Ciência Rural
ISSN: 0103-8478
cienciarural@mail.ufsm.br
Universidade Federal de Santa Maria
Brasil

Martini, Daiane; Feliciani Barbosa, Giselle; Matias, Rosemary; Camargo Marques Filho, Wolff; Trombini Garcia, Nayara Zielasko
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Ciência Rural, vol. 47, núm. 3, 2017, pp. 1-6
Universidade Federal de Santa Maria
Santa Maria, Brasil

Ciência Rural, Santa Maria, v.47: 03, e20160312, 2017
http://dx.doi.org/10.1590/0103-8478cr20160312
ISSNe 1678-4596
BIOLOGY

Seasonality on the antifungal potential of green propolis collected in Campo Grande – MS, Brazil

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ABSTRACT: ***Apis melífera*** *L. propolis is a resinous and balsamic material whose biological effects are related to its chemical composition. This chemical composition is greatly influenced by seasonality, so propolis from different seasons and regions has a different chemical composition. The increasing need for natural options to control fungi that cause damage to food crops makes propolis an alternative that deserves more research. In this context, the aim of this study was to evaluate the effect of seasonality on the antifungal potential of propolis collected in Campo Grande, Mato Grosso do Sul, Brazil, on the fungus* ***Lasiodiplodia theobromae****. Effects of different concentrations of ethanolic extracts of green propolis on the mycelial growth of the pathogen were evaluated. Concentrations of flavonoids and phenolic compounds in the samples were also determined by spectrophotometric methods. Results showed that the propolis extracts have a different chemical composition, potential fungi static effects on the tested fungus, and that there is interference of seasonality on the mycelial growth of the fungus, pointing to the concentration of 1250µg 100mL^{-1} of the samples collected in the summer, in a first moment, as the most efficient.*
Key words: ***Lasiodiplodia theobromae****, alternative control, time of collection, green propolis.*

Sazonalidade sobre o potencial antifúngico da própolis verde coletada em Campo Grande – MS

RESUMO: *A própolis da* ***Apis mellifera*** *L. é um material resinoso e balsâmico, cujos efeitos biológicos estão relacionados a sua composição química, e esta, sofre grande interferência da florada e da sazonalidade. Por isso, o própolis de regiões diferentes possuem composição química diferente. A busca crescente por opções naturais no controle de fungos que causam danos às culturas de alimentos torna a própolis uma alternativa a ser pesquisada. Nesse contexto, o objetivo deste trabalho foi avaliar o efeito da sazonalidade sobre potencial antifúngico da própolis verde coletada em Campo Grande – MS sobre o fungo* ***Lasiodiplodia theobromae****. Foram avaliados os efeitos de diferentes concentrações dos extratos etanólicos de própolis verde, coletadas em diferentes períodos do ano, sobre o crescimento micelial do fitopatógeno. Determinaram-se as concentrações de flavonoides e compostos fenólicos das amostras por meio de métodos espectrofotométricos. Os resultados demonstraram que os extratos de própolis verde possuem composição química diferente, potencial fungistático sobre o fungo testado, e que há interferência da sazonalidade no crescimento micelial do fungo, apontando para a concentração de 1250µg 100mL^{-1} das amostras coletadas no verão, em um primeiro momento, como a de maior eficiência.*
Palavras-chave: ***Lasiodiplodia theobromae****, controle alternativo, época de coleta, própolis verde.*

INTRODUCTION

Propolis is a resinous substance, produced from material from various plant species collected by a range of bee species, among them ***Apis mellifera*** L. It has a pleasant aroma and a greenish yellow to dark brown color, depending on its origin and the time of collection (MARCUCCI, 1995; BANKOVA et al., 2000; TEIXEIRA et al., 2010; TORETI et al., 2013). Propolis is used by bees for its biological properties, protecting them against attacks from fungi and bacteria; they also use it in preparing aseptic places for the queen bee to lay eggs and in the mummification of invasive insects (MARCUCCI, 1995; BANKOVA et al., 2000; SILVA et al., 2006; LONGHINI et al., 2007; LUSTOSA et al., 2008; BANKOVA, 2009).

More than 300 substances have already been identified in different samples of propolis (KOC et al., 2005; ABUBAKAR, et al., 2014; KUREK-GÓRECKA et al., 2014), including fatty acids, phenolic acids, phenolic esters, terpenes, β-sterols, aromatic aldehydes, alcohols, sesquiterpenes and naphthalenes (MARCUCCI, 2001; KUREK-GÓRECKA et al., 2014).

The propolis produced in the Brazilian Cerrado regions, which contains fragments of the plant ***Baccharis dracunculifolia*** DC (called "wild rosemary" in Brazil) is internationally known as green propolis. It is recognized as having biological activities, such as anti-microbial (bactericidal and fungicidal), anti-

Received 03.25.16 Approved 10.17.16 Returned by the author 12.02.16
CR-2016-0312.R2

inflammatory, cicatrizing, anesthetic, photo-protective, antibiotic, antiviral, antioxidant, immunomodulatory, hypotensive, anti-HIV, anti-carcinogenic and even anti-neoplasic (PEREIRA et al., 2002; BANKOVA, 2005; BANKOVA, 2009; TEIXEIRA et al., 2010). Biological activities of this kind of propolis are attributed to phenolic substances, such as flavonoids, prenylated p-coumaric acids, and lignans (BANKOVA et al., 2000). Due to its tropical climate, production of propolis in Brazil by bees occurs throughout the year, which leads to variation in the chemical composition of propolis produced in different seasons of the year (BANKOVA et al., 1998).

In a study carried out by SILVA et al. (2006) it was observed that propolis sampled in periods of high rainfall had lower values waxes and consequently higher bioactive compounds. This occurs because in this period, bees had less available resins in plants, and lower contents of waxes and consequently higher contents of bioactive. Difference in chemical composition of propolis such as flavonoids and phenolic compounds is explained by the different plants where the bees collect the resin material to be used for production (CHAILLOU et al., 2004). These substances secreted by plants are present as secondary metabolites, for which are attributed the biological action of propolis (BANKOVA, 2005).

The fungus ***Lasiodiplodia theobromae*** is a phytopathogen of great importance for Brazilian fruit-growing (PEREIRA et al., 2006). It causes cankers, perishing and root rot in more than 500 species, including perennial crops, fruit, vegetables and ornamental plants (ÚRBEZ-TORRES et al., 2008). The pathogenicity of this fungus has been increasing over the years, mainly due to deforestation and the destruction of environments with monocultures, which dramatically reduce natural diversity, restricting biological interactions.

In the search for new organic formulations that help to control phytopathogens, and taking into consideration that the composition of propolis varies with flowering and harvesting period, the objective of this research was to check the effect of seasonality on the antifungal potential of different concentrations of ethanolic extract of green propolis on the post-harvest phytopathogen ***Lasiodiplodia theobromae***.

MATERIALS AND METHODS

The propolis was collected on the experimental farm "Fazenda Escola Três Barras" at the Anhanguera Uniderp University in Campo Grande - MS (S 20º 34' 04" W 54º 32' 16"). To observe the effect of seasonality on the characteristics of green propolis, samples were chosen that came from months distributed in the four seasons of the year - spring (September), summer (February), autumn (April) and winter (July). The three beehives in the area that were observed to be most productive were selected, and from these one sample per season was collected, except for the seasons of spring and autumn, in which there was no production in one of the hives in the collection month, thus totaling 10 samples.

The raw ethanolic extract was prepared according to methodology described by PARK et al. (2002). The concentration obtained was 2.0g $30mL^{-1}$. The phytopathogen used was ***Lasiodiplodia theobromae***, the causal agent of stalk rot. The fungus was provided by the Maria Menezes Culture Collection at the Universidade Federal Rural de Pernambuco (UFRPE).

To evaluate the antifungal activity 20 treatments were tested, in a 4x5 factorial scheme: four collection periods (summer, autumm, winter and spring) and five concentrations of ethanolic extract of propolis (500, 750, 1000 and 1250µg $100mL^{-1}$, plus a control without the extract).

For each concentration, the evaluation was quadruplicated. Two controls were also prepared: one with DMSO (dimethyl sulfoxide), and a blank control. DMSO was used at a ratio of 5µL in each 25mL ethanolic extract of propolis. Measurements were carried out daily of the surface of the fungal growth, until that on the control reached the edge of the Petri dishes. The evaluation of mycelial growth was carried out using the mean of two perpendicular measurements of the diameter of the mycelial border, done daily. Quantification of the antifungal activity of the extracts was carried out based on the percentage inhibition of diameter growth (PIDG), which measures the activity of each extract in relation to the advance of the mycelial border, according to the equation below, in which D_c is the diameter of the colony on the control plate and D_t is the diameter of the colony on the test plate (HIMRATUL-AZNITA et al., 2011).

$$\text{PIDG}\ (\%) = \frac{Dc - Dt}{Dc} * 100$$

The percentage was determined for the daily growth rate (TX), by means of the following formula:

$$TX = \frac{\text{Final Diameter of Colony}}{\text{Number of Days to Incubation}}$$

Values for the percentage mean growth of the pathogens in each treatment were used to calculate the area under the disease progress curve (AUDPC) (CAMPBELL & MADDEN, 1990).

The concentration of the total phenolic compounds was determined using the Folin-Ciocalteu method, as described by CHAILLOU et al. (2004), using

gallic acid as standard for construction of calibration curve. For the analyses of flavonoids the methodology was from FUNARI & FERRO (2006), using standard curve with quercetin solution as a reference standard. Concentration of flavonoids was obtained by preparing solution of 2.0ml of the propolis extract to 1.0ml of 2.5% aluminum chloride, and the reading was performed in a spectrophotometer at 425nm.

The data were submitted to analysis of variance by the F-test and, when significant, a means comparison was carried out by Tukey test ($P \leq 0.05$) and a regression analysis for the different concentrations of the extracts. For the statistical analyses, we used the software Assistat (SILVA, 2014).

RESULTS AND DISCUSSION

Results of the assays demonstrate that there was a significant interaction between the collection months for propolis and the concentrations of the extracts tested for the inhibition of mycelial growth of ***L. theobromae*** on the first two days of growth, out of three days in total. In table 1, it can be seen that for the first day of evaluation and using the highest concentrations (1000 and 1250mg mL^{-1}), the lowest inhibition of the fungal growth was observed in the extracts taken from propolis collected in winter. For the lowest concentration of the extract, in summer and spring there was a greater inhibition of the growth of this fungus. In summer and autumn, as the extract concentrations increased, there was a tendency for the mycelial growth of the fungus to be increasingly inhibited.

On the second day of evaluation, however, in the control treatment and the concentration of 500mg mL^{-1}, differences were not observed between collection times, as regards antifungal action by the propolis extract. Again, in summer and autumn, there was a tendency to increase antifungal activity by the extracts as concentrations increased. In winter and spring, the best results regarding the inhibition of fungal growth were for concentrations of 750 and 1000mg mL^{-1}, respectively (Table 1).

Tables 2 and 3 show the percentage inhibition of diameter growth (PIDG), daily growth rate (TX) and area under the disease progress curve (AUDPC). It can be seen that there was no significant difference between the concentrations tested and seasonality for the variables PIDG and TX. For the AUDPC there was significant interaction between concentrations and propolis collection times, and the outcome of this interaction is shown in table 3. The AUDPC evaluates the disease severity, and the greater its value, the more aggressive the disease. In the case of this experiment, the greater the AUDPC, the lower the inhibition by the extract, as the fungus showed higher growth. With the increase in the concentrations of the extracts, in summer and autumn, there is a tendency for fungal growth to decrease.

In general, the control of fungal infections depends initially on the complexes and mechanisms of defense in each host. If the disease establishes itself

Table 1 - Outcome of the interaction months x concentrations of propolis for the diameter (cm) of the colony on the first and second day of evaluation in the "*in vitro*" assay of the antifungal activity of different concentrations of green propolis extract collected in seasons of the year, on ***Lasiodiplodia theobromae***. Campo Grande, MS, 2014.

	Concentrations of propolis (mg mL^{-1})	Seasons			
		Summer[(1)]	Autumn[(2)]	Winter[(3)]	Spring[(4)]
Day 01	Control	2.84 a	2.84 a	2.84 a	2.84 a
	500	2.34 b	2.64 a	2.80 a	2.30 b
	750	2.50 b	2.79 a	2.07 d	2.23 c
	1000	2.15 b	2.18 b	2.32 a	2.26 ab
	1250	1.74 c	2.43 b	2.73 a	2.26 b
	Concentrations of propolis (mg mL^{-1})	Seasons			
		Summer[(5)]	Autumn[(6)]	Winter[(7)]	Spring[(8)]
Day 02	Control	7.34 a	7.34 a	7.34 a	7.34 a
	500	6.31 a	6.55 a	6.26 a	6.53 a
	750	6.33 b	7.20 a	5.63 c	6.02 b
	1000	6.11 a	6.50 b	6.16 a	6.24 a
	1250	5.02 b	6.46 a	6.40 a	6.32 a

Means followed by the same letter in the line do not differ statistically by Tukey test at 5% probability. [(1)]$y=2.832\ 0.002x+0.000003921x^2$ ($R^2=0.97$); [(2)]$y=2866-0.0004167x$ ($R^2=0.56$); [(3)]$y=2.848+0.002x-0.000007329x^2+0.000000004364x^3$ ($R^2=0,82$); [(4)]$y=2.248+0.589e^{(-0.05x)}$ ($R^2=0.99$); [(5)]$y=7.338-0.006x-0.00000984x^2+0.00000000546x^3$ ($R^2=0.99$); [(6)]$y=7.250-0.000629x$ ($R^2=0.51$); [(7)]$y=7.362-0.004x+0.0000241x^2$ ($R^2=0.92$); [(8)]$y=7.358-0.003x+0.00000144x^2$ ($R^2=0.95$).

Table 2 - Mean values of the percentage inhibition of diameter growth (PIDG), rate of mycelial growth (TX) and area under the disease progress curve (AUDPC) in the *in vitro* assay of antifungal activity of different concentrations of green propolis extract collected in different seasons of the year, on ***Lasiodiplodia theobromae***. Campo Grande, MS, 2014.

Treatments	PIDG (%)	TX (cm day^{-1})	AUDPC
----------Seasons----------			
Summer	4.32	2.67	134.17
Autumn	1.34	2.76	146.00
Winter	3.12	2.71	138.32
Spring	2.52	2.73	139.81
F Test	0.94^{ns}	1.03^{ns}	2.98*
LSD $^{(1)}$	3.51	0.10	8.65
----------Concentrations of propolis (mg mL^{-1})----------			
Control	3.15	2.70	153.90
500	0.68	2.78	141.88
750	3.14	2.71	137.79
1000	3.37	2.70	136.78
1250	4.69	2.66	133.46
F Test	1.84^{ns}	1.88^{ns}	6.28**
LSD$^{(1)}$	5.05	0.14	12.21
S x C$^{(2)}$	1.43^{ns}	1.43^{ns}	2.96**
CV (%)$^{(3)}$	203.08	6.42	9.69

By F test, **significant at 1% probability; *significant at 5% probability; ^{ns}non-significant. $^{(1)}$Least significant difference. $^{(2)}$Interaction seasons x propolis concentration. $^{(3)}$Coefficient of variation.

when there is a failure in these, it will be necessary to use fungicidal or fungistatic products that act against the pathogen, preventing damage to the host (FARNESI, 2007).

As shown in table 4, the extracts of propolis tested have a high concentration of phenolic compounds and flavonoids, with the exception of the extract collected in autumn, which may have caused the difference in the growth rate in the tests. According to Brazilian legislation (BRASIL, 2001), propolis is classified aby its flavonoid content in: low content (up to 1.0% mm^{-1}), average content (between 1.0 and 2.0%) and high contents (above 2.0%). Thus, samples taken in summer, winter and spring are considered of high content. Before the preparation of the extract, differences were observed in color and consistency of autumn taste of autumn. Samples obtained in summer, winter and spring showed up green brownish with hard texture and dry. Already the sample collected in autumn showed green color and sticky texture. The difference in color remained after preparation of the extract.

Table 3 - Outcome of interaction months x concentrations of propolis for the area under the disease progress curve (AUDPC) in the *in vitro* assay of the antifungal activity of different concentrations of green propolis extract collected in different seasons of the year, on ***Lasiodiplodia theobromae***. Campo Grande, MS, 2014.

Concentrations of propolis (mg mL^{-1})	----------Seasons----------			
	Summer$^{(1)}$	Autumn$^{(2)}$	Winter$^{(3)}$	Spring$^{(4)}$
Control	153.90 a	153.90 a	135.90 a	153.90 a
500	139.28 b	144.80 a	142.22 a	142.34 a
750	140.46 b	153.53 a	127.41 c	133.63 cb
1000	134.50 a	139.25 a	136.51 a	138.14 a
1250	115.85 b	142.48 a	141.95 a	138.11 a

Means followed by the same letter in the line do not differ statistically by Tukey test at 5% probability. $^{(1)}$y=153.894-0.082x+0.00001501x^2-0.00000008706x^3 (R^2=0.99); $^{(2)}$y=153.525-0.010x (R^2=0.49); $^{(3)}$y=154.073-0.030x+0.000002039x^2+0.000000009786x^3 (R^2=0.92); $^{(4)}$y=154.234-0.039x+0.0000206x^2 (R^2=0.92).

Table 4 - Mean values of total phenolic compounds and flavonoids in green propolis collected in different seasons of the year. Campo Grande, MS, 2014.

Treatments	Phenolic compounds (%)	Flavonoids (%)
Seasons		
Summer	12.8 a	7.6 a
Autumn	0.0 c	0.2 c
Winter	12.8 a	7.6 a
Spring	8.8 b	5.1 b
Reference value[(1)]	≥ 5.0%	≥ 0.5%
F Test	11.49**	15.49**
LSD [(2)]	3.79	1.54
CV (%)[(3)]	24.78	19.80

Means followed by the same letter in the columns do not differ statistically by Tukey test at 5% probability. By F test, **significant at 1% probability. [(1)]Reference values under existing legislation (BRASIL, 2001). [(2)]Least significant difference. [(3)]Coefficient of variation.

Many therapeutic activities of phytochemicals are ascribed to biologically active phenolic compounds, such as flavonoids and phenolic acids. As well as being well known for their antioxidant activity, phenolic compounds stand out for their ability to bind to cell receptors and to membrane transporters, and to influence gene expression, cell signaling and adhesion, among other functions. Besides their antioxidant function, there is evidence that proves the antifungal action of phenolic compounds, and one of the mechanisms by which this action may occur is the inactivation of enzymatic systems in the microorganisms involved in producing energy and in the synthesis of natural compounds (CHEN, 2006).

The fungitoxic/fungicidal action of the extracts of propolis is attributed to the presence of phenolic compounds, mainly flavonoids, phenolic acids and their esters (KOC et al., 2005). In the case of propolis collected in autumn, the antifungal activity demonstrate could be attributed for other class of compounds. The microbial activity of propolis may be the result of the synergistic action of several of its components (SIQUEIRA et al., 2009; KUREK-GÓRECKA et al., 2014).

The mechanism by which the flavonoids act on microorganisms is not totally understood yet. It is only known that they act by means of metabolic disturbance, destabilizing the channels of plasmatic membrane ions (FARNESI, 2007).

CONCLUSION

It may be concluded that the extract of green propolis has fungistatic potential against the fungus ***Lasiodiplodia theobromae***, since alterations were observed in the growth rate of the samples growing in different concentrations of the extract. Different growth rates were also noted for each collection period tested, demonstrating that there was interference from seasonality, which also influenced the concentration of phenolic compounds and flavonoids in the extracts tested. This may be the cause of the different growth rates observed in the tests carried out. The concentration of 1250µg $100mL^{-1}$ of samples collected in the summer can be pointed, in a first moment, as the most efficient.

ACKNOWLEDGEMENTS

The authors acknowledge the Fundação Manoel de Barros by their partnership.

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