Phytoprotective film for resistance induction, growth, and yield of organic strawberries

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Key words: Colletotrichum spp., enzymatic activity, Fragaria x ananassa, Induced systemic resistance, K2HPO4.

Abstract: The objective of this work was to evaluate a phytoprotective film of chitosan-pyrolygenic extract in promoting growth, productivity, induction of systemic resistance in strawberry cultivars managed in an organic production system. Treatments consisted of rates (0, 25, 50, and 100 mL L⁻¹) of Chi-Pyro-Film and a reference resistance inducer (dipotassium hydrogen phosphate - K2HPO4), evaluated in three strawberry cultivars (‘Albion’, ‘San Andreas’ and ‘Portola’). Growth, yield, anthracnose incidence, and enzymatic activity were evaluated. The experimental design was a randomized block design with four replications. Chi-Pyro-Film increases the growth, yield, and anthracnose resistance of strawberry plants. The best concentration of Chi-Pyro-Film varies between 50 and 60 mL L⁻¹, according to strawberry cultivar.

1. Introduction

Strawberry (Fragaria x ananassa Duch.) is the most planted red fruit in Brazil. The high demand is due to its sensory characteristics such as color, texture, aroma, and taste (Ventura-Aguilar et al., 2018).

However, according to a report by the Brazilian Health Surveillance Agency (ANVISA) of 2016, 26% of strawberry samples collected between 2013 and 2015 presented nonconformities with pesticide residues. Pesticides detected not authorized for the crop, captan stood out, detected in 20.4% of the samples analyzed, among others such as dithiocarbamates, pyrimethanil, carbendazim, tebuconazole, iprodione, and azoxystrobin (ANVISA, 2016). Most of these fungicides aim to control or prevent anthracnose (Colletotrichum spp.), a disease that is considered main in strawberry fields in Brazil (Kososki et al., 2001; Capobiango et al., 2016).

Currently, there is a search for technologies, which make agriculture sustainable and “smart”, with practices and ways to minimize the excessive use of chemicals (Grewal et al., 2018). In this context, the use of resistance inducing products activates the plant’s natural defenses, enabling disease control in the organic production system.
The resistance is associated with various defense responses, such as protein and phytoalexin synthesis, cell wall changes, and increased activity of various enzymes defense-related (Durrant and Dong, 2004). Responses that are associated with changes in the activity of various enzymes such as peroxidase (POX, EC 1.11.1), polyphenol oxidase (PPO, EC 1.14.18.1), and phenylalanine ammonia-lyase (PAL, EC 4.1.3.5) (Prasannath et al., 2014; Prasannath, 2017).

POXs have been implicated in many defense processes, such as hypersensitive response, lignification, phenolic and glycoprotein cross-linking, suberization, and phytoalexin production (Thakker et al., 2012; Prasannath, 2017). PPOs are a group of enzymes that catalyze the oxidation of hydroxyphenols to their quinone derivatives, which have antimicrobial properties (Prasannath, 2017). PAL (EC 4.1.3.5) is the major enzyme in the phenylpropanoid pathway and acts in the synthesis of defense-related secondary compounds such as phenols and lignins (Hemm et al., 2004; Vanitha et al., 2009).

Among the compounds that have activating properties of defense mechanisms in plants are the chitosan and pyroligneous extract (Di Piero and Garda, 2008; Grewal et al., 2018; Souza et al., 2018).

Chitosan is a polycationic β-1,4 polymer bound to D-glucosamine chemically derived from crustaceans and soluble in organic acids and known to be a natural elicitor and triggers various physiological and biochemical responses in plants that act in the growth, production, and protection against disease (Chandra et al., 2015; Katiyar et al., 2015; Pichyangkura and Chadchawan, 2015). Chitosan has several characteristics that make this polymer advantageous for many applications: (1) has a defined chemical structure; (2) may be chemically and enzymatically modified; (3) is physically and biologically functional; (4) is biodegradable and biocompatible with many organs, tissues and cells; (5) can be processed into various products including flakes, powders, membranes, fibers and films (Badawy, 2012; van den Broek et al., 2015; Porto et al., 2019).

The pyroligneous extract is a liquor with strong smoke flavors, is a crude and acid condensate produced from the distillation of the smoke generated in the carbonization of wood. It consists of a complex mixture of compounds derived from the chemical decomposition of wood components through the condensation of vapors and gases generated during pyrolysis in a low oxygen concentration (Campos, 2018; Pimenta et al., 2018).

The pyroligneous extract is composed of water (80-90%) and more than 200 species of organic compounds (10-20%) (Theapparat et al., 2018). The presence of phenolic compounds in the pyroligneous extract confers growth-promoting and antifungal properties, as found in the literature on *Helminthosporium sativum*, *Cochliobolus sativus*, *Waltz*, *Colletotrichum orbiculare*, *Alternaria mali* (Jung, 2007; Baimark and Niamsa, 2009; Wei et al., 2010; Grewal et al., 2018).

In this context, the cationic character of chitosan in acidic conditions offers the possibility to establish electrostatic interactions with other negatively charged compounds, for example with the pyroligneous extract, considered a raw material obtained from renewable sources, and is a good solvent for chitosan (Campos et al., 2012; van den Broek et al., 2015; Porto et al., 2019).

The phytoprotective film of chitosan-pyroligneous extract (Chi-Pyro-Film) consists of the chitosan diluted in the pyroligneous extract, and its characteristics are the formation of a film with photoprotection capability against radiation (UV-B and UV-C), fungi toxic action, and inducing systemic resistance in plants (Campos et al., 2012).

The objective of the present work was to evaluate the effect of different concentrations of phytoprotective film formulated with chitosan and pyroligneous extract (Chi-Pyro-Film) and dibasic potassium phosphate (K$_2$HPO$_4$) on growth promotion, resistance induction to anthracnose, yield, and defense enzyme activity in strawberry cultivars in the organic production system.

2. Materials and Methods

The study was carried under field conditions, at Embrapa Temperate Climate Experimental Station, in Pelotas city, Rio Grande do Sul state, Brazil (31°40’49” S 52°26’18” O, at 60 m altitude) (Fig. 1 a). The climate of the region, according to the Köppen classification is Cfa type, temperate and humid, with hot summers.

**Raw materials**

The pyroligneous extract was obtained through an extraction procedure of *Eucalyptus grandis* proposed by Campos (2018) and the distillation process was performed according to that described by Porto et al.
Dos Santos Pereira et al. ‐ Resistance induction in strawberries cultivars (2019). Chitosan was supplied by Nutrifarm™, with a 97% degree of deacetylation, determined by proton magnetic resonance (Porto, 2011).

**Plant material**

The seedlings of neutral-day strawberry cultivars, ‘Albion’, ‘San Andreas’ and ‘Portola’, were planted in May and were grown under a low tunnel system with mulching and drip irrigation. The spacing between lines and between plants was 0.30 m, with three lines per bed. The area had a history of many years with severe anthracnose incidence in the strawberry plants (Fig. 1b, 1c). Liming and fertilization were performed according to the recommendation for strawberry organic production. Climatic data from the experiment period are shown in figure 1d.

**Treatments**

The treatments of induction of systemic resistance consisted of four concentrations of the Chi-Pyro-Film and a reference treatment with dibasic potassium phosphate (K₂HPO₄). The Chi-Pyro-Film was registered in the field of Green Chemistry at the National Institute of Intellectual Property in Brazil (PCT/BR2013/000597), United States (US2015036854A1) and Germany (DE112013006230T5) as a phytoprotective for agriculture use.

The Chi-Pyro-Film with a concentration of 30 g L⁻¹ (Fig. 2) was diluted with distilled water in different concentrations (0, 25, 50, and 100 mL L⁻¹). Treatment with K₂HPO₄ was applied at a concentration of 50 mM (Orober et al., 2002; Aleandri et al., 2010). This compound has shown efficacy against e.g. powdery mildew on barley, cucumber, pepper, and tomato (*Blumeria graminis* f. sp. hordei, *Sphaerotheca fuliginea*, *Leveillula taurica*, and *Erysiphe oronti*, respectively), anthracnose (*Colletotrichum lagenarium*) on cucumber, rust (*Puccinia sorgi*) and leaf blight (*Exserohilum turcicum*) on maize and rice blast (*Pyricularia oryzae*), mildew (*Sphaerotheca fuliginea*) (Reuveni et al., 1996; Reuveni and Reuveni, 1998; Manandhar et al., 1998; Reuveni et al., 2000; Ehret et al., 2002; Orober et al., 2002; Hamza et al., 2017).

The spraying of treatments started at the beginning of fruiting, and the application dates were indicated in figure 1d. The spray volume was 4.5 mL per plant (500 liters per hectare), applied through conical jet nozzles, observing a total coverage of the plants until close to the runoff point.

**Analysis of plant growth, damage by anthracnose, and fruit yield**

Vegetative growth evaluations consisted of dry mass (g plant⁻¹) of crown, root, and leaf, by drying to constant weight (65°C) of three plants collected in each experimental unit at the end of the production cycle, 256 days after seedlings transplanting. The productive variables measured were fruit yield (g plant⁻¹) and fruit weight (g fruit⁻¹), obtained by evaluating the total fruits harvested in each experimental unit. The percentage of fruits with anthracnose was obtained by counting the fruits with symptoms at each harvest (Fig. 1c). The harvests were carried out three times a week between October and January.

**Enzymatic activity analysis**

Biochemical evaluations were realized by determination of the specific activity of peroxidase (POX),
3. Results

Chi-Pyro-Film concentrations had a significant effect on the growth, yield, anthracnose resistance induction, and enzymatic activity of strawberry plants.

Growth and development

The dry mass of the plant showed factors interaction. The three cultivars showed a quadratic response to Chi-Pyro-Film concentrations (Fig. 3), but the highest efficiency concentration was different. ‘Albion’ and ‘Portola’ had the highest efficiency concentration estimated at 60 mL L⁻¹ of Chi-Pyro-Film, while for ‘San Andreas’ the concentration was 50 mL L⁻¹ (Fig. 3). The treatment with reference resistance inducer, K₂HPO₄, had also different according to cultivars. In ‘Albion’, it provided a leaf mass slightly higher than the control treatment and similar to the 100 mL L⁻¹ Chi-Pyro-Film concentration, but it was lower than 25 and 50 mL L⁻¹ (Fig. 3).

POX and PPO activity was determined in the enzyme extract mix with a phosphate-citrate buffer composed of 0.2 M sodium phosphate solution and 0.1 M citric acid (pH 5.0).

The mixture was homogenized in vortexed for 15 seconds. POX activity was determined according to Campos et al. (2004).

PPO activity was determined in the enzyme extract with 3.6 mL of 0.05 M phosphate buffer (pH 6.0) and 0.1 mL of 0.1 M catechol. PPO activity was determined according to Campos et al. (2004).

PAL activity was determined in crude leaf extracts according to the methods described by Hyodo and Yang (1971) and Hyodo et al. (1978) modified by Campos et al. (2003).

For extraction, 500 mg of tissue was macerated (below 4°C) with 8 mL of 50 mM sodium borate buffer (pH 8.5) containing 25 g L⁻¹ of mercaptoethanol. The protein extract obtained after filtration (Whatman 1) and centrifuged (5,600 g, 30 min) under refrigeration (below 4°C), was used to test the activity.

Experimental design and statistical analyses

The experimental design was randomized blocks with four replications of nine plants. The results were submitted to variance analysis and the means of the variables with significant effect were compared using the Tukey test (cultivars) or regression analysis (concentrations) at 5% error probability.

The yield also was significantly influenced by Chi-Pyro-Film, with a quadratic response to isolated effect of the film concentration factor (Fig. 4). Regardless of cultivar, the highest efficiency concentration was estimated at 60 mL L⁻¹. Regarding the reference treatment (K₂HPO₄), it was observed that it had a performance similar to 100 Chi-Pyro-Film and higher than control (0 mL L⁻¹ of Chi-Pyro-Film), but less than the 50 mL L⁻¹ (Fig. 4). Between cultivars
effect, ‘Portola’ showed the highest yield than ‘Albion’ and ‘San Andreas’ (Table 1). Results similar to those observed by Carini et al. (2015) in a study of evaluation of strawberry cultivars in an organic system, in which ‘Portola’ was also more productive than ‘Albion’ and ‘San Andréas’.

The fruit weight was not influenced by Chi-Pyro-Film treatments. However, there was an effect of the cultivar factor, with ‘San Andréas’ producing fruits of a higher mass (Table 1). Corroborating with Carini et al. (2015), which evaluated the same three cultivars, found a higher fruit weight of ‘San Andréas’, but with the same weight that ‘Albion’.

The variable fruits with anthracnose showed an isolated effect of the factor Chi-Pyro-Film concentrations, that is, all cultivars had the same response to the application of Chi-Pyro-Film, with a quadratic reduction in the number of fruits attacked, with the maximum efficiency concentration estimated at 60 mL L⁻¹ Chi-Pyro-Film (Fig. 5). The reference treatment with K₂HPO₄ was similar to the 100 mL L⁻¹ of Chi-Pyro-Film, but lower than the 25 mL L⁻¹ and 50 mL L⁻¹ (Fig. 5). Among the cultivars, ‘Portola’ was the most sensitive to the occurrence of anthracnose in fruits, with no difference between ‘Albion’ and ‘San Andréas’ (Table 1).

**Enzymatic activity**

In the present study, there was a significant effect of Chi-Pyro-Film concentrations on the activity of POX, PPO, and PAL enzymes (Figs. 6 and 7). An interaction effect between film concentration and sampling time indicated that 48 hours after application, there was a quadratic effect of film concentrations on the activity of the three enzymes studied. In the case of POX and PPO, an activity reduction effect was obtained up to the estimated concentrations of 54 mL L⁻¹ and 50 mL L⁻¹, respectively, followed by an increase (Fig. 6a and 6b). However, PAL activity increased until the estimated concentration of 36 mL L⁻¹, with a subsequent reduction (Fig. 6c). About the

### Table 1 - Yield, fruit weight and fruits with anthracnose in Albion, Portola and San Andréas cultivars (1)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cultivar</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Albion</td>
<td>Portola</td>
</tr>
<tr>
<td>Yield (g plant⁻¹)</td>
<td>131.90 ± 7.93 c</td>
<td>221.35 ± 12.87 a</td>
</tr>
<tr>
<td>Fruit weight (g fruit⁻¹)</td>
<td>8.60 ± 0.67 b</td>
<td>8.50 ± 0.57 b</td>
</tr>
<tr>
<td>Fruits with anthracnose (%)</td>
<td>5.29 ± 1.71 b</td>
<td>9.13 ± 1.53 a</td>
</tr>
</tbody>
</table>

(1) Means followed by different lowercase letters in the row, differ by Tukey test at 5% error probability. The averages correspond to the isolated effect of cultivar factor to studied variables.
reference treatment, with $K_2PHO_4$, it induced activities similar to Chi-Pyro-Film in the concentration of 100 mL L$^{-1}$ for POX, PPO, and PAL.

The enzymatic activity also had an interaction effect between treatments of resistance induction and strawberry cultivars. POX activity decreased to concentrations of 49 mL L$^{-1}$ and 57 mL L$^{-1}$ in Albion and Portola, respectively (Fig. 7a). For San Andreas, although there was a similar trend, it was not significant (Fig. 7a).

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Fig. 6 - Specified activity of peroxidase-POX (a), polyphenoloxidase-PPO (b) and phenylalanine ammonia lyase-PAL (c) in the leaves, before (BA) and after application (AA) of Chi-Pyro-Film concentrations and reference treatment ($K_2HPO_4$, 50 mM). Interaction effect between film concentration and sampling time (before and 48 hours after application). *, **, ***, significant at p<0.05, p<0.01, p<0.001, respectively.

Fig. 7 - Specified activity of peroxidase-POX (a), polyphenoloxidase-PPO (b) and phenylalanine ammonia lyase-PAL (c) in leaves of Albion, Portola and San Andreas, in response to Chi-Pyro-Film concentrations and reference treatment ($K_2HPO_4$, 50 mM). Interaction effect between film concentration and cultivars. *, **, ***, significant at p<0.05, p<0.01, p<0.001, respectively.
The cultivars Albion and San Andreas also had a quadratic response to Chi-Pyro-Film, with a decrease in PPO activity up to the estimated concentrations of 54 mL L¹ and 55 mL L¹ (Fig. 7b). Concerning Portola, there was a linear increase up to a 100 mL L¹ concentration of Chi-Pyro-Film (Fig. 7b).

Concerning PAL, there was a quadratic response in Albion, with an increase up to the 38 mL L¹ rate of Chi-Pyro-Film, as well as a linear reduction in Portola (Fig. 7c).

The reference treatment with K₂HPO₄ showed a response similar to the 100 mL L¹ film concentration for the enzymes PPO and PAL in the three cultivars studied (Figs. 7b and 7c). For the POX in Albion and San Andreas, the reference treatment was similar to the 25 mL L¹ concentration, while in Portola, it was similar to the 50 mL L¹ film (Fig. 7a).

4. Discussion and Conclusions

In general, the results indicate that Chi-Pyro-Film contributed to the increase of vegetative growth and yield, as well as, to the anthracnose damage reduction in strawberry fruits. Results that are in agreement with those found in the literature, where it is observed an increase in vegetative, productive, and health variables, such as height, number of leaves, leaf area, yield and reduction of incidence of diseases in plants that received chitosan or pyroligneous extract (El-Miniawy et al., 2013; Masum et al., 2013; Mungkunkamchao et al., 2013; Mukta et al., 2017; Kumaraswamy et al., 2018; Ventura-Aguilar et al., 2018).

The effect of Chi-Pyro-Film on strawberry plant growth can be attributed to the role of chitosan as a non-toxic and biodegradable plant growth promoter (Salachna and Zawadzińska, 2014; Ahmed et al., 2020). Some authors suggest that foliar application of chitosan enhances the endogenous concentration of phytohormone such as gibberellic acid and auxin (Uthairatanakij et al., 2007; Ahmed et al., 2016). But the increase in macro and micronutrient accumulation and improved the content of photosynthetic pigments, provided by chitosan are also related to its influence on plant growth (Shehata et al., 2012; Ahmed et al., 2016). On the other hand, pyroligneous extract contributes to plant growth by its phytoprotective effect against pathogens, especially fungi. Was reported antipathogenic effects of the pyroligneous extract on plant pathogenic fungi like *Helminthosporium sativum*, *Cochliobolus sativus*, *Valsa mali*, *Colletotrichum orbiculare*, and *Alternaria mali* (Jung 2007; Wei et al., 2010 a). This antifungal activity has been related to the presence of furaldehydes and phenols in pyroligneous extract (Grewal et al., 2018).

The efficiency of spraying with Chi-Pyro-Film can be attributed to the effects of pyroligneous extract and chitosan individually, and to an interaction effect between both. According to Porto et al. (2019), who studied physicochemical properties of Chi-Pyro-Film, the film showed a semicrystalline structure, which is smooth and stable up to 50°C, being persistent in environmental conditions; it is permeable to water vapor and has high hygroscopicity, in addition to being able to efficiently block incident UVB and UVC radiation. The coverage presented by the Chi-Pyro-Film, as well as its persistence on the leaf surface (Fig. 2), probably provide several days of action, perhaps a large part of the application interval (15 days).

The main effect of chitosan and the pyroligneous extract is attributed to resistance induction by increased defense enzyme activity and accumulation of phenolic compounds acting on reactive oxygen species (ROS) (Wei et al., 2010 b; Katiyar et al., 2015; Pichyangkura and Chadchawan, 2015; Grewal et al., 2018). This effect is in line with the behavior verified by the PAL activity in this study (Figs. 6c, 7c). However, the activity of the POX and PPO enzymes responded differently, with a reduction in activity in the concentrations estimated between 50 and 60 mL L¹, 48 hours after application of treatments (Figs. 6b), especially in the Albion and San Andreas cultivars (Figs. 7a, 7b). This film rate (50-60 mL L¹) had a better performance in the dry mass accumulation, yield, and incidence of anthracnose.

The results indicate that in addition to the increase in systemic resistance, suggested by the PAL response, there is a direct effect of phytoprotection and a reduction of stress condition. Some aspects that can be associated with this second aspect, maybe the block that the film exerts concerning UVA and UVB radiation, as well as its potential action on pathogens, a hypothesis that corroborates the results obtained by Porto et al. (2019).

In this way, it can be suggested that Chi-Pyro-Film has a complex performance, acting both on metabolism with increased plant resistance and reducing cellular damage caused by physical (radiation) and biological stress (pathogens), as well as forming a phytoprotective film that inhibits the direct action of stress...
agents, such as UVA and UVB radiation and inhibiting the attack of pathogens.

In general, Chi-Pyro-Film showed greater efficiency in promoting growth, yield, and resistance to anthracnose than the reference treatment (K$_2$HPO$_4$), mainly in concentrations between 25 and 50 mL L$^{-1}$. K$_2$HPO$_4$ performed similarly to the 100 mL L$^{-1}$ of the film. Different studies indicate the effect of K$_2$HPO$_4$ in subsequent pathogen attack (Mauch-Mani and Métraux, 2018). According to Orober et al. (2002), foliar application of K$_2$HPO$_4$ results in the activation of systemic resistance mechanisms. The positive effect of K$_2$HPO$_4$ is associated with salicylic acid involved in triggering plant cell defense and sensitization responses for a faster and stronger response to subsequent pathogen attack (Mauch-Mani and Métraux, 1998; Orober et al., 2002).

The 100 mL L$^{-1}$ concentration of Chi-Pyro-Film may be high for the strawberry crop. Probably the increase in film thickness, which according to Porto et al. (2019), can significantly reduce the film’s permeability to water vapor. An aspect that can make some physiological processes such as the flowing water, the absorption of nutrients, and photosynthesis less efficient.

This study provides indicates that the phytoprotective film (Chi-Pyro-Film) is effective as a growth promoter and inducer of systemic resistance to anthracnose, resulting in increased growth and fruit production in strawberry plants of different cultivars. The optimal concentration of Chi-Pyro-Film ranges between 50 and 60 mL L$^{-1}$, depending on the strawberry cultivar.

We believe that the tested film can be an important tool for production systems, especially agroecological and organic systems, which require alternatives for disease control. But also, due to its physical-chemical properties and great stability, we believe that the film can be combined with other components, such as nutrients, biostimulants, and plant hormones, to enhance its effect. However, such combinations need to be studied.

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References


CAMPOS A.D., UENO B., PORTO F.G.S., ANTUNES I.F., GARCIA I.T.S, PEREIRA J.F.M., CASTRO L.A.S., SCIUVITTO W.B., 2012 - Processo de obtenção de formulação com capacidade fertilizante e fitoprotetora, Formulação com capacidade fertilizante e fitoprotetora, Uso da for-
mulation com capacidade fertilizante e fitoprotetora. - Registered at Instituto Nacional de Propriedade Intelectual in Brazil (PCT/BR2013/000597), In United States (US20150336854 A1) and in Germany (DE1120130623075).


PORTO F.G.S., 2011 - Caracterização de quitosana em ácido pirolenhoso distilado com potencial uso como coberturas protectoras. - Dissertação, Universidade Federal de Pelotas (in Portuguese).


PRASANNATH K., 2017 - Plant defense-related enzymes against pathogens: A Review. - Int. J. Agric. Sci., 11:
38-48.