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TITLE OF THE ARTICLE WITH UP TO 35 WORDS: SHOULD MENTION THE STUDY DESIGN

Adriane da Fonseca DUARTE[1], Juliano Lessa Pinto DUARTE[1], Liliane Nachtigall MARTINS[1], Lucas Raphael DA SILVA[1], Nicole de Souza CUNHA[2], Fábio Pereira Leivas LEITE[2], Uemerson Silva DA CUNHA[1], Daniel BERNARDI[1]

[1] Department of Plant Health, Graduate Program in Plant Health, Universidade Federal de Pelotas, capão do Leão, Rio Grande do Sul, Brazil.
[2] Technological Development Center, Universidade Federal de Pelotas, Capão do Leão, Rio Grande do Sul, Brazil.

Corresponding author:
Adriane da Fonseca Duarte
adriane.faem@hotmail.com

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Abstract

Bacillus thuringiensis (*Bt*) Berliner has potential for use in insect management. Its use can be an alternative for the management of *Bradysia* aff. *ocellaris* (Comstock), considered one of the main strawberry pests in a soilless system. Therefore, the objective of this work was to evaluate the toxicity of different bacteria on *B*. aff. *ocellaris* in laboratory and greenhouse bioassays. The following isolates were used in the experiments: *Bacillus circulans* (*Bc*), *B. thuringiensis* var. *oswaldo cruzi* (*Bto*) or *B*. *thuringiensis* var. *israelensis* (*Bti*) and *B. thuringiensis* var. *kurstaki* (*Btk*) In the laboratory, *B.* aff. *ocellaris* larvae showed high susceptibility to *Bti* isolate (92 % mortality) 14 days after treatment exposure (DAET). In contrast, the isolates *Bc*, *Bto*, and *Btk* showed less than 32 % mortality, not differing from the control treatment (water – 22 % mortality). According to the concentration-response curves the values of lethal concentration LC_{50} and LC_{90} were 4 x 106 CFU.mL^{-1} and 4 x 10^{15} CFU.mL^{-1}. By applying *Bti* (4 × 10^{12} CFU.mL^{-1}) at the base of strawberry plants growing in plastic pots containing commercial plant substrate, a reduction of 26 % in the emergence of *B.* aff. *ocellaris* was observed. According to these results, the *Bti* isolate is considered promising for the formulation of bioinsecticides based on *Bt* for the management of *B*. aff. *ocellaris* in strawberry culture.

Keywords: Biological Control. *Bti*. Strawberry. Sustainable management.

1. Introduction

Species from the genus *Bradysia* Winnertz (Diptera, Sciaridae), popularly known as Fungus gnats, are considered to be one of the main groups of pest insects in protected cropping systems, especially mushroom, tobacco, onion, and strawberry crops (Cloyd 2008, Shamshad 2010, Zhang et al. 2014, Broadley et al. 2018). In Brazil, *Bradysia* aff. *ocellaris* is considered one of the main pests of strawberry cultivation grown in soilless systems (unpublished data), together with *Bradysia impatiens* (Johannsen 1912) which was also observed in strawberry plants in Japan (Arimoto et al. 2018).

Plant damage is caused by larvae that are responsible for causing damage to the roots, impairing the development of the root system and, consequently, interfering with the ability of plants to absorb water and nutrients, resulting in withering and stunted growth (San-blas et al. 2017; Ye et al. 2017). Likewise, injuries to the underground organs facilitate the entry of pathogens such as *Pythium, Botrytis,*

Verticillium, Fusarium, Thielaviopsis, Cylindrocladium, and *Sclerotinia* that accelerate the process of plant deterioration (Mohrig et al. 2012; Cloyd 2015; San-blas et al. 2017; Arimoto et al. 2018).

In Brazil, there are no chemical insecticides registered for this pest management. This fact means that in high gnat infestations, the control is carried out with insecticides recommended for the management of other pests that infest strawberry crop. Although these insecticides can contribute to the reduction of population levels and are compatible with other pest management strategies (Duarte et al. 2020; Duarte et al. 2021), the use of these chemicals should be avoided. Furthermore, the action of these insecticides is hampered by the location of the larvae inside the soil and because they can select resistant populations (Moreira and Moraes 2015; Chen et al. 2017).

Within this context, it is believed that the use of entomopathogenic bacteria can be considered an alternative for the management of *B.* aff. *ocellaris* (Ben-Dov 2014; Cloyd and Dickinson 2006; Frankenhuyzen 2013; Wang et al. 2019), as it was already testes with the use of entomopathogenic nematodes (EPNs) (San-blas et al. 2017), predator mites (Freire et al. 2007; Castilho et al. 2009), plant extracts (Erler et al. 2009), and entomopathogenic fungi (Andreadis et al. 2016).

Bacillus thuringiensis (Berliner) is a soil bacterium that produces specific Cry or Vip (δ-endotoxin) proteins against various species of insect pests (Schnepf et al. 1998; Frankenhuyzen 2009; Cossentine et al. 2016). It is a Gram-positive, aerobic bacterium with its inclusion body composed of Cry proteins (Insecticidal Crystal Proteins) that have specific insecticidal activities on the target of action (Schnepf et al. 1998; Bravo et al. 2007; Soberón et al. 2013) through the formation of pores in the cell membrane of infected insects and subsequent cell lysis and insect death (Crickmore et al. 1998; Federici 2006; Frankenhuyzen 2013).

Unlike Cry proteins, Vip proteins are exotoxins produced and secreted during the vegetative growth phase of *B. thuringiensis* and have a different mode of action than Cry proteins which are produced during the sporulation phase of *B. thuringiensis* (Estruch et al. 1996). This results in the formation of pores with unique properties, showing low potential for cross-resistance (Sena et al. 2009; Gouffon et al. 2011).

Studies have shown that several species of dipterans are susceptible to *B*. *thuringiensis*, such as *Anastrepha ludens* (Loew) (Toledo et al. 1999; Buentello-Wong et al. 2015), *Bactrocera oleae* (Gmelin) (Alberola et al. 1999; Ilias et al. 2013), *Ceratitis capitata* (Wiedemann) (Aboussaid et al. 2010), *Bactrocera cucurbitae* (Shishir et al. 2015) and *Anastrepha fraterculus* (Wiedemann 1830) (Diptera: Tephritidae) (Martins et al. 2018), *Drosophila suzukii* (Matsumura) (Cossentine et al. 2016), *Zaprionus indianus* (Diptera: Drosophilidae) (Geisler et al. 2019), *Bradysia* sp nr. *coprophila* (Cloyd and Dickinson 2006) and *Bradysia difformis* (Wang et al. 2020). Given this, our study aimed to assess the toxicity of different bacterial isolates (*Bt*) on *B*. aff. *ocellaris* in laboratory and greenhouse conditions.

2. Material and Methods

Insects rearing

The colony of *Bradysia* aff. *ocellaris*, was established with larvae and adults collected in a plastic greenhouse (8 x 10 x 4 m, width, length and height respectively), with strawberry cultivation (*Fragaria* spp.) of the Aromas cultivar (31º48'10.8"S, 52º25'06.24"W), Capão do Leão, Rio Grande do Sul State, Brazil. The insects were maintained in plastic cups (250 mL) containing a mixture of potato pieces, ground black beans, and moist peat moss, which were kept in plastic containers (30 x 20 x 18 cm, height, weight, and depth), which were moistened once a week. Lateral openings were made in the plastic containers for air circulation. *Voil* tissue was used in the openings to prevent the escape of adult insects. Second and third instar larvae were used in all bioassays.

Bacterial strains

Strains used in the bioassays were *Bacillus circulans* (*Bc*)*, B. thuringiensis* var. *israelensis* (*Bti*), *B. thuringiensis* var. *kurstaki* (*Btk*), and *B. thuringiensis* var. *oswaldocruzi* (*Bto*) obtained from the Microorganisms Collection of the Microbiology and Parasitology Department of the Biology Institute, at

Federal University of Pelotas, Rio Grande do Sul, Brazil. Strains were recovered and multiplied in brain heart infusion (BHI-Acumedia) type culture medium, incubated at 28° C, and subcultivated in 1-liter glass flasks, containing 200 ml of media plate on NYSM agar (Nutrient Broth, Yeast extract, $MnCl_2$, $MgCl_2$, and $CaCl_2$) as Yousten (1984). When approximately 90 % of each culture medium had developed spores, the flasks were heated in a water bath at 80 °C for 15 min to eliminate vegetative cells. The average concentration of spores was determined as Colony Forming Units (CFU. ml^{-1} on BHI agar). Later, the bacterial cultures for each strain were stored at 4°C until the bioassays were performed.

Strawberry plants

The strawberry plants with bare-root (Aromas cultivar) were purchased in local floriculture and maintained in greenhouse conditions, where prophylaxis was performed using abamectin spray (0.70 $mL.L^{-1}$ concentration of the active ingredient). After the security period, each bare-root plant was transplanted into a plastic pot, containing 300 g of a commercial substrate (Macplant®). At the moment of transplant, 50 mL of water was added to the substrate. The pots were maintained in laboratory conditions, with an average temperature of 25.2 ºC.

Bioassays

Bioassays were conducted according to the methodology proposed by Chen et al. (2017), Zhang et al. (2014), and Taylor, Willey and Noblet (2007), with modifications which will be mentioned next.

Biological activity against *B*. aff. *ocellaris* larvae

To evaluate the effect of different bacterial strains (Table 1) on the fungus gnats larvae, the bacteria was incorporated directly into the diet (potato pieces). The bioassays were conducted in a completely randomized design, with fifty larvae by treatment. The experimental unit consisted of a transparent plastic Petri dish (2.7 cm diameter, 1.2 cm high), whose base was covered with three filter paper discs, which were moistened with 0.1 ml distilled water, each 48 h. All strains were prepared using distilled water, which was the control treatment. Two potato pieces, with approximately 0.5 cm^2, were put in each experimental unit and covered with 0.1 mL of one of the solutions. Distilled water was used to cover the potato pieces in the control treatment. Five larvae were transferred to each experimental unit. Each one of the larvae was considered as a single replicate, with ten replicates for each treatment (fifty larvae per treatment in total). The evaluations were performed daily for 14 days and larvae were considered dead if they were unable to move when touched by a moist brush.

Table 1. Bacterial strains and their concentrations used in the first bioassay.

Treatment	Concentrations $CFU.ml^{-1}$
Bacillus circulans	4×10^{10}
Bacillus thuringiensis var. *israelensis (Bti)*	4×10^{12}
Bacillus thuringiensis var. *kurstaki (Btk)*	8×10^{12}
Bacillus thuringiensis var. *osvaldocruzi (Bto)*	2×10^{11}
Water/Control	-

Concentration-response curves of the active treatments on *B*. aff. *ocellaris* larvae

Based on the results from this initial test, the most promising treatment (*Bti*) was further evaluated to estimate the concentration required to kill 50 % and 90 % of exposed flies [Lethal concentration (LC), LC_{50} and LC_{90}, respectively]. To evaluate this, different concentrations of *Bti* were incorporated into the diet according to the previous bioassay. Therefore, seven concentrations 4×10^{12}, 4×10^{8}, 4×10^{7}, 4×10^{6}, 4×10^{5}, 4×10^{4}, and 4×10^{3} $CFU.mL^{-1}$ were prepared and spilled over the potato pieces. Five larvae were transferred to each experimental unit. Each larva was considered a single replicate, with sixteen replicates

for each treatment (eighty larvae per treatment in total). The evaluations were performed daily for 14 days and the larvae were considered dead if they were unable to move when touched by a moist brush.

Toxicity of *B. thuringiensis* against *B*. aff. *ocellaris* larvae under greenhouse conditions

This bioassay was conducted in greenhouse conditions with an average temperature of 25.2 ºC and 63 % relative humidity. The experimental unit consisted of a pot containing one strawberry plant, each pot was isolated inside plastic cages. Lateral openings covered with voil tissue were made in the plastic cages for air circulation. The treatments were assembled as follows: T1- artificial inoculation of the larvae and *Bti* solution; T2- inoculation of larvae only; T3- no inoculation, to evaluated possible bare-root plant contaminations. Ten days after transplant, twenty larvae of *B*. aff. *ocellaris* were transferred to the substrate inside the pots containing strawberry plants. Ten pots were used per treatment and each pot was considered one replicate, totalizing 200 larvae per treatment. Twenty-four hours after the larvae inoculations, a second inoculation of 6 mL of *Bti* (4×10^{12} CFU.mL^{-1}) was performed in the treatment with *Bti*. In the other treatments, an equal amount of distilled water was inoculated.

Two yellow adhesive traps (2.5 x 5 cm) were fixed at the top of each experimental unit. The efficiency was evaluated by monitoring the emergence of the insects in the 14 days after the inoculation of larvae when the traps were removed to enable us to count the number of adult fungus gnats stuck to their surfaces.

Statistical analysis

The normality and homoscedasticity of the data were evaluated by Shapiro–Wilk and Bartlett tests, respectively. For the first bioassay, as data did not fit the assumptions for parametric analyses, they were compared by non-parametric tests, using the Kruskal–Wallis test followed by Dunn's test for multiple comparisons between treatments ($p < 0.05$). For the second bioassay, the LC_{50} and LC_{90} were calculated with probit analysis, using the gama ecotoxicology package (Carvalho et al. 2017), compared based on the confidence limit (95 %) and the slope of the line relating probit mortality calculate to the log dose, according to (Carvalho et al. 2017). For the third bioassay, the number of emerged flies were evaluated by variance analysis, followed by the Dunn test ($p < 0.05$), since they did not fit the statistical assumptions. All analyses were performed using the statistical software "R", version 3.5.2 (R Development Core Team 2018).

3. Results

Biological activity against *B.* aff. *ocellaris* larvae

The mortality rate (%) of *B.* aff. *ocellaris* larvae significantly differed from the control group ($\chi^2 = 27.28$; df = 4; $p = 1.74 \times 10^{-5}$) (Figure 1). *B. thuringiensis* var. *israelensis* (*Bti*) treatment provided the higher mortality rate (92 %), differing from all other treatments ($p \leq 0.0059$). *B. circulans, Bt. kurstaki, Bt. osvaldocruzi* and the control group did not differ from each other ($p \geq 0.05$).

Concentration-response curves of the active treatments on *B*. aff. *ocellaris* larvae

The concentration-response curves were evaluated only for *Bti*. By using the curves of lethal concentrations, it was possible to infer the LC_{50} and LC_{90} doses for this strain. In order to control fifty percent of the larvae in 14 days after the exposure to *Bti*, it is necessary a concentration of 4×10^6 CFU.mL^{-1}, while to control ninety percent of the larvae it is necessary a concentration of 4×10^{15} CFU.mL^{-1} (Figure 2).

Toxicity de *B. thuringiensis* against *B*. aff. *ocellaris* larvae under greenhouse conditions

The control efficiency in this bioassay was reduced when compared to the previous ones (36 % compared to 92 % in the first bioassays), although significant differences were found ($p < 0.001$).

In the treatment with *Bti* inoculation, the average number of emerged adults 14 days after the release was 14.8, while in the treatment without *Bti* inoculation, the emergence was 19.3, differed significantly between them ($p = 0.014$) and of the control group ($p = 0.006$) (Figure 3). Nevertheless, in the control groups, no adult were expected, indicating that the bare-root plants are acquired contaminated, demanding preventive control.

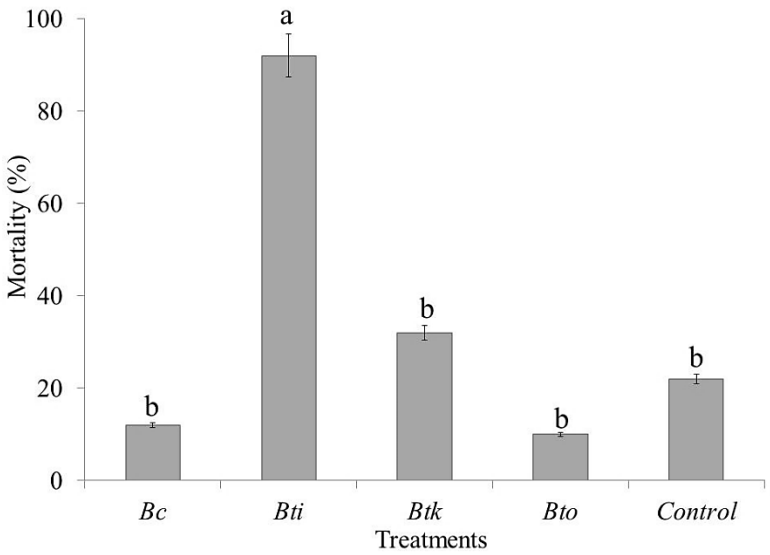

Figure 1. Mortality (± standard error) of *Bradysia ocellaris* larvae, when fed a potato pieces containing *Bacillus (Bc: B. circulans; Bti: B. thuringienses var. israelensis; Btk: B. t. var. kurstaki; Bto: B. t. var. osvaldocruzi)* and control: water, at 14 days after treatment. Significant by the variance analysis and by the Kruskal-Wallis test. Values followed by same letters in the column did not differ significantly (Dunn test $p < 0.05$).

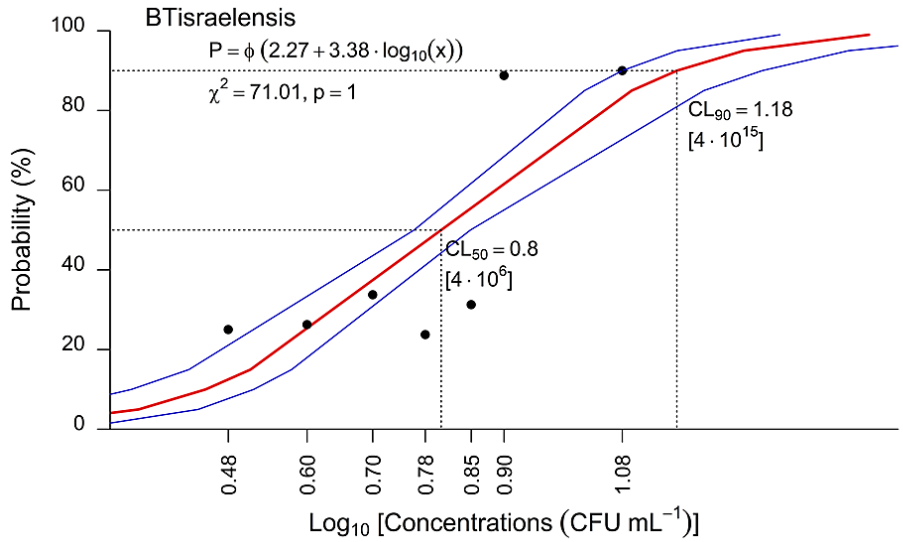

Figure 2. Concentration-mortality curves of *Bacillus thuringiensis israelensis (Bti)* on second/third instar of *Bradysia ocellaris* larvae. Temp.: 25 ± 1ºC, RH 70 ± 10% and in the dark. Blue line is the confidence limit.

4. Discussion

Our study showed the potential use of *Bacillus thuringiensis* var. *israelensis* for the control of *B*. aff. *ocellaris* populations. The isolate *B. thuringiensis* var. *israelensis* (*Bti*) presented the higher toxicity over

second and third instar *B.* aff. *ocellaris* larvae, opposing the studies performed with *B. coprophila* (Cloyd and Dickinson 2006). This difference in the results is probably due to the different methodology used in both studies. Cloyd and Dickinson (2006) allowed the larvae to be exposed to *Bti* for 72 hours. Considering that the toxin acts in the digestive system (Ben-Dov 2014), the greater the exposure to the toxin, the greater the consumption of food contaminated with the bacteria, and therefore, the greater should be the impact on the larvae metabolism (Taylor et al. 2007).

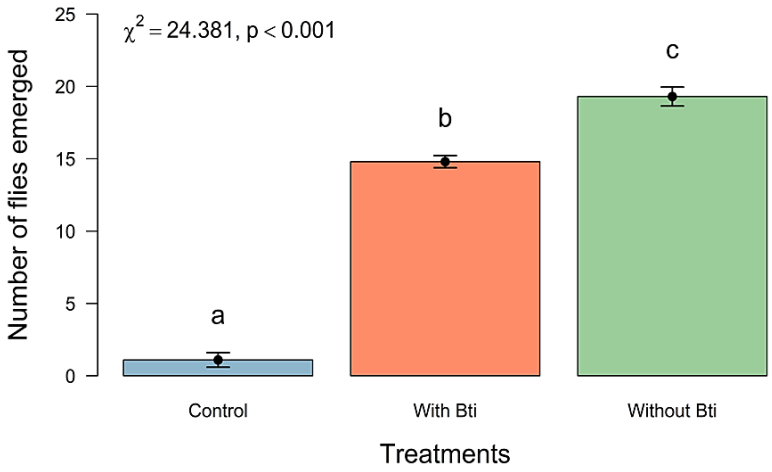

Figure 3. Number (± standard error) of *Bradysia ocellaris* adults emerged in pots of the strawberry plants, at 14 days after the control with and without release of *Bacillus thuringiensis israelensis* (*Bti*) (4×10^{12} CFU.mL^{-1}).

The susceptibility of larvae of dipteran species to *B. thuringiensis* isolates has been demonstrated in several studies (Aboussaid et al. 2010; Molina et al. 2010; Ilias et al. 2013; Shishir et al. 2015; Buentello-Wong et al. 2015; Cossentine et al. 2016; Geisler et al. 2019; Martins et al. 2018), however, for sciarids and some mosquitoes, such as *Aedes*, *Culex*, and *Anopheles*, it is known that some endotoxins are more specific and effective in control (Ben-Dov 2014). Nevertheless, they act similarly to what occurs in lepidopterans. The ingested crystals dissolve in the alkaline intestinal environment releasing soluble proteins (Bravo et al. 2007).

Bacillus thuringiensis is an effective agent for managing insect pests due to its production of multiple toxins (Cyt and Cry) with different modes of action, which synergize combined effects to increase toxicity to the control target (Vidal-Quist et al. 2010; Soberón et al. 2013). In the present study, it was not determined which toxins caused the mortality of *B*. aff. *ocellaris*. However, studies are being conducted to identify which toxins are expressed by the *Bti* isolate based on models proposed by Sauka et al. (2014).

In addition to the high toxicity showed in laboratory bioassays, the *Bti* isolate significantly reduced the emergence of *B*. aff. *ocellaris* when applied on the base of strawberry plants infested with *B*. aff. *ocellaris*. This aspect demonstrates the ability of the isolate to adapt and colonize the application environment. This result is of paramount importance for field applicability. In Brazil, there are still no registered products for the management of this pest insect (Brasil 2020), but the predatory mite *S. scimitus* has demonstrated its potential for use in strawberry cultivation (Duarte et al. 2021,). In other countries, the use of synthetic insecticides via dripping by the irrigation system is a strategy used for the management of some species of *Bradysia* (Diptera, Sciaridae) (Cloyd and Dickinson 2006; Cloyd 2008).

Although the results have been satisfactory, further studies must be carried out in field conditions in order to verify the toxicity of these formulations over time under different thermal and light conditions, factors that can accelerate the degradation of bacterial isolates (Navrozidis et al. 2000; Zahiri et al. 2002; Molina et al. 2010). Considering that in the soilless system, the support base for the substrate may be either tubular plastic packaging, fiber-cement tiles, PVC or wooden gutters, where the substrates remain

with high humidity, these conditions can provide an ideal environment for the multiplication and colonization of *B. thuringiensis* inside the substrate.

Furthermore, our observations in the greenhouse bioassay showed that the bare-root plants were acquired contaminated. Cloyd and Zaborski (2004) found similar results with *Euphorbia pulcherrima* Willd cuttings containing *B. coprophila* larvae. Considering that, greenhouse producers should use prophylactic applications before the transplant. Moreover, it may be a good strategy for the wholesale producer to treat the cuttings, seedlings, or bare-root plans. Biological agents such *Bti,* for example, have short residual activity and need to be applied frequently in order to provide long-term control (Osborne et al. 1985), but it is interesting mainly in the cuttings, seedlings, plug plants and bare-root plants.

Still, further studies must be done to determine the type of endotoxin produced by this *B. thuringiensis* isolate, in order to verify which toxin has the greatest toxic effect on the control target. That would contribute to reduce losses in production fields since there is no product registered within the Ministry of Agriculture, Livestock and Supply (MAPA) for the control of this insect in Brazil.

5. Conclusions

Among the evaluated isolates of *Bacillus* spp, *Bti* was the only isolate that provided significant mortality under laboratory conditions, however, further studies are necessary under greenhouse conditions.

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Ethics Approval: Not applicable.

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