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Effect of encapsulated plant growth promoting microorganisms on soil biochemical parameters and development of fruit tree seedlings

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Abstract

There is a great diversity of microorganisms that participate in biological, biochemical and biogeochemical processes responsible for the formation and maintenance of soil physical structure, quality and fertility. The aim of this study was to evaluate the effect of the inoculation of encapsulated plant growth promoting microorganisms on plant growth, microbial biomass carbon, soil nitrogen and phosphorus, and to estimate the microbial activity in the substrate used for the formation of fruit species seedlings. Microbial inoculum contained the following species: *Azospirillum brasilense, Burkolderia cepacia, Bacillus thuringienses, B. megaterium, B. cereus, B. subtilis, Tricoderma* spp. and Isolate 411. Fruit species evaluated were*: Myrciaria glazioviana*, *Myrciaria dubia, Annona muricata*.; *Chrysophyllum cainito*.; and *Litchi chinensis*. The experimental design was completely randomized in a 3 x 2 factorial scheme (control, sodium alginate and clay) (presence and absence of microbial inoculum) with five replicates (one seedling per replicate), for ninety days. Each plant specie was analyzed separately according to each treatment. At the end of the experimental period, the following parameters were evaluated in soil samples: dehydrogenase enzymatic activity (DEA), ammonium (NH_4^+) and nitrate (NO_3^-), bicarbonate-soluble phosphate (BSP), microbial biomass carbon (MBC) and total number of bacteria (CFU). The results of the present study showed no effective action of encapsulation in promoting plant growth. However, some soil parameters such as ammonium content were positively affected for *Myrciaria dubia*.
Abbreviations: DEA – dehydrogenase enzymatic activity; $NH4^+$ - ammonium; NO_3^- nitrate; BSP – bicarbonate-soluble phosphate; MBC – microbial biomass carbon; CFU – colony forming unit.

Keywords: Fruit propagation, encapsulation, microbial inoculum, plant growth promoting bacteria, dehydrogenase, biomass, carbon.

Introduction

The quality of the substrate is one of the main factors that guarantee successful production of seedlings of fruit tree species. Adequate substrate must be dense and firm, highly decomposed and stable, sufficiently porous to drain excess water, free of pests and diseases, have low salinity, be able to be pasteurized or chemically treated, have high cation exchange capacity (CTC), retain nutrients and be easily available (Hartmann et al., 2010).

In addition, the presence of microorganisms beneficial for species to be propagated can also be advantageous, since there is a great diversity of microorganisms that participate in biological, biochemical and biogeochemical processes responsible for the formation and maintenance of the soil physical structure and fertility. Many of these microorganisms play a role in the interaction with plants, such as: nitrogen fixation, phosphate solubilization and mycorrhization, and act in the antagonistic action to pathogens and in the production of substances that promote or inhibit plant growth, thus contributing to soil fertility and plant nutrition (Andreola and Fernandes, 2007). Microorganisms are responsible for mineralization processes, representing a considerable amount of nutrients potentially available to plants (Hayat et al., 2010). Consequently, the most important soil processes that affect nutrient bioavailability are regulated by microbiota (Frighetto and Valarini, 2000).

In general, shortly after suspensions of bacteria are inoculated into the soil without a proper carrier, the bacteria population declines rapidly for most species of PGPB. This phenomenon, combined with poor production of bacterial biomass, difficulty sustaining activity in the rhizosphere, and the physiological state of the bacteria at application time, can prevent the buildup of a sufficiently large PGPB population in the rhizosphere. A threshold number of cells, which differs among species, is essential to obtain the intended positive plant response, for example, 10^6–10^7 cells·plant^{-1} for the PGPB *Azospirillum brasilense* (Bashan 1986). The inherent heterogeneity of the soil is the key obstacle, where introduced bacteria sometimes cannot find an empty niche in the soil. These unprotected, inoculated bacteria must compete with the often better-adapted native microflora and withstand predation by soil microfauna. Consequently, a major role of formulation of inoculants is to provide a more suitable microenvironment, combined with

physical protection for a prolonged period to prevent a rapid decline of introduced bacteria. Inoculants for field-scale use have to be designed to provide a dependable source of bacteria that survives in the soil and become available to crops, when needed (Bashan 1998).

Encapsulation is considered an important technique for the maintenance of the stability of microorganisms and increases the resistance of bacteria to the substrate conditions, being a technique used for the inoculation of microorganisms. Among the many types of encapsulating agents, calcium and sodium alginate, as well as milk whey proteins and gums have been the most widely used (Schoebitz et al., 2013; Vemmer and Patel, 2013). Alginate beads can protect microbes from biotic and abiotic stresses and improve persistence and physiological activity as well as cell densities (Schoebitz et al. 2013). Encapsulating also allows an easy delivery of the microbial inoculants at the site where they are needed and can be used in seed drills commonly used by farmers (Lójan et al. 2017). Encapsulate agents as alginate has been reported in numerous studies with single isolated microorganisms (e.g. AMF—Declerck et al. 1996 or PGPR while much fewer studies reported on consortia. Buysens et al. (2016) co-inoculated the AMF *Rhizophagus irregularis* MUCL 41833 with the fungus *Trichoderma harzianum* MUCL 29707 into alginate beads for field trials, which resulted in an increased potato yield. Co-immobilization constitutes an unexploited biotechnology for microbial formulation (Vassilev et al. 2001).

Clay is another material that can be added to formulations as an encapsulating agent of microorganisms, being a promising alternative due to its great availability and low cost (Messa et al., 2016). Clay minerals provide expandable space that can be colonized by microorganisms (Park et al., 2013) and therefore are recognized as protective molecules.

Microbial activity evaluation has been considered as an adequate parameter to evaluate the microbiological variables of soil quality. In this way, the influence of microorganisms can be evaluated through the carbon (C), nitrogen (N) and phosphorus (P) biomass content, as well by the enzymatic activity responsible for soil nutrient transformations and availability (Alves et al., 2011; Capuani et al., 2012).

The novelty of this study was to evaluate the effect of the inoculation of encapsulated plant growth promoting microorganisms on plant growth, microbial biomass carbon, soil nitrogen and phosphorus and to estimate the microbial activity in the substrate used for the formation of fruit species seedlings such as *Myrciaria glazioviana* (Kiaersk.) G. Barros & Sobral (cabeludinha); *Myrciaria dubia* (Kunth) Mc Vaugh (camu-camu); *Annona muricata* L. (graviola); *Chrysophyllum cainito* L. (caimito) and *Litchi chinensis* Sonn. (lichia) where normally this kind of procedure is not used by farmers.

Results and Discussion

Encapsulating agent effect on plants

A significant effect of growth promoting microorganisms was observed only on the height of *C. cainito* L. (caimito) plants (Figure 1). Encapsulation with clay was superior to sodium alginate, as it did not differ statistically from control. For the other species, no significant differences were observed for plant height, stem diameter, shoot dry mass and root dry mass, indicating that microorganisms did not influence seedling growth, regardless of encapsulation type (clay or sodium alginate).

Consortium of microorganisms

Some studies have shown that the use of microbial consortium may have a positive effect on plant growth, since different microbial species also have different plant growth promoting abilities. Another interesting aspect of using a microbial consortium is that the probability of one or more microorganisms becoming established in the rhizosphere or endophytically increases greatly. However, problems with the use of microbial consortium may also occur, such as an antagonistic effect and competition among microorganisms. This would lead to a decrease in microbial populations, considerably reducing the chances of these microorganisms to establish in the rhizosphere and the growth promotion effect would not occur. On the other hand, microorganisms were inoculated in an immobilized way by alginate and clay, which would promote less contact among them.

Failure to promote plant growth

The failure of inoculated microorganisms to promote plant growth is probably due to some factors such as: microorganisms should have been inoculated since the formation of seedlings and not when seedlings were 60 days old. Seedlings were in plastic bags used for seedling formation, which may have hampered root development and consequently plant development.

Other factors that may have contributed to the failure to promote plant growth using inoculated microorganisms may have been the low the amount of microorganisms released with alginate, which was insufficient for the establishment of microorganisms in the rhizosphere, consequently not promoting plant growth. Finally, there may have been lack of interaction between microorganisms and fruit trees, since these originate in the Amazonian forest and require specific soil conditions and soil microbiota.

The possibilities presented above are assumptions reporting the likely reasons leading to failure to promote plant growth when microorganisms with growth promoting abilities are inoculated. However, further studies are needed to better understand what may have occurred.

In relation *M. glazioviana* (cabeludinha) seedlings, significant difference was observed among treatments ($p < 0.05$) for dehydrogenase enzymatic activity (DEA) and nitrate levels (NO_3^-) (Table 1).

Encapsulating agent effect on soil

For DEA, differences were observed in both encapsulation factor and microorganism factor, as well as in the interaction between them; however, higher DEA values were observed in the control treatment, when compared to treatments with encapsulants clay or sodium alginate, evidencing that encapsulation interfered in the enzymatic activity of the microorganisms present in a negative way. However, when the presence and / or absence of microorganisms was evaluated, greater enzymatic activity was observed in the presence of microorganisms (Table 1).

When analyzing the DEA interaction, it was observed that in the absence of the microbial inoculum, the control treatment presented higher enzymatic activity when compared to encapsulation using clay and sodium alginate (Table 2). However, in the presence of microorganisms, differences among treatments were observed. Regarding the encapsulation factor, higher DEA value was observed in the presence of the microbial inoculum, for both clay and sodium alginate.

For the other variables: ammonium content, soluble phosphate, microbial biomass carbon and total number of bacteria, no significant difference was observed among treatments (Table 1).

Dehydrogenase is a biontic enzyme, i.e., it is not present in the soil, but within microbial cells, being part of the electron transport chain. This feature allows a close relationship between enzymatic activity and microbial activity (Melero et al., 2011). The results showed that the presence of the encapsulating agent had a negative effect on DEA. This result suggests that the gradual release of microorganisms by encapsulating agents decreased the colonization of the rhizospheric soil by microorganisms compared to soils that received microorganisms directly without encapsulating agents. This property of encapsulating agents to promote a gradual release of encapsulated microorganisms is not necessarily negative since it may allow the colonization of the rhizospheric soil over a longer period of time compared to the application of microorganisms directly to the soil. However, the decreased and gradual release of microorganisms may lead in the short term to lower microbial activity reflecting in lower DEA.

For substrate containing *M. dubia* (camu-camu) seedlings, significant differences were observed among treatments for ammonium (NH_4^+) contents. In this species, higher NH_4^+ levels were identified in substrate when microorganisms were inoculated, regardless of whether or not they were encapsulated (Table 3). For the same variable, the F-test was also significant for the interaction between encapsulation and microorganisms used, indicating that factors A and B act together in the NH_4^+ contents found in the substrate for the seedlings of this species.

Kumar and Singh (2001) also observed that the inoculation of nitrogen-fixing bacteria increased N and P contents, causing a significant effect on the availability of these nutrients in vermicompost production. These findings indicate that multiple inoculations with microorganisms in the rhizosphere can promote plant growth and increase N and P concentrations and uptake in the soil (Wani et al., 2007).

In the presence of the microbial inoculum, higher NH_4^+ content was observed in treatment with sodium alginate, but without difference from treatment with clay (Table 4). These variable microorganism encapsulation efficiency results and the long-term viability of cells are fundamental aspects that need to be taken into account, since the interaction between inoculum, encapsulating agent and rhizosphere is of great importance for the success of the technique (Vilchez and Manzanera, 2011).

The increase of soil ammonium content promoted by the presence of alginate and clay together with microbial inoculation is a positive aspect. Encapsulating agents may have favored the establishment of nitrogen-fixing microorganisms. Nitrogen is an essential element for plant nutrition as part of every macromolecule responsible for maintaining cell life. When nitrogen-fixing microorganisms establish in the soil, an increase of this nutrient in the plant occurs (Di-Salvo et al., 2018).

For substrate containing *A. muricata* (graviola) seedlings, significant difference was observed for the dehydrogenase enzymatic activity (AED). Similarly to cabeludinha species, the enzymatic activity of substrates inoculated and encapsulated with clay did not differ from the control treatment (Table 5). This result leads us to believe that encapsulant sodium alginate interfered with the action of the microorganisms and, therefore, damaged AED in some way, which may also explain non-significant results for the other variables.

On the other hand, when *B. subtilis* were encapsulated in sodium alginate enriched with humic acid, they remained viable for 5 months at different pH conditions, with high capacity to solubilize phosphate *in vitro* and successfully promote the growth of lettuce plants (Young et al., 2006; Rekha et al., 2007). These results suggest that the addition of humic acid to the formulation may increase the perspectives of the encapsulation technique and, consequently, better establishment and survival of microorganisms in the rhizosphere.

Soil biochemical parameters

Nitrogen levels in the form of ammonium (NH_4^+) and nitrate (NO_3^-) showed results with significant differences in the substrate of *C. caimito* (caimito) seedlings (Table 6). Higher NH_4^+ and NO_3^- levels were found in substrate not inoculated by microorganisms (control) (Table 6). In relation to the encapsulating agent, sodium alginate presented better result for NO_3^-values when compared to control, which did not differ from results using clay (Table 6).

Sahim et al., (2004) reported that the effects of soil microbial inoculation can vary significantly according to environmental conditions, bacterial strain and plant and soil conditions. This fact may explain the variation of results observed from one fruit species to another, even using the same substrate and group of microorganisms.

Regarding the results for the interaction between encapsulation and inoculation of microorganisms for the NO_3^- contents in the substrate of caimito seedlings, it could be observed that, in the absence of microbial inoculum, both clay and sodium alginate presented higher NO_3^- levels when compared to control (Table 7). However, in the presence of the inoculum, no difference for NO_3^- levels was found. These results suggest that encapsulation was not sufficiently effective in the biological activity of growth promoting microorganisms on the substrate.

For substrate containing *L. chinensis* (lichia) seedlings, significant differences in the nitrogen (NH_4^+ and NO_3^-) and microbial biomass (MBC) contents were observed, both in the encapsulation and in the inoculation of microorganisms, as well as in the interaction between these factors (Table 8). Higher NH_4^+ levels were observed when substrate was inoculated by microorganisms, both encapsulated with clay and with sodium alginate, evidencing that both encapsulating agents were efficient in inoculum protection and release.

Table 1. Combined analysis to compare dehydrogenase enzymatic activity (DEA), determination of NH_4^+ and NO_3^-, bicarbonate-soluble phosphate (BSP), microbial biomass carbon (MBC) and bacterial suspension (BS) on substrate of cabeludinha seedlings (*Myrcia glazioviana* (Kiaers.) G. Barros & Sobral) inoculated with growth promoting microorganisms in two encapsulation methods, 90 days after inoculation.

Treatments	DEA µg TFF g^{-1} SS $24h^{-1}$	NH_4^+ Amount of N µg SS	NO_3^- Amount of N µg SS	BSP µg P g^{-1} SS	MBC µg g^{-1} of C in soil	BS CFU g^{-1} of SS
Encapsulation (A)						
Control	297.57 a	84.00 a	54.25 a	7.97 a	30.55 a	$2.37x10^6$ a
Clay	226.27 b	77.58 a	48.41 ab	8.36 a	23.79 a	$1.33x10^6$ a
Alginate	219.27 b	81.66 a	47.25 b	8.42 a	10.89 a	$1.27x10^6$ a
Microorganisms (B)						
With microorganism	291.83 a	79.72 a	48.61 a	8.44 a	20.86 a	$2.03x10^6$ a
Without microorganism	203.95 b	82.44 a	51.33 a	8.06 a	22.62 a	$1.28x10^6$ a
VC (%)	17.89	9.96	8.08	25.12	86.10	92.22
f-test F						
Encapsulation (A)	5.68^*	0.97^{ns}	5.17^*	0.08^{ns}	1.71^{ns}	0.98^{ns}
Microorganisms (B)	17.66^*	0.51^{ns}	2.04^{ns}	0.15^{ns}	0.04^{ns}	1.07^{ns}
A x B	5.40^*	0.51^{ns}	1.17^{ns}	0.66^{ns}	0.56^{ns}	1.46^{ns}

Means followed by same letter in the column do not differ by the Tukey test, being * significant at 5% probability, ** significant at 1% probability and ns not significant. SS = dry soil.

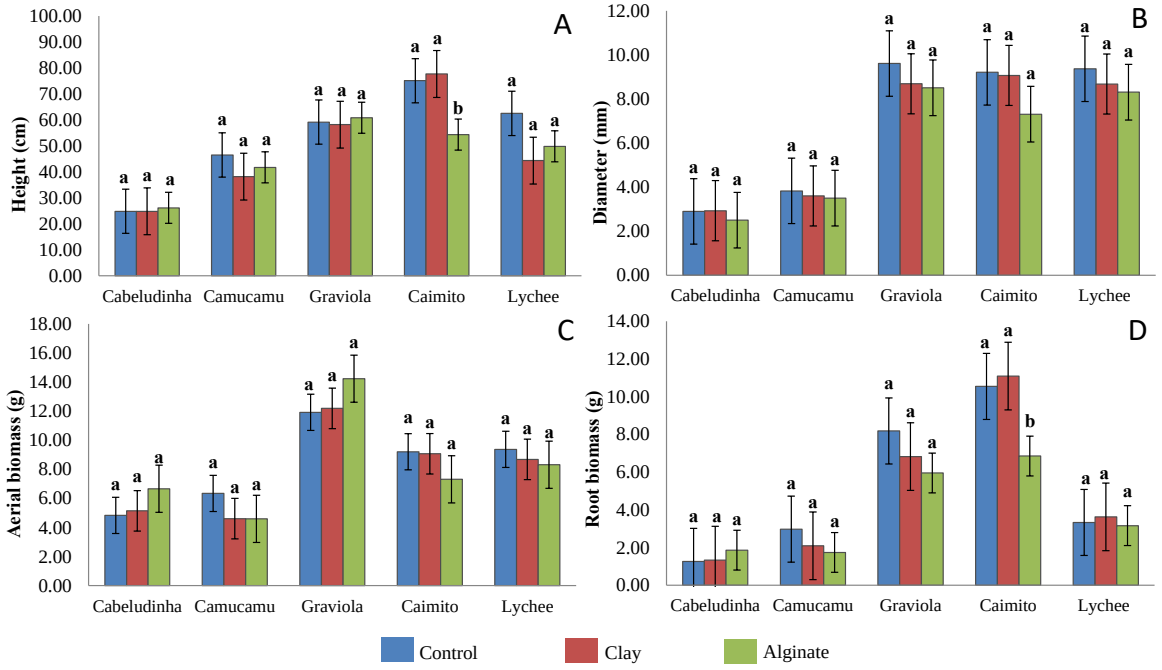

Fig 1. Height (A), diameter (B), Aerial biomass (C) and root biomass (D) of fruit trees that received microbial inoculum encapsulated or not with alginate and clay.

Table 2. Dehydrogenase enzymatic activity (DEA) on substrate of cabeludinha seedlings (*Myrciaria glazioviana* (Kiaers.) G. Barros & Sobral) inoculated with growth-promoting microorganisms as a function of encapsulation, 90 days after inoculation.

Encapsulation	Microorganisms	
	Absence	Presence
	DEA (µg TFF g^{-1} SS $24h^{-1}$)	
Control	302.01 aA	293.13 aA
Clay	154.43 bB	298.12 aA
Alginate	155.43 bB	284.23 aA

Means followed by the same lowercase letters in columns and upper case in row do not differ by the Tukey test (p≤0.05). SS = dry soil.

Table 3. Combined analysis to compare dehydrogenase enzymatic activity (DEA), determination of NH_4^+ and NO_3^-, bicarbonate-soluble phosphate (BSP), microbial biomass carbon (MBC) and bacterial suspension (BS) on substrate of camu-camu seedlings (*Myrciaria dubia* (Kunth) Mc Vaugh) inoculated with growth-promoting microorganisms using two encapsulation methods, 90 days after inoculation.

Treatments	DEA µg TFF g^{-1} SS $24h^{-1}$	NH_4^+ Amount of N µg SS	NO_3^- Amount of N µg SS	BSP µg P g^{-1} SS	MBC µg g^{-1} of C in soil	BS CFU g^{-1} of SS
Encapsulation (A)						
Control	126.81 a	76.41 a	45.50 a	7.46 a	23.51 a	$1.11x10^6$ a
Clay	108.20 a	82.83 a	47.25 a	7.45 a	26.40 a	$9.92x10^6$ a
Alginate	161.08 a	81.08 a	48.41 a	7.93 a	26.11 a	$8.17x10^6$ a
Microorganisms (B)						
With microorganism	144.11 a	84.00 a	48.61 a	7.90 a	25.66 a	$1.11x10^6$ a
Without microorganism	119.95 a	76.22 b	45.50 a	7.32 a	25.03a	$8.29x10^6$ a
VC (%)	29.08	7.90	8.03	18.52	86.13	36.82
F-test						
Encapsulation (A)	2.93^{ns}	1.64^{ns}	0.90^{ns}	0.22^{ns}	0.03^{ns}	1.03^{ns}
Microorganisms (B)	1.78^{ns}	6.78^{*}	3.05^{ns}	0.76^{ns}	0.00^{ns}	2.94^{ns}
A x B	0.79^{ns}	8.15^{**}	2.05^{ns}	0.34^{ns}	1.51^{ns}	2.62^{ns}

Means followed by the same letter in column do not differ by the Tukey test, being * significant at 5% probability, ** significant at 1% probability and ns not significant. SS = dry soil.

Table 4. Determination of NH_4^+ on substrate of camu-camu (*Myrciaria dubia* (Kunth) Mc Vaugh) seedlings inoculated with growth-promoting microorganisms as a function of encapsulation, 90 days after inoculation.

Encapsulation	Microorganisms	
	Absence	Presence
	NH_4^+ (Amount of N µg SS)	
Control	78.16 aA	74.66 bA
Clay	81.66 aA	84.00 abA
Alginate	68.33 aB	93.00 aA

Means followed by the same lowercase letters in columns and upper case in rows do not differ by the Tukey test (p≤0.05). SS = dry soil.

Table 5. Combined analysis to compare the dehydrogenase enzymatic activity (DEA), determination of NH_4^+ and NO_3^-, bicarbonate-soluble phosphate (BSP), microbial biomass carbon (MBC) and bacterial suspension (BS) on substrate of graviola seedlings (*Annona muricata* L.) inoculated with growth promoting microorganisms in two encapsulation methods, 90 days after inoculation.

Treatments	DEA µg TFF g^{-1} SS 24 h^{-1}	NH_4^+ Amount of N µg SS	NO_3^- Amount of N µg SS	BSP µg P g^{-1} SS	MBC µg g^{-1} of C in soil	BS CFU g^{-1} of SS
Encapsulation (A)						
Control	230.70 ab	77.58 a	62.41 a	6.44 a	21.03 a	$2.73x10^6$ a
Clay	252.28 a	78.75 a	63.00 a	5.72 a	32.60 a	$2.64x10^6$ a
Alginate	191.03 b	85.16 a	64.16 a	5.73 a	16.36 a	$2.12x10^6$ a
Microorganisms (B)						
With microorganism	217.23 a	78.94 a	61.44 a	6.55 a	21.00 a	$2.63x10^6$ a
Without microorganism	232.11 a	82.05 a	64.94 a	5.39 a	25.66 a	$2.37x10^6$ a
VC (%)	15.66	10.09	12.10	40.70	56.19	61.10
F-test						
Encapsulation (A)	4.68^{*}	1.52^{ns}	0.08^{ns}	0.17^{ns}	2.44^{ns}	0.28^{ns}
Microorganisms (B)	0.80^{ns}	0.66^{ns}	0.94^{ns}	1.02^{ns}	0.57^{ns}	0.13^{ns}
A x B	1.76^{ns}	1.62^{ns}	2.55^{ns}	1.18^{ns}	0.02^{ns}	1.83^{ns}

Means followed by the same letter in column do not differ by the Tukey test, being * significant at 5% probability, ** significant at 1% probability and ns not significant. SS = dry soil.

Table 6. Combined analysis to compare the dehydrogenase enzymatic activity (DEA), determination of NH_4^+ and NO_3^-, bicarbonate-soluble phosphate (BSP), microbial biomass carbon (MBC) and bacterial suspension (BS) on the substrate of caimito seedlings (*Chrysophyllum cainito* L.) inoculated with growth promoting microorganisms in two encapsulation methods, 90 days after inoculation.

Treatments	DEA µg TFF g^{-1} SS 24 h^{-1}	NH_4^+ Amount of N µg SS	NO_3^- Amount of N µg SS	BSP µg P g^{-1} SS	MBC µg g^{-1} of C in soil	BS CFU g^{-1} of SS
Encapsulation (A)						
Control	394.23 a	75.83 a	39.66 b	9.83 a	32.88 a	$1.92x10^6$ a
Clay	384.72 a	87.50 a	46.08 ab	9.52 a	40.27 a	$3.00x10^6$ a
Alginate	370.61 a	84.58 a	48.41 a	8.27 a	35.97 a	$3.19x10^6$ a
Microorganisms (B)						
With microorganism	387.87 a	74.27 b	40.83 b	9.49 a	36.63 a	$2.39x10^6$ a
Without microorganism	378.51 a	91.00 a	48.61 a	8.92 a	36.12 a	$3.01x10^6$ a
VC (%)	10.07	11.20	12.51	18.91	50.68	45.42
F-test						
Encapsulation (A)	0.57^{ns}	2.58^{ns}	3.93^{*}	1.35^{ns}	0.24^{ns}	1.85^{ns}
Microorganisms (B)	0.26^{ns}	14.67^{**}	8.70^{*}	0.49^{ns}	0.00^{ns}	1.15^{ns}
A x B	0.05^{ns}	1.53^{ns}	3.93^{*}	1.86^{ns}	0.16^{ns}	2.97^{ns}

Means followed by the same letter in the column do not differ by the Tukey test, being * significant at 5% probability, ** significant at 1% probability and ns not significant. SS = dry soil.

Table 7. Determination of NO_3^- in the substrate of caimito seedlings (*Chrysophyllum cainito* L.) inoculated with growth-promoting microorganisms as a function of encapsulation, 90 days after inoculation.

Encapsulation	Microorganisms	
	Absence	Presence
	NO_3^- (Amount of N µg SS)	
Control	38.50 bA	40.83 aA
Clay	51.33 aA	40.83 aB
Alginate	56.00 aA	40.83 aB

Means followed by the same lowercase letters in columns and upper case in rows do not differ by the Tukey test (p≤0.05). SS = dry soil.

Table 8. Combined analysis to compare the dehydrogenase enzymatic activity (DEA), determination of NH_4^+ and NO_3^-, bicarbonate-soluble phosphate (BSP), microbial biomass carbon (MBC) and bacterial suspension (BS) on substrates of lichia seedlings (*Litchi chinensis* Sonn.) inoculated with growth promoting microorganisms in two encapsulation methods, 90 days after inoculation.

Treatments	DEA µg TFF g^{-1} SS 24 h^{-1}	NH_4^+ Amount of N µg SS	NO_3^- Amount of N µg SS	BSP µg P g^{-1} SS	MBC µg g^{-1} of C in soil	BS CFU g^{-1} of SS
Encapsulation (A)						
Control	190.20 a	68.25 b	43.75 a	10.82 a	34.19 a	$5.31x10^5$ a
Clay	160.34 a	75.83 a	43.75 a	10.11 a	29.86 a	$7.03x10^5$ a
Alginate	166.05 a	75.83 a	43.75 a	10.85 a	23.42 a	$7.95x10^5$ a
Microorganisms (B)						
With microorganism	179.63 a	71.55 b	44.72 a	10.35 a	33.73 a	$6.78x10^5$ a
Without microorganism	164.76 a	75.05 a	42.77 a	10.83 a	24.58 b	$6.75x10^5$ a
VC (%)	24.89	3.55	4.61	9.48	25.85	44.93
F-test						
Encapsulation (A)	0.82^{ns}	16.90^{**}	0.00^{ns}	1.03^{ns}	3.10^{ns}	1.16^{ns}
Microorganisms (B)	0.54^{ns}	8.10^{*}	4.17^{ns}	1.03^{ns}	6.63^{*}	0.00^{ns}
A x B	0.05^{ns}	33.30^{**}	4.67^{*}	1.32^{ns}	8.88^{**}	0.68^{ns}

Means followed by the same letter in the column do not differ by the Tukey test, being * significant at 5% probability, ** significant at 1% probability and ns not significant. SS = dry soil.

Table 9. Determination of NH_4^+, NO_3^- and microbial biomass carbon (MBC) on substrates of lichia seedlings (*Litchi chinensis* Sonn.) inoculated with growth promoting microorganisms as a function of encapsulation, 90 days after inoculation.

Encapsulation	Microorganisms	
	Absence	Presence
	NH_4^+ (Amount of N µg SS)	
Control	64.16 cB	72.33 abA
Clay	84.00 aA	67.66 bB
Alginate	77.00 bA	74.66 aA
	NO_3^- (Amount of N µg SS)	
Control	40.83 aB	46.66 aA
Clay	43.16 aA	44.33 aA
Alginate	44.33 aA	43.16 aA
	CBM (µg g^{-1} of C in soil)	
Control	39.80 aA	28.58 aA
Clay	17.68 bB	42.05 aA
Alginate	16.27 bB	30.58 aA

Means followed by the same lowercase letters in columns and upper case in rows do not differ by the Tukey test (p≤0.05). SS = dry soil.

For MBC levels, there was no difference between the type of encapsulation and control; however, better results were observed when microbial inoculum was used (Table 8).

In the interaction for the nitrogen and carbon contents of the microbial biomass in the substrate of lichia seedlings, it was observed that, in the absence of inoculum, higher NH_4^+ values were found using clay as encapsulating agent, followed by sodium alginate, in comparison with control (Table 9). However, in the presence of microorganisms, better results were found using alginate, which did not differ from control. For NO_3^- levels, no differences between encapsulating agents were found, either in the presence or absence of microorganisms.

MBC values were higher when no microbial inoculum was used, suggesting that, in this case, both encapsulation methods were inefficient in the development of microorganisms in the substrate, since when directly inoculated, resulted in higher microbial biomass values.

Materials and Methods

The experimental design

The experiment was conducted between March 16 and June 16, 2015 (90 days) in a protected nursery system belonging to the Department of Plant Production - School of Agrarian and Veterinary Sciences - UNESP, Campus of Jaboticabal - SP (21°14'05S and 48º17'09''W and 590 m a.s.l).

The experimental design was completely randomized design with five replicates at 3 x 2 factorial scheme evaluating the encapsulating effects (control, clay and alginate) with microbial inoculum (with or no inoculum).

The treatments were T1 = control; T2 = microbial inoculum; T3 = clay; T4 = clay+ microbial inoculum; T5 = alginate; T6 =alginate+ microbial inoculum.

Plants species

All fruit species seedlings studied were originated from seeds obtained from mother plants of the UNESP-FCAV Germplasm Active Bank Campus of Jaboticabal. Microorganisms were inoculated on substrates where seedlings of the following fruit species were being conducted: *Myrciaria glazioviana* (Kiaersk.) G. Barros & Sobral (cabeludinha); *Myrciaria dubia* (Kunth) Mc Vaugh (camu-camu); *Annona muricata* L. (graviola); *Chrysophyllum cainito* L. (caimito) and *Litchi chinensis* Sonn. (lichia).

Seedlings

The substrate used was composed of a 3: 1: 1 (v / v / v) mixture of soil, sand and bovine manure disposed in polyethylene bags (22 x 11 cm). Irrigation was manual, daily, according to substrate moisture monitoring. Seedlings were kept under 50% luminosity cover for a period of 90 days.
The seedlings selected for this experiment were standardized according to height and stem diameter before inoculation. At the end of the 90 days, they were evaluated as to plant height (cm), measured from base to plant apex; stem diameter (mm), in the stem region, with the aid of a digital caliper; shoot dry mass (g) and root dry mass (g) (65 ° C, 72 h), as described by Araújo Neto et al., (2015).

Microbial inoculum

The microbial inoculum contained the following microorganisms: *Azospirillum brasilense, Burkolderia cepacia, Bacillus thuringienses, B. megaterium, B. cereus, B. subtilis, Tricoderma* spp. and Isolate 411. All microorganisms are part of the collection of the Laboratory of Soil Microbiology - Department of Plant Production of UNESP-FCAV, Campus de Jaboticabal.
Microorganisms were grown separately in nutrient broth for seven days in vials kept in B.O.D. chamber (Eletrolab, model 347 F, Brazil) at 25°C. After the incubation period, microorganisms were centrifuged separately at 10,000 rpm for 10 min at 28°C (Novatecnica, model MLW K24, Brazil). The inoculum concentration was standardized as recommended by Barry and Thornsberry (1991) and Sahm and Washington II (1991) at 1 x 10^7 CFU mL^{-1}, with spectrophotometer (Micronal, B382 model, Brazil) at 695 nm absorbance.

Encapsulating agent

Sodium alginate solution ($NaC_6H_7O_6$) at concentration of 1% (m / v) was added to each microorganism solution. Microorganism solutions added of sodium alginate were dropped in 0.1 M calcium chloride (CaCl2) solution, giving rise to beads with the microbial inoculum (Sheu and Marshall, 1993; Sultana et al., 2000). Beads were then washed with distilled water using a sieve and submitted to the drying process in a laminar flow chamber for a period of six hours and kept at room temperature (17-28°C). For each 1 mL of solution of one microorganism, 12 beads were generated. Since each vessel contained eight different microorganisms, each vessel received 96 microbial beads in total, which were incorporated into the substrate in orifice of approximately 10 cm in depth in order to ensure greater proximity of the deposited material on the substrate with the roots of seedlings. Treatment containing alginate received only the sodium alginate beads without the presence of the microbial inoculum, and 96 beads were added per vessel.

Microbial inoculation

For the inoculation of microorganisms with clay, suspension containing 1 ml of each microorganism to 1 ml of clay diluted in distilled water at 1: 3 ratio (w / v) was prepared (adapted from Carneiro and Gomes, 1997). Each vessel received a total of 8 mL of microbial inoculum added to 8 mL of clay, which were incorporated into the substrate in orifice of approximately 10 cm in depth, totaling 16 mL per vessel. For treatment containing only clay, the same procedure was performed, except for the addition of the microbial inoculum.

Soil parameters analysis

At the end of ninety days, the soil was separated from the plant and was kept in refrigerator until analysis of the dehydrogenase activity, determination of ammonium and nitrate, soluble phosphate, microbial biomass carbon and total number of bacteria.
Soil samples were collected after ninety days, when plants were removed from vessels. These samples were collected in the region near the rhizosphere, packed in plastic bags and immediately taken to the Laboratory of Soil Microbiology, where they were placed in refrigerator (5ºC) until analyses. The following parameters were evaluated in soil samples: dehydrogenase enzymatic activity (AED), ammonium (NH_4^+) and nitrate (NO_3^-), bicarbonate-soluble phosphate (FSB), microbial biomass carbon (CBM) and total number of bacteria (CFU).
The dehydrogenase activity was evaluated according to method described by Casida (1977) using triphenyltetrazolium chloride (TTC) as an artificial electron acceptor.
For this analysis, 3 g of sieved soil (2 mm mesh) were weighed and placed in test tubes (18 x 180 mm) added of 0.03 g $CaCO_3$ and then 0.5 mL of 3% TTC aqueous solution, 1.3 mL distilled water were added; tubes were then capped with plastic film and stirred and incubated in water bath for 24 h at 37ºC. Subsequently, a 10 mL aliquot of methanol was added to tubes to extract TFF produced from TTC by the enzyme present in the soil. Tubes were then shaken and samples filtered on filter paper and read in spectrophotometer at 485 nm wavelength.
The method of Keeney and Nelson (1982) was used. For determination of the ammonium (NH_4^+) content, approximately 5 g of fine air-dried soil sample was used, 1M potassium chloride solution was added under stirring, after decantation and extraction, an aliquot of 10 mL of this extract was collected and the ammonium content was determined by adding a indicator solution of boric acid to nitrogen. Distillation was performed in a preheated boiler after adding 0.2 g magnesium oxide to the sample. The distillate was collected to the mark of 40 mL in Erlenmeyer flask, and then, the sample was titrated with 0.0025 M sulfuric acid solution until color turned from green to pink.

For determination of the nitrate (NO_3^-) content, the sample was distilled heated boiler adding 1 mL of sulfamic acid. Using the side outlet, 0.2 g of Devarda alloy were added, collecting the distillate in Erlenmeyer flask to the 40 mL mark. The distillate was titrated with 0.0025 M sulfuric acid solution until color turned from green to pink.

To determine the phosphorus content, the method of Watanabe and Olsen (1965) was used. About 0.6 g of fine air-dried soil was added to 0.5 M sodium bicarbonate solution under stirring and, after decantation, the solution was filtered. To determine the phosphorus content, the filtrate was used, which was added of 5 N sulfuric acid and previously prepared reagent B. The solution was then shaken and incubated in water bath at 45°C for 20 min. Phosphorus determination was performed by colorimetry after reading at 820 nm absorbance. The result was expressed in µg of P g^{-1} dry soil.

The methodology described by Mendonça and Matos (2005) was used, which consists irradiating soil using microwaves to break microbial cells, releasing the microbial content, mainly cytoplasmic content, which was extracted with 0.5 M potassium sulfate aqueous solution.

For each sample, two 10 g soil portions were weighed and sieved in a 2 mm mesh sieve, one of which was irradiated with microwaves and the other was not.

Then, the two irradiated and non-irradiated samples were placed in Erlenmeyer flask and added of 40 mL of 0.5 M potassium sulfate (K_2SO_4) solution. Subsequently, Erlenmeyer flasks were taken to the horizontal shaker for 30 min. After this period, flasks were allowed to stand for another 30 min for the decantation of the coarser soil layer. The supernatant was then removed and slowly filtered to obtain extract free of soil and coarse organic matter particles. A 10 mL aliquot was collected from the filtered extract and placed in a 125 mL Erlenmeyer flask added of 2 mL of 0.066 M potassium dichromate ($K_2Cr_2O_7$) to react with the CBM and 10 mL of concentrated sulfuric acid (H_2SO_4), as dichromate only reacts with organic matter in acid medium. Thereafter, the filtrate was cooled and 50 mL of distilled water were added. After further cooling, 4 drops of ferroim indicator were added for titration with 0.03 M ammoniacal ferrous sulfate ($Fe(NH_4)_2(SO_4)_2.6H_2O$).

The microbial carbon was calculated by the difference between carbon extracted from the filtrate of irradiated and non-irradiated samples.

The total number of bacteria was measured by counting colonies, using surface scattering in Petri dishes. From serial dilutions of soil samples, aliquots were transferred and scattered in Agar medium, and after growth (24 hours) in B.O.D. chamber, counting was performed by estimating the colony forming units (CFU), according to Wollum's methodology (1982).

The experimental design was completely randomized (CRD), in a 3 x 2 factorial scheme (control, sodium alginate and clay) (presence and absence of microbial inoculum) with five replicates (one seedling per replicate). Data were submitted to analysis of variance (ANOVA) and the means compared by the Tukey's test ($p \leq 0.05$), using the AgroEstat Software (Barbosa and Maldonado Júnior, 2009).

Conclusion

Plant growth promoting microorganisms that established in the rhizosphere have various abilities to enhance plant development and encapsulation assists these microorganisms in their establishment. However, there are several challenges in the search for adequate microorganisms and conditions for effective fruit tree growth promotion.

The results of the present study did not show significant effect of encapsulation on plant growth. However, some soil parameters such as ammonium content were positively affected for *Myrciaria dubia* (Kunth) Mc Vaugh (camu-camu).

These results of this study provide subsides for studies that seek new microbial interactions with appropriate fruit species and that result in a more effective fruit tree growth promotion.

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