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## **DOCTOR OF PHILOSOPHY**

**Leaf ion concentrations and salt tolerance in barley.**

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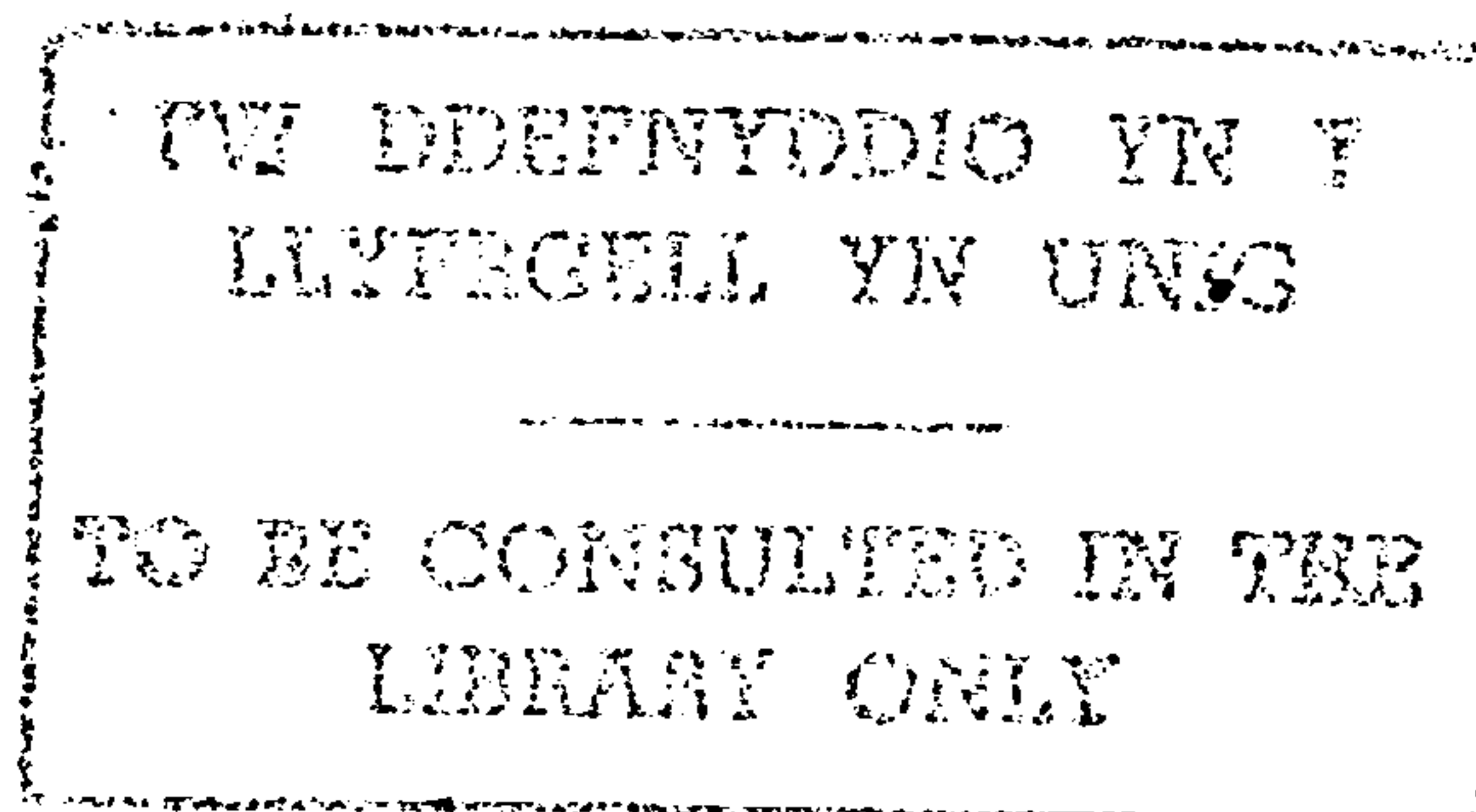
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**LEAF ION CONCENTRATIONS AND SALT TOLERANCE  
IN BARLEY**



A dissertation presented in partial fulfilment of the requirements  
for the degree of Doctor in Philosophy

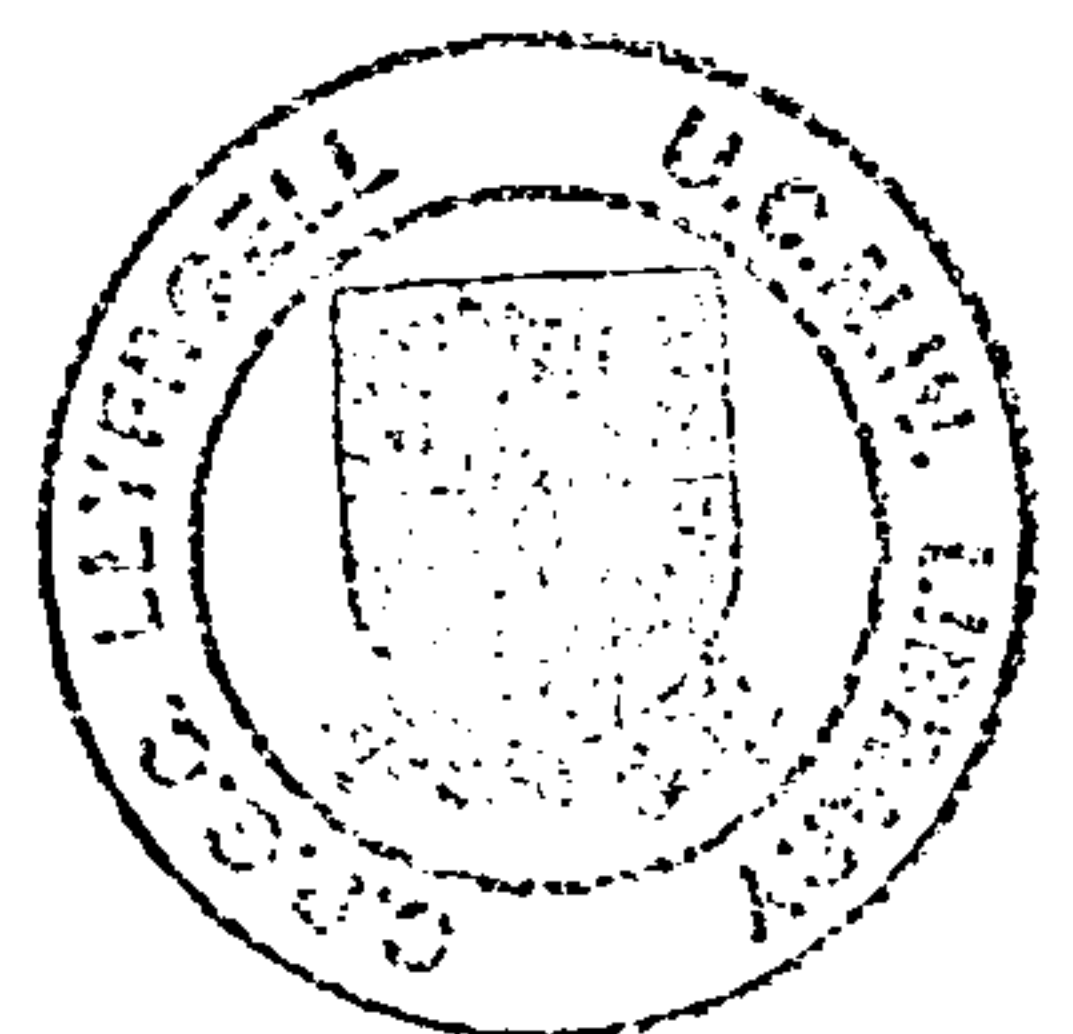
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## SUMMARY

Breeding and selection for salt tolerance has been limited because of the large heterogeneity of natural saline soils and the lack of efficient criteria for measuring salt tolerance. Regulation of salt balances in leaves is an important aspect of salt tolerance. This work analyses the relationship between leaf ion concentrations and salt tolerance with the aim of using these traits as indicators of salt tolerance. This is done both in solution culture (hydroponics) and field trials (sprinkler irrigation with saline water).

Varieties were found to differ in the amounts of ions accumulated in their leaves. However, these differences did not relate directly with their level of salt tolerance. The lack of correlation was partly due to difficulties in estimating salt tolerance in the field. Also, the Triple Line Sprinkler system (TLS) used in the field experiments posed several problems, the most important ones being related to direct ion absorption by the leaves. The high concentrations of  $\text{CaCl}_2$  (in addition to  $\text{NaCl}$ ) used in the irrigation water added a further complication.

In hydroponic experiments, a minimum of  $2 \text{ mol m}^{-3} \text{ Ca}^{2+}$  was enough to prevent an indiscriminate entry of  $\text{Na}^+$  and to ameliorate the growth inhibition of plants growing at  $200 \text{ mol m}^{-3} \text{ NaCl}$ . Higher  $\text{Ca}^{2+}$  concentrations ( $50 \text{ mol m}^{-3} \text{ CaCl}_2$ ) reduced even more the concentrations of  $\text{Na}^+$  in leaves without significantly affecting growth. At these high levels of  $\text{CaCl}_2$  any toxic effect was probably caused by high  $\text{Cl}^-$  concentrations.

It is concluded that the salt tolerance of barley can be improved, and that methods to control soil salinity need to be further developed, since the TLS proved to be an imperfect tool for studying plant responses to soil salinity.

# CONTENTS

SUMMARY . . . . .	I
CONTENTS . . . . .	II
LIST OF TABLES . . . . .	VII
LIST OF FIGURES . . . . .	IX
ACKNOWLEDGEMENTS . . . . .	XI
CHAPTER 1. LITERATURE REVIEW . . . . .	1
1.1. INTRODUCTION . . . . .	2
1.2. SALINE SOILS . . . . .	4
1.2.1. Characteristics of saline and sodic soils . . . . .	4
1.2.2. Saline soils in the Ebro Valley . . . . .	5
1.2.3. Line-Source Sprinkler Systems . . . . .	9
1.3. PHYSIOLOGY OF SALT TOLERANCE . . . . .	11
1.3.1. Effects of salinity on plants . . . . .	11
1.3.2. Osmotic stress . . . . .	12
1.3.3. Responses to osmotic stress . . . . .	13
1.3.3.1. Turgor regulation and osmotic adjustment . . . . .	13
1.3.3.2. Changes in cell wall characteristics . . . . .	14
1.3.4. Ionic stress . . . . .	14
1.3.4.1. Specific ion effects . . . . .	14
1.3.4.2. Toxic effects . . . . .	15
1.3.5. Responses to ionic and toxic effects . . . . .	15
1.3.5.1. Ion exclusion <i>versus</i> ion inclusion . . . . .	15
1.3.5.2. Cellular ion compartmentation . . . . .	16
1.3.5.3. Regulation of salt balances in leaves . . . . .	17
1.3.6. Causes of reduced growth . . . . .	18
1.4. SALT TOLERANCE IN CULTIVATED PLANTS . . . . .	21
1.4.1. Agronomic aspects of salt tolerance . . . . .	21
1.4.2. Measurement of salt tolerance; models . . . . .	23
1.4.3. Comparative salt tolerance of crops . . . . .	25
1.4.4. Mechanisms of salt tolerance in cereals . . . . .	26

1.5. SELECTION AND BREEDING FOR SALT TOLERANCE . . . . .	28
1.5.1. General . . . . .	28
1.5.2. Breeding methods . . . . .	28
1.5.3. Environment for selection . . . . .	30
1.5.4. Selection criteria for salt tolerance . . . . .	31
1.5.5. Physiological traits in breeding for salt tolerance . . . . .	32
1.5.6. Use of new technologies . . . . .	33
1.6. GENERAL OBJECTIVES OF THE PRESENT WORK . . . . .	34
<b>CHAPTER 2. BARLEY IN SALINITY:         ION UPTAKE AND OSMOTIC ADJUSTMENT . . . . .</b>	<b>35</b>
2.1. INTRODUCTION . . . . .	36
2.2. COMPARISON OF BARLEY VARIETIES UNDER SALINITY: ION ACCUMULATION AND GROWTH . . . . .	38
2.2.1. Objectives . . . . .	38
2.2.2. Materials and methods . . . . .	39
2.2.3. Results . . . . .	42
2.2.3.1. Plant growth . . . . .	42
2.2.3.2. Ion concentrations in leaves . . . . .	43
2.2.3.3. Osmotic pressure of leaf sap . . . . .	49
2.2.3.4. Overview of ion data . . . . .	49
2.2.3.5. Relationship between leaf ion concentrations and growth	52
2.2.4. Discussion . . . . .	53
2.3. OSMOTIC ADJUSTMENT OF BARLEY UNDER SALINITY . . . . .	59
2.3.1. Objectives . . . . .	59
2.3.2. Materials and methods . . . . .	60
- <i>Experiment 1</i> . . . . .	60
- <i>Experiment 2</i> . . . . .	63
2.3.3. Results . . . . .	65
- <i>Experiment 1</i> . . . . .	65
2.3.3.1. Inorganic ions . . . . .	65
2.3.3.2. Organic solutes . . . . .	67
2.3.3.3. Osmotic pressure and charge balance . . . . .	68

- <i>Experiment 2</i> . . . . .	70
2.3.3.4. Inorganic ions . . . . .	70
2.3.3.5. Organic solutes . . . . .	72
2.3.3.6. Osmotic pressure and charge balance . . . . .	72
2.3.4. Discussion . . . . .	74
<b>CHAPTER 3. SALINITY - CALCIUM INTERACTIONS . . . . .</b>	<b>79</b>
3.1. INTRODUCTION . . . . .	80
3.2. MINIMUM Ca <sup>2+</sup> REQUIREMENTS IN SALT-STRESSED BARLEY	85
3.2.1. Objectives . . . . .	85
3.2.2. Materials and Methods . . . . .	85
3.2.3. Results . . . . .	86
3.2.3.1. Plant growth . . . . .	86
3.2.3.2. Ion concentrations . . . . .	88
3.2.4. Discussion . . . . .	90
3.3. EFFECTS OF HIGH LEVELS OF Ca <sup>2+</sup> (AS CaCl <sub>2</sub> ) IN BARLEY . . .	93
3.3.1. Objectives . . . . .	93
3.3.2. Materials and methods . . . . .	93
- <i>Experiment 1</i> . . . . .	93
- <i>Experiment 2</i> . . . . .	94
3.3.3. Results . . . . .	95
- <i>Experiment 1</i> . . . . .	95
3.3.3.1. Plant growth . . . . .	95
3.3.3.2. Ion concentrations in young leaves and roots . . . . .	96
- <i>Experiment 2</i> . . . . .	97
3.3.3.3. Plant growth . . . . .	97
3.3.3.4. Ion concentrations in young leaves . . . . .	98
3.3.4. Discussion . . . . .	100
3.4. COMPARISON OF SODIUM AND CALCIUM TOXICITIES . . . . .	104
3.4.1. Objectives . . . . .	104

3.4.2. Materials and methods . . . . .	105
- <i>Experiment 1</i> . . . . .	105
- <i>Experiment 2</i> . . . . .	106
3.4.3. Results . . . . .	108
- <i>Experiment 1</i> . . . . .	108
3.4.3.1. Ion concentrations in leaves . . . . .	108
3.4.3.2. Plant growth . . . . .	110
- <i>Experiment 2</i> . . . . .	112
3.4.3.3. Ion concentrations in leaves . . . . .	112
3.4.3.4. Plant growth . . . . .	115
3.4.4. Discussion . . . . .	118
<b>CHAPTER 4. SALT TOLERANCE IN THE FIELD . . . . .</b>	<b>122</b>
4.1. INTRODUCTION: THE TRIPLE LINE SOURCE SYSTEM . . . . .	123
4.2. 1991/92 FIELD (TLS) EXPERIMENT . . . . .	126
4.2.1. Objectives . . . . .	126
4.2.2. Materials and methods . . . . .	127
- <i>Sampling for sap extraction and ion analysis</i> . . . . .	132
- <i>Statistical methods</i> . . . . .	135
4.2.3. Results . . . . .	137
4.2.3.1. Soil and water salinity . . . . .	137
4.2.3.2. Ion concentrations in leaves . . . . .	139
4.2.3.3. Grain yield . . . . .	149
4.2.3.4. Relationship between leaf ion concentrations and yield .	152
4.2.4. Discussion . . . . .	156
4.2.4.1. Soil and water salinity distribution . . . . .	156
4.2.4.2. Ion concentrations in leaves . . . . .	157
4.2.4.3. Grain yield . . . . .	160
4.2.4.4. Relationship between leaf ion concentrations and yield .	163
4.3. 1992/93 FIELD (TLS) EXPERIMENT . . . . .	164
4.3.1. Objectives . . . . .	164
4.3.2. Materials and methods . . . . .	164
- <i>Statistical methods</i> . . . . .	166

4.3.3. Results . . . . .	169
4.3.3.1. Soil and water salinity . . . . .	169
4.3.3.2. Ion concentrations in leaves . . . . .	171
4.3.3.3. Grain yield . . . . .	178
4.3.3.4. Relationship between leaf ion concentrations and yield .	181
4.3.4. Discussion . . . . .	181
4.3.4.1. Soil and water salinity distribution . . . . .	181
4.3.4.2. Ion concentrations in leaves . . . . .	183
4.3.4.3. Grain yield . . . . .	184
4.3.4.4. Relationship between leaf ion concentrations and yield .	186
<b>CHAPTER 5. GENERAL DISCUSSION . . . . .</b>	<b>189</b>
- Measurement of salt tolerance in the field: the TLS . . . . .	190
- Use of models to measure salt tolerance . . . . .	192
— - Measurement of salt tolerance in nutrient solution . . . . .	193
— - Salt tolerance of barley varieties . . . . .	196
— - Measurement of ion concentrations: leaf age and position . . . . .	197
- - Ion concentrations: effect of high Ca <sup>2+</sup> . . . . .	200
- Osmotic adjustment . . . . .	202
- General conclusions . . . . .	203
<b>APPENDICES . . . . .</b>	<b>207</b>
<b>REFERENCES . . . . .</b>	<b>212</b>



## LIST OF TABLES

### - Literature review:

Table 1.1. Quality of irrigation water in the Ebro Valley . . . . .	8
Table 1.2. Composition of saline soils in the Ebro Valley . . . . .	9
Table 1.3. Salt tolerance of some crops . . . . .	26

### - "Comparison of varieties" experiment:

Table 2.2.1. Plant growth . . . . .	43
Table 2.2.2. Cations in leaf sap . . . . .	44
Table 2.2.3. Anions in leaf sap . . . . .	47
Table 2.2.4. Osmotic pressure in leaf sap . . . . .	49
Table 2.2.5. Principal components analysis (ions) . . . . .	50
Table 2.2.6. Correlations: ions in leaves vs growth . . . . .	53

### - "Osmotic adjustment" experiments:

Table 2.3.1. Ion concentrations in leaf sap (Exp. 1) . . . . .	66
Table 2.3.2. Organic solutes in leaf extracts (Exp. 1) . . . . .	68
Table 2.3.3. Osmotic pressure in leaf sap (Exp. 1) . . . . .	69
Table 2.3.4. Inorganic and organic solutes in leaves (Exp. 2) . . . . .	71
Table 2.3.5. Osmotic pressure in leaf sap (Exp. 2) . . . . .	73
Table 2.3.6. Inorganic ions (Exp. 1) on a dry weight basis . . . . .	77

### - "Minimum Ca<sup>2+</sup>" experiment:

Table 3.2.1. Plant growth . . . . .	87
Table 3.2.2. Ions in leaves and roots . . . . .	89

### - "High Ca<sup>2+</sup>" experiments:

Table 3.3.1. Plant growth (Exp. 1) . . . . .	96
Table 3.3.2. Ions in leaves and roots (Exp. 1) . . . . .	97
Table 3.3.3. Plant growth (Exp. 2) . . . . .	98
Table 3.3.4. Ions in leaves (Exp. 2) . . . . .	99

- "Na<sup>+</sup> versus Ca<sup>2+</sup>" experiments:

Table 3.4.1. Electrical conductivity of external solution (Exp. 1) . . . . .	105
Table 3.4.2. Electrical conductivity of external solution (Exp. 2) . . . . .	107
Table 3.4.3. Ions in leaves (Exp. 1) . . . . .	109
Table 3.4.4. Plant growth (Exp. 1) . . . . .	110
Table 3.4.5. Ions in leaves (Exp. 2) . . . . .	115
Table 3.4.6. Plant growth (Exp. 2) . . . . .	118

- Field (TLS) 1991/92 experiment:

Table 4.2.1. Correlations ions (Cl <sup>-</sup> , Na <sup>+</sup> , K <sup>+</sup> ) in leaves and soil salinity . .	143
Table 4.2.2. Goodness of fit, response models . . . . .	149
Table 4.2.3. Threshold model: values of parameters . . . . .	150
Table 4.2.4. Sigmoidal model: values of parameters . . . . .	152

- Field (TLS) 1992/93 experiment:

Table 4.3.1. Correlations ions (Cl <sup>-</sup> , Na <sup>+</sup> ) in leaves and soil salinity . . . . .	172
Table 4.3.2. Correlations ions (Ca <sup>2+</sup> , K <sup>+</sup> ) in leaves and soil salinity . . . . .	176
Table 4.3.3. Goodnes of fit, response models . . . . .	180
Table 4.3.4. Sigmoidal model: values of parameters . . . . .	180
Table 4.3.5. Ranking of varieties (EC <sub>50</sub> and mean yield) . . . . .	184

## LIST OF FIGURES

### - Literature review:

Figure 1.1. Location of saline soils in the Ebro Valley . . . . .	6
Figure 1.2. Threshold (Maas and Hoffman) model . . . . .	24
Figure 1.3. Sigmoidal (Van Genuchten) model . . . . .	24
Figure 1.4. Classification of crop salt tolerance . . . . .	24

### - "Comparison of varieties" experiment:

Figure 2.2.1. Principal components analysis (leaf ions) . . . . .	51
---	----

### - Na<sup>+</sup> versus Ca<sup>2+</sup> experiments:

Figure 3.4.1. Na <sup>+</sup> in leaves vs Na <sup>+</sup> in external solution (Exp. 1) . . . . .	111
Figure 3.4.2. Plant dry weight vs Cl <sup>-</sup> in young leaves (Exp. 1) . . . . .	113
Figure 3.4.3. Area of leaf 4 vs Cl <sup>-</sup> in young leaves (Exp. 1) . . . . .	113
Figure 3.4.4. Plant dry weight vs Cl <sup>-</sup> in external solution (Exp. 1) . . . . .	114
Figure 3.4.5. Plant dry weight vs external osmotic pressure (Exp.1) . . . . .	114
Figure 3.4.6. Plant dry weight vs external electrical conductivity (Exp. 1) .	114
Figure 3.4.7. Plant dry weight vs Cl <sup>-</sup> in leaves (Exp. 2) . . . . .	116
Figure 3.4.8. Area of leaf 4 vs Cl <sup>-</sup> in leaves (Exp. 2) . . . . .	116
Figure 3.4.9. Plant dry weight vs external Na <sup>+</sup> or Ca <sup>2+</sup> (Exp. 2) . . . . .	117
Figure 3.4.10. Area of leaf 4 vs external Na <sup>+</sup> or Ca <sup>2+</sup> (Exp. 2) . . . . .	117

### - Field (TLS) 1991/92 experiment:

Figure 4.1.1. Design of the Triple Line System . . . . .	124
Figure 4.2.1. Map of the TLS in the 1992 experiment . . . . .	129
Figure 4.2.2. Salinity of the irrigation water . . . . .	131
Figure 4.2.3. Soil salinity for each treatment, variety and replicate . . . . .	138
Figure 4.2.4. Amount and salinity of irrigation water . . . . .	140
Figure 4.2.5. Evolution of soil salinity with time . . . . .	140
Figure 4.2.6. Salinity in the soil profile (depth) . . . . .	141
Figure 4.2.7. Cl <sup>-</sup> concentrations in different leaves . . . . .	144
Figure 4.2.8. Na <sup>+</sup> concentrations in different leaves . . . . .	146
Figure 4.2.9. K <sup>+</sup> concentrations in different leaves . . . . .	148
Figure 4.2.10. Grain yield vs soil salinity; models . . . . .	151
Figure 4.2.11. Relative yield vs soil salinity; models . . . . .	153
Figure 4.2.12. Yield vs Cl <sup>-</sup> in leaves . . . . .	154
Figure 4.2.13. Relative yield vs Cl <sup>-</sup> in leaves . . . . .	155

**- Field (TLS) 1992/93 experiment:**

Figure 4.3.1. Amount and salinity of irrigation water . . . . .	170
Figure 4.3.2. Evolution of soil salinity with time . . . . .	170
Figure 4.3.3. Cl <sup>-</sup> concentrations in leaves . . . . .	173
Figure 4.3.4. Na <sup>+</sup> concentrations in leaves . . . . .	175
Figure 4.3.5. Ca <sup>2+</sup> concentrations in leaves . . . . .	177
Figure 4.3.6. K <sup>+</sup> concentrations in leaves . . . . .	179
Figure 4.3.7. Yield vs Cl <sup>-</sup> in leaves . . . . .	182
Figure 4.3.8. Salt tolerance (EC <sub>50</sub> ) vs yield potential (Y <sub>m</sub> ) . . . . .	187

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# CHAPTER ONE

## LITERATURE REVIEW

### 1.1. INTRODUCTION

Rapid population growth in recent years has resulted in increased food demands. However, food production has not grown at the same rate as world population, in spite of the expansion of cultivated areas and the increase in crop yields. This imbalance has resulted in food shortages in the poorest, over-populated countries, and the situation is expected to worsen if present trends do not change. On the other hand, fertile arable lands are limited, and there is a pressure for bringing into cultivation lands which at present are not used because of their poor quality. A significant proportion of these areas are affected by soil salinity.

Estimates of the extent of land affected by salinity range from 344 million hectares (Ponnamperuma, 1984) to more than 900 million hectares (Szabolcs, 1989, cited by Szabolcs, 1991). Even the lower estimate represents a substantial area of the earth's land surface. From the point of view of agriculture, these salt-affected areas represent between 13% (Flowers & Yeo, 1988) and 23% (Tanji, 1990) of cultivated land, and between 30% (Epstein *et al.*, 1980) and 50% (Flowers & Yeo, 1988) of the land under irrigation.

Most of the world's saline soils occur in arid and semi-arid regions. Crop production in these areas needs irrigation, and irrigation always adds salts to the soil. The amount of salts added in this way depends on the concentration of salts in the irrigation water, and the amount of water entering the soil. However, even good quality waters contain enough dissolved salts to result in substantial amounts of salts being added to the soil at the end of a cropping season. (A high quality water with less than 0.2 g l<sup>-1</sup> of total dissolved salts, applied at a rate of 500 mm a year, adds 1 tonne of salt per hectare a year.) When this water is removed from the soil by evapotranspiration, it leaves the salts behind.

At the same time, as water passes through the soil it also dissolves salts. As a result, drainage water has higher salt concentrations than irrigation water. These drainage waters eventually flow to the rivers, usually downstream from where they had been taken. At each cycle of irrigation and drainage, water becomes more salinized. As a consequence, it is usually observed that the level of salinization of a river increases from its source to its mouth.

Although some plants (halophytes) grow naturally in saline soils, most crop species are affected to some extent by high salt concentrations. A figure of  $40 \text{ mol m}^{-3}$  NaCl is usually given as the level of salt concentration which begins to cause injury to most plants (Ponnamperuma, 1984). Because of decreased crop yields and the concurrent economic losses, salt-affected lands are progressively abandoned as unproductive. This only helps to spread desertification.

Several strategies are available to minimize the detrimental effects of salinity. They include the reclamation of saline soils by leaching the excess of salts, the use of adequate agronomic practices (particularly in relation to irrigation and drainage), and the use of salt tolerant species and varieties.

Leaching involves the application of good quality water to the soil, which dissolves the soluble salts in it and removes them from the root zone by deep percolation. The use of this technique, however, is often limited by the availability of good quality water and adequate drainage.

The adverse effects of salinity on crops may also be reduced by adequate management of irrigation. For example, a high uniformity and efficiency of irrigation may reduce the need for artificial drainage; increased irrigation frequency maintains a high water content in the soil and avoids additional water stress; applying water in excess of the crop requirements contributes to leaching and avoids accumulation of salts in the root zone. Other cultural practices may facilitate germination and seedling establishment, particularly in those crops which are more salt-sensitive at the early stages of growth.



The use of more salt-tolerant species and varieties (i.e. which are able to grow and produce acceptable yields at higher levels of salinity) is another step in the process of reclaiming salt-affected soils. It is particularly interesting for those areas where only poor quality (saline) water is available for irrigation. However, it has to be said that the use of tolerant varieties is not a solution on its own. Unless it is accompanied by other measures to remove salts from the soil, and to avoid their accumulation in the first place, in the long term soil salinity will increase and eventually reach levels too high even for the most tolerant crops.

## 1.2. SALINE SOILS

### 1.2.1. Characteristics of saline and sodic soils

Saline soils influence the growth of plants by osmotic and specific ion effects; that is, they reduce the availability of water to the plant (a physical effect), and may also induce chemical effects due to the presence of certain ions. Thus, not only the total concentration of salts, but also the nature of these salts (as well as many other factors) will influence the responses of plants to salinity. However, some criterion has to be used to distinguish between saline and non-saline soils, even if this criterion is somehow arbitrary.

Saline soils are usually defined as having an electrical conductivity (EC) of the saturation extract greater than  $4 \text{ dS m}^{-1}$  at  $25^\circ\text{C}$  ( $\approx 40 \text{ mol m}^{-3} \text{ NaCl}$ ) (Richards, 1954). Sodicty refers to the accumulation of  $\text{Na}^+$  ions on the exchange phase, which has a direct effect on soil properties, by swelling and dispersion of clays and breaking down of aggregates. These effects result in a lower soil permeability to water and air, and a loss of structure (Shainberg & Singer, 1990). In contrast, the high salt concentrations present in saline soils do not adversely affect the physical properties of the soil (Rhoades, 1990). In this sense, sodic soils are more detrimental to plant growth than saline soils.

Sodicity is estimated by several criteria, the most common ones being the exchangeable sodium percentage (ESP) and the sodium absorption ratio (SAR). The ESP is the percentage of the cation exchange capacity (CEC) of the soil which is occupied by  $\text{Na}^+$ . Soils with ESP greater than 15 are considered sodic (Richards, 1954). The SAR relates the activity of  $\text{Na}^+$  ions to those of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , and is defined as:

$$\text{SAR} = \frac{\text{Na}^+}{[(\text{Ca}^{2+} + \text{Mg}^{2+})/2]^{1/2}}$$

(all concentrations in milliequivalents per litre). Values of SAR exceeding 13 also indicate sodicity (Richards, 1954).

By definition, saline soils have an ESP lower than 15 (Richards, 1954). Saline soils ( $\text{EC} > 4 \text{ dS m}^{-1}$ ) with ESP greater than 15 are termed saline-sodic.

One of the principal characteristics of saline soils is the irregular distribution of salinity in them, both in time and space. This is not only true for natural saline soils, but also for irrigated soils, where salt distribution depends largely on irrigation practices and the extent of leaching and drainage. The salt profile of an irrigated field usually increases with depth, with low concentrations (similar to that of the irrigation water) near the surface, and much higher concentrations at the bottom of the root zone. In some cases, if a shallow saline water table exists or if saline water is used for irrigation, the highest salt concentrations may be found on the top part of the soil, resulting in what is commonly known as an "inverted" profile. Soil salinity also varies with time as a result of changes in the amount of water: dilution by rain and irrigation, and concentration by evapotranspiration.

### 1.2.2. Saline soils in the Ebro Valley

The Ebro Valley, covering about 83000  $\text{km}^2$ , is located in the north-east of Spain, and is limited in the north by the Pyrenees, which are the source of most of its waters. The salt-affected soils of the Ebro Valley are located in its central zone

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(Figure 1.1) and cover an area of more than 300000 ha (Alberto *et al.*, 1986). The main factors that contribute to the salinization of this region are the geology, the climate and the topography.

The Ebro Valley has been formed upon materials rich in salts ( $\text{CaSO}_4$ ,  $\text{MgSO}_4$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{NaCl}$ ,  $\text{MgCl}_2$ ) which act as a centre of salt redistribution. The climate of the central area is arid or semi-arid, with an annual rainfall below 400 mm. With evapotranspiration (ETP) being larger than precipitation, the leaching of salts is not effective, and in places where there is a shallow water table, water and salts ascend to the soil surface. The occurrence of strong winds intensifies the evaporation of water, thus increasing the concentration of salts. Because of all these factors, salinity may develop quite easily, especially in topographically depressed zones due to accumulation of water and its subsequent evaporation.

In addition to these natural factors (geology and climate), salinization is intensified by human action: land cultivation, irrigation, and deforestation are all processes that increase the flow of water and the redistribution of salts. Under the arid climatic conditions of the zone, dryland (rainfed) agriculture is not economically viable, and irrigation is essential. The large engineering works undertaken to bring into irrigation many areas of the Ebro Valley (started in the last century but mainly developed from 1940 onwards) were not accompanied by drainage systems until much later, when flooded and salinized areas had already appeared.

Irrigation water enhances the natural salinization process, because it reaches deeper layers than the small amount of natural rainfall and dissolves new salts; these ascend by capillary action (driven by evapotranspiration) and accumulate on the soil surface. This process is highlighted in low-lying areas where water tends to accumulate, giving rise to the typical irregular distribution of salinity found in even small areas.

The problem is also intensified due to the poor quality of irrigation water. Some of the water comes from reservoirs located upstream (near the Pyrenees) and its salt content is low (EC: 0.2 - 0.4 dS  $\text{m}^{-1}$ ); this kind of water is found in the main irrigation canals (Table 1.1). However, this water is reused several times, so that

increasing salinities are found when looking at minor waterways (Table 1.1). If irrigation water comes from wells, its quality depends on the lithology of their location (Table 1.1).

Table 1.1. Quality of some waters from irrigation canals and wells in the Ebro Valley (after Porta & Boixadera, 1988); (EC in  $\text{dS m}^{-1}$  at  $25^\circ\text{C}$ ; ion concentrations in  $\text{mol m}^{-3}$ ).

Water Origin	EC	$\text{HCO}_3^-$	$\text{SO}_4^{2-}$	$\text{Cl}^-$	$\text{Ca}^{2+}$	$\text{Mg}^{2+}$	$\text{Na}^+$	$\text{K}^+$
Main Canal	0.25	1.8	0.4	0.3	2.1	0.3	0.2	0.1
Minor Course	1.85	5.3	12.1	4.9	6.3	8.1	6.5	0.1
Well-1	2.48	6.2	20.5	3.9	7.8	10.9	11.5	0.2
Well-2	0.75	nd	nd	0.6	2.8	4.5	0.9	0.1

nd - not determined

The salt-affected soils of the Ebro Valley are mainly saline soils; that is, with a high content of soluble salts ( $\text{EC} > 4 \text{ dS m}^{-1}$ ) and a favourable Ca/Na relationship ( $\text{SAR} < 13$ ), due to the presence of gypsum and soluble  $\text{Ca}^{2+}$  in the soils. Some sodification processes have been reported locally (Porta *et al.*, 1986), but these are not important enough to develop a sodic soil (according to the criteria of Soil Taxonomy (USDA, 1975)). Sodidity, when it appears, is accompanied by salinity (saline-sodic soils:  $\text{EC} > 4 \text{ dS m}^{-1}$  and  $\text{SAR} > 13$ ).

The ionic composition of the soils varies depending on the area considered: in some places  $\text{Cl}^-$  is the main anion, and in others  $\text{SO}_4^{2-}$ ; the same happens with  $\text{Na}^+$  and  $\text{Mg}^{2+}$ . The main ions found in the saturated extract of a number of soil samples are shown in Table 1.2. There, the concentrations of individual ions are expressed as percentage of their combined concentrations ( $\text{mol m}^{-3}$ ); the variability between different soils is reflected by the high standard deviations.

Table 1.2. Relative ionic composition (percentage of total main ion concentration, in mol m<sup>-3</sup>, which correspond to each ion) in saturated extracts of saline soils of the Ebro Valley; (mean  $\pm$  standard errors from 254 samples); (after Alberto *et al.*, 1986).

HCO <sub>3</sub> <sup>-</sup>	SO <sub>4</sub> <sup>2-</sup>	Cl <sup>-</sup>	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Na <sup>+</sup>
3 $\pm$ 7	42 $\pm$ 18	55 $\pm$ 19	15 $\pm$ 18	22 $\pm$ 23	63 $\pm$ 22

Some authors have tried to estimate the economic cost of salinity, both of soils and waters, in the agriculture of the region. Thus, Albisu *et al.* (1988) estimated the losses due to irrigating with water which had a mean EC of 2 dS m<sup>-1</sup> to be about 22000 ptas (around £110) per hectare per year. A similar figure was calculated from the data of Zekri *et al.* (1990) for an area where more than 20% of the land was highly saline (EC > 8 dS m<sup>-1</sup>), but irrigated with water of good quality (EC < 0.4 dS m<sup>-1</sup>). These figures represent between 15% and 22% decrease in the gross benefit that could be obtained if the crops were producing at their maximum capacity (yield not reduced by salinity).

In summary it can be said that the general presence of salts in many soils of the Ebro Valley makes it an impossible task to try to leach them by engineering methods, and the area affected by salts is large enough to have an important impact on the economy of the area.

### 1.2.3. Line-Source Sprinkler Systems

It is well recognised that the large heterogeneity, both spatial and temporal, of salinity in soils precludes the use of natural saline soils for testing crop salt tolerance (Shannon & Noble, 1990). That is why most of the research on the salt tolerance of crops has been carried out under "artificial" conditions, using solution (hydroponic) or sand cultures, either in growth chambers or greenhouses. However, the performance of crops in these conditions might be very different from that in the field, especially in regard to yield, so field testing under more "natural"

environmental and edaphic conditions is still necessary. To overcome the problem of the large variability of natural saline soils, systems have been developed in order to experiment with controlled salinity levels in the field. One such system is the Triple Line Source Sprinkler (TLS), developed by Aragüés *et al.* (1992).

The TLS is a modification of the line-source sprinkler system developed by Hanks *et al.* (1976). The original system used a single line of sprinklers along the centre of a plot to produce a continuous gradient of applied water across the plot, and was designed for studies of the response of crops to decreasing levels of irrigation water. By placing other treatment variables randomly in strips at right angles to the irrigation treatment, other factors can be studied. This layout has been used, among others, by Hanks *et al.* (1977) to study interactions of salinity and irrigation, and by Sorensen *et al.* (1980), who compared varieties and cultivation methods in response to decreasing irrigation.

While the single line-source is useful for irrigation studies, it is unsuitable for research with sprinkler-applied treatments, because the gradients of the applied substance and water coincide, and thus their effects cannot be separated. Lauer (1983) extended the original layout by using a triple line-source to experiment with sprinkler-applied N fertilizer; this design produces a gradient of N while supplying a uniform amount of water, by injecting N into the middle line and water in the two outer lines. Other authors have used double line-source sprinklers to study salinity (Morkoc *et al.*, 1985), salinity and N (Broadbent *et al.*, 1988), and a two-crossed triple line system has even been developed for a study of the interactive effects of salinity and N (Magnusson *et al.*, 1989).

The line-source systems have been found suitable for the application of a continuous gradient of water, provided that the system is operated only in low winds, and that the application rates are kept low to avoid ponding and runoff (Hanks *et al.*, 1976; Aragüés *et al.*, 1992). One disadvantage of this layout is related to the non-randomization of the continuous variable treatment. The most usual statistical tests (anova, regression) assume that all non-controlled variability (arising from genetic variations, cultivation procedures, measurement errors and different soil properties)

is randomly distributed. But irrigation, salinization and other treatments applied by the line-source sprinklers are, by the nature of the design, systematically arranged, and those tests are no longer valid. Hanks *et al.* (1980) realised this limitation, and warned about the potential bias in the estimates of the regressions that might be brought about by the systematic arrangement. Johnson *et al.* (1983) suggested the use of multivariate methods to overcome the problem of non-randomization. Morgan and Carr (1988) used analysis of covariance to remove any fertility trends perpendicular to the sprinkler line (the direction in which no randomization is possible). In general, however, good symmetry in the response around the two sides of the centre line is an indicator that there are no fertility trends (unless these are also symmetrical and coincide with the treatment gradient). Therefore, no highly complex statistical analyses are used in practice.

The Triple Line Source (TLS) sprinkler system consists of 3 parallel sprinkler lines which supply fresh water by the 2 outer lines, and a saline solution by the centre line. This results in a continuous gradient of salinity with the same volume of water between each lateral pair, and permits to study the response of crops to controlled levels of salinity under field conditions. The main drawback of the TLS seems to be the additional ion absorption through leaves that may occur by sprinkling with saline water.

### **1.3. PHYSIOLOGY OF SALT TOLERANCE**

#### **1.3.1. Effects of salinity on plants**

The most easily observable effect of salinity on plants not adapted to this condition is a reduction in their growth. The primary cause of this growth reduction is still not clear. High salt concentrations in the medium affect the plants mainly at two levels: in their water relations (osmotic effect), and in their ion balances (ionic effect). The first effect is due to the reduced availability of soil water to the plant, while ionic effects are involved in two other aspects: disorders in mineral nutrition, and toxicities due to excess of some ions. Some authors (e.g. Pasternak, 1987) also consider a third



effect: a change in the energy levels of the plant; but this is more a result of the mechanisms by which the plants adapt to salinity than a direct effect itself.

### 1.3.2. Osmotic stress

The flux of water between a plant cell and the environment depends on two factors: the differences of water potential between the cell and the medium (the "driving force" for water movement) and the hydraulic conductance of the plant tissues (a measure of the resistance to water flow). In a saline soil, the major component of water potential is the osmotic potential, determined by its salt concentration. Inside the cell, the two important components are turgor (positive hydrostatic pressure) and osmotic pressure. The water potential of a cell can be defined as:  $\Psi = P - \pi$ , where  $P$  is the turgor pressure and  $\pi$  the osmotic pressure (Nobel, 1983).

A positive turgor pressure and continued water uptake are necessary for growth. Growth begins by a loosening of some elements in the cell wall. This results in a decrease in turgor and in water potential within the cell. The gradient of water potential across the plasmalemma drives water into the cell, increasing its volume and restoring the turgor, so that the cycle can begin again. However, this water influx tends to dilute the solutes within the cell (decrease in osmotic pressure). To compensate for this dilution and maintain osmotic pressure, solutes must accumulate inside the cell, either by uptake or by synthesis.

The rate of cell volume increase is also related to the wall yielding properties:  $r = \phi (P - Y)$ , where  $r$  is the growth rate,  $\phi$  is the wall plastic extensibility (a measure of the ease with which cells undergo irreversible expansion), and  $Y$  is the yield threshold (a minimum turgor needed before the wall begins to expand) (Lockhart, 1965).

Soil salinity interferes with the plant's water relations and reduces plant growth. However, considering all the parameters involved in cell expansion, growth inhibition under salinity stress could result from alterations in any one of them: turgor pressure, cell wall extensibility, yield threshold or hydraulic conductance. Therefore, plants

growing in saline soils have to regulate their turgor (P) or adjust the wall properties ( $\phi$  and/or Y) to maintain growth.

### 1.3.3. Responses to osmotic stress:

#### 1.3.3.1. Turgor regulation and osmotic adjustment

In response to decreased external water potential in saline soils, the plants have to decrease their internal water potential if water influx is to be maintained. At the same time, they must keep positive turgor pressures, necessary for growth. An increase in the internal osmotic pressure (osmotic adjustment) results in a decreased water potential while maintaining turgor. A higher internal solute concentration can be achieved in three ways: by accumulation of ions absorbed from the medium ( $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ ), by synthesis of organic solutes (aminoacids, sugars), or by a loss of water (partial dehydration). Plants commonly use a combination of these mechanisms.

Halophytes (the plants which naturally grow in saline environments) absorb relatively large quantities of salts from the medium to achieve osmotic adjustment. Dicotyledonous halophytes use mainly  $\text{Na}^+$  and  $\text{Cl}^-$  as osmotica (they have high Na:K ratios), and tend to become succulent (increase their cell volume). Monocotyledonous halophytes use  $\text{K}^+$  and sugars, in addition to  $\text{Na}^+$  and  $\text{Cl}^-$ , for osmotic adjustment, (their  $\text{Na}^+$  and  $\text{K}^+$  concentrations are similar), and their tissue water content may decrease (Flowers *et al.*, 1986).

On the other hand, many glycophytes (except, perhaps, the most salt-sensitive species) when exposed to moderate salinities tend to exclude salts from their leaves, and whatever amount of salt does get into the plant is largely accumulated in roots and stems (Läuchli & Epstein, 1990). Without high concentrations of ions in leaves, non-halophytes have to rely mostly on organic compounds for osmotic adjustment. However, osmotic adjustment by means of organic solutes is an expensive alternative, particularly in mature cells because of their large volume; (in small meristematic cells this is not such a limitation). The synthesis of organic solutes for osmotic adjustment would require large amounts of carbohydrates and/or enzymes, and would thus compete with other processes (e.g. growth) for their supply (Yeo, 1983).

### 1.3.3.2. Changes in cell wall characteristics:

Traditionally, reduced turgor was viewed as one of the main factors that limits growth under salinity; therefore, turgor regulation and osmotic adjustment have been extensively studied. However, the theoretical framework developed by Lockhart (1965) and others permitted consideration of other parameters which take part in the regulation of cell growth. Thus, as Wyn Jones and Pritchard (1989) pointed out, the recovery of growth after a decrease in external water potential could be the result of one or more of the following processes: a) an osmotic adjustment to restore turgor; b) a decrease in yield stress threshold to maintain the effective turgor; and c) an increase in wall extensibility to facilitate growth at a lower turgor.

The effects of water (osmotic) stress on cell elongation, and particularly on the cell wall mechanical properties, have received much attention in recent years (see Hsiao *et al.*, 1985; Lawlor & Leach, 1985; Wyn Jones & Pritchard, 1989; Cramer & Bowman, 1994). In spite of contradictory results, there is some evidence that wall properties (yield threshold and extensibility) change during or after exposure to stress (see Cramer & Bowman, 1994, for references).

### 1.3.4. Ionic stress

#### 1.3.4.1. Specific ion effects

On the nutritional side, excess of certain ions in the soil may interfere with the absorption of other ions and cause deficiencies. There is extensive evidence, particularly from laboratory studies, that high  $\text{Na}^+$  concentrations in the medium interfere with the absorption of  $\text{K}^+$  and  $\text{Ca}^{2+}$ , and that the addition of  $\text{Ca}^{2+}$  to nutrient solutions, above the minimum levels adequate for non-saline conditions, improves growth. (This aspect will be discussed in more detail in Chapter 3.) However, except in the case of sodic soils (where an imbalance between  $\text{Na}^+$  and  $\text{Ca}^{2+}$  does exist), the levels of  $\text{Ca}^{2+}$  in most soils are not limiting, and therefore  $\text{Ca}^{2+}$  deficiency is not an important factor in salinity stress in the field.

Additionally,  $\text{Cl}^-$  reduces  $\text{NO}_3^-$  uptake in plants (Cram, 1973; Aslam *et al.*, 1984). This is usually observed as a decrease in leaf  $\text{NO}_3^-$  concentrations, (although other nitrogen-containing fractions, such as proline and glycine-betaine, may increase (Gorham *et al.*, 1986)). According to Munns and Termaat (1986), while N-deficiency might occur in NaCl-treated plants, this is not likely to be a major limiting effect. Their view is supported by the observation that applications of N fertilizer to saline fields, above the levels considered optimal in non-saline conditions, does not generally improve growth or yield (Grattan & Grieve, 1992). However, the form in which N is supplied ( $\text{NO}_3^-$  or  $\text{NH}_4^+$ ) may be important (Lewis *et al.*, 1989).

#### 1.3.4.2. Toxic effects

The fact that plant growth is not improved by restoring the nutritional imbalances caused by salinity constitutes indirect evidence for some kind of ion toxicity. More direct evidence comes from sensitive species (fruit trees, many legumes) where  $\text{Na}^+$  or  $\text{Cl}^-$  begin to reduce growth at such low concentrations that a water deficit has to be ruled out (Greenway & Munns, 1980). In most of these cases  $\text{Cl}^-$  seems to be the toxic ion. Adverse effects of  $\text{Na}^+$  are mainly indirect, through high Na/Ca ratios and poor aeration in sodic and saline-sodic soils. A case of specific  $\text{Na}^+$  toxicity, however, has been reported for wheat (Kingsbury & Epstein, 1986), where a salt-sensitive variety was adversely affected by nutrient solutions containing high  $\text{Na}^+$  concentrations, but not by iso-osmotic solutions without  $\text{Na}^+$ .

#### 1.3.5. Responses to ionic and toxic effects

##### 1.3.5.1. Ion exclusion *versus* ion inclusion

Because of their high salt uptake, halophytes have usually been referred to as "salt includers"; glycophytes, in contrast, are called "salt excluders". However, this terminology is misleading, since all plants exclude salts from the medium and regulate the accumulation of specific ions. Even in salt-rich halophytes, ion uptake is tightly controlled. If there was no such regulation, the concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in the shoot would increase much faster than they actually do. This can be easily calculated from transpiration rates. Using data from different authors for barley under salinity, transpiration rate values of 4 litres of water per kg fresh weight per day

were estimated; assuming water contents of around 87%, plants growing in a solution of  $100 \text{ mol m}^{-3}$  NaCl would be increasing their internal NaCl concentrations at a rate of  $20 \text{ mol m}^{-3}$  per hour, and in a few days they would be filled with solid NaCl. Clearly, there must be some kind of exclusion.

The differences between halophytes and glycophytes are quantitative rather than qualitative. They differ in the extent to which this exclusion is achieved, and in the levels of salinity they are able to tolerate before their ability to regulate ion concentrations in shoots fails. Some very sensitive species lose this ability at low salinities, rapidly accumulate salts, and die in a relatively short time.

To avoid excessive concentrations of ions in the shoots (particularly, in the photosynthetic tissues),  $\text{Na}^+$  and  $\text{Cl}^-$  may be retained at the roots by different means. The mechanisms for  $\text{Na}^+$  exclusion are better studied. These include the existence of  $\text{Na}^+$  efflux pumps at the plasmalemma of root cells (Jeschke, 1970); a preferential accumulation of  $\text{Na}^+$  in root vacuoles (Jeschke, 1979);  $\text{Na}^+$  reabsorption in the xylem parenchyma (Yeo *et al.*, 1977); and even its extrusion back into the medium (Nassery & Baker, 1972). For  $\text{Cl}^-$ , differences in lipid composition of the root membrane, (which would affect its permeability to  $\text{Cl}^-$ ), have been related to differences in  $\text{Cl}^-$  exclusion in rootstocks of grapevine (Kuiper, 1968) and of citrus (Douglas & Walker, 1983).

#### 1.3.5.2. Cellular ion compartmentation

Except in the halophytic bacteria, whose enzymes are adapted to function at high concentrations of NaCl (Brown, 1983), high cytoplasmic concentrations of monovalent ions are toxic to metabolism, because they inhibit protein synthesis and enzyme activities. In general, the enzymes of halophytes are as sensitive to high salt concentrations as those of glycophytes (Flowers, 1972; Greenway & Osmond, 1972). Thus, the ions involved in osmotic adjustment ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ) have to be excluded to some extent from the cytoplasm, and stored where they do not interfere with metabolism; the best place for them is the vacuole. This sharp contrast between ion concentrations in cytoplasm and vacuole is known as intracellular ion compartmentation, and is an important aspect of salt tolerance.

Ion compartmentation, though, has to be complemented with osmotic compensation between vacuole and cytoplasm, since the tonoplast cannot sustain a gradient of turgor pressure across it. The cytoplasm needs to increase its osmotic pressure using solutes which do not interfere with metabolism. Because of the small volume of the cytoplasm (5%-10% of the total cell volume), a small amount of solutes can compensate the osmotic pressure of the vacuole, without large requirements of energy. A variety of organic compounds (e.g. proline, glycine-betaine, sugars and polyols) have been isolated from halophytes, which are believed to be involved in the maintenance of osmotic balance between vacuole and cytoplasm. They are called "compatible" solutes because they are not inhibitory to metabolism; (although some sugars, like sucrose, are not compatible, and they are probably located in the vacuole).

#### 1.3.5.3. Regulation of salt balances in leaves

Intracellular compartmentation is an important feature of salt tolerance, but not the only one; the regulation of ion transport in relation to growth is also important. At any one moment, the internal ion concentration will be the ratio between net ion import and growth rate. Thus, while plants are growing fast, high rates of ion uptake can be regulated (diluted) by growth. The other possibility is to decrease net uptake. This can be done by reducing the amount of ions that reach the cell (exclusion at the root and xylem level), or re-exporting any excess via the phloem. (Export from the shoot can also be achieved by excretion through salt glands, but this is a feature found only in some halophytic species.) However, concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in the phloem are usually low under saline conditions (see Flowers & Yeo, 1988, for references), reflecting its cytoplasmic nature, and this pathway is not quantitatively important. In fact, the presence of high  $\text{Na}^+$  and/or  $\text{Cl}^-$  concentrations in the phloem has been related to salt sensitivity in some plants (Lessani & Marschner, 1978).

Finally, if excess ions do not accumulate inside the cell (with proper compartmentation), they may remain in the cell wall (apoplast). However, because of the small volume of this compartment compared to that of the protoplast ( $\approx 5\%$ ) and its very low water content (30%-35%), ion concentrations (and thus osmotic pressure) in the apoplast would rise very fast and become imbalanced with the rest

of the cell. In this situation, the cell wall would tend to extract water from inside the cell, and this would lead to loss of turgor and dehydration. This aspect was first discussed by Oertli (1968) and is sometimes known as Oertli's hypothesis. There is some evidence (e.g. Munns & Passioura, 1984, Flowers *et al.*, 1991) that this may happen, at least in some species, in the later stages of the life of a leaf, and would explain the premature senescence of leaves of plants growing in salinity. Significant apoplastic solute concentrations have also been found in some halophytes (Clipson *et al.*, 1985, for *Suaeda maritima*; Richardson, unpublished, for *Atriplex amnicola*).

Thus, excess of Na<sup>+</sup> and/or Cl<sup>-</sup> may accumulate in cell walls of leaves causing loss of turgor and desiccation; or they may accumulate inside the cell, where if not properly compartmented they may lead to ion toxicity. On the other hand, too much exclusion of ions from the shoot may result in insufficient osmotic adjustment and water deficit. Hence the importance of the regulation of ion transport to the shoot.

#### 1.3.6. Causes of reduced growth

Low rates of ion uptake ("exclusion") decrease the danger of ion toxicity, but increase the chances of water stress. Alternatively, high rates of ion uptake ("inclusion") facilitate osmotic adjustment, but may lead to ion toxicity. Traditionally, there had been two main lines of thought about the causes of reduced growth under salinity: the "osmotic school" and the "specific-ion school" (Bernstein, 1975). However, it was often difficult to assess the relative contribution of these two factors to the growth inhibition under salinity.

It is likely that the causes of reduced growth are different for sensitive and tolerant species. Plants in the first group lose their capacity to control ion influx at relatively low levels of salinity; in that situation, too much ion uptake will eventually result in direct toxicity from metabolic interference. At the other extreme, growth of halophytes at very high salinities is probably limited by insufficient ion transport to growing tissues, resulting in a water deficit (Munns *et al.*, 1983). Requirements for solute uptake by extending cells are very large, because they have to keep their

turgor while at the same time increasing their volumes. If  $\text{Na}^+$  and  $\text{Cl}^-$  are to be used for osmotic adjustment, they have to be transported by the phloem, since growing tissues are mostly fed by the phloem; this might be the limiting step (Delane *et al.*, 1982). Jeschke (1984) suggested that poor recirculation of  $\text{K}^+$  may also contribute to insufficient turgor in those tissues.

Reduced shoot growth under salinity is mainly observed as a reduced leaf area and smaller shoots. Since final leaf size depends on the number and the size of the cells, decreased leaf expansion could be due to either reduced cell division (fewer cells) or reduced cell expansion (smaller cells). The latter seems to be more sensitive to salinity than the former, (Kriedemann, 1986; Papp *et al.*, 1983), although both are affected. Unfortunately, we do not know which process is limiting cell growth (be it division or expansion). Many mechanisms are affected by salinity (e.g. photosynthesis, respiration) but it is difficult to prove a causal relationship.

Osmotic adjustment and compartmentation, both of organic and inorganic solutes, are processes that require energy. (This is what some authors (e.g. Pasternak, 1987) refer to when they talk about a change in the energy levels of the plant.) This energy can be obtained by an increase in respiration rate (maintenance respiration) or, alternatively, by diverting assimilates from other processes (e.g. sugars from growth to osmotic adjustment). Maintenance respiration has, indeed, been shown to increase at moderate salinities (Schwarz & Gale, 1981). Several authors (Greenway & Munns, 1983; Yeo, 1983; Raven, 1985) have tried to quantify the metabolic cost of adaptation to salinity. From these studies, it is generally agreed that the extra cost of transport of ions and compartmentation, although high, is not excessive, and that it can only explain part of the observed growth reduction.

Photosynthesis is also reduced by salinity, usually due to decreased stomatal conductance (e.g. Rawson, 1986). However, leaf elongation is affected before photosynthetic processes are (Munns *et al.*, 1982; Papp *et al.*, 1983), which seems to indicate that a decrease in leaf area available for photosynthesis (rather than a decrease in photosynthetic rates) is the major factor for the overall reduction in carbon assimilation. This fact, and the additional observation that in the long term



growth declines more than photosynthesis (Papp *et al.*, 1983), support the prevalent view that photosynthetic rate is not the (major) limiting factor under salinity.

Exposure to salinity causes an immediate reduction (even cessation) of leaf growth, but after a short time (hours) growth is partially recovered, although at lower rates. This short term response is presumed to be related to water stress, and the restoration of growth is probably due to osmotic adjustment. However, even with osmotic adjustment growth is inhibited under salinity in non-halophytes. While many halophytes accumulate  $\text{Na}^+$  and  $\text{Cl}^-$  in order to adjust osmotically, salt tolerance in non-halophytes has usually been correlated with the ability to exclude  $\text{Na}^+$  and/or  $\text{Cl}^-$  from the shoots (Munns, 1990), thus suggesting that specific ion toxicity is an important factor in salt stress. However, as has been pointed out by several authors, high ion concentrations may not only be the cause but the consequence of the injury (accumulation due to reduced growth) (Munns & Termaat, 1986).

Munns and collaborators have tested several hypotheses to elucidate the causes of growth decrease in salinity. After ruling out the possibility of water deficit in the shoot (Termaat *et al.*, 1985), specific ion effects (Munns *et al.*, 1982), and other metabolic processes that could be limiting the growth of the shoot, Munns and Termaat (1986) suggested that in the medium term (days to weeks) the effects of salinity might arise from osmotic effects in roots, via a messenger which regulates metabolic processes in the growing leaves. That messenger would probably be a hormone or growth regulator (cytokinins, abscisic acid, gibberelic acid, or ethylene).

In the long term (weeks to months) the situation is different, and specific ion effects are probably more important. High salt loads are usually found in older leaves, because once growth is finished ion concentrations are not anymore compensated by volume increases, and continued transpiration will cause those concentrations to rise. At some stage, the capacity for compartmentation within the cell will become saturated, and the build-up of salts in the cytoplasm and/or the cell wall will eventually kill the old leaves. Munns and Termaat (1986) noted that if the rate of leaf death is greater than the rate of leaf expansion the supply of carbohydrates will

decrease in proportion to the reduction in photosynthetically active leaf area. With time, the young leaves will be unable to sustain the growth of the whole plant.

In summary, although it is still not clear how salinity affects growth, the current line of thought is as follows. In the very short term, changes in the water status of the root medium (osmotic effect) cause a reduction (and temporarily cessation) of leaf growth. The plant responds by increasing its internal osmotic pressure to restore growth (osmotic adjustment). However, this recovery of growth is only partial, in spite of turgor been fully restored, thus suggesting that the cell wall properties (extensibility, yield threshold) are affected by the stress (Hsiao *et al.*, 1985). Osmotic changes in the root medium might be sensed by the shoot by means of a messenger (probably an hormone) which regulates metabolic processes related to growth. In the long term, excess ion accumulation (ionic effect), either in the protoplast or the apoplast, is probably the main cause of reduced leaf longevity and high rates of leaf death.

## **1.4. SALT TOLERANCE IN CULTIVATED PLANTS**

### **1.4.1. Agronomic aspects of salt tolerance**

Some authors (e.g. Levitt, 1980) distinguish two strategies for resistance to any kind of stress: stress avoidance and stress tolerance. In the first case, the plant is able to "exclude" the stress by some kind of barrier (physical or chemical) so that it does not affect its metabolism. A stress tolerant plant, in contrast, permits the stress to "enter" its tissues, but is able to prevent or repair the injury induced by the stress. In the case of salt resistance, the strategy of salt excluders would be an avoidance mechanism: avoidance of ion toxicity by exclusion, and avoidance of internal water deficits by osmotic adjustment with organic solutes. The strategy of salt includers could be regarded as a tolerance mechanism: "tolerance" to high tissue concentrations is achieved by ion compartmentation, either at the cellular level or at the tissue and organ level. Strict tolerance of enzymes and metabolism to high electrolyte concentrations has only been found in halophilic bacteria (Brown, 1983). Still, since

salt resistance is a more complex issue where several strategies are involved, the word tolerance will be used throughout this work in a broad sense, as a synonym of resistance, and including any kind of mechanism that can help the plant withstand the negative effects of high external salinity.

Salt tolerance can be defined as the plant's capacity to endure the effects of excess salt in the medium of root growth (Maas, 1990). This is not something easily measured, because it depends on many factors, e.g., the type of salts involved, the growth stage of the plant, and the growing conditions (soil fertility, irrigation, climate). Thus, all these factors should be specified when giving an estimation of the salt tolerance of a crop. However, for comparative purposes, general values are commonly used for different species.

The salt tolerance of a plant is usually assessed in one of three ways: a) its ability to survive on saline soils; b) its growth or yield at different levels of salinity; c) its relative growth or yield on a saline soil compared to its growth or yield on a non-saline soil (all other conditions being similar).

Survival is an important ecological criterion, but it has little value from the agronomic point of view. It is usually associated with strategies which limit growth below the minimum economically viable levels, and it does not necessarily correlate well with yield reductions at more moderate salinities. From the point of view of the farmer, the most useful criterion might be the absolute yield under salinity. However, this is not only a function of salt tolerance, since it may be the result of different environmental factors, such as soil fertility, soil moisture, or pest and disease control, for example. Finally, yields can be expressed on a relative basis: that is, the yield of a crop under saline conditions expressed as a fraction of the yield achieved under non-saline, but otherwise similar, conditions. Expressing salt tolerance in such a way has some advantages: it is possible to make comparisons between different management practices, environmental conditions and even between crop species. Also, genotypes with high relative tolerance can be identified; these genotypes are interesting from a breeding point of view, because the trait may later be transferred to other more sensitive, but agronomically better, varieties.

#### 1.4.2. Measurement of salt tolerance; models.

For most crops, the yield response to increasing soil salinity follows a sigmoidal relationship. Maas and Hoffman (1977) proposed that this response could be simplified to two straight lines: a tolerance plateau with zero slope, and a salinity-dependent line where the slope indicates the yield reduction per unit increase in salinity. The intersection of the two lines is the threshold ( $EC_t$ ), the maximum salinity of soil or water that does not reduce yield below that achieved under non-saline conditions. This piece-wise linear model is represented in Figure 1.2.

The usual measure of soil salinity is the electrical conductivity of the saturated soil extract ( $EC_e$ ), in  $dS\ m^{-1}$ . For  $EC_e$  exceeding the threshold, the yield ( $Y$ ) can be estimated with the following equation:

$$Y = Y_m - Y_m \times S \times (EC_e - EC_t)$$

where  $Y_m$  is the yield with no salinity (maximum yield),  $S$  is the slope of the line, and  $EC_t$  is the salinity threshold. Expressed in relative terms, the relative yield ( $Y_r$ ) would be:

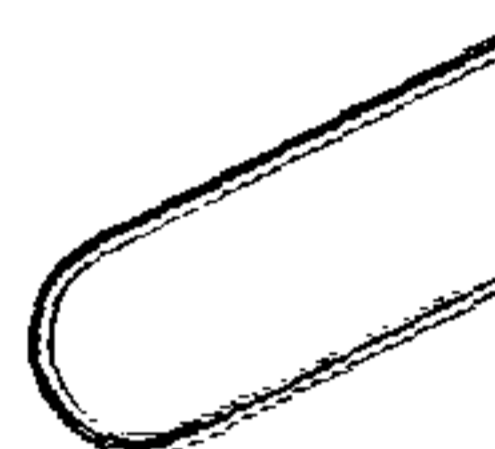
$$Y_r = 100 - S \times (EC_e - EC_t)$$

This kind of relationship is also accurate if the osmotic potential of the soil solution at field capacity ( $OP_{fc}$ ) is used as a measure of soil salinity; ( $OP_{fc}$  is not a linear function of  $EC_e$ , but its deviation from linearity is very small (Richards, 1954)).

This model gives a general indication of the salt tolerance for a crop. However, carefully controlled conditions are needed to obtain meaningful threshold and slope values. The threshold in particular is very sensitive to interaction with other environmental factors (Shannon, 1985), and several points below it (low salinity levels) are necessary to get a good estimation.

If a more accurate description of the response to salinity is needed, (and if data are available for many salinity levels), some non-linear models can be used (see Van

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(Greenway & Munns, 1980). However, when comparisons are made between closely related genotypes with similar degrees of salt tolerance, the above rule no longer applies, and salt tolerance is then associated with low  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations in shoots ("salt exclusion") (Greenway & Munns, 1980; Munns, 1990).

This kind of situation can be found in two of the most important cereal crops, wheat and barley. When grown at low salinities, barley takes up considerably greater amounts of  $\text{Na}^+$  (and, to a lesser extent,  $\text{Cl}^-$ ) than hexaploid wheat (Wyn Jones & Gorham, 1989; Gorham & Wyn Jones, 1993); yet it is more salt tolerant. Nonetheless, in comparisons between barley cultivars,  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations are usually higher in sensitive than in tolerant varieties (Greenway, 1962; Wyn Jones & Storey, 1978). Therefore, salt exclusion (rather than inclusion) is considered to be the major strategy in this species. This apparent discrepancy is related to the degree of control that plants have on their ion uptake and transport. With increasing salinities, this control ("exclusion") is firstly lost in wheat, whilst it is better maintained in barley.

Gorham and collaborators have studied ion uptake in relation to salt tolerance within the tribe Triticeae, by using a wide range of germplasm, from wild relatives to commercial varieties, including interspecific hybrids. In particular, they identified the "enhanced K/Na discrimination character", which is present in hexaploid (bread) wheats, but not in tetraploid (durum) wheats (Wyn Jones *et al.*, 1984; Shah *et al.*, 1987). This trait controls  $\text{K}^+$  and  $\text{Na}^+$  transport from roots to shoots, possibly at the point of xylem loading, and is located on the long arm of chromosome 4D (Gorham *et al.*, 1987). (For an extensive characterization of this character see Gorham, 1993). The higher  $\text{Na}^+$  levels found in leaves of durum wheats has been attributed to the lack of this enhanced selectivity. However, barley has leaf  $\text{Na}^+$  concentrations as high as those of durum wheats, though it is more tolerant (Richards *et al.*, 1987; Rawson *et al.*, 1988). In fact, like tetraploid wheats, barley also lacks the enhanced K/Na discrimination trait (Gorham *et al.*, 1990). Thus, barley must have some other mechanisms which allow it to tolerate high salt loads, probably by efficient compartmentation of ions, both at the cellular level (in vacuoles) and at the tissue level (in older leaves).

## 1.5. SELECTION AND BREEDING FOR SALT TOLERANCE

### 1.5.1. General

If saline soils have to be used for crop production, two major approaches can be considered: a) the agronomic approach (change the environment to suit the plant); and b) the biological approach (adapt the plant to the environment). Both approaches have limitations, and neither of them can provide a solution on its own. The technology to reclaim saline soils is usually very expensive, and sometimes limited by the availability of water resources. The biological approach involves adapting the existing crops to environments different from those where they naturally occur, and/or changing the traditional crops for new crops. It includes the use of crops and varieties with improved salt tolerance for areas of moderate salinity, and the domestication of halophytes to be used as new crops in areas of high salinity.

Although the interest of breeding for salt tolerance has been recognised for a long time, not many results have been obtained till present. Noble and Rogers (1992) cite only 6 commercial varieties specifically bred for improved salt tolerance; (however, this number does not include selected lines developed in "Third World" countries, which are used in local conditions but not registered under plant breeders' rights schemes). The reasons for this lack of success are various: the complexity of saline soils (spatial and temporal variation); the interaction between salinity and other environmental stresses; the inadequate understanding of how plants integrate and respond to salinity at the whole plant level, and throughout their life cycle (variation with ontogeny); and the lack of efficient criteria for rating the salt tolerance of individuals in large segregating populations. Despite these limitations, however, it is generally agreed that salt tolerance of crops can be improved beyond the present phenotypic range. Some of these issues are discussed below.

### 1.5.2. Breeding methods

Several methods may be used for increasing the salt tolerance of existing crops. The most simple approach consists of screening a large number of accessions and directly

using the most resistant ones. However, the range of naturally occurring salt tolerance in many species is limited, because selection pressures in crops have worked against tolerance to poor environments (Shannon & Akbar, 1978; Rosielle & Hamblin, 1981).

Screening only helps to identify the already tolerant phenotypes. To improve the existing varieties, new genotypes have to be created by recombination of different genes involved in salt tolerance. Some of these genes may be found in salt tolerant wild relatives of the existing crops, and they can be introduced into the crop species through interspecific hybridization. This approach has been used in tomato (e.g. Rush & Epstein, 1981a) and in wheat (e.g. Gorham *et al.*, 1986).

Richards (1992), however, questioned the contribution that wild relatives can make to improving the productivity of some crops, particularly when not only salinity but also drought are limiting yield. He argued that the physiological traits identified by growing plants in salinized nutrient solution are likely to be of minor importance for improving salt tolerance and productivity in saline soils, and that manipulating water use and water-use efficiency may be more appropriate. Notwithstanding the risks of extrapolating results obtained through solution culture to field conditions, Richards' (1992) conclusions may only apply to certain crops growing in non-irrigated saline soils.

Another way of improving the existing varieties is by identifying physiological traits which are related with salt tolerance, and then recombining them into a single genotype. This is the "pyramiding" approach suggested by Yeo and Flowers (1986). It is based on the idea that salt tolerance is not conferred by a single factor (i.e. governed by one or few genes), but the result of several independent factors. In the absence of selection pressure for salt tolerance, it is not expected that the current varieties have evolved the optimal combination of these characters. Also, the presence of these traits may not be easily detectable, because of their partial contribution to the overall performance of the phenotype. Thus, the physiological characters which help to confer resistance to salinity have to be identified and independently selected before they can be combined in a single genotype.



### 1.5.3. Environment for selection

Traditionally, plant breeding has been directed towards improving the performance of varieties for a specific environment; this approach results in the selection of the highest yielding variety for that particular environment. With increased international cooperation in the field of plant breeding, the need to develop cultivars with adaptation to a wider range of environments has arisen. In subsistence agriculture, stable performance over a range of environments is more important than high yield *per se*. Because of the high variability of saline soils, these can be regarded as a set of microenvironments. Breeding for salt tolerance could, thus, be regarded as breeding for yield stability over a range of salinities. This is rather difficult, because of the complex interactions between genotype and environment. It is usually found (e.g. Finlay & Wilkinson, 1963; Jana, 1993) that varieties with high stability under various conditions do not produce high yields in favourable environments, while varieties with high yields in non-stress environments have much lower yields in unfavourable conditions.

Discussing whether selection for salt tolerance should be done in saline or non-saline soils, Richards (1983) concluded that breeding for high yields in non-saline environments would be more efficient. His conclusion came after the observation that, because of the high variability in salinity within a field, 80% of the yield came from the 20% of that field with the lowest salinity levels. A small increase in the yields at the highest salinities would not make a large contribution to the overall yield of a particular field. Besides, selection for maximum yield in a favourable environment is easier and cheaper.

However, Richards (1983) based his study on results from moderately salt tolerant species (barley, wheat and triticale); these results cannot be extended to more sensitive species, which have poor yields at relatively low salinities. Additionally, the field that Richards (1983) described as having a "medium" level of salinity had 73% of its land with less than 4 dS m<sup>-1</sup> soil salinity. This high proportion of non-saline soil might be common in salt-affected fields of California, but not necessarily in other

parts of the world. Finally, when the source of salinity is in the irrigation water (not in the soil) his conclusions are not valid anymore.

Another argument against Richards' opinion is the commonly accepted view that maximum potential yield and stability of yield are independent factors, and thus controlled by different genes. Similarly, the genes that determine yield in saline conditions are probably different from those which control high yield under non-saline conditions, (although this aspect has never been investigated in detail (Shannon, 1985)). Consequently, saline environments have to be used in a breeding programme for salt tolerance, to permit the expression of these genes.

#### 1.5.4. Selection criteria for salt tolerance

Different terms have been used to describe the salt tolerance of a genotype. The most common ones are the threshold level (as defined by Maas and Hoffman, 1977), the rate of yield decrease with increasing salinity (the slope in Maas and Hoffman model) and the salinity at which yield is reduced by half ( $EC_{50}$ ). Jana (1991) also suggested a tolerance range, i.e., the range of salinities in which agronomically and economically acceptable yields can be obtained.

The problem with using yield as a measure of tolerance is that final yield is the result of a multitude of factors which interact during the life cycle of the plant. Under field conditions, it is difficult to maintain other environmental variables at an optimal level. Moreover, selection for grain yield is inefficient because of its low heritability (it is a quantitative trait). Still, in the absence of better characters, yield and other agronomic traits cannot be dismissed as part of the selection criteria for salt tolerance.

In view of these limitations, it has often been suggested (Epstein *et al.*, 1980; Ramage, 1980; Shannon, 1985; Tal, 1985) that breeding for increased tolerance might be more successful if selection is based directly on the relevant physiological mechanisms which determine salt tolerance. However, this is not straightforward either. First of all, many mechanisms are involved, and their relative importance can

vary largely between species and even varieties (Noble & Rogers, 1992). Some of these traits are difficult to identify because they may be obscured in the overall expression of the phenotype, and they do not correlate well with yield under salinity (Yeo & Flowers, 1986). There is also a lack of studies on the genetic control of these characters (Shannon, 1985; Tal, 1985).

For these physiological mechanisms to be efficient as selection criteria in breeding for salt tolerance, several conditions have to be met (Tal, 1985; Noble & Rogers, 1992). A basic one is the need for genetic variation in the relevant trait, and a sufficiently high heritability to permit advances through selection. Another requirement is that the trait be easily measured, so that it permits the screening of large number of genotypes without requiring large amounts of resources.

If positive results have to be obtained in the short term, the chosen mechanism should also have a major effect in the overall plant tolerance. The pyramiding approach of Yeo and Flowers (1986) may offer greater improvements in the long term, but it requires more time and resources, and a better knowledge of the specific mechanisms involved in salt tolerance. In other words, if for a given species there exists a major mechanism for salt tolerance (such as salt exclusion at the root level), selection for a secondary trait (such as ion compartmentation at the cell level) will probably be of limited benefit in the short term. An example of such results may be the poor relationship frequently found between the performance of cells selected for salt tolerance in tissue culture and the response of the plants regenerated from them (Dracup, 1991).

#### 1.5.5. Physiological traits in breeding for salt tolerance

Salt sensitivity in some crops has been attributed to the failure of the plant to keep  $\text{Na}^+$  and  $\text{Cl}^-$  out of the transpiration stream and, thus, the cytoplasm of the aerial parts. Varietal differences related to the ability to regulate  $\text{Cl}^-$  and/or  $\text{Na}^+$  transport from root to shoot (ion restriction) have been reported for barley (Greenway, 1965), soybean (Abel & Mackenzie, 1964) and *Elytrigia pontica* (Shannon, 1978).

In contrast to glycophytes, where ion restriction is the major strategy for salt tolerance, halophytes take up large quantities of ions to cope with salinity. As mentioned earlier, ion accumulation has to be complemented by good compartmentation at the cellular and tissue level. A wild relative of tomato, *Lycopersicon cheesmanii*, is thought to be more salt-tolerant than the cultivated species because of its capacity to accumulate ions (Rush & Epstein, 1981b). The interest of these wild halophytes in breeding programmes, either as sources of new genes to transfer into existing crops or for use as new crops after adaptation, has already been noted.

Osmotic adjustment and accumulation of organic solutes (sugars, glycinebetaine) have also been suggested as indices of salt tolerance (Rathert, 1984; Grumet & Hanson, 1986), although if considered on their own they may not be very useful. Other mechanisms that can prevent loss of turgor through better water efficiency (e.g. fewer stomata, increased cuticle thickness) may also help. However, most of these strategies also affect negatively the maximum production of a crop, through reduced photosynthesis.

#### 1.5.6. Use of new technologies

The possibility of increasing salt tolerance by selecting undifferentiated cells in tissue culture has been suggested by many authors (see Shannon and Noble, 1990, for references). However, the relationship between cellular and whole-plant response to salinity is not clear. Usually, plants developed from salt resistant cells do not show improved tolerance (reviewed by Downton, 1984, and Yeo and Flowers, 1989). Only in a few instances (e.g. tobacco, Nabors *et al.*, 1980) has regeneration of salt-tolerant plants from cell culture been successful.

Recent advances in molecular biology have also broadened the possibilities for gene manipulation. Stress may induce changes in gene expression. Indeed, salt-induced proteins have recently been described (reviewed by Shannon and Noble, 1990). If genes for salt tolerance are identified, they may then be transferred to salt-sensitive species by using the new techniques of genetic engineering.

## 1.6. GENERAL OBJECTIVES OF THE PRESENT WORK

This thesis forms part of a broader project studying salt tolerance in barley, one of the final objectives of which is to find physiological traits which can be used as selection criteria in breeding programmes for salt tolerance. In particular, the present work focuses on one such trait: the ion uptake and accumulation of barley under saline conditions. Four main points were investigated:

- 1) the patterns of ion accumulation in response to salinity (field and hydroponics) of several varieties of barley known to differ in their salt tolerance;
- 2) the salt tolerance of the above varieties, based on measurement of grain yield (field) and of plant growth (hydroponic culture);
- 3) the effects of high  $\text{Ca}^{2+}$  concentrations in the nutrient solution (hydroponic) and in the irrigation water (field) on the response of the plants to (NaCl) salinity;
- 4) the validity of the Triple Line System for use in studies of salt tolerance.

The final objective was to see if the measurement of leaf ion concentrations was an indicator of the salt tolerance of a genotype, in which case it might be used as a criterion for selection in breeding programmes.

# CHAPTER TWO

## BARLEY IN SALINITY: ION UPTAKE AND OSMOTIC ADJUSTMENT

### 2.1. INTRODUCTION

In comparison to other cereals (e.g. bread wheat), high concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  are found in leaves of barley when grown in saline media. In that sense barley behaves like a halophyte, using these ions to achieve osmotic adjustment. However, monovalent cations (both  $\text{Na}^+$  plus  $\text{K}^+$ ) at high concentrations (above  $200 \text{ mol m}^{-3}$ ) are inhibitory to enzymes (Flowers, 1972; Greenway & Osmond, 1972), and  $\text{Cl}^-$  is at least as toxic as  $\text{Na}^+$  and  $\text{K}^+$  (Gibson *et al.*, 1984; Gimmler *et al.*, 1984). Thus, it is clear that the ions involved in osmotic adjustment have to be stored mainly in the vacuole, while solutes with less deleterious effects are accumulated in the cytoplasm to maintain osmotic equilibrium across the tonoplast. The term "compatible solutes" has often been applied to these cytosolutes, because of their compatibility with metabolic functions (Brown & Simpson, 1972), although the physiological significance of their accumulation in salt- (and water-) stressed plants is not clear (see Steward & Larher, 1980; Rhodes & Hanson, 1993).

Two of the most studied compatible solutes are glycine-betaine (= N,N,N-trimethyl glycine) and proline. Most halophytes contain levels of glycine-betaine 10 times higher than tolerant non-halophytes, while little or no betaine is found in salt-sensitive species (Storey & Wyn Jones, 1977). In contrast, proline only accumulates, both in salt-sensitive and salt-tolerant species, when growth is severely reduced, and this accumulation may be a consequence of reduced growth (Greenway & Munns, 1980). Increased concentrations of both solutes in leaves of salt-stressed barley plants have often been reported (e.g. Wyn Jones & Storey, 1978a; Delane *et al.*, 1982).

The osmotic pressure of plants growing in saline media can be increased not only by accumulation of ions and organic solutes, but also by a decrease in the water content of the cell. Storey and Wyn Jones (1978) found that a decrease in cell water content was the main mechanism of osmotic adjustment of barley under salinity. It is interesting to note that, although the term osmotic adjustment is used in a wide sense to indicate the changes in internal osmotic pressure in response to a decreased external water potential, some authors (e.g. Yeo, 1983) prefer to use it in a stricter sense. That is, when the plant responds to a change in the external osmotic pressure by a net increase in the quantity of osmotically active solutes (Turner & Jones, 1980). Other responses which can help in turgor maintenance when the external water potential is reduced (such as a decrease in water content or in cell volume) are not considered by these authors to be "osmotic adjustment" in the strict sense.

In spite of relying mostly on ions for its osmotic adjustment, relative differences in salt tolerance between barley varieties have been related to the ability to exclude  $\text{Na}^+$  and  $\text{Cl}^-$  from the young leaves, while maintaining high  $\text{K}^+$  concentrations. This idea dates back to the experiments of Greenway in the sixties (Greenway, 1962a,b), where it was found that the saline-treated shoots of a sensitive variety (Chevron) had higher  $\text{Cl}^-$  and  $\text{Na}^+$ , and lower  $\text{K}^+$ , concentrations than those of two more resistant varieties. Later, similar conclusions were reached by Storey and Wyn Jones (1978), when the greater salt sensitivity of cultivar Arimar, compared to California Mariout, was related to its poorer capacity to regulate  $\text{Na}^+$  and  $\text{Cl}^-$  accumulation in the shoot. A significant correlation between  $\text{Cl}^-$  concentrations in leaves and growth was also found by Rawson *et al.* (1988) when comparing several barley varieties (which included California Mariout) .

At the cellular level, the main mechanisms for K/Na discrimination are related to K/Na exchange at the plasma membrane of root cortical cells (Jeschke, 1984), by selective influx of  $\text{K}^+$  over  $\text{Na}^+$  (Rains, 1972), and by  $\text{K}^+$ -dependent  $\text{Na}^+$  efflux (Jeschke & Stelter, 1973). In young tissues, the selectivity is mainly due to  $\text{K}^+$  retranslocation from leaves to the growing tissues by the phloem (Greenway *et al.*, 1965; Jeschke & Wolf, 1985; Wolf & Jeschke, 1987). The mechanisms for  $\text{Cl}^-$  exclusion are not so well known. However, several new techniques (X-ray



microanalysis, single-cell sap analysis, and isolation and analysis of protoplasts) have provided evidence for the preferential accumulation of  $\text{Cl}^-$  in epidermal cells of barley leaves compared to mesophyll cells, both under "normal" and saline conditions (Dietz *et al.*, 1992; Leigh & Storey, 1993; Williams *et al.*, 1993; Fricke *et al.*, 1994a,b). Preferential accumulation of  $\text{Cl}^-$  in the leaf sheath also contributes to maintain low leaf blade  $\text{Cl}^-$  concentrations, although the relevance of this mechanism in barley is under discussion (see Boursier *et al.*, 1987; Huang & Van Stevenick, 1989).

## **2.2. COMPARISON OF BARLEY VARIETIES UNDER SALINITY: ION ACCUMULATION AND GROWTH.**

### **2.2.1. OBJECTIVES:**

This preliminary experiment was designed to compare the growth of some varieties of barley under salinity stress in nutrient solution, in relation to their ability to regulate ion uptake. In particular, three aspects were investigated:

- a) how well does vegetative growth of plants grown in artificial culture compare with their known salt tolerance in the field?;
- b) does ion accumulation in leaves realistically reflect the ability of a genotype to grow under salinity?;
- c) is there variability in both salt tolerance and ion uptake characteristics between barley varieties, and if so, can we use some of these varieties as reliable "checks" to compare others against?

To assess the first question, the experiment included some cultivars for which there was already some information about their performance in the field under saline conditions. While in some of these cultivars their patterns of ion accumulation were well known, in others this point had never been studied. Their inclusion in the experiment would thus provide some verification of the hypothesis that ion exclusion is related to salt tolerance. Finally, if some "new" tolerant or sensitive varieties could

be identified, they could be used as checks for future experiments, instead of having to rely on the traditional cultivars (CM-67, Chevron) which in some cases do not compare very well with other varieties better adapted to Spanish conditions.

Seven barley cultivars were used for this experiment: CM-67, reputedly salt tolerant (Ayers *et al.*, 1952; Epstein *et al.*, 1980; Richards *et al.*, 1987); CHEVRON, reputedly salt sensitive (Ayers *et al.*, 1952; Greenway, 1962a; Wyn Jones & Storey, 1978b); ALBACETE, a commercial variety widely used in Spain for dryland conditions; BARBARROSA, a commercial variety commonly used in Spain under irrigation; IGRI and DACIL, 2 commercial varieties that had performed well under salinity in previous experiments (Royo, 1989); and a presumed landrace collected by Dr. Wyn Jones in Morocco, where it was grown in a natural saline soil (and referred to as MOROCCO).

#### 2.2.2. MATERIALS AND METHODS:

The experiment was conducted in a glasshouse at the University of Wales, Bangor (Pen-y-Ffridd Field Station) in February/March 1990. The minimum temperatures in the glasshouse were 18/16°C day/night, with a photoperiod of 16 hours light per day (natural daylight supplemented with 400W Son-T high pressure sodium lamps; Osram, UK).

Seeds of the above mentioned varieties were washed in running tap water for 24 hours, and imbibed in aerated distilled water for another 24 hours. After that period they were sown (02.02.90) in rock-wool plugs (Grodan BV, Roermond, Holland) in plastic trays (P84, Plantpak Ltd, Maldon, Essex), one seed per cell and 5 plants of each variety per tray, in a randomized design. The trays were placed over wet vermiculite and covered with black plastic film until emergence.

After 5 days the young seedlings were moved into hydroponic culture. The plug trays were suspended in 25 dm<sup>3</sup> containers (W6, Mailbox International Ltd, Stalybridge, Cheshire) aerated from underneath, and containing a solution of 1 mol m<sup>-3</sup> Ca(NO<sub>3</sub>)<sub>2</sub>

and  $0.5 \text{ mol m}^{-3} \text{ MgSO}_4$ , to help with root establishment. Five days later a Phostrogen-based (Phostrogen Ltd, Corwen, Clwyd) nutrient solution (for details see Gorham *et al.*, 1984a) and micronutrients (as in Hoagland & Arnon, 1950) were added.

Three levels of salinity were used: 0 (control), 100 and 200  $\text{mol m}^{-3} \text{ NaCl}$ .  $\text{CaCl}_2$  was added at a molar ratio of 20:1 (0, 5 and 10  $\text{mol m}^{-3} \text{ CaCl}_2$ , respectively). (The final Na:Ca ratio, however, was slightly different, since the Phostrogen solution already contained  $0.5 \text{ mol m}^{-3} \text{ Ca}^{2+}$ .) Two containers (tubs) with 5 plants of each variety (randomly distributed) were used for each salinity level, resulting in 6 tubs with 35 plants, and 10 replicated plants for each variety and treatment.

Twelve days after sowing, salt stress was commenced by adding  $50 \text{ mol m}^{-3} \text{ NaCl}$  daily until the appropriate final concentration was reached (2 and 4 days). The salt solution was replaced weekly, together with the nutrient solution. The ECs in the salinity treatments were around 10 and 18  $\text{dS m}^{-1}$  for treatments of 100 and 200  $\text{mol m}^{-3} \text{ NaCl}$ , respectively.

One month after the first salt was added (13.03.90) 6 plants of each cultivar and treatment were harvested. These replicates were chosen at random (3 from each tray). Two leaves per plant were sampled for sap extraction and analysis: the youngest expanded leaf (referred to as "young" leaf), and the second leaf below that one ("old" leaf). Whole plant fresh weight was recorded. Because of time and space limitations, only 3 plants of each cultivar and treatment were oven-dried to measure dry weight; their average water content was afterwards used to calculate the dry weight of the remaining 3 plants.

Individual leaves were stored in Eppendorf tubes and frozen in a commercial freezer ( $-18^\circ\text{C}$ ) for a minimum of 24 hours. Cell sap was extracted following the method of Gorham *et al.*, (1984b). This consists of crushing the thawed samples with a metal rod, making a small hole at the top and bottom of the Eppendorf tube, placing it inside another empty one, and centrifuging. The cell sap is collected in the second tube.

Major ions ( $\text{Cl}^-$ ,  $\text{NO}_3^-$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{Na}^+$  and  $\text{K}^+$ ) were analyzed by ion-exchange HPLC (Dionex 2000i, Dionex (UK) Ltd, Camberley, Surrey), after dilution with 10% isopropanol (which acts as a preservative and protein precipitant) and a second dilution with an appropriate eluent. For anion analysis, the eluent was a solution of  $\text{Na}_2\text{CO}_3$  ( $3.77 \text{ mol m}^{-3}$ ) and  $\text{NaHCO}_3$  ( $1.31 \text{ mol m}^{-3}$ ). The HPLC was fitted with an AS4A anion-exchange column and an Anion Micro-Membrane Suppressor regenerated with diluted  $\text{H}_2\text{SO}_4$  ( $0.68 \text{ ml l}^{-1}$ ). For the analysis of monovalent cations, the HPLC was fitted with a CS1 cation-exchange column and a Cation Micro-Membrane Suppressor regenerated with  $\text{KOH}$  ( $64 \text{ mol m}^{-3}$ ); the eluent was diluted  $\text{HCl}$  ( $0.80 \text{ ml l}^{-1}$ ). (For more details see Gorham, 1987).  $\text{Ca}^{2+}$  was analyzed by atomic absorption spectrophotometry (SP 2900, Pye Unicam Ltd, Cambridge, England), after dilution with 0.2%  $\text{LaCl}_3$  to minimize interferences. Osmotic potential of the extracted sap was measured with a vapour pressure osmometer (5100B, Wescor Inc, Logan, Utah).

Statistical analysis was performed using the Genstat-5 statistical package (Lawes Agricultural Trust, Rothamsted Experimental Station). The whole experiment might be regarded as a triple factorial (7 varieties x 3 salinities x 2 leaves), with the last factor (leaf) being nested within the combination of the other two. An analysis of variance (anova) for such a model was initially carried out for all characters studied. This revealed the existence of significant interactions between all factors for almost all the traits. Whenever interactions are significant, the comparison of main effects (i.e. overall means for variety, salinity or leaf) has little relevance. Furthermore, the effects of salinity and leaf age on ion accumulation are already well documented. Since the main interest of the experiment was to compare the varieties at a similar level of salinity, the results will be presented as if they came from 3 separate experiments, one for each salinity treatment.

Within each level of salinity, a two-factor anova was carried out using varieties and leaves as factors. The interaction between variety and leaf was still significant in many cases; therefore, the comparison of varieties was done independently for the two leaves at each level of salinity. Separation of means (varieties) was performed using Tukey's test.

In all these statistical analyses, the individual values of the 6 replicates (plants) were used. These might not be considered as proper replications since, as described before, the minimum unit to which a treatment was assigned was the 25 dm<sup>3</sup> container with 5 plants of each variety; (see Mead, 1988, pp. 112-122, for a discussion of this topic). However, they had to be used in this case, because the 6 similar samples had not been identified according to their origin (container). The use of means of 6 plants (instead of individual values) would have resulted in not enough degrees of freedom to perform any analysis.

Principal component analysis was carried out to study all the ions at the same time. This was done with the Minitab statistical package (Minitab Inc.). The relationships between ion concentrations and plant growth were examined, and linear correlation was used to study these relationships.

### 2.2.3. RESULTS:

#### 2.2.3.1. Plant growth (Table 2.2.1):

The reduction in shoot dry weight with salinity was not linear: the effect was proportionally less at 200 than at 100 mol m<sup>-3</sup> NaCl. At 100 mol m<sup>-3</sup> NaCl, shoot dry weights ranged between 48% and 65% of those of the controls, and at 200 mol m<sup>-3</sup> between 31% and 40%.

In general, there were no large differences between varieties in dry weight (Table 2.2.1). This may partly be attributed to the way dry weights were calculated (see section 2.2.2), where half of the values came from estimations rather than actual measurements. In all 3 treatments, Albacete had the lowest dry weight (followed by Igri), and Chevron the highest ones. The values for Albacete were always significantly lower than those of Chevron ( $p < 0.05$ ; Tukey's test).

In addition to studying the absolute values in the stress conditions, it is interesting to see if the varieties responded differently to salinity. One way to do this is by ranking them for their dry weights: any change in the ranking between the control and the

saline treatments may be regarded as an indication of the relative tolerance or sensitivity of that variety. Overall, the ranking of the varieties in the saline and non-saline conditions did not change very much. The exceptions were Dacil, which performed relatively better under salinity than in the control, and Morocco, which ranked lower with increasing salinity.

Table 2.2.1. Shoot dry weight (g) and fresh weight to dry weight ratios (FW:DW) of 7 varieties grown for one month at different NaCl concentrations; (means of 6 and 3 plants, respectively).

VARIETY	0 mol m <sup>-3</sup>		100 mol m <sup>-3</sup>		200 mol m <sup>-3</sup>	
	DW	FW:DW	DW	FW:DW	DW	FW:DW
ALBACETE	0.46	10.3	0.25	13.1	0.15	13.0
BARBARROSA	0.71	11.8	0.37	11.5	0.28	9.3
CHEVRON	0.76	12.1	0.49	10.9	0.30	10.7
CM-67	0.69	13.8	0.34	14.1	0.24	12.2
DACIL	0.68	11.4	0.39	11.2	0.27	13.2
IGRI	0.56	13.6	0.29	13.8	0.22	10.7
MOROCCO	0.72	12.7	0.34	11.8	0.22	11.8
L.S.R.*	0.27	3.0	0.14	4.9	0.11	4.8

\*L.S.R. = Least Significant Range,  $\alpha=0.05$  (Tukey's test).

No significant differences were detected between varieties in the ratios of fresh to dry weights (FW:DW) (Table 2.2.1), except for the control treatment, where Albacete had a lower ratio than CM-67 and Igri ( $p < 0.05$ ; Tukey's test). Large standard errors resulted from the small number of replicates used (3 plants).

#### 2.2.3.2. Ion concentrations in leaves:

##### i) Sodium (Table 2.2.2.a):

As expected, Na<sup>+</sup> concentrations in leaves (young and old) increased with increasing salinity, although in some varieties (Albacete, Chevron) more so than in others. Concentrations were also higher in older leaves than in young ones, in accordance

Table 2.2.2. Na<sup>+</sup> (a), K<sup>+</sup> (b) and Ca<sup>2+</sup> (c) concentrations (mol m<sup>-3</sup> sap) in young (YL) and old (OL) leaves of 7 varieties grown for one month at different NaCl concentrations; (means of up to 6 samples).

VARIETY	0 mol m <sup>-3</sup>		100 mol m <sup>-3</sup>		200 mol m <sup>-3</sup>	
	YL	OL	YL	OL	YL	OL
<i>a) Sodium:</i>						
ALBACETE	5.5	7.8	164	223	240	407
BARBARROSA	4.8	5.8	134	223	268	319
CHEVRON	5.3	15.2	210	253	295	430
CM-67	6.3	6.8	122	216	94	266
DACIL	5.7	7.5	190	219	186	303
IGRI	7.5	6.0	139	190	182	290
MOROCCO	5.5	5.2	198	271	218	345
L.S.R.*	3.6	7.0	41	53	66	142
<i>b) Potassium:</i>						
ALBACETE	159	156	100	42	129	60
BARBARROSA	166	163	120	37	115	37
CHEVRON	169	210	74	31	43	26
CM-67	173	169	123	52	163	82
DACIL	182	217	96	70	133	108
IGRI	180	186	139	57	151	64
MOROCCO	173	167	89	49	117	68
L.S.R.*	24	37	34	24	48	39
<i>c) Calcium:</i>						
ALBACETE	3.6	9.8	1.0	0.7	0.7	0.6
BARBARROSA	2.9	6.3	2.2	1.7	1.6	2.5
CHEVRON	6.4	12.1	8.1	10.2	6.3	6.3
CM-67	3.2	5.7	0.6	1.2	0.5	2.8
DACIL	9.6	12.6	7.8	3.3	3.1	2.8
IGRI	3.9	9.1	2.5	3.9	3.6	4.0
MOROCCO	2.4	6.8	1.5	0.6	1.3	1.2
L.S.R.*	3.4	6.0	5.5	3.1	3.9	4.2

\* L.S.R. = Least Significant Range,  $\alpha=0.05$  (Tukey's test)

with previous reports (Greenway, 1962a,b), with these differences being larger at 200 mol m<sup>-3</sup> NaCl than at 100 mol m<sup>-3</sup>.

For the youngest leaf, some differences between varieties were already apparent at 100 mol m<sup>-3</sup> NaCl. At this salinity level, the varieties could be arranged in two groups: one having less than 140 mol m<sup>-3</sup> Na<sup>+</sup> (CM-67, Barbarrosa and Igri), and the other having around 200 mol m<sup>-3</sup> Na<sup>+</sup> (Dacil, Morocco and Chevron); (Albacete ranked somewhere in between). At 200 mol m<sup>-3</sup> NaCl, however, CM-67 had significantly ( $p < 0.05$ ; Tukey's test) lower concentrations of Na<sup>+</sup> (less than 100 mol m<sup>-3</sup>) than all other varieties. In Barbarrosa and Chevron Na<sup>+</sup> concentrations increased to almost 300 mol m<sup>-3</sup> Na<sup>+</sup>. In relative terms, Na<sup>+</sup> concentrations in some varieties (CM-67, Dacil, Morocco) hardly changed from 100 to 200 mol m<sup>-3</sup> NaCl, whereas others showed a large increase (in particular, concentrations in Barbarrosa doubled).

In older leaves the differences were not so clear-cut, but the varieties ranked in a similar order. At 200 mol m<sup>-3</sup> NaCl, CM-67 had the lowest concentrations and Chevron the highest; these two varieties were significantly different at the 5% level (Tukey's test). Again, the smallest increases in Na<sup>+</sup> levels from 100 to 200 mol m<sup>-3</sup> NaCl were observed in CM-67 and Morocco, while the largest ones were found in Albacete and Chevron.

#### ii) Potassium (Table 2.2.2.b):

In general, K<sup>+</sup> concentrations were lower in both NaCl treatments than in the control, irrespective of the level of salinity applied. This was not the case, however, for variety Chevron, where concentrations were still lower at 200 than at 100 mol m<sup>-3</sup> NaCl. This reduction of K<sup>+</sup> concentrations in NaCl-treated plants has long been known (Greenway, 1962a,b; Storey & Wyn Jones, 1978), and is explained by a partial substitution of K<sup>+</sup> by Na<sup>+</sup> (Flowers & Läuchli, 1983). For most varieties, K<sup>+</sup> concentrations were very similar in the two types of leaves in the control, but much higher in younger than older leaves under salinity. In some varieties, though, the differences between leaves were larger (and of the opposite sign) in the control than under salinity (Chevron and Dacil).



In the control,  $K^+$  concentrations in young leaves were very similar for all varieties (no significant differences at the 5% level), with values between 160 and 180 mol  $m^{-3}$ . In the two salinity treatments, however, Chevron always had the lowest  $K^+$  concentrations; this was particularly noticeable at 200 mol  $m^{-3}$ , where this variety had values significantly lower ( $p < 0.01$ ; Tukey's test) than all the others; (compare the 40 mol  $m^{-3}$   $K^+$  of Chevron to the values above 100 mol  $m^{-3}$  for the rest of the varieties). CM-67 and Igri were the cultivars that maintained the highest  $K^+$  concentrations in their young leaves under salinity; reductions from their control values were below 20%.

In the older leaves,  $K^+$  concentrations decreased more with salinity than in the young ones (over 60% reduction). The highest concentrations were found in variety Dacil, while Chevron again had the lowest ones (except in the control).

### iii) Calcium (Table 2.2.2.c):

In general,  $Ca^{2+}$  concentrations were lower under salinity than in the control, except for variety Chevron which maintained similar levels of  $Ca^{2+}$  with increasing salinity. In fact, Chevron had larger concentrations of  $Ca^{2+}$  than any other variety, in both young and old leaves and at the 2 salinity treatments. Differences between leaves were only found with no salinity: older leaves had more  $Ca^{2+}$  than young ones.

### iv) Chloride (Table 2.2.3.a):

As expected, the amounts of  $Cl^-$  in leaves (both old and young) increased in all varieties with increasing salinity. Concentrations were always higher in older leaves than in younger ones, in agreement with the idea that young, growing leaves are well protected from an excess of ions in the substrate (Greenway, 1962b). The differences between younger and older leaves were more pronounced at the highest salinity level.

For the young leaves, differences between varieties were not very large at 0 or 100 mol  $m^{-3}$  NaCl. At the highest salinity, however, CM-67 had significantly ( $p < 0.05$ ; Tukey's test) less  $Cl^-$  than any of the other varieties (all of them above 200 mol  $m^{-3}$ ).

Table 2.2.3.  $\text{Cl}^-$  (a),  $\text{H}_2\text{PO}_4^-$  (b) and  $\text{SO}_4^{2-}$  (c) concentrations ( $\text{mol m}^{-3}$  sap) in young (YL) and old (OL) leaves of 7 varieties grown for one month at different NaCl concentrations; (means of up to 6 samples).

VARIETY	0 mol $\text{m}^{-3}$		100 mol $\text{m}^{-3}$		200 mol $\text{m}^{-3}$	
	YL	OL	YL	OL	YL	OL
<i>a) Chloride:</i>						
ALBACETE	62	94	160	183	231	368
BARBARROSA	70	86	151	181	270	332
CHEVRON	72	100	152	173	230	285
CM-67	60	77	123	179	148	215
DACIL	57	64	140	163	205	252
IGRI	82	98	156	153	206	310
MOROCCO	68	63	155	192	241	276
L.S.R.*	18	30	29	41	56	116
<i>b) Phosphate:</i>						
ALBACETE	7.3	9.7	15.7	32.0	33.7	61.0
BARBARROSA	5.5	4.0	13.5	9.2	21.8	17.8
CHEVRON	5.8	4.2	13.5	9.0	22.8	25.8
CM-67	6.2	2.8	20.2	12.3	31.8	27.0
DACIL	6.2	6.2	13.0	14.5	23.2	28.8
IGRI	5.7	3.7	11.3	10.7	25.2	26.8
MOROCCO	6.3	7.7	21.0	34.2	33.5	61.0
L.S.R.*	2.5	3.4	5.0	13.1	7.6	12.8
<i>c) Sulphate:</i>						
ALBACETE	2.7	3.8	1.0	0.7	1.0	1.0
BARBARROSA	1.2	1.7	1.2	4.8	1.3	1.2
CHEVRON	2.7	3.3	2.5	2.7	1.8	1.8
CM-67	2.8	1.8	3.3	2.0	4.0	2.2
DACIL	1.3	1.7	1.0	0.8	0.8	1.0
IGRI	1.8	1.5	1.2	0.7	1.2	0.2
MOROCCO	2.0	1.0	2.0	1.8	1.2	1.0
L.S.R.*	1.2	1.5	1.2	3.0	1.2	1.5

\* L.S.R. = Least Significant Range,  $\alpha=0.05$  (Tukey's test)

The increase in  $\text{Cl}^-$  from 100 to 200  $\text{mol m}^{-3}$  NaCl was quite similar for all varieties (average: 45%); the exceptions were Barbarrosa, which accumulated more  $\text{Cl}^-$ , and CM-67 which hardly changed at all. In fact, the  $\text{Cl}^-$  concentrations in young leaves of CM-67 at the highest salinity (around 150  $\text{mol m}^{-3}$ ) were quite similar to those found in young leaves of other varieties growing at only 100  $\text{mol m}^{-3}$  (between 140 and 160  $\text{mol m}^{-3}$ ).

Similarly, in older leaves differences between varieties were found mostly at the highest salinity. There,  $\text{Cl}^-$  concentrations ranged from just over 200  $\text{mol m}^{-3}$  for CM-67 (only 20% more than at 100  $\text{mol m}^{-3}$  NaCl), to over 350  $\text{mol m}^{-3}$  for Albacete (which, together with Igri, doubled its concentration in relation to the 100  $\text{mol m}^{-3}$  NaCl treatment).

v) Phosphate (Table 2.2.3.b):

Phosphate concentrations increased with increasing salinity. The extra  $\text{H}_2\text{PO}_4^-$  was localized preferentially in older leaves. Not many differences between varieties were found in  $\text{H}_2\text{PO}_4^-$  concentrations of younger leaves; CM-67, Albacete and Morocco were the varieties with the highest levels. These last two varieties also had very large concentrations in their older leaves, especially in the two saline treatments (significantly higher than all other varieties;  $p < 0.05$ , Tukey's test).

vi) Sulphate (Table 2.2.3.c):

Concentrations of  $\text{SO}_4^{2-}$  in sap did not exhibit a clear response to salinity. In most varieties, they either did not change or tended to decrease, except for CM-67 where  $\text{SO}_4^{2-}$  levels showed a slight increase with increasing salinity (even accounting for the change in FW:DW ratios). No significant differences (at 5% level) between leaves were found, although in CM-67 the concentrations tended to be higher in younger leaves than in the older ones.

vii) Nitrate:

No results for  $\text{NO}_3^-$  could be obtained, due to deterioration of the samples.

### 2.2.3.3. Osmotic pressure of leaf sap (Table 2.2.4):

The measured osmotic pressure of leaf sap increased in response to salinity for all varieties and leaves. Differences between leaves were only important at the highest salinity, where older leaves had higher osmotic pressures than the youngest ones. This was not the case, however, for CM-67, which had similar values for both types of leaves (even slightly lower in older leaves).

Table 2.2.4. Osmotic pressure (mOsmol kg<sup>-1</sup> sap) in young (YL) and old (OL) leaves of 7 varieties grown for one month at different NaCl concentrations; (means of up to 6 samples).

VARIETY	0 mol m <sup>-3</sup>		100 mol m <sup>-3</sup>		200 mol m <sup>-3</sup>	
	YL	OL	YL	OL	YL	OL
ALBACETE	391	419	620	632	860	1194
BARBARROSA	450	444	650	614	916	1035
CHEVRON	467	617	709	673	900	1081
CM-67	440	424	680	620	929	827
DACIL	535	569	707	678	847	1016
IGRI	401	468	585	534	814	882
MOROCCO	505	470	720	761	897	982
L.S.R.*	114	135	102	122	109	431

\* L.S.R. = Least Significant Range,  $\alpha=0.05$  (Tukey's test).

Differences between varieties were not consistent across salinities or leaves. Igri and Albacete tended to have low osmotic pressures, while Dacil generally had higher values; however, these trends changed at the highest salinity treatment.

### 2.2.3.4. Overview of ion data:

To study the ion accumulation patterns of the varieties from a global point of view, only the data from the highest salinity treatment (200 mol m<sup>-3</sup> NaCl) will be considered, since at lower levels the differences between varieties were not very large. In order to reduce the number of variables, principal component analysis was

used. This method calculates linear combinations of the original variables with the aim of finding a small set of new variables (indices) which account for a large proportion of the total variance.

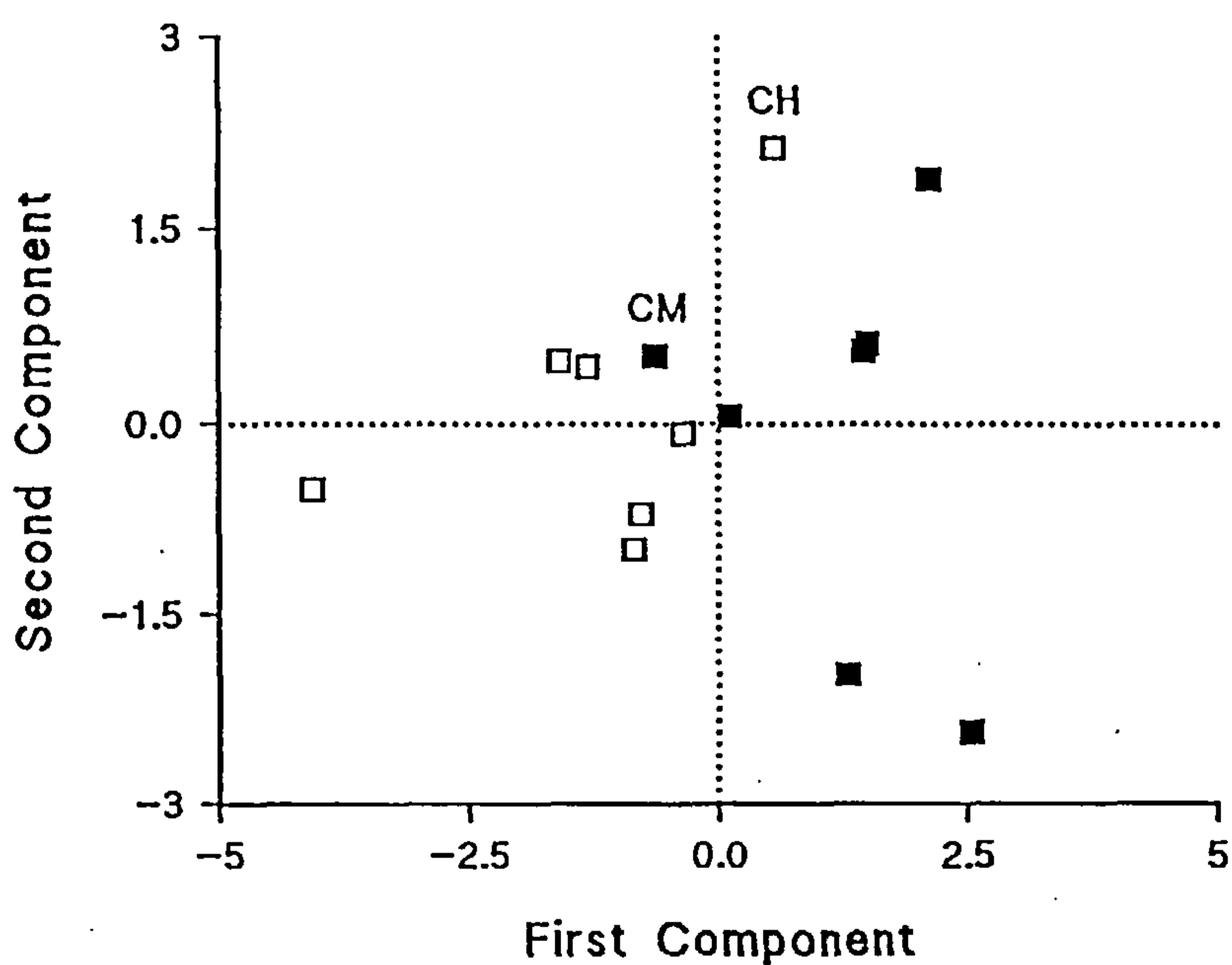
Principal component analysis was applied to the ion data of all varieties and leaves at the highest salinity. The variables considered were all the ions analyzed:  $\text{Cl}^-$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$ . Results are summarized in Table 2.2.5a. Only the two first principal components had variances (eigenvalues) larger than any one of the original variances after standardization (data not shown), and together they accounted for 78% of the total variance. Thus, only these two components will be considered.

Table 2.2.5. Principal component analysis for the ion concentrations of young and old leaves (a), and young leaves only (b), of 7 varieties of barley growing at  $200 \text{ mol m}^{-3} \text{ NaCl}$ . Only the first 2 principal components (PC) are shown.

	a) Young + Old		b) Young leaves	
	PC1	PC2	PC1	PC2
eigenvalue	3.068	1.618	3.514	1.360
proportion	0.511	0.270	0.586	0.227
cumulative	0.511	0.781	0.586	0.812
$\text{Cl}^-$	0.520	-0.178	0.428	-0.463
$\text{H}_2\text{PO}_4^-$	0.172	-0.653	-0.330	-0.431
$\text{SO}_4^{2-}$	-0.337	0.100	-0.351	0.451
$\text{Na}^+$	0.544	-0.004	0.503	-0.176
$\text{K}^+$	-0.506	-0.220	-0.444	-0.217
$\text{Ca}^{2+}$	0.187	0.695	0.366	0.565

The first principal component is mainly a contrast between  $\text{Na}^+$  and  $\text{Cl}^-$  on one hand (with high, positive coefficients) and  $\text{K}^+$  on the other (high, negative coefficient), and it effectively discriminates between young and old leaves. Figure 2.2.1a clearly illustrates these findings, and also points to some exceptions: old leaves of CM-67 are classified with the young leaves of the other varieties, because of their low  $\text{Na}^+$  and  $\text{Cl}^-$  contents. The opposite happens with young leaves of Chevron, which are grouped with the rest of the old leaves due to their high  $\text{Na}^+$  and low  $\text{K}^+$

a) young and old leaves



b) young leaves

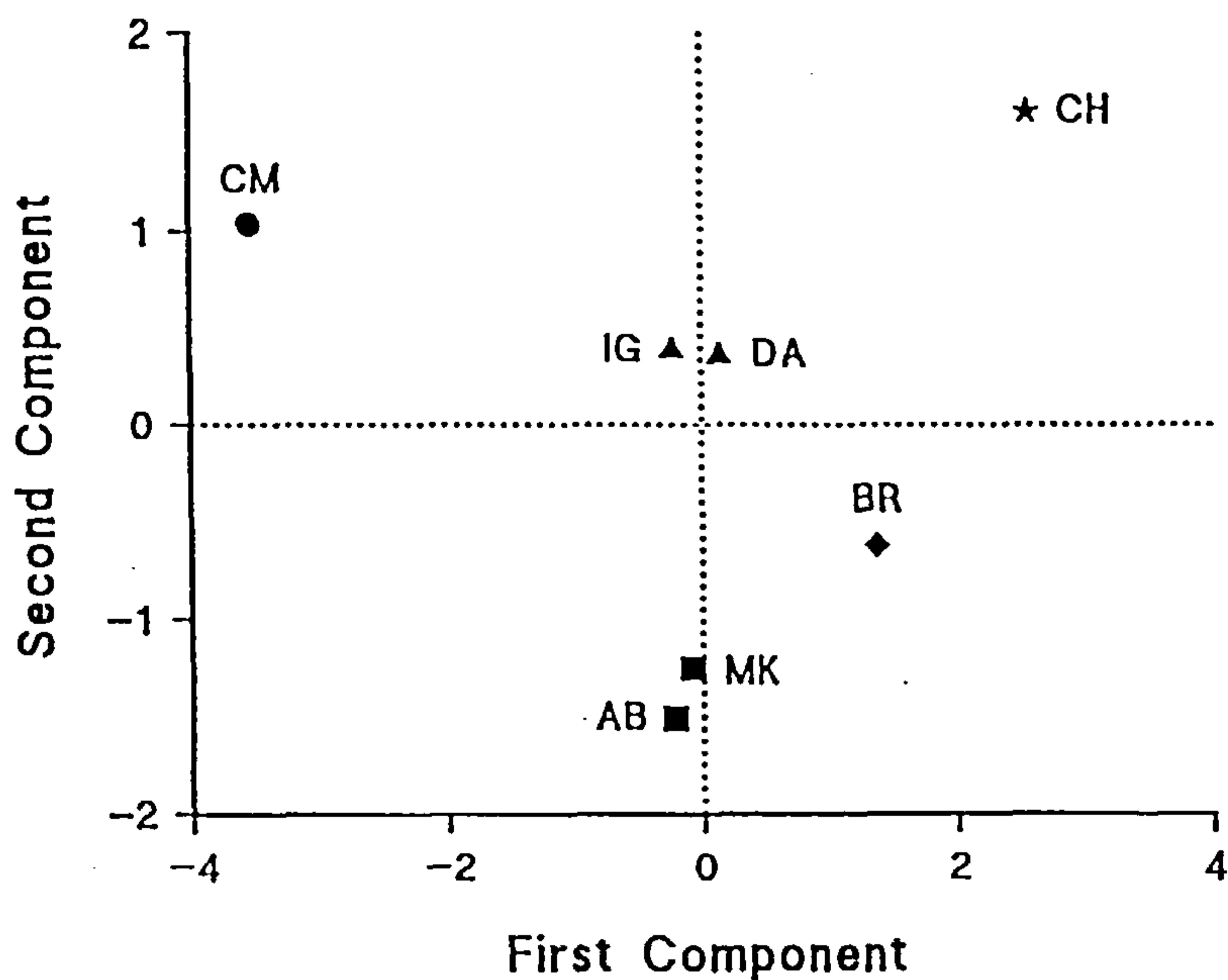


Figure 2.2.1. Plot of values of the first two principal components (from principal components analysis) of 7 barley varieties grown at  $200 \text{ mol m}^{-3} \text{ NaCl}$ ; a) using data from young (open symbols) and old (closed symbols) leaves; b) using data from young leaves only.

concentrations. The second principal component is a contrast between  $\text{Ca}^{2+}$  and  $\text{H}_2\text{PO}_4^-$ , and it mainly separates Albacete and Morocco, with low  $\text{Ca}^{2+}$  and high  $\text{H}_2\text{PO}_4^-$  concentrations, from the rest of the varieties.

Since the first component of the previous analysis only segregates between young and old leaves, the same analysis was applied to the ion data for the young leaves only (at  $200 \text{ mol m}^{-3} \text{ NaCl}$ ). In this case, (Table 2.2.5b) the first component accounts for almost 60% of the variation, and contrasts  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  (positive coefficients) against  $\text{K}^+$ ,  $\text{SO}_4^{2-}$  and  $\text{H}_2\text{PO}_4^-$  (negative coefficients). It splits the varieties in 3 groups (Figure 2.2.1b): CM-67 in one extreme, Barbarrosa and Chevron in the other side, and Igri, Albacete, Morocco and Dacil in between (values around zero). In this case the second component is more difficult to interpret, but again it separates Albacete and Morocco from the rest of the varieties, since  $\text{Ca}^{2+}$  and  $\text{H}_2\text{PO}_4^-$  are two of the main factors contrasted.

#### 2.2.3.5. Relationship between leaf ion concentrations and growth:

Values of the linear correlation coefficient between leaf ion concentrations and plant dry weight were calculated using the means of the 6 plants for each variety and treatment (because the individual plant samples were not identified). When considering the three treatments at the same time, some significant correlations were found, such as a decrease in dry weight with increasing  $\text{Cl}^-$  and  $\text{Na}^+$  in all types of leaves, or a positive correlation with  $\text{K}^+$  concentrations in older leaves (data not shown). These correlations are well known, and they are detected because of the large changes that occur with increasing salinity, but they do not detect differences between varieties.

When restricting the analysis to any one of the saline treatments (which would allow for a comparison between varieties) few significant correlations were found (Table 2.2.6). This is due to both the small number of values used for the calculations (which demand a large coefficient to be significant) and to the small differences found in the dry weights of the plants. The only cases where a significant correlation was detected (positive for  $\text{Ca}^{2+}$ , negative for  $\text{H}_2\text{PO}_4^-$ ) can be explained by the

to  $250 \text{ mol m}^{-3}$  NaCl; that is, root dry weight was less affected than shoot dry weight from 0 to  $100 \text{ mol m}^{-3}$  NaCl. It might be that these opposite trends for shoot and root with increasing salinity partly compensate each other, and explain the linear relationship found by those authors when considering the whole plant.

Another factor that may explain the lack of linearity in the response in the present experiment is the relatively late application of the stress. Since the plants had already accumulated some dry matter at the time the stress was begun, any later differences in growth rate would have been attenuated.

In absolute terms, Albacete had the lowest dry weights in all treatments. The poor performance of Albacete in hydroponic culture contrasts with its known tolerance to harsh conditions in the field (it is the preferred cultivar grown under severe drought conditions in Spain (Lasa *et al.*, 1991)); but it is in accordance with previous observations by other researchers (J. Abadía, personal communication). This apparent discrepancy might be due to a lower adaptation of Albacete to the better conditions of the hydroponic culture, where water is not limiting. The results can also be explained by the characteristic slow development of this variety during the early stages, when the measurements of dry weight were taken.

At the other extreme, Chevron had the highest dry weights in all salinities. This does not agree with the literature, where it is usually reported as a salt-sensitive cultivar (Ayers *et al.*, 1952; Greenway, 1962a). One of the reasons for these results might have been, again, the late application of the stress. Since Chevron was also the best variety in the non-saline treatment (it has a lush vegetative development, producing a great number of large leaves), the earlier (pre-stress) growth of this variety would have had a large influence in its final dry weight. Another suggested reason is related to the sub-optimal conditions in the greenhouse: relatively low temperatures and, particularly, low light intensity. These conditions probably favoured a variety like Chevron, adapted to colder climates (it originated in Switzerland (Greenway, 1962a)), in opposition to CM-67 and others, better adapted to warmer conditions (California, Spain, etc). However, the present results for Chevron, expressed relative to the control, are not very different from some of those reported in the literature:



68% dry weight (whole plant) after 15 days in 100 mol m<sup>-3</sup> (Greenway, 1962b), and 42% in 150 mol m<sup>-3</sup> NaCl (Greenway, 1962a); (65% and 39% in 100 and 200 mol m<sup>-3</sup> NaCl respectively in this experiment).

The presumably salt tolerant variety, CM-67, did not perform very well in any of the salinity treatments (49% and 35% of control dry weights at 100 and 200 mol m<sup>-3</sup> NaCl, respectively). Storey and Wyn Jones (1978) reported, for California Mariout (a variety from which CM-67 was derived), 50% and 27% of control fresh weights at the same salinities. Taking into account the decrease in FW:DW ratio reported by these authors, the above values correspond (approximately) to 61% and 44% of control dry weight, (higher than in the present experiment). Again, the poorer performance of CM-67 in this experiment may be partly attributed to the environmental conditions in the greenhouse.

Another way to look at the results is by considering the ranking of the varieties in the different treatments (control vs salinity). Dacil moved up in the rank under salinity, which may indicate a higher salt tolerance, and Morocco moved down, suggesting a lower tolerance. For all the other varieties, though, the rankings were very similar with or without NaCl. This might be interpreted as an indication that the growth (measured as dry weight) of a variety under saline conditions depends largely on its potential growth under non-stressed conditions (intrinsic growth rate). A similar conclusion was reached by Rawson *et al.* (1988) using a wider range of genotypes (which included barley, wheat and triticale).

For monocotyledonous plants, one of the usual ways to cope with the lower external water potential brought about by salinity is by reducing the water content of their tissues. This automatically increases their internal solute concentration and, thus, their osmotic pressure. In the present experiment, reduced water contents (or reduced FW:DW ratios) were not observed at 100 mol m<sup>-3</sup> NaCl, but there was a general decrease in FW:DW ratios at 200 mol m<sup>-3</sup> NaCl (except in Albacete and Dacil). Some authors (e.g. Storey & Wyn Jones, 1978) have found a progressive decrease in water content of barley at all levels of salinity; others (e.g. Boursier *et al.*, 1987) only found it above a certain level (50 mol m<sup>-3</sup> NaCl). However, this trait depends

largely on the environmental conditions in which the plants are growing, and thus discrepancy between authors is not uncommon. Even no changes at all have been reported sometimes for water content of leaf tissue between 0 and 180 mol m<sup>-3</sup> NaCl (Delane *et al.*, 1982).

Salt tolerance in barley has been positively correlated with Na<sup>+</sup> and Cl<sup>-</sup> exclusion from the shoot (Greenway, 1973). Ion data for the two varieties used as checks in this experiment (CM-67 and Chevron) are in accordance with previous reports (Greenway, 1962a,b; Wyn Jones & Storey, 1978b) at least in relative terms; (the actual concentrations reported by various authors vary, depending on the particular conditions of each experiment). Thus, CM-67 restricted the accumulation of Na<sup>+</sup> and Cl<sup>-</sup>, both in young and old leaves, and maintained high concentrations of K<sup>+</sup> under salinity. In contrast, Chevron was not as efficient in this regulation, and leaf concentrations, particularly those of Na<sup>+</sup>, built up steadily with increasing salinity, while those of K<sup>+</sup> fell dramatically.

While the two control cultivars had the expected ion concentrations, the rest of the varieties had values in between those two most extreme ones, and no clear-cut patterns were observed. Only two of them, Dacil and Igri, had consistently lower concentrations of Cl<sup>-</sup> and Na<sup>+</sup> in their young leaves than the other cultivars, (although they still had more than CM-67). In addition, Igri also had high concentrations of K<sup>+</sup> in its youngest leaves. Dacil and Igri might, thus, be considered similar to CM-67 in their ion accumulation characteristics. It might be interesting to remember that these two varieties had been chosen because they had performed well under salinity in previous field experiments.

At the other extreme, Barbarrosa might be compared to Chevron: it had the highest Cl<sup>-</sup> concentrations in young leaves (even higher than Chevron), and also rather high Na<sup>+</sup>, although it maintained higher concentrations of K<sup>+</sup> than Chevron. Thus, this variety might be expected to be rather salt sensitive, according to the ion exclusion hypothesis. Finally, Albacete and Morocco had intermediate values for the major ions (intermediate tolerance), but both had high H<sub>2</sub>PO<sub>4</sub><sup>-</sup> contents and relatively low Ca<sup>2+</sup>. It is worth noting that this classification is the same as that obtained by the principal

component analysis when this was applied to the data for ion concentrations in young leaves only.

As the external salinity increases, plants adjust the osmotic pressure in their cells by increasing the amounts of ions and/or decreasing their water content. In the present experiment, the water content did not change very much (see FW:DW ratios), but the osmotic pressure of the sap did increase considerably with salinity, and this was mainly due to the accumulation of ions (and perhaps other compounds, although this aspect was not studied in the present experiment).

The change in osmotic pressure of the external solution from 0 to 100 mol m<sup>-3</sup> NaCl was about 195 mOsmol kg<sup>-1</sup> (0.48 MPa), and about 395 mOsmol kg<sup>-1</sup> (0.98 MPa) for the 200 mol m<sup>-3</sup> NaCl; (these figures include the 1/20 CaCl<sub>2</sub> added to the NaCl solution). The changes in osmotic pressure of the leaf saps were, in general, similar or even slightly higher, indicating that these barley varieties do adjust their internal osmotic pressure in response to salinity. The exception was Dacil, where the increase in internal osmotic pressure was slightly smaller than that of the external solution. This fact did not affect its growth, though, since this was one of the varieties with highest dry weights under salinity, and the only one which improved its ranking with salinity compared to the control. In general, thus, osmotic adjustment does not seem to be limiting growth in barley; (this subject will be considered in more detail in the next experiments, section 2.3).

When relating the ion data to the growth results, a further reason to explain the high dry weight of the sensitive cultivar (Chevron) may be suggested: the large quantities of ions accumulated by this cultivar might have accounted for a great proportion of its dry weight. To check this hypothesis, the contribution of the ions to the shoot dry weight was calculated. In Chevron, the weight of the main ions (Cl<sup>-</sup>, Na<sup>+</sup> and K<sup>+</sup>) at 200 mol m<sup>-3</sup> NaCl represented between 16% and 20% of its dry weight; this would give an "adjusted" dry weight of around 0.25 grams per plant. (A more accurate calculation was not possible because fresh and dry weights were taken for the whole shoot, while ion concentrations were measured in two different individual leaves.) Still, the same calculations gave values between 15% and 19% for CM-67, the most

contrasting variety from Chevron in terms of ion contents. It is expected that these percentages would be similar for the rest of the cultivars, with intermediate ion concentrations. Thus, the higher weight of Chevron cannot be explained only by its higher content of inorganic ions.

Another factor might have been the short duration of the experiment and the relatively late application of the stress. It is considered (e.g. Storey & Wyn Jones, 1978) that a minimum of two weeks under salinity is needed for the plants to adapt to salinity and reach a steady-state. More than that may be needed, however, for the long term effects of ion accumulation to be manifest. On the other hand, the longer the plants are grown, the more apparent their differences in growth habits (cycle) become, and the more difficult it is to make comparisons. In the present case, with varieties differing considerably in their growing cycles (as observed in a parallel experiment not reported here), a compromise had to be taken. Maybe if the plants had been left for another 1 or 2 weeks, the effects of high  $\text{Na}^+$  (and low  $\text{K}^+$ ) concentrations in Chevron would have affected its growth much more drastically.

One of the objectives of this experiment was to see how well the measurement of the dry weight of young plants grown in hydroponics agreed with their known salt tolerance under field conditions. After seeing the results of the varieties used as checks it was clear that a few things had to be changed in order to get results more in accordance with field salt tolerance. In particular, the environmental conditions in the greenhouse should be improved (higher light intensity, and more realistic temperatures). Maybe the experiments should run for a longer period (and the stress applied at earlier stages), in order to allow the high ion concentrations of leaves to reveal their effects (as would happen under field conditions). In this case it would be necessary to use varieties of more similar agronomic characteristics (winter or spring types, 2-row or 6-row, similar growing season) to allow for reasonable comparisons. The problem is then in finding varieties of the desired characteristics which, at the same time, respond differently to salinity. With most of the present commercial varieties not differing in their response to salinity (as has been seen after a few years of field trials in Spain (A. Royo, personal communication)), there does not seem to be much scope for choice.

Another objective was to relate the data on ion accumulation to salt tolerance and somehow confirm the "ion exclusion" theory. In view of the results for the check varieties, it should be concluded that ion accumulation in leaves is not a trait simply related to salt tolerance, but this statement has to be taken with care, since in this experiment plant dry weight was not a reliable measure of salt tolerance (as already discussed).

Which brings us again to the previous subject, and the third objective of this experiment: what about varieties other than the traditional CM-67 and Chevron? Is there variability in both ion accumulation and salt tolerance? The results for the ion contents of leaves did not find any variety which was more extreme than the 2 already mentioned; most of them had intermediate values.

In summary, this experiment served as an introduction to the methodology used for salinity studies, pointing to some deficiencies (which were improved in later experiments), and confirming the different patterns of ion accumulation found in the two check varieties (CM-67 and Chevron). Thus, it is clear that under NaCl salinity (up to  $200 \text{ mol m}^{-3}$ ) CM-67 maintains high concentrations of  $\text{K}^+$  and restricts the accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  in its young leaves, while Chevron readily accumulates large amounts of  $\text{Na}^+$  and does not maintain high concentrations of  $\text{K}^+$ .

## **2.3. OSMOTIC ADJUSTMENT OF BARLEY UNDER SALINITY**

### **2.3.1. OBJECTIVES:**

These experiments were designed to study the degree of osmotic adjustment in barley under salinity, together with the nature of the solutes that contribute to it. In the first of these experiments the analysis of soluble sugars could not be done for several reasons. However, the contribution of all inorganic and organic solutes determined only explained around 80% of the measured sap osmotic pressure. Therefore, the

experiment was repeated to see if that difference could be accounted for by sugars. The term osmotic adjustment is used here in a broad sense, unless it is indicated otherwise.

### 2.3.2. MATERIALS AND METHODS:

#### *Experiment 1.*

The experiment was carried out in a rain-shelter at the University of Wales, Bangor (Memorial Building), during August 1992. No supplemental heating or light were provided. Two varieties of barley contrasting in salt tolerance, CM-67 and Chevron, were grown in hydroponic culture with or without (control) the addition of  $100 \text{ mol m}^{-3}$  NaCl and  $50 \text{ mol m}^{-3}$  CaCl<sub>2</sub>; (this is, approximately, the proportion of Na:Ca used in the field experiments). Each treatment was replicated 4 times, with 12 plants of each variety per replication.

Seeds were sown (10.08.92) on plastic plug trays containing a mixture (1:1) of sand and compost (John Innes Compost, n<sup>o</sup> 1). Germinated seeds were moved into hydroponics (17.08.92), on top of  $25 \text{ dm}^3$  tubs containing Phostrogen and micronutrients solution. A week later the stress was begun by adding the equivalent of  $50 \text{ mol m}^{-3}$  Cl<sup>-</sup> per day, until the final concentration was reached, 4 days later. All solutions were replaced weekly.

Plants were harvested when they had been under stress for two weeks, one month after sowing. Half of the plants were used to analyze inorganic ions and some other compounds, and the other half were used for the analysis of soluble sugars. In the first group (6 plants per variety and replicate), the 3 youngest leaves were sampled individually, put into Eppendorf tubes and frozen; the sap was later extracted by centrifugation (as described in section 2.2.2). In the other group, the 3 youngest leaves were also sampled, but in order to obtain a larger volume of sample, the corresponding leaves of the 6 plants were taken together. After cutting the leaves, these pooled samples were weighed, and put into pots with liquid nitrogen; this had to be done in the shortest time possible to prevent the degradation of sucrose by

sucrose-invertase. These fast-frozen samples were crushed with a glass rod, and sugars (and other organic solutes) were extracted with a methanol mixture (methanol-isopropanol-water, 7:1:2).

Concentrations of inorganic anions ( $\text{Cl}^-$ ,  $\text{NO}_3^-$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{SO}_4^{2-}$ ) were measured in the expressed saps by HPLC (Dionex 2000i), using the dilution described in section 2.2.2. Then, the saps of the 6 replicated plants per tub (combination of variety, treatment and leaf) were pooled together (same amount of each one) for the other determinations. These included inorganic cations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ), proline, quaternary ammonium compounds (QACs), free amino acids and osmotic pressure.

Main cations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) were also analyzed by HPLC (Dionex), diluting first with water and later with methane-sulphonic acid solution ( $20 \text{ mol m}^{-3}$ ). This dilution is different from the one described in section 2.2.2. The reason for it was the new cation-exchange column (CS12) that had been fitted to the HPLC. This new column allows for the analysis of mono- and divalent cations at the same time, but needs a different suppressor (Self-Regenerating Cation Suppressor), which does not accept the use of organic solvents or HCl.

Proline was measured by spectrophotometry (Jenway 6100 spectrophotometer) according to the method of Bates *et al.* (1973). This consists in reacting the sample with acid-ninhydrin and acetic acid in a hot bath ( $100^\circ\text{C}$ ) for 1 hour, mixing it with toluene to extract the chromophore, and reading the absorbance in the toluene phase at 515 nm.

Quaternary ammonium compounds (QACs; mainly glycinebetaine, and some choline) were determined by spectrophotometry using a modification of the method of Grieve & Grattan (1983). The QACs are precipitated as periodides using  $\text{H}_2\text{SO}_4$  ( $1 \text{ mol l}^{-1}$ ) and a I-KI reagent in cold conditions ( $0-4^\circ\text{C}$ ) for some hours (overnight). The supernatant is then separated from the periodides by aspiration, and those are dissolved in methanol; the absorbance of this solution is read at 360 nm.

Free amino acids (primary amines) were analyzed with fluorescamine, a reagent for the fluorometric assay of primary amines (Udenfried *et al.*, 1972). Sodium borate buffer (pH=9, 0.2 mol l<sup>-1</sup>) is added to the sample in a test tube. This test tube is then placed on a vortex mixer and, while mixing, 0.5 ml of fluorescamine solution (25 mg in 100 ml acetone) are rapidly added. The fluorescence is then read on a Perkin-Elmer LS-5 luminescence spectrometer at between 475-490 nm, with the excitation wavelength set at 390 nm.

Osmotic pressure was measured on the extracted sap with a vapour pressure osmometer (5100B, Wescor Inc.)

For several reasons, the determination of sugars in the methanol extracts could not be done until a few months later, and by then the samples had deteriorated (sucrose did not appear in the chromatograms). The results were judged to be incorrect, and they were ignored.

The approximate contribution of different solutes to the osmotic pressure of the sap was calculated according to the expression: Osmolality = Molality x N° particles x Osmotic coefficient (Wyn Jones & Gorham, 1983). At low concentrations (such as those found in the sap), the molality of a solution is almost equal to its molarity; thus, the latter was used for the calculations. Na<sup>+</sup> and K<sup>+</sup> chloride salts dissociate into 2 particles, while Ca<sup>2+</sup> and Mg<sup>2+</sup> chloride salts dissociate into 3 particles. As osmotic coefficients, the values of 0.92 and 0.88 were taken for Na<sup>+</sup> and K<sup>+</sup>, and Ca<sup>2+</sup> and Mg<sup>2+</sup>, respectively (Weast, 1971). (The exact values depend on the type of salts present, which we do not know. Those values were taken as an approximation, and they correspond to the osmotic coefficients of NaCl and CaCl<sub>2</sub>, presumably the major salts, at low concentrations.) Proline and glycinebetaine have osmotic coefficients close to 1 (Weast, 1971). Concentrations of free amino acids were so low (<0.01 mol m<sup>-3</sup>) that their contribution to the sap osmotic pressure was considered to be irrelevant. The sum of all calculated contributions to the osmotic pressure was then compared to the measured osmotic pressure.



The increase in the internal osmotic pressure of the salt-treated plants (in relation to those in the control) was compared to the increase in the external osmotic pressure. This last one was calculated to be around 316 mOsmol kg<sup>-1</sup> (186 mOsmol kg<sup>-1</sup> due to NaCl + 130 mOsmol kg<sup>-1</sup> due to CaCl<sub>2</sub>).

Charge balance between inorganic ions (cations vs anions) was also calculated. Inorganic phosphate was assumed to be mainly in the form of H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, because this is how it dissociates in an acidic medium such as that of the vacuole; (it is assumed that most of the "sap" comes from the large compartment which is the vacuole).

Statistical analysis was performed using the Genstat-5 statistical package. Values of 6 plants of each variety in each replication (tub) were averaged for the calculations. The anova for a triple factorial design (variety x salinity x leaf) was carried out, and it revealed the existence of many significant interactions in almost all characters. It was decided, therefore, to present the results in terms of means and standard errors for each combination of variety, salinity and leaf.

### *Experiment 2.*

This experiment was carried out in a rain-shelter (no extra heat or light) in the University of Wales, Bangor (Memorial Building), during August 1993. The experimental design was very similar to the previous one. The same two varieties (CM-67, Chevron) were tested, with or without 100 mol m<sup>-3</sup> NaCl and 50 mol m<sup>-3</sup> CaCl<sub>2</sub>, with 3 replications (12 plants of each variety in each 25 dm<sup>3</sup> tub).

Plants were harvested when they were 6 weeks old and had been in stress for 21 days. Only the youngest expanded leaf was sampled, except in those cases where this was the flag leaf (in some plants of CM-67 in the saline treatment), where the leaf below that one was sampled. Half of the plants were again used for sap extraction and analysis of inorganic ions, proline and measurement of osmotic pressure. The other half were used for the determination of soluble sugars and glycinebetaine. These samples were made of 2 pooled leaves, which were cut, weighed and frozen in liquid nitrogen. The extraction was done with acetone (instead of methanol). Free

amino acids were not determined, since their concentrations had been found to be almost negligible in the first experiment.

Determinations on the extracted sap were done using the same methods as in the previous experiment: inorganic ions by HPLC (Dionex), proline with ninhydrin by spectrophotometry, and osmotic pressure was measured with a vapour pressure osmometer (Wescor).

The acetone extracts were filtered through glass-wool and water was added to make up a known volume (10 ml). A smaller volume (5 ml) was transferred to the sample concentrator (70°C) until all the liquid had evaporated. The residue was diluted in 2 ml water, mixed and centrifuged; it was then injected into a vial by filtering through 0.45 µm pore size Whatman syringe filter, to remove any residual particles. Soluble sugars and glycinebetaine were analyzed by HPLC (Dionex) with a Sarasep Carbohydrate column (CAR-Na<sup>+</sup>) operated at 80°C, and detected with a Shodex refraction index detector. The eluent was Na<sub>2</sub>SO<sub>4</sub> (25 mol m<sup>-3</sup>). A sample containing sucrose, fructose, glucose and glycinebetaine (1 g l<sup>-1</sup>) was used as a standard for calibration.

The contribution of the different solutes to the measured osmotic pressure was calculated as before. Osmotic coefficients of the identified sugars (sucrose, glucose and fructose) are very close to 1 (Weast, 1971), and these solutes do not dissociate in water (at least, they do not separate into different particles). The sum of the contributions of all measured solutes was compared to the measured osmotic pressure. The changes in external (solution) and internal (sap) osmotic pressures from control to saline conditions were also compared. Finally, charge balance was calculated as the difference between cations and anions.

Analysis of variance for 2 factors (variety and treatment) was carried out using the Genstat-5 package; means of 6 plants of each variety in each tub were used. Results will be presented, as before, in the form of means and standard errors within each combination of those two factors.

### 2.3.3. RESULTS:

#### *Experiment 1.*

##### 2.3.3.1. Inorganic ions:

###### i) Anions (Table 2.3.1a):

In the control treatment,  $\text{Cl}^-$  concentrations were similar for the 2 varieties and the 3 leaves studied ( $\approx 54 \text{ mol m}^{-3}$ ). Under salinity, however, they were much higher in Chevron than in CM-67, and in older leaves than in younger ones, thus confirming the results of the previous experiment (Section 2.2.3.2).

Concentrations of  $\text{NO}_3^-$  decreased significantly in the saline treatment in relation to the control. This kind of response is commonly found under salinity (e.g. Gorham *et al.*, 1990), and is due to the replacement of  $\text{NO}_3^-$  by  $\text{Cl}^-$  in the vacuole, where it acts as an osmoticum. There was a different response to leaf age in the 2 varieties (interaction significant, with  $p < 0.001$ ), particularly without salinity: in CM-67 the levels of  $\text{NO}_3^-$  increased with age while in Chevron they decreased.

Like  $\text{Cl}^-$ ,  $\text{H}_2\text{PO}_4^-$  concentrations were quite similar for the 2 cultivars and 3 leaves in the control. The response to salinity, however, was different for the 2 varieties: in CM-67 the concentrations of  $\text{H}_2\text{PO}_4^-$  in treated plants were almost twice those of untreated plants, especially in the younger leaves, whilst in Chevron they tended to decrease, particularly in older leaves.

The response was also different for the 2 varieties regarding  $\text{SO}_4^{2-}$  concentrations. Differences were already present in the control, where amounts of  $\text{SO}_4^{2-}$  were similar for all leaves in CM-67, but they increased with leaf age in Chevron. The ensuing decrease under salinity was, as a consequence, relatively larger for Chevron than for CM-67.

###### ii) Cations (Table 2.3.1.b):

Concentrations of  $\text{Na}^+$  were, as expected, higher under salinity than in the control, and higher in Chevron than in CM-67. Also, in this latter variety, the concentrations

Table 2.3.1. Anion (a) and cation (b) concentrations ( $\text{mol m}^{-3}$ ) in the 3 youngest leaves of 2 varieties of barley growing with or without the addition of  $100 \text{ mol m}^{-3}$  NaCl and  $50 \text{ mol m}^{-3}$   $\text{CaCl}_2$ ; (means  $\pm$  standard errors of up to 24 plants).

		CONTROL		SALINE	
Ion		CM-67	Chevron	CM-67	Chevron
a) <i>anions:</i>					
L-6	$\text{Cl}^-$	$48 \pm 2$	$50 \pm 4$	$165 \pm 8$	$241 \pm 4$
	$\text{NO}_3^-$	$48 \pm 4$	$64 \pm 5$	$10 \pm 2$	$10 \pm 1$
	$\text{H}_2\text{PO}_4^-$	$29 \pm 1$	$29 \pm 1$	$57 \pm 3$	$27 \pm 1$
	$\text{SO}_4^{2-}$	$19 \pm 1$	$20 \pm 2$	$14 \pm 1$	$7 \pm 1$
-----					
L-5	$\text{Cl}^-$	$57 \pm 3$	$56 \pm 4$	$255 \pm 10$	$308 \pm 5$
	$\text{NO}_3^-$	$85 \pm 4$	$56 \pm 5$	$9 \pm 1$	$2 \pm 1$
	$\text{H}_2\text{PO}_4^-$	$23 \pm 1$	$29 \pm 2$	$43 \pm 3$	$18 \pm 1$
	$\text{SO}_4^{2-}$	$18 \pm 1$	$26 \pm 2$	$9 \pm 1$	$5 \pm 1$
-----					
L-4	$\text{Cl}^-$	$61 \pm 4$	$55 \pm 5$	$308 \pm 7$	$311 \pm 6$
	$\text{NO}_3^-$	$93 \pm 3$	$44 \pm 4$	$7 \pm 1$	$2 \pm 1$
	$\text{H}_2\text{PO}_4^-$	$22 \pm 1$	$31 \pm 2$	$24 \pm 2$	$17 \pm 1$
	$\text{SO}_4^{2-}$	$16 \pm 1$	$34 \pm 3$	$5 \pm 1$	$6 \pm 1$
-----					
b) <i>cations:</i>					
L-6	$\text{Na}^+$	$3 \pm 1$	$4 \pm 1$	$52 \pm 4$	$164 \pm 7$
	$\text{K}^+$	$232 \pm 3$	$209 \pm 8$	$249 \pm 11$	$146 \pm 9$
	$\text{Mg}^{2+}$	$6 \pm 1$	$11 \pm 1$	$5 \pm 1$	$3 \pm 1$
	$\text{Ca}^{2+}$	$7 \pm 1$	$14 \pm 2$	$9 \pm 1$	$10 \pm 1$
-----					
L-5	$\text{Na}^+$	$4 \pm 1$	$5 \pm 1$	$104 \pm 4$	$173 \pm 6$
	$\text{K}^+$	$251 \pm 7$	$220 \pm 11$	$232 \pm 13$	$155 \pm 8$
	$\text{Mg}^{2+}$	$7 \pm 1$	$17 \pm 1$	$4 \pm 1$	$7 \pm 1$
	$\text{Ca}^{2+}$	$11 \pm 3$	$23 \pm 3$	$10 \pm 1$	$36 \pm 1$
-----					
L-4	$\text{Na}^+$	$5 \pm 1$	$6 \pm 1$	$133 \pm 7$	$120 \pm 4$
	$\text{K}^+$	$240 \pm 9$	$245 \pm 11$	$206 \pm 12$	$178 \pm 5$
	$\text{Mg}^{2+}$	$10 \pm 1$	$19 \pm 4$	$6 \pm 1$	$15 \pm 1$
	$\text{Ca}^{2+}$	$18 \pm 4$	$23 \pm 5$	$25 \pm 2$	$51 \pm 1$

increased with leaf age, while this was not so clear for Chevron (especially in leaf 4 under salinity).

In the control,  $K^+$  concentrations were slightly higher in CM-67 than in Chevron. No differences between leaves were detected in the first variety, but increasing levels of  $K^+$  were found in older leaves of Chevron. Under salinity, CM-67 always had higher concentrations than Chevron (as in the previous experiment), and these concentrations were not much lower than those in the control. The highest amounts of  $K^+$  were found, in this cultivar, in the youngest leaf. In contrast, Chevron had significantly lower concentrations in the treated plants than in the untreated ones, and again the largest concentrations were in older leaves rather than in the young ones.

Without salinity, concentrations of  $Mg^{2+}$  increased with leaf age, and the levels found in Chevron were about twice those of CM-67. The concentrations in this latter variety under salinity were only slightly lower than those without salinity, but in Chevron they were much more reduced.

Calcium concentrations increased with leaf age, and they were always higher in Chevron than in CM-67. Differences between treated and untreated plants were also larger in the oldest leaves of Chevron.

#### 2.3.3.2. Organic solutes (Table 2.3.2):

The concentrations of QACs (mostly glycinebetaine) were higher in the saline-treated plants than in those without salt, and higher in CM-67 than in Chevron. In the first variety, the amounts in younger leaves were higher than in the older ones, particularly under salinity; this did not happen in Chevron. Proline concentrations also increased with salinity. In the control plants, these concentrations were similar for the 2 varieties; however, under salinity they were larger in CM-67 than in Chevron. No large differences were found between leaves of different age. Finally, concentrations of free amino acids were also higher in the saline-treated plants than in those without salt. Their amounts tended to increase with increasing leaf age. No overall differences between varieties were detected.

Table 2.3.2. Concentrations ( $\text{mol m}^{-3}$ , except for amino acids, where they are  $\text{mmol m}^{-3}$ ) of some organic solutes in the 3 youngest leaves of 2 varieties of barley growing with or without the addition of  $100 \text{ mol m}^{-3} \text{ NaCl}$  and  $50 \text{ mol m}^{-3} \text{ CaCl}_2$ ; (means  $\pm$  standard errors of 4 replicates, each made up of 6 leaves).

		CONTROL		SALINE	
Solute		CM-67	Chevron	CM-67	Chevron
L-6	QACs*	$26.7 \pm 2.3$	$21.3 \pm 1.2$	$35.5 \pm 0.4$	$26.2 \pm 0.6$
	Proline	$2.0 \pm 0.1$	$2.5 \pm 0.6$	$7.5 \pm 0.3$	$5.1 \pm 0.4$
	Aminoac.**	$37.3 \pm 4.7$	$34.0 \pm 4.1$	$65.5 \pm 10.9$	$92.3 \pm 7.2$
L-5	QACs	$26.9 \pm 1.7$	$20.9 \pm 0.9$	$31.2 \pm 1.3$	$24.2 \pm 1.9$
	Proline	$1.2 \pm 0.6$	$2.1 \pm 0.2$	$10.6 \pm 0.9$	$5.5 \pm 0.5$
	Aminoac.	$60.8 \pm 6.8$	$45.3 \pm 7.0$	$76.5 \pm 12.3$	$72.3 \pm 8.2$
L-4	QACs	$22.4 \pm 1.1$	$20.0 \pm 0.8$	$25.2 \pm 1.9$	$25.9 \pm 1.3$
	Proline	$1.2 \pm 0.6$	$1.7 \pm 0.2$	$9.2 \pm 0.7$	$4.1 \pm 0.6$
	Aminoac.	$82.3 \pm 6.7$	$58.5 \pm 8.7$	$81.0 \pm 10.6$	$72.8 \pm 9.1$

\* QACs - Quaternary Ammonium Compounds

\*\* Aminoac. - amino acids ( $\text{mmol m}^{-3}$ ).

### 2.3.3.3. Osmotic pressure and charge balance (Table 2.3.3):

The contribution of the measured solutes to the osmotic pressure of the leaves sap was calculated as detailed in section 2.3.2. Without salinity, a very high proportion ( $\approx 80\%$ ) of the osmotic pressure was due to  $\text{K}^+$  salts. Their contribution, though, was higher in CM-67 than in Chevron; in this latter variety,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  salts compensated for the lower proportion of  $\text{K}^+$ . In the saline-treated plants, not only  $\text{K}^+$  but also  $\text{Na}^+$  salts were the major contributors to the sap osmotic pressure. The proportion of  $\text{Na}^+$  was higher in Chevron (almost 50% in the youngest leaf) than in CM-67. In the older leaves of Chevron,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  salts also had some importance (23%). Although the concentrations of organic solutes (betaine and proline) increased with salinity (particularly in CM-67), their contribution to the osmotic pressure was not proportionally larger in the treated plants; (around 5% in both treated and untreated plants).

Table 2.3.3. Calculated osmotic contributions (mOsmol kg<sup>-1</sup>) of the measured solutes and comparison with measured osmotic pressures (a), "osmotic adjustment" (b) and charge balance (c) in the 3 youngest leaves of the plants in the first "Osmotic adjustment" experiment.

		CM-67			Chevron		
		L-6	L-5	L-4	L-6	L-5	L-4
<i>a) calculated osmotic contributions:</i>							
CONTROL	Na <sup>+</sup> salts <sup>(1)</sup>	5	8	10	8	9	11
	K <sup>+</sup> salts <sup>(1)</sup>	428	461	441	385	406	450
	Mg <sup>2+</sup> salts <sup>(2)</sup>	15	18	28	29	44	49
	Ca <sup>2+</sup> salts <sup>(2)</sup>	19	28	49	37	60	62
	Organic <sup>(3)</sup>	29	28	24	24	23	22
Total OP		496	543	552	483	542	594
Measured OP		611	656	615	551	543	547
Accounted (%)		81	83	90	88	100	109
-----							
SALINE	Na <sup>+</sup> salts <sup>(1)</sup>	96	191	245	302	318	221
	K <sup>+</sup> salts <sup>(1)</sup>	459	427	380	270	285	327
	Mg <sup>2+</sup> salts <sup>(2)</sup>	15	11	16	8	19	39
	Ca <sup>2+</sup> salts <sup>(2)</sup>	24	26	65	27	94	135
	Organic <sup>(3)</sup>	43	42	34	31	30	30
Total OP		637	697	740	638	746	752
Measured OP		830	926	907	795	869	817
Accounted (%)		77	75	82	80	86	92
=====							
<i>b) "Osmotic adjustment":</i>							
EXCESS/DEFICIT (*) OP		-97	-46	-24	-72	10	-46
=====							
<i>c) Charge balance:</i>							
CONTROL	Cations (+)	260	290	303	263	305	334
	Anions (-)	164	201	208	183	193	199
	Difference (+/-)	+96	+89	+95	+80	+112	+135
-----							
SALINE	Cations (+)	331	364	401	338	414	429
	Anions (-)	259	325	363	292	339	342
	Difference (+/-)	+72	+39	+38	+46	+75	+87

<sup>(1)</sup> Na<sup>+</sup> and K<sup>+</sup> salts: mol m<sup>-3</sup> x 2 x 0.92

<sup>(2)</sup> Ca<sup>2+</sup> and Mg<sup>2+</sup> salts: mol m<sup>-3</sup> x 3 x 0.88

<sup>(3)</sup> organic solutes (betaines + proline): mol m<sup>-3</sup> x 1 x 1

(\*) Difference between change in leaf measured osmotic pressure (from control to saline) and change in external osmotic pressure (316 mOsmol kg<sup>-1</sup>).

The contributions of the different solutes were added up, and these calculated osmotic pressures were compared with those measured with the osmometer. In the control conditions between 80% and 90% of the measured osmotic pressure of CM-67 was accounted for in this way, and between 90% and 100% of that of Chevron. Under salinity, however, a higher proportion of the measured osmotic pressure was not explained by the contribution of the measured solutes; (between 18% and 25% in CM-67, and up to 20% in Chevron).

The increase in the osmotic pressure of sap, from non-saline to saline conditions, was also compared to the increase in the external osmotic pressure ( $316 \text{ mOsmol kg}^{-1}$ ) (Table 2.3.3b). In almost all cases (specially in the younger leaves) there was a small deficit of osmotic pressure (up to 10%).

Charge balance between anions and cations is shown in Table 2.3.3c. In general, there was an excess of positive charges; this was larger in the control plants than in those in salinity.

## *Experiment 2.*

### 2.3.3.4. Inorganic ions (Table 2.3.4a):

In the control plants, the concentrations of the measured anions were quite similar for the 2 varieties. Concentrations of  $\text{NO}_3^-$  and  $\text{SO}_4^{2-}$  were also similar for the 2 varieties under salinity. These two ions decreased considerably (96% and 60% respectively) in the treated plants, in relation to the untreated ones. On the other hand,  $\text{Cl}^-$  concentrations increased with salinity, and much more so in Chevron than in CM-67, (as in all previous experiments). Finally, the concentrations of  $\text{H}_2\text{PO}_4^-$  in CM-67 increased with salinity, while they tended to decrease (although not significantly at the 5% level) in Chevron.

Concentrations of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  were also similar for the 2 varieties in the control, while  $\text{Mg}^{2+}$  was slightly higher in Chevron, and  $\text{K}^+$  was higher in CM-67. More differences between varieties were found in the saline-treated plants. There,  $\text{Na}^+$  and



$\text{Ca}^{2+}$  concentrations increased more, in relation to the untreated ones, in Chevron than in CM-67. On the other hand, concentrations of  $\text{Mg}^{2+}$  decreased with salinity, particularly in CM-67, where its levels were inappreciable. Finally,  $\text{K}^{+}$  concentrations only decreased slightly in CM-67 under salinity, but were much more reduced in Chevron.

Table 2.3.4. Concentrations ( $\text{mol m}^{-3}$ ) of inorganic ions (a) and of some organic solutes (b) in the youngest expanded leaf of 2 barley varieties grown with or without the addition of  $100 \text{ mol m}^{-3}$  NaCl and  $50 \text{ mol m}^{-3}$   $\text{CaCl}_2$ ; (means  $\pm$  standard errors of up to 18 plants for ions, and of 9 replicates, each one made up with 2 similar leaves, for organic solutes).

Solute	CONTROL		SALINE	
	CM-67	Chevron	CM-67	Chevron
<i>a) Inorganic ions:</i>				
Chloride	$71 \pm 4$	$68 \pm 5$	$247 \pm 20$	$417 \pm 30$
Nitrate	$41 \pm 5$	$52 \pm 4$	$1 \pm 1$	$3 \pm 1$
Phosphate	$35 \pm 2$	$26 \pm 1$	$54 \pm 5$	$22 \pm 2$
Sulphate	$16 \pm 1$	$19 \pm 2$	$6 \pm 1$	$8 \pm 1$
<hr/>				
Sodium	$6 \pm 1$	$6 \pm 1$	$76 \pm 10$	$239 \pm 11$
Potassium	$262 \pm 6$	$234 \pm 6$	$213 \pm 9$	$100 \pm 4$
Magnesium	$13 \pm 2$	$18 \pm 1$	$1 \pm 1$	$6 \pm 1$
Calcium	$18 \pm 1$	$18 \pm 1$	$33 \pm 4$	$50 \pm 4$
<hr/>				
<i>b) Organic solutes:</i>				
Sucrose	$0.7 \pm 0.1$	$0.3 \pm 0.1$	$22.8 \pm 3.3$	$5.8 \pm 1.1$
?	$4.6 \pm 0.3$	$4.3 \pm 0.3$	$12.8 \pm 0.8$	$6.0 \pm 0.4$
Glucose	$30.9 \pm 2.7$	$25.3 \pm 1.3$	$28.1 \pm 1.7$	$21.9 \pm 1.3$
Fructose	$35.0 \pm 2.8$	$32.4 \pm 1.2$	$28.6 \pm 1.6$	$26.5 \pm 1.3$
Glycinebetaine	$9.7 \pm 1.1$	$6.0 \pm 0.7$	$30.3 \pm 1.7$	$28.5 \pm 1.9$
??	$11.1 \pm 2.0$	$4.8 \pm 0.3$	$31.4 \pm 3.0$	$7.2 \pm 0.9$
Proline	$0.07 \pm 0.01$	$0.07 \pm 0.04$	$0.51 \pm 0.08$	$0.12 \pm 0.01$

? - compound eluting between sucrose and glucose, expressed in sucrose equivalents.

?? - compound eluting after glycinebetaine, expressed in glycinebetaine equivalents.

### 2.3.3.5. Organic solutes (Table 2.3.4b):

Glucose and fructose were the major organic solutes found under control conditions, with similar concentrations for the 2 varieties. These concentrations tended to be slightly, although not significantly (at the 5% level), lower under salinity. The levels of sucrose in untreated plants were very low ( $< 1 \text{ mol m}^{-3}$ ). However, this was one of the major organic solutes in saline-treated plants of CM-67; (sucrose concentrations in Chevron in the saline treatment were only slightly higher than in the controls). Glycinebetaine was also one of the most important organic solutes under salinity, with a large increase in relation to the concentrations in the control. No significant differences (at the 5% level) between varieties were found for this compound. Finally, although levels of proline increased slightly with salinity (at least for CM-67), they were very low in all cases ( $< 1 \text{ mol m}^{-3}$ ).

Two other compounds were separated by the chromatographic column, one eluting between sucrose and glucose, and the other eluting after glycinebetaine. Their areas were transformed into equivalent concentrations using the response factors for the previously eluted compound (sucrose and glycinebetaine). These 2 compounds increased their concentrations in salinized plants of CM-67, but only minor increases were observed in Chevron.

### 2.3.3.6. Osmotic pressure and charge balance (Table 2.3.5):

The contribution of the measured solutes to the osmotic pressure was calculated as before. Most ( $\approx 70\%$ ) of the osmotic pressure in the control was due to  $\text{K}^+$  salts. Organic solutes also had some importance (12% to 14%). These results were similar for the 2 varieties. Under salinity, however, the main osmolytes differed in the 2 cultivars. In CM-67, 50% of the osmotic pressure was accounted for by  $\text{K}^+$  salts, with organic solutes and  $\text{Na}^+$  salts accounting for another 19% each. The role of the  $\text{K}^+$  salts was taken over by  $\text{Na}^+$  salts in salinized Chevron plants, providing 50% of the osmotic pressure. Potassium salts only accounted for 21% of the osmotic pressure, and  $\text{Ca}^{2+}$  salts contributed another 15%. Organic solutes had less importance in this cultivar (11% of the osmotic pressure) than in CM-67.

In non-salinized plants, a good proportion of the measured osmotic pressure was explained by the measured solutes, especially in Chevron. Under salinity, however, there was still a high proportion (24% to 35%) of the measured osmotic pressure which was not accounted for by the measured solutes.

The degree of osmotic adjustment was assessed by comparing the change in the sap osmotic pressure with the increase in the external osmotic pressure. Both varieties increased their osmotic pressures more than was needed to maintain the same difference between internal and external osmotic pressures; i.e., there was an excess of osmotic pressure, particularly in Chevron.

Table 2.3.5. Calculated osmotic contributions (mOsmol kg<sup>-1</sup>) of the measured solutes (a), comparison between measured and calculated osmotic pressures (b) and charge balance (c) in leaf sap of plants in the second "Osmotic adjustment" experiment.

	CONTROL		SALINE	
	CM-67	Chevron	CM-67	Chevron
<i>a) Calculated O.P.</i>				
Na <sup>+</sup> salts <sup>(1)</sup>	11	11	140	440
K <sup>+</sup> salts <sup>(1)</sup>	482	431	392	184
Mg <sup>2+</sup> salts <sup>(2)</sup>	34	48	3	16
Ca <sup>2+</sup> salts <sup>(2)</sup>	48	48	87	132
Organic solutes <sup>(3)</sup>	92	73	155	96
<i>b) Comparison of O.P.</i>				
Total OP	667	611	777	868
Measured OP	826	640	1190	1136
Accounted for (%)	81	95	65	76
Excess <small>(increase above external change)</small>			48	180
<i>c) Charge balance</i>				
Cations (+)	330	312	357	451
Anions (-)	179	184	314	458
Difference (+/-)	+151	+128	+43	-7

<sup>(1)</sup> Na<sup>+</sup> and K<sup>+</sup> salts: mol m<sup>-3</sup> x 2 x 0.92

<sup>(2)</sup> Mg<sup>2+</sup> and Ca<sup>2+</sup> salts: mol m<sup>-3</sup> x 3 x 0.88

<sup>(3)</sup> Organic compounds: mol m<sup>-3</sup> x 1 x 1

Charge balance (calculated from inorganic ions) is shown in Table 2.3.5c. There was an excess of cations in control plants. This balance was better under salinity, especially for Chevron.

#### 2.3.4. *DISCUSSION:*

In non-saline conditions, the major inorganic ions that contribute to osmotic regulation and charge balance in the plant cells are  $\text{Cl}^-$ ,  $\text{NO}_3^-$  and  $\text{K}^+$  (Marschner, 1986). From the analysis of inorganic ions it can be seen that these were the most important ones in the control plants (both experiments). Under salinity, however, most of the  $\text{NO}_3^-$  was replaced by  $\text{Cl}^-$ , which was the predominant anion in salt-treated plants. Phosphate also had some (minor) importance in CM-67 under salinity, where the concentrations of this ion increased in relation to the control plants.

As for cations, the predominant one in saline-treated plants continued to be  $\text{K}^+$  in CM-67, with  $\text{Na}^+$  coming only second in importance. In Chevron, though, both  $\text{Na}^+$  and  $\text{K}^+$  had similar relevance, with Na/K ratios being around 1. These results contrast with those found in the "Comparison of varieties" experiment (section 2.2.3.2), where under NaCl salinity alone  $\text{Na}^+$  had replaced  $\text{K}^+$  to a much larger extent in both varieties. This discrepancy is probably due to the mixture of salts used in the present experiments (NaCl and  $\text{CaCl}_2$  at 2:1 molar ratio). It has to be noted that the saline treatment here is equivalent, in terms of  $\text{Na}^+$  concentrations, to the medium treatment before ( $100 \text{ mol m}^{-3}$ ). Still, the  $\text{Na}^+$  levels in salinized plants were lower in the present case, while  $\text{K}^+$  concentrations were maintained high. Only in old leaves of Chevron was the decrease in  $\text{Na}^+$  accumulation (in relation to NaCl-alone salts) compensated for by an increase in  $\text{Ca}^{2+}$  (the other externally applied salt).

Relative differences between varieties and treatments were similar in the two "Osmotic adjustment" experiments, although absolute concentrations were different. Particularly, in saline-treated plants, levels of  $\text{Cl}^-$ ,  $\text{Na}^+$  and  $\text{Ca}^{2+}$  were higher in the second one, while  $\text{NO}_3^-$  and  $\text{K}^+$  were slightly lower. This is probably due to the fact that the plants in experiment 2 had been under stress for a longer period, and the

leaves sampled were older; (in some plants of CM-67 the flag leaf was already fully expanded). As a result of extended transpiration, these older leaves had accumulated more salts ( $\text{Cl}^-$ ,  $\text{Na}^+$  and  $\text{Ca}^{2+}$ ), which had replaced the usual osmolytes ( $\text{NO}_3^-$ ,  $\text{K}^+$ ) to a larger extent. It has been found, under non saline conditions, that  $\text{Ca}^{2+}$  partially compensates for loss of  $\text{K}^+$  as the leaf ages (Hinde *et al.*, 1992), and  $\text{Cl}^-$  compensates for  $\text{NO}_3^-$  (Richardson *et al.*, 1992). In the second "Osmotic adjustment" experiment, higher concentrations of  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  and lower amounts of  $\text{NO}_3^-$  were already found in the control plants, confirming the idea that the differences in relation to the first "Osmotic adjustment" experiment might have been due to different leaf ages.

From the organic solutes analyzed, proline and betaines increased with salinity, especially in CM-67. Concentrations of free amino acids (experiment 1) were very low ( $<0.1 \text{ mol m}^{-3}$ ) and they did not clearly increase with salinity. In similar experiments with barley (e.g. Delane *et al.*, 1982) amino acid concentrations were reported to be about two orders of magnitude higher than those found here. It is possible that the sap samples had deteriorated (the temperature at which they were stored might not have been sufficiently low to prevent deterioration).

Proline concentrations were much lower in the second experiment than in the first one, in both control and stressed plants. These differences between experiments might be due to the time of day when the plants were harvested. Proline accumulates in all water-stressed tissues (Hsiao, 1973), and it can do so at very high rates (Singh *et al.*, 1973); it also declines very fast after rehydration (Singh *et al.*, 1973). It has been suggested (Wyn Jones & Storey, 1978a) that its concentrations may fluctuate during the day following diurnal changes of water stress (maximum at midday). Higher levels of proline at dusk than at dawn were found by Weimberg and Shannon (1988) in *Thinopyrum elongatum*. Here, plants in experiment 1 were harvested in the early afternoon (higher stress), while those in experiment 2 were harvested in the morning (lower stress).

Concentrations of glycinebetaine under salinity were similar in both experiments, but in the un-stressed plants they were lower in the second experiment than in the first. The results from experiment 2 are more in accordance with the reported betaine

accumulation in salt-stressed plants (e.g. Wyn Jones & Storey, 1978a). The reasons for the high levels of glycinebetaine in the control plants in experiment 1 are not known.

Among the measured soluble sugars (experiment 2), glucose and fructose were the predominant ones in the control plants, with concentrations of sucrose being very low ( $< 1 \text{ mol m}^{-3}$ ). Under salinity, levels of glucose and fructose were slightly lower, but sucrose increased, particularly in CM-67. As a result, total soluble sugars (glucose + fructose + sucrose) increased with salinity for CM-67, but they decreased slightly for Chevron; (although this increase might be just a reflection of reduced water contents). It has to be noted that the concentrations of sugars in photosynthetic tissues change over a 24 hours period; therefore, their absolute concentrations will vary depending on the time of harvesting. However, since the plants in experiment 2 were sampled within a few hours, no bias due to different time of day is expected.

When the osmotic contributions of the different solutes are calculated (Tables 2.3.3 and 2.3.5), it is evident that most of the osmotic pressure of the expressed sap is due to inorganic ions, either  $\text{K}^+$  salts in non-saline conditions, or  $\text{K}^+$  and  $\text{Na}^+$  salts under salinity. The contribution of organic solutes is mainly due to soluble sugars, while the "compatible" solutes only contribute 4-5% to the total osmotic pressure. It is clear, then, that in order to have an osmotic role these must be located predominantly in the cytoplasm.

Because of time limitations, fresh and dry weights were not measured in these experiments. However, using data from similar experiments, the concentrations in leaf sap were transformed so that they could be expressed on a dry weight basis. Fresh weight to dry weight ratios of 12 and 7 for control and saline-treated plants, respectively, were used. Data for experiment 1 is shown in Table 2.3.6. These results show that the only ions that did accumulate (net increase) under salinity were  $\text{Na}^+$  and  $\text{Cl}^-$  (and  $\text{H}_2\text{PO}_4^-$  in CM-67). In spite of this, total ion concentrations under salinity were lower than without salinity. Similar results were found for experiment 2 (data not shown). This means that a good proportion of the increased osmotic pressure was due to the lower water content, and not to increased ion accumulation.

That is, there was no osmotic adjustment in the strict meaning of the term, although the solutes used to generate the osmotic pressure were different. (Always assuming that the estimations of a 40% decrease in water content, which were obtained in other experiments for whole young plants, can be applied to the present one).

Table 2.3.6. Calculated inorganic ion concentrations (mmol kg<sup>-1</sup> dry weight) in the youngest leaf of the plants in the first "Osmotic adjustment" experiment, assuming FW:DW ratios of 12 for the control and 7 for the saline-treated plants.

	CONTROL		SALINE	
	CM-67	Chevron	CM-67	Chevron
Cl <sup>-</sup>	522.7	549.1	989.4	1446.1
NO <sub>3</sub> <sup>-</sup>	529.3	706.5	57.5	59.3
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	320.2	323.5	341.6	162.4
SO <sub>4</sub> <sup>2-</sup>	213.5	214.6	52.1	40.8
Total	1585.7	1793.7	1470.6	1708.6
Na <sup>+</sup>	27.5	45.1	314.0	983.5
K <sup>+</sup>	2557.5	2302.2	1494.7	878.0
Mg <sup>2+</sup>	60.5	118.9	33.0	19.2
Ca <sup>2+</sup>	78.1	153.0	55.1	62.3
Total	2723.6	2619.2	1896.8	1943.0

The measured inorganic and organic solutes accounted for most of the observed osmotic pressure, particularly in the control plants. Between 20% and 35% of the measured osmotic pressure was not accounted for in the salinized plants. The solutes responsible for the remaining osmotic pressure are not known, but it is suggested that fructans of low molecular weight might play a role, since they are found in significant amounts in plants that store sucrose (as opposed to starch) as the end product of photosynthesis, such as barley (Sicher *et al.*, 1984; Farrar & Farrar, 1985).

Charge balance revealed an excess of positive charges in most cases. This excess was probably compensated for by organic acids (not determined), since this is how electrical neutrality is usually maintained (Osmond, 1976).

In experiment 1, the increase in sap osmotic pressure from control to saline treatments was a bit less than the change in the external osmotic pressure. This deficit was larger in younger than older leaves. In the second experiment, however, osmotic adjustment was complete; Chevron even over-adjusted (excess of osmotic pressure). There are examples in the literature where osmotic adjustment of barley under salinity has been found to be either complete (e.g. Storey & Wyn Jones, 1978) or incomplete (e.g. Termaat *et al.*, 1985). Delane *et al.* (1982) found both situations: full adjustment in mature tissues and incomplete adjustment in rapidly elongating tissues. Absolute adjustment may not be necessary, providing that the difference between internal and external water potentials does not become too small. In experiment 1, this difference was around 500 mOsmol kg<sup>-1</sup> for all types of leaves, which is considered to be high enough to maintain influx (500 mOsmol kg<sup>-1</sup> is a normal osmotic pressure for plants without stress (Wyn Jones & Gorham, 1983)).

In summary, these two varieties of barley (CM-67 and Chevron), when subjected to a certain degree of salinity (100 mol m<sup>-3</sup> NaCl and 50 mol m<sup>-3</sup> CaCl<sub>2</sub>), adjust their internal osmotic pressure by a reduction in their water content (as will be seen in other experiments) and a net accumulation of Cl<sup>-</sup> and Na<sup>+</sup>. Under these conditions of salinity, the contribution of the different solutes to the osmotic pressure varies depending on the cultivar. In CM-67, K<sup>+</sup> (50%) and Na<sup>+</sup> (19%) salts, together with organic compounds (soluble sugars and glycinebetaine; 19%) are the main osmotica. In Chevron, salts of inorganic ions (Na<sup>+</sup> 50%, K<sup>+</sup> 21%, and Ca<sup>2+</sup> 15%) contribute mostly to the osmotic pressure, with organic solutes (glucose, fructose and glycinebetaine) being less important (11%). The degree of osmotic adjustment seems to be sufficient to maintain the water influx into the plant.



# CHAPTER THREE

## SALINITY - CALCIUM INTERACTIONS

### 3.1. INTRODUCTION

The first observations on the interactions between  $\text{Na}^+$  and  $\text{Ca}^{2+}$  go back to the beginning of this century: Kearney and Cameron, (1902; quoted by LaHaye & Epstein, 1971) reported that the addition of  $\text{Ca}^{2+}$  would neutralize the harmful effects of  $\text{Na}^+$  in various plants. Later, Ratner (1935) suggested that the tolerance of plants to high levels of  $\text{Na}^+$  depended on the availability of  $\text{Ca}^{2+}$  in the soil. In the early 60s, the experiments of Epstein (1961) and Jacobson *et al.* (1961) in barley demonstrated the essentiality of  $\text{Ca}^{2+}$  for selective cation absorption. In the absence of  $\text{Ca}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$  were absorbed in a non-selective manner from a solution containing a mixture of these ions; but the addition of  $\text{Ca}^{2+}$  drastically altered the ratio of their absorption, increasing the uptake of  $\text{K}^+$  and decreasing that of  $\text{Na}^+$ .

Hyder and Greenway (1965) noted that NaCl reduced growth of barley much more at low nutrient concentrations (1/40 Hoagland) than at higher dilutions (1/10), and that growth was restored (to that of 1/10 nutrient) when  $\text{Ca}^{2+}$  was added; addition of other ions, however, did not improve growth. Thus, they concluded that the adverse effects of  $\text{Na}^+$  were partly due to a low Ca:Na ratio. Similarly, LaHaye and Epstein (1969, 1971) working with beans, a rather NaCl-sensitive species, reported that between 1 and 3 mol  $\text{m}^{-3}$   $\text{Ca}^{2+}$  were needed to improve the growth of plants in 50 mol  $\text{m}^{-3}$  NaCl to almost that of the controls. They proposed that the site of this Na/Ca interaction was the plasmalemma of the root cells.

Nowadays, the beneficial effect on salt stress of added  $\text{Ca}^{2+}$  is generally recognized (Rengel, 1992). In the very few studies where no significant effect was found (e.g. Leidi *et al.*, 1991) it was probably because the control conditions already had relatively high levels of  $\text{Ca}^{2+}$  (6 mol  $\text{m}^{-3}$   $\text{Ca}^{2+}$  in the above paper).

The effects of higher  $\text{Ca}^{2+}$  concentrations in the saline medium were not restricted to improved growth; as had been shown with very low concentrations of  $\text{Na}^+$ , they also influenced the absorption of other ions. Thus LaHaye and Epstein (1971) observed that increased  $\text{Ca}^{2+}$  depressed  $\text{Na}^+$  absorption by bean roots, and its translocation to the leaves. The decrease in  $\text{K}^+$  uptake caused by the  $\text{NaCl}$  treatments was also reduced by the addition of higher concentrations of  $\text{Ca}^{2+}$  (Elzam, 1971; Lynch & Läuchli, 1985). As a result,  $\text{K/Na}$  ratios in the plant were increased. Uptake of  $\text{NO}_3^-$  under salinity has also been reported to increase with the addition of  $\text{Ca}^{2+}$  (Ward *et al.*, 1986).

It is worth noticing that the plants in these experiments, which were performed in solution culture, never exhibited any symptoms of  $\text{Ca}^{2+}$  deficiency under "normal" (control) conditions. That is,  *$\text{Ca}^{2+}$  levels that were adequate for growth in a balanced nutrient solution proved insufficient when the  $\text{Na/Ca}$  ratio in the growth medium was increased* (Bernstein, 1975). This statement was to be confirmed in many other studies (e.g. Kent & Läuchli, 1985; Maas & Grieve, 1987). It seems that high external  $\text{Na}^+$  strongly reduces the chemical activity of  $\text{Ca}^{2+}$  ions in the root medium, and thus decreases the amount of  $\text{Ca}^{2+}$  that is available for uptake by the plant (Cramer & Läuchli, 1986; Cramer *et al.*, 1986).

That  $\text{Na}^+$  interferes with normal  $\text{Ca}^{2+}$  nutrition was proved by Lynch and Läuchli (1985) with barley plants growing in rather low salinity ( $30 \text{ mol m}^{-3} \text{ NaCl}$ ):  $\text{Ca}^{2+}$  levels in shoots of those plants decreased, in comparison to the control, due to a lower transport of  $\text{Ca}^{2+}$  from roots to shoots. (This was not simply a result of decreased transpiration, since the reduction in transpiration was less than the reduction in  $\text{Ca}^{2+}$  transport, and  $\text{Ca}^{2+}$  transport to non-transpiring organs was reduced too.) They suggested that  $\text{Na}^+$  probably inhibits  $\text{Ca}^{2+}$  transport to the shoot at the root level, before it enters the xylem; (it seems unlikely that its movement would be restricted by  $\text{Na}^+$  once it had entered the xylem). Later, Cramer *et al.* (1985) observed that  $\text{Na}^+$  displaced  $\text{Ca}^{2+}$  from the plasmalemma of the root cells, and postulated that this was the primary response to salt stress. However, other authors (Lynch & Läuchli, 1988; Martinez & Läuchli, 1993) believe that it is intracellular (tonoplast)  $\text{Ca}^{2+}$ , rather than that at the plasma membrane, which is affected.

The essentiality of  $\text{Ca}^{2+}$  for preserving the structural and functional integrity of plant membranes has been recognised (Hanson, 1984). Considering that these membranes constitute the physiological barrier to free movement of ions in plants, it is not surprising that any disturbance in the membrane-associated  $\text{Ca}^{2+}$  will also affect the status of other ions. This would explain the observed changes in  $\text{Na}^+$  and  $\text{K}^+$  concentrations: with the loss of  $\text{Ca}^{2+}$  the membranes become more permeable, inducing  $\text{K}^+$  to leak out of the cytoplasm, and increasing the entry of  $\text{Na}^+$ . The beneficial effect of high  $\text{Ca}^{2+}$  concentrations in a saline environment would then be due to the maintenance of K/Na selectivity through an improved  $\text{Ca}^{2+}$  status of the roots (Kent & Läuchli, 1985; Cramer *et al.*, 1987).

It is not clear whether the displacement of  $\text{Ca}^{2+}$  from binding sites in the membranes is  $\text{Na}^+$ -specific (Cramer *et al.*, 1985) or may also be induced by other monovalent ions (Lynch *et al.*, 1987). Depending on the exact mode of action of  $\text{Na}^+$  (and other?) ions, the external  $\text{Ca}^{2+}$  concentration required to compensate for these effects might increase with increasing external salinity or, alternatively, be constant for any level of salinity (Zidan *et al.*, 1991). That is, it is not known whether what is needed is a minimum concentration of  $\text{Ca}^{2+}$  or a minimum ratio of  $\text{Ca}^{2+}$  relative to  $\text{Na}^+$ . However, even though some aspects are still uncertain, it is quite generally accepted that injury to membranes, through changes in  $\text{Ca}^{2+}$  status, is one of the initial effects of NaCl stress (Leopold & Willing, 1984; Rengel, 1992).

It is important to remark that although  $\text{Ca}^{2+}$  plays an important role in salt tolerance, it is not the sole factor involved in salt stress. Additional  $\text{Ca}^{2+}$  certainly ameliorates the effects of NaCl and partly restores growth in most plant species, but not always to the levels of plants in normal conditions. Calcium deficiency cannot, therefore, be the main cause of growth inhibition.

At the other extreme, high  $\text{Ca}^{2+}$  concentrations in the medium may also cause nutritional imbalances. Thus, saline irrigation water made up with  $\text{CaCl}_2$  and NaCl increased absorption of  $\text{Ca}^{2+}$  and decreased that of  $\text{K}^+$  in carrots, compared to the controls (Bernstein & Ayers, 1953). The same authors (1951), noticing the high levels of  $\text{Ca}^{2+}$  accumulated by bean plants in a similar experiment, suspected that a

nutritional imbalance (additional to any osmotic effect) was the cause of their poor performance. Furthermore, reduced leaf  $\text{Mg}^{2+}$ , together with increased leaf  $\text{Ca}^{2+}$ , has usually been reported in plants growing at high external  $\text{Ca}^{2+}$  concentrations (Nassery *et al.*, 1979; Grieve & Maas, 1988; Plaut & Grieve, 1988).

A Ca/Mg imbalance may lead to a deficiency of  $\text{Mg}^{2+}$ , which has been suggested as the reason for the lower growth of sesame under  $\text{CaCl}_2$  salinity (Nassery *et al.*, 1979), and also for the poorer performance of some sorghum genotypes at high (almost 4:1) Ca:Na ratios (Grieve & Maas, 1988). Under non-saline conditions,  $\text{Mg}^{2+}$  deficiency has been shown to reduce photosynthesis in several plant species, including maize (Peaslee & Moss, 1966), spinach (Bottrill *et al.*, 1970) and sugar beet (Terry & Ulrich, 1974).

In most experiments, high  $\text{Ca}^{2+}$  treatments are supplied with  $\text{CaCl}_2$  because of the difficulty of using other salts of  $\text{Ca}^{2+}$ : some of them (e.g.  $\text{CaSO}_4$ ) are highly insoluble, and other anions can be more toxic than  $\text{Cl}^-$  at high concentrations. In these conditions it is difficult to establish the origin of the toxic effects, i.e. whether high internal  $\text{Ca}^{2+}$  is toxic to plants (either directly or through an induced  $\text{Mg}^{2+}$  deficiency), or the accompanying high  $\text{Cl}^-$  concentrations are toxic. Nassery *et al.* (1979) tried to determine which ion ( $\text{Ca}^{2+}$  or  $\text{Cl}^-$ ) was the cause of the large (65%) growth reduction observed with sesame growing with only  $15 \text{ mol m}^{-3} \text{ CaCl}_2$ . They did so by comparing different ratios of  $\text{Ca}(\text{NO}_3)_2$  and  $\text{NaCl}$ , and found that  $\text{Ca}(\text{NO}_3)_2$  alone suppressed growth more than  $\text{NaCl}$  or any combination of the two salts. Since  $\text{NO}_3^-$  had previously been found to be the least detrimental of the anions tested ( $\text{Cl}^-$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ , with  $\text{Na}^+$  as accompanying cation), they suggested that the reduction in growth was due to high  $\text{Ca}^{2+}$  concentrations.

The same authors suggested that it was not high levels of  $\text{Ca}^{2+}$  or  $\text{Cl}^-$  which caused necrosis in  $\text{CaCl}_2$ -treated plants, but the low  $\text{Mg}^{2+}$  concentrations; ( $\text{Ca}^{2+}$  levels were similar to those in control plants, and  $\text{Cl}^-$  levels were lower than in uninjured  $\text{NaCl}$ -treated plants). Plaut and Grieve (1988) reached a similar conclusion when they observed that maize plants grown with high  $\text{CaCl}_2$  ( $\approx 55 \text{ mol m}^{-3}$ ) and normal levels of  $\text{Mg}^{2+}$  developed chlorosis, but when 1/3 of the  $\text{Ca}^{2+}$  was replaced with  $\text{Mg}^{2+}$  the

plants remained green and uninjured. This substitution also restored in part the decrease in  $\text{CO}_2$  fixation that had been found with high  $\text{Ca}^{2+}$ , which is in accordance with the observation that  $\text{Mg}^{2+}$  deficiency reduces photosynthesis. These authors concluded that, at high external  $\text{CaCl}_2$ , part of the inhibition of photosynthetic activity was due to  $\text{Mg}^{2+}$  insufficiency, but part was also due to high  $\text{Ca}^{2+}$  *per se*. They based this statement on the fact that  $\text{Na}^+$  concentrations in the treatment where  $\text{Ca}^{2+}$  was partly replaced with  $\text{Mg}^{2+}$  were similar to those in the control; they did not discuss, though,  $\text{Cl}^-$  concentrations, which were as high as those in other saline treatments. Since rates of  $\text{CO}_2$  fixation were also similar to those in the high  $\text{NaCl}$  treatments, it might well have been the  $\text{Cl}^-$  ion which was affecting photosynthesis.

It is difficult to interpret the results when several factors are changing at the same time. In many experiments comparing different  $\text{Na}:\text{Ca}$  ratios the external osmotic pressure is kept constant by changing the absolute concentrations of the ions considered. The same problem is found in the last two treatments of the above-mentioned experiment (Plaut & Grieve, 1988), where the effects of decreasing  $\text{Ca}:\text{Mg}$  ratios are confounded by those due to decreased  $\text{Ca}^{2+}$ . A treatment where the lower  $\text{Ca}:\text{Mg}$  ratio was obtained by adding  $\text{Mg}^{2+}$ , instead of by replacing part of the  $\text{Ca}^{2+}$  by  $\text{Mg}^{2+}$ , might have clarified the response.

It is generally agreed that the level of free  $\text{Ca}^{2+}$  in the cytoplasm is very low, around  $1 \mu\text{mol m}^{-3}$  or less (Wyn Jones & Pollard, 1983). Such low levels have to be maintained in order to prevent interferences with other ions and with enzymes. In particular, excess  $\text{Ca}^{2+}$  might react with inorganic phosphate forming an insoluble precipitate, and phosphate-based energy metabolism would then be severely inhibited (Hepler & Wayne, 1985). A large proportion (up to 60%) of cytoplasmic  $\text{Ca}^{2+}$  is sequestered in organelles (mitochondria, chloroplasts). Any excess  $\text{Ca}^{2+}$  that enters the cytoplasm is actively pumped out back to the apoplast, or into the vacuole which acts as a sink for excess  $\text{Ca}^{2+}$  (Hanson, 1984). Thus, even though  $\text{Ca}^{2+}$  is usually reported as a non toxic ion, if it is not properly compartmented (in the same way as  $\text{Na}^+$  and  $\text{Cl}^-$ ) it may have damaging effects.

## 3.2. MINIMUM $\text{Ca}^{2+}$ REQUIREMENTS IN SALT-STRESSED BARLEY.

### 3.2.1. OBJECTIVES:

In a previous experiment (not reported here) no differences were found in the growth of barley when Na:Ca molar ratios ranging from 2:1 to 20:1 were used at  $10 \text{ dS m}^{-1}$  ( $30$  to  $5 \text{ mol m}^{-3} \text{ Ca}^{2+}$ ,  $60$  to  $95 \text{ mol m}^{-3} \text{ NaCl}$ ), suggesting that the proportion used routinely in the experiments in Bangor (20:1) covers the minimum requirements needed to ameliorate the adverse effects of salinity. The present experiment was set up to determine what was the minimum amount of  $\text{Ca}^{2+}$  needed to improve the growth of barley under a more severe ( $200 \text{ mol m}^{-3}$ ) NaCl salinity stress, and to confirm that the levels conventionally used in our hydroponic experiments were sufficient. Because it was suspected that this minimum level might be dependent on the degree of tolerance of the genotype involved, two contrasting varieties (one tolerant, one sensitive) were used.

### 3.2.2. MATERIALS AND METHODS:

The experiment was conducted in a glasshouse at the University of Wales, Bangor (Memorial Building), in August 1991. The minimum temperature was  $15^\circ\text{C}$ , and natural light was supplemented with 400W Son-T high pressure sodium lamps (Osram) for a minimum of 12 hours per day. Two varieties of barley, CM-67 and Chevron, were used. Five Na:Ca molar ratios were used as treatments (20:1, 40:1, 100:1, 200:1, 400:1) at a constant NaCl concentration of  $200 \text{ mol m}^{-3}$ , with the corresponding  $\text{CaCl}_2$  concentrations being 10, 5, 2, 1, and  $0.5 \text{ mol m}^{-3}$  respectively.

Seeds of the 2 varieties were washed in running tap water for 24 hours, and sown (06.08.91) on rock-wool in plastic plug trays, one seed per cell and 12 seeds of each variety per tray. Trays were suspended on  $9 \text{ dm}^3$  tubs (Z210, Mailbox International), containing a solution of  $1 \text{ mol m}^{-3} \text{ Ca}(\text{NO}_3)_2$  and  $0.5 \text{ mol m}^{-3} \text{ MgSO}_4$ ; a total of 5 tubs (one for each treatment) were used. Three days later, when 70% of the seedlings had already emerged, Phostrogen and micronutrients were added, as in previous experiments (e.g. section 2.2.2).

On 11.08.91, trays were "thinned" to leave 5 plants of each variety (randomly distributed) per tub. At the same time, the stress was started by adding 25 mol m<sup>-3</sup> NaCl (plus the corresponding concentration of CaCl<sub>2</sub>) twice a day, until the final concentration was reached (4 days later). The concentration of Ca<sup>2+</sup> in the Phostrogen solution (0.5 mol m<sup>-3</sup>) was taken into account when preparing the different Na:Ca ratios. All solutions were replaced at weekly intervals; their ECs were around 19 dS m<sup>-1</sup>.

Plants were harvested 19 days after the final concentration was reached, when they were 4 weeks old (02.09.91). Harvested plants were divided into shoot and root. For the shoot, fresh weight and stem length (from the base of the stem to the top of the sheath of the youngest expanded leaf) were recorded, and leaves number 2 ("old") and 4 (youngest expanded) from the base were sampled for sap extraction; the rest of the shoot was oven-dried to obtain dry weight. The roots were washed for 2 minutes in a MgSO<sub>4</sub> solution of the same osmolality as 200 mol m<sup>-3</sup> NaCl (to avoid losses of salts), and dried with tissue paper; fresh weight was recorded, and the whole root sampled for sap extraction (same procedure as with leaves).

On the extracted sap, major ions (Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup>) were analyzed by HPLC (Dionex 2000i); Ca<sup>2+</sup> was determined by atomic absorption spectrophotometry (SP2900, Pye Unicam); (methods as detailed in section 2.2.2).

Statistical analysis was performed using the Genstat-5 package. A 2-factor analysis of variance (calcium level and variety) was carried out, using individual values of 5 plants (since only one tub had been used for each treatment). Because there were significant interactions (calcium x variety) in many traits, the treatments were compared within each variety. Separation of means was done using Tukey's test.

### 3.2.3. RESULTS:

#### 3.2.3.1. Plant growth (Table 3.2.1):

The two varieties behaved rather differently in relation to the growth traits measured. With decreasing Ca<sup>2+</sup> levels, CM-67 increased its dry weight and, especially, the



stem length, down to 1 mol m<sup>-3</sup> Ca<sup>2+</sup>; only at lower Ca<sup>2+</sup> concentrations (0.5 mol m<sup>-3</sup>) was its growth severely reduced. On the contrary, Chevron began to decrease both dry weight and stem length earlier (around 2 mol m<sup>-3</sup> Ca<sup>2+</sup> and below).

It is worth noting that decreasing Ca<sup>2+</sup> availability induced earliness in CM-67; (a few plants already had the flag leaf out in the treatment with 1 mol m<sup>-3</sup> Ca<sup>2+</sup>). It would be interesting to see how greatly grain yield was affected (decreased) by the reduction in the period left for ear development. On the other hand, the same conditions caused a sort of dwarfing effect on Chevron (quite noticeable by observation, although not in all plants).

The water content in the plant, measured as the ratio of fresh weight to dry weight, was slightly higher in Chevron than in CM-67. This ratio was very similar for all treatments in CM-67, but in the lowest Ca<sup>2+</sup> treatment of Chevron it decreased significantly ( $p < 5\%$ ; Tukey's test).

Table 3.2.1. Shoot dry weight (mg), stem length (mm), and fresh weight to dry weight (FW/DW) ratio of 2 varieties (CM=CM-67, CH=Chevron) grown at 200 mol m<sup>-3</sup> NaCl and decreasing levels of CaCl<sub>2</sub>; (means of 5 plants).

Ca <sup>2+</sup> conc. (mol m <sup>-3</sup> )	DRY WEIGHT		STEM LENGTH		FW/DW RATIO	
	CM	CH	CM	CH	CM	CH
10	252	251	154	85	5.66	6.41
5	283	257	193	78	5.30	6.03
2	300	228	234	60	5.45	6.26
1	291	179	256	59	5.29	5.65
0.5	183	137	88	46	5.38	4.89
L.S.R.*	123	49	122	18	0.98	1.07

\* L.S.R. - Least Significant Range,  $\alpha=0.05$  (Tukey's test)

### 3.2.3.2. Ion concentrations (Table 3.2.2):

#### i) in leaves (young and old):

In both varieties, chloride concentrations (young and old leaves) did not increase with decreasing levels of  $\text{Ca}^{2+}$  until the lowest concentrations were reached (less than  $2 \text{ mol m}^{-3} \text{ Ca}^{2+}$ ); (Table 3.2.2a). Concentrations of  $\text{Cl}^-$  were quite similar for both varieties, except at  $0.5 \text{ mol m}^{-3} \text{ Ca}^{2+}$ , where concentrations in Chevron tended to be higher than in CM-67 (although not significantly at the 5% level).

Like  $\text{Cl}^-$ ,  $\text{Na}^+$  concentrations did not increase until the lowest levels of  $\text{Ca}^{2+}$  (below  $2 \text{ mol m}^{-3}$ ) were reached, both in young and old leaves; (Table 3.2.2b). This behaviour was common to both varieties, although the concentrations of  $\text{Na}^+$  in Chevron were about twice those of CM-67.

Potassium concentrations did not change very much with decreasing  $\text{Ca}^{2+}$  in the external solution, particularly in older leaves; (Table 3.2.2c). Only in young leaves of CM-67 were the concentrations at the lowest level much lower ( $p < 0.05$ ; Tukey's test) than in the other treatments. The concentrations of  $\text{K}^+$  in Chevron were only half those in CM-67.

As might have been expected,  $\text{Ca}^{2+}$  concentrations in leaves (both young and old) decreased with decreasing availability in the external solution; (Table 3.2.2d). This was true for both varieties, and particularly in the young leaves of Chevron, where the amounts of  $\text{Ca}^{2+}$  were extremely low (less than  $1 \text{ mol m}^{-3}$ ) in the lowest treatments.

#### ii) in roots:

Not many differences were found in root  $\text{Cl}^-$  concentrations between the 2 varieties, except for the 2 lowest  $\text{Ca}^{2+}$  levels where the concentrations for CM-67 were higher ( $p < 0.05$ ; Tukey's test); in Chevron, however, they did not change significantly (at the 5% level); (Table 3.2.2a). On the other hand, the concentrations of  $\text{Na}^+$  in roots

Table 3.2.2.  $\text{Cl}^-$  (a),  $\text{Na}^+$  (b),  $\text{K}^+$  (c) and  $\text{Ca}^{2+}$  (d) concentrations ( $\text{mol m}^{-3}$  sap) in young leaves, old leaves and roots of 2 varieties (CM=CM-67; CH=Chevron) growing at  $200 \text{ mol m}^{-3}$  NaCl and decreasing levels of  $\text{CaCl}_2$ ; (means of up to 5 plants).

$\text{Ca}^{2+}$ conc. ( $\text{mol m}^{-3}$ )	YOUNG LEAVES		OLD LEAVES		ROOTS	
	CM	CH	CM	CH	CM	CH
<i>a) Chloride:</i>						
10	268	281	371	410	131	142
5	301	275	393	565	142	147
2	276	285	363	417	145	133
1	ns	404	408	663	182	150
0.5	595	942	764	988	179	151
L.S.R.*	91	374	171	317	29	33
<i>b) Sodium:</i>						
10	125	269	302	450	166	156
5	145	297	361	711	189	163
2	158	323	347	509	203	159
1	ns	437	450	842	257	177
0.5	578	1082	893	1270	272	187
L.S.R.*	53	477	216	478	34	29
<i>c) Potassium:</i>						
10	255	121	132	74	51	56
5	247	73	104	49	32	36
2	204	65	79	30	23	27
1	ns	56	83	37	21	21
0.5	103	98	102	73	14	9
L.S.R.*	32	80	59	43	9	7
<i>d) Calcium:</i>						
10	6.7	7.7	9.6	8.3	3.3	3.6
5	4.7	4.6	9.5	8.3	2.8	2.8
2	3.6	1.8	5.3	6.3	2.3	2.3
1	ns	0.6	3.9	3.6	2.2	2.3
0.5	2.0	0.3	3.1	2.5	2.1	2.1
L.S.R.*	3.3	2.5	5.5	2.7	0.4	0.6

ns - data not available (no sap)

\* L.S.R. - Least Significant Range,  $\alpha=0.05$  (Tukey's test)

of CM-67 were generally higher than those in Chevron, and they increased noticeably with decreasing the availability of  $\text{Ca}^{2+}$  in the external solution, while they hardly changed in Chevron; (Table 3.2.2b). Potassium concentrations in the roots of both varieties were very similar for a given treatment, and they decreased considerably with decreasing the amounts of  $\text{Ca}^{2+}$  in the solution; (Table 3.2.2c). A similar behaviour was found for root  $\text{Ca}^{2+}$  concentrations; (Table 3.2.2d).

#### 3.2.4. DISCUSSION:

In order to determine the minimum level of  $\text{Ca}^{2+}$  needed to ameliorate the growth reduction caused by  $200 \text{ mol m}^{-3}$  NaCl, in the present experiment the proportion of  $\text{Ca}^{2+}$  in the saline solution was progressively decreased. The growth data shows that this minimum depends on the variety considered: the growth of Chevron (salt-sensitive) was affected at higher  $\text{Ca}^{2+}$  concentrations than that of CM-67 (salt-tolerant). That is, the former needed higher levels of  $\text{Ca}^{2+}$  (minimum  $2 \text{ mol m}^{-3}$ ) than the latter ( $1 \text{ mol m}^{-3}$ ) to maintain its growth at that salinity. Different responses to supplemental  $\text{Ca}^{2+}$  between genotypes have also been reported for growth in sorghum (Grieve & Maas, 1988), and for germination in triticale (Norlyn & Epstein, 1984).

Not many similar step-down experiments with  $\text{Ca}^{2+}$  concentrations at a given salinity are found in the literature; most studies only deal with the addition of moderate amounts of  $\text{Ca}^{2+}$  to the basic nutrient solution. For instance, Cramer *et al.* (1989) found that  $10 \text{ mol m}^{-3}$   $\text{Ca}^{2+}$  partly ameliorated the growth of barley at  $150 \text{ mol m}^{-3}$  NaCl in relation to the  $0.4 \text{ mol m}^{-3}$   $\text{Ca}^{2+}$  present in their control conditions, but no intermediate concentrations were studied. Only in the paper by Yeo and Flowers (1985) were  $\text{Ca}^{2+}$  concentrations reduced in a gradual way. These authors found that the growth of rice at  $50 \text{ mol m}^{-3}$  NaCl only decreased below  $0.2 \text{ mol m}^{-3}$   $\text{Ca}^{2+}$  (a Na:Ca ratio of 250:1), although  $\text{Na}^+$  concentrations in the shoot began to increase earlier (below  $1 \text{ mol m}^{-3}$   $\text{Ca}^{2+}$ ).

In many experiments (e.g. Maas & Grieve, 1987; Plaut & Grieve, 1988; Grieve & Maas, 1988) the Na:Ca ratios are progressively changed by substituting  $\text{Ca}^{2+}$  for  $\text{Na}^+$ , in order to maintain a constant osmotic potential or  $\text{Cl}^-$  concentration in the

external solution; but then it is difficult to separate the effects of increased  $\text{Ca}^{2+}$  with those due to reduced  $\text{Na}^+$ . This is the case in the experiments of Subbarao *et al.* (1990) with pigeonpea, where a positive response to decreasing Na:Ca ratios was found up to  $\text{Ca}^{2+}$  concentrations of 10 and 15  $\text{mol m}^{-3}$ , at a salinity of 6  $\text{dS m}^{-1}$  (corresponding to 40 and 30  $\text{mol m}^{-3}$  NaCl, and Na:Ca ratios of 4 and 2, respectively).

Ward *et al.* (1986), working with CM-72 (a barley variety developed from CM-67), compared elongation rates of the second leaf of plants grown at 150  $\text{mol m}^{-3}$  NaCl and either 0.5 or 3  $\text{mol m}^{-3}$   $\text{Ca}^{2+}$ . They found that the latter concentration (3  $\text{mol m}^{-3}$   $\text{Ca}^{2+}$ ) improved growth. In a review by Clarkson and Hanson (1980) it is mentioned that  $\text{Ca}^{2+}$  concentrations between 1 and 5  $\text{mol m}^{-3}$  are generally required to protect the roots of plants from the deleterious effects of, among other things, salinity and ion imbalance. The present results ( $\approx 2$  and 1  $\text{mol m}^{-3}$  for Chevron and CM-67, respectively) fall within this range.

The data for ion concentrations are in general agreement with those for growth: they do not change very much until the lowest  $\text{Ca}^{2+}$  treatments are reached. An exception, however, is found in roots, where  $\text{K}^+$  concentrations decreased continuously with decreasing external  $\text{Ca}^{2+}$  levels for both varieties. The role of  $\text{Ca}^{2+}$  in maintaining membrane selectivity has already been mentioned: with low external  $\text{Ca}^{2+}$  membranes become more permeable and there is a leakage of  $\text{K}^+$  (efflux) out of the cell. This would explain the observed decreasing  $\text{K}^+$  concentrations in roots with decreasing  $\text{Ca}^{2+}$  availability. It is interesting to note that these changes cover the whole range of  $\text{Ca}^{2+}$  concentrations used, something not observed in the other traits studied where only the two lowest treatments are affected (except, maybe, root  $\text{Na}^+$  concentrations in CM-67). According to these data,  $\text{K}^+$  levels in roots respond to increasing external  $\text{Ca}^{2+}$  at least up to 10  $\text{mol m}^{-3}$  (no higher concentrations were investigated).

Elzam (1971) reported that  $\text{K}^+$  uptake by barley roots growing in 100  $\text{mol m}^{-3}$  NaCl was reduced by 95% relative to the non-saline control when external  $\text{Ca}^{2+}$  concentrations were low (0.5  $\text{mol m}^{-3}$ ), but this reduction was smaller (72%) with 4  $\text{mol m}^{-3}$  external  $\text{Ca}^{2+}$ . She did not try, though, higher levels of  $\text{Ca}^{2+}$ . Similar results

were reported for cotton (Kent & Läuchli, 1985) when comparing  $K^+$  concentrations in roots of plants growing without NaCl or with  $200 \text{ mol m}^{-3}$  NaCl and either  $0.4$  or  $10 \text{ mol m}^{-3}$   $Ca^{2+}$ . But again there is no information on intermediate levels.

If we consider all the ion data for leaves and roots, particularly in the 3 treatments with the highest  $Ca^{2+}$  levels, it is worth noticing the differences between the two varieties: in CM-67, less  $Na^+$  is going into the plant, and a higher proportion of it is being retained in roots and old leaves, than in Chevron. The opposite happens with  $K^+$ : more  $K^+$  is going into CM-67, and there it is better directed towards young leaves, than in Chevron. This fact is well reflected in the K:Na ratios of the two varieties: in CM-67, young leaves had higher K:Na ratios ( $> 1$ ) than roots ( $< 1$ ) (except in the lowest  $Ca^{2+}$  treatment), while in Chevron these ratios were similar for young leaves and roots ( $< 1$ ). This higher K:Na selectivity has been proposed as one of the reasons for the higher salt tolerance of CM-67 (Jeschke & Wolf, 1985).

In view of the extreme levels of salt sensitivity/tolerance of the 2 varieties studied, and the fact that they were subjected to a rather high NaCl salinity, it seems quite safe to extrapolate and conclude that a  $Ca^{2+}$  concentration of  $2 \text{ mol m}^{-3}$  is enough for most barley varieties at the range of salinities usually employed. As mentioned before, it is not known whether this minimum requirement is going to be constant for any salinity level or if, alternatively, it depends on the NaCl concentration. If the later case was true, then we should talk about Na:Ca ratios rather than absolute concentrations. This aspect was not considered in the present experiment, because the initial interest was in deciding if the amounts of  $Ca^{2+}$  in the hydroponic experiments were adequate. Typical levels of soluble  $Ca^{2+}$  in saline soils are around  $15 \text{ mol m}^{-3}$  (Richards, 1954); this is well above the  $2 \text{ mol m}^{-3}$  required to protect membranes from injury and, therefore, addition of  $Ca^{2+}$  is not necessary. (The case of sodic soils is different. There, application of gypsum - $CaSO_4$ - has a double effect: it improves soil structure and aeration, and it increases the Ca:Na ratio.) In hydroponic experiments, however, a minimum amount of  $Ca^{2+}$  ( $2 \text{ mol m}^{-3}$ ) needs to be added to the nutrient solution. This minimum is well covered by the 20:1 Na:Ca molar ratio used in the Bangor experiments, where the highest salinities do not usually exceed  $200 \text{ mol m}^{-3}$  NaCl (i.e.  $10 \text{ mol m}^{-3}$   $Ca^{2+}$ ).

### 3.3. EFFECTS OF HIGH LEVELS OF $\text{Ca}^{2+}$ (AS $\text{CaCl}_2$ ) IN BARLEY.

#### 3.3.1. OBJECTIVES:

The first of the next two experiment was set up to explore the toxicity of  $\text{CaCl}_2$ . As this might depend on the genotype, two varieties known to differ in their response to  $\text{NaCl}$  were used. The results showed a large decrease in the dry weight of plants growing in  $50 \text{ mol m}^{-3} \text{ CaCl}_2$ , which was the lowest salinity tested. Thus, a second experiment was set up to investigate the effect of lower concentrations and determine when  $\text{CaCl}_2$  begins to reduce the growth of barley.

#### 3.3.2. MATERIALS AND METHODS:

##### *Experiment 1.*

The first experiment was carried out in a glasshouse in the University of Wales, Bangor (Memorial Building), during July and August 1991. Conditions in the greenhouse were the same as in the previous experiment (section 3.2.2). Two varieties of barley, CM-67 and Chevron, were grown at 5 levels of  $\text{CaCl}_2$  salinity: 0 (control), 50, 100, 150, and  $200 \text{ mol m}^{-3} \text{ CaCl}_2$ .

Seeds of the 2 varieties were washed in running tap water for 24 hours, and sown (23.07.91) in plastic plug trays suspended on  $9 \text{ dm}^3$  tubs (see section 3.2.2 for details). A few days later, nutrient solution was added, and plants were thinned to leave 5 seedlings of each variety per tub; only one tub was used for each treatment.

Stress was begun on 28.07.91 by adding  $25 \text{ mol m}^{-3} \text{ CaCl}_2$  twice a day, until the highest concentration was reached (4 days). Solutions were replaced at weekly intervals. The approximate ECs of the different treatments were: 1, 9, 16.5, 24, 31.5  $\text{dS m}^{-1}$ . Plants were harvested when they were about 3 weeks old, and had been growing for 2 weeks under salinity; at this stage, the plants of variety Chevron at the highest salinities were almost dead.

The youngest expanded leaf was sampled for sap extraction, and the shoot was weighed and oven-dried to obtain dry weight. Roots were washed for 2 minutes in sorbitol solutions of the same osmolality as the corresponding treatment (to avoid losses of ions), dried with tissue paper, weighed and sampled for sap extraction.

On the extracted sap the following ions were analyzed:  $\text{Cl}^-$  with a chloride-meter (Corning-Eel 920, Evans Electroelenium Ltd, Halstead, Essex),  $\text{Ca}^{2+}$  by atomic absorption spectrophotometry (SP2900, Pye Unicam) and  $\text{K}^+$  by flame emission spectrophotometry (SP90, Pye Unicam).

A two-factor analysis of variance (salinity level and variety) was done using individual values of 5 plants (because of lack of proper replication). Although not many significant interactions were detected (i.e. the 2 cultivars responded similarly to increasing  $\text{CaCl}_2$  concentrations), the effects of increasing  $\text{CaCl}_2$  were still studied within each variety. Separation of means was carried out using Tukey's test.

### *Experiment 2.*

This experiment was conducted in a glasshouse at the University of Wales, Bangor (Pen-y-Ffridd Field Station) in November 1992. Environmental conditions in the greenhouse were as described in section 2.2.2. The same two varieties of the previous experiment (CM-67 and Chevron), were grown at 4 levels of  $\text{CaCl}_2$  salinity: 0 (control), 15, 30 and 45  $\text{mol m}^{-3}$   $\text{CaCl}_2$ , the highest treatment being similar to the lowest one before.

Seeds of the 2 varieties were soaked overnight and sown (05.11.92) in compost (John Innes n° 1, L & P Peat Ltd, Carlisle) in plastic trays (P84, Plantpak). Four days later, young seedlings were moved onto hydroponics: 9  $\text{dm}^3$  containers (Z210, Mailbox), with Phostrogen and micronutrients solution; four plants of each variety per tub, and 5 replicated tubs per treatment were used.

On 11.11.92 stress was begun by adding 15  $\text{mol m}^{-3}$   $\text{CaCl}_2$  per day, until the highest concentration was reached (3 days). Solutions were replaced at weekly intervals. The



ECs of the different treatments were: 0.5, 2.8, 4.6 and 6.1 dS m<sup>-1</sup>. On one occasion, samples of the solutions were taken for analysis of anions, because by using relatively high concentrations of CaCl<sub>2</sub> there might be a risk of phosphates being precipitated. Levels of soluble phosphate in the CaCl<sub>2</sub> treatments were found to be about 75% those in the control solution (from 0.40 to 0.48 mol m<sup>-3</sup>, compared to 0.60).

Plants were harvested when they had been growing for 3 weeks under salinity (4 weeks after sowing). The youngest expanded leaf was sampled for sap extraction; shoot fresh and dry weights were recorded. Extracted saps of 2 plants (same variety) from each tub were combined for the chemical analysis.

Main anions (Cl<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup>) were analysed by HPLC (Dionex 2000i) as previously described (section 2.2.2). Main cations (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup>) were also analysed by HPLC, using the same dilution as in the experiments of section 2.3.2.

The analysis of variance for 2 factors with interaction was done using Genstat-5. Mean values of the 4 plants of each variety in each tub (replication) were used. Separation of means was done using Tukey's test.

### 3.3.3. RESULTS:

#### *Experiment 1.*

##### 3.3.3.1. Plant growth (Table 3.3.1):

With only 50 mol m<sup>-3</sup> CaCl<sub>2</sub> (the lowest concentration) shoot dry weight was already reduced to almost half of that in the control, and it decreased a further 40% at 100 mol m<sup>-3</sup> CaCl<sub>2</sub>. These values were quite similar for the 2 varieties, (no significant differences, at the 5% level, within a given treatment). Fresh weight to dry weight ratios were also similar for the 2 cultivars, and they decreased with increasing CaCl<sub>2</sub> concentrations. This decrease in plant water content (FW:DW) is a well known response of some plants (particularly monocots) to salinity.

Table 3.3.1. Shoot dry weight (mg) and Fresh Weight to Dry Weight ratio (FW:DW) of 2 varieties grown at increasing concentrations of  $\text{CaCl}_2$ ; (means of 5 plants).

$\text{CaCl}_2$ (mol m <sup>-3</sup> )	DRY WEIGHT		FW:DW	
	CM-67	Chevron	CM-67	Chevron
0	601	560	9.5	9.0
50	352	253	7.5	6.2
100	201	163	5.3	4.2
150	114	95	4.7	4.3
200	105	61	4.3	3.6
LSR*	102	120	1.3	1.2

\* LSR = Least Significant Range,  $\alpha=0.05$  (Tukey's test)

### 3.3.3.2.- Ion concentrations in young leaves and in roots (Table 3.3.2):

As expected,  $\text{Cl}^-$  concentrations increased with increasing  $\text{CaCl}_2$ , both in leaves and in roots. For a given treatment (external  $\text{CaCl}_2$  concentration), there were no significant differences (at the 5% level) between varieties in the amounts of  $\text{Cl}^-$  in roots. In leaves, Chevron seemed to increase  $\text{Cl}^-$  concentrations faster than CM-67 above 50 mol m<sup>-3</sup>  $\text{CaCl}_2$ ; unfortunately, there is no data available for leaves of Chevron at the highest  $\text{CaCl}_2$  concentrations, where plants were almost dead and no sap could be extracted.

Like  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$  concentrations increased with increasing  $\text{CaCl}_2$ , both in roots and in leaves. There were no significant differences (5% level) between varieties in the amounts of  $\text{Ca}^{2+}$  in leaves or in roots at a given level of  $\text{CaCl}_2$ .

Concentrations of  $\text{K}^+$  in roots tended to increase in variety CM-67, especially at the highest treatments, but they did not change significantly (at the 5% level) in Chevron. In leaves, and up to 100 mol m<sup>-3</sup>  $\text{CaCl}_2$ ,  $\text{K}^+$  concentrations were similar for both varieties and did not change very much; they even increased for CM-67 at higher  $\text{CaCl}_2$  concentrations; (no data for Chevron).

Table 3.3.2. Chloride, calcium and potassium concentrations ( $\text{mol m}^{-3}$  sap) in the youngest leaves (a) and roots (b) of 2 varieties (CM=CM-67, CH=Chevron) grown at increasing concentrations of  $\text{CaCl}_2$ ; (means of up to 5 plants).

$\text{CaCl}_2$ ( $\text{mol m}^{-3}$ )	$\text{Cl}^-$		$\text{Ca}^{2+}$		$\text{K}^+$	
	CM	CH	CM	CH	CM	CH
<i>a) young leaves:</i>						
0	105	86	9	18	296	289
50	214	187	30	36	304	224
100	275	392	65	89	286	295
150	584	ns	174	ns	342	ns
200	876	ns	267	ns	404	ns
LSR*	193	55	44	16	53	42
<i>b) roots:</i>						
0	18	14	1.9	2.2	141	161
50	91	89	6.7	6.0	175	151
100	131	132	9.4	10.3	190	179
150	171	183	19.9	16.5	225	185
200	238	206	24.5	30.0	239	184
LSR*	31	25	5.9	6.9	54	37

ns - data not available (no sap)

\* LSR - Least Significant Range,  $\alpha=0.05$  (Tukey's test)

## Experiment 2.

### 3.3.3.3. Plant growth (Table 3.3.3):

Shoot dry weight tended to decrease with increasing external  $\text{CaCl}_2$ , although not very much for CM-67. Only in Chevron were there some significant differences: the dry weight of the highest treatment was lower than those in the control and with 15  $\text{mol m}^{-3}$   $\text{CaCl}_2$  ( $p < 0.05$ ; Tukey's test). Fresh weight to dry weight ratios also decreased, and slightly faster in Chevron than in CM-67.

Table 3.3.3. Shoot dry weight (mg) and Fresh Weight to Dry Weight ratios (FW:DW) of 2 varieties grown at low concentrations of  $\text{CaCl}_2$ ; (means of 20 plants).

$\text{CaCl}_2$ ( $\text{mol m}^{-3}$ )	DRY WEIGHT		FW:DW	
	CM-67	Chevron	CM-67	Chevron
0	296	309	13.9	13.9
15	317	304	12.8	11.8
30	280	272	10.9	10.2
45	270	240	10.0	8.8
LSR*	96	62	1.3	1.1

\* LSR = Least Significant Range,  $\alpha=0.05$  (Tukey's test)

#### 3.3.3.4. Ion concentrations in young leaves (Table 3.3.4):

As expected,  $\text{Cl}^-$  concentrations tended to increase with increasing external  $\text{CaCl}_2$ , particularly in Chevron; as a result, this variety always had higher concentrations of  $\text{Cl}^-$  than CM-67 for a given treatment (except in the control). On the other hand, concentrations of  $\text{NO}_3^-$  did not change significantly (at the 5% level) with increasing  $\text{CaCl}_2$  in CM-67, but they tended to decrease in Chevron. The latter variety always had lower amounts of  $\text{NO}_3^-$  than the former for a given level of  $\text{CaCl}_2$ , except in the control. This replacement of  $\text{NO}_3^-$  by  $\text{Cl}^-$  has already been seen in previous experiments (e.g. "Osmotic adjustment", section 2.3.3).

Phosphate concentrations did not change significantly (5% level) between treatments in CM-67, but in Chevron they decreased steadily with increasing external  $\text{CaCl}_2$ . Sulphate concentrations decreased slightly in the  $\text{CaCl}_2$  treatments in comparison to the control. A reduction in  $\text{SO}_4^{2-}$  levels was already observed with NaCl salinity in previous experiments (e.g. "Osmotic adjustment", section 2.3.3) which might be due to interferences caused by the high  $\text{Cl}^-$  concentrations. Overall, no significant differences between varieties were detected.

Concentrations of  $\text{K}^+$  did not change significantly (5% level) in Chevron, but they increased with increasing  $\text{CaCl}_2$  in CM-67. This variety had, in general, higher levels

of  $K^+$  than Chevron, particularly at the 2 highest treatments. Sodium concentrations were very low, as expected from an experiment which did not deal with NaCl salinity. The small differences found between treatments or varieties are probably not important.

Concentrations of calcium were similar for the 2 varieties in the control, but they increased very fast in Chevron as soon as  $CaCl_2$  was added to the external solution, whilst they only increased slightly in CM-67. On the other hand,  $Mg^{2+}$  concentrations hardly changed at all with increasing  $CaCl_2$ , but Chevron always had higher levels of  $Mg^{2+}$  than CM-67 at a given treatment.

Table 3.3.4. Main anion (a) and cation (b) concentrations ( $mol\ m^{-3}$ ) in young leaves of 2 varieties (CM=CM-67, CH=Chevron) grown at low concentrations of  $CaCl_2$ ; (means of 10 samples, each made up of 2 plants).

a) anions:

CaCl <sub>2</sub> (mol m <sup>-3</sup> )	Chloride		Nitrate		Phosphate		Sulphate	
	CM	CH	CM	CH	CM	CH	CM	CH
0	68	70	102	104	26.6	21.0	8.4	12.2
15	121	141	98	85	17.9	14.4	6.0	8.5
30	131	158	91	81	20.6	10.3	5.5	6.0
45	147	183	91	75	23.6	8.4	6.4	5.9
LSR*	21	14	12	9	11.5	11.0	7.2	5.8

b) cations:

CaCl <sub>2</sub> (mol m <sup>-3</sup> )	Potassium		Sodium		Calcium		Magnesium	
	CM	CH	CM	CH	CM	CH	CM	CH
0	207	199	3.4	4.9	2.2	3.0	2.4	4.6
15	226	218	3.7	2.7	3.2	6.9	2.3	3.6
30	245	222	2.7	3.1	4.6	13.1	2.3	4.7
45	258	224	3.6	3.7	7.8	25.0	2.9	5.3
LSR*	23	19	0.9	1.9	2.9	7.4	0.8	1.7

\* Least Significant Range,  $\alpha=0.05$  (Tukey's test)

### 3.3.4. DISCUSSION:

These two experiments were set up to investigate the toxicity of  $\text{CaCl}_2$ . The results on dry weight clearly show that  $\text{CaCl}_2$  strongly decreases the growth of barley. With only  $50 \text{ mol m}^{-3}$  (experiment 1), shoot dry weight was reduced by almost 50% in relation to the control. In experiment 2 the reductions were not as large; only 15% and 22% for CM-67 and Chevron respectively at  $45 \text{ mol m}^{-3} \text{ CaCl}_2$ . This discrepancy can be explained by the different environmental conditions experienced by the two experiments: the first one was done during the summer months, with plenty of natural daylight and high temperatures, while the second was carried out during winter. The combination of lower temperatures and lower light intensity (plants were almost completely dependent on artificial light) would have reduced growth in all treatments of experiment 2. This reasoning is supported by the fact that even the control plants grew only half as much in the second experiment as in the first one, in spite of having been harvested when they were one week older.

The considerable effect of  $\text{CaCl}_2$  on growth should not be very surprising: after all,  $50 \text{ mol m}^{-3} \text{ CaCl}_2$  is equivalent to  $100 \text{ mol m}^{-3} \text{ Cl}^-$ , and similar growth reductions are found with corresponding levels of  $\text{NaCl}$  (e.g. section 2.2.3). Further reductions in dry weight (with  $100 \text{ mol m}^{-3} \text{ CaCl}_2$ ) are also not much different from those found with  $200 \text{ mol m}^{-3} \text{ NaCl}$ . Higher  $\text{CaCl}_2$  concentrations do not have much practical interest, and they will not be discussed in detail.

In the first experiment, both varieties doubled their leaf  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  concentrations at  $50 \text{ mol m}^{-3} \text{ CaCl}_2$  in relation to the control. Above that, concentrations of  $\text{Cl}^-$  increased faster in Chevron than in CM-67, as in the case of  $\text{NaCl}$  salinity (see section 2.2.3). That is, above  $100 \text{ mol m}^{-3}$  external  $\text{Cl}^-$ , CM-67 restricts the accumulation of  $\text{Cl}^-$  in young leaves, while Chevron does not have such a tight control. This regulation of ion uptake by CM-67 seems to operate even at the highest treatments, (above  $200 \text{ mol m}^{-3}$  external  $\text{Cl}^-$ ) where, in spite of very high  $\text{Cl}^-$  concentrations in their leaves, plants of this variety were still alive, whereas those of Chevron were almost dead. It is easy to see that, even with such high  $\text{Cl}^-$  levels in the leaves, some regulation of  $\text{Cl}^-$  uptake must exist: with transpiration rates of 4 l

water per kg fresh weight per day (estimated from Kalaji & Nalborczyk, 1991) and FW:DW ratios of around 5 (Table 3.3.1),  $\text{Cl}^-$  concentrations in the plants growing in  $100 \text{ mol m}^{-3} \text{ CaCl}_2$  would increase by about  $500 \text{ mol m}^{-3}$  per day if  $\text{Cl}^-$  was not "excluded" from the transpiration stream.

CM-67 also restricted the accumulation of  $\text{Ca}^{2+}$  in leaves slightly better than Chevron, at least up to  $100 \text{ mol m}^{-3}$  external  $\text{CaCl}_2$  (experiment 1). Above that,  $\text{Ca}^{2+}$  concentrations in young leaves of CM-67 were very high (they increased almost exponentially), contrasting with the generally accepted idea that cereals contain low levels of  $\text{Ca}^{2+}$  (Loneragan & Snowball, 1969). But Gorham *et al.* (1980) already showed that, in saline habitats,  $\text{Ca}^{2+}$  levels in monocotyledonous halophytes were not lower than those of dicotyledonous. Therefore, it seems that, although in normal conditions monocots take up lower amounts of  $\text{Ca}^{2+}$  than dicots, given ample supply of this mineral they may accumulate it in substantial amounts. In these conditions it is probably used as an osmoticum in the vacuole since, as already mentioned, cytoplasmic  $\text{Ca}^{2+}$  concentrations have to be maintained within very restricted limits.

On the other hand,  $\text{K}^+$  concentrations in young leaves apparently did not change with increasing  $\text{CaCl}_2$  salinity, unlike what is usually observed with  $\text{NaCl}$  salinity (particularly in Chevron), where  $\text{K}^+$  concentrations decrease due to both competition with, and replacement by,  $\text{Na}^+$ . This does not happen with  $\text{Ca}^{2+}$ , because  $\text{Ca}^{2+}$  ions are (physically) too different from those of  $\text{K}^+$  to either compete with, or substitute for, them. In fact, in both experiments,  $\text{K}^+$  concentrations in leaf sap tended to increase at the highest treatments. However, this is only a reflection of the changes in FW:DW ratios with increasing external  $\text{CaCl}_2$ . Since FW:DW ratios decreased faster than the increase in  $\text{K}^+$  concentrations in leaf sap, when expressed on a dry weight basis  $\text{K}^+$  concentrations did tend to decrease (data not shown). Thus, although  $\text{K}^+$  is still the main cation used for osmotic adjustment in the vacuole, high external concentrations of  $\text{Ca}^{2+}$  seem to reduce the levels of  $\text{K}^+$  accumulated in young leaves.

In the second experiment, the patterns of ion accumulation with increasing external  $\text{CaCl}_2$  were similar to those seen in experiment 1, although the concentrations found both in the control and at the highest treatment ( $45 \text{ mol m}^{-3} \text{ CaCl}_2$ ) were lower. This

can be partly a result of the lower light and temperature, which would have reduced transpiration rates and, therefore, the uptake and translocation of those ions whose uptake is largely related to transpiration (particularly  $\text{Ca}^{2+}$  (Marschner, 1986)). In general, the differences between the two experiments were larger in CM-67 than in Chevron, reflecting the fact that the former is a fast-growing variety adapted to warm climates, whilst Chevron, preferring colder conditions, was not so badly affected by the limiting conditions of the winter experiment.

Concentrations of  $\text{Mg}^{2+}$  in sap (experiment 2) increased slightly with increasing external  $\text{Ca}^{2+}$ , but this was again a reflection of the decreased water contents of the leaves. If expressed on a dry weight basis,  $\text{Mg}^{2+}$  concentrations tended to decrease, although not very much in CM-67 (data not shown). This decrease, however, was not dependent on the  $\text{Ca}^{2+}$  concentrations (whether internal or external), but similar for all  $\text{CaCl}_2$  treatments. Thus, although some competition between  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  may exist, no clear effect of  $\text{Ca}^{2+}$  on leaf  $\text{Mg}^{2+}$  concentrations was observed here. This is in opposition to some results found for other species (e.g. Nassery *et al.*, 1979, with sesame; Plaut & Grieve, 1988, with maize) where a  $\text{Ca}^{2+}$ -induced  $\text{Mg}^{2+}$  deficiency was claimed. This might be due to the relatively low levels of  $\text{CaCl}_2$  used as treatments in the present experiment, combined with the low ion uptake experienced by the plants. ( $\text{Mg}^{2+}$  concentrations in all treatments were rather low compared to those reported elsewhere, and some  $\text{Mg}^{2+}$  deficiency might, indeed, have occurred, but not as a result of high  $\text{Ca}^{2+}$  levels). It is interesting to notice that Chevron had higher concentrations of  $\text{Mg}^{2+}$  than CM-67, in spite of having rather high  $\text{Ca}^{2+}$  concentrations too. High concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , though, are common in this variety when grown in hydroponics (see section 2.3.3).

Concentrations of  $\text{H}_2\text{PO}_4^-$  (experiment 2) did not change very much in CM-67, but they decreased steadily in Chevron, and at  $45 \text{ mol m}^{-3}$   $\text{CaCl}_2$ ,  $\text{H}_2\text{PO}_4^-$  concentrations in this variety (approximately  $65 \mu\text{mol g}^{-1}$  dry weight) were approaching deficiency levels (the minimum necessary for adequate growth is reported to be around  $60 \mu\text{mol g}^{-1}$  dry weight (Marschner, 1986)). This might have been another reason why Chevron plants died at the highest salinities of the first experiment (though  $\text{H}_2\text{PO}_4^-$  was not analysed there). This nutritional imbalance was not observed in CM-67, nor



in the maize experiment reported by Plaut and Grieve (1988), where  $\text{H}_2\text{PO}_4^-$  even increased slightly with increasing external  $\text{Ca}^{2+}$  in relation to the control. In maize, however, salinity may disrupt control of  $\text{H}_2\text{PO}_4^-$  uptake and lead to toxicity levels (Nieman & Clark, 1976), especially under conditions of high external  $\text{H}_2\text{PO}_4^-$ , typical of solution culture. Thus,  $\text{H}_2\text{PO}_4^-$  deficiency might be another of the particular features of variety Chevron related to its poor ability to regulate ion uptake under saline conditions. In the present experiment this might have been enhanced by the low levels of ion uptake and the lower availability of phosphate in the external solution after the addition of  $\text{CaCl}_2$  (see section 3.3.2).

Concentrations of ions in roots (experiment 1) were generally in good agreement with the results so far discussed.  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  increased regularly as their concentrations increased in the external solution, and the same happened with  $\text{K}^+$  for CM-67, but not for Chevron. This, combined with its inability to restrict  $\text{Cl}^-$  uptake, might be an indication about the failure of this variety at the highest  $\text{CaCl}_2$  treatments. Older leaves not only accumulate harmful ions (such as  $\text{Cl}^-$ ) but they also provide most of the  $\text{K}^+$  for the younger leaves and growing tissues. If the rate of leaf death (due to an excess of  $\text{Cl}^-$ ) is too fast, the supply of  $\text{K}^+$  to growing tissues will be at risk, especially if there is not an increase in  $\text{K}^+$  uptake by the roots to compensate. That might have been another reason for the poor performance of Chevron.

It may be worth mentioning that at the highest treatments of the first experiment the plants of CM-67 were still alive, in spite of the high  $\text{Cl}^-$  concentrations found in their leaves, and in contrast with those of Chevron which were almost dead. This fact might indicate that CM-67 is more resistant to  $\text{CaCl}_2$  salinity than Chevron, in the same way that it is more tolerant to  $\text{NaCl}$  salinity, (and this is probably due to its better regulation of ion uptake). This statement, however, cannot be maintained without further examination: how long would these plants have survived with those levels of  $\text{Cl}^-$  in their leaves? This aspect was not investigated, because such high concentrations of  $\text{CaCl}_2$  are not usually found under natural conditions. The only safe conclusion to be drawn from the present data is that  $\text{CaCl}_2$  salinity decreases growth depending on the environmental conditions and genotype (as happens with  $\text{NaCl}$ ), and that concentrations as low as 30 to 45 mol  $\text{m}^{-3}$  may already affect some varieties.

### 3.4. COMPARISON OF SODIUM AND CALCIUM TOXICITIES

#### 3.4.1. OBJECTIVES:

The decrease in growth observed in plants under salinity is usually attributed to a combination of osmotic effects and toxic effects. The latter could be due to either  $\text{Na}^+$  or  $\text{Cl}^-$ . From the results of the experiments with  $\text{CaCl}_2$  alone (section 3.3), where high concentrations of  $\text{Ca}^{2+}$  in leaves were found, we may speculate on a toxic effect of  $\text{Ca}^{2+}$  itself. In the present experiments, 3 types of salt ( $\text{NaCl}$ ,  $\text{CaCl}_2$ , and a mixture of both) were used in order to compare the relative toxicity of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ .

Since  $\text{Na}^+$  is a monovalent cation and  $\text{Ca}^{2+}$  is a divalent one, it is impossible to have the same concentrations of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  at any one time for the different salts, and this complicates the comparison. Sodium and calcium salts of other anions ( $\text{SO}_4^{2-}$ ,  $\text{CO}_3^{2-}$ ,  $\text{NO}_3^-$ ) could be included in the experiment as a reference, but then a new factor (the toxicity of the anion) would need to be considered. Many  $\text{Na}^+$  salts ( $\text{Na}_2\text{CO}_3$ ,  $\text{Na}_2\text{SO}_4$ ) are more toxic than  $\text{NaCl}$ , while many  $\text{Ca}^{2+}$  salts ( $\text{CaCO}_3$ ,  $\text{CaSO}_4$ ) are highly insoluble. Nitrate cannot be used as the anion either, since it may have a beneficial effect (nitrogen is a macronutrient). Thus, only  $\text{Cl}^-$  salts were used in this experiment.

The two salts ( $\text{NaCl}$  and  $\text{CaCl}_2$ ) can be compared at either the same osmotic pressure, the same electrical conductivity, or the same concentration of  $\text{Cl}^-$ . This last criterion was adopted in the present experiment. The comparison between the effects of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  would be done by contrasting plant growth at similar levels of  $\text{Cl}^-$  in the leaves. The hypothesis was that, if either of the two cations was more toxic than the other, a different degree of growth reduction would be observed at similar levels of  $\text{Cl}^-$  in leaves. By plotting growth against internal  $\text{Cl}^-$  concentrations, any differences in the toxicity of the cations would be detected by different response lines. A higher degree of growth inhibition could be interpreted as a greater toxicity of the particular cation.

A first experiment did not give enough evidence to decide which was the most toxic ion. Thus, a second one was set up in order to obtain more precise information, particularly at low  $\text{Cl}^-$  concentrations.

### 3.4.2. MATERIALS AND METHODS:

#### *Experiment 1.*

The first experiment was carried out in a greenhouse in the University of Wales, Bangor (Memorial Building) during September and October 1991. Conditions in the greenhouse were the same as described in previous experiments (e.g. section 3.2.2). Three types of salt were used:  $\text{CaCl}_2$ ,  $\text{NaCl}$  (with 1/20  $\text{CaCl}_2$ ) and a mixture (2:1 molar) of  $\text{NaCl}$  and  $\text{CaCl}_2$ . Each salt was applied at four  $\text{Cl}^-$  concentrations: 50, 100, 150 and 200  $\text{mol m}^{-3}$ . Only one variety (CM-67) was used, with 8 plants per treatment.

After being soaked overnight in running tap water, seeds of CM-67 were sown (10.09.91) in plastic plug trays on top of 9  $\text{dm}^3$  tubs containing Phostrogen and micronutrients, as in previous experiments. A week later, stress was begun by adding 50  $\text{mol m}^{-3}$   $\text{Cl}^-$  a day (corresponding to 25  $\text{mol m}^{-3}$   $\text{CaCl}_2$ , 45  $\text{mol m}^{-3}$   $\text{NaCl}$ , and the equivalent for the 2:1 mixture), until the highest concentrations were reached (4 days). All solutions were replaced at weekly intervals. The electrical conductivities and osmotic pressures of the different treatments are shown in Table 3.4.1.

Table 3.4.1. Electrical conductivities ( $\text{dS m}^{-1}$ ) and osmotic pressures ( $\text{mOsmol kg}^{-1}$ ) of the 12 treatments (4 for each salt) in the first  $\text{Na}^+$  vs  $\text{Ca}^{2+}$  experiment.

$\text{Cl}^-$ ext. ( $\text{mol m}^{-3}$ )	Electrical Conductivity			Osmotic Pressure		
	$\text{CaCl}_2$	$\text{NaCl}$	( $\text{Na}+\text{Ca}$ )	$\text{CaCl}_2$	$\text{NaCl}$	( $\text{Na}+\text{Ca}$ )
50	5.5	5.9	5.7	64	89	78
100	9.3	10.1	9.7	128	181	157
150	13.5	14.3	13.9	191	270	235
200	17.5	18.4	18.0	255	360	314

Plants were harvested when they were five weeks old and had been growing for 25 days in stress. Shoot fresh and dry weights were recorded, and leaves number 3 (old) and 5 (youngest expanded) were sampled for sap extraction. Dimensions of leaf number 4 were measured to estimate leaf area; this was calculated as the product of the length of the leaf blade by its width at half length, multiplied by a coefficient (0.85) that had been previously calculated for the same variety.

In the extracted sap,  $\text{Cl}^-$ ,  $\text{Na}^+$  and  $\text{K}^+$  were analysed by HPLC (Dionex 2000i) and  $\text{Ca}^{2+}$  by atomic absorption spectrophotometry (SP2900 Pye Unicam), as described earlier (see section 2.2.2).

As in previous experiments, a 2-way analysis of variance (type of salt and level of salinity) was done, using individual values of 8 plants per treatment (since the treatments themselves were not replicated). The main interest, however, was in the comparison of the different salts at similar levels of internal (leaf)  $\text{Cl}^-$ . This was done by plotting growth (and other traits) against  $\text{Cl}^-$  concentrations in leaves. Means of 8 plants per treatment, rather than individual values, were used to make the graphs more intelligible. All statistical analysis were performed using the Genstat-5 package.

### *Experiment 2.*

This experiment was conducted in a greenhouse in the University of Wales, Bangor (Pen-y-Fridd Field Station) in January - February 1993. The conditions in the greenhouse were similar to those described in previous experiments (e.g. section 2.2.2). Only 2 types of salts were used this time:  $\text{CaCl}_2$  and  $\text{NaCl}$  (with 1/20  $\text{CaCl}_2$ ); (the mixture of the two salts used in the previous experiment was omitted, because it did not add any further information). Eight treatments were applied, corresponding to four levels of  $\text{CaCl}_2$  (10, 25, 50, and 100  $\text{mol m}^{-3}$   $\text{CaCl}_2$ ) and four levels of  $\text{NaCl}$  (25, 50, 100 and 200  $\text{mol m}^{-3}$   $\text{NaCl}$ , all with 1/20  $\text{CaCl}_2$ ). The same variety as before (CM-67) was used, in 3 replicated tubs per treatment with 8 plants each.

Imbibed seeds were sown (21.01.93) in compost (John Innes N<sup>o</sup> 1) in plastic plug trays (Plantpak P84) and later transferred to 9  $\text{dm}^3$  tubs containing Phostrogen and

micronutrients. A week after sowing, stress was begun by applying 50 mol m<sup>-3</sup> Cl<sup>-</sup> (or less, if required) per day to each tub (up to 4 days). All solutions were replaced weekly. The ECs and OPs of the different treatments are shown in Table 3.4.2.

Table 3.4.2. Electrical conductivities (dS m<sup>-1</sup>) and osmotic pressures (mOsmol kg<sup>-1</sup>) of the 8 treatments (4 for each salt) in the second Na<sup>+</sup> vs Ca<sup>2+</sup> experiment.

Cl <sup>-</sup> ext. (mol m <sup>-3</sup> )	Electrical Conductivity		Osmotic Potential	
	CaCl <sub>2</sub>	NaCl	CaCl <sub>2</sub>	NaCl
20/25*	2.9	3.3	26	45
50	5.8	6.0	64	90
100	10.1	10.5	128	181
200	18.0	19.0	255	362

(\*) 20 mol m<sup>-3</sup> for CaCl<sub>2</sub>, 25 mol m<sup>-3</sup> for NaCl.

Plants were harvested after having been under stress for 25 days, when they were 5 weeks old. Shoot fresh and dry weights were recorded, and the second youngest leaf was measured (length and width at half length) for leaf area estimation. Having seen (in all previous experiments) that the youngest leaf is very well protected from "toxic" ions, especially in this variety, it was decided to consider an older leaf in the hope of finding larger differences between types of salt. Thus, the second youngest leaf (the same where the area had been measured) was sampled for sap analysis. On the extracted sap, major ions (Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>) were analysed by HPLC (Dionex 2000i). (Other anions (NO<sub>3</sub><sup>-</sup>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup>) were not properly separated by the chromatographic column, which was later replaced.)

A 2-way analysis of variance was applied to all traits, using the means of 8 plants for each tub. The Anova, however, can only be applied to the external Cl<sup>-</sup> concentrations. To study the response to internal Cl<sup>-</sup> levels, regression analysis (linear regression) was used; this was based on the 3 replicates of each treatment. The comparison between regression lines was made following the method described in Snedecor & Cochran (1989; pp: 390-393).

### 3.4.3. RESULTS:

#### *Experiment 1.*

##### 3.4.3.1. Ion concentrations in leaves (Table 3.4.3):

With increasing external  $\text{Cl}^-$  concentration, the amounts of this ion found in leaves (either young or old) also increased; (Table 3.4.3a). Although the plants growing in NaCl tended to have higher  $\text{Cl}^-$  concentrations (except at the lowest treatment), no significant overall differences (at the 5% level) were detected between types of salt. Chloride concentrations were always higher in older leaves (overall mean  $270 \text{ mol m}^{-3}$ ) than in younger ones (mean  $183 \text{ mol m}^{-3}$ ). Differences between young and old leaves (measured as the ratio of concentrations: YL/OL) were similar for all salt types.

Concentrations of  $\text{K}^+$  did not change very much with increasing external  $\text{Cl}^-$ , but there were differences in  $\text{K}^+$  levels between the different salts (Table 3.4.3b). Thus, plants growing in NaCl had lower concentrations of  $\text{K}^+$ , in both young and old leaves, than those growing in  $\text{CaCl}_2$ ; the plants in a mixture of the two salts had intermediate levels. The reduced uptake of  $\text{K}^+$  in presence of  $\text{Na}^+$  due to competition has already been seen in previous experiments (e.g. in section 2.2.3). Partitioning of  $\text{K}^+$  between young and old leaves also varied depending on the type of salt. Plants in  $\text{CaCl}_2$  had similar  $\text{K}^+$  concentrations in young and old leaves, while plants in NaCl maintained higher levels of  $\text{K}^+$  in young leaves (overall mean  $153 \text{ mol m}^{-3}$ ) than in old leaves (mean  $88 \text{ mol m}^{-3}$ ). Plants growing in the mixture of salts had an intermediate behaviour.

Differences in  $\text{Na}^+$  and  $\text{Ca}^{2+}$  concentrations between different salts were as expected: high  $\text{Ca}^{2+}$  and low  $\text{Na}^+$  levels were found in the  $\text{CaCl}_2$ -alone treatments, and the opposite was true for the NaCl (+1/20  $\text{CaCl}_2$ ) treatments; the plants in the mixture of salts had intermediate values for the 2 ions; (Table 3.4.3c,d). In almost all cases the levels of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  in leaves tended to increase as their concentration in the external solution increased. The two ions accumulated preferentially in older leaves. However, in those cases where high amounts of these ions were entering the shoot,

(e.g.  $\text{Na}^+$  in the NaCl treatments), a larger proportion of them accumulated in the younger leaves, (as measured by the ratio of concentrations YL:OL).

Table 3.4.3. Chloride (a), potassium (b), sodium (c) and calcium (d) concentrations ( $\text{mol m}^{-3}$  sap) in young and old leaves of plants growing at different levels of  $\text{CaCl}_2$ , NaCl or a 2:1 mixture of both ("Na+Ca"); (means of up to 8 plants).

Cl <sup>-</sup> ext. ( $\text{mol m}^{-3}$ )	YOUNG LEAVES			OLD LEAVES		
	$\text{CaCl}_2$	NaCl	(Na+Ca)	$\text{CaCl}_2$	NaCl	(Na+Ca)
<i>a) Chloride:</i>						
50	174	142	151	214	228	226
100	150	165	148	240	236	224
150	165	212	182	269	343	285
200	234	278	191	317	350	305
LSR*	49	60	33	32	87	73
<i>b) Potassium:</i>						
50	219	145	193	204	92	180
100	189	143	177	177	74	141
150	191	147	163	183	82	119
200	195	178	182	180	105	132
LSR*	17	30	30	18	30	21
<i>c) Sodium:</i>						
50	<1 <sup>(1)</sup>	101	32	<1	225	81
100	<1	140	41	<1	253	103
150	<1	146	63	<1	304	128
200	<1	154	58	<1	278	142
LSR*	<1	47	17	<1	59	29
<i>d) Calcium:</i>						
50	15	6	11	40	10	24
100	17	6	11	58	10	25
150	24	5	17	67	11	41
200	51	5	19	87	9	52
LSR*	17	2	9	13	3	25

<sup>(1)</sup> <1 = not detected

\* Least Significant Range,  $\alpha=0.05$  (Tukey's test)

It is interesting to notice the effect of high  $\text{Ca}^{2+}$  added to NaCl: for a similar external  $\text{Na}^+$  concentration, the amounts of  $\text{Na}^+$  found in leaves (young and old) were much less when  $\text{Ca}^{2+}$  was present at high concentrations ("Na+Ca" treatments) than when they were kept to a minimum ("NaCl" treatments, which included 1/20  $\text{CaCl}_2$ ); (Figure 3.4.1). This effect was already observed when comparing the results of the "Osmotic adjustment" experiments (section 2.3), where a 2:1 mixture of NaCl to  $\text{CaCl}_2$  was used, with those from the "Comparison of varieties" experiment (section 2.2), with only a minimum (1/20)  $\text{CaCl}_2$  added to NaCl.

### 3.4.3.2. Plant growth (Table 3.4.4):

Shoot dry weight decreased with increasing external  $\text{Cl}^-$  concentration. Overall, the plants growing in  $\text{CaCl}_2$  had the lowest weights, and those growing in the mixture of salts had the largest weights, although this pattern did not hold for all levels of  $\text{Cl}^-$  concentrations. Leaf area also decreased with increasing salinity, although this time the plants growing in  $\text{CaCl}_2$  tended to have the largest leaves, (except in the lowest  $\text{Cl}^-$  treatment). This apparent discrepancy in the data (plants with biggest leaves had the smallest dry weights) can be explained because of the generally higher water contents of the plants growing in  $\text{CaCl}_2$ .

Table 3.4.4. Shoot dry weight (mg), area of leaf 4 ( $\text{cm}^2$ ), and shoot fresh weight to dry weight ratios (FW:DW) of plants growing at different concentrations of  $\text{CaCl}_2$ , NaCl or a 2:1 mixture of both ("Na+Ca"); (means of up to 8 plants).

$\text{Cl}^-$ ext. ( $\text{mol m}^{-3}$ )	DRY WEIGHT			LEAF - 4 AREA			FW:DW RATIO		
	$\text{CaCl}_2$	NaCl	(Na+Ca)	$\text{CaCl}_2$	NaCl	(Na+Ca)	$\text{CaCl}_2$	NaCl	(Na+Ca)
50	645	812	898	29.1	30.3	30.1	9.3	9.0	8.7
100	497	594	565	26.3	25.0	23.7	8.1	7.5	7.6
150	415	357	420	24.4	18.0	19.8	7.1	6.1	6.2
200	296	284	352	17.5	15.2	16.0	5.6	6.0	5.6
LSR*	147	156	207	6.4	8.2	6.5	0.6	0.8	1.1

\* Least Significant Range,  $\alpha=0.05$  (Tukey's test)



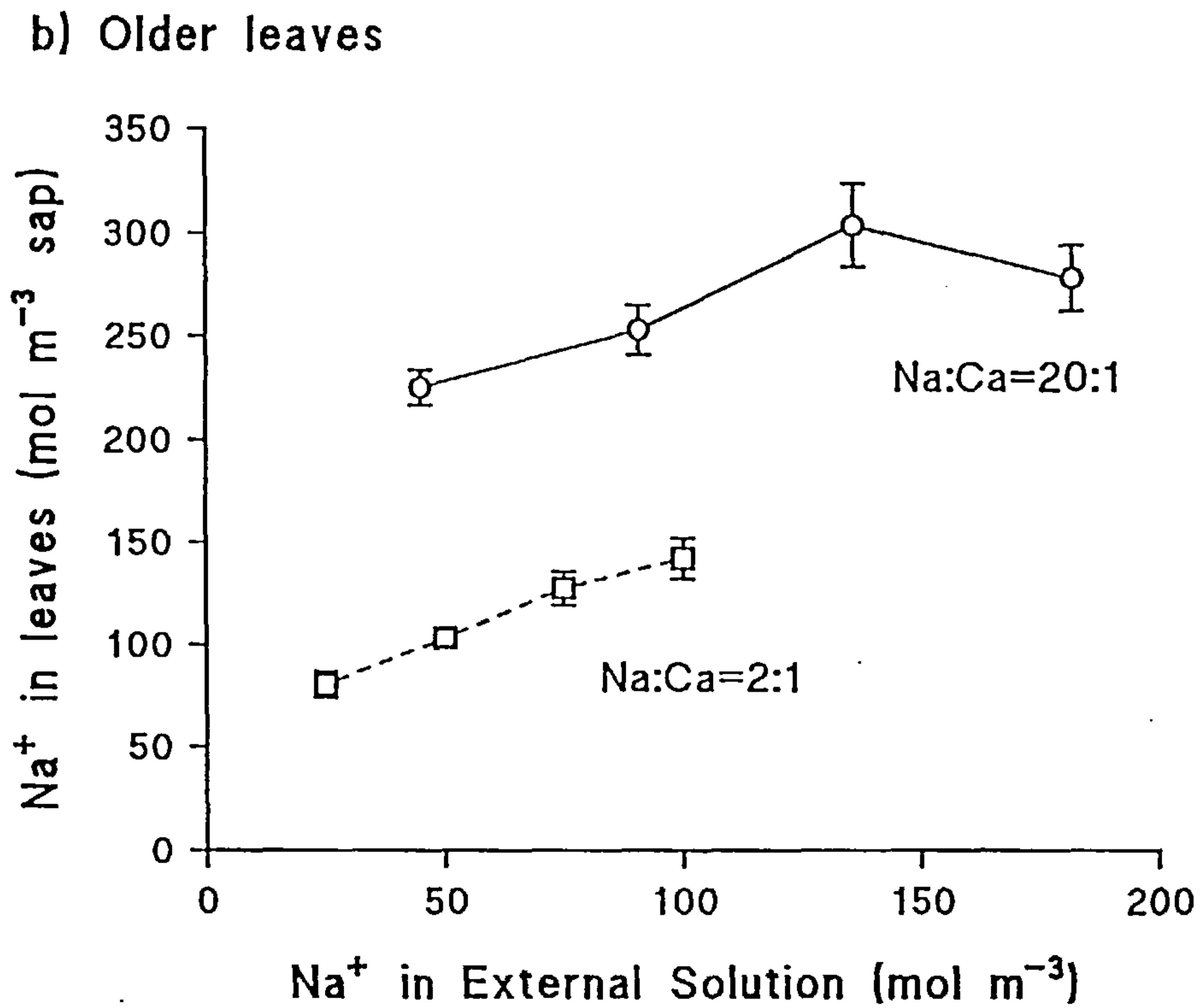
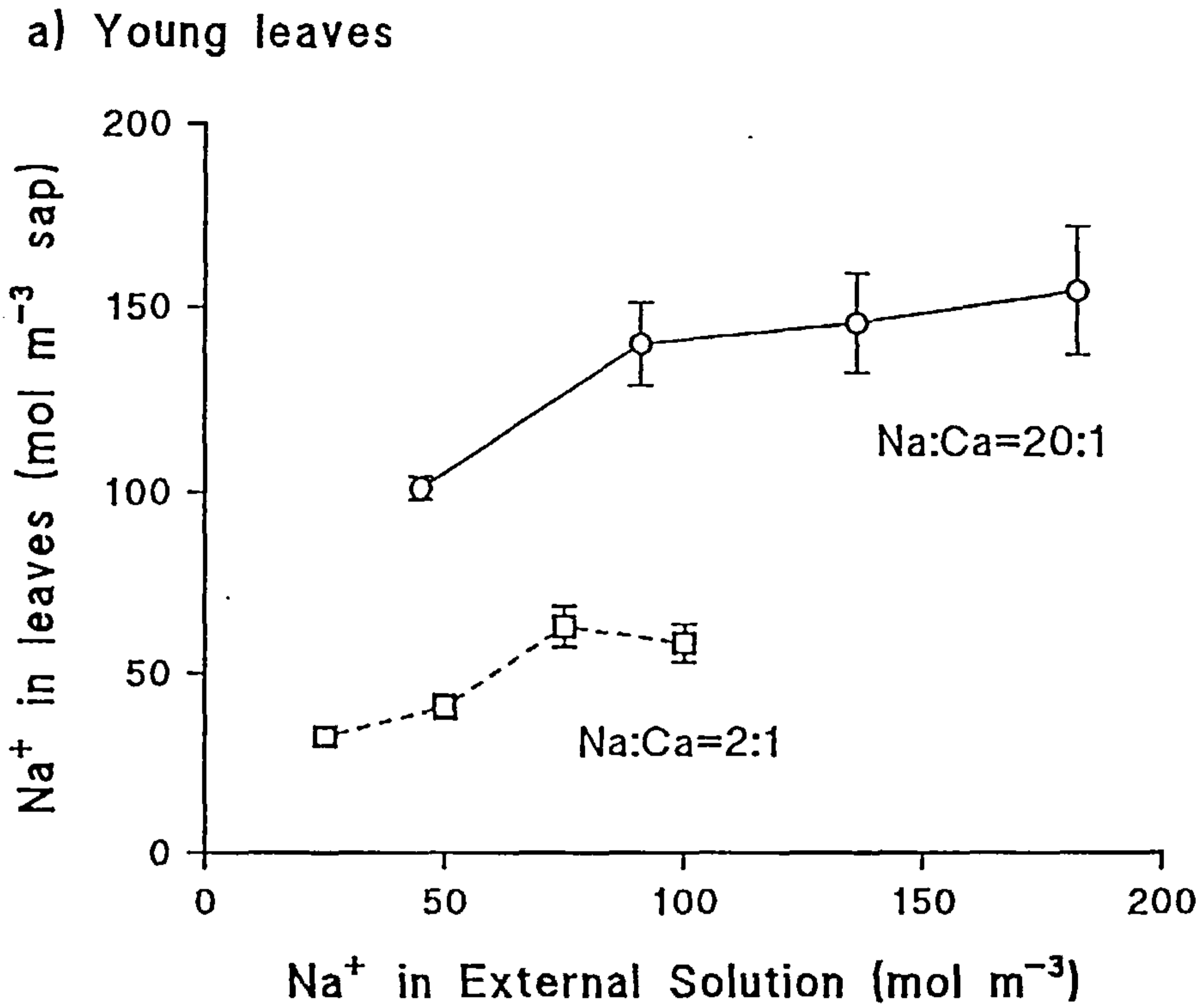


Figure 3.4.1. Sodium concentrations in young (a) and old (b) leaves of plants growing at several levels of salinity and different Na:Ca ratios.

The growth responses of the plants as a function of the  $\text{Cl}^-$  concentrations in their youngest leaf (leaf 5) are presented in Figures 3.4.2 and 3.4.3. Similar graphs were obtained when dry weight and leaf area were plotted against  $\text{Cl}^-$  concentrations in leaf 3 (data not shown). From these figures, no different responses seem to exist for the different types of salt. Dry weights of plants were also plotted against some characteristics of the external solution, such as  $\text{Cl}^-$  concentration (Figure 3.4.4), osmotic pressure (Figure 3.4.5) and electrical conductivity (Figure 3.4.6). These figures illustrate the different responses that may be obtained depending on what basis the comparison is made.

## *Experiment 2*

### 3.4.3.3. Ion concentrations in leaves (Table 3.4.5):

With increasing external levels of  $\text{Cl}^-$ , the concentrations of this ion in the second youngest leaf also tended to increase, particularly at the highest salinities. The rate of increase was not different for the 2 types of salt (regressions not significantly different at the 5% level; data not shown).

As in the previous experiment,  $\text{K}^+$  concentrations of plants in  $\text{NaCl}$  were lower than those in  $\text{CaCl}_2$ , due to the reduced  $\text{K}^+$  absorption in the presence of high  $\text{Na}^+$  concentrations.

Differences between types of salt were also present for  $\text{Na}^+$  and  $\text{Ca}^{2+}$  levels, as expected. Sodium concentrations increased when external  $\text{NaCl}$  levels were raised from 25 to 50  $\text{mol m}^{-3}$ , but remained more or less constant above that. Calcium concentrations tended to increase with increasing external  $\text{CaCl}_2$ , especially above 100  $\text{mol m}^{-3}$ .

Magnesium concentrations were similar for the 2 types of salt, and did not significantly change (5% level) with different levels of salinity (data not shown).

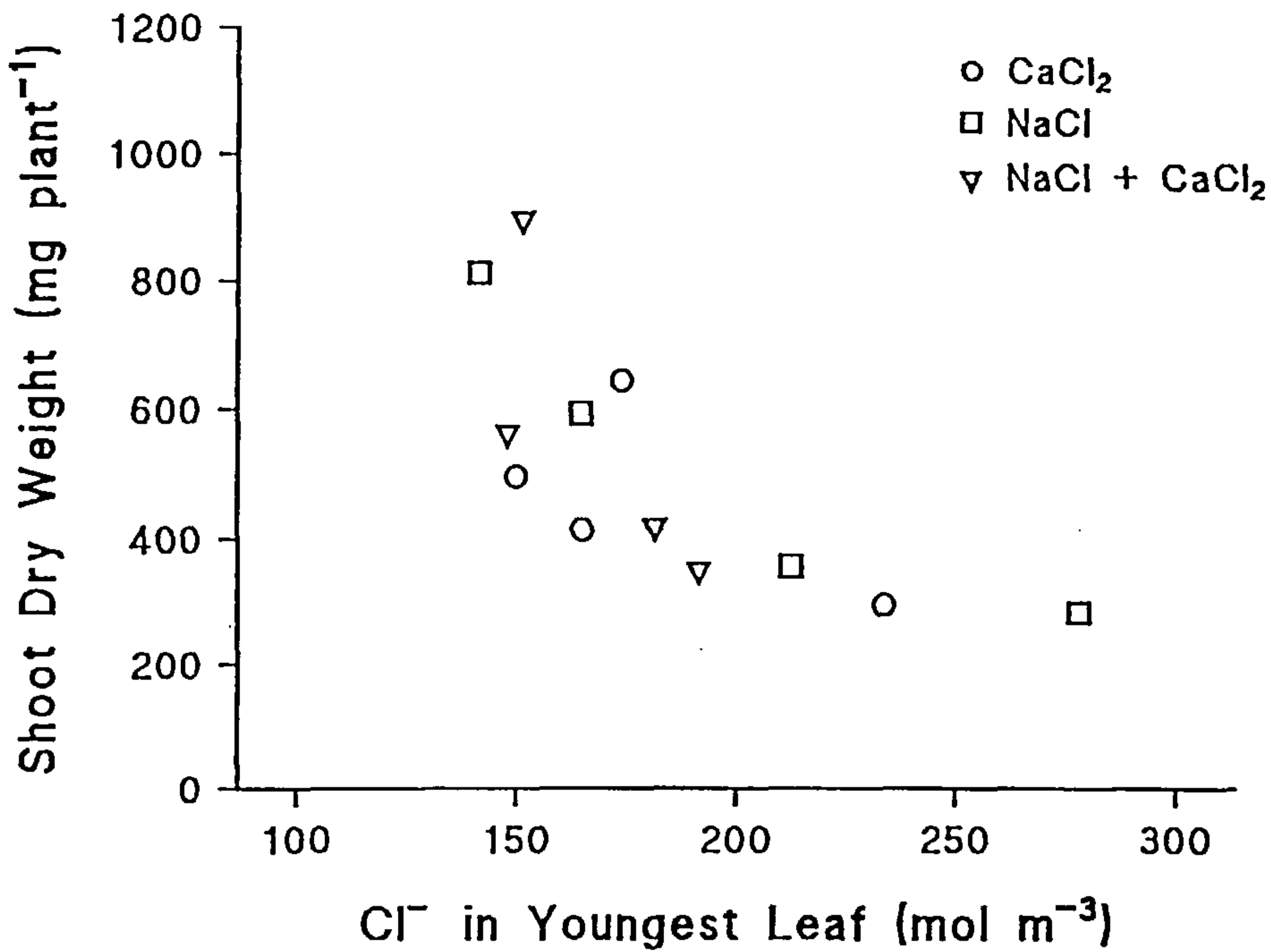


Figure 3.4.2. Dry weight of plants growing with different types of salt in relation to the concentrations of Cl<sup>-</sup> in their youngest leaf; (Na<sup>+</sup> vs Ca<sup>2+</sup>, experiment 1).

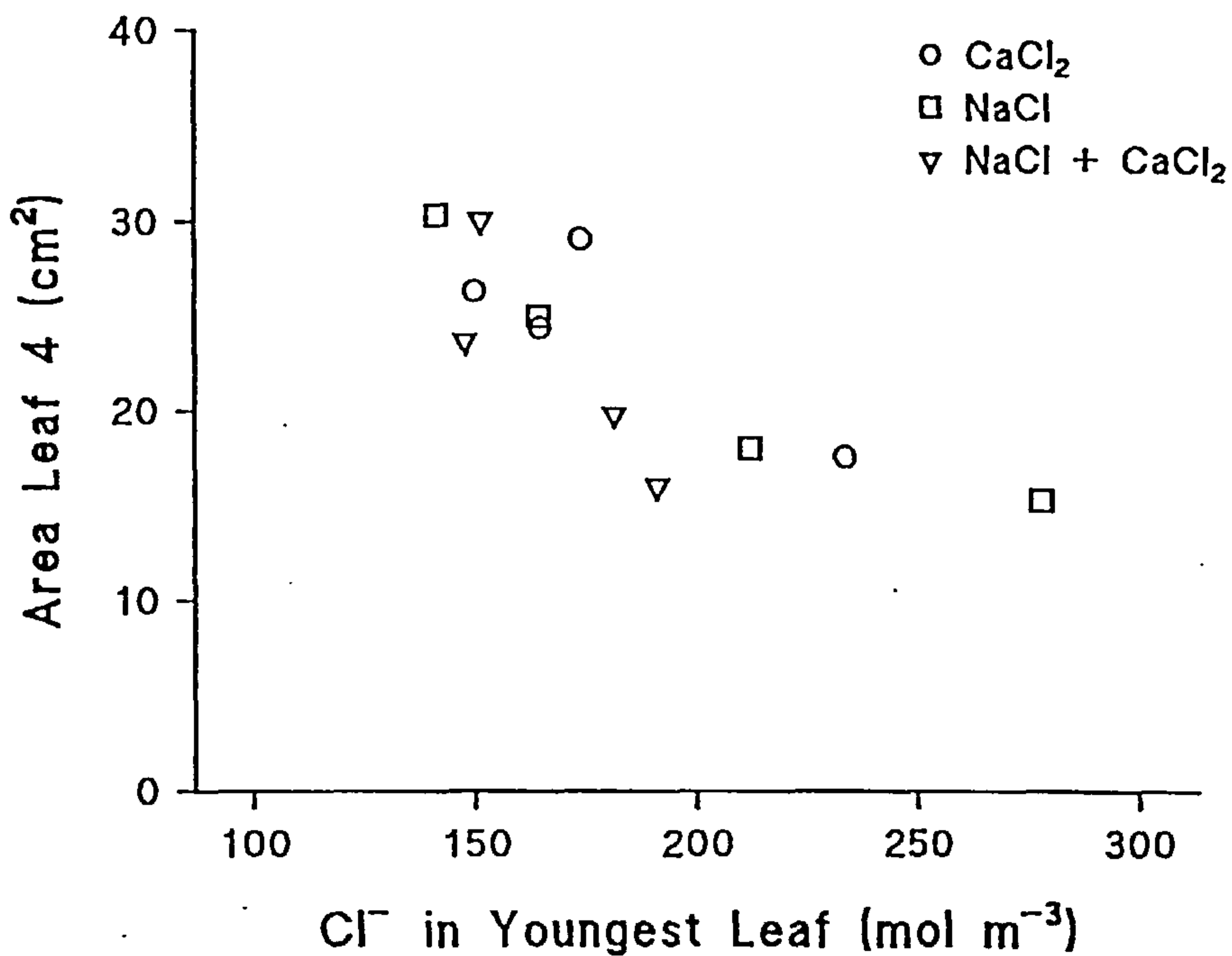


Figure 3.4.3. Area of leaf number 4 of plants growing in different types of salt, in relation to the concentrations of Cl<sup>-</sup> in their youngest leaf; (Na<sup>+</sup> vs Ca<sup>2+</sup>, experiment 1).

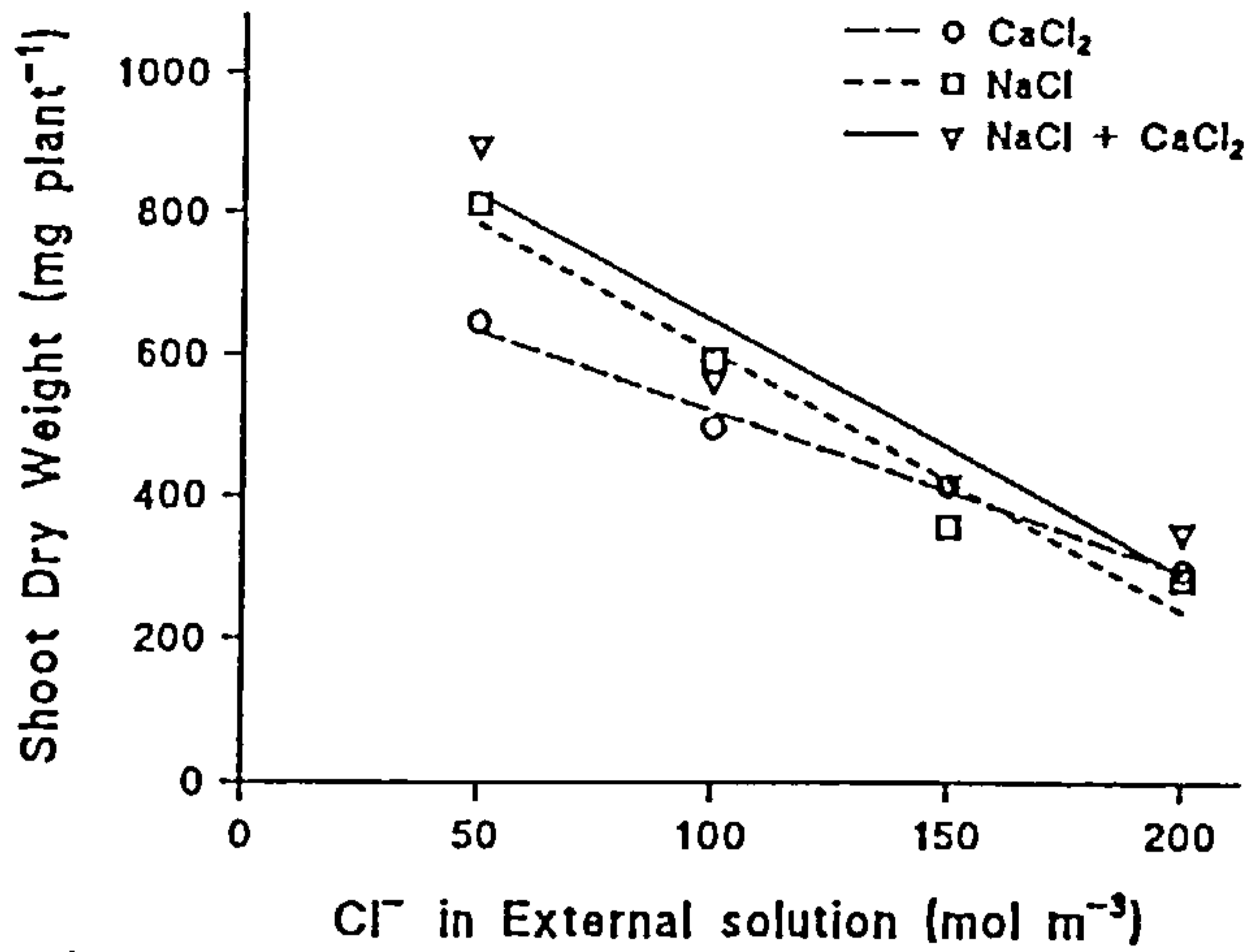


Figure 3.4.4. Dry weight of plants growing in different salts, in relation to the concentrations of  $\text{Cl}^-$  in their external solutions; ( $\text{Na}^+$  vs  $\text{Ca}^{2+}$ , experiment 1).

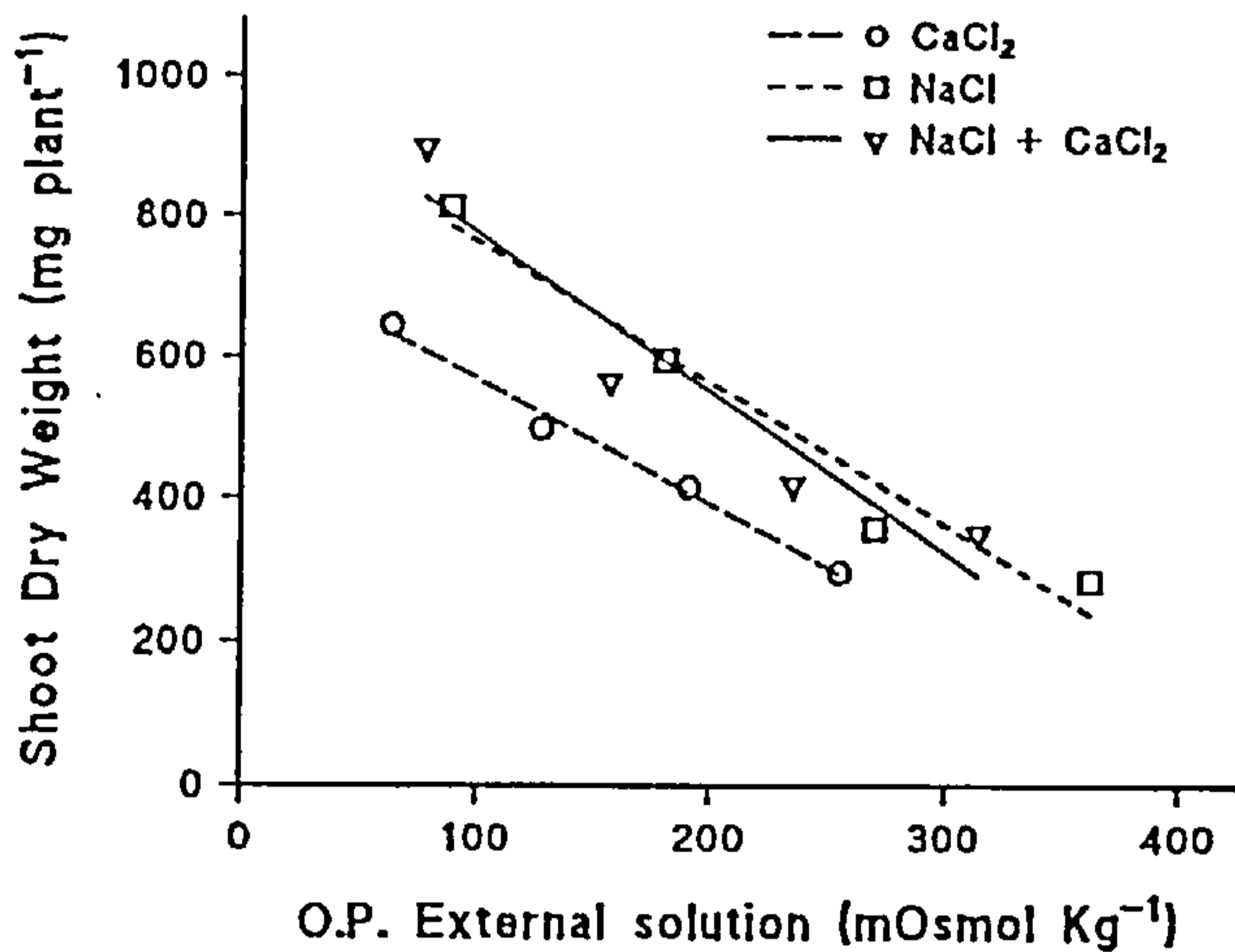


Figure 3.4.5. Dry weight of plants growing in different salts, in relation to the osmotic pressure (OP) of their external solutions; ( $\text{Na}^+$  vs  $\text{Ca}^{2+}$ , experiment 1).

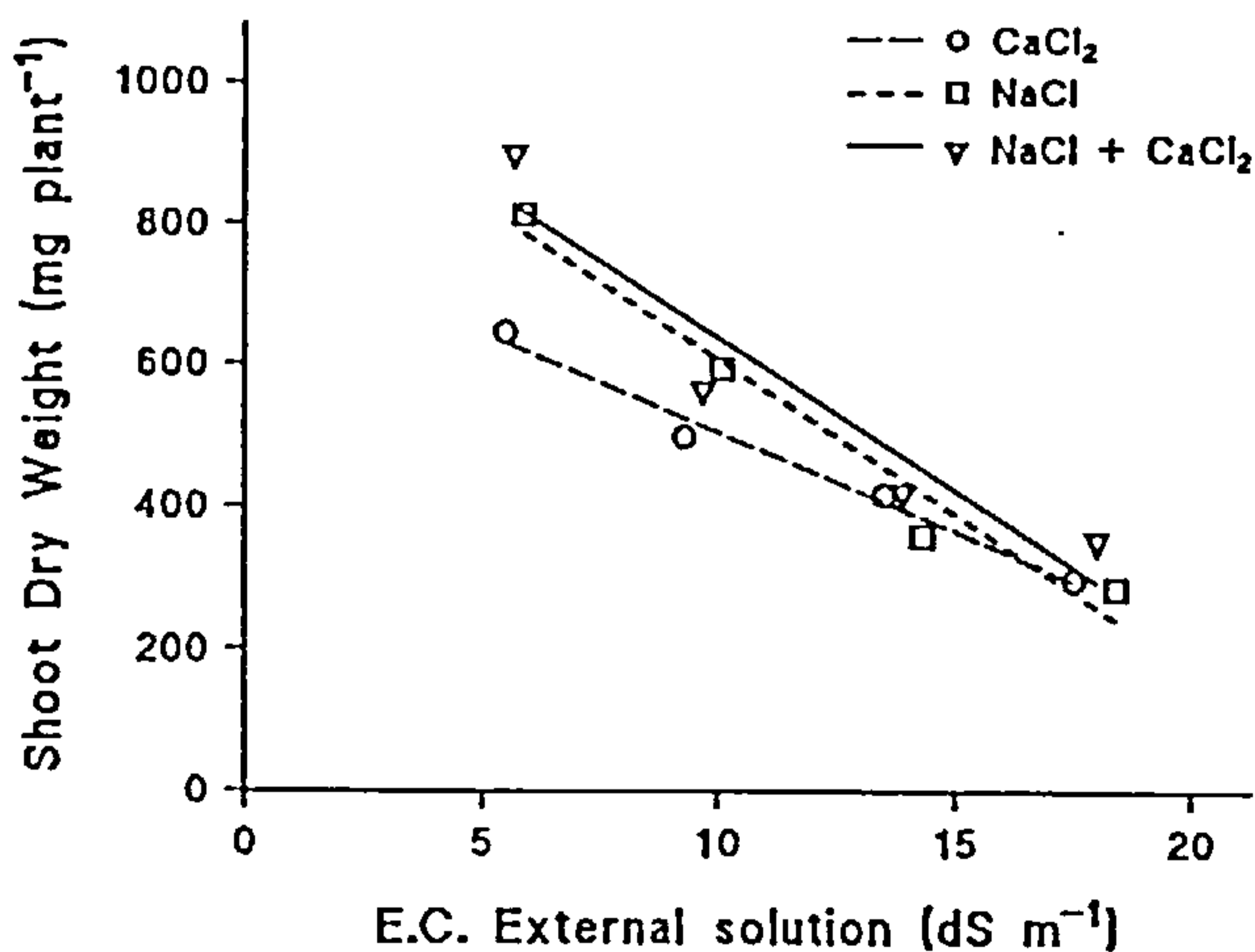


Figure 3.4.6. Dry weight of plants growing in different salts, in relation to the electrical conductivity (EC) of their external solutions; ( $\text{Na}^+$  vs  $\text{Ca}^{2+}$ , experiment 1).

Table 3.4.5. Ion ( $\text{Cl}^-$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ) concentrations ( $\text{mol m}^{-3}$  sap) in the second youngest leaf of plants growing at different levels of either  $\text{CaCl}_2$  or  $\text{NaCl}$ ; (means of up to 24 plants).

$\text{Cl}^-$ ext. ( $\text{mol m}^{-3}$ )	CHLORIDE		POTASSIUM		SODIUM		CALCIUM	
	$\text{CaCl}_2$	$\text{NaCl}$	$\text{CaCl}_2$	$\text{NaCl}$	$\text{CaCl}_2$	$\text{NaCl}$	$\text{CaCl}_2$	$\text{NaCl}$
20/25 <sup>(1)</sup>	156	158	264	210	4.1	88	7.0	3.6
50	153	171	274	190	3.2	168	6.3	4.0
100	180	173	300	202	3.6	195	10.9	5.8
200	198	216	278	243	2.3	183	29.7	5.8
LSR*	38	30	39	46	1.5	63	12.4	4.7

<sup>(1)</sup> 20  $\text{mol m}^{-3}$  for  $\text{CaCl}_2$ , 25  $\text{mol m}^{-3}$  for  $\text{NaCl}$ .

\* Least Significant Range,  $\alpha=0.05$  (Tukey's test)

#### 3.4.3.4. Plant growth (Table 3.4.6):

Shoot dry weight and area of the second youngest leaf both decreased with increasing external  $\text{Cl}^-$  concentrations for the 2 types of salt. They also decreased with increasing amounts of  $\text{Cl}^-$  in the second youngest leaf, but the study of the regressions (dry weight and leaf area vs internal  $\text{Cl}^-$ ) did not find significant differences (5% level) between the 2 salts (Figures 3.4.7 and 3.4.8).

When both dry weights and leaf areas were compared at similar levels of either  $\text{Na}^+$  or  $\text{Ca}^{2+}$  in the external solution, (instead of similar  $\text{Cl}^-$  concentrations),  $\text{CaCl}_2$  tended to give smaller plants than  $\text{NaCl}$ , particularly at the highest treatments; (Figures 3.4.9 and 3.4.10). This might suggest a higher toxicity of  $\text{CaCl}_2$ , compared to  $\text{NaCl}$ . However, it might just be a result of an increased  $\text{Cl}^-$  uptake with  $\text{CaCl}_2$ . If  $\text{Cl}^-$  concentrations in leaves are compared at corresponding levels of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  in the external solution, they also tend to be higher for  $\text{CaCl}_2$ , especially at the highest treatments (see Table 3.4.5).

The water contents (FW:DW ratios) of the plants decreased with increasing external  $\text{Cl}^-$  concentrations, but they were similar for the two types of salt at each treatment level.

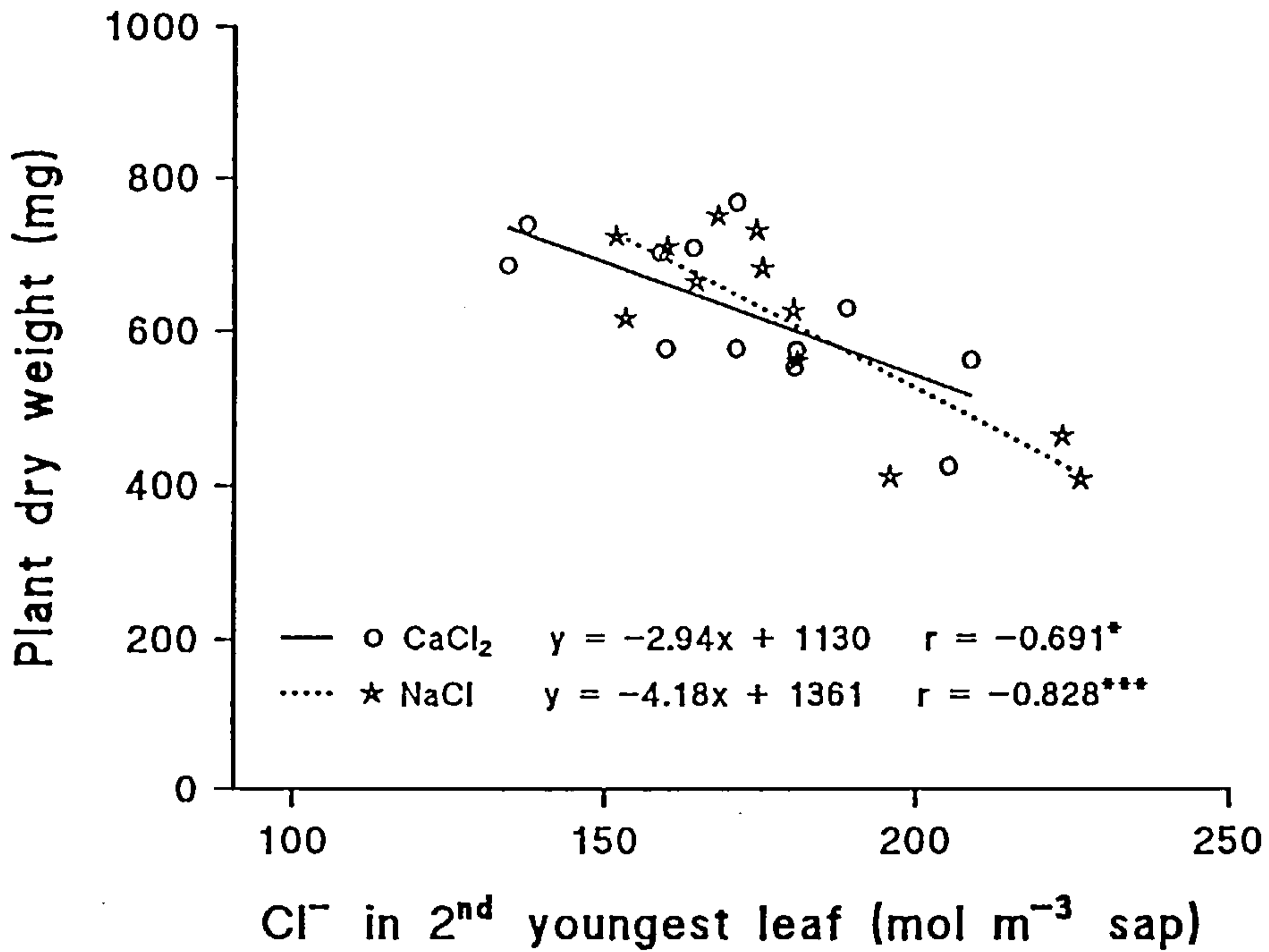


Figure 3.4.7. Dry weight of plants growing with different types of salt, in relation to the concentrations of Cl<sup>-</sup> in their second youngest leaf; (Na<sup>+</sup> vs Ca<sup>2+</sup>, experiment 2).

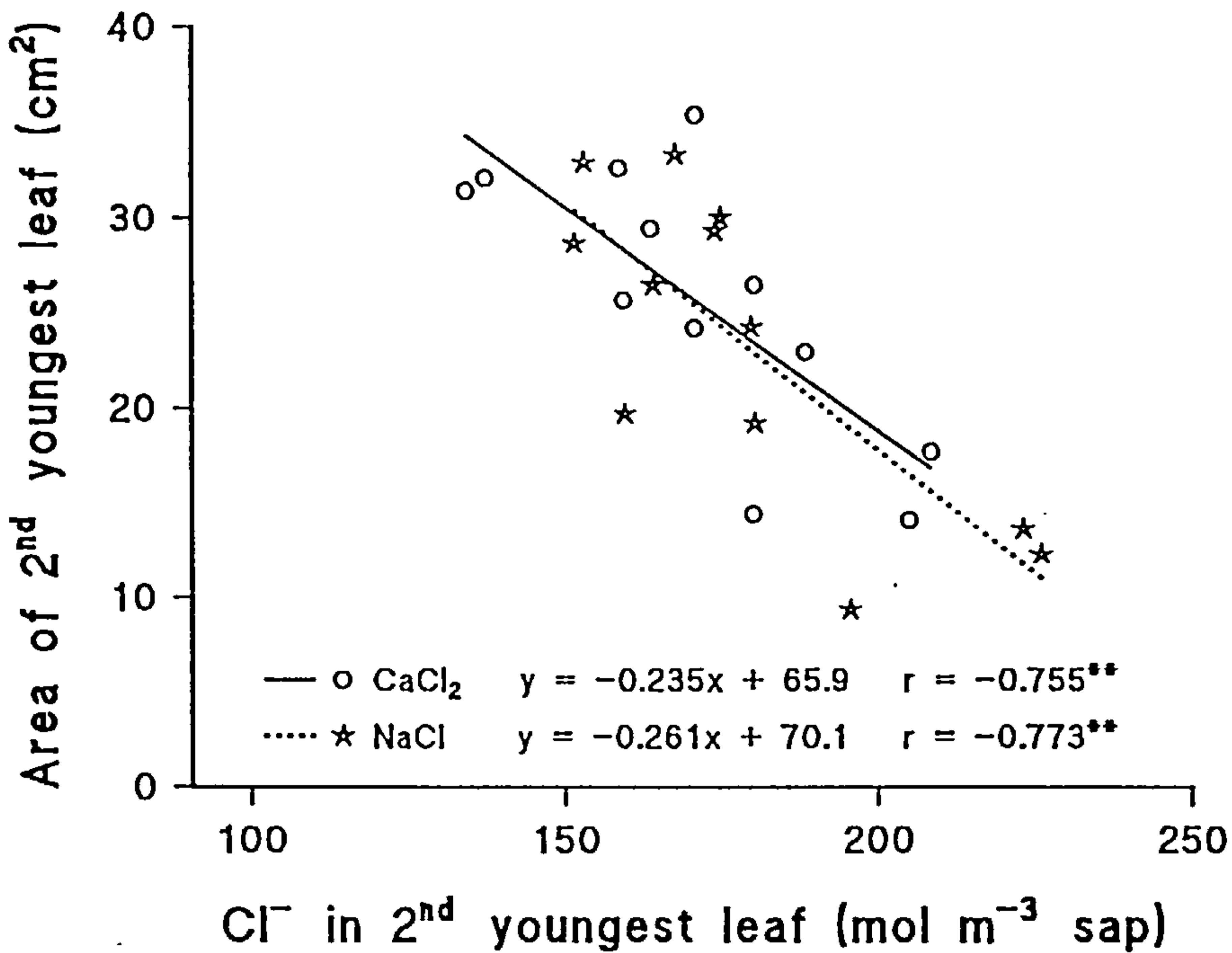


Figure 3.4.8. Area of the second youngest leaf of plants growing in different types of salt, in relation to the concentrations of Cl<sup>-</sup> in the same leaf; (Na<sup>+</sup> vs Ca<sup>2+</sup>, experiment 2).

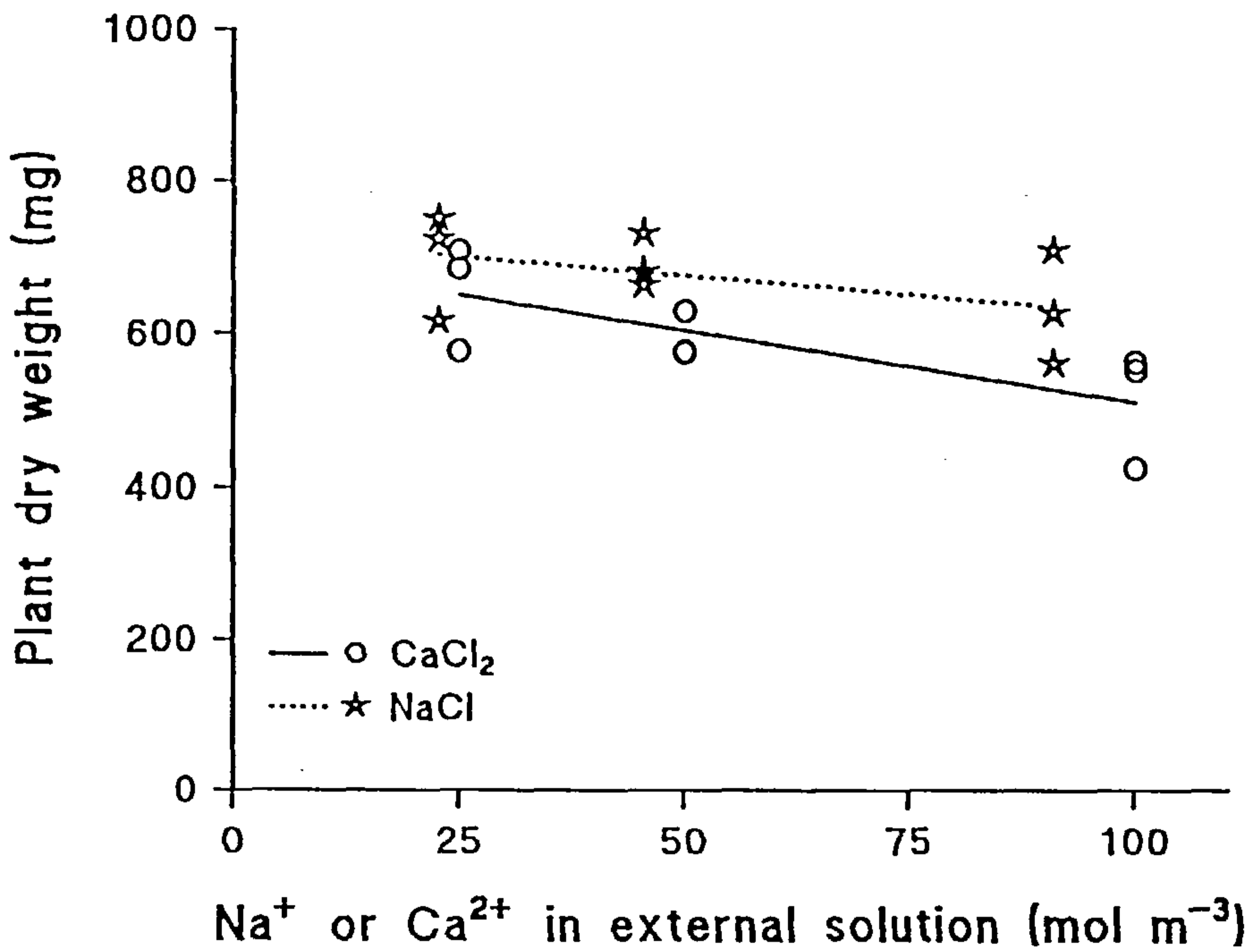


Figure 3.4.9. Dry weight of plants growing in different types of salt, in relation to the external concentrations of either  $\text{Na}^+$  (NaCl salt) or  $\text{Ca}^{2+}$  ( $\text{CaCl}_2$  salt); ( $\text{Na}^+$  vs  $\text{Ca}^{2+}$ , experiment 2).

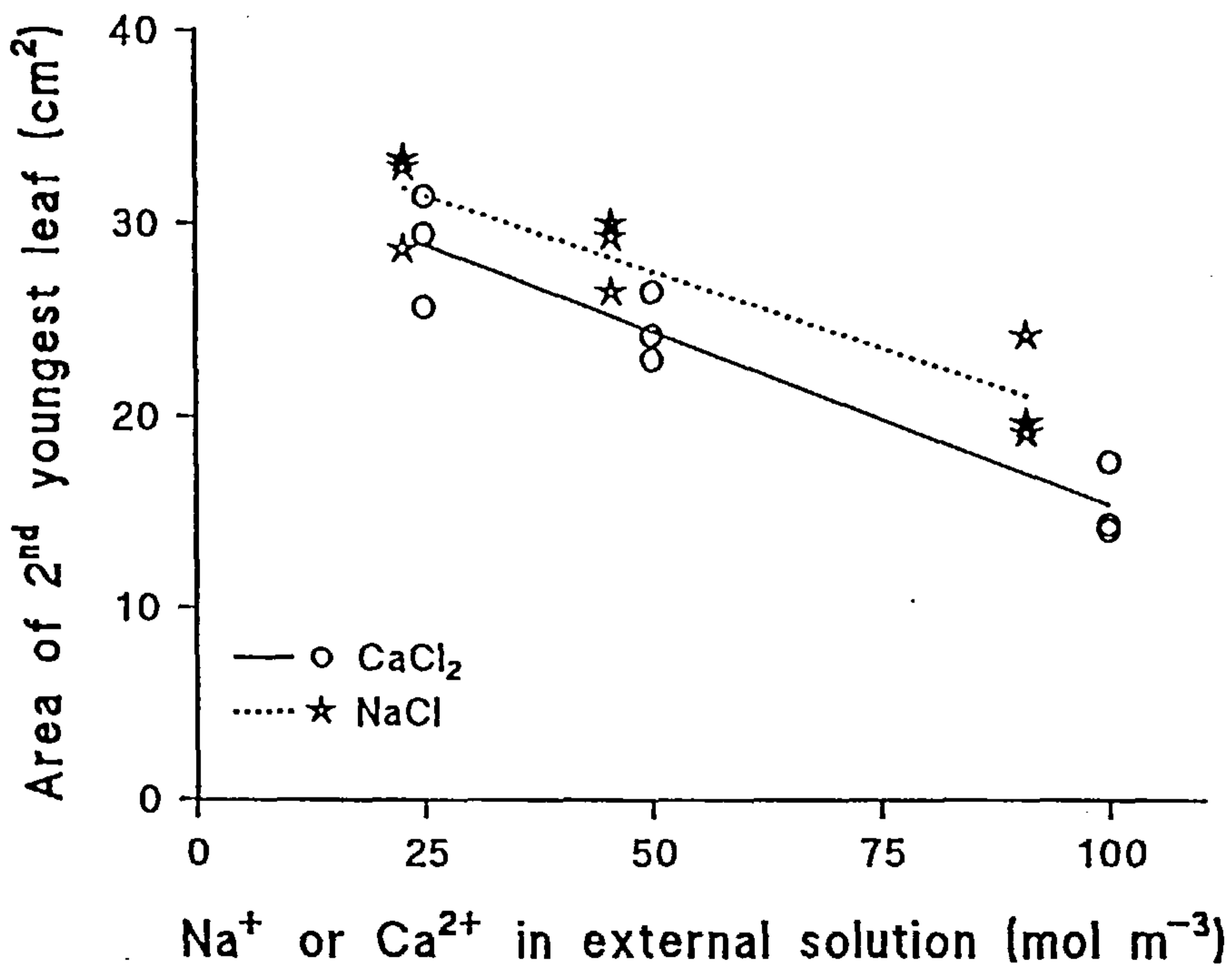


Figure 3.4.10. Area of the second youngest leaf of plants growing in different types of salt, in relation to the external concentrations of either  $\text{Na}^+$  (NaCl salt) or  $\text{Ca}^{2+}$  ( $\text{CaCl}_2$  salt); ( $\text{Na}^+$  vs  $\text{Ca}^{2+}$ , experiment 2).

Table 3.4.6. Shoot dry weight (mg) and area of second youngest expanded leaf (cm<sup>2</sup>) of plants growing at different levels of either CaCl<sub>2</sub> or NaCl; (means of 24 plants).

Cl <sup>-</sup> ext. (mol m <sup>-3</sup> )	DRY WEIGHT		LEAF AREA		FW:DW	
	CaCl <sub>2</sub>	NaCl	CaCl <sub>2</sub>	NaCl	CaCl <sub>2</sub>	NaCl
20/25 <sup>(1)</sup>	737	696	33.4	31.6	11.87	11.33
50	657	692	28.9	28.6	10.52	10.25
100	594	632	24.6	21.0	9.04	8.76
200	514	428	15.4	11.7	7.01	7.29
LSR*	148	148	5.7	6.2	1.32	1.41

<sup>(1)</sup> 20 mol m<sup>-3</sup> for CaCl<sub>2</sub>, 25 mol m<sup>-3</sup> for NaCl

\* Least Significant Range,  $\alpha=0.05$  (Tukey's test)

#### 3.4.4. DISCUSSION:

The objective of these two experiments was to compare the growth of plants under different types of salinity (NaCl and CaCl<sub>2</sub>) at similar levels of Cl<sup>-</sup> in their leaves, in order to establish the relative toxicity of Na<sup>+</sup> and Ca<sup>2+</sup> ions. This was done in Figures 3.4.2 and 3.4.3 (experiment 1) and 3.4.7 and 3.4.8 (experiment 2). If one of the two ions had been more toxic than the other, two different response curves would have been expected in those figures. For instance, had Na<sup>+</sup> been more toxic than Ca<sup>2+</sup>, the response line for the NaCl treatments would have been expected to be below and/or steeper than that of the CaCl<sub>2</sub> treatments. Reciprocally, higher values of dry weight or leaf area for NaCl than for CaCl<sub>2</sub>, at similar levels of Cl<sup>-</sup>, (response line of NaCl above and less steep than that of CaCl<sub>2</sub>) would have indicated a greater toxicity of Ca<sup>2+</sup>. However, no different responses for the different types of salt were seen in the above figures.

In fact, no significant differences were found in growth (dry weight and leaf area) of plants in different salts, no matter on which basis this was compared: internal Cl<sup>-</sup> concentrations, or properties of the external solution (Cl<sup>-</sup>, OP, EC). That is, both salts (and their mixture) seem to have similar effects on the growth of plants. A more detailed study of the data might throw some light on the reason for these results. The following discussion will concentrate only on the measurements of dry weight, and



particularly those from experiment 1, since the results for leaf area were influenced by the water content of the leaves (which changed for the different salts), and are more difficult to interpret.

At the 2 lowest treatments (50 and 100 mol m<sup>-3</sup> external Cl<sup>-</sup>) the amounts of Cl<sup>-</sup> in the youngest leaf were quite similar for the 3 types of salt, and the levels of the other ions (Na<sup>+</sup> and Ca<sup>2+</sup>) were generally too low to suspect any toxic effect. Only in NaCl-treated plants were Na<sup>+</sup> concentrations high enough (above 100 mol m<sup>-3</sup>) to be potentially dangerous if not properly compartmented. (Although it is not clear what levels of Na<sup>+</sup> are actually toxic to the cytoplasm and organelles; values between 100 and 140 mol m<sup>-3</sup> Na<sup>+</sup> have been found in salinity (Cheeseman, 1988; Munns, 1993)). Since Cl<sup>-</sup> was the ion which was present at the highest concentrations in all cases, it might well be that this (Cl<sup>-</sup>) is the most toxic ion.

Chloride alone, though, is not the only factor affecting growth. Chloride concentrations in the youngest leaves of all plants at 100 mol m<sup>-3</sup> external Cl<sup>-</sup> were quite similar to those of plants in the lower (50 mol m<sup>-3</sup> Cl<sup>-</sup>) treatment. In spite of this, dry weights were reduced between 23% (CaCl<sub>2</sub> treatments) and 37% (mixture of salts). Again, concentrations of other ions (Na<sup>+</sup> and Ca<sup>2+</sup>) were in general too low to be toxic, with perhaps the exception of Na<sup>+</sup> in NaCl-treated plants. Thus, some other factor (external osmotic stress sensed by the roots?) must be affecting growth at these relatively low levels of salinity.

At the two highest external treatments (150 and 200 mol m<sup>-3</sup> Cl<sup>-</sup>) the situation was different: there was large variability in the Cl<sup>-</sup> concentrations in the youngest leaf, but not many differences in dry weight of the plants. Sodium concentrations in NaCl-treated plants were again high enough ( $\approx$ 150 mol m<sup>-3</sup>) to have some toxic effect, although they were not significantly higher than in the preceding treatment. The CaCl<sub>2</sub>-treated plants had very low dry weights at the highest treatment (200 mol m<sup>-3</sup> external Cl<sup>-</sup>), which coincided with a large increase in the concentrations of both Cl<sup>-</sup> and Ca<sup>2+</sup> in relation to the previous treatment. It is, therefore, difficult to suggest which one of these ions was most responsible for that decrease in dry weight, since some kind of toxic effect of Ca<sup>2+</sup> at those levels (> 50 mol m<sup>-3</sup> Ca<sup>2+</sup>) cannot be ruled

out. Finally, plants growing in the 2:1 mixture of NaCl and CaCl<sub>2</sub> had the lowest Cl<sup>-</sup> concentrations in the two highest treatments, and their Na<sup>+</sup> and Ca<sup>2+</sup> concentrations were too low to be toxic. These plants had the largest weights of all (although not significantly higher), and thus this mixture was the least toxic salt.

In relation to this, it is interesting to remark the different levels of Cl<sup>-</sup> accumulation for the different salts (Table 3.4.3). This might be due to a different rate of Cl<sup>-</sup> uptake: according to Marschner (1986), at high external concentrations, ions with lower uptake rates (such as Ca<sup>2+</sup>) depress the uptake rate of Cl<sup>-</sup> considerably, due to limitations in charge compensation. This would explain the lowest Cl<sup>-</sup> levels found in leaves of plants growing in CaCl<sub>2</sub> in relation to those in NaCl; (although without measurements of rate of ion uptake this hypothesis cannot be proved). On the other hand, it might be that the high Cl<sup>-</sup> concentrations found in leaves are not the cause but the result of reduced growth; that is, growth would be reduced first, and as a consequence ions would accumulate in leaves. Without measurements of growth rate, this cannot be proved. However, if this had been the case (the concentrations of Cl<sup>-</sup> reflecting the reduction in growth), larger leaf areas might have been expected for the plants in the mixture of salts (see Table 3.4.4).

It is interesting to notice the effect of Ca<sup>2+</sup> in reducing Na<sup>+</sup> uptake (Figure 3.4.1). We have to remember that the NaCl treatments included a minimum level of Ca<sup>2+</sup> (from 2.5 to 10 mol m<sup>-3</sup>, depending on the NaCl concentration) to prevent rapid Na<sup>+</sup> uptake (see section 3.2). It is clear from the present data that this decrease in Na<sup>+</sup> uptake is even larger in the presence of higher Ca<sup>2+</sup> concentrations. Similar results were found for cotton growing in 100 mol m<sup>-3</sup> NaCl and varying concentrations (up to 50-100 mol m<sup>-3</sup>) of CaCl<sub>2</sub> (Gorham, unpublished results).

In experiment 2, an older leaf (second youngest one) was sampled for analysis. Also, lower external Cl<sup>-</sup> concentrations and more replicates were used. With these changes it was expected to detect more differences than in experiment 1. However, these differences were not found. There was still large variation in dry weight (or leaf area) for a similar level of leaf Cl<sup>-</sup>, though that variation did not consistently correspond to any one of the two salts studied.

It is interesting to notice that leaf  $\text{Cl}^-$  concentrations in experiment 2 were not much different from those of corresponding treatments in experiment 1. Knowing the pattern of  $\text{Cl}^-$  accumulation in older leaves, higher concentrations were expected in experiment 2 in comparison to the young leaves of experiment 1, but this was not so. The reason for this lower  $\text{Cl}^-$  levels might be related to different environmental conditions (lower temperatures in the greenhouse during winter). Something similar happened with  $\text{Ca}^{2+}$  concentrations in  $\text{CaCl}_2$ -treated plants: they were lower in older leaves of experiment 2 than in younger leaves of experiment 1. As a result,  $\text{Ca}^{2+}$  concentrations never reached high (potentially toxic) levels in the second experiment (maximum  $30 \text{ mol m}^{-3}$ ). This may have contributed to the lack of differences between the two salts. This was not the case, however, with  $\text{Na}^+$  concentrations in the  $\text{NaCl}$ -treated plants of experiment 2. In this case, concentrations of  $\text{Na}^+$  in older leaves were higher than in young leaves of corresponding treatments in experiment 1. This apparent discrepancy can be explained by the more efficient compartmentation at the organ level (young vs old leaves) of  $\text{Na}^+$  compared to  $\text{Cl}^-$  (see sections 2.2.3 and 3.2.3).

As a summary, it seems that at relatively low salinities (up to  $100 \text{ mol m}^{-3}$  external  $\text{Cl}^-$ , which corresponds to around  $150 \text{ mol m}^{-3}$  leaf  $\text{Cl}^-$ ) the amounts of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  found in leaves are not high enough to be the main cause of reduced growth, and thus it is difficult to find differences in their relative toxicities. Any toxic effect that may exist (in addition to the osmotic stress) is probably caused by  $\text{Cl}^-$ . At higher salinities (around  $200 \text{ mol m}^{-3}$  external  $\text{Cl}^-$ ), concentrations of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  in leaves begin to be high enough to be potentially toxic, although neither of them seems to be clearly more damaging than the other. The effect of  $\text{Cl}^-$  is probably the primary one, and this overshadows the lower toxicity of any other ion (at these concentrations).

# CHAPTER FOUR

## SALT TOLERANCE IN THE FIELD

### 4.1. INTRODUCTION: THE TRIPLE LINE SOURCE SPRINKLER SYSTEM

The Triple Line System (TLS) consists of 3 parallel sprinkler lines with a lateral spacing of 15 m (equal to the sprinkler's wetted radius); the in-line sprinkler spacing is 4.5 m (30% of the wetted radius). The sprinkler heads are Wright, model MPL-75 (Hydro-riego Wright, Barcelona, Spain). An equal quantity of water is applied through each sprinkler line: fresh water into the 2 outer lines, and a saline solution into the centre line. This results in a continuous gradient of salinity, with the same volume of water, between each pair of sprinkler lines.

The saline solution is prepared in a 3100 l tank by adding equal amounts of NaCl and hydrated CaCl<sub>2</sub> (CaCl<sub>2</sub>·2H<sub>2</sub>O) to the tank and mixing with well water until complete dissolution; this mixture has an EC of around 50-60 dS m<sup>-1</sup>. The solution in the tank is then injected, using a diesel pump, into the centre line, where it mixes with well water (EC around 2 dS m<sup>-1</sup>). By regulating the motor's revolutions and the outlet valve of the tank, the salinity of the water delivered by the central line can be adjusted as desired; it is usually set at around 19 dS m<sup>-1</sup>. The mixture of NaCl and CaCl<sub>2</sub> salts is set at a ratio 1:1 in weight (approximately 2:1 molar ratio), in order to get an acceptable SAR (maximum 15 equivalents m<sup>-3</sup>) and thus avoid an alkalization effect on the soil (loss of structure and permeability) (Ayers & Westcot, 1985).

Figure 4.1.1 schematically shows the design of the TLS.

Although the salinity gradient is continuous between each pair of sprinkler lines, for practical reasons this area is divided into ten individual plots of 1.4 m wide; these plots will be referred to as "salinity treatments". The different varieties are then placed in rows at right angles to the sprinkler lines.

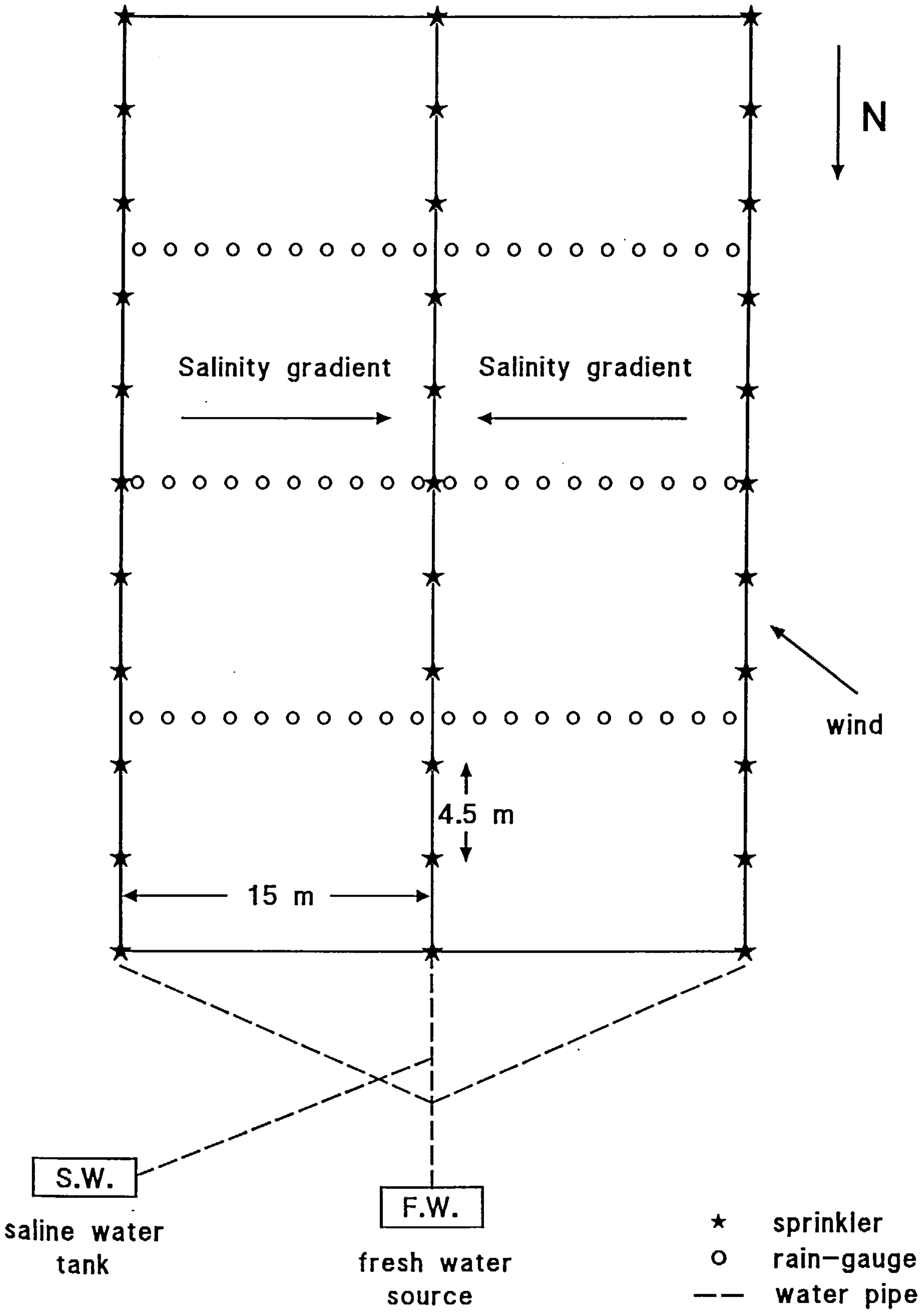


Figure 4.1.1. Schematic design of the Triple Line Sprinkler System.

Reference evapotranspiration ( $ET_0$ ) is determined from the measurements taken in a Class-A evaporation pan located at an adjacent site, with daily values averaged over a 10 day period. Crop evapotranspiration ( $ET_c$ ) is then calculated by applying a crop factor ( $K_c$ ) which depends primarily on the developmental stage of the crop. These measurements are used for scheduling the irrigations. In practice, 2 to 3 irrigations per week are given.

The duration of each irrigation is usually limited to 30 min, because of the high application rate of the system, and to avoid flooding and runoff. Irrigation is started only when the wind speed is less than  $2 \text{ m s}^{-1}$  (usually in the early morning), to minimize the influence of wind on the uniformity of the water and salinity applied. Before and after each saline irrigation a supplemental 3 min irrigation is given using only fresh water, to reduce the risk of direct salt absorption through leaves.

The volume and salinity of water received by the plots (salinity treatments) are monitored by rain-gauges placed in the centre of each plot in 3 lines along the field; (previous research had shown that salinity and amount of water applied are fairly uniform along the sprinkler lines (Aragüés *et al.*, 1992)). After each irrigation, the volume of water collected in these rain-gauges is measured, and its EC determined.

Soil salinity is measured periodically during the growing season with a portable Geonics EM-38 electromagnetic sensor (EMS) (Geonics Limited, Mississauga, Ontario), placing it horizontally in the middle of each plot, at alternate rows of varieties. Soil samples of plots of different salinities are taken on several occasions each season, and the EC of their water extracts (either saturation or 1:5 soil:water) are used to calibrate the readings obtained by the EMS.

The electromagnetic sensor method is based on the linear relationship that exists between the soil apparent EC ( $EC_a$ ) and the change of intensity of an electromagnetic wave generated by a magnetic coil positioned on the soil; (for full details of the method see Rhoades & Corwin, 1981). The soil  $EC_a$  is a function not only of the number of electrolytes in the soil solution but of several other factors, such as soil water content, soil temperature and some physico-chemical soil characteristics.

Nevertheless, for a given soil with a given water content and at a reference temperature, the  $EC_a$  may be considered to be only a function of the salinity of the soil solution (or a dilution of it, such as a saturated extract solution). It is important, therefore, always to make the EMS measurements at the same water content (in practice, one day after irrigation, when the soil is approximately at field capacity), and to record the soil temperature.

To obtain the EC of the saturated extract (the usual standard measure of soil salinity) from the EMS readings a calibration is needed. The soil samples for this calibration are usually taken at depth intervals of 25 cm down to 1 m (4 samples); this is the depth that the electromagnetic wave reaches when the EMS is placed horizontally on the soil, and corresponds with the zone where most of the roots are found. The EC of the saturation extract (and/or that of a 1:5 soil/water extract) is determined for these samples, and a weighted mean calculated to compare with the EMS readings obtained in the same place where the samples were taken. The weighted mean takes into account the contributions of the different depths to the EMS reading, and was established by the manufacturers of the instrument. Aragüés and Millán (1986) have translated that relationship into the following expression:

$$EC_{se} = [36 \times EC_{(0-25)} + 21 \times EC_{(25-50)} + 11 \times EC_{(50-75)} + 8 \times EC_{(75-100)}] / 76$$

where  $EC_{se}$  is the EC of the saturation extract, and the figures in brackets refer to depth intervals in cm.

## 4.2. 1991/92 FIELD (TLS) EXPERIMENT

### 4.2.1. OBJECTIVES:

The main objective of the 1991/92 experiment was to study the accumulation of ions in different leaves during vegetative growth, and to relate these data to final yield. To do that, an extensive calendar of sampling was established, which covered the period from early tillering to near heading time. Similar leaves were sampled at two stages, first as young leaves, and later as old leaves; changes in ion concentrations



with leaf ageing could, in this way, be followed. The results obtained would also be useful in deciding the most appropriate growth stage for sampling in order to discriminate between varieties.

#### 4.2.2. *MATERIALS AND METHODS:*

The experiments using the TLS were conducted at Zaragoza (Spain), in the central part of the Ebro River Basin (0°35'W, 42°05'N), on land belonging to the Agronomic Research Service of the Aragón Autonomous Government (SIA-DGA). The 0.5 ha field where the TLS is installed is a well-drained, levelled terrace, with slope less than 1%; the soil has a silt-loam texture, and is described (J.M. Salamero, personal communication) as a mixed, mesic, Typic Torrifuvent, according to the U.S. Soil Survey System (USDA, 1975).

The field had been used for other salinity experiments with the TLS in the previous 3 years; all these experiments were done with barley and, to a much smaller extent, wheat. After each season's harvest, a few irrigations with fresh water were given to leach the salts accumulated during the previous year; (natural rainfall in summer is too low to rely on it for this leaching process). It was found, however, that by starting each new season with very low levels of salts in the soil, the salinity gradient took too long to develop in the profile, and the plants were growing with less stress than desired. This resulted in those varieties with rapid growth and shorter life cycles escaping from the stress; that is, their roots were growing in areas of the soil that the salts had not yet reached.

To solve this problem it was decided, in summer 1991, to cover half of the field with plastic film after the harvest, until the land was prepared for the following crop. This would prevent the salts being leached (either by fresh water irrigation or by rainfall) in that half of the field; the other half was treated as usual, for comparison. Thus, when this experiment (1991/92) was begun, part of the field already had some salts accumulated in the soil. Although no detailed measurements of soil salinity at the time of sowing are available, readings with the EMS in a few plots confirmed that salinity was generally higher in the unleached area (R. Isla, personal communication).

To compensate for this high initial salinity, the few first irrigations were given with fresh water, in order to help with seedling emergence. Barley is more sensitive to salinity at these early stages than at the adult stage (Maas & Hoffman, 1977), and tolerance at germination and emergence does not usually correlate well with tolerance at later stages (Royo *et al.*, 1991). An irregular seedling establishment may influence the later growth of the plants and interfere with the varieties' response to salinity, making it more difficult to interpret the results. It is thus important to get a uniform crop establishment. (It might be argued that this procedure of helping plant emergence by means of fresh water irrigation does not seem very realistic. In practice, though, farmers will only sow their crops in a saline soil after some rain has fallen and diluted the salts, so that they can expect a reasonably good germination. Thus, the approach adopted here is not that far from reality.)

Seed bed preparation was done following usual cultivation practices (ploughed and cultivated). Fertilizer was provided in a split application: 200 kg ha<sup>-1</sup> of 15-15-15 complex to the seed bed, and 100 kg ha<sup>-1</sup> NH<sub>4</sub>NO<sub>3</sub> at beginning of tillering. This gave a total of 65 kg of N, 12.9 kg of P, and 24.9 kg of K per hectare. Pests and diseases were kept under control using agrochemicals as appropriate.

On 21.11.91, three varieties of barley (Albacete, CM-67 and Chevron) were sown by plot-drill along the salinity gradient of the TLS in 10 plots, and 2 replicates for each variety; (the number of replicates was limited by the availability of space in the TLS). Each plot consisted of 6 rows 1.20 m long with 65 seeds/row. Row spacing was 28 cm, resulting in a plot 1.4 m wide. Sowing depth was 2-4 cm. Most of the plots belonging to this experiment were sown in the part of the field that had been leached, with the rest of the field where the TLS is installed being used for other experiments. One replicate of two varieties (CM-67 and Chevron, replicate-I), however, fell in the higher salinity area; this is reflected in the measurements of soil salinity (see Results section).

In Figure 4.2.1 a sketch map of the field is presented. It can be seen from there that the distribution of varieties within block was not randomized, but systematic. In one of the replicates (Rep-I), the "block" was divided in 3 sub-plots ("bands") along the

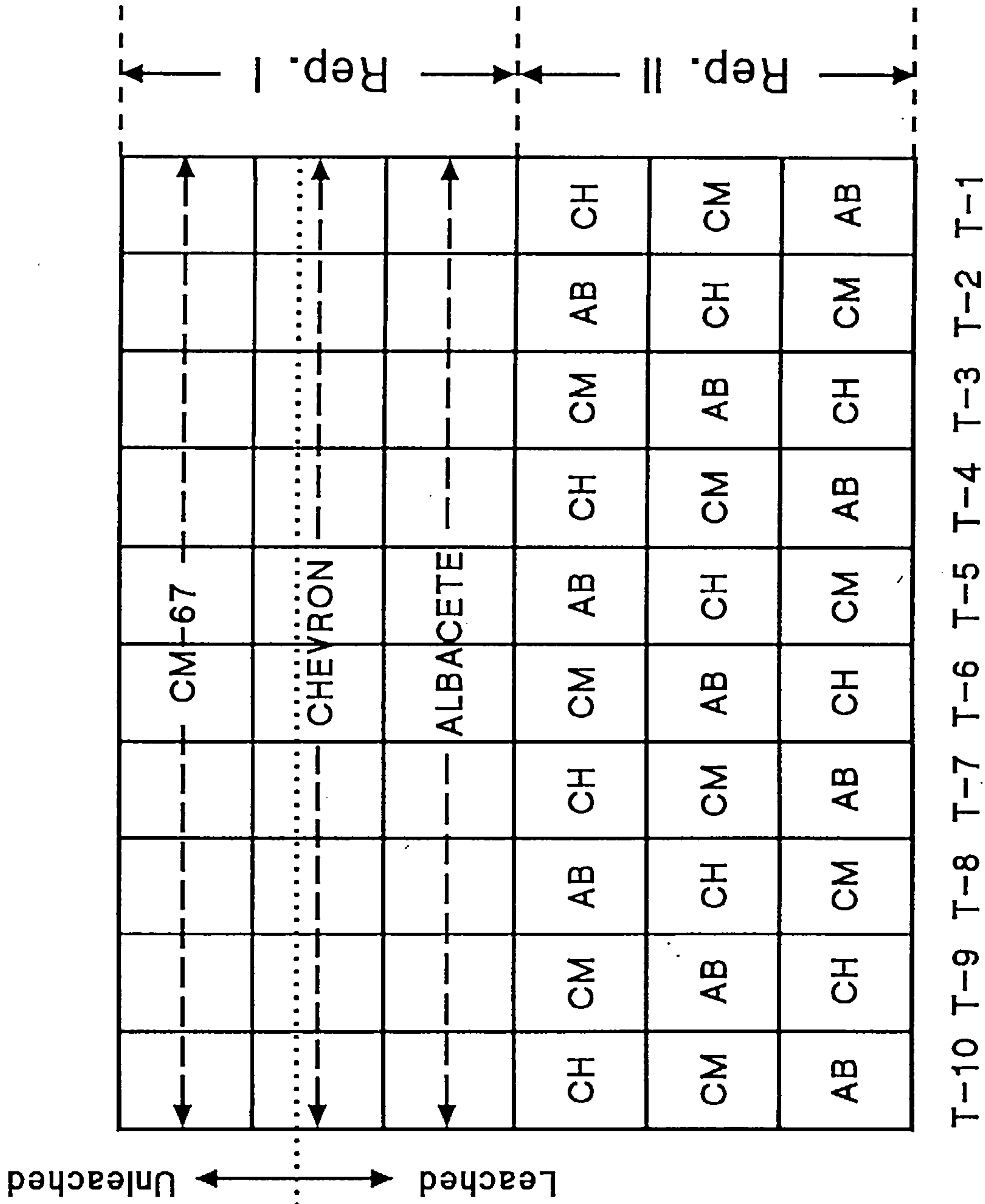


Figure 4.2.1. Map of the field of the TLS 1991/92 experiment (arrangement of varieties and replicates).

gradient of salinity, and each variety occupied one of these bands. In the other replicate (Rep-II), the 3 varieties were systematically arranged within the 3 sub-plots, with each cultivar covering all 10 levels of salinity.

The 3 varieties were chosen after previous experiments in Bangor had revealed large differences in ion accumulation between CM-67 and Chevron (see section 2.2); the purpose of this experiment was to see if those differences corresponded with salt tolerance in the field. Albacete was included as a check; it is a Spanish drought-tolerant cultivar, and has been used in many other experiments in the TLS.

Dates of each irrigation, together with amount of water applied and its range of salinity (measured on the line of rain-gauges closest to the plots), are given in Appendix 1. It is seen there that the first irrigation was done with fresh water, as already mentioned, to help get a uniform plant establishment. Later in the season, some more irrigations were also done with fresh water. This was brought about by the high salinity levels found in the soil (measured with the EMS) at the time. (The salinity applied with irrigation water had been similar, at the beginning of the season, to the levels used in previous years. However, because of the residual salinity from the previous season, soil salinity increased faster, at least in that part of the field where no leaching had been allowed.) To lower these excessive levels of salinity, a few short irrigations with fresh water were given. Afterwards, the EC of the irrigation water was decreased slightly. A summary of water salinity across the treatments is also presented in Figure 4.2.2.

As mentioned before, to minimize the risk of salt absorption through leaves, 3 minutes of pre- and post-irrigations with fresh water are given. This is done by closing the valve at the exit of the tank which contains the saline mixture, and leaving the central sprinkler line connected only to the fresh water source. Thus, a normal irrigation would consist of: 3 min fresh water, 25-35 min saline+fresh water, and 3 min fresh water. However, it was realized that at the end of this cycle, when the pump was switched off and the pipes were left to drain the residual water, some leaking occurred in the junctions between two pipes (probably due to the low pressure under these conditions, since no leakages were ever found while the system was

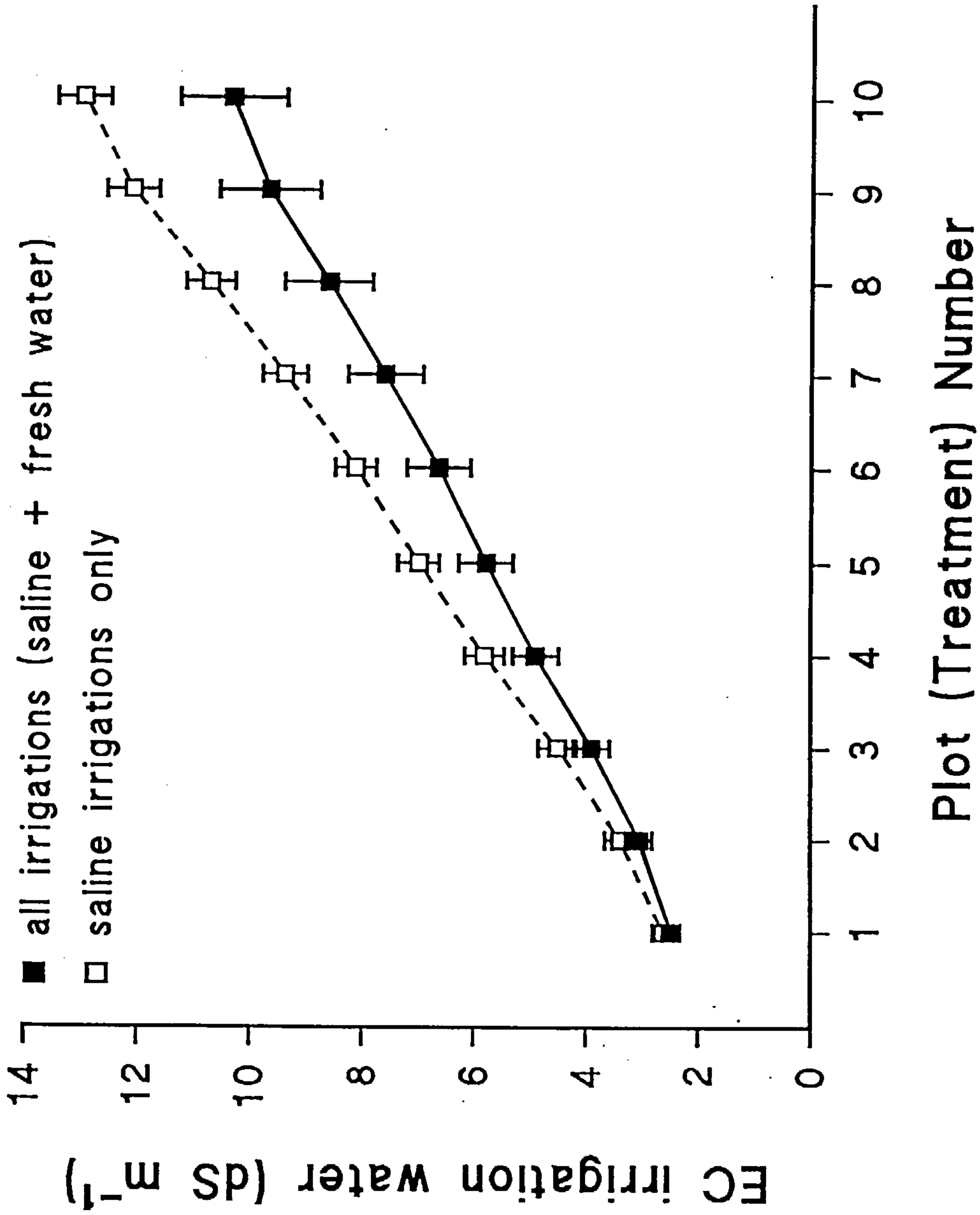


Figure 4.2.2. Salinity of irrigation water applied to the 10 treatments of the TLS in the 1991/92 experiment. Means and standard errors of 22 saline irrigations (open symbols) and total 29 (saline and fresh water) irrigations (closed symbols).

working at normal pressure). Because the leaked water is fresh water (which is the last applied through the central line), it results in a dilution of the soil salinity in the plots at the highest treatment. (Any leaks that may occur in the 2 outer lines would not affect soil salinity, since these lines always provide fresh water.) The effects were first noticed as the plants in treatment 10 (highest) were more vigorous than those at treatment 9 (slightly lower); later it was confirmed by measurements of soil salinity. As a result of this irregularity it was decided not to include treatment 10 in the analysis.

From the time of ear emergence until harvest, the whole field was covered with netting, installed at about 2 m above the ground, to protect the developing grains from birds. All plots were harvested at maturity with a plot harvester (Hege 125), on 02.07.92. The grain collected in each plot was weighed. This grain weight (g) per plot was used as the main measure of yield for all calculations, although it was later transformed to other units ( $\text{g m}^{-2}$ ) for presentation purposes. In some cases, yield was also transformed to the more standard units of  $\text{kg ha}^{-1}$ , which may be more indicative of the actual yields achieved; (although it is considered that yields obtained in small, experimental plots tend to overestimate the real potential of the genotypes tested, because of the optimal conditions usually provided in these cases). No correction for moisture content of the grain was made; (in the Spanish weather, natural drying of cereals in the field does not pose any problem).

*- Sampling for sap extraction and ion analysis:*

Observations from previous years had shown that some of the varieties tested in the TLS develop at very different rates, because they have different origins and are adapted to different conditions. One extreme case is found when comparing the short-duration, fast-growing CM-67 with the long-duration, non-dwarf Chevron, whose heading times may differ by as much as 3 weeks. Salinity also affects development rate so that, for a given cultivar, the 10 treatments are not always at the same growth stage, and by heading time they may differ by more than a week.

The result of this is that, at any one moment, the plants in the field are at different development stages. Thus, when sampling the different varieties and treatments, two

comparisons are possible: a) at the same time but different development stage; or b) at the same development stage but different time. In the first case the environmental conditions for all the plants until the moment of sampling are the same (days from sowing, number of irrigations, climate, etc), but it may not be possible to use the same leaf for sampling in all varieties. Selecting the youngest fully expanded leaf will result in later leaves being sampled in short duration varieties than in long-duration ones, particularly in the latest samplings, since differences increase with time. On the other hand, with the second method we can compare similar leaves (say leaf number 4, or flag leaf) at similar age (recently expanded), but the environmental conditions between different sampling dates may have changed with increasing time (lower or higher temperatures, number of irrigations, etc). Still, it is practically impossible to sample everything on a single day (or even in a few days), and thus some differences in time will always exist. In the present experiment it was decided to follow the second method, and sample each plot when a given leaf was fully expanded.

Each plot (combination of variety, treatment and replicate) was sampled 3 times between early growth and heading time, when leaves number 4, 6 and flag were fully expanded. At each sampling date, one young leaf (the youngest fully expanded) and one old leaf (2-3 insertions below the youngest) of 7 plants in each plot were sampled; this corresponds to leaves number 4 and 2 (first sampling), 6 and 4 (second sampling), and flag leaf and 6 (third sampling). Sampled leaves were put into plastic bags and taken to the lab, where they were washed in distilled water (3 times x 10 seconds), dried with tissue paper, put individually into Eppendorf tubes and frozen in a commercial freezer (-18°C).

The time of day when any given plot was sampled varied depending on several factors (weather conditions, irrigation being applied, amount of work to do, etc). To check that these differences in the time of sampling did not affect the results obtained, some extra leaves (of 3 salinity treatments) were sampled and analyzed. No significant differences in ion concentrations were found for leaves sampled from early morning (9 am) until mid afternoon (4 pm) (the normal times when samples were taken). Also, several samples were usually taken in the morning, with some of them being left in plastic bags inside the fridge for a few hours before being prepared

for storage. No significant differences in ion concentrations were found, either, for samples kept in the refrigerator for up to 9 hours after collection (by which time all samples had always been processed).

Since the measurements of soil salinity are taken in the middle of each plot, (and the rain-gauges for the measurement of water salinity are also placed in this way), it was decided to sample one of the central rows of plants for ion analysis. The third row from the left was taken, and plants at the appropriate stage of development were chosen at random. In addition to leaves for sap extraction and ion analysis, whole shoots were sampled, to study fresh and dry (oven-dried) weights, and leaf area. These data are not relevant to the present work, and are not presented. However, the row where the samples came from was left with almost no plants by the time the 3rd sampling was finished. The few remaining plants were cut, in order to keep similar plots with 5 whole rows. This was later taken into account for the transformation of yield data per plot into yield per unit area.

In a few cases during the second sampling it was not possible to take leaf number 6 as the youngest recently expanded (because of accumulation of work on the same day), and leaf number 7 was sampled instead, when it had recently expanded. Consequently, in the third sampling leaf number 7 was also taken as the old leaf (instead of number 6). This did not really affect the comparisons between young leaves (second sampling), but it did affect comparisons of older leaves (third sampling). This is because youngest, fully expanded leaves are quite similar at that stage, no matter whether they are number 6 or number 7, while later in the season, when the flag leaf appears, leaves number 6 are rather older than number 7, and have been accumulating ions for a longer time. This effect was observed in the results, with concentrations in "old" leaves 7 being lower than equivalent "old" leaves 6. Therefore, it was decided not to include in the regression analysis the values which came from leaves number 7 in the third sampling. This affected treatments 3 to 5 of varieties Albacete and Chevron.

Details of dates of sampling are given in Appendix 2. It can be seen from there that some of the high salinity treatments were not sampled, particularly at the earlier



harvests. The plants at these higher treatments were badly affected by frost and very low temperatures experienced in mid February, when soil salinity was already quite high, and many of them died. (This was another reason for giving some fresh water irrigations at that time.) These cold conditions affected especially plants of CM-67 and Chevron in Rep-I, which had the highest soil salinities (because they were in the unleached area). With only a few plants left in some plots, and with no clear prospects of recovery, it was decided not to sample them. Some of these plots were excluded altogether from the rest of the experiment, due to the small number of plants that survived and their irregular growth.

It has to be stressed that these plants did not die just because of excess salinity, but were killed by a combination of stresses, mainly chilling. Since these stresses probably interact, and because the different varieties may respond differently to them, the decision of not taking into account the most affected plots seemed justifiable.

The frozen leaf samples were taken to Bangor for sap extraction and chemical analysis. The methodology for extracting the sap has been described in previous experiments.  $\text{Na}^+$  and  $\text{K}^+$  concentrations were determined either by flame emission spectrophotometry, (using a Pye Unicam SP90 spectrophotometer or a Jenway PFP7 flame photometer), or by atomic absorption spectrophotometry (Pye Unicam SP9).  $\text{Cl}^-$  was determined using a Jenway PCLM3 chloride-meter. Because the samples came from individual leaves, not enough sap was always available for carrying out all the measurements (especially in leaves number 2 and flag leaves, due to their small size); determination of  $\text{Ca}^{2+}$ , in particular, was not done for this reason.

- *Statistical methods:*

To study the changes in the amounts of ions in leaves with increasing salinity, ion concentrations were plotted against soil salinity. By examination of these plots it appeared that the relationship between the two parameters was quite linear. Thus, the linear correlation (Pearson's coefficient) was calculated, and a linear regression line was fitted. This was done for each plot, (that is, each combination of sampling time, leaf, variety and replicate), using means of 7 plants per plot. The soil salinity considered was the mean EC of the soil saturation extract (estimated from the EM-38

measurements) up to the time when that particular plot was sampled (or to the nearest day to that when an EMS measurement was taken). In practice, means up to 2<sup>nd</sup> of March were used for the first sampling, 12<sup>th</sup> of March for the second sampling, and 28<sup>th</sup> of April for the last one.

For formal reasons, the 2 replicates of each variety were first considered separately, and the slopes and intercepts of their regression lines were compared to see if there was any "block" effect. In general, this was not the case, and thus the 2 replicates were pooled and used to derive a single regression line for each variety; these were used to compare the response of the 3 varieties.

For all these comparisons of regression lines, the method described in Snedecor and Cochran (1989; pp:390-393) was followed. Slopes (change in ion concentration with increasing salinity) were tested first and, if they were not different, y-intercepts (equivalent to average concentrations) were then compared. (If the slopes are significantly different, the intercepts cannot be compared, since the test, an analysis of covariance, assumes that the slopes are parallel.) Also, whenever a variety did not have a significant correlation (at the 5% level) its coefficients were not included in the comparison. In the case of the 3 varieties not having a significant correlation with salinity for a given ion in a given sampling, then their mean ion concentrations were directly compared, (since in that case the concentrations did not depend on the level of salinity).

In addition to absolute ion concentrations, comparisons between leaves of different age were done by using the ratio of the concentrations of old to young leaves (OL/YL) for any given sampling time. To follow the changes with leaf ageing, the difference between the concentrations in a similar leaf at two consecutive sampling dates was calculated. Thus, the changes in leaf 4 were calculated as the difference between concentrations in older leaves at the second sampling, and younger leaves at the first sampling. Similarly, differences between older leaves at the third sampling and younger leaves at the second sampling would give an indication of the changes in ion concentrations in leaf 6 with time.

For the study of yield response to increasing salinity, yields were plotted against soil salinity for each variety. Again, this was first done for the 2 replications independently, and later pooling the 2 replicates of each variety. The soil salinity considered this time was the EC of the soil saturation extract averaged over the whole growing season.

The plots of yield vs salinity showed relationships quite linear within the range of salinities studied. However, since it is known that yield responses to salinity are not linear, but decrease faster above a certain value (threshold), three different models were fitted to the yield data: a simple linear regression (without threshold); the threshold model of Maas and Hoffman (1977); and a sigmoidal curve defined by  $Y = Y_m / [1 + (EC_e/EC_{50})^p]$  (Van Genuchten, 1983); (see section 1.4.2 for a detailed description and a graphical representation of these models). This was done using the SALT computer program of Van Genuchten (1983).

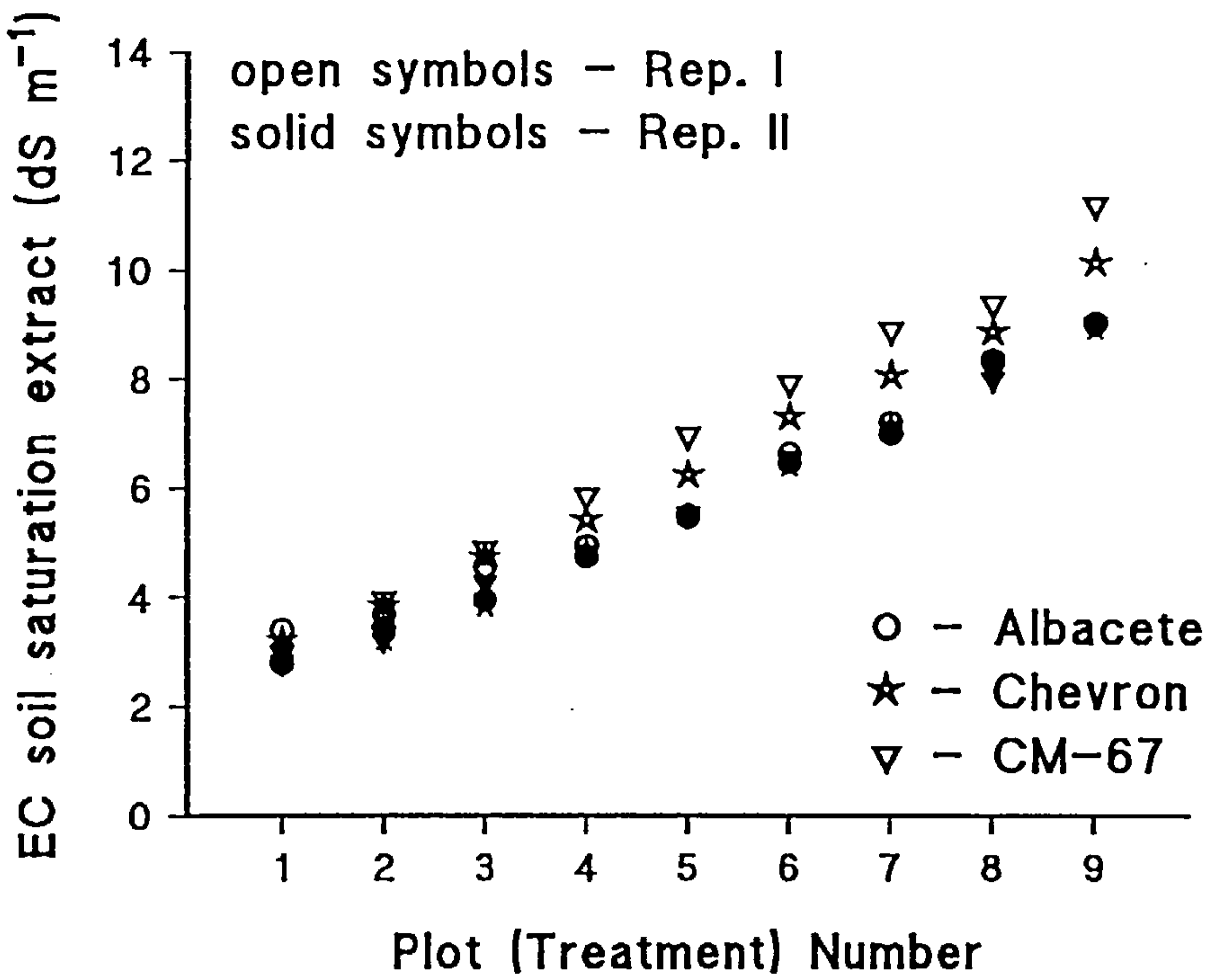
To decide which model gave the best fit, the coefficients of determination between observed and fitted values were calculated, and the residuals studied by plotting them against soil salinity. Once the best model was chosen, the varieties were contrasted by comparing the parameters that define them under such a model. This was done by means of pairwise comparisons using a t-test, since the SALT program provides standard errors for the estimates of the parameters. Although this method is not usually recommended (see, for example, Carmer & Walker, 1982), no alternative seemed to be available in the present case (multiple comparison procedures cannot be easily modified for use in the threshold or exponential models). Varieties were also compared by using their relative yields, expressed as a percentage of their yields without salinity.

### 4.2.3. RESULTS:

#### 4.2.3.1. Soil and water salinity:

The soil salinity, measured with the EMS and calibrated with saturated extracts of soil samples, is presented in Figure 4.2.3. The salinities of the 3 sampling dates for

a) mean of 3 sampling times (ion analysis)



b) mean over the whole season

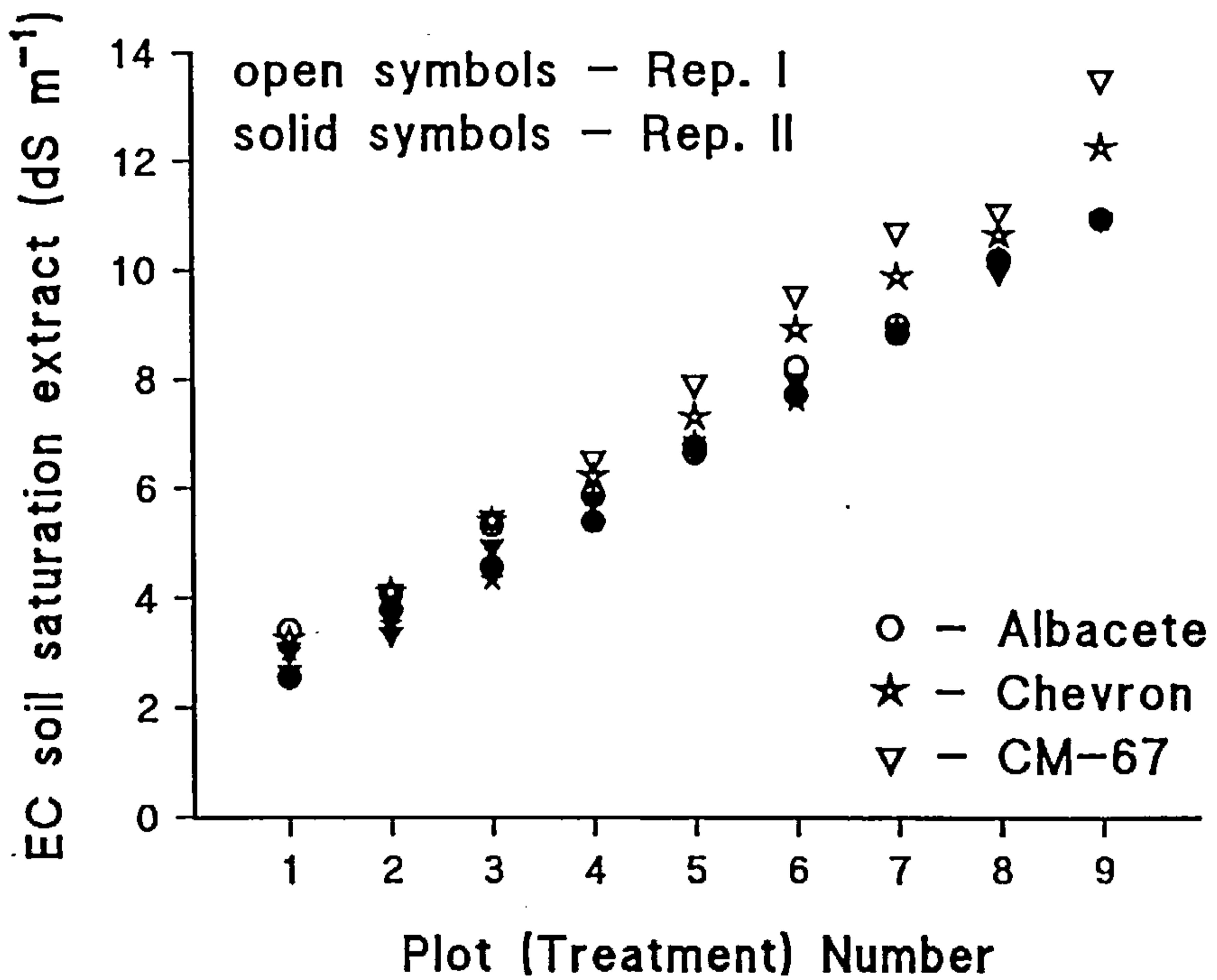


Figure 4.2.3. Soil salinity for each variety and replicate; a) mean of 3 sampling times for ion analysis; b) mean over the whole season.

each plot have been averaged, since they were very similar. In that figure, the higher salinity levels in one replicate of CM-67 and Chevron are clearly seen. The regression lines (soil salinity vs plot position) for these two cases were significantly higher (at the 5% level) than those for the other varieties and replicates. This was because these plots corresponded with a part of the field that had not been leached prior to this experiment.

The total amount of irrigation water applied by the TLS and its mean salinity are presented in Figure 4.2.4. The decrease in salinity with distance from the central sprinkler line was linear ( $r^2=0.998$ ), particularly in the central treatments; it might be slightly less so at the 2 extremes. The total amount of water received by the different treatments was also fairly regular, although a tendency towards lower amounts applied with increased distance from the centre line can be detected. However, since the coefficient of variation for this trait was less than 5%, it was assumed that the differences between the 10 treatments were small and unlikely to affect growth and yield.

Figure 4.2.5 shows the increase in soil salinity with continued saline irrigations. The effect of fresh water irrigations in late February is clearly noticed in the lower salinities found at the fourth EMS measurement (12<sup>th</sup> March). A later irrigation with fresh water (early May) probably prevented soil salinity from increasing too fast, but only effectively decreased it in the 2 lowest treatments.

Salinity in the soil profile at 2 different dates in some plots (taken from the whole field) is presented in Figure 4.2.6. Continued saline irrigations increased soil salinity with time, although this was limited to the upper layers (down to 50 cm). In the treatments with lower salinities, salt distribution in the profile was quite uniform; in higher treatments, salinity decreased with depth.

#### 4.2.3.2. Ion concentrations in leaves:

For each sampling date and leaf, the relationship between concentrations of ions (means of 7 plants) and soil salinity (measured on each plot and averaged over time)

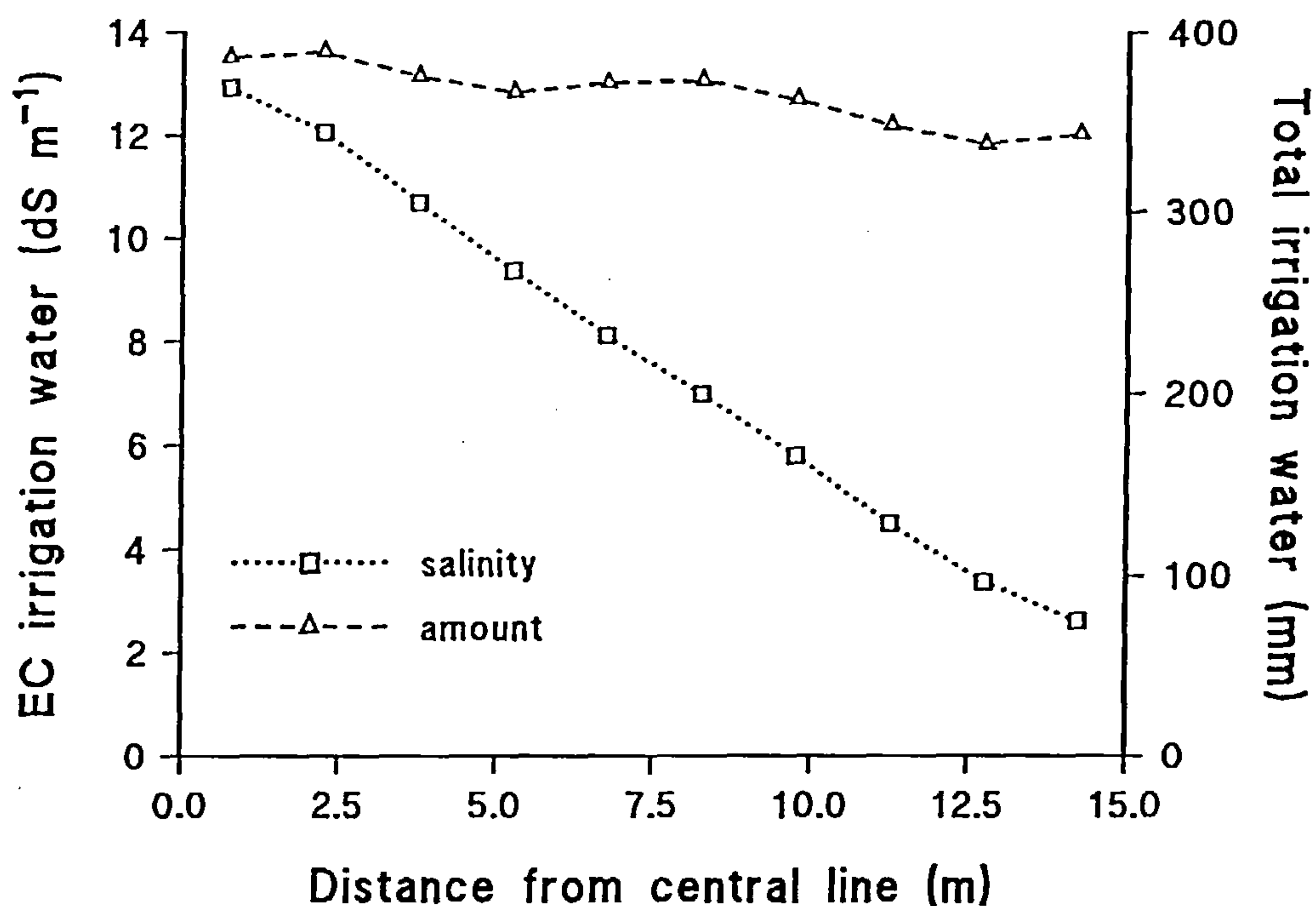


Figure 4.2.4. Salinity (mean of 22 saline irrigations) and amount of water (total of 29 irrigations) with distance from the source of saline water; (1991/92 season)

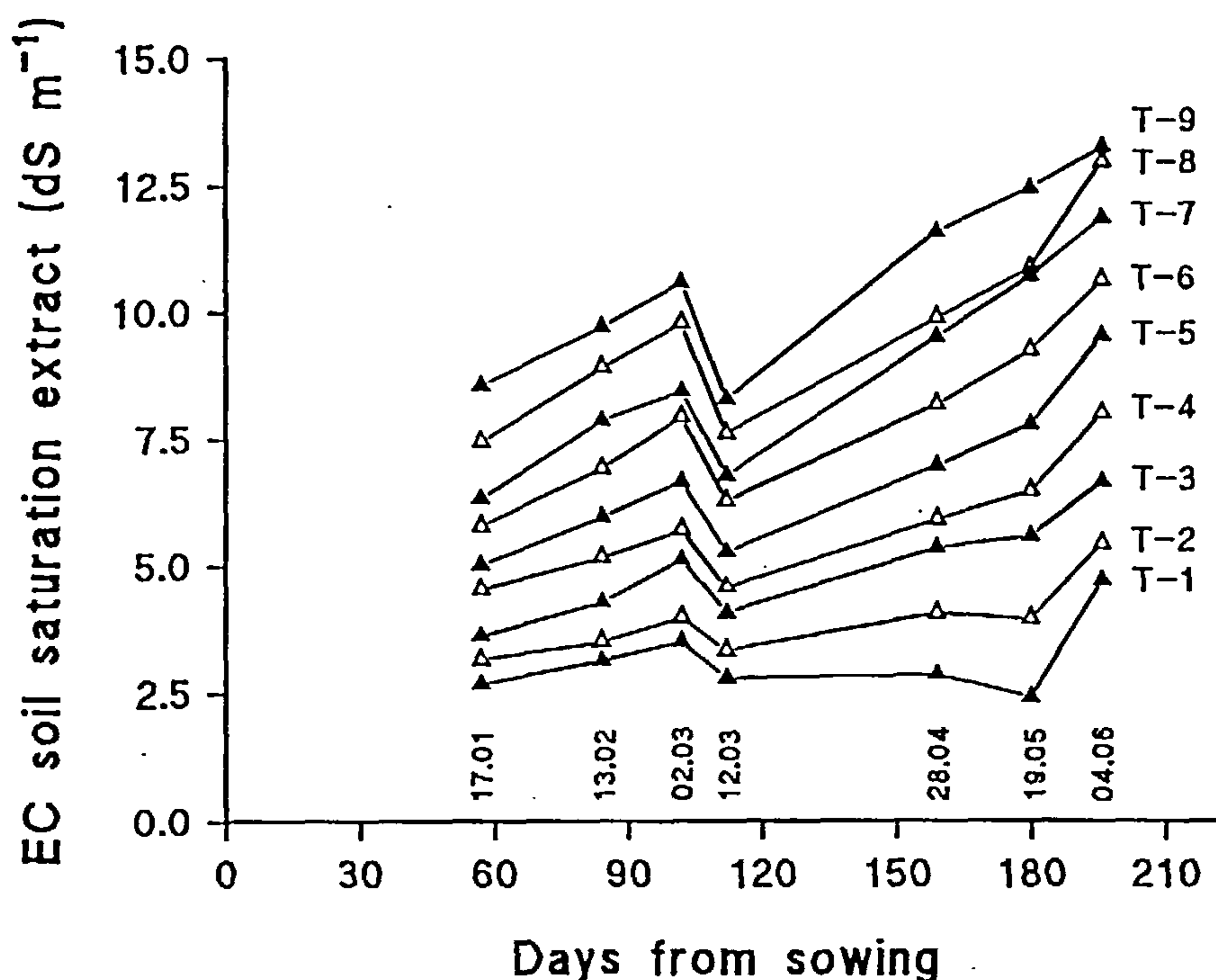


Figure 4.2.5. Evolution of soil salinity with time: values calibrated from the EM-38 readings; means of 6 plots per treatment; (1991/92 season)

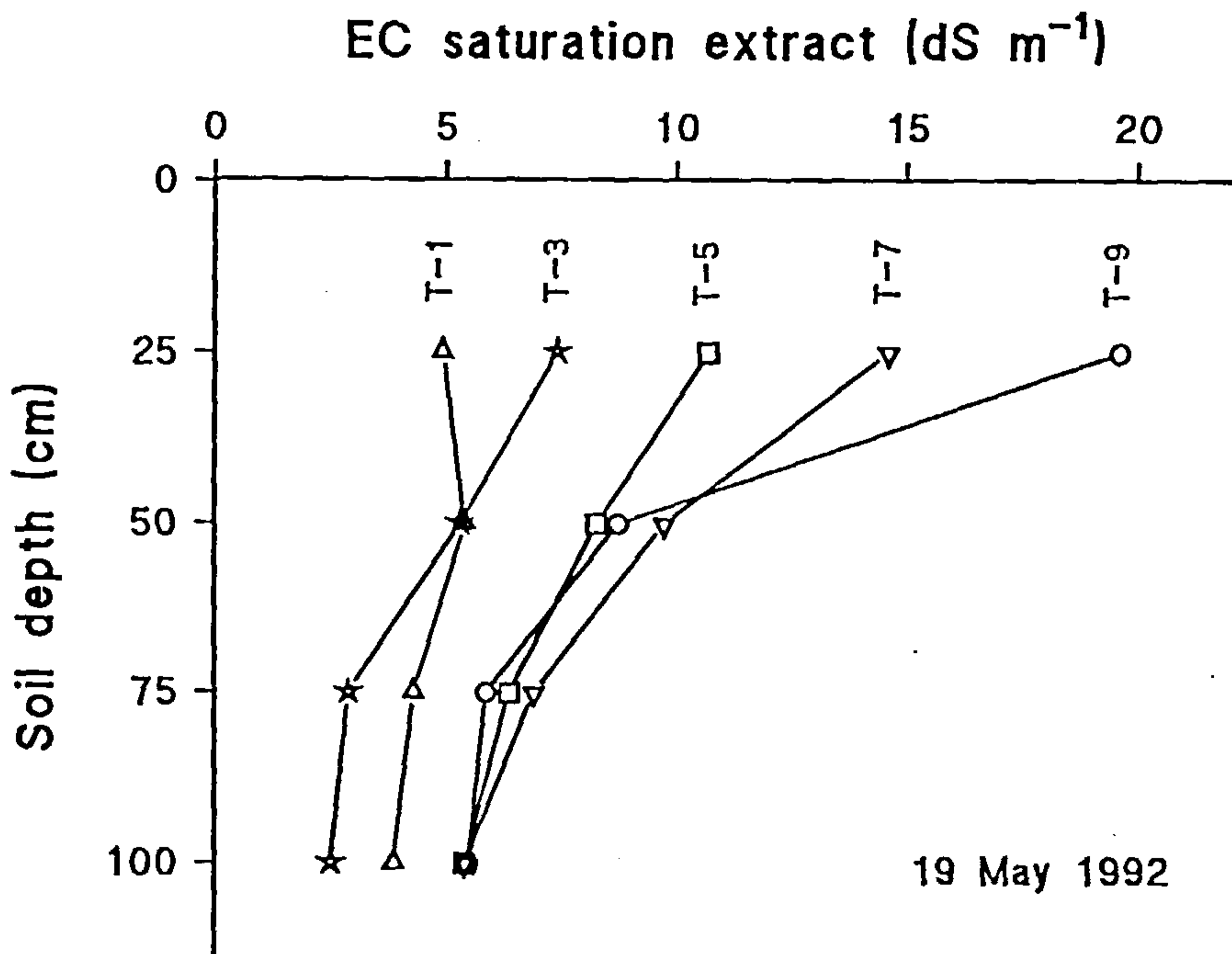
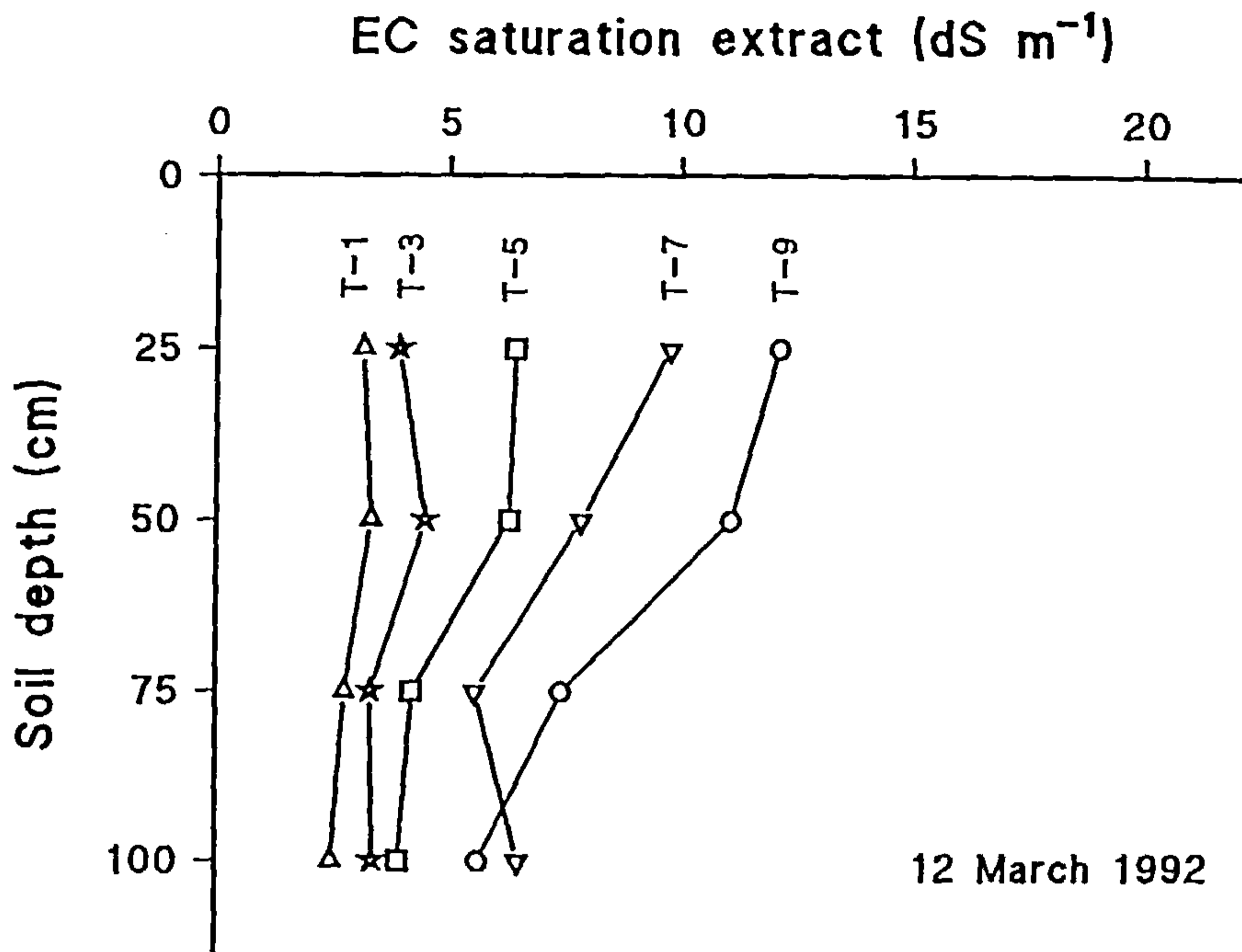


Figure 4.2.6. Soil salinity profile of some treatments at 2 sampling dates; (1991/92 season).

was studied by means of linear regression. However, some very high ion concentrations were occasionally found at the highest treatments, which significantly influenced the values of the correlation and regression coefficients. On the other hand, some of these highest treatments were not sampled because plants had been killed by frost; thus, some varieties and replicates lacked data on leaf ion concentrations at the highest salinities. In these conditions, the inclusion of those extreme figures in some cases and not in others (simply because they were not available) might have resulted in larger apparent differences between varieties and replicates. For comparative purposes, it was decided to include, within each sampling time, only those observations which fell within a similar range of soil salinities for all the varieties. That is, the upper limit of the treatments included in the regressions was determined by the highest common soil salinity.

In general, no significant block effects were found (data not shown). Only in a few cases, most of them in variety CM-67, were the 2 replicates significantly different. This was attributed to the history of the field (see above). Although the regressions were calculated using the values of soil salinity for each plot (i.e. the data in Appendix 1), and not with a hypothetical "salinity level" (e.g. treatment number), the higher early salinity in Replicate-I might have differently affected the plants growing there; otherwise a similar response function should have been obtained for the same variety. The following results are based on the regressions obtained after pooling the 2 replicates of each variety. (Original data on ion concentrations is not presented here.)

a) Chloride (Table 4.2.1a and Figure 4.2.7):

In general, the correlations were positive ( $\text{Cl}^-$  increased with increasing salinity), although in the 3rd sampling they were very low, and sometimes not significantly different from zero. In young leaves, the rate of leaf  $\text{Cl}^-$  increase with salinity was similar for all varieties (slopes not significantly different), but CM-67 tended to have less  $\text{Cl}^-$  (lower intercept) than Chevron and Albacete. In older leaves, the rate of  $\text{Cl}^-$  increase was faster (steeper slope) in Albacete than in CM-67, Chevron usually being



Table 4.2.1. Linear correlation (r) and regression coefficients between Cl<sup>-</sup> (a), Na<sup>+</sup> (b) and K<sup>+</sup> (c) concentrations in leaves (mol m<sup>-3</sup> sap) and soil salinity (EC saturation extract) for the 3 varieties in the TLS 1991/92 experiment. Within each leaf type (number), estimates of slopes and of intercepts with the same letter are not significantly different (p < 0.05). (Units: slope: mol m<sup>-3</sup> ion per dS m<sup>-1</sup> soil salinity; intercept: mol m<sup>-3</sup> ion.) (Correlation: \* - p < 0.05; \*\* - p < 0.01; \*\*\* - p < 0.001.)

Variety	YOUNG LEAVES				OLD LEAVES			
	Leaf	r	slope	interc.	Leaf	r	slope	interc.
<i>a) Chloride:</i>								
Albacete	4	0.787***	24.8 a	85.3 a	2	0.962***	110.2 a	-126.7 #
Chevron		0.799**	21.7 a	81.6 a		0.920***	72.3 b	-10.1 a
CM-67		0.806**	19.1 a	46.6 b		0.860***	62.7 b	-7.4 a
Albacete	6/7	0.747**	26.7 a	101.9 a	4	0.787***	65.1 a	66.7 a
Chevron		0.708**	22.5 a	93.3 a		0.832***	92.0 a	-52.3 a
CM-67		0.461 ns	10.5 +	82.7 +		0.649*	24.4 b	92.1 #
Albacete	flag	0.581*	8.0 a	33.0 a	6/7	-0.180 ns	-3.9 +	432.8 +
Chevron		0.560*	4.6 a	45.7 a		0.475 ns	13.3 +	251.4 +
CM-67		0.714**	9.1 a	37.1 a		0.658**	20.0	186.1
<i>b) Sodium:</i>								
Albacete	4	-0.794***	-9.0 a	118.8 a	2	0.085 ns	1.2 +	105.0 +
Chevron		0.544 ns	6.1 +	92.0 +		0.667*	9.2 a	81.1 #
CM-67		-0.775**	-8.9 a	90.7 b		-0.802**	-12.8 b	149.6 #
Albacete	6/7	-0.042 ns	-0.6 +	101.1 +	4	-0.220 ns	-5.9 +	153.0 +
Chevron		0.810***	22.7	14.5		0.829***	28.3 b	34.3 #
CM-67		-0.450 ns	-3.0 +	51.9 +		-0.853***	-10.4 a	122.8 #
Albacete	flag	0.402 ns	1.7 +	24.5 +	6/7	-0.382 ns	-4.7 +	162.5 +
Chevron		0.900***	7.4	2.0		0.866***	15.0 b	88.8 #
CM-67		-0.113 ns	-0.4 +	35.4 +		-0.712**	-12.2 a	170.7 #
<i>c) Potassium:</i>								
Albacete	4	-0.358 ns	-4.1 +	199.8 +	2	0.132 ns	1.2 +	104.0 +
Chevron		-0.868***	-12.4 a	178.5 b		-0.340 ns	-2.7 +	82.4 +
CM-67		-0.789**	-10.7 a	235.9 a		0.505 ns	3.1 +	125.5 +
Albacete	6/7	0.223 ns	4.3 +	172.5 +	4	0.587*	14.5 a	61.8 b
Chevron		-0.826***	-12.8	207.9		0.538*	7.1 a	47.4 a
CM-67		0.431 ns	7.5 +	174.5 +		0.246 ns	3.9 +	149.1 +
Albacete	flag	-0.223 ns	-3.6 +	261.8 +	6/7	0.056 ns	0.6 +	177.6 +
Chevron		-0.485 ns	-8.1 +	265.6 +		-0.039 ns	-0.4 +	120.0 +
CM-67		0.125 ns	1.5 +	217.9 +		0.651**	10.2	142.8

+ - coefficients not compared: correlation not significant.

# - no comparison possible (test for intercepts assumes that the slopes are parallel).

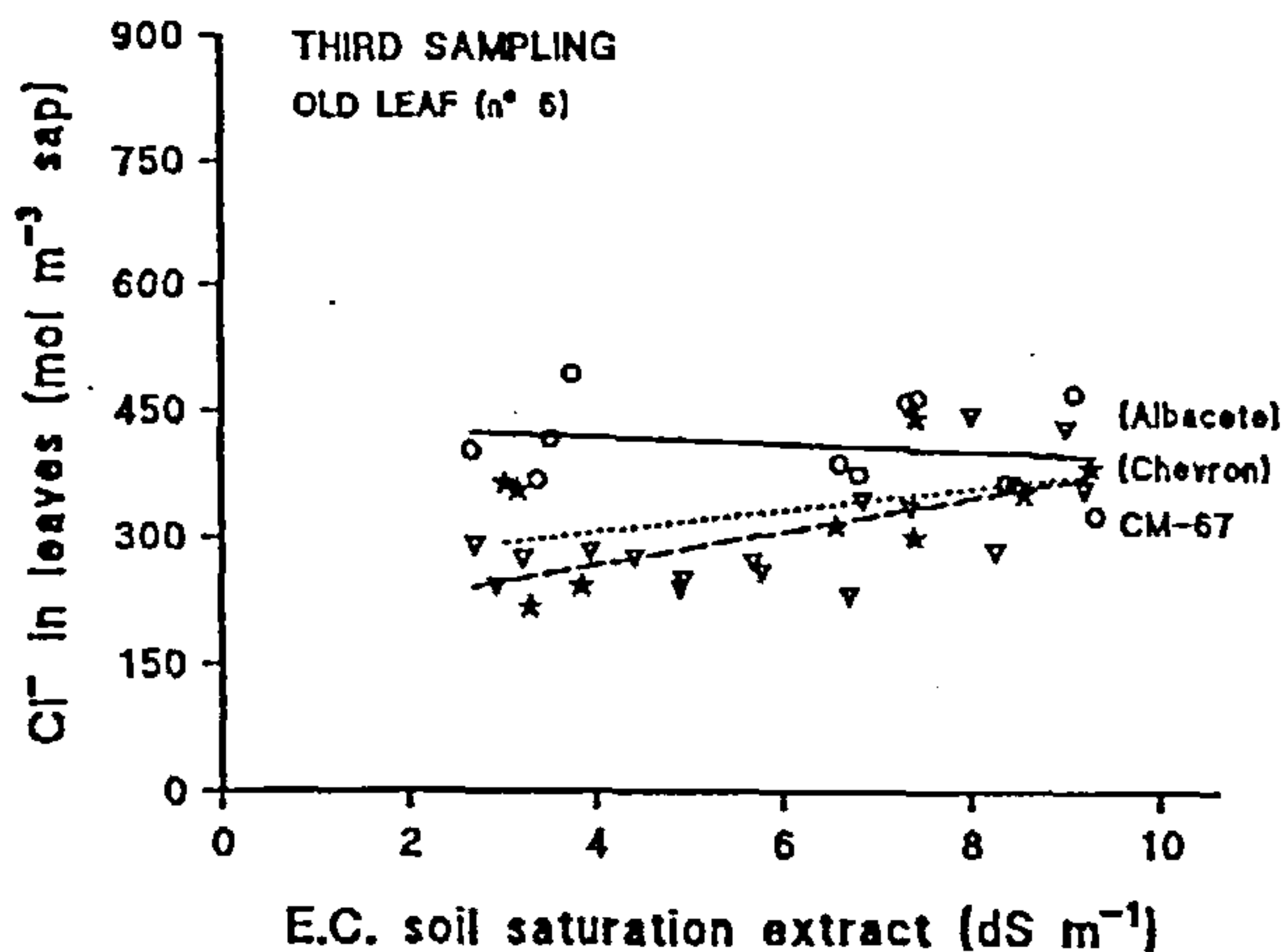
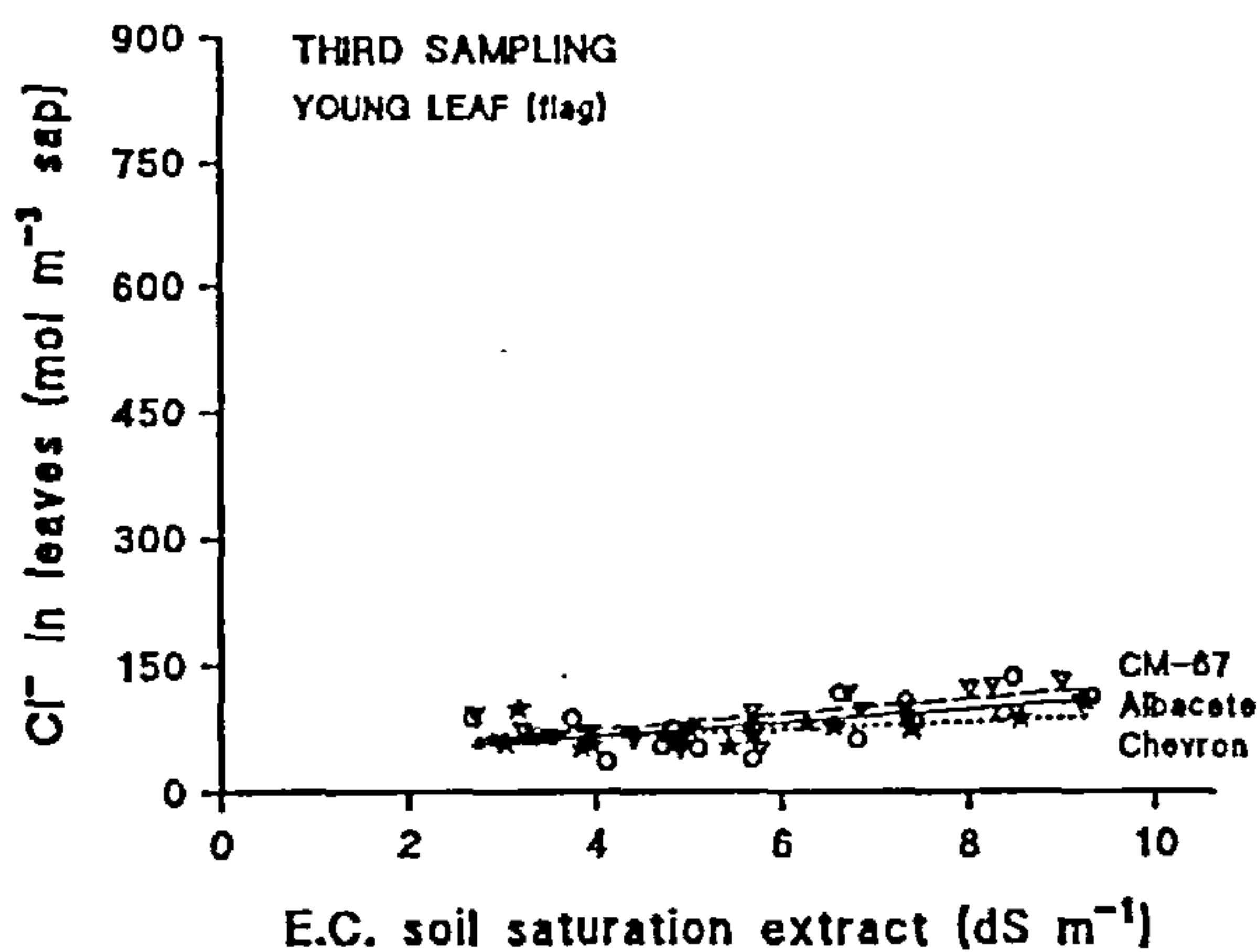
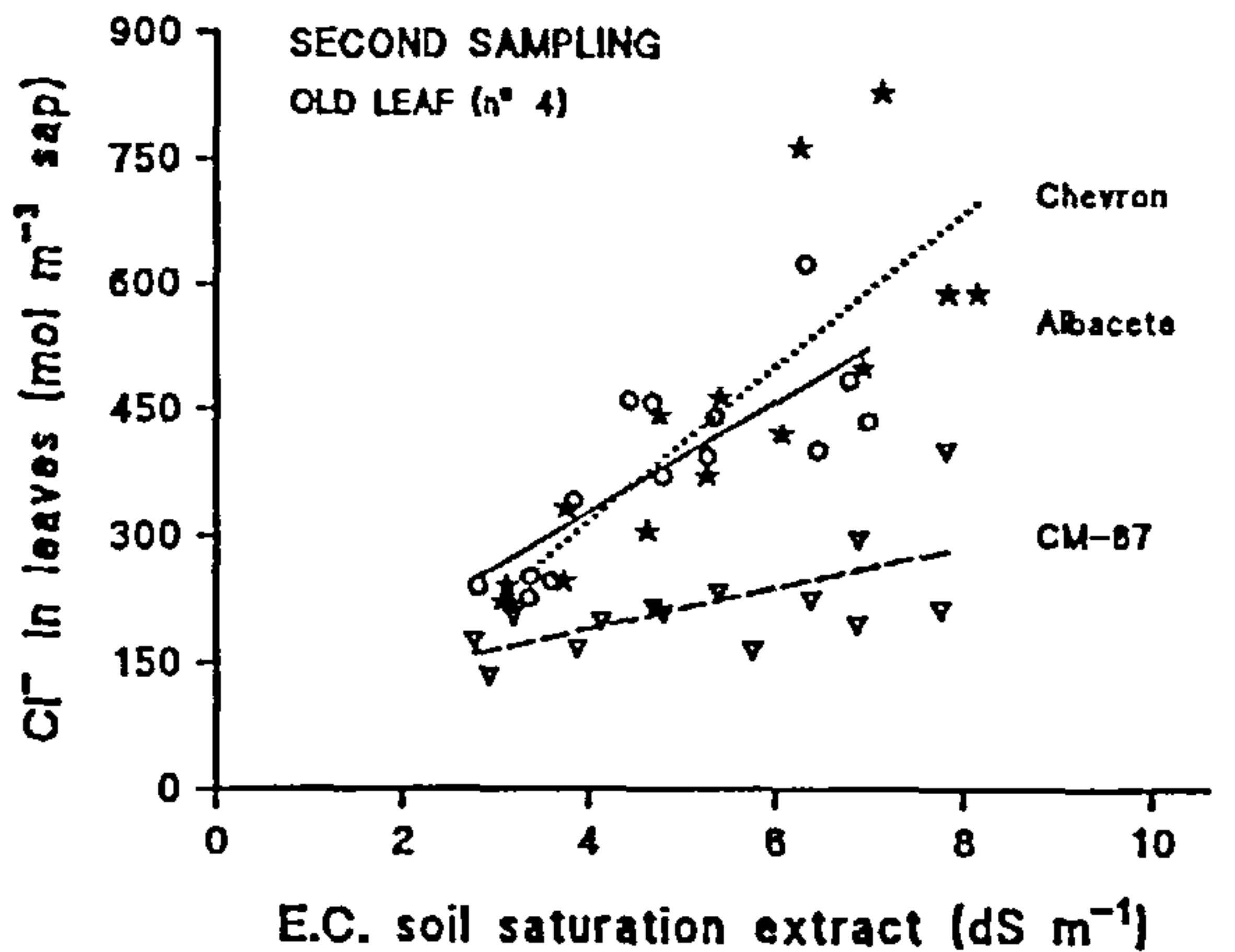
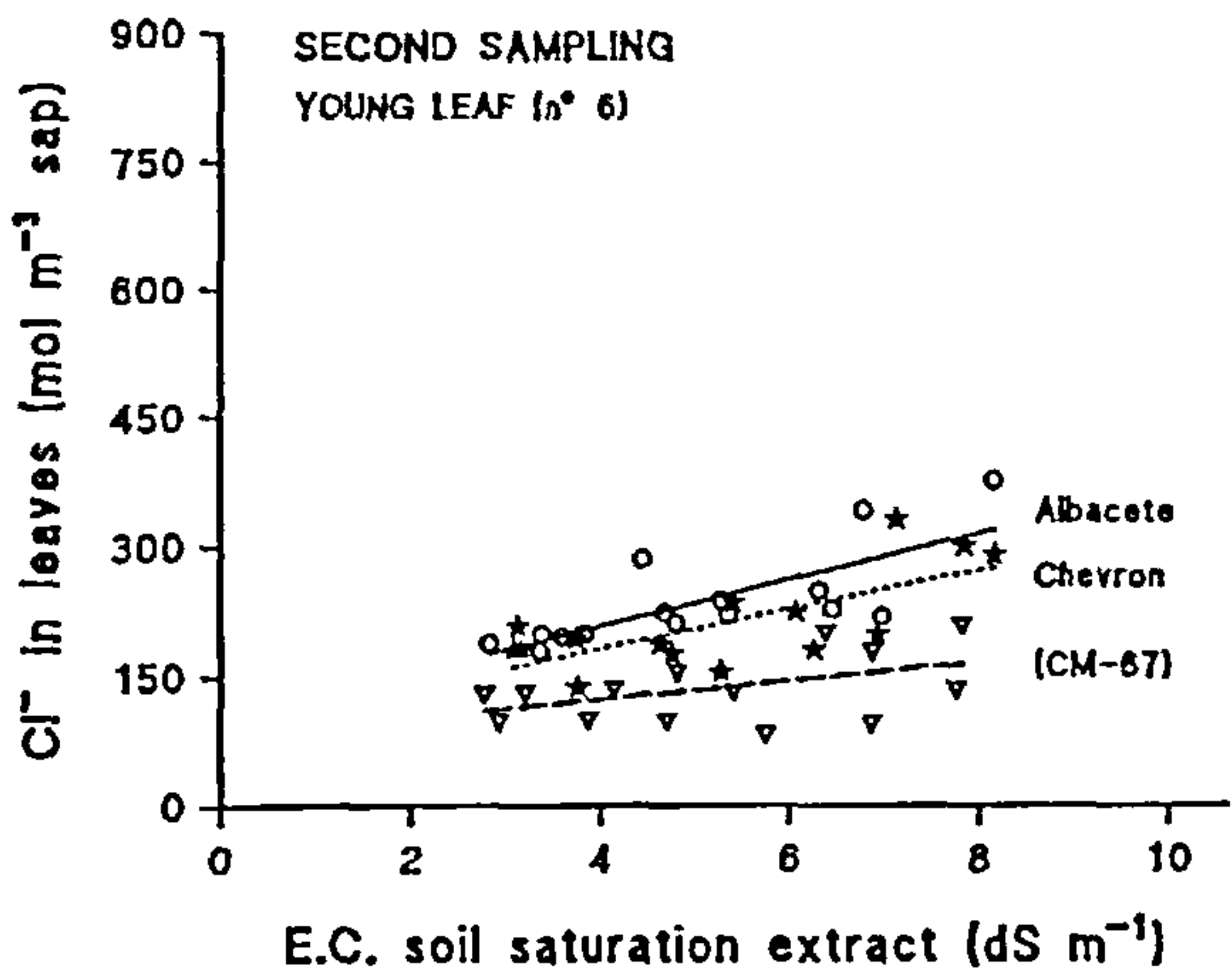
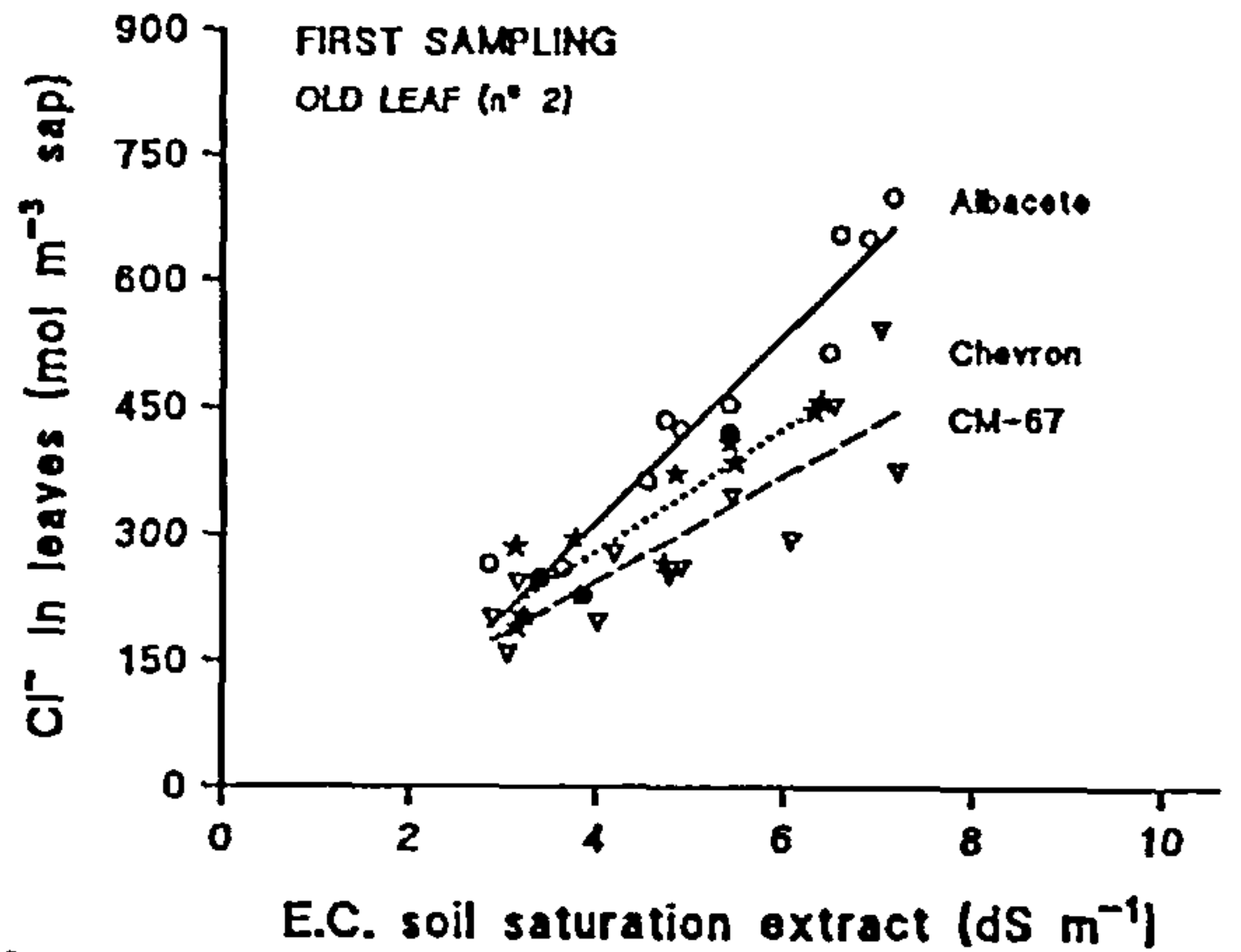
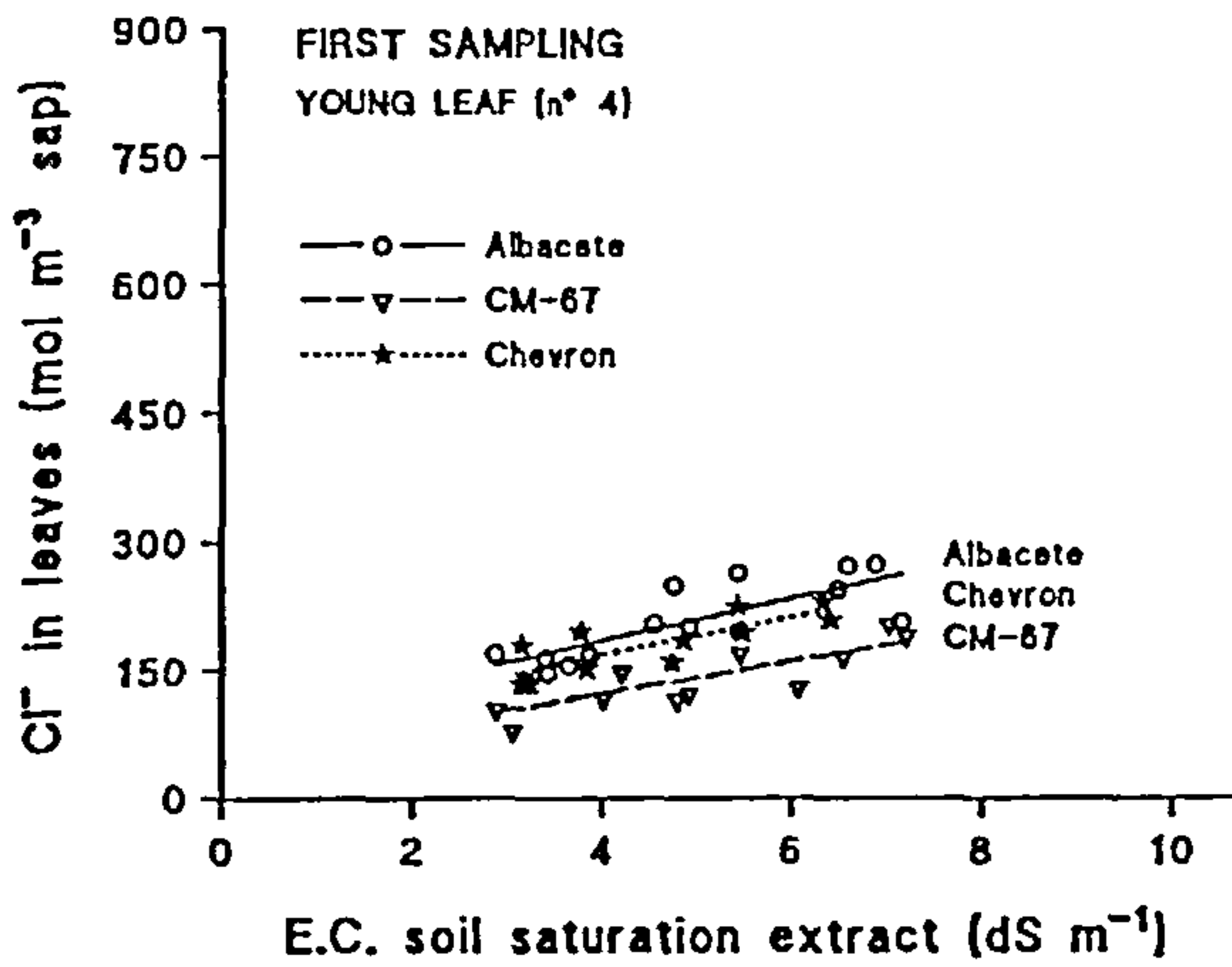


Figure 4.2.7. Cl<sup>-</sup> concentrations in leaves of plants in the TLS (1992) as a function of soil salinity. A variety name in brackets indicates a non-significant correlation.

intermediate. In the 3rd sampling there were hardly any differences between varieties (slopes or intercepts) in the few cases when the regressions were significant.

Comparisons between young and old leaves were made by means of ratios of their concentrations (old over young). For a given sampling time,  $\text{Cl}^-$  concentrations in older leaves were usually much higher than in younger leaves. These differences were least pronounced in variety CM-67, particularly at the second sampling. The highest correlations of these new values with soil salinity were found for the first sampling (data not shown). However, no better information was obtained with these ratios, in terms of discrimination between varieties; this was as a result of the large variability found between treatments, and the inconsistency of results between sampling dates (data not shown).

Increased  $\text{Cl}^-$  concentrations with leaf age can also be seen if the same leaf is compared across samplings, e.g. leaf 4 in the first sampling (young) and at the second (old). The increase of leaf  $\text{Cl}^-$  with time (old minus young for the same leaf) was also plotted against soil salinity, but correlations were very low (leaf 4) or not significant at all (leaf 6) (data not shown). No overall differences (mean of all treatments) between varieties were found either; again, this was due to the large variability and inconsistency of results between the 2 leaves (data not shown).

b) Sodium (Table 4.2.1b and Figure 4.2.8):

Chevron was the only variety in which the correlations were positive, that is, where  $\text{Na}^+$  concentrations in leaves increased with increasing salinity. For Albacete and CM-67,  $\text{Na}^+$  in leaves either did not change (correlations not significantly different from zero) or it decreased with salinity (negative correlations). In the latter case, the rate of change (slope) was similar for both varieties. However, amounts of  $\text{Na}^+$  in leaves of Albacete were usually higher (larger intercepts) than in CM-67. Chevron had the highest  $\text{Na}^+$  concentrations at almost all salinities. The young leaf in the 3rd sampling (flag leaf) had very low levels of  $\text{Na}^+$ , and again did not reveal differences between varieties.

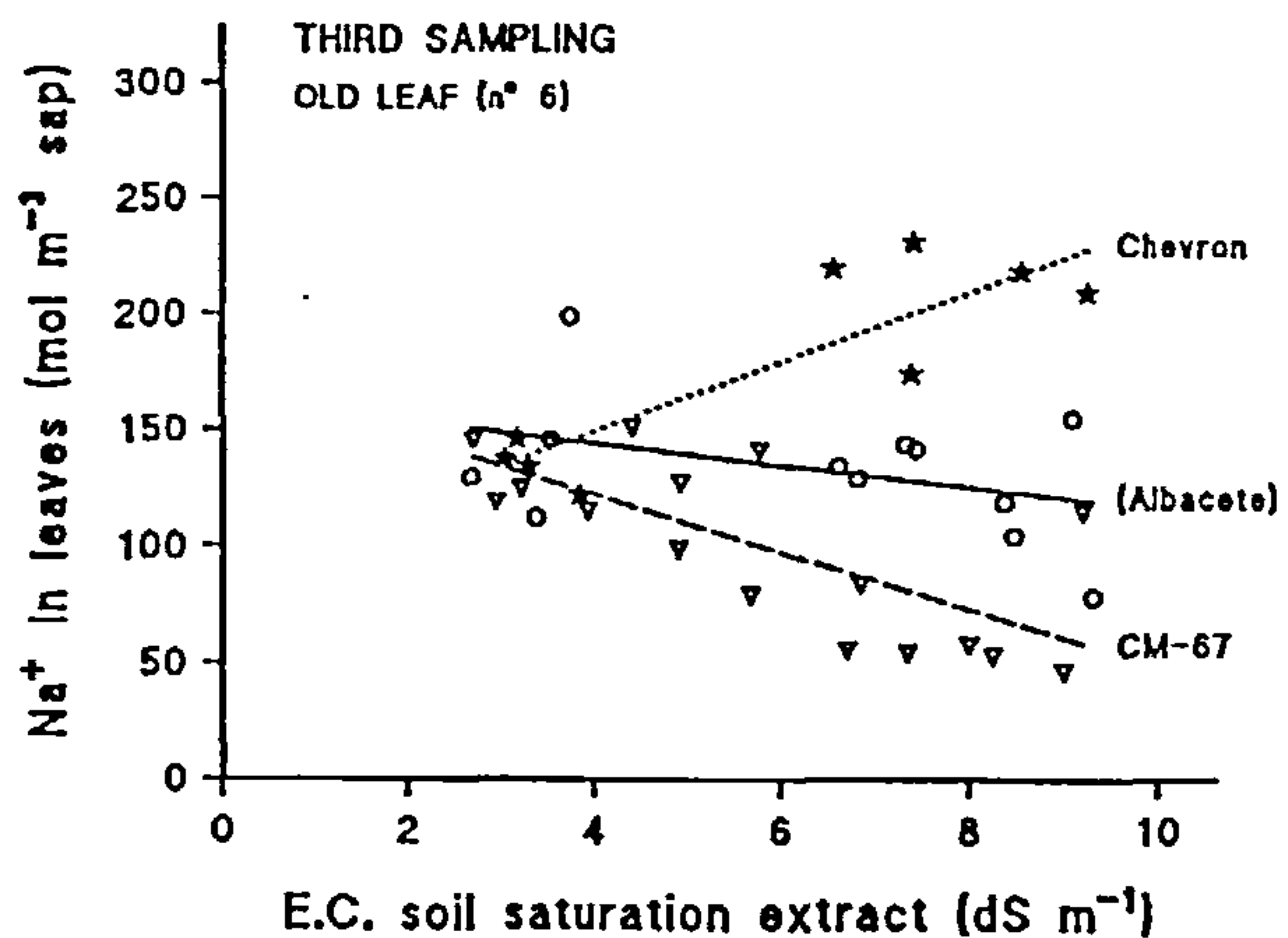
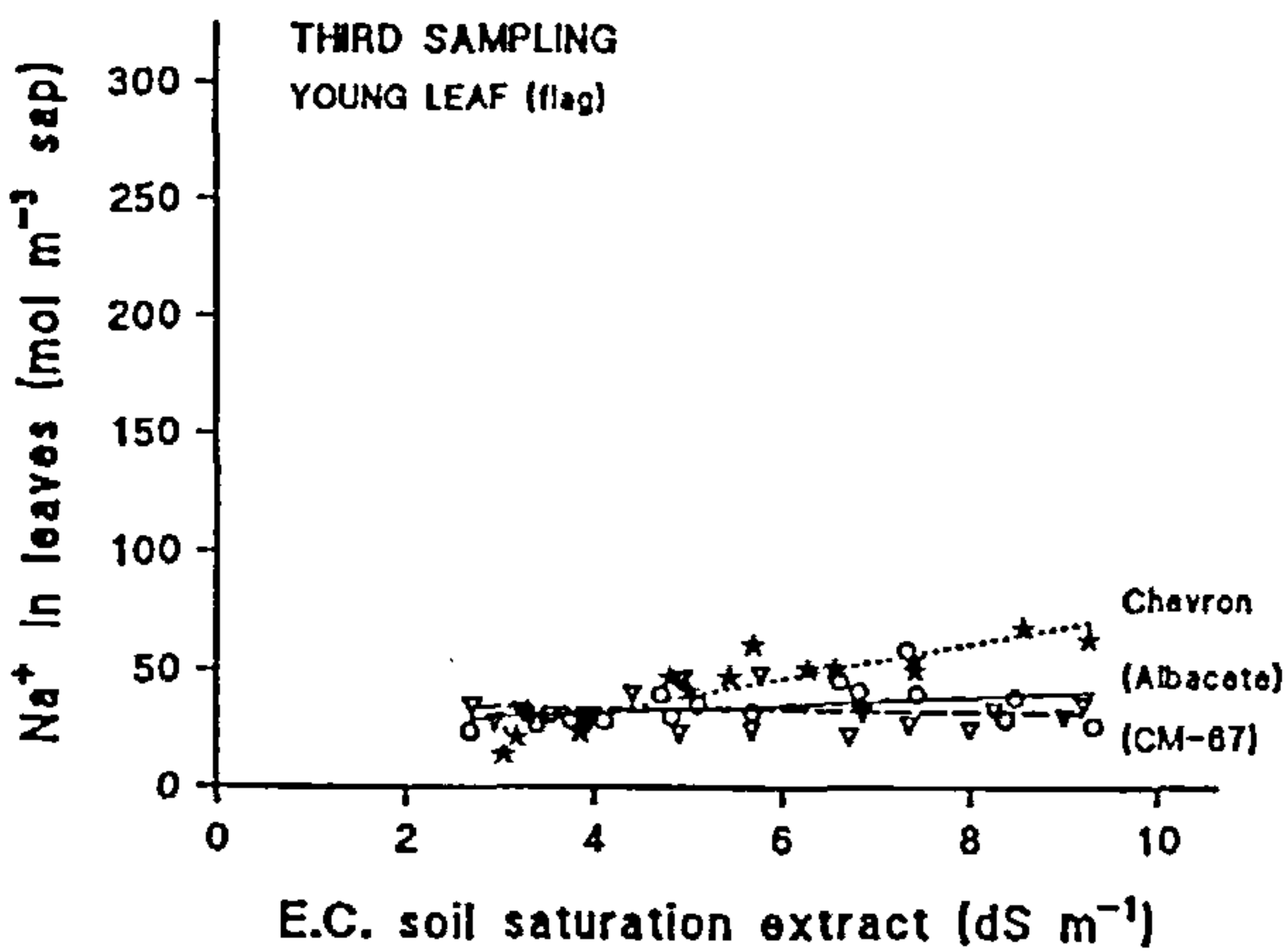
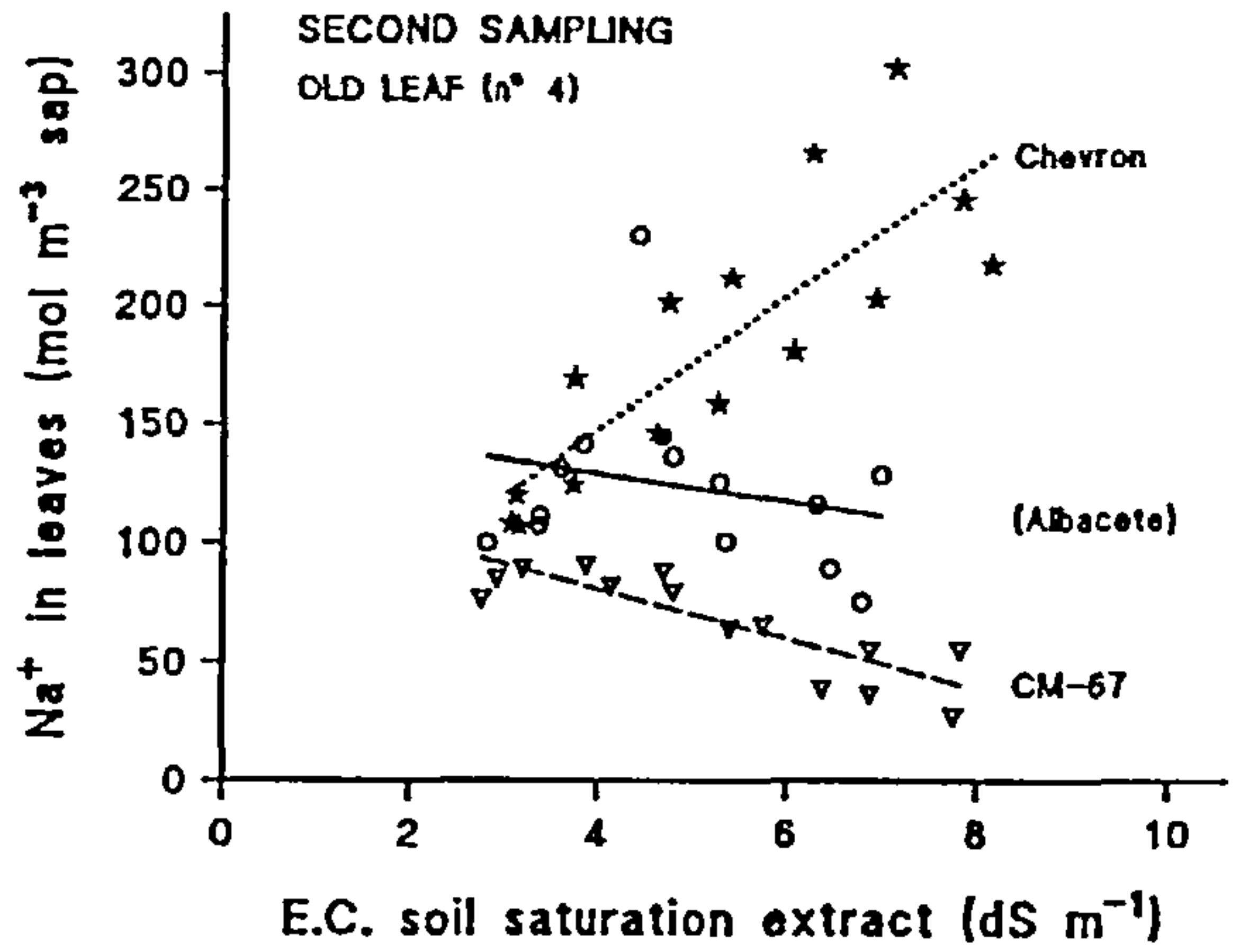
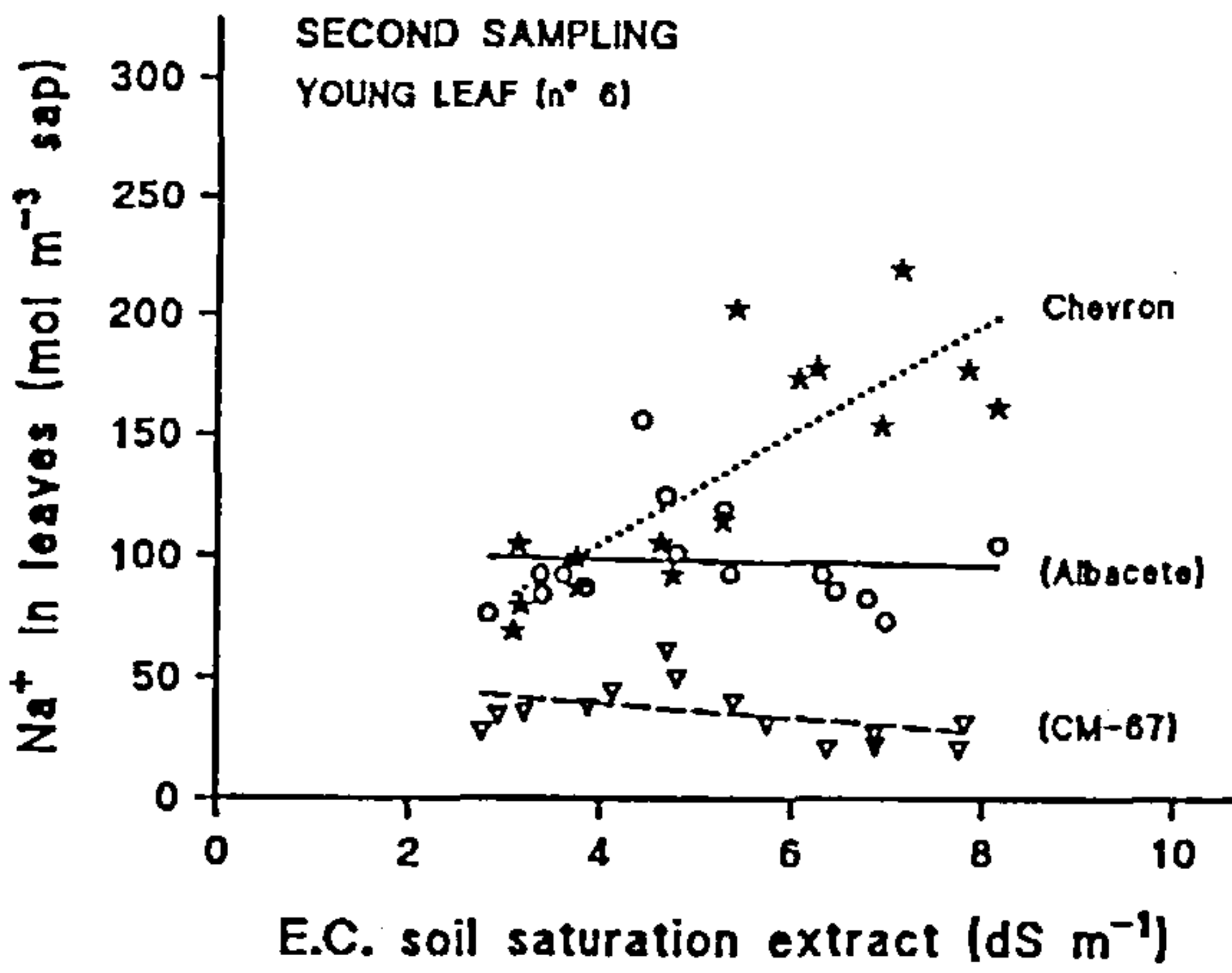
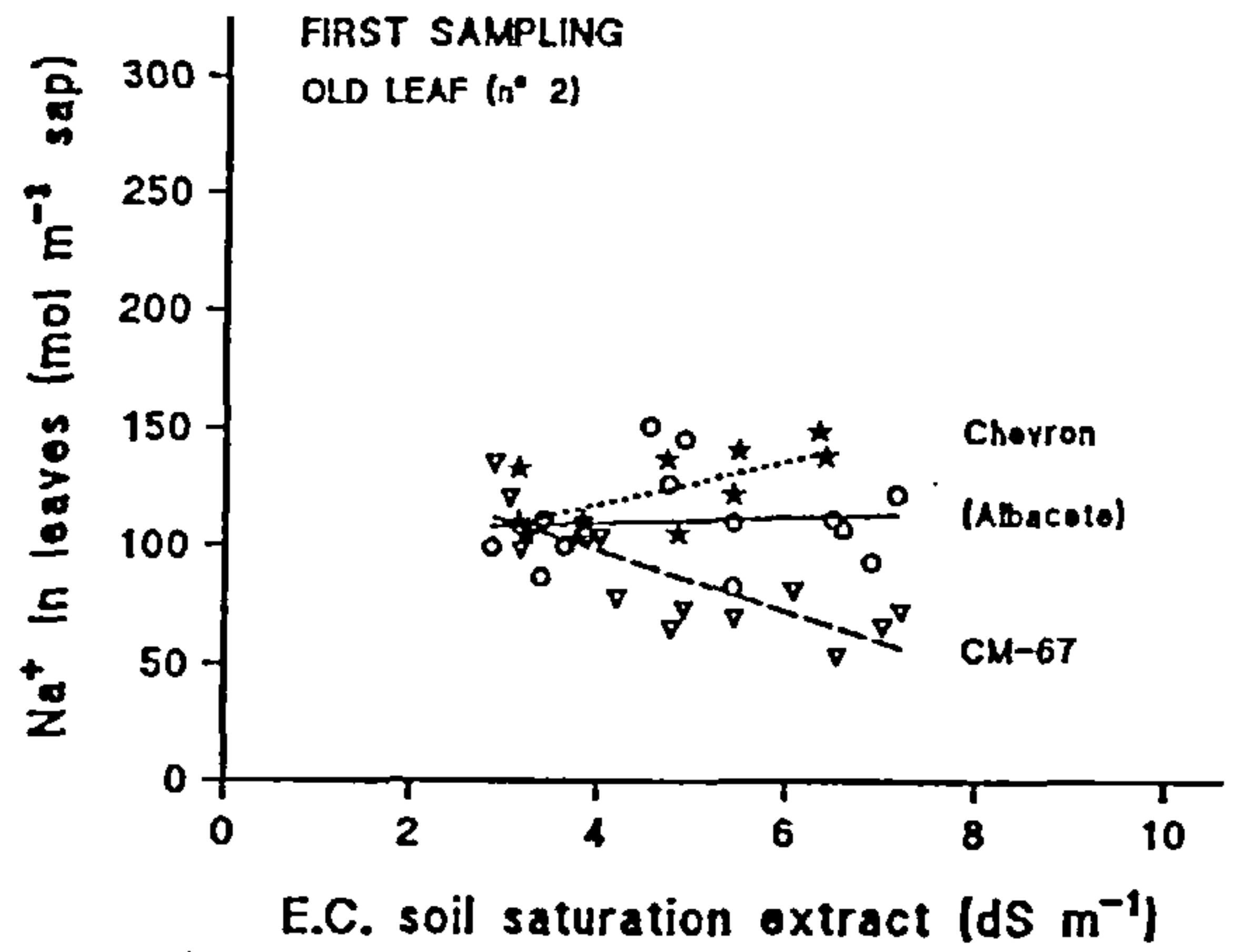
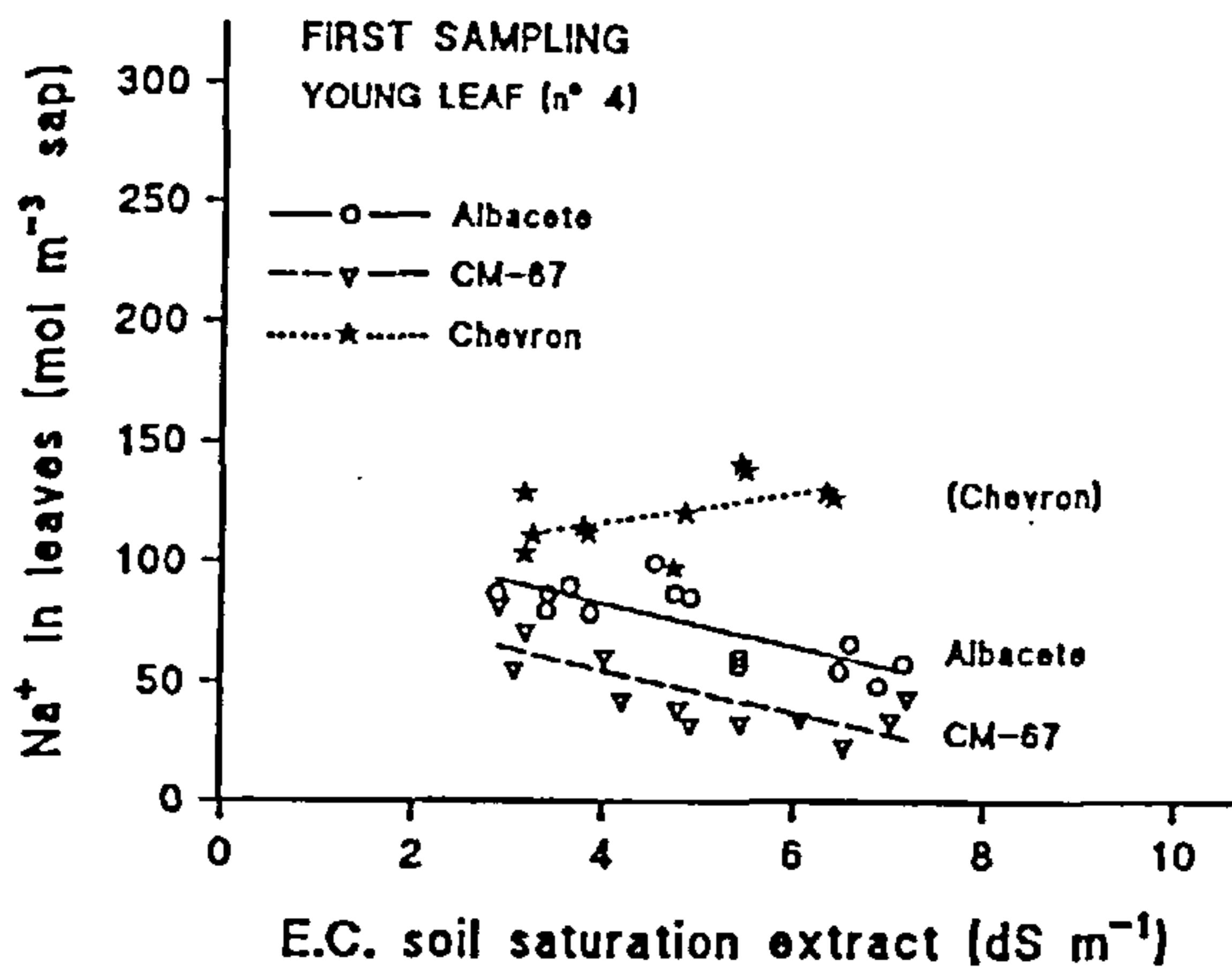


Figure 4.2.8. Na<sup>+</sup> concentrations in leaves of plants in the TLS (1992) as a function of soil salinity. A variety name in brackets indicates a non-significant correlation.

For a given sampling date, differences between young and old leaves were not very large, except in the last sampling (due to low  $\text{Na}^+$  concentrations in the flag leaf). This was particularly true for Albacete and Chevron, while CM-67 tended to partition  $\text{Na}^+$  better towards older leaves, protecting the younger ones. This can be measured as the ratio of concentrations between old and young leaves, which was usually higher in CM-67 than in the other cultivars (data not shown). Interestingly, in CM-67 this ratio tended to decrease with increasing salinity, suggesting that its ability to partition  $\text{Na}^+$  between leaves is less at higher salinities. However, this might have been a reflection of decreased total (young plus old)  $\text{Na}^+$  concentrations with salinity (they tended to decrease in older leaves but were kept more or less constant in the younger ones). No other clear tendencies or differences between varieties were found when studying the ratios in concentrations between leaves (data not shown).

The changes in  $\text{Na}^+$  concentrations with time in a given leaf (number 4, or number 6) can be seen if comparisons are made across sampling dates. Quantitatively, this is the difference between concentrations in the same leaf with time. These values were positive (increased concentrations with time) in almost all occasions, but this increase was not proportional to the level of salinity (correlations not significantly different from zero, data not shown). Because of the large size of the standard errors, no overall differences between varieties (mean over all treatments) were found for this trait either (data not shown).

c) Potassium (Table 4.2.1c and Figure 4.2.9):

Most correlations were either not significantly different from zero or very low; that is,  $\text{K}^+$  concentrations did not change very much in response to salinity. Wherever there was a significant correlation, it was negative for young leaves ( $\text{K}^+$  decreased with salinity) and positive for old leaves ( $\text{K}^+$  increased with salinity). In all cases, Chevron was the variety with lowest  $\text{K}^+$  concentrations, while CM-67 had the highest levels and Albacete was intermediate. The flag leaf had, in general, slightly higher  $\text{K}^+$  concentrations than earlier young leaves.

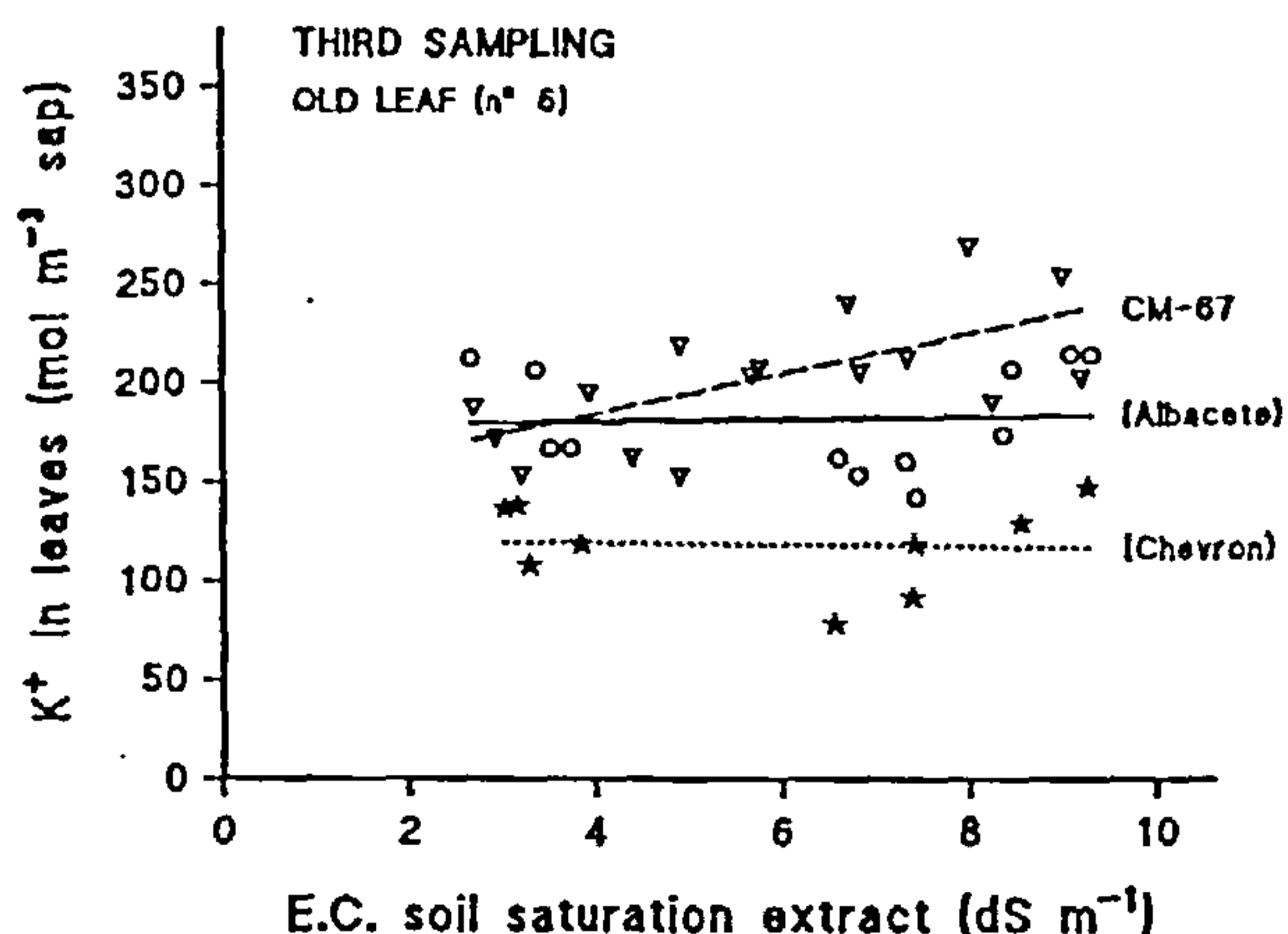
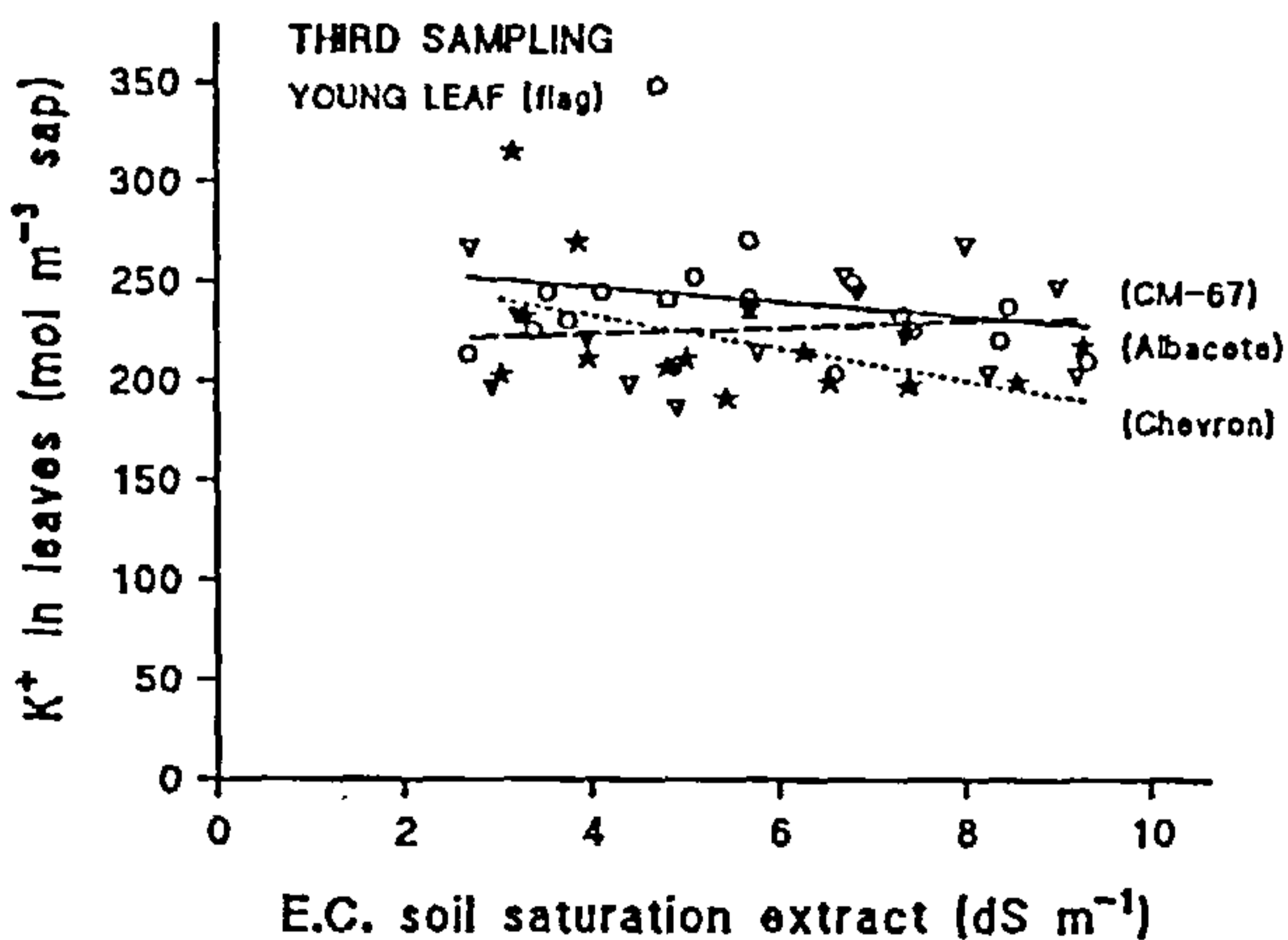
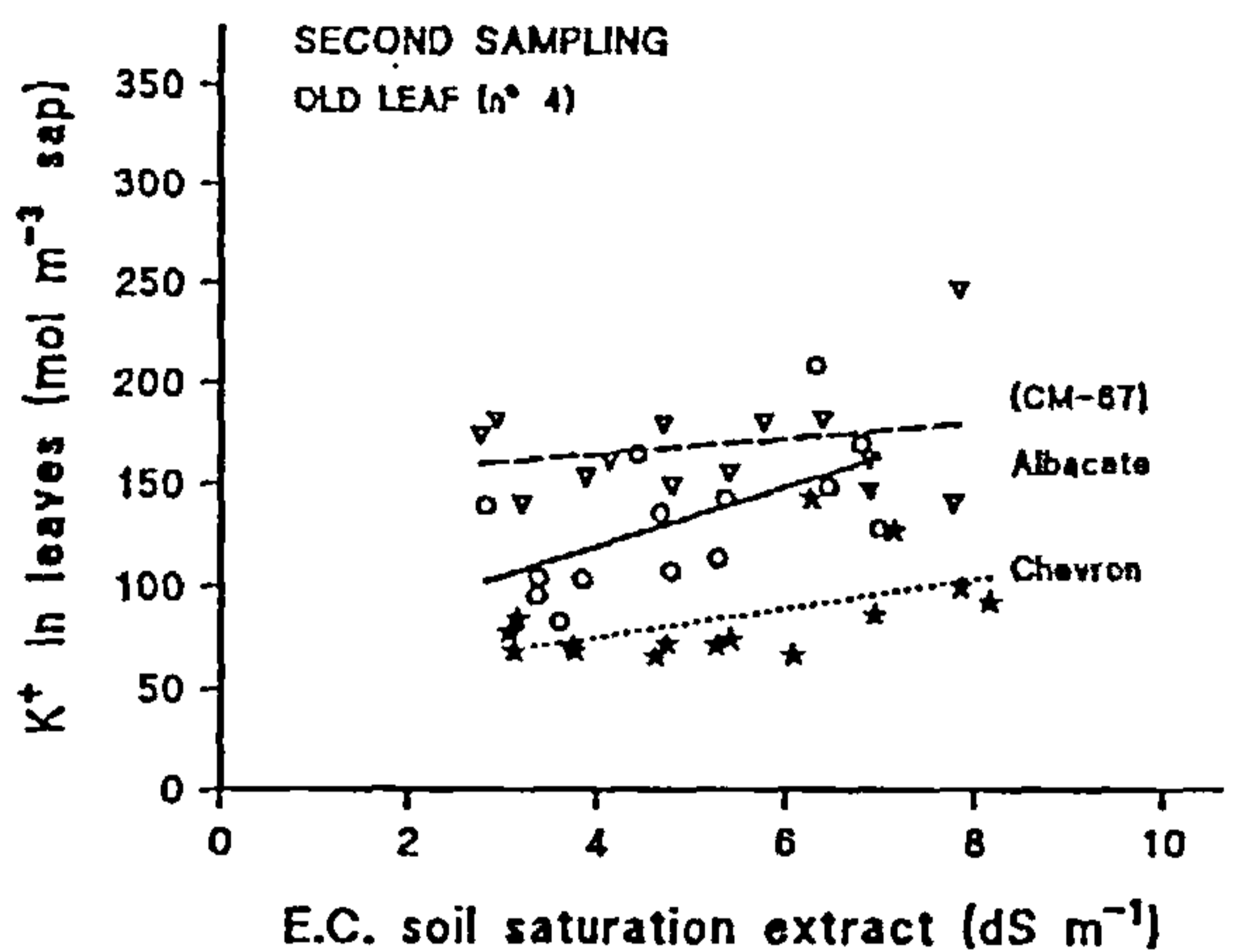
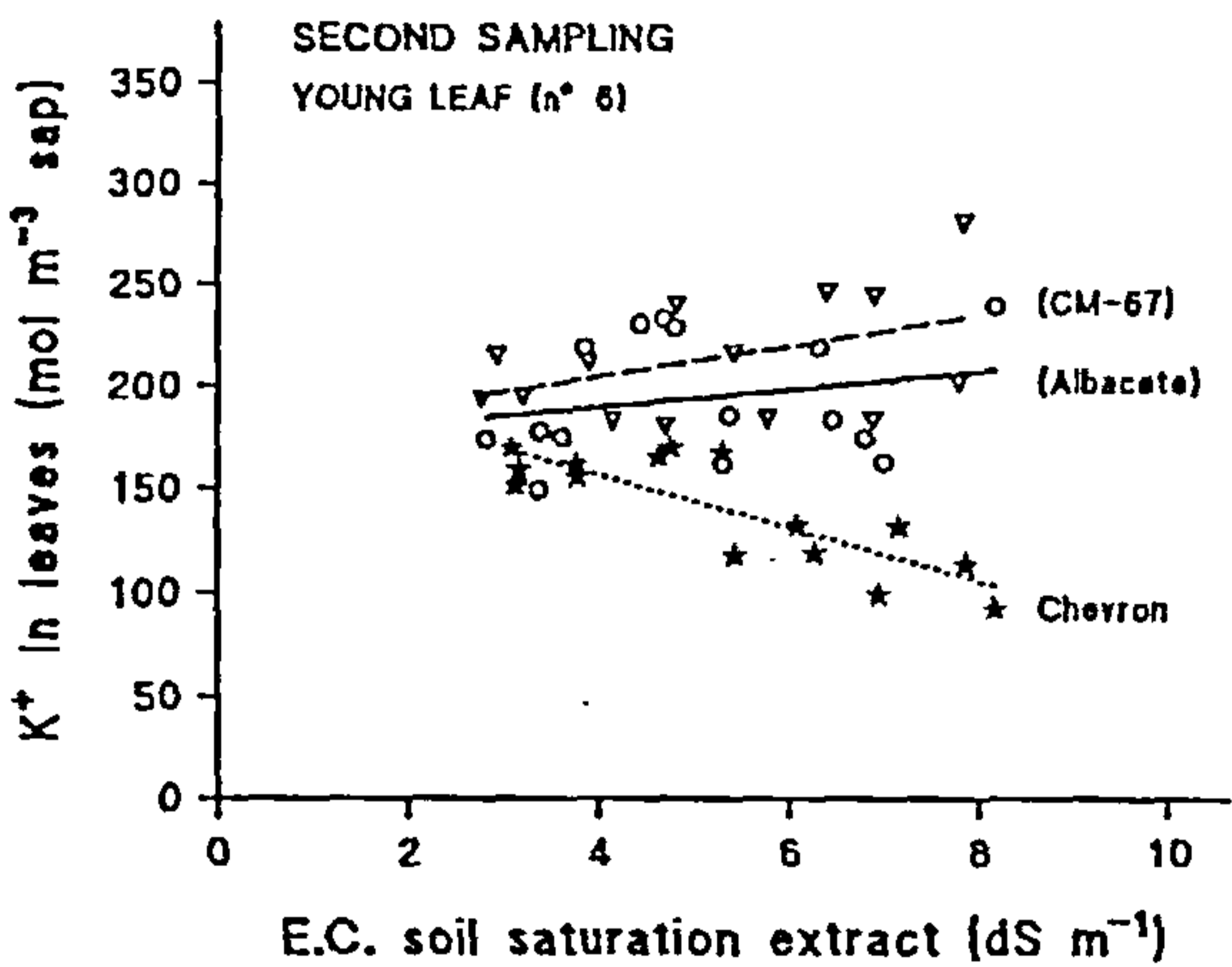
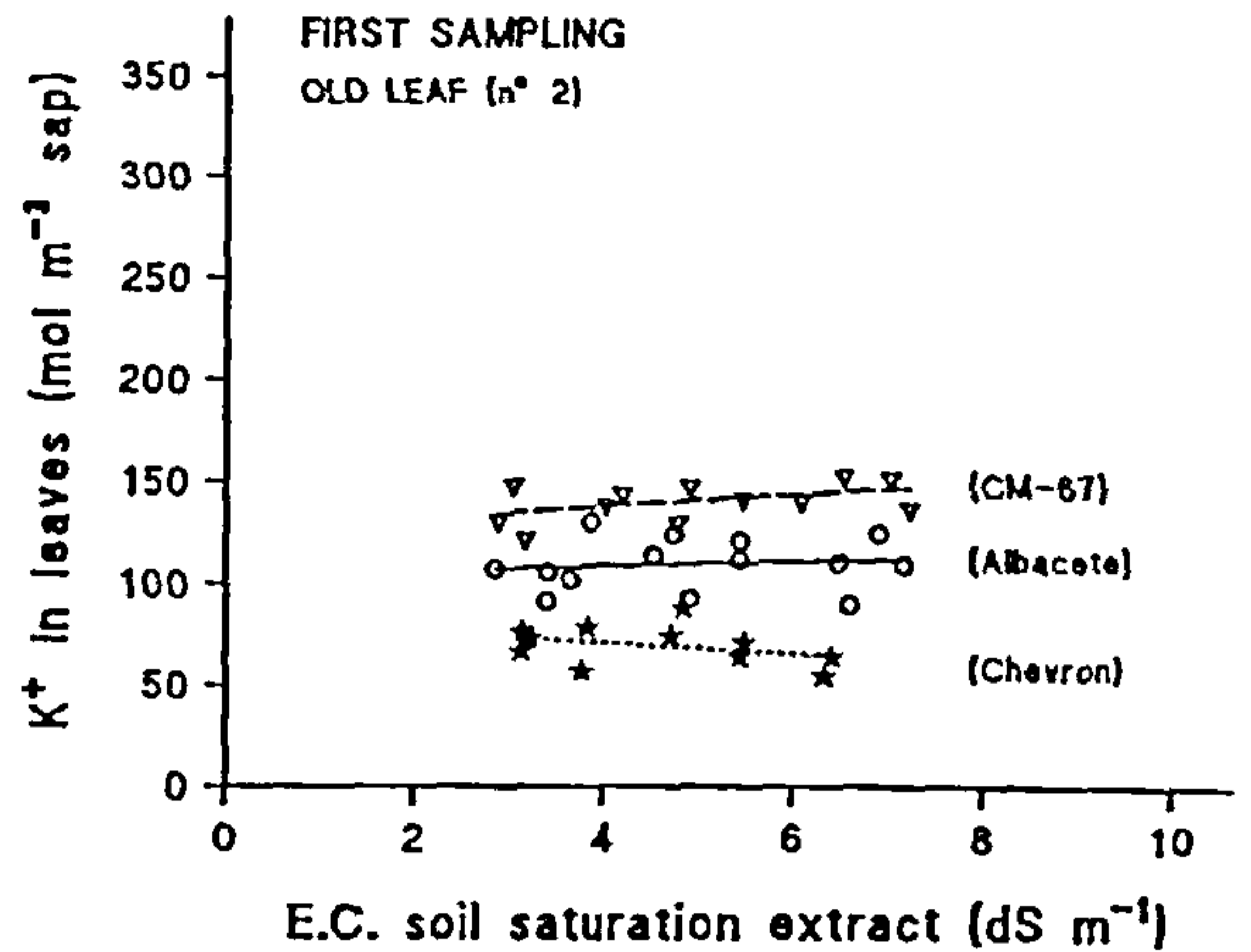
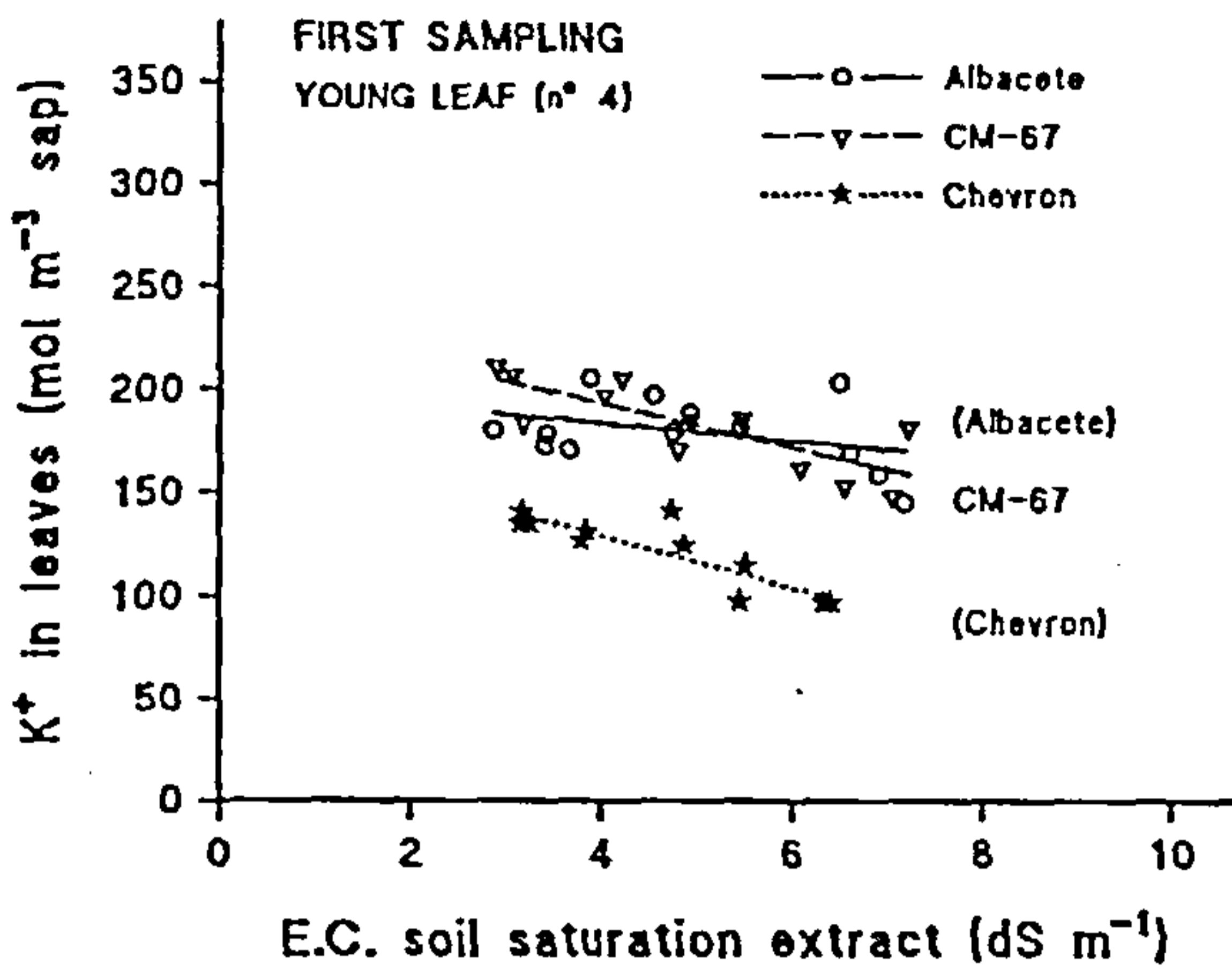


Figure 4.2.9.  $K^+$  concentrations in leaves of plants in the TLS (1992) as a function of soil salinity. A variety name in brackets indicates a non-significant correlation.

In general, concentrations of  $K^+$  were higher in younger than in older leaves, both when comparing leaves of different ages for a given sampling date, or when considering the evolution of the same leaf with time (successive samplings). However, as in the case of absolute  $K^+$  concentrations, most of these changes were not dependent on the level of salinity (correlations not significant, data not shown). No new information, regarding differences between varieties, was obtained when considering these traits, because of large variability within variety, and inconsistent responses between sampling dates.

#### 4.2.3.3. Grain yield:

As with the results for ions, the 2 replicates of each variety were first treated separately to see if there was any "block" effect. No significant differences were found between the 2 replicates of each variety (data not shown), so they were pooled together.

The 3 models (linear regression, threshold and sigmoidal) were fitted to the data for each variety, and they were compared by calculating the coefficient of determination ( $r^2$ ) between observed and fitted values. All models gave quite good fits ( $r^2 > 0.77$ ), but in all cases the sigmoidal curve had higher  $r^2$  than the other models (Table 4.2.2).

Table 4.2.2. Coefficient of determination ( $r^2$ ) for observed vs fitted values of the response models (yield vs soil salinity) in the TLS 1991/92 experiment. All correlations are highly significant ( $p < 0.001$ ).

VARIETY	model 1*	model 2*	model 3*
Albacete	0.894	0.921	0.933
Chevron	0.771	0.765	0.783
CM-67	0.961	0.968	0.975

\* model 1: linear regression; model 2: threshold; model 3: sigmoidal curve.

The residuals of the different models were also studied by plotting them against soil salinity. In general, no trends were observed in any of the models (data not shown), except for variety Albacete when the simple linear regression was fitted: the residuals were larger at extreme salinities (low and high) than at intermediate ones. This might have been expected, since yield responses to changing salinities are attenuated at the two extremes of the scale. This finding also suggests that models 2 and 3 are better, because they take into account these decreased responses at low and high levels. These 2 models are discussed next. Results are presented in Figure 4.2.10.

In the Maas and Hoffman (1977) model the varieties are defined by their salinity threshold ( $EC_0$ ), their slope ( $s$ ) and their maximum yield ( $Y_m$ ); (Figure 4.2.10a). The values of these parameters for each variety are shown in Table 4.2.3. No significant differences in thresholds were found between varieties; however, Chevron had a lower ( $p < 0.001$ ) maximum yield and slope than CM-67 or Albacete.

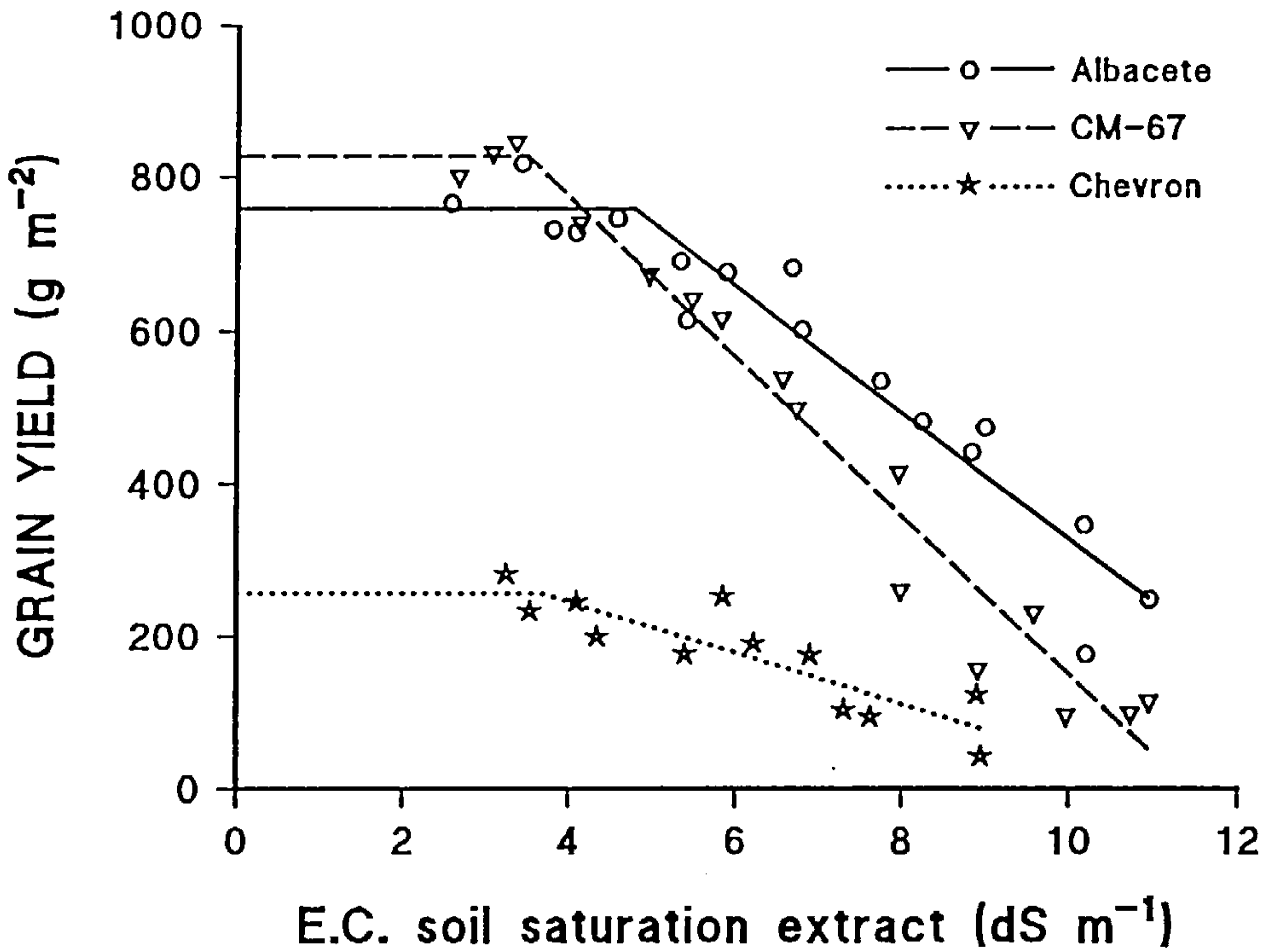
Table 4.2.3. Values of the parameters ( $\pm$  standard errors) that define the varieties' response to salinity, according to model 2, in the TLS 1991/92 experiment. Units: yield ( $Y_m$ ) in  $g\ m^{-2}$ ; salinity ( $EC_0$ ) in  $dS\ m^{-1}$ ; slope in  $g\ m^{-2}$  per  $dS\ m^{-1}$ .

VARIETY	slope	threshold	max. yield
Albacete	$82.2 \pm 9.2$	$4.76 \pm 0.50$	$758 \pm 25$
Chevron	$33.5 \pm 8.5$	$3.65 \pm 1.12$	$257 \pm 28$
CM-67	$103.8 \pm 7.8$	$3.48 \pm 0.43$	$827 \pm 31$

In the sigmoidal curve model the varieties are defined by their  $EC_{50}$  (salinity at which yield is reduced to half), their  $Y_m$ , and the exponential coefficient ( $p$ ) (Figure 4.2.10b). The values of these parameters for each variety are shown in Table 4.2.4. Again, Chevron had a  $Y_m$  lower ( $p < 0.001$ ) than Albacete or CM-67. In this case, however, Albacete also had a higher ( $p < 0.01$ )  $EC_{50}$  than the other 2 varieties; that is, it was able to withstand higher salinities before its maximum yield was reduced by 50%. No significant differences between varieties were found in the parameter  $p$ .



## a) Maas and Hoffman (1977) model



## b) Van Genuchten (1983) model

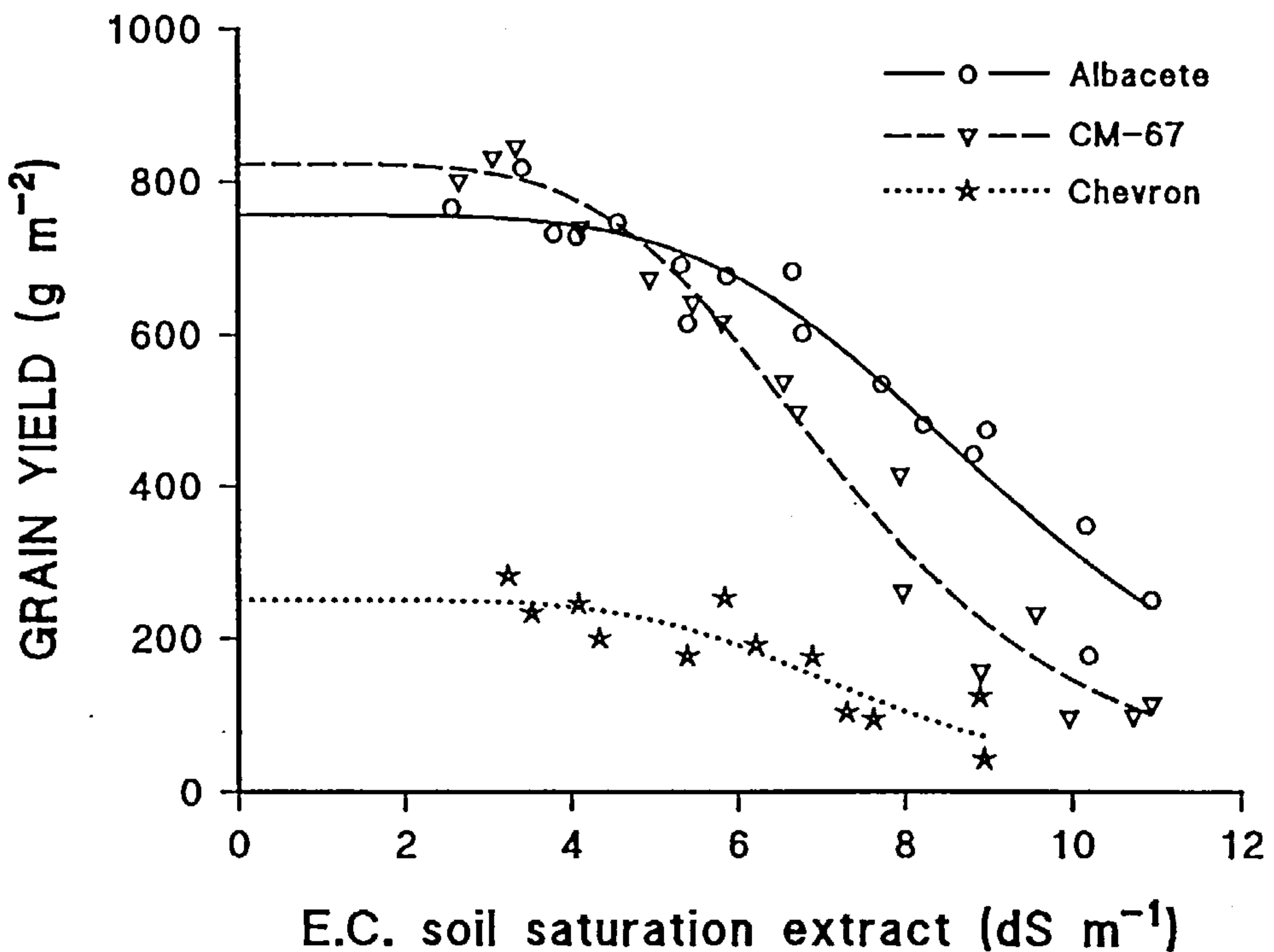


Figure 4.2.10. Actual yields (symbols) and fitted models (lines) of plants in the TLS (1991/92 season); a) threshold model; b) sigmoidal model.

Table 4.2.4. Values of the parameters ( $\pm$  standard errors) that define the varieties' response to salinity, according to model 3, in the TLS 1991/92 experiment. Units: yield ( $Y_m$ ) in  $g\ m^{-2}$ ; salinity ( $EC_{50}$ ) in  $dS\ m^{-1}$ ; p (exponent) has no dimensions.

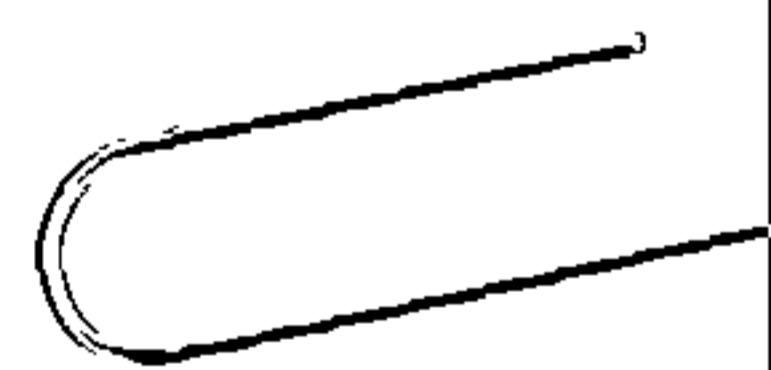
VARIETY	$EC_{50}$	p-exp	max. yield
Albacete	$9.32 \pm 0.26$	$4.80 \pm 0.8$	$756 \pm 27$
Chevron	$7.50 \pm 0.52$	$5.21 \pm 2.1$	$250 \pm 26$
CM-67	$7.27 \pm 0.20$	$4.82 \pm 0.5$	$823 \pm 29$

Yield results with increasing salinity can also be expressed relative to their yield without salinity. In this way, the responses of the varieties to salinity can be compared without the bias introduced by differences in yield potential. In this experiment, because of the good fits of the two models, maximum yields were taken to be those calculated by the program ( $Y_m$ ). The response of the varieties to salinity in terms of relative yields (expressed as percent of the maximum yield) is shown in Figure 4.2.11. It can be seen there that the responses of CM-67 and Chevron, when expressed in this way, were very similar, while Albacete was slightly better (higher relative yields) in both models.

#### 4.2.3.4. Relationships between leaf ion concentrations and yield:

One way to study if there is any close relationship between the two types of trait is by plotting the yields of each variety as a function of their corresponding ion concentrations. This was done for all ions, leaves, and sampling times, using both absolute and relative yields. No clear trends were found. As an example, this kind of relationship is shown in Figures 4.2.12 and 4.2.13, for  $Cl^-$  concentrations in young and old leaves at the second sampling. If yield was a simple function of  $Cl^-$  concentrations in leaves, we would expect similar yields for the different varieties at a given level of  $Cl^-$ . This is not the case, even when differences in yield potential (maximum yield) are taken into account (Figure 4.2.13).

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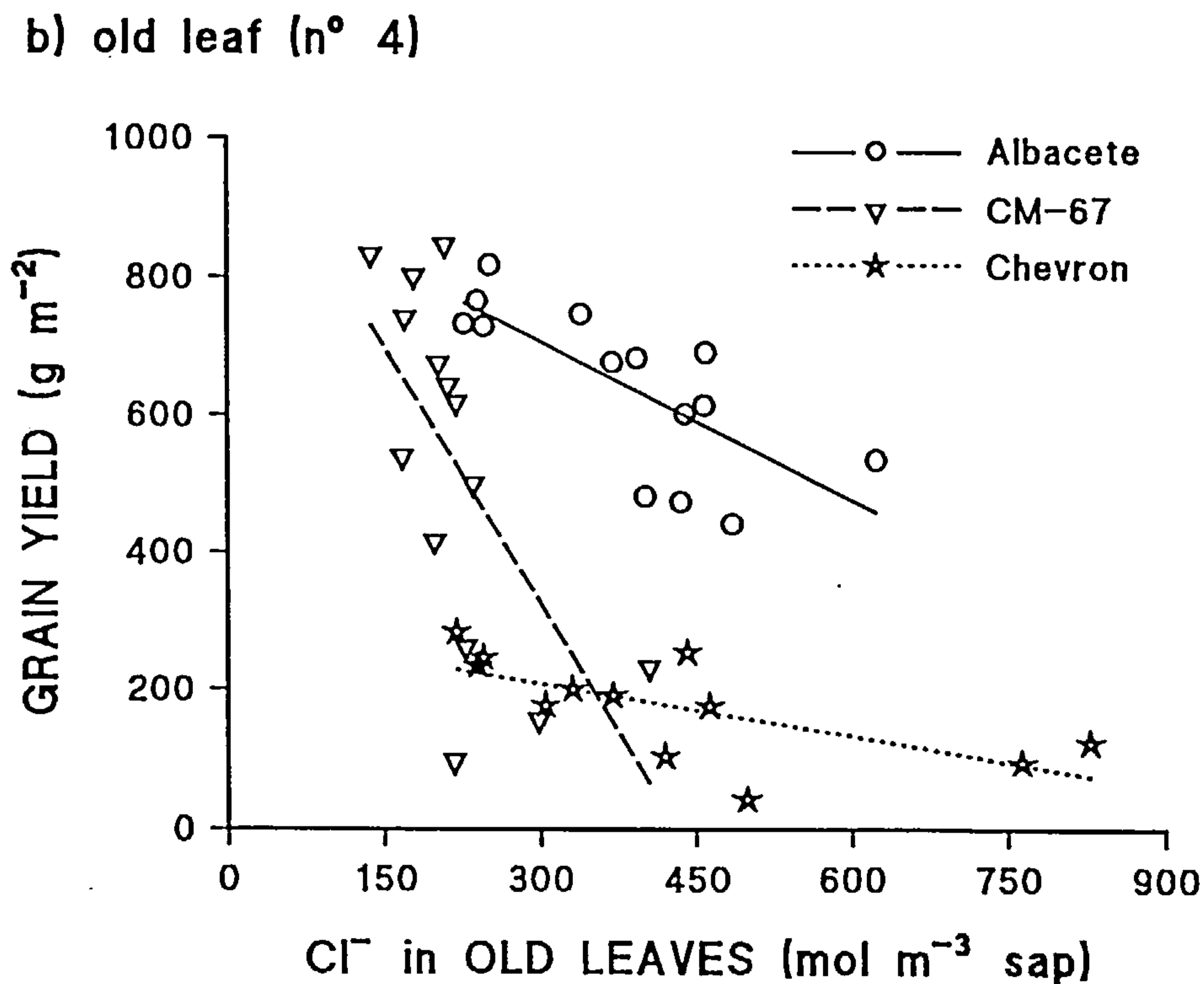
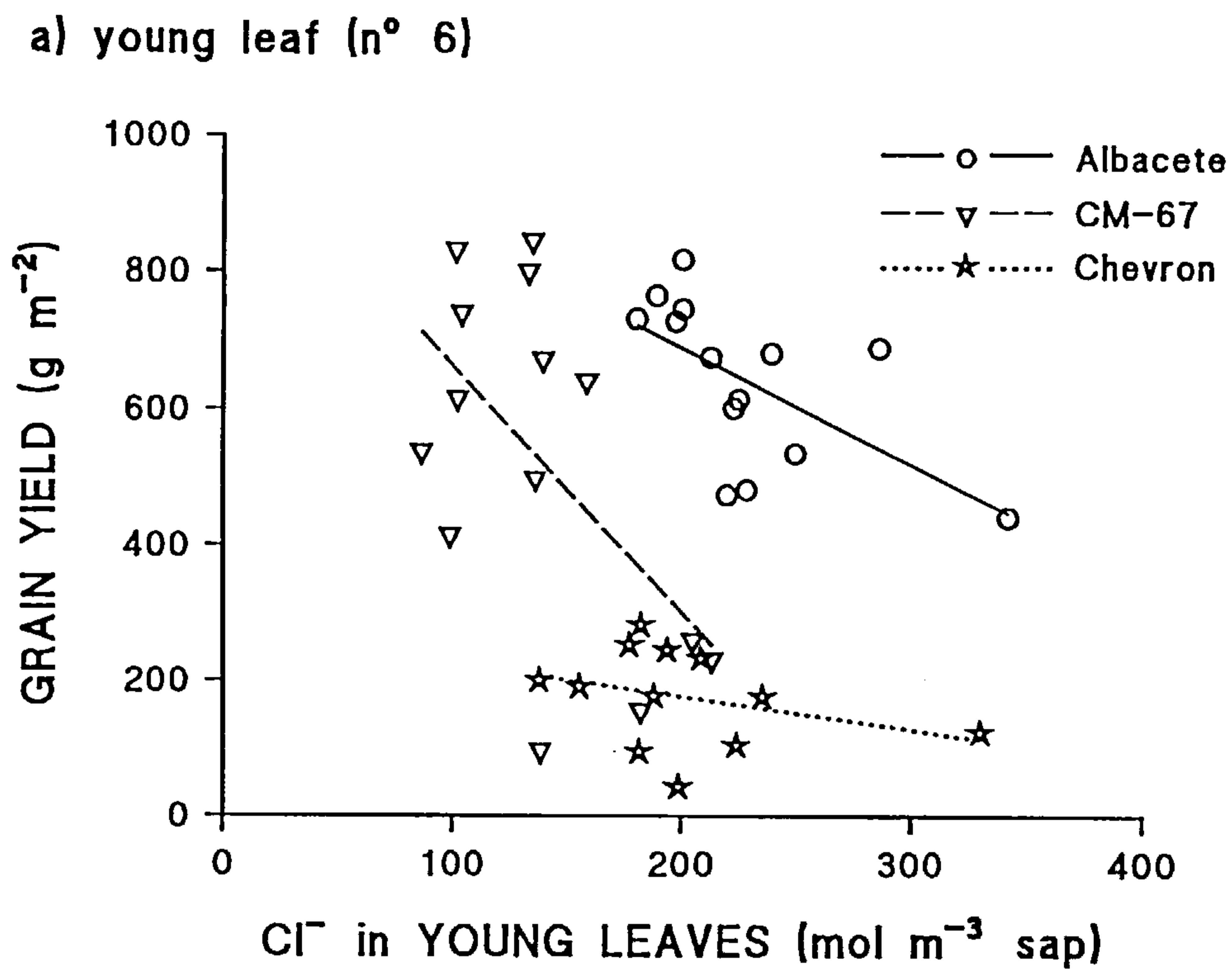
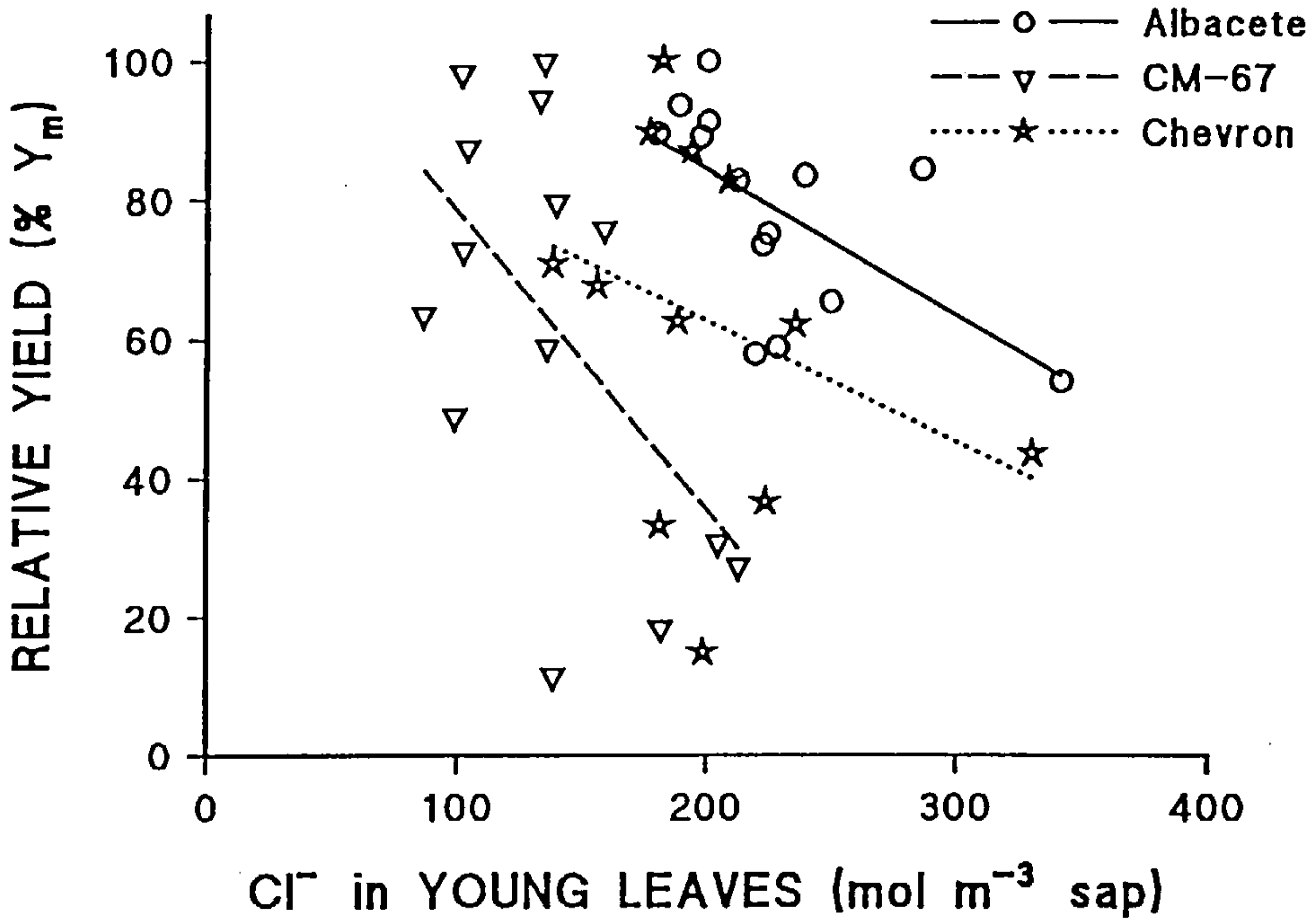


Figure 4.2.12. Grain yield as a function of  $\text{Cl}^-$  concentrations in young (a) or old (b) leaves in the second sampling time; (TLS, 1991/92 experiment).

a) young leaf (n° 6)



b) old leaf (n° 4)

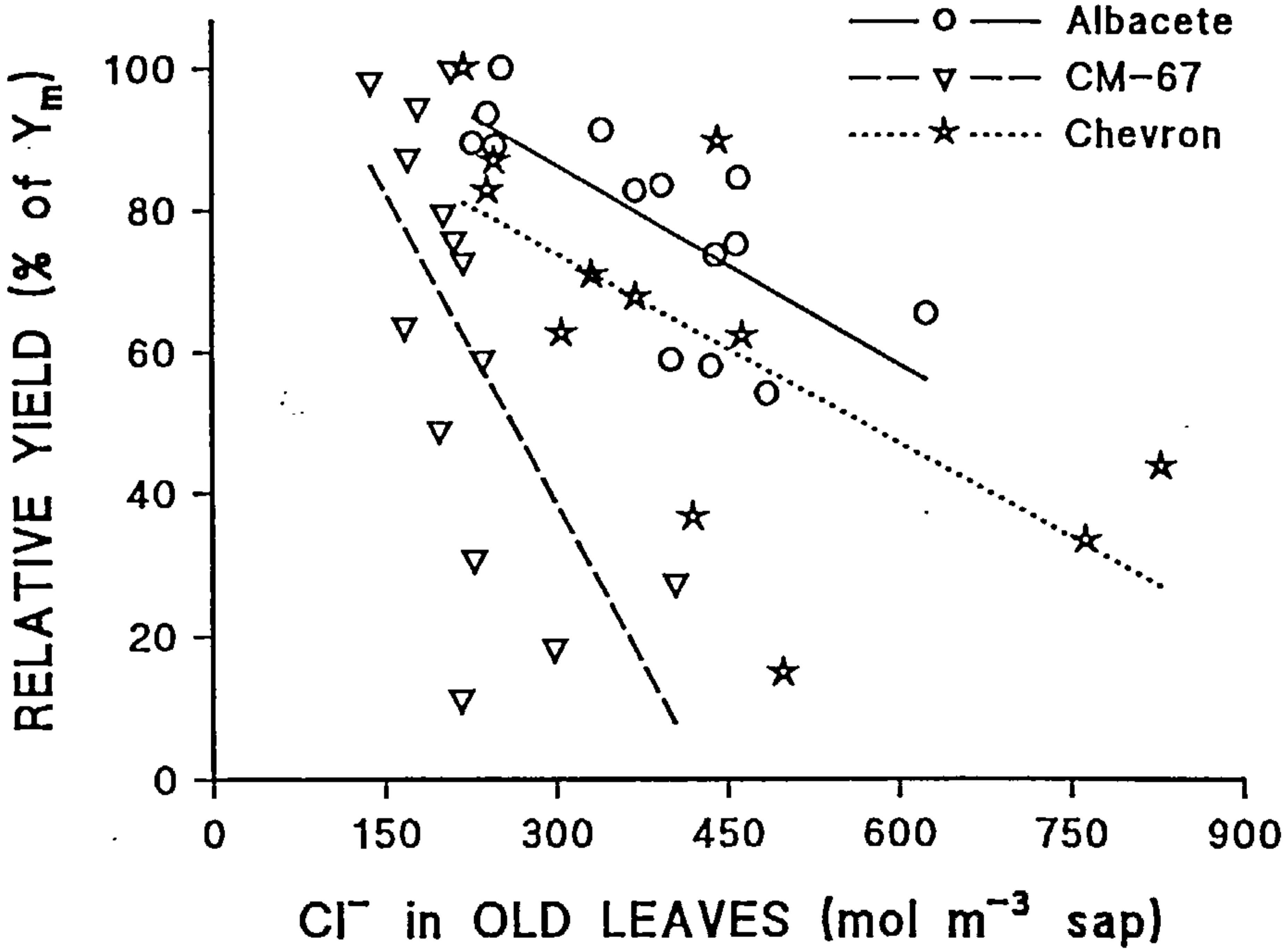


Figure 4.2.13. Relative yield as a function of Cl<sup>-</sup> concentrations in young (a) or old (b) leaves in the second sampling time; (TLS, 1991/92 experiment).

In some cases, correlations of opposite sign were found for different varieties in the same trait. This was the case, for instance, of  $\text{Na}^+$  concentrations in older leaves, where increasing amounts of  $\text{Na}^+$  corresponded with lower yields in Chevron, but were associated with higher yields in CM-67. However, the existence of correlations does not prove a causal relationship. In this example, the results are only reflecting the opposite responses of  $\text{Na}^+$  to increasing salinity in these 2 varieties. Since yield decreased with salinity in all cases, when it is plotted against any trait which is highly correlated with salinity (e.g.  $\text{Cl}^-$ ), it will exhibit the same kind of response. Consequently, these new plots of yield vs ion concentrations in most cases are just describing the same effect of salinity on yield, but in another (indirect) way.

#### 4.2.4. DISCUSSION:

##### 4.2.4.1. Soil and water salinity distribution:

The distribution of water and salinity by means of the TLS was quite good, as measured from the water collected in the rain-gauges after each irrigation (Figure 4.2.4). The smaller quantity of water received by the plots at the outer side of the TLS (treatments 1 to 3) can be attributed to the prevailing wind, which blows almost perpendicular to the sprinkler lines. This results not only in the lower precipitation observed at those treatments, but also in the salinity gradient having its maximum slightly displaced to the left of the central line (Aragüés *et al.*, 1992). Hence the importance of irrigating only in days with very low wind.

Distribution of salinity in the soil, measured as the weighted mean down to the first 1 m (as integrated by the EMS), was also quite good across treatments (Figure 4.2.3). If the higher salinities (due to their position in the unleached area) in Replicate I of CM-67 and Chevron are not considered, differences between similar plots along the TLS were larger at low than at high salinities. This might be partly due to the more irregular distribution of water (lower quantities) at that side of the TLS (see above).

Soil salinity increased with time (Figure 4.2.5), due to the continued irrigation with saline water. A relatively high salinity gradient across treatments was found in the early stages (first 3 readings with the EMS), after only 5 saline irrigations. In previous years it had been found that it took a longer time (10 to 15 irrigations) to develop an appropriate gradient (Aragüés *et al.*, 1992). This was why it was decided not to leach the soil between experiments, in order to begin each season with a higher soil salinity. That this approach did work can be seen in Figure 4.2.3, where the gradient across treatments was larger in the non-leached plots (Rep.I of CM-67 and Chevron) than in the leached ones.

The salinity distribution in depth (soil profile) was not very uniform (Figure 4.2.6). Although that figure is based on soil samples from a single plot for each treatment (no replication), it is still representative of what is commonly found in the TLS, (see Aragüés *et al.*, 1992). That is, only in the top 50 cm is salinity relatively high, showing substantial differences between treatments. At greater depths, salinity is rather low and similar for all treatments. To obtain more uniform profiles (vertical lines) the amount of saline water provided by irrigation should be increased, but this is limited by the rate of infiltration of the soil. No leaching between crops may also help, although this was not examined in this experiment; (the samples used for soil analyses came from a few plots located in different parts of the field, with no distinction between leached and non-leached; they were selected on the bases of the EMS readings in those plots).

Although some aspects of the TLS are still not satisfactory (such as the irregular salt distribution in depth, or the late establishment of the gradients) from a theoretical point of view it does provide what was expected from its design: a linear gradient of salinity with similar amounts of water across treatments. Other aspects of the validity of the TLS as an experimental tool will be discussed in more detail in Chapter 5.

#### 4.2.4.2. Ion concentrations in leaves:

In a system like the Triple Line, where saline water is applied by sprinkler irrigation, the concentrations of ions in leaves will be the result of salt absorption via the roots

(soil salinity) plus any absorption through the leaves that may occur by direct contact with the saline water. In order to minimize salt absorption through leaves, plants in the TLS are routinely pre-wetted with fresh water (3 min) prior to saline irrigation, and again at the end of each irrigation (3 min post-washing). When this experiment was done, there was some evidence indicating that ion absorption via the leaves was reduced by these pre- and post-washings (Adouni, 1991) and the subject of leaf absorption was not considered in great detail. However, some recent data (Aragüés *et al.*, 1994; Gorham *et al.*, 1994) indicates that the extent of ion absorption by leaves can still be considerable under the TLS. Although this source of salinity has been recognised as a cause of foliar injury and decreased yields (Maas *et al.*, 1982), little information is yet available to quantify these effects. This aspect will be further discussed in Chapter 5.

Of the ions determined,  $\text{Cl}^-$  had the highest positive correlations with soil salinity, while low or no correlations were found for  $\text{Na}^+$  and  $\text{K}^+$ . This lower response may be attributed to the high proportion of  $\text{Ca}^{2+}$  used in the irrigation water. If a higher Na:Ca ratio had been used (e.g, 20:1, as in some of the hydroponic experiments) an increase in  $\text{Na}^+$  concentrations with salinity, together with a decrease in  $\text{K}^+$ , might have been expected. However, with the 1:1 NaCl to  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  ratio (around 2:1 molar) the lack of response of  $\text{K}^+$  concentrations was, somehow, expected (see section 2.3.3).

Surprisingly, with the exception of Chevron (where  $\text{Na}^+$  concentrations increased with salinity) no significant changes in  $\text{Na}^+$  concentrations (Albacete), or even negative correlations (CM-67) with salinity were found. This could be another effect of the high  $\text{Ca}^{2+}$  levels used, since it has already been shown (section 3.4.3) that high  $\text{Ca}^{2+}$  in the solution decreases  $\text{Na}^+$  absorption. It may be speculated that with increasing salinity more  $\text{Ca}^{2+}$  was being taken into the plant by the roots in place of  $\text{Na}^+$ ; without measurements of  $\text{Ca}^{2+}$  concentrations, however, this cannot be demonstrated.

For all ions, differences between varieties were larger in older leaves than in younger ones. This probably reflects not only the ability for leaf-to-leaf partitioning



(sequestering of harmful ions in older leaves), but also the effect of ion absorption by leaves (prolonged exposure to saline irrigation by older leaves). It is worth stressing that partitioning between leaves is only possible for those ions which are absorbed by the roots and transported by the xylem. Transport of  $\text{Na}^+$  and  $\text{Cl}^-$  by the phloem is very limited (Munns *et al.*, 1986; Flowers & Yeo, 1988); thus, if these ions are absorbed directly by the leaf, they have less chance of being re-exported, and will remain there.

The flag leaf had remarkably low concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in all varieties, while its  $\text{K}^+$  concentrations were the highest of all leaves studied. It seems, therefore, that this leaf, which is very important for the later filling of the grain, is well protected from any excess of toxic ions. Greenway *et al.* (1965) also found lower concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in flag leaves of barley growing in  $125 \text{ mol m}^{-3} \text{ NaCl}$ , compared to other (young) leaves developed earlier.

Similar results (very low  $\text{Na}^+$  and relatively high  $\text{K}^+$  in the top 2 leaves) were also found by Wolf *et al.* (1991) when studying  $\text{Na}^+$  and  $\text{K}^+$  fluxes along the stem and into different leaves of salt-treated barley. These authors observed that the composition of the xylem sap changed along the stem, with concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  decreasing and those of  $\text{K}^+$  increasing (to a lesser extent) as the sap ascended. These gradients in concentration in the xylem sap would result in a low import of  $\text{Na}^+$  and  $\text{Cl}^-$  in the top leaves, and a higher supply of  $\text{K}^+$ . (Retranslocation of  $\text{K}^+$  from older leaves was also an important component of  $\text{K}^+$  supply to the top leaves.) The model proposed by Wolf *et al.* (1991) fits well with the observed low  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations in flag leaves in the present experiment.

It is important to remark that, because the flag leaf (as all other "young" leaves considered) was sampled when it was just fully emerged (appearance of the ligule), it had not been directly exposed to saline irrigations for any significant length of time (maybe 1 or 2 irrigations). Thus, it can be assumed that the (low) ion concentrations found in the flag leaves resulted largely from root absorption and transport along the stem; foliar absorption was probably very small at that time. It is in these conditions that the above model can be applied.

Although those authors used only one barley variety (salt-tolerant California Mariout), it seems from the present results that the restricted import of  $\text{Na}^+$  and  $\text{Cl}^-$  into the flag leaf is a rather widespread feature, since the same pattern was found in the salt-accumulating cultivar Chevron. This mechanism of protection of the flag leaf may be of significance for the later development of the ear and grain.

Unfortunately, the flag leaf was only sampled as a "young" (recently expanded) leaf in this experiment. A later sampling, after having received several saline irrigations, would have provided information on the degree of salt absorption by flag leaves. It would be interesting to see if any mechanism exists to protect the flag leaves against high salt concentrations which can arise from direct foliar absorption. At the moment there is no evidence to suggest that this is the case. In fact, data from Grattan *et al.* (1994) in a TLS experiment seem to indicate the opposite. In flag leaves sampled after the ears had emerged (i.e. after they had been sprinkler-irrigated with saline water for some time),  $\text{Cl}^-$  concentrations at increasing levels of salinity were significantly higher than in the corresponding leaves of plants which had received saline water only through the roots.

Comparing the different sampling times and leaves, it can be concluded that early sampling (during tillering) is the best time to detect differences between varieties. In the flag leaf these differences were reduced to a minimum. Although older leaves exhibited larger differences between varieties than younger ones, the added effect of salt absorption by leaves may obscure the interpretation of results.

Due to the type of salinity used in the TLS,  $\text{Cl}^-$  is the ion which more readily responds to increasing salinity (since it is the predominant one in the mixture of salts). Larger differences between varieties may, thus, be expected to be found (as it was here) when comparing their  $\text{Cl}^-$  concentrations.

#### 4.2.4.3. Grain yield:

All three models adjusted to the yield data fitted the results very well, even the simplest one (linear regression). This is because the lowest levels of salinity applied

were already relatively high ( $> 2.5 \text{ dS m}^{-1}$ ), and not many data-points were available at salinities below the threshold. Had there been more observations at lower salinities, the existence of a threshold might have been more apparent, and then the linear model would have resulted in poorer fits. As a comparison, Richards *et al.* (1987), expressing grain yield of barley as a simple linear function of soil salinity (no threshold), obtained values for the coefficient of determination ( $r^2$ ) ranging from 38% to 86% (average 71%); that is, much lower than here.

With the threshold model of Maas & Hoffman (1977) Chevron had a lower maximum yield than Albacete and CM-67, but also a smaller slope (less decrease in yield with increasing salinity). In relative terms, this smaller slope might have compensated for its lower maximum yield. In fact, if relative yields are plotted (Figure 4.2.11a), this appears to be the case: all varieties have very similar slopes. However, from an agronomic point of view, we have to consider the actual yields, and Chevron had very low yields in all treatments.

The poor performance of Chevron in the field is not only the result of salinity. This is an old variety, with a very long growing cycle, and large height (non-dwarf). It does not compare with the modern dwarf, high yielding varieties, particularly if grown with the same cultural methods. The high doses of N fertilizer applied to the field, which stimulate vegetative growth, result in problems of lodging in non-dwarf varieties like Chevron (all the others were dwarf). Strong winds in the site of the TLS also affect this cultivar more than the shorter ones, increasing the chances of lodging. Finally, its grains tend to shed from the ears before they are ripe; (old varieties were harvested before complete maturity). This shedding problem is also aggravated by the strong winds. In the present experiment, an additional accident affected this variety: a plague of ants infested the field just before harvesting, feeding on the grains of Chevron; (because of its late maturity, this was the only variety which still had soft grains at the time). All these factors contributed to the very low yields of Chevron, not only under salinity, but in all treatments.

No significant differences (at the 5% level) were found between the other two varieties using the threshold model. Still, Albacete had a moderately higher threshold

and a slightly smaller slope (see Figure 4.2.11a), which would indicate a higher tolerance. In spite of having a (not significantly) lower maximum yield, its overall yield (mean of all treatments) was higher than in CM-67. This is also an important feature from an agronomic point of view, since natural saline soils are very heterogeneous in their salinity distribution. In these conditions, a variety with a good overall performance might be more suitable than others which only respond well at low or high salinities.

Using the sigmoidal model of Van Genuchten (1983), Chevron also had a lower maximum yield than CM-67 and Albacete, but it did not differ significantly from them in the other parameters. The exponent  $p$ , (which relates to the shape of the curve and might be considered equivalent to the slope in the previous model), had a large standard error in Chevron, and this was the reason for the lack of significance. That large standard error was brought about by the poorer fit of the model in this variety (see Table 4.2.4).

Significant differences were found with the sigmoidal model for the 2 other varieties: Albacete had a higher  $EC_{50}$  than CM-67. This is not surprising, since this parameter integrates the two concepts of threshold and slope, without taking into account the absolute maximum yield. (Maximum yield is considered in the calculation of  $EC_{50}$ , but only in relative terms.) In this way, the small advantage that Albacete already had in the previous model, in terms of threshold and slope, is combined here in the  $EC_{50}$ , and results in significant differences. A slightly better fit of this model, compared to the threshold one, may have resulted in smaller standard errors for the estimates of the parameters, and thus helped in finding significant differences.

From the above results it can be seen that the choice of model may influence the conclusions relating differences between varieties. Royo *et al.* (1991) applied 4 different response models to their salinity data: the same 3 used here, plus a further, exponential one. They found that the threshold and sigmoidal models always gave the best fits, and that their results were very similar. They also concluded that the parameter which best estimates the salt tolerance of a variety is the  $EC_{50}$ , since it does not depend on the model used. (Values of  $EC_{50}$  estimated from different models

were very similar -not significantly different- between the models.) This is again due to the fact that the  $EC_{50}$  integrates the two parameters of threshold and slope needed to characterise a variety in the Maas & Hoffman (1977) model. (Notice that the  $EC_{50}$  can also be calculated from the threshold model. The advantage of the sigmoidal model is that it provides the estimate of the  $EC_{50}$  together with its standard error, and this is useful for the statistical comparison of the varieties.)

#### 4.2.4.4. Relationships between leaf ion concentrations and yield:

Considering the two types of data together (ions in leaves and grain yield) there does not seem to be a simple relationship between them. Albacete and Chevron both tended to have very high  $Cl^-$  and  $Na^+$  concentrations in their leaves, but the former had the highest yields and the latter the lowest ones. CM-67 had low ion concentrations (characteristic of this variety) but it was not more tolerant than Albacete.

As mentioned earlier (section 4.2.3.4), the existence of negative correlations between (some) ion concentrations and grain yield when studied over all salinity treatments (as in Figures 4.2.12 and 4.2.13) does not imply a causal relationship. In the present case it is only an indirect effect of the simultaneous change of both yield and ion concentrations with salinity. A more direct relationship might have been suggested by the existence of such correlations at a constant salinity. However, with only 3 varieties (3 data-points) this could not be investigated.

In summary, no direct relationship between leaf ion concentration and yield was found. However, this subject needs further testing, using a wider range of genotypes (three varieties is not enough). Also, the poor performance in the field of some varieties (like Chevron, a non-dwarf, late-ripening, old cultivar) calls for the use of better adapted genotypes. These two limitations were taken into account when planning the field experiment on the following year (see section 4.3).

### 4.3. 1992/93 FIELD (TLS) EXPERIMENT

#### 4.3.1. *OBJECTIVES:*

Using the 1991/92 results in the TLS, it was possible to decide on the best growth stage for sampling to detect differences in ion concentrations between varieties. In order to determine whether a good relationship exists between this trait and the varieties yield response to salinity a larger number of genotypes was included in the present experiment. The ultimate aim was to determine whether leaf ion concentrations is a trait closely related to the salt tolerance of a genotype, in which case it might be useful as a selection criterion in breeding programs.

#### 4.3.2. *MATERIALS AND METHODS:*

This experiment was done on the same site as the 1991/92 experiment. No leaching of salts with fresh water irrigation was done during summer 1992, so that at the beginning of the present experiment some residual salinity already existed in the soil. However, no data is available about the level of salinity at that time (no measurements were taken). Because some rain fell soon after sowing and during the first month ( $\approx 20$  mm), there was no need to give the first irrigations with fresh water to help seed germination and seedling establishment.

Cultural practices (seed bed preparation, fertilization, etc.) were the same as in 1991/92. On 21.11.92, twelve varieties of barley were sown by plot-drill in 10 plots along the salinity gradient of the TLS. Because of space limitations, only one replicate per variety was sown, and the elementary unit was also reduced in relation to the previous year. Plot size was still the same 1.20 m x 1.40 m, but 3 rows of 2 varieties were sown in each half of this plot (instead of 6 rows of the same variety). Previous research had shown that the response to salinity is largely independent of the plot size (Royo & Aragüés, 1993). Plant density was as in the previous year: 65 seeds/row, and 28 cm between rows. Sowing depth was about 3 cm.

The varieties used were: Albacete (AB), Barbarrosa (BR), Begoña (BE), Berta (BT), CM-67 (CM), Critter (CR), Forrest (FR), Igri (IG), Mogador (MO), Olivia (OL), Pané (PA), and Viva (VI). Albacete, Barbarrosa and Igri had been used as standard checks in the TLS in the 4 previous years. Most of the other varieties (except Forrest) had also been tested in the TLS at least once before. Their  $EC_{50}$ s for yield were calculated from their response to the EC of the applied water (irrigation corrected for rainfall), and they were compared to the mean of the 3 checks. According to this analysis, Begoña was rather sensitive, Berta and Olivia very tolerant, Pané and Viva quite tolerant, and CM-67, Critter and Mogador were similar to the checks; (Aragüés and collaborators, unpublished results).

Dates of each irrigation, together with amount of water applied and its range of salinity (measured on the rain-gauges closest to the plots), are given in Appendix 3. As in the previous year, some leakage of fresh water at the completion of the post-saline irrigation was observed at the pipe junctions in the central sprinkler line. Again, this resulted in a dilution of the soil salinity in treatment 10, which was subsequently discarded.

Measurements of soil salinity with the EM-38 were taken on 7 occasions, from late January until early June. At all those dates, except for the first one, (25 January), soil samples were taken to calibrate the readings obtained with the EMS. The samples were taken at 6 depth intervals down to 1 m, and the weighted mean was calculated as:

$$EC = \frac{18 \times EC_{(0-10)} + 14 \times EC_{(10-20)} + 20 \times EC_{(20-40)} + 12 \times EC_{(40-60)} + 7 \times EC_{(60-80)} + 5 \times EC_{(80-100)}}{76}$$

where the figures in brackets refer to depth intervals in cm.

The field was covered with netting from heading time until harvest. All varieties were harvested at maturity with a plot harvester (Hege 125), on 05.07.93.

The extensive sampling of the previous year had shown that the best growth stage for sampling to detect differences in ion concentrations between varieties was during vegetative growth, from tillering to stem elongation. This corresponded with the first two samplings in the 1991/92 season. For practical reasons (to space the sampling time evenly and to obtain larger leaves), the "first" sampling (i.e. leaves 2 and 4) was rejected, and the "second" sampling was chosen. Thus, leaf number 6 completely expanded was chosen as the young leaf, while leaf number 4 was sampled as an older leaf. The criterion used was, again, to sample a plot when the selected leaf (n° 6) was fully expanded (instead of trying to sample all varieties at the same time).

Four replicated samples per half-plot (elementary unit) were taken, each sample made up of several leaves of similar size from different plants (3 for leaf 6, 4 for leaf 4); this would provide enough sap to run the full range of chemical analyses automatically. For each variety, the samples came from plants in the same row within the plot. No other destructive samples were taken; so, at harvest the plots still had 3 complete rows of each variety. Leaves were put into plastic bags, taken to the lab, washed in distilled water, transferred into vials, and frozen. The sampling took place between the 9<sup>th</sup> and the 26<sup>th</sup> of March 1993; (detailed dates are presented in Appendix 4).

Frozen leaf samples were taken to Bangor, where sap was extracted and the ions determined. Chemical analysis was carried out using ion exchange chromatography (Dionex 2000i). For anions ( $\text{Cl}^-$ ,  $\text{NO}_3^-$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{SO}_4^{2-}$ ), the extracted sap was diluted (1/110) with 4.5% isopropanol. For cations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ), the dilution (1/85) was with de-ionized water. Details of the methods have already been given in previous sections (e.g. 2.2.2 and 2.3.2).

- *Statistical methods:*

Statistical analysis of the ion data was done, as in the previous year, by means of linear correlation between leaf ion concentrations (for each variety and leaf) and soil salinity. This was measured as the mean of the EM-38 readings, transformed to EC



of soil saturation extract, up to the 25<sup>th</sup> of March. Means of 4 replicated samples per plot were used in all calculations.

Whenever these correlations were significant ( $p < 0.05$ ), the regression lines (one for each variety) were compared for their slopes by fitting a regression model with an interaction term; this allows for different slopes (and also intercepts) for each variety. A significant F-statistic for the interaction term indicates different slopes (see Snedecor & Cochran, 1989). In order to separate the varieties by their slopes, a series of pairwise comparisons (t-tests) might be used. However, this is not recommended, since the overall significance level for the several tests combined is greater than the significance level of each separate test, and there is more chance of rejecting a true null hypothesis (i.e. of considering different two slopes which are equal). To overcome this problem, multiple comparison procedures can be used. These are similar to those used for comparing variety or treatment means following an analysis of variance, although not many have been developed for application to regression analysis. One such method is the Simultaneous Test Procedure described by Sokal and Rohlf (1969, pp 456-458). However, when a large number of varieties are to be compared, these methods usually yield a large number of overlapping groups and complicate the interpretation of the results.

An alternative is to use cluster analysis techniques, which divide the varieties into relatively homogeneous groups, so that varieties within a same group are similar and those from different groups are dissimilar. Cluster analysis has sometimes been used for comparison of variety means (e.g. Scott & Knott, 1974; Gates & Bilbro, 1978). A limitation of these methods (see Willavize *et al.*, 1980) is that no significance statements can be made, although in some cases a "probability scale" can be added (Jolliffe *et al.*, 1989). The main use of cluster analysis in this context is as a descriptive tool rather than as a formal test of hypotheses.

In the present case, when the preliminary test (regression with an interaction term) indicated the presence of significant differences between regression lines (i.e. different responses), two approaches were used to further compare the varieties. The main aim was to reduce the rather large number of varieties (12) to a smaller number

of groups of similar varieties. As a first approach, the values for the slopes and intercepts of the regression lines were first plotted on a bi-dimensional graph to examine any "natural" grouping of the varieties (those with similar slopes and intercepts). This was accompanied by cluster analysis of the slopes and intercepts. The distance between pairs of varieties was measured as the Euclidean distance between them, and similarity between groups was assessed using the centroid method, where the mean of each group is used to calculate the distance. The second approach was to use a method very similar to the "backwards elimination" from the multiple regression theory. This method is described below.

From a model which contains all (significant) varieties ( $n$ ), those which have the 2 most similar slopes are pooled as if they were only one. This results in a new, reduced model with  $(n-1)$  varieties. An F-test is then made between these two models, which tests for the significance of pooling two similar slopes in comparison to the full model (all varieties). A non-significant F is taken as an indication of two slopes not being significantly different. The next 2 most similar slopes are then pooled, and the new reduced model compared to the previous one. This proceeds until no more varieties (slopes) can be pooled (i.e. the F-statistic comparing the two models is significant). (It has to be noted that this method, as with the backwards elimination in multiple regression, has not a unique answer. It can happen that a variety with a slope value lying half-way between two groups could equally be classified with any one of them.)

In a second step, varieties with similar slopes were compared for their intercepts, using analysis of covariance (Ancova) as described in Snedecor and Cochran (1989). This method has the limitation that it assumes that the lines are parallel (i.e. only varieties with similar slopes can be compared). This is somehow reasonable, since in many cases there is not much point in comparing the intercepts of lines which have different slopes. However, the assumption of parallelism forces the lines to have exactly the same slope, and this changes their original intercept. The result is that the grouping obtained by comparing the means in the Ancova (which is equivalent to comparing the intercepts, since the lines are now parallel) does not always agree with

that obtained by cluster analysis, where the slopes and intercepts are considered simultaneously.

In those cases where there was no significant correlation between leaf ion concentrations and soil salinity (i.e. ion concentrations did not change with salinity), the varieties were contrasted by comparing their means. This was done with an Anova and, if this was significant, means were separated using Tukey's test. However, since the latter usually resulted in several overlapping groups, the means were also separated using the procedure described above for the comparison of slopes, supplemented with cluster analysis (centroid method). This results in a smaller number of non-overlapping groups, and facilitates the graphical representation of the results.

For the study of yield response to salinity, the 3 models used in the previous year (see section 4.2.2) were again fitted, and the coefficients of determination between observed and fitted values calculated to decide on the best fit. Varieties were then compared by means of t-tests (pairwise comparisons) applied to the parameters which define them under the different models.

The relationships between leaf ion concentrations and yield were studied by plotting the yield of each variety against ion concentrations in its leaves at a given salinity treatment. This was done to remove any bias due to the simultaneous change in yield and ion concentrations with increasing salinity.

### 4.3.3. RESULTS:

#### 4.3.3.1. Soil and water salinity:

Figure 4.3.1 shows the distribution of water and salinity applied with the TLS. Salinity decreased linearly ( $r^2=0.997$ ) with increasing distance from the central line (source of saline water), while the total amount of water received by the different

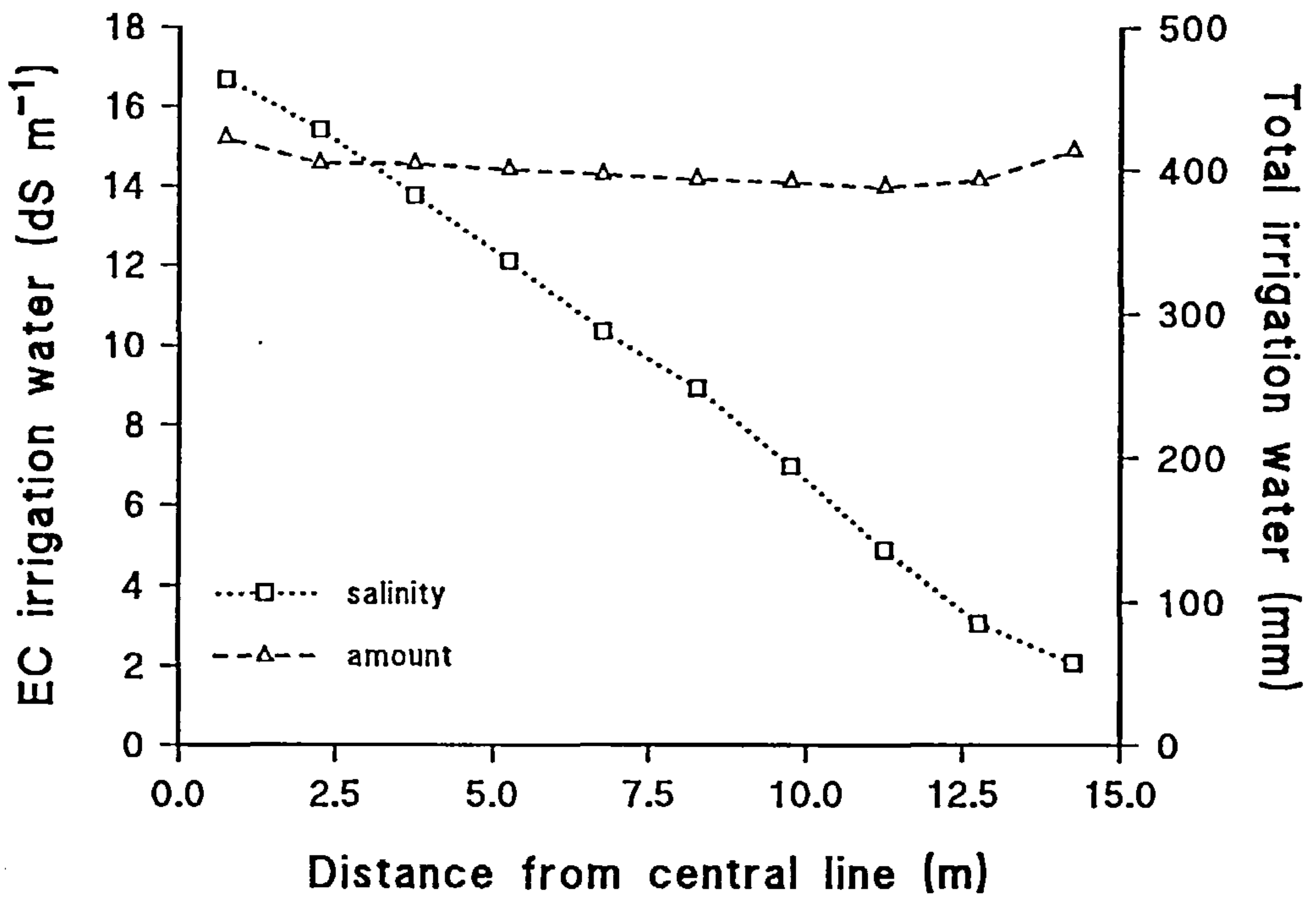


Figure 4.3.1. Salinity and amount of irrigation water (mean and standard errors of 31 irrigations) with distance from the source of saline water; (1992/93 season).

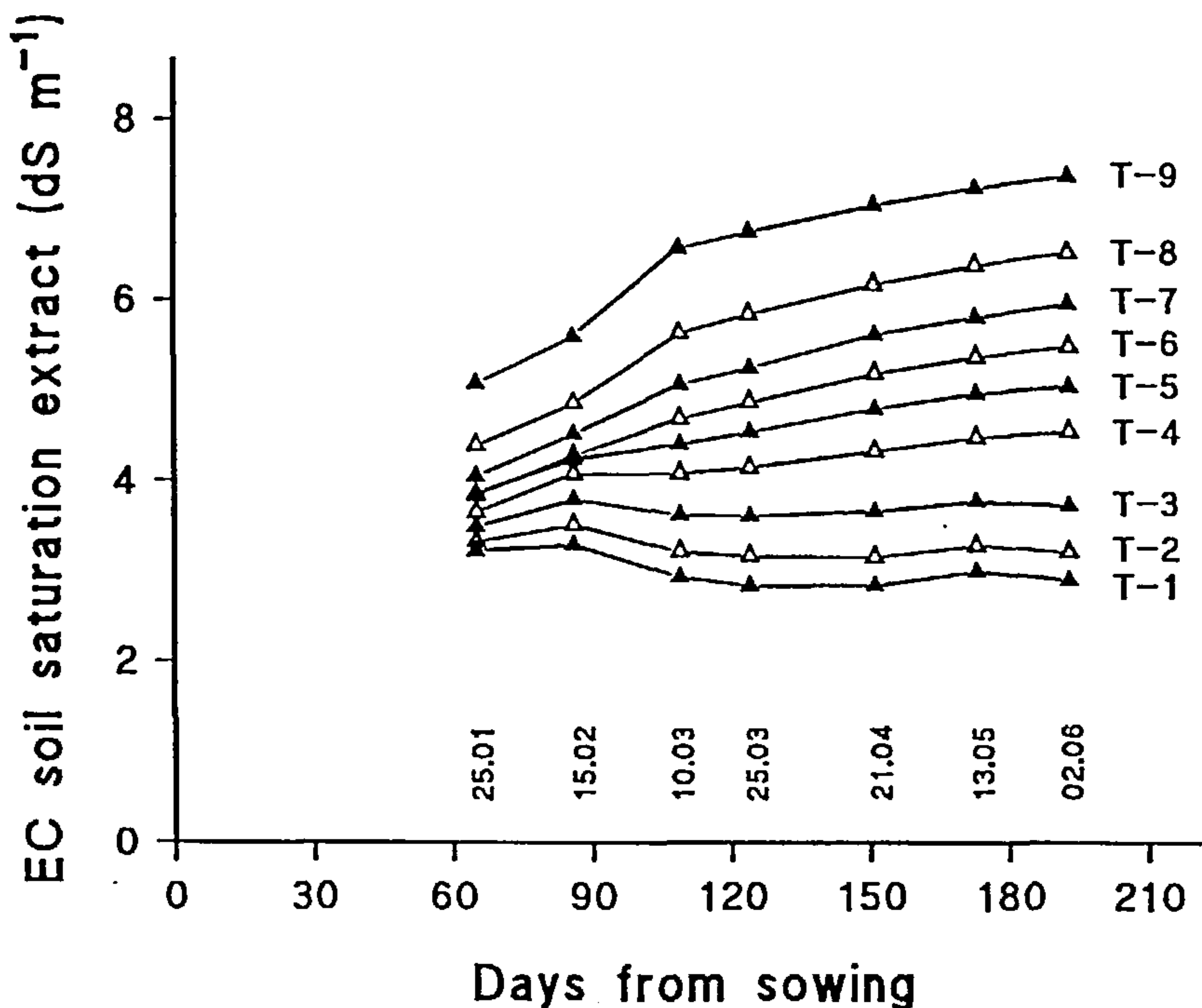


Figure 4.3.2. Evolution of soil salinity with time: values calibrated from the EM-38 readings; means of 6 plots per treatment; (1992/93 season).

treatments was very similar (c.v. = 5%). Differences between plots of corresponding treatments were quite small, as indicated by the standard errors in Figure 4.3.1.

Steady levels of salinity in the soil were achieved early in the season (Figure 4.3.2). From March onwards, soil salinity only increased slightly in the highest saline treatments. The apparent decrease in soil salinity from January until March in the low saline treatments (Figure 4.3.2) might be attributed to the more regular distribution of salts in the profile at that time (see Figure 4.2.6 in the TLS-1992 experiment), and the way the EMS measures soil salinity (integrating down to 1 m depth). However, these changes with time at the low saline treatments (T-1 to T-3) were probably not important, since the overall coefficient of variation was lower than 6%.

#### 4.3.3.2. Ion concentrations in leaves:

Ion concentrations were studied in relation to both soil salinity and irrigation water salinity. The varieties' response to these two measures of salinity was in general very similar. In a few cases, particularly when considering the older leaves, the correlations of ion concentrations with irrigation water salinity was slightly higher than with soil salinity; the differences, however, were not generally significant. The following results are based on correlations with soil salinity (mean EC of soil saturated extract up to the time of sampling).

##### a) Chloride (Table 4.3.1a and Figure 4.3.3):

Chloride concentrations in leaves increased with increasing salinity. Only in young leaves of variety Igri was this correlation not significant. The rate of increase (slope) was, in general, higher in older leaves than in the youngest ones.

Differences between varieties in young leaves were only found in the average  $\text{Cl}^-$  concentrations (different intercepts), not in the rate of  $\text{Cl}^-$  increase (slopes not significantly different) (Figure 4.3.3a). In older leaves varieties differed not only in their mean  $\text{Cl}^-$  concentrations, but also in the rate at which this ion changed with

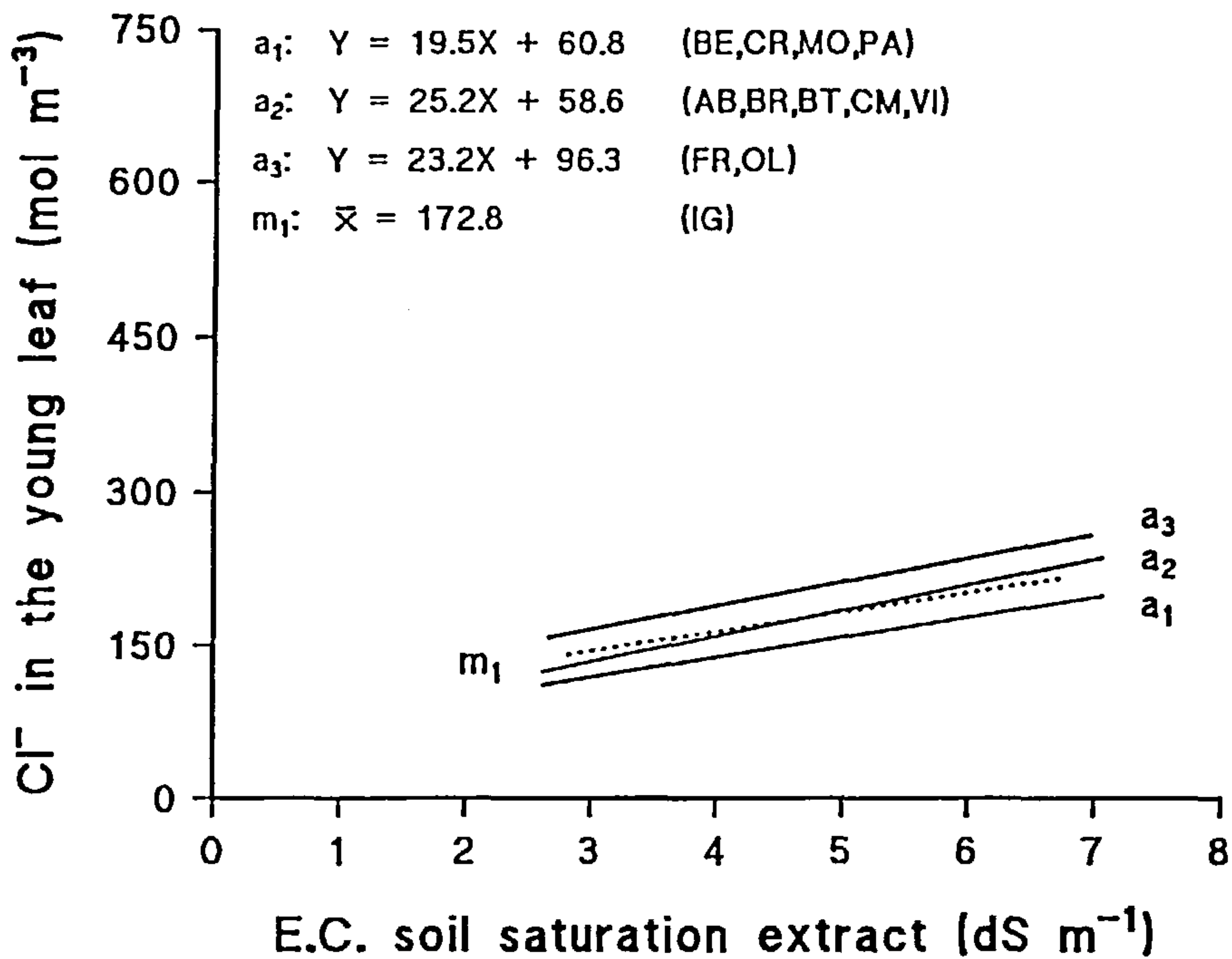
Table 4.3.1. Linear correlation (r) and regression coefficients between Cl<sup>-</sup> (a) and Na<sup>+</sup> (b) concentrations in leaves (young and old) and soil salinity (EC saturation extract) for the 12 varieties in the TLS 1992/93 experiment. Within each type of leaf, estimates of slopes and of intercepts with the same letter are not significantly different (see text). (Units: slopes in mol m<sup>-3</sup> ion per dS m<sup>-1</sup> soil salinity; intercepts in mol m<sup>-3</sup> ion.)

VARIETY	YOUNG LEAF (N° 6)			OLDER LEAF (N° 4)		
	r	slope	interc.	r	slope	interc.
<i>a) Chloride:</i>						
AB Albacete	0.790*	24.0 a	56.8 a <sub>1</sub>	0.891**	56.0 a	27.3 a
BR Barbarrosa	0.926***	32.5 a	30.2 a <sub>2</sub>	0.962***	73.4 b	-51.9 b
BE Begoña	0.807**	17.3 a	72.7 a <sub>1</sub>	0.901***	67.7 b	-28.0 b
BT Berta	0.980***	25.7 a	54.6 a <sub>2</sub>	0.956***	76.2 b	-65.5 b
CM CM-67	0.777*	26.1 a	49.5 a <sub>2</sub>	0.948***	80.1 b	-47.0 b
CR Critter	0.750*	20.3 a	67.4 a <sub>1</sub>	0.917***	50.8 a	33.4 a
FR Forrest	0.827**	25.8 a	88.7 a <sub>3</sub>	0.921***	107.7 c	-80.4 c
IG Igri	0.651 ns	19.1 +	87.3 +	0.823**	60.3 a	11.4 a
MO Mogador	0.684*	16.7 a	65.1 a <sub>1</sub>	0.929***	49.8 a	22.5 a
OL Olivia	0.839**	20.3 a	105.6 a <sub>3</sub>	0.961***	111.8 c	-109.1 c
PA Pané	0.879**	25.7 a	28.3 a <sub>1</sub>	0.891**	70.9 b	-54.9 b
VI Viva	0.802**	20.0 a	90.3 a <sub>2</sub>	0.939***	55.6 a	25.1 a
<i>b) Sodium:</i>						
AB Albacete	-0.054 ns	-0.35 +	89.9 +	0.247 ns	4.8 +	1045 +
BR Barbarrosa	0.856**	7.89 a	48.7 a	0.852**	10.3 a	70.3 a
BE Begoña	0.305 ns	1.31 +	57.5 +	0.857**	18.2 b	20.9 b <sub>1</sub>
BT Berta	-0.380 ns	-5.37 +	95.4 +	0.792*	21.3 b	25.9 b <sub>2</sub>
CM CM-67	-0.001 ns	-0.01 +	84.8 +	0.862**	20.1 b	56.6 b <sub>3</sub>
CR Critter	-0.133 ns	-1.22 +	72.3 +	0.843**	10.3 a	70.7 a
FR Forrest	0.420 ns	3.82 +	62.7 +	0.873**	33.1 c	-10.4 c
IG Igri	0.647 ns	5.10 +	60.1 +	0.600 ns	8.4 +	840 +
MO Mogador	-0.056 ns	-0.42 +	68.6 +	0.785*	7.5 a	77.1 a
OL Olivia	0.763*	4.55 a	68.6 a	0.955***	35.4 c	-1.6 c
PA Pané	-0.017 ns	-0.13 +	70.7 +	0.792*	18.1 b	47.7 b <sub>2</sub>
VI Viva	0.915***	8.98 a	39.6 a	0.920***	9.6 a	75.2 a

ns - not significant; \* - p < 0.05; \*\* - p < 0.01; \*\*\* - p < 0.001.

+ - coefficients not compared: correlation not significant.

## a) YOUNG LEAF



## b) OLD LEAF

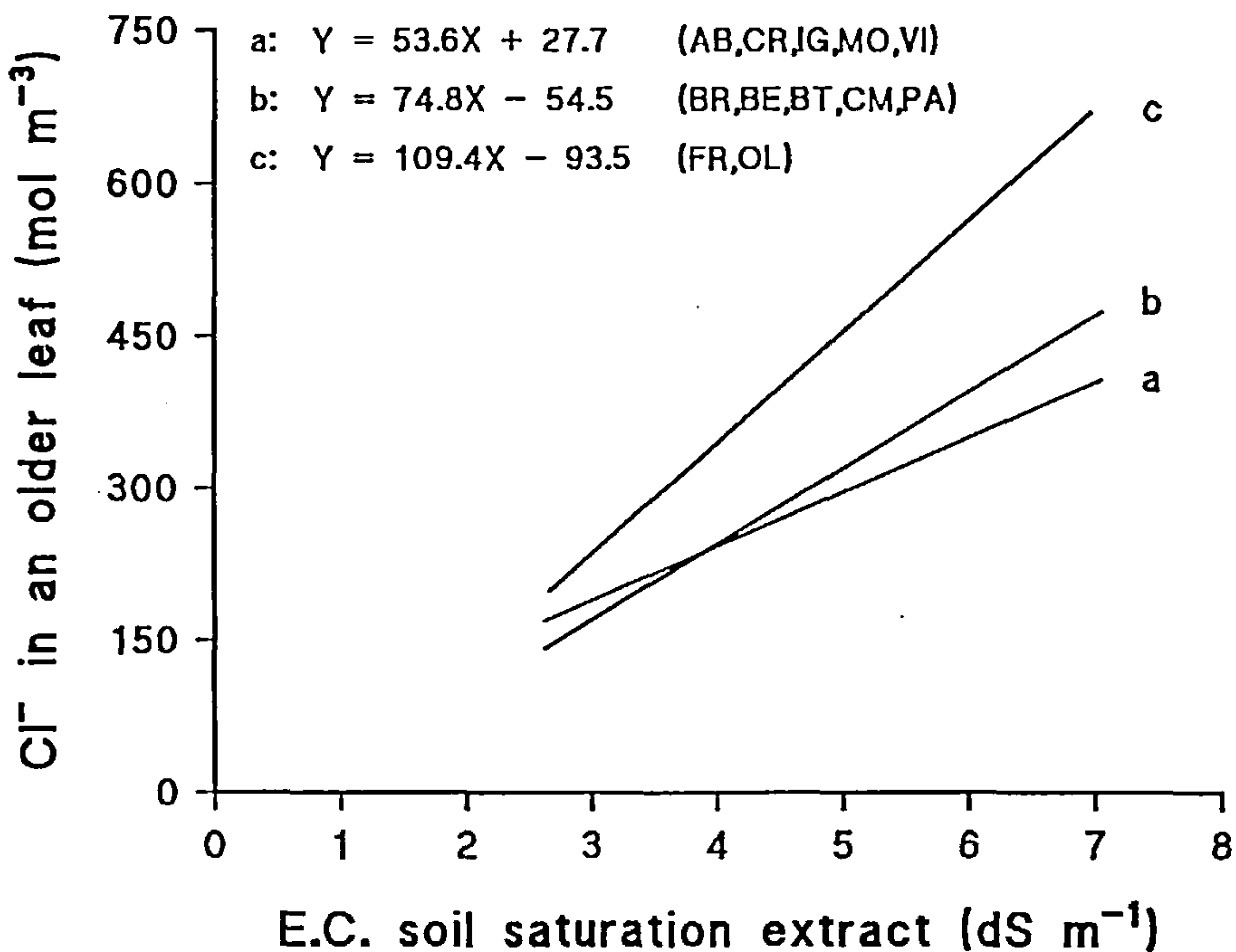


Figure 4.3.3. Cl<sup>-</sup> concentrations in leaves of plants in the TLS (1993) as a function of soil salinity. Varieties with a similar response (see text) have been grouped. For non-significant correlations the mean value is indicated.

salinity (Figure 4.3.3b). Two varieties, Forrest and Olivia, always had higher concentrations of  $\text{Cl}^-$  and higher rates of  $\text{Cl}^-$  increase with salinity than the rest.

b) Sodium (Table 4.3.1b and Figure 4.3.4):

Concentrations of  $\text{Na}^+$  in the youngest leaf did not generally change with salinity. Only in varieties Barbarrosa, Olivia and Viva were these correlations significant and positive ( $\text{Na}^+$  concentrations increased with salinity). A positive correlation was, however, found for most varieties in the older leaves, except in Albacete and Igri. Concentrations of  $\text{Na}^+$  were always higher in older leaves than in younger ones.

In young leaves, some differences between varieties were found in the mean  $\text{Na}^+$  concentrations; these could be classified in two main groups (Figure 4.3.4a). In older leaves, varieties differed not only in their average  $\text{Na}^+$  concentrations, but also in the rate at which this ion changed with salinity (slope) (Figure 4.3.4b). Varieties Forrest and Olivia again had higher mean  $\text{Na}^+$  concentrations and higher slopes than the rest.

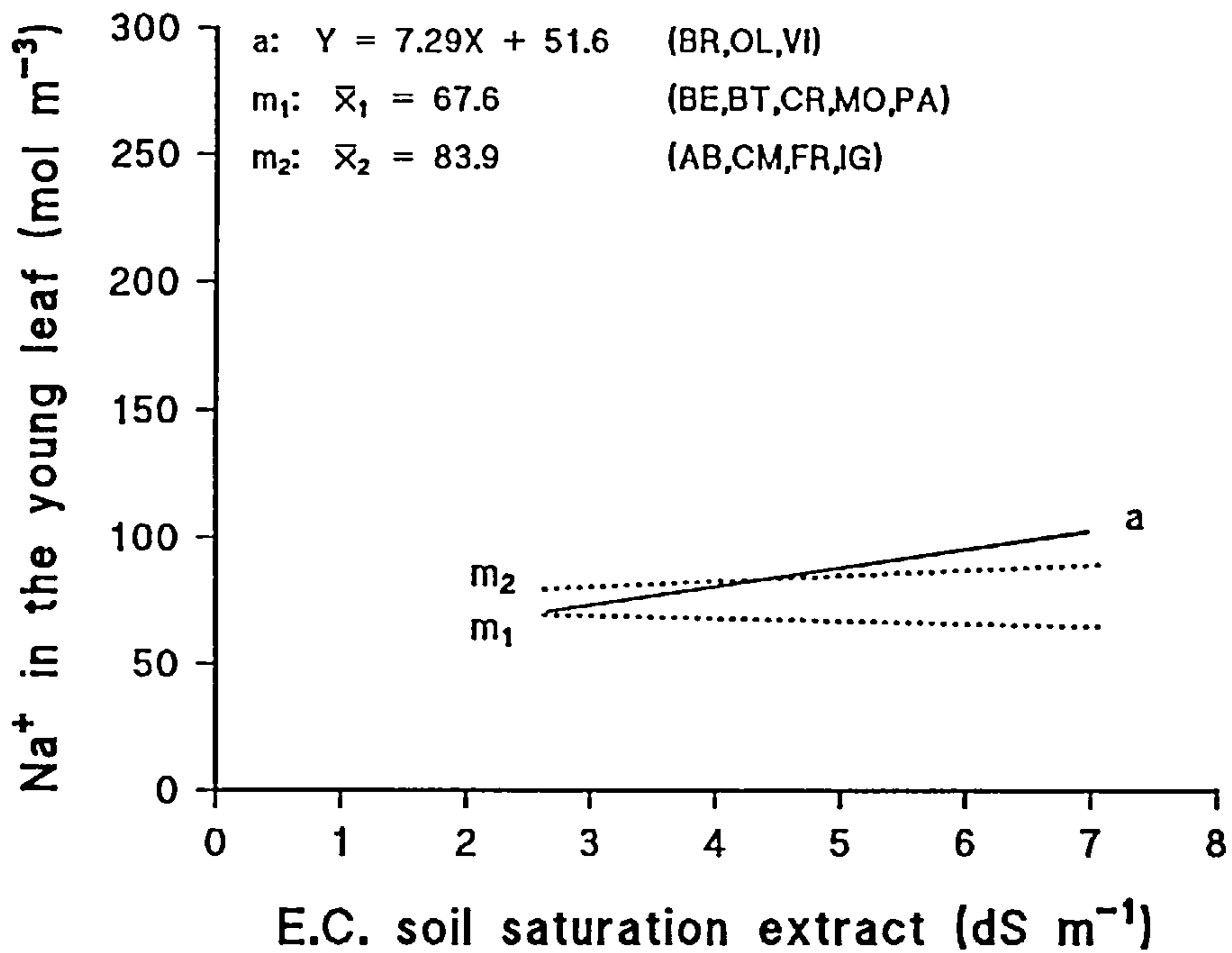
c) Calcium (Table 4.3.2a and Figure 4.3.5):

As with  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  concentrations in young leaves did not generally change with salinity. Only in varieties Forrest and Olivia was there a positive correlation, while in Albacete the correlation was negative ( $\text{Ca}^{2+}$  concentrations decreased with salinity). In older leaves, concentrations of  $\text{Ca}^{2+}$  increased with salinity in all varieties. Calcium concentrations in older leaves were always higher than in the youngest ones.

The rate of increase in  $\text{Ca}^{2+}$  concentrations in the young leaves of varieties Forrest and Olivia were similar, as were their absolute concentrations. Some differences in the average  $\text{Ca}^{2+}$  concentrations of the other varieties were found; they were classified in 4 groups (Figure 4.3.5a). Regarding the older leaves, the varieties differed both in their rate of increase in  $\text{Ca}^{2+}$  concentrations, and in their mean concentrations (Figure 4.3.5b). Forrest and Olivia were again the varieties with higher  $\text{Ca}^{2+}$  concentrations, and higher rates of increase (slopes).



## a) YOUNG LEAF



## b) OLD LEAF

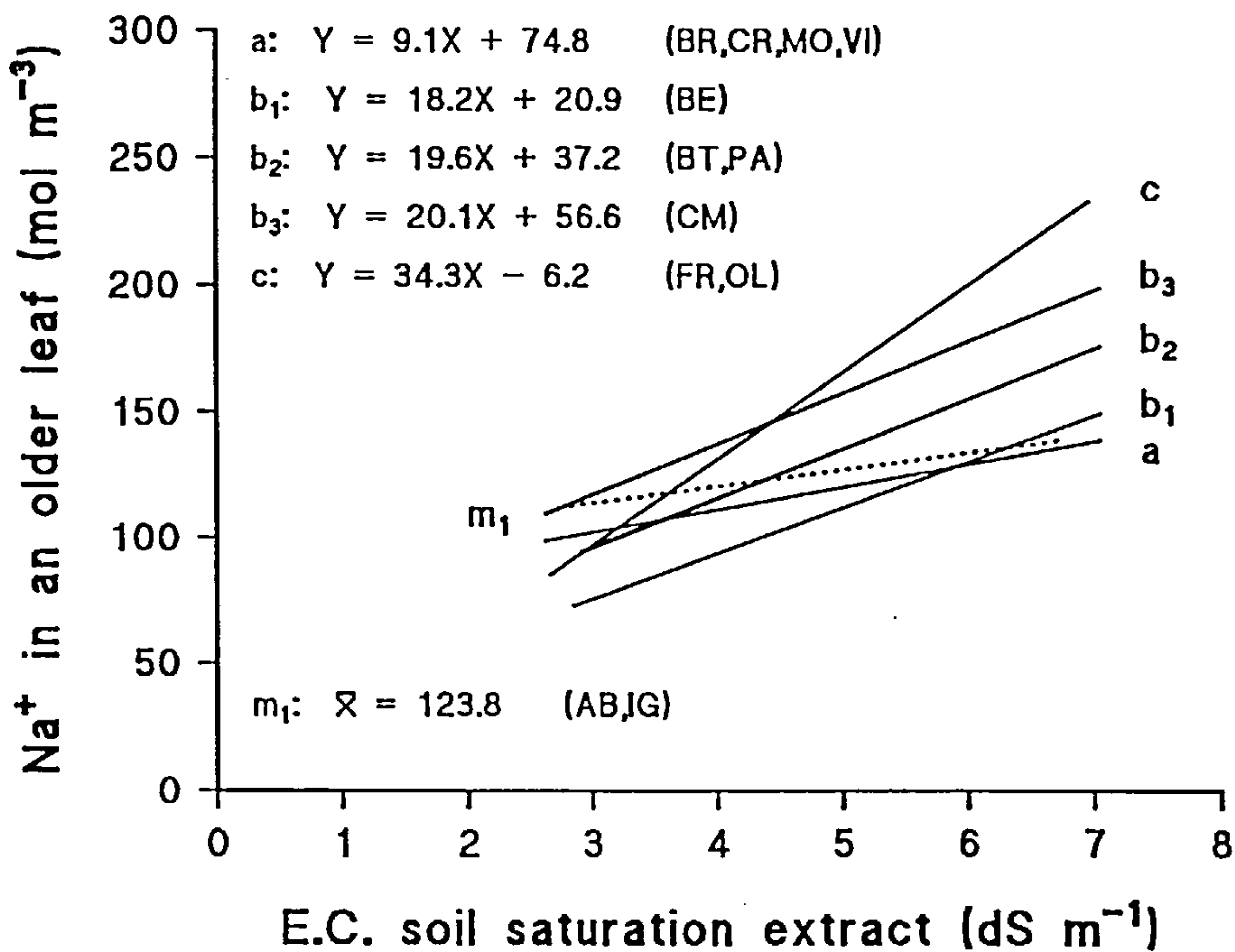


Figure 4.3.4. Na<sup>+</sup> concentrations in leaves of plants in the TLS (1993) as a function of soil salinity. Varieties with a similar response (see text) have been grouped. For non-significant correlations the mean value is indicated.

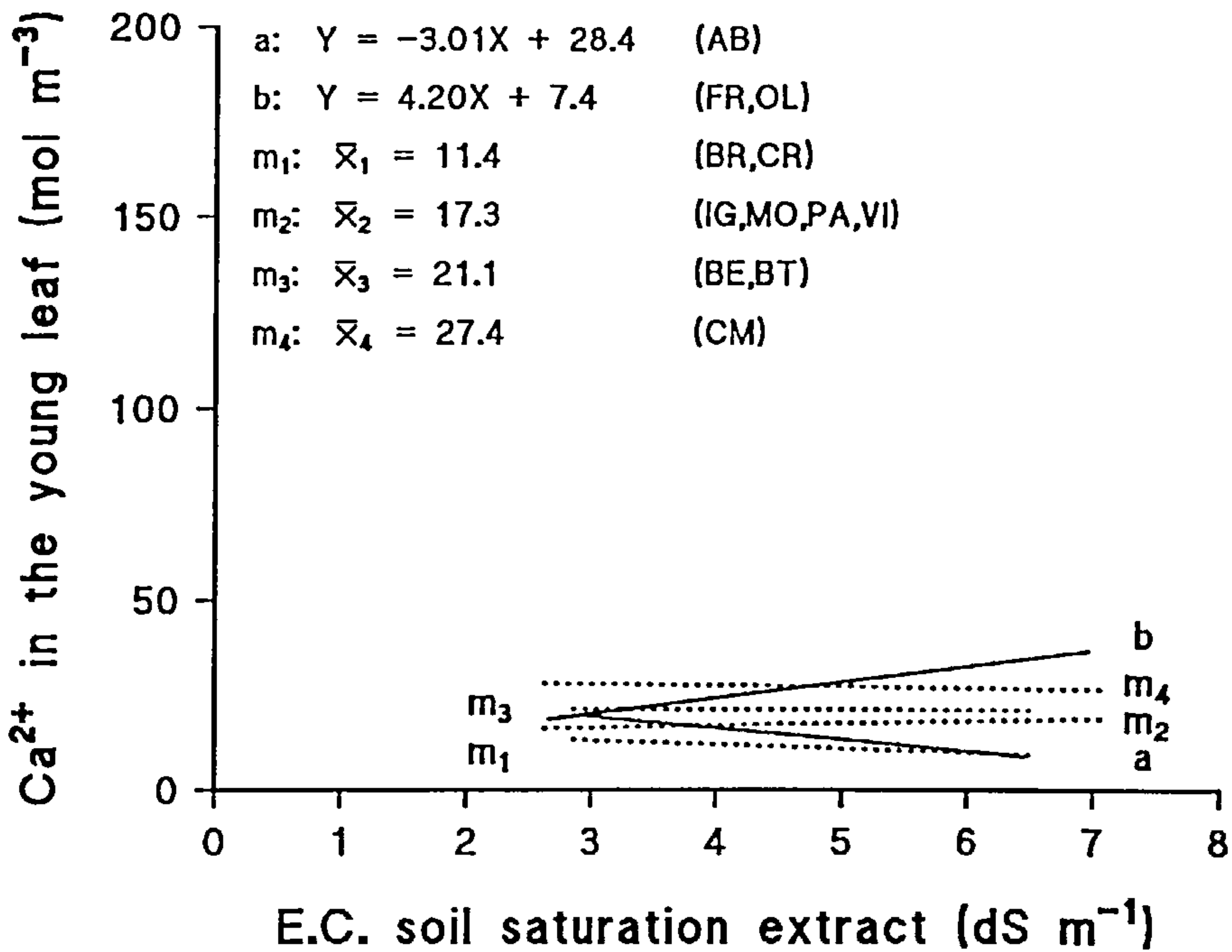
Table 4.3.2. Linear correlation (r) and regression coefficients between  $\text{Ca}^{2+}$  (a) and  $\text{K}^+$  (b) concentrations in leaves (young and old) and soil salinity (EC saturation extract) for the 12 varieties in the TLS 1992/93 experiment. Within each type of leaf, estimates of slopes and of intercepts with the same letter are not significantly different (see text). (Units: slopes in  $\text{mol m}^{-3}$  ion per  $\text{dS m}^{-1}$  soil salinity; intercepts in  $\text{mol m}^{-3}$  ion.)

VARIETY	YOUNG LEAF (N° 6)			OLDER LEAF (N° 4)		
	r	slope	interc.	r	slope	interc.
<i>a) Calcium:</i>						
AB Albacete	-0.753*	-3.01 a	28.4 a	0.758*	9.72 a	104.5 a <sub>2</sub>
BR Barbarrosa	-0.496 ns	-0.75 +	15.5 +	0.852**	10.77 a	70.3 a <sub>1</sub>
BE Begoña	-0.235 ns	-0.78 +	24.5 +	0.814**	12.98 a	20.9 a <sub>3</sub>
BT Berta	0.303 ns	0.82 +	17.4 +	0.859**	14.78 b	25.9 b
CM CM-67	-0.080 ns	-0.36 +	29.0 +	0.934***	10.92 a	56.6 a <sub>3</sub>
CR Critter	-0.441 ns	-1.33 +	16.8 +	0.813**	8.14 a	70.7 a <sub>1</sub>
FR Forrest	0.669*	4.52 b	7.2 b	0.930***	25.79 c	-10.4 c
IG Igri	-0.458 ns	-1.58 +	24.4 +	0.760*	11.66 a	84.0 a <sub>2</sub>
MO Mogador	-0.085 ns	-0.39 +	19.6 +	0.958***	8.30 a	77.1 a <sub>1</sub>
OL Olivia	0.671*	3.87 b	7.6 b	0.966***	22.63 c	-1.6 c
PA Pané	0.636 ns	3.16 +	4.4 +	0.687*	11.71 a	47.7 a <sub>2</sub>
VI Viva	0.325 ns	1.41 +	9.2 +	0.921***	15.18 b	75.2 b
<i>b) Potassium:</i>						
AB Albacete	0.651 ns	13.27 +	91.4 +	0.635 ns	9.64 +	352 +
BR Barbarrosa	-0.285 ns	-3.21 +	174.7 +	-0.188 ns	-2.91 +	1111 +
BE Begoña	0.669*	11.70 a	110.3 a <sub>2</sub>	0.253 ns	2.36 +	854 +
BT Berta	0.516 ns	6.76 +	115.1 +	-0.724*	-4.92 a	91.2 a
CM CM-67	0.875**	10.51 a	101.7 a <sub>1</sub>	0.949***	20.67 c	17.5 c
CR Critter	0.919***	13.90 a	102.2 a <sub>2</sub>	0.482 ns	5.86 +	631 +
FR Forrest	-0.218 ns	-2.54 +	164.9 +	0.941***	27.38 d	2.5 d
IG Igri	0.870**	12.24 a	92.6 a <sub>1</sub>	0.569 ns	6.47 +	491 +
MO Mogador	0.574 ns	7.71 +	108.6 +	0.663 ns	5.99 +	514 +
OL Olivia	0.006 ns	0.08 +	128.3 +	0.322 ns	3.32 +	419 +
PA Pané	0.399 ns	4.51 +	113.4 +	0.910***	5.53 b	40.6 b
VI Viva	0.698*	4.70 a	141.9 a <sub>2</sub>	0.301 ns	2.10 +	738 +

ns - not significant; \* -  $p < 0.05$ ; \*\* -  $p < 0.01$ ; \*\*\* -  $p < 0.001$ .

+ - coefficients not compared: correlation not significant.

## a) YOUNG LEAF



## b) OLD LEAF

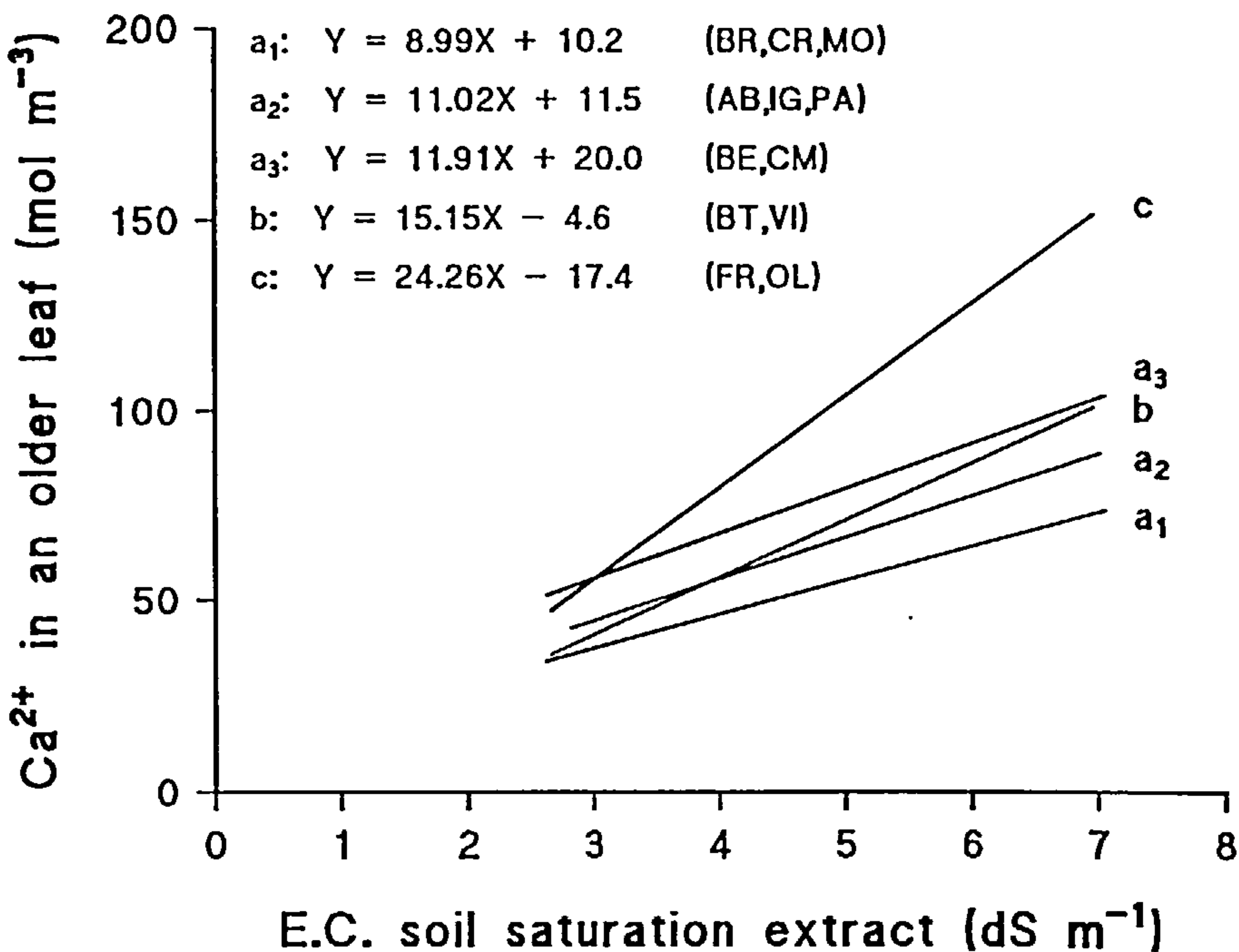


Figure 4.3.5. Ca<sup>2+</sup> concentrations in leaves of plants in the TLS (1993) as a function of soil salinity. Varieties with a similar response (see text) have been grouped. For non-significant correlations the mean value is indicated.

#### d) Potassium (Table 4.3.2b and Figure 4.3.6):

Concentrations of  $K^+$  in most varieties did not change with increasing salinity, in either young or old leaves. In those varieties where they did change, the correlations tended to be positive, although relatively low ( $K^+$  concentrations increased slightly with salinity). The only exceptions were in the older leaves of CM-67 and Forrest, where this increase was considerable (high slope values), and in old leaves of Berta, where concentrations of  $K^+$  tended to decrease (negative correlation). Potassium concentrations were generally higher in the youngest leaves than in the older ones.

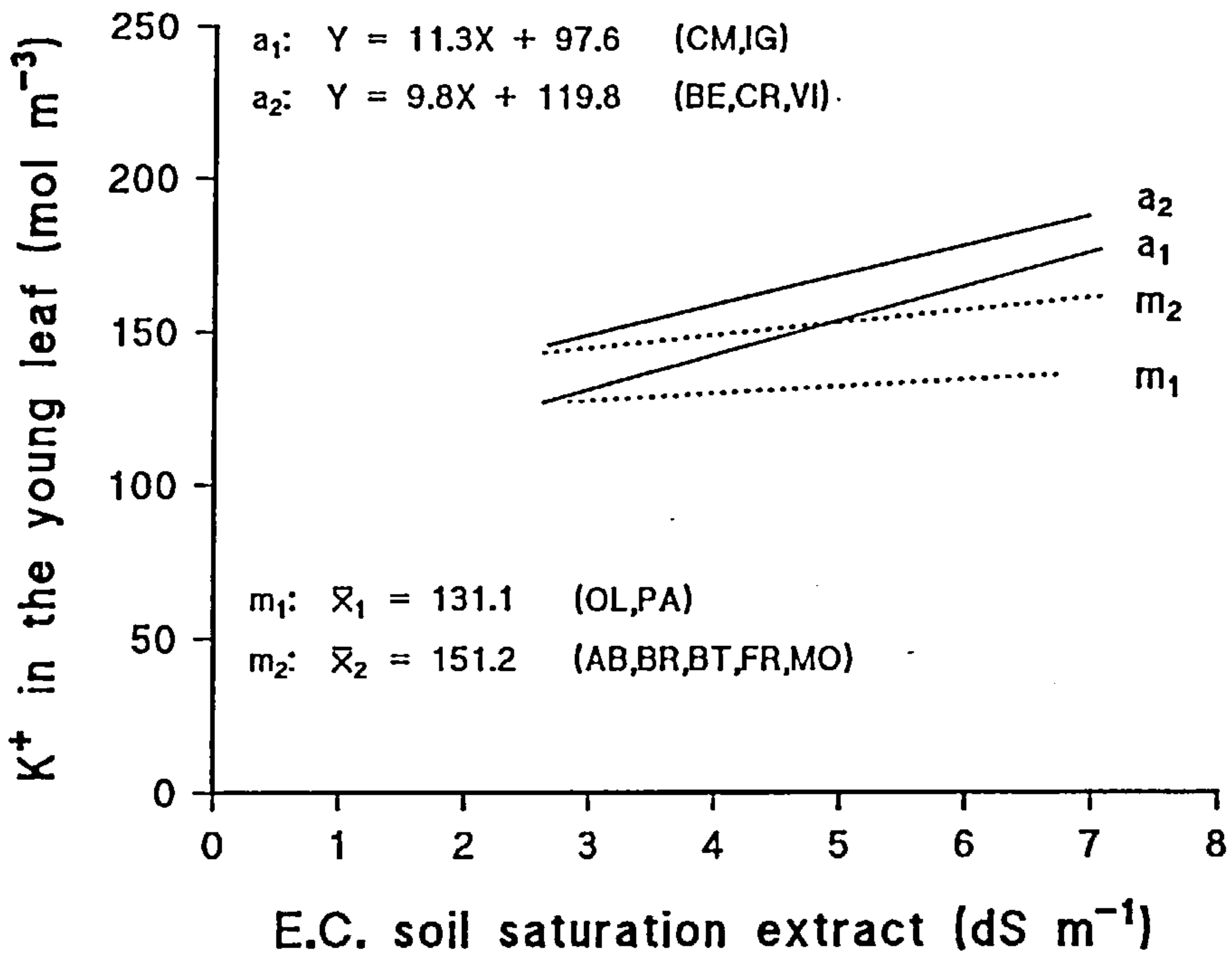
Some differences between varieties were found in the  $K^+$  concentrations in young leaves (Figure 4.3.6a), although these were not very large (mean values ranged from 130 to 165 mol  $m^{-3}$  sap). Differences between varieties in older leaves (Figure 4.3.6b) were mostly in average concentrations (from 55 to 125 mol  $m^{-3}$  sap), although varieties CM-67 and Forrest had larger slopes and higher mean  $K^+$  concentrations than all the others.

#### 4.3.3.3. Grain Yield:

The 3 models (linear regression, threshold and sigmoidal) were fitted to the data for each variety, and they were compared by calculating the coefficient of determination ( $r^2$ ) between observed and fitted values. Except in a few cases, all 3 models gave significant fits (Table 4.3.3), although these were generally lower than in the previous year (Table 4.2.2, section 4.2.3.3). The sigmoidal curve (model 3) usually provided the best fit; in those cases where this model was not clearly superior, (Berta, CM-67 and Olivia), it was at least as good as the best one. The threshold model (number 2) generally gave the poorest fits; it was with this model that non-significant fits were found in some varieties (Albacete, Barbarrosa and Pané).

For the comparison of the varieties in terms of yield response to salinity, only the sigmoidal model was used, since this was the model which best fitted the observed values. The values of the parameters which define the varieties' response with this model ( $Y_m$ ,  $EC_{50}$  and  $p$ ) are listed in Table 4.3.4. Because of the large standard

## a) YOUNG LEAF



## b) OLD LEAF

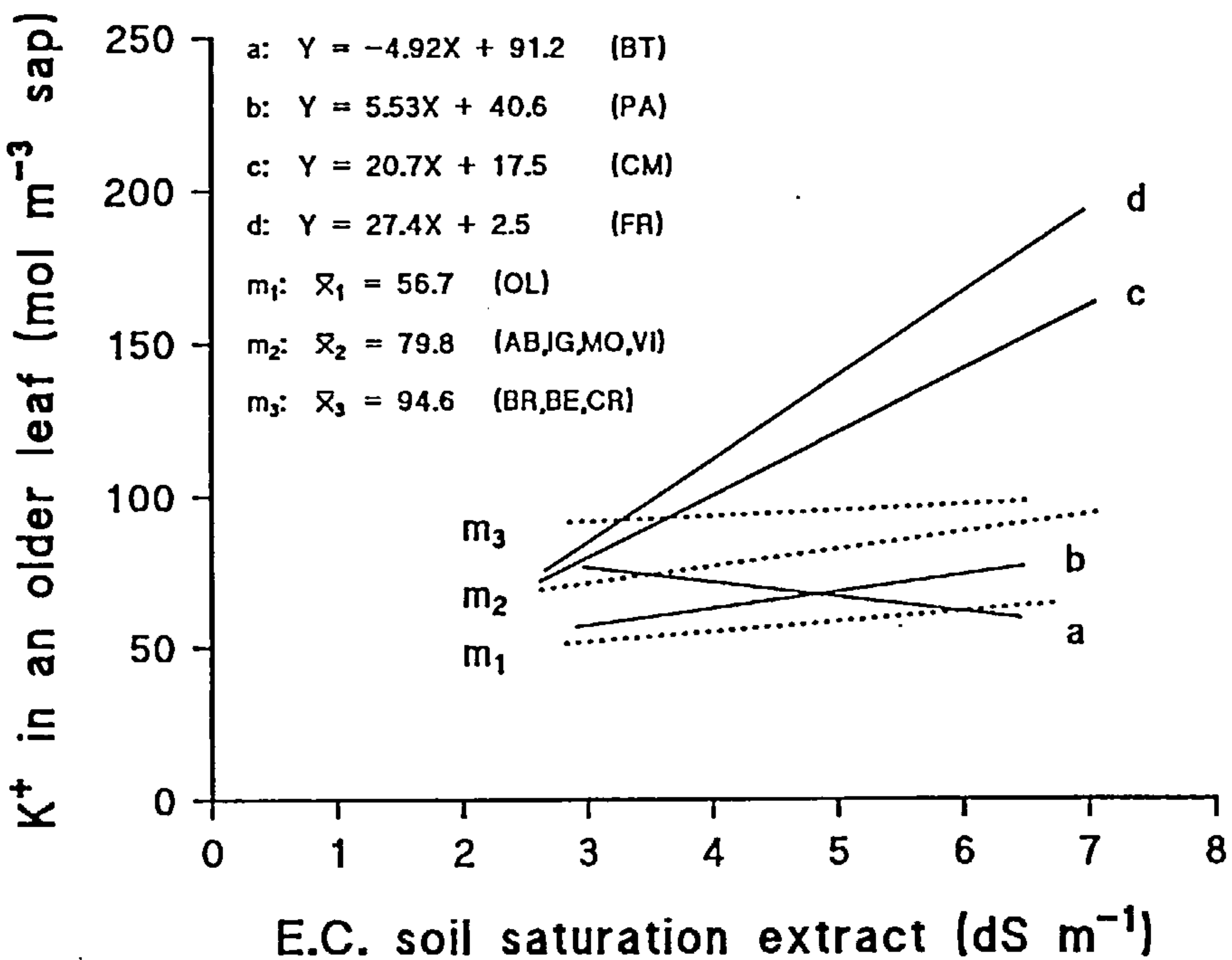


Figure 4.3.6.  $K^+$  concentrations in leaves of plants in the TLS (1993) as a function of soil salinity. Varieties with a similar response (see text) have been grouped. For non-significant correlations the mean value is indicated.

Table 4.3.3. Coefficients of determination ( $r^2$ ) for observed vs fitted values of the response models (yield vs soil salinity) in the TLS 1992/93 experiment.

VARIETY	model 1+	model 2+	model 3+
Albacete	0.519*	0.440 ns	0.576*
Barbarrosa	0.476*	0.315 ns	0.519*
Begoña	0.811***	0.792**	0.815***
Berta	0.473*	0.482*	0.457*
CM-67	0.715**	0.691*	0.707**
Critter	0.711**	0.713**	0.721**
Forrest	0.764**	0.683*	0.765**
Igri	0.698**	0.563*	0.703**
Mogador	0.590*	0.593*	0.658**
Olivia	0.770**	0.743*	0.760**
Pané	0.572*	0.396 ns	0.621*
Viva	0.732**	0.733**	0.739**

+ model 1: linear regression; model 2: threshold; model 3: sigmoidal curve.  
 ns - not significant; \* -  $p < 0.05$ ; \*\* -  $p < 0.01$ ; \*\*\* -  $p < 0.001$ .

Table 4.3.4. Values of the parameters ( $\pm$  standard errors) that define the varieties' response to salinity according to model 3 in the TLS 1992/93 experiment. Units: yield ( $Y_m$ ) in  $\text{gr m}^{-2}$  and salinity ( $EC_{50}$ ) in  $\text{dS m}^{-1}$ ; (p has no dimensions).

VARIETY	$EC_{50}$	p-exp	max. yield*
Albacete	$6.42 \pm 1.01$	$1.25 \pm 0.47$	505
Barbarrosa	$6.73 \pm 1.23$	$1.78 \pm 0.92$	438
Begoña	$6.00 \pm 0.49$	$1.84 \pm 0.43$	590
Berta	$9.35 \pm 2.76$	$2.58 \pm 2.03$	297
CM-67	$7.70 \pm 1.73$	$2.11 \pm 1.77$	609
Critter	$8.71 \pm 1.21$	$2.76 \pm 1.89$	439
Forrest	$7.70 \pm 0.87$	$2.07 \pm 0.63$	491
Igri	$7.32 \pm 0.85$	$2.06 \pm 0.69$	493
Mogador	$5.52 \pm 0.74$	$1.31 \pm 0.42$	692
Olivia	$6.50 \pm 0.70$	$1.77 \pm 0.51$	727
Pané	$8.96 \pm 1.92$	$1.39 \pm 0.55$	512
Viva	$7.47 \pm 2.79$	$1.73 \pm 1.61$	605

\* standard errors not available

errors (due to the relatively poor fit), no significant differences between varieties were found in any of the parameters.

#### 4.3.3.4. Relationship between leaf ion concentrations and grain yield:

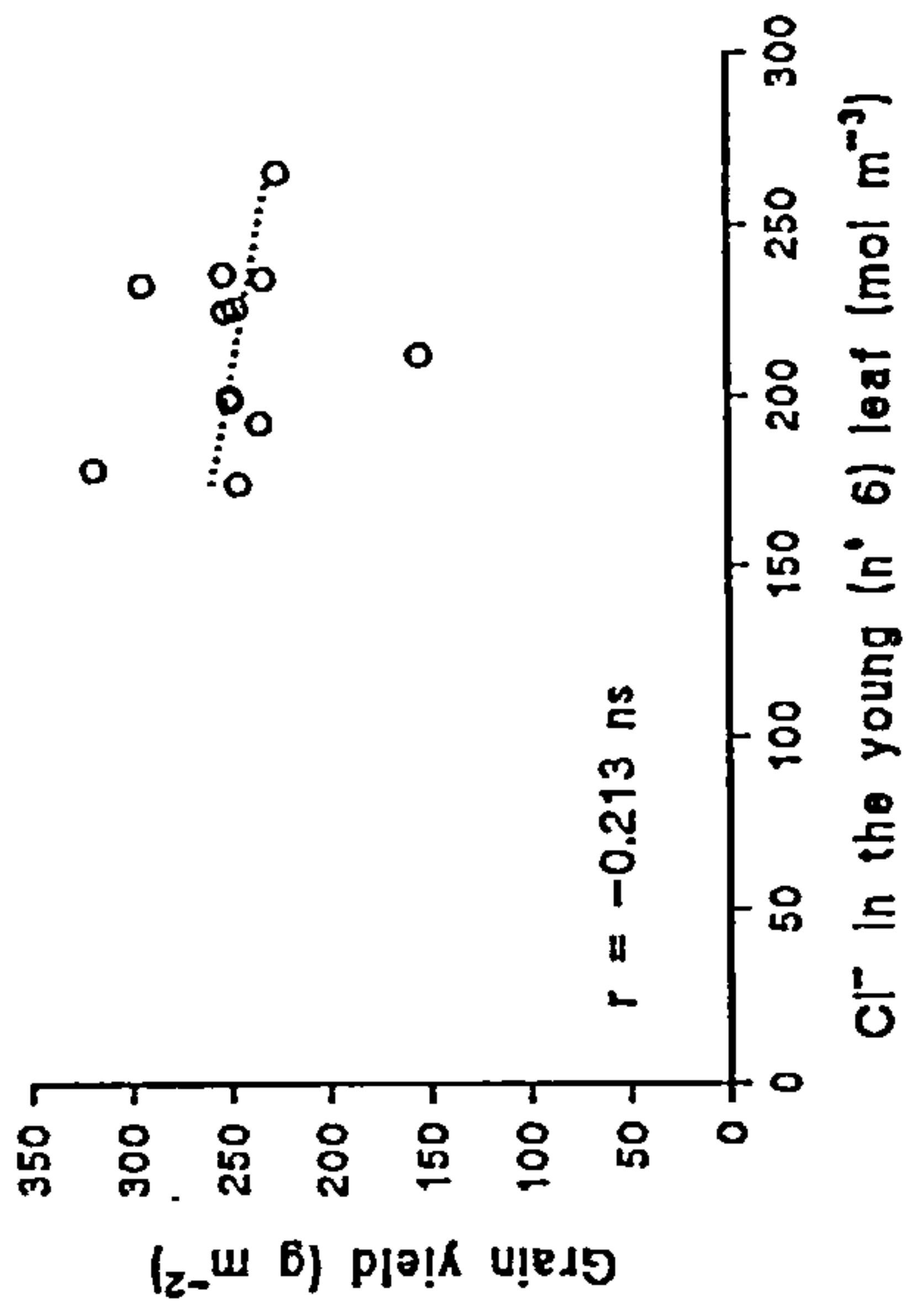
To study the relationship between leaf ion concentration and grain yield, for a given salinity treatment (similar soil and water salinity), the yields of each variety were plotted against the individual ion concentrations in the leaves of that variety. This was done with both absolute and relative yields, and with ion concentrations in young and old leaves. Some examples of the types of graphs obtained for the highest treatment are given in Figure 4.3.7. In general, no significant relationships were found (data not shown). Only in a few cases was the correlation significant, but very low (just at the 5% level). It seems, thus, that no simple relationship exist between these two parameters.

### 4.3.4. *DISCUSSION:*

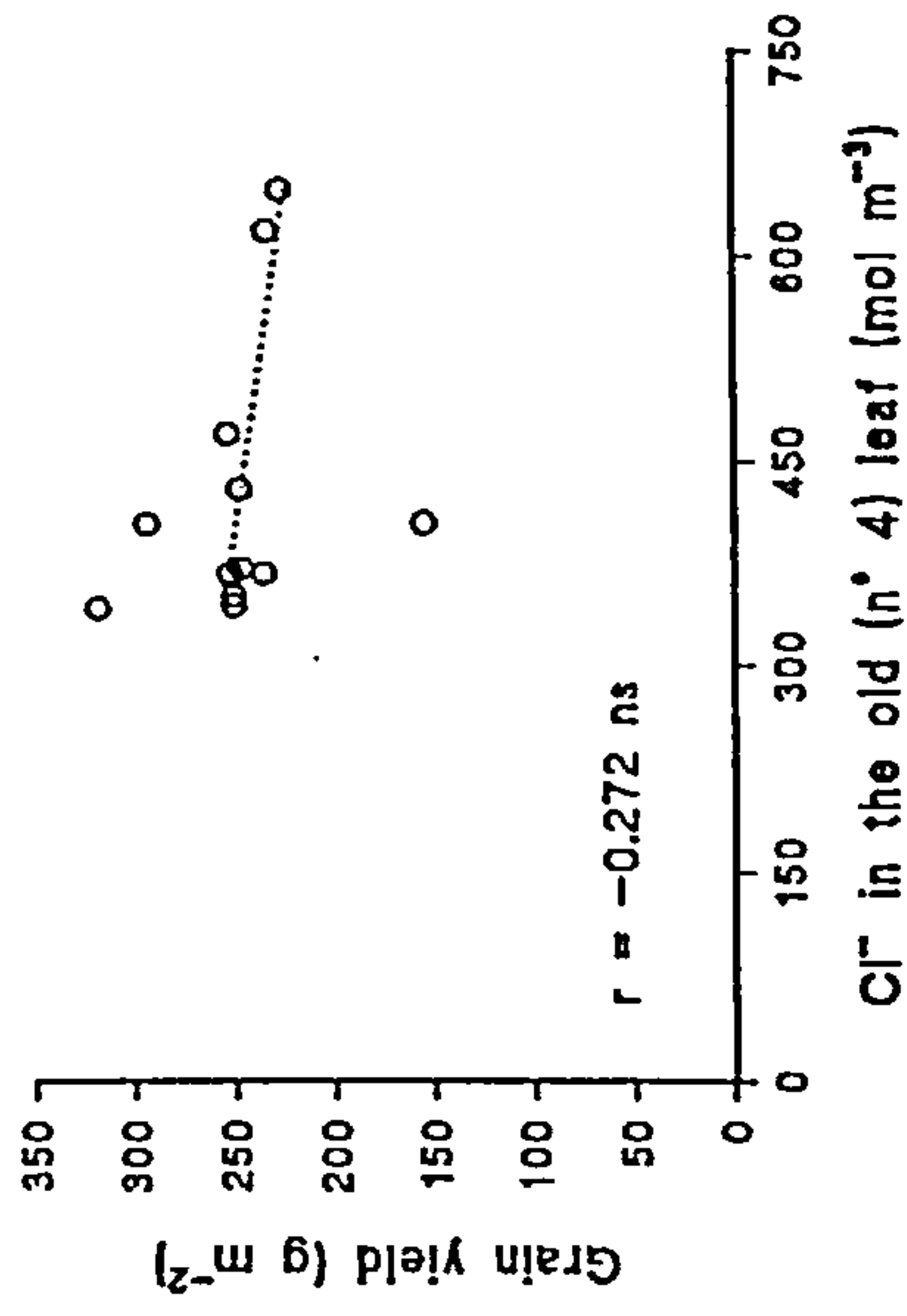
#### 4.3.4.1. Soil and water salinity distribution:

One of the most notable features of this experiment was the low salinity reached in the soil relative to the previous year (compare Figures 4.2.5 and 4.3.2). This was in spite of the slightly higher levels of salinity in the irrigation water in the 1992/93 season (Figures 4.2.4 and 4.3.1, respectively). The discrepancies may be attributed to the way the electrical conductivity of the saturated extract was calculated in the present experiment. Since the preparation of the soil saturated extracts is a time-consuming technique, 1:5 (soil:water) extracts were used to measure the EC of the samples taken for the calibration of the EMS readings. Only in one sampling date was the EC of the saturation extract (as well as that of the 1:5 extract) measured. The 20 pairs of values were correlated, and a linear regression was fitted. This was quite similar to the one obtained in the previous season, but because the latter was based on more samplings (80 pairs of values), it was thought to be more accurate. Hence, the equation obtained in 1991/92 was used to transform the  $EC_{1:5}$  to  $EC_{se}$  in

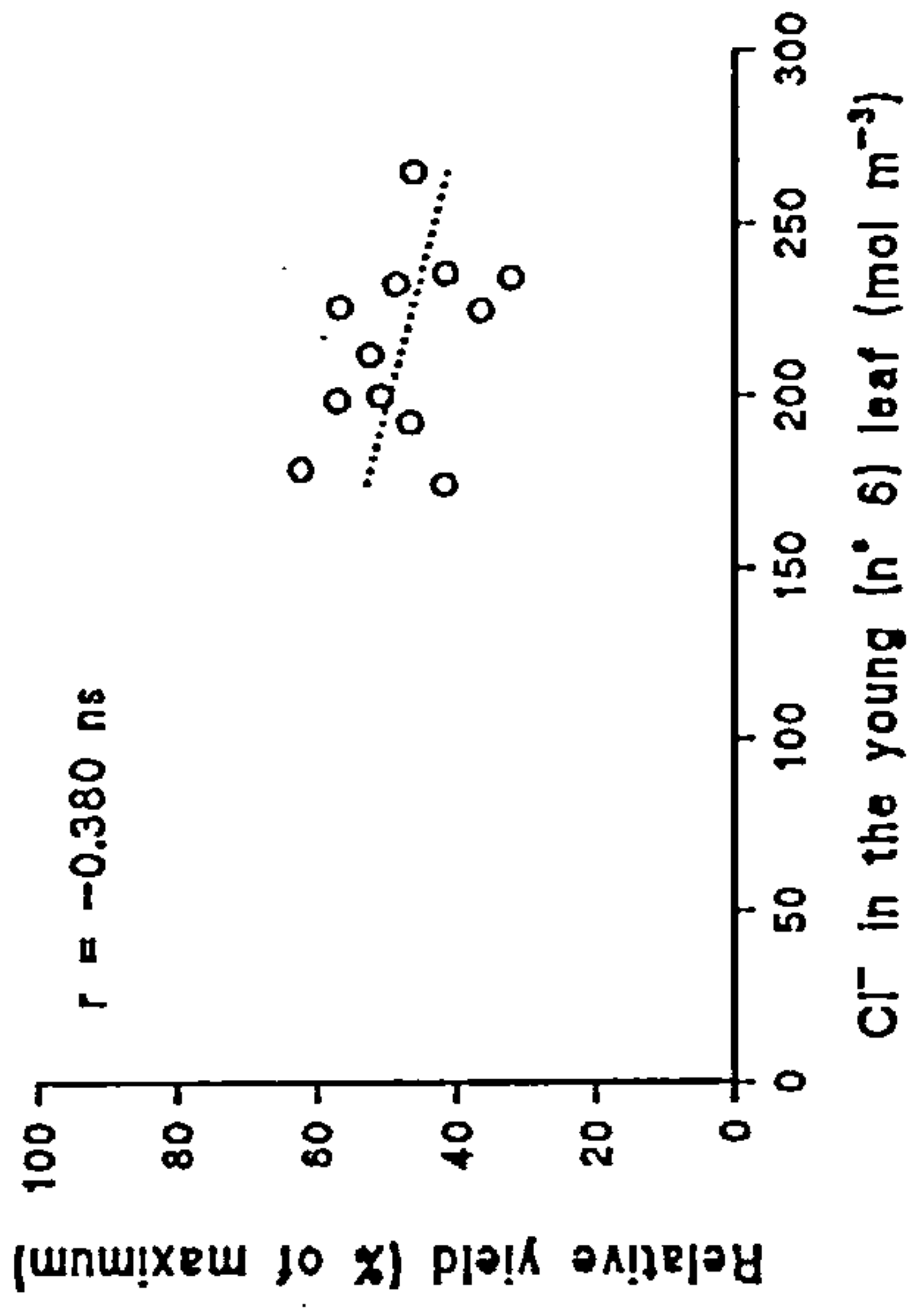
a) Absolute yield, young leaves



b) Absolute yield, old leaves



c) Relative yield, young leaves



d) Relative yield, old leaves

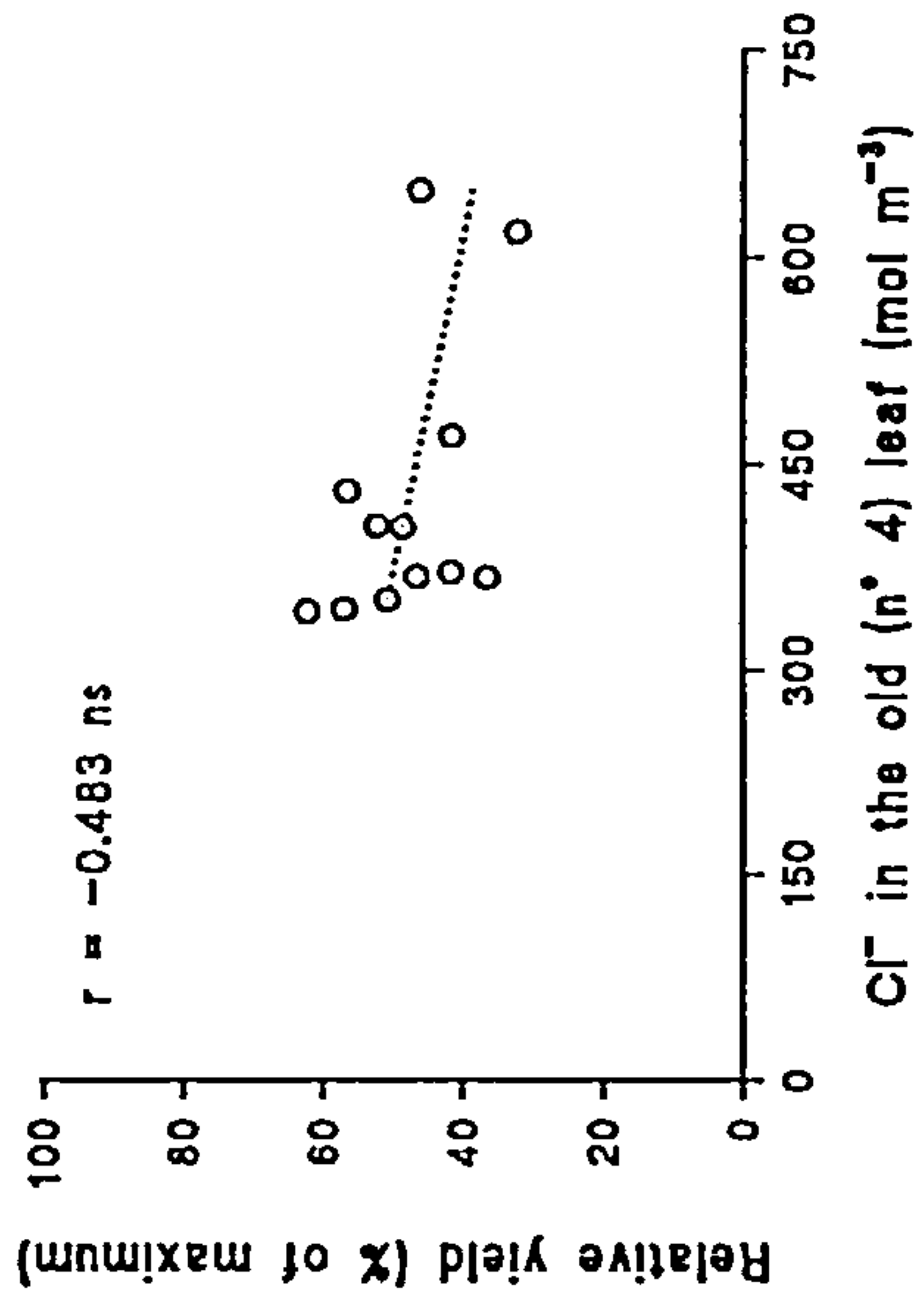


Figure 4.3.7. Grain yield (actual and as percent of maximum) at the highest salinity treatment ( $\text{EC}_{\text{sc}}$  from 7.98 to 8.51  $\text{dS m}^{-1}$ ) as a function of the concentrations of  $\text{Cl}^-$  in leaves (young and old), in the TLS 1992/93 experiment.



the 1992/93 season. In view of the results, however, the relevance of that calibration in this particular season is in doubt.

#### 4.3.4.2. Ion concentrations in leaves:

Except for  $\text{Cl}^-$ , the ion concentrations in the youngest leaves did not seem to depend very much on salinity, whether measured as soil salinity or as irrigation water salinity. The few exceptions were Barbarrosa, Olivia and Viva, which increased their  $\text{Na}^+$  concentrations with increasing salinity, and Forrest and Olivia which increased their  $\text{Ca}^{2+}$  concentrations (in Albacete they decreased). In some varieties (Begoña, CM-67, Critter, Igri and Viva),  $\text{K}^+$  concentrations in young leaves increased with salinity.

Correlations in older leaves were generally higher than in the youngest ones. All the ions applied through the irrigation water ( $\text{Cl}^-$ ,  $\text{Na}^+$  and  $\text{Ca}^{2+}$ ) increased their concentrations in older leaves with salinity. Only in a few cases were these correlations not significant ( $\text{Na}^+$  concentrations in Albacete and Igri). In some varieties (CM-67, Forrest and Pané) concentrations of  $\text{K}^+$  in older leaves did also increase with salinity; in others (Berta), they decreased.

Of the ions applied with the irrigation,  $\text{Cl}^-$  was the one found in higher concentrations in the leaves, both young and old, and which changed (increased) most with salinity. This is not surprising, since this is also the ion present in the highest proportion in the irrigation water. At the other extreme,  $\text{K}^+$  concentrations were maintained quite constant across salinities. Concentrations of  $\text{Na}^+$  in leaves either did not change with salinity or they increased (old leaves); the negative correlation found in the previous year between salinity and  $\text{Na}^+$  concentrations in old leaves of CM-67 was not found this time.

In general terms the present results are in good agreement with those found in the TLS in 1991/92, although the precise concentrations of ions in leaves for a particular variety did change between the two seasons.

#### 4.3.4.3. Grain yield:

The 3 models used to study the yield response to salinity did not fit the observed data as well as in the previous year. This was probably due to the smaller size of the plots (3 rows per variety instead of 6), which presumably resulted in larger errors in the measurement of grain yield.

In the present experiment, and partly as a consequence of the poor fit of the models and the large standard errors of the estimates, no significant differences were detected between varieties in any of the parameters which define the yield response. Still, the varieties can be compared by ranking them according to their response. The  $EC_{50s}$  (calculated from model 3) were used for this comparison, because this is the parameter which best estimates the salt tolerance of a genotype, as discussed in the previous experiment (section 4.2.4.2). The rankings based on the  $EC_{50}$  of the soil saturated extract and that of the irrigation water are shown in Table 4.3.5 (a and b).

Table 4.3.5. Ranking of varieties according to their  $EC_{50}$  ( $dS\ m^{-1}$ ) of the soil saturation extract (a), their  $EC_{50}$  ( $dS\ m^{-1}$ ) of the irrigation water (b), and their mean yield over all salinity treatments (c) in the TLS 1992/93 experiment.

a) Variety	$EC_{50}$ soil	b) Variety	$EC_{50}$ water	c) Variety	mean yield
1 - Berta	(9.35)	1 - Berta	(19.84)	1 - Olivia	(457)
2 - Pané	(8.96)	2 - Critter	(18.93)	2 - CM-67	(432)
3 - Critter	(8.71)	3 - Pané	(17.96)	3 - Viva	(406)
4 - CM-67	(7.70)	4 - CM-67	(14.74)	4 - Mogador	(386)
5 - Forrest	(7.70)	5 - Viva	(14.34)	5 - Pané	(356)
6 - Viva	(7.47)	6 - Forrest	(14.09)	6 - Begoña	(353)
7 - Igri	(7.32)	7 - Igri	(13.29)	7 - Critter	(350)
8 - Barbarrosa	(6.73)	8 - Barbarrosa	(11.34)	8 - Forrest	(348)
9 - Olivia	(6.50)	9 - Olivia	(10.71)	9 - Igri	(336)
10 - Albacete	(6.42)	10 - Albacete	(10.12)	10 - Albacete	(294)
11 - Begoña	(6.00)	11 - Begoña	(10.06)	11 - Barbarrosa	(279)
12 - Mogador	(5.52)	12 - Mogador	(8.92)	12 - Berta	(240)

The two measures of  $EC_{50}$  are very similar in ranking the varieties. The second column ( $EC_{50}$  of irrigation water) was included to compare with results from previous experiments in the TLS, as compiled by Aragüés and collaborators (unpublished data). Some varieties ranked in similar positions in these two lists (that in Table 4.3.5b and that of Aragüés *et al.*): Begoña was one with the lowest  $EC_{50}$ s, Pané and Berta were among the highest ones, and Barbarrosa ranked in the middle. Others, however, ranked very differently in the two cases: Mogador had an intermediate  $EC_{50}$  in the results of those authors, while it had the lowest one in this experiment; at the other extreme, Olivia had the highest  $EC_{50}$  ( $21.5 \text{ dS m}^{-1}$ ) in the list of Aragüés' group, while it was one of the worst varieties in the present experiment. The actual values of  $EC_{50}$  are rather different for the two lists, although this might have been expected since they come from different experiments and years, and salt tolerance is very sensitive to changes in environmental factors (Maas, 1990). It is usually considered that, in field experiments, results from a single season are not very representative. Thus, the lack of total agreement between the present results and those found in previous years might just be due to different environmental conditions.

In Table 4.3.5, Berta is the variety with the highest  $EC_{50}$  in columns *a* and *b*, but it has to be remembered that this variety had the poorest fits with all models, just significant at the 5% level (Table 4.3.3). Thus, its high  $EC_{50}$ s have to be regarded with some caution. Also, from an agronomical point of view it is worth noting that its maximum yield was very low (only half that of other varieties). This means that, in spite of its greater tolerance, in conditions of low and medium salinity this variety will be of no advantage over others with higher yield potential. In fact, in column *c* of Table 4.3.5, where the varieties are ranked by their overall yield (averaged over the 9 salinity treatments), Berta ranks in the last position, with just over half the total yield of the best varieties (Olivia, CM-67), illustrating this point.

At the other extreme, Mogador, with the lowest  $EC_{50}$ s, had one of the highest maximum yields. These results suggest that a negative correlation between these two parameters might exist. The idea of an inverse relationship between maximum yield (or yield potential) and salt tolerance is not new (e.g. Richards, 1983; Shannon,

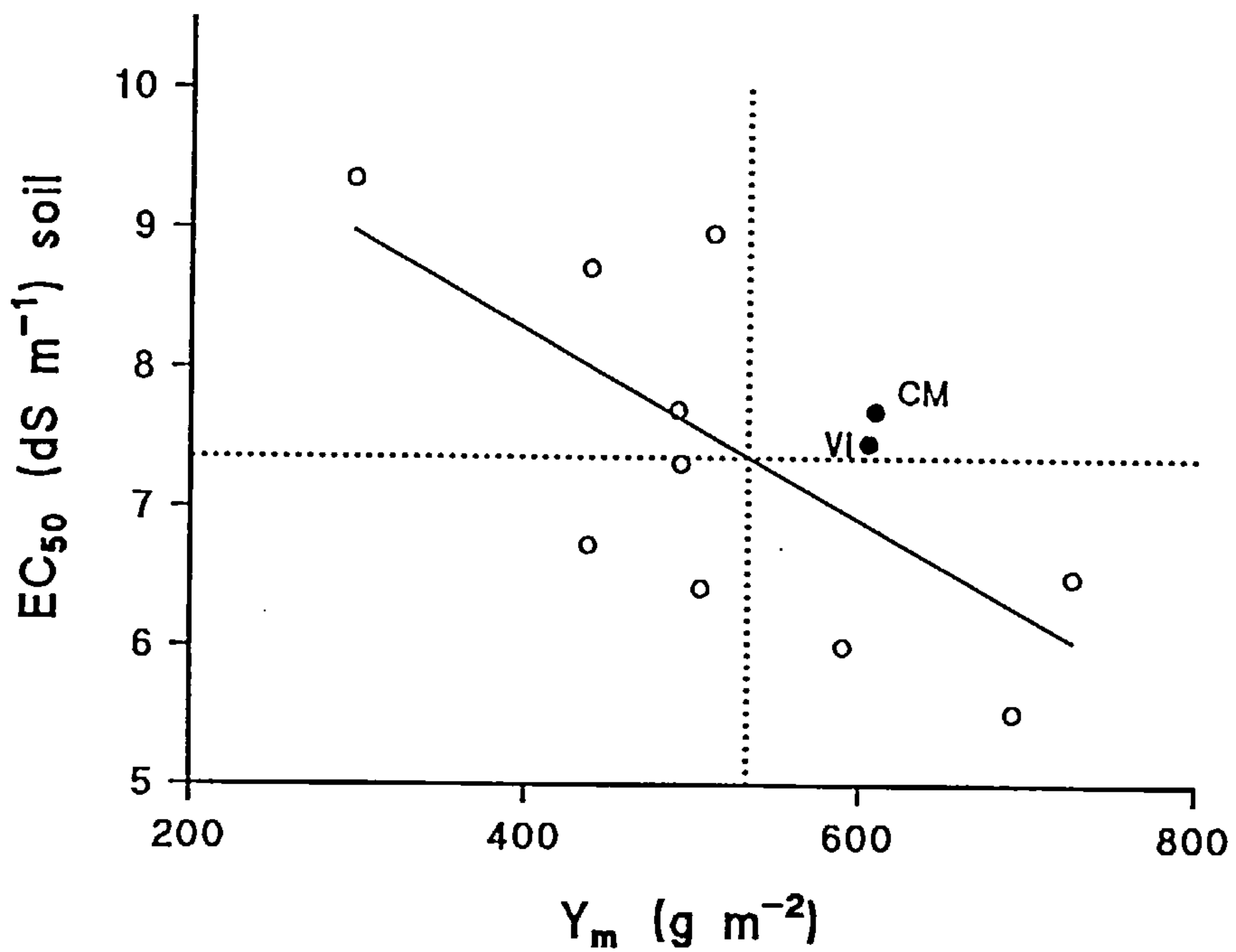
1985). To explore this relationship in more detail, the values of the  $EC_{50}$  and  $Y_m$  for each variety were plotted and correlated; results are presented in Figure 4.3.8. Although some negative correlation does exist between the two parameters, this is not very strong (only significant at the 5% level). Therefore, the development of high yielding varieties which are also salt resistant should be possible. This conclusion is in agreement with the findings of Richards *et al.* (1987).

The dotted lines in Figure 4.3.8 show the average values for maximum yield and  $EC_{50}$ . Any varieties having both values ( $Y_m$  and  $EC_{50}$ ) above the mean can be considered salt tolerant in a broad sense, since they will have not only high relative tolerance, but also good yield potential. It can be seen in Figure 4.3.8 that CM-67 and Viva are the two varieties which comply with this condition. Varieties with high  $Y_m$  but low  $EC_{50}$  (Olivia, Mogador and Begoña) may be of advantage in conditions of low salinity, while those with high  $EC_{50}$  but low  $Y_m$  (Berta, Pané, Critter and Forrest) have high relative tolerance and might be interesting in breeding programs as a source of salt tolerance. Finally, varieties which have both  $Y_m$  and  $EC_{50}$  below the average (Igri, Barbarrosa and Albacete) can be considered the least tolerant of those studied. Again, though, these conclusions have to be taken with caution, since they are based in only one year of field results. In the previous season ("TLS 1991/92" experiment, section 4.2.3), Albacete was the most tolerant variety, with a higher  $EC_{50}$  than CM-67 and a similar maximum yield.

#### 4.3.4.4. Relationship between leaf ion concentrations and yield:

The plots of yield vs leaf ion concentrations at a given salinity, such as those presented in Figure 4.3.7, were drawn in the hope that they would reveal the existence of some correlations between these traits. However, no clear relationships were found. This lack of correlation does not necessarily imply that the two traits are not related. There is enough evidence in the literature to prove that a strict regulation of ion uptake and accumulation is a key feature of salt tolerance. However, the control of ion uptake and accumulation can be accomplished at different levels, from salt exclusion in the root, to proper compartmentation at the tissue and cellular level. A single measure of ion concentrations in the bulk leaf does not give any information

## a) Soil saturation extract



## b) Irrigation water

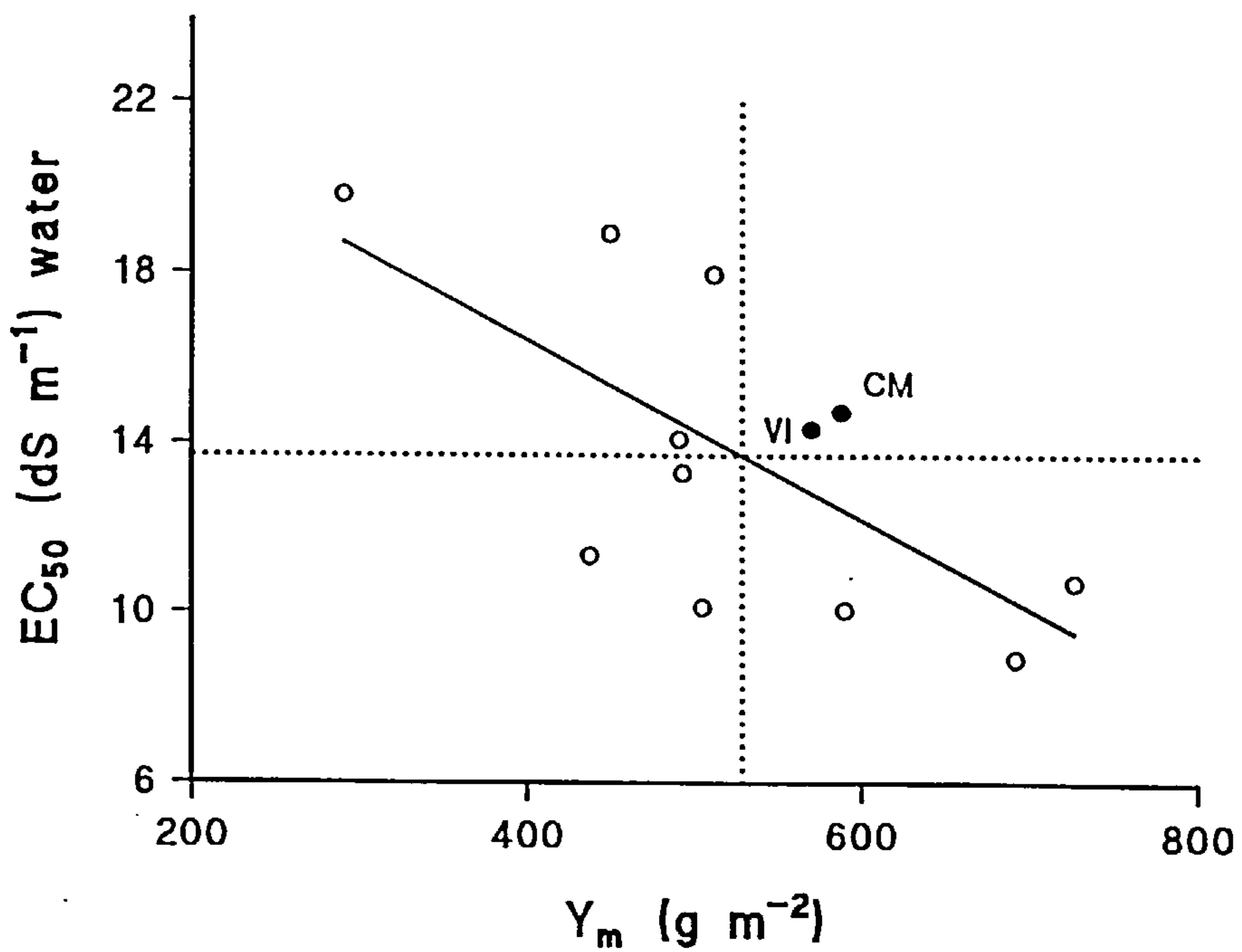


Figure 4.3.8. Relationship between  $EC_{50}$  of soil (a) and irrigation water (b) and maximum yield ( $Y_m$ ) for the 12 varieties in the TLS 1992/93 experiment.

on the distribution of these ions within the leaf (different cells) or within the cells (different compartments).

Another aspect considered was whether differences in grain yield and in leaf ion concentrations were related to differences in rate of development between varieties. (Although the extremely slow growing variety Chevron had been excluded from this experiment, some differences were still found for this trait. This is reflected in the different dates of sampling (Appendix 4), and also in their average heading times, which stretched for over 2 weeks (data not shown).) A high growth rate can help in maintaining low ion concentrations (particularly in young leaves) simply by a dilution effect. It might, thus, be expected that faster growing varieties would have lower ion concentrations than those with a slower development. On the other hand, later maturing varieties tend to be higher yielding, and differences in growth cycle may, in this way, overshadow any effects due to higher ion concentrations.

No relationships, however, were found between development rate (as measured both by the dates when varieties were sampled and by their days to heading) and either leaf ion concentrations or grain yield. As an example, the two varieties with the highest ion concentrations (Forrest and Olivia) were among those with the shortest growing cycles, thus contradicting the above hypothesis. Other fast growing and early maturing varieties (CM-67, Pané) did have low or intermediate ion concentrations, but so had some of the later maturing ones. A similar situation was found with yield: Viva had both one of the longest growing cycles and the highest overall yield; however, at the other extreme of the scale, those varieties with the lowest yields (Barbarrosa, Berta) differed largely in their rate of development (and the ion concentrations in their leaves were not very different). Therefore, it seems that the differences found in the degree of leaf ion accumulation between varieties are not just a consequence of the different growing cycles of these varieties.

# CHAPTER FIVE

## GENERAL DISCUSSION

The ultimate objective of this work was to determine whether the measurement of ion concentrations in leaves of barley is a good indicator of the salt tolerance of a particular genotype, in which case these traits (leaf ion concentrations) could be used as selection criteria in breeding programmes for salt tolerance. The approach used to answer this question was to compare different varieties of barley for both their salt tolerance and their leaf ion concentrations. While the latter is, in principle, a relatively straightforward measurement involving techniques of chemical analysis, the assessment of the salt tolerance of a genotype poses more of a problem, if only because there is no general agreement on how to quantify tolerance.

### *- Measurement of salt tolerance in the field: the TLS*

Ultimately, if the assessment of salt tolerance is to be of any value, plants need to be grown under environmental conditions comparable to those they will experience when grown as a crop, and this entails growing the plants in the field. The limitations of naturally saline soils (large heterogeneity both in space and in time) for experimental purposes are well recognized. To overcome these problems, the Triple Line Sprinkler System was used for the field experiments of the present work. Other alternatives involve using experimental designs and statistical techniques designed to reduce the (extremely large) environmental variation, such as nearest neighbour models (Bartlett, 1978; Wilkinson *et al.*, 1983). However, some of these analyses are rather complicated to perform, and they have not been properly tested under saline conditions (P.A. Hollington, personal communication).

The TLS was designed to supply a uniform gradient of salinity, with a constant amount of water, between the sprinkler lines. This is actually achieved with great precision at the ground level, as testified by the water collected in the rain-gauges. However, the movement of water and salts in the soil is a rather complex matter, and



the distribution of salinity in the soil resulting from the use of the TLS is not completely satisfactory.

One of the limitations of the system (which in this work was partly overcome by not leaching the soil between seasons) is the relatively late establishment of the salinity gradient in the soil profile. This is not very different from the situation found in many salt-affected soils of arid and semi-arid regions. There, the growing season begins with low salinity after the autumn rainfall has diluted the salts, and salinity levels in the soil increase with time as a result of continued evapotranspiration. However, for salt tolerance studies, a uniform salinity during the growing season would be more appropriate, so that conditions can be standardized.

The irregular distribution of salinity with depth in the TLS (where only the top 50 cm has relatively high salinity) is another limitation. It is usually said that roots tend to extract water from the least saline areas of the soil (see Meiri, 1984, for references). A recent (1993) survey of root growth patterns of one barley variety (Albacete) in the TLS showed that between 95% and 98% of the roots were found in the top 50 cm in treatments 1 and 5, while this proportion decreased to 73% at treatment 9 (Cantero, unpublished results). In this last treatment, another 20% of the root volume was found between 50 and 75 cm, which agrees with the above idea of roots growing in the areas of lower salinity. Nevertheless, this type of distribution of salt with depth (inverted profile) is found in some natural salt-affected soils when a saline water table exists close to the surface and salts are transported upwards by capillary flow. A similar situation occurs when crops are irrigated with low quality (saline) water: depending on the level of leaching and drainage salts may accumulate in the top layers (as in the case of the TLS). This effect is intensified by evaporation of water from the surface between irrigations; therefore, more frequent irrigations may help to alleviate the problem. (In the present case, however, the frequency of irrigation is usually limited by the weather, in particular strong winds.)

Two other problems were found in the TLS when comparing results from the 2 years of experiments. First, there were inconsistencies in the measurement of soil salinity depending on whether this was based on the EC of the saturated extract or that of a

1:5 soil:water extraction. An examination of the results over the last few years revealed that the correlation between these two types of measurement ( $EC_{se}$  and  $EC_{1.5}$ ) varied from year to year. This is probably due to the variability associated with the preparation of the saturated extract samples. It is well recognized (e.g. Aragüés & Millán, 1986) that it is difficult to obtain consistent samples of saturated extracts, particularly when the preparation is carried out by different people.

A second problem was related to the poor fit of the models in the 1992/93 experiment, compared to the previous year. This was attributed to the smaller size of the plots. Similar results had been found by Royo and Aragüés (1993) when comparing yields from different plot sizes: smaller plots (2-3 rows) had lower  $r^2$  (an indication of the goodness of fit) than larger plots (6 rows). Still, these authors did not find significant differences in the  $EC_{50}$ s estimated from plots of different sizes, and concluded that 2-row and 3-row plots can be used with reliable results. A more critical look at their results, however, reveals rather large differences between the estimates of  $EC_{50}$  obtained from the different plots, at least in some varieties (up to  $4.4 \text{ dS m}^{-1}$  -c.v. > 16%- in Albacete); the lack of significance of these differences was due to the large standard errors of the estimates (from  $0.9$  to  $3.0 \text{ dS m}^{-1}$ ). Thus, the assumption made by those authors that the size of the plot does not affect the results has to be taken with some caution.

*- Use of models to measure salt tolerance:*

As mentioned before, different authors do not agree on the best measurement of salt tolerance. Maas and Hoffman (1977) proposed to simplify the sigmoidal response of plants to salinity by the use of a threshold model. In this model, two parameters are necessary to define a variety's response: the threshold and the slope. In the sigmoidal model of Van Genuchten (1983), not two but three parameters are needed. Nevertheless, the introduction by these authors of the concept of the  $EC_{50}$  was helpful, since this is a measure of tolerance in relative terms: the salinity which reduces yield to half of that without salinity (maximum yield). (The Maas and Hoffman model can also be expressed in relative terms, and the  $EC_{50}$  calculated from the slope and threshold.) This parameter ( $EC_{50}$ ) might be useful for an overall

comparison between varieties, when no reference is made to particular field conditions (levels of salinity). However, once the environment where the variety is to be grown is known (range of salinities), the concepts of threshold and slope (or the shape of the curve in the Van Genuchten model) are still needed, since varieties with a similar  $EC_{50}$  may perform very differently at more extreme salinities. That is, for a given environment, where the range of soil salinities might be relatively small, the yields of the varieties at different salinities need to be considered to decide on the best one for those particular conditions, and these cannot be estimated from the  $EC_{50}$  alone.

In the 1991/92 experiment, the  $EC_{50}$  was the best parameter at discriminating between varieties. Royo *et al.* (1991), comparing different response models, also concluded that this is the best parameter for evaluating the salt tolerance of crops, since the values of  $EC_{50}$  estimated from different models were very similar. The precision in the estimation of the threshold depends on the number of data-points studied above and around it. Since the lowest salinity levels in the TLS are already relatively high ( $EC_{se} = 2.5-3 \text{ dS m}^{-1}$ ) it is difficult to get an accurate estimate of the threshold in those conditions. In the case of the slope, poor estimates result from inaccuracies at the two ends of the straight line; at high salinities because the slope tends to decrease, and at low salinities because of uncertainties in the threshold. Additionally, the use of only one parameter ( $EC_{50}$ ) to measure salt tolerance has obvious advantages over the need for two (or more) parameters. However, these advantages do not seem to be widely recognised as yet, since most authors still use the better known concepts of threshold and slope. Even in a paper where the sigmoidal model (and the SALT programme) of Van Genuchten were used (Janzen & Chang, 1987), the authors described their results by using the concept of threshold.

*- Measurement of salt tolerance in nutrient solution:*

Since it is difficult to test numerous genotypes for salt tolerance in the field (whether in natural saline soils or under artificially salinized plots), most studies on salt tolerance are done by growing the plants in nutrient solution to which NaCl has been

added. Tolerance is then often based on vegetative growth rather than on yield, because of the difficulty of obtaining reliable estimates of yield under such artificial conditions. However, vegetative growth does not necessarily correlate well with yield under saline conditions. In many crop species (including barley) vegetative growth is more sensitive to salinity than reproductive growth (Läuchli & Epstein, 1990). In barley in particular, Lynch *et al.* (1982) reported a different ranking of cultivars for salt tolerance depending on the development stage considered: biomass production at early growth, or grain yield at harvest.

In this work, discrepancies between assessments of salt tolerance by means of vegetative growth and grain yield can also be found when comparing results from hydroponic experiments (e.g. "Comparison of varieties", in Chapter 2) and from field experiments (Triple Line System, Chapter 4). In hydroponics, varieties Chevron and Barbarrosa had the largest dry weights under salinity, while CM-67 was intermediate, and Igri and Albacete had the smallest dry weights. On the other hand, grain yield at high salinities (treatments 7 and above in the TLS) was highest for CM-67, intermediate for Igri and Albacete, and lowest for Barbarrosa and Chevron. Clearly, the ranking of varieties by early growth (hydroponics) and final yield (field) do not agree.

Some of the causes for these differences have already been mentioned: Chevron has a lush vegetative growth but poor grain yield (resulting in very low harvest index); Albacete does not seem to grow well in hydroponics, while it is one of the preferred varieties for dry conditions in Spain. Differences in rate of development between cultivars is another factor affecting comparisons during the vegetative stage. A further complication arises from the fact that some varieties are winter types (i.e., require vernalization) while others are spring types (no vernalization required). Growing plants up to maturity in the greenhouse would entail artificially vernalizing them at the seedling stage, which is not always feasible. As an example, Albacete and Barbarrosa grow rather slowly initially (as reflected in their dates of sampling in the TLS 1992/93 experiment, Appendix 4), while CM-67 is much faster. Since the latter does not require vernalization, in the warm conditions of the greenhouse it was sometimes found to be booting in just 5 or 6 weeks after sowing (e.g. "Osmotic

adjustment" experiment 2). Comparisons of varieties based on dry matter production after only a few weeks of growth are, thus, heavily biased by these differences in intrinsic growth rate. At the final harvest, however, these differences do not seem to be that important, since no clear relationship between length of cycle and grain yield was found (see section 4.3.4.4).

Rawson *et al.* (1988) have already addressed the question of how well genotypes grown in artificial conditions (sand culture) reflect their salt tolerance in the field. In that study final grain yield was not considered, because differences in development rate between varieties were too large and they would have biased the results. Comparisons were based on biomass yield (dry weight) of plants harvested at the 7-leaf stage and at ear emergence (i.e., later than in the experiments reported here). They found that the ranking of varieties correlated well with published field data only when plants were allowed to follow their normal phenologic development (in spite of large differences between genotypes). On the other hand, when plants were given vernalization and long photoperiods in order to accelerate floral development (and hence make the cultivars more similar in their development), the ranking obtained was rather different. According to these authors, differences in development rate do not seem to be an obstacle in the evaluation of salt tolerance through measurements of dry weight. (However, in the 'normal development experiment', where their results agreed with the literature, only 5 varieties of barley were tested, the rest being wheats and triticales. The overlapping of species in the ranking makes the direct comparison with published field data more difficult.)

An interesting result of the work of Rawson *et al.* (1988) was the finding that cultivars ranked similarly regardless of treatment; that is, with or without salinity. The authors concluded that the amount of biomass produced by a genotype in saline conditions was largely dependent on its intrinsic growth rate. Similar results were found in the comparison of varieties in hydroponics in this work (Chapter 2). However, in the TLS 1992/93 experiment (Chapter 4), where more varieties were studied, some changes in the rankings between low and high salinities were found. This was done by comparing rankings based on maximum yields (and also on mean yields in treatments 1 to 3) and those based on average yields at high salinities

(treatments 7 to 9). Thus, although yield potential probably determines the yield under salinity in many genotypes, a degree of salt tolerance also exists in some varieties.

#### - Salt tolerance of barley varieties

The need to select for salt tolerance in crops has sometimes been questioned (e.g. Richards, 1983). He argued that because most of the yield from patchy saline soils comes from the least saline areas, breeding for high yield potential would result in higher overall yields than specifically breeding for salt tolerance. His arguments were already discussed in Chapter 1 (section 1.5.3). The fields that he considered to be of 'medium' salinity had 73% of the land with an EC of the soil saturation extract less than 4 dS m<sup>-1</sup> (i.e., not saline); even a 'badly' salinized field had more than 50% of its land with EC<sub>se</sub> below 4 dS m<sup>-1</sup>. Under these conditions it is easy to see why any increase in yield at low salinities, however small, will soon outweigh any yield gains at higher salinities.

Richards (1983) also based his calculations on the assumption that selection for salt tolerance alone might increase the threshold or decrease the slope, but that it would not change the maximum yield. If increases in yield potential could be obtained at the same time than increases in salt tolerance (joint selection for salt tolerance and maximum yield), then the expected yield gains would be equal or greater than those obtained by selecting only for yield potential. He argued, then, that this is not only more difficult to achieve in terms of breeding effort, but also that it might not even be possible, because of a "yield penalty" associated with higher salt tolerance. This conclusion was based on a previous finding of a significant inverse relationship between yield at low salinities and the slope of the response line (Richards *et al.*, 1987).

This type of negative relationship has been observed a few times (McColl, 1987, cited by Jana, 1991,1993). This does not imply, however, that the two traits (yield potential and salt tolerance) are irreconcilable. In the present work this aspect was considered in section 4.3.4 (Figure 4.3.8). Only a weak (although significant)

correlation was found. This is in agreement with the above-mentioned comparison of cultivar rankings for yield at high and low salinities. And the conclusion is, again, that the ability to grow (and yield) under saline conditions is not directly linked to yield potential *per se*. Thus, there is scope for improving salt tolerance in barley.

*- Measurement of ion concentrations: leaf age and position*

Compared to the assessment of salt tolerance, the measurement of ion concentrations in leaves is an easier task, particularly under the standard conditions of hydroponic culture. Results from the "Comparison of varieties" experiment (Chapter 2) were in good agreement with previously published data. However, when the first results from leaves sampled in the field (TLS) were obtained (from experiments not reported in this work) some discrepancies were observed. These were traced back to differences in the age (position) of the leaves sampled, and the proportion of  $\text{Ca}^{2+}$  in the saline water, and prompted an investigation into the effects of these factors.

In the TLS 1991/92, one of the objectives was to study ion concentrations in different leaves, from the early ones (number 4, 6), which corresponded to the type of leaves analyzed in the hydroponic experiments, to the last one (flag leaf). In all varieties studied, independently of their level of salt tolerance, the flag leaf was found to be very well protected from toxic ions (low concentrations of  $\text{Cl}^-$  and  $\text{Na}^+$ ), while it maintained high concentrations of  $\text{K}^+$  ( $\approx 250 \text{ mol m}^{-3}$ ). On the other hand, differences between varieties were consistent when other leaves, sampled at earlier stages (leaves 4 and 6), were compared.

The role of the stem in ion partitioning between leaves under salinity was studied by Wolf *et al.* (1991). These authors found that, after stem elongation, lower concentrations of  $\text{Na}^+$  were being delivered to the leaves located at higher positions. (Before stem elongation, similar concentrations were found in the xylem sap reaching different leaves (Wolf *et al.*, 1990).) Their model agrees with the pattern of ion concentrations found in the flag leaf. The practical implication of these differences between leaves at different positions was the realisation that, if differences in ion concentrations between varieties were to be detected, lower leaves had to be sampled

at an early stage of development. This was applied in the following field experiment (1992/93).

Compounded with leaf position is the effect of leaf age. A non-uniform distribution of  $K^+$  and  $Na^+$  between leaves of plants growing under salinity is usually found, with low concentrations of  $Na^+$  and high concentrations of  $K^+$  in young, developing leaves, and the opposite trend for mature leaves. In barley, these differences result from a high  $K/Na$  selectivity of phloem loading, which allows for a significant retranslocation of  $K^+$  from old to young leaves while limiting the export of  $Na^+$  from mature leaves (Wolf & Jeschke, 1987). With time,  $Na^+$  accumulates in the older leaves, resulting in the observed differences between young and old leaves. The salt tolerance of variety California Mariout has been attributed to this ability in partitioning  $Na^+$  and  $K^+$  between leaves. Thus, it seemed interesting to study ion concentrations in both young and old leaves, to see if differences between varieties existed. In order to do that, the leaves in the 1991/92 TLS experiment were sampled at two stages, as young (recently expanded) leaves, and as old (mature) leaves.

In the hydroponic experiments, differences between young and old leaves were as expected: low  $Na^+$  (and  $Cl^-$ ) concentrations in young leaves, high concentrations in older ones, and the opposite for  $K^+$ . CM-67 proved to be very efficient in this partitioning of ions between leaves, while in Chevron differences between leaves of different age were not so distinct (e.g. "Osmotic adjustment" experiments, Chapter 2). The inability of this variety to maintain high concentrations of  $K^+$  under salinity is probably one of the main factors contributing to its poor salt tolerance.

Differences between young and old leaves in the TLS experiments were generally more pronounced than in the hydroponic experiments, particularly in some varieties. In this case, however, this was probably the result of ion absorption directly through the leaves, rather than efficient partitioning between leaves. Some recent experiments (Aragüés *et al.*, 1994; Gorham *et al.*, 1994) have shown that the extent of ion absorption via leaves in the TLS can be considerable, in spite of the pre- and post-washings with fresh water. At the time of sampling, older leaves had been exposed to saline irrigations for a longer time, and it has to be remembered that the ions



present in the saline irrigation water ( $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{Ca}^{2+}$ ) are very immobile in the phloem.

To further complicate matters, the degree of leaf ion absorption was found to vary widely for different genotypes; as an example, varieties CM-67 and Chevron showed completely opposite patterns of salt absorption via leaves than via roots (Gorham *et al.*, 1994). In a more recent experiment designed to compare ion absorption via leaves and via roots in the same varieties as in the TLS 1992/93 experiment (Aloy-Lleonart and Gorham, unpublished), it was found that the accumulation of  $\text{Na}^+$  in leaves of Forrest, CM-67 and Olivia was double when they received the salts ( $200 \text{ mol m}^{-3} \text{ NaCl} + 100 \text{ mol m}^{-3} \text{ CaCl}_2$ ) by leaf spray than when they were applied to the soil. It is interesting to note that these varieties (particularly Forrest and Olivia) always had the highest rates of ion accumulation in older leaves in the TLS experiment. In the other varieties, the rate of ion absorption by leaves was similar to or lower than that via roots, but in no case was the amount absorbed by leaves negligible.

The above-mentioned experiment involved several differences from the TLS, which make a direct comparison of the results difficult. First, the salinity applied was higher than the levels used in the TLS, and the leaves were not pre-wetted nor post-washed with fresh water. This probably resulted in higher rates of absorption by leaves than in the TLS, since the rinsing of the leaves with fresh water was found to significantly reduce foliar absorption in the TLS (Aragüés *et al.*, 1994). Secondly, the plants experienced only one type of salinity stress, either in the roots (soil) or in the leaves (spray), whilst in the field both types are acting, and it seems reasonable to expect some interactions between the two (see Grattan *et al.*, 1994). However, the results are still significant from the point of view of varietal differences.

Another source of differences between measurements of leaf ion concentrations in the field and the hydroponic (greenhouse) experiments is related to the environmental conditions in the two cases. Although the effect of sampling at different times of day was found to be negligible in the TLS (see section 4.2.2), sampling on different days may be a source of variation. This aspect was not considered in the TLS

experiments, since it was not possible to do all the samplings on a single day. It does not seem unreasonable to expect differences in ion concentrations between those leaves sampled soon after a saline irrigation and those sampled after a period of rain (dilution of salts), particularly when considering the importance of ion absorption by leaves.

More general climatic effects will also add to these differences. High temperatures, low humidities and, particularly, strong winds at the site of the TLS will increase transpiration rates in the field-grown plants and may indirectly increase the rate of absorption of ions. That the water relations of plants in the field were different from those in the greenhouse can be seen from the data on water contents. Fresh weight to dry weight ratios of around 10-12 and 6-8 were found for the control and saline-treated plants respectively in the hydroponic experiments. In the TLS, these values decreased to 3-4 in almost all treatments (data not presented here).

*- Ion concentrations: effect of high  $Ca^{2+}$ :*

Another source of differences in leaf ion concentrations between plants grown in the field and in hydroponics is related to the higher proportion of  $Ca^{2+}$  in the saline irrigation waters used in the TLS (around 2:1 Na:Ca molar ratio). As mentioned earlier (section 4.1), this is done in order to maintain a SAR below 15 equivalents  $m^{-3}$  and to avoid the accumulation of  $Na^{+}$  in the soil (the same field has been used for the TLS experiments for several years). On the other hand, a 20:1 Na:Ca ratio is routinely used in the hydroponic experiments in Bangor, to cover the extra amount of  $Ca^{2+}$  needed in saline conditions to prevent an indiscriminate entry of  $Na^{+}$  (Rengel, 1992).

Although this 20:1 ratio was found to be enough for this purpose (see "Minimum  $Ca^{2+}$ " experiment, section 3.2), the effect of even higher external  $Ca^{2+}$  in decreasing  $Na^{+}$  levels in the shoot was not expected. It has to be said, though, that the effects of high concentrations of  $Ca^{2+}$  on  $Na^{+}$  absorption have not been reported in much detail in the literature. Most studies investigating the effects of high external  $Ca^{2+}$  concentrations on salinity have been done by replacing  $Na^{+}$  by  $Ca^{2+}$  (i.e., changing

Na:Ca ratios) to maintain a constant level of salinity. In these cases, the effect of increasing  $\text{Ca}^{2+}$  is confounded with that of decreasing  $\text{Na}^+$ . One of the exceptions is the work by Imamul Huq and Larher (1984) with cowpea (*Vigna sinensis*), where several NaCl concentrations (up to  $150 \text{ mol m}^{-3}$  NaCl) were studied in the presence or the absence of added  $\text{CaCl}_2$  (at a constant Na:Ca ratio of 5:1). In that work, concentrations of  $\text{Na}^+$  in the shoot were always lower in the "added  $\text{Ca}^{2+}$ " treatments; (treatments without extra  $\text{Ca}^{2+}$  only had the concentration of  $\text{Ca}^{2+}$  already in the nutrient solution, which was  $1.5 \text{ mol m}^{-3}$ ).

Growth of plants was also improved at all levels of salinity in the above mentioned work (Imamul Huq and Larher, 1984). In the present study, growth can be compared at similar levels of external  $\text{Na}^+$  and different amounts of external  $\text{Ca}^{2+}$  in the first "Sodium versus Calcium" experiment (sections 3.4.3.1 and 3.4.3.2). There, this response was not found. One of the reasons for these differences might be related to the fact that the above authors worked with a dicotyledonous plant, and it is well known (Loneragan & Snowball, 1969) that these need more  $\text{Ca}^{2+}$  for optimum growth than monocotyledons, at least under normal (non-stressed) conditions. It is not unreasonable to think that a similar situation may be found in salinity, although no direct comparisons between monocots and dicots seem to exist in this respect.

This effect of high  $\text{Ca}^{2+}$  may have some practical implications for field conditions. It is usually said that no extra  $\text{Ca}^{2+}$  is needed in saline (non-sodic) soils, since their levels ( $5\text{-}15 \text{ mol m}^{-3} \text{ Ca}^{2+}$ ) are above that minimum considered necessary under salinity ( $2 \text{ mol m}^{-3} \text{ Ca}^{2+}$ ). However, if higher  $\text{Ca}^{2+}$  concentrations are found to improve growth in salt-affected soils, the addition of extra  $\text{Ca}^{2+}$  might need to be considered. This subject, though, needs further investigation, since no evident beneficial effects of added  $\text{Ca}^{2+}$  under saline field conditions have been reported (see review by Grattan & Grieve, 1992).

An interesting result was reported by Gorham *et al.* (1994) in relation to the interactions between  $\text{Na}^+$  and  $\text{Ca}^{2+}$ : high  $\text{CaCl}_2$  concentrations (in addition to NaCl) had the opposite effect when they were applied by means of leaf sprays than when they were supplied by the nutrient solution (via roots). That is, they increased  $\text{Na}^+$

concentrations in leaves, instead of reducing them. This finding adds another complication in the comparison between the hydroponic experiments and those in the TLS.

- *Osmotic adjustment:*

Two of the experiments investigated the degree of osmotic adjustment of barley under salinity and the type of solutes used. The two varieties considered (CM-67 and Chevron) differ largely in their salt tolerance, but both of them were found to adjust their internal osmotic pressure in response to external salinity, suggesting that this is not the main cause of reduced growth in these conditions.

Although the osmotic pressures of the extracted saps increased with salinity, as did the concentrations of  $\text{Cl}^-$  and  $\text{Na}^+$ , in most experiments reported in this work a large reduction in the plant's water content was observed. It was concluded, in accordance with other authors (e.g. Storey & Wyn Jones, 1978), that this is the main mechanism of osmotic adjustment in barley. When the ion concentrations were expressed on a dry weight basis it was found that the total concentrations of ions did not change. Only a net accumulation of  $\text{Cl}^-$  and  $\text{Na}^+$  was found (see table 2.3.6), but at the expense of other ions (mainly  $\text{NO}_3^-$  and  $\text{K}^+$ ). The main contribution to the osmotic pressure of leaf sap was calculated to be that of  $\text{Na}^+$  and  $\text{Cl}^-$  (see section 2.2.3). Other ions ( $\text{K}^+$  and  $\text{Ca}^{2+}$ ), sugars and, to a lesser extent, glycinebetaine also contributed to the measured osmotic pressure.

It is worth noting that proline, one of the putative "compatible solutes", does not seem to have an important role in the response of these varieties to salinity. It has been reported that proline begins to accumulate when the concentrations of  $\text{Na}^+$  plus  $\text{K}^+$  are above  $200 \mu\text{mol g}^{-1}$  fresh weight (Weimberg *et al.*, 1982, 1984 for sorghum; Weimberg & Shannon, 1988, for *Thinopyrum elongatum*). If the content of dry matter in barley leaves is about 10%, that value would be around 220 mol  $\text{Na}^+$  plus  $\text{K}^+$  per  $\text{m}^3$  sap. Since concentrations of  $\text{K}^+$  in young leaves of non-salinized barley plants already reach similar levels, any increase in leaf  $\text{Na}^+$  resulting from high external salinity would trigger the accumulation of proline, unless it is accompanied

by a concurrent decrease in  $K^+$ . In the experiments reported here (section 2.3), concentrations of  $Na^+$  plus  $K^+$  were around  $300 \text{ mol m}^{-3}$ , but no accumulation of proline was observed. It seems, thus, that the above threshold level ( $200 \text{ mol m}^{-3}$ ) does not apply to barley. Voetberg and Stewart (1984) already remarked that unstressed barley leaves had concentrations of  $Na^+$  plus  $K^+$  which were very close to that threshold.

- *General conclusions:*

This study was set up to determine whether the concentrations of ions in leaves of plants grown under salinity were a reliable indicator of their salt tolerance. Although the regulation of salt balances in leaves is an important aspect of salt tolerance, no clear relationships between ion concentrations in leaves and salt tolerance were found in the experiments reported here. In the field experiments this lack of correlation was partly due to the system used (sprinkling with saline water), which introduced a new factor (salt absorption by leaves) and complicated the original model of salt "exclusion" vs salt "inclusion". This difficulty did not arise when plants were grown in hydroponics where leaf ion concentrations resulted only from selective absorption by roots and controlled transport to the shoot. Still, no correlations were found.

One of the reasons for this lack of correlation may be that ion concentrations were determined on whole leaf extracts, and these measurements do not give any indication about the location of these ions within the leaf or the cell. The varieties studied might have differed in their ability to compartment ions at a lower level than the leaf unit considered here (e.g. by accumulating them in the epidermal cells, or in different cell compartments: cytoplasm, vacuole, cell wall), and these differences cannot be detected by analysis of whole leaf extracts. It has to be noted, however, that this type of measurement was chosen for its simplicity, the final aim being its use as a criterion for selecting salt tolerant varieties. Even though finer measurements (at the tissue and cellular level) are now possible, the need for sophisticated techniques and apparatus (X-ray microanalysis, single-cell sampling, etc) may preclude their use in the screening of large numbers of genotypes.

According to the bi-phasic model of Munns and Termaat (1986), ion toxicity in older leaves is the cause of premature death of plants in salinity. However, in salt-tolerant species such as barley, this might only happen at very high salinities, where plants do actually die of excess ions. No such extreme cases were found in the experiments reported here (except in the case of Chevron at  $200 \text{ mol m}^{-3} \text{ CaCl}_2$ , section 3.3, experiment 1), and this may be another reason why ion concentrations in leaves did not correlate well with yield. At moderate salinities, the accumulation of ions in the cytoplasm and/or the cell wall of old leaves may kill a few leaves, but not the whole plant. Growth is the main parameter affected in these conditions, and this is probably the result of the osmotic (rather than the toxic) component of salt stress. The reduction in leaf area available for photosynthesis, resulting from decreased growth and aggravated by the premature senescence of older leaves, would then be the main cause of decreased yields in salinity.

Whatever the reason for the lack of correlation between leaf ion concentrations and yield under saline conditions, it has to be concluded from the present results that this trait (ion concentrations in leaves) is not a reliable indicator of the salt tolerance of a genotype. Since one of the conditions for indirect selection to be effective is that a high correlation exists between the two related traits, the measurement of whole leaf ion concentrations in either solution culture or field grown plants cannot be recommended as a selection criterion in breeding for salt tolerance.

On the other hand, even though reduced vegetative growth is probably the main cause of yield reductions in salinity, no clear relationships were found between early growth and final yield. This trait, however, was not studied in detail in this work, partly because of the very different environmental conditions between the greenhouse facilities in Bangor and the field trials in Spain. Under more standardized conditions, a better relationship might have been obtained, since some degree of correlation probably exists between vegetative growth and grain yield. The difficulty lies in obtaining accurate estimations of salt tolerance based on yield, so that these correlations can be detected. More work needs to be done in this area before rejecting the measurement of early growth (e.g. leaf area) as a criterion to select for in breeding for salt tolerance. Rawson *et al.* (1988) already suggested that the area

of leaf 3 was a good indicator of salt tolerance. It has to be remembered, though, that they measured salt tolerance as biomass yield at ear emergence. Although this trait is probably correlated with final grain yield, those authors did not prove this relationship.

The interest in physiological traits related to salt tolerance came from the realization that yield is not a good measurement of tolerance, since many other factors affect final yield, particularly in field experiments. However, in the absence of better indicators of salt tolerance, yield might need to be the criterion used. And yield has to be measured both in saline and non-saline conditions, since it is not clear (from the results presented here) that yield under salinity depends only on yield potential.

For yield to be measured at different levels of salinity, the TLS used in these experiments did not prove to be a useful tool. The different problems encountered with this system have already been discussed, and only the most important ones will be outlined here.

- 1) it does not provide a uniform salinity over time (late establishment of salinity gradient), nor in depth (inverted profile);
- 2) the lowest level of salinity obtained depends on the salinity of the fresh water used, and if this is relatively high (as in Zaragoza) not enough treatments would be placed below the threshold level, making the estimation of this parameter difficult.
- 3) in spite of the pre- and post-washings with fresh water, a considerable degree of leaf absorption results from sprinkling with saline water. This problem would not be so crucial if ion absorption by leaves was similar in all varieties, but this is not the case.
- 4) the use of high concentrations of  $\text{CaCl}_2$  (in addition to  $\text{NaCl}$ ) in the irrigation water to avoid alkalization of the soil adds a further complication for the comparison with natural saline soils.  $\text{Ca}^{2+}$  applied by sprinkler irrigation has a different effect on the ion relations of the plant than the addition of  $\text{Ca}^{2+}$  to the soil.

The TLS may still be useful in those cases where the source of salinity is the irrigation water, and where sprinkler systems are used for irrigation. However, if the interest is in comparing the varieties' response to soil salinity, the TLS should not be used, unless the problems listed above are solved.

If breeding for salt tolerance is to be done in the field at different levels of salinity and line-sprinkler systems cannot be used, then other ways to control soil salinity have to be devised. Drip-irrigation systems with water of known levels of salinity may be a solution, although they might prove a bit difficult to implement. Some studies are currently being done into this subject in the Agronomic Research Service of the Aragón Autonomous Government (SIA-DGA) in Zaragoza. The development and testing of statistical procedures to overcome the problems associated with high heterogeneity in saline soils is another useful approach to dealing with the present limitations. If they have not yet been properly tested is because they need to be complemented with detailed monitoring of soil salinity and the establishment of salinity maps for each field where trials are conducted. However, with the equipment currently available for the measurement of soil salinity this aspect should not be a great limitation. The application of these methods to naturally saline soils of India and Pakistan is presently being investigated in the Centre for Arid Zone Studies of the University of Wales, Bangor.



# **APPENDICES**

Appendix 1. Irrigation data for the 1991/92 TLS experiment. The amount of applied water is the mean (and standard errors) of 10 rain-gauges. Minimum and maximum ECs correspond to the lowest and highest of the 10 salinity treatments. When a fresh-water irrigation was given the EC was not measured, and thus it is not shown. (These data do not include rainfall.)

Irrig. N°	Date (DD.MM.YY)	Amount (mm)	Duration (min)	EC (dS m <sup>-1</sup> )	
				min.	max.
1	25.11.91	8.8 ± 0.5	25	F.W.*	F.W.
2	21.01.92	15.9 ± 0.4	41	2.0	15.7
3	27.01.92	19.0 ± 0.4	48	1.7	14.5
4	30.01.92	15.9 ± 0.5	38	2.3	15.6
5	06.02.92	15.9 ± 0.8	42	1.9	17.5
6	11.02.92	13.0 ± 0.7	36	1.7	18.1
7	14.02.92	12.2 ± 0.9	40	F.W.	F.W.
8	18.02.92	9.8 ± 0.6	28	F.W.	F.W.
9	24.02.92	5.2 ± 0.5	15	F.W.	F.W.
10	28.02.92	7.3 ± 0.5	20	F.W.	F.W.
11	04.03.92	10.9 ± 0.7	30	2.1	13.1
12	10.03.92	12.6 ± 0.4	36	4.4	13.2
13	16.03.92	5.2 ± 0.4	20	F.W.	F.W.
14	17.03.92	9.7 ± 0.2	30	2.6	13.2
15	20.03.92	15.0 ± 0.5	44	2.4	10.3
16	26.03.92	14.2 ± 0.5	39	1.6	10.0
17	31.03.92	15.3 ± 0.4	42	1.7	11.5
18	03.04.92	11.9 ± 0.4	32	3.8	11.8
19	07.04.92	12.3 ± 0.5	35	2.0	12.9
20	14.04.92	15.8 ± 0.3	44	3.2	11.8
21	20.04.92	15.3 ± 0.6	42	2.0	11.0
22	24.04.92	13.2 ± 0.4	39	3.0	12.8
23	27.04.92	13.5 ± 0.6	39	2.2	10.9
24	04.05.92	12.2 ± 0.5	35	2.1	10.8
25	08.05.92	10.6 ± 0.5	30	F.W.	F.W.
26	12.05.92	14.7 ± 0.4	40	3.5	12.3
27	15.05.92	12.7 ± 0.4	38	4.5	13.2
28	18.05.92	15.2 ± 0.3	46	3.8	12.0
29	01.06.92	12.8 ± 0.3	15	3.1	12.9

\* "Fresh" water (EC of well water ≈ 2 dS m<sup>-1</sup>).

Appendix 2. Dates of sampling for the different varieties and treatments in the TLS 1991/92 experiment. Dates are given as DD.MM (day-month); all dates refer to the year 1992.

VARIETY Treatment	1st sampling		2nd sampling		3rd sampling	
	Rep-I	Rep-II	Rep-I	Rep-II	Rep-I	Rep-II
<b>ALBACETE</b>						
T-1	25.02	25.02	16.03	09.03	23.04	23.04
T-2	27.02	27.02	23.03	18.03	21.04	21.04
T-3	26.02	26.02	24.03	24.03	21.04	19.04
T-4	27.02	27.02	25.03	25.03	19.04	15.04
T-5	02.03	02.03	28.03	30.03	17.04	15.04
T-6	03.03	03.03	19.03	23.03	19.04	15.04
T-7	03.03	03.03	23.03	24.03	21.04	21.04
T-8	06.03	n.s.*	28.03	n.s.	23.04	24.04
T-9	06.03	n.s.	16.03	24.03	23.04	24.04
<b>CHEVRON</b>						
T-1	25.02	25.02	16.03	13.03	16.04	16.04
T-2	27.02	27.02	19.03	18.03	15.04	15.04
T-3	25.02	25.02	23.03	23.03	17.04	17.04
T-4	02.03	27.02	25.03	25.03	17.04	15.04
T-5	02.03	02.03	30.03	30.03	22.04	19.04
T-6	n.s.	03.03	06.04	06.04	24.04	23.04
T-7	n.s.	n.s.	13.04	08.04	n.s.	24.04
T-8	n.s.	n.s.	n.s.	13.04	n.s.	27.04
T-9	n.s.	n.s.	n.s.	n.s.	n.s.	28.04
<b>CM-67</b>						
T-1	26.02	26.02	11.03	09.03	08.04	10.04
T-2	27.02	27.02	13.03	11.03	09.04	09.04
T-3	02.03	27.02	16.03	09.03	10.04	08.04
T-4	02.03	02.03	11.03	11.03	08.04	06.04
T-5	03.03	03.03	12.03	12.03	06.04	02.04
T-6	n.s.	03.03	18.03	18.03	06.04	01.04
T-7	n.s.	06.03	n.s.	18.03	10.04	06.04
T-8	n.s.	06.03	n.s.	19.03	n.s.	09.04
T-9	n.s.	06.03	n.s.	19.03	n.s.	06.04

\* n.s. - not sampled.

Appendix 3. Irrigation data for the 1992/93 TLS experiment. The amount of applied water is the mean (and standard error) of 20 rain-gauges. Minimum and maximum ECs correspond to the lowest and highest of the 10 salinity treatments. (These data do not include rainfall.)

Irrig. N°	Date (DD.MM.YY)	Amount (mm)	Duration (min)	EC (dS m <sup>-1</sup> )	
				min.	max.
1	03.02.93	13.1 ± 0.4	36	1.9	12.6
2	05.02.93	13.3 ± 0.3	36	1.8	11.0
3	08.02.93	12.8 ± 0.2	36	1.8	13.5
4	12.02.93	13.5 ± 0.2	36	1.9	16.5
5	16.02.93	13.1 ± 0.2	36	2.0	18.2
6	19.02.93	12.4 ± 0.6	36	2.0	16.8
7	26.02.93	14.4 ± 0.4	41	2.1	20.0
8	02.03.93	13.3 ± 0.3	39	2.1	18.8
9	04.03.93	12.0 ± 0.3	37	2.1	19.6
10	08.03.93	12.7 ± 0.5	36	2.2	19.1
11	11.03.93	12.9 ± 0.6	36	2.4	21.1
12	15.03.93	12.1 ± 0.4	37	1.9	21.6
13	18.03.93	13.4 ± 0.2	36	2.3	20.0
14	23.03.93	13.8 ± 0.3	38	1.9	18.7
15	30.03.93	13.8 ± 0.4	38	1.8	20.3
16	01.04.93	12.8 ± 0.4	35	1.8	19.6
17	06.04.93	15.8 ± 0.7	44	3.1	17.1
18	08.04.93	13.4 ± 0.4	36	1.9	20.1
19	14.04.93	12.9 ± 0.5	36	1.8	18.8
20	19.04.93	13.4 ± 0.4	39	1.9	21.5
21	22.04.93	11.0 ± 0.3	37	1.9	20.5
22	29.04.93	12.1 ± 0.3	34	2.0	22.4
23	03.05.93	12.7 ± 0.3	38	2.5	21.7
24	06.05.93	13.1 ± 0.3	39	2.5	22.7
25	10.05.93	11.7 ± 0.2	36	3.5	24.4
26	12.05.93	13.2 ± 0.4	39	2.0	20.3
27	17.05.93	12.0 ± 0.4	38	5.2	24.8
28	21.05.93	12.6 ± 0.3	37	2.5	23.9
29	24.05.93	12.0 ± 0.3	36	5.6	25.4
30	28.05.93	13.1 ± 0.3	39	2.9	22.0
31	31.05.93	12.8 ± 0.2	37	2.7	23.6

Appendix 4. Dates of sampling for the different varieties and treatments in the TLS 1992/93 experiment. Dates are given as DD.MM (day-month); all dates refer to the year 1993.

VARIETY	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9
Albacete	09.03	10.03	11.03	22.03	22.03	24.03	25.03	24.03	25.03
Barbarrosa	09.03	10.03	11.03	23.03	26.03	25.03	26.03	26.03	26.03
Begoña	09.03	10.03	11.03	16.03	16.03	21.03	21.03	22.03	24.03
Berta	09.03	09.03	11.03	15.03	15.03	16.03	17.03	21.03	21.03
CM-67	09.03	09.03	10.03	12.03	12.03	12.03	12.03	12.03	12.03
Critter	09.03	10.03	11.03	22.03	22.03	26.03	25.03	24.03	25.03
Forrest	09.03	09.03	10.03	12.03	12.03	12.03	12.03	12.03	12.03
Igri	09.03	10.03	11.03	22.03	22.03	24.03	24.03	26.03	25.03
Mogador	09.03	10.03	11.03	17.03	17.03	16.03	16.03	21.03	22.03
Olivia	09.03	09.03	10.03	13.03	13.03	13.03	17.03	21.03	21.03
Pané	09.03	09.03	11.03	16.03	17.03	17.03	16.03	21.03	21.03
Viva	09.03	10.03	11.03	23.03	24.03	24.03	25.03	26.03	26.03

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