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Iron Deficiency May Result in Interveinal Chlorosis of Shamrock Plant (*Oxalis regnellii*)

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ABSTRACT

Oxalis regnellii is a geophytic ornamental pot plant grown primarily for its clover-like leaves. During greenhouse production, the leaves often become chlorotic for unknown reasons, possibly including virus infection, iron (Fe) and/or manganese (Mn) deficiencies, and improper greenhouse forcing temperatures. We conducted a series of experiments to address these hypotheses. *Shamrock chlorotic ringspot virus* (SCRV) has been reported before in *Oxalis regnellii*. Oxalis plants exhibiting virus-like symptoms were analyzed and a potyvirus was detected, although this virus was not further confirmed to be SCRV. To confidently test other hypotheses, any suspected viral infected material was discarded. Plants grown at 13°C exhibited slowed growth and development; however, the incidence of leaf chlorosis did not increase compared with plants grown at warmer temperatures of 21/16°C (day/night); 22°C constant; or 22 to 16°C (plants were moved to 16°C when 50% of the plants were in first flower). To assess the ability to correct an iron (Fe) deficiency, a media drench of ferric ethylenediaminedi (*o*-hydroxyphenylacetic) acid (Fe-EDDHA) was applied to chlorotic, Fe-deficient oxalis plants and plants successfully re-greened within 5 days.

Keywords: floriculture, greenhouse production, iron chlorosis, micronutrient chelate, virus
Abbreviations: Fe-DTPA, diethylenetriaminepentaacetic acid; **Fe-EDDHA**, ferric ethylenediaminedi (*o*-hydroxyphenylacetic) acid; **Fe-EDTA**, ferric ethylenediamine tetraacetic acid; **$FeSO_4$**, ferrous iron sulfate; **INSV**, *Impatiens necrotic spot virus*; **SCRV**, Shamrock Chlorotic Ringspot Virus; **TEM**, transmission electron microscopy; **TMV**, *Tobacco mosaic virus*; **SPAD or chlorophyll-meter**, Soil Plant Analysis Development

INTRODUCTION

Oxalis regnellii (oxalis), also known as "The Shamrock Plant", is a specialty potted bulb crop grown for its clover-like leaves and marketed primarily for the St. Patrick's Day holiday in the United States (Miller 1997; Dole and Wilkins 2005). A second species, *Oxalis triangularis* (purple shamrock) is primarily cultivated for use in the landscape and in mixed containers. *Oxalis regnellii* is susceptible to interveinal chlorosis, which has perplexed oxalis forcers. The exact cause for this symptom has been unclear. Several factors can contribute to yellowing or chlorosis in plants. One major factor is nutrient availability, directly (quantity of the element available) and indirectly (as affected by pH). Viral infection is another potential cause, as infected plants may exhibit chlorotic symptoms. A third potential factor is an indirect temperature effect. As temperature decreases, plant metabolic activity decreases, including potential nutrient uptake. These factors are all plausible reasons for leaf chlorosis noted as a production problem in oxalis, and are the basis for the studies reported in this paper.

Two plausible hypotheses have been reported to explain this phenomenon. The first is that the chlorosis is a result of a deficiency of iron (Fe) and/or manganese (Mn). Iron is often the first micronutrient that becomes limiting in greenhouse media (Nelson 1994). Iron deficiency is most often encountered with high media pH, as the solubility of Fe (and other micronutrients, with the exception of molybdenum) decreases with increasing pH. Iron is a major component of many photosynthetic pigments and is involved in the development and synthesis of chloroplasts and chlorophyll (Marshner 1995). Iron deficiency leads to smaller chloroplasts and chlorophyll reduction, and subsequent chlorosis. Manganese is involved in the electron transport system; deficient levels result in reduced or stunted growth and interveinal chlorosis of young leaves, very similar to Fe deficiency.

The second hypothesis is that virus has infected some of the *O. regnellii* population (De Hertogh 1996; Dole and Wilkins 2005). Oxalis is asexually propagated, which carries a high risk of perpetuating viral infection. However, no research has been conducted to substantiate these claims.

A series of studies were conducted to investigate potential factors for their effect on chlorosis in oxalis. First, a screening of oxalis plants was conducted to assess the plausibility of a viral infection. *Shamrock chlorotic ringspot virus* (SCRV), suspected to be a potyvirus, was first reported in 1981 by Coyier and is the only virus reported in *Oxalis regnellii*. Virus symptoms may look very similar to interveinal chlorosis resulting from a nutritional deficiency; the most identifiable virus symptom is the characteristic

Fig. 1 Chlorotic ringspot symptoms associated with a potyvirus in oxalis (*Oxalis regnellii*).

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chlorotic ring spot surrounding an island of green tissue (**Fig. 1**). As SCRV infection progresses, the chlorotic ring spots fade into indistinct chlorotic blotches and streaks and infected rhizome scales become dark brown or black. The virus is thought to be transmitted by aphids and possibly through mechanical contact between diseased roots and healthy plant roots, along with asexual propagation techniques. Ultimately the plants may die within two years after being infected with SCRV (Coyier 1981). Careful and immediate rouging of symptomatic plants is the most effective control of SCRV. In order to accurately determine and characterize interveinal chlorosis and confidently test the Fe deficiency hypothesis, identification and disposal of any potential virus infected oxalis was necessary.

A second study investigated the effects of growing temperatures on chlorosis incidence. Optimal greenhouse forcing temperatures are important to produce high quality, marketable ornamental plants. Current forcing recommendations for *O. regnellii* suggest growing at 21-24°C until plants are well rooted and then reducing the night temperature to 18-21°C (De Hertogh 1996; Miller 1997). Oxalis is forced in the winter months, late December to early March; *O. triangularis* can be forced any time from December to June, as it is typically used as a pot plant and/or for mixed containers. Since *O. regnellii* is produced primarily during cooler months, the potential ability to reduce greenhouse heating costs is important to growers. Further understanding and information on forcing temperature in relation to leaf chlorosis incidence would be valuable.

There are two main procedures to correct Fe deficiency in greenhouse production. The first is pH management. Adjusting media pH during crop production can be difficult; thus it is often more common to manipulate potting media pH before planting. A second strategy to correct Fe deficiency is Fe chelate foliar sprays and/or media drenches. In both corrective procedures, the Fe form applied has a significant effect on the application effectiveness. Common Fe chelate forms used (in order of increasing availability at pH solutions above 6.5) are ferrous iron sulfate ($FeSO_4$), ferric ethylenediamine tetraacetic acid (Fe-EDTA), diethylenetriaminepentaacetic acid (Fe-DTPA), and ferric ethylenediaminedi (o-hydroxyphenylacetic) acid (Fe-EDDHA) (Fisher *et al*. 2003). Hammer (2006) described successful re-greening of yellow oxalis plants using foliar Fe chelate applications. Other research has shown that both foliar and soil applications of Fe chelates were beneficial in correcting Fe deficiency in *Calibrachoa* and in fruit crops (Swietlik and Faust 1984; Fisher *et al*. 2003). A preliminary study was conducted to assess micronutrient chelate applications and their effects on re-greening chlorotic *O. regnellii* leaves.

MATERIALS AND METHODS

Virus screening

O. regnellii plants with virus-like symptoms were first tested at the Cornell University Diagnostic Laboratory for two common viruses that infect greenhouse ornamentals, *Tobacco mosaic virus* (TMV) and *Impatiens necrotic spot virus (*INSV*)*. Tissue samples of putative virus infected plants (n = 4), which were showing chlorosis and characteristic ringspots, and plants (n = 26) suspected to be virus-free (showing none of the symptoms previously described) were examined. Tissues were tested for presence of virus by transmission electron microscopy (TEM) at the University of Minnesota, using partially purified extracts. Leaf tissue samples (1.5-2.0 g) were powdered in liquid nitrogen in a mortar and then extracted with 17 ml extraction buffer (500 mM NaK-PO_4, pH 7.5, 1M urea, 5% w/v polyvinyl pyrrolidone (PVP-T40), 0.5% (v/v) 2-mercaptoethanol). The mixture was filtered through Miracloth (Calbiochem/EMD Millipore, Billerica, MA), the filtrate centrifuged at 27,000 × *g* for 15 min and the pellet discarded. The supernatant was layered over 5 ml 30% (w/v) sucrose in 100 mM Na-PO_4 pH 7.0 and centrifuged at 148,000 × g for 90 min at 10°C. The pellet was resuspended in 100 µl 100 mM Na-PO_4, pH 7.0 and clarified by vortexing with an equal volume of chloroform and centrifugation in a micro-centrifuge for 10 min at 15,000 rpm. The upper aqueous phase constituted the partially purified extract, and was examined by TEM following negative staining with 2% sodium phosphotungstate, pH 7.0. Virus particles observed in positive samples were flexuous filamentous particles measuring 750-800 × 12 nm.

Greenhouse forcing temperature

O. regnellii and *O. triangularis* rhizomes supplied from a commercial Dutch supplier (Leo Berbee Bulb Co., Marysville, OH) were used for this experiment. Eight rhizomes of each species that had been stored for several months at 3°C were planted on 5 February. One rhizome was planted per 10 cm pot using a commercial greenhouse media substrate (LC1; Sun Gro Horticulture Ltd., Vancouver, Canada) and grown under four glasshouse temperature regimes: 21/16°C (day/night); 22°C constant; 22 to 16°C (plants were moved to 16°C when 50% of the plants showed first flower); and 13°C constant. Plants were fertilized at each irrigation with 250 mg $N{\cdot}L^{-1}$ 20N–2.2P–16.6K (Jack's Professional LX Water Soluble Fertilizer 21-5-20 All Purpose; J. R. Peters Inc., Allentown, PA). The date of shoot emergence (DTE) was recorded when the first leaf was visible above the media surface. Flower data recorded included the number of flower cymes per plant and the number of days to flower (DTF), recorded when the first floret fully opened. Ten weeks after planting, plant height was measured from pot rim to top of the foliage canopy; Soil Plant Analysis Development (SPAD) meter readings (Minolta Chlorophyll Meter SPAD-502; Spectrum Technologies, Plainfield, IL) were taken to the right of the mid-vein on each leaflet of *O. regnellii.* Substrate pH and electrical conductivity (EC) measurements using the pour through method (Cavins *et al*. 2000) were obtained. Plant tissues were oven dried at 70°C for at least 48 h and dry weight (DW) determined.

Media drench chelate application

Five *O. regnellii* plants exhibiting induced interveinal Fe chlorosis due to high media pH (unpublished data) and five plants with no visible signs of Fe deficiency (controls) were selected. Chlorotic plants were drenched with an Fe-EDDHA chelate (Sprint 138; Becker Underwood; Ames, IA) drench solution (0.375 $g{\cdot}L^{-1}$) until the solution leached through the pot. Control plants were drenched with reverse osmosis water. SPAD readings were obtained (as described above) immediately before chelate application and five days post application. SPAD meter values were averages of six readings from two different leaves per pot.

Statistical analysis

One-way analysis of variance tests were conducted to identify differences in the measured parameters in response to temperature treatments and Tukey's HSD method ($P = 0.05$) was used to conduct pairwise comparisons for each species. Statistical analyses were conducted with JMP v. 8 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Virus screening

In our research stock, we initially observed virus-like symptoms in *O. regnellii* plants obtained from three rhizome suppliers. Plants tested by ELISA for INSV and TMV were negative for both viruses. Examination by TEM indicated that all 26 plants thought to be virus free by visual assessment were negative for any virus, while filamentous potyvirus-like particles were observed in three of the four plants suspected to be virally infected. The viral particles were not however, conclusively identified as SCRV. Based on this information, plants with any suspected virus-like symptoms were discarded, rather than being included in subsequent studies.

Table 1 Temperature effects on several plant growth parameters in *Oxalis regnellii* and *Oxalis triangularis*.

Temperature (˚C)	Days to emerge	Days to flower	No. of flower cymes	Height (cm)	Dry weight (g)	SPAD	pH	EC
O. regnellii								
13	17 a [z]	54 a	2 a	3.1 a	0.18 a	36.6 a	4.61 a	10.5 a
21/16 [y]	17 a	44 b	5 a	6.6 b	0.79 b	31.9 b	5.74 b	5.6 b
22 to 16 [x]	15 a	42 b	8 ab	6.7 b	0.82 b	36.6 a	5.26 c	7.5 c
22	12 b	37 b	13 b	6.9 b	1.03 b	37.2 a	4.97 d	7.4 c
O. triangularis								
13	19 a	dnf [w]	dnf	2.3 a	0.10 a	-	-	-
21/16	13 b	52 a	3 a	6.4 b	0.64 b	-	-	-
22 to 16	15 ab	48 a	3 a	5.8 b	0.48 b	-	-	-
22	13 b	49 a	4 a	6.9 b	0.66 b	-	-	-

[z] Day/Night temperature
[y] Plants were moved from 22 ˚C to 16 ˚C when 50% of plants were in flower.
[x] Letters after values in each column for each species represent mean separation using Tukey's honestly significant difference (HSD) at $P = 0.05$.
[w] Did not flower

Fig. 2 Effects of greenhouse forcing temperatures on growth and development of *Oxalis regnellii* (green leaved) and *O. triangularis* (purple leaved). L to R 21/16°C (D/N); 22°C constant; 22 to 16°C (plants were moved to 16°C when 50% of the plants showed first flower); and 13°C constant.

Greenhouse forcing temperature

Temperature had a significant effect on growth parameters in both *Oxalis* species (**Table 1; Fig. 2**). The warmest temperature (22°C) significantly decreased DTE and DTF for both species as compared to the coldest temperature (13°C). *O. triangularis* did not flower by the end of the experiment in the coldest temperature treatment. There was little temperature effect on flower cyme number for *O. triangularis* plants that did flower. However, in *O. regnellii*, significantly more flower cymes were observed at the higher temperature. Plants were 50% and ~33% shorter for *O. regnellii* and *O. triangularis*, respectively, at 13°C compared to other temperature treatments. Plant DW was lowest for both species in the coldest temperature treatment. SPAD readings were lowest for *O. regnellii* plants grown under the diurnal 21/16°C temperature treatment. The highest pH reading (5.74) and lowest EC reading (5.58) were obtained in the diurnal temperature treatment, while the lowest pH (4.61) and highest EC reading (10.46) were observed in the coldest temperature treatment for *O. regnellii*.

It has been often reported that *O. regnellii* frequently develops interveinal chlorosis before market. De Hertogh (1996) suggested reducing the greenhouse night temperature to 18-21°C until flowering and market. It is well known that colder temperatures can significantly reduce plant growth and development. Cooler forcing temperatures (13°C) did not significantly increase chlorosis levels in our study; plants forced at temperature regimes above 13°C had similar growth and development. Miller (1997) reported that interveinal chlorosis on *O. regnellii* was more prevalent at lower growing temperatures. There was little visual

Table 2 Effects of ferric ethylenediaminedi (*o*-hydroxyphenylacetic) acid (Fe-EDDHA) media drenches on regreening of iron (Fe) deficient oxalis (*Oxalis regnellii)* after 5 days.

Treatment	Initial SPAD[z]	Ending SPAD[y]	SPAD difference
Fe Deficient	15.4 a	27.5 a	+12.1 a
Control	28.0 b	27.1 a	-0.9 b
Signficance	***[x]	NS	***

[z]SPAD meter values were averages of six readings from two different leaves per pot before chelate application. Means followed by different letters within each column are significantly different by Student's *t* test at $P \leq 0.05$.
[y]SPAD values 5 days after chelate drench.
[x]NS, *, **, *** Not significant or significant at $P \leq 0.05$, 0.001, or <0.0001

Fig. 3 The effect of iron (Fe) chelate drenches 5 days after application on iron deficient oxalis (*Oxalis regnellii*). Control plants are shown on the right. Oxalis plants five days after drenching are shown on the left.

difference in chlorosis among treatments; however, SPAD readings were significantly lower in the diurnal temperature treatment.

Based on this study, greenhouse forcing temperatures should be at least 21°C for optimal growth of *O. regnellii* and *O. triangularis*. Increasing temperatures above 21°C is beneficial for growth and development and further reduce the time to market. However, increasing temperatures increases greenhouse heating costs, and appropriate only if other crop species in production simultaneously require similar warm temperatures. Further studies investigating greenhouse temperatures between 16 and 21°C could be beneficial for fine-tuning production recommendations.

Media drench chelate applications

Miller and Miller (2011) found foliar Fe-chelate applications were not effective in re-greening oxalis leaves and chlorophyll levels did not increase after micronutrient chelate applications. This could be due to limited metal mobility even with chelated micronutrient forms (Swietlik and Faust 1984). Unlike foliar sprays, chelate drenches were effective in re-greening of oxalis (**Table 2**). **Fig. 3** shows chlorotic *O. regnelii* plants and plants 5 days after drenching with Fe chelate. Chlorotic plants that received Fe-chelate drenches had nearly double the chlorophyll content (time frame). SPAD measurements also increased significantly five days after chelate application. Chlorophyll content did not increase in control plants.

The data obtained in this study provides evidence that Fe chelate drenches can be effective in re-greening chlorotic oxalis plants that are Fe deficient. The best way to counter Fe deficiency (or other nutrient problems) is through a program monitoring and adjusting pH and nutrition appropriately during production. However, problems are sometimes unavoidable and, thus, corrective procedures need to be employed. Additional studies on different Fe chelate rates and chelate forms for both foliar sprays and drenches should be conducted to further elucidate ideal preventative and rescue treatments for *O. regnellii*.

CONCLUSIONS

This study provides more information about the chlorosis phenomenon in *Oxalis regnellii*. Virus symptoms and nutrient deficiency symptoms may be difficult for growers to distinguish. The hypothesis that some of the foliar disorders reported in *O. regnellii* over recent years, could be a direct result of a viral infection is certainly plausible. Because *Oxalis* species produce several daughter rhizomes and are vegetatively propagated, it is important that rhizome producers continually monitor for symptoms and rogue any suspected plants to have viral infection, to reduce dissemination of the virus. Our research also suggests that cooler forcing temperatures do not translate into increased chlorosis. However, factors such as media type and irrigation (quantity and method), coupled with cooler forcing temperatures could have a significant effect on growth and development of oxalis and chlorosis and need to be considered. Our investigations also showed that if leaf chlorosis occurs due to a high pH, iron chelate drench applications are more effective than foliar applications in view of the results of Miller and Miller (2011). Additional studies for both correction techniques would be beneficial. The chlorosis phenomenon is complex and does not appear to be attributable to any one specific cultural practice.

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