CRANFIELD UNIVERSITY

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EFFECT OF HEAVY METAL STRESS IN PLANT METABOLISM OF SOLANACEOUS PLANT SPECIES WITH EMPHASIS ON NITROGEN ASSIMILATION

CRANFIELD HEALTH

PhD THESIS

2011

CRANFIELD UNIVERSITY

School of Health

PhD Thesis

Academic Years 2007-2011

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February 2011

This thesis is submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

ABSTRACT

Several plant species are able to accumulate and withstand large quantities of heavy metals in their tissues without dramatic alterations in their growth usually observed in plants. Such metal accumulating plants are tested and used for remediation of contaminated soils and waters. Although the literature provides extensive information on the effect of heavy metals in growth and development of several metal-accumulating and non accumulating plants, nitrogen metabolism and the regulation of related enzymes have not been widely studied. In an effort to better understand the responses of plants species under heavy metal stress, a comparative study was held between two solanaceous species, *Nicotiana tabacum* and *Nicotiana glauca*, plants with promising properties for phytoremediation. Plants of the two species were grown in the presence of different concentrations of the heavy metals zinc (Zn), nickel (Ni) and cadmium (Cd) in the following experimental systems in the greenhouse: in pots containing artificial substrate for 6 weeks and hydroponically for 8 days. In order to get a global picture for the heavy metal –induced variations concerned the examined species, the accumulation and distribution of metals in various plants tissues (lower and upper leaves, lower and upper shoots, roots) was determined. Also, several morphological parameters related with plants growth, physiological related with light and dark reactions of photosynthesis and water balance, biochemical related with nitrogenous compounds metabolism and the enzymes involved in ammonia assimilation, were followed. The presence of the referenced metals in the culture medium induced alterations, in all the parameters examined. In particular, strong positive correlations exhibited between the accumulation of Zn, Ni and Cd in the examined tissues and the supplied metals concnentrations. Our data revealed different accumulation patterns for each examined metal at tissue and species level. Furthermore, both growth and physiological parameters were negatively affected at least by the higher concentration of the three metals tested. However, differential responses were observed between *N. tabacum* and *N. glauca*, where the latter seemed to be more sensitive at the higher concentration of the metals, especially for Ni and Cd, showing heavier symptoms. In addition, proline accumulation was positively affected over the range of the three metals concentrations. Alterations

induced in quantitive and qualitive profile of soluble proteins corresponding to Zn, Ni and Cd treatments. Furthermore, the concentration of Zn, Ni and Cd differentially affected the specific activities and the protein levels of ammonia assimilating enzymes glutamine synthetase (GS), glutamate synthase (Fd-GOGAT) and glutamate dehydrogenase (GDH).

ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation and thanks to my supervisors Prof. Konstantinos Loulakakis, Dr. N. Primikirios and Dr. David Aldred for their valuable guidance throughout this project and consideration of this thesis. I am grateful to Dr. Dimitrios Lydakis and Prof. Naresh Magan for giving me the opportunity to acquire this scientific experience.

I specially thank Cranfield University and the Technological Institute of Crete for their support and of letting me use all the necessary equipment needed for this project.

Grateful thanks to my family, friends and colleagues for supporting and encouraging me throughout the long period of this study.

LIST OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF PLATES

CHAPTER 1

CHAPTER 3

NOTATION

$^{\cdot}OH$	Hydroxyl Radicals
Ag	Silver
APX	Ascorbate Peroxidase
As	Arsenic
ATP	Adenosine-5'-triphosphate
BciP	Bromochloroindolyl Phosphate
BSA	Bovine Serum Albumin
Ca^{+2}	Calcium
$CaCl_2$	Calcium Chloride
$CaCl_2$	Calcium Chloride
CAT	Catalase
Cd	Cadmium
CDF	Cation Diffusion Facilitor
CEC	Cation Exchange Complex
Chl	Chlorophyll
Ci	Intracellular CO_2
CO	Carbon Monoxide
Co	Cobalt
CO_2	Carbon Dioxide
Cr	Chromium
Cu	Copper
$CuSO_4$	Copper Sulphate
CW	Cell Wall
DDT	p,p'-Dichloro-diphenyl-trichloroethane
DHAR	Dehydroascorbate Reductase
DNA	Deoxyribonucleic Acid
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic acid
F_0	Minimal Fluorescence
$FeCl_3$	Iron(III) Chloride
F_m	Maximal Fluorescence

FOREGS	Forum of European Geological Surveys
Ftr	Terminal Fluorescence
F_{v}/F_{m}	Ratio of variable fluorescence to maximal fluorescence
GDH	Glutamate Dehydrogenase
GOGAT	Glutamate Synthase
GR	Glutathione Reductase
gs	Somatal Conductance
GS	Glutamine Synthetase
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulphuric Acid
H_3BO_3	Boric Acid
HCl	Hydrogen Chloride
$HClO_4$	Perchloric Acid
Hg	Mercury
Hg(O)	Mercury Oxide
HM	Heavy Metal
HMAs	Heavy Metal ATPases
HNO_3	Nitric Acid
Hsps	Heat Shock Proteins
K	Potassium
KH_2PO_4	Mono Potassium Phosphate
KNO_3	Potassium Nitrate
M	Metal
MA	Mugineic Acid
MAPK	Mitogen-activated Protein Kinase
MDHAR	Monodehydroascorbate Reductase
MeHg	Methyl Mercury
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
Mn	Manganese
$MnSO_4$	
Mo	Molybdenum

N	Nitrogen
N. glauca	*Nicotiana glauca*
N. tabacum	*Nicotiana tabacum*
NA	Nicotianamine
NAD(P)H	
NADH	
NBT	Nitroblue Tetrazolium
NH_4Cl	Ammonium Chloride
Ni	Nickel
NO_x	Nitrogen Oxides
Nramp	Natural Resistance Associated
$O_2^{\cdot -}$	Superoxide
O_2^1	Oxygen Singlet
O_3	Tropospheric Ozone
ODS	Ozone Depleting Substances
P	Phosphorus
PAHs	Polycyclic Aromatic Hydrocarbons
Pb	Lead
PBS	Phosphate Buffered Saline
PC	Phytochelatins
PMSF	Phenylmethylsulfonyl Fluoride
Pn	Net Photosynthesis Rate
POPs	Persistent Organic Pollutants
ppm	parts per million
PSI	Photosystem I
PSII	Photosystem II
PVPP	Polyvinylpolypyrrolidone
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
S	Sulfur
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	Selenium

Sn	Tin
SO_2	Sulfur Dioxide
SOD	Superoxide Dismutases
SPM	Suspended Particulate Matter
T	Transpiration Rate
TCA	Trichloroacetic Acid
UV/VIS	Ultraviolet and Visible
UV-B	Ultraviolet Radiation
F_v	Variable Fluorescence
VOCs	Volatile Organic Compounds
WUE	Water Use Efficiency
Zn	Zinc

1. INTRODUCTION

1.1 Abiotic Stress

Recently, soil, water and air pollutants generated through human activities have added to the list of factors that plants must cope with in their environment. Extremes in environmental parameters create stressful conditions for plants, which may have a significant impact on their physiology, development and survival. Higher plants are continuously exposed to different stress factors without any protection. Studies of plant responses to environmental stress have long been a central theme for plant environmental physiologists, physiological ecologists and agricultural scientists, because stress invariably leads to reduced agricultural productivity. Plant stress can be divided into two primary categories. Abiotic stress is a physical or chemical insult (e.g. pollutants) that the environment may impose on a plant. Biotic stress is a biological insult (e.g. insects) to which a plant may be exposed its lifetime. The most common stress factors are shown in Table 1.1 and include drought, salinity, light, deficient or excess nutrients, heavy metals and pollutants etc. (Rao, 2006).

Table 1.1: Principal environmental stresses to which plants may be subjected (Hopkins and Huner, 2003).

High temperature	Heat
Low temperature	Chilling, Freezing
Excess Water	Flooding, Anoxia
Water deficit	Drought, Low water potential
Radiation	Visible, Ultraviolet
Chemical	Pesticides, Heavy metals, Air pollutants
Biotic	Pathogens, Competition

1.1.1 Effects of Abiotic Stress Factors on Plants

Plants are sessile and therefore cannot escape from stress factors. This has enabled them to develop unique molecular mechanisms to cope with different stress factors. Alterations occur in their physiologies, metabolic mechanisms, gene

expression and developmental activities to cope with stress effects. Those plants that have better tolerance, resistance, protective and acclimation mechanisms alone can survive while others cannot. These factors can cause diverse effects on growth, development and productivity of plants, either individually or in combinations (Rao, 2006). Some of the common effects of abiotic stresses on plants are:

Growth: germination inhibition, growth reduction, premature senescence, reduction in productivity.

Physiology: reduction in water uptake, altered transpiration uptake, reduction in photosynthesis, altered respiration, decrease in nitrogen assimilation, metabolic toxicity, accumulation of growth inhibitors.

Molecular biology: altered gene expression, breakdown of macromolecules, reduced activity of vital enzymes, decreased protein synthesis, disorganization of membrane systems.

1.1.2 Plants Response to Abiotic Stress

The immobile nature of plants enables them to develop more than one strategy to survive under stress conditions (Fig. 1.1). Some plants, which are considered to be susceptible, may be injured by a stress. This means that they exhibit one or more metabolic dysfunctions which may lead to death. Other plants, which are called ephemeral plants, may escape the stress altogether.

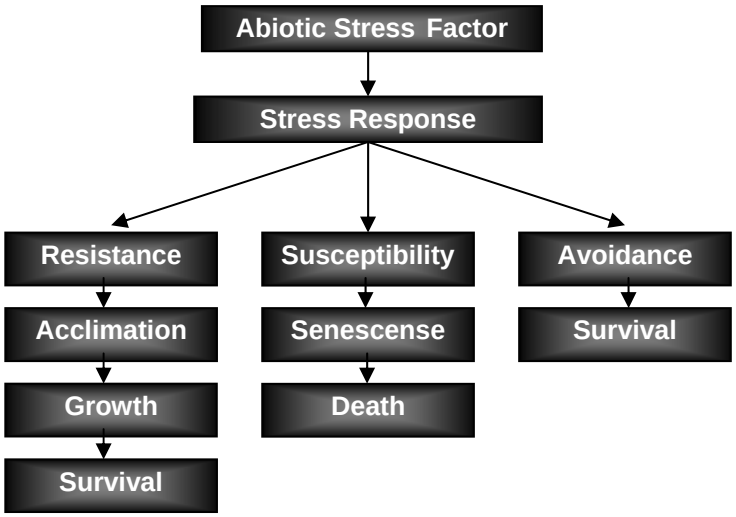


Figure 1.1: The relationship between environmental stress and either plant survival or death (Hopkins and Huner, 2003).

One example is short-lived, desert plants. They germinate, grow and flower very quickly following seasonal rains. They complete their life cycle during a period of adequate moisture and form dormant seeds before the onset of the dry season. Avoidance mechanisms reduce the impact of stress, even though the stress is present in the environment. Another example is plants of *alfalfa* (*Medicago sativa*) which ensure an adequate water supply under dry conditions by sending down deep root systems that penetrate the water table, in conditions where more shallow-rooted plants experience drought. Other plants develop thick cuticles or pubescence to help reduce evaporation, or other modifications that help either conserve water or reduce water loss. Another example of drought avoiders are is the Cacti, with their freshly photosynthetic stems and leaves reduced to simple thorns (Rao, 2006).

The immediate emphasis is on the development of tolerance mechanism in plants. Plants exhibit great variations in their tolerance mechanisms, within species, between species and among the plants of different groups, which are highly significant in developing stress tolerance in plants. Many plants have the capacity to tolerate a particular stress and hence are considered to be stress resistant. Resistance requires that the organism come to thermodynamic equilibrium with the stress, to maintain homeostasis. Adaption and acclimation are a means of achieving tolerance to a particular stress. Adaption refers to heritable modifications in structure or function that increase the fitness of the organism in the stressful environment (e.c. CAM plants). Acclimation on the other hand, refers to nonheritable physiological modifications that occur over the life of an individual.

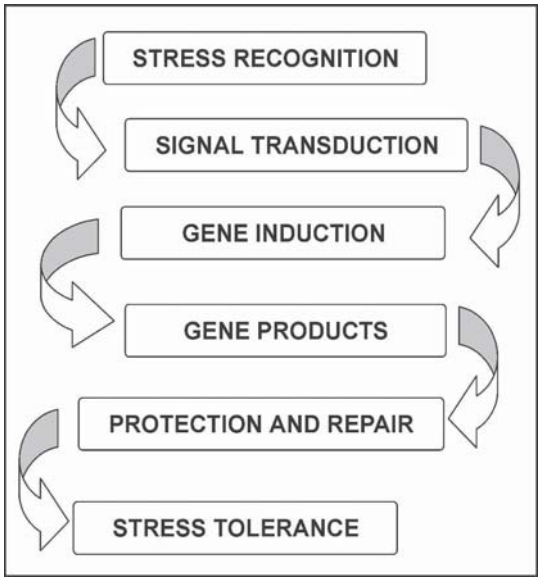


Figure 1.2: The path of stress tolerance in plants (Rao, 2006).

Gene products play a key role in the molecular mechanisms of stress tolerance. To combat stress effects plants develop some common tolerance mechanisms as well as stressor specific mechanisms to cope up with stress. However the degree of tolerance varies from plant to plant, from low

to high. Stress tolerance mechanisms start with stress perception followed by the formation of gene products that are involved in cellular protection and repair (Fig. 1.2). The signal transduction pathways that detect stress play a crucial role in the induction of stress tolerance in plants (Rao, 2006).

1.2 Heavy Metals

Heavy metals are conventionally defined as elements with metallic properties (ductility, conductivity, stability as cations, ligand specificity etc.) and an atomic number >20. They are chemical elements with a specific gravity that is at least 5 times the specific gravity of water. For example, the specific gravity of cadmium is 8.65, of iron 7.9, of lead 11.34, of mercury 13.5. 53 of the 90 naturally occurring elements are heavy metals (Schutzendubel and Polle, 2002), but not all of them are biologically important Based on their solubility under physiological conditions, 17 heavy metals may be available for living cells and of importance for organisms and ecosystems (Schutzendubel and Polle, 2002). Based on metal coordination chemistry, Nieboer and Richardson (1980) provided a biologically relevant classification of metals into three categories namely class A with affinity for oxygen-containing ligands, class B with affinity for nitrogen- or sulphur-containing ligands, and borderline, i.e. intermediate between the two with affinity for all three groups of ligands with definite preferences. This categorization reflects a general manner in which different metal ions interact with biological systems (Gasic and Schuyler, 2006). These elements are localized mainly in a dipersed form in rock formations. As a consequence of the industrial revolution there is an enormous and increasing demand for heavy metals that leads to high anthropogenetic emission of heavy metals into the biosphere. The most common heavy metal contaminants are cadmium, chromium, copper, molybdenium and zinc (Zenk, 1996).

1.2.1 Heavy Metals in the Environment

Currently, contamination of soil and water by heavy metals represents a major environmental hazard to human health. Toxic metal pollution in the environment has accelerated rapidly since the onset of the industrial revolution and is continuously growing (during the last century) due to anthropogenetic activities. Due to their

mobilization into the biosphere, heavy metals circulation through soil, water and air has greatly increased (Clemens, 2006). The main sources of heavy metal contaminants in soils include metal mining and smelting, agricultural and horticultural materials, such as fertilizer and pesticide application, sewage sludges, fossil fuel combustion, metallurgical industries, electroplating, chemical and other industrial sources (energy and fuel production), and waste disposal.

Large areas of cultivated land in many countries have been contaminated by As and Cd due to agricultural and industrial practices such as application of pesticides and chemical fertilizers, waste water irrigation and smelter wastes and residues from metal mining (Papazoglou et al., 2005). The use of copper is frequent in Mediterranean agriculture as a fungicide, a practice that has increased the levels of copper in the cultivated soils and the extensive use of fertilizers that contain toxic metal as impurities (such as phosphate fertilizers, sewage biosolids, composted solid wastes and ashes from combustion of coal) have elevated the concentrations at least of cadmium, zinc and lead in the recipient soils (Singh and Agrawal, 2007). Cd in phosphorus- fertilizers is of great concern, and individual European Union Member States have recently begun to assess the risks arising from fertilizer – derived soil accumulation of Cd (Lougon-Moulin et al., 2004). Heavy metals are natural elements that are found at various high background levels (Table 1.2) at different places throughout the world, due to various concentrations in the bedrock.

A geostatistical analysis of the FOREGS Geochemical database on heavy metal concentrations, sampled from 26 European countries revealed that the administrative units (NUTS level3) with highest overall concentrations are: (1) Liege (Arrondissement) (BE), Attiki (GR), Darlington (UK), Coventry (UK), Sunderland (UK), Kozani (GR), Grevena (GR), Hartlepool & Stockton (UK), Huy (BE), Aachen (DE) (As, Cd, Hg and Pb) and (2) central Greece and Liguria region in Italy (Cr, Cu and Ni).

Table 1.2: Background levels in natural water and sediment and the upper limit of non-polluted soil (Prasad, 2010).

Metal	Natural Water, μg l^{-1}		Soil, μg g^{-1}		Sediment, μg g^{-1}	
	Seawater	**Freshwater**	**Sandy soil**	**Loam**	**Lake**	**Sea**
Cd	0.01-0.07	0.07	1	1	0.14-2.5	0.02-0.43
Cr	0.08-0.15	0.5	15	30	7-77	11-90
Co	0.04	0.05	5	15		0.1-74
Cu	0.04-0.1	1.8	15	25	16-44	4-250
Hg	0.01	0.01	0.15	0.15	0.004-0.2	0.001-0.4
Mn	0.2	<5	500	800		390-6700
Mo	10	1	5	5		0.2-27
Ni	0.2-0.7	0.3	1	1	34-55	2-225
Pb	0.001-0.015	0.2	50	50	14-40	7-80
Zn	0.01-0.62	10	100	150	7-124	16-165

1.2.2 Heavy Metals- Roles and Phytotoxicity

It is important to realize that the soil is both a source of metals (natural components in soil) and a sink for metal contamination. It is well known that most of the microelements are not all indispensable for the growth of plants; beyond certain threshold concentration some of the elements show toxic effect on plants. Several transition metals are essential for plants, algae and most living organisms (Clemens, 2006). Living organisms require trace amounts of some heavy metals (including iron, cobalt, manganese, copper, zinc) but excessive levels can be detrimental to the organisms (Zenk, 1996). These elements have been acquired in the course of evolution because of their chemical properties such as redox-activity under physiological conditions (Cu, Fe) or Lewis acid strength (Zn) (Clemens, 2006). These same properties that make transition metal ions indispensable for life, however, are also the reason why they can easily be toxic when present in excess. Tight control and regulation of essential metal accumulation are vital at the organnelle as well as at the cellular level (31). The physiological range for essential transitional metals between deficiency and toxicity is therefore extremely narrow and tightly controlled metal homeostasis networks exist to adjust to fluctuations in micronutrient availability for all organisms (Clemens, 2006). Furthermore, the imperfect control of accumulation, and the lack of specificity of uptake and distribution systems, leads the organisms to

cope with exposure to unwanted elements, such as Cd, which is generally considered nonessential (Clemens et al., 2002).

Therefore, in low concentrations several heavy metals are essential micronutrients (trace elements) for plants, although elevated concentrations of both essential and non-essential metals can result in growth inhibition and toxicity problems (Fig. 1.3). Heavy metals that are considered essential for at least some forms of life include V, Cr, Mn, Fe, Cu, Zn, Mo, and possibly Ni, while others have no biological function. The phytotoxicity of such relatively common heavy metal as Cd, Cu, Hg and Ni is substantially greater than that of Zn. (Raskin et al., 1994). Ni has been considered to be among non-essential elements for the healthy growth of organisms, however recent literature has suggested that it is present in many species of plants and animals, where it stimulates metabolism and may be regarded as a key metal in several enzyme systems (Jadia and Fulekar, 2009).

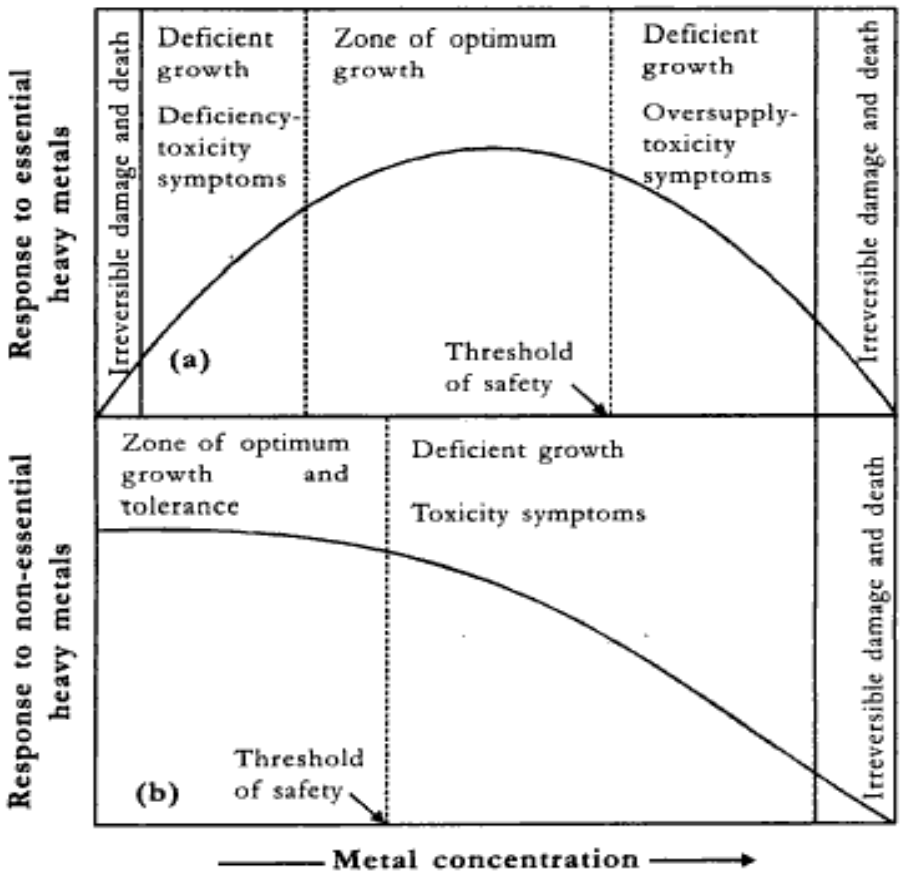


Figure 1.3: Hypothetical Dose-Response Curves depicting the effect of heavy metal in plants (Prasad, 2010) .

Copper contributes several physiological processes in plants including photosynthesis-related plastocyanin and membrane structure, respiration, carbohydrate distribution, nitrogen and cell wall metabolism, and it is a necessary cofactor for oxidative enzymes. Zinc is an activator of numerous enzymes and plays an essential role in DNA transcription. Manganese is an enzyme cofactor as well as part of the oxygen evolving complex in the chloroplast. Molybdenium is a key component of nitrogen metabolism (Salisbury and Ross, 1992).

Elevated concentrations of both essential and non-essential heavy metals in the soil can lead to toxicity symptoms and the inhibition of growth of most plants. The toxicity symptoms seen in the presence of excessive amounts of heavy metals may be due to a range of interactions at the cellular – molecular level (Hall, 2001). They cannot be broken down and when concentrations inside the plant cells accumulate above certain threshold levels, it can cause direct or indirect toxicity. In general, three different molecular mechanisms of metal toxicity can be distinguished: (a) blocking of essential functional groups in biomolecules, (b) displacement of essential metal ions from biomolecules and (c) production of reactive oxygen species by autoxidation and the Fenton reaction, which is typical for transition metals (Fig. 1.4). (Schutzendubel and Polle, 2002).

Heavy metals produce their toxicity by forming complexes or ¨ligands¨ with organic combounds. These modified biological molecules lose their ability to function properly and result in malfunction or death of the affected cells. The most common groups involved in ligand formation are oxygen, sulfur and nitrogen. Uncontrolled high affinity binding to these groups can cause inactivation of important enzyme systems or affect protein structure.

Many enzymes contain metals in positions important for their activity. The displacement of one metal by another will lead to inhibition or loss of enzyme activities. Divalent cations such as Co^{+2}, Ni^{+2} and Zn^{+2} are found to displace Mg^{+2} in ribulose-1,5-bisphosphate-carboxylase/oxygenase and result in loss of activity. Displacement of Ca^{2+} by Cd^{2+} in the protein calmodulin, important in cellular signaling, leads to an inhibition in the calmodulin-depedent phosphodiesterase activity in radish (Schutzendubel and Polle, 2002).

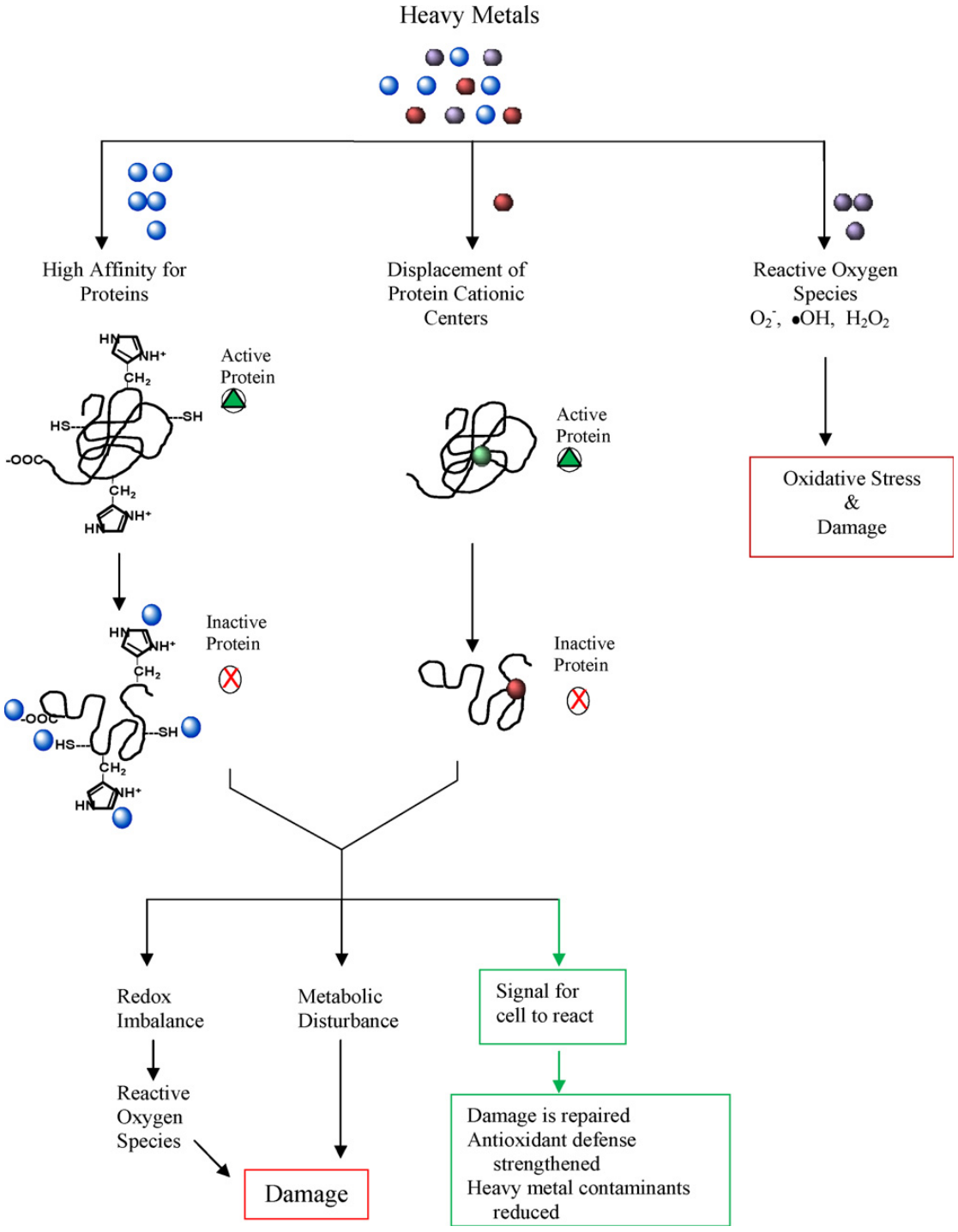


Figure 1.4: Heavy metal toxicity in plants. Purple spheres indicate redox active metals and red and blue are redox inactive metals. The green sphere is a metal center that is displaced by a heavy metal (red). The affinity for heavy metals will alter the activity of the protein and create imbalances and disruption that will lead to macromolecular damage. However the cell may adjust to the toxic metals and signal for responses to prevent damage (Peralta-Videaa et al., 2009).

In addition, exposure to heavy metals and other abiotic stress factors results to the generation of reactive oxygen species (ROS). Redox- active metal ions can participate in Haber-Weiss and Fenton reactions and thereby trigger the formation of hydroxyl radicals. The ROS, such as superoxide ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), oxygen singlet (O_2^1) and hydrogen peroxide (H_2O_2), are produced in different cellular components including chloroplasts, peroxisomes, glyoxysomes, the cell wall, plasma membrane and apoplasts. However, the chloroplasts, mitochondria and the microbodies are the main sources of ROS in the plant cell.

1.2.3 Effects of Heavy Metals on Plants

The literature provides extensive information on the effects of heavy metals in plant growth and development (Barazani et al., 2004). The effects of heavy metals on plant development vary according to different soil characteristics, the type of plant and the metal. Alterations to fundamental physiological processes of plant metabolism have been attributed to heavy metals, including plant water relationships, photosynthesis, cell respiration and nitrogen metabolism (Maksymiec et al., 2007; Romanowska et al., 2006; Monni et al., 2001). These effects have been correlated, at cellular and molecular levels, with oxidative damage that causes enhanced lipid peroxidation, DNA damage, oxidation of protein and reductants in the cell (Dring, 2006).

The first 'living' structural target for heavy metal toxicity is the plant plasma membrane. In the presence of high concentrations of heavy metals, the plasma membrane function may be rapidly affected by an increased leakage of contents from cells. For example, it was shown that Cu, caused increased K^+ efflux from excised roots of *Agrostis capillaries*. Various mechanisms could cause such damage, including the oxidation and cross-linking of protein thiols, inhibition of key membrane proteins such as H^+-ATPase, or changes to the composition and fluidity of membrane lipids (Hall, 2001). Direct effects on the lipid composition of membranes of Cu and Cd treatments have been reported (Hall, 2001), which may have a direct effect on membrane permeability. Cd treatments have been shown to reduce the ATPase activity of the plasma membrane fraction of wheat and sunflower roots (Hall, 2001).

Metals affect the gene transcription, expression and activation of numerous signaling proteins including mitogen-activated protein kinase (MAPK) proteins and nuclear transcription factors, proteins involved in calcium and lipid signaling as well as hormone signaling pathways. The effects of metal stress on intracellular signal transduction may be direct through the interaction of metals with proteins or indirect through the formation of metal-induced ROS.

The photosynthetic apparatus appears to be very sensitive to the toxicity of heavy metals (Prasad, 2010). The scale and character of changes observed in plants after heavy metal application was shown to be dose- dependent and it can vary for different plant species even for identical metal treatments depending on individual plant tolerance. A critical examination of the literature reveals that heavy metals react with photosynthetic apparatus at various levels of organization and architecture:

- → Accumulation of metals in leaves (main photosynthetic organ)
- → Portioning in leaf tissues, like stomata, mesophyll and bundle sheath
- → Metal interaction with cytosolic enzymes and organics
- → Alteration of the functions of chloroplast membranes
- → Supramolecular level action, particularly on PSII, PSI, membrane acyl lipids and carrier proteins in vascular tissues
- → Molecular level interactions, particularly with photosynthetic carbon reduction cycle enzymes, xanthophyll cycle and adenylates.

The deleterious effects of heavy metals on the physiological processes of plants are summarized in Table 1.3.

Table 1.3: Physiological processes affected in plants exposed to elevated metal concentrations (Prasad, 2010).

Physiological effect on		Metal	Plant
Photosynthesis	Chloroplast and thylakoid membrane, PSI/II activity	Cd	*Hordeum vulgare*
	Pigment content and thylakoids	Cd	*Oryza sativa*
	Chlorophyll fluorescence parameters	Cd	*Thlaspi caerulescence*
	Net photosynthesis rate, calvin cycle enzymes	Cd/Cu	*Cucumis sativus*
	CO_2 fixation	Cu/Mn	*H. vulgare*
	Carbohydrate metabolism	Cu/Ni	*Pinus sylvestris*
	Chlorophyll content and chloroplast ultrastructure	Ni	*Brassica oleracea*
	Phosystem II acivity, CO_2 fixation	Zn	*Lolium perenne*
Mineral nutrition	Fe, Zn, Mn, Cu, Mg contents	Cd	*O. sativa*
	Fe translocation	Cd	*Nicotiana tabacum*
	Na, Mg, P, S, K, Ca, Mn, Fe, Cu, Zn contents	Cd	*A. thaliana*
	Mn, Mg, Cu, Zn, Fe, Ca contents	Cd/Ni	*Zea mays*
	Mg, Mn contents	Zn	*L. perenne*
	Fe, Mn contents	Zn	*Brassica rapa*
	Ca, Fe, Mn, Zn, Cu contents	Pb	*Phaseolous vulgaris*
Water balance	Stomatal conductance	Cd/Cu	*C. sativus*
	Transpiration rate	Cr	*L. perenne*
	Water content	Hg	*Lupinus albu*

1.2.4 Zinc

Zinc is the last element in the first transition series with a completely filled 'd' orbital. Plants take up zinc mainly as a divalent cation (Zn^{2+}). Zinc is bluish-white, soft metal extracted from ore. It is used in alloys, for galvanizing iron to prevent corrosion and oxidation and in numerous compounds including those for use in cosmetics, pharmaceuticals and dry cell batteries.

Zinc is one of the most common elements in the Earth's crust and present in nearly all foods. Also, found naturally in air, water and soil. Zinc is reported to interact with soil organic matter to form both soluble and insoluble zinc complexes. The zinc content of most soils is in the range of about 10-30 ppm. However, the level of zinc in soil is very much related to the parent material. Soils originating from basic igneous rocks are high in zinc, while the soils derived from more siliceous parent materials are particularly low. Soils associated with zinc deficiency are usually neutral to alkaline in reaction; alkali soils tend to have lower levels of available Zn. Excessive soil zinc levels may occur on extremely acid (<pH 5.0).

The biochemical functions of zinc are based on its strong tendencies to form tetrahedral complexes with N, O_2 and S ligands and it therefore plays an important structural and functional role in many enzyme reactions. Zinc is an essential element for plant growth because it controls the biosynthesis of indoleacetic acid. It is also necessary for chlorophyll synthesis and carbohydrate formation. Zinc is closely involved in the N-metabolism of the plant. It plays a vital role in the synthesis of nucleic acids and proteins and helps in the utilization of phosphorus and nitrogen. A close correlation between the zinc supply and nitrogenous fractions and RNA content has been observed.

Because zinc is not readily translocated within the plant, deficiency symptoms first appear on younger leaves. A general guide for zinc concentration in mature leaf tissue is as follows: deficient less than 20 ppm, sufficient 25 to 150 ppm, excessive or toxic 300 ppm or more. Zinc toxicity induces stunted growth, interveinal chlorosis, rolling of leaf margins and brownish and necrotic roots. Excessive absorption of zinc

can suppress copper and iron absorption. It has been reported that the addition of Zn in the soil solution reduces also the phosphorus availability in soils (Yadav, 2010).

1.2.5 Nickel

Nickel is silvery-white, hard, malleable, and ductile metal. It is of the iron group and it takes on a high polish. It is a fairly good conductor of heat and electricity. In its familiar compounds nickel is bivalent, although it assumes other valences. It also forms a number of complex compounds. Most nickel compounds are blue or green. Nickel dissolves slowly in dilute acids but, like iron, becomes passive when treated with nitric acid. Finely divided nickel adsorbs hydrogen. It is closely related to iron and cobalt in chemical and physiological properties. Nickel may exist in the oxidation state of Ni (I) and Ni (II), but the most preferred oxidation state in biological systems is Ni (II).

Nickel is a recently discovered micronutrient that can form chelation compounds and can replace other heavy metals from physiologically important centers of metabolism. Ni is an essential element that can however, be toxic and possibly in high concentrations (Drazkiewicz, 2010). Most soils contain small quantities of nickel. The nickel content in the soil can be as low as 0.2 ppm or as high as 450 ppm in some clay and loamy soils. The average is around 20 ppm. Nickel occurs in some beans where it is an essential component of some enzymes. Another relatively rich source of nickel is tea which has an average of 7.6 mg/kg of dried leaves (Yadav, 2010).

Nickel is regarded as an essential element for plant growth. Nickel is a component of the enzyme urease, microbial dehydrogenases, hydrogenases and methyl reductase. It plays a significant role in urea and ureide metabolism, iron absorption, nitrogen fixation and seed development. Nickel deficiency is generally associated with the accumulation of nitrate and amino acids. Nickel deficient plants show interveinal chlorosis and necrosis in leaves. However, the requirement of nickel in plants is very low (Rao, 2006).

Symptoms of Ni toxicity can be observed between 0.19 and 0.85 mmol kg^{-1} (Ni) in plant dry biomass. Plants suffering with nickel toxicity show necrosis on the leaf tips and margins. Ni is rapidly taken up by the plant root system and the research with different plant species has shown that Ni is able to inhibit a large number of plant enzymes such as those of the Calvin cycle and chlorophyll biosynthesis and consequently decreases the photosynthetic activity. Besides this, Ni alters the plant water relations and increases the antioxidant system (Ali et al., 2008; Pandey and Sharma, 2002).

1.2.6 Cadmium

Cadmium is a divalent chemical element with atomic number 48, atomic weight 112.41 and melting point 320 ^{o}C, low enough compared to other transition metals. It is soft, malleable, ductile, bluish-white, toxic metal. It is similar in many respects to zinc but forms more complex compounds. It was first discovered in Germany in 1817 as a by-product of the zinc refining process. Its name is derived from the Latin *cadmia* and the Greek *Kadmeia*. Thanks to its unique chemical and physical properties, Cadmium is widely used in special alloys, pigments, stabilisers, coatings and above all (almost 70% of its use), in rechargeable nickel-cadmium batteries.

Cadmium is a naturally occurring minor element, one of the metallic components in the earth's crust and oceans and presents everywhere in our environment. It is derived in soils from both natural and anthropogenetic sources (Prasad, 19950. Cd occurs naturally in the environment from the gradual process of erosion and abrasion of rocks and soils, and from singular events such as forest fires and volcanic eruptions. It is therefore naturally present everywhere in air, water, soils and foodstuffs. The average natural abundance of cadmium in the earth's crust has most often been reported from 0.1 to 0.5 ppm, but much higher and much lower values have also been cited depending on a large number of factors. Anthropogenetic activities such as the non-ferrous metal industry, mining and energy production from coal, use and disposal of batteries and other products with high Cd content, metal-contaminated wastes and sludge disposal, and the application of pesticides and

especially phosphate fertilizers are important sources of Cd contamination in many areas of the world (Vasiliadou and Dordas, 2009; Lombi et al., 2000).

Among the heavy metals, cadmium is a potential environmental hazard. It is a toxic trace metal pollutant for humans, animals and plants. As a widespread trace pollutant with a long biological half-life, Cd can enter the food chain through uptake into plant tissues and pose a serious hazard to human health and ecosystem function. Cadmium accumulation in crop plants such as tobacco can lead to human exposure to this carcinogenic metal. Subsequently it travels to and accumulates in the liver and kidneys of animal and fish tissues. In addition, the high concentration of Cd in tobacco smoke has long been associated with various diseases (Vasiliadou and Dordas, 2009). Tobacco leaves naturally accumulate and concentrate relatively high levels of cadmium.

Special attention should be paid to Cd pollution in the soil-plant system, due to its mobility and the small concentration at which its toxic effect begins to show (Vasiliadou and Dordas, 2009). It is easily taken up by plants and with no essential function known to date (Lehoczky, 2000). Cd is accumulated mainly in the plant leaves and to a smaller extent in the other parts of the plant such as fruits and grains. Within the plant cell Cd can be found in various cell components (e.g., cell wall, cytoplasm, chloroplast, nucleus, vacuole; (Ramos et al., 2002; Lugon-Moulin et al., 2004). Numerous factors, such as soil characteristics, agronomic practices, environmental conditions, plant species and genotype impact the uptake of Cd by plants (Vasiliadou and Dordas, 2009; Lugon-Moulin Critical, 2004).

Cadmium is one of the most widely studied toxic metal in plants. Although Cd is not an essential nutrient metal for plants, it is taken up rapidly by the roots and on most occasions causes inhibited growth (Vasiliadou and Dordas, 2009). A number of toxic effects of Cd on metabolism have been reported, such as decreased uptake of nutrients, changes in nitrogen metabolism, interaction with the water balance of the plant and inhibition of stomatal opening. Cd^{2+} ions are known to cause alterations in the functionality of membranes by affecting the lipid composition and certain enzymatic activities associated with membranes, such as H^{+}-ATPase. Net

photosynthesis is also sensitive to Cd because it directly affects chlorophyll biosynthesis and the proper development of chloroplast ultra structure (Li et al., 2008; Wang et al., 2007). Early studies indicated that Cd ions affect the oxidizing side of photosystem 2 (PSII) and lead to the uncoupling of electron transport in the chloroplasts. The negative effects of Cd can also be observed in the carboxylating phase of photosynthesis (Gouia et al., 2003). Although there is no definitive understanding of how plants respond to Cd, increasing evidence indicates that the toxicity of Cd may be associated with oxidative damage caused by so-called reactive oxygen species (ROS) (Radetski et al., 2004; Piqueras et al., 1999). Cd stress might cause oxidative damage such as lipid peroxidation and induce alterations of the antioxidant system in various plants (Qadir et al., 2004; Ercal et al., 2001; Foyer et al., 1997).

1.3 Phytoremediation

1.3.1 The Phytoremediation Concept

Recognition of the ecological and human health hazards of some toxic pollutants has led to development of reliable and cost-effective technologies such as bioremediation capable of reducing heavy metals in soils and wastes to environmentally acceptable levels. The use of biological materials to clean up heavy metal contaminated soils has been focused on as an efficient and afforable form of remediation, and this can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition.

The concept of using plants to clean up contaminated environments is not new. About 300 years ago, plants were proposed for use in the treatment of wastewater. At the end of the 19th century, the first plant species documented to accumulate high levels of metals in leaves were *Thlaspi caerulescens* and *Viola calaminaria* (Lasat, 2000). Phytoremediation is an emerging, low cost technology that utilizes plants to remove, transform or stabilize contaminants located in water, sediments or soils, at a fraction of the cost of conventional technologies, such as soil replacement, solidification and washing strategies. Phytoremediation is based on the

use of some plants termed as phytoremediators that are capable of absorbing large amounts of several heavy metals from the soil and accumulating these metals in plant tissues.

Significant progress in phytoremediation has been made with metals and radionuclides. One particular process, concerned with the aquatic environment, involves rising of plants hydroponically and transplanting them into metal-polluted waters where plants absorb and concentrate the metals in their roots and shoots. When they become saturated with the metal contaminants, they are harvested for disposal. Several aquatic species have the ability to remove heavy metals from water, such as water hyacinth (*Eichhornia crassipes*), pennywort (*Hydrocotyle umbellata*), and duckweed (*Lemna minor*), (Prasad and Freitas, 2003). The process of phytoremediation can be divided into a few approaches, as shown in (Fig. 1.5), each having a different mechanism of action for the remediation of metal-polluted soil, sediment or water.

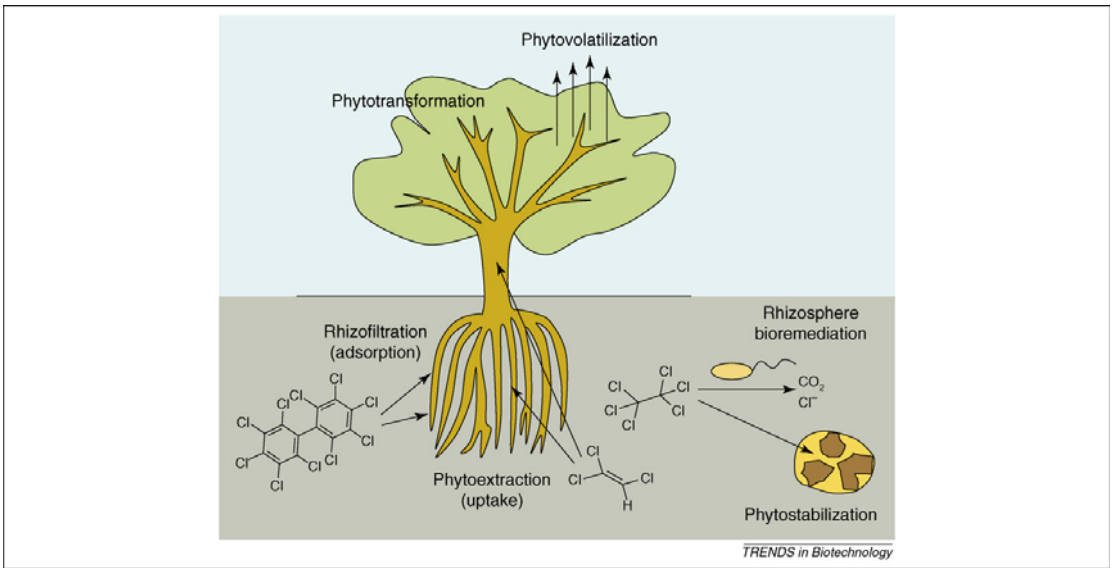


Figure 1.5: Processes of Phytoremediation include phytoextraction: pollutants in soil and groundwater can be taken up inside plant tissues, rhizofiltration: pollutants adsorbed to the roots, phytotransformation: pollutants inside plant tissues can be transformed by plant enzymes, phytovolatilization: pollutants can volatilize into the atmosphere, rhizosphere bioremediation: pollutants in soil can be degraded by microbes in the root zone, phytostabilization: pollutans incorporated in soil material (Aken, 2008).

a. Phytoextraction

The phytoextraction of heavy metals and radionuclides represents one of the largest economic opportunities for phytoremediation, because of the size and scope of environmental problems associated with metal-contaminated soils, and the competitive advantage offered by plant based remediation technology (Raskin et al., 1997). Phytoextraction involves transport of metals from the soil into the plant and their accumulation in harvestable parts of roots and shoots. For this purpose, a plant species and its specific genotype should not only accumulate and tolerate high levels of toxic metals, but should also have rapid growth rate to produce high level of biomass. However, even *Thlaspi caerulescens* and *Brassica juncea*, which in field trials have been found to be the best metal accumulators, would take 13 to 14 years of continuous cultivation to clean a particular site contaminated with toxic metals.

b. Phytostabilization

Phytostabilization is a plant-based remediation technique that reduces the mobility of substances in the environment, for example by limiting the leaching of substances from the soil. Plants chosen for phytostabilization should be poor translocators of metal contaminants to aboveground plant tissues that could be consumed by human or animals (Prasad and Freitas, 2003). *Festuca rubra* and *Agrostis tenuis* are now commercially available for the phytostabilization of Pb-, Zn- and Cu- contaminated soils. Phytostabilization is more effective at sites having fine-textured soils with high organic-matter content (Prasad and Freitas, 2003).

c. Phytotransformation

Phytotransformation includes the chemical modification of environmental substances as a direct result of plant metabolism, often resulting in their inactivation, degradation (phytodegradation) or immobilization (phytostabilization). It involves conversion of pollutants into nontoxic materials, as is the case with some herbicides. Enzymes like oxidoreductases, dehalogenases, nitroreductases, peroxidases and nitrilases may be involved, for and rhizosphere activity.

d. Phytovolatilization

Phytovolatilization includes the removal of substances from soil or water with release into the air, sometimes as a result of phytotransformation to more volatile and/ or less polluting substances. It is a unique method of dealing with metals contaminants that may exist as gaseous species in environment such as mercury or selenium. It has been known for a long time that microorganisms play an important role in the volatilization of Se, and a plant with the ability to perform the same function was recently discovered. Se volatilization in the form of methyl selenate has been proposed as a major mechanism for Se removal from soil. *Brassica juncea* was identified as a valuable plant for removing Se from soils (Raskin et al., 1997).

e. Rhizofiltration

Rhizofiltration involves absorption, precipitation and accumulation of toxic substances or excess nutrients, in roots only, there being no involvement of shoots. A plant should have rapidly growing roots to be able to remove toxic metals from water and soil for an extended period of time. A number of plant species including rapeseed mustard (*Brassica juncea*), rye, corn and sunflower can have rapidly growing roots and can be used for rhizofiltration.

Advantages and limitation of phytoremediation summarized in Table 1.4.

Table 1.4 : Advantages and limitations of the phytoremediation technology (Alkorta et al., 2004)

Advantages	*Limitations*
o Applicable to a wide variety of inorganic contaminants	• Limited by depth (roots) and solubility and availability of the contaminant
o Reduces the amount of waste going to landfills	• Although faster than natural attenuation, it requires long time periods (several years)
o Does not require expensive equipment or highly specialized personnel	• Restricted to sites with low contaminant concentration
o It can be applied *in situ.* Reduces soil disturbance and the spread of contaminants	• Plant biomass from phytoextraction requires proper disposal as hazardous waste

o Early estimates of the costs indicate that phytoremediation is cheaper than conventional remediation methods	• Climate and season dependent. It can also lose its effectiveness when damage occurs to the vegetation from disease or pests
o Easy to implement and maintain. Plants are a cheap and renewable resource, easily available	• Introduction of inappropriate or invasive plant species should be avoided (non-native species may affect biodiversity)
o Environmentally friendly, aesthetically pleasing, socially accepted, low-tech alternative	• Contaminants may be transferred to another medium, the environment, and/or the food chain
o Less noisy than other remediation methods. Actually, trees may reduce noise from industrial activities	• Amendments and cultivation practices may have negative consequences on contaminant mobility

1.3.2 The Hyperaccumulation Concept

One type of metal tolerant plant is termed a 'hyperaccumulator'. This kind of plant is known to be capable of both growing on soils contaminated with toxic metals and accumulating extraordinary high levels of them (Lim et al., 2006). To date approximately 400 plant species from 45 families have been identified. The capacity for accumulation is due to hypertolerance which is the result of adaptive evolution of the plants to hostile environments along multiple generations (Fitter and Hay, 2002; Salt et al., 1998). The metal-accumulating plants can be divided into three groups on the basis of their tendency to accumulate different metals: (1) Cu/Co, (2) Zn/Cd/Pb, and (3) Ni accumulators (Alkorta et al., 2004; Mejare and Bulow, 2001). There are four main types of metallliferous soils that host hyperaccumulators: (a) serpentine soils derived from Fe- and Mg-rich ultramafic rocks that are enriched with Cr, Co and Ni, (b) seleniferous soils derived from Se-rich rock types, (c) calamine soils enriched with Zn, Cd and Pb, (d) Co- and Cu-containing soils derived from argillites and dolomites (Callahan et al., 2005).

Metal concentrations in the shoots of accumulating plants can be 100–1,000-fold higher than in non-accumulating plants: 1% for Zn (up to 4%) and Mn); 0.1% for Co; (up to 1.2%), Cu, Ni; up to 3.8%, As up to 0.75% and Se (up to 0.4%); and 100 ppm for Cd (up to 0.2%). One definition proposes that a plant containing more than 0,1% of Ni, Co, Cu, Cr and Pb or 1% of Zn in its leaves on a dry weight basis can be

called as hyperaccumulator, irrespective of the metal concentration in the soil (Raskin et al., 1994).

The problem of these hyperaccumulators is that they have small biomass and are slow-growing, therefore it could take many years to decontaminate a polluted place (Hirata et al., 2005; Qadir et al., 2004). An approach to find metal-tolerant hyperaccumulating plants for phytoremediation involves searching for and studying natural hyperaccumulators, or developing genetically engineered plants that possess these traits. The introduction of metal-binding proteins and peptides into plants to enhance metal tolerance and/or accumulation is a compelling strategy. These metal-binding peptides or proteins should be preferentially metal specific such that only the toxic metals are sequestered (for example Cd and Pb) and not essential trace metals such as Zn (Mejare and Bulow, 2001).

A successful phytoremediation system would require plants that (Martinez et al., 2006):

- → could survive on the contaminated soil (i.e. they must be resistant)
- → could absorb large quantities of the toxin into the roots, even when the toxin was present in soil in a unavailable form
- → could transport the toxin to the shoots, so that it could be harvested and removed
- → had a high growth rate, so that the absolute amounts of metal transported were large.

A relatively small group of hyperaccumulator plants is capable of sequestering heavy metals in their shoot tissues at high concentrations. In recent years major scientific progress has been made in understating the physiological mechanisms of metal uptake and transport in these plants (Yang et al., 2005). A comparative study between *Thlaspi caerulescens*, one of the best known zinc and cadmium huperaccumulators, and the nonaccumulator *Thlaspi arvense* found an enhanced uptake of metals into the root symplast in *T. caerulescens* compared with the latter (Mijovilovich et al., 2009; Lasat, 1998), and a reduced sequestration into the root

vacuoles was associated with the higher root-to-shoot translocation efficiency of *T. caerulescens* (Mijovilovich et al., 2009; Lasat, 1998).

Hyperaccumulation of heavy metals by higher plants is a complex phenomenon. It involves several steps, such as: (a) transport of metals across the plasma membrane of root cells; (b) xylem loading and translocation; and (c) detoxification and sequestration of metals at the whole plant and cellular levels (Yang *et al.*, 2005). Several authors have proposed that hyperaccumulators have different strategies of detoxification for metals that hyperaccumulated compared with nonhyperaccumulated metals. For the hyperaccumulated metals, detoxification is mainly based on active sequestration into the vacuoles of the epidermis, where they stored only loosely associated with organic acids that are abundant in this organelle. Strong ligands like phytochelatins and metallothioneins that detoxify heavy metals in nonaccumulator plants do not play a major role in the detoxification of hyperaccumulated metals in hyperaccumulator plants (Mijovilovich et al., 2009). However, the nonproteogenic amino acid nicotianamine (NA) seems to play an important role in metal homeostasis of plants. According to several studies, it binds Fe, Zn and Cu, mainly for long-distance transport in the vascular bundle (Mijovilovich et al., 2009), and NA synthase has been shown to be highly over expressed in hyperaccumulators compared with nonaccumulator plants (Mijovilovich et al., 2009).

Several additional resistance mechanisms may become activated, when metal concentrations reach toxic levels in hyperaccumulator plants. An increase of the Mg content was found in the mesophyll of metal-stressed hyperaccumulator plants, which was interpreted as a defense against the replacement of Mg^{2+} in chlorophyll by heavy metals (Kupper et al., 2004).

1.3.3 Phytoremediation and Biotechnology

The first goal in phytoremediation is to find a plant species which is resistant to or tolerates a particular contaminant with a view to maximizing it's potential for phytoremediation. Once a tolerant species has been selected traditional breeding

methods, agricultural methods, such as the application of fertilizers, chelators and pH adjusters can be utilized to improve the potential for phytoremediation.

Biotechnology is an important means to increase the efficacy of heavy metals phytoextraction, through the genetic engineering (GE) of plants (Lugon-Moulin et al., 2004), especially plants that have been genetically engineered to accumulate and resistant higher levels of metals (Lugon-Moulin et al., 2004). GE approaches can be used to over-express the enzymes involved in the existing plant metabolic pathways or to introduce new pathways into plants. Phytoremediation strategies that have recently been put into practice include the genetic manipulation of GSH and phytochelatine production in plant tissues (Peuke and Rennenberg, 2005).

Plants of *Arabidopsis thaliana* have been genetically modified with bacterial organomecurial lyase (*MerB*) and mercuric reductase (*MerA*). These plants absorb elemental Hg(II) and methyl mercury (MeHg) from the soil and release volatile Hg(O), which is approximately 100 times less toxic, from leaves into the atmosphere (Prasad and Freitas, 2003). Plants of F2 generation also, showed a greater resistance to organic mercury, compared to wildtype plants. The same *MerA/MerB* inserts have been used in other plant species including *Nicotiana tabacum* (tobacco) and *Liriodedron tulipifera* (yellow poplar). Wetland species (bulrush and cat-tail) and water tolerant trees (willow and poplar) have been targetted for transformation.

1.4 Plant Responses to Heavy Metal Toxicity

1.4.1. Tolerance and Resistance Responses

Some plant species have evolved tolerant races that can survive and thrive on metalliferous soils, presumably by adapting mechanisms that may also be involved in the general homeostasis of, and constitutive tolerance to, essential metal ions as found in all plants (Hall, 2001). The level of accumulation of elements differs between and within species, suggested that plants could exhibit three basic responses for growing on soils with potentially toxic levels of metal ions (Fig. 1.6):

- *Metal excluders*: These plants do not translocate metal ions to the above-ground tissues, a trait which enables them to grow on soils toxic to most plants; however

they can still contain large amounts of metals in their roots (Memon et al., 2001 ; Callahan *et al.*, 2005). The exclusion strategy, comprising avoidance of metal uptake and restriction of metal root-to-shoot transport, and which minimize the cellular accumulation of metals, is usually used by pseudometallophytes. These plants are therefore currently used to revegetate bare soils areas (e.g. in phytostabilisation technology), i.e. where the lack of vegetation results from excessively high metal concentration (Papazoglou et al., 2004).

- *Metal accumulators*: These plants accumulate metals in their above-ground tissues and the metal levels in the tissues of these plants generally reflect metal levels in the soil (Memon *et al.*, 2001).
- *Metal hyperaccumulators*: The accumulation strategy allows plants to survive, while accumulating strong concentrations of metals in their tissues. These plant species exhibit higher metal concentrations in their tissues than are present in the soil or in hydroponic solution or in the non-accumulating species. They can tolerate much higher metal concentrations before showing symptoms of toxicity (Callahan et al., 2005).

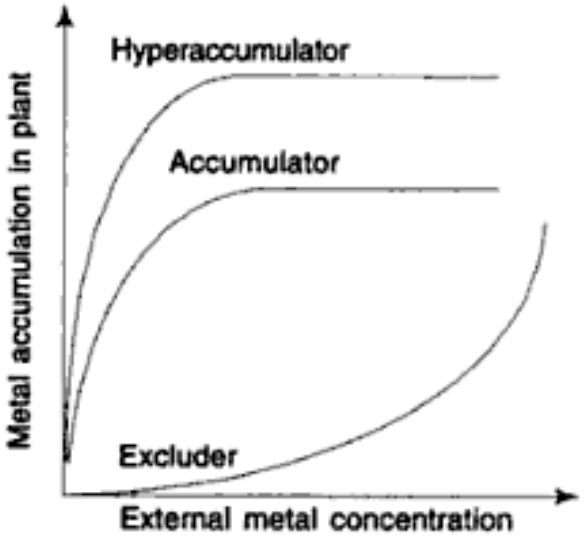


Figure 1.6: Plants with different uptake characteristics (Ghosh et al., 2005)

1.4.2. Mechanisms behind Tolerance to Metal Toxicity

Plants experience oxidative stress upon exposure to heavy metals that leads to cellular damage. In addition, plants accumulate metal ions that disturb cellular ionic homeostasis. To minimize the detrimental effects of heavy metal exposure and their accumulation, plants have evolved detoxification mechanisms. The mechanisms by which plants bind and thus minimize the toxic effect of metals are unique to each metal (Fitter and Hay, 2002).

Plants respond to heavy metal toxicity in a variety of different ways. The sensitivity of plants to heavy metals depends on an interrelated network of physiological and molecular mechanisms such as (i) uptake and accumulation of metals through binding to extracellular exudates and cell wall constituents; (ii) efflux of heavy metals from cytoplasm to extranuclear compartments including vacuoles; (iii) complexation of heavy metal ions inside the cell by various substances, for example, organic acids, amino acids, phytochelatins, and metallothioneins; (iv) accumulation of osmolytes and osmoprotectants and induction of antioxidative enzymes (v) activation or modification of plant metabolism to allow adequate functioning of metabolic pathways and rapid repair of damaged cell structures (John et al., 2009). Understanding the molecular and genetic basis for these mechanisms will be an important aspect of biotechnological implications (Clemens et al., 2002) as well as of developing plants as agents for the phytoremediation of contaminated sites (Cobbett, 2000).

a. Metal uptake accumulation transfer to plants

The alleviation of heavy metals stress affecting higher plants includes uptake, translocation, and efflux. Plants accumulate essential and non-essential elements from soils in response to concentration gradients induced by selective uptake of ions by roots, or by diffusion of elements in the soil (Peralta-Videaa et al., 2009). Within the cortex the metals are transported in the apoplastic space according to their concentration gradient and also accumulate in the cell walls (Schutzendubel and Polle, 2002). Toxic effects are exerted at the plasma membrane and within the cell. Two different uptake routes have been reported: (a) passive uptake, only driven by the concentration gradient across the membrane and (b) inducible substrate-specific and energy-dependent uptake (Schutzendubel and Polle, 2002). Root accumulation of Cd is both passive (covalent or ionic bonding to functional groups on cell walls) and transmembrane (using transporters for Zn or Ca) (Vliet et al., 2007). Active and passive transport systems have also been reported for Cd and Ni in roots of spruce and soybean (Schutzendubel and Polle, 2002). So, non-essential heavy metals may effectively compete for the same transmembrane carriers used by essential heavy metals and toxic heavy metals may effectively compete for the same transmembranic

carrier as used by micronutrient heavy metal. This relative lack of selectivity in transmembrane ion transport may partially explain why non-essential heavy metals can enter cells, even against a concentration gradient (Ghosh et al., 2005). Fig. 1.7 presents the molecular mechanisms proposed to be involved in transition metal accumulation by plants.

Transport processes have been recognized as a central mechanism of detoxification and tolerance (Sharma and Dietz, 2006; Hall, 2002). The application of powerful genetic and molecular techniques has now identified a range of gene families that are likely to be involved in transition metal transport. These include the heavy metal ATPases (HMAs), the natural resistance-associated macrophage protein (Nramp) family, the cation diffusion facilitor (CDF) family, the ZIP family and the cation antiporters. Transition metal transporters have a central importance in the plant metal homeostasis network, which maintains internal metal concentrations within physiological limits. A common transmembrane transporter was found for Cd, Cu, Ni and Zn (Schutzendubel and Polle, 2002). Resistance to Zn appears to be associated with ion transport, when *Arabidopsis* was engineered to overexpress a Zn transporter, analogous to those found in animals, its resistance to Zn was enhanced (Fitter and Hay, 2002).

Resistance to Zn appears to be associated with ion transport. Resistance to Zn was enhanced in *Arapidopsis* when it was engineered to overexpress a Zn transporter. Zn can also be sequestered in vacuoles, for example in the zinc hyperaccumulator *Thlaspi caerulescens* and inactivated by precipitation as zinc phytate, although this would be a very expensive mechanism, immobilizing six moles of P for every mole of Zn removed (Fitter and Hay, 2002).

b. Factors affecting the uptake of heavy metals by plants.

Metals in soil are present as free metal ions, soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates and hydroxides, or they may form part of the structure of silicate minerals (indigenous soil content).

Toxicity of heavy metals to plants depends on the bioavailability of the elements in the soil, the length of the exposure period and the physiological activity of plant roots and their associated microorganisms (Daisei et al., 2008).

The process of metal uptake and accumulation by different plants depend on the concentration and solubility of available metals in soil, and plant species growing on these soils (Clemens et al., 2002).

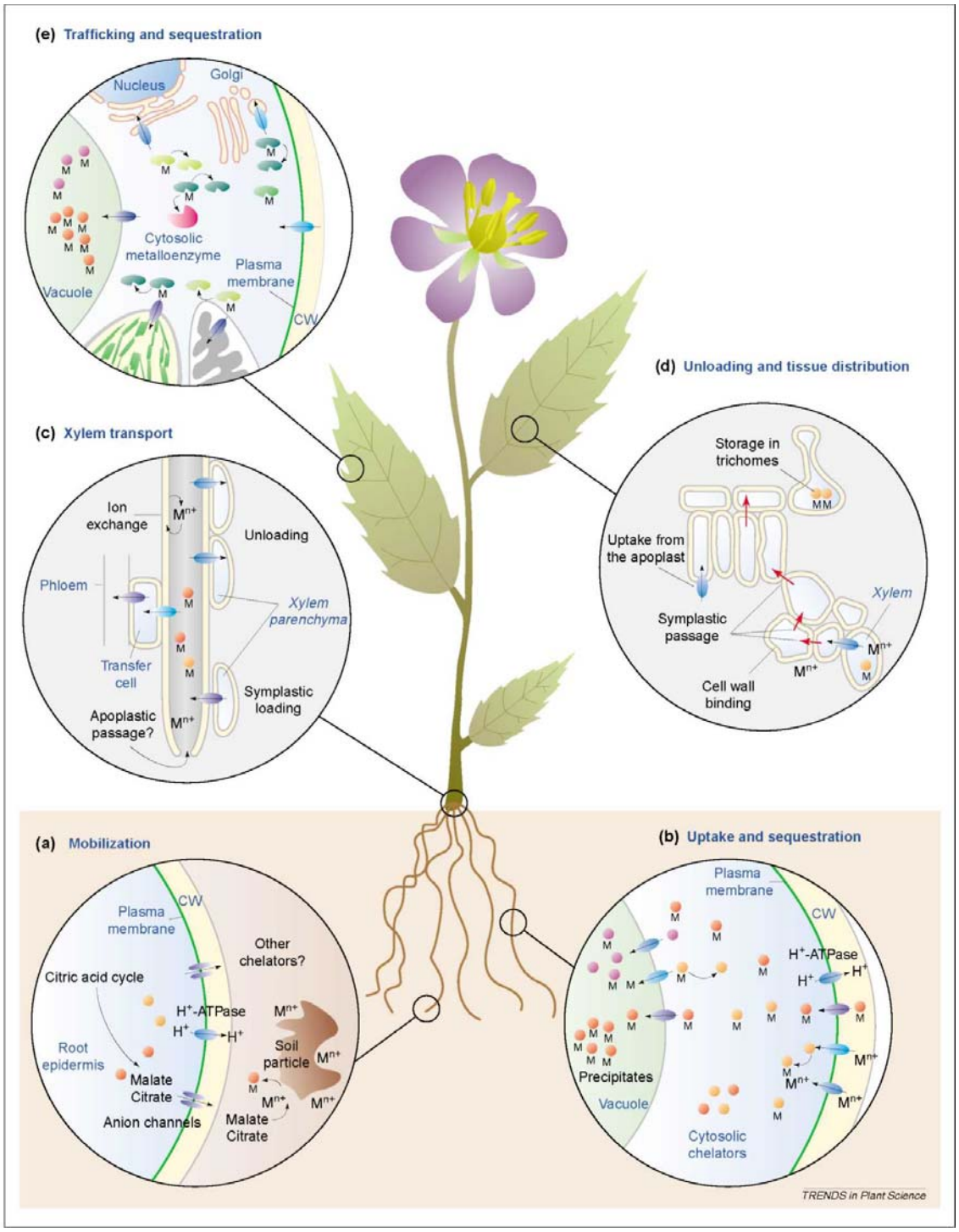


Figure 1.7: Transition metal accumulation involved molecular mechanisms**:** (a) Mobilization of metal ions by secretion of chelators and by acidification of the rhizosphere. (b) Uptake of hydrated metal ions or metal-chelate complexes is mediated by various uptake systems residing in the plasma membrane. Inside the cell: metals chelation and excess metal sequestration transport into the vacuole. (c) From the roots, transition metals are transported to the shoot via the xylem. Presumably, the larger portion reaches the xylem via the root symplast. Apoplastic passage might occur at the root tip. Inside the xylem, metals are present as hydrated ions or as metal-chelate complexes. (d) After reaching the apoplast of the leaf, metals are differentially captured by different leaf cell types and move cell-to-cell through plasmodesmata. Storage appears to occur preferentially in trichomes. (e) Uptake into the leaf cells again is catalyzed by various transporters [not depicted in (e)]. Intracellular distribution of essential transition metals (= trafficking) is mediated by specific metallochaperones and transporters localized in endomembranes (please note that these processes function in every cell). Abbreviations and symbols: CW, cell wall; M, metal; filled circles, chelators; filled ovals, transporters; bean-shaped structures, metallochaperones (Clemens et al., 2002)

The solubility and mobility of metals is affected by adsorption, desorption and complexation processes, which in turn are dependent on the soil type (Schutzendubel and Polle, 2002). The mobility of As in soil is mainly controlled by adsorption/desorption processes and co-precipitation with metal oxides. Therefore the most extensively studied amendments for As immobilization are oxides of Fe and, to a lesser extent, Al and Mn.

Overall there is no correlation between soil metal content and plant metal content. Some metals are practically not available for plant uptake because of their low solubility in soil such as Cr, Ag or Sn. Other metals such as Pb can be major pollutants and locally present in enormous quantities yet are hardly taken up into plants because of low solubility and strong interaction with soil particles (Clemens, 2006). Different techniques are used to estimate the bioavailability of metals in soil, including chemical extractions and biological tests using plants or micro-organisms (Leyval et al., 1997;).

The toxicity of metals in soil depends on their bioavailability, defined as their ability to be transferred from a soil compartment to a living organism. According to Bertheline et al. (1995), metal bioavailability is a function not only of their total concentration but also of physico-chemical (e.g. pH, Eh, organic matter, clay content)

and biological (e.g. biosorption, bioaccumulation and solubilisation) factors (Leyval et al., 1997). Numerous factors such as soil characteristics, agronomic practices and environmental conditions impact the uptake of heavy metal by plants. Agronomic practices include: sludge amendments, liming, fertilizers, irrigation water, other practices (crop rotation), and factors such as climate conditions (temperature, moisture), atmospheric deposition on leaves, and other variations associated with the crop year (Lugon-Moulin et al., 2004).

Also, the availability of heavy metals to plants and thus, their toxicity depends on complex rhizospheric reactions involving not only exchange processes between soil and plants but also microbial activities. In this respect, mycorrhizal fungi appear to play a central modulation role (Schutzendubel and Polle, 2002). Under natural conditions, roots of many plants species, especially those of trees are associated with mycorrhizal symbionts. This modifies the response of plants to heavy metals significantly (Schutzendubel and Polle, 2002). Studies have shown that the effects of maize root colonization by arbuscular mycorrhiza could either reduce the heavy metal content of the plants or increase metal absorption from polluted soils, depending on growth conditions, the fungus and the metal (Hall, 2001).

c. Distribution/ Sub-cellular Localization/ Interactions with plant nutrients

The physiology of metal toxicity in plants is mainly concerned with metal movement from soil to root and metal absorption and translocation (Rout and Das, 2003). In addition to uptake, an efficient root to shoot translocation is thought to be one of the most important characters of metal hyperaccumulators (Daisei et al., 2008). Plants distribute metals internally in many different ways. They may localize selected metals mostly in roots and stems, or they may accumulate and store other metals in nontoxic form for latter distribution and use (Memon et al., 2001). Within the plant cell, heavy metals can be found in various cell components (e.g. cell wall, cytoplasm, chloroplast, nucleus, vacuoles (Lugon-Moulin et al., 2004). Distribution may vary according to the species, organ, tissue (Lugon-Moulin et al., 2004) and the metal concerned (Mari and Leburn, 2005). Under metal-induced stress, the heavy metal accumulation pattern changes. For example, in a study conducted under such conditions, heavy metal (Cd, Ni) accumulation was enhanced in a few cells of the

mesophyll (Mijovilovich et al., 2009). As seen in Fig. 1.8, heavy metals differ substantially according to their accumulation in plant tissues.

Recent progress in the study of toxic metals and their interactions with essential elements has greatly increased our understanding of the mechanism of toxicity at the biochemical level (Rout and Das, 2003). According to the classical definition "Interactions between nutrients occur when the supply of one nutrient affects the adsorption, distribution or function of another". Thus, depending on nutrient supply, interactions between nutrients can both induce deficiencies or toxicities and modify growth responses. Competitive interactions can also occur between essential nutrients and heavy metals, which are easily taken up plants.

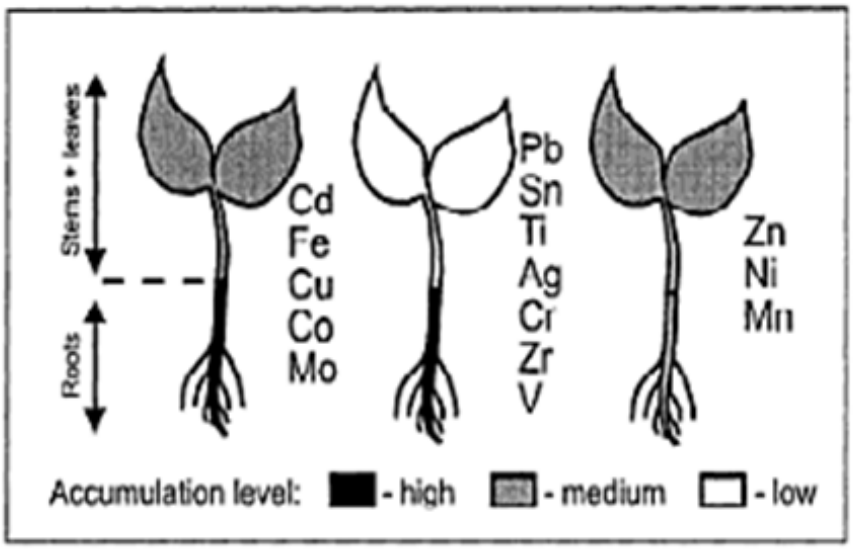


Figure 1.8: Heavy metal distribution between plant organs (Prasad, 2010).

For example, Zn toxicity induces Fe deficiency due to Fe/Zn interaction and competition that might be explained by similar ionic radii of hydrated cations and cell regulatory mechanisms. Cd, Cu, Pb and Zn show a wide range of interactions. Cadmium interacts with Cu and Zn at the root surface, whilst Pb interacts with Zn and Cd within the plant. Some of these interactions are antagonisms (Zn with Cu and Cd), others are synergistic (Cd with Zn at the root surface, Pb with Cd within the plant). Synergism and additivity of Cd-Zn interaction might be related to inadequate compartmentation of Cd and Zn, and is generally unfavorable to plant growth.

Heavy metal uptake might be also inhibited or limited by some essential nutrients. Usually Ca, P, Zn, Cu, Mn and Fe are supported to act antagonistically against Cd, but it seems to depend on plant species and variety. Manganese is widely recognized as an antidote to elevated uptake and distribution of some heavy metals. It has been reported to have a protective role against Cd toxicity in relation to the photosynthetic apparatus. In contrast, increased supply of some essential metallic elements may appear dangerous for heavy metal-treated plants. Nickel toxicity was increased in the presence of Co, Zn, Mn and Mo, leading to intensified Ni-type chlorosis (Prasad and Strzalka, 2002; Prasad, 2010).

d. Chelation/ sequestration

Passage through the plasma membrane by metals is enhanced by intracellular binding and sequestration. Once metal ions enter the cell, they are bound by chelators and chaperones. Chelators contribute to metal detoxification by buffering cytosolic metal concentrations, while chaperones specifically deliver metal ions to organelles and metal-requiring proteins. So, upon exposure to metals, plants often synthesize a set of diverse metabolites that accumulate to concentrations in the millimolar range, particularly specific amino acids, such as proline and histidine, peptides such as glutathione and phytochelatins (PCs), and the amines spermine, spermidine, putrescine, nicotianamine and mugineic acids (Fig. 1.9).

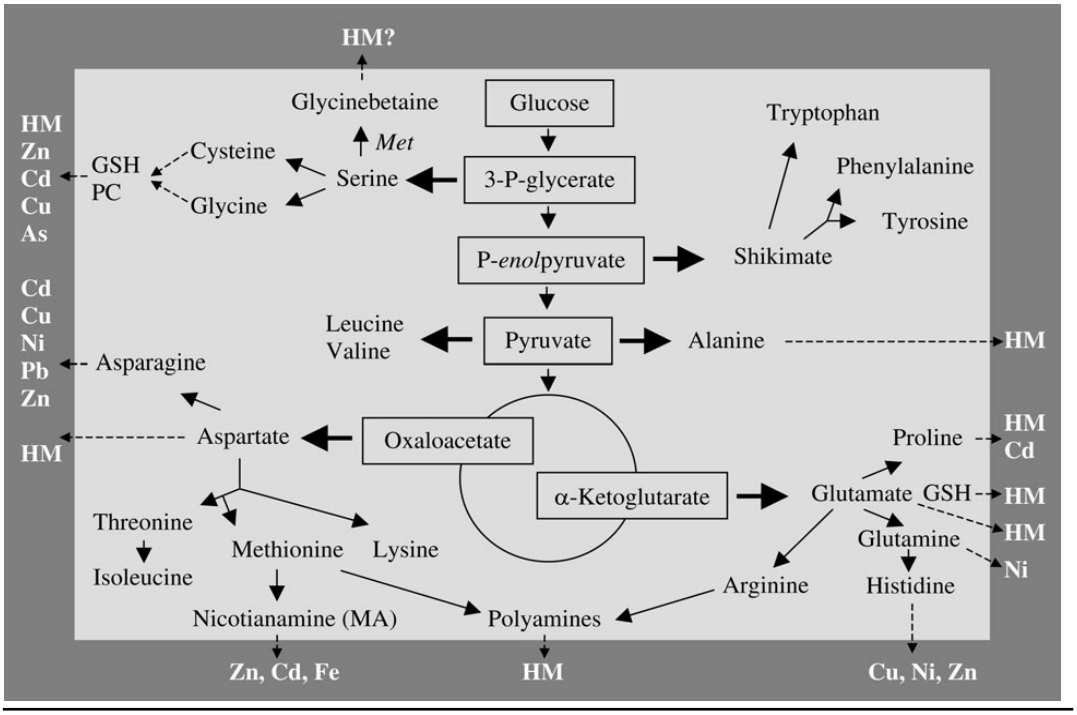


Figure 1.9: Schematic depiction of synthetic pathways involved in the synthesis of amino acids and heavy metal-related N-metabolites in plants. The inner area shows the central pathways of glycolysis and citric acid cycle and the linking metabolites to amino acid synthesis. Also shown is the branching to glutathione (GSH), phytochelatin (PC), polyamine, nicotianamine, and mugineic acid (MA) synthesis. In the outer frame, examples are given showing the involvement of N-metabolites in metal metabolism. HM: heavy metal (Gasic and Schuyler, 2006).

Thus, nitrogen metabolism is central to the response of plants to heavy metals (Sharma and Dietz, 2006). Generally, it is assumed that the major sites of metal sequestration in the roots are vacuoles. Extensive research, performed in vacuolar sequestration, has revealed a range of gene families involved in intracellular metal transport.

e. Phytochelatins (PCs)

Phytochelatins are the best known example of metal-induced metabolites and are implicated in cellular metal detoxification. Phytochelatins are unusual peptides with the general formula (γ-glutamic acid-cysteine)$_n$-glycine, where n=2-8. The unusual structure arises from the fact that the peptide bond between glutamate and cysteine utilizes the side chain, or γ-carboxyl group of glutamate rather than the α-carboxyl group characteristic of proteins. This bonding arrangement suggests that phytochelatins are not synthesized on ribosomes and are thus not a direct gene product but are the product of some biosynthetic pathway. The structure of Phytochelatins is similar to that of the tri-peptide glutathione (GSH) γ-glutamyl-cysteinyl-glycine, the most abundant thiol (-SH compound) in plants. Glutathione plays an important role in detoxification of peroxides that are generated in the presence of active oxygen species. GSH also accumulates when plants are fed sulfur compounds, suggesting that glutathione may also serve as a storage pool for cysteine. When sulfur is in short supply, GSH is slowly degraded and used as a source of sulfur (Cobbett, 2000).

Significant amounts of phytochelatins are found only when toxic levels of cadmium, copper, mercury, lead zinc and other metalloid elements are present. Although there is little doubt that PCs readily sequester metallic elements to the

cysteine thiol groups, their exact role is not clear. They may serve as a shuttle, binding metals in the cytosol and carrying them into the vacuole. Once in the vacuole, the acidic pH would displace the metal, allowing the peptide to return to the cytosol. The metal would then be sequestered by organic acids that are usually present at high concentrations in the vacuole. (Hopkins and Huner, 2003).

Most of the heavy metals which are toxic to plants can be complexed in the plant in some way. Cd can certainly be rendered chemically inactive in cells in the form of complexes with phytochelatins, whose synthesis is induced by exposure to Cd^{2+} ions in the root environment. The complexes can then be excluded from cytoplasm by transport across the tonoplast into the vacuole, where they are probably stabilized by binding to sulphide (Fitter and Hay, 2002).

f. Antioxidant defense mechanisms

A wide range of protective mechanisms exist in plants that serve to remove ROS before they can damage sensitive parts of the cellular machinery. These mechanisms can be divided in two groups, that is non-enzymatic antioxidants such as glutathione, ascorbate, tocopherols, carotenoids etc and enzymatic antioxidants like catalase (CAT), superoxide dismutases (SOD), as well as enzymes of ascorbate-glutathione cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). H_2O_2 can be directly metabolized by peroxidases, particularly those from the cell wall and by CAT in the peroxissome (Fig. 6). In the chloroplast O_2^- is converted by SOD into H_2O_2, which is then detoxified to H_2O and O_2 by the ascorbate-glutathione cycle. Several studies have been carried out with plants to evaluate the effect of heavy metals on the activity of antioxidant enzymes such as SOD, CAT and GR (*Crotalaria juncea*, Ni).

g. Heat shock proteins

Plant cells often produce heat shock proteins, after being subjected to metal stress. These molecular chaperones are general stress proteins involved in the

protection, repair and degradation of damaged cell components, especially proteins, during most abiotic stresses. According to several reports, the increased production of Hsps is indeed a general plant response to heavy metal accumulation (Heckathorn et al., 2004).

1.5 Heavy Metals and Nitrogen Metabolism

Previous studies provide extensive information on the effect of some heavy metals in growth and development of several metal-accumulating and non accumulating plants. However, nitrogen metabolism and the regulation of related enzymes have not been widely studied, especially in metal-accumulating species, although nitrogen plays the most important role of all nutrients in plant growth, it is correlated with biomass production and its metabolism is directly connected with carbon metabolism (Kumar and Joshi, 2008; Lim et al., 2006; Chaffei et al., 2004). Nitrogen metabolism is important for the response of plants to heavy metal toxicity (Fig. 1.9; Sharma and Dietz, 2006; Wang et al., 2007). Upon exposure to metals accumulating plants often synthesize a set of N-containing metabolites through N metabolism, such as proline, glutathione and phytochelatins (Wang et al., 2007; Sharma and Dietz, 2006).

1.5.1 Proline

Proline is predominantly synthesized from glutamate. It is an extremely studied molecule in the context of plant response to abiotic stresses. Proline is considered as a strong indicator of environmental stress (Zengin and Munzuroglu, 2005). Many plants accumulate compatible solute under water deficit, low and high temperature, salinity heavy metal stress and other environmental stresses. Proline is a nitrogen compound whose accumulation in heavy metal resistant plants is detected. Its accumulation in plant tissues may be due to a decrease in proline degradation, an increase in proline biosynthesis, a decrease in proline synthesis or utilization and hydrolysis of proteins (Zengin and Munzuroglu, 2005; Sharma and Dietz, 2006). Proline was demonstrated to protect glucose-6-phosphate dehydrogenase and nitrate reductase in vitro against Zn- and Cd-induced inhibition (Sharma and Dietz, 2006).

1.5.2 Ammonium Assimilating Enzymes

In particular, knowledge on the ammonia assimilating enzymes, namely glutamine synthetase, glutamate synthase and glutamate dehydrogenase and their regulation under heavy metal stress conditions is restricted. In plants, inorganic nitrogen is reduced to ammonia and then assimilated into amino acids via the enzymes glutamine synthetase (GS, EC 6.3.1.2), glutamate synthase (Fd-GOGAT, EC 1.4.7.1 or NAD(P)H-GOGAT, EC 1.1.1.14) and probably glutamate dehydrogenase (GDH, EC 1.4.1.2) (Fig. 1.10). Although the operation of the GS/GOGAT cycle in ammonia assimilation is well established by biochemical, molecular and genetic studies (Loulakakis et al., 2009; Lea et al., 1990), the exact physiological role of GDH in plants remains obscure and two apparently conflicting functions have been proposed for the enzyme.

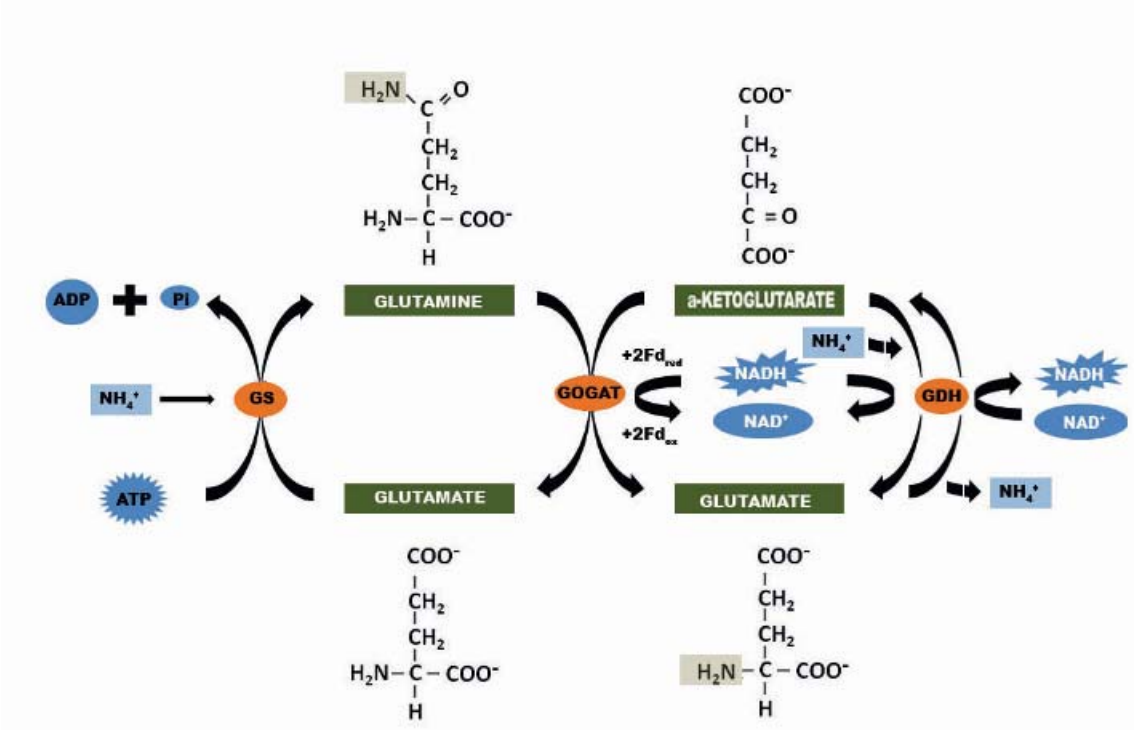


Figure 1.10: Biochemical reactions catalyzed by ammonia assimilating enzymes in plants. GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase (Loulakakis et al., 2009).

The enzyme may play a complementary role to GS/GOGAT cycle in the assimilation or reassimilation of ammonia, especially under stress conditions or during specific stages of development (Loulakakis et al., 2009; Oaks, 1994). The observations that during darkness, natural senescence, or fruit ripening there is an induction of GDH, together with an alteration in its isoenzymes (Loulakakis et al., 1994), tent to support this hypothesis. Under such conditions the limitation of the GS/GOGAT pathway, as well as excessive protein degradation result in increased ammonia levels. Alternatively, GDH could catalyses the oxidation of glutamate ensuring sufficient carbon skeletons for effective functioning of the Crebs cycle under conditions of carbon limitation (Masclaux-Daubresse, 2006; Loulakakis et al., 1994; Oaks, 1994).

1.6 Morphological and Botanical Characteristics of *Nicotiana tabacum* and *N. glauca*

1.6.1 *Nicotiana tabacum*

Nicotiana tabacum or cultivated Tobacco is a perennial or herbaceous plant of the *Solanaceae* family. It is a very variable, tall, sticky-hairy, strong smelling annual, up to 2 m. Leaves are large, elliptical to lanceolate, untoothed, with a winged base that runs onto the stem. Flowers are pale green or creamish, often with a pink tinge, trumpet-shaped, 35-55 mm long, many borne in a broad terminal panicle. Fruits are green capsules. It occurs in cultivated land, waste places, occasionally on arable land. It is sensitive to temperature, air ground humidity and the type of land. For adequate growth *N. tabacum* requires temperatures of 20 to 30 ^{o}C, an atmospheric humidity of 80 to 85% and soil without a high level of nitrogen.

Nicotiana tabacum is native in South America, but is now commercially cultivated worldwide. It has been and continues to be one of the most commonly used and socially important plants. Ethnobotanists surmise that its use began 8000 years ago, in prehistoric America. Tobacco is obtained from the partially fermented leaves of this species in particular it contains nicotine, and is smoked, chewed, or inhaled as snuff by most of the peoples of the world. Every part of the plant except the seed contains nicotine, but the concentration is related to different factors such as species,

type of land, culture or weather conditions. The concentration of nicotine increases with the age of the plant. Tobacco leaves contain 2 to 8 % of nicotine combined as malate or citrate.

The distribution of the nicotine in the mature plant is widely variable: 64% of the total nicotine exists in the leaves, 18% in the stem, 13% in the root and 5% in the flowers. Nicotine is manufactured in the roots and transported via the xylem to the leaves.

Plate 1.1: View of *Nicotiana tabacum* plant.

Nicotine stimulates the nervous system primarily by mimicking a natural compound in the body called acetylcholine, a neurotransmitter. It is broken down by the liver and its action lasts about half an hour. Lethal doses of nicotine may be estimated in 0,5 mg to 1 mg/kg body weight. Surely, Tobacco has been and continues to be one of the most important plants in our history.

1.6.2 *Nicotiana glauca*

Nicotiana glauca is sometimes referred to as Mustard Tree or Brazilian Tree Tobacco or simply Tree Tobacco and belongs to the family *Solanaceae*. A woody plant from South America with long tubular yellow flowers in a loose head, and egg-shaped to elliptic, pointed blue-gray leaves. It is A shrub 2-3 m high, with erect sparsely branched, hairless blue-gray stems. Leaves are tough, hairless, on a long stalk which is longer than the blade. Flowers have a corolla tube 3-4 cm long and about 5 mm in diameter, hairy on the outside and with short teeth. The Calyx is hairless, tubular with 5 pointed teeth and more or less enclosing the fruit. It is thoroughly naturalized on old walls, rocks and waste ground throughout the Mediterranean region. *N. glauca* needs full sun to partial shade with deep rich soil and flowers nearly all the year round. It is commonly planted as an ornamental for its flowers, but it also has medicinal uses. Care should be used, because all plant parts are extremely poisonous. Tree tobacco, while related to tobacco, does not contain nicotine.

Plate 1.2: View of *Nicotiana glauca* plant.

Nicotiana glauca is frequently a pioneer plant in many disturbed ecosystems and waste damping sites. It is the only perennial plant found in industrial, agricultural and municipal solid waste disposal site. Therefore, it was assumed that *N. glauca* might tolerate exceeding concentration of heavy metals. It has a wide distribution world wide, with rapid growth and high biomass production. Therefore, many experiments have conducted to test the phytoremediation potential of *N. glauca*,

which is considered to be a potential candidate for phytoremediation of contaminated soils.

1.7 Aims and Objectives

In an effort to better understand the physiological and biochemical responses of plants under heavy metal stresses, a comparative study is proposed between two solanaceous species, the *Nicotiana tabacum* and.*N. glauca.* More specifically, *N. glauca,* a plant with promising properties for phytoremediation, is compared with the cultivated relative *N. tabacum,* an economically important plant. The main aim of this work is to correlate heavy metal concentrations with the alterations of several morphological and physiological parameters (plant morphology, photosynthesis, transpiration, stomatal conductance, soluble proteins etc) and with the levels of some central molecules related with nitrogen metabolism and assimilation (soluble proteins, proline, ammonium assimilating enzymes). The comparison between the two plant species is expected to shed light on the mechanisms plants use to tolerate heavy metals and reveal the participation of nitrogen metabolism on the phenomenon.

The experimental approach included the study of the effect of Zn, Ni and Cd in greenhouse cultures of the two plant systems and particularly:

1. pot cultured plants in solid substrate provided with the aforementioned metals in a long term experiment for 6 weeks.
2. seedlings cultured hydroponically in the presence of different concentrations of Zn, Ni and Cd, in a short time experiment for 8 days.

Various morphological and biochemical parameters were determined, including:

1. Plant characteristics such as shoot length, number of leaves, fresh weight, biomass production etc.
2. Several parameters related with photosynthesis and transpiration such as chlorophyll content, photosynthesis and transpiration rates, chlorophyll fluorescence, stomatal conductance etc.

3. Endogenous heavy metal concentrations.
4. Soluble proteins levels.
5. Proline levels
6. Specific activities.
7. Protein levels of the enzymes GS, Fd-GOGAT and GDH.

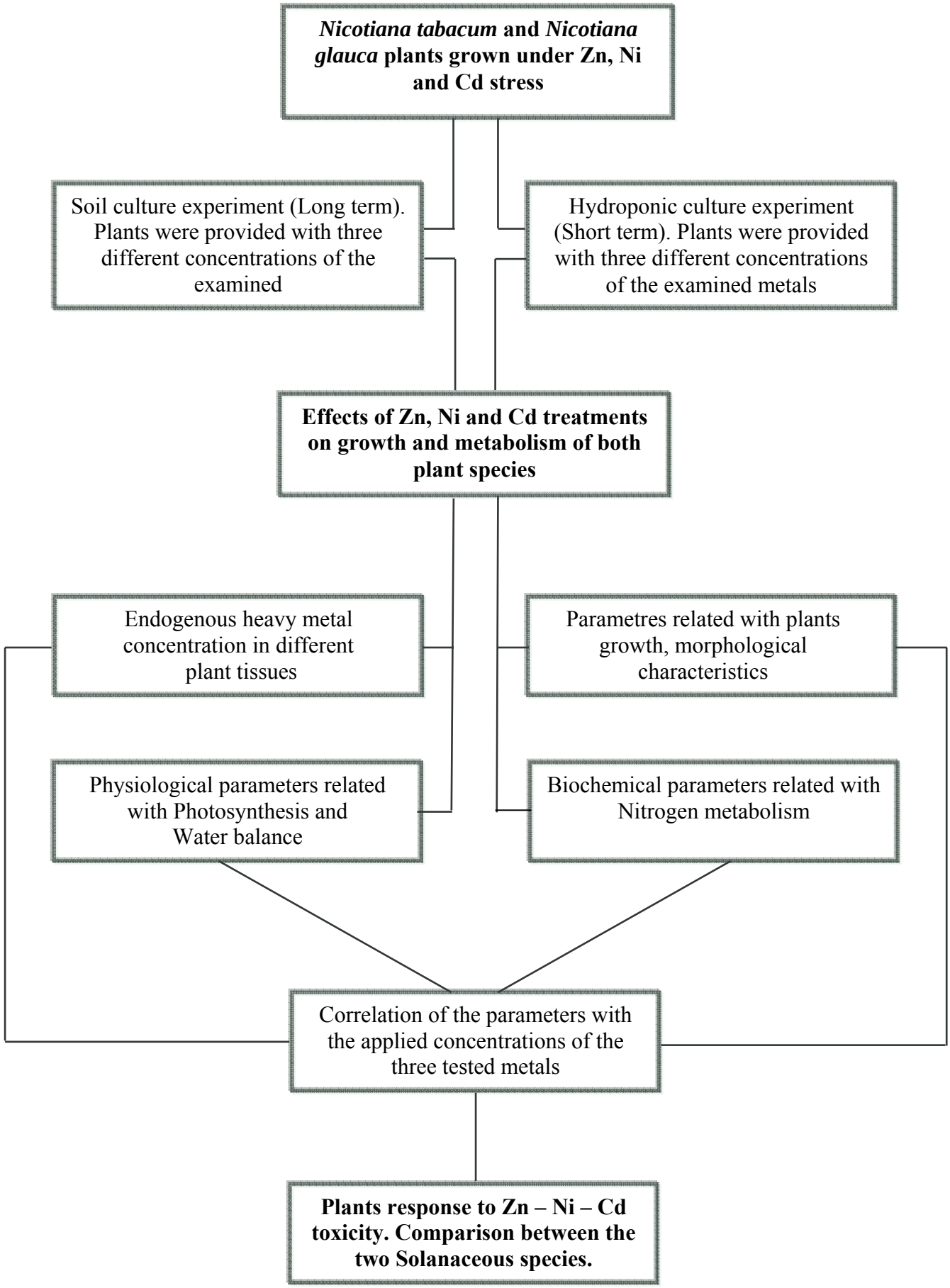


Figure1.11 : Diagram of the experimental work carried out in this thesis.

2. MATERIAL AND METHODS

The experiments presented in this work were established in a greenhouse at the farm of the Technological Education Institute of Crete (TEI). They were arranged in a complete randomized design with 16 replicates (Soil-culture experiment) and 20 replicates (Hydroponic culture experiment). At the end of the experiments tissue samples were harvested and further analyzed at the Plant Physiology and Biotechnology laboratory of the School of Agricultural Technology of TEI.

2.1 Experimental Design

2.1.1 Soil-Culture Experiment

Seeds of *Nicotiana tabacum* and *Nicotiana glauca* were sown in seed-plots for germination. The seed-plots were installed for about 1 month on a heated bench, in a mist propagation room of the greenhouse, with an automated irrigation system. After germination when the seedlings were about 15 cm in length, they were transplanted into plastic pots and transferred to the greenhouse. Each pot was filled uniformly with 5 L homogenous substrate that contained peat and perlite in a ratio of 3:1. The adaption period (acclimation phase) for the plants lasted 1 month. During that period the plants were irrigated 3 times per week. Also, every 2 weeks the plants were irrigated with a commercial nutritional solution (Green leaf, N:P:K 20:20:20 + micronutrients, 100 mL of a 0,2 % w/v solution per plant). Sixteen plants of each plant species were used for each heavy metal treatment.

Three concentrations of Zn, Ni and Cd supplied as solutions of $ZnSO_4$ $7H_2O$, $NiSO_4$ $6H_2O$ and $CdSO_4$ 2.67 H_2O, respectively, were used for the experiment (Table 2.1). The treatments of each metal will be referred to as 0.0, 0.5, 2.5, 5.0 mM Zn, 0.0, 0.1, 0.5, 1.0 mM Ni and 0.0, 0.2, 1.0, 2.0 mM Cd. As control of the experiment was used water, resulting in a total of ten treatments for each plant species. Once a week, for 6 weeks, plants (pots) were provided with 500 mL of metal solution or 500 mL water (control plants). During this period, measurements of growth parameters, chlorophyll fluorescence and of other photosynthesis parameters were logged weekly for each plant. Photosynthetic parameters were followed in the leaves of two zones of

each plant, the lower (5th-6th leaves from the base) and the upper (the youngest fully expanded leaf near the tip).

Table 2.1: Concentration of the heavy metals used in the treatments of Soil culture experiment

Treatments	Substances	Final molar concentration (mM)	Final concentration (g/L)
Control	H_2O	0.0	
Zn 0.5 mM	$ZnSO_4\ 7H_2O$	0.5	0.14
Zn 2.5 mM	$ZnSO_4\ 7H_2O$	2.5	0.71
Zn 5.0 mM	$ZnSO_4\ 7H_2O$	5.0	1.43
Ni 0.1 mM	$NiSO_4\ 6H_2O$	0.1	0.02
Ni 0.5 mM	$NiSO_4\ 6H_2O$	0.5	0.13
Ni 1.0 mM	$NiSO_4\ 6H_2O$	1.0	0.26
Cd 0.2 mM	$CdSO_4\ 2.67\ H_2O$	0.2	0.05
Cd 1.0 mM	$CdSO_4\ 2.67\ H_2O$	1.0	0.25
Cd 2.0 mM	$CdSO_4\ 2.67\ H_2O$	2.0	0.51

At the end of the growth period samples of leaves, shoots and roots were harvested in order to be used for further analysis. For each treatment, leaves and shoots were harvested separately from the above mentioned lower and upper zone of the plants. For root samples, roots were carefully separated from the substrate, washed with tap water, rinsed with distilled water and dried with filter paper.

Parts of the harvested samples (roots, shoots and leaves) were frozen in liquid nitrogen and then kept at a –80 ^{o}C ultra-refrigerator in order to be used for further plant physiological and biochemical analyses. Other parts of the harvested samples were dried at 80 ^{o}C. Then dried tissues were ground in a ball mill to a fine powder and used for the determination of endogenous heavy metal concentrations.

2.1.2 Hydroponic-Culture Experiment

Seedlings of *N. tabacum* and *N. glauca* germinated as in soil culture experiment were cultured hydroponically in the presence of different concentrations of Zn, Ni and Cd, in a short time experiment. When the seedlings were seven centimeters long, they were transferred in plastic beakers and cultured for seven days in 200 mL of 0.1 x Hoagland solutions (Table 2.2) in the greenhouse. After one week adaptation, the seedlings were treated with different concentrations of Zn, Ni and Cd in 0.1 x Hoagland's nutrient solution for 8 days. To avoid root necrosis and nutrient deficiencies in plants, the metal solutions was completely replaced every three days with new solutions.

Table 2.2: Composition of the modified Hoagland solution used in this experiment**.**

Ingredients	Final concentration for 1x solution
Macronutrients	
$CaCl_2$ $6H_2O$	0.3 g/L
$MgSO_4$ x $7H_2O$	0.52 g/L
KNO_3	1.6 g/L
KH_2PO_4	1.42 g/L
EDFeS	0.03 g/L
Micronutrients	
H_3BO_3	2.8 mg/L
$MnSO_4$ x H_2O	3.4 mg/L
$CuSO_4$ x $5H_2O$	0.1 mg/L
$ZnSO_4$ x $7H_2O$	0.22 mg/L
$(NH_4)_6$ Mo_7O_{24} x $4H_2O$	0.1 mg/L
H_2SO_4 (concentrated)	5 ml/L

Twenty seedlings, one per plastic beaker, were grown for each treatment from both plant species. Each heavy metal was used in different concentrations, which were:0.0, 0.1, 0.5 and 2.0 mM for Zn, 0.0, 0.02, 0.05 and 0.15 mM for Ni and 0.0,

0.02, 0.05 and 0.2 mM for Cd. Control seedlings for each plant species were cultured in 0.1 x Hoagland solutions without any metal treatment. The heavy metals were supplied as solutions of $ZnSO_4$ $7H_2O$, $NiSO_4$ $6H_2O$ and $CdSO_4$ 2.67 H_2O, as in the first experiment.

Visual analysis and observations were conducted throughout the experiment. Height measurements and the number of leaves were taken to access growth. At the end of the trial the plants were harvested and divided into shoots and leaves. Growth parameters of harvested samples were recorded. Samples of shoot and leaves were immediately immersed in liquid nitrogen and stored at –80 °C for further plant physiological and biochemical analyses.

2.2 Methodology

2.2.1 Atomic Absorption Spectrophotometer

Dried plant tissues (leaves, shoots, roots) were ground using a ball mill to a fine powder in order to determine the heavy metal concentrations. From the fine powder 1 g of each sample was used and the humidity was determined by drying them at 104 oC for 24 h. Then, wet oxidation was used with digesting solution: HNO3 / H2SO4 / HClO4 (5:1:2, v/v). 20 ml of the digesting solution was added in each sample. Filtration was followed and the received solutions adjusted to 100 ml. Zinc (Zn), nickel (Ni) and cadmium (Cd) were determined with an Atomic Absorption Spectrophotometer (Perkin Elmer, Germany, 2100).

2.2.2 Total Chlorophyll Content and Photosynthetic Pigments

Total chlorophyll content was followed *in vivo* and *in vitro*. The *in vivo* total chlorophyll content was estimated as SPAD values using a SPAD-502 apparatus (Minolta Co. Ltd., Japan). Total chlorophyll content was recorded, by measuring four leaves from each lower and upper zone of plants. The chlorophyll meter (SPAD meter) is a portable diagnostic tool that measures the greenness or the relative chlorophyll concentration of leaves. The meter makes instantaneous and non-

destructive readings on a plant based on the quantification of light intensity (peak wavelength: approximately 650 nm, red LED) absorbed by the tissue sample. A second peak (peak wavelength: 940 nm, infrared LED) is emitted simultaneous with red LED for to compensate the thickness leaf (Minolta Camera Co. Ltd., 1989).

Total chlorophyll concentration is a unifying parameter for indicating the effect of specific interventions. However it is important to record changes in the two components of chlorophyll, chlorophyll a (chl a) and chlorophyll b (chl b) and especially their ratio (chl a/b). This is due to the fact that heavy metals could affect each component at a different level creating changes in some part of plants physiology and not in others (Manios et al., 2003). Fresh leaf tissue, 0.2 g were extracted with a mortar and pestle in 2 mL of 80% acetone. The extracts were centrifuged at 2000g for 10 min. and then, absorbance concerned part of the supernatant, was determined at 663 and 646 nm. Measurements were carried out using spectrophotometer UV/VIS (UV mini 1240, Shimadzu, Japan). Concentrations of chlorophyll a, b and total chlorophyll content were calculated by the following equations:

Total Chlorophyll Content (μg/mL) = $17.3\ A_{646} + 7.18\ A_{663}$

Chlorophyll a (μg/mL) = $12.21\ A_{663} - 2.81\ A_{646}$

Chlorophyll b (μg/mL) = $20.13\ A_{646} - 5.03\ A_{663}$

(A: Absorbance)

2.2.3 Gas Exchange Parameters

After the first application of the heavy metals the net photosynthesis rate (*Pn*), the stomatal conductance (*gs*), the intercellular CO_2 concentration and the transpiration rate (*T*) were measured (between 09:00 and 11:00 h), using a portable infrared gas analyzer (LI-COR, LI-6400). At the first measurement these physiological parameters were recorded for the youngest fully expanded leaf (Upper), but at the second measurement, the same parameters were recorded also for the sixth leaf from the base (old leaf-Lower). The gas analyzer was equipped with a clamp-on

leaf cuvette that exposed 6 cm^2 of leaf area. Measurements were held at ambient temperature and humidity and with a light intensity 1000 μmol m^{-2} s^{-1}, CO_2 was maintained at a constant level of 400 μmol L^{-1} using an L1-6400-01 CO_2 injector with a high pressure liquefied CO_2 cartridge source. From these data, the water use efficiency was estimated (WUE= *Pn/T)* (Avola et al., 2008)

2.2.4 Chlorophyll Fluorescence

The same leaves were used for chlorophyll fluorescence measurements the sixth leaf from the base (Lower) and the youngest fully expanded leaf (Upper). Chlorophyll fluorescence of the leaves was measured using a Chlorophyll Fluorometer (Os-30p, OPTI-SCIENCES) after 15 min dark acclimation. The following parameters: i) Minimal fluorescence (arbitrary units) F_0, fluorescence occurring when all antenna sites are assumed to be open (dark adapted), ii) maximal fluorescence (arbitrary units) F_m, fluorescence intensity under exposure to saturation flash. All antenna sites are assumed to be closed iv) terminal fluorescence, Ftr, fluorescence quenching value at the end of the test v) T1/2 (mS or uS), half rise time from F_0 to F_m. Also, it was calculated the ratio of variable fluorescence to maximal fluorescence, F_v/F_m. Calculated as $F_v/F_m = (F_m - F_0)/ F_m$. Variable fluorescence (F_v) is the change in fluorescence due to initial electron backup in the antenna complex. Additionally, the ratio F_v/F_0 was calculated.

2.2.5 Proline Content

Proline concentration was determined according to Bates *et al*., (1973). Particularly, 0.2 g of leaf tissue was pulverized with liquid nitrogen and extracted in 1 mL TCA 10% w/v. The extracts were centrifuged at 2000g for 15 min. Following, 300 μL of the supernatant were treated with 300 μL of glacial acetic acid and 300 μL of ninhydrin solution and then, boiled at 95 ^oC for 60 min. Proline content was measured at 546 nm by using a UV/VIS spectrophotometer (UV mini 1240, Shimadzu, Japan) and calculated as μmol g^{-1} FW. Nynhidrin solution contained: 2.5% w/v ninhydrin, in solution of acetic acid, H_2O and orthophosphoric acid (CH_3COOH: H_2O: H_3O_4P, 6:3:1 v/v).

2.2.6 Protein Extraction and Determination of Specific Enzyme Activities

Frozen tissue samples stored at -80 ^{o}C were pulverized to fine powder in liquid nitrogen with a mortar and pestle. The powder from leaves was suspended in 4 volumes (w/v), of ice-cold grinding medium consisting of 100 mM Tris-HCl, pH 8.0, 14 mM β-mercaptoethanol, 10 μM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 3 mM $MgCl_2$, 0.5% (v/v) Triton X-100 and 0.2 g polyvinylpolypyrrolidone (PVPP) g^{-1} fresh weight. After homogenization in an Ultra-Turrax for four times, 20 s each and incubation on ice for 15 min, debris were removed by centrifugation at 15.000 g for 30 min and the supernatants were used for enzyme assays and protein determination. All steps were carried out at 4 ^{o}C.

a. Protein Determination

The protein content of the extracts was determined following 10% (w/v) trichloroacetic acid precipitation by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as the protein standard. Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan and cysteine react with Folin-Ciocalteau reagent, a mixture of phosphotungstic and phosphomolybdic acid in phenol, to produce an unstable product that becomes reduced to molybdenum/tungsten blue. The latter can be detected colourimetrically by absorbance at 625 nm.

b. Determination of Enzyme Activities

Aminating GDH activity was determined following the NADH-dependent absorption change at 340 nm (Loulakakis and Roubelakis-Angelakis 1990). The standard amination reaction mixture contained 100 mM Tris-HCl, pH 8.0, 20 mM α-ketoglutarate, 200 mM NH_4Cl, 1.0 mM $CaCl_2$, 0.2 mM NADH, enzyme solution and deionized water to a final volume of 1.0 mL. All assays were performed at 30 ^{o}C. The absorption change at 340 nm was measured using a UV/VIS spectrophotometer (UV mini 1240, Shimadzu, Japan). Specific activity of GDH was calculated in nmol NADH oxidized per min. mg protein at 30 ^{o}C.

GS activity was determined as described by Loulakakis et al. (2002). The reaction mixture (4 mL) contained 0.1 M Tris–malate buffer (pH 7.5), 1 M hydroxylamine (pH 7.0), 100 mM glutamate (pH 7.2), 10 mM ATP (pH 7.2), 1 M $MgSO_4$ and 0.2 mL of properly diluted enzyme extract. The reaction was started by adding hydroxylamine and the mixture was incubated for 20 min at 30 ^oC. The reaction was stopped by the addition of 1 mL of $FeCl_3$ reagent, prepared by mixing equal volumes of 10 % $FeCl_3$. $6H_2O$ in 0.2 M HCl, 24% TCA and 5 % HCl. After 10 min, the protein precipitate was removed by centrifugation and the absorbance of the supernatant was measured on a UV/VIS spectrophotometer (UV mini 1240, Shimadzu, Japan), at 540 nm. Specific activity of GS was calculated in nmol of γ-glutamylhydroxamate formed per min. mg protein at 30 ^oC.

Activity of Fd-GOGAT was assayed using ferredoxin as the electron donor, by determining the formation of glutamate in the reaction as previously described (Matoh and Takahashi 1982). Specific activity of Fd-GOGAT was calculated in nmol of glutamate formed per min. mg protein at 30 ^oC.

2.2.7 Polyacrylamide Gel Electrophoresis and Immunoblot Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Bio-Rad mini Protean II Dual Slab Cells gel apparatus as described by Loulakakis et al. (1994). The analysis of Fd-GOGAT was performed on 7% SDS-polyacrylamide gels and of GS and GDH on 10% gels. For western blot analyses the electrophoretically separated proteins were transferred onto nitrocellulose filters (0.2 μm pore size; Scheicher and Schuell, Keene, NH). After blocking the sites with 2% BSA in PBS the filters were incubated for 2 h at room temperature with the Fd-GOGAT (Loulakakis et al., 2002) or GS (Loulakakis and Roubelakis-Angelakis, 1996) or GDH (Loulakakis and Roubelakis-Angelakis, 1990) antibodies and following washing of the membranes with the alkaline phosphatase-conjugated second antibody. All antibodies were diluted 1:2000 in PBS containing 1% w/v BSA and 0.5% w/v Tween-20. Fd-GOGAT- or GS or GDH-antibody complexes were visualized by the bromochloroindolyl phosphate (BciP, 0.05 mg/mL) color

development reaction in 100 mM diethanolamine pH 9.6 and 0.1 mg/ml nitroblue tetrazolium.

2.2.8 Statistical Analysis

Statistical analysis employed SPSS ver. 17.0 and Microsoft Office Excel 2003. In order to detect differences in the determined parameters concerned growth, physiology and biochemistry of plants treated with different concentrations of heavy metals, analysis of variance (ANOVA) was performed. Significant differences between the mean values of variables of treatments were determined according to Duncan's test and Least Significant Difference (LSD) test at P=0.05 level. In order to study the relationships among the variables Pearson's Rank Correlation was carried out. Microsoft Office Excel 2003 was used for the calculation of Standard Error (SE).

3. RESULTS

3.A Soil Culture Experiment

Nicotiana tabacum and *Nicotiana glauca* plants were grown in the greenhouse and irrigated with media containing different concentrations of Zn, Ni and Cd for 6 weeks. The plants were assorted per treatment (Plate 3.1). During that period, several parameters related to plant growth were monitored. At the end of the experiment, leaf and shoot tissue samples, from the upper and lower zone of the plants (as described in section Material and Methods) as well as from the roots were harvested and used for further analysis. Data for the parameters followed and presented in tables and charts.

Plate 3.1: General view *N. tabacum* and *N. glauca* plants cultured in the greenhouse, in the presence of increasing concnentrations of Zn, Ni and Cd,one week before harvesting.

3.1 Effect of Zinc on Morphological, Physiological and Biochemical Parameters of *N. tabacum* and *N. glauca*

3.1.1 Zinc in Plants Tissues

Zinc is essential for human diet, but can also be toxic to human and plants in high concentration. The physiology of metal toxicity in plants involves mainly with the movement of metal from soil to root, as well as metal absorption and translocation (Rout and Das, 2003). In this study, accumulation of Zn in five different parts of *N. tabacum* and *N. glauca* plants was investigated at three Zn concentrations. The endogenous Zn concentration in plant tissues of both species was affected significantly as Zn concentration increased in the culture medium.

Particularly, as presented in Fig. 3.1 Zn concentration increased 4.4-fold in lower leaves of *N. tabacum* and 3.5-fold in upper leaves compared to untreated plants, as a response to higher exogenous concentration of Zn,. Lower and upper shoot accumulated 5-fold at the highest exogenous concentration of Zn, compared to control plants, whereas Zn treatment increased Zn content by 5.6- and 4.5-fold at 2.5 and 5 mM treatments, respectively, compared to untreated plants. *N. tabacum* exhibited maximum accumulation in root and lower leaves.

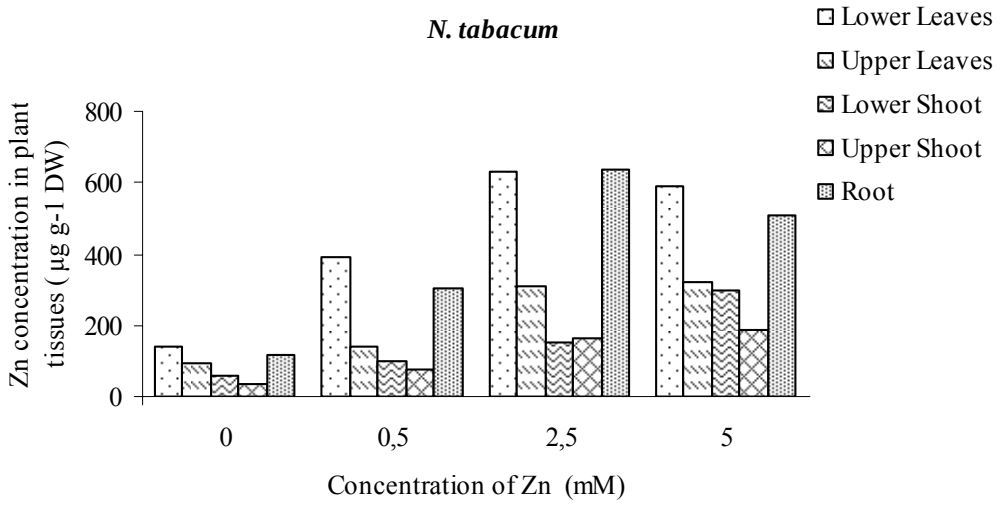


Figure 3.1: Accumulation of Zn in plant tissues of *N. tabacum*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Statistical significant differences of plant tissues between control and treatments are presented in Table 3.1.

Table 3.1. Statistical significant differences of the endogenous Zn concentration in plant tissues of *N. tabacum*. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Zn, mM	Plant Tissue				
	Lower leaves	Upper leaves	Lower shoot	Upper Shoot	Root
0.0	a	a	a	a	a
0.5	b	a	a,b	a	b
2.5	c	b	b	b	c
5.0	c	b	c	b	c

In lower leaves of *N. glauca*, the total amount of endogenous Zn increased by 4-fold at 0.5 mM, by 8-fold at 2.5 mM and by about 10-fold at 5 mM exogenous Zn concentration, compared to control plants (Fig. 3.2, Table 3.2). Treatments by all Zn concentrations increased the Zn content in upper leaves, as far as 3.5-fold at the highest concentration. Zn removal by the lower shoots increased by 6.3-, 13.4- and 15.5-fold with the respective 0.5, 2.5 and 5mM treatments. Also, in upper shoots Zn content increased by 5.3-, 9.8- and 13-fold at the lower to the highest concentration respectively. Zn content in roots of *N. glauca* increased by 15-fold at 0.5 mM and reached 28-fold at higher concentrations of Zn compared to untreated plants. *N. glauca* showed maximum accumulation in root, while excessive levels accumulated in lower leaves as well.

Comparatively, in both plant species, Zn uptake was positively affected by exogenous Zn treatments. The accumulation of Zn in treated plants was significantly higher, than in control plants. Similarly, both plant species accumulated more in lower parts (leaves, shoots) than in upper (Fig. 3.1, 3.2).

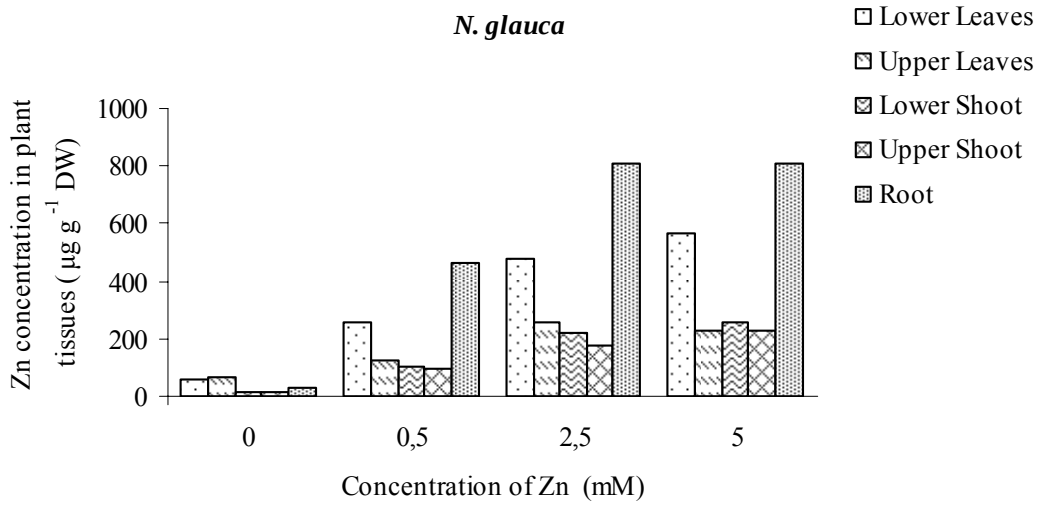


Figure 3.2: Accumulation of Zn in plant tissues of *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Statistical significant differences of plant ttissues between control and treatments are presented in Table 3.2

Table 3.2. Statistical significant differences of the endogenous Zn concentration in plant tissues of *N. glauca*. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Zn, mM	**Plant Tissue**				
	Lower leaves	Upper leaves	Lower shoot	Upper Shoot	Root
0.0	a	a	a	a	a
0.5	b	b	b	b	b
2.5	c	c	c	c	c
5.0	c	c	c	d	c

3.1.2 Effect of Zn on morphological characteristics and growth of *N. tabacum* and *N. glauca*

The morphological characteristics of *N. tabacum* and *N. glauca* were monitored for the duration of the experimental period and the symptoms were observed (Plates 3.2 and 3.3). The data obtained revealed a significant effect of Zn treatments on both plant species. Zinc toxicity has been known to induce stunted growth and interveinal chlorosis in leaves, which later become dry and papery. Sometimes the affected leaves show rolling of leaf margins, whereas roots turn brownish and necrotic (Rao et al., 2006). In this study, plants of all treatments showed visible toxicity symptoms. The gradation of the Zn concentration was related with chlorosis, typical symptom of Zn toxicity. The higher Zn treatments caused severe chlorosis at lower leaves of *N. tabacum*, while upper leaves merely began to turn chlorotic. Visual symptoms of prolonged exposure to Zn treatments of *N. glauca* were related more with chlorosis of upper expanded leaves spreading to the interveinal space, while lower leaves developed dark brown necrotic spots. In general, Zn induced stunted growth in both plant species and symptoms, which were related not only with the physiological senescence. The extent and magnitude of symptoms and damage of the plant parts increased with increasing treatment concentration of Zn.

Plate 3.2: Plants of *N. tabacum* (left) and *N. glauca* (right) grown in greenhouse conditions in presence of 0.0, 0.5, 2.5, 5.0 mM Zn^{2+}.

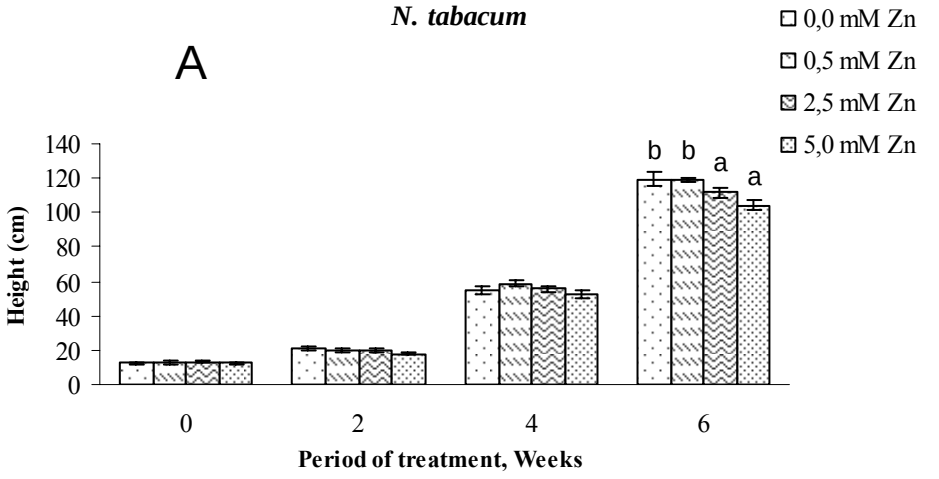
Plate 3.3:. Visual symptoms of Zn treatment on *N. tabacum* (left) and *N. glauca* (right) leaves. Plants were grown in plastic pots in greenhouse.

Figure 3.3 illustrates results from the height of both plant species during the experiment, under Zn stress. In this study, statistically significant differences ($P<0.05$) were observed only after 6 weeks exposure to Zn treatments of both plant species. Particularly, the supply of excess Zn resulted to reduction of height of *N. tabacum* by 13%, compared to control plants. *N. glauca* appeared more affected, since the supply of 2.5 and 5 mM Zn caused height inhibition approximately 13% and 20% respectively, compared to untreated plants. Another growth parameter followed during Zn treatment was the number of leaves of *N. tabacum* and *N. glauca* (Fig. 3.4). Differences were observed only at 6 week treated plants. Both plant species were similarly affected by the highest Zn concentration, where considerable reduction was observed in the number of leaves.

Differences in fresh weight of leaves, shoots and roots of both plant species were determined under Zn stress. As presented in Fig. 3.5, a reduction trend was observed at fresh weight of leaves of *N. tabacum*, however no significant difference was present, compared to untreated plants. In contrast, the inhibitory effect was greater on shoot and root weight with increasing Zn doses in the culture medium. Specifically, shoot weight began to reduce by 19% and 30% at 2.5 and 5mM Zn,

respectively, compared to controls, while reduction in root weight was up to 15% and 34% in respect to the relative treatments.

The reduction observed in fresh weight of leaves, shoot and root of *N. glauca* followed a dose-dependent manner. As in the case of *N. tabacum*, the inhibitory effects on fresh weight tissues of *N. glauca* became more pronounced at the higher Zn treatments. Particularly, weight of leaves and shoots was decreased by 28% and 44%, respectively, at the highest Zn concentration, compared to untreated plants. Also, as with *N. tabacum*, the greatest reduction occurred in root fresh weight, where the addition of 2.5 and 5 mM Zn led to significant reduction by 26% and 39%, respectively, compared to controls.



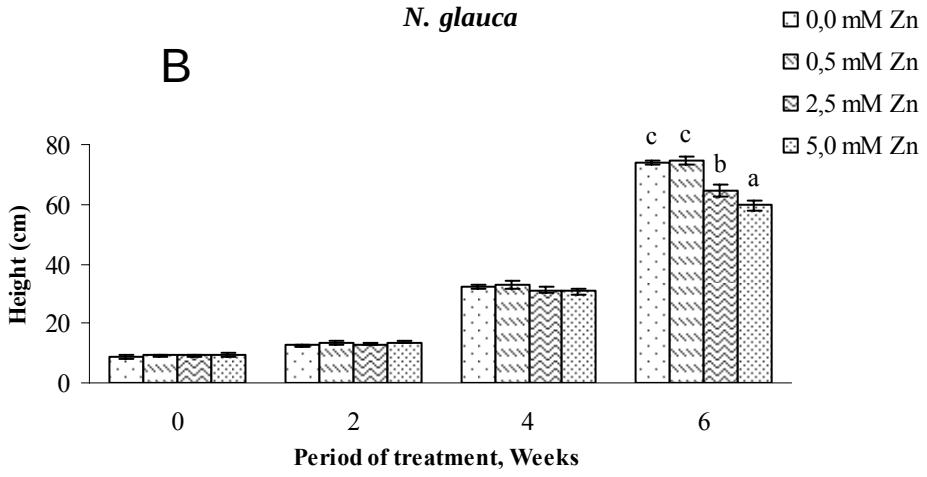
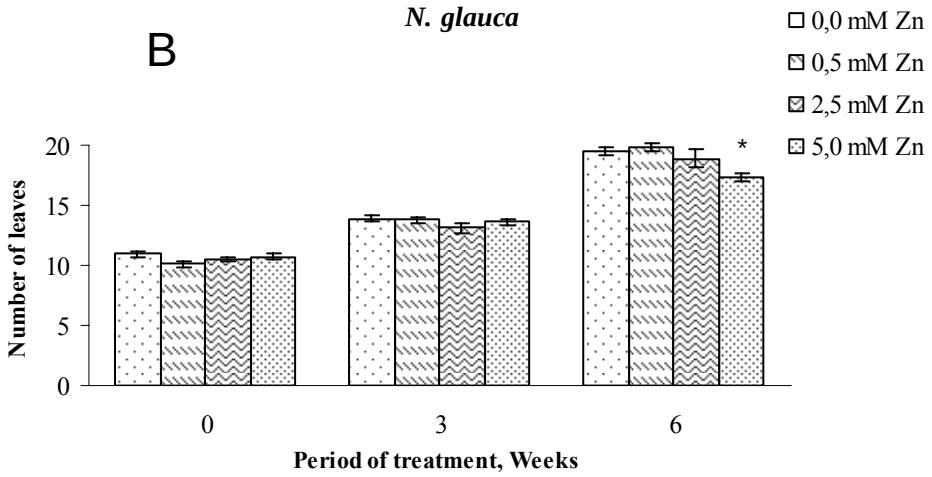


Figure 3.3: The effect of Zn concentration on plant height during the treatment period A) *N. tabacum*, B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05)



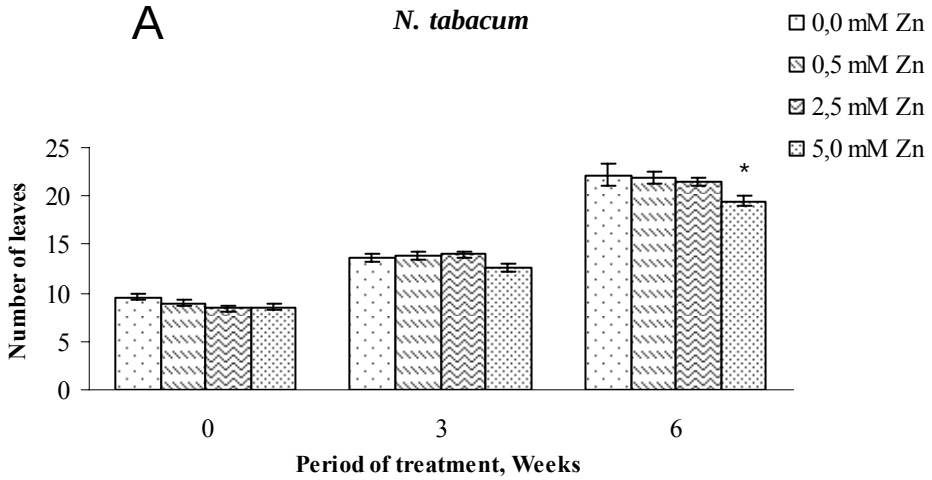


Figure 3.4: The effect of Zn concentration on the number of leaves of A) *N. tabacum*, B) *N. glauca*, during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE). Asterisks indicate significant differences between treatments and control (only for data obtained at 6th week, LSD, P=0.05)

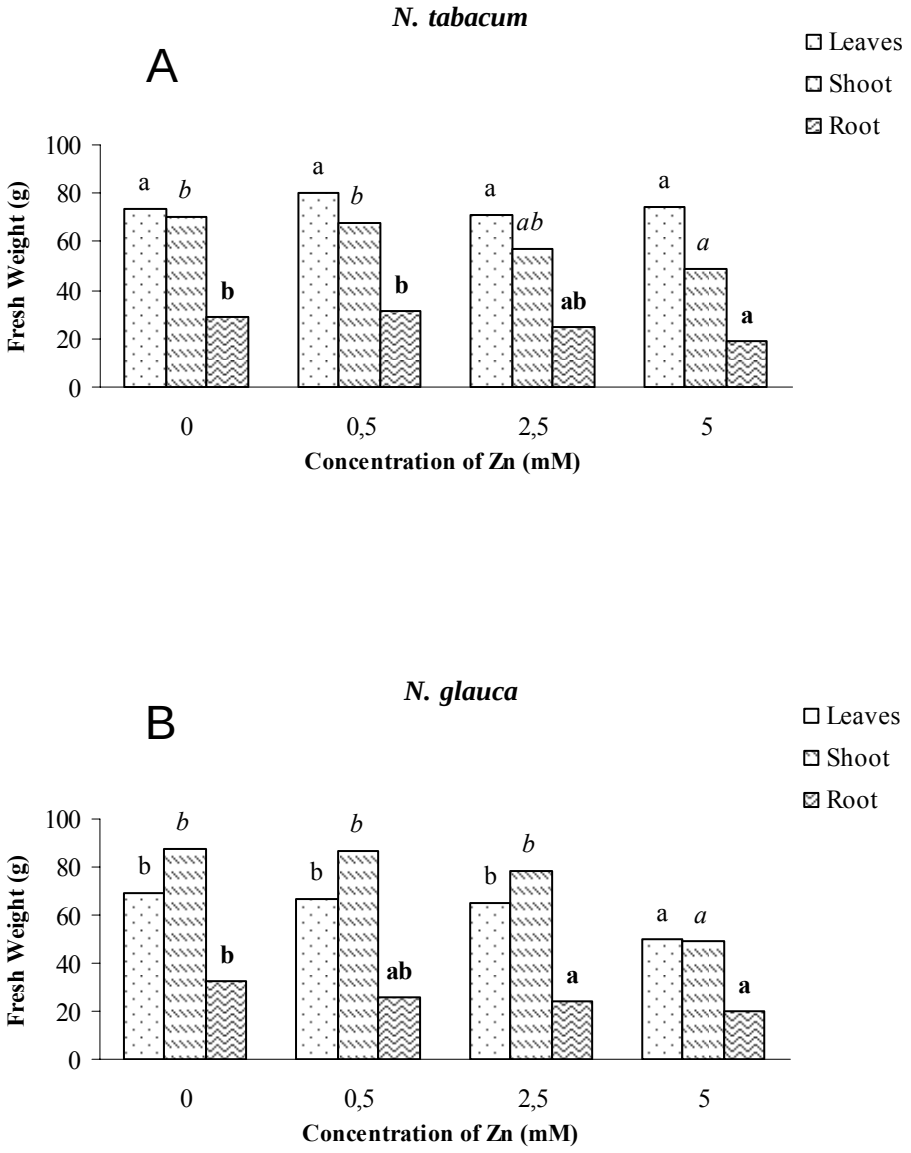


Figure 3.5: The effect of Zn concentration on plant fresh weight of A) *N. tabacum*, B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (regular for Leaves, italics for Shoot, bold for Root, Duncan, P=0.05)

3.1.3 Effect of Zn on Photosynthetic Parameters of *N. tabacum* and *N. glauca*

Photosynthesis is a highly sensitive process significantly affected by heavy metals in a number of plant species. The degree of heavy metals effect on photosynthesis depends on the growth stage, plant conditions as well as on the duration of the stress. Heavy metal application was shown to affect photosynthetic functions directly or indirectly (Prasad, 2010).

a. Chlorophyll Pigments

The chlorophyll contents of lower and upper leaves of *N. tabacum* and *N. glauca* were investigated in dependence on the Zn^{2+} concentrations and presented in the Figures below. Exposure of *N. tabacum* to Zn resulted to the reduction of photosynthetic pigments in lower and upper leaves (Fig. 3.6A). Particularly, the intermediate Zn concentrations lowered chlorophyll a content in lower leaves, while the highest Zn treatment led to statistically significant decline ($P<0.05$) of the relevant pigment by about 30% and 38% in upper leaves, compared to control plants. Chlorophyll b of lower leaves decreased approximately 25% at the highest Zn concentration, but the decline was not statistically significant. On the contrary treatment with 5 mM Zn led to statistically significant reduction of chlorophyll b in upper leaves, by about 48%, compared to intact plants.

In lower leaves of *N. glauca*, the mean values of both photosynthetic pigments of plants grown in the presence of Zn were lower than those of control plants; however, the decrease was not statistically significant (Fig. 3.6B). In upper leaves chlorophyll a and b slightly increased with the addition of 0.5 mM in the culture medium, rise significantly differed compared with the higher concentrations. The extent of pigments depression at 5 mM reached up to 33% and 45% for chlorophyll a and b, respectively, compared to controls.

Figure 3.7 summarizes the effect of Zn treatments on total chlorophyll content of lower and upper leaves of *N. tabacum* and *N. glauca* plants. Obviously, Zn excess negatively affected total chlorophyll content in both examined tissues of *N. tabacum*. In lower leaves the content was gradually reduced over the range of Zn concentrations

and reached up to 30% at the most severe treatment, while in upper leaves 5 mM Zn led to significant decline, 42% compared to controls. The chlorophyll content was similarly affected, measured as SPAD values (Fig. 3.7B). Comparatively, the total chlorophyll content of *N. glauca* showed similar response to *N. tabacum*, however the latter appeared more affected. The extent of chlorophyll content decrease was greater at the higher Zn treatment, in both tested tissues.

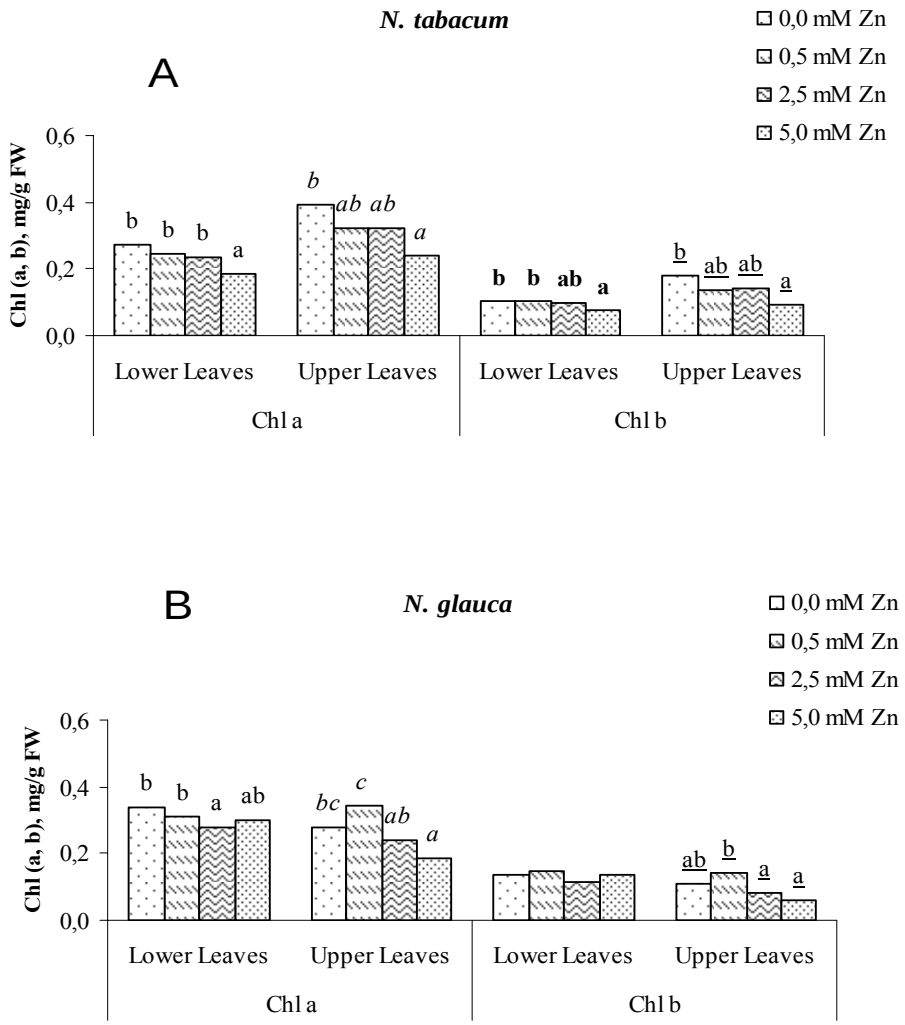
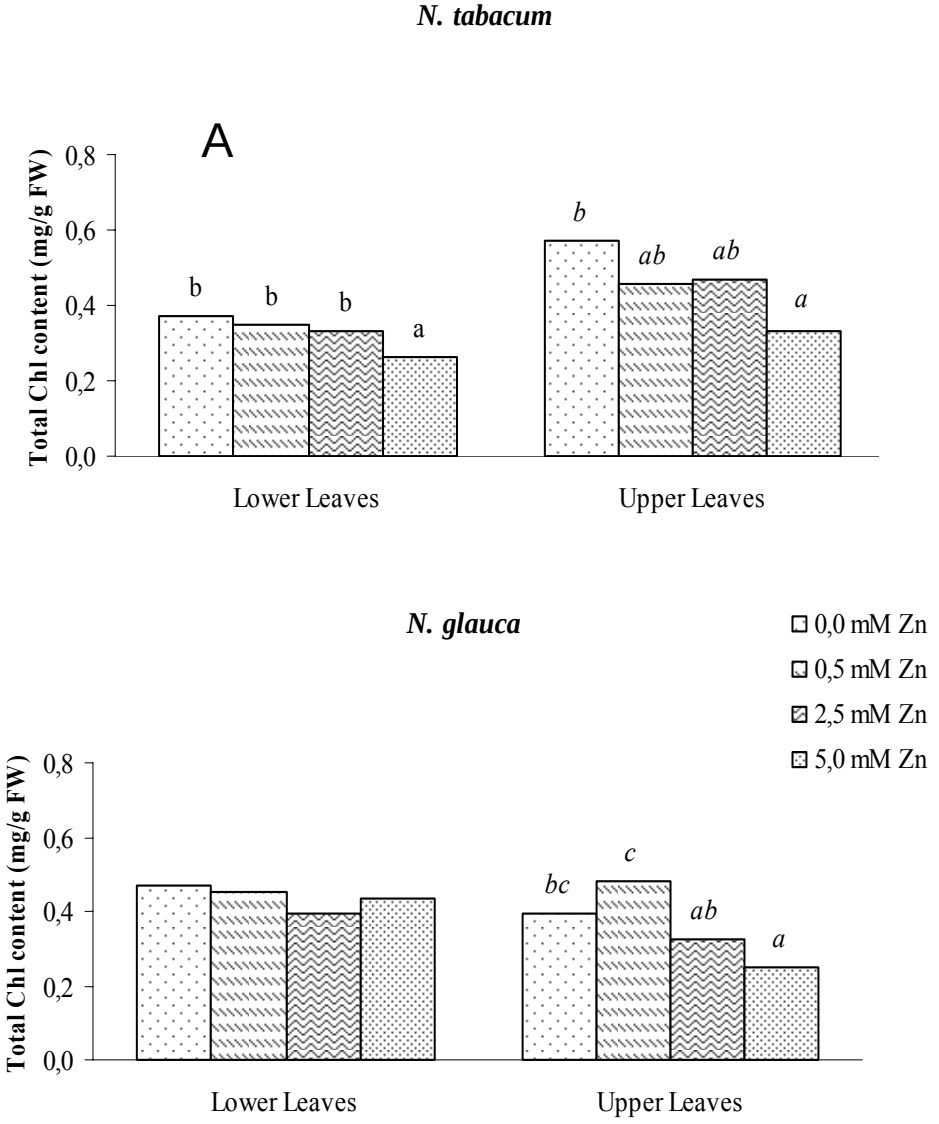


Figure 3.6: The effect of Zn concentration on photosynthetic pigments, chl a and chl b of lower and upper leaves of A) *N. tabacum*, B) *N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Columns with significant differences are marked with a different letter (regural for chl a of lower leaves, italics for chl a of upper leaves, bold for chl b of upper leaves, Duncan, P=0.05). Columns without letters on, do not differ significantly.

Table 3.3 illustrates the change of the chlorophyll ratio a to b under Zn stress in lower and upper leaves of both plant species. In lower leaves the chlorophyll ratio showed a depleted trend in response to Zn exposure, indicating that chlorophyll a was more affected than b. On the other hand, the Zn- induced decline of photosynthetic pigments a and b, resulted to rise the chlorophyll ratio in upper leaves of both plant species.



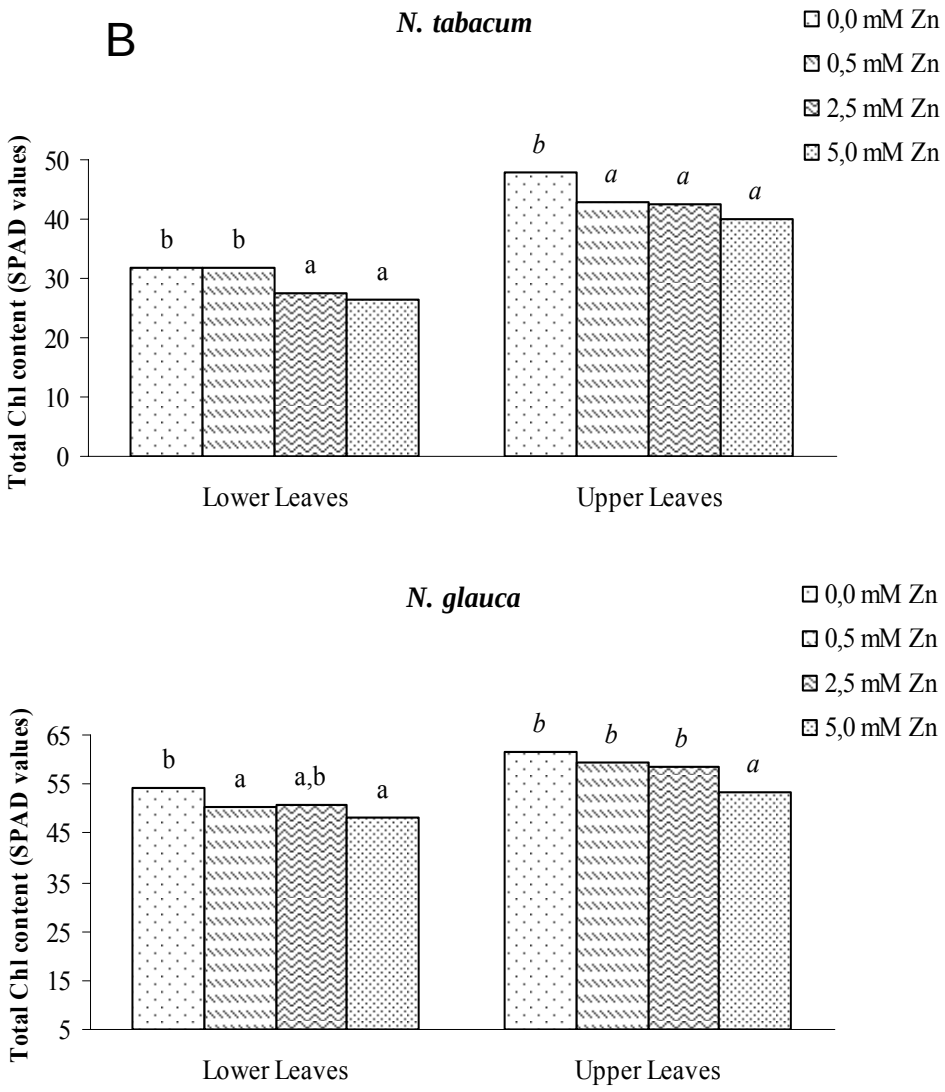


Figure 3.7: The effect of Zn concentration on total chlorophyll contents A) mg/g FW and B) SPAD values of lower and upper leaves of N. *tabacum* and *N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (regular for lower leaves, italics for upper leaves, Duncan, P=0.05). Columns without letters on do not differ significantly.

Table 3.3. The effect of Zn concentration on the chlorophyll *a/b* ratio of lower and upper leaves of *N. tabacum* and *N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Zn, mM	*N. tabacum*		*N. glauca*	
	Lower leaves	Upper leaves	Lower leaves	Upper leaves
0.0	2.70 a	2.24 a	2.52 a	2.56 a
0.5	2.35 a	2.43 ab	2.19 a	2.52 a
2.5	2.37 a	2.29 ab	2.46 a	2.89 ab
5.0	2.48 a	2.52 b	2.24 a	3.19 b

b. Photosynthetic Rate and Gas Exchange Parameters

Net photosynthetic rate (Pn) in lower leaves of *N. tabacum* decreased linearly, in respect to the period of Zn treatments, as well as in plants grown in control medium (Fig. 3.8). Pn of the 3 weeks treated plants was lowered by 25% at 0 mM, 34% at 0.5 mM, 36% at 2.5mM and reached by 41% at 5mM Zn compared to untreated plants. The deleterious effect on Pn of lower leaves became more pronounced at the 6^{th} week of Zn treatments. Particularly, Pn was approximately 53% at control plants, 65% and 61% at the intermediate treatments and reached 67% at the highest Zn concentration, compared with the mean values of the first period of treatment. Statistical significant differences ($P<0.05$) were also observedafter comparing the mean values of the harvesting period. Particularly, 0.5 mM and 5 mM led to significant decline by 23% and 28%, respectively, compared to control plants. In addition, a reduced trend (linear) observed in Pn of upper leaves of *N. tabacum*, in respect to the period of Zn treatment. However, the measurements obtained the last week of Zn treatment differed when compared to the first one.

As shown in Fig. 3.9, Pn in lower leaves of *N. glauca* decreased linearly, with the exposure period of the plants at Zn treatments, as well as at control medium. After

3 weeks exposure to Zn, the reduction of Pn observed in lower leaves was approximately 34% at control plants and reached up to 43% at the highest treatment. Pn was dramatically affected the last period of treatment, where it was lowered by 71% in lower leaves of plants grown at control medium, 0.5 and 2.5 mM Zn and reached up to 76% at 5mM Zn. Also, a decline about 18% observed at the highest Zn treatment, comparing the mean values with untreated plants, at 6th week. On the other hand, Pn in upper leaves of *N. glauca* remained almost unaffected by the exposure period of Zn, except at the highest treatment; where as seen in Fig 3.9. a linear decline of about 10% and 23% was observed at the 3rd and 6th week, respectively. Comparing the mean values of the harvesting period (6th week), statistical significant reduction ($P<0.05$) was observed at the highest Zn treatment, by about 25%, compared to control plants.

Stomatal conductance in lower and upper leaves of *N. tabacum* and *N. glauca* affected similarly to Pn (Fig. 3.9), by the exposure of plants to Zn treatments (Fig. 10) Particularly, in lower leaves of *N. tabacum* a reduced trend observed in stomatal conductance and reached up to 39% at the highest treatment, compared to controls, however the decline was not statistical significant. In addition, in upper leaves, stomatal conductance did not exhibit significant differences, however an increased trend was observed at the higher Zn concentrations. The relevant parameter in lower leaves of *N. glauca*, increased at 0.5 mM Zn, and then decreased, however there was no statistical significant difference. On the other hand, stomatal conductance in upper leaves of *N. glauca* showed a reduced trend with the supply of Zn concentration in the culture medium, however statistical significant decline ($P<0.05$) was observed only at the highest Zn treatment, where it was lowered by 38%, compared to control plants.

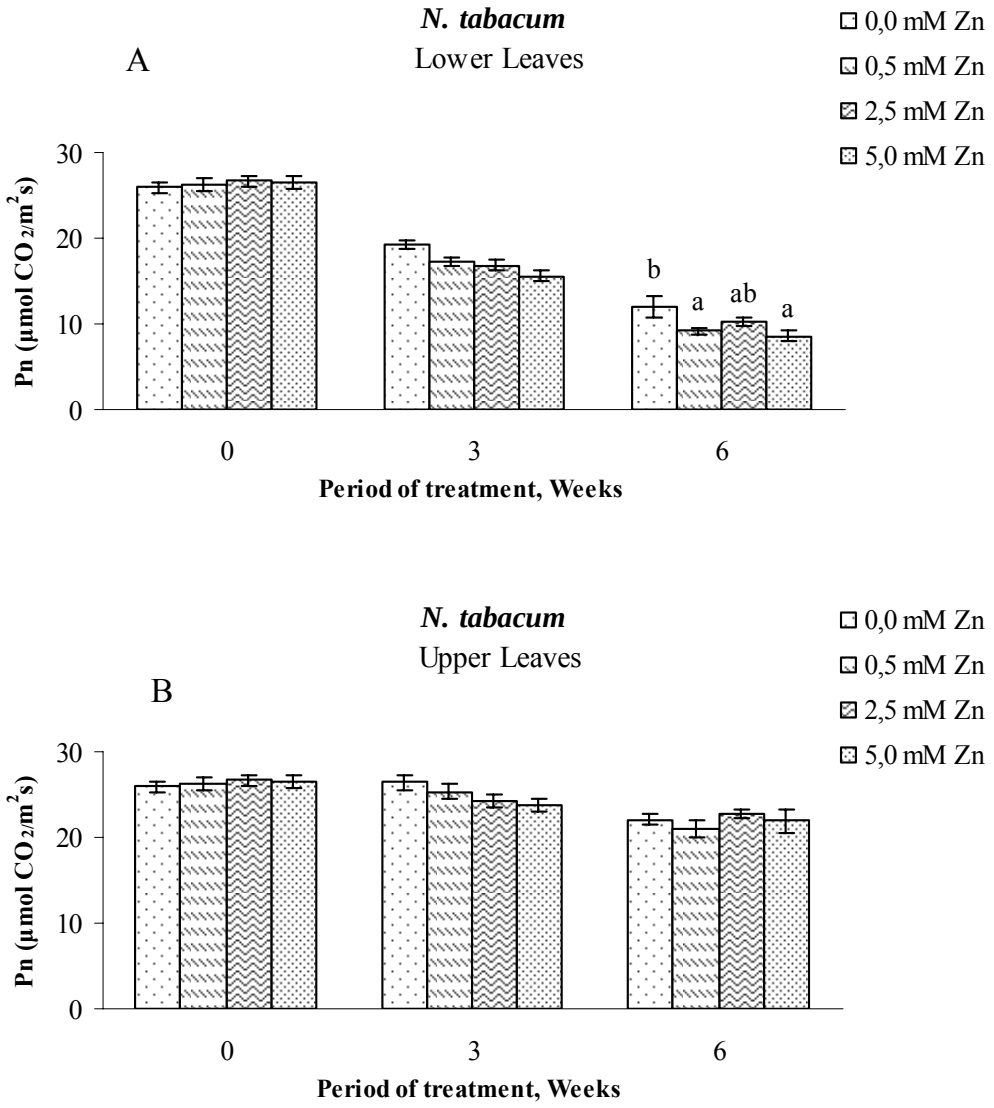


Figure 3.8: The effect of Zn concentration on net photosynthetic rate of: A) lower and B) upper leaves, of *N. tabacum* during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

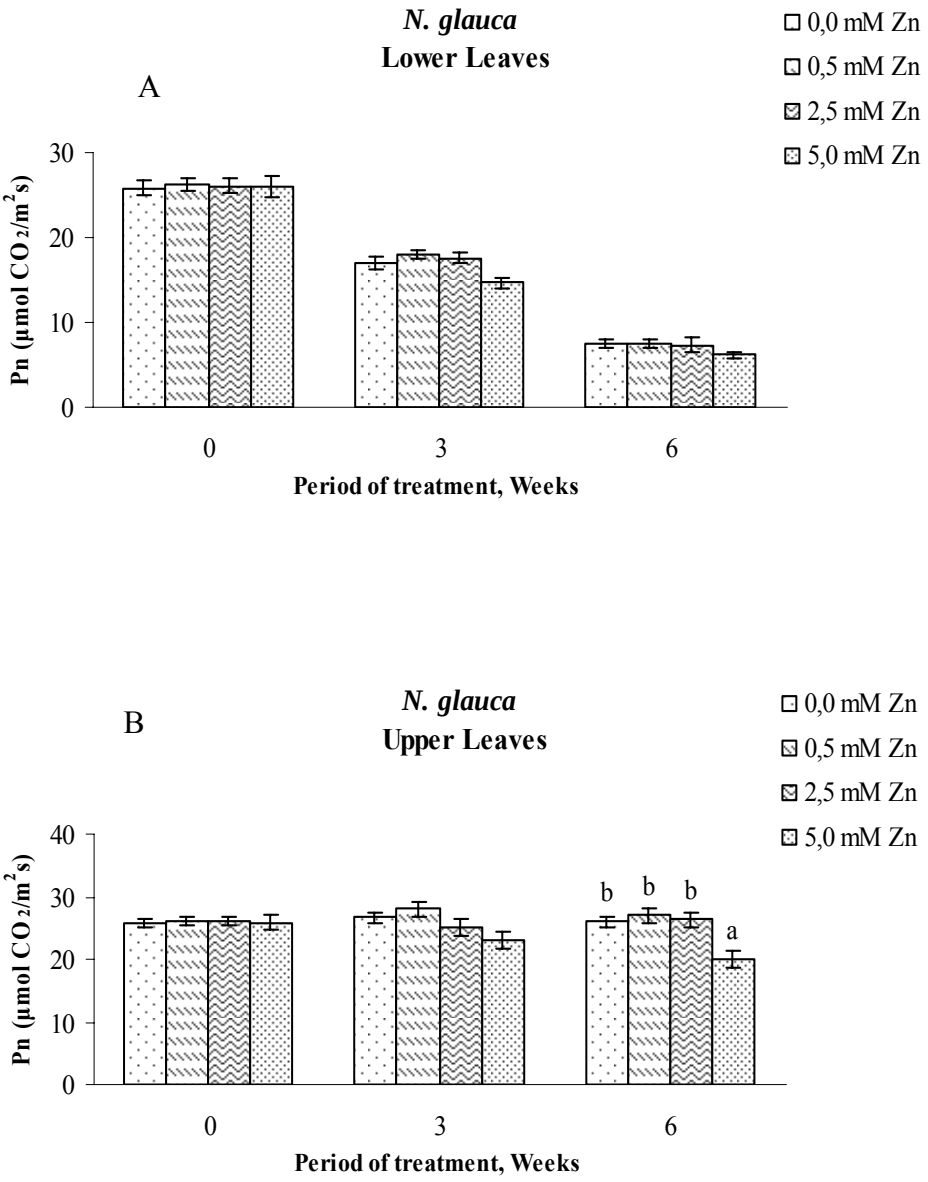


Figure 3.9: The effect of Zn concentration on net photosynthetic rate of: A) lower and B) upper leaves, of *N. glauca* during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6^{th} week, Duncan, P=0.05).

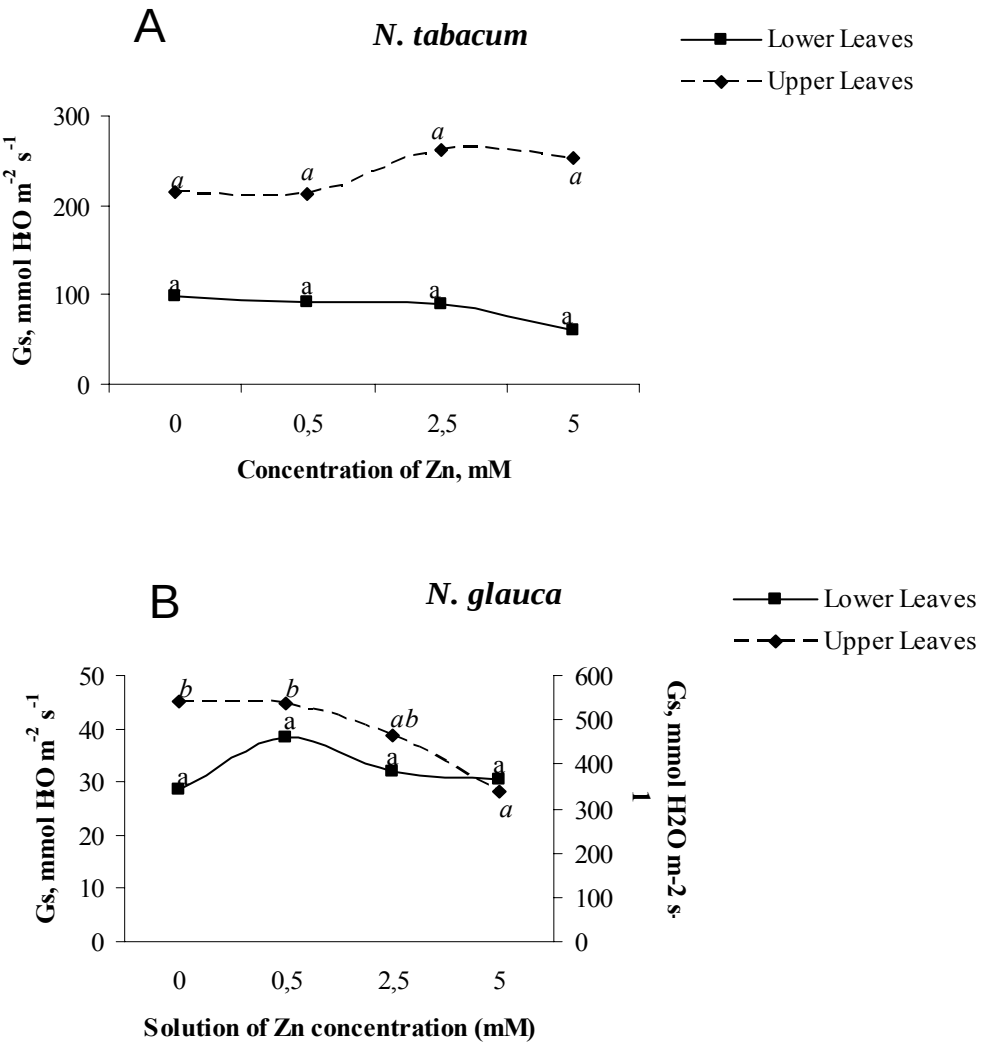


Figure 3.10: The effect of Zn concentration on stomatal conductance of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca* (left axis for lower leaves, right axis for upper leaves). Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

As with stomatal conductance, transpiration rate in lower leaves of *N. tabacum* was reduced with the addition of Zn concentrations in the growth medium, especially at the most severe treatment (Fig. 3.11). In contrast, transpiration rate in upper leaves exhibited an increasing trend, following exposure of plants to Zn, compared to controls. Comparated to *N. tabacum*, in lower leaves of *N. glauca* transpiration rate after an initial increase, at 0.5 mM Zn, it decreased, however no statistical significant difference was observed. Transpiration rate in upper leaves of *N. glauca* showed a

reduction trend in respect to the supply of all Zn concentrations in the culture medium and reached approximately 12% at the highest treatment, compared to control plants.

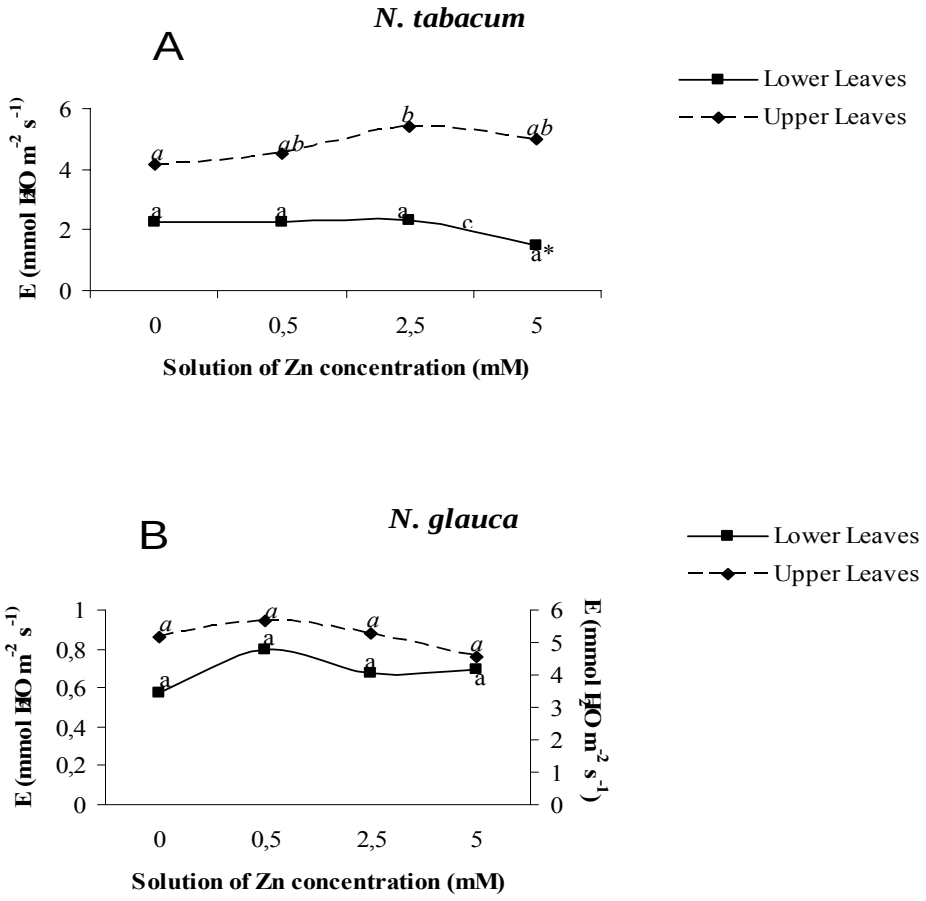


Figure 3.11: The effect of Zn concentration on transpiration rate of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca* (left axis for lower leaves, right axis for upper leaves). Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Water use efficiency (WUE) was calculated as the ratio of net carbon dioxide uptake to transpiration. WUE can be useful parameter as it's correlated with the physiological and biochemical processes of higher plants. Fig. 3.12, presents the WUE in lower and upper leaves of *N. tabacum* and *N. glauca*. Interestingly, in lower leaves of *N. tabacum* the WUE values were higher at the highest Zn treatment compared to the intermediate concentrations. This emphasizes its limited water loss due to transpiration. In upper leaves, WUE was reduced, with the addition of Zn

concentrations in the culture medium, which was accompanied with an increase in transpiration rate. In contrast, the lower leaves of *N. glauca* treated plants had lower WUE values, compared to control plants, while the upper leaves remained almost unaffected.

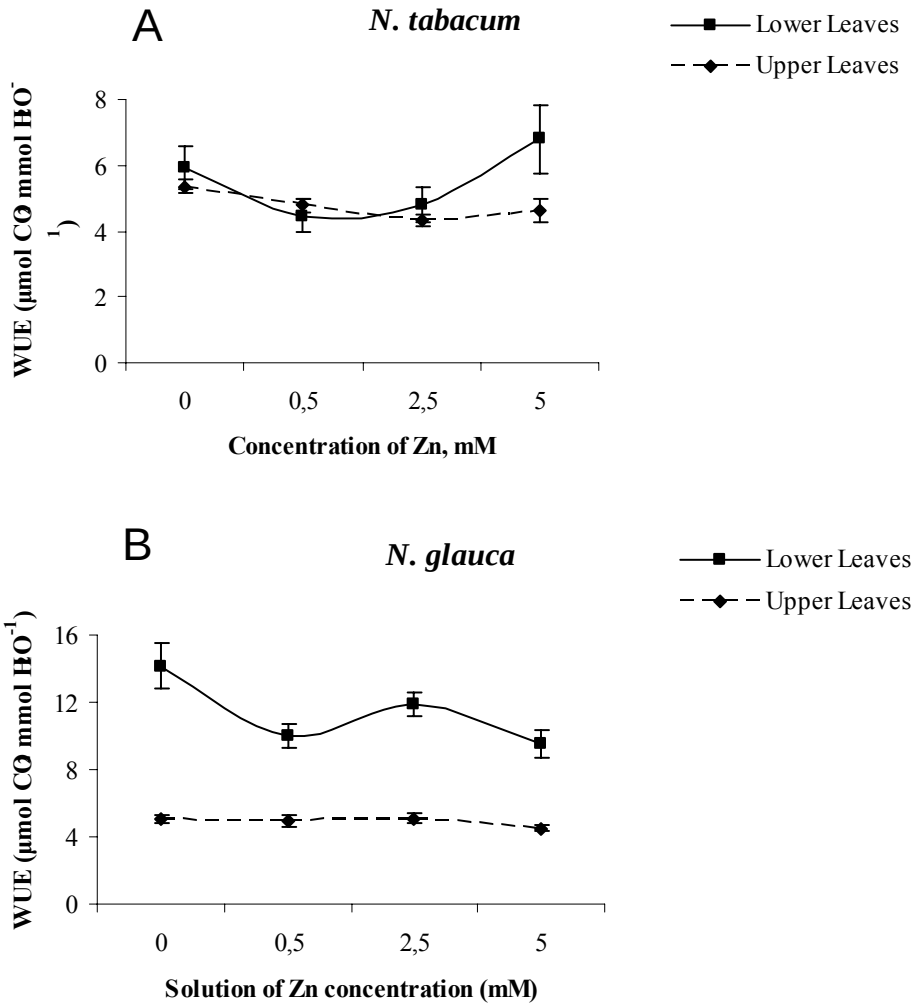


Figure 3.12: The effect of Zn concentration on Water Use Efficiency (WUE) of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE).

The effect of Zn treatments on photosynthetic rate of both plant species was previously presented in Fig. 3.8 and 3.9, however it is interesting to investigate it in parallel with intracellular CO_2 concentration as shown in Fig. 3.13. In upper leaves of *N. tabacum* the intracellular CO_2 concentration and photosynthetic rate showed similar responses in respect to the exposure of plants to Zn treatments. Both parameters remained almost unaffected by the presence of Zn in the culture medium

and no statistical significant differences were evident. The intracellular CO_2 concentration in upper leaves of *N. glauca* was reduced corresponding to increasing Zn concentrations in the culture medium, however no statistical significant differences were obtained from the data analyzed. Similarly, photosynthetic rate remained almost steady by the addition of Zn concentrations in the growth medium, except at the highest treatment, where a reduction by 24% observed.

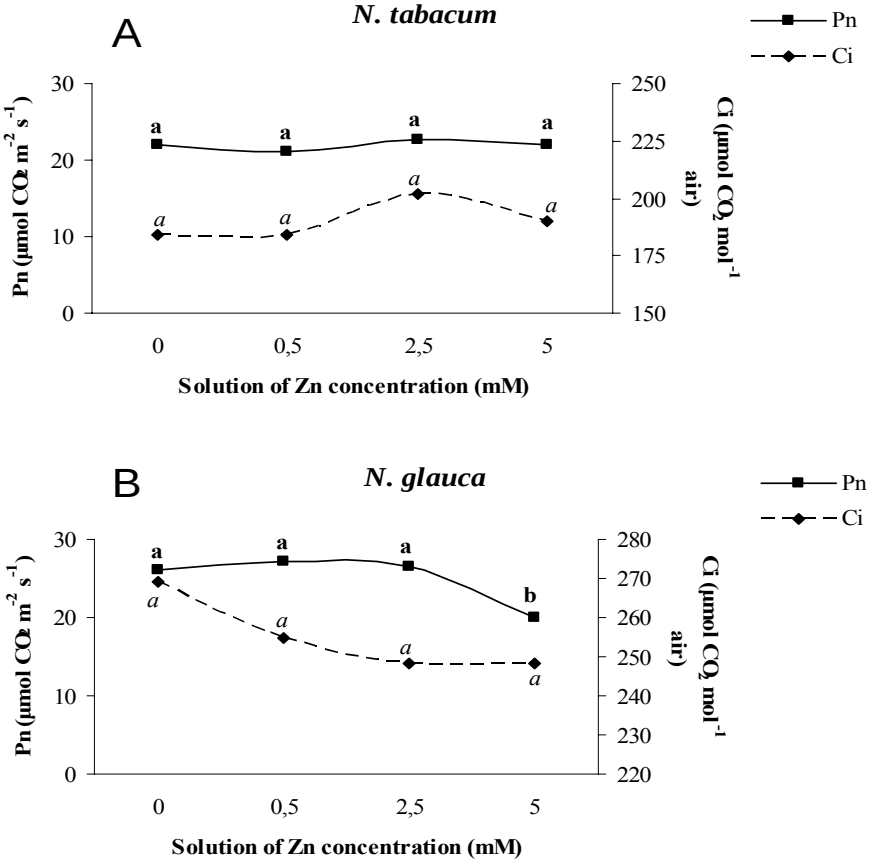


Figure 3.13: The effect of Zn concentration on net photosynthetic rate (left axis) and intracellular CO_2 concentration (right axis) of upper leaves of: A) *N. tabacum* and B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (bold for net photosynthetic rate, italics for intracellular CO_2 concentration, Duncan, P=0.05).

c. Chlorophyll fluorescence parameters - photochemical efficiency of PSII

Chlorophyll fluorescence parameter Fv/Fm, the ratio of variable to maximum fluorescence after dark adaptation, represents the maximum quantum yield of PSII. The majority of studies concerning the effect of heavy metals on the light phase of photosynthesis are devoted to PSII (Prasad, 2010). Chlorophyll fluorescence measurements are nondestructive and can reveal the detailed properties of PSII activity, including the fraction of open PSII centers, energy dissipation via antennae and photoinhibition of PSII. In healthy leaves the value of Fv/Fm is always close to 0.8, independently of the plant species studied. A lower value indicates that a proportion of PSII reaction centers are damaged due to photoinhibition, often observed in plants under stress conditions. The most used stress measurement parameters include Fv/Fm, Fm and F_0 and were described in more detail in the following Table 3.4 (Mijovilovich et al., 2009).

Table 3.4. Explanation of technical terms

F_0	Minimal fluorescence yield of a dark- adapted sample, fluorescence in nonactinic measuring light.
Fm	Maximum fluorescence yield of a dark- adapted sample after supersaturating irradiation pulse
Fv	Variable fluorescence
Fv/Fm	Maximal dark- adapted quantum yield of PSII photochemistry

In parallel to the measurement of the gaseous exchange, the photochemical parameters of photosynthesis were analyzed after 6 weeks in response to zinc excess. As shown in Figure 3.14, all photochemical parameters in lower and upper leaves of *N. tabacum* exhibited statistical significant differences ($P<0.05$). Specifically, F_0 in lower and upper leaves of *N. tabacum* increased with increasing Zn concentration in the culture medium. In contrast, the value of Fm decreased significantly especially at the higher Zn treatments, compared to control plants. Similarly with Fm, Fv of the examined tissues showed a reducing trend, but the effect wasgreater at the higher Zn concentrations. Also, the Fv/ F_0 ratio decreased as the Zn stress intensity increased, however significant differences were present only at lower leaves, reflecting earlier structural dysfunctions of the PSII (Vaillant et al., 2005).

N. glauca and *N. tabacum* showed similar response of photochemical parameters of photosynthesis with the latter appearing more affected (Fig. 3.15). In particular, in lower and upper leaves of *N. glauca*, F_0 slightly increased with the supply of Zn in the culture medium. A reducing trend was present in the tested tissues of Fm under Zn stress, while only 5mM Zn decreased significantly Fm of lower leaves. Fv was affected similarly with Fm in lower and upper leaves of *N. glauca*. The Fv/ F_0 ratio strongly dropped by the presence of 5mM Zn in the growth medium, compared to intact plants.

The maximal dark-adapted yield of PSII photochemistry, Fv/Fm, of both plant species was also followed during the Zn treatment period. In general, for the most measurements obtained during the first and third week, the ratio remained almost unaffected, regardless of the Zn concentration applied with values close to 0.8, a normal value for healthy leaves. The Fv/Fm ratio, a known stress marker, declined significantly, especially by the 6^{th} week of plant exposure to Zn stress. Specifically, in lower leaves of *N. tabacum* the Fv/Fm ratio decreased significantly by about 17%, 20% and 34% at 0.5, 2.5 and 5mM Zn respectively, compared to control plants, while in upper leaves a minimal decrease was observed (Fig. 3.16)

In lower leaves of *N. glauca* the Fv/Fm ratio reduced progressively as the stress intensity increased, however the most severe treatment resulted to significant decline approximately 16%, compared to controls (Fig. 3.17). Similar responses appeared in upper leaves of *N. glauca*. In lower leaves of both plant species the ratio was much lower than 0.8, not only for treated plants, but for controls as well. The decrease observed in Fv/Fm ratio of leaves of both plant species was paralleled with the increase of the basic fluorescence (F_0).

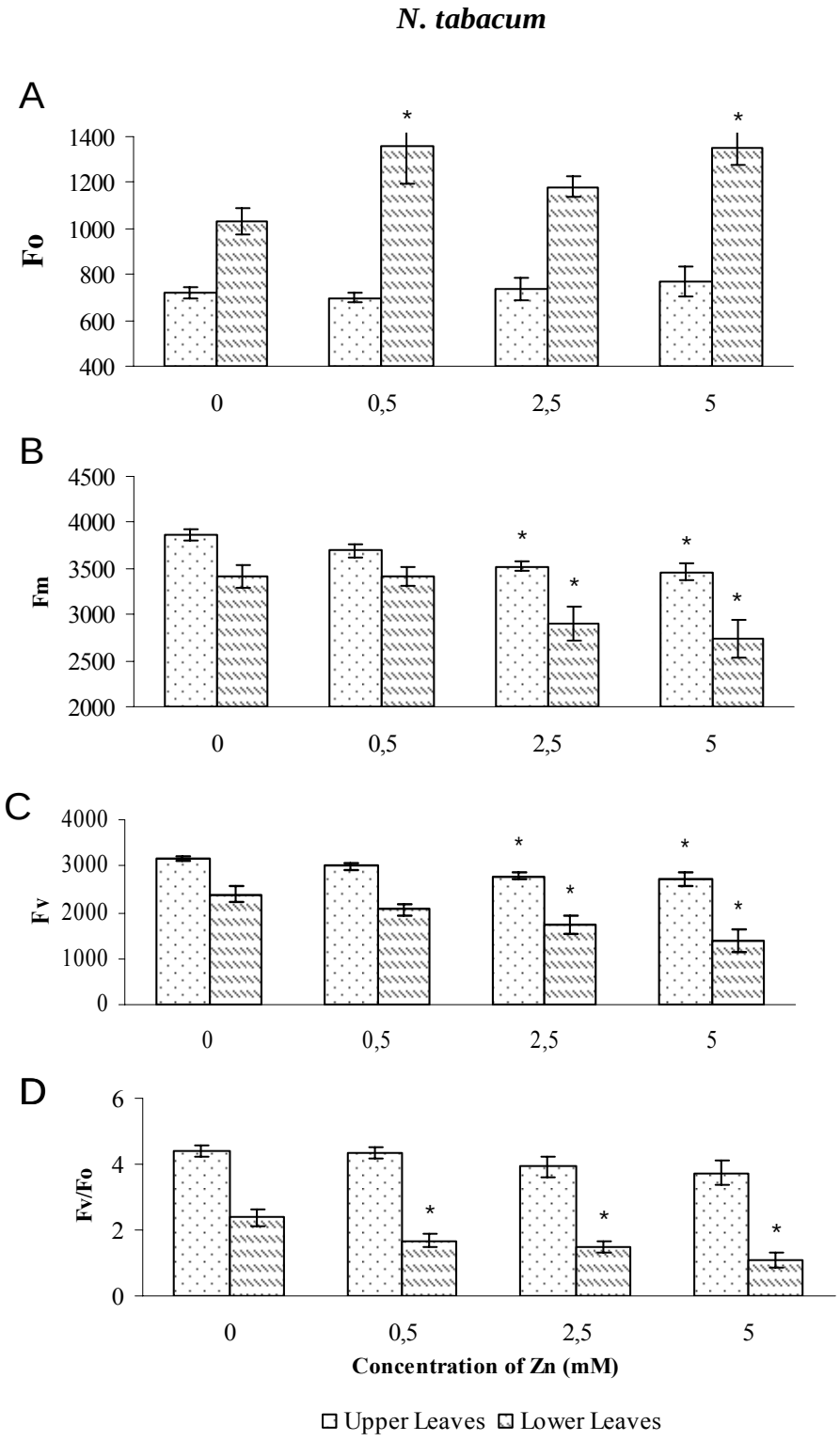


Figure 3.14: The effect of Zn concentration on Chl fluorescence parameters of PSII in dark – adapted leaves: A) initial, F_o B) maximum, Fm C) variable, Fv D) ratio F_v/F_o, of lower and upper leaves of *N. tabacum*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE). Asterisks indicate significant differences between treatments and control (only for data obtained at 6th week, LSD, P=0.05).

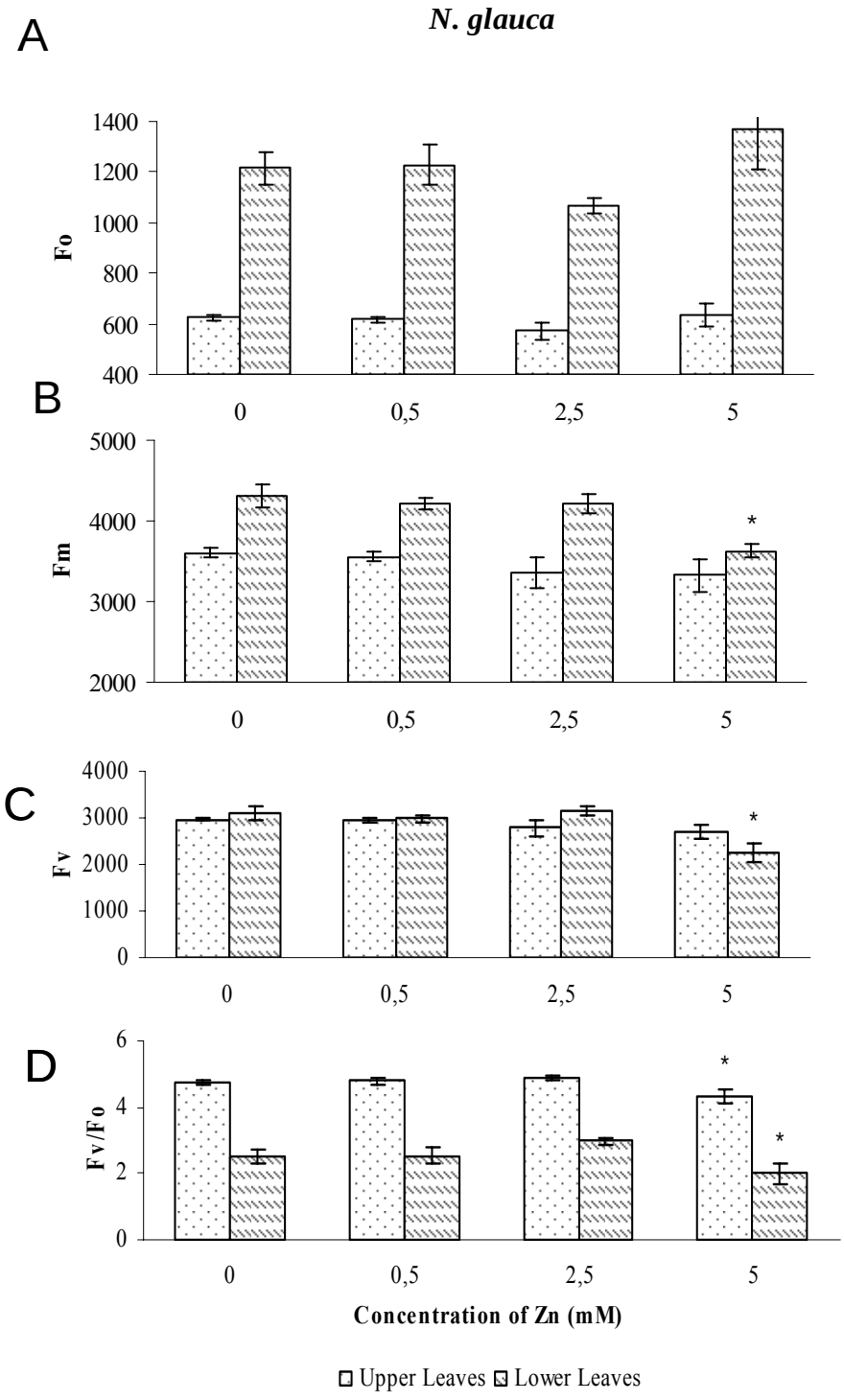


Figure 3.15: The effect of Zn concentration on Chlorophyll fluorescence parameters of PSII in dark – adapted leaves: A) initial, F_0 B) maximum, Fm C) variable, Fv D) ratio F_v/F_o , of lower and upper leaves of *N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE). Asterisks indicate significant differences between treatments and control (only for data obtained at 6th week, LSD, P=0.05).

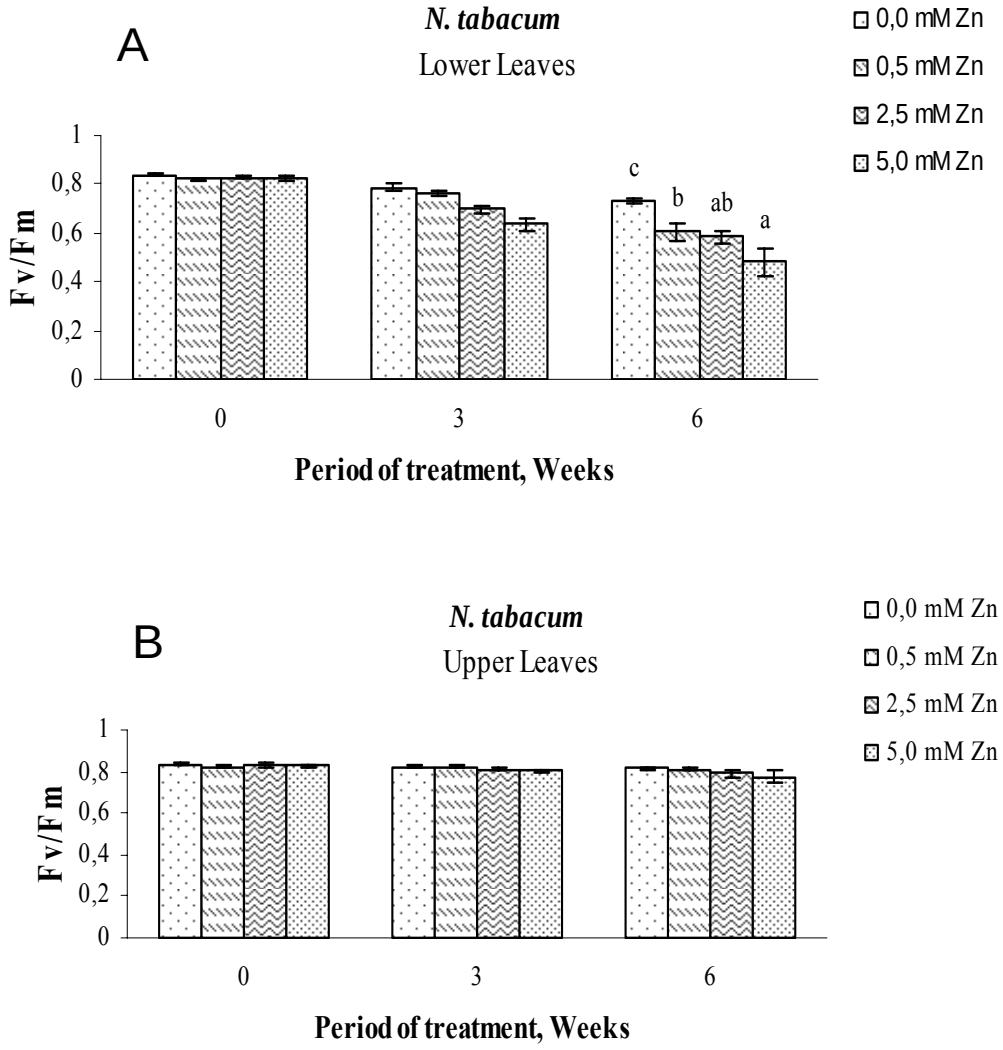


Figure 3.16: The effect of Zn concentration on the photochemical efficiency of PSII (F_v/F_m) in dark – adapted leaves of *N. tabacum*: A) lower leaves B) upper leaves, during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

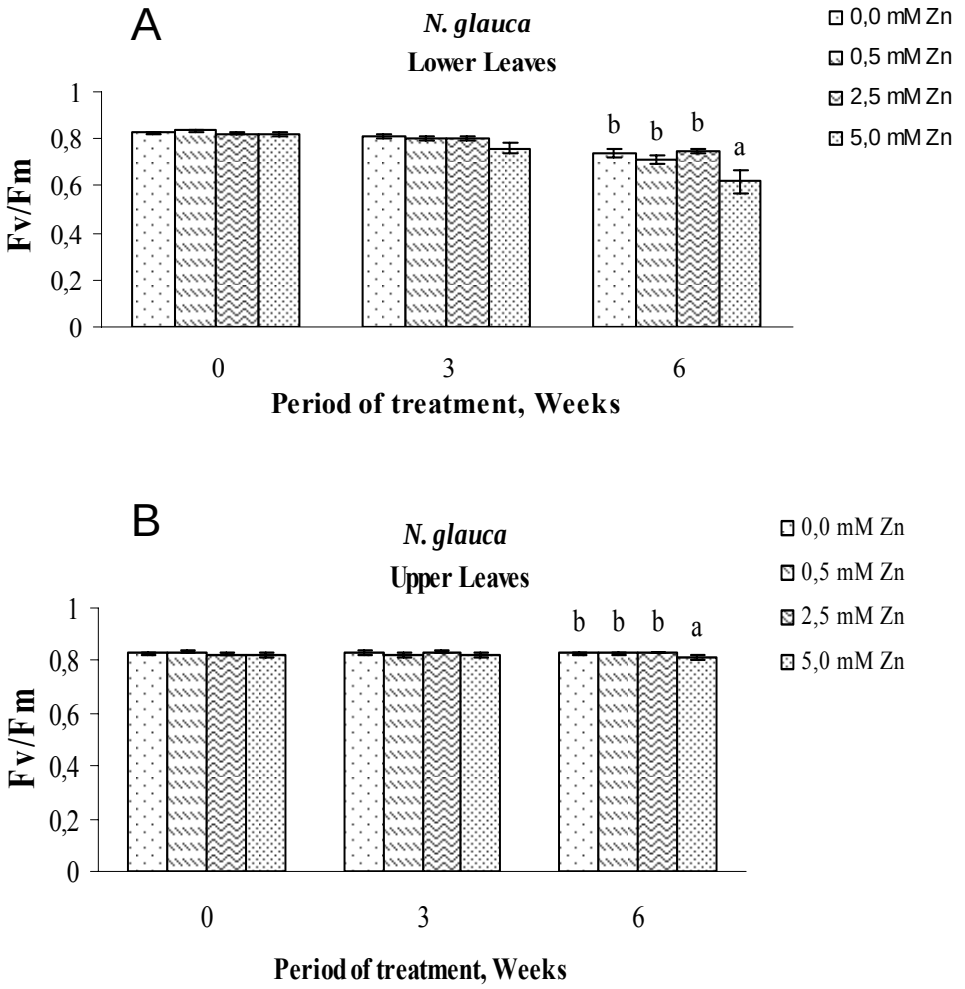


Figure 3.17: The effect of Zn concentration on the photochemical efficiency of PSII (F_v/F_m) in dark – adapted leaves of *N. glauca*: A) lower leaves B) upper leaves, during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

3.1.4 Effect of Zn on Parameters Related with Nitrogen Metabolism

a. Proline

The proteinogenic amino acid proline functions as an osmolyte, radical scavenger, electron sink, stabilizer of macromolecules and a cell wall component. Proline is an extensively studied molecule in the context of plant responses to abiotic stresses (Sharma and Dietz, 2006).

Fig. 3.18 illustrates the effect of Zn concentrations on proline accumulation of lower and upper leaves of *N. tabacum* and *N. glauca*. The accumulation pattern was similar in both plant species. The amount of proline was positively affected by Zn treatments, while significant differences ($P<0.05$) exhibited by all applied Zn concentrations. Both plant species accumulated maximum at the highest treatment.

b. Total Soluble Protein Content

Total soluble protein content was differentially affected by the presence of Zn concentrations in the culture medium (Table 3.5). In lower leaves of *N. tabacum* the protein content pattern did not change under Zn excess, while in lower leaves of *N. glauca* show a depleted trend. In upper leaves of *N. tabacum* appeared an increased trend, while in upper leaves of *N. glauca* remained fairly steady. However, in lower leaves of both plant species detected considerable lower protein content, than in upper.

c. Specific Activities Determination

Specific activities were determined and presented in Tables 3.6 and 3.7. Immunoblot analysis presented in Plates 3.3 and 3.4.

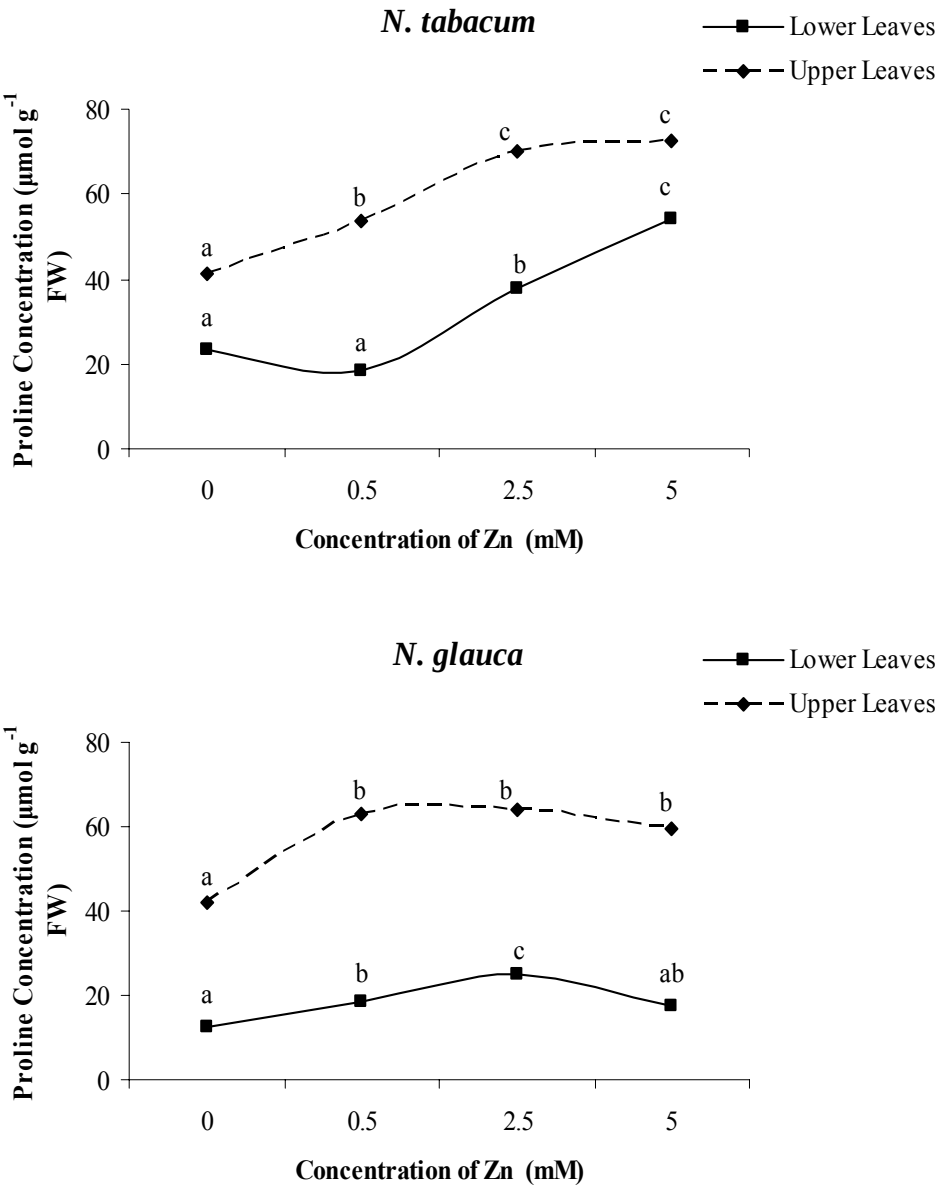


Figure 3.18: The effect of Zn concentration on proline concentration of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Table 3.5: The effect of Zn concentration on protein content of lower and upper leaves of *N. tabacum and N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. Values are means ± standard error (SE).

Treatment Zn, mM	*N. tabacum*		*N. glauca*	
	Lower leaves	Upper leaves	Lower leaves	Upper leaves
0.0	6,14 ± 0,19	7,59 ± 013	6,38 ± 0,4	10,79 ± 0,2
0.5	5,50 ± 0,07	7,75 ± 0,28	5,13 ± 0,2	10,22 ± 0,4
2.5	6,90 ± 0,09	10,10 ± 0,29	5,72 ± 0,1	10,50 ± 0,2
5.0	6,97 ± 0,13	10,17 ± 0,45	5,21 ± 0,1	10,04 ± 0,3

Table 3.6: The effect of Zn concentration on specific activity of the enzymes of nitrogen assimilation of lower and upper leaves of *N. tabacum.* Values concerned controls and plants treated with 5 mM Zn. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Plant tissues	Treatments Zn, mM	Nitrogen Assimilating Enzymes (Enzyme activity: nmol mg^{-1} protein min^{-1})		
		GS	Fd-GOGAT	GDH
Lower leaves	**0.0**	420.33 ± 20.6	89,18 ± 4.7	398,92 ± 14.3
	5.0	247.84 ± 20.0	52,12 ± 5.2	492.58 ± 16
Upper leaves	**0.0**	777.80 ± 43.2	119,28 ± 7.2	165.63 ± 11.8
	5.0	460.92 ± 25.3	112,14 ± 9.1	187.19 ± 21.1

Table 3.7: The effect of Zn concentration on specific activity of the enzymes of nitrogen assimilation of lower and upper leaves of *N. glauca.* Values concerned controls and plants treated with 5 mM Zn. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Plant tissues	Treatments Zn, mM	Nitrogen Assimilating Enzymes (Enzyme activity: nmol mg^{-1} protein min^{-1})		
		GS	Fd-GOGAT	GDH

Lower leaves	**0.0**	333.11 ± 19.2	52.15 ± 3.8	474,70 ± 24.0
	5.0	302,93 ± 21.2	39.23 ± 5.7	513.93 ± 16.9
Upper leaves	**0.0**	619.63 ± 31.6	86.58 ± 8.8	136.43 ± 13.9
	5.0	480.25 ± 29.7	78.44 ± 6.1	144.43 ± 17.7

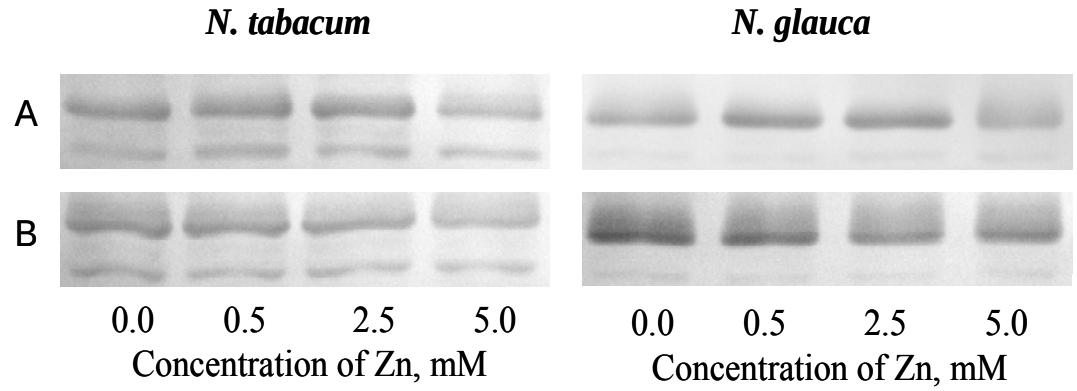


Plate 3.4: Western blot analysis of GS in A) lower leaves B) upper leaves of Zn treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti-GS serum, as described in section "Materials and Methods"

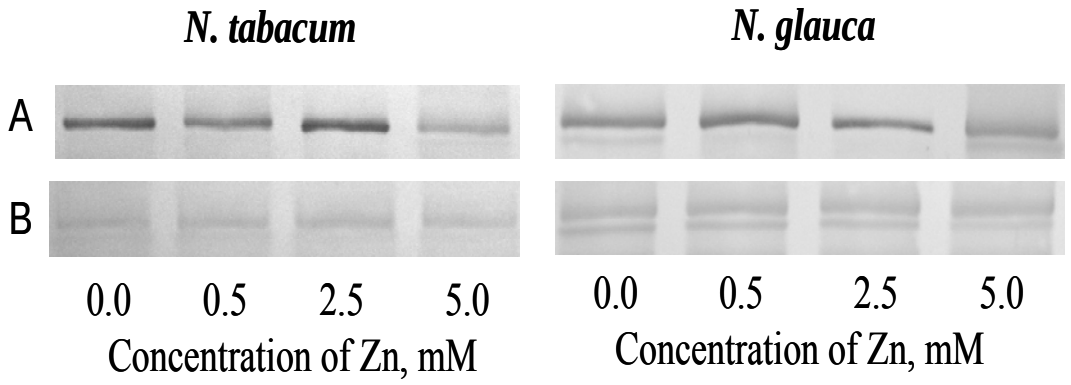


Plate 3.5: Western blot analysis of Fd-GOGAT in A) lower leaves B) upper leaves of Zn treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants grown in plastic pots containing homogenized soil in the presence of different concentrations of Zn^{2+} for 6 weeks. For immunoblot analysis proteins were resolved in 7% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti- GOGAT serum, as described in section "Materials and Methods"

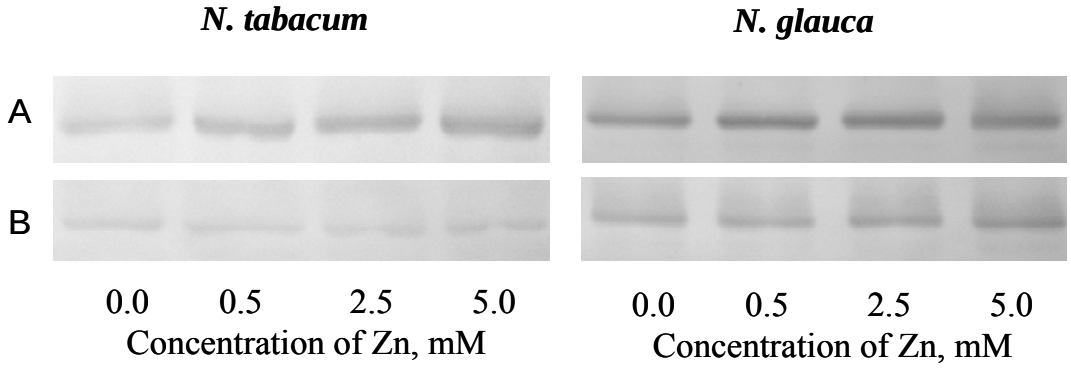


Plate 3.6: Western blot analysis of GDH in A) lower leaves B) upper leaves of Zn treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni for 6 weeks. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti-GDH serum, as described in section "Materials and Methods"

3.2 Effect of Nickel on Morphological, Physiological and Biochemical Parameters of *N. tabacum* and *N. glauca*

3.2.1 Nickel in Plants Tissues

Literature suggests that, Ni is a recently discovered essential microelement for many plant and animal species. Ni is rapidly taken up by plant root system; however literature suggests that the accumulation of nickel in the roots is higher compared to the aboveground biomass. In this study, accumulation of Ni in five different parts of *N. tabacum* and *N. glauca* was investigated at three Ni concentrations. The addition of Ni in the culture medium, significantly affected the endogenous concentration in the examined tissues of both plant species.

In general, the accumulation of Ni in root and aboveground tissues of *N. tabacum* increased significantly with the increasing doses of the metal in the culture medium (Fig. 3.19, Table 3.8). Particularly, lower and upper leaves exhibited the same accumulation pattern. Thus, statistically significant increases observed in leaves, where the Ni accumulated by about 2-fold and 4-fold at 0.5 mM and 1 mM Ni, respectively, compare to control plants. Ni concentration in lower and upper shoot of plants treated with 0.5 mM and 1mM Ni was 2 and 3 times higher, respectively than untreated plants. While mean values for plants grown in 0.1 mM were higher for all

aboveground tissues, compare to control plants, no significant differences were found. Also, Ni treatment increased progressively the Ni amount in root, where the maximum accumulation observed. Root accumulated by 3.6-, 4- and 5- fold at 0.1, 0.5 and 1 mM Ni, respectively, compare to intact plants.

N. glauca showed similar accumulation pattern with *N. tabacum* (Fig. 3.20, Table 3.9). As the Ni concentration increased in the culture medium, the higher concentration detected in examined tissues. Statistical analyses of the obtained data resulted in a 2.5- and 3.5- fold increase of Ni concentration in lower leaves of plants treated with 0.5 and 1 mM Ni, compare to control plants. Higher rise observed in upper leaves, where Ni content increased 1.7-, 3.2- and 4- fold, by the presence of the three examined Ni concentrations in the culture medium.

Comparatively, in both plant species, Ni uptake was positively affected by Ni treatments. The accumulation of Ni in plants grown by the presence of the three different concentrations of Ni, was higher than control plants. Also both plant species showed maximum accumulation in root, at the highest Ni treatment. However, Ni accumulated differently in leaves of the two plant species. *N. tabacum* showed higher accumulation in lower leaves, compare to upper leaves. On the other hand, in upper leaves of *N. glauca* detected more Ni ions than in lower leaves, at all Ni exogenous concentrations. However, both plant species, in all examined tissues accumulated much higher than the critical toxicity level. Normally, the requirement of nickel in plants is very low, in the range of 1-10 μg/g dry matter. Toxicity occurs at concentrations in the range of 10 μg /g in sensitive and 50 μg/g in tolerant plants (Assunção et al., 2003).

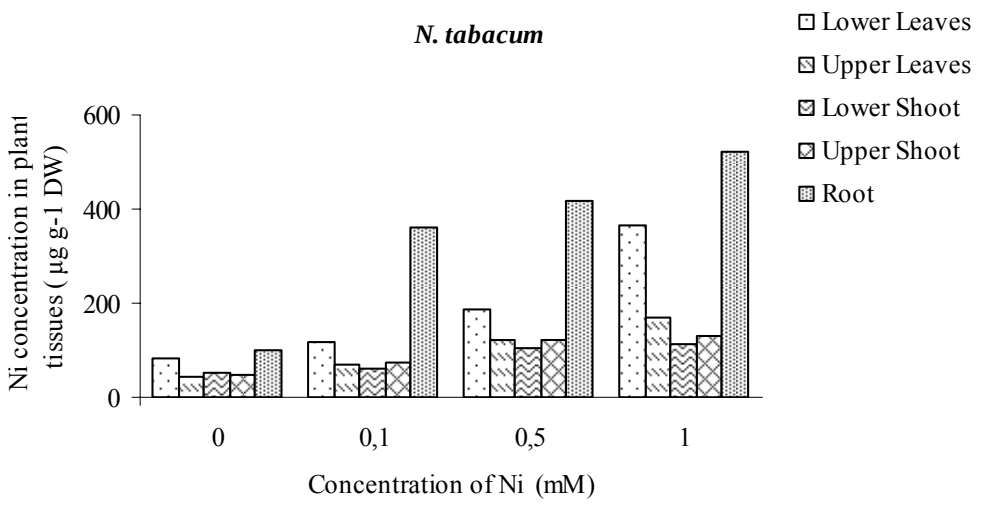


Figure 3.19: Accumulation of Ni in plant tissues of *N. tabacum*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Statistical significant differences of plant ttissues between control and treatments are presented in Table 3.8.

Table 3.8. Statistical significant differences of the endogenous Ni concentration in plant tissues of *N. tabacum.* Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Ni, mM	**Plant Tissue**				
	Lower leaves	Upper leaves	Lower shoot	Upper Shoot	Root
0.0	a	a	a	a	a
0.1	a	a	b	b	b
0.5	b	b	c	c	b
1.0	c	c	d	c	b

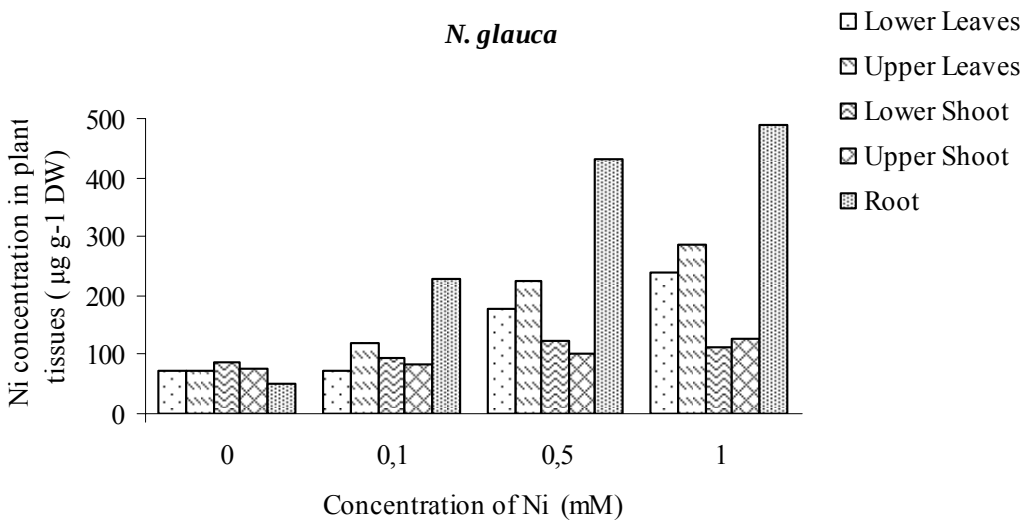


Figure 3.20: Accumulation of Ni in plant tissues of *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Statistical significant differences of plant ttissues between control and treatments are presented in Table 3.9.

Table 3.9. Statistical significant differences of the endogenous Ni concentration in plant tissues of *N. glauca*. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Ni, mM	**Plant Tissue**				
	Lower leaves	Upper leaves	Lower shoot	Upper Shoot	Root
0.0	a	a	a	a	a
0.1	a	b	a	a,b	b
0.5	b	c	b	b	c
1.0	c	d	a,b	c	d

3.2.2 Effect of Ni on Morphological Characteristics and Growth of *N. tabacum* and *N. glauca*

The morphological characteristics of *N. tabacum* and *N. glauca* were followed during the experiment period and the symptoms were observed (Plates 3.7. and 3.8). The determinations occurred, have provided data showing non-negligible effects of Ni treatments on both plant species. Plants suffering with Ni toxicity show necrosis on the leaf tips and margins, and young leaves may become distorted while the terminal shoot bunds may die (Rao, 2006). In this study, plants of all treatments showed visual toxicity symptoms. The gradation of the Ni concentration in *N. tabacum* was related with chlorosis. The higher Ni treatments caused severe chlorosis at lower leaves of *N. tabacum*, while upper leaves began to turn chlorotic. *N. glauca* appeared more affected by the prolonged exposure to Ni treatments. The higher Ni concentration caused interveinal chlorosis in lower and upper leaves, development of dark brown necrotic spots, fringed margins. Plant showed stunted growth and wilted appearance. The extent and magnitude of symptoms and damage of the plant parts increased with increasing treatment concentration of Ni.

Plate 3.7: . Plants of *N. tabacum* (left) and *N. glauca* (right) grown in greenhouse conditions in the presence of 0.0, 0.1, 0.5 and 1.0 mM Ni^{2+}.

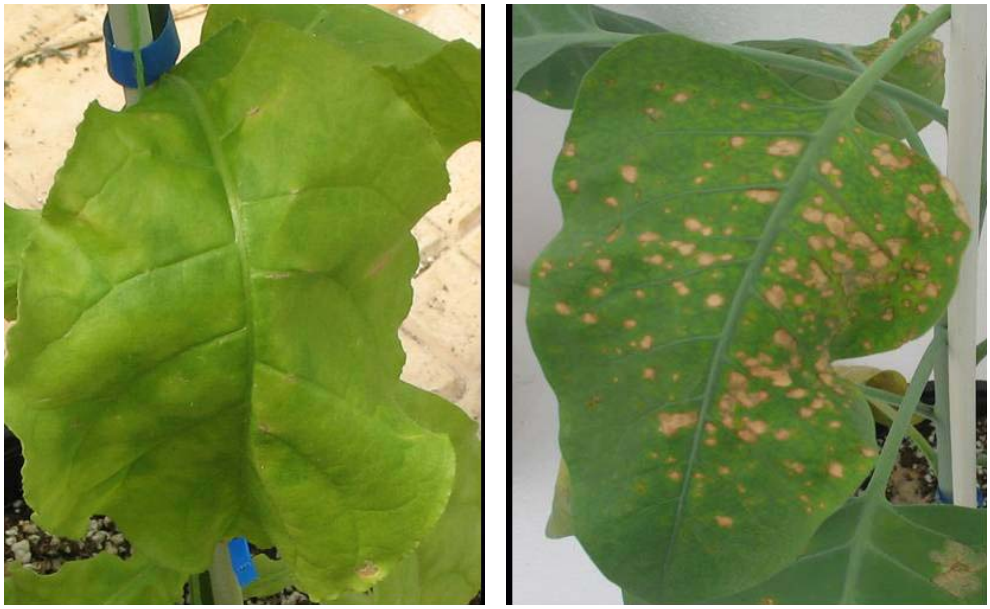


Plate 3.8: Visual symptoms of Ni stressed *N. tabacum* (left) and *N. glauca* (right) leaves. Plants were grown in plastic pots in greenhouse.

As recommended, during the period of Ni treatments of *N. tabacum* and *N. glauca* plants, several parameters related with plant growth were followed. Statistically significant differences of plants height exhibited mainly, for the 6 week treated plants of both plant species, as shown in Fig. 3.21. Interestingly, the 4 week supply of 0.1mM Ni resulted to increase significantly the height of *N. tabacum*, compared to controls and to treated plants with higher Ni concentrations. Remarkable reduced of height caused the presence of 0.5 mM Ni in the culture medium at the 6th week. Similarly, height of plants of *N. glauca* positively affected by the intermediate Ni concentrations at the 4th week, while significant inhibition ($P<0.05$) by 14% was resulted by the highest Ni treatment the last week, compared to controls.

Another growth parameter was followed during Ni treatment period was the number of leaves of *N. tabacum* and *N. glauca* as presented in Figure 3.22. Leaves number of *N. tabacum* showed reduced trend by the presence of Ni in the culture medium, however no significantly differences were exhibited. *N. glauca* appeared more affected, since considerable reduction was observed after 3 weeks with Ni treatments.

Fresh weights of leaves, shoots and roots of both plant species were determined under Ni stress. As presented in Figure 3.23, a reduced trend was

observed at fresh weight of leaves and shoots of *N. tabacum*, reached approximately 15%, however no significant difference was exhibited, compared with untreated plants. In contrast, the inhibitory effects increased on root weight with increasing Ni doses in the culture medium, where the reduction reached up to 15% and 30%, at 0.5 and 1mM Ni, respectively. The reduction was observed in fresh weight of leaves, shoot and root of *N. glauca* followed a dose-dependent manner. Similarly, with *N. tabacum*, the inhibitory effects on fresh weight tissues of *N. glauca* became more pronounced at the highest Ni treatment. Specifically, weight of leaves, shoot and root was significantly decreased by 28%, 39% and 31%, respectively, at the highest Ni concentration, compared to intact plants. Differently with *N. tabacum*, where the greatest reduction occurred in root fresh weight, *N. glauca* exhibit the highest decline in shoot fresh weight.

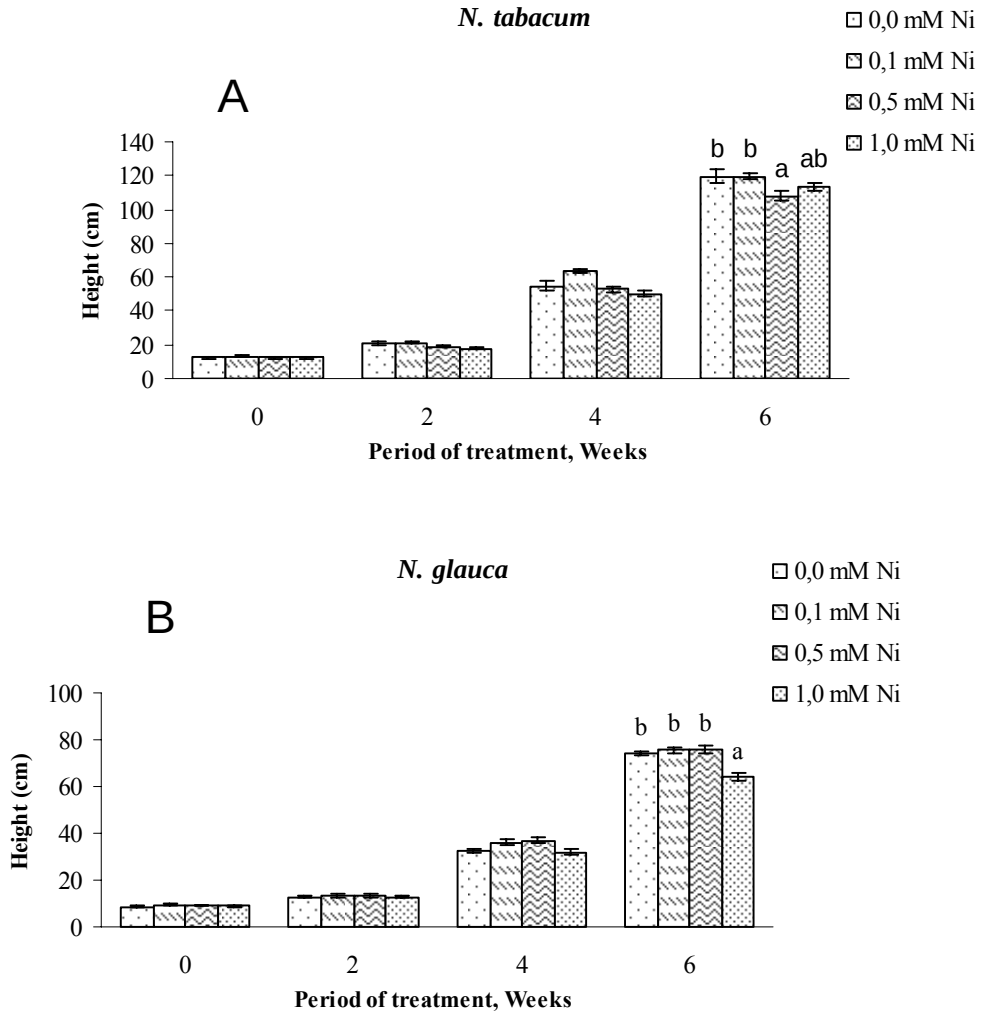


Figure 3.21: The effect of Ni concentration on plant height during the treatment period A) *N. tabacum*, B) *N. glauca*. Plants were grown in plastic pots containing

homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05)

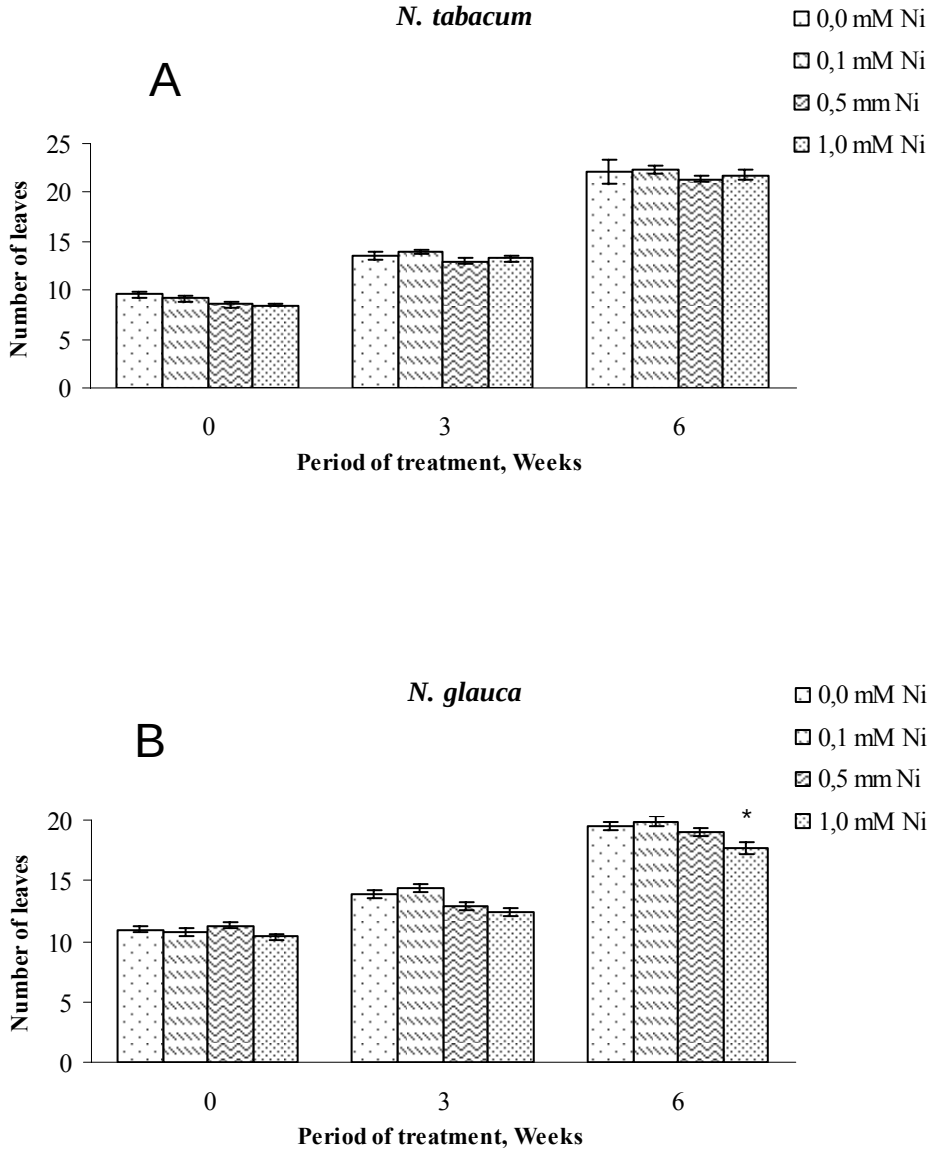


Figure 3.22: The effect of Ni concentration on the number of leaves of A) *N. tabacum*, B) *N. glauca*, during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE). Asterisks indicate significant differences between treatments and control (only for data obtained at 6th week, LSD, P=0.05).

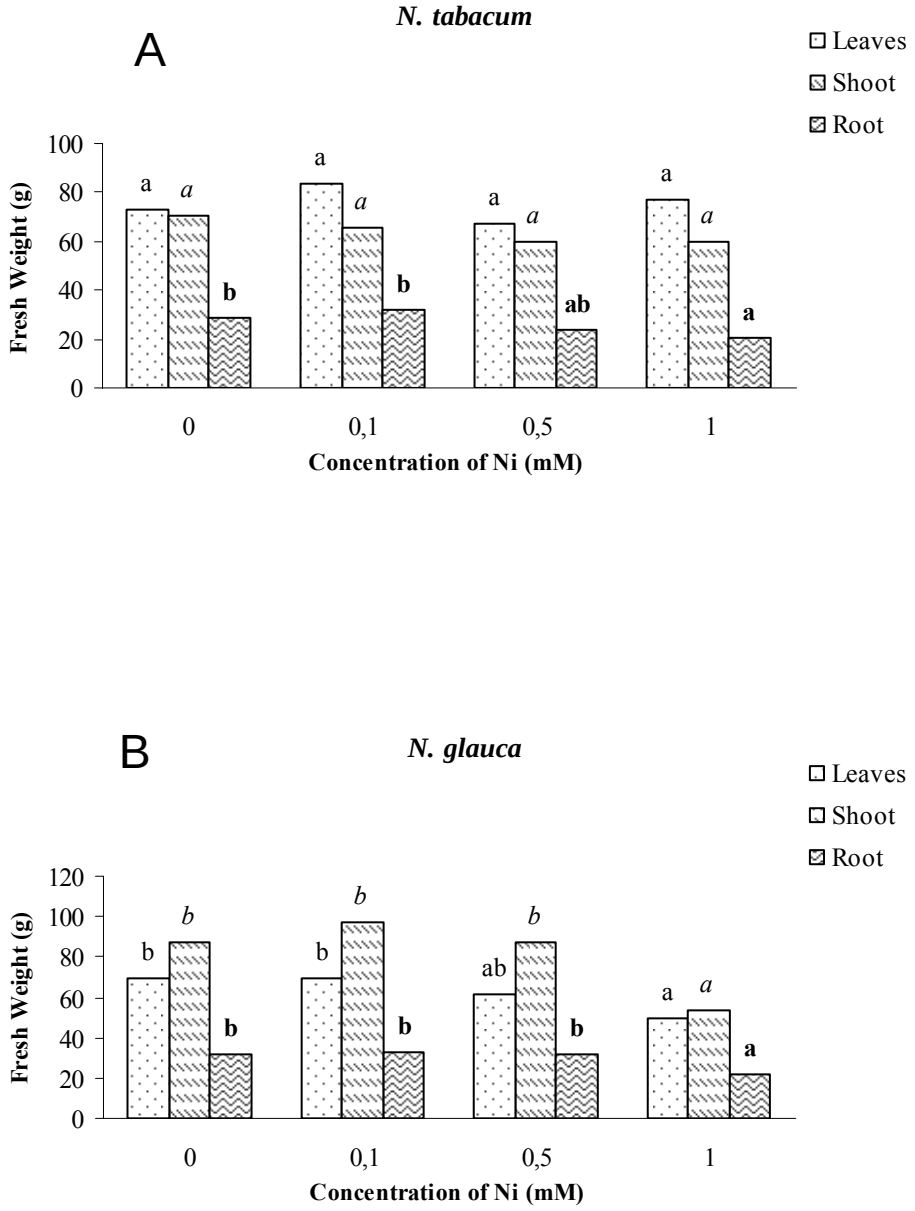


Figure 3.23: The effect of Ni concentration on plant fresh weight of A) N. tabacum, B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (regular for leaves, italics for shoot, bold for root, Duncan, P=0.05).

3.2.3 Effect of Ni on Photosynthetic Parameters of *N. tabacum* and *N. glauca*

a. Chlorophyll pigments

The contents of chlorophylls of lower and upper leaves of *N. tabacum* and *N. glauca* were investigated in dependence on the Ni^{2+} concentrations and presented in Figures below. Particularly, chlorophyll a and b in lower leaves of *N. tabacum* remained relatively constant over the range of Ni concentrations investigated (Fig. 3.24 A). In contrast, statistically significant differences observed in upper leaves, by the presence of Ni in the culture medium, compared to intact plants. The deleterious effect of Ni became more pronounced with increasing concentrations. Chlorophyll a and b were progressively reduced and reached up to about 28% and 37%, respectively, in plants exposed to 1mM Ni, compare to control plants. In the case of *N. glauca* (Figure 3.24 B), the recommended photosynthetic pigments in lower and upper leaves were slightly affected by the presence of Ni in the culture medium and similarly with *N. tabacum*.

Figure 3.25 summarized the effect of Ni treatments on total chlorophyll content (mg/g FW and SPAD values) of lower and upper leaves of *N. tabacum* and *N. glauca*. Similarly in both plant species upper leaves appeared more affected than lower. Specifically, total chlorophyll content decreased significantly ($P<0.05$) approximately 30% and 26% in upper leaves of *N. tabacum* and *N. glauca*, respectively, by the highest Ni treatment.

Table 3.10 presented the change of the chlorophyll ratio a to b under Ni stress in lower and upper leaves of both plant species. In lower leaves the chlorophyll ratio showed a depleted trend in response to Ni exposure, indicating that chlorophyll a was more affected than b. On the other hand, the Ni- induced decline of photosynthetic pigments a and b, slightly increased the chlorophyll ratio in upper leaves of both plant species.

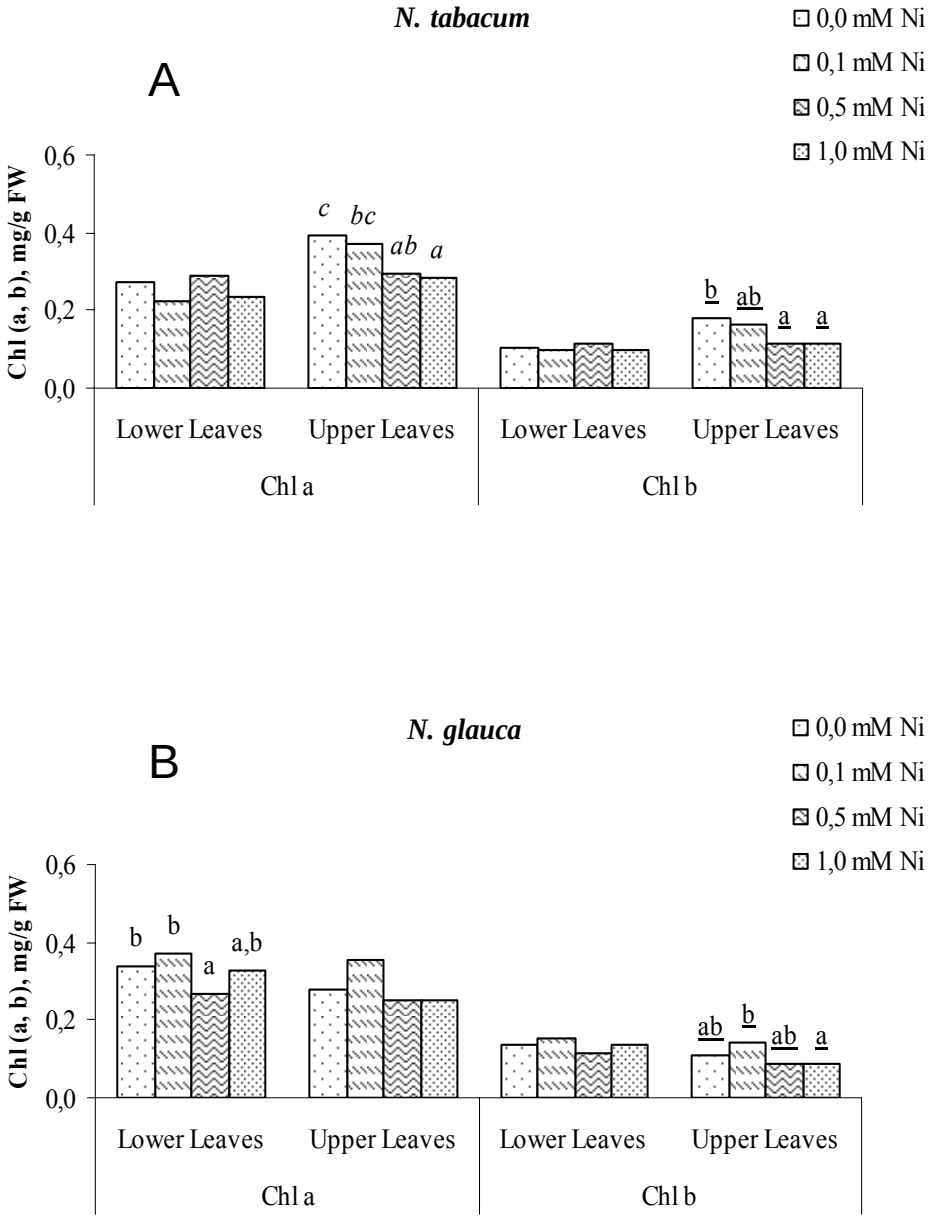
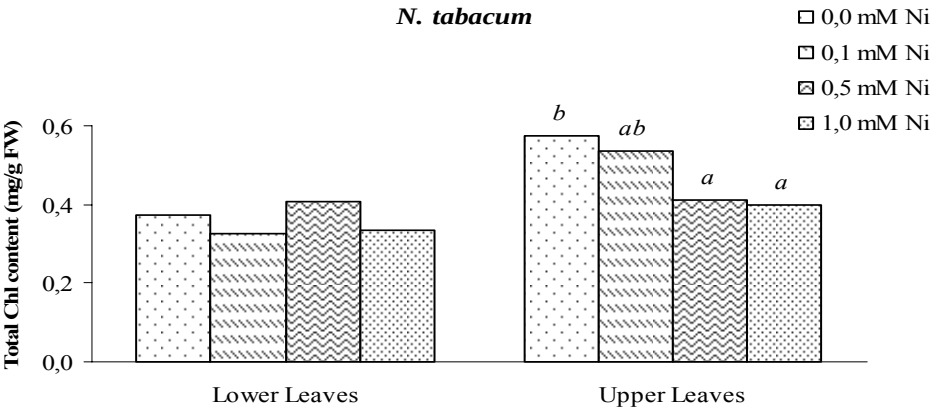
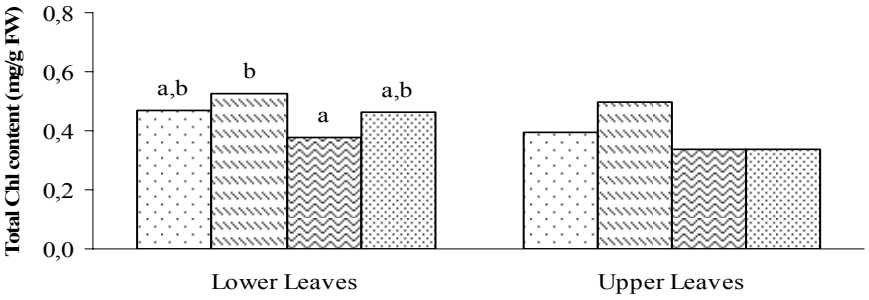
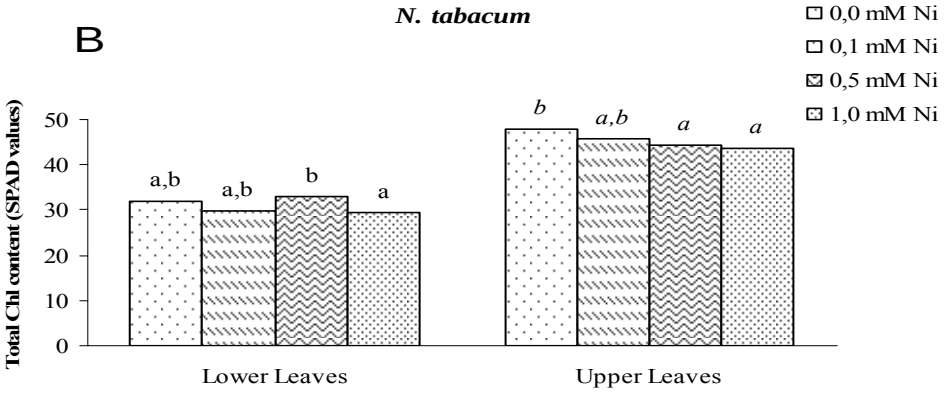


Figure 3.24: The effect of Ni concentration on photosynthetic pigments, chl a and chl b of lower and upper leaves of A) *N. tabacum*, B) *N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (regural for chl a of lower leaves, italics for chl a of upper leaves, Duncan, P=0.05). Columns without letters on do not differ significantly.


N. tabacum
0,0 mM Ni
0,1 mM Ni
0,5 mM Ni
1,0 mM Ni
Total Chl content (mg/g FW)
0,6
0,4
0,2
0,0
b
ab
a
a
Lower Leaves
Upper Leaves


N. glauca
Total Chl content (mg/g FW)
0,8
0,6
0,4
0,2
0,0
a,b
b
a
a,b
Lower Leaves
Upper Leaves


B
N. tabacum
0,0 mM Ni
0,1 mM Ni
0,5 mM Ni
1,0 mM Ni
Total Chl content (SPAD values)
50
40
30
20
10
0
a,b
a,b
b
a
b
a,b
a
a
Lower Leaves
Upper Leaves

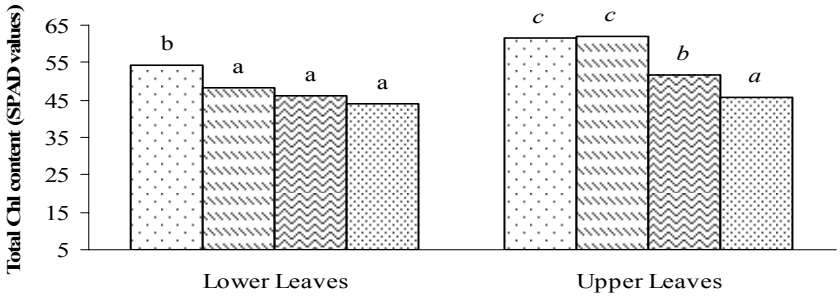

N. glauca
Total Chl content (SPAD values)
65
55
45
35
25
15
5
b
a
a
a
c
c
b
a
Lower Leaves
Upper Leaves

Figure 3.25: The effect of Ni concentration on total chlorophyll contents A) mg/g FW and B) SPAD values of lower and upper leaves of *N. tabacum* and *N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (regular for lower leaves, italics for upper leaves, Duncan, P=0.05). Columns without letters on do not differ significantly.

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Table 3.10: The effect of Ni concentration on the chlorophyll *a/b* ratio of lower and upper leaves of *N. tabacum* and *N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Ni, mM	***N. tabacum***		***N. glauca***	
	Lower leaves	Upper leaves	Lower leaves	Upper leaves
0.0	2.70 b	2.24 a	2.52 a	2.56 a
0.1	2.30 a	2.35 a	2.42 a	2.57 a
0.5	2.48 ab	2.53 a	2.41 a	2.80 a
1.0	2.34 a	2.48 a	2.40 a	2.97 a

Photosynthetic rate and gas exchange parameters

As presented in Fig. 3.26, Pn in lower leaves of *N. tabacum*, decreased linearly, in respect to exposure period of Ni treatments, as well as in plants grown in control culture medium. The 3 weeks exposure to Ni treatments led to significant decline in Pn values, by about 25% at 0 mM and reached up to 33% at 1 mM. The deleterious effect became more pronounced the last period of treatment, where Pn was lowered by about 53% at control culture medium and reached up to 60% at the highest Ni treatment, compared with the mean values of the first period. A reduced trend (linear) observed in upper leaves of *N. tabacum*, as well, in respect to the exposure period to Ni treatments, however statistical significant differences observed only at the measurements obtained the last week. Particularly, the 6 week Ni treatments resulted to a reduction by 14% at 0mM and reached up to 37% at 1mM.

Significant differences observed comparing the mean values between treatments at the last week, where the highest Ni concentration led to significant decline ($P<0.05$) by 25%, compared to control plants.

Pn in lower leaves of *N. glauca* was progressively decreased, with the exposure period of the plants at Ni treatments, as well as at control medium.(Fig. 3.27). After 3 weeks exposure to Ni, the reduction of Pn observed in lower leaves was 34% at control plants and reached up to 44% at the highest treatment. The deleterious effect became more pronounced the last period of treatment, where Pn was lowered by 71%, 74%, 80% and 85%, at 0, 0.1, 0.5 and 1 mM Ni, respectively, comparing with the values of the first period. In addition, the presence of 0.5 and 1mM Ni in the medium culture, the last week before harvesting, led to statistically significant decline ($P<0.05$) of Pn, in lower leaves approximately, 30% and 47%, respectively, comparing to control plants. Pn in upper leaves of plants grown in control medium and at the presence of 0.1mM Ni, remained almost constant irrespective of the period of treatment. On the other hand, Pn in upper leaves of plants grown in the presence of 0.5 and 1mM Ni decreased linearly. Particularly, the highest Ni concentration led to significant decline of Pn values in upper leaves of 3 and 6 weeks treated plants by 30 and 54%, respectively.

Stomatal conductance of lower and upper leaves of both plant species appeared similarly affected by Ni treatments (Fig. 3.28). In lower leaves of *N. tabacum*, stomatal conductance remained steady by the presence of Ni concentrations in the culture medium, while a slight increase observed at the higher treatments. In contrast, in upper leaves, the presence of 0.5 and 1 mM Ni resulted to the reduction of stomatal conductance, by 20% and 44%, respectively, compared to control plants. On the other hand, in lower leaves of *N. glauca* significant depression by 28% and 39%, resulted by the presence of 0.5 and 1 mM Ni, compared with intact plants. Similarly, in upper leaves, the supply of 0.5 and 1 mM Ni, led to statistical significant decline ($P<0.05$) of stomatal conductance, by 26% and 72%, compared to control plants.

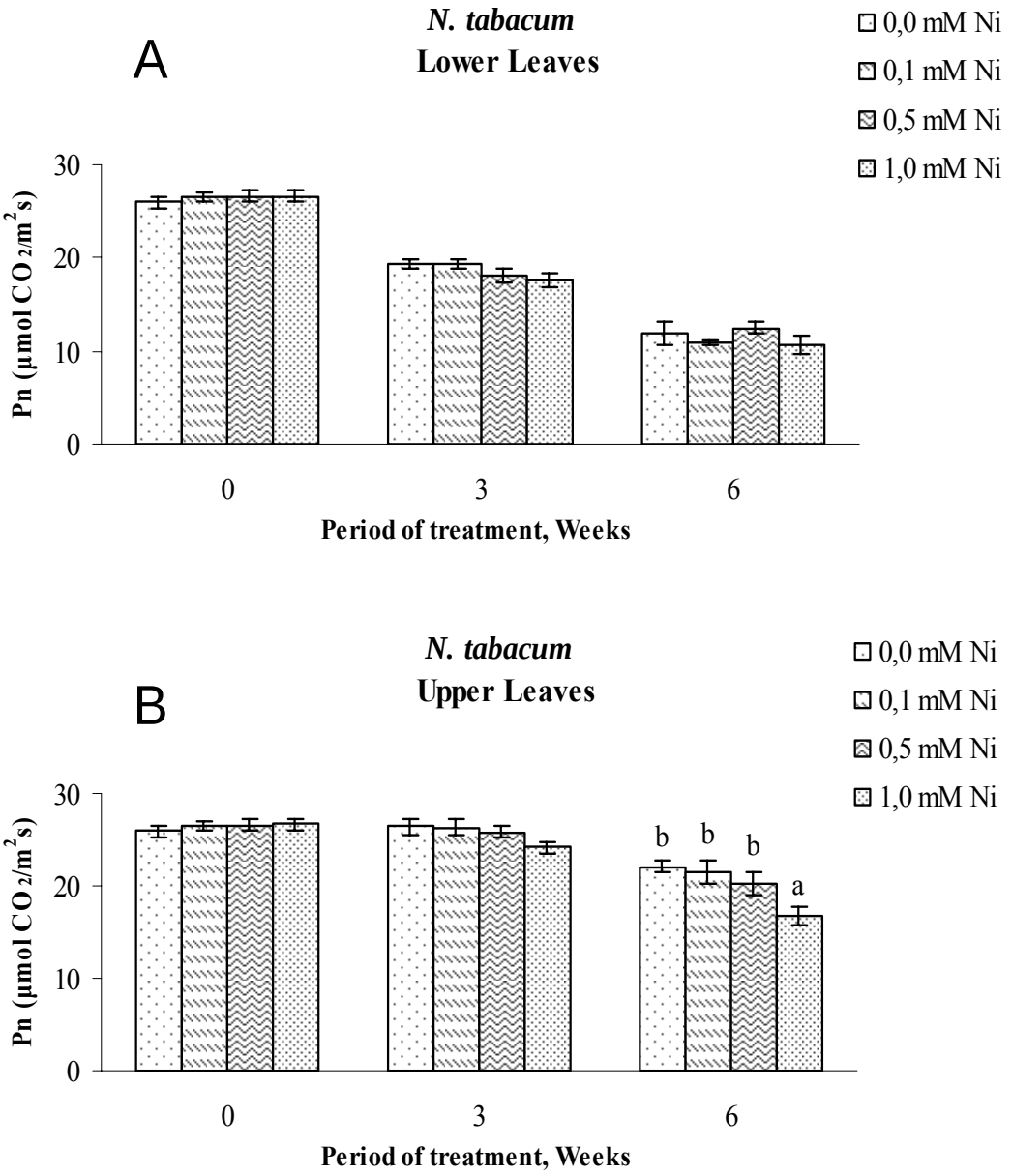

Figure 3.26: The effect of Ni concentration on net photosynthetic rate of: A) lower and B) upper leaves, of *N. tabacum* during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

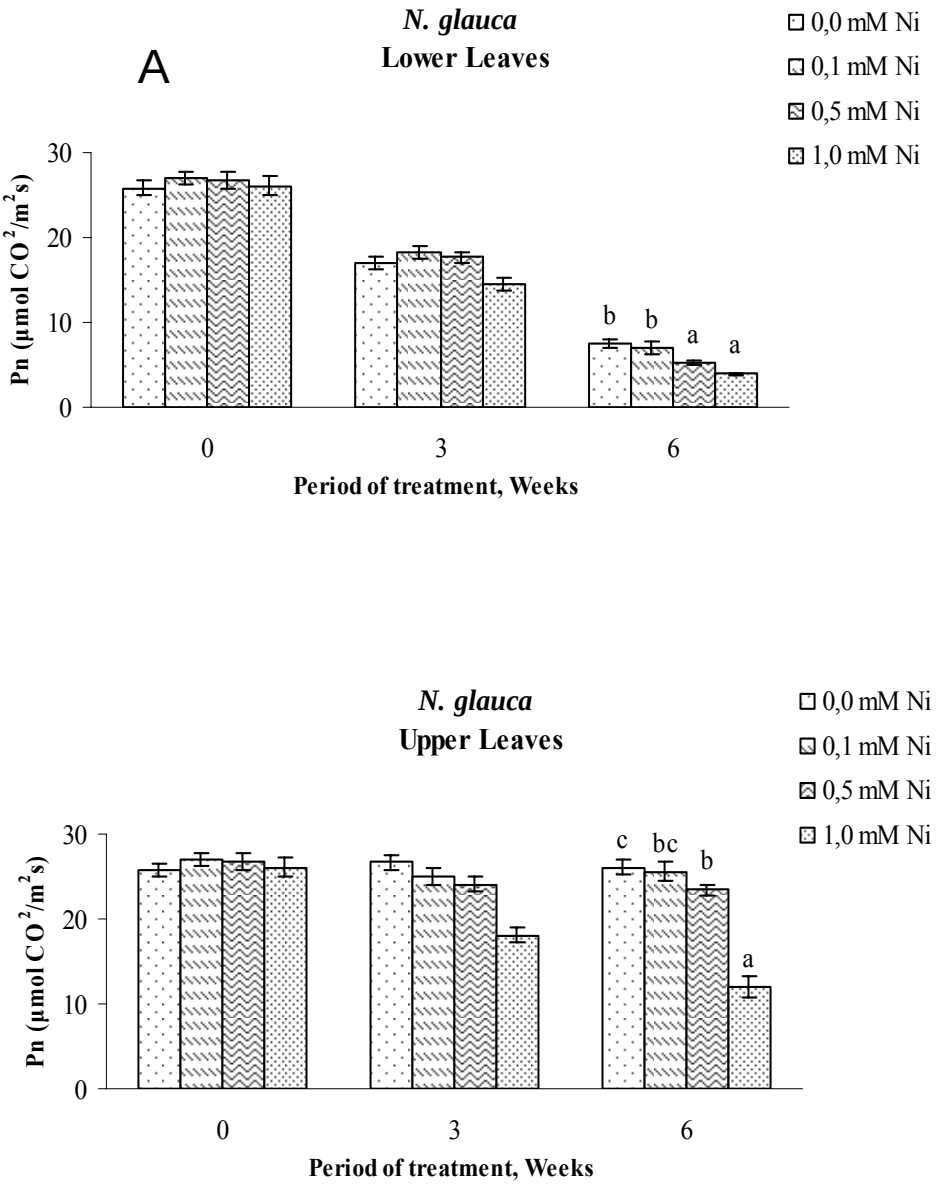

Figure 3.27: The effect of Ni concentration on net photosynthetic rate of: A) lower and B) upper leaves, of *N. glauca* during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

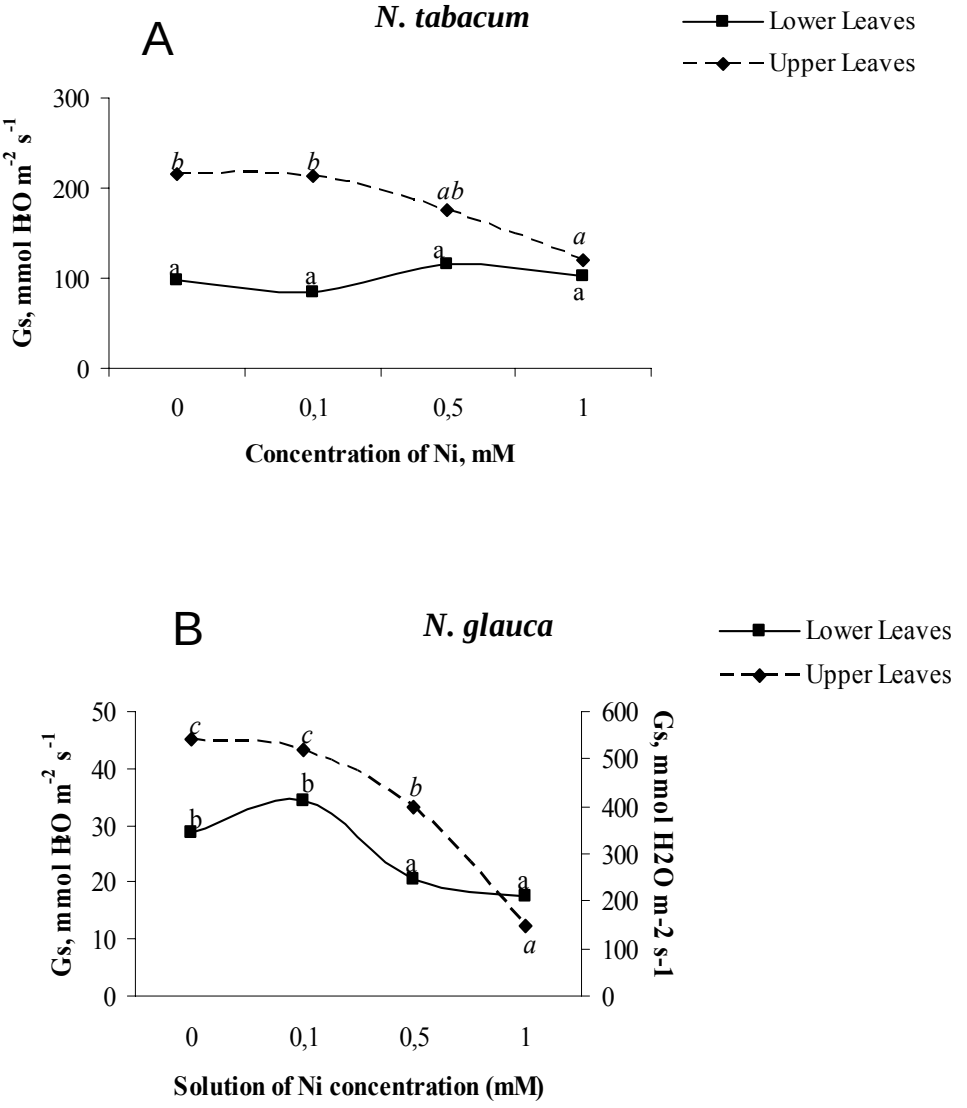

Figure 3.28: The effect of Ni concentration on stomatal conductance of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca* (left axis for lower leaves, right axis for upper leaves). Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Fig. 3.29 presented the Ni-induced effect on transpiration rate in lower and upper leaves of *N. tabacum* and *N. glauca*. Transpiration rate appeared was affected accordingly to stomatal conductance. In lower leaves of *N. tabacum* transpiration rate remained almost unaffected by the presence of Ni concentration, while a slight increase observed at the higher concentration. In upper leaves, transpiration rate appeared a reduced trend at the higher Ni treatments, and reached up to 24%,

compared to controls, however the reduction was not statistically significant. In lower leaves of *N. glauca* transpiration rate remained almost steady in respect to Ni treatments, while a slight decrease observed by about 20% at 1mM Ni, compared to controls, however the reduction was not statistical significant. Additionally, the presence of Ni concentrations affected the transpiration rate in upper leaves of *N. glauca* and led to significant reduction ($P<0.05$) by about 42% at the highest treatment, compared to control plants.

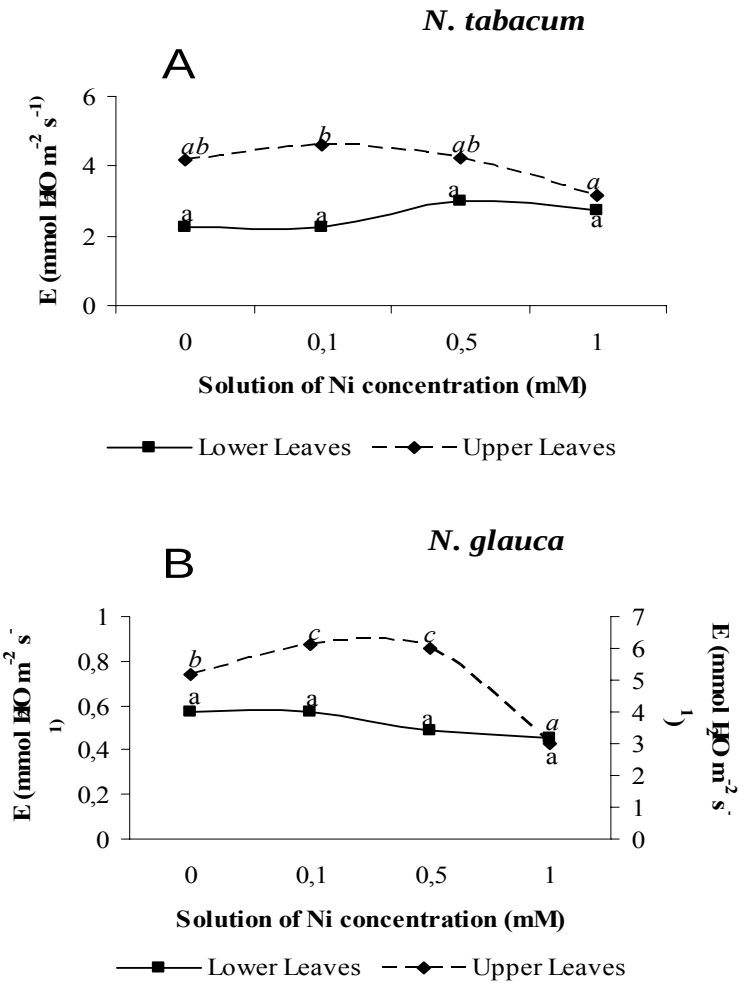

Figure 3.29: The effect of Ni concentration on transpiration rate of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca* (left axis for lower leaves, right axis for upper leaves). Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Exposure of lower leaves of *N. tabacum* to Ni treatments resulted to lower WUE slightly, especially at the higher concentrations (Fig. 3.30). This may ascribed

to the increased trend of transpiration rate at these treatments. Constantly, upper leaves of treated plants appeared an increased trend at the higher Ni concentrations, emphasizing the limited water loss due to transpiration. WUE in lower leaves of *N. glauca* decreased linearly in respect to the increase of Ni concentrations in the growth medium, indicating that photosynthetic rate was more affected than transpiration rate, especially at the highest Ni treatment, compared with intact plants. On the other hand, while photosynthetic and transpiration rate variations induced by Ni treatments did not change WUE in upper leaves of *N. glauca*, however a reduced trend observed.

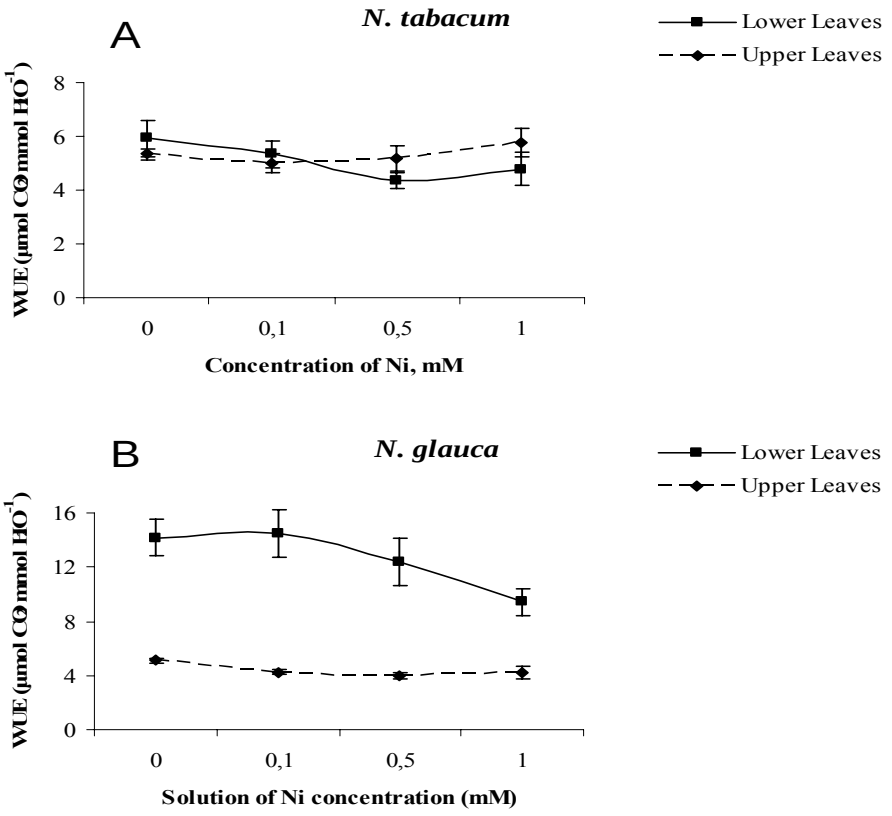

Figure 3.30: The effect of Ni concentration on Water Use Efficiency (WUE) of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE).

The effect of Ni treatments on photosynthetic rate of both plant species has previously presented in Fig. 3.26 and 3.27, however it is interesting to investigate it in parallel with intracellular CO_2 concentration as shown in Fig. 3.31. The intracellular

CO_2 concentration decreased with increasing Ni concentrations in the culture medium, irrespective of cultivar. Particularly, the intracellular CO_2 concentration in upper leaves of *N. tabacum* decreased progressively and reached up to 44% at the highest Ni treatment, compared with control plants. Also, photosynthetic rate appeared similar pattern, with the greatest reduction at the highest Ni concentration. Similarly, the presence of 1mM Ni in the culture medium resulted to significant decline of intracellular CO_2 concentration in upper leaves of *N. glauca* by approximately 23%, compared with intact plants, as well as of photosynthetic rate by 54%.

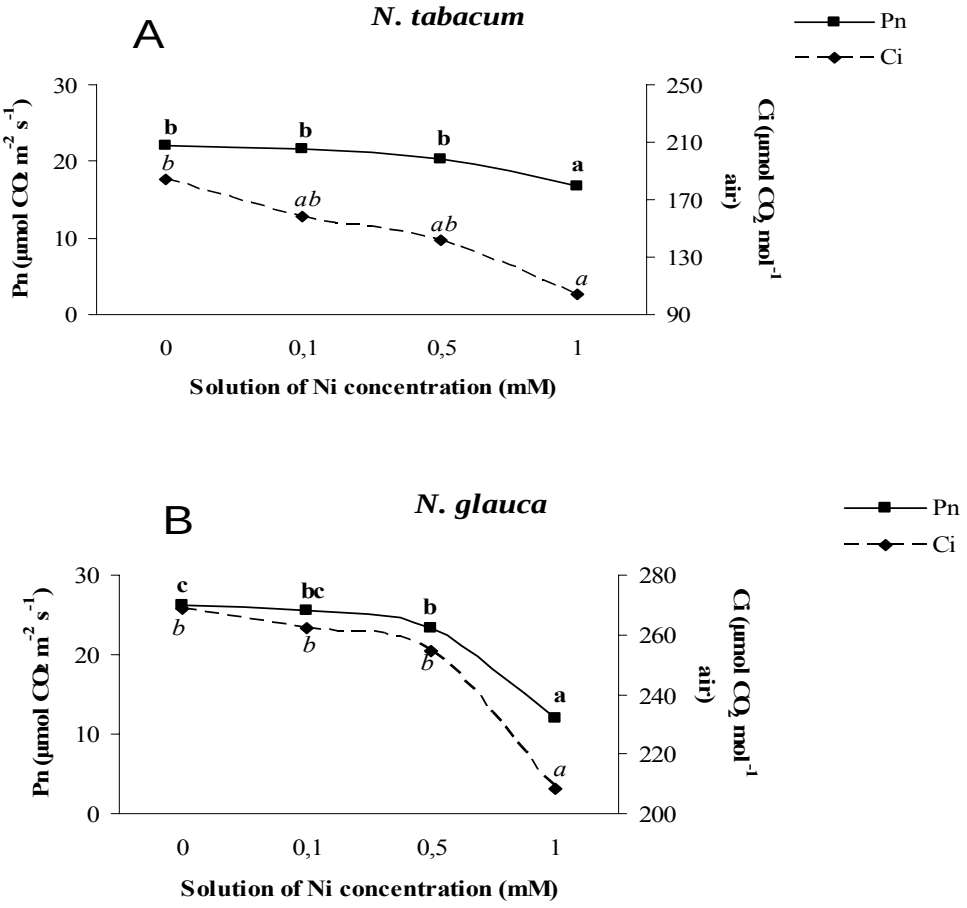

Figure 3.31: The effect of Ni concentration on net photosynthetic rate (left axis) and intracellular CO_2 concentration (right axis) of upper leaves of: A) *N. tabacum* and B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (bold for net photosynthetic rate, italics for intracellular CO_2 concentration, Duncan, P=0.05).

b. Chlorophyll fluorescence parameters - photochemical efficiency of PSII

In parallel to the measurement of the gaseous exchange, the photochemical parameters of photosynthesis were analyzed after 6 weeks in response to Ni excess. As shown in Fig. 3.32, all photochemical parameters in lower and upper leaves of *N. tabacum* seemed differently affected by the addition of Ni concentration in the growth medium. Specifically, F_0 in lower and upper leaves of *N. tabacum* increased slightly as the Ni stress intensity increased, while statistical significant ($P<0.05$) rise exhibited only for measurements concerned lower leaves at 0.1 and 1mM, compared to intact plants. In contrast, the value of Fm of lower and upper leaves decreased in a dose-dependent manner, compared to controls. Similarly with Fm, the value of Fv of the examined tissues was lower in treated plants than controls, however the differences were not statistical significant. Also, the Fv/F_0 ratio, which reflects earlier structural dysfunctions of the PSII (Vaillant et al., 2005), showed a reduced trend under Ni excess in lower leaves of *N. tabacum*, while in upper leaves remained almost unaffected.

Fig. 3.33 presented the Ni induced response of photochemical parameters of photosynthesis in lower and upper leaves of *N. glauca*. In particular, in lower leaves of *N. glauca*, F_0 appeared more affected than in upper, as the highest treatment led to statistical reduction ($P<0.05$) of the parameter. Considerable reduction of Fm in lower leaves was resulted by all Ni treatments, while the parameter in upper leaves declined significantly only by the presence 0.5mM Ni. Fv was affected similarly with Fm in lower and upper leaves of *N. glauca* under Ni stress. No differences resulted at the Fv/F_0 ratio of the examined tissues by Ni exposure.

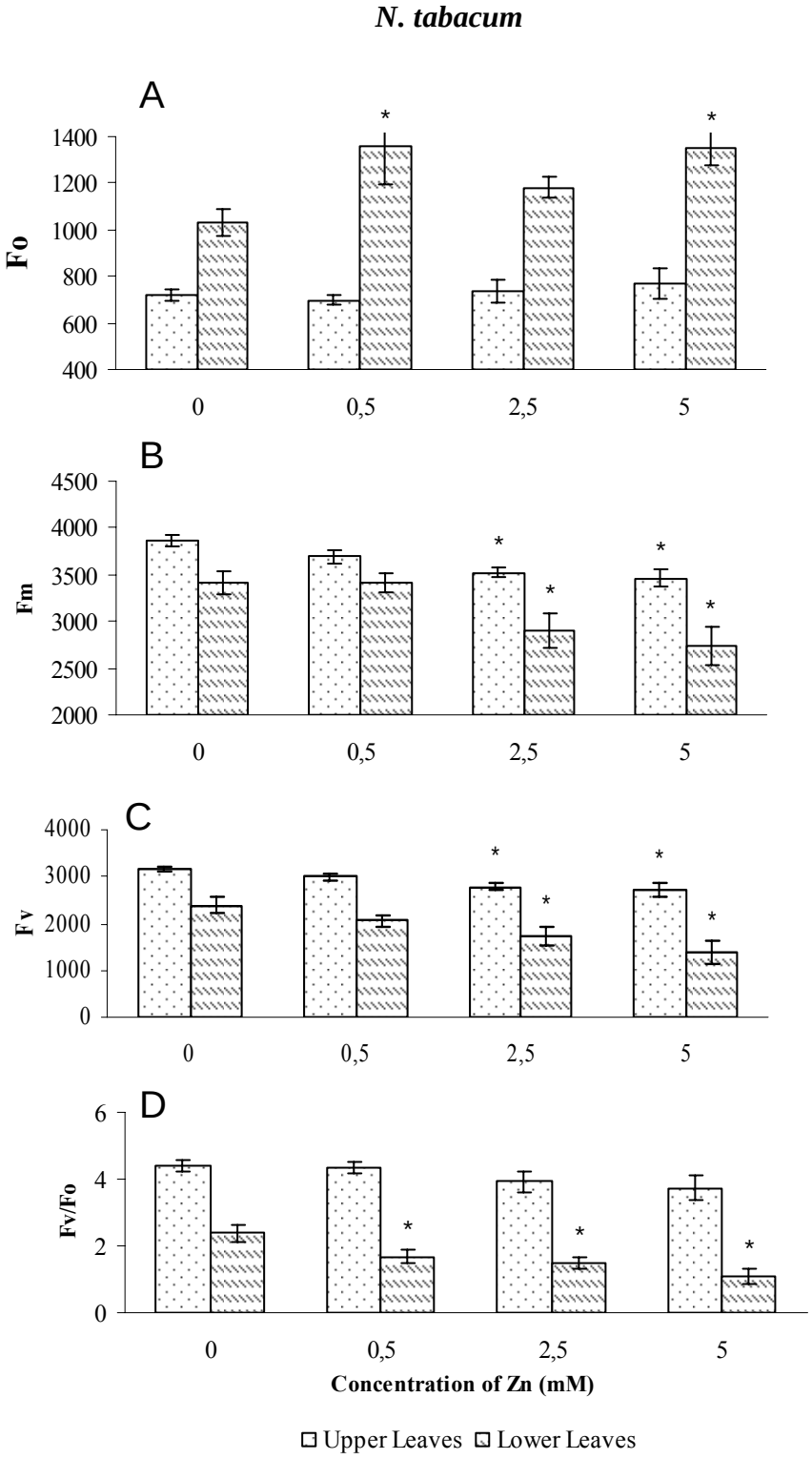

Figure 3.32: The effect of Ni concentration on chlorophyll fluorescence parameters of PSII in dark – adapted leaves: A) initial, F_0 B) maximum, Fm C) variable, Fv D) ratio F_v/F_o , of lower and upper leaves of *N. tabacum.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE). Asterisks indicate significant

differences between treatments and control (only for data obtained at 6th week, LSD, P=0.05)

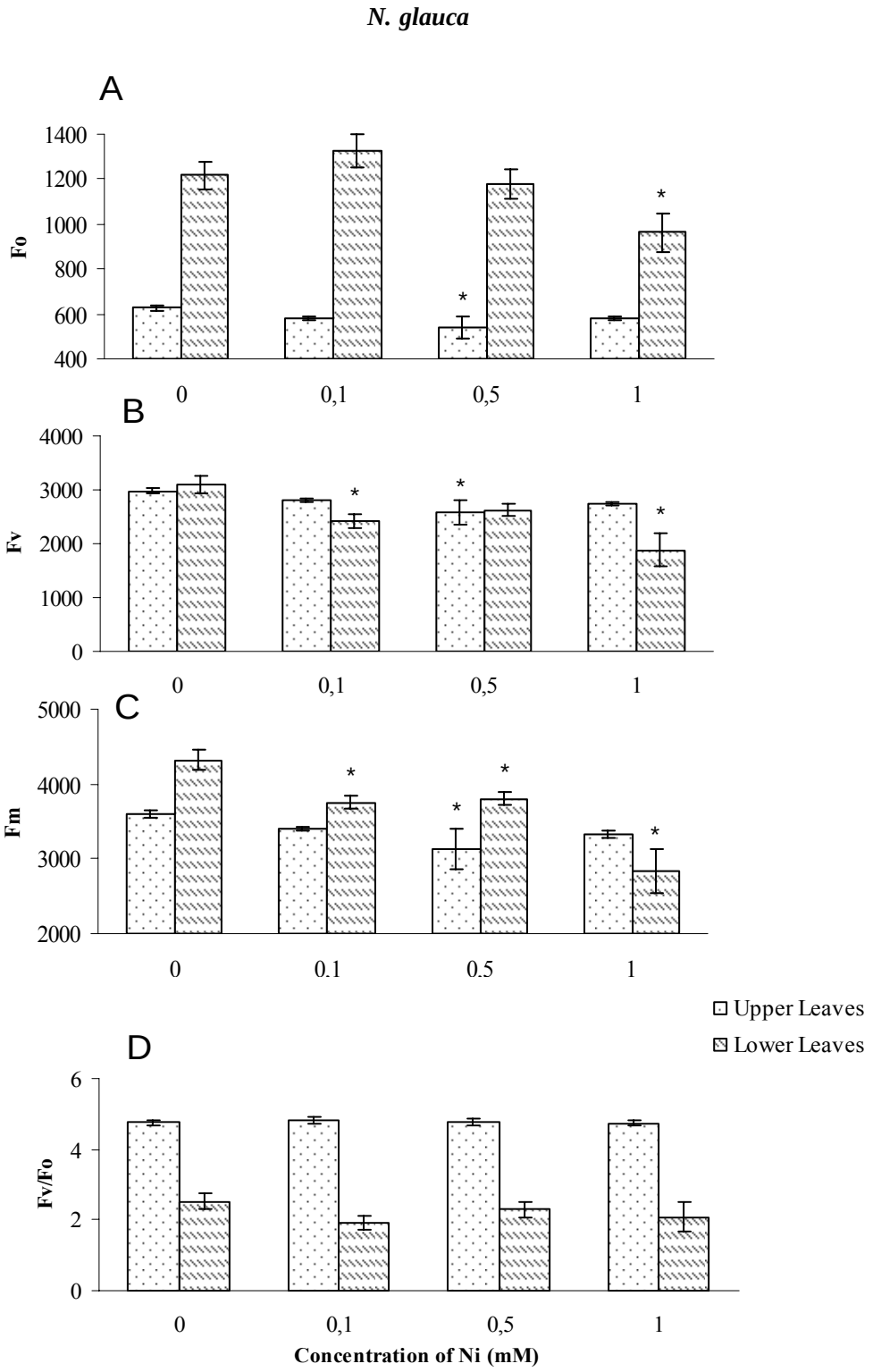

Figure 3.33: The effect of Ni concentration on chlorophull fluorescence parameters of PSII in dark – adapted leaves: A) initial, F_0 B) maximum, Fm C) variable, Fv D) ratio F_v/F_0 , of lower and upper leaves of *N. glauca.* Plants were grown in plastic pots

containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE). Asterisks indicate significant differences between treatments and control (only for data obtained at 6th week, LSD, P=0.05)

The maximal dark-adapted yield of PSII photochemistry, Fv/Fm, of both plant species was followed during the Ni treatment period. In general, for the most measurements obtained during the first and third week, the ratio remained almost unaffected, whatever the Ni concentration applied and the values were close to 0.8, normal value for healthy leaves. The Fv/Fm ratio, which can indicate stress, declined significantly, especially by the 6 week exposure of plants to Ni stress. Specifically, in lower leaves of *N. tabacum* the Fv/Fm ratio decreased approximately 15% and reached up to 25% at 0.1 and 1mM Ni respectively, compared to control plants, while in upper leaves remained almost steady, regardless the Ni concentration applied (Fig. 3.34). The decrease was observed in Fv/Fm ratio was in parallel with the increase of the basic fluorescence (F_0).

In lower leaves of *N. glauca* the Fv/Fm ratio reduced progressively as the stress intensity increased (Fig. 3.35). Particularly, the addition of 0.1, 0.5 and 1mM Ni in the culture, led to significant decline by 12%, 6% and 14%, respectively, compared with untreated plants. The reduction of the recommended ratio was in parallel with the fact that the decrease in Fm preceded that of F_0. On the other hand, the Fv/Fm ratio in upper leaves of treated plants did not differ with controls. In lower leaves of both plant species the values of the ratio were much lower than 0.8, not only of treated plants, but of controls as well.

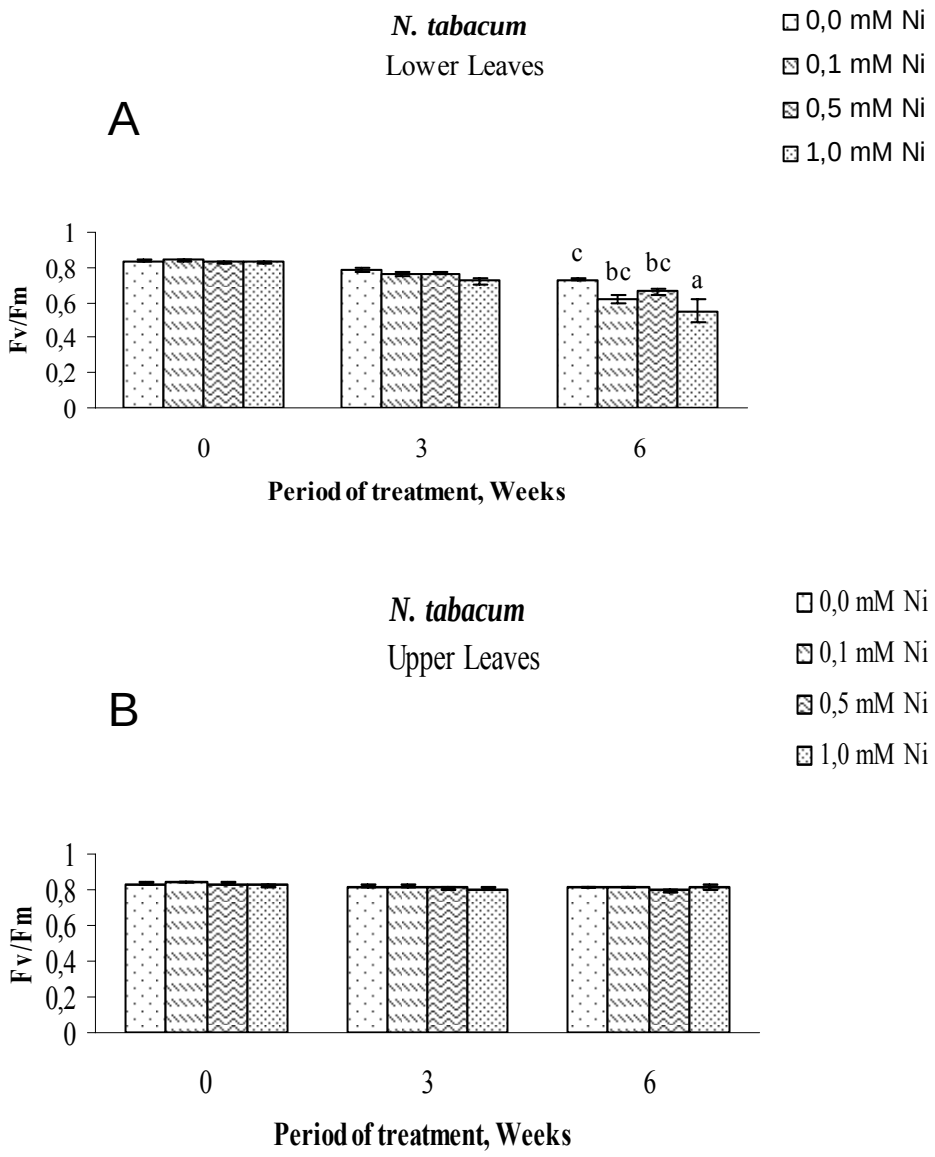

Figure 3.34: The effect of Ni concentration on the photochemical efficiency of PSII (F_v/F_m) in dark – adapted leaves of *N. tabacum*: A) lower leaves B) upper leaves, during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

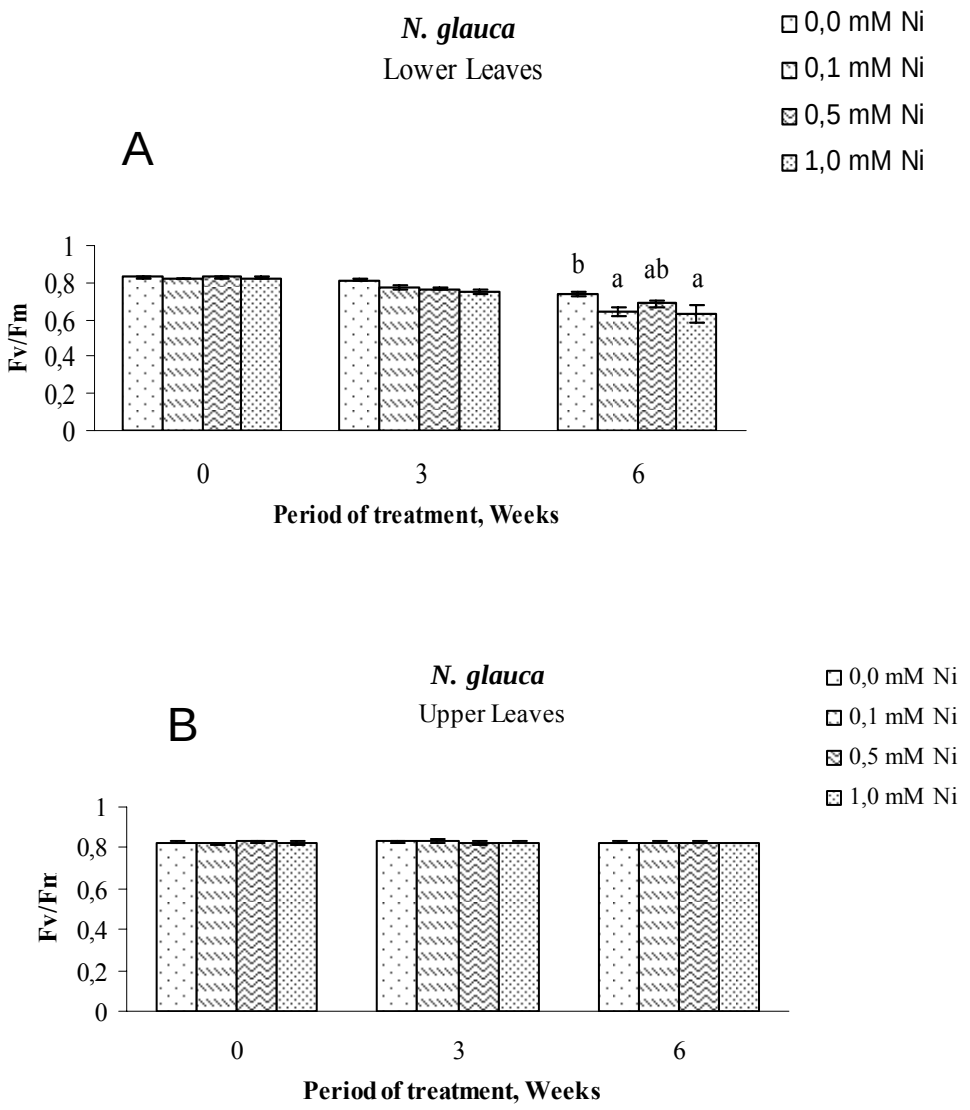

Figure 3.35: The effect of Ni concentration on the photochemical efficiency of PSII (F_v/F_m) in dark – adapted leaves of *N. glauca*: A) lower leaves B) upper leaves, during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6^{th} week, Duncan, P=0.05).

3.2.4 Effect of Ni on Parameters related with Nitrogen Metabolism

a. Proline

Fig. 3.36 illustrates the effect of Ni concentrations on proline accumulation of lower and upper leaves of *N. tabacum* and *N. glauca*. The amount of proline was positively affected by Ni concentrations in leaves of both plant species, while significant differences ($P<0.05$) exhibited over the range Ni treatments.

b. Total soluble protein content

Total soluble protein content was determined in response to Ni stress (Table 3.11) Similarly, in both plant species the protein level was lower than in upper leaves. Moreover, while in lower leaves the protein content remained almost steady, over the range of Ni treatments, differential response observed in upper leaves of the two plant species. Interestingly, in upper leaves of *N. tabacum* a considerable increased observed in the protein content at the highest Ni treatment, while concerned *N. glauca* remained unaffected.

c. Specific activities and immunoblotanalysis of the ammonium assimilating enzymes

Results presented below in Tables and Plates.

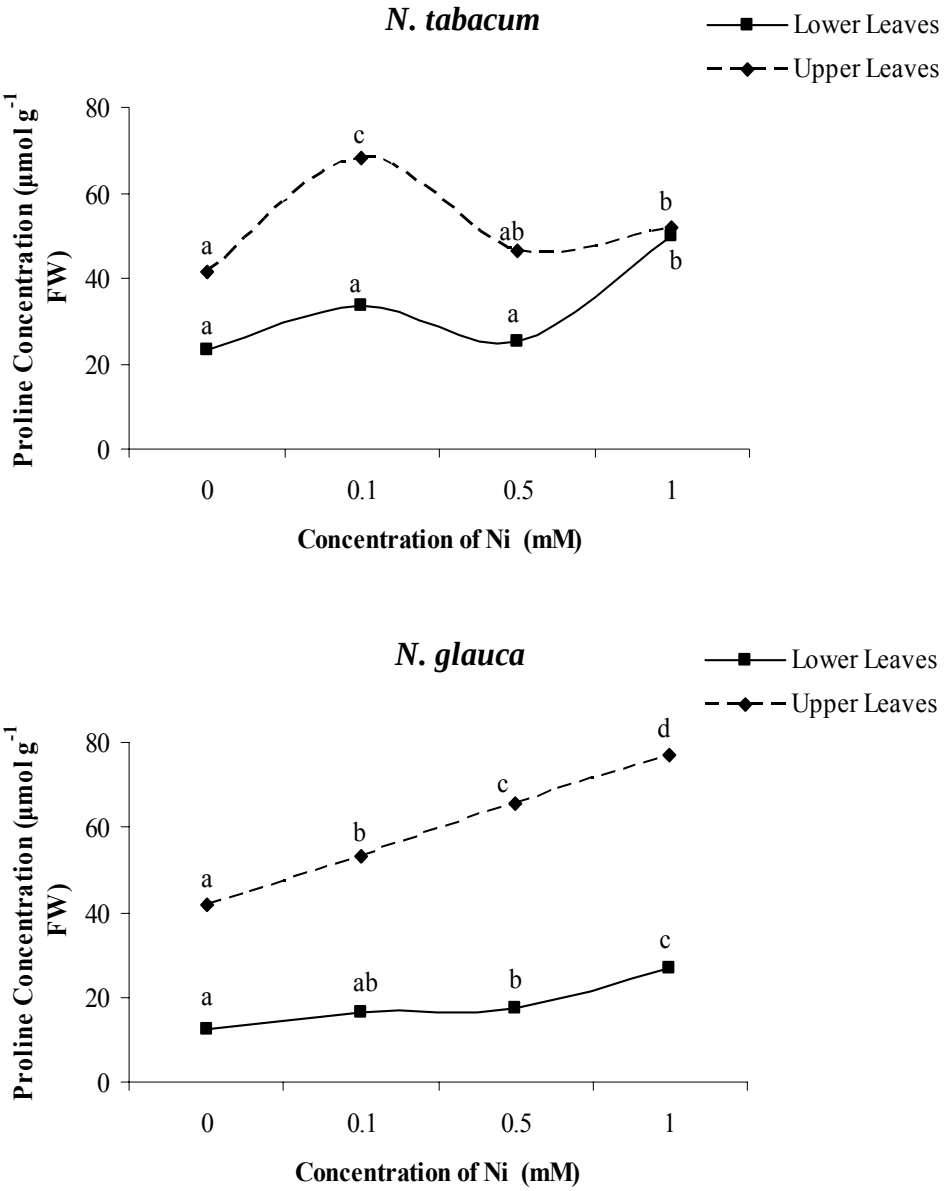

Figure 3.36: The effect of Ni concentration on proline concentration of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Table 3.11: The effect of Ni concentration on protein content of lower and upper leaves of *N. tabacum and N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni^{2+} for 6 weeks. Values are means ± standard error (SE).

Treatment Ni, mM	*N. tabacum*		*N. glauca*	
	Lower leaves	Upper leaves	Lower leaves	Upper leaves
0.0	5,81 ±	10,52 ±	7,80 ± 0, 21	11,27 ± 0,25
1.0	6,07 ±	11,29 ±	7,59 ± 0,39	10,90 ± 0,33
0.5	6,65 ±	11,34 ±	5,09 ± 0,34	11,56 ± 0,69
1.0	7,27 ±	13,95 ±	7,74 ± 0,11	11,76 ± 0,49

Table 3.12: The effect of Ni concentration on specific activity of the enzymes of nitrogen assimilation of lower and upper leaves of *N. tabacum.* Values concerned controls and plants treated with 1 mM Ni. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Plant tissues	Treatments Ni, mM	Nitrogen Assimilation Enzymes (Enzyme activity: nmol mg^{-1} protein min^{-1})		
		GS	Fd-GOGAT	GDH
Lower leaves	**0.0**	420.33 ± 20.6	89.18 ± 4.7	398.92 ± 14.3
	1.0	310.98 ± 15.3	84.8 ± 6.4	403.05 ± 22.7
Upper leaves	**0.0**	777.80 ± 43.2	119.28 ± 7.2	165.63 ± 11.8
	1.0	677.03 ± 23.9	76.01 ± 5.9	202.23 ± 12.3

Table 3.13: The effect of Ni concentration on specific activity of the enzymes of nitrogen assimilation of lower and upper leaves of *N. glauca.* Values concerned controls and plants treated with 1 mM Ni. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Plant tissues	Treatments Ni, mM	Nitrogen Assimilation Enzymes (Enzyme activity: nmol mg^{-1} protein min^{-1})		
		GS	Fd-GOGAT	GDH
Lower leaves	**0.0**	333.11 ± 19.2	52.15 ± 3.8	474.70 ± 24.0
	1.0	311.34 ± 20.2	42.24 ± 2.6	582.97 ± 23.3
Upper leaves	**0.0**	619.63 ± 31.6	86.58 ± 8.8	136.43 ± 13.9
	1.0	500.82 ± 28.1	56.17 ± 4.6	181.57 ± 26.6

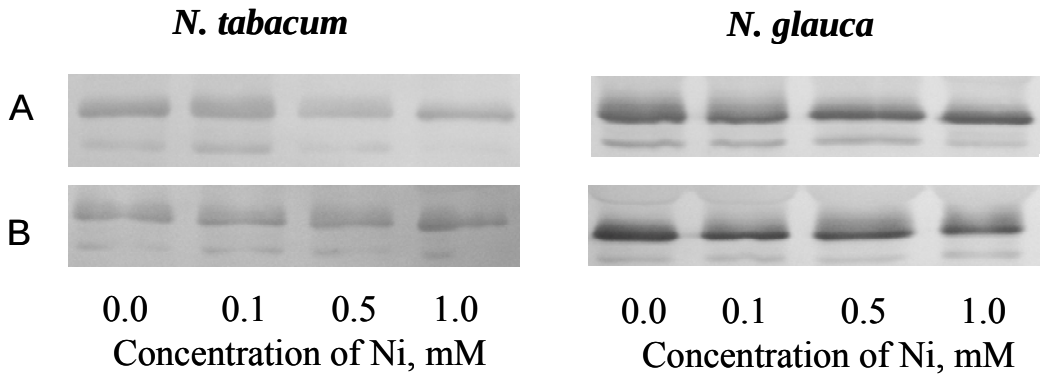

Plate 3.9: Western blot analysis of GS in A) lower leaves B) upper leaves of Ni treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni for 6 weeks. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti-GS serum, as described in section "Materials and Methods"

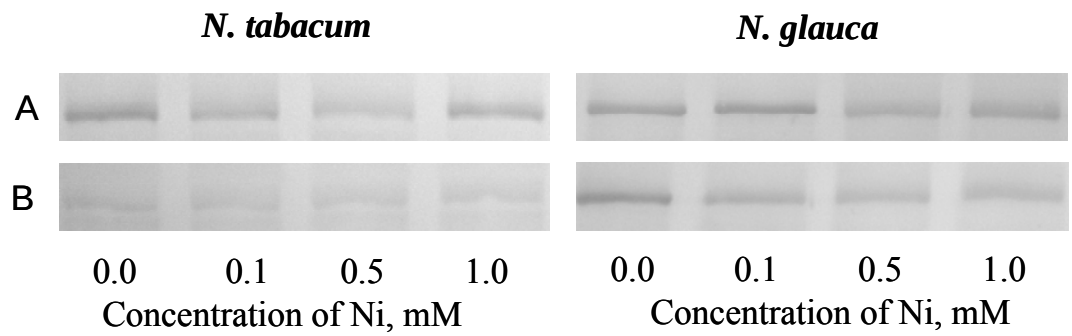

Plate 3.10: Western blot analysis of Fd-GOGAT in A) lower leaves B) upper leaves of Ni treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni for 6 weeks. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti- GOGAT serum, as described in section "Materials and Methods"

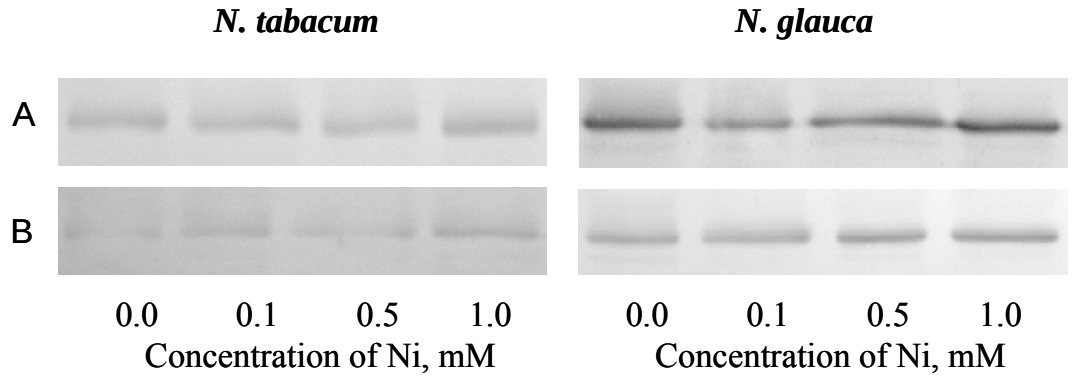

Plate 3.11: Western blot analysis of GDH in A) lower leaves B) upper leaves of Ni treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants grown in plastic pots containing homogenized soil in the presence of different concentrations of Ni for 6 weeks. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti-GDH serum, as described in section "Materials and Methods"

3.3 Effect of Cadmium on Morphological, Physiological and Biochemical Parameters of *N. tabacum* and *N. glauca*

3.3.1 Cadmium In Plants Tissues

Cadmium is vey toxic for plants and animals and its toxicity considered to be 2 -20 times greater than other heavy metals. It is readily taken up by the root system of plants, where in many species detected the highest accumulation. In this study, Cd accumulation was investigated in different plant tissues of *N. tabacum* and *N. glauca*, in response of three concentrations of Cd in the culture medium. As expected, no traces of Cd^{2+} were detected in control plants, whatever the considered species.

The accumulation of Cd in root and the aboveground tissues of *N. tabacum*, was increased in a dose-dependent manner (Fig.3.37, Table 3.14). Particularly, in lower leaves, Cd uptake increased significantly and reached up to 836 and 1163 ppm at 1 and 2 mM Cd, respectively. Cd removal by lower and upper shoots significantly increased at all treatments, compare to control plants, and reached up to 572 and 464 ppm, respectively, at the highest concentration. Significant increases occurred, also, in root where the concentration detected at 1 mM was 1076 ppm and 1149 ppm at 2 mM Cd.

Similarly, all supplied concentrations of Cd in the culture medium, resulted to statistically significant increases in plant tissues of *N. glauca* (Fig. 3.38, Table 3.15). The inadvertent uptake of Cd led to raise the endogenous concentration in lower and upper leaves and reached up to 538 and 336 ppm, respectively at the highest treatment. While, at the intermediate concentrations the accumulation of Cd was also high. The translocation of the metal in lower and upper shoots, led to statistically significant increases. Root showed the maximum accumulation at all treatments comparing the tissues, where detected 266, 598 and 1269 ppm, at 0.2, 1 and 2 mM Cd, respectively.

In general, Cd increased in all investigated plant tissues, with increasing concentration in the culture medium. Both plant species accumulated more in root, especially at the highest Cd treatment, fact considered as a first barrier to Cd toxicity,

in order to restrict its transport to the shoot. Comparing the two plant species, *N. tabacum* accumulated in lower leaves by about 2.7-, 2.5- and 2- fold more, at 0.2, 1 and 2 mM Cd, respectively, than *N. glauca*. However, all the concentrations detected were above the permissible limits (5-30 ppm).

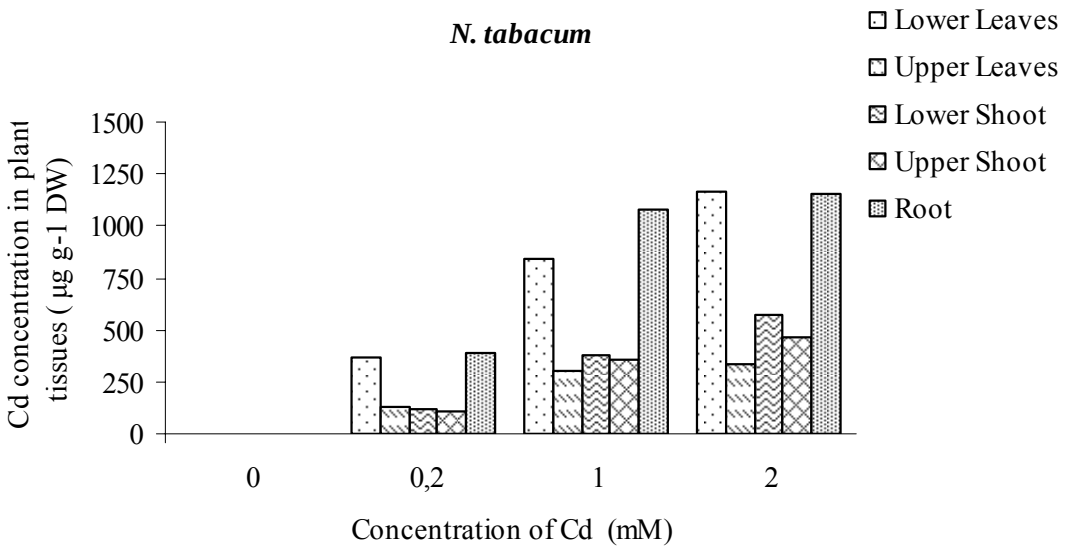

Figure 3.37: Accumulation of Cd in plant tissues of *N. tabacum*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Statistical significant differences of plant ttissues between control and treatments are presented in Table 3.14.

Table 3.14. Statistical significant differences of the endogenous Cd concentration in plant tissues of *N. tabacum*. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Cd, mM	**Plant Tissue**				
	Lower leaves	Upper leaves	Lower shoot	Upper Shoot	Root
0.0	a	a	a	a	a
0.2	a	b	b	b	a
1.0	b	c	c	c	b
2.0	b	c	d	d	b

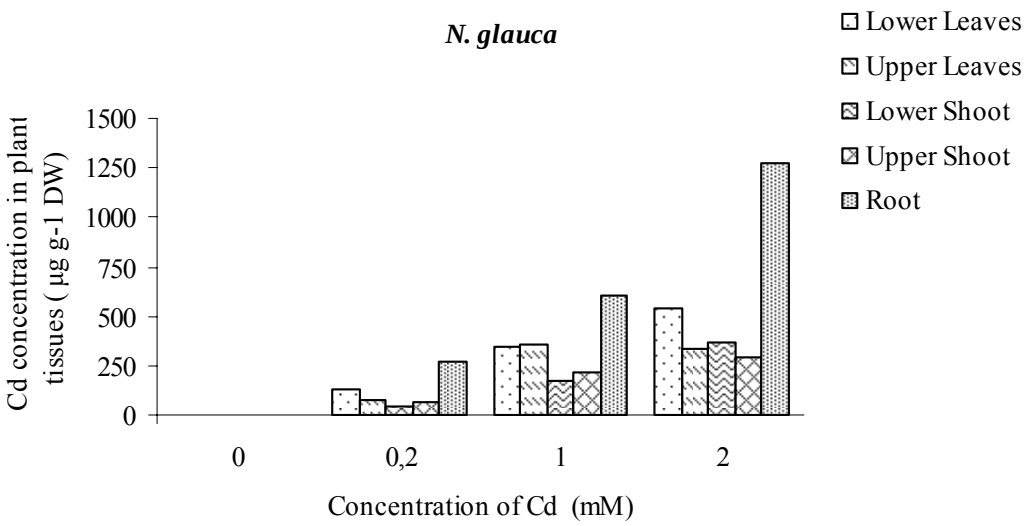

Figure 3.38: Accumulation of Cd in plant tissues of *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Statistical significant differences of plant ttissues between control and treatments are presented in Table 3.15.

Table 3.15: Statistical significant differences of the endogenous Cd concentration (ppm) in plant tissues of *N. glauca.* Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Cd, mM	**Plant Tissue**				
	Lower leaves	Upper leaves	Lower shoot	Upper Shoot	Root
0.0	a	a	a	a	a
0.2	b	a	a	b	b
1.0	c	b	b	c	c
2.0	d	b	c	d	d

3.3.2 Effect of Cd on Morphological Characteristics and Growth of *N. tabacum* and *N. glauca*

The morphological characteristics of *N. tabacum* and *N. glauca* were followed during the experiment period and the symptoms were observed (Plates 3.12 and 3.13). The determinations occurred, have provided data showing non-negligible effects of Cd treatments on both plant species. Plants of all treatments showed visual toxicity symptoms. The gradation of the Cd concentration in *N. tabacum* was related with chlorosis and wilt. The higher Cd treatments caused severe chlorosis at lower leaves of *N. tabacum*, while upper leaves began to turn chlorotic. Generally, plants showed wilted appearance. *N. glauca* appeared more affected by the prolonged exposure to Cd treatments. The higher Cd concentration caused interveinal chlorosis in lower and upper leaves, development of dark brown necrotic spots, dissolution of stem and leaf detachment, symptoms related not only with the physiological senescence. Plant showed stunted growth, loss of turgor and appearance. The extent and magnitude of symptoms and damage of the plant parts increased with increasing treatment concentration of Cd.

Plate 3.12. Plants of *N. tabacum* (left) and *N. glauca* (right) grown in greenhouse conditions in presence of 0.0, 0.2, 1.0, 5.0 mM Cd^{2+}.

Plate 3.13: Visual symptoms of Cd treatment on *N. tabacum* (left) and *N. glauca* (right) leaves. Plants were grown in plastic pots in greenhouse.

Figure 3.39 presented the height of both plant species during the experiment, under Cd stress. Interestingly, 0.2 mM Cd exhibited stimulatory effect on height of both plant species, in 4 weeks treated plants, compared to controls Statistically significant differences exhibited only after 6 weeks exposure to Cd treatments of both plant species. Specifically, the supply of 1 and 2mM Cd resulted to reduction of height of *N. tabacum* by 22% and 25% respectively, compared to control plants. Similarly affected was *N. glauca*, where 1 and 2mM Cd caused height inhibition approximately 14% and 23% respectively, compared to intact plants.

Another growth parameter was followed during Cd treatment period was the number of leaves of *N. tabacum* and *N. glauca*. (Fig.3.40). Leaves number of *N. tabacum* showed reduced trend by the presence of Ni in the culture medium, however no significantly differences were exhibited. *N. glauca* appeared more affected, since considerable reduction was observed after 3 weeks with Ni treatments.

Fresh weights of leaves, shoots and roots of both plant species were determined under Cd stress, which progressively decreased with increasing concentration of applied Cd. As presented in Fig. 3.41, significant reduction ($P<0.05$) obtained for all examined tissues at 1 and 2mM Cd for both plant species. Specifically, fresh weight of leaves of *N. tabacum* was reduced by 20% at the

recommended concentrations, compared to controls. The inhibitory effects became more pronounced for shoot and root weight, which decreased 48% and 58% at 1mM Cd, 53% and 69% at 2 mM Cd respectively, compared to untreated plants. Similar response was observed at fresh weight of tested plant tissues of *N. glauca*. A reduced trend was observed at fresh weight of leaves of *N. glauca* by 20% at the highest Cd treatment; however no significant difference was exhibited, compared with untreated plants. In contrast, the inhibitory effects increased on shoot and root weight with increasing Cd doses in the culture medium. Specifically, shoot weight began to reduce by 45% and 56% at 1 and 2mM Cd, respectively, compared with controls, while root weight decline reached up to 46% and 56% in respect to the recommended treatments.

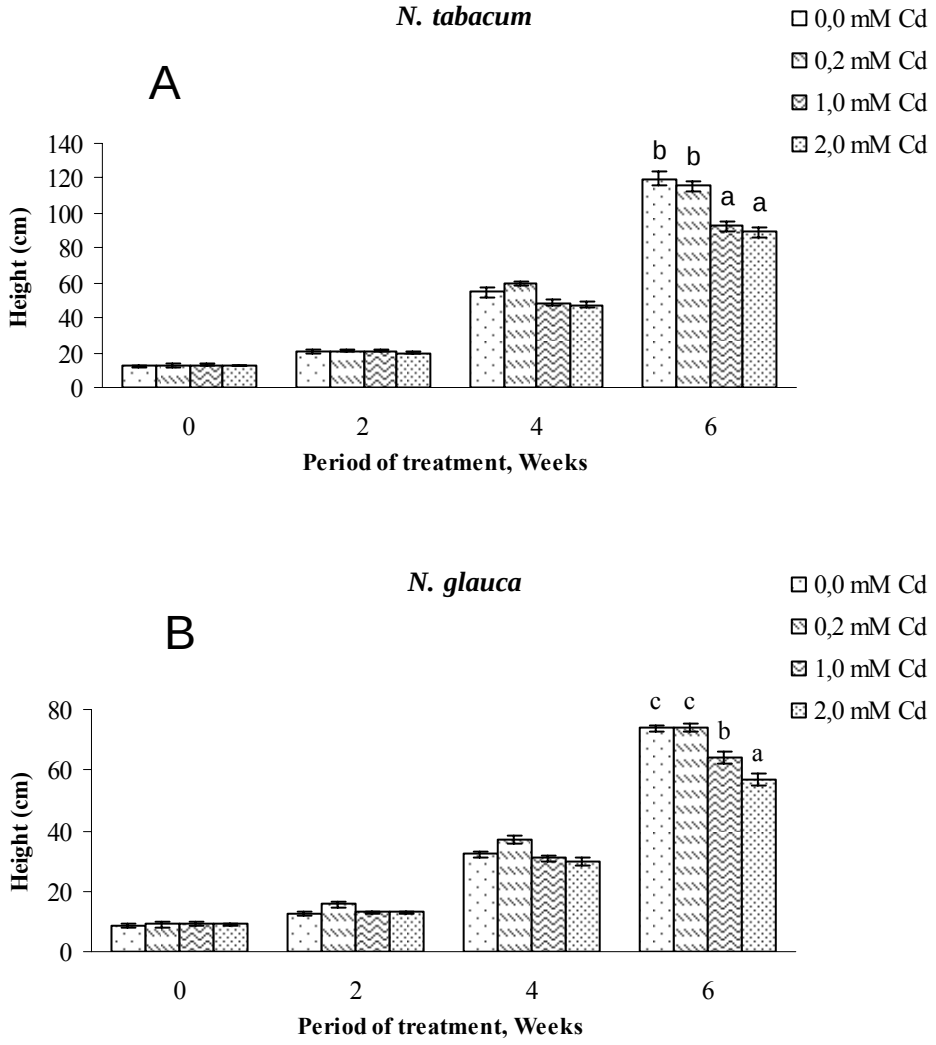

Figure 3.39: The effect of Cd concentration on plant height during the treatment period A) *N. tabacum*, B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one

another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

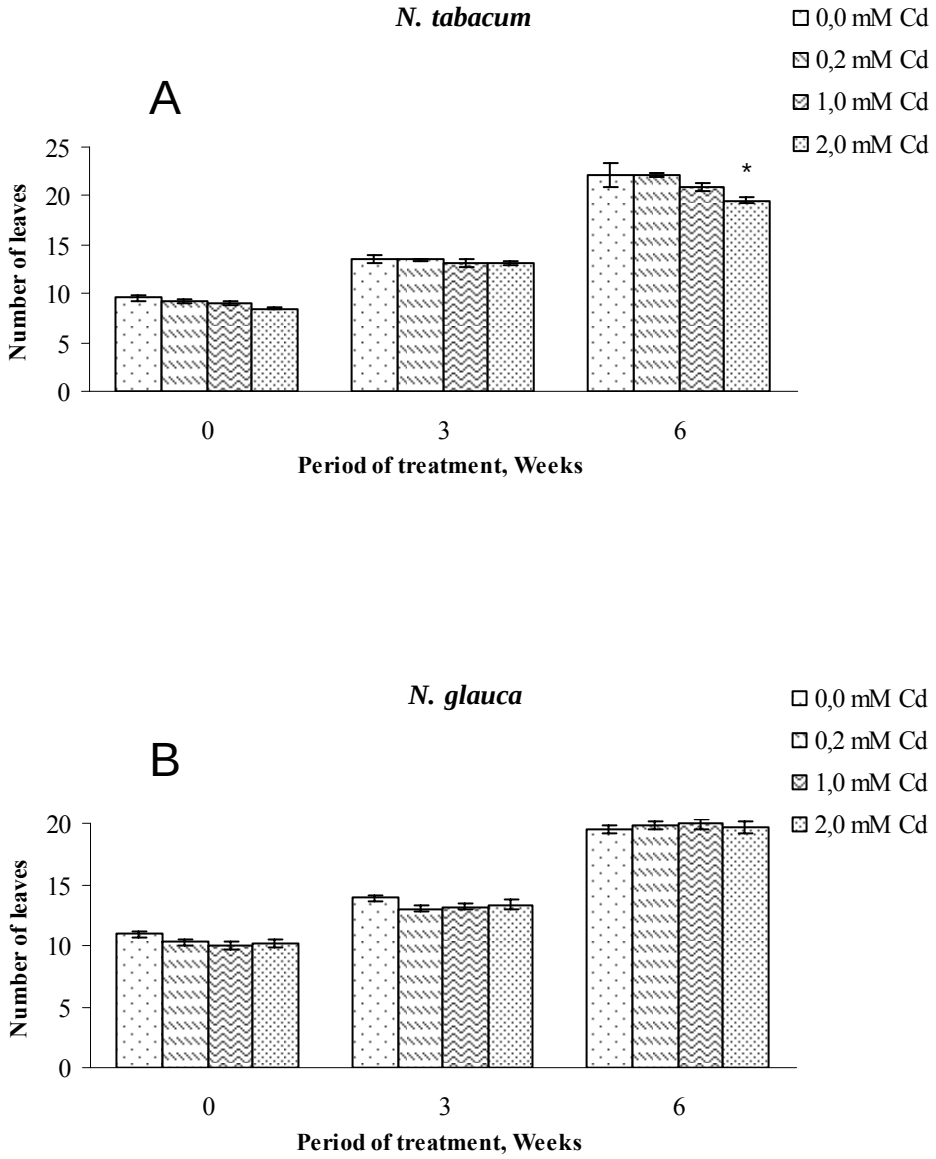

Figure 3.40: The effect of Cd concentration on the number of leaves of A) *N. tabacum*, B) *N. glauca*, during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE). Asterisks indicate significant differences between treatments and control (only for data obtained at 6th week, LSD, P=0.05).

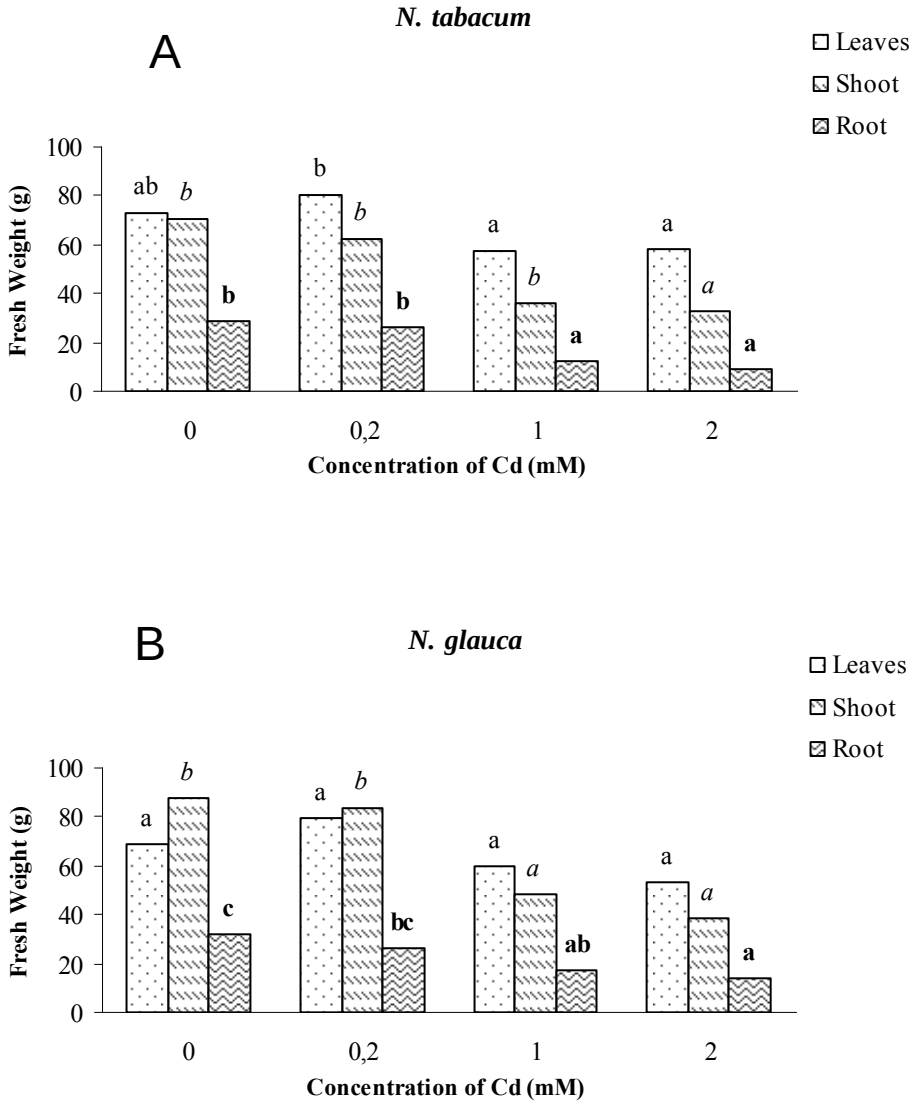

Figure 3.41: The effect of Cd concentration on plant fresh weight of A) *N. tabacum,* B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (regular for leaves, italics for shoot, bold for root, Duncan, P=0.05),

3.3.3 Effect of Cd on Photosynthetic Parameters of *N. tabacum* and *N. glauca*

a. Chlorophyll pigments

Cadmium primarily affected the photosynthetic pigments before photosynthetic function (Prasad, 1995). The contents of chlorophylls of lower and upper leaves of *N. tabacum* and *N. glauca* were investigated in dependence on the

Cd^{2+} concentrations and presented in Figures below. Cd treatments affected negatively and the photosynthetic pigments in lower and upper leaves of *N. tabacum* (Figure 3.42A). Particularly, in lower leaves, chlorophyll a and b gradually decreased with increasing Cd concentration in the culture medium, while the highest treatment led to significant decline ($P<0.05$) by about 45% and 44%, respectively, compared to untreated plants. In upper leaves, significant decrease exhibited at all Cd treatments, with the greatest concentration resulted to reduce chlorophyll a and b by about 33% and 44%, respectively, compared to untreated plants. Exposure of *N. glauca* to Cd treatments did not induce significant variations in photosynthetic pigments of lower and upper leaves (Figure 3.42B). However, in lower leaves, chlorophyll a and b was reduced approximately 23% and 15% by the highest concentration of Cd, compared to untreated plants. In addition, a slight increase of pigments was noticed in plants exposed to 0.2 mM Cd, compared to control and the higher treatments.

Figure 3.43 summarizes the effect of Cd treatments on total chlorophyll content (mg/g FW and SPAD values) of lower and upper leaves of *N. tabacum* and *N. glauca*. Obviously, Cd concentrations progressively decreased chlorophyll content in lower leaves of *N. tabacum* and reached up to 44% at the greatest treatment, compared to controls. In upper leaves, the chlorophyll content strongly dropped at all Cd treatments ($P<0.05$). On the other hand, chlorophyll content remained almost steady across treatments in the tested tissues of *N. glauca*. However, significant difference exhibited between 0.2 mM and 1 mM, where the fist increased slightly the chlorophyll content and the latter decreased it by 20%, compared to intact plants. The increased trend observed in chlorophylls, in leaves of *N. glauca* at 0.2 mM Cd, may be regarded as an adaptive mechanism to the toxic conditions. Total chlorophyll content showed similar response as SPAD values for both plant species.

Table 3.16 presents the change of the chlorophyll ratio a to b under Cd stress in lower and upper leaves of both plant species. A depleted trend showed the chlorophyll ratio in lower leaves of both plant species, indicating that chlorophyll a was more affected. Contrastly, in upper leaves the ratio was differentially affected in the tested species. Chlorophyll a and b variations induced by Cd treatments led to

increase the chlorophyll ratio in upper leaves of *N. tabacum*, while in *N. glauca* remained almost unaffected.

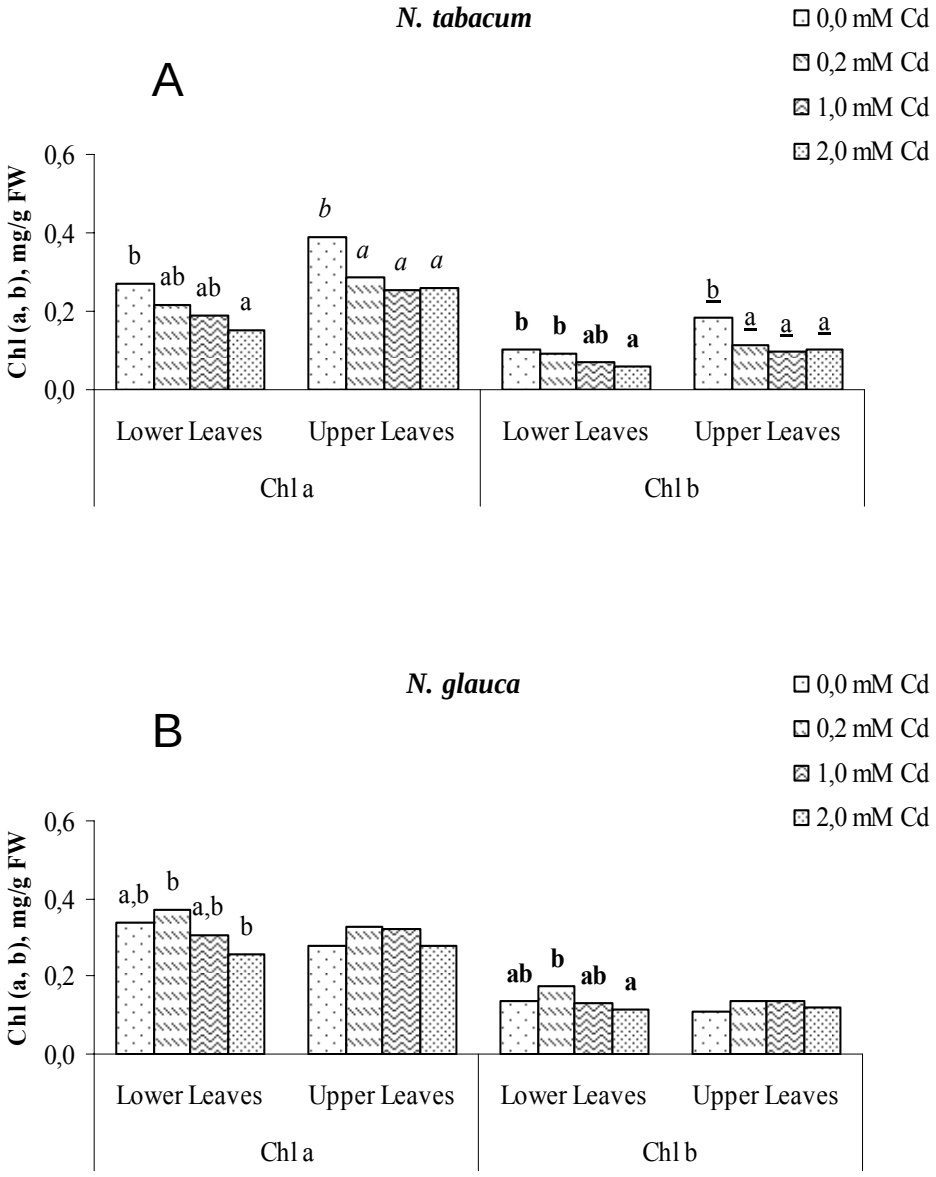

Figure 3.42: The effect of Cd concentration on photosynthetic pigments, chl a and chl b of lower and upper leaves of A) *N. tabacum*, B) *N. glauca* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (regural for chl a of lower leaves, italics for chl a of upper leaves, bold for chl b of lower leaves, underlined for chl b of upper leaves, Duncan, P=0.05). Columns without letters on do not differ significantly.

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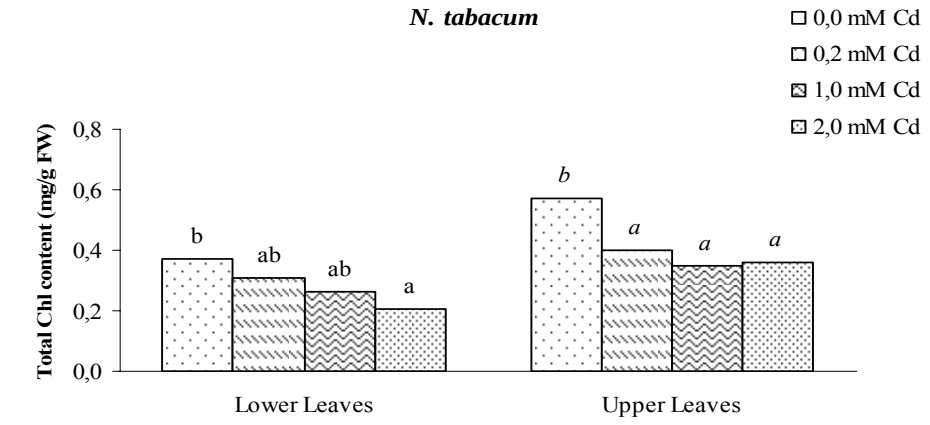
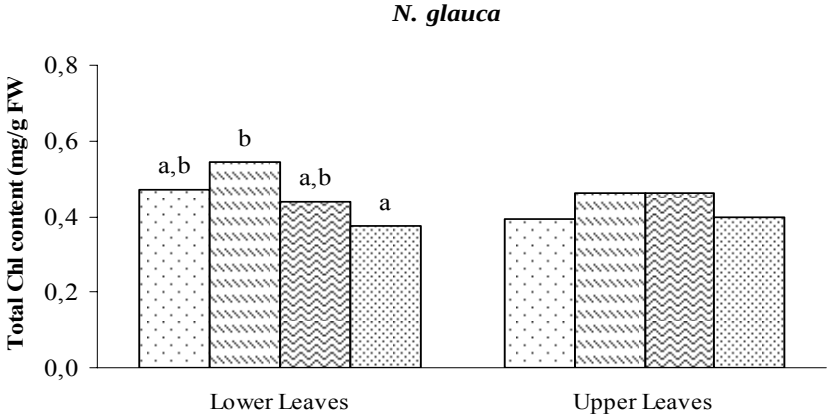
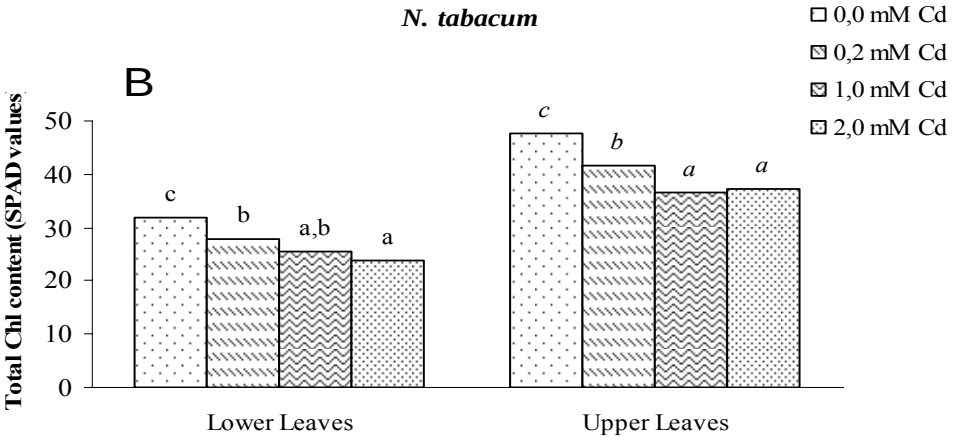

N. tabacum
0,0 mM Cd
0,2 mM Cd
1,0 mM Cd
2,0 mM Cd
Total Chl content (mg/g FW)
0,8
0,6
0,4
0,2
0,0
b
ab
ab
a
b
a
a
a
Lower Leaves
Upper Leaves


N. glauca
Total Chl content (mg/g FW
0,8
0,6
0,4
0,2
0,0
a,b
b
a,b
a
Lower Leaves
Upper Leaves


N. tabacum
0,0 mM Cd
0,2 mM Cd
1,0 mM Cd
2,0 mM Cd
B
Total Chl content (SPAD values)
50
40
30
20
10
0
c
b
a,b
a
c
b
a
a
Lower Leaves
Upper Leaves

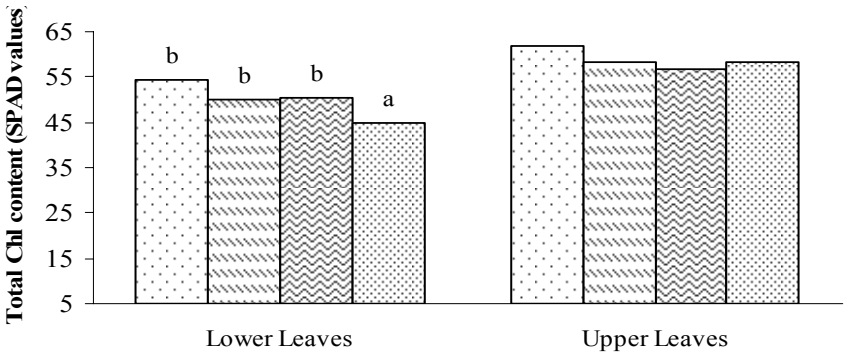

N. glauca
Total Chl content (SPAD values)
65
55
45
35
25
15
5
b
b
b
a
Lower Leaves
Upper Leaves

Figure 3.43: The effect of Cd concentration on total chlorophyll contents A) mg/g FW and B) SPAD values of lower and upper leaves of N. *tabacum* and *N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (regular for lower leaves, italics for upper leaves, Duncan, P=0.05). Columns without letters on, do not differ significantly.

Table 3.16. The effect of Cd concentration on the chlorophyll *a/b* ratio of lower and upper leaves of *N. tabacum* and *N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Cd, mM	*N. tabacum*		*N. glauca*	
	Lower leaves	Upper leaves	Lower leaves	Upper leaves
0.0	2.70 a	2.24 a	2.52 b	2.56 a
0.2	2.40 a	2.56 b	2.10 a	2.51 a
1.0	2.58 a	2.74 b	2.40 ab	2.49 a
2.0	2.60 a	2.61 b	2.23 ab	2.42 a

b. Photosynthetic Rate andGas Exchange Parameters

As presented in Fig. 3.44, Pn in lower leaves of *N. tabacum* was progressively decreased in respect to the exposure period of the plants at Cd treatments. Statistical significant reduction observed comparing the mean values of measurements obtained at the three periods of treatments. Pn in lower leaves of the 3 week treated plants reduced approximately 25% at control plants, 30% and 33% at the intermediate concentrations of Cd and at the highest treatment, respectively. The deleterious effect became more pronounced the 6^{th} week of treatments, where the reduction of Pn was about 53% at 0mM and reached up to 64% at 2mM Cd.

Also, comparing the mean values obtained the last period, Pn decreased approximately 20%, compared to untreated plants, however the reduction was not statistically significant. Similarly, a reduced trend observed in Pn of upper leaves of

N. tabacum, with the exposure period of the plants to Cd treatments. The reduction of Pn observed at the 3rd and 6th week reached up to 25% and 40% by the presence of 2mM Cd in the culture medium. Statistical significant decline ($P<0.05$), observed also, comparing the mean values of Pn, obtained the last week of Cd treatment. Particularly, the presence of 0.2, 1 and 2 mM Cd in the culture medium led to significant decline of Pn by 14%, 10% and 28%, compared to untreated plants.

Pn in lower leaves of *N. glauca* decreased linearly, with the exposure period of the plants at Cd treatments, as well as in plants grown in control medium (Fig. 3.45). Pn of the 3 weeks treated plants was lowered by 34% at 0 mM, 31% at the intermediate treatments of Cd and reached up to 40% at 2 mM Cd. The deleterious effect on Pn of lower leaves became more pronounced at the 6th week of Cd treatments. Particularly, Pn was reduced approximately 71% at control plants and reached up to 78% at the highest Cd treatment compared to the first period of treatment.

Comparing the mean values of the harvesting period, in plants treated with 2 mM Cd, Pn decreased by 25%, compared with controls. In upper leaves of *N. glauca* Pn was not affected as much as in lower leaves, however showed significant decrease with increasing Cd concentrations in the culture medium. As shown in Figure ,Pn in upper leaves of plants grown in control medium and at the presence of 0.2 mM Cd, remained almost unaffected irrespective of the period of treatment. In contrast, Pn in upper leaves of plants grown at the presence of 1 and 2 mM Cd decreased linearly in respect of the period of treatment. Statistical significant decline of Pn values observed at the 6 week treated plants, where Pn was reduced approximately 18% and 30% at 1 and 2 mM Cd, respectively, compared to control plants.

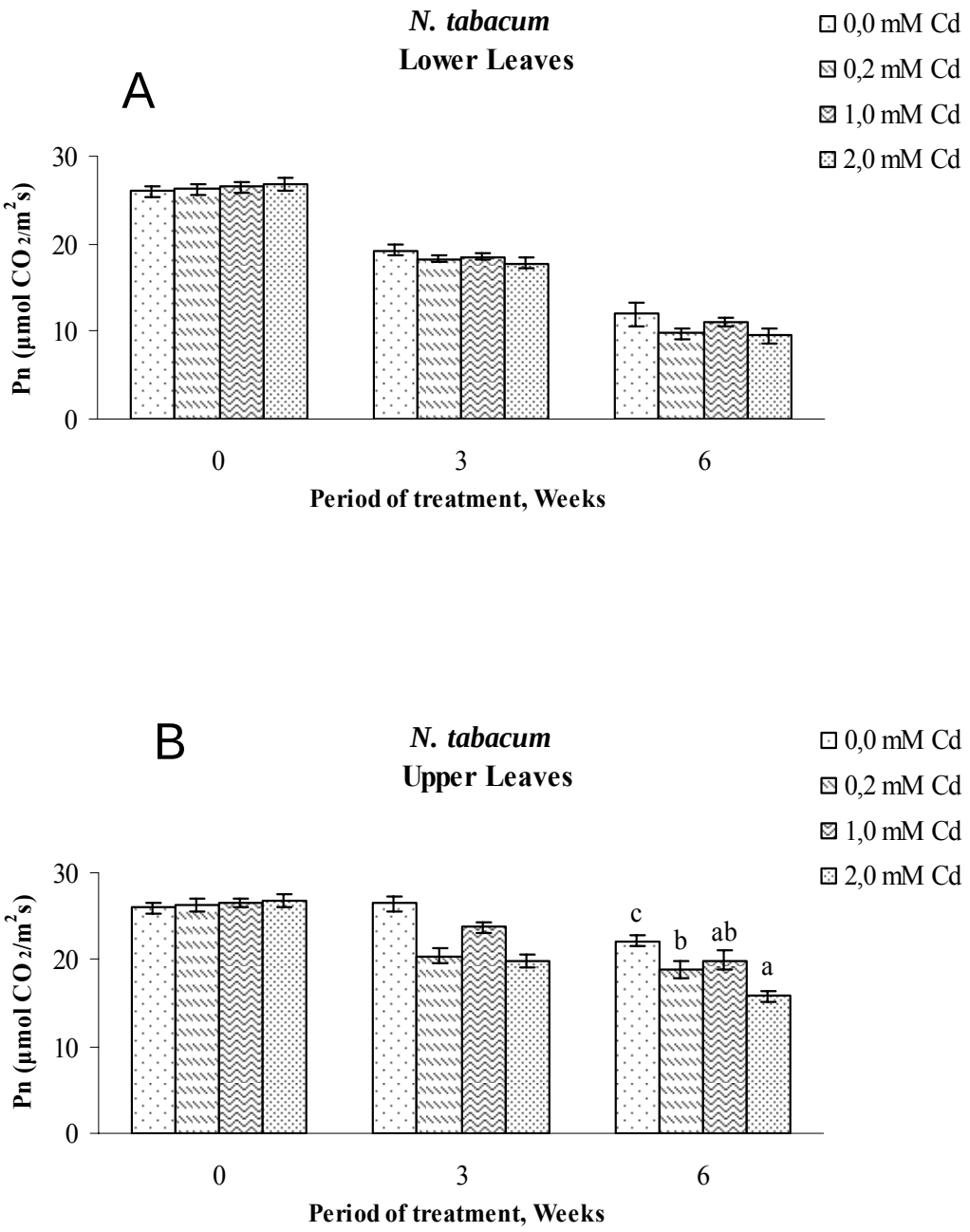


Figure 3.44: The effect of Cd concentration on net photosynthetic rate of: A) lower and B) upper leaves, of *N. tabacum* during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

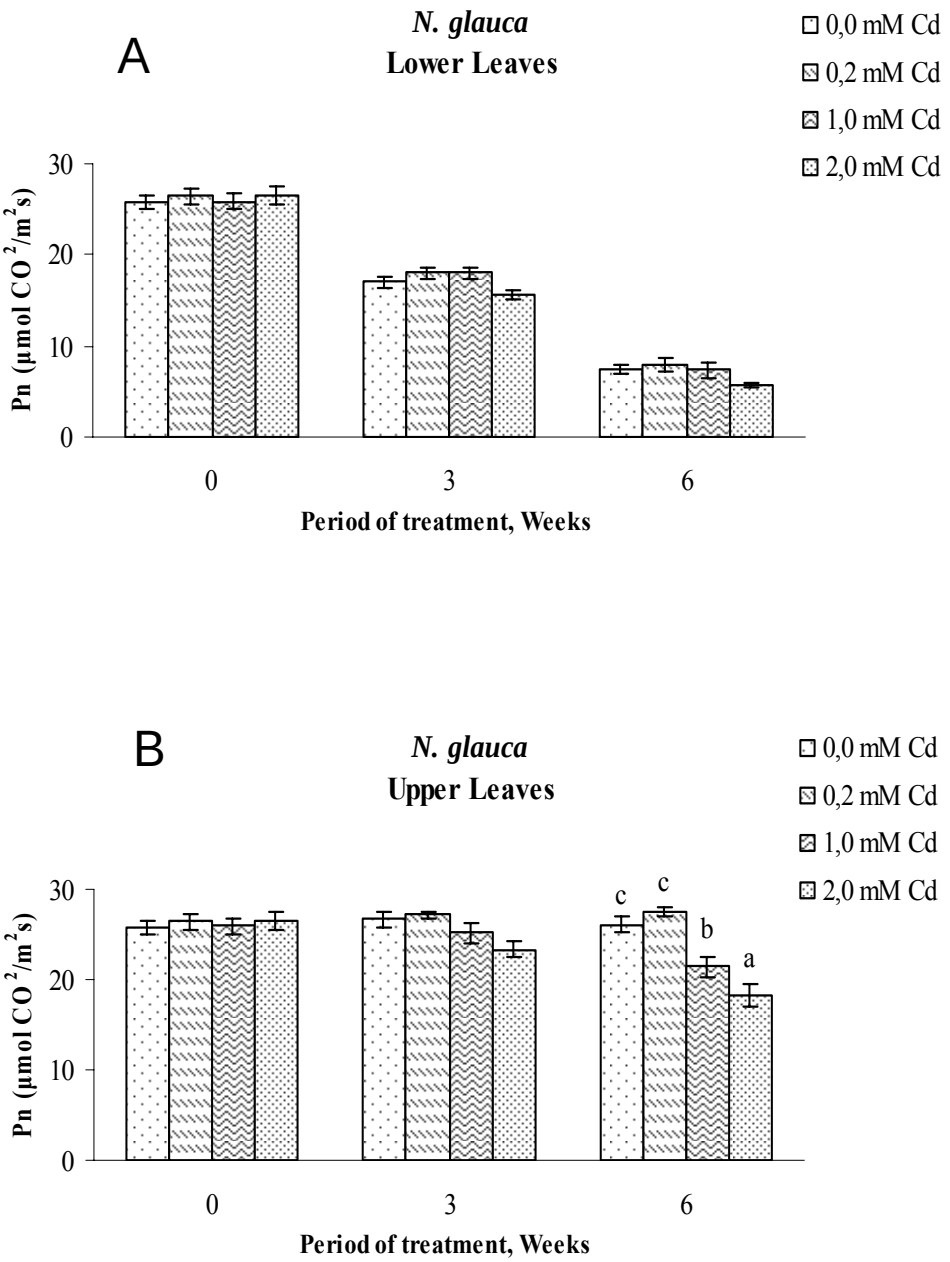


Figure 3.45: The effect of Cd concentration on net photosynthetic rate of: A) lower and B) upper leaves, of *N. glauca* during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

As shown in Figure 3.46, stomatal conductance remained fairly steady at all Cd treatments in lower leaves of *N. tabacum*, compared to control plants. On the other

hand, upper leaves appeared more affected, especially at the highest Cd treatment. In particular, 0.2 and 2 mM Cd resulted to significant reduction ($P<0.05$) of stomatal conductance by 30% and 50%, respectively, compared to controls. The stomatal conductance was significantly affected by the presence of Cd concentrations in the growth medium, in lower and upper leaves of *N. glauca* as well. Particularly, the depression observed ($P<0.05$) reached up to 60% and 50% at the highest Cd treatment, in lower and upper leaves respectively, compared to the intact plants. In spite of a different evolution according to species, an excess in Cd led to the closing of stomates in both plant species (R).

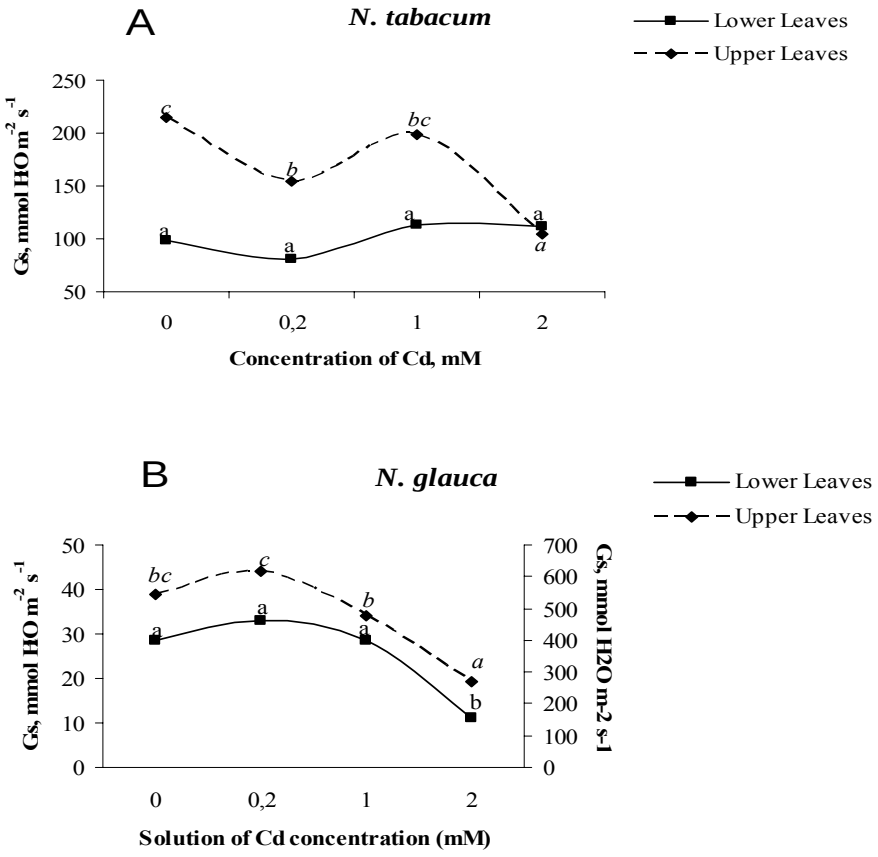


Figure 3.46: The effect of Cd concentration on stomatal conductance of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca* (left axis for lower leaves, right axis for upper leaves). Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Accordingly to stomatal conductance transpiration rate of *N. tabacum* and *N. glauca* was similarly affected in respect to Cd treatments and presented in Fig. 3.47.

In lower leaves of *N. tabacum* transpiration rate remained almost unaffected by the presence of Cd in the culture medium, with a slight increase at the highest treatment. In contrast, in upper leaves, it exhibited significant different and decreased by 37%, at the highest Cd concentration, compared to control plants. Comparatively, transpiration rate in lower and upper leaves of *N. glauca*, was similarly affected by the presence of Cd in the growth medium. Particularly, an increasing trend firstly observed at the intermediate Cd concentrations and it decreased then at the highest treatment.

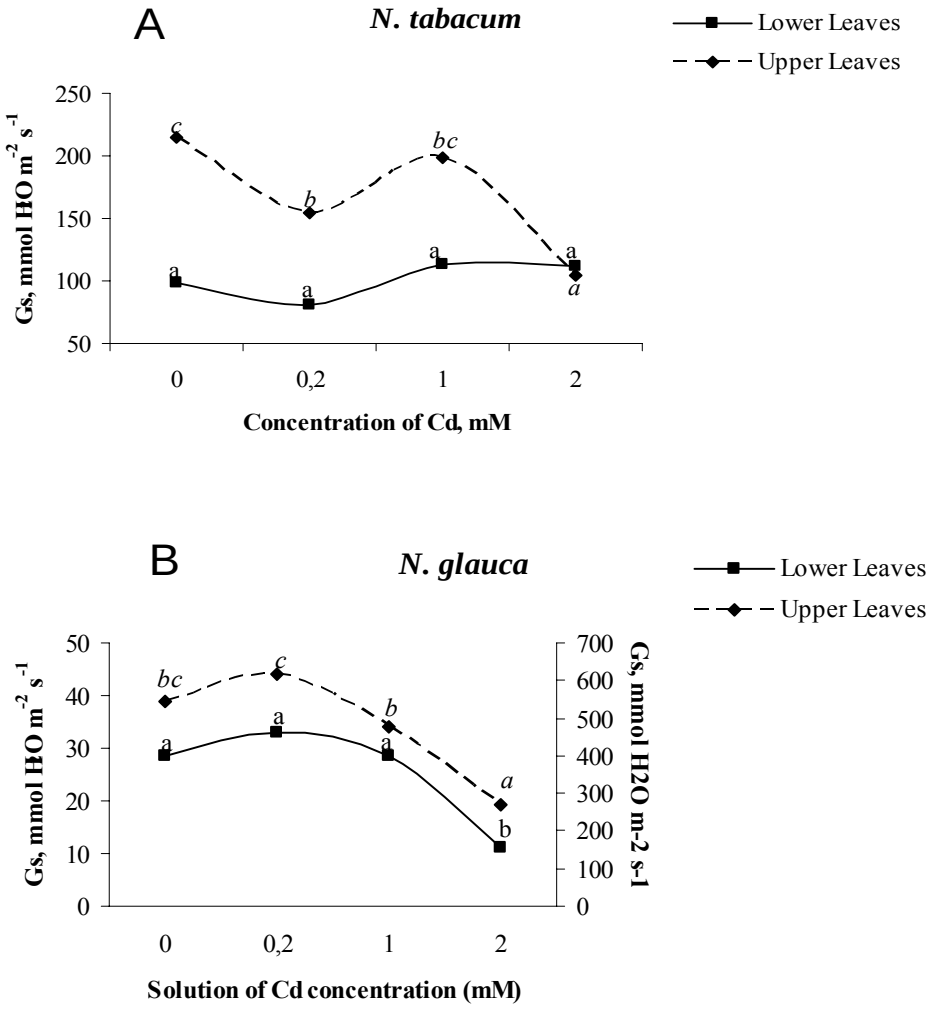


Figure 3.47: The effect of Cd concentration on transpiration rate of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca* (left axis for lower leaves, right axis for upper leaves). Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

As recommended, Water use efficiency (WUE) was calculated as the ratio of net carbon dioxide uptake to transpiration. As presented in Fig. 3.48, in lower leaves

of *N. tabacum*, WUE was lowered by increasing Cd concentration in the culture medium. Interestingly, WUE in upper leaves appeared an increased trend, especially at the highest Cd treatment, indicating that photosynthetic rate was more affected than transpiration rate. WUE in lower leaves of *N. glauca* started to show a reduced trend at the intermediate Cd concentrations, which is accompanied with the increase of transpiration rate at these treatments, while it increased at 2 mM Cd, indicating the water loss limitation due to transpiration rate. In upper leaves of *N. glauca* treated plants had lower WUE than controls, which is ascribed to the Cd induced variations in photosynthetic rate.

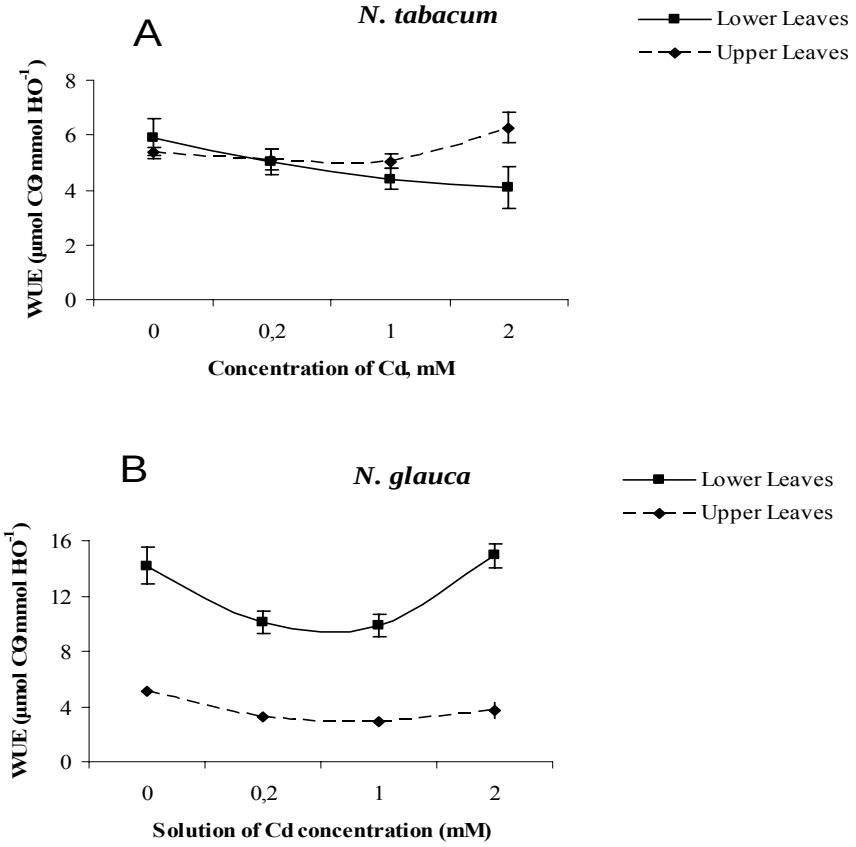


Figure 3.48: The effect of Cd concentration on Water Use Efficiency (WUE) of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE).

The effect of Cd treatments on photosynthetic rate of both plant species has previously presented in Fig. 3.44 and 3.45, however it is interesting to investigate it

with intracellular CO_2 concentration as shown in Fig. 3.49. The intracellular CO_2 concentration decreased with increasing Cd concentrations in the culture medium, irrespective of cultivar. Particularly, the intracellular CO_2 concentration in upper leaves of *N. tabacum* decreased linearly and reached up to 44% at the highest Cd treatment, compared with control plants. Also, photosynthetic rate appeared similar pattern, with the greatest reduction at the highest Cd concentration. Similarly, the presence of 2mM Cd in the culture medium resulted to significant decline of intracellular CO_2 concentration in upper leaves of *N. glauca* by approximately 28%, compared with intact plants, as well as of photosynthetic rate by 30%.

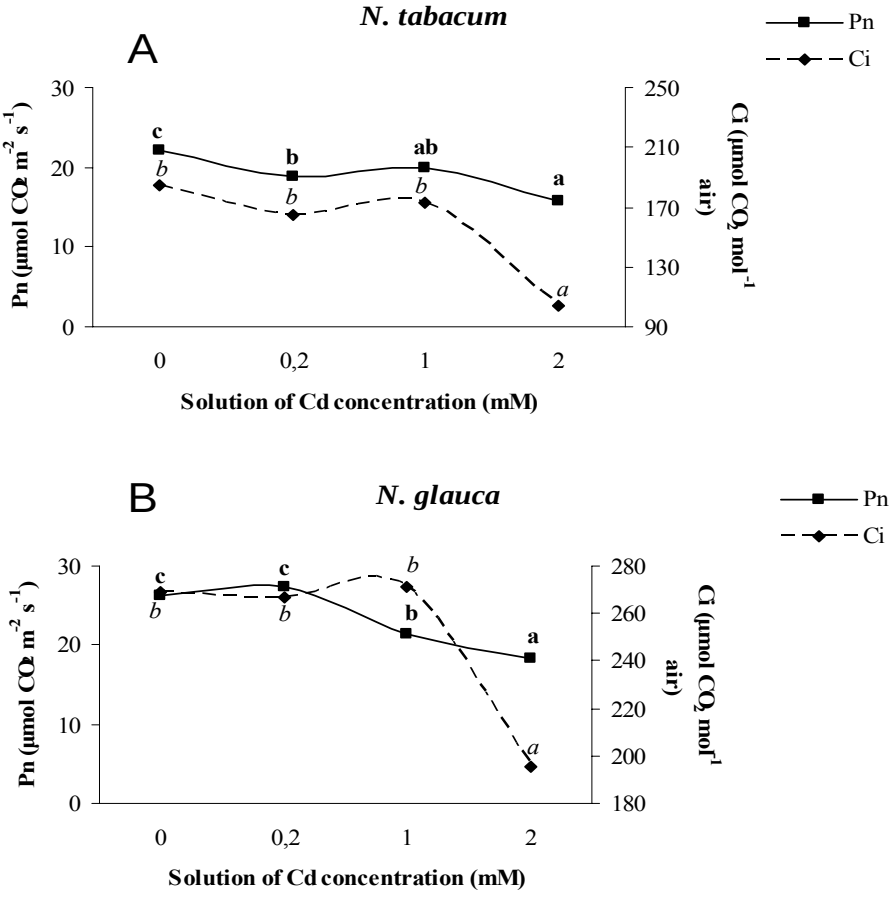


Figure 3.49: The effect of Cd concentration on net photosynthetic rate (left axis) and intracellular CO_2 concentration (right axis) of upper leaves of: A) *N. tabacum* and B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (bold for net photosynthetic rate, italics for intracellular CO_2 concentration, Duncan, P=0.05)

c. Chlorophyll Fluorescence Parameters - Photochemical Efficiency of PSII

In parallel to the measurement of the gaseous exchange, the photochemical parameters of photosynthesis were analyzed after 6 weeks in response to Cd excess. As shown in Fig. 3.50, all photochemical parameters in lower and upper leaves of *N. tabacum* exhibited statistical significant differences ($P<0.05$). Specifically, F_0 in lower leaves of *N. tabacum* decreased as the stress intensity increased, while in upper leaves remained almost unaffected, whatever the Cd concentration applied. Fm in lower and upper leaves decreased considerably by the exposure of plants to Cd treatments. Fv was affected similarly with Fm in examined tissues of *N. tabacum* under Cd stress. Also, the Fv/ F_0 ratio in upper leaves appeared more affected than lower as the Cd stress intensity increased, reflecting earlier structural dysfunctions of the PSII (Vaillant et al., 2005).

N. glauca showed similar response of photochemical parameters of photosynthesis with *N. tabacum*, however the latter appeared more affected (Fig.3.51). In particular, in lower leaves F_0 decreased significantly irrespective the Cd concentration applied, while in upper leaves the parameter remained almost unchanged. Fm and Fv appeared similar affected by the exposure of plants to Cd treatments, where statistical significant decline ($P<0.05$) exhibited at all Cd concentrations applied in both examined tissues, compared to intact plants. Fv was affected similarly with Fm in lower and upper leaves of *N. glauca*. The Fv/ F_0 ratio in lower leaves dropped by the presence of 1 and 2 mM Cd in the growth medium, compared to intact plants, while in upper leaves remained unchanged over the range of Cd treatments.

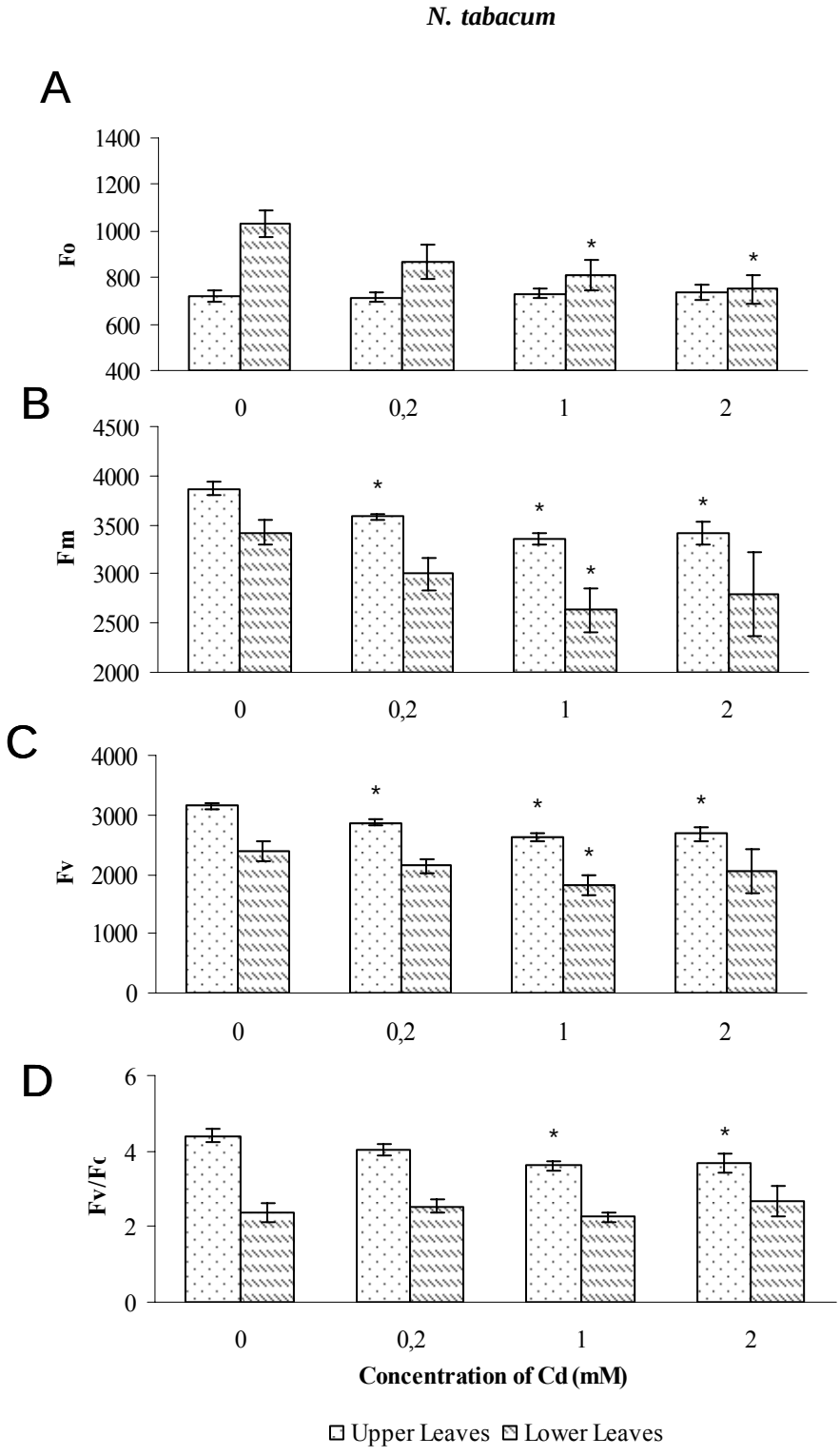


Figure 3.50: The effect of Cd concentration on chlorophyll fluorescence parameters of PSII in dark – adapted leaves: A) initial, F_0 B) maximum, Fm C) variable, Fv D) ratio F_v/F_0 , of lower and upper leaves of *N. tabacum.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE). Asterisks indicate significant

differences between treatments and control (only for data obtained at 6th week, LSD, P=0.05)

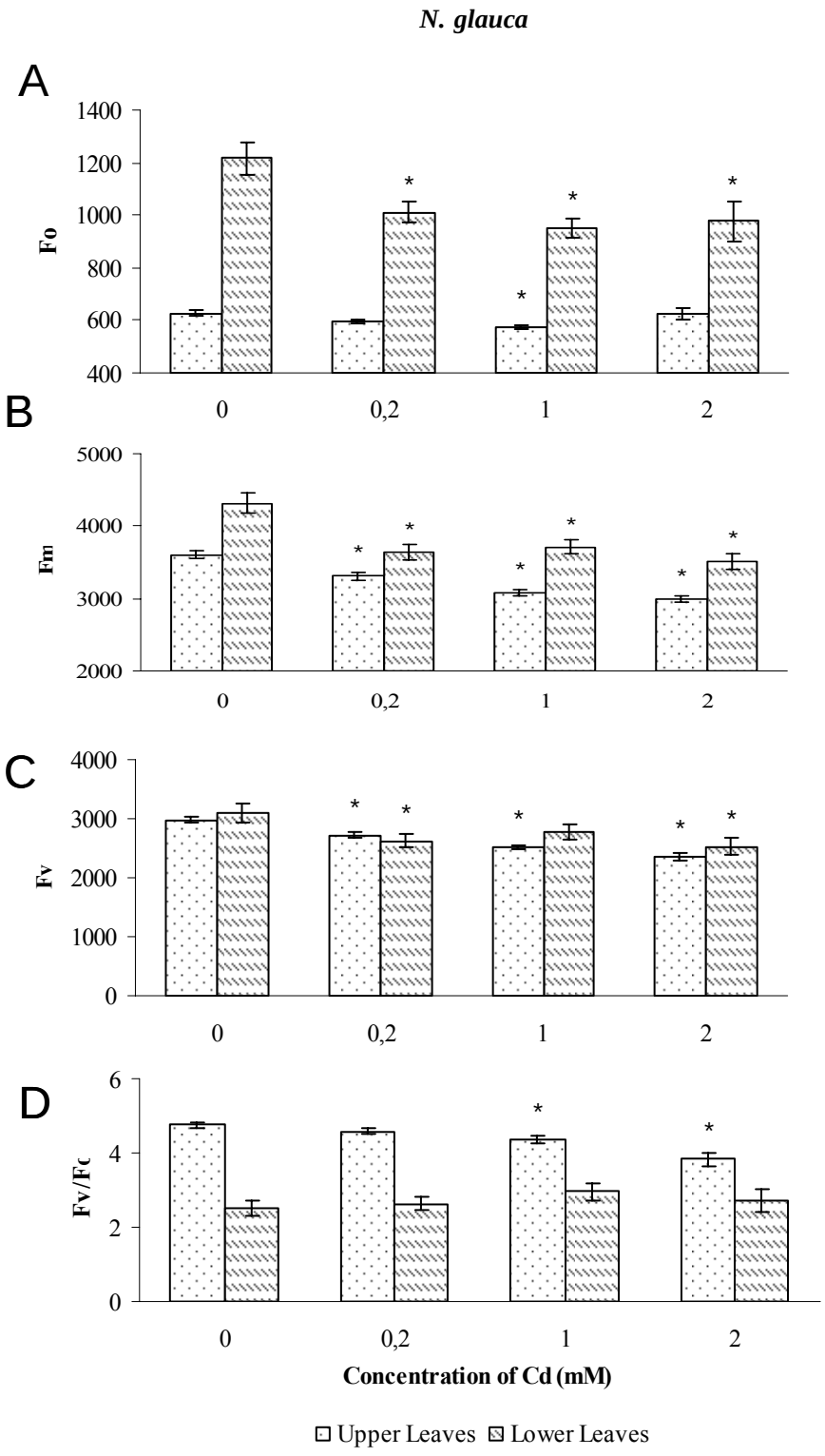


Figure 3.51: The effect of Cd concentration on chlorophyll fluorescence parameters of PSII in dark – adapted leaves: A) initial, F_0 B) maximum, Fm C) variable, Fv D) ratio F_v/F_0 , of lower and upper leaves of *N. glauca.* Plants were grown in plastic pots

containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE). Asterisks indicate significant differences between treatments and control (only for data obtained at 6th week, LSD, P=0.05)

The maximal dark-adapted yield of PSII photochemistry, Fv/Fm, of both plant species was followed during the Cd treatment period and presented in Fig. 3.52. In general, for the most measurements obtained during the first and third week, the ratio remained almost unaffected, whatever the Cd concentration applied and the values were close to 0.8, normal value for healthy leaves. The Fv/Fm ratio, which can indicate stress as recommended, declined especially by the 6 week exposure of plants to Cd stress. Specifically, in lower and upper leaves of *N. tabacum* the Fv/Fm ratio gradually decreased in response to Cd concentrations, however significant differences exhibited only from measurements concerned upper leaves.

Similar response observed in *N. glauca*, where in lower leaves the values of the Fv/Fm ratio were lower in treated plants than controls and the remarkable Cd induced reduction of the ratio in upper leaves, resulted by the highest treatment compared to controls (Fig. 3.53). However, similarly both plant species kept the Fv/Fm ratio closely to 0.8 in upper leaves, while in lower leaves the values were lower of 0.8, not only of treated plants, but of controls as well. The decrease was observed in Fv/Fm ratio of leaves of both plant species was paralleled with the increase of the basic fluorescence (F_0).

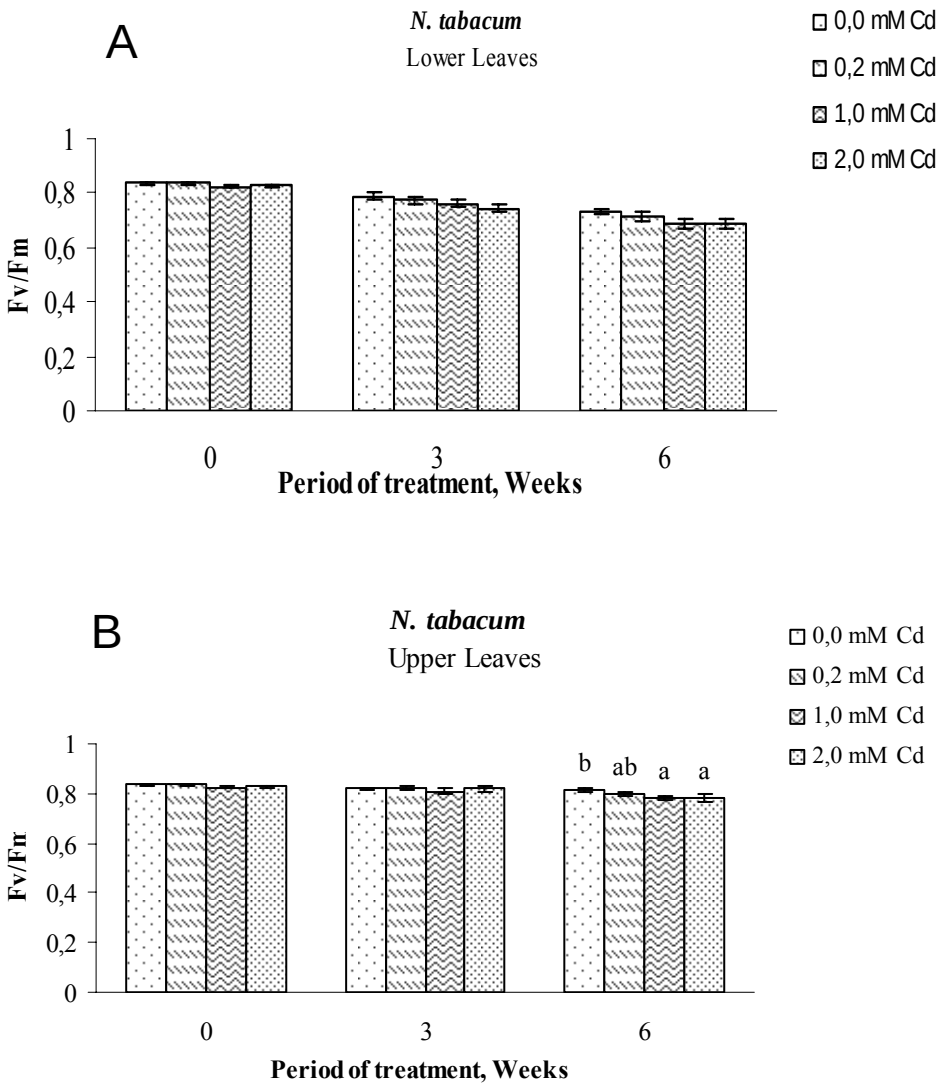


Figure 3.52: The effect of Cd concentration on the photochemical efficiency of PSII (F_v/F_m) in dark – adapted leaves of *N. tabacum*: A) lower leaves B) upper leaves, during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

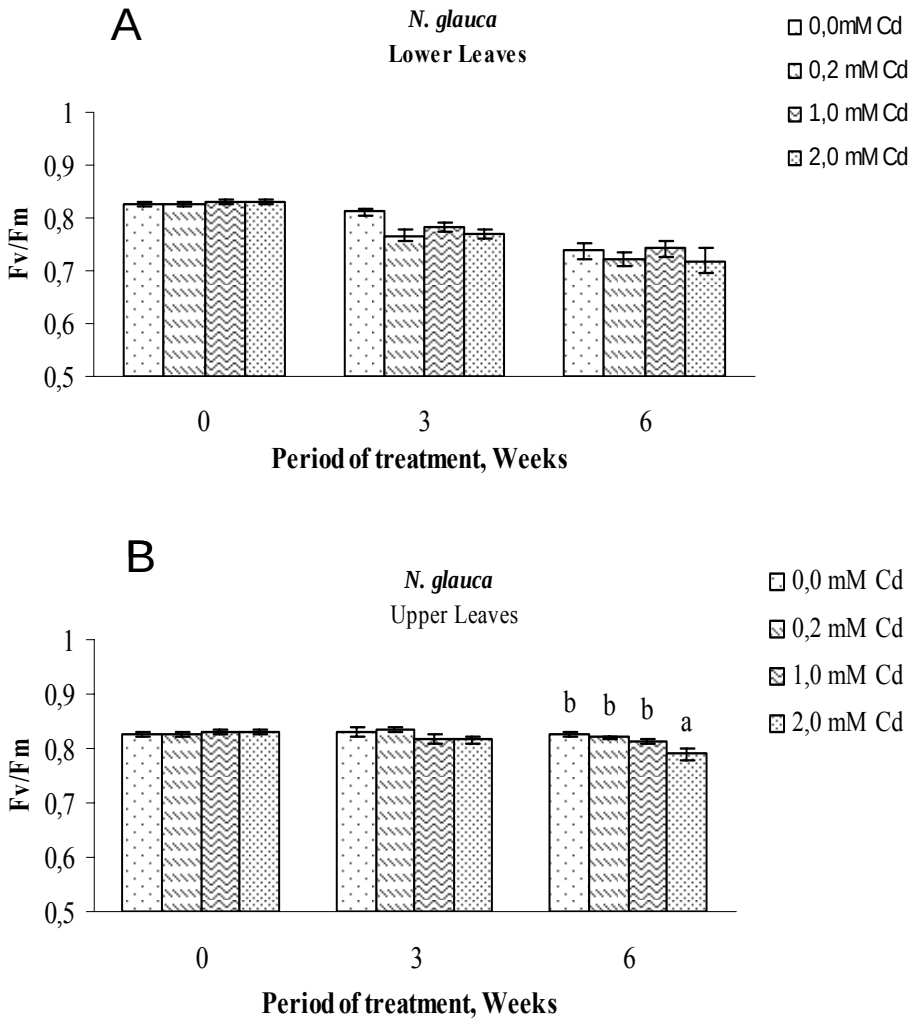


Figure 3.53: The effect of Cd concentration on the photochemical efficiency of PSII (F_v/F_m) in dark – adapted leaves of *N. glauca*: A) lower leaves B) upper leaves, during the treatment period. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE). Columns which differ significantly from one another are marked with a different letter (only for data obtained at 6th week, Duncan, P=0.05).

3.3.4 Effect of Cd On Parameters Related With Nitrogen Metabolism

a. Proline

Figure 3.54 illustrates the effect of Cd concentrations on proline accumulation of lower and upper leaves of *N. tabacum* and *N. glauca*. The accumulation pattern was similar in both plant species. The amount of proline was positively affected by Cd treatments, while significant differences (P<0.05) exhibited over the range of

applied Cd concentrations. Both plant species accumulated maximum at the highest Cd treatment.

b. Total Protein Content

Total soluble protein content was determined corresponding to Cd excess (Table 3.17). A slight enhancement of protein level observed in lower leaves of both plant species. A considerable increase of total chlorophyll content exhibited in upper leaves of *N. tabacum* at the highest Cd treatment, while a slight enhancement observed in upper leaves of *N. glauca*. However, in lower leaves of both plant species detected considerable lower protein content, than in upper.

c. Specific activities of Ammonium assimilating enzymes

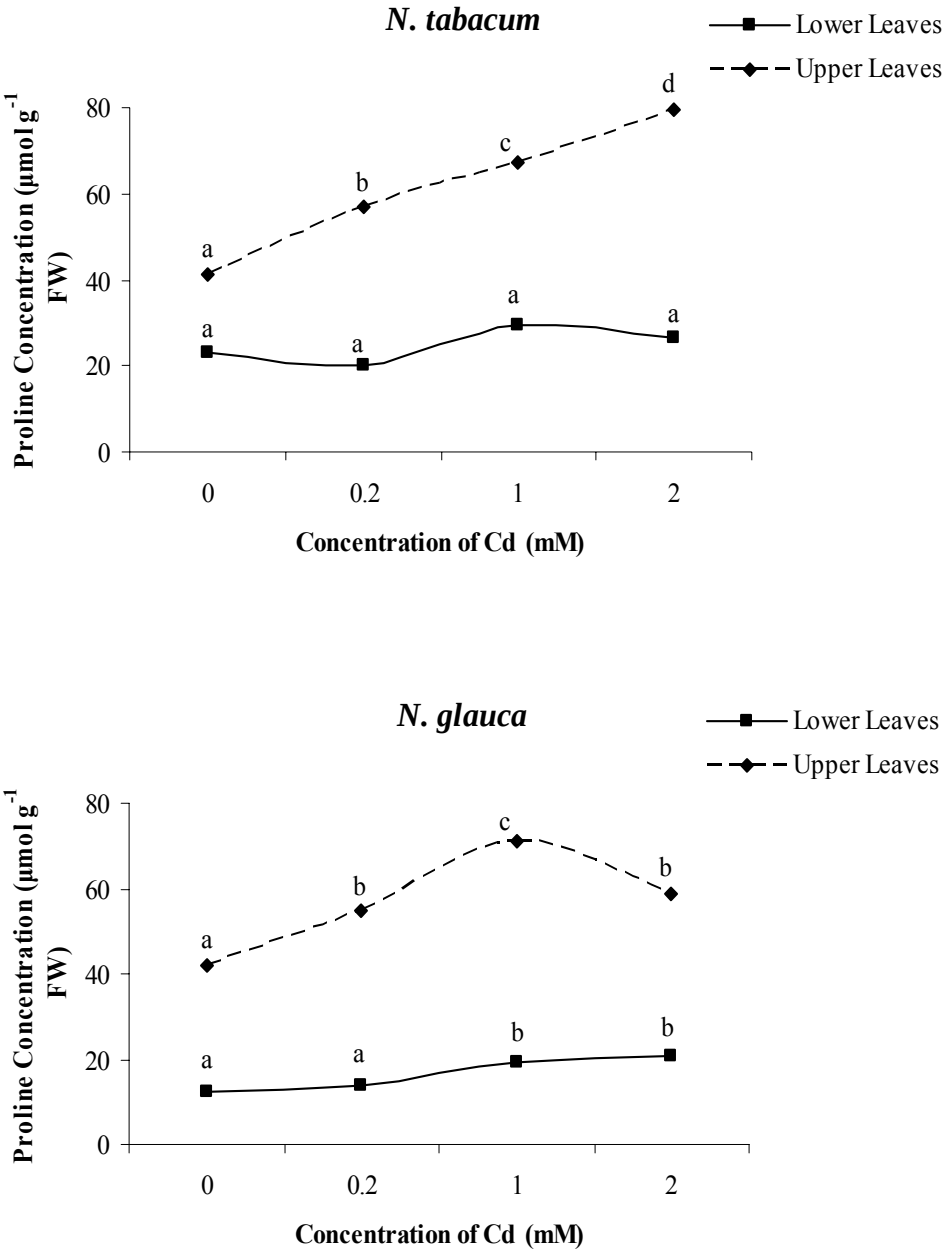


Figure 3.54: The effect of Cd concentration on proline concentration of lower and upper leaves of: A) *N. tabacum* and B) *N. glauca*. Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Table 3.17: The effect of Cd concentration on protein content of lower and upper leaves of *N. tabacum and N. glauca.* Plants were grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd^{2+} for 6 weeks. Values are means ± standard error (SE).

Treatment Cd, mM	*N. tabacum*		*N. glauca*	
	Lower leaves	Upper leaves	Lower leaves	Upper leaves
0,0	6,94 ± 0,41	9,56 ± 0,57	4,93 ± 0,04	9,68 ± 0,64
0,2	5,69 ± 0,45	9,93 ± 0,77	6,23 ±0,19	10,83 ± 0,07
1,0	6,24 ± 0,18	11,34 ± 0,75	5,88 ± 0,05	11,16 ± 0,12
2,0	7,42 ± 0,23	13,77 ± 0,30	6,12 ± 0,37	10,32 ± 0,35

Table 3.18: The effect of Cd concentration on specific activity of the enzymes of nitrogen assimilation of lower and upper leaves of *N. tabacum*. Values concerned controls and plants treated with 2 mM Cd. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Plant tissues	Treatments Cd, mM	Nitrogen Assimilating Enzymes (Enzyme activity: nmol mg^{-1} protein min^{-1})		
		GS	Fd-GOGAT	GDH
Lower leaves	**0.0**	420.33 ± 20.6	89.18 ± 4.7	398.92 ± 14.3
	2.0	266.73 ± 14.3	84.68 ± 5.7	454.67 ± 29.1
Upper leaves	**0.0**	777.80 ± 43.2	119.28 ± 7.2	165.63 ± 11.8
	2.0	517.77 ± 21.7	94.16 ± 6.6	238.36 ± 20.5

Table 3.19: The effect of Cd concentration on specific activity of the enzymes of nitrogen assimilating of lower and upper leaves of *N. glauca.* Values concerned controls and plants treated with 2 mM Cd. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Plant tissues	Treatments Cd, mM	Nitrogen Metabolism Enzymes (Enzyme activity: nmol mg^{-1} protein min^{-1})		
		GS	Fd-GOGAT	GDH
Lower leaves	**0.0**	333.11 ± 19.2	52.15 ± 3.8	474.70 ± 24.0
	2.0	269.75 ± 25.1	50.21 ± 2.2	565.40 ± 36.1
Upper leaves	**0.0**	619.63 ± 31.6	86.58 ± 8.8	136.43 ± 13.9
	2.0	406.52 ± 31.2	61.43 ± 8.2	277.48 ± 21.4

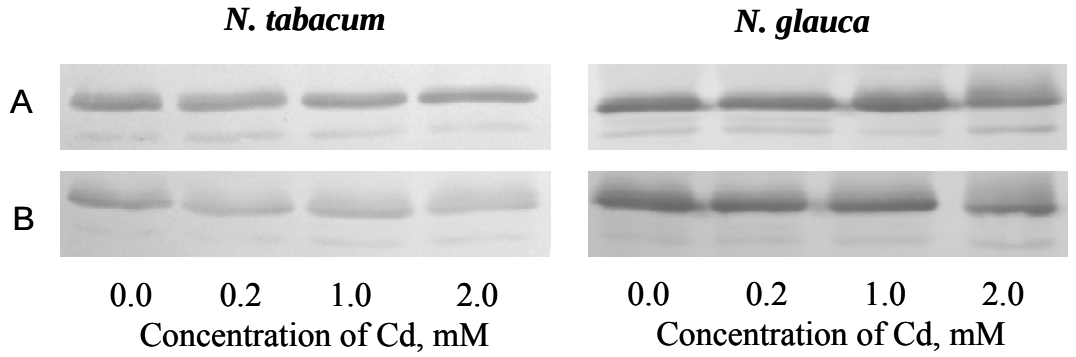


Plate 3.14: Western blot analysis of GS in A) lower leaves B) upper leaves of Cd treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd for 6 weeks. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti-GS serum, as described in section "Materials and Methods"

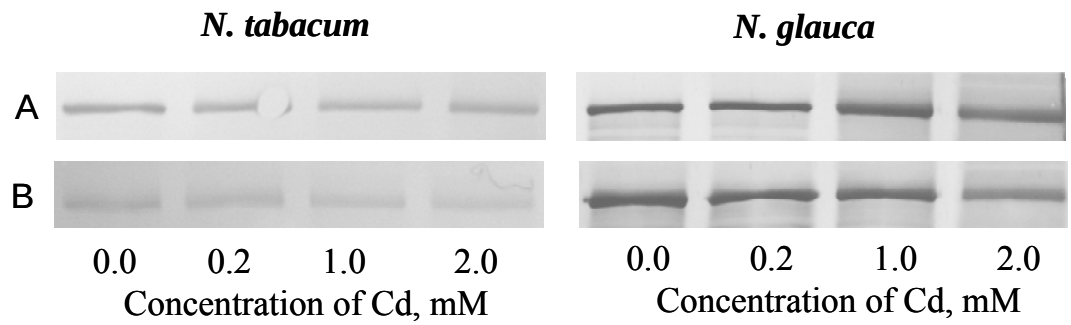


Plate 3.15: Western blot analysis of Fd-GOGAT in A) lower leaves B) upper leaves of Cd treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd for 6 weeks. For immunoblot analysis proteins were resolved in 7% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti- GOGAT serum, as described in section "Materials and Methods"

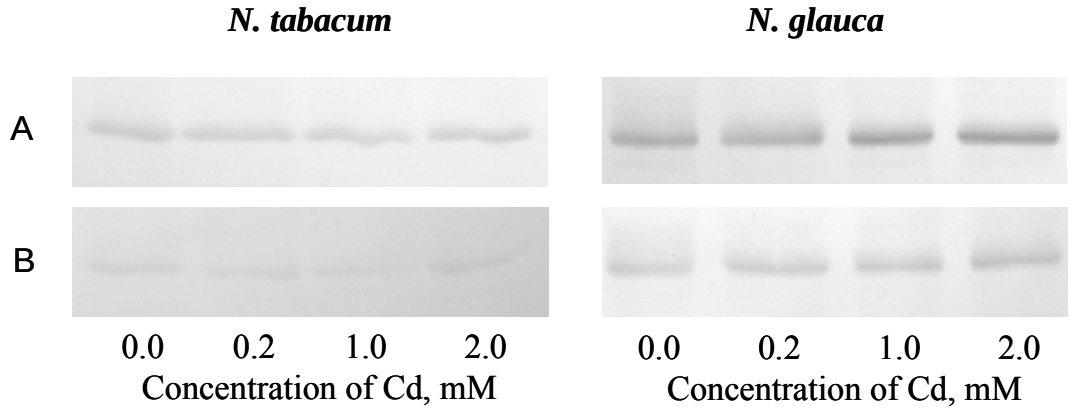


Plate 3.16: Western blot analysis of GDH in A) lower leaves B) upper leaves of Cd treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants grown in plastic pots containing homogenized soil in the presence of different concentrations of Cd for 6 weeks. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti-GDH serum, as described in section "Materials and Methods"

3. B. Hydroponic Culture Experiment

In this experinment seedlings of *N. tabacum* and *N. glauca* were cultured hydroponically in the presence of different concentration of Zn, Ni and Cd for 8 days. In these conditions the entire metal pool was accessible in plants. Studies often use this way of cultivation for a quick identification and selection of species with regard to their tolerance to metals (Malgorzata et al., 2005). Several morphological parameters of the plants were recorded and at the end of the experiment samples of shoots and leaves were collected for further analysis presented here.

3.1 Zinc Effects of Different Concentrations of Zn, Ni and Cd on *N. tabacum* and *N. glauca* Plants.

The results obtained of this experiment are presents below in Tables and plates.

Plate 3.17: Plants of *N.tabacum* (left) and *N.glauca* (right) grown in Hoagland solution in the presence of different concentrations of Zn for 8 days.

Table 3.20: The effect of Zn concentration on the height, number and fresh weight of leaves of *N. tabacum.* Seedlings were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of $ZnSO_4$ $7H_2O$ for 8 days. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Zn, mM	*N. tabacum*		
	Height (cm)	Number of leaves	Fresh Weight of leaves (g)
0.0	14.58 b	8.45 a	4.60 c
0.1	12.78 a	7.85 a	3.75 b
0.5	13.20 a	7.85 a	3.75 b
2.0	12.62 a	7.96 a	2.97 a

Table 3.21: The effect of Zn concentration on the height, number and fresh weight of leaves of *N. glauca.* Seedlings were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of $ZnSO_4$ $7H_2O$ for 8 days. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05

Treatments Zn, mM	*N. glauca*		
	Height (cm)	Number of leaves	Fresh Weight of leaves (g)
0.0	8.94 a	8.20 a	5.41 b
0.1	8.72 a	8.10 ab	5.15 b
0.5	8.69 a	7.35 ab	4.97 b
2.0	8.50 a	7.25 b	3.72 a

Table 3.22: The effect of Zn concentration on the SPAD values of leaves of *N. tabacum* and *N. glauca.* Plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of $ZnSO_4$ $7H_2O$ for 8 days. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Zn, mM	*N. tabacum*	*N. glauca*
	SPAD values	
0.0	26.56 ab	42.26 a
0.1	26.88 ab	39.90 a
0.5	27.76 b	40.72 a
2.0	25.59 a	40.70 a

Plate 3.18: Plants of *N.tabacum* (left) and *N.glauca* (right) grown in Hoagland solution in the presence of different concentrations of Ni for 8 days.

Table 3.23: The effect of Ni concentration on the height, number and fresh weight of leaves of *N. tabacum*. Plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of $NiSO_4$ $6H_2O$ for 8 days. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Ni, mM	***N. tabacum***		
	Height (cm)	Number of leaves	Fresh Weight of leaves (g)
0.0	14.58 b	8.45 b	4.60 c
0.02	14.50 b	8.75 a	3.92 b
0.05	15.20 b	8.55 a	3.75 b
0.15	12.42 a	7.75 a	3.33 a

Table 3.24: The effect of Ni concentration on the height, number and fresh weight of leaves of *N. glauca*. Plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of $NiSO_4$ $6H_2O$ for 8 days. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Ni, mM	***N. glauca***		
	Height (cm)	Number of leaves	Fresh Weight of leaves (g)
0.0	8.94 a	8.20 a	5.41 b
0.02	8.43 a	7.70 a	5.01 b
0.05	8.90 a	7.67 a	4.05 a
0.15	8.11 a	7.89 a	3.98 a

Table 3.25: The effect of Ni concentration on the SPAD values of leaves of *N. tabacum* and *N. glauca.* Plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of $NiSO_4$ $6H_2O$ for 8 days. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Ni, mM	*N. tabacum*	*N. glauca*
	SPAD values	
0.0	26.56 a	42.26 a
0.02	26.01 a	40.38 a
0.05	26.32 a	40.08 a
0.15	28.31 b	41.89 a

Plate 3.19: Plants of *N.tabacum* (left) and *N.glauca* (right) grown in Hoagland solution in the presence of different concentrations of Cd for 8 days.

Table 3.26: The effect of Cd concentration on the height, number and fresh weight of leaves of *N. tabacum.* Plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of $CdSO_4$ 2.67 H_2O for 8 days. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Cd, mM	*N. tabacum*		
	Height (cm)	Number of leaves	Fresh Weight of leaves (g)
0.0	14.58 b	8.45 a	4.60 b
0.02	13.79 ab	8.24 a	4.21 ab
0.05	14.45 b	8.27 a	4.62 b
0.2	12.54 a	8.08 a	3.98 a

Table 3.27: The effect of Cd concentration on the height, number and fresh weight of leaves of *N. glauca.* Plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of $CdSO_4$ 2.67 H_2O for 8 days. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Cd , mM	*N. glauca*		
	Height (cm)	Number of leaves	Fresh Weight of leaves (g)
0.0	8.94 b	8.20 b	5.41 c
0.02	8.82 b	7.40 ab	4.37 b
0.05	8.32 ab	7.40 ab	4.53 b
0.2	7.61 a	6.40 a	3.44 a

Table 3.28: The effect of Cd concentration on the SPAD values of leaves of *N. tabacum* and *N. glauca.* Plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of $CdSO_4$ 2.67 H_2O for 8 days. Columns which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments Cd, mM	*N. tabacum*	*N. glauca*
	SPAD values	
0.0	26.56 c	42.26 a
0.02	25.32 b	40.84 a
0.05	25.82 cb	43.90 a
0.2	23.95 a	42.84 a

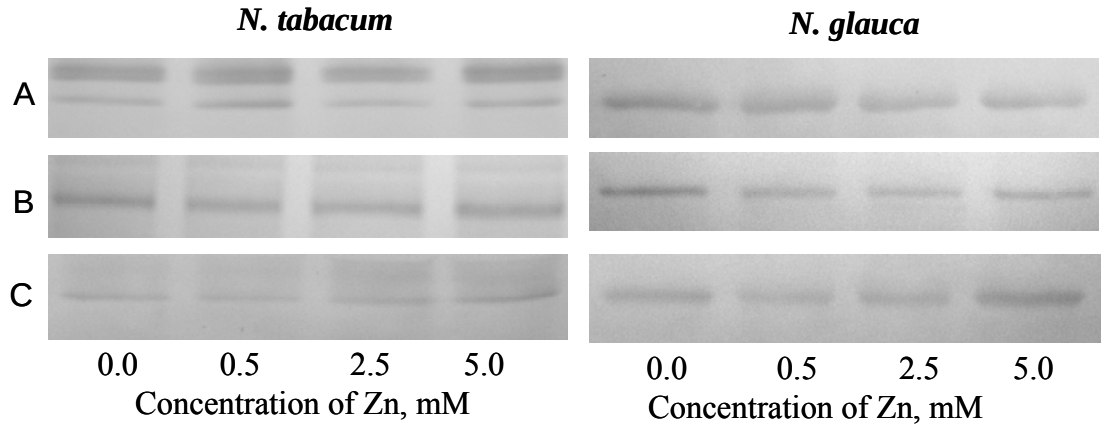


Plate 3.20: Western blot analysis of A) GS, B) Fd-GOGAT and C) GDH of Zn treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of Zn^{2+} for 8 days. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti-GS -GOGAT -GDH serum, as described in section "Materials and Methods

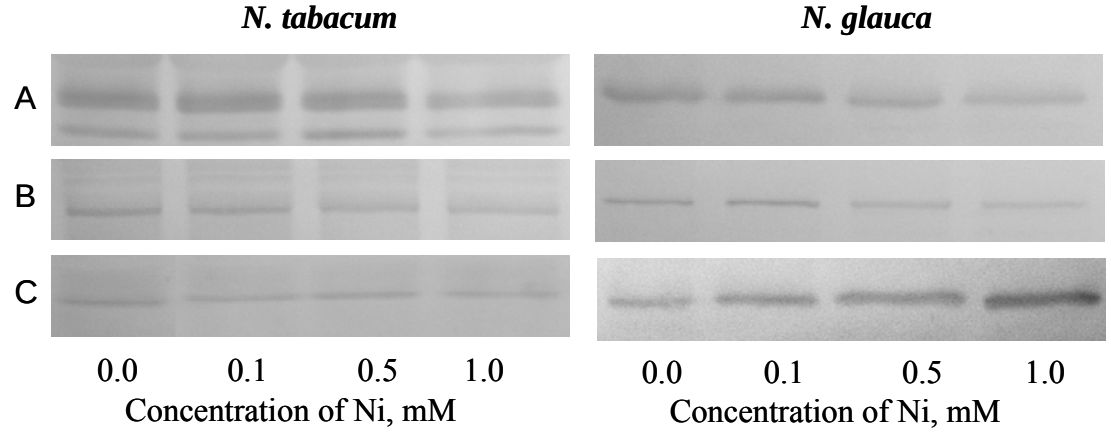


Plate 3.21: Western blot analysis of A) GS, B) Fd-GOGAT and C) GDH of Ni treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of Ni^{2+} for 8 days. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti-GS -GOGAT -GDH serum, as described in section "Materials and Methods"

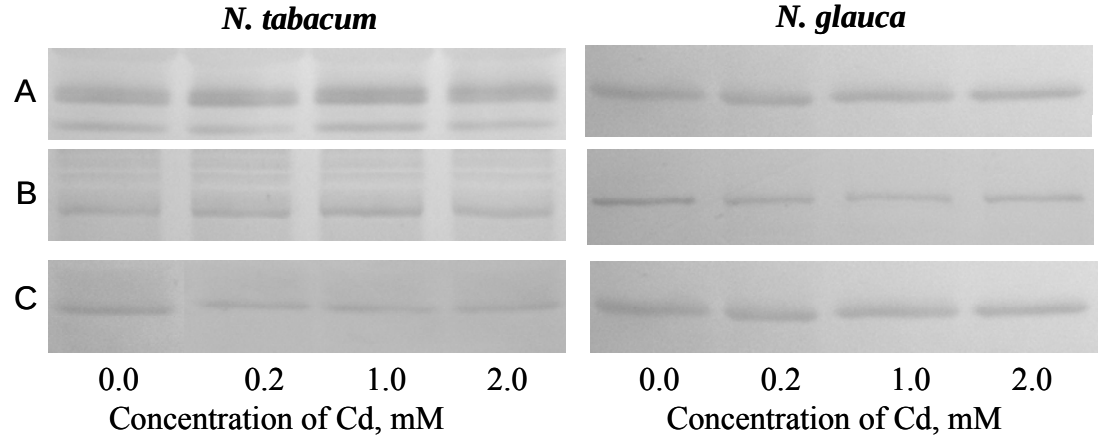


Plate 3.22: Western blot analysis of A) GS, B) Fd-GOGAT and C) GDH of Cd treated *N. tabacum* and *N. glauca* plants. Protein extracts were obtained from plants were grown in polyethylene pots containing Hoagland solution in the presence of different concentrations of Cd^{2+} for 8 days. For immunoblot analysis proteins were resolved in 10% SDS polyacrilamide gels, transferred onto nitrocellulose membranes and probed with anti-GS -GOGAT -GDH serum, as described in section "Materials and Methods"

4. DISCUSSION

The physiology of metal toxicity in plants was mainly concerned with metal movement from soil to root and metal translocation (Rout, 2009). Studies with heavy metals have shown that they can be either stored in root or transported to shoot (Barazani *et al*., 2004). Some plants tolerate metals by having a low metal translocation rate to the shoot and in this way protecting photosynthesis from damages.

In this study, the analysis of heavy metal accumulation revealed significant differences and strong correlations between treatments and endogenous metal concentration in the plant tissues of the examined species ($P<0.05$, $P<0.01$, Appendix). Generally, both plant species accumulated Zn, Ni and Cd in a dose-dependent manner. The endogenous concentrations of the examined metals in treated plants of *N. tabacum* and *N. glauca* were several times higher than the values in control plants, with greater accumulation at the highest metal treatment. However, the accumulation pattern differed between species and supplemented metals.

In the case of Zn, *N. tabacum* accumulated more in all the above ground tissues than *N. glauca.* On the other hand, *N. glauca* accumulated more in roots than *N. tabacum*. Similarly, in lower tissues of both plant species detected more Zn ions than in upper. Higher Zn concentration in old leaves, corresponding to the metal restriction in young leaves (Baccio *et al.,* 2009). Moreover, *N. tabacum* showed maximum accumulation in lower leaves, while *N. glauca* accumulated maximum in root, indicating more efficient root to shoot translocation of the metal in plants of *N. tabacum*.

Similarly, under Ni stress, both plant species showed maximum accumulation in root, while their accumulation pattern differed in the above ground tissues. Particularly, *N. tabacum* accumulated more in lower leaves than in upper. In contrast, in upper leaves of *N. glauca* detected more Ni ions than in lower. Nickel is highly mobile in plants and can be easily retranslocated from old to young leaves. It has been

reported, also, that the translocation to the aerial parts including stem and leaves occurs through the phloem (Ahmad *et al.,* 2011).

Although Cd is not an essential or beneficial element for plants, excessive concentrations were detected particularly in roots, but also in leaves of both plant species. Comparing the two plant species, *N. tabacum* accumulated much more amount of Cd in lower leaves than *N. glauca*. It has been reported by many authors that more Cd accumulates in lower leaves than in medium or upper leaves, suggesting a permanent Cd accumulation mechanism (Westcott and Springer, 1974; Wagner and Yeargan, 1986; Frank *et. al*., 1991; Miele *et. al*., 2002; Lugon-Moulin, 2004). In agreement with these authors, was presented *N. tabacum*, which absorbed maximum in lower leaves at the highest Cd treatment. Contrary, *N. glauca* showed maximum accumulation in roots. As a first barrier to Cd toxicity, most species accumulate Cd in the roots in order to restrict its transport to the shoots (Vazqueza, *et al*., 2006). In response to Cd stress, the plant cell relies on a number of defense systems, such a immobilization, exclusion and compartmentalization, as well as the synthesis of organic cheletes, stress proteins and ethylene.

Cd is chemically similar to Zn and is easily taken up by plants. Its translocation to the shoot appears, also to be rapid (Lugon-Moulin, 2004). Plants are not expected to have specific uptake mechanisms for non-essential metal ions, such as Cd (Palmgren, 2008). Instead, Cd may effectively compete for the same transmembrane carriers used by essential elements. This relative lack of selectivity in transmembrane ion transport may partially explain why non- essential heavy metals can enter cells, even against a concentration gradient (Ghosh et al., 2005). A common transmembrane transporter was found for Cd, Ni and Zn. Transport processes have been recognized as a central mechanism of metal detoxification and tolerance (Sharma and Dietz, 2006)

It is interesting, to compare the heavy metal concentration detected in the plant tissues with the range of toxicity level for each metal tested. Normal foliar Zn concentrations are around 100 ppm, while 300 ppm considered toxic (Pence *et al*., 2000). Ni-induced toxicity occurs at concentrations in the range of 10 ppm in

sensitive and 50 ppm in tolerant plants (Assuncao et al., 2003). Also, foliar Cd levels above 1 ppm usually are considered toxic (Pence *et al*., 2000). Combined the above concentrations with the analysis of heavy metal accumulation in both plant species, inferences that, the detected concentrations in all the examined tissues in both plants species were considerably above the permissible limits for each metal, respectively, especially at the higher concentrations.

One definition proposes that metal hyperaccumulation is defined based on certain metal-specific thresholds of metal levels detected in the above ground biomass. Thus, plant with the ability to accumulate 1% of Zn, 0.1% of Ni and 0.01% of Cd on a dry weight basis are considered as hyperaccumulators for these metals (Chaney *et. al*., 1997; Clemens, 2001; Sharma, 2006). While some plants, can survive in environments containing extremely high concentrations of metals, they may not show high ability of accumulating metals. Furthermore, the definition of metal hyperaccumulation has to take in consideration not only the metal concentration in the above ground biomass, but also the metal concentration in the soil. Translocation factor (TF) has to be considered while evaluating whether a particular plant is a metal hyperaccumulator (Isidora *et al.*, 2006). The term TF defined as the ratio of metal concentrations in plant aerial parts to those in the roots shows the effectiveness of plants in translocating metals from roots to the shoots (Isidora *et al.*, 2006). For hyperaccumulator plants, TF should be higher than 1.

In this study, even though *N. tabacum* and *N. glauca* accumulated significant amounts of both Zn and Ni, did not either succeed the recommended percentage for each metal, respectively, or the value of TF, for being considered hyperaccumulators. In the case of Cd, both plant species accumulated more than 0.01% of Cd on a dry weight basis, especially at the higher concentrations, however they kept TF lower than 1.

The accumulation of the three metals in root and above ground tissues indicated that tolerance mechanisms of both plant species could not solely be related to avoidance mechanism (Barazani *et al*., 2004). Nevertheless, the high absorption, above the critical toxicity level for each metal, and content in leaves demonstrated active translocation of metals from below- to above- ground tissues, although not as efficiently as in hyperaccumulator plants (Baccio *et al*., 2009).

The effects of heavy metals applied were visible at both plant species, especially at the higher treatments, where chlorosis and other phytotoxicity symptoms induced.

Photosynthesis, an important process for plant growth and biomass production, was used as a bioindicator of early stress (Sheoran). The photosynthetic apparatus appears to be very sensitive to the toxicity of heavy metals, while reduced photosynthetic activity is an effect commonly noticed in plants exposed to heavy metals stress. This has been ascribed to the metal disturbing action on chlorophyll synthesis, activity of photochemical enzymes and plant water balance (Ming et al., 2008). According to literature, the scale and character of changes observed in plants after heavy metal application was shown to be dose-dependent, while it can vary for different plant species even for identical metal treatment depending on individual plant tolerance.

In our study, net photosynthetic rate was negatively affected by metal treatments, while the deleterious effect became more pronounced by the higher concentrations. In lower leaves of both plant species, the presence of excess metals combined to natural senescence kept the values considerably lower than in upper leaves. Photosynthetic rate in upper leaves of *N. tabacum* seemed more affected by Ni and Cd stress, than Zn. On the other hand, the referenced parameter in upper leaves of *N. glauca* reduced by all tested metals, especially at the highest treatment. In general, *N. glauca* appeared more affected than *N. tabacum*.

The content of chlorophylls was reduced by the presence of Zn, Ni and Cd in the growth medium, especially by the highest treatment. The decline in chlorophyll content is believed to be a consequence of the substitution of the central Mg in chlorophyll by heavy metals. This in vivo substitution prevents photosynthetic light harvesting in the affected chlorophyll molecules and results in a breakdown of photosynthesis. The replacement of Mg ion of chlorophyll pigment causes the inhibition of enzymes associated with chlorophyll biosynthesis, such as *δ*-aminolevulinic acid (ALA) synthetase, ALA dehydratase and porphobilinogenase. The decrease in chlorophyll content also may be attributed to increased activity of

chlorophyll-degrading enzyme chlorophyllase and/or the elevated ROS production (John et al., 2009). The reduction in chlorophylls is in parallel with the toxicity symptoms observed in lower and upper leaves of both plant species.

In the case of Ni the decrease in photosynthesis activity was more significant than the measured effect on the chlorophyll concentration. The Pn inhibition cannot be only allotted to chlorophyll reduction.

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The variations of the photosynthetic pigments induced by metal treatments, similarly affected the chlorophyll a to b ratio in both plant species. Though, the recommended ratio was differentially affected in lower and upper leaves. Specifically, in lower leaves the ratio was progressively decreased over the range of metal treatments, indicating that chlorophyll a was more affected than b. Contrary, the ratio in upper leaves of both plant species showed an increased trend, except of the case of Cd concerned *N. glauca*. Both chlorophyll a and b are present in PSII, while chlorophyll a is the main light- harvesting pigment of PSI. Literature suggests, that variations in chlorophyll a/b ratio can indicate possible changes in PSI/PSII ratio. Higher chlorophyll a/b may show a higher ratio of PSI to PSII, as a consequence of an adaptive mechanism in chloroplasts due to exposure to stress, seemingly a positive strategy to maintain the maximum quantum yield (Fv/Fm) inside of normality (Lage-Pinto *et. al*., 2008, Takabayashi *et.al*., 2005).

The study of gas exchange represents a tool for evaluating the impact of environmental conditions on crop productivity as well as for suggesting technical or genetic means to improve plant water and carbon use efficiency (Avola *et al*., 2007). Stomata play a pivotal role in controlling the balance between water loss and carbon gain, i.e. biomass production. Thus, measurements of stomatal conductance and intercellular CO_2 concentration provide a useful tool to qualify the effects of stress factors on stomatal opening (Papazoglou *et. al*., 2007). The inhibition of stomatal conductance in plants exposed to heavy metal stress may depend on metal concentration, exposure time and the degree of toxicity suffered by plants (Papazoglou *et. al*., 2007).

Consequently, in this study, severe reduction in stomatal conductance exhibited by the higher applied metal concentrations, especially in cases of Ni and Cd. The simultaneous decrease of net photosynthetic rate and transpiration of leaves of both plant species, in response to heavy metal stress was in direct relation with the reduction in stomatal conductance. These results are in agreement with literature (Vaillant *et al*., 2005; Ying *et al*., 2010). Stomatal limitation is recognized to reduce both photosynthetic rate and intercellular CO_2 concentration (Ying *et al*., 2010). Moreover, the decrease in stomatal conductance may be related to an alteration in the K^+/Ca^{2+} ratio in the guard cells and/or to the alteration in the abscisic acid concentration, which controls the stomatal movement (Vaillant *et al*., 2005).

Photosynthetic water use efficiency (WUE) was calculated as the ratio of net carbon dioxide uptake to transpiration (Avola *et al*., 2007). WUE can be a useful parameter as it's correlated with the physiological and biochemical processes of higher plants. The conventional view holds that plant biomass production is linearly coupled with the amount of water used and WUE is a conservative parameter (Kang and Zhang, 2004). Heavy metal stress produced disturbances in water balance and thus reduction of the recommended parameter (Januskaitiene, 2010). However, Zn, Ni and Cd differential affected the ratio. The reduction observed in leaves of treated plants, might be due to the inhibition of absorption and translocation of water. Meanwhile, the Zn- and Cd- induced increase of the ratio was caused by a higher decrease in transpiration rate compared to the photosynthetic rate of those plants and emphasizes its limited water loss due to transpiration (Avola *et al*., 2007 Januskaitiene, 2010).

Previous studies have shown that a decrease in CO_2 assimilation could result from the influence of heavy metals Calvin-cycle enzymes, localized in chloroplast stroma (Ying *et al*., 2010). Furthermore, increase in intercellular CO_2 in Cd treated barley plants suggests that enzymatic dark reaction of photosynthesis was strongly affected (Januskaitiene, 2010). Contrary, in this study, the supply of greater Ni and Cd concentrations led to decrease the referenced parameter in upper leaves of both plant species. However, Kupper *et al*., (2007) reported that heavy metals (Cd) inhibited light phase rather than the Calvin cycle.

A third category of photosynthesis limitations, concerned heavy metal stress, affects the photochemistry of photosynthesis. The indicator function of chlorophyll fluorescence arises from the fact that fluorescence emission is complementary to alternative pathways of de- excitation which are primarily photochemistry and heat dissipation. Generally, fluorescence yield is highest when photochemistry and heat dissipation are lowest. Therefore changes in the fluorescence yield reflect changes in photochemical efficiency and heat dissipation. The potential photochemical efficiency (Fv/Fm) is a good indicator of the photosystems state, but, the Fv/F_0 ratio rather reflects earlier structural dysfunctions of the PSII. The F_0 variable represents the fluorescence emission from the antenna complex, even before the arrival of the photon energy to the reaction center of the PSII. In this state all the reaction centers are oxidized (open) (Lage- Pinto et. al., 2008; Krause and Weis, 1991).

The photochemical parameters were differentially affected in response to Zn, Ni and Cd stress. Wherever increase in F_0 may result from some damage in the reaction PSII reaction center, which reduces the ability to transfer energy from the LHCII antenna complex to the reaction center (Lage- Pinto et. al., 2008). The increase in the Fm values may be associated with an elevated difficulty in electron transport, which decreases the ability of reoxidation of the primary QA electron acceptor (Lage- Pinto *et. al*., 2008).

5. CONCLUSIONS

In this study, the effect of Zn, Ni and Cd concentration on *N. tabacum* and *N. glauca* plants was examined. The plants of both plant species produce rich biomass production and root system, while studies have shown that they appear resistance in heavy metals.

The response of the two Solanaceous species was examined in two plant systems, while morphological, physiological and biochemical parameters correlated with the concentration of Zn, Ni and Cd. Our data revealed that the three metal tested significantly affected all the parameters tested. The main conclusions are:

- ⇛ Exogenous heavy metal concentration strong correlated with the accumulation in both plant species. Both Zn and Cd accumulated more in roots and lower leaves, while Ni appeared differential accumulation pattern.
- ⇛ Reduction in all photosynthetic parameters observed, especially at the higher concentration metal applied.
- ⇛ Alterations in parameters related with Nitrogen metabolism occurred by the three metals. Variations in prline accumulation and in protein profile induced. The expression of Ammonium assimilating enzymes was differently affected. Specific activities and protein levela of GS and Fd-GOGAT showed a reduced trend. On the other hand, GDH appeared an increase trend.

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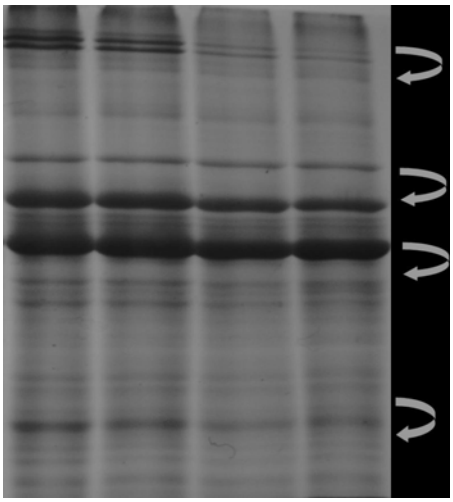
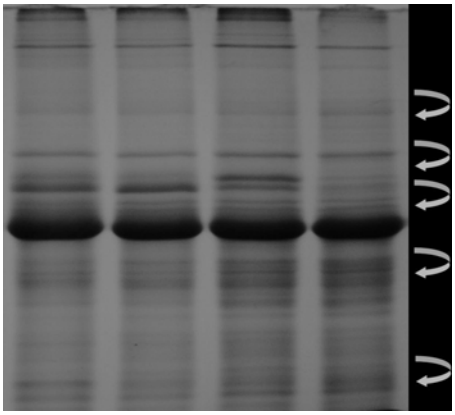
APPENDIX A

SDS-PAGE Electrophoresis of protein extracts of treated N. tabacum and N. glauca plants





APPENDIX B

EXPERIMENT 1

Zink

Statistical Analysis for Figure : 3.1. and Table: 3.1.

Zn aas Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Lower Leaves	Between Groups	454690,936	3	151563,645	20,172	,000
	Within Groups	60107,371	8	7513,421		
	Total	514798,307	11			
Upper Leaves	Between Groups	125501,376	3	41833,792	37,417	,000
	Within Groups	8944,301	8	1118,038		
	Total	134445,677	11			
Lower Shoot	Between Groups	99361,967	3	33120,656	31,742	,000
	Within Groups	8347,364	8	1043,421		
	Total	107709,331	11			
Upper Shoot	Between Groups	44008,616	3	14669,539	18,616	,001
	Within Groups	6303,993	8	787,999		
	Total	50312,608	11			
Roots	Between Groups	478013,586	3	159337,862	24,763	,000
	Within Groups	51476,570	8	6434,571		
	Total	529490,156	11			

Statistical Analysis For Figure: 3.2. and Table: 3.2.

Zn aas Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.

Lower Leaves	Between Groups	474869,839	3	158289,946	19,937	,000
	Within Groups	63514,873	8	7939,359		
	Total	538384,712	11			
Upper Leaves	Between Groups	70412,364	3	23470,788	32,593	,000
	Within Groups	5760,880	8	720,110		
	Total	76173,244	11			
Lower Shoot	Between Groups	111317,017	3	37105,672	89,747	,000
	Within Groups	3307,572	8	413,447		
	Total	114624,590	11			
Upper Shoot	Between Groups	78174,754	3	26058,251	73,061	,000
	Within Groups	2853,296	8	356,662		
	Total	81028,050	11			
Roots	Between Groups	1235631,044	3	411877,015	66,038	,000
	Within Groups	49895,614	8	6236,952		
	Total	1285526,659	11			

Statistical Analysis for Figure: 3.3.

Height (cm) Zn Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1761,902	3	587,301	7,345	,000
Within Groups	3438,056	43	79,955		
Total	5199,957	46			

Statistical Analysis for Figure: 3.3.

Height (cm) Zn Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2458,331	3	819,444	37,655	,000
Within Groups	1218,657	56	21,762		
Total	3676,988	59			

Statistical Analysis for Figure: 3.4

Number of Leaves Zn

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Number of Leaves Tabacum	Between Groups	69,606	3	23,202	6,111	0,001
	Within Groups	227,804	60	3,797		
	Total	297,409	63			
Number of Leaves Glauca	Between Groups	58,834	3	19,611	5,685	0,002
	Within Groups	206,983	60	3,45		
	Total	265,817	63			

Statistical Analysis for Figure: 3.5.

Fresh Weight (gr) Zn Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Leaves	Between Groups	196,241	3	65,414	,261	,853
	Within Groups	4014,768	16	250,923		
	Total	4211,009	19			
Shoot	Between Groups	1488,682	3	496,227	3,591	,037
	Within Groups	2210,776	16	138,174		
	Total	3699,458	19			
Roots	Between Groups	447,205	3	149,068	3,229	,050
	Within Groups	738,720	16	46,170		
	Total	1185,925	19			

Statistical Analysis for Figure: 3.5.

Fresh Weight (gr) Zn Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.

Leaves	Between Groups	1169,624	3	389,875	4,950	,011
	Within Groups	1417,702	18	78,761		
	Total	2587,326	21			
Shoot	Between Groups	4916,459	3	1638,820	6,515	,004
	Within Groups	4275,988	17	251,529		
	Total	9192,447	20			
Roots	Between Groups	404,108	3	134,703	4,609	,017
	Within Groups	467,592	16	29,225		
	Total	871,700	19			

Statistical Analysis for Figure: 3.6.

Chlorophyll Content Zn Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Content a Lower Leaves	Between Groups	0,015	3	0,005	8,022	0,003
	Within Groups	0,008	12	0,001		
	Total	0,023	15			
Chlorophyll Content a Upper Leaves	Between Groups	0,046	3	0,015	2,155	0,146
	Within Groups	0,085	12	0,007		
	Total	0,131	15			
Chlorophyll Conten b Lower Leaves	Between Groups	0,002	3	0,001	2,795	0,086
	Within Groups	0,003	12	0		
	Total	0,005	15			
Chlorophyll Conten b Upper Leaves	Between Groups	0,016	3	0,005	2,531	0,106
	Within Groups	0,024	12	0,002		
	Total	0,04	15			

Statistical Analysis for Figure: 3.6.

Chlorophyll Content Zn Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Content a Lower Leaves	Between Groups	0,007	3	0,002	1,472	0,272
	Within Groups	0,019	12	0,002		
	Total	0,026	15			
Chlorophyll Content a Upper Leaves	Between Groups	0,051	3	0,017	5,35	0,014
	Within Groups	0,038	12	0,003		
	Total	0,089	15			
Chlorophyll Content b Lower Leaves	Between Groups	0,002	3	0,001	0,693	0,574
	Within Groups	0,012	12	0,001		
	Total	0,014	15			
Chlorophyll Content b Upper Leaves	Between Groups	0,014	3	0,005	4,512	0,024
	Within Groups	0,013	12	0,001		
	Total	0,027	15			

Statistical Analysis for Figure: 3.7.

Total Chlorophyll Content Zn Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Total Chlorophyll Content Lower Leaves	Between Groups	0,027	3	0,009	6,821	0,006
	Within Groups	0,016	12	0,001		
	Total	0,043	15			
Total Chlorophyll	Between Groups	0,114	3	0,038	2,316	0,127

Contant	Within Groups	0,197	12	0,016		
Upper Leaves	Total	0,312	15			

Statistical Analysis for Figure: 3.7.

Total Chlorophyll Content Zn Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Total Chlorophyll Content Lower Leaves	Between Groups	0,013	3	0,004	0,943	0,45
	Within Groups	0,057	12	0,005		
	Total	0,07	15			
Total Chlorophyll content Upper Leaves	Between Groups	0,119	3	0,04	5,137	0,016
	Within Groups	0,093	12	0,008		
	Total	0,212	15			

Statistical Analysis for Table: 3.3.

Chlorophyll Content a/b ratio Zn Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Conten ab Lower Leaves	Between Groups	0,312	3	0,104	0,96	0,443
	Within Groups	1,302	12	0,108		
	Total	1,614	15			
Chlorophyll Conten ab Upper Leaves	Between Groups	0,325	3	0,108	2,438	0,115
	Within Groups	0,533	12	0,044		
	Total	0,857	15			

Statistical Analysis for Table: 3.3.

Chlorophyll Content a/b ratio Zn Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Content ab Lower Leaves	Between Groups	0,324	3	0,108	1,223	0,344
	Within Groups	1,06	12	0,088		
	Total	1,385	15			
Chlorophyll Content ab Upper Leaves	Between Groups	1,179	3	0,393	6,297	0,008
	Within Groups	0,749	12	0,062		
	Total	1,928	15			

Multiple Comparisons

LSD

Dependent Variable	(I) mMZn	(J) mMZn	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
FvmLoZn	,00	,50	,00815	,03834	,833	-,0704	,0867
		2,50	-,03031	,03834	,436	-,1088	,0482
		5,00	,09665*	,03834	,018	,0181	,1752
	,50	,00	-,00815	,03834	,833	-,0867	,0704
		2,50	-,03846	,03834	,324	-,1170	,0401
		5,00	,08850*	,03834	,029	,0100	,1670
	2,50	,00	,03031	,03834	,436	-,0482	,1088
		,50	,03846	,03834	,324	-,0401	,1170
		5,00	,12696*	,03834	,003	,0484	,2055
	5,00	,00	-,09665*	,03834	,018	-,1752	-,0181
		,50	-,08850*	,03834	,029	-,1670	-,0100
		2,50	-,12696*	,03834	,003	-,2055	-,0484
Fv0LoZn	,00	,50	,08335	,31951	,796	-,5711	,7378
		2,50	-,36414	,31951	,264	-1,0186	,2904
		5,00	,73640*	,31951	,029	,0819	1,3909
	,50	,00	-,08335	,31951	,796	-,7378	,5711
		2,50	-,44748	,31951	,172	-1,1020	,2070
		5,00	,65305	,31951	,050	-,0014	1,3075
	2,50	,00	,36414	,31951	,264	-,2904	1,0186

		,50	,44748	,31951	,172	-,2070	1,1020
		5,00	1,10054*	,31951	,002	,4460	1,7550
	5,00	,00	-,73640*	,31951	,029	-1,3909	-,0819
		,50	-,65305	,31951	,050	-1,3075	,0014
		2,50	-1,10054*	,31951	,002	-1,7550	-,4460
FoLoZn	,00	,50	-12,50000	132,30866	,925	-283,5220	258,5220
		2,50	151,25000	132,30866	,263	-119,7720	422,2720
		5,00	-151,25000	132,30866	,263	-422,2720	119,7720
	,50	,00	12,50000	132,30866	,925	-258,5220	283,5220
		2,50	163,75000	132,30866	,226	-107,2720	434,7720
		5,00	-138,75000	132,30866	,303	-409,7720	132,2720
	2,50	,00	-151,25000	132,30866	,263	-422,2720	119,7720
		,50	-163,75000	132,30866	,226	-434,7720	107,2720
		5,00	-302,50000*	132,30866	,030	-573,5220	-31,4780
	5,00	,00	151,25000	132,30866	,263	-119,7720	422,2720
		,50	138,75000	132,30866	,303	-132,2720	409,7720
		2,50	302,50000*	132,30866	,030	31,4780	573,5220
FmLoZn	,00	,50	106,25000	147,62820	,478	-196,1527	408,6527
		2,50	93,75000	147,62820	,531	-208,6527	396,1527
		5,00	683,75000*	147,62820	,000	381,3473	986,1527
	,50	,00	-106,25000	147,62820	,478	-408,6527	196,1527
		2,50	-12,50000	147,62820	,933	-314,9027	289,9027
		5,00	577,50000*	147,62820	,001	275,0973	879,9027
	2,50	,00	-93,75000	147,62820	,531	-396,1527	208,6527
		,50	12,50000	147,62820	,933	-289,9027	314,9027
		5,00	590,00000*	147,62820	,000	287,5973	892,4027
	5,00	,00	-683,75000*	147,62820	,000	-986,1527	-381,3473
		,50	-577,50000*	147,62820	,001	-879,9027	-275,0973
		2,50	-590,00000*	147,62820	,000	-892,4027	-287,5973
FvLoZn	,00	,50	118,75000	203,28524	,564	-297,6609	535,1609
		2,50	-57,50000	203,28524	,779	-473,9109	358,9109
		5,00	835,00000*	203,28524	,000	418,5891	1251,4109
	,50	,00	-118,75000	203,28524	,564	-535,1609	297,6609
		2,50	-176,25000	203,28524	,393	-592,6609	240,1609
		5,00	716,25000*	203,28524	,001	299,8391	1132,6609
	2,50	,00	57,50000	203,28524	,779	-358,9109	473,9109
		,50	176,25000	203,28524	,393	-240,1609	592,6609
		5,00	892,50000*	203,28524	,000	476,0891	1308,9109
	5,00	,00	-835,00000*	203,28524	,000	-1251,4109	-418,5891
		,50	-716,25000*	203,28524	,001	-1132,6609	-299,8391
		2,50	-892,50000*	203,28524	,000	-1308,9109	-476,0891

Statistical Analysis for Figure:

Proline Zn

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Proline Lower Leaves Tabacum	Between Groups	2380,593	3	793,531	70,708	0
	Within Groups	89,781	8	11,223		
	Total	2470,374	11			
Proline Upper Leaves Tabacum	Between Groups	1947,022	3	649,007	21,945	0
	Within Groups	236,589	8	29,574		
	Total	2183,611	11			
Proline Lower Leaves Glauca	Between Groups	253,25	3	84,417	11,414	0,003
	Within Groups	59,168	8	7,396		
	Total	312,417	11			
Proline Upper Leaves Glauca	Between Groups	947,55	3	315,85	19,416	0
	Within Groups	130,138	8	16,267		
	Total	1077,689	11			

Nickel

Statistical Analysis for Figure: 3.18. and Table: 3.5.

Nickel aas Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Lower Leaves	Between Groups	141383,599	3	47127,866	113,376	,000
	Within Groups	3325,428	8	415,678		
	Total	144709,026	11			

Upper Leaves	Between Groups	27583,003	3	9194,334	19,872	,000
	Within Groups	3701,450	8	462,681		
	Total	31284,454	11			
Lower Shoot	Between Groups	8318,995	3	2772,998	8,126	,008
	Within Groups	2729,893	8	341,237		
	Total	11048,888	11			
Upper Shoot	Between Groups	14709,097	3	4903,032	21,344	,000
	Within Groups	1837,681	8	229,710		
	Total	16546,778	11			
Roots	Between Groups	290436,811	3	96812,270	13,062	,002
	Within Groups	59293,346	8	7411,668		
	Total	349730,157	11			

Statistical Analysis for Figure: 3.19 and Table: 3.6.

Nickel aas Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Lower Leaves	Between Groups	61891,871	3	20630,624	74,779	,000
	Within Groups	2207,100	8	275,888		
	Total	64098,972	11			
Upper Leaves	Between Groups	86844,365	3	28948,122	79,048	,000
	Within Groups	2929,688	8	366,211		
	Total	89774,052	11			
Lower Shoot	Between Groups	2363,287	3	787,762	4,425	,041
	Within Groups	1424,180	8	178,022		
	Total	3787,466	11			
Upper Shoot	Between Groups	4724,303	3	1574,768	16,651	,001
	Within Groups	756,593	8	94,574		
	Total	5480,896	11			
Roots	Between Groups	363975,682	3	121325,227	245,730	,000

	Within Groups	3949,876	8	493,734		
	Total	367925,558	11			

Statistical Analysis for Figure: 3.20.

Height (cm) Nickel Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1293,561	3	431,187	4,309	,009
Within Groups	4602,919	46	100,063		
Total	5896,480	49			

Statiatical Analysis for Figure: 3.20.

Height (cm) Nickel Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1392,787	3	464,262	20,495	,000
Within Groups	1268,543	56	22,653		
Total	2661,330	59			

Statistical Analysis for Figure: 3.21.

Number of Leaves LSD Nickel

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Number of Leaves Tabacum	Between Groups	8,967	3	2,989	,893	,450
	Within Groups	200,775	60	3,346		
	Total	209,743	63			
Number	Between Groups	41,701	3	13,900	6,023	,001

of Leaves Glauca	Within Groups	138,483	60	2,308		
	Total	180,184	63			

Statistical Analysis for Figure: 3.22.

Fresh Weight (gr) Nickel Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Leaves	Between Groups	725,926	3	241,975	1,824	,183
	Within Groups	2122,760	16	132,672		
	Total	2848,686	19			
Shoot	Between Groups	411,228	3	137,076	2,103	,140
	Within Groups	1042,780	16	65,174		
	Total	1454,008	19			
Roots	Between Groups	397,453	3	132,485	3,698	,034
	Within Groups	573,292	16	35,831		
	Total	970,745	19			

Statistical Analysis for Figure: 3.22

Fresh Weight (gr) Nickel Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Leaves	Between Groups	1059,828	3	353,276	4,760	,015
	Within Groups	1187,580	16	74,224		
	Total	2247,408	19			
Shoot	Between Groups	5686,746	3	1895,582	9,200	,001
	Within Groups	3296,572	16	206,036		
	Total	8983,318	19			
Roots	Between Groups	392,600	3	130,867	3,725	,033
	Within Groups	562,172	16	35,136		
	Total	954,772	19			

Statistical Analysis for Figure: 3.23.

Chlorophyll Content Nickel Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Content a Lower Leaves	Between Groups	0,011	3	0,004	2,135	0,149
	Within Groups	0,021	12	0,002		
	Total	0,032	15			
Chlorophyll Conten a Upper Leaves	Between Groups	0,035	3	0,012	4,549	0,024
	Within Groups	0,031	12	0,003		
	Total	0,067	15			
Chlorophyll Content b Lower Leaves	Between Groups	0,001	3	0	0,616	0,618
	Within Groups	0,006	12	0		
	Total	0,007	15			
Chlorophyll Content b Upper Leaves	Between Groups	0,014	3	0,005	3,134	0,066
	Within Groups	0,017	12	0,001		
	Total	0,031	15			

Statistical Analysis for Figure: 3.23.

Chlorophyll Content Nickel Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Content a Lower Leaves	Between Groups	0,022	3	0,007	4,537	0,024
	Within Groups	0,02	12	0,002		
	Total	0,042	15			
Chlorophyll Content a	Between Groups	0,029	3	0,01	2,213	0,139

Upper Leaves	Within Groups	0,052	12	0,004		
	Total	0,08	15			
Chlorophyll Content b Lower Leaves	Between Groups	0,003	3	0,001	1,852	0,191
	Within Groups	0,007	12	0,001		
	Total	0,011	15			
Chlorophyll Content b Upper Leaves	Between Groups	0,008	3	0,003	2,353	0,124
	Within Groups	0,014	12	0,001		
	Total	0,022	15			

Statistical Analysis for Figure: 3.24.

Total Chlorophyll Content Nickel Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Total Chlorophyll Conten Lower Leaves	Between Groups	0,017	3	0,006	1,478	0,27
	Within Groups	0,047	12	0,004		
	Total	0,064	15			
Total Chlorophyll Content Upper Leaves	Between Groups	0,093	3	0,031	4,005	0,034
	Within Groups	0,093	12	0,008		
	Total	0,186	15			

Statistical Analysis for Figure: 3.24.

Total Chlorophyll Content Nickel Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Total Chlorophyll Content Lower Leaves	Between Groups	0,043	3	0,014	3,635	0,045
	Within Groups	0,047	12	0,004		
	Total	0,09	15			
Total	Between	0,067	3	0,022	2,332	0,126

Chlorophyll Content Upper Leaves	Groups					
	Within Groups	0,115	12	0,01		
	Total	0,182	15			

Statistical Analysis for Table: 3.7.

Chlorophyll Content a/b ratio Nickel Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Content ab Lower Leaves	Between Groups	0,405	3	0,135	2,711	0,092
	Within Groups	0,598	12	0,05		
	Total	1,003	15			
Chlorophyll Content ab Upper Leaves	Between Groups	0,358	3	0,119	2,136	0,149
	Within Groups	0,671	12	0,056		
	Total	1,029	15			

Statistical Analysis for Table: 3.7.

Chlorophyll Content a/b ratio Nickel Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Content ab Lower Leaves	Between Groups	0,036	3	0,012	0,141	0,933
	Within Groups	1,021	12	0,085		
	Total	1,058	15			
Chlorophyll Content ab Upper Leaves	Between Groups	0,463	3	0,154	2,461	0,113
	Within Groups	0,752	12	0,063		
	Total	1,215	15			

Statistical Analysis for Figure:

Proline Nickel

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Proline Lower Leaves Tabacum	Between Groups	1349,013	3	449,671	12,898	0,002
	Within Groups	278,9	8	34,862		
	Total	1627,913	11			
Proline Upper Leaves Tabacum	Between Groups	1205,05	3	401,683	31,921	0
	Within Groups	100,668	8	12,583		
	Total	1305,718	11			
Proline Lower Leaves Glauca	Between Groups	344,319	3	114,773	19,251	0,001
	Within Groups	47,694	8	5,962		
	Total	392,013	11			
Proline Upper Leaves Glauca	Between Groups	2080,474	3	693,491	143,648	0
	Within Groups	38,622	8	4,828		
	Total	2119,095	11			

Cadmium

Statistical Analysis for Figure: 3.35 and Table: 3.8.

Cd aas Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Lower Leaves	Between Groups	2363554,746	3	787851,582	21,075	,000
	Within Groups	299062,744	8	37382,843		
	Total	2662617,490	11			
Upper Leaves	Between Groups	216207,912	3	72069,304	55,138	,000
	Within Groups	10456,479	8	1307,060		
	Total	226664,392	11			
Lower Shoot	Between Groups	593561,131	3	197853,710	54,358	,000
	Within Groups	29118,822	8	3639,853		
	Total	622679,953	11			

Upper Shoot	Between Groups	410252,858	3	136750,953	90,570	,000
	Within Groups	12079,157	8	1509,895		
	Total	422332,015	11			
Roots	Between Groups	2770656,791	3	923552,264	11,067	,003
	Within Groups	667618,481	8	83452,310		
	Total	3438275,272	11			

Statistical Analysis for Figure: 3.36 and Table: 3.9.

Cd aas Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Lower Leaves	Between Groups	503186,600	3	167728,867	57,428	,000
	Within Groups	23365,582	8	2920,698		
	Total	526552,183	11			
Upper Leaves	Between Groups	288616,715	3	96205,572	42,361	,000
	Within Groups	18168,646	8	2271,081		
	Total	306785,361	11			
Lower Shoot	Between Groups	247109,980	3	82369,993	40,968	,000
	Within Groups	16084,768	8	2010,596		
	Total	263194,749	11			
Upper Shoot	Between Groups	162943,322	3	54314,441	51,409	,000
	Within Groups	8452,088	8	1056,511		
	Total	171395,410	11			
Roots	Between Groups	2699489,005	3	899829,668	101,085	,000
	Within Groups	71213,691	8	8901,711		
	Total	2770702,696	11			

Statistical Analysis for Figure: 3.37.

Height (cm) Cd Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.

Between Groups	9358,425	3	3119,475	26,408	,000
Within Groups	5906,334	50	118,127		
Total	15264,759	53			

Statistical Analysis for Figure: 3.37.

Height (cm) Cd Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3053,473	3	1017,824	45,998	,000
Within Groups	1239,145	56	22,128		
Total	4292,618	59			

Statistical Analysis for Figure: 3.38.

Number of Leaves LSD Cd

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Number of Leaves Tabacum	Between Groups	74,672	3	24,891	9,020	,000
	Within Groups	165,563	60	2,759		
	Total	240,234	63			
Number of Leaves Glauca	Between Groups	1,622	3	,541	,250	,861
	Within Groups	129,858	60	2,164		
	Total	131,480	63			

Statistical Analysis for Figure: 3.39.

Fresh Weight (gr) Cd Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Leaves	Between Groups	1900,200	3	633,400	3,611	,037
	Within Groups	2806,820	16	175,426		

	Total	4707,020	19			
Shoot	Between Groups	5245,612	3	1748,537	22,683	,000
	Within Groups	1233,388	16	77,087		
	Total	6479,000	19			
Roots	Between Groups	1490,078	3	496,693	12,407	,000
	Within Groups	640,528	16	40,033		
	Total	2130,606	19			

Statistical Analysis for Figure: 3.39.

Fresh Weight (gr) Cd Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Leaves	Between Groups	1970,502	3	656,834	1,948	,160
	Within Groups	5730,821	17	337,107		
	Total	7701,323	20			
Shoot	Between Groups	9815,432	3	3271,811	9,986	,001
	Within Groups	5570,040	17	327,649		
	Total	15385,472	20			
Roots	Between Groups	1023,532	3	341,177	6,672	,004
	Within Groups	818,180	16	51,136		
	Total	1841,712	19			

Statistical Analysis for Figure: 3.40.

Chlorophyll Content Cd Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Content a Lower	Between Groups	0,031	3	0,01	3,661	0,044
	Within Groups	0,034	12	0,003		

Leaves	Total	0,066	15			
Chlorophyll Content a Upper Leaves	Between Groups	0,048	3	0,016	5,224	0,015
	Within Groups	0,037	12	0,003		
	Total	0,085	15			
Chlorophyll Content b Lower Leaves	Between Groups	0,005	3	0,002	3,912	0,037
	Within Groups	0,005	12	0		
	Total	0,01	15			
Chlorophyll Content b Upper Leaves	Between Groups	0,02	3	0,007	7,945	0,003
	Within Groups	0,01	12	0,001		
	Total	0,03	15			

Statistical Analysis for Figure: 3.40.

Chlorophyll Content Cd Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Conten a Lower Leaves	Between Groups	0,026	3	0,009	3,584	0,047
	Within Groups	0,029	12	0,002		
	Total	0,055	15			
Chlorophyll Content a Upper Leaves	Between Groups	0,008	3	0,003	0,545	0,661
	Within Groups	0,061	12	0,005		
	Total	0,069	15			
Chlorophyll Conten b Lower Leaves	Between Groups	0,008	3	0,003	2,845	0,082
	Within Groups	0,011	12	0,001		
	Total	0,019	15			
Chlorophyll Content b Upper	Between Groups	0,002	3	0,001	0,287	0,834
	Within Groups	0,027	12	0,002		

Leaves	Total	0,029	15			

Satistical Analysis for Figure: 3.41.

Total Chlorophyll Content Cd Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Total Chlorophyll Content Lower Leaves	Between Groups	0,06	3	0,02	3,829	0,039
	Within Groups	0,062	12	0,005		
	Total	0,122	15			
Total Chlorophyll Content Upper Leaves	Between Groups	0,129	3	0,043	6,23	0,009
	Within Groups	0,083	12	0,007		
	Total	0,212	15			

Statistical Analysis for Figure: 3.41.

Total Chlorophyll Content Cd Glauca

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Total Chlorophyll Content Lower Leaves	Between Groups	0,06	3	0,02	3,282	0,058
	Within Groups	0,073	12	0,006		
	Total	0,134	15			
Total Chlorophyll Content Upper Leaves	Between Groups	0,018	3	0,006	0,445	0,725
	Within Groups	0,16	12	0,013		
	Total	0,178	15			

Statistical Analysis for Table: 3.10.

Chlorophyll Content a/b ratio Cd Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.

Chlorophyll Content ab Lower Leaves	Between Groups	0,189	3	0,063	0,945	0,449
	Within Groups	0,802	12	0,067		
	Total	0,991	15			
Chlorophyll Content ab Upper Leaves	Between Groups	0,788	3	0,263	3,671	0,044
	Within Groups	0,859	12	0,072		
	Total	1,646	15			

Statistical Analysis for Table: 3.10.

Chlorophyll Content a/b ratio Cd Tabacum

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Chlorophyll Conten ab Lower Leaves	Between Groups	0,406	3	0,135	2,499	0,109
	Within Groups	0,651	12	0,054		
	Total	1,057	15			
Chlorophyll Content ab Upper Leaves	Between Groups	0,045	3	0,015	0,096	0,961
	Within Groups	1,878	12	0,156		
	Total	1,923	15			

Statistical Analysis for Figure:

Proline Cd

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Proline Lower Leaves Tabacum	Between Groups	142,964	3	47,655	1,197	0,371
	Within Groups	318,421	8	39,803		
	Total	461,385	11			

Proline Upper Leaves Tabacum	Between Groups	2362,761	3	787,587	38,035	0
	Within Groups	165,655	8	20,707		
	Total	2528,416	11			
Proline Lower Leaves Glauca	Between Groups	143,903	3	47,968	14,174	0,001
	Within Groups	27,073	8	3,384		
	Total	170,976	11			
Proline Upper Leaves Glauca	Between Groups	1310,502	3	436,834	66,16	0
	Within Groups	52,822	8	6,603		
	Total	1363,323	11			

EXPERIMENT 2

Zink

Statistical Analysis for Table:

Height (cm) Zn Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	49,724	3	16,575	3,606	,017
Within Groups	372,349	81	4,597		
Total	422,072	84			

Statistical Analysis for Table:

Height (cm) Zn Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10,485	3	3,495	3,389	,024
Within Groups	62,900	61	1,031		

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10,485	3	3,495	3,389	,024
Within Groups	62,900	61	1,031		
Total	73,385	64			

Statistical Analysis for Table:

Fresh Weight (gr) Zn Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19,162	3	6,387	15,004	,000
Within Groups	22,137	52	,426		
Total	41,298	55			

Statistical Analysis for Table:

Fresh Weight (gr) Zn Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28,139	3	9,380	6,770	,000
Within Groups	91,446	66	1,386		
Total	119,585	69			

Statistical Analysis for Table:

SPAD Zn Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	53,772	3	17,924	3,001	,035
Within Groups	483,832	81	5,973		
Total	537,604	84			

Nickel

Statistical Analysis for Table:

Height (cm) Nickel Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	84,576	3	28,192	4,807	,004
Within Groups	469,171	80	5,865		
Total	553,747	83			

Statistical Analysis for Table:

Number of Leaves Nickel Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12,880	3	4,293	5,282	,002
Within Groups	66,655	82	,813		
Total	79,535	85			

Statistical Analysis for Table:

Fresh Weight (gr) Nickel Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12,412	3	4,137	17,012	,000
Within Groups	12,403	51	,243		
Total	24,815	54			

Statistical Analysis for Table:

Fresh Weight (gr) Nickel Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28,657	3	9,552	5,895	,001
Within Groups	111,802	69	1,620		
Total	140,459	72			

Statistical Analysis for Table:

SPAD Nickel Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	73,052	3	24,351	6,488	,001
Within Groups	307,777	82	3,753		
Total	380,829	85			

Cadmium

Statistical Analysis for Table:

Height (cm) Cd Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	61,091	3	20,364	3,584	,017
Within Groups	477,338	84	5,683		
Total	538,429	87			

Statistical Analysis for Table:

Height (cm) Cd Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.

Between Groups	20,165	3	6,722	3,912	,012
Within Groups	120,287	70	1,718		
Total	140,451	73			

Statistical Analysis for Table:

Number of Leaves Cd Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1,537	3	,512	,812	,490
Within Groups	52,963	84	,631		
Total	54,500	87			

Statistical Analysis for Table:

Number of Leaves Cd Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8,150	3	2,717	3,505	,040
Within Groups	12,400	16	,775		
Total	20,550	19			

Statistical Analysis for Table:

Fresh Weight (gr) Cd Glauca

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	36,148	3	12,049	13,868	,000
Within Groups	57,346	66	,869		
Total	93,495	69			

Statistical Analysis for Table:

SPAD Cd Tabacum

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	82,971	3	27,657	7,943	,000
Within Groups	292,476	84	3,482		
Total	375,446	87			

Statistical Analysis-Correlations for Fig. : 3.1. and Table: 3.1.

CORRELATIONS

		mM Zn	Lower Leaves	Upper Leaves	Lower Shoot	Upper Shoot	Roots
mM Zn Tabacum	Pearson Correlation	1	,747**	,882**	,946**	,881**	,707*
	Sig. (2-tailed)		,005	,000	,000	,000	,010

**. Correlation is significant at the 0.01 level (2-tailed).

Statistical Analysis-Correlations for Fig.: 3.2. and Table: 3.2.

CORRELATIONS

		mM Zn	Lower Leaves	Upper Leaves	Lower Shoot	Upper Shoot	Roots
mM Zn Glauca	Pearson Correlation	1	,867**	,781**	,912**	,935**	,808**
	Sig. (2-tailed)		,000	,003	,000	,000	,001

**. Correlation is significant at the 0.01 level (2-tailed).

Statistical Analysis-Correlations for Fig.: 3.5.

Fresh Weight (gr) Zn Tabacum

CORRELATIONS

		mM Zn	Fresh Weight of Leaves	Fresh Weight of shoot	Fresh Weight of Roots
mM Zn	Pearson Correlation	1	-,057	-,628**	-,591**
	Sig. (2-tailed)		,810	,003	,006

**. Correlation is significant at the 0.01 level (2-tailed).

Statistical Analysis-Correlations for Fig.: 3.5.

Fresh Weight (gr) Zn Glauca

CORRELATIONS

		mM Cd	Fresh Weight of Leaves	Fresh Weight of Shoot	Fresh Weight of Roots
mM Cd	Pearson Correlation	1	-,432	-,762**	-,696**
	Sig. (2-tailed)		,050	,000	,001

**. Correlation is significant at the 0.01 level (2-tailed).

Glauca

CORRELATIONS

		mM Zn	Total Chlorophyll Content Upper Leaves	SPAD Lower Leaves	SPAD Upper Leaves
mM Zn	Pearson Correlation	1	-,663**	-,468**	-,645**
	Sig. (2-tailed)		,005	,007	,000

**. Correlation is significant at the 0.01 level (2-tailed).

Glauca

CORRELATIONS

		mM Zn	Pn Lower leaves	Pn Upper Leaves	Gs Lower Leaves	Gs Upper Leaves	Ci Upper Leaves
Mm Zn	Pearson Correlation	1	-,279	-,585**	,019	-,499**	-,211
	Sig. (2-tailed)		,095	,000	,913	,002	,211

**. Correlation is significant at the 0.01 level (2-tailed).

Nickel

Statistical Analysis-Correlations for Fig.: 3.18. and Table: 3.5.

CORRELATIONS

		mM Ni	Lower Leaves	Upper Leaves	Lower Shoot	Upper Shoot	Roots
mM Ni Tabacum	Pearson Correlation	1	,977**	,926**	,810**	,861**	,773**
	Sig. (2-tailed)		,000	,000	,001	,000	,003

**. Correlation is significant at the 0.01 level (2-tailed).

Statistical Analysis-Correlations for Fig.: 3.19 and Table: 3.6.

CORRELATIONS

	mM Ni	Lower Leaves	Upper Leaves	Lower Shoot	Upper Shoot	Roots

mM Ni Glauca	Pearson Correlation	1	,967**	,958**	,564	,924**	,903**
	Sig. (2-tailed)		,000	,000	,056	,000	,000

**. Correlation is significant at the 0.01 level (2-tailed).

Statistical Analysis-Correlations for Fig.: 3.22.

Fresh Weight (gr) Nickel Tabacum

CORRELATIONS

		mM Ni	Fresh Weight of Leaves	Fresh Weight of Shoot	Fresh Weight of Roots
mM Ni	Pearson Correlation	1	-,079	-,459*	-,600**
	Sig. (2-tailed)		,742	,042	,005

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Statistical Analysis-Correlations for Fig.: 3.22

Fresh Weight (gr) Nickel Glauca

CORRELATIONS

		mM Ni	Fresh Weight of Leaves	Fresh Weight of Shoot	Fresh Weight of Roots
mM Ni	Pearson Correlation	1	-,679**	-,710**	-,573**
	Sig. (2-tailed)		,001	,000	,008

**. Correlation is significant at the 0.01 level (2-tailed).

Cadmium

Statistical Analysis-Correlations for Fig.: 3.35 and Table: 3.8.

CORRELATIONS

		mM Cd	Lower Leaves	Upper Leaves	Lower Shoot	Upper Shoot	Roots
mM Cd Tabacum	Pearson Correlation	1	,906**	,887**	,961**	,949**	,809**
	Sig. (2-tailed)		,000	,000	,000	,000	,001

**. Correlation is significant at the 0.01 level (2-tailed).

Statistical Analysis-Correlations for Fig.: 3.36 and Table: 3.9.

CORRELATIONS

	mM Cd	Lower Leaves	Upper Leaves	Lower Shoot	Upper Shoot	Roots

mM Cd Glauca	Pearson Correlation	1	,962**	,845**	,968**	,942**	,979**
	Sig. (2-tailed)		,000	,001	,000	,000	,000

**. Correlation is significant at the 0.01 level (2-tailed).

Statistical Analysis-Correlatins for Fig.: 3.39.

Fresh Weight (gr) Cd Tabacum

CORRELATIONS

		mM Cd	Fresh Weight of Leaves	Fresh Weight of Shoot	Fresh Weight of Roots
mM Cd	Pearson Correlation	1	-,532*	-,791**	-,832**
	Sig. (2-tailed)		,016	,000	,000

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Statistical Analysis-Correlations for Fig.: 3.39.

Fresh Weight (gr) Cd Glauca

CORRELATIONS

		mM Zn	Fresh Weight of Leaves	Fresh Weight of Shoot	Fresh Weight of Roots
mM Zn	Pearson Correlation	1	-,628**	-,701**	-,612**
	Sig. (2-tailed)		,002	,000	,004

**. Correlation is significant at the 0.01 level (2-tailed).