An innovative green iron-fertilizer, produced biotechnologically, for correcting iron chlorosis of soybean plants grown in calcareous soils

Ferreira C.[1,2], Lopez-Rayo S.[3], Lucena J.[3], Soares E.[2,4], Soares H.[1,*]

[1] REQUIMTE/LAQV, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Portugal
[2] CEB-Centre of Biological Engineering, University of Minho, Braga, Portugal
[3] Departamento de Química Agrícola y Bromatología, Facultad de Ciencias, Universidad Autónoma de Madrid, Spain
[4] Bioengineering Laboratory-CIETI, Department of Chemical Engineering, ISEP, Porto, Portugal

*corresponding author: Helena MVM Soares: e-mail: hsoares@fe.up.pt;

Abstract
Iron deficiency is one of the main causes of chlorosis in plants, which leads to the loss in the field crops quality and yield. Iron-deficiency is a worldwide problem, particularly sever in calcareous soils (about 30% of world´s land surface). The current use of synthetic iron-chelates to prevent or correct iron-deficiency in plants raises environmental concerns due to their poor biodegradability. Thus, new, more environmentally-friendly efficient solutions are needed to solve iron deficiency-induced chlorosis (IDIC) in crops grown in calcareous soils. In this work, a new green freeze-dried iron fertilizer was produced (patent submitted) from a culture of *A. vinelandii* containing siderophores of a natural source able to bind iron at pH 9. Soybean plants cultivated under calcareous soils and treated with the green iron-fertilizer responded more significantly and comparable to the positive control, ethylenediaminedi(o-hydroxyphenylacetic) acid, than those treated with the negative control, when evaluated by their growth (dry mass) and chlorophyll concentration (SPAD index). On average, iron content was also greater on green iron-fertilizer treated plants than on negative control treated ones. Results suggest that the freeze-dried product, prepared from *A. vinelandii* culture, can be a viable alternative for mending IDIC of soybean plants grown in calcareous soils.

Keywords: Freeze-dried iron-bacterial siderophores products; Environmental-friendly iron-chelates; Iron chlorosis correction of soybean plants; Calcareous soils

1. Introduction

Iron (Fe) deficiency is one of the main causes of chlorosis in plants due to the low solubility and dissolution kinetics of Fe compounds, which limits the absorption of Fe by plants. Chlorotic plants produce less biomass, flowers and fruits and ultimately can lead to a complete breakdown of the crop. To prevent the problem of Fe deficiency in plants, synthetic iron-chelates are currently used but they are not satisfactory mainly due to its poor biodegradability, which raises environmental concerns. Therefore, the search for cheap, environmental-friendly and suitable Fe chelators, with specific properties to be used, as Fe fertilizers, became a great challenge. Among the compounds considered, siderophores constitute an interesting object of study since they form extremely stable octahedral complexes with Fe(III) ion (Hider and Kong, 2010) and significant amount can be biologically produced in Fe-deficient cultures (Tschierske et.al., 1996; Santos et.al., 2014).

The present work deals with the preparation of a freeze-dried product obtained by a biotechnological-based process (filtrate of *Azotobacter vinelandii* culture). Subsequently, the ability of this new iron fertilizer to prevent chlorosis of soybean plants grown in calcareous soils was evaluated by their growth (dry mass), chlorophyll development (SPAD index) and iron content.

2. Materials and Methods

In this study, a culture of *Azotobacter vinelandii* Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), grown in Fe depleted Burk´s medium during 72 h, was used. Then, culture was centrifuged and the supernatant was filtered (0.45 µm pore size filter). The filtrate was mixed with corn starch 15 g L^{-1}, which was used as anti-caking agent, and freeze-dried. Soybean sprouts, in a chlorotic-induced state, were planted in pots filled with 0.6 kg of a mixture of the sandy clay soil (70%) from Picassent (Valencia, Spain) and calcareous sand (30%) and irrigated until 80% of the soil water capacity was achieved. A total of three pot sets were prepared: one without any treatment (negative control, C-), one for *o,o*- ethylenediaminedi(*o*-hydroxyphenylacetic acid, *o,o*-EDDHA (Positive control, C+) and another for freeze-dried iron fertilizer (A). Each pot set had five pots. To each pot (unless negative control pots), enough volume of chelate was applied in order to have 2.5 mg of Fe(III) per kg of soil. After 21 days of treatment application, plants were dissected into leaves, stems and roots and placed to dry in a force air oven at 60 °C for 72 h. Once dried, the plant parts were weighted for the dry mass determination and calcined at 480 °C. The Fe concentration was analysed after acid digestion of the resulting ashes by atomic absorption spectroscopy with flame atomization. The level of plant "greenness" was evaluated using a soil and plant analyser development (SPAD) chlorophyll meter. SPAD index was evaluated in all leaf stages from the cotyledons to the last developed plant stage.

3. Results and Discussion

The direct application of microbial cultures containing siderophores for fertilizing soils is not feasible due to the large volume needed. On the other hand, siderophores being natural bioproducts are potentially biodegradable. To overcome these problems, a freeze-dried product of the filtrate of *Azotobacter vinelandii* culture was prepared by mixing the filtrate with 15 $g.L^{-1}$ corn starch, which was used as an anti-caking agent.

The impact of the plant treatment with the freeze-dried preparation, given by the average dry weight of different plant parts, can be seen in figure 1. It is possible to observe that plants treated with freeze-dried preparation presented a higher dry-weight mass for leaves, stem, or shoots (stem plus leaves) comparatively to plants not exposed to iron-chelate (negative control). This positive effect is comparable to the obtained with the positive control (*o*,*o*-EDDHA) (Figure 1).

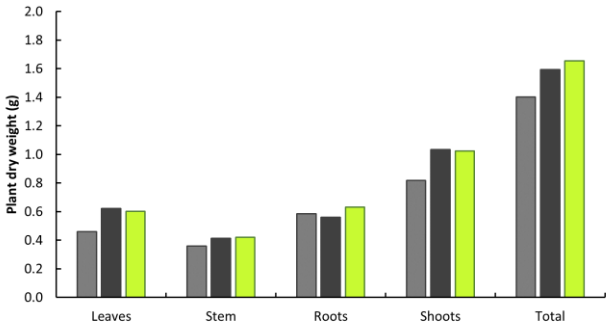

Figure 1. Dry-weight of soybean plant, after 21 days of treatment, planted in calcareous soil. Plants were treated with the freeze-dried preparation (light green) or with o,o-EDDHA (positive control) (dark grey). As negative control, no iron-chelate was applied (light grey). Shoots: leaves plus stem. Total: all parts of the plant.

The greenness of the plants, which reflects the chlorophyll amount, was evaluated using the SPAD value at the end of the treatment (21 days) in the four stages of the plant (Table 1). For all leave stages, soybean plants treated with the freeze-dried fertilizer presented a higher level of chlorophyll compared with plants non treated with iron- chelate (negative control) (Table 1). Chlorophyll content in the second and third leave stages of plants treated with the freeze-dried preparation was similar to the positive control (o,o-EDDHA) (Table 1).
Moreover, the freeze-dried preparation promoted an increase of iron in the leaves, comparatively to plants non treated with iron-chelate (negative control) (data not shown).

Table 1. Average SPAD levels, measured at the 21st day after treatment in different plant leaf stages.

Treatment	Stage 2	Stage 3	Stage 4	Stage 5
A	40.1	34.0	24.1	22.2
C-	28.5	17.3	3.1	6.7
C+	42.0	38.8	41.6	37.9

Plants were treated with the freeze-dried preparation (A) or with o,o-EDDHA (positive control, C+); as negative control, no iron-chelate was applied (negative control, C-).

4. Conclusions

The results presented above demonstrated that the treatment of soybean plants, cultivated in calcareous soil, with the freeze-dried preparation of bacterial origin (*A. vinelandii*) originated more developed plants (with higher biomass) and with greener leaves (high chlorophyll content), which demonstrates that this freeze-dried fertilizer can be used in chlorosis amendment of plants cultivated under calcareous soils.

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