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RESEARCH PAPER

Elemental composition of strawberry plants inoculated with the plant growth-promoting bacterium *Azospirillum brasilense* REC3, assessed with scanning electron microscopy and energy dispersive X-ray analysis

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Keywords

Azospirillum brasilense; energy dispersive X-ray spectroscopy; plant mineral nutrition; scanning electron microscopy; strawberry.

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ABSTRACT

The elemental composition of strawberry plants (*Fragaria ananassa* cv. Macarena) inoculated with the plant growth-promoting bacterium *Azospirillum brasilense* REC3, and non-inoculated controls, was studied using scanning electron microscopy (SEM) and energy dispersive X-ray (EDS) analysis. This allowed simultaneous semi-quantification of different elements in a small, solid sample. Plants were inoculated and grown hydroponically in 50% or 100% Hoagland solution, corresponding to limited or optimum nutrient medium, respectively. Bacteria-inoculated plants increased the growth index 45% and 80% compared to controls when grown in 100% and 50% Hoagland solution, respectively. Thus, inoculation with *A. brasilense* REC3 in a nutrient-limited medium had the strongest effect in terms of increasing both shoot and root biomass and growth index, as already described for *Azospirillum* inoculated into nutrient-poor soils. SEM-EDS spectra and maps showed the elemental composition and relative distribution of nutrients in strawberry tissues. Leaves contained C, O, N, Na, P, K, Ca and Cu, while roots also had Si and Cl. The organic fraction (C, O and N) accounted for over 96.3% of the total chemical composition; of the mineral fraction, Na had higher accumulation in both leaves and roots. *Azospirillum*-inoculated and control plants had similar elemental quantities; however, in bacteria-inoculated roots, P was significantly increased (34.33%), which constitutes a major benefit for plant nutrition, while Cu content decreased (35.16%).

INTRODUCTION

Strawberry cropping requires high inputs of fertilisers and pesticides to enhance fruit yields, both of which may be hazardous to the environment and to human health when applied incorrectly (Ajwa *et al.* 2003). Therefore, the use of plant growth promoting bacteria (PGPB), such as *Azospirillum*, as inoculant provides a good and environmentally sound biotechnological alternative for plant nutrition (Bashan & de-Bashan 2010). *Azospirillum brasilense* REC3 was isolated from strawberry roots and shown to have beneficial effects such as N fixation, auxin and siderophore production (Pedraza *et al.* 2007). The bacterium showed positive chemotaxis toward strawberry root exudates, biocontrol activity against anthracnose disease, colonisation of different strawberry tissues and growth promotion effects on strawberry plants under controlled and field conditions (Pedraza *et al.* 2010; Tortora *et al.* 2011, 2012; Guerrero-Molina *et al.* 2012). However, the elemental composition of plant tissues resulting from the *Azospirillum*–strawberry interaction has not yet been assessed.

Generally, studies of plant nutrition are carried out using methods that require wet chemical extraction, homogenisation and dissolution of plant components to determine one element at a time, *e.g.* total N and organic C content are usually determined with the Kjeldahl (Bremner & Mulvaney 1982) and Walkey-Black (Nelson & Sommers 1982) methods, respectively. Even when methods are used that allow the quantification of several elements at the same time, *e.g.* inductively coupled plasma spectrometry (Esitken *et al.* 2010) and atomic absorption spectroscopy (Rojas-Tapias *et al.* 2012), samples still need to be subjected to ashing, wet extraction and digestion procedures. Scanning electron microscopy (SEM) coupled to energy-dispersive X-ray analysis (EDS) is a potent method, based on the characteristic X-radiation emitted from samples that can yield both qualitative identification and quantitative elemental information, thus also allowing the direct observation, comparison and characterisation of different materials (Goldstein *et al.* 2003). The technique requires a small solid sample, is relatively fast, non-destructive and allows determination of different elements simultaneously (Goldstein *et al.* 2003). This technique has been used in plant tissues mainly to study bioaccumulation, biodegradation, metal immobilisation and bioavailability (Küpper *et al.* 2000; Rivera-Becerril *et al.* 2002; Chen *et al.* 2007).

Our working hypothesis was that *A. brasilense* REC3 can positively contribute to strawberry plant growth and nutrition. Hence, the aim of this work was to assess these phenomena using SEM coupled to EDS.

MATERIAL AND METHODS

Plant origin and growth

In vitro micropropagated plants of strawberry (*Fragaria ananassa*, Duch) cv. Macarena (20 plants in total) were grown separately in pots containing sterile humus:perlome (2:1) under phytotron conditions (28 °C, 70% relative humidity, photoperiod of 16 h at 250 μmol·photons·m^{-2}·s^{-1}). After 60 days of cultivation, plants were carefully removed from each pot; roots were washed thoroughly with sterile distilled water to remove adherent soil particles and plants transplanted to aerated (pO_2 = 20 kPa) and disinfected trays containing either 50% or 100% (w/v) Hoagland solution, corresponding to limited or optimum nutrient medium, respectively. Optimised Hoagland solution contained 6 mM KNO_3, 4 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4{\cdot}7H_2O$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4{\cdot}H_2O$, 2 μM $ZnSO_4{\cdot}7H_2O$, 0.5 μM $CuSO_4{\cdot}5H_2O$, 0.5 μM H_2MoO_4 and 26 μM FeEDTA (Epstein 1972). Hydroponic medium volume was periodically measured and maintained constant by adding distilled water to retain optimum nutrient concentration.

Bacterial inoculation

Ten days after transplantation of strawberry plants into aerated hydroponic solution, ten plants were inoculated by submerging their roots in a suspension of *A. brasilense* REC3 (about 10^6 CFU·ml^{-1} water) for 30 min, as described in Pedraza *et al.* (2010), and ten control plants were maintained in a different tray without bacterial inoculation. Four treatments with five plants each were placed in different trays during a further 10 days, corresponding to: (i) control in 100% Hoagland; (ii) *A. brasilense* REC3 in 100% Hoagland; (iii) control in 50% Hoagland and (iv) *A. brasilense* REC3 in 50% Hoagland. Plants were then harvested and small samples of young roots and leaves fixed in 3% (v/v) glutaraldehyde solution for SEM-EDS. The same sampled plants were used to evaluate dry mass of roots and shoots.

Plant dry matter quantification and growth index determination

Roots and shoots were oven-dried at 65 °C for 72 h (constant weight) and dry weight of each tissue recorded. Total biomass was calculated as the sum of root and shoot dry weights, and the growth index as (final biomass – initial biomass) divided by the initial biomass.

Scanning electron microscopy (SEM) and energy dispersive X-ray spectrometry (EDS)

The SEM-EDS method was used to examine morphological changes on the surface of REC3-inoculated plants and to identify the chemical composition and relative distribution of macro- and micronutrients. Samples of roots and leaves were prepared for SEM according to Guerrero-Molina *et al.* (2012). Briefly, fixed samples were post-fixed in 1% (v/v) osmium tetroxide overnight. Specimens were then washed three times in distilled water and treated with 2% uranyl acetate (w/v) for 40 min. After fixation, samples were step-wise dehydrated in an ethanol (series from 30% to 100%) and in acetone (100%), critical point dried and coated with gold. SEM micrographs and EDS spectra were obtained with a Zeiss Supra 55VP (Carl Zeiss, Oberkochen, Germany) SEM equipped with an Oxford INCA EDS; the electron energy used was 20 keV. Elemental composition values correspond to the average of three replicate spectra obtained from each tissue and each treatment. This method is a relative determination, and the amount of each element is a relative percentage in relation to the other elements detected in the sample, consequently the sum of all elements is equal to 100%.

Statistical analysis

Statistical analysis of data (one-way ANOVA) was carried out with the software Infostat (version 2008 for Windows; GraphPad, La Jolla, CA, USA) and significant differences were reported at $P < 0.05$ using Tukey multiple comparison test. Data are expressed as mean ± SD.

RESULTS AND DISCUSSION

Growth promotion

Root dry weight of plants grown in 50% Hoagland solution and inoculated with *A. brasilense* REC3 showed the highest mean values (Fig. 1a; $P < 0.05$), probably due to the increment of root area through augmentation of root hair number and length (Fig. 1e). This was in agreement with our previous work, where REC3 also increased strawberry root dry weight and root area by stimulating root hair proliferation (Pedraza *et al.* 2010), and also observed in other crops inoculated with *Azospirillum* (Okon & Kapulnik 1986; Morgenstern & Okon 1987). In this work, mass values of leaves (Fig. 1b) were 75% higher than those of roots (Fig. 1a), and thus leaf mass had more influence on total biomass and growth index determination (Fig. 1c). Also, biomass of strawberry leaves was significantly increased after inoculation with *A. brasilense* REC3 when plants were grown in either limited or optimum nutrient solution (Fig. 1b; $P < 0.05$). Among non-inoculated control plants, those grown in 100% Hoagland had higher values for leaf mass than controls in 50% Hoagland (Fig. 1b; $P < 0.05$) as expected, considering that the first solution (100%) is an optimum nutrient culture while the second had only half of the amount of nutrients considered as ideal for plant growth. However, this difference was not observed among inoculated plants (Fig. 1b; $P < 0.05$).

Although REC3 promoted plant growth under both hydroponic medium concentrations, the effect of bacterial inoculation was better in 50% Hoagland solution, where REC3-inoculated plants had 80% more growth than their controls, while REC3 with 100% Hoagland was only 45% higher than controls with 100% Hoagland. Similar results were observed in eroded and nutrient-poor soil for several crops, where *Azospirillum* inoculation had a larger effect on plant growth promotion rather than in soils without nutrient limitation (Bacilio *et al.* 2006; Çakmakçi *et al.* 2006).

Microscopy and microanalysis with SEM-EDS

Scanning electron micrographs showed that the mineral concentrations of Hoagland solution did not affect the bacterial colonisation capacity. We observed a similar density of bacteria

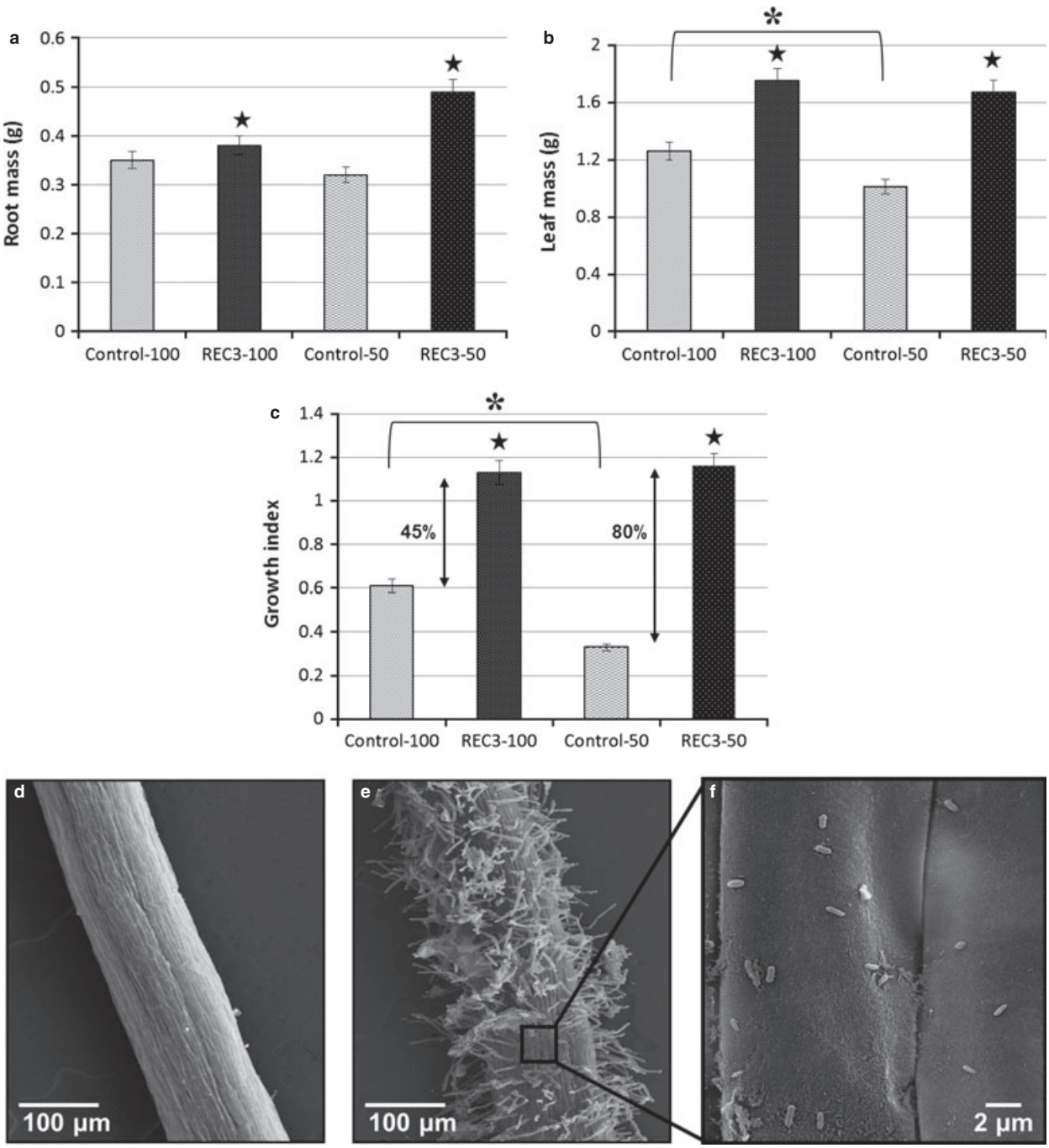

Fig. 1. Dry weight measurements of (a) roots and (b) leaves of control and *A. brasilense* REC3- inoculated strawberry plants grown in 50% or 100% Hoagland solution (*, $P < 0.05$). c: Growth index and percentage growth increment after REC3 inoculation in comparison to controls (*, $P < 0.05$). d: SEM micrographs of control, non-inoculated root. e: SEM of REC3-inoculated root showing root hair proliferation of plants growth in nutrient-limited medium. f: Magnification of (e), note bacteria attached to the root surface of inoculated plants (a similar pattern was observed in optimum medium).

attached to the root surface of strawberry plants grown in both Hoagland concentrations: 50% and 100% (Fig. 1f). Figure 1d and e also show the differences in root hair proliferation observed between *Azospirillum*-inoculated plants and the control, as reported elsewhere (Bashan & de-Bashan 2010; Pedraza *et al.* 2010).

The results of SEM-EDS microanalysis of leaves and roots of REC3-inoculated and control non-inoculated strawberry plants grown with limited and optimum nutrient media are shown in Figs 2a,b and 3a,b. Also, Figs 2d and 3d show EDS maps with the relative distribution of Na in leaves and roots, respectively. EDS spectra allowed the quantification of plant nutrients C, O, N, Na, P, K, Ca, Cu, Si and Cl; moreover, EDS mapping also allowed studies of the relative distribution of these elements and other nutrients not determined in the spectra, *e.g.* Fe and Zn (see Figures S1 and S2: SEM-EDS Leaves and SEM-EDS

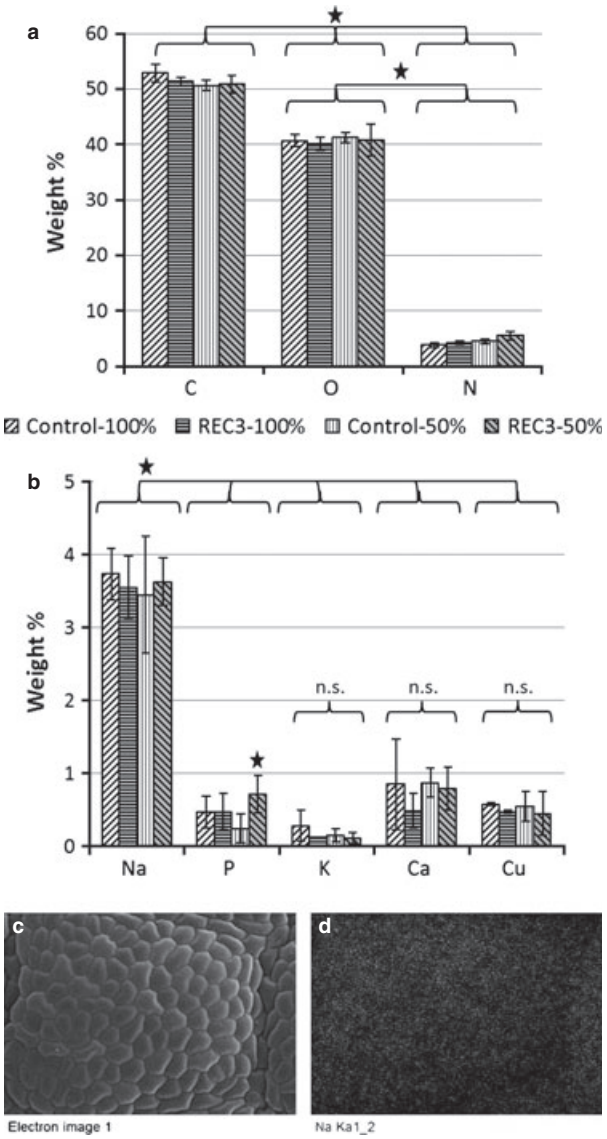

Fig. 2. SEM-EDS microanalysis of leaf mineral composition of *Azospirillum*-inoculated and not inoculated control strawberry plants grown in 50% or 100% Hoagland solution (average of three replicates). a: Organic fraction represented by C, O and N. b: mineral fraction represented by Na, P, K, Ca and Cu. (*, $P < 0.05$; n.s., not significant). c: Scanning micrograph of analysed leaf area (1000× magnification). d: EDS map of the relative distribution of Na in strawberry leaves of inoculated plants grown in 50% Hoagland solution.

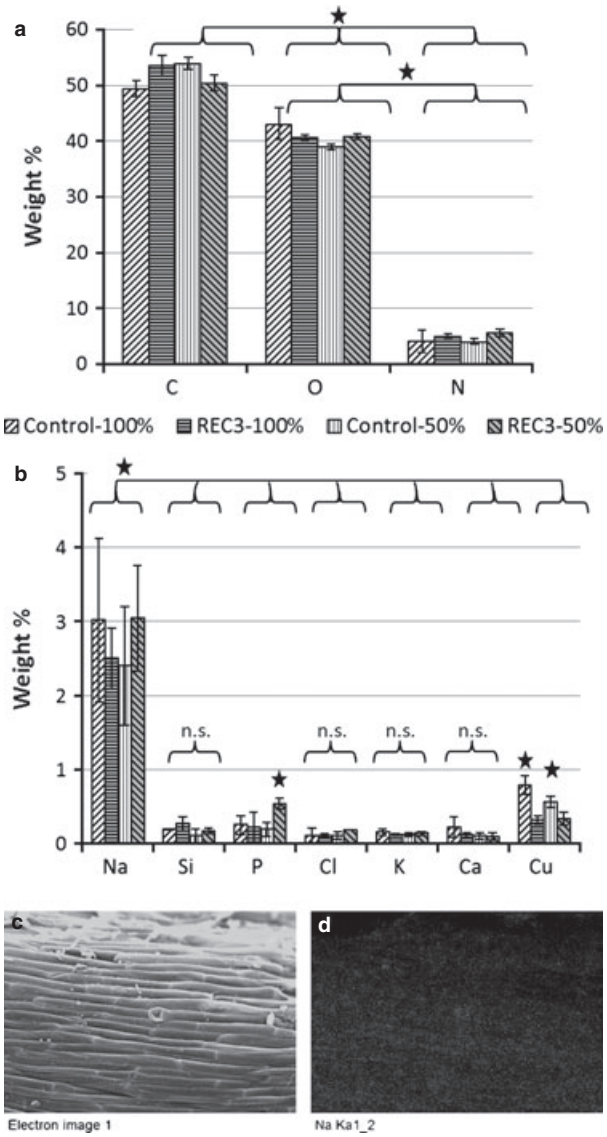

Fig. 3. SEM-EDS microanalysis of root mineral composition of *Azospirillum*-inoculated and not-inoculated control strawberry plants grown in 50% or 100% Hoagland solution (average of three replicates). a: Organic fraction represented by C, O and N. b: mineral fraction represented by Na, Si, P, Cl, K, Ca and Cu *(*, $P < 0.05$*; n.s., not significant). c: Scanning micrograph of analysed root area (1000× magnification). d: EDS map of the relative distribution of Na in strawberry roots of inoculated plants grown in 50% Hoagland solution.

Roots, respectively). Results showed that the elements detected were ubiquitous and uniformly distributed in both tissues.

The EDS leaf spectra showed a similar chemical composition to root samples and no significant differences among the mean values of the content of each major element (expressed as Weight %) were observed. This failure to determine differences was probably due to a limitation of the technique, since results are shown as relative values (Weight %) rather than absolute numbers. Nonetheless, qualitatively, it was possible to observe differences in the elemental composition of the two tissues: leaves were composed of C, O, N, Na, P, K, Ca and Cu, while roots also contained Si and Cl. However, in a previous X-ray microanalysis (Bashan *et al.* 1990) with wheat and soybean plants inoculated with different *Azospirillum* strains there were inconsistencies in the plant mineral changes.

The organic fraction, represented here by the non-mineral elements C, O and N, was significantly higher than the others macro- and micronutrients in the two tissues: means of C and O being higher than N (Figs 2a and 3a; $P < 0.05$). According to EDS spectra, these three elements accounted for 96.3% of the total plant mineral composition. It is well known that C, O and H are obtained from H_2O and CO_2 and represent 95% of the plant mineral composition. Hydrogen was not identified with the EDS because the detector is not capable of detecting

elements with atomic numbers below six (Goldstein *et al.* 2003; Kutchko & Kim 2006).

The EDS spectra showed the presence of N, although to a lesser extent than expected considering that REC3 is a N-fixing bacterium. However, this experiment was carried out in aerated ($pO_2 = 20$ kPa) hydroponic medium, which probably inhibited N_2 fixation since nitrogenase activity is negatively regulated by pO_2 over 7 kPa and in the presence of combined N. The hydroponic culture conditions used here were designed to favour plant growth regardless of the strict regulation of bacterial nitrogenase by O_2 and fixed N; under these conditions it is more likely that this enzyme would be inhibited. Kapulnik *et al.* (1985) also reported that there was no N fixation activity in wheat roots inoculated with *A. brasilense* under hydroponic condition. Furthermore, the slight differences (not statistically significant) observed in N content between inoculated and non-inoculated roots can perhaps be due to improved nutrient uptake through the growth promotion of inoculated roots.

Sodium was the most accumulated mineral element in leaves and roots (Figs 2b and 3b). In leaves, Na was 11.36%, 28.84%, 9.65% and 7.46% higher than P, K, Ca and Cu, respectively, and in roots it was 19.45%, 24.43%, 26.90%, 7.88%, 26.52%, and 29.89% higher than P, K, Ca, Cu, Si and Cl, respectively. Even though Na is classified as a beneficial element (Epstein 1972) and not as an essential nutrient for plant growth, strawberry plants require relatively higher levels. Esitken *et al.* (2010) also observed this in control, non-inoculated strawberry plants cv. Fern that accumulated 841.6 $mg \cdot l^{-1}$ Na, determined using inductively coupled plasma spectrometry. When plants are exposed to excess Na they become chlorotic and necrotic and no further growth occurs, despite a high K concentration in the plants (Broadley *et al.* 2011). These symptoms were not observed in strawberry plants used in this work despite the high content of Na (Figs 2 and 3). Strawberry is considered to be a crop that is very sensitive to salinity; however, Martínez Barroso & Alvarez (1997) found that toxicity symptoms observed in strawberry plants (cv. Toro and Douglas) irrigated with different content of salts (NaCl, KCl, Na_2SO_4, $NaHCO_3$) were due to the specific action of Cl^- and not Na^+. The toxicity was due to excess anion levels in the leaves and not an increased in the presence of Na^+. This may explain the high tolerance of strawberries plants to Na. Moreover, Na was shown to exert a beneficial effect on the growth of strawberry cv. Korona under saline stress (Saied *et al.* 2005), which could be related to the substitution of K for Na, which helps in osmoregulation to maintain the water content of plant tissues and ultimately increase fresh weight (Turhan & Eris 2004).

Sulphur is present in all organic tissues as a component of proteins, but in this study it was not detected; we speculate that this is because SEM samples were gold-coated, which might interfere with detection of S. Carbon-coating is recommended for EDS analysis of organic matter.

Regarding the macronutrients Ca and P, they were found in both root and leaf tissues, with mean values higher in leaves than in roots. The mean percentage weight of P was highest in both tissues of plants inoculated with REC3 and grown in nutrient-limited medium ($P < 0.05$; Figs 2b and 3b), being 34.33% and 42.26% higher in inoculated roots and leaves than their respective controls. K was also found in both tissues but without differences among tissues or treatments. The increment of P in both strawberry leaves and roots and Si in roots of REC3-inoculated plants, as compared to non-inoculated controls, could be explained as the stimulatory effect on roots exerted through *Azospirillum*-produced auxins. Esitken *et al.* (2010) observed a similar increment in P uptake in strawberry plants inoculated with others PGPB strains: *Pseudomonas* BA-8, *Bacillus* OSU-142 and *Bacillus* M-3. It is well known that *Azospirillum* mainly colonises root tissues (Bashan & de-Bashan 2010; Guerrero-Molina *et al.* 2012) and produces its major effect at this level by increasing the root area and hence improving plant nutrition, as observed in this work.

The micronutrient Cu was also found in roots and leaves; however, statistical analysis of this element showed a significant decrease (35.16%) in REC3-inoculated roots (Fig. 3b), but no differences in leaves (Fig. 2b). Recently, it was reported that two strains of *A. brasilense*, Sp7 and Sp245, were able to take up Cu when grown in the presence of Cu II (0.1 mM Cu^{2+}), and this induced the biosynthesis of poly-3-hydroxybutyrate (PHB) in strain Sp7 (Kamnev *et al.* 2012). In line with these findings, we deduce that the REC3 strain of *A. brasilense* might also be capable of Cu uptake and could compete with other plants for this element. During this experiment no plants shows any symptoms of nutrient deficiency.

CONCLUSIONS

Scanning electron microscopy and EDS microanalysis allowed assessment of the mineral nutrition of strawberry plants inoculated with *A. brasilense* REC3 and grown in two concentrations of Hoagland solution. The EDS spectra and maps showed the elemental composition and relative distribution of nutrients in inoculated and control plants. There were differences in the quality of elements: leaves contained C, O, N, Na, P, K, Ca and Cu, while roots also contained Si and Cl. Inoculation with *A. brasilense* REC3 increased both the biomass and growth index of strawberry plants, as compared to non-inoculated controls, especially when the plants were grown in a nutrient-limited medium. Moreover P uptake was improved after REC inoculation, which represents a major benefit for plant nutrition.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. SEM-EDS Leaves (a–j) EDS map of the relative distribution of elements in *Azospirillum*-inoculated strawberry leaves: (a) SEM of analysed leaf area of inoculated strawberry plants grown in 50% Hoagland solution (1000× magnification); (b) maps of C, (c) O, (d) P, (e) Na, (f) N, (g) Fe, (h) Zn, (i) K, (j) Ca.

Figure S2. SEM-EDS Roots (a–j) EDS map of the relative distribution of elements in *Azospirillum*-inoculated strawberry roots: (a) SEM of analysed root area of inoculated strawberry plants grown in 50% Hoagland solution (1000× magnification); (b) maps of C, (c) O, (d) P, (e) Na, (f) N, (g) Fe, (h) Zn, (i) K, (j) Ca.

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