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Microbial natural bioactive formulations in citrus development

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ABSTRACT

Efficient Microorganisms (EM) are commonly used in organic crops; however, there are no studies on their effects on the production of citrus seedlings. The work aimed to evaluate the impact of applying the inoculants Native Efficient Microorganisms (NEM) and the commercial product EM1® in forming the seedling of the rootstock *Poncirus trifoliata* (L.) Raf and in the development of young plants of Sweet Orange "Valência" (*Citrus sinensis* (L.) Osbeck) and Murcott tangor (*Citrus sinensis* x *Citrus reticulata* Blanco). The inoculant based on efficient microorganisms from the homemade technology of capture and multiplication, native efficient microorganisms (NEM), showed greater microbial diversity when compared to the commercial product EM1®. The results obtained from the dry mass analysis of the Valência orange and Murcott tangor plants indicate that positive effects resulting from the use of EM1® and NEM inoculums can be obtained by cultivating the respective crops in a system with oat straw cover. It was observed that the use of efficient microorganisms, as microbial natural bioactive formulation, has potential use in citrus and that farmers with fewer resources will be able to produce the microorganisms on their properties.

1. Introduction

Citrus fruits are appreciated worldwide owing to their high nutritional importance. Brazil is the world's largest citrus producer, emphasizing the standard group of dual-purpose oranges, fresh consumption, and juice. Its industrial processing generates several underexploited by-products that are generally disposed into the environment or used as animal feed. On average, 90% of Brazilian orange juice production is exported, with the European Union and the United States among the largest buyers of Brazilian orange juice, coming mainly from the State of São Paulo, which accounts for 60% of the Brazilian planted area and 74.5% of the total national production [1,2,3,4].

The rootstock most used to obtain citrus seedlings for the Southern region of Brazil is *Poncirus trifoliata*. The preference for seedlings formed from this rootstock results mainly from the significant tolerance to cold that it can give to the canopy cultivars grafted into it. When cultivated in a greenhouse, *P. trifoliata* is characterized by a slower growth rate than the other rootstocks frequently used for citrus, increasing the seedling time needed to form [5]. In addition, one of the most important varieties of this group for producers in southern Brazil is the Valência orange (*Citrus sinensis*), with fruits of good commercial acceptance and plants with significant tolerance to periods of low temperatures [6,7]. As well as the Valência orange, the Murcott tangor (*Citrus sinensis* x *Citrus reticulata*) also presents a critical characteristic of the dual aptitude of the fruits. The Murcott tangor is a hybrid that has been gaining more and more space in growing areas of citrus since when compared to commercial orange varieties, including Valência, it has shown more excellent resistance to "Citrus blight" and "Citrus variegated chlorosis" (CVC). These diseases have been severely affecting orange plantations [8,9].

In an adequate climate and soil conditions, citrus plants begin to produce from the third year of age onwards, gradually increasing production until the tenth year, when production tends to stabilize and maintain itself until the twentieth year when production declines [10]. According to Koller [11], one of the main factors limiting the production of citrus in young orchards is the size of the plants, which must have adequate aerial and root vegetative structure to support fruit production in the first years of life. To anticipate the return resulting from the

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implementation of citrus orchards, the sector has adopted the growing use of significant amounts of chemical fertilizers over time. Such practices have compromised the sustainability of production units, increasing citrus growers' dependence on external inputs, reducing profits, and causing severe environmental problems.

The existing alternatives to improve crop development and reduce the excessive use of agricultural inputs are the so-called Efficient Microorganisms (EM) [12,13]. This technology has proven to be an essential ally for organic crops as a whole, i.e., accelerating the decomposition of organic matter present in the soil and providing adequate release of nutrients and other beneficial substances for plant growth and health [14,15]. Brazilian legislation establishes that the production of citrus seedlings must be carried out in a protected environment, a practice that can facilitate the inoculation of microorganisms of interest, accelerate plants' development, and bring economic and environmental advantages to the producer [16].

One of the main groups of microorganisms used to increase plant development is the so-called Efficient Microorganisms (EM) [17]. The EM can be obtained commercially or produced with some ease and low cost by the rural producer.

Some studies have already demonstrated the benefits of using EM in citrus plants. According to Higa [12] and Hussain et al. [18], the use of EM in cultivated areas allows an optimized increase in plant development, improving the rates of decomposition and release of nutrients in the organic matter present in the soil. In addition, to protect plants against the attack of pests and diseases. The use of EM promoted an increase in germination, emergence, vigor, and survival of Cleopatra tangerine (*Citrus reshni* Hort.) seedlings [19]. Trees of Fruits of orange trees of the Pera variety (*Citrus sinensis* Osbeck) grafted to clove lemon (*Citrus limonia* Osbeck) rootstock, treated with EM, produced fruits with showed an 8% increase in the amount of orange juice, Paschoal et al [20]. observed an 8% increase in the amount of juice from orange fruits treated with EM. Among the microorganisms present in the EM group, Kupper et al [21]. reported that the yeast *Saccharomyces cerevisiae* effectively controls green citrus mold caused by the agent *Penicillium digitatum*.

Although several studies show the beneficial effects of EM, studies on the production of citrus seedlings in an organic system are still scarce. Thus, the present work aimed to evaluate the impact of Native Efficient Microorganisms (NEM) and the commercial product EM1®: i) on the root and aerial development of seedlings of the *Poncirus trifoliata* rootstock; ii) the influence of NEM and EM1® inoculants on the persistence of oat straw and fallow associated with the cultivation of Valência orange and Murcott tangor; iii) in the development of young orange plants of the Valência variety (*Citrus sinensis*) and the hybrid Murcott tangor (*Citrus sinensis* x *Citrus reticulata*) under oat straw coverage (*Avena sativa*) and fallow (spontaneous vegetation) situations.

2. Material and methods

2.1. Plant material and experimental conditions

The experiments were carried out under greenhouse conditions at the Federal University of Fronteira Sul, Campus Erechim, Erechim, RS, Brazil (27 ° 43′ 37.97 "S and 52 ° 17′ 16.23″ O, altitude 783 m). The municipality is located in a temperate zone, with a humid subtropical climate and well-distributed rains throughout the year, with hot summers and cold winters [22].

Experiment 1:

Seeds from the rootstock *Poncirus trifoliata* (L.) Raf was planted in August 2019 in tubes with a capacity of 150 cm^3, filled with a commercial substrate for seedling production. In the tube stage, twice as many plants as necessary were cultivated to ensure uniformity and a better selection of seedlings recommended by [23]. Therefore, 24 tubes were used per experimental unit, each sown with three seeds at approximately 2 cm depth. Fifty-two days after sowing (DAS), seedling thinning was performed, and only one seedling from a nucellar embryo per tube was left. The plants grew in the tubes for a period of 96 DAS.

After this period, 12 (twelve) of the 24 (twenty-four) tubes were selected for uniformity in size. The seedlings were then transplanted into polyethylene pots (6.2 L), with dimensions of 15 cm in diameter and 35 cm in height in height, filled with a commercial substrate for seedling production.

The plants were drip irrigated using dechlorinated water. The tubes and pots were kept on benches 40 cm from the floor. All lateral shoots of the plant stems were removed as they appeared to maintain the plant's growth in a single and erect stem.

The experimental design used was completely randomized (DIC), with 3 (three) treatments, 3 (three) replicates per treatment and 12 (twelve) plants per experimental unit. The treatments used were: Control (CT, without EM application); the commercial product EM1®; and native efficient microorganisms (NEM).

Experiment II:

Valência orange seedlings propagated using rootstock citrumelo Swingle (*Citrus paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.) and the Murcott tangor seedlings using the mandarin rootstock Cleópatra (*Citrus reshni* Hort.) were obtained commercially from a plant nursery. Seedlings free of pests and diseases were used, with good development according to the minimum quality standards for citrus seedlings [24].

The seedlings were transplanted in August 2019 in pots with a capacity of 25 dm3 containing a mixture consisting of 1 liter of tanned poultry manure, 150 gs of simple superphosphate, and sieved soil until the pot was filled. The general planting rule was performed according to Koller [8], and fertilization was described for citrus cultivation in pots [25]. The soil used was collected at a depth of 0 – 20 cm, on the premises of the experimental area of UFFS - Campus Erechim, in an area free from pesticides, close to where the native efficient microorganisms (NEM) were captured. The soil was nutritionally corrected 90 days before collection [26].

Immediately after planting, the seedlings were pruned at the height of 50 cm to form the canopy of the plants. Three branches were selected and distributed in a spiral around the stem after the emergence of the first shoots of the single stem (seedling stick). The other shoots formed below the selected branches were removed as suggested by Ledo et al. [27]. Other technical aspects related to planting, topdressing and general management of the plants were developed in compliance with the recommendations suggested by Souza et al [3]. and Koller [11].

The collection of oat straw (Avena sativa) and fallow (spontaneous vegetation) was carried out on the premises of the experimental area of UFFS – Campus Erechim/RS. Each pot received a layer of lightly compacted 7 cm straw, placed on the soil surface, a procedure carried out immediately after planting the seedlings. The fallow straw was mainly composed of Plantain Signal Grass (*Brachiaria plantaginea*) at the end of a cycle. The straws were harvested around 60 days before the beginning of the experiments, dried in the shade, and shredded with the aid of an organic waste shredder (model TR-200, TRAPP, Jaraguá do Sul, Brazil) with a sieve/outlet diameter of 5 cm.

The experimental design used was a completely randomized design (CRD), with treatments arranged independently for each crop. (Valência orange and Murcott tangor). The factorial scheme for each citrus crop was three by two (3 × 2). The first factor refers to the 3 (three) treatments: commercial product EM1®; native efficient microorganisms (NEM); and control (C). The second factor is composed of 2 (two) mulch situations: oat straw (*Avena sativa*) and straw from fallow (spontaneous vegetation). Five (5) replicates per treatment, and one (1) plant per experimental unit was used.

2.2. Capture and multiplication of native efficient microorganisms – NEM

Native efficient microorganisms (NEM) were captured and multiplied in culture medium using the methodology proposed by Leite and Meira [28]. The baits for capture were collected inside the forest area,

using cooked rice, at the Federal University of Fronteira Sul, Erechim campus, Erechim, RS, Brazil. The colonies were separated from microorganisms using sterile plastic spatulas. Only the yellow, pink and white rice bait portions were used for the EM multiplication process.

2.3. NEM activation

The activation of NEM occurred previously at each moment of application of treatments; 50 ml of stabilized NEM, 50 ml of cane molasses, and 900 ml of dechlorinated water were added in a container, as described by Leite and Meira [28]. The mixture was kept in an expandable and air-tight container for five days, and the gasses were released once a day as recommended by Zhong et al [29]..

2.4. EM1® activation

The activation of the commercial product EM1® was performed according to the manufacturer's recommendations. The product was mixed in the proportion of 1 part (5%) of EM1®, 1 part (5%) of cane molasses, and 18 parts (90%) of dechlorinated water; the solution was stirred until homogeneous. The mixture remained in a sealed container for seven days. The container lid was opened daily just enough to remove excess gas during the fermentation.

2.5. Microorganisms identification

For the EM 1® inoculant, the microbial composition information provided by the manufacturer was considered.

The evaluation of the microbial diversity of the MNE was carried out using the methodology of analysis and identification NGS (New Generation DNA Sequencing). Samples (100 ml) of activated NEM were centrifuged at 560 g for 5 min, and 100 mg of pellet from each sample was transferred to microtubes in NeoZ solution. The identification of microorganisms present in the fermentation process was performed from the extraction of genetic material using 1 g of the biomass sample by the PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA), the manufacturer's instructions. The V3-V4 regions were amplified with primers 341F/806R, according to Caporaso et al [30]. and Wang and Qian [31]. The libraries were sequenced in a MiSeq system, using the Illumina standard primers provided in the kit. The operational taxonomic units (UTOs) were grouped sequences subjected to taxonomic classification in tree service databases, considering at least 99% identity with the reference database.

2.6. Treatment application

For both experiments, the application solution was prepared from the mixture of activated EM1® or NEM in water in the proportion of 16,65/500, according to Zydlik and Zydlik [32]. In experiment I, the treatments was applied monthly, starting at 52 DAS, at a dose of 16,65 l/ha of activated inoculant for both inoculants. The inoculants were applied using a watering can directly on the plants and the substrate surface present in the tubes or polyethylene bags. Treatment applications started in October 2019 and ended in February 2020. In experiment II, the application of the treatments EM1® and NEM occurred between 30 and 390 days after planting (DAP) of the plants, with monthly frequency and dosage per application, for both EM1® and NEM 16.65 l/ha of activated inoculant. The application of NEM and EM1® was carried out using a manual sprayer directly on the straw covering present in the pot. Applications were always carried out after irrigation, as Andrade et al [13]. indicated.

2.7. Morphological analysis

Morphological analyzes were performed at 217 DAS for the plants of experiment I. For experiment II, the persistence of the straw was measured monthly until 240 days after the planting of seedlings, the PH and SD were measured monthly until 300 days after planting, and NL, TLA, LAI, CV, DMSB, LDM, SDM, RDM, TDM, TRV, TRSA e RCD were performed at 419 DAP (days after planting).

Plant height (PH), considering the neck to the apex, was obtained using a measuring tape. The stem diameter (SD) at the grafting point's height (10 cm from the neck) was evaluated using a digital caliper. Leaf number (LN) was determined considering all fully expanded leaves. Digital images of the leaves were obtained to determine the total leaf area (LA), performed using the ImageJ software (Image Processing and Analysis in Java, v. 1.52d, USA). The leaf area index (LAI) was calculated by the ratio between the total leaf area (TLA) and the occupied surface area (OSA), according to Eqs 1 and 2:

$$\mathrm{LAI} = \frac{\mathrm{TLA}}{\mathrm{OSA}}$$

$$OSA = \pi\left(\frac{CRD + CID}{4}\right)^2$$

The canopy volume (CV) was analyzed according to Zekri et al. [30], according to Eq 3:

$$CV = \pi\left(\frac{\pi}{6}\right) \times H \times CRD \times CID$$

Where H is the height of the plant, CRD is the canopy diameter in the row direction, and CID is the canopy diameter in the interrow direction.

The roots were removed from the substrate and carefully washed using fine sieves. The root collar diameter (RCD) was determined in the pivoting root using a digital caliper. Photographs of the roots were obtained using a digital camera (13MP; 3264 × 1836px) for further processing of the images using the Safira v1.1 software (Fiber and Root Analysis System, Embrapa Instrumentação Agropecuária, São Paulo, Brazil). From these analyzes, the following variables were obtained: total root volume (TRV, cm^3), total root length (TRL, cm) and total root area (TRA, cm^2); very thin root volume (VTRV, cm^3), very thin root length (VTRL, cm) and very thin root area (VTRA, cm^2), with a diameter of less than 1 mm, ($\varnothing < 1$ mm); thin root volume (THRV, cm^3), thin root length (THRL, cm) and thin root area (THRA, cm^2), with a diameter between 1.1 and 2.0 mm ($1.1 < \varnothing < 2.0$ mm); medium root volume (MRV, cm^3), medium root length (MRL, cm) and medium root area (MRA, cm^2), with a diameter between 2.1 and 3.0 mm ($2.1 < \varnothing < 3.0$ mm); and thick root (TCRV, cm^3), thick root length (TCRL, cm) and thick root area (TCRA, cm^2), with a diameter greater than 3.1 mm ($\varnothing > 3.1$ mm), according to the methodology described by Böhm [34], with adaptations.

Leaf dry matter (LDM, g), stem dry matter (SDM, g), and root dry matter (RDM, g) were obtained by weighing the vegetal material on a semi-analytical scale. The leaves, stem, and roots were then dried separately in an oven with forced ventilation, at 65 °C, until constant weight. The shoot dry matter (SDM, g) was obtained by the sum of LDM and SDM. Total dry mass (TDM, g) was obtained by APDM and RDM. Specific root length (SRL, cm/g) was calculated by the ratio between TRL and RDM.

The straw column decrease was measured monthly with the aid of a digital caliper, a ruler, and a 2.5 cm x 10 cm acrylic base. For each pot (plot), four monthly readings were collected at pre-established points, distributed equidistantly over the surface of the straw present in the pot.

2.8. Statistical analysis

All the data obtained were subjected to analysis of variance by the F test followed by Tukey test's, using the statistical software Past v. 3.24.

In experiment II, to evaluate the development effect of the Valencia and Murcott tangor orange plants, at 419 DAP, the straw factor was disregarded, resulting in three treatments: control without straw factor

(CS); EM1® without straw factor (EM1®S); and NEM without straw factor (NEMS). The treatments contained the data obtained both with oat straw and fallow straw.

3. Results

3.1. Microbial composition of EM1® and NEM inoculants

In the NEM samples, four species of fungi and 33 species of bacteria were identified. The totals of sequences of the species of fungi and bacteria found are shown in Table 1.

The EM1 inoculant has one fungus species (yeast) and two species of bacteria (lactic acid). The microorganisms' concentrations were reported by the company Ambien Indústria e Comércio Ltda and are shown in Table 2.

The NEM showed a greater microbial variety concerning the commercial product EM1®. The species *Lactobacillus acidophillus* and *Lactobacillus casei* were identified in the commercial product EM1® and NEM.

3.2. Experiment I: morphological traits of Poncirus trifoliata

Plant height (PH), number of leaves (LN), and total leaf area (LA) did not show significant differences between treatments. The *P. trifoliata* plants grown under EM1® and NEM treatments showed 17% and 15% increments in the stem diameter (SD) compared to the control plants (Table 3).

Table 1
Fungi and bacteria found in a sample of Native Efficient Microorganisms – NEM.

Species	Total sequences	%
Fungus		
Zygotorulaspora florentina	44,240	53.66
Pichia nakasei	37,907	45.98
Hanseniaspora uvarum	271	0.32
Mortierella sp. bc_besc_211a	15	0.01
Total sample	**82,433**	**100**
Bacterium		
Gluconobacter cerinus	13,257	78.53
Lactobacillus casei	1352	8.01
Gluconobacter frateurii	1145	6.78
Streptococcus thermophilus	212	1.26
Methylophilus methylotrophus	175	1.04
Sphingomonas melonis	134	0.79
Novosphingobium subterraneum	101	0.60
Acidovorax delafieldii	62	0.37
L. acidophilus	52	0.31
Gluconobacter oxydans	43	0.25
Sinorhizobium fredii	40	0.24
Afipia genosp.	27	0.16
Bifidobacterium animalis	26	0.15
LactoBacillus brevis	26	0.15
Flavobacterium terrigena	22	0.13
Lactococcus lactis	22	0.13
Cupriavidus pampae	19	0.11
Arcicella rosea	18	0.11
Bosea minatitlanensis	16	0.09
Janthinobacterium agaricidamnosum	16	0.09
Duganella zoogloeoides	15	0.09
Acidovorax temperans	14	0.08
Novosphingobium taihuense	14	0.08
Pelomonas saccharophila	12	0.07
Variovorax paradoxus	12	0.07
Caulobacter vibrioides	9	0.05
Acidovorax radicis	7	0.04
Bacillus cereus sp. group	7	0.04
Caulobacter henricii	6	0.04
Neisseria flavescens	5	0.03
Pseudomonas koreensis	5	0.03
Pseudomonas putida	5	0.03
Rhodoferax ferrireducens	5	0.03
Total sample	**16,881**	**100%**

Table 2
Fungus and bacteria found in the commercial inoculant EM1®.

Espécie	CFU/ml	%
Saccharomyces cerevisae	$> 1.5 \times 10^6$	92.25
Lactobacillus acidophillus	$> 7.9 \times 10^4$	4.86
Lactobacillus casei	$> 4.7 \times 10^4$	2.89

Table 3
Plant height (pH), stem diameter (SD), leaves number (LN), leaf area (LA), root diameter at the neck level (RDNL), weighted average root diameter (WARD), and specific root length (SRL) of *P. trifoliata* plants grown without inoculant (Control, CT) and with a commercial product (EM1®) and native efficient microorganism (NEM) inoculants, at 217 days after sowing.

	PH (cm)	SD (cm)	LN	LA (cm^2)
CT	871.46 ± 31.88 [a,†]	52.23 ± 2.33 [b]	474.66 ± 12.68 [a]	1830.62 ± 179.11 [a]
EM1®	950.20 ± 58.24 [a]	60.32 ± 3.15 [a]	500.33 ± 15.32 [a]	1916.09 ± 203.21 [a]
NEM	890.40 ± 48.25 [a]	59.65 ± 1.99 [a]	494.00 ± 25.13 [a]	1984.83 ± 101.26 [a]
	RDNL (mm)	WARD (mm)	SRL (cm/g)	
CT	60.17 ± 2.40 [b,†]	0.11 ± 0.01 [a]	290.88 ± 11.39 [a]	
EM1®	67.49 ± 3.30 [a]	0.13 ± 0.04 [a]	290.25 ± 32.34 [a]	
NEM	62.60 ± 2.12 [ab]	0.13 ± 0.03 [a]	288.67 ± 24.91 [a]	

[†] Data represent means ± SD ($n = 3$). Means followed by different letters between treatments differ by Tukey test ($p < 0.05$).

The root diameter at the neck level (RDNL) was increased by 12% when the plants grew with EM1®, compared to the control treatment (Table 3). The variables weighted average root diameter (WARD) and specific root length (SRL) was not influenced by the treatments (Table 3).

The total root volume (TRV) of *P. trifoliata* plants treated with the

Table 4
Total root volume (TRV), very thin root volume (VTRV), thin root volume (THRV), medium root volume (MRV), thick root volume (TCRV), total root length (TRL), very thin root length (VTRL), thin root length (THRL), medium root length (MRL), and thick root length (TCRL) of *P. trifoliata* plants grown without inoculant (Control, CT) and with a commercial product (EM1®) and native efficient microorganism (NEM) inoculants, at 217 days after sowing.

	TRV (cm^3)	VTRV (cm^3)	THRV (cm^3)	MRV (cm^3)	TCRV (cm^3)
CT	75.30 ± 3.20 [b,†]	23.17 ± 1.38 [a]	19.72 ± 3.51 [a]	9.30 ± 0.43 [b]	23.09 ± 1.59 [b]
EM1®	85.18 ± 2.38 [a]	24.59 ± 0.96 [a]	20.41 ± 2.78 [a]	11.50 ± 0.65 [a]	28.67 ± 1.43 [a]
NEM	79.54 ± 3.78 [ab]	23.52 ± 1.20 [a]	19.56 ± 1.53 [a]	10.40 ± 0.69 [ab]	26.06 ± 2.14 [ab]
	TRL (m)	VTRL (m)	THRL (cm)	MRL (cm)	TCRL (cm)
CT	41.97 ± 2.28 [a,†]	37.35 ± 1.99 [a]	282.71 ± 26.00 [a]	96.41 ± 4.58 [a]	82.78 ± 6.58 [a]
EM1®	47.40 ± 3.36 [a]	42.08 ± 3.43 [a]	340.48 ± 28.61 [a]	100.63 ± 6.74 [a]	91.23 ± 12.62 [a]
NEM	45.08 ± 4.21 [a]	39.74 ± 3.87 [a]	336.44 ± 44.16 [a]	106.91 ± 4.78 [a]	91.18 ± 10.83 [a]
	TRA (cm^2)	VTRA (cm^2)	THRA (cm^2)	MRA (cm^2)	TCRA (cm^2)
CT	316.83 ± 29.38 [a,†]	176.55 ± 23.33 [a]	54.55 ± 2.94 [a]	23.43 ± 0.92 [b]	62.30 ± 3.39 [b]
EM1®	345.26 ± 14.39 [a]	184.06 ± 19.78 [a]	59.21 ± 1.84 [a]	27.82 ± 2.08 [a]	74.15 ± 4.20 [a]
NEM	326.80 ± 25.64 [a]	179.23 ± 14.56 [a]	55.70 ± 2.95 [a]	25.58 ± 1.34 [ab]	65.60 ± 3.99 [ab]

[†] Data represent mean ± SD ($n = 3$). Means followed by different letters between treatments differ by Tukey test ($p < 0.05$).

commercial product EM1® was 21% higher than the control treatment (Table 4). The treatments EM1® and NEM did not differ, and the NEM did not differ in the control treatment (CT). The volume of very thin roots (VTRV) and volume of thin roots (THRV) were not influenced by any of the treatments with efficient microorganisms (Table 4).

The EM1®-treatment caused an increase of 23% in the volume of medium roots (MRV) and 24% in the volume of thick roots (THRV) compared to CT-treatment. There were no differences in the volume of medium roots (MRV) and volume of thick roots (TCRV) between the treatments EM1® and NEM, as well as there was no difference between the NEM and the control treatment (Table 4).

Although EM1® has shown an increase in the TRV compared to the CT treatment, a detailed analysis of the roots, according to the diameter range, has demonstrated that EM1® significantly increased only medium and thick roots (Table 4).

No differences were observed in the total root length (TRL), very thin root length (VTRL), thin root length (THRL), medium root length (MRL), and thick root length (TCRL) of *P. trifoliata* plants submitted to control, EM1® and NEM treatments (Table 4).

Total root surface area (TRA), very thin root area (VTRA), and the thin root surface area (THRA) were not influenced by the treatments (Table 5). The EM1®-treatment increased the medium root area (MRA) by 18% and the thick root surface area (TCRA) by 19% when compared to the CT treatment (Table 4).

Inoculants NEM- and EM1®-treatment did not affect the leaf dry matter (LDM), stem dry matter (SDM), root dry matter (RDM), aerial part dry matter (APDM), and total dry matter (TDM) (Table 5).

3.3. Experiment II: evolution and persistence of straw columns

At 60 days after the start of inoculant applications, it was observed that the treatment with NEM (VNEMO, MNEMO, VNEMF, and MNEMF) for both straws, and with EM1® and oat straw (VEM1®O and MEM1®O)) positively influenced the decrease of the straw column compared to treatments without the application of any of the inoculants (VOS, MOS, VFS, and MFS) (Table 6).

However, after 120 days, the accumulated persistence of both oat and fallow straw regardless of the application of efficient microorganisms (EM1® or NEM) did not differ between treatments for both citrus, Valência orange, and Murcott tangor (Table 6).

3.4. Experiment II: morphological traits of Valência orange e tangor Marcott

The Valencia orange plants submitted to treatments with efficient microorganisms and oat straw (EM1®O and NEMO) showed higher accumulated growth in height than treatments without efficient microorganisms (OS and FS) between 180 and 240 days after the start of treatments (Fig. 1A). The height growth of Murcott tangor plants submitted to efficient native microorganisms treatment with fallow straw (NEMF) was superior to the control treatment with oat straw (OS) at 180 days (Fig. 1B). There were no differences in plant height for the other treatments and periods evaluated (Figs. 1).

Treatment with NEM and oat straw (NEMO) showed more significant stem diameter growth of Valência orange plants compared to treatment with oat straw (OS) only at 90 days (data not shown). The Murcott tangor plants treated with EM1® and fallow straw (EM1®F) showed positive results in the monthly growth of the stem diameter 60 days earlier when compared to the association with oat straw (data not shown). The cumulative development of stem diameter was higher in Valência orange plants submitted to treatments with EM1®, with both oat and fallow straw (EM1®O and EM1®F) and to treatment with NEM with fallow straw (NEMF) compared to the control treatment with oat straw (OS), mainly from 150 days onwards and keeping them until 240 days (Fig. 2A). As for the Murcott tangor plants, the accumulated growth of the stem diameter was higher with the treatments EM1®O and EM1®F, concerning the OS, at 150 days (Fig. 2B).

No differences were observed between the EM1® and NEM treatments for the variables number of leaves (NL), total leaf area (TLA), leaf area index (LAI), canopy volume (CV) for the Valência orange and Murcott tangor plants (Table 7).

The dry mass of stem and branches (DMSB) was increased by an average of 23.3% in the Valencia orange plants treated with EM1®O, EM1®F, and NEMF; and 26.5% in Murcott tangor plants in treatments with EM1®O and NEMF, concerning the respective control treatments with oat straw (OS) (Table 7). When analyzing the DMSB and leaf dry mass (LDM) data, without considering the straw factor, for both citrus plants evaluated, the treatments with EM1® without straw factor (EM1®S) and NEM without straw factor (NEMS) were, on average, 16.5% and 13% higher, respectively than the control treatment without straw factor (CS) (Table 7). Similar to the DMSB, the (LDM) of the Valência orange plants had an increase, on average, of 25.3% when comparing the treatments EM1®O, NEMO, and NEMF, concerning the OS treatment (Table 7). For the Murcott tangor plants, no effects on LDM were observed with the treatment with EM1®, regardless of the straw used, while NEM presented higher values for LDM when associated with oat straw (OS) (20%) and fallow straw (24%) (Table YY). Disregarding the straw factor, the LDM of the Murcott tangor plants was higher with the treatments EM1®S (13%) e NEMS (16%), concerning the control treatment without straw factor (CS) (Table 7).

The root dry mass (RDM) and total dry mass (TDM) variables of the Valência orange and Murcott tangor plants were not influenced by the evaluated treatments (Table 8). The SDM of Valência orange plants had significant increases, on average 9.3%, for both inoculants associated with oat straw (EM1®O e NEMO) and for NEM associated with fallow straw (NEMF), concerning the control treatment with oat straw (OS). As for the Murcott tangor plants, the NEMF treatment was the only positive effect on SDM, increasing 11% compared to the control treatment with oat straw (OS). Disregarding the straw factor, the SDM of the Valência orange plants were 7% and 8% higher in the treatments EM1®S and NEMS, respectively, and 6% (EM1®S) and 9% (NEMS) for the Murcott tangor plants, concerning the respective control treatments without straw factor (CS) (Table 8).

The variables total root volume (TRV), total root surface area (TRSA), and root collar diameter (RCD) of both evaluated citrus, Valência orange and Murcott tangor, were not influenced by the tested treatments (Table 8). It was possible to verify an increase in DM of the Valência orange plants and Murcott tangor plants treated with the inoculants EM1® and NEM when associated with oat straw alone (Table 8).

Table 5
Leaf dry matter (LDM), stem dry matter (SDM), root dry matter (RDM), shoot dry matter (SHDM), and total dry matter (TDM) of *P. trifoliata* plants grown without inoculant (Control, CT) and with a commercial product (EM1®) and native efficient microorganism (NEM) inoculants, at 217 days after planting.

	LDM (g)	SDM (g)	RDM (g)	SHDM (g)	TDM (g)
CT	19.80 ± 1.89 [a†]	42.34 ± 2.10 [a]	16.18 ± 0.71 [a]	62.14 ± 3.46 [a]	78.33 ± 3.12 [a]
EM1®	21.80 ± 1.72 [a]	47.29 ± 2.93 [a]	18.10 ± 0.88 [a]	69.10 ± 3.69 [a]	87.20 ± 4.40 [a]
NEM	20.36 ± 1.53 [a]	44.62 ± 2.17 [a]	17.29 ± 0.96 [a]	64.99 ± 4.63 [a]	82.28 ± 4.26 [a]

[†] Data represent mean ± SD ($n = 3$). Means followed by different letters between treatments differ by Tukey test ($p < 0.05$).

4. Discussion

4.1. Diversity and possible microbial interaction in inoculants with efficient microorganisms and its effects on plant development

The greater diversity of microbial species found in the NEM

Table 6
Accumulated persistence of the oat straw column (O) and fallow (spontaneous vegetation, F) associated with the cultivation of Valência orange (V) and Murcott tangor (M) evaluated up to 240 days after the start of applications with a commercial product (EM1®) and native efficient microorganism (NEM).

Treatments[¥]	Days after the start of treatment application							
	30	60	90	120	150	180	210	240
VOS	0.02[a,†]	0.50[b]	0.96[c]	1.82[a]	2.44[a]	3.48[a]	3.98[a]	4.58[a]
MOS	0.00[a]	0.52[b]	1.00[bc]	1.74[a]	2.52[a]	3.60[a]	4.10[a]	4.62[a]
VFS	0.02[a]	0.50[b]	0.94[c]	1.78[a]	2.46[a]	3.38[a]	4.04[a]	4.74[a]
MFS	0.06[a]	0.57[b]	1.06[bc]	1.74[a]	2.54[a]	3.62[a]	4.24[a]	4.82[a]
VEM1®O	0.02[a]	0.86[a]	1.40[a]	1.90[a]	2.66[a]	3.66[a]	4.22[a]	4.76[a]
MEM1®O	0.00[a]	0.85[a]	1.42[a]	1.90[a]	2.54[a]	3.40[a]	3.90[a]	4.42[a]
VEM1®F	0.00[a]	0.66[ab]	1.16[ac]	1.84[a]	2.68[a]	3.72[a]	4.20[a]	4.72[a]
MEM1®F	0.00[a]	0.66[ab]	1.22[ac]	1.98[a]	2.76[a]	3.70[a]	4.28[a]	4.82[a]
VNEMO	0.02[a]	0.88[a]	1.34[ab]	1.96[a]	2.88[a]	3.84[a]	4.40[a]	4.98[a]
MNEMO	0.02[a]	0.86[a]	1.40[a]	2.00[a]	2.58[a]	3.54[a]	4.02[a]	4.58[a]
VNEMF	0.08[a]	0.93[a]	1.37[ab]	2.03[a]	2.85[a]	3.87[a]	4.45[a]	4.97[a]
MNEMF	0.02[a]	0.85[a]	1.41[a]	1.87[a]	2.59[a]	3.53[a]	4.11[a]	4.61[a]

[¥] Valência orange with oat straw (VOS), Murcott tangor with oat straw (MOS), Valência orange with fallow straw (VFS), Murcott tangor with fallow straw (MFS), Valência orange with oat straw under application of a commercial product EM1® (VEM1®O), Murcott tangor with oat straw under application of a commercial product EM1® (MEM1®O), Valência orange with fallow straw under application of a commercial product EM1® (VEM1®F), Murcott tangor with fallow straw under application of a commercial product EM1® (MEM1®F), Valência orange with oat straw under application of native efficient microorganisms (VNEMO), Murcott tangor with oat straw under application of native efficient microorganisms (MNEMO), Valência orange with fallow straw under application of native efficient microorganisms (VNEMF) e Murcott tangor with fallow straw under application of native efficient microorganisms (MNEMF).

[†] Data represent mean ± SD ($n = 5$). Means followed by different letters between treatments differ by Tukey test ($p < 0.05$).

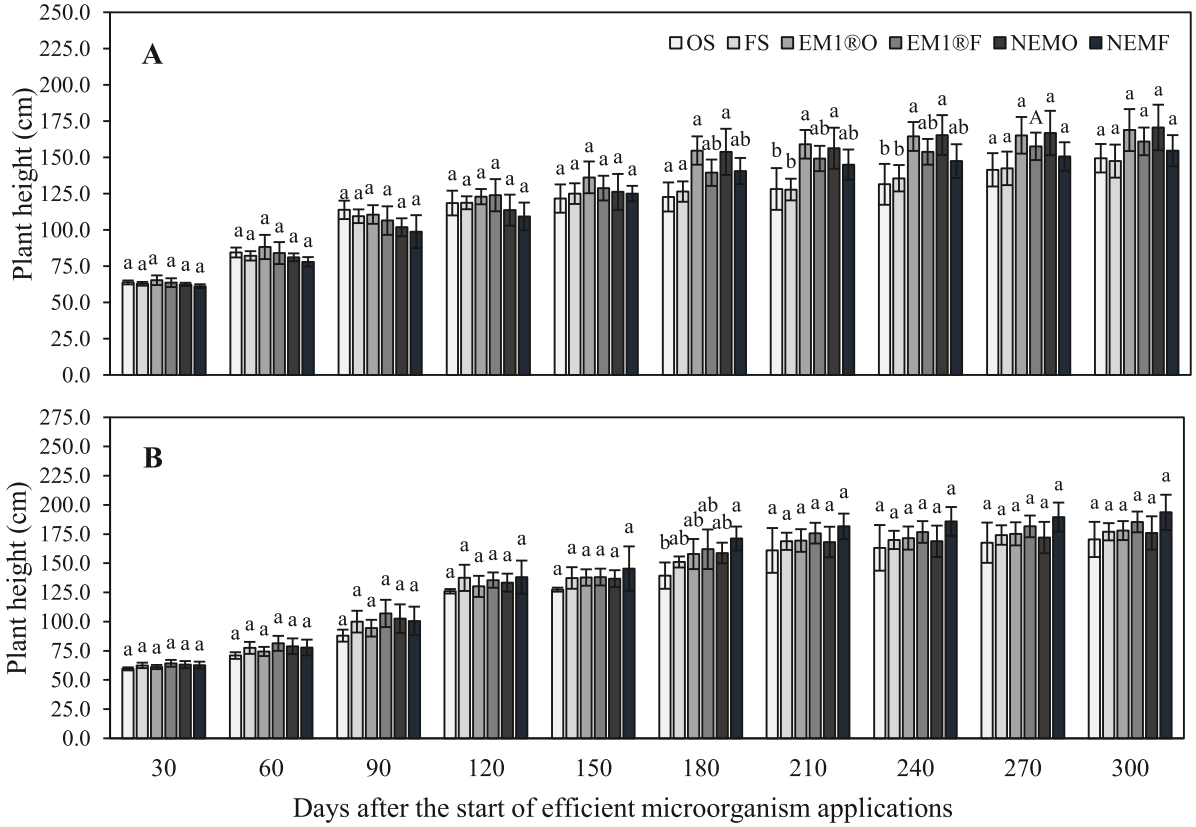

Fig. 1. Growth in the height of the Valência orange (A) and Murcott tangor (B) plants under control with oat straw (OS), control with fallow straw (FS), commercial product EM1® with oat straw (EM1®O), commercial product EM1® with fallow straw (EM1®F), native efficient microorganisms with oat straw (NEMO) and native efficient microorganisms with fallow straw (NEMF), evaluated at 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 days after the beginning of the application of efficient microorganisms (EM1® and NEM). Bars represent mean ± SD (n= 5). Means followed by different letters between treatments differ by Tukey test ($p < 0.05$).

(Tables 1) compared to the commercial product EM1® (Table 2) is verified by the impossibility of selecting species during capture, which will be multiplied for the production of the inoculant. In industries, inoculants' preparation occurs with few species, using microorganisms compatible with each other, with beneficial effects already known in plants and soil, and adapting to different climate and soil conditions. This means that homemade inoculant preparations, based on efficient microorganisms, can have diverse species among themselves. For example, Higa and Wididana [35] observed more than 80 species from 10 different genera of microorganisms in an EM-based preparation. Despite the more significant number of species, the authors observed a lower diversity of genera than our work, which showed 37 species and 27 genera. Even in works carried out in Brazil, there is a variation in the microbial diversity of inoculants. Santos et al [36]. identified the genera,

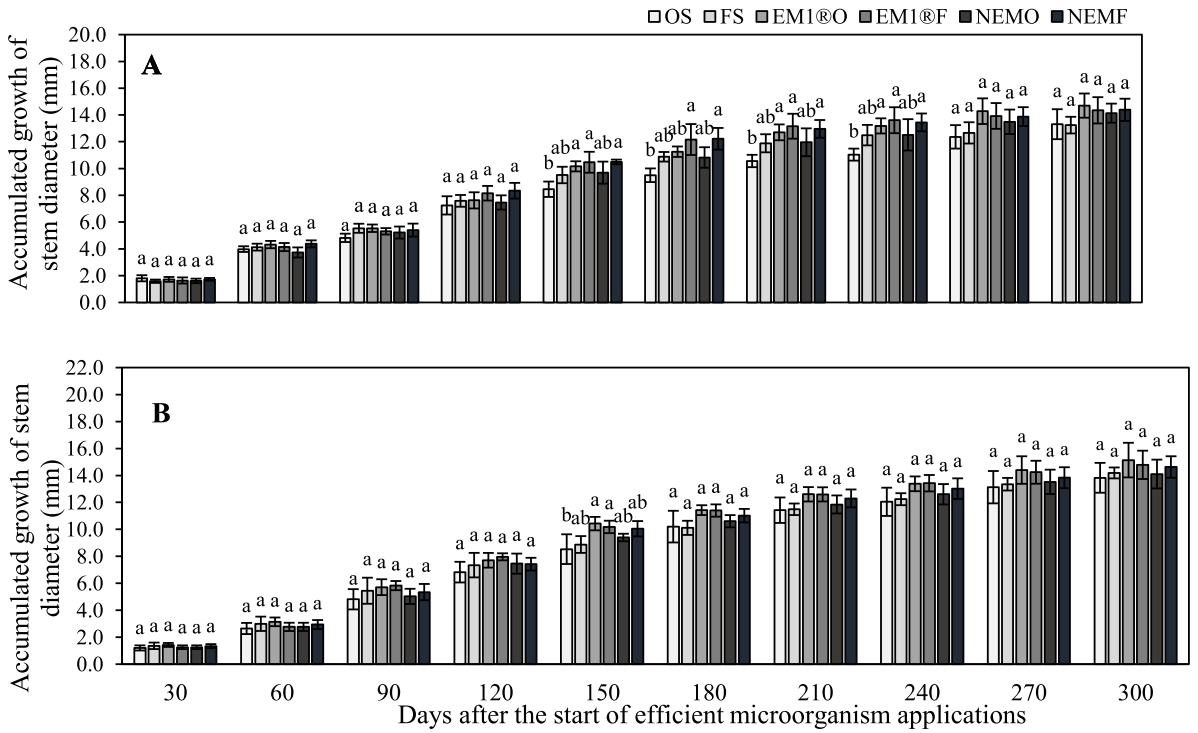

Fig. 2. Accumulated growth in stem diameter of the Valencia orange (A) and Murcott tangor (B) plants under control treatment with oat straw (OS), control with fallow straw (FS), commercial product EM1® with oat straw (EM1®O), commercial product EM1® with fallow straw (EM1®F), native efficient microorganisms with oat straw (NEMO) and native efficient microorganisms with fallow straw (NEMF) evaluated at 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 days after the beginning of the application of efficient microorganisms (EM1® and NEM). Bars represent mean ± SD (n= 5). Means followed by different letters between treatments differ by Tukey test ($p < 0.05$).

Candida, Peniophora, Penicillium, Uwebraunia, and *Fusarium*, inoculant prepared from EM the Zona da Mata region of Minas Gerais, in southeastern Brazil. No microorganisms of these genera were identified in the inoculant prepared in the present study from EM in southern Brazil.

The efficiency of inoculants depends on the beneficial interaction between the microorganisms. Different species of bacteria and fungi were observed in the NEM, while the EM1® consists of only two species of bacteria of the genus *Lactobacillus* and the fungus *Saccharomyces cerevisiae*.

Species of the genus *Lactobacillus* are present in both EM1® and NEM. *Lactobacillus* species, in addition to showing positive responses in the control of *Phytophthora nicotianae* – a fungus that causes gummosis or colon rot that compromises the root system–, have been described for improving the development of the root system in citrus rootstocks [16] and favor the growth of tomato seedlings [37].

In addition to lactic acid bacteria, gram-negative bacteria were found in the NEM samples. Several of these bacteria are described for their beneficial effects on plants, such as species of the genus *Methylophilus* and *Sphingomonas melonis*, commonly found in the rhizosphere. These organisms are associated with the decomposition processes of organic matter, in addition to being able to metabolize a vast range of carbon compounds and promote plant growth [38]. Also, bacteria of the genus *Pseudomonas* are described for their ability to solubilize and make phosphorus (P) available to plants [39]. The proteobacterium *Variovorax paradoxes* is also a species of interest in agriculture identified in the NEM used in this study. Regardless of the bacterial load, *V. paradoxes* have been identified as promoting root growth in tomato and potato crops [40,41], in addition to presenting good levels of root colonization [42].

Fungi are also present in the microbial composition of both EM1® and NEM. The EM1® has in its formulation only the yeast *Saccharomyces cerevisiae*, a species known and documented for its beneficial effects on plants. The NEM presented four fungal species: *Zygotorulaspora florentina, Pichia nakasei, Hanseniaspora uvarum,* and *Mortierella* sp. Despite being commonly found in homemade EM-based preparations, the yeast S. cerevisiae was not identified in the NEM inoculant due to its natural occurrence in soils and quickly multiplied. This may be due to non-Saccharomyces fungi, such as *Z. florentina, P. nakasei, H. uvarum*, and *Mortierella* sp., the NEM during fermentative processes, *Saccharomyces* and non-*Saccharomyces* yeasts do not usually passively coexist. Species of both genera *Pichia* [43,44] and *Zygotorulaspora* [45] affect the growth of *S. cerevisiae*, both by competition and by the production of inhibitory compounds.

Despite being antagonistic to *S. cerevisiae*, the species *P. nakasei*, found in NEM, also has beneficial effects for plants. Species of both genera *Saccharomyces* and *Pichia* showed potential responses in inhibiting growth in pathogenic fungi in vines [46]. Platania et al [47]. found that toxic proteins' production appears to be a general characteristic of yeast species of different genera, including *Saccharomyces, Hansenula, Kluyveromyces,* and *Pichia*. According to the authors, in *S. cerevisiae*, it is possible to identify at least three different toxins with fungicidal potential on *Penicillium digitatum*, a citrus rot agent.

However, the high microbial diversity can cause interaction and competition between the microorganisms, reducing the inoculant's effectiveness. Previous studies have reported the presence of *Streptococcus thermophilus*, for example, found in NEM, can be harmful to *Lactobacillus acidophilus* [48]. The presence of genus *Gluconobacter*, such as *Gluconobacter cerinus* –the most abundant bacterium in MNE–is also considered undesirable in crops since these microorganisms are often associated with plant rot [49]. According to He et al [50]., the pathogen *G. cerinus* develops well in environments rich in sugar, causing putrefaction of plant tissues and positively influencing the fruit fly's hatching rate (*Bactrocera dorsalis*) due to their symbiotic association. Giassi et al. [16] also emphasize the difficulty in elucidating lactic acid bacteria's effects on plants due to the high complexity of interactions between microorganisms present in the soil or substrate for cultivation.

Table 7
The number of leaves (NL), total leaf area (TLA), leaf area index (LAI), canopy volume (CV), dry mass of stem and branches (DMSB), and dry mass of leaves (LDM) of Valência orange and Murcott tangor plants grown under oat straw (OS), fallow straw (FS), commercial product EM1® without straw factor (EM1®S), commercial product EM1® with oat straw (EM1®O), commercial product EM1® with fallow straw (EM1®F), native efficient microorganisms without straw factor (NEMS), native efficient microorganisms with oat straw (NEMO) and native efficient microorganisms with fallow straw (NEMF), up to 419 days after planting.

	NL	TLA (m^2)	LAI	CV (cm^3)	DMSB (g)	LDM (g)
Valência orange						
OS	190.00 ± 17.18[a,†]	0.99 ± 0.17[a]	3.58 ± 0.26[a]	462.27 ± 104.85[a]	263.00 ± 24.18[b]	110.20 ± 8.30[b]
FS	196.00 ± 25.72[a]	1.05 ± 0.04[a]	3.95 ± 0.69[a]	478.51 ± 99.77[a]	285.40 ± 17.20[ab]	118.40 ± 15.60[ab]
EM1®O	205.80 ± 18.29[a]	1.13 ± 0.12[a]	4.21 ± 0.55[a]	473.29 ± 53.85[a]	330.40 ± 15.86[a]	148.20 ± 15.15[a]
EM1®F	208.00 ± 22.49[a]	1.12 ± 0.20[a]	4.07 ± 0.99[a]	500.11 ± 95.30[a]	325.60 ± 26.75[a]	137.60 ± 17.86[ab]
NEMO	206.00 ± 23.62[a]	1.09 ± 0.17[a]	4.04 ± 1.13[a]	507.67 ± 108.91[a]	292.20 ± 17.66[ab]	144.50 ± 19.00[a]
NEMF	212.40 ± 19.73[a]	1.12 ± 0.09[a]	3.81 ± 0.75[a]	535.28 ± 98.52[a]	322.00 ± 27.97[a]	149,80 ± 18.01[a]
CS	193.00 ± 22.08[a]	1.02 ± 0.13[a]	3.77 ± 0.55[a]	470.39 ± 102.66[a]	274.20 ± 23.78[b]	114.30 ± 13.15[b]
EM1®	206.90 ± 20.53[a]	1.12 ± 0.17[a]	4.14 ± 0.80[a]	486.70 ± 78.55[a]	328.00 ± 22.12[a]	142.90 ± 17.39[a]
NEMS	209.20 ± 21.99[a]	1.10 ± 0.14[a]	3.92 ± 0.97[a]	521.48 ± 104.76[a]	307.10 ± 27.73[a]	147.20 ± 18.53[a]
Murcott tangor						
OS	275.80 ± 20.39[a,†]	0.78 ± 0.17[a]	2.94 ± 0.69[a]	524.17 ± 148.55[a]	251.80 ± 28.16[a]	103.20 ± 15.38[b]
FS	274.00 ± 24.28[a]	0.81 ± 0.15[a]	3.15 ± 0.85[a]	552.95 ± 62.57[a]	287.60 ± 32.00[ab]	112.80 ± 16.63[ab]
EM1®O	282.60 ± 23.95[a]	0.84 ± 0.19[a]	3.26 ± 0.81[a]	604.68 ± 99.52[a]	318.80 ± 20.18[b]	128.20 ± 20.84[ab]
EM1®F	284.20 ± 28.51[a]	0.83 ± 0.12[a]	3.35 ± 0.62[a]	581.32 ± 97.56[a]	308.40 ± 29.61[ab]	132.60 ± 12.95[ab]
NEMO	290.40 ± 25.27[a]	0.80 ± 0.04[a]	3.18 ± 0.66[a]	618.82 ± 11.60[a]	294.60 ± 38.99[ab]	140.80 ± 20.75[a]
NEMF	293.80 ± 24.47[a]	0.84 ± 0.09[a]	3.60 ± 0.94[a]	587.84 ± 101.04[a]	319.00 ± 27.01[b]	144.00 ± 17.87[a]
CS	274.90 ± 22.44[a]	0.80 ± 0.16[a]	3.05 ± 0.78[a]	538.56 ± 114.89[a]	269.70 ± 35.06[b]	108.00 ± 16.72[b]
EM1®	283.40 ± 26.34[a]	0.83 ± 0.16[a]	3.30 ± 0.73[a]	593.00 ± 99.23[a]	313.60 ± 25.86[a]	130.40 ± 17.49[a]
NEMS	292.10 ± 24.93[a]	0.82 ± 0.08[a]	3.39 ± 0.84[a]	603.33 ± 107.58[a]	306.80 ± 35.68[a]	142.40 ± 19.43[a]

† Data represent mean ± SD ($n = 5$). Means followed by different letters between treatments differ by Tukey test ($p < 0.05$).

Table 8
Shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM), total root volume (TRV), total root surface area (TRSA), and root collar diameter (RCD) of Valência orange and Murcott tangor plants grown under oat straw (OS), fallow straw (FS), commercial product EM1® without straw factor (EM1®S), commercial product EM1® with oat straw (EM1®O), commercial product EM1® with fallow straw (EM1®F), native efficient microorganisms without straw factor (NEMS), native efficient microorganisms with oat straw (NEMO) and native efficient microorganisms with fallow straw (NEMF), up to 419 days after planting.

	SDM (g)	RDM (g)	TDM (g)	TRV (cm^3)	TRSA (cm2)	RCD (mm)
Valência orange						
OS	398.20 ± 8.30[b,†]	413.20 ± 48.47[a]	811.40 ± 52.32[a]	370.49 ± 53.39[a]	1520.87 ± 151.76[a]	14,76 ± 1.67[a]
FS	406.40 ± 15.60[ab]	452.00 ± 46.61[a]	858.40 ± 39.19[a]	370.34 ± 25.71[a]	1560.96 ± 184.84[a]	15.40 ± 0.61[a]
EM1®O	436.20 ± 15.15[a]	465.20 ± 77.07[a]	901.40 ± 84.81[a]	392.97 ± 61.72[a]	1561.2 ± 178.25[a]	16.00 ± 1.04[a]
EM1®F	425.60 ± 17.86[ab]	445.60 ± 59.09[a]	871.20 ± 60.00[a]	385.84 ± 33.12[a]	1584.52 ± 119.22[a]	16.06 ± 1.12[a]
NEMO	435.80 ± 19.36[a]	455.20 ± 118.00[a]	891.40 ± 118.32[a]	399.14 ± 48.47[a]	1612.49 ± 112.44[a]	15.20 ± 0.82[a]
NEMF	437.80 ± 18.01[a]	461.00 ± 67.43[a]	898.80 ± 61.31[a]	408.16 ± 58.76[a]	1565.32 ± 171.54[a]	16.01 ± 1.20[a]
CS	402.30 ± 13.15[b]	432.60 ± 51.35[a]	834.90 ± 67.14[a]	370.41 ± 41.90[a]	1540.91 ± 170.30[a]	15.08 ± 1.29[a]
EM1®	430.90 ± 17.39[a]	45,540 ± 69.36[a]	886.30 ± 70.10[a]	389.40 ± 49.66[a]	1572.86 ± 152.08[a]	16.03 ± 1.08[a]
NEMS	436.80 ± 18.72[a]	458.10 ± 96.14[a]	894.90 ± 110.26[a]	403.65 ± 54.05[a]	1588.90 ± 146.94[a]	15.60 ± 1.10[a]
Murcott tangor						
OS	354.20 ± 15.38[b,†]	312.40 ± 58.19[a]	666.60 ± 45.37[a]	336.01 ± 42.09[a]	1400.11 ± 212.97[a]	14.89 ± 1.33[a]
FS	363.80 ± 16.63[ab]	309.60 ± 34.65[a]	673.40 ± 20.14[a]	353.12 ± 22.86[a]	1410.40 ± 83.06[a]	15.08 ± 0.83[a]
EM1®O	379.20 ± 20.84[ab]	342.40 ± 51.44[a]	721.60 ± 57.71[a]	361.8 ± 25.53[a]	1502.78 ± 156.07[a]	15.86 ± 1.13[a]
EM1®F	383.60 ± 12.95[ab]	369.20 ± 34.38[a]	752.80 ± 39.53[a]	362.58 ± 41.99[a]	1458.56 ± 147.73[a]	15.58 ± 0.80[a]
NEMO	387.20 ± 26.30[ab]	341.40 ± 50.91[a]	728,60 ± 67.56[a]	370.15 ± 42.96[a]	1474.31 ± 112.26[a]	15.28 ± 0.76[a]
NEMF	395.00 ± 17.87[a]	347.20 ± 38.4[a]	742.20 ± 48.33[a]	365.92 ± 34.51[a]	1467.74 ± 153.71[a]	15.53 ± 0.83[a]
CS	359.00 ± 16.72[b]	311.00 ± 47.91[a]	670.00 ± 35.26[a]	344.57 ± 34.94[a]	1405.25 ± 161.72[a]	14.98 ± 1.05[a]
EM1®	381.40 ± 17.49[a]	355.80 ± 45.76[a]	737.20 ± 51.86[a]	362.19 ± 34.76[a]	1480.67 ± 153.56[a]	15.72 ± 0.99[a]
NEMS	391.10 ± 22.82[a]	344.30 ± 45.19[a]	735.40 ± 59.15[a]	368.03 ± 39.02[a]	1471.03 ± 134.63[a]	15.40 ± 0.81[a]

† Data represent mean ± SD ($n = 5$). Means followed by different letters between treatments differ by Tukey test ($p < 0.05$).

4.2. *Efficient microorganisms stimulate the growth of P. trifoliata plants*

The aerial part's development, mainly by increasing the leaf area, is essential for capturing CO_2 and the synthesis of starch and sugars during the photosynthetic process. However, tree plants show slower growth than cultivars; thus, no significant change was observed in the variables of plant height, leaf area, and dry mass of leaves in plants of *P. trifoliata* treated with commercial and homemade inoculant based on EM.

The stem diameter is one of the characteristics most observed by citrus seedlings producers during the rootstock cultivation phase, as it

constitutes a limiting factor for grafting. However, it was possible to verify a significant increase of, on average, 15% in the stem diameter of P. trifoliata when the plants were treated with EM1® and NEM; as also observed by Zydlik and Zydlik [32] when evaluating rootstock plants for M9 apple trees submitted to EM applications. In citrus seedling nurseries, the increase in the rootstock stem's diameter can mean the anticipation of the grafting procedure and the consequent reduction in the time required for the seedling production.

In addition to the SD, the root system was responsive to the inoculants' application in direct contact with the substrate and microorganisms. There was an increase in the volume, length, and area of the medium and thick roots of *P. trifoliata*, mainly in plants treated with EM1®. These data corroborate the more significant development of the pivotal system of *Prunus campanulata* Maxim (cherry) plants treated with EM [51] and in *Prunus dulci* (almond tree) treated with EM and exposed to water deficit [52].

First-order roots have support as their primary function. These roots also function as reserve mechanisms for the plant, especially in deciduous trees such as *P. trifoliata*, which may be favorable in abiotic stress conditions. Besides, Wu et al [53]. state that the most prominent plant root system is its ability to uptake nutrients and water available in the substrate. The authors also infer that changes in the root structure of citrus rootstocks, mainly related to their volume, can positively interfere in the uptake and storage of water and nutrients, especially for the less mobile nutrients. Citrus rootstocks have poorly developed absorbents in their root systems, making them highly dependent on microorganisms such as arbuscular mycorrhizal fungi to uptake low mobility nutrients. This is also facilitated by the increase in the surface area occupied by the roots, contributing to the movement of ions towards the roots, caused by the concentration gradient difference generated on the roots' surface [54,55,56]. The uptake of micronutrient B, for example, in rootstocks citrange Carrizo, tangerine Cleopatra, sour orange, and *P. trifoliata*, was positively correlated with the root surface area [57].

It must also be considered that the increase in the root surface area caused by EM1® can strengthen the reciprocal relationships between plants and microorganisms present in the substrate, improving the conditions for their establishment and permanence in the cultivation environment. According to Rengel and Marschner [58], the exudation of organic compounds through the root surface and the surface itself means the permanence and multiplication of certain microorganisms beneficial to plants.

The lesser development of pivoting roots in citrus seedlings during the nursery phase has favored the appearance in the field of the "decline of citrus," an anomaly that causes damage in citrus orchards of sweet orange that use the Clove lemon tree, *P. trifoliata*, the lemon tree Rugoso and the citranges Morton and Troyer [59]. Another point of interest resulting from the increase in the pivoting system may be concerning the sap transportation between the root system and the plants' aerial part. Eissenstat and Achor [60] evaluated the relationship between the hydraulic conductivity of the roots and the root architecture in rootstocks of *P. trifoliata*, rough lemon (Citrus jambhiri), Swingle citrumelo (*Citrus paradisi* x *Poncirus trifoliata*), and sour orange (*Citrus aurantium*). They found that the first order (more extensive) roots' diameter was positively correlated with the number and size of the cells passing through the plants' vascular cylinder, both for plants grown in pots and plants in the field.

According to Pineda et al [61]., the changes in root systems perceived when inoculating EM are, in most situations, the result of changes in phytohormone levels due to the marked microbial colonization. Phytohormones such as gibberellins act in cell division and stretching, increasing their number and length. Describe gibberellins as regulators of cell wall plasticity, influencing certain enzymes' activity, which may favor cell water movement during their growth [62].

Even if not significantly, the dry mass of *P. trifoliata* treated with efficient microorganisms showed a slight increase concerning the control plants, on average, 10% and 5% when treated with EM1® and NEM, respectively.

4.3. Evolution of the persistence of straw columns

The treatments with NEM and EM1® positively influenced the decomposition of oat straw and fallow, mainly at 60 and 90 days. The most significant reduction in the straw column occurred at 60 days in treatments with efficient microorganisms, probably the period of most excellent establishment of the microbial population, which also allowed the early availability of carbon resources concerning treatments without the application of EM. Efficient microorganisms accelerate the decomposition processes of organic matter present in the soil in agricultural areas [63,64]. The use of EM1® associated with fallow straw did not favor its decomposition process. This could have been due to the composition of this straw, predominantly composed of Plantain Signal Grass (*Brachiaria plantaginea*). This species is part of a genus already described by the high persistence of mulch on the soil surface [65]. When comparing NEM and a commercial product EM1®, Xin et al. [63] found a 37% and 20% higher reduction rate for corn and rice straw by applying NEM concerning the commercial product. Thus, the restricted microbial diversity of the commercial product may have contributed to the lower decomposition rate and consequent decrease in the fallow straw column in the situations studied. This can justify the more significant decomposition of the straw column, both oat and fallow when combined with NEM, which presented a tremendous microbial variety.

The more excellent composition of microorganisms allows a species to be quickly replaced due to the dynamism of the processes, allowing the continuity of biological processes [67]. In addition, there is an interrelationship between plant species and the microbial community, and there may be qualitative and quantitative changes in the microorganisms present in the rhizosphere due to variations resulting from the root exudates produced by each plant [68,69]. However, other studies also found an increase in the decomposition rates of plant residues from maize [70] and rice straw [71,72] after applying EM. The rapid decomposition of straw is beneficial to make available the nutrients present in the straw; however, the vegetation cover must have a sufficient useful life to protect the soil against erosive agents until the establishment of the subsequent crop [73]. Although the acceleration of the decomposition process by the EM applied, from 120 days onwards, there were no differences in the accumulated persistence of the straw column between the treatments, demonstrating that the use of EM did not compromise the permanence time of the oat and fallow straw layers and arranged on the ground in the cultivation of Valência orange and Murcott tangor.

4.4. Growth of Valência orange and Murcott tangor plants under straw effect and efficient microorganisms

EM1® and NEM positively influenced the growth in height and stem diameter of Valencia orange plants when associated with oat straw. NEM favored the development of Murcott tangor plants when associated with fallow straw. The NEM, in addition to containing a greater diversity of microorganisms, may also have benefited from the diversity observed in the straw evaluated, allowing for greater availability of nutrients from the vegetation cover for citrus plants. This is an essential fact for crops, where microbial diversity is dependent on the variety of organic matter present in the soil [74,75,76] describe in this sense, stating that a greater diversity of plant substrates results in a greater diversity of organic compounds present in the rhizosphere, which in turn can better contribute to the survival and development of different groups of microorganisms in the soil. Although there are no limiting factors related to soil characteristics, citrus trees still present a cyclical growth rate, alternating between the shoot and the roots [77]. It is essential to maintain the microbial composition and plant material, especially during vegetative growth. On the other hand, the less expressive results observed for the accumulated development of the Murcott tangor may

have been due to the early flowering of these plants. Although the flowers were removed as they emerged, flowering is a process that drains the plant's resources for flowering and consequent fruit production, thus reducing the resources allocated to the vegetative growth of plants [78,79], minimizing the effects of the applied treatments. Stem growth has been reported by using EM to other fruit trees, such as M9 rootstocks for apple trees [32]; and also by the application of isolates of bacteria of the genus *Bacillus* and lactic acid from the stem diameter of three citrus rootstocks: Swingle citrumelo [*Citrus* × *paradisi* Macfad cv. Duncan × *Poncirus trifoliata* (L.) Raf.], Sunki mandarin (*Citrus sunki* Hort. ex Tan), and rangpur (*Citrus* × *limonia* Osbeck) [16], and in the height of plants of *Moringa oleífera* Lam. by the application of lactic acid bacteria [80], demonstrating the importance of EM in citrus plant development. Positive effects on the height of plants subjected to the use of bacteria of the genus *Pseudomonas*, also present in the NEM used in the present study, were observed in Coffee seedlings (*Coffea* sp) [81] and Caucasian fir (*Abies nordmanniana*) [82].

Lactic acid bacteria, such as the *Lactobacillus* genus present in NEM and EM1® in the present study, are reported to act as phosphorus (P) solubilizers [83]. P was previously reported to favor the growth of SDM of mandarin lime seedlings (*Citrus limonia*) [84] and probably contributed to the most significant accumulation of DMSB and LDM of young plants of Valência orange and tangor. Murcott Furthermore, the increases in DMSB, LDM, and SDM of young Murcott tangor plants submitted to NEMF treatment may be associated with greater availability of nitrogen (N) since the bacteria *Bacillus cereus*, verified in the NEM, had detected the activity of the enzyme nitrogenase [85], being described as a nitrogen-fixing bacterium of the associative type [86]. Associative-type atmospheric nitrogen-fixing bacteria are also defined by Baldani and Baldani [87] and Moreira et al. [88] as synthesizers of phytohormones that positively influence nitrogen metabolism in plants.

There are few studies on the effects of EM on the dry matter of citrus plants. Still, for other crops, an increase in these variables with the application of EM has been demonstrated, such as an increase in the TDM of cucumber (*Cucumis sativus*) by the application of yeast *S. cerevisiae* [89], soy (*Glycine* max) after application of the bacteria L. *acidophilus* [90], species present in the inoculants used in the present study. The main benefits arising from the increase in dry mass accumulation in perennial plants such as citrus are that the reserves contained, mainly in the woody portion of the tree, contribute a significant amount of the nutrients needed for spring growth, flowering, and fruiting [91,92]. This is due to the low nutrient uptake by the roots of citrus plants in early spring [93].

5. Conclusions

The inoculant based on efficient microorganisms from the homemade technology of capture and multiplication, native efficient microorganisms (NEM), showed greater microbial diversity when compared to the commercial product EM1®.

The results obtained from the dry mass analysis of the Valência orange and Murcott tangor plants indicate that positive effects resulting from the use of EM1® and NEM inoculums can be obtained by cultivating the respective crops in a system with oat straw cover.

It can be concluded the viability of using efficient microorganisms in citriculture as microbial natural bioactive formulation and that farmers with less financial resources can produce it on their properties with low costs. These bioproducts present a high quality that can be used in agroecological production after scaling up the process, especially NEM obtention.

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7. Authors' contributions

NLD, AU, TS, and IGG: experimental procedures, results and discussion, and data treatment. CM, FWRJ, HT, and AJM: research coordinators.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Supplementary materials

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