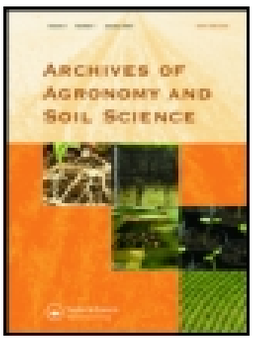

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Effect of living cells of microalgae or their extracts on soil enzyme activities

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Abstract

The enzymatic activity of the soil and potential plant growth can both be affected by the addition of different substances, such as biostimulants. The final objective of this work was to evaluate the effect of *Chlorella vulgaris*, *Scenedesmus quadricauda* or their extracts applied directly into the soil, by monitoring the complex soil-microorganism-plant system. Experiments were conducted in pots using agricultural soil and tomato plants in a growth chamber at 25 ± 2 °C illuminated by artificial light with a 16-h photoperiod. The soil biological activity was analyzed by monitoring fluorescein diacetate hydrolysis, dehydrogenases, acid and alkaline phosphomonoesterase and urease activities. Dry weight and chlorophyll index values of tomato plants were also investigated. Results showed that both the microalgae and their extracts positively affected soil biological activity by increasing values of the biochemical index of potential soil fertility (Mw) both in cultivated and uncultivated soils. The highest Mw value was reached in cultivated soil treated for 11 days with the lower concentration of *S. quadricauda* extract (Mw 9.47). All treatments significantly increased the growth of tomato plants with respect to untreated soil. These results are very promising with a view to improving soil biological activity as well as increasing plant growth.

Key words: biostimulants, *Chlorella vulgaris*, *Scenedesmus quadricauda*, tomato, Mw index.

Introduction

Soil plays a crucial role in the functioning and long-term sustainability of ecosystems. Soil enzymes are produced by the soil microorganisms and perform an important function in maintaining the ecological, physical and chemical properties of soil as well as its fertility and health. The study of soil enzymology is a very useful method to study the soil fertility since soil enzyme assays are simple, accurate, cheap and generally based on short term laboratory assessment (Nannipieri et al. 2012). Therefore, soil enzymology is still considered of practical importance in evaluating the effect on soil fertility management (Utobo and Tewari 2015). In particular, fluorescein diacetate hydrolysis (FDA) and dehydrogenase activities (DHA) are considered to be related to the oxidative processes of organic molecules and reflect the metabolic state of the soil (Nannipieri et al. 2012; Fernández et al. 2009). Therefore, using biostimulant substances to improve enzyme activities is of considerable importance.

It was observed that the application of seaweeds and their extracts can enhance soil health by increasing moisture holding capacity and promoting the growth of beneficial soil microbes (Moore 2004; Khan et al. 2009). The structure and enzyme activities of soils can also be improved by many substances produced by cyanobacteria. Caire et al. (2000) observed that the incorporation of cyanobacteria biomass and their exopolyssaccharides into the soil promoted the growth of other microorganisms and increased the activity of soil enzymes that participate in liberating the nutrients required by plants. Furthermore, these results were confirmed by Mahmoud et al. (2007), who observed that cyanobacterial inoculation generally enhanced soil biological activity.

Microalgae have been the subject of various studies due to their great adaptability to various nutrient substrates and their ability to grow in different environmental conditions (Mata et al. 2010; Safi et al. 2014). Formulations of photosynthetic organisms such as microalgae-based ones are promising because of the significant contributions they make to the maintenance

of soil fertility and enhancing crop yields (Li et al. 2017). Faheed and Abd El Fattah (2008) showed that *Chlorella vulgaris* used as a liquid extract positively affected the growth parameters and physiological responses of *Lactuca sativa*. It has been documented that *C. vulgaris* contains high amounts of macro and micronutrients, constituents or metabolites, such as carbohydrates and proteins as well as growth promoting factors, such as cytokinins (Wake et al. 1992; Stirk et al. 2002; Ördög et al. 2004). Elhafiz et al. (2015) found that living cells of *C. vulgaris* distributed in irrigation water may be a promising sustainable biofertilizer, in terms of both dry weight of plant and chlorophyll content, for growing rice, lettuce, cucumber and aubergine. Furthermore, a consortium of *Stenotrophomonas maltophilia* and *C. vulgaris* used as a soil amendment was shown to improve the root and leaf area of meadow clover plantlets (Raposo and Morais 2011). The function of green microalgae as bioactive compounds in the soil and their ability to improve plant growth makes them a suitable biofertilizer (Renuka et al. 2018). Coppens et al. (2016) found that the application of microalgae to an organic growing medium positively affected tomato plant growth at a level comparable to that of a commercial organic fertilizer. Moreover, it was recently shown that living cells of *C. vulgaris* and *Scenedesmus quadricauda* added to a Hoagland solution positively affect the growth of hydroponically-cultivated tomato plantlets (Barone, Puglisi et al. 2018). Unfortunately, there is no literature concerning the treatment of agricultural fields with microalgae which focuses on their effect on soil enzymatic activity. Moreover, in the majority of studies, the addition to the soil of substances with a putative biostimulant effect was evaluated in long-term treatments and mainly concerned with plant growth.

The aim of this work was therefore to evaluate soil enzymatic activity in the short term in response to the addition of living cells of *C. vulgaris*, *S. quadricauda* or their extracts. The soil enzymology approach was applied by monitoring FDA, DHA, acid and alkaline phosphomonoesterase (ACP and ALP, respectively) and urease (URE) activities, which were useful for calculating the potential biochemical index (Mw) of soil fertility, taking into account

DHA, ACP, ALP and URE activities, as well as organic carbon content. The effects of microalgae or their extracts on soil cultivated with tomato plants were also investigated and the preliminary effects (leaf dry weight and chlorophyll content) on plant growth were evaluated.

Materials and methods

Microalgae culture and preparation of extracts

The microalgae used in this study were *C. vulgaris* (Beijerinck (1890) CCAP 211/11C) and *S. quadricauda* (isolated from an algal company raceway pond, in Borculo, Gelderland, the Netherlands in 2011) obtained by and maintained in the algal collection of Swansea University (Wales, UK). The microalgae were cultivated in a growth chamber for 30 days using standard BG11 algae culture medium at pH 8.4 (Stanier et al. 1971), bubbled with air and incubated on a mechanical shaker (100 rpm) at 25–30 °C, illuminated by a 3500-lx average photon flux (PPF) 100-μmol m^{-2} s^{-1} light source (Philips SON-T AGRO 400) with a 12 h photoperiod. The biomass of each species (referred to as Cv and Sq for *C. vulgaris* and *S. quadricauda,* respectively) was recovered by centrifugation and the biomass was washed with distilled water (until conductivity < 200 μS cm^{-1}) and used to inoculate the soils. The characterization of the microalgae was detailed in Barone, Baglieri et al. (2018) and Puglisi et al. (2018).

Extracts were prepared according to Barone, Baglieri et al. (2018). In brief, the final microalgal pellets obtained from the biomass were added to methanol to lyse the cell wall so as to obtain the intracellular extracts. After centrifugation and evaporation of the organic solvent, the extract was collected with distilled water, to obtain a microalgal extract stock solution. The characterization of the microalgal extracts was given in details in Barone, Baglieri et al. (2018).

Experimental conditions and soil characterization

The experiment was conducted on agricultural top soil under laboratory conditions in a growth chamber at 25 ± 2 °C, illuminated by artificial light with a 16-h photoperiod. The soil was classified as a *Typic xerofluvents* soil according to the USDA Soil Taxonomy (Soil Survey Staff, 2014). The soil samples were air dried, sieved at 2 mm and characterized for pH, texture,

organic carbon, phosphorus and Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} cations content. Following the procedures described in Violante et al. (2000), pH, organic carbon, Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} were routinely performed for each soil sample throughout the experimental period. Briefly, the texture of the soil was evaluated using the pipette method, the organic carbon was determined by the oxidation-titrimetric method and cations in the soil were determined by atomic absorption spectrometry (Perkin Elmer, 3110), after extraction with a $BaCl_2$ solution. Phosphorus was determined by the Olsen method (Olsen et al. 1954).

One kg of sandy loam soil (sand, silt and clay were measured only prior to the experiment and were 73.2%, 18.5% and 8.3%, respectively) was placed in a plastic pot (15x15x10 cm) and hydrated to 50% water holding capacity (WHC). After two days of acclimatization at room temperature (25 °C ± 1), the pots were treated. The extract stock solutions of *C. vulgaris* or *S. quadricauda* (characterized in Barone, Baglieri et al. 2018) were added to the soil in a single dose to obtain a final concentration of 0.5 mg (referred to as CvC1 and SqC1) and 1 mg (referred to as CvC2 and SqC2) of dry organic matter of the extract per kg of soil (w/w), corresponding to 1 mg Corg L^{-1} and 2 mg Corg L^{-1} in the free water of the soil, respectively. These two concentrations were chosen on the basis of the results obtained in a hydroponic cultivation of sugar beet (Barone, Baglieri et al. 2018).

The pots treated with living cells of *C. vulgaris* or *S. quadricauda* (referred to as Cv and Sq, respectively) were supplemented with fresh microalgal biomass obtained as described above. The quantity of microalgal biomass added to the soil (68 mg and 55 mg biomass of fresh Cv and Sq, respectively) corresponded to the amount necessary to obtain an extract concentration of 1.5 mg Corg L^{-1}.

For each treatment (both extracts and living cells of microalgae), 5 replicates were performed in 5 independent pots, for both cultivated and uncultivated soils. Four young (four-leaf stage) tomato plants (*Lycopersicon esculentum* cv. Missouri), were transplanted to cultivated soils immediately after the soil had been treated.

Sampling was performed, both in cultivated and uncultivated soils, by randomly extracting a cylinder of soil 10 cm long and 1 cm diameter extending for the whole depth of the pot, from 5 different points of the soil, then the soil samples from the same pot were homogeneously mixed. Time 0 was referred as the day on which the treatments were performed. Homogeneous soil samples were collected from each pot at 0, 1, 4, 11 and 18 days' post-treatment and immediately processed for enzymatic analysis as suggested by Gianfreda and Ruggiero (2006). Irrigation was applied when needed to maintain 50% WHC.

Soil enzymatic activities

FDA activity was assayed according to Green et al. (2006), immediately after the soil sampling. Briefly, 1 g of soil was dissolved in 60 mM sodium phosphate buffer, pH 7.6. Reaction was started by adding 4.9 mM fluorescein diacetate as substrate and the samples were incubated for 3 h at 37 °C. Hydrolysis reaction was stopped by adding 2 mL of acetone. The samples were centrifuged at 8820 x g for 5 min. Absorbance of the filtered supernatant was then measured by spectrophotometry (Jasco V-530 UV-vis spectrophotometer) at 490 nm. The fluorescein concentration hydrolyzed during the reaction was calculated from a fluorescein standard calibration curve.

DHA (EC 1.1) was assayed according to von Mersi and Schinner (1991), immediately after the soil sampling. DHA was determined in 1M Tris(hydroxymethyl) aminomethane (TRIS buffer), pH 7, by mixing 1g of soil with 9.88 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazoliumchloride (INT) solution, prepared daily. Samples were incubated for 2 h at 40 °C, then a mixture of ethanol and dimethylformamide (1:1) was used to extract the reduced iodonitrotetrazolium formazan (INTF). Quantification was performed photometrically (Jasco V-530 UV-vis spectrophotometer) at 464 nm and the concentration of INTF in the samples was calculated from an INTF standard calibration curve.

Acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphomonoesterase activities (ACP and ALP, respectively) were determined using a modified version of the two original methods

(Tabatabai and Bremner 1969; Eivazi and Tabatabai 1977), immediately after the soil sampling. The buffer was made of 28 mM TRIS, 28 mM maleic acid, 19 mM citric acid and 28 mM boric acid pH 6.5 and pH 11 for ACP and ALP, respectively. Soil samples (1 g) were incubated at 37 °C for 1 h using 115 mM p-nitrophenyl phosphate as the substrate, prepared daily. Reaction was stopped with 0.5 M calcium chloride and 0.5 mM sodium hydroxide, then the sample was filtered. The resulting colour was determined photometrically (Jasco V-530 UV-vis spectrophotometer) at 400 nm. The concentration of p-nitrophenol (PNP) released in the samples was calculated from a p-nitrophenol standard calibration curve.

Urease activity (EC 3.5.1.5) was performed using a modified Berthelot method (Kandeler and Gerber 1988). The soil samples (5 g) were mixed in a 720 mM buffered urea solution and incubated for 2 h at 37 °C. The samples were treated with 2 M potassium chloride and were filtered in order to prevent the interference of possible precipitates. Under alkaline pH conditions (pH 10), a green-coloured complex was formed as a result of the reactions between NH_3 and sodium salicytate in the presence of sodium dichloroisocyanurate. Urease activity was expressed as N released in the reaction and was determined photometrically (Jasco V-530 UV-vis spectrophotometer) at 690 nm, calculated from a NH_4Cl standard calibration curve. Sodium nitroprusside was used as a catalyst and increased the sensitivity of the method about tenfold.

FDA, DHA, ACP, ALP and URE activities were performed on three replicates of mixed soil samples for each pot.

Potential biochemical index of soil fertility (Mw)

The potential biochemical index of soil fertility (Mw) was calculated as proposed by Kalembasa and Symanowicz (2012) to include ACP, ALP, DHA and URE activities, as well as organic C content, using the following formula:

$$Mw = (ACP + ALP + DHA + URE \times 10^{-1}) \times \% C$$

where C is organic carbon.

Physiological parameters of tomato plants

Tomato plants were divided into roots and leaves, and the leaves were weighed separately from the roots. The samples were placed in a drying oven at 70 °C until constant weight was reached and allowed to cool for 2 h inside a closed bell jar, then the dry weight was measured per plant.

The chlorophyll content represented by the SPAD index was measured using the SPAD-502 Leaf Chlorophyll Meter (Minolta Camera Co., Ltd., Osaka, Japan) on three points of the last expanded leaf of each tomato plant for all the replicates.

Statistical analysis

Data were analyzed by one-way ANOVA ($p < 0.05$) followed by the Tukey's test for multiple comparison procedures.

Results and discussion

Soil enzymatic activities

Results showed that the addition of *C. vulgaris*, *S. quadricauda* or their extracts affected FDA within just a few days after treatment (Figure 1). The results seem to be quite similar in cultivated and uncultivated soils. One day after treatment, FDA activity slightly decreased in both experimental soils (cultivated and uncultivated soils) in comparison to the untreated soil, mainly as regards living cells of *C. vulgaris* and its extracts (Figures 1 A and B). The decrease in activity was probably due to the autochthonous microorganisms of the soil which need to adapt their metabolism to the new environmental condition of the soil modified by the treatments. The only exception was the treatment of cultivated soil with SqC1 in which FDA activity increased with respect to the untreated soil. In 4-day treated samples, FDA activity increased in all the treatments, reaching the highest value with Cv that was 2 and 2.7 times higher than the untreated soil in the uncultivated (Figure 1 A) and cultivated (Figure 1 B) soils, respectively. After 11 days, in all the treatments the values of FDA activity remained higher than the untreated soils (Figure 1) except for soils treated with CvC2 (Figure 1 A and B) and Cv for uncultivated soil (Figure 1 A). Finally, after 18 days, in the uncultivated soil only the treatment

with living cells of *S. quadricauda* (Sq) and CvC2 showed an evident increase with respect to the untreated soil (Figure 1A). Putatively *S. quadricauda* needs more time than *C. vulgaris* to release those substances that have a bioactive effect on FDA activity in uncultivated soil. Interestingly, in cultivated soils at the end of the experimental period all the treatments determined an increase in FDA activity in the soils with respect to untreated soil, except for SqC2 (Figure 1 B). In this latter case the negative effect on FDA activity may be due to the plant-soil interaction, since this reduction was not observed in the corresponding experiment with uncultivated soil (Figure 1A).

Considering that Gaspar et al. (2001) found that FDA activity may function as a sensitive estimator of fungal biomass, it could be envisaged that the addition of living cells of *C. vulgaris* may somehow be involved in the evolution of the soil microbial community to the benefit of fungi. This effect was more evident in the cultivated soils than the uncultivated ones. Moreover, the global net quantity of FDA hydrolyzed in the cultivated soils was greater than that detected in uncultivated soils, thus implying that plant-rhizosphere interaction positively contributed to the FDA activity until the end of the experimental period. One hypothesis may be that the plant exudates produced in the rhizosphere determined a suitable environment for the microbial population involved in FDA hydrolysis. In fact, root exudates play a key role in determining the selection of rhizobacterial populations (Brimecombe et al. 2001).

As already observed for FDA activity, DHA was affected quite similarly both in cultivated and uncultivated soils (Figure 2). Over the whole experimental period, all the treatments stimulated dehydrogenase activity in the cultivated and uncultivated soils, with respect to the untreated soils (Figure 2), except in uncultivated soils for the SqC1 and Sq treatments after only 1 day of treatment and SqC1 and SqC2 at the end of the experimental period (18 days), which in any case maintained similar values to the untreated soil (Figure 2 A). The highest values of DHA activity were observed after 4 days of treatment, when the treatment with Cv proved to be the best treatment, showing an increase of around 3.5 and 4.3 times higher than the untreated soils for uncultivated and cultivated soils, respectively (Figure 1).

It is worth observing that the treatment with Cv proved to be very efficient in all the soils (cultivated and uncultivated) as regards both FDA and DHA activities, probably because both these enzymatic activities are involved in the oxidative processes of organic molecules (Oliveira and Ferreira 2014). Our results suggest that *C. vulgaris* may produce some substances in its attempt to survive in the soil, as already observed in Liu et al. (2016). In fact, an adaptive response of *C. vulgaris* to the change of the environmental conditions might be modulated by the production of both intra and extracellular polysaccharides. Moreover, different high-molecular-weight biopolymers may be secreted from microalgae into the surrounding adverse environment during their growth or propagation (Delbarre-Ladrat et al. 2014, Xiao and Zheng 2016). These compounds probably protect microalgae cells from unfavorable stress in the natural environment (Prajapat and Patel 2013). It cannot be excluded that these different substances produced by *C. vulgaris* when adapting to the new environmental condition (soil) may induce a higher microbial activity in the soil, involved in the oxidative processes of organic molecules. Therefore, these results proved to be very interesting.

Figures 3 and 4 show ACP and ALP activities in cultivated and uncultivated soils subjected to the different treatments. ACP activity (Figure 3) remained comparatively constant at each sampling except in soils treated for 4 days. In effect, at this sampling, ACP activity sharply increased in uncultivated soils treated with SqC1 and CvC1, reaching values of up to 6 and 5.5 fold higher than those of the untreated soil, respectively (Figure 3 A). Similarly, in the cultivated soils the treatments with SqC1, CvC1 and Sq, recorded values about 4.3, 3.4 and 2.4 fold higher than the untreated soil, respectively (Figure 3 B).

ALP (Figure 4) showed a quite dissimilar trend to that observed for ACP activity. The greatest increase was registered likewise after 4 days of treatment, the treatment with Sq being the best activator, reaching a value about 3.6 and 4.8 times higher than the untreated soil, in uncultivated and cultivated soils, respectively. Moreover, in uncultivated soil (after 4 days) the treatments with Cv and CvC2 also induced activity of around 2.9 and 2 times higher with respect

to the untreated soil, respectively (Figure 4 A). Surprisingly, after 11 days of treatment ALP activity was positively affected by SqC2 and CvC2 in the uncultivated soils and by SqC1 and Cv in the cultivated soils. Finally, at the end of the experimental period (18 days) all the treatments showed values of ALP activity similar to the untreated soil, except for the CvC2 in both cultivated and uncultivated soils (Figure 4) and the Cv treatment in cultivated soil (Figure 4 B).

Interestingly, these results suggest that ACP activity seems to be more stimulated, in quantitative terms of PNP released, by microalgal extracts but only 4 days after the treatment. Overall, these results are in agreement with a soil pH around 7.7 (Table 1), which is closer to the optimal value for ACP (pH6.5) than that for ALP (pH11.0). Conversely ALP activity reached the highest values in treatments with living microalgae cells, especially in cultivated soils, in which Cv was still bioactive until the end of the experimental period (Figure 4 B). Although, in uncultivated soil CvC2 seems to be the only treatment which maintained its effect until the end of the experimental time (Figure 4 A). According to Dick et al (1997) ACP and ALP activities may be affected by soil pH, the availability of macro and micro elements and microbial biomass, however being the soil pH and macro elements quite constant throughout the experimental period (table 1), these effects may be due to the variation that living microalgae or their extracts exerted on micro element availability as well as microbial biomass.

Extracellular urease activity constitutes around 63% of the total activity of this enzyme found in soil (Martinez-Salgrado et al. 2010). It catalyzes the hydrolysis of urea into CO_2 and NH_3, through carbamate as an intermediate, resulting in a rapid loss of nitrogen to the atmosphere through NH_3 volatilization (Tabatabai 1982; Simpson et al. 1984; Simpson and Freney 1988).

Figure 5 shows urease activity in uncultivated and cultivated soils subjected to different treatments. The most relevant result was observed in uncultivated soil 4 days after treatment (Figure 5 A). In this latter case the soil treated with *C. vulgaris* extract at a lower concentration (CvC1) showed the highest value reaching a value 2.4 times higher than untreated soil, whereas

the SqC1 and Cv treatments slightly decreased URE activity with respect to the untreated soil (Figure 5 A). This finding was in contrast with those observed for all the other activities, which always showed higher values for treatment with Cv after 4 days of treatment. However, it is equally true that the variability of enzymatic activities in soil changes according to the availability of the substrate. The presence of *C. vulgaris* probably increases the availability of urea in the uncultivated soil immediately after its addition (1 day), although this is the only case in which Cv increased URE activity. It is interesting to note that in cultivated soils URE activity was reasonably constant throughout the experimental period, showing a value lower than the untreated soil only 1 day after treatment with Sq (Figure 5 B).

Soil contains many free enzymes, that play a critical role in catalyzing reactions leading to the decomposition of organic matter and which serve as bioindicators of biochemical and microbial soil activities. The addition of biostimulant substances to the soil may affect enzymatic activities and thus potential plant growth. Noteworthy the organic carbon values (Table 1) were kept at constant level throughout the experimental period (0.6%). Putatively the treatments did not affect the organic carbon level since the soil was characterized by a low level of organic carbon (Table 1). In order to evaluate soil fertility, a potential biochemical index (Mw) was calculated, following Kalembasa and Symanowicz (2012). Mw was calculated throughout the experimental period for all the treatments (Table 2). This index is very useful as it takes into account all the enzymatic activities calculated in the present study, in order to establish the best treatment in terms of soil fertility. In fact, each enzymatic activity was differently affected by the treatments throughout the experimental period.

Interestingly, the Mw values were quite different in the cultivated and uncultivated soils (Table 2). These results are in accordance with the evidence that crops influence rhizosphere biological or biochemical activity (Pinton et al. 2007; Pii et al. 2015). In the uncultivated soils the Mw values greatly increased with respect to the untreated soils 4 and 11 days after all the treatments. At t4 the lower concentrations of the extracts from both microalgae species (SqC1,

Mw 7.53; CvC1, Mw 7.99) proved to be the best treatments. However, the effect of the treatments differs according to the microalgal species used (Table 2). In fact, the results showed that *S. quadricauda* as well as its extracts, positively affected Mw values until 11 days after treatment (SqC1, Mw 7.58; SqC2, Mw 7.92; Sq, Mw 7.26), thereafter at the end of the experimental period (18 days) Mw values sharply decreased (SqC1, Mw 4.67; SqC2, Mw 3.86; Sq, Mw 5.38). Conversely, *C. vulgaris* and its extracts increased the values of Mw throughout all the experimental period reaching at this time, especially in the case of the extracts, the highest Mw values (Table 2). Therefore, in the uncultivated soils the treatments that proved to be most efficient, as referred to Mw, for the whole experimental period seem to be *C. vulgaris* and its extracts.

In the cultivated soils all the treatments at each sampling time showed Mw values always greater than the untreated soils (Table 2), the lowest concentration of *S. quadricauda* extract (SqC1) 11 days after treatment having the greatest Mw value (9.47). It should be noted that, at the end of the treatments (18 days) all the Mw values were lower than the values observed after 11 days. However, in this latter case (18 days) the most efficient treatment proved once again to be *C. vulgaris* cells and its extracts, as observed in the uncultivated soils.

On the basis of our results, it seems that both microalgae and their extracts positively affect soil functioning as evaluated by enzymatic activities. Taking all these results together, it is possible to hypothesize that the direct use of living cells of *C. vulgaris*, may be a more easily handled and cheaper treatment method in order to obtain the same beneficial effects. Furthermore, microalgae added to the soils seem to have a bioactive effect inducing enzyme activities, above all FDA, dehydrogenase and partially urease, using a small amount of biomass. Therefore, our results are very promising if compared to the dose of other substances applied to the soil to obtain similar Mw values (Kalembasa and Symanowicz 2012; Symanowicz et al. 2014).

The presence of tomato plants positively influenced the Mw values probably due to the action of the rhizosphere activities (Brimecombe et al. 2001). Nevertheless *C. vulgaris* and its extracts seem to be less sensitive to the action of the plants than *S. quadricauda* and its extracts in the long period of 18 days, although the effect of these treatments (especially for CvC1) in the uncultivated soils showed a greater positive action on the Mw values.

Physiological parameters of tomato plants

In order to evaluate the overall growth of the tomato plants subjected to treatment with *C. vulgaris* and *S. quadricauda* as well as their extracts, the dry weight and SPAD index were determined (Table 3). It is interesting to note that all the treatments enhanced leaf dry weight. These results seem to be very interesting, since applying living cells of microalgae species or their extracts (in particular, *C. vulgaris*) may result simultaneously in a better biochemical functioning in soil, as well as increased plant growth.

In accordance with leaf dry weights, the chlorophyll content (in relative SPAD units) increased in each treatment if compared to the control (Table 2). Numerous studies have shown that various biostimulant substances increase the content of chlorophyll pigments in different plant species. Increments of chlorophyll content and dry weight of maize plants were obtained by Shaaban (2001) after soil application of *C. vulgaris*. Higher chlorophyll content and net photosynthesis activity was also found after *Chlorella* sp. application in maize (Grzesik and Romanowska-Duda 2015), while another study observed a pigment content increase in *Lactuca sativa* seedlings grown in fertilized soils with *C.vulgaris* (Faheed and Abd El Fattah 2008). Increments of 19% were observed by Khan et al. (2009), in grapevines after application of seaweed extract and amino acids, while Spinelli et al. (2009) noticed a 12% increase in “Fuji” apple after seaweed extract treatment. Recently, a chlorophyll content increase was observed in *Salix viminalis,* after biofertilization using cyanobacteria and green algae (Grzesik and Romanowska-Duda 2015).

Our results are also in accordance with those obtained by Barone, Baglieri et al. (2018), who found that extracts obtained from *C. vulgaris* and *S. quadricauda* were promising biostimulants in the early stages of plant growth in sugar beet. Moreover, Barone, Puglisi et al. (2018) showed that *C. vulgaris* and *S. quadricauda* enhanced the growth of tomato seedlings in hydroponic culture. The application of a low dosage of microalgae (max 68 mg of biomass kg^{-1} of soil) to the soil makes them a strong biostimulant for tomato plants. Coppens et al. (2016) needed to apply to the organic growing medium a quantity of microalgae (*Nannochloropsis oculata*) one thousand times greater to obtain a biofertilizer effect on tomato plants. Putatively, an interesting, economical and direct application of microalgae to the soil may be obtained by the addition of biomass obtained from the purification of wastewater as described in Baglieri et al. (2016). Although further experimental studies are necessary, e.g. it would be useful to investigate the potential effect of the incorporating microalgae and their extracts into the soil in long-term-trials under greenhouse- and field conditions, this idea may be a very cheap and useful application to help farmers improve soil functioning as well as an increase in the growth performance of tomato plants.

Conclusion

This is the first work regarding the direct application of living cells of *S. quadricauda* or *C. vulgaris* to the soil, which aims to evaluate the soil enzymatic activities as well as their bioactive effect on tomato plants in a short-term period of treatment. Successfully, the treatments of soils with the microalgae or their extracts positively affected both the soil biological activity and the growth of tomato plants. Considering the increased world production of microalgae and their wide use in different fields, the fate of microalgae biomasses may be a critical point. Therefore, when cultivating tomato plants the application of microalgae onto the soil may be a useful way of reusing waste microalgal biomass, helping to reduce disposal costs and at the same time improving soil biological activity. Therefore, although the microalgae extracts seem to have a greater influence on soil biochemical fertility, the direct use of living microalgae cells in the

soil may be an easier-to-handle and cheaper method of treatment in order to obtain the dual benefits of sustainable cultivation of tomato plants and a reduction in the cost of chemical fertilizers.

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Table 1. Characterization of the soils. NCS: uncultivated soil; CS: cultivated soil; SqC1: *S. quadricauda* extract, 1 mg Corg L^{-1}; SqC2: *S. quadricauda* extract, 2 mg Corg L^{-1}; Sq: living cells of *S. quadricauda*, 55 mg in biomass; CvC1: *C.vulgaris* extract, 1 mg Corg L^{-1}; CvC2: *C.vulgaris* extract, 2 mg Corg L^{-1}; Cv: living cells of *C.vulgaris*, 68 mg in biomass. No letters indicate no significant differences ($p < 0.05$).

Treatment	Sampling	Soil properties													
		pH		OC (%)		P (%)		Ca (%)		K (%)		Mg (%)		Na (%)	
		NCS	CS	NCS	CS	NCS	CS	NCS	CS	NCS	CS	NCS	CS	NCS	CS
Untreated soil	**0**	7.70	7.63	0.60	0.61	0.26	0.26	1.24	1.26	0.46	0.45	0.18	0.16	0.57	0.56
	1	7.61	7.69	0.63	0.62	0.24	0.25	1.20	1.26	0.43	0.48	0.17	0.19	0.55	0.58
	4	7.63	7.61	0.59	0.61	0.26	0.27	1.29	1.24	0.44	0.46	0.19	0.20	0.60	0.56
	11	7.80	7.63	0.62	0.60	0.25	0.27	1.24	1.23	0.45	0.44	0.17	0.19	0.55	0.56
	18	7.72	7.64	0.58	0.59	0.28	0.26	1.25	1.23	0.46	0.44	0.18	0.17	0.60	0.55
SqC1	**0**	7.65	7.75	0.60	0.60	0.25	0.28	1.25	1.26	0.47	0.43	0.16	0.18	0.56	0.57
	1	7.63	7.71	0.58	0.61	0.24	0.26	1.23	1.22	0.45	0.45	0.18	0.16	0.55	0.54
	4	7.72	7.68	0.63	0.60	0.22	0.20	1.24	1.25	0.46	0.44	0.20	0.17	0.57	0.62
	11	7.80	7.65	0.62	0.62	0.25	0.26	1.22	1.26	0.45	0.46	0.17	0.17	0.60	0.57
	18	7.71	7.66	0.59	0.61	0.29	0.25	1.24	1.24	0.40	0.45	0.17	0.16	0.54	0.56
SqC2	**0**	7.70	7.72	0.60	0.61	0.27	0.26	1.27	1.25	0.44	0.45	0.17	0.18	0.55	0.60
	1	7.75	7.68	0.58	0.60	0.25	0.25	1.22	1.26	0.46	0.47	0.18	0.20	0.56	0.57
	4	7.71	7.69	0.61	0.62	0.26	0.28	1.24	1.24	0.43	0.44	0.16	0.19	0.61	0.57
	11	7.69	7.70	0.59	0.61	0.28	0.27	1.23	1.23	0.46	0.47	0.18	0.18	0.55	0.56
	18	7.68	7.72	0.64	0.59	0.24	0.25	1.25	1.26	0.44	0.46	0.19	0.17	0.55	0.55
Sq	**0**	7.75	7.73	0.60	0.58	0.26	0.26	1.22	1.26	0.46	0.47	0.16	0.19	0.57	0.53
	1	7.68	7.70	0.63	0.61	0.27	0.25	1.28	1.20	0.48	0.44	0.20	0.18	0.63	0.58
	4	7.65	7.70	0.60	0.62	0.23	0.26	1.24	1.26	0.43	0.49	0.18	0.20	0.55	0.59
	11	7.72	7.71	0.58	0.62	0.28	0.27	1.23	1.24	0.46	0.45	0.16	0.17	0.54	0.60
	18	7.71	7.68	0.61	0.60	0.26	0.25	1.23	1.25	0.43	0.42	0.18	0.16	0.55	0.57
CvC1	**0**	7.69	7.73	0.58	0.60	0.25	0.24	1.25	1.23	0.44	0.44	0.13	0.16	0.56	0.58
	1	7.70	7.69	0.63	0.58	0.26	0.28	1.22	1.26	0.44	0.46	0.22	0.20	0.57	0.60
	4	7.70	7.69	0.56	0.61	0.27	0.27	1.24	1.25	0.45	0.48	0.20	0.18	0.56	0.58
	11	7.67	7.67	0.63	0.59	0.26	0.26	1.26	1.23	0.46	0.46	0.17	0.18	0.57	0.55
	18	7.69	7.72	0.59	0.60	0.25	0.25	1.24	1.24	0.44	0.43	0.16	0.16	0.58	0.56
CvC2	**0**	7.70	7.75	0.59	0.63	0.27	0.26	1.24	1.24	0.45	0.46	0.15	0.15	0.60	0.57
	1	7.65	7.68	0.61	0.61	0.24	0.24	1.23	1.25	0.44	0.43	0.18	0.20	0.55	0.57
	4	7.70	7.70	0.63	0.59	0.26	0.25	1.27	1.25	0.46	0.45	0.22	0.17	0.56	0.57
	11	7.72	7.69	0.59	0.57	0.25	0.26	1.24	1.24	0.43	0.49	0.20	0.18	0.60	0.56
	18	7.68	7.72	0.57	0.62	0.27	0.27	1.23	1.25	0.46	0.44	0.16	0.18	0.57	0.58
Cv	**0**	7.78	7.70	0.64	0.56	0.26	0.27	1.25	1.26	0.43	0.44	0.20	0.16	0.58	0.57
	1	7.68	7.72	0.56	0.64	0.27	0.26	1.23	1.25	0.46	0.46	0.17	0.19	0.56	0.56
	4	7.65	7.70	0.64	0.59	0.28	0.26	1.24	1.22	0.44	0.45	0.20	0.17	0.57	0.58
	11	7.68	7.74	0.58	0.59	0.24	0.25	1.26	1.25	0.48	0.46	0.16	0.18	0.56	0.55
	18	7.75	7.72	0.62	0.64	0.26	0.24	1.25	1.24	0.43	0.45	0.17	0.18	0.57	0.57

Table 2. Biochemical index of potential soil fertility (Mw), in uncultivated and cultivated soils treated with living cells of *C. vulgaris* or *S. quadricauda* and their extracts (SqC1: *S. quadricauda* extract, 1 mg Corg L^{-1}; SqC2: *S. quadricauda* extract, 2 mg Corg L^{-1}; Sq: living cells of *S. quadricauda*, 55 mg in biomass; CvC1: *C.vulgaris* extract, 1 mg Corg L^{-1}; CvC2: *C.vulgaris* extract, 2 mg Corg L^{-1}; Cv: living cells of *C.vulgaris,* 68 mg in biomass). The values were calculated using the following formula: Mw= (ACP + ALP + DHA + URE $\times 10^{-1}$) × % C, considering average values of activities.

	Mw									
	Uncultivated soil					**Cultivated soil**				
	0	**1**	**4**	**11**	**18**	**0**	**1**	**4**	**11**	**18**
Untreated	1.14	1.89	2.91	4.77	4.30	1.13	1.86	2.62	4.58	3.64
Sq C1	1.15	2.12	7.53	7.58	4.67	1.15	3.13	7.57	9.47	5.04
Sq C2	1.14	2.38	5.68	7.92	3.86	1.15	2.42	5.60	8.31	4.64
Sq	1.13	1.49	6.35	7.26	5.38	1.14	2.45	7.10	7.71	4.46
Cv C1	1.14	3.03	7.99	8.62	8.72	1.14	2.90	7.62	7.92	6.16
Cv C2	1.15	3.32	6.12	6.82	7.01	1.13	3.39	4.84	6.02	5.52
Cv	1.14	3.38	6.73	8.68	7.07	1.14	3.00	6.23	8.21	6.58

Table 3. Leaf dry weight and SPAD index in leaves of tomato plants grown for 18 days in soils treated with living cells of *C. vulgaris* or *S. quadricauda* and their extracts. Values followed by different letters are significantly different ($p < 0.05$).

	Leaf Dry weight (g)		**SPAD**	
Untreated	1.87 ± 0.14	b	35.1 ± 2.14	b
Sq C1	2.34 ± 0.21	a	42.3 ± 3.73	a
Sq C2	2.29 ± 0.11	a	40.2 ± 2.51	a
Sq	2.19 ± 0.17	a	39.1 ± 1.82	a
Cv C1	2.48 ± 0.23	a	40.6 ± 3.27	a
Cv C2	2.37 ± 0.15	a	39.4 ± 2.84	a
Cv	2.22 ± 0.17	a	41.2 ± 3.14	a

Figure Captions:

Figure 1. FDA activities (µg FDA per g of soil) in uncultivated (A) and cultivated (B) soils treated with living cells of *C. vulgaris* or *S. quadricauda* and their extracts. The values are means of data from 5 pots and three replicates each. Values of the same soil incubation time followed by different letters are significantly different ($p < 0.05$).

Figure 2. Dehydrogenase activity (µg INTF per g of dry matter in 1 h) in uncultivated (A) and cultivated (B) soils treated with living cells of *C. vulgaris* or *S. quadricauda* and their extracts. The values are means of data from 5 pots and three replicates each. Values of the same soil incubation time followed by different letters are significantly different ($p < 0.05$).

Figure 3. Acid phosphomonoesterase activity (µg PNP per g of dry matter in 1 h) in uncultivated (A) and cultivated (B) soils treated with living cells of *C. vulgaris* or *S. quadricauda* and their extracts. The values are means of data from 5 pots and three replicates each. Values of the same soil incubation time followed by different letters are significantly different ($p < 0.05$).

Figure 4. Alkaline phosphomonoesterase activity (µg PNP per g of dry matter in 1 h) in uncultivated (A) and cultivated (B) soils treated with living cells of *C. vulgaris* or *S. quadricauda* and their extracts. The values are means of data from 5 pots and three replicates each. Values of the same soil incubation time followed by different letters are significantly different ($p < 0.05$).

Figure 5. Urease activity (µg N per g of dry matter in 2 h) in uncultivated (A) and cultivated (B) soils treated with living cells of *C. vulgaris* or *S. quadricauda* and their extracts. The values are means of data from 5 pots and three replicates each. Values of the same soil incubation time followed by different letters are significantly different ($p < 0.05$).

Figure 1

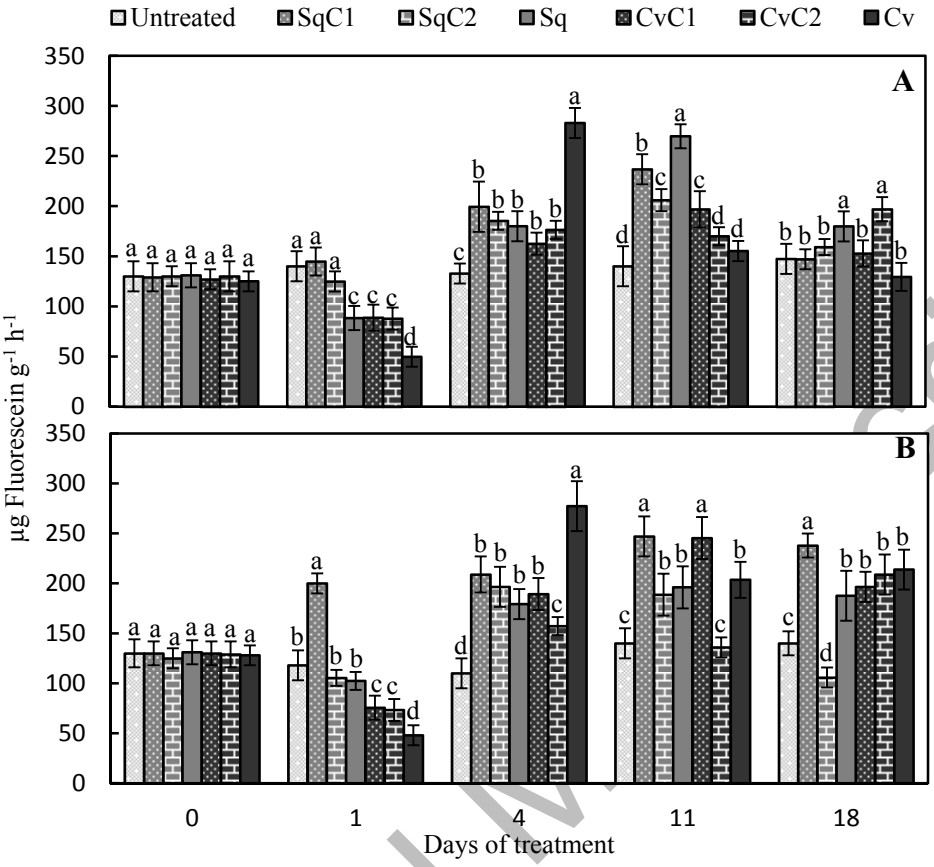

Figure 2

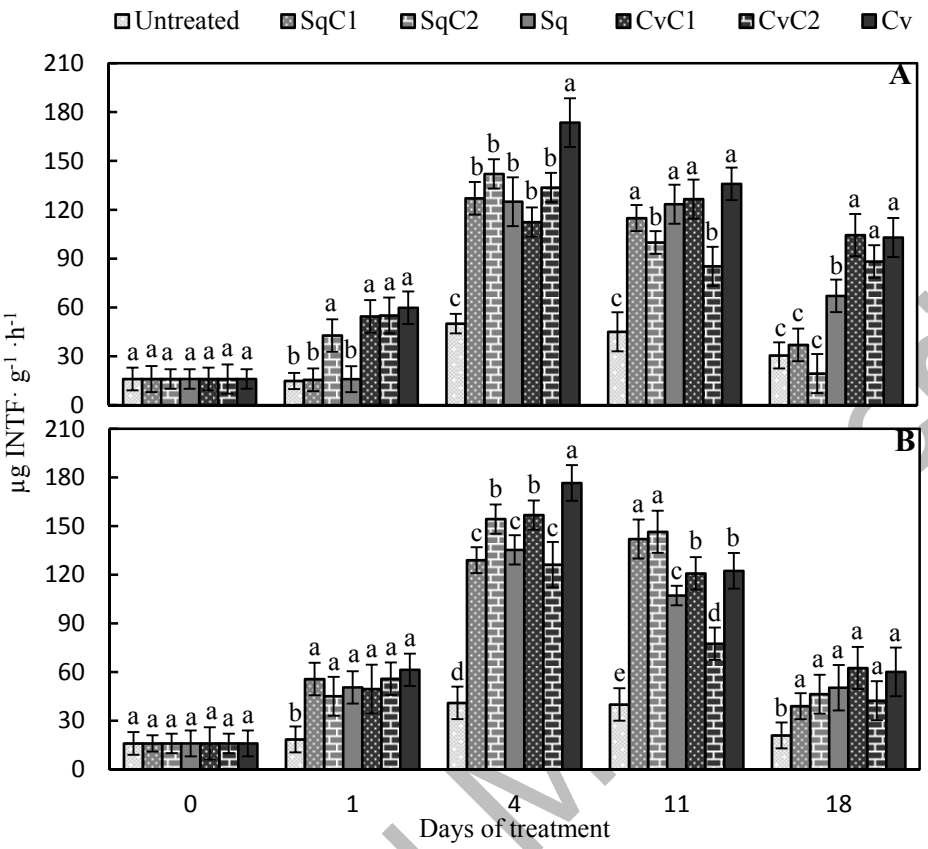

Figure 3

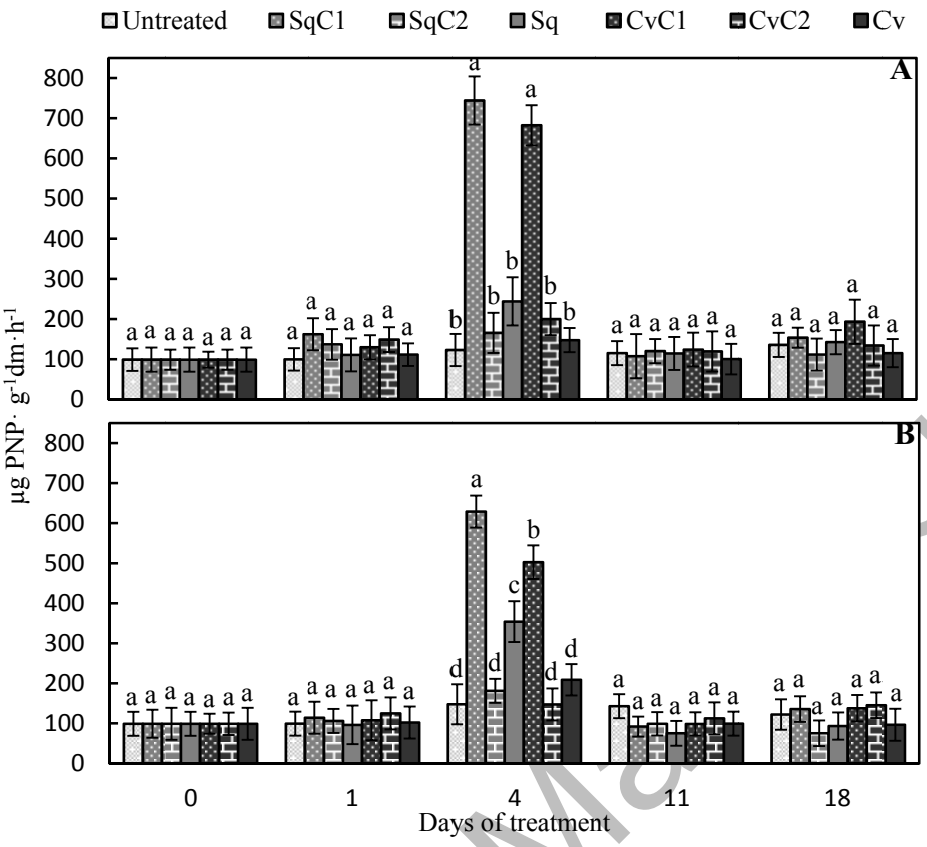

Figure 4

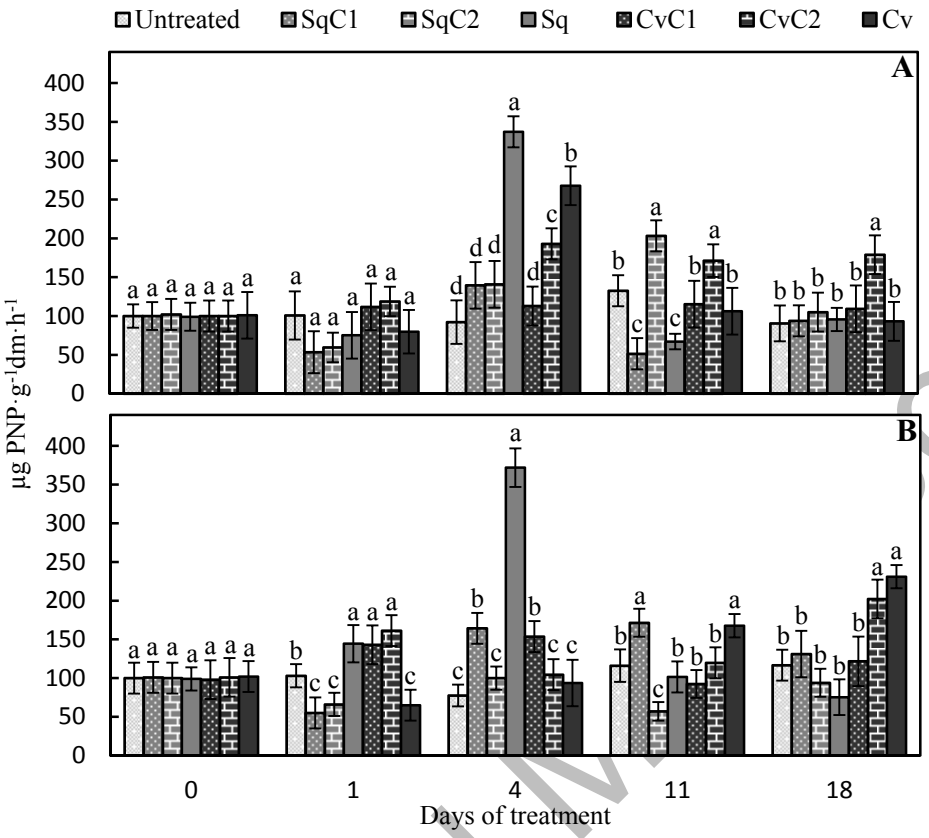

Figure 5