

## Delivery systems for agriculture: Fe-EDDHSA/CaCO<sub>3</sub> hybrid crystals as adjuvants for prevention of iron chlorosis

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Experimental Section	p. S2
Table S1	p. S5
Figure S1	p. S6
Figure S2	p. S6
Figure S3	p. S7
Figure S4	p. S7
References	p. S8

## Experimental Section

**Synthesis of Fe-EDDH(A, MA or SA)/CaCO<sub>3</sub> hybrid crystals.** Synthetic calcium carbonate composites were grown in a bulk assay at room temperature. In this process 50 ml of a 50 mM Na<sub>2</sub>CO<sub>3</sub> aqueous solution was injected into 50 ml of 50 mM CaCl<sub>2</sub> aqueous solution by using an NE-300 Just Infusion™ syringe Pump with a pumping rate of 50 ml/hour under continuous mechanical stirring. The CaCl<sub>2</sub> aqueous solution contained Fe-EDDH(A, MA or SA) at the concentration of 10<sup>-3</sup>, 10<sup>-4</sup> or 10<sup>-5</sup> M. At the end of the pumping process, the solutions were mechanically stirred for 12 hours in beakers sealed with Parafilm M® to prevent evaporation. Finally the calcium carbonate crystals formed in solution were harvested by filtration, washed with milliQ (18.2 MOhm-cm) water and air-dried. The mixing procedure was carried out at least 10 times for each additive concentration. Only freshly prepared solutions were used. Reference samples for the formation of pure CaCO<sub>3</sub> were prepared from solutions containing no additive. In the following experiments only the Fe-EDDH(A, MA or SA)/CaCO<sub>3</sub> hybrid crystals that were grown from 10<sup>-3</sup> M Fe-EDDH(A, MA or SA) solutions were used.

**Characterization of Fe-EDDH(A, MA or SA)/calcite hybrid crystals.** The optical microscope observations of CaCO<sub>3</sub> precipitates were made with a Leica microscope equipped with a digital camera. The scanning electron microscope (SEM) observations were conducted using a Phenom microscope (FEI) for uncoated samples and a Hitachi FEG 6400 microscope for samples after coating with gold. Iron and calcium ions content of calcium carbonate crystals was determined by plasma spectrometer (Ametek Spectro, Arcos, Kleve, Germany). X-ray powder diffraction patterns were collected using a Panalytical X'Pert MPD diffractometer provided with Cu K $\alpha$  radiation. The diffraction patterns were collected within a 2 $\theta$  range from 20° to 60°. A quantitative analysis of the crystalline phases was performed using the software “Quanto” (see [www.ic.cnr.it/Varie/quantohelp/help/geninfo.htm](http://www.ic.cnr.it/Varie/quantohelp/help/geninfo.htm)), which is based on the Rietveld method. The agreement index GoF was used as a figure of merit of the refinement process, it was lower than 3.15.

Thermal gravimetric analysis of samples was carried out using a TA Instruments SDT 2960, at heating rate of 10 °C/min in nitrogen atmosphere from 30 to 600 °C. Samples weight were 3–5 mg and nitrogen flow rate of 100 ml/min.

***In vitro culture.*** Micropropagation is an *in vitro* vegetative propagation technique, which uses small parts of plant tissues or organs as initial explants, grown in liquid or solid, synthetic substrates, in ideally aseptic and controlled conditions (i.e.: light, photoperiod, temperature). This technique allows the rapid multiplication of stock plant material to produce a large number of plants identical to the mother plant (clones). It can be used to propagate plants that are difficult or even impossible to propagate by traditional methods, and selected plant genotypes obtained through traditional breeding techniques or *in vitro* biotechnological procedures. In addition, this technique does not depend on seasonal changes. The production of plantlets goes through three *in vitro* main steps: preparation of an aseptic culture, shoot multiplication and rooting of the shoots, followed by plantlet acclimatization in the greenhouse (George, 1993). *In vitro* culture has been suggested as a tool for rapid evaluation of plant responses to variable substances (Marino *et al.*, 2006), biotic and abiotic stresses (Marino and Bertazza, 1998; Capuana, 2011; Lombardi *et al.*, 2003). *In vitro* plant behavior has been found in several cases to resemble that of *ex vitro* plant growth (Karam *et al.*, 1998; Houshmand *et al.*, 2005). In particular, some kiwifruit somaclones, able to grow at high *in vitro* pH, were also fairly tolerant to lime when cultured in pots and/or in the field (Marino *et al.*, 1998). Furthermore, two quince A somaclonal variants that showed good tolerance to iron (Fe) chlorosis in *in vitro* culture, also showed similar behavior in the greenhouse (Bunnag *et al.*, 1996).

***Plant material and growth conditions.*** Donor shoot cultures of kiwifruit (*Actinidia deliciosa* A, Chev.), cv. Hayward, were acquired from a commercial laboratory and maintained through repeated 5-week subcultures on a shoot multiplication medium (SM). The medium consisted of basal MS (Murashige and Skoog, 1962) macro and microelements; among them Fe (100.17  $\mu\text{M}$   $\text{Fe}^{2+}$ ) was supplied as iron sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) together with ethylenediaminetetraacetic acid disodium salt dihydrate ( $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ ), as a chelating agent. In addition, medium was supplemented with ( $\mu\text{M}$ ): 555 *myo*-inositol, 4.06 nicotinic acid, 2.43 pyridoxine hydrochloride, 26.6 glycine, 2.96 thiamine hydrochloride, 4.4 6-benzyladenine (BA), 0.58 gibberellic acid, and ( $\text{g l}^{-1}$ ): 30 commercial sucrose and 6.5 ‘type A’ bacteriologic agar (7178-01-A Biokar, Diagnostic International Distribution, Milan, Italy). All the chemicals were from Sigma (Sigma-Aldrich, Milan, Italy), except for sucrose and agar. The pH was adjusted to 5.6 before autoclave-sterilization at 120 °C for 20 min. Five shoots were grown in 500-ml glass jars, each containing 40 ml medium. The jars were closed with twist-off screw metal caps, wrapped with polyvinyl chloride (PVC) transparent film for food (allowing gas exchange), and placed in a growth chamber under Standard Growth Conditions (SGC):  $22 \pm 2$  °C, 16-h-light photoperiod at  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic active radiation (supplied by Philips TLD 36 W/33 lamps).

Well-developed shoots, about 20-mm long, were dissected from donor cultures at the end of a maintenance subculture, weighed and randomly transplanted into 250-ml jars (with 30 ml medium and two shoots each) on the two different multiplication media. The jars were closed as reported above and placed under SGC. The shoot fresh (fw) and dry (dw) weight, the growth rate [(final shoot fw – initial fw)/initial fw], and the mineral composition of the shoots (as reported below) were determined after 45 days in culture on five jars (replicates) per treatment. The final pH and the pH decrease ( $\Delta$ pH) of the culture media were assessed after heating to 50 °C to obtain agar liquefaction.

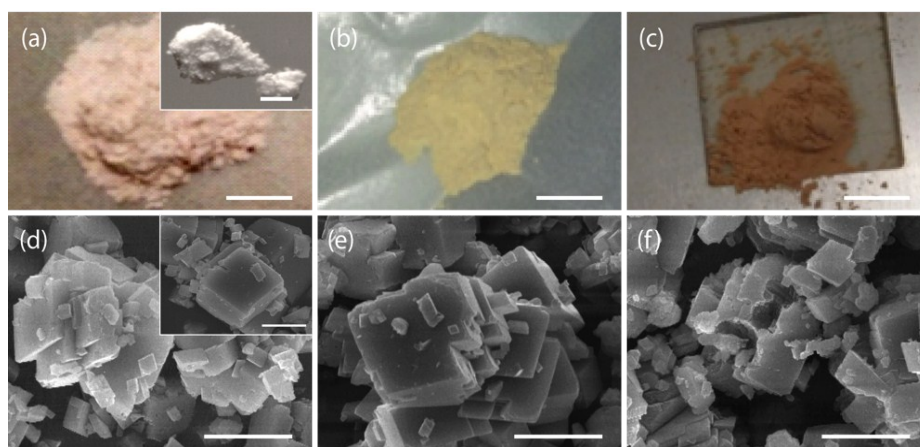
***Determination of the shoot mineral composition.*** Phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) concentration were determined by plasma spectrometer (Ametek Spectro, Arcos, Kleve, Germany) on a 0.2 g dry sample previously mineralized (US EPA Methods 3052; Kingston 1988) in an Etos TC microwave lab station (Milestone, Bergamo, Italy).

***Statistical analysis.*** The data were subjected to the analysis of variance as in a complete randomized experimental design; when analysis of variance showed a statistical effect of treatments ( $P \leq 0.05$ ), means were separated by Student Newman Keuls test.

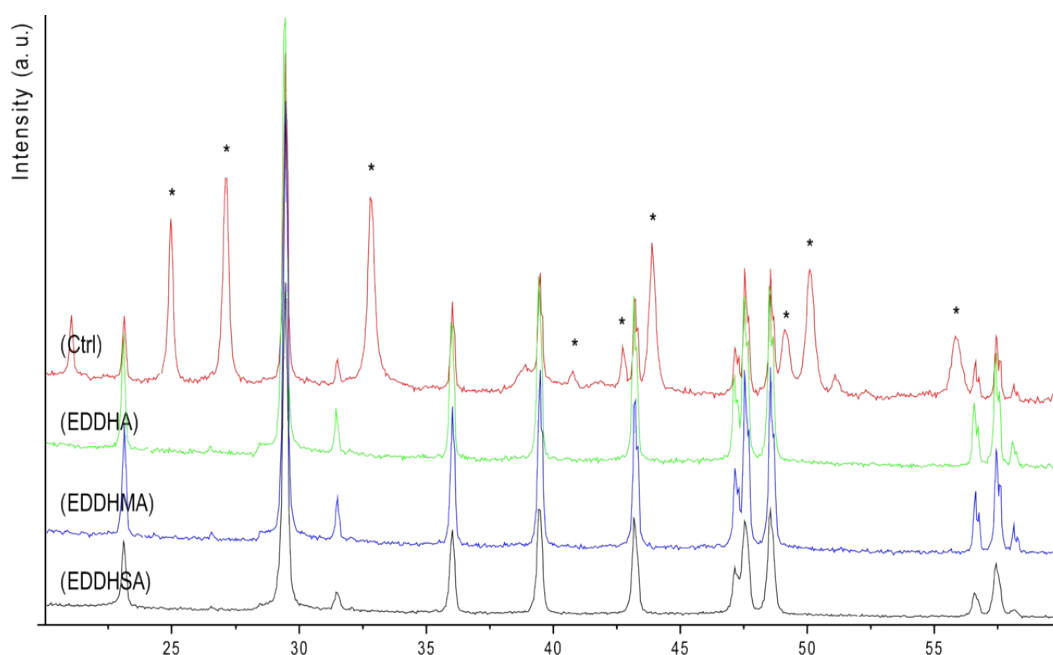
**Table S1.** Features of the calcium carbonate precipitates obtained in the presence of different concentrations, namely  $1.0 \cdot 10^{-3}$ ,  $1.0 \cdot 10^{-4}$  or  $1.0 \cdot 10^{-5}$  M of Fe-EDDHSA, Fe-EDDHA or Fe-EDDHMA.

Sample	mineral phase <sup>§</sup> (wt.%)	Calcite <sup>§</sup> habitus	calcite cryst. (FWHM / $2\Theta$ ) <sup>#</sup>	Fe (wt.%) <sup>&amp;</sup>
Ctrl	C (49.3) - V (50.7)	rhomb. aggr. / {10.4}	0.16	-
Fe-EDDHSA				
$1.0 \cdot 10^{-5}$ M	C	rhomb. aggr. / {10.4}	0.16	0.005
$1.0 \cdot 10^{-4}$ M	C	rhomb. aggr. / Hopper {10.4}	0.16	0.059
$1.0 \cdot 10^{-3}$ M	C	rhomb. aggr. / Hopper {10.4}	0.18	0.616
Fe-EDDHMA				
$1.0 \cdot 10^{-5}$ M	C (97.5) - V (2.5)	rhomb. aggr. / {10.4}	0.16	0.002
$1.0 \cdot 10^{-4}$ M	C (98.0) - V (2.0)	rhomb. aggr. / {10.4}	0.16	0.008
$1.0 \cdot 10^{-3}$ M	C	rhomb. aggr. / {10.4}- {hk.0}	0.16	0.028
Fe-EDDHA				
$1.0 \cdot 10^{-5}$ M	C (51.6) - V (48.4)	rhomb. aggr. / {10.4}	0.16	0.009
$1.0 \cdot 10^{-4}$ M	C (13.9) - V (86.1)	rhomb. aggr. / {10.4}	0.16	0.053
$1.0 \cdot 10^{-3}$ M	C	rhomb. aggr. / {10.4}- {hk.0}	0.16	0.502

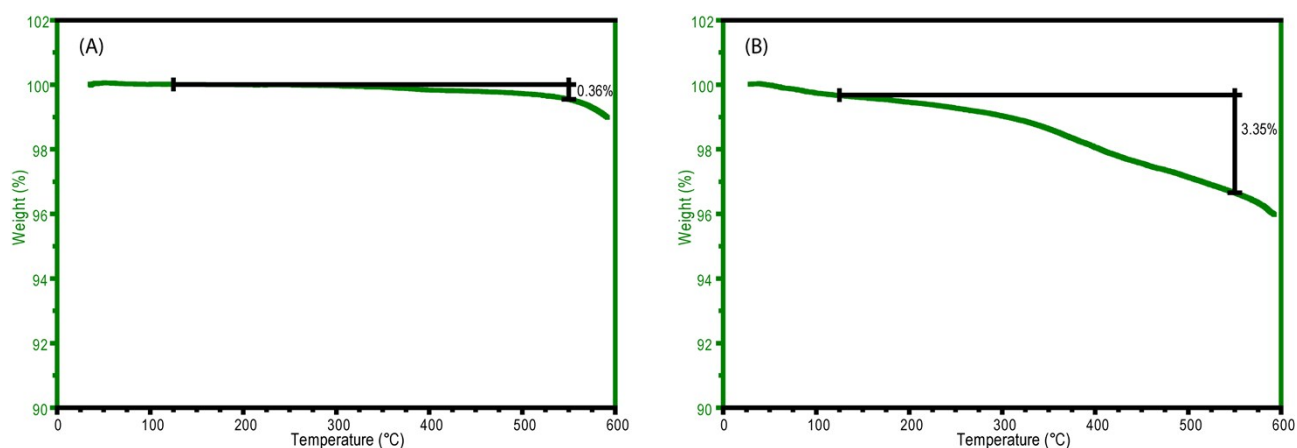
<sup>§</sup> C and V indicate calcite and vaterite, respectively. <sup>§</sup> rhomb. aggr. Indicates aggregates of {10.4} rhombohedral crystals. Hopper {10.4} indicates crystal with holes on the {10.4} faces. <sup>&</sup>The iron concentration is reported as weight percentage with respect to the calcium carbonate (calcite and vaterite) mass. <sup>#</sup>The crystallinity of the calcite samples was evaluated by the full width half maximum (FWHM) of the {10.4} diffraction peak of calcite. A higher value indicates a lower crystallinity.



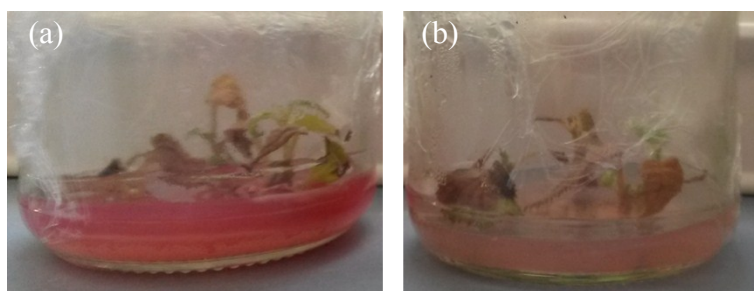
**Figure S1.** Camera (a-c) pictures and SEM (d-f) images of the calcite crystals precipitated in the presence of  $1.0 \cdot 10^{-3}$  M of EDDHMA (a, d), EDDHA (b, e) or EDDHSA (c, f). The insets in (a) and (d) show calcium carbonate precipitated in the absence of additives. Scale bar in (a-c) 1 cm and scale bar in (d-f) 5 microns.



**Figure S2.** X-ray powder diffraction patterns of the calcium carbonate precipitates obtained in the absence of additive (Ctrl) and in the presence of  $1.0 \cdot 10^{-3}$  M of EDDHA, EDDHMA or EDDHSA. The star indicates the diffraction peaks of vaterite, all the others are assigned to calcite.



**Figure S3.** Thermogravimetric profile of (A) pure calcite crystals and (B) Fe-EDDHSA/CaCO<sub>3</sub> hybrid crystals.



**Figure S4.** Plant growth with phenanthroline 1 mg/l, in the presence (a) or without (b) Fe-EDDHSA/calcite hybrid crystals. It is noticeable that the redder color is due to EDDHSA/calcite hybrid crystals dissolution (left); while the light red color visible on right jar is a consequent of some Fe release by plants.

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