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Effect of iron chlorosis-inducing factors on the pH of the cytoplasm of sunflower (*Helianthus annuus*)

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Summary Growth chamber experiments with sunflower in nutrient solution were performed to investigate the effect of phosphorus and bicarbonate in inducing iron chlorosis.

Iron chlorosis as proved by lower dry matter yield and reduced chlorophyll content was induced by bicarbonate alone and more pronounced by a combination of bicarbonate and phosphate, but not by phosphate alone.

Iron content of roots and aerial plant parts was reduced by bicarbonate in all experiments, but only in one experiment by phosphate alone.

Bicarbonate in the nutrient medium increased the pH of the cytoplasm in leaf cells, while phosphate had no effect.

A daily adjustment of the pH in the nutrient medium to a value comparable to that in the bicarbonate trial, did not affect the pH of the cytoplasm.

It is concluded that the pH of the cytoplasm plays an important role in establishing plant resistance or susceptibility to Fe chlorosis.

Introduction

Contrary to many previous investigations, which concentrated on the cause and appearance of absolute Fe-deficiency in different plant species[4,15], it has been shown by our research under field conditions over several years, that chlorosis on calcareous soils is neither accompanied by reduced Fe-concentration in the soil nor by low Fe-content in the leaves[3].

The grape-vine, used in these experiments, is considered an iron efficient species.

The lowering of the pH in the rhizosphere by increased proton excretion and the excretion of reducing or chelating substances at the root surface are considered main criteria of Fe-efficiency[13,20]. However, wih regard to Fe-chlorosis under field conditions as in our experiments, we believe them to be only secondary processes, useful only indirectly for solving the problem.

The new research of Bienfait *et al.*[2] clearly suggest that probably the activity of a Fe-reducing enzyme located in the plasmalemma of root-cells, is lowered due to high pH.

Following these observations, the purpose of our experiments was

to investigate more precisely the effect of phosphate, bicarbonate and pH of the nutrient medium both on the alkalinity of the cytoplasm and on its pH.

Materials and methods

Growth chamber experiments under controlled conditions: 20 000 Lux, light: dark = 16:8 hrs; temperature, day: 20°C, night' 15°C; rel. humidity in air, 60%. Experimental plants: Sunflower (*Helianthus annuus* L., c.v. Semi 27/17), 15 plants per 61 pot, 3 replicates. Nutrient solution (modified Hoagland No. 3): 5 m*M* $Ca(NO_3)_2 \cdot 4H_2O$, 5 m*M* KNO_3, 2 m*M* $MgSO_4 \cdot 7H_2O$, 1 m*M* KH_2PO_4, 1 μM $CuSO_4 \cdot 5H_2O$, 1 μM $NaMoO_4 \cdot 2H_2O$ 50 μM H_3BO_3, 1 μM $MnSO_4 \cdot H_2O$, 1 μM $ZnSO_4 \cdot 4H_2O$, 10 μM Fe in form of FeEDTA.

Germination of seeds between filter paper, pre-culture in tap-water, transfer to half concentrated nutrient solution at 14 days after a further week in full nutrient solution. For the first and second experiments the treatments were:

1. Control = normal nutrient solution
2. Bicarbonate = + 600 ppm HCO_3^- as $NaHCO_3$
3. Phosphate = + 4 m*M* P as KH_2PO_4
4. Bicarbonate + phosphate = + 600 ppm HCO_3^- + 4 m*M* P

In the third experiment the treatments were:

1. Control = normal nutrient solution: pH 6.05
2. Bicarbonate = normal nutrient solution + 600 ppm HCO_3^- as $NaHCO_3$: pH 7.5
3. Bicarbonate with pH adjustment = normal nutrient solution + 600 ppm HCO_3^- daily pH adjustment to pH 6.05
4. pH-adjustment trial = normal nutrient solution, daily pH adjustment to pH 7.5.

Plants were harvested after appearance of symptoms of chlorosis, about 2 to 3 weeks after transfer to full nutrient solution.

Analysis

Determination of the relative values of pH in the cytoplasm using ^{14}C-DMO-technique (^{14}C-DMO = 5.5 Dimethyl (2-$^{14}C^1$) oxazolidine-2.4-dione)[11]. For this purpose, 0.5 g fresh leaf material for each sample was cut into 2 to 3 mm strips, incubated in 5 ml incubation medium with ^{14}C-DMO (MES-TRIS-buffer, pH 6.2; 10^{-6} *M* ^{14}C-DMO with 50 μCi ^{14}C in 500 ml). After 2 hrs reaction time pH measured in the external solution and measurement of DMO-concentration in inner and outer medium by catalytic destruction of the plant material using an oxidizer, and subsequently counting ^{14}C-activity in a LSC (Liquid Scintillation Spectrometer). Calculating the relative value for pH inside the cytoplasm according to the following formula:

$$pH_i = pK_i^{DMO} + \log \frac{c_i}{c_o} (10\ pH_o - pK^{DMO} + 1) - 1$$

$pK_{DMO} = 6.3$

c_i = $^{14}C'$ – concentration (dpm) inside (leaves)

c_o = $^{14}C'$ – concentration (dpm) outside (incubation medium)

Mineral analysis: After drying (105°C), wet ashing of the plant material (leaves and roots washed several times with distilled H_2O) using a mixture of $HNO_3 : HClO_4 : H_2SO_4 = 40:10:1$. Determinations of the concntrations of Fe, using AAS (Atomic Absorption Spectrometer); P-determination using vandate-method[8]. Alkalinity measured according to Jungk[9].

Table 1. Yield mineral composition and chlorophyll content of leaves and roots of the first experiment

	Leaves				Roots		
	Yield (g/dm)	Chloro-phyll (mg/g fm)	Fe (ppm/dm)	P (%/dm)	Yield (g/dm)	Fe (ppm/dm)	P (%/dm)
Control	10.77	1.84	315	0.68	6.8	496	0.98
Phosphate	10.6	1.84	183***	0.75	8.5	444	1.34*
Bicarbonate	7.4	0.19***	226**	0.61	6.3	464	1.06
Bicarb. + Phosph.	8.1	0.19***	165***	0.62	5.1	705**	1.81***

Significance level compared with the control * = P 5% ** = p 1% *** = p 0.1% n = 3

Table 2. Yield and mineral composition of leaves and roots in the second experiment

	Leaves				Roots		
	Dry matter (g)	Chloro-phyll (mg/g fm)	Fe (ppm/dm)	P (%/dm)	Dry matter (g)	Fe (ppm/dm)	P (%/dm)
Control	8.05	1.49	97	1.00	2.83	345	1.42
Phosphate	8.08	1.72*	110	1.25***	2.76	343	1.35
Bicarbonate	8.51**	0.37***	53***	0.79***	3.57***	201***	2.26***

n = 3

Table 3. pH of the nutrient solution, and relative pH in the cytoplasm of the leaves in the second experiment

	pH/nutrient solution	pH/cytoplasm
Control	6.36	6.80
Phosphate	5.75	6.85
Bicarbonate	7.68	6.98***

n = 12

Results

In the first experiment the addition of bicarbonate and of bicarbonate + phosphate to the nutrient medium reduced plant growth, as can be seen by the fresh and dry weight of all plant parts (Table 1). The most severe depression of growth was caused by the combined bicarbonate and phosphate. Visible symptoms of chlorosis were recorded in the bicarbonate and bicarbonate + phosphate trials only; this is supported by the chlorophyll contents (Table 1). All treatments reduced the Fe-content of the leaves. The combination HCO_3^- + P gave the most pronounced decrease. Higher P-concentrations in the nutrient medium increased the P-concentration of the leaves, but combined P + bicarbonate and bicarbonate also, reduced the P-content

of the leaves (Table 1). The same responses were obtained in the stem. In the roots of all 3 trials an enrichment of P was observed, most marked in the combination trial.

Alkalinity as an indicator of excess cations did not result in significant differences. Therefore these data are omitted.

The results of the first experiment point to an enhancement effect of phosphate on the chlorosis inducing effect of bicarbonate. The aim of a second experiment therefore was to get more information on the effect of both factors on the pH of the cytoplasm. In this second experiment there was no growth depression at the time of chlorosis apperance, as indicated by reduced chlorophyll-concentrations (Table 2).

As in the previous experiment, the Fe-concentration in the leaves was clearly lowered by bicarbonate. Here, however, bicarbonate also reduced the Fe-content of the roots. It also led to an increased P-concentration in the roots, while the P-concentration of the leaves was reduced. Phosphorus neither reduced the Fe-content of leaves nor increased P in the roots. The relative values of pH in the cytoplasm exceeded those of the nutrient medium in all trials (Table 3). Comparing the cytoplasm pH-values of the trials (with each other) there was a rise of 0.2 pH units above the value of control due to bicarbonate.

A third experiment seemed necessary to see whether or not the rise of cytoplasm pH was primarily due to the bicarbonate itself or to the high pH in the nutrient medium which increased the pH of the cytoplasm compared with the control.

Even with daily adjustment of the pH to that of the bicarbonate medium (Table 4), without bicarbonate in the solution, there was no change in the cytoplasm pH.

Furthermore, it can be seen (Table 5) that chlorosis was most severe in trials with bicarbonate and high pH of the nutrient solution. In all 3 trials the Fe-content of the leaves was reduced below that of the control. The roots, however, showed a lowered Fe-content only at high pH-levels in the nutrient solution.

Bicarbonate caused a lower P-content in the leaves; the higher P-content of the roots was obviously due to the high pH-value. As in the previous experiments, the alkalinity of the plant parts remained unaltered.

Discussion

The results of the first experiment, a reduced transference of iron into the upper plant parts and its concentration in the roots of chlorotic

Table 4. pH of nutrient solution, and relative pH in cytoplasm of the leaves in the third experiment

	pH/nutrient solution	pH/cytoplasm
Control	6.05	6.82
Bicarbonate	7.5	7.02***
high pH without bicarbonate	7.5	6.87
low pH with bicarbonate	6.05	7.04***

n = 9

Table 5. Yield and mineral composition of leaves and roots of the third experiment

	Leaves					Roots		
	Dry matter (g)	Chloro-phyll (mg/fm)	Fe (ppm/ dm)	P (%/dm)	Alkalinity (mval/ 100g dm)	Dry matter (g)	Fe (ppm/ dm)	P (%/dm)
Control pH 6.05	8.57	1.70	84	0.98	235	2.56	283	1.52
Bicarbonate pH 7.5	8.35	0.84***	43***	0.73***	215	3.72***	138***	1.95***
High pH without bicarbonate pH 7.5	8.83	1.53*	74*	1.00	234	2.44	164***	1.66*
Low pH with bicarbonate pH 6.5	8.60	1.51*	69**	0.87***	224	2.22*	297	1.56

n = 3

plants, confirm previously published data from numerous experiments[6,7,16,19]. However, as shown by the change in chlorophyll content (in the first, but also in the second and third experiments), the symptoms of chlorosis are primarily caused by bicarbonate, while phosphate exhibited only an enhancing effect. There is clearly a disagreement between the results represented here and those of field trials, particularly concerning Fe- and P-contents of the leaves[3,17]. Therefore it must be concluded that these pot experiments, although helpful for evaluating the role of bicarbonate and phosphorus as inducing factors of chlorosis, are not suitable for discovering the primary effect on plant metabolsim.

There is convincing evidence in the literature that chlorotic plants are characterized mostly by increased cation excess and subsequently enhanced production of organic acids[5]. These observations, together with reports on the enhancement of chlorosis by high nitrate supply in the culture medium[1], strengthen the idea that processes guiding the alkalinity of cytoplasm may probably initiate iron chlorosis.

Price[14] has already reported a reduced activity of all Fe-containing enzymes under chlorosis conditions; these enzymes, normally operating over a limited range of pH, will react with severe disturbances in the metabolic pathways when the cytoplasm-pH is changed. These ideas are confirmed by the results of the second experiment (Tables 2 and 3) in which the chlorosis induced by bicarbonate is accompanied by a rise in cytoplasm-pH of the chlorotic leaves. However, as the pH of the nutrient medium had been varying over a wide range and that in the nutrient solution of the bicarbonate trial had reached a high value, it could not be decided whether the increase in cytoplasm-pH was due to bicarbonate or to the pH of the nutrient medium. This question was answered by separating the two factors in a third experiment. The results (Table 4) of this experiment show that the rise in pH of the cytoplasm is caused alone by the bicarbonate in the medium. It follows that a high nitrate supply and the subsequently enhanced OH^--production by nitrate reduction in the cells[1] may increase the severity of chlorosis by providing optimum conditions for high bicarbonate levels.

A second important aspect arises from consideration of the contents of Fe and P in leaves and roots (Table 2). Changes in some cases are due only to a change in the pH of the nutrient solution, particularly when the root is considered. In contrast, there seems to be a sole effect of bicarbonate on the concntration of Fe and P in the leaves.

The results presented here lead to the conclusion that an increase in the pH of cytoplasm, which is assumed to exist also in root cells, reduces the activity of a Fe-reducing-enzyme[2]; this may be the reason for the lower Fe^{III}-reduction in the roots with increasing pH, as has been observed by the above mentioned authors.

We therefore believe that besides other factors, the pH of the cytoplasm plays an important role in establishing plant resistance or susceptibility to Fe-chlorosis.

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