Egypt. J. Soil Sci. **Vol. 53,** No. 1 pp. 67- 74 (2013)

Induced Fe-Deficiency-Chlorosis Severity in Soybean using EDTA-Buffered Nutrient Solutions

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CHELATOR-buffered nutrient solutions have been used to control Fe phytoavailability for dicots in a number of studies, but use of diethylenetriaminepentaacetic acid (DTPA) required adding Zn, Cu and other trace elements at levels much higher than traditional nutrient solutions. In order to have lower levels of other trace element cations in the buffered solutions, ethylenediaminetetraacetic acid (EDTA) was used to impose varied levels of Fe stress to soybean (*Glycine max* L.Merr., cv Williams-82). In addition to a control treatment (no FeEDTA added), FeEDTA was supplied at 0.32, 1.00, 3.16, 5.00, 10.0, 20.0 and 50.0 µM in pH buffered 0.5 Johnson solutions. Enough EDTA was added to chelate all of the micronutrient cations plus 100 µM of "excess" EDTA which is largely chelated with some of the Ca^{2+} in the nutrient solution. Plant dry matter significantly corresponded with Fe activity in solution. Highest dry matter was obtained with 20 and 50 µM Fe (plants remained green throughout the experiment). At intermediate levels (1-10 µM FeEDTA) plants had mild or moderate chlorosis and remained at a steady chlorosis rating until harvest. Over time, the leaves with 0 and 0.32 µM FeEDTA became severely chlorotic. Analysis showed that trifoliolate leaf Fe corresponded with chlorosis severity, while all other microelements were present at normal concentrations of healthy soybeans, although somewhat increased due to smaller biomass dilution. The EDTA-buffered Johnson solution offers a strong control of Fe stress at varied severity and should provide a valuable tool in study of microelement plant nutrition.

Claims have been made that spray application of glyphosate may interfere with plant's ability to obtain soil Mn and Fe (*e.g.*, Bernards *et al.*, 2005, Bott *et al.*, 2008, Eker *et al.*, 2006, Huber, 2007, Huber *et al.*, 2004 and Ozturk *et al.*, 2008). Although the bulk of evidence refutes this claim for glyphosate-resistant (GR) genotypes (Ebelhar *et al.*, 2006 and Duke *et al.*, 2012), non-GR genotypes may suffer Fe or Mn deficiency because glyphosate is translocated to roots and root physiological adaptations are important in obtaining soil Fe and Mn (*e.g.*, Ozturk *et al.*, 2008). To date, there appear to be no controlled experiments testing this hypothesis which involved cultivars resistant to Fe or Mn deficiency grown on soils which are likely to induce Fe or Mn deficiency. The present study was conducted to establish a test system with controlled intermediate deficiency to allow testing any of effect of glyphosate on plant adaptation to low Fe supply.

Nutrient solution tests of whether glyphosate interferes with plant processes to obtain soil Fe and Mn are difficult if traditional nutrient solutions are used. In traditional solutions the nutrient for which one wishes to induce deficiency is omitted from the solution. When the internal supply (seed) and solution contamination are exhausted, the plant becomes deficient and then becomes increasingly severely deficient until it dies. There is no "steady state" Fe or Mn stress that one could use to test the effect of another factor such as glyphosate application. The deficiency induced by omitting Fe is not at all like that caused by low phytoavailable element in soils.

Fortunately, chelator-buffering offers a method to induce relatively constant intermediate levels of Fe and other essential microelements stress such that interacting factors may be tested to see if they inhibit the normal plant adaptations to obtain soil Fe or Mn (Parker *et al.,* 1995). Plants of non-*Poaceae* species use a combination of acidification around young roots, increased expression of a membrane bound Fe^{3+}-chelate reductase and of a Fe^{2+}-transporter (IRT1) to adapt to the availability of Fe around their young roots. Until plants can no longer up-regulate the expression of these activities to match the phytoavailability of Fe, they remain green. Chaney *et al.* (1992) demonstrated the use of a chelator-buffered nutrient solution to test the Fe-stress-responses of tomato and showed that the primary responses (proton secretion, reductase and Fe^{2+}-transporter) were up-regulated at intermediate stress before any chlorosis was evident, but when chlorosis became evident dense root hairs and more frequent lateral roots were also induced. They concluded that Fe-chlorosis induced root hairs of tomato were an effect of chlorosis rather than a plant response to obtain more Fe. During those experiments, the severity of chlorosis was maintained at intermediate levels of severity over a 10 day period at varied levels of severity from very mild to severe chlorosis.

Although the paper by Chaney *et al.* (1992a) illustrated the utility of such chelator-buffered solutions in study of Fe-deficiency-stress, few have used this approach at least partially because they were concerned about the high levels of CuDTPA and ZnDTPA needed to supply the activity of Cu^{2+} and Zn^{2+} required by dicots, 20 and 50 μM total Cu and Zn, respectively. If care is taken to limit root breakage, there is little evidence that uptake of metal chelates or free chelator is occurring, and traces of EDTA found in plants are not sufficient to interfere with element bioavailability within the plant tissues. The DTPA-buffered solutions were adapted to screen for Fe-chlorosis-resistance of soybean (Chaney *et al.,* 1992b) and chickpea (Chaney *et al*., 1992c) by including bicarbonate which inhibits the ability of chlorosis susceptible species to up regulate the Fe-stress-responses to obtain Fe from wet calcareous soils.

Soybean is the crop reported to suffer adverse effects in some papers (Huber *et al.,* 2004 and Bott *et al.,* 2008). Because I wanted to test the effect of glyphosate on the ability of soybeans to obtain Fe and Mn, this study tests use of the chelator ethylenediaminetetraacetic acid (EDTA) to induce Fe and Mn deficiency stress to see if more acceptable test systems could be developed.

Material and Methods

Seeds of soybean (*Glycine max* L.Merr., cv. Williams-82) were germinated in standard germination papers wetted with 0.5 Johnson macronutrients solution under controlled conditions at 25°C. Uniform seedlings, 6 days old, with healthy primary leaves and roots were transferred to a temperature-controlled greenhouse at 25±3°C, to grow in continuously aerated solutions (2.5 L polyethylene beakers, 6 seedlings per beaker) with a modified 0.5 Johnson solution (Johnson *et al.*, 1957). The solution had the following composition of major nutrients: 2.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 1.0 mM $MgSO_4$ and 0.1 mM KH_2PO_4. Micronutrients were held constant/buffered with varied activities of Fe in all solutions. Micronutrients were added at the following total microelement concentrations (µM): 10 Mn ($MnCl_2$), 5 Cu ($CuSO_4$), 10 Zn ($ZnSO_4$), 1 Ni ($NiSO_4$, 1 Cd ($CdSO_4$) and 1 Co ($CoSO_4$) with equimolar EDTA plus the "excess" 100 µM EDTA that provides the buffering action for all of the strongly chelated microelements. H_3BO_3 (10 µM), Na_2MoO_4 (0.1 µM) and KCl (100 µM) were included as non-chelated micronutrients. Treatments of FeEDTA were added at 0.32, 1.00, 3.16, 5.00, 10.0, 20.0 and 50.0 µM. A control treatment (no Fe added) was also included. In this study, ethylenediaminetetraacetic acid (EDTA) was used to buffer the free metal activity, referred to as –log free ion activity (pM). The basal concentration of EDTA in all solutions was 128 µM. By adding Fe as the FeEDTA chelate, changing Fe had no effect on activity of other micronutrients (Parker *et al.*, 1995). GEOCHEM-PC was used to calculate the activities of minerals added with the buffer in all solutions. Values of Fe^{3+} activity (pFe^{3+}) and EDTA concentration in solutions treated with the above mentioned Fe concentrations are presented in Table 1.

TABLE 1. Fe and EDTA concentrations and calculated pFe in the nutrient solutions .

Treatment No	1	2	3	4	5	6	7	8
Fe (µM)	0	0.32	1.00	3.16	5.00	10.0	20.0	50.0
EDTA (µM)	128	128	129	131	133	138	148	178
Fe level (pFe^{3+})	-21.88	-21.35	-20.89	-20.39	-20.19	-19.89	-19.59	-19.19

The activities of Zn, Cu, Mn, Ni, Co and Cd were -10.44, -13.04, -7.84, -13.34, -11.44 and -11.44 µM, respectively. It should be recognized that the activity of Fe^{3+} is not the controlled variable which induces Fe deficiency stress in dicots, but rather the competition of EDTA with IRT1 for Fe^{2+} generated by reduction at the young root membrane. Catalysis of Fe^{2+} oxidation by EDTA competes with uptake of Fe^{2+} by IRT1 and regenerates Fe^{3+}EDTA in the solution.

During the course of the experiment, additional P was added daily; 10 µM in the first 10 days and 20 µM daily until harvest. The pH of all solutions was adjusted to 6.5 and buffered with 2 mM 2-[N-Morpholino]ethanesulfonic acid (MES) and adjusted every second day using solutions of HCl or KOH to keep the solution pH within ± 0.2 pH units. Three replicates of each treatment were

arranged in randomized blocks. Chlorosis severity was scored for the second trifoliolate leaf and younger leaves, using the scale of Weiss (1943): 1 = full green, 2 = light green, but no interveinal pattern, 3 = interveinal chlorosis, 4 = full yellow, veins light green, 5 = severe chlorosis with necrosis.

The treatment period was terminated by removing all six plants from the beaker (day 23) and separating the shoot and root at the transition zone. Shoots (primary, first trifoliolate and stems were separated from and remaining trifoliolate leaves) and roots were transferred to paper bags and placed in a 70℃ forced draft oven for 24 hr. The dry weight of shoots and roots was recorded. Shoots were then dry ashed overnight at 480°C and the ash dissolved in acids (concentrated HNO_3 heated to dryness and ash dissolved in 3N HCl) for analyses of nutrient composition using ICP-AES (inductively coupled plasma – atomic emission spectroscopy).

Data was subjected to analysis of variance and Tukey test was carried out using GenStat for Windows 7.0 (VSN Int. Ltd, UK, 2005). In Tables, values are means (n=3) and in figures error bars are standard errors.

Results

Plant Growth

A wide range of FeEDTA concentrations was used to test soybean response to Fe nutrition. Plant growth, hereafter expressed as dry matter (DM), increased with increasing Fe supply in the solutions (Fig. 1). It was highest in the nutrient solution with 20 µM Fe (pFe^{3+}=19.58); addition of 50 µM Fe caused no further increase in plant yield. Compared to that with 20 µM Fe, plant DM was reduced by approximately 50 and 20% in the solutions with 3.16 and 10 µM Fe, respectively.

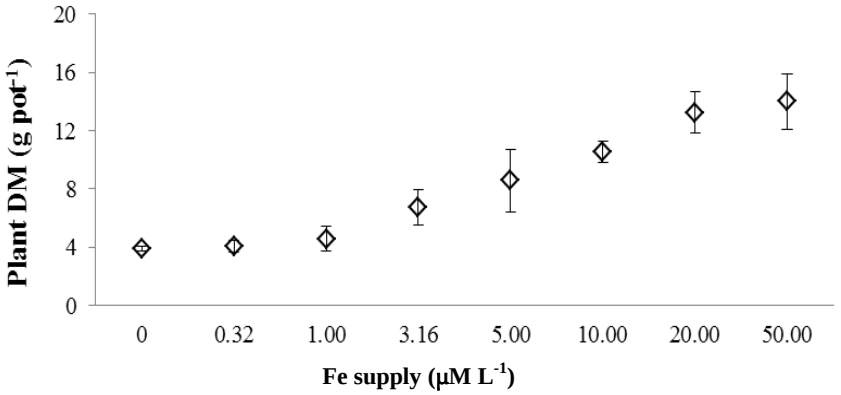

Fig. 1. Soybean dry matter (g pot^{-1}) after 23 days of growth in the nutrient solutions with varied total FeEDTA supply 0.0, 20.0 and 50.0 µM L^{-1}.

Trifoliate nutrient composition and chlorosis severity

Table 2 presents the nutrient composition and chlorosis score of the second and younger trifoliate leaves of soybean (the composition of first trifoliolate leaves is more strongly affected by seed supplies than are younger leaves, so the nutrient levels in younger trifoliolates are used to indicate nutritional status related to treatments).

The concentration of Fe correlated closely with FeEDTA/pFe^{3+} in the solution; Fe concentration in leaves increased with increasing Fe concentration in solution, but addition of more than 20 µM had no additional effect. Concentrations of P, K, Ca, Mn, Mo, Ni and Cd were at the optimal concentration with the higher Fe supply, whereas in the solutions without (no Fe added), or with lower Fe, most nutrients were higher due to the lower biomass production under Fe deficiency. Total solution Fe had no effect on soybean uptake of Mg, Zn or Cu.

The soybean plants remained green until the end of growth of the first trifoliolate leaves due to the seed Fe supply. Soybean second trifoliate leaves started to show chlorosis on day 9 after transplanting. Thereafter, severity of chlorosis of the second and younger trifoliolate leaves was closely related to the amount of FeEDTA supplied. Over time, the leaves with 0 and 0.32 µM FeEDTA became severely chlorotic. At intermediate levels (1-10 µM FeEDTA) leaves showed intermediate severity of chlorosis; the plants kept growing and remained mildly or moderately chlorotic during the remaining growth. Chlorosis score of soybean leaves with 20 µM was insignificantly different from that with 50 µM FeEDTA.

TABLE 2. Nutrient composition and chlorosis score of the second and younger trifoliolate leaves of soybean grown in solutions with EDTA-buffered Fe supplied at 0, 0.32, 1.00, 3.16, 5.00, 10.0, 20.0 and 50.0 µM L^{-1}.

FeEDTA	P	K	Ca	Mg	Fe	Zn	Mn	Cu	Mo	Ni	Cd	Chlorosis score*
µM	----------%----------				----------mg kg^{-1} DM----------							
0.00	0.89f	4.7a	1.67a	0.36c	15a	55b	282a	9.6e	4.0f	1.8d	0.60d	5.0f
0.32	1.01f	4.6a	1.66a	0.39d	13a	62d	286a	11.2e	3.7e	1.6d	0.64e	4.6f
1.00	0.70e	5.5b	1.69a	0.33bc	27b	51b	285a	7.8d	2.8d	1.3c	0.77f	3.6e
3.16	0.53d	4.7a	1.76ab	0.30b	34c	48b	215b	4.5c	1.7c	1.3c	0.70f	3.0de
5.00	0.39c	4.3a	1.83b	0.31b	42d	38c	161c	5.6b	1.5bc	0.9b	0.59d	2.5c
10.0	0.39c	3.1c	1.66a	0.36c	58e	47b	128d	7.4d	1.3b	1.0b	0.44c	2.0b
20.0	0.34b	4.6a	1.33c	0.31b	88f	49b	81e	5.0b	0.7a	0.6a	0.30b	1.5a
50.0	0.29a	2.9c	1.07d	0.26a	85f	34a	89e	3.2a	0.7a	0.6a	0.23a	1.5a

* Chlorosis score is an average of visual rating of second and younger trifoliolate leaves of 6 plants per beaker on days 9, 15, 18 and 21 after transplanting .

Discussion

In contrast with traditional nutrient solutions, using EDTA buffered solutions can supply varied levels of Fe stress, which remains relatively constant over time. This varied severity has the appearance of the varied severity of chlorosis one sees in the field with varied phytoavailability of soil Fe due to varied bicarbonate or inactive Fe oxides in calcareous soils. In most field observations, soybean

chlorosis severity varies across the field from green to mild to moderate to severe chlorosis depending on soil chemistry and water content.

The EDTA buffered nutrient solution system allows one to supply strongly buffered adequate levels of the other micronutrient cations (Zn, Cu, Mn, Ni, Co) and control levels of other cations such as Cd. Buffering also prevents other chelatable elements from displacing Fe from the FeEDTA as occurs in traditional nutrient solutions (Parker *et al.*, 1995). These elements are buffered at activities that allow the plant to obtain normal levels for growth with a constant supply over time rather than the higher supply when solutions are renewed in traditional solution culture. As indicated in the work of Chaney *et al.* (1992a), using buffered Fe^{3+} supply allows plants to express their inherent Fe-stress-responses while remaining green as occurs in normal soils. And if study of physiological changes in response to chlorosis is desired, lower Fe availability can be supplied to induce steady severity of chlorosis rather than simply letting the plant run out of Fe and move toward death. Because much lower levels of buffered total Zn and Cu are required in EDTA buffered solutions, concerns raised by the DTPA-buffered system of Chaney *et al.* (1992a,b, c) may be avoided. As long as the nutrient solution is kept in the dark to avoid photo-decomposition of the FeEDTA, the buffering will remain steady during growth. It is still reasonable to replace solutions periodically during longer growth periods. In all cases, when a micronutrient cation is supplied by seeds, deficiency cannot be induced until the seed supply is diluted by growth.

It could be concluded that EDTA-buffered Johnson solution offers a strong control of Fe stress at varied severity and should provide a valuable tool in study of microelement plant nutrition.

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(*Received* 6/2/2013;
accepted 28/2/2013)

الحكم فى ظاهرة الإصفرار الناتج عن نقص الحديد فى نبات فول الصويا بإستخدام محاليل Fe EDTA

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تم تجربة كثير من المخلبيات فى المحاليل المغذية فى التحكم فى تيسر وإمتصاص النباتات ذات الفلقتين لعنصر الحديد ولم تنجح المحاولات لذلك فقد أقيمت هذه التجربة لدراسة قدرة مركب EDTA على التحكم فى تيسر وامتصاص الحديد عند وجوده بتركيزات منخفضة ومتوسطة ومرتفعة فى المحلول الغذائى . وقد تم استخدام نبات فول الصويا فى هذه الدراسة. وقد أضيف الحديد فى صورة EDTA بتركيزات 0,32 –1,00 – 3,16 – 5,00 – 10,00 – 20,00 – 50 ,00 ميكرومول بالأضافة إلى معاملة الكنترول مع إضافة 100 ميكرومول أضافية من EDTA للتحكم فى تيسر العناصر الأخرى فى جميع المعاملات. وقد أقيمت التجربة لمدة 23 يوم داخل الصوبة تحت الظروف المثلى للمحاليل الغذائية ونمو نبات فول الصويا.

وقد أمكن الحصول على أعلى وزن جاف من فول الصويا من المعاملة 20 ميكرومول حديد مع عدم وجود فرق معنوى باضافة المعاملة 50 ميكرومول. كما لوحظ ظهور أعراض الأصفرار بدرجات متفاوتة عند مستويات 1-10 ميكرومول FeEDTA وقد استمر هذا الإصفرار عند نفس الدرجة حتى نهاية التجربة. أما فى معاملة الكنترول فقد لوحظ أعراض الذبول على النبات. وبتحليل أوراق النبات وجد أن تركيزات عنصر الحديد تتناسب مع أعراض نقصه فى النبات والتى تم تقييمها من خلال تقييم درجة الإصفرار فيما كان تركيز بقية العناصر الصغرى فى الحدود المناسبة.

ومن النتائج أتضح أن مركب EDTA كمادة منظمة فى المحاليل الغذائية لها قدرة عالية على التحكم فى تيسر وإمتصاص الحديد عند وجوده بتركيزات منخفضة ومتوسطة ومرتفعة فى المحاليل المغذية كما أنه يمكن إستخدامه للتحكم فى تغذية النبات بالعناصر الصغرى الأخرى.