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Research article

Effectiveness of synthetic calcite doped with Fe-EDDHSA as a slow-release Fe source: *In-vitro* experiment on kiwifruit (*Actinidia chinensis* var. *deliciosa*) plants

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Abstract: Doped calcite (Fe-EDDHSA/$CaCO_3$) was experimentally produced. The hypothesis of the present experiment is that, when roots get in contact with Fe-EDDHSA/$CaCO_3$, the extrusion of H^+ decreases the pH and dissolves calcite with subsequent release of Fe that becomes available for roots. The aim of the experiment was to determine whether doped calcite might represent a slow-release Fe source for *in-vitro* grown kiwifruit plantlets.

The root elongation media used in the experiment had pH 8.0 and differed from each other for Fe supply as follow: Control medium that contained complete Murashige and Skoog salt mixture, including $FeSO_4$ and Na_2EDTA; calcite medium enriched with Fe-EDDHSA/$CaCO_3$ as the only Fe source; −Fe medium without Fe.

The absence of $FeSO_4$ in the medium caused a reduction of plantlet growth. The final pH was higher with calcite medium than in control and −Fe. The addition of Fe-EDDHSA/$CaCO_3$ increased Fe shoot concentration when compared with the −Fe medium. The data of the present experiment show the potential Fe slow release ability of Fe-EDDHSA/$CaCO_3$; however, further investigation on Fe containing fertilizers should be conducted on potted plants to validate our results.

Keywords: Fe-chelates; *in-vitro* culture; pH; alkaline growing media; EDDHSA/$CaCO_3$

1. Introduction

Lime-induced iron (Fe) chlorosis is a frequent issue of several fruit plant species; in Italy, many orchards are located on calcareous or alkaline soils, where the reduced Fe availability impairs fruit yield and quality [1]. The high pH of these soils causes Fe precipitation as hydroxides (i.e. $Fe(OH)_2$ and $Fe(OH)_3$ [2]) and the reduction of the activity of Fe-chelate-reductase at the cell membranes of the root cortical zone. This enzyme is responsible for the reduction of Fe^{3+} to Fe^{2+}; the latter is the form that roots of plants characterized by "strategy I" can uptake [3]. These plants respond to Fe deficiency with protons (H^+) excretion in order to reduce soil pH to around 6.5 and activate the enzyme [4]. However, this strategy is not sufficient to counteract the effect of calcareous or alkaline soils. To prevent the occurrence of Fe deficiency, growers often apply synthetic Fe-chelates to soil that, however, are expensive and can cause pollution of ground water due to their high water solubility [1,5]. Therefore, alternative means for controlling the Fe chlorosis should be developed to reduce cost and improve environmental sustainability.

Recent research in medicine [6,7] developed drug carriers able to release specific molecules into the systemic circulation of the body. Analogous systems for programmed release of nutrients in soils could be of relevant interest in agriculture [8] and contribute to reduce the impact of fertilization on the environment. Fertilizers carriers have the ability of slowly releasing a specific molecule upon a stimulus (pH, temperature, light).

Plant tissue culture techniques allow a rigorous monitoring and precise manipulation of plant growth and development. *In vitro* experiments proved to be a useful mean for rapid evaluation of plant responses to different substances [9] and to biotic and abiotic stresses [10,11]. Previous experiments [12,13] demonstrated that plants grown *in vitro* and *in vivo* had similar behaviors. In particular, some quince BA 29 [14] and quince A [15] somaclones, that were tolerant to *in-vitro* Fe deficiency conditions, also showed good tolerance to Fe chlorosis in hydroponic culture and in the greenhouse, respectively. Moreover, some leaf-derived kiwifruit somaclones *in vitro* selected for their better growth at high pH, were also moderately tolerant to lime when cultured in pots and/or in the field [16].

Kiwifruit shoot cultures were used as model plant, since this species is sensitive to lime-induced Fe-chlorosis both in fields [5] and *in vitro* at high medium pH [17].

In our preliminary experiment [18] the effect of ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) ferric-sodium complex (Fe-EDDHSA) combined with calcite ($CaCO_3$) was tested on kiwifruit proliferating shoots (without roots) grown on modified Murashige and Skoog (MS) media [19] with pH adjusted to 8. This high pH avoided calcite dissolution. The release of H^+ ions by the cultures caused the decrease of the external pH; consequently, calcite crystals were dissolved and Fe became available for plant uptake. The red colour of phenanthroline added to the medium evidenced Fe^{2+} release. Moreover, comparative SEM images of Fe-EDDHSA/$CaCO_3$ crystals before inclusion in the culture medium and near the shoot bases proved crystals dissolution during culture. Therefore, the increased Fe availability improved shoot growth compared to a culture medium lacking Fe.

To evaluate the effect of roots on calcite crystals, the present experiment was conducted to test the hypothesis that, when roots get in contact with Fe-EDDHSA/$CaCO_3$, the extrusion of H^+ decreases the pH and dissolves calcite with release of Fe, which becomes available for plant uptake.

If the results of the previous experiment on proliferating shoots [18] are here confirmed, this will reinforce the hypothesis that synthetic calcite could act, in sub-alkaline soils, as a carrier and stable source of Fe until it is absorbed by plants.

2. Materials and methods

2.1. Donor cultures, standard growth conditions and rooting procedures

As previously reported [18], donor *in-vitro* shoot cultures of kiwifruit [*Actinidia chinensis* var. *deliciosa* (A. Chev.) A.Chev.] cv. Hayward, were maintained through repeated 5 week subcultures on a modified MS [19] shoot multiplication medium. Four shoots were grown in 500 mL glass jars, each containing 40 mL of medium; jars were closed with twist-off screw metal caps and wrapped with polyvinyl chloride (PVC) transparent film (allowing gas exchange). Standard growth conditions were: 22 ± 2 °C, 16-h-photoperiod at 30 μmol m^{-2} s^{-1} photosynthetic active radiation (supplied by Philips TLD 36 W/33 lamps).

A two-step rooting procedure was used providing a root-induction and a root-elongation phase. At the end of the maintenance subculture about 20 mm well developed shoots were dissected and transplanted on a root induction medium (in 250 mL jars, with 30 mL medium and two shoots each) and placed five days in complete darkness, at 22 ± 2 °C. The root induction medium had the same composition of the multiplication medium but the addition of 2.5 μM indole-3-butyric acid (IBA) as the only plant growth regulator. Afterward, two shoots were transplanted in each jar on root-elongation media with the same basal composition as the induction medium but lacking IBA and added with different Fe source as reported below. Plantlets were grown for additional 50 days under standard growth conditions. The jars were closed as reported above in both rooting phases.

2.2. Fe-EDDHSA/$CaCO_3$ crystals

Calcite crystals entrapping Fe-EDDHSA were obtained from a controlled precipitation process of solutions of $CaCl_2$ and Fe-EDDHSA mixed with sodium carbonate [18,20]. The obtained Fe-EDDHSA/$CaCO_3$ hybrid crystals are stable at sub-alkaline pH values, while at sub-acid pH they dissolve releasing the Fe-EDDHSA complex. The maximum concentration of Fe entrapped within the calcite lattice was 0.616 wt% when the precipitation occurred in the presence of 1.0×10^{-3} M of Fe-EDDHSA [18].

2.3. Experimental conditions

The IBA-induced shoots were randomly transferred to three root-elongation media with pH adjusted to 8.0 and the same basal composition except for Fe, differing as follows: 1) control medium containing complete MS [19] salt mixture; 2) calcite medium enriched with Fe-EDDHSA/$CaCO_3$ as the only Fe source, at the concentration of 1861 mg l^{-1} in order to match Fe content of control medium; 3) −Fe medium without any Fe supply. In detail, control and calcite medium both contained Fe but while control was added with $FeSO_4$ chelated with Na_2EDTA, calcite medium was enriched with Fe-EDDHSA/$CaCO_3$ hybrid crystals providing a slow release source of Fe. Five jars (replicates), each containing two shoots, were used for each treatment.

2.4. Tissue sampling and element analysis

At the end of the rooting phase (55 days), mean fresh (fw) and dry weight (dw) of shoot and roots + callus, the final medium pH and the mineral composition of plants were determined on five jars (replicates) per treatment. Rooting occurred on shoots through the formation of a basal callus before root emergence. Since it was impossible to separate callus from roots, they were weighed together. The culture media were heated to about 50 ℃ in order to obtain agar liquefaction for a better accuracy in pH determination.

Phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), copper (Cu), Fe, manganese (Mn) and zinc (Zn) concentrations were determined by plasma spectrometer (ICP-OES Ametek Spectro, Arcos, Kleve, Germany) on a 0.2 g dry sample previously mineralized (US EPA Methods 3052) [21] in an Etos TC microwave lab station (Milestone, Bergamo, Italy). Element concentration of different plant portions was referred to dw. Organ macro and micronutrients content was calculated by multiplying nutrient concentration by dw; shoot and root-callus nutrient content was summed to calculate total uptake.

2.5. Statistical analysis

All data were subjected to the analysis of variance as in a complete randomized experimental design considering each jar as a replicate; when analysis of variance showed a statistical effect of treatments ($p \leq 0.05$), means were separated by Student Newman Keuls (SNK) test.

3. Results

3.1. Plant biomass and medium pH

The discriminant canonical analysis performed on plant fresh weight (Figure 1) separated the three different media, indicating that control had the highest total plant and roots + callus growth, followed by calcite medium and −Fe (Figure 1). Shoot fresh weight was higher in control plants than in those grown on calcite medium; −Fe showed intermediate values (Figure 1).

Medium pH was significantly ($p \leq 0.001$) decreased by plants in control and −Fe (4.72 and 4.40, respectively), while remained near 7 (7.22) in calcite.

3.2. Nutrient concentration in shoot

The addition of doped calcite increased Ca concentration (Table 1). Phosphorous, K, Mg, S, Cu and Zn concentration was not statistically influenced by different media (Table 1). Iron concentration was highest in control; calcite medium showed intermediate values, lower than control but higher than −Fe (Table 1). Manganese concentration was higher in control than −Fe, calcite induced intermediate values not different from the other media (Table 1).

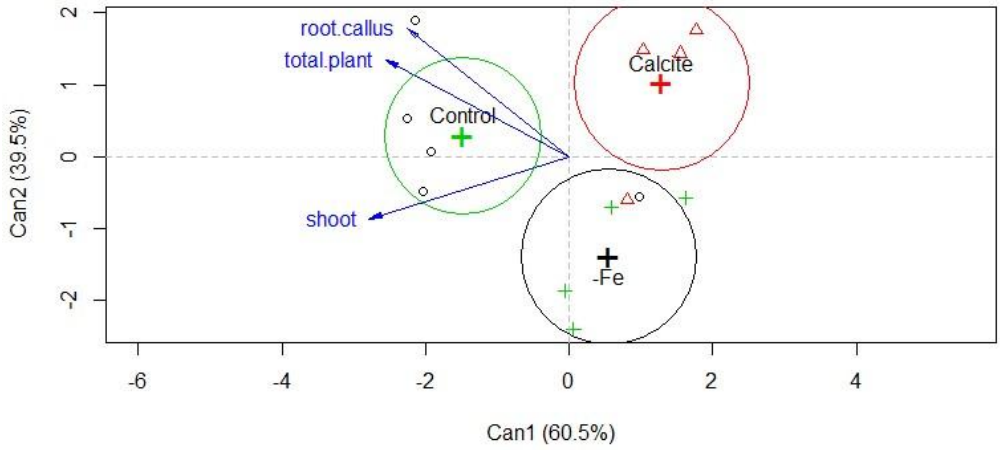

Figure 1. Discriminant canonical analysis on roots + callus, shoot and total plant biomass fresh weight. Control stands for control medium containing complete MS salt mixture; Calcite for calcite medium without $FeSO_4$ and enriched with Fe-EDDHSA@$CaCO_3$; −Fe for medium with any Fe supply. Data refer to five jars (replicates) with two shoots each.

3.3. Nutrient concentration in roots + callus

Potassium concentration was enhanced by Fe, no matter the source (Table 2). Magnesium concentration was higher on calcite medium if compared to −Fe; control showed intermediate values not different from calcite medium and −Fe (Table 2). Iron concentration was higher in control than other media (Table 2). Phosphorous, S, Cu, Mn and Zn concentrations were higher in -Fe than calcite medium and control (Table 2).

3.4. Total nutrient uptake

The total content of K, Mg, S, Cu, Fe and Mn was higher in control plants than in other media that showed comparable values (Table 3). Calcium total uptake was similar for control and calcite medium and higher than −Fe; no significant differences were observed for P and Zn total plant content (Table 3).

Table 1. Effect of the addition of doped calcite (Fe-EDDHSA/$CaCO_3$) on shoot macro and micronutrients concentration at the end of the experiment.

Treatment	P (% dw)	K (% dw)	Ca (% dw)	Mg (% dw)	S (% dw)	Cu (mg kg^{-1} dw)	Fe (mg kg^{-1} dw)	Mn (mg kg^{-1} dw)	Zn (mg kg^{-1} dw)
Control	0.122	1.18	0.236 b	0.076	0.155	1.27	82.3 a	111 a	50.6
Fe-EDDHSA/$CaCO_3$	0.080	1.26	0.463 a	0.072	0.132	0.446	19.6 b	74.4 ab	29.6
−Fe	0.060	0.618	0.077 b	0.032	0.058	0.285	5.45 c	25.8 b	20.1
Significance	*n.s.*	*n.s.*	*	*n.s.*	*n.s.*	*n.s*	*	*	*n.s.*

Within the same column, values followed by the same letter are not statistically different according to Student Newman Keul test ($p \leq 0.05$). n.s., *: Effect not significant or significant at $p \leq 0.05$, respectively. Control = medium containing complete MS salt mixture; Fe-EDDHSA/$CaCO_3$ = calcite medium enriched with 1861 mg l^{-1} Fe-EDDHSA/$CaCO_3$; −Fe = medium without Fe. dw = dry weight. Data are average of five jars (replicates) with two shoots each.

Table 2. Effect of the addition of doped calcite (Fe-EDDHSA/$CaCO_3$) on roots + callus macro and micronutrient concentration at the end of the experiment.

Treatment	P (% dw)	K (% dw)	Ca (% dw)	Mg (% dw)	S (% dw)	Cu (mg kg^{-1} dw)	Fe (mg kg^{-1} dw)	Mn (mg kg^{-1} dw)	Zn (mg kg^{-1} dw)
Control	0.168 b	3.04 a	0.310 b	0.193 ab	0.218 b	1.70 b	262 a	188 b	182 b
Fe-EDDHSA/$CaCO_3$	0.152 b	3.59 a	0.754 a	0.245 a	0.226 b	1.71 b	35.0 b	107 b	157 b
−Fe	0.498 a	2.24 b	0.220 b	0.124 b	0.271 a	2.62 a	25.0 b	387 a	514 a
Significance	*	**	***	*	**	*	***	**	**

Within the same column, values followed by the same letter are not statistically different according to Student Newman Keul test ($p \leq 0.05$). *, **, ***: Effect significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

Control = medium containing complete MS salt mixture; Fe-EDDHSA/$CaCO_3$ = calcite medium enriched with 1861 mg l^{-1} Fe-EDDHSA/$CaCO_3$; −Fe = medium without Fe. dw = dry weight. Data are average of five jars (replicates) with two shoots each.

Table 3. Effect of the addition of doped calcite (Fe-EDDHSA/$CaCO_3$) on total plant macro and micronutrient content at the end of the experiment.

Treatment	P (mg)	K (mg)	Ca (mg)	Mg (mg)	S (mg)	Cu (μg)	Fe (μg)	Mn (μg)	Zn (μg)
Control	0.701	10.6 a	1.33 a	1660 a	0.913 a	0.722 a	87.0 a	73.7 a	58.6
Fe-EDDHSA/$CaCO_3$	0.257	5.65 b	1.38 a	617 b	0.405 b	0.258 b	6.16 b	42.1 b	21.6
−Fe	0.627	2.85 b	0.298 b	366 b	0.338 b	0.311 b	3.15 b	18.4 b	58.6
Significance	*n.s.*	*	**	***	*	*	**	*	*n.s.*

Within the same column, values followed by the same letter are not statistically different according to Student Newman Keul test ($p \leq 0.05$). n.s., *, **, ***: Effect not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Control = medium containing complete MS salt mixture; Fe-EDDHSA/$CaCO_3$ = calcite medium enriched with 1861 mg l^{-1} Fe-EDDHSA/$CaCO_3$; −Fe = medium without Fe. Data are average of five jars (replicates) with two shoots each.

4. Discussion

Auxins in the medium induce the release of H^+ ions from cells and K^+ influx; therefore, the medium pH will decrease while that of the cell sap will rise. Callus growth and root formation are affected by medium pH [22]. The two-step rooting procedure used in the present experiment should prevent the interference of IBA protonic pumps activation of the cell membranes on the potential ability of the shoots to acidify the culture medium. In the present experiment, callus formation at the base of most shoots should be the consequence of a combined effect of residual IBA from root-induction and the initial high pH of root-elongation media, both of them enhancing H^+ secretion, with consequent cell expansion.

As expected, the highest shoot growth was found with the complete MS salt mixture (control); roots + callus growth on calcite medium was higher than −Fe. Plants grown on control also had the highest Fe concentration in shoots, roots and callus. Changes in mineral uptake can strongly affect *in vitro* morphogenesis; in particular, optimal Fe availability was found to improve growth of all types of plant cultures [22]. Iron deficiency during bud formation in strawberry nodal cultures resulted in significant reduction of explant growth [23], as also observed in our experiment, in particular for roots and callus.

On the other hand, the higher shoot Fe concentration on calcite medium than −Fe indicates that plants were able to partially dissolve the $CaCO_3$ hybrid crystals. Since in control and −Fe media pH decreased, a release of H^+ by roots can be supposed. The lower pH reduction of calcite medium (from initial pH 8.0 to 7.22 at the end of the experiment) also suggests a release of H^+, although $CaCO_3$ coming from Fe-EDDHSA/$CaCO_3$ masked plant cell activity toward a decrease of the external pH.

In addition, the dissolution of Fe-EDDHSA/$CaCO_3$ brought about an increase of Ca concentration in the whole plant on calcite medium with respect to the other two media. The higher K concentration in roots + callus of plants grown on control and calcite with respect to −Fe should be the final consequence of the extrusion of H^+ ions and the contemporary penetration of K^+ into the cells characterized by a better growth in the presence of Fe, as a part of cell pH and osmotic balance regulation process [22].

Surprisingly, in roots + callus, P, S, Cu, Mn and Zn concentrations were higher in −Fe than calcite and control. Probably, this was due to the reduced growth in −Fe with consequent lower nutrient dilution.

Control plants showed a higher total K, Mg, S, Cu, Fe and Mn content mainly due to their higher weight. However, the same nutrients except for Cu were higher on calcite medium than in −Fe, although not significant.

High agar concentrations lead to medium hardening that reduce water and nutrients uptake by the cultures and inhibit their growth [22]. Thus, the lower plant growth and nutrient uptake on calcite medium with respect to control could be due to the hardening of the culture medium observed after $CaCO_3$ hybrid crystals addition. Moreover, considering that only the basal part of the explant was in contact with the medium, gradients of nutrients might be formed between the medium and plants tissues. In addition, since a basal callus is produced by shoots before roots development, vascular connections between roots and shoot might be incomplete [22]. Therefore, the presence of callus at the shoot base might be in part responsible of some unclear results on nutrient uptake.

In conclusion, the present research, on *in-vitro* grown entire plants with roots showed that doped calcite, although not being a Fe source as effective as $FeSO_4$ chelated with Na_2EDTA, was able to release Fe for plant uptake. Therefore, the results of this and the previous experiment [18] support the hypothesis that synthetic calcite hybrid crystals could act in sub-alkaline substrates as a carrier and stable source of Fe until it is absorbed by plants, being an environmental friendly alternative to chelates. In contrast, iron chelates, traditionally used in agriculture, are not slow release fertilizers and since they are water soluble they could be leached and pollute ground water with unpredictable effects on the food chain. However, future research should focus on the use of Fe-EDDHSA/$CaCO_3$ crystals under *in-vivo* conditions (potted or field grown plants).

Conflict of interest

All authors declare no conflict of interest in this paper.

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