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Article *in* Journal of Soil Science and Plant Nutrition · February 2019

DOI: 10.1007/s42729-019-0017-9

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https://doi.org/10.1007/s42729-019-0017-9

RESEARCH ARTICLE

Evaluation of FE-heme Applications or Intercropping for Preventing Iron Deficiency in Blueberry

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Received: 26 March 2018 / Accepted: 26 October 2018

Abstract
The aim of this investigation was to study the effectiveness and physiological implications of sustainable strategies to correct Fe chlorosis in blueberries, based on Fe-heme applications or intercropping with graminaceous species. The experiment was conducted in a blueberry orchard established on a sub-alkaline soil. The Fe-heme applications increased shoot length without increasing the leaf chlorophyll concentration, gaseous exchange, and fruit yield components in comparison with control plants. On the other hand, intercropping with graminaceous species increased the leaf chlorophyll concentration, photosynthetic activity, and fruit yield, with similar effectiveness to the Fe-EDDHA treatment. However, this management technique reduced the shoot length and leaf N, P, and K concentrations in the plants. The results obtained highlight the potential of intercropping with graminaceous species as a sustainable management technique to correct Fe chlorosis in blueberry. Further studies will need to select new graminaceous species characterized by low nutrient requirements in order to optimize the effectiveness of this management technique.

Keywords Intercropping · Fe-heme · Iron chlorosis · Photosynthesis · Leaf chlorophyll · *Vaccinium* spp.

1 Introduction

In recent decades, the cultivation and human consumption of blueberries (*Vaccinium* spp.) have acquired significant relevance in the world, mainly due to the high concentration of phenolic compounds in their skins which significantly contribute to the prevention of several human diseases (Brewer 2011). Blueberries are classified as a calcifuge species since they are adapted to acidic soil conditions, and the best plant growth and fruit yield are obtained when it grows in soil with pH in the range of 4.5 to 5.5 (Retamales and Hancock 2012). In fact, most of the surfaces cultivated with blueberries in South America are located in acid or sub-acid soils. In the last few years, however, due to some agronomical factors that provide better fruit commercialization, like early fruit harvests and a lower incidence of fungal diseases in plants, an expansion of blueberry cultivation has been verified in lower rainfall zones, often characterized by alkaline or sub-alkaline soils. In such areas, blueberries frequently manifest severe symptoms of iron (Fe) chlorosis. Iron deficiency can cause a reduction in blueberry orchard longevity and productivity, in root and shoot growth, and losses in berry yield and quality (Retamales and Hancock 2012). In other fruit crops such as grapevine, Fe scarcity causes modifications in fruit quality, such as peroxidase activity depression, trans-resveratrol increases, and higher phenolic compound accumulation in the berries (Bavaresco et al. 2010). Moreover, although the anthocyanin concentration in skins from Fe-deficient grapes should be increased, the synthesis of these compounds—also present in blueberries—might be decreased by a reduction in the activity of anthocyanidin synthase, whose activity requires Fe (Wilmouth et al. 2002).

On the other hand, the photosynthetic apparatus is one of the most Fe-rich cell systems in plants, since about 80% of the Fe is located in chloroplasts, and of this, about 60% is located in the thylakoid membranes (Varotto et al. 2002). In fact, plants growing in conditions of Fe depletion may exhibit changes in the chloroplast structure and functioning due to a decrease in the concentration of thylakoid membrane components in leaves and, in particular, photosynthetic pigments

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Published online: 01 February 2019

(Briat et al. 2015). In addition, Fe is a structural component of electron transport molecules such as ferredoxins and cytochromes and aids in the regulation of enzymes linked to the Calvin cycle (Briat et al. 2015). In this context, given the numerous physiological processes related to the photosynthetic apparatus functioning in which Fe participates, the lack of this element leads to a marked reduction in the carbon-binding capacity of plants (Bashir et al. 2015). However, the effects of Fe deficiency on leaf gaseous exchange in a calcifuge species such as blueberry have scarcely been described and reported in the scientific literature.

Strategy I plants, corresponding to dicots like blueberry and non-graminaceous monocots, uptake Fe from the soil as Fe^{2+}. In alkaline and sub-alkaline soils, Fe^{2+} is commonly oxidized to the less soluble Fe^{3+} (Golshahi et al. 2018; Granja and Covarrubias 2018). In this case, Fe deficiency-tolerant strategy I species are able to extrude protons into the rhizosphere by root plasma-membrane ATPase enzyme activity, lowering the pH of the soil solution and increasing the solubility of Fe^{3+}, and/or increasing the root ferric-chelate reductase (FCR) enzyme activity to reduce Fe^{3+} to Fe^{2+} (Granja and Covarrubias 2018). In addition, Fe deficiency-tolerant genotypes react to Fe scarcity by increasing the concentration of some organic acids (mainly citric and malic acid) in root cell and xylem sap (Covarrubias and Rombolà 2015; Covarrubias et al. 2016) and the root exudation of phenolic compounds in *Arabidopsis thaliana* (Fourcroy et al. 2015). Considering the mechanisms by which phenolic compounds can regulate the mobility of Fe in the rhizosphere, it is widely accepted that the Fe mobilization capacity of these root exudates is associated with the formation of Fe^{3+} complexes with catechols (Fourcroy et al. 2015). In the case of blueberry, a highly Fe chlorosis-sensitive species, a low-FCR activity was recorded in plants submitted to Fe deficiency (Poonnachit and Darnell 2004), and to our knowledge, none of the other abovementioned mechanisms have been previously identified in any commercial blueberry genotype. Possibly, the absence of these response mechanisms to Fe deficiency in blueberry is linked to its origin in wetland areas, which are characterized by acidic soils with high organic matter and Fe^{2+} concentrations, conditions, all of them, favorable to Fe uptake (Poonnachit and Darnell 2004).

In blueberry orchards located in calcareous soils, growers usually try to solve Fe deficiency by acidifying the soil with inorganic acids, such as sulfuric acid, applied through irrigation. However, this management technique is hazardous and difficult for farm operators to use, and repeated applications may be detrimental to microbial biomass and mycorrhizal fungi populations. Moreover, sulfuric acid reacts with calcium carbonate and sodium bicarbonates, which are often abundant in these soils, increasing the CO_2 emissions from the soil to the environment. On the other hand, the prevention/cure of Fe chlorosis with Fe chelates is a widespread agronomical practice in orchards (Bastani et al. 2018). Nevertheless, such an approach involves high costs and environmental and health risks (Granja and Covarrubias 2018). Iron chelates are expensive (representing 60% of total fertilization costs in orchards located in calcareous soils) and, therefore, their use is economically justified only for extremely high-value crops (Granja and Covarrubias 2018). In addition, Fe chelates require repeated applications and, due to their high stability and solubility, increase the risk of metal leaching and chelating agents in the deep soil layers and in the water table (Granja and Covarrubias 2018). In this sense, the use of natural Fe sources can represent an economic and sustainable strategy to control Fe deficiency as compared to synthetic Fe chelates.

Formulations derived from animal blood compounds are widely considered as nitrogen (N) fertilizers; however, these products also contain a high-Fe concentration (20–30 g Fe kg^{-1}) chelated by a heme group related to hemoglobin (López-Rayo et al. 2015). Innovative formulations based on fluid bovine blood (Fe 0.125%; N 5%) have been previously investigated, demonstrating high stability and ability to maintain Fe available for plants in calcareous soils (Yunta et al. 2013). In different graft/rootstock combinations and micropropagated rootstocks of grapevine, the addition of Fe-heme to the soil increased leaf chlorophyll content with effectiveness similar to Fe chelates (López-Rayo et al. 2015). This evidence suggests that the use of these natural formulations may be an interesting and sustainable alternative to overcome Fe chlorosis in blueberry orchards located in calcareous soils.

On the other hand, several studies have shown that the intercropping of kiwifruit, citrus, grapevine, and olive with graminaceous species contributes to preventing Fe chlorosis with similar effectiveness to synthetic Fe chelates (Ammari and Rombolà 2010; Cañasveras et al. 2014; Covarrubias et al. 2014). The improvement of Fe nutrition in intercropped fruit tree crops induced by intercropping with grasses as *Festuca rubra*, *Poa pratensis*, *Hordeum vulgare*, and *Brachypodium distachyon*, is partially due to their abilities to exudate mugineic acid family phytosiderophores (MAs), which are chelating compounds characterized by a high affinity with Fe^{+3}, promoting Fe solubility in alkaline soil pH conditions (≥ 7.0) (Ma et al. 2003; Xiong et al. 2013). It has been shown that Fe chlorosis prevention depends on the graminaceous species used in the intercropping system, with some species being highly effective, whereas others are not very effective or even ineffective (Ammari and Rombolà 2010). It has been suggested that such varied behavior is mainly due to the effectiveness of grasses to secrete phytosiderophores into the rhizosphere and keep them stable and mobile in the soil, among other factors. Some authors described that the main phytosiderophore secreted by *Festuca rubra* is 2′-deoxymugineic acid (DMA), whereas the graminaceous species *Poa pratensis* exudes DMA, avenic acid A (AVA) and 2'hydroxyavenic acid A (HAVA) (Ma et al. 2003; Ueno et al. 2007). Such evidences indicate that crop systems

adopting intercropping with grasses properly selected for phytosiderophore release may represent a sustainable tool for controlling Fe chlorosis, avoiding or reducing Fe chelates applications. However, these aspects have scarcely been investigated in a calcifuge crop like blueberry and certainly deserve further efforts for scientific, practical, and environmental reasons.

The present investigation endeavors to evaluate the effectiveness of sustainable management techniques to control Fe chlorosis, based on Fe-heme applications or intercropping with graminaceous species, on vegetative, productive, and physiological variables in blueberry cv. Emerald cultivated in a sub-alkaline soil.

2 Materials and Methods

2.1 Plant Material, Experimental Conditions, and Treatments

The experiment was undertaken from May 2014 to April 2015 (2014–2015 season) in a blueberry orchard located in the Valparaiso Region, Chile (32° 42′ S and 70° 54′ W) in an alluvial soil, classified as Aeric Calciaquolls (Gleyic Kastanozems). The field soil was composed of calcium carbonates 4% and active lime 2%, which provided sufficiently favorable chemical conditions to induce Fe chlorosis in the cultivated blueberries (Table 1). The climate in the field area is of local steppe and a few rainfalls occur during winter (278 mm per year on average). During the spring-summer season, the maximum temperature reached 36.3 °C, whereas the minimum temperature was 12.8 °C.

The trial was conducted on highbush blueberries cv. Emerald (interspecific origin based largely on *Vaccinium corymbosum* L. with some genes from *Vaccinium darrowi* Camp) planted in September 2011 at a distance of 3 m between the rows and 0.8 m between plants along the row (4166 plants ha^{-1}). The fertilization management in the orchard consists of ammonium nitrate, potassium nitrate, and phosphoric acid applications through the irrigation system in variable doses according to the plants yield on each season. The phytosanitary control is conventional since the experimental orchard does not apply to organic production guidelines. At the beginning of the experiment, the plants evidenced clear chlorosis symptoms in young leaves. To corroborate that the observed symptoms were due to Fe deficiency, the greenness intensity of two leaves in ten randomly selected plants was measured using a SPAD portable greenness meter (SPAD Minolta 502, Konica Minolta, Osaka, Japan) before and after 10 days of a foliar application of Fe-EDTA 2 mM and pH 6.0 solution. The results showed that the foliar treatment with the Fe solution significantly increased leaf greenness, indicating that the cause of the leaf chlorosis was Fe deficiency.

Table 1 Chemical properties of the soil utilized in the experiment

Fertility		Unit	Value
pH (water, ratio 1:2, 5)		1:2.5	7.7
Electric conductivity (in extract)		dS/m	3.3
Organic matter		%	1.1
Available nitrogen (N)		mg/kg	20
Available phosphorus (P)		mg/kg	22
Available potassium (K)		mg/kg	122
Exchangeable cations			
Calcium (Ca)		cmol+/kg	21.1
		% CEC	70
Magnesium (Mg)		cmol+/kg	4.1
		% CEC	14
Potassium (K)		cmol+/kg	0.31
		% CEC	1.0
Sodium (Na)		cmol+/kg	0.29
		% CEC	0.9
CEC (cation exchange capacity)		cmol+/kg	30.3
Available microelements			
Iron	(Fe)	mg/kg	15.3
Manganese	(Mn)	mg/kg	47.2
Zinc	(Zn)	mg/kg	19.3
Copper	(Cu)	mg/kg	23.9
Boron	(B)	mg/kg	1.0
Carbonates			
Total carbonates	$CaCO_3$%		4.0
Active lime	$CaCO_3$%		2.0

*For available microelements and exchangeable cations determinations, DTPA and ammonium acetate were used as extractants, respectively

In autumn 2014, five contiguous rows homogeneous in size and Fe chlorosis degree in plants were selected with no apparent phytosanitary problems. The treatments tested were: (1) control, bare soil; (2) soil-applied Fe-ethylenediamine-N,N′-bis(2-hydroxyphenyl)acetic acid (Fe-EDDHA) chelate; (3) soil-applied bovine blood compound (Fe-heme); (4) intercropping with *Festuca rubra* (graminaceous species), and (5) intercropping with *Poa pratensis* L. (graminaceous species). A Latin square design (5 × 5) was used to take into account the slope and drip line as possible independent sources of variance, so each treatment was replicated five times. The experimental plot for each treatment was composed of six plants, with similar Fe chlorosis (SPAD value) and vegetative development. The treatments along the same row were separated by two plants between them.

The Fe chelate was applied to the soil from August 2014 according to the SPAD value in order to maintain an intensive green color in the leaves (SPAD index > 35). Doses of 500 mL per plant of a 5% Fe-EDDHA solution (4 g Fe-EDDHA L^{-1}) were occasionally applied to the soil, reaching 1 g of Fe applied per plant at the end of the season (autumn 2014). The Fe-

heme was applied through a dried bovine blood formulation composed of 2675 mg Fe kg^{-1}. Bovine blood was diluted in distilled water to a concentration of 20 g L^{-1} and applied to the soil at doses of 500 mL per plant, every 15 days, reaching 0.27 g of Fe applied per plant at the end of the season. The Fe-EDDHA and Fe-heme applications were carried out manually, from buds break beginning (spring 2014) to the summer flush growth. The graminaceous species *Festuca rubra rubra* L. and *Poa pratensis* L were sown over the rows in autumn 2014 at a density of 20,000 seeds m^{-2}. During the season, the graminaceous species were cut manually to a height of 5 cm every time they reached 15 cm. The plants were irrigated daily through two 2 L h^{-1} in-line microdrip emitters, maintaining a constant soil moisture level, close to field capacity (40% saturation). The soil water content was daily measured using one tensiometer per experimental plot. In intercropped plants, an additional water supply was added in order to maintain similar soil moisture among treatments, according to the tensiometers records. Weeds were removed manually and pest and disease protection was regularly carried out. The agronomic management of pruning, phytosanitary control, and fertilization with macro and micronutrients, except for Fe, were performed regularly throughout the season.

2.2 Leaf Chlorophyll Concentration and Shoots Length

Leaf chlorophyll concentration was measured every 15 days during the experiment on five points of the first completely expanded leaf of 12 shoots per experimental unit (two shoots per plant) using the SPAD meter. The SPAD value was previously calibrated with leaf chlorophyll concentration (R^2 = 0.97; $p < 0.0001$; data not reported) in 26 leaves with different degrees of chlorosis according to Wellburn (1994).

During the season, the length of the same shoots selected for the chlorophyll determinations was determined every 15 days. During the spring flush growth, the length of 12 shoots in each experimental unit was determined, whereas, during the summer flush growth, the length of all the lateral shoot branches from the spring shoots was measured.

2.3 Leaf Mineral Concentrations

In February 2015, at 175 days of treatment, samples of 100 healthy and fully expanded leaves were collected from each experimental unit. The leaves were collected from the middle third of shoots developed during the season and without berries. The samples were oven-dried at 75 °C, ground to pass a 40-mesh screen, and 200 mg of each sample were submitted to acid digestion with nitric acid 8 mL and hydrogen peroxide 2 mL at 180 °C per 20 min in a Microwave Reaction System (Multiwave PRO, Anton Paar 3200, Austria). The concentration of P, K, Ca, Mg, Fe, Mn, Zn, B, and Cu was determined by Microwave Plasma Atomic Emission Spectroscopy MP-AES 4200 (Agilent Technologies, USA). Total leaf N concentrations were determined by the Kjeldahl method.

2.4 Leaf Gas Exchange

Leaf gas exchange was measured on one plant per replicate using an infrared gas analyzer (IRGA, LCi-ADC, London, United Kingdom). During the experiment, measurements were performed on the first mature leaf inserted in the middle third of one shoot per plant. Net photosynthesis (μmol CO_2 m^{-2} s^{-1}) was measured when foliar CO_2 uptake was steady. Measurements were taken at 9:00–10:30 a.m., 11:30 a.m.−12:30 p.m., 1:30–2:30 p.m., and 3:30–4:30 p.m. at the beginning (66 days after bud break DABB) and the end (123 DABB) of harvest. In addition, the total leaf carbon fixed during the day was calculated by integrating the area under the curve from net photosynthesis data.

2.5 Crop Load, Fruit Weight, and Plant Production

The fruit was harvested when berries reached full color on their skins (marketable criteria). In total, the harvest was executed in five different days, according to the fruit maturation rate. At each harvest, the fruit number and yield per plant were recorded for six plants in each replicate. At the end of the season, the total yield and fruit number recorded at each harvest were determined and the average berry fresh weight was calculated.

3 Statistics

The analysis of variance (ANOVA) was performed under the framework of mixed linear models (MLM). Data related to chlorophyll concentration, shoot growth, and leaf gas exchange were analyzed, considering possible temporal correlations, using the function "lme" from the package "nlme" in *R*. In case of significant differences between treatments, in measurements with temporal correlations, the multiple comparisons DGC test ($\alpha = 0.05$) was used. For yield components and leaf mineral concentrations, Fisher's LSD test for multiple comparisons was used ($\alpha = 0.05$). The statistical software used was InfoStat v. 2013.

4 Results

4.1 Leaf Chlorophyll Concentration and Shoots Length

The treatments assessed in the present experiment influenced the leaf chlorophyll concentration from 71 DABB (Fig. 1). During the season, the plants treated with Fe-EDDHA and

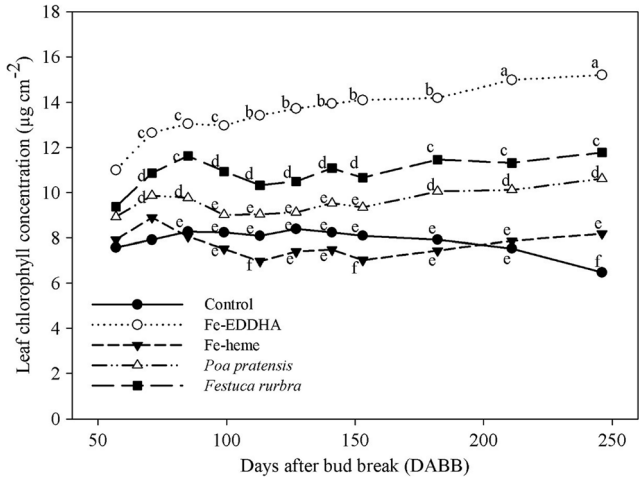

Fig. 1 Leaf chlorophyll concentration during the season in 'Emerald' blueberries under different treatments to prevent Fe deficiency. Adjusted means with different letters indicate significant differences according to DGC test ($p < 0.05$)

those intercropped with grasses showed a higher leaf chlorophyll concentration than the Fe-heme and control plants. Between intercropping treatments, the association with *Festuca rubra* was a more effective treatment to increase the leaf chlorophyll concentration than with *Poa pratensis*.

The shoot growth (centimeters) of plants lasted for 245 days (Fig. 2). During the spring growth flush, the treatments did not significantly influence shoot length. Later, in the summer growth flush, data obtained revealed that, from 182 DABB, the highest shoot growth was recorded in plants treated with Fe-heme in comparison with the other treatments (Fig. 2), whereas the intercropping with *Festuca rubra* reduced the shoot length in comparison with plants treated with Fe-EDDHA intercropped with *Poa pratensis* and the control.

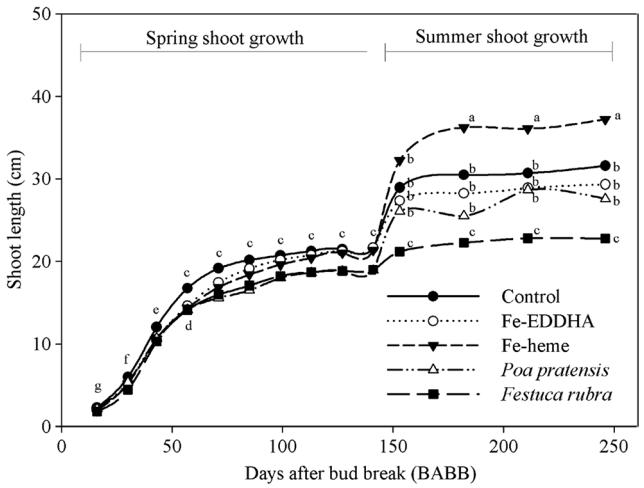

Fig. 2 Shoots length during the season of 'Emerald' blueberries under different treatments to prevent Fe deficiency. Adjusted means with different letters indicate significant differences according to DGC test ($p < 0.05$)

4.2 Leaf Mineral Concentrations

Data obtained showed changes in the leaf mineral concentrations in blueberries according to the treatments. The application of Fe-heme and Fe-EDDHA significantly increased the leaf N concentration in comparison with the control (Table 2). On the other hand, the association of blueberries with *Festuca rubra* reduced the N concentration in leaves, whereas the association with *Poa pratensis* had no influence on this variable. Regarding the leaf P and K levels, the treatments based on Fe-heme applications and *Poa pratensis* did not influence these concentrations, whereas lower values were recorded in plants intercropped with *Festuca rubra* and treated with Fe-EDDHA than in the control. Treatments did not influence the concentration of Ca and Mg in leaves.

Concerning the effect of treatments on micronutrient concentrations, the application of Fe-heme increased the Mn concentration in comparison with control plants, whereas the application of Fe-EDDHA significantly reduced it (Table 3). The association with grasses did not influence the leaf Mn concentration. No significant differences were recorded between treatments in Fe, Zn, and Cu concentrations in leaves.

4.3 Leaf Gas Exchange

At 66 and 123 DABB, blueberries reached the highest net photosynthesis activity between 9:30 and 10:30 a.m. and this variable gradually decreased until reaching the lowest values at 3:30–4:30 p.m. (Fig. 3a, c).

At the beginning of the harvest (66 DABB), data indicate that Fe-EDDHA and intercropping with both grasses increased the leaf net photosynthesis and, consequently, the total leaf carbon fixed during the day as compared to Fe-heme and the control (Fig. 3a, b). After the harvest (123 DABB), a similar trend was recorded for these variables (Fig. 3c, d).

4.4 Crop Load, Fruit Weight, and Plant Production

The sustainable management techniques assessed in our experiment significantly influenced some fruit traits. The association with both graminaceous species and the Fe-EDDHA applications increased the crop load as compared to the control plants (Table 4), and a higher crop load was recorded in plants intercropped with *Festuca rubra* than in those fertilized with Fe-EDDHA (Table 4). Fertilization with Fe-heme did not modify the crop load in comparison with the control. In addition, fertilization with Fe-EDDHA significantly increased the fruit weight compared to the plants treated with Fe-heme, whereas the plants associated with both grasses and control plants showed intermediate values, with no statistically significant differences among them (Table 4). In blueberries associated with the two grasses and in those treated with Fe-EDDHA, a higher yield per plant was recorded than in those

Table 2 Leaf concentration (%) of macroelements (N, P, K, Ca, Mg) in 'Emerald' blueberries under different treatments to prevent Fe deficiency. Sampling was carried out during the summer 2015

Treatment	Nitrogen (%)	Phosphorus (%)	Potassium (%)	Calcium (%)	Magnesium (%)
Control	1.61 ± 0.04 c	0.14 ± 0.01 ab	0.67 ± 0.03 ab	0.83 ± 0.06	0.17 ± 0.02
Fe-EDDHA	1.66 ± 0.05 b	0.12 ± 0.01 c	0.49 ± 0.03 c	0.67 ± 0.06	0.16 ± 0.02
Fe-heme	1.79 ± 0.06 a	0.15 ± 0.01 a	0.68 ± 0.03 a	0.87 ± 0.06	0.18 ± 0.02
Poa pratensis	1.60 ± 0.04 cd	0.13 ± 0.01 bc	0.58 ± 0.03 bc	0.81 ± 0.06	0.17 ± 0.02
Festuca rubra	1.57 ± 0.05 d	0.12 ± 0.01 c	0.49 ± 0.03 c	0.80 ± 0.06	0.19 ± 0.02
Significance	$p < 0.0001$	$p = 0.0004$	$p < 0.0004$	n.s.	n.s.

In each column the adjusted mean ± standard error is presented. Adjusted means with different letters in the same column indicate significant differences between treatments, according to Fisher LSD test ($p < 0.05$)

treated with Fe-heme, whereas the control plants showed an intermediate production between the treatments (Table 4).

The association with both graminaceous species influenced the fruit-ripening rate, increasing the proportion of berries collected in the first two harvest dates as compared with the other treatments, whereas in the third harvest date, the contrary result has been recorded (Fig. 4). No differences among treatments were recorded in the last two harvest dates.

5 Discussion

Our results reveal that the soil chemical properties of the experimental field clearly induced Fe deficiency in the blueberries, which is verified by the positive response related to leaf chlorophyll concentration shown by the blueberries treated with Fe-EDDHA as compared with the control plants (Fig. 1). In fact, Fe chlorosis symptoms similar to those observed in the control plants have been previously reported for other fruit crops subjected to Fe deficiency, such as grapevine, pear, peach, citrus, kiwifruit, and olive, and these were attributed mainly to a reduction in chlorophylls and carotene synthesis, in particular, of neoxanthin, β-carotene, and chlorophyll *a* (Bashir et al. 2015). Such evidences indicate that the leaf chlorosis manifested by the control plants was caused by Fe deficiency and confirm the high effectiveness of Fe-EDDHA applied to the soil as a treatment to correct Fe chlorosis, in this case in blueberry, a calcifuge species. Indeed, Fe-EDDHA has been extensively employed in several investigations as a standard treatment for contrasting with other Fe-rich fertilizer formulations (López-Rayo et al. 2015).

At the end of the season, the blueberries treated with Fe-heme showed a higher shoot length in comparison with the control and the other treatments (Fig. 2). Such an effect induced by bovine blood could be related to the additional supplement of minerals contained in the formulation linked with shoot elongation in plants, such as Fe (2675 mg kg^{-1}), Zn (15.8 mg kg^{-1}), S (0.49%), and particularly N (14.4%). Also, Fe-heme is a source of organic matter, which gives significant benefits in some physical properties of the soil (Seguel et al. 2013). However, the effect of Fe-heme on the shoot growth strongly contrasts with the low leaf chlorophyll concentrations registered in the plants, which was similar to those recorded in the control (Fig. 1). This suggests that the Fe supply by bovine blood applications was probably not enough to maintain a leaf chlorophyll synthesis according to the shoot growth rate. In fact, the Fe dose per plant supplied through the bovine blood applications during the season (0.27 g) was significantly lower than that supplemented by Fe-EDDHA (1 g). It is possible, however, that the chemical soil properties of the field used in our experiment negatively impacted on the effectiveness of the Fe-heme treatment. In an experiment conducted on grapevine, positive effects of bovine blood applications on plants growth and leaf chlorophyll concentration were recorded in plants cultivated in hydroponic conditions, whereas no effects were registered in plants cultivated in pots filled with calcareous soil (López-Rayo et al. 2015). In addition, a high reactivity (Yunta et al. 2013) and interaction of Fe-heme

Table 3 Leaf concentration (mg kg-1) of microelements (Fe, Mn, Zn, and Cu) in 'Emerald' blueberries under different treatments to prevent Fe deficiency. Sampling was carried out during the summer 2015

Treatment	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	Cu (mg kg^{-1})
Control	122 ± 14.3	95 ± 7.0 b	17 ± 2.5	17 ± 1.0
Fe-EDDHA	128 ± 14.3	75 ± 5.4 c	15 ± 2.0	17 ± 1.1
Fe-heme	126 ± 14.3	140 ± 10.9 a	15 ± 2.0	18 ± 1.1
Poa pratensis	117 ± 14.3	100 ± 7.2 b	18 ± 2.8	18 ± 1.1
Festuca rubra	101 ± 14.3	101 ± 7.1 b	18 ± 2.9	16 ± 1.1
Significance	n.s.	$p = 0.0001$	n.s.	n.s.

In each column the adjusted mean ± standard error is presented. Adjusted means with different letters in the same column indicate significant differences between treatments, according to Fisher LSD test ($p < 0.05$)

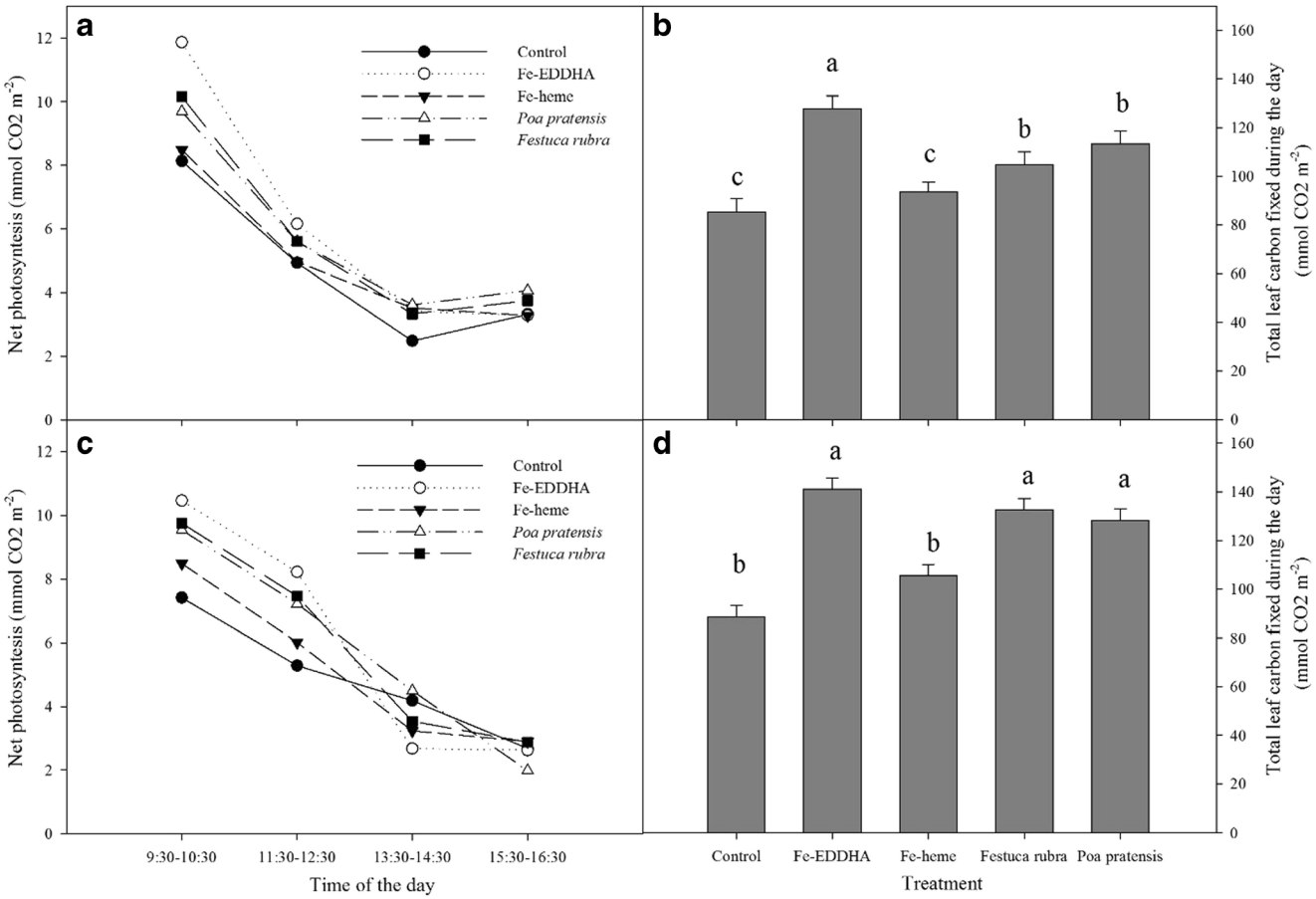

Fig. 3 Daily evolution of net photosynthesis (A, C), and total leaf carbon fixed during the day (B, D) in 'Emerald' blueberries under different treatments to prevent Fe deficiency at 66 DABB (A, B) and 123 DABB (C, D). Adjusted means with different letters between treatments indicate significant differences according to DGC test ($p < 0.05$). Vertical bars indicate the standard error

with soil microorganisms have been reported (López-Rayo et al. 2015) in soils characterized by high Fe oxides and hydroxides and a low Ca concentration. These soil biochemical processes probably occurred in our experiment, reducing the effectiveness of Fe-heme as a ferric fertilizer. Nevertheless, this strategy should not be discarded as a possible sustainable alternative for controlling Fe deficiency in other field-grown fruit crops, since its effectiveness has been demonstrated in species such as grapevine (López-Rayo et al. 2015) and pear (Sorrenti et al. 2012). It is possible that some experimental conditions such as the Fe-heme source, the application mode, and the Fe-heme dose applied in the present study will need to be adjusted to improve the ferric nutrition in plants.

Conversely, the presence of both graminaceous species on the row significantly increased the leaf chlorophyll concentration of blueberries compared to the control (Fig. 1). Between the two grasses assessed, the association with *Festuca rubra* was slightly more effective at increasing the leaf chlorophyll content, which is in line with the results obtained from an experiment conducted by Ammari and Rombolà (2010). It

Table 4 Fruit load (fruit plant-1), fruit weight (g), and plant production (g plant-1) at harvest in 'Emerald' blueberries under different treatments to prevent Fe deficiency

Treatment	Crop load (fruits plant^{-1})	Fruit weight (g)	Plant production (g plant^{-1})
Control	585 ± 47 c	1,23 ± 0,05 ab	802 ± 55 bc
Fe-EDDHA	663 ± 53 b	1,35 ± 0,06 a	984 ± 70 ab
Fe-heme	629 ± 47 bc	1,12 ± 0,05 b	770 ± 51 c
Poa pratensis	749 ± 54 ab	1,20 ± 0,06 ab	934 ± 64 ab
Festuca rubra	844 ± 55 a	1,19 ± 0,06 ab	993 ± 66 a
Significance	$p = 0.0033$	$p = 0.0490$	$p = 0.0105$

In each column the adjusted mean ± standard error is presented. Adjusted means with different letters in the same column indicate significant differences between treatments, according to Fisher LSD test ($p < 0.05$)

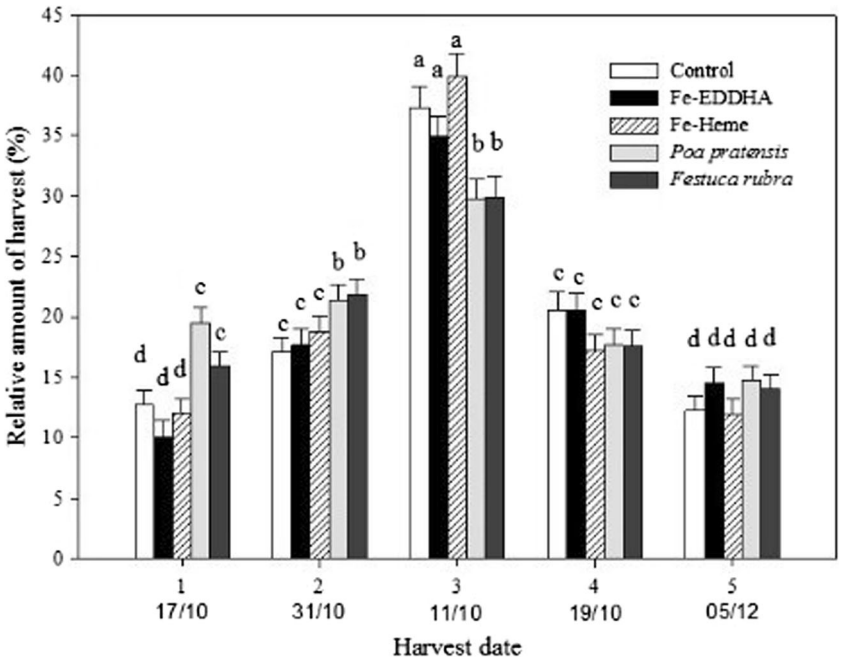

Fig. 4 Harvest distribution in 'Emerald' blueberries under different treatments to prevent Fe deficiency. Adjusted means with different letters per harvest date indicate significant differences according to DGC test ($p < 0.05$). Vertical bars indicate the standard error

has been proposed that, in intercropping systems, the Fe deficiency correction depends on the ability of graminaceous species to release phytosiderophore compounds (Ammari and Rombolà 2010). In this context, the greater effectiveness of *Festuca rubra* to increase the leaf chlorophyll concentration in blueberries could be attributed to its higher root phytosiderophore release rate as compared to *Poa pratensis*.

However, the association with *Festuca rubra* promoted a significant reduction in the shoot length of blueberries in comparison with the control, whereas the association with *Poa pratensis* did not modify this plant growth variable (Fig. 2). This effect has been noted in other studies on intercropping systems (Cañasveras et al. 2014; Covarrubias et al. 2014), and it has been attributed to the strong competition for water and nutrients between some grasses and the main crop. In fact, our results show lower leaf N, P, and K concentrations in blueberries associated with *Festuca rubra* than with the other treatments, whereas this effect was not observed in blueberries associated with *Poa pratensis* (Table 2). In an experiment conducted on grapevines cultivated in calcareous soil, a strong reduction in grapevine biomass induced by the association with *Festuca rubra* was also recorded (Covarrubias et al. 2014). However, contrasting results were observed in citrumelo 'Swingle' and grapevines cultivated in calcareous soil and associated with grasses since no reductions in the growth rate were recorded (Ammari and Rombolà 2010; Bavaresco et al. 2010). These evidences suggest that the effect of graminaceous species on the main crop's growth could vary according to the graminaceous species, water and nutrient availability, sowing density, and soil volume availability, among others. Consequently, our results highlight the importance of adapting strategies focused on controlling the vegetative growth of grasses in an intercropping system in order to reduce the potential competition for water and nutrients with the blueberries that could affect the long-term productivity and orchard life. Furthermore, a possible allelopathic interference induced by the graminaceous species used in our experiment should not be discounted. Allelopathic phenomena can coexist in an ecosystem with competition for resources between two plant species and the magnitude of these is stimulated by the level of plant stress caused by factors such as pests and diseases, extreme temperatures, water restrictions, nutritional deficiencies, and solar radiation (Einhellig 1996). The study of possible allelopathic effects induced by different grasses on the associated crop is an intriguing line of research that should be considered in future experiments.

It is important to note that, in spite of the clear influence of treatments on the chlorophyll concentration and shoot growth of the blueberry leaf, no differences were found in the leaf Fe concentration (Table 3). It has been well established that the leaf Fe concentration does not represent a reliable and representative index to diagnose the ferric status in plants cultivated in calcareous soils, and this phenomenon has been called the "chlorosis paradox" in the literature (Römheld 2000). On the other hand, the Fe chelates applied to the soil generally has a negative effect on the Mn uptake by plants, which has been observed in several species (Moosavi and Ronaghi 2010) and in our investigation (Table 3). In this context, the application of Fe-heme in crops cultivated in calcareous soils may be a better strategy for maintaining the plant's Fe and Mn nutritional balance than the application of Fe-EDDHA.

It is clear from our results that those treatments resulting in higher leaf chlorophyll concentration, such as intercropping with *Festuca rubra* and *Poa pratensis*, together with Fe-

EDDHA applications, were also capable of increasing the capacity for CO_2 assimilation, before and after the fruit harvest (Figs. 1 and 3). It is well known that Fe nutrition is closely linked to the photosynthetic capacity of plants since it is involved in the synthesis of cytochromes, the electron transport system, the construction of Fe-S clusters, and the synthesis of chlorophyll molecules (Briat et al. 2015). Therefore, a poor Fe status in crop plants has been suggested as a serious limitation to productivity (Briat et al. 2015). Not surprisingly, blueberries grown in association with graminaceous species and those treated with Fe-EDDHA, achieved the largest crop load, fruit weight, and plant production per plant in comparison with the plants treated with Fe-heme and the control (Table 4). Even though the Emerald cultivar is considered a southern highbush blueberry (Retamales and Hancock 2012), meaning it evolves in a climate with hot summers, it seemed that the experimental site was restrictive for the plants, with their photosynthetic activity declining throughout the day (Fig. 3a, c). In this sense, Fe deficiency, further affecting the photosynthetic capacity, is expected to have a strong effect on fruit yield in commercial orchards.

It must be emphasized that there was apparently a negative correlation between the leaf chlorophyll concentration, photosynthetic activity, and yield components compared to shoot length (Figs. 1 and 3 and Table 4). Blueberries have been shown to be highly sensitive to the sink-source balance (Jorquera-Fontena et al. 2014) and the higher shoot growth observed overall for the Fe-heme-treated plants (Fig. 2) could be the result of a reduced crop load, caused by a more severe Fe deficiency. Also, and particularly for the Fe-heme-treated plants, the higher shoot growth observed might be associated with its more abundant N content (Table 2) which, in turn, is known to mediate carbon allocation to vegetative growth through sucrose and cytokinins (van der Werf and Nagel 1996). Interestingly, even after the fruit removal, the blueberries associated with graminaceous species showed a lower shoot growth rate than the other treatments despite maintaining a high leaf photosynthesis rate. This may face address the water and nutrient stress induced by the grass. The behavior described likely occurred to a lesser extent in the blueberries associated with *Poa pratensis*, which is apparently a less competitive graminaceous species than *Festuca rubra*.

Likewise, our results reveal a significant effect of intercropping with both grasses on the berry ripening rate, leading a higher fraction of fruits collected in the first two harvest days. Increases in berry sugar accumulation rate and skin anthocyanin concentrations have been also observed in grapes submitted to controlled water stress management after veraison (Deluc et al. 2009). In addition, some authors have been reported that water and/or some nutrients deficit accelerates berry ripening and induces changes in the expression of genes that regulate flavonoid biosynthesis in berries (Castellarin et al. 2007). However, although these evidences contribute to confirm that grasses employed could have induced water and/or nutrient stress in the associated blueberries, despite the additional supplement they received during the season, the fruit ripening acceleration in plants could lead to some positive commercial and agronomic aspects for growers related to the period of fruit availability in the market.

6 Conclusions

The results obtained indicate that the application of Fe-heme increases the shoot length, possibly as a consequence of the additional nutrient supply to the plants, without modifying the leaf chlorophyll concentration, carbon fixation, and the yield components in comparison with the control. On the other hand, the association with the graminaceous species utilized in this experiment increases the leaf chlorophyll concentration, the photosynthetic activity, and the yield components per plant, with an effect similar to the Fe-EDDHA treatment. However, this management technique maintains or reduces shoot growth according to the associated graminaceous species and may reduce the leaf N, P, and K concentrations in comparison with the control. Data obtained suggest that intercropping with graminaceous species in blueberry orchards may be an effective and sustainable strategy to control Fe deficiency, whereas the use of Fe-heme requires further studies to improve its efficiency. In future investigations, it will be necessary to select new grasses characterized by a low water and nutrient consumption in order to optimize the efficiency of this management technique to improve the nutritional status of blueberries.

Funding information This work was supported by the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) of Chile (FONDECYT project no. 11130328).

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