ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating 2017: 3.53
JMPS 2017; 5(5): 39-45
© 2017 JMPS
Received: 13-07-2017
Accepted: 14-08-2017

Mythali Lima Garcia
Laboratório de PD & I de Bioprodutos, Faculdade de Farmácia, Universidade Federal de Goiás, Goiânia, GO, Brazil

Nayara Luiza Oliveira Ferreira
Laboratório de PD & I de Bioprodutos, Faculdade de Farmácia, Universidade Federal de Goiás, Goiânia, GO, Brazil

Rejanne Lima Arruda
Laboratório de PD & I de Bioprodutos, Faculdade de Farmácia, Universidade Federal de Goiás, Goiânia, GO, Brazil

Marta Cristina Corsi De Filippi
Departamento de Fitopatologia, Embrapa Arroz & Feijão, Santo Antônio de Goiás, GO, Brazil

Edemilson Cardoso Da Conceição
Laboratório de PD & I de Bioprodutos, Faculdade de Farmácia, Universidade Federal de Goiás, Goiânia, GO, Brazil

Correspondence
Mythali Lima Garcia
Laboratório de PD & I de Bioprodutos, Faculdade de Farmácia, Universidade Federal de Goiás, Goiânia, GO, Brazil

Standardized rosemary extract induces host plant defenses and suppresses rice leaf blast

Mythali Lima Garcia, Nayara Luiza Oliveira Ferreira, Rejanne Lima Arruda, Marta Cristina Corsi De Filippi and Edemilson Cardoso Da Conceição

Abstract
The efficiency of rosemary (*Rosmarinus officinalis* L.) liquid extract (RLE), standardized as rosmarinic acid (RA) on the suppression of rice (*Oryza sativa*) leaf blast (*Magnaporthe oryzae*) (LB) were determinate. RLE (3.3, 6.6, and 10 mg·mL^{-1}), SRA (3.1, 4.2, and 6.3 mg·mL^{-1}), and water (control) were sprayed on leaves of Primavera rice cultivar, at 48 hours before inoculation with *M. oryzae* (MO). The activities of chitinase, β-1, 3-glucanase, peroxidase, lipoxygenase, phenylalanine ammonia-lyase, and phenolic compounds and of salicylic acid levels were determined in samples collected before and after inoculation with MO. The highest concentrations of RLE and RA reduced the LB severity by more than 92%. Except for Peroxidase, all enzymes increased their activity after spraying RLE and RA, prior and after the challenge inoculation. Our results suggest that RLE standardized as RA can induce the activity of enzymes related to plant defense.

Keywords: *Magnaporthe oryzae*, *Oryza sativa*, *Rosmarinus officinalis*, induction of resistance, rosmarinic acid

Introduction
Leaf blast is caused by *Magnaporthe oryzae* B. Couch [anamorph *Pyricularia oryza Cav.*] and is one of the most important diseases affecting rice, resulting in up to 100% loss of plantations. Fungicide treatments account for approximately 65% of production costs, and the use of vegetal extracts to reduce resistant pathogens development and prevent environment and human health disturbances is an alternative to be incorporated on integrated disease [1, 2, 3, 4].
Vegetal extracts are phyto complexes, which means they are composed by a mixture of active compounds. Because of that, their chemical and physical characteristics have to be monitored and reproducible to attest their effective activity. Thus, extract standardization is required, and it is performed based on one or more substances contained in the extract that are used as biomarkers [5].
Rosmarinus officinalis L., popularly known as rosemary, has been widely studied since its antioxidant, antimicrobial, antitumoral and anti-inflammatory properties were first reported. Rosmarinic acid is the species biomarker and one of the main components found in rosemary extract. Its biosynthetic pathway also produces important compounds related to plant defense, which are used in response to phytopathogens [6, 7, 8, 9, 10].
Vegetal extracts have the potential to control phytopathogens; their secondary metabolites have been studied for their ability to induce different plant defense mechanisms that may interfere in disease developmental stages, such as pathogen germination and penetration [11, 12, 13]. Studies on disease control by vegetal extracts show they may have a direct or indirect activity on the pathogen by inducing host plant defense mechanisms [14]. Resistance induction involves the activation of several mechanisms, such as phenolic compounds production; increased activity of pathogenesis-related proteins (PPR's), such as CHI (Chitinase), GLU (β-1,3-glucanase), POX (peroxidase), LOX (lipoxygenase) and PAL (phenylalanine ammonia-lyase); and increased production of defense hormones like SA (salicyclic acid), which may act alone or in association with other signalling substances, such as jasmonic acid and ethylene [15, 16, 17, 18].
The present study aimed to develop *Rosmarinus officinalis* L liquid extracts standardized as rosmarinic acid, and evaluate their potential to induce resistance and suppression of leaf blast disease in rice experimentally cultivated in greenhouses.

Material and Methods

Preparation and characterization of plant material

Leaves of *Rosmarinus officinalis* L. were obtained from the company Paladar Condimentos e Especiarias Ltda., batch A0008, in Goiânia, Goiás, Brazil. The material was processed and characterized according to the Brazilian Pharmacopeia in terms of powder microscopy, content of volatile substances, total ash, acid insoluble ash, intumescence index, granule size distribution and chromatography profile through thin-layer chromatography [19].

Characterization and standardization of extract

The hydro alcoholic extract was obtained by percolation and concentrated with a rotary evaporator until the solid content was above 40% (m/m). Concentrated extract was then characterized in terms of solid content, pH, bulk density, viscosity and chromatography profile, through thin-layer chromatography, according to the Brazilian Pharmacopeia [19]. The biomarker rosmarinic acid was quantified using a Waters® high-performance liquid chromatographer, model e2695 and Empower 2.0 software was used to process data obtained. Linearity, precision and accuracy parameters were evaluated according to the Guide of Herbal Medicines and Resolution nº 899 of 2003. The characterization essays were run in triplicate [19, 20, 21].

Plant growth conditions and treatment

Rice seeds from 'BRS Primavera' cultivar were disinfected with ethanol 70% and sodium hypochlorite, and subsequently planted in trays containing approximately 3 kg of NPK fertilized soil complemented with 1 g of zinc sulfate and 3 g of ammonium sulfate, in eight grooves of approximately 4 cm long. Eighteen days after planting, plants were covered with a 3 g ammonium sulfate layer.

Inoculation and disease evaluation

The experimental design was fully randomized and consisted of seven treatments (rosemary extract: T1: 3.3; T2: 6.6 and T3: 10 mg·mL^{-1}; standardized rosmarinic acid T4: 3.1; T5: 4.2 and T6: 6.3 mg·mL^{-1}; and experimental control with distilled water T7) in triplicates, performed in a greenhouse under controlled conditions. The concentrations used are of data not shown.

Rice plants in the V3 stage (third leaf stage) were sprayed with *R. officinalis* extract (T1, T2, and T3), standard rosmarinic acid (T4, T5, and T6), and water (T7) 48 hours before challenging inoculation with *M. oryzae*, conidial suspension (isolate BR 10900) was prepared according to Sena *et al* [22], and the final concentration was adjusted to 3×10^5 conidia.mL^{-1}. After spray inoculation, rice plants were incubated in a wet chamber for 24 hours, at 24°C and 26°C. Next, rice plants were kept between 28°C and 30°C under high humidity in a greenhouse conditions. Leaf blast severity was evaluated using a grade scale according to Notteghem [23].

Enzyme activity assays

Two essays (E1 and E2) were conducted in a fully randomized experimental design in triplicates. The difference between than them was the sampling moment. Both, E1 and E2 consisted of seven treatments (rosemary extract: T1: 3.3; T2: 6.6 and T3: 10 mg·mL^{-1}; standardized rosmarinic acid: T4: 3.1; T5: 4.2 and T6: 6.3 mg·mL^{-1}; and control with distilled water: T7). E1 samples were collected 4, 8, 24 and 48 hours after all 7 treatments application and before *M. oryzae* challenge inoculation. E2 samples were collected after all 7 treatments application and 24 and 48 hours after of *M. oryzae* challenge inoculation. The collected leaves were stored in a freezer at −80°C until analysis.

Protein extraction and assessment: E1 and E2 samples were macerated in liquid nitrogen, and the resulting powder was placed in microtubes, and kept in an ice bath. Protein extraction buffer as described by Bradford [24]. The total protein concentration was measured on the basis of a standard curve of bovine serum albumin (BSA).

Estimation of CHI (EC 3.2.1.14): estimated according to Babu *et al* [25], with adaptations. The N-acetylglucosamine production rate was measured using colloidal chitin in sodium acetate buffer solution as a substrate. The reaction product was quantified through the dinitrosalicylic acid (DNS) method in a spectrophotometer (540 nm). Specific enzymatic activity was defined as the micrograms of reduction sugar per hour per milligram of protein based on a standard glucose curve.

Estimation of GLU (EC 3.2.1.6): estimated on the basis of the reduction sugar production rate with 1% laminarin in sodium acetate buffer as a substrate[26]. Readings were performed in a spectrophotometer at 540 nm, and DNS was used as the colorimetric agent.

Estimation of POX (EC 1.11.1.7): estimated on the basis of the 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) oxidation rate and its own colorimetric property in ABTS solution (0.4 mg·mL^{-1}) in 0.1 M sodium acetate buffer (pH 4.0) and 3% hydrogen peroxide. Readings were performed in a spectrophotometer at 405 nm, and specific enzymatic activity was defined as the variation in absorbance produced per second per milligram of protein[27].

Estimation of LOX (EC 1.13.11.12): estimated using 1% linoleic acid as the substrate. A 50-µL aliquot of each sample was added to 2 mL of 50 mM phosphate buffer (pH 6), homogenized, and incubated for 12 hours at room temperature. The reaction medium was interrupted after the samples were stored in a freezer at 0°C for 20 minutes. Enzymatic activity was determined at 234 nm, and the result was expressed in the micrograms of linoleic acid hydroperoxide oxidized per minute per milligram of protein[28].

Estimation of PAL (EC 4.3.1.5): 50 µL of plant extract was added to 2 mL of L-phenylalanine/sodium borate buffer solution (100 mM, pH 8.8). This mixture was homogenized, and the result was expressed by the quantification of trans-cinnamic acid, the resultant product obtained from the hydrolytic reaction of L-phenylalanine, was quantify by a spectrophotometer at 290 nm [29].

Estimation of PC: rice leaves (300 mg) from each treatment were solubilized in 2:1 methanol/water (v/v) and incubated for 30 minutes in an ultrasonic bath. The resulting extract (120 µL) was homogenized with 220 µL of water and 300 µL of Folin–Ciocalteu reagent and incubated for 5 minutes. Subsequently, 300 µL of Na_2CO_3 was added, followed by 600 µL of distilled water. Sample reading was performed in a spectrophotometer at 720 nm using a gallic acid standard curve as reference. Results were expressed as the milligrams of PC per gram of dry mass [30].

Estimation of SA: vegetal material (200 mg) from each sample was macerated in liquid nitrogen, and placed in 15 mL Falcon tubes. After the addition of 1.5 mL of 90% methanol, samples were vortex and centrifuged for 5 minutes at 10,000 rpm at 25°C. In total, 1 mL of supernatant was removed and added to 500 µL of 5% trichloroacetic acid and 500 µL of ethyl acetate: cyclopentane: isopropanol (50:50:1 v/v) solution. The mixture was mildly homogenized through immersion, and 500 µL of the upper phase was removed. In total, 100 mg of polyvinylpolypyrrolidone (PVPP) was added to this, and after vortex, the samples were centrifuged for 5 minutes at 10,000 rpm. The resulting supernatant from each sample was removed and lyophilized until total drying. The resulting powder was re-solubilized in 100 µL of methanol:acetate buffer (20 mM, pH 5.0) (23:77 v/v) and filtered using a 0.45-µm pore filter. Sample quantification was performed in a high-performance liquid chromatographer with a Perkin Elmer C18 column and mobile phase composed of methanol: 20 mM acetate buffer (pH 5.0) at a proportion of 23:77 v/v. Flow was controlled at 0.2 mL/min under isocratic conditions. The wavelength was 283 nm, oven temperature was kept at 35°C, running time for samples and standards was set to 15 minutes, and injection volume was 20 µL [31].

Statistical analysis

For leaf blast severity analysis, mean values of treatments were calculated, data were converted to $\sqrt{x+0.5}$, submitted to variance analysis and the mean values were compared by Tukey's test ($p< 0.05$).

For enzymatic activity, salicylic acid and phenolic compounds analysis, mean values for each treatment and each collecting time were calculated and submitted to variance analysis. Interactions between treatments and collecting time, when significant, were further analyzed so that differences in the activity of each plant defense-related enzyme were identified among samplings from the same treatment and among treatments from the same sampling. The same method was used to salicylic acid and phenolic compounds analysis. Mean values were compared by Tukey's test ($p<0.05$).

Results

Vegetal material characterization and extract standardization

Epidermic fragments with stomates, vessel elements, straight contoured polygonal cells, and branched tector trichomes were found in rosemary leaf powder on microscopic analysis. Vegetal material a had volatile substance content of 6.6 ± 0.3% (m/v) and total ash and acid insoluble ash contents of 4.52 ± 0.01% (m/m) and 0.22 ± 0.06% (m/m), respectively. The intumescence index was 1.2 ± 0.01 mL/g, powder was classified as moderately coarse on the basis of granule size distribution, and rosmarinic acid retention factor determined by CCD was 0.5 for both vegetal material and liquid extract. The extract had a solid content of 45.17% (m/m), pH of 4.64, bulk density of 1.129 ± 0.003 $g \cdot mL^{-1}$, and viscosity of 148.2 mPas. Parameters to verify the suitability of our study system were in agreement with the guidelines of Food and Drug Administration (FDA) [30]. The method used in this study was linear, precise, and accurate, and the content of rosmarinic acid in the extract was 2.93%.

Leaf blast severity

All treatments significantly reduced the severity of leaf blast (Table 1) at day seven after challenge inoculation with *M. oryzae*. T6 was the best treatment, 98,02% (Figure 1), however, T2, T3 and T5 suppressed leaf blast by 97.9%, 97.80% and 92.9% respectively, and were superior to the other treatments (T1 and T4).

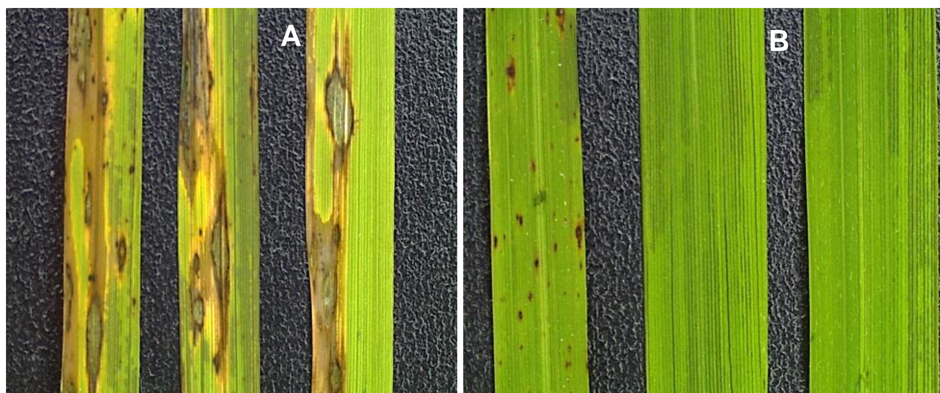

Fig 1: Leaf blast severity. A - Positive control with rice leaves representing how disease severity, B - after treatment with T6 (6.3 mg/mL^{-1}).

Table 1: Leaf blast severity (LBS) at 10 days after challenge inoculation, Phenolic compounds and SA content 24 hours and 48 hours after *M. oryzae* challenge. Values expressed in mg/g^{-1} and ng/g^{-1}, respectively.

Treatments (mg/mL^{-1})	LBS (%)	PC		SA	
		24	48	24	48
T1	4,21 ± 1.07 b**	7,91dAB	7,59bAB	116 f B	112 f A
T2	0,83 ± 0.59 a	7,69bcA	8,95cdB	115 e B	110 d A
T3	0,79 ± 0.4 a	9,81eA	9,02dA	115 e B	111 e A
T4	4,85 ± 1.12 b	6,06aAB	5,97aAB	111 d B	103 a A
T5	2,67 ± 1.13 ab	7,00bcA	8,33c B	110 c B	107 b A
T6	0,75 ± 0.62 a	8,27dB	6,13aA	105 a A	106 b A
T7/CTR*	36,86 ± 1.09 c	7,51bcdA	7,06 bA	108 bA	109 dA
CTR**	-	6,81abA	5,82 aA	105 aA	107 cA

Legend: T1 - 3.3 mg/mL^{-1}; T2 - 6.6 mg/mL^{-1}; T3 - 10 mg/mL^{-1}; T4 – 2.1 mg/mL^{-1}; T5 - 4.2 mg/mL^{-1}; T6 - 6.3 mg/mL^{-1}; T7 – Control, plants inoculated with *M. oryzae.*

*Control treated with *M. oryzae*; **Control treated with water. Treatment with the same small letter in the column does not differ, same capital letter in the line does not differ from each other (ANOVA) according to Tukey's test $p<0.05$. The values in bold presented significant differences when compared to control treated with *M. oryzae*.

Enzyme activity before *M. oryzae* inoculation (E1)

Statistical significance was observed for treatments, samplings, and interaction between treatments and sampling time, except for the POX enzyme. T2 (6.6 $mg \cdot mL^{-1}$) and T5 (4.2 $mg \cdot mL^{-1}$) showed CHI increased activity at 4 and at 8 and 24 hours, respectively (Table 2). T5, T1 (3.3 $mg \cdot mL^{-1}$) and T3 (10 $mg \cdot mL^{-1}$) increased GLU activity at 4, 8 and 24-hours after its application. LOX activity responded to T3, T2 and T1 at 4 and 48, 8 and 24-hour time point, respectively. T1 and T3 increased PAL activity at 4 and 24 and at the 8-hours time point, respectively. T5 and T3 increased PC levels at 4 and 8, 24, and 48 hours, respectively. T5, T1 and T3 increased SA content at the 4, 8 and 24 and 48-hours time point (Table 2).

Table 2: CHI, GLU, LOX and PAL enzyme activity and contents of phenolic compounds (PC) and SA before *M. oryzae* challenge at 1 (0 hour), 2 (4 hours), 3 (8 hours), 4 (24 hours) and 5 (48 hours). Values expressed in $U.mg^{-1}$ for enzymatic activity and in mg/g^{-1} and ng/g^{-1} for PC and SA respectively.

Collections		Treatments (mg/mL^{-1})						
		Extract (mg/mL^{-1})			Standard (mg/mL^{-1})			*CTR
		T1	**T2**	**T3**	**T4**	**T5**	**T6**	**Water**
	0 h	2809 a A	2780 a A	3320 a A	3364 a A	3364 a A	3026 a A	2807 a
C	4 h	5541 ab B	8299 b B	5989 ab B	6560 ab B	5278 ab A	5686ab B	4603 a
H	8 h	5457abcB	7123bcAB	4966abAB	5429 ab AB	7964 c B	4558 a A	4416 a
I	24 h	5021abcB	5710bcAB	5217abAB	4571abcAB	6001c AB	4252 ab A	3727 a
	48 h	5585 a B	4971 a A	5841 a AB	5406 a AB	5301a AB	4960a AB	4527 a
	0 h	30072 aB	2478aAB	23645 a A	30072 a E	30072 a C	30072 a D	3172 a
G	4 h	26252bcB	2901bc B	18662aAB	31336 c E	35021cC	27769abC	2008 ab
L	8 h	30751cB	28255bcB	17561aAB	25612bcCD	3001bcBC	22524abB	2296 ab
U	24 h	21686abA	1633abAB	23602AB	1633ab BC	20230abA	16081abA	13871a
	48 h	19220 aA	19220a A	21336 a B	21448 a A	19168a A	20482aAB	16519 a
	0 h	1364 a A	1364 a A	1295 a A	1295 a A	1364 a A	364 a AB	1364 a
L	4 h	3583 d F	1669 c A	3889 e C	1021 a A	1483 b B	1437 b B	1511 b
O	8 h	1669 c B	2980 e C	2006 d B	1746 c B	1497 b B	1398 b AB	1022 a
X	24 h	3145 e C	1609abcA	475 ab B	1185 ab A	1659 cd C	2193 d D	1060 a
	48 h	1933 c E	2424 e B	4234 g D	2688 f C	1698 b C	1994 d C	1532 a
	0 h	145 a A	136 a A	129 a A	129 a A	134 a A	145 a A	136 a
P	4 h	1087c BC	331 ab B	720 ab C	6560ab AB	327 a B	249 a AB	199 a
A	8 h	468 a AB	412 a B	1171 d D	613 b AB	723 c B	424 a AB	590 b
L	24 h	1786 d D	997 b D	1772 cd E	1355 c AB	1052 b D	1214 b D	121 a
	48 h	315 a AB	603 a C	574 a B	1742 a AB	609 a C	607 a C	148 a
	0 h	6,049 a A	5,051aA	5,467aA	5,051a A	5,051a A	4,455 a A	4.468 a
P	4 h	7,314abB	7,505bcB	8,229cdB	8,380dC	9,534e D	8,600dD	6,687 a
C	8 h	8,461dCD	7,618 c B	9,802e B	7,063bcB	6,643abB	7,334cBC	6,029 a
	24 h	8,356bCD	8,881b C	9,055b B	6,975a B	6,479 a B	7,066aBC	6,037 a
	48 h	8,967d E	8,385d B	9,886e B	6670abB	7, 535c C	7,115bcBC	6,029 a
	0 h	1096 a A	1096 a A	1096 a A	1096 a D	1096 a C	1096 a C	1096 a
S	4 h	1099 ab A	1100bcAB	1097 bc A	1092 ab C	1101 c D	1087 a C	1095abc
A	8 h	1138 f C	1123 e C	1126 e D	1060 a B	1079 c C	1069 b AB	1096 d
	24 h	1115 g B	1104 e AB	1110 f B	1038 a A	1071 b B	1078 c C	1083 d
	48 h	1133 d B	1137 de B	1139 e E	1095 b CD	1049 a A	1106 c D	1093 b

*Control.

Treatments with the same small letter in the line do not differ and with the same capital letter in the column do not differ from each other (ANOVA) according to Tukey's test $p< 0.05$. The values in bold presented significant differences when compared to control treated with water.

Enzymatic activity after inoculation with *M. oryzae* (E2)

The activities of CHI, GLU, OX, LOX, and PAL (Table 3) and contents of PC and SA (Table 1) increased when compared to the control treatment. CHI activity increased in T1 (3.3 $mg \cdot mL^{-1}$), and T5 (4.2 $mg \cdot mL^{-1}$), T1 and T7 (*M. oryzae*), at 24 and 48-hours time point, respectively. GLU activity increased in T1, T2 (6.6 $mg \cdot mL^{-1}$), and T5 at 24- and 48-hour time points. POX activity increased after 48 hours in T6 (6.3 $mg \cdot mL^{-1}$), T1, T4 (3.1 $mg \cdot mL^{-1}$), and T5. LOX activity increased at the 24-hour time point in T1 and 48-hour time point in T3. PAL activity increased at 24 hours in T6 and at 48 hours in T4, T1 and T3. PC contents increased in T3 after 24 and 48 hour time points. With regard to the content of SA an increase was observed in T1, at both time points.

Table 3: CHI, GLU, POX, LOX and PAL activity 24 hours and 48 hours after *M. oryzae* challenge. Values expressed in $U.mg^{-1}$.

Treatments (mg/mL^{-1})	CHI		GLU		POX		LOX		PAL	
	24	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**
T1	5558dAB	6447cdB	2426cAB	2153cdA	605a A	469ab A	3764 B	2602eA	1409deA	2415bcB
T2	4910abcA	5386cAB	2331bcAB	1893abcdAB	327 a A	342 a A	2189d A	2199dA	1284 cA	2108bB
T3	4698abA	5386cAB	1552a A	184abAB	335 a A	414 a A	1376bA	2943fB	1775 f A	2564bc B

T4	5063abcAB	4808abAB	1594a A	1555abA	610 a A	400 ab AB	1513c A	2216dB	1350cAB	1973bAB
T5	5992dAB	4962abA	2285bcA	2303dA	868 a A	743 ab A	1892d A	1803cA	1445eA	1899 b B
T6	4448aA	5521cB	690 a A	2178cdA	1002a A	1133 b A	1272a A	1657bB	2293 g A	2850 c A
CTR*	5335 abcA	7268 dB	2091 abA	1576 abcA	262 aA	311 aA	2494 fA	2858 fA	325 aA	467 aA
CTR**	5090 abcAB	3837 aA	1809 abA	1283 aA	725aAB	174 aA	1584 cA	1402 aA	1099 bA	2355 bAB

*Control treated with *M. oryzae*; **Control treated with water. Treatment with the same small letter in the column does not differ, same capital letter in the line does not differ from each other (ANOVA) according to Tukey's test $p< 0.05$. The values in bold presented significant differences when compared to control treated with *M. oryzae*.

Discussion

The process described in this study produced a standardized rosemary extract whose main chemical component (rosmarinic acid) was determined, in addition to its pharmacotechnical characteristics, enabling the monitoring of its biological activity based on the determination of concentrations that ensured reproducibility and safety.

Rice leaf blast control through plant extracts is a modern, alternative and apparently risk-free method of management. Several studies have demonstrated the effective management of extracts as a biocontrol against a wide range of plant pathogens [13, 33, 34, 35, 36, 37]. In this study, our results showed that T2 (6.6 mg·mL^{-1}), T3 (10 mg·mL^{-1}), T5 (4.2 mg·mL^{-1}), and T6 (6.3 mg·mL^{-1}), sprayed before the challenge inoculation suppressed the severity of leaf blast. We believe that these treatments helped to increase the activity of enzymes related to plant defense, interrupting pathogen developmental stages such as penetration and colonization [12, 13]. Characteristics open blast lesions were observed in the control treatment, while small brown dots, characteristic of hypersensitivity reaction, were observed in the other treatments.

Similar to rosemary extract standardized as rosmarinic acid, extracts of *Azadirachta indica, Chromolaena odorata*, *Allium sativum*, and *Anadenanthera peregrina* have been successfully used to inducer resistance in *Oryza. Sativa,* and *Carica papaya*, when sprayed before challenger[38, 39]. Nonetheless, nonstandardization of these ethanolic extracts prevents proper monitoring of diseases suppression and affects reproducibility [40].

Four hours after extract administration, increased activities of CHI (T2), GLU (T5), LOX (T3), and PAL (T1) as well as increased levels of PC (T5) and SA (T5), were observed. Extract spraying may have trigged its defensive ability. Besides their structural barriers and secondary metabolites, plants can develop inducible defense mechanisms to repel possible phytopathogenic threats. This phenomenon, known as priming, allows activated cells to respond to a very low levels of stimuli faster than non-activated cells, and, in the presence of a challenge, it triggers a faster and more intensive defense response, frequently related to the development of local and systemic immunity and stress tolerance [40].

At 24 and 48 hour time points, CHI, GLU, and LOX activities and SA levels increased in T1 (3.3 mg·mL^{-1}), followed by T5 (4.2 mg·mL^{-1}), T3 (10 mg·mL^{-1}), and T6 (6.3 mg·mL^{-1}). In T6, POX and PAL activities were also increased. The highest PC content was observed in T3 (10 mg·mL^{-1}).

Based on rosmarinic acid standardization, it can be said that 3.3 mg·mL^{-1} concentration has the lowest content of the main chemical component; however, vegetal extracts, besides the biomarker compound, have other bioactive molecules such as tannins, flavonoids, flavones, phenolic acids and coumarins in a complex mixture that, besides antiicrobial effect, act in synergy to increase plant defense against pests and pathogenic infections[41]. With regard to the 4.2 mg·mL^{-1} concentration, a dose–response relationship can be determined, considering it is the intermediate standardized rosmarinic acid concentration and is sufficient to increase the enzyme activity levels and control pathogen penetration[42]. Kalage *et al* [43] observed the accumulation of these enzymes starting at a 24-hour time point after *R. solani* and *Alternaria solani* infection in rice and tomatoes pretreated with *Ipomoea carnea* and *Datura metel* extracts, respectively.

POX activity increased in T6 (6.3 mg·mL^{-1}) at 48 hours after challenge inoculation. Nisha *et al* [42] also observed that the highest POX expression in rice plants susceptible to bacterial burn caused by *Xanthomonas oryzae* occurred 48 hours after the challenge. Probably, POX increased activity induced lignin production through hydroxycinnamic alcohol polymerization in the presence of hydrogen peroxide, strengthening its mechanical support and blocking pathogen growth and rosmarinic acid oxidation [44].

Increased LOX activity was particularly observed only in plants treated with concentrations of 3.3 mg·mL^{-1} (T1) and 10 mg·mL^{-1} (T3) at 24 and 48 hour time points, respectively. These treatments probably activated enzymes responsible for lipids degradation, supplying the substrates polyunsaturated fatty acids to LOX enzyme and fatty acid peroxides, LOX is a key enzyme for the metabolism of biologically active compounds such as jasmonic acid, a hormone related to several cell responses, and highly reactive aldehydes with antimicrobial activity[44]. Sandhu *et al* [45] studied the increase in LOX in eight rice lineages and observed a positive correlation with this enzyme activity and blast disease reduction.

PAL activity particularly increased in T6 (6.3 mg·mL^{-1}) 24 hours and 48 hours after challenge with *M. oryzae*. Containing the highest concentration of rosmarinic acid, this treatment probably increased of constitutive levels phenylpropanoids, causing an increase in PAL activity for the synthesis of PC [46].

The content of PC increased in plants treated with T3 (10 mg·mL^{-1}) collected 24 hours and 48 hours after the challenge. Both, marker molecule (rosmarinic acid) and the rosmarim extract are in their most concentrated form, and they may act in synergy with plant intrinsic PC. PC contents enhance its defense mechanisms for effective pathogen isolation in the beginning of the infection [44, 46].

SA contents increased 24 and 48 hours after challenge with *M. oryzae* in plants treated with T1 (3.3 mg·mL^{-1}). Based on the standardization of rosmarinic acid, this treatment contains the lowest concentration of this marker, but because the extract is a complex mixture of several components, most of them being active, when adding the phenolic acid, they probably increased SA and PAL contents as soon as the pathogen settled [48].

Conclusions

1. In greenhouse assays, the treatments evaluated in this study were able to suppress leaf blast, triggering biochemical mechanisms of rice plant defense, probably inducing resistance, considering that they increased SA

levels and activities of enzymes related to induction routes, such as LOX and PAL.
2. The rosemary extract standardized as rosmarinic acid offered an alternative, easy to prepare and more sustainable approach to for rice leaf blast control and its use can be associated with integrated management techniques.

Acknowledgments
We would like to thank the Fundação de Amparo a Pesquisa de Goiás (Fapeg), Coordenação de Aperfeiçoamento de Nível Superior (Capes) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the financial support to this project.

References
1. Scheuermann KK, Eberhardt DS. Avaliação de fungicidas para o controle da brusone de panícula na cultura do arroz irrigado. Revista de Ciências Agroveterinárias. 2011; 10:23-28.
2. Gonçalves FJ, Araújos LG, Silva GB, Filippi MCC. Controle químico da brusone em arroz de terras altas: efeitos nos fungos não alvos do filoplano. Pesquisa Agropecuária Tropical. 2012; 42:77-81.
3. Tang X, Zhang ZY, Zhang WJ, Zha XM, Li x, Zhang D *et al*. Global gene profiling of laser-captured pollen mother cells indicates molecular pathways and gene subfamilies involved in rice meiosis. Plant Physioloy. 2010; 154:1855-1870.
4. DEPEC, Departamento de Pesquisas e Estudos Econômicos. Arroz e produtos. Bradesco. http://www.economiaemdia.com.br, 2015.
5. Klein T, Longhini R, Bruschi ML, Mello JCP. Fitoterápicos: um mercado promissor. Revista de ciências farmacêuticas básica e aplicada. 2009; 30:241-248.
6. Schwan-estrada KRF, Stangarlin JR, Cruz MÊS. Uso de extratos vegetais no controle de fungos fitopatogênicos. Floresta. 2000; 30:129-137.
7. Porte A, Godoy RLO. Alecrim Rosmarinus officinalis L. Propriedades antiimicrobiana e química do óleo essencial. B. CEPPA. 2001; 19:1-18.
8. Erkan N, Ayranci G, Ayranci E. Antioxidant activities of rosemary Rosmarinus Officinalis L. extract black seed Nigella sativa L. essential oil, carnosic acid, rosmarinic acid and sesamol. Food Chemistry. 2008; 110:76-82.
9. Ferraz S, Lopes EA, Amora DX Controle de fitonematoides com o uso de extratos e óleos essenciais de plantas. In: Poltronieri L,S.; Ishida, AKN (Eds). Métodos alternativos de controle de insetos-praga, doenças e plantas daninhas. Panorama atual e perspectivas na agricultura. Belém: EMBRAPA Amazônia Oriental. 2008, 308.
10. Yosr Z, Hnia C, Rim T, Mohamed B. Changes in essential oil composition and phenolic fraction in Rosmarinus officinalis L. var. typicus Batt. organs during growth and incidence on the antioxidant activity. Industrial Crops and Products. 2013; 42:412-419.
11. Itako AT, KRF. Schwan-Estrada KRF, Stangarlin JR, Tolentino Júnior JB, Cruz MES. Controle de Cladosporium fulvum em tomateiro por extratos de plantas medicinais. Arquivos do Instituto Biológico. 2009; 76:75-83.
12. Reis KB, Côrtes MCCB, Martins FS, Filippi MCCF, Paula JR, Conceição ED. *et al.* Characterization of rue extract and its potential for controlling rice blast. Pesquisa Agropecuária Brasileira. 2015; 50:1121-1130.
13. Rodríguez MDCH. Utilização de extrato padronizado de Rosmarinus officinalis L. (Lamiaceae) no controle da brusone (Magnaporthe oryzae) do arroz (Oryza sativa L. – Poaceae). Dissertação (Mestrado). Programa de Pós-Graduação em Ciências Biológicas. Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, 2015, 31.
14. Amadioha AC. Controlling rice blast in vitro and in vivo with extracts of Azadirachta indica. Crop Protection. 2000; 19:287-290.
15. Pieterse CMJ, Pelt JAV, Van Wees SCM, Ton J, Verhagen BWM, Léon-Kloosterziel K, *et al*. Indução de resistência sistêmica por rizobactérias e comunicação na rota de sinalização para uma defesa redefinida. Revisão Anual de Patologia de Plantas. 2005; 13:277-295.
16. Filippi MCC, Silva GB, Silva-Lobo VL, Côrtes MVCB, Moraes AJG, Prabhu AS. Leaf blast (Magnaporthe oryzae) suppression and growth promotion by rhizobacteria on aerobic rice in Brazil. Biological Control. 2011; 58:160-166.
17. Carvalho JCB, Sousa KCI, Brito DC, Chaibub AA, Luzini AP, Côrtes MVCB *et al*. Biocontrol potential of Waitea circinata, an orchid mycorrhizal fungus, against the rice blast fungus. Tropical Plant Pathology. 2015; 40:151-159.
18. Sena APA, Chaibub AA, Côrtes MVCB, Silva GS, Silva-Lobo VL, Prabhu AS *et al*. Increased enzymatic activity in rice leaf blast suppression by crude extract of Epicoccum sp. Tropical Plant Pathology. 2013; 38:387-397.
19. Brazil. Agência Nacional de Vigilância Sanitária. Farmacopéia Brasileira, 5.ed., Brasília, DF, 2010.
20. Brazil. Agência Nacional de Vigilância Sanitária. Guia de orientação para registro de fitoterápicos e registro e notificação de produto tradicional fitoterápico. Brasília: Anvisa. 2014, 37.
21. Sena APA, Chaibub AA, Côrtes MVCB, Silva GS, Silva-Lobo VL, Prabhu AS *et al*. Increased enzymatic activity in rice leaf blast suppression by crude extract of Epicoccum sp. Tropical Plant Pathology. 2013; 38:387-397.
22. Notteghem JL. Cooperative experiment on horizontal resistance to rice blast. In: Blast and upland rice: report and recomendation from the meeting for international collaboration in upland rice improvement. Los Baños, IRRI. 1981, 43-51.
23. Bradford MA. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analitical Biochemistry. 1976; 7:248-254.
24. Babu RM, Sajeena A, Seetharaman K, Reddy MS. Differential induction of chitinase and β-1,3 glucanase in rice in response to inoculation with bacterial leaf blight pathogen (Xanthomonas oryzae pv. Oryzae). Journal of plant diseases and protection. 2003; 110:105-112.
25. Pan SQ, Ye XS, Kuc J. Association of a β-1,3-glucanase activity and isoform pattern with systemic resistance to blue mold in tobacco induced by stem injection with Peronospora tabacinaor leaf inoculation with tobacco mosaic virus. Physiological and molecular plant pathology. 1991; 39:25-39.
26. Côrtes MVCB, Viana HF, Silva FR, Lobo VLS, Silva GB, Prabhu AS *et al*. Quantificação da atividade enzimática de proteínas relacionadas a patogênese no patossistema Oryza sativa/ Magnaporthe oryzae. Boletim de pesquisa e

desenvolvimento. 2008, 1-16.
27. Chewe, *et al*. Methods in enzymology. Phytotherapy research. 1981; 71:430-441.
28. Hyodo H, Kuroda H, Yang SF. Induction of phenylalanine ammonia-lyase and increase in phenolic in lettuce leaves in relation to the development of russet spotting caused by ethylene. Plant physiology. 1978; 62:31-35.
29. Dicko MH, Hilhorst R, Traores AS, Van Bwekwl WJ, Voragen AG. Comparison of content in phenolic compounds, polyphenoloxidase, and peroxidase in grains of fifty sorghum varieties from Burkina Faso. Journal of agricultural and food chemistry (2002); 50: 3780-3788.
30. Côrtes MVCB, Filippi MCC, Silva-Lobo VL. Método Validado para a Determinação de Ácido Salicílico em Folhas de Arroz por Cromatografia Líquida de Alta Eficiência (CLAE). Boletim de pesquisa e desenvolvimento. 2015; 40:5-14.
31. FDA - FOOD AND DRUG ADMINISTRATION: Guidance for Industry: Analytical Procedures and Methods Validation, 2000. Disponível em: <www.fda.gov/downloads/Drugs/Guidances/ucm122858.pdf>. Acesso em, 2015.
32. Joseph B, Darand MA, Kumar V. Bioefficacy of plant extracts to control Fusarium solani f. sp. melangenae incitant of brinjal wilt. Glob J Biotechnol Biochem. 2008; 3:56-9.
33. Sowjanya N, Manohara C. Effect of plant extracts on the growth of Microsorum gypseum. J Phytopathol. 2012; 4:41-4.
34. Dey U, Harlapur SI, Dhutraj DN, Suryawanshi AP, Jagtap JP, Apet KT, *et al*. Effect of fungicides, botanicals, bioagents and Indigenous Technology Knowledge (ITK's) on germination of urediniospores of Puccinia sorghi in vitro. Afr J Agric Res- 2013; 8:4960-71.
35. Elsharkawy MM, El-Sawy MM. Control of bean common mosaic virus by plant extracts in bean plants. Int J Pest Manag. 2015, 1-6.
36. Nascimento LCN, Nery AR, Rodrigues LN. Controle de Colletotrichum gloeosporioides em mamoeiro, utilizando extratos vegetias, indutores de resistências e fungicidas. Acta scientiarium agronomy. 2008; 30:313-319.
37. Khoa DC, Thúy PTH, Thuy TTT, Collinge DB, Jorgenses HJL. Disease-Reducing Effect of Chromolaena odorata Extract on Sheath Blight and Other Rice Diseases. Phytopatology (2011); 101:231-240.
38. Marques LC, Vigo CLS. Preparação e Padronização de extratos vegetais. In: LEITE, J.P.V. (Eds.). Fitoterapia: bases científicas e tecnológicas. Rio de Janeiro: Atheneu. 2009, 169-204.
39. Conrath U. Molecular aspects of defence priming. Trends In Plant Science. 2011; 16:524-531.
40. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Ver. 1999; 12:564-82.
41. Nisha S, Revathi K, Chandrasekaran R, Kirubakaran SA, Sathish-Narayanan S, Stout MJ, Senthil-Nathan S. Effect of plant compounds on induced activities of defense-related enzymes and pathogenesis related protein in bacterial blight disease susceptible rice plant. Physiological and molecular plant patology. 2012; 80:1-9.
42. Kalage S, Marimuthu T, Kalage J, Thayumanavan B, Samiyappan R. Induction of systemic resistance in rice by leaf extracts of Zizyphus jujube and Ipomoea carnea against Rhizoctonia solani. Plant Signaling & Behavior. 2011; 6:919-923.
43. Stangarlin JR, Kuhn OJ, Toledo MV, Portz RL, Shwan-Estrada KRF, Pascholati SF. A defesa vegetal contra fitopatógenos. Scientia Agraria Paranaenis. 2011; 10:18-43.
44. Sandhu SS, Mazzafera P, Azini LE, Bastos CRB, Colombo CA. Lipoxygenase activity in Brazilian rice cultivars with variable resistance to leaf blast deases. Bragantia. 2007; 66:27-30.
45. Gibert S. Two phenylalanine ammonia lyase isoforms are involved in the elicitor-induced response of rice to the fungal pathogen *Magnaporthe oryzae.* Journal of plant physiology. 2012; 169:249-254.
46. Joshi JR, Burdman S, Lipsky A, Yedidia I. Effects of plant antimicrobial phenolic compounds on virulence of the genus Pectobacterium. Research in microbiology. 2015; 166:535-545.
47. Venturoso LR, Bacchi LMA, Gavassoni WL, Conus LA, Potim BCA, Bergamin AC *et al.* Atividade antifúngica de extratos vegetais sobre o desenvolvimento de fitopatógenos. Summa Phytopathologica. 2011; 37:18-23.