Original Research Article

# Liquid *Bacillus subtilis* Formulation in Rice for the Control of *Meloidogyne javanica* and Lettuce Improvement


**ABSTRACT**

**Aim:** To develop a liquid formulation based on *Bacillus subtilis*-34 using rice to evaluate the shelf life under refrigerated and room conditions and to evaluate the effect of different addition times of the formulation on the control of *Meloidogyne javanica* and growth of lettuce plants.

**Statistical Design:** The design was completely randomized, with five treatments and eight replicates. The results were submitted to analysis of variance and the means were compared by the Scott Knott test with 5% error probability.

**Location and Duration of the experiment:** The experiment was set up during the period from 02/13/2018 to 03/20/2018 in a greenhouse located at the State University of Montes Claros, municipality of Janaúba, MG, Brazil.

**Methodology:** Treatments consisted of drench in the substrate of tubes at 8 and 15 days; drench in the substrate of tubes at 8 and 15 days and in pot at 25 and 35 days; irrigation in pot only at 25 and 35 days after transplanting and 2 controls (Onix® and absolute control). All pots with plants were inoculated with 5000 nematode eggs. At 45 days of transplanting, the following nematological variables were evaluated: number of galls, number of egg mass, number of eggs per gram and reproduction factor, and agronomic variables fresh and dry biomass.

**Results:** Additions in the tube and pot and in the pot only were efficient for the reduction in the reproduction of *M. javanica* and for the improvement of lettuce plant growth.

**Conclusion:** *B. subtilis*-34 remains viable until 9 months in formulation stored under the refrigerator and up to 7 months under room conditions.



*Keywords: Lactuca sativa L; rhizobacteria; root-knot nematode, technology; shelf life*


## 1. INTRODUCTION

*Lactuca sativa* L. (Lettuce) is a vegetable economically important for Brazil, being cultivated in almost all regions of the country [1]. Lettuce is a good source of fibre, vitamins, especially B, A, C and K, low in calories and a rich source of pigments beneficial to human health [2]. In addition to the nutritional aspect, it is also a culture of great importance from the social point of view, being cultivated mainly by family farmers near large urban centers in the so-called "green belts" [3].

Lettuce has numerous phytosanitary problems, among which phytonematodes stand out. Nematodes of the genus *Meloidogyne* are considered as limiting factor of the commercial cultivation of several vegetables, since they have a short cycle and are always cultivated in the same area, favouring the increase in the nematode population. Losses caused by phytonematodes in vegetable crops are estimated at 12.3% in developed countries and 14.6% in developing countries [4]. Lettuce plants, attacked by nematodes, are less improved due to the dense formation of galls in the root system and their control is a difficult task.

Several strategies have been used for the control of nematodes, among them: crop rotation, solarization, use of resistant cultivars and chemical control. Most lettuce cultivars present high susceptibility to *Meloidogyne* species [5]. The species of this genus most important to lettuce and other leafy crops are *M. javanica* and *M. incognita* [6]. The damage caused by agrochemicals, crop rotation infeasibility in small areas and the cost of plastic for solarization evidences the necessity of the use of biological control agents, among them rhizobacteria [7].

Among rhizobacteria, the genus *Bacillus* stands out, which has the capacity to produce antibiotics, enzymes and toxins, which act directly, causing the mortality of juveniles and/or indirectly affecting their behaviour, feeding or reproduction. Plant-host recognition, resistance induction and/or plant growth promotion processes can also be performed [8]. In addition, *Bacillus* produces endospores, that survive under conditions of nutrient deprivation and of high temperature, which favours the maintenance of the viability of formulations [9].

Promising results have been obtained in the control of *M. javanica* by *B. subtilis* in banana and tomato plants [10]. *Bacillus* has been described for producing hydrolytic enzymes such as lipases, chitinases and proteases capable of degrading structural components of *Meloidogyne* [11, 12, 13].

The formulation of biocontrol agents such as rhizobacteria or another microorganism is an essential step for their commercial use [14]. Formulations should improve shelf life since biocontrol agents are living organisms. In addition, formulations should be economical and contain a sufficient number of viable colony forming units (CFUs) and be easily applied to soil or plants [15]. Several farmers have multiplied bacteria from organic products on their properties. Thus, the development of low-cost formulations for large-scale use is essential.

In this context, the aims of this work were to evaluate the application of a liquid formulation of *B. subtilis* produced in rice broth and determine its efficiency for the control of *M. javanica* and for the promotion of lettuce growth.

## 2. MATERIAL AND METHODS

### 2.1 Development of *Bacillus subtilis* formulation in rice broth and establishment of the growth curve

*Bacillus subtilis*-34 isolate maintained in mineral water in glass tubes under room conditions was used. From this suspension, a volume of 1.85 ml was collected. This suspension was placed in 1 liter erlenmeyers and the components were added in g.L$^{-1}$ of distilled water: 185 g of raw rice, 185 g of sugar, 55.55 g of sodium chloride (NaCl), 46.29 g of phosphate of potassium monobasic ($KH_2PO_4$). Chemical compounds, sugar, water and rice were autoclaved at 1.0 atm at 120 ° C for 30 minutes. The formulation had final pH of 7 (± 0.2).

For determination of the initial number of CFUs, a one-milliliter aliquot of the liquid formulation was submitted to a $10^{-5}$ dilution and 100 µL were collected for plating in Petri dishes containing Tryptic Soy Agar (TSA). Petri dishes were incubated at 25ºC for 24 hours when the initial number of CFU.mL$^{-1}$ was evaluated. For the establishment of the growth curve, the formulation was incubated for 44 hours on an orbital shaker at 28°C at 220 rpm and at 4-hour intervals; the same procedure was performed to determine the number of CFUs. The growth curve of the bacteria was also determined in Tryptic Soy Broth (TSB) medium, following the same methodology as that used for the liquid rice formulation.

## 2.2 Evaluation of different addition times of the liquid *Bacillus subtilis* formulation in rice broth in the control of *Meloidogyne javanica* and in lettuce improvement

The experiment was carried out in the greenhouse at the State University of Montes Claros, Janaúba, MG, with the following geographical coordinates (43 ° 16'18.2 "W and 15 ° 49'51.5" S) and an average altitude of approximately 540 m a.s.l.). For the evaluation of growth promotion and reduction of nematological variables, *B. subtilis*-34 bacterial isolate was used. The formulation was made as reported in item 2.1.

The isolate growth was interrupted 28 hours after the beginning of incubation. At this time, dilution at $10^{-5}$ and TSA plating were performed to determine the initial number of CFUs per ml. To evaluate the survival period of the bacterium in the liquid formulation, a volume of 50 ml was kept at room temperature in the laboratory of Phytopathology on the bench with mean temperature of 26.05°C (maximum of 29.1°C and minimum of 23°C) and another 50 ml kept in refrigerator at 9°C. At one-month intervals for a period of 10 months, $10^{-5}$ dilution and TSA plating were performed to determine the number of CFUs.

Lettuce seedlings 'Aurélia' cultivars were obtained from the sowing in styrofoam tubes containing Bioplant® substrate. After 17 days, seedlings were transplanted to 3-liter pots containing: substrate composed of soil (heavy clay, 26.6% silt, 60% clay, 13.4% sand, pH = 5) in 3:1 proportion, respectively, previously autoclaved at 1 atm and 120°C for 30 minutes, three times at 24-hour intervals. The substrate was fertilized as recommended for the culture. Prior to assay setup, the substrate was submitted to liming and incubated for 30 days.

The experiment was set up in a completely randomized design with five treatments and eight replicates. Treatments consisted of: T1- drench of the formulation to the substrate in tubes at 8 and 15 days, T2 - added via drench to the substrate of tubes at 8 and 15 days and in the pot at 25 and 35 days, T3- drench of the formulation to the soil of pots at 25 and 35 days after transplanting and 2 controls; T4-Onix® (Commercial product based on *Bacillus methylotrophicus* - Isolated UFPEDA 20) and T5- without bacterium addition and without commercial product. In Onix® treatment, each plant received 250 ml of the commercial product, previously diluted in water in the proportion of 4 mL.L$^{-1}$ one day after transplanting. Regarding the rice formulation, the volume used by addition in tubes and in pots, was two milliliters and 150 ml, respectively. At each addition, a new formulation was produced.

The inoculation of *M. javanica* was carried out 24 hours after the transplanting of seedlings to the pot, each one received 5 ml of suspension containing 5000 eggs and eventual J2 calibrated in Peters chamber, applied in three holes around the roots of each plant. At 45 days after transplanting, the number of galls per gram of root (NG / g), egg mass per gram of root (MO / g), number of eggs per gram of root (NO / g) were evaluated, as well as the reproductive factor, calculated by the following formula: FR = Pf / Pi, where Pf is the final nematode population and Pi is the initial population added to the plant [16]. To count the number of egg masses, roots were immersed in floxin B solution (150 mg.L$^{-1}$). The number of eggs was determined after root extraction [17, 18]. For the number of J2 in the soil, samples 200cm$^3$ were processed [19]. Eggs and J2 of *M. javanica* were quantified in Peters counting chamber in invert objective microscope.

The number of leaves, height, head diameter, fresh shoot mass, dry shoot mass and root weight were also evaluated. In order to determine the shoot dry mass, leaves were placed in paper bags, which were placed in a drying oven with forced air circulation at 65ºC for 72 hours. Data were submitted to analysis of variance and means were compared by the Scott-Knott test at 5% error probability. Statistical analysis was performed using the "Sisvar" software [20].

## 3. RESULTS AND DISCUSSION

### 3.1 Development of *Bacillus subtilis*-34 formulation in rice broth and establishment of the growth curve

Figure 1 and 2 show the growth curves of *B. subtilis*-34 in the liquid rice formulation and TSB, respectively. In the liquid rice formulation, it was observed that the bacteria remained in the adaptation phase up to 20 hours after plating. After 24 hours, the exponential growth phase begins, culminating with higher number of CFU (6.14x10$^8$) at 32 hours after incubation. From 36 hours, the decline phase began. The sudden drop in this value is justified by the depletion of nutrients in the culture medium and by the increase of toxic products from bacterial metabolism [21].

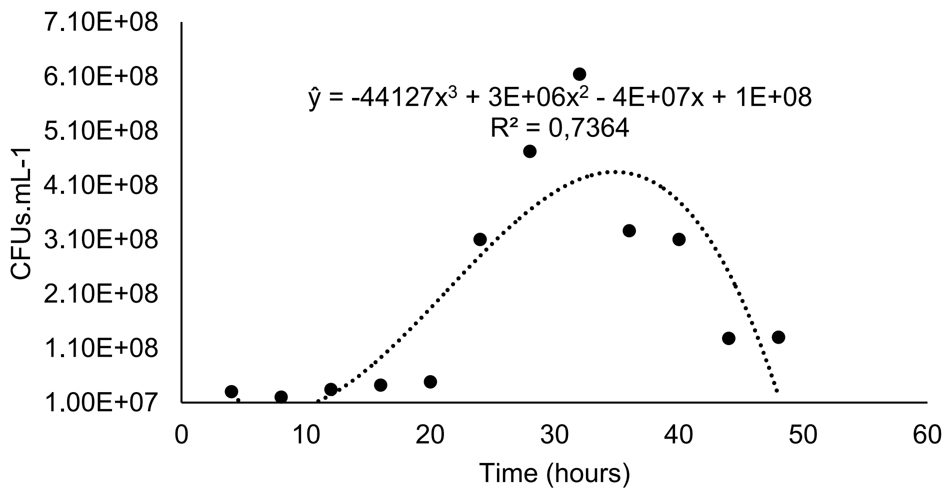


**Figure 1. Growth curve of *Bacillus subtilis*-34 in liquid rice formulation.**

In TSB, the highest number of CFUs occurred at 40 hours after incubation (6.41x10$^7$), that is, eight hours after the occurrence in the liquid formulation and with a difference in the number of CFUs of 5.5x10$^8$ in relation to the liquid rice formulation (Figure 2). For the greenhouse experiment, the formulation was incubated for up to 28 hours because it was already in the logarithmic growth phase. It is important to highlight that 28 hours from the start of incubation, the liquid rice formulation provided CFU of 4.72x10$^8$, while in the TSB medium, CFU was 3.49x10$^7$.

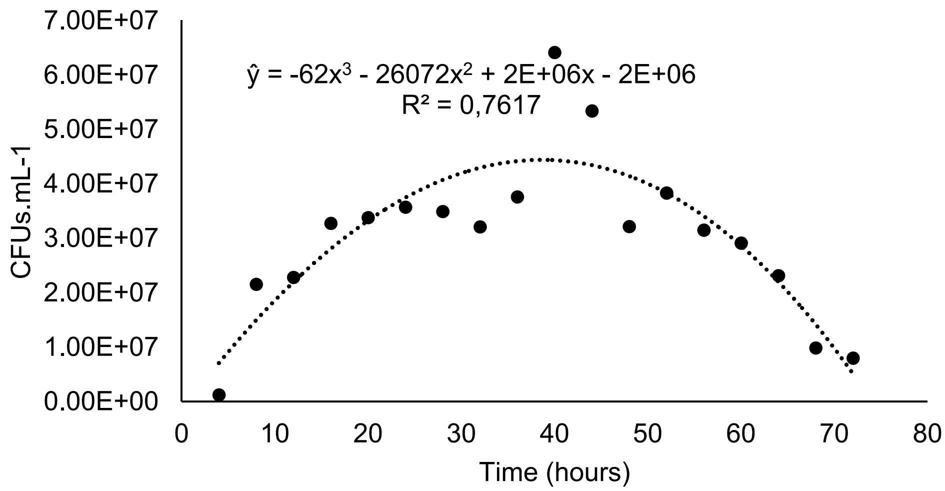


**Figure 2. Growth curve of *Bacillus subtilis*-34 in TSB medium.**

### 3.2 Evaluation of different addition times of the liquid *Bacillus subtilis* formulation in rice broth in the control of *Meloidogyne javanica* and lettuce improvement

Lettuce plants that received the liquid *B. subtilis*-34 formulation added to the pot and tube + pot showed a lower number of galls per gram of root and lower number of egg mass per gram of root (Table 1). The number of eggs per gram of root was significantly lower in all treatments that received the liquid *B. subtilis*-34 formulation compared to Onix® and control. The reproduction factor of *M. javanica* was lower in treatment of formulation drench added to the tube followed by addition in the pot and pot + tube. Addition in tube reduced the reproduction factor of the nematode by 30.55% and 36.07% in relation to Onix® and control, respectively (Table 1). Positive *B. subtilis* results in reducing nematode populations, mainly of *Meloidogyne* species, in plants such as rice, tomato and banana have been observed in other studies [22, 23, 24]. There was no occurrence of juveniles of the second stage in the soil in any of treatments; probably the J2 that hatched and infected the roots again.

**Table 1. Number of galls per gram root (NG/g), number egg mass per gram of root (EM / g), number of eggs per gram of root (NE / g) and reproduction factor (RF) of *Meloidogyne javanica* in lettuce inoculated by *B. subtilis*-34 via liquid formulation at different times**

| Treatments | NG/g | EM/g | NE/g | RF |
|---|---|---|---|---|
| Pot | 8.00a | 2.25a | 1,686.52a | 2.90b |
| Pot + Tube | 8.12a | 3.00a | 1,562.07a | 3.07b |
| Tube | 21.75b | 7.75b | 2,173.50a | 2.41a |
| Absolute control | 36.50c | 11.62b | 4,784.71b | 3.77c |

| | | | | |
|---|---|---|---|---|
| Onix® | 47.00c | 9.62b | 5,800.99b | 3.47c |
| Coefficient of variation | 41.85 | 61.54 | 40.00 | 15.37 |

Averages followed by the same letter in the column do not differ from each other by the Scott-Knott test at 5% error probability.

*Bacillus* species interrupt the nematode life cycle through the production of toxic metabolites that restrict their mobility, prevent juvenile hatching and penetration into plant roots [25]. Some authors have demonstrated reduction of the *M. incognita* population in tomato inoculated with *Bacillus* species [26], others have observed that metabolites produced by *B. subtilis* trigger hypersensitivity reactions in plant cells and affect oviposition, preventing nematode females from obtaining sufficient energy to produce eggs [27].

The genus *Bacillus* is described as one of the main microbial groups capable of acting in the control of phytopathogens through the synthesis of secondary metabolites, which, in general, present a wide range of inhibition to different phytopathogen species [28]. *Bacillus* secretes many secondary metabolites, including antibiotics, antifungals and siderophores. Metabolites produced by *Bacillus* may also affect the microflora in the rhizosphere, providing an environment antagonistic to pathogens, or may trigger host defence responses [29]. Cry proteins produced by *Bacillus* species are toxic to nematodes, both of free-living and phytoparasites and the production of proteases by this group of bacteria have been proposed as virulence factors in their pathogenesis against nematodes [30].

*Bacillus subtilis*-34 added by soil drenching and via pot + tube promoted higher number of leaves, head diameter and dry shoot mass significantly higher than additions in a tube, control and Onix® commercial product (Table 2). Additions in pot and pot + tube increased the number of leaves compared to control by about 80.02 and 73.56%. In relation to Onix®, the increase was 83.52 and 76.94%. Variable head diameter increased 81.98 and 75.42% when applying the formulation in pot and pot + tube, respectively, in relation to control; when compared with Onix®, the increase was 94 and 87.73%. In variable dry shoot mass, considering applications in pot and pot + tube, the increase was 21.74 and 17.94% in relation to control, and in relation to Onix®, the increase was 23.38 and 19.54%.

The height and fresh shoot mass of lettuce plants that received *B. subtilis*-34 in the pot were significantly higher than the other treatments, with an increase of about 119 and 322.22%, respectively, in relation to control. On the other hand, addition via pot and pot + tube provided an increase of 98.61 and 155% of root weight in relation to control and Onix® commercial product. The *in situ* effects by exposure of *B. subtilis* living cells may also lead to an increase in plant biometry [31], reflecting productivity gains, with the bacterium being commercially used for both purposes [32, 33].

**Table 2. Number of leaves (NL), height (H), head diameter (HD), fresh shoot matter (FSM) (g), shoot dry matter (SDM) (g) and fresh root matter (FRM) (g) of lettuce infected by *Meloidogyne javanica* and submitted to application of *Bacillus subtilis*-34 via liquid rice formulation at different times.**

| Treatments | NL | H (cm) | HD(cm) | FSM(g) | SDM(g) | FRM(g) |
|---|---|---|---|---|---|---|
| Pot | 34.87a | 7.12a | 41.62a | 76.00a | 11.87a | 8.62b |
| Pot + Tube | 33.62a | 6.25b | 40.12a | 64.12b | 11.50a | 10.50a |
| Tube | 26.25b | 5.00c | 28.12b | 23.50c | 9.87b | 6.25c |
| Absolute control | 19.37c | 3.25c | 22.87c | 18.00c | 9.75b | 4.37d |

| | | | | | | |
|---|---|---|---|---|---|---|
| Onix® | 19.00c | 3.62c | 21.37c | 17.12c | 9.62b | 3.37d |
| Coefficient of variation | 11.39 | 15.32 | 5.53 | 22.235 | 9.76 | 25.41 |

Averages followed by the same letter in the column do not differ from each other by the Scott-Knott test at 5% error probability.

*Bacillus subtilis* has been commercially used for the biocontrol of plant diseases and to increase crop yields [32, 33]. *B. subtilis* (PRBS-1) added to tomato plants reduced the reproduction of root-knot nematode and promoted the growth of plants under greenhouse conditions [34]. Nemathel® treated banana seedlings reduced reproduction of *Radopholus similis, Meloidogyne* spp., *Pratylenchus* ssp. and *Helicotylenchus* spp. with efficiency similar to nematicide Carbofuran [35]. Tomato plants that received *B. subtilis* additions showed higher shoot growth, characterizing the bacterium as a plant growth promoter, and this effect may be due, in part, to the production of plant phytoregulators in the rhizosphere [36].

### 3.3 Evaluation of the viability of liquid *Bacillus subtilis* rice formulation under room and refrigerator conditions.

The initial CFU was $4.72 \times 10^8$. Over the storage period under both conditions, oscillations in the concentration of bacterial cells were observed, sometimes with higher and sometimes with smaller values (Figure 3). At 4 months, the number of CFU of formulation stored under room and refrigerator conditions was $12.5 \times 10^8$ and $6.3 \times 10^8$, respectively. At 6 months, the number of viable cells was similar in both storage conditions 7.7 x $10^8$ at room temperature and 6.7 x $10^8$ at refrigerator temperature. At 7 months, it was verified that the number of CFUs remained approximately constant 7.8 x$10^8$ under room conditions, while under refrigeration conditions, reduction to 3.8 x$10^8$ UFC was observed, and this value remained approximately constant until 9 months.

From 8 months, the number of CFUs stored under room conditions becomes minimal, while in the refrigerator, the number of CFUs was approximately $4 \times 10^8$ at 9 months. The refrigerated environment extended the "shelf life" of the bacterium in two months. Low temperatures are generally used to preserve microorganisms by ensuring metabolism at low activity and preventing contamination with other microorganisms from affecting the stability of the biological control microorganism [37].

A biological control product to be economically viable needs to have minimum concentration of $1 \times 10^8$ CFU / mL with 85% viability [38], which was achieved by the liquid *B. subtilis*-34 formulation stored under room conditions for up to seven months and under refrigerator conditions for up to nine months.

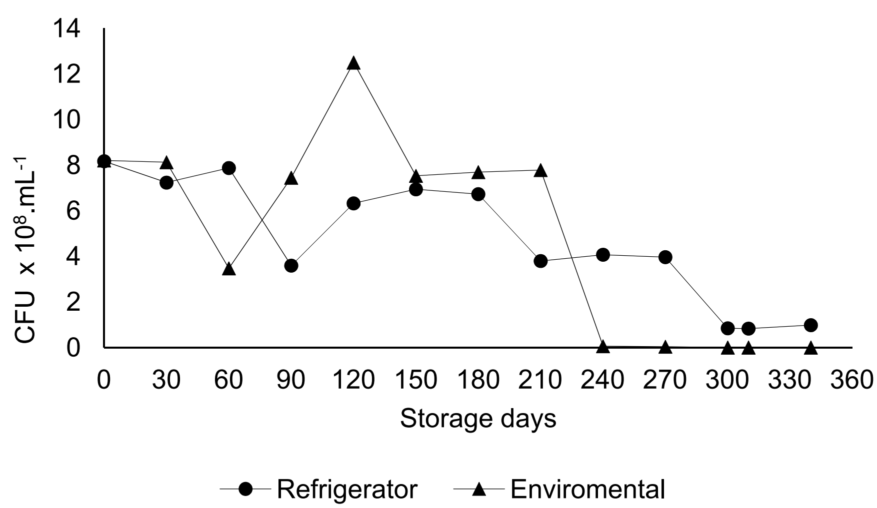

**Figure 3. Number of CFU of *Bacillus subtilis*-34 in liquid formulation stored under room and refrigerator conditions for twelve months.**

The results verified in the nematode control and in the promotion of lettuce growth demonstrate that the liquid bacterium formulation was effective despite the lower initial number of CFU.$mL^{-1}$ ($4.72x10^8$) compared to Onix, which had $1x10^9$ CFU. It also presented lower cost when compared to TSB synthetic culture medium since US$ 128.00 are necessary for the production of one liter of TSB, whereas the liquid rice formulation requires only US$ 11.64.

## 4. CONCLUSION

Higher promotion of lettuce growth and control of *Meloidogyne javanica* was were obtained by adding the liquid *Bacillus subtilis*-34 formulation twice to the soil in the pot. *Bacillus subtilis*-34 remains viable until nine months in formulation stored under refrigerator conditions and up to seven months under room conditions in northern state of Minas Gerais.

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