SCREENING FOR IRON CHLOROSIS TOLERANCE OF *IN VITRO PYRUS* ROOTSTOCK GERMPLASM

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ABSTRACT

Fruit trees grown in calcareous soils in arid and semiarid regions are iron (Fe) deficient, producing symptoms known as Fe-chlorosis. *In vitro* screening for pear rootstocks to determine which are tolerant to the Fe-chlorosis from diverse *Pyrus* germplasm could be major importance to fruit growers for preventing chlorosis, reducing economic losses and the need for expensive and environmentally unfavorable soil amendments. This study was to develop a standardized *in vitro* testing and screening method for resistance to Fe chlorosis. *In vitro* Fe-stress conditions were induced for the plantlets of each rootstock by modifying the Fe^{3+}chelate ethylendiamine di (*o*-hydroxyphenylacetic) acid (EDDHA) and potassium bicarbonate ($KHCO_3$) concentrations in pear rootstock (PRS) medium. Data was evaluated for six categories; quality, shoot length, percent dead leaves, color, and SPAD index. Photos were taken for the visual assessment. The most effective treatment was PRS medium with 1 mM each of Fe-EDDHA and KCO_3 at pH 7.0. The plant responses indicated that this treatment can be used as a definitive protocol with evaluation using the SPAD readings to determine tolerant or susceptible genotypes. Medium pH reduction for each genotype was not effective for determining tolerant cultivars, but the SPAD index and the visual assessments were definitive. The most tolerant was *P. communis* 'OHxF87' followed by *P. communis* 'Horner 4', *P. betulifolia* 'OPR 260' and *P. spinosa*. Susceptible cultivars were 'Winter Nelis', OPR 113 and 'Pyriam'. This *in vitro* screening tool can be used to determine which rootstocks should be considered as possible candidates for field trials, reducing time and resources needed to test all available rootstocks

INTRODUCTION

Fruit trees grown in calcareous soils in arid and semiarid regions are iron (Fe) deficient, producing symptoms known as Fe chlorosis. The nutritional status of field plants can be detected by leaf mineral analysis; however, leaf analysis has serious limitations for evaluating actual iron deficiency. There is often no correlation between iron content in

leaves and the degree of Fe-chlorosis, making it one of the most complex nutritional deficiencies.

Massive application of synthetic Fe chelates to soils are the most common treatment for correcting Fe-chlorosis in fields, but this creates the risk of negative environmental impacts(Pestana et al., 2003), and is expensive (Álvarez-Fernández et al., 2005). In Europe, an increase in Fe-chlorosis symptoms directly correlated with sharp reductions in fruit yield per tree, fruit size and quality and there is a serious need for early diagnosis of this condition (Abadía et al., 2000).

Fortunately, *in vitro*-grown pear and quince shoots with no root system are able to respond to iron deficiency and bicarbonate enriched conditions and provide biochemical and physiological responses that can be measured (Donnini et al., 2008). *In vitro* responses of micropropagated shoots to low Fe conditions are similar to those of mature trees in the field. Obvious differences exist among species in the ability to reduce Fe and to acidify the growth medium (Dolcet-Sanjuan et al., 1990). *Pyrus amygdaliformis* adapted more easily to Fe-limiting conditions found with alkaline soils, and had a higher ability to reduce Fe and to acidify the medium than the more sensitive quince rootstocks, *Cydonia oblonga* (Lombard and Westwood 1987). Roots and shoots showed similar adaptive responses to Fe stress, indicating that Fe deficiency can be readily expressed in shoots as well as in the roots. A species that adapts well to Fe-limiting conditions such as alkaline soils exhibits enhanced ability to reduce Fe and to acidify the medium compared to sensitive species (Dolcet-Sanjuan et al., 1992). A SPAD chlorophyll meter provides an unbiased, quantitative measure of leaf chlorosis (Álvarez-Fernández et al., 2005) and chlorosis-tolerant *Prunus* rootstocks in the field showed high or very high SPAD values (Jiménez et al., 2008).

In vitro screening for pear rootstocks to determine which are tolerant to the Fe chlorosis from available *Pyrus* germplasm could be of major importance in regions with calcareous soils for preventing chlorosis, reducing economic losses and reducing the need for soil amendments.

OBJECTIVES

Develop a standardized *in vitro* testing and screening method for Fe chlorosis for *Pyrus* rootstocks. Test a wide range of pear rootstock germplasm. Disseminate findings and protocols to growers and commercial labs.

PROCEDURES

***Plant Materials*:** Pear rootstock cultures were established on *Pyrus* rootstock (PRS) medium developed in earlier studies (Wada et al., unpublished). Fully grown shoot cultures (4 week subculture) were planted with five shoots per container without removing the base. Three replications and three separate experiments were run (October / November / December) for 'Winter Nelis', OHxF87, 'Horner 4' and OPR 260 (n=45). Due to slower multiplication, two experiments were run for *P. spinosa* set in Oct. and Dec.(n=30), and one for 'Pyriam' in Oct. and one for OPR113 in Dec.(n=15) (Table 1).

Table 1. Genotypes tested October through December, 2014.

Genotype	Local number	Species (subfamily)	October	November	December
Winter Nelis	1164.001	*P. communis*	X	X	X
OHxF 87	1345.002	*P. communis*	X	X	X
Horner 4	2955.001	*P. communis*	X	X	X
OH-11 Pyriam	2700.001	*P. communis*	X		
OPR 260	1379.001	*P. betulifolia*	X	X	X
OPR 113	655.001	*P. betulifolia*			X
P. spinosa	634.001	*P. spinosa* (*Amygdaloideae*)	X		X

Induction of Fe chlorosis: *In vitro* Fe-stress conditions were induced for the plantlets of each rootstock by modifying the Fe^{3+}chelate ethylendiamine di (*o*-hydroxyphenylacetic) acid (EDDHA) and potassium bicarbonate ($KHCO_3$) concentrations in PRS medium. Two initial tests with six *P. communis*: OHxF69, 87,97, 513, Pyro 2-33, and Horner 10 for the first, the second for OHxF 87, 97, 513, Pyro 2-33 Horner 10 and Fox 11, were used to identify a definitive testing range for the chemicals and pH.

Fe-deficient to Fe-sufficient conditions: To compare responses to Fe-deficient or Fe–sufficient conditions by adding 1 mM each of Fe-EDDHA and $KHCO_3$, shoot cultures of each genotype were grown on three containers of each treatment for 4 weeks (n=15). Data were taken at 4 weeks. Each genotype have three replicates and the experiment were done three times (n=45). Testing continued using the six modified treatments (all at 1 mM) with more genotypes including other species.

Trt 1: MS-Fe concentration with no $KHCO_3$ at pH 5.7 (Control 1)
Trt 2: MS-Fe concentration with 1 mM $KHCO_3$ at pH at 6.3
Trt 3: 1 mM Fe-EDDHA with no $KHCO_3$ at pH at 5.7 (Control 2)
Trt 4: 1 mM Fe-EDDHA and 1 mM $KHCO_3$ at pH at 5.7

Trt 5: 1 mM Fe-EDDHA and 1 mM $KHCO_3$ at pH at 6.3
Trt 6: 1 mM Fe-EDDHA and 1 mM $KHCO_3$ at pH at 7.0

The pH range: The medium pH was adjusted to 5.7 for T1, T3, and T5; 6.3 for T2 and T5; and 7.0 for T6. High or low target pH was achieved using 4N hydrochloric acid (HCL) or 1N sodium hydroxide (NaOH). The pH of the medium was measured before and after autoclaving and at the end of the growth period.

Data taken: Categories evaluated were quality ratings (1 low, 3 high), shoot length, percent dead leaves, color ratings (1 low, 3 high), and SPAD readings (< 25 yellow or light green, > 25 green, >30 dark green) (data not shown). Mean responses of 15 plants from three replicates (n=45) were taken 4 weeks after planting. Data were pooled and analyzed using SAS (ver. 9.3 Cary, NC: SAS Institute). Models were determined for six responses (quality, shoot length, percent dead leaves, color rating, and SPAD index).

RESULTS

The initial tests showed that the pH range should be adjusted to 5.7 or 6.3 with the initial pH no higher than 7.0 as the most extreme pH condition for *in vitro* system. An adjusted pH at 8.0 was too high for shoot survival. Potassium bicarbonate at 1 mM should be the highest concentration. It was not meaningful to test a high concentration such as 10 mM $KHCO_3$ for this *in vitro* system as all the cultures died. The standard MS iron concentration or 1 mM Fe EDDHA were both within range for continued testing. Removing the base of shoots when planting was too harsh on the high $KHCO_3$ media and growth was poor for all.

The final screening tests resulted in data from six categories and all had significant P-values between 0.02 to <0.0001. Medium pH substantially changed after autoclaving when $KHCO_3$ was added. Control media remained close to the original pH. Spent medium pH largely varied by genotype (Table 2). The highest pH was originally at 7.0 but reached 9.58 after autoclaving the medium . Reduction of pH in the spent medium ranged from a decrease of 0.34 in T6 by ‘OHxF87’ to a decrease of 1.95 in T5 by ‘Winter Nelis’ (Figure 1). These decreases were not useful for evaluating the pear rootstock tolerance.

Table 2. Medium pH values at three points in the experiment.

Treatment	Adjusted medium pH	Fresh medium pH after autoclaving	Spent medium pH After 4 wks (average)
1 MS iron (EDTA)	5.7	5.49	4.23
2 MS iron+$KHCO_3$	6.3	9.28	7.82
3 Fe-EDDHA	5.7	5.52	4.28

4 Fe-EDDHA+$KHCO_3$	5.7	8.43	7.57
5 Fe-EDDHA+$KHCO_3$	6.3	9.29	7.65
6 Fe-EDDHA+$KHCO_3$	7.0	9.58	8.66

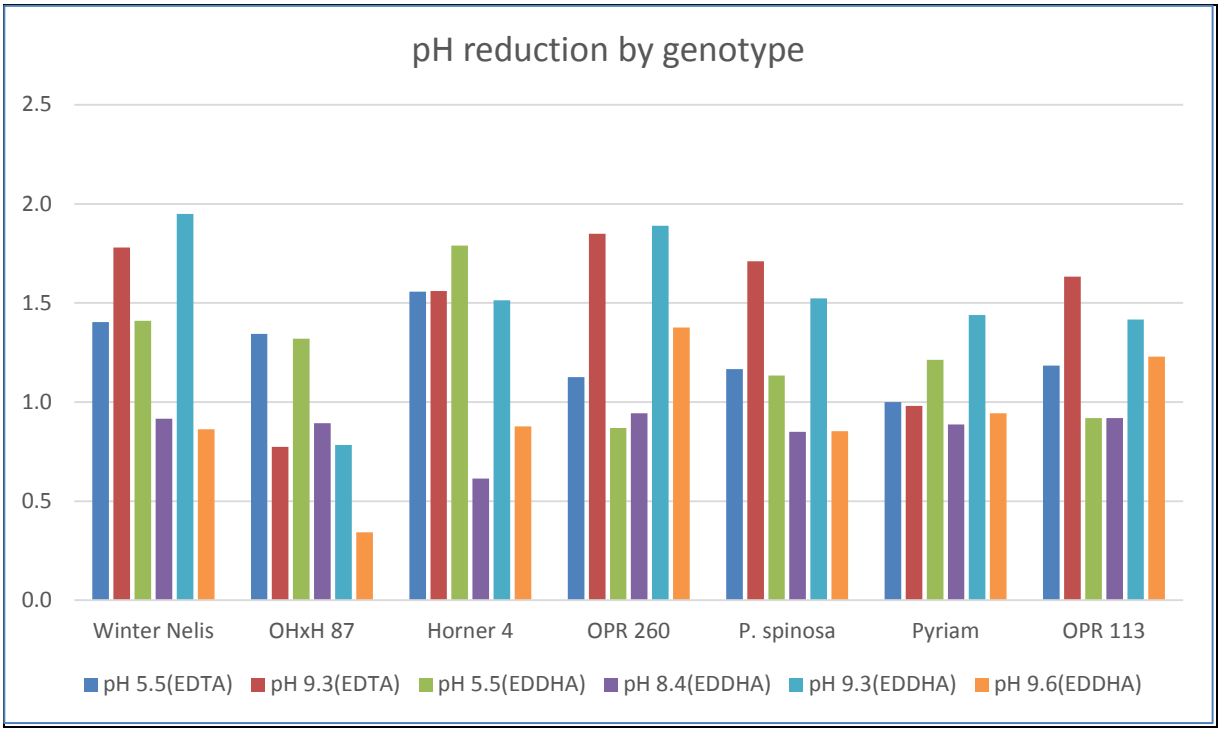

Figure 1. The medium pH reduction by each genotype 4 week after planting. The pH values represent the fresh medium pH after autoclaving. Final medium pH for each treatment is shown Table 2.

Chlorophyll content

Chlorophyll content in leaves was determined by the SPAD readings (Figure 2). The higher the chlorophyll content, the healthier the leaf, even under stress on the high pH medium (T2 and T6). Treatment 6, the EDDHA medium with the highest pH (9.6), could be used to determine tolerant or susceptible genotypes by the SPAD readings. The most tolerant was *P. communis* 'OHxF87' followed by *P. communis* 'Horner 4', *P. betulifolia* 'OPR 260' and *P. spinosa*. Susceptible cultivars were 'Winter Nelis', 'OPR 113' and 'Pyriam'.

The visual assessment evaluated the overall condition of the shoot cultures (Figure 3). Shoots for all genotypes on the MS control treatment T1 (MS iron) were green and actively growing and all except OPR 113 were good on T3 (EDDHA iron). Higher pH treatments T2, T4 and T5 produced acceptable growth except for 'OPR 113'. The main differences in plant growth were seen on T6 with the highest pH. This treatment

resulted in distinct visual changes in the shoots on some genotypes and could be used to differentiate the tolerance or susceptibility of the genotypes.

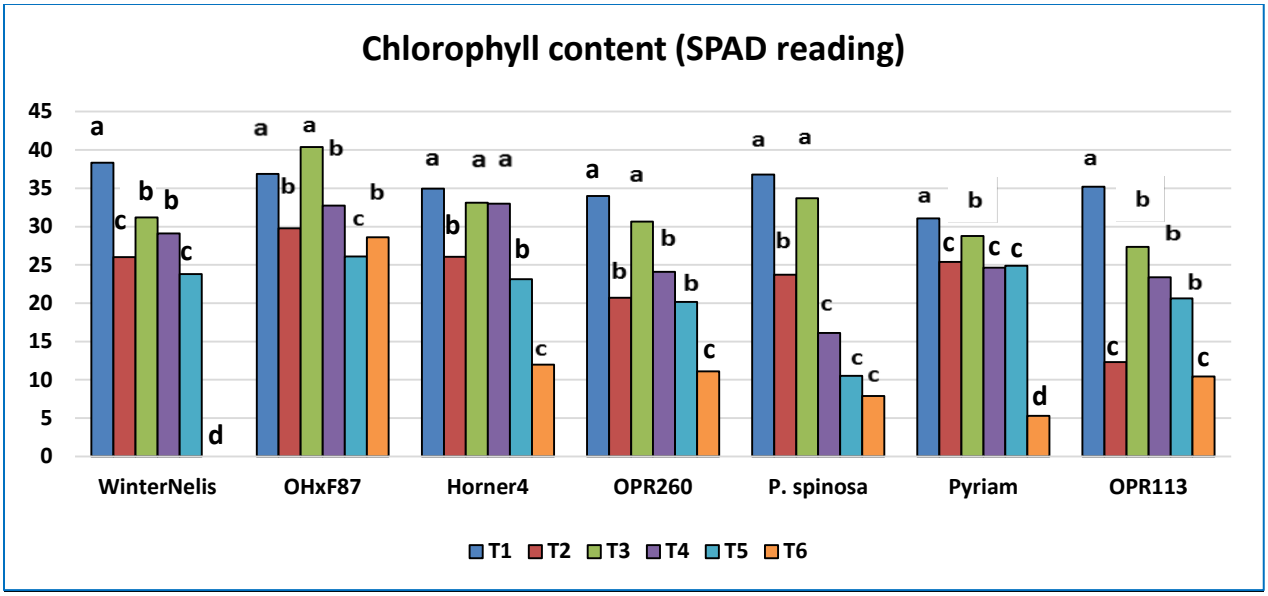

Figure 2. Chlorophyll content of the six pear rootstocks after growth on six treatments. The means separation values are by T-test (SAS 9.3).

SPAD data (Figure 2) combined with visual assessment (Figure 3) indicated that 'OH×F 87', 'Horner 4' and 'OPR 260' were tolerant; 'OPR 113' was moderately tolerant and 'Pyriam' and 'Winter Nelis' were susceptible.

DISCUSSION

This study identified Fe-chlorosis tolerant and susceptible rootstocks using an *in vitro* system. The combination of 1 mM each of Fe-EDDHA and $KHCO_3$ created the sufficient medium pH range from 5.49 to 9.58 after autoclaving for this screening test. The most effective treatment was 1 mM Fe-EDDHA with 1 mM $KHCO_3$ at pH 7.0 and it can be used as a definitive protocol for pear rootstock Fe-chlorosis screening. Medium pH was reduced at different rates by each genotype (Figure 1) but it did not definitively indicate tolerance. The SPAD readings and the visual assessments used together were definitive. This study defining an *in vitro* screening tool for pear rootstocks can be used to determine which rootstocks should be considered as candidates for field trials, reducing the time and cost needed to test all available candidates.

Treatment	T1. MS-Fe -$KHCO_3$	T2. MS-Fe +$KHCO_3$	T3. Fe-EDDHA -$KHCO_3$	T4. Fe-EDDHA +$KHCO_3$	T5. Fe-EDDHA +$KHCO_3$	T6. Fe-EDDHA +$KHCO_3$
Autoclave - Before	pH 5.70	pH 6.30	pH 5.50	pH 5.70	pH 6.30	pH 7.00
- After	pH 5.49	pH 9.28	pH 5.52	pH 8.43	pH 9.29	pH 9.58
Winter Nelis						
OH×F 87						
Horner 4						
OPR 260						
P. spinosa						
OH-11 Pyriam						
OPR 113						

Figure 3: Appearance of seven genotypes cultured for 4wks on the six treatments.