

## Effect of Endophytic Fungal Associations on the Chemical Profile of *in vitro* *Vochysia divergens* Seedlings

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*Vochysia divergens* (Vochysiaceae) is considered an invasive species in the wetlands of the Brazilian Pantanal, which hinders the cultivation of agricultural species. In this study, we evaluated the chemical profile by HPLC-DAD (high-performance liquid chromatography-diode array detector) of leaves extracts from *V. divergens* seedlings inoculated with endophytic fungi isolated from *V. divergens* roots. These fungi were collected on dry (D) and wet (W) seasons in the Pantanal. The presence of tannin hexahydroxydiphenoyl (HHDP)-galloyl-glucose and flavone 3',5'-dimethoxy-luteolin were predominant in the seedlings inoculated with endophytic fungi W experiments at 100 and 80%, respectively. Likewise, flavone 3',5'-dimethoxy-luteolin-7-O- $\beta$ -glucoside showed a similar representation in the two evaluated periods, compared with 5-methoxy-luteolin, which was detected only in seedlings inoculated with W endophytic fungi. This approach is new to *V. divergens*, which has no scientific data on its *in vitro* elicitation, in the search for a better understanding of the ecological relationships of this species.

**Keywords:** *Vochysia divergens*, Pantanal, endophytic fungi, 5-methoxy-flavones

### Introduction

The Pantanal of Mato Grosso State, Brazil (16-20°S, 55-58°W) is a large wetland in the center of South America; it covers approximately 160,000 km<sup>2</sup>, of which approximately 140,000 km<sup>2</sup> belong to Brazil. Seasonal flooding is the most important ecological phenomenon in the Pantanal.<sup>1-3</sup> This large continental savanna wetland is strongly affected by its hydrology and is characterized by wet (October to April) and dry (May to September) seasons. *Vochysia divergens* Pohl (Vochysiaceae), also named Cambará, is a native species from the Amazon Basin and the Cerrado (Brazilian savanna) biomes, and it is considered an invasive species in the wetlands of the Brazilian Pantanal. *V. divergens* has a curious ability to quickly and extensively spread under the extreme water stress of the Pantanal, both in prolonged flooding or dryness, which results in extensive monospecific forests known as Cambarazal.<sup>4</sup> Several

researchers studied the physiological aspects, phenology, vegetative structure, soil nutrient content and energetic balance correlated with climate variation of *V. divergens*.<sup>5-9</sup> Machado *et al.*<sup>9</sup> studied 14 species in the Pantanal flooding season and found an absolute predominance of *V. divergens* at 73%. However, the reason for the invasion of this flood-adapted species and how this species survives and persists in habitats with broadly differing hydrology remains poorly understood.<sup>9</sup> The invasion and predominance of *V. divergens* become a serious ecological problem because it replaces areas of natural pastures, damaging the livestock sector in the region and hinder the cultivation of agricultural species.<sup>10</sup> The competitive abilities of weedy plants may be increased when mutualistic associations are established with symbiotic microbes.<sup>11,12</sup> Endophytes are non-pathogenic bacteria and fungi that colonize and grow within the interior spaces or cells of healthy plants.<sup>13</sup> These microorganisms benefit plants through auxin production,<sup>14</sup> N<sub>2</sub> fixation or increased mineralization of soil nutrients,<sup>15,16</sup> which results in plant growth promotion. They also help

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plants increase their tolerance to stresses, including soils contaminated by heavy metals.<sup>17</sup> Natural products synthesized by endophytic bacteria can induce resistance to plant pathogens<sup>18</sup> or biocontrol phytopathogens by lipopeptides that result in plant growth.<sup>19</sup> Previous studies by our research group<sup>20</sup> found that the endophytes colonization of *Hyptis marrubioides* seedlings Epling results in a qualitative and quantitative modification of the phytochemical profile of the host. In this study, we decided to investigate the relationship of *V. divergens* and their endophytic fungi collected in dry and wet seasons in Pantanal. With this purpose, initially, *in vitro* *V. divergens* seedlings were obtained and then inoculated with different endophytic fungi collected from this species in both seasons in the Pantanal. The chemical profile by HPLC-DAD (high-performance liquid chromatography-diode array detector) of methanol extracts of the seedlings was compared to control samples. This approach is new to studying *V. divergens*, which characterizes the importance of this work in the search for a better understanding of the ecological relationships of this species.

## Experimental

### Chemicals and reagents

The MeOH used in the experiments was of HPLC grade and was obtained from J. T. Baker. Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system from Millipore.

### Plant materials and sample preparations

*Vochysia divergens* seeds were collected in the Pantanal region (S16°35'22.90" and W56°47'83.40"). A voucher specimen was deposited in the Herbarium of Federal University of Mato Grosso, Brazil (UFMT 39559). Surface disinfected seeds (disinfected with 2.5% sodium hypochlorite for 5 min and then rinsed 5 times with autoclaved distilled water) were germinated on plates that contained mineral medium (MM: 0.68 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.95 g KNO<sub>3</sub>; 0.22 g (CaCl<sub>2</sub>)<sub>2</sub>H<sub>2</sub>O; 0.18 g MgSO<sub>4</sub>·7H<sub>2</sub>O; 0.08 g KH<sub>2</sub>PO<sub>4</sub>; 9 g agar). Plates were incubated for 15 days at ambient laboratory temperature in 12 h alternating light/dark cycle. Seedlings from these plates without presence of visible microbe growth were considered endophyte-free (E-)<sup>21,22</sup> and were used for inoculation experiments. It was used 14 strains of endophytic fungi (Table 1) that were previously isolated from the Cambará roots.<sup>21</sup> DNA was extracted from a representative strain of each morphological group with an Axxygen Biosciences (Union City, USA) kit according

to the manufacturer's recommendations. The ITS5 and ITS4 primers were used for the amplification of the ITS region.<sup>23</sup> PCR (polymerase chain reaction) products were purified and bidirectionally sequenced using the Sanger method. Sequences were compared to sequences deposited in the UNITE database (<https://unite.ut.ee/analysis.php>) and GenBank using the BLASTn tool (<http://www.ncbi.nlm.nih.gov>). The strains were activated on PDA (potato dextrose agar) for seven days at 28 °C. Seedlings were transplanted to new plates, and after four days, fragments of mycelium were inoculated close to the roots' seedlings. The plates were incubated at ambient laboratory temperature in the 12 h alternating light/dark cycle. Root colonization of the host was evaluated, seedlings were collected after 30 days, sufficient time for the host's root system to be superficially colonized by fungal lineages. The material was kept in a drying oven at 60 °C until dry. Dry powder samples from the leaves of each seedling (20 mg) were dissolved in 3 mL methanol HPLC grade (J. T. Baker), sonicated in an ultrasonic bath (Unique®, Ultra Cleaner 1400A, Brazil) for 30 min and filtered through a 0.45 nylon membrane prior to the HPLC analysis. The same procedure was repeated for *V. divergens* leaves in nature (Vd). The experiment was performed only once.

### HPLC analysis conditions

The analytical HPLC analyses were carried out on a Shimadzu Prominence LC-20AD binary system equipped with a DGU-20A5 degasser, an SPD-20A series diode array detector, a CBM-20A communication bus module, an SIL-20A HT autosampler, and a CTO-20A column oven. The chromatographic separations of the microplants extracts were performed on a Phenomenex Gemini C18 (particle diameter 5 µm, 250 × 4.60 mm) column equipped with a pre-column with the same material. The mobile phase used was a linear gradient CH<sub>3</sub>OH/H<sub>2</sub>O/CH<sub>3</sub>COOH (5:94.9:0.1 v/v/v) to 100% methanol for 30 min, followed by elution with 100% methanol for 10 min, oven at 40 °C, flow 1.0 mL min<sup>-1</sup>, and a 10 µL injection volume. The total analysis time was 60 min, including returning to the initial condition and equilibration. The detector wavelength was set at 254 nm. Data were analyzed using LC solution, 1.25 version software (Shimadzu, Japan).

### Nuclear magnetic resonance (NMR) and MS analysis

<sup>1</sup>H and <sup>13</sup>C NMR (nuclear magnetic resonance) spectra were recorded in methanol-*d*<sub>4</sub> for compounds **2** (methyl gallate), **3** (3',5-dimethoxy-luteolin-7-*O*-β-glucoside), **4** (5-methoxy-luteolin) and **6** (bis (2-ethylhexyl) phthalate);

dimethyl sulphoxide- $d_6$  for **1** (HHDP-galloyl-glucose) and in pyridine- $d_5$  for compound **5** (3',5'-dimethoxy-luteolin) on a Bruker® DRX-500 spectrometer using TMS as the internal standard. The electrospray ionization mass spectrometry (ESI-MS) mass spectrometry analyses were performed in a micrOTOF-Q II ESI-TOF Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA) by direct infusion. Experimental conditions: nitrogen was used as dry gas (temperature of 180 °C, flow of 4 L min<sup>-1</sup>) and as nebulizer gas (pressure of 0.4 bar). The capillary voltage was set up to 3500 volts. Internal calibration was performed with sodium trifluoroacetate solution 10 mg mL<sup>-1</sup>.

### Standard compounds

The compounds **1-6** were previously isolated from leaves in ethanol extract of *in natura* *V. divergens* by our research group and their spectral data<sup>24</sup> are in agreement with published data.<sup>25-30</sup>

### Structural identification of the compounds

According to the ESI-MS data presented in Table 2, compound **1** was identified as an isomer of HHDP-galloyl-glucose [M – H]<sup>-</sup> at  $m/z$  633.0748. The identification was corroborated by the presence of the fragment  $m/z$  481, confirming the loss of a galloyl moiety from this precursor ion, the fragment  $m/z$  463 associated with the loss of a gallic acid unit and the fragment  $m/z$  301 corresponding to the hexahydroxydiphenoyl (HHDP) unit after lactonization to ellagic acid.<sup>31</sup>

### Data analysis

The software R version 3.2.1 (The R Foundation for Statistical Computing) was used to perform hierarchical clustering analysis (HCA) (details can be found in the Supplementary Information). The calculation of the degree of similarity between the elements of the Cartesian space was done based on the Euclidean distance equation:

$$d_{ii'} = \sqrt{\sum_j (X_{ij} - X_{i'j})^2} \quad (1)$$

where  $d_{ii'}$  is the Euclidean distance between the pair of individuals  $i$  and  $i'$ ,  $X_{i'j}$  and  $X_{ij}$  the numerical values of the  $j^{\text{th}}$  coordinates  $i'$  and  $i$ , respectively. The calculation of distance was applied until all elements of a group were more similar to each other and dissimilar to the elements of different groups. Ward's method<sup>32</sup> allowed the rearrangement of the formed clusters. The results were presented as a hierarchical

tree, a two-dimensional graph also known as a dendrogram, in which the lengths of the branches represent the degree of similarity between the objects.<sup>33</sup>

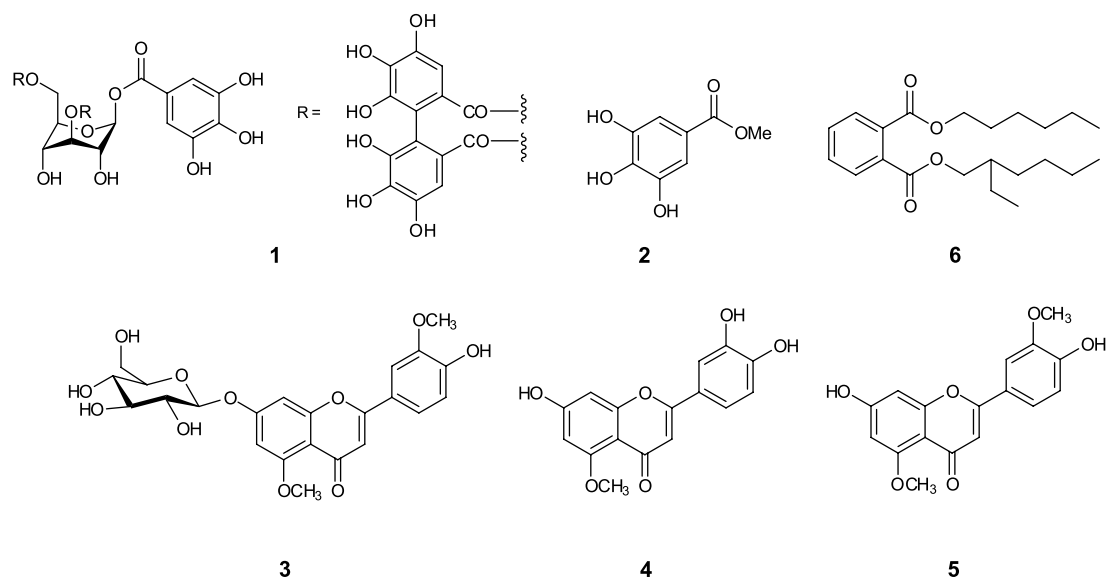
## Results and Discussion

The HPLC-DAD chromatographic analyses of crude extracts from *V. divergens* seedlings inoculated with endophytic fungi isolated from *V. divergens* roots collected on dry and wet seasons in the Pantanal allowed the detection of at least thirteen chromatographic bands at 254 nm (Table 1); six of these bands were identified as the tannin HHDP-galloyl-glucose (**1**, retention time (RT) 12.67 min); methyl gallate (**2**, RT 17.63 min), the flavones 3',5-dimethoxy-luteolin-7-*O*- $\beta$ -glucoside (**3**, RT 19.210 min); 5-methoxy-luteolin (**4**, RT 22.14 min), 3',5'-dimethoxy-luteolin (**5**, RT 23.85 min) and bis (2-ethylhexyl) phthalate (**6**, RT 36.69 min) by comparison of retention time, UV spectra with authentic standards obtained from *in natura* *V. divergens* by our research group in previous studies<sup>24</sup> and are in accordance with literature data (Figure 1, Table 2).

Based on the cluster analysis using Ward's method, the seedlings inoculated with endophytic fungi from the dry period (D) and wet period (W) could be ranked according to their HPLC chemical profile in four and three groups, respectively (Figures 2 and 3).

Tannin **1** occurred in all the samples, including the control. In contrast, the methyl gallate **2** (C6-C1 phenolic compound) was observed only in *V. divergens* in nature. Comparing the flavones occurrence in seedlings inoculated with fungi collected from both seasons, the data revealed that flavone **3** had the same representation in both fungi groups (20%). However, flavone **4** occurred only in seedlings inoculated with fungi isolated on the wet period with 20% of occurrence. In contrast, flavone **5** had a predominance of 80% of the *V. divergens* seedlings inoculated with endophytic fungi collected on the wet season compared with 30% of occurrence of strains from the dry period.

Regarding the HPLC analysis, the chromatographic band with RT 36.69 min was observed in all samples except for the 2D strain and was associated with the compound bis (2-ethylhexyl) phthalate (**6**). This phthalate have been isolated previously from tubers of *Humirianthera ampla*;<sup>34</sup> bis (2-ethylhexyl) phthalate was also isolated from *Nauclea officinalis* leaves.<sup>30</sup> Although several phthalates were found in the *Burkholderia cepacia* bacterium, including dibutyl and dioctyl phthalate,<sup>35</sup> and bis (2-ethylhexyl) phthalate was isolated from fungal strain No. 7088, associated with the plant *Erica arborea*;<sup>36</sup> in this work, we are unsure whether this compound is a natural



**Figure 1.** Chemical structures of compounds 1-6.

**Table 1.** Chromatographic bands observed in HPLC analysis of *in vitro* *V. divergens* seedlings inoculated with endophytic fungi from the dry and wet periods compared with the control

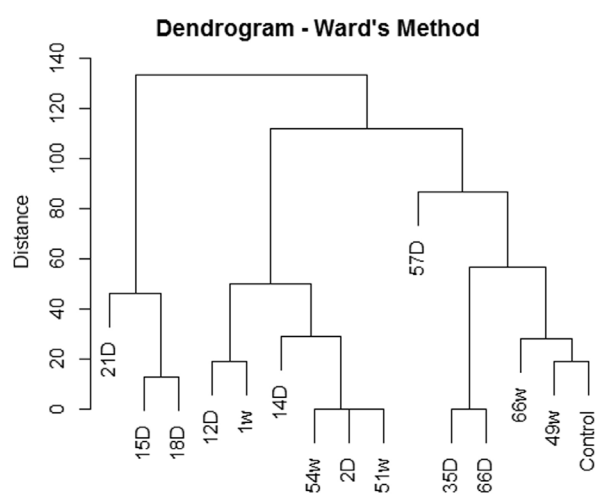
Code	Endophytic fungi identification (GenBank access number, Similarity)	Chromatographic bands RT / min											
		12.67 (1)	15.64	17.63 (2)	19.21 (3)	21.41	22.14 (4)	23.85 (5)	29.21	33.80	36.14	36.69 (6)	38.02
Dry period													
2D	<i>Eutypella scoparia</i> (KY083415, 98%)	+						+			+	+	+
12D	<i>Paraconiothyrium brasiliense</i> (KY083416, 12%)	+									+	+	+
14D	<i>Paraconiothyrium cyclothyrioides</i> (KY083417, 99%)	+			+			+			+	+	+
15D	Fungal sp. ASR-179 (KY083418, 99%)											+	+
18D	<i>Leptosphaeria senegalensis</i> (KY083419, 99%)	+										+	+
21D	<i>Melanconiella elegans</i> (KJ173701, 96%)	+											+
35D	Uncultured <i>Pleosporales</i> (KY083420, 99%)	+								+	+	+	+
57D	Fungal sp. ARIZ L80 (KY083421, 92%)	+			+	+		+	+	+	+	+	+
66D	<i>Wuestneia molokaiensis</i> (KY083422, 99%)	+								+	+	+	+
Wet period													
1W	<i>Neosartorya fischeri</i> (KY083410, 99%)	+			+						+	+	+
51W	Fungal sp. ARIZ L80 (KY083411, 99%)	+						+			+	+	+
49W	<i>Eupenicillium</i> sp. (KY083412, 98%)	+						+	+	+	+	+	+
54W	<i>Gongronella butleri</i> (KY083413, 99%)	+						+			+	+	+
66W	<i>Chaetomium</i> sp. (KY083414, 99%)	+						+	+		+	+	+
	Control <sup>a</sup>	+			+			+	+	+	+	+	+
	Vd <sup>b</sup>	+	+	+	+	+	+	+	+	+	+	+	+

<sup>a</sup>Control: *V. divergens* without fungi inoculation; <sup>b</sup>Vd: *V. divergens* in nature.

**Table 2.** Identified compounds in *V. divergens* microplants infected with D and W strains

Compound	Identification	RT / min	UV $\lambda_{max}$ / nm	Molecular formula	MW	ESI-MS ( $m/z$ )
<b>1</b> <sup>25</sup>	HHDP-galloyl-glucose <sup>a,b</sup>	12.67	194, 225, 270	C <sub>27</sub> H <sub>22</sub> O <sub>18</sub>	634	657.0461 [M + Na] <sup>+</sup> 633.0748 [M - H] <sup>-</sup>
	unknown	15.64	195, 228, 278			
<b>2</b> <sup>26</sup>	methyl gallate <sup>b,c</sup>	17.63	241, 264, 339	C <sub>8</sub> H <sub>8</sub> O <sub>5</sub>	184	
<b>3</b> <sup>27</sup>	3',5-dimethoxy luteolin- 7-O- $\beta$ -glucoside <sup>a,b,c,d</sup>	19.21	245, 265, 339	C <sub>23</sub> H <sub>24</sub> O <sub>11</sub>	476	477.14115 [M + H] <sup>+</sup>
	unknown	21.41	196, 252, 243			
<b>4</b> <sup>28</sup>	5-methoxy luteolin <sup>a,b,c,d</sup>	22.14	243, 284, 339	C <sub>16</sub> H <sub>12</sub> O <sub>6</sub>	300	301.07404 [M + H] <sup>+</sup>
<b>5</b> <sup>29</sup>	3',5-dimethoxy luteolin <sup>a,b,c,d</sup>	23.85	244, 264, 338	C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>	314	315.08903 [M + H] <sup>+</sup>
	unknown	29.20	197, 243, 279			
	unknown	33.80	198, 251			
	unknown	36.14				
<b>6</b> <sup>30</sup>	bis (2-ethylhexyl) phthalate <sup>a,b,c,d</sup>	36.69	267, 330	C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>	390	
	unknown	38.02	268, 330			
	unknown	39.77	257, 316			

<sup>a</sup>Identification by MS; <sup>b</sup>identification by literature data; <sup>c</sup>identification by NMR spectra; <sup>d</sup>identification by comparison with reference standards. HHDP: hexahydroxydiphenyl; RT: retention time; MW: molecular weight; ESI-MS: electrospray ionization mass spectrometry.

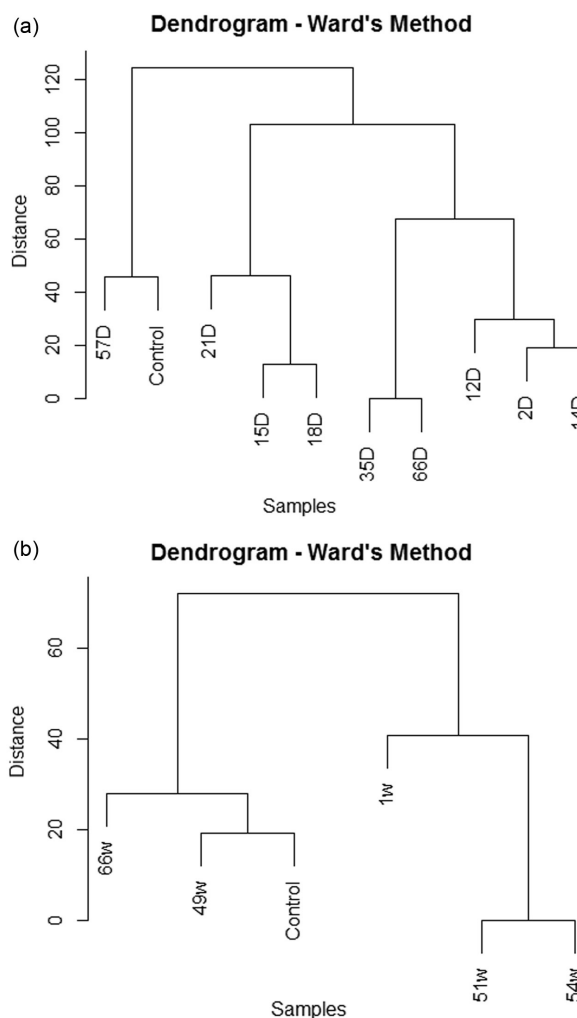


**Figure 2.** Cluster analysis using Ward's method of endophytic fungal strains collected in the dry (D) and wet (W) seasons from the Pantanal Biome associated with *in vitro* *V. divergens* seedlings. The codes are described in Table 1.

product or a contaminant; therefore, additional studies are necessary to gain a better understanding of these results.

We observed that the effect of inoculation with endophytic fungi in *in vitro* microplants varied according to the season and the strain used. For example, the production of flavones **3** and **4** seems to have been favored by inoculation with strains acquired in the wet period.

Concerning the metabolite diversity, seedlings inoculated with endophytic fungi 57D (Fungal sp. ARIZ L80) and 49W (*Eupenicillium* sp.) strains exhibit a higher number of produced metabolites. The endophyte-plant interaction leads to alterations in the phytochemical profile of the host.<sup>37</sup>



**Figure 3.** Cluster analysis using Ward's method of endophytic fungal strains collected in (a) dry (D) and (b) wet (W) seasons from the Pantanal Biome. The codes are described in Table 1.



Studies regarding the interactions of *V. divergens* and their endophytic microbiota are rare; however, Savi *et al.*<sup>38</sup> describe the isolation of an endophytic actinomycete strain from *V. divergens*.

The influence of *V. divergens* leaves extracts on the germination and growth of lettuce and tomato were investigated by Oliveira *et al.*<sup>39</sup> and they suggest a possible allelopathic effect of this species associated with the presence of phenolic compounds, which include flavonoids. Several authors reported that the presence and increase of flavonoids in plants may be associated with several abiotic factors including UVB, temperature and drought among others, such as environmental stress.<sup>40,41</sup> In line with this, Guidi *et al.*<sup>42</sup> studied the interactions of water stress and solar irradiance on the physiology and biochemistry with special emphasis on flavonoid production of *Ligustrum vulgare*. They observed that the content of quercetin and luteolin derivatives increased in response to full sunlight irrespective of the water treatment; however, the phenylpropanoid concentrations increased in response to water stress only in shaded leaves.

Ahuja *et al.*<sup>43</sup> mention that environmental stress factors such as drought, elevated temperature, salinity and rising CO<sub>2</sub> or multiple environmental stress in combination affect plant growth, and they reprogram the plant to survive in a changing climate. Responses to perturbations are usually accompanied by major changes in the plant transcriptome, proteome and metabolome.

In this work, the re-isolation of the endophytic fungi from the *in vitro* seedlings was not carried out to effectively confirm the endophyte-host interaction.

Comparing the flavone structures reveals that all of them have a methoxy group at C-5 position, and **3** and **5** have one additional methoxy group at C-3' carbon. In addition, 5-methoxy flavones and flavones with no substitution in this position are shown active as growth inhibitors of navel orange worm (NOW), a citrus pest that also attacks other cultures, especially walnuts and almonds. These flavones appear to be associated with the resistance of citrus to this pathogen, whereas flavones with hydroxyl-substitution at the 5-position are inactive. Since a 5-hydroxy substituent is strongly hydrogen bonded to the 4-carbonyl oxygen, the growth inhibition is correlated with the availability of this carbonyl.<sup>44</sup>

In contrast, polymethoxy flavones are related to the defense mechanisms of the plant itself, and they have an antiviral and antimicrobial capacity that confers resistance against microbial infections in citrus.<sup>45,46</sup>

The 5-methoxy flavonoids 5-methoxy-luteolin and 3',5-dimethoxy luteolin have been reported as active nod gene inducers in *Rhizobium meliloti* together with the

flavonoids luteolin, luteolin-7-*O*-glucoside and 3-methoxy-luteolin (chrysoeriol). This gene is responsible for the nitrogen fixation in alfalfa.<sup>47</sup>

## Conclusions

Therefore, the presence of 5-methoxy flavones in *V. divergens* leaves suggests the possible correlation of this class of substances with the *V. divergens* resistance to flooding, pathogens attack and allelopathic action. This approach is new to *V. divergens*, with no scientific data on *in vitro* fungi elicitation; however, further studies are necessary to gain a better understanding of the ecological relationships of this species.

## Supplementary Information

Supplementary data, including <sup>1</sup>H NMR, <sup>13</sup>C NMR, mass spectra and HPLC chromatogram, are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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