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ANIMAL SCIENCE

Hydroalcoholic extract of pitaya (*Hylocereus guatemalensis*) supplied in the diet for blue *Betta splendens* and its effects on fish pigmentation

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Abstract: The experiment was conducted to evaluate the composition of the hydroalcoholic extract of pitaya bark (*Hylocereus guatemalensis*) and its effects on the diet of male blue *Betta splendens* in terms of skin color, concerning the content of digestive enzymes, liver metabolism, and antioxidant activity. The fish were supplemented with hydroalcoholic extract of pitaya bark (HEPB) at the doses of 0.24, 0.45, and 0.90 mg kg^{-1} of feed for 21 days. The inclusion of 0.24 and 0.45 mg kg^{-1} of the HEPB promoted the best weight gain. and did not change the coloration of blue beta fish (P>0.05). The digestive activity showed an increase in alkaline phosphatase activity only in fish fed 0.45 mg kg^{-1} HEPB. The inclusion of 0.45 and 0.90 mg kg^{-1} from the HEPB promoted a decrease in hepatic ALT activity. An increase in CAT activity was observed in the skin of fish fed diets with added HEPB. In conclusion, the addition of hydroalcoholic extract of pitaya bark in the diet of *Betta splendens* is beneficial for the weight gain parameter and has a hepatoprotective effect but does not affect skin coloration.

Key words: Antioxidant activity, hepatoprotectant, aggregation of value to waste, ornamental fish.

INTRODUCTION

Color is one of the main factors that promote sales and expand choices for consumers in the market of ornamental fish (Celli & Brooks 2017). Therefore, betta fish (*Betta splendens*) displays a variety of colors that are not sensitive to pH variations (6.0 – 8.0), which makes it a highly sought-after species among other aquarium species (Froese & Pauly 2021). One of the ways to maintain and improve the color of ornamental fish species is through the use of pigments in their food (Maiti et al. 2017), which prospects natural sources as in growing demand (Gomes et al. 2021) to improve the appearance of ornamental fish intended for aquariums by intensifying their coloration (Gouveia et al. 2003, Eaton et al. 2016).

Because of the presence of biologically active substances in plant residues (Neves et al. 2012), the development of products with biological activities, and concomitantly added value to these raw materials (Vanegas-Espinoza et al. 2019), is valuable. The activity of nutritional additives in fish diets can influence the utilization of the diet and the metabolization process (do Carmo Ota et al. 2019). The action of antioxidant enzymes in the organism may not fully minimize the damage caused by free radicals produced during metabolic processes or by the organism's exposure to exogenous factors (Venturini et al.

2019). The pitaya plant (*Hylocereus polyrhizus*) belongs to the Cactaceae family and is a climbing fruitful cactus; its fruit has an intense red pulp and rind derived from pigments known as betalains (Wong & Siow 2015), which makes it interesting as a source of natural colorings.

Thus, this study evaluated the composition of the hydroalcoholic extract of pitaya bark (*Hylocereus guatemalensis*) and the effects of its inclusion in blue male betas (*Betta splendens*).

MATERIALS AND METHODS

The pitaya (*Hylocereus guatemalensis*) hulls were acquired in the municipality of Itaporã (Itaporã, Mato Grosso do Sul, Brazil) (south latitude 21° 25 59' 41.8", longitude west W 55° 19' 24.9", and altitude 429 m) in February 2019. The shells were dried, lyophilized for 48 hours and then ground in a Wiley-type mill, with a 2 mm mesh sieve. Hydroalcic extract was obtained by macerated, using 20 mL of hydrocolic solution (50:50) for every 2 g of ground dry matter. The material remained in macerated for seven days, under room temperature (25°C).

In the extract, the antioxidant activity (AA) was determined by: ability to reduce free radical DPPH (1,1-diphenyl-2-picril-hydrazil) and by the capture of free radical ABTS (2,22–azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), according to (Rufino et al. 2007). According to Ramli et al. (2014), the extract contains betanin (17.64 ± 0.03 mg/100 g dry extracts), flavonoids (145.88 ± 2.33 mg% dry basis), and Total phenolic content (65.16 ± 1.52 mg% dry basis), while Lira et al. (2020) reports, in addition to the compounsds already mentioned, the presence of isobetanin, isophyllocactin, sucrose, maltotriose, maltotetraose, phyllocactin, 2'-O-Apiosyl-phyllocactin, 6'-Malonyl-2'-descarboxy-betanin, Isorhamnetin, triglycosides isomer and Quercetin-3-O-hexoside.

The in vivo assay was performed with juvenile male *Bettas splendens*, acclimated to the environment for three days. Fish were divided into 20 experimental units (5 L water) containing one fish each, totaling 5 fish per treatment with photoperiod of 12 hours of light. Water quality was monitored during the assay (2000 µS cm^{-1} of conductivity; pH 7.2; 5.2 ±0.8 mg L^{-1} for dissolved oxygen; water temperature 28.27 ±1.20 °C; 150 mg L^{-1} total hardness as CaCO3, Godoy et al. 2021).

Fish were fed a commercial feed (40.88% PB e 4374.8 kcal kg^{-1}) supplemented with hydroalcoholic extract of pitaya peel to obtain the doses (control; 0.24; 0.45 and 0.90 mg kg^{-1} of HEPB - Table I). The assay has been approved by the Ethics Committee for Animals Use of Universidade Federal da Grande Dourados (Protocol 03/2019).

The growth was measured as: Weight Gain (WG) = (final body weight - initial body weight); Length Gain (LG) = (final body length - initial body length); Specific Growth Rate (SGR) = ([(ln final body weight - ln initial body weight) / number of experimental days] × 100); Diet Consumption (DC) = feed intake (g) day^{-1}; Feed Conversion Ratio (FCR) = feed intake (g) weight $gain^{-1}$ (g); Protein Efficiency Ratio (PER) = weight gain (g) crude prot00ein intake $(g)^{-1}$. The Condition Factor (K) of the fish was calculated by the allometric method, from the expression $K= W/L^b$, where, W represents the total mass, L the standard length of the individuals and b the regression coefficient. To estimate the value of the coefficient b, a single equation of weight-length ratio ($W = aL^b$) was adjusted, from the set of all individuals collected, using the least squares method applied to the data converted in their respective natural logarithms, according to the methodology suggested by Lima-Junior et al. (2002).

The color gain of fish skin was performed using a portable photocolorimeter Chroma

Table I. The chemical composition of diets analyzed and the antioxidant characteristics of the extract.

	Diets (mg kg^{-1})			
	Control	**0.24**	**0.45**	**0.90**
Composition (%)				
Commercial Diet (g)	100	100	100	100
Extract of pitaya (ml)	_	12	25	50
Chemical composition (%)				
Dry matter	90.38	90.38	90.38	90.38
Mineral matter	10.54	10.54	10.54	10.54
Crude protein	40.88	40.88	40.88	40.88
Lipid	10.31	10.31	10.31	10.31
Fibre	2.46	2.46	2.46	2.46
Crude energy (kcal kg^{-1})	4374.8	4374.8	4374.8	4374.8
Antioxidant characteristics				
ABTS mmol trolox	24.25[a]	26.60[b]	29.15[c]	29.60[d]
DPPH mmol trolox	5.90	6.15	6.20	5.85

*** (ABTS) = 2,2-azinobis free radical (3-ethylbenzothiazoline-6-sulfonic acid); (DPPH) = 2,2- Diphenyl (1-picrylhydrazyl) free radical.**
[a, b, c, d]*Averages followed by different letters report a difference by one-way analysis of variance and Tuckey's test (P< 0.05).

Meter CR-400 (Konica Minolta®), by the Hunter's coordinate system L*, a*, b*, which measured the intensity of L* representing brightness or luminosity (-100, black and + 100, white), the chromaticity of the a*, represented by shades of green (-100) and red (+100) and chromaticity of b*, represented by shades blue (-100) and yellow (+100) (Rezende et al. 2012). The quantification was performed in the dorsal region, just below the dorsal fin, in the fish in vivo.

At the end of the experiment, the fish remained in food restriction for 24 hours. All fish were anesthetized with clove oil (50 mg L^{-1}) for one minute (Honorato & Nascimento 2016), and then euthanized, weighed, and measured. The samples of fish liver, intestine, and skin for enzymatic analysis were immediately stored at -80 °C. The digestive tract, liver, and skin were excised and homogenized to measure enzyme activity. The intestine homogenates were done into 0.02 M Tris / 0.01 M phosphate buffer pH 7.0 mixed with anhydrous glycerol v/v under ice-bath, with a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 12,000 × g for 3 minutes and the supernatants (crude homogenates) were used as enzyme source. The digestive enzymes are nonspecific protease, lipase, amylase, and alkaline phosphatase.

Non-specific proteolytic activity was assayed with 1% casein as substrate (Walter, 1984). The pH values were previously optimized for intestine, and 0.1 M Tris - HCl (pH 9.0) for middle. Reactions were carried out at 25 °C for 1 h, stopped with 15% TCA, and the optical density of the supernatant recorded at 280 nm against tyrosine as standard. Both a substrate free control and an enzyme free control were run. Specific activity was expressed in micromole of hydrolyzed substrate protein (UI mg^{-1} protein).

Amylase was assayed according to Bernfeld (1955), slightly modified. Briefly, the reactions were done in 0.2 M citrate/phosphate buffer

pH 7.0 with 5% starch solution as substrate and 0.5% NaCl as cofactor. The amount of crude homogenate was previously adjusted. Reactions were performed at 25 °C for 35 min and stopped by addition of 5% $ZnSO_4$ and 0.3 N $Ba(OH)_2$. Precipitate was discarded after centrifugation at 1000 87 × g for 3 minutes and the reaction product was measured at 690 nm (Park 1949).

Lipase determination was adapted from Albro et al. (1985) method. Reactions were incubated with 0.4 mM r-nitrophenyl myristate in 24 mM ammonium bicarbonate (pH 7.8) with 0.5% Triton X-100. Control reactions were run without enzyme and without substrate. The reaction was stopped by the addition of NaOH to a final concentration of 10 mM and the optical density was registered at 405 nm for 30 min. One unit was defined as micromole of substrate hydrolyzed per min and expressed per milligram protein (UI mg^{-1} protein). The Alkaline Phosphatase (AP) was measured using colorimetric methods (Alkaline phosphatase OSR6004), analysis by spectrophotometry (semi-automatic spectrophotometer Bioplus S-200).

The liver sub-samples were homogenized in buffer as described earlier. Homogenates were centrifuged at 600 g for 3 min, and the supernatants centrifuged at 6000 g for 8 min at 3 °C. Resultant supernatants were used as crude enzyme source. Enzyme protocol assays were: Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assays were end-point type, read at 430 nm following the protocol: ALAT - mM alanine or ASAT - 44.4 mM aspartate, 11.6 mM a-ketoglutarate, 0.22 mM arsenate, 0.27 mM pyridoxal phosphate and phosphate buffer pH 7.0 (Reitman & Frankel 1957). The albumin was measured using colorimetric methods (albumine OSR6102), analysis by spectrophotometry (semi-automatic spectrophotometer Bioplus S-200). Protein concentrations in enzyme crude extracts were determined as Bradford (1976) method at 450nm, and 1.0 mg mL^{-1} of albumin was used as standard.

The skin samples were homogenized in buffer as described earlier. Homogenates were centrifuged at 12,000 g for 3 min, and the supernatants (crude homogenates) were used as enzyme source. SOD was assayed by pyrogallol self-oxidation, which is inhibited in the presence of SOD (Beutler 1984) modified. Absorbance readings were performed at 420 nm, considering that 1.0 UI inhibits 50% the pyrogallol self-oxidation. CAT activity was assayed by reading H_2O_2 decay at 230 nm (Beutler 1984). One unit of CAT was defined as the amount of enzyme required in 1.0 µmol of H_2O_2 min^{-1} oxidation, and the molar absorptivity used was (H_2O_2) $\epsilon\lambda 230$ = 0.071 mM cm^{-1}. Protein concentrations in enzyme crude extracts were determined as (Bradford 1976) at 450nm, and 1.0 mg mL^{-1} of albumin was used as standard.

Statistical analyses were performed using RStudio software (R Core Team 2019). Data normality and variance was checked by the Shapiro-Wilk test. It was performed a one-way analysis of variance (ANOVA); and when differences were significant ($P< 0.05$), the means were compared by Tukey test. The results were expressed as means ± standard deviation (SD).

RESULTS

The pitaya bark extract in different doses increased antioxidant activity (AA) measured by the DPPH (2,2-difenil-1-picril-hidrazil) and ABTS (2,2 AZINO BIS [3-ethylbenzo thiazoline 6 sulfonic acid]) methods (Table I).

Dietary inclusion of 0.24 and 0.45 mg kg^{-1} HEPB improved weight gain compared to control fish (Table II). The lowest growth rates were observed in fish in the control group and supplemented with 0.24 mg kg^{-1} HEPB. No significant differences ($P> 0.05$) were observed

Table II. Zootechnical performance, skin coloration, the activity of digestive, metabolic, and antioxidant enzymes of *B. splendens* fed with different levels of inclusion of hydro-alcoholic extract of pitaya bark.

	Diets (mg kg^{-1})				
Zootechnical parameters	**Control**	**0.24**	**0.45**	**0.90**	**P-value**
Weight gain (g)	0.46 ±0.15^{c}	0.84 ±0.11^{a}	0.93 ±0.40^{ab}	0.44 ±0.33^{bc}	0.02*
Total Length (cm)	66.92 ±2.90	66.36 ±4.45	69.47 ±7.21	65.21 ±3.95	0.58ns
Standard Length (cm)	39.51 ±1.63	39.56 ±1.35	40.37 ±3.48	35.89 ±5.07	0.17ns
Height gain (cm)	14.49 ±1.83^{ab}	14.87 ±0.86^{a}	15.72 ±1.44^{a}	12.97 ±0.61^{b}	0.02*
Food Conversion	4.02 ±2.20	2.42 ±0.36	2.94 ±1.77	3.40 ±1.52	0.47ns
Specific Growth Rate (% day^{-1})	34.15 ±0.39^{b}	2.09 ±0.23^{a}	35.13 ±0.80^{ab}	34.06 ±1.01^{b}	0.03*
Relative Condition Factor	6.62 ±0.00^{b}	1.70 ±0.01^{a}	1.94 ±0.02^{ab}	4.69 ±0.01^{b}	0.04*
Color parameters					
L*	34.83 ±3.55	32.07 ±4.14	31.75 ±3.89	31.79 ±2.84	0.49ns
a*	-0.91 ±4.65	-0.06 ±4.61	-0.70 ±3.60	0.57 ±2.31	0.93ns
b*	-10.64 ±4.94	-15.32 ±3.71	-12.31 ±5.11	-12.24 ±3.75	0.43ns
Digestive enzyme (U mg^{-1} prot)					
Nonspecific Protease	398.33 ±155.15	314.83 ±162.10	317.95 ±172.12	381.94 ±121.13	0.2078
Amylase	0.36 ±0.05	0.36 ±0.10	0.37 ±0.08	0.35 ±0.07	0.82
Lipase	46.19 ±4.10	43.75 ±4.45	40.65 ±5.33	48.79 ±5.39	281
Alkaline phosphatase	42.00 ±5.40^{b}	40.75 ±11.39^{b}	171.00 ±80.02^{a}	49.20 ±3.89^{b}	0.0001
Metabolic enzyme					
ALT (U mL^{-1})	892.00 ±31.14^{a}	813.75 ±19.64^{a}	633.00 ±29.47^{b}	603.75 ±14.62^{b}	0.0001
AST (U mL^{-1})	859.60 ±31.19	879.50 ±82.96	774.00 ±21.41	761.25 ±23.95	96
Albumin (g dL^{-1})	0.13 ±0.01	0.12 ±0.01	0.12 ±0.02	0.12 ±0.03	97
Antioxidant enzyme (U mg^{-1} prot)					
SOD	4.13 ±0.75	3.65 ±0.54	4.20 ±0.55	4.12 ±0.29	0.083
CAT	55.67 ±9.02^{b}	88.5 ±6.36^{a}	73.33 ±2.08^{a}	77.00 ±2.88^{a}	0.0001

*** Values are the mean ± SE.$^{a, b, ab, c, bc*}$ Averages followed by different letters report a difference by one-way analysis of variance and Tuckey's test (P< 0.05). Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), catalase enzymes (CAT) and superoxide dismutase (SOD).**

in the mean total length, standard length, and feed conversion, regardless of the used HEPB dose (Table II).

Increasing the inclusion of HEPB did not change the coloration of blue beta fish (Figure 1 and Table II). Among the digestive enzymes, an increase in alkaline phosphatase (AF) activity was observed only with 0.45 mg kg^{-1} HEPB in the feed. However, no differences were observed in digestive protease, amylase, and lipase at any level of HEPB inclusion (Table II). Among the evaluated liver enzymes, no significant difference was observed in AST and albumin in any of the testing concentrations. The inclusion of 0.45 and 0.90 mg kg^{-1} HEPB promoted a decrease in hepatic ALT activity (Table II).

For enzymes related to antioxidant metabolism, an increase in CAT activity was observed in the skin of fish fed diets with added HEPB, however, no change in SOD activity was observed, regardless of the level of HEPB inclusion (Table II).

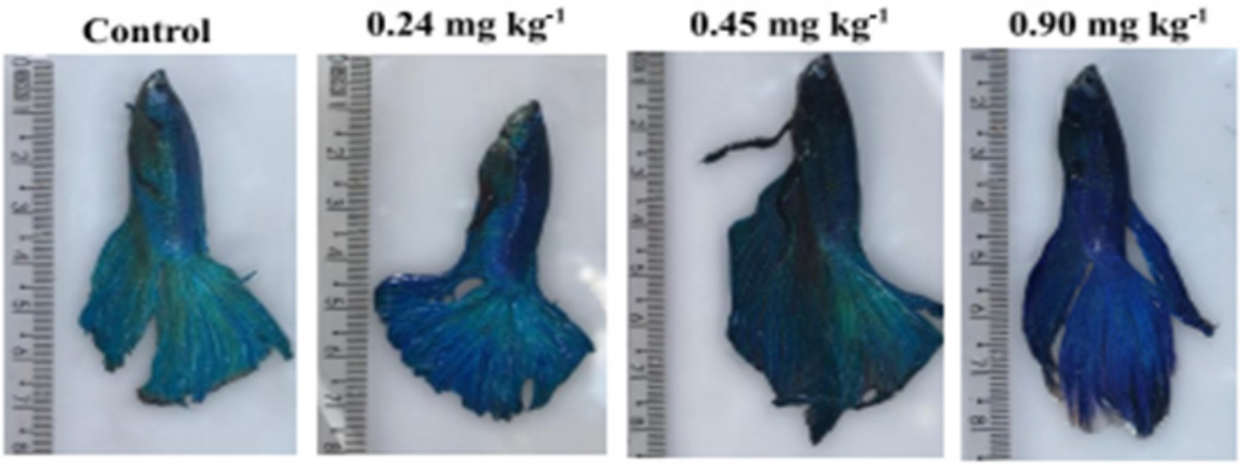


Figure 1. Effect of inclusion of pitaya bark hydroalcoholic extract on skin pigmentation of male blue *Betta splendens*.

DISCUSSION

The beneficial effect provided using plants in fish feeding is one of the reasons for the inclusion of these actives (Oetting et al. 2006, do Carmo Ota et al. 2019, Gomes et al. 2021). The action of plants and their derivatives, when included in diets, is attributed to the presence of many components in the active principle (Awad et al. 2013) this mode of action is related to the assimilation of dietary compounds (Vanegas-Espinoza et al. 2019), the presence of anthocyanins (Guimarães et al. 2012), and the presence of betalains (Abreu & Reis 2020).

An interesting characteristic of pitaya is the presence of the two categories of betalain (Harivaindaran et al. 2008), the betaxanthins (Chew et al. 2019) and betacyanins (Wong & Siow 2015), which both present high antioxidant activities. These pigments are soluble in water, which represents an attractive characteristic for potential uses such as in food coloring, antioxidants, and/or pharmacological applications (Das et al. 2021). Products that are rich in betacyanins are usually red in color, derived from the Betalamic Acid (Marañón-Ruiz et al. 2011). This acid shows biological activity in scavenging free radicals (Celli & Brooks 2017) and benefits to animal health (Sawicki et al. 2020). The action of compounds such as dyes containing nutritional actives in animal feed can have a beneficial effect on the zootechnical performance of fish, promoting enhanced development (Nhan et al. 2019, Vanegas-Espinoza et al. 2019, Rashidian et al. 2020). In our study, the use of 0.24 and 0.45 mg kg^{-1} HEPB in the feed of *B. splendens* promoted improvement in weight gain (Table II). It is noteworthy that there was no difference in feed conversion, which guarantees that the diet consumption was effective during the trial and efficiently metabolized by the digestive tract of these fish.

No alterations in the color of fish were observed with the inclusion of HEPB in their diets. The use of nutritional actives from natural sources is still incipient in the field of feed development for ornamental fish. Despite the presence of pigments in the composition of some products, they do not alter the skin color when added to fish diets. Among these are the use of pink pepper essential oil (Porto et al. 2020) and the addition of bacuri pulp oil (*Attalea phalerata*) (Porto et al. 2020) in the diets for *Hyphessobrycon eques*. The pigmentation of fish is associated with the species, ability of the dye to pigment, its dose in the diet (Li et al. 2017) and

especially the form of inclusion (Dethlefsen et al. 2016). The search for dyes capable of increasing the blue color is still scarce; it is noteworthy that anthocyanins at neutral to alkaline pH tend to be blue-violet in color (Guimarães et al. 2012).

In this study with young male bettas, the inclusion of 0.45 mg kg^{-1} HEPB promoted an increase in alkaline phosphatase activity, which shows the action of betalains in the digestive tract. It is noteworthy that the increase in alkaline phosphatase (AP) activity corresponds to greater absorption of nutrients by the digestive tract. The increase in intestinal AP activity stimulated by the inclusion of natural products has been reported in *Sprague Dawley* rats (Iahtisham-Ul-Haq et al. 2019). Other studies reveal that this enzyme participates in the absorption of nutrients such as lipids, glucose, calcium, and inorganic phosphate (Tengjaroenkul et al. 2000). Some substances can function as gastric protectors or even as stimulants in the production of digestive enzymes (Ayyat et al. 2018, El-Gendy et al. 2022), which have benefits for the health and well-being of fish. The stimulation of enzyme production by the presence of specific foods is associated with both the production and modulation of gene expression in the hepatopancreas (Fuentes-Quesada et al. 2018, Giri et al. 2023).

In this study, a hepatoprotective effect of HEPB of decreasing ALT activity was observed. The increase in AST and ALT activity is considered the organism's response to stressors and nutritional metabolism; this increase is indicative of an injured or damaged organ (Meng et al. 2018). Therefore, the decreased activity of these enzymes can be attributed to the presence of compounds in the diet that have antioxidant activity, such as betalains.

Some plants are reported in the literature as efficient in promoting improvements in the antioxidant response and immune status (Song et al. 2017, Wang et al. 2018, Nunes et al. 2019, Chu et al. 2021, El-Gendy et al. 2022). The knowledge on the importance of natural dyes, such as carotenoids, and their role in coloring and eliminating free radicals is still very preliminary (Hõrak et al. 2006). However, it has been reported that they influence the healthy functioning of systems and the prevention of oxidation (Gomes et al. 2021).

Endogenous antioxidant enzymes play a crucial role in eliminating harmful reactive oxygen derivatives, which were important indicators of the antioxidant response (Aliko et al. 2018). The increased antioxidant activity of the diets with added HEPB is linked to the presence of bioactive in the pitaya bark, the betalains (Das et al. 2021). In the present study, the HEPB supplementation significantly increased CAT activity. Increased SOD and CAT activities were observed with the use of astaxanthin in Channa argus (Li et al. 2017, Lili et al. 2020). Despite the increased antioxidant activity in the betta's skin, no non-standard color alteration was observed in this evaluation. The increased expression of skin color requires an increase in the availability of other antioxidants (non-pigmenters) (Pérez et al. 2008).

CONCLUSIONS

The results obtained in this study indicate that the addition of HEPB in the diet of young male blue bettas helps the digestive function and has a hepatoprotective effect that can be attributed to the antioxidant action of compounds present in the pitaya bark. Also demonstrates beneficial for zootechnical performance, affecting the Weight gain, but does not demonstrate affect skin coloration when utilizing these doses.

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PATRICIA C. GAUER: performed and analyzed the experiments, wrote the manuscript, designed the study and analyzed the experimental data, read and approved the final manuscript; ANNYE C.V. FERREIRA: correction and adaptation of the article for the journal (Anais da Academia Brasileira de Ciências), read and approved the final manuscript; MAYARA S. SIQUEIRA and HIGO A. ABE: performed the experiments, analyzed the experimental data; HERIBERTO GIMÊNES-JUNIOR: designed the study, read and approved the final manuscript; LETICIA FANTINI-HOAG and ANTONIO CESAR GODOY: analyzed the experimental data, read and approved the final manuscript; CLAUCIA APARECIDA HONORATO: conceived the study, designed the study, wrote the manuscript, read and approved the final manuscript.

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