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Chlorella vulgaris as Soil Amendment: Influence of Encapsulation and Enrichment with Rhizobacteria

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ABSTRACT

Several trials with five plant growth-promoting rhizobacteria (PGPR) and the chlorophyte *Chlorella vulgaris* were carried out in order to look for the consortia that could show the best interactions, giving rise to improved growth of mixed cultures. *Pseudomonas putida, Serratia proteomaculans* and *Stenotrophomonas maltophilia* were the chosen bacteria for the consortia with *Chlorella*, while the proportions of microalgae/bacteria tested were 2:1, 3:1 and 3:1, respectively. Three replicates of 20 treatments were performed and studied, after sowing 20 seeds per replicate, for each of the consortia. Plantlets were left to grow for a two-week period. Maltodextrin (MD) and arabic gum (GA) or gelatine (G) were used as coats for the freeze-dried biomass microbeads. Longest roots were obtained with the consortium *Chlorella:Serratia* but encapsulates of *Chlorella:Stenotrophomonas* gave rise to meadow clover plantlets with the highest root and shoot system dried biomass, especially with coating proportions of 1:1 MD:G and MD:GA. Results obtained with this last consortium suggested some interactions with the plant metabolism, as well as some synergistic effects between *Chlorella* and bacteria. © 2011 Friends Science Publishers

Key Words: *Chlorella*; Microencapsulation; *Pseudomonas; Serratia;* Soil substrate; *Stenotrophomonas*

INTRODUCTION

Biofertilizer is a relatively new term that is increasingly being used when referring to soils that are enriched with microalgae, bacteria and/or fungi, usually in consortia, to promote germination and plant growth, including crops. As noted by Vessey (2003), biofertilizer can be any substance that contains living microorganisms, which can colonize rhizosphere or the interior of the plants and promote growth of plants by improving their nutrient status.

It is well known that freshwater algae, as is the case of *Chlorella vulgaris*, contain high amounts of macro and micronutrients, as constituents or metabolites, like carbohydrates and proteins (Wake *et al.,* 1992), but growth-promoting factors, such as cytokinins, have already been found in several strains of microalgae (Stirk *et al.,* 2002; Ördög *et al*., 2004).

In addition, inoculants of plant growth-promoting bacteria (PGPB) and plant growth-promoting rhizobia (PGPR) have also been used in agriculture (Bloemberg & Lugtenberg, 2001), to enhance plant growth (Lucy *et al.,* 2004). They increase the availability of nutrients for the plant in the rhizosphere (Rodriguez & Fraga, 1999). Besides, these bacteria can be useful, helping to increase production of microalgae, important in agriculture as biofertilizers, as for *Chlorella*, whose growth is increased when co-immobilized and co-cultured in alginate beads with a plant growth-promoting bacterium (Hernandez *et al.,* 2009).

Some of the bacteria present in the rhizosphere, which can be entrapped along with *Chlorella* to improve soil fertilization and therefore, plant growth, are *Klebsiella oxytoca, Herbaspirillum chlorophenolicum, Pseudomonas putida, Serratia proteomaculans* and *Stenotrophomonas maltophilia*. There is evidence that *P. putida* acts by solubilising P in the rhizosphere (Richardson, 2001) and by inducing denitrification (Prescott *et al.,* 2002), increasing nutrients availability to host plants. Besides, some strains can also act by influencing on or by phytohormones (Mayak *et al.,* 1999; Belimov *et al.,* 2001). *S. proteomaculans* is a Gram negative bacillus, but is an anaerobic facultative *Enterobacteriaceae*. *Serratia* is able to reduce nitrates to nitrites (Brenner, 1981). *S. maltophilia* is an almost ubiquitous Gram negative aerobic bacillus, found in soils, water, animals and plants (Palleroni & Bradbury, 1993). There is not much information on *Stenotrophomonas*, but De Freitas *et al.* (1997) referred to *Xanthomonas maltophilia* as a rhizobacterium with positive effects on plant growth. In addition, Palleroni and Bradbury (1993) proposed *Stenotrophomonas* as a new bacterial genus for *X. maltophilia*. Moreover, Hoflich and Metz (1997) referred

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Stenotrophomonas as a free-living plant growth promoting rhizobacterium that was used for phytoremediation, stimulating the growth of maize, while absorbing heavy metals.

Entrapment of microorganisms (microalgae & bacteria), besides protecting them from the different types of environmental stress, can gradually supply soils with those microorganisms. Besides, encapsulates of inoculants can be easily stored under room temperature (Bashan *et al.*, 2002).

Alginate is probably the most used polymer to encapsulate microorganisms (Yabur *et al.*, 2007; Bashan *et al.*, 2002), but other polysaccharides have already been used (Farias *et al.*, 2007; Kanakdande *et al.*, 2007; Leach *et al.*, 1998). Considering all the above mentioned studies and reasons, we found of interest to investigate the effect on the development of meadow clover plantlets after inoculating their growth substrate with *C. vulgaris*, alone or in consortia with some strains of rhizobacteria. The effect of encapsulation of microorganisms was also tested.

MATERIALS AND METHODS

Bacteria and bacterial growth conditions: Rhizobacteria *Herbaspirillum chlorophenolicum, Klebsiella oxytoca, Pseudomonas putida, Serratia proteomaculans* and *Stenotrophomonas maltophilia* were isolated from soils and gently supplied by the Microbiology Laboratory of College of Biotechnology (Catholic University, Porto – Portugal). Bacteria were cultivated onto Nutrient agar (Biokar Diagnostics) at 30ºC for 48 h. Identical colonies were used as inoculants for the consortia with *C. vulgaris*.

Microalgae and growth conditions: *C. vulgaris* was isolated in our laboratory as previously described (Lima *et al.*, 2004). Microalgae were axenically grown in OHM (Fabregas *et al.*, 2001), for a two-week period, in a walk-in chamber, under constant light (37 mmoles s^{-1} m^{-2}) and temperature (25ºC). Mixing of cultures was undertaken by bubbling sterile air into the flasks.

Screening for the microalgae/bacteria consortia and proportions between microalgae:bacteria: Growth of *C. vulgaris* and different bacteria consortia was tested in small wells (total volume 2 mL), first with just one bacterium species, then two and three bacterial species, under different proportions of microalgae/bacteria (1:1, 1:2, 1:3, 2:1 & 3:1). Base units were one colony for bacteria and 1 x 10^6 microalgae cells/mL culture medium. After seven days, growth was evaluated by counting microalgae and bacterial CFUs. Tests were carried out in triplicates. Microalgae density (number of cells/mL culture) was measured by direct counting, each culture twice, in a Neubauer improved haemocytometer. To evaluate bacterial growth, strains were sequentially diluted (till 10^{-5}) in a Ringer (Merck 15525) solution and grown onto NA plates, under 30ºC, in duplicates for each well. CFUs were counted after 48 h.

After testing consortia (microalgae:bacteria) growth for each bacterium species, under the conditions indicated before, consortia with *P. putida* (2:1), *S. proteomaculans* (3:1) and *S. maltophilia* (3:1) were chosen for performing the next tests (relative proportions of microalgae:bacteria are written in brackets), this time with two and three different species of bacteria. Amongst the four consortia tested (*Chlorella:Pseudomonas:Serratia, Chlorella:Pseudomonas:Stenotrophomonas, Chlorella:Serratia:Stenotrophomonas* & *Chlorella:Pseudomonas:Serratia:Stenotrophomonas* at the proportions 2:1 and 3:1 (microalgae:bacteria), *Chlorella:Pseudomonas:Serratia* (2:1) was the consortium with more than one species of bacteria that presented the best results, while growing altogether.

From the above results, four consortia were chosen to fertilize the soil and test for the influence on the seed germination and plant development. Such consortia, *Chlorella:Pseudomonas, Chlorella:Serratia, Chlorella:Stenotrophomonas* and *Chlorella:Pseudomonas:Serratia* were then cultivated altogether in OHM liquid medium in order to obtain the needed biomass.

Preparation of biomass to be encapsulated: Cultures of *Chlorella* and consortia that were grown for a seven-day period under the referred conditions, were centrifuged (Sorval Instruments RC5C) at 3500 x g, 10 min, 4-10ºC. Biomass obtained was then freeze-dried (Christ Alpha 1-4, B. Braun Biotech Int). Dried powder was resuspended in sterile deionised water to 0.7% (w:v) just before encapsulation.

Obtaining microbeads by spray-drying: Maltodextrin (DE 12, Fluka/Biochemika 31410), gum Arabic (Merck 4228) and gelatine (type B from bovine skin, Sigma), the polymers used to entrap the microorganisms were suspended in deionised water to 0.7% (w:v) and mixed as MD:GA and MD:G, 1:1 and 1:2.

Suspensions of MD:GA and MD:G (1:1 & 1:2) were then mixed with suspensions of *Chlorella* alone or included in referred consortia, as MD:GA or G:algae or consortia in the proportions 1:1:1, 1:1:2 and 1:2:1 (v:v:v). Frozen-dried *Chlorella* (in the same proportions as in consortia) and consortia were also suspended in deionised water before being spray-dried as it was done with encapsulates. Beads of MD:GA and MD:G, either in 1:1 and 1:2 proportions were also prepared, to be used as controls.

Conditions for the production of microencapsulates: Microencapsulates and powders of *Chlorella* and consortia were accomplished using a mini spray-dryer device (Büchi B-191, Switzerland). Drying conditions were: Inlet temperature 150ºC, Outlet temperature 80-90ºC, aspirator output 80-85%, flow of compressed air (5-8 bar) 500-600 l h^{-1}, peristalsis pump speed 24-26%.

Viability tests to encapsulates: Encapsulates in microbeads and biomass powders were tested for their viability, after having been spray-dried. Plates with solidified OHM (1.5% agar, w:v), inoculated with respective powders (encapsulates & simply dried biomass), were incubated in a

walk-in chamber under 25ºC. Growth of microorganisms was recorded. Despite the longer period for *Chlorella* to form colonies (as it was expected), the spray drying temperatures did not seem to affect as much the viability of microorganisms.

Preparation of soil and dispersion of seeds: Composition of growth substrate was: 30% sphagnum acid turf (Combo Austria GmbH), 1% $CaCO_3$, 0.06% simply dried or encapsulated powders and sand to complete 100% (w/w). Experiments were carried out in small plastic boxes, with 170 g soil. Seeds (20) of meadow clover plants were spread over the soil. During the different trials soil was watered every other day. There was also a control without any fertilizer and another one whose boxes were watered once with 1 mL Substral (Henkel Iberica, SA), a known fertilizer from the market.

Composition of used turf, as it is indicated on the plastic pack: 40% dried matter:total weight; 35% organic matter:total weight, 52% organic carbon (C), 0.8% organic nitrogen (N). Every condition was always performed in triplicates, each of the replicates having 20 seeds to germinate. Boxes (10 cm length x 7.5 cm wide x 5.5 cm height) were loosely closed till germination of 50% seeds.

Meadow clove plantlets grew in a walk-in chamber for a two-week period, under constant light (58 mmoles $s^{-1}m^{-2}$) and temperature (25ºC); root system and stem were measured. Plantlets were then dried during 24 h, in an oven at 30ºC; root system and stem were weighed.

Statistical analysis: Data were analysed by the analysis of variance (ANOVA) and treatment means separated by the Fisher's Least Significant Difference test with STATISTICA 6.0© (StatSoft Inc., 1984-2001). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Main advantage of using rhizobacteria in the fields is that they do not cause damages to the environment and, therefore, can be used as an alternative to chemicals and pesticides, enhancing plant growth and crop yield. Other advantages are their different modes of action. As stated by Cattelan *et al.* (1999), they can promote plant growth indirectly by affecting symbiotic N_2 fixation, nodulation, or nodule occupancy, but affect directly by providing the bioavailability of phosphorus and iron, nitrogen fixation, or production of plant hormones (Lucy *et al.*, 2004) and thus influencing positively the growth and morphology of roots and shoots, by simply colonizing rhizosphere or because they may attach to the surface of the roots or colonize their interior (Vessey, 2003).

P. putida (2:1), *S. proteomaculans* (3:1) and *S. maltophilia* (3:1) (microalgae:bacteria) were identified as the three strains of rhizobacteria that could grow in consortia with *C. vulgaris*, having also promoted the growth of the microalgae. Actually, when consortia with these bacteria were tested against commercial fertilizers and simple water, high changes in the morphology of the plant roots could be observed, mainly when using encapsulates of *Chlorella+Stenotrophomonas*.

In what relates to the consortium *Chlorella+Serratia* (Figs. 1 & 2), results were not conclusive when roots and shoots were used to evaluate the effects of different fertilizers. Nonetheless, when referring to the association *Chlorella+Pseudomonas*, despite being shorter than the roots obtained with *Chlorella+Stenotrophomonas*, the positive influence of consortium could be observed, since length of roots increased significantly ($p<0.05$) in relation to the plants whose substrate was inoculated with the other fertilizers (Fig. 1). However, there was no evident influence of the encapsulation. The longest roots were obtained with the consortium *Chlorella+Stenotrophomonas*, especially when microorganisms were encapsulated in MD:GA 1:2, followed by MD:G 1:1 and MD:GA 2:1 (Fig. 1), differences being significant ($p<0.05$).

Besides length that shows how far roots went for the exploration of the substrates, the other parameter evaluated was dry mass, which indicates the volume of soil that was explored, through the increasing of root surface (Vessey, 2003). In fact, plantlets, whose substrate was inoculated with this last consortium, presented a significant increase in the root and leaf dried biomass, 1:1 being the best proportions for the chemicals used to encapsulate microorganisms, either MD:GA or MD:G (Fig. 3). Moreover, when considering dry weight, 6:7 of the fertilizers with the association *Chlorella:Stenotrophomonas* changed substantially the morphology of roots, giving rise to roots whose dry weights were significantly higher than the ones obtained with the other consortia, encapsulated or not (Fig. 3). This increase in the surface of the roots could improve plant growth as volume of soil explored also increased, thus enhancing bioavailability of nutrients to be used by the plants. These results were in agreement with what was stated by Lucy *et al.* (2004) who also noted the increase of leaf area as a benefit due to addition of PGPR. Here we observed that shoots suffered modifications as well, showing an increase in their dry weight, when the association *Chlorella:Stenotrophomonas* was used. Despite the results obtained with the consortium *Chlorella+Serratia proteomaculans*, encapsulated or not, which produced higher stem-leaves biomass than *Chlorella+P. putida*, plants from the last consortium presented a significantly higher root biomass ($p<0.05$) (Fig. 3).

Another mechanism that could have improved the availability of nutrients was the increase of root length. Actually, plantlets that grew in soils fertilized with encapsulates of consortia either with *Stenotrophomonas* or *Serratia* presented the highest length of roots (Fig. 1). These results could simply be due to the slowest release and action of microorganisms when out of the beads, but Lucy *et al.* (2004) obtained similar results when working with strains related to the rhizobacteria tested in this study.

Fig. 1: Root length of plantlets according to the fertilizers used. Graphic on the top shows the results for the experiment where consortium of *C. vulgaris+P. putida* (T3) was used; in the middle, consortium of *Chlorella+S. proteomaculans* (E1) was used; and at the bottom the consortium was *Chlorella+S. maltophilia* (E2). Bars represent the standard deviation

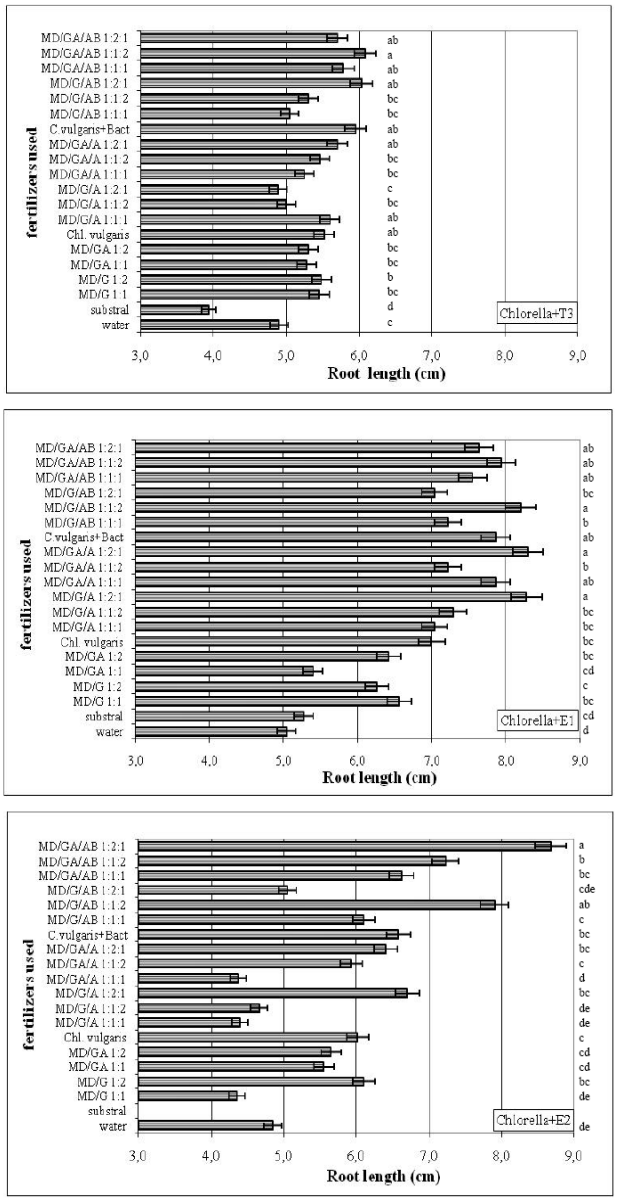
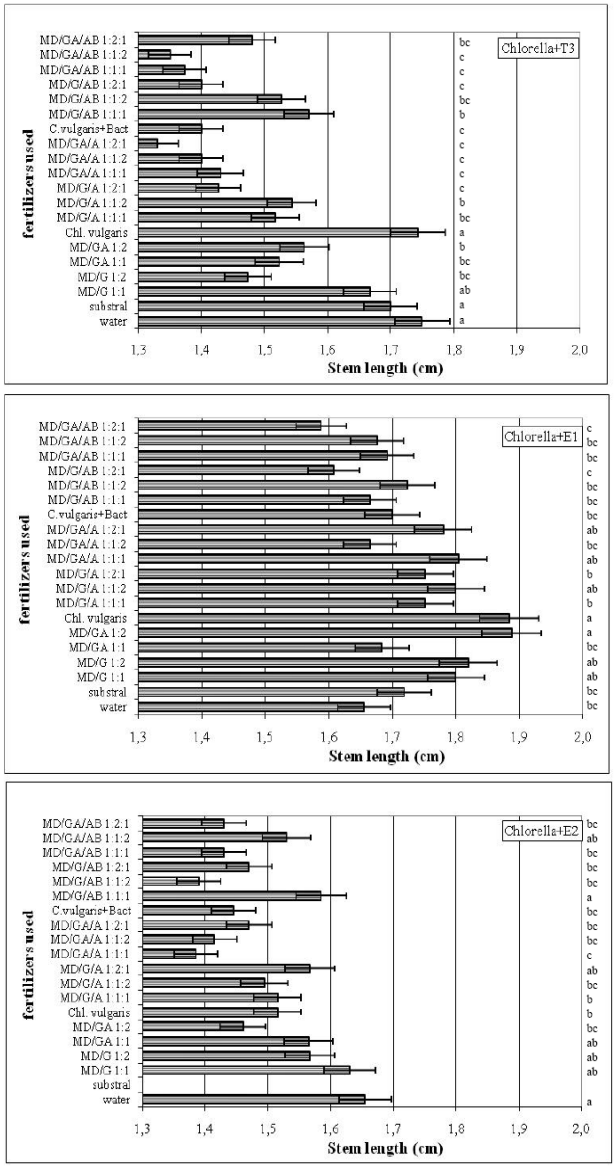


Fig. 2: Stem length of plantlets according to the fertilizers used. Graphic on the top shows the results for the experiment where consortium of *C. vulgaris+Pseudomonas putida* (T3) was used; in the middle, consortium of *Chlorella+S. proteomaculans* (E1) was used; and at the bottom the consortium was *Chlorella+S. maltophilia* (E2). Bars represent the standard deviation

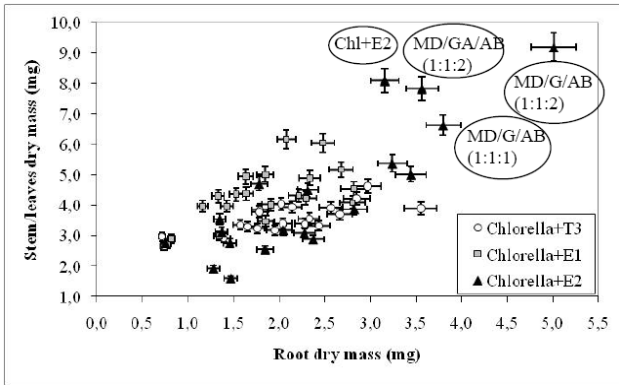


Another aspect that has to be emphasized is the benefit that rhizobacteria provided when in the consortia with *Chlorella*. As a matter of fact, the non-encapsulated consortia always promoted a root growth (Fig. 1) longer than the ones verified when simple powder of microalgae was used ($p<0.05$).

These changes in the morphology of the roots and/or shoots can simply be related to the humidity retention in the growth substrates or because microorganisms within the consortia improved a wet aggregate stability of the soil, or could even be associated to the production or influence on the concentration of auxins or other plant promoters, either by the bacteria (Glick *et al.,* 1999; Patten & Glick, 2002) or the microalgae (Stirk *et al.,* 2002; Ördög *et al.,* 2004). But shorter shoots were also observed (Fig. 2).

Perhaps a small decrease of *Chlorella*+bacteria clusters (with all the consortia), during the first days of germination, could give rise to stems shorter than those of

Fig. 3: Comparison of the development of plantlets from the three experiments as a function of shoots system and roots biomass. Open circles represent plantlets of the consortium *Chlorella+Pseudomonas* (T3); open squares represent plantlets of the consortium of *Chlorella+Serratia* (E1); and filled triangles represent plantlets of the consortium *Chlorella+Stenotrophomonas* (E2). Bars represent the standard deviation for both shoot system and roots biomass



the plantlets fertilized with *Chlorella*. Similar results were observed by Gonzalez and Bashan (2000) when *Chlorella* was co-immobilized and co-cultured with *Azospirillum brasilense*. They also noticed that when *Chlorella* was incubated alone, there were more clusters in the interior of the beads. Nevertheless, as stated above, when considering relative growth of plants (dried biomass of stem/leaves & roots; Fig. 3) instead of length, the positive influence of the consortia utilization is obvious, especially when encapsulates of consortium of *C. vulgaris+S. maltophilia* were used. We could also observe that best proportions of microbeads coating were 1:1 for MD and G or GA, respectively, perhaps because these conditions of encapsulation could protect both microalgae and bacteria and also their traits/biochemical characteristics. In addition, it seems that the consortium *Chlorella+Serratia* induced a longer growth in the stem/leaves and shorter roots, whilst *Chlorella+Pseudomonas* produced longer roots and shorter stems. These results could be associated, eventually, with the production of cytokinins by *S. proteomaculans* and auxins by *P. putida*, respectively.

CONCLUSION

Combinations of MD:G and MD:GA were good materials, especially in 1:1 proportions, to encapsulate *S. maltophilia* and *C. vulgaris* as a consortium: this association improved the root and leaf area of meadow clover plantlets, influencing positively the growth of the plants. Nevertheless, further studies are imperative with these bacteria, in order to determine and understand the mechanisms and/or the traits that induce/promote the plant growth.

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