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Full Length Research Paper

Reuse of anaerobic reactor effluent on the treatment of poultry litter

Michael Steinhorst Alcantara[1,2*], Maria Hermínia Ferreira Tavares[2] and Simone Damasceno Gomes[2]

[1]Federal University of Technology – Paraná, road to Boa Esperança, km 04, Zip code 85660-000, Dois Vizinhos, Paraná State, Brazil.
[2]Postgraduate Agricultural Engineering Program, Western Parana State University, Universitaria Street, 2069, Zip code 85819-110, Cascavel City, Parana State, Brazil.

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Anaerobic digestion of poultry litter was studied with reutilization of its effluent in the process by pumping into reactor feeding, contributing to the moisture content and making part of the feeding organic load: 0.5 and 1.0 kg VS/m^3/day, at evaluations 1 and 2, respectively. The hydraulic residence time lasted 10 days for both evaluations and the useful volume of reactor was 35 m^3, with a semi-continuous reactor feeding, under field conditions. The stability of anaerobic digestion was verified through Shewhart control chart. Average efficiency of biogas production was 0.0119 m^3/(kg VS$_{added}$) at evaluation 1 and 0.0429 m^3/(kg VS$_{added}$) at evaluation 2. In the second evaluation, the study revealed that biogas produced more energy as methane than spent with electric energy in reactor feeding. According to Lower Process Capability Index (C$_{pl}$), measure developed for convenience engineering to quantify the performance of a process, the anaerobic digestion in the second evaluation was capable in its energy operations.

Key words: Biogas, Lower Process Capability Index, operational energy viability index, Shewhart control chart, statistical process control.

INTRODUCTION

The intensive production system for broiler production has promoted poultry industry in Brazil, which is the world's third largest producer according FAOSTAT database (FAO, 2015), but also brought on generation of large amounts of waste, poultry litter (PL) and dead birds. PL is composed of animal waste and the material used as bed for broilers (e.g., wood shavings), dietary waste (Sharma et al., 2013) and broiler feathers. As there are high concentrations of poultry farms in producing regions, it would be an attractive alternative to farmers finding different applications for such residue, despite its direct use as fertilizer on soil. In this context and considering current environmental problems related to global warming, anaerobic digestion of solid wastes has attracted more interest (Nasir et al., 2012). Anaerobic digestion has been successfully used in many applications and has conclusively demonstrated its ability to recycle biological wastes biomass (Dahiya and Joseph, 2015). Its scope

*Corresponding author. E-mail: eamsa_1@hotmail.com. Tel: 55 046 9912 1516. Tel: 55 046 30551914. Fax: 55 045 3220 3262.

has been spread in a wide range of operating conditions: the process is implemented at psychrophilic, mesophilic, and thermophilic temperatures, and even extreme conditions like high salt concentrations can currently effectively be tolerated in anaerobic reactors provided that adequate operational measures are taken (Kleerebezem et al., 2015).

According Labatut et al. (2014), the temperature and influent substrate may be the most important parameters determining performance and stability of the anaerobic digestion process. However, to heat the feedstock for anaerobic digestion is need the source of power.

The C/N ratio is an important indicator for controlling biological treatment systems (Wang et al., 2012). However, the optimum C/N range in feedstock for anaerobic digestion remains highly debated, although 20/1 to 30/1 is a most acceptable range (Zhong et al., 2012).

The methanogenic bacteria involved in AD have a low growth rate and are sensitive to inhibitors such as low pH caused by excessive concentrations of volatile fatty acids (VFA) (Brown et al., 2012; Jiang et al., 2012). The pH value increases by ammonia accumulation during degradation of proteins, while the accumulation of VFA decreases the pH value (Weiland, 2010). However, the pH also depends on the buffer capacity of the substrate.
There is also a wide variety of inhibitory substances are the primary cause of anaerobic digestion failure, since they are present in substantial concentrations in wastes, as ammonia, sulfide, light metal ions (Na, K, Mg, Ca e Al), heavy metals, and organics (Chen et al., 2008). However, such inhibitors are not controlled in most anaerobic digestion processes under field conditions because of the difficulty and complexity of the determination of these substances. Therefore, the process design must be well adapted to the substrate properties for achieving a complete degradation without process failure (Weiland, 2010).

A limitation for anaerobic digestion of PL is its low moisture content (about 20 to 40%), relative to the water amount required for the process (about 90 94%). This problem can be solved with the liquid waste anaerobic co digestion or with the mixture with fresh water. For example, studies have been reported on anaerobic co-digestion of PL and stillage (Sharma et al., 2013), on anaerobic co digestion of PL and carcasses of dead birds (Orrico Júnior et al., 2010), on anaerobic digestion from PL with water for biogas production (Espinosa-solares et al., 2009; Gangagni Rao et al., 2013; Markou, 2015). However, there are environmental concerns with the use of fresh water to treat waste.

Other alternative for this would be the process effluent reuse with PL into substrate mixture of the reactor feeding, which contributes also to recirculate the microorganisms and to take advantage of the organic load of effluent by the process of effluent recirculating in the reactor. So, to recirculate the effluent with PL for inlet feedstock in reactor by pumping also allows circulating partially the slurry in reactor.

Thereby, this study aims at evaluating the effluent reuse of the PL anaerobic digestion in the process to dilute the PL in the reactor feeding, on a pilot scale.

MATERIALS AND METHODS

Poultry litter (PL)

The PL under study consists of wood shavings, sawdust, poultry manure and feathers remains, obtained from poultry houses and a result from 13 lots of 45 fattening days of broilers with an 11-day interval.

Treatment system

This trial was carried out in a rural farm in Francisco Beltrão city, in Parana, Brazil, Latitude 25° 59'1.18" S and Longitude 53 ° 6'10.37" W.

The PL treatment system was formed by three units, according to Figure 1: Station 1, PL storage in a shed; station 2, PL anaerobic digestion; station 3, three effluent storage tanks. Each tank contained a hydraulic stirring system.

The horizontal reactor was formed by the union of two fiberglass boxes, with dimensions 3.60 m × 3.30 m × 2.60 m (largest diameter × smallest diameter × height) and then it was placed in horizontal direction within a 2.80 m-depth trench. PVC pipes of 200 mm diameter were connected on each side of the boxes for the inlet/outlet of the reactor.

Inoculum

The anaerobic digestion was started with 3 m^3 of inoculum from reactor of swine wastewater plus 32 m^3 of PL diluted in water at 0.5% volatile solids (VS). The total and useful volumes of reactor were 40 and 35 m^3, respectively.

Operational procedures

Two feeding organic load were evaluated with the stabilized reactor, 0.5 and 1.0 (kg VS)/m^3/day during 142 to 174 days and 210 to 241 days, forming evaluation 1 and 2, respectively. Since, the evaluation period was determined by period when anaerobic digestion was considered stable.

The reactor feeding volume was set at 3.5 m^3/day and controlled by calibrated volumetric graduation in a flow control box, corresponding to 10 days of hydraulic residence time (HRT). Thus, according to feeding organic load and the feeding daily flow rate and the useful volume of reactor, the reactor was feeding daily with 17.5 and 35 kg VS/day, respectively.

The PL was used as complement of effluent during reactor feeding or to make part of the feeding organic load, since PL amount depended on VS effluent content. The effluent was reused to feed the reactor with a new amount of PL, according to Figure 2.
So, for each reactor feeding, the effluent and PL stored an amount that could supply almost one reactor feeding were characterized, according to Figure 3.

Analyses regarding characterization were carried out in triplicate and daily obtained to determine the total solids (TS) and VS contents of effluent and PL. Prior to reactor feeding the stored effluent was stirred in order to prevent the supernatant build-up in

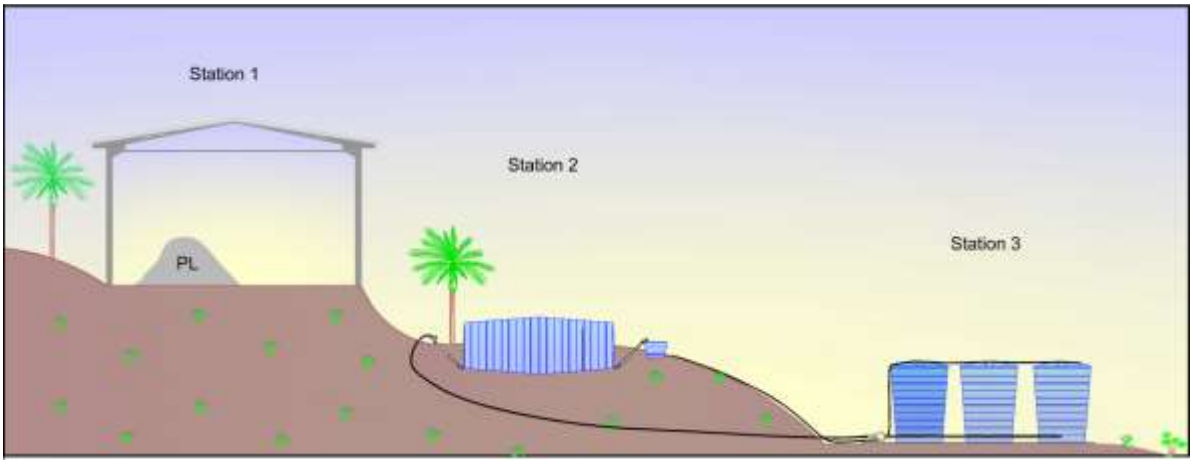

Figure 1. Poultry litter system treatment.

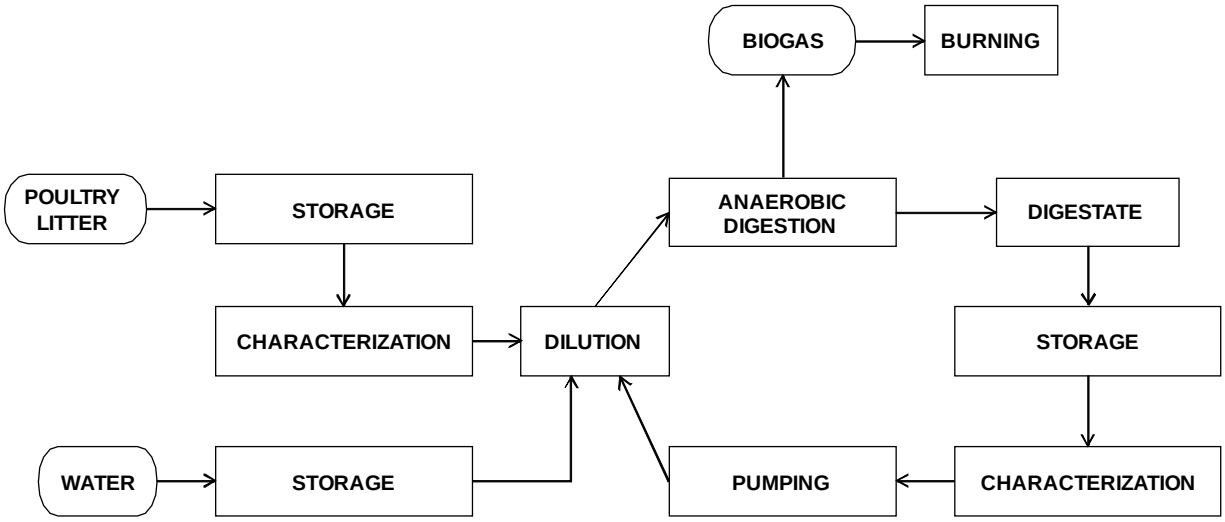

Figure 2. Flow chart of anaerobic digestion system.

the tank.

During the evaluations, data as pH and electrical conductivity of effluent, minimum and maximum room and anaerobic digestion temperatures as well as the TS and VS reduced content of effluent were periodically monitored.

The volume of the biogas was daily quantified by a gas meter LAO brand (model G 0.6) and corrected for Standard Temperature and Pressure of 10^5 Pa and 0°C. In each evaluation, biogas samples were tested with the analyzer GEM 5,000 Plus, Landtec brand, to investigate the concentrations of methane (CH_4)

Analytical methods

In order to analyze physicochemical parameters, the procedures described by APHA (1998) were applied for TS (2540B Method) and VS (2540E method) and by Silva (1977) to obtain volatile fatty acidity, total and partial alkalinity and pH.

Stability of anaerobic digestion

The reactor was stabilized according to biogas production and considered as so when it was under statistical process control by Shewhart control chart for individual measurements, with three average standard deviations, created in MINITAB® 17.1.0 (2013) software, according to Montgomery (2009). Prior to the creation of Shewhart control chart, its assumptions were tested in the variables analysis: Normality by Anderson Darling test (5% significance), sample independence by autocorrelation graph (5% significance and limits of two standard deviations) and sample randomness, observed in the Shewhart control chart.

Among the checking criteria of non-random patterns of control charts, some were chosen to determine the stability process: One or more points outside of the control limits (three average standard deviations); eight points in a row on both sides of the center line with none point inside one average standard deviations; and six points in a row steadily increasing or decreasing (Montgomery, 2009).

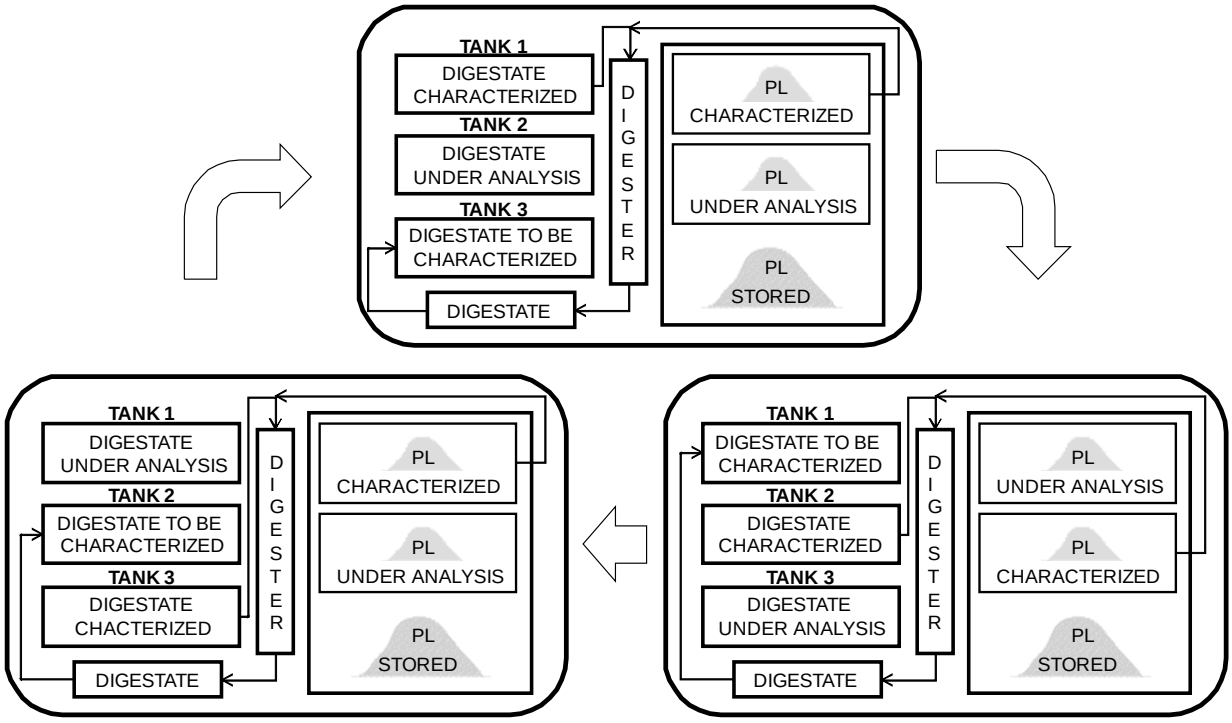

Figure 3. Reactor feeding system with poultry litter (PL) and effluent.

The ratio between VFA, total alkalinity (TA) and partial alkalinity (PA) were also monitored to obtain a better record of the process concerning the reactor potential withstand the evaluated loads.

Data analysis

As this process requires energy, a new index was created in order to relate the energy produced as methane ($E_{produced}$) by the electric energy expended to stir and to pump the effluent into reactor feeding system ($E_{expended}$): Average Index of Operational Energy Viability (AIOEV) shown in Equation 1.

$$AIOEV = \frac{E_{produced}}{E_{expended}} = \frac{P_{biogas} * [\,]\,CH_4 * \rho * c}{H * P} > 1 = \text{feasible} \quad (1)$$

Where: P_{biogas} = average production of biogas (m^3/day); [ ] CH_4 = average concentration of methane (percentage rate, volume); ρ = lower heating value of methane (CH_4), equal to 50,156 J/g CH_4 (Rendeiro et al., 2008); H = number of daily hours of pumping operation (h/day); P = power of effluent recirculation pump: 5 Hp * 746 J/s/Hp = 3,730 J/s; c = constant, [16 g CH_4/mol * (1,000 L/m^3 / 22.4 L/mol) / 3,600 s/h] = 0.1984 g/m^3.

The Lower Process Capability Index (C_{pl}) was also used to check the process capability in each evaluation for a Lower Specification Limit of biogas production (P_{biogas}), which was determined prior to C_{pl} calculation with methane content value and the operating hours of the pump, respectively to the ones obtained during the evaluations, according to Equation 2.

$$LSL = \frac{P_{biogas}}{VS_{added}} = \frac{\frac{H * P}{1 * [\,]\,CH_4 * \rho * c}}{VS_{added}} \quad (2)$$

Where: LSL = Lower Specification Limit to P_{biogas} (m^3/kgVS_{added}); P_{biogas} = average biogas production (m^3/day); VS_{added} = amount of added volatile solids (kg VS/day); [ ] CH_4 = average methane concentration, percentage rate (volume); ρ = lower heating value of methane (CH_4), equal to 50,156 J/g CH_4 (Rendeiro et al., 2008); H = number of daily hours of pumping operation (h/day); P = power of effluent recirculation pump: (5 Hp * 746 J/s/Hp = 3,730 J/s); c = constant, [16 g CH_4/mol * (1,000 L/m^3 / 22.4 L/mol) / 3,600 s/h] = 0.1984 g/m^3.

So, in order to determine C_{pl}, the Lower Specification Limit (LSL) was determined by P_{biogas} resulting in an AIOEV equal to one, value that relates the limit in which the process is feasible in its energy operations, according to Equation 3.

$$C_{pl} = \frac{\bar{X} - LS}{k\,\sigma} \quad (3)$$

Where: C_{pl} = Lower Process Capability index; $\bar{X}$ = sampling average to P_{biogas} (m^3/kg VS_{added}); LSL = lower specification limit to P_{biogas} (m^3/kgVS_{added}); k = number of sampling standard deviations; σ = sampling standard deviation to P_{biogas} (m^3/kg VS_{added}).

Finally, the classifications were associated to the process according to C_{pl} and AIOEV.

RESULTS AND DISCUSSION

Process monitoring

Differences of pH, VFA and alkalinity between PL and inoculums were observed, according to Table 1.

However, this did not cause instability in process during

Table 1. Values of pH and of the ratio between volatile fatty acidity (VFA), total alkalinity (TA) and partial alkalinity (PA) from poultry litter and Inoculum.

Material	pH	VFA/PA	VFA/TA	PA/TA
Poultry litter	6.76	5.29	0.39	0.07
Inoculum	8.33	0.24	0.09	0.38

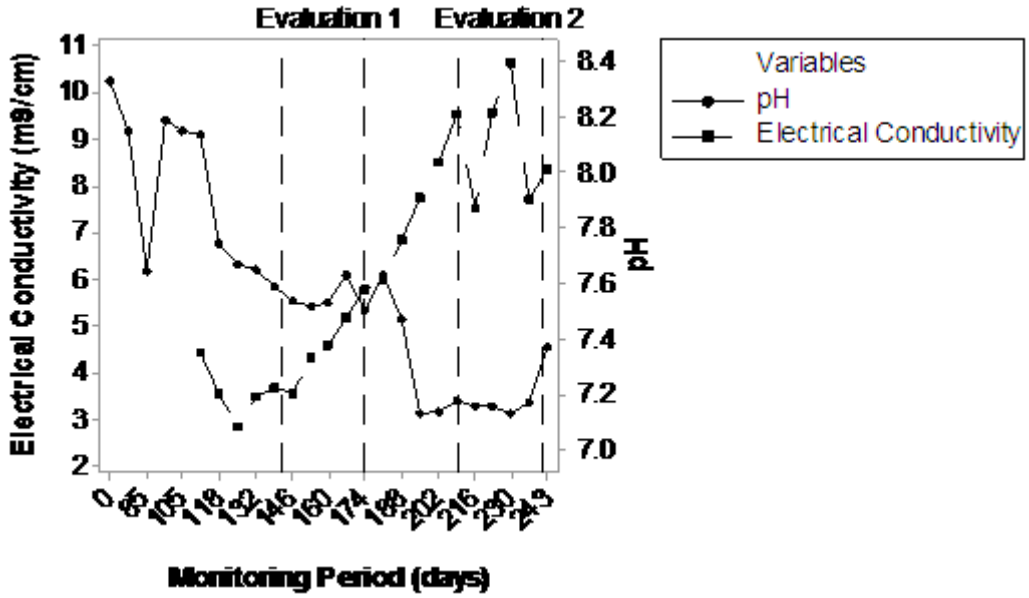

Figure 4. Values of pH and electrical conductivity of effluent.

its start because, for the following parameter concerning reactor operation, effluent pH remained similar to the inoculum (superior to 8.15) until 78 days, in accordance with Figure 4. The gradual increase of feeding organic load rate during the 113 initial days contributed to this answer. Methane production occurred at 135th day, fact observed by the biogas burning.

According Zuo et al. (2013), effluent recirculation from the methanogenic stage to the acidic stage can help buffer the rapidly produced VFA from hydrolysis and maintain a suitable pH, which was characteristic this process.

Unlike pH, electric conductivity tended to increase until the beginning of evaluation 2. The light metal ions including sodium, potassium, calcium, and magnesium are present in the influent of anaerobic reactors (Chen et al., 2008), therefore the increase of electric conductivity is due to effluent recirculation into reactor and by addition daily of PL in process, which contributes to the accumulation of salts inside the reactor. VFA/PA, VFA/TA and PA/TA rates presented the lowest fluctuations during the periods of evaluations 1 and 2. This fact has indicated a stable process during the evaluations. According to Zickefoose and Hayes (1976), VFA/TA ratio can vary from less than 0.1 to almost 0.35 without any significant changes in digestion. Volatile fatty acidity and alkalinity rates are commonly used to verify the anaerobic digestion stability, however, in this study the rates do not express differences between the period that did have the feeding organic load increase (unstable) and the period that did have a single feeding organic load (stable), according to Figure 5a. This highlights the importance to use the Shewhart control chart to verify the anaerobic digestion stability.

According to variation range regarding daily values of maximum and minimum anaerobic digestion temperature, the highest answer was 2.3°C, so, there was a good thermal stability in the process. Reactor design kept stable the anaerobic digestion temperature, because daily room temperature varied in almost 20°C. Consequently, it can be pointed out that only seasonality influenced on greater ranges in anaerobic digestion temperature, according to Figure 5b.

Stability

Statistical assumptions, based on Shewhart control chart

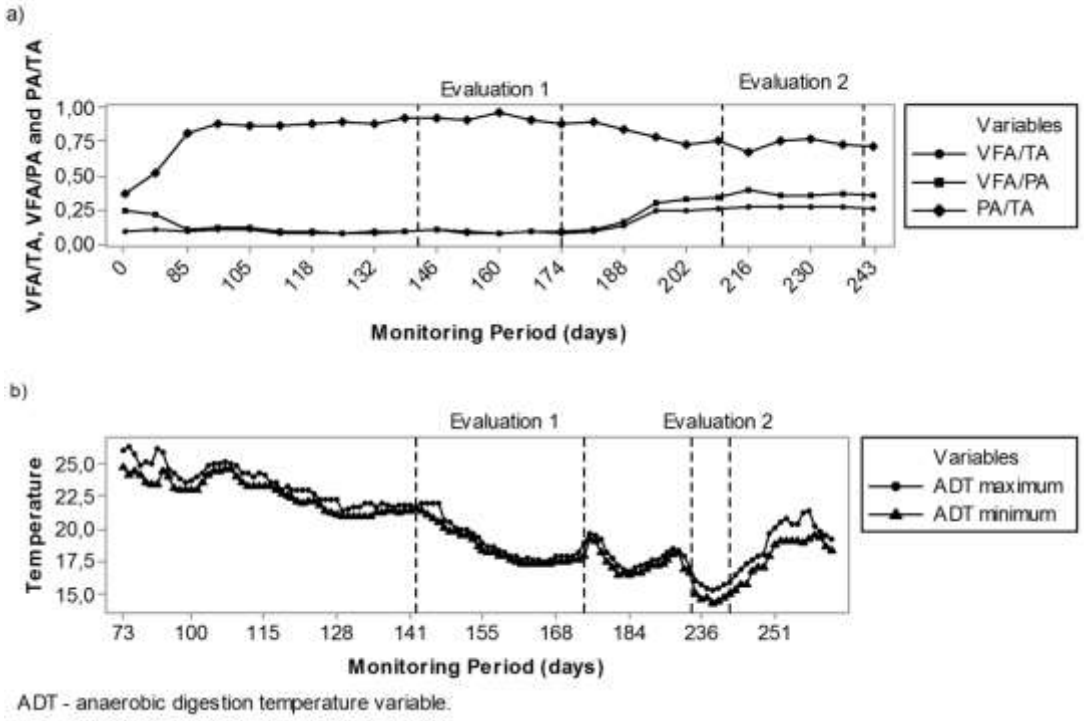

Figure 5. (a) Values of the ratio between volatile fatty acidity (VFA), total alkalinity (TA) and partial alkalinity (PA) from effluent; (b) Maximum and minimum temperature of anaerobic digestion.

for individual measurements, were met in evaluations 1 and 2. According to normality test, biogas production values showed normal distribution with 0.966 p-value for evaluation 1 and 0.192 p-value for evaluation 2.

Values of both evaluations are independent according to the chart of sampling autocorrelation function. Randomness was confirmed at Shewhart control chart since the values are nearby their average, without any trends. So, since statistical assumptions have been met, Shewhart control chart was drawn using biogas production values to check reactor stability in each evaluation, according to Figure 6.

Shewhart control charts met the criteria of non-random patterns of control charts, so, the process was considered stabilized during the reactor evaluation periods. The average efficiency of biogas production was 0.0119 m^3/(kg VS_{added}) in evaluation 1 and 0.0429 m^3/(kg VS_{added}) in evaluation 2.

Energy production

Augusto (2011) recorded biogas production values close to the ones registered in this trial, 0.0185 m^3/(kg VS_{added}), when he evaluated a 10 L of PL batch reactor, diluted in water, for 50 days at 3.91% VS rate. Santos (2001) has also obtained production biogas average of 0.0336 m^3/(kg VS_{added}) when he evaluated anaerobic digestion of PL, diluted in water, in sequenced batch system with 9.5% TS rate over a period of 15 production days. By comparison, biogas production efficiency concerning evaluation 2 was higher and stood at the lowest HRT (10 days) and its feeding rate was only 1.0% VS.

Average methane content (CH_4) was 49.25% in the evaluation 1 and 42.40% in the evaluation 2. So, the average efficiency of methane production was 0.0059 m^3/(kg VS_{added}) in the first evaluation and 0.0182 m^3/(kg VS_{added}) in the second evaluation.

Gangagni Rao et al. (2013) has also evaluated the anaerobic digestion of PL with effluent reuse in self-mixed anaerobic reactor under high-organic loading rate (4 kg VS/m^3/day and 24 HRT days) and in conventional fixed dome anaerobic reactor (2.15 kg VS/m^3/day and a 40 HRT days). They recorded the following answers to production of biogas and methane: 0.23 m^3/(kg VS_{added}) and 0.15 m^3/(kg VS_{added}), 0.128 m^3/(kg VS_{added}) and 0.083 m^3/(kg VS_{added}), respectively. Nevertheless, the authors applied a higher feeding organic load as well as a higher HRT when compared to the one used in this trial, which contributed to its biogas production.

AIOEV and C_{pl}

According to the results, AIOEV of each evaluation was calculated and has shown that both evaluations were

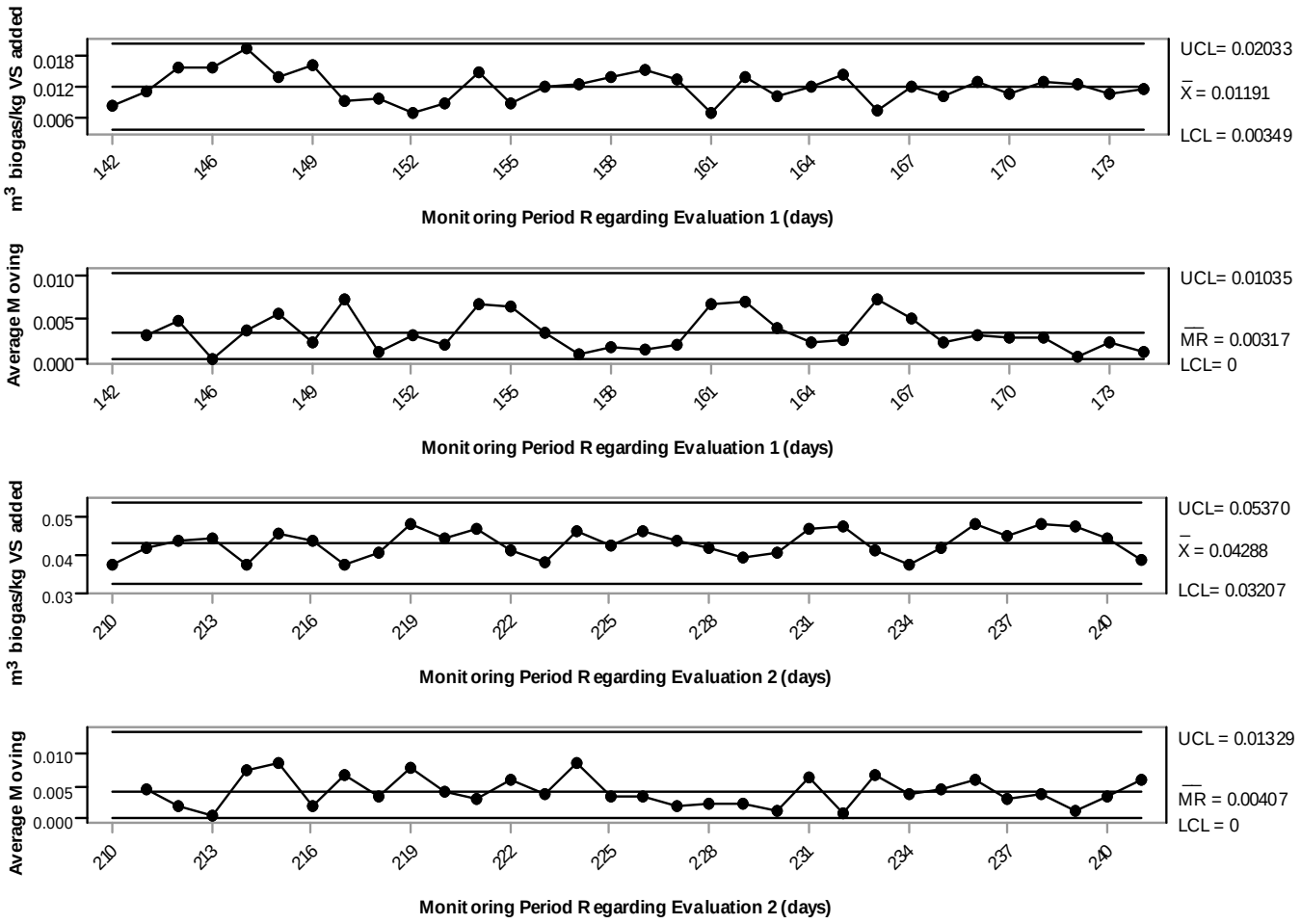

Figure 6. Values of biogas production from evaluations 1 and 2. Where: UCL = upper control limit; LCL = lower control limit; $\bar{X}$ = sampling average to P_{biogas} ($m^3/kgVS_{added}$) or estimate of the population means; $\overline{MR}$ = estimate of the average moving range.

Table 2. Average index of operational energy viability (AIOEV).

Treatments	P_{biogas} (m^3/day)	[ ] CH_4 (%)	H (h/day)	AIOEV
Evaluation 1	0.2084	49.25	0.26	1.05
Evaluation 2	1.5010	42.40	0.39	4.35

feasible. However, evaluation 2 stood out with energy production in methane form 4.35 times greater than electric power used in the treatment system operations with the pump (Table 2). Evaluation 1 was classified as feasible, though; the energy produced in methane form was only 1.05 times greater than the operational power expended.

According to Montgomery (2009), a process considered new (e.g., research on anaerobic digestion) is capable when its C_{pl} is greater than 1.45, according to Table 3. In this context, it is important to mention that AIOEV and C_{pl} indexes are related to the factors that affect biogas production, that is, factors that affect anaerobic digestion: temperature, C/N ratio, pH, volatile fatty acidity, alkalinity, inhibitors, solids, HRT, volume reactor, others. Thus, for a larger useful volume of reactor is possible to obtain a larger production of biogas. However, in this case the pumping time to feed the reactor is also higher, because the feeding daily flow rate also increases to maintain the HRT. Thus, the AIOEV index relates the energy produced in the form of methane with the spent energy in the anaerobic digestion operations.

Solids

Solid load in effluent influenced on the time of pump use, which increased from 15.56 min in the first evaluation to

Table 3. Lower Process Capability Index (C_{pl}) versus average index of operational energy viability (AIOEV).

Treatment	LSL $\left(\frac{m^3 \text{ biogas}}{kg\ VS_{added}}\right)$	AIOEV	Classification	C_{pl}	Classification
Evaluation 1	0.0113	1.05	Feasible	0.07	Incapable
Evaluation 2	0.0099	4.35	Feasible	3.04	Capable

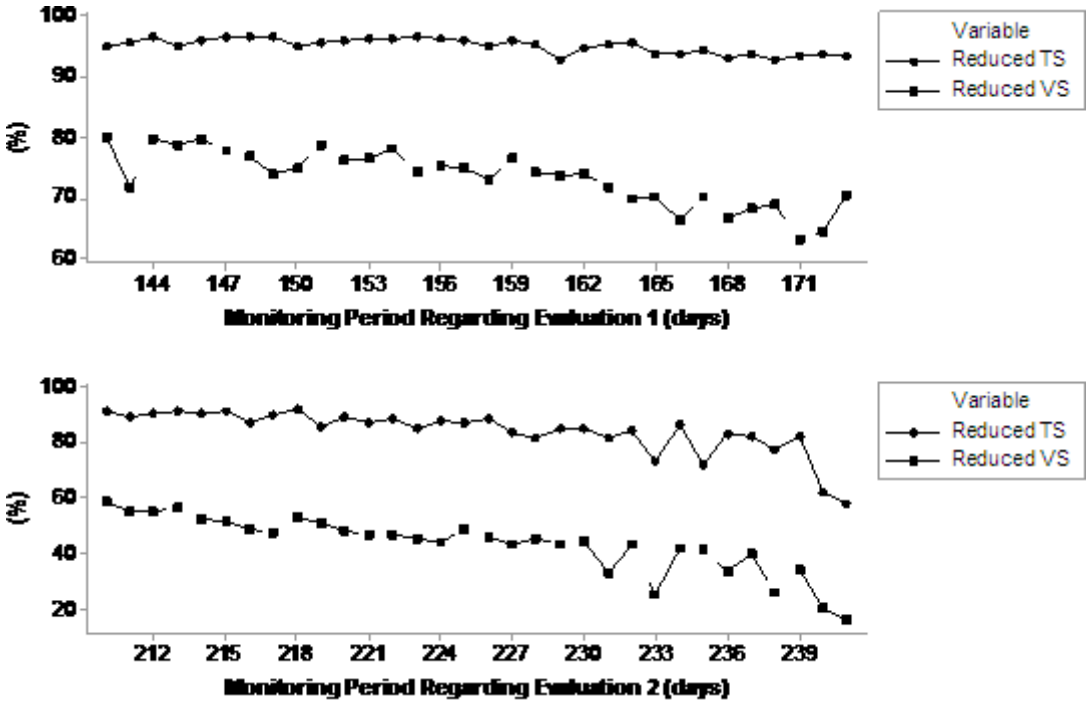

Figure 7. Values of total solids (TS) and volatile solids (VS) reduced for evaluations 1 and 2.

23.40 min in the second one, due to the need of a greater stirring of effluent and to the flow rate decrease of pump with the solids. After second evaluation, the effluent began to show greater solids content, which indicated solids' deposition into reactor. Since, the stirring and feeding time was 50 min and the pump had major reduction in its flow rate.

The averages on solids reduction were 95 and 73% in evaluation 1 and 84 and 43% in evaluation 2 for TS and VS, respectively, according to Figure 7. The high TS removed value can be attributed to the solid fraction of PL that has settled at the bottom of the digester because, according to Farias et al. (2012), the solid fraction of the bird waste is rapidly sedimented in the digester and its determination is always subject to be underestimated. This implies in use the volatile solids added (VS_{added}) in the calculation of the specific biogas production instead of the volatile solids removed to avoid values not representative in the biogas production efficiency analysis: $m^3/kg\ VS_{added}$. So, from 1 to 239 days, solid content into effluent was low, and the values of TS reduction varied from 70 to 99%. Later on, TS reduction decreased by 20% at 289 days, showing that the reactor has gotten saturated by sludge, with higher output of solids in effluent.

Conclusion

The effluent of the PL anaerobic digestion can be reused in the own process to dilute the PL in the reactor feeding, on a pilot scale, contributing to the moisture content and making part of the feeding organic load.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Evaluation of *in vitro* inhibition of mycelial growth of *Fusarium solani* f. sp. *piperis* by different products in Brazil

Verônica D'Addazio[1]*, Renata Aparecida Ahnert dos Santos[2], Arthur Salomão Bastos Leitão[2], Marcelo Barreto da Silva[2], Adriano Alves Fernandes[2] and Antelmo Ralph Falqueto[2]

[1]Universidade Federal do Espírito Santo (UFES) - Av. Fernando Ferrari, 514 – Goiabeiras, 29075-910 - Vitória, ES, Brazil.
[2]Universidade Federal do Espírito Santo (UFES) - Centro Universitário Norte do Espírito Santo – CEUNES, Rodovia BR 101 Norte, Km 60 - Bairro Litorâneo, 29932-540 - São Mateus, ES, Brazil.

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The black pepper (*Piper nigrum* L.) is one of the most popular peppers in the world. Nonetheless, there are several limitations on cultivation, resulting in reduced production or a complete loss of the crop. The major disease affecting this crop is fusarium root rot caused by *Fusarium solani* f. sp. *piperis*, which is responsible for decimating whole crops in Brazil, with losses reaching millions of dollars per year. So far, there is no effective control measure against this fungus and no cultivars resistant to it. In this study, *in vitro* effects of different products on colony growth was evaluated. Carbendazim, chitosan, silicon, and phosphate were tested against *F.solani* f. sp. *piperis* isolates CML-2466, CML-2353, E-637, and E-596. Chitosan and silicon did not inhibit mycelial growth of any of the isolates, while phosphite inhibited mycelial growth by 100%. Carbendazim was found to be fungitoxic for isolates CML-2353 and E-596 and fungistatic for CML-2466 and E-637, inhibiting the mycelial growth of these isolates by 60 and 80%, respectively. There were no dose effects of the products tested.

Key words: *Fusarium solani*, Black pepper, chitosan, silicon, phosphite, carbendazim

INTRODUCTION

Among the major factors limiting agricultural production are infections with fungi, bacteria, viruses, and nematodes, insects, mites, and weeds (Kreyci and Menten, 2013). It is estimated that, agricultural losses due to pest attacks reach US $1.4 trillion, or almost 5% of global gross domestic product (GDP) worldwide. According to Nojosa et al. (2015), the losses for the Brazilian agribusiness can be as high as $55 billion annually due to diseases of crops, which is equivalent to the average annual loss of 7.7% or 25 million tons of agricultural produce. According to the Food and Agriculture Organization(FAO) of the United Nations (UN)

*Corresponding author. E-mail: veronicadaddazio@yahoo.com Tel: +55 (27) 3312-1548.

pathogens are responsible for 13.3% of such damages.

The black pepper (*Piper nigrum* L.), which accounts for ~57,800 tons of national agricultural produce (IBGE, 2016), has been affected by root rot, also known as fusariosis (Kimati et al., 1997). This severe disease can cause a lot of damage to the crop, with an annual reduction of 3% in the cultivated area and production. A healthy pepper crop has a productive cycle potential of 12 years on average. This disease reduces the cycle by 5 to 6 years (Tremacoldi, 2014). The causal agent is *Fusarium solani* (Mart.) Appel & Wr. emend. Snyd. & Hans.f. sp. *piperis*, Albuquerque (Teleomorphs *Nectria haematococca* Berk. & Br. f. sp. *piperis* Albuq.). The *Fusarium* species are widely distributed in the soil, and in an adverse environment, form a resistant structure called *chlamydospore*, which can remain viable for more than 20 years (Pfenning and Lima, 2007). After infection, the fungi settlesdown in the vascular system of the plant, hindering the absorption of water and nutrients (Bedendo, 1995). Once inside the root system, the fungi are initially limited to the root or the plant base and, at some point, begin to spread to the vascular system. The damage is due to colonization of xylem vessels by hyphae and microconidia; hypertrophy and hyperplasia of the cambium, xylem, and phloem; destruction of xylem fibers and amyloplasts in parenchymatous cells; and production of gels by the plant (Ortiz et al., 2014). Eventually, the combination of the fungal growth in the vascular system, fungal toxins such as naphthoquinones and fusaric acid (Rocha et al., 2016), and defense structures produced by the plant hamper the absorption and transport of water, causing wilt and death of the plant (Wheeler and Rush, 2001).

Strategies for *F. solani* sp. *piperis* control are limited because there is still no information on resistant cultivars and an effective fungicide does not exist (or is not officially approved in Brazil). *In vitro*, studies are needed to identify the products with the possible ability to control the fungus. To this end, various compounds are being tested. Benzimidazole fungicides are used extensively in agriculture due to strong systemic activity against a great number of fungal species (Reis et al., 2001). Carbendazim, a systemic fungicide with a benzimidazole chemical group, exerts both preventive and curative action (Kus and Altanlar, 2003). Among the products with great potential antifungal stand out chitosan, silicon and phosphite. Such products have not been tested effectively in black pepper as an alternative method of controlling fusarium or were used on a small scale without scientific evidence. Thus, various control methods are used to minimize the severity of the disease. Chitosan, a high-molecular-weight polysaccharide, has many physicochemical and biological properties (El-Ghaouth et al., 1994), e.g., antimicrobial activity against some fungi (yeasts) and bacteria (Allan and Hadwiger, 1979; Roller and Covill, 1999). Among mineral nutrients used in pest management, silicon (Si) stands out as an element that reduces severity of major diseases in several crops (Epstein, 1999). Silicon can act on the constitution of the physical barrier to prevent penetration of fungi and affects the signals between the host and the pathogen, resulting in more rapid and extensive activation of pre-and post-formed defense mechanisms in the plant (Chérif et al., 1994; Epstein, 1999), e.g., by increasing the synthesis of phenolic compounds and peroxidase, polyphenoloxidase, chitinase, and β-glucosidase (Fauteux et al., 2005). Phosphites are characterized by their effectiveness in controlling downy mildew and various diseases caused by genus *Phytophthora* (Ouimette and Coffey, 1989), exert acropetal and basipetal systemic action and suppress foliar and root diseases (Guest and Grant, 1991). Furthermore, they have high stability in plants and may remain active for substantial periods (Smillie et al., 1989). Regarding the mechanism of action of phosphites, some authors discuss direct action on the pathogen (Fenn and Coffey, 1984; Fenn and Coffey, 1985; McGrath, 2004). Others suggest that the mechanism is indirect, via activation of plant defense mechanisms (Nemestothy and Guest, 1990; Saindrenan et al., 1990) or a combination of direct and indirect effects (Smillie et al., 1989; Jackson et al., 2000).

The objective of the present study was to evaluate the effects of alternative antifungal agents such as chitosan, silicon, and phosphite as well as the known fungicide carbendazim on mycelial growth of *F. solani* f. sp. *piperis in vitro*.

MATERIALS AND METHODS

F. solani f. sp. *piperis* isolates

The isolates that we tested were CML-2466 and CML-2353 (Coleção Micológica de Lavras, Universidade Federal de Lavras - Minas Gerais State) and E-637 and E-596 (Incaper– Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural - Espírito Santo State). The isolates were maintained in Petri dishes containing potato dextrose agar (PDA) in refrigerator at 4°C. Every month, an agar disk (5mm) from a pure culture of *F. solan i* was placed in the center of a PDA plate containing the same medium. The plates were incubated at 25°C in biochemical oxygen demand (B.O.D.), with the photoperiod of 12 h.

Preparation of chitosan, silicon, phosphite, and carbendazim concentrations

Chitosan was added to the PDA medium at concentrations of 0.5, 1.0, 1.5, 2.0%, 2.5, and 3.0%. Chitosan (Fagron®) was extracted in acetic acid and diluted in water to a concentration of 2% at pH 4.4. This substance has high viscosity and was diluted with sterilized distilled water to obtain the desired concentrations. The other products added to the culture medium at the following concentrations were: silicon (SiO_2) at 0.25, 0.50, 1.0, 1.5, 2.0, or 3.0 g L^{-1}; phosphite (Phosethyl Al) at 1.0, 2.0, 3.0, 4.0, 5.0, or 6.0 g L^{-1}; and carbendazim (Carbomax 500®) at 0.83, 1.67, 2.50, 3.34, 4.16, or 5.0 ml L^{-1}. As controls, we used Petri dishes containing PDA medium supplemented with 2% of acetic acid for the treatment with

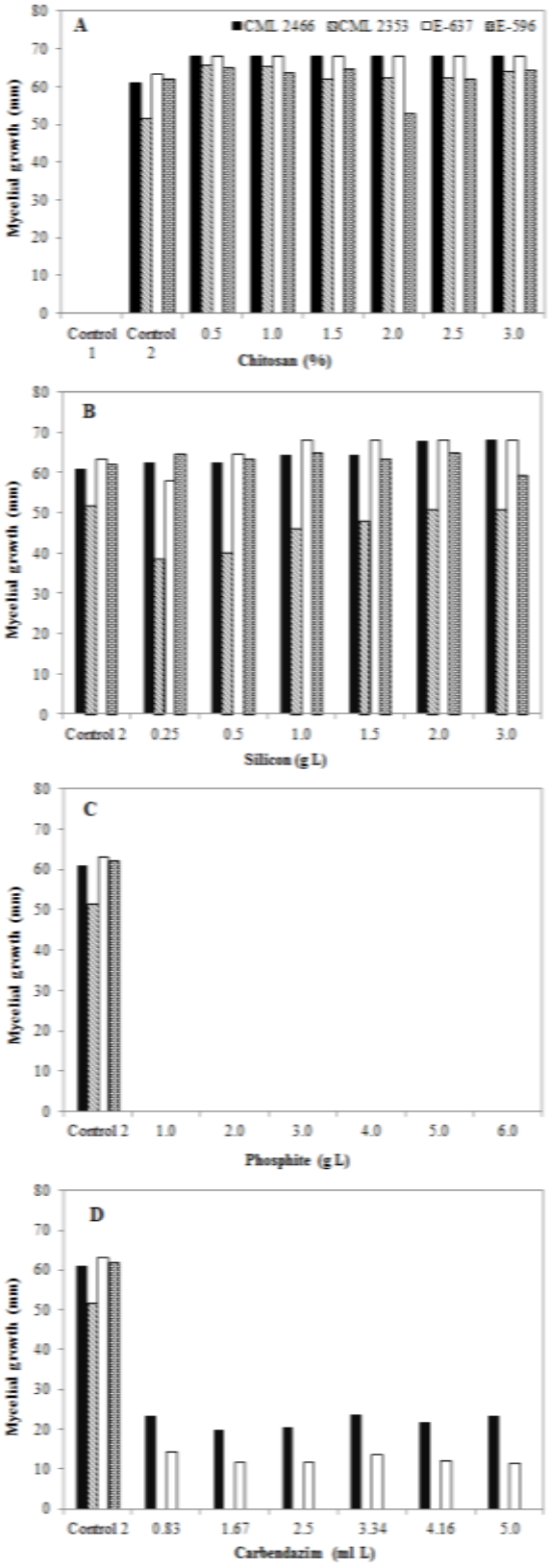

Figure 1. Effect of chitosan (A), silicon (B), phosphite (C) and carbendazim (D) on the mycelial growth of *F. solani* isolates. Control 1: PDA medium supplemented with 2% of acetic acid; Control 2: only PDA medium.

chitosan (Control 1) and/or only the PDA medium (Control 2). After solidification, a fungal mycelial disc 5mm in diameter, 15 days old, was transferred to the center of each Petri dish (68-mm diameter). This procedure was performed for each *F.solani* isolate. The plates were sealed with parafilm and maintained in B.O.D at 25°C, with a photoperiod of 12 h.

Effect of different products on mycelial growth of *F. solani* isolates

The mycelial growth of *F. solani* isolates was assessed daily by measuring the diameter of the colonies in orthogonal directions by means of a pachymeter, until the colonies in control treatments reached the edge of the board. The percent growth inhibition was calculated according to Guo et al. (2006), using the following formula: antifungal index (%) = (1 – Da/Db) × 100, where Da was the diameter of the zone of growth in the test plates, and Db was the diameter of growth zone in the control plate.

Statistical analysis

The experiment was performed using randomized block design (RBD) with 28 treatments and five repetitions for each isolate of *F. solani*. Each repetition consisted of a Petri dish. Each experiment was repeated three times. The significance of treatment effects on radial growth among isolates was tested with analysis of variance (ANOVA). Where significant F values were obtained, Tukey's all pairwise comparison test, which includes a correction for multiple comparisons, was used to assess the significance of differences between meansin the statistical software ASSISTAT 7.1 beta (Silva and Azevedo, 2009).

RESULTS

The mycelial growth of *F. solani* was not inhibited by any chitosan concentrations tested except for the test plate PDA with added acetic acid. The presence of chitosan favored the growth of the four fungal isolates: CML-2466 and E-637 reaching the edge of the plate after 9 days of incubation, and CML-2353 and E-596 showed maximal growth after 11 days (Figure 1A). The fungi were also seeded on agar-agar with added chitosan (same concentrations) or agar-agar only and all reached the edge of the plate after 9 days of growth (data not shown). Silicon, at the concentrations tested, did not inhibit fungal growth (Figure 1B). For CML-2353, at concentrations of 0.25 and 0.5 gL^{-1}, the inhibition rate was 25.28 and 22.07%, respectively. At other concentrations, the inhibition rate was below 11%. The other strains grew normally at all concentrations of silicon tested (Table 1B). Phosphite proved to be effective in inhibiting the mycelial growth of fungi under all our experimental conditions (Figure 1C). None of the plates showed mycelial growth at the tested doses of phosphite.

Carbendazim was 100% effective against two of the four isolates tested. The mycelial growth of CML-2353 and E-596 were completely inhibited at the various concentrations of carbendazim. Carbendazim exerted a fungistatic effect on the isolates CML-2466 and E-637,

Table 1. Colony diameter (C.D.) and percent growth inhibition (P.I.) of F. solani isolates in chitosan (A), silicon (B), phosphite (C) and carbendazim (D).

A

Chitosan (%)	CML 2466**		CML 2353**		E-637**		E-596**	
	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)
Control 1	0[c]	100	0[c]	100	0[b]	100	0[b]	100
Control 2	60.95[b]	0	51.58[b]	0	63.16[a]	0	62.03[a]	0
0.5	68.00[a]	-11.57	65.56[a]	-27.10	68.00[a]	-7.66	65.04[a]	-4.86
1.0	68.00[a]	-11.57	65.53[a]	-27.05	68.00[a]	-7.66	63.76[a]	-2.79
1.5	68.00[a]	-11.57	62.16[a]	-20.51	68.00[a]	-7.66	64.64[a]	-4.21
2.0	68.00[a]	-11.57	62.49[a]	-21.15	68.00[a]	-7.66	52.95[a]	14.63
2.5	68.00[a]	-11.57	62.21[a]	-20.61	67.85[a]	-7.43	61.96[a]	0.12
3.0	68.00[a]	-11.57	64.17[a]	-24.41	68.00[a]	-7.66	64.49[a]	4.30
V.C. (%) =	4.72		7.96		4.12		12.71	

B

Silicon (g L^{-1})	CML 2466 n.s.		CML 2353*		E-637**		E-596 n.s.	
	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)
Control 2	60.95[a]	0	51.58[a]	0	63.16[ab]	0	62.03[a]	0
0.25	62.48[a]	-2.51	38.54[b]	25.28	58.00[b]	8.17	64.63[a]	-24.22
0.5	62.50[a]	-2.54	40.20[ab]	22.07	64.57[ab]	-2.24	63.43[a]	-21.92
1.0	64.33[a]	-5.54	46.15[ab]	10.53	68.00[a]	-7.66	64.90[a]	-24.73
1.5	64.43[a]	-5.72	47.82[ab]	7.29	68.00[a]	-7.66	63.38[a]	-21.81
2.0	67.78[a]	-11.20	50.78[ab]	1.56	68.00[a]	-7.66	65.13[a]	-25.18
3.0	68.00[a]	-11.57	50.66[ab]	1.78	68.00[a]	-7.66	59.33[a]	-14.03
V.C. (%) =	9.82		12.47		5.98		12.05	

C

Phosphite (g L^{-1})	CML 2466**		CML 2353**		E-637**		E-596**	
	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)
Control	60.95[a]	0	51.58[a]	0	63.16[a]	0	62.03[a]	0
1.0	0[b]	100	0[b]	100	0[b]	100	0[b]	100
2.0	0[b]	100	0[b]	100	0[b]	100	0[b]	100
3.0	0[b]	100	0[b]	100	0[b]	100	0[b]	100
4.0	0[b]	100	0[b]	100	0[b]	100	0[b]	100
5.0	0[b]	100	0[b]	100	0[b]	100	0[b]	100
6.0	0[b]	100	0[b]	100	0[b]	100	0[b]	100
V.C. (%) =	34.0		13.35		28.70		10.34	

D

Carbendazim (ml L^{-1})	CML 2466**		CML 2353**		E-637**		E-596**	
	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)	C.D. (mm)	P.I. (%)
Control 2	60.95[a]	0	51.58[a]	0	63.16[a]	0	62.03[a]	0
0.83	23.45[b]	61.52	0[b]	100	14.18[b]	77.55	0[b]	100
1.67	19.77[b]	67.57	0[b]	100	11.81[b]	81.30	0[b]	100
2.50	20.61[b]	66.19	0[b]	100	11.65[b]	81.55	0[b]	100
3.34	23.67[b]	61.16	0[b]	100	13.50[b]	78.63	0[b]	100
4.16	21.89[b]	64.09	0[b]	100	12.09[b]	80.86	0[b]	100
5.0	23.49[b]	61.46	0[b]	100	11.40[b]	81.96	0[b]	100
V.C. (%) =	16.56		13.35		14.73		10.34	

Averages followed by the same letter are not statistically different among themselves, by Tukey test. V.C. = Variation coeficient; ** significant at 1% probability ($p < 0.01$); * significant at 5% probability ($0.01 = < p < 0.05$); [ns] not significant ($p > = 0.05$).

and the effect was not dosedependent. The inhibition of growth of these isolates was 60 and 80%, respectively (Figure 1D, Table 1D).

DISCUSSION

The absence of inhibition of mycelial growth by chitosan suggested that the *F .solani* f. sp. *piperis* isolates can use this substance as anadditional carbon source. This is possibly because chitosan is a polysaccharide, and probably, the fungus uses it as a source of nutrients for its growth. Nascimento et al. (2007), studying fungi causing grapevine trunk diseases, found that chitosan inhibited the growth of all fungi tested except *Neonectria liriodendri*, which grew at all the concentrations analyzed. According to Baños et al. (2004) and Bhaskara-Reddy et al. (1998), mycelial growth and sporulation of *Penicillium digitatum* and *Alternaria alternata*, respectively, were stimulated by the presence of chitosan. These authors believed that such behavior may be a response to stress caused by the chitosan. Several studies have shown that the biological activity of chitosan is significantly dependent upon its molecular weight, acetylation degree (Alfredsen et al., 2004; Wu et al., 2004; Torr et al., 2005), pH of the medium (Devlieghere et al., 2004), and the microorganism membrane characteristics (Qi et al., 2004). In general, the lower the molecular weight and degree of acetylation of chitosan, the greater the efficacy atreducing the growth and multiplication of microorganisms (Goy et al., 2009). The other possibility is the unusual pH of the culture medium, which remained acidic (about 4.0). The ability of fungi to grow in wider pH ranges is associated with the presence of pH-regulatory systems. These regulatory systems are mediated by differential production of extracellular enzymes and metabolites as a function of pH of the medium (Denison, 2000). It is likely that this pH adjustment mechanism also exists in *F. solani*. This phenomenon may be associated with fungal survivability for long periods in the soil, even under adverse conditions. In control plates where we added acetic acid, there was no growth for any of the isolates tested. Sholberg et al. (2000) reported that the inhibitory effect of acetic acid on microorganisms is due to the reduction in pH as well as the ability of the coupled molecules of acetic acid to pass easily through the membrane of conidia, exerting its toxic effect by reducing the cellular protoplasm.This mechanism may explain the inhibition of mycelial growth of *F. solani* in control plates containing only PDA with added acetic acid. Chitosan's effects on growth of microorganisms are well known, but the mechanisms underlying its antifungal action have not been fully elucidated. The response to this possible antifungal agent may vary depending on the pathogen (El-Ghaouth et al. 1992).

Our results suggest that silicon does not have direct action on the *F. solani* isolates tested because it induced mycelial growth at all concentrations. Silicon probably acts as a resistance inducer in the plant. Similar results were reported by Carré-Missio et al. (2010), who studied the effect of silicon on Pestalotia leaf spotin cultivated strawberry. *In vitro* results showed that silicon at the dose of 8 g L^{-1}does not inhibit mycelial growth of*Pestalotia longisetula*. In another study, the growth of *Fusarium* spp. and *Verticillium* spp. were enhanced at silicon concentrations of 5 and 10 ml L^{-1}, respectively (Kaiser et al. 2005). Generally, silicates do not act directly on microorganisms that cause diseases in plants, but have alternative mechanisms of action, which in some cases—because of their beneficial effect on the plant—may reduce abiotic and biotic types of stress (Zambolim et al. 2012). In the literature, there are reports of reduced and increased intensity of diseases in plants after treatment with silicon (Zambolim and Ventura, 1996). Silicon can act locally by inducing defensive reactions in cells and can also contribute to systemic resistance by increasing the production of stress hormones. Nonetheless, the exact mechanism by which silicon modulates signaling in plants remains unclear. Evidence suggests that silicon can act as an enhancer of plant defense responses or as a strategic signaling proteins. Silicon can therefore interact with several key components of the plant stress response-related signaling pathways, leading to effective resistance to pathogenic fungi.

In agreement with the results of our study, Araújo et al. (2008), while studying *Colletotrichum gloeosporioides*, showed that potassium phosphite (Fitofós K®) has a direct effect on this fungus, almost completely inhibiting the mycelial growth *in vitro*. Potassium phosphite was tested against *Penicillium expansum*, which causes postharvest blue mold infections on apple fruits; this compound completely inhibited the mycelial growth (Amiri and Bompeix, 2011). In a study made by Lobato et al. (2010), phosphate exerted a fungicidal effect on pathogens of potatoes: *F. solani*, *Rhizoctonia solani*, and *Streptomyces scabies*. According to Guest and Grant (1991), phosphites inhibit the growth of pathogens in plants via a complex mechanism of action. The first stage is a direct fungistatic effect, which is dependent on the concentration of phosphite that accumulates in the fungus. This, in turn, is influenced by the concentration of phosphate, and the effectiveness of the phosphite oxidation system. The second step is a change in the metabolism of the pathogen, such that a faster and more effective defensive response by the plant can develop. These alterations imply a reduction in the amount of suppressor molecules on the pathogen's surface or an increase in the number of receptors exposed to agonists in host cells, or both, suggesting that phosphites may have multiple modes of action. As for the direct action on the pathogen, it is known that phosphorous acid and its derivatives act by inhibiting the process of oxidative phosphorylation in Oomycetes (McGrath, 2004). In general, the effects of phosphites on the phytopathogens are mediated by the formation of membrane pores due to

damage to the plasma membrane and cell wall of the hyphae, probably because of transcription changes in genes that encode proteins involved in the biosynthesis of their components and other parts of the overall cellular metabolism. These changes compromise the morphology, physiology, and sporulation of the fungus, interfering with the parasitism (Smillie et al., 1989; King et al., 2010). The indirect action of phosphate involves activation of plant defense mechanisms such as stimulation of the production of phytoalexins (Guest and Grant, 1991; Daniel and Guest, 2006) or lignification and production of phenols (Nojosa et al., 2005).

The biological activity of benzimidazoles (such as carbendazim) is mediated by interference with the formation and functioning of microtubules in eukaryotic cells. The affinity of benzimidazole for tubulin is the main factor determining its fungicidal activity. The stronger the binding affinity of the compound for tubulin, the more sensitive is the organism to the fungicide. Also, resistance to carbendazim is described as a change in the□□-tubulin protein, which reduces the binding of the fungicide to this protein (Osmani and Oakley, 1991). In our study, carbendazim showed different effects when administered to the fungus. In two *F. solani* isolates, CML-2466 and E-637, this compound had a fungistatic effect at all the doses analyzed, whereas for CML-2353 and E-596 isolates, this compound showed a fungitoxic effect. According to Sultana and Ghaffar (2013), carbendazim completely inhibits colony growth of *F. oxysporum*. When tested on mycelial growth of *Rhizoctonia solani*, carbendazim reduced it by 86% *in vitro* (Schurt et al., 2013). In addition to *in vitro* results, chitosan (El-Ghaouth et al., 1994), silicon (Epstein, 1999) and phosphites (Ouimette and Coffey, 1989) due to resistance induction characteristics are being field tested by us in Black pepper plants, to evaluate the behavior of such plants inoculated with *F. solani*.

In summary, chitosan and silicon did not inhibit the growth of *F. solani* and instead promoted the growth of most isolates. Carbendazim exerted growth control in 50% of the isolates and in the other 50%, had a fungistatic effect, and these effects were not dosedependent. Among the products tested for possible inhibition of the mycelial growth of *F. solani* f. sp. *piperis in vitro*, the action of phosphite stands out: 100% inhibition in isolates CML-2466, CML-2353, E-637, and E-596.

Conflict of Interests

There is no conflict of interest of any kind related to this work.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Survival of *Salmonella* Enteritidis and *Escherichia coli* in cactus cladodes under domestic marketing conditions in Mexico

José Luis Zárate-Castrejón[1], Talina Olivia Martínez-Martínez[1]*, Juan Gabriel Angeles-Núñez[1], Fanny Guadalupe Concha-Valdez[2], José Ramón Verde-Calvo[3] and Martha Elva Ramírez-Guzmán[4]

[1]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Campo Experimental Bajío. Km. 6.5 Carretera Celaya-San Miguel de Allende, C.P. 38110, Celaya, Guanajuato, México
[2]Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Universidad Autónoma de Yucatán. Mérida, Yucatán Avenida Itzáes # 490 x Calle 59, Colonia Centro C.P. 97000 Mérida, Yucatán, Mexico.
[3]Universidad Autónoma Metropolitana. Depto. de Biotecnología, Laboratorio de Enología y Alimentos Fermentados. México, D.F. México. Av. San Rafael Atlixco No. 186, Col. Vicentina, Del. Iztapalapa. C.P. 09340. México, D.F. México.
[4]Colegio de Postgraduados, Campus Montecillo, Colegio de Postgraduados, Carretera México-Texcoco, Km. 36.5, Texcoco, México.

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In Mexico, during domestic marketing of cactus cladodes, called "nopalitos", there is a tendency not to provide refrigerated storage, and sometimes dealers do not take care of the hygienic conditions of the product during commercialization. Therefore, the objective of this study was to evaluate the survival of *Salmonella* Enteritidis and *Escherichia coli* on cladodes of *Opuntia ficus-indica* Var. Atlixco under conditions associated with domestic marketing in Mexico. Some phenolic compounds present in cladodes, which could influence the survival of these pathogens in the vegetable were analyzed at the same time. For the survival experiment, a 2x2 factorial design was used; treatments included two presentations of cactus cladodes (without spines and with spines) and two storage temperatures. Viable cells were counted during 16 days of storage using specific culture media. Phenolic compounds were determined using HPLC. *E. coli* did not survive on cactus without spines during 16 days at 4°C, while *S.* Enteritidis was able to survive until 16 days in all the treatments. The analytic results obtained indicated higher contents of caffeic and protocatechuic acid. The results showed the importance of refrigerated storage of nopalitos during their commercialization to reduce the risk of presence of foodborne illness and provide good practices during marketing.

Key words: *Opuntia ficus-indica*, human pathogens, handling, storage temperature.

INTRODUCTION

Cactus cladodes (*Opuntia ficus-indica*) are a crop of great economic importance for Mexico; the species is cultivated in an area of 12,038 ha, with a production value of $1,617.645 MXN (SIAP, 2015). Most of the fresh cactus cladodes are sold with spines in domestic markets; their storage time is longer than for cladodes without spines,

where periods of commercialization are about three to five days (Valencia-Sandoval et al., 2010). Although, there is no information on outbreaks of foodborne illnesses associated with the consumption of cactus cladodes, some bad practices in the handling of this vegetable, especially in storage and elimination of spines, can involve the risk of product contamination by foodborne pathogens (Angeles-Núñez et al., 2014). The survival and growth of these pathogens in other vegetables have been associated with temperature, time and presentation of product (Corbo et al., 2005).

Foodborne pathogens have mechanisms to protect themselves within the plant and continue their proliferation. Studies have demonstrated the ability of *Salmonella* to survive in cactus leaves through the formation of biofilms after 24 h of incubation (De los Santos et al., 2012). This bacterium was found in the tissue of the cactus leaf cladodes, persisting for up to 14 days at room temperature (Landa-Salgado et al., 2013). Particularly, *Salmonella* was able to survive and proliferate under refrigerated storage conditions, increasing its population up to 3 log CFU at 4°C for periods longer than three weeks (Kroupitski et al., 2009; Liao et al., 2010); while *Escherichia coli* increased its population up to 5 log CFU at < 8°C for more than three weeks (Corbo et al., 2005, Liao et al., 2010).

Some authors have suggested that the survival of pathogens depends on the availability of nutrients in foods and the presence of secondary metabolites that may inhibit the survival of bacteria. Pad extracts of *Nopalea cochenillifera* have flavonoids and tannins that inhibit the growth of *E. coli* and *Salmonella Typhimurium* (Gómez-Flores et al., 2006). According to some studies with cladodes of *O. ficus indica,* the presence of protocatechuic, gallic, 4-hydroxybenzoic feluric, chlorogenic, syringic and sinapic acids and the epicatechin and quercetin flavonoids have been detected (Qiu et al., 2003; Guevara- Figueroa et al., 2010). These phenolic compounds have antimicrobial action through enzymatic inhibition processes and protein transport; some compounds and destabilization of cell membranes have the ability to inhibit biofilm formation (Othman et al., 2010).

In order to determine the survival ability of *S.* Enteritidis and *E. coli* in cactus cladodia under temperature conditions associated with marketing in Mexico, both bacteria were inoculated into cactus leaves with spines and without spines to assess survival for 16 days under two temperatures, refrigeration (4°C) and environmental (18°C). Also, the presence of phenolic compounds in cladodes with antagonistic potential for these bacteria was determined. The results of this study demonstrated higher survival of *S*. Enteritidis and *E. coli* on cactus cladodes at 18°C, suggesting the importance of refrigerated storage during commercialization to reduce the risk of the growth of foodborne pathogens. Additionally, the low survival of pathogens in cactus without spines suggests an antimicrobial effect provided by the leakage of phenolic compounds from plant tissue due to the peeling process.

MATERIALS AND METHODS

Inoculum

The *S.* Enteritidis isolate (C-4153) was obtained from the bacterial culture collection of the Regional Research Center Dr. Hideyo Noguchi of Universidad Autonóma de Yucatán (UADY); this isolate was serotyped by the Institute of Epidemiological Diagnosis and Reference "Dr. Manuel Martinez Baez", Secretary of Health of Mexico (INDRE-SSA). *E. coli* (ATCC 10536) was provided by the Microbiology Laboratory, Department of Agroindustrial Engineering, Universidad Autónoma Chapingo. All cultures were maintained in nutrient broth (Difco Laboratory, U.S.A) at 35°C for 27 h. Bacterial solution of *S.* Enteritidis was prepared at 2.96 log CFU mL^{-1}, while the *E. coli* solution was at 2.89 log CFU mL^{-1}.

Cactus cladode preparation and inoculation

Cactus cladores of var. Atlixco were purchased from a local market (State of Mexico), and carried to the laboratory in a cooler box. Cactus cladodes were immersed in 1% NaClO solution for two minutes to disinfect them, and then dried on a disinfected surface. The cactus cladodes were divided into two groups. In the first, spines were removed using sterile gloves and knives, while the second group was preserved with spines. Later, cladodes with and without spines were stored at 4 or 18°C; 4 lots with 36 cladodes each were used. Circles of 2 cm in diameter were inoculated with bacterial solutions. Cladodes were packed individually in plastic zipper bags. Survival evaluations of *S.* Enteritidis and *E. coli* were made by triplicate on days 0, 3, 6, 8, 10, 12, 14 and 16.

Bacterial enumeration

The inoculated area of the cactus cladode was cut and diluted with 50 mL of sterile peptone water (0.8%) in sterile plastic bags and homogenized with a stomacher for 1 min. Serial dilutions were prepared, the bacterial dilution 10^{-5} was inoculated (1 mL) onto a Petri dish containing specific media. Hektoen Enteric Agar (Difco, BBL) was used for identification of *S.* Enteritidis and Agar Eosin-methylene blue (Merck) for *E. coli*. Incubation was carried out at 37°C for 24 h. Colony forming units (CFU) were enumerated. The results were expressed in log CFU mL^{-1}.

Extraction of phenolic compounds for HPLC analysis

Physiologically mature cladode tissue (18-20 cm long) was used to prepare extracts by the method of conventional extraction (reflux distillation). Different conditions were evaluated to determine the optimal conditions for extraction of total phenols: amount of tissue

*Corresponding author. E-mail: martinez.talina@inifap.gob.mx.

Table 1. Comparison of means of survival of *Salmonella Enteritidis* and *E. coli* in cactus leaf with and without spines to 18 and 4°C.

Storage (days)	*Salmonella* (log UFC)				*E. coli* (log UFC)			
	18°C		4°C		18°C		4°C	
	CE	SE	CE	SE	CE	SE	CE	SE
3	3.09^{e}	3.29^{c}	2.9^{c}	2.82^{b}	4.02^{b}	3.94^{g}	3.18^{a}	1.93^{c}
6	3.33^{c}	3.30^{c}	3.05^{c}	2.38^{d}	4.39^{a}	4.25^{c}	3.15^{a}	2.17^{b}
8	3.39^{b}	3.30^{c}	3.20^{b}	2.57^{c}	4.37^{a}	4.19^{b}	2.74^{b}	1.62^{d}
10	3.82^{a}	3.75^{a}	3.35^{a}	2.31^{d}	3.74^{c}	3.61^{d}	2.53^{c}	1.43^{e}
12	3.80^{a}	3.70^{b}	3.32^{a}	2.15^{e}	3.40^{d}	3.23^{e}	2.39^{d}	1.25^{f}
14	3.22^{d}	3.16^{d}	2.56^{d}	2.05^{f}	3.30^{e}	3.08^{f}	2.28^{e}	0.21^{g}
16	2.29^{g}	2.15^{b}	1.75^{e}	1.45^{g}	2.53^{f}	2.20^{h}	1.82^{f}	0.00^{h}

CE=With spines, SE=Without spines, same letters are not significantly different.

(2, 6 and 10 g), 30 mL of solvent (water, methanol and ethanol), extraction temperature (40 and 60°C) and time (1, 2, 3, 4 and 5 h). Quantification of total phenols was performed in triplicate by the Folin–Ciocalteu method (Kuskoski et al., 2005) and expressed in terms of equivalent amounts of gallic acid.

HPLC analysis of phenolic compounds

The phenolic compounds in the extracts were determined by HPLC using a UV detector (Thermo Separations Products, USA) at 280 nm (Agilent 1100 series, Hewlett Packard Co., USA). The separation was conducted in an Alltech Lichrosorb C18 column (250 x 4.6 mm), all extracts and solvents were filtered through a 0.47 µm filter (Varian) prior to analysis. In accordance with the methodology of Ndhlala et al. (2007), two mobile phases we used: A: water : acetic acid (98:2 v/v) and B: water : acetonitrile : acetic acid (68:30:2). The flow rate was 2 mL min^{-1} and 20 µL of each sample were injected. Standard solutions (0.02 mg mL^{-1}) of gallic, protocatechuic, 4-hydroxybenzoic, caffeic, feluric, chlorogenic, syringic, ρ-coumaric and sinapic acids, and (-) epicatechin and quercetin were dissolved using methanol as the solvent (HPLC degree). All phenolics were identified by comparing the UV spectral properties and retention times to those of authentic standards.

Experimental design and statistical analysis

Two statistical test were performed, a 2x2 factorial design was used to determine the influence of the cladode cactus presentation (without spines and with spines), and temperature storage (4 and 18°C) on the survival of *S.* Enteritidis and *E. coli* every other day for 16 days (three repetitions in three individuals each time), an analysis of variance for repeated measures was used ($p \leq 0.05$) and Tukey Mean Difference tests. A 3x3x2x6 factorial design was used to determine the optimal conditions for the extraction of phenolic compounds, using ANOVA ($p \leq 0.05$) and Tukey mean difference tests. The SAS 9.1 program was used to perform the analyses.

RESULTS

Evaluation of *S.* Enteritidis and *E. coli* survival

The populations (CFU) of *S.* Enteritidis and *E. coli* on cactus cladodes stored at 4°C were lower than at 18°C; the minimal survival was on cactus cladodes without spines stored at 4°C. The analysis of variance showed interaction between storage time, temperature and presentation of cactus cladodes in the survival of *S.* Enteritidis and *E. coli* (Table 1). The maximum growth of *S.* Enteritidis was observed at 10 days after inoculation; the population of *Salmonella* in cactus cladodes without spines at 4°C declined with respect to the initial concentration 0.15 log CFU after 3 days of storage and 1.5 log CFU at final storage (16 days) (Figure 1). In cactus cladodes with spines, the population of *S.* Enteritidis increased approximately 0.21 log CFU from 3 to 10 days, and declined 1.2 log CFU at the end of storage (Figure 2).

S. Enteritidis was able to survive in the storage times and temperatures of domestic marketing in cactus cladodes with and without spines. The ability to survive at 4°C has been observed from nine days to eight weeks of storage; in this time, the populations declined approximately 0.5 to 2 log CFU (Liao et al., 2010). *Salmonella* has been able to survive at temperatures lower than 4°C. Strawn and Dayluk (2010) demonstrated viability of the bacteria in papaya and mango after 180 days at -20°C, and Kimber et al. (2012) found *Salmonella* on almonds and pistachios stored at -19 and 4°C at least one year after inoculation. In contrast, at 18°C, *S.* Enteritidis increased by 0.45 log CFU after 3 to 14 days in both presentations of cactus cladodes (Figures 1 to 2). Liao et al. (2010) observed a similar situation in jalapeño pepper, where populations of *Salmonella* Saintpaul increased around 3 log CFU at 20°C in just 48 h. *E. coli* presented a similar behavior to *S.* Enteritidis; the population of *E. coli* decreased significantly ($p < 0.001$) at 4°C in both presentations of cactus cladodes. However, the population of this bacterium decreased to 0.94 log CFU at 3 days, and viable cells were not found at 16 days on cactus cladodes without spines (Figure 3). On cladodes with spines, the population increased by 0.3 log CFU on days 3 and 5, followed by a gradual decrease:

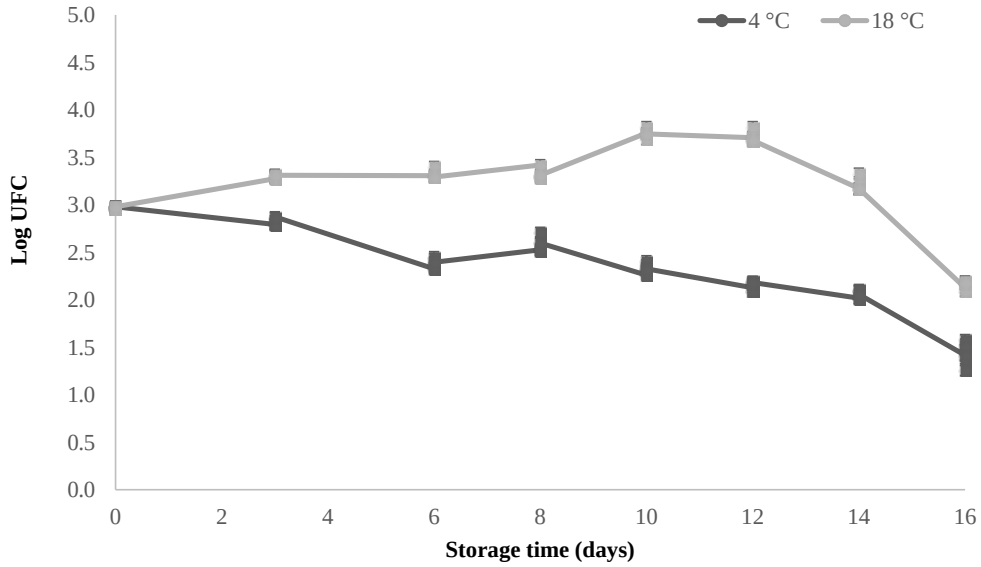

Figure 1. Survival of *S.* Enteritidis in cactus cladodes without spines, stored at 4 and 18°C. Bars denote standard deviation, n = 9.

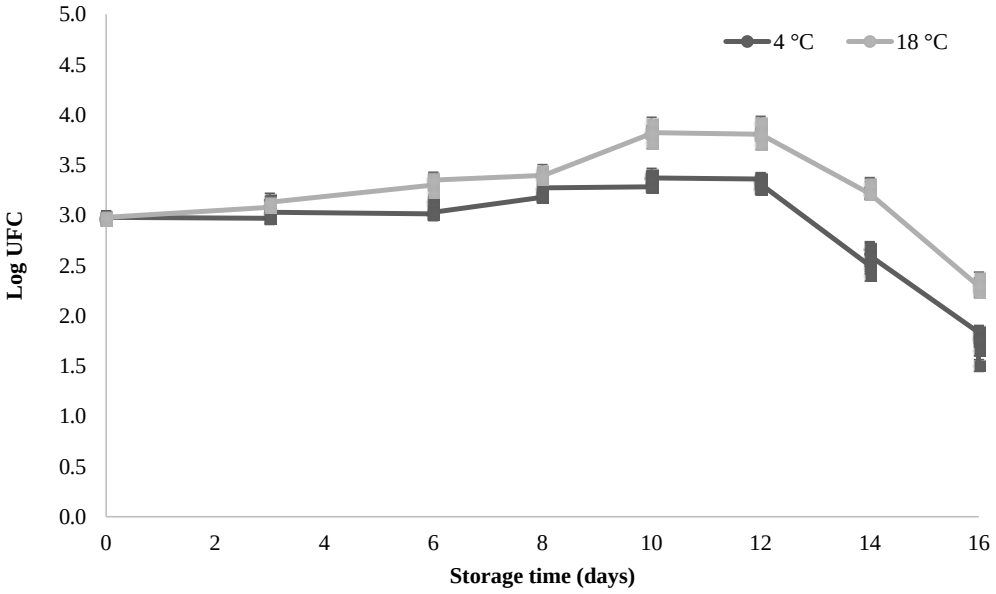

Figure 2. Survival of *S.* Enteritidis in cactus cladodes with spines, stored at 4 and 18°C. Bars denote standard deviation, n=9.

1.06 log CFU was detected on day 16, nearly the initial concentration (Figure 4). At 18°C, in cactus cladodes without spines and with spines, the bacterial population had increased at day 3; maximum growth was found on day 6, after this time the population decreased considerably, with remnants of 2.20 and 2.53 log CFU without and with spines, respectively, on day 16. The survival ability of *E. coli* on cactus cladodes has not been documented; however, on fruits of prickly pear without peel, *E. coli* O157: H7 was able to survive and increase its population to 4.5 and 5 log CFU g^{-1} in storage at 4 and 8°C, respectively (Corbo et al., 2005). In other vegetables at <8°C, this bacterium showed decreased growth but was able to survive (Khalil and Frank, 2010; Liao et al., 2010; Corbo et al., 2005). The ability of both pathogens to survive at the same time was studied by Hsu et al. (2006) at 4°C on aromatic herbs; the populations of both bacteria decreased to about <0.8 log at 5 days of

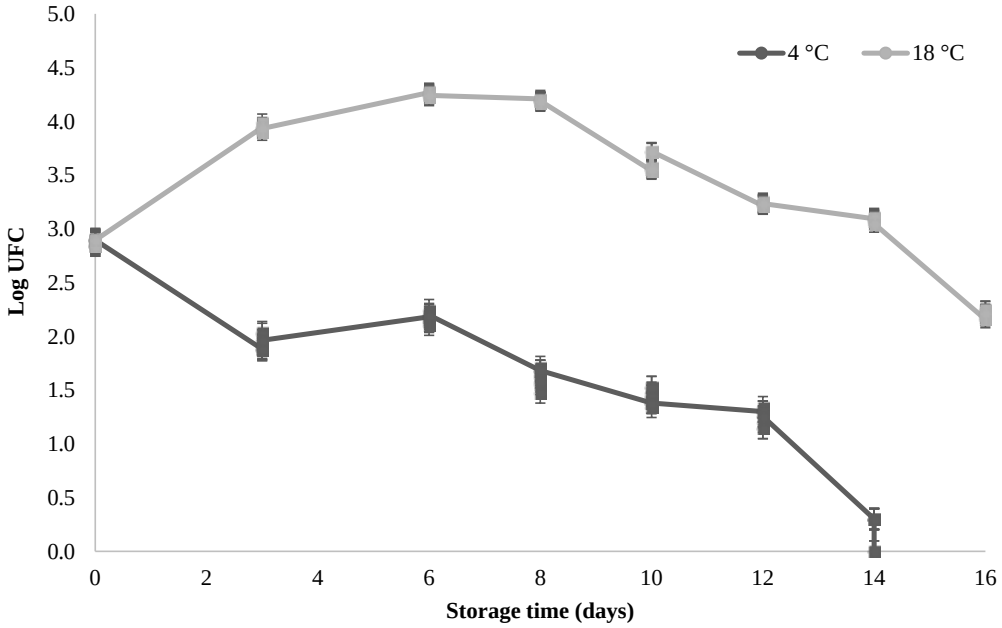

Figure 3. Survival of *E. coli* in cactus cladodes without spines stored at 4 and 18°C. Bars denote standard deviation, n = 9.

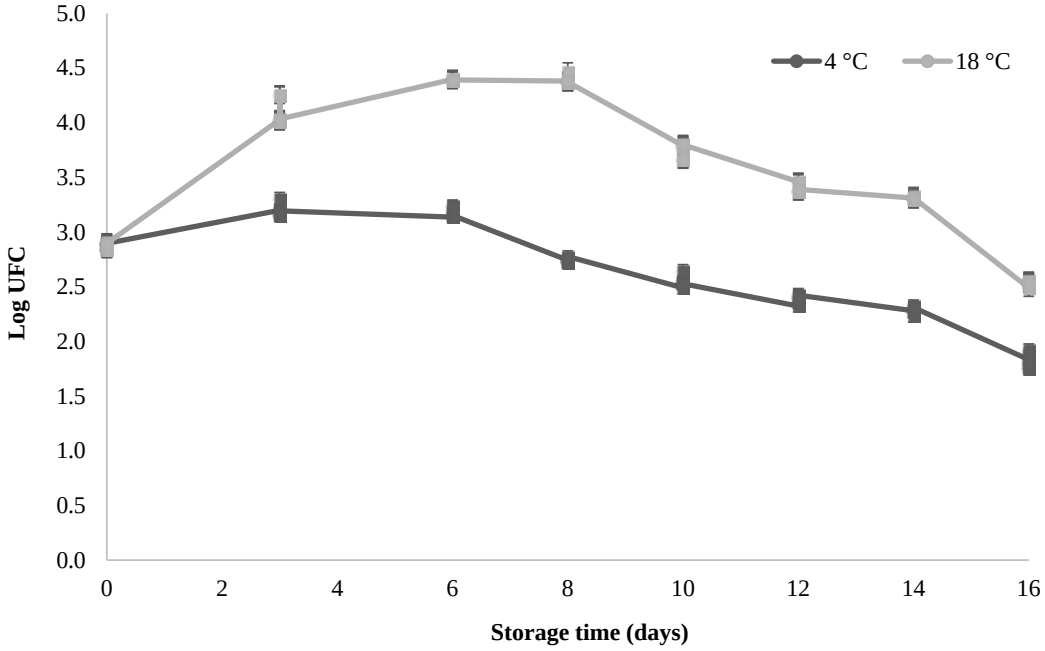

Figure 4. Survival of *E. coli* in cactus cladodes with spines stored at 4 and 18°C. Bars denote standard deviation, n = 9.

storage; however, *E. coli* O157: H7 decreased rapidly over time; bacteria were detected even after 24 days on rotting tissue. Other studies reported that the ability to survive was related to type of tissue. Khalil and Frank (2010) observed major growth on spinach leaves at 8°C (1.18 log CFU), while in lettuce, cilantro and parsley leaves, the bacterium did not grow at 8°C.

With the information obtained in this study on

Table 2. Comparison of means from the content of total phenols of aqueous and methanolic extracts. The means of total phenolics are shown according to statistical analysis.

Treatment	Total phenols[&] gallic acid mg g^{-1}	Tukey mean separation			
60°C, water, 10 g, 6 h	1.127[a]	A			
60°C, water, 10 g, 3 h	1.084[b]	A	B		
40°C, water, 10 g, 5 h	1.058[c]	A	B	C	
40°C, water, 10 g, 6 h	1.051[c]	A	B	C	
60°C, water, 10 g, 5 h	1.039[c]	A	B	C	D
60°C, methanol, 10 g, 6 h	1.039[c]	A	B	C	D
60°C, methanol, 10 g, 4 h	1.031[c]	A	B	C	D
60°C, water, 10 g, 4 h	1.026	A	B	C	D

Means with the same letter are not significantly different

temperature and time of survival, there is a latent risk of the presence of foodborne pathogens in cactus cladodes, particularly when they are transported or kept in poor hygienic conditions or unrefrigerated, and consumed fresh in salads and juices.

The presentation of cactus cladodes during storage was another important factor in the survival of both bacteria; survival was significantly greater ($p \leq 0.001$) in cactus cladodes with spines. The surface wax of the cladodes controls the transpiration and reflects solar radiation, and prevents the penetration of microorganisms into the surface tissue. The bacteria probably produce biofilms that allow them to survive on this wax. Some bacteria have the ability to form biofilms on the surface of the epidermis of fruits, leaves, stems and flower organs, as an adhesion and protection mechanism, also to trap nutrients for feeding (Ávila-Quezada et al., 2010). Hernandez et al. (2009) documented biofilm formation by *Salmonella* Typhimurium and *S.* Javanica 24 h after inoculation; their results showed faster adhesion. Few studies have been conducted to estimate the survival of both bacteria on the surface of this vegetable; however, in other products such as cucumber, mango, guava and tomato, *S.* Enteritidis was capable of developing biofilms on surfaces after inoculation (Tang et al., 2012). If these bacteria are established on the cuticle of cactus cladodes with biofilms, there is a risk of internalization of foodborne pathogens into tissue during the removal of the spines, which would remain there until consumption. The survival of *S.* Enteritidis and *E. coli* in spineless cactus cladode was significantly less at 4°C; this behavior is probably attributable to the decrease in bacterial growth influenced by the temperature; however, the hypothesis of liberation of metabolites as result of mechanical damage in the spine removal process should not be rejected. The production of these compounds is activated as a defense mechanism against a wide variety of microorganisms and increases their survival. The presence of metabolites with microbiological properties has been documented in several species of the genus *Opuntia.* Some extracts of *O. cochenillifera* (Syn.: *Nopalea cochenillifera*) showed *in vitro* inhibition of the growth of *Candida albicans, Candida glabrata*, *E. coli*, *Salmonella* Typhimurium, *S.* Typhi, *Micrococcus* sp., *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Saccharomyces cerevisiae* and others (Gomez-Flores et al., 2006; Necchi et al., 2012). Also, extracts of *O. stricta* presented antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *Bacillus* sp., *Pseudomonas aeruginosa* and *Enterococcus faecalis* (Koubaa et al., 2015), while extracts of *O. ficus-indica* presented bactericidal activity against *Campylobacter jejuni*, *Campylobacter coli*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *Proteus mirabilis*, *Salmonella* spp., *E. faecalis*, *Citrobacter freundii*, *Acinetobacter baumannii*, *Streptococcus pneumoniae*, *Enterococcus faecium* and *Enterobacter cloacae* (Wasnik and Tumane, 2016). Hayek and Ibrahim (2012) determined the antimicrobial potential of *Opuntia matudae* (xoconostle) against *E. coli* O157: H7. The authors attributed this inhibition to organic acids and polyphenols, especially flavonoids and tannins. Phenolic compounds caused cell wall degradation and disruption of the cytoplasmic membrane (Cetin-Karaca and Newman, 2015). Other mechanisms are enzyme inactivation, inhibition of DNA and RNA synthesis, electron transport chain and biofilm formation, and neutralization of toxins (Gutiérrez-Larraínzar et al., 2012).

Determination of phenolic compounds in cladodes of cactus *O. ficus-indica* var. Atlixco

The comparison of means showed water as the best solvent to extract phenolic compounds. However, this solvent yielded an excessive amount of mucilage which obstructed the passage of the sample in the HPLC columns; therefore, the second option was methanol as solvent. The phenolic compound extraction used 10 g of tissue, solvent methanol, extraction time and temperature of 60°C for 6 h (Table 2).

Gallic (tR=3.8), protocatechuic (tR=6.6), 4-hydroxybenzoic

Table 3. Concentration of phenolic compounds analyzed by HPLC in extracts of cactus cladodes var. 'Atlixco'.

Phenolic Compounds	Cactus cladodes var. Atlixco (mg 100 g^{-1})
Gallic acid	1.58±0.05
Protocatechuic acid	24.03±0.21
Hydroxybenzoic acid	0.02±0.01
Caffeic acid	41.32±0.28
Chlorogenic acid	0.12±0.01
Syringic acid	0.24±0.1
ρ-coumaric acid	0.33±0.7
Feluric acid	0.74±0.03
Sinapic acid	3.22±0.24
4-hydroxybenzaldehyde acid	0.16±0.01
Quercetin	5.31±0.61
Epicatechin	0.17±0.01

(tR=11.6), caffeic (tR=16.9), feluric, (tR=32.6), chlorogenic (tR=18.9), syringic (tR=19.9), ρ-coumaric (tR=26.7), sinapic (tR=37.2), 4- hydroxybenzaldehyde (tR=14.1) acids and (-) epicatechin (tR=24.4) and quercetin (tR=37.7) were detected (Table 3).

The major compound was caffeic acid (41.32 mg 100 g^{-1} fresh weight), followed by protocatechuic acid (24.03 mg 100 g^{-1}). Both acids are recognized to have anti-inflammatory, anti-glycemic, antioxidants, anti-cancer, anti-mutagenic and anti-microbial properties; they are also precursors of lignin formation in plant tissues. The concentrations of phenolic compounds were similar to those of Guevara-Figueroa et al. (2010) who determined the presence of protocatechuic (0.06-2.5 mg 100 g^{-1}), gallic (0.64 mg 100 g^{-1}) 4-hydroxybenzoic (0.5 -3.19 mg 100 g^{-1}), feluric (0.56-4.32 mg 100 g^{-1}) acids and quercetin (iso-quercetin form: 22.9-32.21 mg 100 g^{-1}) in the Blanco, Manso, Amarillo and Cristalino varieties. Ginestra et al. (2009) documented quercetin (isoquercetin form: 7 mg 100 g^{-1}), traces of 4-hydroxybenzoic, trans-ferulic and trans and cis p-coumaric acids in a mix of Surfarina, Muscaredda and Sanguigna cultivars of *O. ficus-indica*. Qiu et al. (2003) determined protocatechuic (0358 mg 100 g^{-1}), 4-hydroxybenzoic (2 mg 100 g^{-1}) and feluric (0.47 mg 100 g^{-1}) acids in *O. dillenii*,. The amount of phenolic compounds obtained in this study with the Atlixco variety was similar to other studies except for 4-hydroxybenzoic acid (0.02 mg 100 g^{-1}), known for its antimicrobial and antioxidant activity (Yang et al., 2009). In this study, chlorogenic, syringic, sinapic acids and the flavonoid (-) epicatechin were documented for first time in the Atlixco variety of *O. ficus-indica*; they are important due to their antioxidant, antibacterial, antiviral, anticancer, anti-mutagenic and anxiolytic properties (Othman et al., 2010).

Conclusions

The effect of temperature and presentation of cactus cladodes was significant on the survival of *S* Enteritidis and *E. coli*. *S.* Enteritidis and *E. coli* were able to grow and survive for 16 days at 4 and 18°C in cactus leaves with spines. In cactus leaves without spines, *S.* Enteritidis survived for 16 days at 4 and 18°C, while *E. coli* only survived during this period at 18°C. *Opuntia ficus-indica* (L.) Mill var. Atlixco presents phenolic compounds with antimicrobial potential that could reduce the pathogenicity of *S.* Enteritidis and *E. coli* associated with consumption of fresh cactus.

Conflict of Interests

The authors have not declared any conflict of interests.

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Effect of compaction of soil on the development and production of cowpea inoculated with rhizobium

Núbia Leite da Silva, Salomão Lima Guimarães*, Edna Maria Bonfim-Silva, Erineudo Lima Canuto and Helon Hébano de Freitas Sousa

Universidade Federal de Mato Grosso - (UFMT) Brasil.

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Cowpea is considered as one of the most important species in Brazil, for presenting essential components to the feeding process, which makes it relevant to identify the physical, chemical and biological conditions that benefit or cause damage to its development. Thus, the objective of this study was to evaluate the effect of inoculation with rhizobia strains in compacted soil on the development and production of cowpea. The experiment was conducted in a greenhouse, in a completely randomized design with six replications, in a factorial 3 × 2, with two inoculation treatments (BR3267 - *Bradyrhizobium* sp. and the combination of the strains MT8 - *Rhizobium tropici* and MT15 - *R. tropici*) and a control with nitrogen fertilization - 150 mg dm^{-3}, using urea as a source, in compacted and uncompacted soil. The evaluations were carried out during the crop cycle (35, 45, 52, 58 and 90 days) after the emergence of plants, determining the Falker chlorophyll content, dry mass of grains, nitrogen content, nitrogen accumulation and crude protein in shoots and grains. All data were subjected to statistical analyses using the SISVAR program, performing the analysis of variance and the Tukey test at the level of 5% probability. The results showed positive effect on plants inoculated with the combination of the strains MT8 + MT15 in compacted soil, in all parameters evaluated, being observed, values similar to or greater than in plants grown in uncompacted soil. Therefore, the use of the combination of strains becomes a promising alternative for the cowpea development in soil with a density of 1.6 Mg m^{-3}.

Key words: Biological nitrogen fixation, cerrado, soil density.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) has several favorable characteristics for cultivation in the Midwest of the country, especially the existence of genotypes with increased tolerance to drought, lower requirement

*Corresponding author. E-mail: slguimaraes@cpd.ufmt.br

Table 1. Chemical and granulometric characterization of the 0-0.2 m layer of Dystrophic Red Latosol (Rondonópolis-MT, 2015).

pH	P	K	Ca	Mg	H	Al	SB	CTC	V	O.M.	Sand	Silt	Clay
$CaCl_2$	mg dm^{-3}		---------- cmolc dm^{-3} ----------						%	g dm^{-3}		g kg^{-1}	
4.0	1.4	23	0.4	0.2	5.4	0.8	0.7	6.8	9.7	27.1	423	133	444

pH = Hydrogen ionic potential; P = phosphorus; K = potassium; Ca = calcium; Mg = magnesium; H = hydrogen; Al = aluminum; SB = sum of bases; CTC = cation exchange capacity; V = base saturation; O.M. = organic matter.

increased tolerance to drought, lower requirement of nutrients and the possibility of use of agricultural machinery in all production processes (Odutayo et al., 2005; Locatelli et al., 2014).

However, the use of agricultural machinery in soil above the optimal humidity may cause changes in the physical properties of the soil and consequently promote compaction (Horn et al., 2003). When there is soil compaction, the plant growth is hindered due to the formation of a cohesive layer, which causes increased resistance to penetration of roots, reduced macroporosity and consequently, there is a decrease in the absorption of water and nutrients (Shittu and Amusan 2015).

Despite the negative effects to the soil when used in inadequate conditions, agricultural machinery has become indispensable because of the large areas, the exploitation of several annual crops and the agricultural modernization (Collares et al., 2011; Streck et al., 2004).
In addition to the physical alterations of the soil, which affect the formation of roots and the grain production, increased density can reduce the activity of microorganisms. In a study by Farias et al. (2013) with dwarf pigeonpea grown in compacted soil, the formation of nodules showed a reduction of up to 76.82%, and this effect is explained by the change in root mass due to the reduction of the pore spaces, which increases the resistance to the penetration of roots in the soil profile (Hamza and Anderson, 2005).

Nonetheless, microorganisms play an important role in the physical characteristics of the soil, due to the ability to unite the mineral fraction of the soil in stable aggregates, favoring the establishment of plants against adverse conditions in the long term (Harris et al., 1966); regarding the short term, bacteria provide the supply of nitrogen and growth hormones, a process that would stimulate the growth of secondary roots, resulting in greater absorption of nutrients and water (Glick, 2012)

This way, tests carried out under controlled conditions may be used for an indication of the responses of crops to stressful conditions, such as inadequate management leading to soil compaction. In this context, the objective was to evaluate the effect of inoculation with rhizobia strains in compacted soil on the development and production of cowpea.

MATERIALS AND METHODS

Study site

The experiment was conducted in a greenhouse at the Institute of Agricultural Sciences and Technology of the Federal University of Mato Grosso, Rondonópolis Campus, Brazil, located at Latitude 16°27'49.62" S, Longitude 54°34'47.78" E, with an average altitude of 227 m.

Experimental design

The design was completely randomized with six replications, in a factorial 3 x 2, with two inoculation treatments (BR3267 - (*Bradyrhizobium* sp.) and the combination of the strains MT8 - *Rhizobium tropici* and MT15 - *R. tropici*), in addition to a control with nitrogen fertilization - 150 mg dm^{-3}, using urea as source, in two soil conditions (compacted and uncompacted soil), totaling 36 experimental plots.

The soil used was collected in a fragment of Cerrado and classified as Dystrophic Red Latosol (Embrapa, 2013). Later, it was sieved on a 4 mm sieve and then, based on chemical analysis, liming with dolomitic limestone (PRNT 80%) was performed, raising the base saturation to 60% for 30 days.
The results of the physical and chemical analysis of the soil shown in the experimental area can be found in Table 1.

Experimental plot

Each experimental plot consisted of three rigid polyvinyl chloride (PVC) rings with a diameter of 0.15 cm and 10 cm in height (Figure 1). At the bottom of the experimental unit, it was inserted, a polyethylene screen with a 1 mm mesh, fixed with a rubber ring obtained through the cross-section of the air chamber. The assembly of the experimental plot was performed by fitting the rings, using an adhesive tape (Silver Tape) to ensure resistance to the transport and the experimental evaluation procedures. Plastic dishes of 100 mm diameter were used, aiming to provide support for the experimental plots and to facilitate the irrigation.

The upper and lower rings referring to layers A and C, respectively, were filled with soil mass without adding compaction, while the central ring (Layer B) was compacted, increasing the density to 1.6 Mg m^{-3}, according to the methodology described by Fagundes et al. (2014).

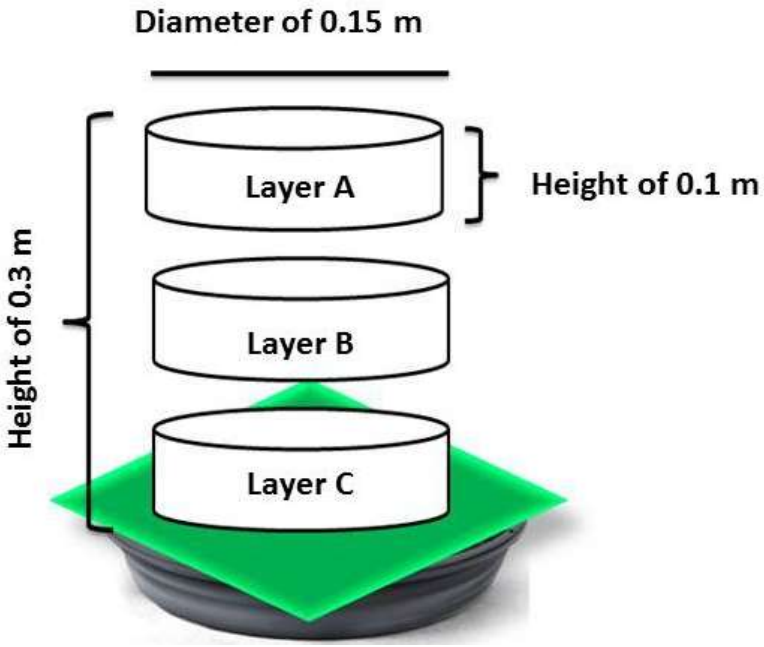

Figure 1. The experimental plot comprising three PVC rings cut with a height of 0.1 m and outer diameter of 0.15 m.

Soil compaction

The ideal moisture for compaction was determined in a laboratory test (Proctor normal test), in accordance with NBR 7182 (ABNT, 1986), establishing the value of 16% on the basis of mass and the weight of the dry soil to be compacted was determined according to the Equation 1:

$$Ds = \frac{\text{MSS}}{\text{VT}}$$

In which Ds- soil density, (kg dm^{-3}); MSS (DSM)- dry soil mass (g); VT- total layer volume (dm^{-3}). The calculation of the mass of the wet soil to be used in the compacted layer was determined according to Equation 2:

$$MSU = MSS + (1 - \theta m)$$

In which: MSU (MSM)- Moist soil mass (g); MSS (DSM)- dry soil mass (g); θm- mass-based moisture content (%).

Fertizer application and seed sowing

Fertilizer was applied at the time of compaction, with the application of 240 mg dm^3 phosphorus (P_2O_5) and 200 mg dm^3 potassium (K_2O) in all plots. The nitrogen dose was 150 mg dm^3, applied only to the control. Urea, simple superphosphate and potassium chloride were used as source.

Five seeds of the cultivar, BRS Tumucumaque were sown directly in the vessels, and after seven days, thinning was held, leaving only two plants per experimental plot. Soil moisture was maintained by the addition of water on the surface and by capillarity when water was added at the bottom of the experimental plot.

Inoculation

To prepare the inoculum, strains of rhizobia isolated from cowpea (MT8 - *Rhizobium tropici* and MT15 - *R. tropici*) and BR3267 from *Bradyrhizobium japonicum*, a strain recommended by the Laboratory Network to Recommendation, Standardization and Diffusion of the Technology of Microbiological Inoculants of Agricultural Interest - RELARE for cowpea inoculation (Martins et al., 2003) were used.

The bacteria were grown in YM liquid culture medium according to the methodology of Fred and Waksman (1928). Incubation was continued for about 96 h at 28°C. Thereafter, the inoculation with rhizobia was performed 7 days after plant emergence, with the application of 10 mL of the inoculant in each experimental plot.

Collection of samples for analysis

At 35, 45, 52 and 58 days after sowing, assessments of the Falker chlorophyll content were carried out in the leaves collected in the middle third of the plants, through the portable meter Clorofilog 1030.

At 90 days, the cutting of plants was performed at ground level, to evaluate the variables: dry mass of grains, nitrogen and crude protein content in shoots and grains. Initially, the plant material was packed in paper bags and identified, and shortly thereafter, dried in a forced-air oven at 65°C for 72 h or until constant weight, subsequently, weighing was performed on a precision scale.

Table 2. Effect of inoculation with rhizobia strains on the Falker chlorophyll content in cowpea grown in compacted and uncompacted soil.

Treatments	Compacted soil	Uncompacted soil
Falker chlorophyll content at 35 days		
BR3267	65.0^{Aa}	66.2^{Aa}
MT8+MT15	63.7^{Aa}	64.8^{Aa}
Control	66.8^{Aa}	69.8^{Aa}
CV(%) = 9.04		
Falker chlorophyll content at 45 days		
BR3267	68.4^{Aa}	65.4^{Aa}
MT8+MT15	71.2^{Aa}	68.4^{Aa}
Control	63.2^{Aa}	65.1^{Aa}
CV(%) = 9.04		
Falker chlorophyll content at 52 days		
BR3267	66.9^{Ab}	73.0^{Aa}
MT8+MT15	73.8^{Aa}	73.6^{Aa}
Control	72.5^{Aa}	72.7^{Aa}
CV(%) =6.50		
Falker chlorophyll content at 58 days		
BR3267	68.4^{Aa}	67.1^{Aa}
MT8+MT15	71.2^{Aa}	68.0^{Aa}
Control	64.3^{Aa}	66.4^{Aa}
CV(%) = 10.47		

Means followed by the same letter - uppercase, vertically, and lowercase, horizontally - do not differ by Tukey test at 0.05 significance level.

The determination of the total nitrogen content in leaves and grains followed the micro-Kjeldahl method described by Malavolta et al. (1997). The content of crude protein (CP) was calculated using the factor 6.25 for the conversion of total nitrogen to crude protein and the nitrogen accumulation was determined according to the following equation:

$$QNTA = \frac{\text{RMS} * \text{N}}{1000} \; X \; 1000$$

Where, QNTA (TAN)- Total accumulated nitrogen (mg plant^{-1}); RMS (DMY)- dry mass yield (g plant^{-1}); N- plant nitrogen content (g kg^{-1}). All results were subjected to statistical analyses using the SISVAR program (FERREIRA, 2011), performing the analysis of variance and the Tukey test at the level of 5% probability.

RESULTS AND DISCUSSION

Soil compaction resulted in changes in the following variables: Falker chlorophyll index, after 52 days; dry mass of grains and nitrogen accumulation in the grains. Notwithstanding, regarding the Falker chlorophyll index at 35, 45 and 58 days, the nitrogen content in shoots and grains and the crude protein in shoots and grains, no statistical difference was observed between the treatments.

The highest averages for the Falker chlorophyll index in the evaluation performed at 35 days after sowing were observed in control plants, which received nitrogen fertilization, but in the assessments made at 45, 52 and 58 days, the combination of the strains MT8 + MT15 provided the best values, regardless of soil compaction, being observed, an increase of up to 11.23% in relation to nitrogen fertilization.

The plants inoculated with the strain BR 3267 showed a significant difference in relation to soil compaction. It was possible to observe a reduction of 8.35% in the Falker chlorophyll content when plants were grown in compacted soil (Table 2).

The results may have the same hypothesis described by Artursson et al. (2006), Smith and Read (2008) and Bashan (1998), in which the authors emphasized that the combination of microorganisms in inoculants may be

Table 3. Effect of inoculation with rhizobia strains on the dry mass of grains of cow pea grown in compacted and uncompacted soil.

Treatments	Compacted soil (g plots $^{-1}$)	Uncompacted soil (g plots^{-1})
BR3267	12.5^{Ab}	16.2^{Aa}
MT8+MT15	16.1^{Aa}	14.5^{Aa}
Control	12.4^{Ab}	15.7^{Aa}
CV(%) = 17.5		

Means followed by the same letter - uppercase, vertically, and lowercase, horizontally - do not differ by Tukey test at 0.05 significance level.

better at promoting greater uptake of nutrients by plants because of the ability of microorganisms to improve some beneficial aspects of the physiology of both, such as, for example, the greater affinity with the host plant, thus increasing the efficiency of the inoculation, besides promoting a greater development of the plants.

The averages achieved by the plants grown in compacted soil were due to the lack of soil densification in layers A and C, which would facilitate the growth and development of roots in this region, not changing the Falker chlorophyll index.

According to the authors, Hamza and Anderson (2005), the sowing of plants that have a deep and aggressive root system, which can grow even with the soil structure changed by increasing density, which is one of the main characteristics required for the management of compacted soil. Furthermore, Reinert et al. (2008) emphasized that the root system develops in the soil zone that has lower resistance to penetration, which favors the absorption of nutrients and water and thus provides increased nodulation.

For dry mass of grains, it was observed that the soil compaction provided a reduction in grain yield of 22.83 and 21.01% for treatments BR3267 and nitrogen fertilization, respectively, when compared with treatments without compaction. However, the plants inoculated with the combination of the strains MT8 + MT15 showed better performance in compacted soil, being observed, an increase of 9.93% as compared to uncompacted soil (Table 3). In uncompacted soil, the strain BR3267 provided the highest grain yield, with up to 10.49% increase as compared to the other treatments (Table 3). These results can be explained by the change in root growth, affecting the absorption of water and nutrients, and by the decrease in macropores, which consequently interferes with soil aeration (Grath and Hakansson, 1992). The soil conditions created by increased density have a direct and indirect effect on the microbial population. A smaller development of the root system results in a smaller amount of substrate for biological activity and, moreover, the remaining roots decrease the release of substances that attract the bacteria to start the infection and, subsequently, the formation of nodules (Flores et al., 1999).

Nevertheless, the strains exhibit different levels of competitiveness and adaptability in compacted soil conditions, where there is excess CO_2, O_2 deficiency and the presence of toxic elements produced by the environment (Miransari et al., 2007). This information is a hypothesis for the result of the treatment with the combination of the strains MT8 + MT15 and the performance of the strain BR3267 in uncompacted soil.

The results agree with Carvalho et al. (2005), who found that soybeans inoculated with the strain SEMIA 5019, along with three variants generated through spontaneous mutations (1A, 2A and 3A), showed better symbiotic efficiency in a test conducted in a greenhouse, using sand and vermiculite as substrate, in the ratio of 3:1.

The authors Cregan and Berkum (1984) pointed out that the plant nitrogen uptake increases in the vegetative growth stage and in the reproductive stage of the crop, decreasing in the grain filling stage, this information leads to assume that the inoculation with the combination of the strains MT8 + MT15 in plants grown in compacted soil provided nitrogen in an adequate quantity to meet a grain production similar to that of plants grown in soil without densification.

Similar to the results presented by plants inoculated with the strain BR3267 and fertilized with nitrogen, Collares et al. (2008) found in a study that the beans grown under field conditions, in soil with different densities (continued no-till- NT; no-till with additional compaction – NTc; and scarification - Sca), had their productivity decreased by 17%, as compared to the planting without compaction. According to these authors, the compaction altered the root system of plants leading to restriction in the absorption of nutrients and water.

For the variable nitrogen content in shoots and grains, the results were similar to the Falker chlorophyll index. The treatments that stood out in compacted soil were those that received nitrogen fertilization and those inoculated with the combination of the strains MT8 + MT15, respectively. It was observed that the combination

Table 4. Effect of inoculation with rhizobia strains on the nitrogen content in shoots and grains of cowpea grown in compacted and uncompacted soil.

Treatments	Compacted soil	Uncompacted soil
Nitrogen content in the shoots		
BR3267	21.6^{Aa}	21.7^{Aa}
MT8+MT15	22.0^{Aa}	22.8^{Aa}
Control	22.5^{Aa}	21.3^{Aa}
CV(%) = 16.8		
Nitrogen content in the grains		
BR3267	45.1^{Aa}	45.0^{Aa}
MT8+MT15	46.3^{Aa}	43.4^{Aa}
Control	43.1^{Aa}	43.1^{Aa}
CV(%) = 9.5		

Means followed by the same letter - uppercase, vertically, and lowercase, horizontally - do not differ by Tukey test at 0.05 significance level.

Table 5. Effect of inoculation with rhizobia strains on the nitrogen accumulation in shoots and grains of cowpea grown in compacted and uncompacted soil.

Treatments	Compacted soil	Uncompacted soil
Nitrogen accumulation in the shoots		
BR3267	245.3^{Aa}	288.7^{Aa}
MT8+MT15	252.1^{Aa}	303.9^{Aa}
Control	257.3^{Aa}	295.7^{Aa}
CV(%) = 19.3		
Nitrogen accumulation in the grains		
BR3267	281.0^{ABb}	367.9^{Aa}
MT8+MT15	377.0^{Aa}	315.3^{Aa}
Control	270.6^{Ba}	338.6^{Aa}
CV(%) = 9.5		

Means followed by the same letter - uppercase, vertically, and lowercase, horizontally - do not differ by Tukey test at 0.05 significance level.

of the strains MT8 + MT15 provided a 6.91% increase in the nitrogen content in grains as compared to the treatment with nitrogen fertilization in compacted soil (Table 4).

In uncompacted soil, the highest contents observed were obtained with the treatments with inoculation, being found, an increase of 6.57 and 4.22% in the nitrogen content in shoots and grains of the treatments MT8 + MT15 and BR3267, respectively. The results indicate that the rhizobia and cowpea symbiosis may have provided nitrogen in similar amounts as compared to nitrogen fertilization, benefiting the crop development.

Alves et al. (2003) conducted a study using three soil types (Dystrophic Red-Yellow Latosol of medium texture, Dystrophic Red-Yellow Latosol, clayey, and Typical Distroferric Red Latosol) and five compaction levels (50, 62.5, 75, 87.5 and 100% of maximum density) and found that the compaction did not alter the nitrogen uptake and the nitrogen content in bean plants, the best values being found at the density of 2.12 Mg m^{-3} in the Dystrophic Red-Yellow Latosol of medium texture.

Mandal et al. (1990), using three compaction levels (moderate - 1.79 kg dm^{-3}, highly compacted - 1.85 kg dm^{-3} and uncompacted - 1.52 kg dm^{-3}) in a sandy loam soil (680 g kg^{-1} sand, 240 g kg^{-1} silt and 80 g kg^{-1} clay), found that the loss of nitrate through leaching was three to four times lower in the highly compacted soil, as compared to the uncompacted soil.

Ferreira et al. (2000) proved the symbiosis efficiency in a experiment carried out with beans under field conditions, with Dystrophic Red Latosol. The results showed foliar nitrogen contents of inoculated plants similar to those found in nitrogen fertilization, which can be an indication that there was biological nitrogen fixation and that it was enough to meet the demand of plants for this nutrient (Martins et al., 2003).

For Figueiredo et al. (2008) and Meghvanshi et al. (2010), the success of the symbiosis depends on the compatibility with the cultivar analyzed, which enables the root invasion and thus provides nitrogen in a sufficient time to meet the plants' needs, a characteristic that was presented by the strains MT8 + MT15 in all variables analyzed.

The variable nitrogen accumulation in shoots of cowpea plants showed no difference between treatments, but the plants inoculated with the combination of the strains MT8 + MT15 provided increments of up to 5% when grown in uncompacted soil. Regarding the nitrogen accumulation in the grains, there was statistical difference between treatments. It was found that the inoculation with the combination of the strains MT8 + MT15 provided the best result in compacted soil, being verified the increase of 28.22% as compared to the control (Table 5).

The soil compaction negatively influenced the plants that received inoculation of the strain BR 3267, a reduction of 23.62% was observed in relation to the plants grown in uncompacted soil. Even with no significant difference, the treatment MT8 + MT15 showed higher nitrogen accumulation in compacted soil, where it was found, an increase of 16.36% as compared to the uncompacted soil.

Work conducted by Brito et al. (2011) showed that the biological nitrogen fixation provided most of the accumulated nitrogen in bean and cowpea plants, followed in descending order by the soil and the nitrogen

Table 6. Effect of inoculation with rhizobia strains on the crude protein in shoots and grains of cowpea grown in compacted and uncompacted soil.

Treatments	Compacted soil	Uncompacted soil
Crude protein in the shoots (mg. plant^{-1})		
BR3267	135.0^{Aa}	135.6^{Aa}
MT8+MT15	137.6^{Aa}	142.5^{Aa}
Control	140.9^{Aa}	133.6^{Aa}
CV(%) = 19.3		
Crude protein in the grains (mg. plant-1)		
BR3267	282.3^{Aa}	281.0^{Aa}
MT8+MT15	289.5^{Aa}	271.4^{Aa}
Control	269.9^{Aa}	269.8^{Aa}
CV(%) = 21.0		

Means followed by the same letter - uppercase, vertically, and lowercase, horizontally - do not differ by Tukey test at 0.05 significance level.

fertilization in an experiment carried out in a greenhouse, using the analysis of the isotopic content as a method. In an experiment carried out by Soares et al. (2006), the authors also found that the rhizobia strain INPA 03-11b provided the highest value for nitrogen accumulation in grains, in a field study, with a Typical Dystrophic Red Argisol. The results observed for the analysis of crude protein in the shoots and grains of cowpea were similar to the nitrogen content. The averages showed no significant difference, but the plants that received nitrogen fertilization and the combined inoculation of the strains MT8 + MT15 reached the highest values for these variables in compacted soil respectively (Table 6). In uncompacted soil, the strains MT8 + MT15 and BR3267 provided higher crude protein in shoots and grains, respectively, being found, a value 6.24 to 3.98% higher than those found in plants fertilized with nitrogen. This result confirms the data related to nitrogen content and yield found in studies by Ferreira et al. (2000), where the authors demonstrated that the bean does not depend on nitrogen fertilization when subjected to inoculation.

Thus, the strains studied in this test have demonstrated the ability to fix nitrogen, by promoting a result similar to that of plants fertilized with nitrogen (150 mg dm^{-3}).

Conclusion

The inoculation of the BR3267 strain contributed positively to the development and production of cowpea when grown on uncompacted soil, becoming more efficient in a management system that has the concern of preserving macropores of the soil. Inoculation with the combination of the MT8 + MT15 strains provided satisfactory results in both compacted soil and non-compacted soil favoring the production of cowpea in soil with a higher density.

Conflict of interest

The authors have not declared nay conflict of interest.

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