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PHYSICOCHEMICAL INTERACTIONS AND VIABILITY OF *Cordyceps fumosorosea* ASSOCIATED WITH ADJUVANTS

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Abstract
Adding adjuvants to crop protection sprays is common practice; however interactions among formulations must be better understood to prevent reducing efficacy, especially for those containing microorganisms for biological control. This study aimed to assess physicochemical interactions, droplet formation, and viability of the fungus *Cordyceps fumosorosea* mixed with an adjuvant. The experiments followed a completely randomized design with four replications and six treatments. The treatments included the fungus associated with five adjuvants: polyether/silicone copolymer (1 - PSC), alkyl phosphate ester (2 - PAE), soybean oil methyl ester (3 - SME), orange peel oil (4 - OPO), lecithin/propionic acid mixture (5 - LPA), and a control (only fungus) (6). The LPA adjuvant was physically compatible with the fungus, unlike others that showed phase separation. All adjuvants reduced the contact angle and surface tension compared to the control, with treatments PSC and LPA presenting the lowest values, respectively. All adjuvants reduced droplet size compared to the control. Treatment LPA produced the smallest droplet size, the highest risk of drift, and the most droplet uniformity. The highest viscosity values originated from solutions in treatment LPA, followed by PAE. Formulations with the LPA adjuvant plus the bioinsecticide yielded the lowest pH and the highest electrical conductivity values, followed by OPO. Even with the low pH, the LPA treatment did not affect the viability of the entomopathogenic fungus. It is evident that the adjuvants affected the physicochemical characteristics of the solutions, and treatment LPA yielded the best results for physicochemical compatibility, as it did not reduce the viability of the entomopathogenic fungus.

Keywords: Agricultural pest management. Biological control. Entomopathogenic fungus. Pesticide application technology. Phytosanitary sprays. Tank mixture.

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1. Introduction

Entomopathogenic fungi naturally control phytophagous insects (Roberts and Hajek 1992; Chandler 2017). Despite the existence of various entomopathogenic fungi, only a few genera are widely commercialized, and the most abundant on a commercial scale are *Beauveria bassiana* (Deuteromycotina: Hyphomycetes), *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae), *Cordyceps* spp. (Hypocreales: Cordycipitaceae), and *Lecanicillium* spp. (Hypocreales: Cordycipitaceae) (Skinner et al. 2014; Sharma et al. 2023). The development of mycoinsecticides with these microorganisms has produced active ingredients with an increasingly prominent role in integrated pest management (IPM) (Shah and Pell 2003; Li et al. 2010).

Numerous crops are plagued by caterpillar complexes that inflict significant damage (Formentini et al. 2015; Michereff-Filho et al. 2021). These insects can be managed with commercially available mycoinsecticides. The exploration and development of *Cordyceps fumosorosea* species is particularly promising, showing potential in controlling pests of the order Lepidoptera (Corrêa et al. 2020; Du et al. 2021). *Cordyceps fumosorosea* has been effective against various species, including *Spodoptera* spp. (Lepidoptera: Noctuidae), *Helicoverpa armigera* (Lepidoptera: Noctuidae), and *Plutella xylostella* (Lepidoptera: Plutellidae) (Zimmermann 2008; Lagogiannis et al. 2020; Lei et al. 2021). All these species are phytophagous insects that infest crops of considerable economic relevance. Given its potential to regulate populations of *H. armigera*, a polyphagous species notorious for causing economic losses in various crops such as soybeans, corn, and cotton (Czepak et al. 2013), the utilization of *C. fumosorosea* could become more widespread, as it demonstrates consistent and effective control levels.

Although formulations based on entomopathogenic fungi are efficient, factors may limit the action of fungi in the field, affecting the preservation time and viability of conidia (Silva et al. 2015). The action and efficiency of crop protection products depend on the constituents of the spray solution (Cunha and Alves, 2009). Therefore, many adjuvants have been developed to integrate the spray solution, increasing the stability, virulence, and efficacy of the entomopathogenic agent (Silva et al. 2015; Arnosti et al. 2019; Holka and Kowalska 2023).

Adjuvants are non-phytosanitary substances that alter the physical and chemical properties of spray mixtures to enhance the effectiveness of an active ingredient (Green and Beestman 2007). The inclusion of adjuvants may improve the product's coverage, retention, and absorption onto the target, enhancing spray quality (Avery et al. 2013; Cunha et al. 2017; Ferreira et al. 2020). Nevertheless, achieving optimal performance from mycoinsecticides requires specific tests to assess each combination and application method. Many commonly employed adjuvants lack information regarding their compatibility with spray mixtures, which potentially causes adverse effects depending on the application method (Ryckaert et al. 2007; Bueno et al. 2013; Assunção et al. 2019). Therefore, this study aims to evaluate physicochemical interactions, droplet formation, and viability of the fungus *Cordyceps fumosorosea* (isolate IBCB 130) when mixed with adjuvants. Investigating these interactions will hopefully provide relevant insights to improve the effectiveness and practical application of mycoinsecticides in agricultural pest management.

2. Material and Methods

The study was conducted from September to November 2021 at the facilities of the Research and Development Center for Application Technology (NEDTA) at UNESP, Campus Jaboticabal, SP, Brazil.

Production of the entomopathogenic fungus

The fungus *C. fumosorosea* (isolate IBCB 130) came from the collection bank of the Biological Institute of Campinas, SP, Brazil. It was cultivated in potato dextrose agar (PDA, KASVI) and incubated in a biological oxygen demand (BOD) air-conditioned chamber at 26 ± 1°C during 12 hours of photophase, for 7 to 10 days. The structures in the PDA culture medium were scraped to prepare the conidia suspension. The scraped conidia were placed in a glass tube, and then underwent serial dilutions using 0.01% Polysorbate 80 (Tween® 80). The number of conidia was quantified in a Neubauer chamber under a light microscope. All fungal suspensions were adjusted to a standardized concentration of $1x10^8$ conidia/mL.

Experimental design

The applied treatments included an insecticide mixture composed of the entomopathogenic fungus with five adjuvants at the concentrations recommended by the manufacturers (Table 1), totaling six treatments.

The parameters evaluated for the physical and chemical properties of the spray solutions were I) spray solution stability, II) pH, III) electrical conductivity, IV) surface tension, V) droplet contact angle, VI) droplet size, and VII) viscosity. The applied doses represent the maximum levels recommended for soybean cultivation and were taken as the reference for this study, considering a concentration equivalent to an application volume of 100 L/ha.

Table 1. Active ingredient of the adjuvants and respective dosages used to evaluate the physicochemical interactions with *Cordyceps fumosorosea* (isolate IBCB 130).

	Active ingredient*	Treatment code	Indicated application	Dose/100 L water
1	Polyether and silicone copolymer	PSC	Spreading adhesive	1 ml
2	Phosphate alkyl ester	PAE	Spreading adhesive	62.5 mL
3	Soybean oil methyl ester	SME	Spreading adhesive	125 mL
4	Orange peel oil	OPO	Surfactant and spreading adhesive	75 mL
5	Lecithin and propionic acid	LPA	Surfactant and spreading adhesive	500 mL
6	Only the fungus	Control	-	1 L

*Information obtained from product labels.

Assessment of the physical and chemical properties of the spray liquid

Physical stability of the spray liquid

A standard water reagent with a total hardness of 20 mg/L in $CaCO_3$ equivalent per liter of water was used to prepare the spray solutions (ABNT 2014). Then, the adjuvants, the fungus, and the polysorbate were added. Each repetition included a 250mL graduated cylinder with a lid, a metallic sieve with a nominal opening of 149 µm, and a graduated pipette. After preparing the spray solution, the beaker was covered and inverted 10 times for homogenization (ABNT 2014).

The spray solutions were evaluated at four intervals: immediate separation after mixing (0 hours) and after 2, 6, and 24 hours of rest. Thus, the possible effects of interactions between products regarding homogeneity/heterogeneity included flocculation (appearance of small dispersed aggregates), sedimentation (accumulation of solid particles at the bottom), phase separation (e.g. between oil and water), oil suspension (free oil layer on the surface), lump formation (formation of large compact particles), crystal formation (appearance of crystalline solids), creaming (accumulation of cream material), and foaming (formation of foam on the surface).

Electrical conductivity and pH

The hydrogen potential (pH) was determined using a bench pH meter (model Q400AS, Quimis, Diadema, Brazil), and electrical conductivity was established using a conductivity meter (Marth® MP11P, Santa Rita do Sapucaí, Brazil). Each evaluation immersed the sensor in the spray solution, and the readings were captured after the values stabilized. Both equipment were calibrated using standard solutions. Measurements were taken at four intervals: after the formation of the spray liquid mixture (0 hours) and after 2, 6, and 24 hours of rest.

Surface tension and contact angle

Static surface tension was determined using an OKA 15EC tensiometer (Dataphysics, Filderstadt, Germany), and contact angle measurements were obtained with Contact Angle System OCA 15-plus software (DataPhysics Instruments GmbH, Filderstadt, Germany). Automation and computer image processing were performed with SCA20 software (DataPhysics Instruments GmbH, Filderstadt, Germany). The equipment produced drops of 3 µL in volume that were deposited on a paraffinized plastic film surface (Parafilm M, Bemis NA). Thus, the surface tension (pending drop) and contact angle on the paraffin film surface were obtained at 1, 5, 10, 20, 40, and 60 seconds of drop formation/deposition.

Droplet spectrum analysis

A TT110015 spray nozzle (TeeJet Technologies South America, São Paulo, Brazil) with a flow rate of 0.600 mL/min was used to determine the droplet size for each treatment. The diameters of the produced droplets were determined by a Mastersizer S particle size analyzer (Malvern Ltd. Malvern, United Kingdom), version 2.19, using the laser diffraction method. The pressure of 312 kPa (equivalent to 45.25 PSI) was set and maintained. Water was sprayed between each treatment to clean the spray system. The droplet diameter (µm) was measured and represented by the values of $D_{V0.1}$, $D_{V0.5}$, and $D_{V0.9}$, where 10, 50, and 90% of the sprayed volume, respectively, comprises drops smaller than that diameter. The analysis of droplets with high drift potential considered the volume percentage of drops smaller than 100 µm (% V <100 µm). Furthermore, the uniformity coefficient (span), which represents the distribution of the droplet size spectrum, was analyzed using the equation: $Span = (D_{V0.9} - D_{V0.1}) / D_{V0.5}$. This analysis included a water-only treatment.

Viscosity

The viscosity of spray solutions was evaluated using a Brookfield rotational viscometer, model DV-I Prime, at a temperature of 25°C and an agitation speed of 60 rpm. The experiment followed a completely randomized design, with four replications.

Fungal viability

The adjuvant with the best result in previous evaluations was selected to verify its effect on fungal viability over time. Thus, solutions were prepared for the treatment (the adjuvant combined with *C. fumosorosea*) and the control (only fungus with water), as specified in Table 1.

The solutions were thoroughly mixed in 250mL Erlenmeyer flasks by shaking them on an orbital shaker for 0, 2, and 6 hours, with the agitation set to 120 rpm to simulate the conditions in a tractor tank in the field. Samples and 10µL drops of the mixtures were taken at each interval of 0, 2, and 4 hours and inoculated into Petri dishes containing PDA medium. Each treatment and time interval underwent four replications.

The inoculated plates were incubated at 28 ± 2°C in a BOD incubator. After 15 hours of incubation, the selected dilution was the one that allowed optimal visualization of the conidia, and their viability was assessed under a light microscope using a 40× objective lens (400× magnification). A conidium was viable in case of swelling or germination. In three different drops per treatment, at least 100 conidia per drop were counted and classified as viable or non-viable. The viability percentage was calculated for each drop using the formula: Viability (%) = (number of viable conidia / total number of conidia) × 100

The final viability for each treatment was determined by calculating the arithmetic mean of the three drops.

Data analysis

Regarding the pH and electrical conductivity analyses, the experiments followed a completely randomized design in a 6x4 factorial scheme (mixtures vs. analysis times), with four replications. Surface tension and contact angle evaluations used a 6x6 factorial scheme (grouting x analysis times), with four replications. Repeated-measure analyses were performed over time for these parameters using the mixed models procedure.

The data on droplet size and viscosity parameters underwent the Bartlett test to verify homoscedasticity and the Cramer von Mises test for normality. The data that presented these requirements were subjected to the Analysis of Variance. Significant mean values were compared using Tukey's test ($p > 0.05$). All of these analyses relied on SAS v.9.4 software (SAS Institute, Cary, NC, USA).

The effects of treatments (fungus alone and fungus plus adjuvant) and time (0, 2, and 6 hours) on fungal viability were analyzed using generalized linear models (GLM) with a binomial distribution. Model adequacy was assessed with an overdispersion analysis. Hence, the following were calculated: residual deviation, the number of residual degrees of freedom, and the Pearson index. The conventional criterion considered that a Pearson index significantly higher than 1 indicates overdispersion. These analyses occurred in the R statistical environment (version 3.6.2).

3. Results

Physical stability of the spray solution

The physical stability assessment presented incompatibilities regarding phase separation and foam formation (Table 2). Phase separation occurred at all evaluation times for all treatments, except for LPA, which only showed phase separation after six hours. Foam formation occurred in spray solutions with the PSC, OPO, and LPA adjuvants only immediately after agitation (0 hours).

Table 2. Physical stability and compatibility of mycoinsecticide spray solutions at different times (0, 2, 6, and 24 hours) for each treatment.

	Observation time (h)			
Parameter	0	2	6	24
Flocculation	-	-	-	-
Sedimentation	-	-	-	-
Phase separation	PSC, PAE, SME, OPO, and control	PSC, PAE, SME, OPO, and control	PSC, PAE, SME, OPO, LPA, and control	PSC, PAE, SME, OPO, LPA, and control
Oil separation	-	-	-	-
Clumping	-	-	-	-
Crystal formation	-	-	-	-
Cream formation	-	-	-	-
Foam formation	PSC, OPO, and LPA	-	-	-

Note: "-" indicates no visible changes for the parameter.
PSC: Polyether and silicone copolymer; PAE: Phosphate alkyl ester; SME: Soybean oil methyl ester; OPO: Orange peel oil; LPA: Lecithin and propionic acid.

Hydrogen potential (pH) and electrical conductivity (EC)

The pH and EC results varied ($p < 0.0001$) depending on the adjuvant (Table 3) and rest time (Figure 1). At all rest times, the formulations with treatment LPA plus the bioinsecticide presented the lowest pH and the highest EC values, followed by OPO. The PSC, PAE, and SME adjuvants presented the highest pH and the lowest EC values. However, the pH of the solution plus SME did not differ from the control.

Regarding the pH variation over time, after preparing the mixture, the pH decreases considerably and stabilizes after 24 hours, with values around 7, except for treatment LPA (Figure 1).

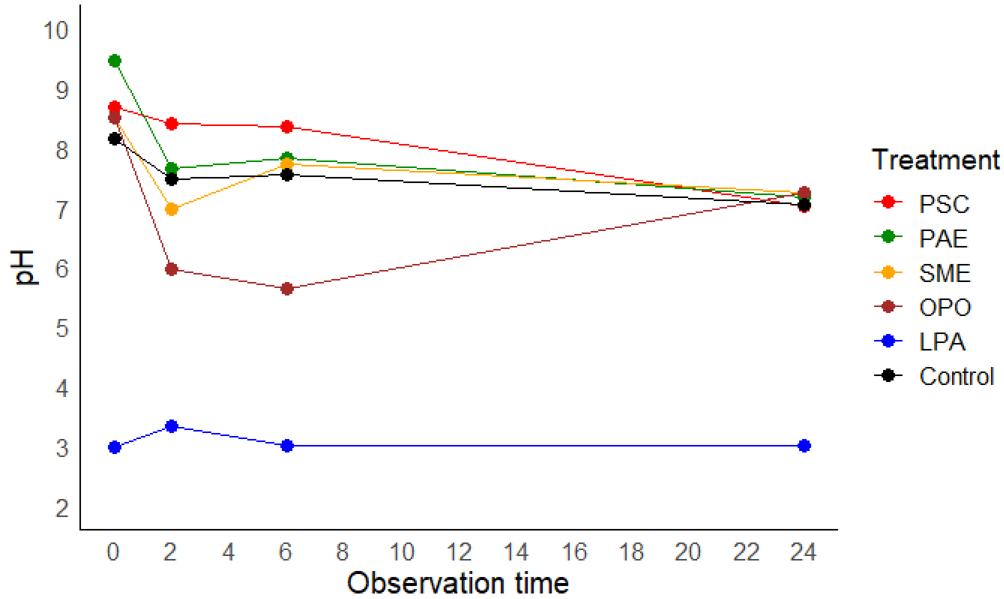

Figure 1. The pH variation of the *Cordyceps fumosorosea* solution for all treatments during the rest periods.

Table 3. The pH and electrical conductivity values (p-value <0.0001) for each treatment.

Treatment	pH[1]	Electrical conductivity (µS cm $^{-1}$)
Polyether and silicone copolymer	8.12[a]	35.85[d]
Phosphate alkyl ester	8.03[a]	36.29[cd]
Soybean oil methyl ester	7.62[b]	37.51[c]
Orange peel oil	6.84[c]	51.52[b]
Lecithin and propionic acid	3.08[d]	206.09[a]
Control	7.57[b]	32.07[e]

[1]Means followed by distinct letters in the columns differ at α = 0.05.

Surface tension and contact angle

All adjuvants reduced the contact angle and surface tension compared to the treatment without an adjuvant. It was impossible to calculate the contact angle of treatment PSC, as its drops did not produce an angle, meaning total spread (Table 4). The addition of adjuvants in treatments PSC and LPA showed the lowest results (higher spread on the surface - hydrophilic effect). The sprays with treatments SME, PAE, and OPO showed neutral results (close to no hydrophobic or hydrophilic effect). The control treatment showed surface hydrophobicity. The surface tension in treatment OPO did not differ from that in treatment LPA.

Table 4. Surface tension and contact angle results (p-value <0.0001) for each treatment.

Treatment	Contact angle (°)[1]	Surface tension (mN m[1])
Polyether and silicone copolymer	0[e]	22.37[e]
Phosphate alkyl ester	84.85[c]	43.68[c]
Soybean oil methyl ester	90.40[b]	46.50[b]
Orange peel oil	83.43[c]	38.18[d]
Lecithin and propionic acid	76.77[d]	39.01[d]
Control	106.26[a]	56.84[a]

[1]Means followed by distinct letters in the columns differ at α = 0.05.

Droplet spectrum analysis

The adjuvants reduced the droplet size relative to the control (Table 5). The smallest droplet sizes were verified in treatment LPA. The other treatments produced droplets with a size closer to the control. The largest droplet sizes were obtained with adjuvants OPO and PSC.

The mixtures with adjuvants presented a higher risk of drift (%V <100 µm), except for treatment PSC, which was similar to the control. Treatment LPA presented the highest volume of droplets smaller than 100 µm. Regarding droplet uniformity, treatment LPA presented the best results and differed from the others.

Table 5. Droplet size in µm ($D_{V0.1}$, $D_{V0.5}$, $D_{V0.9}$), percentage of droplets smaller than 100 µm, and uniformity coefficient (span) of all sprays tested at a constant pressure of 3.12 bar.

Treatment	$D_{V0.1}$[1]	$D_{V0.5}$	$D_{V0.9}$	%V <100 µm	Span
Polyether and silicone copolymer	96.53[a]	241.73[bc]	530.33[b]	10.92[c]	1.79[a]
Phosphate alkyl ester	92.60[ab]	232.22[c]	503.30[c]	12.11[bc]	1.77[a]
Soybean oil methyl ester	93.73[ab]	232.46[c]	507.30[c]	11.76[bc]	1.78[a]
Orange peel oil	94.49[ab]	245.43[b]	539.10[b]	11.46[bc]	1.81[a]
Lecithin and propionic acid	83.97[c]	202.39[d]	422.44[d]	15.07[a]	1.67[b]
Fungus and water	97.68[a]	256.89[a]	553.79[a]	10.59[c]	1.78[a]
Only water	90.24[b]	239.4[bc]	534.20[b]	12.74[b]	1.86[a]

[1]Means followed by distinct letters in the columns differ at α = 0.05.

Viscosity

The highest viscosity values appeared in treatment LPA, followed by PAE (Table 6). The lowest viscosity values resulted from mixtures with adjuvants PSC, SME, and OPO. It was impossible to measure viscosity in the control treatment.

Table 6. Mean viscosity values (mPa s) (p-value <0.0001) for each treatment.

Treatment	Viscosity (mPa s)[1]
Polyether and silicone copolymer	0.62[e]
Phosphate alkyl ester	1.02[b]
Soybean oil methyl ester	0.94[d]
Orange peel oil	0.97[c]
Lecithin and propionic acid	1.10[a]
Control	-

[1]Means followed by distinct letters in the columns differ at α = 0.05.

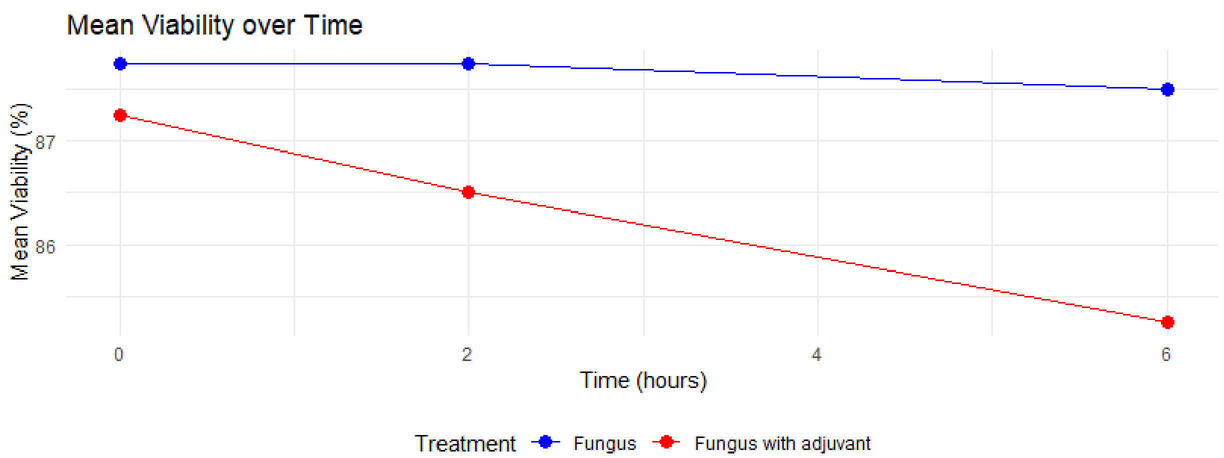

Figure 2. Viability of the fungus *Cordyceps fumosorosea* with and without adjuvant addition during the rest periods.

Fungal viability

Adding the LPA adjuvant did not significantly alter fungal viability over time (Figure 2). Fungal viability did not differ regarding treatment (p = 0.3313), time (p = 0.4964), or the interaction between time and treatment (p = 0.6259).

4. Discussion

The LPA treatment demonstrated the best physical compatibility with the entomopathogenic fungus, while conidia did not mix with adjuvants (phase separation) in the other treatments. Consequently, hydraulic sprayer systems with efficient solution agitation await treatment LPA to maintain a uniform mixture during application. Additionally, LPA exhibited the highest uniformity in droplet size. The closer the uniformity coefficient (span) is to zero, the higher the uniformity of sprayed droplets, indicating minimal diameter variations (Costa et al. 2017). Thus, these solutions enable applications with droplets closer to the desired diameter, suggesting higher-quality applications that are more standardized and uniform, allowing a more controlled droplet size selection. Droplet size can be adjusted by properly selecting the spray nozzle model, working pressure, and liquid used (Matuo 1990). In this context, the lecithin-based adjuvant yielded results that contributed to the quality of application.

All adjuvants increased the susceptibility to drift compared to the treatment without an adjuvant. Solutions containing treatments PSC and OPO showed the lowest susceptibility to drift, while treatment LPO exhibited the highest susceptibility. The found values influenced the volume percentage, with droplets smaller than 100 µm attributed to the generalized droplet size reduction, as expressed in the volume median diameter. Even in cases of higher uniformity, diameter reductions significantly influenced the percentages of droplets smaller than 100 µm, affecting drift rates (Oliveira and Antuniassi 2012; Oliveira et al. 2015; Griesang et al. 2017; Griesang et al. 2022).

Increasing the viscosity of the spray solution is associated with the production of larger droplets and a decrease in the potential risk of drift (Cunha and Alves 2009; Oliveira et al. 2015). Treatments LPA and PAE presented the highest viscosity values. These treatments yielded the smallest droplets and, consequently, the highest potential for risk of drift. Viscosity worked with surface tension to form drops, and apparently, the drop sizes and drift potential were more affected in the present study by surface tension than by viscosity.

Water has a high surface tension, which most often reduces the spread of droplets on plant surfaces after droplet deposition (Ferreira et al. 2020). Low surface tension and contact angle values indicate higher scattering levels on the treated surface (Iost and Raetano 2010). The addition of adjuvants in this study reduced surface tension, highlighting treatment PSC with the lowest values. Previous studies reported similar results, with a marked decrease in surface tension in grouts under treatment PSC (Iost and Raetano 2010; Costa et al. 2017). Regarding the contact angle, treatment LPA yielded the best results in reducing surface tension. Therefore, the mixtures composed of these adjuvants (PSC and LPA) have higher spreading capacity over the plants' leaf surfaces.

After solution formation, all treatments produced an alkaline pH between 8 and 9.5, except for LPA. However, after some time, these values decreased and ranged between 7 and 8. The optimal pH range for the germination of *C. fumosorosea* conidia is between 5 and 8 (Zhang et al. 2013). Thus, two hours after preparing the mixture, all tested adjuvants fell within this pH range. Treatment LPA was the only adjuvant outside this range, with a pH from 2.5 to 3.5. These low pH values are expected, as this adjuvant is indicated as an acidifier. Electrical conductivity (EC) in phytosanitary solutions measures the solution's ability to conduct an electrical current, determined by the concentration and composition of mineral salts dissolved in water as ions (Cortes et al. 2024). In addition to pH, the EC of spray solutions also varied among treatments, reflecting differences in the concentration of dissolved ions (Cunha et al. 2017). The LPA treatment showed notably higher EC values than the others, which may be related to its chemical composition. These physicochemical characteristics, such as pH and EC, may influence conidial survival and germination and should be considered when selecting adjuvants for use with entomopathogenic fungi (Souza et al. 2022).

Only treatment LPA exhibited physical compatibility with the entomopathogenic fungus. Thus, an additional test was conducted to evaluate whether the acidic pH would impact the survival of the biological agent. Although fungal viability decreased over time, probably due to the solution's acidification caused by adjuvant addition, this effect did not cause a significant reduction compared to the control. Therefore, despite its low pH and high EC values, the adjuvant used in treatment LPA presented favorable characteristics for application alongside the fungus *C. fumosorosea*. Previous research has reported that propionic acid and lecithin-based adjuvants are compatible with bioinsecticides based on *Bacillus thuringiensis* (gram-positive bacteria), facilitating bacterial growth and sporulation (Dos Santos et al. 2021). That supports the potential use of such adjuvants with bioinsecticides.

The findings help advance mycoinsecticide applications and agricultural pest management. Using adjuvants in the formulation of products containing the entomopathogenic fungus *C. fumosorosea* and their addition at the time of application in sprayer tanks may improve features that enable a more efficient application of the biological agent. Additional field studies are still recommended, along with efficacy verification in pest control.

5. Conclusions

Lecithin and propionic acid (LPA) and polyether and silicone copolymer (PSC) were the tested adjuvants that showed physicochemical compatibility with the entomopathogenic fungus *C. fumosorosea* (isolate IBCB 130). LPA revealed the best compatibility, as it did not reduce entomopathogen viability and exhibited higher uniformity and standardization of solution drops. PSC showed the lowest risk of susceptibility to drift and higher dispersion capacity across the plants' leaf surface.

Authors' Contributions: OLIVEIRA, S.C.: conception and design, acquisition of data, analysis and interpretation of data and drafting the article; VIGNA, F.B.: conception and design, acquisition of data and critical review of important intellectual content ; PACHECO, D.R.: conception and design, acquisition of data and critical review of important intellectual content ; FERREREIRA, M.C.: conception and design, acquisition of data and critical review of important intellectual content. All authors have approved the final version of the manuscript.

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