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Activation of biochemical defense mechanisms in bean plants for homeopathic preparations

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To evaluate the potential elicitor of homeopathic preparations on bean plants cv Carioca, homeopathics of *Corymbia citriodora*, *Calcarea carbonica*, *Silicea* and *Sulphur* in dynamisations 12, 24, 30 and 60CH were applied by pulverization throughout the aerial part. Samples of leaf tissue were taken at 6, 12, 24, 48 and 126 h after the treatment (HAT) to analyze the activity of peroxidase (POX), catalase (CAT), chitinase (CHI) and β-1.3-glucanase (GLU), and fifteen days after the application for the total contents of chlorophyll a and b. For the induction of phytoalexin phaseolin, seeds of the same genotype were germinated in the presence of respective treatments, and the production of phytoalexin quantified spectrophotometrically. All treatments increased the activity of POX, CAT, QUI and GLU, in at least one of the schedules evaluated in comparison to the control. The treatments *C. citriodora* and *C. carbonic* did not alter the contents of chlorophyll but induced the accumulation of phaseolin. *Silicea* and *Sulphur* caused significant reduction in the levels of chlorophyll a and b. The induction values were superior to the trading inductor (harpin), indicating that these homeopathies may come to be utilized as elicitor treatments on bean plants. The results indicate the potential of the treatments applied in the induction of biochemical mechanisms of defense in the bean plants.

Key words: Resistance induction, homeopathy, enzymes, phytoalexins.

INTRODUCTION

The plants exhibit a range of effective defense mechanisms against phytopathogens regardless to the arrival of the pathogen on the infection site. However, there are other defense mechanisms even further efficient that apparently remain inactive or dormant, only being actuated or activated after exposure of the plants to inducing agents. In this case, the resistance is said to be induced, that is, the plants realize the attacks, and in its high capacity for adaptation, active defense mechanisms allow that they survive (Pascholati et al., 2011).

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The inducible defense mechanisms during the process of resistance induction may be structural and biochemical. Among the biochemicals, it highlights the production of phytoalexins, proteins-RP, hydroxyproline rich glycoproteins, protease inhibitors, and peroxidases, among others. These can act as barriers for the production of toxic substances or repellents, creating adverse conditions for the establishment of the pathogen in the plant (Cavalcanti et al., 2005; Pascholati et al., 2011). Activation of latent defense mechanisms may be related to action agents of origin biotic or abiotic called elicitors. The presence of the elicitors renders the plant resistant to posterior infections for several weeks, with effective protection against a diversified range of pathogens (Agrios, 2005).Among the compounds that may act by inducing plant defense mechanisms can be cited in the fungal extracts, plant extracts, essential oils, and homeopathic preparations, among others. The use of homeopathy in plants can act as abiotic inducers of induced resistance, as well as being an easy technique to apply and low cost, being used in all types of living beings, thus reducing the need for agrochemicals and contributing to the conservation of the environment and human health (Rossi et al., 2007).

The classic homeopathies, or already tested in humans and animals, or prepared from different compounds have shown effects on the activation and / or inhibition of secondary metabolic compounds. Coqueiro et al. (2008) to streamline the essential oil of *Corymbia citriodora* that verified the effects of homeopathies elicitors on peroxidase activity and increased production of phytoalexins in soybean. Das Dores (2007) reports the effects of the application of homeopathies of *Sulphur* and *Phosphorus* 12CH in plants fava d'anta (*Dimosphandra mollis*) observing increased activity of phenylallanine ammonia lyase and phenolic compounds, as well as an increase of the synthesis of the flavonoid rutin. Already Fonseca et al. (2006) with a single application of *Sulphur*, *Natrium muriaticum*, *Kalium phosphoricum*, *C. carbonic*, *Silicea terra* and *Magnesium carbonicum* in dinamization 4CH, reported an increase in tannin content in cabbage-clove leaves (*Porophyllum ruderale*) when compared to the control. Thus, the present study aimed to evaluate the potential of homeopathies *Calcarea carbonic*, *Silicea*, *Sulphur* and oil *C. citriodora* (dinamized) on the induction of pathogenesis-related protein, phaseolin and chlorophyll contents in bean plants.

MATERIALS AND METHODS

Choice and preparation of treatments

The choice of treatments used in this work was primarily based on analogies with the medical references used in homeopathy for humans (Carneiro, 2011). The choice of treatments *C. carbonic*, *Silicea* and *Sulphur* was based on reports of the effects of these drugs on various plant species.

The choice of *C. citriodora* was justified by reports of the potential inducer of defense compounds in various plant species.For the preparation of homeopathic remedies the essential oil of *C. citriodora* was extracted by hydrodistillation and passed by trituration in lactose until the third dynamization (3CH) (centesimal hahnemanian scale). The fourth dynamization was prepared by diluting 1% (w/v) of 3CH, using 30% grain alcohol, followed by 100 sucussions (agitations) made in mechanical arm, obtaining the dynamization 4CH. The following dynamizations were prepared by dissolving 1% (v/v) of previous dynamization until 60CH (ABFH, 2003).

The dynamizations of *C. carbonic*, *Silicea* and *Sulphur* were prepared from the matrices 4CH. The fifth dynamization was prepared of the previous dynamization at 1% (w/v) in 30% grain alcohol, followed by 100 sucussions obtaining the dynamization 5CH. The following dynamizations were prepared on the same scale by diluting 1% (v/v), with the process of succussion done with the aid of the mechanical arm until 60CH (ABFH, 2003). The dynamizations utilized in bioassays were 12, 24, 30 and 60CH.

Plant materials

Bean seeds (*Phaseolus vulgaris*) cv Carioca, provided by the Agronomic Institute of Paraná (IAPAR) were sown in pots containing 5 L substrate prepared with soil, sand and humus (3:1:1). The soil was sieved and sequentially autoclaved three times during 1 h at 121°C with 24 h intervals. Twenty days after the end of the autoclaving process, it was sowed and maintained in two plants / pot.

The application of homeopathy 12, 24, 30 and 60CH diluted in distilled water (1% v/v) of *C. citriodora* (EC), *C. carbonica* (CC) *Silica* (Sl), *Sulphur* (SU) and eucalyptus essential oil (0.5%) was performed on plants at the V4 stage (twenty days after emergence). As a negative control, we used grain alcohol (0.3%) and distilled water, and as positive control harpin (Messenger ®, 873 mg L^{-1}).

Treatments were applied with sprayer to the point of straining on adaxial and abaxial surfaces throughout the leaves. For the biochemical analyzes, samples of the first and second trifoliate leaves were taken at 6, 12, 24, 48 and 216 h after treatment. The samples were stored at -20°C for subsequent analysis of enzymatic assays.

The experimental design was completely randomized, with 20 treatments with four replications; each experimental unit consisted of two plants. The results were submitted to analysis of variance and averages compared by the test of Scott-Knott ($p \geq 0.05$).

Obtaining the protein extracts

The leaf samples were macerated in liquid nitrogen and homogenized in 4 mL of potassium phosphate buffer 50 mM (pH 7.0) containing 0.1 mM EDTA and 1% (w/w) PVP (poly vinyl pyrrolidone), in a porcelain mortar. The homogenized was centrifuged for 30 min at 7300 g at 4°C, and the supernatant obtained considered enzyme extract, and stored at -20°C. The extract was used for determination of protein content using the Bradford method (1976) and the activity of peroxidase, catalase, chitinase and β-1,3-glucanase.

Determination of the activity of guaiacol peroxidase (EC 1.11.1.7)

The guaiacol peroxidase activity was determined directly by measuring the conversion of guaiacol in tetraguaiacol by mixing 0.5 mL of the enzyme extract to 2.5 mL of prepared substrate (250 µL

and 306 µL guaiacol and hydrogen peroxide in 100 mL of 0.01 M phosphate buffer pH 6.0).

The reaction occurred at 30°C and the reading activity was performed in a spectrophotometer at 470 nm for 2 min with 10 s intervals (Lusso and Pascholati, 1999). The difference between the readings on the linear increment period was used to determine the activity. The specific activity results are expressed as absorbance $min^{-1}.mg^{-1}$ protein.

Determination of the activity of catalase (EC 1.11.1.6)

Catalase activity was determined by the stable complex formed by ammonium molybdate with hydrogen peroxide. A aliquot of 50 µL of the enzyme extract was incubated in 0.5 mL of reaction mixture containing 60 mM hydrogen peroxide in potassium phosphate buffer 60 mM pH 7.4 at 38°C for 4 min. After this time the addition of 0.5 mL of 32.4 mM ammonium molybdate stopped the consumption of hydrogen peroxide present in the extract. A blank for each sample was prepared by adding ammonium molybdate to the reaction mixture, omitting the incubation period (Góth, 1991; Tománková et al., 2006). The reaction tubes were removed and 0.1 mL material transferred plates for ELISA absorbance reading. Reading the yellow complex formed by molybdate and hydrogen peroxide which was measured at 405 nm in ELISA reader VersaMax ® Microplate Reader Molecular Divicers. The difference between the absorbance of the blank and the sample incubated indicated the amount of hydrogen peroxide used by the enzyme. H_2O_2 concentration was determined using the molar extinction coefficient ε = 0.0655 mM^{-1} cm^{-1}.

Determination of chitinase activity (EC 3.2.1.14)

The chitinase activity was assessed using the methodology described by Silva et al. (2008). To this, 600 µL of sodium acetate buffer 100 mM pH 5.2 was mixed with 200 µL protein extract and 200 µL "CM-chitin-RBV" (2 mg L^{-1}). After incubation at 40°C for 20 min, the drying was made with 200 µL 1M HCl, followed by cooling on ice and centrifugation at 10,000 rpm for 5 min. The absorbance of the supernatant was determined at 550 nm in the Elisa VersaMAx® Microplate Reader Molecular Divicers, with reference 800 µL of extraction buffer + 200 µL "CM-chitin-RBV + 200 µL HCl 1.0 M. The results are expressed in absorbance units min^{-1} mg^{-1} protein.

Determination of the activity of β-1, 3 - glucanase (EC 3.2.1.6)

The determination of the activity of β-1,3-glucanase was conducted analogously to the procedure chitinase, but using carboxymethyl-curdlan marked with Remazol Brilliant Blue - "CM-curdlan-RBB"4 mgL^{-1} as substrate. The absorbance of the supernatant was determined at 600 nm at reader VersaMAx® Microplate Reader Molecular Divicers, with reference 800 µL l of extraction buffer + 200 µL "CM-curdlan-RBB + 200 µL HCl 1.0 M. The results are expressed in absorbance units' min^{-1} mg^{-1} protein (Costa et al., 2000).

Determination of total polyphenols

Thirty days after the treatments were withdrawn from the aerial part of the plants and dried in a forced ventilation oven at 60°C to constant weight and ground. The concentration of total polyphenols was determined spectrophotometrically by the Folin-Ciocalteau accordingly Bucic-Kojic et al. (2007) from the powder of dried leaves. One gram of this powder was homogenized with 50 mL of 80% ethanol in a mixer for 2 min. After 5 min centrifugation at 5000 rpm, it was transferred to 0.2 mL of this test tube extract, adding 1.8 mL of distilled water, 10 mL of Follin 10%, and after 8 min adding 8 mL of sodium carbonate (Na_2CO_3) 7.5% and it remained in the dark for 2 h. The reading was held in the 765 nm spectrum, using white as all reagents without the sample aliquot centrifuged. The results were expressed in mg of gallic acid equivalents per gram of dry weight (mg GAE / gdw).

Determination of chlorophyll content

Fifteen days after the first treatment application, two leaf discs of 2 mm in diameter were removed from the plant for the quantification of chlorophyll. Plant tissue samples were weighed and placed in glass vials containing 10 mL of 80% acetone for 7 days in the dark at 25°C. After this period, it was held reading the spectrophotometer at 663 and 645 nm for chlorophyll *a* and *b*, respectively. The determination of chlorophyll *a* was given by the formula $(0.0127.A_{663})-(0.00269.A_{645})$ and chlorophyll *b* by the formula $(0.0229.A_{645})-(0.00468.A_{663})$ (Viecelli et al., 2009). The total chlorophyll content was obtained by summing the results of the contents of chlorophyll *a* and *b*. The values were expressed in mg g^{-1}fresh tissue.

Accumulation of phytoalexin

Bean seeds cv. Carioca after disinfection were sown in plastic boxes containing germination paper moistened with 5 mL of the same treatments described above. The gerbox with seeds and their treatments were incubated in a growth chamber at 25°C in the dark for seven days. After this period, they were highlighted with 5 cm segments of etiolated hypocotyls, washed in sterile water, wiped dry and weighed. Later, they were transferred to tubes containing 10 mL of ethanol and kept at 4°C for 48 h to extract the phytoalexin. After stirring for 1 h, the measurement was performed spectrophotometrically at 280 nm. The results were expressed in units of absorbance per fresh $weight^{-1}$ ($ABS.fw^{-1}$) (Brand et al., 2010).

The experimental design was completely randomized, with 20 treatments with four replications, each experimental unit consisting of a tube containing approximately 1 g of hypocotyls. The results were submitted to analysis of variance and the averages compared by the test of Scott-Knott ($p \geq 0.05$).

RESULTS

Activity of guaiacol peroxidase (POX) and catalase (CAT)

All treatments at least one point, resulted in changes in the activity of these enzymes, with the oscillatory behavior of induction and reduction in POX activity. Six hours after the first application of homeopathy, it was possible to observe the effects of inducing peroxidase in plants treated with EC 24CH, 60CH and SU and CC 60CH, with increases of 100, 75 and 16%, respectively (Table 1). Twelve hours after treatment (HAT) there was a trend of reduced enzyme activity in plants treated with oil 0.5%, EC 30 and 60CH. The potencies of CC, SI 12 and 60CH and SU 12CH, presented statistically lower

than the control water. This trend was maintained at the time of evaluation 24 HAT, when the inhibition of POX activity was observed in all treatments. In 48 HAT all dynamizations CC increased the POX activity, reaching 29-fold increased in dynamisations 12CH, this being the only time to differ from control alcohol; *Silicea* 30 and 60CH increased activity of POX, 4 and 5 times, respectively compared to the water control. *Sulphur* resulted in increases ranging between 3.3 and 8.3 times higher than in water control in dynamisations 60 and 24CH, respectively (Table 1).

In time 216 HAT activity of POX in plants treated with EC 12CH, CC 24CH, SI 12, 30 and 60CH, SU 12, 24 and 30CH were higher compared to the water control. The application of harpin increased enzyme activity only after 6 h of the first application treatment, increasing 2.3 times the activity of POX, with the same pattern observed for the reduction of homeopathy in 24 HAT.

The patterns of CAT activity were altered by all treatments. At 6 HAT EC 12CH inhibited CAT activity with values statistically similar to alcohol control, different from that observed in CC 12CH, when activity increased 2.6 times, as well as the dinamizations of SI 24 and 30CH which promoted increases of 2.4 to 3.6 times. The application of SU 12 and 24CH also increased the activity of CAT, exceeding water control of 2.4 and 3.2 more times, respectively (Table 1). At 24 and 48 HAT CC 24 and 30CH, SI 12CH, it increased the enzymatic activity of CAT. Already SI 60CH, 216 HAT was increased by 29% CAT activity, different from what occurred with other treatments that had significantly lower compared to the water control.

Total polyphenols

Thirty days after the treatments, the amount of phenols reduction was observed when plants were treated with dinamizations EC 30CH, 24CH CC and SU SL, compared to the water control (Figure 1).

The mean EC 30CH treated plants were 16% smaller than control plants, as well as CC 24 and 60CH reduced 42 and 21%, respectively in the amounts of phenols. SU 12CH resulted in values 18% lower compared to the water control. The other treatments did not have their values changed significantly.

Activity of chitinase (CHI) and glucanase (GLU)

The homeopathy of EC 24, 30 and 60CH reduced or were statistically equal to the water control in the first evaluations. Already at 48 and 126 HAT EC 30CH showed an increase in the activity of 30 and 56%, respectively, in the activity in relation to the water control (Table 2). Plants treated with CC only at 48 HAT showed no increase in the activity of CHI which was statistically higher than the control water, reaching 130% increase enzyme activity by CC 60CH. For SI at 48 HAT, it was observed that the dynamizations 24, 30 and 60CH showed statistically higher values than the control, with increases of 223, 282 and 312%, respectively. When plants received applications of SU, enzymatic activity increased at 6 HAT (60CH), 48 and 126 HAT with dynamizations 12, 24 and 30CH (Table 2).

All homeopathies applied, presented for purposes of elicitors' glucanase for at least one of the evaluations. The evaluation showed 6 HAT inducing effects by EC 12CH with increased activity GLU at 54% and DC 12CH increased by 63%. The application of SU in all dynamizations had inducing effects at the times 6 and 12 HAT, with increments ranging from 70% (24CH) and 318% (60CH) in 6 HAT and 47% (30CH) and 131% (12CH) at 12 HAT. At 12 HAT, SI 30 and 60CH also promoted induction in the activity of GLU (Table 2).

In the time 24 HAT, CC 12CH increased 3 times the activity of GLU, other dynamizations (24, 30 and 60CH) significantly reduced enzyme activity when compared to untreated controls, as well as all treatments SI and dynamizations 12, 30 and 60CH of SU which also reduced the activity of GLU.

After 48 h of the application of DC 12 and 30CH, and all dynamizations of SI and SU 12, 24, 30CH observed increased activity of β-1,3-glucanase. Treatments with CC 12CH increased 98% of enzymatic activity. The best promotion of SI at this time was 60CH which increased over 200%. SU 12, 24 and 30CH promoted increases of over 100%. These inducing effects were also observed in the treatment by EC 216 HAT and 30 60CH with means 4 and 3 fold higher than the untreated control values in SI 4 times greater, and SU 12, 24 and which also promote increase 30CH enzyme activity, reaching 480% in 30CH. The control treatment with harpin also induced the activity of POX, CAT and GLU, and also showed the same oscillatory pattern that homeopathy.

Chlorophyll

The amounts of chlorophyll were not affected by treatment with the dinamizations of EC (Table 3), unlike observed in plants treated with SU and SI the values of total chlorophyll *a* and *b* were reduced. Plants treated with homeopathy SI had a 33% reduction in chlorophyll content as well as the 24 and 30 CH, in 60CH in the reduction was 26%. The dynamizations of SU resulted in reductions ranging from 18% (12CH) and 26% at24 and 60CH.

Production phaseolin

All treatments altered the production of phaseolin. The hypocotyls treated with essential oil of EC increased

Table 1. Specific activity of peroxidase and catalase (absorbance min^{-1} mg^{-1}protein) in bean leaves treated with homeopathy *Corymbia citriodora* (EC), *Calcarea carbonica* (CC) *Silica* (12CH) and *Sulphur* (SU).

Tratamento	Peroxidase					Catalase				
	6 HAT**	12 HAT	24 HAT	48 HAT	216 HAT	6 HAT	12 HAT	24 HAT	48 HAT	216 HAT
Control (H_2O)	0.037[a]*	0.049[b]	0.092[b]	0.086[a]	0.085[a]	2.061[b]*	0.000[a]	0.650[a]	2.615[a]	8.972[c]
Alcohol 0.3%	0.022[a]	0.027[b]	0.106[b]	0.356[b]	0.233[b]	1.312 [a]	4.661[b]	0.501[a]	4.337[b]	7.344[c]
Harpin	0.070[c]	0.031[b]	0.034[a]	0.063[a]	0.050[a]	0.000[a]	3.860[b]	0.000[a]	4.763[b]	11.741[d]
OE 0.5%	0.024[a]	0.023[a]	0.041[a]	0.070[a]	0.068[a]	2.691[b]	16.011[c]	0.000[a]	9.003[c]	7.434[c]
EC 12CH	0.015[a]	0.039[b]	0.036[a]	0.107[a]	0.122[a]	0.057[a]	0.710[a]	0.000[a]	5.066[b]	0.000[a]
EC 24CH	0.074[c]	0.032[b]	0.050[a]	0.100[a]	0.172[a]	3.054[b]	0000[a]	0.000[a]	1.804[a]	2.510[b]
EC 30CH	0.009[a]	0.022[a]	0.015[a]	0.035[a]	0.224[b]	2.390[b]	0.000[a]	0.000[a]	3.749[b]	3.780[b]
EC 60CH	0.031[a]	0.014[a]	0.017[a]	0.045[a]	0.081[a]	2.466[b]	1.443[b]	0.603[a]	0.604[a]	5.783[c]
CC 12CH	0.032[a]	0.025[a]	0.014[a]	2.519[c]	0.145[a]	5.517[c]	0.211[a]	0.521[a]	9.334[c]	3.463[b]
CC 24CH	0.009[a]	0.014[a]	0.012[a]	0.315[b]	0.382[b]	3.166[b]	1.792[b]	4.773[c]	1.430[a]	8.287[c]
CC 30CH	0.007[a]	0.009[a]	0.010[a]	0.475[b]	0.091[a]	3.095[b]	1.892[b]	2.963[b]	12.473[c]	3.292[b]
CC 60CH	0.044[b]	0.008[a]	0.019[a]	0.544[b]	0.269[b]	2.853[b]	0.000[a]	0.000[a]	8.331[c]	4.785[b]
SI 12CH	0.012[a]	0.030[b]	0.028[a]	0.159[a]	0.278[b]	3.285[b]	0.000[a]	2.007[b]	14.501[d]	0.000[a]
SI 24CH	0.025[a]	0.008[a]	0.019[a]	0.173[a]	0.164[a]	7.434[d]	0.000[a]	0.000[a]	6.196[c]	3.885[b]
SI 30CH	0.017[a]	0.021[a]	0.013[a]	0.373[b]	0.381[b]	5.103[c]	0.000[a]	0.000[a]	18.564[e]	7.952[c]
SI 60CH	0.019[a]	0.045[b]	0.016[a]	0.483[b]	0.273[b]	1.815[b]	0.000[a]	1.101[a]	2.602[a]	11.607[d]
SU 12CH	0.024[a]	0.017[a]	0.016[a]	0.378[b]	0.276[b]	5.001[c]	0.000[a]	0.129[a]	0.000[a]	0.000[a]
SU 24CH	0.021[a]	0.040[b]	0.040[a]	0.719[b]	0.244[b]	6.581[d]	0.000[a]	0.000[a]	0.000[a]	2.973[b]
SU 30CH	0.035[a]	0.016[a]	0.010[a]	0.084[a]	0.268[b]	2.582[b]	0.000[a]	1.360[a]	0.000[a]	2.827[b]
SU 60CH	0.066[c]	0.012[a]	0.037[a]	0.284[b]	0.182[a]	2.415[b]	2.370[b]	0.756[a]	0.000[a]	0.000[a]
cv%	52.55	47.49	65.39	57.34	66.49	43.5	122.55	128.96	53.26	53.98

*Means followed by different letters in the same column differ by Scott-Knott ($p < 0.05$). **HAT = hours after treatment application.

0.5% in 23% of the production of phytoalexin when compared to the water control, and homeopathics 12 and 24CH increased an average of 10 and 23% more than the control, respectively, but with values statistically similar to the control with grain alcohol (Figure 2). The application of harpin does not alter the production of phaseolin.

The application of CC increased 27% phaseolin for the treatment 12CH, 26% for 24CH, 18% for 30CH and 21% to 60CH (Figure 2). The values observed for the test, although different potencies reported for the control of water to grains alcohol which were similar (0.3%). There were no significant differences between the results of the potencies used.

The homeopathics SI and SU caused inhibition of the production of phaseolin. The application of SI 12CH inhibited 36% the formation of phaseolin, SI 24CH 36%, 20CH 28% and 60CH 24% (Figure 2). Similarly, the potencies of SU resulted in 41%, average inhibition in 24CH values were equivalent to control.

DISCUSSION

All treatments showed potential elicitor of defense compounds in beans, showing oscillatory behavior of the activity of enzymes induced. The oscillation observed in CAT and POX activity may be related

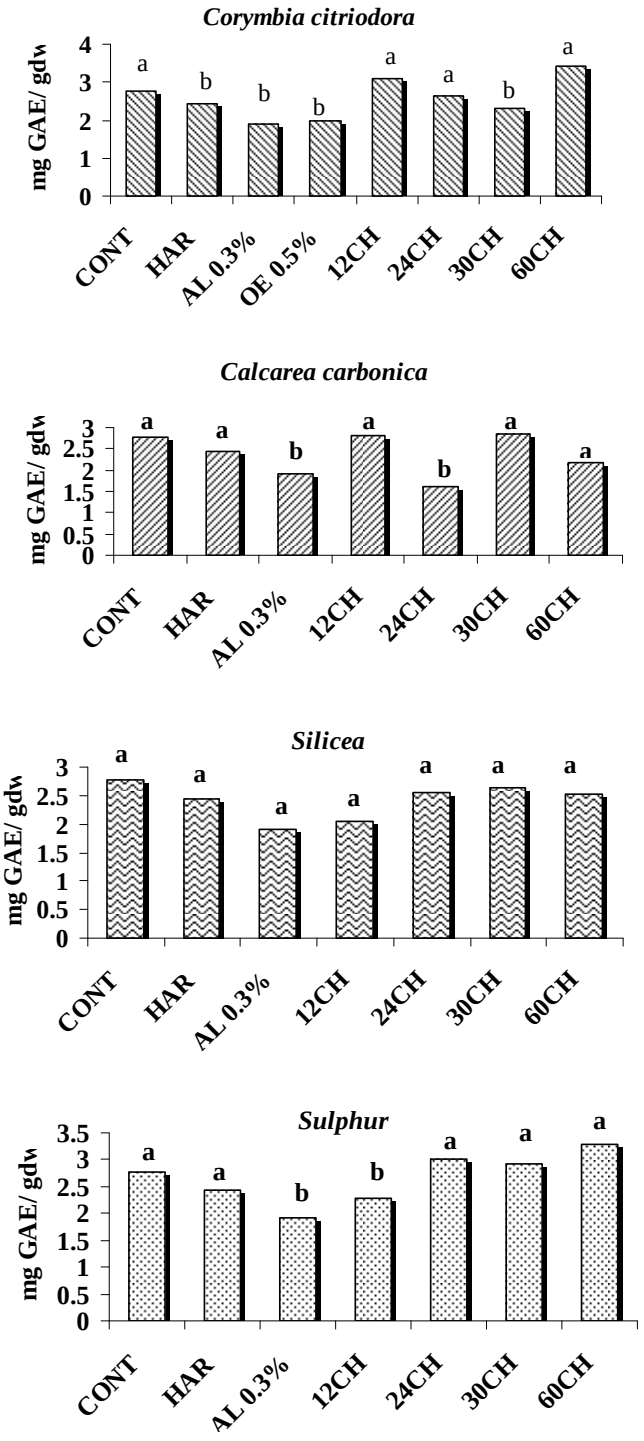

Figure 1. Total phenol content in leaves of bean cv Carioca after treatment with homeopathy *Corymbia citriodora* (EC) and eucalyptus essential oil (EO), *Calcarea carbonica* (CC) *Silica* (SI) and *Sulphur* (SU), averages of four replicates. Means followed by the same letter do not differ by Scott-Knott ($p < 0.05$).

Table 2. Specific activity of Chitinase and Glucanase (absorbance min^{-1} mg^{-1} protein) in leaves of bean cv Carioca treated with homeopathy *Corymbia citriodora* (EC). *Calcarea carbonica* (CC) *Silica* (SI) and *Sulphur* (SU).

Tratamento	Quitinase					Glucanase				
	6 HAT**	12 HAT	24 HAT	48 HAT	216 HAT	6 HAT	12 HAT	24 HAT	48 HAT	216 HAT
Control (H_2O)	0.059[b]*	0.216[a]	0.142[c]	0.039[a]	0.128[a]	0.011[c]	0.080[c]	0.055[b]	0.057[c]	0.026[d]
Alcohol 0.3%	0.058[b]	0.216[a]	0.136[c]	0.031[a]	0.226[b]	0.010[c]	0.096[c]	0.054[b]	0.045[c]	0.054[d]
Harpin	0.055[b]	0.215[a]	0.127[c]	0.038[a]	0.145[a]	0.009[c]	0.102[c]	0.053[b]	0.055[c]	0.056[d]
OE 0.5%	0.060[b]	0.243[b]	0.171[d]	0.049[b]	0.138[a]	0.006[c]	0.073[c]	0.051[b]	0.061[c]	0.035[d]
EC 12CH	0.050[b]	0.225[a]	0.106[c]	0.016[a]	0.165[a]	0.004[c]	0.077[c]	0.050[b]	0.032[c]	0.054[d]
EC 24CH	0.037[a]	0.230[a]	0.089[b]	0.051[b]	0.169[a]	0.002[c]	0.062[c]	0.049[b]	0.053[c]	0.069[d]
EC 30CH	0.032[a]	0.229[a]	0.073[b]	0.042[a]	0.200[b]	0.004[c]	0.060[c]	0.048[b]	0.057[c]	0.104[c]
EC 60CH	0.045[a]	0.226[a]	0.091[b]	0.027[a]	0.156[a]	0.017[b]	0.093[c]	0.041[b]	0.040[c]	0.089[c]
CC 12CH	0.044[a]	0.224[a]	0.079[b]	0.070[b]	0.162[a]	0.018[b]	0.059[c]	0.190[a]	0.113[b]	0.074[d]
CC 24CH	0.028[a]	0.219[a]	0.046[a]	0.057[b]	0.161[a]	0.008[c]	0.059[c]	0.020[c]	0.082[c]	0.052[d]
CC 30CH	0.036[a]	0.211[a]	0.057[a]	0.090[b]	0.169[a]	0.006[c]	0.050[c]	0.031[c]	0.117[b]	0.053[d]
CC 60CH	0.030[a]	0.222[a]	0.056[a]	0.054[b]	0.139[a]	0.008[c]	0.062[c]	0.021[c]	0.079[c]	0.062[d]
SI 12CH	0.038[a]	0.216[a]	0.033[a]	0.031[a]	0.165[a]	0.011[c]	0.072[c]	0.013[c]	0.052[c]	0.071[d]
SI 24CH	0.031[a]	0.224[a]	0.041[a]	0.126[c]	0.161[a]	0.009[c]	0.072[c]	0.005[c]	0.149[a]	0.076[d]
SI 30CH	0.031[a]	0.211[a]	0.032[a]	0.149[c]	0.246[b]	0.009[c]	0.132[b]	0.012[c]	0.173[a]	0.118[b]
SI 60CH	0.050[b]	0.230[a]	0.046[a]	0.161[c]	0.154[a]	0.011[c]	0.200[a]	0.009[c]	0.176[a]	0.082[b]
SU 12CH	0.067[b]	0.247[b]	0.062[a]	0.146[c]	0.204[b]	0.019[b]	0.185[a]	0.022[c]	0.174[a]	0.089[b]
SU 24CH	0.075[b]	0.255[b]	0.064[a]	0.081[b]	0.218[b]	0.018[b]	0.130[b]	0.041[b]	0.115[b]	0.151[a]
SU 30CH	0.065[b]	0.245[b]	0.057[a]	0.056[b]	0.208[b]	0.041[a]	0.118[b]	0.031[c]	0.117[b]	0.064[d]
SU 60CH	0.119[c]	0.299[c]	0.061[a]	0.003[a]	0.157[a]	0.046[a]	0.142[b]	0.030[c]	0.050[c]	0.091[b]
cv%	23.26	6.35	29.51	35.01	19.52	44.07	33.39	41.64	32.66	26.91

*Means followed by different letters in the same column differ by test of Scott-Knott test ($p < 0.05$). ** HAT = hours after treatment application.

to characteristics of these enzymes. A point to be analyzed was the times that there was a reduction in activity because they coincide largely with opposite effects on the activity of another enzyme. Possibly, this is due to the fact that these enzymes are involved in the production of reactive oxygen species (ROS). During this process, the superoxide radical can undergo redox reactions transformed into H_2O_2 is converted to O_2 and H_2O by the action of catalase, or it may be converted to H_2O by the action of peroxidase (Soares and Machado, 2007).

The reduction of POX activity may be related to the activation of CAT, which might explain the alternation observed in the activities, and higher activities of catalase and peroxidase inactivation and vice versa, as both work as the same substrate (Marafon et al., 2009). Cavalcanti et al. (2005) showed that the peroxidase enzyme is related to events involving induction of resistance, there is a definite pattern to their behavior, which depends on the type of inducer, its concentration, time after application and plant pathosystem studied. The possible increase in the quantity of

Table 3. Concentration of chlorophyll *a*, *b* and total in bean plants cv Carioca treated with homeopathic remedies *Corymbia citriodora*, *Calcarea carbonica*, *Silicea* and *Sulphur*. Values expressed in mg g^{-1} fresh tissue.

Treatment	Chlorophyll *a*	Chlorophyll *b*	Chlorophyll total
Test H_2O	0.121[a]*	0.044[a]	0.165[a]
Álcool 0.3%	0.133[a]	0.051[a]	0.184[a]
Harpina	0.121[a]	0.042[a]	0.164[a]
OE 0.5%	0.115[a]	0.043[a]	0.158[a]
OE 1%	0.111[a]	0.045[a]	0.156[a]
EC 12CH	0.111[a]	0.043[a]	0.155[a]
EC 24CH	0.117[a]	0.045[a]	0.162[a]
EC 30CH	0.117[a]	0.044[a]	0.162[a]
EC 60CH	0.100[b]	0.039[a]	0.139[a]
CC 12CH	0.106[a]	0.041[a]	0.147[a]
CC 24CH	0.109[a]	0.042[a]	0.152[a]
CC 30CH	0.108[a]	0.042[a]	0.151[a]
CC 60CH	0.121[a]	0.046[a]	0.168[a]
SI 12CH	0.109[a]	0.041[a]	0.150[a]
SI 24CH	0.080[b]	0.021[b]	0.102[b]
SI 30CH	0.080[b]	0.024[b]	0.105[b]
SI 60CH	0.089[b]	0.018[b]	0.108[b]
SU 12CH	0.099[b]	0.021[b]	0.121[b]
SU 24CH	0.090[b]	0.019[b]	0.109[b]
SU 30CH	0.096[b]	0.021[b]	0.118[b]
SU 60CH	0.090[b]	0.020[b]	0.111[b]
cv%	16.58	17.09	15.54

*Means followed by different letters vertically differ by Scott-Knott test ($p < 0.05$).

phenolic compounds may contribute to reducing levels of POX (Brum, 2010).

This effect on the amount of phenolic compounds has been verified by Das Dores (2010) in bean plants treated with homeopathic *Sulphur* 12CH in which it was found that there was an increase in the production of phenolic compounds evaluated, different that observed in this study. The reduction in the accumulation of phenolic in treated plants may be due to an imbalance between the production and consumption of phenolic intermediates, contributing to reduce the amount of phenolic compounds. More than that, higher activities of enzymes downstream in the phenylpropanoid pathway, such as chalcone synthase (CHS), and cinnamyl alcohol dehydrogenases, that could have an effect promoting drain on soluble phenolic substrates. There were reports of lower levels of phenolic compounds in tissues which were observed by POX activity in the similarity observed in this study (Cavalcanti et al., 2006). As observed for the CAT and POX homeopathies tested, it increased the activity of CHI and GLU. These enzymes have received attention as important components of the arsenal of plant defense proteins, that hydrolyse the major carbohydrate cell wall of fungi (chitin and β-1.3-glucan), with direct action on fungi inhibiting their growth and releasing oligosaccharide elicitors that induce the production of phytoalexins (Di Piero and Garda, 2008).

In this study we observed a similar pattern of increase for both enzymes in the same time of evaluation. Typically, chitinases and β-1.3-glucanases exhibit patterns of regulation, that are co-regulated which would explain the decrease in activity at certain moments as the period of gene transcription. Costa et al. (2000) verified that the deletion of chitinase activity, demonstrated in his work with transgenic plants was due to the duration of the process of gene transcription.

The control treatment with harpin also induced the activity of POX, CAT and GLU, and also showed the same oscillatory pattern of homeopathy. The increases caused by harpin were significant in the initial assessments between 6 and 24 HAT. This response is in agreement to that described by Barbosa-Mendes (2007) which the application of harpins within minutes after application, changed the membrane potential, causing oxidative burst and changes in ion flux. The harpins are also related to increases in the activity of phenylalanine ammonia-lyase, responsible for the synthesis of phytoalexins (Danner et al., 2008).

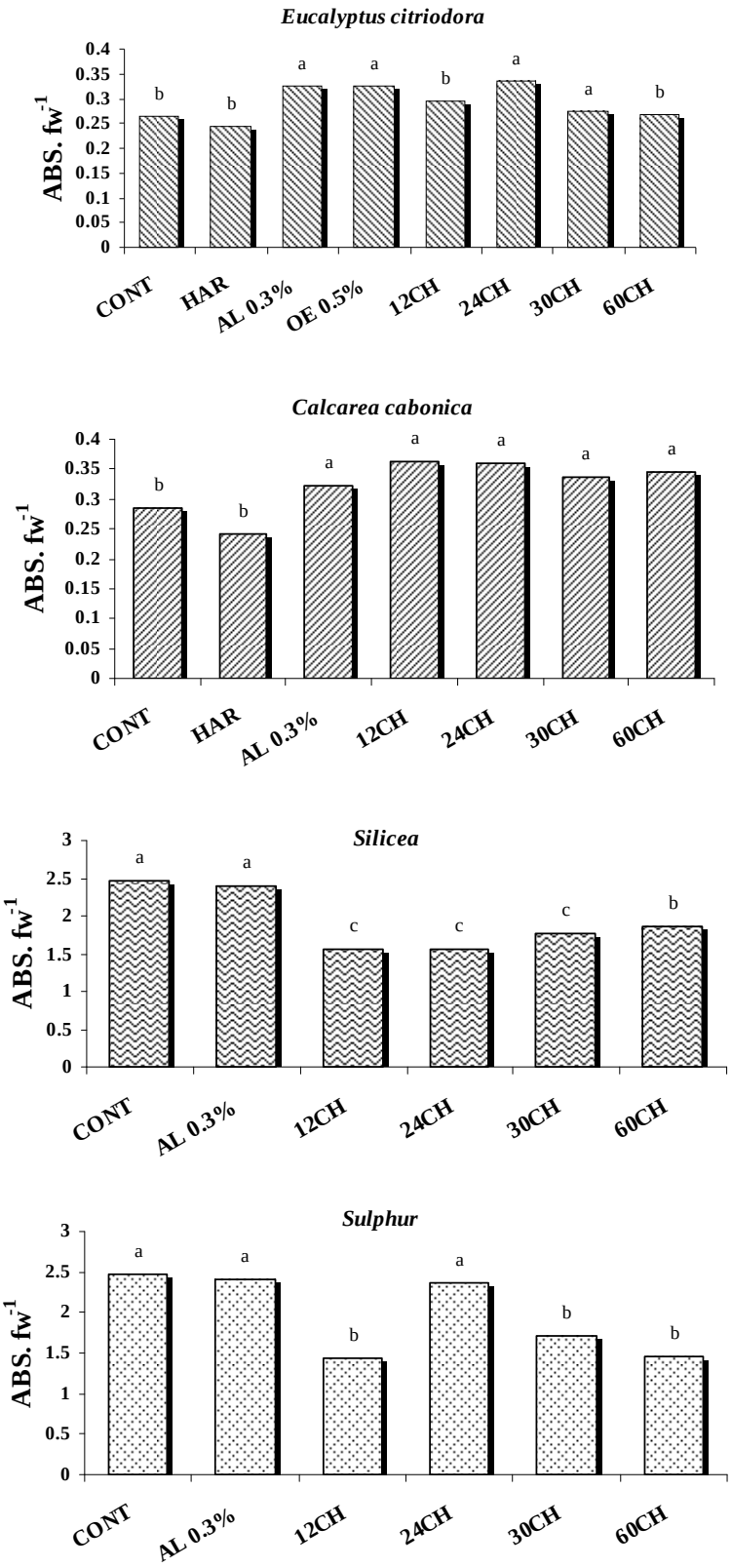

Figure 2. Production of Phaseolin in hypocotyls etiolated bean cv Cariocaafter treatment with homeopathy *Corymbia citriodora* (EC) and eucalyptus essential oil (EO). *Calcarea carbonica* (CC) *Silica* (SI) and *Sulphur* (SU).averages of four replicates, means followed by the same letter do not differ by the Scott-Knott test ($p < 0.05$).

However, this research was not induced with the production of chitinase by phaseolin and application of harpin. The comparison between the mean values, the result of the application of homeopathy and those resulting from the application of harpin show that the homeopathy when provoked induction were more efficient compared to the commercial product, in quantities as in the period of sustained elicitation. As the molecules of chlorophyll *a* and *b* are the two pigments responsible for the absorption and transfer of radiant energy (Viecelli et al., 2009), these results suggest a shift in the production of primary energy for synthesis of plant defense compounds.

It can be seen that the treatments applied in this work which reduced the amount of chlorophyll (SI, SU) were the same with the highest results in induction of catalase and peroxidase. These results suggest a deviation of carbon skeletons that directed the prime route to the sideline plant metabolism.

Regarding the production of phaseolin, the inhibition observed in plants treated with SI and the potencies 12, 30 and 60CH of SU may be related to resumption of homeostatic balance, which would require less production of defensive compounds and consequently lower metabolic cost due the balance of the plants. The metabolic cost required to produce phytoalexins, can result in disfavoring the primary pathways for the production of defense compounds, resulting from the activation of latent defense mechanisms (Barros et al., 2010).

However, increased production of these compounds as found in treatments with CE, CC and 24CH of SU, is important in the process of plant defense, justifying the expense required for its production. Considering that the antimicrobial phytoalexin substances are widely associated with vertical resistance or immunity (Matiello et al., 1997).

As observed in this study, there is a variation in the response to homeopathic treatments, which can act both in induction or reduction in the synthesis of secondary compounds. Fonseca et al. (2006) found effects of inducers and reducers of homeopathy on the tannin content in *P. ruderale* (cauliflower cloves), which varied according to homeopathy, promotion and treatment period. CC and SU single application increased tannin content assessed as SU and SI under multiple acted in the opposite direction.

The absence of universal responses described for homeopathic treatments was also observed in this study. According to the results observed, the effects are of variations between the different potencies of the same drug, but with no dose effect dependence.

It was found that for peroxidase activity, catalase, and production of phaseolin, the best treatments were potencies CC (*C. carbonica*); in the induction of chitinase and β-1.3-glucanase, applications of SU (*Sulphur*) were higher values relative to the water control and consistency between the hours.

Conclusion

In this study, the *C. citriodora* homeopathy, *C. carbonica*, *Silicea* and *Sulphur* showed potential in the elicitation of peroxidase, catalase, quitinase, β-1.3-glucanase and phytoalexin. These results show the potential of these preparations in the process of plant protection that can be a tool in the search for mechanisms for controlling plant pathogens, which are less harmful to the environment.

Conflicts of interest

The author(s) have not declared any conflict of interests.

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