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Endemic Atlantic Forest species of Caryophyllales as inhibitors of viral infection in zucchini

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ABSTRACT

In Brazil, zucchini (*Cucurbita pepo*) is a socioeconomically important vegetable affected by damage caused primarily by zucchini yellow mosaic virus (ZYMV). Although the occurrence of cucumber mosaic virus (CMV) is less frequent, in *C. pepo* 'Caserta' plants it can cause symptoms such as mottle, mosaic, leaf and fruit distortion, as well as reduced plant development. To minimize the damage, the most widely used management technique is the preventive, albeit inefficient, application of insecticides, aimed at controlling aphids, the vectors of this virus. Thus, the search for more effective and less environmentally harmful control methods has been the target of investigations. The purpose of the present study was to assess the action of the extracts of four native Caryophyllales species, as inhibitors of infection by CMV and ZYMV in *C. pepo*, in addition to evaluating the possible induced resistance in this species. Fresh leaf extracts (LEs) of *Guapira opposita*, *Pisonia ambigua* (Nyctaginaceae), *Gallesia integrifolia* and *Seguieria langsdorffii* (Phytolaccaceae), previously assessed in the tobacco mosaic virus / *Nicotiana glutinosa* pathosystem, were submitted to progressive dilutions sprayed on cotyledonary *C. pepo* leaves 30 min before inoculation with CMV and ZYMV. Leaf extracts of *G. integrifolia* did not induce inhibition in any of the pathosystems assessed. *Guapira opposita* LEs inhibited the infection of plants inoculated with ZYMV below 50% but inhibited CMV infection by 70% at a concentration of 1:40. Given that leaf extracts of *P. ambigua* and *S. langsdorffii* induced high percentage inhibition, evident in the number of asymptomatic plants and confirmed by serological tests, these species were selected to assess induced resistance in pre-treatment experiments. The LEs were efficient in inhibiting ZYMV and CMV infection in *C. pepo* when applied up to 48 h before inoculation. The LEs of *S. langsdorffii* and *G. opposita*, also tested for this system, were efficient when applied up to 72 h before CMV inoculation. The LEs can be prepared from dry leaves and maintained at -20°C for at least three years, conserving their inhibitory activity. These results expand the possibilities for producers and consumers alike in the sustainable management of the main zucchini viruses, without damaging the environment.

Keywords: *Cucurbita pepo*, *Cucumovirus*, *Potyvirus*, alternative control, antiviral.

RESUMO

Espécies de Caryophyllales endêmicas da Mata Atlântica como inibidoras de infecção viral em abobrinha-de-moita

No Brasil, a abobrinha-de-moita (*Cucurbita pepo* L.) é uma olerícola de importância socioeconômica que tem sofrido prejuízos crescentes, devido principalmente aos danos causados pela infecção pelo zucchini yellow mosaic virus (ZYMV). Embora a ocorrência do cucumber mosaic virus (CMV) seja menos frequente, em plantas de *C. pepo* 'Caserta' ele pode causar sintomas como mosqueado, mosaico, distorção de folhas e frutos, além de redução do desenvolvimento das plantas. Para minimizar o prejuízo, a medida de manejo mais utilizada é a aplicação preventiva, porém ineficiente, de inseticidas, visando ao controle da população de afídeos, vetores desses vírus. Assim, a busca por formas de controle ambientalmente menos danosas tem sido alvo de investigação. Este estudo visou avaliar a ação de extratos foliares de espécies nativas de Caryophyllales, como inibidoras da infecção pelo CMV e ZYMV em *C. pepo* e como indutoras de resistência. Extratos de folhas frescas (EFs) de *Guapira opposita*, *Pisonia ambigua* (Nyctaginaceae), *Gallesia integrifolia* e *Seguieria langsdorffii* (Phytolaccaceae), previamente avaliados no patossistema tobacco mosaic virus / *Nicotiana glutinosa*, foram submetidos a diluições progressivas e pulverizados em folhas cotiledonares de *C. pepo*, 30 min antes da inoculação com o CMV e ZYMV. EFs de *G. integrifolia* não induziram qualquer inibição em nenhum dos patossistemas. EFs de *G. opposita* induziram inibição da infecção das plantas inoculadas com o ZYMV inferior a 50%. Na concentração de 1:40, inibiu a infecção das plantas pelo CMV em 70%. Como os extratos foliares de *P. ambigua* e *S. langsdorffii* induziram altas porcentagens de inibição, essas espécies foram selecionadas para avaliação de indução de resistência, em experimentos de pré-tratamentos. Os EFs inibiram a infecção de ZYMV e CMV em *C. pepo*, quando aplicados até 48 h antes da inoculação. Os EFs de *S. langsdorffii* e *G. opposita*, também testada para esse sistema, foram eficientes quando aplicados até 72 h antes da inoculação do CMV. Constatou-se que os EFs podem ser preparados a partir de folhas secas e mantidos a -20°C por, pelo menos três anos, conservando a sua atividade inibidora. Esses resultados expandem as possibilidades de manejo das principais viroses da abobrinha-de-moita, de maneira sustentável, sem causar danos ao ambiente, aos produtores e aos consumidores.

Palavras-chave: *Cucurbita pepo*, *Cucumovirus*, *Potyvirus*, controle alternativo, antiviral.

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According to a Brazilian Federal Official Gazette (DOU) publication, among the 474 agrochemicals authorized for commercialization, only 40 (8.4%) are classified as biological and legally sanctioned as organic agricultural inputs (DOU, Act No. 82, November 25, 2019). Given this scenario, Brazil and other countries that import Brazilian agricultural products find themselves at a serious impasse since the population is increasingly consuming healthier foods and prioritizing less industrialized products with as few agrochemicals as possible. Following this trend, the United Nations (UN) declared 2020 as the international year of plant health, making agribusiness increasingly visible and inducing producers to adopt alternative pest and disease control techniques, to guarantee phytosanitary and food security, thereby meeting the demands of the internal and external consumer market (UN, 2020).

Despite UN recommendations (2020), Brazil is one of the world's leading consumers of agrochemicals. São Paulo state reported the highest number of poisonings over the last ten years, totaling more than 8,000 cases (Tavares *et al*., 2020). One of the consequences of this excessive use is reflected in the induced resistance of insects and phytopathogens as well as the high residual concentrations of insecticide and fungicide molecules in the final product (Pedlowski *et al*., 2012). This has prompted debate and studies among specialists who warn of the harmful effects of these molecules on health and the environment (Mata, 2013; Starling *et al*., 2019).

The situation is worrisome, primarily in the vegetable segment, since most of the foods produced (leaves, flowers, and fruits) are consumed fresh or minimally processed (Sediyama *et al*., 2014).

Zucchini (*Cucurbita pepo*, Cucurbitaceae), one of the ten most widely produced seed-propagated crops in Brazil, stands out for its considerable socioeconomic importance yielding 65,711.4 t in 39 municipalities in São Paulo state, encompassing an area of 4,125.71 ha (IEA, 2019). Unfortunately, this crop is also noteworthy for the intensive application of insecticides, which are often recommended as an insect/vector control strategy to prevent virus infections. This control strategy, however, produces adverse environmental effects and has been increasingly questioned (Castle *et al*., 2009). Unfavorable environmental conditions, in addition to viral diseases and the lack of resistant cultivars, have negatively influenced zucchini production, discouraging farmers from maintaining production (Lecoq & Katis, 2014).

Among the ten virus species reported in cucurbits that can affect Brazilian zucchini production are zucchini yellow mosaic virus (ZYMV, family *Potyviridae*, genus *Potyvirus*) and cucumber mosaic virus (CMV, family *Bromoviridae,* genus *Cucumovirus*) (Lima & Alves, 2011; ICTV, 2017). The ZYMV and CMV are non-persistently transmitted by around 60 aphid species, which means that the viral particles are acquired and transmitted in seconds or minutes, during bite tests, adhering to the inner tip of the sucking apparatus of aphids (stylets). As such, insecticide application is not an efficient strategy to prevent viral dispersion in this crop (Eigenbrode *et al*., 2018).

ZYMV is restricted to cucurbits and was described in *Citrullus lanatus* (watermelon, Cucurbitaceae), in São Paulo state, in the early 1990s (Pavan *et al*., 2016). The virus has been increasingly reported in all cucurbit producing regions of Brazil (Yuki *et al*., 2000; Lima, 2011; Spadotti *et al*., 2015). Intense yellowing between the veins, mosaic pattern, blistering and extremely reduced and deformed leaf blades are symptoms that emerge after infection, causing the plant to atrophy. Early infection in the zucchini interferes in flowering and fruit production (Pavan *et al*., 2016). In addition, fruit deformations may cause damage during production and compromise seed quality.

Despite infecting several species of plants and spontaneous vegetation, the incidence of CMV is lower than ZYMV in cucurbits (Pavan *et al*., 2016). In general, CMV induces mottle, mosaic and leaf distortion symptoms in *C. pepo* 'Caserta' plants, as well as yellowing, reduced plant development and fruit distortion (Lima, 2011).

Given that insecticide application has proved inefficient in controlling ZYMV and CMV transmission by aphids (Eigenbrode *et al*., 2018), alternative methods such as the application of plant extracts with inhibitory action against viral infection may be a sustainable strategy for the zucchini (*C. pepo*) cultivation. This strategy can minimize the damage caused by the virus, protects producer health, prevent environmental waste, and enable the production of healthy foods. In this scenario, studies conducted with leaf extracts of Caryophyllales species have produced promising results.

Caryophyllales is one of the largest orders of eudicots (APG IV, 2016), with approximately 12,500 species distributed in 39 families (Hernández-Ledesman *et al*., 2015), including Nyctaginaceae and Phytolaccaceae, which contain species with widely-studied inhibitory activity against viral infection due to antiviral proteins (Grasso & Shepherd, 1978; Duarte *et al*., 2021). In addition to *Bougainvillea spectabilis* and *Phytolacca thyrsiflora*, which exhibited excellent results (Noronha *et al*., 1989; Duarte *et al*., 1990), viral inhibitors have been extensively studied only in *Celosia argentea* (Amaranthaceae), *Dianthus caryophyllus* (Caryophyllaceae), *Boerhaavia difusa*, *Mirabilis jalapa* and *B. spectabilis* (Nyctaginaceae) and *Phytolacca americana* (Phytolaccaceae) (Awasthi *et al*., 2015). Tozetto *et al.* (2002) described 60% inhibitory action of *Mirabilis jalapa* (Nyctaginaceae) leaf extract in *C. pepo* 'Caserta" / ZYMV pathosystem when applied up to 120 h before virus inoculation, and virus-infection inducible resistance has been associated with these extracts. Elbeshehy (2017) found 90% inhibition of ZYMV in watermelon (*Citrullus lanatus*, Cucurbitaceae) treated with LEs from *B. spectabilis* 48 h before inoculation, while Tozetto *et al.* (2002) demonstrated inhibitory activity of *M. jalapa* LE sprayed up to 120 h before ZYMV inoculation in *C. pepo* 'Caserta'. Bharathi (1999) showed that *M. jalapa* extract inhibits CMV infection in

Solanum melongena (eggplant) by 81, 64 and 44%, when applied 72, 96 and 120 h before inoculation, respectively.

Extracts obtained from several species of Caryophyllales were also efficient in reducing local symptoms caused by the following viruses: Amaranthaceae and Basellaceae / tobacco necrosis virus (TNV) / *Phaseolus vulgaris* (Smookler, 1971), Amaranthaceae, Nyctaginaceae, Petiveriaceae and Talinaceae / TMV / *N. glutinosa* (Noronha *et al*., 1980, 1983), Caryophyllaceae / TNV / *P. vulgaris* (Barakat & Stevens, 1980), and Amaranthaceae / TMV / *N. glutinosa* (Choi & Jung, 1984).

In the Atlantic Forest, an important biome in São Paulo state, Brazil (Ribeiro *et al.*, 2009), several Caryophyllales families can be found. However, few studies on these native species as inhibitors of viral infection have been conducted. Excellent results with *B. spectabilis* (Nyctaginaceae) and *Phytolacca thyrsiflora* (Phytolaccaceae) (Noronha *et al*., 1989; Duarte *et al*., 1990) have been reported. As such, other native species belonging to these families should be studied in depth as potential alternatives in the control of plant viruses affecting economically important crops, aimed at sustainable and eco-friendly production. In this context, the main goal of the present study was to assess the efficacy of leaf extracts from the species *Guapira opposita* and *Pisonia ambigua* (Nyctaginaceae) and *Gallesia integrifolia* and *Seguieria langsdorffii* (Phytolaccaceae), all endemic to the Atlantic Forest, as inhibitors of infections caused by CMV and ZYMV in *Cucurbita pepo* 'Caserta'. It should be noted that ZYMV was selected for inhibition tests, since it is one of the potyviruses that cause the most damage to zucchini culture in Brazil. Although the occurrence of the CMV is less frequent, in *C. pepo* 'Caserta' plants it can cause severe symptoms. In addition, CMV is also a model for understanding plant – virus interactions (Lima, 2011; Jacquemond, 2012).

MATERIAL AND METHODS

Species collection and leaf extract (LE) preparation

The investigation and species collection of *G. integrifolia*, *G. opposita*, *P. ambigua,* and *S. langsdorffii* (Figure 1) were conducted based on IAC Herbarium data (http://herbario.iac.sp.gov.br), with records from the municipality of Campinas, São Paulo state. Two Atlantic Forest fragments from the semideciduous seasonal forest physiognomy were selected for sampling effort, namely Mata Santa Elisa (22°51'S, 47°04'W) and Bosque dos Jequitibás (22°55'S, 47°03'W). Exsiccates that registered species occurrence in the area were deposited in the IAC herbarium, under numbers 46457 to 46459 (*G. opposita),* 46560 (*G. integrifolia),* 36471 and 46559 *(P. ambigua)* and 56571 and 56722 *(S. langsdorffii).*

The extracts were prepared from 1 g fresh leaves ground with 5 mL 0.01M phosphate buffer, pH 7.0, added with 0.5% sodium sulfite (inoculation buffer - IB), using an ultra-turrax homogenizer. The crude extracts were filtered through gauze.

Preliminary assessment of the viral inhibition effect using the half leaf test

In order to assess the viral inhibition potential of aqueous extracts in a virus x local host system, the half leaf experiment was conducted by mechanically inoculating the indicator species *Nicotiana glutinosa* (Solanaceae) with viral isolate from tobacco mosaic virus (TMV, *Tobamovirus*). This system was used because *N. glutinosa* plants develop a hypersensitivity reaction when infected with TMV isolates, inducing local necrotic lesions. To perform the test, the aqueous extracts (1:5 dilution, w/v) of the four species were added with an equal volume of purified TMV solution (200 µg/mL), in order to obtain a final inhibitor extract concentration of 1:10 (v/v) and 100 µg/mL of purified solution of the virus (treatment). Fifty µL aliquots of leaf extract + TMV were rubbed on the left half of the *N. glutinosa* leaf. For controls, 50 µL of the purified virus (100 µg/mL) were applied to the right half. After local necrotic lesions appeared, they were counted to determine the percentage inhibition (PI), calculated based on the formula: 100 − (T/C x 100), where T = number of lesions on the left (treatment) and C = number of lesions on the right (controls).

Obtaining viral isolates and inoculum preparation

Isolates used for viral infection inhibition tests in a virus x systemic host system, previously identified and characterized, are part of the collection of the Laboratório de Fitovirologia e Fisiopatologia Karl M. Silberschmidt, Instituto Biológico. ZYMV and CMV isolates were obtained from naturally infected *Lagenaria siceraria* (calabash) and *Melothria pendula* (creeping cucumber) plants, respectively, intercropped with *Cucurbita pepo* 'Caserta' (zucchini) in the municipality of Monte Alegre do Sul-SP (22°40'55"S, 46°40'51"W). To conduct the inhibition tests, the viral inocula were standardized at a concentration (w/v) of 1 g infected leaves per 3 mL of IB.

Determination of the highest dilution with leaf extract (LE) activity

The crude extracts, prepared from the leaves of four Caryophyllales species, were submitted to progressive dilutions up to 1:1,280 (v/v), for the ZYMV / *Cucurbita pepo* pathosystem and 1:640 (v/v), for CMV / *C. pepo* with IB.

Cucurbita pepo 'Caserta' seedlings, obtained after planting, were used for the test. The different extract dilutions were sprayed on the cotyledonary leaves and after 30 min, 30 µL of viral inoculum were applied, using 10 plants for each dilution. The cotyledonary leaves of 10 control plants were applied with 30 µL of inoculum. The plants with visible systemic symptoms were counted fifteen days after inoculation in order to establish percentage inhibition, calculated based on the formula: 100 − (T/C x 100), where T = number of symptomatic plants treated with LEs, and C = the number of symptomatic control plants. In addition, leaf samples from treated plants and controls were collected for subsequent serological

testing.

Assessment of induced resistance in *C. pepo*

With a view to assessing induced resistance in *C. pepo*, the LEs from the species exhibiting the greatest viral inhibition potential were sprayed on the cotyledonary leaves of 10 *C. pepo* plants 24, 48, 72 and 96 h before mechanical inoculation with 30 µL of ZYMV and CMV isolates at a concentration of 1:3 (w/v), which was rubbed on each plant.

The plants were kept in a greenhouse and visually inspected for 15 days to observe the induction or not of symptoms and determine percentage inhibition, calculated as explained in the previous item. Leaf samples were collected for subsequent serological testing.

Assessment of maintenance of the inhibitory properties of leaf extracts at different temperatures and preparation methods

Pisonia ambigua and *S. langsdorffii* LEs, the most efficient in all treatments in both pathosystems, were selected to evaluate a number of properties, such as the maintenance of antiviral activity when prepared using different methods and stored at different temperatures. Thus, *P. ambigua* and *S. langsdorffii* leaves were stored at room temperature away from light and humidity for around 3 months. After drying, aqueous extracts were prepared at a concentration of 1:40 (w/v), diluted at 1:80 and 1:160 and tested in the *C. pepo* / ZYMV pathosystem. In addition, we tested fresh leaf extracts at a concentration of 1:40 (w/v), kept in sealed flasks at room temperature away from light and humidity for 7 days. LEs stored at -20ºC for 3 years were also tested. Six plants per treatment and six controls were used.

The plants were kept in a greenhouse and symptoms assessed for 15 days to determine the percentage inhibition. At the end, leaf samples from all plants were collected for subsequent serological detection.

Serological detection

Viral infection was confirmed by the enzyme-linked immunosorbent assay (ELISA), using the specific panel of polyclonal antiserum against the coat protein of ZYMV and CMV. In all treatments, analyses were carried out in the symptomatic and asymptomatic *C. pepo* leaf extracts, as well as those of control plants. ZYMV was detected by Double Antibody Sandwich - ELISA (DAS-ELISA), according to the manufacturer's protocol (AGDIA®) and CMV by Plate Trapped Antigen – ELISA (PTA-ELISA) with antiserum belonging to the serum bank of the Laboratório de Fitovirologia e Fisiopatologia, Instituto Biológico. Samples with absorbance reading values three times higher than those of negative controls (healthy samples) were considered positive.

Statistical analyses

Statistical tests using a completely randomized design were conducted to compare the absorbance values of an ELISA assay of *C. pepo* 'Caserta' leaf samples treated with extracts from different Brazilian native species at different dilutions and periods, 15 days after CMV and ZYMV inoculation. Sampling involved 10 replicates per treatment. The absorbance values of the uninfected plants (negative results) were used in statistical analyses and treatments, where fewer than three uninfected plants were not considered. Multiple comparisons were performed by Welch's Analysis of Variance (ANOVA) followed by the Games-Howell test. P-values below 0.05 were considered significant. All statistical analyses were carried out using R software (version 4.0.0) and bar graphs were constructed using Excel.

RESULTS AND DISCUSSION

The half leaf experiment in the *N. glutinosa* / TMV pathosystem revealed the high inhibitory power of the extracts from the four native species used in this study, with values above 70% (Table 1). Efficient reduction of local symptoms has been described for several extracts of Caryophyllales species (Smookler, 1971; Noronha *et al*., 1980, 1983; Barakat & Stevens, 1980; Choi & Jung, 1984).

Due to the high efficiency observed in the local system, the LEs from the four species were tested at different dilutions, in two systemic pathosystems involving zucchini (*C. pepo* 'Caserta') and the CMV and ZYMV viral isolates.

Extracts prepared with *P. ambigua* and *S. langsdorffii* were the most efficient in the two pathosystems. In *C. pepo* 'Caserta' / ZYMV, viral infection percentage inhibition greater than or equal to 70% was observed up to a 1:640 dilution (Figure 2A). Similar inhibitions were obtained by Tozetto *et al.* (2002). In the case of the *C. pepo* 'Caserta' / CMV pathosystem, the same efficiency as *P. ambigua* and *S. langsdorffii* LEs was detected only at 1:40 dilution (Figure 2B). In contrast to that observed with ZYMV infection, the LE of *G. opposita* was efficient in inhibiting CMV infection in *C. pepo* in 70% of the plants, but only at 1:40 dilution. Awasthi *et al*. (2015) reported that ten-fold dilutions of the extracts dramatically reduced the inhibitory activity of viral infection. The results found in the present study contradict this finding. The distinct efficiency of *G. opposita* LE in inhibiting CMV and ZYMV infections in *C. pepo* reinforced the hypothesis that the inhibitory effect of any LEs is highly dependent on the virus/host interaction. The LE of *G. integrifolia* was the less efficient in the two pathosystems, reaching a maximum percentage inhibition of 10% (Figure 2A).

Table 1. Percentage inhibition (PI) of local lesions induced in the tobacco mosaic virus (TMV)/*Nicotiana glutinosa* pathosystem applying aqueous leaf extracts from native species. São Paulo, IB, 2019.

Species/Family	Average no. of lesions		PI (%)
	Control	Treatment	
Guapira opposita/Nyctaginaceae	36	1.7	95.3
Pisonia ambigua/Nyctaginaceae	11.5	3.2	72.0
Gallesia integrifolia/Phytolaccaceae	19.2	4.9	74.5
Seguieria langsdorffii/Phytolaccaceae	34.4	4.6	86.6

The percentage inhibition results of CMV and ZYMV were reinforced when compared to those obtained by DAS-ELISA (Figures 2C and D), which is highly sensitive in detecting minimal amounts of antigen. The absorbance readings of the viral load in plants were significantly lower in the samples treated with LEs when compared to controls. Serological assay (DAS-ELISA) results also showed lower absorbance values for pokeweed (*P. americana* treated *N. tabacum* samples when compared to PVY-infected control plants, indicating lower levels of PVY infections in the former (Alishiri & Rakhshandehroo, 2015). The results of ZYMV inhibition by extracts of the four species revealed that *S. langsdorffii* was more efficient in inhibiting viral infection, since only individuals treated with 1:640 and 1:1,280 diluted extracts were found to be infected.

The LEs at 1:20 dilution of *S. langsdorffii* and *P. ambigua* were assessed for possible induced resistance in the two pathosystems, while the LE of *G. opposita* was evaluated only for the CMV / *C. pepo* interaction. Since no inhibitory effect on viral infection was observed with *G. integrifolia* LE, it was not evaluated as a resistance inducer in *C. pepo* plants.

For the ZYMV / *C. pepo* pathosystem, the LEs of *S. langsdorffii* and *P. ambigua* obtained similar results at time intervals of 24 and 48 h, with inhibitions greater than or equal to 80%. However, when applied 72 or 96 h before inoculation, inhibition was less than 30% (Figure

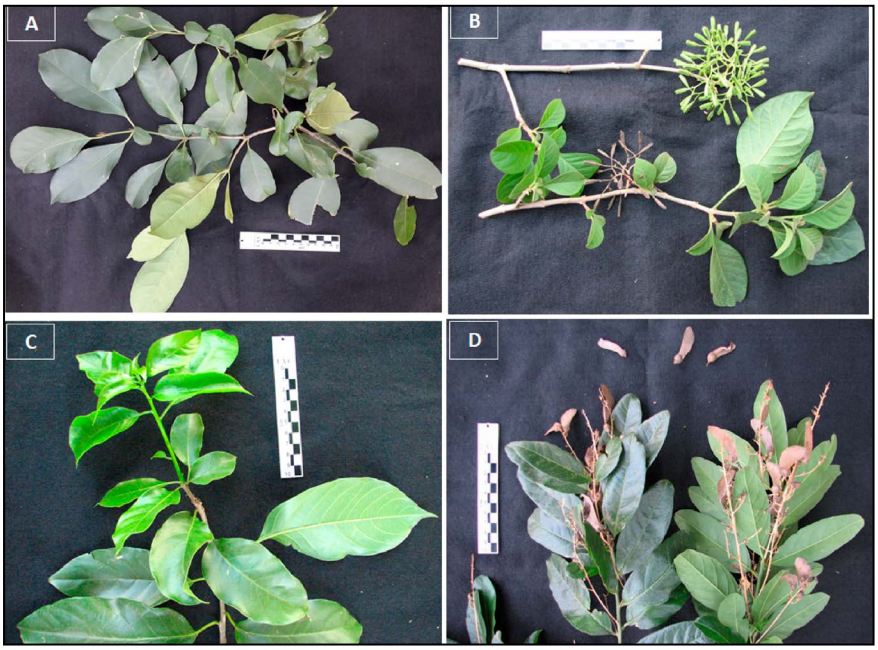

Figure 1. Branches of Caryophyllales species endemic to the Atlantic Forest selected to obtain an aqueous solution for viral inhibition tests. **(A)** *Guapira opposita* and **(B)** *Pisonia ambigua* (Nyctaginaceae), **(C)** *Gallesia integrifolia*, and **(D)** *Seguieria langsdorffii* (Phytolaccaceae). Ruler = 10 cm. São Paulo, IB, 2019.

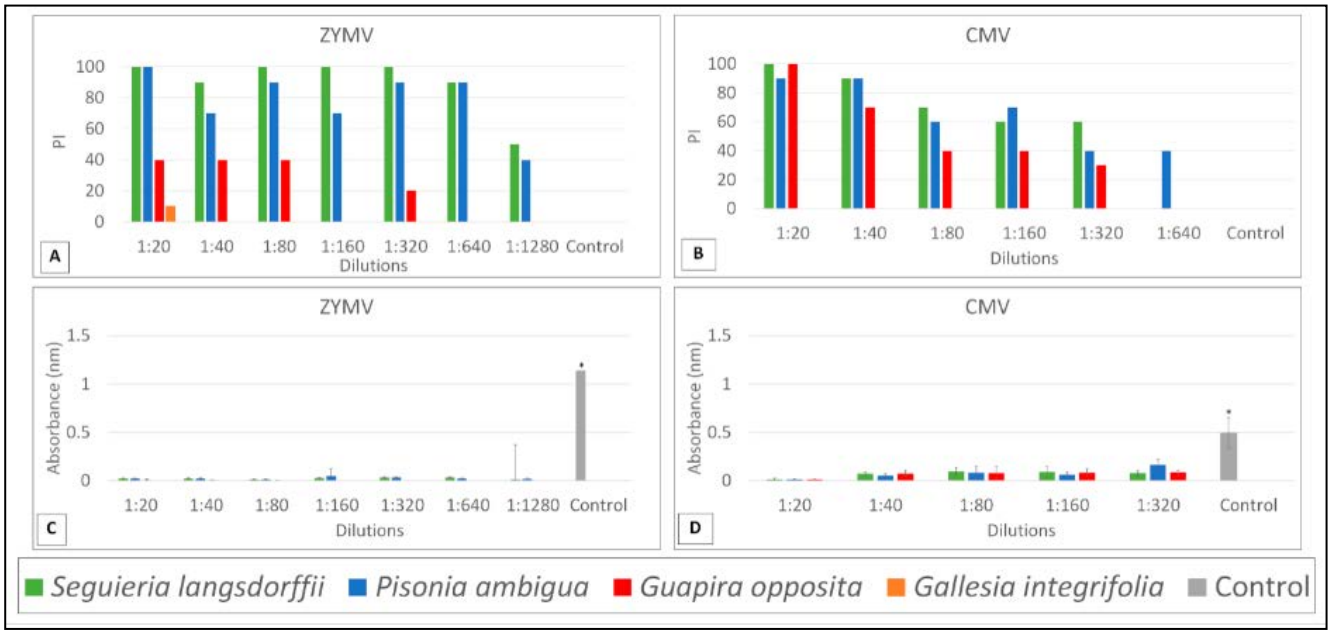

Figure 2. Percentage inhibition (PI) of leaf extracts from *Gallesia integrifolia*, *Guapira opposita*, *Pisonia ambigua* and *Seguieria langsdorffii* in different dilutions against systemic viral infection: **(A)** zucchini yellow mosaic virus (ZYMV) / *Cucurbita pepo* 'Caserta' pathosystem, **(B)** cucumber mosaic virus (CMV) / *C. pepo* 'Caserta' pathosystem. Absorbance readings values at 405 nm, after serological tests of *C. pepo* samples treated with leaf extracts from native species, in different dilutions and inoculated with **(C)** ZYMV and **(D)** CMV. Statistical analysis was performed only for treatments that presented at least three non-infected individuals compared to those of the infected individuals of the control. For multiple comparison, Anova-Welch followed by Games-Howell test was applied. The asterisks indicate significant differences. São Paulo, IB, 2019.

3A). A similar result was reported by Elbeshehy (2017) with watermelon (*Citrullus lanatus*, Cucurbitaceae) / ZYMV pathosystem. On the other hand, *M. jalapa* LE was more efficient against ZYMV inoculation in *C. pepo* 'Caserta' (Tozetto *et al*., 2002).

In addition, as observed in the test to determine maximum dilution, significantly lower absorbance readings were found in samples with high percentage inhibition (24 and 48 h) for the ZYMV / *C. pepo* pathosystem when compared to controls (Figure 3C), corroborating viral infection inhibition.

Similar to the ZYMV / *C. pepo* pathosystem, *S. langsdorffii* extract was very efficient in inducing resistance to CMV, with 85, 70 and 57% inhibition at time intervals of 24, 48 and 72 h, respectively (Figure 3B). High inhibitory efficiency with longer time intervals was observed with *M. jalapa* extract in the *Solanum melongena* (eggplant) / CMV pathosystem (Bharathi, 1999). In the case of *P. ambigua* LE, the highest percentage inhibitions were observed in samples pre-treated 24 and 48 h before viral inoculation, similar to that observed for the ZYMV / *C. pepo* pathosystem. The LE from *G. opposita* exhibited significant results in the CMV / *C. pepo* pathosystem, similar to that obtained with LE from *S. langsdorffii* when applied 24, 48 and 72 h before viral inoculation. In the 96-h pre-treatment, the percentage inhibition obtained with this extract (41%) was higher than that of the two other species (Figure 3B).

Although the multiple comparison performed between absorbance values from zucchini leaves pre-treated with LEs of *S. langsdorffii*, *P. ambigua* and *G. opposita* and non-treated leaves (control) 48 (24, 48 and 72 h) 72 h before CMV inoculation detected differences (Figure 3D), the Games-Howell post-hoc test was not robust enough to detect where these differences occurred. The *p*-values between the LEs from the three species and the controls were marginal ($0.07 \leq p \leq 1.00$), but higher compared with *p*-values obtained from the comparisons between LEs. As such, the differences detected by Welch's ANOVA were more likely to occur between LEs and the controls. Comparison of this result with LE application 24 and 48 h before ZYMV inoculation (Figure 3C) demonstrated a more pronounced difference, which was confirmed by the lower *p*-values (≤ 0.0001). The comparisons between different times only detected a difference between the 24 and 72 h values of leaves pre-treated with *S. langsdorffii* LEs; however, the *p*-value ($p = 0.044$) was close to the 0.05 value that is usually adopted in statistical analyses as the threshold for a significant difference.

The decline in the number of infected plants in the two pathosystems (ZYMV / *C. pepo* and CMV / *C. pepo*) after pre-treatment with the LEs from these native Caryophyllales species, indicates possible induction of the plant defense system, increasing the resistance of these plants to a pathogen. Elbeshehy (2017) previously suggested that LEs from *B. spectabilis* must perform an essential function in the acquired systemic resistance mechanism, with likely pathogenesis-related (PR) protein production. Analyses are being conducted to determine whether PR protein production occurs in plants pre-

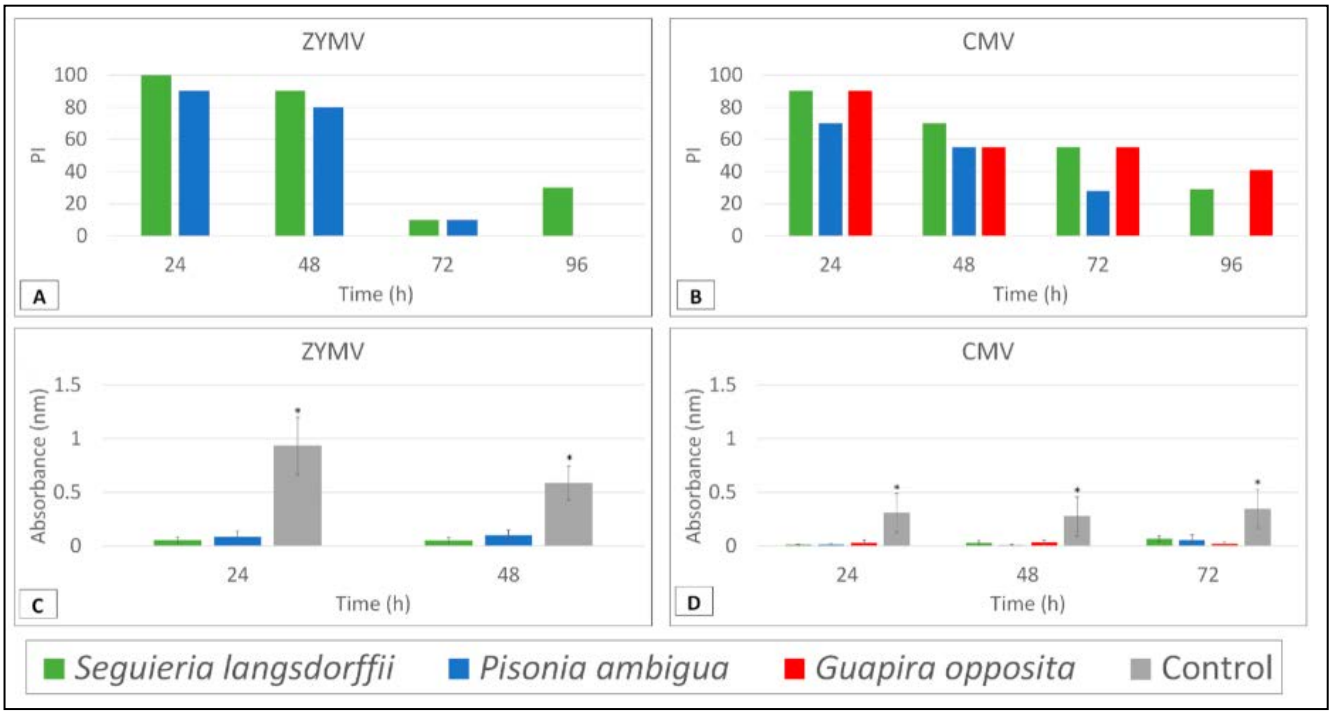

Figure 3. Percentage inhibition of leaf extracts from native species sprayed at different times before inoculation with **(A)** zucchini yellow mosaic virus (ZYMV) and **(B)** cucumber mosaic virus (CMV) in *Cucurbita pepo* 'Caserta'. Average absorbance readings at 405 nm, after serological tests of *C. pepo* samples treated with leaf extracts from *P. ambigua* and *S. langsdorffii*, at different times and inoculated with **(C)** ZYMV and **(D)** CMV. Leaf extract of *G. opposita* was also applied at different times before inoculation with CMV **(B, D).** The asterisks indicate significant differences. São Paulo, IB, 2019.

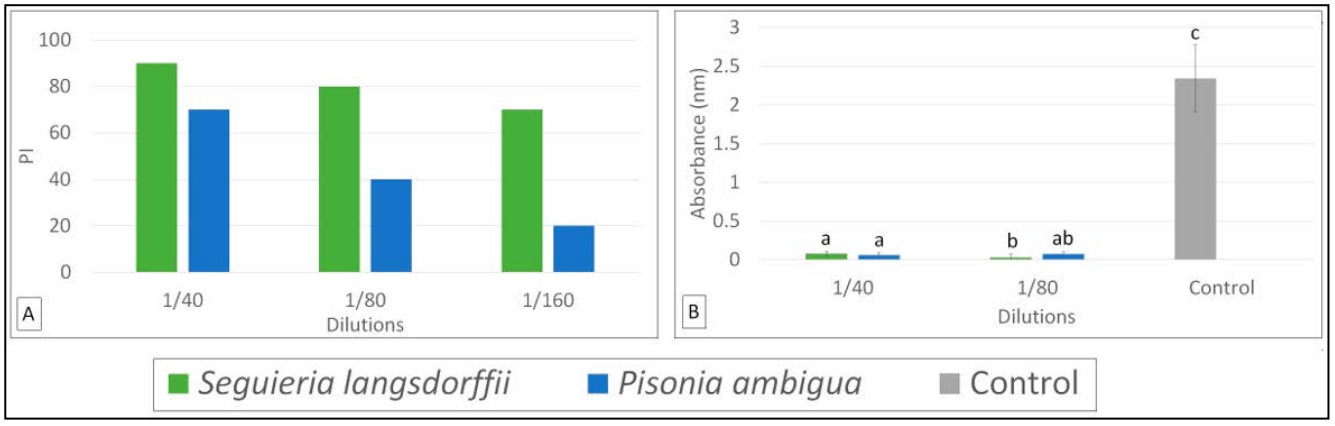

Figure 4. (A) Percentage inhibition of leaf extracts from native species sprayed 30 minutes before inoculation with zucchini yellow mosaic virus (ZYMV) in *Cucurbita pepo* 'Caserta'. Extracts prepared with dry leaves and applied in different dilutions. **(B)** Average absorbance readings at 405 nm, after serological tests of *C. pepo* samples treated with leaf extracts from *P. ambigua* and *S. langsdorffii*. Different letters over each bar correspond to significant differences at 0.05%, according to Games-Howell test. São Paulo, IB, 2019.

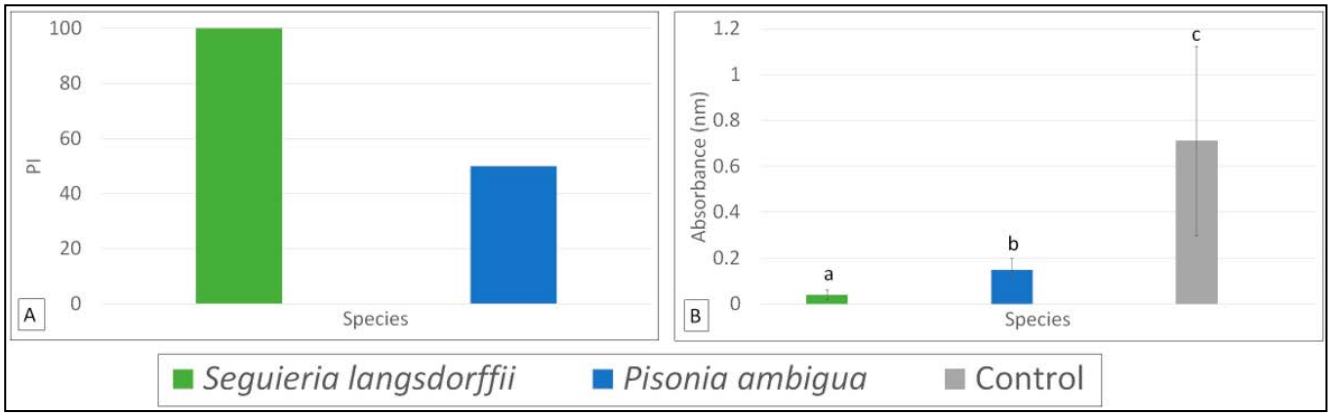

Figure 5. (A) Percentage inhibition of leaf extracts from native species stored for 3 years at -20°C and applied 30 minutes before inoculation with zucchini yellow mosaic virus (ZYMV) in *Cucurbita pepo* 'Caserta'. Extracts prepared with fresh leaves. **(B)** Average absorbance readings at 405 nm, after serological tests of *C. pepo* samples treated with leaf extracts from *P. ambigua* and *S. langsdorffii*. Different letters over each bar correspond to significant differences at 0.05%, according to Games-Howell test. São Paulo, IB, 2019.

treated with extracts from native species.

Surveys in Brazil have shown that ZYMV occurrence is uninterrupted and prevalent in zucchini (Lima & Alves, 2011). Thus, stability and storage tests were conducted to provide information to zucchini producers on obtaining and storing leaf extracts. The LEs prepared from dry leaves of *P. ambigua* and *S. langsdorffii*, at 1:40, 1:80 and 1:160 dilutions (w/v) were evaluated in the ZYMV / *C. pepo* pathosystem. The LE from *S. langsdorffii* exhibited inhibitory activity in the three dilutions (90, 80 and 70%, respectively), while the LE from *P. ambigua* induced 70% inhibition at a concentration of 1:40 and lost inhibition capacity as dilution increased (Figure 4A). While the inhibition performance of dry leaf extract of *S. langsdorffii* was the same as that observed for fresh leaf extract (Figure 2A), it was not detected for *P. ambigua*. Rasoulpour *et al*. (2018) also found that the inhibitory effect of the aqueous extract obtained from the dry clades of *Opuntia ficus-indica* (Cactaceae, Caryophyllales) against CMV infection in the fava bean was maintained.

Similar to fresh leaf extracts, there was a reduction in viral particles in samples with great inhibitory effect using dry leaf extracts. No significant difference between the 1:40 and 1:80 dilutions of *P. ambigua* LEs was observed, and the 1:80 dilution of *S. langsdorffii* LE was lower than 1:40 (Figure 4B). Thus, extracts prepared from the dry leaves of these two species at a dilution of up to 1:80 can be recommended to zucchini producers as a management strategy for disease caused by ZYMV.

With respect to storage tests, the inhibitory effect of ZYMV infection of zucchini was maintained for at least 3 years in extracts stored at -20°C (Figure 5). The efficiency of *S. langsdorffii* LE was as good as that of fresh crude extract (Figure 2A), but a decrease in virus-infection inhibition of more than 50% was detected with stored *P. ambigua* LE. On the other hand, *S. langsdorffii* LE kept for 7 days at room temperature inhibited ZYMV infection in *C. pepo* only up to 40%, demonstrating a significantly lower viral load than that of controls (data not shown). *P. ambigua* LE kept at room temperature oxidized, precluding this

test. Comparison of the absorbance values from non-infected samples of plants treated with LEs revealed that plants treated with *P. ambigua* LEs obtained higher values than those treated with their *S. langsdorffii* counterparts. However, both LEs showed lower absorbance values when compared to the controls (Figure 5B), reinforcing virus inhibition.

According to Awasthi *et al*. (2015), inhibitory activity was lost after one week storage for several of the extracts tested. However, *M. jalapa* LE remained active for up to one month, reaching percentage inhibition of eggplant infection by CMV of 100, 88, 75, 50, 44 and 0% when stored for 4, 8, 12, 16, 20 and 24 days at room temperature, respectively (Bharathi, 1999).

Our results indicate that three of the four native species exhibited excellent viral infection inhibitory potential for the two pathosystems tested. For the ZYMV / *C. pepo* pathosystem, it is recommended that leaf extracts from *P. ambigua* and/or *S. langsdorffii* should be applied at a concentration of 1:40, at 48 h intervals. On the other hand, for the CMV / *C. pepo* pathosystem, leaf extracts from *G. opposita* and *S. langsdorffii* can be applied at 72 h intervals. In addition, the extracts can be prepared with dry leaves from *S. langsdorffii* in dilutions of up to 1:160, kept at -20°C for up to 3 years, without losing their capacity to inhibit ZYMV infection in *C. pepo.* Thus, when the two pathosystems were jointly assessed, the *S. langsdorffii* extract obtained the best results.

Field experiments confirmed the economic feasibility of applying Caryophyllales extract in virus disease control. Zuchini plants treated with *B. spectabilis* and *M. jalapa* leaf extracts at cotyledon emergence and sprayed at 72 h intervals until flowering, achieved significantly higher fruit yield than that of control plants, even after ZYMV infection (Duarte *et al*., 2021).

Although not all the mechanisms of action of viral infection inhibitory compounds are known, the results presented here showed that the leaf extracts from *P. ambigua*, *S. langsdorffii* and *G. opposita*, not previously tested, exhibit excellent potential as resistance inducers and deserve more in-depth field study, in order to assess the viability of their application in minimizing the damage caused by plant viruses.

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