Nitric Acid- and O-Phenanthroline-Extractable Iron for Diagnosis of Iron Chlorosis in Citrus Lemon Trees

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ABSTRACT

Plant analysis for total iron (Fe) is frequency used for diagnosis of Fe-deficiency chlorosis. However, chlorotic plants frequency contained similar or higher amount of total Fe than the healthy green plants. The objectives of this study were to (i) determine if Fe chlorosis in citrus lemon can be diagnosed by total or active Fe and can be related to the degree of chlorosis, and (ii) determine the optimum extraction time and ratio of extracting solution to plant sample for extracting the active Fe. Leaf samples of different degrees of Fe chlorosis were sampled from different citrus lemon trees from three different sites. Total Fe was extracted with nitric acid (HNO₃) and active Fe with o-phenanthroline from lemon leaves. An extraction time of 20 and 45
hours and the ratios of the extractor to the sample of 5:1, 10:1, and 20:1 were investigated. The results indicated that an extraction time of 20 hours is enough for extracting the active Fe from citrus lemon leaves by o-phenanthroline. The amount extracted by all ratios (5:1, 10:1, and 20:1) were detectable and at the same time similarly and consistency showed the differences in degrees of chlorosis in all plant samples. Total Fe content was always higher in moderately and severely chlorotic leaves compared to the green leaves and was not related to the degree of chlorosis. Therefore, total Fe cannot be used as a criteria to differentiate between the Fe-deficient and non-deficient plants. On the other hand, active Fe tended to decrease with the increase in the degree of chlorosis. The ratio of active to total Fe was calculated and was found to be closely correlated with the degree of chlorosis. This clearly illustrates the failure of plant analysis for total Fe and the effectiveness of active Fe and/or the ratio of active to total Fe for diagnosing Fe chlorosis.

**INTRODUCTION**

Iron is present in soil in a higher concentration than any other nutrient, yet Fe deficiencies occur because so little of the element is in a form available to plants. Iron-deficiency chlorosis has been observed in plants growing in many of the world’s calcareous soils (Wallace and Lunt, 1960; Miller et al., 1984; Bloom and Inskeep, 1986) where the presence of bicarbonate (HCO₃⁻) has been recognized as being the main factor inducing Fe chlorosis (Patel et al., 1977). Under these conditions, a high concentration of HCO₃⁻ in plant tissues increases the pH of the apoplast which inactivates Fe and prevents its entry into the cytoplasm where it is needed for chlorophyll synthesis (Mengel, 1994).

Chlorotic plants cannot produce chlorophyll, and therefore unable to produce photosynthates for growth and development (Miller et al., 1984). Knowing that most of the Fe is present in the chlorophyll, Longnecker (1988) found that chlorophyll synthesis was inhibited in Fe-deficient plants. It was also found that as the Fe-deficiency chlorosis develops, the amount of Fe and chlorophyll decreases proportionately (Terry and Abadia, 1986).

Iron chlorosis is considered a common nutritional problem in nearly all citrus orchards all over the world (Vose, 1982), especially those orchards on calcareous soils (Longnecker, 1988). Plant analysis for total Fe is frequently used to diagnosis Fe-deficiency chlorosis. However, in studies on Fe chlorosis for other plant species, chlorotic plants frequently contained similar amounts of ferric (Fe³⁺)-Fe (Olsen et al., 1981) or higher amounts (Hamze and Nimah, 1982) than the normal green plants. Therefore, the Fe plant status cannot always be diagnosed by determining total Fe. This has lead many scientists to suggest the estimating of physiologically-active Fe rather than total Fe in plant tissues for diagnosis of Fe deficiency (Mengel
et al., 1994; Sudahono et al., 1994). Active Fe has been found to be correlated better with Fe chlorosis in cotton (Katyal and Sharma, 1980), sorghum (Mohammad, 1986), sunflower (Mengel et al., 1994), and peach (Koseoglu and Acikgoz, 1995).

Little research has been conducted relating Fe chlorosis to both total and active Fe concentrations in plant tissues. Moreover, the results from research conducted in other plant species or crops are controversial and not consistent. Sudahono et al. (1994) in a sand culture study, reported that active Fe, but not total Fe, was well correlated with chlorophyll content and with visual ratings of chlorosis in citrus rootstock seedlings. On the other hand, Rao (1993) found that neither total Fe nor active Fe extracted by 0.1N hydrochloric acid (HCl) indicated the Fe-deficient level. The objectives of this study were to (i) diagnose Fe-deficiency chlorosis in citrus grown on highly calcareous soils, (ii) determine if total or active Fe is related to the degree of chlorosis in citrus lemon trees, and (iii) determine the optimum extraction time and optimum ratio of extracting solution to plant sample for extracting active Fe with o-phenanthroline.

MATERIALS AND METHODS

Sampling sites were located in the Jordan Valley. Climate in the Mediterranean area is characterized by hot, dry summers and moderately cool, wet winters. The average temperatures range from 0°C in January to 43°C in August. The soils are highly calcareous, alkaline, and frequently low in phosphorus (P) and Fe (Dow, 1984). Citrus trees growing on these artificially irrigated soils are frequently Fe deficient (Dow, 1984).

Leaf samples of different degrees of chlorosis (green, moderately chlorotic, and severely chlorotic) were taken from citrus orchards located in three different sites: North Shuna, Site 1; Wadi Yabis, Site 2; and Kraimeh, Site 3. All three sites are located in the Jordan Valley where citrus is one of the most important horticultural crops and is the major citrus-growing area in Jordan. Five normal green, five moderately chlorotic, and five severely chlorotic lemon trees of similar age were sampled from each orchard. Trees infested with pest and diseases were excluded from sampling. From each tree, 20 leaves were sampled equally from the four sides of the tree canopy. A total of 100 leaves were collected from the five trees of the same category to make one composite sample. The leaves sampled were 5 to 6 months old.

The samples were immediately transported to the laboratory where they were washed with tap water followed by rinsing with 0.1N HCl and then distilled water. After drying, the sample of dry tissue was randomly divided into two subsamples, one chopped into about 2-mm pieces using stainless steel scissors for determining active Fe. The other subsample was oven dried at 68°C to a constant weight. The moisture content was determined and used to express all analytical results on a
dry weight basis. The oven-dried subsample was ground to pass a 20-mesh sieve in a Wiley Mill.

A wet HNO₃ method (Havlin and Soltanpour, 1980) was used to determine the plant content for total Fe. A 0.5±0.1 g aliquot of the oven-dried ground sample was weighed directly into a tuff container. Ten mL concentrated HNO₃ was added to each container, covered tightly, and placed in an 85°C oven overnight. On the following day, the containers were taken out of the oven and cooled for two hours. Two mL of 30% hydrogen peroxide (H₂O₂) was added to each container and placed back in the oven for two hours. The digest was cooled, transferred quantitatively to 50-mL volumetric flasks, brought to volume with deionized water, and analyzed for Fe content by atomic absorption spectrophotometry (AAS).

The method suggested by Katyal and Sharma (1980) was used to determine the active Fe in the chopped fresh citrus leaves. The procedure involves extraction of fresh plant by o-phenanthroline (pH 3.0, concentration 1.5%), a selective extractant for ferrous (Fe²⁺)-Fe, and measurement of the Fe²⁺ in the filtrate. About 1 g of the fresh-chopped sample was immediately weighed to the nearest 0.1 mg and transferred to 125-mL Erlenmeyer flask. Five, 10, or 20 mL o-phenanthroline solution according to the desired ratio of the extracting solution to the fresh weight was added and the contents of the flask were mixed gently to bathe the plant samples with the extractant. The flasks were stoppered and allowed to stand for about 20 or 45 hours, according to the time of extraction to be evaluated, at room temperature. The contents were filtered through a Whatman No.1 filter paper. Iron (Fe²⁺) was measured directly in the filtrate by AAS. All samples were analyzed for both total and active Fe in triplicates and only the mean values are presented.

<table>
<thead>
<tr>
<th>Site</th>
<th>Extraction time, hours (ratio = 10)</th>
<th>Degree of iron chlorosis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Green</td>
<td>Moderate</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>23.0 (1.3)*</td>
<td>15.3 (0.9)</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>25.6 (1.5)</td>
<td>16.4 (1.1)</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>32.7 (1.5)</td>
<td>17.1 (1.2)</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>30.1 (1.8)</td>
<td>15.8 (0.9)</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>36.5 (1.9)</td>
<td>24.2 (1.8)</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>38.1 (1.8)</td>
<td>21.8 (1.9)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses represent ± the standard deviation.
TABLE 2. Concentration of active Fe (mg kg⁻¹) extracted with o-phenanthroline solution as affected by the ratio of the extracting solution to the fresh plant sample.

<table>
<thead>
<tr>
<th>Site</th>
<th>Ratio of extracting solution to fresh plant sample (extraction time = 20 hours)</th>
<th>Degree of iron chlorosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Green</td>
</tr>
<tr>
<td>2</td>
<td>5:1</td>
<td>24.0 (1.7)*</td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>32.7 (1.5)</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>38.8 (2.1)</td>
</tr>
<tr>
<td>3</td>
<td>5:1</td>
<td>28.0 (1.7)</td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>36.5 (1.9)</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>48.7 (2.2)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses represent ± the standard deviation.

RESULTS AND DISCUSSION

The effect of time of extraction on the extractability of active Fe from rice by o-phenanthroline studied by Katyal and Sharma (1980) found that extractable Fe increased with increasing extraction time up to 12 hours. However, extraction time greater that 12 hours did not result in a significant increase in extractable Fe from rice. Therefore, since citrus leaves are thicker and waxy, more extraction time would be needed. In this study, 20 and 45 hours for the extraction time were investigated. The results indicated that increasing extraction time from 20 to 45 hours did not result in a significant increase in extractable active Fe (Table 1). This phenomenon was observed in all samples of different degrees of chlorosis and at all three sample sites. This suggests that an extraction time of 20 hours is enough for extracting active Fe from citrus lemon leaves by o-phenanthroline. Moreover, this time period is convenient for running a routine laboratory analysis where the extraction time can be achieved by leaving samples soaking with the extracting solution overnight.

Increasing the ratio of extracting solution to plant sample at an extraction time of 20 hours increased the extractability of active Fe from the plant tissues (Table 2). It was found by other researchers that ratios higher than 10 resulted in only a slight and negligible increase in the extractable Fe from rice tissues (Katyal and Sharma, 1980). The results of this study indicated that increasing the ratio from 5:1 to 10:1 and then to 20:1 continued to increase the amount of active Fe extracted by the extracting solution. Unlike the extraction of Fe from rice observed by
Katyal and Sharma (1980), increasing the ratio for extracting Fe from citrus leaves continued to extract Fe. This may be attributed to the fact that citrus leaves are thicker and waxy, and therefore, a higher amount of extracting solution was able to continue extracting Fe from the tissues. However, the amount extracted by all three ratios (5:1, 10:1, or 20:1) was detectable, and at the same time, similarly and consistently differentiated plant samples of different degrees of chlorosis.
FIGURE 2. Ratio of active to total Fe (mg kg⁻¹) in citrus leaves with different degrees of Fe chlorosis (numbers at the top of each bar represent ± standard deviation).

Therefore, any of the ratios can be recommended for extracting and analysis for active Fe in citrus lemon leaves. If one is intended to extract the maximum amount of Fe from the leaves, however, a ratio higher than the 20:1 should be investigated to determine that ratio which extracts the maximum amount of active Fe.

The values for total and active Fe in citrus lemon leaves (Figure 1) indicates that the total Fe content was always higher in moderately and severely chlorotic leaves. This phenomenon was observed in all samples taken from the three sampling sites. This also was observed for the normal green leaves except for those taken from Site 2 where the total Fe was lower than the active Fe in the green leaves.

Total Fe obtained in all plant samples with different degrees of chlorosis were very much similar, and therefore, cannot be used as a criteria to differentiate between Fe-deficient and non-deficient plants. On the other hand, the amount of active Fe always tended to decrease with the increase in the degree of chlorosis. This agrees with the findings of other researchers on other crops (Katyal and Sharma, 1980; Mohammad, 1986; Mengel et al., 1994; Koseoglu and Acikgoz 1995). This clearly illustrates the failure of plant analysis for total Fe as a tool for diagnosis of Fe deficiency in citrus lemon trees, and the effectiveness of active Fe extracted with the o-phenanthroline for separating the Fe-deficient from non-deficient plants or even for using it as a criteria for screening different cultivars for Fe efficiency.
The ratio of active to total Fe was calculated and found to be closely correlated with degree of chlorosis (Figure 2). This suggests that this ratio can also be effectively used to distinguish between Fe chlorotic and healthy normal green plants. It was found that enhancing Fe availability by partial acidification of calcareous soils with sulfur (S) and/or sulfuric acid (H$_2$SO$_4$) markedly increased the proportion of active to total Fe in sorghum plants (Mohammed, 1986) and apple trees (Kastori et al., 1985). This will make the active Fe content in plant tissues acting not only as a criteria for diagnosis of Fe chlorosis, but also as a variable parameter to be considered for the evaluation of plant responses to various factors affecting Fe availability in the soil and/or Fe nutrition in plants.

**CONCLUSIONS**

In the light of our results, it can be concluded that the total Fe content in plant tissue extracted by HNO$_3$ was not associated with chlorosis while active Fe extracted by o-phenanthroline and/or the ratio of active to total Fe were closely related to the degrees of chlorosis in citrus lemon trees. Therefore, active Fe, the fraction of Fe involved in the synthesis of chlorophyll, and/or the ratio of active to total Fe, is recommended to be used as a criteria for diagnosis of Fe chlorosis in citrus lemon trees.

**REFERENCES**


NITRIC ACID- AND O-PHENANTHROLINE-EXTRACTABLE IRON


