Mestrado em Engenharia Agronómica

Deficiência de fósforo no milho (*Zea mays* L.) e no grão-de-bico (*Cicer arietinum* L.). Efeitos na absorção, transporte e distribuição do fósforo, potássio e nitrato considerados em relação à produção de ácidos orgânicos nas raízes.

Dissertação de mestrado elaborada por:
Paulo Jorge de Matos Vicente
Orientadora
Prof.ª Doutora Alexandra Rosa da Costa

Universidade de Évora
2009

Mestrado em Engenharia Agronómica

Deficiência de fósforo no milho (*Zea mays* L.) e no grão-de-bico (*Cicer arietinum* L.). Efeitos na absorção, transporte e distribuição do fósforo, potássio e nitrato considerados em relação à produção de ácidos orgânicos nas raízes.

Dissertação de mestrado elaborada por:
Paulo Jorge de Matos Vicente
Orientadora
Prof.ª Doutora Alexandra Rosa da Costa

Universidade de Évora

2009

À minha família

Enquadramento do trabalho

A presente dissertação é apresentada no âmbito do Mestrado em Engenharia Agronómica, frequentado ao abrigo do Programa "Vale a Pena ser Mestre". A mesma havia sido por mim apresentada, como tese final de Licenciatura em Engenharia Agrícola, concluída na Universidade de Évora.

A presente tese resultou de um trabalho de investigação realizado na Universidade de Leeds, enquanto estagiário ao abrigo do Programa Erasmus, tendo no desenvolvimento do mesmo tido como orientadores o Sr. Ernest A. Kirkby, por parte da Universidade de Leeds, e o Professor Tomaz Moreira, por parte da Universidade de Évora.

O trabalho que a seguir se apresenta é o mesmo então defendido, com as devidas correcções e actualização gráfica.

Deficiência de fósforo no milho (*Zea mays* L.) e no grão-de-bico (*Cicer arietinum* L.). Efeitos na absorção, transporte e distribuição do fósforo, potássio e nitrato considerados em relação à produção de ácidos orgânicos nas raízes.

Resumo

Plantas de milho *(Zea mays* L. cv. Earliking) e de grão-de-bico *(Cicer arietinum* L. cv. CPS-I) foram cultivadas em solução nutritiva e atmosfera controlada durante um período de 8 e 16 dias respectivamente, e sujeitas a dois tratamentos: solução contendo fósforo (P+) e solução da qual o fósforo estava ausente (P-).

A deficiência de fósforo (P) levou a um aumento do peso da raiz relativamente à parte aérea. Traduziu-se também num aumento do Comprimento Especifico da Raiz (CER).

A deficiência de P reflectiu-se na absorção e distribuição quer do P quer de outros nutrientes, como o nitrato (NO_3^-) e o potássio (K^+). Originou uma redução na concentração de P nas plantas, e uma maior percentagem do P total passou a estar distribuída nas raízes. A distribuição e transporte do K^+ foram também distintamente afectados pelos tratamentos. O NO_3^- foi o ião mais sensível à deficiência de P.

A deficiência de P promoveu um acréscimo na quantidade de ácidos orgânicos presentes nos exsudados radiculares, particularmente no grão-de-bico. Induziu igualmente uma acidificação relativa da rizosfera.

Phosphorus deficiency in maize (*Zea mays* L.) and chickpea (*Cicer arietinum* L.). Effects on acquisition, transport and distribution of phosphorus, potassium and nitrate considered in relation to organic acids production in the roots.

Abstract

Plants of maize *(Zea mays* L. cv. Earliking) and chickpea *(Cicer arietinum* L. cv. CPS-I) were cultivated in nutrient solution and controlled atmosphere for a period of 8 and 16 days respectively, and two treatments were considered: solution containing phosphorus (P+) and solution from which phosphorus was absent (P-).

Phosphorus (P) deficiency resulted in an increase in root weight relatively to the shoot. The Specific Root Length (SRL) also increased.

P deficiency was reflected not only in the absorption of P itself, but also of other nutrients, such as nitrate (NO_3^-) and potassium (K^+). It originated a reduction in the concentration of P in plant tissues and a bigger percentage of the total P was distributed to the roots. The distribution and transport of K^+ were also pronouncedly affected by the treatments. NO_3^- was the most sensitive to P deficiency

P deficiency promoted an increase in the amounts of organic acids present in the root exudates, mainly in chickpea. It also induced a relative acidification of the rhizosphere.

"You may depend upon my bare word, reader, without and further security, that I could wish this offspring of my brain were as ingenious, sprightly and accomplished as yourself could desire; but the mischief of it is, nature will take its course: every production must resemble it's author, and my barren and unpolished understanding can produce nothing but what is very dull, very impertinent, and extravagant beyond imagination. "

(Miguel de Cervantes, Don Quixote)

Acknowledgements

I'm most indebted to Prof. Tomaz Moreira from the Department of Ecology of the University of Évora and to Mr. Ernest Kirkby for making it possible for me to have this unique opportunity to come to such an extraordinary city as Leeds. My many thanks to Prof. Alexandra Costa for being so patient with me and this work, and for her wise and pertinent advices.

I'm also particularly grateful to everyone in the Department of Pure and Applied Biology at the University of Leeds, particularly to Dr. Pilbeam, Dr. Sanders, Ramadam, Khaled and Chris for helping me with their valuable suggestions and constructive criticism.

Also to Mark for helping me to find the reagents, to Ken for germinating the seeds and to Martin for looking after the growth rooms.

This would not be completed without my thanks to my parents, my brothers and sister, and all my friends.

General

Genotypical differences, translated into phenotypical, physiological (type, age and state of the cells) and physic-chemical (water, temperature, pH and the ion concentration in the nutrient medium) differences have an important influence not only over the rate of nutrient uptake but also in the efficiency with which plants use those nutrients in the production of dry matter.

Evolution, driven by the force of natural selection, has furnished plants with efficient mechanisms that permit them to selectively acquire nutrients from the medium were they are growing, resulting that the composition of the plants is different from the composition of the medium were they are developing.

It is quite possible that the most difficult function for early root systems to perform was the acquisition of non mobile resources, especially phosphate (Pirozynsky and Malloch, 1975) and, being so, this nutrient can have played a very important role not only in the evolution of the root systems and the hole plant itself, but also in the distribution of vegetation throughout the world, so that the origin and diversity of the root systems of modem plants can be seen as achieving the more effective performance of these functions.

No wonder that variations among plant species in the ability to absorb soil and fertilizer phosphate have received ample attention in the literature and continue to attract the interest of many researchers.

A further stimulus to this field of research was given some years ago. Because of the increasing problems with pollution caused by the high amounts of chemical fertilizers used in agriculture (due to an agricultural policy that pointed in the direction of high productivity per unit area, using high amounts of inputs) lead to the introduction of the concept of sustainable agriculture. This meant that most of the inputs should/could be replaced by another input: knowledge. This knowledge should provide the clear understanding of how plants transform the inputs into outputs and, of the efficiency with which they do it, so that the ability to manage a farm in a way as profitable as possible could be increased. It became clear that this could only be achieved with the understanding of the mechanisms used by the plants in the acquisition of those inputs and, in this particular case, mineral

nutrients.

The ability of plants to modify the conditions of the medium that surrounds them, particularly the conditions of the rhizosphere, as a response to nutrient stress, is an interesting phenomena surrounded with controversy. The work that is now presented pretends to give its own contribution to the understanding of the wonderful and mysterious world of plant nutrition.

Contents

List of Figures

List of Photos

List of Tables

I - INTRODUCTION:

1.1 - **Chickpea** (*Cicer arietinum* L) (2n=16)

Chickpea, *Cicer arietinum* L., also known as garbanzo bean, Bengal gram or gram is a plant that belongs to the family of Fabaceae. It's an herbaceous, annual, semi-erect plant, 25-50 cm tall. All parts of the plant are covered with clavate glandular hairs. Leaves are imparpinnate. Two types of germination, epigeal and hipogeal, might occur, and the plants are completely self pollinated.

Chickpea is not known in the wild state, but is found as an escape in Mesopotamia and Palestine. The first certain records of cultivation, from Turkey, are dated of 7400 years old. The area of origin of the specie is assumed to be the North East of Africa (Van der Maesen, 1972). The progenitor of the present day *C. arietinum* must have been spread by the Aryans, and around 2000 BC the plant was introduced to India, a country which now produces about 79% of the word's crop. Outside India cultivation is mainly centred around the Mediterranean (Greece, Italy, Portugal, Spain, Morocco, Algiers, Iraq, Iran).

In India, where it is the most important pulse, the whole dried seeds are eaten cooked or boiled in the form of *dhal*, a tradicional Indian recepy. Flour (*basin*) is made by grinding the seeds and is one of the most important ingredients of Indian confectionery. Green pods and tender shoots are used as a vegetable. The dry stems and leaves are fed to the livestock. An acid liquid produced by the glandular hairs is collected by spreading a cloth over the crop at night, which absorbs the exudation that contains about 94% malic acid and 6% oxalic acid. The collected exudate has a reputation in Indian folk medicine as being good for digestive upsets and sunstroke (it's also used as vinegar).

Chickpeas are a nutritious food legume which could well become a much more important high protein food in the future.

1.2 - **Maize** (*Zea mays* L.) (2n=20)

Maize, (*Zea mays* L.), also known as "I*ndian corn*" and in America simply as corn is a plant that belongs to the family of Poaceae, subfamily Panicoidea tribe Maydeae. The name is derived from the arawak-carib word "mahiz".

Maize is a tall annual grass (2-3 m high) with thick, solid stems with clearly defined nodes and internodes, usually supported by prop roots. The leaves are broad and smooth with a conspicuous midrib. Very few tillers are produced. The plant bears separate male and female inflorescences (monoecious plant) which is a very important characteristic for the production of hybrid maize, that is perhaps one of the greatest achievements in crop improvement during the past century. Leaves are born alternately on either side of the stem at the nodes. They are glabrous or pubescent, usually with hairs along upper margins. Maize is cross pollinated and exhibits great heterozygosity.

Of the most important modern cereal grains, maize is the most efficient in converting water and carbon dioxide into foodstuffs (Simpson, 1986), but the maize grain is deficient in the aminoacids tryptophan and lysine, and it's relatively low in total protein, but it contains more oil than any other cereals.

One of the best records of maize domestication comes from archaeological records in the Tehuacan Valley of Mexico. Pollen 80000 years old has been collected from oldlake beds near Mexico City. Maize was unknown to the New World until the time of Columbus.

In terms of yield produced throughout the world maize is at present the third most important grain crop following wheat and rice (Langer and Hill, 1982). The USA (particularly the Corn Belt region of the North Central states) produces almost half of the annual world production, followed by China, Brazil, Mexico, Argentina, and India. (FAOSTAT, 2009)

Over 300 commercial products can be obtained from maize grain. Maize starch, antibiotics, salad oils, gumlike polymers and animal feed are just some of the many possible examples. In South America, maize beer, or *chicha*, is produced by fermenting hydrolysed maize starch. In the United States, maize is also a major ingredient in the production of *Bourbon* and industrial alcohol.

1.3 - Phosphorous, phosphorus deficiency and plants:

Just after nitrogen, phosphorus (P) is considered the second most important nutrient, not only in plant nutrition but also for people and animals who feed themselves on those same plants. The relatively high amounts of P required by plants in association with its extremely low concentration in the nutrient solution leaves us to consider phosphorus as a major plant nutrient (also called macro nutrient). Just to give an idea, the concentration of P in soils is at least 10-2 lower than the concentration of any other major nutrient ions (NO_3^-, NH_4^+, SO_4^{2-}, Ca^{2+}, Mg^{2+} and K^+). From the point of view of plant nutrition, most of the phosphate in soils is virtually inaccessible to plants ("non-labile" fraction) and "available" P represents only a small amount of the total P, which is represented by the phosphate in the soil solution and in the "labile pool" (solid phosphate which is held by surfaces so that it is in rapid equilibrium with the soil solution phosphate) (Kirkby and Le Bot, 1995).

Phosphorus is taken up by plants as $H_2PO_4^-$ or HPO_4^{2-} depending on the pH of the nutrient medium (at physiological pH the dominant form is $H_2P0_4^-$) and after its uptake by plants it remains either as inorganic phosphate (Pi), (its esterified through a hydroxyl group to a carbon chain as a simple phosphate ester (e.g., sugar phosphate)) or attached to another phosphate by the energy-rich pyrophosphate bond P~P (e.g., in ATP). Another type of phosphate bond is the diester (C-P-C), which mainly occurs in more complex or macromolecular structures (Marschner, 1995).

In plants phosphorus is found in higher concentrations in young leaves and their petioles.

The main fractions/functions of phosphorus in plants are:

1-Phospholipids (mainly in membranes, important for the separation of different compartments in the cell);

2-Nucleic acids (carriers of the genetic information);

3-Sugar and nucleoside phosphates (in the cytoplasm, essential in energy metabolism and active transport);

4-Inorganic phosphate (in the cytoplasm (enzyme regulator, exchange processes) and in the vacuole (storage pool));

5-Phytate (storage form, e.g., in seeds).

Plants differ not only in the ability to compete for phosphorus but also in the phase of the vegetative cycle when they most need of it. Normally, a young plant that has produced about 25% of its total fresh weight might have already absorbed/accumulate around 75% of its total phosphorus.

The ability of plants to acquire nutrients from a given nutrient medium may be very different according to genus, species, or even variety and, it's essential for the plants that the root systems have the ability to react to the heterogeneity of the root environment, in other words, they should present phenotypic plasticity.

The availability of nutrients is governed by a complex of soil and plant properties.

This term includes at least two different aspects: availability in a chemical and in a positional sense. Most of the interactions determining the availability of nutrients to plants occur in the root-soil interface, conveniently regarded as the rhizoplane (the root surface). The rhizosphere (for some authors also referred as the hidden half of the hidden half that are the roots), is considered the zone of soil influenced by the roots. The interactions that occur between plants and the nutrient medium make it possible for roots to perform one of their many functions: the acquisition of soil based resources (principally water and dissolved ions).

Although the chemical properties of the nutrient medium (e.g., the pH) are very important for root growth and mineral nutrients availability, the conditions in the rhizosphere and the extent to which roots can modify these conditions playa very important role in mineral nutrient uptake in general (Marschner *et al.*, 1986). By depleting the soil solution to a very low level the roots create almost the maximum possible gradient and thus initiate a strong diffusive flux toward their surface. Hence it can be stated that it is the plant that affects the availability of soil nutrients.

Conditions in the rhizosphere are also of importance for the adaptation of plants to adverse soil chemical conditions, as occur, for example in acid mineral soils (Marschner, 1991b). Being phosphorus supply to plants a major determinant of growth in many environments efficient uptake mechanisms and accumulation of P, for instance in vacuoles, serve to maintain plants with deficient and/or variable P supply.

Hewinkel (1991) outlined the "strategies" for phosphorus acquisition under low supply proposed in the literature. He placed them into four categories:

1 - Higher P efficiency in the plant, by

- using less phosphorus per gram of dry matter produced;
- remobilizing phosphorus from other plant parts (i.e., old leaves).

2 - Adaptation of the uptake mechanism, by

-lowering C_{min} and K_m i.e. better uptake from low concentrations;

- rising V_{max}.

3 - Exploitation of a larger soil volume, by

- reducing the shoot root ratio;
- increasing root hair formation;
- profiting from symbiosis with VA-Mycorrhiza;
- forming proteoid roots.

4 - Influence on soil chemistry in order to increase the solubility of certain P-fractions, by

- releasing chelators for Fe and Al;
- increasing root hair formation;
- elevating Ca^{2+} uptake to balance Ca-phosphate solubility;
- increasing the phosphatase activity on the root surface to make organic phosphorus available;

-lowering the pH (organic acids, protons H^+).

These morphological, physiological and metabolic changes that occur in response to phosphorus deficiency are part of an "adaptation strategy".

The work that is now presented will try to determine how two such different plants as maize and chickpea respond when in the presence of a P-deficient nutrient medium, in terms of morphological and physiological adaptations that are enhanced under phosphate deficiency. This can give us an idea of how efficient these plants can be when it comes to acquire phosphorus from soils with a low P-content. A very special attention will be given to the importance of organic acids not only in maintaining the charge balance in the plant but also to the important role that they might perform in enhancing the acquisition of mineral nutrients, particularly phosphorus, by plants.

II - MATERIALS AND METHODS:

2.1 - Plant Cultivation:

Chickpea (cv. CPS-I) and maize (cv Earliking) seeds were germinated in plastic trays for a period of 12 days using perlite as germination medium. Daily the seedlings received about 2 L of water per tray and in the first day each one of the trays received also 2 g of $CaSO_4$. Research done during the eighties indicates that calcium is very important to obtain a good development of the root system, because it acts as a messenger in signal transduction in plants, particularly in transducting gravity and light signals in plant roots (Pickard, 1985; Poovaiah *et al.*,1987; Evans *et al.*, 1991).

Thirty six seedlings of both species were then selected on a weight basis (Wm=4,26 g, SD=0,233 g ; Wc=3,24 g, SD=0,412 g) and then grown in a growth room for 4 days as a pre-culture period. The first two days in half and the other two in full strength nutrient solution, in 50 L polythene tanks (Photo 1 and 2). The full strength nutrient solution was as follows:

-Calcium nitrate $Ca(NO_3)_2H_2O$ – 1,4 mM;

-Potassium sulphate K_2SO_4 – 0,8 mM;

-Magnesium sulphate $MgSO_4.7H_20$ – 0,7 mM;

-Potassium dihidrogen orthophosphate KH_2PO_4 – 0,5 mM;

-Fe-EDTA -0,1 mM for chickpea;

-0,25 mM for maize.

In the case of the nutrient solution without P (-P), 0,5 mM KH_2PO_4 was omitted and replaced by 0,5 mM of K_2SO_4. In both treatments the micro nutrients were supplied according to the Long Ashton formula (Hewitt, 1966).

The pH of all the nutrient solutions was adjusted to 6.0 at the beginning of the experiment using H_2SO_4 (0,05 M) or a saturated solution of $Ca(OH)_2$ (Photo 3).

After the pre-culture period 24 seedlings of both maize and chickpea were again selected on a weight basis (Wm=5,91 g, SD=1,586 g; Wc=4,06 g, SD=0,340 g). Four 50 L polythene tanks were used, each one containing 12 plants.

So, at day 0 we had four 50 L tanks inside the growth room, each one containing 12 plants of maize or chickpea, two of them with a "-P" and the other

two with a "+P" nutrient solution. The plants were grown over the following 8 and 16 days, for maize and chickpea respectively, in the same growth room (day temperature 25 °C; night 16 °C; photoperiod 16 hours.day^{-1}).

In order to maintain the nutrient concentration throughout the experiment the nutrient solutions were completely renewed every two days and the induced change in their pH was registered.

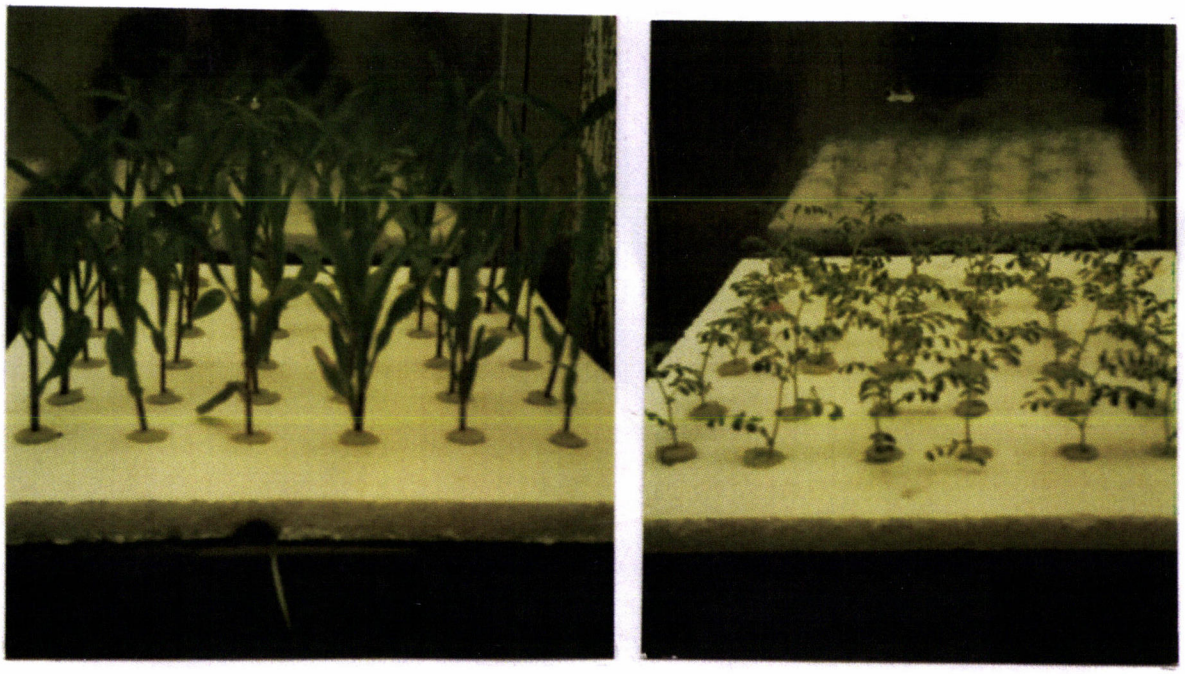


PHOTO 1 and 2 – The thirty six seedlings of maize and chickpea growing in the nutrient solutions.

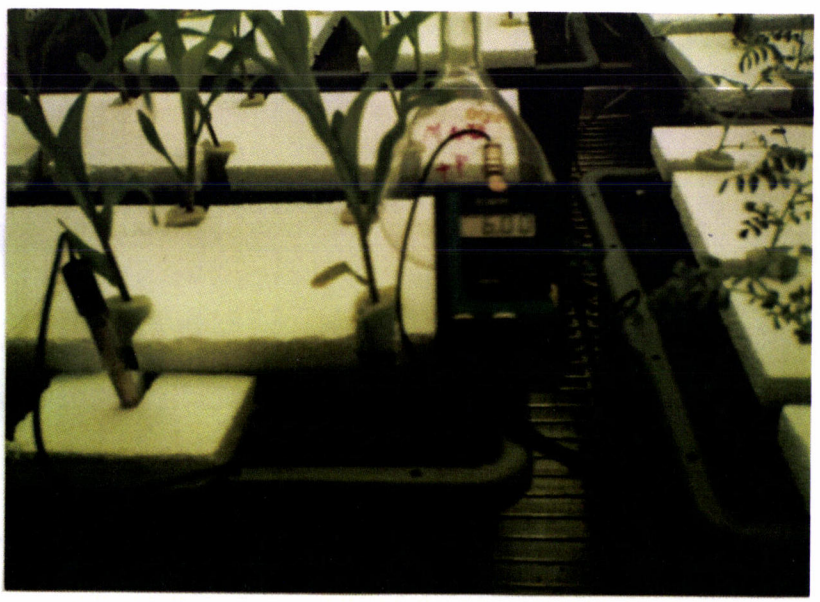


PHOTO 3 – Ajustment of the pH to 6,0.

2.2 - Harvest Procedure:

For both maize and chickpea four replicate plants per treatment were harvested at every harvest time. Three harvests were made: for chickpea on day 0, 4 and 16; for maize on day 0, 2 and 8.

The shoot was separated from the root about 2 cm above the surface to allow for the collection of the xylem sap. The fresh weights of roots and shoots were recorded using a digital balance (Sartorius 1216 MP), and the roots were then sub sampled for determination of the organic acids content and root length measurement using the line intercept method (Tennant, 1975). After this, both roots and shoots were dried in a oven at 80 °C for 24 hours to obtain the respective dry weights.

2.3 - Collection of the xylem sap:

The xylem sap was collected over a period of one and a half hours in maize and two hours in chickpea, using a 1 ml syringe. The collected xylem sap was placed in individual 1,5 mL eppendorfs and was kept at 0 °C using a recipient with ice. Sap was obtained individually from all plants, on day 0, 4 and 16 except from some of the chickpea P stressed plants.

2.4 - Acidification of the rhizosphere:

To permit the visualisation of the acidification ofthe rhizosphere the roots of the plants were first placed over a glass tray.

One L of + P and -P nutrient solution was boiled with plain agar (1%) for a period of 10 minutes. Bromocresol purple was then added to the agar solution to achieve a concentration of 0.075%. The pH ofthe solution was adjusted to 5.8 using NaOH (0.05M), and it was kept liquid at 45°C until it was pored over the roots. Before covering the roots with the agar solution, small plastic rings were placed over the roots, in the apical zone and near the root base (3 cm). 0,25 mL of -P or +P nutrient solution was pipetted inside the plastic rings.

The photographic results and the exudates were obtained after one hour of incubation at room temperature. Four treatments were considered for each specie: for chickpea they were:C++ (chickpea plants grown in P sufficient medium in P sufficient solution); C+- (chickpea plants grown in P sufficient medium in P defficient solution); C-+ (chickpea plants grown in P defficient medium in P

sufficient solution) Unfortunately it was not possible to obtain the results from the C++ plants; for maize they were: M—(P-stressed plant in P-deficient agar); M-+ (P-stressed plant in P-sufficient agar); M++ (P-unstressed plant in P-suficient agar) and M+- (P-unstressed plant in deficient agar)

2.5 - Determination of Organic Acids:

The determination of the organic acids (malic, citric and aconitic acid) content was carried out on a HPLC (High Performance Liquid Chromatography: Chromatography detector - Waters milipore Lambda Max Model 481 LC Spectrophotometer; Pump - Waters milipore Model 510) operating with a solvent flow rate of 2 mL/minute, at a pressure of 1500 psi and using a wavelength of 210 nm (electronic absorption band for the carboxylic group). The determination of the organic acids was made on the roots, on the roots exudates and on the xylem sap.

2.5.1 - Sample preparation:

After being weighted the fresh plant material was transferred to a blender. 30 mL of 70% ethanol in distilled water were then added and the plant material was reduced to very small pieces. The solution was then filtered through a 10mm Whatman nº1 filter paper to a 100 mL volumetric flask and diluted to 100 mL with 70% ethanol.

To make possible the determination of the organic acids content in the plant material that we want to analyse we first prepared the diluted samples to eliminate all the anions and cations that are present in the material so that they do not interfere with the HPLC.

a - Preparation of the SCX column:

1 - Turn on the vacuum pump (AASP VAC-Elut Ana Lytichem Int.)

2 - Put the column into the vacuum box

3 - Wash the column with 2 mL of methanol

4 - Wash the column with 2 mL of distilled water

5 - Insert a plastic container to collect the sample

6 - Put 2 mL of sample into the column

7 - Wash with 2 mL of distilled water.

b - Preparation of the SAX column:

1 - Put the SAX column into the vacuum box

2 - Wash the column with 2 mL of methanol

3 - Wash the column with 2 mL of distilled water

4 - Take 2 mL of the sample obtained from the SCX and put into the SAX column

5 - Wash with 2 mL of distilled water

6 - Insert a plastic container to collect the sample

7 - Wash with 2 mL of formic acid (8 M)

The samples were then dried inside a oven until all the formic acid had evaporated. A 0,10 mL syringe was then used to inject the standard solution containing 0.1341, 0.1921 and 0,0217 g/L of malic, citric and aconitic acid respectively. The absorbance values were recorded with the use of a chart recorder (Kipp and Zonen BD8 multi range) adjusted to a velocity of 2 $mm.min^{-1}$ and a sensitivity of 20 mV. After the readings for the standard solution had been obtained the samples were injected, after being diluted in 1ml of distilled water. The amounts of organic acids in the samples were determinated by measuring and comparing the height of the peaks obtained from the samples with the height of the peaks obtained with the standard solution (Fig. 1).

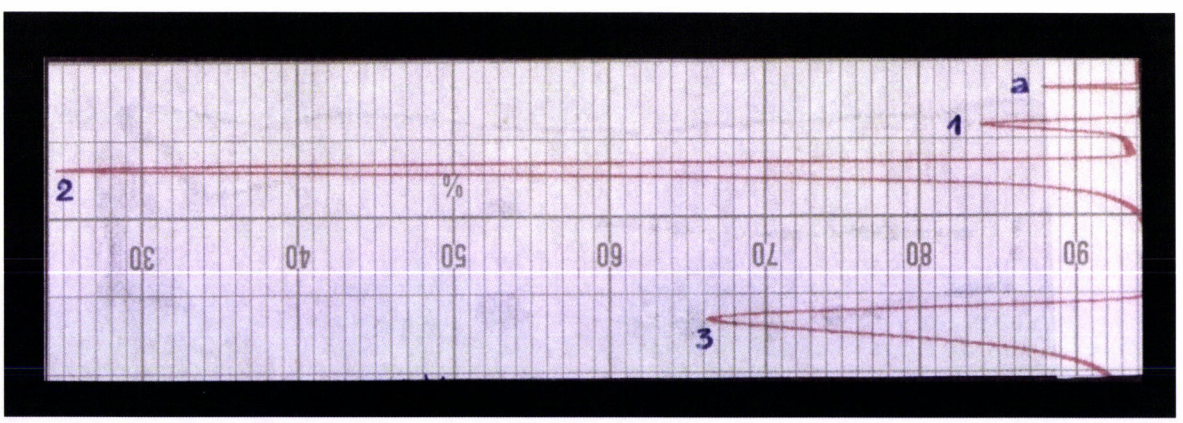


FIGURE 1– Standard graph for the Organic Acids (**a** - Solvent peak; 1 – Malic acid peak; **2** – Citric acid peak; **3** - Aconitic acid peak)

2.6 - Determination of Phosphorus and Potassium in plant material and in the xylem sap:

2.6.1 - Sample preparation:

To make possible the determination of the content of these nutrients in plant material (shoots and roots) the organic matter must first be destroyed. This is

achieved preparing the samples by dry combustion and solubilizing the ash mineral constituents in hydrochloric acid.

In the case of the xylem sap, the amounts of sap that were collected were diluted to complete 1 mL using distilled water.

2.6.1.1 - Procedure:

After being weighted, the dried plant material was placed inside 30 mL silica flasks that were then transferred to a muffle furnace and the temperature was adjusted to 450 °C and maintained over a period of 24 hours, until a whitish-grey ash remained, indicating that all the organic matter had been destroyed. The flasks were then removed from the muffle furnace and 10 mL of hydrochloric acid 6 M (equal volumes of hydrochloric acid 36% and destilled water) were added to each one. These were then placed over a hot plate at 102 °C until all the liquid had evaporated. The residue was moistened with 2 mL of hydrochloric acid (36% approx.) and left to boil for 2 minutes.

After this 10 mL of destilled water were added and left to boil again. After cooling the contents of the flasks were filtered through a 10 mm Whatman nº541 filter paper into 50 mL volumetric flasks and diluted to 50 mL.

2.6.2 - Determination of Potassium:

The concentration of potassium in the diluted ashed plant material and in the xylem sap was determined by flame photometry (CORNING 400). The first step to make this possible consists on the preparation of the potassium stock and standard solutions.

2.6.2.1 - Reagents:

-Potassium stock solution: To prepare the stock solution 0.1908g of potassium chloride were dissolved in 1 L of destilled water. This solution contains 100 ppm

-Potassium standard solutions: 10 mL of the stock solution were diluted to 100 mL. This solution contains 10 ppm of potassium.

To calibrate the flame photometer appropriate dilutions of this standard solution were made to give a range of concentrations from 0 to 10 ppm of potassium.

Before analysing the samples the flame photometer was adjusted until a steady zero and maximum readings were obtained, using the potassium working standard solutions. The readings obtained from each concentration were used to draw the standard/calibration curve for potassium (Fig. 2).

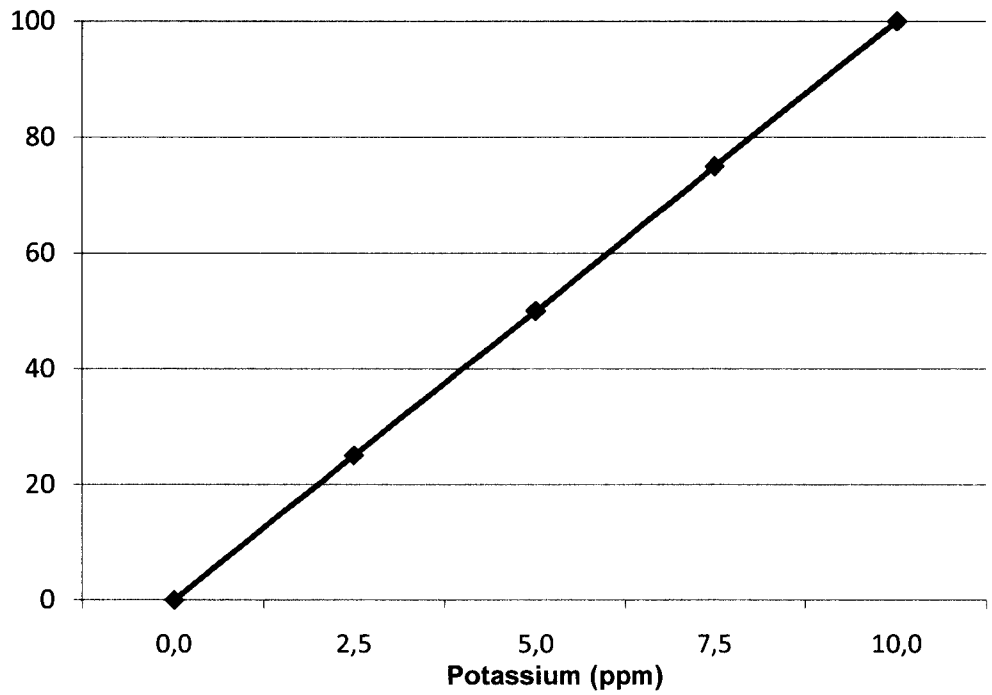


FIGURE 2– Standard/calibration curve for potassium.

2.6.2.2 - Examination of the sample solutions:

1 mL of each sample solution was taken and dilluted with distilled water until the concentration of potassium felt into the range of 0-10 ppm. In the case of the xylem sap the same procedure was used using 0,1 mL of diluted sap.

2.6.3 - Determination of Phosphorus:

The determination of the phosphorus concentration in the diluted sample solutions was carried out spectrophotometrically as the yellow phospho-vanado-molybdate complex.

2.6.3.1 - Reagents:

-Ammonium-molybdate-ammonium metavanadate reagent: Prepared by adding 25 g of ammonium molybdate and 1,25 g of ammonium metavanadate to

approximately 300 mL of distilled water. The solution was then heated to dissolve and after cooling was diluted to 500 mL.

-Hidrochloric acid 5 M: Prepared by diluting 215 mL of hydrochloric acid (approx.36% m/m HCl) to 500 mL with distilled water.

-Phosphorus stock standard solution: Potassium dihydrogen ortho phosphate was dried at 102 °C for one hour and left to cool in a desiccator. 0,879 g of the dried salt were dissolved in distilled water and 1 mL of hydrochloric acid (approx. 36% m/m HCl) was added to the solution that was then diluted to 200 mL and one drop of toluene was also added to the solution.

This solution contains 1 $mg.mL^{-1}$ (1000ppm) of phosphorus.

-Phosphorus standard solutions: These were prepared on the day of use and contained 0, 10, 20, 30, 40 and 50 $mg.mL^{-1}$ of phosphorus.

2.6.3.2 - Preparation of the standard graphic:

10 mL of each phosphorus working standard solutions were pipetted into a 50 mL volumetric flask. To each one 5 mL ol 5 M hydrochloric acid and 5 mL of ammonium molybdate-ammonium metavanadate reagent were added, after which they were diluted to 50 mL and allowed to stand for 30 minutes.

The standard graph was drawn by measuring the absorbance for the different standard solutions in a 10 mm optical cell at 400 nm (LBK Biochrom Ultrospec II) (Fig. 3).

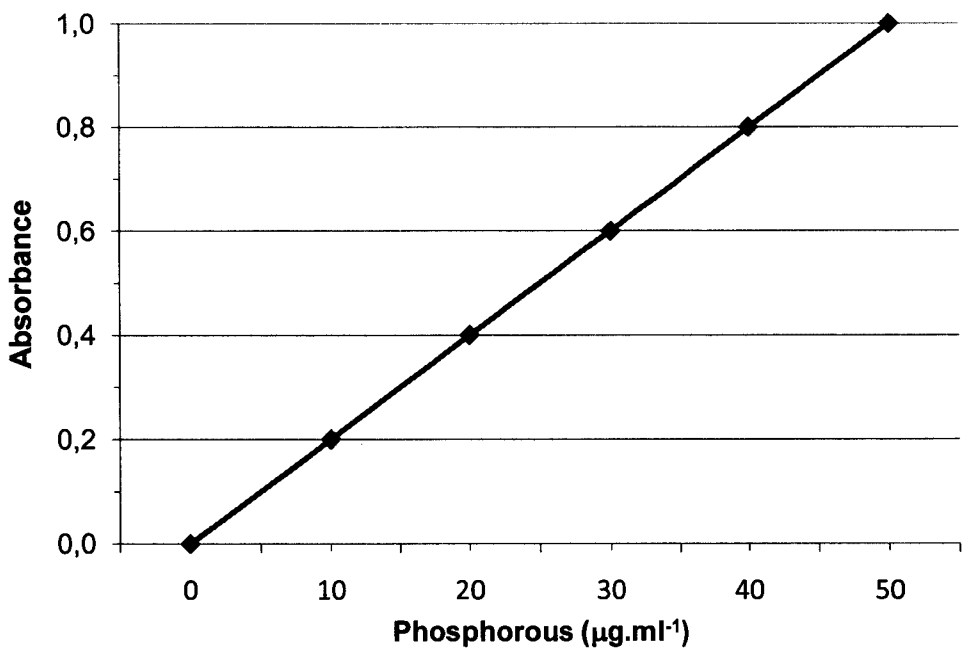


FIGURE 3 – Standard/calibration curve for phosphorus.

2.6.3.3 - Determination of the phosphorus content in the sample solution:

10 mL of each sample solution were treated like for the preparation of the standard graph. The determination of the phosphorus content was made by reading from the standard graphic the number of mg of phosphorus equivalent to the absorbances of the samples.

2.7 - Determination of nitrate in plant material and in the xylem sap:

The nitrate concentration was measured in 0,5 g of dried plant material using the method of Cataldo *et al.* (1975).

2.7.1 - Sample preparation:

The preparation of the sample started with the drying of the plant material (shoots and roots) in a oven at 85 °C over a period of 24 h. After this the plant material was reduced to very small pieces using a blender and 0,5 g were weighted into 100 mL volumetric flasks. Boiling distilled water was then added to complete 100 mL. 0,05 g of activated charcoal were also added to each flasks. The flasks were then shaked in a hot bath for a period of 15 minutes using an electric shaker (Gallenkamp) after which the suspensions were filtered through a 10 mm Whatman n° 1 filter paper into 50 mL volumetric flasks.

2.7.2 - Reagents:

-Salicylic acid in concentrated sulphuric acid: Prepared diluting 5 g of salicylic acid in sulphuric acid in a 100 mL volumetric flask.

-Sodium hydroxide 2 N: Prepared by diluting 80 g of sodium hydroxide (NaOH) in distilled water to complete 1 L.

-Nitrate stock standard solution: 0,4077 g of potassium nitrate (KNO_3) were dissolved in distilled water to 1 L. This solution contains 250 mg of $NO_3^-N.cm^{-3}$.

-Nitrate standard solutions: Using the stock solution a serie of standard solutions were prepared in 100 mL volumetric flasks containing 0, 5, 10, 20, 30, 40 and 50 mg of $NO_3^-N.cm^{-3}$.

2.7.3 - Preparation of the standard graphic:

To prepare the standard curve each one of the standard solutions were treated as follows:

1 – 0,2 mL of each solution were transferred to a boiling tube;

2 – 0,8 mL of salicylic acid in concentrated sulphuric acid were added and the tubes were left to cool for 20 minutes;

3 – 19 mL of NaOH were slowly added to each one of the tubes to raise the pH above 12 (yellow colour);

4 - The tubes were then allowed to cool and the absorbance was measured at 410 nm using a spectrophotometer (LBK Biochrom Ultrospec II). The values obtained from the readings were used to draw the standard/calibration curve for nitrate (Fig. 4).

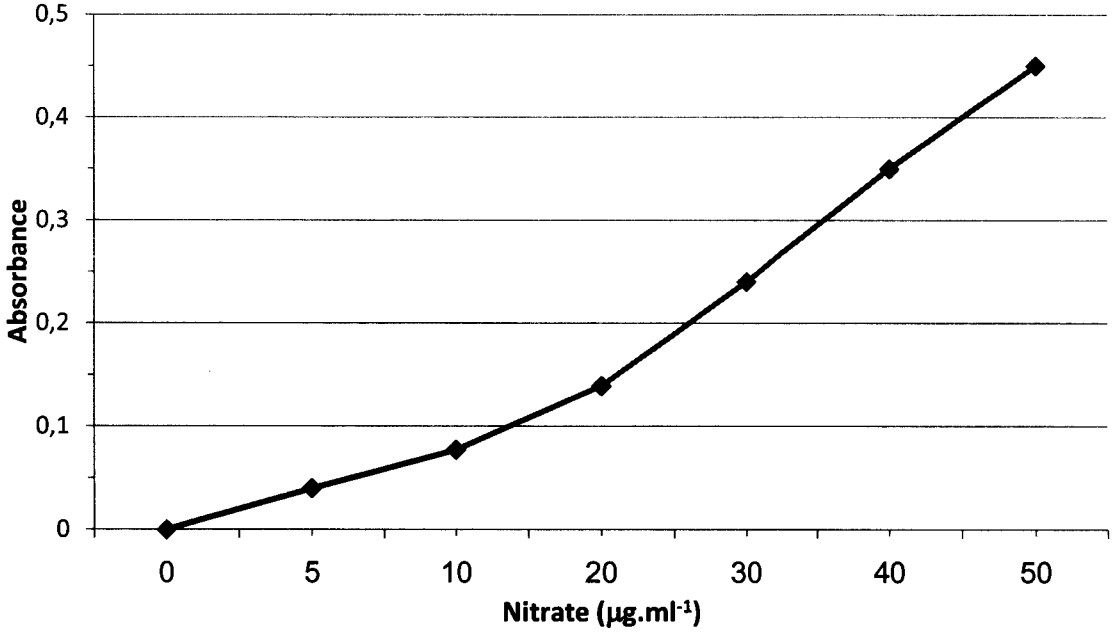


FIGURE 4 – Standard/calibration curve for nitrate.

III - RESULTS

3.1 - Morphological responses to phosphorous stress:

For both maize and chickpea significant differences in plant growth were observed as a result of phosphorous starvation. The difference in plant growth between the stressed and the unstressed plants increased with the duration of the phosphorous starvation period and, considering the last harvest there was a reduction of 57 and 58% (for maize and chickpea respectively) in the total fresh weight of the stressed plants when compared with the unstressed ones (Fig. 5 and 6).

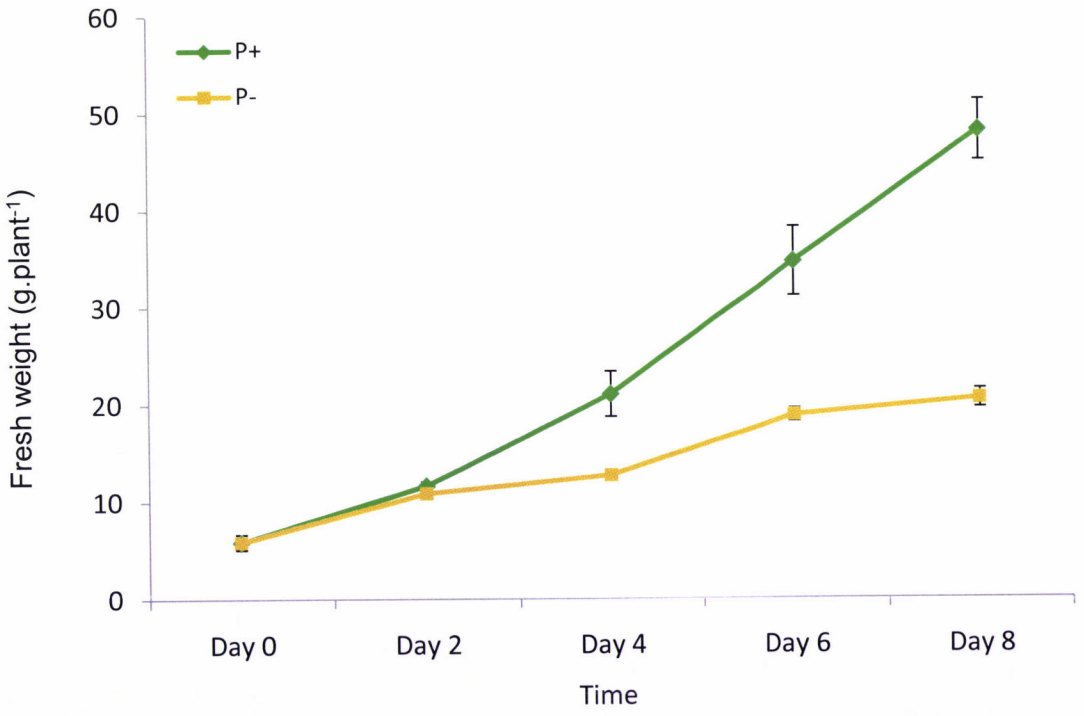


FIGURE 5 - Effect of phosphorous stress on the growth of maize (P+ = unstressed plants, P- = stressed plants; bars represent the standard errors of means, n=4)

As expected, this reflected itself in the evolution of dry weight of the -P and +P plants with time (fig. 7 and 8). The distribution of the dry weight between root and shoot was also very influenced by the treatments (Fig 7, 8, 9 and 10).

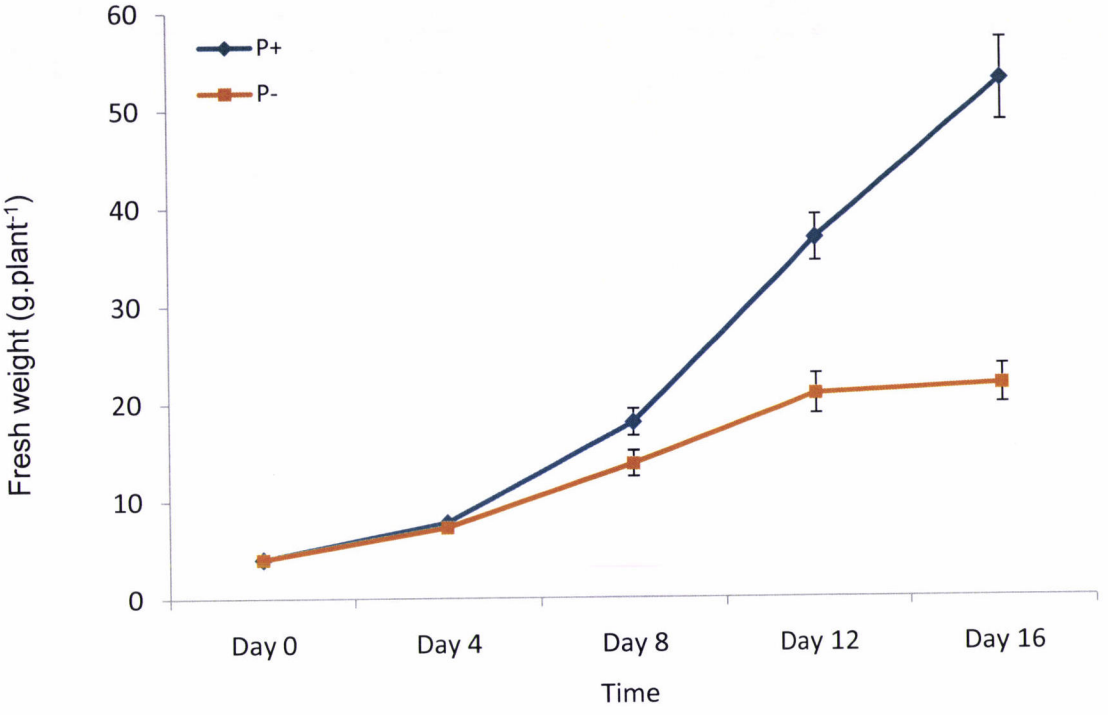


FIGURE 6 - Effect of phosphorous stress on the growth of chickpea (P+ = unstressed plants, P- = stressed plants; bars are standard errors of means, n=4).

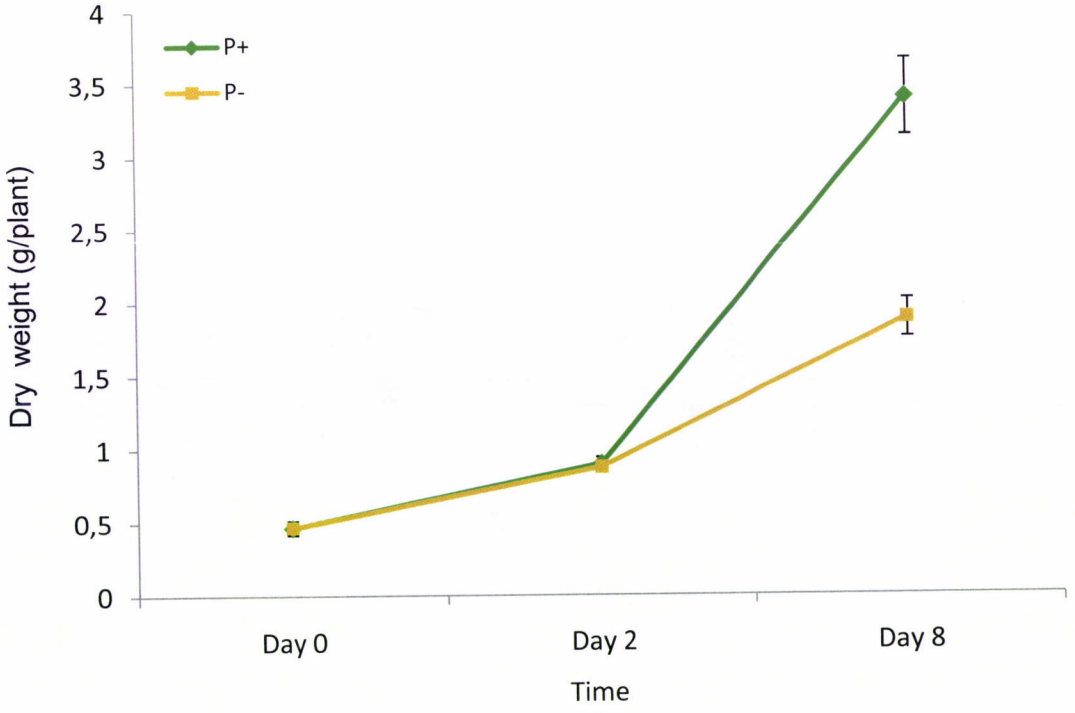


FIGURE 7- Effect of phosphorous stress on the evolution of the dry weight in maize.

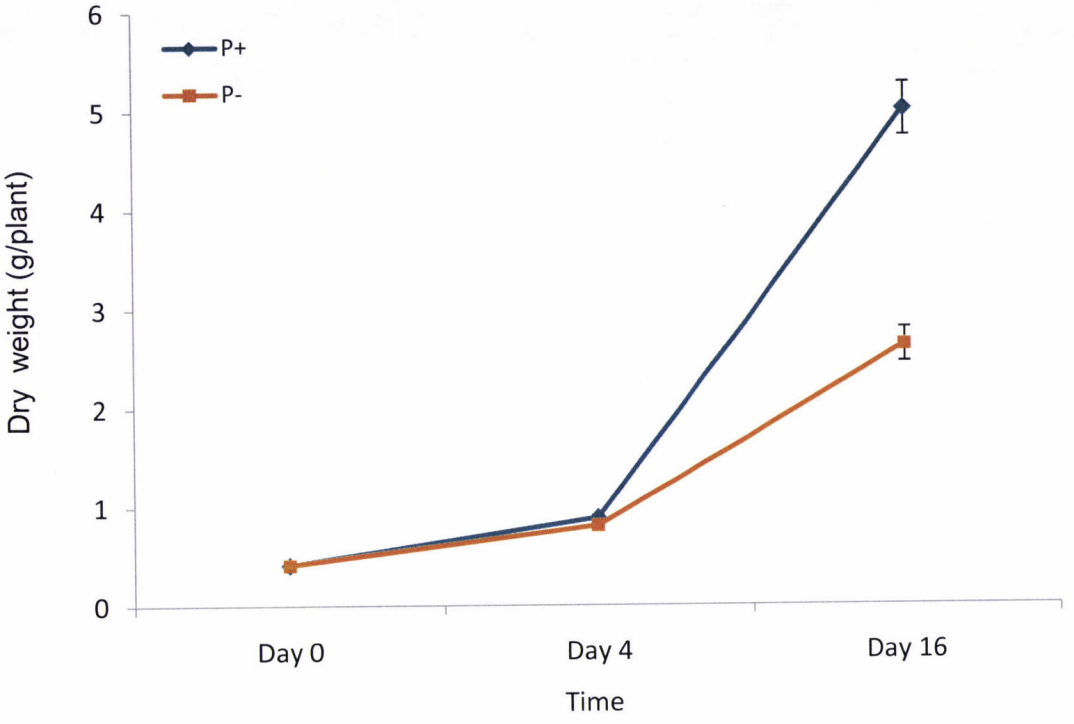


FIGURE 8 - Effect of phosphorous stress on the evolution of dry weight in chickpea.

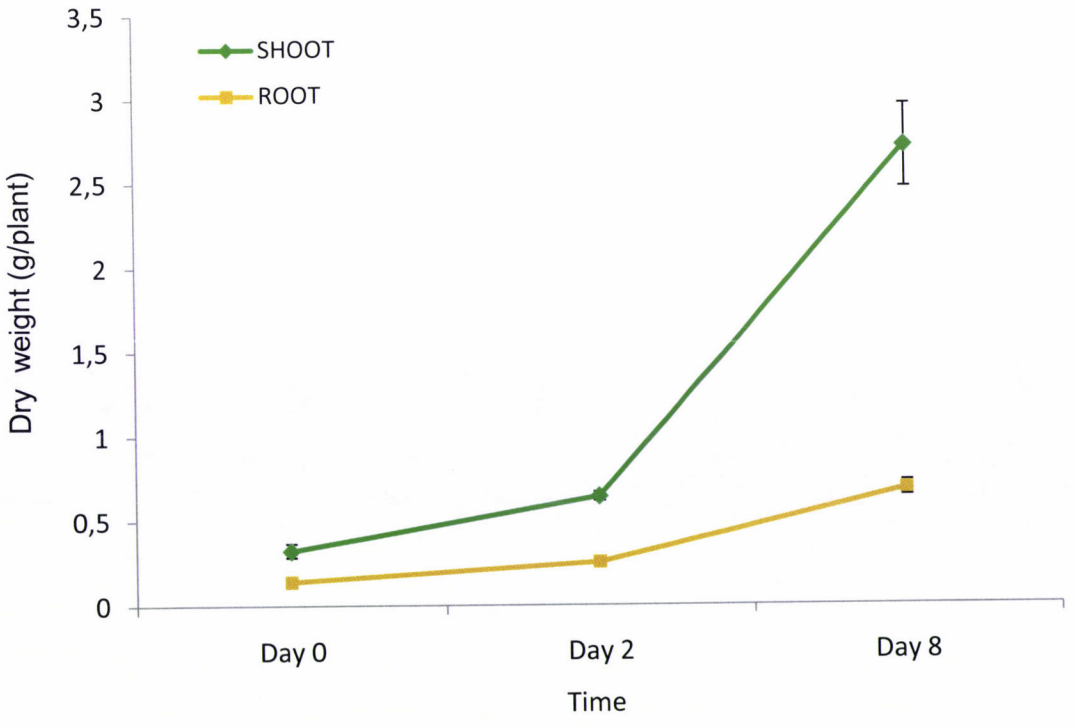


FIGURE 9 - Distribution of dry weight in unstressed (+P) maize plants.

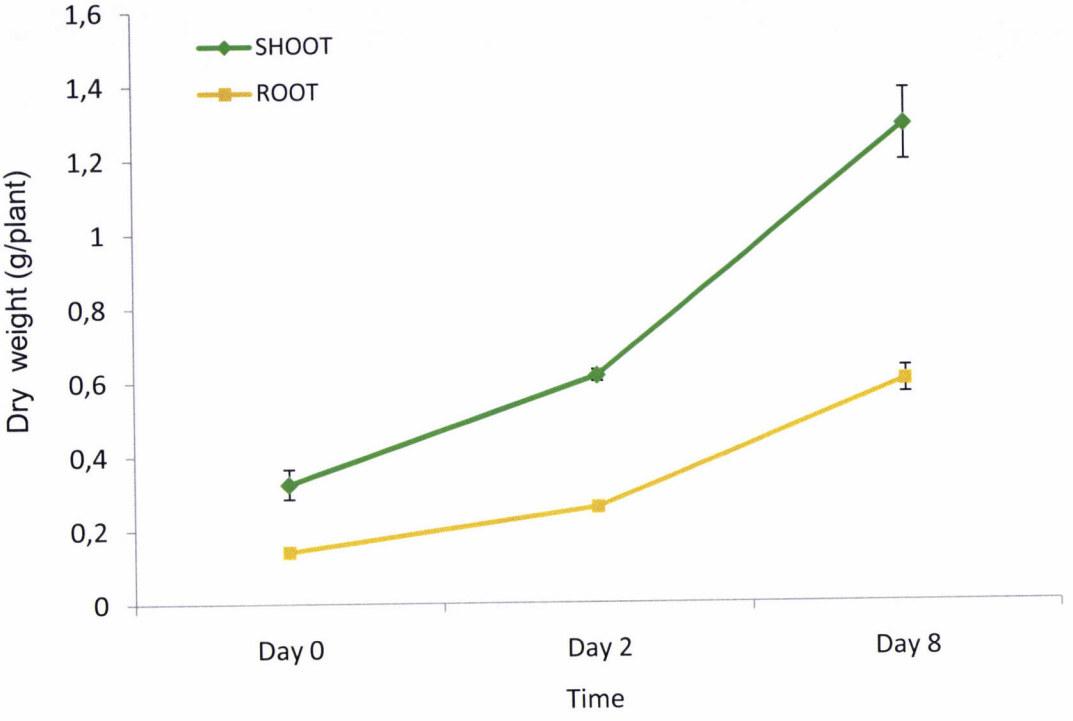


FIGURE 10 - Distribution of dry weight in stressed (-P) maize plants.

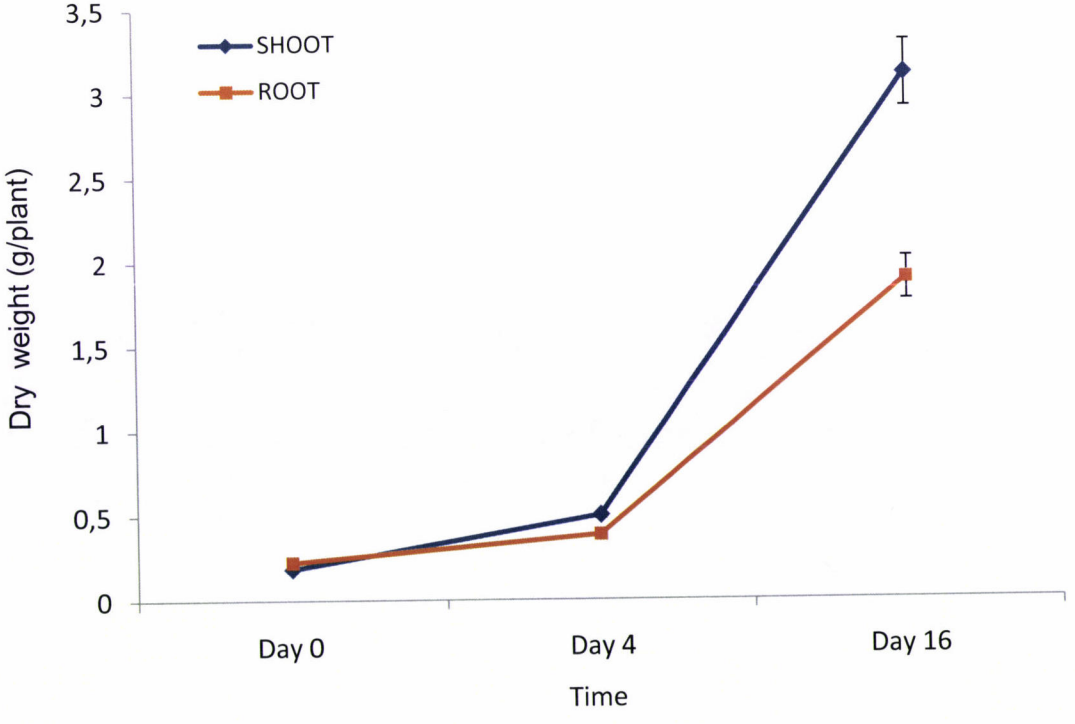


FIGURE 11 - Distribution of dry weight in unstressed (+P) chickpea plants.

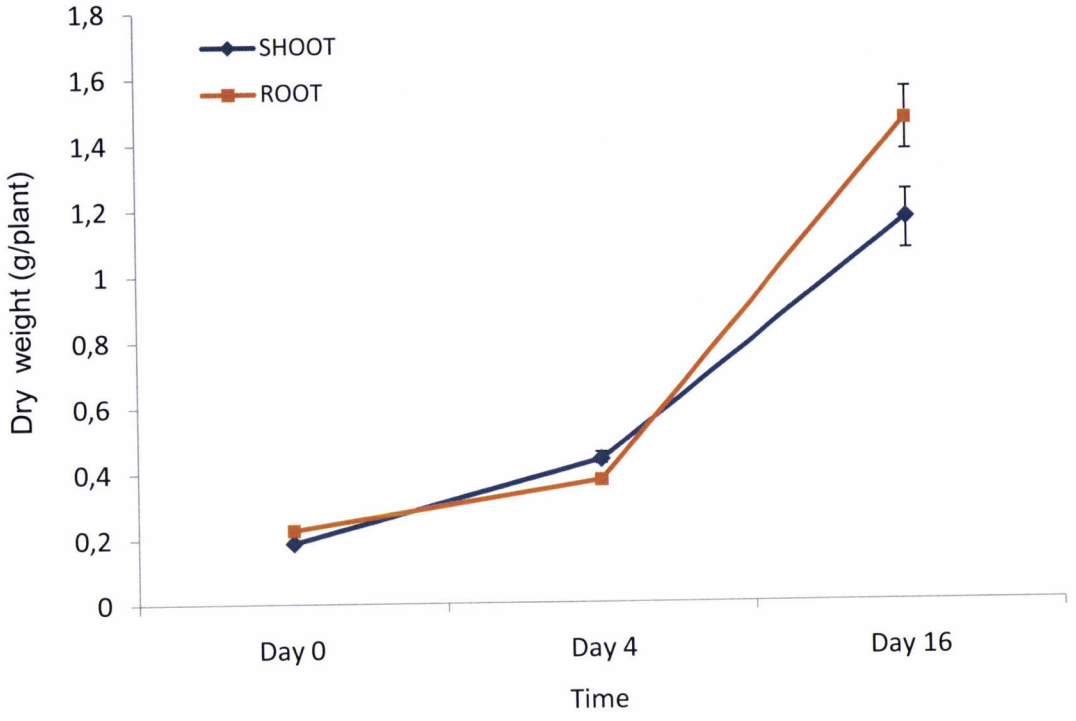


FIGURE 12 - Distribution of dry weight in stressed (-P) chickpea plants.

Contrasting with shoot growth, root growth was much less inhibited under phosphorus deficiency, leading to a decrease in the shoot-root dry weight ratio (Table 1 and 2).

Looking at the shoot-root ratios we see that when comparing maize with chickpea at day 0 it can be observed that while in chickpea the root weight was superior to the shoot weight (shoot-root ratio < 1), in maize happened exactly the contrary (shoot-root ratio > 1). This was mainly due to the bigger seed weight in the case of chickpea.

In both maize and chickpea the value of the shoot-root ratio for the last harvest was inferior to the value corresponding to day 0 in the case of the plants suffering from phosphorus deficiency. This was particularly pronounced in chickpea. The reduction that was observed in the shoot-root ratio was mainly due to the decrease of the shoot dry weight, as a consequence of a lower Absolute Growth Rate (AGR).

TABLE 1 – Evolution of fresh weight, dry weight and shoot/root ratio in maiz

Time (days)	Treatment	Total Fresh weigth (g)	Shoot fresh weigth (g)	Root fresh weigth (g)	Total dry weigth (g)	Shoot dry weigth (g)	Root dry weigth (g)	Shoot/Root ratio	Root length (m)	SRL (m.g root dw^{-1})
0		5,91	3,66	1,99	0,47	0,33	0,14	2,36		
2	P+	11,66	8,19	3,47	0,9	0,64	0,26	2,46		
	P-	10,94	7,71	3,24	0,88	0,62	0,26	2,38		
8	P+	48,15	37,07	11,08	3,4	2,72	0,69	3,94	7,2	10,43
	P-	20,56	14,24	6,32	1,88	1,29	0,6	2,15	10	16,66

TABLE 2 – Evolution of fresh weight, dry weight and shoot/root ratio in chickpea.

Time (days)	Treatment	Total Fresh weigth (g)	Shoot fresh weigth (g)	Root fresh weigth (g)	Total dry weigth (g)	Shoot dry weigth (g)	Root dry weigth (g)	Shoot/Root ratio	Root length (m)	SRL (m.g root dw^{-1})
0		4,06	1,31	2,75	0,42	0,19	0,23	0,83		
4	P+	7,77	3,17	4,6	0,89	0,5	0,39	1,28		
	P-	7,28	2,63	4,65	0,81	0,44	0,38	1,16		
16	P+	52,91	19,36	33,55	5,01	3,11	1,9	1,64	17,75	9,34
	P-	21,8	6,11	15,69	2,63	1,17	1,47	0,8	15,34	10,44

TABLE 3 - Absolute Growth Rate (g dry matter.day^{-1}) for maize and chickpea

		Shoot	Root	Total
Maize	P+	0,299	0,069	0,368
	P-	0,12	0,058	0,178
Chickpea	P+	0,182	0,104	0,287
	P-	0,061	0,078	0,138

As presented in Table 3, the smaller AGR for the phosphorous stressed plants was mainly a reflection of the reduction in the shoots AGR.

Looking back at Table 1 and 2 it can also be observed that the root length was another root morphological characteristic that was significantly affected by the treatments. When comparing maize with chickpea we can see that while in maize phosphorous starvation induced the increase in root length in the case of chickpea it was exactly the contrary and, a decrease in root length could be observed.

Perhaps more important than this is to look at the Specific Root Length (SRL) which gives us a idea of how efficient plans are when it comes to respond adaptatively to phosphorous deficiency. As can be seen both maize and chickpea responded to phosphorus starvation by producing a bigger root length per gram of root dry weight.

Phosphorus deficiency also reflected itself on the colour of the root system. Plants growing in a nutrient medium without phosphorus presented darker roots when compared with the plants that were supplied with phosphorus. This was particularly visible in the case of the chickpea plants (Photo 8).

The symptoms of phosphorus deficiency were more visible in the shoots then in the roots. In maize, the leaves of the phosphorus deprived plants presented a purplish colour (Photo 9) mainly along the central region and on the lower stalk of the plant. In these plants there was also a premature senescence of the older leaves (Photo 7). In the phosphorus sufficient plants the leaves presented inter venial chlorosis (Photo 10).

In the phosphorus stressed chickpea plants the tips of the older leaves started by getting chlorotic few days after the withdrawal of phosphorus from the nutrient medium and by day 16 they were necrotic (Photo 11). In these plants there was also a marked inhibition of branching and lateral bud development (Photo 5).

PHOTO 4 – View of a phosphorus unstressed chickpea plant (P+) on day 16.

PHOTO 5– View of a phosphorus stressed chickpea plant (P-) on day 16.

PHOTO 6 – View of a phosphorus unstressed maize plant (P+) on day 8.

PHOTO 7– View of a phosphorus stressed maize plant (P-) on day 8.

PHOTO 8 – View of the roots from the stressed (left) and unstressed (right) chickpea plants.

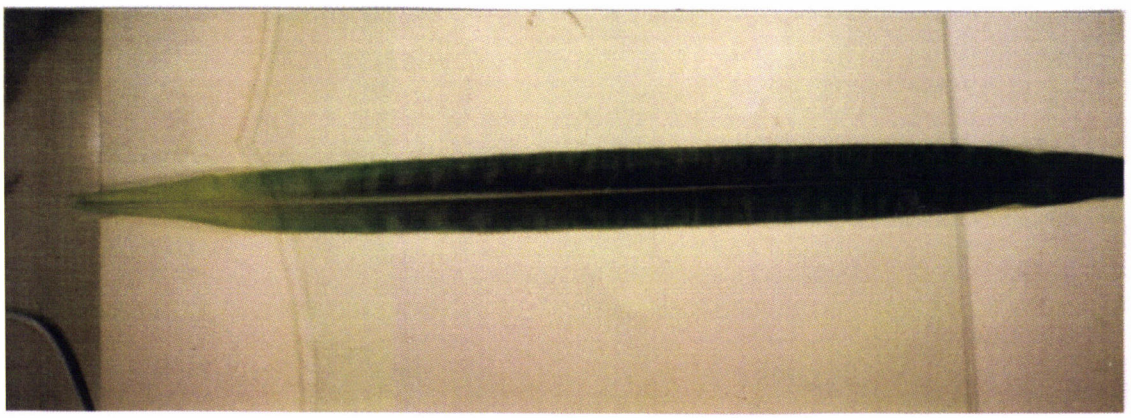


PHOTO 9 – Leaf of a phosphorus deficient (P-) maize plant.

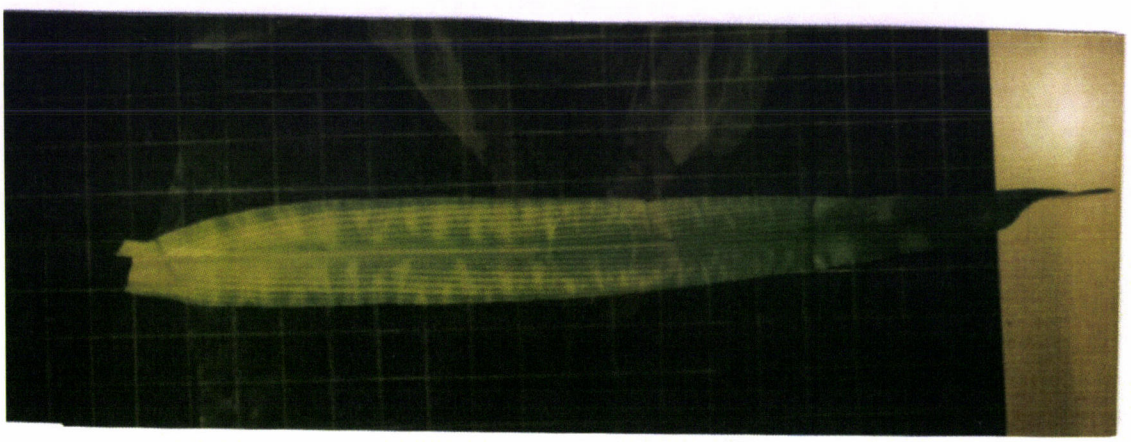


PHOTO 10 – Leaf of a phosphorus sufficient (P+) maize plant.

PHOTO 11 – View of the older leaves of a P-defficient chickpea plant, showing the necrotic tips.

3.2 - Physiological responses to phosphorus stress:

As presented in figures 13 and 14 one of the most important and noted responses of plants growing in a phosphorus deficient medium and supplied with nitrate-N is the induced relative acidification of the nutrient medium, due to a decrease in OH^- release, when compared with the unstressed plants.

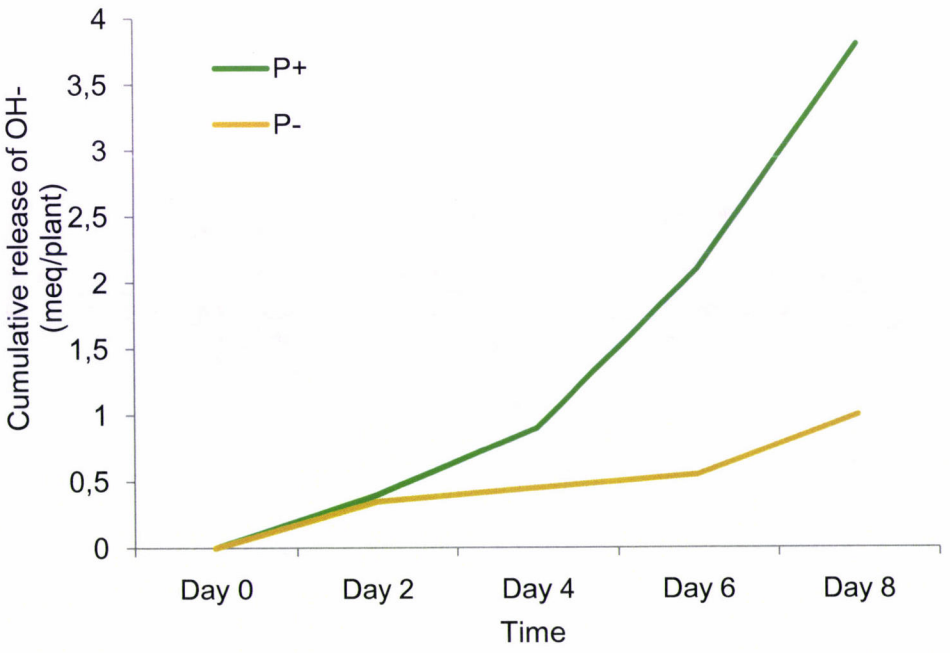


FIGURE 13 - Cumulative release of OH^- in P-stressed (P-) and P-sufficient (P+) maize plants growing in nutrient solution.

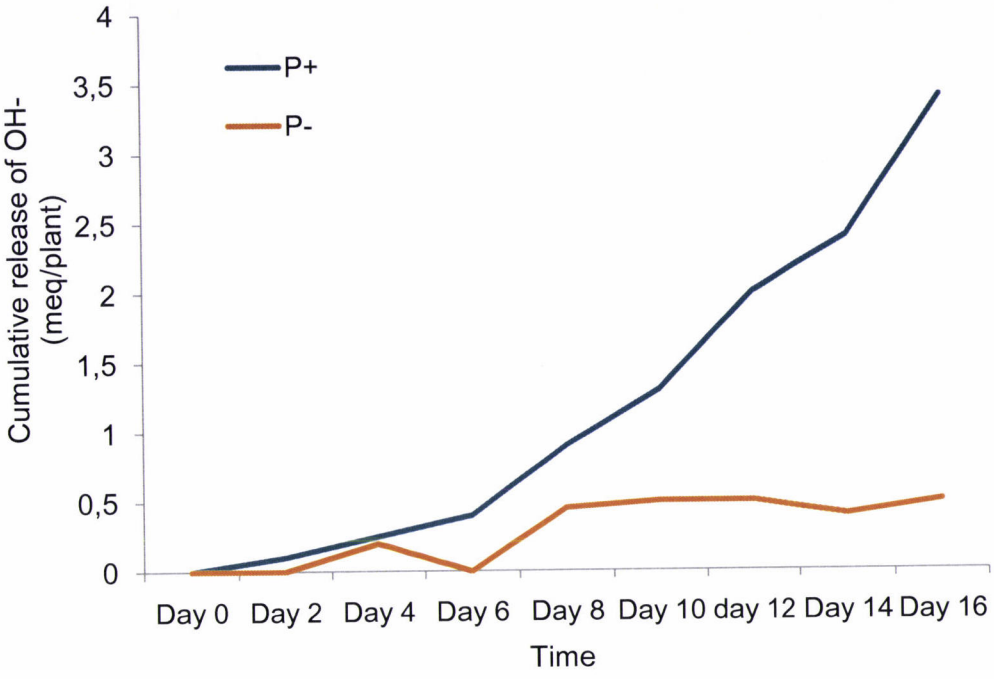


FIGURE 14 - Cumulative release of OH^- in P-stressed (P-) and P-sufficient chickpea plants growing in nutrient solution.

The cumulative amounts of OH^- released by the roots of the unstressed plants were nearly the same for maize and chickpea but, in the phosphorus deficient chickpea plants the induced relative acidification of the nutrient medium was bigger when compared with the P-deficient maize plants (this can be due to the diference in the duration of the experiment).

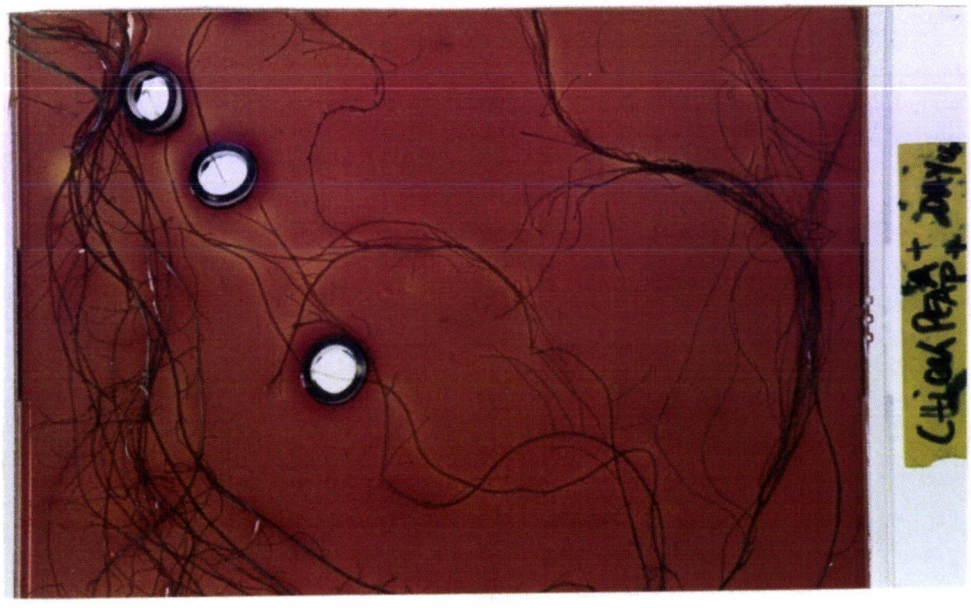


PHOTO 12 – Visualization of the induced change in the pH of the rhizosphere by a P-sufficient chickpea plant treated with agar prepared from a P-sufficient solution (C++)

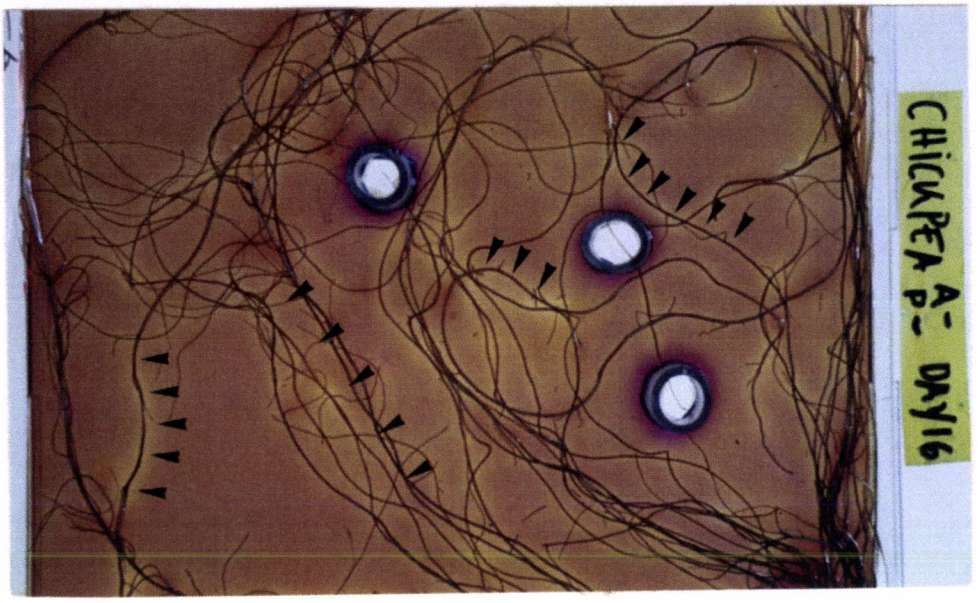
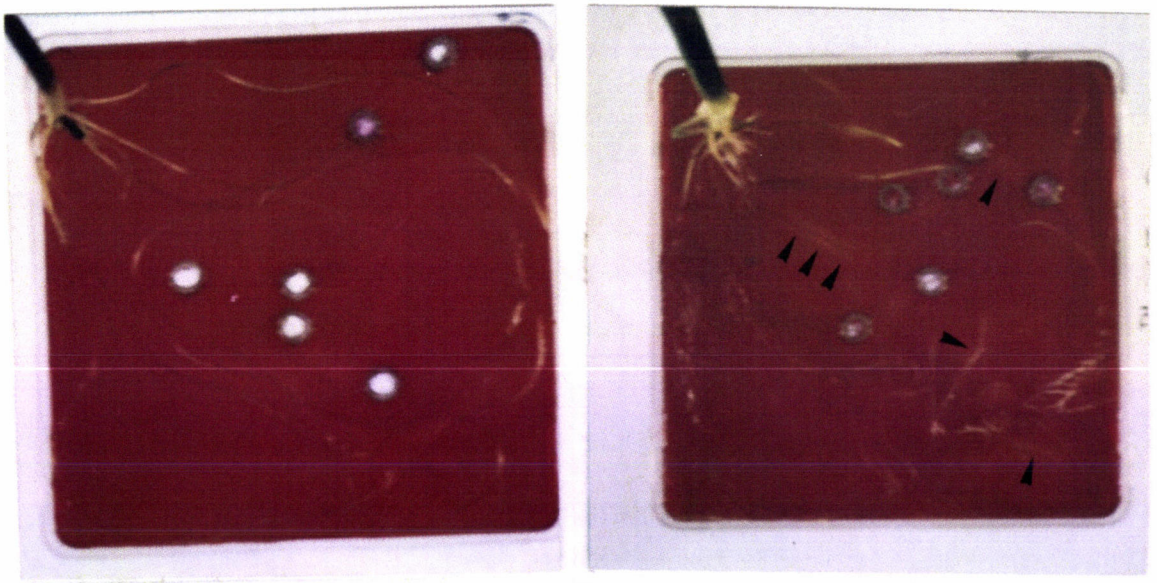


PHOTO 13 – Visualization of the induced change in the pH of the rhizosphere by a P-stressed chickpea plant treated with agar prepared from a P-deficient solution (C--).



PHOTOS 14 and 15 – Visualization of the induced change in the pH of the rhizosphere in maize.

Photos 12, 13, 14 and 15 confirm the results presented in figures 9 and 10. In the plants treated with P-deficient agar it was visible a yellow colour

(acidification) along almost the entire roots, and this was particularly noted in the P-stressed chickpea plants. In the plants treated agar prepared using a P-sufficient solution the acidification was only visible around the root tips.

Looking now at Table 4 we can see that, as expected the amount of phosphorus in the phosphorus deficient plants was significantly lower than in the sufficient ones and, about ten times bigger in chickpea when compared with maize.

TABLE 4 - Differences in the amount of phosphorus (as $H_2PO_4^-$) between shoots and roots of P-stressed (P-) and P-unstressed (P+) maize and chickpea plants in meq.plant^{-1} (the values between parenthesis represent the % of phosphorus in shoots and roots).

Maize

	Day 0	Day 2		Day 8	
		P+	P-	P+	P-
Shoot	0.041(71,6)	0,098(61,3)	0,048(73,5)	0,364(75,2)	0,036(60,5)
Root	0.061(28,4)	0,052(38,7)	0,023(26,5)	0,12(24,8)	0,024(39,5)

Chickpea

	Day 0	Day 4		Day 16	
		P+	P-	P+	P-
Shoot	0,37(64,6)	0,573(55,7)	0,262(44,9)	2,729(44,9)	0,31(44,1)
Root	0,203(35,4)	0,456(44,3)	0,348(57,1)	3,349(55,1)	0,393(55,9)

In maize, the amount of phosphorus in the shoots was always bigger then in the roots and, while on day 2 the P-stressed plants had a bigger percentage of phosphorus in the shoots, on day 8 happened exactly the contrary.

In chickpea the same only happened on day 0 and on day 4 in the phosphorus unstressed plants. On day 16 there was no difference in the distribution of phosphorus between shoots and roots of the P-stressed plants when compared with the unstressed plants, although there were large differences in the amount of phosphorus present in the shoots and roots.

From the results presented it can also be taken that, as a result of phosphorus deficiency, a bigger percentage of phosphorus was allocated to the roots, and this increase in the distribution of phosphorus to the roots was mainly visible in maize plants.

The data presented in Table 5 show us that contrary to the amount of phosphorus found in the tissues, the concentration of this element was bigger in maize, particularly in the shoots (with exception of day 0). These results also show decrease in the concentration of phosphorus was particularly noted in the shoots.

TABLE 5 - Effect of phosphorus stress in the concentration of phosphorus (as $H_2P0_4^-$) in shoots and roots of stressed (P-) and unstressed (P+) maize and chickpea plants in meq.100 g dry weight^{-1}.

Maize

	Day 0	Day 2		Day 8	
		P+	P-	P+	P-
Shoot	13,15	12,62	10,18	13,8	2,88
Root	12,08	19,83	8,44	16,95	4,06

Chickpea

	Day 0	Day 4		Day 16	
		P+	P-	P+	P-
Shoot	20,15	11,35	5,93	8,62	2,7
Root	9,07	11,67	9,24	17,63	2,75

In Table 6 the amounts of potassium in shoots and roots are are presented as a function of the treatments. In maize the amount of potassium was always bigger in the shoots. Phosphorus deficiency reduced the amount of potassium in plant material. Phosphorus stressed plants also allocated a bigger percentage of their total potassium into the roots. This reflected itself over the concentration of potassium in both shoots and roots according to the treatments (Table 7). This concentration was always smaller in the roots, and this was particularly

pronounced in the P-stressed plants on day 8. These values have to be considered in relation to the change in the shoot-root ratio.

TABLE 6 - Effect of phosphorus deficiency on the evolution of the potassium content in shoots and roots of maize and chickpea in meq K^+.plant^{-1} (the values between parenthesis represent the % of phosphorus in shoots and roots).

Maize

	Day 0	Day 2		Day 8	
		P+	P-	P+	P-
Shoot	0,473(83,9)	0,81(79,9)	0,767(78,9)	3,073(83,5)	1,413(79,1)
Root	0,092(16,2)	0,204(20,1)	0,204(21,1)	0,606(16,5)	0,373(20,9)

Chickpea

	Day 0	Day 4		Day 16	
		P+	P-	P+	P-
Shoot	0,134(53,3)	0,36(46,4)	0,246(36,2)	2,018(44,4)	0,547(18,78)
Root	0,117(46,7)	0,416(53,6)	0,433(63,8)	2,525(55,6)	2,363(81,22)

By the contrary, in chickpea, with the exception of day 0, the amount of potassium was always bigger in the roots. This increase in the allocation of potassium into the roots was more pronounced in the plants suffering from phosphorus deficiency (81,22 % in the P-stressed plants against 55,6 % in the P-unstressed plants on day 16). Looking at Table 7 we can also see that during the time of the experiment the concentration of potassium was decreased in shoots and increased in roots, particularly in the stressed plants.

The results presented above in Table 8 show us that between day 0 and day 2 maize plants growing in a P-deficient nutrient medium not only produced a bigger amount of organic acids but also had a bigger concentration of organic acids in the roots, particularly malic and aconitic acid. By day 8 while the amounts of citric and aconitic were increased in the P-stressed plants the amount of malic acid was depressed with the concentrations having a similar behaviour.

TABLE 7 - Effect of phosphorus deficiency on the evolution of the potassium concentration in shoots and roots of P-stressed (P-) and P-unstressed (P-) maize and chickpea plants (values in meq.100g dry weight^{-1}).

Maize

	Day 0	Day 2		Day 8	
		P+	P-	P+	P-
Shoot	148,5	126,5	124,75	115,5	113,25
Root	67,75	78,5	79	86,5	61,25

Chickpea

	Day 0	Day 4		Day 16	
		P+	P-	P+	P-
Shoot	71,23	71,6	56,8	66,25	46,5
Root	52,63	106,93	115,75	134,5	160

TABLE 8 - The effect of phosphorus deficiency on the amount and concentration of organic acids in the roots of maize stressed (P-) and unstressed (P+) plants.

Maize

	Day 0	Day 2		Day 8	
		P+	P-	P+	P-
			(μmol.plant^{-1})		
Malic	16	8,43	20	111,75	55,05
Citric	13,9	3,75	5,14	24,25	33,98
Aconitic	8,7	1,84	6,32	11,16	16,78
			(mmol.100 g^{-1} dw)		
Malic	11,45	3,24	7,67	16,43	9,47
Citric	9,92	1,44	1,98	3,62	5,69
Aconitic	6,22	0,71	2,43	1,67	2,81

TABLE 9 - Effect of phosphorus deficiency on the amount and concentration of organic acids in the roots of chickpea.

	Chickpea				
	Day 0	Day 4		Day 16	
		P+	P-	P+	P-
		(μmol.plant^{-1})			
Malic	93,4	50,6	26,9	392,4	206
Citric	19,7	10,7	11,4	81,3	138,08
Aconitic	0,62	2,86	0,37	6,66	11,01
		mmol.100 g^{-1} dw)			
Malic	40,6	12,97	7,09	23,16	14,09
Citric	8,59	2,74	2,99	4,17	9,55
Aconitic	0,27	0,73	0,09	0,3	0,45

The results presented for chickpea (Table 9) show us a pattern of organic acid production that is different from the one we could see for maize. At day 4 only the amount and concentration of citric acid was bigger in the roots of the P-stressed plants and, the same could be observed at day 16.

Comparing maize with chickpea we can see that in both plants malic acid was the organic acid that was present in the bigger amount and concentration and that the concentrations of malic and citric acid were always bigger in chickpea, with the biggest difference occurring in the P-stressed plants. The amounts and concentration of aconitic acid were always bigger in maize.

Table 10 shows us the volume flux of the xylem sap exudate. We can see that the volume collected per plant and per hour was always bigger for maize and, with exception of day 2 in maize the volumes were always bigger in the P-unstressed plants.

In Tables 11 and 12 are presented the results of the phosphorus deficiency in the amounts of phosphorus, nitrate and potassium in the xylem sap exudate of maize and chickpea respectively. Of the three ions we can see that, with some exceptions in the case of chickpea, potassium was the ion that was present in the bigger amounts and concentrations.

TABLE 10 - Volume flux of the xylem sap exudate in P-stressed (P-) and P-unstressed (P+) maize and chickpea plants (ml.plant^{-1}.hour^{-1}).

Maize

Day 0	Day 2		Day 8	
	P+	P-	P+	P-
0,17	0,23	0,3	0,47	0,12

Chickpea

Day 0	Day 4		Day 16	
	P+	P-	P+	P-
0,006	0,06	0,006	0,24	0,008

In maize both the amounts and concentrations were noticeably higher in the P-unstressed plants when compared with controls. The amounts of both potassium and nitrate changed quite similarly to one another, and while on day 2 they were bigger in the P-stressed plants on day 8 happened exactly the contrary. This was not reflected in the evolution of the concentration of these two ions.

In chickpea the reduction in the amounts and concentration of phosphorus in the xilem sap exudate was followed by a similar behaviour of nitrate and potassium.

TABLE 11 - The effect of phosphorus deficiency in the amounts and concentrations of phosphorus, nitrate and potassium in the xylem sap exudate of maize.

Maize

	Day 0	Day 2		Day 8	
		P+	P-	P+	P-
			(μmol.plant^{-1}.hour^{-1})		
Phosphorous	1,3	1,8	0,4	2,8	0,3
Nitrate	2,1	1,6	3,3	3,4	0,6
Potassium	6,2	8	9,5	10,5	0,6
			(mM)		
Phosphorous	7,6	7,8	1,3	5,9	2,7
Nitrate	12,3	6,9	10,8	7,2	4,8
Potassium	36,6	33	30,9	37,8	51,5

TABLE 12 - The effect of phosphorus deficiency in the amounts and concentrations of phosphorus, nitrate and potassium in the xylem sap exudate of chickpea.

	Chickpea				
	Day 0	**Day 4**		**Day 16**	
		P+	**P-**	**P+**	**P-**
		(μmol.plant^{-1}.hour^{-1})			
Phosphorous	0,4	0,2	0,03	1,3	0,08
Nitrate	0,04	0,07	n.d.	0,05	0,05
Potassium	0,4	7,7	0,3	23,9	0,4
		(mM)			
Phosphorous	6	3,3	5,1	5,41	10
Nitrate	6,4	1,2	n.d.	0,8	0,6
Potassium	6	25,5	10,2	21,1	10,2

In Tables 13 for maize and 14 for chickpea are presented the results of the effect of phosphorus starvation in the amount and concentration of malic citric and aconitic acid in the xylem sap exudate. These values were always higher in maize when compared with chickpea, and in both species malic acid was the organic acid that was in bigger amounts and higher concentrations in the xilem sap exudate.

In maize on day 2 the amounts and concentrations of all organic acids were bigger in the P-stressed plants when compared with controls. The reverse situation was found on day 8, due to a general increase in the sufficient plants and a decrease in the deficient ones.

In chickpea, as a consequence of the withdrawal of phosphorus from the nutrient medium both the amounts and concentrations of all organic acids were lower in the phosphorus deficient plants (P-).

TABLE 13 - The effect of phosphorus deficiency in the amounts and concentrations of malic, citric and aconitic acid in the xylem sap exudate of maize.

Maize

	Day 0	Day 2		Day 8	
		P+	P-	P+	P-
			(μmol.plant^{-1}.hour^{-1})		
Malic	0,138	0,058	0,150	0,193	0,049
Citric	0,011	0,012	0,020	0,032	0,008
Aconitic	0,015	0,015	0,029	0,066	0,015
			(mM)		
Malic	0,810	0,250	0,500	0,410	0,410
Citric	0,065	0,050	0,067	0,069	0,066
Aconitic	0,087	0,065	0,097	0,141	0,123

TABLE 14 - The effect of phosphorus deficiency in the amounts and concentrations of malic, citric and aconitic acid in the xylem sap exudate of chickpea (n.d.: not detected).

Chickpea

	Day 0	Day 4		Day 16	
		P+	P-	P+	P-
			(μmol.plant^{-1}.hour^{-1})		
Malic	1,62	18	0,70	86	0,62
Citric	0,19	1,5	0,01	7,44	0,06
Aconitic	0,19	2,1	0,2	13	n.d.
			(mM)		
Malic	0,270	0,300	0,115	0,360	0,077
Citric	0,032	0,025	0,009	0,031	0,008
Aconitic	0,031	0,034	0,031	0,055	n.d.

In figures 15 and 16 are presented the amounts of organic acids released by the roots of chickpea and maize respectively. The exudates were collected in two sites along the root axis: in the tip (apical) and about 3 cm above the tip (3

cm). As can be seen there were large differences not only in the type but also in the amounts of organic acids that were identified. While in chickpea it was possible to detect the presence of both malic, citric and aconitic in maize only malic acid was present in a detectable amount, and this was much smaller when compared with chickpea.

In chickpea malic acid was the main organic acid that was exudated with the amounts being higher 3 cm above the tip (3 cm). The contrary happened with malic and citric. The results that were obtained show us that when a plant that has been deprived of phosphorus is transferred to agar containing phosphorus there is a decrease in the exudation of citric and aconitic acid while the exudation of malic shows a small increase in the tip. It can also be taken from these results that when a plant that has been growing in a P-sufficient nutrient medium is transferred to a P-deficient agar the amounts of namely citric and aconitic acid are increased and are bigger then the ones of the C-- plant. Unfortunately it was not possible to obtain the results from the C++ plants.

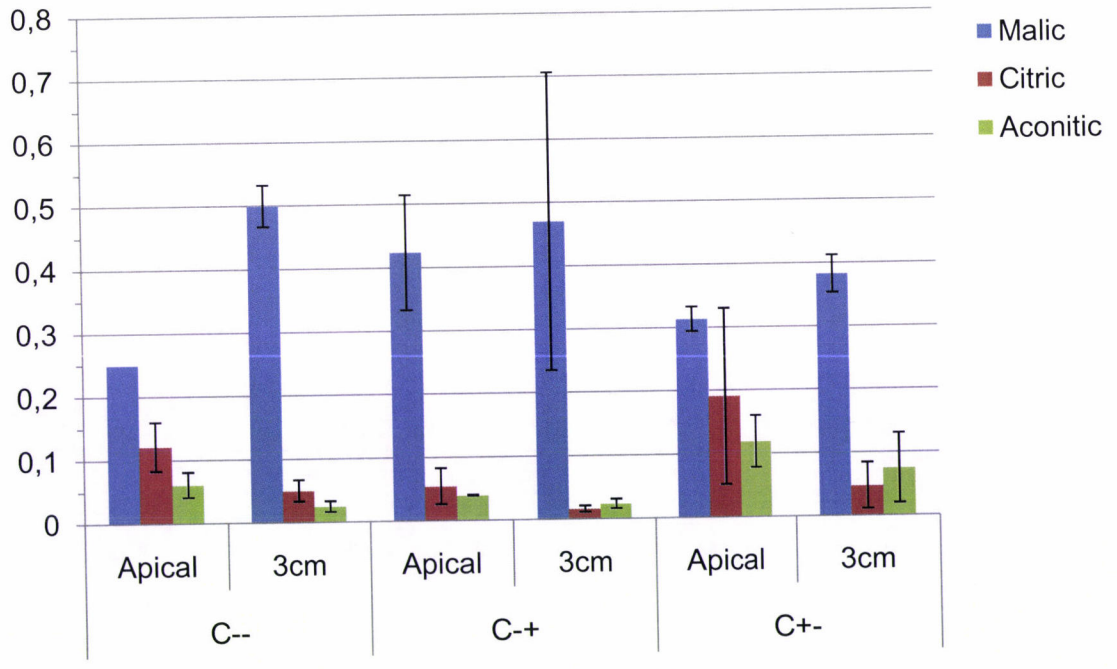


FIGURE 15 – Organic acids in the root exudates of chickpea at day 16.
(C--: P-stressed plant in P-deficient agar; C-+: P-stressed plant in P-sufficient agar; C++: P-unstressed plant in P-suficient agar; C+-: P-unstressed plant in defficient agar)

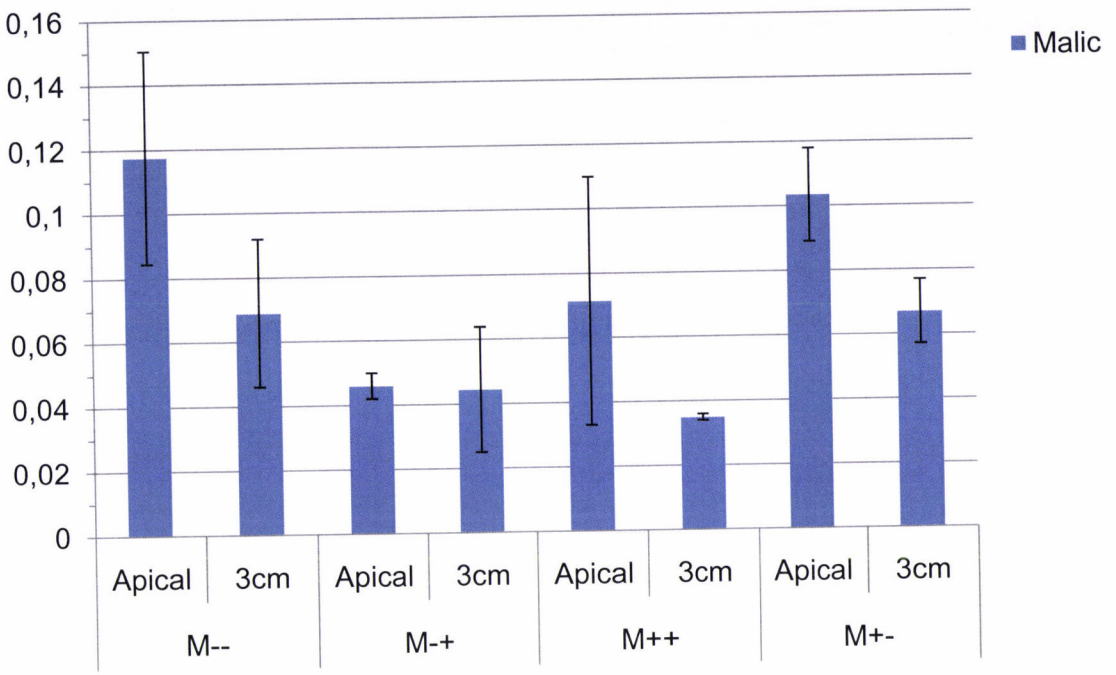


FIGURE 16 – Organic acids in the root exudates of maize at day 8.
(M--: P-stressed plant in P-deficient agar; M-+: P-stressed plant in P-sufficient agar; M++: P-unstressed plant in P-suficient agar; M+-: P-unstressed plant in deficient agar)

In maize the amounts of malic acid in the exudates were always higher in the tips (apical) and a decrease in the amount that was exudated could be observed when a plant tha was growing in a P-deficient medium was transferred to a P-sufficient agar, and were increased when a plant growing in a P-sufficient medium was transferred to a P-deficient agar.

IV - DISCUSSION

The results presented in this work confirm that plants exhibit numerous morphological and physiological adaptations to phosphorous starvation. One of the most pronounced effects of phosphorus deficiency is the reduction in shoot growth while root growth is unaffected or even stimulated. The results show that plant growing under phosphorus deficiency, tend to present a smaller shoot/root ratio. According to Pilbeam *et al.* (1993) this is probably because of a stronger sink competition by roots for photosynthates and possibly by changes in the phytohormone balance between shoots and roots. However Quiu and Israel (1992) defended that this decrease in the shoot/root ratio is not caused by higher export of photosynthates to roots, but by a more efficient utilization of carbohydrates in the roots of P-deficient plants. It is also possible that a combination of these two mechanisms might function together as part of an adaptative "strategy". Low phosphate plants allocate more of their photosynthates to roots and use them more efficiently, at the expense of shoot growth so as to maximize their capacity to absorb phosphate (Fredeen *et al.*, 1989) and this optimization is dependent on plant species, environmental conditions and time in the growing season (Klepper, 1991). The capacity to distribute a higher proportion of the photosynthates to the roots is obviously under genetic control and is an important aspect or phosphorus efficiency for plants grown in deficient soils (Marschner, 1995). This partionizing of photosynthates, the source sink relationships and its controlling mechanisms are therefore of crucial importance in crop production.

In chickpea, the fact that the reduction in shoot growth affected mainly the development of primary and secondary branches seems to indicate that roots were the main sinks. This reduction in the development of lateral branches can have important implications in terms of final seed production because collectively these produce about 83 to 89% or the total flowers ill the plant (Zaifer and Bakarat, 1995).

In maize the shoot/root ratio was not so depressed and this was perhaps related with the fact that the young leaves might have also acted as sinks, wich might also be associated with the duration of the experiment (8 days compared with 16 days for chickpea). The fact that the old leaves behaved as sources

explains the premature senescence (in maize) and tip necrosis (in chickpea) that was observed in these leaves. In maize was also noted the purpling of the leaves wich is known to be associated with the formation of anthocianin pigments.

Another consequence of the reduction in shoot growth is that by decreasing the expansion of the photosynthetic/leaf surface (due to an insufficient supply of phosphorus for the expansion of epidermal cells) phosphorus deficiency decreases plant photosynthetic products, which is further pronounced by a reduction in the photosynthetic rate per unit area as found by Quiu and Israel (1994). The small size and dark green colour of the leaf blades in phosphorus deficient plants are the result of impaired cell expansion and a correspondingly larger number of cells per unit surface area (Hecht-Buchholz, 1967).

Relatively to root growth the increase in the SRL (Specific Root Length) that was observed under phosphorus deficiency (particularly in maize) can be related not only with the increase in the root surface area but also with the possibility that plants have to explore deeper horizons in the soil and/or a bigger soil volume. Root systems are characterized by a very high adaptability and their growth and development involves complex interactions between both the soil environment and the shoots. Since the environment in wich root systems develop is highly heterogeneous, both in space and time, the root system has to have the ability to react to heterogeneity and, thus, must posses high phenotipic plasticity (Fitter, 1991). This might contribute to an improvement of the phosphorus acquisition by the plants because it is known that when the roots of plants that are growing in media deficient of phosphate are exposed to ample concentrations of this nutrient the rate of uptake of the ion previously in short supply is much increased compared with control plants maintained with an adequate provision of this ion (Hoffman, 1968; Cartwright, 1972; Clarkson and Scategood, 1982). Because maize plants developed a bigger SRL we may speculate that they were more efficient than chickpea in determining the spatial availability of phosphorus.

Roots not only act as sinks for mineral nutrients supplied by the soil via mass flow or diffusion but they can also change the rhizhosphere in a variety of ways (Marschner *et al.*,1986; Marschner and Cakmark, 1987). When nutrients are strongly bound to the soil the exposure of large root surfaces to the soil may not be enough to absorb nutrients from very low concentrations. Nutrient acquisition in these cases depends on a variety of "strategies" that increase the solubility of

nutrients by changing the chemical environment of roots. It was observed that phosphorus stress induced a relative acidification of the rhizosphere. Other studies have reported a net acidification of the rhizosphere in response to P-stress, and this is associated with a shift from excess anion over cation uptake by the P-sufficient plants to an excess cation uptake by the P-deficient plants (Nye *et al.*, 1982; Moorby *et al.*, 1988; Hofftand *et al.*, 1989; Le Bot *et al.*, 1990). This highly localized acidification might enable the roots to decrease the rhizosphere pH in apical zones even in calcareous soils to enhance phosphorus mobilization. The form of nitrogen supply (ie, NO_3^-, NH_4^+ or symbiotic N_2 fixation) is a major determinant of the rhizosphere pH (Marschner and Romheld, 1983). However there is increasing evidence that in many instances phosphorus deficiency-induced acidification is either exclusively, or at least to a high extent caused by excretion of organic acids (Marschner, 1995). Striking differences in the rhizosphere pH exist between plant species growing in the same soil and supplied with nitrate nitrogen. Buckweath (Raij and van Diest, 1979) and chickpea (Marschner and Romheld, 1983) have a very low rhizosphere pH compared, for example, with that of weath and maize. These genotipical differences reflect differences in cation/anion uptake ratio (Bekele *et al.*, 1983).

Phosphorus deficiency not only reflected itself in the uptake but also in the distribution of, not only phosphorus but also other nutrients such as nitrogen (NO_3^--N in this case), and potassium.

TABLE 15 - Ionic balance in the roots of P-stressed and P-unstressed maize and chickpea plants on day 8 and 16 respectively (in mmol).(*values from Le Bot *et al.*,1990II).

	K^+	$H_2PO_4^-$	NO_3^-	(C-A)	Total Org. Acids
			Maize		
P+	86,5	17	4,18	64,42	21,7
P-	61,25	4,1	23,28	33,87	17,7
			Chickpea		
P+	134	17,63	14*	102,4	27,6
P-	160	2,75	3*	154,25	24,1

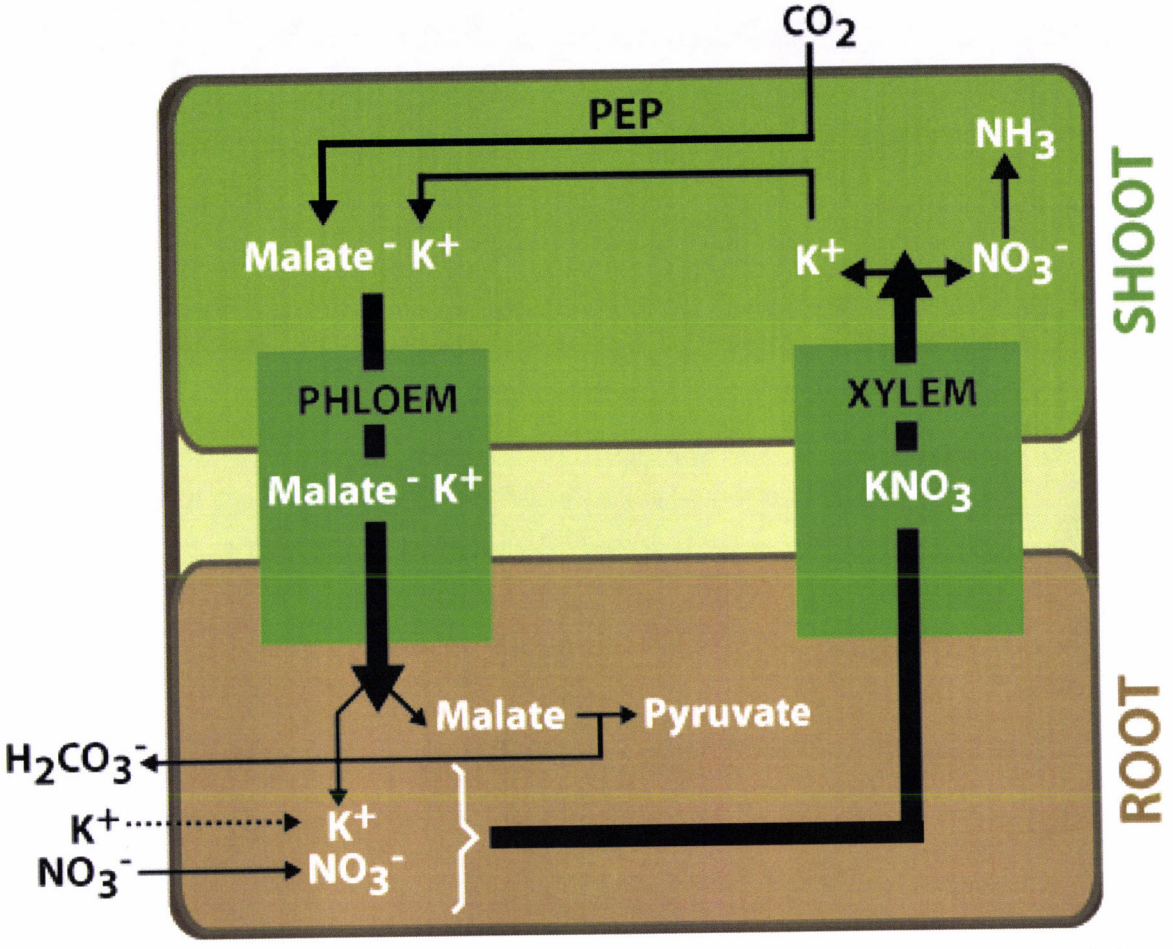


FIGURE 17 - Model for the recirculation of potassium between root and shoot in relation to nitrate and malate transport (PEP-Phosphoenol Piruvate). (Based on Ben-Zioni *et al*,1971 and Kirkby and Knight, 1977).

The concentrations of P (as $H_2PO_4^-$) in the shoot and root of P-stressed plants were noticeably lower than controls. Phosphorus deficiency resulted in an increase in the percentage of the total phosphorus being allocatted in to the root system, and this difference was particularly visible in maize. The fact that the concentration of phosphorus was smaller in the roots of the P-stressed plants can be related not only with the phosphorus deficiency itself but also with the decrease in the shoot/root ratio. These results are consistent with the theoretical generalization (Chapin, 1983) that plants allocatte resources optimally when all processes are equaly limiting to growth. For phosphorus higher use efficiency in certain genotypes may be related to better use of stored Pi (Caradus and Snaydon,1987; Hart and Colville,1988) either within a given tissue or by better retranslocation between shoot organs (Youngdahl, 1990).

The concentration of potassium in both maize and chickpea roots was quite

differently affected by the two treatments. The results that were found can be discussed in relation to Figure 18. Potassium plays an important role as counterion for nitrate transport in the xylem (Van Beusichem *et al.*,1988). In maize the lower concentration of K^+ can be related with the decline in the concentration of organic acids (namely malate), and can explain the higher accumulation of NO_3^- in the roots of the maize P-deficient plants. According to Keltjens (1986) the reduced concentration of K^+ in the roots of P-deficient maize plants can also be due to a lack of a K^+-recirculation in the maize plant.

By the contrary, in chickpea the higher concentration of K^+ in the roots of the Pstressed plants can not be related with the need to maintain electroneutrality in plant tissues. After prolonged periods of phosphorus deficiency plants are thought to produce large amounts of oxalic acid (Ramadam. pers. com.), and the presence of this organic acid migth explain this high K^+ concentration.

Nitrate however seems to be the most sensitive ion to P-stress probably because it is most readily affected by influx and efflux processes (Le Bot *et al.*, 1990). The results resumed in Table 15 show indirectly that P-deficiency has a large influence over the rates of NO_3^- uptake and assimilation, and these results are in agreement with the ones of Rufty et al. (1993).

In maize the fact that the concentration of nitrate was bigger in the P-stressed plants seems to indicate that the assimilation of nitrate was proportionaly more affected than uptake. Working with tomato plants Pilbeam *et al.* (1993) have reported a depression in the rate of nitrate reductase activity (NRA) after 3-5 days of withdrawal of P from the nutrient solution while PEPcarboxilase showed a marked increase over the same period.

In chickpea the concentration of nitrate was smaller in the roots of the P-stressed plants. By reducing the assimilation of nitrate into proteins phosphorus deficiency might have a negative feed-back on NO_3^- influx and/or stimulate NO_3^- efflux. One obvious possibility for the decreased uptake is the decreased availability of energy (metabolic Pi and ATP), required for active uptake of NO_3^- across the plasma membrane of root cells (Glass, 1988).

It is well known that assimilation of nitrate leads to organic acid accumulation (Mengel and Kirkby, 1982). In Table 15 we can also see that phosphorus deficiency by inducing a limitation in the assimilation of nitrate lead to a reduction in the concentration of organic acids in the roots, so that the

electrochemichal balance could be maintained.

The composition and concentration of mineral elements and organic solutes in the xylem sap depends on a various factors such as plant species, mineral element supply to the roots, assimilation of mineral elements in the roots and nitrogen recycling (Marschner, 1995). As can be taken from the tables showed bellow P stress induced a reduction in the concentration of phosphorus in the xylem sap of maize while in chickpea the concentration was bigger in the P-stressed plants. This can be explained by differences in the in the xylem sap volume fluxes for both plant species. The concentration of K^+ in the xylem sap exudate was smaller in the chickpea stressed plants and bigger in the maize stressed plants. Nitrate concentration was also reduced but in maize that only happened after day 2, showing an inverse relation with the concentration of K+ in the roots.

TABLE 16 - Ionic balance in the xylem sap exudate of P-stressed (P-) and P-unstressed (P+) maize plants (in mM).

		K^+	$H_2PO_4^-$	NO_3^-	(C-A)	Total Org. Acids
Day 2	**P+**	33	7,8	6,9	18,3	0,365
	P-	30,9	1,3	10,8	18,8	0,664
Day 8	**P+**	37,8	5,9	7,2	24,7	0,62
	P-	51,5	2,7	4,8	44	0,599

TABLE 17- Ionic balance in the xylem sap exudate of P-stressed (P-) and P-unstressed (P+) chickpea plants (in mM).

		K^+	$H_2PO_4^-$	NO_3^-	(C-A)	Total Org. Acids
Day 4	**P+**	25,5	3,3	1,2	21	0,359
	P-	10,2	5,1	n.d.	5,1	0,155
Day 16	**P+**	21,2	5,4	0,8	14,9	0,466
	P-	10,2	10	0,6	-0,4	0,085

As in ion accumulation in root cells, maintenance of cation-anion balance is necessary in the xylem exudate (Allen *et al.*,1988; Findenegg *et al.*,1989). The corresponding difference in negative charges in the exudate is aproximately compensated by the elevated concentrations of organic acid anions. Once again the concentration of organic acids was a function of the necessity of plants to maintain this ionic balance between cations and anions. There was an exception to this in maize at day 8 were in the P-stressed plants a bigger proportion of the K^+ relativelly to nitrate and phosphorus was not balanced with the production of organic acids. This can mean that in the late stages of phosphorus deficiency other organic acids (such as oxalic acid) might also play an important role in maintainig the ionic balance in these plants.

On average 30-60% of the net photosynthetic carbon is allocated to the roots and of this carbon an apreciable proportion is released as organic carbon into the rhizosphere. This release of carbon, also named rhizodeposition, is highly variable (Lynch and Whipps, 1990). For a given plant species rates of rhizodeposition vary much and can be, for example, 2-4 times higher for soil-grown plants than for plants grown in nutrient solution (Trofymow *et al.*,1987). Organic acids are part of the Low Molecular Weight Root Exudates and enhanced root exudation of organic acids is often observed under phosphorus deficiency in dicots in general and in legumes in particular, and in some plants like white lupin they can be accounted for 23% of the net photosynthesis after 13 weeks growth (Dinkelaker *et al.*, 1989). Once again it is of great importance the high adaptability ("phenotipic plasticity") of plants and root systems in particular to the heterogeneity of the rhizosphere environment, a dinamic microenviromnent continually renewed by root growth and the substances relleased by the root. Organic acids may also be produced by microbial activity stimulated by the release of organic carbon from roots (Marschner, 1995), and certain constituents of the LMW root exudates migth also be transformed by rhizosphere microorganisms to highly physiologicaly active compounds (e.g. phytohormones). The results presented in this work allow us to conclude that the increased exsudation of organic acids may be an important component in the strategies of plant adaptation to acid mineral soils for both increasing efficiency in phosphorus acquisition and avoidance of alluminium toxicity.

Marschner (1991a) has drawn attention to the fact that organic acid release

from roots may be an efficient alternative to V A-Mycorrhiza in the acquisition of phosphorus from the soil.

Citric and malic acid form relatively stable chelates with Fe(III) and Al, thereby increasing the solubility and rate of phosphorus uptake and due to this they can have an important role in bringing inorganic phosphates into solution. Citric acid is the dominant compound in the proteoid root exudates of white lupin (Gardner *et al.*,1983) and effective in the mobilization of phosphorus from both acid and calcareous soils. The highly local citric acid exudation acidifies the rhizosphere even in calcareous soils and mobilizes sparingly soluble calcium phosphates by dissolution and subsequent formation of sparingly soluble calcium citrate in the rhizosphere.

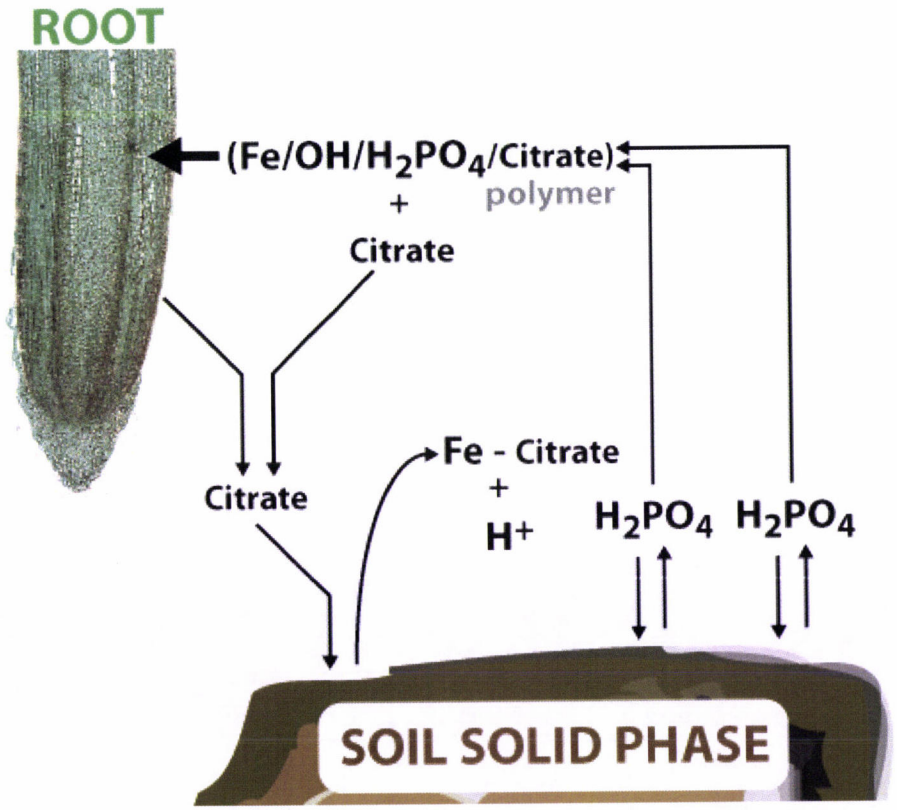


FIGURE 18 - Illustration of the possible reaction sequence between citrate iron and phosphate in soil (From Gardner *et al.*, 1983)

The model presented in figure 18 is an adaptation of the one presented by Gardner *et al.* (1983) and is a representation of the reaction of citrate after it has been exudated in the rhizosphere. According to this model after citrate has been exudated from the roots it reacts with Fe and P leading to the formation of

polymerised ($Fe/OHPO_4$/Citrate) particles that diffuse towards the root surface were, due to the reducing activity of roots, they are degradated and phosphate is absorbed by the plants. This also explains the reported change in the colour of the root system in the P-stressed plants (particularly in chickpea) that is though to be related with the accumulation and/or precipitation of Fe in the root system.

The results obtained and presented in Fig. 15 and 16 seem to be in agreement with this model and with the possibility that citric acid has a very important role in phosphorus acquisition under conditions of low supply. The fact that the plants that were growing in a P-deficient nutrient solution seem to produce less citrate when transferred to a P-sufficient nutrient solution seems to be a confirmation of this. Furthermore when a P-sufficient plant is transferred to a P-deficient medium there seems to be an increase in the exudation of this organic acid. The higher rates of organic acid exudation in chickpea plants can mean that chickpea plants are more efficient than maize plants in affecting the chemichal availability of phosphorus in the nutrient medium were its supply is limited. On acid soils the release of organic acids offers a number of advantages to the plant for it allows the mobilization of P from sparingly soluble Fe and Al compounds wich can then be absorbed by the root. The chelation of Fe and Al by the organic acids also prevents the phytotoxic effects of these elements. It is therefore likely that the secretion of citrate by the roots will result in the formation of ferric hidroxil phosphate polycation kept in solution by a coating of citrate molecules. Thus, the action of citrate would be to effectivelly increase the concentration of phosphorus in solution around the root by formation of the polymeric ($Fe/OH/PO_4$/Citrate) particles wich (in addition to the phosphate ions in solution) can diffuse towards the root surface were, due to the reducing activity of the root), they will be degradated and phosphate will be made available for absorption by the plant.

But in both maize and chickpea it was malic acid the main organic acid to be exudated from the roots, with bigger amounts in chickpea, and if in chickpea there seemed to exist no relation between the rate of its exudation and phosphorus stress the same can not be said for maize, were the behaviour of malic acid was equal to the one reported for citric acid in chickpea. Malic acid is thought to stimulate the microbial activity in the rhizosphere (Sanders, *per. com.*), which may affect the acquisition of mineral nutrients by roots either directly via effects on mobilisation and/or immobilisation or indirectly via effects on root

morphology and/or physiology (Marschner, 1991a). The excretion of organic acids in the rhizosphere is probably coupled with H cotransport, and Ratnayake et al. (1978) suggests that the main factor responsible for the stimulation of exudation under P deficiency is the increase in membrane permeability. Since phospholipids are essential components of the plasma membrane it may be supposed that if phosphate is witheld from the nutrient medium, membrane function should be impaired and permeability increased, as observed by Ratnayake *et al.* (1978), wich results in a net release not only of organic acids but also of other metabolites wich are supposed to promote the infection of the roots by V A-Mycorrhiza (Graham et al., 1981).

The nitrogen fixing microorganism *Azospirillum* has been isolated from maize roots (von Bullow and Dobereiner, 1975) and has been found to use malic acid preferentially as carbon source (von Berkum and Bohool, 1980), and it is possible that some other microorganisms which might present a important role in phosphorus balance in soil can also be stimulated by the presence of malic acid.

The effects of aconitic acid are unknown and further research is necessary not only to understand the effects of and the mechanisms ("strategies") that are enhanced under phosphorus deficiency but also to permit the selection of species or even cultivars according to their ability to respond adaptativelly to nutrient mediums with a low supply of phosphorus.

V - REFERENCES:

-Alien S., Raven, J. A. and Sprent, J. I. (1988). "The role of long distance transport in intracellular pH regulation in *Phaseolus vulgaris* growth with ammonium and or nitrate as nitrogen source, or nodulated." J. Exp. Bot. 39, 513-528.

-Andreeva, T. F. and Persakov, B. M. (1970). "Effect of duration of phosphorus deficiency on photosynthesis intensity and leaf growth in connection with productivity of horse beans." Fiziol. Rast. 17,478-484.

-Barry, D. A. J. and Miller, M. H. (1989). "Phosphorous nutritional requirement of maize seedlings for maximum yeld ". Agon. J. 81:95-99.

-Becard, G. and Pielu, Y. (1989)."Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mychorrizal symbiosis." Appl. and Env. Microbiology. 55:2320-2325.

-Bekele, T. , Cino, B.J. , Ehlert, P.A.I. , van der Mass, A. A. and van Diest, A. (1983). "An evaluation of plant born factors promoting the solubilization of alkaline rock phosphates". 75: 361-378.

-Ben Zioni, A. , Vaadia, Y. and Lips, S. H. (1971). "Nitrate uptake by roots as regulated by nitrate reduction products in the shoot." Physiol. Plantarum. 24:288-290.

-Bethenfalvay, Gabor J. , Deyes-Solis, M. G. , Camel, S. B. and Ferrera-Carrato, R. (1991). "Nutrient transfer between the root zones of soybean and maize plants connected by a common mycorrhyzal mycelium." Physiol. Plantarum. 82: 423-432.

-Bould, C. and Parfitt, R. I. (1973). "Leaf analysis as a guide to the nutrition of fruit crops. X. Magnesium and phosphorous sand culture experiments with apple." J. Sci. Food Agri. 24: 175-185.

-Caradus, J. R. and Snaydon, R. W. (1987). "Aspects of the phosphorus nutrition

of white clover populations. I- Inorganic phosphorus content of leaf tissue." J. Plant Nutr. 11,189-207.

-Cartwright, B. (1972). "The effect of phosphate deficiency on the kinetics of phosphate absorption by sterile excised barley roots, and some factors affecting the ion uptake efficiency of roots." Soil Sci. Plant Anal. 3: 313-322.

-Chapin, F. S. ID (1983). "Adaptation of selected trees and grasses to low availability of phosphorus." Plant Soil. 72,283-297.

-Clarkson, D. T. and Scattergood, C. B. (1982). "Growth and phosphate transport in barley and tomato plants during the development of, and recovery from, phosphate stress." J. Exp. Botany. 33: 865-875.

-Cramer, M. D. , Lewis, O.A. and Lips, S.H. (1993). "Inorganic carbon fixation and metabolism in maize roots as affected by ammonium and nitrate nutrition." Physiol. Plantarum. 89: 632-639.

-Davies, D. D. (1986). "The fine control of cytosolic pH." Physiol. Plantarum. 67: 702-706.

-Dinkelaker, B. , Romheld, V. and Marschner, H. (1989). "Citric acid excretion and precipitation of calcium citrate in the rhizosphere of white lupin *(Lupinus albus* L.)." Plant Cell Environ. 12: 285-292.

-Drew, M. C. , Saker, L. R. , Barber, S. A. and Jenkins, W. (1984). "Changes in the kinetics of phosphate and potassium absorption in nutrient deficient barley roots maesured by a solution depletion technique." Planta. 160: 490-499.

-Dunlop, James and Gardiner, Sarah (1993). "Phosphate uptake, proton extrusion and membrane electropotentials of phosphorous deficient *Trifolium repens* L." J Exp. Botany.44: 1801-1808.

-Engels, C. and Marschner, H. (1993). "Influence of the form of nitrogen suply on root uptake and translocation of cations in the xylem exudate of *maize (Zea mays*

L.)." Journal of Exp. Botany. 44: 1695-1701.

-Findenegg, G. R. , Nelemans, J. A. and Arcozis, P. A. (1989). "Effect of external pH and Cl on the accumulation of NH_4-ions in the leaves of sugar beet." J. Plant Nutr. 12, 593-601.

-Fitter, A. H. (1991b) "Charactheristics and functions of root systems." *In* "The Plant Root, the Hidden Half." (Y. Waisel , A. Eschel and V. Kafkafi, eds.). pp. 3-25. Marcel Dekker, New York.

-Fitter, A. H. and Hay, R. K. M. (1983). "Environmental physiology of plants. " 2nd edition, Academic Press, London, UK.

-Fredeen, A. L. , Rao, I. M. and Terry, N. (1989). "Influence of phosphorus nutrition on growth and carbon partionizing in *Glicine wax."* Plant Physiol. 89,225-230.

-Gahoonia, T. S. , Classen, W. and Jungk, A. (1992). "Mobilization of phosphate in different soils by ryegrass suplied with ammonium or nitrate." Plant Soil. 140: 241-248.

-Gardner, W. K. , Barber, D. A. and Parberry, D, G. (1983). "The acquisition of phosphorous by *Lupinus albus* L. III- The probable mechanism by wich phosphorous movement in the soil/root interface is enhanced. " Plant and Soil. 70, 107-124.

-Glass, A. D. M. (1988). "Nitrogen uptake by plant roots." ISI Atlas of Sci. Anirn. Plant Sci. 1,151-156.

-Hart, A. L. and Colville, C. (1988). "Differences among attributes of white clover genotypes at various levels of phosphorus suply." J. Plant Nutr. 11,189-207.

-Hecht-Buchholz, C. (1967). "Uber die Dunkelfarbung des Blattunks bei Phosphorrnangel." z. Pflanzenernahr Bodenk. 118,12-22.

-Hedley, M. J. , Nye, P. H. and White, R.E. (1982). "Plant induced changes in the rhizosphere of rape seedlings II- Origin of the pH change." New Phytologist. 91: 31-44.

-Hewinkel, H. (1991). "Auswirkungen von P-mangel auf das waschtum und die nahrsloffan nahrne von tomatenpflanzen in nahrslosung." (Diplomarbeit, Hohenheim 1991).

-Hewinkel, H. et al. (1992). "Phosphorous deficiency enhances molibdenium uptake by tomato plants." J. Plant Nut., 15(5), 549-568.

-Hoffland, Ellis ,Findenegg, G. R. and Nelemans, J. A. (1989a). "Solubilization of rock phosphate by rape. I -Evaluation of the role of the nutrien uptake pattern." Plant and Soil, 113, 155-160.

-Hoffman, W. E. (1968). "Mineralstofftransport in die xilengefasse der wurzel in abhangigkeit von inrem K- und P-status." Land-wirtsch. Forsch. 21: 203-212.

-Jones Jr, J. Benton (1991). "Plant analysis Handbook. " Micro-Macro Publishing Inc., USA.

-Keltjens, W. G. (1886). "Nitrogen metabolism and K recirculation in plants." International Plant Nutrition Colloquium. Washington. pp283-287.

-Khamis, S., Chaillon, S. and Lamaze, T. (1990a). "CO_2 assimilation and partionizing of carbon in maize plants deprived of orthophosphate." J. Exp. Bot., 41, 1619-1625.

-Kirkby, E. A. and Le Bot, J. (1995). "Phosphorus in the vegetal kingdom." Unpublished paper.

-Kirkby, E. A. and Mengel, K. (1967). "Ionic balance in different tissues of the tomato plant in relation to nitrate, urea, or ammonium nutrition." 42,6-14.

-Klepper, Betty (1991). "Root-shoot relationships." In Plant roots: the hidden half

(Ed Waisel, Yoav; Eschel, Amram; Kafkafi, Uzi) pp 265-286.

-Le Bot, J. , Alloush, G. A. , Kirkby, E. A. and Sanders, F. E. (1990). "Mineral nutrition of chickpea plants suplied with N03 or NIIa-N. II-Ionic balance in relation to phosphorous stress." 1. Plant Nutrition, 13(12),1591-1605.

-Lewis, D. W. (1987). "Evolutionary aspects of mutualistic associations between fungi and photosyntetic organisms." In Evolutionary Biology of the Fungi." (Ed. Rayner, A. D. M., Brasier, C. M. and Moore, D.). Cambridge University Press, Cambridge, pp 161-178.

-Linskens, H. F. and Jackson, J. F. (1987). "High Performance Liquid Chromatography in plant sciences." Springer Verlag, Berlin, Germany.

-Lipton, Doug S. , Bauchar, R. H. and Blevins, D. G. (1987). "Citrate, malate and succinate concentratin in exudates from P-sufficient and P-stressed *Medicago sativa* L. seedlings." Plant Physiology, 85, 315-317.

-Lynch, J. M. and Whipps, J. M. (1990). "Substrate flow in the rhizosphere." Plant Soil. 129, 1-10.

-Marschner, H. (1991a). "Root induced changes in the rhizosphere." *In* "The Plant Roots, the Hidden Half" (Y. Waisel, A. Eshel and V. Kafkafi, eds.) pp. 503-528. Marcel Dekker, New York.

-Marschner, H. (1991b). "Mechanism of adaptation of plants to acid soils." Plant Soil. 134: 1-20.

-Marschner, H. (1995). "Mineral nutrition of higher plants." Academic Press. 2nd edition. Cambridge, Great Britain.

-Marschner, H. and Romheld, V. (1983). "In vivo measurements of the root induced pH changes at the soil-root interface: effect of plant species and nitrogen source. " Z. Pflanzenphysiol. 111: 241-251.

-Marschner , H. , Romheld, V. , Horst, W. J. and Martin, P. (1986b). "Root induced changes in the rhizosphere: importance for the mineral nutrition of plants. "Z. Pflanzenernahr. Bodenk.. 149: 441-456.

-Marinus, L. Van Bensichem, Kirkby, E. A. and Baas, Robert (1988). "Influence of nitrate and ammonium nutrition on uptake, assimilation, and distribution of nutrients in *Ricinus cummunis.* "Plant Physiology, 86, 914-921.

-Martin-Prevel, P. Gagnard, J., Gaultier, P. (1987). "Plant analysis as a guide to the nutrient requirements of temperate and tropical crops." Lavoisier Publishing inc., France.

-Mengel, K. and Kirkby, E.A. (1982). "Principles of plant nutrition." International Potash Institute Worblaufen-Bem, Switzerland.

-Miller, Raymond W. and Donhaue, Roy L. (1990). "Soils: an introduction to soils and plant growth." 6th edition, Prentice Hall International Editions, USA.

-Moorby, H. , White, R. E. and Nye, P. H. (1988) "The influence of phosphate nutrition on H ion efflux from the roots of young rape plants." Plant and Soil, 105,247-256.

-Ohwaki, Yoshinari and Hirata, Hiroshi (1992). "Differences in carboxilic acid exudation among P-starved leguminous crops in relation to carboxilic acid contents in plant tissues and phospholipid level in roots." Soil Sci. Plant Nutrition. 38: 235-243.

-Pilbeam, D. J. , Cakmak, I. , Marschner, H. and Kirkby, E. A. (1993). "Effect of withdrawal of phosphorus on nitrate assimilation and PEP carboxilase in tomato . "Plant and Soil. 154: 111-117.

-Pirozynski, K. A. and Malloch, D. W. (1975) "The origin of land plants: a matter of mycotrophism." Biosystems. 6: 153-164.

-Quiu, Justin and Israel, D. W. (1992). "Diurnal starch accumulation and utilization

in phosphorus deficient soybean plants." Plant Physiol. 98: 316-323.

-Quiu, Jinshu and Israel, Daniel W. (1994). "Carbohidrate accumulation and utilization in soybean plants in response to altered phosphorus nutrition." Physiol. Plantarum. 90: 722-728.

-Raij, B. van and van Dies, A. (1979). "Utilization of phosphate from different sources by six plant species." Plant Soil. 51,577-589.

-Ratnayake, M. , Leonard, R.T. and Menge, A. (1978). "Root exudation in relation to supply of phosphorus and its possible relevance to mycorrhizal infection." New Phitol. 81: 543-552.

-Roberts, Justin K. M. (1981). "Extent of intracellular pH changes during Ir extrusion by maize root-tip cells." Planta. 152: 74-78.

-Rossiter, R. C. (1978). "Phosphorous deficiency and flowering in subterraneum clover *(Trifolium subterraneum* L.)." Ann. Botany,(London) [NS] 42, 325-329.

-Rychter, Anna M. and Randall, Douglas D. (1994). "The effect of phosphate deficiency on carbohydrate metabolism in bean roots." Physiol. Plantarum. 91: 383-388.

-Rufty, Thomas W. Jr. , Charles, T. Mackown and Daniel, W. Israel (1990) "Phosphorous stress effects on assimilation of nitrate. " Plant Physiology, 94, 328-333.

-Rufty Jr., Thomas W. , Siddigi, M. Y. , Glass, A. D. M. and Ruth, T. J. (1991). "Altered $N0_3$- influx in phosphorus limited plants." Plant Science. 76: 43-48.

-Rufty Jr., Thomas W., Israel, D. W. , Volk, R. J., Qin, J. and Sa, T. (1993)."Phosphate regulation of nitrate assimilation in soybean." 1. Exp. Botany. 44:879-89l.

-Schjorring, J. K. (1986). "Nitrate and ammonium absorption by plants growing at

a sufficient or insufficient level of phosphorus in nutrient solutions." Plant and Soil. 91: 313- 318.

-Stitt, Mark and Quick, W. Paul (1989). "Photosynthetic carbon partionizing: its regulation and possibilities for manipulation." Physiol. Plantarum. 77: 633-641.

-Stuart Chapin, F. and Wardlaw, Ian F. (1988). "Effect of phosphorus deficiency on source-sink: interactions between the flag leaf and developing grain in barley." J. Exp. Botany. 39: 165-177.

-Theodorou, Maria E. and Plaxton, William C. (1993) "Metabolic adaptation of plant respiration to nutritional phosphate deprivation If. Plant Physiology, 101,339-344.

-Trofymow, J. A. , Coleman, D. C. and Cambardella, C. (1987). 'Rates of rhizodeposition and ammonium depletion in the rhizosphere of axenic oat roots." Plant Soil. 97, 333-344.

-Usuda, Hideaki and Shimogawara, H. (1993). "Phosphate deficiency in maize.IV-Change in the amounts of sucrose phosphate synthase during the course of phosphate deprivation." Plant Cell Physiol. 34: 767-770.

-von Berkum, P. and Bohlool, B. B. (1980). "Evaluation of nitrogen fixation by bacteria in association with roots of tropical grass." Microbiological Reviews. 44: 491-517.

-von Bulow, J. F. and Dobereiner, J. (1975). "Potential for nitrogen fixation in maize genotypes in Brazil." Proceedings of the National Academy of Sciences, USA. 72: 2389- 2393.

-Youngdahl, L. J. (1990). "Differences in phosphorus efficiency in bean genotypes." J. Plant Nutr. 13, 1381-1392.

-Zaifer, H. Z. and Bakarat, S. G. (1995) " Flower and pod abortion in chickpea as altered by sowing date and cultivar." Can. J. Plant Sciences. 75: 321-327.