Growth and nutritive value of lucerne (*Medicago sativa* L.) and Melilotus (*Melilotus albus* Medik.) under saline conditions

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ABSTRACT

Dryland salinity is a major and expanding threat to agricultural land in Australia. Animal production from forages grown on saline land is perhaps its most promising economic use. Glycophytic forage legumes have been evaluated under saline conditions mainly for agronomic characteristics and, to a lesser extent, for nutritive quality to animals. Plant growth and its nutritive quality are interrelated, but a decline in yield in response to salinity may be associated with effects on the chemical constituents of the plant since soil salinity affects plant metabolism. This research aimed to investigate changes in the components of yield and nutritive value of two legumes species. Lucerne (*Medicago sativa*) and Melilotus (*Melilotus albus*) were exposed to different levels of NaCl in the range of 0 to 110 mM NaCl. The research tested the hypothesis that the components of plant nutritive value are not as sensitive to salinity as shoot biomass production since the adaptive mechanisms of the plant lessen harmful effects of the salts.

For both plant species, salinity decreased leaf and stem dry matter production, but increased leaf-to-stem ratio. In addition, salinity resulted in earlier flowering in Melilotus. Mineral composition was the most sensitive component of forage quality. Calculated sodium chloride concentrations were up to 125 g/kg DM in lucerne and 39 g/kg DM in Melilotus when irrigated with 110 mM NaCl. The concentrations of calcium and magnesium decreased in both species and approached the marginal range for animal production. Zinc concentration also decreased while potassium decreased in stems of lucerne only. The digestible organic matter (DOMD) in response to salinity varied between species. At the highest salt concentration, the whole shoot (i.e., leaf and stem) of lucerne decreased up to 4 percentage units while Melilotus increased by 6 percentage units. In lucerne, DOMD was influenced by a high concentration of soluble ash in leaf and stem and, in Melilotus, by an increase in the organic matter content of leaf and a reduction in lignin concentration in stem, which favoured higher digestibility. These results were supported by a histological study in which an increase in starch in Melilotus leaf, and a lower proportion of xylem in relation to parenchyma in stems, was measured. Crude protein concentration was not compromised and, in relation to Melilotus, coumarin concentration did not increase with salinity.

In conclusion, the reduction in DM production of species with similar salt tolerance does not necessarily correspond to an equivalent reduction in nutritive value. This research represents the most detailed study into effects of salinity on glycophytic forage legumes. Results show that

while some aspects of forage quality (e.g., minerals composition and energy) are strongly influenced by salinity, other aspects (e.g., protein) remain relatively unaffected. These findings have implications for development of productive grazing systems on saline agricultural land.

DECLARATION

I declare that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the University Library, being made available in all forms of media, now or hereafter known.

21- May - 2006

Juan de Dios Guerrero-Rodríguez

Date

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CHAPTER I
INTRODUCTION

A number of strategies are being considered in an attempt to achieve economic returns from the increasing area of salt-affected land in agricultural regions. One of them is the implementation of agricultural systems where salt-tolerant plant species are used as a source of forage for grazing livestock (Masters *et al.*, 2001; Barret-Lennard, 2003a; Masters *et al.*, 2005). Nonetheless, even though ruminants tolerate a relatively high dietary load of salts, when salt consumption surpasses a biological threshold, several detrimental effects are induced that decrease animal production. Among them are feed intake depression, reduced intestinal feed retention time due to an accelerated flow of fluids from the reticulum-rumen, and mineral imbalances, especially calcium and magnesium. Thus, reduction of salt intake to more acceptable levels becomes an important feature in order to improve animal production.

Plant species that meet the low salt concentration requirement for ruminants include most of the glycophytic fodder crops, which have good palatability and nutritive value, but their main limitation is poor tolerance to soil salinity. It is recognized that there is scope for use of their genetic variability to develop more salt-tolerant and productive cultivars (Wyn Jones and Gorham, 1989; Winicov, 1998; Bohnert et al 2001; Sharma and Goyal, 2003) and for identifying alternative species, especially legumes where there are a limited number of choices (Rogers *et al.*, 1997; Cocks, 2001; Rogers *et al*., 2005).

One question that arises is whether glycophytic forage species can still provide a valuable source of nutrients for grazing animals even though biomass production decreases under saline conditions. The general assumption is that the nutritive value will be similar or better to the non-stressed plants, as generally, factors that depress plant growth tend to mantain nutritive value (Van Soest *et al*., 1978) and osmotic stress (i.e., water deficit) improves nutritive quality (Wilson, 1982; Buxton and Fales, 1994). But there are several physiological and morphological changes which are caused by direct or indirect effects of salinity and by the intrinsic responses of the plant, increasing the likelihood that nutritive value could be negatively altered. The precise answer is complex and involves a knowledge of the main components of nutritive value that might be affected and the magnitude of change that may occur. These issues have not been

extensively investigated and information about effects of salinity on forage quality is fragmentary.

The present research was aimed at evaluating forage production and nutritive value at different levels of sodium chloride in the irrigation water, taking as model species lucerne (*Medicago sativa*) and Melilotus (*Melilotus albus*). These species are considered as being moderately sensitive and moderately tolerant to salinity, respectively. It was expected that the components of nutritive value may not be as sensitive to salinity as shoot biomass, because the adaptive mechanisms of plants are likely to protect cellular components and minimize otherwise harmful effects imposed by salts.

The outcome of this research should result in a better understanding of the influence of salinity on forage quality, which is a key component of animal production, knowledge that will have at least two benefits. First, this new knowledge may be used in plant breeding programs and, second, this knowledge will be useful in development of grazing systems for animal production from saline land.

CHAPTER II
LITERATURE REVIEW

2.1 Introduction

The purposes of this review are to provide (i) the definition of salinity, forage nutritive value and their importance in relation to animal production; (ii) an overview of the main effects of salt-stress on glycophytic plants; and (iii) plant responses at the whole-plant and cellular level to cope with salt-stress and the relationships with its nutritive value. The focus is on the proteins, carbohydrates and minerals, key components of nutritive value. Due to the emphasis of recent research on lucerne, there is more information to review on this species than with Melilotus. The review concludes with an overview of the implications of changes in nutritive value on animal production and the aims of this research.

2.2 Salinity definition and importance

Salinity implies a high concentration of soluble salts in the soil solution where inhibitory or potentially damaging agents (e.g. Na^{+}, Cl^{-}, SO_4^{--}, CO_3^{--} ions among others) affect growth of most crop species (Marschner, 1995). When the electrical conductivity (EC) of the saturation extract of a soil is greater than 4 dS/m, the soil is considered to be saline (Shannon and Grieve, 1999; Hillel, 2000). This situation is present world wide and major affected areas are found in Australasia, North, South and Central Asia, North and South America, Africa and Europe (Flowers, *et al.*, 1977; Szabolcs, 1979; Blum, 1988; McKersie and Leshem, 1994; Marschner, 1995; Clark and Baligar, 2000; Larcher, 2003).

In Australia, of its approximately 20 million hectares of cropping area, around 16% is likely to be affected by shallow water table-induced salinity and a further 67% by transient salinity (Rengasamy, 2002). In this area, the composition of salts in the ground water and soil is dominated by NaCl (Peirce, 1966; Mehanni and Rengasamy, 1990; Kennewell, 1999; Rengasamy, 2002) and this imposes a restriction on reaching the potential yield of agricultural crops. A plausible option for making productive use of salt-affected land is through the use of halophytic and moderately salt-tolerant glycophytic plant species with acceptable nutritive and feeding value for ruminant livestock.

2.3 Nutritive value definition

The quantity and quality of the forage on offer are two important factors in livestock systems. Both are determined by the intrinsic characteristics of the plant species, the influence of the environment and their interactions. When the animal is considered, quantity is evaluated according to the availability of the forage on offer, whereas quality is related to nutritive value.

Nutritive value is defined as the presence and availability of nutrients in the forage that the animal requires for maintenance and production, and it can be described in terms of concentration of nutrients, or as animal production response, or response per unit of feed ingested (Ulyatt, 1973; Kellaway *et al.,* 1994; Baker and Dynes, 1999; Masters *et al.,* 2001). It is a function of the digestibility of nutrients and efficiency of their utilization for maintenance and production (Kellaway *et al.,* 1994; Baker and Dynes, 1999; Masters *et al.,* 2001). The nutritive value concept is incomplete if voluntary feed intake is not included as; together, these two terms comprise feeding value (Figure 2.1). Therefore, the feeding value of a forage species is a biological assessment through the productive response of an animal under no limitations of feed availability, and it is a function of voluntary feed intake, as well as nutritive value (Baker and Dynes, 1999; Masters *et al.,* 2001).

Baker and Dynes (1999) noted that there is a range of factors related to availability and acceptability of forages that may limit nutrient intake and their utilization. In relation to acceptability, the presence of electrolytes or secondary compounds in species grown under saline conditions may influence palatability and digestibility, which could affect the nutritive value and, consequently, the feeding value. According to these authors, forages with good nutritive value may be found, but if these species have poor palatability they will have low feeding value because they will be eaten in small amounts. Hence, it would be better to evaluate the quality of forage in relation to its voluntary intake, concentration of nutrients, anti-nutritional factors and digestibility. For forage plant species under saline conditions, there is little research on possible detrimental effects on nutritive value. This deficiency in knowledge is especially so for glycophytic species, where nutritive value will be their main advantage over biomass production on saline land.

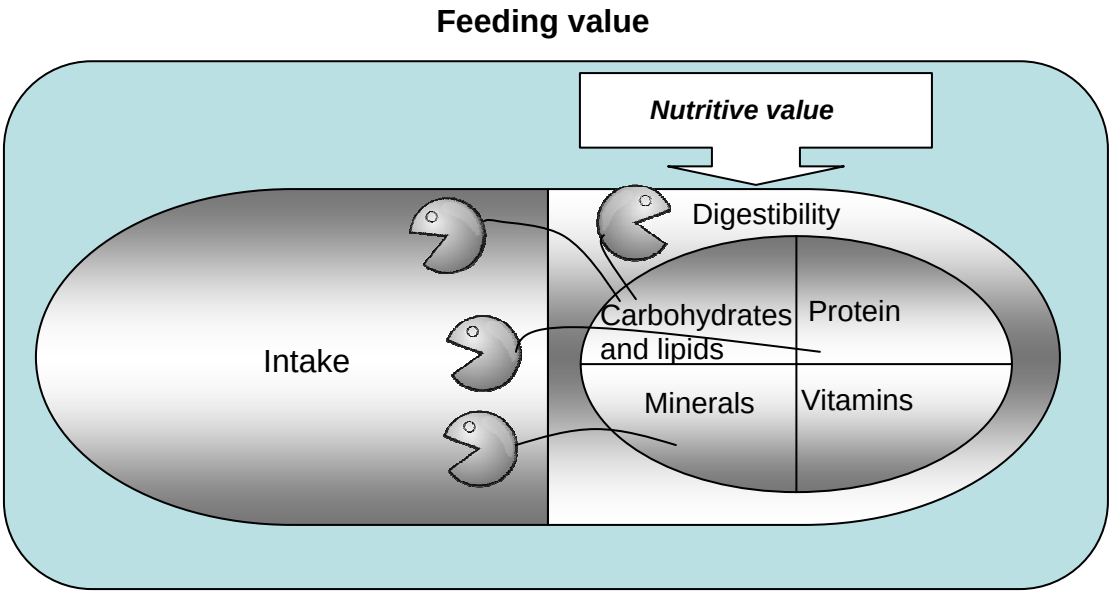


Figure 2.1. The feeding value concept as a function of intake and nutritive value. The heads represent some examples of anti-nutritional factors (from minerals: high salt concentration; from total nitrogen fraction: cynogens, alkaloids, nitrates; from carbohydrate fraction: glucosides) that can affect intake and digestibility which consequently affect the feeding value of a forage. Adapted from Ulyatt (1973).

2.4 Overview about the effects of salt-stress on glycophytic plants

Salinity affects plants in several ways and these have been discussed extensively in several comprehensive reviews (e.g., Bernstein and Hayward, 1958; Levitt, 1972; Lessani and Marschner, 1978; Epstein *et al.,* 1980; Greenway and Munns, 1980; Wyn Jones, 1981; Yeo, 1983; Gorham *et al.,* 1985; Munns and Termaat, 1986; Shannon *et al.,* 1994; Yeo, 1998; Shannon and Grieve, 1999; Hasegawa *et al.,* 2000; Zhu, 2001; Munns, 2002; Tester and Davenport, 2003; Bennett and Khush, 2003). Overall, the main effects can be summarized as a high osmotic pressure that reduces water availability, an ion imbalance that can induce nutrient deficiencies, and an excessive ion accumulation that produces toxicity to the plant (Figure 2.2). Through these processes, salinity causes metabolic alterations that can affect respiration, photosynthesis, the synthesis of protein, nucleic acids, chlorophyll, carotene and phytohormones, as well as on carbohydrate metabolism and enzyme activity (Levitt, 1972; Strogonov, 1973; Tal, 1985; Aspinall, 1986; Munns and Termaat, 1986; Blum, 1988; Amzallag, 1997). Additionally,

due to the alteration in some metabolic processes, oxidizing species may accumulate, which impose additional stress to the plant grown under saline conditions (Levitt, 1972; Strogonov, 1973; Zhu, 2001; Santa-María, 2003; Penna 2003). Thus, the disturbances and their energetic costs caused by either direct or indirect effects of salts, result in a reduction in plant growth or even death of the plant.

To balance negative effects of salinity, plants have several responses that allow them to tolerate, to certain degrees, the presence of high salt concentrations in soil. Plants that show salt resistance are those that maintain cellular homeostasis that allows them to persist and grow under saline conditions where sensitive genotypes cannot prosper (Yeo, 1983; Winicov, 1998). This salt resistance, as Levitt (1972) pointed out, may be achieved through either avoidance or tolerance. In the former situation, salts can be excluded passively or extruded actively from cells or organs or be diluted by an increment in water concentration in the tissue producing succulence. The tolerance mechanism is manifested when a plant under salt stress can endure dehydration (i.e., dehydration tolerance) and be able of re-hydrate again, absorbing salts or producing organic solutes (e.g., proline, betaine, sugars) in order to adjust osmotic pressure (i.e., dehydration avoidance) and maintain positive turgor.

According to Levitt (1972), plant characteristics associated with salt tolerance include functional compartmentation where the absorbed salts are transported into vacuoles, and protoplasmic substances and organelles are able to maintain their functions under a salt-induced change in ionic balance (Figure 2.3). Several authors agree that effects of salinity on plant physiology and biochemistry operate simultaneously and may not be independent. In addition, plants show a mixture of functional adaptive and indicative responses, which makes it difficult to delineate clearly between direct or indirect effects of salinity (Bernstein and Hayward, 1958; Wyn Jones, 1981; Yeo, 1983; Aspinall, 1986; Winicov and Deutch, 1994). Hence, salt resistance in the plant is the expression of a number of processes that can operate and interact at the level of cell, tissue, organ and whole plant structure (Figure 2.4) intended to reduce the amounts of damaging ions entering plant tissues and/or reduce negative effects of accumulated salts (Lazof and Bernstein, 1999; Subbarao and Johansen, 2002; Tester and Davenport, 2003). As most of these plant adaptations involve components of nutritive value, it is important to know and understand these changes in order to asses their impact on grazing animals.

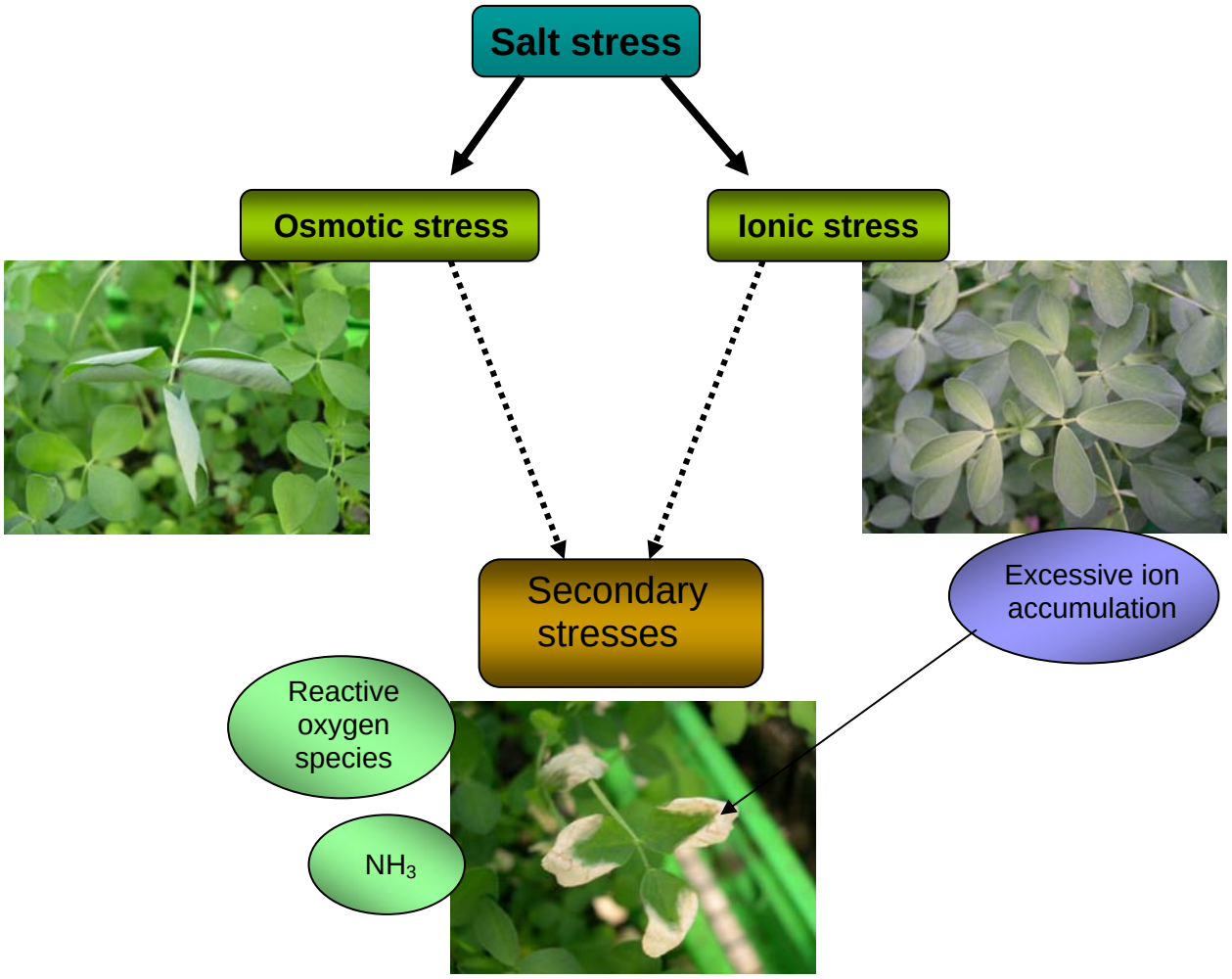


Figure 2.2. Main effects of salinity on glycophytic plant species.

As shoot biomass is the component of the plant that is consumed by ruminants, changes in the sodium and chloride concentration in tissues, leaf-to-stem ratio, leaf and stem anatomy, proteins, carbohydrates and minerals, may re-define the nutritive value for a glycophytic plant species grown under saline conditions. The following points are intended to give an overview of the extent of the possible changes mentioned.

2.5 Salt accumulation and distribution in plants

There is no perfect salt-excluding plant (Cheeseman, 1988), and hence sodium and chloride will accumulate in plant tissue when soil salinity reaches high levels. In legumes, which have some capacity to retranslocate sodium from leaves (Läuchli, 1984; Jeschke and Wolf, 1993), the mechanisms involved in sodium exclusion, and the organs of salt retention, can become saturated

with long duration and/or high salt concentrations (Läuchli, 1984). Yeo (1983) suggested that a discontinuous ion distribution at the tissue, cellular and intracellular levels could occur in plants as a strategy to cope with salinity. This graduated concentration of sodium and chloride between plant compartments is important, because it helps to maintain a limited uptake of these elements into expanding leaves, inflorescences and seeds, using the vacuoles of mature leaves as storage areas (Munns, 1993; Marschner, 1995).

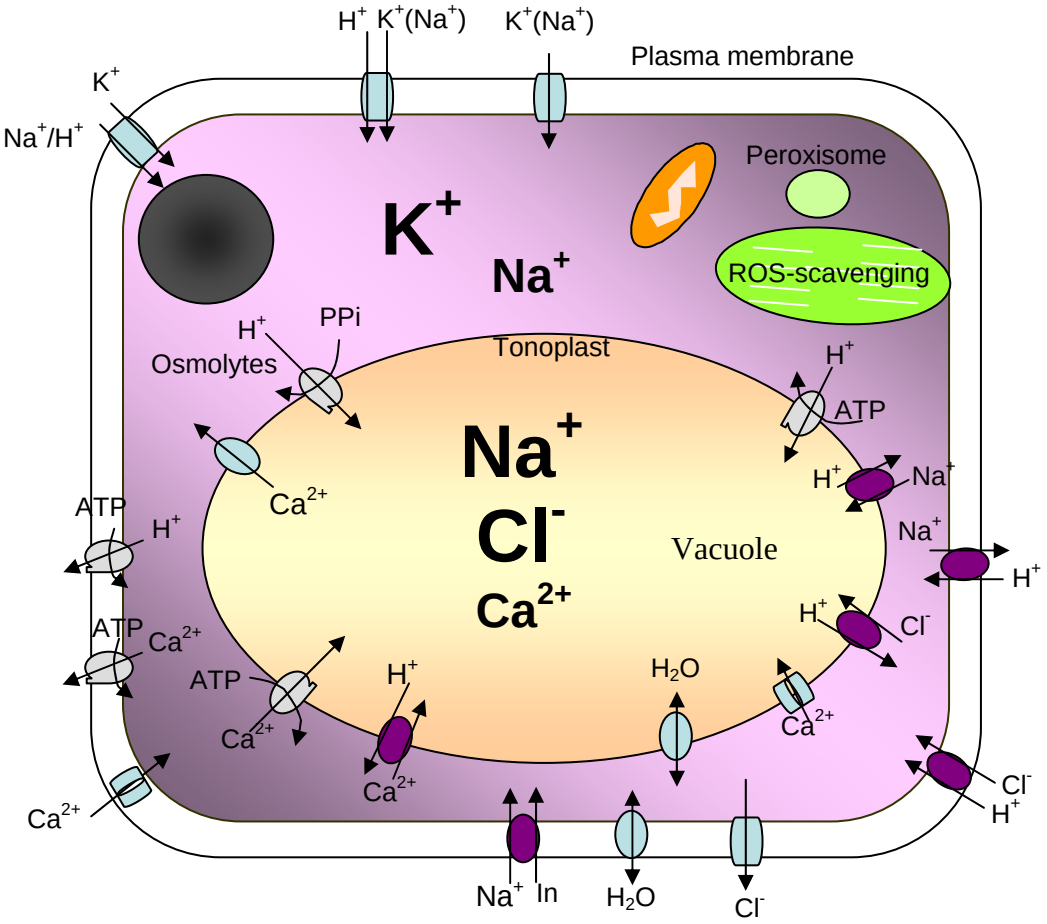


Figure 2.3. Sodium and chloride compartmentalization in cytoplasm and vacuole after the cell has re-established homeostasis under salt-stress conditions. This is achieved through the contribution of transport proteins, water channels, the production of osmoprotectants substances (osmolytes and reactive oxygen species scavengers) and other contributors (from Hasagawa *et al.*, 2000).

In forage legumes grown under saline conditions, a higher salt concentration is more commonly found at the base than at the top of plants (Ashraf and O'Leary, 1994; Boughanmi *et al.,* 2003). This could be because fully expanded leaves have transpired for longer than young leaves, and therefore are more likely to accumulate higher concentrations of salts (Munns and Termaat, 1986; Munns, 2002; Barret-Lennard, 2003b). Expanding leaves often start to show salt symptoms long after the old leaves have already died (Munns and Termaat 1986). Old leaves with a high load of salt eventually die, and can thus prevent salts in these leaves fro becoming toxic to other parts of the plant (Sen *et al.,* 2002). Differences in salt concentration across the plant (e.g., stem versus leaf, old versus new leaves) are likely to influence animal grazing selectivity, but this issue has not been investigated with forage legumes or other forages species in saline conditions.

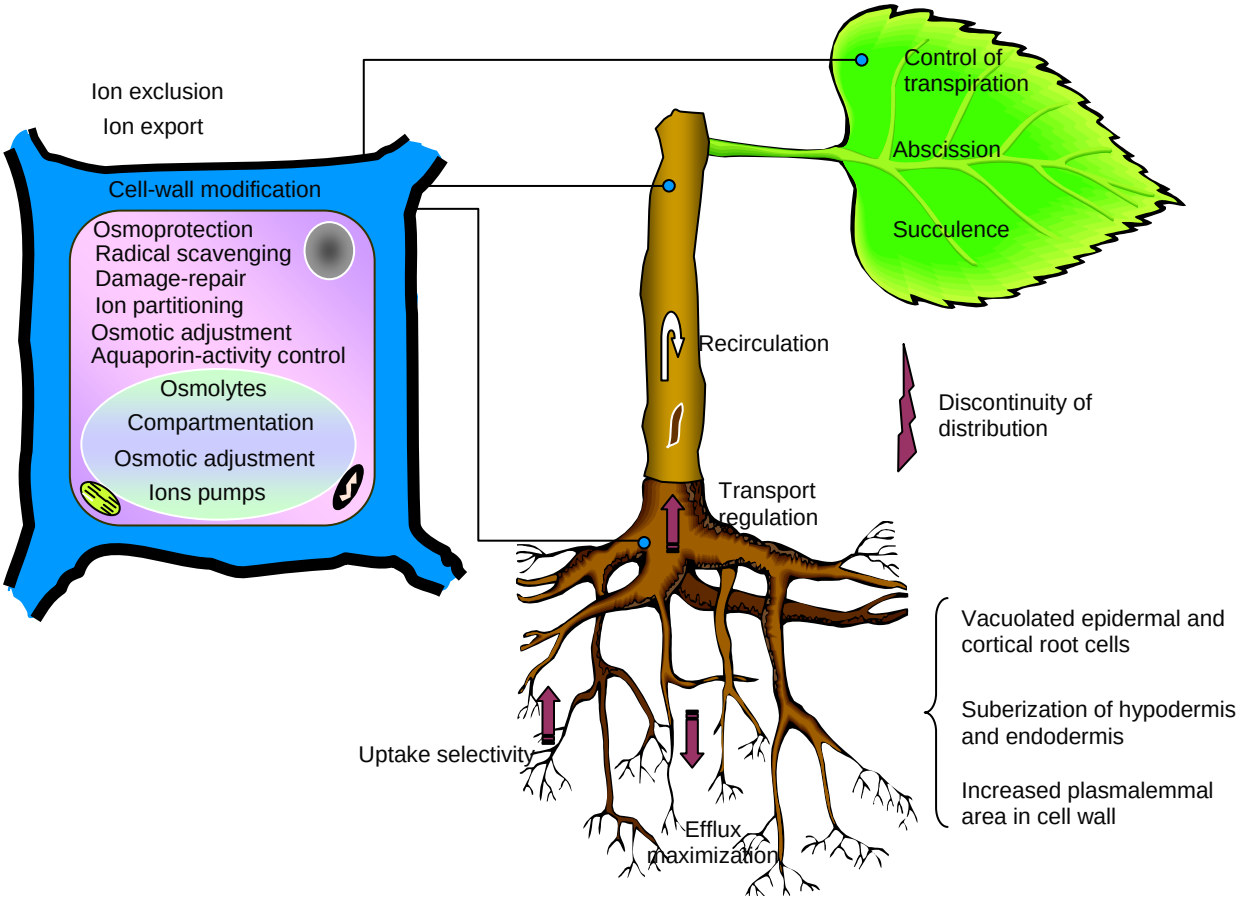


Figure 2.4. Cellular and whole-plant responses to salinity. Adapted from Munns and Termaat (1986); Flowers and Yeo, 1989; Shannon *et al*. (1994); Bohnert and Jensen (1996); Tester and Davenport (2003).

2.6 Effects of salinity on the morphology and anatomy at the whole plant level

The general visual plant symptoms caused by salinity are stunting, succulence, dark green leaf colouration, leaf area reduction, leaf burning, necrosis, accelerated leaf senescence, defoliation, and chlorosis (Bernstein and Hayward, 1958; Repp *et al.*, 1959; Jennings, 1968; Mass and Hoffman, 1977; Smillie and Nott, 1982; Noble, *et al.*, 1984; Shanon, 1985). Salinity reduces the number and size of leaves, stem growth, branching and tillering (Mass, 1993). Biomass accumulation can be irregular and differences in dry matter partitioning may increase leaf to stem ratio (Mass and Hoffman, 1977; Keck *et al.*, 1984). Root growth is also inhibited (Neumann, 1997), although it is less suppressed than shoot growth (Munns and Termaat 1986; Richards, 1992).

Another effect of salts is the variation in phenology, especially in the reproductive stages, where differences among species, and even among cultivars, can be expected (Mass, 1993; Dhingra and Varghese, 1997). In some legumes, salinity affects the production, viability and germination of pollen and, consequently, the number of fruits and seeds per plant (Dhingra and Varghese, 1997). Most of these characteristics are related directly to nutritive value and therefore they may affect animal performance. A high leaf-to-stem ratio is almost always a desirable characteristic for forage plants as generally has a strong positive influence on nutritive value, but this advantage can be lost if leaf abscission and senescence increase with time. The phenology for a grazing system is important, especially when biennial or annual species are included, because seed set needs to be achieved in order to keep the system functioning. Indeed, it is related to forage nutritive value because negative changes in quality genrally occur as maturity approaches. In this regard, little is known about the extent of change in the nutritive value for legumes under saline conditions that reach flowering or maturity earlier than the non-salt stressed plants.

2.7 Effects of salinity at tissue and cellular level

The important components of nutritive value in forages are the amount and types of carbohydrates, protein, minerals and vitamins (Figure 2.1). A limited availability or poor digestibility of these components negatively affects productivity in any animal production system. Total nitrogen in plants is in both true protein and non-protein nitrogen, compounds that microbes in the rumen can utilize to produce microbial protein, which then is digested post-ruminally and incorporated into animal tissues. It is known that the process of nitrogen utilization by rumen microbes is efficient if there is a sufficient availability of energy, mainly

from soluble carbohydrates and digestible structural carbohydrates. The following sections deal with changes induced by salinity in the cellular structure of the plants as will as concentrations of protein, carbohydrates and minerals.

2.7.1. Tissue and cellular structure

At the cellular level, several changes in response to salinity occur, although it is not clear if these changes are signs of adaptation or susceptibility to salinity (Poljakoff-Mayber, 1975; Shannon *et al.,* 1994). Salt-induced succulence can be found on leaves where the epidermal, palisade and stomatal guard cells are bigger and with fewer stomata per unit of surface area than non stressed plants (Repp *et al.,* 1959; Jennings 1968; Tal, 1985; Bahaji *et al.,* 2002). In leaf veins, modification of xylem and phloem parenchymal cells into transfer cells has been observed in forage species like *Trifolium alexandrinum*, *Medicago sativa* and other leguminous species (Winter, 1982; Boughanmi *et al.,* 2003). In Lucerne leaves, for instance, Boughanmi *et al*. (2003) reported increases in wall ingrowth and enlarged plasmalemma area in transfer cells, as well as reduced area of sieve elements in the phloem of main veins. Even in halophytes, leaf epidermal cells show salt-induced changes. Niu *et al*. (1996) found abundant small polyphenol globuli in the cytoplasm near the cell wall and between the plasma membrane and cell wall, as a result of NaCl treatment in *Atriplex nummularia*. These authors also found that chloroplasts of the bundle-sheath cells were devoid of starch and had many plastoglobuli.

In roots, cell wall modifications are often induced by salinity, which promotes deposition of suberin in cell walls of endodermis and, in species like cotton, formation of exodermis (Reinhardt and Rost, 1995). Little is known whether some of these effects produce negative or positive effects on ruminal fibre digestibility when plants are subjected to salinity.

2.7.2 Effects on protein

Saline conditions have a marked effect on nitrogen metabolism in plants, producing direct effects on the synthesis rate of nucleic acids and proteins (Langdale and Thomas, 1971; Strogonov, 1973). During severe or prolonged salt stress, protein synthesis in leaves may decline and net protein degradation through proteolysis of storage proteins can occur (Levitt, 1972; Strogonov, 1973; Aspinall, 1986; Marschner, 1995). In contrast, synthesis of alternative proteins and polypeptides may increase as a result of changes of gene expression as a salt stress response (Luo *et al*., 1992; Winicov and Deutch, 1994; Bohnert *et al.,* 2001; Weretilnyk *et al.,* 2001; Zhu, 2001; Yang and Yen, 2002; Tester and Davenport, 2003).

The net decline in overall protein synthesis may be linked to several factors that possibly act together. One of them is the antagonism between Cl^- and NO^-_3 uptakes (Kafkafi, 1984; Grattan and Grieve, 1992; Grattan and Grieve, 1999) that can limit availability of nitrogen to the plant. Additionally, it has been suggested that chloride toxicity, especially in sensitive species, interferes with DNA synthesis, thereby producing dissociation on the DNA-histone bonds, leaving DNA unprotected and exposed to enzymatic action (Strogonov, 1973; Marschner, 1995). Another cause may be the dominance of sodium in the Na^+/K^+ ratio, where sodium may replace some functions of potassium (Marschner, 1995; Epstein, 1998). Potassium, apart from its role in neutralizing effects of anions, participates in membrane transport and starch synthesis. It also activates enzymes and maintains turgor pressure in cells (Subbarao *et al.,* 2002) which is linked to cell expansion and, when potassium is replaced, cell turgor is negatively affected. This decline in potassium has also been correlated with decreases in protein synthesis (Munns and Termaat 1986).

It has also been suggested that a decrease in protein synthesis could be related to negative effects on specific enzymes and organelles. Nitrate reductase is a key enzyme in reduction of nitrate to ammonium, which is further used for synthesis of amino acids (Moorhead *et al.*, 1996; Chung *et al.*, 1999; Forde, 2000). Under water restriction, decreased activity of this enzyme has been linked to reductions in the polyribosomal content that may decrease the rate of its synthesis (Morilla *et al.,* 1973). It has been found also that NaCl can produce a direct effect on depression of activity of this enzyme thereby decreasing NO_3^- reduction in species such as bean, lucerne, cotton and sugar beet (Gouia, *et al.,* 1994; Khan *et al.,* 1997; Ghoulam *et al.,* 2002).

In addition to the decrease in protein content, saline conditions can lead to accumulation of non-protein nitrogen compounds and can alter the properties of proteins. A range of non-protein nitrogen compounds produced by proteolysis or synthesis accumulate in leaves. Examples include ammonia, imino acids, amides, polyamines and quaternary ammonium compounds (Levitt, 1972; Strogonov, 1973; Blumm, 1988; Mansour, 2000; Sen *et al.,* 2002; Ashraf, 2004; Parida and Das, 2005). Some of these substances maintain optimum cellular osmotic pressure (i.e., are osmoprotectants) when the plant is challenged by salinity and other stresses and their positive effects can be reflected in the stabilization of nucleic acids, proteins and membranes under salt stress (Yancey *et al.,* 1982). Specific nitrogen-containing compounds are glycine

betaine, proline betaine, proline and trigonelline among others (Ashraf, 2004; Ashraf and Harris, 2004; Parida and Das, 2005).

Changes in the properties of proteins, especially the readily-soluble proteins in response to NaCl, have been found in several plant species. In legumes, increases have been detected in *Pisum sativum* (Strogonov, 1973) and decreases in *Vicia faba*, *Lens culinaris*, *Sesbania aculeata* and *Phaseolus vulgaris* (Gadallah, 1999; Ashraf and Bashir, 2003; Ashraf and Harris, 2004). In leaves, about 75% of total protein is in chloroplasts where half of this percentage is the soluble protein ribulose-1, 5-biphosphate carboxilase, which is extensively degraded in the rumen (Mangan, 1982; Skinner *et al.*, 1994). Salinity affects chloroplasts (Poljakoff-Mayber, 1975; Winter, 1982; Mengel et al 2001) and this may explain in part why changes in levels of soluble protein occur. Little is known about whether salinity affects the fractions and, consequently ratios of the protein (i.e., soluble and insoluble) of the major forage crops such as lucerne. Overall, it seems that salinity has a negative influence on total protein concentration of plants and the synthesis of new proteins may modify the total amino acid profile found normally under non-stress growing conditions.

2.7.3 Salt effects on carbohydrates

In relation to carbohydrates, according to Greenway and Munns (1980), Munns and Termaat (1986), and Munns (1993), plants respond differently to salinity in the short-term (i.e., days) versus in the long term (i.e., weeks, months). During short-term exposure, when effects of salinity are on growth and cell division, plants respond by increasing sugars and starch content, but decreases can occur if there is a direct effect of salts on photosynthesis.

Although there are differences among plant species, the sugars that tend to accumulate are glucose, fructose, sucrose, trehalose, and the sugar alcohols (polyols) inositol, D-sorbitol, D-mannitol, D-ononitol and D-pinitol (Aspinall, 1986; Fougère *et al.*, 1991; Nuccio *et al.*, 1999; Rontein *et al.*, 2002; Martínez-Ballesta *et al.*, 2004). It has been suggested that accumulated carbohydrates function as osmoprotectants and some of them, such as the sugar alcohols, scavenge reactive oxygen species such as superoxide radicals, hydrogen peroxide and hydroxyl radicals to prevent oxidative damage to proteins, nucleic acids and membrane lipids (Yeo 1998; Holmberg and Bülow 1998; Nuccio *et al.*, 1999; Apse and Blumwald 2002; Hasegawa *et al.*, 2000). It is not completely clear for sugars whether their accumulation is a direct response of the plant against salinity or an indirect consequence of other effects of the salt-stress.

Several explanations have been proposed to account for sugar accumulation. One of them is that disturbances in the glycolic cycle, mainly through decreases in the intermediate organic acids such as pyruvic, citric, malic, oxalic, aconitic and fumaric may lead to sucrose accumulation (Strogonov, 1973). A decline in plant sugar use may also lead to increasing amounts of sugars, as has been found in leaves of peas and barley (Greenway and Munns, 1980; Munns and Termaat, 1986; Munns, 1993). Other contributors may be the increased enzyme activity for starch hydrolysis and sucrose synthesis (e.g., starch phosphorylase; sucrose phosphate synthase) and abscisic acid, which have been observed to increase in plants grown under salt-stress (Dubey and Singh, 1999; Ashraf, 2004).

Under long-term exposure to salinity, depletion in reserve carbohydrates in plants can occur because the plant invests additional amounts of energy, excluding the excess of Na or secreting it into the vacuoles (Mengel *et al.,* 2001). In addition, the energy status of the plant can be worse due to reduced leaf area arising from accelerated senescence that cannot sustain the high supply of carbohydrates imposed by the high demand from the roots, an organ that generally has more biomass than the shoot under saline conditions (Munns and Termaat, 1986). Several studies indicate sugar depletion, as decreases in sugar concentration have been found in sugarcane (Lingle and Wiegand, 1997), rice (Sultana *et al.,* 1999), faba bean (Gadallah, 1999) and melon (Sivritepe *et al.,* 2003). However, increases in soluble sugars have been reported for tomato (Gao *et al.,* 1998), wheat (Sairam *et al.,* 2002) and for muskmelon (Carvajal *et al.,* 1998). Recovery from grazing, and speed of regrowth, depends on the reserve carbohydrates in forage plant species, but little is known about this fraction or variability among species. Furthermore, the effect of salinity on plant carbohydrate concentration is rarely evaluated in terms of it being a source of energy for rumen microbes and for the grazing animal.

2.7.4 Minerals

Minerals in animal nutrition are of crucial importance because they are components of biological fluids, bones, proteins and vitamins, and participate in many metabolic reactions. Their deficiency or excess in the plant may lead to deficiencies or toxicities in the animal and, consequently, diminish productivity. In the plant, salinity has been shown to influence uptake, translocation and availability of nutrients, mainly due to the ionic strength of the substrate and the competition imposed by the dominant ions (Grattan and Grieve, 1992). Thus, some mineral

imbalances can be induced in shoots, which may have negative effects on plant growth. Of crucial importance are the salt-induced deficiencies of potassium, calcium and magnesium (Grattan and Grieve, 1992; Rengel, 1992; Lazof and Bernstein, 1999). Important as well are deficiencies in phosphorus and microelements such as iron, zinc, copper, manganese, boron and molybdenum. However, concentrations of these minerals have been shown to vary in magnitude, either increasing, decreasing or remaining unchanged, depending on salinity level, type of salt, plant organ, plant species, temperature and other components of the environment (Grattan and Grieve, 1992).

Effects of salinity and responses of the plant are diverse and there are variations among plant species that need to be considered to gain a better picture of salinity impacts. The remaining sections of this review will focus on lucerne as it is one of the most studied forage legume species under saline conditions and is grown in many regions of Australia in which salinity management is required.

2.8 Lucerne under salinity

Lucerne is considered to be a moderately sensitive species growing at electrical conductivities of saturated soil extracts from 8 to 16 dS/m at 25 °C (Mass, 1985). It is capable of survival with 50% seawater irrigations, but yields have low commercial value because its low production (Mass, 1993), although wide variability in responses suggests further genetic improvement is possible (McCoy, 1987; Smith, 1994; Chaundhary, *et al.,* 1996; Humphries and Auricht, 2001). In this species, and other 'alternative' species of this genus, several authors have indicated that it is feasible to find genotypes with enhanced tolerance to NaCl in order to create new cultivars (Rogers *et al.,* 1994; Rogers *et al.,* 1997; Cocks 2001; Dear *et al.,* 2003; Evans and Kearney 2003). It is concluded that NaCl tolerance at different stages of plant development can be heritable (Noble *et al.,* 1984; Allen *et al.,* 1985; Ashraf *et al.,* 1987; Winicov, 1991; Al-Khatib *et al.,* 1993), and this knowledge has encouraged researchers to develop improved lucerne genotypes with forage potential under saline conditions.

NaCl affects lucerne germination (Rumbaugh *et al.,* 1993; Smith, 1994), seedling growth and mature plant growth (Smith *et al.,* 1981; Rogers *et al.,* 1998). Detailed information has been reported by Smith (1994) who summarized salinity effects on lucerne at all these stages. Symptoms of salinity in leaves can be bleaching of leaflets, chlorosis, marginal necrosis, changes

in colour (i.e., dark green or blue-green colour), succulence and abscission of the oldest leaves. The stems are shortened and can become succulent, chlorotic and necrotic.

Lucerne shows partial stomatal closure in response to salinity that limits the rate of CO_2 diffusion (Gale and Zeroni, 1985; Khan *et al.*, 1994b; Anand *et al.*, 2000) and reductions in the amounts of chlorophyll "a" and "b", as well as xanthophylls (Khavary-Nejad and Chaparzadeh, 1998). Therefore there is a decrease in the photosynthetic rate due to stomatal and non-stomatal factors. Transpiration generally is reduced and less water is used per unit net CO_2 assimilation or biomass produced (Hoffman *et al.*, 1975; Richards, 1992; Khan *et al.*, 1994b; Anand *et al.*, 2000; Zhang *et al.*, 1999). Shone and Gale (1983) and Gale and Zeroni (1985) suggested that lucerne under salt stress has an increased energy demand that induces an increase in plant respiration. Thus the produced assimilates are used mainly for maintenance and less are used for growth. These effects are manifested in reduced relative growth rate (Khan *et al.*, 1994a; Chaudhary *et al*. 1996; Khan *et al.*, 1997; Khavary-Nejad and Chaparzadeh, 1998) and therefore limited biomass production.

2.8.1. Shoot biomass production

Decreases in shoot biomass production in response to salinity for lucerne have been described by the equation of Maas and Hoffman (1977) as: $Y=100-7.3(EC_e-2)$. This equation was obtained from experiments conducted mostly in soil and gravel cultures and the yields were averaged from four or more harvests. "Y" is the relative forage yield for any given soil salinity when the EC_e in mmho/cm exceeds the threshold of 2 mmhos/cm. It is clear that a value of 15.7 mmho/cm in the EC_e may represent the limit for lucerne with values in Y close to 0. Data from other experiments show that there are conditions where the yield of the plants can be higher than the Maas and Hoffman prediction. A compilation of these data show a linear response [Y= 0.98-0.0034 (mM NaCl), r^2=81] as described in Figure 2.5. The difference between equations widens as salinity reaches values higher than 100 mM NaCl. Although many factors modify the response of lucerne grown under saline conditions, it is likely that differences in methodology, mainly in nutrient solutions and length of the salinization period, could be major contributors to these differences. About 70% of the data after Maas and Hoffman comes from only the first harvest.

Components of forage yield are also modified by saline growing conditions. Even when leaf area (McKimmie and Dobrenz, 1991; Khan *et al.*, 1994b; Khavary-Nejad and Chaparzadeh, 1998;

Esechie and Rodriguez, 1999; Zhang *et al.*, 1999) and leaf numbers are reduced (McKimmie and Dobrenz, 1991) the leaf-to-stem ratio increases (Hoffman *et al.*, 1975; Al-Khatib *et al.*, 1993; Khavary-Nejad and Chaparzadeh, 1998). Increasing this ratio generally has a substantive positive contribution to nutritive value.

Effects of salinity on lucerne phenology, seed production and nutritive value in the long term have been little studied. It has been reported that salinity produces variations in the number of days to reach specific stages of flowering in several genotypes. Flowering time can be delayed (Kapulnik *et al.*, 1989) or be early (Brown and Hayward, 1956; Rogers, 1998). This characteristic could be important in lucerne forage harvest or grazing as nutritive value is modified when plants change phenological stages.

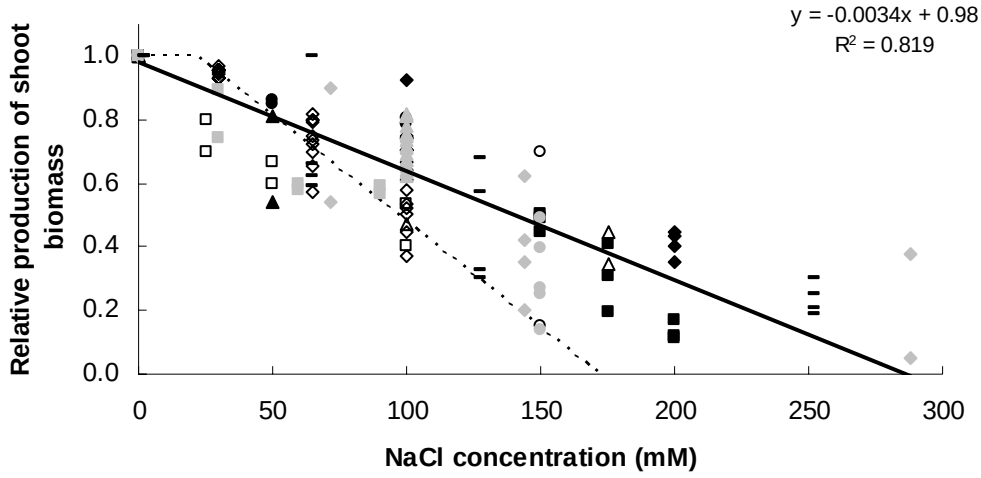


Figure 2.5. Shoot biomass of lucerne as affected by increasing NaCl salinity in the irrigation water (data sourced from several authors and calculated as salinity/control ratio). Legend: ▬ Smith and McComb, 1981; ▲Gale and Zeroni, 1985; Δ Ashraf *et al.*, 1986; ♦Kapulnik *et al.*, 1989; ○ Mckimmie and Dobrenz, 1991; ♦ Zhou *et al.*, 1992; ■ Al-Khatib *et al.*, 1993; ◊ Khan *et al.*, 1994a; ▲ Khan *et al.*, 1997; □ Serraj and Drevon, 1998; ■ Khavari-Nejad and Chaparzadeh, 1998; ● Djilianov *et al.*, 2003; ● Rogers *et al.*, 2003. The solid line represents the linear regression of all data and the dotted line represents the general equation of Maas and Hoffman (1977) for lucerne (assuming 1 mmho≈-0.03608 MPa and 1 mM of NaCl≈-0.00329MPa according to Smith, 1994).

2.8.2. Nutritive value

Although few animal production studies have been conducted to describe the nutritive value of lucerne grown under saline conditions, information from physiological and agronomic studies is available, which gives good indications about how plant nutritive value can be modified under such conditions. Lucerne is considered a good source of animal feed due to its high digestibility and high protein concentration. If salinity affects nitrogen metabolism in the plant (see section 2.7.2), plant quality may decline, although changes in carbohydrate metabolism (section 2.7.3) may partially offset detrimental responses.

2.8.2.1. Nitrogen concentration in shoot

Shoots of lucerne contain ammonium compounds such as prolinebetaine, pipecolabetaine, hydroxyprolinebetaine and glycinebetaine (Wyn Jones and Storey, 1981; Wood *et al.,* 1991). Prolinebetaine is found in the highest concentrations and it accumulates in plant biomass under saline growing conditions (Wyn Jones and Storey, 1981; Safarnejad *et al.*, 1996; Petrusa and Winicov, 1997). Safarnejad *et al*. (1996) reported up to 0.18 g of proline per kg of fresh weight when seedlings were exposed to 200 mM of NaCl for 14 days, compared to the control that had about 0.01 g of proline/ kg fresh weight. Petrusa and Winicov (1997) have reported larger increases from 0.11 to 1.8 g of proline/kg FW, in response to 171 mM NaCl in the short term (0-8 days). While total nitrogen concentrations were not reported, data indicate that there were changes in the nitrogenous compounds in the plant.

The total nitrogen concentration, or its equivalent “crude protein” (total nitrogen x 6.25), of lucerne shoots in response to salinity has been shown to remain unchanged, increase or decrease across a range of studies (Table 2.1). In comparison to controls, increases in total nitrogen have been reported to range from 13 to 37% and the decreases have ranged from 14 to 49%. In most cases where plants had depended exclusively on nitrogen fixation a decrease in nitrogen content of shoots has been reported, attributable in part to suboptimal symbiosis. One factor that can introduce variation to these results is the leaf-to-stem ratio, which generally has not been reported, as there are big differences in the concentration of nitrogen between leaf and stem, organs that seem to be affected differently by saline growing conditions. Little is known about the proportions of true protein to non-protein nitrogen, or soluble to insoluble protein, both of which can affect the ruminal utilization of nitrogen.

Table 2.1. Effect of salinity on the shoot total-N content in *Medicago sativa*.

Salt	Salinity range (dS/m)[1]	Range of shoot total-N content (g/kg DM)	Comment	Reference
NaCl	0-13.5 0-6.7	32.6-36.6 (N-fed) 31.4-22.7 (N-fixation)	Decrease in plants dependent on N fixation. Did not change in plants receiving NO_3	Shone and Gale (1983)
NaCl	0-17.9	41.5-34.4 (45-day-old) 40.8-39.5 35.4-40.3 39.9-40.5 30.9-32.8 (20% bloom)	Decrease in one population and increase in other. No change when plants were at 20% bloom. Range for different populations at 45-day-old seedlings	Kapulnik *et al.* (1989)
NaCl	0-12.9	21.2-18.2 17.7-22.9	Decrease in one population and increase in the other.	Pessarakli and Huber (1991)
NaCl	0-13.5	23.4-16.4 24.7-14.3 31.5-15.9	Decrease in N content. Each range corresponds to a different strain of Rhizobium.	Mashhady *et al.* (1998)
NaCl	0-9.0	29.4-25.0 (N-fed) 26.0-19.0 (N-fixation)	Decrease only at 100 NaCl mM (≈ 9 dS/m) in NO_3 fed plants. Decrese in plants dependent on N fixation.	Serraj and Drevon (1998)
NaCl	0-14.8	34.7-43.7 (N-fed) 39.8-41.9 (N-fixation)	Increase in NO_3 fed plants No change in plants dependent on N fixation.	Bernstein and Ogata (1966)
Mixture	0.3-5.8	30.6-31.6 (Cuts avge.)	Only in the 3rd cutting was there an increase. No change in the fist two cuttings.	Kagawa *et al.* (1989)
Mixture	0-43.5	23.4-20.4 (P 0) 26.2-26.1 (P 50) 31.5-29.8 (P 100) 35.1-23.7 (P 150) 44.6-27.2 (Mycorrhizal inoculation)	Decrease in treatments with higher phosphate and in mycorrhyzal dependent. Each range corresponds to a different P concentration in the substrate.	Azcón and El-Atrash (1997)
Mixture	15-25	40.6-45.6 (Cuts avge.) 40.4-42.7	No significant change in the cultivars screened.	Robinson *et al.* (2004)
Mixture	3.1-12	31.5-36.2	No significant change	Pasternak *et al.* (1993)
Mixture	2.1-7.8	35.0-36.0 31.4-38.6 26.9-36.9	Increase in N content as salinity increased. Range for different cultivars.	Hussain *et al.* (1995)
Seawater	5.75-23	22.0-25.0	No consistent effect	Ashour *et al.* (1997)

[1] Estimated based on the assumptions that 1 dS/m ≈ -0.03671 MPa and 1 mM of NaCl ≈ -0.00329 MPa at 25 °C (Smith, 1994) or seawater equals to 46 dS/m (Maas, 1993). The control (for most of the cases 0) is possible that its EC is around 1 dS/m.

2.8.2.2. Mineral concentrations

Most published research on lucerne grown under saline conditions has focused mainly on sodium, chloride, potassium and, to a lesser extent, on calcium and magnesium. Most of the studies have been completed under different conditions, especially nutrient solutions, substrates and salinizing media, but general trends can be observed.

When the predominant salt in the medium has been NaCl, the sodium content of lucerne shoots tends to increase linearly as the concentration increases, and a similar response occurs for chloride (Figure 2.6). From this information, the average concentration of sodium in lucerne shoots increases by 2.0 units per unit increase in the EC or salt added. Whereas for chloride, the increase in one unit of EC corresponds on average to 2.8 units in chloride content in lucerne shoots.

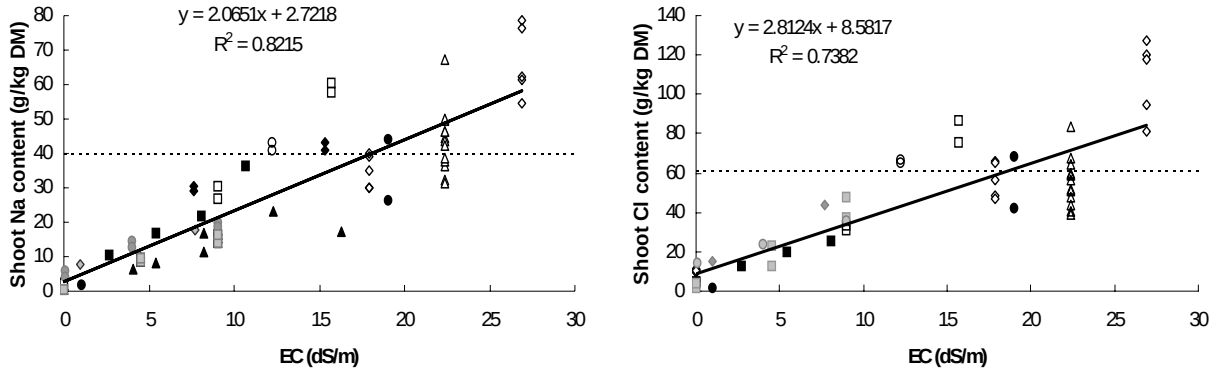


Figure 2.6. Sodium and chloride concentration in lucerne shoots as affected by salinity where the main salt in the irrigation solution was NaCl (data sourced from several authors: ▲ Shone and Gale, 1983; Δ Noble *et al.*, 1984; □ Ashraf *et al.*, 1986; ● Mckimmie and Dobrenz, 1991; ♦ Winicov, 1991; ◊ Chaudhary *et al.*, 1996; ■ Banet *et al.*, 1996; ○ Esechie and Rodriguez, 1998; ● Rogers 2001; ♦ Mezni *et al.*, 2002; ■ Rogers *et al.*, 2003). Estimation based on the assumptions that 1 dS/m ≈ -0.03671 MPa and 1 mM of NaCl ≈ -0.00329 MPa at 25 °C (Smith, 1994). The dotted line represents the concentration at which Na and Cl becomes restrictive (10% of NaCl) for animal production (sheep value).

For ruminants, a dietary NaCl concentration of over 10% DM will limit animal production by suppressing voluntary feed intake. This NaCl percentage is equivalent to 39.3 g of Na/kg DM and to 60.7 g of Cl/kg DM, which represents a Cl/Na ratio of 1.5 on a weight basis and 1:1 on a molar basis. It is then likely that Na^+ and Cl^- concentrations become limiting for sheep at EC

concentrations about 18 dS/m. Nevertheless, there are studies that show that these threshold concentrations may be reached at around 12 dS/m of EC, whilst a few others are not reached until about 22 dS/m. The underlying reasons for this range are not clear, but warrant further investigation to develop improved cultivars suitable for growth in moderately saline soils. Even though lucerne is a glycophytic species, the figures suggest that Na and Cl can accumulate in the plant tissue to concentrations that may limit animal production. Thus, on the basis of the Na and Cl concentrations in Figure 2.6, the relationship between dry matter production and exclusion of these ions needs to be considered when developing new cultivars in order to retain acceptable forage quality.

The magnitude of Na and Cl accumulation in shoots differs when salinity is the result of a mixture of salts (Figure 2.7), especially when calcium and magnesium salts are included in the mixture. The response depends on the main salt being used. For example, in experiments using Na_2SO_4 in the growth media (Rogers *et al.,* 1998; Grieve *et al.,* 2004), both sodium and sulphur in plant biomass may reach limiting or toxic figures to animals.

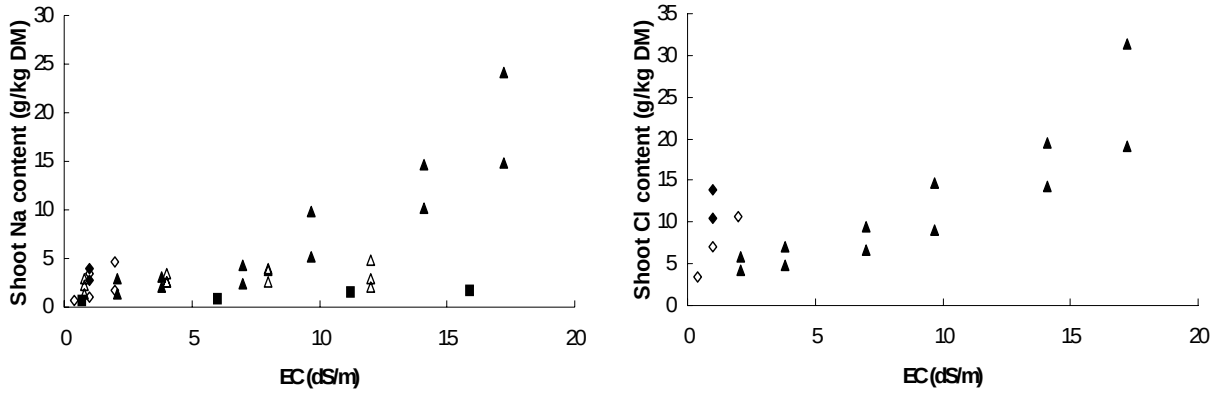


Figure 2.7. Sodium and chloride concentration in shoot of lucerne as affected by salinity of a mixture of salts [data sourced from several authors: ■ Brown and Hayward, 1956 (using sodium and calcium chloride); ◊ Bernstein and Francois, 1973 (using sodium and calcium chloride); ♦ Francois, 1981 (using sodium, calcium and magnesium chloride; ▲ Rogers *et al.,* 1998 –maximum and minimum values of cultivars- (using sodium and magnesium sulphate and sodium and calcium chloride); Δ Anand *et al.,* 2000 (using sodium, calcium and magnesium chloride and sodium sulphate)].

Reports on shoot potassium content show a wide distribution among experiments, especially in the range of 0 to 10 dS/m (Figure 2.8). Decreases in the concentration of this mineral have been

reported under saline growth conditions; but this effect appears to occur more markedly at higher salinities and stems seem to be more affected than leaves. For instance, Rogers *et al*. (1998) using Na_2SO_4 as a major salt, found that potassium concentration decreased by an average of 17% in 16 varieties of lucerne after 9.7 dS/m EC and by 42% at 17.2 dS/m. Ashraf *et al.* (1986) and Kapulnik *et al*. (1989), using NaCl as the main salt at 175-200 mM (≈16-18 dS/m), found that potassium concentration decreased by 33-56% in leaves and by 44-76% in stems, respectively. Interactions with other elements can occur. Shone and Gale (1983) reported that potassium concentrations in lucerne shoots decreased by 46% from the control of 27.4 g/kg DM when lucerne was not inoculated and exposed to 75 mM NaCl (≈ 6.7 dS/m); but that potassium concentration did not decrease when plants were supplied with nitrate in a range of salinity from 0 to 150 mM NaCl (≈ 16.3 dS/m). Potassium deficiency is considered when the whole lucerne shoot has values lower than 13 g/kg DM, or less than 18 g/kg DM when the plant is at vegetative and in early flowering stage (Pinkerton *et al*., 1997). Marginal concentrations are likely to occur at high salinity as Noble *et al*. (1984) found in several populations of lucerne grown at 250 mM NaCl (≈ 22.4 dS/m) where the K concentrations were marginal (i.e., 13.5-15.8 g/kg DM).

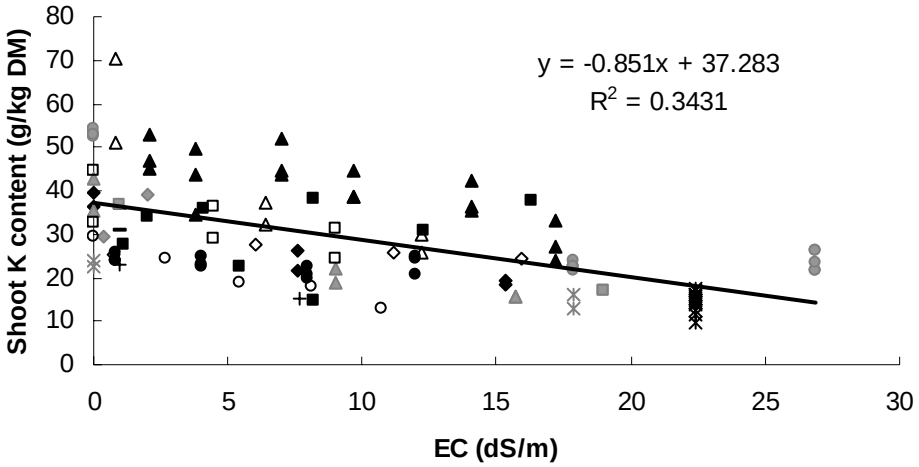


Figure 2.8. Potassium concentration in lucerne shoots as affected salinity, mainly using NaCl (data sourced from several authors: ◊ Brown and Hayward, 1956; ♦Bernstein and Francois, 1973; - Francois, 1981; ■ Shone and Gale, 1983;* Noble *et al.,* 1984; ▲ Ashraf *et al.,* 1986; * Kapulnik *et al.,* 1989; ■ Mckimmie and Dobrenz, 1991; ♦ Winicov, 1991; ○ Banet *et al.,* 1996; ● Chaudhary *et al.,* 1996; Δ Esechie and Rodriguez, 1998; ▲ Rogers *et al.,* 1998 –minimum, average and maxium values-; ● Anand *et al.,* 2000; + Mezni *et al.,* 2002; □ Rogers *et al.,* 2003).

For calcium, Ashraf *et al*. (1986) reported reductions of approximately one half in stems and leaves, with the exception of one lucerne line that had a decrease in leaves of only 13% at 100 mM of NaCl (the controls had on average 25 g/kg DM for leaves and 13 g/kg DM for stems). These previous results contrast with those of Kapulnik *et al*. (1989), where Ca content did not change in four lucerne populations at 200 mM NaCl, although the concentration of 9.9 g/kg of DM was in the marginal range for this species. Calcium content in the lucerne is sensitive when Na_2SO_4 is the dominant salt in the external medium. Rogers *et al*. (1998) reported that shoot calcium concentration decreased by an average of 25% at 9.7 dS/m and 49% at 17.2 dS/m for 16 varieties, which contrasted with the controls that averaged 16.9 g Ca/kg DM. A similar trend was found by Grieve *et al*. (2004) where calcium concentrations in shoots decreased despite slight increases in nutrient solution over 12 harvests at monthly intervals under salinity levels of 15 and 25 dS/m. From Figure 2.9, the overall trend for shoot calcium concentration in response to salinity decreased by 0.47 g/kg DM per unit of salinity increase.

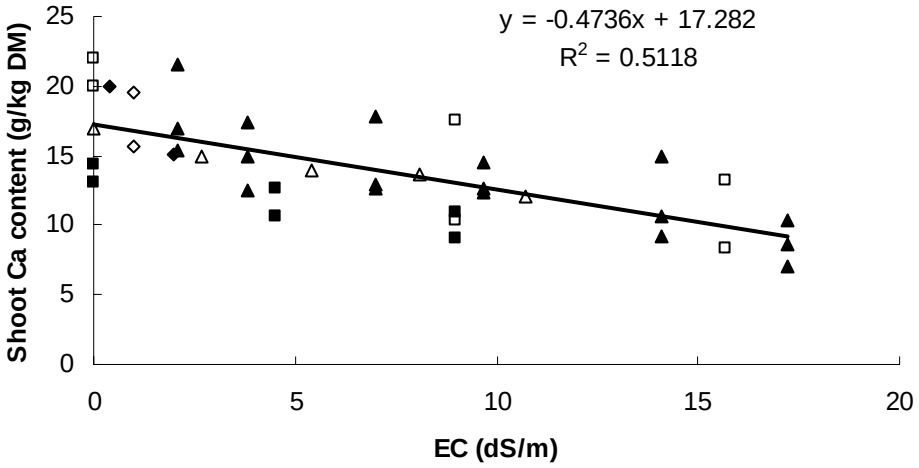


Figure 2.9. Calcium concentration in lucerne shoots as affected by salinity (data sourced from several authors where for most of the cases the main salt was NaCl: ♦ Bernstein and Francois, 1973; ◊ Francois, 1981; □ Ashraf *et al.*, 1986; Δ Banet *et al.*, 1996; ▲Rogers *et al.*, 1998 –minimum, average and maximum values-; ■ Rogers *et al.*, 2003). Estimation based on the assumptions that 1 dS/m ≈ -0.03671 MPa and 1 mM of NaCl ≈ -0.00329 MPa at 25 °C (Smith, 1994).

Few papers deal with magnesium concentration in salinity experiments and plant responses have been variable. With NaCl as the main salt in the growth media, decreases in magnesium concentration in plant biomass have been reported to reach 35%, and leaves seem to be more sensitive than stems (Ashraf *et al*., 1986; Esechie and Rodriguez, 1998). The previous two investigations contrast with Kapulnik *et al*. (1989) where no change in magnesium concentration occurred in any of the four lucerne populations screened, when using NaCl as a main salt. Rogers *et al*. (2003) found a similar pattern. In the cultivars 'Moapa' and 'CUF-101' the Mg concentration decreased by approximately 32% when plants were subjected to 0.02 and 0.2 mM of P with 100 mM NaCl (≈ 9 dS/m) for 24 days, whereas the cultivar 'Aurora' during 43 days growth under salinity at 100 mM of NaCl with 0.5 and 5 mM of P did not have any change in Mg concentration. There is evidence that chlorophylls in lucerne decrease when exposed to salinity (see section 2.8) and it would be expected that Mg concentration may drop, as it is a core component of these substances. However it seems that there are interactions with other chemical elements and cultivar responses that can modify such pattern.

In relation to trace element concentrations in plants as a result of growth under saline conditions, few works have been published. There are suggestions that in saline and alkaline soils, the availability of trace elements in the soil solution is limited. This is mainly due to the pairing of Cu, Fe, Mn and Zn with the dominant ligands, such as Cl^-, SO_4^- or CO_3^{2-}, which affects adsorption, precipitation and concentration inducing deficiencies in the plant (Page and Chang, 1990). Thus, when ruminants consume plants deficient in these trace elements they are at risk of developing metabolic deficiencies. In this regard, Miller *et al.* (1996) have reported that in saline sites zinc, copper and selenium in several plant species including lucerne, are likely to be deficient for growing beef cattle.

In animal nutrition it is important to consider the mineral concentration in the plant and the mineral balance. For instance, hypomagnesaemic tetany, a condition where pregnant or lactating cattle and ewes have a concentration of magnesium less than 2.0 mg/dl blood (Mayland and Wilkinson, 1989), develops when diets with less than 2.0 g Mg/kg DM are consumed (Grunes and Welch, 1989). But the safe concentration of magnesium can rise if the milliequivalent ratio of K/(Ca+Mg) reaches values equal or higher than 2.2 in the feed or if potassium and nitrogen surpass 3 and 4%, respectively in the plant (Grunes and Welch, 1989). Under saline growing conditions, although the concentration of potassium decreases, calcium and magnesium

concentrations may decrease to a greater extent, thereby increasing the risk of hypomagnesemia. Calculated K/(Ca+Mg) ratios from the available data are lower than the critical value of 2.2, but it is more common to find values of K about 3% (Figure 2.8) and values of N closer to or about 4% (Table 2.1). Aspects such as forage intake, the availability of the absorbable magnesium and calcium, which are likely to form oxalates, and the interaction of high nitrogen and low water-soluble carbohydrates concentrations have been shown to interfere with magnesium absorption (Robinson *et al.,* 1989). Little is known about mineral absorption and bioavailability in forages grown under saline conditions and the extent of the variation in other important mineral relationships.

2.8.2.3 Fibre and digestibility

Ashour *et al*. (1997) reported increases of 12% in carbohydrate concentrations in lucerne grown under saline conditions, but this is not a consistent characteristic as results of Kagawa *et al*. (1989) showed only a slight increase in only the third harvest.

Crude fibre concentration of the whole plant has been reported to decrease (Kagawa *et al.,* 1989; Ashour *et al.,* 1997) and the same trend has been found for NDF and ADF (dry matter basis) (Pasternak *et al.* 1993; Robinson *et al.,* 2004) and for lignin (Kagawa *et al.,* 1989). For i*n vitro* digestibility of the whole plant, there has been no response to salinity (Kagawa *et al.,* 1989; Robinson *et al.,* 2004), although an increase of 16% at 21.3 dS/m using NaCl as a main salt was found by Boyd and Rogers (2004) where controls had on average 74% of IVD of the dry matter.

It is not clear whether these effects are the result of an increased leaf-to-stem ratio, disturbances in the phenological stage as a response to salinity or a direct effect of salts on the biochemical processes in the plant. It is also worthwhile to note that for digestibility, organic matter and adjustments for soluble ash are more indicative of impacts and so more able to distinguish clear effects from salts (Masters *et al.,* 2001; Norman *et al.,* 2002) and prevent plant energy values to be overestimated (Figure 2.10). Norman *et al*. (2002) found overestimations on IVD of DM in non-halophytic species that ranged from 1 to 14 percentage units, values that depended on plant species, season of the year and salinity level. Values corrected for soluble ash should apply also for fibre measurements in order to have more accurate estimations of them. This information is needed in order to obtain a better understanding of the implications for animal production, and to have better information when making selections of superior palnt genotypes with acceptable or improved nutritive value for ruminants.

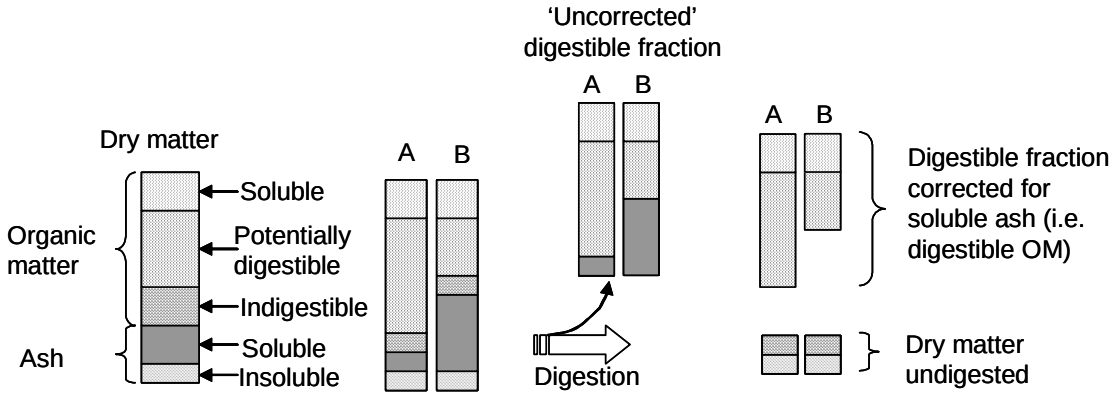


Figure 2.10. Hypothetical example of dry matter components and the influence of soluble ash in the digestibility value. The "A" element represents a feed with low soluble ash content, while "B" represents a high soluble ash fraction.

2.8.3. Rhizobia symbiosis

The consequence of a deficient symbiosis between N_2-fixing bacteria and the host plant may result in less forage produced and less nitrogen concentration in shoots (Table 2.1) and this can be detrimental to its nutritive value. Rhizobia are generally more tolerant of salinity than their host legumes (Wilson, 1970; Bhardwaj, 1975; Singleton *et al.,* 1982; Craig *et al.,* 1991; Mohammad *et al.,* 1991), but factors related to root growth disturb the symbiosis. It is well documented that NaCl salinity can reduce growth of roots by decreasing length, branching and dry weight in the main roots (Subba Rao *et al.,* 1972; Keck *et al.,* 1984; Hoffman *et al.,* 1975; Khan *et al.,* 1994a; Serraj and Drevon, 1998) and by shortening and changing the appearance (i.e., stubby and bulbous morphology) of the root-hairs, effects that lead to a degradation of the 'rhizosphere' (Lakshmi-Kumari *et al.,* 1974; Mohammad *et al.,* 1991; Banet *et al.,* 1996). These effects impair establishment of an effective N_2-fixing nodule (Lakshmi-Kumari *et al.,* 1974; O'hara *et al.,* 1988; Zahran, 1991; Banet *et al.,* 1996) which results in a reduced number of nodules (Subba Rao *et al*., 1972; Fougère *et al.,* 1991; Banet *et al.,* 1996; Mashhady *et al.,* 1998) whilst nodule mass and size are less affected (Bernstein and Ogata, 1966; Keck *et al.,* 1984; Fougère *et al.,* 1991; Banet *et al.,* 1996).

Nitrogen fixation per nodule is also affected by salinity (Fougère *et al.,* 1991; Zhou *et al.,* 1992; Mashhady *et al.,* 1998; Serraj and Drevon, 1998), and this is believed to occur mainly due to a

reduction of nitrogenase activity per nodule, possibly because of both a drop in bacteroid respiration a product of a shortage of assimilates, and a decrease in the nodule permeability to O_2 diffusion (Bekki *et al.*, 1987; Serraj and Drevon, 1998). Studies on translocation of assimilates to the root under saline conditions or selection of genotypes with enhanced root growth, which could lead to an improvement in symbiosis, are lacking, but there are indications that when root growth is improved, salt tolerance in lucerne improves (Winicov, 2000).

2. 9 Implications of salinity-induced changes in forage nutritive value to ruminants

Salt tolerance in ruminants is variable and factors like animal species, breed, physiological status, diet and environmental conditions can influence the limits to which production can be sustained (Singh and Taneja, 1981; Gihad, 1993). The dietary content of sodium chloride that sheep can tolerate without depressing production is no more than about 10% (Wilson, 1966; Kromann and Ray, 1967; Jackson *et al.*, 1971; Moseley and Jones, 1974). High levels of dietary NaCl induce several physiological responses that influence animal production. Water consumption and kidney glomerular filtration rate increase as an adaptive response to excrete excess sodium and chloride and, consequently, urine volume is increased (Tomas *et al.*, 1973; Moseley and Jones, 1974; Godwin and Williams, 1986; Hamilton and Webster, 1987; Arieli *et al.*, 1989). This is accompanied by reduced retention of minerals, apparently due to reduced proportional reabsorption, which increases excretion of elements such as calcium, magnesium, potassium, phosphorus and nitrogen (Tomas *et al.*, 1973; Moseley and Jones, 1974). Thus, a mineral imbalance can be induced and, for example serum concentrations of magnesium and calcium may decrease whilst potassium and chloride concentration in plasma increase (Tomas *et al.*, 1973; Moseley and Jones, 1974; Moseley, 1980).

In the rumen and abomasum there is an increase in the osmotic pressure and outflow of fluids with high NaCl diets (Potter *et al.*, 1972; Hemsley *et al.*, 1975; Godwin and Williams, 1986; Arieli *et al.*, 1989; Garg and Nangia, 1993) and some rumen microorganism populations are reduced (especially protozoa and selenomonads) (Potter *et al.*, 1972; Hemsley *et al.*, 1975; Garg and Nangia, 1993), and this changes the production and proportions of volatile fatty acids primarily due to a reduction in ruminal organic matter degradation (Jackson *et al.*, 1971; Potter *et al.*, 1972; Hemsley *et al.*, 1975; Godwin and Williams, 1986; Toha, *et al.*, 1987; Garg and

Nangia, 1993). The animal reduces food intake and overall, the animal performance is affected negatively.

From such effects of salinity on the animals, the main characteristics of a forage plant grown under saline conditions should include: a) acceptable low levels of sodium and chloride; b) high ruminal degradation rates of organic matter to maintain high ruminal digestion in the free of the increased rate of passage of digesta; and c) acceptably low concentrations of macro and micro-minerals.

It is likely that under saline growing conditions, the content of non-protein nitrogen compounds in plants increase. These kinds of compounds, when degraded to ammonia in the rumen under limitations of energy can lead to a nitrogen loss (Figure 2.11). This is because incorporation of ammonia into microbial protein is limited, and excess ammonia is absorbed into the bloodstream and mostly converted to urea for excretion in urine (Casler, 2001; Masters *et al.,* 2001; Hristov and Ropp, 2003). Nonetheless, fibre-degrading ruminal bacteria (mainly cellulolytic and hemicellulolose-degrading bacteria) depend on ammonia as a primary nitrogen source (Nocek and Russell, 1988; Russell *et al.*, 1992; Tedeschi *et al.,* 2000; Nagadi *et al.*, 2000). In addition, in the rumen of animals consuming high salt diets where dry matter intake has not been depressed, ammonia concentration was decreased by 36 to 57% (Godwin and Williams, 1986; Toha *et al.*, 1987). These authors attributed the effect to an increase in the rumen turnover rate, and possible decline in proteolysis and deamination. A shortage of ammonia to fibre-degrading bacteria may decrease fibre digestibility, but less so if readily fermentable carbohydrates are available. A decline in ruminal proteolysis may have positive effects on amino acid supply to the host animal, possibly offsetting some of the detrimental effects on rumen microbial protein synthesis.

Forage legumes grown under saline conditions tend to show a reduction in carbohydrate content in the long term. If so, a reduction in protein availability may be expected for ruminants consuming diets high in non-protein nitrogen (NPN), due to poor capture of ammonia as microbial protein. Indeed high levels of NPN may occur in plants grown under saline conditions (see section 2.7.2).

Other compounds that produce beneficial effects on ruminants, and are synthesized by plants grown under saline conditions, have been reported. For instance proline feed at 80 g/day to

lactating cows, when it is not degraded in the rumen, can be used for casein synthesis, diminishing the use of other amino acids such as arginine and ornithine that are utilized to produce proline (Bruckental *et al.*, 1991). Betaine, when it is not degraded in the rumen, caused a reduction in subcutaneous fat thickness in Manchego lambs (Fernández *et al.*, 1998) especially in females (Fernández *et al.*, 2000). For example, lambs fed with 2 g of betaine anhydrous per kilogram of feed had a reduction in backfat of 10% (Fernández *et al.*, 1998). This compound is also an important methyl group donor for synthesis of other amino acids, proteins, phospholipids, hormones and nucleic acids (Virtanen and Rumsey, 1996; Bianchi *et al.*, 1999; Hageman and Stierum, 2001). All these beneficial effects depend on the extent of their degradation in the rumen but, as rumen turnover is likely to be high in animals on high-salt diets, sufficient quantities may scape the rumen to be effective.

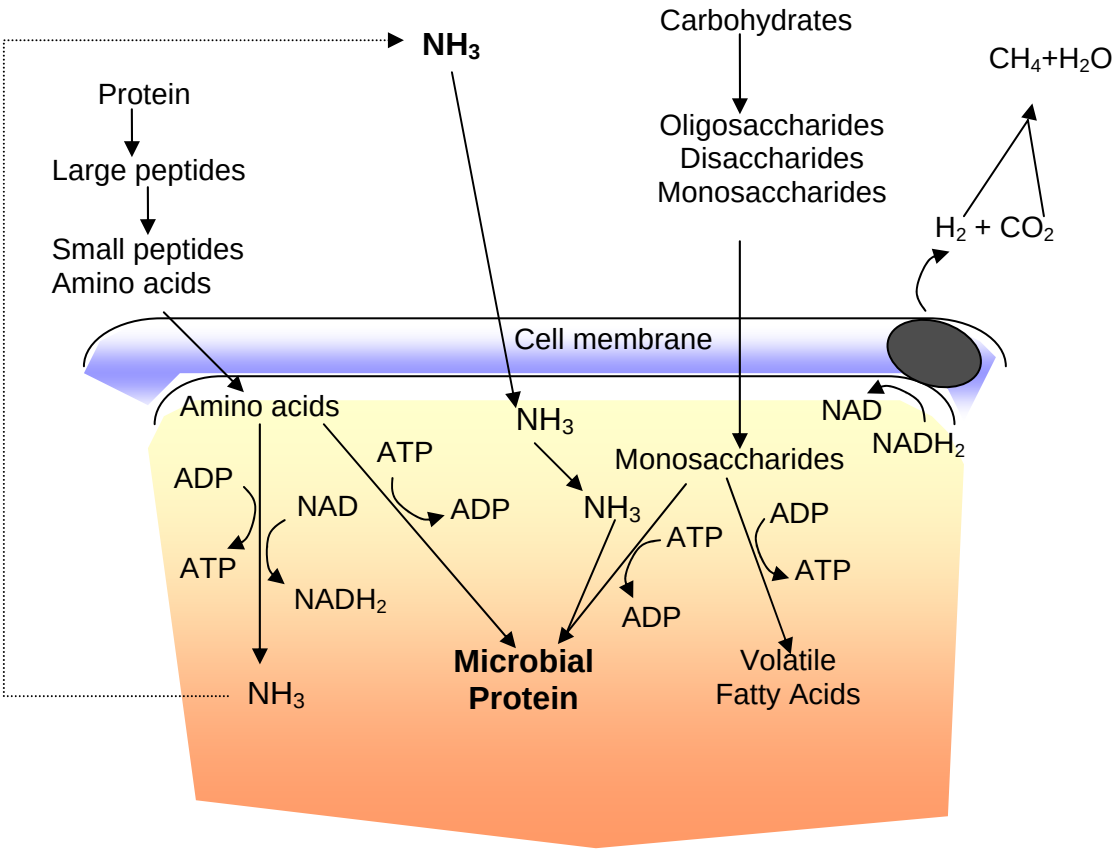


Figure 2.11. The utilization of protein and carbohydrate by rumen bacteria. From Nocek and Russell, 1988.

2.10 Concluding remarks and thesis aims

Most of the research completed on effects of salinity on forage species has been focused mainly on agronomic aspects. However, since grazing animals are the main economic option for the productive use of the forage products of saline land, it is necessary to consider aspects related to nutritive and feeding value of forages. There is limited information on the mechanisms that could be involved in forage legume salt tolerance and the consequent effects on animal production.

It is clear that soil salinity can have a strong influence on several agronomic aspects related to plant persistence and productivity, but several characteristics related to forage quality are also modified. Even though sodium and chloride can be excluded in glycophytic species, these elements can reach concentrations in shoots above recommended levels for ruminant diets at higher concentrations of NaCl (above 10 dS/m) and undesirable mineral ratios are likely to result. It seems that the amounts of proline or betaine in lucerne are quite low and unlikely to have a significant effect on animal performance. There is limited information on changes in the proportions of true protein and non-protein nitrogen fractions that constitute crude protein.

In the long term, salinity affects the carbohydrate concentration and this has a direct relationship with energy reserves for regrowth and persistence. In this regard, there is a lack of information in relation to grazing or harvest strategies under saline conditions. Grazing strategies have implications for plant growth and nutritive value of the forage on offer. Additionally, there are differences in salt concentration across the plant (e.g., stem versus leaf, old versus new leaves) and this could influence animal selectivity, an issue that has not been investigated with forage legumes grown in saline conditions. However, there are other plant responses to salinity that can be positive, such as increased leaf-to-stem ratio and reductions in fibre content, aspects that may modify digestion rates, which are important in maintaining digestion with the high ruminal turnover rates of digesta associated with high-salt diets. Both leaves and stems usually do not have the same patterns of mineral concentrations, fibre, protein and other characteristics and, therefore, it is important to know the proportions of leaf-to-stem rather than only measuring whole shoot material.

There are a number of highlighted issues that indicate our understanding of the complex interactions between salinity-plant responses, nutritive value and animal nutrition is far from complete. Some basic information required is:

- The extent in changes of mineral concentration and ratios of particular elements,
- carbohydrate (including fibre) characteristics,

- protein content,
- digestibility and presence of secondary plant compounds.

Thus, the purpose of this thesis is to investigate changes in legume forage nutritive value associated with salinity. This thesis reports production of lucerne and Melilotus forage under three salinity concentrations as described in Chapter III. The quality of the forage produced is described in Chapter IV and V. Chapter IV includes the mineral analyses and element relationships for the plant and for the animal, and Chapter V describes the components of dry matter related to plant fibre composition and digestibility. Chapter VI is a study of the histology of the leaf and stems of the species under study in order to further explain the results from the gravimetric determinations. Chapter VII is an overview of secondary compounds in Melilotus under salinity and finally, Chapter VIII provides the general discussion and conclusions, including implications for animal production from saline land and future research.

CHAPTER III
THE EFFECT OF SALINITY ON THE GROWTH OF LUCERNE AND MELILOTUS

3.1 Introduction

There is a need for forage legume species to be productive on salt-affected land to complement available halophytic plants, and thereby improve the diet of grazing ruminants. Currently, livestock represents the main productive option for saline land. The need for tolerance to salinity and tolerance to waterlogging, in its permanent or temporary form, makes it difficult to find well adapted plant species. In addition, a perennial or long-season forage is needed to cover the gaps in forage supplies when annuals do not grow. The number of choices is reduced, as most of the species of economic importance of this genus are either sensitive or only moderately tolerant to salinity (Läuchli, 1984; Rogers *et al.*, 1997). Some species with potential have been detected and a number of others represent plausible alternatives (Cocks, 2001; Rogers *et al.*, 2005).

Lucerne and Melilotus can produce forage over an extended period of time and have moderate tolerance to salinity, but their tolerance to flooding and waterlogging is low (up to 14 days according to McKenzie, 1951). In the case of lucerne, flooding tolerance can be doubled if this period coincides with cool temperatures (i.e., 13 - 18°C) (Heinrichs, 1972; Cameron, 1973; Thompson and Fick, 1981; Teusch and Sulc, 1997; Teusch *et al.*, 2000) or if the water table does not reach 45 cm of the surface (Rai, *et al.*, 1971; Slavich *et al.*, 2002; Mueller *et al*., 2005). For Melilotus, this situation seems to be the same or even better than lucerne (Ahi and Powers, 1938; Evans and Kearney, 2003). These characteristics and the genetic variability within these species make them attractive for evaluation. For both species, little information exists on the description of the components of forage yield that could influence nutritive value under relatively long periods of salinity and this chapter will describe production and components of forage yield.

3.2 Methodology

Salt-stressed forage was obtained from two experiments where salinity was imposed in the irrigation water using sodium chloride as the main salt. Initially, the first experiment consisted of three forage legume species chosen according to their tolerance to salinity: Melilotus (*Melilotus albus*) cultivar "El Domador", a biennial species considered moderately tolerant; lucerne (*Medicago sativa*) cultivar "Eureka" (winter activity = 8, highly resistant to the blue green and the spotted aphids and semi-postrated stems), a perennial species considered moderately sensitive (Maas and Hoffman, 1977; Johnson *et al*., 1992; Humprey and Auricht, 2001); and Red clover (*Trifolium pratense*) cultivar "Astred", a perennial species considered as sensitive. However, Red clover could not tolerate a treatment of 70 mM of NaCl and, before the first harvest, was eliminated from the experiment.

This first experiment was conducted following the protocol for determining forage production of lucerne under sodium chloride (NaCl) salinity proposed by Smith (1996), where harvests occurred chronologically over a five month period. Based on results of the initial experiment, a second experiment was completed, but in this experiment harvests were over a period of eight months, at times that provided plant material considered to be of similar phenological stages across all the treatments to allow species comparisons of nutritive value at similar stages of plant maturity.

The seed of all species was kindly supplied by the Australian Medicago Genetic Resource Centre located at the Waite Campus, The University of Adelaide, South Australia.

3.2.1 Establishment

The substrate used was the University of California Mix (Waite version) made of sterilized coarse washed sand (company name, Golden Grove S.A.) and Eurotorf peatmoss in a ratio 1.6-to-1 by volume respectively, fertilized with 0.5 g of calcium hydroxide, 0.9 g of calcium carbonate and 1.0 g of Nitrophoska 12-5-14, per liter of the mixture. The pH was 6.8, $EC_{1:5}$ of 621 µS and total dissolved solids of 299 mg/l, with measurements taken at 25 °C.

Free-draining nursery tree-tubes with dimensions of 50x50x120 mm (200 ml effective capacity) were filled with substrate and arranged in free-draining trays with a capacity for 36 pots.

Before sowing, Melilotus and lucerne seeds were inoculated with Group AL commercial *Rhyzobium*. Three seeds were sown per pot and five days after germination the plants were

thinned to one. Trays were located in a glasshouse equipped with automatic cooling system at Roseworthy Campus, The University of Adelaide, South Australia.

The first experiment was conducted predominantly over the summer and the glasshouse was covered with shade cloth to give 70% shade in order to help the cooling system maintain a more stable temperature. The second experiment was conducted mainly during winter and spring and no shade cloth was used after 60 days of establishment. The temperature was recorded using a data logger (Tinytag plus model) and the average temperature and standard deviation for the first experiment was 19.2 °C ± 3.0 and for the second 15.9 °C ± 4.5.

3.2.2 Nutrient solution

The base nutrient solution consisted of quarter-strength Hoagland's solution, except for Ca and Mg, which were at full strength. This concentration was according to the protocol for forage production under salt stress (Smith, 1996). All fertilizers used were analytical reagent grade and their concentrations in the irrigation solution were as: 0.25 mM KH_2PO_4, 1.25 mM KNO_3, 5.0 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2.0 mM $MgSO_4 \cdot 7H_2O$, 11.3 µM H_3BO_3, 2.3 µM $MnCl_2 \cdot 4H_2O$, 0.2 µM $ZnSO_4 \cdot 7H_2O$, 0.08 µM $CuSO_4 \cdot 5H_2O$, 0.004 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and, 30.0 µM Fe-EDTA. Stock solutions were prepared with ultrapure water obtained from an EASY pure LF Barnstead model, and the irrigation solution with rainwater that was filtered through a carbon filter. Table 3.1 shows the water characteristics for the two experiments.

Table 3.1. Characteristics[§] of the rainwater and the irrigation solutions (mean ± S.D.) used in Experiments 1 and 2.

Water	pH	EC (mS)	Salinity (‰)	TDS[†] (mg/L)
Rainwater	7.2 ± 0.33	0.12 ± 0.02	0.1 ± 0.0	57.3 ± 8
Nutrient solution 0 mM of NaCl	6.6 ± 0.10	1.62 ± 0.19	0.8 ± 0.1	796 ± 97
Nutrient solution 55 mM of NaCl[1]	6.6 ± 0.05	7.24 ± 0.61	3.9 ± 0.3	3870 ± 375
Nutrient solution 70 mM of NaCl	6.6 ± 0.06	7.54 ± 0.29	4.1 ± 0.1	4050 ± 171
Nutrient solution 110 mM of NaCl	6.6 ± 0.07	11.60 ± 0.81	6.5 ± 0.6	6420 ± 604
Nutrient solution 130 mM of NaCl[2]	6.5 ± 0.07	12.62 ± 0.24	7.2 ± 0.1	7040 ± 127

[§] Measurements were performed with a pH meter ATi Orion model 320 and an EC meter ORION model 115 and correspond to the average of seven samples for rainwater, 0, 55, 70 and 110 mM of NaCl, and four for the 130 mM.
[†] TDS means total dissolved solids , [1] Experiment 2 only, [2] Experiment 1 only.

In the first experiment there were four levels of NaCl (0, 70, 110 and 130 mM), allocated according to the salt tolerance of the species. Lucerne had three levels (0, 70 and 110); and Melilotus four (0, 70, 110 and 130 mM). In total there were seven treatments, aiming to achieve reductions in shoot biomass of about 50% at the highest salt concentration for each species. The second experiment consisted of lucerne and Melilotus, each grown at three salinity levels (0, 55 and 110 mM of NaCl).

Sowings were on the 21st of November 2002 for Experiment 1 and on the 1st of February 2004 for Experiment 2. Five days after sowing, germination occurred (Figure 3.1). For the first experiment, all pots were watered with rainwater for 11 days after germination. Irrigation with nutrient solution started at day 17 and saline irrigation started at day 21, increasing progressively over the following eight days until the target concentrations of NaCl were reached. For the second experiment, rainwater irrigation was maintained for 17 days after germination, followed by four days of irrigation with nutrient solution and, at day 26, salt treatments were gradually imposed until the desired concentrations were reached. The frequency of watering was once every two days for the first 10 days after germination and post-harvest and after that once per day, always allowing free drainage of the solution to avoid salt accumulation. Irrigations occurred by placing a copper pipe trident in the pot medium at the base of the plants in the tray, watering 36 pots at a time, with the flow rate calibrated to 5 litres/min via a 12 volts pressure regulated Flojet pump.

3.2.3 Harvesting

According to the protocol for screening lucerne under salinity (Smith, 1996), the first harvest from both experiments was not used for any of the measurements. Three subsequent harvests were conducted; during the first experiment harvests occurred at intervals of 29 days, harvesting all the treatments at the same time. At all harvests lucerne was at the late vegetative stage, but the Melilotus phenological stage in this experiment had marked differences. The control plants were on average in pre-bud, while the plants salt affected were at bud and flowering (stages shown in Plate 3.1). Early flowering was considered when at least five inflorescences on a plant had all their flowers open.

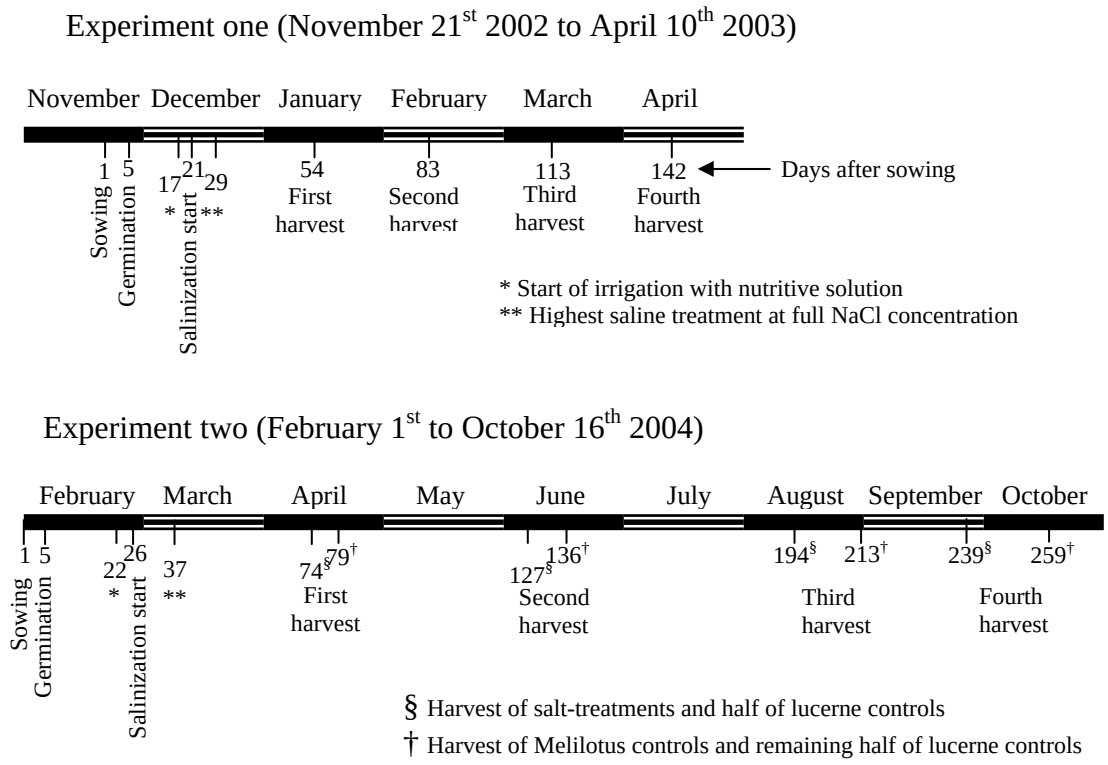


Figure 3.1. Time elapsed (days after sowing) in the two glasshouse experiments.

In the second experiment, the harvest interval was dictated by the phenology of Melilotus, where the harvests occurred when 90% of plants had started flowering. Melilotus plants in the salt treatments started to flower early, while control plants were late. As a consequence, lucerne had two control treatments, one that was harvested when all the salt treatments of both species were harvested, and the other when the Melilotus control was harvested. This was in order to have a comparison of both plant species at all times and to determine what effect, if any, harvest time had on the nutritive quality of lucerne controls. For the Melilotus control, days elapsed to harvest were 57, 77 and 46 for the second, third and fourth harvest, respectively; whereas for the plants in salt treatments the elapsed days between harvests were 53, 67 and 45 in the same order of harvest.

After each harvest, all pots were flushed with fresh water (rainwater) at 0.1 liter per pot and then watered again with their respective salt treatments. In the first experiment, the height of harvest was three centimeters above the pot surface for both species, but in the second experiment

Melilotus was harvested at seven centimeters. This change was made after consideration of a possible negative effect on the activation of the stem vegetative buds in Melilotus.

The harvested fresh biomass was placed in plastic sealable bags, weighed and immediately frozen at -18 to -20 °C. The plant material was later freeze-dried in a CHRIST BETA 1-8 freeze-dryer, weighed and sorted into leaf and stems, including the petioles in the leaf fraction in both species.

3.2.4 Experimental design and measurements

Initially the first experiment was planned as an unbalanced block design, but the Red clover treatments were eliminated and the 130 mM treatment of Melilotus yielded insufficient biomass for most of the measurements of nutritive value, and so that it was decided to remove it from the analysis. Therefore, this experiment was analyzed as a randomized complete-block design with four replications. The second experiment was completed as a randomized complete block design (Plate 3.2). The block design was employed to account for any sources of variability in the plant growth environment within the glasshouse (e.g., light distribution during the day and the position of the ventilators of the cooling system).

In both experiments, data was analyzed by ANOVA, using the GENSTAT 6 program. The residuals were checked for normality and homogeneity and the least significant difference (LSD) was used for means separation. The experimental unit consisted of 36 pots with one plant per pot. Total stems and branches were analyzed with randomized block structure, but shoot biomass production (including leaf and stems), shoot length, basal diameter and plant survival were analyzed as a split-plot array, where the salinity treatments were the main plots and harvests the subplots.

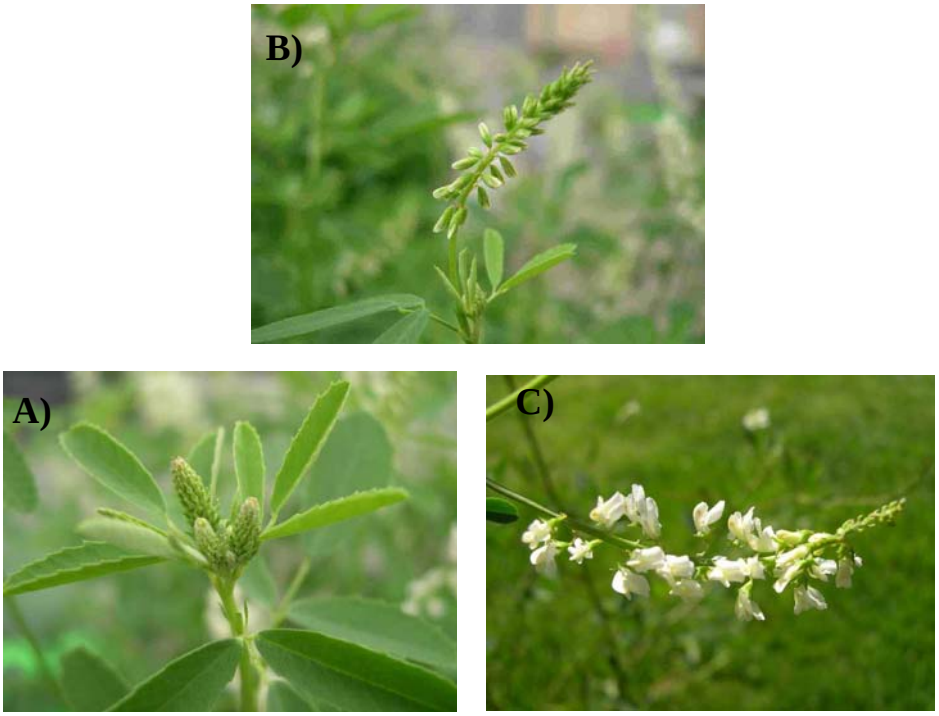


Plate 3.1. Reproductive stages of Melilotus: A) pre-bud, B) bud and C) flowering.

Plate 3.2. View of the experiment. Upper picture, partial view of one replicate.

3.3 Results

3.3.1 Shoot biomass production

A salinity effect occurred in most of the variables measured for both species. Shoot biomass decreased ($P<0.001$) as the concentration of NaCl in the nutrient solution increased (Figure 3.2). Dry matter production per plant in both species was reduced by approximately 70 % at 110 mM of NaCl. The exception was Melilotus in experiment 2, when harvest intervals were extended at the time of Melilotus flowering, where this species had a decrease of 88% (Figure 3.2 b). It was noted that at the highest salinity level the biomass production between species did not differ.

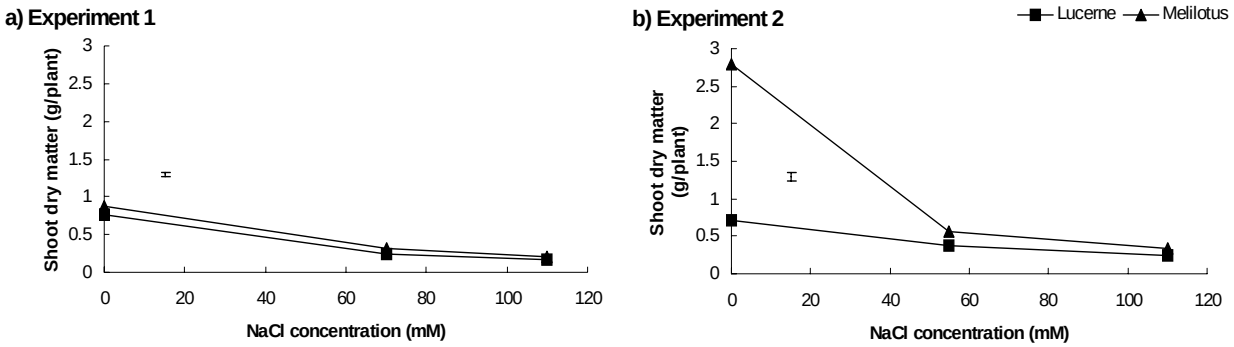


Figure 3.2. Dry matter production per plant (average of three harvests) in lucerne and Melilotus as affected by NaCl salinity. In Experiment 1, harvests were performed at the same chronological age between plant species. In Experiment 2, harvests for Melilotus were at 90% of plants at flowering, harvesting lucerne at the same time than the former species. The bars in both figures represent the LSD at 5% level.

In addition to the effect of NaCl, there was an effect of harvest number ($P<0.001$) which, for plants in salt treatments in both experiments, there was less biomass produced at harvest four (Figure 3.3). Shoot biomass of control plants also declined with successive harvests, especially for Melilotus, an effect that was more noticeable in Experiment 1 (harvested chronologically and also cut lower). Lucerne controls tended to re-grow after harvesting with more vigour than Melilotus, especially in Experiment 2 with longer harvest intervals.

The decrease in total shoot biomass imposed by salinity corresponded to decreases in both leaf and stem dry matter. However the contribution of each organ to total dry matter differed ($P<0.001$), indicating differences in dry matter partitioning at the whole-plant level (Figure 3.4).

Leaves contributed a greater proportion than stems and consequently, the leaf-to-stem ratio was modified (Table 3.2). This ratio increased ($P<0.001$) as salinity increased. At harvest four, the salt-stressed plants had the biggest increase in this ratio.

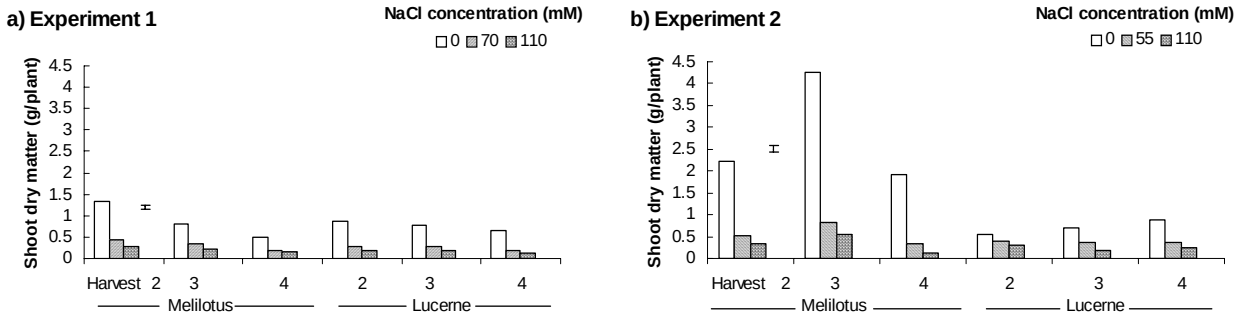


Figure 3.3. Shoot dry matter production of lucerne and Melilotus at different harvests as affected by NaCl. The bars represent the LSD at 5% level.

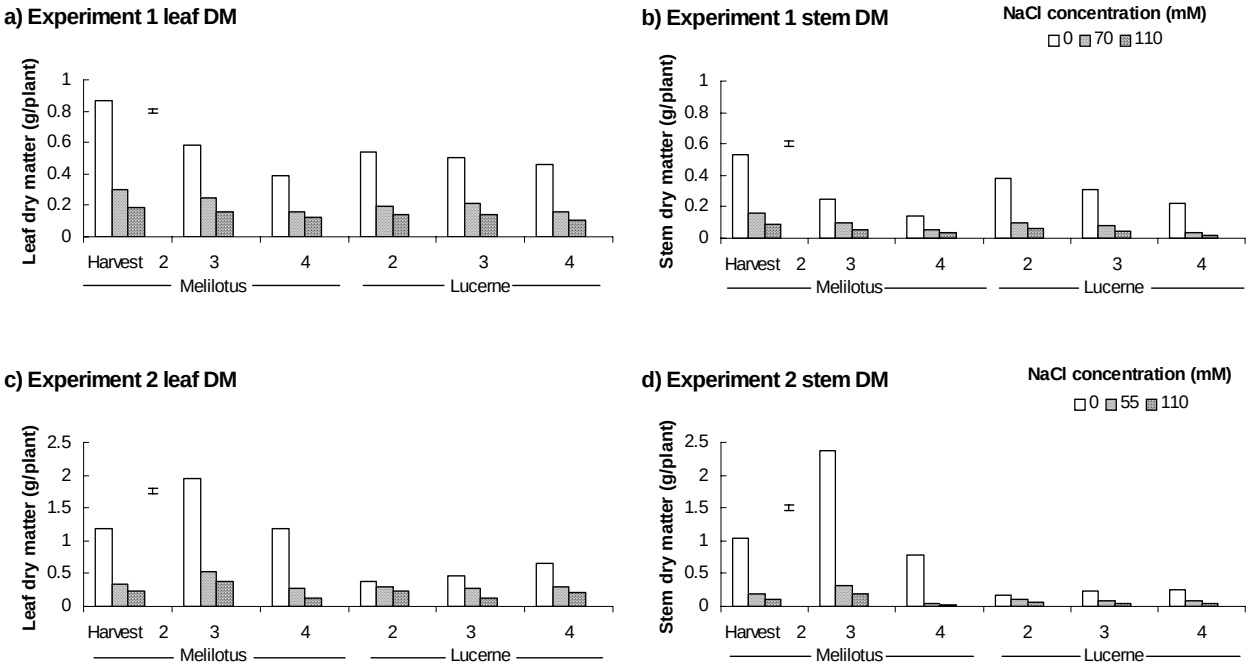


Figure 3.4. Dry matter of leaves and stems of lucerne and Melilotus (g/plant) at different harvests as affected by NaCl salinity. The bars represent the LSD at 5% level.

Table 3.2. Leaf-to-stem ratio in lucerne and Melilotus at different harvests as affected by NaCl salinity.

NaCl (mM) and experiment	Lucerne			Melilotus		
	Harvest 2	Harvest 3	Harvest 4	Harvest 2	Harvest 3	Harvest 4
Experiment 1						
0	1.4	1.6	2.0	1.6	2.4	2.8
70	2.0	2.7	4.4	1.8	2.6	3.2
110	2.4	3.2	5.5	2.2	2.9	3.2
L.s.d.[1]	0.4					
Experiment 2						
0	2.3	1.9	2.6	1.2	0.8	1.5
55	2.9	3.1	3.3	1.7	1.7	5.5
110	3.2	3.6	4.5	2.1	2.2	8.9
L.s.d.[1]	0.6					

[1] Least significant difference of means (5% level) for plant species, salinity level or harvest.

Stem characteristics (i.e., length, basal diameter, number of stems in lucerne and number of branches in Melilotus) were taken only for the first experiment and results are in Figures 3.5 and 3.6 and Table 3.3.

In both species, the decrease in shoot dry matter was accompanied by a decrease ($P<0.001$) in shoot length as salinity increased. Lucerne shoot length decreased by up to 55% at 110 mM NaCl and Melilotus by 40%. A strong harvest effect occurred for Melilotus, especially in the control treatment where there was a 21% of reduction in shoot length over time. Lucerne controls were more stable over time.

Stem basal diameter decreased ($P<0.001$) in response to salinity in both species (Figure 3.6). At each harvest, the treatments grown under salt stress had thinner stems ($P<0.001$). Lucerne stem diameter decreased by 29% and Melilotus stems by 39% in response to 110 mM of NaCl.

The number of stems per plant in lucerne was affected negatively by NaCl ($P<0.001$). In lucerne, at 110 mM NaCl, the number of stems per plant decreased by 46%, and the proportion of dead stems increased by 20%. In Melilotus, the cultivar used had (in most of the cases) only one stem and, for this reason branch number was quantified instead. At the highest salinity level, the production of branches decreased by approximately 50%.

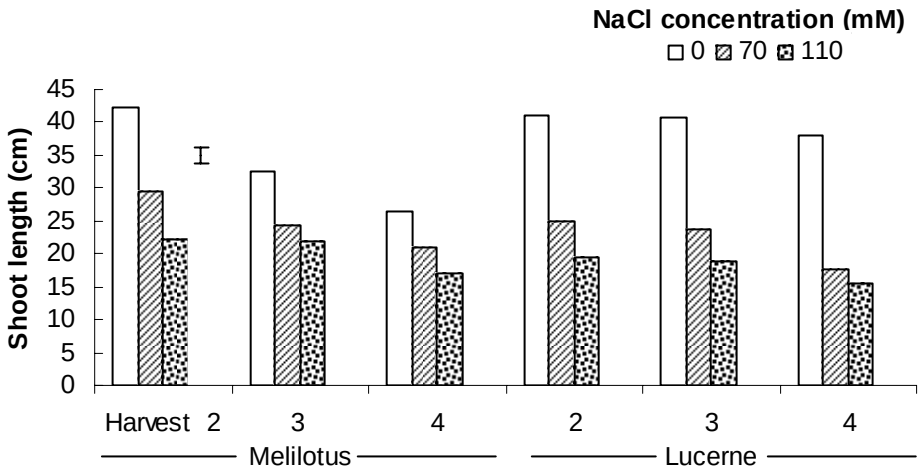


Figure 3.5. Shoot length of lucerne and Melilotus at different harvests as affected by NaCl salinity. The bar represents the LSD at 5% level.

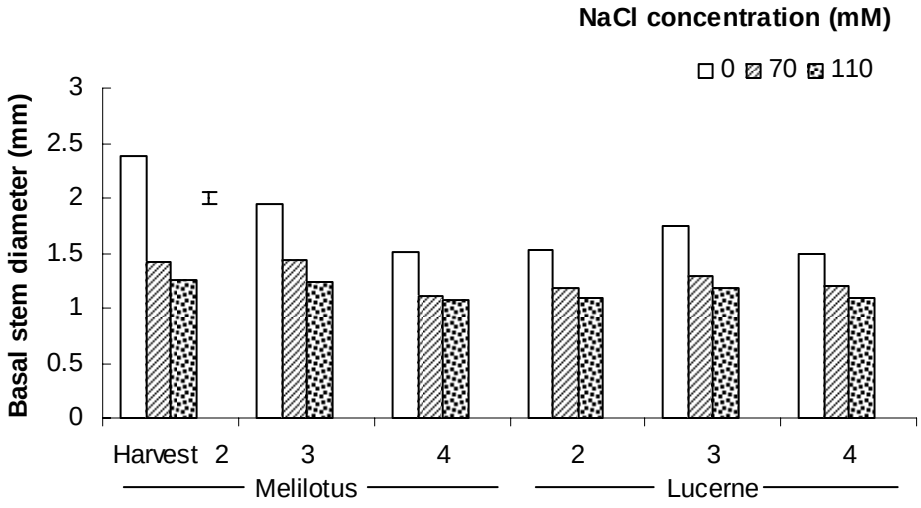


Figure 3.6. Stem diameter (base) of lucerne and Melilotus at different harvests and NaCl concentrations. The bar represents the LSD at 5% level.

Table 3.3. Effects of NaCl on stem number and dead stems of lucerne and total branches in Melilotus at harvest fourth.

mM of NaCl	Lucerne		Melilotus
	Total stems	Dead/total	Total branches
0	12.9	0.44	13.2
70	8.4	0.48	8.0
110	7.0	0.53	6.7
L.s.d. (P=5%)	1	0.05	0.6

The dry matter content was affected by salinity (P<0.001), but the pattern differed between experiments (Figure 3.7). In general for experiment 1 the dry matter content increased with salinity, the exception was with lucerne at harvest four where all treatments had similar DM content. In experiment 2 differences occurred between species (P<0.001). All the salt-stressed treatments for lucerne had a bigger decrease in DM content (more succulence) than the controls and for Melilotus this happened only at harvest four.

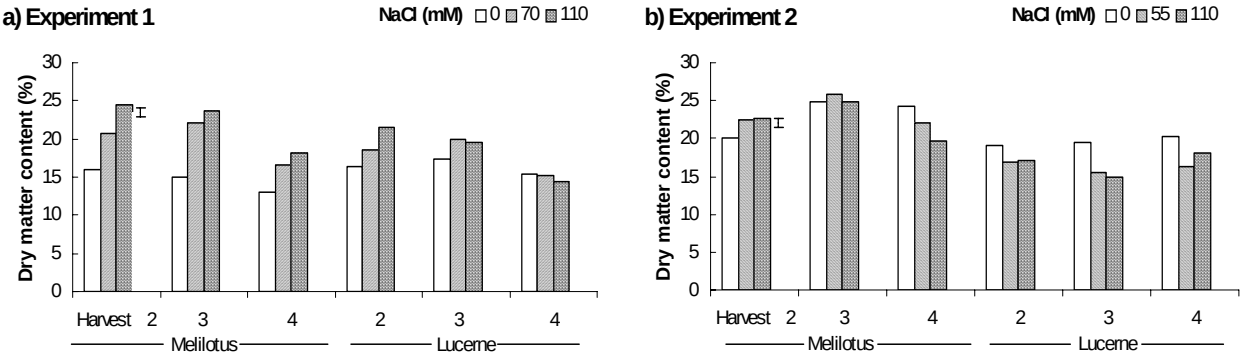


Figure 3.7. Dry matter content (%) of lucerne and Melilotus at different harvests as affected by NaCl in two experiments with different harvest interval. The bars represent the LSD at 5% level.

Salinity advanced flowering time in Melilotus (P<0.001). Although there were differences among harvests, the control always had a lower proportion of flowering plants than those in the salt-stressed treatments (Table 3.4). The differences were more noticeable at harvest two, which had the longest pre-harvest interval, where most of the plants grown under salinity had more developed their floral structures.

Table 3.4. Effect of NaCl on the phenology of Melilotus treatments (% of plants in specific stages) at different harvests in Melilotus.

mM of NaCl	Harvest								
	Second			Third			Fourth		
	Phenological stage			Phenological stage			Phenological stage		
	Pre-bud	Bud	Flower	Pre-bud	Bud	Flower	Pre-bud	Bud	Flower
0	87.8	7.8	4.3	95.2	3.9	0.9	91.8	5.1	3.2
70	0.0	83.2	16.8	80.8	10.0	9.2	74.3	12.2	13.4
110	0.0	85.2	14.8	76.1	10.7	13.2	62.7	7.0	30.2
L.s.d. (P=5%)	11.9	10.0	11.7						

3.4 Discussion

3.4.1 Biomass production

Salinity reduced shoot biomass in both plant species in both of the experiments. This is consistent with findings with lucerne from other authors (Smith and McComb, 1981; Gale and Zeroni, 1985; Ashraf *et al.*, 1986; Kapultnik *et al.*, 1989; Mc Kimmie and Dobrenz, 1991; Zhou *et al.*, 1992; Al-Kathib *et al.*, 1993; Khan *et al.*, 1994a; Khan *et al.*, 1997; Serraj and Drevon, 1998; Khavari-Nejad and Chaparzadeh, 1998; Djilianov *et al.*, 2003 and Rogers *et al.*, 2003). However, in the present investigation, the magnitude in the decrease of shoot biomass production was much lower than the reported in most of the cited references, but closer to the prediction equation of Maas and Hoffman (1977), which was derived from soil, sand and gravel cultures and included data from more than four harvests, where, in some of them, the first harvest was discarded.

There are several factors that can modify the response of a plant species under salinity (e.g., nutrient solutions, substrates, length of salinization period), but it is likely that the number of harvests could increase parameter differences among investigations. The first harvest in the present research was not included for any of the measurements, whereas from the 13 references cited, nine of them considered only the data of the first harvest, three included the first and second harvest, and only one included values for the third. After the first harvest, remnant foliage in the salt-treatments would be loaded with salts, and the regrowth would depend on reserves accumulated and the photosynthetic capability of the salt-affected leaves. Consequently, regrowth of salt-affected plants will be compromised relative to control plants, leading to reduced yields with successive harvests. In contrast, control plants can gain vigor as harvest number increases (e.g., lucerne in experiment 2), thus exacerbating differences between control

and salt treatments. At the salinity levels examined, lucerne decreased its dry matter production by 70%.

Unlike with lucerne, there are few published studies that deal with responses of *Melilotus albus* to salinity. The general trend of a reduced shoot biomass as salinity increases, as found in the current study, has also been reported by Repp *et al.* (1959) and Rogers and Evans (1996), although the magnitude of responses differed. With the "El Domador" cultivar used by Rogers and Evans (1996), control plants produced similar dry matter (g/plant) as measured in the present research but, for their salt treatments, they obtained plants that produced 30% more dry matter than obtained here. These differences can be attributed to several factors probably linked to the nutrient solution and to the harvest time which implies differences in exposure to salinity. Rogers and Evans (1996) used half-strength Hoagland's solution and intervals of 21 days to harvest, whereas in the present research a ¼ Hoagland's solution with Ca and Mg at full strength was used and the harvest interval was longer.

In Melilotus, the effect of phenology produced marked differences in shoot dry matter between the control and salt-affected plants, and this accounted for differences between experiments. This species under salt stress prompted flowering and had a strong trend to reduce plant growth with each successive harvest (noted in the control). In field conditions Melilotus has showed poor tolerance to frequent defoliation (Evans *et al*., 2004). Thus, these conditions make difficult to do precise comparisons in relation to its tolerance. Repp *et al.* (1959), in their review, stated that Melilotus (cultivar "Spanish") had better salt resistance at the beginning of the vegetative period than lucerne, but after a period lucerne became more vigorous than Melilotus. In contrast Evans and Kearney (2003) found Melilotus to be more productive than lucerne under saline conditions.

Based on the reduction in shoot dry matter production in the current research, Melilotus was as sensitive as lucerne to increasing salt and produced similar amount of herbage. This was unexpected and suggests further research is needed to clarify the actual salt tolerance of this reputedly salt tolerant legume. In addition, this research highlights the need to have protocols for plant salt screening to facilitate comparisons among studies.

Components of forage yield

In both plant species the partitioning of dry matter was strongly affected by salinity. Increases in the leaf-to-stem ratio for lucerne have been reported to vary from 0.4 to 1 unit in the range of 0 to 200 mM NaCl (Hoffman *et al*., 1975; Al-Khatib *et al*., 1993; Khavari-Nejad and Chaparzadeh, 1998). In the present research, lucerne had increases in the leaf-to-stem ratio from 1 to 3.3 units

and Melilotus from 0.4 to 7.4 units. This ratio can have a strong influence on the determination of elements (e.g., calcium, sulphur, nitrogen, micro-minerals) and other characteristics (NDF, CP, lignin) at the whole plant level, because both organs generally have different accumulation, or concentrations, of these components. Because of the sensitivity of leaf-to-stem ratio to salinity, when comparing the effects of salinity on nutritive value, changes in characteristics for both leaf and stem should be reported separately.

Other characteristics of stems, such as diameter, shoot height and number of stems in lucerne and number of branches in Melilotus decreased in response to salinity, particularly evident in experiment 1. These characteristics relate indirectly to nutritive value in different ways. Thinner stems are likely to require less shear force to break (i.e., chew) the material. Usually, the height of the plant has been considered to influence animal intake, especially if very short plants dominate the canopy, through effects on the amount of forage obtained per bite.

In lucerne, according to Noble *et al.* (1984), shoot length is a characteristic that has genetic variation under saline conditions and this characteristic, in conjunction with leaf damage, has helped to identify plants with enhanced salt tolerance. Ashraf *et al.* (1987) and Al-Khatib *et al.* (1993) found that when lucerne was exposed to NaCl concentrations up to 300mM of NaCl during two weeks, shoot length decreased, but the plant lines selected for improved salt tolerance were taller than the unselected lines. Al-Khatib *et al.* (1993) showed that in tall lines there was more shoot dry matter produced and the stems contributed a higher proportion to the dry weight than the unselected lines. Therefore it is possible to use plant height as a selection criterion for salt tolerance in lucerne, but it is likely that nutritive value may be affected as stem is generally less digestible than leaves.

The results at harvest 4 in experiment 1 showed that production of stems or development of new buds was restricted by salinity. This is consistent with Kapulnik *et al.*, (1989) who found that the number of stems per plant in some lucerne populations tended to decrease in seedlings grown for 79 days at 200 mM of NaCl. Additionally, in the present study, there was an increase in the death of the harvested stems as time passed, which was evidenced by higher dead-to-total stems ratio. This characteristic for lucerne seems to have importance because it may be a way to extend plant survival. The dead stems were those that were harvested, which suggest that these functioned as retention organs to help the new stems to have lower loads of salts. Thus the characteristic of multiple stems can be helpful when selecting more tolerant genotypes.

Dry matter accumulation by stems seems to be more affected by salinity than leaves, but it remains to be investigated if this is a plant response to decrease energy requirements for transportation of products (i.e., translocation and retranslocation), stronger capture of metabolites from leaves and roots than stems or it is due to higher stem cell sensitivity to the salt effects than cell of root and leaves.

Succulence

The content of dry matter appeared to be related to the length of exposure to salinity for both plant species. In the first experiment, although both species increased DM content in response to salinity, the differences between treatments as the harvests progressed, tended to be smaller. When the length of salinity exposure was increased (experiment 2), it was noted that the trend to increase DM reversed. This shift does not seem to be related to the phenology of the species, as lucerne in both situations was at late vegetative stage.

It has been suggested that increases in dry matter are the result of osmotic adjustment (Shannon *et al.*, 1994), but a different strategy may be used by some plants in some circumstances in order to avoid salt stress, thereby resulting in succulence. Succulence is meant to help through diluting entering salt, which results in less Na and Cl per unit of plant water (Levitt, 1972). In order to do that, plants increase cell size, having more space for storing the excess of the entering salt (Flowers and Yeo, 1989). Both lucerne and Melilotus seem to have both mechanisms available, but the result for Melilotus, indicates that succulence is retarded in comparison to lucerne.

Increases in DM content in lucerne have been found by Al-kathib *et al.* (1993) only at 200 mM NaCl by about 5% compared to the control, whereas the treatments of 0, 150 and 175 mM NaCl did not cause changes during the 6 weeks of salinization. Smith and McComb (1981), in an experiment with lucerne varieties completed by three weeks of salt exposure, observed a decrease in DM content only in the cultivar ‘Hunter River’ at 62.5 mM of NaCl, but increases at salinities of 125 and 250 mM. Khavari-Nejad and Chaparzadeh (1998) did not detect any change in a range of salinities from 0 to 90 mM of NaCl, when lucerne was exposed to salinity by 6.5 weeks. On the contrary, Melilotus has been reported to increase succulence in a lesser proportion than lucerne.

The importance of succulence in the nutritive value of a forage for a ruminant is linked to voluntary dry matter intake and the amount of Na and Cl that could be consumed. If the plant material ingested has high water content, voluntaty intake will be reduced and the energy

requirements of the animal may not be met. However, intake of Na and Cl (on a DM basis) is likely to remain high and that could be detrimental to animal production. Thus, increased DM, provided it is mainly organic matter, is a useful characteristic of a forage as it can increase forage intake dry matter per bite and per unit time.

In summary, salinity reduced shoot dry matter and plants were shorter and had fewer stems. Succulence was variable but, in Melilotus, it was more favorable in terms of dry matter than in lucerne. Plants were leafier and with thinner stem thereby implying high nutritive value. Although both species seem to have similar salt tolerance, and have similar yield (on an actual weight basis) under salinity, other differences may be decisive. In this regard, salinity reduced the time to flowering in Melilotus, but not in lucerne, and this phenological response may have implications for the nutritive value of Melilotus.

An unexpected result was the lack of difference in salt tolerance of these two species. Previous research led to an expectation that Melilotus would be more tolerant to salinity than lucerne, but this was not supported by results obtained under these growing conditions.

CHAPTER IV
EFFECT OF SALINITY ON THE MINERAL COMPOSITION OF LUCERNE AND MELILOTUS

4.1 Introduction

Under saline growth conditions, several processes related to ion movement in the plant are activated including competition, substitution, exclusion, accumulation and compartmentation, which have repercussions on plant mineral composition. Thus, in the salinity-plant interaction, an excess of some minerals can occur, whilst others can fall into marginal or deficient ranges, which produce disturbances in plant metabolism. It is important to know which elements in the plant are affected by salinity, including changes in relative proportions (i.e., mineral imbalances) to explain effects on biomass production and nutritive value.

For the animals consuming salt-affected herbage, those disturbances in plant mineral composition can cause direct effects on rumen microflora and the animal, which can modify animal production. This can occur through an insufficient supply of an essential element to meet animal requirements or through disturbances in the proportion, or ratios, of essential elements which can determine their availability in animal metabolism. This highlights the importance of considering the close interrelationships of chemical elements for plant and animal production. In this regard, information is scarce for most forage legume species. This chapter contributes information on the mineral profile of lucerne and Melilotus grown under saline conditions.

4.2 Methodology

For each experiment described in Chapter III, the freeze-dried plant material used was pooled among harvests of lucerne and Melilotus, but keeping leaves and stems separate. They were then ground with a Cyclotec mill (1093 Sample mill FOSS TECATOR) to pass a 1 mm screen. They were then analyzed for Na, K, Ca, Mg, P, S, Zn, B, Mn, Fe, Cl, N and C. For mineral content, samples were analyzed by Inductively Coupled Plasma Atomic Emission Spectrometry (Waite Campus, The University of Adelaide). Chloride was determined colourimetrically using the LabConco Digital Chloridometer and nitrogen and carbon were analyzed with a LECO CN-2000 analyzer (AOAC International, 1997).

Data was analyzed by ANOVA as a randomized complete-block design with four replications using GENSTAT 6. The residuals were tested for normality and homogeneity and, where necessary, data was log-transformed. For some element relationships the values were expressed on a milliequivalent basis and the molecular weights were as follow: Na^{+} =22.9898, K^{+}=39.0983, Cl^{-}=35.4527, S^{2-}=32.066, Ca^{2+}=40.078, P^{3-}=30.9738 and Mg^{2+}=24.305.

4.3 Results

Sodium and chloride

In both experiments, the sodium concentration in the shoot dry matter of both plant species increased ($P<0.001$) as irrigation water salinity increased, but plant species responded to differing extents ($P<0.001$). Lucerne leaf and stem Na concentrations at 110 mM NaCl, were 2 and 3-fold higher than Melilotus (on average, 29 g/kg DM vs 13 g/kg DM in experiment 1, and 51 g/kg DM vs 17 g/kg DM in experiment 2, respectively). Between organs in both species, stems had higher Na concentrations than leaves ($P<0.01$).

Chloride concentration in shoots followed a similar trend to sodium, increasing ($P<0.001$) as irrigation water salinity increased (Figure 4.2). Both plant species and their respective organs had different magnitudes of response ($P<0.001$). The average concentration of chloride for lucerne leaf and stem reached up to 55 g/kg DM in experiment 1 and up to 85 g/kg DM in experiment 2. Melilotus accumulated chloride differentially ($P=0.05$) between organs. In experiment 1, the chloride concentration in Melilotus leaf was 22 g/kg DM and in stem was 29 g/kg. In experiment 2, the chloride concentration for Melilotus leaf was 27 g/kg DM and stem 41 g/kg DM.

Based on the values for the Na concentration and considering the leaf-to-stem ratio, the calculated NaCl concentration per gram of whole shoot DM at 110 mM was, for lucerne in experiment 1, 67 g of NaCl/kg DM and, for experiment 2, 125 g NaCl/kg DM. For Melilotus the values were 32 and 39 g of NaCl/kg DM for experiment 1 and 2, respectively.

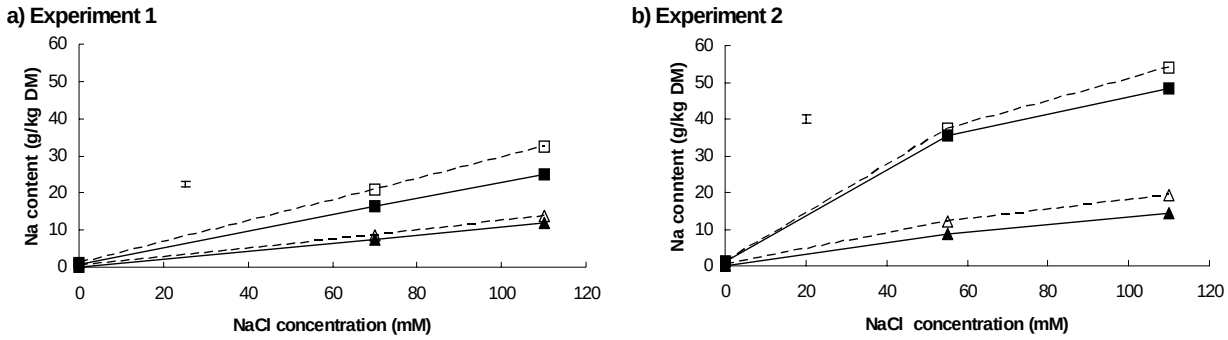


Figure 4.1. Sodium concentration in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) as affected by NaCl salinity. The bars are the LSD (P=5%).

Macro-elements

The potassium concentration was affected by salinity (P<0.001), but the effect differed between organs (Figure 4.3). Leaves in both species were barely affected, but for stems there were decreases, mainly in lucerne (P=0.05), where the potassium stem concentration in experiment 2 had a pronounced decrease in potassium as salinity increased.

The potassium concentration contrasted between species (P<0.001), with lucerne having higher values than Melilotus, especially in leaves where the concentrations in lucerne were 30-50% higher.

The concentration of phosphorus in the shoot of both plant species increased (P<0.001) as salinity increased, an effect that was very distinctive in stems (Figure 4.3). Overall, among salinity treatments, Melilotus maintained higher phosphorus concentrations in leaves than stems, but lucerne stems surpassed leaf concentrations (P=0.05).

The calcium concentration in both plant species decreased (P<0.001) as irrigation water salt concentration increased, but organs reacted differently (P<0.001). Leaves were more sensitive to salinity and calcium concentration declined by about two-thirds in both plant species in both experiments. In the control conditions, leaves had double the concentration of calcium than

stems, but this difference was essentially lost at high salinity levels, such that both organs had a similar concentration at the highest salinity level. Differences between experiments were observed mainly for calcium leaf concentration, which in experiment 1, both species had different values.

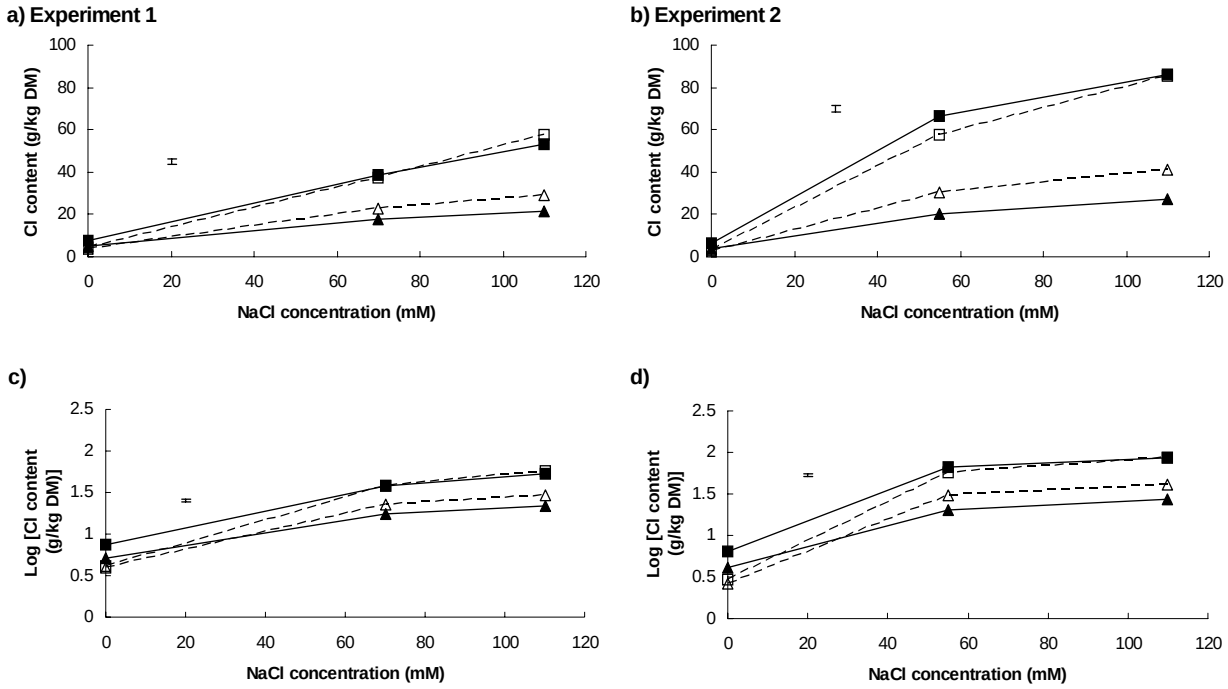


Figure 4.2. Chloride concentration in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) as affected by NaCl salinity. Graphs a and c correspond to the results (actual and log-transformed, respectively) obtained in the first experiment and graphs b and d correspond to the results obtained in the second experiment. The bars are the LSD (P=5%).

The magnesium concentration also declined in response to salinity ($P<0.001$) in both plant species. There were differences between species ($P<0.001$) where both leaf and stem of lucerne had bigger decreases than Melilotus organs. At the highest salinity level, both leaves and stems had similar concentrations of this element. Between experiments in experiment 2, Melilotus stem magnesium concetration remained unchanged, a difference that was not detected in experiment 1.

Sulphur concentration was affected by salinity ($P<0.001$), although the species responded differently ($P<0.001$). In both experiments the sulphur concentration of lucerne was stable but, in Melilotus, there were differences in how organs in responded to salinity, the most marked response being in leaves. Overall, Melilotus leaves decreased in sulphur concentration by 30% ($P=0.05$). Melilotus stems in contrast, had a less defined trend and its response to salinity was variable. Observed differences in sulphur concentrations between organs ($P<0.001$) were evident in both experiments, with leaves having double the concentration of stems.

Micro-elements

There were differences in effects of salinity on micro-mineral concentrations (Figure 4.4). Zinc concentration decreased ($P<0.001$) as salinity increased. Even though there were differences between experiments in zinc concentration at 0 mM NaCl, at the highest salinity level the concentration of this element was about the same, between 35 and 88 mg/kg DM.

The iron concentration decreased as irrigation water salinity increased ($P<0.001$), but the extent in the effect varied between experiments, especially in experiment 2 where there was a stronger effect of salinity than in experiment 1. The difference in iron between organs was large ($P<0.001$), with leaves having twice the overall concentration vs stems (83 vs 41 mg/kg DM).

The manganese concentration in response to salinity varied between experiments. The only consistent response to salinity was in lucerne stem, where, in both experiments at the highest salinity level, the manganese concentration increased with salinity by 2-fold ($P=0.05$). There were the differences between species ($P<0.001$), lucerne having higher concentrations than Melilotus (44 vs 26 mg/kg DM), and between organs ($P<0.001$) where leaves had higher values than stems (52 vs 17 mg/kg DM).

For boron there was an interaction ($P<0.001$) between organs and salinity in both experiments. In both species, leaf had a decrease in concentration and stem an increase. The distinction between organs was maintained and, overall, leaves had about twice the concentration of stems (55 vs 22 mg/kg DM).

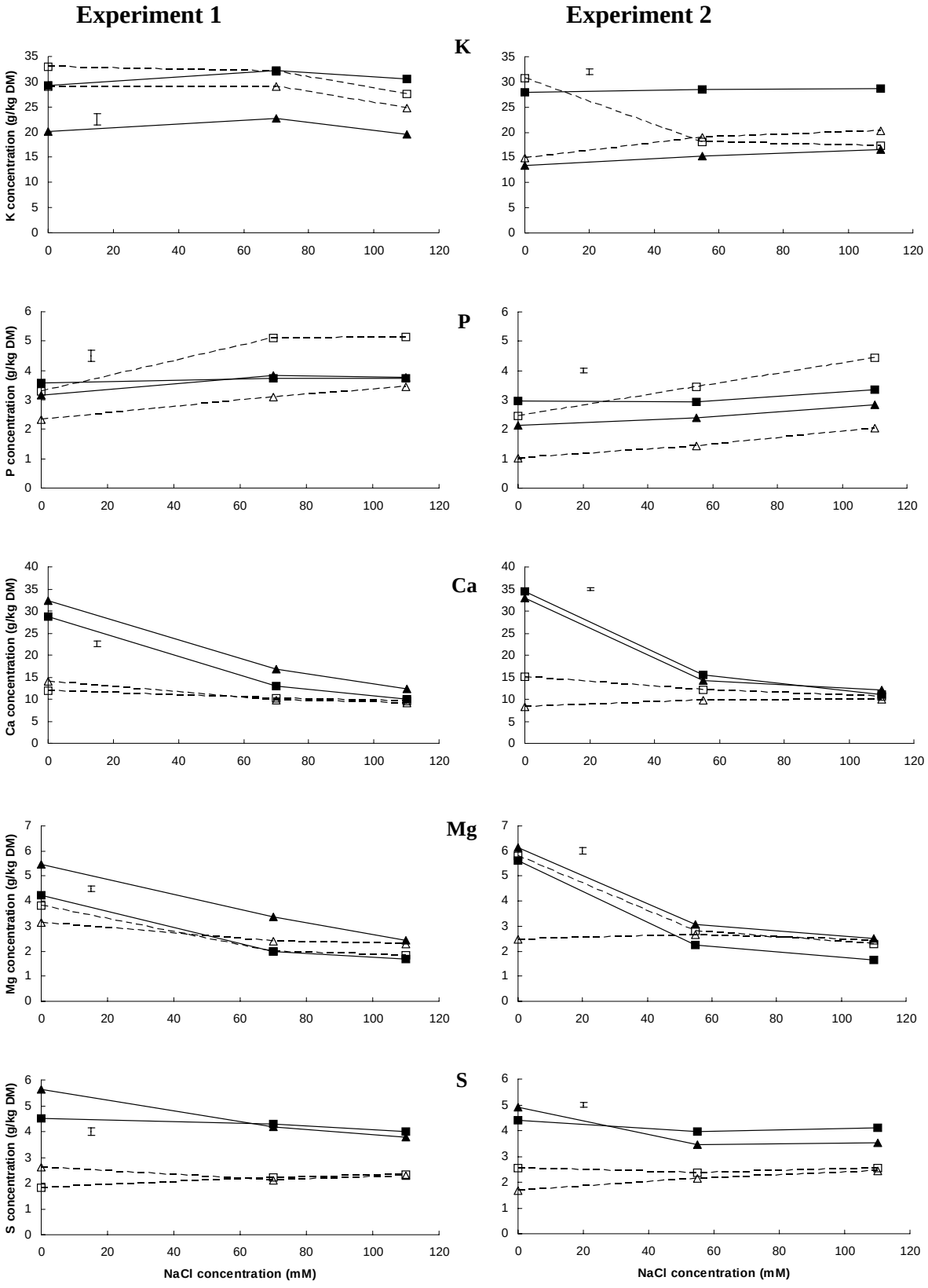


Figure 4.3. Concentration of macro-minerals in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) as affected by NaCl. The bars are the LSD (P=5%).

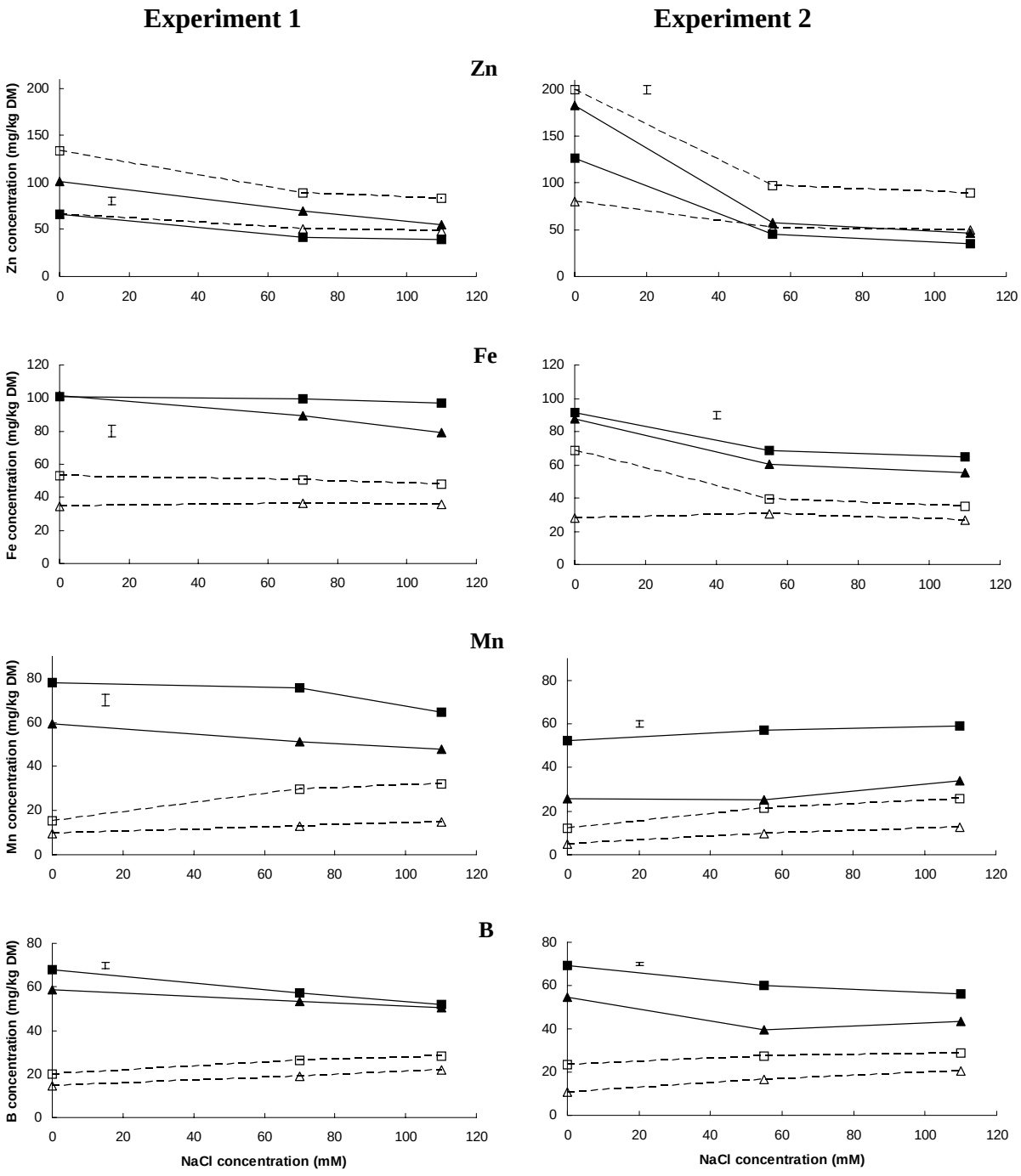


Figure 4.4. Concentration of micro-minerals in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) as affected by NaCl. The bars are the LSD (P=5%).

Nitrogen and Carbon

Nitrogen concentration decreased with increased salinity in the irrigation water (P<0.001), but the reduction was small. In the first experiment, leaf nitrogen concentration of both species, at 110 mM of NaCl, decreased from 46 to 41 g/kg DM, whereas in stems there was no change (Figure 4.5). In the second experiment, only lucerne had a small decrease (P=0.05) from 48 to 46 g/kg DM in leaves and from 32 to 28 g/kg DM in stems. Melilotus leaves did not have any change, but stems had an increased concentration of nitrogen (60%; from 14 to 22 g/kg DM). The main differences in nitrogen concentration were due to leaf and stem (P<0.001) and between species (P<0.001). Leaves contained about twice the nitrogen concentration of stems and lucerne had a slightly higher concentration (P=0.05) than Melilotus. Differences between species were more distinctive in the second experiment.

Carbon concentration decreased (P<0.01) as salinity increased, but the response of species and organs differed (P<0.001). In both experiments (Figure 4.5), Melilotus leaves were stable in carbon concentration, lucerne leaves had a decrease similar to Melilotus stem (around 10%), but lucerne stem was the most affected (decreased by 15%).

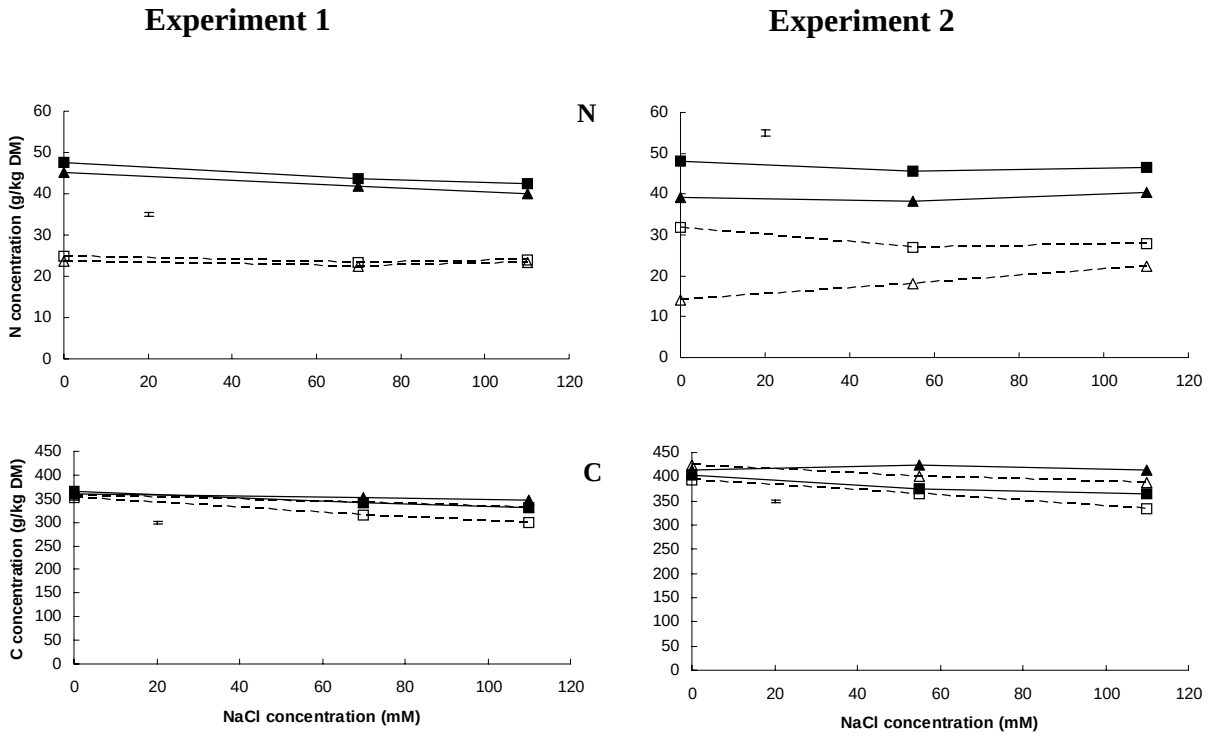


Figure 4.5. Concentration of nitrogen and carbon in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) as affected by NaCl. The bars are the LSD (P=5%).

Element relationships

The Na-to-Cl ratio on a milliequivalent basis increased ($P<0.001$) as the NaCl in the irrigation solution increased (Figure 4.6). With the exception of lucerne stems in experiment 2, changes in the values of this ratio were dominated by change in chloride. In Melilotus organs and lucerne leaf, values of this ratio, at the highest salinity level, ranged from 0.72 to 0.87.

The Na-to-K ratio on a milliequivalent basis increased ($P<0.001$) similarly for both species as irrigation water salinity increased (Figure 4.6). Melilotus consistently had lower values than lucerne and the ratio of the elements was close to one-to-one in both experiments. Between experiments, lucerne had greater increases in experiment 2 when the harvest interval was increased.

The ratio of the monovalent ions (Na+K)/Cl (milliequivalent basis) decreased as salinity increased ($P<0.001$). Sodium and potassium were slightly dominant over chloride, a result that indicates chloride entered in high amounts (Figure 4.7). No differences occurred between species at the highest irrigation water salinity level and, in both experiments, the response was similar. The same ions expressed as a balance (Na+K-Cl) resulted in a slight variation in species. Melilotus leaf had stable values, whereas lucerne organs and Melilotus stems decreased with increasing salinity (Table 4.1).

The ratio (Na+K)/(Cl+S) on a milliequivalent basis was affected by salinity ($P<0.001$). Overall, leaves were less affected than stems, and between species, Melilotus leaves were essentially unaffected. Differences that existed between organs and species at 0 mM of NaCl did not occur at the highest salinity level. In both experiments a strong trend towards a 1-to-1 ratio as salinity increased was evident. These elements expressed as a balance [(Na+K)-(Cl+S)], showed that Melilotus leaves moved from values close to neutrality to more positive values (Table 4.1), whereas lucerne organs and Melilotus stems had the opposite response.

Salinity decreased ($P<0.001$) the ratio of the major cations over anions [(Ca+Mg+K+Na)/(P+S+Cl)], a response of a well defined trend for both organs in both species (Figure 4.7). A major contribution from anions reduced the ratio as NaCl in the irrigation solution increased, moving this ratio from values of 3.2 and 3.9 in the controls in the first and second experiment, respectively, to 1.3 at the highest salinity level. Expressing these elements as

the balance [(Ca+Mg+K+Na)-(P+S+Cl)], there was a consistent decrease with salinity ($P<0.001$). Overall, leaf balances in both species decreased in higher proportion than stems and, at the highest salinity level, differences that were observed between organs and species did not occur (Table 4.1). It is noteworthy that in most of the previous ratios described, there was trend towards the neutrality (or 1-to-1) as salinity increased.

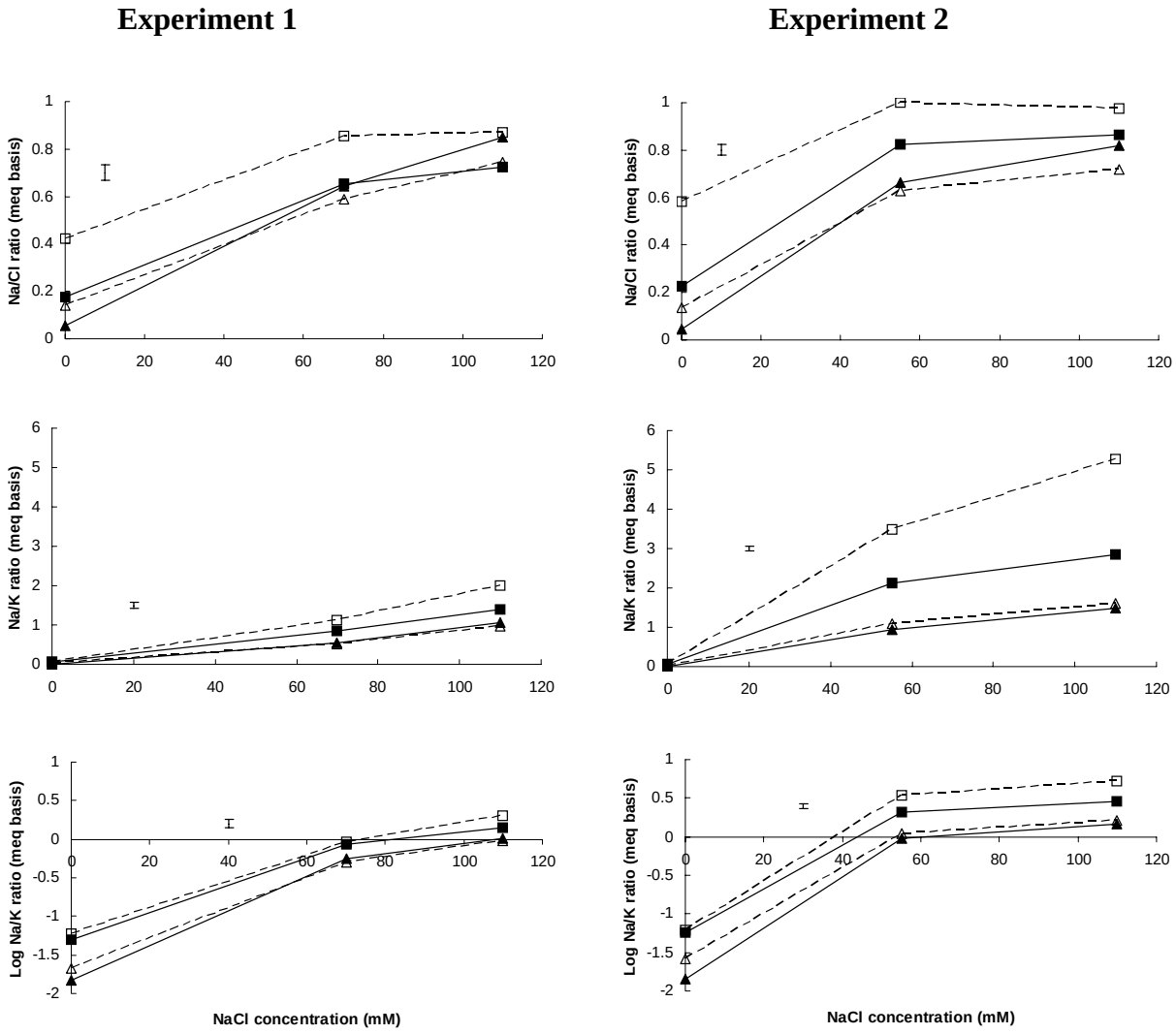


Figure 4.6. Sodium-to-chloride and sodium-to-potassium ratio (actual and log-transformed data) in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) as affected by sodium chloride. The bars represent the LSD at 5% level.

The K/(Ca+Mg) ratio increased ($P<0.001$) as salinity increased, and this effect was more pronounced in leaves than stems ($P<0.01$). Lucerne leaves had higher values ($P=0.05$) than Melilotus (Figure 4.8) but maximum values were only about 1.2. The increase for leaves was due mainly to the pronounced drop of Ca and Mg, elements that were more stable in stems. Between experiments, the organ that had the greatest variation was stem which, in the experiment 2, had lower values in this ratio than in experiment 1.

The Ca-to-P ratio decreased ($P<0.001$) as irrigation water salinity increased (Figure 4.8). The ratio in leaves decreased to a higher extent than stems, mainly due to the stronger effect of increased salinity on leaf calcium concentration. The decrease in this ratio in both lucerne and Melilotus leaves in both experiments was about 70% and, for stems on average, was half ($P=0.05$). These differences between organs and between species observed in the controls did not occur as salinity increased.

The N/S ratio changed due to the interaction between species and salinity ($P<0.001$). The differences observed in the control treatment between organs did not occur when the species were exposed to high salinity, reaching a steady value of about 10 (Figure 4.8). Lucerne leaves remained stable, while Melilotus leaves had an increased ratio ($P=0.05$). For lucerne, stems consistently decreased ($P<0.05$), whilst Melilotus stems were more variable, increasing in the first experiment ($P=0.05$) and remaining stable in the second.

The C/N varied very little as salinity in the irrigation water increased. The only the significant change was observed in stems of Melilotus during the second experiment when plants of this species were harvested at same phenological stage (early flowering). Organs were very distinctive in this ratio, where stems had higher values than leaves.

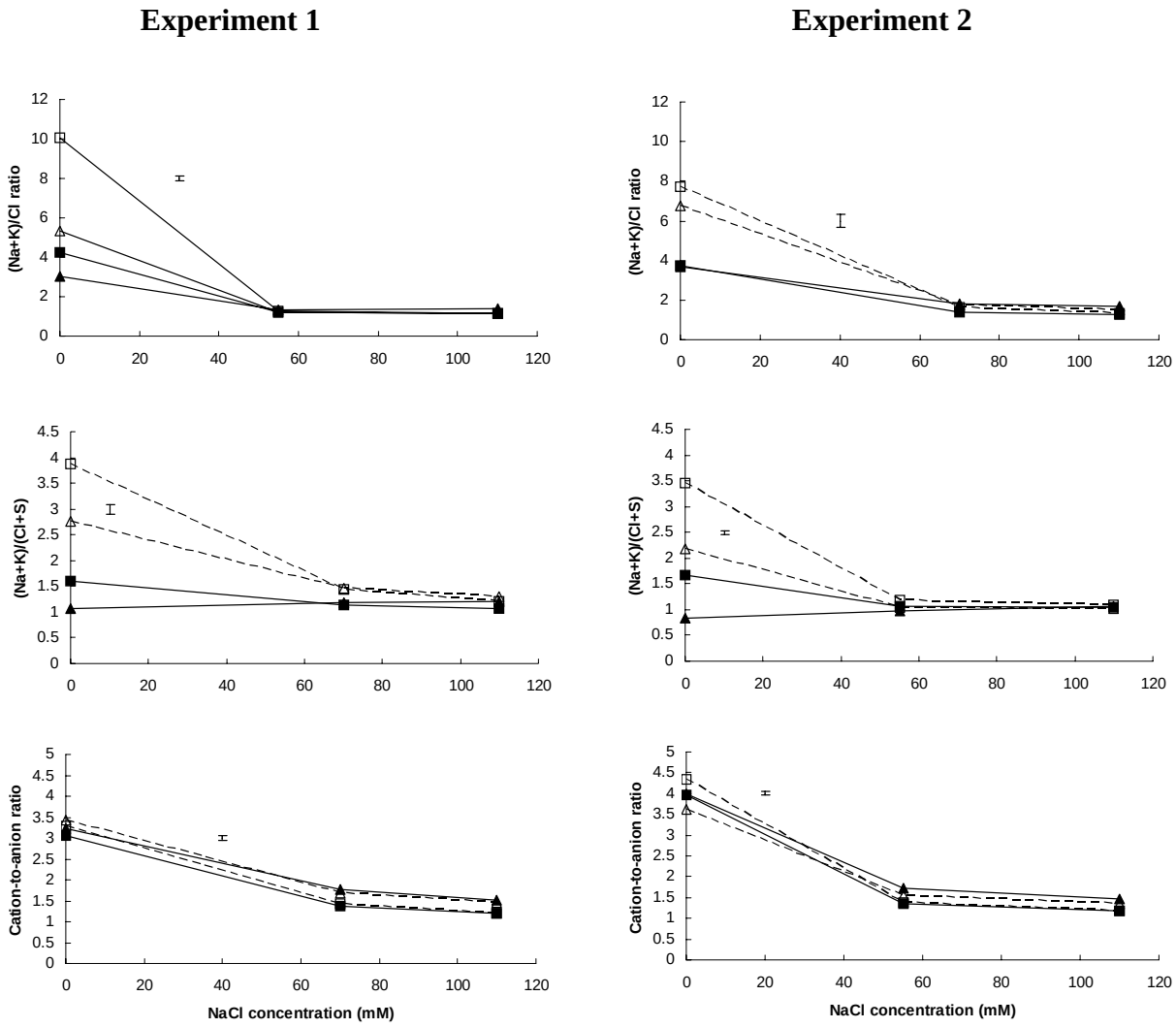


Figure 4.7. Sodium plus potassium-to-chloride, sodium plus potassium-to-chloride plus sulphur and cations-to-anions ratios on a milliequivalent basis in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) as affected by sodium chloride. The bars represent the LSD at 5% level.

Table 4.1. Cation-anion balances (in milliequivalents) in lucerne and Melilotus as affected by NaCl in the irrigation water.

Organ and NaCl (mM)	Experiment 1					
	Na+K-Cl		(Na+K)-(Cl+S)		(Ca+Na+Mg+K)-(Cl+P+S)	
	Lucerne	Melilotus	Lucerne	Melilotus	Lucerne	Melilotus
Leaf						
0	573	380	293	27	1728	1783
70	446	401	179	141	631	880
110	372	407	123	171	396	619
Stem						
0	776	647	662	482	1251	1221
70	666	481	528	349	713	732
110	495	424	350	282	483	598
L.s.d. (P=0.05)	53		55		82	
	Experiment 2					
Leaf						
0	578	234	304	-72	2194	1875
55	395	202	147	-13	824	723
110	400	287	143	66	505	603
Stem						
0	752	317	597	212	1590	726
55	467	171	320	37	821	602
110	381	192	222	39	510	541
L.s.d. (P=0.05)	95		93		100	

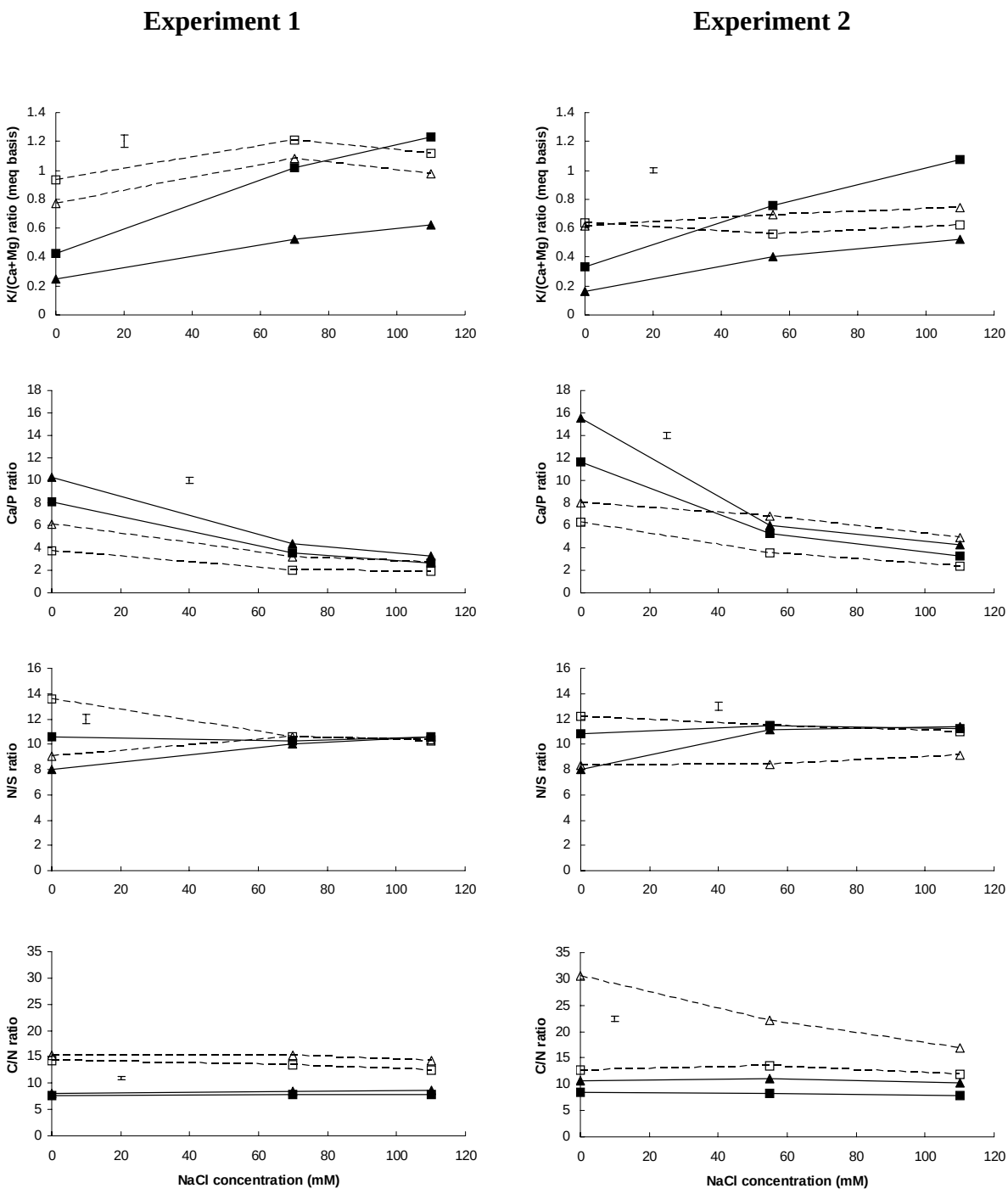


Figure 4.8. Potassium-to-calcium plus magnesium on a milliequivalent ratio and calcium-to-phosphorus, nitrogen-to-sulphur and carbon-to-nitrogen ratios in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) as affected by sodium chloride. The bars represent the LSD at 5% level.

4.4 Discussion

Sodium and Chloride

Uptake of sodium by roots of plants is passive at moderate to high soil salinity levels, whereas uptake of chloride may be either active or passive (McKersie and Leshem, 1994; Tyerman and Skerret, 1999; Kabata-Pendias and Pendias, 2001; White and Broadley, 2001; Mengel *et al*., 2001). Therefore, the concentration of these elements in plant tissues increases as the external salt concentration increases, but the degree of accumulation depends on the species as the large differences between lucerne and Melilotus show.

The pattern of the differences in sodium and chloride concentrations between species was similar and remained so among the salinity treatments independent of the length, or other conditions, of the two experiments.

On a milliequivalent basis, Na was more restricted than Cl which was present in high amounts in both organs. However on a dry matter basis, for lucerne, Na accumulation was higher in stems than in leaves. This situation did not occur with Cl, which accumulated in equal amounts in both organs. These results agree with some of the findings of Kapulnik *et al.*, (1989) and Esechie and Rodriguez (1998). Nonetheless, there is evidence that leaf can accumulate equal or higher amounts of Na and Cl per unit DM than stem (Ashraf *et al.*, 1986; Kapulnik *et al.*, 1989; McKimmie and Dobrenz, 1991; Mezni *et al.*, 2002). In Melilotus DM the response to salinity was to accumulate Na in similar amounts for leaf and stems, but Cl accumulated more in stem than leaf.

Results in the present research suggest that differences in element partitioning occurred, and it is probable that both species restricted accumulation of these elements in leaves (Na in lucerne and Cl in Melilotus) by using stems as retention organs. In addition, results from other investigations indicate that, at least for lucerne, important differences in partitioning of Na and Cl exist among cultivars. Although specific information on genetic variation for Na and Cl accumulation in lucerne is lacking, evidence from some investigations (e.g., Noble *et al*., 1984) suggests that Na and Cl accumulation in the plant may be under genetic control (see Figure 2.6).

Between species, Melilotus had less Na and Cl than lucerne at the highest irrigation water salinity level, yet the reductions in dry matter production in both species were similar. This suggests that it is not only the concentration of these elements that produce negative effects on growth, but that other factors are also involved.

The higher value in accumulation of Na and Cl in the plants in experiment 2 may be related to the increased time to harvest. This is consistent with the explanation of Munns and Termaat (1986), who suggested that the longer the time a leaf transpires, the higher the accumulation of these elements. This also agrees with results of Ashraf and O'Leary (1994) who concluded that in lucerne the accumulation of Na and Cl increases with age. Thus, when working with pasture species it will be necessary to account for the grazing intervals to assure lesser amounts of Na and Cl in plant tissues. The fact that lucerne accumulated more Na and Cl may be linked to the different strategies that these plants use to ameliorate the effect of both ions. It is likely that succulence has a strong influence over the final concentration of these ions, probably due to a higher storage capacity of cells.

Lower concentrations of Na and Cl on a DM basis in Melilotus vs lucerne, is a desirable characteristic for animal production because a concentration of NaCl higher than 10% has been reported to limit animal performance. This characteristic does not preclude lucerne having potential for moderately saline lands, but strengthens the need to select populations with low Na and Cl concentration as the amounts detected may restrict animal production. For Melilotus, the ability to flower earlier means less time at the vegetative stage and that the nutritive value may decline faster as the reproductive stage progresses. Nonetheless, flowering could account for re-establishment, as this species is capable of seed set in saline conditions (Evans and Kearney, 2003).

Macro-elements

According to Mengel *et al*. (2001), antagonistic effects can occur among cation species when the supply of one of them increases in the nutrient medium, resulting in a decreased concentration of other cation species in the plant. This situation has been observed in most of the research reviewed and variable responses have been found, as was the case in the present research.

Potassium

It was expected that potassium concentration would be affected by salinity, although it only occurred in lucerne stems. Melilotus, even though it had lower K concentration than lucerne, was not affected. Thus, the result for Melilotus suggests that uptake and/or translocation of K was effective, and that the relationship of this element with other elements, rather than its

concentration *per se*, may be more important in the decrease of biomass of this species under the salinity conditions examined.

Under saline conditions lucerne has been found to decrease substantially its K concentration in both stems and leaves (Ashraf *et al.*, 1986; Kapulnik *et al.*, 1989; Esechie and Rodriguez, 1998 and Mezni *et al.*, 2002), an effect where sodium seems to have produced impairment in potassium uptake as its decreased concentration in roots suggested by the first three investigations implied. Contrasting results for potassium were reported by Ashraf and O'Leary (1994), where lucerne grown at 100 mM NaCl using half-strength Hoagland's solution, did not have decreases in leaves or stems and, on the contrary, one of their selected lines had an increase in K concentration in stems. The conditions under which each experiment was conducted make it difficult to establish clear comparisons but, according to Kafkafi (1984), steady levels and decreases in K may occur depending on the level of exchangeable sodium present in the soil solution. He reported that in whole shoots of legumes such as beans, potassium can be maintained at steady concentrations even though the concentration in roots may decline. However, at levels of exchangeable sodium higher than 45%, the potassium concentration in shoots can be severely reduced, which may have been the situation with Ashraf *et al.* (1986) and Kapulnik *et al.* (1989) who worked with higher concentrations of NaCl (175 and 200 mM of NaCl, respectively) than in the present research. Esechie and Rodriguez (1989) and Mezni *et al.* (2002) worked with lower concentrations than the previous investigators, but still found decreases in potassium in leaves, stems and roots. However as they worked with soil as a substrate, this could indicate that interactions between the substrate and potassium uptake may be present.

Species differences in the concentration of potassium were evident where lucerne leaves contained much more potassium than Melilotus leaves. An adequate concentration of potassium in lucerne at the whole plant level in the vegetative and first flowering stages has been reported to range between 25-38 g/kg DM and values lower than 18 g/kg DM for the whole shoots and 12.3 g/kg DM for stems are considered deficient (Pinkerton *et al.*, 1997). In the present research, when the time to harvesting was increased, lucerne stems reached values that were in the marginal range. Thus, as stem is an important contributor of K to the whole shoot and its DM production was reduced, lucerne potassium concentration at the whole shoot level decreased compared to the control.

In animals, total dietary requirements of potassium vary depending mainly on the animal species and physiological age. For cattle, requirements range from 4.7–8.4 g/kg DM and for sheep from 3.0-3.6 g/kg DM (Grace, 1983; Underwood and Suttle, 1999). In terms of concentration, these levels of potassium can easily be supplied from both plant species and, according to the Figures 2.8 and 4.3, potassium deficiency in animals would be unlikely to occur. Decreases in potassium have been seen as a positive characteristic for non-lactating dairy cows near parturition and pregnant ewes in reducing the incidence of hypocalcaemia, milk-fever and hypomagnesaemic tetany. However this would depend on the concentration and availability of the other elements involved, such as calcium and magnesium.

Calcium

According to Rengel (1992), effects of salinity on calcium concentration in glycophytic plant species are related to displacement of this element from cell membranes and to disturbances in its flux in the cell (i.e., reduced influx and enhanced efflux), especially impacted by sodium. In addition, there is evidence that uptake of calcium from the soil solution is mainly by root tips (Mengel *et al*., 2001) and salinity has a considerable effect on this organ and its structure (Lakshmi-Kumari *et al.*, 1974; Banet *et al.*, 1996). Thus, this factor, and cation antagonism (especially with Na), may be the main reasons for calcium depletion in the plant.

In the present research, calcium concentration was more affected in leaves than in stems, results that agree with Esechie and Rodriguez (1998) for lucerne. However, there are cases where salinity has had stronger reductions on calcium concentration than those found in the present research in lucerne leaves and stems (Ashraf *et al.* 1986; Ashraf and O'Leary, 1994) or, in contrast, where there has not been any variation (Kapulnik *et al.*, 1989; Mezni *et al.*, 2002). Such result variations may be attributed to differences in nutrient solutions and, possibly, to differences in cultivars.

Under non-saline conditions, differences in calcium concentration between leaf and stems are normal. According to Mengel *et al*. (2001), organs that are supplied with nutrients by the phloem sap, such as stems, show relatively low concentrations of calcium and high potassium, which agrees with results from 0 mM NaCl treatment. Nonetheless, when salinity was imposed, results show that leaves and stems ended with similar levels of calcium in both species, which suggests that uptake and translocation were affected.

For the whole shoot of lucerne deficient levels of calcium at the vegetative stage and first flowering have been reported to be lower than 2.5 and 10 g/kg DM, respectively (Pinkerton *et al.*, 1997). These low levels were not reached in this research, although there was a reduction, but it is likely that lower values would occur at high salinity (>15 dS/m) as the values in Figure 2.9 suggest.

Depending on the physiological stage of the animal, its dry matter intake and diet digestibility, calcium requirements for sheep range between 1.4 to 7.0 g/kg DM and for cattle from 2.1 to 10.8 g/kg DM (Underwood and Suttle, 1999). For animals with high requirements, such as those growing and lactating, it is likely that the required supply of this element may be compromised by salinity, especially at high levels (for the present research at 110 mM of NaCl). True digestibility of this element in forages grown under saline conditions is unknown, but it could be an issue due to reports that NaCl may cause decreases of plama calcium in the ruminant (Moseley, 1980). In lucerne, part of the calcium (20 to 33%) is in the form of oxalate which is largely unavailable to ruminant metabolism (Ward *et al.*, 1979), but no information is available for either lucerne or Melilotus grown under saline conditions.

Magnesium

Concentrations of magnesium declined with salinity and lucerne was more sensitive than Melilotus. For lucerne, results agree with Ashraf *et al.* (1986); Ashraf and O'Leary (1994) and Esechie and Rodriguez (1998) who found similar reduction patterns in both leaf and stem biomass.

Plant deficiencies in magnesium can be attributed to impaired uptake due to an excess of other cation species in the soil solution (e.g. Na) and to restricted translocation from roots to the shoots imposed by potassium and calcium antagonisms (Mengel *et al*., 2001). Magnesium deficiency in whole shoots of lucerne at early flowering occur at levels less than 2 g/kg DM, whereas the adequate concentrations range is between 2.5 to 5.0 g/kg DM (Pinkerton *et al.*, 1997). In the current research, the salt treatments for lucerne caused values which fell into the marginal and deficient range, especially when time to harvesting was increased, indicating that this mineral was likely a limiting factor to growthr. For Melilotus there is no available information, but its response to salinity was quite similar to lucerne.

Magnesium requirements for grazing sheep and cattle range from 0.7 to 1.4 and from 1.3 to 2.2 g/kg DM, respectively (Underwood and Suttle, 1999). At least for sheep, the requirements would

be met by these plant species, although it is noteworthy that these values have been determined for non-salt stressed animals. Magnesium is mostly absorbed from the rumen with an absorptive efficiency of 35% (Underwood and Suttle, 1999), and it is known that the ionic strength increases in the rumen due to increases in Na and Cl and that the outflow rate also increases, conditions that might decrease magnesium absorption. In this regard, Poe *et al.* (1985) and Grace (1988) have reported that magnesium absorption from the rumen can be adversely affected by high NaCl concentrations and this can be accentuated if potassium is high (i.e., above 3%). Another aspect is that magnesium can be associated with indiffusible anions such as oxalate and pectate (Mengel *et al*., 2001) compounds that reduce its availability to animals. Little is known about the extent of the association of this element in forage species under saline conditions.

Phosphorus

Phosphorus is readily mobile in the plant, its uptake is active and it is metabolically involved in energy transfer for synthesis of several substances including cellulose, phospholipids and nucleic acids (Mengel *et al*., 2001). Under saline growth conditions, some plant species increase their requirements for phosphorus (Grattan and Grieve, 1992), especially because energy is required to transport the excess of ions into the vacuoles (Mengel *et al*., 2001). Findings of the present research are consistent with this theory, as phosphorus increased as salinity increased and this was more noticeable in stems; organs that had the higher Na and Cl concentrations. Nonetheless, there are results, at least for lucerne, showing that phosphorus concentration in shoots can be variable. Esechie and Rodriguez (1998) found that in two cultivars of lucerne grown in sandy soil at different temperatures and exposed to salinity for 21 days, salinity tended to decrease phosphorus concentration and temperatures at 35-46 ºC promoted higher phosphorus concentrations vs temperatures at 20-25 ºC. Under hydroponic conditions, the phosphorus concentration of lucerne shoot has been found unaffected by salinity (Rogers *et al.*, 2003), even when there were contrasting phosphorus concentrations. These results suggest that growing conditions and cultivars influence phosphorus concentration of the plant biomass.

Phosphorus requirements for sheep have been reported to range from 1.0 to 3.9 g/kg DM and for cattle from 0.9 to 4.8 g/kg DM (Underwood and Suttle, 1999). On the basis of results of this research, these requirements can be met by both plant species.

Sulphur

Overall, sulphur concentration of leaves was approximately twice that of stems and only Melilotus leaves had a significant decrease with increased salinity. Levels of sulphur in lucerne were practically did not change. For the requirements of sheep and cattle according to Underwood and Suttle (1999), the minimum sulphur concentration can range between 1.1 to 1.6 g/kg DM, concentrations that are easily met by both plant species. This element is very important in nutrition of sheep and cattle because the margin to reach toxic concentrations (3-4 g/kg DM in the forms of elemental sulphur or as sulphate) is narrow (Kandylis, 1984). Rogers *et al*. (1998) and Grieve *et al.* (2004) have found that if the availability of sulphur increases in saline water (e.g., such as those dominated by sodium sulphate), lucerne sulphur can range from 4 to 9 g/kg DM. Grieve *et al.* (2004) concluded that sulphur may be a limiting factor for using forages as a source of ruminant feed, as some species reach toxic levels, as was the case of *Paspalum vaginatum* cv Polo that reached concentrations around 14 g/kg DM. In salinity dominated by NaCl some plant species (e.g., saltbush species) can have near toxic levels (Norman *et al*., 2002), but this was not the case of lucerne and Melilotus in the current research. In Melilotus, the decrease in sulphur concentration in leaves could be a topic for further studies, especially if the salinity is dominated by sulphates.

Nitrogen

In experiment 2, under longer harvest intervals, lucerne had higher concentrations of N than Melilotus, but this may be because lucerne was still in the vegetative stage, whereas Melilotus was flowering. Melilotus stems tended to increase in N concentration, at least in experiment 2, possibly due to the slow development of stems under saline growth conditions, as will be shown further.

Nitrogen concentrations in the whole plant are modified by differences in the leaf-to-stem ratio, due to the contrasting concentration of this element between stems and leaves. In lucerne, the slight nitrogen decrease that occurred with salinity in leaves (experiment 1) and in stems (experiment 2), were counteracted by differences in leaf-to-stem compared with the controls. Because of this when the nitrogen concentration of salt-stressed leaf and stem were pooled, no differences occurred. However, in Melilotus the nitrogen concentration increased as the differences in leaf-to-stem were higher than in lucerne, especially in the second experiment. Overall, under the conditions tested, although nitrogen concentration was affected, the decreases were small and it may be considered that values are not substantially compromised by salinity.

Data from the literature (Table 2.1) indicates that substantial decreases in N are more likely when plants rely on symbiosis but, in the current research, N was supplied in the nutrient solution and so this aspect was not tested.

Although not measured, the proportion of true-protein nitrogen-to-non-protein nitrogen, which may influence the efficiency of ruminal utilization of this element, may be altered by salinity. In response to salinity, proteolysis may occur, new proteins and peptides may be synthesized, some enzymes (e.g., nitrate reductase) may be disturbed (see section 2.7.2) and, according to Mengel *et al*. (2001) magnesium deficiency tends to increase non-protein nitrogen in relation to protein nitrogen. It would not be surprising that concentrations of the forms of nitrogen may change in lucerne and Melilotus, but information under saline conditions is lacking in relation to both, the extent in the changes in the proportions of nitrogen and the extent of the resultant impact in animal performance.

Micro-minerals

Comparing the concentrations of the microelements measured in this research with those reported by Pinkerton *et al.* (1997), in lucerne none of them were in a marginal or deficient concentration for the plant. For Melilotus, no data was available to compare and all that can be said is that Melilotus micro-mineral trends were quite similar to lucerne.

All these microelements are involved in a diverse range of physiological functions in the plant and some are important to defining the nutritive value of the forage. Zinc deficiency, for instance, disturbs ribosomes and the activity of the enzymes RNA polymerase and RNase, impairs protein synthesis and amino acids and amides accumulate (Mengel *et al*., 2001). Zinc has been observed to influence starch formation the in legumes like *Phaseolus vulgaris*, where a deficiency decreased the size and number of starch grains (Jyung *et al.*, 1975). As boron and manganese are involved in cell membrane stability and participate in phenol and lignin synthesis, their deficiency in the plant can lead to an increase in phenolic compounds and to a decrease in lignin concentration (Graham and Webb, 1991; Shuman, 2000). Although a decrease in lignin of a forage is of benefit to ruminants eating it, because there is a likely increase in the potential digestibility of the forage, this would be offset by a limited source of energy (e.g., starch). Nonetheless, according to estimations at the requirement for microelements for sheep and cattle reported by Underwood and Suttle (1999), their concentrations were well above minimum animal requirements in both plant species.

Element relationships

The chloride-to-sodium ratio

In the plant, the presence of sodium was highly correlated to the presence of chloride (0.98) in both experiments, result that supports the view that these ions counteract each other (Tyerman and Skerret, 1999). Expressed in a ratio (on a milliequivalent basis), Cl accumulated to a higher extent than Na, suggesting that there was discrimination in translocation against Na, and/or Cl uptake was less regulated. Studies at a cellular level with cultured lucerne cells indicated that Cl was more abundant than Na (Croughan *et al.,* 1978), which is consistent with results of the present research. Symptoms of Cl injury in plants appear earlier than those of Na (McKersie and Leshem, 1994) and, in lucerne, leaf damage correlates with toxic effects of Cl (Noble *et al.*, 1984). How the plant controls the reactivity of these ions is still obscure, but chloride has a wide ability to combine with other elements to produce toxic compounds such as NaCl, KCl, HCl and HOCl, which could contribute to toxicities that the plant faces.

In the current research, the association of Na and Cl with reductions in DM varied between experiments. In experiment 1, the Na correlation factor was -0.75 and for Cl -0.73. In experiment 2, Na had -0.58 and Cl -0.60. However, the decreases in DM correlated in higher extent with the Na/Cl ratio (-0.87) in both experiments. This suggests that negative effects on dry matter production were accentuated by the interaction between Na and Cl.

A 1:1 proportion in the Na/Cl ratio has been the basis for most of the studies on the effect of salt-rich diets in ruminants. In the plant species tested, the total amount of both ions was not in a 1:1 proportion, but the potential amount of salt contained in plant tissues could induce similar physiological effects, even though Cl was slightly in excess.

Na-to-K ratio

This ratio is one of the most important in glycophytic plant species, mainly because many of the physiological processes occur at high K and low Na concentrations. When Na is introduced to the cell system at stressful levels, imbalances in the ratio are produced and the plant suffers several disturbances. Similarities in the physicochemical characteristics between these two elements are suggested to be the cause of poor discrimination in the K-transport sites that increases the competition of Na and may lead to a K deficiency (Atmann and Sanders, 1999; Maathuis and Atmann, 1999; Blumwald, 2000). Once Na is taken up by the plant, at the cytoplasmic level, Na competition continues for K-binding sites, which disturbs plant metabolic processes in which K is essential (Maathuis and Atmann, 1999).

In plant species examined here at the salt concentration tested, this ratio decreased drastically, reaching values that were close to a one-to-one, or even lower in favor of Na. This response was driven mainly from the increase of Na concentration, because potassium was relatively constant.

In the animal, K and Na are very important elements for glucose and amino acid uptake, a highly energy demanding physiological process mediated by the Na^+-K^+ ATP-dependant pump (Block, 1994; Underwood and Suttle, 1999; Blumwald, 2000), but little is known if these processes could be directly affected by a high Na/K ratio in the diet. Nutritively, this ratio has been associated with the carbohydrate concentration as in some leguminous species, such as *Phaseolus vulgaris*, high Na/K ratios in the presence of Cl have influenced the carbohydrate concentration in leaves, where one characteristic has been starch and soluble sugars accumulation (Rathert *et al.*, 1981). For ruminants a source of dietary energy is critical, and of particular benefit if that energy can be used by microorganisms in the ruminal capture of N, which is usually high in legume species. In the current research, the increase in DM per gram of fresh weight could be an indication of carbohydrate accumulation, which appeared to be more favorable in Melilotus, but this issue will be discussed in Chapter V and VI.

Relationships among dietary cations and anions

An excess of dietary cations or anions influence the acid-base homeostasis of animal fluids that can induce acidosis or alkalosis, conditions that can produce negative effects in the animal (Underwood and Suttle, 1999). When animals are fed diets with nutritivelly innappropiate concentrations of Na, K and Cl, factors related with their absorption from the gastrointestinal tract influence the animal acid-base balance, mainly because Na and K are exchanged by a proton and Cl is exchanged for a bicarbonate ion (Tucker *et al.*, 1988). With diets high in NaCl, several changes in blood composition have been reported to happen such as increases in chloride (Meyer and Weir, 1954; Hemsley *et al.*, 1975) and glucose concentrations (Garg and Nangia, 1993) and decreases in urea (Godwin and Williams, 1986; Garg and Nangia, 1993) and serum calcium and magnesium (Moseley and Jones, 1974; Moseley 1980). It is not clear to what extent such blood alterations can influence blood pH through blood bicarbonate, CO_2 or their partial pressures, components that several authors (Patience, 1990; Block, 1994; Leblanc, 2004) consider very important in the buffer system of blood acid-base balance. Acid-base status influences metabolism of amino acids, vitamins and minerals and so, it has a relationship to the incidence of metabolic disorders (such as hypocalcaemia), appetite and, consequently, growth

(Patience, 1990). Although all animals are prone to negative effects due to a disturbed acid-base balance, dairy cows and reproductive ewes have received the most research attention relative to disrupted calcium homeostasis.

Commonly, K, Na and Cl are considered in the cation-anion balance of a diet, but the contribution of the cations Ca and Mg and anions S and P can also be included (Block, 1984; Patience, 1989; Block, 1994).

From results of mineral concentrations in the current research, saline conditions altered proportions of several of the main cations such as Ca and Mg, and Na became dominant. Nonetheless, Na increases were counteracted to some extent by increases in Cl, which was the main anion contributing to the anion pool (72% at the highest salinity level including P and S).

When Cl was expressed as a proportion of Na+K, the ratio showed that the 'alkalogenic' characteristic of the DM (i.e., dominance of cations over anions) was strongly affected. The ratio values were displaced towards neutrality (ratio 1:1), but Na and K still dominated the ratio by approximately 20%. When sulphur was added to the ratio, both organs of both plant species were moved closer to neutrality as irrigation water salinity increased. Including Ca, Mg and P did not change the trend of the cation-to-anion ratio, and it maintained the shift towards neutrality. The 'alkalogenic' character of the DM was kept, but anions (mainly Cl) and loss of the divalent cations (Ca and Mg) were determinants in displacing the ratio towards neutrality.

For ruminants generally, use of legumes during the prepartum period is restricted because their high cation ratios have been associated with the predisposition of dairy cows and reproducing ewes to hypocalcaemia (Block, 1994; Goff and Horst 1997; Espino *et al.*, 2003). Ratios found in these plant species under saline conditions indicate a positive change as a feed because the cationic strength was reduced. However, further studies are needed to determine if these ratios in the plant, or their interactions with Na and Cl concentrations, have importance or if it is just the Na and Cl concentrations that account for negative effects on animals fed high salt diets.

The K/(Ca+Mg) ratio

This element relationship is considered to influence magnesium absorption from the rumen and values above 2.2 have been reported to promote hypomagnesaemic tetany, a metabolic disorder that, according to Underwood and Suttle (1999), can reduce production and even be fatal. Because of the high calcium and magnesium concentrations in legumes, it is uncommon to observe this disorder. Nonetheless, it can appear, as it has been found in beef cattle fed lucerne hay (Grunes and Welch, 1989; Sleper *et al.*, 1989).

As calcium and magnesium decreased in the plant due to the effects of NaCl, the possibility of reaching ratio values close to 2.2 increased. Overall, Melilotus had lower values at this ratio than lucerne due to the lower concentration of potassium at the phenological stages that were harvested. Stems had smaller changes in this ratio than leaves because decreases in calcium and magnesium were also smaller. In addition, potassium, at least in lucerne stems, was more affected than leaves, but this organ contributed proportionally less to biomass production. Thus, leaves, at least in lucerne, were the organs that increased the K/(Ca+Mg) ratio to a greater extent, although the ratio values were still lower by one unit than the critical value of 2.2. This ratio seems to have a limit because, at higher salinity levels, K concentration tended to drop and both magnesium and calcium concentrations tended to reach a steady state that may hold this ratio in a relatively fixed range. From values in the present research, it seems that there is little risk that this ratio could be adverse to animals, but there is no information on whether the values of this ratio are still valid when animals are fed forages grown under saline conditions. There is evidence that Mg, and even Ca concentration, decreases in ruminants when they are fed diets high in NaCl (Tomas *et al.*, 1973; Moseley and Jones, 1974; Potter and McIntosh, 1974; Moseley, 1980; Godwin and Williams, 1986; Grace, 1988; Wachirapakorn *et al.*, 1996), therefore it is possible that the critical values for this ratio could be lower than 2.2.

The Ca-to-P ratio

According to McDowell (2003), the importance of this ratio to ruminants is because the deficiency or excess in one of them interfere the utilization of the other, which negatively affects feed efficiency and growth. Underwood and Suttle (1999) and McDowell (2003) maintained that, in general, ruminants with high vitamin D status, which promotes Ca and P absorption, can tolerate a wider range than the recommended 1-to1 or 2-to-1.

Salinity decreased this ratio to values between 4:1 to 5:1, a remarkable improvement from 10 and 12-to-1 for leaves grown at 0 mM NaCl. This effect was due to a decrease in the calcium concentration in leaves and an increase in phosphorus in stems. Although higher than the recommended ratio, it is likely that bioavailable Ca-to-P ratio is lower than 4:1, at least in lucerne, where about 20% of calcium is in the form of oxalate according to Ward *et al.* (1979).

The N-to-S ratio

It has been suggested that sulphur requirements need to be expressed in relation to those of nitrogen, in addition to sulphur requirements *per se*, a condition that is related to efficiency in

synthesis of rumen microbial protein where S-containing amino acids are an important component (Underwood and Suttle, 1999). An ideal rumen degradable N:S is suggested to be about 14.3-to-1 for ruminants diets (Underwood and Suttle, 1999).

This ratio varied in response to salinity. Under the control conditions, Melilotus had lower values than lucerne, independent of time to harvest. This was attributed mainly to marked species differences in sulphur concentrations; Melilotus had higher values than lucerne. However, when salinity was imposed, values for both plant species were similar, ranging from 10-11. In Melilotus leaves, sulphur decreased with salinity and this was the reason the N:S ratio increased in relation to the control, whereas lucerne appeared unresponsive. It seems that this ratio in plant biomass is not compromised by salinity dominated by NaCl.

The C-to-N ratio

This ratio is related to the quality of the forage, especially for digestibility where low ratios may promote microbial growth, provided that part of the carbon is readily metabolizable. This ratio varied greatly between organs and the main difference was the concentration of nitrogen. The vascular tissue in stems is essentially devoid of the cellular structures that are rich in nitrogen which cause such differences. It seems that this ratio is very tight in leaves and values fell in a narrow range. Contrasting both times of harvest in the experiments, Melilotus had marked differences influenced by time at harvesting. When harvests where at chronological intervals, stems of this species were less mature and, consequently, their C/N ratio was lower when compared to the experiment where harvest was at a similar phenological stage. Although small, nitrogen accumulation in stems appears to increase when this plant species is at full flower, which reduced the ratio C/N in the salt treatments. This response did not occur when harvests were at chronological intervals, possibly because not all plants had reached flowering. The nutritive quality of lucerne stems was much higher than Melilotus, mainly because this plant species was still at the vegetative stage. As no major variations occurred in the leaves of both plant species, it can be assumed that this ratio is not affected by salinity.

In summary, there was a large increase in Na and Cl in both plant species as salinity increased. There was a clear distinction between species where, lucerne accumulated higher amounts of both ions than Melilotus. Between experiments, there were major differences also; in experiment 2 the concentration of Na and Cl was doubled compared to experiment 1. This effect was attributed in part to the extended harvest interval. In the case of lucerne, concentrations of Na

and Cl in experiment 2 indicated that values that animal production could be detrimentally affected.

The elements that were reduced in concentration in the plant as a response to salinity were calcium, magnesium and zinc.

The mineral ratios in the plant of importance in animal nutrition were the N/K and K/(Ca+Mg) which were negatively affected and Ca-to-P which improved. The cation-anion balance in both plant species shifted to values that were less ‘alkalogenic’ to the animal that eats them.

CHAPTER V
EFFECT OF SALINITY ON DRY MATTER QUALITY DESCRIPTORS

5.1 Introduction

In the previous chapters the effects of salinity on plant biomass accumulation, mineral concentrations and mineral ratios were described. In addition to these effects, other physiological plant responses might induce changes in cell organic components which may influence the nutritive value of the herbage to ruminants. Digested nutrients and the efficiency by which they are absorbed and utilized within the tissues of the animal, determine nutritive value (Ulyatt, 1981), thereby making digestibility an important plant characteristic. Nonetheless, fibre characteristics are widely used to describe forage quality, and are a major determinant of digestibility, as digestibility is the result of the degradation of different components of cells and tissues in the plant. Hence, the quantification of chemical traits (e.g., structural carbohydrates, lignin and others) can provide insights into the underlying reasons for changes in digestibility an important part of nutritive value.

Salinity can affect traits such as structural and non-structural carbohydrates and protein (Munns and Termaat, 1986; Marschner, 1995). It has been suggested that the mineral components of the cell wall can coat the digestible organic matter and depress digestibility (McManus *et al*., 1977; McManus *et al*., 1979). In addition, it has been observed that salinization of rumen fluid with NaCl (>200 mM) reduces digestibility (Paggi *et al*., 2004). The current research found an excess of Na and/or Cl, and substitution of some minerals (i.e., Ca, Mg and Zn and in lesser extent K) in plant tissues. Mineral substitution, and high concentrations of Na and Cl in the cell, could exert an effect on enzymatic digestion of the organic matter. Information on the extent to which salinity may affect quality descriptors of the organic matter of Melilotus and lucerne is scarce, and this chapter intends to contribute to its quantification and understanding.

5.2 Methodology

The forage production conditions were described in Chapter III and the preparation of the freeze-dried material for analyses was described in Chapter IV.

All the measurements included in this section were cmpleted in duplicate and are described as:

1) Particle density of the material expressed in g/cm^3 was determined using pre-weighed vials of 1.5 mL, filled with ground dry material and pressed manually with a crystal rod until the vial was full. After that, vials were covered with their respective lids and weighed.

2) The neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined sequentially using the fibre analyzer ANKOM 200/220. Samples of material were placed in ANKOM Technology-F57 Filter Bags, following the ANKOM protocols (Ankom Technology, 2003), but omitting the alpha-amylase and acetone steps. After ADF extraction was done, acid detergent lignin (ADL) was determined using the ANKOM method. Hemicellulose was calculated as the difference between NDF-ADF and cellulose as ADF-ADL.

3) Water-soluble carbohydrates (WSC) were analyzed using a Technicon Autoanalizer 11 with 620 nm cell, following the procedure of Yemm and Willis (1954).

4) Organic matter (OM) and soluble and insoluble ash were determined according to Faichney and White (1983).

5) Gross energy is reported only for plant material from the second experiment as there was insufficient material generated from the first experiment. Gross energy was determined using a Parr 1281 Bomb Calorimeter following the protocol for use of the instrument (Parr Instrument Company, 1996).

6) *In vitro* digestibility was determined through a modified pepsin-cellulase procedure (Klein and Baker, 1993) using ANKOM Technology-F57 Filter Bags. It was expressed in different ways according to the equations described in the Feeding Standards for Australian livestock (Standing Committee on Agriculture, 1990) as:

- Dry matter digestibility = (DM in feed – Indigestible fraction)/DM in feed
- Organic matter digestibility = (OM in feed – Indigestible OM)/OM in feed
- Digestible organic matter in dry matter = (OM in feed – Indigestible OM)/DM in feed

Dry matter digestibility was also adjusted by soluble ash component (IVDSA) and calculated as:
IVDSA = [(DM in feed – soluble ash) – indigestible fraction]/DM in feed

Influence of soluble ash on digestibility

In order to determine if the soluble ash fraction affected DM digestibility, two digestibility experiments were completed. These experiments are termed "**a**" and "**b**" for distinguise them. In Experiment "**a"**, lucerne and Melilotus material collected from the 0 mM NaCl treatment in experiment 2 was artificially salinized. NaCl was added at the concentrations of 0, 10 and 15%, values close to the potential NaCl concentration obtained in the salt-stressed plant treatments. To achieve this, 0.6 gram of dry matter was placed in 35x35x8 mm plastic trays, and appropiate amounts of NaCl were added and mixed. After that, 4 mL of distilled water were poured onto each sample in order to allow penetration of Na and Cl into the tissue. Once moistened, samples were left in that condition for 15 hrs and then placed in an oven to dry at 55°C for 48 hrs. After this, all contents of each tray were placed in ANKOM Technology-F57 Filter bags and analyzed following the pepsin-cellulase procedure for digestibility (Klein and Baker, 1993).

In Experiment "**b"**, the dry matter obtained from each treatment (0, 55 and 110 mM NaCl) of experiment 2 was treated for soluble ash. After soluble ash was washed out, the material was dried at 55°C for 48 hrs. Then 0.6 grams of each sample were placed into the ANKOM Technology-F57 Filter bags and put through the pepsin-cellulase procedure for digestibility analysis.

For experiments 1 and 2, data was analyzed by ANOVA as a randomized complete-block design with four replications. In the digestibility experiments, the artificially salinized material (exp. 'a') was analyzed as a completely randomized design with four replicates; whereas for the digestibility experiment where soluble ash was washed out from the dry matter (exp. 'b'), data was analyzed as randomized complete-block design. Residuals were checked for normality and homogeneity and the least significant difference was used for means separation. All statistical analyses were performed using GENSTAT 6.

5.3 Results

The particle density of leaves and stems increased (P<0.001) as salinity increased (Table 5.1) for both plant species. Melilotus leaves grown under saline conditions had a higher density than lucerne leaves grown under the same concentration of salinity (0.80 vs 0.74 g/cm^3). As expected, leaves had a higher density than stems (P<0.001).

Increasing salinity resulted in noticeable changes (P<0.001) in plant organic matter concentrations in both experiments, although each species responded differently (P<0.001). Melilotus consistently had an increase (P=0.05) in leaf organic matter concentration at 110 mM of NaCl in both experiments (Figure 5.1). These increases, although small (3 percentage units in experiment 1 and 1.6 percentage units in experiment 2), contrasted with those of lucerne leaf, where its organic matter content decreased by 4 and 6 percentage units at the highest salinity level (P=0.05). Stem organic matter in both species consistently decreased (P=0.05) with increasing salinity in both experiments. Lucerne stem was more affected than Melilotus stem as salinity increased. The decrease in lucerne stem organic matter corresponded to 7.5 and 10 percentage units and in Melilotus stem to 2.5 and 6.7 percentage units in the experiments 1 and 2, respectively.

Table 5.1. Particle density (g/cm3) for leaves and stems of lucerne and Melilotus as affected by NaCl.

mM of NaCl and experiment	Lucerne		Melilotus	
	Leaf	Stem	Leaf	Stem
Experiment 1				
0	0.61	0.58	0.61	0.58
70	0.75	0.62	0.82	0.60
110	0.76	0.66	0.82	0.62
L.s.d. (P=5%)	0.02			
Experiment 2				
0	0.67	0.66	0.66	0.57
55	0.70	0.67	0.76	0.68
110	0.72	0.71	0.81	0.71
L.s.d. (P=5%)[1]	0.03			

[1] For the comparison between species, NaCl and organs.

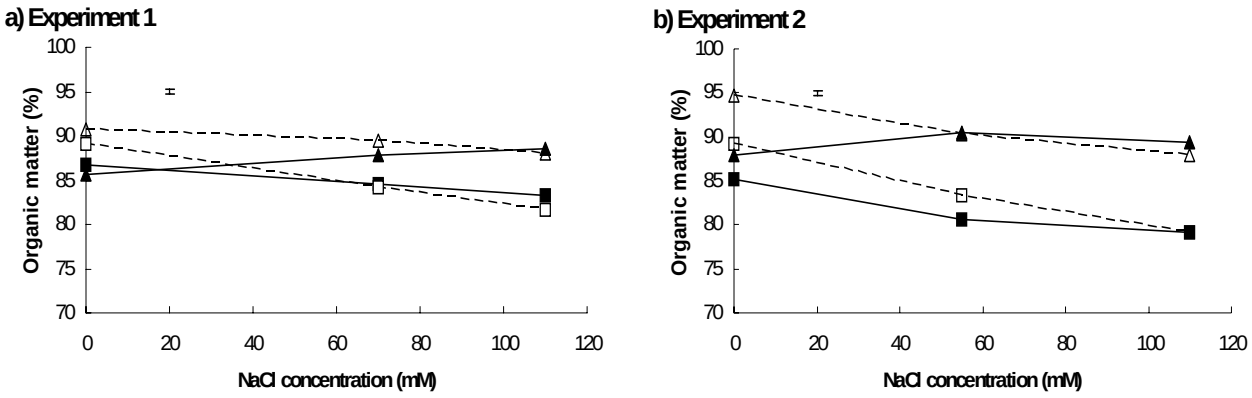


Figure 5.1. Organic matter concentration in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) at different concentrations of NaCl. The bars are the LSD (P=5%).

The range in insoluble ash concentration was narrow in both species (from 1.4 to 2.8 % of total dry weight). It decreased (P<0.001) in leaves of both species, and in stems, only lucerne had a consisten increase (Table 5.2).

Soluble ash was the inorganic fraction most affected by salinity (Table 5.2), but species and organs responded differently (P<0.001) to salinity. For lucerne leaf and stem, soluble ash increased (P<0.001) about 4 to 9 percentage units more than the controls as salinity increased, an effect that was more pronounced in experiment 2. Melilotus organs exhibited a different response, where leaf had a small decrease (P<0.05) and stems an increase in both experiments in response to salinity.

Determination of gross energy, for the second experiment only, confirmed values obtained for organic matter. Salinity affected the gross energy value (P<0.001) with the two species responding differently (Figure 5.2). Gross energy values of Melilotus leaves increased from 16.8 to 17.5 MJ/kg DM, whilst lucerne leaves decreased from 16.5 to 15.7 MJ/kg DM. The stems had lower gross energy values than leaves and decreased further as salinity increased. In relation to the controls, these decreases corresponded to 1.7 MJ/kg DM for lucerne stems and 1.2 MJ/kg DM for Melilotus stems.

Table 5.2. Soluble, insoluble and total ash percentages in lucerne and Melilotus as affected by NaCl.

Organ and NaCl (mM)	Lucerne			Melilotus		
	Soluble ash	Insoluble ash	Total ash	Soluble ash	Insoluble ash	Total ash
Experiment 1						
Leaf						
0	10.8	2.5	13.3	11.6	2.8	14.4
70	13.2	2.2	15.4	9.8	2.5	12.3
110	14.9	1.9	16.8	9.2	2.2	11.4
Stem						
0	9.0	2.0	11.0	7.2	2.1	9.3
70	13.7	2.2	15.9	8.9	1.8	10.7
110	16.3	2.2	18.5	10.3	1.6	11.9
L.s.d. (P=5%)	0.7	0.2	0.7			
Experiment 2						
Leaf						
0	12.7	2.4	15.1	10.0	2.2	12.2
55	17.3	2.2	19.5	7.8	1.8	9.6
110	18.7	2.3	21.0	8.7	1.8	10.5
Stem						
0	8.6	2.1	10.7	4.2	1.2	5.4
55	14.3	2.4	16.7	8.4	1.4	9.8
110	18.4	2.4	20.8	10.5	1.5	12.0
L.s.d. (P=5%)[1]	0.7	0.1	0.7			

[1] For the comparison between species, NaCl and organs.

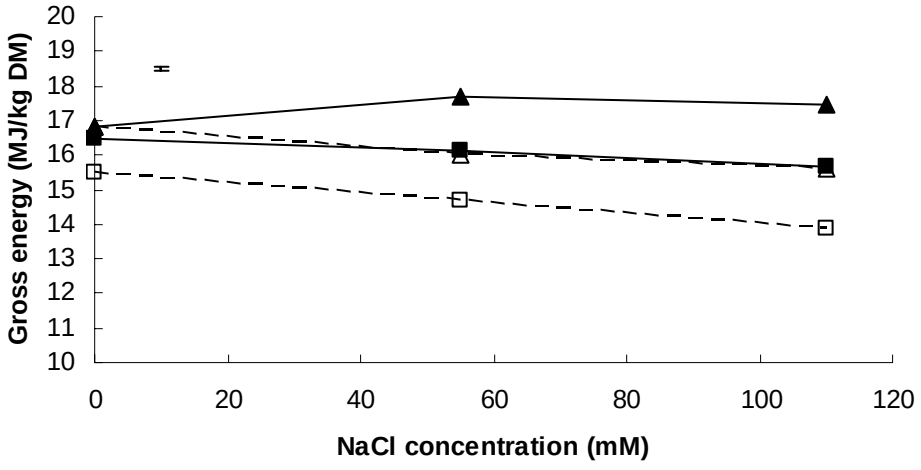


Figure 5.2. Gross energy concentration in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) as affected by NaCl salinity. The bar is the LSD (P=5%).

Salinity affected the concentration of water-soluble carbohydrates (P<0.001) in both plant species. Stems of both species decreased (P<0.05) at the highest salinity level (Figure 5.3). The water-soluble carbohydrate concentration of leaves showed little variation and, only in experiment 1, Melilotus leaf had a small increase from 8.3 to 9.4% (P=0.05). The major differences in water-soluble carbohydrate concentration occurred between species (P<0.001), where lucerne had lower concentrations than Melilotus. Between experiments, Melilotus had contrasting differences in water-soluble carbohydrate concentration, particularly in experiment 2, where the concentration was about 2-fold higher than in experiment 1.

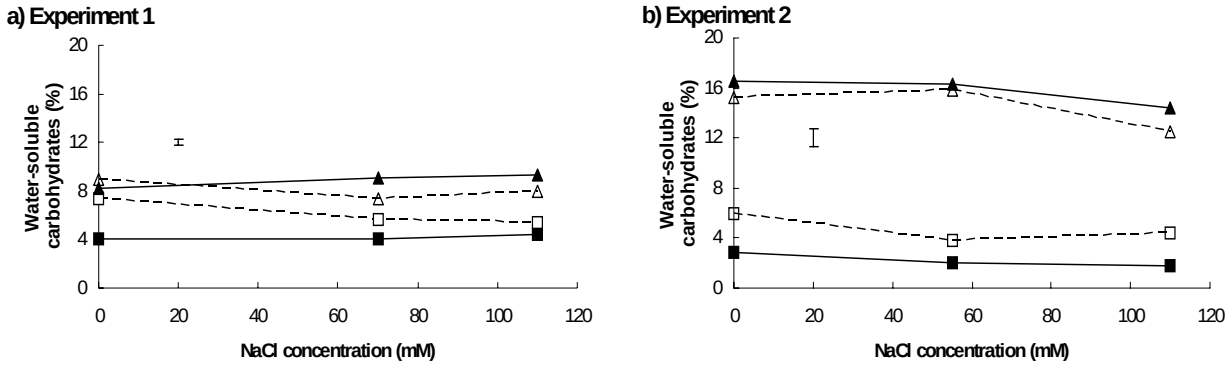


Figure 5.3. Water-soluble carbohydrate concentration in leaves (solid line) and stems (dotted line) of lucerne (squares) and Melilotus (triangles) at different concentrations of NaCl. The bars are the LSD (P=5%) for the comparison between species, NaCl and organs.

Neutral detergent fibre (NDF) decreased (P<0.001) as the concentration of NaCl increased (Table 5.3). Overall, and in both species, stems had a higher decrease than leaves.

The two species differed in their acid detergent fibre (ADF) concentrations (P<0.001). Melilotus had lower values for leaves and higher values in stems than lucerne. Nonetheless, salinity caused a decrease (P<0.001) in the concentration in both species and in both experiments, corresponding on average to a decrease of 3 percentage units in leaves and 13 percentage units in stems.

The sulfuric acid lignin concentration (ADL), either on a DM or OM basis, decreased (P<0.001) as salinity increased. In both species, this effect was observed mainly in stems, such that

differences between species that existed in the control groups did not occur at high salinity (Table 5.3). The species differed in leaf lignin concentration and overall, Melilotus leaf had lower concentrations than lucerne (about 2-fold), and its concentrations under saline growht remained stable. However, lucerne leaf had a sharp drop in lignin at the highest salinity level under the conditions of experiment 2.

Cellulose concentration was negatively affected by salinity ($P<0.001$), an effect that was stronger in stems than in leaves (Figure 5.4 and 5.5). Hemicellulose concentration was variable between experiments and only in the first experiment did it appear that there was a salinity effect, mainly a decrease in concentration in lucerne and in the leaves of Melilotus. Overall, hemicellulose was less affected by salinity than cellulose.

Table 5.3. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and sulfuric acid lignin (ADL) in lucerne and Melilotus as affected by NaCl. Values expressed in percentage on both dry matter and organic matter basis.

Organ and NaCl (mM)	Lucerne						Melilotus					
	NDF		ADF		ADL		NDF		ADF		ADL	
	DM	OM	DM	OM	DM	OM	DM	OM	DM	OM	DM	OM
Experiment 1												
Leaf												
0	27.5	31.1	17.2	18.8	5.1	6.8	27.0	31.3	12.1	13.9	2.5	3.5
70	23.4	27.0	14.7	17.1	5.2	6.6	22.8	25.9	10.6	11.5	2.3	3.0
110	21.9	25.7	13.7	16.5	4.8	5.7	17.0	19.0	9.8	10.4	2.3	2.6
Stem												
0	48.6	54.1	36.8	40.4	4.4	5.0	47.6	52.2	39.7	43.0	5.5	6.1
70	38.8	45.6	28.0	32.3	3.4	4.1	42.0	46.6	31.5	34.4	3.9	4.3
110	32.8	39.9	23.5	27.9	2.9	3.5	38.1	42.9	28.3	31.2	3.1	3.6
L.s.d.[1]	1.5	1.7	1.4	1.4	0.7	0.9						
Experiment 2												
Leaf												
0	21.1	24.7	16.7	19.4	5.4	6.4	16.8	18.5	9.1	10.0	1.6	1.8
55	19.5	24.1	16.6	20.4	6.1	7.5	17.3	18.6	7.4	8.0	1.5	1.7
110	15.3	18.7	9.2	11.4	1.7	2.1	16.1	17.4	7.2	7.8	1.6	1.7
Stem												
0	38.3	42.8	28.0	29.9	3.8	4.3	55.6	58.9	42.2	43.4	5.2	5.4
55	32.5	38.5	22.8	25.9	2.8	3.3	43.8	48.5	27.6	29.8	2.8	3.1
110	27.1	33.6	18.9	22.2	2.3	3.0	36.7	41.3	23.9	26.0	2.5	2.8
L.s.d.[1]	1.3	1.3	1.0	1.0	0.4	0.5						

[1] Least significant difference (P=5%) for the comparison of species, NaCl level and organ.

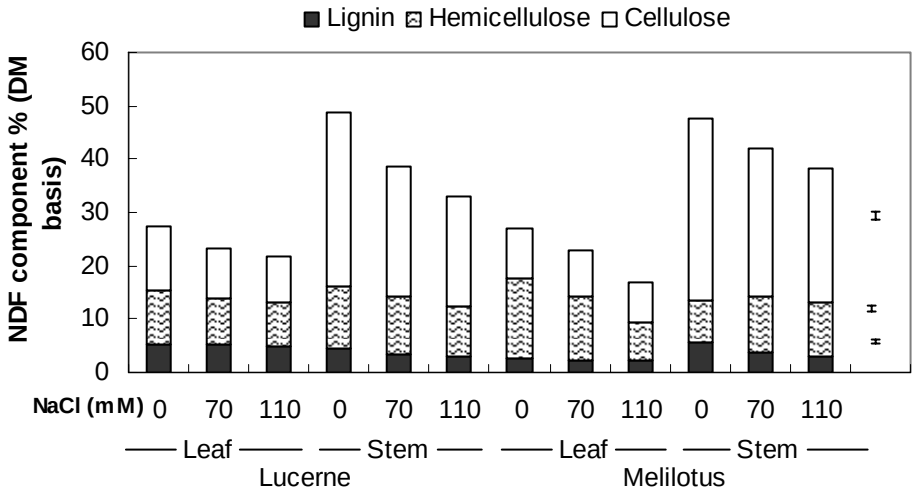


Figure 5.4. Fractioning of NDF into cellulose, hemicellulose and lignin as affected by salinity in Experiment 1. The bars correspond to the LSD (P=5%) for the comparison between species, NaCl concentration and organs.

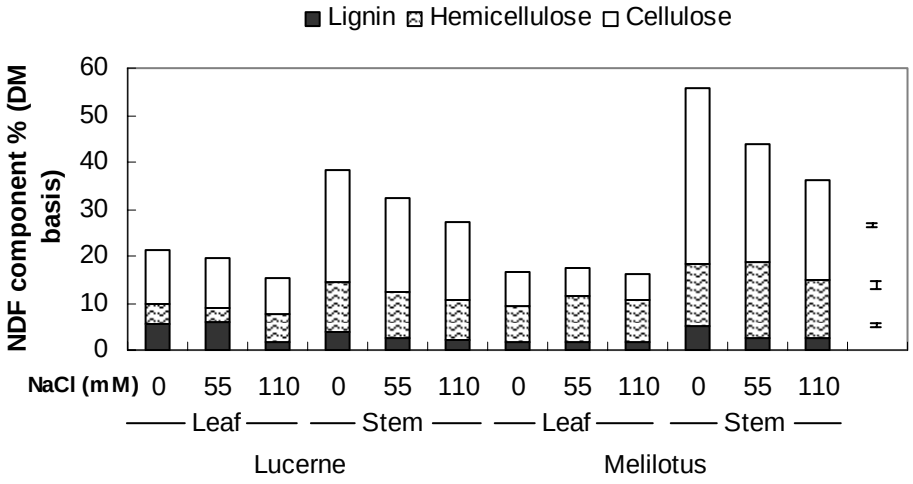


Figure 5.5. Fractioning of NDF into cellulose, hemicellulose and lignin as affected by salinity in Experiment 2. The bars correspond to the LSD (P=5%) for the comparison between species, NaCl concentration and organs.

Salinity affected digestibility, but there was a different response in leaf vs stem ($P<0.001$). In both species, leaf digestibility had a smaller response to salinity than stem (Table 5.4). On a dry matter and organic matter basis, leaf digestibility in both species, and in both experiments, did not change. However, when the soluble ash adjustment was applied (IVDSA) or when expressed as digestibility of organic matter in dry matter basis (DOMD), lucerne leaf on average had small ($P=0.05$) decreases, and Melilotus leaf increases.

Stem digestibility expressed in terms of dry or organic matter increased ($P=0.05$) in both species and in both experiments. However, when digestibility was expressed in terms of soluble ash or DOMD, only Melilotus stems increased ($P=0.05$) with increasing salinity. In constrast, lucerne stems had not any change (e.g., experiment 1) or had a decrease (e.g., experiment 2) when digestibility was expressed as DOMD.

Considering the leaf-to-stem ratio, and the whole shoot, lucerne DOMD remained stable in experiment 1 and leafiness compensated increases in ash. However, under the conditions of experiment 2, lucerne DOMD at the highest salinity level decreased by 4.8 percentage units compared to the control. In this situation, leafiness in lucerne could not compensate increases in ash. In contrast, the whole shoot of Melilotus had increased DOMD in both experiments, by 2.7 and 9.5 percentage units vs the controls for experiments 1 and 2, respectively.

For the digestibility experiments, the *in vitro* digestibility of the artificially salinized dry matter of lucerne and Melilotus (experiment "**a"** –material grown in the absence of salinity) increased as NaCl concentration increased (Table 5.5). When the dry matter of the salinized samples was adjusted by subtracting the weight of NaCl, there was no change in digestibility, indicating that external addition of NaCl to forage samples did not have an effect on *in vitro* digestibility.

The *in vitro* digestibility of material subjected to the procedure to eliminate soluble ash from the different treatments for NaCl of experiment 2, confirmed that, in leaves, salinity has little effect on fibre digestibility but, in stems, there was an improvement in both species (Table 5.6). Lucerne stems had relatively lower extent in improvement of fibre digestibility than Melilotus (8 percentage units vs 14 percentage units). Differences between species were maintained; Melilotus leaf fibre was more digestible than that of lucerne leaves by 11 percentage units, while lucerne stem fibre was more digestible than that of Melilotus stems by 12.6 percentage units.

Table 5.4. *In vitro* digestibility of lucerne and Melilotus as affected by NaCl concentration, expressed as digestibility of dry matter, digestibility of organic matter, digestible organic matter in dry matter and digestibility of dry matter adjusted for soluble ash (all values in percentage).

Organ and NaCl (mM)	Lucerne				Melilotus			
	IVD	OMD	IVDSA	DOMD	IVD	OMD	IVDSA	DOMD
Experiment 1								
Leaf								
0	94.4	93.5	83.6	81.1	97.1	96.6	85.6	82.8
70	94.2	93.0	81.0	78.8	96.7	96.1	86.7	84.5
110	94.5	93.3	79.6	77.7	96.3	95.7	87.0	84.8
Stem								
0	76.6	73.6	67.6	65.7	75.1	72.4	67.9	65.8
70	82.5	79.0	68.9	66.7	77.3	74.5	68.4	66.7
110	85.2	81.7	68.9	66.8	79.5	76.6	69.2	67.6
L.s.d. (P=5%)	1.1	1.2	1.2	1.1				
Experiment 2								
Leaf								
0	92.7	92.0	80.0	78.0	96.6	96.3	86.6	84.6
55	94.0	93.0	76.7	74.8	96.6	96.4	88.8	87.2
110	94.1	93.0	75.4	73.3	96.3	96.0	87.6	85.9
Stem								
0	83.0	81.2	73.2	72.3	63.6	61.7	59.7	58.4
55	86.5	84.0	72.0	70.0	76.2	73.7	67.8	66.5
110	88.8	86.1	70.4	68.2	79.6	77.3	69.0	67.6
L.s.d. (P=5%)	1.1	1.2	1.0	1.0				

IVD= *In vitro* digestibility in dry matter basis; OMD= *In vitro* digestibility of the organic matter; IVDSA= *In vitro* digestibility adjusted by soluble ash; DOMD= *In vitro* digestible organic matter in dry matter. The least significant difference is for the comparison between species, NaCl concentration and organs.

Table 5.5. *In vitro* digestibility of dry matter of plant material grown in the control group with different concentrations of NaCl added during the *in vitro* digestibility assays.

Organ and NaCl (%)	Lucerne		Melilotus	
	IVD in DM basis (%)	IVD adjusted by the NaCl added (%)	IVD in DM basis(%)	IVD adjusted by the NaCl added (%)
Leaf				
0	89.6	89.6	92.5	92.5
10	90.5	89.6	93.2	92.5
15	91.3	90.0	93.4	92.4
Stem				
0	70.6	70.6	58.5	58.5
10	72.6	69.6	61.8	57.9
15	74.1	70.1	64.6	59.2
L.s.d. (P=5%)[1]	1.3	1.5		

[1]Least significant difference for the comparison between species, NaCl concentration and organs.

Table 5.6. *In vitro* digestibility of dry matter of the residue treated for soluble ash in lucerne and Melilotus.

Organ and NaCl (mM)	Lucerne IVD (% DM basis)	Melilotus IVD (% DM basis)
Leaf		
0	73.3	84.0
55	73.6	85.4
110	74.7	85.7
Stem		
0	61.8	44.9
55	66.6	56.5
110	69.7	58.8
L.s.d. (P=5%)[1]	1.7	

[1]Least significant difference for the comparison between species, NaCl concentration and organs.

5.4 Discussion

Effects of salinity on components of dry matter, especially those related to the organic fraction, were diverse. Some traits were negatively affected, others were slightly improved and some were unaffected. Thus, the suitability of either plant species to saline conditions depends on its ability to produce dry matter in the first instance and, secondly on the extent to which any detriment was found and the compensation (and benefits) of the characteristics that improved.

Organic matter

It is known that osmotic stress is alleviated by plants through absorption of minerals or increased production of organic solutes (Levitt, 1972; Flowers and Yeo, 1989). It is probable that the response in organic matter for both plant species in the current research could be related to the strategies employed to cope with salt stress. In response to salinity, Melilotus leaf increased organic matter concentration, a situation contrasted to lucerne leaf, which had higher concentrations of Na and Cl, and therefore lower organic matter concentration than Melilotus. In Melilotus, the increase in organic matter when salinity increased may have produced a dilution effect, thereby decreasing Na and Cl concentration per unit DM. These results seem to be associated with the DM content, or degree of succulence. Increases in DM content in Melilotus corresponded in part to accumulation of organic matter and, in lucerne, with the accumulation of salts, with the consequent decrease in organic matter. It is not clear if lucerne makes use of salts in higher proportion or lacks the capacity to exclude them from accumulation in plant tissue than Melilotus. Nonetheless, succulence in lucerne is activated earlier than in Melilotus.

Stems had higher values of organic matter than leaves but, when salinity increased, both stems and leaves had similar concentrations. For controls, difference in organic matter between organs may be explained by the thicker cell walls in stems, especially those of the vascular tissues, and the higher degree of vacuolation in leaves, where some minerals accumulate. For stems of salt-affected plants, organs that stored more ash than leaves per unit of DM, it is possible that changes in cell wall and/or vacuolation had occurred in order to aid in salt storage. This may be an explanation for why both organs were equal in organic matter.

Even though increases in organic matter are small, as in Melilotus leaves, the determination of this measurement may be a useful indicator of the quality of the forage, as high organic matter may represent higher energy concentration per unit DM than minerals.

Soluble and insoluble ash

In both experiments, lucerne had higher values of soluble ash than Melilotus, a trend that agrees with increases in concentrations of Na and Cl. This indicates that the former species was more liberal in allowing entry of both ions into the shoot. In contrast, for Melilotus, leaves had lower soluble ash content and only the stem increased, an effect associated with the organic matter increase in Melilotus leaves.

In plant species that have been grown under saline conditions, an adjustment by soluble ash is recommended (Masters *et al*., 2001; Norman *et al*., 2002). Without this adjustment, a high concentration in soluble ash, such as NaCl, leads to an overestimate of digestibility (Figure 2.11). Ash does not contribute to the energetic value of dry matter and this is why it should be discounted from calculations in order to provide more realistic estimations of digestible energy.

For lucerne leaves and stems an adjustment by soluble ash is required because the increase was considerable. For Melilotus, an adjustment may only be necessary if the leaf-to-stem ratio was low, as stem had higher soluble ash accumulation than leaf in this species, although, this organ tended to contribute a low proportion of the plant biomass DM.

Insoluble ash was not as affected by salinity as soluble ash, and its variation in both species was less than one percentage unit. High values in cell wall ash (>5%) may negatively affect digestibility, as some minerals constituting the cell wall can coat potentially digestible organic material (McManus *et al.*, 1977; McManus *et al*., 1979). The ash of cell wall is a major constituent of insoluble ash, but salinity levels used in the current studies did not seem to have a major impact.

Soluble ash is a major contributor to the decrease of energetic value of salt-stressed herbage, a situation that was detrimental to quality of lucerne. In contrast, this characteristic was favorable in Melilotus leaf which maintained its gross energetic value.

Water-soluble carbohydrates

Water-soluble carbohydrates include monosaccharides (e.g., fructose, glucose) and oligosaccharides (e.g., sucrose, maltose), which the plant uses to meet its energy requirements, with any excess stored in reserves (Fulkerson and Donaghy, 2001). For the animal, this fraction constitutes a ready source of carbon skeletons and energy for microbial protein synthesis in the rumen (Stern *et al.*, 1994).

The two plant species studied had very different concentrations of water-soluble carbohydrates. Melilotus had higher percentages than lucerne, a difference that may be attributed in part to the ability of Melilotus to accumulate some glycosides (Stoker and Bellis, 1962; Poulton *et al.*, 1980; Oba *et al.*, 1981) and it is likely that when the plant material was treated with ethanol, as the procedure for determination of water-soluble carbohydrates requires, glucose was released. In addition, an effect due to differences in phenology may have widened the concentration between both species. In plant species like lucerne, a trend to increased soluble sugars as the plant approach bloom has been observed (Nordkvist and Åman, 1986; Stefanon *et al.*, 1996) and this may be similar to Melilotus. Comparing experiments, when Melilotus plants were harvested at same chronological age, the concentration of water-soluble carbohydrates increased and, when plants were harvested at the same phenological stage, no major changes attributed to salinity occurred. This suggests that phenology was the major factor affecting water-soluble carbohydrate concentration. The increase in water-soluble carbohydrates in response to salinity in experiment 1 could be related in part to the fact that the control plants were still at the beginning of the bud stage, while the salt-treated plants had started flowering.

Studies on water-soluble carbohydrates are well documented in other plant species (see section 2.7.3). The trend of this measurement under saline conditions has been shown to be variable, increasing or remaining unchanged in some and decreasing in others but, for forage legume species, this information is scarce. In the current research, the changes in water-soluble carbohydrates for lucerne and Melilotus indicate there was no major effect, but phenological effects were strong.

Fibre descriptors

Generally, fibre concentration is quantified as the insoluble material in neutral and acid detergents and 72% sulfuric acid (NDF, ADF and ADL, respectively). In this fraction, several polysaccharides [e.g., cellulose, rhamnogalacturonans, xylans, galactans, glucomannans and others (Duffus and Duffus, 1984)] that form the cell walls are an important source of energy to the microbes in the rumen. Fibre is important because values of NDF concentration higher than 30-35% restrict dry matter intake in ruminants (Dado and Allen, 1995), whereas values lower than 20-25% decrease bacterial yield in a proportion of 2.5% for every 1% decrease in NDF (Russell *et al.*, 1992).

In the current research it was found that NDF concentration decreased in response to salinity. This was because of three factors. First, the concentration of soluble cell contents increased more

under saline conditions than did the cell wall concentration. This means that more soluble ash or soluble organic solids were in each cell. When values of NDF and ADF were expressed in terms of organic matter, the effect of salinity was still visible, and the trend was to decrease with salinity, especially at the highest NaCl treatment.

Second, significant decreases in the hemicellulose fraction and lignin occurred mainly in stems. These differences could be related to differences in thickness and/or composition of the cell walls that may lead to changes in the proportions of fibre in the plant.

Third, at the whole shoot level, the NDF, ADF and ADL are highly influenced by the leaf-to-stem ratio because stems usually contain more of these components than leaves. Thus, a high leaf-to-stem ratio may indicate lower proportions of all three measurements. Salinity increased the leaf-to-stem ratio and, consequently, if all treatments are compared at the whole shoot level, the salt-treated plants had lower values than controls.

In the present research, NDF of the whole plant (i.e., leaf and stem) reached values of less than 20% only in lucerne under the conditions of the second experiment (18% for lucerne and 20% for Melilotus at 110 mM of NaCl). Values in lucerne were expected as it was at vegetative stage, but the values for Melilotus suggest that it is also likely to reach levels of NDF which could fall into the limiting range for bacterial yield when grown under highly saline conditions.

Digestibility

Digestibility is the result of degradation of a wide diversity of cells and their contents in the different tissues and organs of a plant. It can be expressed in different ways and care is required to use the most appropriate value, especially for plant species grown under saline conditions.

In leaves, despite decreases in fibre in salt-stressed plants, values of IVD of DM did not change. This result may be attributed to the stable concentration of both lignin and hemicellulose in leaves. Having a high impact on digestibility were the adjustments by soluble ash and by organic matter per dry matter unit (DOMD). The high total ash concentration in lucerne leaf of the salt-stressed plants widened the differences in relationship to the control. Due to this effect, digestibility values of lucerne leaf were lower than those of Melilotus leaf.

In stems, the fibre concentration was reduced in both plant species and, when the organic solids in NDF increased, the digestibility expressed as IVDSA or as DOMD also increased. This was the case for both plant species in experiment 1 (harvested at chronological intervals) and for Melilotus stems in experiment 2 (harvested at same phenological stage). One of the components that had a strong reduction in stems was lignin and, to a lesser extent, hemicellulose. Lignin and its association with structural polysaccharides, like hemicellulose, are known to limit digestion (Chesson, 1988; Jung and Deetz, 1993; Grabber, 2005). Thus, their reduction in stems is likely to have helped improve digestibility. Higher digestibility values for the stems coming from the high salt treatments were confirmed with the digestibility of organic matter (DOM), which improved in all cases.

Changes in response to salinity in IVD as dry matter or organic matter were similar and always greater than those of IVDSA and DOMD. This indicates that the energy values may be misleading, between 9 to 20 percentage units, if IVD value is not adjusted for ash. In addition, DOM did not detect the decreases attributed to ash, as in lucerne leaves (and in stems in the second experiment) showed when adjusted for ash.

Effects of salinity on digestibility were a function of the amount of the potentially digestible fibre and the amount of soluble cell contents in dry matter. At the levels of salinity used, there were no negative effects on fibre enzymatic digestion produced by a direct effect of salts (i.e, inhibition of digestion). This was verified when external salinization was applied to the control material that was grown in the absence of salinity. Moreover, it was found that values of digestibility of fibre devoid of soluble cell content, especially soluble ash, increased in salt affected plants in response to salinity. This indicated that the modifications that occurred to the fibre composition (e.g., decreases in lignin and hemicellulose) were responsible for the differences in relation to the controls. This is consistent with the determination of DOM from the intact samples Table 5.5.

It is noteworthy that the phenological stage did not exert a strong impact on leaf digestibility, at least for lucerne, which, even though it was in the vegetative stage, showed little improvement compared to Melilotus which was flowering. In contrast, stems were more influenced by stage of development and lucerne stems were more digestible than Melilotus stems.

Overall, digestibility of lucerne adjusted for ash content, was lower under saline growing conditions. In contrast, Melilotus had a slight improvement, values that may produce higher metabolisable energy concentrations. These results suggest that the improvement in leafiness in the salt-stressed plants was not enough to improve quality (at least for lucerne) to the extent required to counteract the negative effect that salinity imposed on dry matter production.

CHAPTER VI
LEAF AND STEM HISTOLOGY IN LUCERNE AND MELILOTUS AS AFFECTED BY SALINITY

6.1 Introduction

In the previous chapter it was shown that fibre concentration of lucerne and Melilotus decreased under saline growing conditions. As concentrations of NDF, ADF and lignin were modified, it suggests that there were modifications related to the cell wall and/or cell contents (i.e., organic and inorganic soluble solids).

At a cellular level, changes in the cell wall have been observed in some tissues in some forage legume species, as a direct or indirect effect of salinity. In the leaf veins of Trifolium alexandrinum, saline growing conditions promoted modification of xylem and phloem parenchyma cells into transfer cells, and wall ingrowths occurred in the xylem parenchyma (Winter, 1982). Increases in wall ingrowth and plasmalemma area in transfer cells, as well as reductions in sieve tube area in the phloem of main veins, have been reported to occur in lucerne leaves under saline growth conditions (Boughanmi et al. 2003). Therefore, these changes could influence fibre concentration.

Even though changes related to cell wall occur, gravimetric estimation is not enough for clarifying what happens in the plant organs. Additionally, factors such as heterogeneity in cell types and size and tissue proportions can be additional source of variation that influences NDF concentration and composition. In this regard, there is little information focused on the likely implications of nutritive value.

In order to have a more complete idea about the changes detected by the gravimetric measurements, a histological study was conducted on leaves and stems of lucerne and Melilotus, aimed at supporting the conclusions drawn from results of previous chapters.

6.2 Methodology

At day 192 and 193 after sowing, one and two days prior to the third harvest in the second experiment, leaf and stem sections were obtained from lucerne and Melilotus. Melilotus control plants were selected from those that were at a similar flowering stage to those that were salt-stressed. Sample collection was from 09:00-12:00 am on a sunny day. Leaf samples were obtained first and, from the same site, stems were identified for sampling on the following day.

The leaf positions considered were the upper and bottom parts of the longest stem in both species. In Melilotus, the upper position corresponded to the leaf of the highest inflorescence with opened flowers (Plate 6.1), while the basal leaf was identified as the one at the cutting height of harvest. In lucerne, the upper leaf was the fourth expanded leaf, counting from the top towards the bottom (Plate 6.1), and the basal leaf was at the height of cutting (at the fourth internode from the substrate). Leaves were fully expanded and only the mid-leaflet was selected for sampling. Stem samples were obtained only from the basal part, again taken at the height of cut harvest.

Plate 6.1. Leaf top position in Melilotus (left) and lucerne (right).

The processing schedule for plant tissue and preparation of chemical solutions was according to O'Brien and McCully (1981) and Ruzin (1999).

After removal from the plant, every leaflet was placed onto three or four drops of fixative in a Petri dish. The fixative was of 3% glutaraldehyde in 0.025 M phosphate buffer saline (PBS) to pH 7. Then, from the middle of the right part of the leaflet, a tissue piece no longer than 5 mm in any dimension was obtained, and cut in such a way as to ensure a part of blade and midrib was included in the section.

Every piece of plant tissue cut was placed in plastic vials filled with fixative (see Ruzin, 1999 for details on chemical solutions preparation) and, after identification, vials were placed in a refrigerator at 4°C for 36 hrs. After removing the vials from the refrigerator, the fixative solution was replaced by PBS and left again at 4°C for 36 hrs. Dehydration was then completed by immersion of the tissue through a series of several alcohols. Tissue was left for at least two hours in each of two changes of methoxy-ethanol, ethanol, propanol and butanol.

Infiltration commenced at the last change of butanol. In this step, a mixture of one part of butanol and one part of glycol methacrylate mix (GMA) was added and samples were left overnight at 4°C. Following this step, the infiltration solution was replaced with GMA only and kept for 2 days at the same temperature and, after the two days, the solution was changed and the process repeated, leaving samples for a further 2 days.

Samples were embedded in gelatine capsules in fresh GMA and polymerized for 3 days at 55°C.

Cross sections of the sample were cut at 3 microns with a Reichert Jung ultramicrotome with glass knives, placed in slides and dried overnight at 60°C. Staining was with periodic acid-Schiffs stain and Toluidine Blue O as described by O'Brien and McCully (1981) and then mounted in DPX for observation.

Slides were viewed under microscopy at 20, 40 and 60 times magnification and images were obtained. Data was obtained using the Image analyses program iTEM Universal TEM imaging Platform Soft Imaging System based on analySIS FIVE.

Two leaves per plant and two plants per treatment (0, 55 and 110 mM NaCl) were sampled. Data was analyzed by ANOVA as a randomized complete-block design with four replications using GENSTAT 6. Residuals were checked for normality and homogeneity and the least significant difference was used for means separation.

In leaves, the measurements included were as:

- Blade thickness, which included three measurements at both extremes of the picture and one in the middle. The measurement included the upper and lower epidermis (Plate 6.2 includes the illustrations).
- Palisade and spongy parenchyma and epidermis thicknesses, which included three measurements taken from the same places where the blade thickness was measured.
- Vascular area of the midrib which included the phloem and xylem areas of the main midrib.
- Xylem cell wall thickness included measurements of three pairs of adjacent cells divided by two to obtain the average per cell.
- Mesophyll starch area included a section of the palisade and the spongy parenchyma of no less than 20 cells with an average area of 18,900 μm^2 ± 7,100 μm^2 where starch granules were highlighted and their area measured and expressed by 10,000 μm^2.
- Mesophyll cell number in a cell column was measured from three sections of the slide taking as limits the epidermises.

In stems, the measurements taken included:

- Radial width, which was taken from the epidermis to the end of the xylem. The xylem end was considered at the start of the pith cells (large parenchyma cells with large intercellular spaces).
- Xylem and phloem width, which was measured taking the widest part of each of them (including primary and secondary xylem and phloem) starting from the limits of the cambial cells to the start of the pith cells.
- Parenchyma width, which included the cells between the phloem and epidermis.
- Epidermis cell width.
- Xylem and phloem cell wall thicknesses, which included the measurement of 6 cells.
- Parenchyma starch area, which included no less than 25 cells with an average area of 16,700 μm^2 ± 5035 μm^2.

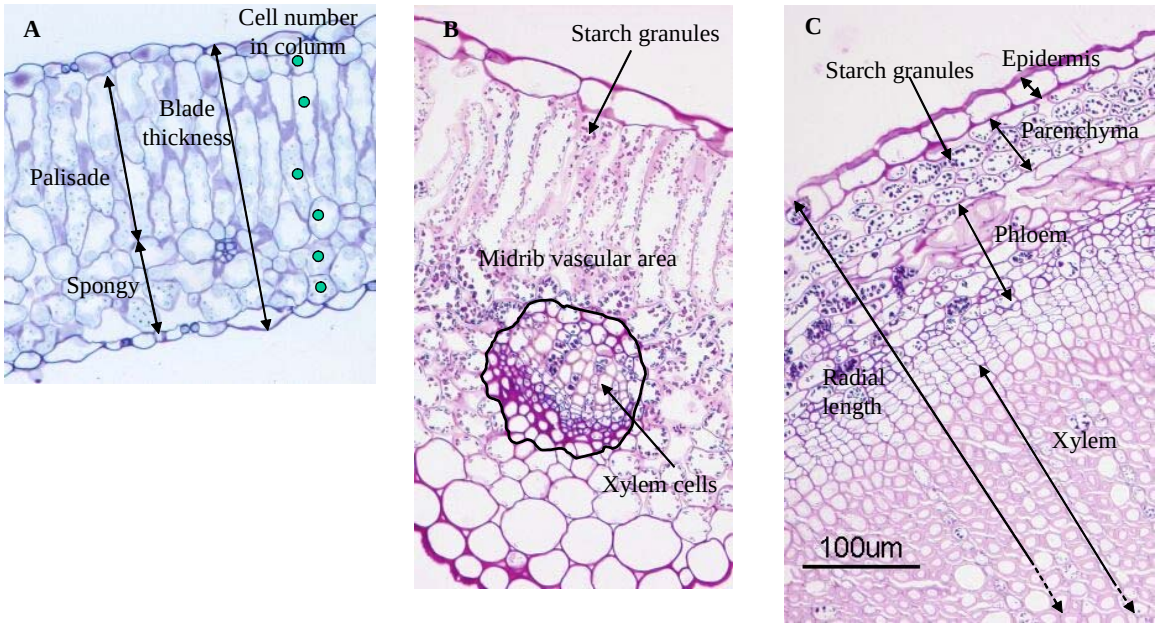


Plate 6.2. Measurement description in leaf blade (A), leaf midrib (B) and stem (C).

6.3 Results

Several measurements were made in order to determine the effects of salinity at the tissue level in the leaf and stems of both plant species. Measurements were aimed at supporting the results obtained by the gravimetric methods (Chapter V) and to have a better understanding of the phylogenic differences between plant species.

6.3.1 Leaves

In leaves, salinity affected ($P \leq 0.03$) blade thickness, especially in lucerne where the top and basal leaves had increases in thickness of 31% in relation to the controls (Table 6.1 and Plates 6.3, 6.4, 6.5 and 6.6). Melilotus leaves were less responsive than lucerne leaves and only those in the basal position increased thickness in response to salinity. The increase of blade thickness was primarily related to an increase in the palisade mesophyll due to the effect of NaCl ($P \leq 0.003$). Palisade mesophyll in lucerne increased by 60% compared to the controls.

It was noticed that the spongy mesophyll did not vary in thickness, and that differences corresponded mainly to differences between plant species. The spongy cell layer in Melilotus was about 30% thicker than lucerne.

In lucerne, the increase in palisade thickness resulted in an increased palisade-to-spongy parenchyma ratio, whereas in Melilotus it remained stable (Figure 6.1).

There were differences in the number of cells in the mesophyll (in cross section), but these were due to differences between species, lucerne had on average a cell count of 5.6 and Melilotus 7.6. Most of the differences between the species could be accounted for by higher cell count of the spongy parenchyma cells in Melilotus (Figure 6.2).

The epidermis thickness showed slight variation, but only in the basal leaves of lucerne was there an increase (P=0.05) in response to salinity (Table 6.1).

Table 6.1. Leaf tissue measurements of the top and base position in the plant of lucerne and Melilotus at different NaCl concentrations in the irrigation water.

Plant species and NaCl (mM)	Blade thickness (µm)		Palisade thickness (µm)		Spongy thickness (µm)		Epidermis thickness (µm)	
	Top	Base	Top	Base	Top	Base	Top	Base
Lucerne								
0	178.9	177.8	81.7	77.8	73.5	72.9	11.8	13.6
55	205.6	199.4	103.6	95.5	74.7	70.5	13.6	16.7
110	235.4	236.0	131.6	128.7	73.4	70.3	15.2	18.4
Melilotus								
0	277.0	295.3	132.6	147.3	113.6	105.5	15.4	21.3
55	256.0	312.6	124.2	153.3	103.3	118.5	14.2	20.4
110	274.9	331.0	134.3	168.6	111.2	118.1	14.7	22.2
L.s.d. (P=5%)[1]	43.2		26.4		19.3		4.4	

[1]Least significant difference for the comparison between species, NaCl concentration and leaf position

Both plant species differed (P<0.001) in starch concentration (quantified as area in μm^2 per 10,000 μm^2 in the parenchyma cells), with Melilotus having higher values than lucerne (Table 6.2). There was an interaction (P≤0.02) between species and leaf position with increasing salinity wherein Melilotus tended to increase starch area with salinity, whereas lucerne tended to decrease starch area. The concentration of starch in the top leaves of lucerne was lower than in the bottom leaves, whereas in Melilotus this trend was the reverse.

In relation to the conductive tissue of the leaf, NaCl did not affect the vascular area of the main midrib (Table 6.2). The variations observed were mainly due to the position of the leaf in the plant where the bottom leaves tended to have lower area values than the top leaves. A similar trend occurred in the thickness of the xylem cell walls where the bottom leaves tended to have slightly thinner cell walls.

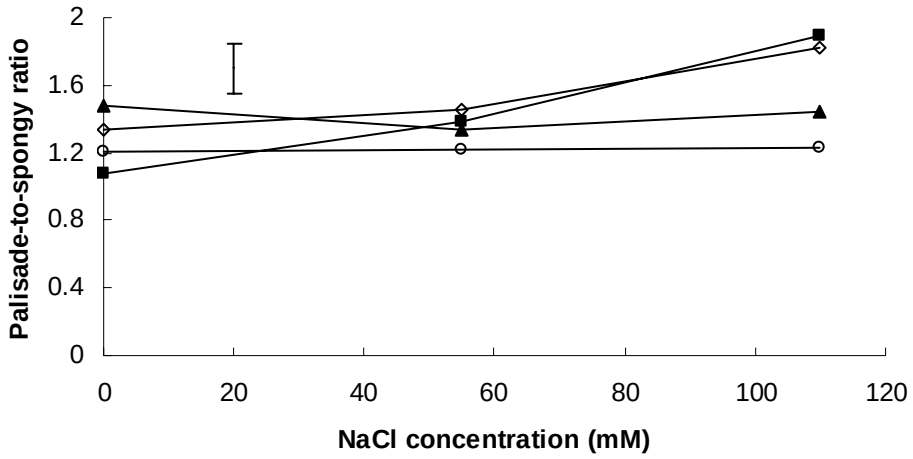


Figure 6.1. Palisade-to-spongy parenchyma ratio (from thickness) in top (open symbols) and bottom (solid symbols) leaves of lucerne (◊, ■) and Melilotus (○, ▲). The bar represents the least significant difference at P=5%.

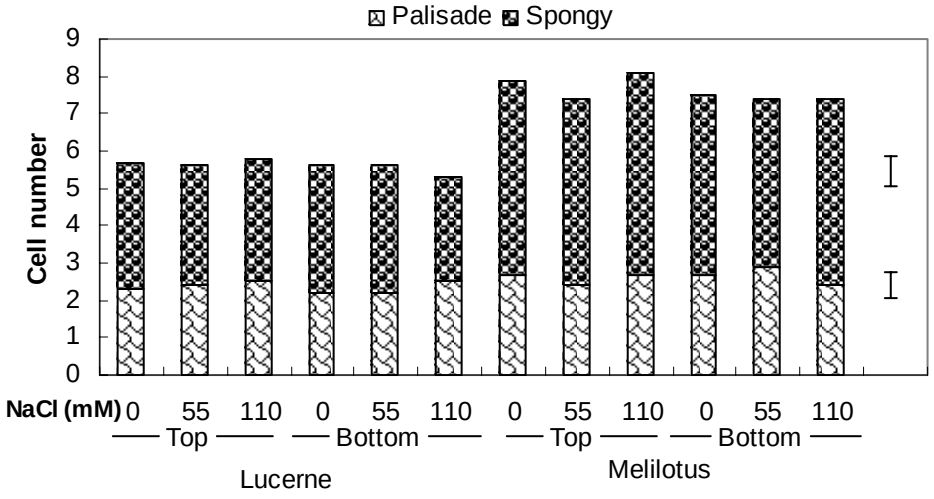


Figure 6.2. Mesophyll cell number in lucerne and Melilotus leaves as affected by salinity. The bars represent the least significant difference at P=5%.

6.3.2 Stems

The thickness of the stems expressed partially (i.e., pith was not included) as radial width was reduced by effects of NaCl ($P<0.001$). Melilotus stems had reductions of about 60% compared to values obtained from the control (Table 6.3). Lucerne stem thickness was 6% lower than in the control.

Xylem was the tissue that contributed most to the decrease in stem diameter. In both plant species, salinity induced decreases in the width of this tissue that, at the highest salinity level, corresponded to a 42 and 54% decrease for lucerne and Melilotus, respectively. Among the other tissues, phloem width and epidermis thickness were not affected, but this was not the case for parenchyma. As salinity increased, lucerne parenchyma tissue thickened by about 90% at the highest salinity level compared with the control, contrasting with values measured in Melilotus where this tissue barely changed (Table 6.3 and Plate 6.7 B). These changes in width modified the vascular tissue-to-parenchyma ratio in both species, decreasing as salinity increased (Figure 6.3).

No effect due to salinity was recorded for the xylem cell wall thickness, and differences found could be attributed to species differences wherein Melilotus had thicker cell walls than lucerne (Table 6.4). Melilotus also had thicker phloem cell walls compared to lucerne but, for this measurement, lucerne was more affected by salinity, especially at the highest salinity level where the cell wall thickness was 40% lower than the control.

Both plant species responded differently to salinity in terms of starch area in the parenchyma cells of the stems. The trend for lucerne was to show a decrease in starch area, while in Melilotus there were no changes (Table 6.4 and Plate 6.7).

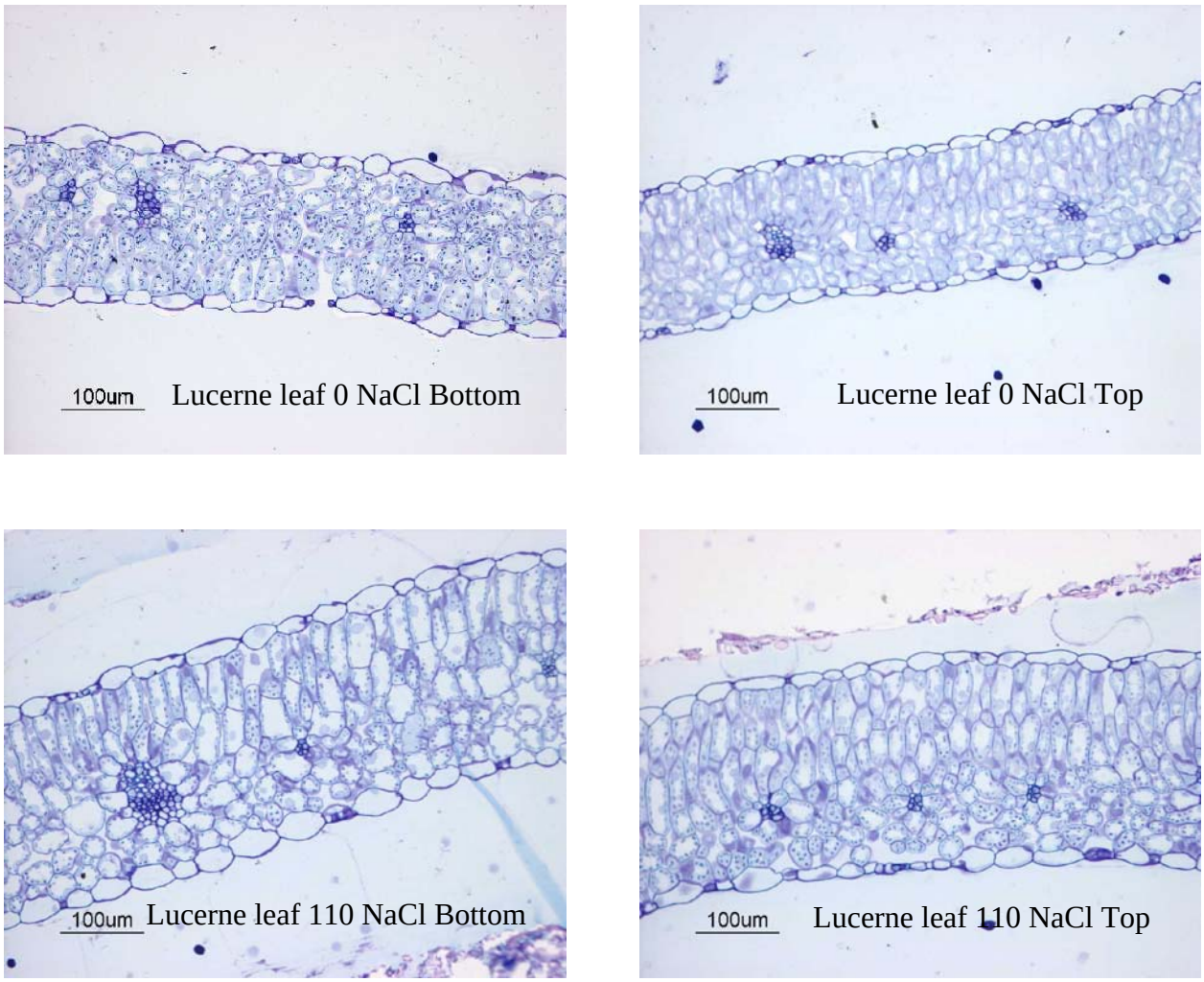


Plate 6.3. Cross sections of lucerne blades in top and bottom leaves of the control and 110 mM of NaCl treatments (20 times magnification).

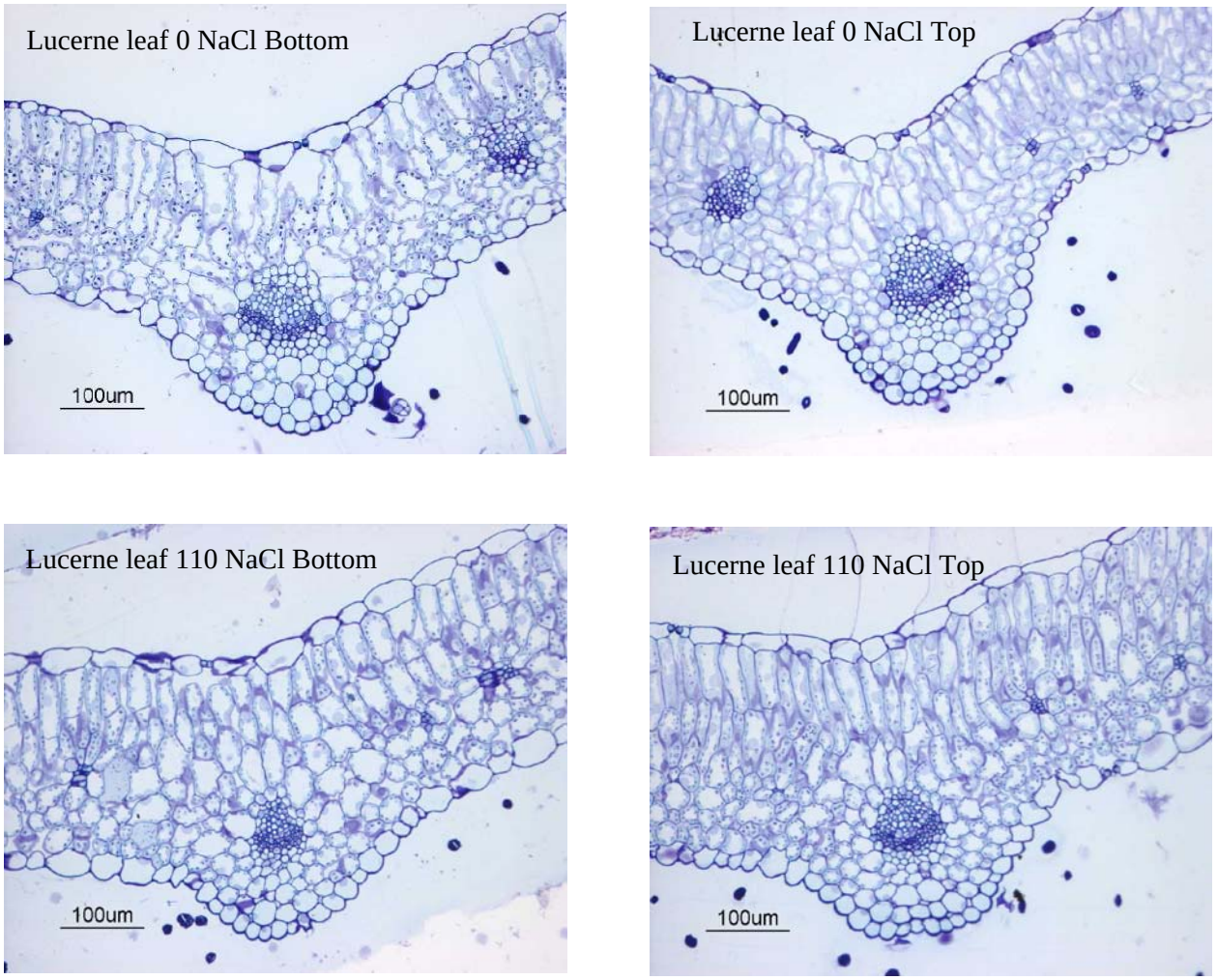
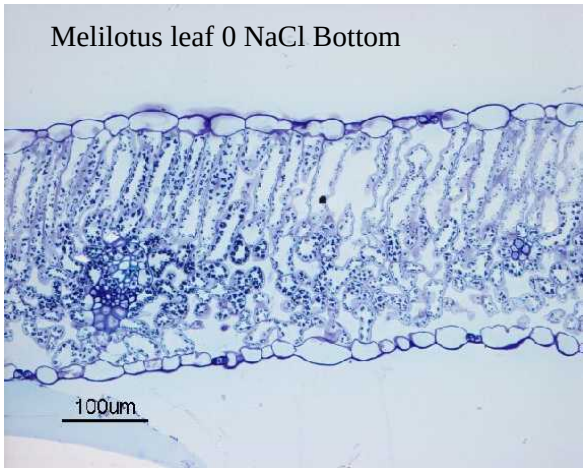
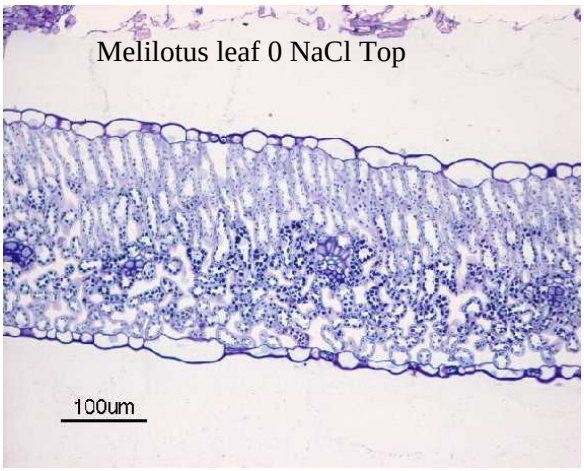
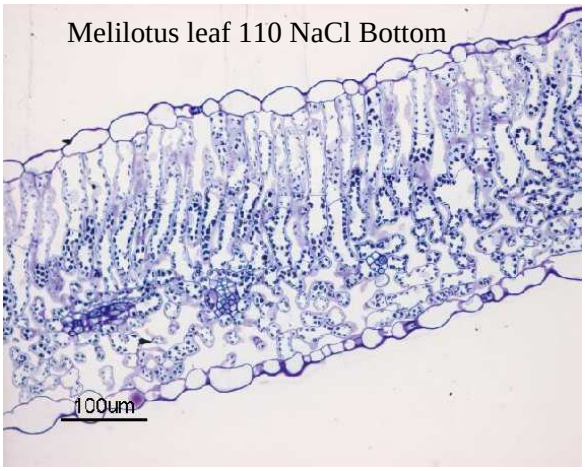


Plate 6.4. Cross sections of the midrib in lucerne top and bottom leaves of the control and 110 mM of NaCl treatments (20 times magnification).







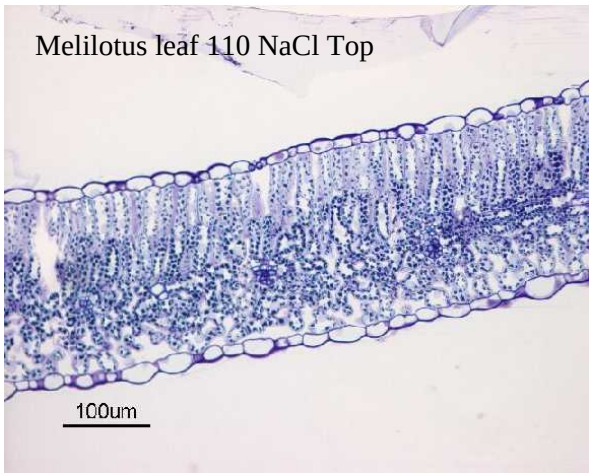


Plate 6.5. Cross sections of Melilotus blades of top and bottom leaves of the control and 110 mM of NaCl treatments (20 times magnification).

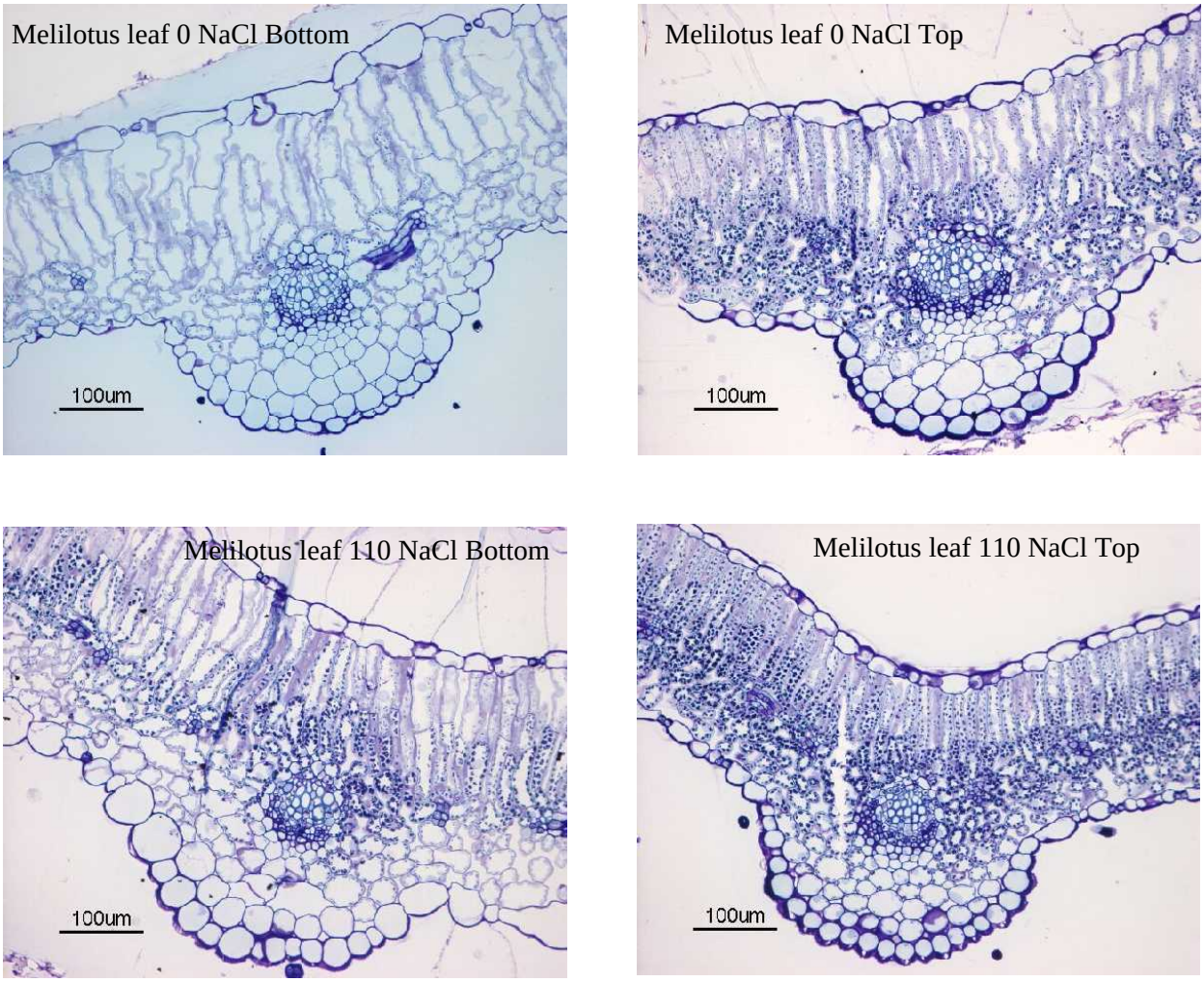


Plate 6.6. Midrib cross sections of Melilotus top and bottom leaves of the control and 110 mM of NaCl treatments (20 time magnification).

Table 6.2. Vascular tissue (phloem and xylem) description of the main midrib and starch area in the leaf mesophyll (palisade and spongy parenchyma) in lucerne and Melilotus as affected by NaCl in the irrigation water.

Species and NaCl (mM)	Vascular area (μm^2)		Xylem cell wall thickness (µm)		Mesophyll starch area (μm^2) per 10,000 (μm^2)	
	Top	Base	Top	Base	Top	Base
Lucerne						
0	10197	7569	1.29	1.05	157 (1.95)*	420 (2.59)
55	16869	8805	1.26	1.13	111 (1.87)	312 (2.42)
110	10824	9922	1.24	1.21	102 (1.45)	210 (2.17)
Melilotus						
0	15527	12992	1.36	1.09	709 (2.80)	346 (2.35)
55	14578	9499	1.25	1.08	1327 (3.04)	534 (2.64)
110	10951	11032	1.31	1.06	1641 (3.16)	860 (2.83)
L.s.d. (P=5%)	3418		0.22		464.3 (0.48)	

* Figures in brackets are the log transformed data.

Table 6.3. Stem tissue description in lucerne and Melilotus as affected by NaCl in the irrigation water.

Species, NaCl (mM)	Radial width (µm)	Xylem width (µm)	Phloem width (µm)	Parenchyma width (µm)	Epidermis thickness (µm)
Lucerne					
0	390.8	168.3	105.2	74.7	21.4
55	335.4	102.9	87.5	112.2	19.7
110	367.2	96.5	95.4	143.8	22.1
Melilotus					
0	748.6	536.8	107.7	59.8	24.7
55	506.1	283.1	104.5	74.3	28.4
110	449.9	246.2	92.1	72.8	24.0
L.s.d. (P=5%)	51.0	35.6	20.6	29.8	6.7

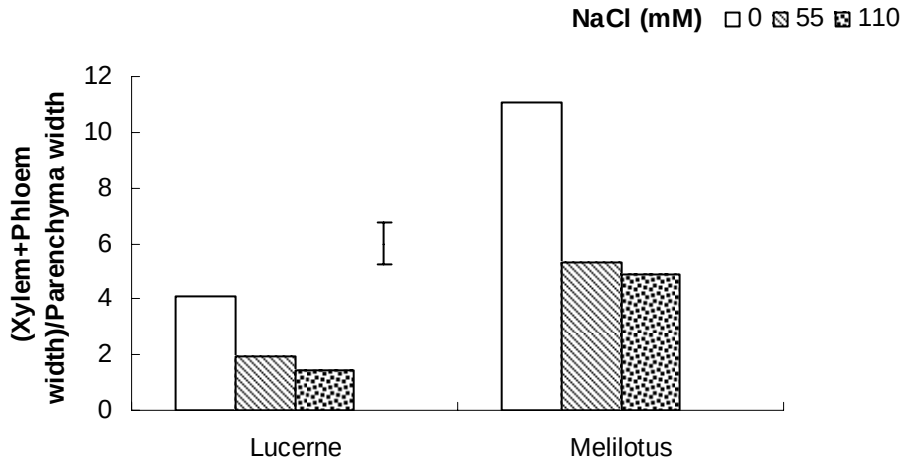


Figure 6.3. Xylem+Phloem width-to-parenchyma width ratio in stems of lucerne and Melilotus as affected by salinity. The bar represents the LSD at P=5%.

Table 6.4. Cell wall thickness of xylem and phloem and starch area in the parenchyma cells in the stems of lucerne and Melilotus as affected by NaCl in the irrigation water.

Species and NaCl (mM)	Xylem cell wall thickness (µm)	Phloem cell wall thickness (µm)	Parenchyma starch area(µm^2) per 10,000 (µm^2)
Lucerne			
0	1.81	3.14	468 (2.58)*
55	1.38	2.26	85 (1.78)
110	1.58	1.89	33 (1.38)
Melilotus			
0	2.03	4.08	964 (2.97)
55	2.13	4.51	987 (2.96)
110	2.07	5.43	1402 (3.09)
L.s.d. (P=5%)	0.38	1.18	615.1 (0.386)

* Figures in brackets are the log transformed data.

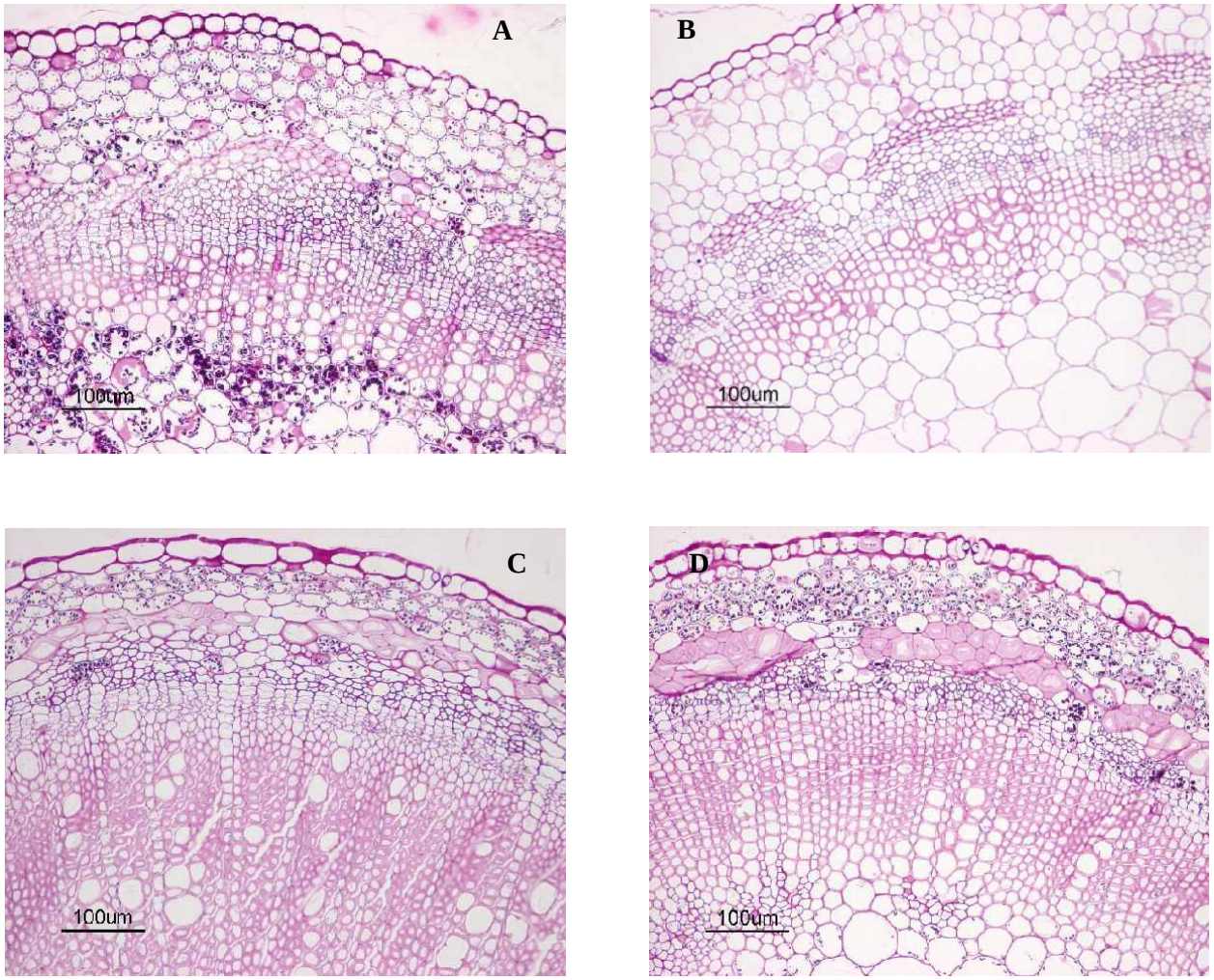


Plate 6.7. Cross sections of stems in lucerne (A and B) and in Melilotus (C and D) at 0 (left) and 110 mM of NaCl (right). The sections represent the base of the stem (at harvest height). In Plate C, the xylem of the control treatment represents approximately ⅔ of its total length. 20 time magnification.

6.4 Discussion

Variations in concentrations of soluble organic compounds, reserve polysaccharides, structural polysaccharides and lignin may result in enhanced, or reduced, digestibility. These variations are related to plant morphology, where tissue proportions and chemical composition may influence nutritive value.

Several characteristics of tissues in the plant were affected by salinity and it is likely to have influenced several measurements of nutritive value.

Leaf

The increase in blade thickness under saline growth conditions was related to succulence. Succulence, according to Shannon *et al.* (1994), is a typical response to salinity in dicotyledonous species. Mainly in lucerne, the increase in the palisade parenchyma cells supports this view and may be associated with the measured increase in water content, as there was a greater volume per leaf area compared to controls. Enlargement of the palisade cells in lucerne may also explain, in part, why the density of the DM increased. The increase in soluble organic solids in relation to cell wall (Table 5.4) in lucerne may be the result in the increase of organic content per cell wall unit due to growth of palisade cells, layer that comprised about 50% of the thickness of the leaf.

When NDF and ADF values were expressed corrected for organic matter, the effect of salinity was still visible, showing a decrease in NDF/ADF concentration, especially at the highest NaCl treatment. Thinner cell walls could be an explanation for this tendency, which could be the result of decrease in cell wall thickness in the palisade cells, due to the enlargement produced. However, this characteristic was difficult to measure in the parenchyma cells under microscopy, mainly because cell distribution and cell wall position are not uniform. Additionally, it is difficult to differentiate the soluble fibre in the cell wall using a microscope, a fraction that accounts for some of thickness of the cell wall, but it is not included in NDF. Instead, plant cell wall thickness was measured in the leaf xylem cells which were more visible. However as no change was found, it appears that cell wall thickness may not be a decisive factor in decreasing NDF/ADF concentration.

The decrease in fibre concentration could be related to the change in the tissue proportions in the organs. The parenchyma-to-vascular tissue ratio increased, and the number of cells did not vary to a great extent. Parenchyma cells have thinner cell walls and more soluble organic content than

the cells of the conductive tissue. Thus, this characteristic may contribute to the decrease of NDF/ADF concentration, which adds support the view that the effect of density could be the major contributor to the reductions in NDF/ADF concentration in leaf.

The increase in starch area in Melilotus leaves in response to salinity agrees with the organic matter increase observed. Similarly, the opposite trend in lucerne could explain in part why this species had much higher ash values under saline growth conditions as there was no diluting effect, as was observed in Melilotus. In leaves of other dicotyledonous plant species where starch is the main reserve carbohydrate, quantitative changes of this compound have been shown to vary under saline conditions. For instance when salinity increased, leaves of bushbeans (*Phaseolus vulgaris* L.) and mangrove (*Aegiceras corniculatum*) accumulated starch (Rathert *et al.*, 1981; Parida *et al.*, 2004); leaves of grapevines (*Vitis vinifera* L.), tomato (*Lycopersicon esculentum* L.) and saltbush (*Atriplex nummularia* L.) starch concentration decreased (Downton, 1977; Gao *et al.*, 1998; Niu *et al.*, 1996), and in sugarbeet leaves (*Beta vulgaris* L.) starch concentration remained unchanged (Rathert *et al.*, 1981). Thus, it is not surprising that lucerne and Melilotus had contrasting responses in starch concentration to salinity. Unfortunatelly, quantitative starch determination for lucerne and Melilotus was not made in the dry matter obtained in the experiments of this research, because a lack of enough plant material. Therefore, the result observed at histological level is still speculative. However, high starch concentration can be a desirable characteristic for ruminants as it is a source of energy that can be utilized by the rumen microbes.

Between species, and even though lucerne was at vegetative stage, its leaf digestibility was lower than Melilotus leaf that was at early flowering. This result was related to a higher ADF and lignin concentration in lucerne leaf. In addition, there was evidence that Melilotus leaf had a higher starch concentration. It is suggested that these characteristics contributed to widen differences in digestibility. Although lignin concentration can be the result of the integration of lignin synthesis rate, and the number and area of veins in the leaf, the proportions of other tissues that contribute to leaf thickness (i.e., the palisade and spongy parenchyma) may have diluted the lignin concentration per unit DM. There were no differences in both the vascular area of the main midrib and epidermis thickness between plant species. Additionally, wider palisade and spongy parenchyma were found for Melilotus, including the cell number for the spongy parenchyma. This means less epidermis and less vascular tissue (which is indigestible tissue

according to Akin, 1989), and therefore less lignin, could be present per unit of leaf DM of Melilotus vs lucerne. These morphological differences in leaf could have contributed to enhance digestibility in Melilotus leaf, a difference that was maintained when the leaf DM without soluble ash was subjected to *in vitro* digestion.

Stems

In stems, both plant species had many similar characteristics, such as phloem width, epidermis thickness, parenchyma width, and xylem and phloem cell thickness but, for most of them, the effect of salinity or the plant response to salinity was different. The most contrasting characteristics between species were xylem width which influenced the radial length and starch area. Xylem width may be the major contributor to increases in ADF and ADL in Melilotus which had higher values than lucerne.

Overall in both plant species, the xylem+phloem width-to-parenchyma width ratio decreased. This is thought to have influenced the digestibility of stem, mainly because xylem width decreased, a response that was more evident in Melilotus. In stems, xylem and phloem, especially the primary phloem and secondary xylem, are tissues with lower digestibility than parenchyma, epidermis and cambium (Akin, 1989; Engels and Jung, 1998, Jung and Engels 2002). Thus, a low contribution of them to NDF, as the reduction in the xylem+phloem-to-parenchyma ratio showed, could be reflected in an improvement in digestibility. Between species, lucerne stem had higher values for parenchyma width and lower values for phloem cell wall thickness, characteristics that could favour higher stem digestibility in lucerne than Melilotus.

The starch area in stems differed between species, with lucerne starch area decreasing under salinity. This is probably linked to the K concentration which, in lucerne decreased, whereas in Melilotus it was not affected. Potassium is involved in enzyme activation (e.g., starch synthetase) (Mengel *et al.*, 2001), and possibly this element reached concentrations that were deficient. Low starch concentration in stems, gives a glimpse of reserve levels of this compound, which concentration could have been affected in the main storage organs (i.e., crown and taproot). Generally, starch is used for regrowth, but crowns and taproots were not studied in this research. Thus, it remains to be seen if a poor regrowth in glycophytic forage species under saline growing conditions is directly related to levels of starch in crowns and taproots.

In summary, there were some characteristics detected in the histological study which provide evidence supporting some of results obtained with the gravimetric determinations as shown next (Table 6.5).

Table 6.5. Summary of the histological study and probable relationships with the gravimetric measurements.

Measurements	Gravimetric quantification	Microscopy characteristics
Succulence	Increase in lucerne	Palisade cells size increase
Organic matter	Increase in Melilotus leaf	Starch increase in Melilotus leaf
Particle density	Increase	Related to palisade size increase (increase in the storage capacity of salts), accumulation of starch in Melilotus.
Fibre concentration	Decrease	Reduction of xylem contribution to total DM. Increased contribution of parenchyma cells
Digestibility	Increase in stems Highervalues for Melilotus	Related to the previous effects on tissues (parenchyma cell width-to-vascular tissue width ratio, starch)

CHAPTER VII
COUMARIN IN MELILOTUS AND ITS PERFORMANCE UNDER SALINITY

7.1 Introduction

One area of concern in forage plants is the presence of secondary compounds with anti-nutritional properties which can affect the nutritive value of the forage. These compounds can cause the animal to reduce feed intake, restrict digestive processes and nutrient utilization and/or cause damage to vital animal organs (Kumar and D'Mello, 1995).

In Melilotus, the compound of concern is the micotoxin dicoumarol, a potent blood anticoagulant that is formed by fungi if plant material aerobically ferments. For example, if the drying process for making hay (or silage) is not well controlled, and excessive moisture (>180 g/kg) and a rise in temperature (about 60ºC) occur, fungal activity can produce the toxin (Emery and Gear, 1970; Alstad *et al.*, 1985; Sanderson *et al.*, 1986). It is believed that the main precursor of dicoumarol is coumarin (Stahmann *et al.*, 1941; Sanderson *et al.*, 1986), although it has been suggested that *o*-coumaric acid (*trans*-2-hydroxycinnamic acid) may be the precursor (Davies and Ashton, 1964; Bellis *et al.*, 1967; Muir and Goplen, 1992).

In intact plant cells, coumarin is thought to be present in small amounts, if any, but when cells are disrupted, production occurs due to action of a β-glucosidase on coumarinyl glucoside (Figure 7.1), which liberates the coumarinic acid from the glucose molecule and then lactonizes to coumarin (Brown, 1979; Poulton *et al.*, 1980; Oba *et al.*, 1981). The presence of high concentrations of either *o*-coumaric acid or coumarin may pose a risk of dicoumarol poisoning to ruminants consuming the forage.

Salinity promotes production of reactive oxygen species (Dionisio-Sese and Tobita, 1998; Sudhakar *et al.*, 2001; Garratt, *et al.*, 2002; Vaidyanathan *et al.*, 2003) and, among the compounds used for antioxidant activities, are the hydroxy-cinnamic acids (Wenfeng *et al.*, 1995; Rice-Evans *et al.*, 1996; Chalas *et al.*, 2001). Most of the compounds related to coumarin in Melilotus are derivatives of hydroxycinnamic acid and have anti-nutritional properties, but little is known on the effect of salinity on coumarin and 2-*trans*-hydroxycinnamic acid synthesis.

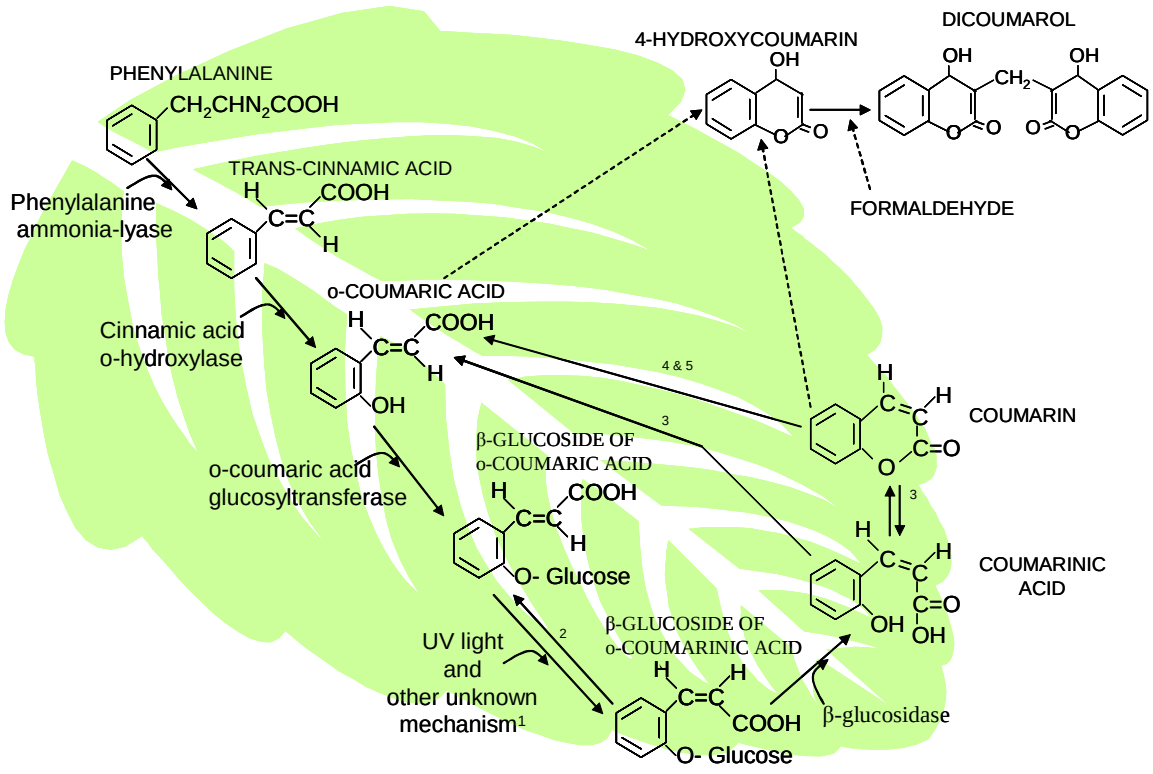


Figure 7.1. Biosynthesis of coumarin and dicoumarol (Adapted from [3]Haskins and Gorz, 1957; [4]Booth *et al.*, 1959; Kosuge and Conn, 1961; [2]Stoker and Bellis, 1962; Bellis *et al.*, 1967; Spring and Stoker, 1968; Poulton *et al.*, 1980; [1]Rataboul *et al.*, 1985; [5]Lake, 1999).

7.2 Methodology

The Melilotus forage samples were from experiments 1 and 2 (Chapter III). Additionally, two new experiments were conducted following the same procedures and materials from the previous experiments. Plants were grown inside the glasshouse and outside it. The objective was to determine if sunlight had an effect on coumarin or on 2-*trans*-hydroxycinnamic acid concentration in leaves, as UV light modifies concentrations of hydroxycinnamic acids (Haskins and Gorz, 1973).

The sowing of these two experiments was on the 18th of March 2004 and germination occurred five days later. At day 26 after sowing, the saline treatments (0, 55 and 110 mM of NaCl) started and, at day 37, the highest concentration had been reached. Two conditions of growth were tested, direct sunlight exposure and glasshouse conditions with no shade cloth over the roof of the glasshouse. For the experiment exposed to direct sunlight, trays were placed on a mobile

metallic table and left outside of the glasshouse from approximately 9:00 to 16:00 hrs and returned overnight to the glasshouse. During the days with rain or very windy conditions (24 days in total), plants remained in the glasshouse to avoid plant injuries or dilution of the salinity treatments. During the experimental period, temperatures inside the glasshouse ranged between 10 to 21ºC with an average of 14ºC. Outside of the glasshouse, temperatures ranged between 7 to 16ºC with an average of 12ºC.

Two harvests were made, but results for only one harvest are reported, because the samples of the second harvest were lost due to freezer failure. The material used was harvested at 151 days after sowing when all plants were at full flowering and only the leaf fraction was analyzed for coumarin and 2-*trans*-hydroxycinnamic acid.

Coumarin analysis included freeze-drying the material which was subsequently ground to pass through a 1 mm screen. Samples of 0.2 ± 0.006 grams were weighed into plastic tubes of 50 ml capacity with screw caps and a rounded base. Each tube received 20 ml of ethanol (100%) and 0.5 ml of vanillin solution (0.1 % vanillin dissolved in methanol as internal standard). Tubes were placed in racks and left overnight in an orbital mixer incubator at 28°C and 150 RPM.
Supernatants were poured into 20 mL syringes coupled with syringe filters (0.20 μm) and the filtered liquid was transferred to polypropylene tubes, which were kept at room temperature and in darkness. A sub-sample of 0.5 mL was poured into a vial, only to assure a sufficient volume for a microinjection of 5 μL. Amounts of two ppm of vanillin, coumarin (CAS 614-60-8) and 2-*trans*-hydroxycinnamic acid (CAS 614-60-8) (SIGMA) were included as controls for determining the concentration in the analyzed samples. A Hewlett Packard Agilent 1100 Series HPLC was used for the determinations of these substances.

The data from all experiments were analyzed by ANOVA using the GENSTAT 6 program and residuals were checked for normality and homogeneity. The first experiment was analyzed as a randomized complete block design arranged as a split plot, where the main plots were the salinity treatments and the subplots were the harvests. In the second experiment, the dry matter produced in all harvests was pooled and the data was analyzed as a randomized complete design. The two additional experiments were analyzed as a completely randomized design, with three replicates, where the experimental unit consisted of 12 plants.

7.3 Results

Experiment 1 (harvests performed chronologically)

The coumarin concentration in Melilotus was not influenced by salinity. The plant components accounted for most of the variation (P<0.01), where leaves had about twice the concentration of stems (Figure7.2). However, the concentration of 2-*trans*-hydroxycinnamic acid (tHCA) increased progressively as the salinity increased (P<0.001). Differences occurred between organs, where leaves had about twice the concentration than stems (Figure 7.3). Considering the total amount of both compounds (i.e., coumarin plus tHCA) responses to salinity (P<0.001) occurred in this experiment, because tHCA was considerably high and responsive to salinity.

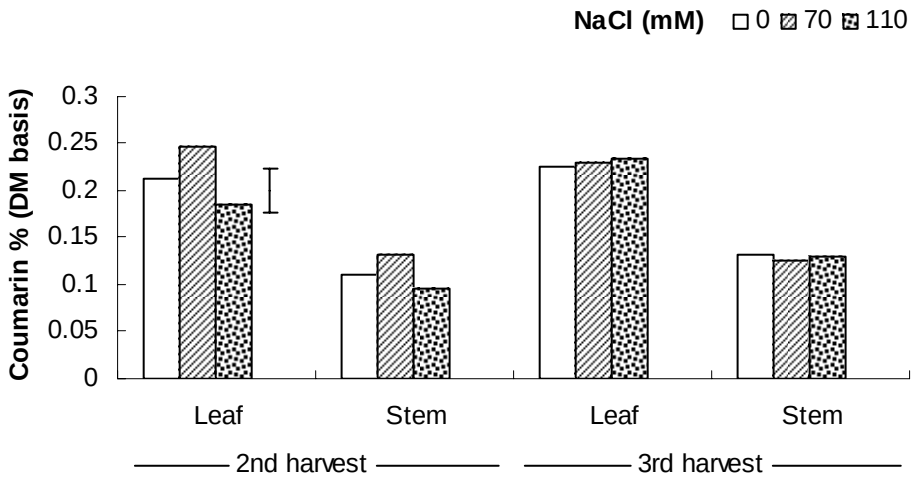


Figure 7.2. Coumarin concentration in leaves and stems of Melilotus in two harvests as affected by NaCl (data from experiment 1). The bar represents the least significant difference at P=5% for the comparison between salinity level and organ.

Experiment 2 (Harvests performed at similar phenological stage)

At harvests performed at similar phenological stage, coumarin concentration was not affected by salinity (Figure 7.4). Both leaf and stem, had similar coumarin concentrations to the first experiment. The tHCA increased with salinity (P<0.001) in the same way as in the first experiment, but the biggest differences, when compared to experiment 1, were that the concentration of tHCA was very low and both organs had similar concentrations (Figure 7.5).

Considering the total amount of both compounds (i.e., coumarin plus tHCA) salinity did not affect the total concentration.

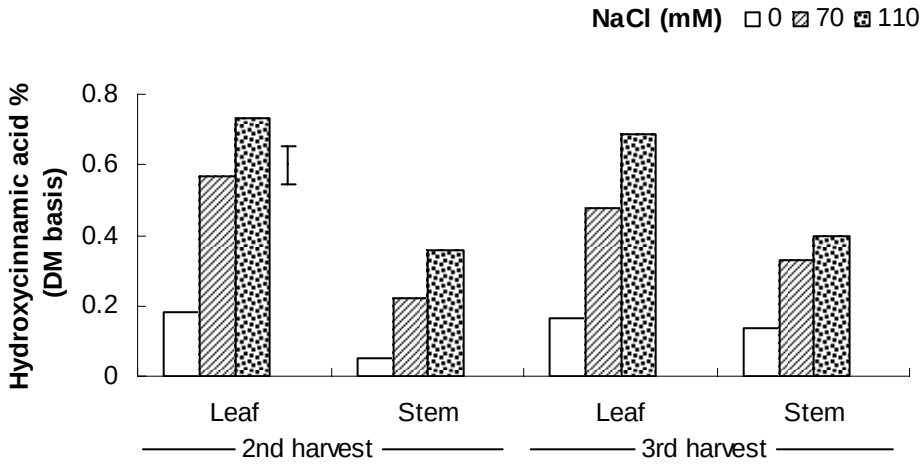


Figure 7.3. Concentration of 2-*trans*-hydroxycinnamic acid in Melilotus as affected by NaCl in leaves and stems in two harvests performed chronologically (data from experiment 1). The bar represents the least significant difference at P=5% for the comparison between salinity level and organ.

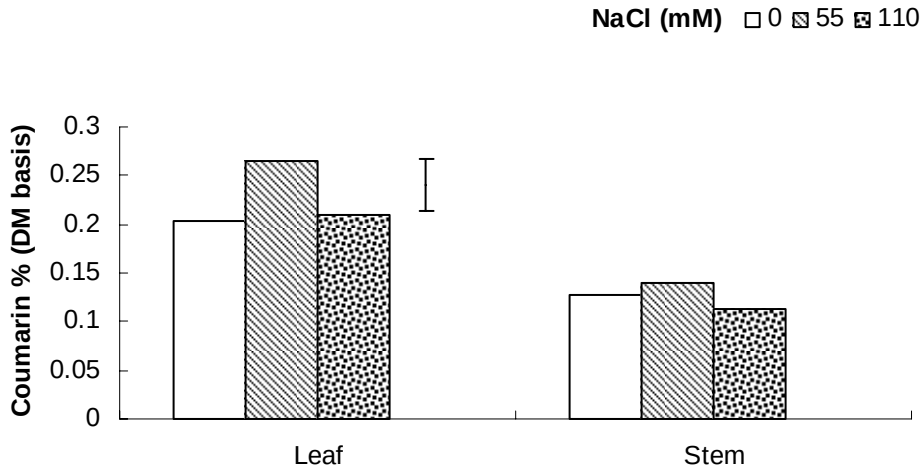


Figure 7.4. Coumarin concentration in leaves and stems of Melilotus at flowering (pooled harvests) as affected by NaCl (data from experiment 2). The bar represents the least significant difference at P=5% for the comparison between salinity level and organ.

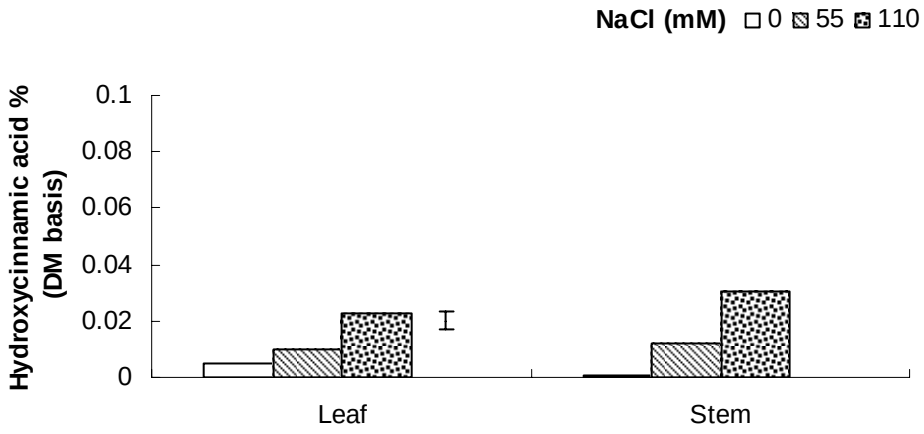


Figure 7.5. Concentration of 2-*trans*-hydroxycinnamic acid in Melilotus as affected by NaCl in leaves and stems in pooled harvests performed at same phenological stage (data from experiment 2). The bar represents the least significant difference at P=5% for the comparison between salinity level and organ.

For plants harvested in the experiments designed to evaluate the effect of sunlight, salinity had a negative impact on the coumarin concentration (Figures 7.6), an effect that was more noticeable in plants that received sunlight directly (P<0.001). In control plants at 0 mM of NaCl in sunlight, the coumarin concentration was double that of the control plants grown in the glasshouse. Plants in salt treatments in direct sunlight had a drop in coumarin vs the control (P=0.05). At the highest level of salinity (110 mM NaCl), the coumarin concentration was the same for plants grown in direct sunlight and in the glasshouse. In relation to tHCA, there were contrasting effects of salinity. Treatments in the glasshouse increased (P<0.001) the tHCA concentration as salinity increased (Figure 7.6. but, in the plants exposed to direct sunlight, this compound was not detected.

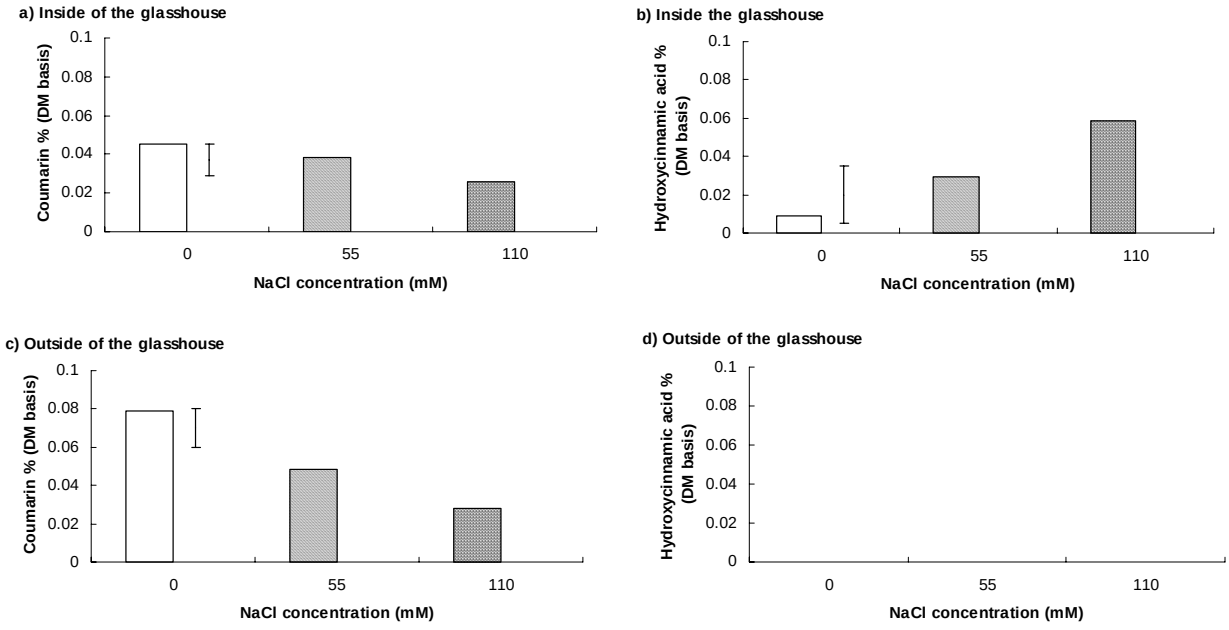


Figure 7.6. Coumarin and 2-*trans*-hydroxycinnamic acid concentrations in Melilotus leaf as affected by salinity. Plants were grown under glasshouse conditions (graphs a and b); plants grown outside of the glasshouse (graphs c and d). The concentration of 2-*trans*-hydroxycinnamic acid was nil in all the treatments for graph d. The bars represent the LSD at P=5%.

7.3 Discussion

Cultivars or genotypes with a high content of total hydroxycinnamic acids (coumarin and 2-*trans*-hydroxycinnamic acid) can range from 4 to 7 % of leaf dry matter and those with low concentration may range from 0.09 to 0.21% (Haskins and Gorz, 1970). According to these criteria, the cultivar "El Domador" could be considered to be in the medium category.

Variation in the measured coumarin and tHCA concentrations in plant biomass could be attributed in part to changes in light quality whilst conducting the experiments. This agrees with findings of Haskins and Gorz (1973), who found that the concentration of hydroxycinnamic acids was linked to the amounts of UV radiation that the plants received. They found that chamber-grown plants contained most of the hydroxycinnamic acids (90%) in the *trans* form,

glasshouse-grown plants contained about 35%, whereas field-grown plants contained only 10% in this form.

It has been observed that after defoliation the content in *o*-hydroxycinnamic acid increases (Kleinhofs et al, 1966) and this could explain differences in coumarin between the earlier experiments that received more harvests vs those that were harvested only once. There were higher concentrations of coumarin plus tHCA in the first experiment than in the second. This phenomenon may be linked to plant phenology, where plants in experiment 1 had not reached complete flowering. Haskins and Gorz (1961) suggested that *o*-hydroxycinnamic acid is synthesized most rapidly during the period of leaf expansion.

According to Gorz and Haskins (1969), stressful conditions that tend to reduce plant vigor, such as insect or disease damage, inadequate nodulation, drought, shading or poor nutrition tend to decrease the content of *o*-hydroxycinnamic acid. Overall, in the experiments conducted, salinity did not increase coumarin concentration, either under restricted sunlight or full exposure. In addition, when plants are under direct sunlight, coumarin tends to decrease under saline growth conditions and the 2-*trans*-hydroxycinnamic acid to be absent. This indicates that coumarin does not have a role in salinity tolerance and, one of its precursors (e.g., 2-*trans*-hydroxycinnamic acid) may not be a threat to livestock, as it is only detected under restricted conditions of light.

CHAPTER VIII
GENERAL DISCUSSION AND CONCLUSIONS

The main question addressed in this research was whether glycophytic legume forage species can maintain high nutritive quality characteristics and remain an option for animal production even though biomass production decreases under saline growing conditions. This question required an investigation into which traits associated with nutritive value would be more affected under saline conditions, and if positive effects could be found in the plant species at the levels of salinity examined. It was expected that the components of nutritive value would not be as sensitive to salinity as shoot biomass, because the adaptive mechanisms of plants aim at protecting the cellular components and minimizing the otherwise harmful effects imposed by salts. However, the degree of change in nutritive value is likely to depend on the salt tolerance of the plant and its physiological responses to salt. For this reason, the experiments reported in the previous chapters used two legume species, lucerne as a model species for being moderately sensitive to salt, and Melilotus as a species that is moderately tolerant.

It was found that salinity modified several physical characteristics of the plant. Salt-affected plants had reduced shoot dry matter, were shorter, had thinner stems, but were leafier than plants grown under no salinity. Leafiness is a desirable characteristic for improving nutritive value, as leaf has a higher quality than stem and it could partly compensate for the reduction in herbage yield. However, leafiness is a general description of a plant that does not describe changes in chemical composition that affect its utilization by animals.

Another characteristic which was modified was time of flowering, although only in Melilotus, which flowered earlier when grown in the presence of saline growing conditions. In Melilotus, earlier flowering did not affect digestibility, in contrast to expectations based on other forage legumes, such as lucerne and birdsfoot trefoil, that decline in digestibility as they mature (Buxton *et al.*, 1985; Buxton *et al.*, 1987; Albrecht *et al.*, 1987). Early flowering in this species means that the plant will reach its maximal yield earlier, with most metabolites subsequently channeled into supporting floral structures as a response to ensure survival under such conditions. This

implies that forage production will slow or stop prematurely, but there is an opportunity to set seed and ensure further re-establishment.

The main findings on the influence of salinity on nutritive value were:

- Crude protein

This component was not compromised by salinity and could be considered stable, as variations were slight. This was the case for both species and for both leaves and stems. When values of crude protein were analyzed at the whole-shoot level, considering the leaf-to-stem ratio, no differences occurred for lucerne in both experiments. In Melilotus, in the second experiment only, a 5% increase in crude protein per unit DM compared to the controls was attributed to increased leafiness. There may have been changes in proportions of true protein and non-protein components as some authors have indicated (Mansour, 2000; Sen *et al.*, 2002; Ashraf, 2004; Parida and Das, 2005), but this was not measured.

- Energy

Despite the similarities in the response to reduce NDF concentration as salinity increased, the species differed in digestibility. Digestibility of DM of lucerne leaves and stems were more affected by salinity than Melilotus, largely attributed to increases in soluble ash in lucerne. In this species at the whole-shoot level in the first experiment, increased leafiness of the plants exposed to salinity helped to maintain similar values to control plants. However, under the conditions of the second experiment, leafiness in lucerne was not enough to improve quality.
The slight increments (around 2% in leaves and up to 9% in stems) in digestibility in Melilotus could be important to animals. According to Casler (2001), small increases in digestibility can result in enhanced improvement in animal weight gain. This improvement, although small, may benefit grazing livestock.

- Minerals

Mineral concentrations were the components of nutritive value most affected by salinity. Although in both species there was a sharp increase in Na and Cl, the magnitude of increase differed between species. Lucerne accumulated more Na and Cl than Melilotus. The potential amounts of NaCl at the whole-shoot level in the first experiment for lucerne at 110 mM of NaCl treatment averaged 67 g of NaCl/ kg DM and for the second experiment 125 g NaCl/ kg DM. In Melilotus, 32 and 39 g of NaCl/kg DM were measured in the first and second experiments

respectively. High amounts of NaCl (>10% DM) in diets have been shown to impose a series of limitations on optimal animal production. Melilotus salt concentration fell into an acceptable range but, in the case of lucerne, evidence is provided that its herbage could reach high NaCl concentrations that may fall outside this range under certain conditions. Differences in total amounts of Na and Cl between experiments were attributed to the length of the harvest interval.

In both species, calcium, magnesium and zinc were reduced by effects of salinity. Magnesium is essential for all the rumen micro-organisms, as it maintains the integrity of cell membranes, activates bacterial enzymes, promotes protein synthesis and supports bacterial growth (Durand and Kawashima, 1980). Therefore a deficiency in this cation can seriously affect animal production. The mineral ratios of importance in animal nutrition affected negatively were the Na/K and K/(Ca+Mg), with values higher than 1-to-1. There was an improvement in the Ca-to-P ratio (values around 5-to-1), but the decrease in calcium may be more important that the ratio itself, as an appropriate ratio from deficient amounts of the elements can be meaningless. Overall, the cation-anion balance was shifted to less 'alkalogenic' values and this could have possible effects in the mineral nutrition of ruminants.

- Secondary compounds

In relation to anti-quality factors, it was shown that coumarin, which is related to bitterness of the forage in Melilotus and is involved in dicoumarol formation, was not increased under growth salinity. This is a favorable characteristic for this species, as one of the concerns about Melilotus is that its coumarin could be converted into dicoumarol upon fungal spoilage, with dicoumarol acting as an anticoagulant in animals. In addition, the bitterness of coumarin may reduce the voluntary intake of the plant. These issues, however, were not exacerbated by growth salinity.

It can be concluded that species with similar salt tolerance do not necessarily have an equivalent reduction in nutritive value as some traits may, to a certain extent, compensate for reductions in biomass accumulation that growth salinity causes.

Areas of further research

Possible areas of further research may include:

1) Development of salt-tolerant forage legume cultivars with improved nutritive value.

It was observed that both plant species turned leafy and there was a slight increase in organic matter in Melilotus under saline growing conditions. The increase in organic matter corresponded to non-structural material (probably starch), which can improve the energy value of the herbage. Both leafiness and increased starch concentration may show variability within other forage species and, in conjunction with other characteristics related to forage yield and survival, could make it possible to select cultivar with improved nutritive value. The presence of atypical or mutant plants (see Appendix A) may be a likely starting point. Both, lucerne and Melilotus are cross-pollinated species and show potential to withstand soil salinity. Thus, screening of large population is likely to identify such plants. It appears that specific plant characteristics in Melilotus may play an important role in salinity tolerance, as evidenced in root growth in the atypical plants, which could be of benefit. Attributes to measure should include, at least, concentration of Na and Cl (looking for acceptable levels), determination of organic matter and digestibility adjusted for soluble ash or expressed as DOMD. Inclusion of a control species would be helpful in order to establish comparisons.

2) Research for better understanding of components of nutritive value.

A considerable effect of salinity on some key minerals (e.g., reductions in calcium and magnesium) occurred. It could be important to research the bioavailability of these elements to animals in forages grown under saline conditions. High NaCl concentrations in the diet modify several parameters in the rumen (e.g., increased flow of fluids and shortened retention time of feeds), that could reduce mineral absorption. In addition, calcium and magnesium combine with oxalates, forms in which the element availability to the animal is sharply reduced (Masters *et al*., 2001). As calcium and magnesium concentrations are already low in plant tissue, any additional effect on their absorption and utilization could worsen mineral deficiencies. Little is known on the potential effects on production and if these minerals could represent a serious problem under saline growth conditions.

Vitamins are another topic where information under saline conditions is lacking. Some vitamins are involved in protection of the plant against effects of reactive oxygen species (Munné-Bosch, 2005), compounds that increase with salinity (Holmberg and Bülow, 1998; Apse and Blumwald, 2002; Garrat *et al*., 2002). Tocopherols (vitamin E) and carotenes

(provitamin A pigments) in the forms as α-tocopherol and β-carotene have been shown to participate in the protection of the photosystem II in chloroplasts from the damage caused by $^1O_2$ and $OH^{\cdot}$ (Munné-Bosch, 2005). These vitamins play an important role in animal nutrition and little is known about the influence of soil salinity on them in glycophytic forage species.

Starch accumulation in salt-affected herbage is an important characteristic in animal nutrition. The accumulation of this compound in the plant seems to be important as it can produce a dilution effect on the concentrations of Na and Cl on DM basis. Little is know about the physiology and variation at the whole-plant level and characteristics between genus and species under saline conditions.

In addition, in the plant-ruminant interface, rumen microbes play an important role. Thus, studies on *in vitro* digestibility might need to use microbes instead of enzymes. Information on this aspect is still scarce and it is possible that some interactions need to be taken into account to improve the understanding of the possible effects of the NaCl accumulated in the cell, and disturbed mineral ratios in the microbial populations.

3) Evaluate nutritive value under field conditions.

Plants under field conditions find different grades of salinity throughout the soil horizons (Ingvalson *et al*., 1976; Richards, 1992; Zhang *et al*., 1999) and root proliferation can differ accordingly (Vaughan *et al*., 2002). Such differences could introduce variations in nutritive value that may not be reflected when plants are exposed to constant salinity as in glasshouse experiments. Graduation of salinity levels in moderately salt-affected soil cores could be an approach to start with in evaluating nutritive value.

4) Animal feeding with legume salt-affected plant species.

According to the results of the present research, the concentration of crude protein and metabolisable energy meet the animal requirements (at least for sheep). It would be interesting to evaluate live weight changes in sheep fed a diet based on a salt-tolerant grass (or other forage material or halophytic species) and salt-affected herbage from a legume. This would be aimed at verifying the potential of legumes under saline conditions.

APPENDIX A

Atypical plants

There were five plants in a population of 720 plants of Melilotus that had different plant characteristics. These plants had greener leaves, stem nearly suppressed and instead there was a large crown-like structure on the base and very thick roots (Plate 3.3 and 3.4), probably a mutation.

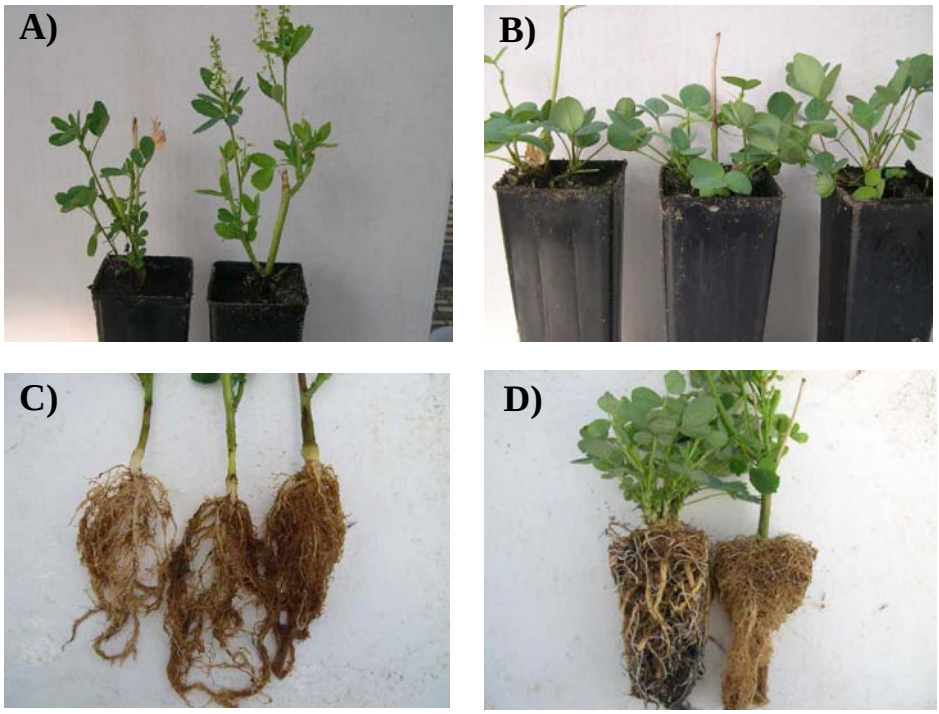


Plate 3.3. Typical and atypical plants of Melilotus. A) normal plants grown at 110 mM of NaCl; B) atypical plants grown at 110 mMof NaCl; C) roots of normal plants grown at 110 mM of NaCl; and D) left, atypical salt stressed plant grown at 110 mM NaCl and, right, normal plant grown at 0 NaCl mM.

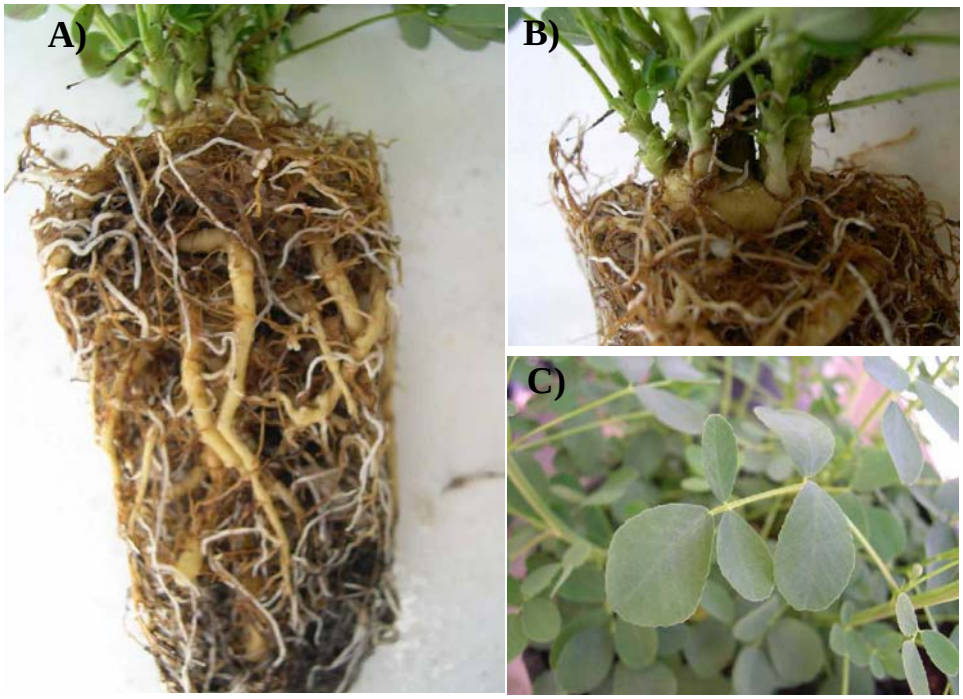


Plate 3.4. Root, stem and leaf of an atypical plant of Melilotus grown at 110 mM NaCl. A) thickened root system; B) close-up of the crown-like structure practically substituting the stem and perhaps functioning as a retention organ; and C) basal leaf of the same plant after harvest four when recovered from salt treatment, which could be the result of a mutation.

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