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Fruit collapse incidence and quality of pineapple as affected by biopesticides based on *Pseudomonas fluorescens* and *Trichoderma harzianum*

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Abstract: In this study the effect of *Pseudomonas fluorescens* and *Trichoderma harzianum* based biopesticides on fruit collapse disease incidence and pineapple quality was investigated. The experiment was implemented in a split-plot design with two factors, one involving two inoculation methods (spray and inject), and a second factor involving four treatments, A (control: no biopesticides used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest) and D (Bio P32 + Bio T10 from 13 weeks before harvest). The inoculated pathogen was *Dickeya zeae*. The incidence of fruit collapse, total soluble solids, total acidity, sucrose, ascorbic acid, mineral content, and electrolyte leakage were determined. The inject method caused more fruit collapse incidence than the spray method. Treatments C and D provided the best results having a low incidence of fruit collapse (spray: 5 and 1.7 %, inject: 20 % in both cases), high antioxidant capacity (regarding ascorbic acid), high mineral nutrient content (in terms of Ca and Mg), and low electrolyte leakage content (< 70 % in average), with a healthier cell wall characteristic. Meanwhile, treatments A and B were less efficient in these aspects and promoted the incidence of fruit collapse, especially when the inject method was used, as this was more harmful regarding the fruit physiology. In conclusion, the biopesticides employed can reduce the incidence of fruit collapse and positively affect the fruit quality.

Key words: biopesticide; *Dickeya zeae*; fruit quality; disease incidence; *Pseudomonas fluorescens*; *Trichoderma harzianum*

Vpliv uporabe biopesticidov na osnovi bakterije *Pseudomonas fluorescens* in glive *Trichoderma harzianum* na propad in kakovost plodov ananasa

Izvleček: V raziskavi je bil preučevan učinek uporabe biopesticidov na osnovi vrst *Pseudomonas fluorescens* in *Trichoderma harzianum* na propad in kakovost plodov ananasa. Poskus je bil izveden kot faktorski poskus z deljenkami, kjer je prvi dejavnik obsegal dva načina vnosa patogena (pršenje in injeciranje), drugi pa naslednja štiri obravnavana: A (kontrola: brez uporabe biopesticidov), B (uporaba Bio P32 13 tednov pred pobiranjem), C (uporaba Bio T10 13 tednov pred pobiranjem) in D (uporaba Bio P32 + Bio T10 13 tednov pred pobiranjem). Inokuliran patogen je bila bakterija *Dickeya zeae*. Po obravnavanjih so bili določeni naslednji parametri: pojav propada plodov, vsebnost topnih snovi v plodovih in njihova celukopna kislost, vsebnost saharoze, askorbinske kisline in mineralov ter puščanje elktrolitov iz plodov. Injeciranje patogena je povzročilo večji propad plodov kot pršenje. Obravnavanji C in D sta dali najbojše rezultate z najmanjšim propadanjem plodov (pri pršenju 5 in 1,7 %, pri injeciranju 20 % v obeh primerih), veliko vsebnostjo antioksidantov (vsebnost askorbinske kisline), mineralov (kot vsebnost Ca in Mg), manjšo vsebnost elektrolitov v iztoku (v poprečju manj kot 70 %) in bolj zdrave celične stene. Obravnavanji A in B sta bili glede na prej naštete parametre manj učinkoviti in sta pospešili propadanje plodov, posebej še pri injeciranju patogena, kar je bilo tudi bolj škodljivo glede na fiziološke lastnosti plodov. Zaključimo lahko, da uporaba biopesticidov zmanjša propadanje plodov in pozitivno vpliva na njihovo kakovost.

Ključne besede: biopesticidi; *Dickeya zeae*; kakovost plodov; pojav bolezni; *Pseudomonas fluorescens*; *Trichoderma harzianum*

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1 INTRODUCTION

Pineapple diseases are common problem affecting fruit quality, with infections usually beginning in the field and before harvest (Rohrbach and Johnson, 2003; Sipes and Pires de Matos, 2018). Fruit collapse is caused by the bacterium *Dickeya zeae* (formerly *Erwinia chrysanthemi* (Peckham et al., 2010; Sueno et al., 2014), which is characterized by exudation of sap and gas in the form of bubbles, an olive-green skin color and cavities within the skeletal fibers that show up in the flesh of the fruit (Aeny et al., 2020; Cano-Reinoso et al., 2021). *D. zeae* can infect the plant via infection vectors coming from the field, such as already infected plants, ants, beetles, and flies that attack during flower induction, or directly affecting the developed fruit when high temperatures weeks before harvest increase transpiration and allow the bacterium to penetrate directly through the stomata of the skin (Pires de Matos, 2019; Cano-reinoso et al., 2021). For this reason, fruit collapse symptoms usually occur just before harvest or during postharvest handling, as *D. zeae* can remain latent for a long time (Rohrbach and Johnson, 2003; Pires de Matos, 2019).

Recently, low acidity pineapple hybrids have been reported to be more susceptible to this disease (Soteriou et al., 2014; Cano-Reinoso et al., 2021). Currently, these hybrids are the most commonly exported by the industry as fresh fruit because they are attractive to consumers (Chen et al., 2009; Kleemann, 2016). Therefore, a solution must be found to address this problem. In addition, the solution should preserve the quality of the fruit and protect the environment, as the use of chemical pesticides is expected to be reduced worldwide in the near future. In this context, the use of biopesticides presents itself as an alternative, as they are environmentally friendly and easier to apply. These can interact with the plant and fruit during development through the plant stomata, lenticels, and natural cracks, and can also be applied after harvest (Soesanto et al., 2011, 2018).

Bio P32 is a biopesticide derived from *Pseudomonas fluorescens*, a strain of *P. fluorescens* isolated from the rhizosphere of wheat. Bio T10 is another biopesticide base on *Trichoderma harzianum*, a soil-borne fungus used for biological control of plant pathogens (Soesanto et al., 2011, 2018). These biopesticides have been widely used on various crops, both preharvest and postharvest, to reduce the incidence of bacterial diseases and improve crop characteristics, such as dragon fruit (Hamarawati et al., 2017), eggplant (Soesanto et al., 2011), and cucumber (Soesanto et al., 2020). However, since few studies has been reported on the effect of these products on fruit collapse and pineapple quality, this experiment aims to evaluate the effect of *Pseudomonas fluorescens* and *Trichoderma harzianum* based biopesticides on fruit collapse disease incidence and pineapple quality. This will involve a comparison of different inoculation methods that represent how *D.zeae* infect the plant during flowering or in the weeks just prior to harvest, and investigating different variables that could characterize the optimal fruit traits for future consumption in a low acid hybrid.

2 MATERIAL AND METHODS

2.1 EXPERIMENT DESIGN AND TREATMENTS

The research was set in pineapple fields located in Lampung, Sumatra island of Indonesia, between February and June of 2020. A pineapple low acid hybrid (MD2) was used for this experiment. MD2 is known for its exceptional sweetness, consistency and uniformed size at harvest; currently is one of the most exported fresh cultivars, with a price tree times higher than any acid hybrid (Bin Thalip et al., 2015). The fruits were harvested in 148 days after flowering, considered an optimal time in MD2 to obtain the best physico-chemical characteristics for a future consumption (Bin Thalip et al., 2015; Ding and Syazwani, 2016).

The soil was previously fertilized with 200 kg ha^{-1} of di-ammonium phosphate, 1000 kg ha^{-1} K_2SO_4, and 200 kg ha^{-1} kieserite crystal. Several foliar applications were carried out after the planting using 700 kg ha^{-1} urea, 700 kg ha^{-1} $(NH_4)_2SO_4$, 1000 kg ha^{-1} K_2SO_4, 170 kg ha^{-1} $MgSO_4$, 60 kg ha^{-1} $FeSO_4$, 60 kg ha^{-1} $ZnSO_4$, in intervals of 30 days during three months; besides, after flowering borax was sprayed on the plant in doses of 30 kg ha^{-1}. The pedological and mineral characteristic of the soil where the experiment was implemented are presented in Table 1. Furthermore, during the experiment, a weather station (LSI Lastem; equipped with a CR6 data logger from Campbell Scientific; Italy) measured an average relative humidity (RH) of 89.34 %, an ambient temperature of 26.8 °C, solar radiation of 16.83 w m^{-1}, and a monthly average rainfall of 133.77 mm.

The experiment was arranged in a split-plot design, with two factors. One factor concerning two methods of inoculation of *D. zeae* bacterium and a second factor about four treatments implemented. Each treatment had three replications with 44 fruits. Field rows where the treatments were administrated consisted of 0.4 m width and 3.75 m length. Pineapple plants were arranged in two lines of 22 plants in the rows with a separation of 0.25 m. Observations were carried out once every two weeks, from six weeks before harvest. The arrangement of the experiment factors used with their respective characteristics are presented in Table 2.

Table 1: Pedological and mineral nutrients characteristics of the soil in the experiment

Texture	
Clay (%)	18.56
Loam (%)	13.01
Sand (%)	68.43
Chemical composition	
pH (H_2O)	6.8
C (%)	0.7
N (mg kg^{-1})	800
P (mg kg^{-1})	43.75
K (mg kg^{-1})	319.8
Ca (mg kg^{-1})	638
Mg (mg kg^{-1})	235.2
Na (mg kg^{-1})	4.6

*The N, P, K, Ca, Mg and Na represent the available mineral nutrients content in the soil

Table 2: The organization of the experiment design employed in the research

Factor one (Inoculation method)
1. Spraying before the open-heart stage.
2. Injection into the fruit flesh from six weeks before harvest.
Factor two (Treatments)
A. Control (No biopesticides used)
B. Bio P32 from 13 weeks before harvest
C. Bio T10 from 13 weeks before harvest
D. Bio P32 + Bio T10 from 13 weeks before harvest

The control had only inoculated the bacterium for each of the methods used (sprayed or injected, respectively). For both inoculation methods, juice of previously infected fruits extracted from the flesh (including *D. zeae*) was employed. For the spray method, doses of 20 ml juice/plant-fruit were employed using a hand sprayer. These doses were selected after field trails before the beginning of this experiment demonstrated that with their employment the fruits exposed symptoms of fruit collapse just after flowering. Also, those trials proved that sprays applications during flowering were more effective to cause fruit collapse than injections. The plants were sprayed at night, in two and one week before flowering and one week thereafter (13, 12 and 11 weeks before harvest). The sprayings moment tried to represent the typical field infection during flower induction, where a latent bacterium in the environment enters the plant through the nectarthodes (Wang et al., 2011; Sipes and Pires de Matos, 2018).

On the other hand, for the inject method were administrated doses of 0.2 ml juice/fruit with a syringe. These doses were implemented after previous field trials exposed that with these doses a fruit can present fruit collapse symptoms during advance stage of development, close to harvest. Also, these doses were employed by Barral et al. (2017). They demonstrated that injections with these doses in pineapple are enough to inoculate a disease before harvest. Moreover, these trials demonstrated that for an advance fruit development, *D. zeae* inoculations with injections were more effective than sprayings. The sprayed inoculations on the plant were administrated in six, four, and two weeks before harvest. For this method, four eyes of the pineapple shell were inoculated by pushing a syringe through them. Two eyes were inoculated on the upper part and two on the lower part of the shell, similar to the technic described in Barral et al. (2017). The inoculation time selected for the inject method intend to replicate another typical moment of infection by *D. zeae*, in this case close to harvest, entering through the shell stomata, as described in Sipes and Pires de Matos (2018).

Concerning the biopesticides applications, from 13 to 10 weeks before harvest, those were employed weekly. Later after ten weeks, those were applied one time every two weeks until harvest. Bio P32 [in 1 l of product solution: 10 % of snail meat, 2 g of fermented Shrimp, and 10 ml of *P. fluorescens* - (10^{12} cell ml^{-1}) - strain 32] and Bio T10 [in 1 l of product solution: 10 g of rice flour and white sugar, and 10 ml of *T. harzianum* (10^8 conidia ml^{-1}) - strain 10] were used in doses 20 ml/per plant-fruit (v/v: 20 ml l^{-1}) during night time. Furthermore, where the fruits had an advance maturation, the biopesticides were not only sprayed in the leaves, also directly into the shell and crown, understanding that the stomata and lenticels available in those structures could permit their absorption and assimilation, as recommend by Soesanto et al. (2011) and (2020).

2.2 DETECTION OF THE TOTAL SOLUBLE SOLIDS (TSS), TOTAL ACIDITY (TA) AND FRUIT COLLAPSE INCIDENCE

The TSS and TA were calculated following the procedures described in Shamsudin et al. (2020), in a composition of four fruits per replication of each treatment arranged. TSS was measured by implementing a handheld refractometer (MASTER-53 α; Atago: Japan), while the TA was detected by titration to pH 8.1 with 0.1 M NaOH using phenolphthalein as an indicator and revealed as a percentage of citric acid. The incidence of fruit collapse was measured by detecting and collecting the percentage of fruits presenting the disease symptoms described in Cano-Reinoso et al. (2021).

2.3 ASCORBIC ACID (ASA) AND SUCROSE CONTENT DETERMINATION

The AsA and sucrose content of the fruits was measured by the method reported in Siti Roha et al. (2013), using a High-Performance Liquid Chromatography (model L-2000 instrument; Hitachi: USA) with a Refractive Index detector model L-2490. A juice extracted from the fruit flesh adjacent to the core was used. The samples were obtained from a composition of four fruits per replication in each of the treatments arranged. Standard solutions of AsA and sucrose were dissolved in distilled water and filtered through a Millipore 0.45 µm membrane filter. The AsA and sucrose content were quantified, comparing the peak area by a chromatographic procedure.

2.4 MINERAL NUTRIENTS DETERMINATION

The calcium and magnesium content of the fruits was calculated using atomic absorption spectrometry (AAS 932 Plus; GBC scientific equipment: USA), employing a composition of four fruits per replication in each of the field treatments. The method applied was the one described in Benton-Jones (2001). Juice samples were put in a digestion tube with 5 ml of 65 % nitric acid and left overnight. Later, the samples were heated with a block digester at 125 °C for one hour. After that, 3 ml of 30 % hydrogen peroxide (H_2O_2) were added and reheated for one hour; thereafter, HNO_3 was used (1 ml residue) and 5 ml of nitric acid with distillate water (1:10) were added and shaken. Finally, the samples were move to a 25/50 ml flask quantitatively and pitched with distillate water, with the goal of creating an extract ready to determine the calcium and magnesium content. As the water content of the samples were previously detected, the results are expressed in a dry basis content.

2.5 DETECTION OF THE ELECTROLYTE LEAKAGE (EL)

Following the EL calculation in pineapple fruit reported in Chen and Paull (2001), the EL of the fruit flesh was obtained from the composition of four fruits per replication of the treatments implemented. Plugs were taken with a cork borer applying a longitudinal cut and then slides into a disk of 2 mm of thickness. Around 6 g of the disk were washed three times to remove any lysed material from the cell. For two hours, the disks were shaken and incubated in 60 ml of 0.3 M mannitol solution. Later on, the conductivity of the previous solution was obtained with a radiometer. After that, the samples were boiled for two hours to release all the electrolytes, and the conductivity was determined. The EL is shown as the percentage of the total conductivity.

2.6 SCANNING ELECTRON MICROSCOPE (SEM) EVALUATION

SEM analysis was performed using a similar method reported in Hu et al. (2012). A piece of tissue adjacent to the core ($5 \times 5 \times 2$ mm^3) was split from the middle of the flesh with a tweezer. Before scanning, the slices were dehydrated in a series of ethanol solutions and dried at a critical point of liquid CO_2 using a desiccator. The samples were mounted onto aluminum specimen stubs employing conductive silver glue and sputter-coated with gold. SEM was executed with a scanning electron microscope (ZEISS/EVO MA 10: German) equipped with an energy dispersive spectroscopy (EDS) at 15.00 kV.

2.7 STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS Version 22.0 software (SPSS Inc.; Chicago, IL: USA). All data were analyzed by a two-way ANOVA. Mean significant differences at $p < 0.05$ were determined by Duncan's multiple range tests and Kruskal-Wallis test (in case of the fruit collapse incidence data).

3 RESULTS AND DISCUSSION

3.1 TOTAL SOLUBLE SOLIDS (TSS), TOTAL ACIDITY (TA), AND SUCROSE CONTENT IN THE FRUIT

The TSS presented significant differences in the interaction results. The treatment D obtained the highest value (14.87 %), when the spray method was employed; however, the same treatment in the case of the inject method delivered the lowest outcome (12.33 %). Lower TSS content was associated with a higher fruit collapse incidence (Table 3). Previous studies reported that the value of the TSS for commercial consumption of pineapple low acid hybrids should be at least close to 12 % (Lu et al., 2014; Bartholomew and Sanewski, 2018; Cano-Reinoso et al., 2022a); this requirement was assessed in the treatment results of both inoculation methods; also, this circumstance could have been promoted by the treatments used as previous authors have reported a positive effect on the TSS content by the administration of biopesticides based on of *P. fluorescens* and *T. harzianum* (Jiang et al., 2019; Carillo et al., 2020). Besides, it has been demonstrated that pathogens interfere with the metabolism of the host by increasing their sugar uptake, especially at the phloem level, decreasing the final TSS content in sink organs like the fruit (Morkunas and Ratajczak, 2014; Naseem et al., 2017). This fact explains why the TSS treatment results of the inject method were lower than the spray one, due to the most critical case of infection in this method causing fruit collapse.

In the case of the TA, there were no significant differences delivered in the interaction outcomes; However, The TA values were higher in the inject method while lower in the spray method (0.69 % and 0.51 %, respectively) (Table 3). This more superior TA content was linked to a higher fruit collapse incidence. In pineapple, TA mainly is a measuring of the citric acid level of the fruit (Saradhuldhat and Paull, 2007; Paull and Chen, 2018). In MD2, the total TA value range between 0.4–0.7 % (Lu et al., 2014; Paull and Chen, 2018). Values inside this range were represented in the interaction results at harvest. However, the higher content of TA in the inject method could have been provoked by a more superior citric acid accumulation. Nevertheless, further studies should be done on this matter.

Concerning the sucrose content, in the interaction results there were significant differences evidenced. The treatment D with inject method had the most reduced outcome (4.31 %); on the contrary, the highest result was observed in the same treatment but when the spray method was employed (9.85 %) (Table 3). A higher content of sucrose was noticeably associated with a more reduced incidence of fruit collapse. The most crucial sugar in pineapple is sucrose. Previous research reported that in low acid hybrids the sucrose should be between 7–9 % at harvest (Nadzirah et al., 2013; Lu et al., 2014). Values among that range were reflected in this research outcomes. It has been proved that cell wall invertase (CWI) is one of the enzymes highly correlated with the sucrose accumulation in pineapple (Saradhuldhat and Paull, 2007; Paull and Chen, 2018). Recently evidence indicated that pathogens generated the induction of CWI activity, producing more hexose as sugars to support their metabolic activities, interfering the normal sugar accumulation in the fruit (Yamada et al., 2016; Naseem et al, 2017). These previous facts inferred that *D.zeae* influencing the CWI activities affected the sucrose accumulation, especially with the inject method, causing a more superior fruit collapse incidence. However, despite reports explaining the increase of sucrose under biopesticides applications of *P. fluorescens* and *T. harzianum* in several fruits (Jiang et al., 2019; Carillo et al., 2020); this phenomenon was not fully evidenced in the inject method, as this was more harmful to the fruit, nullifying this positive characteristic, especially in treatment D. More studies could be elaborated to determine the relation of the biopesticides used in this experiment with the inoculation methods influencing sugar enzymes activities.

Table 3: Effects of the interaction between the treatments and the inoculation methods implemented on pineapple quality and fruit collapse incidence

Treatments*Inoculation methods	TSS (%)	TA (%)	Sucrose (%)	AsA (mg kg^{-1})	Fruit collapse Incidence (%)
A*Spray	13.87 ± 0.18 ab	0.52 ± 0.02 a	9.59 ± 0.21 ab	188.61 ± 28.09 a	3.33 bc
B*Spray	13.93 ± 0.18 ab	0.50 ± 0.02 a	9.22 ± 0.25 abc	91.35 ± 69.37 b	0.00 c
C*Spray	14.13 ± 0.24 ab	0.53 ± 0.01 a	9.11 ± 0.11 abc	53.07 ± 1.39 b	5.00 bc
D*Spray	14.87 ± 0.35 a	0.50 ± 0.01 a	9.85 ± 0.06 a	78.96 ± 15.37 ab	1.67 c
A*Inject	12.80 ± 0.61 ab	0.71 ± 0.13 a	7.93 ± 0.25 c	98.99 ± 5.75 ab	20.0 ab
B*Inject	12.80 ± 1.44 ab	0.65 ± 0.12 a	8.43 ± 1.05 bc	233.42 ± 72.41 a	23.3 a
C*Inject	12.33 ± 0.71 b	0.70 ± 0.16 a	8.77 ± 0.19 abc	132.10 ± 1.05 ab	20.0 ab
D*Inject	12.33 ± 0.27 b	0.70 ± 0.12 a	4.31 ± 0.06 d	181.75 ± 21.63 a	20.0 ab

**Each value represents a mean ± standard error. Mean values in each column followed by the same lower-case letters are not statistically different by Duncan's multiple range test and Kruskal-Wallis test (for the fruit collapse incidence and severity data) ($p < 0.05$)
***A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest). TSS (Total Soluble Solids), TA (Total Acidity), AsA (Ascorbic Acid)

3.2 ASCORBIC ACID (ASA) CONTENT IN THE FRUIT

Observations of the AsA results exposed significant differences in the interaction outcomes. The highest value was obtained in treatment B with the inject method (233 mg kg^{-1}), and the lowest outcome in treatment C with the spray one (53.07 mg kg^{-1}) (Table 3). Overall, higher val-

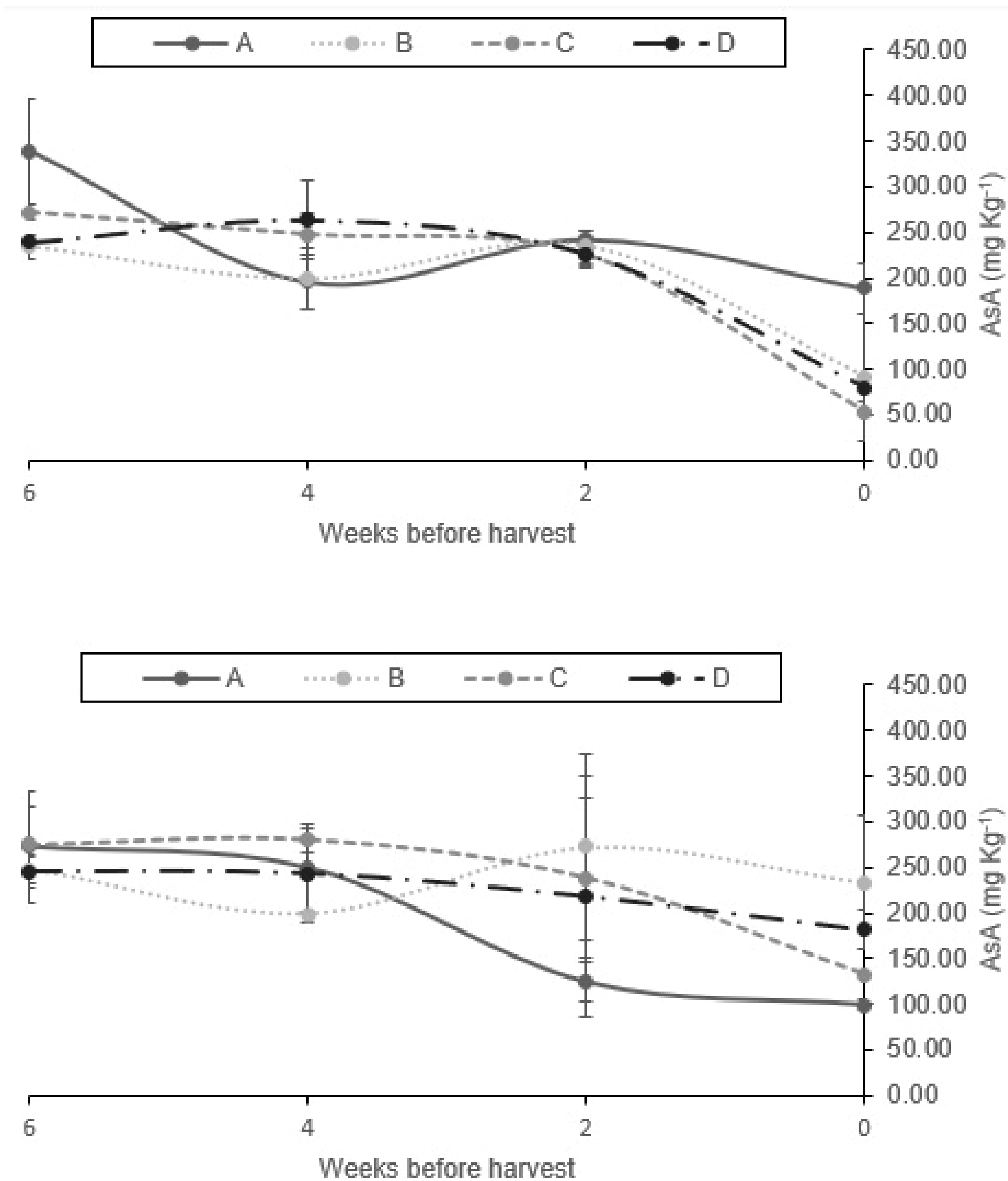

Figure 1: Trend of the ascorbic acid (AsA) content during the experiment for the treatments applied in both inoculation methods. A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest). Values are the mean of three replicates, and error bars represent the standard error

ues of AsA were linked to a more superior incidence of fruit collapse. The trend of the AsA content trough the experiment is presented in Figure (1). This figure shows that in four weeks before harvest there is a remarkable change in the trend of AsA in both inoculation methods, particularly in the inject one, which could have caused a physiological impact generating the final content at harvest.

The AsA in MD2 pineapple usually range between 300–600 mg kg^{-1} at harvest (Lu et al., 2014; Paull and Chen, 2018; Cano-Reinoso et al., 2022a). Besides, previous researches reported a positive correlation between the AsA content in pineapple and its antioxidant activity. The AsA values obtained in this research were lower than the range formerly determined; however, this could be ascribed to the plant's environmental conditions through the experiment time. It is possible to identify that from four weeks before harvest, when the organic acids accumulations start to happen, the AsA content never reach values close to 300 mg kg^{-1} (Figure 2). Seemingly, the irradiation, rainfall and resulting temperature could have affected the AsA accumulation in the fruit, as described in Ferreira et al. (2016) and Paull and Chen (2018).

As has been proved to encourage the activities of several scavenger enzymes like catalase (CAT), peroxidase (POD) and ascorbic peroxidase (APX) (Akram et al., 2017; Noichinda et al., 2017). Pathogen infections cause an increase in the reactive oxygen species (ROS); this circumstance creates a rise of AsA and subsequent scavenging activities to cope with these ROS generation in fruits (Lu et al., 2014; Noichinda et al., 2017). Furthermore, *T. harzianum* and *P. fluorescens* in different liquid culture applications have proved to enhance the antioxidant capacity, scavenger enzyme activities, and resistance mechanisms like hypersensitive responses (HR), in fruits and vegetables (Garcia-Seco et al., 2015; Sood et al., 2020).

The past information demonstrated why the treatments having biopesticides applications in the inject method increase substantially their AsA content. Besides, due to its more harmful impact, this method could have promoted a higher activity of scavenger enzymes, AsA accumulation, and HR to mitigated the fruit injure; a phenomenon that could have occurred also in treatment A without biopesticides used. However, the exhibition of this situation was not enough to reduce the damage created by the pathogen infection, which is evidenced in the higher fruit collapse incidence associated with the more superior AsA content, also in the inject method. Moreover, the insufficient AsA content detected weeks before harvest could have made more difficult to generate an optimal physiological respond of the fruit on these circumstances.

3.3 MINERAL NUTRIENTS CONTENT AND ELECTROLYTE LEAKAGE (EL)

Mineral nutrients interaction outcomes exposed significant differences at harvest. Nonetheless, the observation of the results exposed that the method of inoculation impacted these variables. For the calcium, the most elevated value was detected in treatment C with the inject method (2522.27 mg kg^{-1}); meanwhile, the lowest one was observed in the same treatment but using the spray method (1575.63 mg kg^{-1}). In the case of magnesium, the most elevated result was determined in treatment C using the inject method (2526.31 mg kg^{-1}) and the most reduced in treatment D with the spray one (1837.48 mg kg^{-1}) (Table 4). For the result of both mineral nutrients, the higher content was associated with a more superior incidence of the fruit collapse.

Calcium has been proved to rise the resistance of fruits and vegetables to pathogens attacks by increasing the cellular responses to biotic signals and reducing the cell wall breakdown (Madani et al., 2016; De Freitas and Resender Nassur, 2017). Concerning magnesium, this is a component of the middle lamella and also has been reported to activate calcium-dependent protein kinases (CPDKs) (Waraich et al., 2011; Huber and Jones, 2013); proteins that translated the Ca^{2+} signature into specific phosphorylation events generating signaling responses as part of plant defense mechanisms (Gao et al., 2014; De Freitas and Resender Nassur, 2017; Cano-Reinoso et al., 2022b). Evidently, due to the more severe infection generated by the inject method, the fruit as a protection mechanism could have promoted the increase in the uptake of calcium and magnesium to maintain the cell wall structure, encouraging more molecular ions assimilation and enzyme activities (Ca^{+2}, CDPKs, respectively). Besides, *T. harzianum* and *P. fluorescens* have been associated with a more remarkable assimilation of mineral nutrients content in terms of N, P, K, Ca and Mg in plants and fruits (Pérez-Rodriguez et al., 2020; Sood et al., 2020). These facts make clearer that the biopesticides may have an influence on the plant and fruit defense mechanism when a certain high degree of affectation is reached, in this case, triggering the respective calcium and magnesium increase. However, like the situation observed in the AsA results, these effects were not enough to decrease the incidence of fruit collapse in the inject method.

The interaction results for the EL content at harvest presented significant differences. The most elevated value was observed in treatment D using spray method of inoculation (72.10 %), while the most reduced value was obtained in treatment A with the inject method (54.19 %) (Table 4). A trend of the EL content trough the experi-

Table 4: Effects of the interaction between the treatments and the inoculation methods implemented on pineapple mineral nutrients content, and the electrolyte leakage (EL)

Treatments*Inoculation methods	Ca (mg kg^{-1})	Mg (mg kg^{-1})	EL (%)
A*Spray	1853.55 ± 106.34 bc	2077.48 ± 106.54 abc	65.27 ± 5.48 abcd
B*Spray	1736.12 ± 107.81 c	1993.41 ± 102.55 bc	67.99 ± 6.61 abc
C*Spray	1575.63 ± 90.16 c	1875.93 ± 94.90 c	70.81 ± 1.39 ab
D*Spray	1780.47 ± 29.35 c	1837.48 ± 81.18 c	72.10 ± 3.92 a
A*Inject	2335.41 ± 306.44 ab	2335.25 ± 237.64 ab	54.19 ± 1.70 d
B*Inject	2474.59 ± 233.43 a	2442.74 ± 195.10 ab	58.92 ± 2.59 bcd
C*Inject	2522.27 ± 188.09 a	2526.31 ± 96.49 ab	57.47 ± 2.63 cd
D*Inject	2399.15 ± 52.38 a	2451.99 ± 42.28 ab	57.69 ± 2.70 cd

** Each value represents a mean ± standard error. Mean values in each column followed by the same lower-case letters are not statistically different by Duncan's multiple range test ($p < 0.05$)
***A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest). EL (Electrolyte Leakage)

ment is presented in Figure (2). In this figure it is possible to observe that the EL had a noticeable increase in both methods of inoculation between four and two weeks before harvest, more remarkable in the treatment B of the inject method (around 80 %), which had a EL content much higher than those of the spray method. This outcome can provide a broader understanding concerning the relation of EL with the fruit collapse incidence at harvest, especially for treatment B.

The EL reflects a loss of integrity in cell membranes, common during a pathogen infection (Demidchik et al., 2014). In pineapple fruit, the EL speeds up from six weeks before harvest in concomitance with the sucrose accumulation (Paull and Chen, 2003, 2018). This research exposed that treatment using the spray method had higher EL, which should be correlated with a more superior incidence of fruit collapse; however, this only happened in the treatments employing the inject method. The differences in EL percentage between the two methods were more related to the experiment design and unique status of the sample analyzed. In the Figure (2) it is possible to observe that fruits of the inject method two weeks before harvest had an EL percentage almost like those of the spray method at harvest, especially in treatment B. This situation means that at this time, the fruits gathered from the inject method were predominantly affected by fruit collapse, while at harvest, the number of fruits with disease symptoms were highly reduced. Therefore, it is possible to infer that the EL in the inject method can be correlated to a more superior fruit collapse incidence, analyzing the results from two weeks before harvest. Furthermore, the higher EL percentages of the spray method can be more associated with the normal process of sugar accumulation in pineapple than a physiological response to the stress induced by the bacterium attack; because of that, the lower fruit collapse incidence. Concerning *T. harzianum* and *P. fluorescens*, there is still insufficient information of their influence on the EL in plants and fruits; however, their recognized beneficial impact on calcium uptake could suggest that these biopesticides would help to decrease the percentage of EL under a disease infection. High calcium accumulation has been related to a leakage reduce when a plant is subjected to an abiotic or biotic factor (Demidchik et al., 2014; De Freitas and Resender Nassur, 2017). More experiments could be done on this aspect.

3.4 SEM ANALYSIS

SEM analysis was conducted at harvest time in the treatments A, B, C, and D of the inject inoculation method (Figure 3). The sample of the treatments A and B showed characteristics of a low cell wall integrity, identified by arrows with lack of significant thickness, and an undulated shape not attached to the vascular bundles of the cell. On the contrary, in treatments C and D it was possible to observe symptoms of membrane well-function, with arrows presenting more significant thickness and turgor.

During infections bacteria can cause an increase in the activities of pectolytic and polygalacturonases (PG) enzymes, which are known for their the cell wall degrading properties (Hocking et al., 2016; De Freitas and Resender Nassur, 2017). The activities of these enzymes can be mitigated by minerals like calcium, which binds to the cell wall and increases its strength, making the cell wall matrix less accessible to them (De Freitas and Resender Nassur, 2017). This information suggested the treatments C and D of the inject method caused a lower

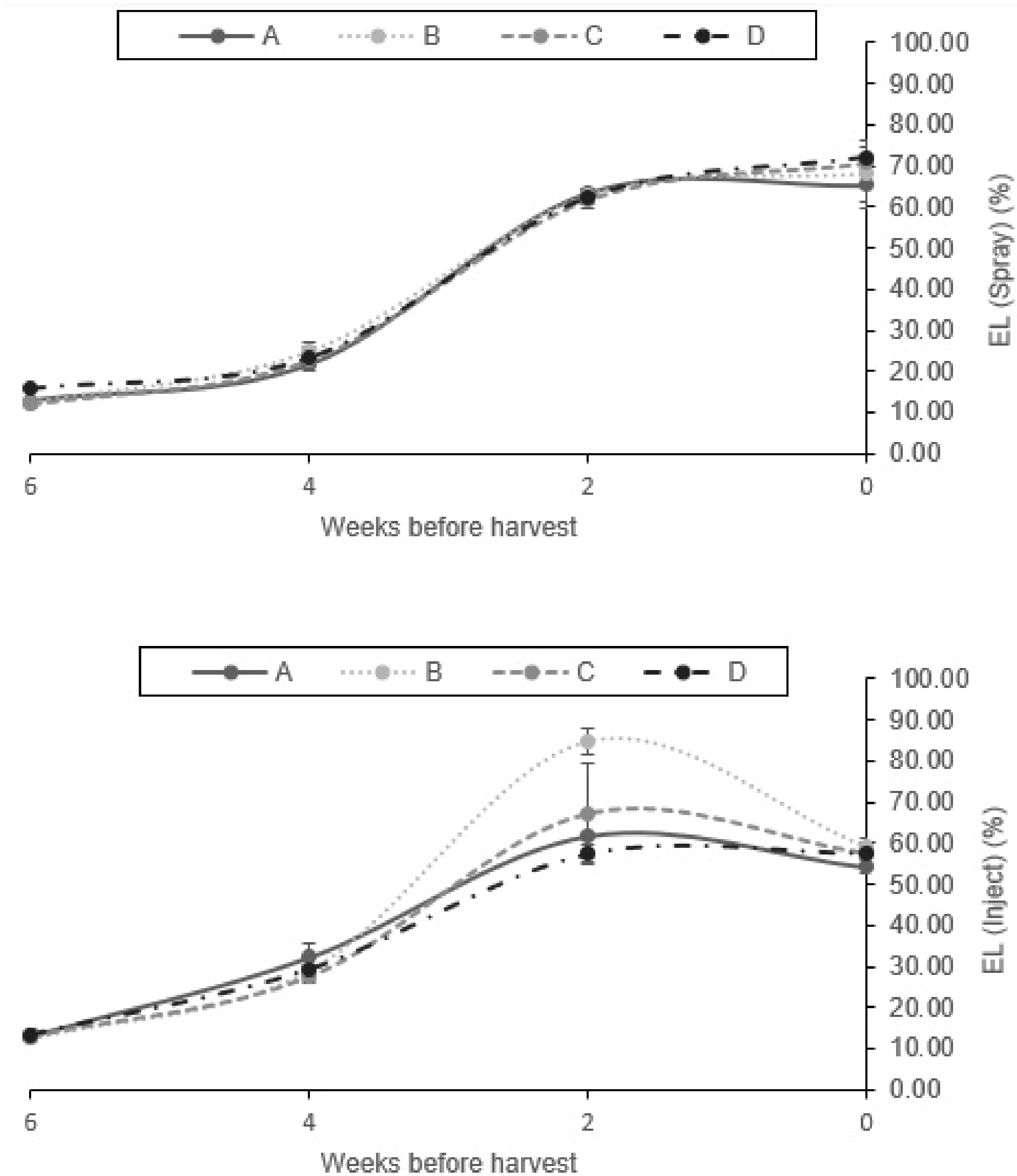

Figure 2: Trend of the Electrolyte leakage (EL) content during the experiment for the treatments applied in both inoculation methods. A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest). Values are the mean of three replicates, and error bars represent the standard error

activity of these enzymes, generating a healthier cell wall status, opposite to treatments A and B, as exposed in the SEM analysis. Despite of the high concentration content of calcium in the treatments A and B, their cell wall primary layer displayed unhealthy characteristics. This situation could be attributed to the lower assimilation

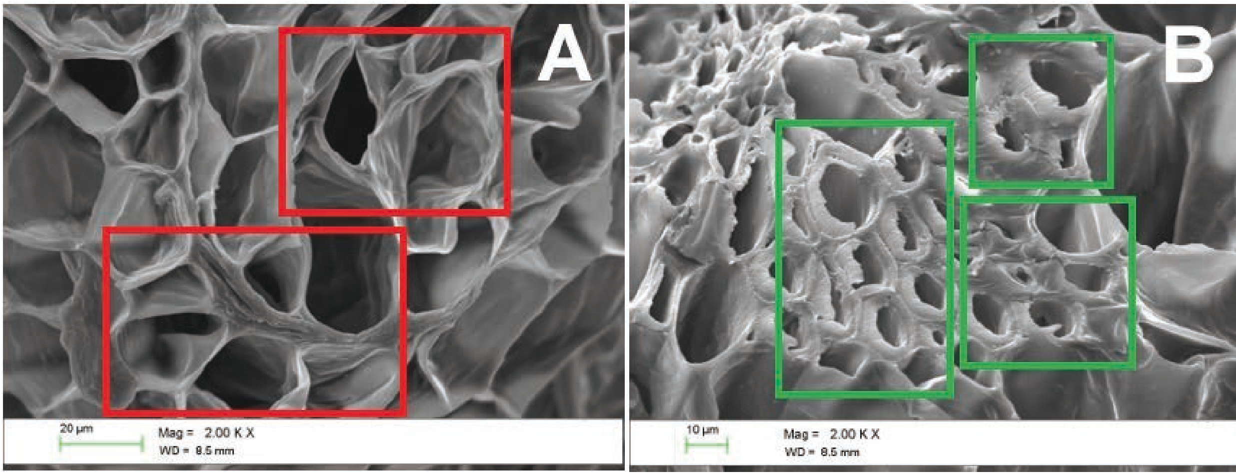

Figure 3: Effects of the treatments A, B, C, and D in the cell walls of the inject method of inoculation detected by SEM (20 and 10 µm size, respectively; with 2000 x of magnification). The smaller thickness and undulated arrows of the cell wall (red square) and more significant thickness and non-undulated arrows (green square) were examined. Treatments, A (Control: No biopesticide used), B (Bio P32 from 13 weeks before harvest), C (Bio T10 from 13 weeks before harvest), D (Bio P32 + Bio T10 from 13 weeks before harvest)

of calcium ions (Ca^{2+}) into the cell wall matrix produced by the harmful impact of the inject method when treatments A and B were implemented. Besides, the calcium ions of these treatments could have also been employed in other calcium-influenced-process like sugar production and fruit respiration (Hocking et al., 2016; Meeteren and Aliniaeifard, 2016); decreasing its sensing activity into the cell wall.

3.5 FRUIT COLLAPSE INCIDENCE

There were significant differences evidenced in the interaction outcomes of the fruit collapse incidence. The treatment B in the spray method obtained the lowest incidence (0 %), while the same treatment but in the in the inject method had the highest one (23.7 %) (Table 4). Moreover, the inject method delivered in average a higher incidence than the spray one for all the treatments (20.93 and 2.50 %, respectively). This evidence finally proves that the inject method was more effective in causing symptoms of this disease. On the other hand, the observation of the significant differences and the mean values of the interaction outcomes can provide the insight that C and D can be considered as the best options to control fruit collapse disease; meanwhile, A and B could be taken as less effective in this aspect. This affirmation can be supported by the examination of the influences of these treatments on the quality variables studied (specially the mineral nutrients content, AsA content, and EL), the cell wall status by the SEM analysis previously described, and their relation with the fruit collapse incidence in both inoculation methods. C and D despite not exposing always the highest outcomes, those delivered mostly optimal results in the previous parameters mentioned, primordially a healthy cell wall, which could help to predict that under a more harmful conditions of infection than the implemented in this experiment, these treatments could satisfactorily mitigate the fruit collapse occurrence. On the contrary, A and B, although displayed high outcomes, regarding antioxidants and resistance parameters, like AsA and Ca content, especially in the case of B, their high EL from weeks prior to harvest, together with their unhealthy cell wall status, suggested that under elevated infections these treatments could not provide enough protection to the fruit.

Furthermore, due to the characteristics of both inoculation methods used in this research, where the juice had to be extracted from previously infected fruits, it was complicated to determine in every juice concentration the number of colonies forming unit (CFU) existed. Previous laboratory trials before the beginning of this experiment demonstrated that the minimal number of CFU required to inoculate *D. zeae* in pineapple should be around 10^7–10^9 CFU ml^{-1}, which was in agreement with former experiments described by Sueno et al. (2014) and Aeny et al. (2020)HI, on a pineapple cultivar (Ananas comosus ‘PRI 73-114’. This information could help to support the explanation about why the spray method was less effective in showing fruit collapse symptoms. Because of the characteristic of this method, the number of CFU ml^{-1} required to cause a *D. zeae* infection could be higher than the inject method. Moreover, because of the number of colonies necessary to produce an infection in the inject method, the doses of biopesticides employed (20 ml/plant-fruit), together with the concentration number of cell ml^{-1} and conidia ml^{-1} in those products (*P. fluorescens* and *T. harzianum*, respectively), could not be enough to mitigate the impact of fruit collapse. For future experiments, the doses and the concentration number of cells and conidia per ml of *P. fluorescens* and *T. harzianum* should be increased in the case that this experiment wants to be replicated in pineapple. On top of that, those future trials could also implement a chemical pesticide treatment as positive control. These future arrangements could help to stablish the differences between the biopesticides administrated in this research and any conventional pesticide, essentially concerning pineapple quality and fruit collapse occurrence. As the employment of chemical agents were outside of the scope of this experiment, this should be a point to be observed eventually.

4 CONCLUSIONS

The biopesticides based on *Pseudomonas fluorescens* and *Trichoderma harzianum* affected the fruit collapse disease incidence and pineapple quality. Treatment C (Bio T10 from 13 weeks before harvest), and D (Bio P32 + Bio T10 from 13 weeks before harvest) delivered the best results having an ideal AsA, EL, mineral nutrients content, healthier cell wall characteristics, and a low fruit collapse incidence, essentially after analyzing their outcomes in both inoculation methods. Meanwhile, treatments A (Control: No biopesticide used), and B (Bio P32 from 13 weeks before harvest) were less effective in these aspects. Finally, the inject inoculation method caused more fruit collapse incidence than the spray one. The number of CFU of *D. zeae* were considered as the reasons why the inject method was more severe affecting the fruit physiology and effective in generating the higher incidence.

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