Plant and Soil 119, 191–197 (1989).
© Kluwer Academic Publishers. Printed in the Netherlands.
PLSO 8088

Effect of root environment on proton efflux in wheat roots

Y. BASHAN and HANNA LEVANONY[1]
Department of Agronomy, The Ohio State University, Columbus, OH 43210, USA and Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel. [1] Address for correspondence

Received 28 December 1988. Revised April 1989

Key words: antimony microelectrodes, associative bacteria, Azospirillum, nitrate and ammonium uptake, pH indicator, rhizosphere pH, proton pump, *Triticum aestivum* L.

Abstract

Proton net efflux of wheat (*Triticum aestivum* L.) roots growing in sand culture or hydroponics was determined by measuring the pH values of the solution surrounding the roots by pH microelectrodes, by base titration and by color changes of a pH indicator in solid nutrient media. The proton net efflux was dependent on light, aeration, and source of nitrogen (NH_4^+, NO_3^-). Ammonium ions caused the highest proton efflux, whereas nitrate ions decreased the proton efflux. Iron deficiency had no significant effect on proton efflux. Replacement of ammonium by nitrate inhibited proton efflux, whereas the reverse enhanced proton extrusion. A lag period between changes in plant environment and proton efflux was observed. The proton net efflux occurred at the basal portion of the roots but not in the root tips or at the elongation zone. Under optimal conditions, proton efflux capacity reached a maximum value of 5.7 μmole H^+ g^{-1} fresh weight h^{-1} with an average (between different measurements) of 3.4 μmole H^+ g^{-1} fresh wt h^{-1} whereas the pH value decreased to 3.2–3.7 and reached a minimal value of 2.9. Inhibition of ATPase activity by orthovanadate inhibited proton efflux. The results indicate that proton efflux in wheat roots is ammonium ion and light dependent and probably governed by ATPase activity.

Introduction

Changes in pH as a result of imbalances in cation/anion uptake were demonstrated in the rhizosphere of several plant species (Marschner *et al.*, 1986; Nye, 1981; Riley and Barber, 1971). Proton extrusion through root cell membranes resulting in acidification of the rhizosphere was suggested to be instrumental in the mobilization of ions, such as phosphate (Riley and Barber, 1971), and iron (Fe^{III}) (Gardner *et al.*, 1982; Römheld and Marschner, 1981; 1984). Proton extrusion was found in roots of several plant species under conditions of iron deficiency (Römheld and Marschner, 1984; Römheld *et al.*, 1984). However, the capability of iron-stressed plants to decrease rhizosphere pH is not common among all plants; for example, grasses have not demonstrated this characteristic (Bienfait, 1985). Recently, it was shown that wheat plants inoculated with the diazotroph rhizosphere bacteria *Azospirillum brasilense*, increased proton efflux of roots over non-inoculated roots (Bashan *et al.*, 1989).

The aims of this study were (i) to define the main environmental and nitrogen nutrition conditions altering proton efflux in wheat roots potentially related to root colonization by Azospirillum, (ii) to localize the site of proton efflux along the root system, and (iii) to measure proton efflux capacity.

Manuscript No. 245-88 from the Ohio Agricultural Research and Development Center.

Materials and methods

Plant growth conditions

Wheat (*Triticum aestivum* L.) seed cv. Deganit

(Zeraim Gedera Co., Israel) were surface disinfected with 1% NaOCl for 5 min and thoroughly rinsed with tap water. Seeds were then transferred to a hydroponic system (100 seeds per system), consisting of the following: a flat 5-L plastic container containing a nutrient solution which was renewed daily. The nutrient solutions used in this study were: (final pH 5.8, composition in m*M*): A- $NH_4H_2PO_4$,1; $Ca(NO_3)_2$,4; $MgSO_4$,2; KNO_3,6; FeEDTA,0.1; H_3BO_3,1.10^{-2}; $MnCl_2.4H_2O$,1.10^{-4}; $ZnSO_4.7H_2O$,$0.5.10^{-5}$; $CuSO_4.5H_2O$,$0.5.10^{-5}$; $H_2MoO_4.H_2O$,$0.5.10^{-6}$ (Hoagland and Arnon, 1938); B- $Ca(NO_3)_2$,2; K_2SO_4,0.75; $MgSO_4$,0.65; KH_2PO_4,0.1; FeEDTA,0.1 + microelements from solution A (Marschner *et al.*, 1982); C- Similar to solution B minus FeEDTA; D- $(NH_4)_2SO_4$,0.5; $CaSO_4$,0.5; K_2SO_4,0.75; $MgSO_4$,0.65; KH_2PO_4,0.1; FeEDTA,0.1 + microelements from solution A (Marschner *et al.*, 1982); E- Similar to solution D minus FeEDTA; nitrogen-free nutrient solution (F) served as control. A plastic stand with cheese-cloth on the upper surface immersed in the solution; and an air ventilation system (a 25-cm glass tube with 10 air exits) provided the aeration. Other plants were grown in quartz sand maintained in plexiglass flat boxes as described by Marschner and Römheld (1983). All experiments were conducted in a growth chamber (Conviron, model EF7H, Controlled Environments Co., Canada) at 22 ± 1°C, 10 h light 150 $\mu E\,m^{-2}\,sec^{-1}$ (fluorescent and incandescent lamps) or fluorescent and incandescent lamps (62 $\mu E\,m^{-2}\,sec^{-1}$) or incandescent lamps only (17 $\mu E\,m^{-2}\,sec^{-1}$) and 14 h darkness or, alternatively at 22 ± 3°C with photosynthetic lamps (150 $\mu E\,m^{-2}\,sec^{-1}$, Sylvania).

Recording pH changes using pH electrodes

Measurements in nutrient solutions. Four seedlings (two leaves, 6–8 days old) were placed in a glass beaker (50 mL) equipped with a small magnetic stirrer at the bottom and containing 10–20 mL of one of the nutrient solutions. The beaker was wrapped with aluminum foil to prevent illumination of roots and aerated as described later. The beaker was placed in a controlled water bath at 25 ± 0.5°C and illuminated with a photosynthetic lamp. A pH electrode (5 mm in diameter) (Broadley James Corp.) was inserted into the solution and pH changes were automatically recorded by a Unicorder U-228 (Pantos, Japan) at a rate of 10 mm h^{-1}. Additionally, the pH was checked manually with a portable pH meter.

The quantity of protons released by the roots was determined at the end of the experiment by titrating the nutrient solution with 0.01 *M* NaOH to the initial pH. The same nutrient solution without plants, maintained under the same conditions, served as control. Excess water was blotted from the roots prior to fresh weight determination. Dry weight of roots was obtained by drying the roots in a forced-air oven at 60°C for 48 h and weighing immediately after cooling.

Measurements in quartz sand. The rhizosphere pH was measured by antimony microelectrodes (0.5–0.8 mm in diameter) according to Häussling *et al.* (1985). A fluid agar solution containing the pH indicator, as described by Marschner *et al.* (1982), was poured into flat plastic containers which produced thin agar sheets (3–5 mm thickness after cooling). These agar sheets were placed onto the surface of the root-soil interface. The rhizosphere pH was measured by inserting the microelectrodes into the agar sheet at a maximum distance of 2 mm from the roots (10 separate measurements in every root zone).

The nitrogen source in the sand was changed by complete replacement of the sand media. Sand was washed from the roots by a light water stream. The washed roots were transferred to a new plexiglass growth cell containing new sand, and maintained in a horizontal position. Roots were spread on the new sand surface with a glass rod and then covered with an additional amount of sand. After the plexiglass growth cell was filled and sealed, to prevent any movement of the sand, it was returned to a vertical position in the growth chamber for further plant growth.

Localizing the proton efflux along wheat roots

The proton extrusion along the wheat root system was determined by the agar-indicator technique (Marschner *et al.*, 1982). The optimal conditions for this test in wheat were: 0.5% agar; 100 mg L^{-1} bromocresol purple in solution E; light intensity of 150 $\mu E\,m^{-2}\,sec^{-1}$ and 7 cm deep glass

Petri dish incubated at a constant 25 ± 1°C. Changes in color (from red to yellow, corresponding to a decrease in pH from 5.8 to 4.5 or lower) were visibly detected after 2 to 4 h and continued until 24 h after embedding.

Aeration of roots

All seedlings submerged in nutrient solutions were subjected to constant aeration (20 cm^3 air-min^{-1}). In one treatment the beakers were not aerated and left open. Roots submerged in thin solid layers of agar (up to 6 mm depth) were aerated by leaving the plate open.

Experimental design and statistical analysis

Each experiment was carried out at least five times in a randomized design consisting of at least five replicates. A replicate consisted of five seedlings, five petri dishes, five flasks, five plants five hydroponic systems, or ten different pH determinations with microelectrodes. Graphs presented are from one experiment in each case. Means of proton efflux are from all experiments performed. Significance is given by $P \leqslant 0.05$.

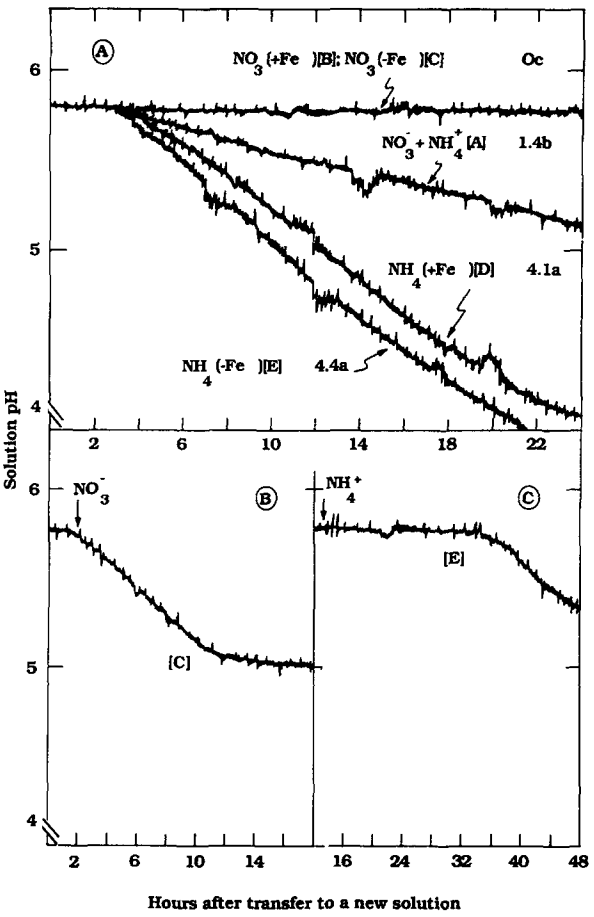

Fig. 1. Effect of nitrogen source and Fe supply on proton extrusion of wheat roots in nutrient solution. **A** – Effect of five nutrient solutions listed in Materials and methods; **B** – Effect of replacing ammonium by nitrate in nutrient solution; **C** – Effect of replacing nitrate by ammonium in nutrient solution. *Arrows* indicate time of solution changes. Light intensity was 150 μE $m^{-2} sec^{-1}$. Letters in square parenthesis represent the type of nutrient solution used. Numbers followed by different letters differ significantly at $P \leqslant 0.05$ and represent (in μmole H^+ fresh weight h^{-1}) the mean proton extrusion measured in all replicate experiments after 24 h. *Lines* are from one experiment.

Results and discussion

The capacity of plants to lower their rhizosphere pH by exchange of extruded protons for potassium and ammonium ions is a known phenomenon (Marschner *et al.*, 1986). This activity is carried out mainly by electrogenic proton pumps localized in plasma membrane (Churchill and Sze, 1983). Such proton effluxes may act as a driving force for energy-dependent transmembrane uptake of essential substances, such as amino acids, sugars, and various ions.

The effect of plant nutrition on medium acidification has been demonstrated in several plant species such as peanut, corn (Schaller and Fischer, 1985) and sunflower (Römheld *et al.*, 1984). In the present study, proton efflux of four-days-old wheat seedlings grown in the absence of a nitrogen source was 1.1 μmole H^+ g^{-1} fresh wt h^{-1}. Supply of nitrate lowered proton extrusion whereas ammonium enhanced it (Fig. 1A). Contrary to sunflower in which ammonium nutrition was the secondary factor for enhanced proton extrusion after iron deficiency, iron deficiency in wheat plants had no significant effect on proton extrusion. Repression of proton extrusion in wheat roots by nitrate under conditions of iron deficiency indicates iron to be of secondary importance. Supply of both nitrate and ammonium ions (solution A) resulted in intermediate effects. Replacing nitrate by am-

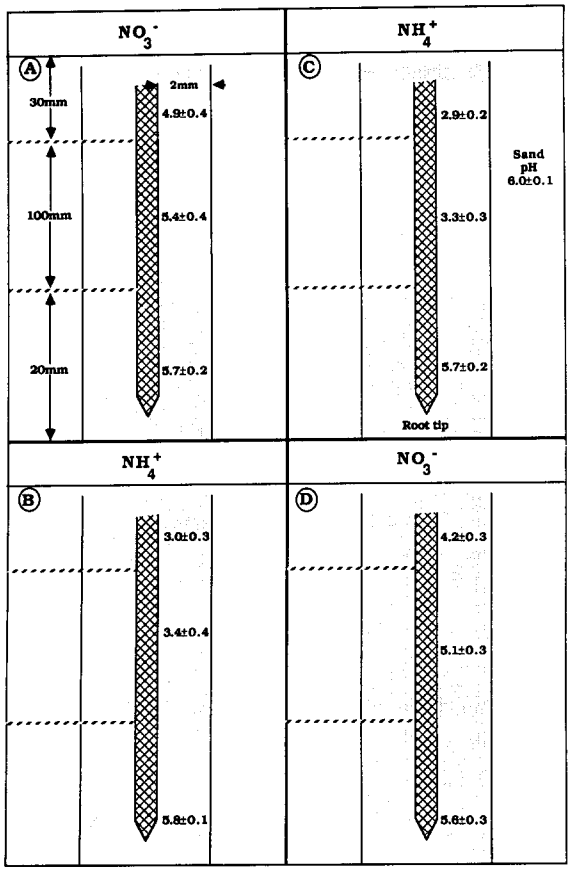

Fig. 2. Schematic presentation of the rhizosphere pH (measured by microelectrodes) along the roots of 10-days-old wheat seedlings grown in sand initially supplied with $Ca(NO_3)_2$ (solution C) (**A**) and $(NH_4)_2SO_4$ (solution E) (**C**) and after replacement of the NO_3-sand with NH_4-sand (**B**) and replacement of the NH_4-sand with NO_3-sand (**D**). ▩ – represents wheat root; ▨ – the measured area 4 h after application of the agar sheet onto sand surface. Diagrams are not in the same scale; area sizes are as indicated by *arrows*. Numbers followed by standard error indicate the mean pH of each of the indicated areas in five plants each measured by 10 different measurements near each root portion.

monium resulted in enhanced proton extrusion after a lag of 16 h, whereas the reverse change stopped proton extrusion within 10 h (Fig. 1B, C). In sand, wheat roots showed similar proton efflux activity as that in agar (Fig. 2A, C). Replacing of these ions one by the other reversed the regular trend of proton efflux (Fig. 2B, D). Therefore, it was concluded that alterations in proton efflux in wheat roots are directly induced by the type of nitrogen source available to the plant. In addition, lack of aeration reduced proton extrusion and consequently decreased the change in pH (Fig. 3A).

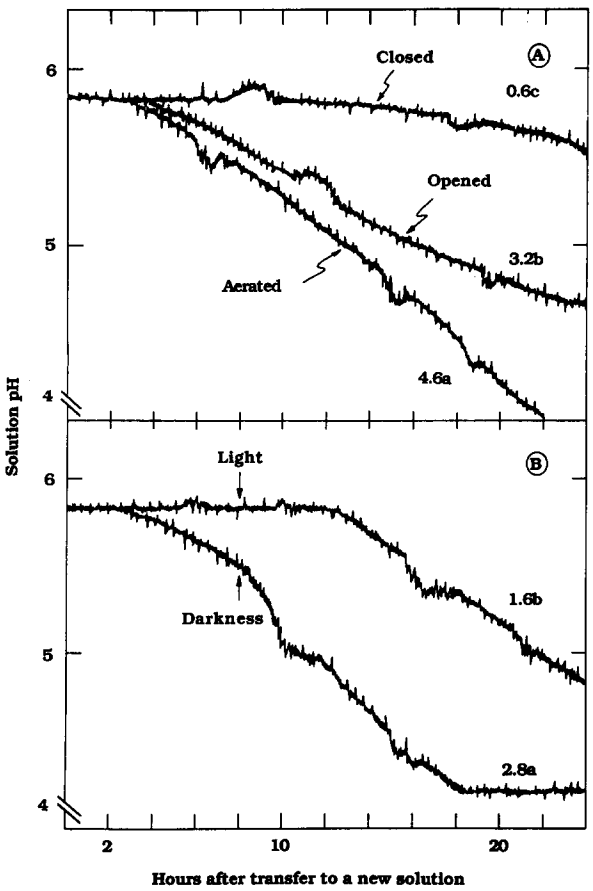

Fig. 3. (**A**) – Effect of aeration of the nutrient solution (solution E) on proton extrusion of wheat roots; (**B**) – Proton efflux in dark-grown plants transferred to light (↓) and in light-grown plants transferred into darkness (↑). Other details as for Fig. 1.

Therefore, the feature of proton extrusion in wheat roots found in this study is the iron deficiency independence, unlike the case of sunflower (Römheld *et al.*, 1984), and the dependence of proton extrusion on nitrogenous compounds as shown for other graminaceous species.

Light is known to stimulate the efflux of K^+ and Cl^-, which were suggested to be dependent on ATP and photosynthetic electron flow, respectively (Raven, 1967; Spanswick, 1974). This study demonstrated a light involvement in proton efflux in wheat. In the absence of light no significant release of protons was detected, whereas a constant illumination resulted in proton efflux (Table 1). Alternating periods of light and darkness showed intermediate proton extrusion values. Increased light intensity from 17 $\mu E\,m^{-2}\,sec^{-1}$ to 150 $\mu E\,m^{-2}$

Table 1. Illumination effects on proton extrusion of wheat roots in nutrient solution (solution E)

Treatment	Light ($\mu E\, m^{-2}\, sec^{-1}$)	Proton extrusion ($\mu mole\, H^+\, g^{-1}$ fresh wt h^{-1})	pH after 24 h[d]
Darkness	—	0.04d[e]	5.8a
Illumination[b]	17	2.3c	4.5b
Illumination[a] and darkness interval	62	2.6c	4.8b
Illumination[a]	62	3.2b	3.3c
Illumination[c]	150	4.6a	3.1c

Roots of four seedlings were immersed in 10 mL nutrient solution and then subjected to various illumination treatments for 24 h.

[a] Combination of fluorescent and incandescent lamps.

[b] Incandescent lamps.

[c] A photosynthetic lamp.

[d] Initial pH 5.8.

[e] Numbers in each column (separately) followed by a different letter differ significantly at $P \leqslant 0.05$.

sec^{-1} increased protein extrusion and decreased pH (Table 1). Following changes in the light exposure, plant response was preceded by lag period: when dark-grown plants were illuminated at least 4 h elapsed before measurable changes in proton extrusion were detected (Fig. 3B). On the other hand, when illuminated plants were transferred to darkness, a lag period of 10 h passed before the proton efflux ceased. Thus, stimulation by light does not operate directly via net photosynthesis, as evidenced by the relatively long lag period between exposure to light and proton extrusion. The data suggested that proton efflux of wheat roots is a primary event, providing the driving force for cation uptake, and controlled through the nitrogen metabolism of the plant.

Two aspects of proton efflux are ecologically significant: its localization along the roots and the amount of protons extruded. Proton efflux was confined to the root subapical zones of maize and barley where extension growth occurs (Pilet *et al.*, 1983; Weisenseel *et al.*, 1979). The present study

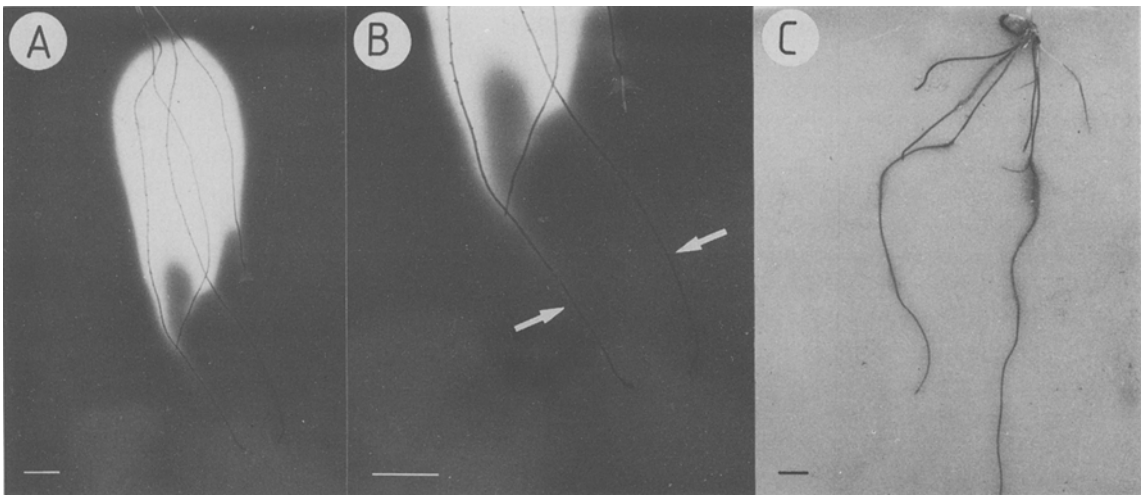

Fig. 4. Localization of proton extrusion along roots of 10-day-old wheat seedlings in different nutrient solutions. **(A)** – solution containing NH_4^+ and lacking iron; **(B)** – localization of the site of proton efflux along the basal portion of the roots embedded in solution E. *Arrows* indicate the absence of proton extrusion in the root tips and in the elongation region; **(C)** – solution contained nitrate and iron ions. Roots of intact plants (precultured in similar liquid nutrient solution) were embedded in agar medium (pH 7.0) with the pH indicator bromocresol purple (which gives purple color in this pH). The yellow color (white in the photographs) indicates acidification of the agar medium to pH 4.5 and lower. *Bars* represent one cm. The intensity of the dark background was reduced during picture processing in order to distinguish the roots from the background.

Table 2. Effect of ATPase inhibitor, orthovanadate, on proton efflux of wheat roots in nutrient solution E

Addition of orthovanadate (μM)	Proton efflux (μmole H^+ g^{-1} fresh wt h^{-1})	pH after 24 h (Units)[a]
0	4.4a[b]	3.4c
50	3.9a	4.1b
100	1.3b	4.8b
200	0c	5.8a

[a] Initial pH 5.8.

[b] Numbers in each column (separately) followed by a different letter differ significantly at $P \leqslant 0.05$.

has shown that in a solidified agar medium containing ammonium ions, proton efflux of wheat roots was restricted to the basal portion of the roots (Fig. 2 and Fig. 4). Root tip and the elongation zone of wheat did not extrude protons, unlike the case of peanut (Schaller, 1987). This finding is of particular ecological importance for wheat root colonization by associative plant beneficial bacteria, since it has been shown that the main site of root colonization by these bacteria is the root elongation zone (Bashan *et al.*, 1986; Okon and Kapulnik, 1986). Acidification of the soil rhizosphere close to the basal portion of the root markedly increased adsorption of bacteria to soil particles (Bashan and Levanony, 1988), which may prevent the bacterium cell from reaching the target root. On the other hand, a non-acidic root segment may target the soil-migrating bacteria towards its surface. Therefore, it can be suggested that the non-acidic portion of the wheat root represents an ecological mechanism by which the plant is targeting rhizosphere bacteria to the proper site for root colonization.

In a nutrient solution that contained nitrate ions, proton extrusion from wheat roots could not be detected during the first 16 h after embedding. Prolonged incubation in this nutrient solution resulted in proton efflux along the basal portion of the root. As demonstrated in this study, wheat roots resembled barley roots (Weisenseel *et al.*, 1979) in having a non-acidic elongation zone. However, unlike barley, (Weisenseel *et al.*, 1979), the elongation zone of wheat did not increase the pH of its surroundings over the initial pH value. Therefore, it is suggested that proton efflux in wheat is not coupled with efflux of substances which are basic-in-nature. Perhaps proton efflux in wheat roots is associated with ion uptake, as suggested by many studies with other plant species (Spanswick, 1981).

The amount of released protons is of particular importance. Rates of proton extrusion of 1 μmole H^+ up to 6 μmole H^+ g^{-1} fresh wt h^{-1} up to an upper limit of 28 μmole H^+ g^{-1} fresh wt h^{-1} were reported (Glass and Siddiqi, 1981; Mengel and Malissiovas, 1982; Pitman *et al.*, 1975; Römheld *et al.*, 1984). Results reported here showed that under optimal conditions, the amount of proton extrusion of wheat roots ranged from 2.6 to 5.7 μmole H^+ g^{-1} fresh wt h^{-1} with an average of 4.2 μmole H^+. Simultaneously, the pH level of the plant root environment decreased from 5.8 to a minimum of 2.9 with an average pH value of 3.2 for 63 determinations. Thus, the level of proton efflux in wheat roots corresponds to the levels found in other plant species.

Orthovanadate is known as an inhibitor of ATPase activity (Cocucci *et al.*, 1980). Cocucci *et al.* (1980) found proton efflux inhibition by orthovanadate in radish seedlings. In our study addition of orthovanadate at a concentration of 100 μM to wheat roots submerged in solution E significantly inhibited proton efflux of the roots. At a concentration of 200 μM, proton efflux was completely inhibited. Concomitantly, no decrease of solution pH was detected at the highest inhibitor concentration (Table 2). These effects with intact plants were interpreted as an indication of proton efflux inhibition mediated by ATPase(s).

It is concluded that the basal zones of wheat roots release protons – a phenomenon which is ammonium and light dependent and probably driven by ATPase(s).

Acknowledgements

This study was written for the memory of the late Mr Avner Bashan. We thank Mr A Aronheim for excellent technical assistance and Prof P R Hender-

long, from the Department of Agronomy, Ohio State University, Columbus, Ohio USA for critical reading of the manuscript. Salary and research support to Y.B. were provided by State and Federal Funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

References

Bashan Y and Levanony H 1988 Adsorption of the rhizosphere bacterium *Azospirillum brasilense* Cd to soil, sand and peat particles. J. Gen. Microbiol. 133, 3473–3480.

Bashan Y, Levanony H and Klein E 1986 Evidence for a weak active external adsorption of *Azospirillum brasilense* Cd to wheat roots. J. Gen. Microbiol. 132, 3069–3073.

Bashan Y, Levanony H and Mitiku G 1989 Changes in proton efflux of intact wheat roots induced by *Azospirillum brasilense* Cd. Can. J. Microbiol. 35 (*In press*).

Bienfait H F 1985 Regulated redox processes at the plasmalemma of plant root cells and their function in iron uptake. J. Bioenerg. Biomembr. 17, 73–83.

Churchill K A and Sze H 1983 Anion-sensitive, H^+-pumping ATPase in membrane vesicles from oat roots. Plant Physiol. 71, 610–617.

Cocucci M, Ballarin-Denti A and Marrè M T 1980 Effects of orthovanadate on H^+ secretion, K^+ uptake, electric potential difference and membrane ATPase activities of higher plant tissues. Plant Sci. Lett. 17, 391–400.

Gardner W K, Parbery D G and Barber D A 1982 The acquisition of phosphorus by *Lupinus albus* L. II. The effect of varying phosphorus supply and soil type on some characteristics of soil/root interface. Plant and Soil 68, 33–41.

Glass A D M and Siddiqi M Y 1982 Cation-stimulated H^+ efflux by intact roots of barley. Plant, Cell Environ. 5, 385–393.

Häussling M, Leisen E, Marschner H and Römheld V 1985 An improved method for non-destructive measurements of the pH at the root-soil interface (rhizosphere). J. Plant Physiol. 117, 371–375.

Hoagland D R and Arnon D I 1938 The water culture method for growing plants without soil. Circular 347. University of California, Berkeley.

Marschner H and Römheld V 1983 *In vivo* measurement of root-induced pH changes at the soil-root interface: Effect of plant species and nitrogen sources. Z. Pflanzenphysiol. 111, 241–251.

Marschner H, Römheld V and Ossenberg-Neuhaus H 1982 Rapid method for measuring changes in pH and reducing processes along roots of intact plants. Z. Pflanzenphysiol. Bodenkd. 105, 407–416.

Marschner H, Römheld V, Horst W J and Martin P 1986 Root-induced changes in the rhizosphere: Importance for the mineral nutrition of plants. Z. Pflanzenernaehr. Bodenkd. 149, 441–446.

Mengel K and Malissiovas N 1982 Light dependent proton excretion by roots of entire vine plants (*Vitis vinifera* L.). Z. Pflanzenernaehr. Bodenkd. 145, 261–267.

Nye P H 1981 Changes of pH across the rhizosphere induced by roots. Plant and Soil 61, 7–26.

Okon Y and Kapulnik Y 1986 Development and function of Azospirillum-inoculated roots. Plant and Soil 90, 3–16.

Pilet P-E, Versel J-M and Mayor G 1983 Growth distribution and surface pH patterns along maize roots. Planta 158, 398–402.

Pitman M G, Schaefer N and Wildes R A 1975 Stimulation of H^+ efflux and cation uptake by fusicoccin in barley roots. Plant Sci. Lett. 4, 323–329.

Raven J A 1967 Light stimulation of active transport in *Hydrodictyon africanum*. J. Gen. Physiol. 50, 1627–1640.

Riley D and Barber S A 1971 Effect of ammonium and nitrate fertilization on phosphorus uptake as related to root-induced pH changes at the root-soil interface. Soil Sci. Soc. Am. Proc. 35, 301–306.

Römheld V and Marschner H 1981 Iron deficiency stress induced morphological and physiological changes in root tips of sunflower. Physiol. Plant. 53, 354–360.

Römheld V and Marschner H 1984 Plant induced pH changes in the rhizosphere of "Fe-efficient" and "Fe-inefficient" soybean and corn cultivars. J. Plant Nutr. 7, 623–630.

Römheld V. Müller C and Marschner H 1984 Localization and capacity of proton effluxes in roots of intact sunflower plants. Plant Physiol. 76, 603–606.

Schaller G 1987 pH changes in the rhizosphere in relation to the pH-buffering of soils. Plant and Soil 97, 439–444.

Schaller G and Fischer W R 1985 pH-Änderungen in der Rhizophäre von Mais- und Erdnusswurzeln. Z. Pflanzenernaehr. Bodenkd. 148, 306–320.

Spanswick R M 1974 Evidence for an electrogenic ion pump in *Nitella translucens*. II. Control of the light-stimulated component of the membrane potential. Biochim. Biophys. Acta 332, 387–398.

Spanswick R M 1981 Electrogenic ion eflux. Annu. Rev. Plant Physiol. 32, 267–289.

Weisenseel M H, Dorn A and Jaffe L F 1979 Natural H^+ currents transverse growing roots and root hairs of barley (*Hordeum vulgare* L.). Plant Physiol. 64, 512–518.