2 mg l^{-1} 2,4-D and incubated in the dark or light at 26 °C for 4 weeks. The callus differentiation and growth rate of callus after subculture was investigated.

2.2.1.6 Effect of embryo orientation on callus development

The immature corn seeds were surface sterilized as previously described in 2.2.1.1. Aseptically, 10 immature corn embryos were horizontally (placed with the scutellum side on the medium surface) or vertically (the agar was slit with scalpel and each immature embryos was gently placed vertically in a slit) oriented on the petri dish of 10 ml N6 medium containing 2 mg l^{-1} 2,4-D (3 replicates per orientation; 10 immature embryo per plate) as shown in figure 13. The culture plates were incubated in the dark at 28 °C for at least 2 weeks. Changes during the development of embryogenic callus on the culture medium were observed.

(a) (b)

Figure 13 Horizontal (a) and vertical (b) orientation of immature embryo on culture media (10 ml N6 medium per plate)

2.2.1.7 Growth rate of cell suspension culture

The suspension cultures were initiated from friable Type II callus cultures. Two to three weeks before initiating a suspension culture, the Type II calluses were subcultured every 7 day cycle. This short subculture cycle reduces embryoid development and aids in the dispersion of the Type II callus in liquid growth medium. The liquid medium containing N6 salts and 2, 3 and 4 % sucrose were prepared for comparison of % weight increase of culture. Before autoclaving, the pH of medium was adjusted to 5.8 and then the 20 ml of each medium was dispensed into 250 ml erlenmeyer flasks. The flasks were covered with two layers of aluminum foil or other appropriate material to ensure sterility and yet allow air exchange. Type II calluses (2 g weighed in a sterile preweighed petri dish) was selected and dispensed into 250 ml flasks containing 20 ml of prepared medium (3 replicates for each sucrose concentration in medium). The flasks were placed on the shaker at 120 rpm in the dark at 26-28 °C.

The suspension cultures in different sucrose concentrations were cultured for 2 months. Every week, the media were drained and the calluses were weighed and investigated on a sterile plate.

2.2.2 Nitrate reductase assays

2.2.2.1 Preparation of cell suspension for NR and protein assay

Aseptically, the suspension cells were collected on pieces of tissue paper in the morning, 3 h after the onset of light, and weighed before the extraction. The calluses used for determination of fresh weight, NO_2^- and NO_3^- content, enzyme activity and protein content were immediately frozen in liquid nitrogen and stored at –80 °C prior to analysis. In all experiments, a minimum of three replicates was used.

2.2.2.2 Crude enzyme extraction

One gram of cell suspension was ground in liquid nitrogen with mortar and pestle then mixed with 4 ml extraction buffer (Grinding buffer) containing 50 mM MOPS buffer, pH 7.5, 1 mM EDTA, 5 mM DTT, 1 mM PMSF and 10 µM chymostatin (Redinbaugh *et al.*, 1996). The ground calluses were put into syringes plugged with a 8 layers of cotton pads and the filtrate was collected in a plastic tube. The filtrate was poured into 2 ml microfuge tubes and centrifuged at 8,000 rpm in an Eppendorf microfuge at 4°C, for 25 minutes to remove the insoluble materials. The supernatant was transferred into a graduated tube, and stored on ice for further analysis of protein content and enzyme activities. All subsequent operations were carried out on ice.

2.2.2.3 Enzyme activity assays

A. *In vitro* assay

The NADH and NADPH NR activities of corn callus in the supernatant were determined by modified method of Redinbaugh, *et al.* (1996). The NR reaction was started by mixing 100 µl sample with 400 µl *in vitro* assay buffer containing: 50 mM MOPS buffer, pH 7.5, 1 mM EDTA, 10 mM KNO_3 and 2.5 mM NADH or 2.5 mM NADPH. All tubes, containing in vitro assay buffer, were placed at 30 °C for 5 minutes to equilibrate them to the reaction temperature. Sequentially, aliquots of extract (100 µl) were added at timed intervals (*e.g.* 20 second intervals). Each reaction was stopped

sequentially in the same time interval that used to initiate the reactions. A "Color Development Reagent" containing of 1% sulfanilamide in 1.5 N HCl and 0.02% N-1 (naphthyl)-ethylenediamine hydrochloride in water was used to stop the reaction and determine the amount of nitrite (enzyme product) in each reaction tube and each standard tube. Immediately before used to stop the reaction and determine nitrite, the color reagent was combined to equal amounts of 1% sulfanilamide (in 1.5N HCl) and 0.02% N-1(naphthyl)-ethylenediamine hydrochloride (in water). 500 µl of the color reagent was added to each reaction or standard curve tube, mixed and incubated for 15 min at room temperture. The spectrophotometer was set zero at 540 nm using the zero nitrite control. The nitrite colourimetric reaction is shown in figure 14. The fine precipitate formed was eliminated by centrifugation at 8,000 rpm for 5 minute then 1,000 µl of each supernatant was transferred to a fresh 1.5 or 2.0 ml microfuge tube. A standard curve of nitrite was prepared from a stock solution of 0.1 mM KNO_2 using distilled water as a zero nitrite control.

B. *In vivo* assay

Corn callus NR activity could be directly assayed in a solution containing nitrate, MOPS buffer and propanol, as nitrate can readily enter cells (Ross, 1974). In this case, the cell supplies NR and the reductant NADH. The propanol in the assay medium increases cell permeability and makes the callus segments essentially anaerobic. Under anaerobic conditions, the reduction of nitrite to ammonia is inhibited, and the conversion of nitrate to nitrite is enhanced. Because of the sensitivity of the reaction to molecular oxygen all operations have to be carried out in dim room light to reduce photosynthetic activity (Harley, 1993). About 0.25 g calluses were weighed and cut into small segments and transferred into 25 x 100 mm screw-caped test tubes. The reactions were set for time of 0 (t_o), 20(t_{20}), 40 (t_{40}) and 60 minute (t_{60}). Ten ml of *in vivo* assay solution containing 50 mM MOPS buffer, pH 7.5, 30 mM KNO_3 and 5%(v/v) propanol were added to each tube. The reactions were allowed to proceed for 0, 20, 40 and 60 minutes at room temperature. At the end of reaction period, the tubes were placed in a boiling water bath for 5 minutes, then cooled to room temperature. The concentration of nitrite in the assay tubes were determined by adding 10 ml of colour

development reagent containing 1% sulfanilamide in 3N HCl and 0.02% N-(1-naphthyl) ethylenediaminehydrochloride and the tubes were mixed thoroughly and placed in the dark at RT for 15 minutes. OD 540 nm was measured and compared with standard curve. The standard curve was constructed using a stock solution of 0.1 mM KNO_2 by making a series of at least five sequentiall dilutions (20, 40, 60, 80 and 100 µM). The distilled water was placed in a microfuge tube to serve as a "zero nitrite control".

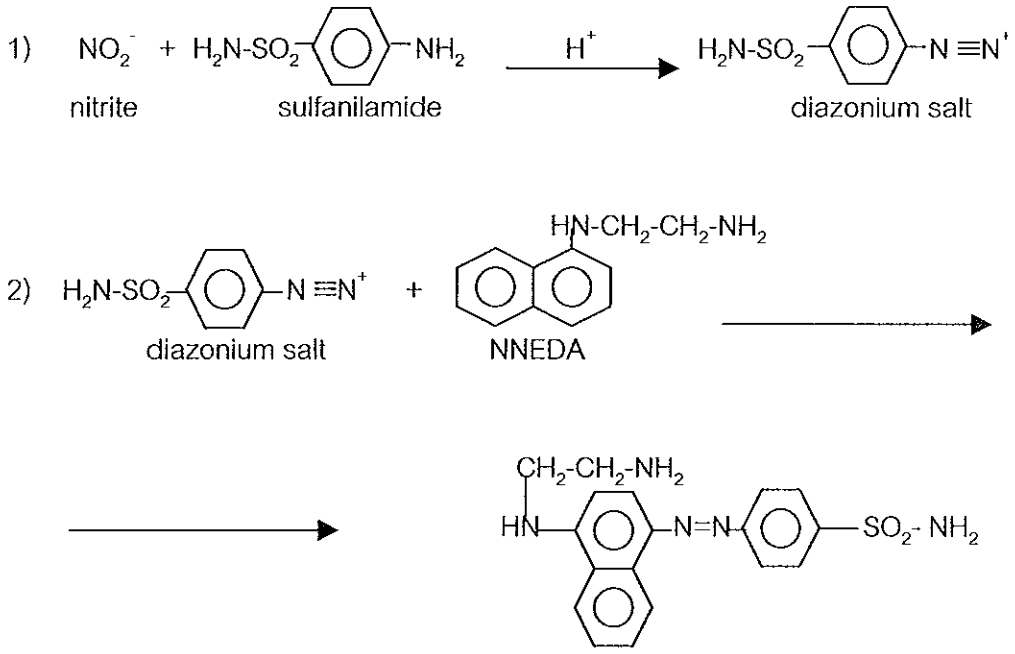

Figure 14 Two steps of nitrite colourimetric reaction (Coomb and Hall, 1982)
NNEDA = *N*-(1-napthyl) ethylenediamine dihydrochloride

2.2.2.4 Determination of protein concentration (Lowry method)

The method was modified from that of Lowry *et al.*, (1951). In the Lowry assay a blue color is formed as a result of a reaction of protein with copper ions, and subsequent reduction of the Folin-Ciocalteu reagent by the protein-copper complex. The sensitivity of the Lowry assay ranges from 1 to 500 µg of protein. The sample

protein was extracted as described in 2.2.2.2 and serial diluted with distilled water. Protein samples 100µl (in duplicates) were transferred to the test tubes and 400 µl of 10% deoxycholate was added to each test tube and mixed immediately. 3 ml of 2% Na_2CO_3 in 0.1 N NaOH solution was added, followed by 100 µl of an equal mixture of 2% w/v potassium sodium tartrate and 1% w/v $CuSO_45H_2O$ (freshly mixed). The contents of the test tube were mixed thoroughly and left at RT for 10 minutes. Then, 300 µl of Folin-Ciocalteu reagent (1:1 Folin's Reagent: distilled water) was added to each tube, again mixed immediately after each addition. After 30 minutes, absorbance at 600 nm of each sample was measured in a spectrophotometer using the 0 mg test tube as the blank. A series of protein standards (in duplicates) which contain 20, 40, 60, 80, 100 and 120 µg of BSA in 100 µl were allowed to react with alkaline cooper, and Folin-Ciocalteu as previously described . The absorbance values from the standards were used to plot a "Standard Curve" of Protein. The linear regression equation was used to determine the protein content of the samples based upon their absorbance.

2.2.3 Determination of NR activity in corn and rice seedling

Sweet corn (*Zea mays* var. Olympia99) and rice (*Oryza sativa* var. khaodokmali 105) seeds were soaked in distilled water for 5 to 6 h and allowed to germinate on 2 layers of thick tissue paper moistened with distilled water. The seedlings were grown at RT under dark condition for 1 week, then seedlings were supplied with 5 and 10 mM KNO_3 or NH_4NO_3. They were grown for another day in 16/8 h light/dark (about 270 µmol m^{-2} s^{-1}) at RT. After 24 h of induction, leaves and roots were harvested 3-5 h after exposure to light and dissected into 1 cm long segments from the base to the tip of leaf and root. Ten seedlings of each plant species were analyzed. The enzyme activity in each part (leaf, stem and root) was determined.

2.2.4 Comparison of *in vivo* and *in vitro* assays for NR activity

The suspension cultures were established as previously described in 2.2.1.2. One g of calluses were weighed and transferred into fresh induction medium [N6 medium supplemented with 2 mg l^{-1} 2,4-D, 3% fructose and 28 mM KNO_3 without 3.5 mM $(NH_4)_2SO_4$)] to induce NR; then the sample flasks were incubated in 16/8 h light (345 µ mole m^{-2} s^{-1})/dark periods at 28 °C on a shaker at 120 rpm. On day 4 after NR induction,

the calluses were collected at 10.00-12.00 am and ground in liquid nitrogen by using pestle and mortar. The ground calluses were mixed and extracted with extraction buffers pH 7.5 as described in 2.2.2.1 and 2.2.2.2. Both *in vivo* and *in vitro* NR activity were determined as described in 2.2.2.3 A. and B., respectively.

2.2.5 Influence of extraction buffers on stability of NR activity

2 g of calluses were weighed and transferred into 20 ml of fresh induction medium (N6 medium supplemented with 2 mg l^{-1} 2,4-D and 3% sucrose) to induce NR; then the sample flasks were incubated in 16/8 h light/dark period at 28 °C on a shaker at 120 rpm. On day 4 after NR induction, the calluses were collected at 10.00-12.00 am and ground in liquid nitrogen by using pestle and mortar. The ground calluses were mixed and extracted with extraction buffers A, B, C, D and E pH 7.5 as described in table 1. The NR activities of the samples were determined every 4 or 5 h during the day. The stability of NR extracted from corn calluses using several extraction buffers were compared.

Table 1. Component of 5 different extraction buffers

Extraction buffer	50 mM MOPS	5 mM DTT	1 mM EDTA	1mM PMSF	1 μM chymostatin	10 mM $MgCl_2$
A	+	+	+	-	-	-
B	+	+	+	+	-	-
C	+	+	+	-	+	-
D	+	+	+	+	+	-
E	+	+	+	+	+	+

2.2.6 Effect of environmental factors to NR activity in suspension cultures

2.2.6.1 Determination of NR activity in culture for a period of 7 days

The calluses (2 g) were transferred to 250 ml Erlenmeyer flask containing 20 ml N6 medium supplemented with 3% sucrose and incubated in dark

condition for 1 week. Then, the suspension cultures were subcultured by transferring to fresh media and incubated in 16/8 h light/dark photoperiod for 1 week on a shaker at 120 rpm. Aseptically, the corn calluses were weighed (about 0.25 g) and collected for NR activity assay everyday at 10.00-12.00 am for 7 days.

2.2.6.2 Effect of the photoperiod to NR

Two weeks old calluses were weighed (1 g) and transferred to 250 ml Erlenmeyer flask containing 10 ml N6 medium supplemented with 3% sucrose. The suspension cultures were incubated in dark condition for 1 week. Then, the cultures were subcultured by transferring to fresh media with or without nitrate and incubated in 16/8 h light/dark photoperiod. On the 4^{th} day, for dark condition, the cultures were covered with aluminum foil before on set of light. Aseptically, the 0.25 g suspension calluses of all treatment were collected every second hour during the 4^{th} day. The extraction, NR activity assay and protein contents were determined as described previously in 2.2.2.

2.2.7 Effect of nitrate and ammonium ions

About 2 g of 2 weeks old calluses were weighed and cultured in 20 ml N6 medium N-free medium and incubated in the dark for 4 days, then the cultures were transferred to 250 ml Erlenmeyer flask containing 20 ml of fresh media with varying components as described below. The cultures were incubated in 16/8 h light/dark photoperiod for 1 week.

The culture N6 media were modified for each treatment containing:

1. N-free media: N6 medium without KNO_3 and $(NH_4)_2SO_4$
2. N6 medium [without $(NH_4)_2SO_4$]: N6 medium supplemented with several KNO_3 concentrations (5, 10, 15, 30, 50 and 80 mM) without $(NH_4)_2SO_4$.
3. N6 medium [with $(NH_4)_2SO_4$]: N6 medium in the presence of 3.5 mM $(NH_4)_2SO_4$ and several KNO_3 concentrations (5, 10, 15, 30, 50 and 80 mM).

Aseptically, after all cultures were incubated in 16/8 h light/dark photoperiod, on the 4^{th} day after subculture, about 0.25 g suspension calluses were collected and weighed every second day at 3 h after the onset of light. The calluses were

extracted for NR activity assay and protein determination as described previously in 2.2.2.

2.2.8 Effect of sugars on NR

About 1 g of 2 weeks old calluses were weighed and cultured in N6 medium without sucrose (sugar free medium) and incubated in the dark for 4 days. Then, the cultures were transferred to 250 ml Erlenmeyer flask containing 20 ml of fresh modified media as described below and incubated in 16/8 h light/dark photoperiod for 1 week.

The modified culture N6 media as follows:

1. Sugar free media: N6-sugar free medium in the presence of 28 mM KNO_3 without 3.5 mM $(NH_4)_2SO_4$.

2. Sugar media: N6 media in the presence of 28 mM KNO_3 without 3.5 mM $(NH_4)_2SO_4$ and supplemented with 1, 2, 3, 4, 5 and 7% sugar (sucrose, glucose or fructose).

Aseptically, calluses were collected and weighed on the 4^{th} day in the morning (3 h after the onset of light) for 1 week. The calluses were extracted, NR activity assay and protein content of the extract from cultured in N6 medium containing verying concentration of sugars were determined as described previously in 2.2.2.

2.2.9 Effect of light intensity on NR

2 weeks old calluses were weighed (1 g) and transferred to 250 ml Erlenmeyer flask containing 10 ml N6 medium. The suspension cultures were incubated in dark condition for 4 days, then the calluses were subcultured by transferring to fresh media (supplemented with 3% sucrose) and incubated in 16/8 h light/dark photoperiod by using 0-345 µmole $m^{-2}s^{-1}$ of light intensities.

Aseptically, calluses were collected and weighed on the 4^{th} day after subcultured in the morning (3 h after the onset of light) for 1 week. The extraction, NR activity assay and protein contents were determined as described previously in 2.2.2.

2.2.10 NR-NAD(P)H activities from cultured callus and corn seedling

The calluses were induced in N6 medium supplemented with 2 mg 2,4-D and 3% fructose then 2 weeks old calluses were weighed (1 g of callus per 10 ml culture

media) and transferred to 250 ml Erlenmeyer flask containing N-free medium. The suspension cultures were incubated in dark condition for 4 days, then the calluses were subcultured by transferring to fresh media (supplemented with 28 mM KNO_3, without 3.5 mM $(NH_4)_2SO_2$ and 3% fructose) for induction of NR activity and incubated in 16/8 h light/dark photoperiod. Aseptically, the calluses were weighed and collected on the 4th day after subculture at 10.00-12.00 am. The NADH and NADPH NR activity were determined as described in 2.2.2.

NADH- and NADPH-NR activities from corn seedling were also assayed. Sweet corn (*Zea mays* var. Olympia99) seeds were soaked in distilled water for 5 to 6 h and then allowed to germinated in pots (15x15 cm) containing a mixture of sand and soil in a greenhouse. The corn seedlings were watered every day with distilled water for 1 week. The seedlings were grown and supplied with 2.74 g/pots of KNO_3 and watered every day with 100 ml of distilled water in light condition for 1 week. Every 2 days, the leaves and roots were harvested at 3-5 h after exposure to light. Each sample was analyzed from 10 different plants. The samples were ground in liquid nitrogen by using pestle and mortar; then the samples were kept at -80 °C before NR assay.

2.2.11 NAD(P)H-NR kinetics

NAD(P)H-NR kinetics were studied from callus suspension. The 2-week-old calluses were suspended in N6 medium in the presence of 28 mM KNO_3 without 3.5 mM $(NH_4)_2SO_4$) and supplemented with 2 mg l^{-1} 2,4-D and 3% fructose on a shaker at 120 rpm for 4 days in darkness after which the suspended calluses were transferred to fresh N6 medium and incubated in 16/8 h light/dark photoperiods on a shaker at 120 rpm for NR induction. Aseptically, 4 days after subculture, the calluses were collected and weighed at 10.00-12.00 am. NR activity and kinetics of NADH and NADPH-NR were determined using crude extract from calluses as described previously by using 10 mM KNO_3 as the substrate and 0-1.0 mM of NADH and NADPH as an electron donor.

Chapter 3

RESULTS

3.1 Induction of callus and suspension cultures from sweet corn

3.1.1 Influence of 2,4-D on callus induction

For induction of corn calluses, immature embryos were cultured in modified N6 media from Chu *et al.*, 1975 and several types of corn callus were found as shown in figure 15. The corn callus from immature embryo was classified as Type I (compact callus and regenerated callus) and Type II (friable callus). For Type II, this characteristic much easier to establishment of suspension cultures.

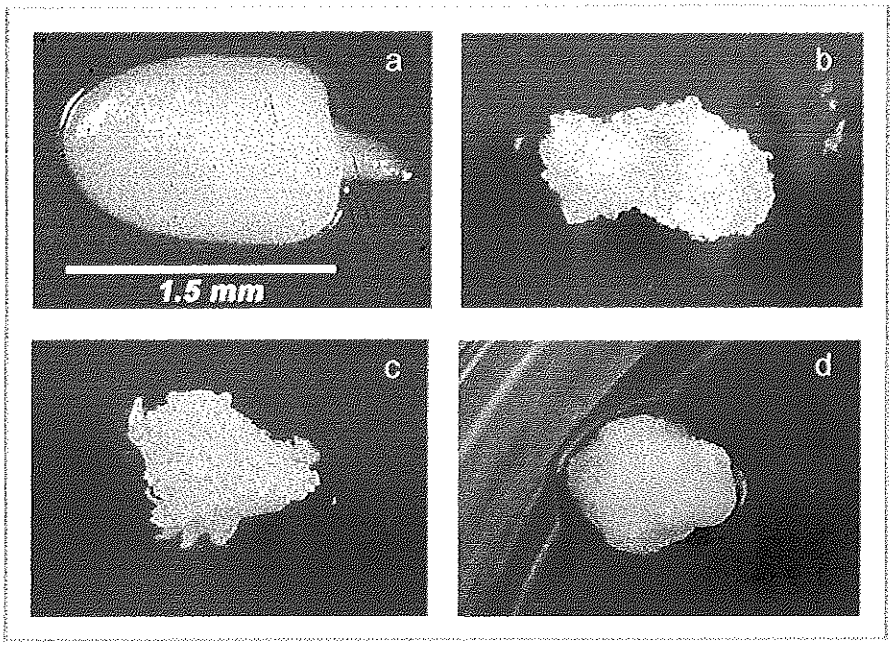

Figure 15 Immature corn embryo (a), friable callus (b), shoot and root developed callus (c) and compact callus (d).

The influence of the concentration of 2,4-D on the development of cultured embryos was shown in figure 16a for mature embryo and in figure 16b for immature embryo. During culture on N6 media supplemented with a range of 2,4-D

concentrations, the optimal concentration for the induction of Type II callus was 2 mg l^{-1} 2,4-D.

For callus induction from mature embryos, calluses were initiated from 5.0 mm immature embryos of corn. The mature embryos failed to develop callus at all concentrations of 2,4-D (Figure 16a). In the first week, failed to develop into callus or gave callus and germinated in some embryos. When the concentration was raised from 1 to 2 mg l^{-1}, less of type II callus was formed. At 5 and 10 mg l^{-1} 2,4-D mature embryo did not form callus at all, and became static. When no 2,4-D was added in medium, the embryos germinated and no callus was formed. In the second week, at 1 to 2 mg l-1 2,4-D, the embryoids grew rapidly within this week and transited from induction of callus to germination. After 4 weeks of culture, shoots and roots were established from mature embryos in all cultures of with different 2,4-D concentrations ceased to grow and develop and finally turned to a brown color.

For callus induction from immature embryos, calluses were initiated from 2.0 mm immature embryo of corn cultured on solid N6 media enriched with various concentrations of 2,4-D. The most effective 2,4-D level was 2 mg l^{-1}, which provided the highest percentage of callus formation. The inclusion of 1 to10 mg l^{-1} 2,4-D in the medium improved callus growth, but declined growth and development of shoot and root after 4 weeks in the culture media. Immature embryos from 2.5 month old plant were more suitable than those from 3 months old plant (Figure 12) for callus induction. In the first week, the immature embryo culture resulted in calluses with shoot at all concentrations of 2,4-D (Figure 16b). When the concentrations of 2,4-D was raised from 1 to 10 mg l^{-1}, embryo transited from immature embryo growth to development of callus. When no 2,4-D was added in media, the embryos germinated and did not form callus. In the second week, at all concentrations of 2,4-D, the calluses rapidly grew within this week. After 4 weeks of culture, the calluses established from immature embryos in the media with 1, 5 and 10 mg l^{-1} 2,4-D decreased in size and turned to brown color more rapidly than calluses induced with 2 mg l^{-1} 2,4-D.

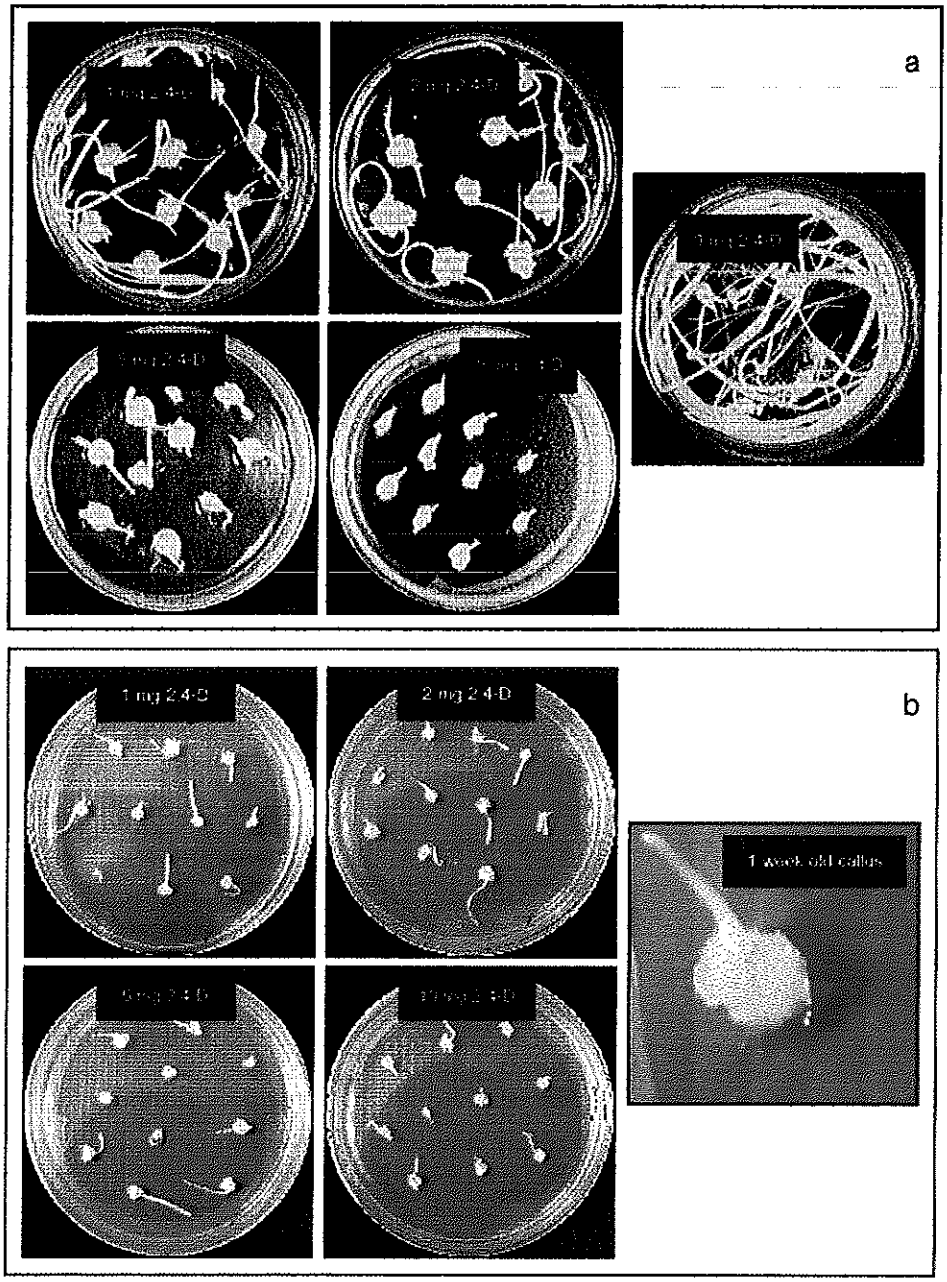

Figure 16 Influence of 2,4-D on callus induction in Olympia 99 corn embryos mature (a) and immature (b).

3.1.2 Callus formation stages

The formation of calluses from corn immature embryos was determined. The result in previously described in 3.1.1 suggested that for induction of corn callus from immature embryo the optimal size was 2.0 mm and the optimal concentration of 2,4-D was 2 mg l^{-1}. Therefore, in this study, immature embryos (1.5-2.0 mm) were cultured in N6 media supplemented with 2 mg l^{-1} 2,4-D. The cultures were maintained in the dark and the formation of Type II callus was observed.

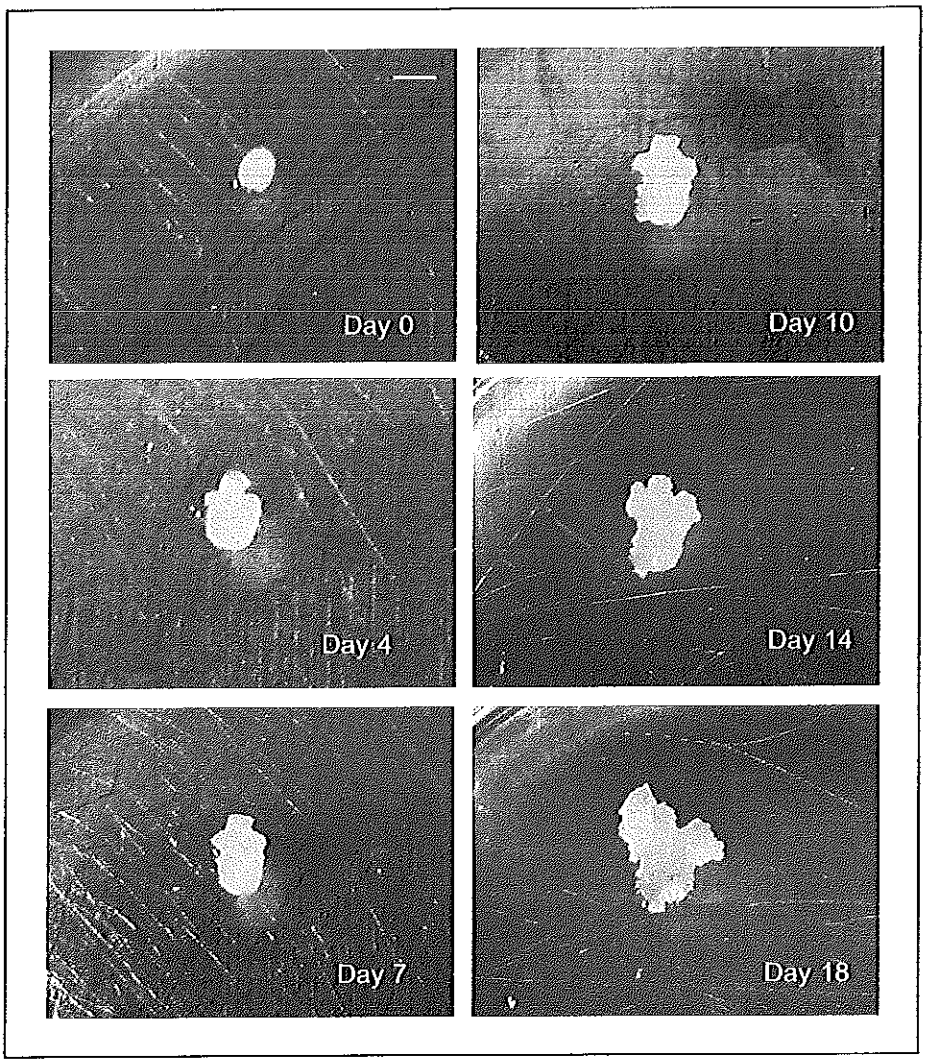

Figure 17 Callus development during culture period in N6 media supplemented with mg l^{-1} 2,4-D.

— = 2.0 mm

The development of corn callus was very rapid during the first two weeks. Callus originated from the scutellum in day 4 after that the Type II callus was formed in 2 week. Type II callus was distinguishable as soft and friable with numerous individual small clumps protruding from the callus surface (Figure 17). In the case of immature embryos with the sizes ranging from 1.5 to 2.0 mm, the influence of the concentration of 2,4-D on the development of cultured embryos was shown as described previously in figure 16b. The medium with 2 mg l^{-1} 2,4-D gave better growth than other concentrations, confirming the optimal concentration at 2 mg l^{-1}. However, shoot development was not successfully obtained by subculture these calluses on N6 media every 2 weeks

3.1.3 Growth rate of callus in light and dark condition

In order to obtain the best growth condition, the increase of fresh weight and differentiation of calluses in light and dark conditions were determined. The callus fresh weight in the light and dark conditions slowly increased in the first week after subculture in N6 medium supplemented with 2 mg l^{-1} 2,4-D and 2% sucrose (Figure 18a). Moreover, 1 week after subculture, corn callus grown in light was similar in dark condition (Figure 18a). There was no significant difference in the % weight increase of cultured in both conditions. In both light and dark condition, the immature embryos on N6 media showed similar behavior at the beginning, independently of their initial size. After 2 weeks of callus initiation, in the light, callus developed normally to form shoot and root length, after which all of them germinated and green spots were observed as shown in figure 18b. The friable Type II callus was rapidly induced in the first week of culture period under dark condition (Figure 18c). After 2 weeks of callus initiation in the dark, calluses rapidly grew but germination and green spots were not detected.

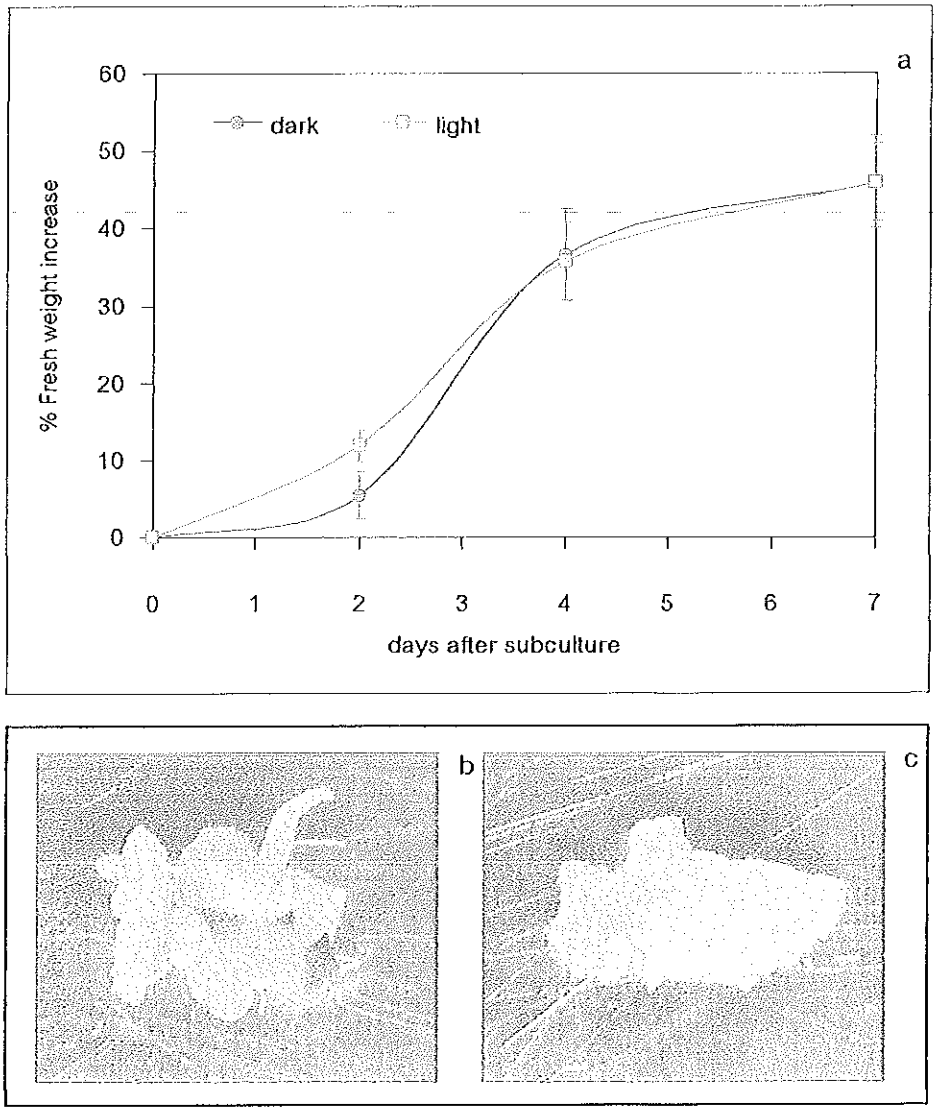

Figure 18 Effect of light and dark condition on callus induction.

a. % Weight increase after the first subculture on N6 medium supplemented with 0.25% phytagel.

b. Induced callus in light condition (2 weeks old callus).

c. Induced callus in dark condition (2 weeks old callus).

3.1.4 Effect of embryo orientation to embryogenic callus development on nutritionally complete growth medium

An efficient mode of placement of the embryo to enhance callus induction frequency was identified in this study. The callus induction medium was N6 medium supplemented with 2 mg l^{-1} 2,4-D, 200 mg l^{-1} casein hydrolysate, 100 mg l^{-1} myoinositol, sucrose 2% and solidified with 0.25 % phytagel. The culture was kept under dark conditions at 26±1 $^{\circ}C$ for 14 days and callus induction frequency was scored visually. Ten immature or mature seeds per replication with three replications were used for this experiment. Two different embryo placements (Figure 13) were taken into consideration. The results indicated that the mature embryo when placed laterally and half inserted in the media (vertically), showed better callus induction frequency and callus growth as compared to horizontal (plumule-radicle side placed downward) place on media. When mature embryo was placed facing the medium, the plumule starts to germinate quickly, pushing the explant up and disconnecting it from the media surface, thereby restricting the callus induction. Immature embryo has been used extensively to obtain embryogenic callus and regeneration in corn tissue culture. For callus induction from immature embryo, the orientation of the embryo on the media has small effect. The plumule-radicle side of the immature embryo, when placed downward, gave a small greater frequency of induction and amount of callus induction than the scutellar side placed downward (data not shown). Hence, placement of embryos on the medium plays a critical role in successful cultures in mature embryo more than in immature embryo.

3.1.5 Growth rate of cell suspension

The effect of sucrose on fresh weight of calluses, 2,3 or 4% sucrose in N6 medium supplemented with 2 mg l^{-1} 2,4-D was studied. The fresh weight of callus in N6 media supplemented with 3% was higher than that in N6 media supplemented with 2% and 4% sucrose (Figure 19). The best callus growth (100 %) was obtained using N6 medium supplemented with 2.0 mg/L 2,4-D, and 3% sucrose. Increase in sucrose concentration of 4% in the N6 medium reduced callus growth by about 30 % in the first week after subculture (Figure 19).

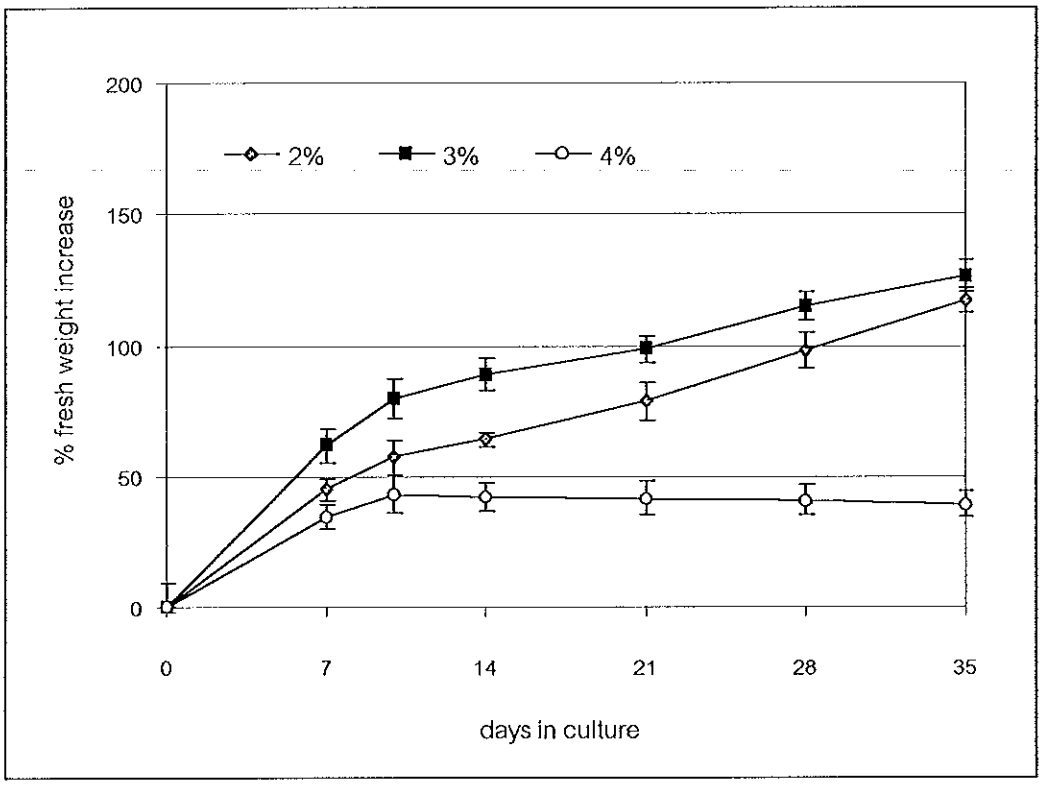

Figure 19 The % fresh weight of corn calluses in suspension culture increases as affected by sucrose levels in liquid N6 medium supplemented with 2 mg l^{-1} 2,4-D under 16/8 h in light/dark. Bars in data represent ± SE.

The fresh weight of calluses rapidly increased in medium with 2 and 3% sucrose in the first week after subculture. After the second week, the fresh weight of calluses slightly increased and the white or yellow calluses turned to brown color on the forth week after subculture. Both in 2 and 3% sucrose, the calluses ceased to grow and develop after the forth week. In addition, the growth rate of callus in 3% sucrose increased higher than the calluses that with 2% sucrose. Moreover, after 2 weeks of the culture, the formation of shoots and roots were obtained from primary callus in all cultures of the N6 medium with different sucrose additives. In addition, the light intensity altered callus to be small roots and shoots with green spot and led finally to a loss in totipotency of the callus. Moreover, during culture in liquid medium, some calluses

germinated with both shoot and root liked organs. Higher sucrose (4% sucrose) concentration, the fresh weight of corn calluses slowly increased and reached the maximum weight on day 10 (Figure 19). Moreover, in 4% sucrose, the white callus turned to yellow callus in the first week and ceased to grow and finally changed to a brown color. Concentrations of sucrose affected the survival of the youngest calluses more than the oldest ones.

3.2 Nitrate reductase determination

3.2.1 Determination of NR activity in corn and rice seedling

1 week old seedlings of rice and corn were treated with 5 mM KNO_3 and 5 mM NH_4NO_3 and then, the activities of NR in leaves, stems and roots were compared. In rice seedlings, activities of NR were detected in green leaves after treatment with 5 mM KNO_3 and 5 mM NH_4NO_3, the specific activities were 0.363±0.058 and 0.174±0.018 μ mole/min/mg protein, respectively. The results indicated that NR activity of KNO_3-treated leaves were induced more than that of NH_4NO_3-treated leaves. In the contrast, in roots of rice seedlings, no activity of NR was detected when treated with both KNO_3 and NH_4NO_3. In corn seedlings treated with 5 mM KNO_3, low levels of NR activity were detected in both green leaves and roots and their specific activities of NR were 0.184±0.022 and 0.0334±0.019 μmole/min/mg protein, respectively. In corn seedlings treated with 5 mM NH_4NO_3, activities of NR were detected in green leaves, but not in stems and roots as found in rice seedlings. The specific activity of NR in leaves treated with NH_4NO_3 was 0.106±0.017 μmole/min/mg protein (Figure 20).

The result showed that NR activities were induced in rice more than in corn seedlings both in treated with 5 mM KNO_3 and 5 mM NH_4NO_3. In addition, both in rice and corn seedlings, KNO_3 could induce NR activity better than NH_4NO_3. In conclusion, rice leaves showed markedly higher nitrate reduction than corn leaves in the absence as well as the presence of NH_4^+ ion. The reduction of NR in corn treated with 5 mM KNO_3 and 5 mM NH_4NO_3 were 50.68% and 60.92% that of rice. In the presence of NH_4^+, rice leaves reduced 52.07% of the NR activity relative to 42.39% in corn leaves when compared with in the absence of NH_4^+ of both seedlings. In roots of corn seedlings, in

the presence of NH_4^+ NR activity at 5 mM KNO_3 reduced 100% when compared with in the absence of NH_4^+. Leaves and roots NR activities of 0.231±0.0098 and 0.1049± 0.0226 μmole/min/mg protein in corn were achieved at a 10 mM KNO_3 and slightly increased 0.25 and 3.2 fold higher than that leaves and roots of corn seedlings treated with 5 mM KNO_3, respectively. As can be seen from figure 20, NR activity increased with increasing nitrate concentrations in corn seedlings. However, the NR activity of both species the presence of NH_4^+ ions was greatly reduced in both species.

The protein contents of both seedlings were evidently under the influence of N source (data not shown). Higher values were found in the presence of in 5 mM NH_4NO_3 of both seedlings. The protein of both seedlings was in the same externals KNO_3 or NH_4NO_3 concentrations approximately the same. However, the protein contents of both seedlings treated with NH_4NO_3 were higher than that treated with KNO_3. Moreover, in corn seedlings, the protein levels of both leaves and roots were approximately the same in both 5 and 10 mM KNO_3.

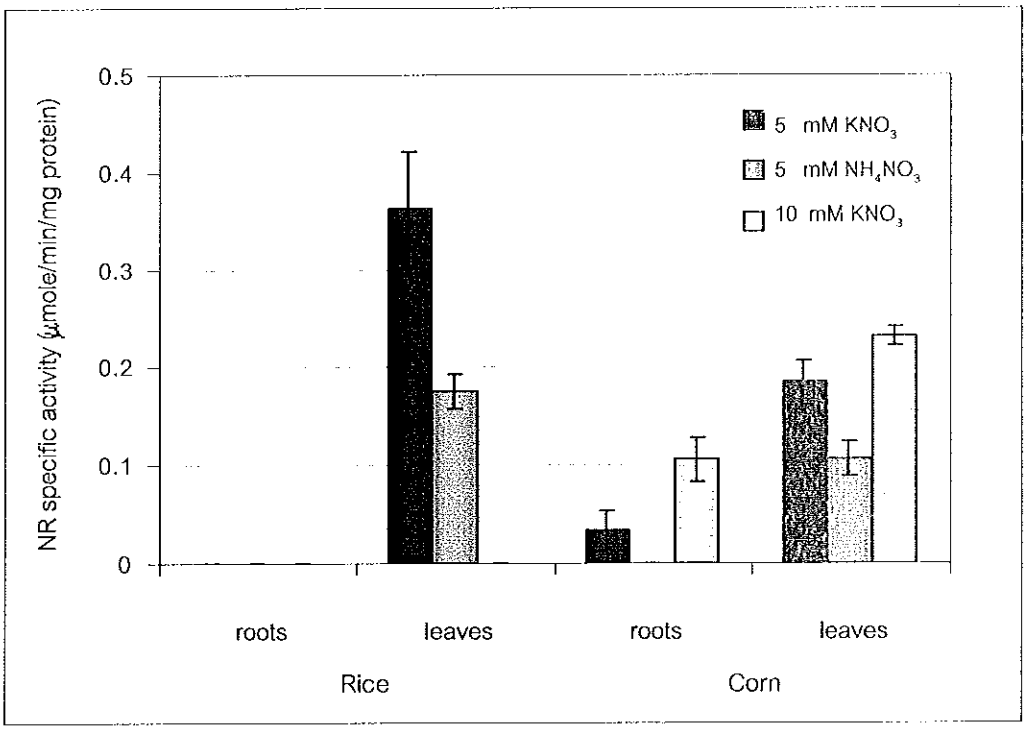

Figure 20 NR specific activities in leaves and roots of rice seedlings supplied with 5 mM KNO_3 or 5 mM NH_4NO_3 and corn seedlings supplied with 5 and 10 mM KNO_3 or 5 mM NH_4NO_3. Values are means from three replications ±SD.

3.2.2 Comparison of NR *in vivo* and *in vitro* assays

Callus suspension cultures were used to initiate callus to ensure cellular homogeneity and *in vivo* and *in vitro* assays for NR was compared. After 4 days in the dark, calluses were transferred to fresh medium and a 16/8 h light /dark photoperiod and assayed on the 4th day after subculture for NR activity induction using the optimised conditions for *in vivo* and *in vitro* assays. NR activity measured by the two assays showed similar trends. The specific activities of NR by using *in vivo* and *in vitro* assays were 0.998± 0.158 and 0.979± 0.181 µmole/min/mg protein, respectively. No significant differences occurred between the two assayed.

3.2.3 Influence of extraction buffer on stability of NR activity

For NR activity induction, calluses were cultured in N6 medium supplemented with 28 mM KNO_3 and 3.5 mM $(NH_4)_2SO_4$ and incubated in 16/8 h light/dark periods for 4 days. When, the corn calluses were harvested and extracted by using 50 mM MOPS buffer pH 7.5 containing several compounds (protease inhibitors etc. PMSF and chymostatin) as described in table 1, then crude extracts were kept on ice and NR activities were determined every 4 h. The experiment was repeated three times with different enzyme crude extraction samples. Using NADH as an electron donor and KNO_3 as the substrate, the NR activities, assayed in the presence of both chymostatin and PMSF without Mg^{2+} in extraction buffer D, were highest among other buffers as shown in figure 21. In contrast, the sensitivity of corn NR activity to Mg^{2+} inhibition was shown clearly by calluses extracted with buffer E, in the presence of both chymostatin and PMSF with Mg^{2+}, the NR activities of which remained lower than other extraction buffers but decreased slightly throughout the incubation on ice. The MOPS buffer containing 5 mM DTT and 1 mM EDTA pH 7.5 (extraction buffers A) was used as control in this experiment, NR activities of corn calluses extracted in it were lower than that extracted in buffer B, C and D but higher than that extracted in buffer E. In addition, in calluses extracted in buffer A, NR activities in corn calluses decreased slightly and were sustained at a low level throughout the 12th h after extraction and kept on ice. It rapidly lost NR activity within 5 h after that. In calluses extracted in buffer C supplemented with 10 µM chymostatin and extracted in buffer B supplemented with 1 mM PMSF, NR

activities of both crude extract were similar and lower levels than that extracted in buffer D that supplied with both chymostatin and PMSF. Either PMSF or chymostatin were affected to stability of NR, but activities of NR were almost totally activated by using both chymostatin and PMSF in extraction buffer. However, the NR activity was expectedly inhibited by 28.57% in the presence of Mg^{2+} in buffer E when compared with the activities of NR in crude extract D that supplied both chymostatin and PMSF in extraction buffer without Mg^{2+}. In contrast, in figure 21, the remaining of NR activities of all crude extracts were similar and slightly decreased after extraction and kept on ice, excepted in crude extract A (control) that lost in activity after 17 h of incubation on ice. Figure 21 showed that the presence of both PMSF and chymostatin in extraction buffer, although it did allow some increase in the activity and stability of NR from callus, only partially prevented its inactivation in vitro.

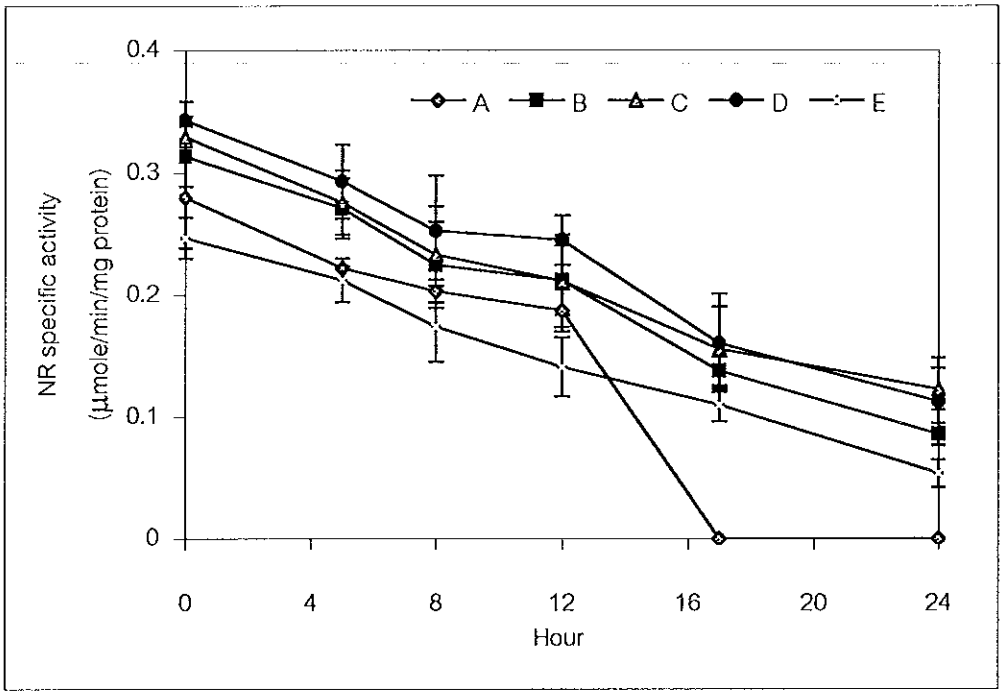

Figure 21 Influence of extraction buffers on stability of NR activity. Values are means from three replications ±S. The NR specific activities of corn callus that extracted by using extraction buffer containing:

A: 50 mM MOPS buffer, pH 7.5 + 5 mM DTT + 1 mM EDTA

B: 50 mM MOPS buffer, pH 7.5 + 5 mM DTT + 1 mM EDTA + 1mM PMSF

C: 50 mM MOPS buffer, pH 7.5 + 5 mM DTT + 1 mM EDTA + 1 µM chymostatin

D: 50 mM MOPS buffer, pH 7.5 + 5 mM DTT + 1 mM EDTA + 1mM PMSF + 1 µM chymostatin

E: 50 mM MOPS buffer, pH 7.5 + 5 mM DTT + 1 mM EDTA + 1mM PMSF + 1 µM chymostatin + 10 mM $MgCl_2$

3.2.4 Effect of environmental factors to NR activity in suspension culture

3.2.4.1 Effect of culture period on plant growth, NR specific activity, protein and nitrite content in corn callus

In the corn callus suspension cultures, corn calluses were cultured in N6 medium supplemented with 2 mg l^{-1} 2,4-D in darkness condition for 1 week after that subcultured in fresh N6 media and incubated in 16/8 h light/dark photoperiod. In three independent experiments with calluses transferred to N6 media, the % fresh weight increase, NR activity, protein and nitrite accumulations were investigated in 7 days of culture period. Corn calluses grew more slowly. In figure 22a, the corn callus weight highest increased only about 25 % within 7 days of culture. During the 3 days after transfer to fresh N6 media containing 28 mM KNO_3^- and 3.5 mM $(NH_4)_2SO_4$, there was only a small effect of media N on fresh weight of corn cell culture. The fresh weight slightly increased about 8% in 3 days after subculture and then rapidly increased about 16% on the 4^{th} day after subculture. Callus fresh weight continued to increase throughout the 7 days culture period, suggesting the cultures did not reach stationary phase in this period. Media N also affects enzyme activities associated with nitrate assimilation (e.g. NR). In calluses transferred to N6 media containing 28 mM KNO_3^- and 3.5 mM $(NH_4)_2SO_4$, NADH: NR specific activity did not change significantly over the 3 days period after subculture, then increased relatively highest on the 4^{th} day after transfer. After that NR activities decreased somewhat on day 5 by about 73.85 % (Figure 22b). In calluses transferred to N-free media, NR activity was not detected throughout the culture period (data not shown). However, the protein contents did vary consistently throughout 7 days culture period. When calluses were transferred to N6 media, the protein contents were slightly increased on the 1^{st} day after subculture, then remained constant (Figure 22c). In addition, to test nitrite contents, which produced by nitrate assimilation enzyme (NR) in calluses transferred to fresh N6 media, the data showed that the nitrite content slightly increased in 3 days after subculture. After that nitrite contents were rapidly increased on the 4^{th} day after subculture (Figure 22d). Total nitrite increase was about 0.14 to 0.16 mmole/mg protein then, nitrite contents were highest and constant on the 6^{th} and on the 7^{th} day. The data indicated that the calluses maintained

the ability to rapidly induced nitrate assimilation enzyme (NR) on the 4th day after subculture. However, NR activities were inhibited by nitrite content that increased in the calluses after on the 4th day of subculture.

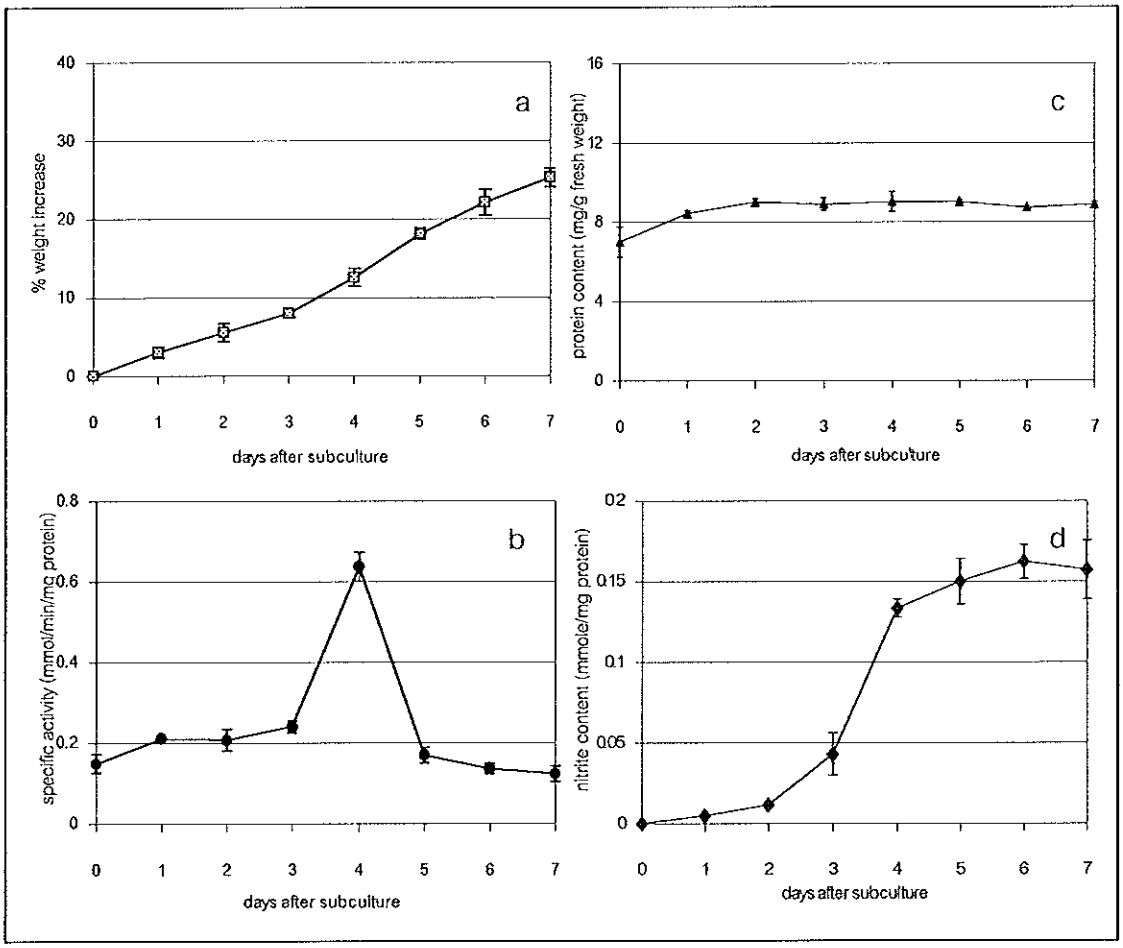

Figure 22 Changes of corn callus during 7 days sudculture period, % callus fresh weight increases (a), protein contents (b), specific activities of NR (c) and nitrite accumulations (d) of corn callus on the 7 days culture period. Values are means from three replications ±SD.

3.2.4.2 Diurnal changes of NR activity in the cultured callus

In this study, on the 4th days after subculture, calluses were cultured in either continuous darkness or long day conditions (standard photoperiod; 16 h light: 8 h dark) in the presence and absence of nitrate. NR activities were measured in corn calluses over the course of time. Diurnal variations of NR activities over a 16 h day and 8 h night growth cycle were detected. In a long day condition, no NR activity was detected in the absence of nitrate in N6 medium (data not shown). In the presence of nitrate, the maximum of NR activities of calluses was highest in light condition, but much lower in dark condition as shown in figure 23a. The NR activities increased when the light was switched on. In light period, the specific activity of NR was not much different (0.5 versus 0.7 μmole/min/mg protein) for this treatment. After onset of light, the NR activities increased during the first hour of illumination in cultured calluses and the increase was more rapid. NR activity remarkably increased during 2-4 h of the light period, then rapidly decreased in the dark phase. Callus NR reached maximum activity after 4 h of illumination, and their specific activity was about 0.7 μmole/min/mg protein. Thereafter, activity decreased gradually until, at the end of the light period, it was about the same as at the beginning of the light period. Levels of NR activities remained constant throughout the dark period. In the dark phase, the activities of NR and protein contents (data not shown) remained almost constant during the day; however, they increased in the light phase in the long day photoperiod exposed calluses, indicating net NR degradation during the day. Little difference of NR activity between light and continuous dark condition was indicated in the result of sugar (Larios *et al.*, 2001).

In continuous darkness condition, no activity of NR was also detected in the absence of nitrate (data not shown). In the presence of nitrate, the level of NR activities remained constant throughout the dark period and their specific activities were 0.35 μmole/min/mg protein (Figure 23b). These results suggested that NR activities were also limited by NO_3^- availability in darkness. In the absence of NO_3^-, however, the addition of sucrose to the assay medium had no effect on NR activity, whereas it enhanced enzyme activity when NO_3^- was present in darkness (Figure 23b). Under dark condition, the NR activity of corn callus, which cultured in N6 medium

supplemented with 3% sucrose, was generally only about 55% of the activity of under light phase in long-day condition supply.

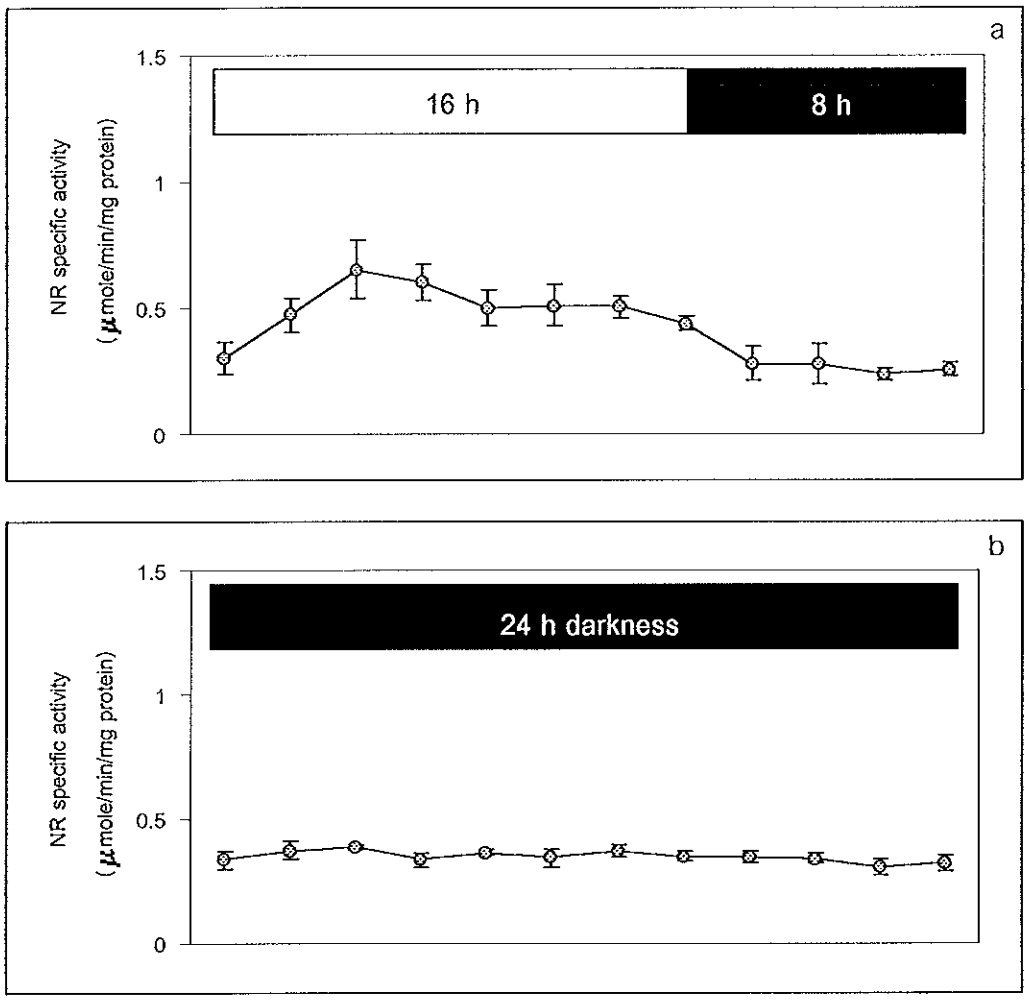

Figure 23 Diurnal change of NR specific activity in corn callus cultured in N6 medium supplemented with 3% sucrose in the presence of nitrate. Values are means from three replications ±SD.

a. Long-day condition

b. Continuous dark condition

3.2.5 The effect of N supply on corn callus NR

In preliminary experiments with corn callus initiated from cell suspension cultures, a decrease in enzyme activity in the callus was accompanied by substantial depletion in medium nitrate (N-free media). Therefore, NR activity regulation and protein content of corn callus during cultured on N6 medium supplemented with several concentrations of KNO_3 and with or without $(NH4)_2SO_4$ were investigated. Media N affected enzyme activities associated with NO_3^- assimilation. As described previously, in callus transferred to standard N6 media supplemented with 28 mM KNO_3 and 3.5 mM $(NH_4)_2SO_4$, NADH: NR remained relatively high for 4 days after transfer, then decreased and sustained in low levels throughout the culture period. NR activity increased during development in calluses transferred to N6 media (Figure 22b). To test NO_3^- induction of enzyme activities in N-starved calluses, cultures were transferred to N-free media for 4 days after that subcultured to fresh N6 medium supplemented with several concentrations of KNO_3 (5, 10, 15, 20, 30, 50, 60 and 80 mM) and with or without 3.5 mM $(NH_4)_2SO_4$. The NR activities shown in figure 24a were evidently under the influence of both N sources and N levels. For 4 days after subculture, the positive effect of nitrate on NR activity was observed both in the absence and presence of ammonium in N6 media. Higher values of NR activity were found for higher nitrate with out ammonium and nitrate predominated when presented of ammonium. NR activity reached to maximal levels on the 4^{th} day for 30 mM KNO_3 without NH_4^+. In low (5 and 10 mM KNO_3) concentrations of nitrate both with or without ammonium (3.5 mM $(NH_4)_2SO_4$), no NR activities were detected on the 4^{th} day after subculture. In addition, the result showed that, in high levels of nitrate, differences in NR activity (Figure 24a) between the two nitrogen treatments were not statistically significant, indicating that the developmental regulation of NR by ammonium during corn callus culture was not affected when cultured in high nitrate levels.

On the 4^{th} day after subculture, in the absence of ammonium, induction of NR activities were highest at 30 mM external KNO_3, even though NR activities increased with increasing external nitrate. Treatment with ammonium absolutely inhibited the induction of NR activity at external KNO_3 concentrations below 15 mM. Moreover, in callus

supplied with 3.5 mM $(NH_4)_2SO_4$ at 30 mM NO_3^-, the level of the enzyme activity was 70 to 80% lower than in calluses not supplied with ammonium. NR activities of corns calluses treated with ammomiun at all exogenous KNO_3 concentrations were lower than in those not treated with ammonium. In contrast, ammonium had little effect on NR activity of corn calluses induced with 30 mM KNO_3. However, as shown in figure 24b, the protein contents of nitrate plus ammonium treated calluses were higher than that only nitrate treated calluses during the same period. In all results indicate that the optimal concentration of exogenous nitrate was 30 mM KNO_3 and in the presence 3.5 mM $(NH_4)_2SO_4$, ammonium ions inhibited of NR activity.

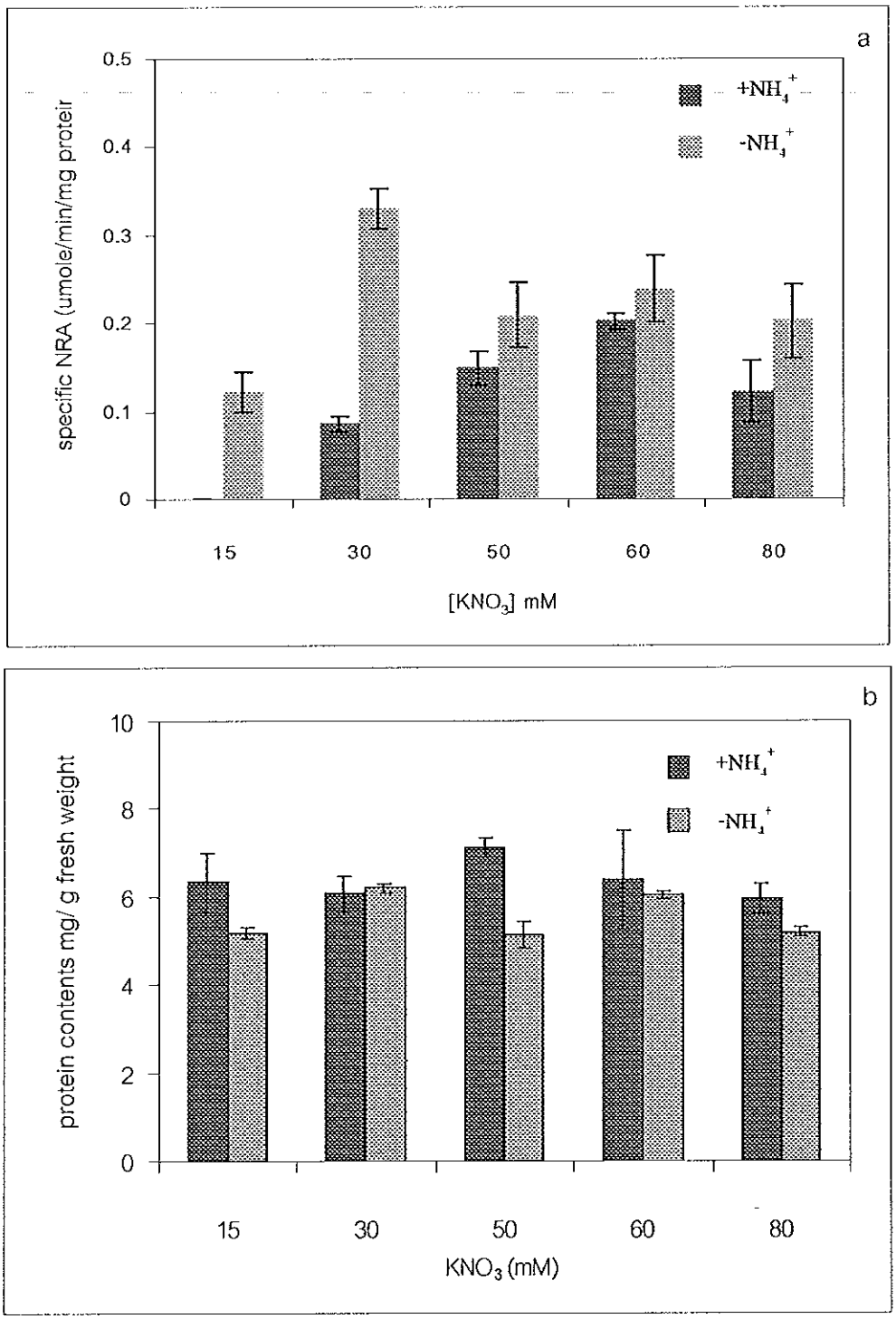

Figure 24 Influence of nitrate and ammonium ion on NR activity (a) and protein contents (b) in callus that cultured in liquid N6 medium supplemented with KNO_3 [with or with out 3.5 mM $(NH_4)_2SO_4$]. Values are means from three replications ±SD.

3.2.6 Influence of sugars on NR

To test the hypothesis that sugar increased NR activity independently, feeding studies with sugars were performed and the activities of NR were determined 4 days after sugar supplementation. The effect of exogenous sugars on NR was studied on corn calluses cultured in N6 medium containing different concentrations of sugars such as sucrose, fructose or glucose. Sugars were important for the regulation of nitrogen metabolism and optimal sugar concentration was 2-3% in N6 medium. In the presence of 28 mM KNO_3 without ammonium ions in N6 media, fructose markedly increased the levels of NR activity more than glucose and sucrose (Figure 25a). In sugar-free medium no NR activity was detected in the cells (data not shown). High sugar levels in medium led to decreases in NR activity, probably due to high osmolarity (Figure 25a). Sugars (sucrose, glucose and fructose) induced NR activity in the presence of nitrogen. Under nitrate depletion, NR activity was not affected by sugar feeding (data not shown). By contrast, NR activity was increased by sugar feeding only in the presence of nitrate. Maximal activity was detected 4 days after sugar addition, and then NR activity decreased. These changes in NR activity were similar to the ones obtained in the presence of nitrate (Figure 25a) and are consistent with the idea that sugars regulated NR expression independently from nitrate assimilation. The addition of fructose led to a higher response in NR induction. In contrast, the increase of NR activity level in the presence of both nitrate and sugars was greater than that in the absence of sucrose, suggesting that NR activity was limited more by the availability of nitrate than by energy supply. In corn calluses cultured in N6 medium supplemented with sucrose as carbon source and presented of nitrate, the NR activities were highest at 3-4% sucrose. In contrast, using glucose as carbon source, the NR activities were highest at 2 % glucose but at other concentrations only a small increase of NR activity was found. In calluses cultured in N6 medium supplemented with fructose as carbon source and in the presence of nitrate, NR activities were highest at 3% sucrose. It can be noted that on supplement, increase in sugar led to a small increase in the protein contents reaching a saturation level at 3-4 %sugar (Figure 25b).

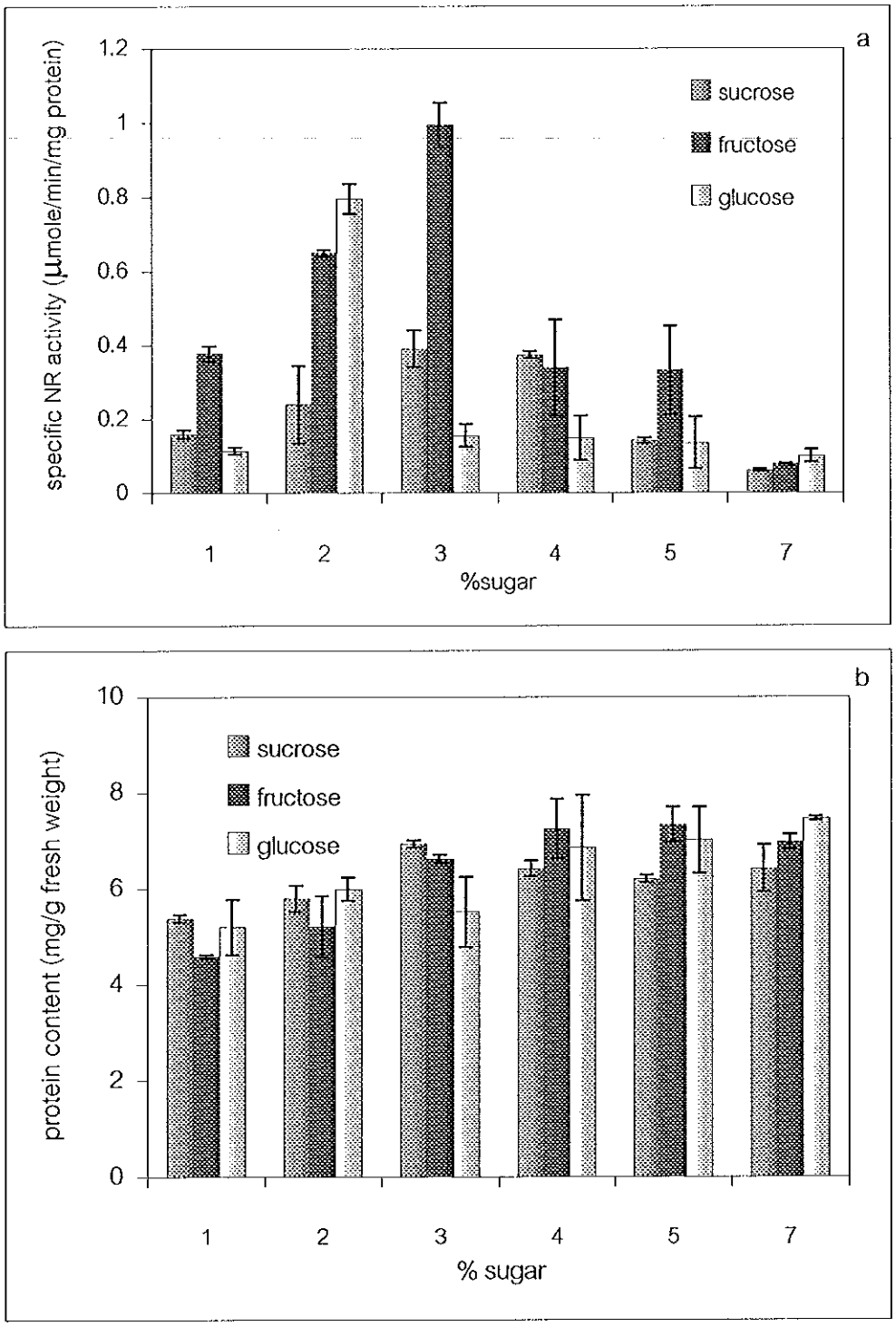

Figure 25 Influence of sugar (sucrose, fructose and glucose) contents on NR activity (a) and protein content (b) of corn calluses. Values are means from three replications ±SD.

3.2.7 Effect of light intensity on NR

The increase in NR activity in response to photoperiod conditions (Figure 22) was striking evidence for the importance of light in NR regulation during culture in N6 media. Two weeks old dark grown calluses were induced with 28 mM KNO_3 without NH_4^+ ions and 3% fructose in N6 medium. On the 4th day after subculture under 16/8 h light/dark photoperiod, calluses were collected 4 hous after the onset of light. To investigate the activation of light intensities for the maximum activation of NR, a separate set of experiments was carried out where calluses were transferred from darkness to various light intensities from low of 110 µmole m^{-2} s^{-1} to high of 345 µmole m^{-2} s^{-1}, keeping 3 flasks of calluses in the dark as the control. After 4 h illumination, when the activities were at maximum in the light, the values of NR activity in high light intensity differed from those in low light. The influence of light intensities on NR activities and protein contents in corn calluses was demonstrated in figure 26. The nitrate reductase activity increased as the light intensity increased but no saturation level was reached (Figure 26a). The light intensity at which highest NR activity of the calluses was estimated to be 345 µmol m^{-2} s^{-1}. On the basis of all experiments in this work, an irradiance of 345 µmol m^{-2} s^{-1} was maximal light intensity used to illuminate the callus suspension cultures. At this intensity limitation (of the instrument) it might not be sufficient to fully activate the NR protein pool, and thus it might produce a lower level of NR activation in corn calluses. In addition, the NR activities and protein levels were largely correlated under the same light intensity. So far, the calluses were cultured under irradiance of this intensity, induced highest protein contents (Figure 26b). In long period of dark condition, no NR activity was detected.

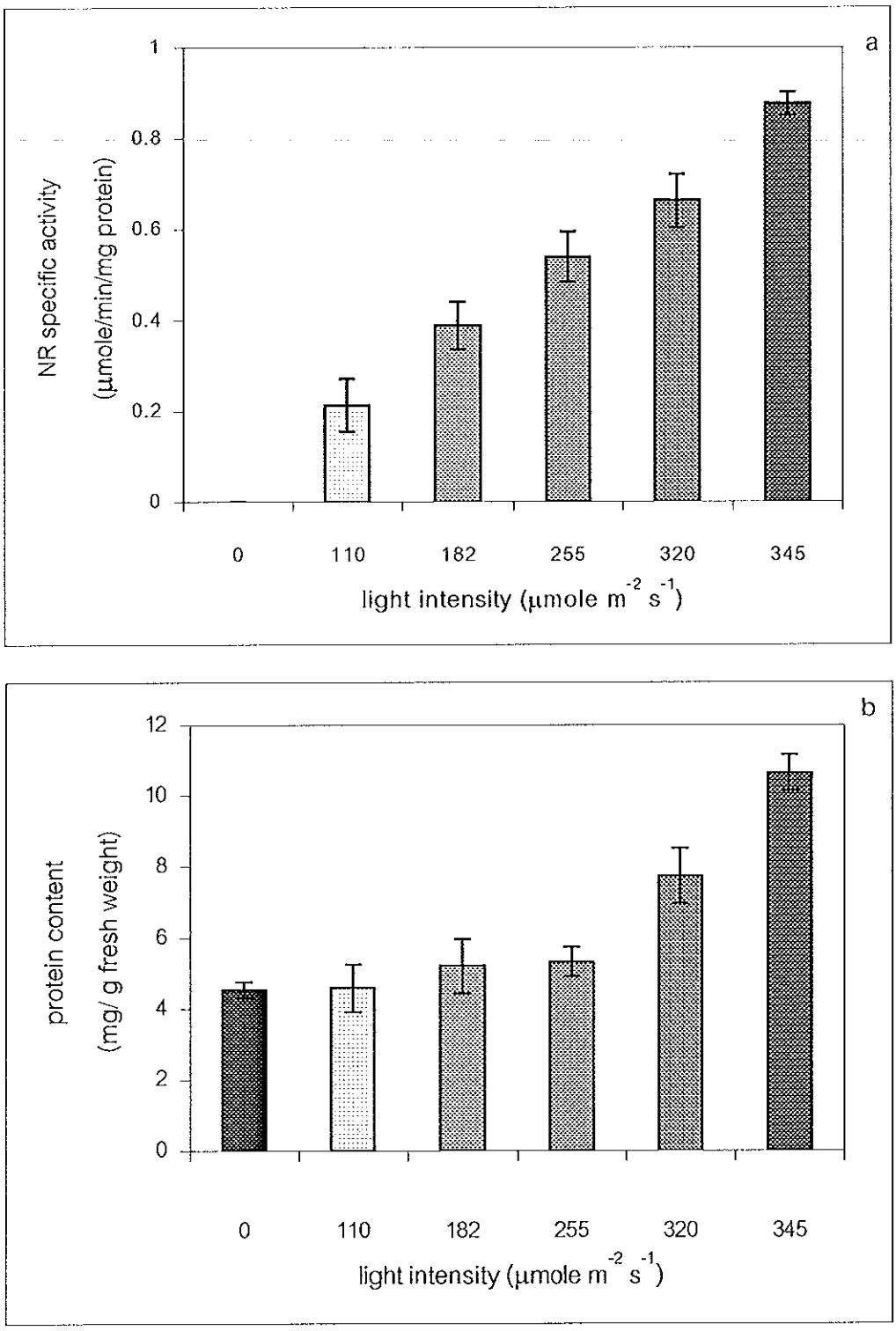

Figure 26 Effect of light intensity on NR specific activity (a) and protein content (b) of corn calluses. Values are means from three replications ±SD.

3.2.8 Comparison of NR activity in cultured calluses and corn seedlings

The corn callus suspension cultures, were shaken in N6 medium supplemented with 3 % fructose and 28 mM KNO_3 without 3.5 mM $(NH_4)_2SO_4$ in dark condition for 1 week, then subcultured in fresh N6 media and incubated in 16/8 h light/dark photoperiod. In three replications with calluses were transferred to N6 media, NADH-NR and NADPH-NR activities were investigated every second day of culture period. As shown in figure 27a, NADH-NR specific activities increased rapidly on the 2nd day, then increased to the highest level on the 4th day after the transfer; the highest activity was about 5.35-fold higher than that on day 0. NR activities decreased rapidly on day 6 by about 68.63 %. Similar to NADH-NR, the NADPH-NR specific activities increased rapidly on the 2nd day, then increased to relatively highest on the 4th day after transfer which was about 4.2-fold higher than that on day 0. The NADPH-NR activity also decreased rapidly on day 6. However, the specific activities of NADPH-NR were lower than those of NADH-NR.

In vitro assays of NR were carried out in leaf and root samples of 2 weeks old seedlings with the results given in figure 27b. The activities of NR in corn seedlings induced with KNO_3 were detected 2 days after induction. The maximum activities of leaf and root NADH-NR were reached by the 2nd day, then the leaf NR declined (Figure 27b). In contrast, no root NADH-NR activity was detected after 4 days of induction with nitrate. For NADPH-NR, the activities were detected only in the root and NADPH-NR activities were lower than NADH-NR activities. The seedling leaf and root NR activities were always lower in 1-2 weeks old seedlings (early old seedling), as described previously in figure 20. This coincided with the period of rapid depletion of endosperm nitrogen reserves during seedling growth. The NR activity was higher in the leaves than in roots. It was also found that the NR activities in corn calluses were higher than in both leaf and root of corn seedlings (Figure 27c). In many ways, the response of corn suspension cultures to environmental nitrate was similar to that previously observed in roots, leaves and other cell cultures (Redinbaugh *et al.*, 1996).

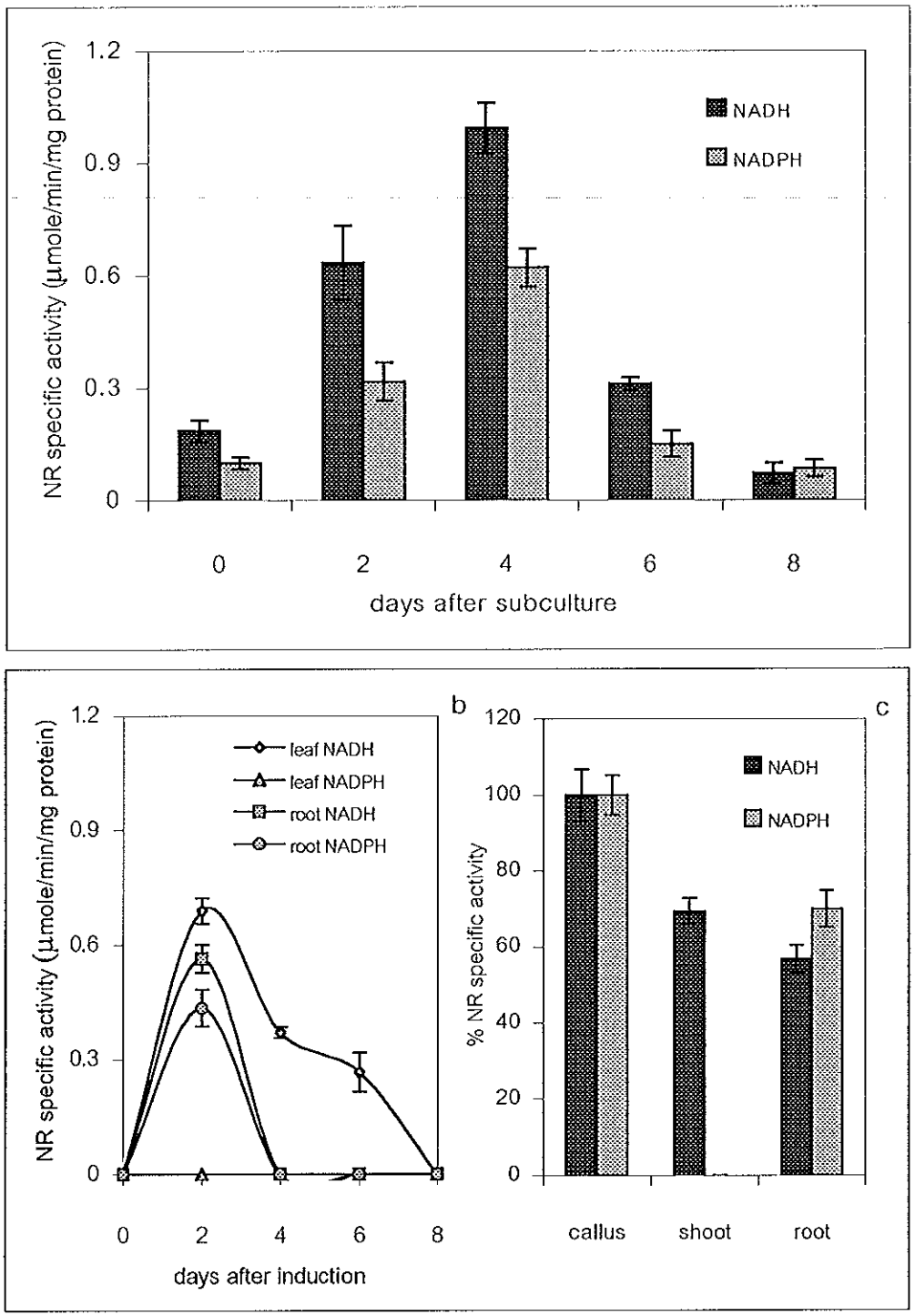

Figure 27 NR specific activities of corn callus cultured in N6 media supplemented with 3% fructose, without NH_4^+(a), specific activity of NADH-NR in shoot and NAD (P)H-NR in root of 2 weeks old corn seedlings (b) % NR specific activities of shoots and roots of corn seedlings after treatment with KNO_3 on the 2nd day compared with corn callus (c). Values are means from three replications ±SD

3.2.9 NAD(P)H-NR kinetics

Calluses were harvested, extracted and assayed for NADH and NAPDH NR activities as previously described. Similar kinetic experiments to determine the K_m of NR for NADH and NADPH, respectively in the presence of 10 mM KNO_3 as substrate were performed. NR assays were performed with crude extract and either NADH or NADPH at varying concentrations was used as an electron donor. The concentrations of NADH or NADPH used varied between 0.005 and 1 mM. Plots of 1/*v versus* 1/*s* were drawn. For the kinetics of NADH-NR, the result from Lineweaver-Burk double reciprocal plot (Figure 28) indicated that $-1/K_m$ and K_m values of NADH were –3.70 $(mM)^{-1}$ and 0.270 mM, respectively. In figure 29, for the kinetic of NADPH-NR, the result from Lineweaver-Burk double reciprocal plot indicated that $-1/K_m$ and K_m values of NADPH were –3.305$(mM)^{-1}$ and 0.302 mM, respectively.

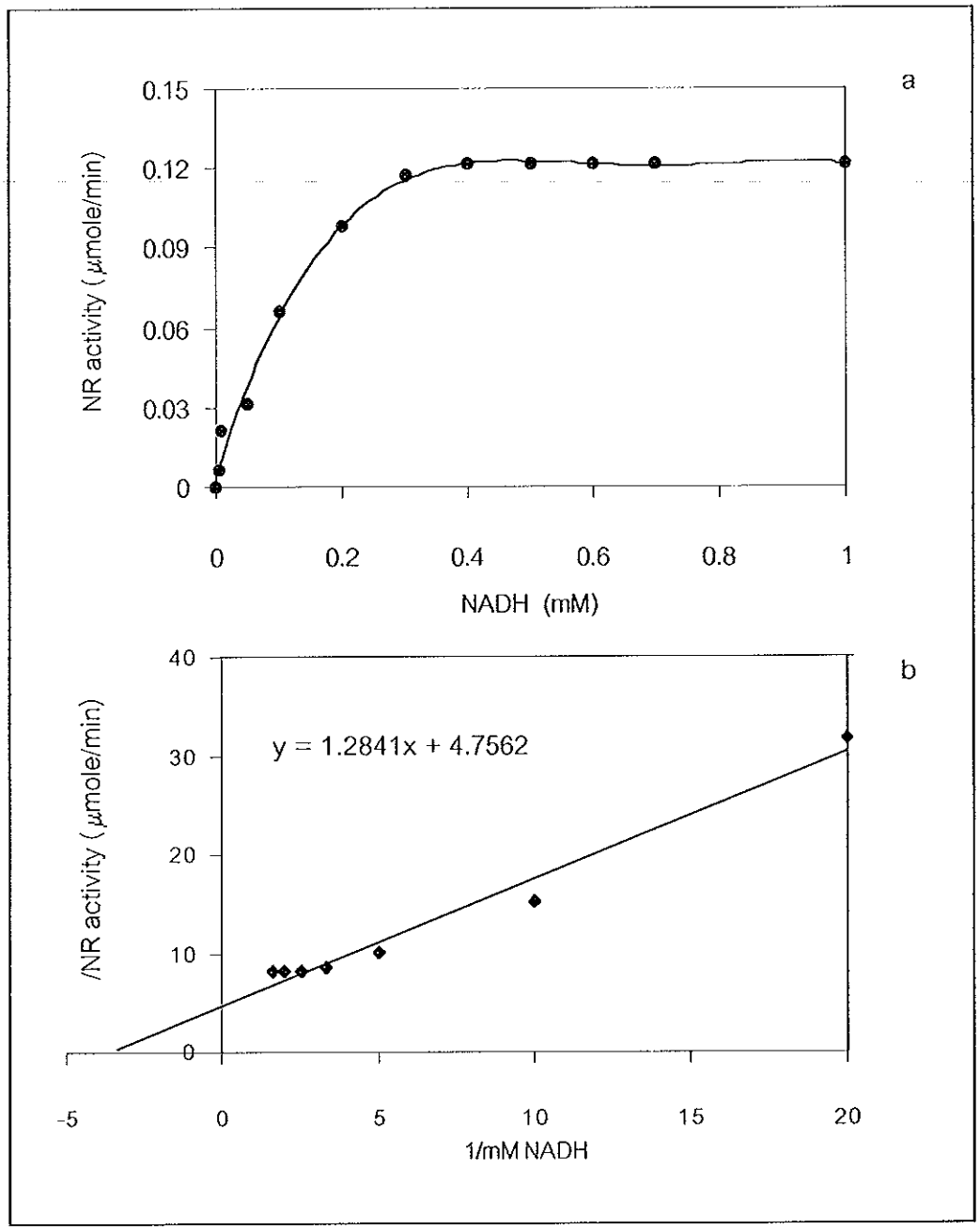

Figure 28 Determination of K_m for NR using NADH as an electron donor. NR assays were performed with crude extract (0.266 mg protein/assay) and varying concentrations of NADH. Saturation curve (a "Michaelis-Menten" curve) of nitrate reduction used NADH as an electron donor and 10 mM KNO_3 as the substrate (a). Lineweaver-Burk double reciprocal plot (b).

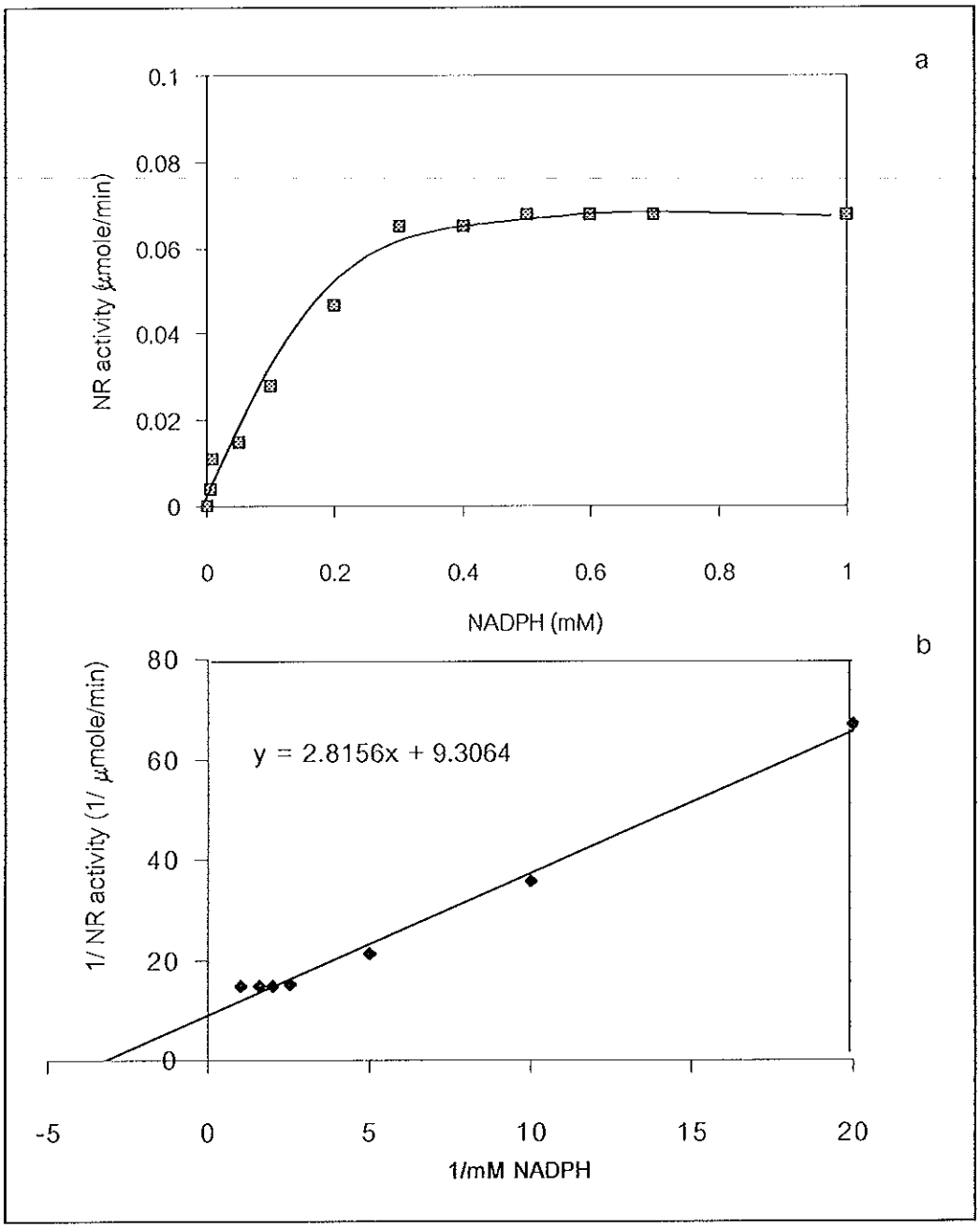

Figure 29 Determination of K_m for NR using NADPH as an electron donor. NR assays were performed with crude extract (0.266 mg protein/assay) and varying concentrations of NADPH. Saturation curve (a "Michaelis-Menten" curve) of nitrate reduction used NADPH as an electron donor and 10 mM KNO_3 as the substrate (a). Lineweaver-Burk double reciprocal plot (b).

Chapter 4

DISCUSSIONS

4.1 Induction of callus and suspension cultures from sweet corn

4.1.1 Callus induction

The establishment of callus from explants was necessary in many tissue culture experiments. Roots, stems, buds, and aseptically germinated seedlings are a few of the sources of tissue that can be used to induce callus formation. Generally, young and actively growing tissues (i.e. young leaves, roots, meristems) are the best sources of explants and should be preferentially used, unless they are not available (Chu, 1981). Callus formation from immature corn embryo is controlled by growth regulating substances (auxins) present in the medium (Green and Phillip, 1975). The specific concentration of plant regulators, needed to induce callus formation, varies from species to species and can even depend on the source of explant. In some instances, it is advised to use different growth substances (i.e. 2, 4-D) or a combination of them during tests, since some species may not respond to a specific growth regulator (Bronsema *et al.*, 1997). In addition, culture conditions (i.e. light, temperature, etc...) are important factors that determine the establishment of callus. Once established, callus cultures can be used to obtain protoplasts, or study somatic embryogenesis, organogenesis, and secondary metabolite production (Roberts *et al.*, 1996).

For initiation and maintenance of corn cell cultures, friable, embryogenic corn callus cultures were initiated from immature embryos produced from sweet corn inbred line Olympia 99. While corn callus cultures could be initiated from a number of different plant tissues, the cultures useful herein were preferably derived from immature corn embryos which were removed from the kernels of an ear when the embryos had reached a length of 1.5 to 2.0 mm. This length generally occurs about 12-14 days after pollination. The immature

kernels were surface sterilized in 70% ethanol for 90 second and 1:5 v/v commercial bleach (6% w/v sodium hypochlorite) for 20 min. Immature embryos were aseptically isolated and placed on nutrient initiation/maintenance media. Under aseptic conditions, the immature embryos were placed on conventional solid media with the embryo axis down (scutellum up). Initiation/maintenance media consisted of N6 basal media Chu *et al.* (1975) as described in Bronsema *et al.*, 2001 with 2% (w/v) sucrose, 2.0 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.25% Phytagel. The pH was adjusted to 5.8 prior to autoclaving. Unless otherwise stated, all tissue culture manipulations were carried out under sterile conditions. The immature embryos were incubated at 26 °C, in the dark. Cell proliferations from the scutellum of the immature embryos were evaluated for friable consistency and the presence of well-defined somatic embryos. Tissue with this morphology was transferred to fresh media 14 days after the initial plating of the immature embryos. The calluses were then subcultured on a routine basis every 14 days. The corn callus initiation media was solid because callus could not be readily initiated in liquid medium (Bronsema *et al.*, 2001). Supplements such as L-proline and casein hydrolysate have been found to improve the frequency of initiation of callus cultures, morphology, and growth (Emos *et al.*, 1993). The cultures were generally maintained in the dark. The level of synthetic hormone 2,4-D, necessary for maintenance and propagation, should be generally about 2.0 mg/l.

4.1.2 Influence of 2,4-D on callus induction

This experiment dealt with the influence of the growth regulator 2,4-D on the induction of callus in cultured immature embryo of *Zea mays* L. The optimal concentration of 2,4-D for callus induction from both immature and mature embryos of corn was 2 mg l^{-1}. In corn, two types of embryogenic callus can be induced in immature zygotic embryos. Type I callus, which is compact in appearance and hard to subculture, and type II callus, which is friable, and is well suited for subculture without loosing its capability to produce somatic embryos (Green and Phillips, 1975). Friable embryogenic callus can be used for the production of suspension cultures. Immature embryo of corn formed embryogenic callus

when they were excised 12-14 days after pollination and cultured with growth regulators like 2,4-D (Green and Phillips, 1975) or Dicamba (Dancan *et al.*, 1985). The uptake of 2,4-D and the biochemical fate after uptake were studied previously in corn callus for both the embryogenic inbred line A188 and the non-embryogenic inbred line A632 by Bronsema *et al.* in 1996. Under exhaustive conditions embryos took up 2,4-D rapidly during the first 3 days of culture but the embryogenic line accumulated less 2,4-D than the non-embryoginic line and metabolisation rates differed as well as culture responses such as germination, callus formation and somatic embryogenesis (Bronsema *et al.*, 2001). When the embryos were exposed to 2,4-D for 1-14 days, the effect of 2,4-D became pronounced with increasing incubation periods. It was shown that during culture 2,4-D accumulated in embryos (Bronsema *et al.*, 1996) and suggested that immature embryos need a certain amount of 2,4-D to form embryogenic callus. The presence of 2 mg l^{-1} 2,4-D in a culture media was a prerequisite for callus induction on immature embryos of corn (Green and Phillips, 1975).

Exogenous auxin is important for callus cells in order to confer embryogenic potential and to induce mitotic divisions, but auxin deprivation is necessary for further progress of embryogenic morphogenesis (Felle *et al.*, 1991). Samaj *et al.* (2003) assumed that the cytoskeleton plays a crucial role in these developmental switches driving somatic embryogenesis. In corn callus, addition of 2,4-D to the culture medium leads to embryogenic induction of callus cells (Samaj *et al.*, 1995) accompanied by secretion of a distinct subset of AGPs (arabinogalactan proteins) into the outer cell walls of embryogenic cells (Samaj *et al.* 1999a, b). During this process, nuclei are actively positioned at the centers of embryogenically competent cells. Both microtubules and actin filaments radiating from nuclear surfaces are involved in the centering of the nuclei and also in maintaining and sensing this central nuclear position via interactions with the cortical cytoskeleton (Grolig 1998; Collings and Allen, 2000). This proposes that both radiating perinuclear microtubules and actin filaments allow rapid signalling to the nucleus, which enables these cells to

respond to changing extracellular stimuli including different levels or absence of exogenous auxin (Samaj *et al.* 2003).

4.1.3 Callus formation

The embryos were isolated and placed on the culture media with the rounded scutellar side exposed and the flat plumule radicle axis side in contact with the medium. The embryos were maintained at 26-28°C in darkness for 18 days. Callus appeared from the scutellum after several days to a few weeks. After the callus has grown sufficiently, the cell proliferations from the scutellum may be evaluated for friable consistency and the presence of well-defined embryos. By "friable consistency" it was meant that the tissue was easily dispersed without causing injury to the cells (Frame *et al.*, 2000). Tissue with this morphology was then transferred to fresh media and subcultured on a routine basis about every two weeks. Somatic embryogenesis in corn proceeds through well-defined cellular structures and growth patterns (Armstrong and Green, 1985). In early embryogenic stages, both induction of cell division and suppression of cell growth are necessary. On the other hand, polarized cell growth is required for advanced embryogenic stages (Samaj *et al.* 1995).

4.1.4 Induction of callus in light and dark conditions

After 2 weeks of callus initiation, in the light, immature embryos developed callus normally up to a determined shoot and root length, after which all of them germinated and green spots were observed. However, in the dark, immature embryos formed calluses and rapidly grew but germination and green spots were not detected. The result showed that light inhibited callus induction due to the degradation of exogenous 2,4-D in medium by light and light induced shoot and root elongation in the role of auxin in cell (Padua *et al.* 1998). Nhut *et al.* (2000) showed that darkness was generally observed to stimulate more somatic embryogenesis than light conditions when 2,4-D is present in rice culture. Light inhibited somatic embryogenic formation in plants such as barley (Kott and Kasha, 1984).

However, other possibilities are not excluded, such as the interaction of light with auxin on shoot production, as can be found in rice (Padua *et al.* 1998).

4.1.5 Influence of embryo orientation on culture medium

An efficient mode of placement of the embryo to enhance callus induction frequency was identified in immature and mature embryos. For callus induction from immature embryo, the placement fashion of the embryo on the medial slant plays an important role. For horizontal placement, the plumule-radicle side of the immature embryo, when placed downward gave a greater frequency of induction and amount of callus induction than the scutellar side placed downward (Green and Phillips, 1975). However, in this experiment both horizontal (plumule-radicle side placed downward) and vertical placements (the embryo axis side directed towards the media) were not important for callus induction from immature embryos. When immature embryos were cultured with their axis side towards (horizontal) the medium and plumule-radicle side placed downward (horizontal), the immature embryos were exposed to 2,4-D in media and they showed maximal callus formation in both placements. However, immature embryos oriented horizontally grew more rapidly than that oriented vertically. In contrast, when mature embryo was horizontally placed facing the medium, the plumule started to germinate quickly, pushing the explant up and disconnecting it from the medial surface, thereby restricting the callus induction. However, when mature embryo was vertical placed facing, embryos formed both callus and germinated plumule. Horizontally, possible reasons include gravity influences, embryo germinated rapidly, but this response damaged by vertical implantation (Green and Phillips, 1975). Hence, placements of embryo on the medium in this experiment played a critical role in mature embryos but not immature embryos for callus induction from corn cultures.

4.1.6 The effect of sucrose contents on callus growth

In order to determine an adequate culture medium for obtaining the best growth of callus suspension culture, calluses were cultured in N6 medium (Chu *et al.*, 1975),

with the addition of 3 different concentrations of sucrose, 2%, 3% and 4%, respectively. The media were adjusted to pH 5.8. Sucrose gave the best cell growth in medium containing 3 percent sucrose. Concentrations of sucrose affected the survival of the youngest embryos more than the oldest ones. It seems likely that sucrose concentration increases osmotic value in the medium, which is necessary for growth induction in proembryos of several species, probably to allow an effective flow of metabolites (Raghavan and Srivastava, 1982). However, increase in sucrose concentration reduced callus growth possibly due to osmolarity. From the report of Mihaljivic *et al.*, 2002, the best callus induction in both kinds of primary explants of *Taxus baccata* was achieved using MS medium supplemented with 3 mg/L 2,4-D, 0.5 mg/L kinetin and 2% sucrose. Sucrose at 3 % reduced induction and growth of *T. baccata* callus, possibly due to osmotic shock or repression of biosynthetic pathways (Mihaljivic *et al.*, 2002).

4.2 Nitrate reductase

4.2.1 NR activity in corn and rice seedling

The inducibility of nitrate reductase of 7-day-old seedlings of rice and corn in relation to nitrogen source (NO_3^-or NH_4^+) was investigated. Nitrate was absolutely and specifically required for NR induction, and activated NR activity more effectively than NH_4^+. The inhibition of NR activity by NH_4^+ application to seedlings indicated that NO_3^--dependent enzyme induction was elicited by NO_3^- and not by a product of its assimilatory reduction, e.g. NH_4^+. In the presence of NO_3^-, light remarkably enhanced the appearance of NR activity (Huber *et al.*, 1994,1996). Moreover, in 1998 Cabello *et al.* suggested that chloroplasts are required for the inductive effect of light and NO_3^- and/or for the accumulation of newly formed enzymes in the organelle. Nitrate reductase gene expression is well known to be controlled by nitrate and, at least in green tissues, by carbohydrates which are somehow responsible for the light effect on NR induction (Somers *et al.*, 1983; *Deng et al.*, 1990; Bowsher *et al.*, 1991; Vincentz *et al.*, 1991). Previous study of Ouko *et al.* (2002) has suggested that rice is ammonophilic and, as such, not capable of assimilating

nitrate-N at the early seedling stage. In corn, the activity of NR during early seedling growth is low or not detected which coincides with the period of rapid hydrolysis of endosperm nitrogen reserves (Sivasankar and Oaks, 1995). Asparagine (Asn) and Glutamine (Gln), potential products of endosperm hydrolysis in corn, are predominant in the plant tissue and in root exudates during early seedling growth (Sivasankar *et al.*, 1997). Of the two amides, Gln is known to be the effector molecule responsible for nitrogen catabolite repression of the transcription of NR in the filamentous fungus *Neurospora crassa* (Marzluf and Fu, 1989). This amide, together with Asn, constitutes more than 50% of the reduced nitrogen in the root exudate of 7-day-old corn seedlings (Sivasankar and Oaks, 1995). Taken together, the above facts suggested that the observed constraint on NR activity during early seedling growth resulted from an inhibition by Asn and/or Gln derived either from the hydrolysis of protein reserves in the endosperm or from their synthesis within the seedling itself (Sivasankar and Oaks, 1995). Moreover, in corn seedlings, root tissues were more sensitive to the inhibitory effect of amides than shoot tissues. Therefore, the lower levels of inhibition of NR in the shoot may have been due to either a reduced sensitivity of the corn shoot enzyme to the inhibitors or differences in compartmentation in shoot and root tissues. In addition, in corn, the shoot NR is NADH dependent, whereas the roots posses an NADH-NR as well as an NAD(P)H-bispecific NR (Long *et al.*, 1992). Thus, it is possible that the NAD(P)H-bispecific NR appears to be the differential response to amides in shoots and roots (Li and Oaks, 1995). As described previously, it was concluded that the low capacity for assimilation of external nitrate by growing tissues such as seedlings (or meristems or metabolic sinks) could result, in part, from an inhibition of the induction of NR by nitrogenous compounds that are actively imported from senescing plant parts. A repression of NR in early seedling would prevent an unnecessary drain of reductant, energy, and carbon into the nitrate assimilation pathway, resources that would otherwise be utilized for new growth (Sivasankar and Oaks, 1995).

4.2.2 Comparison of NR activity *in vivo* and *in vitro* assays

NR activity is frequently estimated from plant material using one of three assays (Roberts *et al.*, 1996), namely the *in vivo* (Watt *et al.*, 1986), the *in vitro* (Hageman and Reed, 1980) and the *in situ* (Larsson *et al.*, 1985) assays. Each has been subjected to valid criticisms and each requires optimization according to the type of material under study. The *in vitro* and *in vivo* assays were selected for studies in this experiment, because they were easier to perform than the *in situ* assay and large numbers of samples could be handled simultaneously. In this experiment, the two assays generally gave similar results, indicating that physiological levels of reductant and substrate were probably non-limiting during most of corn callus culture period. On the basis of these results, a single assay system was deemed sufficient to determine regulatory aspects for callus NR induction. The *in vitro* assay was the most suitable method for reasons of simplicity and efficiency and selected for all subsequent studies. The *in vitro* assay was assumed to measure NR activity under optimal conditions, and hence provided a measure of the amount of potentially active enzyme (Padidam *et al.*, 1991; Roberts *et al.*, 1996). For this reason, many studies have obtained *in vitro* NR activity values several times higher than *in vivo* NR activity (Duke and Duke, 1984). In contrast, other workers have found that *in vivo* activity was consistently higher than *in vitro* activity, attributing this to inactivatiors and organic compound inhibition during extraction in the latter (Jones *et al.*, 1976).

4.2.3 Influence of extraction buffers on stability of NR

It is well known that NR protein is extremely unstable during *in vitro* extraction from different tissues, a potential target for specific proteolysis (Yaneva *et al.*, 2000) Proteases are ubiquitous enzymes in every cell of all organisms. They are released during disruption of cells, and quickly degrade any proteins. When there is a need to save, or recover or detect a given protein, protease inhibitors are also useful to protect proteins during further analysis (for enzyme assay) and storage. Different types of proteases exist.

Serine proteases are present in almost all cells, dominating in plants with cysteine proteases or in bacteria with metalloproteases (www. Interchim.com). Occasionally, aspartic proteases can interfere too in animal tissue isolations notably when a low pH is required. In this experiment, for investigating the stability of NR in extraction buffers, PMSF and chymostatin as most important general serine and cysteine proteases inhibitors were included in 50 mM MOPS buffer pH 7.5 containing 1 mM EDTA and 5 mM DTT. In buffer B, the presence of 1 mM PMSF was shown to inhibit the inactivating enzyme and, when employed in the extraction buffer, prevented the inactivation of NR in corn calluses (Figure 25). Although, it did allow some increase in the stability of NR from callus, it only partially prevented its inactivation in vitro. Wallace and Shannon (1981) observed that, in corn roots, higher levels of PMSF (up to 20 mM), which were not completely soluble, only slightly improved the recovery and stability of NR in preparations of this older root tissue. The failure of PMSF to protect NR in this case suggested that other factors, in addition to the inactivating enzyme, were responsible for the in vitro loss of NR activity in extract of this tissue. In buffer C, the presence of 10 µM chymostatin was shown to inhibit the inactivating enzyme as well as PMSF. However, the presence of both PMSF and chymostatin in extractiion buffer D, could inhibit the inactivation of NR better than the presence of either PMSF or chymostatin alone. It has been demonstrated that the inclusion of 10 µM chymostatin in extraction buffer markedly increased the yield and stability of NR from spinach leaf (Huber *et al.*, 1992c). The result showed that the effect of chymostatin on the *in vitro* NR activity in corn calluses was probably due to the higher susceptibility to proteolysis of the phosphorylated NR, which is known to predominate in dark-exposed plants (Yaneva *et al.*, 2000). This suggestion agrees with the results of Kaiser and Huber (1997) which showed that the phosphorylated form of NR was a better substrate for proteolytic degradation than the dephosphorylated NR form. As shown by the study of Appenroth *et al.*, (2000) showed that EDTA led to a rapid activation of the enzyme but Mg^{2+}inhibited the activation of NR. In the presence of Mg^{2+}in extraction buffer E, the activity of NR was lower than that in other extraction buffers. Spinach

NR was inactivated in the dark, and this inactivation was best revealed when NR activity was assayed in a magnesium-containing buffer (Kaiser *et al.*, 2000). However, the presence of EDTA in this buffer partially inhibited the inactivation of NR by Mg^{2+}. The assay in the presence of both EDTA and Mg^{2+} was estimated as the *in situ* NR activity (Kaiser and Huber, 1997). The activation was reversible in vitro by adding EDTA to the reaction buffer (Kaiser and Brendle-Behnish, 1991). The activity of the existing NR protein is modulated by phosphorylation on ser543. In the presence of divalent cations (Mg^{2+}), phospho-NR forms a catalytically inactive complex by binding to a 14-3-3 protein. If cations are chelated by EDTA, NR becomes fully active (Kaiser *et al.*, 1999). Stimulation of NR by nonmetabolite EDTA suggested a chelatation mechanism. Chelators of metals could increase nitrate reductase activity by preventing the metals from inhibiting the enzyme. Therefore, the NR activation state, or percentage of active NR, can be estimated by calculating the ratio between NR activity assayed without EDTA (which corresponds to the activity of the dephosphorylated NR in the extract) and NR activity assayed with EDTA (reactivated NR) (Pigaglio *et al.*, 1999).

4.2.4 Effect of environmental factors to NR activity in suspension cultures

4.2.4.1 Determination of growth, NR activity, protein and nitrite contents of callus during one week culture period

Preliminary studies in this study have indicated that the presence of nitrate in the culture medium is essential for induction NR during culture periods. Higher plant species differ in ability to grow and differentiate on different sources of nitrogen, and a combination of nitrate- and ammonium-nitrogen has been found to be required for in vitro growth and differentiation in various higher plant species (Grimes and Hodges, 1990). The concentration of inorganic nitrogen used for in vitro tissue culture affect growth, with optimal levels being species dependent (Roberts *et al.*, 1996). In this experiment, the weight of calluses slowly increased in the first week after subculture, but on the 4^{th} day, the start of rapid callus growth was observed. Soluble protein levels slightly increased on the 1^{st} day then were constant at high level throughout the culture period. NR activities reached

maximal levels on the 4th day then rapidly decreased. However, the protein contents were usually high and did not match NR activity, which declined particularly after the 4th day. The protein content was much less responsive to light than NR activity, suggesting the possibility that post-translational modification of NR is an important regulatory factor, caused by enzyme phosphorylation (Roberts *et al.*, 1996). There was a direct relationship between callus growth and NR activation. In addition to its incorporation into organic nitrogen via nitrate assimilation, nitrate may have an osmoregulation role during plant cell growth (Talouizite and Champigny, 1988). However, the increase of nitrite accumulation in callus inhibited the callus growth and NR activation. Ogawa *et al.*, (1999) showed that callus browning or necrosis could be caused by buildup of nitrite ions in cell. Hardy and Thorpe (1990) have shown that, under certain circumstances, nitrite reductase (NiR) may limit nitrogen assimilation during *in vitro* culture. Simultaneous regulation of NR and NiR inductiion by nitrate and light has been reported by Neininger *et al.*, (1992).

4.2.4.2 Diurnal fluctuations of *in vivo* NR activity

Diurnal fluctuations of NR activity have been reported in a number of plant species (Lillo, 1983; Huber *et al.*, 1992,1994 and Yaneva *et al.*, 2000). The rapid modulation of leaf NR activity during light/dark transitions has been established for many herbaceous plants, including spinach (Kaiser and Spill, 1991), barley (Lillo *et al.*, 1996), corn (Li and Oaks, 1995), *N. plumbaginifolia* (Nussaume *et al.*, 1995) and *Arabidopsis* (Su *et al.*, 1996). Analysis of NR activity in cultured callus over the 16 h light: 8 h dark photoperiod showed light/dark-dependent variations. The result indicated that light stimulated the NR activity. The results presented by Yaneva *et al.* (2000), in winter wheat that the light modulation of leaf NR activity is also a rapid process, detected soon after the onset of illumination and controlled in part by the phosphorylation/dephosphorylation mechanism. The synthesis/degradation of the enzyme can play a major role in regulation of diurnal variations of NR activity and supports the assumption that increase in NR mRNA may be explained by increase in transcriptional activity (Kaiser and Huber, 2001; Huber *et al.*, 2002). Decrease of

enzyme activity at the end of the photoperiod under long day conditions is satisfactorily explained by degradation of the protein. Similarly, the decline in the enzyme activity during the latter part of the day was due to the decreased availability of nitrate. Interestingly, the activation state of root NR showed slight fluctuations without light/ dark dependent changes (Haba *et al.*, 2001). Light stimulates synthesis as well as activation of higher plant NR protein, and the protein is rapidly deactivated in the dark. The rapid reversible activation and inactivation by light/dark transitions is strongly correlated with protein phosphorylation and dephosphorylation (Huber *et al.*, 1992a, 1994). Likewise, Gojon *et al.* (1991) reported that the xylem flux of nitrate in light is the main determinant of the actual rate of nitrate reduction in soybean [*Glycine max* (L.) Merr.] leaves. These reports suggest that the diurnal fluctuation in NR activity may be controlled by nitrate flux in the plant. If the light/dark transition effect on NR activity *in vivo*, is through the regulation of substrate availability and/or the generation and supply of reductant (Aslam *et al.*, 2001b). However, light affected NR expressions of nitrate transporter genes (*Nrt*), both *Nrt2* and *Nrt1* are diurnally regulated in photosynthetically active *A. thaliana* plants. Both increase during the light period and decrease in the first hours of the dark period (Lejay *et al.*, 1999). Sucrose supply prevents the inhibition of *Nrt2* and *Nrt1* expressions in the dark (Lejay *et al.*, 1999). In contrast, changes in the *Nrt1* mRNA level are not always associated with similar changes in the activities of high- or low-affinity nitrate transport systems (Vidmar *et al.*, 2000 and Glass *et al.*, 2001).

4.2.5 Effect of N supply on NR

In this experiment, the change patterns of nitrate reductase (NR) activity in response to N source with rising concentration of nitrate were determined. Corn calluses, which culture in N-free media for 4 day after that subcultured in N6 media supplied with only nitrate or both nitrate and ammonium in N6 medium as their sole N source. After 4 days, calluses were harvested, and NR activity measured. The studies revealed that the induction of NR calluses by nitrate was higher than nitrate plus ammonium and that ammonium

partially inhibited nitrate reductase. NR activities were reduced in callus cultured in N6 media supplement with both N sources, but did not change in high concentrations of both mediums. NR activity was unaffected at low concentrations of both N sources. Scaling this response to corn calluses, NR activity was reduced in N6 medium containing ammonium ions. However, because of high concentration of nitrate with constant ammonium concentration, total NR activity was unaffected.

Root nitrate uptake and expression of two root *Nrt2;1* and *Nrt1* genes were investigated in response to changes in the N- or C-status of hydroponically grown *Arabidopsis thaliana* plants (Lejay *et al.*, 1999). Expression of *Nrt2;1* is up-regulated by nitrate starvation in wild-type plants and by N-limitation in a nitrate reductase (NR) deficient mutant transferred to nitrate as sole N source. These show that expression of *Nrt2;1* is under feedback repression by N-metabolites resulting from nitrate reduction (Lejay *et al.*, 1999). Shaner and Boyer (1976) reported that a decrease in nitrate flux to corn leaves resulted in a rapid loss of NR activity, although leaf nitrate content was unchanged. It concluded that NR activity is regulated by nitrate flux rather than nitrate content of the tissue (Aslam *et al.*, 2001b).

Moreover, expression of the nitrate assimilation activity is negatively regulated by ammonium. Genes encoding the nitrate transport system (*NRT1,* and *NRT2*) and NR (*NIA)* are inhibited by the addition of ammonium to the Arabidopsis cultures (Crawford *et al.*, 1998). Ammonium inhibits transcription through its fixation into Gln, but Gln is not the direct regulator of transcription (Forde, 2000). Nitrate assimilation is subjected also to post-translational regulation, being inhibited upon addition of ammonium to the cultures (Crawford *et al.*, 1998). The major rate-limiting step of nitrate assimilation is nitrate transport into the cell, which has been shown to be inhibited by ammonium. Because inhibition of glutamine synthetase by L-methionine-DL-sulfoximine abolishes the negative effect of ammonium on nitrate transport, fixation of ammonium to Gln is clearly required for the regulation (Crawford

et al., 1998). However, the metabolic signal leading to the inhibition of nitrate transport and the molecular mechanism of the regulation remain to be elucidated.

4.2.6 Influence of sugars on NR

NR activity in corn calluses was higher after feeding fructose than glucose and sucrose in N6 media supplied with 28 mM KNO_3. However, in sugar-free medium supplemented with 28 mM KNO_3, no activity of NR was detected. The results presented in this experiment indicated that sugars, especially fructose, enhanced nitrate reduction in corn callus by stimulating the synthesis and the activation of NR, and that acted as positive regulatory metabolites. The role of sugars in the induction of the NR gene was also inferred from the observation that exogenously supplied sugars led to an increase in NR transcripts in the dark (Cheng *et al.*, 1992; Vincentz *et al.*, 1993; Sivasankar *et al.*, 1997). When sugars were given via roots to carbohydrate and nitrate depleted intact cucumber plants for 7h and NR-gene expression was examined, fructose markedly stimulated the production of NR mRNA both in leaves and root (Larios *et al.*, 2001). In *Nicotiana plumbaginifolia*, sucrose had more effect than glucose or fructose on NR-gene expression, when sugars were supplied to detached leaves in the dark (Vincentz *et al.*, 1993). Since sucrose (Cheng *et al.*, 1992) and glucose also induced nitrate reductase transcription, the effect of carbohydrates on NR activity might be triggered by the accumulation of products of nitrate assimilation.

The existence of sugar-regulated gene expression in plant has become apparent in the past few years. Because of sugar metabolism in the plant, it is difficult to ascertain which sugar is directly involved in the control of NR-gene transcription and whether hexokinase acts or not in the kexose-sensing system (Koch 1996; Jang and Sheen, 1997). Nitrate assimilation by plant, which leads to the synthesis of amino acids, requires carbon skeletons derived from carbohydrates synthesized though photosynthesis (Larios *et al.*, 2001). Carbohydrate depletion up-regulates the expression of gene for photosynthesis and reserve mobilisation, whereas high sugar levels enhance the expression of genes for carbon storage and utilisation (Koch, 1996). Then, enhanced CO_2 fixation, and concomitant

increased sugar avalability, would stimulate nitrate utilization by enhancing NR expression and activity, thus maintaining an adequate C/N ratio in the plant. It has been reported (Seith *et al.*, 1991; Cabello *et al.*, 1998) that signal or factor originating from the plastids (chloroplasts) is required for proper light-induction of NR. This has been proposed for those nuclear genes encoding chloroplastic proteins, including nitrite reductase (Neininger *et al.* 1992), or choroplast cytosolic enzymes whose functions are related to chloroplast metabolism (Thompson and White, 1991), as is the case of NR. Moreover, sugars are implicated in the post-translational regulation of NR. Darkening leads to post-translational regulation of ser_{543} and magnesium-dependent binding of an inhibitory 14-3-3 protein (Bachmann, *et al.*, 1996; Moorhead *et al.*, 1996). Dark-inactivation is reversed if sugars are supplied to leaves in the dark (Kaiser and Brendle-Behnisch, 1991; Kaiser and Huber 1994) and light-activation does not occur if carbon fixation is prevented by water stress or low carbon dioxide (Kaiser and Forster 1989; Kaiser and Brendle-Behnisch 1991; Lejay *et al.*, 1999). It has been shown that in the leaf NR increases in response to light whereas the root enzymes responds to additions of sucrose and are only marginally influenced by light. So it looks like this could be two basic responses to light, one via a plastidic factor which might be under the control of phytochrome and the second where the light may be replaced by sugars (Larios *et al.*, 2001).

4.2.7 Effect of light intensity on NR

The activity of NR in corn calluses highest was in the maximum light intensity applied in this study. The values of NR activities were determined in corn calluses illuminated with 354 $\mu mol\ m^{-2}\ s^{-1}$. In other plant species, the range of light modulations in the NR activation state may depend on the light intensity as a growth factor. For instance, in *Brassica campestris* the activation state of leaf NR increased from 70 % under illumination at 120 $\mu mol\ m^{-2}\ s^{-1}$ up to 95% at 1000 $\mu mol\ m^{-2}\ s^{-1}$ (Kojima *et al.* 1995). On the other hand, several examples showed that the values of NR activation state might greatly differ among species irrespective of the similar light conditions for plant growth. In *Ricinus communis*, for

example, an unusually low activation state of leaf NR (2 %) has been found in comparison with the NR activation state in spinach (88 %) grown under the same light intensity (Kandlbinder and Kaiser 2000). Thus the modulation range level of NR activation state appears to depend not only on light conditions for plant growth, but is also a species-specific feature (Yaneva *et al.*, 2000).

It is now well established that NR is rapidly phosphorylated in response to various stimuli, for instance darkness, and after phosphorylation members of the 14-3-3 family of proteins bind to NR which causes inactivation of NR (Huber *et al.* 1996; Moorhead *et al.* 1996; Kaiser *et al.* 1999 and Campbell 1999). In the light NR is rapidly activated by dephosphorylation (Agüera *et al.*, 1999). Photosynthesis apparently triggers a signal transduction chain leading to activation of NR (Chandok and Sopory 1996). Sugars transported from the chloroplasts into the cytosol (where NR is located) are likely to be involved (Provan and Lillo 1999), whereas for instance phytochrome does not appear to play any role in this signaling (Appenroth *et al.* 2000). Phytochromes are the best characterized photoreceptors known to mediate light-dependent responses in higher plants (Furuya 1993), and regulate the transcription of specific genes (Terzaghi and Cashmore 1995). The report by Sheen (1993) on the role of protein phosphatases in light-inducible gene expression in corn is one piece of direct evidence for the importance of phosphorylation status of proteins in Pfr signal transduction. Nitrate reductase is a substrate-inducible enzyme and light influences the accumulation of NR mRNA, appearance of NR protein and NR activity (Sopory and Sharma 1990 and Raghuram and Sopory 1995). The NR is regulated by phosphorylation/ dephosphorylation event with the dephosphorylated form of NR being the active enzyme (Kaiser *et al.* 1993 and Huber *et al.* 1992a). In the same system, a role for protein phosphatases in chlorophyll accumulation and photosynthetic gene activation has been demonstrated (Sheen 1993), suggesting that light could transduce signals by activating either kinases or phosphatases or both. As described previously NR activity in corn is also regulated by light via phytochrome (Rao *et al.* 1980; Sharma and Sopory 1984).

Chandok and Sopory (1996) showed that the level of NR transcript cannot in all cases be correlated with NR activity.

4.2.8 Comparison of NR in corn callus and seedling

In many ways, the response of corn suspension cultures to environmental NO_3^- was similar to that previously observed in root, leave and other cell culture (Redinbaugh *et al.*, 1996). Corn roots and scutella contain a bispecific NAD(P)H: NR (Gowri *et al.*, 1992 and Li and Oaks, 1995). The enzyme isolated from root, NAD(P)H bispecific, uses NADPH and NADH as the electron donor with about equal efficiency whereas, in leaves, NADH: NR is specific for NADH (Redinbaught and Campbell, 1980). As in corn roots, NADPH: NR activity in corn cells may be attributed to expression of NAD(P)H: NR. Because NR activity in cells was greater with NADH than with NADPH, it appears that corn cells were similar to roots and scutellum in expressing both NR forms (Li and Oaks, 1995). The somewhat different patterns seen for NADH: and NADPH: NR may indicate some distinct regulation of activation of NADH and NAD(P)H NR. In this experiment, the result indicated that the response of NR activity in corn callus was similar in root more than in shoot of corn seedlings. However, the NAD(P)H-NR activity of corn calluses was estimated about equal of NADH-NR activity of shoot plus NAD(P)H-bispecific NR activity of root of corn seedlings. The response of corn calluses to nitrate was similar to that of corn seedling tissues, especially roots (Redinbaugh *et al.*, 1996). These results indicated that the cellular components required for the recognition of, and response to, external nitrate are either the same or similar in corn roots and cultured corn cell. However, the cell culture has the advantage of being more uniform and more easily manipulatated than higher plant tissues. Because small aliquots of a cell culture can be reproducibly and uniformly treated, the effects of compounds that influenced in plant systems (e.g. inhibitors and activators of protein kinases and/or phosphatases) on NR expression in corn cells can be tested. In leaves of corn seedlings, NADPH-NR activity was not detected. Moreover, Barlaan *et al.*, 1998 showed that NADPH-NR activity was not detected in early seedling stage of rice seedling, but the activity

gradually increased from 15 to 45 days after planting. Result indicated differences in the activity of NADH-NR and NADPH-NR. Some possible reasons could be the limited expression of genes for low or deficient NADPH-NR at very early seedling stages, or activation of NADPH-NR genes during later growth stages, as in cases of seedling susceptibility and adult resistance in crop plants (Barlaan *et al.*, 1998).

4.2.9 NAD(P)H-NR kinetics

NR is unusual among oxidoreductase since it exists in NADH- and NADPH-specific forms as well as bispecific forms that accept electrons form either NADH or NADPH. Since bispecfic NR forms were the first identified in soybeans and as secondary NR in monocots like corn, rice and barley. Corn seedlings appear to express three forms of NR: NADH-specific, NAD(P)H-bispecific with some similarity to barley, and a unique NAD(P)H-bispecific with some similarity to green algal NR (Campbell, 1999). More detialed studies of various forms of NR including the unusual bispecific forms should provide a clearer understanding of pyridine nucleotide specificty (Campbell and Kinghorn, 1990; Campbell, 1996; Crawford, 1995). Only small changes in the fine structure of the pyridine nucleotide domain of NR are required to change from an NADH-specific form to an NADPH-specific form or perhaps even fewer to make a bispecific NR from a monospecific one, which may explain the existence of so many bispecific NR forms in nature (Campbell, 1999). K_m values for NADH or NADPH (1 to 5 µM) and Nitrate (20 to 40 µM) are in the low micromolar range (Campbell, 2001). NR activity is usually determined at substrate saturation, i.e. with NADH between 0.2 and 0.5 mM, and nitrate concentrations between 1 mM and 10 mM. In spinach leaf, the apparent K_m-values of NR are 7 µM for NADH and 40 µM for nitrate (Kaiser and Spill, 1991). In all experiments, for NR activity assay in both corn seedlings and calluses, the NR assay buffer contained 0.5 mM NADH or 0.5 mM NADPH as described in Redinbaugh *et al.*, 1996 for determine NADH and NADPH: NR activity, respectively. These concentrations of NADH and NADPH were sufficiently saturated for determination of NAD(P)H: NR activities.

Chapter 5

CONCLUSIONS

5.1 Callus induction

5.1.1 Influence of 2,4-D on callus induction

The induction of callus in immature embryos of corn using 2,4-D concentrations ranging from 1 to 10 mg l^{-1} and optimal concentration for the induction was 2 mg l^{-1} and optimal size of embryo for induction of callus was 2.0 mm.

5.1.2 Callus formation stage

The development and growth of corn callus was very rapid in during the first two weeks. Callus originated from the scutellum in day 4. The friable embryogenic callus was formed after 7 days of culture on medium with 2 mg l^{-1} 2,4-D.

5.1.3 Callus induction in light and dark condition

Dark condition optimized for callus induction, but light induced the formation of shoot and root and inhibited callus formation.

5.1.4 Influence of embryo orientation on callus induction

For immature corn embryos, the orientation of embryos both horizontal and vertical placement on N6 medium culture were a small effected for induction of callus. However, in the case of mature embryos, placement of embryos on the medium plays a critical role for induction of calluses and the vertical placement showed better callus formation than horizontal placement.

5.1.5 Cell suspension growth

The callus growth in N6 media supplemented with 3% sucrose was higher than that in N6 media supplemented with 2% sucrose and the lowest was 4% sucrose. This concentration was used for callus suspension culture.

5.2 Nitrate reductase determination

5.2.1 NR activity in corn and rice seedling

Rice seedlings showed markedly higher nitrate reduction than corn seedlings in the presence of nitrate at the same concentration. NR activity increased with increasing nitrate concentrations in corn seedlings. However, the NR activity of both species it was greatly reduced in the presence of NH_4^+ ions.

5.2.2 Comparison of NR *in vivo* and *in vitro* assays

NR activity measured by the *in vitro* and in vivo assays showed similar trends. The *in vitro* assay was the most suitable method for reasons of simplicity and efficiency and was selected for all subsequent studies.

5.2.3 Influence of extraction buffer on stability of NR activity

Using NADH as an electron donor and KNO_3 as the substrate, the NR activities, assayed in the presence of both chymostatin and PMSF without Mg^{2+} in extraction buffer D, were very stable and highest among other buffers. In contrast, the sensitivity of corn NR activity to Mg^{2+} inhibition was shown clearly by calluses extracted with buffer E, in the presence of both chymostatin and PMSF with Mg^{2+}, the NR activities of which remained lower than other extraction buffers but decreased slightly throughout the incubation on ice.

5.2.4 Effect of environmental factors on NR in suspension culture

5.2.4.1 Plant growth, NR activity, protein and nitrite content in corn callus during culture period

The rapid increase of NR activity was observed on the 4^{th} day, causing the growth of callus. There was a direct relationship between callus growth and NR activation. The protein content was much less responsive to light than NR activity, suggesting the possibility that post-translational modification of NR is an important regulatory factor during culture period. However, the increase of nitrite accumulation in callus inhibited the callus growth and NR activation.

5.2.4.2 Diurnal fluctuations of *in vivo* NR activity

Nitrate and light induced NR. Analysis of NR activity in cultured callus over the 16 h light: 8 h dark photoperiod showed light/dark-dependent variations.

However, the absence of nitrate, no NR activity was detected. In dark condition, sucrose supply in the presence of nitrate partially prevented the inhibition of NR activation.

5.2.5 Effect of N supply on NR

The optimal nitrate concentration for induction high NR activation was about 30 mM of KNO_3 in N6 media in the absence of 3.5 mM $(NH_4)_2SO_4$. NR activity was inhibited by ammonium ions in N6 media.

5.2.6 Influence of sugars on NR

NR activity in corn calluses was higher after feeding fructose than glucose and sucrose in N6 media supplied with 28 mM KNO_3 and indicated that sugars, especially fructose, enhance nitrate reduction in corn callus by stimulating the synthesis and the activation of NR, and that acted as positive regulatory metabolites. However, in sugars free medium supplemented with 28 mM KNO_3, no activity of NR was detected.

5.2.7 Influence of light intensity on NR

The activity of NR in corn calluses highest increased in the maximum light intensity (354 µmol m^{-2} s^{-1}) applied in this study. When increase light intensity, the NR activity and protein level increased.

5.2.8 Comparison of NR in corn callus and seedling

The NR activity in corn callus was higher than that in corn seedlings. However, the NAD(P)H-NR activity of corn calluses was estimated as NADH-NR activity of shoot plus NAD(P)H-bispecific NR activity of root of corn seedlings. The response of corn calluses to NR induction was similar to that of corn seedling tissues, especially roots.

5.2.9 NAD(P)H-NR kinetics

In corn calluses crude extract, the apparent K_m-values of NR are 0.27 mM for NADH and 0.302 mM for NADPH. This showed that in corn callus, NR was NAD(P)H-NR bispecific. In all experiments, for NR activity assay in both corn seedlings and calluses, the NR assay buffer contained 0.5 mM NADH or 0.5 mM NADPH for determine NADH and NADPH: NR activity, respectively. These concentration of NADH and NADPH saturated for determine NAD(P)H: NR activities.

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Appendix

1.Tissue culture

1.1 Materials

Beakers, scalpels, forceps, needles and Petri dishes were sterilized in the autoclave at 121 °C for 20 min.

1.2 Reagents

70% ethanol for sterilizing tissue samples

70% ethanol was prepared by adding distilled or deionized water to 370 ml of 95% ethanol to make 500 ml in a graduated cylinder.

95% ethanol for use to flame the material

1. Normally, ethanol taken from the reagent bottle is 95% pure. This solution was used to flame the forceps and scalpels during the process of removing the embryos from the cotyledons.

2. The ethanol was placed in a bottle with a lid that can be closed when not in use.

1:5 Haiter solution for sterilizing tissue samples

1:5 Haiter solution was prepared from commercial bleach (6% sodium hypochlorite solution). 10 ml of the commercial bleach was diluted with 40 ml of distilled or deionized water.

Sterile distilled/deionized water

Distilled and deionized water was autoclaved at 121°C for 20 minutes in Duran bottles.

1.3 Growth Media

N6 medium (Chu *et al.*, 1975) containing:

Inorganic nutrient

1. Macronutrient	mg l^{-1}
KNO_3	2.83X 10^3
$(NH_4)_2SO_4$	463
KH_2PO_4	400
$MgSO_4.7H_2O$	185
$CaCl_2.2H_2O$	166
2. Micronutrient	mg l^{-1}
$MnSO_4.H_2O$	3.3
$ZnSO_4.7H_2O$	1.5
H_3BO_3	1.6
KI	0.8
$FeSO_4.7H_2O$	28
Na_2EDTA	37
*$Na_2MoO_4.2H_2O$	0.25
*$CoCl_2.6H_2O$	0.025

Organic nutrient

1. Amino acid	mg l^{-1}
L-proline	2.88
Glycine	2.0
Casamino acids	100
Thiamine HCl	1.0
Pyridoxine HCl	0.5
Nicotinic acid	0.5
2. Carbon source	g l^{-1}
Sucrose	20
3. Agar	g l^{-1}
Phytagel	2.5

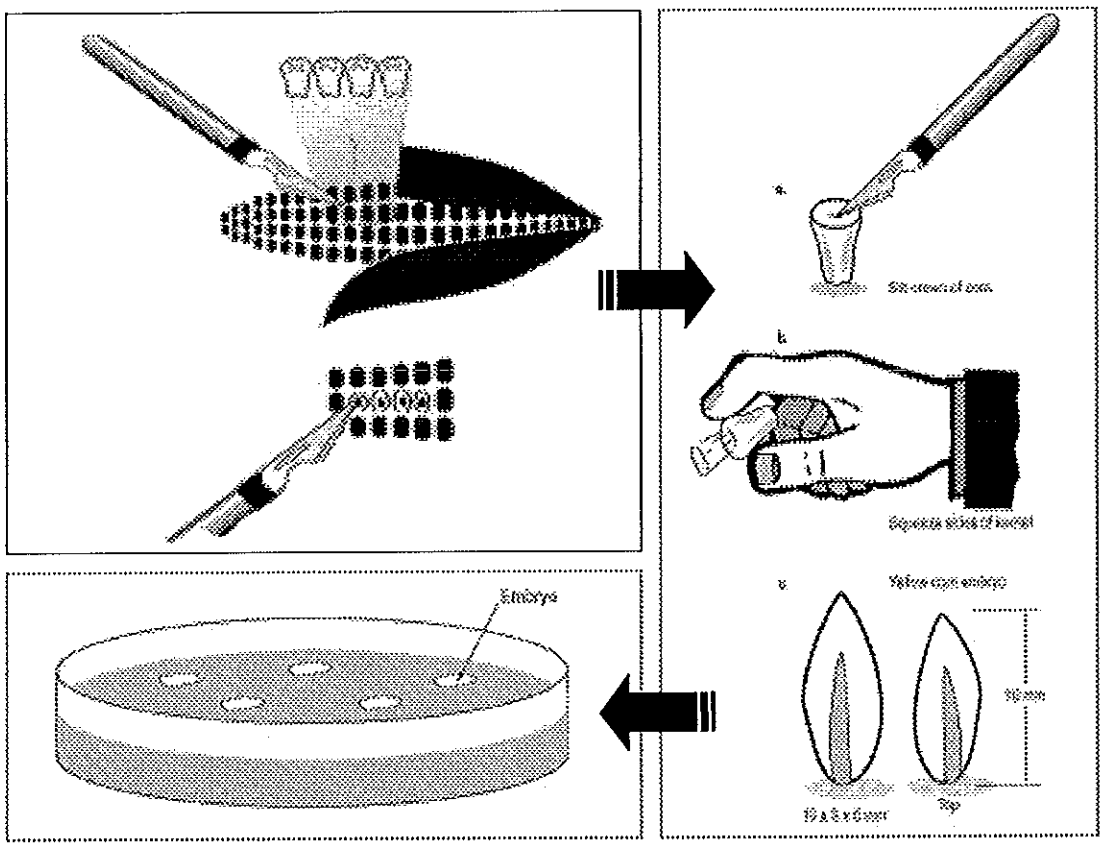

Figure 30. Diagram of corn embryo culture.

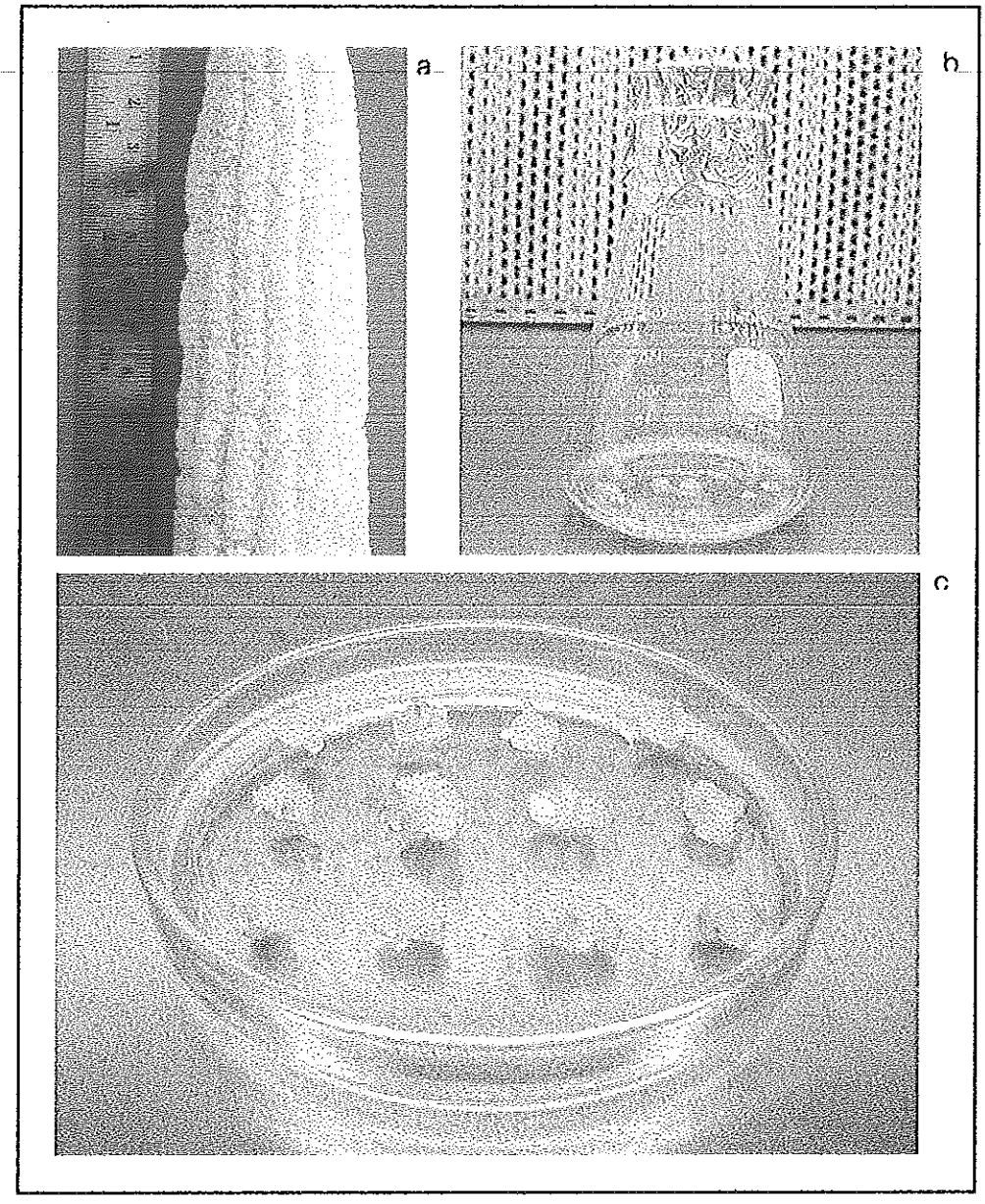

Figure 31 Corn callus culture. 4-week-old calluses (a), Callus suspension culture (b and immature ear for callus induction (c).

2. Nitrate reductase Activity Measurement

The measurement of NR activity was modified from Redinbaugh *et al.* (1996).

2.1 Nitrite standard curve establishment

1. Standard 0.1 mM potassium nitrite (KNO_2) was diluted with distilled water to concentrations of 20, 40, 60, 80 and 100 µM respectively and 500 µl was placed in 1 ml Eppendorf tubes.

2. 500 µl aliquots of 1:1 dilution mixture of 1% (w/v) sulfanilamide in 1.5 M HCl and 0.02% (w/v) N-(1-napthylenediamine dihydrochloride) in distilled water were added.

3. The solution was mixed and placed at RT for 20 minutes, then OD.540. was measured.

4. The amount of nitrite released was obtained from a standard curve of nitrite.

2.2 NR activity mesurement

Solution	Assay	Blank
Distilled water	-	0.5
Assay buffer	0.4	-
Sample	0.1	-

Assay buffer containing: 50 mM MOPS buffer, pH 7.5, 1 mM EDTA, 10 mM KNO_3 and 0.5 mM NADH or NADPH.

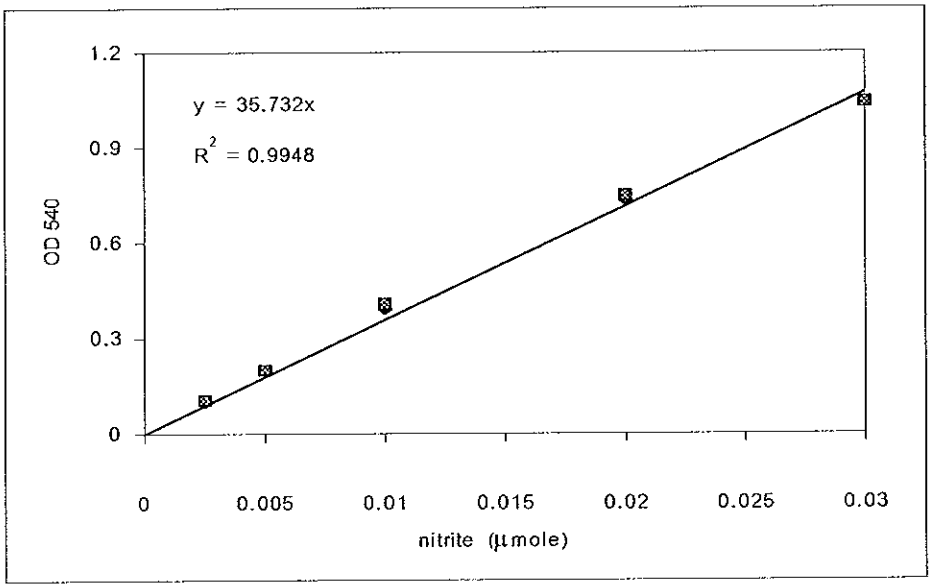

Figure 32. Standard curve of NO_2^- at absorbance 540 nm.

3. Protein Measurement

The protein measurement was modified from Lowry *et al.*(1951).

Component	Assay (ml)	Blank (ml)
Distilled water	-	0.1
Standard protein solution	0.1	-
10% Deoxycholate	0.4	0.4
2% Na_2CO_3 in 0.1 N NaOH	3.0	3.0
1% $CuSO_4$+ 2% Na/K tartate (1:1)	0.1	0.1
Incubate in RT for 10 minutes		
1:1 Folin cioculteu' s phenol reagent in distilled water	0.3	0.3
Incubate in RT for 30 minutes		
measured OD.540		

The amount of protein was determined from a standard curve of BSA.

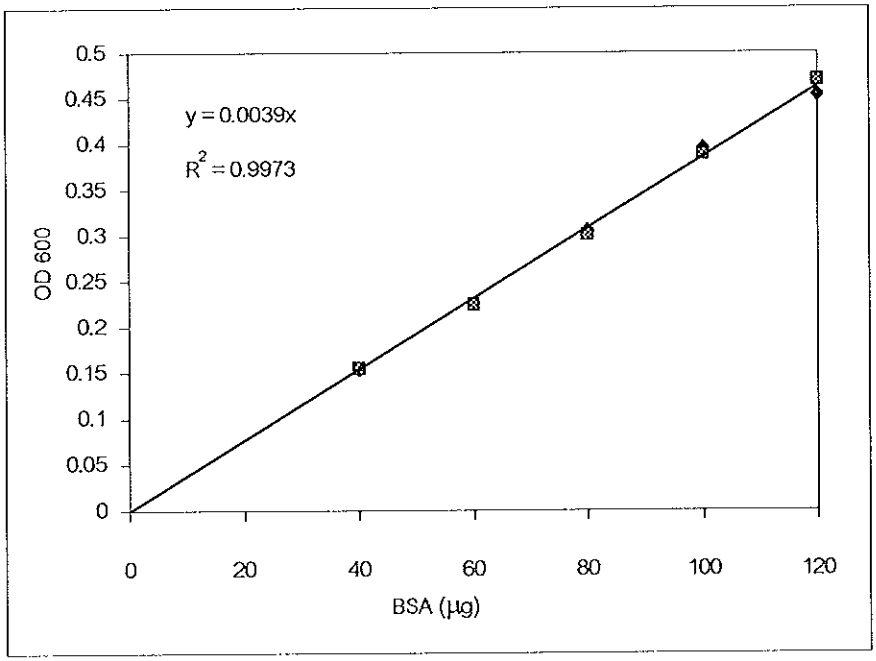

Figure 33 Standard curve of Bovine Serum Albumin at 600 nm using the Lowry method.

4. Nitrate Measurement

The determination of nitrate level was modified from Cataldo *et al.* in 1975.

4.1 Standard curve establishment

1. Prepare test tubes containing dilutions of KNO_3 to concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 µmole, respectively.

2. Transfer several solutions as described below to the tubes, respectively.

Component	Assay (ml)	Blank (ml)
Distilled water	-	0.2
KNO_3 in several concentrations	0.2	-
5% Salicylic acid in conc. H_2SO_4	0.8	0.8
Incubate in RT for 20 minutes		
4 N NaOH	9.5	9.5
Mix and incubate in RT		

4.2 nitrate measurement

Diluted samples and transfer several solutions as described below to the tubes, respectively.

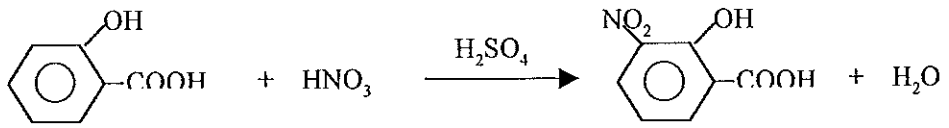

Figure 34 Nitrosalicylic formation reaction.