American Journal of

Plant Nutrition and Fertilization Technology

ISSN 1793-9445

Academic
Journals Inc.

www.academicjournals.com

American Journal of Plant Nutrition and Fertilization Technology 1 (1): 36-47, 2011
ISSN 1793-9445 / DOI: 10.3923/ajpnft.2011.36.47

Comparisons between Effects of Bicarbonate and High pH on Iron Uptake, Fe^{III} Reducing Capacity of the Roots, PEP Carboxylase Activity, Organic Acid Composition and Cation-anion Balance of the Xylem Sap of Maize Seedlings

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ABSTRACT

Maize plants were grown in nutrient solution at pH 8 achieved either with bicarbonate (7.5 mM $NaHCO_3$) or HEPES buffer with a comparative treatment, a conventional nutrient solution at pH 5.5, as a control. Measurements were made of growth, Fe concentration and root activities of Fe^{III} reductase and PEP carboxylase. In comparison with the pH 5.5 control, the two high pH treatments depressed growth but the influence of bicarbonate was greater. Shoot and root growth were decreased at both harvests (2 days and 8 days) for bicarbonate and HEPES; in comparison to the control shoot dry weights were lowered by 33 and 19% and root dry weights by 36 and 18%, respectively at the final harvest at day 8. Plant leaves of the bicarbonate treatment were lowest in Fe concentration, with greatest visual evidence of Fe deficiency, both features evident after only 2 days from the start of the treatments. The roots of the bicarbonate plants showed lowest activities of Fe^{III} reductase but highest activities of PEP carboxylase. The xylem sap collected from the bicarbonate treated plants at the final harvest at 8 days showed a lower efflux rate with a slightly higher sap pH (5.31 versus 5.22) as compared with the HEPES treatment. The sap contained higher concentrations of malate, citrate and aconitate and higher concentrations of inorganic cations. The results are discussed in relation to external bicarbonate supply and pH in inducing Fe deficiency in the leaves and on pH regulation in the roots and bleeding sap.

Key words: Reducing capacity, iron deficiency, maize xylem sap, organic acid campasition

INTRODUCTION

The adverse effect of high concentrations of bicarbonate (HCO_3^-) in the rooting medium on nutrient uptake and induced chlorosis is well established (Brown, 1960, 1961). High concentrations of bicarbonate appear to disturb plant metabolic processes which ultimately affect growth and the uptake of nutrients (Marschner, 1995; Mengel and Kirkby, 2001). This is of special relevance to the micronutrients, iron (Fe) in particular, in relation to high pH calcareous soils, which are renowned for so called lime induced chlorosis (Marschner, 1995; Alhendawi *et al*., 2008). Within the roots, HCO_3^- promotes dark fixation of CO_2 which is of consequence in relation to mineral nutrition since the primary products of dark fixation in the roots are malate and other organic acids (Rhoads and Wallace, 1960; Lee and Woolhouse, 1969b). The mode of action of bicarbonate, however, is not yet fully understood. It is still not clear whether the effects of bicarbonate result from the HCO_3^- ion itself or from the high pH that it induces in the rhizosphere or a combination of both.

Porter and Thorne (1955) showed in an experiment on common bean (*Phaseolus vulgaris* L.) grown at constant rate of HCO_3^- supply but with varied or constant pH and plants grown with varying rates of HCO_3^- supply, that high rates of HCO_3^- lowered chlorophyll concentration whereas the comparative effect of high nutrient solution pH was less pronounced. Subsequently Falade (1972) showed that Fe absorption by barley, pea and runner bean was inhibited by high pH but was even stimulated by HCO_3^-. Kolesch *et al.* (1984), supplying bicarbonate at either pH 6.05 or 7.5, demonstrated increased cytoplasmic pH in sunflower above the value measured when the plants were grown without HCO_3^- at pH 6.05. However, this difference was not observed without HCO_3^- with a rhizosphere pH of 7.5.

By contrast, activity of plasma membrane Fe^{III}-chelate reductase isolated from tomato roots had a pronounced pH optimum, being at a maximum at about pH 6.5 and being depressed at higher and lower pH values (Holden *et al.*, 1991). This finding implies that HCO_3^- could exert some influence through the chemistry of the ion itself as well as by raising rhizosphere pH.

The purpose of this study was to separate out the effects of HCO_3^- and high pH on acquisition of iron in maize. This was achieved by comparing the response of plants to bicarbonate (pH 8) with that of the organic buffer HEPES [N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid] at a similarly high pH with a comparative control treatment of pH 5.5. Measurements were made on solution culture experiments comparing plants grown at pH 8 in either a nutrient solution containing HCO_3^- or a nutrient solution containing HEPES with a nutrient solution at pH 5.5. The effects of HCO_3^- and high pH as compared with the control (pH 5.5) are studied in relation to plant growth, iron acquisition, Fe^{III} reductase activity of the roots, PEP carboxylase activity of the roots, accumulation of organic acids in the xylem sap and cation-anion balance of the sap. Results are also recorded of the effects of the treatments on the appearance of iron chlorosis.

MATERIALS AND METHODS

Plant growth: Maize (F1 Earliking) seeds were germinated over a 7 day period in a moist Perlite and peat mixture and then transferred to a half strength nutrient solution for 2 days and a complete nutrient solution for a further 3 days. The composition of the nutrient solution was as follows: (mM) Ca $(NO_3)_2$, 2; K_2SO_4, 0.75; $MgSO_4$, 0.65; KH_2PO_4, 0.5; (μM) Fe-EDDHA, 250; H_3BO_3, 10; $MnSO_4$, 1.0; $ZnSO_4$, 0.5; $CuSO_4$, 0.5; $Na_2MoO_4.H_2O$, 0.05. Plants were then grown for a further 8 days in 3 nutrient regimes as described below and harvested at days 0, 2 and 8.

Treatment regimes: Three treatments each with 12 plants per 50 L tank were set up using the nutrient solution described above. These treatments were the two high pH treatments using the same nutrient solution but buffered to pH 8.0 by adding either $NaHCO_3$ (7.5 mM) or HEPES and a control at pH 5.5. In all cases, the maximum concentration of Na^+ as obtained by $NaHCO_3$ (7.5 mM) was replaced by Na_2SO_4 at an equivalent Na^+ ion concentration. Nutrient solutions were changed every two days and pH adjustment was carried out daily using a few drops of 0.1 M H_2SO_4 or NaOH. All plants were grown in a controlled environment chamber (day/night, 16 h/8 h; light intensity 228 $\mu mol.m^{-2}sec^{-1}$; temperature 25°C/22°C; relative humidity 70-80%).

Shoot and root measurements and mineral analysis: Harvested plants (4 replicates per treatment per harvest) were separated into roots and shoots and fresh and oven dried (24 h at 75°C) weights were determined. Oven dried plant parts were prepared for ion analysis by inductively-coupled plasma-spectrometry (ICP) after ashing at 500°C using the method outlined

in MAFF (1986). Xylem sap of maize plants (n = 4) were collected at day 8. The techniques used for organic acids and for mineral analysis in this volume, including xylem sap pH, were according to Armstrong and Kirkby (1979).

Fe^{III} reduction by roots: Assays for Fe^{III} reduction were made on the roots of 8 intact plants in each treatment and carried out on day 2 and 8 after applying treatment using BPDS regent as modified according to Barrett-Lennard *et al.* (1983). Graphs of amounts of Fe^{III} reduced per unit dry weight against time were plotted and the slope of the line over the first two minutes was used to give an initial rate of Fe^{III} reduction.

PEP carboxylase assay: The assays of this enzyme in each treatment were made on roots that were severed from 8 intact plants immediately prior to estimation. This was carried out on day 0, 2 and 8. Samples of root were then immediately frozen in liquid nitrogen and stored at -20°C until analysis were made according to the method of Schweizer and Erismann (1985). In the assay of this enzyme, oxaloacetate produced by the carboxylation of PEP is reduced to malate via the enzyme malate dehydrogenase, with the concomitant oxidation of NADH. The rate of use of NADH is then recorded in a spectrophotometer and related to the activity of the enzyme.

The data shown are all from one experiment, although similar patterns were seen in a duplicate experiment.

RESULTS

Plant growth: In both HCO_3^- and HEPES treatments (pH 8), shoot and root growth were markedly reduced as compared with plants grown at pH 5.5 (control) (Fig. 1). More severe depression was caused by HCO_3^-. At the final harvest (day 8), shoot dry weights were reduced by 33 and 19% compared with the control for plants grown with HCO_3^- and HEPES, respectively (Fig. 1a). Bicarbonate dramatically depressed root growth as early as day 2 of treatment, an effect which became more distinct with time. At day 8 root dry weight decreases comparative with the control were 36% for HCO_3^- and 18% for HEPES (Fig. 1b).

Symptoms of iron deficiency: Maize in the HCO_3^- treatment showed slight interveinal discoloration of the younger leaves as soon as day 4. Intensity of the symptoms was more

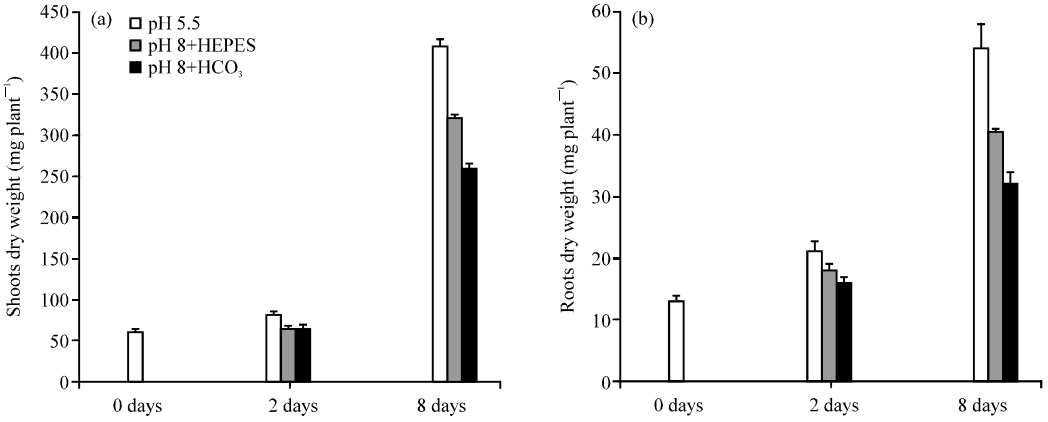

Fig. 1: The effects of an external pH 5.5 or pH 8 as obtained by HEPES or bicarbonate on (a) shoot and (b) root dry weight of maize plants from (0 to 8 days) after the onset of treatment (12-20 DAS). All the data are means of three replicates. Vertical bar = ±SD

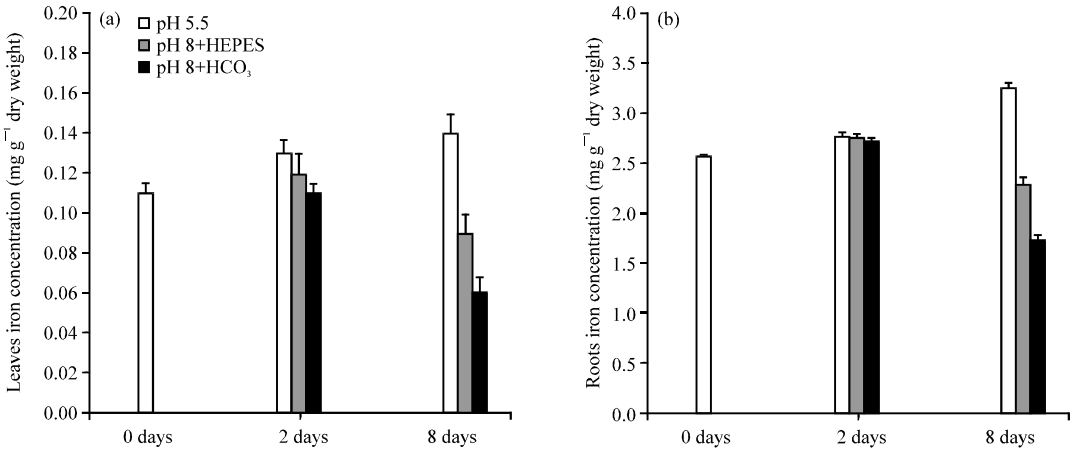

Fig. 2: The effects of an external pH 5.5 or pH 8 as obtained by HEPES or bicarbonate on the concentrations of iron in (a) leaves and (b) roots of maize plants from (0 to 8 days) after the onset of treatment (12-20 DAS). All the data are means of three replicates. Vertical bar = ±SD

pronounced by day 8, when the Fe concentration in the leaves was 0.062 mg g^{-1} dry weight (a value 56% lower than in the control) (Fig. 2a). In the HEPES treatment Fe concentration in the leaves at day 8 was 0.096 mg g^{-1} dry weight higher than the HCO_3^- treatment but 32% lower than the control plants (Fig. 2a). About 90% of the Fe in all three treatments occurred in the roots (Fig. 2b).

Fe^{III} reduction by roots: The rates of Fe^{III} reduction were less for both HCO_3^- and HEPES treatments and especially for HCO_3^-, than in the control (pH 5.5) plants. This inhibition of Fe^{III} reduction was noticeable as soon as day 2 and by day 8 the inhibitory effects of high pH and HCO_3^- on Fe^{III} reduction were even more pronounced (Fig. 3a).

PEP carboxylase activity in roots: Bicarbonate and high pH, especially HCO_3^-, markedly increased the activity of PEP carboxylase in roots at day 2 as compared with the pH 5.5 treatment. At day 8 the activity of PEP carboxylase in the roots of the HCO_3^- supplied plants was also almost three times greater than the control and those in the HEPES treatment more than double that of the control (Fig. 3b).

Organic acids in the xylem sap: Increases in organic acid concentrations in the xylem sap of maize plants harvested at day 8 mirrored the increase in activity of PEP carboxylase (Table 1). In the HCO_3^- treatment there was a 10-fold increase in total organic acid and for HEPES a 7-8-fold increase compared with the control. Total concentrations of organic anions were extremely low (<0.4 meq L^{-1}). The main anions increased were malate and citrate, with a relatively small amount of aconitate (Table 1).

Charge balance in the xylem sap and ion translocation: The volume flux of the xylem sap in maize after detopping was generally lower at day 8 in plants grown with HCO_3^- and HEPES compared with the control plants (Table 1). Furthermore, volume flow was markedly lower in

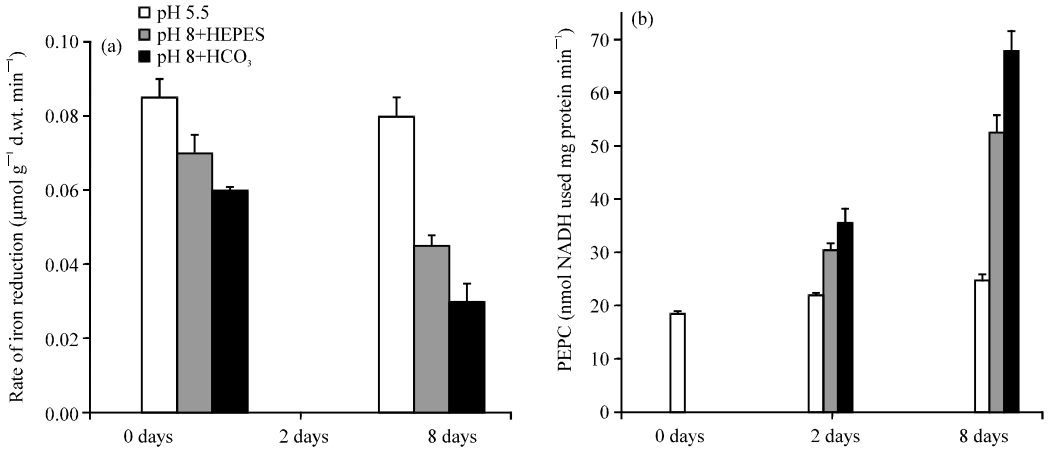

Fig. 3: The effects of an external pH 5.5 or pH 8 as obtained by HEPES or bicarbonate on root reduction of (a) Fe^{+3} and (b) on PEP carboxylase activity by root intact plants of maize at different time after the onset of treatment (12-20 DAS). All the data are means of three replicates. Vertical bar = ±SD

Table 1: The effects an external pH of 5.5 or pH 8 as obtained by HEPES or bicarbonate on the rate of volume flow (mL plant^{-1} 2 h^{-1}), pH and the chemical composition of the xylem sap of decapitated maize plants after 8 days of treatment (20 DAS)

Substance	pH 5.5	pH 8+HEPES	pH 8+HCO_3^-	SEM
Volume flow	1.4	1.25	1.1	0.060
Xylem sap pH	5.1	5.22	5.31	0.070
«meq.dm^{-3}»				
NO_3^-	20.7	19.5	18.8	0.200
P (1)	1.8	1.7	1.6	0.020
S (1)	1.1	1.1	1	0.010
∑ Inorganic anions	23.6	22.3	21.4	0.400
Malate	0.02	0.16	0.18	0.010
Citrate	0.01	0.05	0.1	0.010
Aconitate	<0.01	0.02	0.03	0.001
∑ Organic anions	0.03	0.23	0.31	0.010
K^+	20.4	21.2	21.6	0.200
Ca^{2+}	1.5	1.6	1.8	0.020
Mg^{2+}	1.2	1.3	1.3	0.010
Na^+	1	1.1	1.1	0.010
∑ Inorganic cations	24.1	25.2	25.8	0.200
∑ anions	23.6	22.5	21.7	0.200

Results in columns are means of three replicates. SEM = Standard error of mean. (1) = Total element concentrations of (S) and (P) assigned charges of 2 and 1, respectively

HCO_3^- as compared with HEPES supply. Of the total anion concentrations, NO_3^- was the dominant anion (about 87%) and of the total cations K^+ was the dominant cation (about 84%). In general anion concentrations (NO_3^-, P and S) were slightly lower in both HCO_3^- and HEPES treatments (due to the lower NO_3^- concentrations) compared with the control. In both the saps of HCO_3^- and HEPES treated plants, total cation concentrations exceeded total anion concentrations, suggesting that some anion charge may have been omitted from the balance (e.g., Cl^-, HCO_3^-, amino acids,

etc). The pH of the xylem sap was slightly higher in the HCO_3^- (0.21 units) and the HEPES treatment (0.12 units) than in the control plants (Table 1).

DISCUSSION

The depressed shoot and root dry weight, depressed leaf and root Fe concentration, depressed Fe^{III}-reducing capacity and increased PEP carboxylase activity in both the pH 8 treatments indicates a big effect of high nutrient solution pH on maize plant growth. However, the fact that all of these effects were more extreme with HCO_3^- than with HEPES shows that there was a different response to HCO_3^- than to high pH alone.

Low Fe concentrations occurred in the leaves and at the final harvest the Fe concentrations in the shoots of the HCO_3^- and HEPES treatments were 56 and 32% of the low pH treatment, respectively. The much higher Fe concentrations in the roots indicate that Fe may not have been available for uptake into the symplast and may mainly represent an extracellular fraction. At day 2 there was little effect, but by day 8 there were decreased root concentrations of Fe in the pH 8.0 treatments, particularly where HCO_3^- was supplied. These results partially confirm the negative effect of HCO_3^- on Fe uptake in sunflower found by Kolesch *et al.* (1984). In experiments on runner bean, pea and barley kept in pH 6 or 8 and plus or minus added HCO_3^- for one hour and then fed ^{59}Fe for 6 h, the presence of HCO_3^- actually stimulated absorption of Fe, but the absorption was much less at pH 8 than at pH 6 (Falade, 1972).

In the current experiment, as the depressed Fe concentration in the roots was not apparent at day 2, yet many of the other effects of HCO_3^- and pH were already obvious, the lowered uptake by day 8 was probably a secondary effect arising from metabolic changes within the plants.

Fe^{III}reductase activity was depressed by 2 days after application of high pH or HCO_3 (Fig. 3a), despite the fact that the nutrient solution was not Fe-deficient. The decrease was particularly pronounced in the HCO_3^- treatment. This finding is supported by work on soybean (Dofing *et al.*, 1989) and sunflower and cucumber (Romera *et al.*, 1992). The latter authors found a bigger effect of pH than HCO_3^- and plants grown without Fe (so that Fe^{III}-reducing capacity had already been increased) in pH 6.6 HEPES and pH 6.6 HCO_3^- had a lower Fe^{III}-reducing capacity than plants grown at pH 5 and plants grown in pH 7.1 HEPES and pH 7.1 HCO_3^- had an even lower reducing capacity. There were no differences between the HEPES and HCO_3^- treatments for each pH value, although in a separate experiment there was a lower Fe^{III}-reducing capacity in sunflower and cucumber in HCO_3^- at pH 8.0 than in HEPES at pH 8.0. However, at pH 8.0 sunflower plants supplied HCO_3^- plus Fe had more Fe^{III}-reducing capacity than plants grown at pH 6.0 without HCO_3^- (Romera *et al.*, 1992). These responses to pH and HCO_3^- were localized, as in split root experiments the lower reducing capacity with HCO_3^- was only seen in the half of the root system to which HCO_3^- was supplied.

As the Fe^{III} reductase involved in Fe uptake by plants is located in the plasmalemma (Holden *et al.*, 1991), normal functioning of membranes must be important for Fe nutrition. In experiments by Alhendawi *et al.* (1997) in which barley, maize and sorghum were grown in HCO_3^- for short periods there was enhanced net efflux of K^+ and NO_3^- from the roots, indicating damage to the root plasma membranes. When membranes are damaged the activities of enzymes in them are likely to be disturbed, as appears to be the case in the current study (Fig. 3a). Why HCO_3^- should have this damaging effect is not clear but it may not be simply the result of high external pH affecting the membrane ATPase-H^+ pump, as there was little effect on Fe absorption at day 2. Marschner and Romheld (1994) concluded that high HCO_3^- concentrations impair the effectivity

of the membrane-bound reductase by scavenging H^+ and thereby preventing acidification at the plasma membrane/cell wall interface. A similar study by Mengel (1995) indicated that HCO_3^- present in the root apoplast neutralizes the protons pumped out of the cytosol and hampers uptake of nitrate by H^+/NO_3^- co-transport.

Another internal change in the root that occurred as a result of the HCO_3^- and high pH treatments was an increase in PEP carboxylase activity. Bialczyk and Lechowski (1992) showed higher concentrations of malate in roots of tomato with supply of HCO_3^- and Lopez-Millan *et al.* (2000) showed a 16-fold increase in malate concentration in Fe-deficient sugar beet root tips. These authors showed an even larger increase in citrate concentration and a large increase in total organic anion concentration and they linked this to a large increase in PEP carboxylase activity. This was 40 times higher in Fe-deficient plants than Fe-sufficient plants grown at pH 7.3 and 60 times higher in Fe-deficient plants grown at pH 8.5. In the current study there was a noticeable increase in PEP carboxylase activity in the roots of the plants even at day 2 and this increase was large in both the high pH and the high pH+HCO_3^- treatments by day 8 (Fig. 3b). Early work showed that in the grasses *Deschampsia flexuosa* and *Arrhenatherum elatius* the main product of assimilation of exogenous HCO_3^- in the roots was malate, indicative of PEP carboxylase activity (Lee and Woolhouse, 1969b). PEP carboxylase activity in roots of cucumber was increased by addition of 5 or 10 mM $CaCO_3$ to the rooting medium at pH 6.0-7.0 (Roosta and Schjoerring, 2008). In two grapevine genotypes grown in nutrient solution with Fe supplied at pH 6.05 and with 10 mM $NaHCO_3$ supplied to half the tanks (giving a nutrient solution pH of 7.95), PEP carboxylase activity was four times higher in a genotype tolerant of Fe deficiency with HCO_3^- than without HCO_3^-, whereas in a genotype that was sensitive to Fe deficiency the activity did not vary significantly with HCO_3^- treatment, nor from the no-HCO_3^- treatment of the tolerant variety (Ksouri *et al.*, 2007). The tolerant genotype slightly acidified the rooting medium in the presence of HCO_3^-, but in the other three treatments the rooting medium became more alkaline with time. This difference in response between plants varying in their susceptibility to Fe deficiency indicates that the stimulation of PEP carboxylase with high root zone pH/HCO_3^- is likely to be an important response to such deficiency.

Isolated protoplasts of sycamore (*Acer pseudoplatanus*) were found to accumulate malate when kept at pH 8.0 or 9.0, but not at pH 6.0, 7.0 or 7.5 (Gout *et al.*, 1993). However, they accumulated malate and citrate at all of these pH values if HCO_3^- was included in the perfusing solution. Malate, citrate and aconitate have been shown to increase in maize roots with HCO_3^- treatment (Alhendawi *et al.*, 1997). Increases in concentrations of malate, citrate and fumarate in roots of Zn-inefficient rice have also been seen with supply of HCO_3^- at pH 8.0 or through growing the plants at pH 8.0 in HEPES (Yang *et al.*, 2003). These increases were slightly larger with supply of HCO_3^- than where the high pH was provided from HEPES. As seen in earlier studies, the increased PEP carboxylase activity in the current study is due to the high pH or the HCO_3^- supply, or both and not Fe deficiency in the nutrient solution.

Bicarbonate and HEPES in the nutrient medium both depressed root pressure so that volume flow of xylem exudates was markedly decreased in de-topped maize plants (Table 1). As with the other effects, this was more pronounced in the HCO_3^- treatment than where pH in the nutrient solution was raised with HEPES. Not only was a lower volume of sap obtained but this sap had a higher pH (5.31 and 5.22 for HCO_3^- and HEPES treatments, respectively, compared with 5.10 for the plants grown at pH 5.5).

The HCO_3^- and HEPES treatments both increased the concentration of organic anions in the xylem sap (Table 1). Bicarbonate increased their concentration more than HEPES, but in both treatments malate was the most abundant organic anion. Increased concentrations of malate in xylem sap have been seen previously in tomato (Bialczyk and Lechowski, 1995). Despite the increases in organic anions there appeared to be an overall decrease in total anion concentrations in the xylem sap with the HEPES and HCO_3^- treatments, although it could have been the case that anions not measured increased in amount.

Carboxylation of PEP may come about because of higher cytoplasmic pH in accordance with the pH stat mechanism (Davies, 1986), in which PEP carboxylase is stimulated by increase in pH, leading to enhanced carboxylation of PEP to oxalacetate. At normal cytoplasmic pH of 7.5 (Gout *et al*., 1993), most of the dissolved inorganic carbon (DIC) in the root cells will be present as HCO_3^- and this may indeed be the form of DIC taken up by the plants in the current experiment. Although membranes are readily permeable to dissolved CO_2, at pH 8.0 in the nutrient solution DIC would be almost entirely in the HCO_3^- form. Furthermore, there is carbonic anhydrase in the apoplast of root cells and as this enzyme converts CO_2 to HCO_3^- and as the apoplastic form has been shown to be involved in anion uptake (Van der Westhuizen and Cramer, 1998), it is at least possible that carbon crosses the plasmalemma as an anion. The HCO_3^- ion certainly seems to cross the plasmalemma in the aquatic macrophyte *Elodea nuttallii* by an active anion/H^+ symport mechanism (Eighmy *et al*., 1991).

However, if HCO_3^- ions enter plant cells by active transport they would need to be accompanied by H^+ ions, so this would lower the cytoplasmic pH. Such an effect would not increase PEP carboxylase activity. Where else could the potential for a rise in cytoplasmic pH and an increase in PEP carboxylase activity arise from? One possibility is a decrease in uptake of other anions through competition with the HCO_3^- ion and a decrease in NO_3^-concentration in the roots of maize with supply of HCO_3^- has been seen previously (Alhendawi *et al*., 1997). However, in that study there was also a decrease in K^+ concentrations, so if cation uptake is also inhibited there should be no net effect on pH across the plasmalemma.

Carbon dioxide released during respiration must dissolve in the aqueous phase of the root cells, lowering the pH. At a cytoplasmic pH of 7.5 much of the solvated CO_2 would be converted to H_2CO_3 and then to HCO_3^-, with the release of H^+ ions. Furthermore, the cytoplasm also contains Carbonic Anhydrase (CA), which helps facilitate this reaction and CA and PEP carboxylase have been shown to be located together in the root tips and root central cylinder of soybean (Dimou *et al*., 2009). Early work on root assimilation of $H^{14}CO_3^-$ showed that the maximum amount of incorporation occurs 2-3 mm behind the root tips in the grasses *Deschampsia flexuosa* and *Arrhenatherum elatius* (Lee and Woolhouse, 1969a). Carbonic anhydrase activity has been shown to be considerably in excess of PEP carboxylase activity in maize root tips (Chang and Roberts, 1992). There therefore appears to be the mechanism in roots whereby the release of respiratory CO_2 into the cytoplasm gives the potential for a decrease in cellular pH, but provides HCO_3 ions at the location of PEP carboxylase. If this HCO_3^- were to accumulate, even temporarily, because the CA keeps the carbonic acid concentration low it is apparent from the Henderson-Hasselbach equation:

$$pH = pK + \log \frac{[HCO_3^-]}{[H_2CO_3]}$$

where, pK is 6.1 that the intracellular pH would rise. This would stimulate PEP carboxylase activity.

As discussed above, an influx of HCO_3^- from the rooting medium ought to lower intracellular pH unless there is a corresponding depression in uptake of other anions. However, once inside the cell the rapid removal by CA of any H_2CO_3 that would arise at normal cytoplasmic pH would make the intracellular pH rise. This indicates that external HCO_3^- may act to stimulate PEP carboxylase through altering intracellular pH, but does not discount the possibility that it is the external pH that has the key effect. In their experiments on sycamore protoplasts Gout *et al.* (1993) perfused the protoplasts in solutions containing no DIC or DIC at 0.5 mM concentration of CO_2 + HCO_3^- at pH values of 6.0, 7.0, 7.5, 8.0 and 9.0. They found that intracellular pH was maintained close to 7.5 between pH 6.0-7.5, above which value of external pH it was higher. Intracellular pH in the +DIC treatment was lower than in the-DIC treatment at pH 6.0 and pH 7.0, but not at the higher pH values. This implies that either HCO_{3-} ions entered the protoplasts or CO_2 did and was then subsequently converted to HCO_3^-, in either case lowering cytoplasmic pH. However, these protoplasts showed accumulation of malate and citrate whereas the-DIC treatment protoplasts did not, so possibly the pH stat mechanism had adjusted intracellular pH but in too extreme a manner. That would seem unlikely as the mechanism proposed by Davies (1986) gives accurate control. At a solution pH of 8.0 the protoplasts maintained intracellular pH at 7.7, irrespective of whether or not DIC was supplied, although in the + DIC treatment protoplasts there was 11 times more malate than in the-DIC protoplasts. However, the-DIC protoplasts did contain some malate, unlike the-DIC protoplasts in the more acid pH solutions, so it is possible that at higher external pH internal pH is increased and organic anions accumulate. The protoplasts at pH 8.0 + DIC had higher accumulation of malate (and also citrate) than the protoplasts not supplied with DIC (Gout *et al.*, 1993). If this response to external pH occurs in root cells also, it is possible that in our experiment there were separate intracellular responses to both external pH and HCO_3^-.

Other workers have suggested that the effect of HCO_3^- in reducing root weight may be due to inhibited respiration (Hutchinson, 1968; Lee and Woolhouse, 1969a) and root respiration is known to be decreased by increased rhizosphere CO_2 (Qi *et al.*, 1994). Decreased respiration would certainly account for the lowered Fe^{III}-reducing capacity of our plants as there would be less reducing power available to fuel such a reaction. There are at least two possible mechanisms whereby decreased root respiration could occur. As most of the DIC in the cytoplasm of root cells is in the HCO_3^- form, both high external HCO_3^- concentration and high external pH would lower its ability to diffuse out of the cells. However, it seems unlikely that such accumulation of HCO_3^- ions would occur without PEP carboxylase activity increasing and removing them. A second possible mechanism is that perhaps respiration is directly inhibited. Irrespective of whether or not any inhibition of respiration occurs, if there is more synthesis of organic anions in a root than can be derived from any increased uptake of DIC, overall respiratory deficiency decreases and Van der Westhuizen and Cramer (1998) attributed lower root respiration with enhanced DIC supply in nitrate-grown plants to this fact.

In conclusion, it seems as if Fe-deficiency and lowered growth of the maize seedlings grown at pH 8.0 came about as a consequence of changes to root metabolism. Uptake of Fe was probably lowered due to the decrease in reducing capacity, itself possibly caused by the shortage of available H^+ ions within the root, or the immediate scavenging of H^+ ions by the high pH outside of the root cells. Any Fe^{3+} ions getting into the roots may have remained in the apoplast, attached to COO^- groups in the cell walls. Inside the roots there was much higher PEP carboxylase activity at high pH than at pH 5.0, although this seemed to be even higher with HCO_3^- supply. There was less flux of xylem sap, with lower concentrations of total anions and higher concentration of total cations moving to the shoots.

Despite the differences between the HCO_3^- and HEPES treatments, it should be noted that the nutrient solutions were in exchange with the atmosphere, so in all three treatments some atmospheric CO_2 would have dissolved in the nutrient solution. In the two pH 8.0 treatments this DIC would have been mainly in the HCO_3^- form and at that pH it is likely that the concentration of HCO_3^- arising from the atmosphere could have been as much as 0.5 mM (Deutsch, 1997). Therefore, the two pH 8.0 treatments represented a contrast between 0.5 mM HCO_3^- and 7.5+0.5 mM HCO_3^-. The solubility of CO_2 in the pH 5.5 nutrient solution would have been less and at that pH most of the DIC would have remained as solvated CO_2 (Deutsch, 1997). In all three nutrient solutions Fe was supplied as Fe-EDDHA, which would have maintained its solubility across these two pH values, so the responses seen in the two pH 8.0 treatments were due to pH or HCO_3^- (or both) and not to Fe deficiency in the rooting environment. Future research should attempt to separate out the pH and HCO_3^- effects further by removing CO_2 (and HCO_3^- arising from it) from the nutrient solution completely.

ACKNOWLEDGMENTS

The author are much indebted to Dr. D.J. Pilbeam, for endless help and advice in planning this experiment during my sabbatical study (2009/2010) at Faculty of Biological Sciences, University of Leeds-UK. I would also like to thank my dearest friends Dr. Ernest A. Kirkby (UK) and Volkar Romheld (Germany) for technical advice and valuable discussions on PEP carboxylase assays and organic acid determination.

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