NET PROTON FLUXES ALONG MAIZE ROOTS AS A CONSEQUENCE OF NITROGEN UPTAKE

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RÉSUMÉ

Les flux de protons chez de jeunes racines de maïs (*Zea mays* L. cv Volga) sont étudiés à l'aide du système pH-stat et de la méthode de vidéodensitomètrie d'indicateur coloré de pH. Les flux de protons varient d'intensité et de localisation le long de la racine en fonction de la source d'azote. Ces flux ne sont pas uniformément distribués le long de la racine mais sont localisés en des zones préférentielles où leurs valeurs sont souvent importantes. Lorsque la source d'azote est NH_4^+, une acidification de la rhizosphère a lieu au niveau de la zone subapicale. Les valeurs moyennes de l'efflux de protons pour tout le système racinaire sont de 173.17 nmol. H^+ g^{-1}P.F. h^{-1}. Toutefois, les mesures vidéodensitomètriques, montrent des valeurs de 3 nmol H^+ h^{-1} mm^{-1}, au niveau des zones apicales. L'azote sous forme NO_3^- entraîne une alcalinisation de la rhizosphère avec des valeurs moyennes d'influx de H^+ pour tout le système racinaire de -2579 nmol. H^+ g^{-1}P.F. h^{-1}. Les mesures obtenues par vidéodensitomètrie montrent que l'influx de protons est plus important au niveau des dix premiers millimètres de la zone apicale. Les flux nets de protons sont comparés aux prélèvements d'azote et indiquent un rapport $H^+/NO_3^- > 1$, alors que H^+/NH_4^+ est proche de 1.

SUMMARY

Proton fluxes by roots of intact maize (*Zea mays* L. cv Volga) seedlings were studied in nutrient solutions using pH-stat system; or in agarose media containing bromocresol purple, using pH-indicator dye videodensitometry method. H^+ fluxes differ in both, localisation along the root axis and intensity. As they vary considerably according to the form of nitrogen supplied to the plants. Proton fluxes are not uniformly distributed along the roots but are localized in preferential regions where they were sometimes considerable. Under NH_4^+, acidification occurs in the subapical zone. The average values of proton efflux for the whole root system are 173.17 nanoromoles H^+ per gram fresh weight per hour; however, for the apical root zones, videodensitometric measurements give values about 3 nmol H^+ h^{-1} mm^{-1}. While, NO_3^- nutrition leads to an alkalinisation with an average values of H^+ influx for the whole root system of -2579 nanoromoles H^+ per gram fresh weight per hour, when videodensitometric measurements show that the apical root zone took up H^+ in the first 10 mm . Net H^+ fluxes are compared to nitrogen uptake rates and results seem to indicate a H^+/NO_3^- stoichiometry > 1, while H^+/NH_4^+ was close to 1 .

Key words : Ammonium, H^+ flux, Nitrate, Nitrogen uptake, pH-stat, Videodensitometry, *Zea mays*

INTRODUCTION

Proton fluxes play an important role in many physiological processes and have important consequences for nutrient solubility and uptake. The intensity and direction of proton fluxes and pH changes may vary markedly within the rooting environment. The origin of this increase or decrease in pH may be related to both the form and quantity of ions entering the plant as well as to their subsequent metabolism (RAVEN *et al.*, 1992). pH changes vary considerably according to environmental conditions, physiological status, and position along the root (PLASSARD *et al.*, 1999). Changes in the pH of the rhizosphere depend particularly on the form of nitrogen supplied to the plants. While NO_3^- nutrition leads to an alkalinisation of the growth media, NH_4^+ utilisation is characterized by an acidification (RAVEN *et al.*, 1992 ; Plassard *et al.*, 1999). For the ecological role of proton extrusion two main factors are important, the localisation along roots and the rate of proton extrusion (RÖMHELD *and al.*, 1984). Root-induced acidification of the rhizosphere is an important mechanism for mobilisation of mineral nutrients from sparingly soluble sources such as phosphorus from rock phosphates (RILEY and BARBER, 1971) or iron from inorganic Fe^{III} compounds (GARDNER *et al.*, 1982). Several methods have been developed for measuring ionic fluxes along roots. Net proton flux can be rapidly quantified by the pH-stat technique (DAVIDIAN, 1986). A pH-stat prevents any change in pH in the incubation medium by continuous back-titration. Quantification of the consumption of titrator allows the calculation of net proton transport rates. The pH-indicator dye videodensitometry enables pH around roots to be mapped and the amount of protons released by various root regions to be determined (RUIZ and ARVIEU, 1990 ; JAILLARD *et al.*, 1996). In this paper, we report on results with intact maize plants on both the intensity and direction of proton fluxes and localisation along roots as induced by various nitrogen source supplies.

MATERIAL AND METHODS

Plant material

Seeds of maize (*Zea mays* L. cv. Volga) were surface sterilized for 10 min with 0.3 % sodium hypochlorite, washed in distilled water and placed between two sheets of filter paper. Seeds were germinated in the dark at 25 °C in a filter paper moistened with 0.2 mM $CaSO_4$. On the third day seedlings were transferred to hydroponics' tanks containing 5 l of aerated nutrient solution containing : 0.75 mM K_2SO_4, 0.65 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.1 mM FeEDTA, 10^{-2} mM H_3BO_4, 10^{-4} mM $MnSO_4$, 5 10^{-5} mM $CuSO_4$, 5 10^{-5} mM $ZnSO_4$, 5 10^{-6} mM Na_2MoO_4. Plants were grown for 5 days in a growth chamber with a 16/8 h photoperiod, a light intensity of 550 µmol m^{-2} s-1, a temperature of 25/20 °C and 75/80 % relative humidity. Nitrogen was supplied as either 2 mM Ca $(NO_3)_2$ or 0.5 mM $(NH_4)_2SO_4$ and 0.5 mM $CaSO_4$. Solution pH was adjusted to 6.5 by adding 0.1 N Ca $(OH)_2$.

Preparation of plants for H+ flux measurement using videodensitometry

For the videodensitometry technique, the distribution of pH around seminal roots was measured on thin films of agarose gel (PLASSARD *et al.*, 1999). The films were prepared by adding 10 g of agarose powder (Fluka ref. 05068) (CALBA *et al.*, 1995) and 30 mg of bromocresol purple per litre of nutrient solution containing nitrogen as either NO_3^- or NH_4^+. The mixture was boiled for several minutes, then cooled to 37 °C in a water bath and pH was adjusted to 6.5. The solution was then poured into a trough made of two 100 200 mm rectangular sheets of

glass separated and kept parallel to one another by 3 mm thick strips of PVC .The seedling's root system was first positioned between the two glass sheets, then the gel was poured over it. The upper glass sheet was then removed so as not to confine the root environment. Two saturated calibration standards (basic and acidic respectively) also had to be produced for each series of measurements.

Measurement of proton fluxes

Net proton fluxes were measured in a growth chamber at 25 °C, using a pH-stat adjusted to pH 6.5 with continuous aeration. In this system the pH was held constant by titration during the experiment. In the pH-stat, proton excretion is measured by registration of the amount of titrator needed to maintain a constant pH. The fresh weight of roots was determined after removing the excess water by blotting with filter paper.

Measurement and Localization of the proton fluxes along Roots

To localise the proton extrusion along single roots, a videodensitometry technique was applied, using a pH dye indicator dissolved in an agarose medium (JAILLARD *et al.*, 1996). The agarose medium contained the complete nutrient solution with nitrogen as NO_3^- or NH_4^+. Measurements were achieved in a darkroom to avoid stray light. The two acidic and basic standards and plant were then placed one after the other in the camera field for acquiring the images of optical density. At the end, the images of pH and total H^+ concentration (C_H) were computed from images of optical density according to equations :

$$pH = pK - \log_{10}\left(\frac{D - D_B}{D_A - D}\right) \quad (1)$$

Where pK is the pK value of the dye indicator (4.69 for bromocresol), D is the optical density of the medium, and D_A and D_B are the optical densities of acidic and basic standards respectively ;

$$C_H = \int_{pH} dC_H = -\int_{pH} \beta(pH)\ dpH \quad (2)$$

Where β(pH) is the buffering capacity of the medium.

The H^+ fluxes (J_L) profiles along roots were computed from images of total H^+ concentration using equations :

$$J_L = \frac{\Delta Q_H}{\Delta t\ L_R} \quad (3)$$

Where DQ_H is the variation of the total H^+ in the medium ; Dt, the time interval ; L_R, the length of root segment ;

$$\Delta Q_H = \sum_{i=1}^{n} v_i\ \Delta C_{H,i} = v \sum_{i=1}^{n} \Delta C_{H,i} \quad (4)$$

Where v is the elementary volume of medium associated with each pixel in the image ($v = L\ l\ e$, where L, *l* and e are pixel length, pixel width and medium thickness).

Measurement of nitrogen uptake

Determination of net uptake rates of NO_3^- and NH_4^+ was based on nitrogen disappearance from the solution per unit time and per gram fresh weight. The NO_3^- and NH_4^+ concentrations were determined by an automatic analyser (Technicon, RA-2000).

RESULTS AND DISCUSSION

Results

H^+ fluxes measured with pH-stat

With pH-stat system, net H^+ fluxes of maize roots supplied with either NH_4^+ or NO_3^- were measured directly in the nutrient solution (table 1).

Table 1 : pH-stat measurements of net H^+ fluxes of maize roots supplied with NH_4^+ or NO_3^-.

Time (min)	H^+ added (µmol g-1 FW)		H^+ flux (µmol g-1 FW)	
	under NH_4^+	under NO_3^-	under NH_4^+	under NO_3^-
0	0.000	0.000	0.000	0.000
10	0.067	-0.339	0.067	-0.339
20	0.085	-0.868	0.018	-0.529
30	0.110	-1.396	0.025	-5.528
40	0.130	-2.113	0.020	-0.717
50	0.142	-2.641	0.012	-0.528
60	0.158	-3.245	0.016	-0.604
70	0.175	-3.773	0.017	-0.528
80	0.187	-4.377	0.012	-0.604
90	0.199	-4.981	0.012	-0.604
100	0.210	-5.584	0.011	-0.603
110	0.219	-6.000	0.009	-0.416
120	0.237	-6.758	0.018	-0.758

In both cases, the pH was set constant at pH 6.5. Figure 1 shows net H^+ efflux (acidification) with NH_4^+ nutrition (1a), while alkalinisation (negative values) took place with NO_3^- nutrition (1b). Those negative values obtained when N_3^- is the only nitrogen source may be related to net H^+ influx, which is experimentally indistinguishable from OH^- efflux (RAVEN *and al.*, 1992).

Figure 2 shows pH-stat registration of the amounts of titrators needed to maintain a constant pH. It point out an increase in H^+ added (H^+ uptake) into the medium of NO_3^- -fed plants with time (2a), while OH^- added increased in NH_4^+-grown plants (2b) .

H^+ fluxes measured with videodensitometry

Figure 3 shows videodensitometric measurements of H^+ fluxes released by maize roots cultivated at pH 6.5 in nutrient solution with either NH_4^+ (3a) or NO_3^- (3b). The H^+ flux measurements using videodensitometry confirmed the acidification of the rhizosphere (H^+efflux) when NH_4^+ is the nitrogen source, while alkalinisation (H^+ influx) occurred in the medium of NO_3^--grown plants . H^+ flux raised up to 3 nmol h^{-1} mm^{-1}, when NH_4^+ is the nitrogen source, while the maximal proton uptake of -0.2 nmol h^{-1} mm^{-1} is reached with NO_3^- supply.

Localisation of the H^+ fluxes along roots

Figure 4 shows different localisations of H^+ fluxes along roots. Depending upon the form of nitrogen present in the culture solution, H^+ fluxes are localised in different zones along the roots of maize.

Nitrogen at NH_4^+ form, leads to a first acidification zone in the subapical region between 3 and 20 mm from the root tip (4a), followed by a region where H^+ efflux was very low. Thereafter, other acidification zones occurred along the roots. When NO_3^- is the sole nitrogen source, videodensitometry showed that H^+ influx occurred along the whole root, with a maximal proton uptake between 2 to 8 mm from the root tip, followed by a region where

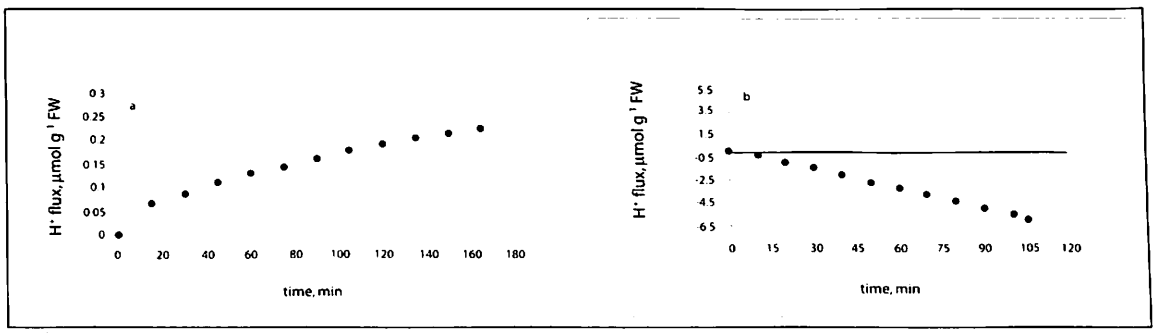

Figure 1 : pH-stat monotoring of the amounts of H^+ produced or consumed by *Zea mays* seedlings with either NH_4^+ (a) or NO_3^- (b).

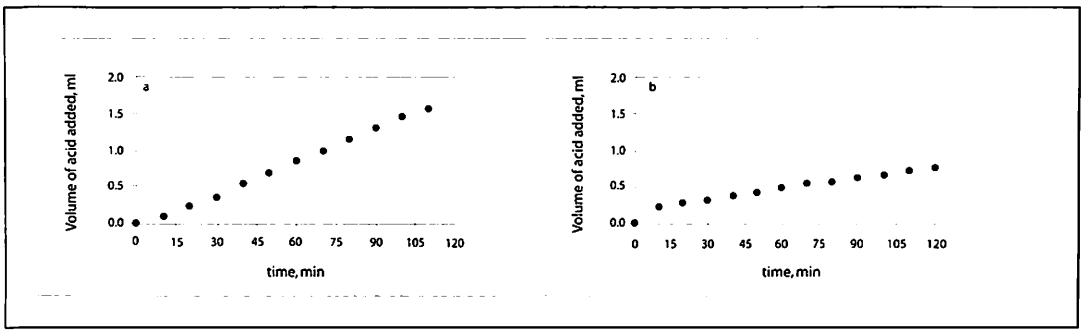

Figure 2 : pH-stat registration of the amounts of titrators needed to maintain a constant pH in the nutrient solution of NO_3^- -grown plants (a) or NH_4^+ -grown plants (b) of *Zea mays*.

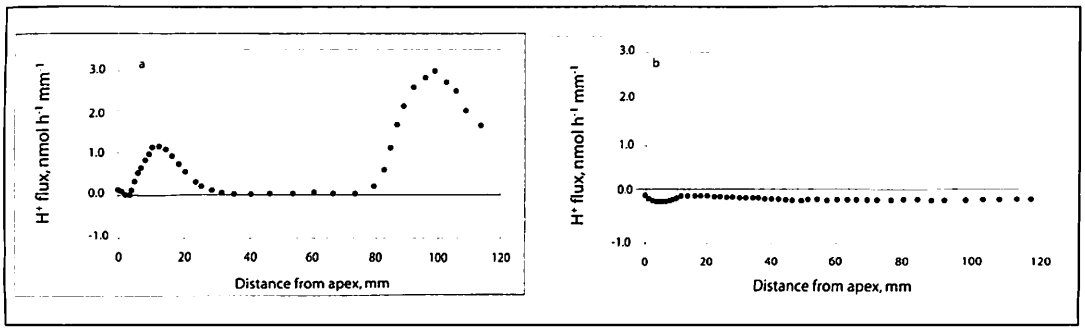

Figure 3 : Measurement of H^+ fluxes along the main root of maize cultivated at pH 6.5 in presence of ammonium (a) or nitrate (b).

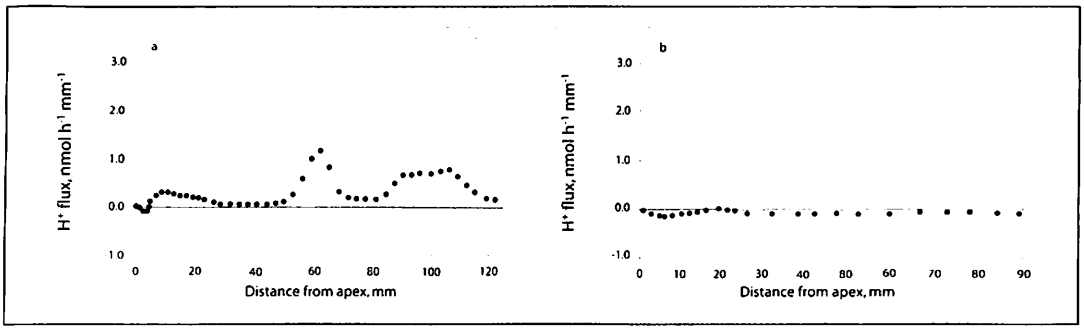

Figure 4 : Videodensitometric localizations of H^+ fluxes along the main root of maize cultivated at pH 6.5 in presence of ammonium (a) or nitrate (b).

H^+ influx decreased considerably leading to an acidification zone. Beyond this zone, the H^+ flux was constant and low (4b).

Net H^+ fluxes and estimate nitrogen uptake rates

A comparison between the rates of nitrogen uptake and H^+ fluxes using intact plants was made. NO_3^- uptake by roots is accompanied by an alkaline effect on the nutrient solution caused by efflux of OH^- (H^+ uptake), while NH_4^+ uptake leads to an acidification caused by efflux of H^+ in the nutrient solution. The results from these experiments demonstrate that, according to the form of nitrogen present in the nutrient

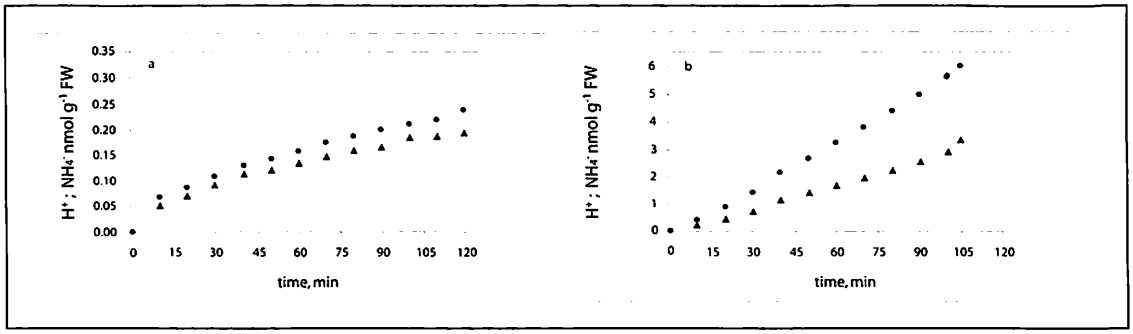

Figure 5 : Time course of the amouts of H^+ produced or consumed (n), and the concentration of N depletion (5) from the nutrient solutions of *Zea mays* supplied with NH_4^+ (a) or NH_3^- (b).

solution, the direction and intensity of the net H^+ fluxes change significantly.

Figure 5 shows the H^+ (OH^-) and nitrogen uptake during 120 minutes experiment. With NH_4^+, in the medium, values of H^+ efflux were not far from the nitrogen taken up values (figure 5a), while, when NO_3^- is the nitrogen source, the amount of nitrogen taken up was different from the OH- efflux (figure 5b) .
The H^+: NH_4^+ ratio determined here is 1, while with nitrate, a ratio OH^- : NO_3^- was about 2 (2 mol H^+ per mol NO_3^-).

DISCUSSION

The results show that, with NH_4^+ in the medium, the maize roots released large amounts of H^+.

The H^+ flux measurements using videodensitometry indicated, however, that acidification is not uniformly distributed along the roots but was localized in preferential regions where fluxes were sometimes considerable. Videodensitometric measurements of proton flux show that H^+ efflux was slowest at the very apex and increased in the zone of elongation, 5 to 20 mm from the apex. A first region of root acidification was clearly identifiable in the subapical zone.

When NO_3^- is the sole nitrogen source, the maize roots released or took up small amounts of H^+, as reported by several authors (MARSCHNER and RÖMHELD, 1983 ; ALLEN and ALLEN, 1987 ; BLOOM, 1997 ; PLASSARD *et al.*, 1999). The videodensitometric measurements, revealed specific and repetitive functioning of the apical region, with alkalinisation of the rhizosphere in the first 20 mm followed sometimes by acidification.

The reason for the differences of H^+ fluxes within a root system is not fully understood. Such root functioning has been reported by several authors (WEISENSEEL *et al.*, 1979; MULKEY et EVANS, 1981 ; O'NEILL and SCOTT, 1983). Possible explanations for the differences may be due to the cation / anion uptake balance (HAYNES, 1990) at specific root zones and physiological characteristics of each plant. The differences in H^+ fluxes between various root zones is presumably due to differences in metabolism along the root axes (MARSCHNER et RÖMHELD, 1983). The systematic alkalinisation occurring in the first 10 mm of the roots indicated that, under these conditions, there was always a higher uptake of anions than of cations in this zone (PLASSARD *et al.*, 1999).
With videodensitometry, the active regions along the root system can then be directly identified and temporal variations in the amounts of H^+ fluxes in the medium determined.

In this study, using pH-stat technique, the rates for H^+ efflux under NH_4^+ nutrition were similar to those for net NH_4^+ uptake. While, under NO_3^- nutrition, influx of 1 mol NO_3^- is accompanied by influx of 2 mol H^+. These data support that NO_3^- transport is followed by a symport with 1 to 2 H^+ (GLASS, 1988 ; MC CLURE *et al.*, 1990 ; BLOOM, 1997). Our result are consistent with the results from Arabidopsis sp (TSAY *et al.*, 1994) and *Hordeum vulgare* (WOLLENWEBER, 1997).

The two methods used in this study gave information on the root functioning as a function of various conditions. While, pH-stat method leads to understand the behaviour of all the root system ; with videodensitometry, the active regions along the root system can be identified, pH around the root mapped and temporal variations in the amounts of H^+ fluxes in the medium determined.

CONCLUSIONS

Results from the experiments presented here demonstrate that direction and intensity of H^+ fluxes depend mainly on the form of nitrogen in the medium. When nitrogen was present in the medium as NH_4^+, roots tend to acidify the rhizosphere, while NO_3^- form leads to an alkalinisation. H^+ fluxes were not uniformly distributed along the root axis and position of different active regions along a root can vary from one plant to another.

The results presented in this paper show that the pH-stat system can be used to study daily nutrient uptake and some aspects of nutrient acquisition on a long term basis. While, pH-indicator dye videodensitometry technique which is easy to use and can rapidly localise the active zones and produce maps of H^+ fluxes intensities along a root system, seems to be appropriate for screening the behaviour of plant species or cultivars in various rooting conditions.

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