REGULATION OF NITRATE REDUCTASE DURING *IN VITRO* DIFFERENTIATION OF *NICOTIANA TABACUM* L. VAR. SAMSUN

by

Michael Austin Roberts

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Abstract

The commencement of *in vitro* differentiation is mediated by genetic changes that result in selective expression of genes and a shift in metabolism. The role of nitrate reductase, a key enzyme of nitrate assimilation, during differentiation was examined in this study using an *in vitro Nicotiana tabacum* (tobacco) callus culture system. In particular, the effects of nitrogen and light/dark regimes on callus differentiation and nitrate reductase were investigated.

Methodology required for the analysis of nitrate reductase regulation during *in vitro* tobacco callus differentiation was established. Optimised *in vivo*, *in situ* and *in vitro* nitrate reductase assays yielded similar values and patterns during tobacco callus culture development, and the *in vivo* assay was selected for nitrate reductase activity measurement during subsequent experiments. Western blot analysis of tobacco callus acetone-extracted protein after sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a spinach polyclonal nitrate reductase antibody yielded major bands at 71 and 48 kD, with numerous minor bands. Extraction of callus protein in the presence of various protectants did not prevent cleavage of putative nitrate reductase polypeptide. Slot blot detection of nitrate reductase mRNA using a [^{32}P]-labelled nitrate reductase cDNA probe isolated from the plasmid pBMC102010 was not possible due to non-specific binding to nitrocellulose filters. Northern blotting of RNA fractionated by agarose gel electrophoresis using a [^{32}P]-labelled nitrate reductase cDNA probe identified a single mRNA species at 3.5 kb, the expected size of tobacco nitrate reductase mRNA.

In vitro tobacco callus differentiation on 60 or 120 mM nitrogen regimes and under light/dark (16/8 h), continuous dark or continuous light treatments were comparable in terms of fresh weight, protein and nitrate uptake. Higher levels of *in vivo* nitrate reductase activity were observed prior to visible shoot primordia in all treatments, suggesting that the developmental status of callus mediated the regulation of nitrate reductase. Putative nitrate reductase protein levels were not correlated with *in vivo* nitrate reductase activity during initial stages of tobacco callus differentiation under various light treatments; nitrate reductase mRNA levels could not be ascertained. These results suggested that post-translational control mechanisms were involved in nitrate reductase regulation during *in vitro* tobacco callus differentiation.

Preface

The experimental work described in this thesis was conducted in the FRD/UND Photosynthetic Nitrogen Metabolism Research Unit at the Biology Department, University of Natal, Durban, under the joint supervision of Professor C.F. Cresswell, Dr B.I. Huckett and Dr M.P. Watt.

All work presented in this study was original research conducted by the author, and has not been submitted in any form to another University. The work of other individuals incorporated into the thesis is acknowledged in the text.

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CHAPTER 1. INTRODUCTION

This introductory literature review provides an historical and current perspective on the two fundamental subjects of the thesis: nitrate reductase (NR) and *in vitro* callus culture systems. Subsequent chapters will describe the development of suitable methods and research undertaken in the examination of NR during tobacco callus differentiation.

1.1 Biochemical, Molecular and Functional Aspects of Higher Plant Nitrate Reductase

1.1.1 Introduction

The acquisition and assimilation of inorganic nitrogen are fundamental biological processes requiring substantial amounts of energy (Guerrero et al. 1981; Solomonson & Barber 1990; Warner & Kleinhofs 1992). Inorganic nitrogen, made available to organisms by nitrification of molecular nitrogen (N_2) or recycling of organic nitrogen through mineralisation, is converted to biologically useful organic nitrogen via the fixation of molecular nitrogen (N_2) or the assimilation of nitrate (Lee & Stewart 1978; Solomonson & Barber 1990). For most higher plants, nitrate is the major source of inorganic nitrogen in the soil, indicating an important role for nitrate assimilation (Hewitt et al. 1979; Beevers & Hageman 1980; Guerrero 1985; Oaks & Hirel 1985; Wray 1988; Crawford & Campbell 1990; Hageman 1990; Warner & Kleinhofs 1992). The process of nitrate assimilation is initiated by the uptake of nitrate (Jackson et al. 1986; Redinbaugh & Campbell 1991; Warner & Kleinhofs 1992), which is reduced to nitrite by NR (EC 1.6.6.1-3) (Campbell 1988; Solomonson & Barber 1990). Nitrite is converted to ammonium by nitrite reductase (NiR - EC 1.7.7.1), and ammonium combines with carbon skeletons to form various nitrogenous compounds, including amino acids (Hageman et al. 1962; Beevers & Hageman 1969; Hewitt 1975; Hewitt et al. 1979; Guerrero et al. 1981; Oaks & Hirel 1985; Solomonson & Barber 1990). NR is regarded as the rate-limiting step of nitrate assimilation (Robinson 1987; Campbell 1988; Solomonson & Barber 1990), thereby avoiding accumulation of nitrite or ammonium, both of which are regarded as toxic at elevated levels (Beevers & Hageman 1980; Guerrero et al. 1981; Ota & Yamamoto 1989). Since the first report of the occurrence and partial purification of NR in higher plants (Evans & Nason 1953), the enzyme has been the subject of numerous specialist and general reviews (including: Beevers & Hageman 1969; Hageman & Hucklesby 1971; Hewitt 1975; Butz & Jackson 1977; Stewart & Rhodes

1977; Lee & Stewart 1978; Aparicio & Maldonado 1979; Garner 1979; Hewitt et al. 1979; Notton & Hewitt 1979; Beevers & Hageman 1980; Hageman & Reed 1980; House & Anderson 1980; Lee 1980; Srivastava 1980; Guerrero et al. 1981; Naik et al. 1982; Abrol et al. 1983; Duke & Duke 1984; Kleinhofs et al. 1985; Oaks & Hirel 1985; Smirnoff & Stewart 1985; Campbell & Smarrelli 1986; Wray 1986; Rajasekhar & Oelmüller 1987; Wallace 1987; Campbell 1988; Wray 1988; Caboche et al. 1989; Cammaerts et al. 1989; Campbell 1989; Crawford & Davis 1989; Kleinhofs et al. 1989; Caboche & Rouze 1990; Campbell & Kinghorn 1990; Solomonson & Barber 1990; Wray & Fido 1990; Redinbaugh & Campbell 1991; Warner & Kleinhofs 1992).

There are various reasons for the substantial interest in NR. Firstly, because NR activity (NRA) appears to limit nitrate assimilation, studying the enzyme is a possible route to enhanced growth and nitrogen content of crop species (Campbell 1988; Solomonson & Barber 1990). NR meets most requirements of a physiological trait for the selection of superior genotypes: NRA is heritable, quantifiable, and normally shows a positive correlation with yield (Lee & Stewart 1978; Peshkova & Khavkin 1980; Naik et al. 1982; Guerrero 1985; Sherrard et al. 1986; Hageman 1990), although some workers have been unable to correlate selection of increasing NRA with improved organic nitrogen or growth rates (Austin et al. 1978; Lee & Stewart 1978; Hageman 1990). Researchers still expect that an increase in the efficiency of NR and nitrate assimilation will improve crop yield (Campbell & Kinghorn 1990). Secondly, because NR is substrate inducible, it has been studied in relation to the regulation of nitrate assimilation (Remmler & Campbell 1986; Campbell 1988; Warner & Kleinhofs 1992). Thirdly, NR has been used as an indicator for plant stress, due to the close association and responsiveness of NR to plant metabolic and physiological status (Srivastava 1980; Campbell 1988). Finally, because NR is one of few plant enzymes that contains a molybdenum (Mo) component, the enzyme might facilitate the identification of plant Mo requirement (Campbell & Smarrelli 1986; Campbell et al. 1987; Campbell 1988).

In this section, the uptake of NR substrate is reviewed prior to a discussion on the location, structure, kinetics, reductant source and molecular genetics of higher plant NR. Regulatory aspects of nitrate assimilation, including nitrate induction of NR, are examined in Chapter 3.

1.1.2 Nitrate uptake by plant roots

Plants have evolved efficient nitrate uptake mechanisms in order to capitalise on transiently available soil nitrate (Warner & Kleinhofs 1992). Differences in the ability to requisition nitrate from the soil may be responsible partially for variability in utilising applied inorganic nitrogen observed in crop species (Jackson et al. 1986; Mattsson et al. 1991). Nitrate transport from the external medium across the root cell plasma membrane and into the cytoplasm is usually against an electrochemical gradient, and thought to be mediated via an energy-dependent nitrate permease (Hewitt 1975; Beevers & Hageman 1980; Guerrero et al. 1981; Jackson et al. 1986; Wray 1988). However, no specific nitrate carrier protein has been identified from higher plants yet (Crawford & Campbell 1990; Warner & Kleinhofs 1992), although McClure et al. (1987) identified a tonoplast polypeptide that was apparently nitrate-inducible, but was not found in other membrane fractions. Nevertheless, there is extensive indirect evidence suggesting the presence of plasma membrane active nitrate carriers (Redinbaugh & Campbell 1991; Warner & Kleinhofs 1992), including sensitivity of nitrate uptake to anaerobiosis (Lee 1978; Wray 1986), inhibition by respiratory, RNA and protein synthesis inhibitors (Heimer & Filner 1971; Wray 1986, 1988; Glass et al. 1990), and the induction and saturation kinetics of nitrate uptake (Jackson et al. 1986; Wray 1986; Aslam et al. 1992; Warner & Kleinhofs 1992).

Apparent nitrate induction of nitrate uptake has been observed in numerous species (Lee & Stewart 1978; Jackson et al. 1986). Induction of nitrate uptake is assumed to be elicited by the nitrate anion itself, rather than an increase in alkaline transmembrane driving force generated by nitrate reduction in the cytoplasm with concomitant hydroxyl ion release (Jackson et al. 1986; Tischner et al. 1990). When *Zea mays* (maize) (Jackson et al. 1973) and *Hordeum vulgare* (barley) (Rao & Rains 1976) roots were first exposed to nitrate, net nitrate uptake increased after a lag period until maximal rates were obtained. Similarly, application of nitrate to nitrate-starved tobacco cells resulted in the appearance of nitrate uptake at low nitrate concentrations that was maximal after a lag period of 3 h (Guy & Heimer 1992). Nitrate induction of nitrate uptake is prevented by RNA and protein synthesis inhibitors, implying a need for specific *de novo* protein synthesis (Wray 1986; Guy & Heimer 1992).

Roots normally have at least two mechanisms of nitrate uptake: (1) the so-called low affinity nitrate transport system (LATS) found in uninduced nitrate-starved

plants is thought to be constitutive, is relatively insensitive to metabolic inhibitors, shows low affinity for nitrate and is therefore active at higher nitrate concentrations; and (2) the high affinity nitrate transport system (HATS), which is inducible in plants previously exposed to nitrate, sensitive to inhibitors, has high affinity for nitrate and is active at low nitrate concentrations (Jackson et al. 1986; Wray 1986, 1988; Glass et al. 1990; Tischner et al. 1990; Redinbaugh & Campbell 1991; King et al. 1992). While both uptake mechanisms in *Arabidopsis thaliana* roots follow Michaelis-Menton kinetics (Wray 1986, 1988), contradictory results have been obtained in barley roots, with either the LATS and HATS showing initial Michaelis-Menton kinetics and then linear uptake after saturation (Aslam et al. 1992), or the LATS following linear kinetics for all nitrate concentrations (Glass et al. 1990; King et al. 1992). The LATS in barley roots possibly could represent an energetically passive channel-mediated system (Glass et al. 1990), but paradoxical characteristics of the system suggest that LATS is active thermodynamically (King et al. 1992).

Different properties of the LATS and HATS has led to the suggestion that there are two independent nitrate permeases, an hypothesis supported by studies on an *Arabidopsis* mutant impaired in only the HATS (Doddema & Telkamp 1979; Warner & Kleinhofs 1992). Alternatively, the different kinetics and affinities of the nitrate uptake systems may represent characteristics of a single multiphasic carrier (Jackson et al. 1986; Warner & Kleinhofs 1992). Additionally, it has been proposed that NR performs the dual function of a nitrate carrier and nitrate reducing enzyme (Butz & Jackson 1977). Anti-NR IgG fragments successfully inhibited NR uptake in barley roots (Ward et al. 1988), and tungstate treatment, which inactivates NR, was found to inhibit nitrate uptake in *Lemna gibba* roots (Ingemarsson et al. 1987a). Conversely, tungstate treatment did not interfere with the nitrate uptake process in tobacco cells (Heimer & Filner 1971), indicating that the extent to which nitrate and reduction are associated depends on plant species and experimental system (Jackson et al. 1986). NR may exist in two forms in some species, one cytoplasmic and involved in nitrate reduction only, the other plasma membrane-associated, mediating nitrate uptake and reduction (Ward et al. 1990). However, it is assumed generally that nitrate uptake and reduction are separate processes, even though the molecular nature of the nitrate permease(s) is not known (Jackson et al. 1986; Wray 1986, 1988; Warner & Kleinhofs 1992). Use of the nitrate analogue chlorate, which shares the nitrate transporter but cannot induce it, may assist in the isolation of nitrate uptake mutants and therefore nitrate permeases (McClure et al. 1986).

Net nitrate uptake is determined by a balance between nitrate influx and efflux. In-

flux rates reportedly are dependent on external nitrate concentrations and independent of internal nitrate concentration, while the variable efflux component is proportional to the internal nitrate concentration (Deane-Drummond & Glass 1983). Passive efflux of nitrate is mediated by a concentration gradient between the cytoplasm and external medium that can be more than 100-fold, and a potential electrochemical gradient of -100 to -150 mV (negative inside) (Jackson et al. 1986). However, in contrast to other studies, Ingemarsson et al. (1987a) showed using ^{13}N-labelled nitrate that in *Lemna* plants nitrate transport above a certain minimum threshold value (the nitrate compensation point) was predominantly unidirectional, with virtually no efflux. Mechanisms for determining relative influx/efflux rates are problematic: ^{13}N radioisotopes can be used only for short-term studies (half-life = 10 min), and chlorate affects NRA, which in turn may influence nitrate uptake (Jackson et al. 1986). Furthermore, cytoplasmic nitrate concentration is difficult to determine accurately (Warner & Kleinhofs 1992). Technical difficulties such as these render nitrate uptake a poorly characterised step of nitrate assimilation (Wray 1988).

Two mechanisms have been proposed for the uptake of nitrate. Firstly, nitrate entry may be accompanied by the excretion of hydroxyl or bicarbonate ions via an antiporter system (Jackson et al. 1986). The discharge of hydroxyl ions would reduce alkalinity associated with nitrate reduction to ammonium within the cytoplasm (Smirnoff & Stewart 1985). Excretion of bicarbonate ions correlates with the hypothesis that nitrate uptake in roots is controlled by shoot nitrate reduction (Ben Zioni et al. 1971). Hydroxyl ions associated with shoot nitrate reduction are neutralised by organic ions such as malate or citrate, which then are decarboxylated so that bicarbonate ions can be excreted in exchange for nitrate (Ben Zioni et al. 1971; Kirkby & Knight 1977; Kirkby & Armstrong 1980; Smirnoff & Stewart 1985). The second proposal for nitrate uptake requires a symporter that cotransports nitrate and hydrogen ions inwards (Jackson et al. 1986). Apparently, the two mechanism are difficult to distinguish experimentally, and support for each is indirect (Jackson et al. 1986). Whichever mechanism operates, the driving force for nitrate uptake is constituted by a transmembrane acidity gradient that is maintained by nitrate reduction, the decarboxylation of organic acids, and the electrogenic extrusion of protons by a plasma membrane ATPase (Jackson et al. 1986). Energy supply for active nitrate uptake and the maintenance of an electrochemical gradient is derived ultimately from photosynthesis or respiration (Breteler & Hänisch ten Cate 1980; Jackson et al. 1986).

Net nitrate uptake by root systems is subject to inhibition by end products of nitrate assimilation, including ammonium and amino acids, although the effect is highly variable (Heimer & Filner 1971; Oaks & Hirel 1985; Deane-Drummond 1986; Jackson et al. 1986). Uptake of nitrate from solutions containing both nitrate and ammonium often commences only when ammonium has been depleted (Guerrero et al. 1981). Deignan & Lewis (1988) showed that ammonium inhibited net nitrate uptake in *Triticum aestivum* (wheat) roots, but that nitrate inhibition of ammonium uptake was more pronounced. For *Raphanus sativus* roots, however, nitrate did not influence ammonium uptake (Ota & Yamamoto 1989). The effect of ammonium on nitrate uptake has been shown to be specifically an inhibition of influx in *Lemna gibba* roots (Ingemarsson et al. 1987b), while in barley roots ammonium reportedly decreased net nitrate uptake by increasing nitrate efflux (Deane-Drummond & Glass 1983). Conflicting results from different species suggest that different mechanisms of nitrate uptake regulation might exist, or that net nitrate uptake is influenced in still undetermined ways by variables such as plant growth, energy supply and nitrogen status (Jackson et al. 1986).

The fate of nitrate transported into the root plasma membrane depends on environmental conditions as well as plant species and/or cultivar: nitrate can be excreted, stored, reduced in the root by NR, or translocated to the shoots (Oaks et al. 1979; Beevers & Hageman 1980; Guerrero et al. 1981; Smirnoff & Stewart 1985; Jackson et al. 1986; Stulen et al. 1990; Gojon et al. 1991; Redinbaugh & Campbell 1991). Processes governing nitrate uptake and translocation are probably distinct (Redinbaugh & Campbell 1991), with translocation under circadian and possibly hormonal control, but nitrate uptake not (Jackson et al. 1986; Steingröver 1986). Nitrate translocation may play some role in regulating provision of reductant for NR, as endogenously stored nitrate is released slowly under natural conditions (Shaner & Boyer 1976a, b; Beevers & Hageman 1980; Guerrero et al. 1981; Jackson et al. 1986). As for other processes of nitrate uptake, translocation is characterised poorly (Wray 1986, 1988).

1.1.3 Location and subcellular distribution of NR

In most higher plants NRA is higher in leaves, although some plants reduce nitrate primarily in roots, with virtually no leaf NRA (Oaks & Hirel 1985; Wallace 1987; Campbell 1988). There is no clear ecological significance of leaf or root nitrate assimilation, although the predominance of the former indicates that leaf NRA may carry a lower energy cost under saturating light conditions (Smirnoff & Stewart

1985). Genera which have shown mainly leaf nitrate assimilation include *Cajanus* (Sainis & Sane 1978), *Cucumis* (Smirnoff & Stewart 1985), *Nicotiana* (Wakhloo & Staudt 1988), *Xanthium* (Smirnoff & Stewart 1985) and *Zea* (Campbell & Remmler 1986). Wheat leaves yielded greater specific NRA, even though higher levels of NR protein (NRP) were found in the roots (Przemeck & Kücke 1986; Soualmi & Champigny 1986). In barley, root and shoot NRA levels were comparable (Gojon et al. 1986). Most woody plants generally translocate little nitrate (Smirnoff & Stewart 1985), although the shoot NRA contribution has been shown in *Prunus persica* to be enhanced when external nitrate availability was increased (Gojon et al. 1991). Differences in experimental plant nitrogen status may account partially for discrepancies in the literature regarding species such as *Phaseolus vulgaris* (bean) - the plant was found to have higher leaf NRA by Sainis & Sane (1978), but higher root NRA by Breteler & Hänisch ten Cate (1980). Also, the contribution of leaf versus root NR may be regulated by certain developmental stages of plant growth (Beevers & Hageman 1969; Oaks 1979; Campbell & Smarrelli 1986).

NR is not restricted to leaves or roots, but can be found in petioles, stems, pods, maize scutella and husks, aleurone cells, pith cells, pollen grains, embryos and cotyledons (Beevers & Hageman 1969; Hageman & Hucklesby 1971; Guerrero et al. 1981). Additionally, in some plants NR may occur predominantly in a particular cell type, eg. in C_4 species, leaf NR is located in mesophyll cells but not in bundle sheath cells (Smirnoff & Stewart 1985; Campbell 1988; Vaughn & Campbell 1988). NR localisation in C_4 mesophyll cells is related possibly to limitations imposed by phosphoenolpyruvate carboxylase malate synthesis to regulate pH (Smirnoff & Stewart 1985), and may also prevent contact with inhibitory nitrate assimilation end-products in xylem vessels (Jackson et al. 1986).

The subcellular distribution of NR has not been established unequivocally (Lee 1980; Guerrero et al. 1981; Solomonson & Barber 1990; Warner & Kleinhofs 1992). Early studies involving density gradient centrifugation suggested that NR was predominantly cytoplasmic, although some association with chloroplast or other membranes could not be ruled out (Beevers & Hageman 1969; Oaks 1979; Smarrelli & Campbell 1979; Guerrero 1985; Wallace 1987; Campbell 1988). Circumstantial evidence favouring a cytoplasmic location of NR includes the presence of nitrite translocators in chloroplasts and cytoplasmic production of NR reductant, reduced nicotinamide adenine dinucleotide (NADH) (Solomonson & Barber 1990). Immunolocalisation studies in maize mesophyll cells showed that NR was located exclusively in the cytoplasm (Campbell 1988; Vaughn & Campbell 1988). However,

disparate results have been obtained from other immunological work, possibly due to different labelling conditions and antibody specificity (Gowri & Campbell 1989; Solomonson & Barber 1990). Microsomal fraction analysis of tobacco cells led Nato et al. (1990) and Hoarau et al. (1991) to conclude that tobacco possessed two different forms of NR, *viz.* a membrane bound and cytosolic form. The implication that can be derived from those results which would explain numerous anomalous findings, ie. that NR forms from certain plant species might exist at different specific locations, is not supported by some workers (Solomonson & Barber 1990).

1.1.4 NR forms and structure

NR belongs to a group of flavoprotein oxidoreductases that includes glutathione reductase (EC 1.6.4.2), NADH dehydrogenase (EC 1.6.99.3) and NADPH ferredoxin reductase (EC 1.18.1.2) (Campbell & Kinghorn 1990). Nomenclature of various forms of NR is according to optimal *in vitro* reductant. The most common form of NR in higher plants, and therefore the best characterised, is NADH:NR (EC 1.6.6.1), which has a pH optimum of 7.5 (Schrader et al. 1968; Beevers & Hageman 1969; Wray & Filner 1970; Guerrero et al. 1981; Wray 1988; Caboche & Rouze 1990; Campbell & Kinghorn 1990; Wray & Fido 1990). NADH:NR was shown soon after its discovery to be a single protein (Wray & Filner 1970), and not a mixture of two separate proteins as had been proposed (Paneque et al. 1965). Some plants, especially monocots, possess a bispecific NAD(P)H:NR (EC 1.6.6.2) that is capable of using either NADH or reduced nicotinamide dinucleotide phosphate (NADPH) at an optimal pH of 6.5 or 7.5 (Guerrero et al. 1981; Campbell 1988, 1989; Caboche & Rouze 1990; Campbell & Kinghorn 1990). NAD(P)H:NR was the form of NR first isolated by Evans & Nason (1953) from *Glycine max* (soybean) leaves. The physiological function of this bispecific form of NR is not clear, and it usually exists with NADH:NR (Campbell 1989). Another form of NR isolated from soybean (no EC number assigned yet) is also bispecific but favours NADH, has optimal activity at pH 6.5, and is thought to be constitutive (Wray 1988; Campbell 1989; Wray & Fido 1989). NADPH:NR (EC 1.6.6.3) that is specific for NADPH is found in fungi and not yet in higher plants (Campbell & Kinghorn 1990; Warner & Kleinhofs 1992).

Soybean possesses all three forms of NR found in higher plants: inducible NADH:NR, constitutive NAD(P)H:NR and constitutive bispecific NADH:NR (Curtis & Smarrelli 1987; Smarrelli et al. 1987; Campbell 1989). In soybean cotyledons, expression of the different NR forms has been related to developmental

stages (Orihuel-Iranzo & Campbell 1980). Species which possess both NADH:NR and NAD(P):NR include *Avena barbata* (Heath-Pagliuso et al. 1984), barley (Campbell 1988), maize (Campbell 1988), *Oryza sativa* (rice) (Shen 1972; Campbell 1988) and wheat (Heath-Pagliuso et al. 1984; Soualmi & Champigny 1986). In *Nicotiana*, only NADH:NR has been reported (Rouze et al. 1990), while in some tropical legumes, only NAD(P)H:NR has been found (Campbell & Smarrelli 1986).

Investigation of the biochemistry of NR has been arduous due to the difficulty of purifying sufficient quantities of intact enzyme (Beevers & Hageman 1980; Wray 1986, 1988; Campbell 1988) - earlier erroneous speculation on aspects of NR quaternary structure has now been dismissed (Notton & Hewitt 1979; Campbell & Smarrelli 1986; Campbell 1989). The NR polypeptide chain is large (105-115 kD), with native enzyme most likely comprising two identical subunits (Wray 1988; Campbell 1989; Campbell & Kinghorn 1990; Warner & Kleinhofs 1992). Each subunit contains three prosthetic groups, *viz.* flavin adenine dinucleotide (FAD), a heme group, and a molybdopterin complex, in the ratio 1:1:1 (Redinbaugh & Campbell 1985; Caboche & Rouze 1990; Campbell & Kinghorn 1990; Rouze et al. 1990; Solomonson & Barber 1990; Warner & Kleinhofs 1992). Limited proteolysis studies on *Spinacea oleracea* (spinach) NR have revealed that the cofactors reside in distinct catalytic domains within the subunit, with hinge regions susceptible to proteolytic cleavage between those domains (Kubo et al. 1988; Caboche & Rouze 1990; Crawford & Campbell 1990; Fido 1991; Shiraishi et al. 1991). Sensitive hinge regions allow for enzyme control by *in vivo* proteases (Guerrero et al. 1981; Campbell & Kinghorn 1990), and also account for the wide range of reported subunit sizes (Campbell & Kinghorn 1990; Warner & Kleinhofs 1992), as is discussed in Chapter 2.

The Mo cofactor is an unique combination of a phosphorylated pterin (a heterocyclic compound found in folic acid) and Mo, with side chains consisting of at least four carbons and two sulphur atoms (Kleinhofs et al. 1985; Wray 1986; Campbell 1989; Rajagopalan 1989). The biosynthesis of the molybdopterin cofactor in higher plants is not understood completely, although pterin is thought to act as a chelator and biological activator of Mo (Wray 1986; Crawford & Campbell 1990). In addition to a catalytic role, Mo-cofactor is responsible for dimerisation of NR subunits (Wray 1986), enabling Mo-containing complexes purified from spinach leaves to be used for the reconstitution of native NR in Mo-deficient plants (Hewitt et al. 1977; Notton & Hewitt 1979). The FAD domain has been crystallised recently and its three-dimensional structure determined (Lu et al. 1992a). The domain has

similar characteristics with the FAD region of $NADP^+$-ferredoxin reductase, whose structure has been determined to 0.22 nm resolution (Karplus et al. 1991). However, mechanisms for insertion of the FAD and heme domains to generate a flavohaemoprotein subunit are not known (Wray 1986, 1988).

Chemical modification studies have revealed that a number of specific amino acid residues are required for higher plant NRA (Solomonson & Barber 1990). Using arginine-specific reagents phenylglyoxal and 2,3-butanedione, Baijal & Sane (1988) demonstrated with *Amaranthus dubious* that NRA function, specifically NADH-binding activity, required this basic amino acid. The other basic amino acids, histidine and lysine, may also play a role in the NADH-binding FAD domain (Rouze et al. 1990; Solomonson et al. 1990). The cysteine (Cys) content of different NR forms varies from 9 to 19, with only four invariant (Campbell & Kinghorn 1990). Two invariant Cys residues probably bind Mo-cofactor, the third Cys may be involved in forming interchain disulphide bonds, and the fourth Cys is found in the heme domain and may be involved in binding NADH (Campbell & Kinghorn 1990; Rouze et al. 1990). Microbial expression of higher plant NR domains, necessary for site-directed mutagenesis experiments that will be useful in determining functional significance of specific amino acid residues (Crawford & Campbell 1990), has been achieved recently for the FAD and heme domains of maize NR using cDNA inserts of the enzyme (Hyde & Campbell 1990; Campbell 1992).

Progress in the isolation of NR cDNA clones has had a major impact on the understanding of the molecular structure of the enzyme (Solomonson & Barber 1990; Warner & Kleinhofs 1992). Genomic and/or cDNA clones for NR genes are available now for *Arabidopsis* (Cheng et al. 1988; Crawford et al. 1988), barley (Cheng et al. 1986), *Cucurbita maxima* (squash) (Crawford et al. 1986), *Lycopersicon esculentum* (tomato) (Daniel-Vedele et al. 1989), maize (Gowri & Campbell 1989), rice (Hamat et al. 1989), spinach (Prosser & Lazarus 1990) and tobacco (Calza et al. 1987; Vaucheret et al. 1989a, b). There is much sequence similarity among higher plant NRs from different species, with the greatest identity existing between genera in the same subclass (monocot/dicot) (Campbell 1988; Cheng et al. 1988; Vaucheret et al. 1989a, b; Warner & Kleinhofs 1992). Furthermore, homology has been established between the deduced amino sequence of NR domains and various other functionally similar enzymes and proteins (Caboche et al. 1989; Crawford & Campbell 1990; Solomonson & Barber 1990). The heme domain, also called the cytochrome b-557 region, shares sequences with mammalian cytochrome b_5, NADH-cytochrome b_5 reductase, sulfite oxidase and yeast flavo-cytochrome b_2 (Calza et al. 1987;

Campbell 1988; Crawford et al. 1989; Campbell 1990; Campbell & Kinghorn 1990). The FAD domain of NR has homology with NADH-cytochrome b_5 reductase (Calza et al. 1987; Crawford et al. 1988; Caboche & Rouze 1990; Campbell 1990). The molybdopterin domain of NR has identity with mammalian sulfite oxidase (Caboche & Rouze 1990; Campbell & Kinghorn 1990; Warner & Kleinhofs 1992). Comparisons of amino acid sequences support the theory that NR is composed of distinct catalytic domains (Campbell & Kinghorn 1990).

1.1.5 NR function and kinetics

NR is classified as a multi-centre redox enzyme, containing several internal electron carriers that are reduced during catalysis (Campbell & Smarrelli 1986; Campbell 1988; Campbell & Kinghorn 1990). Two essentially irreversible half-reactions catalysed by the enzyme are (Beevers & Hageman 1980; Campbell & Smarrelli 1986; Campbell 1989):

(1) $NADH + NR\ (oxidised) \rightarrow NAD^+ + H^+ + NR\ (reduced)$

(2) $Nitrate + H^+ + NR\ (reduced) \rightarrow nitrite + OH^- + NR\ (oxidised)$

Net reaction: $NADH + nitrate \rightarrow NAD^+ + nitrite + OH^-$.

There is much evidence for NR having two separate active sites, indicating a two-site ping-pong steady-state kinetic mechanism, similar to other redox enzymes (Campbell & Smarrelli 1986; Campbell 1988, 1989). Electron transfer proceeds from NADH, bound to the FAD domain, via the heme domain to nitrate, which binds to Mo in the Mo-cofactor domain (Garner 1979; Beevers & Hageman 1980; Ramadoss 1980; Guerrero et al. 1981; Campbell 1988; Campbell 1989; Nakagawa et al. 1990). Partial NR activities that require only one or two of the catalytic domains are classified as diaphorases if NAD(P)H is used as electron donor, or terminal activities if nitrate is used as electron acceptor (Guerrero et al. 1981; Guerrero 1985; Wray 1986; Campbell 1988; Caboche & Rouze 1990; Warner & Kleinhofs 1992). The ability of NR to reduce ferric citrate through diaphorase activity has led some workers to suggest that NR plays a role in iron assimilation in plants (Campbell & Smarrelli 1986; Campbell 1988). However, NR mutants and tungstate-inhibited plants are not chlorotic when supplied with a reduced nitrogen source, indicating no physiological significance of NR partial activities (Deng et al. 1989a, b; Müller & Mendel 1989; Caboche & Rouze 1990).

Specific activities of homogeneous purified NR from higher plants, fungi and algae range from 90 to 120 μmol/mg protein/min (Campbell & Smarrelli 1986). Higher plant NR Michaelis-Menton Km values for nitrate are 0.03-0.2 mM and for NAD(P)H are 0.003-0.06 mM (Hageman & Hucklesby 1971; Beevers & Hageman 1980; Hageman & Reed 1980; Campbell & Smarrelli 1986). The reduction of NR by NAD(P)H thus approaches diffusion limits, whereas the reduction of nitrate by reduced NR is rate-limiting, possibly due to slower electron transfer from the FAD domain to the Mo-cofactor domain via heme (Campbell & Smarrelli 1986; Campbell 1989).

1.1.6 Supply of reducing power for NRA

The physiological source of NADH for NRA has not been established conclusively yet (House & Anderson 1980; Lee 1980; Naik et al. 1982; Abrol et al. 1983; Wallace 1987). It appears that NAD^+ reduction in the cytoplasm could be linked to glycolysis or metabolite shuttles from chloroplasts (in the light) or mitochondria (in the light or dark) (Campbell & Smarrelli 1986; Wallace 1987).

The glycolysis metabolites glucose and phosphorylated hexoses were found to increase nitrite formation in green leaves, while mitochondrial tricarboxylic acid (TCA) cycle substrates were ineffective (Klepper et al. 1971). Therefore, oxidation of glyceraldehyde-3-phosphate by glyceraldehyde-3-phosphate dehydrogenase, and not the TCA cycle or pentose phosphate pathway (PPP), was regarded initially as the sole source of NADH for NRA (Beevers & Hageman 1969; Klepper et al. 1971). However, using a different pH to allow for exogenous substrate entry into the mitochondria, Nicholas et al. (1976b) and Sawhney et al. (1978a) were able to demonstrate that TCA cycle acids stimulated *in vivo* NRA, indicating a possible involvement of mitochondrial NADH production. The transfer of NADH from the mitochondrion to the cytoplasm is mediated by mitochondrial transhydrogenases or via a malate/oxaloacetate shuttle that allows for cytoplasmic oxidation of malate by malate dehydrogenase (Wiskich 1977; Beevers & Hageman 1980; Lee 1980; Woo et al. 1980; Naik et al. 1982). However, competition between mitochondria and NRA for NADH, at least in the dark, has been implicated by numerous studies involving either respiratory inhibitors or stimulation of respiration with concomitant NRA decrease by oxygen (Radin 1973; Canvin & Atkins 1974; Atkins & Canvin 1975; Sawhney et al. 1978a; Canvin & Woo 1979; Hewitt et al. 1979; Subbalakshmi et al. 1979; Beevers & Hageman 1980). Alternatively, chloroplastic photosynthesis, which

would involve exchange of NAD(P)H with the cytoplasm via malate/oxaloacetate or other shuttles, might provide reductant for NRA in the light (Jones & Sheard 1973; Canvin & Atkins 1974; Heber 1974; Atkins & Canvin 1975; House & Anderson 1980). Furthermore, several reports have linked nitrogen nutrition and photorespiration (Sarkissian & Fowler 1974; Platt et al. 1977; Stulen 1979; Hall et al. 1984; Robinson 1987). It is possible that NADH generated by the conversion of glycollate to glyoxylate during photorespiration may be an *in vivo* source of reductant for NRA (Roth-Bejerano & Lips 1973; James & Smith 1979; Lips 1979). In roots, reductant is derived ultimately from photosynthate translocated from the leaves (Lee 1980; Beevers & Hageman 1980; Abrol et al. 1983; Oaks & Hirel 1985; Smirnoff & Stewart 1985). Campbell & Smarrelli (1986) have commented on the confusion regarding NR reductant supply by remarking that the biochemical pathway contributing to the NADH pool is not important for NR, as the enzyme cannot distinguish between NADH molecules.

1.1.7 Molecular genetics of higher plant NR

Selection and characterisation of NR-deficient mutants has contributed substantially to the study and understanding of structure, function and regulation of NR (Caboche & Rouze 1990; Crawford & Campbell 1990; Warner & Kleinhofs 1992). Mutant analysis, which has allowed for the identification of genetic loci of NR and may also allow for the discovery of previously unknown genes, relies on the fact that NR deficiency is conditionally lethal - sources of nitrogen other than nitrate can be successfully metabolised by higher plants (Kleinhofs et al. 1985; Wray 1988; Müller & Mendel 1989). NR mutant work was pioneered by Cove & Pateman (1963) in the fungus *Aspergillus nidulans*. Early higher plant studies showed that NR was heritable, and emphasis was placed on the selection of high NRA strains (Beevers & Hageman 1969; Beevers & Hageman 1980; Gallagher et al. 1980). Contemporary studies have attempted to identify loci associated with different NR forms, structural mutants and regulatory factors (Wray 1986, 1988; Rouze et al. 1990; Warner & Kleinhofs 1992).

Various selection methods have been used to detect NR deficiency. Firstly, NRA assays can be performed on the leaves of plants germinated from mutagenised seeds - a relatively laborious process (Caboche & Rouze 1990). Secondly, mutagenised seedlings or cultures can be screened for chlorate resistance, because individuals lacking functional NR would not reduce chlorate to form the lethal product chlorite (Wray 1986, 1988; Caboche & Rouze 1990). Interestingly, chlorate has been shown

in *Arabidopsis* to induce NR mRNA, but decrease levels of NR protein (NRP) and NRA (Labrie et al. 1991). Chlorate selection of mutagenised plant material can be initiated during culture growth, followed by regeneration of surviving cells, or, with plants difficult to manipulate *in vitro*, during growth of second generation seedlings (Wray 1988; Pelsy et al. 1991). However, some NR^+ plants have expressed chlorate resistance, conferred by unknown mechanisms (Wray 1986; Pythoud & King 1990). Finally, negative selection of NR^- mutants is possible by screening for the inability of mutagenised material to grow on nitrate as sole nitrogen source (Caboche & Rouze 1990). Mutagens utilized to generate NR^- mutants for screening include gamma rays (Hamill et al. 1984; Pelsy et al. 1988; Wilkinson & Crawford 1991), ethylmethane sulfonate (Kuo et al. 1984; Pelsy et al. 1991), N-ethyl-N'-nitro-N-nitrosoguanidine (Strauss et al. 1981) and N-ethyl-N-nitrosourea (Müller & Grafe 1978).

Higher plant NR mutants have yielded a variety of phenotypes, ranging from no detectable NRP to high levels of NRP, with variable levels of total or partial NRA (Müller & Mendel 1989; Warner & Kleinhofs 1992). The physiological consequences of these mutations are either slight or nonexistent when mutants are maintained on non-nitrate nitrogen sources (Müller & Mendel 1989). However, in field conditions, loss of NRA would probably be lethal due to either acidification of soil caused by ammonium utilization or nitrate accumulation (Hamill & Cocking 1986). Two groups of NR mutants are represented: mutants that have defects in the NR structural gene (*nia*), and *cnx* (*c*ofactor for *n*itrate reductase and *x*anthine dehydrogenase) mutants impaired in the biosynthesis of the Mo-cofactor, identified on the basis of simultaneous loss of xanthine dehydrogenase and NR activities (Campbell & Smarrelli 1986; Wray 1988; Caboche & Rouze 1990). Restoration of NRA by intragenic complementation between different classes of *nia* and *cnx* mutants *in vivo* and *in vitro* is possible because mutations can occur in any of the three catalytically distinct NR domains, and because NRP levels usually are excessive (Caboche & Rouze 1990; Crawford & Campbell 1990; Rouze et al. 1990).

Cnx mutant studies have revealed that the Mo-cofactor is controlled by at least six loci in higher plants (Wray 1986, 1988; Kleinhofs et al. 1989; Müller & Mendel 1989; Pythoud & King 1990). Although the precise function of the Mo-cofactor genes are not known, mutant analyses suggest that two different metabolic pathways exist, one involving pterin nucleus modification to produce molybdopterin, and the other related to Mo uptake, translocation, activation and ligand binding to pterin (Kleinhofs et al. 1985; Wray 1986; Pythoud & King 1990; Warner & Kleinhofs

1992). In *Nicotiana plumbaginifolia*, seven *cnx* complementation groups have been described (*cnxA-G*) (Pelsy et al. 1988; Wray 1988; Müller & Mendel 1989). *CnxA* has similar characteristics to Mo-cofactor mutants of *Escherichia coli* and *Aspergillus nidulans* which are not defective in Mo uptake but are unable to insert Mo into the Mo-cofactor moiety, whereas *cnxB-G* gene loci probably encode for functions related to the synthesis of carbon side-chains or the phosphorylation and insertion of sulphur ligands (Wray 1986, 1988; Müller & Mendel 1989).

More *nia* and *cnx* mutants have been isolated from *Nicotiana* than any other higher plant genus (Müller & Grafe 1978; Wallin et al. 1979; Pental et al. 1979; Wray 1986, 1988; Caboche et al. 1989; Cammaerts et al. 1989; Müller & Mendel 1989; Cherel et al. 1990). Complementation of tobacco *nia* mutants with a wild-type *nia* gene has been achieved by co-electroporation and *Agrobacterium tumefaciens*-mediated transformation (Rouze et al. 1990; Vaucheret et al. 1990). In addition to *Nicotiana* species, apoprotein and/or *cnx* mutants have been obtained from *Arabidopsis thaliana*, barley, *Hyoscyamus muticus*, *Petunia hybrida*, *Pisum sativum* and soybean (Strauss et al. 1981; Dailey et al. 1982; Kuo et al. 1984; Kleinhofs et al. 1985; Wray 1986; Kleinhofs et al. 1989; Pythoud & King 1990).

Mutant studies have shown that some higher plant species have a complex array of NR structural genes, while others have only a single gene (Caboche & Rouze 1990; Crawford & Campbell 1990; Warner & Kleinhofs 1992). *Arabidopsis* has at least two *nia* genes which map to different loci, although the major gene (*nia*2) accounts for 95% of NRA (Cheng et al. 1988; Crawford & Campbell 1990; Wilkinson & Crawford 1991). Barley has two genes coding for the chemically distinct NR enzymes NADH:NR (*nar*1) and NAD(P)H:NR (*nar*7), with *nar*7 only expressed in *nar*1 mutants (Kleinhofs et al. 1989). *Nicotiana tabacum*, an amphidiploid species resulting from hybridisation between the progenitor species *N. sylvestris* and *N. tomentosiformis* (Gray et al. 1974), contains duplicate NR structural genes, *nia*1 and *nia*2 (Müller 1983; Müller & Mendel 1989; Vaucheret et al. 1989a, b). Gene dosage experiments have shown that only one of the four *nia* copies in *N. tabacum* is required for optimal growth (Müller & Mendel 1989). Although three types of NADH:NR might be expected - *nia*1 and *nia*2 homodimers and a heterodimer - only one form of NRP has been reported for tobacco, possibly due to inappropriate identification techniques or the high degree of homology between the two gene products (Wray 1988; Müller & Mendel 1989). Species which contain only one NR gene, such as *N. plumbaginifolia*(Crawford & Campbell 1989; Rouze et al. 1990), are not common among higher plants (Kleinhofs et al. 1990).

Genes with NR regulatory functions have not been recognized in higher plants, although they are presumed to exist (Kleinhofs et al. 1985; Wray 1986, 1988; Warner & Kleinhofs 1992). However, NR mutant analysis in the *Aspergillus* and *Neurospora* fungi has identified some genes (*areA* and *nit*2, respectively) that have a positive regulatory role in ammonium repression of NR, and others (*nirA* and *nit*4, respectively) that are required for nitrate induction of NR (Crawford & Campbell 1990). These regulatory genes contain Cys_2/Cys_2 zinc finger coding domains that have been shown to mediate transcription factor protein binding to DNA (Kraulis et al. 1992; Luisi 1992; Marmorstein et al. 1992). Furthermore, significant progress has been made in the isolation of *Chlamydomonas* NR regulatory genes that possess a high degree of similarity with their fungal counterparts (Weeks 1992). It is hoped that the isolation of fungal and algal regulatory genes will facilitate the discovery of higher plant regulatory genes (Crawford & Campbell 1989). Although regulatory perturbations have been found in some *Nicotiana nia* and *cnx* mutants, the nature of these mutants has not been established yet (Wray 1988; Pouteau et al. 1989).

The *nia* gene has provided plant molecular biologists with a unique tool that has a number of applications. The NR^- phenotype can be used for the selection of somatic hybrids (Wray 1986). Thus the combination of a *nia* mutant and a dominant resistance marker in the same genome has been used to select hybrids with nia^+ cells - only hybrids between the marker line and nitrate-utilising cells were able to use nitrate as sole nitrogen source (Wallin et al. 1979; Hamill et al. 1983, 1984; Cammaerts et al. 1989; Caboche & Rouze 1990). Additionally, NR fused to the cauliflower mosaic virus 35S promoter can be used as a negative marker gene, because transformed plants grown on ammonium and chlorate are killed selectively by constitutive NR expression, whereas control plants with wild-type *nia* genes will not express NR in the absence of nitrate (Nussaume 1991). Such a dominant conditional lethal gene reportedly can be applied to studies on chromosomal loss or large deletions or for homologous recombination studies (Nussaume 1991). Nia^- mutants should facilitate identification of new transposable elements also (Caboche & Rouze 1990).

1.1.8 Conclusions

Features of NR that have been described well include quaternary structure, prosthetic group composition and steady state kinetics (Solomonson & Barber 1990). However, numerous gaps in our knowledge of NR still exist, including in-

tracellular location, possible links between transport and reduction, different forms of NR and their roles, details of the catalytic mechanism, three-dimensional structure, and NR regulation (Crawford & Campbell 1990; Rouze et al. 1990; Solomonson & Barber 1990). More information on NR is required before the applied objectives of NR studies, *viz.* to increase NR efficiency and raise crop yield, thereby reducing the need for ecologically and economically damaging nitrogen fertilisers, can be achieved (Beevers & Hageman 1969; Wray 1986; Crawford & Campbell 1990; Lu et al. 1992b). The application of new molecular biology techniques, in combination with traditional selection analysis, provides a mechanism of achieving greater understanding of NR (Solomonson & Barber 1990).

1.2 Plant Differentiation and the Use of in vitro Callus Culture Systems

1.2.1 Introduction

Plant development can be described as the sum total of events leading to sequential elaboration of the plant body (Koornneef 1991). An important component of plant development is differentiation, the process induced by internal and external signals that initiates cell determination and changes in gene expression and morphology (Koornneef 1991; Marks et al. 1991). While phytohormones have been identified as the primary transducing signals for differentiation, the specific mediating mechanisms are unknown (Ross & Thorpe 1973; Owens & Smigocki 1990; Marks et al. 1991). Recent advances in plant differentiation research have revealed a hierarchy of regulatory genes, similar to the homeobox genes discovered in animal systems using *Drosophila melanogaster*, that code for protein transcription factors (Chory et al. 1991; Dawe & Freeling 1991; Hake 1992). Mutations in the maize *Knotted* (*Kn1*) gene affect leaf morphology (Hake 1992), while *A. thaliana det1* mutants grown in the dark have non-etiolated morphology, including chloroplasts and photosynthetic enzymes (Chory et al. 1991). Other systems currently in use to identify genes mediating cell determination and differentiation include trichome formation in *A. thaliana* (Marks et al. 1991), *A. thaliana* flower development (Bowman & Meyerowitz 1991) and a number of vegetative or developmental mutants (Koornneef 1991). Understanding the mechanisms controlling differentiation will create new ideas for enhancing crop productivity in addition to satisfying scientific curiosity (Marks et al. 1991).

Higher plant development and differentiation, initiated by biophysical mechanisms from a group of dividing parenchymatous cells called the meristem (Hake 1992; Selker et al. 1992), is difficult to decipher at the whole plant level due to a number of biological constraints (Tran Thahn Van & Trinh 1990; Koornneef 1991). However, *in vitro* plant tissue culture systems have been developed that facilitate morphological, physiological, cytological, biochemical and molecular work on differentiation (Evenson et al. 1988; Tran Thahn Van & Trinh 1990). All data thus far indicate that *in vitro* systems genetically and morphologically match their whole plant counterparts (Thorpe 1980), making tissue culture more suitable for differentiation studies in terms of ability to manipulate nutritional and environmental parameters (Thorpe & Murashige 1968; Jones et al. 1976; Thorpe 1980; Marion-Poll et al. 1984). Furthermore, cellular differentiation from callus can occur via two routes *in vitro*: (1) by somatic embryogenesis, where cells undergo structural and organisa-

tional changes similar to those occurring during zygote embryogenesis, forming globular, heart and torpedo embryo stages; and (2) by formation of primordia that undergo organogenesis into roots or shoots (Evans et al. 1981; Christianson et al. 1983; Thorpe 1983; White 1984; Ammirato 1986; Aleith & Richter 1990). In the following sections, various aspects of *in vitro* cell and callus culture methods, problems and applications will be assessed briefly before an examination of ultrastructural, biochemical and molecular studies describing *in vitro* plant cellular differentiation in the form of organogenesis. As the aim of the present investigation was to determine the role of nitrate assimilation during differentiation, particular emphasis is placed on the effects of nitrogen nutrition in callus cultures.

1.2.2 In vitro culture methods and conditions

Common types of *in vitro* culture systems include callus cultures, consisting of unorganised tissue grown on solid nutrient medium; cell suspension cultures, usually obtained by dispersion of friable callus in liquid medium and maintained by agitation; protoplasts, which are single cells (sans cell walls) grown in liquid or solid medium; and differentiated cultures, consisting of various organs (eg. embryos) grown on solid or liquid medium (Gamborg & Shyluk 1981; Collin & Dix 1990). Choice of culture procedure and conditions will depend on the purpose of any investigation (Gamborg & Shyluk 1981), but is also limited by species that fail to respond to *in vitro* treatments (Thorpe 1983; Ammirato 1986). In each of the culture systems mentioned above, appropriate inoculum, medium, and physical environment require consideration.

Inoculum selection is important for success in differentiation studies because although plant cells are thought to be totipotent, there are differences between cells or organs in the ability to differentiate in culture (Yamada et al. 1981; Ammirato 1986). Furthermore, endogenous variables such as phytohormone concentration and physiological state of the donor material make reproducible selection difficult (Thorpe 1980; Tran Thahn Van & Trinh 1990). Subcultured callus is used often as inoculum, with only one or two subcultures required to obtain a typical callus for a particular species (Thorpe 1980, 1983). Even with subculturing, however, characteristics of the tissue of origin may persist in culture for years (Thorpe 1980). The manner in which inoculum is excised or transferred may affect subsequent tissue culture also, as found with *Pinus radiata* cotyledons that underwent metabolic changes in culture according to the extent of wounding (Tran Thahn Van & Trinh 1990).

Medium composition is a critical factor for determining *in vitro* differentiation (Ross & Thorpe 1973). Four media ingredients are required for growth and differentiation: (1) inorganic macro- and micronutrients; (2) an energy and carbon source; (3) vitamins; and (4) growth regulators (Thorpe 1980; Gamborg & Shyluk 1981). An organic nitrogen source in the form of various amino acids is included in some media (Murashige & Skoog 1962; Thorpe 1980). If all required mineral nutrients and common organic compounds are supplied in excess, then culture growth variability caused by endogenous compounds should be diminished (Murashige & Skoog 1962). Numerous media suitable for different plant species have been developed (Evans et al. 1981; George et al. 1987). Due to the nature of this investigation, two important media components, growth regulators and nitrogen source, are discussed here in detail.

Growth regulators elicit a complex interaction between plant growth, development and differentiation (Koornneef 1991). Therefore, phytohormones and other types of growth compounds (such as polyamines and ascorbate) are included in media to manipulate differentiation *in vitro* (Thorpe 1980; Evans et al. 1981; Fujimura & Komamine 1975; Tran Thahn Van & Trinh 1991). In some cases, sequential treatment with different phytohormones is necessary to promote organ formation (Thorpe 1980; Chih-hung et al. 1981; Evans et al. 1981). An hypothesis that the ratio, and not absolute concentration, of exogenously applied auxin and cytokinin determines root versus shoot formation has been confirmed in many studies, albeit dependent on factors such as cell sensitivity, endogenous phytohormone levels, the uptake of exogenous phytohormones, and the degree of phytohormone glycosylation and hydrolysis (Murashige & Skoog 1962; Evans et al. 1981; Owens & Smigocki 1990, Tran Thahn Van & Trinh 1990). The precise mechanism of phytohormone action is not understood (Thorpe 1980), although some progress has been achieved in the isolation of an auxin-binding protein and its coding gene, as well as other phytohormone receptors (Lazarus et al. 1991; Tran Thahn Van & Trinh 1991). However, phytohormone receptor antibodies cannot determine active receptor forms and therefore have not been able to confirm whether polarised gradients of phytohormones give rise to polarity in callus cells probably required for differentiation (Thorpe 1980; Tran Thahn Van & Trinh 1991). While auxin and cytokinins appear to play a dominant role in controlling *in vitro* differentiation, other phytohormones such as gibberellic acid and abscisic acid are important also (Ammirato 1986; Owens & Smigocki 1990). Although there is very little data on the effects of endogenous phytohormones, both gibberellic acid and ethylene reportedly change during shoot production in tobacco callus (Thorpe 1980, 1983). The

presence of endogenous phytohormones and other growth regulators accounts for some of the variation in exogenous phytohormone application levels required for optimal *in vitro* differentiation (Murashige & Skoog 1962).

A nitrogen source is essential for *in vitro* growth and differentiation (Thorpe 1983; Ammirato 1986). However, nitrogen utilisation and the ability to grow on different forms of nitrogen is species dependent (Grimes & Hodges 1990; Lumsden et al. 1990). Gamborg (1970) demonstrated that while some plant species were able to grow in culture on nitrate as the sole nitrogen source, others needed reduced nitrogen in the form of ammonium or glutamine. A similar conclusion was reached by Lenee & Chupeau (1989) when comparing the activities of various nitrogen assimilation enzymes of tobacco and *Helianthus annuus* (sunflower) cell suspension cultures: sunflower cells required reduced nitrogen whereas tobacco cells had sufficient NRA to use nitrate as sole nitrogen source. Studies using Paul's Scarlet rose (PSR) suspension cultures have shown that growth occurs in the presence of nitrate-nitrogen (Caldas & Caldas 1976), but is not possible with only ammonium-nitrogen (Jones et al. 1976), although the latter may have been caused by pH inhibition (Kamada & Harada 1984). However, nitrate and ammonium together yielded optimal growth for PSR cells, at rates that correlated well with glutamate synthase (GOGAT) activity (Mohanty & Fletcher 1980). A combination of nitrate- and ammonium-nitrogen was optimal for maize callus growth also, although glutamate synthetase (GS) activity was found to be primarily responsible for nitrogen assimilation (Loyola-Vargas & Sanchez de Jimenez 1986). Those studies illustrated differences in nitrogen utilisation between species at the biochemical level.

The utilisation of different nitrogen forms during culture growth has been investigated by examining comparative activities of NR, the rate limiting enzyme of nitrate assimilation (Section 1.1.1). Jones et al. (1976) found that urea could yield 80% of the growth obtained using nitrate in PSR cell suspension cultures, even though NRA was reduced to trace amounts on urea. Similar results were obtained with PSR cells after addition of ammonium to nitrate medium - although NRA was inhibited, there was a two fold stimulation of growth (Jordan & Fletcher 1980). Furthermore, NRA was adversely affected by various amino acids in culture, possibly via a negative feedback mechanism (Heimer & Riklis 1979; Zink 1982; Kamada & Harada 1984). Yet supplementation of nitrate and/or ammonium growth medium with amino acids either inhibited or enhanced culture growth, depending on both plant species and amino acid used (Gamborg 1970; Zink 1982; Kamada & Harada 1984; Marion-Poll et al. 1984; Bonner et al. 1992). The inhibition of *N. silvestris* suspension culture

growth rates by amino acids apparently was not caused by NRA inhibition, or by ammonium or keto-acid toxicity, but by cell plasmolysis (Bonner et al. 1992). Thus there appears to be no general correlation between nitrate assimilation and culture growth *per se*. However, enhanced nitrogen assimilation, either through oxidised or reduced nitrogen forms depending on media composition and plant species, does seem to be associated with *in vitro* culture productivity (Voronova et al. 1983).

In addition to composition, physical form of medium (liquid or solid) and its pH are important for successful *in vitro* growth and differentiation (Thorpe 1980). Medium pH has not been systematically studied (Thorpe 1980), except for individual species under specific conditions (Wetherell & Dougall 1976). Both solid and liquid medium are set at pH 5.0 to 6.0 usually, at which pH mineral salts remain soluble and growth and differentiation are possible (Murashige & Skoog 1962; Thorpe 1980). Changes in pH during culture depends on plant species and type and status of nitrogen in the medium (Evenson et al. 1988).

Environmental factors such as light and temperature influence differentiation (Thorpe 1983; Ammirato 1986; Tran Thahn Van & Trinh 1990). Light intensity, period and quality are important, although if medium is supplemented with inorganic and organic compounds then differentiation can occur in the dark (Thorpe 1980, 1983). Light may affect organogenesis by destabilising auxin, thereby altering auxin/kinetin ratios (Tran Thahn Van & Trinh 1990). Temperature effects have not been investigated thoroughly, although optima for *in vitro* growth of various species have been established, as well as temperature extremes that allow for plant material storage or altered biochemistry (Thorpe 1980; Ammirato 1986).

1.2.3 Experimental problems associated with in vitro cultures

Variability associated with *in vitro* cultures constitutes a considerable problem for numerous studies employing such systems (Ammirato 1986). Variants have been shown to arise from: (1) physiological, biochemical and morphological changes in undifferentiated cultures; (2) variability in differentiation; (3) changes manifested in differentiated plantlets; and (4) chromosomal and genetic changes (Skirvin 1978). Undifferentiated cultures can develop physiological changes through habituation, involving the loss of phytohormone requirement that usually is developed after long-term culturing (Skirvin 1978; Thorpe 1980, 1983). Ageing through successive cultures of *Daucus carota* (carrot) root callus reduced biochemical sensitivity to various inhibitors (Syono 1965). Other changes occurring in undifferentiated cultures in-

clude growth habit (eg. friability of callus), and modifications of cellular biochemistry such as isoenzyme activity (Syono 1965; Skirvin 1978; Thorpe 1980, 1983). Differences in ability to form roots or shoots within a given culture has been observed in numerous studies, involving species such as *Atropa belladonna* (Thomas & Street 1972), *N. tabacum* (Meins et al. 1982; Bertrand-Garcia et al. 1992), *Trifolium repens* (White 1984), and *Euphorbia esula* (Evenson et al. 1988). While most of those studies associated decreased organogenesis with ageing, aneuploidy (the gain or loss of chromosomes) may induce such changes also (Skirvin 1978). Somaclonal variation in differentiated plantlets, involving morphology, chromosome number or enzyme systems, is fairly common (Skirvin 1978; Evans et al. 1981). Plants which might normally be highly stable in terms of chromosome constitution undergo considerable variation during *in vitro* tissue culture (Skirvin 1978; Gamborg & Shyluk 1981). However, somaclonal variation can be exploited to complement classical breeding programs, as discussed in Section 1.2.4.

Only a few cells participate in the initiation of organogenesis (Thorpe 1980). In tobacco callus, only 1 in 10^3-10^4 cells initiates bud formation, possibly due to position effects (according to the physiological gradient hypothesis, only a few positions within callus favour organogenesis) or caused by the availability of a limited number of cells competent at a given time to respond to inductive signals (Meins et al. 1982). In order to study processes such as *in vitro* differentiation, and considering the agricultural benefits that might be accrued from having enhanced plantlet production, it would be desirable to induce organogenesis in more cells (Bertrand-Garcia et al. 1992). A further complication for studies investigating differentiation is lack of synchrony between culture replicates (Thorpe 1980). Methods assessing the functional status of cultured cells that appear to be morphologically similar might be useful in overcoming the synchrony problem (Thorpe 1980, 1983). However, until an ideal system is developed without problems of variability and low induction frequency, current *in vitro* culture methods still offer an alternative to and many advantages in comparison with whole plant studies (Thorpe & Murashige 1968; Jones et al. 1976; Thorpe 1980; Marion-Poll et al. 1984).

1.2.4 Applications of in vitro callus culture

In vitro tissue culture techniques have wide applications in agriculture, assisting plant breeding analyses to increase plant performance as well as creating a potential for large scale propagation of clonal plants (Ammirato 1986; Collin & Dix 1990). Natural variation associated with *in vitro* cultures and clonally propagated plantlets,

as well as artificial variation induced using chemical (8-azaguanine, diethyl sulfate) or physical (X-rays, gamma rays) mutagens, can be utilised for plant improvement (Skirvin 1978; Evans et al. 1981). Agriculture should benefit from artificial seeds constructed using somatic embryos (Ammirato 1986). For the industrialist, *in vitro* cultures provide potential sources of pharmaceuticals, aromatics and colorants (Collin & Dix 1990).

Plant tissue culture has made important contributions to several fields of discovery in the plant kingdom (Collin & Dix 1990). If plant development and organogenesis were understood sufficiently to allow for greater control, then plant breeding programmes would be enhanced greatly (Ammirato 1986; Tran Thahn Van & Trinh 1990). Furthermore, *in vitro* culture has facilitated the isolation of biochemical mutants that would not have been recognisable or selectable at the plant level (Pythoud & King 1990). *In vitro* techniques have allowed for the isolation and study of NR structural mutants (Müller & Grafe 1978; Wallin et al. 1979; Mendel & Müller 1980; Pental et al. 1982). Tissue culture is a source of standardised plant material for metabolic studies (Collin & Dix 1990), and therefore has been utilised to investigate various aspects of nitrate uptake and NR physiology (Heimer & Filner 1971; Jones et al. 1978; Heimer & Riklis 1979; Nato et al. 1990; Hoarau et al. 1991; Trinity & Filner 1991).

1.2.5 Ultrastructural, biochemical and molecular aspects of in vitro differentiation

In vitro organogenesis has been described well at the histological and ultrastructural level, and is explained here with reference to tobacco callus (Ross et al. 1973; Maeda & Thorpe 1979; Thorpe 1980, 1983). Unorganised callus consists of parenchyma cells that are highly vacuolated with inconspicuous nuclei and cytoplasm, and occasional scattered lignified elements can be observed. Extensive starch grains and numerous paracrystalline bodies are present before cell files of preferential active cell division adjacent to xylem and tracheid elements are formed. Cell files give rise to meristemoids, which are meristem-like aggregates of small, non-polar, nonvacuolate cells that form root or shoot primordia. Meristemoids are more frequent in the lower or central parts of callus tissue, normally at discrete distances from the medium, and are formed in light or dark treatments. During meristemoid formation, starch granules in the callus decrease in size, and paracrystalline bodies disappear. Primordia eventually form roots or shoots, normally from the lower portion of callus. Inversion studies of Ross & Thorpe (1973) support the hypothesis that organogenesis results from quantitative interactions between various growth factors

active over physiological gradients, usually creating optimal induction in callus material closer to the medium.

The peak in starch formation observed prior to meristemoid formation implicated a role for carbohydrate metabolism during organogenesis (Thorpe & Murashige 1968; Thorpe & Meier 1972; Ross & Thorpe 1973; Ross et al. 1973; Thorpe & Meier 1974; Thorpe 1980, 1983). Higher levels of starch degradation and respiration were measured during shoot initiation, indicating that the cells involved in shoot initiation required larger amounts of energy (Thorpe & Murashige 1968; Thorpe & Meier 1972; Ross & Thorpe 1973; Thorpe & Laishley 1973; Thorpe & Meier 1974). The radioactive labelling experiments of Thorpe & Laishley (1973) confirmed respiration enzyme studies and further demonstrated that the pentose phosphate pathway (PPP) was more active than Embden-Meyerhof-Parnas (EMP) glycolysis, although both pathways were enhanced, during shoot formation. The increase in carbohydrate oxidation through PPP and EMP pathways not only leads to increased ATP levels, but also provides reducing power in the form of NAD(P)H, pentose for nucleic acid synthesis, and erythrose-4-phosphate for aromatic compound synthesis (Ross & Thorpe 1973; Thorpe & Laishley 1973; Thorpe 1980, 1983). Furthermore, carbohydrate metabolism may be important for osmotic adjustment in shoot-forming callus (Thorpe 1983). However, levels of the PPP product malate decreased during shoot formation, indicating that malate's osmoregulatory function during cell expansion between cell divisions was not necessary, but rather that malate was converted to pyruvate to form NADH as reducing power (Plumb-Dhindsa et al. 1979).

The disappearance of proteinaceous inclusions in callus cells during organogenesis implies an important role for nitrogen metabolism (Ross et al. 1973; Hardy & Thorpe 1981; Thorpe 1983). Furthermore, the presence or absence of various nitrogen forms have been related to the ability of some cultures to undergo differentiation, or to the type of differentiation found (Thomas & Street 1972; Wetherell & Dougall 1976; Christianson et al. 1983; Loyola-Vargas & Sanchez de Jimenez 1986; Grimes & Hodges 1990). The few studies on nitrogen assimilation enzyme involvement during differentiation have produced equivocal results. In sugarcane (*Saccharum officinarum*) callus, the pre-emergence of shoots was accompanied by increases in NR, GS and GOGAT activities with no change in glutamate dehydrogenase (GDH) activity (Dwivedi et al. 1984), whereas carrot cell shoot emergence was preceded by a 3-fold increase in GDH, with no changes in other nitrogen assimilation enzymes (Werner & Gogolin 1970). Relatively higher levels of NRA in shoot-forming tobacco callus, especially in the lower half of the callus, have

been observed (Thorpe 1983; Hardy & Thorpe 1990), but no correlation between NRA and root formation in *Euphorbia esula* cell suspension cultures was found (Evenson et al. 1988). Evenson et al. (1988) also alleged (data not shown) that root formation in *E. esula* cells was possible in medium without Mo, an essential cofactor for NRA. Interestingly, NRA has been quantified in Paul's Scarlet rose cells grown without Mo at 30% of the NRA in normal cultures (Jones et al. 1976), raising the possibility that residual nutrients were transferred from one culture medium to another upon transferal of pelleted cells. Lack of correlation between NRA and differentiation in some species is perhaps a reflection of preferential assimilation of reduced nitrogen forms (Grimes & Hodges 1990; Lumsden et al. 1990). Various enzymes of the shikimic acid pathway (including shikimate kinase and anthranilate synthetase), which converts erythrose-4-phosphate to aromatic amino acids, increased 24-fold during meristemoid and shoot primordium formation in tobacco callus (Beaudoin-Eagan & Thorpe 1981). Aromatic amino acids are important not only for protein synthesis, but also as precursors for various phytohormones and vitamins (Bonner et al. 1992). Most studies therefore indicate that nitrogen assimilation is fundamental to differentiation (Hardy & Thorpe 1990).

Protein synthesis and protein levels increase during organogenesis (Thorpe & Meier 1974; Thorpe 1980, 1983; Dwivedi et al. 1984). Cultured *Pseudotsuga menziesii* cotyledons showed an increase in the synthesis of low molecular weight proteins associated specifically with adventitious bud formation (Yusada et al. 1980). An analysis of proteins in tobacco callus using two-dimensional PAGE has revealed differences between total, nonhistone chromosomal, and basic chromosomal polypeptides specifically associated with shoot-forming rather than non-shoot-forming callus (Bertrand-Garcia et al. 1992). Furthermore, nonhistone chromosomal proteins, thought to play an important role in eukaryotic gene expression (Guerri et al. 1982), were phosphorylated at 3-4-fold higher levels in shoot-forming callus (Bertrand-Garcia et al. 1992). Guerri et al. (1982) have shown that cell proliferation in shoot formation is accompanied by an increase in transcriptional activity. Modification of mRNA species synthesis, as well as proteins, that occurs during shoot formation indicates that changes in gene expression are correlated with differentiation (Thorpe 1980, 1983; Bertrand-Garcia et al. 1992).

Differentiation arises from progressive developmental interactions between cells and their environment, leading to the initiation of activity of specific genes (Thorpe 1980). The influence of endogenous phytohormone levels has been investigated at the gene level using the crown-gall pathogen *Agrobacterium tumefaciens*, which in its

natural state transfers DNA coding for auxin and cytokinin from the Ti (tumour-inducing) plasmid into a plant (Owens & Smigocki 1990). Studies have shown that alteration of expression of a single phytohormone can induce organogenesis, but also that there is a complex interaction between levels of endogenous phytohormones (Owens & Smigocki 1990). Transgenic plants containing an *A. tumefaciens* cytokinin gene (*ipt*), placed under the control of a heat-inducible promoter from a heat-shock protein gene (hsp70), were used to demonstrate that excess cytokinin caused distinct morphological effects (Medford et al. 1989; Owens & Smigocki 1990). While Owens & Smigocki (1990) deduced from those studies that excess phytohormone results in both differentiation and growth effects, Medford et al. (1989) supported only the latter conclusion. A more recent study by Li et al. (1992) using a different *ipt* construct in transgenic tobacco plants supports the view of Owens & Smigocki (1990) that both growth and differentiation are affected by endogenous phytohormone levels. Although various genes have been shown to be preferentially expressed during differentiation *in vitro* and *in vivo*, it is not clear whether these genes are regulatory or required for enhanced metabolism (Vernet et al. 1982; Aleith & Richter 1990; Greenler & Becker 1990; Györgyey et al. 1991). However, genes that control various aspects of the complex process of plant differentiation have been elucidated (Edwards & Coruzzi 1990).

1.2.6 Conclusions

The initiation of differentiation involves genetic changes that cause modifications of metabolism and enzyme synthesis, with nitrogen playing an important role (Thorpe 1980, 1983; Voronova et al. 1983; Ammirato 1986). A limitation to achieving control of differentiation is the lack of biochemical and molecular knowledge concerning these metabolic changes (Thorpe 1983, Hardy & Thorpe 1990; Owens & Smigocki 1990). With the increase of refined molecular techniques, differentiation studies can advance from descriptive analyses to elucidation of causal mechanisms (Tran Thahn Van & Trinh 1990). *In vitro* tissue cultures can be manipulated to closely approximate the *in vivo* plant situation, greatly facilitating such research.

1.3 Aims of this Investigation

Various studies have indicated that nitrogen assimilation is critical for *in vitro* differentiation of callus material, particularly in *Nicotiana tabacum*. The work presented describes at the biochemical and molecular level the involvement of the key nitrate assimilation enzyme, NR, during tobacco callus differentiation. In order to achieve this objective, methods described in Chapter 2 were developed in order to quantify NR mRNA, NRP and NRA in differentiating tobacco callus. Application of some of these methods are described in Chapter 3, and allowed for delineation of the control of NR expression at translational and post-translational levels. This study endeavored to contribute to a greater understanding of both the differentiation process and the developmental regulation of NR in higher plants.

CHAPTER 2. METHOD DEVELOPMENT FOR BIOCHEMICAL AND MOLECULAR STUDIES ON NITRATE REDUCTASE

2.1 Introduction

NR is considered to be a limiting factor for higher plant growth, development and protein production, and therefore much research involving the delineation of properties associated with NR regulation and catalytic mechanism has been undertaken (Solomonson & Barber 1990). Many workers have attempted to correlate NRA with plant or seed growth, soluble amino acid content, reduced nitrogen or protein accumulation (Brunetti & Hageman 1976; Peshkova & Khavkin 1980; Srivastava 1980; Guerrero et al. 1981; Singh & Vijayakumer 1981; Naik et al. 1982; Guerrero 1985; Sherrard et al. 1986). Notwithstanding the difficulties associated with attempting to utilise a single marker system (Huffaker 1982), those biochemical and correlation studies have been severely hampered by the inability to accurately determine true NRA under normal physiological conditions (Soussana et al. 1989). Additionally, because NR is very highly regulated (Beevers & Hageman 1969), NRP is inherently labile *in vitro* and *in vivo* due to both specific and nonspecific proteolytic and inactivator mechanisms (Jolly & Tolbert 1978; Wallace 1978; Beevers & Hageman 1980; Leong & Shen 1982; Remmler & Campbell 1986; Yoshimura et al. 1992). Therefore, attempts to determine the size or amount of NRP are complicated (Wray 1988). At the nucleic acid level, NR mRNA is relatively unstable due to its very low abundance (0.005-0.01% of the total polyA$^+$RNA [Cheng et al. 1986; Wray 1986]) and large size (3.2-3.5 kb [Crawford et al. 1988; Campbell 1989; Gowri & Campbell 1989; Vaucheret et al. 1989b]). This introduction reviews the theoretical background of techniques that have been utilised for the quantification of NR activity, protein and mRNA.

2.1.1 Assays for NRA estimation

NRA is most frequently estimated using *in vitro*, *in vivo* and/or *in situ* assays, although inconsistencies exist regarding nomenclature of the assay types. Campbell and Smarrelli (1986) state that the true *in vivo* assay requires an analysis at the whole plant level of nitrate disappearance, nitrite appearance and measurement of the increase in total plant reduced nitrogen. Yet the "*in vivo*" term was originally applied to an intact tissue (not whole plant) assay that measured nitrite formation as an indication of NRA (Naik et al. 1982): this usage has conventionally been maintained (among others Streeter & Bosler 1972; Brunetti & Hageman 1976;

Mann et al. 1979; Yoneyama 1981; Lawrence & Herrick 1982; Davies & Ross 1985; Maurino et al. 1986; King et al. 1992) and is continued here. The term "*in situ*" has been applied to certain variations of the *in vivo* assay (Soussana et al. 1989; Corzo & Niell 1991) but will be used here in its original context, involving cell permeabilisation with an aqueous buffer containing substrates and the measurement of nitrite formation for NRA estimation (Herrero et al. 1981; Ramos et al. 1982; Guerrero 1985; Larsson et al. 1985). The term "*in vitro*" used here is consistent with its catholic application to NRA assays performed on cell extracts (among others Evans & Nason 1953; Hageman & Hucklesby 1971; Jones et al. 1976; Hageman & Reed 1980; Neyra et al. 1980; Ingemarsson 1987; Kaiser et al. 1992; Köhler et al. 1992).

Numerous difficulties are encountered when crude enzyme extractions are performed in order to obtain undenatured, active NR for the *in vitro* assay (Wray & Fido 1990). Various forms of NR inhibitory proteins have been reported in higher plant species, including maize (Aslam 1977; Yamada et al. 1980b; Solomonson et al. 1984), rice (Yamada et al. 1980a; Ramarao et al. 1981; Leong & Shen 1982; Solomonson et al. 1984), soybean (Jolly & Tolbert 1978), spinach (Maki et al. 1987; Yoshimura et al. 1992) and wheat (Wallace 1978; Sherrard et al. 1979; Ramarao et al. 1981). Most of the controversy regarding the physiological action of NR inhibitors (Ramarao et al. 1981) has been resolved by the elucidation of different types of inhibitors, viz. those that inactivate NR by reversible or irreversible binding (Jolly & Tolbert 1978; Yamada et al. 1980a, b; Solomonson et al. 1984), and those that have proteolytic activity (Wallace 1978; Solomonson et al. 1984). Although details of the *in vivo* significance of these inactivating proteins and proteases or their specificity for NR are not yet clear (Ramarao et al. 1981; Huffaker 1982), their presence does exert a significant influence on NR stability during extraction (Beevers & Hageman 1980; Hageman & Reed 1980; Campbell & Smarrelli 1986).

In addition to NR inhibitory proteins, plant tissues also contain phenolic compounds and tannins which can affect enzyme stability and hence activity (Ibrahim & Cavia 1975; Rhodes 1977; Beevers & Hageman 1980; Wetter 1984). Normally located in the vacuole, tannins, phenolics and phenolase are mixed with proteins during enzyme extraction. Tannins are capable of linking to proteins through hydrogen bonding or covalent linking, thereby rendering enzymes inactive (Rhodes 1977). Phenolics are hydrolysed by phenolase to form quinones, which are very reactive oxidising agents that cause protein precipitation and enzymic browning (Rhodes 1977) or the oxidisation of essential sulphydryl groups (Beevers & Hageman 1980).

A wide variety of protectants have been used during NR extraction procedures to overcome the problems of inactivator proteins, proteases and reactive plant chemicals (Table 2.1). There are several main types of protectants: metal chelators such as EDTA and EGTA minimise NR inhibition by chelating metal ions, whereas DIECA inhibits phenolase by chelating copper. DIECA is a thiol compound that can react with quinones to form inert products. Thiol compounds such as cysteine, GSH, ME and DTT inhibit phenolase activity and prevent the oxidation of essential sulphydryl groups of NR. Protease inhibitors can be specific for serine proteases (PMSF, chymostatin), thiol proteases (leupeptin), endoproteases (α-macroglobulin) and others (p-aminobenzamide, TLCK, antipain and pepstatin). Thiol proteases seem to be more effective in stabilising NR than serine proteases (Wray & Kirk 1981; Ingemarsson 1987), although chymostatin has proved particularly useful in maize root extracts (Long & Oaks 1990). Polymeric agents like PVP and Polyclar AT absorb tannins and phenolics, while inert proteins like BSA and casein are thought to protect NR from inactivators and proteases, and may prevent binding of NR to various organelles. Proteins such as peptone, gelatin and haemoglobin appear to be less effective than BSA and casein in preventing NR degradation (Schrader et al. 1974a). Cofactors FAD and molybdenum protect NR from proteases and have a stabilising effect. Other protectants that have been used include dithionite, a reducing agent that inhibits phenolase, mannitol, an osmoticum that minimises organelle disruption during extraction, and sucrose which may act as an osmoticum, although it has been shown to induce rapid *de novo* synthesis of NR mRNA (Cheng et al. 1992).

Multiple additions of protective substances for NR extraction are common (Wray & Fido 1990). However, it is apparent from the literature that no single compound or combination of compounds is applicable to all plant species. This is not unexpected considering the wide differences in plant phenolic and protease composition between species (Rhodes 1977). BSA was particularly effective at protecting NRA in *Nicotiana tabacum* (tobacco) leaves (Hageman & Reed 1980), and yet had no beneficial effect with maize roots (Aslam & Oaks 1975) or soybean seedlings (Hageman & Reed 1980). Cysteine was not beneficial to NR extractability in etiolated barley (Remmler & Campbell 1986). Furthermore, within a given species, age also may have a marked effect on inhibitory substances and NR stability (Schrader et al. 1974a, b; Rhodes 1977; Brown et al. 1981; Kenis et al. 1992), and younger plant tissue may not benefit from stabilising factors that enhance NRA in older tissue (eg. casein in soybean extractions - Santoro & Magalhaes 1983). It is therefore important that optimal extraction components be established for specific plant systems.

Table 2.1: Protectants employed during NR protein extraction.

NR protectants employed for *in vitro* assay extraction and purification studies are listed. The percentage number of publications from a total of 104 surveyed that use each protectant and selected examples are given.

Type	%	References Examples
1. Metal chelators		
- DIECA[a]	1	Lenee & Chupeau 1989.
- EDTA	77	Klepper et al. 1971; Aslam & Oaks 1975; Wray & Kirk 1980; Campbell et al. 1987; Curtis & Smarrelli 1987; Amory & Lips 1988; Rajasekhar et al. 1988; Kenis et al. 1992; Lu et al. 1992.
- EGTA	3	Redinbaugh & Campbell 1985; Trinity & Filner 1991.
2. Thiol compounds		
- cysteine	48	Ferrari & Varner 1969; Aslam 1977; Jordan & Fletcher 1980; Funkhauser & Garay 1981; Santoro & Magalhaes 1983; Timpo & Neyra 1983; Oaks et al. 1988; Wray & Filner 1991; Friemann et al. 1992.
- DTT	22	Radin & Trelease 1976; Blahova & Segeta 1980; Dailey et al. 1982; Campbell & Wray 1983; Davies & Ross 1985; Hoarau et al. 1991; Riens & Heldt 1992.
- GSH	3	Jones et al. 1976; Hageman & Reed 1980; Campbell et al 1987.
- ME	8	Smith & Thompson 1971; Lillo 1983, 1984; Nakagawa et al. 1986; Baijal & Sane 1988; Galangau et al. 1988; Lenee & Chupeau 1989.
3. Protease inhibitors		
- antipain	2	Somers et al. 1983; Melzer et al. 1989.
- chymostatin	2	Long & Oaks 1990; King et al. 1992.
- leupeptin	18	Wray & Kirk 1981; Campbell & Wray 1983; Fido & Notton 1984; Kuo et al. 1984; Börner et al. 1986; Cherel et al. 1986; Ingemarsson 1987; Kaiser & Spill 1991; Labrie et al. 1991.
- α-macroglobulin	1	Ingemarsson 1987.
- p-aminobenzamide	1	Redinbaugh & Campbell 1985.
- pepstatin	2	Somers et al. 1983; Labrie et al. 1991.
- PMSF	8	Somers et al. 1983; Redinbaugh & Campbell 1985; Oaks et al. 1988; Nato et al. 1990; Fido 1991; Padidam et al. 1991.
- TLCK	1	Redinbaugh & Campbell 1985.

Table 2.1 (cont.)

4. Polymeric agents		
- BSA	1	Schrader et al. 1974a, b; Gallagher et al. 1980; Hageman & Reed 1980; Heath-Pagliuso et al. 1984; Davies & Ross 1985; Brunswick & Cresswell 1986a; Oaks 1987.
- casein	13	Jordan & Fletcher 1980; Peshkova & Khavkin 1980; Aryan et al. 1983; Voronova et al. 1983; Lillo 1984; Campbell & Remmler 1986; Garate et al. 1989.
- Polyclar AT	7	Streeter & Bosler 1972; Orihuel-Iranzo & Campbell 1980; Cherel et al. 1986; Curtis & Smarrelli 1987; Lenee & Chupeau 1989; Kenis et al. 1992.
- PVP	6	Leidi & Gomez 1985; Kenis & Campbell 1986; Remmler & Campbell 1986; Takio 1987.
5. Cofactors		
- FAD	27	Scholl et al. 1974; Dailey et al. 1982; Brown et al. 1986; Hoarau et al. 1986; Fido 1987; Dean & Harper 1988; de la Haba et al. 1988.
- molybdate	7	Campbell & Wray 1983; Somers et al. 1983; Brunswick & Cresswell 1986a; Campbell et al. 1987; Padidam et al. 1991; Friemann et al. 1992.
6. Miscellaneous		
- dithionite	1	Hoarau et al. 1986.
- glycerol	2	Lillo 1983; Baijal & Sane 1988.
- mannitol	1	Oaks 1979.
- methanol	1	Oaks et al. 1988.
- sucrose	2	Nato et al. 1990; Hoarau et al. 1991.

Note: a) Abbreviations: BSA - bovine serum albumin; DIECA - diethyldithiocarbamate; DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; EGTA - ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; GSH - reduced glutathione; ME - β-mercaptoethanol; PMSF - phenylmethylsufonyl fluoride; Polyclar AT - insoluble PVP; PVP - polyvinylpyrrolidone; TLCK - Nα-p-tosyl-L-lysine chloromethyl ketone.

Once a crude enzyme extract is obtained, *in vitro* NRA is measured usually by the amount of product (nitrite) formed under saturating substrate (nitrate) and reductant (NADH) concentrations, thereby quantifying maximal active enzyme present (Hageman & Hucklesby 1971; Hageman & Reed 1980; Davies & Ross 1985; Guerrero 1985; Hoarau et al. 1991). Nitrite, rather than nitrate, quantification is preferred because the colorimetric assay for nitrite is relatively easy and very sensitive (Scholl et al. 1974). However, the colorimetric nitrite assay is affected by certain interfering factors. Thiol protectants used during extraction (eg. DTT, cysteine, GSH) have been shown to interfere with the nitrite assay, with no treatment examined alleviating this effect (Brunswick & Cresswell 1986b). Pyridine nucleotides, especially NADH, interfere with colour development in the concentration range usually employed for nitrite calibration curves (Hageman & Hucklesby 1971; Scholl et al. 1974; Hageman & Reed 1980; Brunswick & Cresswell 1986b; Schneideler & Ninnemann 1986). A postassay treatment involving the use of phenazine methosulfate (PMS) and/or zinc acetate is effective in alleviating NADH interference (Scholl et al. 1974; Brunswick & Cresswell 1986b; Schneideler & Ninnemann 1986). Finally, endogenous tissue compounds (mainly tannins) have been found to interfere with the nitrite assay (Hageman & Reed 1980; Brunswick & Cresswell 1986b). Between 7% and 73% interference was found using leaf extracts from maize, *Pisum sativum* (pea), sunflower, soybean and wheat (Brunswick & Cresswell 1986b). The interference effect is most likely caused by the coloration of organic material upon addition of sulphuric acid, a component of one of the nitrite assay reagents (Cataldo et al. 1975). A postassay treatment for partial removal of endogenous interference is 5 min boiling of the extract (Brunswick & Cresswell 1986b). With the correct precautions (dependent on plant species and age), stoichiometry of nitrate loss and nitrite formation can be achieved for the *in vitro* NR assay (Brunswick & Cresswell 1986a).

In addition to the physiological reaction catalysed by NR (reduction of NADH and nitrate to form nitrite), the enzyme possesses several partial activities that are useful for *in vitro* analysis (Guerrero et al. 1981; Solomonson & Barber 1990; Wray & Fido 1990), even though they may not play a role *in vivo* (Wilkinson & Crawford 1991). Partial reactions are of two types: (1) diaphorase activity transfers electrons from NADH to artificial acceptors and is independent of NR nitrate binding site; and (2) nitrate reducing activity transfers electrons from artificial donors to nitrate to form nitrite, and is independent of NR NADH binding site (Wray & Fido 1990). Artificial electron acceptors used for partial NRA assays include ferricyanide, cytochrome c and dichlorophenolindophenol, while electron donors include reduced flavin mononucleotide, methylviologen (MV) and various bromophenol blue (BPB)

analogues (Guerrero et al. 1985; Hoarau et al. 1986; Solomonson & Barber 1990). Studies on the electron donor BPB and its analogues have revealed that NRs from different plant species have different kinetic characteristics and electron donor sites, and possibly different structural states (Campbell 1986; Hoarau et al. 1986). Despite this variability, artificial electron donors may give higher NRA rates than NADH (Ingemarsson 1987; Solomonson & Barber 1990), with BPB values up to 15-fold higher (Hoarau et al. 1986). However, NRA rates using some artificial donors or acceptors are not consistently higher or lower: MV:NR compared to NADH:NR was lower in cultured tobacco cells (Hoarau et al. 1986), similar in squash extracts (Campbell 1987) and higher in *Lemna gibba* cultures (Ingemarsson 1987). The use of *in vitro* NR partial activities can be beneficial for the delineation of antigenic sites and description of other NR structural features (Campbell 1986). Unfortunately, partial activity assays may not be appropriate when attempting to determine actual NRA rates.

In view of the precautions necessary to avoid NR degradation during extraction and incubation of the *in vitro* NRA assay, this technique is regarded by some as time-consuming and difficult (Klepper et al. 1971; Corzo & Niell 1991). If appropriate care is taken to avoid enzyme denaturation, then the optimal conditions of the *in vitro* assay may overestimate physiological or calculated reduction rates (Jordan & Fletcher 1980; Naik et al. 1982; Ingemarsson et al. 1987a). An alternative method is the *in vivo* assay, which avoids the problem of extraction, and has been found in some studies to provide a good quantitative approximation of actual NRA and reduced nitrogen status (Brunetti & Hageman 1976; Nicholas et al. 1976a; Breteler & Hänisch ten Cate 1980; Naik et al. 1982; Soussana et al. 1989). Tissue intactness is maintained during the *in vivo* assay, and therefore NRA will depend on cell metabolic status (Lillo 1983; Davies & Ross 1985), unless additional substrates are provided. Various additives such as nitrate, NADH and surfactants, often vacuum-infiltrated into tissue, have been found to enhance *in vivo* NRA (Hageman & Hucklesby 1971; Jaworski 1971; Mann et al. 1979; Yoneyama 1981; Lawrence & Herrick 1982; Lillo 1983). However, the use of these additives is not advisable if NRA values closer to physiological levels are sought (Breteler & Hänisch ten Cate 1980; Soussana et al. 1989).

One of the main effects of surfactants on the *in vivo* assay may be alteration of cellular nitrate compartments (Heuer & Plaut 1978; Soussana et al. 1989). It is widely accepted that nitrate exists in two separate cellular pools (Heimer & Filner 1971; Srivastava 1980; Guerrero et al. 1981; Huffaker 1982; Oaks & Hirel 1985; King et

al. 1992), termed the storage and metabolic pools (Ferrari et al. 1973). The majority of stored nitrate resides in vacuoles (Martinoia et al. 1981; Granstedt & Huffaker 1982), with entry into the cytosol (ie. the metabolic pool) controlled by nitrate transport permeases in the tonoplast (Wray 1988; Stulen et al. 1990; Miller et al. 1992) and dependent on factors such as carbohydrates and light (Wallace 1987). Nitrate flux, either from the storage to the metabolic pool or entry into cells, is reportedly more critical than total cellular nitrate content in determining NRA (Heimer & Filner 1971; Shaner & Boyer 1976a, b), although correlation between total nitrate and NRA has been observed (Santoro & Magalhaes 1983). However, the ability to draw on stored nitrate for metabolic use in short-term experiments is species specific (Soussana et al. 1989). If no substrates or surfactants that disrupt organelle intactness are used, the *in vivo* assay might be an appropriate tool for investigating some of the mechanisms regulating NRA *in vivo*.

In vivo assays require dark, anaerobic conditions - darkness is assumed to avoid depletion of nitrite by NiRA, while anaerobiosis is assumed to prevent competition for reducing power between NR and other metabolic pathways in the dark (Jaworski 1971; Johnson 1976; Hageman & Reed 1980; Lillo 1983; Reed et al. 1983; Duke & Duke 1984; Lillo 1984). NiRA is dependent on reduced ferredoxin generated by photosynthetic reactions or, in dark-grown tissues, on a ferredoxin-like protein reduced by an as yet unknown system (Bevers & Hageman 1980; Lee 1980; Oaks & Hirel 1985; Suzuki et al. 1985). For light-adapted tissue, darkness prevents nitrite reduction either completely (Reed & Canvin 1982) or only partially (Mann et al. 1979; Abrol et al. 1983; Soussana et al. 1989). For non-green tissue, obviously darkness will not affect NiRA, although anaerobiosis may inhibit this enzyme. However, the use of darkness raises theoretical problems for light-grown plant tissue, because in the dark a different NADH-generating system for NRA is probably utilised (Reed & Canvin 1982). Even though Abrol et al. (1983) found that different sources of reductant produce comparable NRA rates in the light and dark, other workers have found that *in vivo* assay conditions caused energy depletion, contributing to NRA loss (Nicholas et al. 1976b). Theoretical justification of the use of darkness to provide reductants for the *in vivo* assay to measure physiological NRA remains uncertain. From another perspective, complications with respect to the use of dark conditions have been revealed by recent papers that demonstrate how NR is rapidly modulated in a light/dark transition by phosphorylation or inactivator proteins (Kaiser & Brendle-Behnisch 1991; Huber et al. 1992b, c; Kaiser et al. 1992; Riens & Heldt 1992).

Anaerobiosis during *in vivo* assays is thought to be required primarily to eliminate competition between nitrate and oxygen for reductant (Atkins & Canvin 1975; Subbalakshmi et al. 1979; Sherrard & Hageman 1980), although it has been suggested recently that anaerobiosis may enhance NRA compared with dark aerobic conditions because NR is activated by the decrease of ATP and increase in AMP associated with anaerobiosis (Kaiser et al. 1992). Oxygen is a better competitor for electrons from glycolysis than NR in the dark (Atkins & Canvin 1975), with less than 1% of air oxygen levels completely preventing dark nitrite formation (Sawhney et al. 1978b). Even in non-photosynthetic tissue, inhibitors of respiration increased nitrite accumulation (Lee 1979). The inhibition of an oxygen effect on NRA by ioxynil and 2,4-dinitrophenyl without affecting respiration rates suggests another possible need for anaerobiosis, ie. to prevent oxygen suppression of NR directly (Mann et al. 1979). However, Mann et al. (1979) have warned that results from inhibitors studies should be viewed with caution because of possible effects of the inhibitors on membranes and therefore nitrate availability. Despite these concerns about the use of anaerobiosis to prevent competition for reducing power, some workers have found that supply of reductant for NRA was not limiting under *in vivo* assay conditions (King et al. 1992).

Anaerobiosis has been shown to prevent NiRA (Glass et al. 1992), although not completely in some cases (Ferrari & Varner 1971; Hageman & Reed 1980; Dry et al. 1981; Gray & Cresswell 1984). It has been postulated that NiRA decline under N_2 may be due to a diversion of glucose-6-phosphate from the pentose phosphate pathway to glycolysis brought about by phosphofructikinase under decreased ATP levels (the Pasteur effect), resulting in lower NADPH concentrations for production of reducing power (Dry et al. 1981). The requirement for anaerobiosis is not universal, however, as comparable rates of NRA in air and N_2 have been observed (Radin 1973; Canvin & Atkins 1974; Maurino et al. 1986). These discrepancies may be resolved by examining the effect of alcohol surfactants and other additives (Soussana et al. 1989).

Non-stoichiometry of nitrate utilisation and nitrite formation during the *in vivo* assay could occur because of nitrogen oxide evolution (Brunswick and Cresswell 1986b), as has been observed in soybean (Harper 1981). Studies on soybean employing ^{15}N-nitrate indicated that acetaldehyde oxime was the main byproduct of the *in vivo* assay, in negligible amounts that did not affect the reliability of the assay (Mulvaney & Hageman 1984). Further work has suggested that nitrite can be reduced to form gaseous NO and NO_2 during the *in vivo* assay by the activity of the constitutive

NAD(P)H-NR in soybean (Dean & Harper 1988), and that this problem is not relevant to other plant species that have other NR forms (Campbell 1989; Soussana et al. 1989). Another limiting factor for achieving stoichiometry in the *in vivo* assay is the interference of endogenous plant compounds with the colorimetric assay for nitrite (Brunswick and Cresswell 1986b), although a boiling treatment will alleviate some of this interference, as discussed previously. Moreover, the *in vivo* assay has been criticised because modifications of nitrogen assimilation and amino acid formation occur in dark, anaerobic conditions (Yoneyama 1981). As with *in vitro* assays, results from *in vivo* assays should be interpreted with caution if physiological NRA values are sought (Radin 1973; Harper 1981).

Another method for NRA quantification, commonly used for blue green algae, is the *in situ* assay (Herrero et al. 1981; Guerrero 1985; Larsson et al. 1985). The *in situ* assay normally employs toluene permeabilisation to allow substrate to enter the cytoplasm, and usually measures partial NRA in the presence of an artificial electron donor like MV or benzyl viologen (Ramos et al. 1982; Larsson et al. 1985). As when used for the *in vitro* assay, MV:NRA rates are often higher than NADH:NRA (Campbell & Smarrelli 1986; Ingemarsson 1987). Dithionite generates the radical cation of MV, which then acts as an electron donor to NR at the molybdenum site, while vortexing in air terminates the reaction by oxidising dithionite and MV (Senn et al. 1976; Campbell & Smarrelli 1986). A formaldehyde treatment prevents dithionite byproducts (bisulfite and thiosufate) from interfering with the nitrite colorimetric assay (Senn et al. 1976), but usually endogenous plant interference factors are not eliminated. The *in situ* assay may not reveal actual NRA because cell intactness is disrupted, partial NRA is measured, and possibly nitrite formation is not quantified accurately due to interference. Furthermore, MV is an efficient artificial electron donor for NiRA (Guerrero et al. 1981), but often no preventative steps are employed to prevent possible nitrite loss, and therefore the assay might underestimate NRA.

The *in situ* assay, like the *in vitro* and *in vivo* assays, has serious theoretical limitations which prevent researchers from establishing actual NRA with certainty. Nevertheless, NRA measurements using each of these methods have in limited cases been able to provide an indication of reduced nitrogen status and plant growth (Brunetti & Hageman 1976; Beevers & Hageman 1980; Srivastava 1980; Guerrero et al. 1981; Huffaker 1982; Naik et al. 1982; Sherrard et al. 1986). NRA assays have been beneficial for the elucidation of NR structure and regulatory mechanisms (Redinbaugh & Campbell 1985; Campbell 1986; Campbell & Kinghorn 1990; Huber

et al. 1992c; Kaiser et al. 1992). With different information provided by each assay, interpretation of experiments has been facilitated by the use of more than one assay in some cases (Blahova & Segeta 1980; Lillo 1983; Davies & Ross 1985; Hoarau et al. 1991). Therefore, even though NR is the best characterised enzyme of the nitrogen assimilation pathway (Wray 1988; Solomonson & Barber 1990), certain methodological problems relating to NRA quantification still require solutions.

2.1.2 Qualitative and quantitative NRP analysis

Analysis of enzymes and other proteins requires extraction procedures that ensure maximum yield and minimum denaturation (Wetter 1984). Hence, common crude homogenisation procedures for quantification or analysis of NR by Western blotting or ELISA (enzyme-linked immunosorbent assay) utilise protective agents as described for the *in vitro* NRA assay previously. Alternatively, acetone powders can be employed to achieve high yields of concentrated undenatured enzymes in extracts free of pigments and low molecular weight contaminants (Wetter 1984). Plant tissues which have high water and low protein content, particularly *in vitro* cultures, are suited to acetone extractions, which obviate the need for high ionic strength extraction buffers (Ibrahim & Cavia 1975). However, to obtain sufficient quantities of homogeneous protein needed for characterisation studies, more sophisticated purification techniques are required.

Existing methods for protein purification, some of which will be discussed here, have been recently reviewed (Weselake and Jain 1992). Immunoaffinity chromatography (IAC) uses antibodies against the protein of interest immobilised covalently on an insoluble support (Weselake & Jain 1992). Originally, polyclonal antibodies were used for NR purification by IAC and yields were low as the enzyme was difficult to release (Notton 1989). Subsequently, monoclonal antibodies (that recognise a specific antigenic site) proved to be very successful: IAC was utilised for NR purification from a crude extract by one-step chromatography on a column of immobilised monoclonal antibodies to obtain a 1500-fold enriched spinach NR fraction (Fido 1987). Moreover, partially purified NR was successfully separated from nicked subunits and other contaminants using IAC (Fido & Notton 1984; Fido 1991), while Sueyoshi et al. (1989) used IAC to isolate *in vivo* degradation products of spinach NR. Another technique, immobilised metal ion affinity chromatography (IMAC), relies on coordination complex formation between surface amino acids and immobilised metal ions (Weselake & Jain 1992). IMAC has been used as a second chromatographic step with a zinc chelate affinity column to purify squash NR 3 300-

fold (Redinbaugh & Campbell 1983, 1985). Other techniques used during NR purification include blue Sepharose chromatography (Orihuel-Iranzo & Campbell 1980; Campbell & Remmler 1986; Nakagawa et al. 1986; Shiraishi et al. 1991), blue dextran Sepharose chromatography (Sherrard & Dalling 1979), hydroxylapatite chromatography (Fido & Notton 1984; Shiraishi et al. 1991) and polyacrylamide gel electrophoresis, followed by band excision (Somers et al. 1983). A survey of the literature has revealed that techniques such as subunit affinity chromatography, used to purify haemoglobins and proteins (Schuder et al. 1979; Chiancone & Winzor 1986), and affinity labelling, which was used to separate auxin-binding proteins (Jones & Venis 1989), have not been applied to NR purification yet.

Pure, undegraded NR is essential for accurate immunological studies, because trace contaminants can be very good immunogens that generate large amounts of non-specific antibodies (Notton 1989). Immunological studies using NR antibodies have been key tools for probing the molecular structure, function and phylogeny of NR (Campbell 1987; Notton 1989). Using techniques such as direct binding assays (Notton et al. 1985; Cherel et al. 1986), rocket immunoelectrophoresis (Snapp et al. 1984), Ouchterlony double immunodiffusion (Nakagawa et al. 1986) and inhibition studies (Snapp et al. 1984; Notton et al. 1985; Nakagawa et al. 1986; Notton et al. 1989), higher plant NRs have been shown to be an homologous family with several antigenic determinants in common (Campbell & Smarrelli 1986). Additionally, NR antibodies have been used for IAC purification as described earlier, and monoclonal NR antibodies are used routinely for the solid-phase ELISA assay for NR (Notton et al. 1985; Campbell & Remmler 1986; Campbell & Smarrelli 1986; Cherel et al. 1986; Maki et al. 1986; Notton et al. 1987; Notton et al. 1988). Binding sites for NADH, MV and BPB have been differentiated using a variety of monoclonal antibodies (Notton et al. 1985; Hoarau et al. 1986), while putative plasma membrane and cytosolic forms of NR were distinguished using monoclonal antibodies (Ward et al. 1988). Also, purified NR was used to screen expression libraries for the isolation of NR cDNA (Cheng et al. 1986; Calza et al. 1987; Gowri & Campbell 1989; Shiraishi et al. 1991). Undoubtedly, immunological techniques made possible by the availability of purified NR have facilitated the elucidation of NR structure and function.

Non-specific protein quantification procedures are essential for most protein studies. Methods for determining protein concentrations include the Dumus and Kjeldahl assays, that provide a quantitative estimate of nitrogen, the Folin phenol method, which depends on colour development of aromatic amino acids, and vari-

ous protein dye-binding assays (Wetter 1984). The widely used Folin assay (Lowry et al. 1951) is subject to interference from potassium and magnesium ions, EDTA, Tris buffer, thiol reagents and carbohydrates (Bradford 1976). Hence suitable controls are required for protein quantification by the Folin assay, especially for plant tissues, although the addition of a precipitation step which separates proteins from endogenous and buffer interfering compounds may minimise the problem (Wetter 1984). Alternatively, the Bradford assay that relies on the conversion of Coomassie Brilliant Blue G-250 dye from red to blue upon binding to proteins, and is apparently less subject to interference yet more sensitive than the Folin assay, can be used (Bradford 1976). The choice of assay eventually depends on an experimenter's needs in terms of desired accuracy and speed of assay, and the number of samples (Wetter 1984).

Electrophoretic techniques have become very useful in solving many protein problems (Wetter & Dyck 1983). Supporting media for electrophoresis include paper, cellulose acetate, silica gel, alumina, cellulose, agarose, starch and polyacrylamide (Hames 1981). While paper and thin-layer materials are inert, agarose, starch and polyacrylamide gels may actively participate in separation, as well as preventing convection and minimising diffusion (Hames 1981). Although starch gels have been used to characterise NR isozymes (Heath-Pagliuso et al. 1984), most analytical electrophoretic work on proteins such as NR is performed using polyacrylamide gels, which are composed of chains of polymerised acrylamide, crosslinked by N,N'-methylene bisacrylamide using N,N,N',N'-tetramethylethylenediamine (TEMED) as polymerisation catalyst (Towbin et al. 1979; Hames 1981; Sinclair & Rickwood 1981; Sambrook et al. 1989). The anionic detergent sodium dodecyl sulphate (SDS) is used widely in combination with a reducing agent to dissociate proteins and provide overall negative charge before loading them onto a polyacrylamide gel. The amount of bound SDS is usually proportional to the molecular weight of any polypeptide (glycoproteins are exceptions), and therefore SDS-polypeptide complexes will migrate through an electrical field in accordance with the molecular weight of the polypeptide (Hames 1981; Sambrook et al. 1989). Variations in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) techniques utilise different buffer or gel systems, and can be used to determine molecular weight or isoelectric points of protein polypeptides (Hames 1981; Wetter & Dyck 1983). The most common SDS-PAGE technique operates via a dissociating discontinuous system, ie. two gel layers (a stacking gel of nonrestrictive large pore size, and a separating or resolving gel) and two separated SDS buffer systems (Laemmli 1970). The Laemmli method, originally used to identify novel proteins in bacteriophage T4 (Laemmli 1970), al-

lows excellent polypeptide resolution because proteins form a narrow stack before entering the separating gel (Hames 1981; Sambrook et al. 1989). SDS-PAGE has been used extensively for NRP work, both on its own and in conjunction with Western blotting, as explained in the ensuing discussion.

Protein polypeptides separated by SDS-PAGE can be transferred electrophoretically to nitrocellulose and detected immunologically by Western blotting (Towbin et al. 1979; Burnette 1981). Western blotting is an extremely sensitive analytical technique that under certain conditions allows for specific detection of a polypeptide of interest (Scott et al. 1988; Sambrook et al. 1989). Sensitivity in Western blotting is achieved partly because of the immobilisation of antigen onto nitrocellulose, which means that immunological detection is possible without the formation of an antigen-antibody precipitate (Towbin et al. 1979). In addition to molecular weight determination by Western blotting, the technique can be used to estimate the relative abundance of a particular protein and its state of degradation (Scott et al. 1988). Although transfer of proteins from SDS-PAGE gels to nitrocellulose was originally not quantitative (Towbin et al. 1979), subsequent adaptations have improved the technique to obtain essentially complete and quantitative elution (Burnette 1981). Immobilised proteins are detected using polyclonal or monoclonal antibodies normally raised in rabbit, with the resultant antigen-antibody complex most commonly visualised using a secondary antibody against the primary antibody (eg. anti-rabbit IgG) linked to small enzymes or tagged with radioisotopes (Towbin et al. 1979; Scott et al. 1988; Sambrook et al. 1989). Antibody-conjugated enzymes employed for Western detection include alkaline phosphatase (Blake et al. 1984; Knecht & Dimond 1984; Scott et al. 1988; Sambrook et al. 1989) and horseradish peroxidase (Towbin et al. 1979; Scott et al. 1988; Sambrook et al. 1989). The sensitivity provided by Western blotting is indispensable for analysis of NRP, which has been estimated to constitute 0.0007 to 0.05% of total extractable protein (Campbell & Smarrelli 1986).

Western blotting techniques have been used extensively to characterise NR structure and regulation. Molecular weight determination of NR from different higher plant species indicates that NR has a molecular weight of about 230 kD, with a subunit size of about 115 kD (Table 2.2). Molecular weight estimation values from SDS-PAGE are roughly comparable to those deduced from amino acid sequences derived from NR clones (Table 2.2). NR is widely regarded as a homodimer (Guerrero et al. 1981; Campbell 1988; Caboche & Rouze 1990; Warner & Kleinhofs 1992), although evidence for this is not yet unequivocal (Wray 1988). NR domain

Table 2.2: Molecular weight values determined for higher plant NADH-NR.

Plant	(Molecular wt, kD) Holo-NR	Apo-NR	Reference
General			
	500-600	-	Bevers & Hageman 1969
	200-300	-	Guerrero 1985
	200-250	-	Kleinhofs et al. 1985
	-	105-115	Campbell 1988
	-	+/-110	Campbell 1989
	-	+/-100	Campbell & Kinghorn 1990
	-	100-120	Caboche & Rouze 1990
Specific species			
Arabidopsis thaliana	-	103*	Crawford et al. 1988
Cucurbita maxima	230	44,75	Redinbaugh et al. 1982
	230	115	Redinbaugh & Campbell 1985
	-	115	Campbell & Smarrelli 1986
	-	115	Langendorfer et al. 1988
	-	115	Campbell 1989
	-	110	Crawford & Davis 1989
Hordeum vulgare	220	110	Kuo et al. 1982
	-	110	Cheng et al. 1986
Nicotiana plumbaginifolia	-	110	Calza et al. 1987
	-	99,107	Vincent & Caboche 1991
N. tabacum	200	110	Mendel & Müller 1980
	-	83-94	Hoarau et al. 1986
	-	102*	Vaucheret et al. 1989a, b
	-	+/-100	Hoarau et al. 1991
Phaseus vulgaris	-	99.1*	Hoff & Henningsen 1990
Spinacea oleracea	-	110-115	Fido 1987
	-	105-110	Fido 1991
	-	120	Shiraishi et al. 1991
Zea mays	160	-	Hageman & Hucklesby 1971
	-	115	Remmler & Campbell 1985
	-	115	Campbell & Remmler 1986
	-	110	Campbell 1989

Note: * = deduced from amino acid sequence.

structure and associated activity have been examined using Western techniques (Campbell & Wray 1983; Kubo et al. 1988; Notton et al. 1990; Shiraishi et al. 1991), and in conjunction with ELISA, Westerns have been used for the quantification of NRP in a variety of studies on NR regulation (Galangau et al. 1988; Langendorfer et al. 1988; Martino & Smarrelli 1989; Deng et al. 1990; Lillo 1991; Kenis et al. 1992).

2.1.3 Isolation, detection and quantification of NR mRNA

RNA molecules are the intermediates of gene expression (Slater 1991). Of all RNA types, ribosomal RNA (rRNA) constitutes from 75-85% whereas mRNA, the molecules responsible for directing the correct assembly of polypeptide amino acids, constitutes only 1-5% (Sambrook et al. 1989; Slater 1991). Analysis of gene expression at the transcriptional level requires extraction procedures that yield undegraded and biologically active mRNA, uncontaminated with other macromolecules (Cox & Goldberg 1988). A major problem is that plants have high levels of endogenous RNases, necessitating employment of RNase inhibitors such as ribonucleoside vanadyl complex, EDTA and EGTA, detergents like SDS and Nonidet-P40, and strong denaturants such as gaunidium thiocyanate (Cox & Goldberg 1988; Slater 1991). Common methods for total RNA isolation use detergent and phenol extraction (de Vries et al. 1989; Nagy et al. 1989; Sambrook et al. 1989; Verwoerd et al. 1989) and guanidine thiocyanate extraction (Chirgwin et al. 1979; Cox and Goldberg 1988; Slater 1991).

SDS/phenol extraction methods have been used successfully to extract RNA for NR mRNA identification (Yamazaki et al. 1986; Smarrelli et al. 1987; Faure et al. 1991; Labrie et al. 1991). However, SDS and phenol alone do not denature RNases sufficiently to prevent loss of yield (Chirgwin et al. 1979). The denaturant guanidium thiocyanate is capable of completely denaturing RNase activity, and therefore has been employed in an RNA extraction procedure which originally was developed using ribonuclease-rich pancreatic cells (Chirgwin et al. 1979). Modifications of the guanidine thiocyanate method specifically for plants have a good reputation for yielding high quantities of total RNA (Slater 1991), and this method has been well utilised for NR analyses (Cheng et al. 1986; Commere et al. 1986; Deng et al. 1989b; Melzer et al. 1989; Pouteau et al. 1989; Deng et al. 1990; Lillo 1991; Shiraishi et al. 1991; Cheng et al. 1992; Kamachi et al. 1992). Extractions employing another guanidium compound, guanidium hydrochloride (Logemann et al. 1987), have been utilised by NR workers (Gowri & Campbell 1989; Kenis et al. 1992). These extrac-

tion procedures often employ caesium chloride or sucrose gradients to purify RNA from polysaccharides, pectins or polyphenols (Nagy et al. 1989). Nucleic acids can be separated from endogenous plant contaminants using anion-exchange resins (Do & Adams 1991). Less prevalent RNA extraction methods include the protease K/phenol method, which relies on a protease from the fungus *Tritirachium album limber* to digest cellular protein (Slater 1991). Recently, RNA has been purified using an anti-RNA monoclonal antibody that was isolated from a mouse with an autoimmune disease (Reines 1991). This new method is limited currently to partially purified systems that are free of RNases (Reines 1991), but could become more useful for biochemical analysis of plant RNA and RNA-complexes.

Alternatively, mRNAs, the majority of which have 3' poly(adenylic acid) tails, can be purified selectively on oligo(dT)-cellulose chromatography after total RNA extraction (Slater 1991). A one-step procedure to purify mRNA using magnetic oligo(dT) microspheres decrease extraction time but also yield (Jakobsen et al. 1990). The choice of RNA extraction protocol from the different types is largely an empirical one, but does depend on the tissue used and the objectives of each particular experiment (Slater 1991).

Analysis of specific mRNAs is possible using a number of blotting and/or hybridisation techniques, including Northern blotting, dot/slot blotting, *in vitro* transcription studies, *in situ* hybridisation and RNA titration. Northern blotting identifies RNA molecules that have been separated by gel electrophoresis, and can be used to estimate both size and amount of a specific mRNA species (Slater 1991). Fractionated RNA is denatured with glyoxal, dimethyl sulfoxide, formamide or methyl mercuric hydroxide prior to transfer to a solid support by capillary action, electroblotting or vacuum (Sambrook et al. 1989). RNA is immobilised onto the solid support by drying, heating or UV crosslinking, depending on the support type. Diazobenzyloxymethyl-paper was used for the original Northern procedure (Alwine et al. 1977), but nitrocellulose and nylon are preferred currently because they are easier to use and more sensitive (Thomas 1980; Sambrook et al. 1989; Slater 1991). Specific immobilised RNAs are then detected by hybridisation with known labelled DNA or RNA probes (Nagy et al. 1989; Sambrook et al. 1989; Slater 1991). There are many variations of the Northern blotting method, with choices of gel electrophoresis protocol, transfer membrane, probe type (DNA/RNA/oligonucleotide), type of label (radioactive/non-radioactive), means of labelling (nick translation, oligo-labelling, *in vitro* transcription, synthetic oligonucleotide synthesis) and hybridisation and washing conditions (Slater 1991). These parameters have been

dealt with extensively elsewhere (Burnette 1981; Meinkoth & Wahl 1984; Keller & Manak 1989; Sambrook et al. 1989), and will be discussed selectively.

Double stranded DNA labelling by nick-translation uses *Escherichia coli* DNA polymerase I to add nucleotides to the 3'-hydroxyl terminus of nicks introduced by pancreatic DNase I into probe DNA (Meinkoth & Wahl 1984). This method produces a population of overlapping radioactive fragments, because nicks are introduced at random sites, and these fragments may hybridise with each other before hybridising to the immobilised nucleic acid (Meinkoth & Wahl 1984). Oligo-labelling uses the Klenow fragment of *E. coli* DNA polymerase I to extend random DNA primers complementary to M13-derived single stranded DNA or denatured double stranded DNA probes (Feinberg & Vogelstein 1983). Although some workers consider Klenow to lack the 5'-3' exonuclease activity of DNA polymerase I (Meinkoth & Wahl 1984), exonuclease activity does exist, and needs to be controlled by labelling conditions such as pH (Feinberg & Vogelstein 1983). A well used adaptation of the oligo-labelling procedure allows for probe DNA fractionated in low melting temperature agarose to be labelled efficiently without purification from the agarose (Feinberg & Vogelstein 1984). Recent techniques for rapid separation of DNA from agarose that yield up to 96% recovery (Mukhopadhyay & Roth 1991; Vaux 1992) have been designed to minimise loss if pure DNA is required for labelling. As an alternative, short synthetic oligonucleotide (14-20 bp) synthesis is possible (Meinkoth & Wahl 1984). However, DNA-DNA duplexes are less stable than RNA-DNA duplexes, and therefore RNA probes that are generated by *in vitro* transcription using cloning vectors are sometimes preferred for more sensitive detection (Meinkoth & Wahl 1984).

Slot/dot blots are easier and quicker than Northern blots, as RNA electrophoresis and blotting are avoided (van Helden & Olliver 1987). The slot/dot blot technique can measure the relative abundance, but not size, of specific mRNA species (Sambrook et al. 1989), but is subject to interference from contaminating proteins that compete for binding sites and contribute towards higher background (Meinkoth & Wahl 1984). Various commercially available slot blotting apparatuses make this method more quantitative than hand dot-blots (Keller & Manak 1989). A further variation of the slot blot technique, called cytoplasmic dot hybridisation, does not require extraction and purification of RNA, but cell debris and nuclei are removed in a single step before application of residual RNA to filters (Keller & Manak 1989).

In vitro transcriptional analysis provides a direct measure of gene control by quantifying relative transcription rates of isolated nuclei, whereas blotting methods measure steady-state levels of mRNAs that are subject to degradation (Cox & Goldberg 1988). Transcription analysis (also termed nucleus run-off transcription analysis) is used routinely to study processes regulating gene expression during plant developmental processes, eg. the effect of hormones on gene expression (Hagen et al. 1984; Lu et al. 1990, 1992b). In addition, the technique was employed to investigate diurnal variations in maize leaf NR (Lillo 1991). *In situ* hybridisation techniques are useful for developmental and differentiation studies, as spatial patterns of specific RNA accumulation at the cellular and subcellular levels can be determined (Cox & Goldberg 1988). In comparison, RNA titration techniques use single-stranded probes as tracers to measure the absolute number of copies of particular mRNA species (Cox & Goldberg 1988). An alternative Northern procedure, termed Northern blot mapping, is a useful technique for determining genomic DNA intron and exon structure using cDNA derived from mRNA species (Hamelin 1988). Northern blot mapping avoids the need for separate steps with numerous probes (Hamelin 1988), and will help to elucidate the process of mRNA splicing in plants, about which our knowledge is limited (Slater 1991). These RNA analysis techniques will provide researchers with powerful tools to examine the role of nitrate assimilation during certain fundamental plant developmental and regulatory processes.

Due to the risks and safety precautions required for radioactive labelling techniques, many workers have attempted to develop non-radioactive techniques (Keller & Manak 1989; Düring 1991). Additionally, non-radioactive nucleotide analogue probes have been developed for their potential use in biomedical research (Langer et al. 1981; Tchen et al. 1984). As a result of these efforts, non-radioactive probes are currently as sensitive as their ^{32}P-labelled counterparts at 1-5x10^8 cpm/μg, but are less sensitive when radioactive probes are labelled at 10^9 cpm/μg or greater (Keller & Manak 1989). There are two major groups of non-radioactive labelling techniques, involving the use of enzymatic or chemical procedures. The first feasible enzymatic labelling procedure used a biotin-labelled deoxyribonucleotide triphosphate that could be detected after enzymatic polymerisation into probe DNA with streptavidin conjugated to alkaline phosphatase (Langer et al. 1981). Biotin-11-dUTP is now the most widely used modified nucleotide, with detection by avidin- or streptavidin-enzyme conjugates (Keller & Manak 1989). Amersham's enhanced chemiluminescence (ECL) system, involving labelling of nucleic acid probes with horseradish peroxidase directly or via a hapten (fluorescein-dUTP) followed by detection using luminol oxidation, has been developed for Northern work (Amer-

sham Life Science 7, 1992). Digoxigenin-dUTP, consisting of the mononucleotide dUTP linked via a spacer arm to a steroid hapten digoxigenin and detected using alkaline phosphatase-conjugated anti-digoxigenin, is another commercially available non-radioactive label (Boehringer Mannheim) that has been utilised successfully for Northern work (Düring 1991).

The other group of non-radioactive labelling procedures make use of chemically modified DNA that can be detected readily. The first practical system employed N-acetoxy-N-2-acetylaminofluorene (AAF) and its 7-iodo derivative (IAAF) bound to guanosine residues, and detected by rabbit anti-AAF or anti-IAAF (Tchen et al. 1984). Numerous other chemical modifications to DNA have been successful, including the use of linker arms to attach desired compounds (see Keller & Manak 1989). Direct detection of probes is possible using enzymes conjugated to oligonucleotides (Keller & Manak 1989). An example is alkaline phosphatase linkage to a synthetic oligonucleotide probe using the hydrophobic linker p-phenylene diisothiocyanate (Urdea et al. 1988). As yet, no reports in the literature indicate the use of non-radioactive probes for NR mRNA detection. This may reflect the high sensitivity required for the detection of low abundance mRNA species (0.00005-0.0005% of total RNA) (data from Cheng et al. 1986).

Gene expression analysed by Northern and slot blotting techniques often involves comparison of specific transcript levels. For these studies to be truly quantitative, RNA concentrations must be determined accurately. Because spectrophotometer methods are not always reliable (Toscani et al. 1987), alternative systems were developed to measure internal standard transcripts which supposedly are not affected by experimental conditions. The most widely used method quantifies so-called "house-keeping" genes that were thought to be constitutively expressed in a particular cell line or tissue (Correa-Rotter et al. 1992). However, many of these house-keeping genes have been shown to be subject to biological variation, including 18S RNA, glyceraldehyde-3-phosphate and α- and β-actin (Bernlohr et al. 1984; Correa-Rotter et al. 1992; Tepper et al. 1992). Members of the commonly used α-tubulin family, represented by at least six genes in *Arabidopsis thaliana* (Kopczak et al. 1992), are expressed preferentially in certain tissues, and thus tubulin genes should be referred to as "house-building" genes (Carpenter et al. 1992). A better external control system might be provided by mitochondrial gene expression, which is subjected to less perturbation than normal house-keeping genes (Tepper et al. 1992). The p72 probe constructed from a 264 bp mitochondrial DNA fragment (Tepper et al. 1992) has the added advantage of sensitivity, as there are 10^3-10^4 copies of

mitochondrial DNA in each cell (Bogenhagen & Clayton 1974).

Due to the possible limitations of the external transcript method, however, alternative approaches may be useful. A method developed by Toscani et al. (1987) uses an internal standard of *in vitro*-prepared control cRNA that is added to cell samples before extraction. The cRNA becomes stably incorporated into the RNA content of each sample, and then can be detected and quantified after extraction. This technique is reportedly universally applicable, independent of final RNA concentration, species/cell type or experimental manipulation (Toscani et al. 1987). A less complicated and quicker method requires measurement of ethidium bromide-stained rRNA bands. These rRNA bands can be quantified using densitometer tracings of photographic negatives (Friemann et al. 1992; Kenis et al. 1992), or by employing computer-assisted densitometry techniques (Correa-Rotter et al. 1992).

Most studies on NR mRNA have used house-keeping genes for external standards. DNA or cDNA probes employed include alcohol dehydrogenase (Privalle et al. 1989), acetolactate synthase (Melzer et al. 1989), rRNA (Bowsher et al. 1991), α-tubulin (Crawford et al. 1986; Lu et al. 1990), β-tubulin (Labrie et al. 1990) and the β-subunit of mitochondrial ATP synthase (Faure et al. 1991). Ethidium bromide-stained rRNA bands have been used by some workers (Friemann et al. 1992). Those confused about how to determine accurately relative RNA amounts, either do not do so, or neglect to report their method of choice (Smarrelli et al. 1987; Oelmüller & Briggs 1990; Gowri et al. 1992; Kamachi et al. 1992).

Recombinant DNA techniques have permitted workers to isolate NR cDNA clones for use as probes in gene research. Higher plant or algal cDNA clones for nitrate assimilation genes have been derived in three ways. Firstly, size-weighted poly(A)$^+$ RNA was inserted into expression vectors and expression libraries were screened using purified monoclonal antibodies (after Young & Davis 1983). Immunoselection was used to isolate partial NR cDNA from barley (Cheng et al. 1986), maize (Gowri & Campbell 1989), spinach (Shiraishi et al. 1991) and tobacco (Calza et al. 1987). Secondly, partial NR cDNA clones of one species were used to select NR cDNA clones of another species. Thus *A. thaliana* NR cDNA was isolated using squash NR cDNA (Crawford et al. 1988), *Chlamydomonas reinhardtii* NR cDNA was isolated using a barley NR cDNA probe (Fernandez et al. 1989) and tomato NR cDNA was cloned using tobacco NR cDNA (Daniel-Vedele et al. 1989). In addition, this method was used to obtain total cDNA sequences from the two different NR genes in tobacco (Vaucheret et al. 1989a, b). Finally, synthetic oligonucleotide DNA probes

representing all possible combinations of a short polypeptide amino acid sequence of NiR were used to isolate the complete cDNA of NiR in spinach (Back et al. 1988). NR cDNA probes, in conjunction with methods for RNA analysis outlined above, have been used for the study of NR regulation at the transcriptional level under environmental conditions such as light (Yamazaki et al. 1986; Gowri & Campbell 1989) and nitrogen (Cheng et al. 1986; McClure et al. 1987; Crawford et al. 1988). Furthermore, NR cDNA sequence comparisons between plant species have confirmed similarities deduced from amino acid studies (Daniel-Vedele et al. 1989). The employment of such molecular techniques is indispensable to our understanding the molecular mechanisms that control nitrogen assimilation (Wray 1988).

The application of biochemical and molecular biological techniques has led to a mass of literature on NR (Warner & Kleinhofs 1992). However, the labile NR was purified to homogeneity by Redinbaugh & Campbell (1985) only 30 years after its discovery by Evans and Nason (1953), and the gene for NR was cloned only recently (Cheng et al. 1986; Crawford et al. 1986). Therefore, it is not surprising that, despite the considerable attention focused on NR research (Campbell 1988), many aspects of NR structure and regulation need further characterisation (Caboche & Rouze 1990; Solomonson & Barber 1990; Warner & Kleinhofs 1992). This chapter describes the development of methods suitable for determining NRA, NRP and NR mRNA so that NR regulation during tobacco callus differentiation could be analysed.

2.2 Materials and Methods

2.2.1 Reagents and biological materials

Routine analytical grade biochemical reagents were obtained from Saarchem (Krugersdorp, SA), except for: DTT, NADH & PMSF (Boehringer Mannheim); PMS, FAD, methyl viologen and PVP (Sigma); ME and formaldehyde (Merck); and N-(1-naphthyl) ethylene diamine dihydrochloride (NED) and kinetin (BDH Chemicals). Molecular biology chemicals were from Sigma, except for the following: SDS, BSA, anti-rabbit IgG-alkaline phosphatase, DIG DNA labelling/detection kit, RNA molecular weight markers and all restriction enzymes (Boehringer Mannheim); Rainbow protein molecular weight standards, Hybond-C nitrocellulose, Hyperfilm-MP X-ray film and [α-^{32}P]dTTP (Amersham); and Ultrapure DNA-grade agarose and low melting temperature agarose (Biorad). Photographs were taken on Kodak T-max 400 black/white film and Kodak Kodacolor Gold colour film.

Tobacco seeds (*Nicotiana tabacum* L. var. Samsun) were obtained from Karl Kunert (Laboratoire de Biologie Cellulaire, INRA, Versailles, France). Fresh spinach leaves were purchased from a local supermarket. Spinach polyclonal NR antiserum raised in rabbit was a gift from Ian Prosser (Long Ashton Research Station, Bristol, UK). The pUC9-derived plasmid pBMC102010 (Vaucheret et al. 1989b) was obtained from Michel Caboche (Laboratoire de Biologie Cellulaire, INRA, Versailles, France).

2.2.2 Establishment and maintenance of in vitro cultures

Tobacco plants were germinated from seeds on potting soil under greenhouse conditions with natural daylight. Callus from surface-sterilised tobacco stem and leaf segments was established in darkness at 25^{o}C on induction medium consisting of MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose, 1% agar, 2 mg/l indoleacetic acid (IAA) and 0.5 mg/l kinetin using glass culture tubes of 89 mm (length) x 23 mm (internal diameter). All media were sterilised by autoclaving at 120^{o}C for 25 min. Callus was maintained in darkness by subculturing every 2-3 weeks, not more than 3 times before use.

Tobacco cell suspension cultures were prepared by Felicity Blakeway as follows: after 2-3 weeks of growth, callus was transferred to growth medium containing MS, 3% sucrose, 0.4% Gelrite, and 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D).

Large, friable callus was transferred after 3-4 weeks to liquid growth medium and placed on an orbital shaker for 21 days to generate single cell suspension cultures. Medium was replenished, or cultures were subcultured, every 7-14 days. During the last few days of the exponential growth phase (7-10 days after subculturing), cell suspension cultures were plated onto solid callus induction medium at a volume of 1.0-1.5 ml per tube.

2.2.3 NRA assays

In vivo assay

Callus samples (0.2-0.35 g f.wt) were placed in glass centrifuge tubes with 0.1 ml dH_2O. Tubes were sealed with Suba-seal bungs and maintained in darkness using aluminum foil. The assay was carried out at 25°C with continuous N_2 (gas) flushing, and terminated after 30 min (Figure 2.1) by adding 3-5 ml boiling dH_2O. Accumulated nitrite was quantitatively extracted from the callus by boiling for 30 min (Lyons et al. 1991). Following clearing by centrifugation for 5 min at 3700 rpm using a Beckman GP benchtop centrifuge, nitrite was determined spectrophotometrically by the addition of 1 ml of supernatant to 1 ml of 1% sulfanilamide in 2N HCl and 1 ml of 0.02% NED (Hageman & Reed 1980). After 20 min, absorbance was read at 540 nm using a Beckman DU 7500 spectrophotometer, and nitrite determined from a calibration curve.

In situ assay

Callus (0.2-0.3 g f.wt) was vortexed for 3 min in 1 ml Hepes-KOH solubilisation buffer (25 mM, pH 7.5) containing 250 mM Na_2EDTA, but without toluene (Table 2.3). One ml incubation buffer (100 mM Hepes-KOH, pH 7.5, 20 mM KNO_3, 4 mM methylviologen) was added, and the reaction was initiated with 0.1 ml of 100 mM sodium dithionite in 300 mM Hepes-KOH (pH 7.5). The assay was terminated after 0 or 10 min at 25°C by vortexing for 30 s to reduce excess dithionite. One volume of formaldehyde (0.6% $^{w}/_{v}$) in Hepes-KOH (300 mM, pH 7.5) was added and the mixture was vortexed for 3 min. After clearing by centrifugation for 5 min at 3700 rpm using a Beckman GP centrifuge, the mixture was assayed colorimetrically for nitrite immediately (Hageman & Reed 1980).

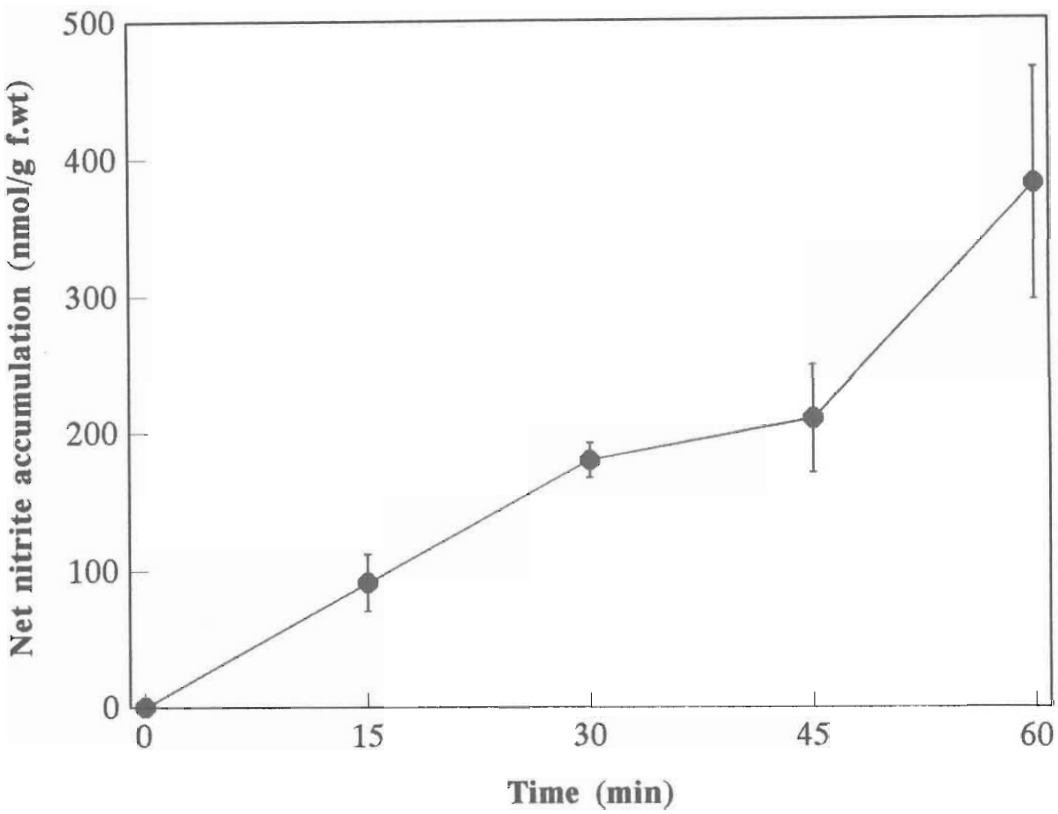

Figure 2.1: Dependence of NRA on time of incubation during the *in vivo* assay.

Tobacco callus (0.23-0.35 g f.wt) was incubated under dark, anaerobic conditions for various periods of time and nitrite accumulation measured. Bars denote standard error of the mean (n = 3).

Table 2.3: Effect of permeabilisation of tobacco callus in the presence of toluene on extractable nitrite measurement during the *in situ* NRA assay.

The *in situ* assay was performed as described in Materials and Methods, except that toluene was included in the permeabilisation buffer as indicated. Toluene had no effect on the nitrite calibration curve (not shown) used to assay nitrite formation after 10 min. Representative results from 3 separate experiments are expressed as mean ± standard error (n = 2). Letters denote significantly different nitrite values ($p<0.05$).

Toluene added (% v/v)	Nitrite extracted (nmol/g f.wt)	
0	70.82 ± 0.435	a
4	61.64 ± 4.38	a
8	43.85 ± 0.330	b

In vitro assay

A crude enzyme extraction was obtained by grinding callus (0.5 g f.wt) at 4°C with mortar and pestle in the presence of acid-washed sand and 2.5 ml potassium phosphate buffer (50 mM, pH 8.5) containing 2% (w/v) BSA. BSA (2% w/v) was found to provide adequate protection to NR (Table 2.4), and did not interfere with the colorimetric assay for nitrite (Figure 2.2). The homogenate was filtered through four layers of muslin cloth and the supernatant collected after centrifugation (3700 rpm) for 10 min at 4°C using a Beckman GPR centrifuge.

Enzyme extract (0.1 ml) was assayed for NRA in the presence of 0.9 ml potassium phosphate buffer (100 mM, pH 7.5) containing KNO_3 (10 mM) and NADH (0.1 mM) for 30 min at 25°C. The NADH concentration used here did not interfere with the nitrite colorimetric assay (Table 2.5). The reaction was terminated by placing tubes in boiling water for 5 min, after which nitrite was determined colorimetrically as for the *in vivo* assay. Nitrite formation was found to be linear for the duration of the incubation period, even in the presence of 0.2 ml enzyme extract (Figure 2.3).

2.2.4 Protein extraction and estimation

Protein was extracted from callus tissue with a Janke & Kunkel Ultra-turrax following the method of Wetter and Dyck (1983), except that octan-2-ol was used to prevent foaming. After precipitation of 100 μl protein extract in the presence of 0.016% (w/v) sodium deoxycholate and 6.06% (v/v) trichloroacetic acid to eliminate water-soluble interfering compounds (Bensadoun & Weinstein 1976), protein was estimated using the Folin-Lowry assay (Lowry et al. 1951), as described by Wetter (1984), using BSA as a standard.

2.2.5 Determination of nitrate utilisation from agar

Agar medium was frozen at -20°C for at least 24 h, thawed at room temperature and centrifuged at 40 000*g* for 60 min using a Beckman J2.21 centrifuge. Supernatant volume was measured and used for further analysis (Lumsden et al. 1990). Nitrate was quantified using the salicylic acid colorimetric method (Cataldo et al. 1975). A calibration curve was constructed using solid induction medium (Section 2.2.2).

Table 2.4: Effect of crude extraction buffer protectants on *in vitro* NRA of tobacco callus.

In two separate experiments (A & B), extraction buffers consisting of various protectants added to potassium phosphate buffer (pH 8.5) were used to obtain a crude enzyme extract. *In vitro* NRA was determined as described in Materials and Methods. Letters denote significantly different assay results ($p<0.05$). Means $\pm$ standard deviation are shown; n = 2-3 for each treatment.

	Protectant	NRA (nmol nitrite/g f.wt/h)
A)	- 2% BSA	583.7 $\pm$ 7.30 a
	- 0.5 mM EDTA & 1 mM DTT	233.3 $\pm$ 2.80 b
	- 1 mM DTT	227.3 $\pm$ 2.50 b
	- 1 mM PMSF, 1 mM ACA & 1 mM BAHC	179.4 $\pm$ 2.00 c
	- 0.5 mM EDTA	166.3 $\pm$ 10.7 c
	- 0.5 mM PMSF, 0.5 mM ACA & 0.5 mM BAHC	113.4 $\pm$ 1.28 d
B)	- 3% BSA	507.0 $\pm$ 16.22 A
	- 2% BSA	490.2 $\pm$ 23.46 A
	- 1% BSA	425.5 $\pm$ 11.56 B
	- 2% BSA & 1 mM DTT	205.9 $\pm$ 13.36 C

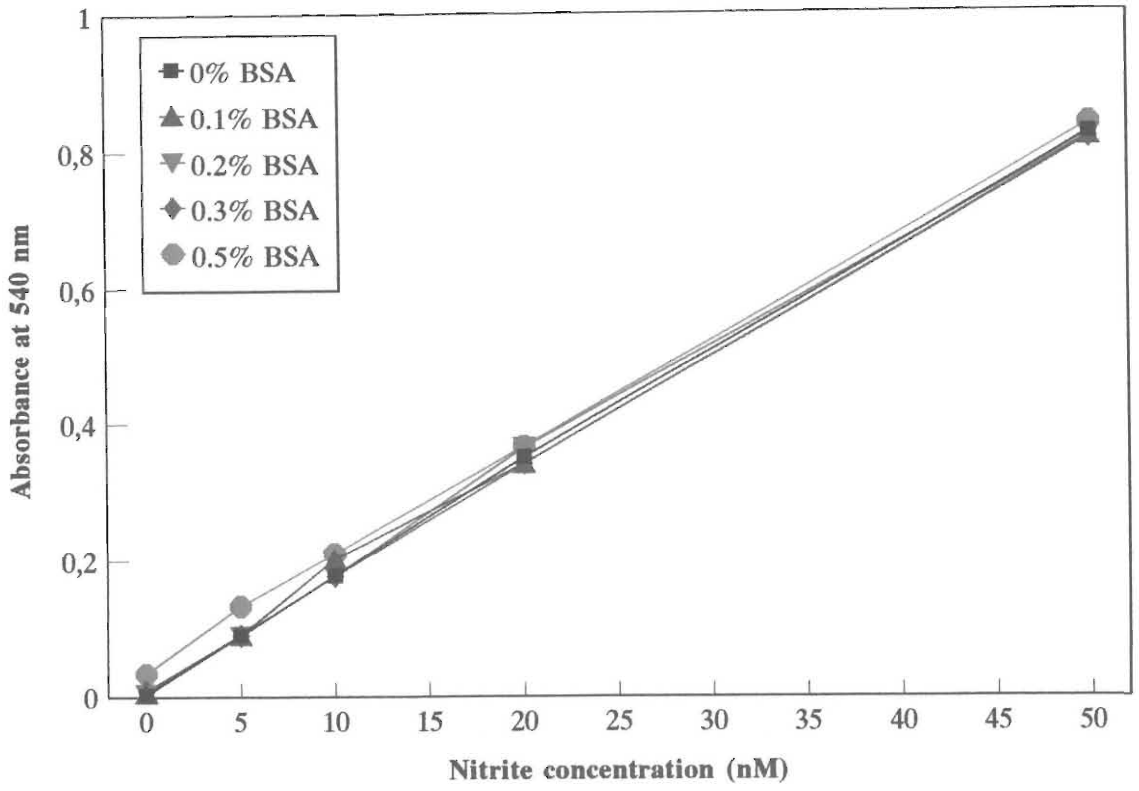

Figure 2.2: Effect of BSA on the nitrite colorimetric assay.

BSA (0.1-0.5% final concentration, corresponding to extraction buffer concentrations of 1-5%) was mixed with nitrite calibration curve standards. After incubation with the colorimetric assay reagents (Section 2.2.3), samples were centrifuged for 5 min at 3 700 rpm to pellet particulate matter, and absorbance at 540 nm was determined. Bars denote standard error of the mean (n = 3). Correlation coefficients (r) for calibration curves were 1.00 for 0-0.3% BSA, and 0.998 for 0.5% BSA.

Table 2.5: Effect of NADH and PMS on observed values for tobacco callus *in vitro* NRA.

In vitro NRA with 0.1 or 1.0 mM NADH as reductant, in the presence or absence of PMS, was determined as described in Materials and Methods. NRAs are given as means ± standard error (n = 3), with letters denoting significantly different values ($p<0.05$).

[NADH] (mM)	[PMS] (μM)	NRA (nmol nitrite/g f.wt/h)
0.1	0	78.43 ± 3.637 a
0.1	33	78.43 ± 5.546 a
1.0	0	29.41 ± 1.109 b
1.0	33	74.51 ± 4.803 a

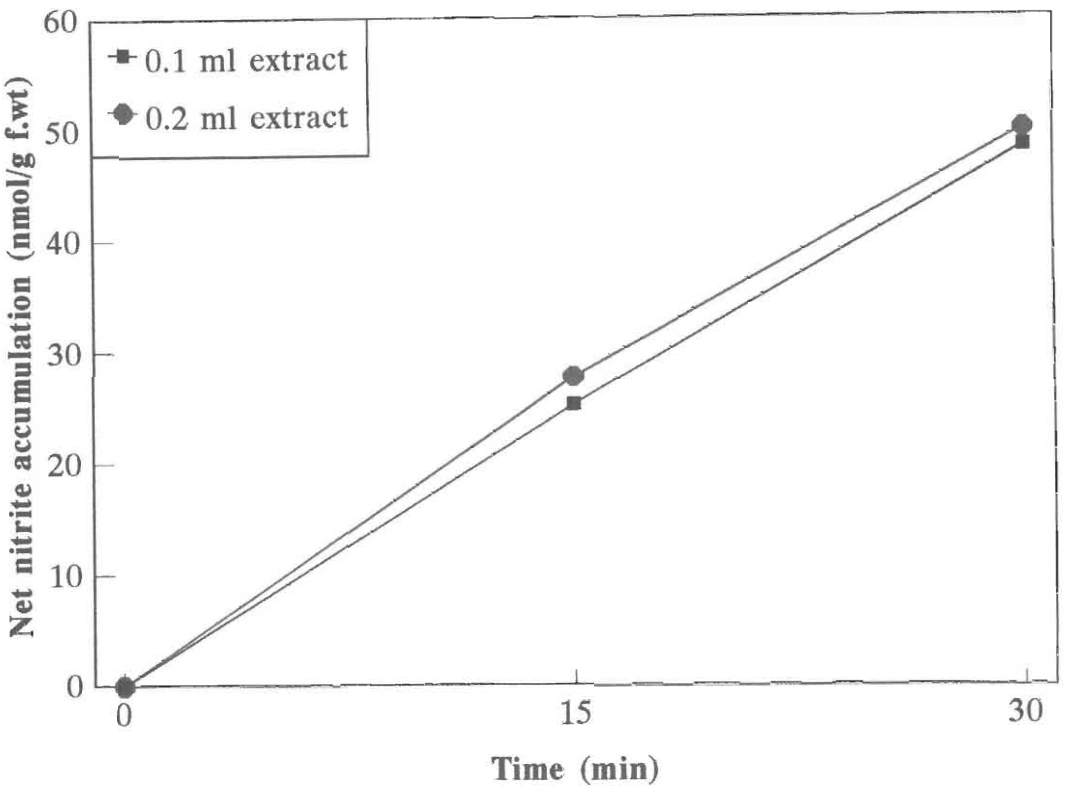

Figure 2.3: Dependence of NRA on time of incubation during the *in vitro* assay.

Tobacco callus (0.5 g f.wt) was ground at 4°C in 50 mM potassium phosphate buffer (pH 8.5) containing 0.5 mM EDTA. The filtered extract (either 0.1 or 0.2 ml) was used to determine *in vitro* nitrite accumulation after 30 min, as described in Materials and Methods. Standard errors (n = 3) were smaller than symbols. Correlation coefficients (r) for kinetic curves were 0.998 (0.1 ml extract) and 0.997 (0.2 ml extract).

2.2.6 Western analysis of NRP

Protein extraction

Crude enzyme preparations were obtained using acetone powders and buffer extractions. Acetone powders were prepared using a modification of the method of Ibrahim & Cavia (1975) as follows: tobacco callus or fresh spinach leaf material (0.3-0.6 g f.wt) was ground to a powder in liquid N_2 using a mortar and pestle, homogenised with cold 75% acetone, and then repeatedly washed under vacuum with very cold 100% acetone. After drying the residue with desiccated air, the powder was collected and stored at -80^{o}C. Crude protein extractions were performed using various buffers (Table 2.6). Frozen callus or leaf material (0.2-0.4 g f.wt) was pulverised in liquid N_2 with mortar and pestle and extracted with 5 ml buffer/g f.wt tissue. The homogenate was filtered through 2 layers of cheese cloth and cleared by centrifugation at 18 000*g* for 10 min at 4^{o}C using a Heraeus Biofuge B microcentrifuge. Supernatants were stored at -80^{o}C.

Gel electrophoresis

Acetone powders were prepared for gel electrophoresis by resolubilisation for 1 h on ice with occasional mixing in 1x SDS-treatment buffer (Hoefer gel electrophoresis protocol, 1992-1993) or in buffers 1-4 (Table 2.6), followed by microcentrifugation at 18 000*g* for 30 min at 4^{o}C. Supernatants (30 μg non-buffer protein) were separated by 9% SDS-PAGE on a Hoefer Vertical Slab Unit (Model SE 600) according to a modification of Laemmli (1970) described by the manufacturer (Hoefer, San Francisco, USA). Rainbow protein molecular weight markers were run as standards.

Western blotting and NR detection

Protein bands were transferred by electroblotting to nitrocellulose (Towbin et al. 1979) at 40-45 mA for 14 h using the Hoefer TE 70 SemiPhor Semi-dry Transfer Unit, or excised gel lanes were stained with Coomassie Blue (Hoefer guide, 1992-1993). Transfer efficiency was checked by staining the blotted gel with Coomassie Blue. Spinach polyclonal NR antiserum raised in rabbit was used as first antibody for NRP detection, with anti-rabbit IgG conjugated to alkaline phosphatase as second (indicator) antibody, according to the Biorad Bio-Dot SF Microfiltration apparatus instruction manual. Indicator antibody was detected as described by Sambrook et al. (1989), except that the toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was utilised (the recommended disodium salt of BCIP was **not** soluble in 100% dimethylformamide).

Table 2.6: Buffer components utilised during extraction and resolubilisation of protein for Western blot analysis of NRP.

Buffers 1 and 2 were adapted from Somers et al. (1983) and Remmler & Campbell (1986), while buffer 4 followed Wetter & Dyck (1983). Buffer component concentrations are indicated for each buffer.

Buffer number	Components
1	500 mM Tris-HCl (pH 8.6), 1 mM EDTA, 3 mM DTT, 25 μM FAD, 5 mM PMSF, 0.1 M phosphate (as orthophosphoric acid, to avoid potassium), 2% Polyclar AT and 0.1% BSA.
2	500 mM Tris-HCl (pH 8.6), 1 mM EDTA, 3 mM DTT, 25 μM FAD, 5 mM PMSF, 0.1 M phosphate, 2% Polyclar AT and 2% BSA.
3	500 mM Tris-HCl (pH 8.6) and 2% BSA.
4	200 mM Tris-HCl (pH 8.5), 0.056 M ME and 1 M sucrose.

2.2.7 Growth and maintenance of bacterial cultures

Stab cultures of *Escherichia coli* strain HB101/pBMC102010 grown on tryptose blood agar base with 50 μg/ml ampicillin (TABA) were stored at room temperature in darkness for periods of 6-12 months. TABA streak plates of HB101/pBMC102010 were stored at 4°C for 1-2 weeks after an initial overnight incubation at 37°C. Single bacterial colonies from TABA streak plates were used as inocula for liquid cultures grown on liquid Luria-Bertani broth medium (Sambrook et al. 1989) with 50 μg/ml ampicillin (LBA) at 37°C overnight.

2.2.8 Nucleic acid procedures

RNA isolation

All equipment used during RNA isolation procedures was made RNase free as follows: glassware and metal instruments were heat-treated at 230°C for at least 8 h, plastic equipment and Tris buffers were autoclaved at 120°C for 25 min, and other solutions were treated with 0.1% (v/v) DEPC overnight with stirring, followed by autoclaving as above. Phenol, chloroform and ethanol were used without pretreatment.

Total RNA was isolated from tobacco callus or leaf tissue according to a modification of Verwoerd et al. (1989). Preliminary experiments (Table 2.7) established the following changes to that protocol: material (0.1-0.2 g f.wt) was homogenised in liquid N_2 for 4 min using a brass mortar and pestle. The powder was transferred to a 1.5 ml microfuge tube and 500 μl of hot (80°C) extraction buffer (phenol : 0.1 M LiCl, 100 mM Tris-HCl [pH8.0], 10 mM EDTA, 1% SDS [1:1]) was added. After homogenising by vortex for 30 s, the mixture was incubated for 30 s at 80°C and then vortexed for another 15 s. RNAs were isolated and collected further as described by Verwoerd et al. (1989). Additional attempts to purify RNA samples using a phenol/chloroform/isoamylalcohol extraction step (Karl Kunert, pers. comm.) were not successful (results not shown). RNA was estimated spectrophotometrically, and samples were stored at -20 or -80°C.

RNA gel electrophoresis

RNA gel electrophoresis followed a modified protocol (Nagy et al. 1989; Sambrook et al. 1989). RNA and RNA molecular weight markers were denatured in formaldehyde buffer (1x 3-[N-morpholine] propanesufonic acid [MOPS] buffer [Sambrook et al. 1989], pH 7.0, 50% deionised formamide, 2M formaldehyde) for 10

Table 2.7: Tobacco callus RNA extraction procedure optimization.

RNA yield and purity (A260/A280) from typical RNA extractions using modified protocols of Verwoerd et al. (1989) are shown (n = 3-5 samples). Other parameters examined include amount of callus material and phenol incubation time (not shown).

Grinding Mechanism	Time (min)	Yield (μg/g f.wt)	A260/A280
- Ceramic mortar & pestle	4	11.3	1.61
- Glass rod in microfuge tube	4	62.4	1.91
- Glass rod in microfuge tube, with sand	4	19.9	1.60
- Metal rod in microfuge tube	4	101	1.82
- Brass mortar & pestle	3	311	1.35
	4	433	1.65
	6	323	1.18

min at 65°C. For Northern analysis, a 1% agarose gel containing 1x MOPS buffer and 2.2 M formaldehyde was prerun for 5 min at 5 V/cm before loading RNA samples mixed with gel loading buffer (Nagy et al. 1989). Fractionation was at 4 V/cm using a Biorad DNA Sub Cell system. After electrophoresis, the RNA marker lane was excised and stained for 10 min with 5 μg/ml ethidium bromide, followed by destaining in water overnight. A 1.5% agarose gel for analysis of RNA degradation was prepared as above using a Hoefer Minnie Submarine Agarose Gel Unit, but stained for 10 min with 5 μg/ml ethidium bromide in 1x MOPS buffer and destained overnight in water.

NR cDNA probe sequence isolation

Plasmid pBMC102010 (Figure 2.4) contains a 1.6 kb EcoRI cDNA insert of tobacco NR derived from the Lambda 13-29 recombinant phage (Calza et al. 1987; Vaucheret et al. 1989b). The plasmid was isolated from liquid LBA cultures of *E. coli* HB101/pBMC102010 (Section 2.2.7) using ethidium bromide/caesium chloride density gradient ultracentrifugation with Tris-EDTA buffer dialysis (Armitage et al. 1988). Plasmid identity was checked by fractionation of restriction enzyme digest fragments on a 1% agarose gel using standard techniques (Sambrook et al. 1989) (Figure 2.5). The 1.6 kb partial NR cDNA sequence to be used as probe was restricted from the purified plasmid with EcoRI and isolated in 1% low melting temperature agarose (Sambrook et al. 1989) prior to labelling.

Northern blot analysis

Agarose gels containing RNA fragments were soaked for 20 min in 0.05 N NaOH, washed several times with DEPC-treated water, and soaked in 20x SSC for 45 min. RNA was transferred to nitrocellulose according to Thomas (1980).

Hybridisation with [^{32}P]-labelled probe was carried out at 42°C in 50% formamide, 6x SSPE (20x SSPE = 3 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7), 5x Denhardt's solution, 0.1% SDS, 10% (w/v) dextran sulfate and 0.1 mg/ml denatured, sheared salmon sperm DNA. The NR probe sequence, a 1.6 kb NR cDNA fragment (Figure 2.4) was radiolabelled to a specific activity of 10^8-10^9 cpm/μg with [α-^{32}P]dTTP by random primer extension in low melting temperature agarose (Feinberg and Vogelstein 1983, 1984) and purified from unincorporated nucleotides by centrifugation on a Sephadex G-50 spun column (Sambrook et al. 1989). Labelled probe was used at 10^6-10^7 cpm/ml (10-20 ng DNA/ml) of hybridisation solution. After hybridisation, filters were washed under very stringent conditions (Nagy et al. 1989) and exposed to X-ray film at -80°C with intensifying screens.

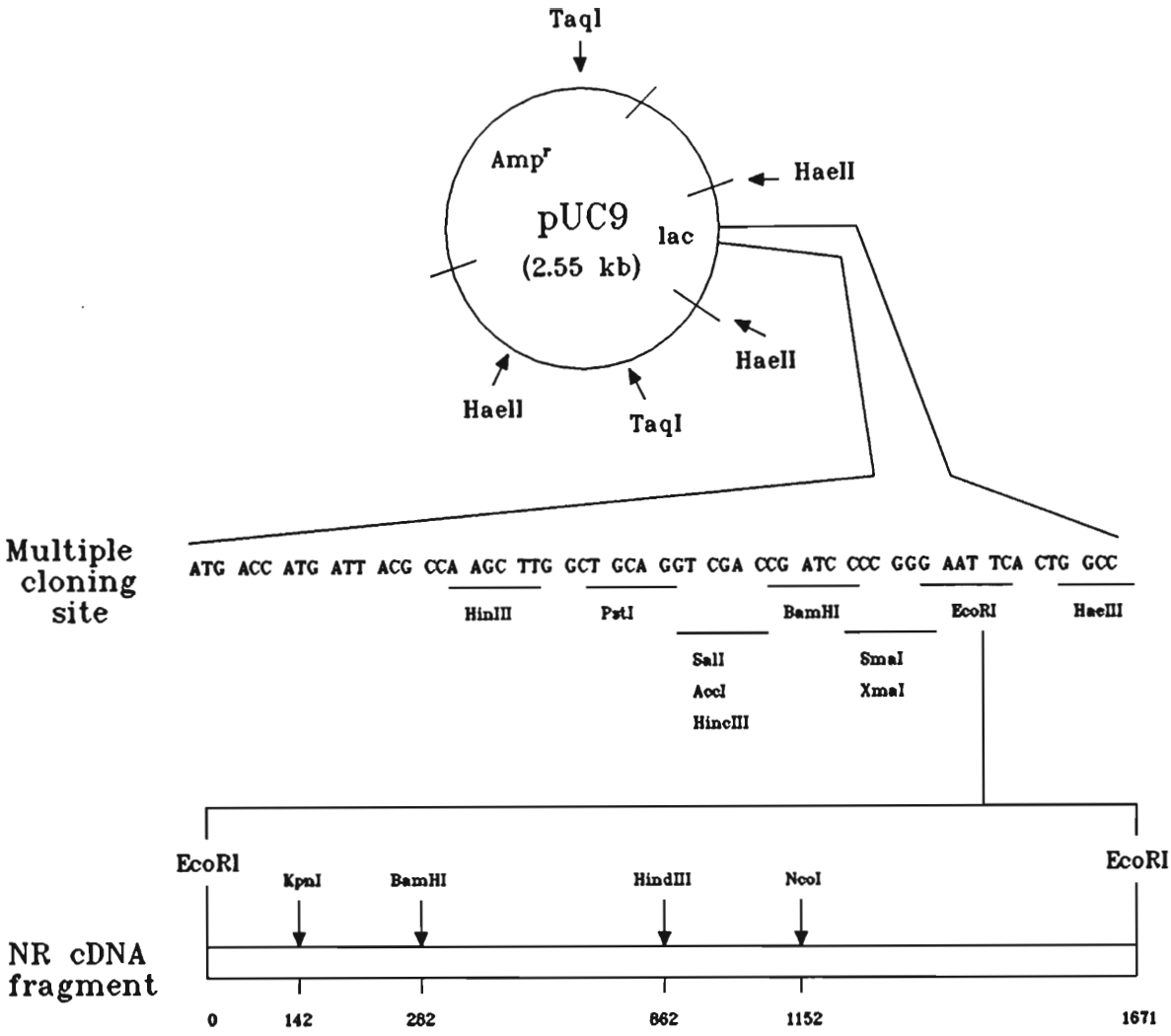

Figure 2.4: Structure of the plasmid pBMC102010 showing pUC9 multiple cloning site and insertion of 1.6 kb NR cDNA fragment.

The 1.6 kb NR cDNA fragment derived from the recombinant phage lambda 13-29 (Calza et al. 1987) was inserted at the EcoRI restriction site into the multiple cloning region of the cloning vector pUC9, a pBR322-derivative (Vieira & Messing 1982), by Vaucheret et al. (1989b). The resultant *E. coli*-based plasmid pMBC102010 carries as a selective marker the gene for resistance to the antibiotic ampicillin (Amp^r).

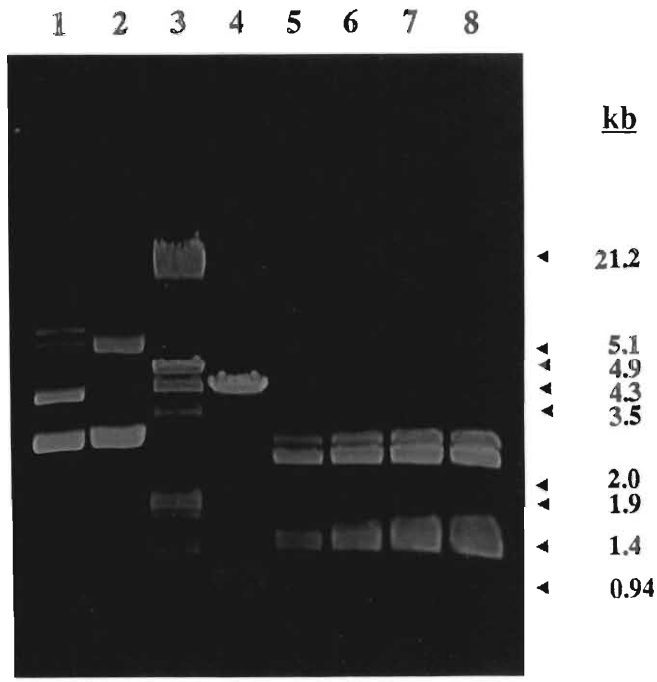

Figure 2.5: Electrophoretic analysis of pBMC102010 and its restriction enzyme fragments.

DNA was fractionated on a 1% agarose electrophoretic gel and stained with 0.5 μg/ml ethidium bromide. Lane 1 is undigested pBMC102010 (0.5 μg), showing a covalently closed circle (ccc), an open circle (oc) and other higher molecular weight multimeric forms (mm). The 3.9 kb DNA fragment in lane 1 is most likely to be partially nicked pBMC102010 (Armitage et al. 1988). Lane 2 is undigested pBR322 (0.3 μg), with covalently closed and open circle forms. Lane 3 contains Lambda DNA/EcoRI/HindIII molecular weight markers. Lane 4 is pBMC102010 linearised with SmaI (0.5 μg), generating a fragment of about 4.5 kb. Low molecular weight DNA in lane 4 (about 1.1 kb) is of unknown identity - it may be fragments resulting from SmaI restriction of partially nicked pBMC102010 (compare lane 1). Lanes 5-8 are EcoRI restrictions of pBMC102010 showing the NR cDNA insert of about 1.6 kb and residual 2.7 kb plasmid fragment (0.1-0.4 μg, respectively). The other DNA fragments observed in lanes 5-8 represent undigested covalently closed pBMC102010.

Autoradiographs of different exposures were scanned using a Hoefer GS 300 densitometer to allow for NR mRNA quantification.

For the nonradioactive DIG system, hybridisation and detection were as specified by the manufacturer (Boehringer Mannheim). The NR cDNA probe fragment was isolated from low melting temperature agarose (Mukhopadhyay & Roth 1991) before labelling, and approximately 160 ng digoxigenin-dUTP labelled DNA/ml was used during hybridisation.

RNA slot blot analysis
An RNA concentration series (0-20 μg) was applied to nitrocellulose filters using the Biorad Bio-Dot SF Microfiltration apparatus, as described by Sambrook et al. (1989). After baking at 80^{o}C for 2 h, filters were hybridised with [^{32}P]-labelled NR probe as for Northerns. Modified washing conditions were as follows: 2x SSC, 0.5% SDS at room temperature for 20 min; 2x SSC, 0.1% SDS at room temperature for 15 min; 0.1x SSC, 0.5% SDS at 37^{o}C for 15 min; 0.1x SSC, 0.1% SDS at 68^{o}C for 20 min; and 0.1x SSC at room temperature for 10 min. Autoradiographs were prepared and analysed as for Northern analysis.

2.2.9 Statistical procedures

Regression analyses, T-tests for equality of means, ANOVAs (Analysis of Variance) and Student-Newman-Keuls (SNK) tests were performed using the Costat statistical package (Cohort Software, Berkeley, USA), while the Kolmogorov-Smirnov test was run from Statgraphics (Statistical Graphics Corporation, Rockville, USA). Outliers were eliminated when detected using Dixon's test statistic, as described by Sokal & Rohlf (1981).

2.3 Results and Discussion

2.3.1 Optimisation of NRA assays for callus material

The *in vivo* assay utilised here did not use additional substrates to enhance NRA or surfactants to assist permeabilisation. Disadvantages of using an *in vivo* assay method employing various additives include membrane disruption (Davies & Ross 1985) and unreliable and non-physiological NRA rates (Soussana et al. 1989). However, if *in vivo* assay conditions exclude sources of reductant normally available to NR (Abrol et al. 1983) and energy becomes limiting for the assay period (Nicholas et al. 1976b), then substrates should be supplied. However, nitrite reduction was found to be linear over 60 min, with a slight lag after 45 min (Figure 2.1). Thus for the duration of the assay period (30 min), there were non-limiting reductant levels in the tissue and no requirement for additional substrates. Sufficient reducing power for NRA during the *in vivo* assay has been observed by other users of the method (King et al. 1992). Furthermore, availability of nitrate may affect nitrite production also, and metabolic nitrate pools have been shown to vary with tobacco cell suspension culture age (Ferrari et al. 1973). Linear nitrite accumulation thus depends on leaf age and species (Soussana et al. 1987). The possibility that linear nitrite production during the standard assay time might not be maintained during a callus growth period, thereby underestimating NRA, could not be excluded.

When performed on algal systems, the *in situ* assay method commonly requires toluene to assist permeabilisation (Herrero et al. 1981; Larsson et al. 1985; Guerrero 1985). However, toluene was omitted from the solubilisation buffer used here after the solvent was found to diminish nitrite yields (Table 2.3). Surfactants such as toluene can affect NRA by influencing respiration and membrane permeability, thereby causing unreliable results (Davies & Ross 1985; Soussana et al. 1987). Also, the addition of formaldehyde prior to nitrite determination is necessary to minimise interference of two *in situ* assay components, sodium dithionite and sulfanilamide (Senn et al. 1976). Whereas some workers have employed formaldehyde for *in situ* NRA measurements (Ramos et al. 1982), those who neglected this postassay treatment may have underestimated NRA (Herrero et al. 1981; Larsson et al. 1985).

For the *in vitro* assay, an extraction buffer containing 2% BSA provided significantly better protection ($p < 0.05$) from endogenous NRA inhibitors/inactivators or phenolics when compared with other protectants, including PMSF, amino-n-caproic acid, bezamidine hydrochloride, DTT and EDTA (Table 2.4). The thiol compound

DTT in conjunction with BSA did not improve *in vitro* NRA, possibly due to DTT's inhibitory effect on the nitrite colorimetric assay (Brunswick & Cresswell 1986b). Addition of 3% BSA (w/v) yielded slightly higher NRA values than 2% BSA (Table 2.4), but the difference was not significant, and therefore 2% BSA was utilised in all subsequent extractions.

Thus the optimal extraction procedure for the *in vitro* assay required a high pH buffer, thought to limit protease activity (Wetter 1984; Wray & Fido 1990), and BSA, which stabilises NR by offering protection from proteolytic enzymes, inhibitors and/or phenolics and tannins (Schrader et al. 1974a, b; Rhodes 1977; Hageman & Reed 1980). Although BSA did not affect the activities of maize or rice inactivating proteins or trypsin (Yamada et al 1980b), the protectant did prevent dissociation of NR in buffers (Schrader et al. 1974a; Yamada et al. 1980b). Nitrite assay calibration curves constructed in the presence of BSA at concentrations used for enzyme extractions here indicated that BSA did not interfere with the nitrite colorimetric assay, while at higher BSA concentrations, slight interference (probably caused by unprecipitated particulate protein matter) was found (Figure 2.2).

Reductant for the *in vitro* assay was in excess supply at 0.1 mM NADH, giving linear NRA rates for the duration of the assay period (Figure 2.3). The possibility that this saturating NADH concentration might be interfering with the nitrite colorimetric assay was investigated by incubating an assay mixture with PMS before adding nitrite assay reagents (Scholl et al. 1974; Brunswick & Cresswell 1986b). PMS treatment alleviated interference of 1.0 mM NADH, but had no effect on calculated nitrite formation when 0.1 mM NADH was utilised, indicating that 0.1 mM NADH did not interfere with the nitrite assay (Table 2.5). Scholl et al. (1974) reported NADH interference with the nitrite colorimetric assay at concentrations as low as 100 nM, and many *in vitro* assays employ NADH in the nM range (Streeter & Bosler 1972; Mohanty & Fletcher 1976; Sherrard & Dalling 1979; Lenee & Chupeau 1989). However, other systems have required more reductant to obtain optimal *in vitro* NRA, from 0.1 to 1.0 mM (Wray & Filner 1970; Blahova & Segeta 1980; Lillo 1983; Kamada & Harada 1984; Hoarau et al. 1991). The use of 0.1 mM NADH for tobacco callus cultures was appropriate because this concentration produced optimal NRA yet did not inhibit the nitrite assay.

2.3.2 Suitability of NRA assays for callus differentiation studies

In order to compare the *in vivo*, *in situ* and *in vitro* assays for NRA, and to examine their usefulness when determining NRA regulation during tobacco callus differentiation, two different experiments using tobacco cell suspension cultures plated onto solid agar medium were performed (Figure 2.6A & B). Although subcultured callus material was used for the establishment of subsequent culture systems (Chapter 3), cell suspension cultures were used to initiate callus here so that variability associated with subcultured callus would be avoided (Skirvin 1978; Meins et al. 1982; Collin & Dix 1990), thereby facilitating NRA assay analysis and choice.

In both experiments, NRA measured by the three assays showed similar trends, with a peak of activity at about 28 days, followed by gradual decline (Figure 2.6A & B). The enzyme assays gave statistically identical values ($p<0.05$) for the entire culture period when compared using the Kolmogorov-Smirnov test for distribution. However, this statistical procedure is not very powerful, and insignificant results should not be used to conclude that sample distributions are identical (Statgraphics manual). Results were therefore compared at each sampling point using a T-test for equality of means (Materials and Methods). In the first experiment (Figure 2.6A), NRA values were generally statistically undistinguishable, except after 42 and 49 days in culture, when *in vitro* NRA was lower than *in vivo* and *in situ* NRA, and day 63, when *in vitro* NRA was higher. In the second experiment (Figure 2.6B) *in vitro* NRA was generally lower than *in vivo* and *in situ* NRA up to day 28, although this difference was not significant ($p<0.05$). However, *in vitro* assay activity was significantly higher than the other activities at day 42 and 49, and the *in situ* assay produced significantly higher values than the *in vitro* assay, but not the *in vivo* assay, at day 35 (Figure 2.6B).

The *in vitro* and *in situ* assays are assumed to measure NRA under optimal conditions, and hence provide a measure of the amount of potentially active enzyme present (Nicholas et al. 1976b; Lillo 1983; Padidam et al. 1991). For this reason, many studies have obtained *in vitro* NRA values several times higher than *in vivo* NRA (Nicholas et al. 1976a, b; Jordan & Fletcher 1980; Huffaker 1982; Lillo 1983; Duke & Duke 1984; Lillo 1984). Other workers have found that *in vivo* activity was consistently higher than *in vitro* activity, attributing this to inactivators and organic compound inhibition during extraction (Jaworski 1971; Johnson 1976; Jones et al. 1976). Streeter & Bosler (1972) reported higher *in vivo* NRA than *in vitro* NRA in soybean leaves due to non-optimal extraction conditions, despite the use of DTT, EDTA,

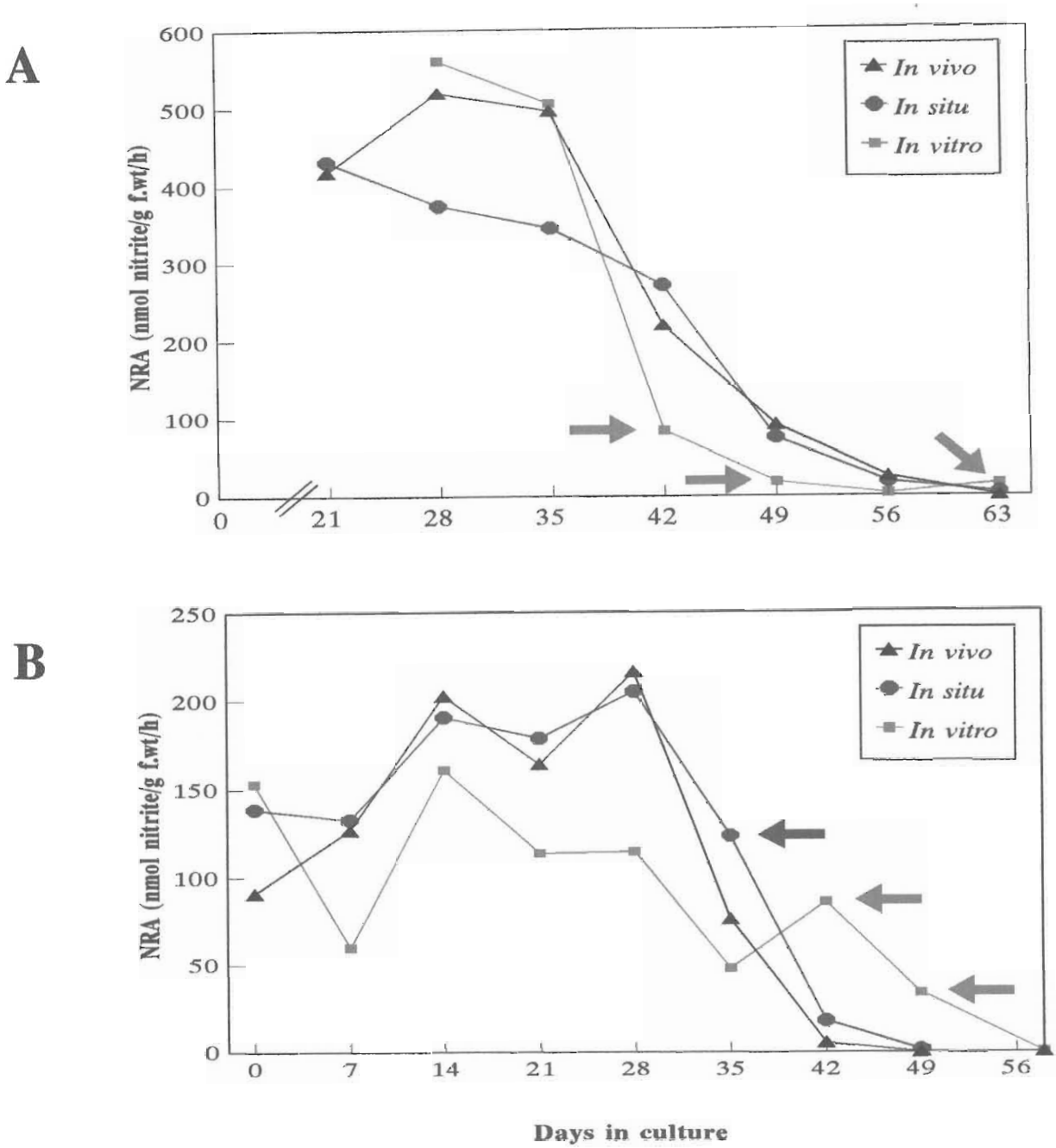

Figure 2.6: *In vivo*, *in situ* and *in vitro* NRA profiles during tobacco callus growth.

In vivo, *in situ* and *in vitro* NRA was measured during growth of two different batches of tobacco cell suspension cultures (A and B) plated onto solid agar induction medium. Cultures were kept in darkness for 5 weeks to enhance callus proliferation, and then transferred to a 16/8 h light/dark period. Significantly different means are indicated by arrows ($n = 3$, $p<0.05$).

Polyclar AT and cysteine as protective agents. Here, *in vivo* NRA was generally at the same level as *in vitro* and *in situ* NRA, indicating that physiological levels of reductant and substrate were probably non-limiting during most of the tobacco callus culture period. Higher *in vitro* NRA at day 42 and 49 in Figure 2.6B and day 63 in Figure 2.6A may be an indication there was insufficient reducing power within the callus tissue for NRA for the duration of the *in vivo* assay at these points. It is not clear why *in situ* assay values were significantly lower than *in vitro* values at these stages.

Conversely, *in vitro* assay values were lower than the other assays at 42 and 49 days in culture in Figure 2.6A. Extractable NRA has generally been observed to decrease with leaf or culture age (Hageman & Hucklesby 1971; Streeter & Bosler 1972; Blahova & Segeta 1980; Jordan & Fletcher 1980; Santoro & Magalhaes 1983; Hoarau et al. 1991). NR from older leaves of maize, oats, soybean and tobacco was found to be less stable *in vitro* than NR from younger leaves (Schrader et al. 1974a, b; Santoro & Magalhaes 1983), although a recent study on maize leaves found NRP to be more stable in older leaves, but synthesised at a lower rate (Kenis et al. 1992). Depending on the species concerned, endogenous inactivators, tannins and polyphenolic compounds that affect NR will vary with age also (Streeter & Bosler 1972). Lower *in vitro* NRA observed with older callus in Figure 2.6 were most likely caused by inhibitory substances exposed to NR during extraction.

Some variability of NRA profiles between the two cell suspension callus batches used for the experiments in Figure 2.6 was evident. However, at least two of the assays used to determine NRA produced statistically similar values at all assay points. While some workers have been able to apply more than one assay method to reveal different aspects of NR regulating systems (Lillo 1984; Hoarau et al. 1991), the results obtained here using three NRA assays on tobacco callus cultures strongly suggested that a single assay system would be sufficient to determine regulatory aspects of callus differentiation. Despite valid criticisms for each of the enzyme assays used here (Section 2.1.1), the *in vivo* assay was selected for all subsequent NRA quantification during differentiation studies. The *in vivo* assay is simple and easier to perform than the other assays, and large numbers of samples can be handled simultaneously (Heuer & Plaut 1978; Hallam & Blackwood 1979; Naik et al. 1982; Padidam et al. 1991) - these attributes are advantageous for studies where tissues exposed to different environmental variables require simultaneous assaying.

Callus soluble protein content and depletion of nitrate from the medium were

quantified in order to assess whether the *in vivo* NRA (the method of choice for subsequent investigations) profiles shown in Figure 2.6 reflected the nitrogen status of the callus cultures. In the first experiment, protein accumulation increased linearly until 49 days in culture, after which levels reached a plateau (Figure 2.7A). Levels of nitrate in the medium decreased correspondingly, reaching a basal level of about 50 μmol/culture after 49 days (Figure 2.7B). Protein levels for the second experiment (Figure 2.7C) increased to higher levels than in the first experiment. Nitrate depletion from the medium was slightly more rapid in experiment two (Figure 2.7D), with almost all the medium nitrate depleted after 42 days in the former. In combination, the results from Figures 2.6 and 2.7 suggested that NRA was correlated with nitrate uptake only when substrate became limiting, resulting in NRA decline. However, most of the nitrate taken up by callus would be stored initially in vacuolar compartments (Martinoia et al. 1981; Granstedt & Huffaker 1982), and released to the metabolic site for NRA depending on various environmental factors (Wallace 1987). It is therefore difficult to determine whether NRA should be correlated with nitrate flux rather than cellular nitrate content (Shaner & Boyer 1976a, b; Santoro & Magalhaes 1983). Comparatively low rates of NRA after 40 days in culture were associated with lower levels of soluble protein (Figures 2.6 and 2.7A, C), indicating that for tobacco callus cultures, nitrate assimilation might limit soluble protein production.

2.3.3 Western analysis of SDS-PAGE NR polypeptide fragment cross-reactivity

General cross-reactivity between eukaryotic NRs has been well established in the literature (Snapp et al. 1984; Notton et al. 1985; Cherel et al. 1986; Notton et al. 1988, 1990). In particular, spinach NR fragments have been shown to have relatively good homology with tobacco NR, with up to 80% reported (Notton et al. 1990; Fido 1991; Shiraishi et al. 1991). It seemed feasible therefore to employ a spinach polyclonal antibody against NR to detect tobacco callus NR. Accordingly, tobacco callus protein samples with relatively high *in vitro* NRA were fractionated by SDS-PAGE and transferred to nitrocellulose, and spinach polyclonal anti-NR was used to detect NR bands (Figure 2.8B). At all protein loadings and antibody dilutions attempted, two major bands with apparent molecular weights of 48 and 71 kD were detected, as well as numerous minor bands. Tobacco NR has a subunit size of 100-110 kD (Mendel & Müller 1980; Hoarau et al. 1991), which means that the major bands detected in Figure 2.8B represent either *in vivo* NR degradation products, *in vitro* cleaved NR subunits, or non-NR proteins cross-reacting preferentially with the spinach polyclonal NR antibody. Comparison of polypeptide bands found in tobacco

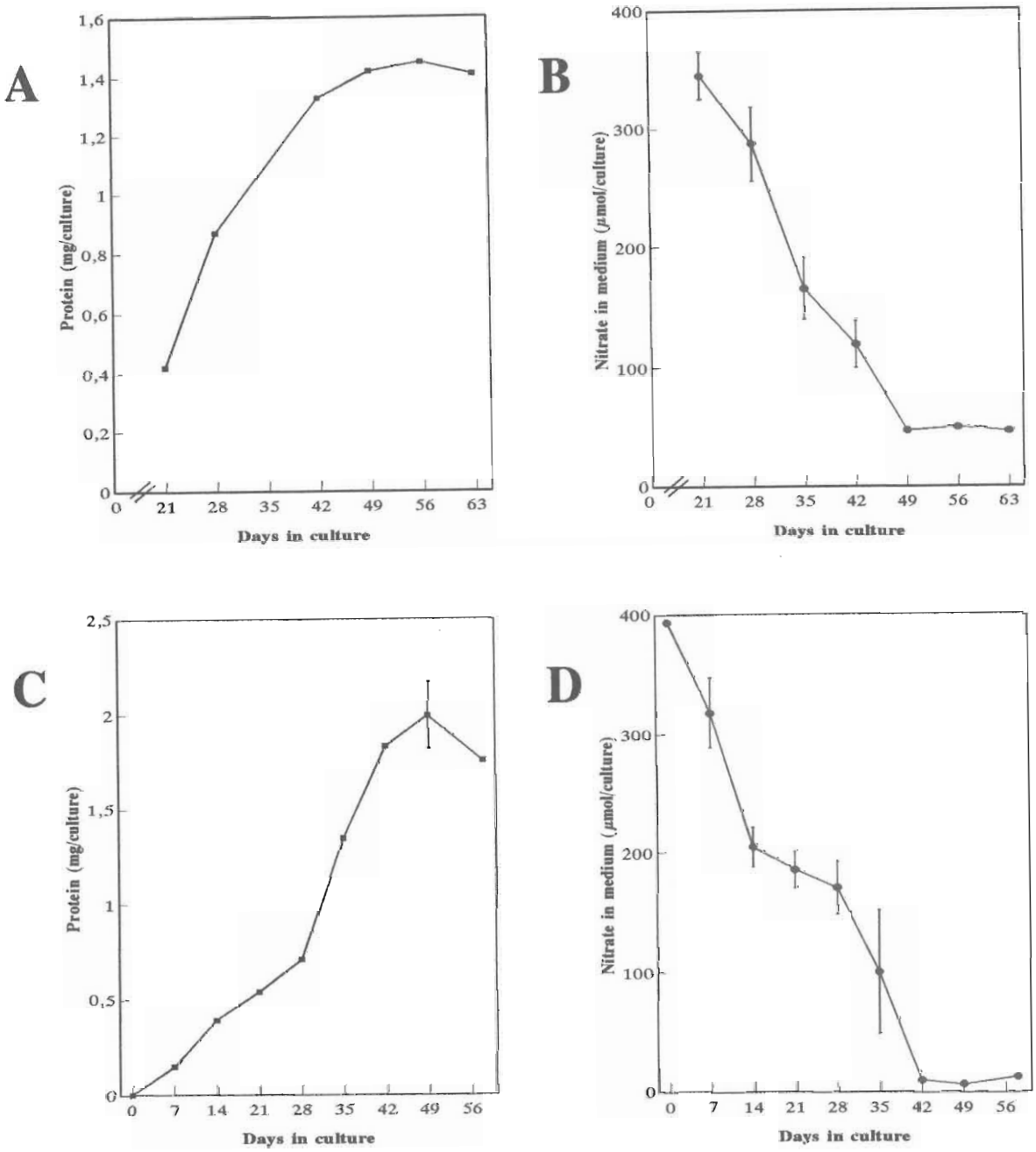

Figure 2.7: Protein accumulation and medium nitrate depletion during tobacco callus growth.

The nitrogen status of tobacco callus derived from two different batches of cell suspension cultures (A,B and C,D, respectively) was monitored using two parameters: protein accumulation (A,C) and nitrate disappearance (B,D). Cultures were kept in darkness for 5 weeks to enhance callus proliferation, and then transferred to a 16/8 h light/dark period. Where larger than symbols, mean standard errors (n = 3-5) are represented by bars.

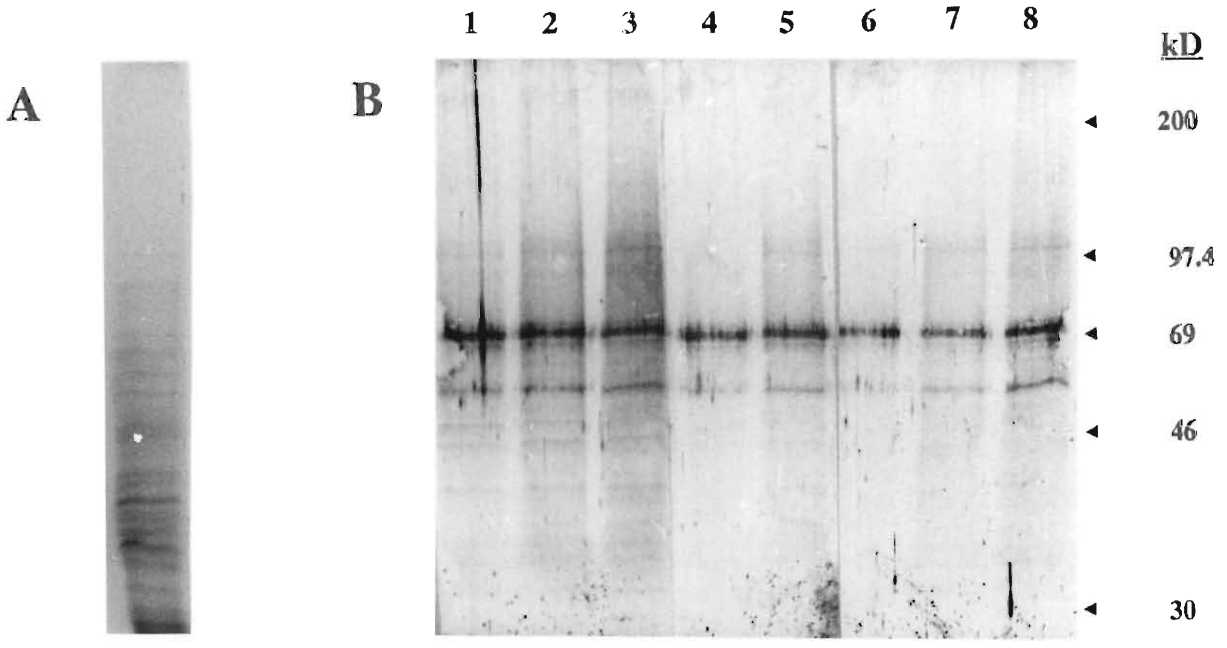

Figure 2.8: SDS-PAGE analysis of tobacco callus protein and Western blot detection of NRP with varying protein loads and antibody titres.

Tobacco callus protein extracted in acetone and resolubilised in SDS treatment buffer was fractionated on a 9% SDS-polyacrylamide gel. (A) Electrophoretic pattern of total protein (100 μg) stained with Coomassie Blue. (B) After transfer by electroblotting to nitrocellulose filters, spinach anti-NR polyclonal antibody diluted to 1/5000 (lanes 6-8), 1/1000 (lanes 4 and 5) or 1/200 (lanes 1-3) was applied. Following second antibody (AP) binding, filters were incubated with chromogenic detection reagents for 1 min (lanes 1-5) or 15 min (lanes 6-8). Lanes 1, 4 and 6 contain 50 μg protein, lanes 2, 5 and 7 contain 100 μg protein, and lanes 3 and 8 contain 150 μg protein.
An *in vitro* NRA of approximately 950 nmol nitrite/mg protein/h was measured using callus acetone powder resolubilised in 50 mM potassium phosphate buffer (pH 8.5) containing 2% BSA.

callus after SDS-PAGE (Figure 2.8A) with anti-NR detected bands after Western blotting (Figure 2.8B) indicated at least that the spinach antibody was not reacting indiscriminately with abundant proteins.

In vivo NR degradation intermediates of 100, 76, 64, 62 and 44 kD in spinach (Sueyoshi et al. 1989), and 80, 55, 42, 40 and 37 kD in barley (Poulle et al. 1987) have been identified, but catabolic products of tobacco NR proteases have not been specified yet. However, numerous studies using purified NR have found that SDS-PAGE generates NR fragments smaller than 110 kD of varying molecular weight, including: 44 and 75 kD in squash (Redinbaugh et al. 1982); 30, 33, 34, 37, 43, 50, 57 and 74 kD in spinach (Notton & Hewitt 1979; Fido & Notton 1984; Fido 1987); 20, 38, 48, 54, 59, 69 and 71 kD in barley (Campbell & Wray 1983); and others (Beevers & Hageman 1980; Campbell & Smarrelli 1986; Hoarau et al. 1986; Fido 1991; Shiraishi et al. 1991). NR appears to be particularly labile in the presence of SDS: Fido (1987) reported that smaller bands found after SDS-PAGE of purified spinach NR were not present when NR was fractionated by native (non-denaturing) PAGE. Furthermore, limited proteolysis studies using *Staphylococcus aureus* endoprotease V8 and/or trypsin, in conjunction with amino acid sequence analysis, have demonstrated that there are particularly susceptible regions between the molybdenum, heme and FAD domains of NR (Kubo et al. 1988; Fido 1991; Shiraishi et al. 1991). Molecular weight estimations of about 30, 15 and 75 kD for the FAD, heme and molybdenum domains have been obtained (Kubo et al. 1988; Fido 1991). Therefore, it is possible that the two bands observed in Figure 2.8B represent the molybdenum domain ($\pm$ 71 kD) and combined heme and FAD domains ($\pm$ 48 kD) of tobacco NR, two fragments often observed as principal bands after SDS-PAGE of purified NR (Notton & Hewitt 1979; Redinbaugh et al. 1982; Campbell & Wray 1983).

Extraction and isolation procedures may partially account for the wide range of NR fragment sizes reported in the literature (Kleinhofs et al. 1985). Protein samples for data shown in Figure 2.8 were prepared by acetone extraction and resolubilised directly in SDS treatment buffer before fractionation. To determine whether the bands detected by spinach anti-NR would differ if other extraction procedures were employed, callus samples were prepared by crude extraction and also by acetone extraction with resolubilisation in various buffers (Table 2.6). Protein from spinach leaves was extracted to provide a species-specific positive control. Western blot results are shown in Figure 2.9.

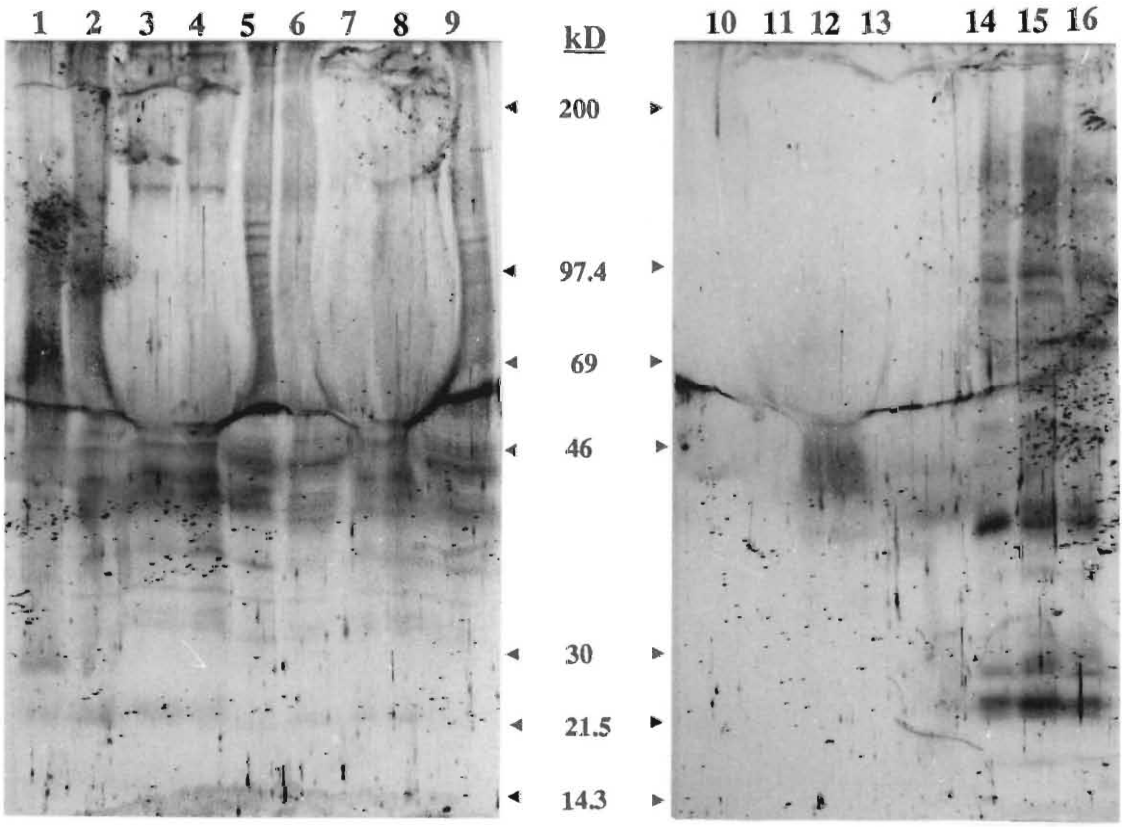

Figure 2.9: Effect of protein extraction technique and buffer composition on Western blot detection of NRP.

Protein from tobacco callus or spinach leaf was fractionated on a 9% SDS-polyacrylamide gel and transferred by electroblotting to nitrocellulose filters before incubation with spinach anti-NR as in Figure 2.8. Lanes 1-5 contain tobacco callus protein extracted in acetone and resolubilised in SDS treatment buffer or buffers 1-4 (Table 2.6), respectively; lanes 6-9 contain tobacco callus protein extracted in buffers 1-4, respectively; lanes 14 and 15 contain spinach leaf protein extracted in acetone and resolubilised in SDS treatment buffer or buffer 1, respectively; and lane 16 contains spinach leaf protein extracted in buffer 1. Lanes 1-9 and 14-16 were loaded with 30 μg endogenous protein. Lanes 10-13 contain buffers 1-4, respectively, at 1 mg protein for buffers 3 and 4, and 50 μg protein for buffer 2. See Materials and Methods for further details.

The most prominent feature of the Western blot shown in Figure 2.9 was severe protein overloading in lanes 3, 4, 7, 8, 11 and 12, which corresponded to samples prepared in the presence of 2% BSA. Besides the smearing effect shown in these lanes, a continuous band was present throughout the blot in the region of 60-70 kD. Samples were loaded according to plant protein levels, at about 30 μg/well, and not according to exogenous buffer protein. Therefore, 200-250 μg of total protein was loaded in lanes 4 and 5, and lanes 7 and 8 contained 440-530 μg of protein. These protein levels, in excess of recommended loadings for SDS-PAGE by 2- to 5-fold (Sambrook et al. 1989), comprised mainly BSA which appeared to react with the spinach NR antibody or with the second antibody, anti-rabbit IgG conjugated to AP (see 40-50 kD region of lanes 11 and 12 of Figure 2.9 which contained only buffers 2 and 3). Bands between 40 and 50 kD for most of the lanes in Figure 2.9 are therefore likely to be artifacts.

Spinach leaf protein extracted by acetone and resolubilised in SDS treatment buffer yielded noticeable bands at $\pm$ 94, 38 and 23 kD (Figure 2.9, lane 14), as well as other faint bands at high and low molecular weight. Similar patterns were observed for other spinach treatments (Figure 2.9, lanes 15 and 16), although the 94 kD band for spinach extracted in buffer 1 (lane 16) was less clear. The 23 kD band was probably not an NR fragment, as the spinach antibody used has reported cross-reactivity with a 23 kD OEC protein of photosystem II (Ian Prosser, pers. comm.). A faint band at $\pm$ 230 kD, which corresponds with the molecular weight of spinach NR holoenzyme (Fido 1987) was obtained when spinach acetone powder protein was resolubilised in buffer 1 (lane 15). Bands present in the spinach control lanes were not as specific as might have been expected from the polyclonal spinach anti-NR antibody. The antibody titre may have been too high in this experiment, and also degradation of NR during extraction may account for many of the reactive bands in Figure 2.9. Although these results do not confirm the specificity for NR, the spinach antibody has been examined for specificity at Long Ashton Research Station, Bristol, UK, and successfully used there for ELISA quantification of NR (Ian Prosser, pers. comm.). On the basis of their results, and assuming that all antigenic sites are not denatured by SDS treatment (Burnette 1981; Sambrook et al. 1989), the polyclonal antibody was regarded as specific for NR at the correct antibody titre.

The effect of extraction procedure on tobacco callus NR was investigated further. Acetone-extracted callus protein resolubilised in SDS treatment buffer (as in Figure 2.8) reacted with NR antibody in a broad band at $\pm$ 70 kD, and also with other polypeptides, including one at $\pm$ 210 kD (Figure 2.9, lane 1). Acetone powders

resolubilised in buffers 1-4 yielded bands in the high molecular weight region (210 kD in lanes 2, 3 and 4 and 140-145 kD in lanes 3 and 4), and several bands below 60 kD (lanes 2-5), some of which may not be NR fragments, as discussed above. Buffer 4 generated noticeable bands in the 110-125 kD region (lane 5), possibly NR subunit fragments, not present in the other buffer treatments. Crude enzyme extractions with buffers 1-4 (lanes 6-9) gave comparable results to the acetone powder treatments in the lower molecular weight area, but less cross-reactivity was found with larger polypeptides. Buffer 4 extract generated faint bands in the 110 kD region (lane 9), as found with the acetone powder equivalent (lane 5).

It was concluded from the results shown in Figure 2.9 that buffers 2 and 3 were most unsuitable for Western blot analysis due to interference caused by excess inert protein included as a protectant. Extraction or solubilisation with buffer 1 did not protect NR sufficiently to yield bands of the expected subunit size, although holoenzyme may have been present in lane 2. Bands in the 110-120 kD region observed after treatment with buffer 4 may have represented NR subunits (lanes 5 and 9). Unfortunately, several cross-reactive polypeptides of NR subunit size made it difficult to evaluate which band(s) to choose for NRP quantification. From Figures 2.8B and 2.9, acetone extraction of tobacco callus with resolubilisation in SDS treatment buffer was considered to be optimal for NR Western analysis for the following reasons: (1) there was no interference by exogenous buffer components; (2) although NR was probably denatured by SDS treatment buffer, the major bands obtained could be quantified easily; and (3) acetone extraction proved to be a simple and rapid technique that avoided contaminating plant compound interference (Ibrahim & Cavia 1975). All subsequent experiments involving NRP quantification (Chapter 3) employed the acetone/SDS method.

2.3.4 Quantification of Northern and slot blot analysis of NR mRNA using radioactive and non-radioactive probes

Successful RNA extraction techniques require complete cell disruption and inactivation of RNases (Sambrook et al. 1989). The modified method of Verwoerd et al. (1989) employed here incorporates a phenol buffer extraction step to isolate RNA from contaminating proteins, including RNases, rapidly. Exogenous RNases are eliminated by the RNase inhibitor DEPC (Nagy et al. 1989; Sambrook et al. 1989), used at low concentrations to prevent chemical modification of RNA components (Leonard et al. 1970). Routine total RNA extractions from tobacco callus and leaf material thus yielded relatively intact RNA (Figure 2.10). Narrow 25S and 18S

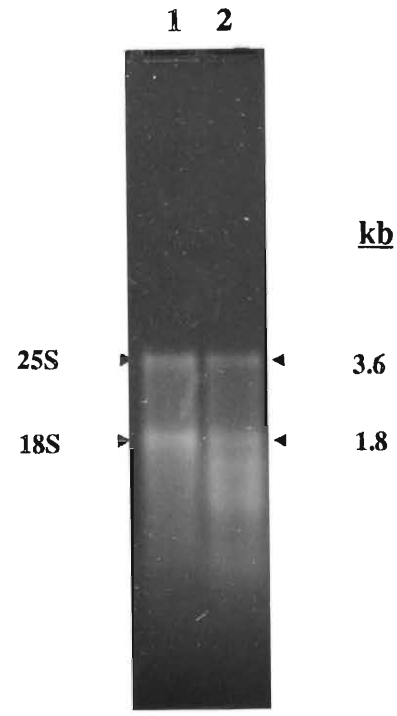

Figure 2.10: RNA agarose gel electrophoresis for assessment of rRNA band integrity.

Total RNA extracted by a modification of Verwoerd et al. (1989) was fractionated on a 1.5% agarose electrophoretic gel. Lane 1 contains 1 μg RNA from dark-grown tobacco callus, and lane 2 contains 1 μg RNA from sterile tobacco plantlet leaves. Bands of lower molecular weight than 18S rRNA in lane 2 represent chloroplast rRNA (Logemann et al. 1987).

rRNA bands at 3.6 kb and 1.8 kb, respectively, are comparable to reported molecular weight values (Grierson & Covey 1984), and indicate good quality RNA (Logemann et al. 1987). However, the ratio of 25S/18S rRNA was assessed qualitatively to be less than 2, implying some degradation of higher molecular weight rRNA (Correa-Rotter et al. 1992).

Northern blot analysis of tobacco callus RNA using a [^{32}P]-labelled cDNA NR probe demonstrated the presence of a specific mRNA species of about 3.5 kb (Figure 2.11A), the reported size for tobacco NR mRNA (Calza et al. 1987; Vaucheret et al. 1989b). Although two different genes are involved in tobacco NR expression (Müller 1983), only a single type of NR has been found (Vaucheret et al. 1989b). Furthermore, both NR gene transcripts are detected at 3.5 kb by the 1.6 kb NR probe used here (Figure 2.4) because there is reportedly a 97% homology of the coding regions of the two NR genes (Vaucheret et al. 1989a, b). NADH-NR from other plant species have mRNA species of comparable size: 3.5 kb for *N. plumbaginifolia*, barley and tomato (Cheng et al. 1986; Calza et al. 1989; Lu et al. 1990; Vaucheret et al. 1990) and 3.2 kb for *A. thaliana*, maize and squash (Crawford et al. 1986, 1988; Gowri & Campbell 1989). Tobacco NR mRNA in Figure 2.11A appeared to be severely degraded: it is not certain whether this state represents predominantly *in vivo* or *in vitro* degradation, although some degradation was evident due to RNA extraction (Figure 2.10). Nevertheless, autoradiograph densitometer tracings revealed that increasing loads of 10, 20 and 30 μg RNA produced relative NR band intensities of 1:2:3. Northern blotting with [^{32}P]-labelled probe was therefore a specific and quantitative technique for NR mRNA analysis.

Attempts to visualise NR mRNA colorigenically with DIG-labelled probe using a duplicate copy of the filter used for [^{32}P] work were unsuccessful (Figure 2.11B). Despite the fact that 600 ng DIG-labelled probe was used in comparison to 100 ng [^{32}P]-labelled probe (specific activity: $2x10^9$ cpm/μg), the non-radioactive technique was not sensitive enough to detect NR mRNA species, as has been described previously by other workers (Keller & Manak 1989). Düring (1991) found that oligolabelling denatured double stranded DNA probe with DIG-dUTP as specified by the manufacturer (Boehringer Mannheim) yielded no detectable signal on a Northern blot, whereas single stranded RNA labelled with the SP6 or T7 RNA polymerase labelling system was successful. It was concluded that the Boehringer DIG DNA labelling and detection kit and recommended protocol were unsuitable in this study for Northern analysis employing nitrocellulose filters and double stranded DNA probe.

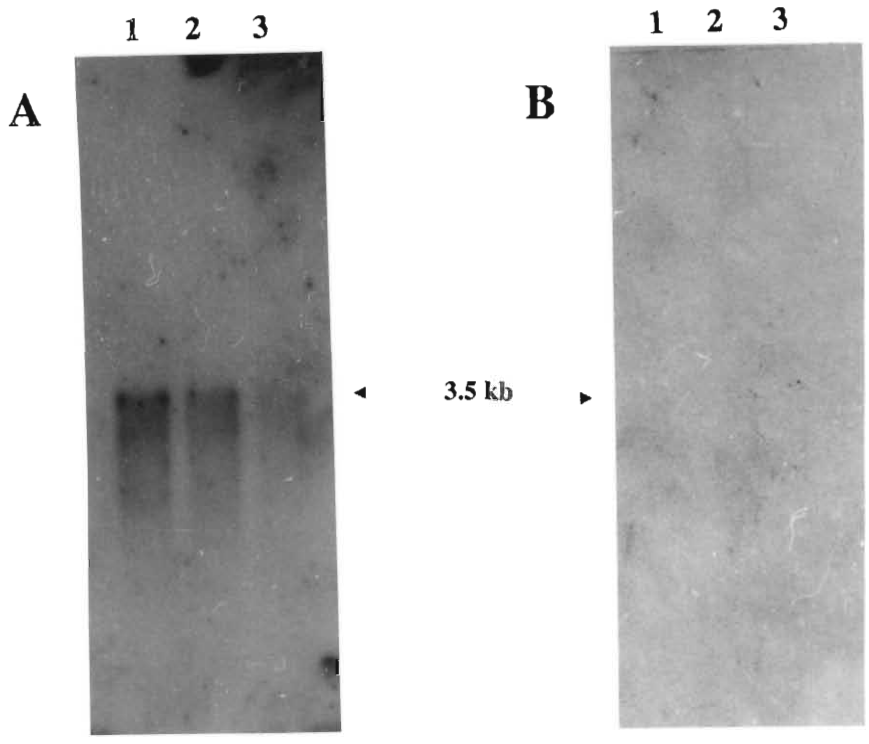

Figure 2.11: NR mRNA detection by Northern blot analysis using radioactive and non-radioactive methods.

Denatured tobacco callus total RNA (30, 20 and 10 μg, lanes 1-3, respectively) was fractionated in duplicate on a 1% agarose gel (A and B), and transferred to nitrocellulose filters. A 1.6 kb NR cDNA fragment isolated from plasmid pBMC102010 (Vaucheret et al. 1989b) and labelled with [α-^{32}P]-dTTP (A) or DIG-dUTP (B) was used to detect NR mRNA species. No bands were detected in (B) using DIG-labelled probe.

RNA slot blots are often more sensitive than Northern blots because both degraded and intact sequence that are vacuum-filtered onto filters contribute to the hybridisation signal (Meinkoth & Wahl 1984). Slot blots allow also for convenient direct quantification of specific mRNA species (van Helden & Olliver 1987). The slot blot protocol of Sambrook et al. (1989) incorporating [^{32}P]-labelled NR probes was followed in order to determine linear ranges of hybridisation signal intensity corresponding to RNA load. Typical results are shown in Figure 2.12. Samples with greater amounts of RNA (10-20 μg) did not pass through the nitrocellulose filter even after prolonged vacuum suction, and hybridisation signal was therefore reduced (Figure 2.12A). Although Sambrook et al. (1989) specify that up to 20 μg RNA can be applied to nitrocellulose filters, other reports indicate that the theoretical binding capacity of a nitrocellulose "slot" is about 8 μg RNA, and 5 μg RNA is the recommended maximum (Melzer et al. 1989; Nagy et al. 1989). Excess loading may have contributed to a relative hybridisation intensity that was not positively correlated to RNA load in the 5-9 μg range (Figure 2.12B). In addition, non-specific binding of radioactive probe was evident at lower RNA values, with 0.01 μg RNA having a 1.8-fold greater signal than 0.5 μg RNA.

Possible causes of the lack of correlation between load and signal observed in Figures 2.12 include interference caused by endogenous proteins in impure RNA samples (Meinkoth & Wahl 1984), and contamination of reagents. However, non-specific signal was present even in the presence of very low quantities of RNA sample, indicating that other factors must have contributed to non-specific signal. Attempts to determine whether the formaldehyde denaturation reagents were responsible were not successful as no radioactive signal was obtained when a Biorad Bio-Dot alkaline denaturation protocol was followed (results not shown). On the basis of these results it was decided that the RNA slot blot technique could not be employed for accurate quantification of NR transcript relative abundance during tobacco callus differentiation. Northern blotting was, therefore, the analytical technique of choice for quantitative and specific detection of NR mRNA.

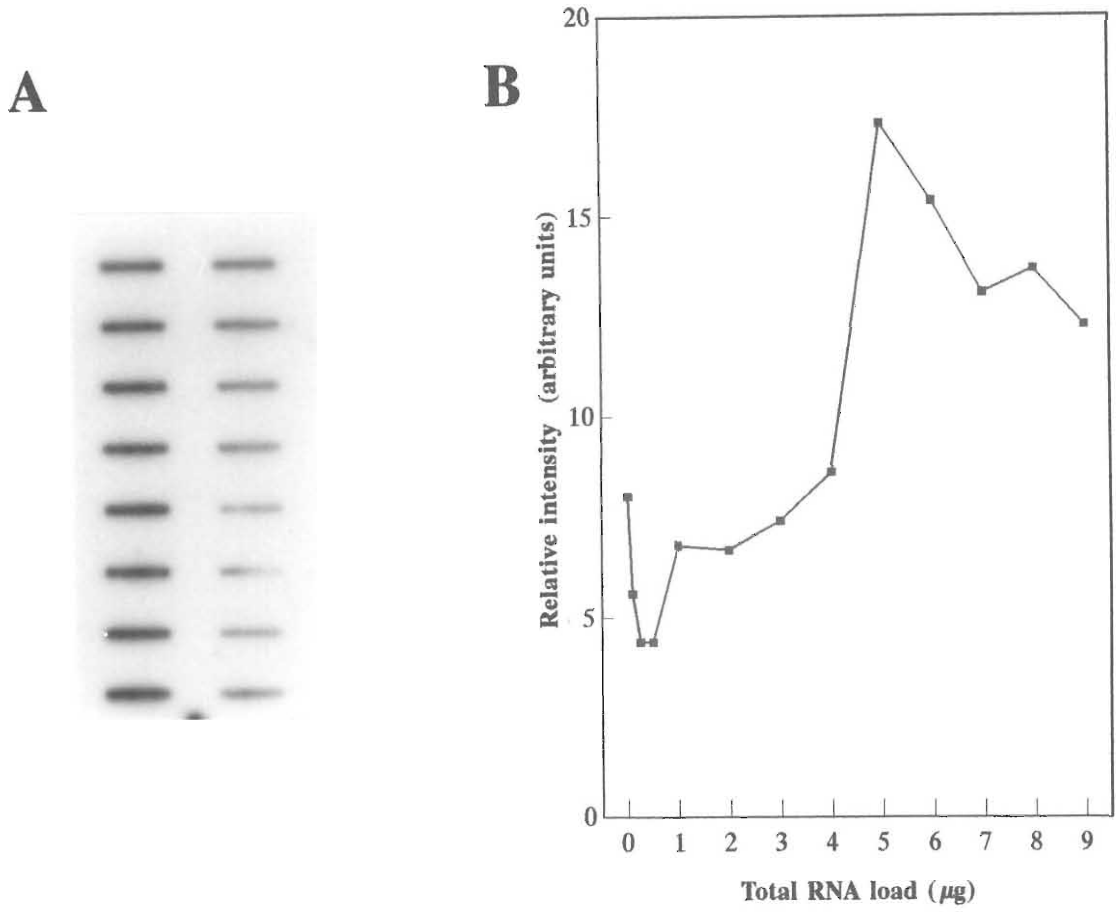

Figure 2.12: Quantification of NR mRNA from a total RNA concentration series by slot blot analysis.

Tobacco callus total RNA slot-blotted onto a nitrocellulose filter was hybridised with [^{32}P]-labelled NR cDNA probe (A). RNA amounts (μg) applied were: (left column, top to bottom) 20, 15, 10, 9, 8, 7, 6 and 5; (right column, top to bottom) 4, 3, 2, 1, 0.5, 0.25, 0.1 and 0.01. Relative hybridisation signal intensity of slots from 0.01-9 μg RNA was quantified by densitometer tracings of two different autoradiograph exposures (B).

2.4 Conclusions

The *in vivo*, *in situ* and *in vitro* NRA assays, optimised for use with tobacco callus, produced similar values and patterns for the enzyme during tobacco callus culture development. The *in vivo* assay was selected for NRA measurement during subsequent callus differentiation experiments. Tobacco NR fragments generated after SDS-PAGE of acetone-extracted callus material were detected after Western blotting using a spinach NR antibody. Acetone extracted callus samples resolubilised in SDS treatment buffer yielded optimal NR bands for quantification in comparison to various other extraction procedures. Tobacco callus NR mRNA was detected by Northern blot analysis using a conspecific partial cDNA probe. Non-specific binding rendered RNA slot blotting unsuitable for quantitative NR mRNA detection. Methods developed for NR regulation studies during tobacco callus development therefore allow for analysis at the gene transcript, protein and activity level (Chapter 3).

CHAPTER 3. REGULATION OF NITRATE REDUCTASE DURING IN VITRO TOBACCO CALLUS DIFFERENTIATION

3.1 Introduction

Plant gene expression has been found to be regulated highly in many cases (Kuhlemeier et al. 1987). In particular, expression of genes involved in nitrate reduction is sensitive to a wide range of environmental conditions, allowing for strict control of nitrogen assimilation (Beevers & Hageman 1969; Guerrero et al. 1981; Crawford & Davis 1989). NR is likely to be the main point where regulation of nitrate reduction is effected because NR is the first enzyme, substrate inducible, unstable and rate-limiting (Beevers & Hageman 1969, 1980; Campbell 1989; Solomonson & Barber 1990). Regulation of NR has been researched extensively due to the potential for increasing the efficiency of nitrogen assimilation (Campbell & Smarrelli 1986; Campbell & Kinghorn 1990; Solomonson & Barber 1990).

Many different modes of regulation act on NR either simultaneously or sequentially (Figure 3.1), yielding three main effects: control of substrate access (nitrate transport and translocation, reductant supply), NR amount (transcriptional and translational control, degradation) and NRA (inactivation/activation, degradation) (Hewitt et al. 1979; Solomonson & Barber 1990). Provision of nitrate, including the processes of uptake, storage and translocation (Section 1.1.2), and the supply of reductant (Section 1.1.6) are important components of NR regulation (Guerrero et al. 1981; Naik et al. 1982; Campbell 1989), but are not elaborated further here. The amount of active NR is a function of the controlled biosynthesis/degradation of NR and the change in catalytic effectiveness of existing enzyme (Hewitt et al. 1979; Beevers & Hageman 1980; Guerrero et al. 1981; Somers et al. 1983; Campbell 1988, 1989). Active enzyme can lose activity by reversible inactivating mechanisms involving NADH, adenine nucleotides, darkness or protein inactivators (Aparicio & Maldonado 1979; Hewitt et al. 1979; Beevers & Hageman 1980; Aryan et al. 1983; Campbell & Smarrelli 1986; Trinity & Filner 1991), although NADH may be involved also in activating NR that has been inactivated by protein inhibitors (Yamada et al. 1980a). Sensitivity of NR thiol sites to oxygen was thought previously to provide a mechanism for reversible inactivation (Hewitt et al. 1979), but more recent studies suggest that oxygen inhibition of NR is mainly at the level of translation (Kenis & Campbell 1989). It has not been demonstrated conclusively whether cyanide, which forms a reversible complex with Mo in *Chlorella* (Solomonson & Spehar 1977, 1979), plays a role in higher plant NR inactivation (Smith &

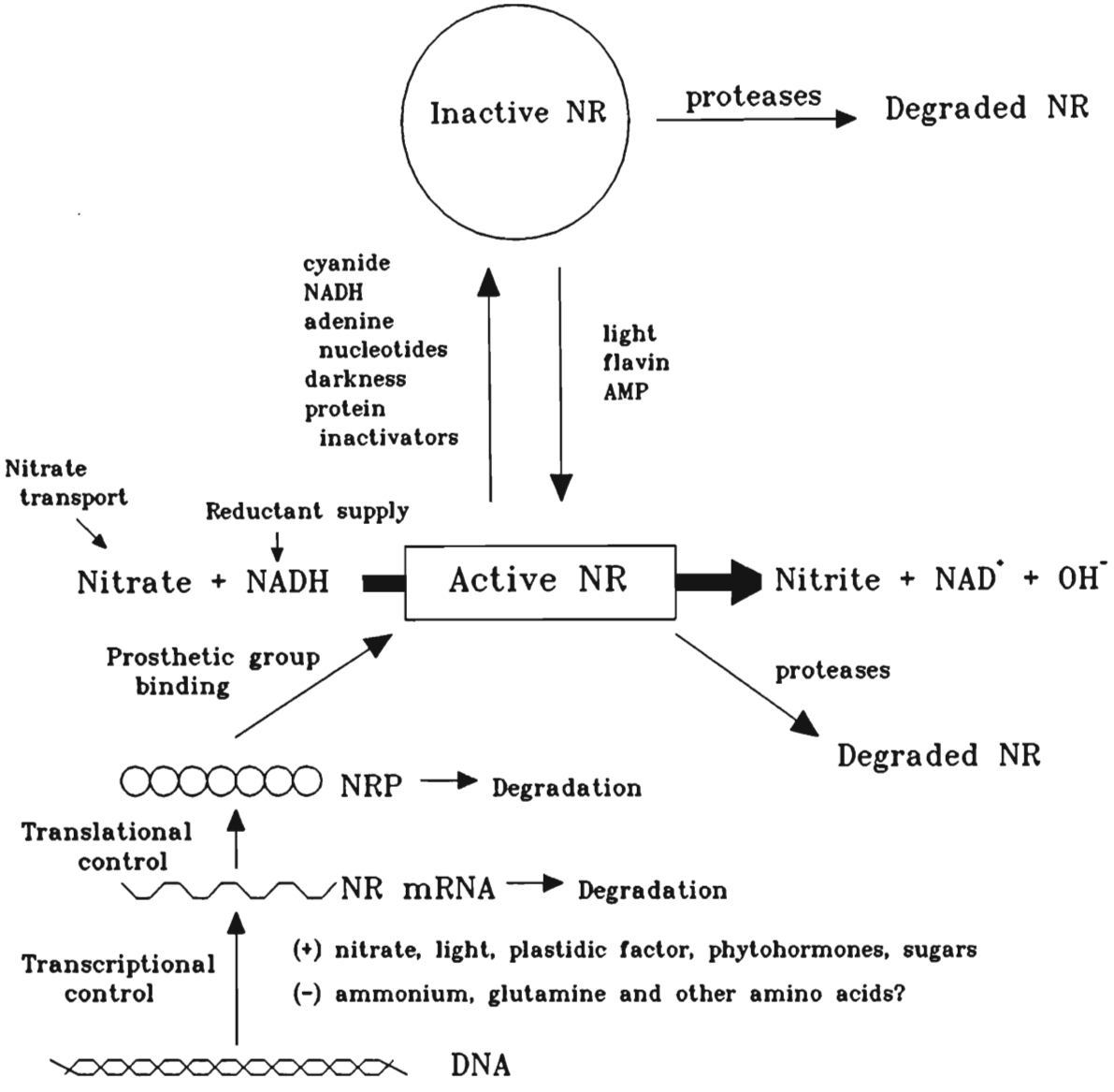

Figure 3.1: Regulation of higher plant NR.

Potential physiological regulatory mechanisms for the control of higher plant NR gene expression at the transcriptional, translational and post-translational level are illustrated. (Adapted from Solomonson & Barber 1990.)

Thompson 1971; Hewitt 1975; Aparicio & Maldonado 1979; Hewitt et al. 1979; Solomonson & Barber 1990). Irreversible loss of NR by proteolytic degradation with specific and general proteases has been researched extensively, as discussed in Section 2.1.1. Enzyme protein turnover, a significant general biological regulatory mechanism (Huffaker & Peterson 1974), is thus an important component of NR regulation.

This introduction examines how NR is influenced by factors that affect NR transcription, translation, inactivation/activation or proteolysis: nitrate, nitrate assimilation products, light, metals, carbohydrates and phytohormones. In addition, the regulation of NR during plant development is reviewed. The rest of the chapter describes experiments which establish aspects of the control of NR expression at translational and post-translational levels during *in vitro* tobacco callus differentiation.

3.1.1 Nitrate induction of NR

Numerous studies involving different higher plant species have shown that nitrate enhances NRA (Beevers & Hageman 1969; Ferrari & Varner 1969; Hewitt 1975; Stewart & Rhodes 1977; Hewitt et al. 1979; Campbell 1987; Wray 1988). Nitrate induction of NRA requires *de novo* protein synthesis, as demonstrated initially by inhibitor studies (Schrader et al. 1968; Beevers & Hageman 1969; Ferrari & Varner 1969; Aslam et al. 1976; Srivastava 1980; Guerrero et al. 1981), and later using specific immunochemical methods (Somers et al. 1983; Campbell & Smarrelli 1986; Remmler & Campbell 1986; Campbell 1987; Privalle et al. 1989; Campbell 1990; Rouze et al. 1990). Furthermore, subsequent to the isolation of NR cDNA clones, workers were able to demonstrate that application of nitrate to nitrate-starved plants resulted in elevated levels of NR mRNA in all species examined, including *Arabidopsis* (Cheng et al. 1986; Crawford et al. 1988), barley (Melzer et al. 1989), maize (Gowri & Campbell 1989; Privalle et al. 1989; Campbell 1990), soybean (Smarrelli et al. 1987), squash (Crawford et al. 1986; Crawford & Davis 1989), tobacco (Calza et al. 1987; Caboche et al. 1989; Rouze et al. 1990) and tomato (Rouze et al. 1990). Using isolated maize (Campbell 1990) and soybean (Callaci and Smarrelli 1991) leaf nuclei, it was established that elevated steady-state NR mRNA levels after nitrate addition was caused by an increase in the rate of transcription, rather than decreased mRNA degradation.

NR mRNA and NRP accumulate more rapidly than observed increases in NRA after application of nitrate (Remmler & Campbell 1986; Campbell 1987; Rouze et

al. 1990; Solomonson & Barber 1990; Friemann et al. 1992). Nitrate-induced NR mRNA can be detected after a few min in tobacco and tomato leaves, whereas NRA enhancement usually occurs after a lag period of at least 30 min (Galangau et al. 1988; Rouze et al. 1990). However, rates of induction differ between plant species and also between different organs of the same species (Beevers & Hageman 1969; Srivastava 1980; Remmler & Campbell 1986; Langendorfer et al. 1988; Crawford & Davis 1989; Martino & Smarrelli 1989; Melzer et al. 1989; Privalle et al. 1989). Maximal induction of NRP and NRA often occurs while NR mRNA levels are decreasing, indicating regulatory mechanisms other than induction control are involved (Melzer et al. 1989; Privalle et al. 1989; Oaks et al. 1990). Feedback control of NR mRNA synthesis by NRA or a product of nitrate assimilation has been proposed (Privalle et al. 1989). That proposal is supported by work with tungstate-grown tobacco plants that are induced by nitrate at the NR mRNA and NRP level but have no NRA: lack of active NR results in the maintenance of high NR mRNA and NRP levels compared with control plants (Deng et al. 1989b). Synthesis of NRP at a faster rate than the appearance of NRA suggests that post-translational regulation by cofactor availability or integration plays some role (Remmler & Campbell 1986; Campbell 1987; Galangau et al. 1988; Campbell 1990; Solomonson & Barber 1990). Additionally, nitrate withdrawal results a rapid decrease of NRA, but a delayed decrease in NR mRNA, indicating that nitrate also influences NR mRNA translation or NRP turnover (Somers et al. 1983; Galangau et al. 1988; Rouze et al. 1990).

Nitrate does not produce general increases in cellular proteins in squash cotyledons (Martino & Smarrelli 1989) or maize roots (McClure et al. 1987). In maize, only a few RNA species, probably related to nitrate assimilation, are induced by nitrate (McClure et al. 1987), although Yamazaki et al. (1986) reported a nitrate stimulation of carbon assimilation enzymes at mRNA level in that species. NiR is induced by nitrate at the transcriptional level in similar ways to NR induction (Rajasekhar & Mohr 1986, 1987; Back et al. 1988; Privalle et al. 1989; Schuster & Mohr 1990; Back et al. 1991; Seith et al. 1991; Friemann et al. 1992; Neininger et al. 1992). Back et al. (1991) showed that if the NiR promoter region was fused to the β-glucoronidase (GUS) reporter gene, then nitrate regulated GUS activity. For NR induction, nitrate probably acts via a regulatory protein that binds to an area flanking the NR gene promoter and induces transcription (Campbell 1988; Gowri et al. 1992). Alternatively, nitrate may induce conformational changes in a repressor protein associated with the NR gene, allowing transcription to proceed (Martino & Smarrelli 1989). Although at least 40 putative transcriptional factors have been isolated from higher plants thus far (Katagiri & Chua 1992), the protein involved in nitrate induction has

not been identified. The proposed nitrate inducer/repressor protein is constitutive (protein synthesis inhibitors do not prevent nitrate induction of NR mRNA) and is converted to an active/inactive form directly by nitrate (Gowri et al. 1992).

Other substances, such as phytohormones, reportedly can induce NADH:NR in the absence of nitrate (Guerrero et al. 1981). Excision of maize scutella from seeds has been shown to induce NR by *de novo* mRNA synthesis (Garate et al. 1989; Gowri et al. 1992). However, NR induction apparently is very sensitive to low levels of nitrate in maize, and nitrate contamination of washed vermiculite and germination paper was sufficient to induce NR without added nitrate (Oaks et al. 1988, 1990). Using a hydroponic system, those authors were able to demonstrate that no detectable NRP was present in maize plants without added nitrate. Oxidation of reduced nitrogen compounds to form nitrate may occur in higher plants under certain conditions, as has been shown for disinfected germinating soybean seeds (Funkhouser & Garay 1981). While endogenous nitrate formation may explain the induction of NR without additional nitrate in some cases, it is not definite yet whether the presence of nitrate is obligatory for induction in all higher plant species possessing non-constitutive NADH:NR.

3.1.2 Effect of nitrate assimilation products on NR

Feedback inhibition of the nitrate assimilation pathway possibly plays a role in some higher plant species (Stewart & Rhodes 1977; Srivastava 1980; Guerrero et al. 1981). While some workers have shown that ammonium inhibits NRA (Smith & Thompson 1971; Srivastava 1980; Guerrero et al. 1981), others have found variable or enhancement effects (Beevers & Hageman 1969; Hewitt 1975; Hewitt et al. 1979; Oaks 1979; Sihag et al. 1979; Mehta & Srivastava 1980; Curtis & Smarrelli 1987; de la Haba et al. 1988). Ammonium did not influence nitrate induction of NR mRNA in maize seedlings (Privalle et al. 1989) or in *Arabidopsis* leaves (Crawford et al. 1988), although ammonium may exert its influence post-translationally (Srivastava 1980). In *Betula pendula*, ammonium suppression of NR expression was reported in roots but not in leaves (Friemann et al. 1992), whereas the converse was found in maize plants (Oaks et al. 1979). Variable results may be attributed to differences in regulation between species, or dissimilar experimental conditions (Beevers & Hageman 1980; Srivastava 1980). Differences between roots and shoots might be due to differences in metabolic patterns, with leaves capable of tolerating high levels of photorespiratory ammonium (Campbell & Smarrelli 1986).

The effects of amino acids on NR, in both whole plant and cultural studies, are variable (Filner 1966; Beevers & Hageman 1969; Gamborg 1970; Heimer & Riklis 1979; Oaks 1979; Zink 1982; Kamada & Harada 1984; Marion-Poll et al. 1984; Bonner et al. 1992). It has been difficult to determine whether amino acid effects were specific for NR or caused by interference with general plant growth and metabolism (Beevers & Hageman 1980). However, glutamine has been shown to inhibit steady-state NR mRNA levels in soybean (Smarrelli et al. 1987) and to diminish NR mRNA, NRP and hence NRA levels in squash cotyledons (Langendorfer et al. 1988; Martino & Smarrelli 1989). An NR regulatory role for glutamine was suggested earlier by Stewart and Rhodes (1977) who reported that glutamine levels and NRA were inversely correlated in some higher plants. While amino acids and ammonium have little effect *in vitro*, they probably play some role in at least short-term regulation of higher plant NR (Solomonson & Barber 1990).

3.1.3 Induction and regulation of NR by light

Light, an important environmental factor for the development of higher plants, is involved in the expression of diverse genes, although activation mechanisms differ (Smith et al. 1977; Tobin & Silverthorne 1985; Fluhr et al. 1986; Kuhlemeier et al. 1987; Redinbaugh & Campbell 1991). The complex and multiple roles of light in higher plant NR regulation have been extensively investigated (Hewitt 1975; Campbell 1988). Levels of NRA are usually higher in the light, but depend also on light duration and fluence rates (Beevers & Hageman 1969; Travis et al. 1970; Nicholas et al. 1976a; Srivastava 1980; Reed & Canvin 1982; Abrol et al. 1983; Reed et al. 1983; Duke & Duke 1984; Bowsher et al. 1991). Nitrate appears to be required for light induction of NR, indicating that light acts as a modulator rather than a primary inducer (Travis et al. 1970; Buczek 1976; Abrol et al. 1983; Rajasekhar & Oelmüller 1987; Campbell 1989, 1990). Light induction of NR is not restricted to photosynthetic tissues, but has been reported in roots (Duke and Duke 1984). It is assumed generally that light control of NRA is necessary to prevent potentially damaging effects of nitrite (Sawhney et al. 1976b; Duke & Duke 1984; Faure et al. 1991). Additionally, nitrite accumulation is prevented by the simultaneous photoinduction of NR and NiR (Rajasekhar & Mohr 1986, 1987; Rajasekhar & Oelmüller 1987; Schuster & Mohr 1990; Seith et al. 1991; Neininger et al. 1992).

A lag phase of 1-2 h in the photoinduction of NRA in various species implied that *de novo* NRP synthesis was required (Duke & Duke 1984). General protein synthesis inhibitors eliminated light induction of NR in different species by 84-100% (Buczek

1976; Sihag et al. 1979; Rao et al. 1980; Gupta et al. 1983; Kakefuda et al. 1983). Light has a stimulatory effect on general protein synthesis (Beevers & Hageman 1980; Naik et al. 1982; Campbell 1987) and increases polyribosome levels, resulting in elevated transcription levels (Travis et al. 1970; Travis & Key 1971; Smith et al. 1977; Tobin & Silverthorne 1985). However, light enhances nitrate induction of NR mRNA levels relative to other mRNA species, indicating that light has a specific regulatory effect on NR transcription (Remmler & Campbell 1986; Campbell 1988; Melzer et al. 1989; Warner & Kleinhofs 1992).

Light induces NR by the following possible mechanisms: (1) an increase in the uptake or availability of nitrate (Beevers & Hageman 1980; Srivastava 1980; Guerrero et al. 1981; Naik et al. 1982; Melzer et al. 1982); (2) enhanced photosynthesis, allowing for the provision of more reductant for NRA (Sawhney et al. 1978b; Naik et al. 1982; Campbell & Smarrelli 1986; Campbell 1988); (3) an effect on the relative activities of specific NR inhibitors and/or activators, as reported in leaves of rice (Leong & Shen 1982), soybean (Jolly & Tolbert 1978) and wheat (Sherrard et al. 1979); (4) an involvement in enhancing production of cofactors for active NR synthesis from NR apo-protein (Campbell 1990); (5) phytochrome stimulation of NR (Jones & Sheard 1972; Srivastava 1980; Crawford & Campbell 1990); or (6) dephosphorylation mechanisms controlled by adenine nucleotide levels for the activation of pre-existing NR (Kaiser & Spill 1991; Huber et al. 1992a, b, c). Details of the extensively researched phytochrome and phosphorylation/dephosphorylation mechanisms are provided in the ensuing discussion.

Phytochrome is a photochromic protein that exists in two photoconvertible isomeric forms, Pr and Pfr, with Pr converted to the biologically active Pfr by a pulse of red light, and Pfr reconverted to Pr by far-red illumination (Smith et al. 1977; Fluhr et al. 1986; Kuhlemeier et al. 1987). Phytochrome involvement in NR regulation was inferred from observations that NRA could be modulated reversibly by red/far-red light pulses in etiolated tissues (Jones & Sheard 1972; Hewitt 1975; Johnson 1976; Srivastava 1980; Abrol et al. 1983; Tobin & Silverthorne 1985; Campbell 1988, 1989; Crawford & Campbell 1990). NR can be induced by two different phytochrome mechanisms, the low irradiance response (LIR) and high irradiance response (HIR) (Duke & Duke 1984). The LIR, first demonstrated in etiolated *Pisum arvense* seedlings (Jones & Sheard 1972), is saturated at low fluence rates of red light, requires short pulse periods (30 min), and is reversed by far-red light (Rao et al. 1980; Duke & Duke 1984; Rajasekhar et al. 1988; Melzer et al. 1989). The HIR requires a minimum radiation period of 2-3 h of continuous far-red or blue light, and can induce

higher levels of NRA *in vitro* than the LIR (Rao et al. 1980; Duke & Duke 1984; Melzer et al. 1989). Different NR induction mechanisms by the LIR and HIR may explain contradictory reports on the effects of mRNA and protein synthesis inhibitors on the phytochrome response (Johnson et al. 1976; Rao et al. 1980).

In etiolated, but not green, barley seedlings, red and blue light enhanced NR mRNA production (Melzer et al. 1989), while NRP and NRA levels were reversibly enhanced by the LIR in etiolated squash cotyledons (Rajasekhar et al. 1988). Those results suggested that phytochrome facilitates NR transcription induction in etiolated but not green leaves. In both barley and squash cotyledons, however, white light was even more efficient at inducing NR than red, continuous far-red or blue light treatments, indicating that light regulates NR by mechanisms other than phytochrome (Rajasekhar et al. 1988; Melzer et al. 1989).

Phytochrome induction of NR and other enzymes depends in some species on a plastidic factor from intact plastids (Duke & Duke 1984; Oelmüller et al. 1986, 1988; Crawford & Campbell 1990; Oelmüller & Briggs 1990). Börner et al. (1986) found very low levels of leaf NRA in *albostrians* (barley) and *iojap* (maize) mutants that lack plastid ribosomes. Using the herbicide norflurazon, which inhibits carotenoid synthesis and thus causes chlorophyll bleaching, it was reported that a chloroplast factor was required for light induction of the constitutive soybean NR form (Kakefuda et al. 1983; Duke & Duke 1984), and for photoinduction of NR mRNA in squash cotyledons (Oelmüller & Briggs 1990). Light was unable to stimulate NR induction in carotenoid-free and photooxidatively treated *Sinapis alba* (mustard) cotyledons (Oelmüller et al. 1988). Loss of functional intact plastids may result in the loss of a specific regulatory signal, required for the cytoplasmic synthesis of many plastid proteins (Bradbeer et al. 1979), or simply inhibit NR induction by causing an accumulation of toxic levels of nitrite (Oelmüller & Briggs 1990). It has been suggested that the three main factors of NR induction, ie. plastidic signal, nitrate and light, regulate enzyme transcription in an hierarchical manner (Rajasekhar & Oelmüller 1987).

Reversible inactivation of NR during a light/dark transition was noticed in maize leaves, where transferal to dark caused a fairly rapid decrease in NRA (30% in 1 h) without affecting NRP (Remmler & Campbell 1986; Campbell 1987). In spinach leaves placed in the dark, an even more rapid inactivation of NRA was observed in the presence of magnesium or calcium ions, with $t_{\frac{1}{2}}$ = 2 min for 85% inactivation (Riens & Heldt 1992). Recent evidence suggests that light/dark modulation of NR

is achieved by phosphorylation (Campbell & Ingemarsson 1992; Huber et al. 1992a, b, c). Phosphorylation of spinach leaf NR seryl residues (two associated specifically with NRA) after transferal to dark was demonstrated using [^{32}P]Pi, and partial dephosphorylation of these sites occurred when the leaves were returned to the light or fed the phosphate-sequestering sugar mannose (Huber et al. 1992a, b). Furthermore, inhibition of type 1 and/or type 2A protein phosphatases with okadaic acid prevented the light reactivation of NR (Huber et al. 1992a, b, c). A comparison of NR with sucrose-phosphate synthase, an enzyme shown to be regulated by protein phosphorylation, revealed many common regulatory features (Huber et al. 1992c). For both enzymes, light-mediated dephosphorylation was prevented by transcription and protein synthesis inhibitors, indicating that the protein phosphatase reactivating both enzymes is activated itself by light, and that protein synthesis is required for reactivation (Huber et al. 1992c).

The involvement of adenine nucleotides in the light regulation of NR was suggested from previous studies by some workers (Hewitt 1975; Hewitt et al. 1979; Sawhney et al. 1978a, b), but not by others (Reed & Canvin 1982). It now appears that adenine nucleotides may be involved in the light/dark phosphorylation-based regulation of NR: dark inactivation of spinach leaf NR was prevented by anaerobiosis or by 2,4-dinitrophenol, an uncoupler of respiratory electron transport, both of which decreased ATP and increased AMP levels (Kaiser et al. 1992). In the presence of the myokinase inhibitor p^1,p^5-di(adenosine-5')pentaphosphate which prevents ATP consumption, *in vivo* dark-inactivated NR was reactivated *in vitro* with AMP (Kaiser et al. 1992). A recent regulation model suggests that when NR is transferred to darkness, protein phophorylation with MgATP (or MgUTP) as substrate induces conformational changes in NR that render the enzyme sensitive to free magnesium or calcium ions, causing inactivation (Kaiser & Spill 1991). When returned to the light, the ATP/AMP ratio decreases, and NR is dephosphorylated, causing an enhancement of NRA (Kaiser & Spill 1991). Involvement of AMP in the light regulation of NR is not certain, however, as cytosolic levels are reportedly very low, at least in wheat leaves (Stitt et al. 1982). In addition to genetic mechanisms involved in light regulation of NR, protein phosphorylation also may play a role (Kaiser & Spill 1991; Campbell & Ingemarsson 1992; Huber et al. 1992a, b, c; Kaiser et al. 1992).

3.1.4 Diurnal fluctuations of NR

Many workers have demonstrated that NR from different plant species grown under

normal light/dark cycles exhibits diurnal rhythmicity (Hageman et al. 1961; Beevers & Hageman 1969; Srivastava 1980; Wallace 1987; Caboche et al. 1989; Rouze et al. 1990; Lillo 1991). In tobacco leaves, NRA was low towards the end of the dark period, increased rapidly to maximum levels shortly after the start of a photoperiod, and then decreased gradually (Galangau et al. 1988; Caboche et al. 1989). Similar fluctuations were observed in other species, although NRA may reach maximal levels up to 6 h after the start of the photoperiod (Lillo 1983; Caboche et al. 1989; Lillo 1991). NRP followed similar diurnal trends to NRA, although some changes in NR specific activity suggest that post-translational activation/inactivation mechanisms are involved (Galangau et al. 1988; Caboche et al. 1989; Rouze et al. 1990; Lillo 1991). NR mRNA, by contrast, accumulated during the latter part of the dark period and declined during the light period in tobacco and tomato (Galangau et al. 1988; Caboche et al. 1989; Deng et al. 1990), whereas corn NR mRNA increased at the start of the photoperiod and then immediately decreased rapidly, maintaining low levels for the rest of the day (Lillo 1991). Disparities between the fluctuations of NR mRNA and NRP indicate that post-transcriptional regulation is involved in NR diurnal rhythmicity.

The amplitude of barley leaf NRA oscillations diminished when light intensity of the photoperiod was decreased (Lillo 1984), and NRA circadian rhythms of barley and other species were completely abolished after continuous light or dark treatments (Lillo 1984; Crawford & Campbell 1990; Rouze et al. 1990). In tobacco leaves transferred to continuous darkness, the rhythmicity of NRP and NRA decreased at a substantially faster rate than NR mRNA, indicating that diurnal rhythms are controlled initially at the RNA level (Deng et al. 1990). Unlike induction of NR by nitrate, NRA is required for diurnal regulation - abolition of NR catalytic activity by biochemical means (eg. tungstate) or via genetic impairment leads to a loss of rhythmicity and an overexpression of NR mRNA (Pouteau et al. 1989; Caboche & Rouze 1990; Deng et al. 1990; Rouze et al. 1990; Faure et al. 1991; Redinbaugh & Campbell 1991). NiR and NR appear to have a common diurnal regulation mechanism, with similar diurnal fluctuations and a comparable overexpression of NiR mRNA in NRA-inhibited *Nicotiana plumbaginifolia* plants (Faure et al. 1991).

Glutamine may be involved in the decrease of NRA and/or NR mRNA during the diurnal cycle (Galangau et al. 1988). Levels of glutamine have been shown to oscillate in opposition to NR mRNA, and elevation of glutamine levels by inhibition of GS caused an increase in NR mRNA levels and abolished rhythmicity (Crawford & Campbell 1990). Also, in tobacco leaves, a relationship has been demonstrated be-

tween NRA and malate, which neutralises hydroxyl ions generated by NRA (Deng et al. 1989c). Those workers found that leaf malate levels, in contrast to NRA, increased during the day and decreased at night, that malate and NRA yielded similar characteristics during a transition from light/dark regimes to continuous light or dark treatments, and that malate accumulation was suppressed if NRA was inhibited by tungstate treatment. Collectively, research on diurnal fluctuations of NR suggests that different biochemical pathways and various levels of regulation are involved; details of those mechanisms are not known.

3.1.5 Regulation of NR by metals

The most widely studied metal in relation to NR regulation is Mo (Beevers & Hageman 1969; Jones et al. 1978; Beevers & Hageman 1980; Srivastava 1980; Notton 1983; Rajagopalan 1989). The Mo-pterin cofactor, a Mo-containing complex that is synthesised separately and added to apo-NR, may be rate-limiting for the conversion of apo-NR to catalytically functional NR (Beevers & Hageman 1980; Remmler & Campbell 1986; Campbell 1987; Solomonson & Barber 1990). The Mo-containing complex (MCC) has a shorter half-life than holo-NR *in vitro*, indicating that MCC is stabilised when bound to apoprotein (Hewitt et al. 1979). When Mo was infiltrated into leaves of plants grown without Mo, NRA was induced in a biphasic manner, possibly representing synthesis and binding of the Mo-pterin cofactor (Hewitt et al. 1979). In comparison, NRA induction by Mo in Mo-limited PSR cells was linear, and was not prevented by transcription or protein synthesis inhibitors (Jones et al. 1978). Although nitrate stimulates the appearance of Mo-cofactor, light has no apparent effect (Campbell et al. 1987; Campbell 1988). While Mo has been shown to exert a specific effect on NRA, regulation of NR by other metal ions such as boron, lithium, manganese, potassium, rubidium and sodium are less well described (Srivastava 1980; Leidi & Gomez 1985; Augsten & Michel 1986; Köhler et al. 1992).

3.1.6 Regulation of NR by carbohydrates

Glucose can induce NRA in some plants, but not in others (Oaks 1979; Srivastava 1980). Glucose may influence NRA indirectly by determining nitrate distribution, particularly by mediating an increase in the size of the metabolic nitrate pool, or through general enhancement of protein synthesis (Aslam & Oaks 1975; Aslam et al. 1976; Oaks 1979; Srivastava 1980). Similarly, sucrose can enhance NRA indirectly by stimulating general RNA and protein synthesis and/or increasing metabolic

nitrate (Sahulka et al. 1975; Sihag et al. 1979; Puranik & Srivastava 1983). However, in dark-adapted *Arabidopsis* plants, glucose and sucrose were able to replace light in causing an induction of NR mRNA, with the 5' flanking sequence of the NR gene sufficient to confer this response (Cheng et al. 1992). Thus, regulation of NR by carbohydrates may be effected directly and/or indirectly.

3.1.7 Hormonal regulation of NR

Changes in phytohormone concentration affect many higher plant enzymes (Varner & Ho 1977). Auxin induced a primary response in soybean hypocotyls (Hagen et al. 1984) and pea epicotyls (Theologis et al. 1985), resulting in a rapid increase in transcription of specific mRNA species, independent of protein synthesis. While various phytohormones have been shown to regulate the tissue levels of NRA (Beevers & Hageman 1969; Hewitt 1975; Beevers & Hageman 1980; Srivastava 1980), the phenomenon has not been thoroughly investigated (Solomonson & Barber 1990). A primary response to cytokinins was reported in etiolated barley seedlings (Lu et al. 1990), whereas protein synthesis inhibitors prevented an increase in NR mRNA transcription induced by benzyl adenine (BA) in etiolated barley leaves (Lu et al. 1992b). Furthermore, in etiolated maize leaves, RNA and protein synthesis inhibitors eliminated a kinetin-induced increase in NRA (Rao et al. 1984), while in *Agrostemma githago* embryos, NRA was induced rapidly by various cytokinins in the absence of added nitrate (Varner & Ho 1977). The modes of action of phytohormones that replace nitrate or light in inducing NR are probably different from those primary inducers (Srivastava 1980; Rao et al. 1984). The developmental state of a particular tissue may be an important factor in determining whether NR responds to exogenously supplied phytohormones (Garate et al. 1989).

3.1.8 Regulation of NR during plant development

The developmental state of germinating seedlings affects inducibility and steady state levels of NR (Srivastava 1980; Rajasekhar & Oelmüller 1987; Privalle et al. 1989). An increase in NRA after the commencement of seed or embryo germination has been observed in barley (Gupta et al. 1979, 1983), *Gossypium hirsutum* (cotton) (Radin 1974), maize (Bowsher et al. 1991), soybean (Radin 1974; Kakefuda et al. 1983), sunflower (de la Haba et al. 1988) and wheat (Disa et al. 1982, 1985). NR did not appear to be associated with germination in *Agrostemma githago* (Köhler et al. 1992), and reportedly did not contribute to cotton germination, even though NRA was enhanced (Radin & Trelease 1976). However, studies on barley (Gupta et al.

1979, 1983), cotton (Radin 1974), soybean (Carelli & Magalhaes 1981) and wheat (Disa et al. 1985) have shown that after an initial non-inducible phase, NR increased during germination even in the presence of transcription inhibitors, but was suppressed by protein synthesis inhibitors. Those results indicated that stored stable NR mRNA was present in dormant seeds or embryos, and suggested that NR was regulated developmentally during the germination process. Conversely, during wheat grain maturation, NRA was observed to decrease as the seed dehydrated and abscisic acid levels increased (Singh & Vijayakumar 1981). Transitory low NRA levels during dormant periods were reported also for *Solanum tuberosum* tubers (Palmer 1979; Kapoor & Li 1982).

NRA changes during leaf development in various species, including barley (Brown et al. 1981), *Cucumis sativus* (Blahova & Segeta 1980), maize (Travis & Key 1971; Kenis et al. 1992), *Phaseolus vulgaris* (Timpo & Neyra 1983), soybean (Santoro & Magalhaes 1983), tobacco (Wakhloo & Staudt 1988) and wheat (Grover et al. 1978; Hallam & Blackwood 1979; Lawlor et al. 1987). Commonly, highest rates of NRA are observed during maximal rates of leaf expansion, when levels of endogenous nitrate are high (Blahova & Segeta 1980; Santoro & Magalhaes 1983; Lawlor et al. 1987). Higher steady-state NRP and NRA in younger maize leaves are due to elevated rates of transcription, even though NR mRNA levels are comparable in younger and older leaves (Kenis et al. 1992). *In vivo* rates of NR turnover are reportedly higher in younger maize leaves (Kenis et al. 1992), even though the converse has been found for barley leaf NR *in vitro* (Brown et al. 1981). Seasonal variation of NRA has been reported in leaves of soybean (Harper & Hageman 1972) and wheat (Jones & Whittington 1982). Not unexpectedly, studies comparable to those on NR have shown that other nitrate assimilation enzymes, *viz.* NiR, GS and GOGAT, are regulated developmentally (de la Haba et al. 1988; Hecht et al. 1988; Swarup et al. 1990; Kamachi et al. 1992; Zehnacker et al. 1992).

3.1.9 Conclusions

NR regulation is achieved by several sensitive and complex control mechanisms, with precise methods differing between higher plant species (Hewitt et al. 1979; Campbell & Smarrelli 1986; Caboche et al. 1989; Rouze et al. 1990). In addition to specific regulatory mechanisms, NRA levels are responsive to cellular conditions in general (Beevers & Hageman 1969; Campbell 1989). Future research in NR regulation will endeavour to identify *cis*- and *trans*-acting factors required for transcriptional control (Campbell 1989; Rouze et al. 1990). Those studies and others should

contribute to the understanding of the molecular control of NR and possible roles of the enzyme during plant developmental processes such as *in vitro* differentiation.

3.2 Materials and Methods

3.2.1 Reagents and biological materials

Sources of biochemical reagents and materials are identified in Section 2.2.1; additional phytohormones (BA and naphthaleneacetic acid [NAA]) were purchased from BDH Chemicals. Tobacco seeds (*Nicotiana tabacum* L. var. Samsun), spinach polyclonal NR rabbit antiserum and the plasmid pBMC102010 containing a 1.6 kb tobacco NR cDNA sequence were obtained from Karl Kunert, Ian Prosser and Michel Caboche, respectively, as specified in Section 2.2.1. An α-tubulin cDNA sample for use as an external RNA standard was a gift from Chong-maw Chen (Biomedical Research Institute, University of Wisconsin-Parkside, Kenosha, USA).

3.2.2 Establishment and maintenance of in vitro tobacco callus cultures

Tobacco callus cultures from surface-sterilised tobacco stem and leaf segments were established in darkness at 25°C on induction medium consisting of MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose, 1% agar, 2 mg/l IAA and 0.5 mg/l kinetin, as outlined in Section 2.2.2. Concentration of different nitrogen sources or phytohormones used for tobacco callus induction experiments (Figure 3.2 and Tables 3.1 and 3.2) are given in figure or table legends. Callus was maintained in darkness by subculturing on induction medium every 2-3 weeks, not more than 3 times before use. Callus material for the experiment represented in Figures 3.11-3.13 and Table 3.3 was subcultured once before use on induction medium with 2,4-D as sole phytohormone.

3.2.3 In vivo NRA assay

Callus samples (0.1-0.35 g f.wt) were placed in glass centrifuge tubes with 0.1 ml dH_2O. Tubes sealed with Suba-seal bungs were maintained in darkness using aluminum foil. The assay was carried out at 25°C with continuous N_2 (gas) flushing, and terminated after 30 min by adding 2-5 ml boiling dH_2O. Accumulated nitrite was quantitatively extracted from the callus by boiling for 30 min. Following clearing by centrifugation, nitrite in the supernatant was determined using a colorimetric assay (Section 2.2.3).

3.2.4 Protein extraction and estimation

Protein was extracted from callus tissue following the method of Wetter and Dyck (1983), as outlined in Section 2.2.4. TCA-precipitated protein was estimated using a Folin-Lowry assay modification of Wetter (1984), with BSA as a standard.

3.2.5 Determination of nitrate utilisation from agar

A modified freeze-thaw method after Lumsden et al. (1990) was used for the analysis of nitrate in agar medium (Section 2.2.5), and nitrate was quantified using the salicylic acid colorimetric method (Cataldo et al. 1975).

3.2.6 Western analysis of tobacco callus NRP

Acetone powders were prepared from tobacco callus material (0.1-0.5 g f.wt) using a modification of the Ibrahim & Cavia (1975) protocol described in Section 2.2.6 and were stored at -80^{o}C. After resolubilisation for 1.5 h on ice with occasional mixing in 1x SDS-treatment buffer (Hoefer gel electrophoresis protocol, 1992-1993), supernatants from pelleted acetone powder samples were separated by 9% SDS-PAGE (Section 2.2.6). Rainbow protein molecular weight markers were run as standards. Protein bands were transferred by electroblotting to nitrocellulose (Towbin et al. 1979) at 45 mA for 14 h using the Hoefer TE 70 SemiPhor Semi-dry Transfer Unit. Transfer efficiency was checked by staining the blotted gel with Coomassie Blue. Spinach polyclonal NR antiserum raised in rabbit was used as first antibody for NRP detection, with anti-rabbit IgG conjugated to alkaline phosphatase as second (indicator) antibody. Indicator antibody was detected using BCIP/nitro-blue tetrazolium chloride substrates (Section 2.2.6).

3.2.7 RNA extraction, electrophoresis and Northern blot analysis

RNase activity was minimised during RNA isolation by appropriate treatment of equipment and chemicals (Section 2.2.8). Total RNA was isolated from tobacco callus according to a modification of Verwoerd et al. (1989), as described in Section 2.2.8. RNA was quantified spectrophotometrically, and samples were stored at -80^{o}C.

Formaldehyde-denatured RNA samples (8 μg total RNA) and molecular weight markers were fractionated on a 1% denaturing agarose gel (Section 2.2.8) before

transferal to nitrocellulose according to Thomas (1980).

The NR probe sequence, a 1.6 kb NR cDNA fragment derived from the plasmid pBMC102010 (Section 2.2.8), and α-tubulin cDNA were radiolabelled with [α-^{32}P]dTTP to specific activities of $2x10^9$ and $1.2x10^8$ cpm/μg, respectively (Feinberg and Vogelstein 1983, 1984). Unincorporated nucleotides were removed by centrifugation on a Sephadex G-50 spun column (Sambrook et al. 1989). NR probe, at $2x10^8$ cpm/ml (10 ng DNA/ml), and α-tubulin probe, at 10^7 cpm/ml (150 ng DNA/ml), were employed simultaneously for hybridisation at 42°C in buffer containing 50% formamide, 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 10% (w/v) dextran sulfate and 0.1 mg/ml denatured, sheared salmon sperm DNA (Solution A). After hybridisation for 16 h, the filter was washed under stringent conditions: 2x SSC, 0.5% SDS at room temperature for 20 min; 2x SSC, 0.1% SDS at room temperature for 15 min; 0.1x SSC, 0.5% SDS at 37°C for 15 min; 0.1x SSC, 0.1% SDS at 68°C for 20 min; and 0.1x SSC at room temperature for 10 min. After an initial exposure to X-ray film at -80°C with intensifying screens for 95 hours, the filter was rewashed in very stringent conditions (as for stringent conditions, but including an additional wash with 0.1x SSC, 0.5% SDS at 68°C for 20 min), and then rehybridised at 40°C for 40 h using hybridisation buffer (Solution A) denatured at 80°C for 20 min. After washing in very stringent conditions, the filter was subjected to autoradiography.

3.2.8 Statistical procedures

T-tests for equality of means, ANOVAs (Analysis of Variance) and Student-Newman-Keuls (SNK) tests were performed using the Costat statistical package (Cohort Software, Berkeley, USA). Outliers were eliminated when detected using Dixon's test statistic, as described by Sokal & Rohlf (1981).

3.3 Results and Discussion

3.3.1 Effect of phytohormones on in vitro tobacco callus growth and differentiation

Phytohormones are included routinely in culture media to manipulate *in vitro* growth and differentiation (Thorpe 1980; Evans et al. 1981; Ammirato 1986; Tran Than Van & Trinh 1991). The ratio of two major classes of phytohormone, auxins and cytokinins, usually determines whether *in vitro* differentiation is in the form of shoots (higher auxin) or roots (higher cytokinin) (Thorpe 1980; Chih-hung et al. 1981; Evans et al. 1981; Ammirato 1986; Owens & Smigocki 1990; Tran Thahn Van & Trinh 1990). However, specific compounds within the auxin and cytokinin classes can elicit different responses that are dependent also on plant species (Fujimura & Komamine 1975; Caldas & Caldas 1976; Thorpe 1980; Chih-hung et al. 1981; Ammirato 1986). Truelsen and Wyndaele (1991) employed NAA and BA at 20 μg/l and 1 mg/l, respectively, to induce shoot formation in tobacco callus cultures. Chih-hung et al. (1981) found that BA up to 2 mg/l without NAA (or at low concentrations) induced tobacco callus shoot formation, whereas higher NAA/BA ratios caused prolific root production. Concentration ranges of IAA and kinetin regularly utilised for tobacco callus shoot formation are 0.3-1.8 mg/l and 1-2.2 mg/l, respectively (Thorpe and Meier 1972; Thorpe & Laishley 1973; Guerri et al. 1982; Joy et al. 1988). For the tobacco variety utilised here, IAA and kinetin were more effective at inducing callus shoot formation than NAA and BA (Table 3.1). IAA and kinetin concentrations in callus induction medium, also used by Thorpe & Laishley (1973) and Evans et al. (1981), were most effective at inducing shoot formation after three weeks in culture (Regime 1 in Table 3.1). Although higher BA concentrations than those examined here would possibly initiate more tobacco callus shoots (Chih-hung et al. 1981; Truelsen & Wyndaele 1991), the amount and rate of bud formation elicited by callus induction medium was suitable for the present study.

Table 3.1: Effect of phytohormone regimes on *in vitro* tobacco callus culture differentiation.

In two separate experiments, sterilised tobacco leaf discs were plated onto MS medium supplemented with 2% sucrose, 1% agar and different auxin (IAA or NAA) and cytokinin (kinetin or BA) concentrations, as indicated (n = 10-30 for each regime). Regime 1 represents standard callus induction medium. Cultures were maintained in darkness for two weeks before transferal to a 16/8 h light/dark regime. After three weeks in culture, differentiation (bud formation) was scored as follows: "A" = greater than 75% differentiation; "B" = less than 75% differentiation.

	Phytohormone treatment				Differentiation rating
	IAA (mg/l)	Kinetin (mg/l)	NAA (mg/l)	BA (μg/l)	
1.	2	0.5	-	-	A
2.	2	0.2	-	-	A
3.	-	-	1	0.5	B
4.	-	-	1	1	B
5.	-	-	2	0.5	B
6.	-	-	2	1	B

3.3.2 Effect of different nitrogen sources on in vitro tobacco callus growth and differentiation

Higher plant species differ in ability to grow and differentiate on different sources of nitrogen, an essential inorganic nutrient (Thorpe 1983; Ammirato 1986; Grimes & Hodges 1990; Lumsden et al. 1990). The nitrogen source in full-strength MS medium, ie. 20 mM ammonium and 40 mM nitrate (Murashige & Skoog 1962), was optimal for *in vitro* tobacco callus growth and differentiation (shoot formation) when compared with other sources of equivalent nitrogen content or no nitrogen (Table 3.2; Figure 3.2). The MS nitrogen regime is the preferred choice for many workers utilising tobacco callus (Thorpe & Laishley 1973; Maeda & Thorpe 1979; Chih-hung et al. 1981; Guerri et al. 1982; Truelsen & Wyndaele 1991; Bertrand-Garcia et al. 1992). A combination of nitrate- and ammonium-nitrogen was required for *in vitro* growth and differentiation in various higher plant species (Gamborg 1970; Caldas & Caldas 1976; Mohanty & Fletcher 1976; Wetherell & Dougall 1976; Jordan & Fletcher 1980; Gamborg & Shyluk 1981; Loyola-Vargas & Sanchez de Jimenez 1986; Evenson et al. 1988; Grimes & Hodges 1990). Reduced callus growth and lack of differentiation with ammonium, glutamine and glutamic acid (Regimes 3-5 in Table 3.2 and Figure 3.2) implies that NRA may be required for differentiation, although pH inhibition is possible (Jones et al. 1976). It has been observed that amino acids applied singly can be inhibitory at high concentrations (Gamborg and Shyluk 1981), while the affects of amino acids on NRA in culture are variable (Section 1.2.2). While Pouteau et al. (1989) were able to grow tobacco *nia* mutants with glutamic acid as sole nitrogen source, growth was prevented in wild-type tobacco callus here (Regime 3 in Table 3.2 and Figure 3.2). The 3-fold higher concentration of glutamic acid used in this study may explain these contradictory results. Lack of callus growth and differentiation in the absence of nitrogen (Regime 2 in Table 3.2 and Figure 3.2) was not unexpected. In addition to the metabolic effects of lack of nitrogen, inhibitory pH levels (below 4.0) may develop (Wetherell & Dougall 1976; Kamada & Harada 1984).

Table 3.2: Effect of different nitrogen regimes on *in vitro* tobacco callus culture growth and differentiation.

Sterilised tobacco leaf discs were plated onto: callus induction medium (1); callus induction medium without inorganic nitrogen (2); or callus induction medium with different nitrogen sources of equivalent nitrogen (60 mM N) content (3-5) (n = 20-23 for each regime). Cultures were maintained in darkness for two weeks and then transferred to a 16/8 h light/dark regime. After seven weeks, differentiated cultures were quantified and callus growth was assessed qualitatively ("+" signs indicate degree of response, "-" indicates no growth).

Nitrogen Source	Differentiation (%)	Callus Growth
1. 20 mM ammonium + 40 mM nitrate	95	+ + +
2. No nitrogen	0	-
3. 60 mM ammonium	0	+
4. 30 mM L-glutamine	0	+ +
5. 60 mM L-glutamic acid	0	-

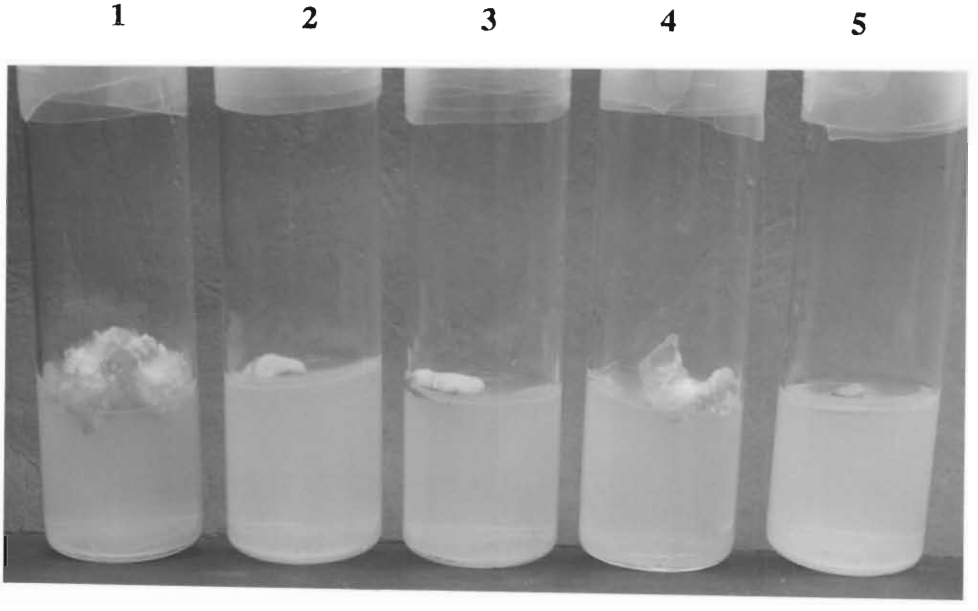

Figure 3.2: *In vitro* tobacco callus culture growth and differentiation on different nitrogen regimes.

Representative tobacco callus cultures plated onto induction medium containing different nitrogen sources, maintained for two weeks in darkness and five weeks in a 16/8 h light/dark regime, are shown. Numbers correspond to nitrogen regimes as described in Table 3.2.

3.3.3 Regulation of NR during in vitro tobacco callus growth and differentiation on 60 mM or 120 mM nitrogen

In preliminary experiments with tobacco callus initiated from cell suspension cultures, NRA increased initially during the culture period, and then declined after 4-5 weeks (Figure 2.6). Nitrate may have been limited NRA during the later stages of the culture period, as medium nitrate was depleted substantially (Figure 2.7C, D). Therefore, NRA regulation and growth parameters during tobacco callus differentiation on 60 mM (standard induction medium containing 40 mM nitrate-nitrogen and 20 mM ammonium-nitrogen) and 120 mM nitrogen (80 mM nitrate-nitrogen and 40 mM ammonium-nitrogen) were investigated.

The concentration of inorganic nitrogen used for *in vitro* tissue culture can affect differentiation, with optimal levels being species-dependent (Gamborg & Shyluk 1981; Ammirato 1986). Murashige & Skoog (1962) found 96 mM nitrogen to be slightly inhibitory for tobacco callus growth in comparison with 60 mM nitrogen. For the tobacco variety and culture conditions in this study, callus fresh weight increases were equivalent for the two nitrogen treatments (Figure 3.3A). Soluble protein levels were significantly higher in the 120 mM nitrogen regime after 3, 4 and 7 weeks in culture, but comparable at other sample points (Figure 3.3B). Fresh weight and protein accumulation growth curves obtained for the two treatments were characteristic for *in vitro* culture development (Zink 1982; Voronova et al. 1983; Loyola-Vargas & Sanchez de Jimenez 1986; Lenee & Chupeau 1989; Hardy & Thorpe 1990). Therefore, nitrogen at 120 mM was not inhibitory for tobacco callus growth in this study.

Typical stages of tobacco callus *in vitro* differentiation (Ross et al. 1973; Maeda & Thorpe 1979; Thorpe 1980, 1983) were obtained (Figure 3.3C). Following initial callus induction and growth, the first visible signs of differentiation were buds (shoot primordia), with differentiated callus then developing into plantlets with shoots and roots. Differentiation was delayed in the higher nitrogen treatment, with 50% of the cultures showing visible bud formation after 30 days, compared with 23 days for 50% differentiation in the 60 mM nitrogen regime. Nevertheless, there was no significant difference ($p < 0.05$) between the number of shoots produced per culture on the two nitrogen regimes after 8 weeks: 9.18 ± 6.14 ($n = 18$) for 60 mM nitrogen and 10.59 ± 5.77 ($n = 17$) for 120 mM nitrogen (mean $\pm$ standard error).

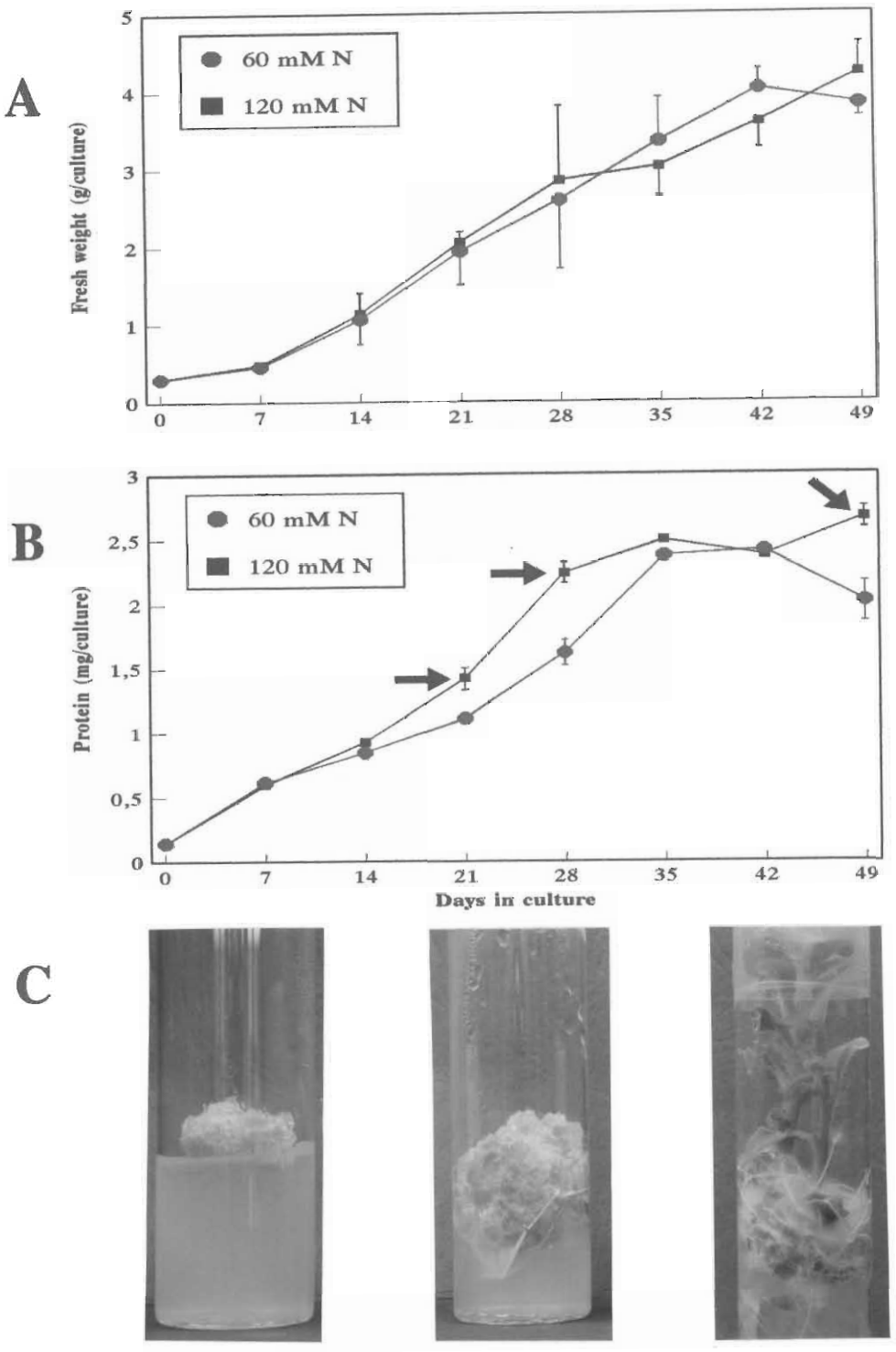

Figure 3.3: Growth parameters and description of tobacco callus cultures during *in vitro* growth and differentiation on 60 mM or 120 mM nitrogen.

Subcultured tobacco callus was plated onto induction medium containing either 60 mM or 120 mM nitrogen (each with nitrate:ammonium at 2:1), maintained in darkness for three weeks and then transferred to a 16/8 h light/dark regime. Fresh weight (A) and soluble protein (B) levels during *in vitro* growth and differentiation are given. Where larger than symbols, standard error of the mean (n = 3-4) is represented by bars. Significantly different means are indicated by arrows ($p<0.05$). For (A) and (B), 50% callus differentiation was observed after 23 days on 60 mM nitrogen and after 30 days on 120 mM nitrogen. In (C), typical stages of *in vitro* tobacco callus growth and differentiation are represented (from left to right): (i) callus induction and growth; (ii) early visible stage of differentiation, with formation of shoot primordia; and (iii) plantlets with shoots and roots.

Differences in *in vivo* NRA per culture (Figure 3.4A) or per callus fresh weight (Figure 3.4B) between the two nitrogen treatments were not statistically significant ($p<0.05$), indicating that the developmental regulation of NR during tobacco callus differentiation was not affected by nitrogen level. NRA per culture, which increased linearly for 35 days and then declined (Figure 3.4A), correlated well with protein accumulation (Figure 3.3B). NRA per unit callus weight reached maximal levels after day 7 for 120 mM nitrogen and after day 14 for 60 mM nitrogen (Figure 3.4B). Single NRA peaks have been obtained for both differentiating and non-differentiating cultures (Jones et al. 1976; Mohanty & Fletcher 1976, 1980; Voronova et al. 1983; Dwivedi et al. 1984; Evenson et al. 1988), but differentiating tobacco callus cultures were found to have higher levels of NRA than non-differentiating cultures, suggesting that different NR control mechanisms (Hardy & Thorpe 1990). Maximum NRA per gram fresh weight of callus was observed prior to the emergence of buds in both nitrogen treatments (Figure 3.4B), when meristemoid formation would be occurring (Ross et al. 1973; Maeda & Thorpe 1979; Thorpe 1980, 1983).

Under certain circumstances, NiR may limit nitrogen assimilation during *in vitro* culture (Hardy & Thorpe 1990). Furthermore, levels of nitrite in differentiating tobacco callus cultures were higher than non-differentiating cultures, and nitrite levels increased in parallel with NRA (Hardy & Thorpe 1990). In this study, callus nitrite levels showed similar tendencies when calli were grown on 60 mM nitrogen, with highest nitrite accumulated after 14 days when NRA per callus weight was maximal (Figures 3.5 & 3.4B). For 120 mM nitrogen, nitrite accumulation was highest after 14 days (Figure 3.5), 7 days after maximal NRA (Figure 3.4B). After day 14, callus nitrite levels were maintained at low levels in both regimes.

Nitrate depletion from the culture medium yielded similar trends for the two nitrogen regimes (Figure 3.6A), despite the availability of excess nitrate in the 120 mM nitrogen treatment. Net nitrate uptake per callus grown on the two treatments during successive weekly culture intervals was also comparable, although net uptake was higher during most intervals for callus grown on 120 mM nitrogen (Figure 3.6B). Rapid net nitrate uptake during the initial stages of tobacco callus growth indicated that the reduced nitrogen source ammonium was not utilised preferentially, as has been observed for other cultures (Guerrero et al. 1981). Whereas maximal NRA coincided with highest net nitrate uptake values for 120 mM nitrogen, this was not observed for the lower nitrogen regime (Figures 3.4B & 3.6B). It is thus not clear whether nitrate entering callus cells were translocated into the storage

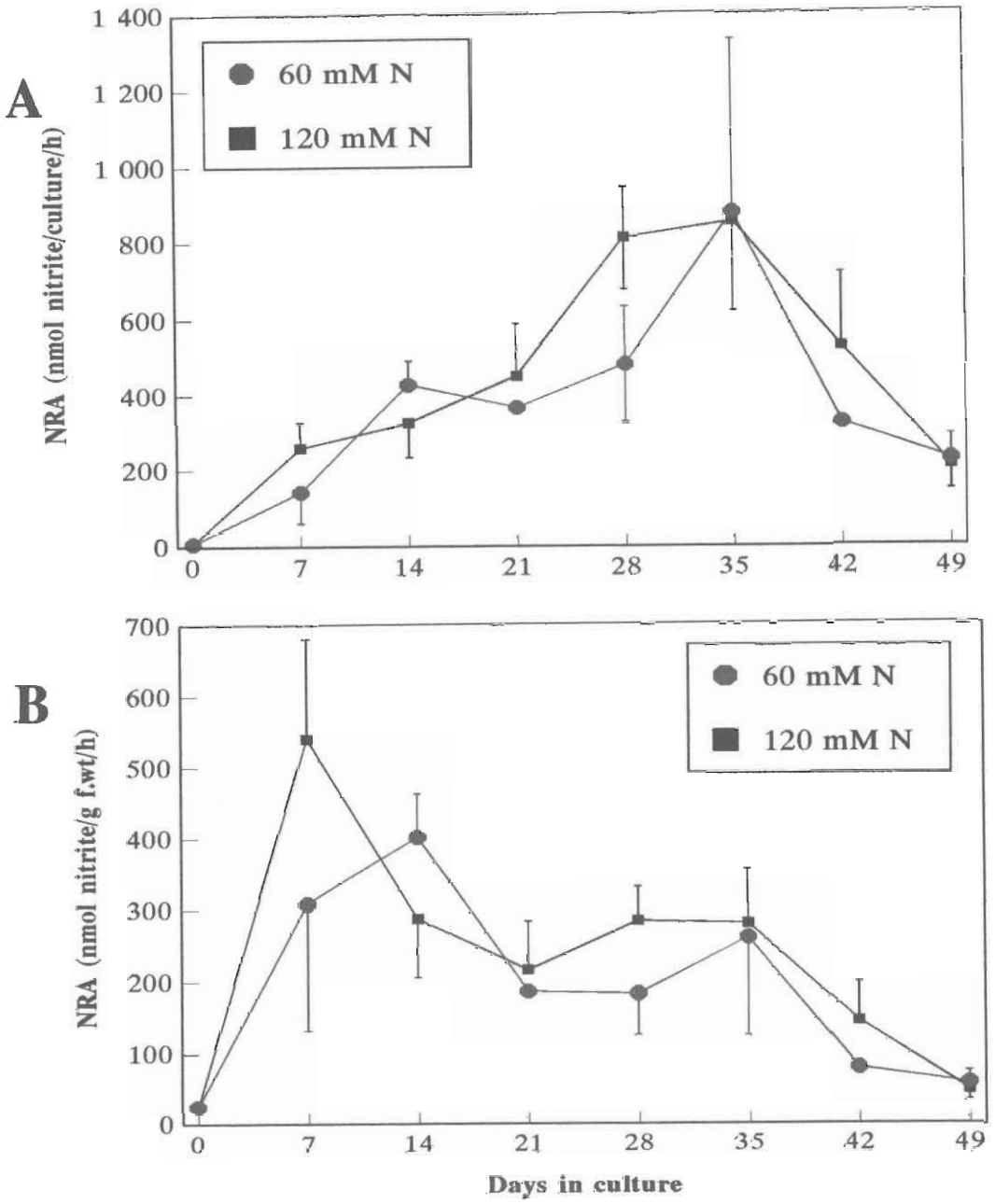

Figure 3.4: *In vivo* NRA during *in vitro* tobacco callus growth and differentiation on 60 mM or 120 mM nitrogen.

In vivo NRA expressed per culture (A) or per g fresh weight (B) during the callus culture period on 60 mM or 120 mM nitrogen are shown. Further details of culture conditions are given in the legend to Figure 3.3. Where larger than symbols, standard error of the mean is represented by bars (n = 3). At all sampling points, no significant difference between mean NRA values of the two nitrogen regimes was observed ($p < 0.05$).

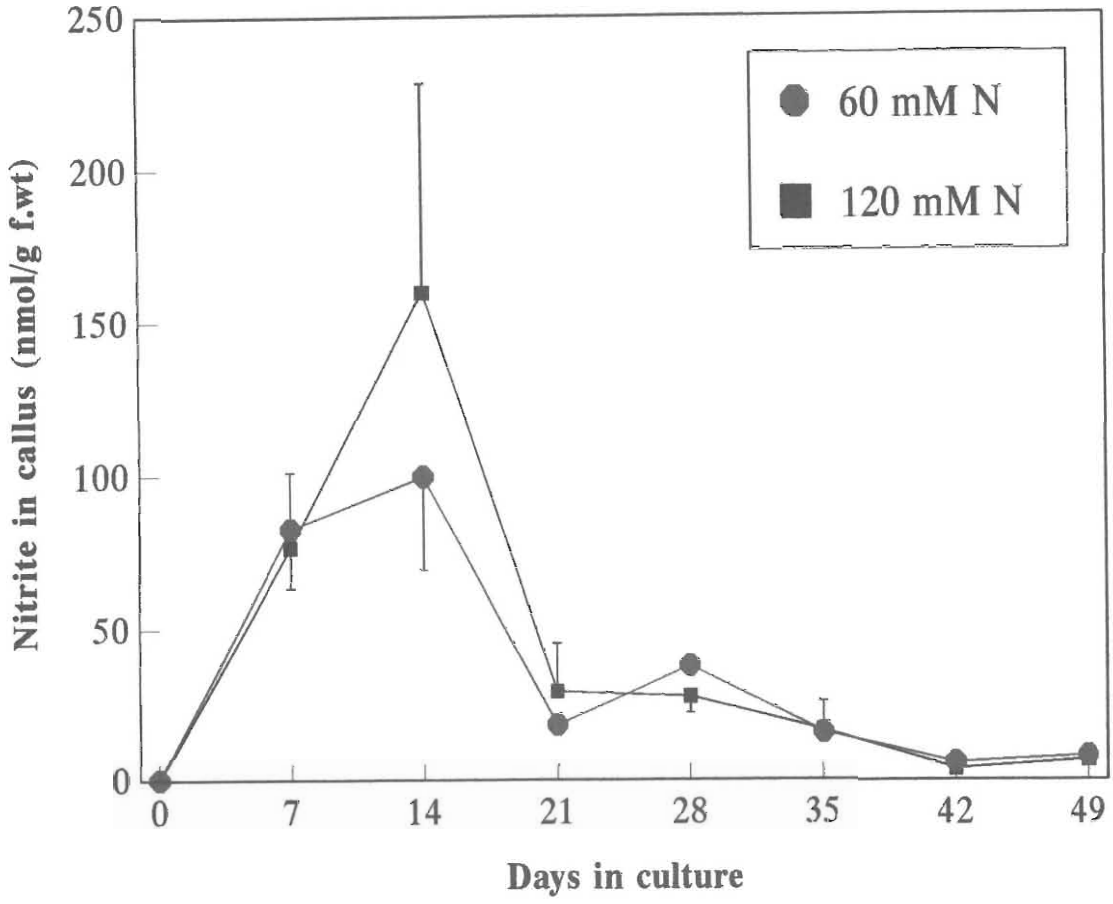

Figure 3.5: Nitrite accumulation in tobacco callus cultures during *in vitro* growth and differentiation on 60 mM or 120 mM nitrogen.

Levels of nitrite accumulated in tobacco callus grown on 60 mM or 120 mM nitrogen are given. Culture conditions are described in the legend to Figure 3.3. Where larger than symbols, standard error of the mean (n = 3) is represented by bars. Mean nitrite accumulation values were not significantly different between the two nitrogen regimes throughout the culture period ($p<0.05$).

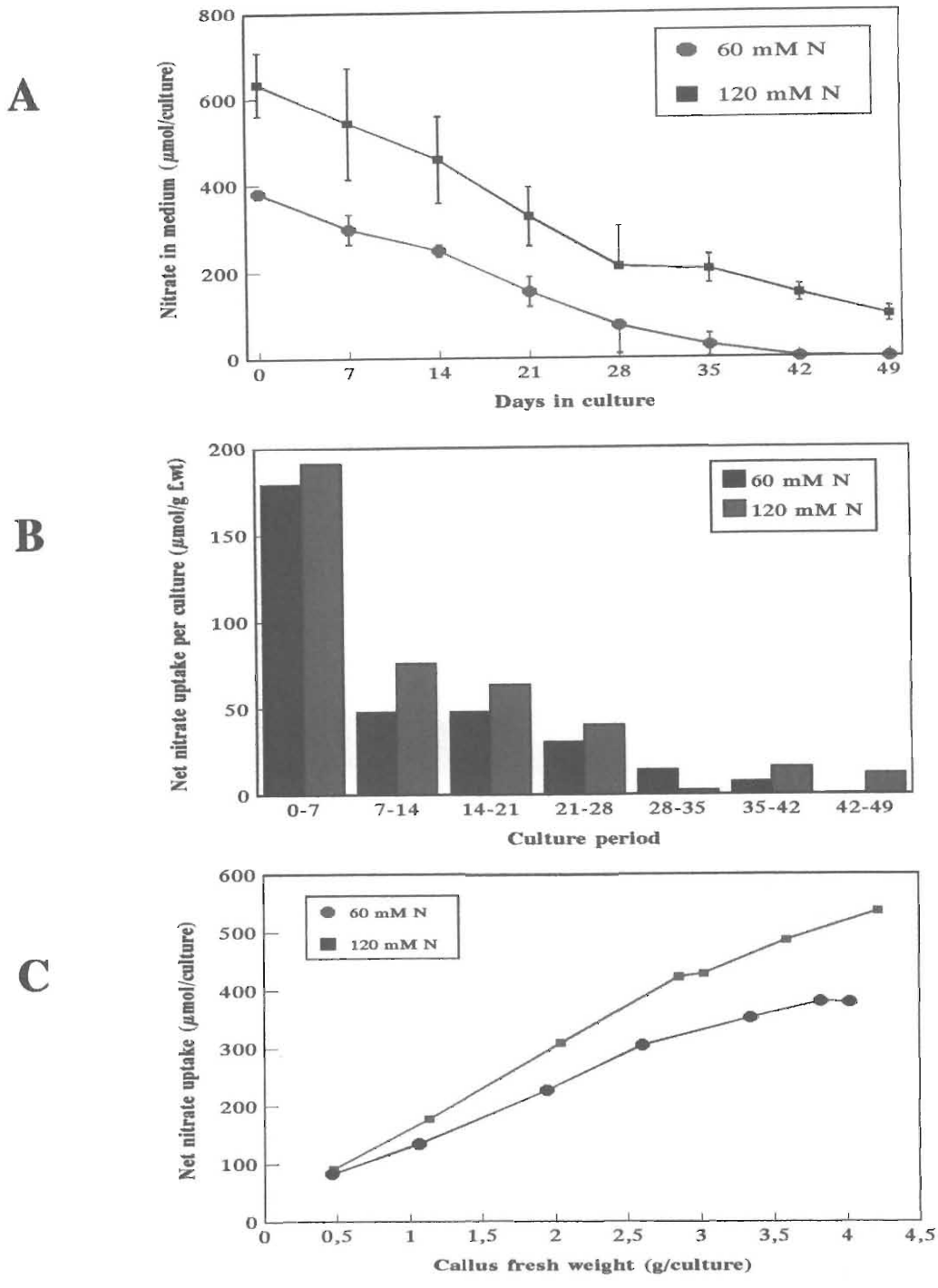

Figure 3.6: Nitrate utilisation by tobacco callus cultures during *in vitro* growth and differentiation on 60 mM or 120 mM nitrogen.

Remaining nitrate levels in culture media (A) and net nitrate uptake per culture for weekly intervals (B) during tobacco callus *in vitro* growth and differentiation on 60 mM or 120 mM nitrogen are shown. The relationship between callus weight and net nitrate uptake for callus grown on the two nitrogen regimes is represented in (C). Where larger than symbols, standard error of the mean (n = 3-5) is represented by bars. Further details of culture conditions are given in the legend to Figure 3.3.

(vacuolar) or metabolic (cytoplasmic) pool (Ferrari et al. 1973; Martinoia et al. 1981; Granstedt & Huffaker 1982). Over the entire culture period, however, NRA was correlated broadly with net nitrate uptake, supporting the hypothesis that nitrate flux into cells regulates NRA (Heimer & Filner 1971; Shaner & Boyer 1976a, b; Kwon 1980).

There was a linear relationship between tobacco callus fresh weight and net nitrate uptake (Figure 3.6C), as has been observed in other species (Lumsden et al. 1990). This proportional relationship between nitrate uptake and growth rate confirms that nitrate is an important nutrient for *in vitro* tobacco callus culture. In addition to its incorporation into organic nitrogen via nitrate assimilation, nitrate may have an osmoregulatory role during plant cell growth (Smirnoff & Stewart 1985; Stienstra 1986; Veen & Kleinendorst 1986; Talouizite & Champigny 1988; Köhler et al. 1992). Different gradients for the relationship between fresh weight and nitrate uptake (Figure 3.6C) indicated that tobacco callus grown on 120 mM nitrogen incorporated more nitrate per gram fresh weight than callus grown on the lower nitrogen level.

In summary, most of the growth parameters examined here did not yield a significant difference between the 60 mM and 120 mM nitrogen regimes, and 120 mM nitrogen was found not to inhibit callus growth or protein accumulation. Although bud formation was delayed slightly in the higher nitrogen regime, differentiation into plantlets was not affected. In subsequent experiments, nitrogen was used at 120 mM nitrogen to ensure that nitrate was not a limiting factor for NR regulation. Subcultured tobacco callus, in preference to cell suspension cultures, was employed as inoculum for the following differentiation experiments because the former were easier to manipulate *in vitro* and shorter culture periods were required to initiate shoot primordia.

3.3.4 Regulation of NR during in vitro tobacco callus growth and differentiation in different light treatments

Although light affects the expression of many genes, including NR (Section 3.1.3), ultrastructural studies on tobacco callus *in vitro* differentiation have shown that meristemoid and bud formation occurred at comparable rates in the light and dark (Section 1.2.5). Therefore, the affects of light on the regulation of NR during tobacco callus differentiation were investigated here, utilising callus cultured in continuous dark or light/dark (16/8 h) regimes. Analyses were restricted to the primary

stages of differentiation, where an increase of NRA had been observed (Figure 3.4B).

Tobacco callus growth in the two light treatments was comparable, except immediately after subculture, where dark-grown callus had significantly higher ($p<0.05$) fresh weight levels (Figure 3.7A). Protein levels appeared to decrease after 7 days in culture for callus grown in the light/dark regime, but were significantly higher ($p<0.05$) than dark-grown callus after 2 weeks (Figure 3.7B). Higher levels of protein after 2 weeks correlated with higher NRA per culture for callus in the light/dark treatment (Figure 3.8A). NRA per callus fresh weight increased slightly over the first two weeks of the culture period in the dark-grown callus (Figure 3.8B), and 50% differentiation occurred after 13 days. In the light/dark treatment, 50% callus differentiation at 16 days was preceded by a marked increase in NRA per callus weight at day 7, not observed in the dark (Figure 3.8B).

High NRA levels in the inoculum used for this experiment (compare day 0 in Figures 3.4B and 3.8B) were due possibly to an advanced state of development in the subcultured callus. Morphologically similar callus is often physiologically disparate, making selection of comparative inocula for different experiments difficult (Thorpe 1980; Tran Thahn Van & Trinh 1990). With starting levels high, changes in NRA during callus differentiation may have been masked. Another problem with the callus experimental system employed here was the high variability of NRA values and other parameters quantified, despite the use of short-term cultures in order to minimise somaclonal variation associated with aging (Syono 1965; Skirvin 1978). Differentiating callus cultures reportedly have a low degree of uniformity for two reasons: (1) new callus growth occurs primarily on the periphery of the callus mass, resulting in cells of different age (Evans et al. 1981; Gamborg & Shyluk 1981); and (2) the incidence of bud formation in tobacco callus is very low (Meins et al. 1982). Quantitative assessment of differentiating tobacco callus cultures was complicated therefore by inherent lack of synchrony and heterogeneity.

After an initial peak at day 2 in the light/dark regime, callus nitrite levels accumulated to relatively high levels in both the dark and light/dark treatments after 7 days (Figure 3.9). Callus nitrite was significantly higher in the dark regime at day 17 and 20 ($p<0.05$), indicating that NiRA may have been restricted in the absence of light. However, callus nitrite accumulation during early differentiation in both light/dark and dark treatments suggests that NiRA was regulated by factors other than the availability of light. Simultaneous regulation of NR and NiR induction by nitrate

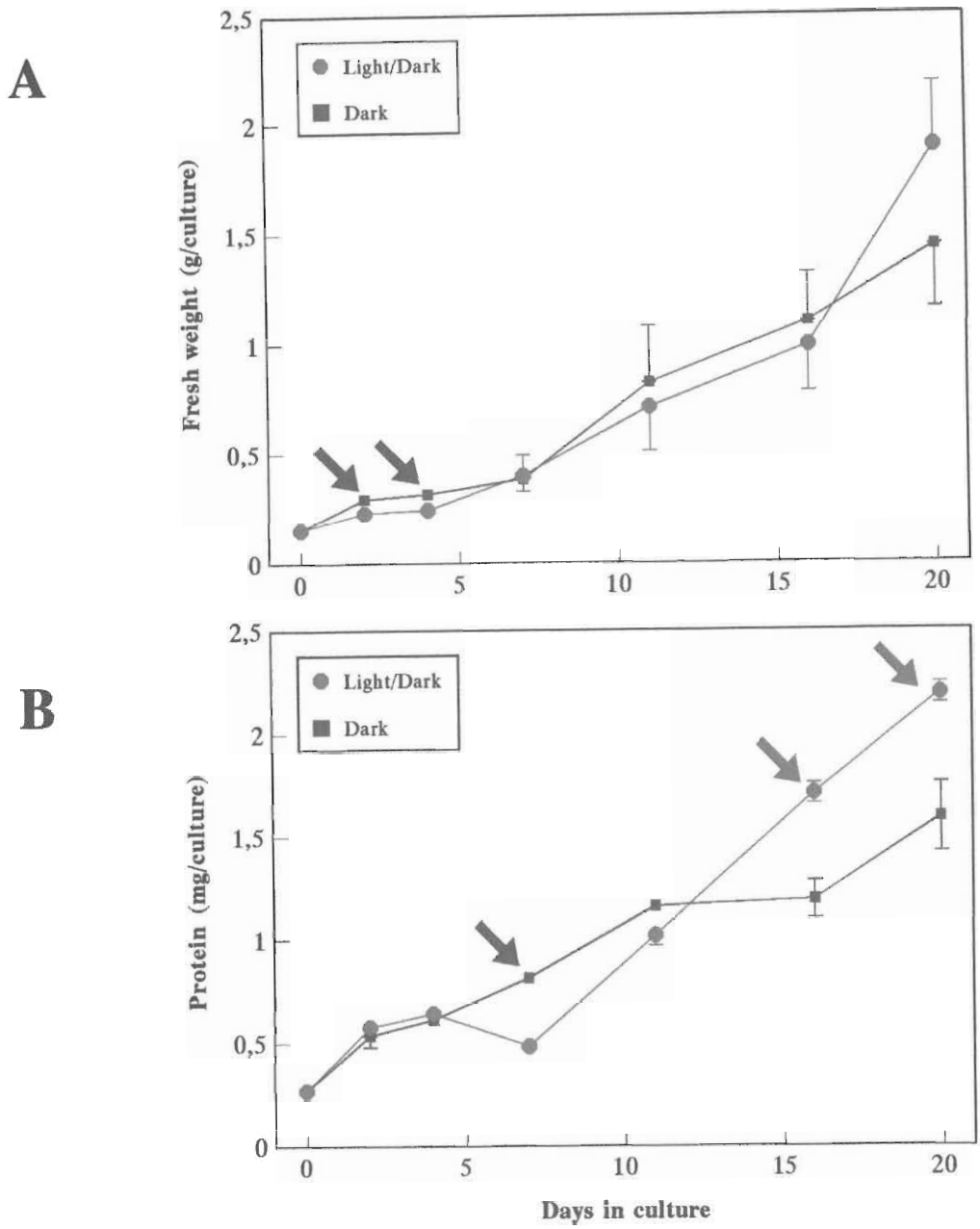

Figure 3.7: Growth parameters for tobacco callus cultures during *in vitro* growth and differentiation in dark or light/dark regimes.

Subcultured tobacco callus was plated onto induction medium containing 120 mM nitrogen (nitrate:ammonium at 2:1), and then maintained either in darkness or in a 16/8 h light/dark regime. Fresh weight (A) and soluble protein (B) levels during *in vitro* growth and differentiation were quantified. Where larger than symbols, standard error of the mean (n = 3-7) is represented by bars. Significantly different means are indicated by arrows ($p<0.05$). Fifty % callus differentiation was observed after 13 days in the dark and after 16 days in the light/dark regime.

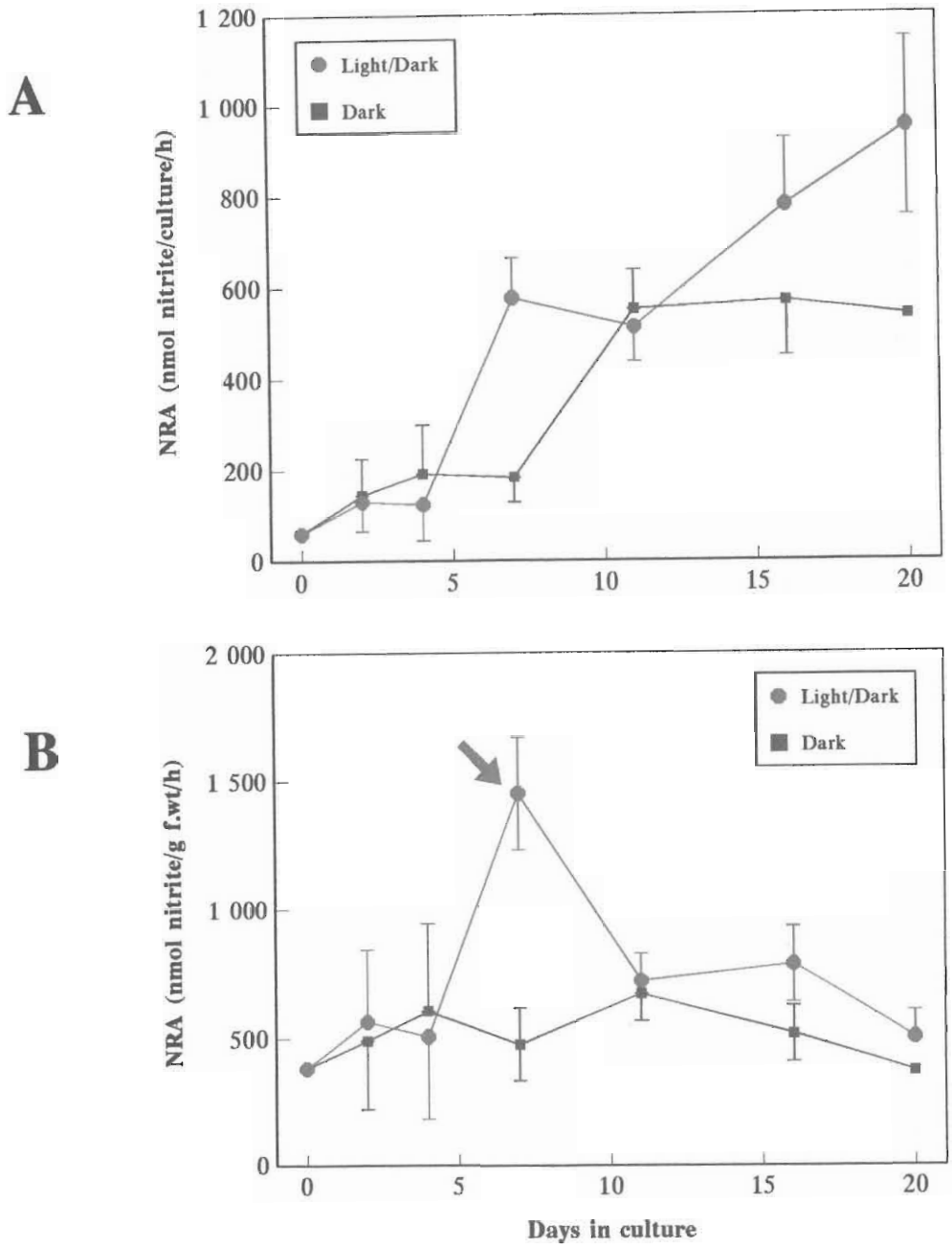

Figure 3.8: *In vivo* NRA profiles during *in vitro* tobacco callus growth and differentiation in dark or light/dark regimes.

In vivo NRA expressed per culture (A) or per g fresh weight (B) during the tobacco callus culture period in darkness or in a light/dark regime are shown. Further details of culture conditions are given in the legend to Figure 3.7. Where larger than symbols, standard error of the mean is represented by bars (n = 3). Significantly different means are indicated by arrows ($p<0.05$).

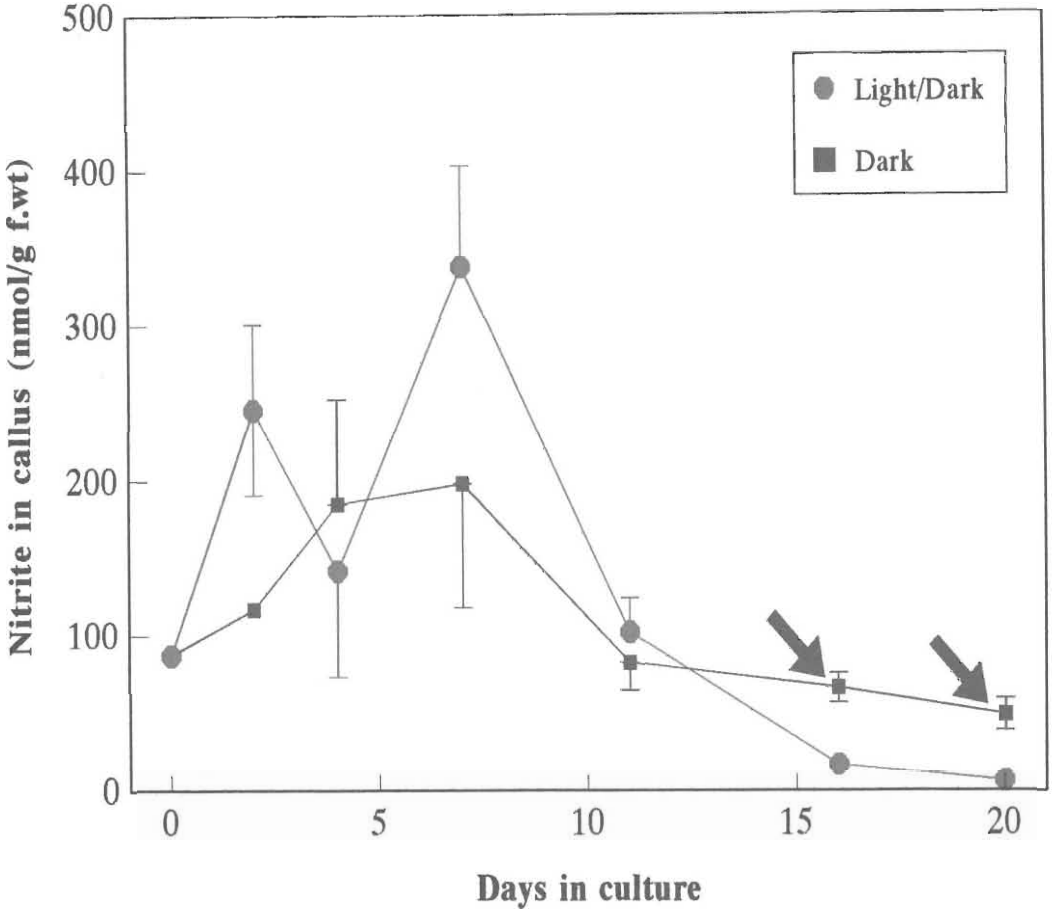

Figure 3.9: Nitrite accumulation in tobacco callus cultures during *in vitro* growth and differentiation in dark or light/dark regimes.

Levels of nitrite accumulated in tobacco callus grown on 120 mM nitrogen in dark or light/dark conditions are given. Culture conditions are described in the legend to Figure 3.7. Where larger than symbols, standard error of the mean (n = 3) is represented by bars. Arrows indicate significantly different mean nitrite values ($p<0.05$).

(Section 3.1.1) and light (Section 3.1.3) has been reported.

Disappearance of nitrate from the culture medium followed similar trends for both treatments (Figure 3.10A). Nitrate was utilised linearly until day 5, after which there was an apparent net efflux of nitrate at day 7. Beyond day 7, nitrate disappearance from the medium displayed linear uptake kinetics (Figure 3.10A). The relationship between net nitrate uptake and callus fresh weight was not linear for initial callus growth, but became proportional after callus attained fresh weights of about 0.4 g in both treatments (Figure 3.10B). The similarity of net nitrate uptake kinetics in the dark and light/dark treatments implied that light did not affect the nitrate uptake process in tobacco callus significantly, and that differences in NRA between the two treatments (Figure 3.8) were the result of regulation of cellular nitrate availability (Wallace 1987; Wray 1988; Stulen et al. 1990).

NRA appeared to be developmentally regulated during *in vitro* differentiation of tobacco callus in low or high nitrogen regimes, and in dark or light/dark treatments. However, measurement of NRA fluctuations does not allow for clarification on the control mechanisms involved (Guerrero et al. 1981). While control of gene expression during differentiation has been described in the literature for several genes (Vernet et al. 1982; Aleith & Richter 1990; Inoguchi et al. 1990; Györgyey et al. 1991), no studies on developmental NR expression during *in vitro* differentiation have been reported. In the following experiments, attempts were made to quantify NRA, NRP and NR mRNA levels during tobacco callus differentiation in different light regimes, using methods developed for tobacco callus in Chapter 2.

Subcultured tobacco callus plated onto callus induction medium was incubated in light/dark, continuous dark or continuous light treatments. Western blot analysis of tobacco callus NRP using a spinach polyclonal NR antibody showed the presence of two major bands, with apparent molecular weights of 71 and 55 kD, at all sample points (Figure 3.11, lanes 1-9). As previously discussed (Section 2.3.3), such bands are possibly cleaved NRP subunits, with the 71 kD band representing the Mo-containing domain. Relative NRP quantities in this study were determined by densitometer quantification of the 71 kD band, which assumed that the 71 kD band was derived from tobacco NRP, and that the ratio of this cleavage product to holo-NR is 1:1. The latter assumption is implicit for the ELISA technique, used routinely for NRP quantification (Section 2.1.2). The ELISA employs monoclonal antibodies specific for a single antigenic site that could be detected on holo-NR, NR subunits or NR degradation products (Campbell & Smarrelli 1986; Remmler & Campbell

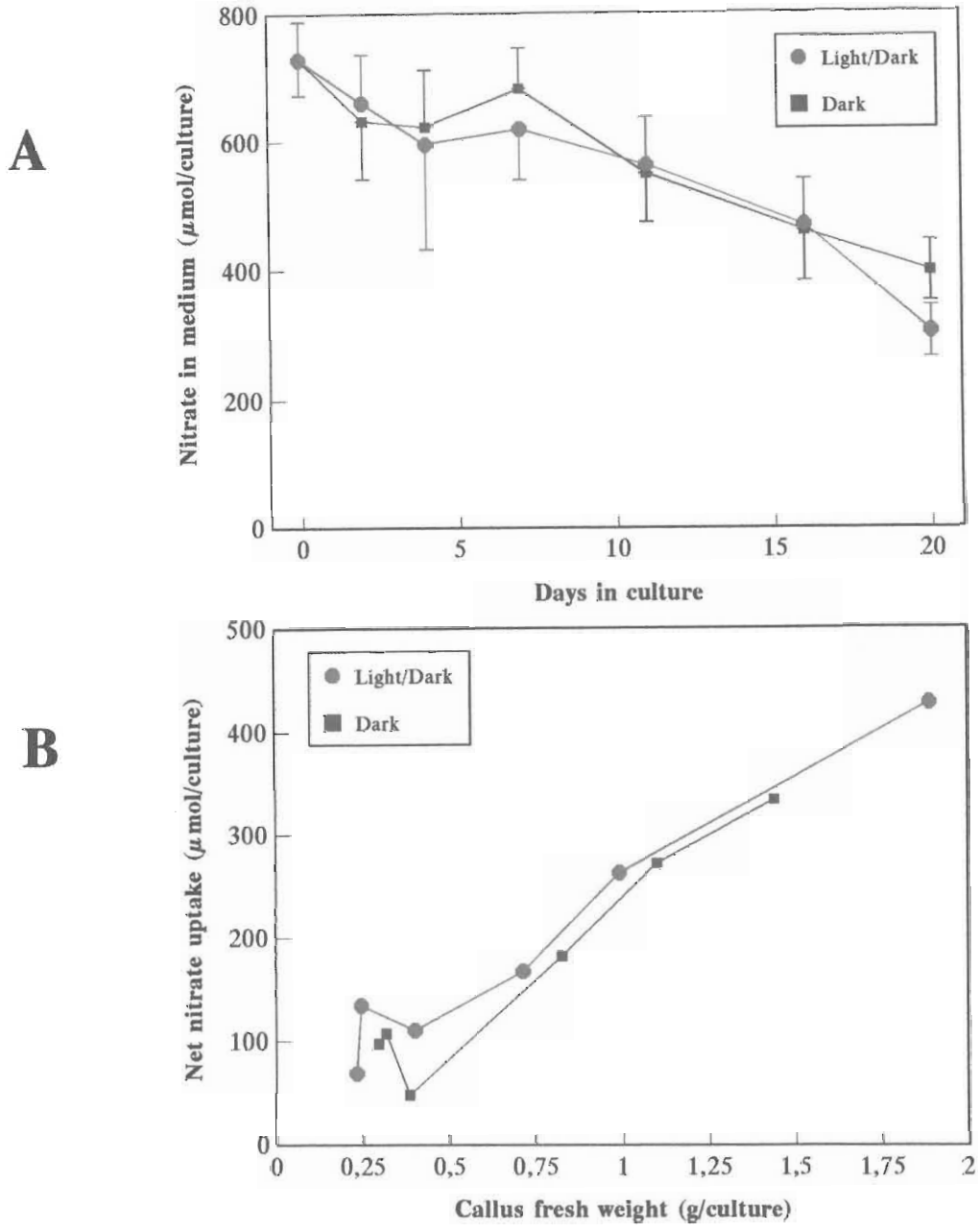

Figure 3.10: Nitrate utilisation by tobacco callus cultures during *in vitro* growth and differentiation in light or light/dark regimes.

Levels of nitrate remaining in culture media during tobacco callus *in vitro* growth and differentiation on 120 mM nitrogen in dark or light/dark regimes are shown in (A), whereas (B) depicts the relationship between callus weight and net nitrate uptake. Culture conditions are described in the legend to Figure 3.7. Where larger than symbols, standard error of the mean is represented by bars (n = 3-7).

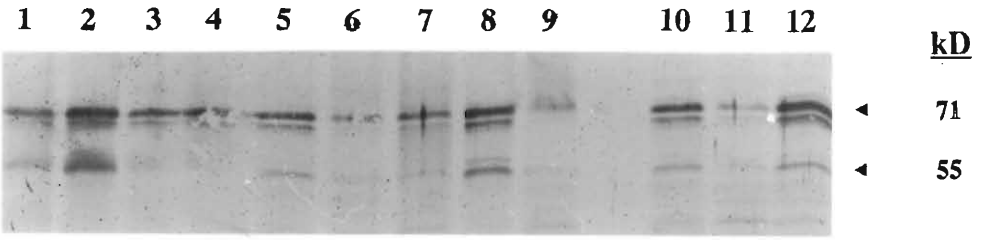

Figure 3.11: Western NRP detection during the initial stages of *in vitro* tobacco callus differentiation in dark, light/dark or light regimes.

Fifty μg of acetone-extracted protein from tobacco callus grown on 120 mM nitrogen (nitrate:ammonium 2:1) in continuous dark (lanes 1-3), light/dark (lanes 4-6 and 10-12) or continuous light (lanes 7-9) was fractionated on a 9% SDS-polyacrylamide gel. After transfer by electroblotting to nitrocellulose, spinach anti-NR polyclonal antibody diluted to 1/3000 was applied. Following second antibody (AP) binding, filters were incubated with chromogenic detection reagents for 3 min. In the first experiment, samples were analysed after 7 (lanes 1,4 and 7), 14 (lanes 2,5 and 8) and 21 (3,6 and 9) days. In a separate experiment (light/dark only), samples were removed for Western blot analysis after 4 (lane 10), 19 (lane 11) and 30 (lane 12) days in culture.

1986; Caboche et al. 1989; Campbell 1990; Rouze et al. 1990). Even when an ELISA is calibrated with blue Sepharose-purified NR, which apparently contains only activeNR (Campbell & Remmler 1986), substantial discrepancies are found between NRP and NRA levels (Campbell 1989), and the ELISA is regarded as useful for detecting inactive NRP (Campbell 1987).

During the initial stages of *in vitro* differentiation in different light regimes, tobacco callus NRA and relative NRP levels were not correlated (Figure 3.12). In the light/dark-treatment, 31% of callus cultures showed visible differentiation (shoot primordia) and 62% were chlorophyllous after 21 days. Relative levels of NRP were high when NRA was low for day 7 and 14, and an increase in NRA at day 21 was accompanied by a decrease in NRP (Figure 3.12A). For the dark-grown callus, NRA levels remained low, and differentiation after 21 days was observed in 13% of the cultures (Figure 3.12B). In comparison, NRP levels increased slightly at day 14, and then decreased (Figure 3.12B). In the continuous light treatment, NRA increased only after 21 days in culture, and was negatively correlated with relative NRP levels (Figure 3.12C). Although only 12% of the cultures grown under continuous light showed visible differentiation after 21 days, an increase in chlorophyllous tissue (41% of the cultures) may explain the observed increase in NRA (Orihuel-Iranzo & Campbell 1980; Kakefuda et al. 1983).

A comparison of relative callus NRP levels between the three regimes revealed similar trends, with an increase after 14 days (except in the light/dark treatment), followed by a decline after 21 days (Figure 3.13). NRP levels were highest in the dark-grown callus cultures at all sampling points, with maximal relative NRP in the dark-grown callus at day 14, where NRA levels were low (Figures 3.13 & 3.12B). Conversely, NRP levels were lowest in callus grown under a light/dark regime at day 21, when high NRA was observed (Figures 3.13 & 3.12A).

Relative NRP levels and NRA were not correlated during a light/dark culture period in a separate experiment (Figure 3.11, lanes 10-12; Figure 3.14). NRA increased after 5 days in culture, reaching maximal levels after 19 days, 10 days before 50% callus differentiation was observed. Relative NRP levels, however, declined after 5 days in culture, but then increased after day 19 to reach maximal levels at day 30. Although NR was induced earlier in the culture period for the second experiment, NRA and NRP values were comparable for the two separate experiments on tobacco callus differentiation in light/dark conditions (Figures 3.12A & 3.14).

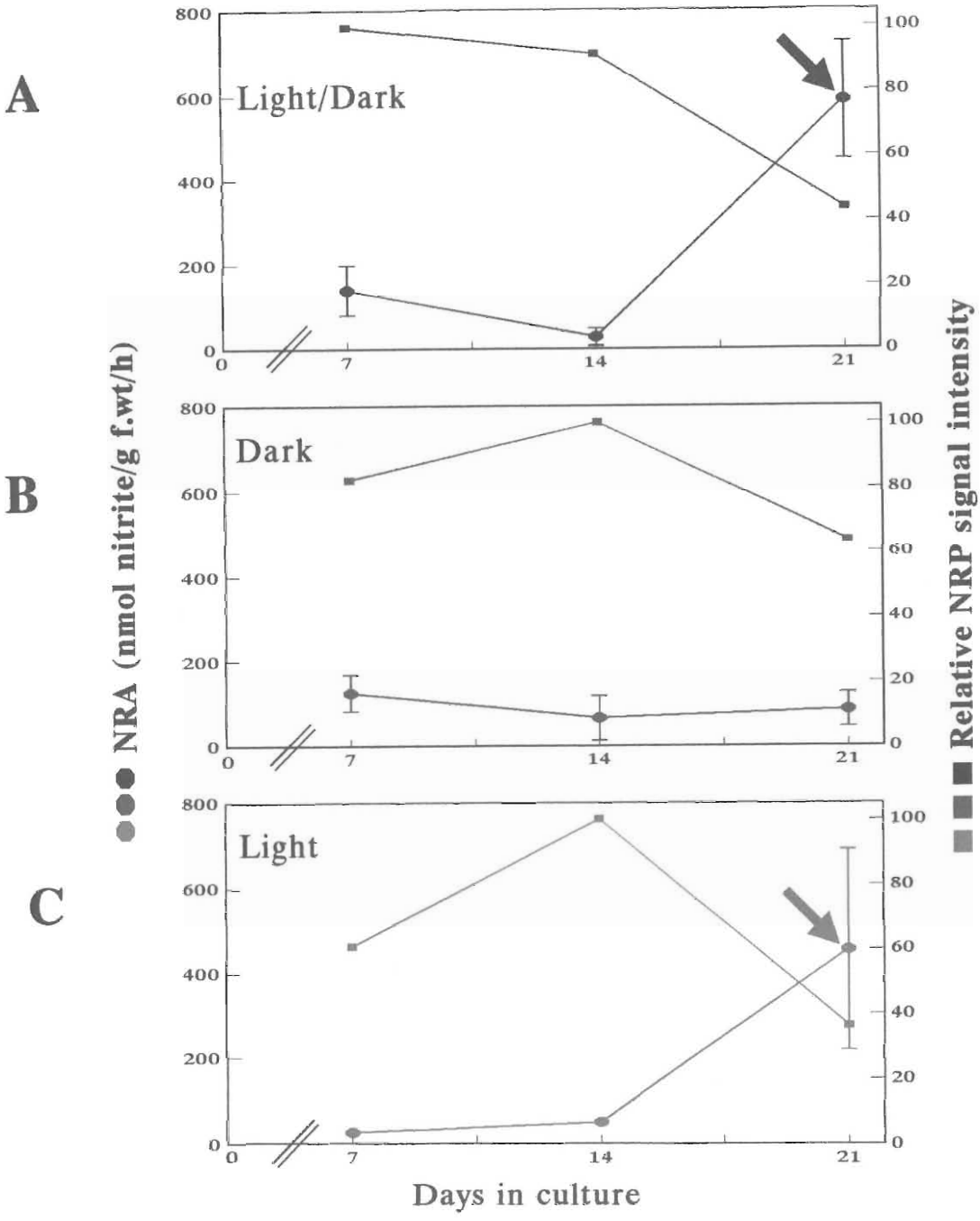

Figure 3.12: *In vivo* NRA and relative NRP levels during initial stages of *in vitro* tobacco callus differentiation in different light regimes.

Relative NRP and *in vivo* NRA values are shown for the initial stages of *in vitro* tobacco callus differentiation on 120 mM nitrogen (nitrate:ammonium at 2:1) in (A) light/dark, (B) continuous dark, or (C) continuous light regimes. Where larger than symbols, standard error of mean NRA is represented by bars (n = 3). Arrows indicate significantly different mean NRA values for each regime ($p<0.05$). Relative NR mRNA and NRP levels were determined by densitometer scanning of relevant Western blot bands (Figure 3.11), with maximum signal in each regime assigned a value of 100%. After 21 days, callus differentiation was observed in 31% (A), 13% (B) or 12% (C) of the cultures.

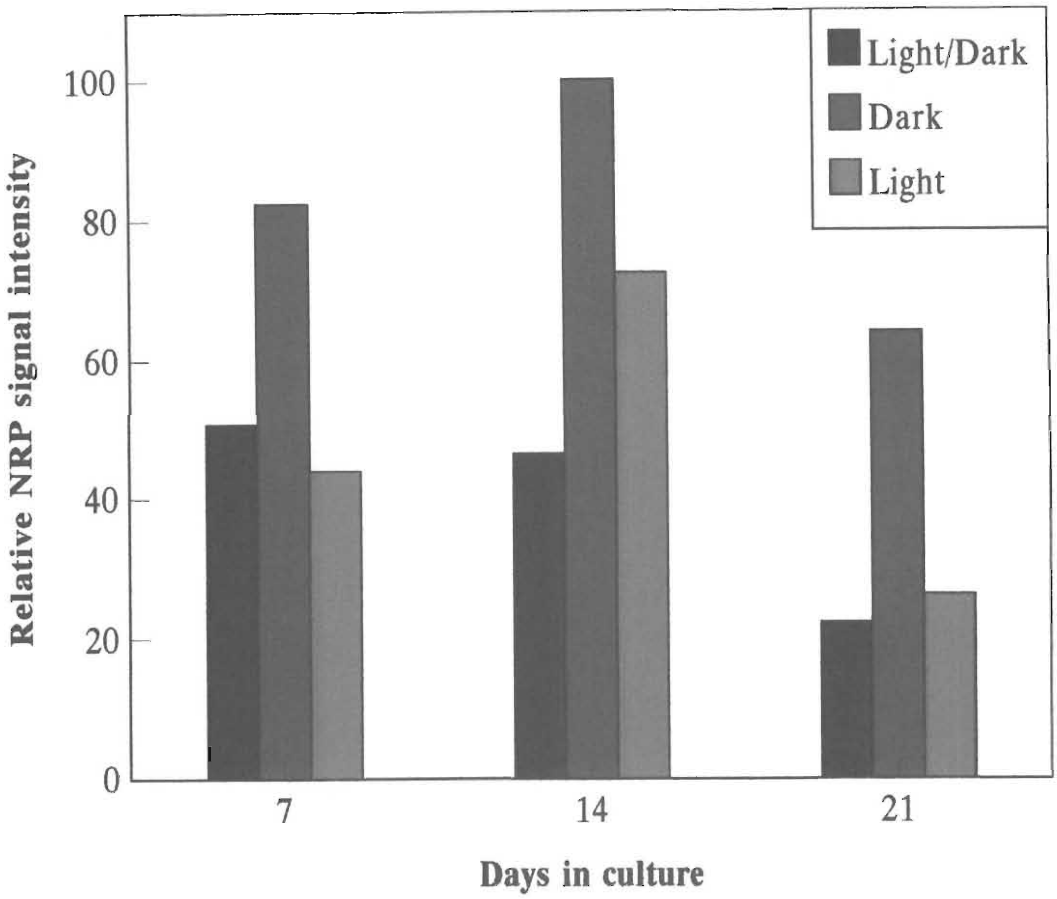

Figure 3.13: Comparative NRP levels for different light regimes during initial stages of *in vitro* tobacco callus differentiation.

Relative NRP levels during *in vitro* tobacco callus differentiation on 120 mM nitrogen are compared between light/dark, continuous dark and continuous light regimes. Relative NRP levels were determined as described in the legend to Figure 3.12, except that maximum signal for the three regimes was assigned a value of 100%.

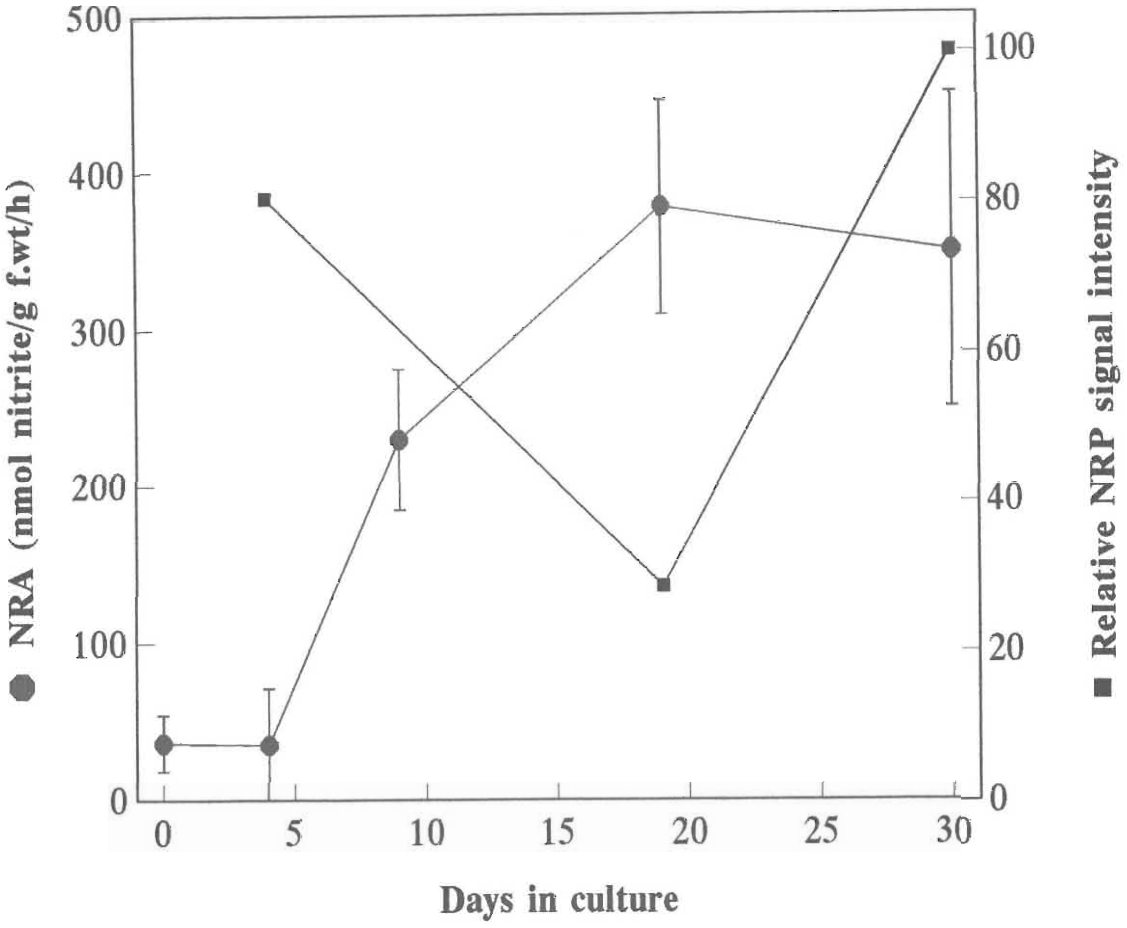

Figure 3.14: *In vivo* NRA and relative NRP levels during *in vitro* tobacco callus differentiation in a light/dark regime.

Relative NRP and *in vivo* NRA values are shown for various stages of *in vitro* tobacco callus differentiation on 120 mM nitrogen (nitrate:ammonium 2:1) in a light/dark regime. Relative NRP levels were calculated from relevant Western blot bands (Figure 3.11), respectively, as described in the legend to Figure 3.12. Fifty % callus differentiation was observed after 29 days in culture.

NR expression during tobacco callus differentiation could be regulated at transcriptional, post-transcriptional, translational and/or post-translational level. Studies on diurnal fluctuations (Deng et al. 1990), NR induction by nitrate (Caboche et al. 1989) and the regulation of NR during leaf senescence (Kenis et al. 1992) have indicated that transcriptional and post-transcriptional control mechanisms are important. Post-translational control of short-term NR expression has been demonstrated in numerous studies showing that NRP and NRA fluctuations often are not correlated (Remmler & Campbell 1986; Galangau et al. 1988; Rouze et al. 1990; Lillo 1991; Friemann et al. 1992; Huber et al. 1992a, b, c). If the 71 kD band detected by spinach NR antibody (Figure 3.11) corresponds with tobacco NRP levels, then the present study suggests that post-translational control mechanisms are involved in the developmental regulation of NR during *in vitro* tobacco callus differentiation (Figures 3.12 & 3.14).

Total RNA content of differentiating tobacco callus changed during the culture period in all three light regimes (Table 3.3). In the light/dark regime, callus RNA content showed a 2-fold increase after 21 days in culture. An increase in transcriptional activity has been reported in differentiating tobacco callus by Guerri et al. (1982). In continuous darkness and continuous light regimes, callus RNA content decreased at day 14 and then increased at day 21 (Table 3.3). As for NRA and NRP levels, differences in developmental stages of callus grown under the three light treatments were reflected at the total RNA content level.

Attempts to quantify NR mRNA specifically by Northern blotting were not successful. An initial hybridisation with [^{32}P]-labelled NR probe and an external mRNA standard ([^{32}P]-labelled α-tubulin probe) produced high non-specific binding, despite stringent washing conditions (results not shown). After very stringent low salt and high temperature washes (Keller & Manak 1989) and a longer hybridisation time (Section 3.2.7), non-specific background was eliminated, but no bands were observed after autoradiography. Underloading was the most likely reason for inability to detect NR mRNA bands, but due to insufficient availability of experimental callus material, more total RNA could not be extracted. Therefore, this work did not ascertain whether transcriptional and/or post-translational regulation were involved in the control of NR expression during tobacco callus differentiation.

Table 3.3: Tobacco callus total RNA levels during initial stages of *in vitro* differentiation in different light regimes.

Total RNA was extracted according to a modification of Verwoerd et al. (1989) from pooled tobacco callus samples during the initial stages of *in vitro* differentiation in different light regimes. Details of the experiment are described in the legend to Figure 3.12.

Light regime	Days in culture	Total RNA content (mg/g f.wt)
1. Light/dark (16/8 h)	7	0.115
	14	0.118
	21	0.261
2. Continuous dark	7	0.172
	14	0.119
	21	0.156
3. Continuous light	7	0.172
	14	0.099
	21	0.151

3.4 Conclusions

Prolific *in vitro* tobacco callus shoot formation occurred on callus induction medium containing IAA (2 mg/l) and kinetin (0.5 mg/l) in the presence of nitrate- and ammonium-nitrogen but not other reduced nitrogen forms. Callus growth and differentiation on 60 mM or 120 mM nitrogen regimes was accompanied by similar trends in NRA fluctuations, with high NRA levels prior to visible shoot primordia. Nitrate depletion data indicated that nitrate flux was apparently correlated with NRA in high and low nitrogen treatments. A comparison of early *in vitro* callus differentiation in different light treatments revealed that the developmental status of callus was more important than the presence of light or darkness in determining callus growth, nitrate utilisation or NRA patterns. Western blot analysis suggested that putative NRP levels were not correlated with NRA during initial stages of tobacco callus in light/dark, continuous dark or continuous light treatments, implicating an important role for post-translational control of NR during tobacco callus differentiation. Involvement of transcriptional regulation of NR expression could not be ascertained as Northern blot analyses were not successful.

CHAPTER 4. CONCLUDING REMARKS

Although short-term mechanisms for NR control are described in the literature relatively well (Section 3.1), little work has been done on the mechanisms involved in higher plant developmental NR regulation. *In vitro* cultures provide a suitable experimental system for examining aspects of plant differentiation that would be difficult in whole plant studies (Section 1.2). Nevertheless, callus material is limited by problems with heterogeneity and hence lack of synchrony during manipulated developmental processes. Despite these difficulties, the role of nitrogen assimilation during the shift in metabolism associated with differentiation was investigated in this thesis.

Suitable methods for NRA, NRP and NR mRNA quantification were developed for use with tobacco callus in Chapter 2. While variations of the *in vivo*, *in situ* and *in vitro* assays are used routinely for NRA estimation, optimised methods here produced similar NRA values during tobacco callus culture development. The *in vivo* NRA assay was selected for NRA estimation. For the estimation of NRP, spinach polyclonal NR antibody was employed in Western blot experiments. Acetone-extracted tobacco callus protein samples resolubilised in SDS treatment buffer, rather than crude buffer extractions, showed clearer bands detected by the spinach antibody. However, whether these bands were derived from tobacco NRP was not determined conclusively. Attempts to quantify tobacco callus NR mRNA by slot blot analysis using a [^{32}P]-labelled NR cDNA probe were not successful due to the presence of non-specific binding. In comparison, Northern analysis of tobacco callus total RNA led to the identification and quantification of an mRNA species hybridising with a [^{32}P]-labelled NR probe at the expected molecular weight. A non-radioactive labelling technique did not provide the required sensitivity to detect fractionated NR mRNA.

In vitro tobacco callus differentiation was affected markedly by phytohormones and nitrogen form. At the two levels of nitrogen examined (60 mM and 120 mM nitrogen) and under different light regimes, however, *in vitro* differentiation proceeded at similar rates. Although NR is induced rapidly by nitrate and light in short-term experiments (Sections 3.1.1 & 3.1.3), NRA in this study was regulated primarily by the level of culture development. Western blot experiments suggested that regulation of NR at the molecular level during tobacco callus differentiation was at the post-translational level. Whether or not translational control mechanisms were affecting NR simultaneously was not resolved.

The work described in this thesis attempted to examine the role of nitrate assimilation during *in vitro* differentiation at the biochemical and molecular level. Future research should focus on the role of transcriptional and post-transcriptional control mechanisms in the developmental regulation of NR during differentiation. Determination of steady state NR mRNA levels and *de novo* transcription rates could be undertaken using northern blot analyses in conjunction with nuclei run-off transcription assays. Total or partial purification of tobacco NR and nondenaturing PAGE analysis would be necessary to verify the specificity of spinach polyclonal NR antibody for the tobacco NR polypeptide. Furthermore, molecular and biochemical studies on the regulation of NR, and hence nitrate assimilation, by environmental and biological factors that affect *in vitro* differentiation could be undertaken. Elucidation of the regulation of key metabolic events in higher plant development would assist researchers with applied objectives to increase plant productivity and minimise fertilizer application.

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