Agricultural University of Athens
Department of Agricultural Biotechnology
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Photosynthetic Characteristics of Representative Plant Species of the Mediterranean Ecosystem

PhD Thesis

Sally Alloh Sumbele

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AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY
FACULTY OF PLANT BIOLOGY
LABORATORY OF PLANT PHYSIOLOGY

PHOTOSYNTHETIC CHARACTERISTICS OF REPRESENTATIVE PLANT SPECIES OF THE MEDITERRANEAN ECOSYSTEM

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Foreword

The writing of this dissertation has been one of the most significant challenges I have ever had to face. This study would not have been realized without the financial support I had from The Greek State Scholarship Board (I.K.Y.) for a 4 year PhD program at the Agricultural University of Athens. I am very grateful for that, and I thank all those who contributed in one way or another to this study or provided logistic help.

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Contents

Abstract

Key leaf properties tend to show predictable relationships with others at a global level, forming the so called “leaf economic spectrum”. Until now however, leaf traits related to protection and defense have not been fully incorporated into this spectrum. Moreover, the relationships between parameters of this spectrum have not been tested in plants thriving in the Mediterannean environment. Here, we tested the hypothesis that plant species of the Mediterannean flora show the same general relationships of the leaf economic spectrum and that parameters related to defence/protection (such as total phenolics and condensed tannins concentration, as well as the nitrogen to total phenolics concentration (N/TP) ratio) could be related to other key traits of leaf economic spectrum.

Functional (photosynthetic capacity, transpiration and dark respiration activity, nitrogen content, total phenolics and condensed tannins concentration, carbon and nitrogen stable isotope ratios, Chlorophyll fluorescence parameters) and structural parameters (leaf thickness and density, leaf mass per area, transparent leaf area in heterobaric leaves) were measured in 30 representative plant species (representing three life forms) from two sites (Parnitha and Domnista) with different climatic conditions.

Most of the relationships of the leaf economic spectrum were observed in the present study as well. Moreover, N/TP ratio was positively related with photosynthetic capacity expressed either per area ($A_{max,a}$) or per dry mass ($A_{max,m}$) and negatively correlated to leaf mass per area (LMA). These relationships were stronger than that between total leaf nitrogen concentration per dry mass (N_m) and $A_{max,m}$ or $A_{max,m}$, indicating that photosynthetic capacity is determined not by nitrogen investments *per se*, but also by the defense/protection demands. Among life forms, herbs showed higher N/TP ratio than trees and shrubs.

The results of the present study showed that N/TP ratio could be considered as an essential component of the leaf economic spectrum, indicating the balance between growth and defense/protection, in addition to its previously proposed roles as an indicator of leaf nutritional value and decomposition rates. The fact that there is a strong relationship between N/TP ratio and photosynthetic capacity, a physiological trait not directly related to herbivory or pathogen attack, indicates that this ratio possibly reflects the need of protection of primary metabolic processes against the side effects of stressful environments and that the levels of leaf phenolics in a certain species may reflect the different risk of photodamage.

1. Introduction

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1.1. Photosynthesis

Autotrophs obtain their energy through photosynthesis. It is a process whereby light energy from sunlight is converted to chemical energy in stable organic compounds, making use of carbon dioxide and water and releasing oxygen. It is the initial process through which energy is stored in plants and animals. Figure 1 shows the process in a simplified reaction. However the whole process is extremely complicated. It is composed of numerous individual light-sensitive and temperature-sensitive reactions which are closely linked and it involves photon absorption, electron transport and carbon metabolism (Ridge, 2002; Rost et al., 1998).

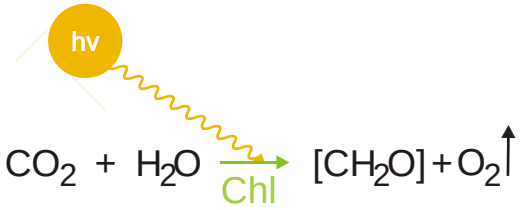

Figure 1. The simplified reaction of photosynthesis.

Photosynthetic reaction appears as the reverse of respiration reaction. Thus, when measuring rates of photosynthesis, consideration must be given to the process of respiration. Since photosynthesis and respiration occur at the same time, what is actually measured is net photosynthesis (NP) which is gross photosynthesis (GP) minus respiration (R), denoted as:

$$NP = GP - R$$

Note should therefore be taken each time the rate of photosynthesis is referred to, whether it is NP or GP. R rate can under some circumstances be evaluated as the dark Respiration (Rd) rate which is the rate of CO_2 release in total darkness and it takes always negative values. At the whole plant level, respiration reduces the rate of photosynthesis because CO_2 uptake is balanced by CO_2 released. At the community level, net gain in dry weight equals to net photosynthesis plus assimilation of all other inorganic elements minus respiration over time (Ridge, 2002).

In photosynthetic eukaryotes, photosynthesis takes place in the subcellular organelle known as the chloroplast. Within the thylakoid membrane of the chloroplast are located chlorophyll molecules that are bound to proteins. During the first stage of photosynthesis (the light-dependent reactions), absorption of photons by chlorophyll molecules leads to the photochemical oxidation of water to molecular oxygen, coupled with the generation of adenosine triphosphate (ATP) and reduced NADPH. Two major types of chlorophylls, *a* and *b* are found in vascular plants. Both types have the same basic structure with slight chemical modification. Chlorophyll appears green because it absorbs the red and blue wavelengths of the visible spectrum, leaving the green portion to be transmitted and

perceived. Their ratio varies considerably in higher plants, with chlorophyll *a* always having a higher concentration than chlorophyll *b*. Since chlorophyll *a* is the primary pigment, it is found in all photosynthetic organisms except anaerobic photosynthetic bacteria. In addition to chlorophyll *a* and *b,* another group of accessory pigments known as carotenoids absorb light of shorter wavelengths. (Northington and Schneider, 1996; Rost et al., 1998; Sestak, 1985; Taiz and Zeiger, 2006).

1.2. Light-dependent reactions of photosynthesis

The light-dependent reactions of photosynthesis take place in the chloroplast grana and stroma thylakoids, which are composed of stacks of membranous vesicles containing photosynthetic pigments. The pigments create complexes with proteins and are organized in a macromolecular structure known as antenna. The light-dependent reactions involve light absorption by pigment molecules within the antenna. The quantum energy absorbed by these pigments in the antenna is transferred to a specific chlorophyll *a* molecule in the photochemical center of photosystem II (PS II). This causes excitation of electrons of the chlorophyll *a* molecule. Consequently, electrons are lost and are transferred from photosystem II (PS II) to photosystem I (PS I) through intermediate electron carriers, namely plastoquinone (Q_A and Q_B), cytochrome b_6f (cyt b_6f) complex and plastocyanin (PC), a mobile carrier (Figure 2). One electron is lost (transferred) for each photon of energy absorbed. The electrons flow through redox pairs consisted of reducing agents (electron donors) and oxidizing agends (electron acceptors). Both photosystems are involved in the trapping of light energy during photosynthesis and each one possesses an antenna (known as light harvesting complexes I and II respectively). PS I has a central chlorophyll *a* molecule that absorbs best at a red wavelength of 700nm. Hence, it is known as the P_{700} molecule and the reaction center of PS I is referred to as P700. PS II also possesses a central chlorophyll *a* molecule. However, it absorbs best at a red wavelength of 680nm and is called the P_{680} molecule and its own reaction center is designated as P680. The electron lost by the chlorophyll *a* molecule is replaced by the donation of another electron from water. The splitting of water results in the release of molecular oxygen and contributed to the development of the proton gradient between the thylakoid lumen and the stroma. At PS I, light absorption causes excitation and transfer of electrons to a carrier, ferredoxin, and then to NADP reductase, the enzyme responsible for the generation of reducing power which is referred to as NADPH. The electron is replaced by the donation of another electron from PS II. This is the non-cyclic transfer of electrons from water to NADP via PSII, PSI and intermediate carriers. PS II is restricted to the inner membrane of grana whereas PS I and the ATP synthase are restricted to the unstacked stroma lamellae, which connects grana across the stroma of the chloroplast. Cytochrome b_6f occurs on both grana and stroma lamellae.

In summary, the absorption of light energy and subsequent transfer of electrons to $NADP^+$ has two important consequences: Water molecules are split through the process of photolysis, releasing electrons, H^+ in the thylakoid lumen and oxygen, and NADPH is formed. The oxygen released diffuses out of the leaf into the atmosphere and is used by aerobic organisms for respiration. Two of the hydrogen atoms produced when water is split

act as reducing power for NADP. The proton gradient generated during electron transport (with protons accumulating in the lumen of thylakoids, see Figure 2) is discharged via the ATP synthase for the production of ATP. All the energy absorbed from sunlight is finally stored as reducing power and in the ATP molecule through the process of photophosphorylation. As a result, the ATP and NADPH molecules provide the energy which is needed to run biochemical reactions (Bjorkman and Demmig, 1987; Lawlor, 2001; Ridge, 2002; Northington and Schneider, 1996; Schulze and Cadwell, 1995).

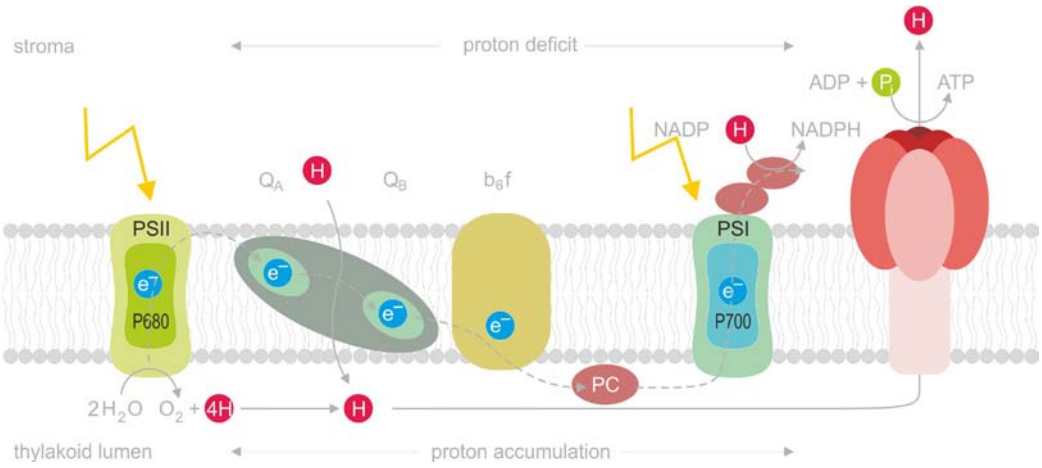

Figure 2. A simplified scheme of the light-dependent reactions of photosynthesis.

1.3. Light-independent (or dark) reactions of photosynthesis

These set of biochemical reactions do not depend directly on light energy. Nevertheless, if ATP and NADPH are available, these reactions are able to take place under both light and dark conditions and can even go up to completion when dark conditions persist. Thus, they are referred to as the "light-independent reactions" since light energy is not required at any stage of the process. Dark conditions do not necessarily imply that reactions must occur in darkness (Northington and Schneider, 1996).

There are four main biochemical pathways that have been distinguished so far, which are representative of the light-independent reactions of photosynthesis (Taiz and Zeiger, 2006):

1) The C_3 cycle or Calvin-Benson Cycle
2) The C_2 cycle (photorespiration)
3) The C_4 pathway or Hatch and Slack cycle
4) The Crassulacean Acid Metabolism (CAM)

1.3.1. C_3 or Calvin-Benson cycle

Carbon fixation is considered the most defining metabolic activity in autotrophs because it converts inorganic carbon to organic compounds and leads to a series of reactions known as the C_3 (because the first product, PGA, contains three carbons) or Calvin-Benson (in honor of its discoverer) cycle which achieves net carbon fixation. These reactions occur in the chloroplast stroma.

Carbon dioxide enters the cycle when it combines with the acceptor molecule ribulose bisphosphate (RuBP) which undergoes carboxylation (Figure 3). This reaction is catalyzed by rubisCO and it results in the formation of 2 molecules of phosphoglycerate (3PGA). PGA is reduced to triose phosphate (a 3-carbon sugar). The plant, by this process, has essentially reduced one molecule of CO_2 and added it to a 5C sugar to produce two molecules of 3C sugar. As these reactions continue, some of the triose phosphate may be transformed into other carbohydrates, including starch. A number of the triose phosphate molecules are used for the regeneration of the first acceptor RuBP. Thus, a cycle of carbon compounds exists, with CO_2 from the atmosphere and water entering the cycle, and various sugars being produced (Huffaker and Miller, 1978; Ridge, 2002; Rost et al., 1998).

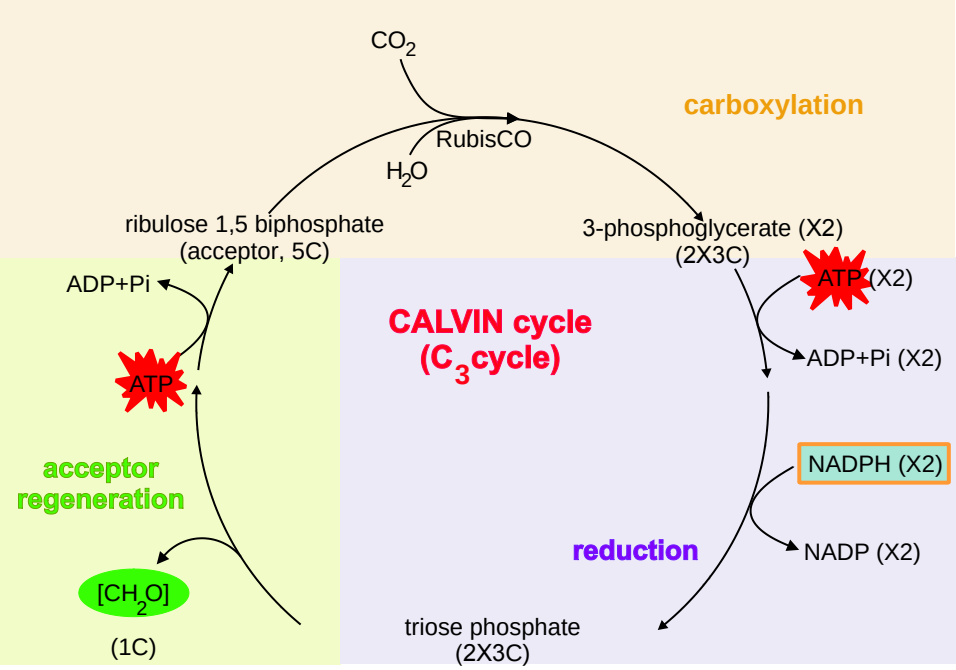

Figure 3. The major steps in the Calvin cycle of photosynthesis.

For the cycle to be driven, it requires a lot of energy which is provided by ATP and reducing power by NADPH. ATP enters the cycle at two stages: during the conversion of 3-PGA to triose phosphate and during the regeneration of RuBP. Thus the light and dark reactions of photosynthesis are interrelated: Light is required for the generation of ATP and NADPH, both consumed by the dark reactions (Figure 4).

RubisCO plays a key role in the C_3 cycle. It is referred to as a premier enzyme because it is involved in the first reaction of the Calvin-Benson cycle, which is the uptake of CO_2. It is the most abundant protein on earth and makes up half of all leaf protein. It carboxylates and may oxygenate RuBP (see below). CO_2 and oxygen are thus competitors for RubisCO. Under normal conditions, the oxygenase activity may reduce the carboxylase activity up to 30%. RubisCO molecule is very large and in green plants is encoded by both chloroplast and nuclear genes. It consists of 8 large subunits and 8 small subunits. It requires a chaperone protein to facilitate assembly of these subunits in the chloroplast (Ridge, 2002).

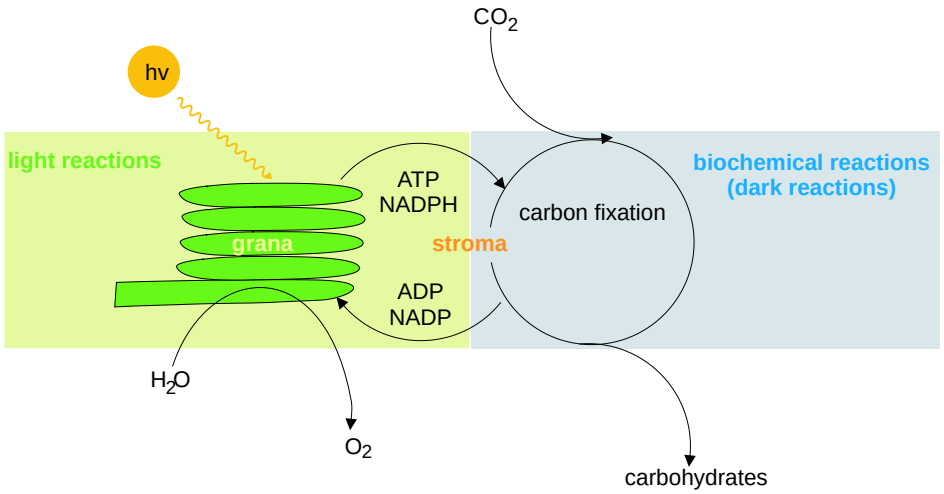

Figure 4. The connection of light and dark reactions in photosynthesis.

Carbohydrates of 3-carbon atoms produced during the operation of the Calvin-Benson Cycle are precursors for the synthesis of major carbohydrateas such as sucrose and starch. Sucrose is the principal form of carbohydrate translocated throughout the plant via phloem. Starch is an insoluble stable carbohydrate reserve that is present in almost all plants. Starch is synthesized in the chloroplast and sucrose in the cytosol.

The process of carbon fixation requires control mechanisms that operate at different levels and have several components which function to create a balance between the light reaction and the C_3 cycle so that the rate of one should match the rate of the other. To accelerate the C_3 cycle, such as in conditions of increasing light flux, or to start up the cycle at sunrise, a large proportion of active enzymes and intermediates are needed. In shade, light is the limiting factor for the operation of the C_3 cycle but in excess light, CO_2 and enzymes are the limiting factors. Sun plants have higher rates of the C_3 cycle and higher levels of C_3 enzymes than shade plants. Environmental factors that influence RubisCO activity are light, carbon dioxide and oxygen concentrations, and temperature. RubisCO is inactivated in darkness and activated in the light. RubisCO activase removes substances that bind to RubisCO, including RuBP, and locks it into an inactive form. This involves hydrolysis of ATP and release of RuBP from inactive RubisCO. Activated RubisCO binds with CO_2 at an allosteric site. CO_2 and oxygen concentrations affect the ratio of carboxylase to oxygenase reaction rates of RubisCO. High temperatures reduce carboxylation and increase oxygenase reaction of RubisCO due to reduced solubility of CO_2 relative to oxygen at high temperatures and because affinity of RubisCO to CO_2 relative to oxygen is also reduced at high temperatures. Global warming has serious effects on RubisCO activity. High CO_2 favors carboxylation and net carbon fixation but high temperature opposes these two increments. Other environmental factors that affect RubisCO activity are altered rainfall patterns and decreased water availability for plant growth (Bjorkman, 1981; Ridge, 2002).

1.3.2. C_2 cycle

Photorespiration (PR) is a light-dependent process which occurs during the oxygenase reaction of RubisCO. It is better referred to as C_2 cycle or oxidative photosynthetic carbon

cycle. It involves the uptake of O_2, the release of CO_2 and partial recovery of carbon lost through photorespiration.

One molecule of oxygen is incorporated into two molecules of 5-carbon RuBP, generating 2-phosphoglycolate and 3-phosphoglycerate. 2-phosphoglycolate formed in the chloroplast by oxygenation undergoes a series of reactions both at the peroxisome and at the mitochondrion including an NADPH-dependent reduction and phosphorylation by ATP. After phosphorylation, 3-PGA enters the chloroplast. In this process, no energy is released. On the contrary, ATP is consumed. Two molecules of phosphoglycolate (4 carbons) lost from the C_3 cycle by the oxygenation of RuBP are converted to one molecule of 3-PGA (3 carbons) and one molecule of CO_2 (1 carbon). At normal atmospheric levels of O_2 and CO_2, the oxygenase reaction of RubisCO reduces net carbon fixation by between 25-50%. Hence, ¾ of carbon is recaptured. This cycle involves oxidation, decarboxylation and amination/deamination.

C_2 cycle uses almost as much ATP and reducing power as the C_3 cycle and plays a key role as a safety valve. It protects plants against photo-inhibition especially in high light and temperature, and low carbon dioxide (Ridge, 2002; Taiz and Zeiger, 2006).

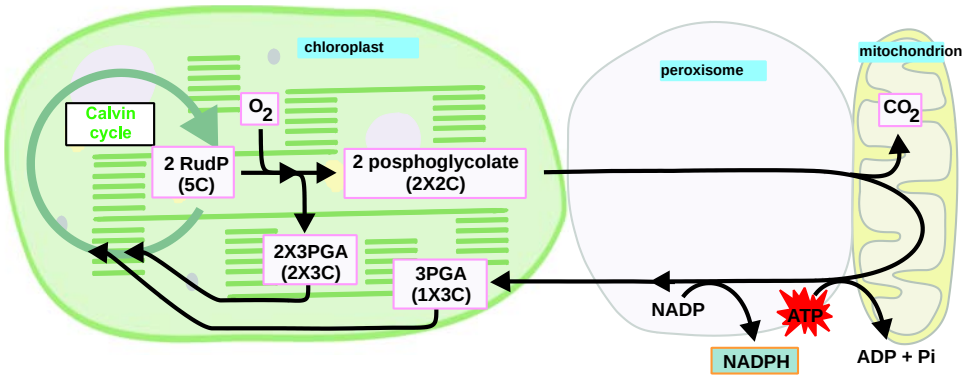

Figure 5. The major reactions of C_2 cycle/photorespiration separated spatially in three organelles.

Though useful, the C_2 cycle reduces net carbon fixation and increases carbon losses. Some biochemical appendances, however, act as CO_2 pumps that accumulate carbon dioxide at the site of RubisCO thus increasing the CO_2/O_2 ratio. Examples of this evolutionary improvement are C_4 plants, algae and cyanobacteria. The function of these biochemical appendances alters the relative amounts of stable carbon isotopes ^{12}C (abundant form) and ^{13}C (rare form) in plant tissues. C_4 plants initially fix carbon dioxide to an intermediate 4-carbon compound and then it is released to RubisCO. There exists another group of land plants with the same principle of carbon dioxide accumulation but with a slightly different mechanism called Crassulacean Acid Metabolism (CAM) because it was first discovered in the Crassulacean family.

1.3.3. C_4 cycle

Structure-function relationship is essential in C_4 plants because in most cases the biochemical steps are spatially separated. C_4 plants are characterized by a special kind of leaf anatomy (Kranz anatomy). The bundle sheath cells surrounding the vascular bundles

possess thicker cell walls and contain more and bigger chloroplasts than any other mesophyll cell. The cell walls may even be suberized at the junction with mesophyll cells although numerous plasmodesmata that facilitate transfer of substances between these two cell types exist at this junction. Bundle sheath chloroplast have relatively fewer grana in many species, hence are deficient in PSII (Ridge, 2002). In C_4 plants, two spatially separated carboxylation steps take place. CO_2 gets into the mesophyll through stomatal pores. It is immediately converted to hydrogen carbonate by carbonic anhydrase. At the first biochemical step it reacts with a 3-carbon compound, phosphoenolpyruvate (PEP), giving a 4-carbon compound oxaloacetate (OAA). This is the fixation stage and is catalyzed by the enzyme PEP carboxylase (Ridge, 2002). OAA is then reduced, depending on the species, to either malate or aspartate. These are both 4-carbon acids. This 4-carbon acid then diffuses through the plasmodesmata to the bundle sheath cells. This is the 1st transport stage. Decarboxylation occurs within the bundle sheath cells. CO_2 is refixed by RubisCO to the C_3 cycle and the 4-carbon acid is converted to a 3-carbon acid. The 2nd transport stage occurs when the 3-carbon acid moves to the mesophyll where regeneration of PEP takes place. C_4 plants require extra energy to fix one molecule of CO_2 to carbohydrates (five ATP plus 2 NADPH molecules).

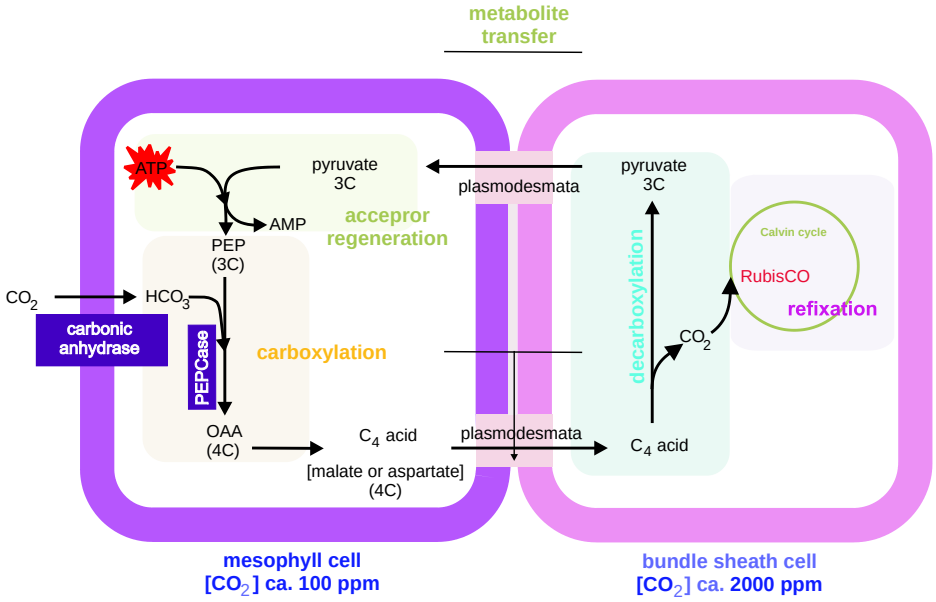

Figure 6. C_4 cycle comprising CO_2 fixation in the mesophyll and bundle sheath cells.

C_4 plants comprise only 1% of known species of flowering plants which include some of the most abundant tropical grasses and weeds and some important crop plants such as maize and sugarcane. Sixteen families of monocots and dicots contain C_4 species, some of which are herbs and shrubs abundant in open, sunny habitats of subtropics and lowland tropics.

The C_4 biochemical appendance gives fitness in hot, sunny habitats where water availability is limited. Under these conditions the stomata open only partially thus contributing to water economy. This however restricts CO_2 from entering the leaf. Despite all these, C_4 plants still achieve considerable rates of photosynthesis. When the

transpiration ratios between C_3 and C_4 plants are compared, an impressive difference is recorded. For plants in very dry habitats, the ratio is even smaller because they have an idiosyncratic photosynthetic metabolism (see below).

1.3.4. Crassulacean acid metabolism (CAM)

CAM mechanism occurs in succulent plants with fleshy leaves and stems such as cacti. These plants share common metabolic features as the C_4 ones, but the two carboxylation steps are temporally rather than spatially separated. CO_2 enters the leaf at night when the stomata open and is fixed again by PEP carboxylase to form malate. This C_4 compound is stored in cell vacuoles at night, creating a sharp acidic taste of the leaf at night which disappears during the photoperiod. Malic acid is released into the cytoplasm during the day and undergoes decarboxylation. CO_2 produced is fixed by RubisCO during the C_3 cycle. During the day, stomata are closed. In addition, these plants possess thick cuticles which prevent water and CO_2 loss from leaves. The CAM is a very effective CO_2 concentrating mechanism since CO_2 acquisition takes place only during the night. At least in some plants, CAM mechanism is quite flexible shifting either to C_2 or C_3 metabolism depending on water supply and temperature. Also, in CAM plants, pyruvate formed in the light is used in the synthesis of carbohydrates which are used for the regeneration of PEP through the process of glycolysis in the dark. Thus, carbohydrate levels rise in the day and fall at night while malate levels follow opposite fluctuations. Malate transport from cytoplasm to vacuole requires energy and is facilitated by a secondary active transport linked to proton pumping. Therefore, CAM is even more energetically expensive than the C_4 cycle resulting in slow growth rates of the CAM plants. CAM plants efficiently use water since they possess a survival strategy appropriate for extreme drought conditions by being able to fix CO_2 while keeping stomata closed during the unfavorable period of the day. Metabolic diversity of CAM plants matches their taxonomic diversity. About 8% of angiosperms exhibit CAM. Desert succulents, orchids (epiphytes on canopy of tropical forests), and some tropical trees are good examples.

1.4. Effect of light on net photosynthesis

Light influences leaf anatomy during development and subsequently photosynthesis. Leaves that develop under full sunlight have thicker palisade structure, large vascular bundles and increased thickness of the epidermal layers compared to shade leaves, which contribute to the increase in LMA for herbaceous and tree species. Light capture is strongly related to the chlorophyll content per unit leaf area and is affected by packaging of pigment molecules within the chloroplast, light scattering by air-cell wall interfaces and mesophyll structure (Evans, 1998). With this anatomical structure, CO_2 diffusion through the intercellular spaces to the sites of carboxylation may limit the rate of CO_2 assimilation. When light is not limiting, net CO_2 assimilation rate depends on factors such as the quantity of light absorbed by the leaf, temperature and CO_2 and O_2 partial pressures at the sites of carboxylation (Evans, 1996). Under low flux densities when photosynthesis is light-limited, net photosynthesis (NP) rises steadily with photosynthetic photon flux density (PPFD). When the curve flattens-off above a certain PPFD, it means that another factor

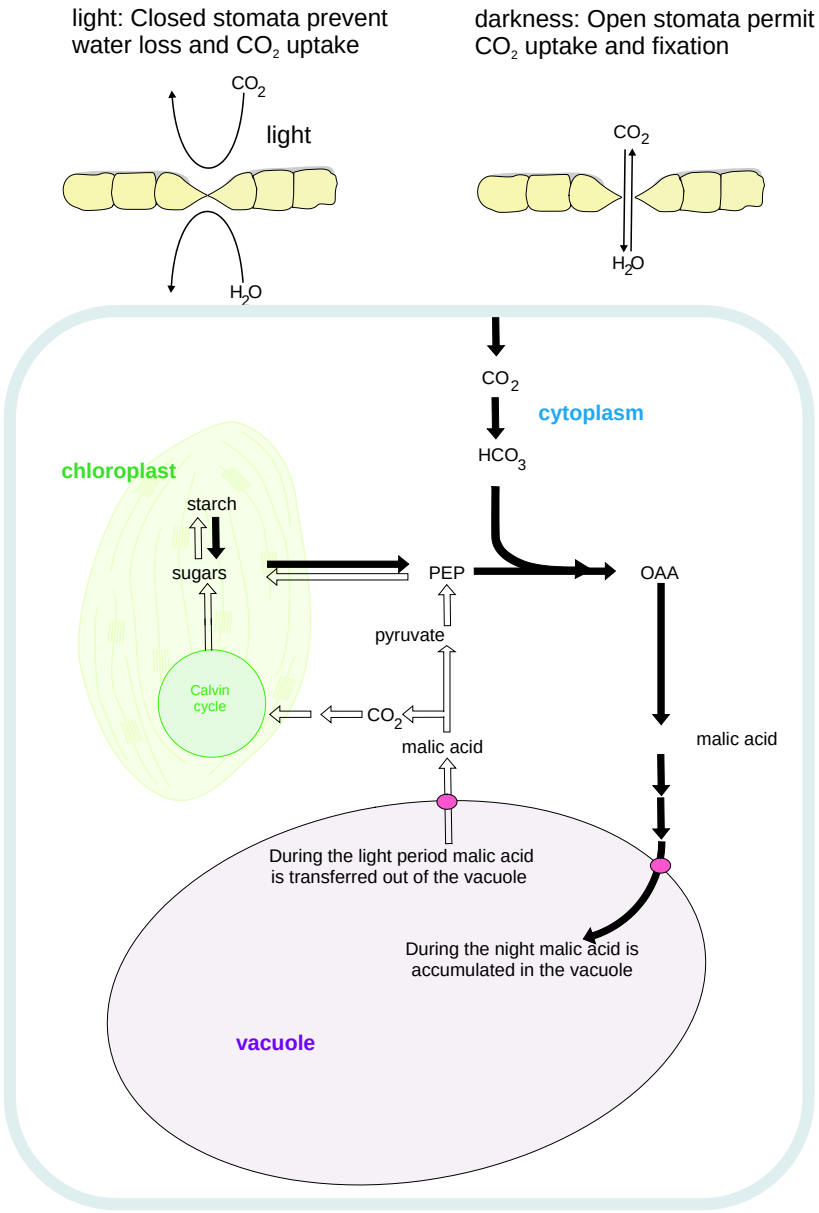

Figure 7. Night and day reactions of CAM photosynthesis.

except light is limiting. In particular, photosynthesis is light saturated or CO_2-limited. At a certain low PPFD, NP appears zero since gross photosynthesis equals respiration (GP=R) or CO_2 uptake equals CO_2 release. This low light flux is called the light compensation point. PPFD below the light compensation point causes R to exceed the GP rate (net release of CO_2). The light compensation point is lower for shade than for sun plants. Consequently, shade plants achieve NP at lower light fluxes than sun plants. Therefore, the former absorb light with much greater efficiency due to special structural and biochemical characteristics. On the other hand, shade plants become saturated at lower light fluxes than sun plants and consequently exhibit relatively lower NP rates at high light intensities compared to sun plants. The same plant species grown (acclimated) at low and high light fluxes exhibit phenotypic plasticity as if they were shade and sun plants respectively. On an area basis, maximum RubisCO activities and rates of photosynthetic electron transport increase with increasing growth irradiance (Niinemets et al., 1998; Hand et al., 1993; Hikosaka and

Terashima, 1995; Larcher, 2003; Dennis, 1992; Terashima and Hikosaka, 1995; Mohr and Schopfer, 1995). 1.5. Effects of CO_2 on photosynthesis

At a saturated light flux and fixed O_2 concentration, there is a CO_2 concentration at which NP equals zero. This concentration is called the CO_2 compensation point. Under these conditions there is no net uptake or net release of CO_2 so GP rate equals the sum of R and PR rates denoted as GP=R+PR. Low CO_2 compensation points show that a plant utilizes CO_2 very efficiently and also has a low PR rate. C_4 plants and CAM plants have low CO_2 compensation points because they operate a CO_2 concentrating mechanism, thereby using CO_2 at an efficient manner. On the contrary, C_3 plants lacking this biochemical appendance exhibit high CO_2 compensation points.

Enhanced rates of photosynthesis in C_3 plants to increased levels of CO_2 compared to the relatively small increase in C_4 plants have been demonstrated. (Ehleringer et al., 2004; Larcher, 2003; Usuda and Shimogawara, 1998; Poorter, 1993; Pearcy and Björkman 1983).

1.6. Photosynthesis and temperature

Temperature is one of the environmental conditions that varies over a wide scale and affects plant growth and survival. Together with rainfall, they determine classification of world vegetation types. Biochemically, they affect membrane properties, enzyme activity and thus rates of chemical reactions. Low temperature and high light lead to photosynthetic damage such as photo-inhibition or even irreversible photo-oxidation. Photosynthetic machinery is damaged by extremes of low and high temperatures due to their action on membrane-bound enzymes. The actual extreme temperature levels depend on the climatic region. For example, a 10 °C temperature is extremely low for tropical species. Plants must therefore be adapted to various temperature conditions in their natural environment ranging from extreme to optimal temperature (temperature at which NP is maximal) levels (Larcher, 2003).

1.7. Role of phenolics in plant defence

1.7.1. Secondary metabolites

Plants exist in contact with a wide variety of living organisms such as animals (mammals, insects, and nematodes), plants (algae, bryophytes, ferns and higher plants), fungi (mycorrhizal associates, pathogens, etc.), and bacteria. One of the characteristics of higher plants is that they do not have the ability to move for avoidance purposes like animals. Thus, in their natural habitats, they are faced with potential enemies because their tissues are a primary food source for countless heterotrophic organisms ranging from pathogens to herbivores (Bennett and Wallsgrove, 1994; Heldt and Heldt, 1997; Ellis, 1997). In order to ensure their survival, they need some form of protection. An essential component of higher plant evolution is the development of an ability to survive the numerous threats or attacks from the environment and other organisms. One form of defence by the plant is the development of physical barriers such as sclerenchymatous tissues, spines, thick bark and hard fruits or seed coats. Superficial tissues and structures

such as the epidermis and the cuticle act as physical barriers against fungal and bacterial entry. Another type of defence exists whereby a group of plant organic compounds known as secondary metabolites defend plants against herbivores and pathogenic microbes (Palo and Robbins, 1991; Rosenthal & Berenbaum, 1991). They are produced from core biosynthetic pathways following primary metabolic routes and are numerous and widespread especially in higher plants. However, very few of these organic chemicals occur universally throughout the plant kingdom because they have a restricted distribution (Bennett and Wallsgrove, 1994; Heldt and Heldt, 1997). Secondary metabolites appear to have no direct function in the growth and development of plants. Previous knowledge about them was elusive since they were initially thought to be metabolic waste and functionless end products, not essential for day-to-day existence of the plant, hence the name 'secondary metabolites' (Bennett and Wallsgrove, 1994; Waterman and Mole, 1994; Heldt and Heldt, 1997). Later they were thought to play a metabolic role in the organisms producing them. Many of them are now thought to have intermediate roles between primary and secondary metabolism. Some of them serve as reserves of energy and as precursors of important organic compounds or sources of nitrogen and may be recycled within the plant itself. Recent studies have however proved that secondary metabolites fulfill important ecological functions in plants (Waterman and Mole, 1994). They have negative effects on herbivores either as toxins or as compounds that reduce the digestibility or palatability of the plant as food resulting in their deterrence (Foley and McArthur, 1994), protection from pathogens, attractants for pollinators and seed dispersing animals, and agents of plant-plant competition, signaling between plants and symbionts and shielding from UV radiation, controlled by a chemistry that is species- and context-specific (Wink, 1999; Luckner, 1990; Ellis, 1997). Thus, the chemical profile of any particular plant at any given time and place is defined by its genetic inheritance which has arisen from an on-going refinement of the optimum chemistry for survival in the face of rapidly evolving populations of insects and microbes. The expression of this genetic potential is conditioned by the plant's developmental state and its environment. Variability thus exists in secondary metabolite patterns across species because of continuous selection of the most useful combination of defence and communication mechanisms from among a vast array of chemical compounds existing in the plant genome. These toxins are effective against herbivores (Iason, 2005) even at moderate concentrations and so they require reasonable investment by the plant. However, selected crop plants have very low levels of these substances which therefore make them susceptible to diseases and herbivory.

The biosynthetic pathways of the secondary metabolism create certain structural units that are diagnostic for the biogenetic origin of each compound. Based on the chemical structure and the biosynthetic origin, three main groups of secondary metabolites are distinguished: Terpenoids (terpenes), phenolics and nitrogen containing compounds. Terpenes constitute the largest group of secondary metabolites and are biosynthesized from primary metabolites in at least two ways: The mevalonic acid pathway and the methylerythritol phosphate (MEP) pathway. The basic structural unit from which all

terpenes are derived is a five-carbon atom precursor (isopentenyl diphosphate or dimethylallyl diphosphate). They function in plant defence against herbivores and in plant growth and development. Phenolic compounds are synthesized by two basic routes: the shikimic acid pathway and the malonic acid pathway. They serve as pollinator attractants, UV-shields, chemical messengers and also in plant defence against herbivores and pathogens. Nitrogen-containing compounds constitute anti-herbivore defence compounds such as alkaloids and cyanogenic glycosides which are biosynthesized from common amino acids. These core patterns are not usually totally exclusive. This means that some compounds are sometimes generated from one or more pathways. The limited number of core biosynthetic sequences signifies that there are relatively few interfaces between secondary and primary metabolism in plants. Most of the photosynthetically-fixed carbon directed towards the formation of secondary metabolites flows either through acetyl-CoA, L-phenylalanine or other aminoacids. Enzymes such as acetyl-CoA carboxylase and reaction sequences in the shikimic acid pathway play important roles in both primary and secondary metabolism (Ellis, 1997; Hall, 2001; Rost et al., 1998; Seigler, 1998; Taiz & Zeiger, 2006).

1.7.2. Phenolic compounds

The basic structure of these compounds is a phenol group which is a hydroxyl functional group attached to an aromatic six-membered benzene ring. Phenolics are the most commonly studied of all secondary metabolites because of their role and ecological impact in chemical ecology. They are widely distributed in plant tissues and organs (Harborne, 1997; Waterman and Mole, 1994). These are a large and chemically diverse group of approximately 10,000 individual compounds ranging from simple phenolic acids to large and complex polymers. They are present at relatively high concentrations in plants (Hartley and Jones, 1997). Some are soluble in organic solvents only; some are water-soluble carboxylic acids and glycosides while others are large insoluble polymers. They have a diversity of roles to perform in plants according to the diversity in their chemical structure. They serve as defense compounds against herbivores and pathogens reducing the digestability and palatability of plants as food, for mechanical support, as attractants to pollinators and seed dispersing animals (involvement in plant/herbivore interactions), in absorbing the harmful ultra-violet radiation and as allelopathic compounds (Jones and Hartley, 1999). They are biosynthesized by several different pathways which leads to their heterogenous nature. The two main pathways are the shikimic acid pathway and the malonic acid pathway (Hopkins, 1999; Taiz & Zeiger, 2006).

The shikimic acid pathway produces most of the phenolic compounds in plants from aromatic amino acids, phenylalanine, tyrosine, and tryptophan. The initial precursors are phosphoenolpyruvate from glycolysis and erythrose-4-phosphate from pentose phosphate pathway, carbohydrate intermediates which are converted to aromatic amino acids through a sequence of reactions which undergo feedback regulation. The shikimic pathway is common to plants, bacteria and fungi but is not present in animals. Shikimic acid is one of the prominent intermediates in this pathway, formed from the condensation of the two carbohydrate intermediates from glycolysis and pentose phosphate pathway. The most

abundant classes of secondary phenols are derived from the aromatic amino acid, phenylalanine, when it undergoes deamination to form cinnamic acid. This reaction is catalyzed by the enzyme phenylalanine ammonia lyase (PAL). It is a key enzyme because it effectively controls the diversion of carbon from primary metabolism to the production of phenolics and it is the most-studied enzyme in plant secondary metabolism. PAL is activated by red and UV radiation, both of which stimulate production of flavonoids. Subsequent phenols formed by the addition of a hydroxyl group and other substituents are trans-cinnamic acid, p-coumaric acid and their derivatives such as caffeic acid. These simple phenolic compounds are called phenyl-propanoids because they contain a hydroxyl-substituted benzene ring (the phenol group) and a 3-carbon side chain. They are important building blocks for complex phenolic compounds or derivatives such as coumarins, lignins, tannins, and flavonoid**s.** They are found everywhere in vascular plants where they function in different capacities (Hopkins, 1999; Taiz & Zeiger, 2006).

Flavonoids are one of the largest classes of plant phenolics. The basic structure of a flavonoid is made up of 15 carbons: 2 aromatic rings connected by a 3-carbon bridge. They are derived from both shikimic and malonic pathways. The flavonoid skeleton has numerous substituents such as hydroxyl groups and sugar molecules attached to it which makes it very soluble in water. If sugars are attached to it, then it exists as a glycoside. The majority of flavonoids occur naturally as glycosides. However, other substituents like methyl ethers or modified isopentyl units render it hydrophobic (lipophilic). Flavonoids are divided into different groups based on the degree of oxidation of the 3-carbon bridge and other modifications. The most important groups are the anthocyanins that serve to attract insect pollinators and the colourless flavonoids which include flavonols, flavones and isoflavones, among others, that function as phytoalexins, UV-protectants and chemical messengers between plants and other organisms, among other roles (Gould and Lister, 2005).

The visible radiation absorbing phenolic compounds help to attract animals to leaves, flowers and fruits by visual and olfactory signals. By so doing, they play a vital role in mutualistic plant-animal interactions in which the animal helps in pollination and seed dispersal. The widest groups of pigmented flavonoids that perform this function are anthocyanins. These are glycosides that have sugars attached at position 3 of the aromatic ring and sometimes in other positions. They are responsible for the red, purple, pink and blue colors observed in plant organs (Ellis, 1997). The aglycons (compounds without the sugars) are known as anthocyanidins. Factors responsible for the specific color of anthocyanins are the type of substituents attached to the B ring of anthocyanidins such as the number of hydroxyl and methoxyl groups, the presence of aromatic acids esterified to the main skeleton and the pH of the vacuole in which these compounds are stored. An increase in the number of hydroxyl groups shifts absorption to a longer wavelength and results in a bluer color. Whereas replacement of a hydroxyl group with a methoxyl group shifts absorption to a slightly shorter wavelength, resulting in a redder color. Anthocyanins may also exist in supramolecular complexes with chelated metal ions and flavone copigments (Hopkins, 1999; Taiz & Zeiger, 2006).

Flavones and flavonols, groups of flavonoids, absorb light at shorter wavelengths than anthocyanins. The patterns or colors expressed by these chemicals are, in most of the cases, only visible to insects such as bees which can see in the UV range of the spectrum that is not perceived by humans. Flavonols in a flower may form symmetric patterns of stripes, spots or concentric circles. These patterns, referred to as nectar guides, help the insects to locate the pollen and the nectar. Flavonoids are also present in the epidermal layers of leaves and stems of all green plants. They not only function as attractants to insects but also protect the cells from excessive UV-B radiation (280-320 nm) by strongly absorbing light in the UV-B region of the spectrum and allowing the photosynthetically active waveband to pass through uninterrupted. Concentration of flavones and flavonols has even been noted to increase when plants are exposed to UV-B light (Jones and Hartley, 1999).

Isoflavones are another type of flavonoids which have one of their aromatic rings shifted to a different position. They are mostly found in legumes and they have different functions. Some act as insecticides while others have anti-estrogenic effects, since they readily bind to estrogen receptors owed to their particular molecular structure, and they cause infertility in sheep that graze on plants rich in such compounds. They are also known for their anti-microbial effect (acting as phytoalexins) to bacterial and fungal infection helping in limiting the spread of the invading pathogen (Taiz & Zeiger, 2006).

Tannins are a group of plant phenolic polymers with defensive properties. There are two categories of tannins: condensed and hydrolysable tannins. Condensed tannins are polymers of flavonoid units linked by strong carbon-carbon bonds. They are common constituents of woody plants. The bonds can be subject to acid-catalyzed hydrolysis and oxidation to anthocyanidins hence, they are also known as pro-anthocyanidins. Hydrolysable tannins are heterogenous polymers containing phenolic acids such as gallic acid, and simple sugars such as glucose, with the hydroxyl group of the sugar esterified to the phenolic acid. They have a lower molecular mass than condensed tannins and are easily hydrolyzed. They are derived from phenylalanine but may also be derived directly from the shikimic acid pathway (Jones and Hartley, 1999). Tannins are toxins which significantly reduce growth and survival of herbivores that feed on plants containing these compounds. Their toxicity can be expressed by their ability to bind protein non-specifically forming a tannin-protein complex in the gut of herbivores, where hydrogen bonds are formed between the hydroxyl group of tannins and the electronegative sites of proteins in herbivores. In addition to this, tannins and other phenolics bond covalently with dietary proteins. There are enzymes present in the leaves of most plants that readily oxidize tannins to the quinone form in the gut of herbivores (Felton et al, 1989). This quinone form is a highly reactive electrophilic molecule and bonds readily with the nucleophilic $-NH_2$ or -SH groups of proteins to form the tannin-protein complex (Appel, 1993). This bonding creates a negative impact on herbivore nutrition because the dietary protein is no longer available for metabolism. Tannins inactivate digestive enzymes in herbivores causing ingested plant material to remain undigested. Herbivores that feed on tannin-rich plants have however come up with several adaptations to remove tannins from their systems

after ingestion. In addition to their capacity as toxins, tannins act as feeding repellents to a diversity of animals such as cattles, apes and deers. This effect could be related to their astringency – a sharp unpleasant sensation in the mouth – for which tannins are noted. This is common especially with unripe fruits. They also play a key role in defense against micro-organisms. The non-living heartwood of many trees contains high levels of tannins that prevent fungal and bacterial infection (Hopkins, 1999; Taiz & Zeiger, 2006).

One disadvantage of these toxins is that they reduce the food value of plants to humans when present in high concentrations. Crop cultivars which have very high concentrations of toxins cannot be eaten by humans but those with low quantities of tannins are preferred and are most popular for use as staple food. In spite of their toxicity and their role in plant defense, tannins are beneficial to the health of humans when their consumption is moderate. The astringent property of tannins is also present in fruits such as grapes, blackberries and apples as well as drinks such as tea and red wine, accounting for their flavor. They possess a level of astringency that is preferred by humans. For example, tannins of red wine block the formation of the signaling molecule responsible for the constriction of blood vessels. Hence, there is a reduction in the risk of heart disease when red wine is moderately consumed (Hall, 2001; Taiz & Zeiger, 2006).

Other groups of simple phenols are phenyl propanoid lactones (cyclic esters). An example is coumarin with a phenyl propanoid skeleton formed by ring closure of hydroxycinnamic acid. Coumarin itself is not toxic. However, certain coumarins known as furanocoumarins (coumarins with a furan ring attached) can be converted to a toxic product when activated by sunlight in the ultraviolet-A region (320-400 nm). These compounds function in the defense of plants against insect herbivores and fungi. At this high-energy electron state**,** they insert themselves to the double helix DNA where they bind with pyrimidine bases, cytosine and thymine, blocking transcription, repair and may eventually cause death of the cell. This is common with plants of the Umbelliferae family such as celery, parsnip and parsley. In celery, the concentration of these compounds increases about 100-fold if the plant is stressed or diseased. Some insects have however adapted to survive on plants that contain furanocoumarins or are phototoxic by living in silken webs or rolled-up leaves which screen out the activating wavelengths of sunlight. Coumarin is also converted by fungi to another toxic product that causes fatal hemorrhaging in cattle by inhibiting vitamin K responsible for blood clotting (Hopkins, 1999; Taiz & Zeiger, 2006).

Lignin is one of the most abundant organic substances in plants and it is a highly branched polymer of the phenyl propanoid group. It is covalently bonded to cellulose and other polysaccharides of the cell wall. It is found in the cell wall of various types of supporting and conducting tissue such as the tracheids and vessel elements of the xylem. It is deposited in the thickened secondary wall but can also be found in the primary cell wall and middle lamellae. It is formed from 3 different phenyl propanoid alcohols: coniferyl, coumaryl, and sinapyl alcohol, all of which are synthesized from phenylalanine through various cinnamic acid derivatives (Ellis, 1997). It provides mechanical strength to stems and vascular tissues because of its mechanical rigidity allowing the plant to grow in

an upward manner and for water and minerals to be conducted through the xylem against the negative pressure without the collapse of the tissue (Jones and Hartley, 1999). Although its principal function is structural, it is implicated in protective functions as well. Its physical toughness and chemical durability deters animals from feeding and makes it relatively indigestible to herbivores. Lignification blocks the growth of pathogens and is a common response to infection or wounding (Hopkins, 1999; Taiz & Zeiger, 2006).

1.8. Role of nitrogen in plant metabolism

1.8.1. Nitrogen assimilation

Nutrients are available in the soil in different forms. However, their availability is dependent on certain factors: the composition of the original rocks, climate, vegetation and management of nutrient supply (Schulze et al., 2005). Nitrogen is a macro-element essential in the structure and metabolism of plants. It is one of the mineral nutrients most limiting to plant growth. Though it is the most abundant element in the atmosphere, the huge amount of atmospheric nitrogen is not directly available to plants. Nitrogen gas is inert and cannot be used directly by plants. It becomes available to plants largely through the recycling of organic matter or through the energy-consuming reduction of nitrogen gas. It is therefore converted to ammonium ions (NH_4^+) or nitrate ions (NO_3^-) or better still to its organic form ($R\text{-}NH_2$) to become usable by plants. In nature, the transformation of gaseous nitrogen to the form available for plants occurs in the atmosphere through lightning or in the soil by nitrogen-fixing free-living bacteria or cyanobacteria under anaerobic conditions or by symbiotic bacteria. The latter create symbiotic relations established in the root nodules of legumes (Marschner 1995; Schulze et al., 2005).

In non-legumes, nitrogen is absorbed from the soil as inorganic ions such as nitrates and ammonium ions, mainly through the roots and is translocated to various parts of the plant. Plant species differ in their preferred forms of nitrogen absorbed, depending on the forms available in the soil. In nutrient-rich soils, plants usually absorb nitrates whereas in nutrient-poor soils, plants tend to take up ammonium ions (Lambers et al, 2008; Larcher, 2003; Marschner, 1995).

1.8.2. Ammonium and nitrate uptake in plant nutrition

Ammonium, in its inorganic form, is not stored in the roots because of its toxic effect on plant cells. Most of the ammonium has to be incorporated into organic compounds in the roots. Ammonium ions are taken up directly through the root hairs and are rapidly assimilated to amino acids (Schenk, 1998; Mauseth, 2009). Nitrate ions, depending on the species, can either be reduced in the roots or transported to leaves through the xylem, where they undergo reduction in the light (Lambers et al, 2008; Pessarakli, 2002). Most woody species assimilate most of the nitrates in the roots (Martins-Lucao & Cruz 1998). Generally, nitrates are stored in the vacuoles of roots, leaves and storage organs. Their accumulation in the vacuole can be of considerable importance for cation-anion balance, for osmoregulation and for the quality of forage and vegetable plants (Roberts and Pang, 1992). A lot more energy is however required when nitrate reduction and assimilation

takes place in the roots than in the leaves (15% as opposed to 2% of plant energy). This is also the case when nitrate reduction occurs in the roots as compared to the uptake, reduction and assimilation of ammonium nitrogen. Due to the lower cost of ammonium rather than nitrate usage by plants, a higher growth rate would be expected from such plants. On the contrary, ammonium-using plants have a lower growth rate than nitrate-using plants which may be as a result of either adjustments in leaf area ratio or a lower efficiency of root respiration. The energy needed for nitrate uptake is obtained through the process of root respiration while that for nitrate reduction in the leaves is provided directly from photosynthesis. Under low light conditions or in the case of fruit-bearing plants, there is a competition for energy between CO_2 and nitrate reduction whereas, under high light conditions nitrate reduction in leaves may even help dissipate the excitation pressure, thus reducing the risk of photoinhibition. Thus, nitrogen and carbon metabolism are closely intertwined. The outputs of the carbon-nitrogen cycles intertwining are: carbohydrate is produced for root formation and growth; carbon skeletons are made available for nitrogen assimilation; influential nitrogen nutrients for protein metabolism; influence of nitrates on plant-hormone balance; feedback coupling of protein availability and plant hormones on photosynthesis and growth of leaves especially (Jones, 2003; Schulze et al, 2005; Touraine, 2004).

Nitrogen serves as a constituent of many plant cell components, including amino acids, proteins, nucleic acids and secondary nitrogenous metabolites**.** Basically, leaf nitrogen is mainly consumed for the biosynthesis of the proteins of the photosynthetic machinery, especially RubisCo. The incorporation of nitrogen into these organic compounds requires energy and molecular structures which are obtained from carbon metabolism. This process is however dependent on photosynthesis and is controlled by the availability of chlorophyll. Therefore, nitrogen supply and increase in biomass have a close relationship which is expressed as the nitrogen use efficiency of production. Its deficiency, however, causes severe metabolic disorders resulting in abnormalities in plant growth, development and reproduction such as stunted growth, increase in lignin synthesis, small and thick-walled individual cells and early reproduction and senescence (Schulze et al, 2005; Wright et al, 2004).

1.8.3. Nitrate reduction and assimilation

Nitrates are first reduced to nitrites by the enzyme nitrate reductase. This reaction occurs in the cytosol and couples glycolysis and nitrate reduction, making use of reduced NAD(P) which is gained via metabolism.

$$NO_3^- + NAD(P)H + H^+ \xrightarrow{\text{nitrate reductase}} NO_2^- + NAD(P)^+ + H_2O$$

Nitrites produced from nitrate reduction are subsequently reduced to ammonium ions by nitrite reductase. Reduction of nitrites occurs in the chloroplast. In photosynthetic tissues, reduced ferredoxin produced during the light-dependent reactions functions as the electron donor (Mohr and Schopfer, 1995; Heldt and Heldt, 2005; Pessarakli, 2002).

$$NO_2^- + 6\text{ferredoxin(red)} + 8H^+ \xrightarrow{\text{nitrite reductase}} NH_4^+ + 6\text{ferredoxin(ox)} + 2H_2O$$

In higher plants, nitrate reductase is a complex enzyme and exists as a dimmer. Each subunit can function separately and contains three prosthetic groups: flavine adenine dinucleotide (FAD), cytochrome 557 (Cyt_c) and molybdenum cofactor (MoCo). On the other hand, nitrite reductase is a monomeric polypeptide containing a siroheme prosthetic group. Nitrate reductase is localized in the cytoplasm whereas nitrite reductase is localized in the chloroplast in leaves and proplastids of roots and other non-green tissues (Marschner, 1995; Baker and Pilbeam, 2006; Schenk, 1998). In green leaves, the electron donor is reduced ferredoxin, generated in the light by photosystem I (Mauseth, 2009). Nitrate reductase activity is very low in molybdenum-deficient plants. Generally, it has a high turn-over rate and its activity is distinctly modulated by effectors such as light, nitrate, or plant hormones. Decrease in the use efficiency of the nitrate reductase transcript for production of the nitrate reductase protein is obviously the main factor responsible for the much lower activity of nitrate reductase in mature compared with young leaves. Maximum nitrate reductase activity occurs when leaf expansion is maximal. After this peak, the activity declines rapidly. Thus, fully expanded leaves have very low nitrate reductase activity and correspondingly high levels of nitrate which are of limited use for the nitrogen metabolism (Schenk, 1998). In roots, nitrate reductase activity is high in the expanding cells of the apical zones and it is rapidly reduced towards the basal zone of the root The proportion of nitrate reduction carried out in roots and shoots depends on factors such as the level of nitrate supply, water supply, light, plant age and plant species (Gissel-Nielsen and Jensen, 1999; Bose and Hemantaranjan, 2005). The proportion of nitrate reduction in the root varies considerably among species of temperate non-legume annuals (Lambers et al., 2008). In herbaceous plants and most deciduous trees of the temperate zone, leaves exhibit the highest rate of nitrate reduction. The majority of this reduction occurs in the juvenile stage and in growing organs (Larcher, 2003; Marschner, 1995; Mengel and Kirkby, 2001). It is promoted by the increasing supplies of nitrate, stimulated by cytokinins and regulated by the change of light to dark. Low levels of nitrate supply however induce nitrate reduction in the roots. The preferential site of nitrate reduction may have ecological consequences for the adaptation of plants to low-light and high-light conditions. Leaf nitrate reductase activity is highest at midday when light intensity is highest (Lambers et al., 2008). The uptake rate of the accompanying cation also affects this proportion. With potassium as the accompanying cation, translocation of both potassium and nitrate is rapid. Consequently, nitrate reduction in the roots is relatively low. But with calcium or sodium as the accompanying cation, nitrate reduction in the roots is considerably higher (Marschner, 1995; Forster and Jeschke, 1993; Givens et al., 2008).

In addition to its function in inducing synthesis of nitrate reductase, together with light, nitrates may act as a signal metabolite altering the partitioning of photosynthetic carbon flow in leaves. In green leaves, there is a strong correlation between light intensity and nitrate reduction. There is a distinct daylight pattern of nitrate reduction in shoots but not in roots. Light has different effects on nitrate reduction in leaves. The daytime rhythm may reflect fluctuation in carbohydrate levels and the corresponding supply of reducing equivalents and carbon skeletons. Light affects enzyme regulation in carbon partitioning or

directly regulates nitrate reductase by enzyme phosphorylation. During the transition from light to dark, nitrate reductase is inactivated in a few minutes and thus nitrite accumulation is prevented. The nitrate content of petioles of many nitrate-storing plant species is higher than that of the leaf blades (Huber et al., 1996; Kaiser and Huber, 1994; Pons and Pearcy, 1994; Foyer et al., 2000).

1.8.4. Amino acid nutrition

Amino acids can also be fixed in the soil by nitrogen fixing bacteria through the direct degradation of litter or in soil solution (Wallenda et al. 2000**).** However, in boreal forests where there is acute nitrogen deficiency, proteins are broken down from litter to release amino acids which are the only source of nitrogen since nitrate and ammonium ions are lacking. This is possible through mycorrhizas where the organisms release an enzyme called protease which breaks down proteins (Schulze et al, 2005). Roots are capable of taking up a broad spectrum of amino acids. According to Nordin et al. (2001), amino acids are taken up even in the presence of nitrate and ammonium ions. The most important transportable amino acids are glutamic acid and aspartic acid and their amides glutamine and asparagine. During nitrogen deficiency, carbohydrate abundance leads to amino acids predominating and when nitrogen is available amides predominate (Taiz and Zeiger, 2006). Most plants however are capable of absorbing any form of nitrogen so far as they get acclimated to its presence (Atkin et al, 1996; Schulze et al. 1994).

1.8.5. Secondary metabolites containing nitrogen

Plants in their natural habitat are susceptible to herbivory and pathogenic attacks. Protection against such biotic stress is achieved not only by phenolics and terpenes, the already mentioned secondary metabolites, but also by the presence of nitrogen-containing compounds. They have no direct function in growth and development and are not directly involved in photosynthesis. Their distribution is restricted in the plant kingdom (Taiz and Zeiger, 2006). Examples of nitrogen-containing secondary metabolites are alkaloids, cyanogenic glycosides, glucosinolates, non-protein amino acids and proteinase inhibitors which are of considerable interest because of their toxicity to humans and their medicinal properties. These metabolites are also relevant in agriculture. In warding off herbivores and pathogens, these defense compounds may make the crop plant undesirable for consumption by humans due to its toxicity. Nitrogen-containing secondary metabolites are biosynthesized from common amino acids.

1.8.6. Photosynthesis – Nitrogen relationship

As noted in the previous section, nitrogen is one of the mineral nutrients most limiting to plant growth. Depending on the plant species, developmental stage, and organ, nitrogen content required for plant growth is in the range of 2-5 % of the plant dry weight (Marschner, 1995, Field and Mooney, 1986). Like other mineral nutrients, it is invested in the construction of leaves, which in turn produce photosynthates that support metabolism and growth (Wright et al, 2004). Nitrogen is deficient in many ecosystems thus being one of the main limiting factors for plant growth and productivity. Its limitation however can be

examined from two perspectives: the relative partitioning among multiple sinks as well as total availability. The relationship between photosynthesis and nitrogen can be considered to be intertwined because photosynthesis provides the energy and structural substrates necessary for reproduction, growth or acquiring additional nitrogen, whereas nitrogen incorporated into essential components and enzymes, two of which are RubisCo (which catalyzes the primary carboxylation reaction in the chloroplasts of C_3 plants) and carbonic anhydrase (which catalyzes the hydration and dehydration of CO_2), play key roles in photosynthesis. These two enzymes have complimentary functions: one fixes CO_2 and the other facilitates the transfer of CO_2 to the site of fixation. The relationship is complex because the integrated series of reactions that make up photosynthesis are sensitive to environmental factors, leaf physiology and leaf structure (Field and Mooney, 1986; Reich et al., 1995; Lambers et al., 2008; Schulze et al., 1994; Schulze et al., 2005; Wright et al., 2004).

Photosynthetic capacity (A_{max}), which is the photosynthetic rate measured under saturating light intensity, optimum temperature, relatively high humidity and CO_2 concentration typical of normal air, is a starting point for analyzing the relationship between photosynthesis and leaf nitrogen because its measurement indicates the maximum rate of CO_2 fixation in nature and the maximum possible benefits from a given investment of nitrogen in the photosynthetic machinery (Field & Mooney, 1986, Marschner, 1995; Taiz and Zeiger, 2006).

All the biochemical and photochemical processes of photosynthesis require nitrogenous compounds. These nitrogenous compounds are chlorophyll (contains 6% nitrogen), proteins creating complexes with chlorophyll (6% nitrogen), electron-transport proteins, the ATP-synthesizing enzyme and the enzymes of the light-independent reactions of the Calvin-Benson cycle. These proteins that catalyze the reactions of CO_2 fixation and regeneration of the CO_2 acceptor are made up of 16% nitrogen. Even though there is no precise knowledge of the nitrogen investment in many of these compounds, the proportion allocated to photosynthesis is large. Approximately 75% of leaf nitrogen in C_3 plants is involved in photosynthesis. Several studies of correlation between photosynthetic capacity (A_{max}) and total leaf nitrogen, highlighting variations within species and among related species have been reported (Evans, 1989; Rundel and Yoder, 2008; Lambers et al., 2008; Reich et al., 2003). Research shows that for any given leaf nitrogen concentration, C_4 plants achieve a higher Amax than C_3 plants. This suggests that in nitrogen-limited habitats, selection may favor C_4 plants. C_3 plants tend to cluster tightly around a single straight line, irrespective of species, growth form or growth conditions, indicating a strong relationship between the two parameters. High $A_{max,m}$ requires high leaf nitrogen per mass at any given leaf mass per area (LMA) but decreases with increasing LMA at any given leaf nitrogen per mass (Reich et al., 1997). Thus, LMA varies inversely with leaf nitrogen on a mass basis. Differences in LMA account for the difference between the two bases of expression. Research indicates that evergreen sclerophylls have the highest LMA and lowest A_{max}, area-based or mass-based when compared with annuals and deciduous species. Leaves with high LMA have longer diffusion paths from the stomata to

chloroplasts, limiting the $A_{max,m}$ for any given leaf nitrogen content. Vulnerability to herbivory is evident in plant species when high leaf nitrogen per mass and LMA are combined. Hence, both leaf structure and leaf nitrogen affect photosynthetic capacity (Wright et al., 2004). Examining one parameter on an area basis and the other on a dry weight basis can be misleading because LMA varies with changes in nutrient availability or changes in light intensity during growth. Analysis of both parameters on a leaf dry weight basis or on an area basis results in a strong positive correlation between A_{max} and nitrogen concentration (Poorter and Evans, 1998; Wright et al., 2004; Warren and Adams, 2006; Evans, 1989). The correlation is sometimes, but not always, considered to be stronger when both parameters are expressed on a mass-basis than on an area basis. The linearity and limited-scatter of the correlation for mass-based A_{max}-Nitrogen relationship is remarkable, with linearity indicating limitations on the relationship between nitrogen-based limitations and other limitations to A_{max}, while the limited-scatter shows that photosynthetic capacity is strongly regulated by leaf nitrogen without significant effects from habitats, growth forms or interspecies differences (Evans, 1983). The strong correlation between A_{max} and leaf nitrogen concentration is also evident in herbaceous plants, with a linear increase that is irrespective of the cause of variation of leaf nitrogen concentration. For non-evergreen species (annuals and deciduous perennials) and evergreen sclerophylls, the correlation coefficient is the same for area-based and mass-based measurements. However, non-evergreen species have higher correlation coefficients than evergreen sclerophylls. Some evergreen sclerophylls have high nitrogen concentration per unit area but low Amax. These are outliers on an area-based but not on a mass-based analysis. Their leaves have been reported to have nitrogen-containing secondary compounds that are not functionally related to Amax but to defense. There are assumptions therefore that sclerophylls allocate less nitrogen to photosynthesis compared to non-sclerophylls. Light capture and CO_2 exchange with the environment are fundamental area-based occurrences which buttress the fact that Amax-nitrogen relationship is better expressed on an area basis for the analysis to be better comprehended. Mass-based analyses, on their own part, generate information on investments related to carbon and nitrogen allocation. Nonetheless, they both provide vital information which leads to a better understanding of the functional and ecological controls on photosynthetic capacity and nitrogen. Thus, converting from mass-based to area-based values of nitrogen or Amax requires multiplication of each value by the LMA of that particular leaf (Cowan, 1986; Evans, 1989; Field & Mooney, 1986).

1.8.7. Nitrogen-based limits to A_{max}

In C_3 plants, photosynthetic capacity (A_{max}) is limited by biochemical and diffusional factors, with the majority of the limitations caused by biochemical factors. Biochemical limitations, on their part, are caused by nitrogen-containing compounds such as RubisCO, carbonic anhydrase and compounds involved in RuBP regeneration. Of all these, limitation by RubisCO is the best documented, owing to its over-abundance (makes up 40% of leaf soluble protein and 15-33% of total leaf protein) and its low catalytic activity. Environmental factors may however alter the balance among these limiting factors. Even

though RubisCO clearly limits photosynthesis under some conditions, variation in RubisCO alone cannot account for the A_{max}-nitrogen relationship. The variation in nitrogen is much too large to be a result of just one or a few enzymes. This implies that the levels of many nitrogenous compounds must be involved. Photosynthesis may also be limited by CO_2 transport, CO_2 reduction capacity or light-driven generation of reducing equivalents and ATP. Thus, it operates near the point of transition between diffusion limitation and biochemical limitation in high and low nitrogen plants. Moreover, low allocation of nitrogen to carbonic anhydrase may depress Amax through effects on CO_2 transport. Components of RuBP (CO_2 acceptor) regeneration also contain nitrogen and limitation on photosynthesis by either CO_2 reduction or the light reactions can be attributed to the effects on the regeneration of RuBP. In conclusion, it can be said that A_{max} is colimited by RubisCO and RuBP regeneration. When environmental factors decrease the gain expected from high nitrogen concentration, nitrogen becomes the major determinant of A_{max} but not the primary environmental constraint. However, when nitrogen availability is limited, nitrogen is then considered as a major limitation to A_{max} and a major environmental constraint (Evans, 1983; Field & Mooney, 1998; Millard, 1988; Stitt and Schulze, 1994; Warren and Adams, 2006).

1.8.8. Nitrogen-use efficiency

There is a strong relationship between photosynthetic capacity and nitrogen content of leaves. The ratio of A_{max}/nitrogen, termed potential photosynthetic nitrogen use efficiency (PPNUE), gives a better understanding of the quantity of A_{max} obtained for a given concentration of nitrogen and allows comparison among species. Research carried out shows that PPNUE is lowest in plants with low A_{max} and increases with A_{max}. There are evidences that PPNUE is positively related to A_{max} and several hypotheses exist in relation to this. Firstly, A_{max} is inversely related to leaf longevity and the structures or factors that promote leaf longevity such as a thick cell wall hinder diffusion of CO_2 into the cells. For this reason, PPNUE is low in evergreen species. Secondly, PPNUE varies with nitrogen allocation. If A_{max} is high, then more nitrogen is being allocated to RubisCO. A portion of 30% of leaf nitrogen is incorporated in RubisCO even though not all of it is active. The excess serves in protecting the photosynthetic machinery under high photon flux. Some of it may be degraded and used for growth during nitrate deficiency. Re-mobilization of nitrogen from leaves makes up 20% of leaf nitrogen. Nonetheless, if A_{max} is low, then more nitrogen is being allocated to leaf longevity (Hikosaka et al., 1998; Field & Mooney, 1986; Hikosaka and Terashima, 1995; Poorter and Evans, 1998; Warren and Adams, 2006).

C_4 plants have lower leaf nitrogen concentrations compared to C_3 plants because they have 3-6 times less RubisCO than C_3 plants. In addition to this, C_4 plants have equal or higher A_{max} than C_3 plants do which results in a higher photosynthetic rate per unit of leaf nitrogen (PNUE). Differences in PNUE between plant species are not caused by a single factor but by several ones. The higher PNUE is probably the result of suppression of the oxygenase activity of RubisCO, lack of photorespiratory enzymes and a higher catalytic rate of RubisCO (Hikosaka et al., 1998; Lambers et al., 2008; Field & Mooney, 1986).

1.9. Mediterranean ecosystems

1.9.1. Mediterranean climate and vegetations

The distinctive Mediterranean-type climate pattern of mild rainy winters and hot dry summers, (which makes the vegetation highly susceptible to fire) is found in five different regions of the world namely the Mediterranean basin, Western and South Australia, Central Chile, Cape Region of South Africa and California (Bennett and Maxted, 2001; Baskin and Baskin, 2007). The mediterranean-type ecosystems cover 1.2% of the earth's surface with 73% of this area lying within the Mediterranean Basin. The Mediterranean Basin extends far to the east across Southern Europe and North Africa to Israel and adjacent parts of the Middle East in Asia. The largest area of the Mediterranean climatic regime in this region is Spain, followed by Turkey, Morocco and Italy. Australia and California have the second largest area of Mediterranean shrublands and woodlands with 10% each of the total area and Cape Region being the smallest with 3% of the total area of the Mediterranean climatic region (Dallman, 1998; Pignatti et al., 2002; Rundel, 1998). Annual mean temperature is mostly in the range of 14^{o}C to 18^{o}C and none of the monthly mean temperatures is below freezing point. Snow is rare except at high elevations. Annual rainfall has a wide range of variation and sometimes it is relatively low (350-400 mm) but generally it is between 600-750 mm while under particular circumstances, it can attain 1000 mm and even more. The important factor for Mediterranean ecosystems however is the distribution of rain during the year rather than the yearly average. Rainfall occurs mostly in spring and autumn and sometimes in winter. The extended period of summer drought is the most striking feature of the Mediterranean climate. It increases plant flammability and fire risk. During this period, plants exhibit conditions of severe water stress. This period of aridity lasts for two or three months during which the monthly mean precipitation is below 20mm (Dallman, 1998; Pignatti et al., 2002; Paula and Pausas, 2006).

Particularly distinctive of this type of climate are evergreen shrublands dominated by species with sclerophyllous leaves. These shrublands are termed maquis, emphasizing a dense shrub formation (Bennett and Maxted, 2001), or garrigue, which is a more open heath, composed of aromatic shrubs in the Mediterranean basin, chaparral in California, matorral in Chile, fynbos in South Africa and kwongan or heathlands in South Western Australia. Woodlands are also widespread in this ecosystem particularly in areas with nutrient-richer soils. Both the Mediterranean Basin and Australia have extensive oak woodlands dominated by the genus *Quercus*. They can be closed canopy woodlands grading into shrublands as in live oak woodlands of Southern California and the maquis of Europe, or open savannas of deciduous oaks that are widespread in both regions, or even evergreen eucalypt woodlands termed mallees that are widespread in semi-arid regions of Western and Southern Australia. In the Mediterranean Basin, the most representative vegetation is the evergreen broadleaved forest dominated by tall shrubs or trees with moderately sclerified leaves. The dominant species is mostly the evergreen oak *Quercus ilex*, locally substituted by *Q. rotundifolia*, *Q. coccifera*, *Q. calliprinus* or *Q. suber*. The high

shrub formation of about 2-5 m, which may even get up to 9 m is sometimes intermingled with small areas of open vegetation of subshrubs (chamaephytes 20-50 cm) or perennial and annual herbs, which arise mostly as post-fire succession species. Drought deciduous shrubs are dominant along arid and drier coastal areas of California and Chile. Similar structural communities in the Mediterranean Basin with mixtures of low evergreen and drought deciduous shrubs are termed phrygana in Greece and the eastern Mediterranean Basin. Of all the five regions, the Cape Region is lacking in woodlands and deciduous shrubs.

1.9.2. Morphological and physiological adaptations of Mediterranean plants

Evergreenness is a recurrent and striking feature in plant assemblages of the Mediterranean area. Evergreen broad-leaved plants survive and even dominate the five Mediterranean-type regions of the world. The peculiarity of evergreen plants of the Mediterranean-type regions is that they are sclerophyllous (Baskin and Baskin, 2007; Paula and Pausas, 2006; Read and Sanson, 2003). Leaf traits associated with sclerophylly are: a relatively low photosynthetic capacity, a high proportion of stored carbon, a low leaf nitrogen concentration, a low surface to volume ratio, thick cell walls and a thick rigid cuticle, with more or less leathery leaves, large development of veins per unit of leaf area, tendency to brittleness when hit or folded. Some are relatively large leaves or pinnately compound. However, most sclerophyllous leaves found in the Mediterranean region are small and undivided. They are all relatively long-lived. The life span of most sclerophyllous leaves exceeds one or two years, averaging three years in holm-oak and olive, and five to six years in kermes oak. A combination of these traits enables the species to maintain leaf shape under conditions of low turgor pressure experienced during water stress (Cunningham et al., 1999; Niinemets, 2001; Lamont et al., 2002). During the period of summer, growth, flowering and fruiting of plant species are limited because of the drought. Cold temperatures in winter may also limit growth and even cause mortality in many plants. Evergreen shrubs, however, can be vigorous throughout the year even though there is a distinct annual growth rhythm as a result of limitations to photosynthesis by a variety of environmental and physiological factors. They show a high degree of stomatal regulation than several deciduous species within these regions, thus can survive periods of summer drought. This regulation strategy employed by evergreen sclerophylls is termed a ‘water-saving’ strategy (Woodward, 2009). Also, the strategy employed in nutrient acquisition and allocation may permit growth in nutrient-poor soils. Thus, evergreen species tend to dominate in nutrient-poor habitats such as in dry and/or sterile environments. The rationale is that a balance between photosynthetic gain and maintenance costs of non-photosynthesizing tissues can only be achieved under limiting environmental conditions by reducing the allocation of photosynthates to leaf growth and by prolonging leaf longevity. These traits have been considered by many authors as adaptations to survival in the Mediterranean-type climate. An ‘anti-herbivory’ effect is evident in evergreen sclerophylls. This deters herbivores from feeding on these plants due to their unpalatability and/possession of secondary metabolites. Evergreen sclerophylls are resilient even when faced with perturbations. That is why they are widespread in the

Mediterranean ecosystems. Despite inadequate knowledge on their ecophysiological functions, Mediterranean-type sclerophylls still differ from their tropical counterparts (Blondel and Aronson, 1999; Archibold, 1995).

Semi-deciduous/deciduous shrubs that shed their leaves during dry periods are also dominant plants in this region. The deciduous leaf habit has evolved as a response to seasonality. These species have a short photosynthetically active period. They are not resilient and so die during any perturbation. Many other leaf types occur in the Mediterranean region in addition to sclerophyllous leaves. Examples are succulent leaves, short-lived leaves, needle-like or scale-like etc. Of remarkable interest are retamoids, species with evergreen stems that are photosynthetically active all year round and which have small deciduous leaves that fall during the drought period. Then, there are aphyllous species, particularly in the drier areas, which also confine their photosynthetic activity to their evergreen stems. Some species however produce long-lived sclerophyllous phyllodes as well as evergreen photosynthesizing stems. Thus, Mediterranean plants exhibit a wide range of leaf types.

Another distinctive trait of the Mediterranean flora is the bulbous life form. These geophytes have a fleshy, subterranean storage organ which is the only part of the plant that survives the summer drought.

On the basis of their root type, Mediterranean plants can be classified into two categories: deep-rooted plant species with root depth greater than 2 m and shallow-rooted plant species with root depth less than 2 m. Mediterranean oaks are among the deepest rooted plants with reports of the deepest California oaks being 24.2 m (*Quercus wisliznii* and *Quercus douglassi*). However lower values were reported for plant species of the Mediterranean Basin (*Pinus halepensis*, 4.5 m; *Arbutus unedo*, 3.5 m) and of the Australian mallee (*Casuarina species*, > 2.4 m; *Banksia* species, 5 m). The deep-rooted plants easily cope with severe water stress and easily survive prolonged drought periods during summer.

Mediterranean ecosystems rival tropical ecosystems in terms of plant biodiversity. The Mediterranean Basin itself hosts 25000 plant species, half of which are endemic (Pignatti and Pignatti, 1999; Thompson, 2005). In this region, plants either avoid or tolerate the prolonged dry and hot summer. Species richness may be high in some parts of the Mediterranean Basin. It is estimated that Greek flora includes 5880 species from which about 20% is endemic (Trigas, 2009). Annuals are more abundant than geophytes in terms of species richness and biomass. They are prominent in the Mediterranean Basin's regional flora and constitute half of the dominant vegetation. Two types of annuals exist. The first type has a relatively small number of large seeds which serve as storage organs that enable survival during the unfavorable dry season. These seeds are relatively short-lived, lasting only a single drought season. The second type has long-lived diaspores that protect the seeds until a favorable season for germination presents itself. Both types of annuals are found in large numbers in Mediterranean habitats. Another efficient adaptation exhibited by many species is the presence of a dense layer of hairs, especially on the abaxial leaf surface, which limits water loss during the arid period and protects against

high UV and visible light intensities (Blondel and Aronson, 1999; Dallman, 1998; Pignatti et al., 2002; Archibold, 1995; Karabourniotis et a1. 1992).

Human occupation which strongly differs between the five regions has important consequences on biodiversity, landscape structure and ecosystem functioning. Fire is considered as a natural disaster in California, Australia and South Africa but it is more linked to human pressure and considered an anthropogenic disturbance in the Mediterranean basin (Joffre et al., 1999).

2. Scope of the study

Several theories exist on the synthesis and accumulation of secondary metabolites, patterns of allocation at metabolic levels including competition between proteins and phenolics for the common precursor L-phenylalanine, and resource partitioning between growth and defence. The defensive role of secondary metabolites has been prominent since plants have been under a constant threat due to herbivory by animals and insect pathogens. Recently, knowledge of their protective role is being highlighted where the secondary metabolites serve as antioxidants, protecting the leaves from photodamage. The function of these metabolites has also been considered at the ecophysiological level. Mediterranean ecosystems are characterized by water-deficient environments, high light intensities and elevated temperatures (Dallman, 1998; Pignatti et al., 2002). Under these conditions, plants have a tendency to experience oxidative damage of their leaves. The potential role of secondary metabolites in protecting plants from such damage needs to be taken into consideration by investigating the size of the phenolic pool of the plants under study in order to understand to what extent these plants can withstand the abiotic stress. Therefore, if the contribution of phenolic compounds is crucial for protection against oxidative damage, it is expected that the concentration of these compounds will be correlated with vital leaf functional parameters such as A_{max}, irrespectively of plant species. Considering that A_{max} is positively correlated with leaf nitrogen content, a fundamental question, that needs to be answered, arises: how does the concentration of the phenolic compounds interact with nitrogen content? Moreover, no data concerning the performance of Mediterranean plant species in relation to the leaf economic spectrum have been reported in literature. Thus, an investigation on the relationship between structural and functional traits of representative Mediterranean plant species is essential.

The main objectives of this study are:

1) The determination of the interrelation between structural and functional leaf traits of Mediterranean plant species that represent different life forms and the confirmation of these relationships consistency with the general trends of the leaf economic spectrum.
2) The verification of probable correlations among nitrogen content, phenolic concentration and functional parameters of leaves, especially photosynthetic activity and transpiration rates.
3) The identification of clear-cut differences among different life forms of Mediterranean plant species concerning their functional and structural leaf traits and their related performance.

To meet these major objectives:

1) Therty Mediterranean plant species growing in their natural habitat on Mount Parnitha and Domnista were chosen and the following parameters were examined.

2) Net carbon dioxide assimilation rate (A_{max}) and related functional parameters such as dark respiration (R_d), stomatal conductance (g_s), and transpiration rate (T_r) in the field. Water Use Efficiency (WUE) and Photosynthetic Nitrogen Use Efficiency (PNUE) were also calculated.e concentration of chlorophylls, total phenolics, condensed tannins and nitrogen in the leaves of all plants sampled.
3) Presence of homobaric or heterobaric leaves among a wide range of species in Mediterranean-type ecosystems in Greece.
4) Structural parameters of the leaves: transparent leaf area (TLA), specific leaf area (SLA), leaf mass per area (LMA), leaf thickness (LT) and leaf density (LD).

3. Materials and Methods

3.1. Study sites

All sampling and field measurements were carried out at two sites, namely Mount Parnitha and Domnista. Mount Parnitha is a densely forested mountain range north of Athens, the highest on the peninsula of Attiki with an elevation of 1,413 metres and a summit known as Karavola. The average annual rainfall at Mount Parnitha is 446 mm. Annual mean minimum and maximum temperatures are 16.5 and 27.8 °C, respectively (Table 1).

Table 1. Study site coordinates and climatic data.

Site	Description	Coordinates of meteorological station (Lat; Lon)	Altitude of meteorological station (m.a.s.l.)	Altitude of study site (m.a.s.l.)	T_{min} (^{0}C)	T_{max} (^{0}C)	Precipitation (mm)
Domnista	Deciduous broadleaf *Quercus frainetto* and *Castanea sativa* forests	N38° 54′ 00″; E21° 48′ 00″	690	1,000	4.5	18	1255
Parnitha	Deciduous (*Quercus macrolepis*) & evergreen (*Quercus coccifera* and *Pistacia lentiscus*) open woodlands	N38° 06′ 05″; E23° 46′ 48″	235	200-400	16.5	27.8	446

Much of the mountain is designated a national park. The summit is located 18 km north of Acharnae and about 30 km north of Athens, while the mountain covers approximately 250 km² of land. Three vegetation zones can be easily identified on Mount Parnitha. The first vegetation zone extends from 300-800 m and is dominated by pine forests, maquis and phrygana. Above 800 m, pines create a mixed forest with firs. The second vegetation zone extends from 900-1.400 m and is dominated by *Abies* forest, brushwood and grasslands. The third vegetation zone is observed on the highest mountain summits. It is vestigial and consists of spiny, cushion-like bushes. It probably originated from the reduction of the *Abies* forest in these areas and as such is not an authentic subalpine zone. About 1,100 taxa (species and subspecies of plants) can be found on the mountain, including crocus and tulips, of which 99 are endemic to Greece (Aplada 2003). The following habitats occur on the mountain: coastline/rocky areas (5%, inland cliffs), forest (55%, native coniferous woodland), grassland (5%, steppes and dry calcareous grassland), shrubland (41%, sclerophyllous scrub, garrigue and maquis). However, a large proportion of the forest, very close to the casino located near the summit, was extensively consumed by wildfire in 2007 destroying approximately 56 km^2 of land. This wildfire did not occur near sampling site. Hence, vegetation at the sampling site was not destroyed. There is a cable car station at an elevation of 485 m above sea level (a.s.l.). Sampling at Mount Parnitha was done in the vicinity of this area, at approximately 50 to 200 m away from the cable car station.

Domnista is one of the largest villages situated in Evritania Prefecture, Central Greece. It is located at an altitude of 1000 m above sea level. The average annual rainfall at

Domnista is 1255 mm. Annual mean minimum and maximum temperatures are 4.5 and 18.0 °C, respectively (Table 1). The vegetation in this region is rich. It comprises a temperate forest with magnificent unique *Abies* forest environment, rare aromatic plants, variety of shrubs and herbs, and shady plane-trees. Sampling in Domnista was done at the following altitudes above sea level: 901 m (38°45′N, 21°51′E), 914 m (38°45′N, 21°51′E), 1,010 m (38°45′N, 21°50′E), 1,303 m (38°44′N, 21°50′E), and 1,145 m (38°45′N, 21°50′E).

Table 2. Plants species and their families, life forms, study sites, seasons and years of sampling, phenological guilts and leaf types.

	Plant Species	Life form	site/season/year of sampling	phonological guilt	leaf type
1	*Pistachia terebinthus*	tree	Parnitha, autumn 06	evergreen	Heterobaric
2	*Quercus ithaburensis*	tree	Parnitha, autumn 06	deciduous	Heterobaric
3	*Platanus orientalis*	tree	Parnitha, autumn 06	deciduous	Heterobaric
4	*Pyrus amygdaliformis*	tree	Parnitha, autumn 06	deciduous	Heterobaric
5	*Castanea sativa*	tree	Domnista, summer 07	deciduous	Heterobaric
6	*Quercus frainetto*	tree	Domnista, summer 07	deciduous	Heterobaric
7	*Ostrya carpinifolia*	tree	Domnista, summer 07	deciduous	Heterobaric
8	*Juglans regia*	tree	Domnista, summer 07	deciduous	Heterobaric
9	*Platanus* sp.	tree	Domnista, summer 07	deciduous	Heterobaric
10	*Cercis siliquastrum*	tree	Parnitha, summer 08	deciduous	Heterobaric
11	*Pistachia lenticus*	tree	Parnitha, autumn 06	evergreen	Homobaric
12	*Olea europaea*	tree	Parnitha, autumn 06	evergreen	Homobaric
13	*Rubus fruticosus*	shrub	Parnitha, autumn 06	semi-deciduous	Heterobaric
14	*Styrax officinalis*	shrub	Parnitha, autumn 06	deciduous	Heterobaric
15	*Clematis vitalba.*	shrub	Domnista, summer 07	deciduous	Heterobaric
16	*Quercus coccifera*	shrub	Parnitha, autumn 06	evergreen	Heterobaric
17	*Phlomis fruticosa*	shrub	Parnitha, autumn 06	evergreen	Heterobaric
18	*Rubus* sp.	shrub	Domnista, summer 07	deciduous	Heterobaric
19	*Cionura erecta*	shrub	Parnitha, summer 08	deciduous	Heterobaric
20	*Arbutus unedo*	shrub	Parnitha, summer 08	evergreen	Heterobaric
21	*Smilax aspera*	shrub	Parnitha, autumn 06	evergreen	Homobaric
22	*Malva sylvestris*	herb	Parnitha, spring 07	deciduous (perennial)	Heterobaric
23	*Bituminaria bituminosa*	herb	Parnitha, spring 07	deciduous (perennial)	Heterobaric
24	*Thapsia garganica*	herb	Parnitha, spring 07	deciduous (perennial)	Heterobaric
25	*Fragaria vesca.*	herb	Domnista, summer 07	deciduous	Heterobaric
26	*Ballota acetabulosa*	herb	Parnitha, summer 08	evergreen	Heterobaric
27	*Securigera securidaca*	herb	Parnitha, spring 07	deciduous	Homobaric
28	*Echinops* sp.	herb	Parnitha, spring 07	deciduous	Homobaric
29	*Lotus ornithopodioides*	herb	Parnitha, spring 07	deciduous	Homobaric
30	*Anchusa* sp.	herb	Parnitha, summer 08	deciduous	Homobaric

3.2. Δειγματοληψία

Measurements were conducted on fully expanded, mature leaves of 30 Mediterranean plant species growing in their natural habitat on Mount Parnitha and Domnista. Five south-facing leaves randomly selected from two or three individual plants of the same species were sampled giving a total of 150 samples. These 30 plant species originate from 19

different families as shown in Table 2. Eleven plant species were sampled in autumn 2006 at Mount Parnitha, six plant species in spring 2007 at Mount Parnitha, eight plant species in summer 2007 at Domnista and five plant species in summer 2008 at Mount Parnitha. These leaves represented different leaf habits (phenological guilt), leaf types and life forms. Out of the 30 plant species sampled, eight plant species were evergreen sclerophylls, 14 plant species were deciduous or semi-deciduous plants, three plant species were perennials and two plant species were annuals. For the purpose of analysis, I considered the two annuals (*Securigera securidaca* and *Lotus ornithopodioides*) and plants species which have not been identified to the species level (*Rubus* sp., *Echinops* sp. and *Anchusa* sp.) as deciduous/semi-deciduous plants. Annuals live for just one growing season and are not woody species. Hence, they cannot be considered as evergreens. I also considered *Ballota acetabulosa* as a herb and not a shrub because it is only a subshrub, even though it is evergreen.

Deciduous species comprise all species that shed their leaves outside the growing season. This group was divided into two groups: herbaceous and woody deciduous species. Semi-deciduous species comprise all species that shed some of their leaves outside the growing season. Evergreen species are all woody species that maintain all their leaves all year round. These species were of two leaf types: 23 plant species with heterobaric leaf type and seven plant species with homobaric leaf type, and consisted of different life forms such as trees, shrubs or perennial/biennial herbs. In total, twelve trees, nine shrubs and nine herbs were sampled. Out of the nine shrubs, three were climbing shrubs.

3.3. Gas exchange measurements

Leaf gas exchange measurements were carried out under field conditions on fully expanded mature intact leaves of healthy plants, exposed to sunlight, using a portable photosynthesis system (LCpro+, ADC Bioscientific Ltd, England). It is equipped with a mini infra-red gas exchange analyzer housed inside a 6.25 cm^2 broadleaf chamber and a LED light source. All gas exchange measurements were recorded during the period of 8 a.m. - 11.30 a.m. To avoid the midday depression of photosynthesis, experienced under strong light intensity and high air temperature, no measurements were carried out after this time. Five leaves per plant species were randomly selected for the measurements.

The LCpro+ has full programmability and large data storage capacity. Hence, gas exchange rates were obtained by direct measurements of maximum net photosynthesis (A_{max}) and dark respiration rate (R_d). Leaf transpiration rate (T_r) and leaf stomatal conductance (g_s) both in the light and in the dark were also obtained by direct measurements. During each sample measurement, the LCpro+ was set to run a sequence of commands. The sequence took eight records, each one including all the above parameters, for every leaf sample. Since there were five samples for every plant species, each plant species ended up with 40 records. Data were transferred to a personal computer running Excel v. 12 (Microsoft Corporation, Redmond, WA) for further processing after which the mean and the standard error of each parameter for each plant sample was obtained. In the case of leaves for which it was not possible to cover the whole sampling area of the leaf chamber (6.25 cm^2), actual sampling area was measured in photographs

using image analysis (Image Pro Plus v. 4.5; Media Cybernetics, Bethesda, MD). Original values of each sample for parameters expressed on a leaf area base, the value recorded by the device, was multiplied by the leaf chamber area and then divided by the leaf surface area within the chamber which had been determined by image analysis. The mean value of maximum net photosynthesis per unit leaf area ($A_{max,a}$) for each sample was then obtained after calculations with the surface area of the leaf. $A_{max,m}$ was calculated as the maximum net photosynthesis per unit leaf dry mass using the $A_{max,a}$ and corresponding leaf mass per area (LMA) values.

From gas exchange measurements during the light period, photosynthetic water use efficiency (WUE) was determined by the ratio of $A_{max,a}$ to transpiration rate (T_r light) (Dang et al., 1991; Zhang and Marshall, 1994). Likewise, photosynthetic nitrogen-use efficiency (PNUE) was determined by dividing $A_{max,a}$ by area-based total leaf nitrogen concentration (Total N_a) (Sheriff, 1992; Ellsworth and Reich,1992).

3.4. Chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters were measured *in vivo* on dark-acclimated leaves (to obtain intrinsic parameters of photochemistry) and on leaves acclimated to varying intensities of ambient light or acclimated to specific artificial actinic light intensities (to obtain light-response curves of photochemical parameters) during the photoperiod (8 a.m. - 11.30 a.m.) using a portable pulse amplitude modulation chlorophyll fluorometer (PAM-2100, Heinz Walz GmbH, Effeltrich, Germany). Each leaf was dark-adapted for at least 20 minutes prior to measurements using a dark leaf clip. Measurements were taken on the same day and on the same or similar leaves on which gas exchange measurements were made. All measurements were taken on the lamina, midway between the base and tip of mature leaves. Upon application of measuring light (655nm, PPFD<0.15 µmole quanta m^{-2} s^{-1}) the base fluorescence yield (F_o) was recorded just before application of a saturation pulse (white light, ca. 10,000 µmole quanta m^{-2} s^{-1} lasting 0.8 s). During the saturation pulse, the maximal fluorescence yield (F_m) was recorded. The intrinsic quantum yield of PSII photochemistry (Φ_{PSIIo}) was calculated as $(F_m-F_o)/F_m$. In the case of light-acclimated leaves (either in ambient or artificial actinic light) the F_s' and F_m' fluorescence yield parameters were recorded and the effective quantum yield of PSII photochemistry (Φ_{PSII}) was calculated as $(F_m'-F_s')/F_m'$. In addition, the following data were automatically measured and recorded within the device: The time of measurement, the number of readings for each light saturation curve, leaf temperature, PPFD of incident radiation (PAR), and the various fluorescence yield (F) parameters stated above. Apparent electron transport rate is determined as ETR= Φ_{PSII} × PAR × 0.84 × 0.5 (where 0.84 is the assumed leaf light absorbance and 0.5 accounts for the even distribution of photons between the two photosystems). Fluorescence quenching analysis (described by the quenching coefficients qP (for photochemical quenching) and qN (for non-photochemical quenching) was performed on-line allowing the instrument to perform the appropriate calculations using the dark-acclimated fluorescence yield parameters (F_o and F_m), therefore not accounting for the quenching of base fluorescence yield (F_o') under light-acclimated conditions. Parameters of yield and ETR at 441 µmol quanta m^{-2} s^{-1} where used for

correlations (ETR441 and Yield441).

3.5. Determination of chlorophyll a and b concentrations

Leaves sampled in the field were harvested and sealed in plastic bags, labeled and placed in a portable icebox until transported to the laboratory for further measurements. Leaf discs were punched out using leaf borers of appropriate dimensions. Number of leaf discs punched out from a particular leaf depended on the size of that leaf. All handlings, including chlorophyll extraction, were performed under a dim light and exposure of chlorophyll extracts to radiant energy was avoided. Leaf extracts were kept in an ice-bath until photometer measurements took place. Chlorophyll extraction was performed by grinding leaf discs in a mortar with 80% acetone in the presence of a small amount of anhydrous calcium carbonate ($CaCO_3$) and extra pure sea sand. The extract was centrifuged at 4000×g for 10 minutes to separate the sediment and the supernatant was obtained. Chlorophyll content was determined spectrophotometrically using a Shimadzu UV-160A double-beam spectrophotometer by measuring absorbance at 720 nm, 663 nm and 646 nm wavelengths. Possible light scattering due to imperfectly clarified extracts was assessed and subtracted from the absorbance at 663 nm and 646 nm. Concentrations of chlorophyll a and b were calculated using the following equations (Lichtenthaler and Wellburn (1983):

$$Chl_a = 12.21 * A_{663} - 2.81 * A_{646}$$

$$Chl_b = 20.13 * A_{646} - 5.03 * A_{663}$$

3.6. Leaf thickness (LTh)

Hand-cut cross sections were made on freshly harvested leaves of all samples. Leaf sections were observed with an Axiolab Zeiss light microscope (Carl Zeiss, Jena, Germany) equipped with an ocular micrometer. Sections were viewed with a magnification lens of either 40× or 10×, depending on leaf thickness. Using the ocular micrometer, three different readings from three different points along the length of the section were taken for each sample and the mean of these values was recorded as the thickness (in µm) of the sample. Five replicates were measured for each plant species.

3.7. Leaf density (LD)

Leaf density (LD) in g cm^{-3} was calculated according to Witkowski and Lamont (1991) as the ratio between leaf mass per area (LMA) and leaf thickness (LTh).

3.8. Specific leaf area (SLA)

The adaxial surface of each sample of freshly harvested mature leaves was photographed with a digital camera (DSC-H5, Sony Corporation, Tokyo, Japan) and the leaf surface area was measured by image analysis (Image Pro Plus, v. 4.5). Dry weight of each leaf was obtained by weighing, after leaves had been dried for a minimum of 48 h at 70^{o}C. Specific leaf area (SLA) in cm^2 g^{-1} for each leaf was calculated as the ratio of the area of each leaf (cm^2) to its dry weight (g). Five replicates were measured for each plant

species.

3.9. Transparent leaf area (TLA)

The transparent leaf area of each leaf sample was determined by image analysis using a custom-made program developed under the Matlab R12 (Mathworks Inc., Natick, MA) environment. A digital camera (DSC-S75) with a Carl Zeiss ACC terminal and adapter ring attached to the Axiolab Zeiss light microscope was used to capture images of portions of intact fresh leaves positioned with the adaxial surface of the leaf lamina facing up while high-intensity light from the microscope was transmitted through the leaf. Images were captured at a magnification of 4×. Three different images were taken for each leaf sample and the best shot used for image analysis. The percentage of transparent leaf area for each leaf sample was determined at a particular gray-scale threshold level (Nikolopoulos et al 2002). TLA was calculated only for heterobaric-type leaf samples while that of homobaric-type leaf samples was taken as 0%. Five replicates were measured for each plant species.

3.10. Leaf mass per area (LMA)

Leaf mass per area (LMA as g m^{-2}) for each leaf sample was calculated by dividing leaf dry weight of that sample by its leaf area.

3.11. Determination of total leaf nitrogen content using the Kjeldahl method

i) Digestion

Leaves collected were kept in the oven to dry for a minimum of 48 hours at 70^{o}C. Dried leaves were finely ground using an analytical mill (A 10, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) and in some cases using a mortar and pestle for species that leaf trichomes imposed difficulties in tissue grinding. A mass of 50mg dry powder of each sample was weighed and placed into micro-kjeldahl tubes. Approximately the same quantity of catalyst (made of 1g copper sulphate ($CuSO_4$), 8g potassium sulphate (K_2SO_4) and 1g selenium dioxide (Se_2O_2)) was mixed with the powder together with 1 ml of 95-97% sulphuric acid (H_2SO_4) and the mixture was shaken thoroughly. The micro-kjeldahl tubes were heated at a boiling temperature of 410^{o}C for 20 minutes on a digestion block (Tecator 2006 Digestor, FOSS NIRSystems, Inc., Laurel, MD). Complete decomposition of the sample was achieved as color of solution changed from the initial black color before heating to an apple-green color.

ii) Distillation

After cooling, each digest solution obtained was poured into a volumetric tube and de-ionized water (approximately 10 ml) which was used to rinse the micro-kjeldahl tube added to it together with 10 ml of 40% sodium hydroxide (NaOH) solution. The estimated 20 ml solution was distilled (Tecator Kjeltec System 1002 Distilling Unit) and the resulting distillate was collected when it reached the 40 ml graduation mark on the conical flask

containing the trapping medium (solution of 20 ml of 2% boric acid (H_3BO_3) containing three drops of the conway solution). Prior to distillation of the digested samples, a blank solution consisting of 10 ml NaOH and 10 ml de-ionized water was distilled. A pink color was obtained after addition of the conway solution to the boric acid which later changed to almost colorless after distillation.

iii) Titration

Each distillate, starting with the control distillate, was titrated with a N/28 hydrochloric acid (HCl) solution which was added volumetrically until the color of solution changed to pink. Three reference curves were drawn from data generated after titration of standard solutions (obtained from mixing a stock solution of 0.04% ammonium sulphate $(NH_4)_2SO_4$ and 250 ml of 4% (v/v) sulphuric acid (H_2SO_4) at different concentrations). . The volumes of HCl used to titrate the control and the standard solutions were used in calculations to generate the final reference curve (r^2 = 0.998). From the regression equation of the final reference curve (y = 15.189 × x – 1.3739) and the volume of HCl used to titrate the distillates of the sample solutions, the percentage of nitrogen present in each sample and subsequently leaf nitrogen content on a mass basis (N_m, mmol/g) were calculated. Leaf nitrogen content on an area basis (N_a) was calculated as the ratio between Nm and SLA.

3.12. Determination of total phenolics (TP) in leaf samples by Folin-Ciocalteu method

Total phenolics were determined in leaves of plants according to the method described in Waterman & Mole (1994).

i) Extraction phase

A mass of 50mg of powder (finely ground) of each plant sample was weighed and put into test tubes. A volume of 6ml 50% methanol solution was pipetted into each test tube containing the powder. Capped test tubes were placed into a shaking bath set at 40^{o}C for one hour (with agitation after 10 minutes), after which they were centrifuged for 10 minutes at 4000×g and the supernatant was collected and used for determinations of both phenolic compounds and tannins (see below).

ii) Reaction phase

For the reaction, 3950 µl of deionised water, 50 µl of the supernatant, 250 µl of Folin-Ciocalteu reagent and 750 µl sodium carbonate (Na_2CO_3) solution were mixed into test tubes. An interval of 1-8 minutes was given for incubation at room temperature between the addition of the Folin-Ciocalteu reagent and the addition of Na_2CO_3. In the blank test tube, 50 µl of 50% methanol solution was added to the de-ionized water in place of the 50 µl of the supernatant and the same procedure was carried out. All test tubes were placed on a vortex stirrer to mix the test tube content into a homogenous mixture. The solutions in the test tubes were all kept for incubation at room temperature for 2 hours and analyzed spectrophotometrically (Shimadzu UV 160 spectrophotometer). Absorbance was measured at 760 nm. A standard curve was constructed using different concentrations of

tannic acid. Total phenolic content was expressed in milligrams of tannic acid equivalent per gram of plant dry mass.

3.13. Proanthocyanidin method for determination of condensed tannin content (CT) of leaves

Condensed tannins were determined in leaves according to the method of Waterman and Mole (1994). Butanol reagent was prepared as follows: 0.7 g of ferrous sulfate heptahydrate was added to 50 ml concentrated hydrochloric acid (HCl) in a 1 litre volumetric flask and n-butanol was added up to 1 litre volume and mixed. An aliquot of 7ml of the butanol reagent and 500μl of the sample (supernatant from leaf extract solutions, see 3.12.i) were put in a screw cap test tube and mixed on a vortex stirrer. A blank was also prepared by mixing 7ml of the butanol reagent and 500μl of 50% methanol solution in a screw cap test tube. All test tubes were put in a bath set at 95^{o}C and heated for ca. 40-60 min. Test tubes were left to cool and absorbance was measured at 550nm. Tannin content was expressed in milligrams of tannin per gram of plant material dry weight.

For the reference curve, a 30ml stock solution of 10mg delphinidin chloride was prepared in a solvent of 0.1% HCl (w/v) in 100% methanol solution. According to the molecular weight of delphinidin chloride which is 338.7g mol-1, the concentration of the stock solution was calculated to be 0.984mM. The standards were then diluted as shown in Appendix III. 500μl of the stock solution was used to carry out the proanthocyanidin method (Waterman & Mole, 1994) and absorbance was read at 550nm. A standard curve was plotted showing the concentration of delphinidin in mM with absorbance readings at 550nm.

3.14. Determination of N/TP, N/CT and N/TP+ CT ratios

Data obtained from the biochemical analyses of leaves for nitrogen per unit mass, total phenolics and condensed tannin contents were used in calculating the N/TP, N/CT and N/(TP+ CT) ratios.

3.15. Υπολογισμός της $A_{max,chl}$

Data obtained from gas measurements of net photosynthetic capacity per unit leaf area ($A_{max,a}$) and total leaf chlorophyll content per unit of surface area were used in calculating the parameter $A_{max,chl}$.

3.16. Determination of stable carbon and nitrogen isotopes

Leaves of plant samples were oven-dried at 60°C for 72 hours and milled to fine powder for carbon and nitrogen isotopes analyses. The determination of stable carbon and nitrogen isotope ratios was performed in the Stable Isotopes Unit (Institute of Material Science) of Democritos Research Institute, Athens, accredited according to EN ISO/IEC 17025:2005.

The samples were analyzed with a ThermoScientific Delta V Plus mass spectrometer after burning at 1020 °C (Flash Elementar Analyzer device). The isotopic ratios are

expressed for carbon as $\delta^{13}C$ versus PDB (a marine carbonate), and for nitrogen as $\delta^{15}N$ versus ‰ air N_2:

$$X = (R_{sample} - R_{standard}) / R_{standard} \cdot 1000$$

where X is the $\delta^{13}C$ or $\delta^{15}N$ value and $R=^{13}C/^{12}C$ and $^{15}N/^{14}N$ respectively. Repeated measurements took place for each of the samples. Analytical precision was 0.1‰ for $\delta^{13}C$, and 0.2‰ for $\delta^{15}N$ values.

3.17. Statistical analysis

A one-way analysis of variance (ANOVA) was conducted to detect differences between means of all parameters for all plant species categorized into 3 distinctive life forms namely: trees, shrubs and herbs. Multiple comparison between means for all pairs of data was done using the Tukey-Kramer HSD ($P<0.05$) (Jmp 7.0, SAS Institute Inc, Cary NC).

In addition, regression analyses were performed to determine the type of relationship that exists between pairs of defined parameters, the strength of the curve and coefficients of determination (r^2) and the statistical significance of correlation coefficients was recorded. Regression analysis was performed using Statgraphics Plus v. 4, (StatPoint Technologies, Inc., Warrenton, VA). Correlations were displayed graphically as scatter graphs using Microsoft Excel v. 12.

4. Results

4.1. Climatic condition of study sites

It is well known that climatic conditions play a key role in defining the distribution and adaptive responses of plants, in combination with other factors. For this reason, we selected the two study sites (Domnista and Parnitha) which portray different climatic conditions (see Table 1); hence species distribution among these sites varies. Domnista, with a typical dense forest formation, is characterized by heavy precipitation and an annual maximum temperature which is slightly higher than the annual minimum temperature for Parnitha, indicating the predominance of a cooler and moister climate. Species distribution at Parnitha is however limited by the combined effect of the long summer drought and higher annual temperatures, resulting to maquis and phrygana vegetation which are more dominant in this area. The present study involved the assessment of key morphological, physiological and biochemical leaf parameters of 30 plant species derived from both study sites and the relationships between these parameters. Moreover, the examined plant species were categorized in to three life forms (trees, shrubs and herbs) and the mean of each parameter within a particular life form was determined. Only the most significant correlations are presented in figures. All correlations including the non- significant correlations in this study are presented in Table 6.

4.2. Parameters studied

a) Morphological parameters

The following morphological parameters were assessed in this study:

1) Specific leaf area (SLA) and its inverse, Leaf mass per area (LMA)
2) Leaf thickness (LT)
3) Leaf density (LD)
4) Transparent leaf area (TLA)

b) Physiological parameters

The physiological parameters studied were as follows:

1) Net photosynthetic capacity per unit leaf area ($A_{max,a}$)
2) Net photosynthetic capacity per unit leaf mass ($A_{max,m}$)
3) Transpiration rate (T_r)
4) Stomatal conductance (g_s)
5) Dark respiration (R_d)
6) Water-use efficiency (WUE = $A_{max,a}/T_r$)
7) Photosynthetic nitrogen-use efficiency (PNUE = $A_{max,a}/N_a$)
8) Electron transport rate (ETR)
9) Quantum Yield of photosystem II (Y)
10) Net photosynthetic capacity per unit chlorophyll concentration ($A_{max,Chl}$)

c) Biochemical parameters

The following biochemical parameters were determined in this study:

1) Total nitrogen content per unit leaf area (N_a)
2) Total nitrogen content per unit leaf mass (N_m)
3) Total phenolic concentration (TP)
4) Condensed tannin concentration (CT)
5) Total Nitrogen/Total phenolics ratio (N/TP)
6) Total Nitrogen/ Condensed tannins ratio (N/CT)
7) Total Nitrogen/Total phenolics + Condensed tannins (N/(TP+CT))
8) Total leaf chlorophyll content (Chl)
9) Stable carbon isotope ratio ($\delta\ ^{13}C$)
10) Stable nitrogen isotope ratio ($\delta\ ^{15}N$)

4.3. Determination of morphological, physiological and biochemical leaf parameters of selected plant species

Data obtained were used to calculate the means and standard errors (se) of parameters for each of the 30 plant species studied. All species showed pronounced differences in morphological, physiological and biochemical leaf parameters measured.

According to assessments of leaf morphological parameters, mean SLA varied from 87.03cm^2 g^{-1} in *Arbutus unedo* to 241.45cm^2 g^{-1} in *Securigera securidaca,* as much as a 3-fold variation across all species sampled. Mean LMA ranged from 41.90g m^{-2} in *Securigera securidaca* to 115.81g m^{-2} in *Arbutus unedo;* Mean leaf thickness ranged from 85μm in *Ballota acetabulosa* to 558.33μm in *Anchusa* sp.; mean leaf density ranged from 157.69 mg m^{-3} in *Anchusa* sp. to 635.38 mg m^{-3} in *Fragaria vesca*; and mean TLA ranged from 2.87% in *Castanea sativa* to 35.46% in *Quercus coccifera* (Table 3).

Table 4 refers to means of physiological parameters in all the plants sampled. Mean $A_{max,a}$ ranged from 2.56 μmol CO_2 m^{-2} s^{-1} in *Rubus fructicosus* to 37.75 μmol CO_2 m^{-2} s^{-1} in *Bituminaria bituminosa.* Mean $A_{max,m}$ ranged from 38.43 nmol CO_2 g^{-1} s^{-1} in *Arbutus unedo* to 653.46 nmol CO_2 g^{-1} s^{-1} in *Malva sylvestris.* The lowest mean T_r (0.59 mmol H_2O m^{-2} s^{-1}) was expressed by *Rubus fructicosus* and the highest mean T_r (8.28 mmol H_2O m^{-2} s^{-1}) by *Bituminaria bituminosa,* thus highlighting the close link between $A_{max,a}$ and T_r. (Table 3). For g_s, the lowest mean (0.01 mol CO_2 m^{-2} s^{-1}) was recorded by *Rubus fructicosus* and the highest mean (0.78 mol CO_2 m^{-2} s^{-1}) by *Bituminaria bituminosa* and *Anchusa* sp. For dark respiration (R_d), mean ranged from -1.34 μmol O_2 m^{-2} s^{-1} in *Pistachia lentiscus* to -0.14 μmol O_2 m^{-2} s^{-1} in *Ballota acetabulosa*; for water use efficiency (WUE), mean ranged from 2.29 μmol CO_2 (mmol H_2O)$^{-1}$ in *Platanus orientalis* to 7.08 μmol CO_2 (mmol H_2O)$^{-1}$ in *Smilax aspera;* for photosynthetic nitrogen- use efficiency (PNUE), mean ranged from 2.63 mmol s^{-1} g^{-1} in *Quercus ithaburensis* to 17.76 mmol s^{-1} g^{-1} in *Phlomis fruticosa*; and for $A_{max,\ Chl}$, mean ranged from 6.54 μmol CO2 g^{-1} Chl s^{-1} in *Smilax aspera* to 107.2 μmol CO2 g^{-1} Chl s^{-1} in *Malva sylvestris.*

In table 5, which represents means of biochemical parameters, lowest and highest means were expressed by *Arbutus unedo* and *Malva sylvestris* respectively for the

following parameters: N_m ranged from 0.39 mmol g^{-1} in *Arbutus unedo* to 1.54 mmol g^{-1} in *Malva sylvestris*; N/TP and N/(TP+CT) ranged from 0.10 mmol mg^{-1} and 0.09 mmol mg^{-1} in *Arbutus unedo* to 2.61 mmol mg^{-1} and 2.54 mmol mg^{-1} in *Malva sylvestris* respectively; N/CT ranged from 1.21 mmol mg^{-1} in *Arbutus unedo* to 92.13 mmol mg^{-1} in *Malva sylvestris*. The mean N/CT ratio for *Olea europaea* was not calculated because condensed tannin was not detected in these leaves. As a result, *Olea europaea* was ranked the lowest in condensed tannins while *Smilax aspera* had the highest mean concentration of 10.29 mg tannins g^{-1} dry weight.

Mean N_a was lowest in *Rubus fructicosus* with 23.82 mmol m^{-2} and highest in *Platanus orientalis* with 90.55 mmol m^{-2}. For TP, the mean ranged from 13.93 mg tannic acid g^{-1} dry weight in *Pyrus amygdaliformis* to 126.09 mg tannic acid g^{-1} dry weight in *Castanea sativa*. Mean total leaf chlorophyll content was lowest in *Fragaria vesca* and highest in *Bituminaria bituminosa.* $\delta^{13}C$ mean ranged from -30.87 ‰ in *Ostrya carpinifolium* to -26.21 ‰ in *Platanus orientalis.* $\delta^{15}N$ mean ranged from -0.55 ‰ in *Quercus frainetto* to 22.85 ‰ in *Styrax officinalis*.

4.4. Correlations between parameters

a) A_{max} vs transpiration rate and stomatal conductance

Across the 30 species studied, simple regression analyses showed that $A_{max,a}$, $A_{max,m}$ and $A_{max,Chl}$ correlated most significantly ($P<0.01$) with T_r and g_s (Figure 8 and Table 6). Transpiration rate and stomatal conductance both increased in a characteristic non-linear manner with $A_{max,a}$ and $A_{max,m}$ indicating that species with higher A_{max} were characterized by higher transpiration rates and stomatal conductance and vice versa. Hence, *Bituminaria bituminosa* which exhibited the highest transpiration rate and stomatal conductance also exhibited the highest net photosynthetic capacity per leaf area. *Anchusa* sp. and *Bituminaria bituminosa* showed considerably distinct stomatal conductance with high $A_{max,Chl}$ and so were displayed as outliers on the graph. The highest $A_{max,Chl}$ was however displayed by *Malva sylvestris* with a considerably distinct stomatal conductance although much lower than that of *Anchusa* sp. and *Bituminaria bituminosa*.

In addition, the correlations between A_{max} and transpiration rate (figures 8a, b & c) and A_{max} and stomatal conductance (figures 8d, e & f) were very strong and positive, regardless of whether A_{max} was expressed on an area basis, on a mass basis or on a chlorophyll basis. Comparatively, leaf area-based net photosynthesis (figures 8a and d) showed a stronger correlation than leaf mass-based net photosynthesis and chlorophyll-based net photosynthesis.

b) A_{max} vs N/TP, N/(TP+CT), N/CT ratios and N_m

Species showed a characteristic set of biochemical parameters that were significantly correlated with A_{max}. Figures 9 and 10 illustrate a significantly strong linear positive relationship ($P<0.01$) between A_{max} on the one hand, and N/TP, N/(TP+CT) and N/CT ratios on the other hand, including $A_{max,m}$-N_m (Table 6).

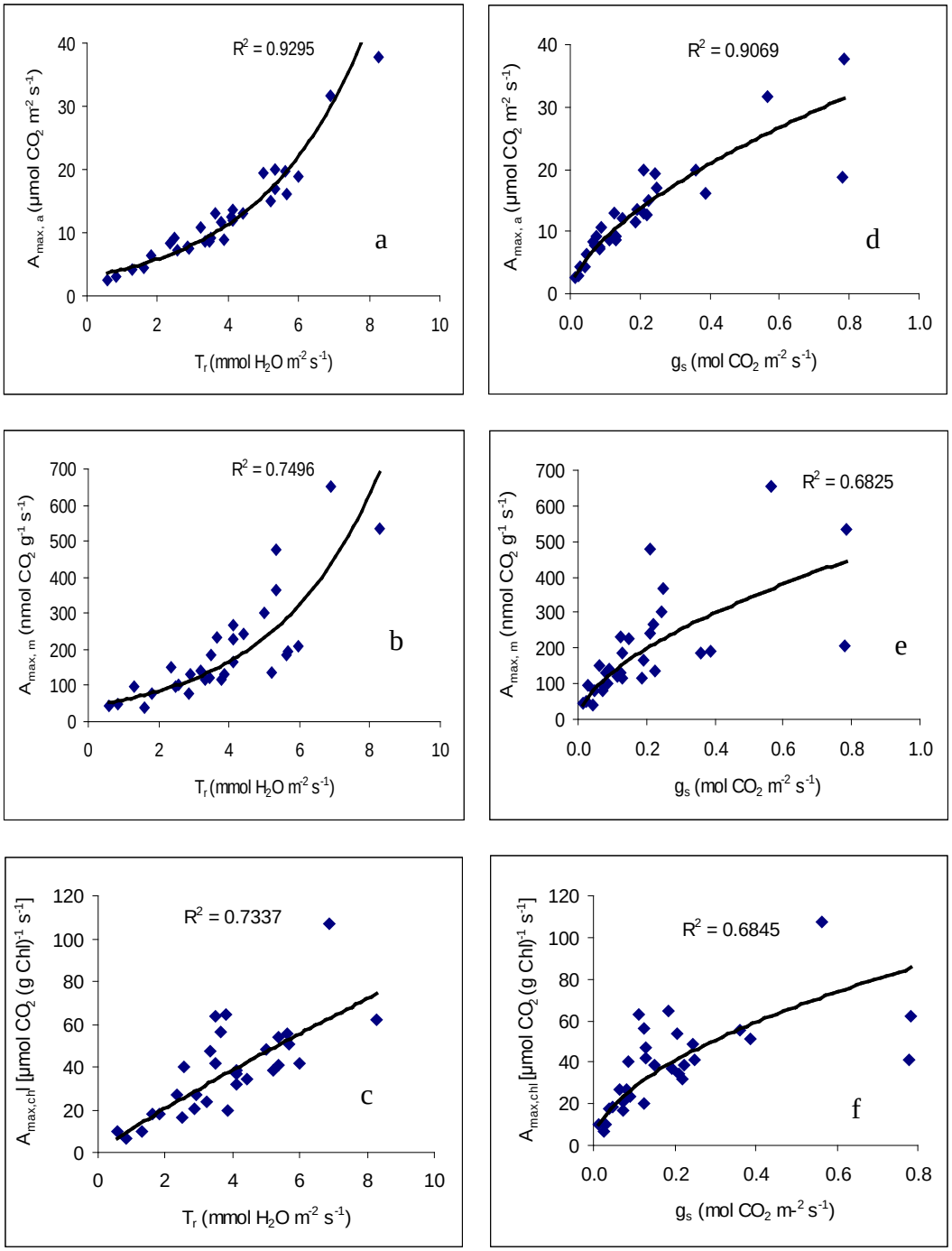

Figure 8. Relationships between net photosynthetic capacity per leaf area, per leaf mass or per chlorophyll concentration and transpiration rate (a, b & c) and stomatal conductance (d, e & f), respectively.

N/TP and N/(TP+CT) were linearly and positively correlated to A_{max} at a significance of $P<0.01$. Plant species with a low net photosynthetic capacity exhibited a low N/TP and N/TP+CT) whereas those with a high net photosynthetic capacity showed a high N/TP and N/(TP+CT) ratios as well. Variation in the mass-based A_{max}-N/TP and A_{max}- N/(TP+CT) relationships showed a more consistent pattern. Hence, mass-based A_{max}-N/TP relationship was stronger, with a higher r^2 (0.789) than area-based A_{max}-N/TP relationship (r^2 = 0.569) and $A_{max,Chl}$ -N/TP relationship (r^2 = 0.474) (Figures 9a, b & c) and mass-based A_{max}-

N/(TP+CT) relationship was also stronger, with a higher r^2 (0.80) than area-based A_{max}-N/TP relationship (r^2 = 0.58) and $A_{max,Chl}$ -N/TP relationship (r^2 = 0.48) (Figures 9d, e & f). Apart from *Malva sylvestris* and *Bituminaria bituminosa* which exhibited a considerably distinct N/TP and N/(TP+CT) mean (above 1.5) and $A_{max,a}$, all the other species exhibited a N/TP and N/(TP+CT) mean below 1.2 (see also Table 6). However, compared to A_{max}-N/TP, the relationship between A_{max} and N/(TP+CT) showed a slightly higher correlation coefficient indicating a stronger relationship.

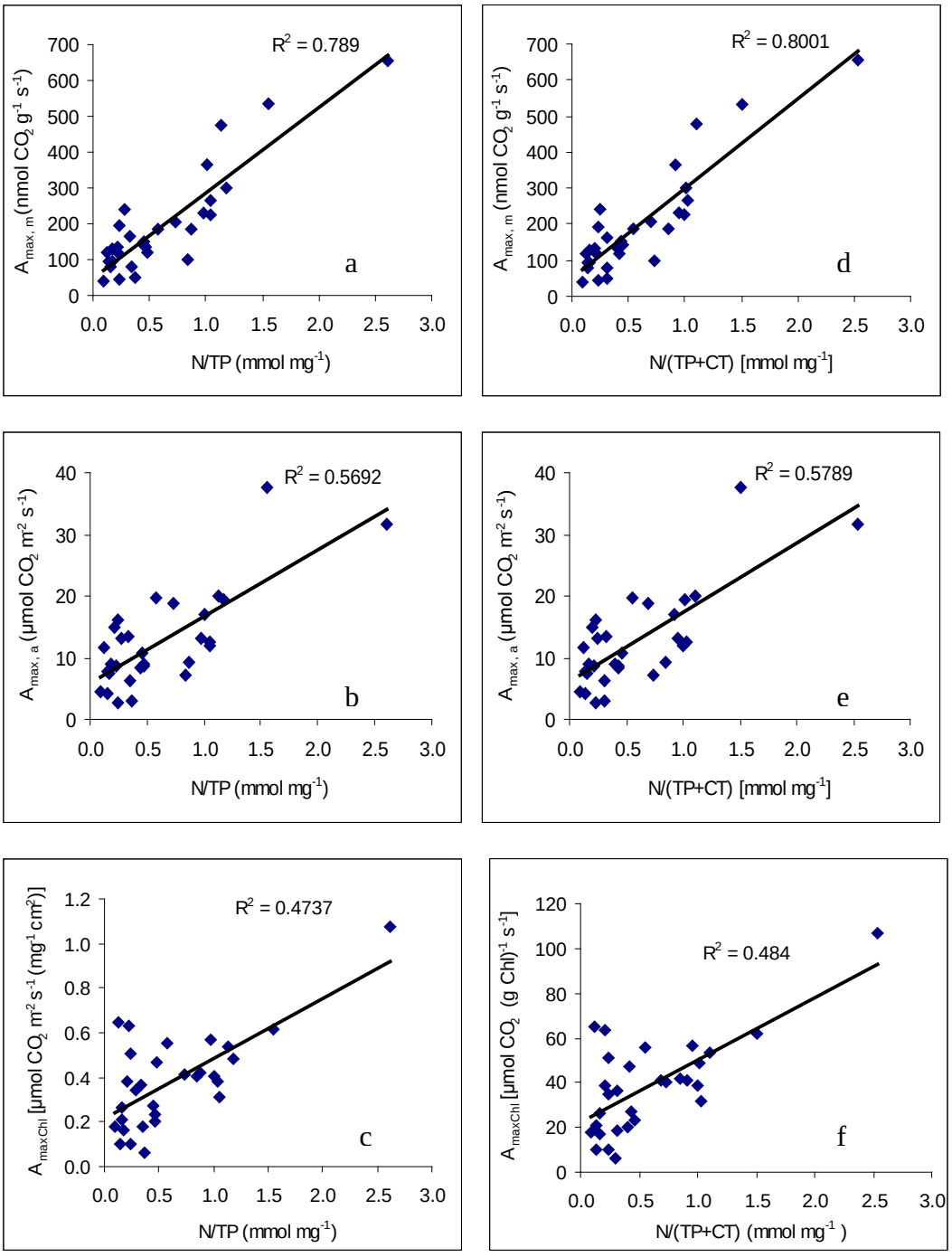

Figure 9. Relationships between net photosynthetic capacity per leaf area, per leaf mass or per chlorophyll content and N/TP (a, b & c), N/(TP+CT) ratios (d, e & f).

Moreover, the same pattern of increase of N/CT mean with increasing A_{max} was observed (Figures 10a, b & c). Plant species with low tannin content exhibited N/CT lower than 6 and appeared to be clustered near the Y-axis despite their differences in A_{max}. There were also a few other plant species with high levels of N/CT and high photosynthetic capacity per leaf area, per leaf mass and per chlorophyll content. These were *Malva sylvestris, Bituminaria bituminosa* and *Securigera securidaca.*

It was also observed that plant species with the lowest $A_{max,m}$ showed the lowest N_m while plant species with the highest $A_{max,m}$ showed the highest N_m (Figure 10d). This pattern was not obvious for $A_{max,a}$ with leaf nitrogen concentration per area which had a relatively weak correlation (see Table 6).

c) T_r vs N/TP and N/(TP+CT) ratios

Transpiration rate was significantly positively correlated ($P<0.01$) with N/TP and N/(TP+CT), (Table 6) with indications of higher N/TP and N/(TP+CT) for species with high transpiration rate and vice versa. *Malva sylvestris* and *Bituminaria bituminosa* showed considerably distinct N/TP and N/(TP+CT) ratio with high transpiration rates (Figures 11a & b).

In addition, the correlations between transpiration rate and N/TP and N/(TP+CT) were very strong and linear. Comparatively, N/(TP+CT) showed a slightly stronger correlation than N/TP.

d) Συσχετίσεις μεταξύ της παραμέτρου PNUE με TP και τους λόγους N/TP, N/(TP+CT), N/CT

PNUE was significantly positively correlated ($P<0.01$) with N/TP and N/(TP+CT) in a non-linear manner (Table 6). As the ratios increased, PNUE increased almost linearly first, then reached a saturation point beyond which PNUE showed little response to increasing ratios (Figures 12a, b & c). Relative to PNUE-N/TP, the relationship between PNUE and N/(TP+CT) showed a slightly higher correlation coefficient indicating a stronger relationship.

PNUE was also significantly correlated ($P<0.01$) with N/CT and TP in a non-linear manner (Table 6). Both correlations were moderately strong with r^2 values of 0.2793 and 0.5836 respectively (Figures 12c & d). While PNUE-N/CT relationship was positive, that of PNUE-TP was negative. N/CT ratio was observed to be very low in certain plant species and appeared clustered near the Y-axis despite increasing PNUE (Figure 12c).

PNUE decreased with increasing TP. Hence, plant species with high concentration of total phenolics tended to exhibit low PNUE while those with low concentration of total phenolics exhibited high PNUE (figure 12d).

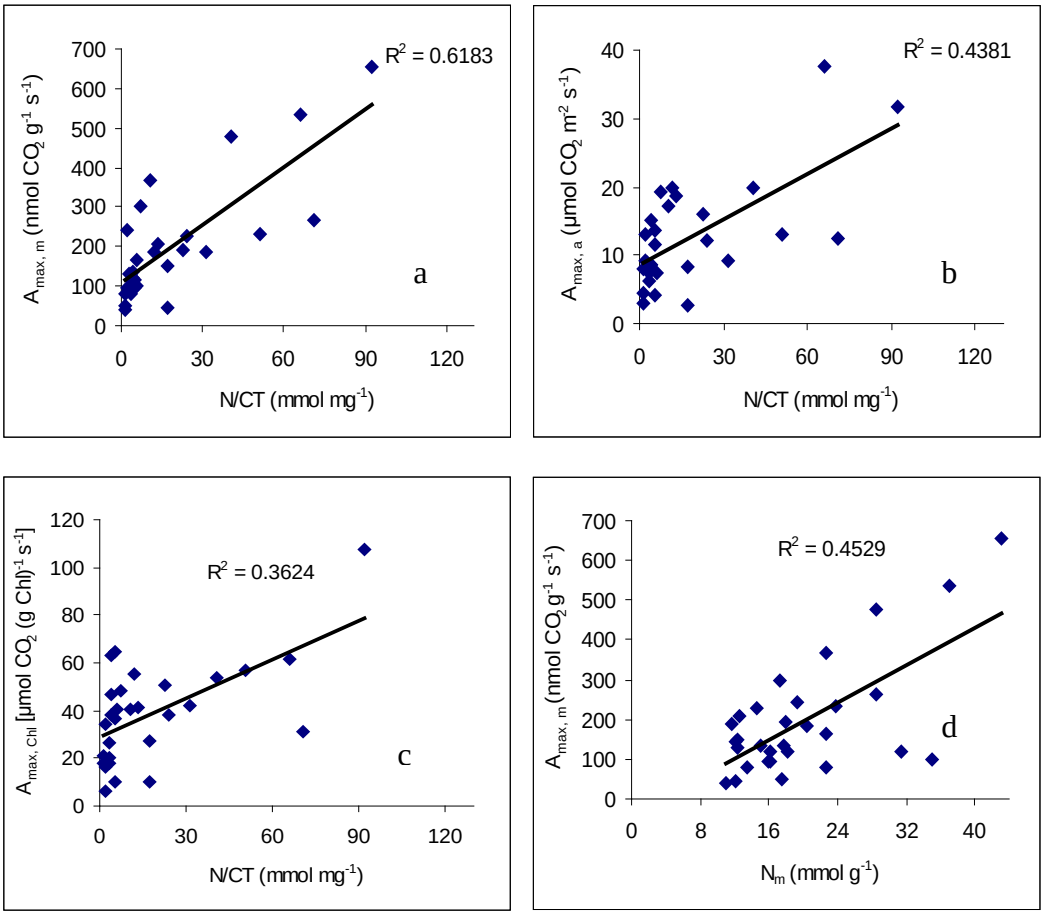

Figure 10. Relationships between net photosynthetic capacity per leaf area, per leaf mass or per chlorophyll content and N/CT ratio (a, b & c) respectively and between net photosynthetic capacity per leaf mass and N_m (d).

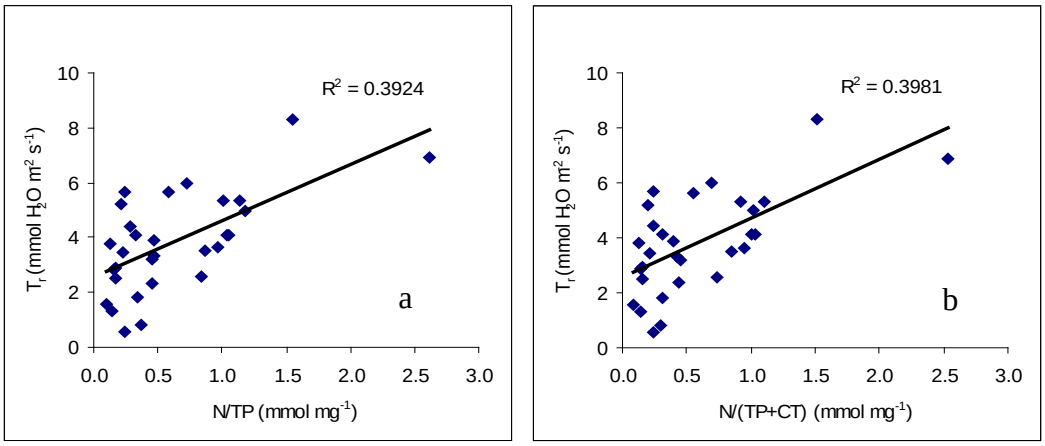

Figure 11. Relationships between transpiration rate and (a) N/TP and (b) N/(TP+CT) ratios

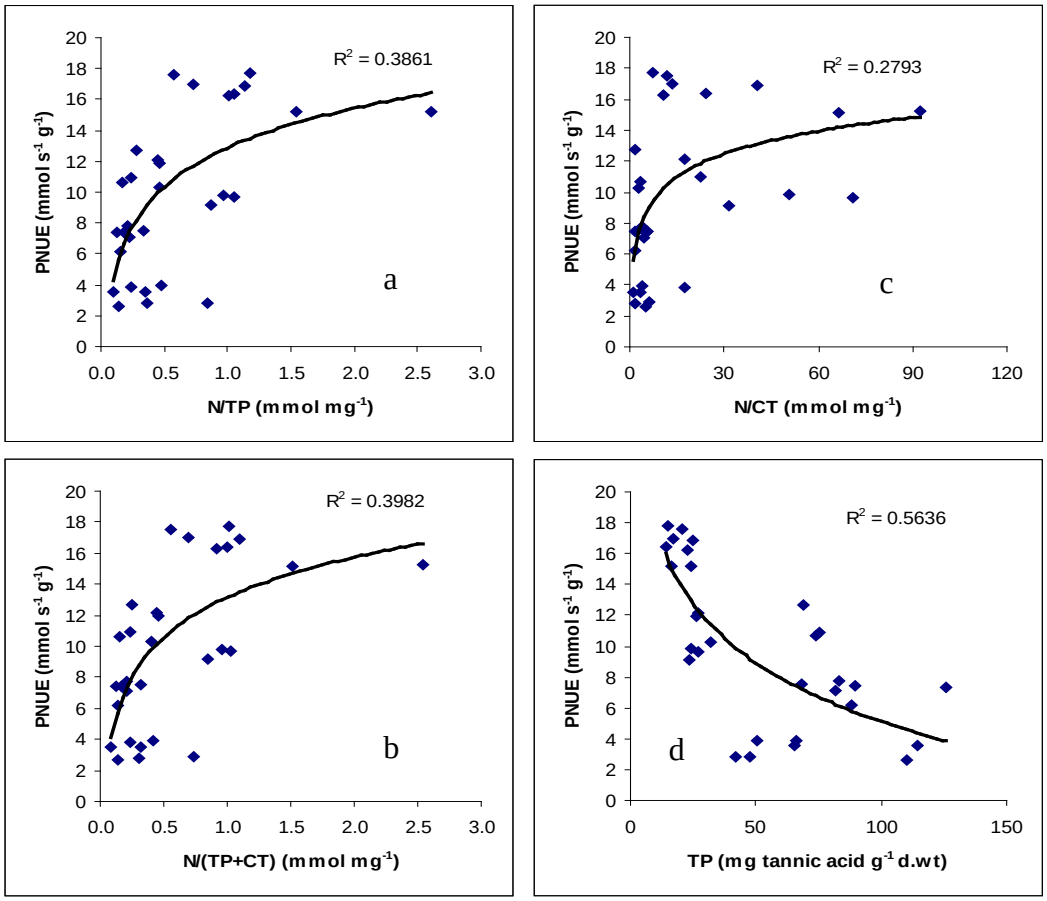

Figure 12. Relationships between photosynthetic nitrogen-use efficiency and (a) N/TP (b) N/(TP+CT) (c) N/CT ratios and (d) TP.

e) Y441 vs N/TP and N/(TP+CT) ratios

A positive but relatively weak relationship was found between the effective quantum yield of photosystem II and N/TP and N/(TP+CT). At a given light intensity of 441 µmol m^{-2} s^{-1} PAR, quantum yield was significantly correlated ($P<0.01$ and $P<0.05$) with N/TP and N/(TP+CT) respectively (Table 6) (Figure 13a & b).

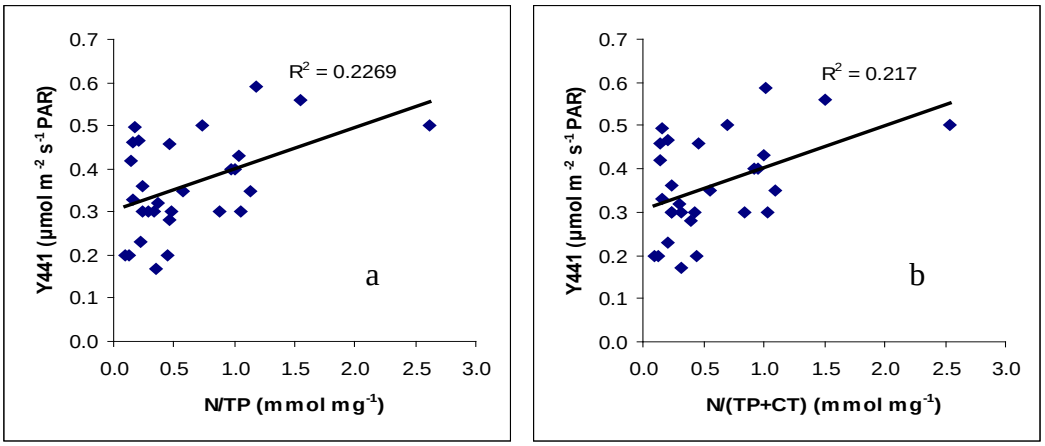

Figure 13. Relationships between effective quantum yield of PSII at light intensity of 441 µmol m^{-2} s^{-1} PAR and (a) N/TP and (b) N/(TP+CT) ratios.

f) LMA vs N/TP and N/(TP+CT) ratios

LMA was significantly correlated with N/TP and N/(TP+CT) ratios at P<0.01 (Table 6). Both relationships were relatively weak and negative, displaying a similar pattern with a slightly higher coefficient of determination for N/(TP+CT) than for N/TP (Figure 14a & b). There was a distinction between *Malva sylvestris* with the highest ratios of N/TP and N/(TP+CT) (2.609 mmol mg^{-1} and 2.537 mmol mg^{-1} respectively) and all the other species below 1.547 mmol $mg^{-1}$and 1.512 mmol mg^{-1} respectively. LMA being the inverse of SLA, N/TP and N/(TP+CT) increased with increasing SLA (graphs not shown).

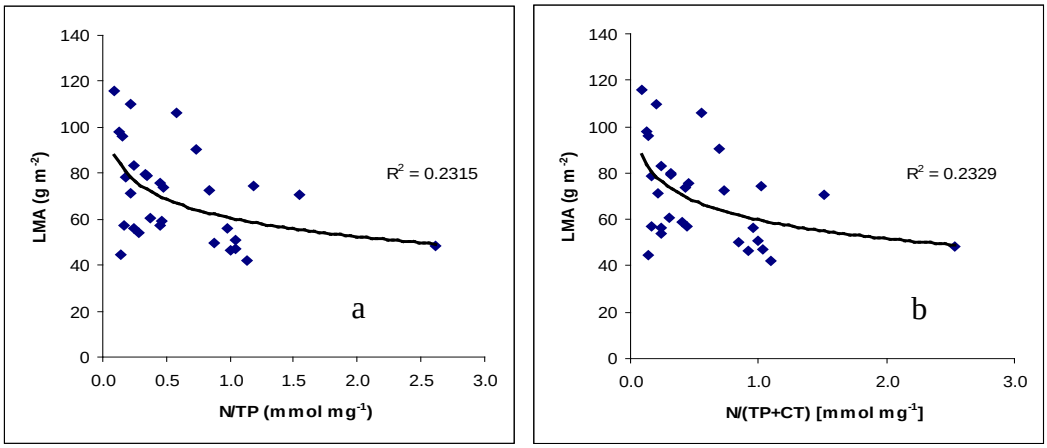

Figure 14. Relationships between LMA and (a) N/TP and (b) N/(TP+CT) ratios.

g) TP vs N/TP and N/(TP+CT) ratios

Total phenolics (TP) was significantly negatively correlated (P<0.01) with N/TP and N/(TP+CT) ratios (Table 6). Both correlations were non-linear, strong and displaying a similar pattern, with a slightly higher coefficient of determination for N/TP than for N/(TP+CT). As total phenolics concentration decreased in plant species, N/TP and N/(TP+CT) ratios increased. Hence, plant species with a low concentration of total phenolics had high N/TP and N/(TP+CT) ratios (Figures 15a & b).

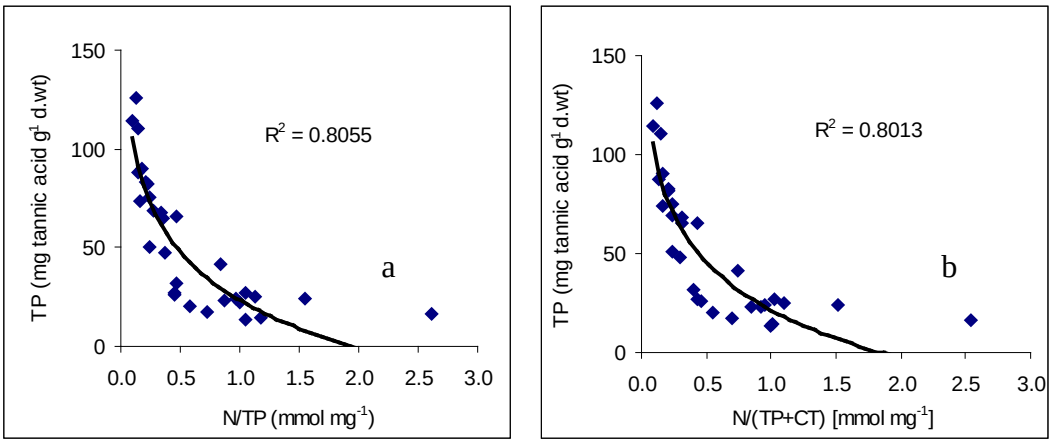

Figure 15. Relationships between LMA and (a) N/TP and (b) N/(TP+CT) ratios.

h) $\delta^{13}C$ vs PNUE

$\delta^{13}C$ was significantly negatively correlated ($P<0.01$) with PNUE (Table 6). The relationship was linear and weak. As PNUE decreased in plant species, $\delta^{13}C$ increased. (Figure 16).

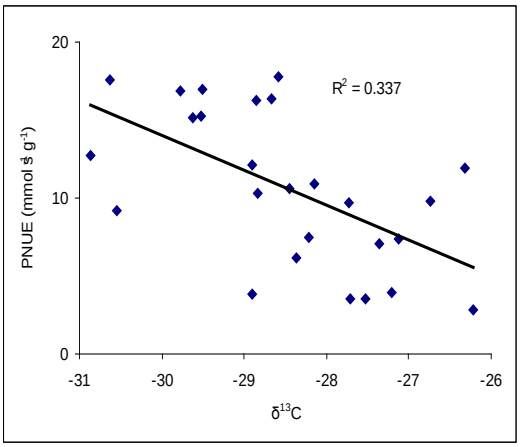

Figure 16. Relationship between $\delta^{13}C$ and PNUE.

i) $\delta^{15}N$ vs N_a and WUE

$\delta^{15}N$ was significantly negatively correlated ($P<0.01$) in a non-linear manner with N_a and positively correlated ($P<0.10$) with WUE (Table 6). An increase in $\delta^{15}N$ resulted to a decrease in N_a but an increase in WUE (figure 17).

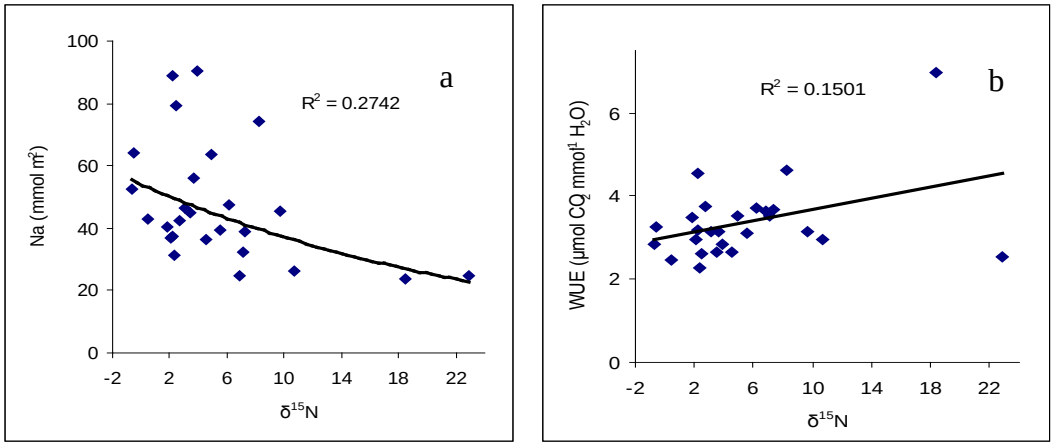

Figure 17. Συσχετίσεις μεταξύ της παραμέτρου $\delta^{15}N$ και των παραμέτρων (a) N_a , (b) WUE

4.5. Variation of morphological parameters across life forms

No significant difference was detected in all the morphological parameters measured between trees, shrubs and herbs in spite of the slight differences in means of parameters between life forms (Table 7). Mean LMA and LD were highest in trees and lowest in herbs. In the case of TLA, only data from heterobaric species were analyzed and were categorized as follows: 10 trees, 8 shrubs and 3 herbs. Shrubs exhibited the highest mean TLA while herbs exhibited the lowest mean TLA. Mean SLA was highest in herbs and lowest in trees. Mean LT was also highest in herbs but lowest in shrubs.

4.6. Variation of physiological parameters across life forms

Mean net photosynthetic capacity differed among life forms. Overall, herbs had a higher mean A_{max} than trees and shrubs, with a 2-fold variation on the average (Table 8). Mean A_{max} for herbs was significantly different from mean A_{max} for trees and shrubs. Mean $A_{max,a}$ for trees was slightly higher than that for shrubs while mean $A_{max,m}$ for shrubs was higher than that for trees. However, means of A_{max} for both trees and shrubs were not significantly different.

Likewise, means of $A_{max,Chl}$, transpiration rate, stomatal conductance and photosynthetic nitrogen use efficiency differed among life forms and were higher for herbs than for trees and shrubs. The means of these parameters for herbs were significantly different from those of trees and shrubs whereas there was no significant difference in means these parameters between trees and shrubs.

R_d, ETR, WUE and Yield showed a different trend, displaying means with no significant differences between trees, shrubs and herbs even though with slight differences in means (Table 8). Trees exhibited the highest R_d and shrubs the lowest. Meanwhile, shrubs exhibited the highest WUE and ETR but the lowest means were observed in trees. The highest yield was displayed by herbs and the lowest by trees.

4.7. Variation of biochemical parameters across life forms

Means of N/TP and N/(TP+CT) differed among life forms and were higher for herbs than for trees and shrubs. Means of N/TP and N/(TP+CT) for herbs were significantly different from those of trees but not for those of shrubs. Likewise, there was no significant difference in means of N/TP and N/(TP+CT) between trees and shrubs. Overall, trees had the lowest means N/TP and N/(TP+CT).

All the other parameters showed a different trend, displaying means with no significant differences between life forms even though with slight differences in means (Table 9). Trees exhibited the highest leaf nitrogen concentration per area whereas herbs exhibited the highest leaf nitrogen concentration per mass. The lowest leaf nitrogen concentrations were observed in shrubs. Trees were also characterized by highest total phenolics and tannin content while herbs displayed lowest total phenolics and tannin content. As a result, the ratios N/TP and N/TP+CT showed significant differences between life forms. Nevertheless, herbs possessed the highest N/CT ratio, total leaf chlorophyll content and $\delta^{13}C$ isotope and the lowest concentrations of these components were observed in trees. $\delta^{15}N$ isotope was highest in shrubs and lowest in herbs.

Table 3. Summary of the morphological parameters (mean ± standard errors) in all 30 species studied.

	Plant species	study site	life form	leaf type	SLA ($cm^2\ g^{-1}$)	LMA ($g\ m^{-2}$)	leaf thickness (LT)	leaf density ($mg\ m^{-3}$)	TLA (%)
1	*Anchusa* sp.	Parnitha	Herb	homobaric	114.02 ± 9.59	90.62± 8.81	558.33± 3.19	157.69± 14.99	na
2	*Arbutus unedo*	Parnitha	Shrub	heterobaric	87.03± 3.83	115.81± 5.18	307.22± 4.67	373.27± 15.62	9.20± 0.39
3	*Ballota acetabulosa*	Parnitha	Herb	homobaric	201.32± 7.21	49.93± 1.80	85.00± 2.03	583.95± 19.28	na
4	*Bituminaria bituminosa*	Parnitha	Herb	heterobaric	149.30± 19.78	70.67± 7.02	301.33± 12.89	234.52± 23.30	9.33± 0.62
5	*Castanea sativa*	Domnista	Tree	heterobaric	102.47± 3.87	98.13± 3.56	231.33± 14.89	424.18± 15.40	2.87± 0.72
6	*Cercis siliquastrum*	Parnitha	Tree	heterobaric	141.40± 14.41	73.52± 6.92	215.56± 9.69	343.78± 28.86	11.76± 0.67
7	*Cionura erecta*	Parnitha	Shrub	heterobaric	179.30± 8.56	56.29± 2.72	169.44± 4.82	331.37± 14.37	7.13± 0.35
8	*Clematis vitalba*	Domnista	Shrub	heterobaric	219.76± 22.33	47.27± 4.67	215.56± 4.01	219.28± 21.66	6.02± 0.49
9	*Echinops* sp.	Domnista	Herb	homobaric	103.85± 15.88	106.03± 16.57	229.33± 5.42	462.34± 72.26	na
10	*Fragaria vesca*	Domnista	Herb	heterobaric	151.56± 19.72	70.99± 9.74	111.72±4.33	635.38± 87.20	6.74± 0.20
11	*Juglans regia*	Domnista	Tree	heterobaric	126.98± 4.12	79.11± 2.79	127.78± 10.56	619.15± 21.87	16.07± 1.52
12	*Lotus ornithopodioides*	Parnitha	Herb	homobaric	217.05± 10.15	46.50± 2.32	221.33± 9.10	210.11± 10.46	na
13	*Malva sylvestris*	Parnitha	Herb	homobaric	206.84± 6.21	48.51± 1.35	232.00± 15.58	209.08± 5.81	na
14	*Olea europaea*	Parnitha	Tree	heterobaric	133.57± 4.63	75.60± 2.65	426.79± 9.01	177.13± 6.20	na
15	*Ostrya carpinifolium*	Domnista	Tree	heterobaric	187.05± 9.99	54.05± 2.76	201.11± 9.76	268.77± 13.74	11.23± 0.48
16	*Phlomis fruticosa*	Parnitha	Shrub	heterobaric	159.86± 13.07	74.36± 3.37	148.60± 18.57	341.50± 14.73	8.14± 0.21
17	*Pistacia lentiscus*	Parnitha	Tree	homobaric	104.62± 2.68	96.11± 2.54	398.36± 14.16	241.25± 6.37	na
18	*Pistacia terebinthus*	Parnitha	Tree	heterobaric	91.76± 3.02	109.89± 3.50	292.38± 3.75	375.87± 11.97	19.30± 1.68
19	*Platanus orientalis*	Parnitha	Tree	heterobaric	171.12± 5.65	59.03± 2.24	203.33± 12.21	292.12± 16.62	10.71± 0.50
20	*Platanus orientalis*	Domnista	Tree	heterobaric	138.49± 4.38	72.52± 2.42	196.11± 6.69	369.77± 12.32	14.69± 0.61
21	*Pyrus amygdaliformis*	Parnitha	Tree	heterobaric	198.77± 5.93	50.70± 1.64	224.61± 6.35	227.46± 11.13	28.13± 1.42
22	*Quercus coccifera*	Parnitha	Shrub	heterobaric	129.36± 5.75	78.56± 3.54	392.24± 14.38	201.45± 10.15	35.46± 1.13
23	*Quercus frainetto*	Domnista	Tree	heterobaric	126.01± 4.89	79.83± 3.22	172.22± 9.80	463.52± 18.71	13.33± 0.80
24	*Quercus ithaburensis*	Parnitha	Tree	heterobaric	99.22± 2.60	44.68± 1.33	202.00± 6.46	221.18± 6.56	15.93± 0.67
25	*Rubus fruticosa*	Parnitha	Shrub	heterobaric	180.98± 8.17	56.19± 2.60	154.53± 6.49	363.62± 16.85	15.68± 0.84
26	*Rubus* sp.	Domnista	Shrub	heterobaric	122.85± 8.53	83.21± 6.56	213.33± 5.09	390.06± 30.77	11.85± 0.69
27	*Securigera securidaca*	Parnitha	Herb	homobaric	241.45± 12.11	41.90± 2.43	231.33± 20.86	181.14± 10.53	na
28	*Smilax aspera*	Parnitha	Shrub	homobaric	165.69± 2.13	60.44± 0.79	172.78± 3.29	350.65± 5.09	na
29	*Styrax officinalis*	Parnitha	Shrub	heterobaric	176.40± 13.96	56.99± 1.52	156.59± 3.28	363.92± 11.88	22.66± 0.86
30	*Thapsia garganica*	Parnitha	Herb	heterobaric	178.20± 11.40	57.00± 3.48	262.67± 6.09	217.01± 4.07	6.66± 0.87

na = not applicable (homobaric leaves do not possess transparent areas in the leaf lamina)

Table 4. Summary of the physiological parameters (mean ± standard errors) in all 30 species studied.

Plant species	study site	life form	$A_{max,a}$ $\mu mol\ m^{-2}\ s^{-1}$	$A_{max,m}$ $nmol\ g^{-1}\ s^{-1}$	$A_{max,Chl}$ $\mu mol\ g\ chl^{-1}\ s^{-1}$	WUE $\mu mol\ CO_2\ mmol^{-1}\ H_2O$	PNUE $mmol\ s^{-1}\ g^{-1}$	R_d $\mu mol\ m^{-2}\ s^{-1}$	g_s $mol\ m^{-2}\ s^{-1}$	T_r $mmol\ m^{-2}\ s^{-1}$	ETR 441 $\mu mol\ m^{-2}\ s^{-1}$ PAR	Yield 441 $\mu mol\ m^{-2}\ s^{-1}$ PAR
Anchusa sp.	Parnith	Herb	18.75±0.11	206.93±1.26	41.3±0.3	3.12±0.06	16.96±0.10	-0.15±	0.78±0.05	5.98±0.14	101.8	0.5
Arbutus unedo	Parnith	Shrub	4.45±1.58	38.43±13.66	17.9±6.4	2.65±0.64	3.52±1.25	-0.39±0.05	0.04±0.01	1.59±0.20	41.5	0.2
Ballota acetabulosa	Parnith	Herb	9.28±1.08	185.94±21.61	41.8±4.9	2.66±0.19	9.15±1.06	-0.14±0.08	0.13±0.01	3.50±0.14	58.5	0.3
Bituminaria bituminosa	Parnith	Herb	37.75±3.47	534.19±49.12	61.9±5.7	4.54±0.16	15.18±1.40	-0.82±0.24	0.78±0.12	8.28±0.55	113	0.56
Castanea sativa	Domnist	Tree	11.56±2.31	117.84±23.53	64.9±13.0	3.14±0.19	7.38±1.47	-0.61±0.25	0.19±0.07	3.80±0.95	43	0.2
Cercis siliquastrum	Parnith	Tree	8.69±0.72	118.13±9.86	46.9±3.9	2.61±0.05	3.92±0.33	-0.93±0.22	0.13±0.03	3.33±0.37	58.9	0.3
Cionura erecta	Parnith	Shrub	13.08±0.14	232.44±2.55	56.6±0.6	3.71±0.47	9.84±0.11	-1.19±0.40	0.13±0.02	3.64±0.49	75.5	0.4
Clematis vitalba	Domnist	Shrub	12.54±1.22	265.33±25.85	31.5±3.1	3.13±0.04	9.66±0.94	-0.36±0.26	0.22±0.03	4.10±0.32	47.5	0.3
Echinops sp.	Domnist	Herb	19.80±2.76	186.74±26.04	55.4±7.7	3.49±0.22	17.56±2.45	-0.67±0.14	0.36±0.10	5.64±0.75	65	0.35
Fragaria vesca	Domnist	Herb	8.53±0.04	120.15±0.54	63.3±0.3	2.48±0.09	7.08±0.03	-0.16±0.05	0.11±0.01	3.46±0.11	42.6	0.23
Juglans regia	Domnist	Tree	6.30±1.06	79.70±13.35	18.2±3.1	3.52±0.15	3.53±0.59	-0.66±0.06	0.05±0.01	1.80±0.24	31	0.17
Lotus ornithopodioides	Parnith	Herb	17.05±1.97	366.62±42.29	40.8±4.7	3.19±0.17	16.27±1.88	-0.51±0.08	0.25±0.03	5.34±0.33	81.4	0.4
Malva sylvestris	Parnith	Herb	31.70±3.59	653.46±73.95	107.2±12.1	4.63±0.24	15.21±1.72	-0.88±0.11	0.56±0.10	6.89±1.03	95	0.5
Olea europaea	Parnith	Tree	10.75±0.51	141.69±6.76	23.4±1.1	3.54±0.13	11.91±0.57	-0.96±0.09	0.09±0.01	3.21±0.22	93	0.459
Ostrya carpinifolium	Domnist	Tree	13.05±0.60	241.40±11.16	34.5±1.6	2.95±0.14	12.70±0.59	nd	0.21±0.03	4.43±0.21	49.6	0.3
Phlomis fruticosa	Parnith	Shrub	19.32±1.94	299.77±29.77	48.5±4.9	3.67±0.24	17.76±1.79	-0.93±0.27	0.24±0.02	5.00±0.20	142	0.589
Pistacia lentiscus	Parnith	Tree	7.91±3.70	79.94±37.45	20.7±9.7	3.15±0.47	6.18±2.90	-1.34±0.17	0.07±0.03	2.86±1.58	95.5	0.46
Pistacia terebinthus	Parnith	Tree	14.99±0.58	134.15±5.23	38.5±1.5	2.89±0.14	7.78±0.30	-0.87±0.29	0.23±0.02	5.20±0.14	102	0.466
Platanus orientalis	Parnith	Tree	8.98±	132.66±	20.0±	2.29±	10.28±0.00	-0.30±	0.13±	3.88±	47	0.28
Platanus orientalis	Domnist	Tree	7.26±0.55	100.16±7.57	40.3±3.0	2.85±0.17	2.86±0.22	-0.62±0.15	0.08±0.01	2.56±0.31	nd	nd
Pyrus amygdaliformis	Parnith	Tree	12.00±1.40	226.32±26.31	38.6±4.5	2.95±0.15	16.40±1.91	-0.33±0.01	0.15±0.01	4.12±0.69	83	0.43
Quercus coccifera	Parnith	Shrub	9.10±0.47	95.04±20.58	16.7±0.9	2.93±0.75	7.41±0.38	-0.67±0.16	0.07±0.01	2.49±0.32	119.5	0.495
Quercus frainetto	Domnist	Tree	13.49±0.62	164.29±7.61	36.7±1.7	3.27±0.06	7.49±0.35	nd	0.19±0.01	4.12±0.12	52.5	0.3
Quercus ithaburensis	Parnith	Tree	4.26±0.60	95.83±13.48	10.0±1.4	3.33±0.45	2.63±0.37	-0.61±0.18	0.03±0.00	1.30±0.11	91.5	0.42
Rubus fruticosa	Parnith	Shrub	2.56±0.50	44.26±8.67	9.9±1.9	6.97±1.91	3.83±0.75	-0.44±0.07	0.01±	0.59±0.25	61.5	0.36
Rubus sp.	Domnist	Shrub	16.02±1.15	192.57±13.81	50.9±3.6	2.83±0.13	10.94±0.78	-0.78±0.44	0.39±0.01	5.67±0.17	59.7	0.3
Securigera securidaca	Parnith	Herb	19.96±5.25	476.32±125.19	53.6±14.1	3.74±0.26	16.88±4.44	-1.00±0.23	0.21±0.06	5.34±1.37	65	0.35
Smilax aspera	Parnith	Shrub	2.98±0.90	49.39±14.89	6.5±2.0	7.08±2.93	2.81±0.85	-0.23±0.12	0.02±0.01	0.82±0.25	54	0.319
Styrax officinalis	Parnith	Shrub	7.39±0.69	129.64±12.16	26.6±2.5	2.54±0.20	10.64±1.00	-0.19±0.05	0.08±0.01	2.91±0.36	92	0.33
Thapsia garganica	Parnith	Herb	8.33±0.98	150.70±18.08	26.9±3.2	3.63±0.87	12.13±1.42	-0.82±0.02	0.06±0.01	2.35±0.53	44.8	0.2

Table 5. Summary of the biochemical parameters (mean ± standard errors) in all 30 species studied.

Plant Species	study site	life form	$N_{,m}$ (mmol g^{-1})	$N_{,a}$ (mmol m^{-2})	TP mg tannic acid g^{-1}.d.wt	CT mg tannins g^{-1} d.wt	N/TP	N/CT	N/(TP+CT)	Chl conc. (μg cm^{-2})	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰
Anchusa sp.	Parnitha	Herb	0.45±0.01	39.49±0.0001	17.27±0.63	0.95±0.08	0.73	13.25	0.69	24.90±0.75	-29.50±0.05	5.52±0.78
Arbutus unedo	Parnitha	Shrub	0.39±0.01	45.16±0.0001	114.22±2.65	9.12±0.12	0.10	1.21	0.09	45.42±0.11	-27.70±0.02	3.48±0.78
Ballota acetabulosa	Parnitha	Herb	0.73±0.02	36.25±0.0001	23.44±0.53	0.65±0.07	0.87	31.45	0.85	22.22±0.42	-30.55±0.18	4.54±0.90
Bituminaria bituminosa	Parnitha	Herb	1.33±0.04	88.82±0.0004	23.99±0.17	0.56±0.08	1.55	66.21	1.51	60.97±0.68	-29.63±0.15	2.24±0.43
Castanea sativa	Domnista	Tree	0.57±0.01	55.97±0.0001	126.09±2.47	3.05±0.08	0.13	5.26	0.12	17.82±0.38	-27.13±.06	3.61±0.47
Cercis siliquastrum	Parnitha	Tree	1.12±0.04	79.11±0.0004	65.84±2.79	8.11±0.46	0.48	3.86	0.42	18.50±0.53	-27.21±0.03	2.47±0.38
Cionura erecta	Parnitha	Shrub	0.85±0.04	47.48±0.0002	24.50±0.48	0.47±0.02	0.97	50.94	0.95	23.10±0.61	-26.73±0.03	6.17±0.37
Clematis vitalba	Domnista	Shrub	1.02±0.03	46.36±0.0002	27.25±1.34	0.40±0.06	1.05	70.94	1.03	39.85±0.19	-27.73±0.74	3.10±.82
Echinops sp.	Domnista	Herb	0.42±0.01	40.26±0.0002	20.29±1.11	0.99±0.07	0.58	11.82	0.55	35.73±0.55	-30.63±0.17	1.85±1.09
Fragaria vesca	Domnista	Herb	0.65±0.01	42.99±0.0002	82.00±2.14	4.29±0.37	0.22	4.26	0.21	13.48±0.38	-27.36±0.09	0.45±0.27
Juglans regia	Domnista	Tree	0.81±0.01	63.82±0.0001	65.22±4.68	6.79±0.61	0.35	3.34	0.32	34.62±0.12	-27.53±0.28	4.91±0.53
Lotus ornithopodioides	Parnitha	Herb	0.81±0.01	37.42±0.0001	22.66±2.64	2.17±0.46	1.00	10.48	0.92	41.77±0.41	-28.84±0.26	2.22±1.54
Malva sylvestris	Parnitha	Herb	1.54±0.02	74.43±0.0001	16.52±2.57	0.47±0.08	2.61	92.13	2.54	29.58±1.07	-29.53±0.03	8.22±0.77
Olea europaea	Parnitha	Tree	0.43±0.01	32.21±0.0001	26.39±0.39	0.00±	0.46	n.d.	n.d.	45.96±0.23	-26.32±0.38	7.11±3.90
Ostrya carpinifolium	Domnista	Tree	0.69±0.01	36.69±0.0001	68.79±1.32	10.14±0.46	0.28	1.90	0.24	37.84±0.21	-30.87±0.23	2.12±0.93
Phlomis fruticosa	Parnitha	Shrub	0.62±0.02	38.86±0.0002	14.75±0.20	2.38±0.05	1.18	7.31	1.02	39.83±0.50	-28.59±.01	7.29±0.43
Pistacia lentiscus	Parnitha	Tree	0.48±0.01	45.69±0.0001	87.90±1.24	8.87±0.15	0.15	1.51	0.14	38.13±0.21	-28.36±0.04	9.68±0.67
Pistacia terebinthus	Parnitha	Tree	0.63±0.02	68.84±0.0002	83.07±2.56	4.31±0.31	0.21	4.10	0.20	38.97±0.48	n.d.	n.d.
Platanus orientalis	Parnitha	Tree	0.53±0.07	31.18±0.0004	32.15±2.02	4.95±0.06	0.46	3.02	0.40	44.97±0.22	-28.83±0.07	2.33±0.61
Platanus orientalis	Domnista	Tree	1.25±0.03	90.55±0.0002	41.82±2.31	5.93±0.46	0.84	5.92	0.74	18.01±0.25	-26.21±0.10	3.88±0.78
Pyrus amygdaliformis	Parnitha	Tree	0.52±0.01	26.15±0.0001	13.93±0.33	0.61±0.03	1.04	23.97	1.00	31.12±0.54	-28.67±0.02	10.72±1.33
Quercus coccifera	Parnitha	Shrub	0.57±0.01	43.87±0.0001	89.92±5.28	8.07±0.19	0.18	1.97	0.16	54.48±0.22	n.d.	n.d.
Quercus frainetto	Domnista	Tree	0.81±0.02	64.32±0.0002	67.92±0.69	4.02±0.46	0.33	5.65	0.32	36.81±0.23	-28.21±0.20	-0.55±0.29
Quercus ithaburensis	Parnitha	Tree	0.57±0.01	57.89±0.0001	110.50±4.59	3.02±0.23	0.15	5.32	0.14	42.59±0.12	n.d.	n.d.
Rubus fruticosa	Parnitha	Shrub	0.43±0.01	23.82±0.0001	50.63±1.47	0.70±0.02	0.24	17.34	0.24	25.85±0.12	-28.90±0.02	18.40±0.62
Rubus sp.	Domnista	Shrub	0.64±0.05	52.34±0.0004	75.44±2.87	0.79±0.05	0.24	22.71	0.24	31.50±0.32	-28.15±0.17	-0.69±0.37
Securigera securidaca	Parnitha	Herb	1.02±0.02	42.23±0.0001	25.19±0.64	0.71±0.07	1.13	40.34	1.10	37.26±0.54	-29.77±0.39	2.69±0.79
Smilax aspera	Parnitha	Shrub	0.63±0.01	37.86±0.0001	47.65±1.17	10.29±0.59	0.37	1.71	0.30	45.52±0.07	n.d.	n.d.
Styrax officinalis	Parnitha	Shrub	0.44±0.01	24.79±0.0001	73.82±1.76	3.81±0.19	0.17	3.21	0.16	27.75±0.34	-28.45±0.10	22.85±1.94
Thapsia garganica	Parnitha	Herb	0.44±0.02	24.54±0.0001	27.28±0.70	0.71±0.08	0.45	17.30	0.44	30.95±0.29	-28.91±0.31	6.84±3.61

Table 6. Correlation coefficients of relationships between physiological, biochemical and morphological parameters of the 30 plant species studied.

	T_r	g_s	R_d	WUE	PNUE	ETR441	Y441	LT	LMA	N_a	N_m	TP	N/TP	CT	N/CT	N/TP+CT	Chl conc.	$\delta^{13}C$	$\delta^{15}N$
A_{maxa}	.96***	.95***	.33*			.45**	.55***	.20ns	-.03ns	.38**	.56***	-.54***	.75***	-.47***	.66***	.76***	.25ns	-.43**	-.40**
A_{maxm}	.87***	.83***	.26ns			.36*	.46**	.02ns	-.42**	.26ns	.67***	-.66***	.89***	-.58***	.79***	.90***	.19ns	-.43**	-.16ns
$A_{maxaChl}$	.86***	.83***	.24ns	-.39**	.63***	.13ns	.20ns	-.09ns	-.02ns	.41**	.60***	-.38**	.69***	-.45**	.60***	.70***		-.15ns	-.29ns
T_r		.97***	.21ns		.79***	.37**	.47**	.22ns	.04ns	.32*	.45**	-.50***	.63***	-.46**	.55***	.63***	.13ns	-.45**	-.48**
g_s			.04ns	-.32*	.77***	.38**	.49***	.40**	.11ns	.33*	.41**	-.46**	.58***	-.42**	.52***	.58***	.13ns	.08ns	-.27ns
R_d				.03ns	.10ns	.33*	.35*	.32*	.16ns	.40**	.27ns	-.01ns	.18ns	-.04ns	.20ns	.18ns	.16ns	.20ns	-.11ns
WUE					-.12ns	.04ns	.18ns	-.11ns	-.22ns	-.08ns	.09ns	-.22ns	.19ns	-.07ns	.23ns	.19ns	.17ns	-.18ns	.39*
PNUE						.43**	.47**	.22ns	-.17ns	-.26ns	.07ns	-.75***	.62***	-.58***	.53***	.63***	.15ns	-.58***	-.05ns
ETR441							.95***	.43**	.04ns	.11ns	.07ns	-.22ns	.35*	-.20ns	.14ns	.34*	.37*	-.15ns	.36*
Y441								.44**	-.04ns	.16ns	.20ns	-.44**	.48***	-.26ns	.30ns	.47**	.39**	-.22ns	.22ns
LT									.42**	-.01ns	-.21ns	.004ns	-.04ns	.01ns	.03ns	-.03ns	.41**	.01ns	-.01ns
LMA										.23ns	-.37**	.45**	-.48***	.32*	-.45**	-.48***	-.04ns	.22ns	-.22ns
N_a												.17ns	.29ns	.14ns	-.24ns	.29ns	-.02ns	.28ns	-.52***
N_m												-.29ns	.73***	-.11ns	.67***	.73***	-.05ns	.04ns	-.37*
TP													-.90***	.62***	-.66***	-.90***	-.04ns	.36*	-.04ns
N/TP														-.54***	.83***	.99***	.05ns	.26ns	-.04ns
CT															-.88***	-.58***	.15ns	.15ns	-.12ns
N/CT (no Olea)																.86***	.07ns	-.18ns	-.02ns
N/TP+CT																	.05ns	-.27ns	-.04ns
Chl conc.																		-.27ns	-.14ns
$\delta^{13}C$																			-.001ns

* 0.10≥P>0.05 ** 0.05≥P>0.01 *** 0.01≥P>0.001 ns not significant, red: power, blue: exponential, green: logarithmic

Table 7. Means and standard errors of morphological parameters of life forms.

life form	number of species	LMA	LT	LD	TLA
trees	11	72.46 ± 6.39[a]	226.66±30.80[a]	343.90±39.80[a]	14.40±2.47[a]
shrubs	11	70.47±6.39[a]	219.42±30.80[a]	341.85±39.80[a]	14.52±2.76[a]
herbs	8	66.53±7.50[a]	268.51±36.12[a]	288.41±46.67[a]	7.58±4.51[a]

Mean values with different letters are statistically different according to ANOVA (P≤0.05).

Table 8. Means and standard errors of physiological parameters of life forms.

life form	number of species	$A_{max,\ a}$	$A_{max,\ m}$	$A_{max,Chl}$	R_d	T_r	g_s	WUE	PNUE	Yield	ETR
trees	11	10.12 ± 1.95[b]	141.11±36.46[b]	0.34±0.06[b]	0.65±0.15 [a]	3.43±0.34[b]	0.13±0.02[b]	3.03±0.33[a]	7.90±1.27[b]	0.33±0.04[a]	65.15±8.87[a]
shrubs	11	9.51±1.95[b]	146.61±36.46[b]	0.30±0.06[b]	0.60±0.19 [a]	3.01±0.39[b]	0.12±0.02[b]	3.76±0.3[a]	8.34±1.27[b]	0.36±0.03[a]	77.02±8.46[a]
herbs	8	20.23±2.28[a]	336.89±42.75[a]	0.56±0.07[a]	0.62±0.12 [a]	5.41±0.60[a]	0.39±0.06[a]	3.60±0.4[a]	14.66±1.49[a]	0.38±0.04[a]	76.08±9.92[a]

Mean values with different letters are statistically different according to ANOVA (P≤0.05).

Table 9. Means and standard errors of biochemical parameters of life forms.

life form	number of species	$N_{,a}$	$N_{,m}$	TP	CT	N/TP	N/CT	N/(TP+CT)	Chl	$\delta^{13}C$	$\delta^{15}N$
trees	11	55.16 ±5.35[a]	0.72±0.02 [a]	63.79±9.36[a]	4.63±0.95 [a]	0.43±0.15[b]	6.23±7.05[a]	0.40±0.14[b]	33.39±3.46[a]	27.89±0.39[a]	4.07±1.71[a]
shrubs	11	40.22±5.35 [a]	0.61± 0.02[a]	57.23±9.36[a]	4.14±0.95 [a]	0.50±0.15[ab]	19.12±6.72[a]	0.47±0.14[ab]	33.92±3.46[a]	28.35±0.39[a]	8.31±1.71[a]
herbs	8	48.77±6.28 [a]	0.83±0.02 [a]	29.40±10.98[a]	1.36± 1.12[a]	1.03±0.18[a]	31.97±7.88[a]	1.00±0.17[a]	36.89±4.06[a]	29.27±0.42[a]	3.75±1.81[a]

Mean values with different letters are statistically different according to ANOVA (P≤0.05).

5. Discussion

5.1. Relationships between leaf traits at inter-specific level

The results of the present study are in accordance with those of previous studies referring to the relationships between key parameters of leaves from different plant species. In agreement with previous findings, results show a significant correlation between A_{max} and g_s (Farquhar and Sharkey, 1982), between A_{max} and N (Ripullone et al, 2003; Field and Mooney, 1986; Evans, 1989; Reich et al, 1994; Mulkey et al. 1996; Quilici and Medina 1998; Reich et al. 1999), and a negative correlation between $A_{max,m}$ and LMA (Gulias et al, 2003; Prior et al, 2003) (see Table 6). Reich et al. (1999) found that regardless of the biome of the species studied, leaf N_m, LMA, g_s and $A_{max,m}$ were all positively related, despite differences in climate and evolutionary history. Therefore, most of the relationships observed in the present study were already known and incorporated in the so-called "leaf economic spectrum". This spectrum refers to predictable relationships between key leaf traits with one another at a global level, and includes a gradient of leaf traits, ranging from species with potential for fast tissue turnover and high resource capture to species with slower tissue turnover (Wright et al, 2004; Poorter and Garnier, 1999; Reich et al, 2003).

Among the relationships presented in Table 6, those of major interest are the ones between PNUE, g_s and T_r. High PNUE values are indicative of photosynthesis occurring with low nitrogen intake causing growth retardation. According to Warren and Adams (2006), stomatal conductance and increasing WUE, decreases PNUE implying that there is a negative correlation between WUE and PNUE, as confirmed by Field et al (1983). Hence, PNUE appears as the parameter with more direct ecological implications. However, significant negative correlation between WUE and PNUE was not observed in the present study.

In this study, $\delta^{13}C$ was shown to be closely related to PNUE. Several previous studies have shown that $\delta^{13}C$ is widely used to assess WUE from leaves at the ecosystem level and across different climate regions (Bonal et al, 2000; Evans 2001; Ponton et al, 2006). Farquhar et al (1989) also identified a remarkably linear relationship between $\delta^{13}C$ and WUE under controlled environmental conditions for different species and genotypes. Some reports, however, show that the interpretation of $\delta^{13}C$ under natural conditions is not straightforward, because this parameter interacts in a complex manner with plant performance and environmental factors (Seibt et al, 2008; Hall et al 1994; Brugnoli and Farquhar 2000; Maguas and Griffiths 2003). Nonetheless, Werner and Maguas (2010) report that $\delta^{13}C$ could be affected by other functional traits and/or changes in environmental conditions, supporting the results of this study. Although their results showed that $\delta^{13}C$ could be affected by both leaf nitrogen content and LMA through its effect on internal CO_2 conductance, no significant relationship was found between $\delta^{13}C$ and the above mentioned parameters in this study.

In this study, $\delta^{15}N$ was shown to be related to transpiration and leaf nitrogen content. Previous studies show that $\delta^{15}N$ values in leaves could explain underlying patterns of nitrogen cycling across ecological gradients (Hogberg and Alexander, 1995; Roggy et al 1999; Ometto et al 2006). Some reports identified a strong relationship between leaf $\delta^{15}N$

values and water availability (Austin and Sala, 1999; Handley et al 1999; Stock and Evans, 2006; Craine et al 2009) and also between $\delta^{15}N$ values and availability of nutrients (Swap et al 2004; Liu et al 2007). Stock and Evans (2006), however, reported that variation in $\delta^{15}N$ is unrelated to transpiration, differential use of nitrogen forms or denitrification. Mycorrhizal plants, on the other hand, were observed to have lower leaf $\delta^{15}N$ compared to non-mycorrhizal plants. This phenomenon may be related to the ability of host-specific ectomycorrhizal fungi to transfer nitrogen to host plants (Hobbie et al 2004). In the current study, although there were no statistically significant differences in $\delta^{15}N$ between different life forms, values of shrub species were two-fold higher compared to species of the other two life forms. This difference may reflect a difference in their ability to form mycorrhizal associations. Evans (2001) states that contrasting nutrient demands, acquisition mechanisms and root morphology may be responsible for isotopic differences between plant species. Recently, in a global level study has indicated that leaf $\delta^{15}N$ increases with increasing leaf nitrogen concentration. However, in the present study, exactly the opposite relationship between leaf $\delta^{15}N$ and leaf nitrogen concentration was observed. This is in conformity with this study where species showed high Amax and lower leaf $\delta^{15}N$.

In conformity to the findings of Holscher et al. (2006) in their assessment of leaf traits in forest trees, physiological parameters showed a closer relation to biochemical parameters than to morphological parameters.

The most important finding of the present study concerns relationships in which phenolic compounds are implicated. This implication becomes more obvious if the concentration of phenolics is expressed per nitrogen content unit (or the opposite, if nitrogen content is expressed per phenolics concentration unit, see Table 6). Results show that both N/TP and N/TP+CT ratios were positively and strongly correlated with photosynthetic capacity expressed either per unit leaf area, per unit leaf mass or per unit of chlorophyll; transpiration rate and photosynthetic nitrogen use efficiency and negatively correlated with LMA, all key parameters of the leaf economic spectrum. These correlations appear stronger than those in which nitrogen or phenolics concentration are correlated with the above mentioned parameters separately. Hence N/TP, N/TP+CT and N/CT ratios may be considered as essential components of the leaf economic spectrum. The implication of key parameters related to defence/protection in the leaf economic spectrum is for the first time suggested in the present study. These results are in agreement with the recent study of 32 co-existing angiosperms (Ishida et al 2008) in Japan, where leaf N/TP ratio was negatively correlated to LMA and positively correlated to $A_{max,m}$ and $A_{max,a}$. Despite the findings, their discussion was not adequately linked to the leaf economic spectrum. Several authors have also occasionally mentioned the N/TP ratio in a number of studies (Laine and Hentonen 1987; Cunningham et al. 1999), without referring to the probable ecological role of this ratio.

The significant negative correlation between LMA and N/TP is also interesting. This correlation becomes stronger when Australian plant species are included in the graph (see below). LMA is widely used as a sclerophylly indicator and its variations are the result of

the combined variations of leaf thickness and density (Groom and Lamont, 1997; Salleo et al, 1997; Lamont et al, 2002; Filella and Penuelas, 2003; Read and Sanson, 2003; Paula and Pausas, 2006). Plant species exhibiting a low LMA value tend to show high $A_{max,m}$ values, due to the effective light absorbance per leaf mass unit (Wright et al, 2001).

Effective parameters of photosystem II photochemical reactions, namely Y441and ETR were correlated with the N/TP, N/CT or N/(TP+CT) ratios probably indicating the relationship between the investment in growth versus defence and the performance of leaves at the level of light-dependent reactions. On the other hand, correlations between A_{max} parameters and the N/TP, N/CT or N/(TP+CT) ratios were comparably stronger implying that while the balance between growth and defence is strongly related with the actual photosynthetic economy of the leaves, it is only marginally related to the photochemical reactions that proceed at a relatively straightforward manner in terms of the Y441and ETR parameters. This is evidence that N/TP, N/CT or N/(TP+CT) ratios are related rather with CO_2 fixation via stomata, rather than with the functional performance of photosystems. Besides, other sinks, additionally to CO_2 fixation, consume photochemical electron flow such as photorespiration and water-water cycle.

5.2. Significance of N/TP and N/(TP+CT) ratios

The results of the present study are in accordance with previous studies, although examined from a completely new perspective. N/TP or N/TP+CT ratios may represent the trade-off in resource allocation between growth (N concentration) and defence/differentiation (TP or TP+CT concentration), as indicated by the Carbon-Nutrient Balance (CNB) and Growth-Differentiation Balance (GDB) hypotheses (Bryant et al, 1983; Herms and Mattson 1992; Appel, 1993; Castells et al, 2002). According to the CNB hypothesis, changes in the carbon source-sink relationship determine variations in the relative partitioning of carbon to growth, to the production of carbon-based secondary compounds and leaf total non-structural carbohydrates. Nutrient deficiency limits growth more than photosynthesis. Hence, plants thriving in nutrient-poor environments have a surplus photosynthetic product, which cannot be invested in growth procedures. As a result, this surplus photosynthetic product is accumulated and the C/N ratio in biomass is increased. CDB theory generalizes the statements of the CNB one and states that every environmental factor inhibits growth more than photosynthesis increases the available sources for secondary metabolism. On the other hand, N/TP or N/TP+CT ratios may represent the cost of defence of each species, as indicated by the Optimal Defence Theory (ODT). This theory suggests that the investment in secondary metabolites is a very expensive metabolic process because it deprives growth from essential resources. Herbivory is considered as an environmental pressure which determines the amount of defensive investment not only among different plant species, but also among different tissues and organs of the same species. Herbivory, as an evolutionary pressure, favors species with low growth rates and optimum defensive investment, because growth is slowed down in favor of defence. Therefore plant species thriving under the same environmental conditions may present different growth rates according to the current herbivory pressure. Organs or tissues easily replaced and with limited role in plant survival

should display low levels of secondary metabolites.

Moreover, N/TP or N/TP+CT ratios may represent the competition between synthesis of proteins and phenolics for the common precursor L-phenylalanine (Jones and Hartley, 1999).

However, the fact that N/TP ratio is strongly correlated with photosynthetic capacity, transpiration and photosynthetic nitrogen use efficiency, physiological processes not directly correlated to herbivory or pathogen attack, indicates that there is a possible need for protection of primary metabolic processes against the side effects of stressful environments. Hence, N/TP probably represents the balance between growth (N) and protection (TP) against abiotic (and not biotic) stress factors, which are common in arid regions such as drought, high temperatures and high ultraviolet/visible radiation. How are both parts of these ratios correlated to photosynthesis?-Concerning the numerator (i.e. nitrogen content of leaves), the correlation is warranted because of the high amounts of nitrogen that are invested in the photosynthetic machinery; for this reason a linear correlation between N and A_{max} is observed (Hikosaka, 2004; Evans, 1989). Concerning the denominator (i.e. the concentration of phenolic compounds or condensed tannins or both), the present study adopts the statement of Close and McArthur (2002) that the increase of phenolic levels in plant tissues and cells is correlated to the protection of photosynthetic apparatus against oxidative damage caused by Reactive Oxygen Species (ROS). If this hypothesis is correct, some processes of secondary and primary metabolism occur in a non-antagonistic manner, such as the regulatory process involved during the synthesis and accumulation of phenolics compounds and the photosynthesis process. This idea was confirmed by Fritz et al (2006), indicating that these regulatory processes are concurrent and do not involve antagonistic changes of carbon and nitrogen metabolites. The theories and the hypotheses concerning plant-herbivore interactions presuppose that plants can not allocate the photosynthetic products simultaneously to growth or defensive metabolic processes. However, there is experimental data indicating that at least in some cases the seasonal variation of phenolics is not retarded by leaf growth and that their levels are continuously increased during leaf expansion (Riipi et al 2002).

Many environmental stresses exert at least part of their effects by causing oxidative damage (Smirnoff and Stewart, 1985). One of the inevitable consequences of water stress is the production of ROS in energy-handling cell compartments such as chloroplasts, mitochondria and peroxisomes (Cruz De Carvalho 2008). If stress is prolonged over a certain extent, ROS production will exceed the scavenging capacity of the antioxidant system, resulting in extensive cellular damage and death (Jaleel et al 2009; Chaves et al 2002; Cruz De Carvalho 2008). Thus, the function and maintenance of protection mechanisms against oxidative damage is an important strategy under water stress conditions. It is expected, therefore, that plants thriving in dry regions (where low water availability is usually combined with high light intensities and high temperatures) will possess a high capacity to detoxify ROS. It is now evident that a range of phenolic compounds exhibit antioxidant capacity and may protect plant tissues from oxidative damage and especially photodamage (Jaleel et al 2009; Close and McArthur 2002).

Sensitive organelles seem to require special protection against ROS damage. Recently, Agati et al (2009) found that the photo-induced generation of ROS was inversely related to the content of flavonoids in mesophyll cells of sun leaves. Flavonoids were located in the chloroplasts, and were likely associated with the chloroplast envelope. However, the majority of cellular phenolic compounds are located to vacuoles of the epidermis whereat they can only exert an indirect antioxidant role acting as optical filters. Moreover, the occurrence of nuclear-bound (Polster et al 2006) or perinuclear flavonoids (Karabourniotis et al 1998) may also be related to the protection of this organelle from ROS damage. This insight substantiates the hypothesis that TP serves as protection against the side effects of abiotic stress factors, such as high ultraviolet/visible radiation.

Photosynthetic rate is correlated with stomatal conductance because increased stomatal conductance is necessary to increase CO_2 diffusion into the leaves. Stomatal closure may cause increased susceptibility to photodamage, indicated by a low N/TP ratio, resulting in the reduction of quantum yield (Muraoka et al, 2000) and in A_{max} (Gratani and Varone, 2004). Low values of N/TP ratio indicate increased needs of protection at the expense of photosynthetic activity and hence of growth. One common feature of many stress responses is the down-regulation of genes encoding many components of the photosynthetic machinery (Roberts and Paul, 2006) and the activation of genes encoding enzymes of phenolic synthesis (Leyva et al 1995; Oh et al 2009; Keles and Oncel 2002). The relationship between A_{max} and N/TP ratio probably shows that the levels of leaf phenolics in a particular species may reflect the different risks of photodamage, as Close and McArthur (2002) proposed.

The results of the present study are not necessarily in contradiction with the statement that the levels of phenolics are an indicator of the defensive ability of leaves against herbivore or pathogen attack. In fact, these secondary metabolites play defensive or allelopathic roles due to their toxicity. Moreover, the occurrence of a certain N/TP value in a plant species could indirectly affect the defensive performance of leaves. There are experimental data supporting the view that this ratio represents the nutritional value of leaves and thus indirectly affects herbivore and pathogen attack (Bryant et al 1987; Lindroth and Bloomer 1991). Water and nutrient shortage and enhanced CO_2 levels negatively affect this ratio and hence the nutrition of enemies (Inbar et al 2001; Mcelrone et al 2005). The significance of N/TP and N/TP+CT ratios becomes more apparent by the fact that they are indicators of litter decomposition potential and nutrient recycle rates in the soil (Aerts and Chapin 2000; Schweitzer et al 2008; Madritch et al 2006).

It should be pointed out that the present study is part of a major research program including a relatively large data set with 60 plant species, derived from contrasting climatic environments (desert to temperate forests) located within Greece and Australia. The examination of all data together confirmed the correlations found by the present study; in some cases the correlations when the whole data set was analyzed were stronger than these of the present study. The emergence of relationships such as that between A_{max} and N/TP requires the determination of the total phenolic concentration in a large data set using plant species from contrasting environments, a prerequisite performed by a few

studies. In general, ecological studies using a large number of species did not include the measurement of phenolics, whereas studies in which phenolics were measured did not include numerous species from contrasting climatic areas. Therefore, the weightiness of N/TP ratio for the leaf economic spectrum was not recognized before now. In the present study, for example, no correlation between N/TP ratio and $A_{max,m}$ or LMA would be detected if only Australian species were included.

5.3. Variability of morphological, physiological and biochemical traits among life forms

Most of the observed differences in the means of key parameters between different life forms have been already mentioned in literature. Most physiological leaf traits of herbaceous species varied significantly from those of shrubs and trees. The results in this topic are also compatible with the leaf economic spectrum, which includes a gradient of leaf traits, ranging from species with potential for fast tissue turnover and high resource capture (herbs) to species with slower tissue turnover (trees and shrubs) (Wright et al, 2004; Poorter and Garnier, 1999; Reich et al, 2003). Herbs had the highest photosynthetic capacity per unit leaf area, per unit leaf mass and per chlorophyll, transpiration rate, stomatal conductance and PNUE. The lower photosynthesizing and transpiration rates of shrubs and trees could be attributed to their possession of safe xylems characterized by short, narrow thick-walled conduits that are resistant to cavitation. They serve plants thriving in areas where water is limiting and evaporative demand is high. Photosynthetic carbon gain is probably limited in such plants (Hubbard et al. 1999; Hacke and Sperry 2001) because they can not easily resupply water to the canopy due to their structure. With the understanding that water moves 10 times faster in herbaceous plants, having efficient xylems with a low resistance and low evaporative demand, than in conifers (Schulze et al, 2005), it gives support to the high photosynthetic and transpiration rate and stomatal conductance in herbs observed in this study.

The life forms studied did not differ significantly in morphological traits. However, there is a trend, the highest and lowest LMA have been observed in trees and herbs respectively. This is similar to earlier observations in morphological studies of plants belonging to different life forms (Wright et al, 2004; Niinemets 2001; Witkowski and Lamont 1991; Groom and Lamnont 1997).

According to Alessio et al 2004, high LMA and low $\delta^{13}C$ in leaves suggest a dense mesophyll structure with strong diffusional constraints. In this study, trees exhibited the highest LMA and lowest $\delta^{13}C$, even though there was no significant difference between the mean values among life forms. It could therefore be said that leaves of trees have a denser mesophyll structure with stronger diffusional constraints when compared to the other life forms.

Means of nitrogen and phenolics considered separately were not significantly different among life forms. However, when tested as a ratio, there was significant variation in N/TP and N/TP+CT ratios among life forms, which may have resulted from the non-significant decrease in nitrogen content and the non-significant increase in total phenolics from herbs

to trees and then to shrubs. Herbs displayed the highest N/TP and N/TP+CT ratios and trees the lowest ratios. Therefore, the hypothesis of the present study that these ratios are essential components of the leaf economic spectrum and that they are useful indicators of the leaf status, was confirmed. The main difference between life forms examined, is that trees have to allocate more carbon to maintain the living sapwood in their woody stems and fine roots, in order to avoid cavitation in taller stems (Taiz and Zeiger, 2006; Ryan and Yoder 1997; Magnani et al 2000; Sperry et al 2008). Moreover, perennial plant species in the Mediterranean region are characterized by appropriate anatomical and physiological traits, so as to confront abiotic stress factors by reducing the impacts of the adverse environment or by repairing damaged tissues and cells (Lange, 1988, Kyparissis et al, 1995; Werner, 2000; Chaves et al, 2002; Munne-Bosch et al, 2003; Levizou et al, 2004). The dry and warm summer period is considered as the most stressful (Mooney, 1983). During the summer period, evergreen sclerophylls and semi-deciduous shrubs are susceptible to photoinhibition phenomena (Harley et al, 1987; Chaves et al, 2002). On the other hand, the biological cycle of herbs is completed within the favourable growth period (Orshan, 1989).

5.4. Conclusion

Although a number of studies have been carried out to demonstrate the relationships between morphological, physiological and biochemical traits at an interspecific level (Wright et al, 2004; Evans, 1989; Rundel and Yoder, 2008; Lambers et al., 2008; Reich et al., 2003), the present study involving ratios of biochemical components and their relationship with net photosynthetic capacity and other key physiological parameters is a novelty in the Mediterranean region and a fundamental contribution incorporating the N/TP, N/TP+CT and N/CT ratios in the leaf economic spectrum. This study shows that photosynthetic capacity is strongly regulated by the balance between leaf nitrogen and phenolic content regardless of habitat, life form and interspecific differences. This indicates that phenolic compounds not only play a role in the defense of plants against herbivores and pathogens, they also protect the plant from photodamage that occurs when stress factors set in. Since this study is the first attempt in the region, it is recommended that more research be carried out with a wide-ranging data set and further analysis conducted, taking into account factors such as the season of sampling, leaf type and other important plant functional groups characteristic of the Mediterranean-type ecosystems such as evergreen and deciduous species.

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